J Ind Microbiol Biotechnol (2014) 41:1027–1034
DOI 10.1007/s10295-014-1430-7

SIMB
Society for Industrial Microbiology and Biotechnology

Overexpression of *Aspergillus tubingensis faeA* in protease-deficient *Aspergillus niger* enables ferulic acid production from plant material

Eunice N. Zwane · Shaunita H. Rose · Willem H. van Zyl · Karl Rumbold · Marinda Viljoen-Bloom

Received: 11 December 2013 / Accepted: 25 February 2014 / Published online: 25 March 2014

Abstract The production of ferulic acid esterase involved in the release of ferulic acid side groups from xylan was investigated in strains of *Aspergillus tubingensis*, *Aspergillus carneus*, *Aspergillus niger* and *Rhizopus oryzae*. The highest activity on triticale bran as sole carbon source was observed with the *A. tubingensis* T8.4 strain, which produced a type A ferulic acid esterase active against methyl *p*-coumarate, methyl ferulate and methyl sinapate. The activity of the *A. tubingensis* ferulic acid esterase (*At*FAEA) was inhibited twofold by glucose and induced twofold in the presence of maize bran. An initial accumulation of endoglucanase was followed by the production of endoxylanase, suggesting a combined action with ferulic acid esterase on maize bran. A genomic copy of the *A. tubingensis faeA* gene was cloned and expressed in *A. niger* D15#26 under the control of the *A. niger gpd* promoter. The recombinant strain has reduced protease activity and does not acidify the media, therefore promoting high-level expression of recombinant enzymes. It produced 13.5 U/ml FAEA after 5 days on autoclaved maize bran as sole carbon source, which was threefold higher than for the *A. tubingensis* donor strain. The recombinant *At*FAEA was able to extract 50 % of the available ferulic acid from non-pretreated maize bran, making this enzyme suitable for the biological production of ferulic acid from lignocellulosic plant material.

Keywords *Aspergillus tubingensis* · Ferulic acid esterase · *Aspergillus niger* · Ferulic acid production

E. N. Zwane · S. H. Rose · W. H. van Zyl · M. Viljoen-Bloom (✉)
Department of Microbiology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa
e-mail: mv4@sun.ac.za

K. Rumbold
School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, Private Bag X3, Johannesburg 2050, South Africa

Introduction

With the increased demand for fuel ethanol and the predicted decline in global crude oil reserves, lignocellulosic biomass currently offers the most sustainable alternative source of transport fuel. However, large-scale commercial cellulosic biofuel production is still unable to compete effectively with fossil fuels on a cost basis and has therefore not yet made a significant breakthrough in the market [4]. A biorefinery concept is considered the most promising to reduce the production cost of cellulosic biofuels [8] owing to value-addition through a range of valuable products in addition to bioethanol (similar to a petroleum refinery).

Lignocellulosic plant material consists of cellulose, hemicellulose and lignin, as well as a range of other compounds, such as phenolics (ferulic acid, syringic acid and *p*-coumaric acid) that are either esterified to α-L-arabinose moieties in arabinoxylans or ether-linked to lignin. This complex interaction is important to protect the structural integrity of the plant cell wall against enzymatic degradation. Knowledge of the type of phenolic ester bonds present in a given feedstock is thus important to inform the choice of hydrolytic enzymes and the general strategy for the production of high-value commodities from a specific feedstock.

Ferulic acid is the major phenolic acid associated with lignocellulosic materials that are released during

pretreatment of biomass [11, 17, 30]. Ferulic acid is best known for its antioxidant properties due to its radical scavenging ability and is widely used as a food preservative and active ingredient in skin lotions and sunscreens [16]. Ferulic acid can also be converted enzymatically to various value-added products, e.g. vinyl guaiacol and vanillin, which are well-known flavouring agents in foods, beverages and perfumes [17]. However, the commercial application of ferulic acid has been limited by its availability and high production cost [16].

Although plant cell walls contain a significant amount of ferulic acid, the complex binding of ferulic acid to hemicellulose and lignin renders it difficult to extract. To date, the commercial production of natural ferulic acid has mainly been from the chemical extraction of ferulic acid from rice bran oil using food grade *n*-hexane, but this poses potential health and environmental hazards [13, 26]. Although alkaline hydrolysis releases the total ferulic acid content from the plant cell wall, purification of ferulic acid from the hydrolysate has also proven difficult because of the presence of many other components. To benefit from ferulic acid production within a biorefinery concept, efficient technologies will therefore be required to enable ferulic acid extraction prior to processing of the lignocellulose. A more natural and specific way of extracting ferulic acid from lignocellulosic material would be based on hydrolytic enzymes that can specifically release ferulic acid from its bound constituents without any adverse side reactions [13].

There is growing interest in ferulic acid esterases (also known as feruloyl esterase) as key enzymes for cell wall hydrolysis and in particular the extraction of phenolic acids from agricultural crops, as they are able to hydrolyse the bond between arabinose and ferulic acid, releasing the covalently bound lignin from hemicellulose. Extraction of ferulic acid also releases other carbohydrates that may be useful for fermentation processes, including bioethanol production. Ferulic acid esterases (FAEs) are classified as type A, B, C or D depending on their substrate specificity. Type A FAEs tend to be induced during growth on cereal-derived substrates, and are active against methyl ferulate (MFA), methyl sinapate (MSA) and methyl *p*-coumarate (MpCA), but not methyl caffeate (MCA). Type B FAEs are active against methyl ferulate, methyl caffeate and methyl *p*-coumarate, but not methyl sinapate. Types C and D are able to act on all four hydroxycinnamic acid methyl esters, but only type D enzymes are able to hydrolyse ferulic acid dimers [13].

A number of recombinant organisms have been developed for the production of FAE, in particular those from *Aspergillus* species. Expression of the *Aspergillus niger* ferulic acid esterase gene in *Saccharomyces cerevisiae* yielded 2 mg/l protein with a specific activity of 8.2 U/μg [38], whereas expression of the *A. niger* cinnamoyl esterase yielded 300 mg/l in *Pichia pastoris* [20] and 1 g/l in *A. niger* [29]. Production of the *A. tubingensis* ferulic acid esterase A (*At*FAEA) in *A. vadensis* was significantly higher than in the donor *A. tubingensis* strain owing to the low proteolytic activity of *A. vadensis* [9]. Furthermore, expression of only the catalytic domain of the *Piromyces equi* cinnamoyl esterase (EstA) in *Trichoderma reesei* yielded 33 mg/l of ferulic acid [13]. The *faeA* genes from *A. niger* and *A. tubingensis* were cloned and overexpressed in *A. niger* and *A. tubingensis* strains [10], with expression of the *A. niger faeA* gene in *A. tubingensis* (3.16 U/ml) yielding the best results. It was concluded that the *At*FAEA was more sensitive to the acid proteases in *A. niger*, as overexpression in an *A. niger* protease-deficient strain yielded 7.71 and 0.97 μM/min for the respective *A. niger* and *A. tubingensis* enzymes.

The application of FAE is currently not considered a practical way of producing commercial ferulic acid in view of the high costs associated with microbial enzyme production and the long reaction time required to release the bound ferulic acid [17]. It has therefore become important to find and/or develop strains that will be able to produce FAE at high levels to ensure the cost-effective extraction of ferulic acid from plant material. The objective of this study was to identify an FAE gene for heterologous expression in *A. niger* D15#26, a protease-deficient strain that does not acidify the growth media, which is desirable for the large-scale production of recombinant FAEs. Four fungal strains, namely *Rhizopus oryzae*, *A. carneus*, *A. nidulans* and *A. tubingensis*, were evaluated for FAE activity, with *R. oryzae* and *A. tubingensis* producing higher levels of FAE than the other two strains. The *A. tubingensis* FAE released the highest levels of ferulic acid from triticale and the *A. tubingensis faeA* gene was therefore selected for cloning and overexpression in *A. niger*.

Materials and methods

Strains and chemicals

The fungal strains *Rhizopus oryzae* MP1, *Aspergillus carneus* ABO374, *Aspergillus nidulans* IFO4342, *Aspergillus tubingensis* T8.4 (PPRI 13401, hereafter referred to as *A. tubingensis*) and *A. niger* D15#26 (*cspA1*, *pyrG1*, *prtT13*, *phmA*, a non-acidifying mutant of AB1.13, ATCC 9029 [15], hereafter referred to as *A. niger* D15) were obtained from the culture collection at the Department of Microbiology, Stellenbosch University, South Africa. Unless stated otherwise, all chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Culturing conditions

Fungal strains were cultivated in minimal media containing 5 g/l yeast extract (Difco), 0.4 g/l $MgSO_4.7H_2O$, 2 g/l casamino acids, 20 ml 50× AspA (300 g/l $NaNO_3$, 26 g/l KCl, 76 g/l KH_2PO_4, pH 6), 1 ml/l of 1,000× trace elements [31], as well as 10 g/l glucose (only where specified) and 0.01 M uridine for cultivation of *A. niger* D15. *Escherichia coli* DH5α was cultured at 37 °C in Luria broth [34] with 100 μg/ml ampicillin added for the selection of transformants.

Triticale (Department Process Engineering, Stellenbosch University) was pretreated (steam exploded, 205 °C, 15 min, 25 bar) and used at 10 g/l as lignocellulosic substrate for quantification of enzyme activities in the native strains, whereas 10 g/l untreated maize bran (Sasko, Paarl, South Africa) was used for characterisation of the recombinant enzymes. Optimisation of FAE expression was done in minimal media without glucose with 10 g/l maize bran prepared at different pH values (pH 4–8) and with different autoclaving periods (30, 60 or 90 min). The growth media was inoculated with 1×10^6 spores/ml and incubated on a rotary shaker at 125 rpm at 30 °C. The cultures were checked daily by microscopy to ensure that there was no bacterial contamination. Following incubation, samples were centrifuged at 8,000 rpm at 4 °C for 15 min and the supernatant used for further analysis.

Enzyme assays

Fungal strains were cultivated in 50 ml minimal media with either 10 g/l triticale or 10 g/l maize bran in Erlenmeyer flasks for 5 days (10 g/l glucose was added where indicated). The 4-nitrophenyl ferulate (4NPF) substrate was synthesized and purified as described by Hedge et al. [18]. The reaction mixture contained nine volumes of 0.1 M potassium phosphate buffer solution (pH 6.5), 2.5 % Triton X-100 and one volume of 11 mM 4NPF in DMSO, followed by immediate vortexing. Enzyme assays consisted of 0.1 ml supernatant and 2 ml 4NPF solution incubated at 37 °C for 1 h, with the release of 4NP measured spectrophotometrically at 410 nm (xMark™ Microplate Spectrophotometer, Bio-Rad, San Francisco, USA). One unit (1 U) of FAE activity was defined as the amount of enzyme that released 1 μmol of 4NP from 4NPF in 1 min.

Ferulic acid was quantified by HPLC analysis [23] on a Nucleosil C18 column (5 μM particle size, Supelco, Bellefonte, USA) with 70 % acetonitrile as mobile phase at a flow rate of 1 ml/min at room temperature, using analytical grade ferulic acid (Sigma-Aldrich, UK) as reference and a Surveyor Plus UV/VIS detector (Thermo Electron Corporation, Elandsfontein, South Africa) at 320 nm. Ferulic acid extraction was expressed as a percentage relative to the total available ferulic acid extracted by chemical means. For the latter, maize bran and triticale (100 mg) were suspended in 1 M NaOH (2 ml) at 55 °C for 5 h. The soluble fraction was then centrifuged (3,600×*g*, 15 min) and acidified to pH 2 with HCl for HPLC analysis [1].

Table 1 Substrate specificity of ferulic acid esterase produced by *A. tubingensis* and *R. oryzae*

Substrate	Abbreviation	Enzyme activity (U/ml)	
		A. tubingensis	*R. oryzae*
Methyl sinapate	MSA	0.2 (±0.21)	1.04 (±0.63)
Methyl caffeate	MCA	0	0.2 (±0.55)
Methyl *p*-coumarate	MpCA	1.5 (±0.42)	0.3 (±0.18)
Methyl ferulate	MFA	1.1 (±0.11)	1.6 (±0.33)

Endoxylanase and endoglucanase activities were quantified with the reducing sugar assay [2] using 10 g/l birchwood xylan and carboxymethyl cellulose (CMC) in 0.05 M sodium citrate buffer (pH 5) as respective substrates. One unit (1 U) was defined as the amount of enzyme releasing 1 μmol of reducing sugar per minute after incubation at 50 °C for 30 min [25]. Total protein concentrations were quantified with the Qubit assay and Qubit 2.0 fluorometer (Invitrogen, Carlsbad, USA) as per the supplier's specification.

Substrate specificity assays were performed with 20 μl supernatant from triticale-induced cultures and 40 μl substrate solutions of 1 mg/ml hydroxycinnamic substrates (Table 1) prepared in MOPS buffer, pH 6. The decline in spectrophotometric absorbance at 335 nm was monitored with the sample compartment kept at 37 °C. One unit of FAE activity (1 U) is defined as the amount of enzyme releasing 1 μmol of free phenolic acid per minute under these conditions [24, 29].

Cloning of *A. tubingensis* ferulic acid esterase gene and expression in *A. niger*

The *AtfaeA* open reading frame (GenBank Y09331) was amplified from *A. tubingensis* genomic DNA using gene-specific primers (AtfaeA-left: 5′–ATGAA GCAATTCTCCG CAAAATACGCCATCGC-3′; Atfae A-right: 5′-CTTACCATGTACAATGTCCGCTCGTC ATC C–3′) and TaKaRaExTaq polymerase (Takara Bio Inc, Otsu, Japan). The open reading frame (ORF) was cloned as a blunt-end fragment into the blunted *Not*I site of the *A. niger* expression vector pGTP, generating pGTP-AtfaeA. Electro-competent *E. coli* DH5α cells [34] were transformed with recombinant plasmids and transformants were selected on LB-Amp agar plates. The DNA sequence of the cloned fragment was verified using the ABI PRISM™ 3100 Genetic Analyzer. Cells of *A. niger* D15 [31] were

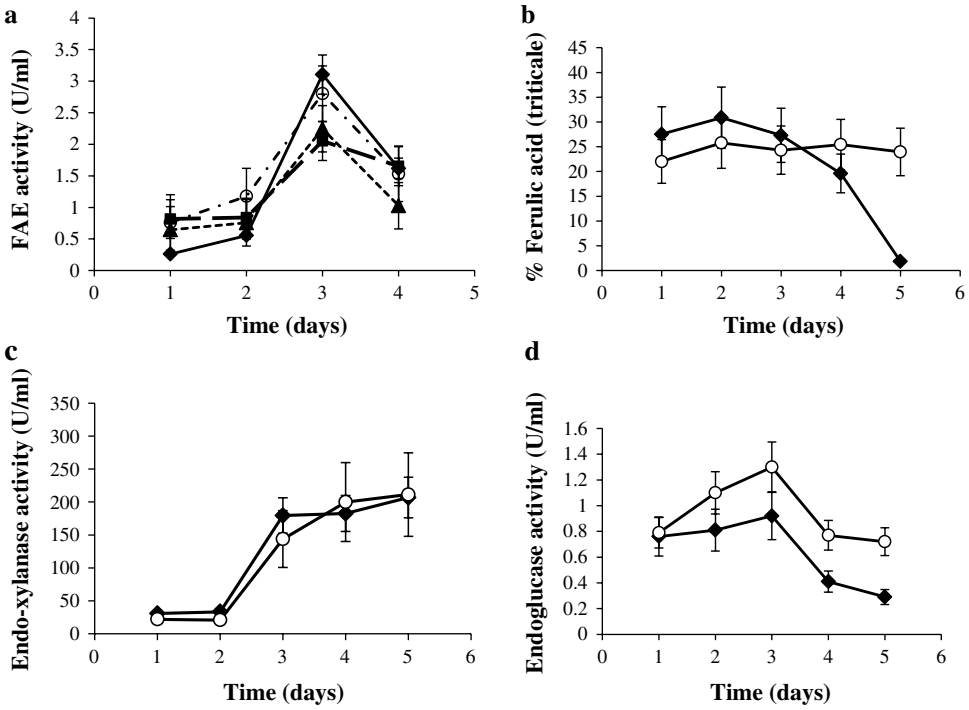

Fig. 1 **a** Extracellular FAE activity, **b** ferulic acid accumulation (as a percentage of the available ferulic acid extracted by 1 M NaOH), **c** endoxylanase and **d** endoglucanase activity displayed by *A. tubingensis* (*filled diamonds*), *R. oryzae* (*open circles*), *A. carneus* (*closed triangle*) and *A. nidulans* (*closed square*) in minimal media containing 10 g/l triticale

transformed with plasmids pGTP and pGTP-*AtfaeA* and transformants were selected on minimal medium plates lacking uridine. Seventy transformants were selected after three purification cycles and screened in minimal medium with maize bran for FAE production.

Protein analysis

SDS-PAGE was used to separate the denatured protein samples, followed by renaturing of the proteins for 4 h at 4 °C using 0.1 M sodium phosphate buffer (pH 6.5). The buffer was replaced with freshly prepared 0.1 M sodium phosphate buffer (pH 6.5) containing 25 µl of a 20 mg/ml Fast Blue solution (Sigma-Aldrich, South Africa). The addition of 50 µl α-naphthyl acetate (1.2 mg/ml acetone) would yield a purple-coloured band in the presence of esterase activity.

Results

Partial characterisation of native enzymes

After culturing for 3 days in the presence of triticale and glucose, the *A. tubingensis*, *R. oryzae*, *A. carneus* and *A. nidulans* strains respectively yielded 3.1, 2.8, 2.25 and 2.05 U/ml of extracellular FAE (Fig. 1a). The release of ferulic acid was evaluated in the two best-performing strains, with ca. 31 and 26 % of the ferulic acid extracted (relative to NaOH treatment) by *A. tubingensis* and *R. oryzae* after 2 days, respectively (Fig. 1b). However, the *A. tubingensis* strain utilised most of the released ferulic acid by day 5.

In minimal media with triticale and glucose, the endoxylanase activity in the *A. tubingensis* and *R. oryzae* supernatant reached 206 and 211 U/ml on day 5 (Fig. 1c). The endoglucanase activity was much lower in both the *A. tubingensis* and *R. oryzae* supernatants, reaching only 0.9 and 1.3 U/ml on day 3, respectively (Fig. 1d).

The hydrolysis efficiency patterns observed for the methyl ester substrates were MpCA > MFA > MSA for *A. tubingensis*, and MFA > MSA > MpCA > MCA for *R. oryzae* (Table 1). This indicated that *A. tubingensis* produced a type A FAE that could hydrolyse C-5 feruloylated substrates, whereas the *R. oryzae* strain produced a type C or D ferulic acid esterase able to hydrolyse C-5 and C-2 feruloylated substrates.

Cloning and expression of *A. tubingensis faeA* in *A. niger*

Since the *A. tubingensis* strain displayed the highest FAE activity among the four fungal strains, it was selected for recombinant expression of its FAE in *A. niger* strain D15#26, a protease-deficient strain that does not acidify the medium [15]. Amplification of the genomic copy of *AtfaeA* generated a 931-bp fragment, which correlated with the published *AtfaeA* ORF [10]. The fragment was subcloned into plasmid pGTP under control of the constitutive *A. niger gpd* promoter [31] and 70 *A. niger* D15 transformants were evaluated in liquid cultures. The *A. niger* D15[pGTP] strain, containing the empty vector, served as

negative control. The *A. niger* D15[*AtfaeA*] transformant that displayed the highest activity in liquid cultures (data not shown) was selected for further study.

Production and partial characterisation of recombinant *At*FAEA

Maize bran contains more bound ferulic acid than triticale (31.28 versus 2.29 mg/g as determined by alkali extraction) [35] and was therefore used in subsequent experiments to ensure detectable levels of ferulic acid. The wild-type *A. tubingensis* and *A. niger* strains displayed similar levels of FAE activity on minimal medium containing 10 g/l glucose, with significant induction of both in the presence of 10 g/l maize bran (Fig. 2a). Under uninduced conditions (i.e. absence of maize bran), the *A. niger* D15[*AtfaeA*] strain displayed more than fivefold higher FAE activity than the native *A. tubingensis* and *A. niger* D15[pGTP] strains, i.e. 5.89 versus 1.07 and 0.97 U/ml FAE, respectively. No induced expression was observed for *A. niger* D15[*AtfaeA*] as the constitutive *A. niger gpd* promoter was used for expression.

Extracellular FAE activity peaked at 5.5 U on day 4 for the wild-type *A. tubingensis* and at 13.5 U on day 5 for *A. niger* D15[*AtfaeA*] (Fig. 2b), with a significant decline in activity for *A. tubingensis* towards day 6, probably due to cell lysis. After 5 days, *A. niger* D15[*AtfaeA*] was able to extract 50 % of the available ferulic acid in untreated maize bran (Fig. 2c). Although there was some ferulic acid accumulation after 3 days, the *A. tubingensis* strain quickly consumed or degraded this to leave no residual ferulic acid by day 5. A similar trend was observed when the native *A.*

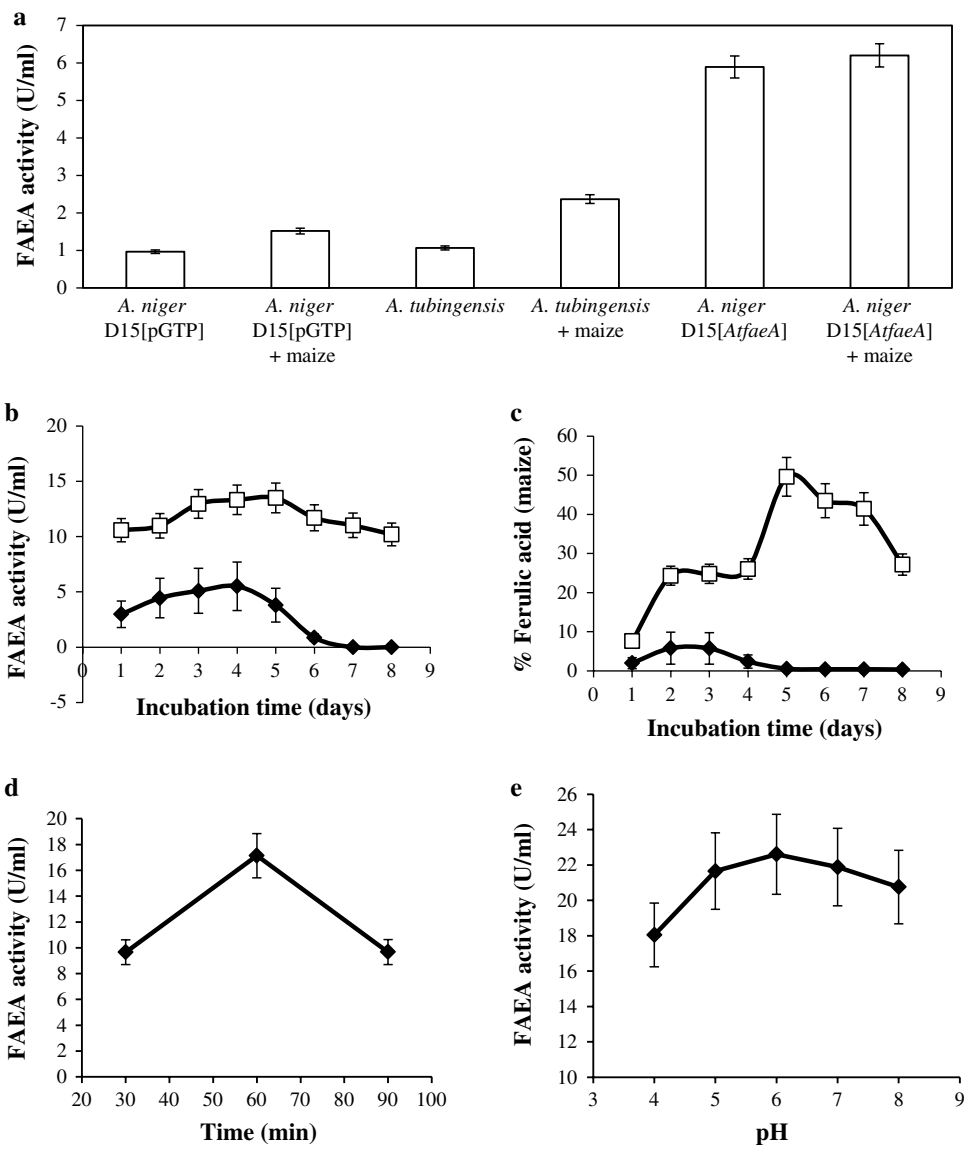

Fig. 2 **a** Extracellular FAE activity produced by *A. tubingensis*, *A. niger* D15[pGTP] and *A. niger* D15[*AtfaeA*] after 5 days of cultivation in minimal media containing 10 g/l glucose with or without 10 g/l maize bran. **b** FAE activity and **c** ferulic acid accumulation (as a percentage of available ferulic acid extracted by 1 M NaOH) by *A. tubingensis* (*filled diamonds*) and *A. niger* D15[*AtfaeA*] (*open squares*) grown in minimal media with 10 g/l maize bran. The effect of **d** autoclaving and **e** pH of the growth medium on ferulic acid esterase production by *A. niger* D15[*AtfaeA*] when grown in minimal media with 10 g/l maize bran for 5 days

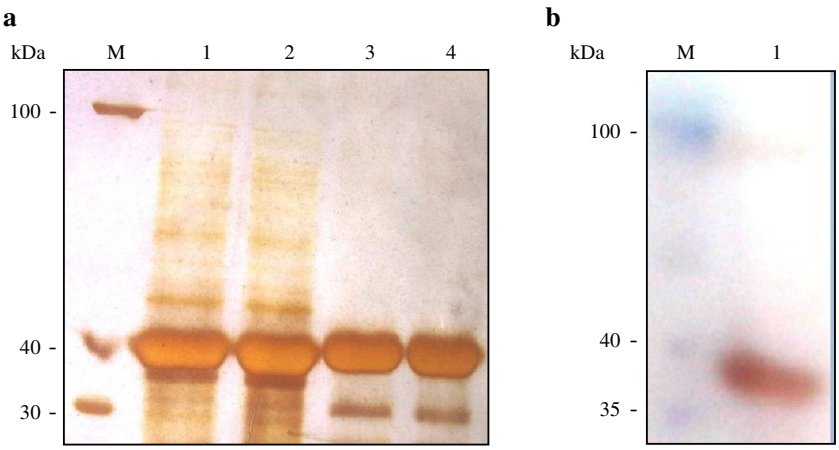

Fig. 3 **a** SDS-PAGE analysis of the extracellular FAE obtained from *A. niger* D15[*AtfaeA*]. Lane *M* molecular weight marker; extracellular proteins (sampled in duplicate) produced in minimal media with maize bran (lanes *1* and *2*) or maize bran plus glucose (lanes *3* and *4*). **b** Zymogram of the extracellular proteins produced by *A. niger* D15[*AtfaeA*] in minimal media with maize bran (lane *1*). Lane *M* prestained molecular weight marker

tubingensis was grown on triticale (Fig. 1b), with very little ferulic acid detected on day 5.

Pretreatment of maize bran by means of autoclaving for 60 min (Fig. 2d) increased FAE activity in *A. niger* D15[*AtfaeA*] almost twofold relative to 30 and 90 min. Although an initial pH of 6 for the culture medium yielded the highest FAE activity (22.6 U/ml) for *A. niger* D15[*AtfaeA*], this was not significantly higher than for the other pH values (Fig. 2e).

Following SDS-PAGE analysis and renaturation of the recombinant *At*FAEA, a zymogram with α-naphthyl acetate revealed a purple halo (confirming FAE activity) that corresponded to a molecular weight of 36 kDa (Fig. 3).

Discussion

The production of ferulic acid esterase by *Aspergillus* strains (*A. nidulans*, *A. carneus* and *A. tubingensis*) is well documented [6, 13]. Previous reports indicated that the soft-rot fungus *R. oryzae* produce significant levels of laccase, endoglucanase and endoxylanase activity [27], suggesting that it should be able to also produce ferulic acid esterases. The four strains evaluated in this study produced 2 to 3 U/ml FAE in liquid minimal medium with triticale, with *A. tubingensis* and *R. oryzae* being able to extract ca. 31 and 26 % of the available ferulic acid after 2 days. Note that these percentages should be treated with caution as alkaline treatment releases both ester and ether-bound ferulic acid, whereas enzymatic extraction only releases ester-bound ferulic acid.

Different types of FAEs have been identified on the basis of their substrate specificity, which is determined by the substitutions on the aromatic ring that can be accommodated by the respective enzymes [39]. The *A. tubingensis* strain was able to hydrolyse all the methyl esters evaluated in this study, except for MCA, indicating that it is a type A FAE, similar to those reported for *A. niger*, *R. oryzae* and *A. awamori* [14, 33]. The *R. oryzae* strain was able to hydrolyse all the methyl esters, similar to the type C or D FAE reported for *Clostridium stercorarium* [36]. The substrate preferences of *A. tubingensis* (MpCA > MFA > MSA) suggested that *At*FAEA would only be able to release ferulic acid from monocots (wheat, oats, maize and barley), as ferulic acid is esterified to the C-5 hydroxyl group of some arabinopyranose residues of arabinoxylans in monocots. In contrast, the *R. oryzae* strain (MFA > MSA > MpCA > MCA) should be able to release ferulic acid from both monocots and dicots, e.g. sugar beet, in which the ferulic acid is esterified to the C-2 hydroxyl group of arabinofuranose.

Access to cellulose is hampered by the complexity of the lignocellulose structure; fungal strains therefore typically produce an array of hydrolytic enzymes, including endoglucanases and endoxylanases when grown on a complex lignocellulosic substrate [4]. In a study by Kumar and Wyman [22], the addition of xylanases to an enzyme cocktail improved hydrolysis and released glucose and xylose in a linear fashion. With triticale as substrate, the endoglucanase and endoxylanase activities of *A. tubingensis* and *R. oryzae* were comparable to other endoglucanase producers [21]. The endoxylanase activity displayed by *A. tubingensis* and *R. oryzae* also correlated with other reports on endoxylanase-producing fungi such as *Penicillium canescens*, *Trichoderma viride* and *Aspergillus tamarri* grown on different carbon sources [3]. The levels of endoglucanase (Fig. 1d) and ferulic acid esterase (Fig. 1a) in both strains peaked on day 3 and then declined, whereas the endoxylanase activities continued to increase up to day 5 (Fig. 1c). The co-production of the ferulic acid esterase, endoxylanase and endoglucanase supports previous reports on the cooperation between these lignocellulose-degrading enzymes [37].

Since the *A. tubingensis* strain showed the highest FAE activity and release of ferulic acid from 10 g/l triticale, its ferulic acid esterase gene (*AtfaeA*) was cloned and successfully expressed in *A. niger* D15. The FAE activity of the recombinant enzyme reached 13.5 U/ml on day 5, which

was threefold higher than for the *A. tubingensis* donor strain (Fig. 2b). The *At*FAEA was produced as a significant portion (estimated at 20 mg/l) of the total extracellular proteins secreted by *A. niger*, which would simplify downstream processing and purification of the recombinant protein.

Induction of FAE is often observed when fungi are grown on complex substrates, most likely exerted by specific components of the hemicellulosic material [12, 24]. A twofold induction of the native FAE activity was observed in *A. tubingensis* after 5 days when 10 g/l maize bran was added as an inducer to glucose-containing minimal medium (Fig. 2a). Both *A. niger* D15[pGTP] and *A. niger* D15[*AtfaeA*] strains displayed an increase of about 0.5 U/ml in FAE activity in the presence of maize bran relative to the absence thereof (Fig. 2a), suggesting that the native *A. niger* FAE was subject to mild substrate induction. However, FAE activity in *A. niger* D15[*AtfaeA*] was twofold higher in minimal medium supplemented with maize bran than in glucose-containing medium (Fig. 2b), indicating glucose repression of the native *A. niger* FAE.

The available carbon source influences FAE production and activity, and some fungal strains produce more than one type of FAE, which can contribute to the total FAE activity measured under a specific set of conditions [24]. The total FAE activity observed for *A. niger* D15[*AtfaeA*] exceeded those previously reported for type A FAE on maize bran [5, 13, 24], suggesting that the *A. tubingensis* FAE performs particularly well in *A. niger* D15. In addition, using the *A. niger* protease-deficient strain and a constitutive promoter resulted in a significant increase in extracellular enzyme levels with no significant degradation, in contrast to FAE expression systems previously reported [10].

HPLC analysis indicated that 50 % of the available ferulic acid was extracted from untreated maize bran by *A. niger* D15[*AtfaeA*] at day 5, i.e. 10-fold more than the native *A. tubingensis* strain's maximum at day 3 (Fig. 2c). The amount of ferulic acid released was significantly higher than the 40 % reported for a native *A. niger* FAE on autoclaved maize bran [7, 28], 30 % for the *Thermoanaerobacter tengcongensis* thermostable FAE on triticale bran [1], and the 7.5 % for expression of the *P. equi* cinnamoyl esterase (*estA*) gene in *Trichoderma reesei* Rut C30 [28].

Release of ferulic acid from maize bran was further enhanced through autoclaving, with treatment of 60 min at 121 °C and pH 6 yielding the best results. Autoclaving of maize bran improves the solubilisation of feruloylated oligosaccharides [7], whilst an optimum of pH 6 corresponds to previous reports on the optimal performance of the *A. niger* D15 host strain [32]. The native *A. tubingensis* strain was able to extract 30 % of the available ferulic acid from steam-exploded triticale (Fig. 1a), but only 5 % from autoclaved maize bran (Fig. 2c). This can be expected as autoclaving is a milder form of pretreatment and maize bran has a more branched structure, which is more recalcitrant than triticale [36]. However, there was a significant decrease in free ferulic acid during prolonged cultivation (Figs. 1a, 2c), which could be ascribed to oxidative coupling of ferulic acid to form larger and more complex molecules, or the metabolism of ferulic acid to a variety of products, such as vanillin [17, 19, 30].

A mixture of extracellular enzymes is required to work synergistically with FAE to liberate ferulic acid from lignocellulosic material, which can induce the expression of the relevant enzymes. On 10 g/l triticale supplemented with glucose, a twofold induction of the native *A. tubingensis* FAE was observed, together with a significant increase in endoglucanase and endoxylanase activities (Fig. 1). Furthermore, the recombinant *A. niger* D15[*AtfaeA*] strain was an effective producer of FAE when compared to the native *A. tubingensis* and *A. niger* strains. *Aspergillus* species tend to acidify the medium and a drop in pH is difficult to control even in automatized fermenters. In the study by de Vries et al. [10], lower levels of ferulic acid esterase activity were obtained because the *A. tubingensis* FAE is highly sensitive to acid proteases. A low pH also triggers the production of extracellular acid proteases, which in turn leads to the degradation of heterologous proteins. The *A. niger* D15 strain used is this study does not acidify the medium, thus representing a better host for recombinant expression of *At*FAEA [31, 32].

We were able to produce extracellular *At*FAE at high levels in an *A. niger* host that is known for its reduced protease activity and that does not acidify the media. The extracellular enzyme cocktail from the recombinant strain extracted 50 % of the available ferulic acid from non-pretreated maize bran—a very recalcitrant substrate—without a pretreatment step. This extraction efficiency was higher than some other feruloyl esterases reported in the literature after a pretreatment step. This confirms that enzymatic ferulic acid extraction is a feasible alternative to chemical processes, but the enzyme production system and conditions need to be further optimised to increase enzyme production by the recombinant *A. niger* D15[*AtfaeA*] strain.

Acknowledgments The authors would like to thank the South African National Energy Research Institute (SANERI), the Department of Science and Technology (DST) and the National Research Foundation (NRF) for financial support. This work is based on the research supported in part by the National Research Foundation of South Africa (Grant 76597).

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