

1 **Fungal feruloyl esterases: functional validation of genome mining based enzyme discovery**
2 **including uncharacterized subfamilies**

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26

27 **Abstract**

28 Feruloyl esterases (FAEs) are a diverse group of enzymes that specifically catalyze the hydrolysis
29 of ester bonds between a hydroxycinnamic (e.g. ferulic) acid and plant poly- or oligosaccharides.
30 FAEs as auxiliary enzymes significantly assist xylanolytic and pectinolytic enzymes in gaining
31 access to their site of action during biomass saccharification for biofuel and biochemical
32 production. A limited number of FAEs have been functionally characterized compared to over
33 1,000 putative fungal FAEs that were recently predicted by similarity-based genome mining,
34 which divided phylogenetically into different subfamilies (SFs). In this study, 27 putative and six
35 characterized FAEs from both ascomycete and basidiomycete fungi were selected and
36 heterologously expressed in *Pichia pastoris* and the recombinant proteins biochemically
37 characterized to validate the previous genome mining and phylogenetical grouping and to expand
38 the information on activity of fungal FAEs. As a result, 20 enzymes were shown to possess FAE
39 activity, being active towards *p*NP-ferulate and/or methyl hydroxycinnamate substrates, and
40 covering 11 subfamilies. Most of the new FAEs showed activities comparable to those of
41 previously characterized fungal FAEs.

42

43 **Keywords**

44 Feruloyl esterase, ferulic acid, genome mining, plant cell wall, fungi

45

46 **Abbreviations**

47 FA, ferulic acid; FAE, feruloyl esterase; SF, subfamily

48

49 **Introduction**

50 Esterified or etherified to polymers within the lignocellulosic matrix, ferulic acid (FA, 4-hydroxy-
51 3-methoxycinnamic acid) and to a lesser extent *p*-coumaric acid (4-hydroxycinnamic acid) are the
52 most abundant hydroxycinnamic acids in plant cell walls [1, 2]. These hydroxycinnamic acids can
53 be linked to arabinoxylans (*O*-5 position of α -L-arabinofuranosyl residues), which are the unique
54 structural components in commelinid monocots (Family Poales, e.g. wheat, rice and barley). They
55 can also be linked to neutral pectic side-chains of rhamnogalacturonan I (*O*-6 position of β -D-
56 galactopyranosyl residues in (arabino)galactan, and *O*-2 or *O*-5 position of α -L-arabinofuranosyl
57 residues in arabinan), which are mainly found in eudicotyledons (Order 'core' Caryophyllales, e.g.
58 sugar beet) [3-8]. FA can form diferulic acids (mainly 5,5'-, 8-*O*-4'-, 8,5'-, 8,8'-diferulic acids)
59 which cross-link two polysaccharide chains or a polysaccharide chain to lignin [7, 9-11]. Phenolic
60 cross-links increase the physical strength and integrity of plant cell walls and reduce their
61 biodegradability by microbial invaders and hydrolytic enzymes [2, 12].

62 Feruloyl esterases (or ferulic acid esterases, FAEs) [E.C. 3.1.1.73] represent a subclass of the
63 carboxylic acid esterases (E.C. 3.1.1) and catalyze the hydrolysis of ester linkage between a
64 phenolic acid and a poly- or oligosaccharide releasing hydroxycinnamic acids from plant cell wall
65 polysaccharides [13, 14]. FAEs are able to release FAs and other phenolic acids from natural plant
66 sources and agro-industrial byproducts. They facilitate the degradation of complex plant cell wall
67 polysaccharides by removing the ester bonds between plant polymers providing accessibility for
68 glycoside hydrolases and polysaccharide lyases [15-17]. Apart from being used as accessory

69 enzymes in the saccharification process, FAEs are also potential biocatalysts for synthesis of a
70 broad range of novel bioactive components for use in the food, cosmetics and pharmaceutical
71 industries [18, 19]. In 2014, an EU collaborative project ‘OPTIBIOCAT’ was granted by the 7th
72 Framework Programme (FP7), which aims to use microbial esterases such as FAEs as biocatalysts
73 for synthesis of potential antioxidants for cosmetic products [19]. As these enzymes are relevant
74 for various industries, different types of FAE are required to fit specific conditions such as pH and
75 temperature.

76
77 Recently, we reported a genome mining strategy for FAE discovery, in which more than 1,000
78 putative fungal FAE sequences were identified and, by using a phylogenetical analysis, classified
79 into 13 subfamilies (SFs) [19]. In contrast to the high number of the putative FAE encoding genes,
80 a limited number of fungal FAEs have been characterized in detail and they only cover SF1, 2, 5,
81 6, 7 and 13. To validate our genome mining strategy and expand the information on activity and
82 properties of fungal FAEs, in this study we report the heterologous expression and biochemical
83 characterization of selected recombinant FAEs discovered through genome mining, and covering
84 the previously uncharacterized SFs.

85

86 **Materials and methods**

87 *Bioinformatics*

88 Genome mining and phylogenetic analysis were performed based on [19]. Signal peptides were
89 predicted using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>; [20]). The gene model
90 correction of selected sequences was performed manually based on BlastX to identify and remove
91 putative introns [21]. Sequence alignment was performed using Multiple Alignment using Fast

92 Fourier Transform (MAFFT) [22]. Theoretical molecular masses and pI were calculated by the
93 ExPASy–ProtParam tool (<http://www.expasy.ch/tools/protparam.html> [23]).

94

95 *Cloning of fae genes*

96 The genes of the selected FAEs without signal peptide and introns were codon optimized and
97 synthesized for expression in *P. pastoris* by NZYTech (Lisbon, Portugal). The gene products were
98 digested by *PsiI* and *NotI* (Thermo Fisher Scientific), and cloned in frame with α -factor secretion
99 signal in pPNic706 (ProteoNic, Leiden, the Netherlands). The obtained plasmids were purified
100 from *Escherichia coli* DH5 α (Invitrogen), transformants selected on Luria Bertani medium
101 supplemented with 50 μ g/mL kanamycin, fully sequenced (Macrogen, Amsterdam, the
102 Netherlands), linearised by *SalI* (Thermo Fisher Scientific), and transformed into *P. pastoris* strain
103 GS115 *his4* according to the manufacturer's recommendation.

104 Ten transformants were selected for the enzyme production screening, which was performed in 96
105 deep-well plates containing 0.8 mL medium. The selected clones were grown first in buffered
106 minimal glycerol medium (1% yeast nitrogen base, 0.1 M potassium phosphate buffer pH 6.5, and
107 1% w/v glycerol). The plates were sealed with AeraSeal™ (Sigma Aldrich) and incubated
108 overnight at 30°C, 900 rpm (INFORS HT Microtron, Bottmingen, Switzerland). A volume of cells
109 equal to an OD₆₀₀ of 1.0 was harvested and resuspended in 0.8 mL buffered minimal methanol
110 medium (1% yeast nitrogen base, 0.1 M potassium phosphate buffer pH 6.5, and 0.5 % methanol)
111 for induction. The induction was performed at 30°C, 900 rpm for 72 h before being harvested. The
112 cultures were supplemented with 80 μ L of 0.5% (v/v) methanol every 24 h.

113

114 *Production and biochemical properties of recombinant FAEs*

115 *P. pastoris* transformants were grown according to [24]. Induction was continued for 96 h at 28°C
116 with methanol being supplemented to 0.5% (v/v) every 24 h. Culture supernatants were harvested
117 (4000 x g, 4°C, 20 min), filtered (0.22 µm; Merck Millipore, Darmstadt, Germany) or concentrated
118 (10 kDa cut off; Merck Millipore) and stored at -20°C prior further analysis. Molecular mass
119 determination and deglycosylation were performed as previously described [24]. Protein
120 concentrations were assessed from SDS-PAGE gels by densitometric method using ImageJ
121 program [25] with bovine serum albumin (Pierce, Thermo Scientific) as a standard.

122

123 *Enzyme activity assay of FAEs*

124 Activity of the recombinant FAEs towards *p*NP-ferulate (Taros Chemicals, Dortmund, Germany)
125 was performed in 275 µL reaction mixtures adapted from [26]. The *p*NP-ferulate substrate solution
126 was prepared by mixing 10.5 mM *p*NP-ferulate (in dimethyl sulfoxide) and 100 mM potassium
127 phosphate buffer, pH 6.5 containing 2.5% Triton-X (1:9, v/v). The reactions were performed in
128 the presence of 250 µL *p*NP-ferulate substrate solution incubated with 25 µL of culture supernatant
129 at 37°C. The release of *p*-nitrophenol was spectrophotometrically quantified by following the
130 absorbance at 410 nm for 30 min with a 2 min interval and calculation according to [26]. All assays
131 were performed in triplicate. One unit of FAE activity is defined as the amount of enzyme releasing
132 1 µmol of *p*-nitrophenol from *p*NP-ferulate per min under the assay conditions.

133

134 Activity towards methyl substrates [methyl caffeate, methyl ferulate, methyl *p*-coumarate, and
135 methyl sinapate (Apin Chemicals Limited, Oxon, United Kingdom)] was assayed in 250 µL
136 reaction mixtures according to [24] at 37°C for 5-30 min. Detection of substrates reduction was
137 performed at 340 nm with a 2 min interval. The activity was determined from the standard curves

138 of the substrates (0.001-0.5 mM). Alternatively, the activities were assayed by HPLC (Agilent
139 1260 Infinity) using Kinetex 2.6u C18 100A column (Phenomenex). The quantification was
140 performed by using calibration curves of the methyl substrates and their corresponding acids, and
141 the detection was performed at 320 nm for methyl caffeate, methyl ferulate and methyl sinapate,
142 and at 308 nm for methyl *p*-coumarate. The 1 mL reaction mixtures contained 850 μ L MOPS
143 buffer (pH 6.0), 100 μ L enzyme and 50 μ L substrate (1 mM). The reactions were stopped by
144 adding 1 vol 0.1% trifluoroacetic acid:acetonitrile solution (80:20). Chromatographic separation
145 was performed by isocratic method with 80% 0.1% trifluoroacetic acid and 20% acetonitrile as
146 solvents. The culture supernatant of *P. pastoris* harboring pPicZ α A plasmid without insert was
147 used as negative control. All assays were performed in triplicate.

148

149 *Enzyme activity assay of tannases*

150 Methyl gallate (Sigma Aldrich, St. Louis, MO) was used for the assessment of tannase activity
151 [27]. The reactions were performed in 125 μ L reaction mixtures containing 6.25 μ L of 100 mM
152 methyl gallate stock solution (in dimethylformamide), 31.25 μ L of 100 mM phosphate buffer, pH
153 6.0, and 25 μ L water incubated with 62.5 μ L of culture supernatant at 30°C for 15 min. To detect
154 the release of gallic acid, 75 μ L of 0.667% rhodanine (in methanol) (Sigma Aldrich) was added to
155 the reaction mixture followed by 5 min incubation at 30°C, addition of 100 μ L of 0.5 M KOH,
156 further incubation at 30°C for 5 min, and addition of 1 mL water prior to quantification by
157 measuring the absorbance at 520 nm. The activity was determined from the standard curves of the
158 substrates (0.006-0.6 mM). Alternatively, tannase activity was assayed by the HPLC method
159 described above by detecting methyl gallate at 280 nm and using InfinityLab Poroshell 120 SB-
160 AQ column (Agilent).

161

162 *Enzyme activity assay of lipases*

163 For assessment of the lipase activity, *p*NP-palmitate (Sigma Aldrich) was used as a substrate. Ten
164 mM *p*NP-palmitate stock solution was prepared in isopropanol and diluted in 100 mM potassium
165 phosphate buffer, pH 6.5. The reaction was performed in 1 mL reaction mixtures containing 900
166 μ L of 0.2 mM *p*NP-palmitate in 100 mM potassium phosphate buffer, pH 6.5, and 100 μ L of
167 culture supernatant at 40°C. The release of *p*-nitrophenol was spectrophotometrically quantified
168 by following the absorbance at 410 nm for 30 min with a 2 min interval and calculation according
169 to [26].

170

171 **Results and discussion**

172 *Discovery of novel fungal FAEs*

173 A genome mining strategy was previously conducted by BLAST analysis with characterized FAEs
174 against published fungal genomes to identify the putative fungal FAEs [19]. In the current study,
175 four additional genomes from *Aspergillus* spp., i.e. *Aspergillus sydowii*, *Aspergillus wentii* DTO
176 134E9, *Aspergillus carbonarius* ITEM 5010 and *Aspergillus tubingensis* [28], were added to the
177 analysis (A.1 in Supplementary materials). Over 1,000 putative fungal FAE sequences were
178 identified, which were classified into 13 SFs. Of these, 33 FAE sequences (six previously
179 characterized and 27 putative FAEs) from both ascomycetes and basidiomycetes, which covered
180 five previously uncharacterized FAE subfamilies (SF3, 8, 9, 10, 12), were selected for biochemical
181 characterization (Table 1). The enzymes from SF11 were not included in this study because they
182 most likely possessed tannase activity.

183

184 *Recombinant enzyme production of selected fungal FAEs*

185 Heterologous expression of putative FAEs was performed using *P. pastoris* as a host organism.
186 FAE sequences from both ascomycetes and basidiomycetes were produced as active enzymes in
187 *P. pastoris*. Three of the selected enzymes, i.e. An09g05120 (*A. niger*), CsFae3 (*Ceriporiopsis*
188 *subvermispota*) and ShFae1 (*Stereum hirsutum*), were produced at a concentration greater than
189 1,000 mg/L and five, namely GmFae1 (*Galerina marginata*), OrpFAE (*Orpinomyces* sp.),
190 AnFaeL, An11g01220 (*A. niger*) and CsFae1 (*C. subvermispota*), were produced at a
191 concentration greater than 100 mg/L. However, the production level of eight and 12 of the enzymes
192 was less than 100 mg/L and 10 mg/L respectively, and five of them were not produced. Almost
193 half of the recombinant enzymes showed higher molecular masses compared to the calculated
194 ones, but the molecular mass reduced to the expected size after treatment with Endoglycosidase H
195 indicating glycosylation by *P. pastoris*.

196

197 *Activity and substrate preference among the selected fungal FAEs*

198 To screen for FAE activity, *p*NP-ferulate was used as a substrate. Among 27 enzymes, 17 were
199 active, with GmFae1 (*G. marginata*) showing the highest activity (Table 1). With respect to the
200 substrate specificity, four methyl substrates (methyl *p*-coumarate, methyl caffeate, methyl ferulate,
201 and methyl sinapate) were used. In agreement with the previous reports [29-31], AnFaeB (*A. niger*)
202 and AsFaeH (*A. sydowii*) from SF1 hydrolyzed three methyl substrates: methyl *p*-coumarate,
203 methyl caffeate and methyl ferulate (Table 2). Docking simulation of methyl sinapate on a
204 structure of a member of SF1, AoFaeB (from *A. oryzae*, PDB: 3WMT), indicated that its narrow
205 active site hindered the binding of the bulky structure of methyl sinapate [32]. AsFaeG (*A. sydowii*)
206 from SF13 was also able to hydrolyze three methyl substrates similarly to the enzymes from SF1,

207 whereas the other FAEs from SF13 showed either low or no activity towards methyl ferulate. The
208 majority of the active FAEs from SF5 and SF6 hydrolyzed all four substrates, except FoFae2
209 (*Fusarium oxysporum* from SF6) and CcFae2 (*Coprinopsis cinerea* from SF6), which did not
210 hydrolyze methyl sinapate, and Settu1| 102085 (*Setosphaeria turcica* from SF5), which was only
211 active towards methyl caffeate and methyl sinapate. Because Settu1| 102085 as well as some other
212 esterases (see below) were not detected to hydrolyze methyl ferulate, they were referred to as
213 hydroxycinnamoyl esterases (HCE) instead of FAEs.

214
215 AnFaeA (*A. niger*) from SF7 hydrolyzed only methyl ferulate and methyl sinapate, which is
216 consistent with the previous reports [29, 31, 33]. The crystal structure of AnFaeA showed a long
217 and narrow cavity displaying hydrophobic residues that stabilize the aromatic moiety of the
218 substrate [34], and replacing the bulky aromatic residues (Tyr80 or Trp260) to smaller residues
219 broadened the substrate specificity of the enzyme [35].

220
221 Three FAEs from SF2, namely FoFaeC (*F. oxysporum*), AwFaeA (*A. wentii*) and GIFae1
222 (*Gymnopus luxurians*), showed no obvious substrate specificity pattern. FoFaeC hydrolyzed all
223 tested substrates with low activity on methyl sinapate, which is consistent with the previous report
224 [36]. AwFaeA also showed low activity towards methyl sinapate, but did not hydrolyze methyl *p*-
225 coumarate. GIFae1 showed the highest activity towards methyl sinapate and lower activity towards
226 methyl ferulate, but was not active towards methyl *p*-coumarate and methyl caffeate. The enzymes
227 from SF3 (Aspca3| 176503 from *A. carbonarius*), SF9 (Asptu1| 30001 from *A. tubingensis*) and
228 SF12 (Galma1| 254175 from *G. marginata*) were not active towards methyl ferulate and *p*NP-
229 ferulate, but showed activity towards only one methyl substrate with no specific pattern. Therefore,

230 these esterases were also referred to as HCEs. The enzyme from SF10 (OrpFAE from *Orpinomyces*
231 sp.) was not active towards methyl substrates, but showed activity towards *p*NP-ferulate.

232
233 Previously, members of SF1, 2, 5, 6, 7 and 13 were shown to possess FAE activity [19]. In the
234 present study, we reported that enzymes from SF8, 9 and 10 showed limited activity towards
235 synthetic FAE substrates, as well as towards methyl gallate for SF9. Recently, an esterase from
236 *Auricularia auricularia-judae* (EstBC), belonging to SF8 was described that acted efficiently on
237 both artificial cinnamic and benzoic acid esters, but was not active on complex natural FAE
238 substrates [37]. Hence, the enzymes from these subfamilies should be further tested towards
239 feruloylated saccharides or natural substrates, e.g. wheat bran or sugar beet pectin, to confirm their
240 true FAE activity.

241
242 *Enzymes with other activity*

243 Five enzymes (CsTan1 – *C. subvermispora*, AnFaeL – *A. niger*, AsFaeL – *A. sydowii*,
244 An11g01220 – *A. niger*, An09g05120 – *A. niger*) were active towards methyl gallate. These
245 enzymes belong to SF9, SF10 and SF13, indicating that the representatives of these SFs may be
246 tannases. Recently, two FAEs from *Schizophyllum commune* were reported to hydrolyse methyl
247 gallate [38]. It is possible that these enzymes are the bridge in the evolution from tannases to FAEs
248 or *vice versa*. Surprisingly, one enzyme (Aspca3| 176503 from *A. carbonarius*) was active towards
249 *p*NP-palmitate, although this enzyme does not share amino acid sequence similarity to known
250 lipases.

251
252 **Conclusions**

253 In the present study, we have confirmed the ability of the genome mining strategy to identify fungal
254 FAE encoding genes, by demonstrating that 20 out of 27 putative fungal FAEs possessed FAE
255 activity towards *p*NP-ferulate and/or methyl hydroxycinnamate substrates. Previously, members
256 of SF1, 2, 5, 6, 7 and 13 were shown to possess FAE activity [19]. In the present study, we also
257 showed that the enzymes from SF8, 9 and 10 are active towards the synthetic FAE substrates.
258 However, it should be noted that most of the enzymes from SF9 possessed tannase activity.
259 Additional experiments are needed to confirm whether the enzymes of these subfamilies are true
260 FAEs. The selected esterases from SF3 and SF12 were active towards methyl caffeate and methyl
261 *p*-coumarate, respectively, but not towards methyl ferulate. In comparison with the previously
262 characterized fungal FAEs, most of the new FAEs showed similar levels of specific activity. Thus,
263 they may potentially be eligible candidates for related biotechnological applications.

264

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267

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271

272 **Appendix A. Supplementary data**

273 **Table A.1** Sequences of putative FAEs from four aspergilli.

274

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Table 1 Molecular mass, production level and specific activity (towards *p*NP-ferulate) of characterized FAEs in this study^a.

Fungal species	Phylum ^b	Accession number	SF ^c	Name ^d	Calculated molecular mass (kDa)	Apparent molecular mass (kDa) ^e	Calculated <i>pI</i>	Production (mg/L)	Specific activity ^f (mU/mg)	Remark ^g
<i>Aspergillus niger</i>	Asco	Q8WZI8.1	1	AnFaeB	55.8	100 (55)**	4.95	55	2	
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 293049	1	AsFaeB	55.4	55*	4.89	<1	4	
<i>Fusarium oxysporum</i>	Asco	jgi Fusox1 5438	2	FoFaeC	59.4	70 (61)**	6.89	6	Active	
<i>Aspergillus wentii</i>	Asco	jgi Aspwe1 156253	2	AwFaeJ	56.0	57	4.99	<1	Active	
<i>Gymnopus luxurians</i>	Asco	jgi Gymlu1 46632	2	GlFae1	56.8	58	4.53	3	na	
<i>Aspergillus carbonarius</i>	Asco	jgi Aspca3 176503	3	-	56.1	58	5.02	4	na	Lipase activity
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 901052	3	-	55.8	nd	4.88	np	na	
<i>Aspergillus nidulans</i>	Asco	EAA62427.1	5	AnidFAEC	25.8	30 (30)**	4.47	30	2	
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 154482	5	AsFaeC	25.9	30 (30)**	4.44	15	2	
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 48859	5	AsFaeE	26.6	nd	3.93	np	na	
<i>Setosphaeria turcica</i>	Asco	jgi Settu1 102085	5	-	26.9	26	8.90	<1	na	
<i>Myceliophthora thermophila</i> (<i>Sporotrichum thermophile</i>)	Asco	AEO62008.1	6	MtFae1a	29.5	35 (30)**	4.44	45	3	
<i>Fusarium oxysporum</i>	Asco	jgi Fusox1 8990	6	FoFae2	29.6	30	8.44	4	Active	
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 1158585	6	AsFaeG	29.5	32*	4.24	7	4	

<i>Stagonospora nodorum</i>	Asco	jgi Stano2 8578	6	-	31.3	30	5.16	12	na	
<i>Coprinopsis cinerea</i>	Basidio	jgi Copci1 3628	6	CcFae2	37.7	38	6.63	<1	Active	
<i>Ceriporiopsis subvermispora</i>	Basidio	jgi Cersu1 68569	6	CsFae1	36.9	70	4.58	280	7	
<i>Galerina marginata</i>	Basidio	jgi Galma1 144217	6	GmFae1	35.2	42	5.83	600	120	
<i>Aspergillus niger</i>	Asco	CAA70510	7	AnFaeA	28.6	40 (35)**	4.19	55	4	
<i>Aspergillus clavatus</i>	Asco	jgi Aspcl1 3045	8	-	39.1	40*	6.34	51	na	
<i>Ceriporiopsis subvermispora</i>	Basidio	jgi Cersu1 89153	9	CsTan1	55.0	90 (60)***	4.59	np	na	Tannase activity
<i>Aspergillus tubingensis</i>	Asco	jgi Asptu1 30001	9	-	57.6	57	4.50	<1	na	
<i>Aspergillus niger</i>	Asco	An15g05280	9	AnFaeL	58.6	68 (60)***	4.93	500	na	Tannase activity
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 41271	9	AsTanA	57.6	60*	4.99	32	na	Tannase activity
<i>Orpinomyces</i> sp.	Neo	AAF70241.1	10	OrpFAE	59.0	68	5.06	600	10	
<i>Aspergillus niger</i>	Asco	An11g01220	10	-	55.0	88	4.11	500	na	Tannase activity
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 194109	10	-	52.7	nd	4.67	np	na	
<i>Dichomitus squalens</i>	Basidio	jgi Dicsq1 136925	12	-	56.3	nd	4.78	np	na	
<i>Galerina marginata</i>	Basidio	jgi Galma1 254175	12	-	56.0	59	6.90	3	na	
<i>Aspergillus sydowii</i>	Asco	jgi Aspsy1 160668	13	AsFaeK	59.4	55*	4.90	<1	4	
<i>Stereum hirsutum</i>	Basidio	jgi Stehi1 73641	13	ShFae1	58.1	67	4.40	2,160	13	
<i>Aspergillus niger</i>	Asco	An09g05120	13	-	53.1	66	4.79	3,400	na	Tannase activity

<i>Ceriporiopsis subvermispora</i>	Basidio	jgi Cersu1 150639	13	CsFae2	55.1	nd	4.28	2,400	1
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^a na, no activity detected; nd, no protein band detected; np, no protein produced or the protein level was lower than detection limit.

^b Asco, ascomycete; Basidio, basidiomycete; Neo, Neocallimastigomycete

^c According to [19]

^d Name in bold indicates the previously reported FAEs [19]. The abbreviation of the enzyme code is based on the convention protein names for different species and not on the types of FAEs.

^e * indicates the protein band was visible only after deglycosylation by Endoglycosidase H. ** indicates molecular mass after deglycosylation by Endoglycosidase H, *** indicates molecular mass after deglycosylation by PNGase F.

^f One unit of enzyme activity is defined as the amount of enzyme releasing 1 μ mol of *p*-nitrophenol from *p*NP-ferulate per min under assay conditions. Active indicates the enzyme was active but the specific activity could not be calculated.

^g Tannase and lipase activities were evaluated using methyl gallate and *p*NP-palmitate as substrate, respectively

Table 2 Relative FAE activity towards four methyl substrates^a.

Fungi	SF ^b	Name ^c	Relative activity (%) ^d			
			Methyl <i>p</i> -coumarate	Methyl caffeate	Methyl ferulate	Methyl sinapate
<i>Aspergillus niger</i>	1	AnFaeB	100	77	59	na
<i>Aspergillus sydowii</i>	1	AsFaeB	100	66	60	na
<i>Fusarium oxysporum</i>	2	FoFaeC	100	67	29	9
<i>Aspergillus wentii</i>	2	AwFaeJ	na	100	88	29
<i>Gymnopus luxurians</i>	2	GlFae1	na	na	18	100
<i>Aspergillus carbonarius</i>	3	-	na	100	na	na
<i>Aspergillus nidulans</i>	5	AnidFAEC	98	42	100	46
<i>Aspergillus sydowii</i>	5	AsFaeC	91	45	100	40
<i>Aspergillus sydowii</i>	5	AsFaeE	na	na	Low*	na
<i>Setosphaeria turcica</i>	5	-	na	100	na	91
<i>Myceliophthora thermophila</i>	6	MtFae1a	100	77	90	36
<i>Fusarium oxysporum</i>	6	FoFae2	100	60	16	na
<i>Aspergillus sydowii</i>	6	AsFaeG	95	53	100	60
<i>Stagonospora nodorum</i>	6	-	na	na	na	na
<i>Coprinopsis cinerea</i>	6	CcFae2	100	41	23	na
<i>Ceriporiopsis subvermispota</i>	6	CsFae1	na	na	na	na
<i>Galerina marginata</i>	6	GmFae1	Low*	Low*	Low*	Low*
<i>Aspergillus niger</i>	7	AnFaeA	na	na	100	81
<i>Aspergillus clavatus</i>	8	-	na	na	Low*	na
<i>Aspergillus tubingensis</i>	9	-	na	100	na	na
<i>Aspergillus niger</i>	9	AnFaeL	na	Low*	Low*	Low*
<i>Aspergillus sydowii</i>	9	AsFaeL	na	na	na	na
<i>Orpinomyces</i> sp.	10	OrpFAE	na	na	na	na
<i>Aspergillus niger</i>	10	-	Low*	Low*	Low*	Low*
<i>Galerina marginata</i>	12	-	100	na	na	na
<i>Aspergillus sydowii</i>	13	AsFaeK	100	65	60	na
<i>Stereum hirsutum</i>	13	ShFae1	na	na	Low*	na
<i>Aspergillus niger</i>	13	-	na	Low*	Low*	Low*
<i>Ceriporiopsis subvermispota</i>	13	CsFae2	na	na	na	na

^a na, no activity detected^b According to [19].^c The abbreviation of the enzyme code is based on the conventional protein names for different species and not on the types of FAEs. Name in bold indicates the previously reported FAEs^d The relative activity was calculated as a percentage of the highest activity for each enzyme that was set to 100%, Low* indicates activity which was lower than the reliable detectable range