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Expanding the feruloyl esterase gene family of *Aspergillus niger* by characterization of a feruloyl esterase, FaeC

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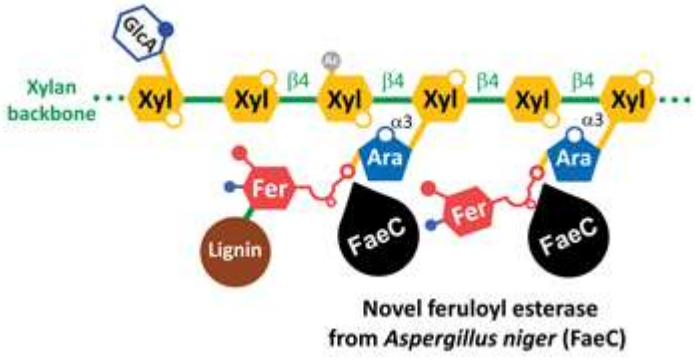
Graphical abstract



Highlights

- New feruloyl esterase from *Aspergillus niger* (FaeC) was produced in *Pichia pastoris*
- FaeC possessed broad substrate specificity towards synthetic FAE substrates
- FaeC was most active at neutral pH 7.0 and 50°C
- FaeC synergistically acted with xylanase to release ferulic acid from xylan
- FaeC showed promising potential for plant biomass processing applications

Abstract

A feruloyl esterase (FAE) from *Aspergillus niger* N402, FaeC was heterologously produced in *Pichia pastoris* X-33 in a yield of 10 mg/L. FaeC was most active at pH 7.0 and 50°C, and showed broad substrate specificity and catalyzed the hydrolysis of methyl 3,4-dimethoxycinnamate, ethyl ferulate, methyl ferulate, methyl *p*-coumarate, ethyl coumarate, methyl sinapate, and methyl caffeate. The enzyme released both ferulic acid and *p*-coumaric acid from wheat arabinoxylan and sugar beet pectin (up to 3 mg/g polysaccharide), and acted synergistically with a commercial xylanase increasing the release of ferulic acid up to six-fold. The expression of *faeC* increased over time in the presence of feruloylated polysaccharides. Cinnamic, syringic, caffeic, vanillic and

ferulic acid induced the expression of *faeC*. Overall expression of *faeC* was very low in all tested conditions, compared to two other *A. niger* FAE encoding genes, *faeA* and *faeB*. Our data showed that the *fae* genes responded differently towards the feruloylated polysaccharides and tested monomeric phenolic compounds suggesting that the corresponding FAE isoenzymes may target different substrates in a complementary manner. This may increase the efficiency of the degradation of diverse plant biomass.

Abbreviations

AcFAE, feruloyl esterase from *Aspergillus clavatus* NRRL1 (GenBank **XP_001274884**); **AN5267,** feruloyl esterase from *Aspergillus nidulans* FGSC A4 (GenBank **EAA62427.1**); **CE1,** Carbohydrate Esterase Family 1; **FaeA,** feruloyl esterase from *Aspergillus niger* (GenBank **CAA70510.1**); **FaeB,** feruloyl esterase from *A. niger* (GenBank **CAC83933.1**); **FaeC,** feruloyl esterase from *A. niger* (GenBank **XP_001395336.1**); **ClFaeA1,** feruloyl esterase from *Myceliophthora thermophila* C1 (GenBank **AEP33616.1**); **ClFaeA2,** feruloyl esterase from *M. thermophila* C1 (GenBank **AEP33617.1**); **FA,** ferulic acid; **FAE,** feruloyl esterase; **Lc-Est1,** esterase from a fosmid library of a leaf-branch compost metagenome (GenBank **KM406415**, PDB 3WYD); **MM,** minimal medium; **NcFaeD,** feruloyl esterase from *Neurospora crassa* OR74A (GenBank **XP_956228**); **SF,** subfamily

Keywords

Aspergillus niger; ferulic acid; feruloyl esterase; pectin; xylan

Introduction

Feruloyl esterases (or ferulic acid esterases, FAEs) [E.C. 3.1.1.73] are a group of enzymes that cleave ester bonds between a phenolic acid and a poly- or oligosaccharide, and release hydroxycinnamic acids from plant biomass (for reviews see [1-5]). Ferulic acid and to a lesser extent *p*-coumaric acid are major plant phenolic compounds. These hydroxycinnamic acids are

linked mainly to arabinoxylans (O-5 position of α-L-arabinofuranosyl residues) and neutral pectic side-chains of rhamnogalacturonan I (O-6 position of β-D-galactopyranosyl residues in (arabino)galactan, and O-2 or O-5 position of α-L-arabinofuranosyl residues in arabinan) and can cross-link xylan chains to each other and to lignin [6-9]. The cross-linking of cell wall polysaccharides and lignin (lignin-carbohydrate complexes) by hydroxycinnamic acids leads to a significant increase in physical strength and integrity of plant cell walls and reduces their biodegradability by microorganisms and enzymatic hydrolysis [7, 10, 11]. FAEs have been shown to act synergistically with other glycoside hydrolases (e.g. xylanases and cellulases) to remove ferulic acid and their cross-links, and facilitate the degradation of complex plant cell wall polysaccharides [12-15]. As they are able to liberate phenolic acids from natural plant sources as well as agro-industrial by-products, FAEs are widely used in the food, feed, pulp and paper, and pharmaceutical industries as well as in biofuel production [1, 4, 16, 17]. These broad application fields require various types of FAEs to fit specific conditions such as pH and temperature.

Previously, two FAEs from *Aspergillus niger* have been reported (FaeA, [18] and FaeB, [19]), which are among the main industrial FAEs used nowadays. FaeA and FaeB possess different substrate specificity. FaeA is active specifically towards methyl ferulate, methyl hydroxybenzoate and sinapate, is more active on wheat arabinoxylan than sugar beet pectin, and can release diferulic acid (5,5, 8-O-4) [6, 19, 20]. In contrast, FaeB is specific for ferulic, *p*-coumaric and caffeic acid methyl esters, and has a preference for pectin over xylan, but cannot release diferulic acids [19]. Phylogenetic analysis of fungal FAEs [1, 5] has revealed numerous novel FAE candidates including an additional putative FAE from *A. niger* (FaeC, An12g02550, Fig. 1). In this study, we report the heterologous production of FaeC in *Pichia pastoris*, its biochemical characterization and potential application in ferulic acid production. We also show induction of the expression of *faeC* by different monomeric and polymeric phenolic compounds in comparison with the genes encoding the previously characterized *A. niger* FaeA and FaeB.

Materials and Methods

Materials

Caffeic acid, caffeic acid dimethyl ether (3,4-dimethoxycinnamic acid), cinnamic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), ferulic acid, sinapic acid, syringic acid, vanillic acid and veratric acid (3,4-dimethoxybenzoic acid) were purchased from Acros (Geel, Belgium). Chlorogenic acid, ethyl coumarate, ethyl ferulate, methyl caffeate, methyl cinnamate, methyl *p*-coumarate, methyl 3,4-dimethoxycinnamate, methyl ferulate, methyl hydroxybenzoate and methyl sinapate were from Apin Chemicals Limited (Oxon, United Kingdom). Wheat bran was from Wageningen Mill (Wageningen, the Netherlands). Sugar beet pulp was from Weiss BioTech (Ascheberg, Germany). Calcium lignin (calcium lignosulphanate) was from Borregaard (Sarpsborg, Norway). Insoluble wheat arabinoxylan (wheat flour) was from Megazyme (Wicklow, Ireland) and sugar beet pectin (Pectin Betapec RU301) was from (Herbstreith & Fox KG, Neuenbürg, Germany). Benzoic acid, *p*-coumaric acid, 3,4-dimethylbenzyl alcohol, *p*-hydroxybenzoic acid, endopolygalacturonase (pectinase from *A. niger*), xylanase (from *Thermomyces lanuginosus*) and other standard chemicals were purchased from Sigma Aldrich (St. Louis, MO). 8-O-4-diferulate and 8-5cyclic (8,5)-diferulate was synthesized according to Ralph et al. [21], followed by saponification and isolation of 8-O-4- and 8,5-diferulic acids by using preparative chromatography. 5-5-diferulic acid was synthesized via dimerization of vanillin [22] followed by the Knoevenagel-reaction with Doebner-modification as published previously to synthesize monomeric phenolic acids [23]. Identity and purity were validated by LC-MS and NMR-experiments.

Bioinformatics

The full-length amino acid sequence of *A. niger* FAE genes (*faeA*, An09g00120; *faeB*, An12g10390; *faeC*, An12g02550) were obtained from Aspergillus Genome Database (AspGD, www.aspergillusgenome.org; [24]). Signal peptides were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/; [25]). The sequence alignment was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/; [26]). The percent similarity of amino acid sequences was calculated using BLASTp suite - 2 (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins; [27]) and visualized using Easy Sequencing in Postscript (http://espript.ibcp.fr/ESPript/ESPript; [28]). Theoretical molecular masses were calculated by ExPASy–ProtParam tool (http://www.expasy.ch/tools/protparam.html; [29]). N-glycosylation was predicted using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc; [30]).

Fungal culture conditions

For gene expression analysis on polysaccharides, *A. niger* N402 was grown in minimal medium (MM; [31]) supplemented with 1% wheat bran or 1% sugar beet pulp and with or without 0.1% calcium lignin at 30°C, 250 rpm. Liquid cultures were inoculated with 10^6 spores/mL. The fungal mycelium was collected over the course of three days, dried between tissue paper, frozen in liquid nitrogen and stored at -45°C prior to RNA extraction [19]. For gene expression analysis on monomeric aromatic compounds, *A. niger* N402 was pre-grown in liquid complex medium [31] overnight at 30°C, 250 rpm, and the mycelium was transferred to MM containing 0.03% phenolic compound (see list of compounds in Results) and incubation was continued for 2 h at 30°C, 250 rpm. The mycelium was collected as described above.

Gene expression assays

RNA was extracted from the mycelium using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA) and RNA isolation kit (NucleoSpin RNA, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's recommendation. The quality of RNA was assessed by gel electrophoresis. cDNA was generated using ThermoScript Reverse Transcriptase (Invitrogen). Gene expression (RT-qPCR) was assayed by real-time PCR (Applied Biosystems 7500 Real-time PCR system) using ABI Fast SYBR Master Mix (Applied Biosystems). The primers were designed using PrimerExpress® 3.0 software (Applied Biosystems) according to the supplier's instructions (Table A.1) and tested to determine the optimal primer concentration and efficiency. The cycling parameters were 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. A dissociation curve was generated to verify that a single product was amplified [32]. Expression levels were normalized against histone as a physiological reference and calculated according to relative quantification $2^{-\Delta\Delta Ct}$ method [33]. Two biological and three technical replicates were analyzed.

Cloning of fae *genes*

The mature *faeA* and *faeB* genes without predicted signal peptide (Met1-Ala21 and Met1-Ala17, respectively) were amplified by PCR from cDNA originating from mycelium grown on wheat bran and ferulic acid, respectively. The mature *faeC* gene sequence without predicted signal peptide (Met1-Gly21) was amplified by PCR from gDNA using the gene-specific primers (Table A.1). The PCR products were digested by the appropriate restriction enzymes (Promega, Madison, WI), and cloned in frame with α-factor secretion signal in pPICZαA (Invitrogen, Thermo Scientific, Carlsbad, CA) using High Fidelity Phusion DNA Polymerase (New England BioLabs, Ipswich, CA). The obtained plasmids were purified from *Escherichia coli* DH5α (Invitrogen) transformants selected on low salt Luria Bertani medium supplemented with 25 μg/mL zeocin, after which they were fully sequenced (Macrogen, Amsterdam, the Netherlands), linearised by *Sac*I (New England

BioLabs), and transformed into *P. pastoris* X-33 strain according to the manufacturer's recommendation.

Production of recombinant FAEs

P. pastoris transformants were grown according to [34]. Induction was continued for 96 h at 22°C with methanol being supplemented to 0.5% (v/v) every 24 h. Culture supernatants were harvested (4000 × g, 4°C, 20 min), filtered (0.22 µm; Merck Millipore, Darmstadt, Germany) and stored at -20°C prior further analysis. The stability of FaeC was assessed by its activity (see below) each time after thawing. FaeC activity remained more than 95% of its original activity while preforming the experiment.

Biochemical properties of FAEs

Molecular mass was estimated by SDS-PAGE (12% w/v, polyacrylamide gel) using Mini-PROTEAN® Tetra Cell (Bio-Rad, Hercules, CA) and Molecular mass standard, Precision Plus Protein™ Unstained Protein Standards (Bio-Rad). Deglycosylation was performed by treating 20 µL of *P. pastoris* culture supernatant with endoglycosidase H (New England BioLabs) as recommended by the manufacturer. Protein concentration was assessed from SDS-PAGE gel by densitometry method using ImageJ program [35] with bovine serum albumin (Pierce, Thermo Scientific) as standard.

Enzyme activity assays of FAEs

Activity assays towards synthetic substrates (chlorogenic acid, ethyl coumarate, ethyl ferulate, methyl 3,4-dimethoxycinnamate, methyl caffeate, methyl ferulate, methyl hydroxybenzoate, methyl

p-coumarate, methyl sinapate) were performed in 250 µL reaction mixtures. The reactions were performed in the presence of 0.12 mM substrate (0.06-0.5 mM for substrate specificity determination), 80 mM phosphate buffer, pH 6.0, and 50 µL of culture supernatant at 37°C for 5-30 min [19]. Detection of initial rates of of hydrolysis was performed at 340 nm (or 280 nm for methyl hydroxybenzoate) with a 2 min interval, using an Optima Plus spectrophotometer. The activity was determined from standard curves of the substrates (0.001-0.5 mM). Culture filtrate from *P. pastoris* harboring pPicZaA plasmid without insertion was used as negative control. All assays were performed in triplicate. The pH optimum was measured towards methyl ferulate with 80 mM Britton-Robinson buffer [36] from pH 2.0 to pH 10.0 under the same conditions as described above. The temperature optimum was determined by measuring the enzyme activity in 80 mM phosphate buffer, pH 6.0 at different temperatures (from 22°C to 80°C) as described above. The pH and temperature stability was deduced from the residual activity towards methyl ferulate after 2 h incubation at 37°C in 100 mM Britton–Robinson buffer from pH 3.0 to 9.0, or at 30-90°C in 80 mM phosphate buffer, pH 6.0, respectively [37].

Hydrolytic activity towards polysaccharides

The activity towards polysaccharides was determined using insoluble wheat arabinoxylan, wheat bran and sugar beet pectin as substrates. 1% of wheat bran or sugar beet pectin was prepared in 50 mM sodium acetate buffer, pH 4.5, with 0.02% sodium azide as described previously [19]. In addition, a NaOH-solubilized insoluble wheat arabinoxylan was prepared following the manufacturer's recommendation (Megazyme). A reaction containing 500 µL 1% substrate and 100 µL culture supernatant (approximately 1 µg FaeC) was incubated with (for co-incubation) or without 1 µg xylanase for wheat bran and wheat arabinoxylan or endopolygalacturonase for sugar beet pectin, at 30°C, 24h, 100 rpm. The enzyme was inactivated by heating at 95°C, 10 min. In case of pretreatment, the substrate mixtures were incubated with 0.1 mg xylanase for wheat bran and

wheat arabinoxylan or endopolygalacturonase for sugar beet pectin, at 30°C, 72h, 100 rpm followed by heat inactivation at 95°C, 10 min prior to incubation with FaeC. An extra batch of sugar beet pectin with and without pretreatment of rhamnogalacturonan hydrolase and rhamnogalacturonan acetyl esterase from [19] was also included for the activity assay. For hydroxycinnamic acid content analysis, the reaction mixture was centrifuged for 15 min at 4°C and the supernatant was mixed with 100% acetonitrile (1:3, v/v). The acetonitrile-mixture was incubated on ice for 10 min and centrifuged for 15 min at 4°C prior to the analysis. For the diferulic acid content analysis, the mixture was acidified to pH <2.0 with HCl followed by ethyl acetate extraction [38]. The extract was vacuum dried and dissolved in 100% acetonitrile.

Hydroxycinnamic acid content analysis

Release of ferulic, *p*-coumaric and diferulic (8-O-4, 5,5, 8,5) acids from wheat arabinoxylan, wheat bran, and two batches of sugar beet pectin was monitored by HPLC (Dionex ICS-5000+ chromatography system; Thermo Scientific, Sunnyvale, CA) equipped with an Acclaim Mixed-Mode WAX-1 LC Column (3×150 mm; Thermo Scientific) and a UV detector (310 nm; Thermo Scientific). The chromatographic separation was carried out according to manufacturer's recommendation using isocratic elution (30°C; flow rate: 0.25 mL/min) with 25 mM potassium monophosphate, 0.8 mM pyrophosphate, pH 6.0, in 50% acetonitrile. Ferulic and *p*-coumaric acids (0.25-50 μM) were used as standards for identification and quantitation. 8,5-, 5-5- and 8-O-4-diferulic acids were used to identify potentially liberated diferulic acids.

Results

Identification of FaeC and its comparison to other A. niger FAEs

An12g02550 from *A. niger* encodes a putative FAE (FaeC), which belongs to subfamily 5 (SF5) according to phylogenetic analysis based classification [1, 5], and Carbohydrate Active enZyme family (CAZy) Carbohydrate Esterase Family 1 (CE1) [39]. In contrast, An09g00120 (FaeA) and An12g10390 (FaeB) are phylogenetically classified to SF7 and SF1, respectively, and are not included in the CAZy database. FaeC shows high amino acid sequence identity to other SF5 FAEs characterized from *Aspergillus* species (Fig. 2), e.g. *A. nidulans* AN5267 (86%, [40]) and *A. clavatus* AcFAE (81%, [41]), and moderate identity to SF5 FAEs characterized from other ascomycete species, e.g. *Neurospora crassa* NcFaeD (49%, [2]), and *Myceliophthora thermophila* ClFaeA1 (47%) and ClFaeA2 (44%, [42]). Currently there is no crystal structure reported for this subfamily enzyme, and FaeC showed only 28% sequence identity to a metagenomics-derived esterase with a crystal structure (LC-Est1, PDB: 3WYD, [43]). However, by correlation to LC-Est1 and conservation among characterized SF5 members, FaeC showed the characteristics of a serine esterase family member by having the G-X-S-X-G signature motif (Gly119-Gly123) and the catalytic residues were predicted to be Ser121, Asp169 and His226 (Fig. 2).

Production and activity of recombinant FaeC

For biochemical characterization, FaeC was produced as a soluble secreted construct with a C–terminal His tag in *P. pastoris*. The recombinant protein production was analyzed by SDS–PAGE (Fig. 3A). FaeC appeared as a band with a molecular mass corresponding to 30 kDa, which was similar to the theoretical molecular mass (29.0 kDa), and treatment with endoglycosidase H did not result in a shift of the protein size. The theoretical p*I* of FaeC was calculated to be 4.58. The protein yield from culture supernatant was estimated to be 10 mg/L using a densitometric method. FaeA and FaeB were produced at similar level (Fig. A.1). In contrast to FaeC, both FaeA and FaeB were glycosylated and after deglycosylation the molecular masses were shifted to 35 and 55 kDa,

respectively, which were similar to the theoretical molecular masses (30.5 and 57.2 kDa, respectively).

The culture supernatant containing FaeC was tested on different monomeric substrates (Table 1). FaeC showed broad substrate specificity and catalyzed the hydrolysis of almost all tested substrates, except for chlorogenic acid and methyl hydroxybenzoate. *A. niger* FaeB also showed broad substrate specificity, but with a preference for methyl *p*-coumarate, ethyl coumarate and chlorogenic acid, whereas FaeA had quite a narrow substrate specificity and could hydrolyze only methyl sinapate, methyl ferulate, ethyl ferulate and methyl 3,4-dimethoxycinnamate. No FAE activity was detected from the culture broth from *P. pastoris* harboring pPICZαA plasmid without insertion.

FaeC was most active at pH 7.0 (Fig. 3B) and retained >85% of its activity from pH 5.0 to 7.0 (Fig. 3C). FaeA and FaeB were most active at pH 5.0 and 6.0, respectively, and retained >85% of their activity from pH 5.0 to 6.0 (Fig. A.1). All three FAEs showed maximal activity at 50°C (Fig. 3D, A.1) and retained >85% of their activity for 2 h up to 45°C (Fig. 3E, A.1).

Release of ferulic acid by FaeC

To investigate the activity of FaeC towards feruloylated polysaccharides, wheat arabinoxylan, wheat bran, and two batches of sugar beet pectin were incubated with (as co-incubation and pretreatment) and without supplement of xylanase, endopolygalacturonase or rhamnogalacturonan hydrolase and rhamnogalacturonan acetyl esterase (Fig. 4). Among the four tested feruloylated polysaccharide-containing materials, the highest ferulic acid amount was released from insoluble wheat arabinoxylan (up to 3 mg/g substrate). High amount of ferulic acid was released from both solubilized as well as non-solubilized samples indicating that FaeC works towards both types of substrates. Pretreatment of the wheat arabinoxylan with a commercial xylanase or co-incubation of

the xylanase with FaeC increased the release of ferulic acid by four to six-fold. However, when using wheat bran as a substrate, the pretreatment with xylanase did not significantly increase the release of ferulic acid. In contrast, co-incubation with xylanase increased the release of ferulic acid by two-fold from this substrate. Wheat bran and insoluble wheat arabinoxylan contain approximately 1 mg and 3 mg of total ferulic acid per 1 g of polysaccharide (analyzed by CCRC) indicating that almost all ferulic acid was released when treated with xylanase. Two batches of sugar beet pectin were used to investigate the FaeC activity. First batch was from Weiss BioTech which was prepared with or without endopolygalacturonase. Second batch from [19] was prepared with or without pretreatment of rhamnogalacturonan hydrolase and rhamnogalacturonan acetyl esterase. FaeC released similar amounts of ferulic acid from both batches of sugar beet pectin (Fig. 4). A small amount (0.07-0.69 mg/g for wheat arabinoxylan/bran and 0.08-0.13 mg/g for sugar beet pectin) of released *p*-coumaric acid was also detected in all samples. No diferulic acids (i.e. 8-O-4-, 5,5- and 8,5- diferulic acids) were detected, although these dimers were demonstrated to occur in wheat and sugar beet earlier [44].

Expression of faeC in the presence of polysaccharides

To determine the expression of the *A. niger fae* genes in the presence of feruloylated polysaccharides, *A. niger* was grown on 1% wheat bran or 1% sugar beet pulp in MM and the expression was assessed by RT-qPCR (Fig. 5). Low expression was observed for *faeC*, which increased over a three day period. In contrast, *faeA* and *faeB* were highly expressed in the early samples and their expression gradually decreased over time. Wheat bran resulted in higher induction of all *A. niger fae* genes compared to sugar beet pulp. Supplementation of lignin (0.1% w/v) to the medium increased the expression of *faeA* on wheat bran and *faeB* on sugar beet pulp, respectively, at the first day after induction, whereas only slightly affect was detected at other time points.

Expression of faeC in the presence of phenolic monomeric compounds

To determine which monomeric phenolic compounds induce the expression of *faeC*, pre-grown *A. niger* mycelium was transferred to MM supplemented with 0.03% phenolic compounds and incubated for 2 h (Fig. 6). The highest expression of *faeC* was observed in the presence of cinnamic acid followed by syringic, caffeic, vanillic and ferulic acid, even though it was very lowly expressed on all tested phenolic compounds. In contrast, *faeB* was highly expressed in the presence of ferulic, caffeic, and *p*-coumaric acid, whereas *faeA* was expressed when *A. niger* was exposed to 3,4-dimethyl benzyl alcohol, vanillic acid, and ferulic acid. These data demonstrated a highly specific expression response for each of the three *A. niger fae* genes.

Discussion

We have shown that FaeC, the third FAE characterized from *A. niger* [18, 19], possesses a broad substrate specificity (Table 1). This is in line with its phylogenetical placement in subfamily SF5 [1, 5], which contains other characterized FAEs with broad substrate specificity, i.e. *A. clavatus* AcFAE [41], *N. crassa* NcFaeD [2] and *M. thermophila* ClFaeA1 [42]. The characterized enzymes from SF5 have low molecular masses, from 28 to 36 kDa [2, 40-42] similar to FaeC with a molecular mass of 30 kDa. The neutral pH optimum for FaeC activity is similar to AcFAE and ClFaeA1 [41, 42], while both *A. niger* FaeA and FaeB have a pH optimum at slightly acidic pH (5.0 and 6.0, respectively) [18, 19, 45]. The temperature optimum of FaeC (50°C) is higher than that of AcFAE and ClFaeA1 (30°C and 45°C, respectively), but similar to FaeB [46, 47]. However, FaeA exhibits a somewhat higher temperature optimum (55-60°C; [18, 45]).

FaeC released ferulic acid from both wheat arabinoxylan and sugar beet pectin, and acted synergistically with xylanase to release ferulic and *p*-coumaric acids from wheat arabinoxylan and

wheat bran. Using sugar beet pectin as a substrate, FaeC did not show cooperative effect with endopolygalacturonase, rhamnogalacturonan hydrolase and rhamnogalacturonan acetyl esterase. This could be because the ferulic acid was attached to the side chains of sugar beet pectin, i.e. arabinan, galactan or type I arabinogalactan, and therefore it was sufficiently distant from the pectin backbone that cleaving the pectin backbone did not significantly change the specific part of pectin FaeC acts on. Debranching enzymes, e.g. endoarabinanase and endogalactanase, could be used to enhance the release of ferulic acid from this substrate as previously tested on FaeA [15]. Compared to the other characterized FAEs from *A. niger*, FaeC resembles FaeA more, which has a higher preference for wheat arabinoxylan over sugar beet pectin. In contrast, FaeB is more active towards sugar beet pectin [6, 19].

In the presence of two feruloylated polysaccharides, wheat bran and sugar beet pulp, *faeC* expression increased over time, whereas the expression of *faeA* and *faeB* gradually decreased after the first day of cultivation. The time-dependent expression pattern points to an intricate gene regulation mechanism in the process of substrate decomposition, affected by substrate presence as well as expression of other genes encoding plant cell wall polysaccharides degrading enzymes [4, 31, 48]. It is also possible that the low expression level of *faeC* and *faeB* was influenced by *faeA* which seemed to be the main FAE enzyme responsible for release of ferulic acid from wheat bran. The *faeA* gene is induced by both xylose through XlnR [31] and by ferulic acid. A possible explanation for the expression profiles is therefore that *faeA* is first induced by xylose and then results in the release of ferulic acid that induces the expression of *faeB* and *faeC*.

Investigation of the induction of *fae* genes in the presence of phenolic compounds revealed that, in contrast to expression profiles on polysaccharides, *faeB* seemed to be the highest expressed gene. All *fae* genes were induced by a broad range of compounds, but showed different response to diverse aromatic monomers. This is also corroborated by the induced *fae* expression at the early stage of the lignin supplemented cultivations. This study also revealed correlation between the

monomeric substrates of FaeA and FaeB and the compounds that induced the expression of their encoding genes [19]. In contrast with those results, the substrate specificity of FaeC included also compounds that did not induce *faeC* expression, such as 3,4-dimethoxycinnamic and sinapic acid. Since FaeC is active on bulky phenolic compounds such as methyl sinapate (with methoxy groups on R_1 and R_3 positions, Fig. 6) and methyl 3,4-dimethoxycinnamate (with methoxy groups on R_1 and R_2 positions), we speculate that the donor binding site (subsite -1) may be less specific toward the donor molecule. However, since FaeC can catalyze the hydrolysis of methyl caffeate but not chlorogenic acid (ester of caffeic acid and quinic acid), its acceptor binding site may be selective, which is different from FaeB that can hydrolyze both substrates. It should be noted that the absorbance spectrum of the released aromatic acids (product) also slightly overlapped with the ester substrates at 340 or 280 nm, which can affect the absolute activity measurement (Table A.2).

Based on our results, it is possible that FaeC may not be the main enzyme responsible for degradation of feruloylated substrates as its corresponding gene was lowly expressed in all tested conditions. However, it is also possible that the substrates that we used for the induction were not the target compounds for FaeC. To further investigate the specific role of FaeC in *A. niger*, construction of a double deletion mutant of *faeA* and *faeB* would be useful to avoid the influence by FaeA and FaeB.

Conclusions

The heterologously produced *A. niger* FaeC possesses a broad substrate specificity towards synthetic FAE substrate compounds. In addition, FaeC acts synergistically with xylanase to release ferulic acid from xylan. Novel FAE isoenzymes with different biochemical properties and substrate range have a high potential for applications in several industrial processes. FaeC showed promising potential particularly because of its broad substrate profile and the maximal activity at neutral pH.

The three *fae* genes from *A. niger* respond differently towards the feruloylated polysaccharides and monomeric phenolic compounds indicating that the FAE isoenzymes may target different substrates in a complementarily manner, contributing to the efficient degradation of diverse plant biomass.

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Appendix A. Supplementary data

Table A.1 Primers used for cloning and RT-qPCR. **Table A.2** The absorbance overlapped ratio between the ester substrates and the correspond acids at 340 nm (or 280 nm for methyl hydroxybenzoate). **Figure A.1** FaeA and FaeB production and the effect of pH and temperature on activity and stability towards methyl ferulate. A) SDS-PAGE of FaeA with (+H) and without (-H) endoglycosidase H treatment, and BSA standard. B) SDS-PAGE of FaeB with (+H) and without (-H) endoglycosidase H treatment, and BSA standard. C) pH-dependence for activity. D) pH-stability after 2 h incubation at 37°C. E) Temperature-dependence for activity. F) Thermal stability after 2 h incubation at pH 6. ●, FaeA; ○, FaeB. Each experiment was made in triplicate. Standard deviations are shown as error bars.

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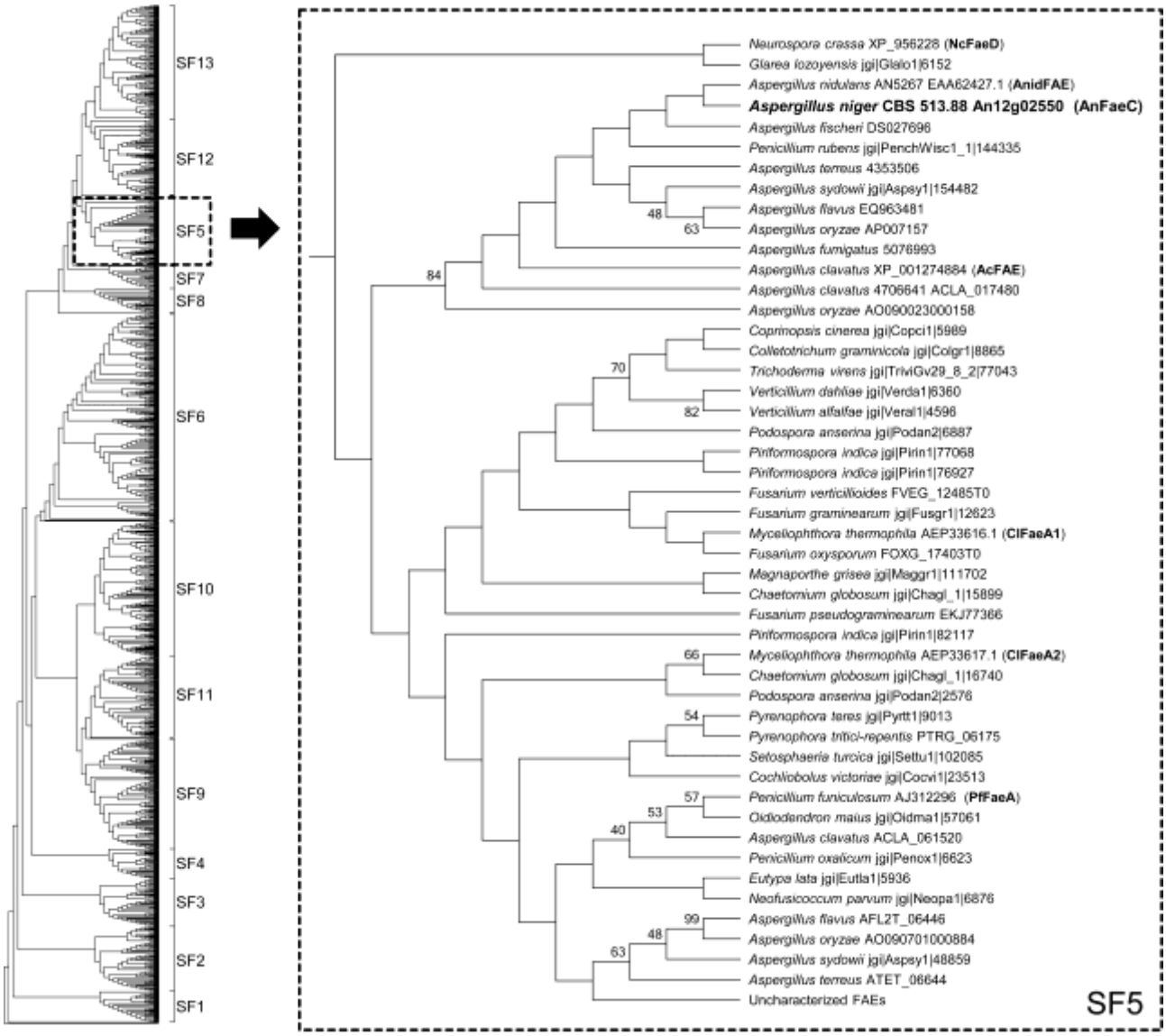


Figure 1 Phylogenetic relationships among the (putative) fungal FAEs from SF5 (modified from [5]). The characterized FAEs are shown in bold including FaeC (An12g02550) described in this paper. 'Uncharacterized FAEs' indicate multiple FAEs in these branches. Only the bootstrap above 40% were shown on the branches.

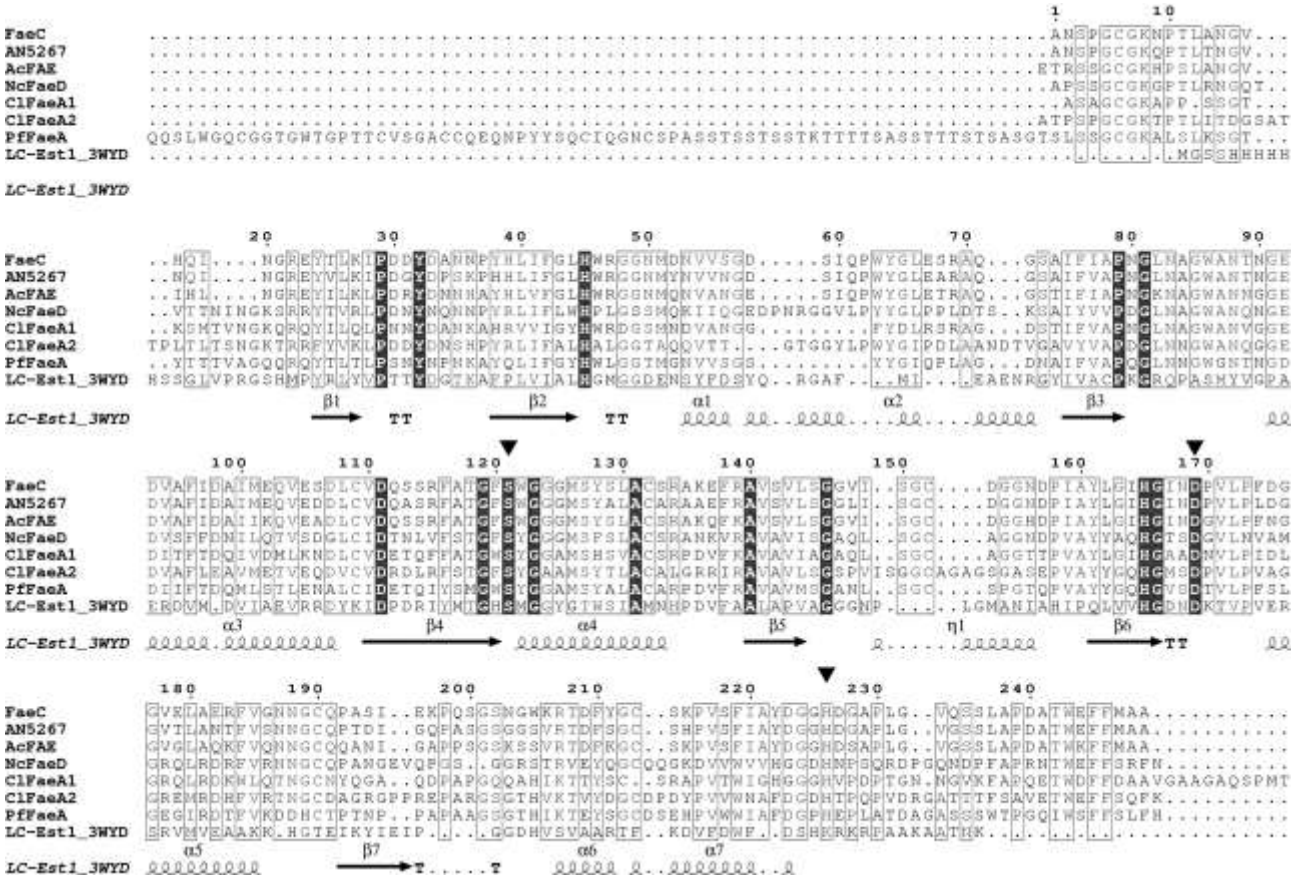


Figure 2 Amino acid sequence alignment of FaeC with related functionally characterized FAEs from SF5. *A. nidulans* AN5267 [40], *A. clavatus* AcFAE [41], *N. crassa* NcFaeD [2], *Myceliophthora thermophila* ClFaeA1, ClFaeA2 [42], metagenome-derived LC-Est1_3WYD [43]. Black triangles indicate putative catalytic residues (Ser121, Asp169, His226).

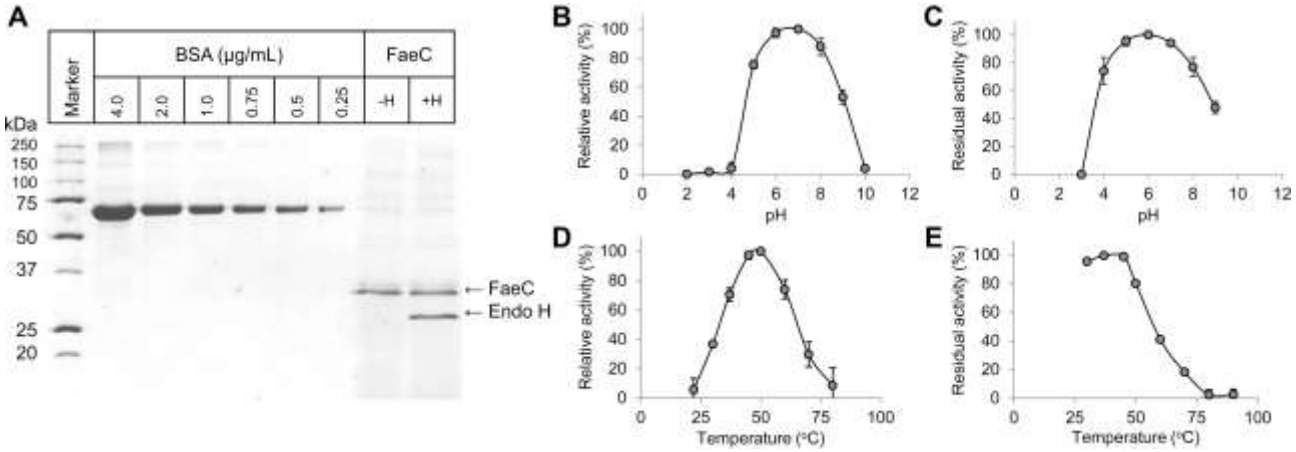


Figure 3 FaeC production and the effect of pH and temperature on activity and stability towards methyl ferulate. A) SDS-PAGE of FaeC with (+H) and without (-H) endoglycosidase H treatment, and BSA standard. B) pH-dependence for activity. C) pH-stability after 2 h incubation at 37°C. D) Temperature-dependence for activity. E) Thermal stability after 2 h incubation at pH 6. Each experiment was made in triplicate. Standard deviations are shown as error bars

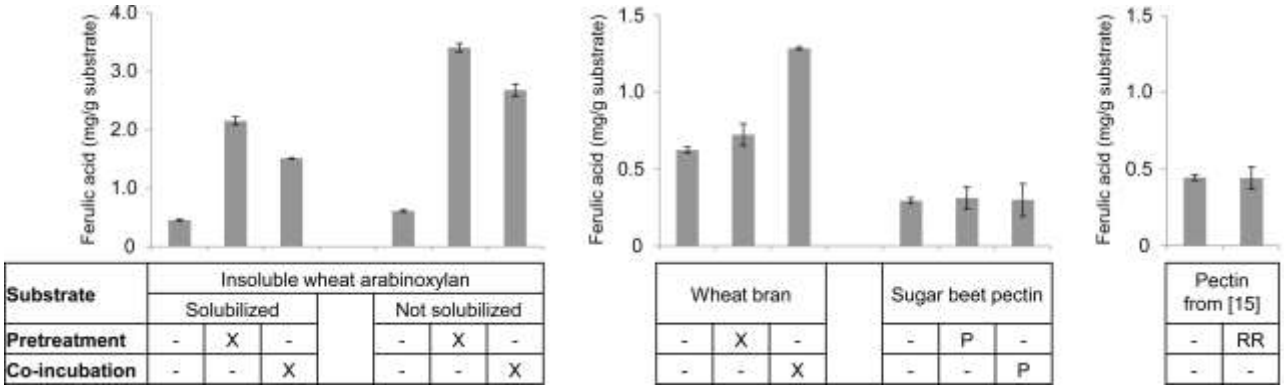


Figure 4 Release of ferulic acid from wheat arabinoxylan, wheat bran and sugar beet pectin using FaeC. The FaeC was used with pretreatment or co-incubation with xylanase (X) for wheat arabinoxylan and wheat bran, with endopolygalacturonase (P) for sugar beet pectin, and with rhamnogalacturonan hydrolase and rhamnogalacturonan acetylesterase (RR) for pectin from [15]. Each experiment was made in triplicate. Standard deviations are shown as error bars. The values were subtracted from the negative control.

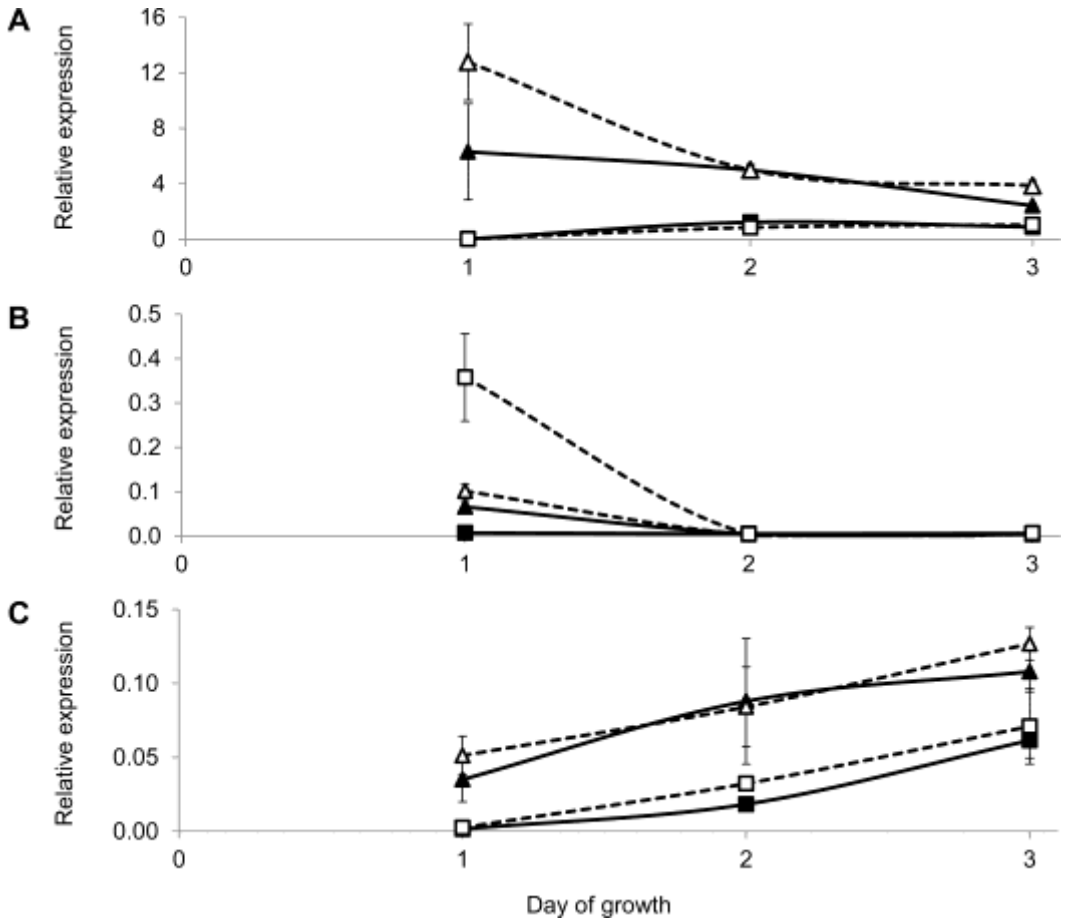
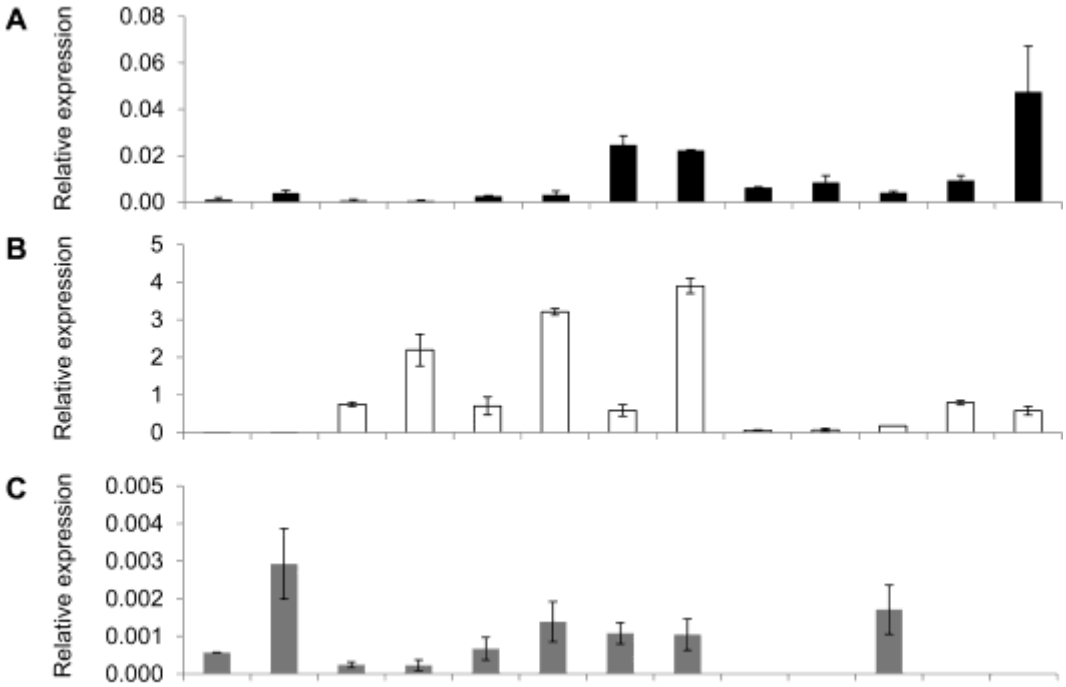
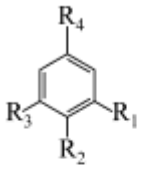


Figure 5 Relative expression patterns for the *fae* genes on feruloylated polysaccharides. A) *faeA*, B) *faeB*, C) *faeC*. The cultures were grown in minimal medium containing 1% wheat bran or 1% sugar beet pulp with or without calcium lignin supplementation in a course of three days, and the gene expression was quantitated using RT-qPCR. ▲, wheat bran; ■, sugar beet pulp; Δ, wheat bran with lignin; □, sugar beet pulp with lignin. Standard deviations from two biological and three technical replicates are shown as error bars.

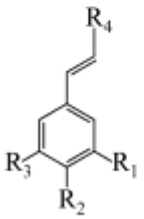


Compound	1	2	3	4	5	6	7	8	9	10	11	12	13
Structure	I	II	I	II	I	II	I	II	I	II	I	II	I

D



Structure I



Structure II

	Compound	Structure	R1	R2	R3	R4
1	Benzoic acid	I	H	H	H	COOH
2	Cinnamic acid	II	H	H	H	COOH
3	*p*-Hydroxybenzoic acid	I	H	OH	H	COOH
4	*p*-Coumaric acid	II	H	OH	H	COOH
5	Protocatechuic acid	I	OH	OH	H	COOH
6	Caffeic acid	II	OH	OH	H	COOH
7	Vanillic acid	I	OCH_3	OH	H	COOH
8	Ferulic acid	II	OCH_3	OH	H	COOH
9	Veratric acid	I	OCH_3	OCH_3	H	COOH
10	3,4-Dimethoxycinnamic acid	II	OCH_3	OCH_3	H	COOH
11	Syringic acid	I	OCH_3	OH	OCH_3	COOH
12	Sinapic acid	II	OCH_3	OH	OCH_3	COOH
13	3,4-Dimethylbenzyl alcohol	I	CH_3	CH_3	H	CH_2COOH

Figure 6 Relative expression patterns for the *fae* genes on monomeric phenolic compounds. A) *faeA*, B) *faeB*, C) *faeC*. The cultures were grown on monomeric phenolic compounds (D) for two hours and gene expression was quantified using RT-qPCR. Standard deviations from two biological and three technical replicates are shown as error bars.

Table 1. Substrate specificity of *A. niger* FAEs towards different synthetic substrates

Substrate	FaeA[a]	FaeB	FaeC
Chlorogenic acid	-	24%	-
Ethyl coumarate	-	36%	42%
Ethyl ferulate	13%	1%	88%
Methyl 3,4-dimethoxycinnamate	6%	0.1%	100%
Methyl caffeate	-	14%	34%
Methyl ferulate	55%	1%	74%
Methyl hydroxybenzoate	-	-	-
Methyl *p*-coumarate	-	100%	66%
Methyl sinapate	100%	-	41%

[a] The highest activity for each enzyme was set to 100% and used to compare relative activities.