Enrichment of maize and triticale bran with recombinant *Aspergillus tubingensis* ferulic acid esterase

Eunice N. Zwane[1], Petrus J. van Zyl[2], Kwaku G. Duodu[3], Shaunita H. Rose[1], Karl Rumbold[4], Willem H. van Zyl[1], Marinda Viljoen-Bloom[1*]

[1] Department of Microbiology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

[2] CSIR Biosciences, PO Box 395, Pretoria, 0001, South Africa

[3] Department of Food Science, University of Pretoria, Private Bag X20, Hatfield, Pretoria 0028, South Africa

[4] School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, Private Bag X3, WITS, 2050, South Africa

*Corresponding author: Prof. M. Viljoen-Bloom: mv4@sun.ac.za; Tel.: +27-21-8085859; Fax: +27-21-8085846

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ENZ was the principal researcher responsible for experimental planning, execution, data collection and analyses, as well as drafting the manuscript. PJvZ assisted with the fermentation protocol; KGD with bran treatments and chemical analyses; SHR and KR with enzyme characterisation. WHvZ provided intellectual input together with MVB who prepared the final manuscript with all authors participating in the final editing.

Research highlights

- *A. tubingensis* FAEA enhanced ferulic acid extraction from maize and triticale bran
- Purified *At*FAEA displayed specific activity of 3 487 U/mg on methyl ferulate
- *At*FAEA production in fed-batch fermentation was superior to batch fermentation
- Fed-batch fermentation yielded 185.14 U/ml FAE activity
- FAE activity peaked at 50°C (stable at 30°C to 60°C) and pH 6 (stable at pH 5 to 7)

Abstract

Ferulic acid is a natural antioxidant found in various plants and serves as a precursor for various fine chemicals, including the flavouring agent vanillin. However, expensive extraction methods have limited the commercial application of ferulic acid, in particular for the enrichment of food substrates. A recombinant *Aspergillus tubingensis* ferulic acid esterase Type A (FAEA) was expressed in *Aspergillus niger* D15#26 and purified with anion-exchange chromatography (3 487 U/mg, K_m = 0.43 mM, K_{cat} = 0.48/min on methyl ferulate). The 36-kDa *At*FAEA protein showed maximum ferulic acid esterase activity at 50°C and pH 6, suggesting potential application in industrial processes. A crude *At*FAEA preparation extracted 26.56 mg/g and 8.86 mg/g ferulic acid from maize bran and triticale bran, respectively, and also significantly increased the levels of *p*-coumaric and caffeic acid from triticale bran. The cost-effective production of *At*FAEA could therefore allow for the enrichment of brans generally used as food and fodder, or for the production of fine chemicals (such as ferulic and *p*-coumaric acid) from plant substrates. The potential for larger-scale production of *At*FAEA was demonstrated with the *A. niger* D15[*AtfaeA*] strain yielding a higher enzyme activity (185.14 versus 83.48 U/ml) and volumetric productivity (3.86 versus 1.74 U/ml/h) in fed-batch than batch fermentation.

Keywords: Ferulic acid esterase; *Aspergillus niger; Aspergillus tubingensis;* triticale; maize

Introduction

The growing global food industry requires the development of new technologies to add value and create new market opportunities for the agricultural sector. Ferulic acid is the most abundant hydroxycinnamic acid in plant cell walls (Balasundram et al. 2006; Mathew and Abraham 2004) and exhibits better inhibition of lipid and protein oxidation compared to other naturally occurring phenolic antioxidants such as gallic acid, caffeic acid, malvidin and epicatechin, among others (Graf, 1992; Heinonen et al. 1998). Due to its low toxicity, ferulic acid has been approved for use as an antioxidant additive in cosmetics, foods and beverage industries (Graf, 1992). In the food industry, ferulic acid is, among others, used as a precursor for the production of vanillin, a flavouring agent (Priefert et al. 2001). Ferulic acid represents up to 1.4% of dry weight of cereal grains and is covalently linked with various carbohydrates as a glycosidic conjugate, ester or amide (Kumar and Pruthi 2014). However, ferulic acid can only be released from these substrates through alkaline hydrolysis or enzymatic hydrolysis. Alkaline treatment is quite effective, with 83% of the bound ferulic acid released from corn bran, but it requires downstream purification of the ferulic acid from the brown hydrolysate (Zhao et al. 2014). The use of microbial enzymes for the fractionation of lignocellulose represents a natural process to release ferulic acid from plant substrates, but poses challenges as most of the ferulic acid in plants is covalently linked with lignin and other biopolymers (Kumar and Pruthi 2014).

Ferulic acid esterases (FAEs; EC 3.1.1.73), also known as feruloyl, cinnamic acid or cinnamoyl esterases, are able to release ferulic acid and other cinnamic acids from plant cells (Benoit et al. 2006; Fazary and Ju 2008). Ferulic acid esterases are classified based on their

substrate specificity for methyl esters, i.e. methyl ferulate [MFA], methyl *p*-coumarate [M*p*CA], methyl sinapate [MSA] or methyl caffeate [MCA], and oligosaccharides, e.g. (*O*-{5-*O*-((*E*)-feruloyl)-*α*-L-arabinofuranosyl}-(1,3)-*O*-β-D-xylopyranosyl-(1,4)-D-xylopyranose (FAXX). Type A FAE typically has a preference for substrates with a methoxy substitution in the meta position (C3 and/or C5) of the aromatic ring (e.g. MSA and MFA) as well as substrates with a hydroxyl group at the C4 position of the aromatic ring (e.g. M*p*CA), as reported for *Aspergillus awamori* (Koseki et al. 2006) and *Aspergillus niger* (Faulds and Williamson 1993; Zwane et al. 2014). The phenolic acids in maize bran, representing about 4-5% of the cell wall on a mass basis, are mostly ferulic acid and lower concentrations of *p*-coumaric acid and other phenolic acids esterified to the cell wall polymers (Lapierre et al. 2001). Triticale, a hybrid of wheat and rye, also contains a variety of cinnamic acids, including ferulic acid and *p*-coumaric acid. The release of these phenolic acids - a natural source of antioxidants - will improve the health benefits of high-fibre brans and therefore represent a significant value-addition to these substrates (Hosseinian and Mazza 2009). In the farming industry, the dimeric and trimeric forms of ferulic acid that are either esterified or etherified to plant residues, have been reported to inhibit digestion of these residues in ruminants (Yu et al. 2005). However, the addition of a multi-enzyme cocktail that included a purified *Aspergillus* FAE increased the cell wall digestibility of oat hulls by 86%.

Given the potential commercial importance of plant-derived ferulic acid and antioxidants in various industries (Benoit et al. 2008; Fazary and Ju 2007; Koseki et al. 2009), it is imperative to develop enzyme production and treatment processes that are both efficient and cost-effective, particularly given the high cost of enzyme production. A genomic copy of the *Aspergillus tubingensis* ferulic acid esterase Type A (*faeA*) gene was previously cloned and expressed in *Aspergillus niger* D15#26 under control of the *A. niger gpdA* promoter (Zwane et al. 2014). The current study describes the evaluation of the crude *A. niger* D15[*AtfaeA*]

extract for the release of phytochemicals from triticale and maize bran, as well as the upscaled production of the recombinant *A. tubingensis At*FAEA in bioreactors.

Materials and methods

Strain and culture conditions

All chemicals were of analytical grade and unless stated otherwise, sourced from Sigma-Aldrich (UK). Recombinant *A. niger* D15 strains were selected and maintained on minimal agar medium (2 g/l neopeptone, 18 g/l agar, 1 g/l yeast extract, 0.4 g/l $MgSO_4.7H_2O$, 10 g/l glucose, 2 g/l casamino acids, 20 ml 50 x AspA [300 g/l $NaNO_3$, 26 g/l KCl, 76 g/l KH_2PO_4, pH 6] and 1 ml/l of 1000 x trace elements (Rose and Van Zyl 2002) at 30°C for 120 h. Spores were harvested with physiological saline solution (NaCl, 0.9% w/v). The *A. niger* D15#26 and recombinant *A. niger* D15[*AtfaeA*] strains were cultivated in liquid minimal medium (5 g/l yeast extract, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 2 g/l casamino acids, 20 ml 50 x AspA, 1 ml/l 1000 x trace elements and 10 g/l maize bran as sole carbohydrate source). The growth media were inoculated with $1x10^6$ spores/ml and incubated on a rotary shaker at 125 rpm at 30°C for 5 days. Samples were centrifuged at 7 600 x *g* for 15 min at 4°C and the crude supernatant used for enzyme treatments.

Enzyme purification

Shake flasks containing 250 ml minimal medium was inoculated with $1x10^6$ spores/ml and incubated on a rotary shaker at 125 rpm at 30°C for 5 days. Cultures were centrifuged at 7 300 x *g* at 4°C for 15 min. The enzyme was purified from the supernatant with anion-exchange chromatography using an ÄKTAPrime system (GE Healthcare Life Sciences, Uppsala, Sweden) and DEAE-Sepharose column (GE Healthcare Life Sciences) pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.5). The bound *At*FAEA was eluted

with 10 column volumes of a 0 to 500 mM NaCl gradient in 50 mM sodium phosphate buffer (pH 6.5). The eluted fractions were heated for 3 minutes at 100°C in the presence of protein loading buffer and separated by SDS-PAGE (12% w/v) (Sambrook et al. 1989), using 5 µl peqGOLD prestained protein marker. Electrophoresis was carried out at 100 V at ambient temperature and the proteins visualised with Fast Blue (Sigma-Aldrich, South Africa). Protein sequencing and identification of protein species, provisionally identified as *At*FAEA, were performed by the Centre for Proteomic and Genomic Research (Cape Town, South Africa).

Characterisation of AtFAEA

For enzyme activity assays, fresh 4-nitrophenyl ferulate (4NPF) substrate solution was prepared by vortexing 0.1 M potassium phosphate buffer (pH 6.5) containing 2.5% v/v Triton X-100 and 11 mM 4NPF (dissolved in DMSO) in a 9:1 ratio. Standard FAE assays (De Vries et al. 2002) were performed with 0.1 ml enzyme and 2 ml 4NPF substrate solution incubated at 37°C for 1 h, with the release of 4NP measured spectrophotomerically 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 µmole 4NP from 4NPF in 1 min (Hedge et al. 2009). Total protein concentrations were quantified with the Qubit assay and Qubit 2.0 fluorimeter (Invitrogen, Carlsbad, USA) as per the supplier's specification.

Similar FAE assay conditions were used to quantify enzyme activity under different pH conditions, using either 100 mM citrate-phosphate buffer (pH 3 to 7) or 100 mM Tris-HCl (pH 7 to 9). For pH stability, the recombinant *At*FAEA was incubated in the respective buffers at 4°C and the residual FAE activity measured after 24 h. The FAE activity was evaluated at 30°C to 70°C in 0.1 M potassium phosphate buffer (pH 6), whilst temperature

stability was determined at 30°C to 70°C after incubation for 1 h in 0.1 M potassium phosphate buffer (pH 6, without substrate).

Substrate specificity was determined by incubating 1.7 µg purified *At*FAEA in a total volume of 2 ml with 2 mg/ml of either hydroxycinnamic acid (MFA, M*p*CA, MSA or MCA) or FAXX prepared in 0.1 M potassium phosphate buffer, pH 6. The reaction was incubated at 37°C for 1 h and terminated by the addition of 500 µl methanol and 400 µl distilled water. Kinetic parameters (K_m and K_{cat}) for MFA were calculated from initial rate data determined for 50 mU *At*FAEA at substrate concentrations ranging from 0.2 to 0.8 mM at 50°C, pH 6 for 15 min. The corresponding hydroxycinnamic acids, i.e. ferulic acid (FA), sinapic acid (SA), coumaric acid (*p*CA) and caffeic acid (CA), were quantified by HPLC with a Nucleosil C18 column with a 5 µM particle size (Supelco, Bellefonte, USA) at a flow rate of 1 ml/min at room temperature with 70% v/v acetonitrile as mobile phase. Detection was done by a Surveyor Plus UV/VIS detector (Thermo Electron Corporation, Elandsfontein, South Africa) at 320 nm.

Enzyme treatment of triticale and maize bran

Triticale bran (*X Triticosecale* Wittmack ex A. Camus cultivar US2007) was provided by the Department of Process Engineering (Stellenbosch University, South Africa), while commercial maize bran was donated by Sasko (Pioneer Foods, Paarl, South Africa). Duplicate batches of 20 mg triticale or maize bran were treated with 0.3 mg/ml (0.2 U FAE) of the crude extract from *A. niger* D15#26 or *A. niger* D15[*AtfaeA*] in 1.0 ml 50 mM potassium phosphate buffer (pH 6) for 24 h at 40°C, while incubated on a rotary platform at 250 rpm (Shin and Chen 2006). The control experiments were treated with growth medium alone (i.e. no crude extract). The supernatant was collected by centrifugation (13 000 x *g*, 10 min) and analysed for ferulic acid, *p*-coumaric acid and caffeic acid with reverse-phase

HPLC using a modification of the method described by Kim et al. (2007). The crude extracts were filtered through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter and the filtrate subjected to HPLC using a Waters 1525 binary HPLC pump, YMC-Pack ODS AM-303 (5 μm particle size) column and 2487 dual wavelength absorbance detector (Waters Associates, Milford, MA, USA). The injection volume of 10 μl was separated at a flow rate of 0.8 ml/min at 25°C and using a mobile phase of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B). The linear gradient of solvent B was increased from 8% to 10% in 2 min, then to 30% in 25 min, followed by an increase to 100% in 1 min, kept at 100% for 4 min, and returned to the initial conditions. Breeze software (Waters Associates) was used for data collection and analysis.

HPLC-grade standards of caffeic acid, *p*-coumaric acid and ferulic acid were prepared in 0.1% acetic acid in acetonitrile at concentrations of 200, 150, 100, 50, 25 and 0 mg/l. Standards of 10 μl aliquots were chromatographed singly and as mixtures by injection into the HPLC system. Standard calibration curves were obtained for each phenolic acid by plotting peak areas versus concentrations. Regression equations that showed high degree of linearity ($r^2 > 0.995$) were obtained for each phenolic acid from the calibration curves. Phenolic acids in the samples were identified by comparing the retention time of the unknown with those of the standard phenolic acids. The concentrations of the identified phenolic acids were calculated using the regression equations.

Batch and fed-batch fermentations

Batch and fed-batch fermentations were conducted in a 2-litre bench-top bioreactor (Inforsag, Switzerland) with a working volume of 1.5 litres. Pre-cultures of *A. niger* D15[*AtfaeA*] were prepared with approximately 10^8 spores/ml inoculated in 500 ml Erlenmeyer flasks containing 100 ml inoculum medium (10 g/l $NaNO_3$, 0.8 g/l $MgSO_4.7H_2O$, 2 g/l KH_2PO_4, 0.1

g/l $CaCl_2.2H_2O$, 5 g/l yeast extract, 5 g/l tryptone, 10% v/v Struktol J673 antifoam and 1 ml/l trace elements) and incubated overnight at 30°C at 125 rpm on a rotary shaker. Duplicate batches of 1 litre minimal medium were inoculated with 100 ml of the pre-culture and the protein concentration, enzyme activity, dissolved oxygen and glucose consumption were monitored. The fermentation temperature was controlled at 30°C (±0.1°C), aeration with oxygen at 0.8 volume per volume per minute (v/v/m) and pH at 6 with 4 M KOH and 2 M H_2SO_4. The dissolved oxygen was maintained above 30% saturation by adjusting the agitation between 400 and 800 rpm. An initial glucose concentration of 30 g/l and 10 g/l glucose was used for batch and fed-batch fermentations, respectively. The glucose feed for fed-batch fermentation was 50 g glucose in 250 ml water (200 g/l), initiated after depletion of the initial glucose charge and fed over 7 h.

Samples were taken every 12 h, centrifuged for 5 min at 37 600 x *g* (Sorval RC-6 Plus, Thermo Scientific) and the supernatant stored at -80°C for ferulic acid esterase assays as described above. Total biomass was based on 10 ml samples taken at the end of the fermentation, vacuum-filtered using pre-weighed filters (Whatman no. 1) and dried to constant weight at 105°C for 24 h. Total protein content was measured with the BioRad Protein Assay (BioRad, Hemel, Hempstead, UK) and residual glucose determined with HPLC (CarboPac™ PA1 column, Dionex, MA, USA).

Results and discussion

Purification and characterisation of recombinant AtFAEA

The *A. tubingensis AtfaeA* gene was previously cloned and successfully expressed in *A. niger* D15#26 under the constitutive *A. niger gpd* promoter (Zwane et al. 2014). In this study, the recombinant *A. niger* D15[*AtfaeA*] strain was cultured in shake-flasks with maize bran as sole carbohydrate source, followed by purification and characterisation of the enzyme. Anion

exchange chromatography enabled 7-fold purification and a 48.2% recovery of FAE activity (Table 1). The specific activity of the purified *At*FAEA on 4NFP as substrate was 77.6 U/mg protein, which was higher than recombinantly expressed feruloyl esterases from *Aspergillus usamii* (44.9 U/mg) (Gong et al. 2013), *A. nidulans* (21.7 U/mg) (Debeire et al. 2012), *A. awamori* (9.01 U/mg) (Koseki et al. 2005) and *A. oryzae (*0.57 U/mg) (Koseki et al. 2009). The purified *At*FAE displayed a 36 kDa protein species on SDS-PAGE (Fig. 1), similar to the *A. niger* FAEA (Faulds et al. 1998), and peptide mass fingerprinting (PMF) confirmed the presence of *At*FAEA with a protein score of 146 and a 100% confidence interval (data not shown).

Table 1 Purification of FAEA from *A. niger* D15[*AtfaeA*]

Purification step	Total FAE activity (U)[a]	Specific activity (U/mg protein)	Purification	Recovery (%)
Culture filtrate	675.0 ± 0.5	11.1 ± 0.7	1-fold	100.0
Anion exchange	325.0 ± 0.8	77.6 ± 0.3	7-fold	48.2

[a]Determined with 4NPF as substrate

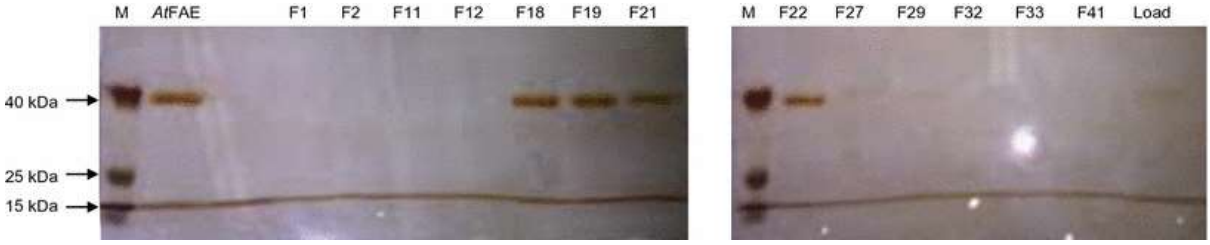

Fig. 1 SDS-PAGE analysis of fractions from anion-exchange chromatography: *Lane M*, peqGOLD protein marker; *AtFAE*, pooled fractions 18-22 that displayed the highest FAE activity; *F1 to F41*, selected fractions; *Load*, culture filtrate

Optimum FAE activity was displayed at pH 5 to 6 (Fig. 2a) and 50°C (Fig. 2b), with the enzyme remaining stable for at least 24 h at pH 5 to 7 and at least 1 h at 30°C to 60°C (data

not shown). Optimum FAE activity at pH 5-6 was also reported for other *Aspergillus* FAEAs (Benoit et al. 2008), whereas the higher enzyme stability in acidic conditions (retaining more than 80% activity at pH 3 to 7) corresponded to an *A. awamori* FAE (Kanauchi et al. 2008). The optimum FAE activity at 50°C is within the temperature range of 35°C to 55°C reported for other fungal FAEAs, including that of *A. niger* (De Vries et al. 2005). However, the native *A. niger* FAE was able to tolerate 80°C (Sundberg et al. 1990), whereas the *At*FAEA was stable only up to 60°C.

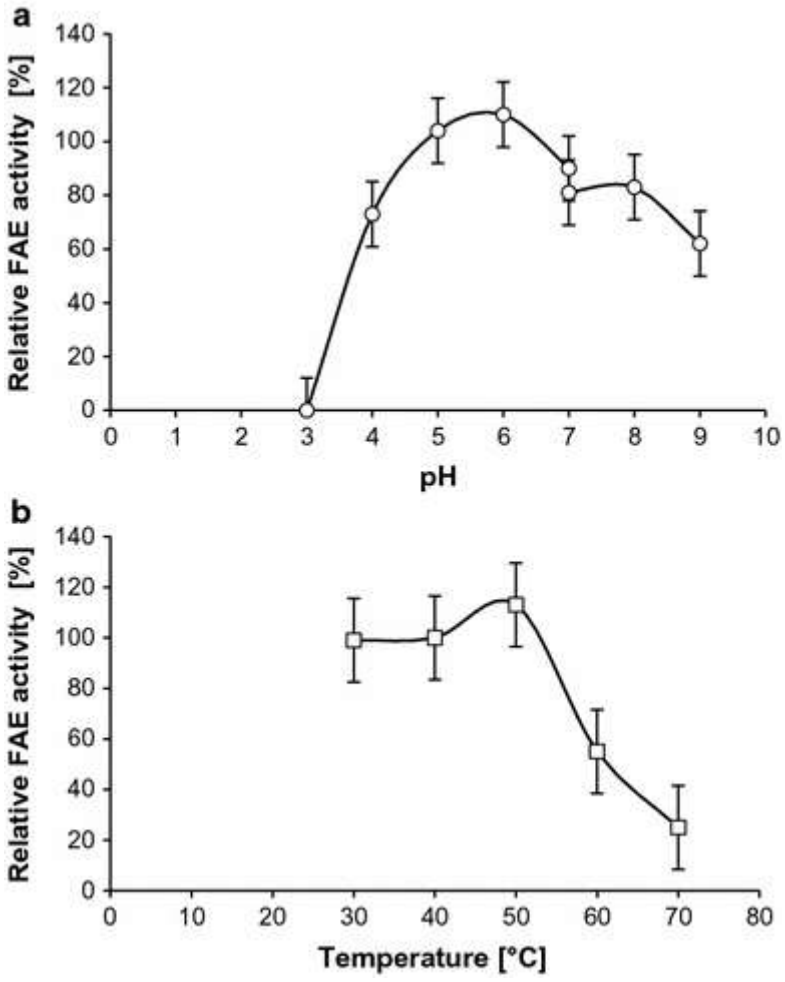

Fig. 2 Effect of pH (**a)** and temperature (**b)** on ferulic acid esterase (FAE) activity in *A. niger* D15[*AtfaeA*]. Error bars represent standard deviations from triplicate experiments.

With MFA as substrate, the purified *At*FAEA displayed a K_m of 0.43 mM (Table 2), compared to 0.76 mM reported for the *A. niger* FAEA (De Vries et al. 1997). The latter also reported low levels of activity (0.97 U/ml) for the *A. tubingensis faeA* expressed in the *A. niger* NW154 strain. Our results indicate that the protease-deficient *A. niger* D15#26 strain was a better host for over-expression of *AtfaeA*, yielding a specific activity of 3 487 U/mg total protein on MFA as substrate.

Table 2 Substrate specificity of purified *At*FAEA

Substrate		Specific activity (U/mg protein)
Synthetic	MFA[a]	3 487 ± 0.5
	M*p*CA	446 ± 0.1
	MSA	432 ± 1.5
	MCA	0
Natural	FAXX	9 837 ± 5.3

[a]MFA, methyl ferulate; M*p*CA, methyl *p*-coumarate; MSA, methyl sinapate; MCA, methyl caffeate; FAXX, *O*-{5-*O*-((*E*)-feruloyl)-*α*-L-arabinofuranosyl}-(1,3)-*O*-β-D-xylopyranosyl-(1,4)-D-xylopyranose

Release of phenolic acids from maize and triticale bran

Corn bran represents one of the best possible source of ferulic acid among agricultural crops, with up to 26 mg/g substrate reported for refined maize bran (Zhao and Moghadasian, 2008). In this study, treatment with a crude *A. niger* D15[*AtfaeA*] extract released 26.56 mg/g ferulic acid from maize bran to, whereas 8.86 mg/g ferulic acid was released from triticale bran (Table 3). Noteworthy is that the *A. niger* D15[*AtfaeA*] preparation also released significantly higher levels of *p*-coumaric and caffeic acid from triticale bran, confirming that these phenolic acids are susceptible to ferulic acid esterases (Poidevin et al. 2009). In contrast, less

p-coumaric and caffeic acid were extracted from the maize bran, relative to the control treatments.

Table 3 Relative concentrations of selected phenolic acids after treatment of maize and triticale bran with crude extracts

Treatment	Phenolic acids (mg/g substrate)		
	Ferulic acid	*p*-Coumaric acid	Caffeic acid
Maize bran			
Control	7.63 ± 0.00*	0.99 ± 0.00	2.65 ± 0.00
A. niger D15[pGTP]	2.71 ± 0.00	0.07 ± 0.00	1.16 ± 0.00
A. niger D15[*AtfaeA*]	26.56 ± 0.00	0.59 ± 0.00	1.21 ± 0.00
Triticale bran			
Control	0.16 ± 0.00	0.15 ± 0.00	0.48 ± 0.00
A. niger D15[pGTP]	3.75 ± 0.00	0.27 ± 0.00	0.98 ± 0.00
A. niger D15[*AtfaeA*]	8.86 ± 0.00	3.83 ± 0.00	3.43 ± 0.00

*Values are the means of duplicate measurements, together with standard deviations

Batch and fed-batch fermentations

The production of FAEA by the recombinant *A. niger* D15[*AtfaeA*] strain was scaled up to 2-litre batch fermentations to increase the FAEA production levels. The maximum biomass reached was 8.68 g/l with an overall growth rate of 0.20 g/l/h. The extracellular protein concentration increased from 106.38 (±5.96) mg/l at the point of sugar depletion (20 h) to 201.31 (±86.30) mg/l after 44 h (Fig. 3a). The extracellular FAE activity followed the same trend and increased from 62.65 (±5.03) U/ml after 20 h to 83.48 (±19.72) U/ml after 44 h. The volumetric productivity of the enzyme production peaked at 6.89 (±0.01) U/ml/h at 10 h before dropping to 1.90 (±0.45) U/ml/h at 44 h (Table 4).

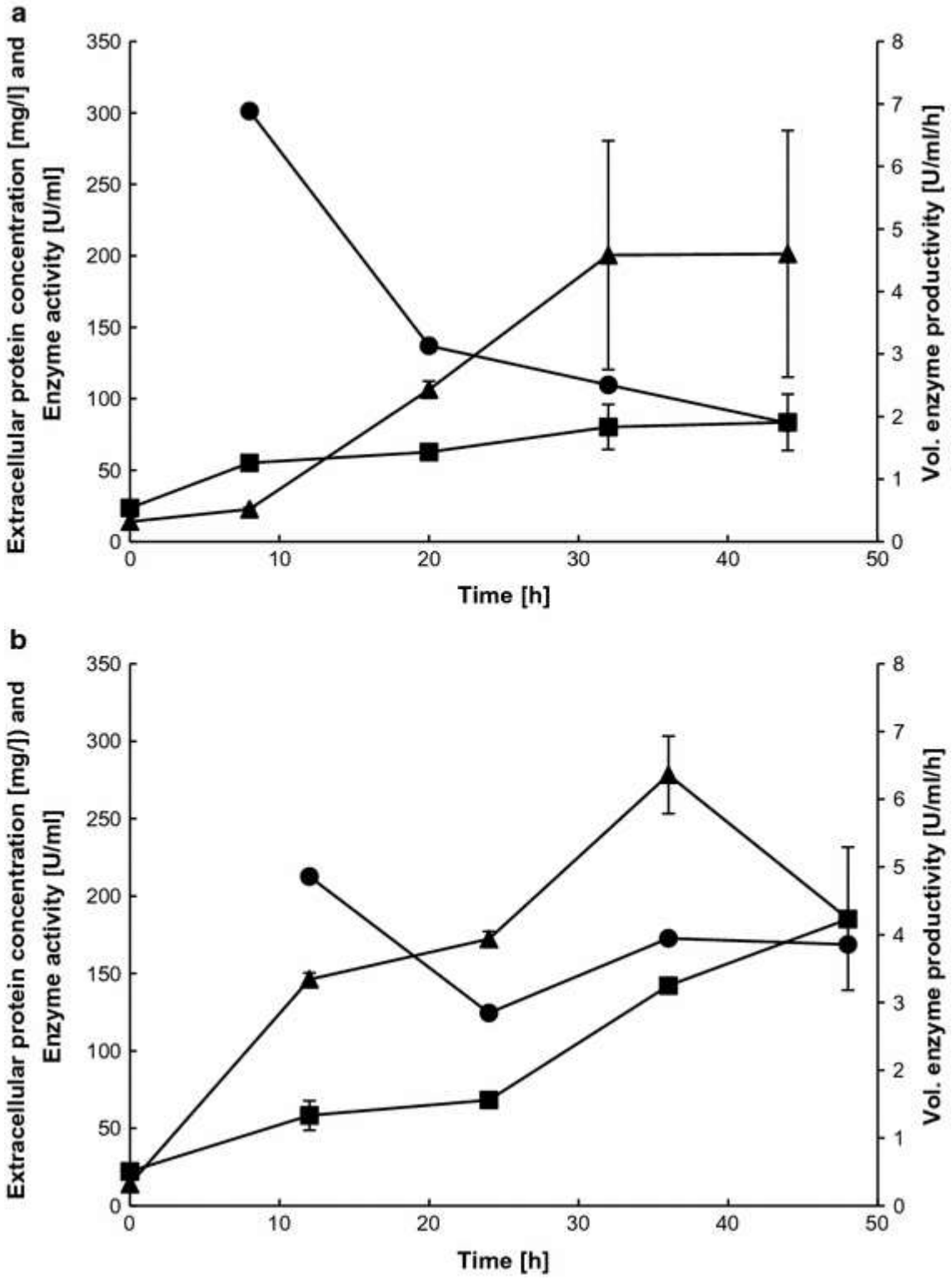

Fig. 3 Time course for the production of FAEA by *A. niger* D15[*AtfaeA*] using a 2-litre bioreactor under batch (**a**) and fed-batch (**b**) conditions: (▲) total extracellular protein concentration (mg/l), (■) FAE activity (U/ml), and (●) volumetric productivity (U/ml/h). Error bars represent standard deviations from duplicate experiments

Table 4 Comparison of *A. niger* D15[*AtfaeA*] performance in batch and fed-batch fermentations at maximum volumetric enzyme activity

	Batch fermentation	Fed-batch fermentation
Run time (h)	44	48
Biomass (g/l)	8.68 ± 0.30*	10.57 ± 0.50
Volumetric enzyme productivity (U/ml/h)	1.90 ± 0.45	3.86 ± 0.03
Yield (g FAE/$g_{glucose}$)	0.29 ± 0.13	0.18 ± 0.01
Volumetric activity (U/ml)	83.48 ± 19.72	185.1 ± 1.33

*Values are the means of duplicate measurements from duplicate treatments, together with standard deviations

The *A. niger* D15[*AtfaeA*] was subsequently evaluated in a fed-batch fermentation with a 10 g/l initial glucose charge and a 20% glucose feed (final glucose concentration of 50 g/l, 40% dissolved oxygen). The batch phase of this fermentation was comparable to the batch fermentation described above with 58.32 (±9.52) U/ml FAE activity after 12 h (Fig. 3b), whereas the volumetric productivity of enzyme production decreased from 4.86 (±0.79) U/ml/h at 12 h to 3.86 (±0.03) U/ml/h at the end of the fermentation (Table 4). The fed-batch process yielded 0.18 g FAEA/$g_{glucose}$ with 10.57 g/l biomass produced at an overall growth rate of 0.22 g/l/h.

Although direct comparisons with other reports on FAE production are difficult due to variations in substrate and culturing conditions, the enzyme production by *A. niger* D15[*AtfaeA*] in the fed-batch reactor (185.14 U/ml) was significantly higher than the 0.97 U/ml reported for the *A. tubingensis* FAEA expressed in *A. niger* NW154 (Table 5). This confirms the suitability of the *A. niger* D15#16 strain for the expression of recombinant proteins, as previously reported (Rose and Van Zyl 2008).

Table 5 Enzyme activity of a selection of recombinant fungal ferulic acid esterases

Enzyme	Enzyme activity	Recombinant host	Reference
A. tubingensis FAEA	185.14 U/ml	*A. niger* D15#26	This study
A. niger FAEA	3.16 U/ml	*A. tubingensis* NW241	De Vries et al. 2002
A. tubingensis FAEA	0.97 U/ml	*A. niger* NW154	De Vries et al. 2002
A. awamori FAEA	9 U/ml	*Pichia pastoris*	Koseki et al. 2006

Conclusions

Based on the hydrolysis profile of the methyl esters (MFA>MpCA>MSA), the recombinant *At*FAEA has affinity for the C-5 feruloylated substrates and would thus be best at releasing ferulic acid from monocots such as wheat, oats, maize, barley, etc. where ferulic acid is esterified to the C-5 hydroxyl group of arabinopyranose residues. Furthermore, the relative stability of *At*FAEA at pH 5 to 7 and 30 to 60°C poses an advantage for applications in the food industry. Of particular importance is that the crude *A. niger* D15[*AtfaeA*] extract was able to release additional phenolic acids from maize and triticale bran without any pre-treatment of the substrate, which would support the cost-effective extraction of ferulic acid from plant substrates.

Enzyme production by the *A. niger* D15[*AtfaeA*] strain was significantly improved when cultured under either batch or fed-batch conditions, relative to shake flasks (Zwane et al. 2014), with the glucose-limited conditions in the fed-batch fermentation yielding higher extracellular FAE activity (185.14 vs. 83.48 U/ml) and volumetric productivity (3.86 vs. 1.90 U/ml/h) than the batch fermentation.

In conclusion, the crude *A. niger* D15[*AtfaeA*] extract was effective at extracting antioxidant-rich phytochemicals, represented in particular by ferulic acid, from triticale and maize bran. Treatment with FAEA can therefore increase the ferulic acid (and antioxidant)

content and thus improve the nutritional value of these brans. Ferulic acid extracted from plant substrates using a natural (enzyme-based) process as opposed to alkaline treatment, can be used for the production of other 'natural' molecules such as vanillin, which is widely used in the food, pharmaceutical and cosmetic industries. An enzymatic process also has potential application in a multi-product biorefinery setup where the product(s) can serve directly as substrate(s) for other enzymes.

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