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Overexpression, purification and characterisation of homologous α-L-arabinofuranosidase and *endo*-1,4-β-D-glucanase in *Aspergillus vadensis*

Helena Culleton · Vincent A. McKie · Ronald P. de Vries

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Abstract In the recent past, much research has been applied to the development of *Aspergillus*, most notably *A. niger* and *A. oryzae*, as hosts for recombinant protein production. In this study, the potential of another species, *Aspergillus vadensis*, was examined. The full length gDNA encoding two plant biomass degrading enzymes, i.e. α-L-arabinofuranosidase (*abfB*) (GH54) and *endo*-1,4-β-D-glucanase (*eglA*) (GH12) from *A. vadensis* were successfully expressed using the *gpdA* promoter from *A. vadensis*. Both enzymes were produced extracellularly in *A. vadensis* as soluble proteins and successfully purified by affinity chromatography. The effect of culture conditions on the expression of *abfB* in *A. vadensis* was examined and optimised to give a yield of 30 mg/L when grown on a complex carbon source such as wheat bran. Characterization of the purified α-L-arabinofuranosidase from *A. vadensis* showed an optimum pH and temperature of pH 3.5 and 60 °C which concur with those previously reported for *A. niger* AbfB. Comparative analysis to *A. niger* AbfA demonstrated interesting differences in temperate optima, pH stability and substrate specificities. The *endo*-1,4-β-D-glucanase from *A. vadensis* exhibited a pH and temperature optimum of pH 4.5 and 50 °C, respectively. Comparative biochemical analysis to the orthologous EglA from *A. niger* presented similar pH and substrate specificity profiles. However, significant differences in temperature optima and stability were noted.

Keywords α-Arabinofuranosidase · Aspergillus · Downstream processing · Endoglucanase · Genetic engineering · Homologous protein production · Industrial biotechnology

Introduction

Over the past two decades, Aspergilli have become the most widely studied group of filamentous fungi due, in part, to their potential in many industrial applications [3, 4, 7, 11, 30]. Aspergilli have adapted to their lifestyle as common soil fungi that are found in many different environments by producing an extensive set of enzyme mixes to degrade the broad range of plant polysaccharides which they encounter [8]. Plant cell walls consist mainly of the polysaccharides cellulose, hemicelluloses (xyloglucans, xylan and galacto(gluco)mannan) and pectin which interact with each other as well as the aromatic polymer lignin to form a network of polymers with linkages and hydrogen bonds that give the plant cell wall its rigidity [13]. Fungi such as Aspergilli degrade these polysaccharides extracellularly by secreting diverse enzymatic mixtures which release utilisable oligo- and monosaccharides from the polysaccharide that is present [20]. The complete degradation of cellulose for instance, requires the action of at least

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H. Culleton (✉) · R. P. de Vries
Fungal Physiology, CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands
e-mail: culleth2@gmail.com

R. P. de Vries
e-mail: r.devries@cbs.knaw.nl

H. Culleton · R. P. de Vries
Fungal Molecular Physiology, Utrecht University, Utrecht, The Netherlands

H. Culleton · V. A. McKie
Megazyme International Ireland, Bray, Co. Wicklow, Ireland

three enzymes: β-1,4-D-glucosidase, cellobiohydrolase and β-1,4-D-endoglucanase. In contrast, the hydrolysis of xylan requires the combined action of at least nine different enzymes: α-L-arabinofuranosidase, α-1,4-D-galactosidase, α-glucuronidase, acetylxylan esterase, arabinoxylan arabinofuranohydrolase, β-1,4-D-xylosidase, feruloyl esterase, β-1,4-D-galactosidase and β-1,4-D-endoxylanase [9]. The ability to produce such a broad range of enzymes combined with their good fermentation capabilities has resulted in many studies being dedicated to the development of *Aspergillus* as hosts for the industrial production of recombinant proteins [15]. To date, much of this research was performed with *A. niger* and *A. oryzae*, but recently *A. vadensis*, a close relative of *A. niger*, has been suggested as a possibly more favourable alternative due to the low levels of extracellular proteases which it produces and the fact that it does not acidify the culture medium [14]. In this study, the potential of *A. vadensis* as a host for recombinant protein production was examined by cloning and expressing two homologous genes encoding cell wall polysaccharide degrading enzymes, i.e. α-L-arabinofuranosidase (*abfB*) (GH54) and *endo*-1,4-β-D-glucanase (*eglA*) (GH12) in this prospective industrial strain.

α-L-arabinofuranosidases (non-reducing end α-L-arabinofuranosidases; EC 3.2.1.55) act by hydrolysing the terminal non-reducing α-L-1,2-, α-L-1,3-, and α-L-1,5-arabinofuranosyl residues in α-L-arabinosides and can act synergistically with other hemicellulases and pectic enzymes for the complete hydrolysis of xylans and pectins [26]. In recent years α-L-arabinofuranosidases have attracted considerable interest due to their potential in industrial applications such as in oligosaccharide synthesis [34, 35], the pre-treatment of lignocelluloses for bioethanol production [17, 37] and in the chlorination of paper products [19, 25]. To date, α-L-arabinofuranosidases have been isolated from various bacterial and fungal organisms such as *Streptomyces* sp. [39]. *Thermotoga maritima* [27] and *A. niger* [36] and their genes have been cloned and expressed in developed expression systems. *A. niger* itself produces two main extracellular α-L-arabinofuranosidases; α-L-arabinofuranosidase A (AbfA) which is specifically active towards *p*-nitrophenyl-α-L-arabinofuranoside and 1,5-α-L-arabinofuranose oligosaccharides, and α-L-arabinofuranosidase B (AbfB) which has a broader activity range and is active on both these substrates but also has activity towards 1,5-α-L-arabinan, beet and apple arabinan and arabinoxylan [36]. Additional genes encoding putative α-L-arabinofuranosidases were detected in the genome of *A. niger*, but have not been biochemically characterised [8]. In this study, a homologous α-L-arabinofuranosidase B encoding gene (*abfB*) from glycosyl hydrolase family 54 (GH54) was cloned and expressed in *A. vadensis* to examine the potential of this expression system. This α-L-arabinofuranosidase B (AvAbfB) was then characterised and compared to the commercially available and biochemically different α-L-arabinofuranosidase A from *A. niger* (AnAbfA) (Megazyme; Cat. No. E-AFASE) from glycosyl hydrolase family 51 (GH51).

Endoglucanases (*endo*-1,4-β-D-glucanases, EC 3.2.1.4) are a group of enzymes which combined with cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) are responsible for the effective degradation of cellulose into glucose. Cellulose exists as highly ordered linear polymers of β-1,4-linked D-glucose residues which are bundled together in microfibrils via hydrogen bonds [18]. It is believed that endoglucanases act by initiating random attacks at multiple sites in the non-crystalline regions of the cellulose fibre [33]. This in turn, opens up sites for subsequent attack by cellobiohydrolases, which cleave cellulose chains at the ends and release cellobiose. These oligosaccharides are then further degraded into D-glucose molecules by the action of β-glucosidases and exoglucanases [9, 33]. Among the most efficient producers of cellulolytic enzymes, in particular *endo*-1,4-β-D-glucanase, is the filamentous fungus *A. niger* which has gained it significant interest especially in the food, textile and pharmaceutical industries [13]. Genes encoding endoglucanases from *A. niger*, such as *eglA*, *eglB* and *eglC*, have been cloned and characterised with EglA demonstrating the highest activity towards β-glucan compared to EglB and EglC [21, 40]. Thus, for this study the *endo*-1,4-β-D-glucanase A encoding gene (*eglA*) from glycosyl hydrolase family 12 (GH12) was cloned and expressed in *A. vadensis* and the activity of the corresponding enzyme (AvEglA) was compared to the commercially available orthologous enzyme, AnEglA from *A. niger* (Megazyme; Cat. No. E-CELAN,), which was also previously expressed through *Pichia* and described by Quay et al. [33].

Materials and methods

Strains, media and culture conditions

Escherichia coli XL1-Blue chemically competent strain (Fisher Scientific) was used as a host for recombinant DNA manipulation. Gene constructs were obtained by PCR from the gDNA of *Aspergillus vadensis* CBS 137441. *Aspergillus vadensis* CBS 113365 (*pyrG*-) was used as the parental strain for transformation.

Aspergillus minimal medium (MM) and complete medium (CM) were described previously [14]. Agar was added at 2 % (w/v) for solid medium. Pre-cultures for protoplast formation were grown overnight at 30 °C in 200 mL MM supplemented with 0.5 % (w/v) yeast extract, 0.2 % (w/v) casamino acids, 2 % (w/v) glucose and 1.22 mg/

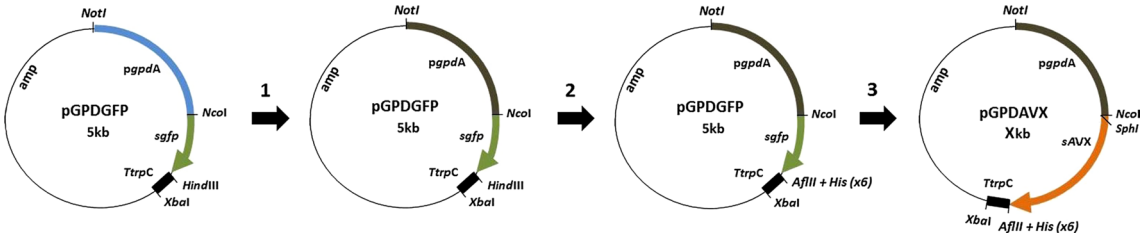

Fig. 1 Construction of the new expression plasmid. *1* Replacement of glyceraldehyde-3-phosphate dehydrogenase promoter (p*gpdA*) from *A. nidulans* with p*gpdA* from *A. vadensis*. *2* Replacement of *Hin*dIII site at 3′ end of gene construct with *Afl*II and addition of 6× His-tag. *3* Insertion of *Sph*I site alongside *Nco*I site and *A. vadensis* genes (*sAVX*) in place of green fluorescence protein gene (*sgfp*)

mL (final) uridine after inoculating with 5×10^6 spores/mL. For the analysis and characterization of enzyme activities, 400 mL liquid cultures (2.5 L baffled flasks) of MM + 1 % (w/v) wheat bran (for production of AvAbfB) or 2 % (w/v) sucrose (for production of AvEglA) + 0.1 % (w/v) TWEEN® 80 were inoculated with 1×10^6 spores/mL (final) and incubated at 35 °C in an orbital shaker at 250 rpm.

Molecular biology methods

Standard methods were used for DNA manipulations, subcloning, DNA digestion reactions and DNA isolations [38].

PCR and expression vectors

Genes were amplified from the then partially sequenced *A. vadensis* genome (Culleton and de Vries, unpublished data) with primers including 5′ *Nco*I and 3′ *Afl*II restriction sites necessary for the cloning of the α-L-arabinofuranosidase encoding *abfB* gene (1,500 bp) and 5′ *Sph*I and 3′ *Afl*II restriction sites necessary for the *endo*-1,4-β-D-glucanase encoding *eglA* gene (836 bp). The nucleotide sequences of the primers utilised (incorporating the restriction sites indicated) are as follows: *abfB* 5′ end oligonucleotide 5′-GGCCATGGTCTCCCGCCGAAACC-3′ and *abfB* 3′ oligonucleotide 3′-GGCTTAAGCGAAGAAAACGCCGTCTC-5′. *eglA* 5′ end oligonucleotide 5′-GGGCATGCAGCTCGCAGTGACAC-3′ and *eglA* 3′ oligonucleotide 3′-GGCTTAAGGTTGACACTAGCGGTCC-5′. PCR reactions were carried out using KOD DNA Polymerase (Merck Biosciences) and conditions supplied. PCR products were inserted into pCR2.1 TOPO vector (Invitrogen) following the instructions provided and plasmids were verified by sequencing.

The pGPDGFP expression vector was used to make the new expression constructs in this study. As described by Lagopodi et al. [24], the pGPDGFP vector is composed of the *gfp* gene under the control of the *gpdA* promoter from *A. nidulans* [31] and terminated with the *trpC* terminator [28]. A new expression vector was built based on the pGPDGFP vector where p*gpdA* from *A. nidulans* was replaced with p*gpdA* from *A. vadensis* [10] using restrictions enzymes *NotI*/*Nco*I. Due to the presence of an internal *Hin*dIII within p*gpdA* from *A. vadensis*, the *Hin*dIII site at the 3′ end of the *gfp* gene was replaced with *Afl*II and a 6× Histidine tag and stop codon were added to assist in the downstream purification of the enzymes once expressed. Due to the presence of an *Nco*I site within the gene sequence of Av*eglA*, an additional *Sph*I site was added alongside the *Nco*I site at 3′ end of promoter to facilitate the cloning of this gene. The *gfp* gene was then replaced with the new gene candidate from *A. vadensis* using restriction enzymes *NcoI*/*Afl*II for Av*abfB* and *SphI*/*Afl*II for Av*eglA* (Fig. 1).

Transformation of *A. vadensis*

The formation of protoplasts by *Aspergillus* strains was based on the protocols by Peraza et al. [29] and de Bekker et al. [12]. Strains were grown for 16 h, after which time the mycelia were gently harvested by filtration over a Büchner funnel with nylon gauze. After washing with 0.9 % NaCl (w/v), 2.5 g (wet weight) mycelium was resuspended in 20 mL stabilisation buffer (0.2 M phosphate buffer (pH 6.0), 0.8 M sorbitol). Lytic enzymes were added to the following final concentrations; 5 mg/mL lysing enzymes from *Trichoderma harzianum* (Sigma; Cat. No. L1412), 460 units/mL β-glucuronidase from *Helix pomatia* (Sigma; Cat. No. G0751) and 0.15 units/mL chitinase from *Streptomyces griseus* (Sigma; Cat. No. C6137). The mixture was incubated in an orbital shaker at 37 °C for 1–2 h with gentle shaking (120 rpm). Protoplasts were separated from the mycelium by filtering over glass wool. The protoplasts were recovered by centrifugation in a swing-out rotor (10 min; ~800 rcf) and were washed twice with STC (1.33 M sorbitol, 50 mM $CaCl_2$ and 10 mM Tris/HCl, pH 7.5). Transformation was performed as described by Kusters-van Someren et al. [22], with 2×10^6 protoplasts, 0.5 µg of pGW635 (carrying the *A. niger pyrG* gene for selection) and 20 µg of the different expression vectors

(carrying the studied genes under the control of the p*gpdA* from *A. vadensis*).

Screening of transformants and selection of expression strains

To test the resulting transformants for *abfB* expression, a fluorimetric ABF screen based on the conversion of the fluorogenic substrate 4-methylumbelliferyl-α-L-arabinopyranoside (0.02 mg/mL (final) in MM + 2 % (w/v) sucrose + 2 % (w/v) agar) when 1000 spores (2 µL) from each transformant were grown at 30 °C for 2 days and viewed under UV light. To test for *eglA* expression, resulting transformants were grown on agar plates containing high purity dyed and crosslinked insoluble AZCL-Barley beta-Glucan (Megazyme; Cat. No. I-AZBGL) (0.1 % (w/v) in MM + 2 % (w/v) sucrose + 2 % (w/v) agar) when 1,000 spores (2 µL) from each transformant were grown at 30 °C for 2 days. In both plate screens, CBS 137441 was grown as a negative control.

Production and purification of recombinant enzymes

Four hundred milliliter liquid cultures were inoculated with 1×10^6 spores/mL (final) and were grown in 2.5 L baffled flasks at 35 °C and 250 rpm with production levels being monitored on a daily basis by SDS polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was done using a 12 % polyacrylamide gel containing 0.1 % (w/v) SDS and protein bands were detected by Coomassie Blue staining [23]. Cultures were grown for the time indicated in results, after which time the mycelia were removed from the culture filtrate by filtration over a Büchner funnel with nylon gauze. Culture filtrates were purified by His-tag affinity chromatography, equilibrated with buffer (10 mM imidazole, 10 mM HEPES and 500 mM NaCl, pH 7.5) and eluted with a stepwise gradient in the same buffer containing 10–500 mM imidazole. Eluent from the column was monitored for levels of protein concentration by testing 10 µL of sample in 150 µL of Bio-Rad protein assay dye reagent (Bio-Rad; Cat. No. 500-0006). 15 µL samples of culture filtrates (C.F.) and column fractions were viewed by SDS-PAGE (as above) and fractions containing purified protein were pooled and precipitated with 50 % ammonium sulphate. Enzyme fractions were then concentrated by centrifugation and the enzyme pellet was resuspended in 3.2 M ammonium sulphate solution for characterization.

Enzyme activity measurement

For the initial measurement of the *A. vadensis* α-arabinofuranosidase B (AvAbfB) and *A. niger* α-arabinofuranosidase A (AnAbfA) activities, serial dilutions of the purified enzymes were carried out in 100 mM sodium acetate buffer, pH 4.0 including BSA (1 mg/mL). 0.2 mL of diluted enzyme was added to 0.2 mL of 10 mM *p*-nitrophenyl α-L-arabinofuranoside (pH 4.0) and incubated at 40 °C for 10 min. The reaction was stopped with the addition of 3 mL of 2 % (w/v) tri-sodium orthophosphate, pH 12.0 and the absorbance was measured at 400 nm. Activities were expressed as Units/mg where one unit is defined as 1 micromole of *p*-nitrophenol liberated per minute per milligram of enzyme.

For the initial measurement of the *A. vadensis endo*-1,4-β-D-glucanase A (AvEglA) and the orthologous *A. niger endo*-1,4-β-D-glucanase A (AnEglA) activities, serial dilutions of the purified enzymes were performed in 100 mM sodium acetate buffer, pH 4.5 including BSA (1 mg/mL). Nelson-Somogyi reducing sugar assays were performed by the addition of 0.2 mL of diluted enzyme to 0.2 mL of 10 mg/mL Barley β-glucan (Megazyme; Cat. No. P-BGBM) (pH 4.5) and incubated at 40 °C for 10 min. The reaction was stopped by the addition of 0.5 mL of Stopping Solution (25 mL of Solution A (2.5 % (w/v) sodium carbonate anhydrous, 2.5 % (w/v) potassium sodium tartrate and 20 % (w/v) sodium sulphate), to which 1 mL of Solution B (3 % (w/v) copper sulphate pentahydrate) was added). Enzyme reactions were then boiled for 20 min and allowed to cool to room temperature for 5 min before the addition of 3.0 mL of a 1:5 dilution of Solution C (5 % (w/v) ammonium molybdate, 4.2 % (v/v) concentrated sulphuric acid and 0.6 % (w/v) sodium arsenate heptahydrate).

pH optima assays were conducted in duplicate measurements using the optimum enzyme dilution as determined from the initial activity assays and using the following pH buffers covering from pH 1.0 to pH 9.0 including BSA (1 mg/mL); potassium chloride (pH 1.0 and pH 2.0), glycine (pH 2.0, pH 2.5, pH 3.0), citrate phosphate (pH 3.0, pH 3.5, pH 4.0, pH 4.5, pH 5.0, pH 5.5, pH 6.0, pH 6.5 and pH 7.0) and sodium phosphate (pH 6.0, pH 7.0, pH 8.0 and pH 9.0). The remaining assay conditions were consistent with the assay conditions used for the measurement of initial activity. For pH stability assays an initial tenfold dilution of the enzyme was carried out in the buffers outlined above (pH 1.0–pH 9.0) including BSA (1 mg/mL) and incubated at 4 °C for 48 h. Activity assays were then conducted in duplicate measurements using the optimum enzyme concentration and pH as determined by previous assays with the enzyme activity at the optimum pH set at 100 %.

For temperature stability assays an initial tenfold dilution of the enzyme in optimum pH buffer was performed and aliquots incubated at the following temperatures for 15 min; 25, 30, 40, 50, 60, 70, 80, 90 and 100 °C. Following incubation, the enzyme was further diluted in optimum pH buffer to an optimum concentration and assayed in

duplicate as described above. Temperature optima assays were performed in duplicate measurements using optimum enzyme concentration and pH as determined in previous assays and at temperatures that the enzyme proved stable.

Aspergillus vadensis α-arabinofuranosidase B (AvAbfB) and *A. niger* α-arabinofuranosidase A (AnAbfA) substrate specificity assays were performed using the following polysaccharide substrates at a final concentration of 5 mg/mL; wheat flour arabinoxylan (Megazyme; Cat. No. P-WAXYL), sugar beet arabinan (Megazyme; Cat. No. P-ARAB) and debranched sugar beet arabinan (Megazyme; Cat. No. P-DBAR). Activities were determined under optimum conditions using Nelson-Somogyi reducing sugar assays as described above. To test *A. vadensis* (AvEglA) and *A. niger* (AnEglA) endoglucanase A for substrate specificity, the following polysaccharide substrates and *p*-nitrophenol substrates were used at final concentrations of 5 mg/mL and 5 mM, respectively; carboxymethyl cellulose 4 M (Megazyme; Cat. No. P-CMC4 M), galactomannan (Megazyme; Cat. No. P-GALML), glucomannan (Megazyme; Cat. No. P-GLCML), pachyman (Megazyme; Cat. No. P-CMPAC), soluble starch (Sigma; Cat. No. S9765), xyloglucan (Megazyme; Cat. No. P-XYGLN), *p*-nitrophenol α-D-glucopyranoside, *p*-nitrophenol β-D-glucopyranoside and *p*-nitrophenol β-D-xylopyranoside. Assays were performed under optimum pH and temperature conditions as determined in previous assays and using methods as described above.

Results

Development of expression strains

The selected genes were PCR amplified from *A. vadensis* gDNA, inserted into pCR2.1 TOPO vector (Invitrogen) and sequenced using the Sanger method at LGC Genomics. Sequencing results confirmed that the correct gene regions were amplified. Identification and comparison of the corresponding α-L-arabinofuranosidase B protein (499 amino acids) and *endo*-1,4-β-D-glucanase A protein (239 amino acids) from *A. vadensis* with other characterised protein sequences were performed using the BLAST program at NCBI. The AvAbfB protein sequence has 99 % identity to *Aspergillus kawachii* IFO 4308 (Q8NK89.1), *Aspergillus awamori* (Q9C4B1.1) and *Aspergillus niger* AbfB (XP_001396769.1). On the basis of the similarities to these other α-L-arabinofuranosidases, *A. vadensis abfB* was assigned to GH54 (CAZy-http://www.cazy.org) [5]. The AvEglA protein sequence showed high identity (96 %) to *A. niger* CBS 513.88 (XP_001400902.1) and 95 % to *Aspergillus usamii* (AEL12376.1), thus providing basis for its inclusion into the GH12 family.

For expression and characterization of the selected genes, the corresponding fragments were cloned behind the *gpdA* promoter from *A. vadensis* [10]. The resulting expression vectors containing the gene sequence were then transformed into *A. vadensis* CBS113365. To test the resulting transformants for *abfB* expression, a fluorimetric screen based on the conversion of the fluorogenic substrate 4-methylumbelliferyl-α-L-arabinopyranoside was applied to select the strongest expressing transformants (Fig. 2a), i.e. transformant no. 7 (*abfB*_t7). To test for *eglA* expression, resulting transformants were grown on agar plates containing high purity dyed and crosslinked insoluble AZCL-Barley beta-Glucan (Megazyme; Cat. No. I-AZBGL), from which the strongest expressing clones were selected based on the ability to hydrolyse the AZCL-Barley beta-Glucan substrate (Fig. 2b), i.e. transformant no. 9 (*eglA*_t9).

Effect of culture conditions on the production of α-arabinofuranosidase (AbfB)

*A. vadensis abfB*_t7 was cultivated under several different conditions for investigation of α-arabinofuranosidase production. Glucose, mannose and sucrose were used as simple carbon sources for comparison purposes, with sucrose giving optimum *abfB* expression with yields of ~7 mg/L. The addition of 0.1 % (w/v) TWEEN® 80 to media doubled these yields to ~15 mg/L. Growth temperatures from 25 to 37 °C were examined with *A. vadensis abfB*_t7 favouring higher incubation temperatures, i.e. 35–37 °C, for the expression of recombinant α-arabinofuranosidase. The use of a complex carbon source such as wheat bran (Odlums) under optimum conditions increased native and recombinant protein production alike, giving a purified AvAbfB yield of 30 mg/L.

α-arabinofuranosidase (AbfB) production and characterization

Liquid cultures were inoculated with 1×10^6 spores/mL (final) from chosen transformants, grown at 35 °C and 250 rpm and expression levels were monitored on a daily basis by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Optimum AvAbfB production was observed after 3 days incubation and the enzyme was purified from these culture filtrates using His-tag affinity chromatography. 15 µL samples of C.F. and column fractions were viewed by SDS-PAGE (Fig. 3a) with purified protein visible in the eluent fractions containing 50, 100 and 500 imidazole. These were pooled and precipitated with 50 % ammonium sulphate. Enzyme fractions were then concentrated and quantified giving a total yield of 30 mg/L purified protein (Table 1 A).

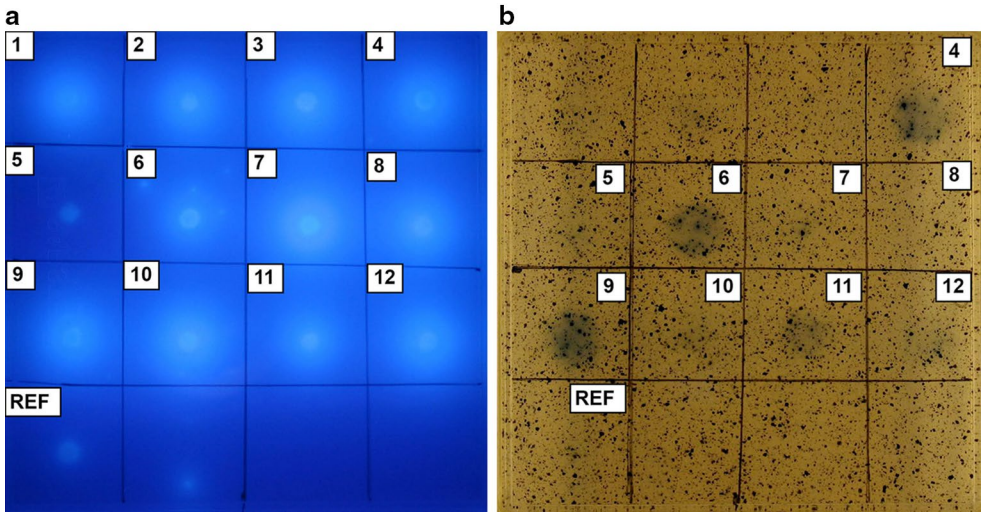

Fig. 2 Substrate plate screens to select the strongest AbfB producers (**a**) and EglA producers (**b**). Fluorimetric ABF screen based on the conversion of the fluorogenic substrate 4-methylumbelliferyl-α-L-arabinopyranoside (0.02 mg/mL (final)) when 1,000 spores (2 µL) from each transformant were grown at 30 °C for 2 days and viewed under UV light. EGL screen based on the ability to hydrolyse the dye particles of the crosslinked and insoluble AZCL-Barley beta-Glucan (I-AZBGL, Megazyme) (0.1 % (w/v)) when 1,000 spores (2 µL) from each transformant were grown at 30 °C for 2 days. CBS 137441 was grown as a negative control. In this study colony no. 7 (*abfB*_t7) was shown to give the strongest fluorescence and colony no. 9 (*eglA*_t9) was shown to give the greatest amount of dye release and hence both were chosen for liquid expression studies

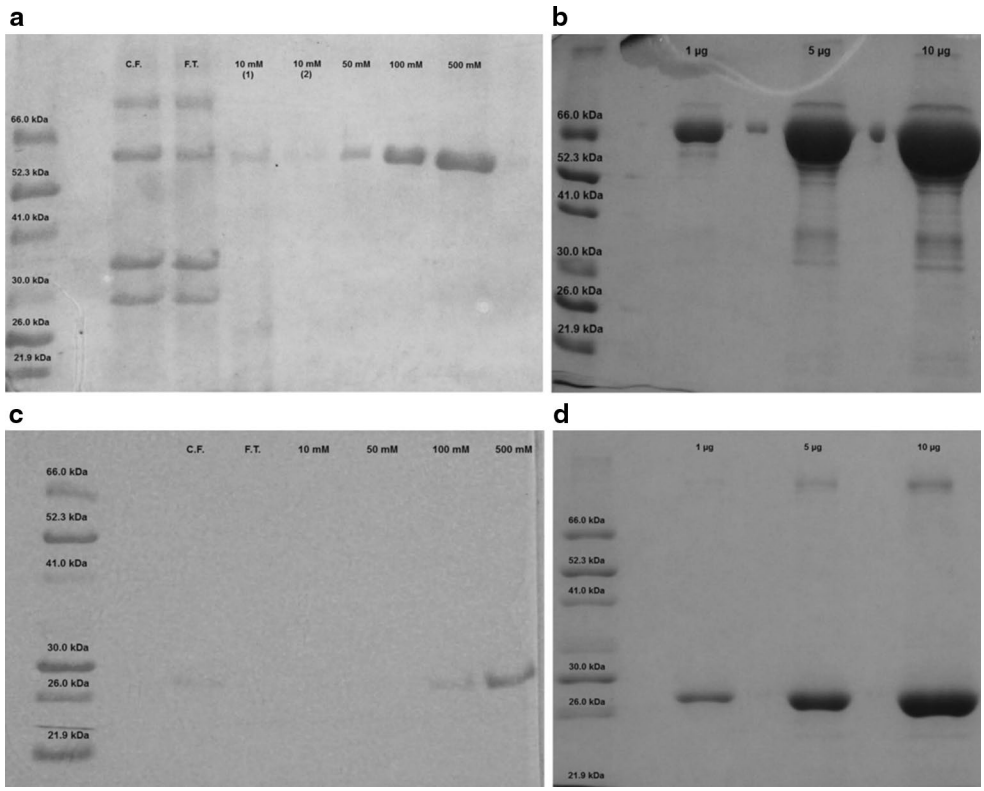

Fig. 3 SDS-PAGE analysis and purification of *A. vadensis* α-L-arabinofuranosidase B (AvAbfB) (**a**) compared to increasing amounts, i.e. 1, 5 and 10 µg, of industrially purified α-L-arabinofuranosidase A from *A. niger* (AnAbfA) (**b**) and *endo*-1,4-β-D-glucanase (AvEglA) (**c**) compared to increasing amounts, i.e. 1, 5 and 10 µg, of industrially purified and orthologous *A. niger* EglA (**d**). Low molecular weight (LMW) ladder produced in house. Culture filtrates (C.F.) were purified by affinity chromatography, equilibrated with buffer (10 mM imidazole, 10 mM HEPES and 500 mM NaCl, pH 7.5) and eluted with a stepwise gradient in same buffer containing 10–500 mM imidazole. Flow through (F.T.) contained proteins which had no specific binding capacity to the resin

Table 1 Purification of α-L-arabinofuranosidase (AbfB) and *endo*-1,4-β-D-glucanase (EglA) from *Aspergillus vadensis*

	Purification-fold	Concentration (mg/mL)	Specific yield (mg/L)	Specific activity (U/mg) 40 °C/50 °C
(A) Purification of α-L-arabinofuranosidase (AbfB)				
Crude (1,500 mL)	–	–	–	–
Purified (20 mL)	75	2.27	30	44/100
(B) Purification of *endo*-1,4-β-D-glucanase (EglA)				
Crude (370 mL)	–	–	–	–
Purified (10 mL)	37	0.92	25	204/280

Specific activities calculated at 40 °C/50 °C under optimum conditions for each enzyme as outlined in "Materials and methods" section

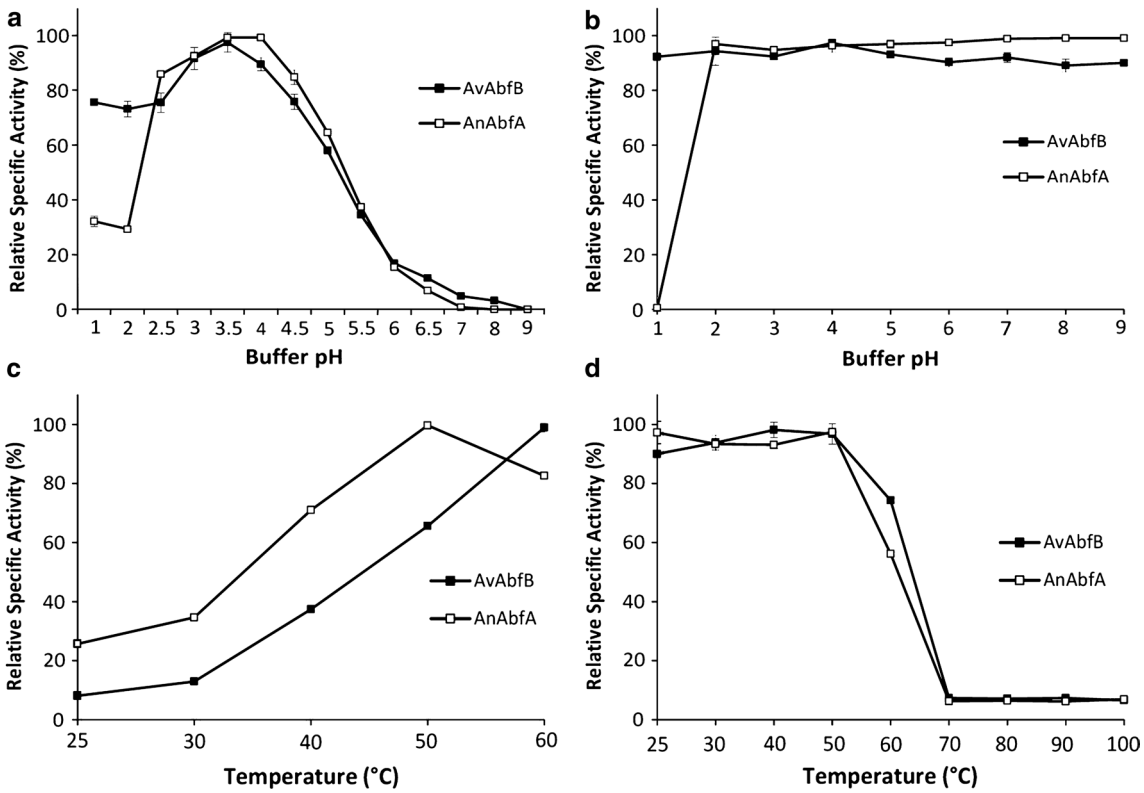

Fig. 4 Biochemical properties of purified recombinant α-arabinofuranosidase from *A. vadensis* (AvAbfB) compared to those of commercially available α-arabinofuranosidase A from *A. niger* (AnAbfA). Values expressed as relative specific activity (%) of maximum activity obtained for that assay. In the case of pH optima and stability, average values were taken where there was an overlap in pH with the use of different buffers. Error bars calculated on standard deviations between technical duplicates. (**a**) pH optima of AvAbfB and AnAbfA; (**b**) pH stability of AvAbfB and AnAbfA; (**c**) Temperature optima of AvAbfB and AnAbfA; (**d**) Temperature stability of AvAbfB and AnAbfA

The enzymatic properties of the recombinant α-arabinofuranosidase B from *A. vadensis* (AvAbfB) compared to that of the commercially available and industrially purified α-arabinofuranosidase A from *A. niger* (AnAbfA) were examined. The optimal pH for both enzymes was pH 3.5 (activity measured at pH 3.5 equalled that at pH 4.0 for AnAbfA) with both retaining ≥90 % activity at pH 3.0 and pH 4.0 (Fig. 4a). AvAbfB demonstrated greater pH stability than AnAbfA, with ≥92 % of AvAbfB activity remaining at all pH's tested, i.e. pH 1.0–pH 9.0, compared to AnAbfA which resulted in 0 % activity at pH 1.0, even with immediate testing after 1 h incubation at this pH (Fig. 4b).

AvAbfB was also more stable at higher temperatures than AnAbfA, with 100 % of activity being preserved for both enzymes up to 50 °C but with 77 % of AvAbfB activity being maintained after a 15 min incubation at 60 °C compared to just 58 % in the case of AnAbfA (Fig. 4d). The temperature optima for these enzymes therefore

Table 2 Relative activities of the purified α-arabinofuranosidase and *endo*glucanase against different substrates

Substrate	Relative activity (%)	
	A. vadensis AbfB	*A. niger* AbfA
p-nitrophenol α-L-arabinofuranoside	100.0	100.0
Wheat flour arabinoxylan	1.5	1.4
Sugar beet arabinan	19.2	5.9
Debranched sugar beet arabinan	3.2	1.0
	A. vadensis EglA	*A. niger* EglA
Barley β-glucan	100.0	100.0
Cellulose	24.0	65.0
Galactomannan	0.0	0.0
Glucomannan	0.2	0.2
Pachyman	0.0	0.0
Soluble starch	0.0	0.0
Xyloglucan	0.0	0.0
p-nitrophenol α-D-glucopyranoside	0.0	0.0
p-nitrophenol β-D-glucopyranoside	0.0	0.0
p-nitrophenol β-D-xylopyranoside	0.0	0.0

All polysaccharide substrates and *p*-nitrophenol substrates were tested at 40 °C and at concentrations of 5 mg/mL (w/v final) and 5 mM (final), respectively, under conditions as described in "Materials and methods" section

differed with AvAbfB giving maximum activity at 60 °C and AnAbfA having an optimum of 50 °C (Fig. 4c). Specific activities for both enzymes in 100 mM citrate phosphate buffer pH 3.5 at 40 °C were 44 U/mg for AvAbfB and 127 U/mg for AnAbfA. These values increased to 100 U/mg for AvAbfB and 221 U/mg for AnAbfA when assayed under optimum pH and at 50 °C.

Additional enzyme activities were also measured for both enzymes on wheat flour arabinoxylan, sugar beet arabinan and debranched sugar beet arabinan relative to the specific activities obtained on *p*-nitrophenyl α-L-arabinofuranoside at 40 °C (Table 2). In these experiments, AvAbfB demonstrated greater activity on all three tested polysaccharides than AnAbfA.

endo-1,4-β-D-glucanase (EglA) production and characterization

Liquid cultures were inoculated with 1×10^6 spores/mL from chosen transformants, grown at 35 °C and 250 rpm and expression levels were monitored on a daily basis by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Optimum AvEglA production was observed after 4 days incubation and resulting culture filtrates were purified by His-tag affinity chromatography. 15 µL samples of C.F. and column fractions were viewed by SDS-PAGE (Fig. 3b) with the 100 and 500 mM fractions containing purified protein. These were pooled and precipitated with 50 % ammonium sulphate. Enzyme fractions were then concentrated and quantified giving a total yield of 25 mg/L purified protein (Table 1B).

The enzymatic properties of the recombinant *endo*-1,4-β-D-glucanase from *A. vadensis* (AvEglA) were compared to the commercially available and industrially purified orthologous *endo*-1,4-β-D-glucanase from *A. niger* (AnEglA). The optimal pH for both enzymes peaked at pH 4.5 with AvEglA demonstrating a narrower optima curve than AnEglA, losing 55 %/40 % relative activity at pH 4.0/pH 5.0, respectively, compared to AnEglA which lost 25 %/6 % activity under the same conditions (Fig. 5a). Both enzymes showed comparable stability at all pH's tested, i.e. stable at pH 1.0–pH 9.0 after 48 h incubation at this pH (Fig. 5b).

The temperature optimum of AvEglA was 50 °C, while AnEglA gave a broader optimum curve, i.e. between 50 and 60 °C but which favoured the higher temperature of 60 °C (50 °C giving ~2.5 % less relative specific activity compared to 60 °C). AnEglA also demonstrating a greater stability profile in maintaining 91 % relative activity at 60 °C compared to AvEglA where activity at this temperature measured only 5 % of that obtained at 50 °C (Fig. 5c, d).

Specific activities for both enzymes in 100 mM sodium acetate buffer pH 4.5 at 40 °C were 204 U/mg for AvEglA and 179 U/mg for AnEglA. These values increased to 280 U/mg for AvEglA and 230 U/mg for AnEglA when assayed under optimum pH and at 50 °C. No activity was detected for either enzyme on galactomannan,

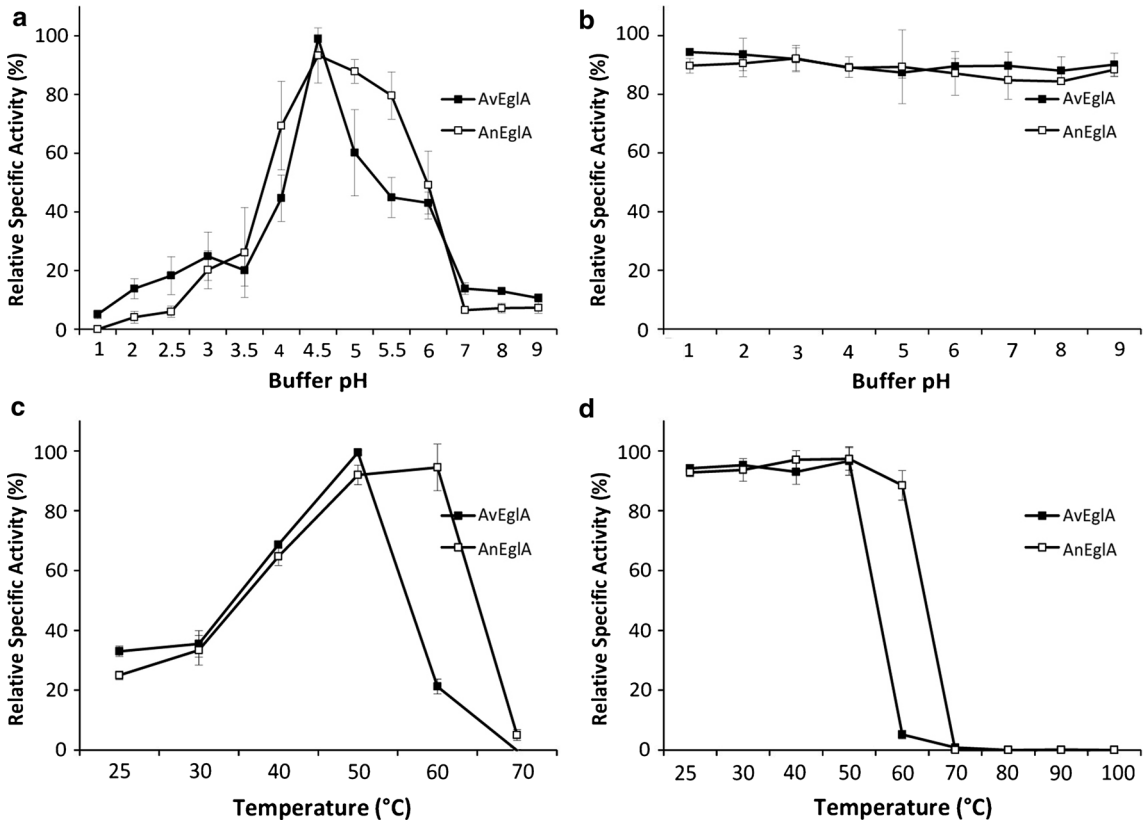

Fig. 5 Biochemical properties of purified recombinant endoglucanase from *A. vadensis* (AvEglA) compared to those of commercially available *endo*-1,4-*β*-D-glucanase from *A. niger* (AnEglA). Values expressed as relative specific activity (%) of maximum activity obtained for that assay. In the case of pH optima and stability, average values were taken where there was an overlap in pH with the use of different buffers. Error bars calculated on standard deviations between technical duplicates. **a** pH optima of AvEglA and nEglA; **b** pH stability of AvEglA and AnEglA; **c** Temperature optima of AvEglA and AnEglA; **d** Temperature stability of AvEglA and AnEglA activity

glucomannan, pachyman, soluble starch, xyloglucan, *p*-nitrophenol *α*-D-glucopyranoside, *p*-nitrophenol *β*-D-glucopyranoside and *p*-nitrophenol *β*-D-xylopyranoside. A lower activity on cellulose was observed for AvEglA than for AnEglA relative to the specific activities obtained on Barley *β*-glucan at 40 °C (Table 2).

Discussion

Over the past 25 years, much research has been devoted to the development of *Aspergillus* as a host for homologous and heterologous protein production. Not only does *Aspergillus* growth excel under fermentation conditions with an exceptional capacity of secreting high levels of homologous product but also their potential in plant polysaccharide degradation and the extensive set of enzyme mixes which they secrete for this purpose is also well recognised [9]. To date, much of this research has been focused on *A. niger* and *A. oryzae*, while the capacity of *A. vadensis*, a close relative of *A. niger,* has remained largely unexplored. In this study, the potential of *A. vadensis* as a host for recombinant protein production was examined by cloning and over-expressing two homologous genes encoding plant biomass degrading enzymes, i.e. *α*-L-arabinofuranosidase (*abfB*) and *endo*-1,4-*β*-D-glucanase (*eglA*).

As shown in Fig. 3a, the *A. vadensis* *α*-L-arabinofuranosidase B (AvAbfB) protein was purified at a molecular weight of ~53 kDa, which corresponds to the calculated predicted mass of 52.5 kDa, based on the obtained amino acid sequence information and to that of the previously recorded 51.0 kDa for the orthologous AbfB enzyme in *A. niger* [16]. Earlier studies reported that the *α*-L-arabinofuranosidases from various *Aspergillus* species had a molecular weight of about 30–118 kDa, depending on the level of *N*- and *O*-glycosylation sites [2]. This appears to be the case for *α*-L-arabinofuranosidase A (AnAbfA) which has a molecular weight of ~62 kDa and is visualised as a single major band on SDS-electrophoresis (Fig. 3b) as is described on the product's data sheet (http://

secure.megazyme.com/Alpha-L-Arabinofuranosidase_A._niger). The biochemical properties of *A. vadensis* α-L-arabinofuranosidase B (AvAbfB) were examined and found to match those of the previously identified orthologous AbfB from *A. niger* with a pH and temperature optimum of pH 3.5 and 60 °C. But interestingly, it was stable at pH values as low as 1.0, at which other α-L-arabinofuranosidases from *A. niger* were not [2]. Comparative biochemical analysis with *A. niger* α-L-arabinofuranosidase A (AnAbfA) demonstrated that AnAbfA had more specific activity towards *p*-nitrophenyl α-L-arabinofuranoside but ~3 times less than AvAbfB on both the branched and debranched sugar beet arabinan. Sugar beet arabinan consists of a 1,5-α-linked backbone to which 1,3-α-linked (and possibly some 1,2-α-linked) L-arabinofuranosyl residues are attached. In previous studies, AbfA from *A. niger* was reported to be incapable of splitting 1,3-α-L- or 1,2-α-L-linked arabinose substituents or arabinose from longer 1,5-α-L-linked arabinose residues whereas AbfB can [36].

Figure 3c shows the purification of *A. vadensis endo*-1,4-β-D-glucanase (AvEglA) at a molecular weight of 26 kDa which too, is in keeping with the calculated predicted mass of 27.72 kDa, based on the obtained amino acid sequence information and with that previously obtained for the purification of the orthologous protein from *A. niger* [33]. This is confirmed with *A. niger endo*-1,4-β-D-glucanase (AnEglA) being visualised at a molecular weight of ~27 kDa and as a single band on SDS-electrophoresis (Fig. 3d), as is described on the products data sheet (http://secure.megazyme.com/Cellulase_endo-1-4-Beta-D-glucanase_A._niger). The biochemical characteristics of *A. vadensis endo*-1,4-β-D-glucanase (AvEglA) are similar to those previously identified in the orthologous enzyme from *A. niger* [33]. Comparative biochemical analysis of AvEglA to the orthologous *endo*-1,4-β-D-glucanase from *A. niger* (AnEglA) showed similar characteristics, both giving equivalent pH optima (pH 4.5) and pH stability (pH 1–pH 9) profiles. Interestingly, a notable difference was displayed for temperature optima and stability, however, with AvEglA having a temperature optimum of 50 °C, compared to AnEglA which gave a broader optimum curve between 50 and 60 °C but which favoured the higher temperature of 60 °C. AnEglA also demonstrated a greater stability profile in maintaining 91 % relative activity at 60 °C compared to AvEglA where activity at this temperature measured only 5 % of that obtained at 50 °C. Both AvEglA and AnEglA demonstrated little to no activity on the additional substrates tested, with the exception of cellulose, where AnEglA demonstrated ~3 times greater activity against this substrate than AvEglA. Cellulose is a polysaccharide of β(1,4)-linked D-glucose units which should be easily degraded by both *endo*-1,4-β-D-glucanases. Sequence alignments for both EglA orthologs studied show a 5 % difference at the amino acid level (Supplemental Figure 1B) which may lead to slight variations in the kinetic properties of the corresponding enzymes. Although both enzymes are visually pure by SDS comparison (Fig. 3c, d), differences in production hosts, glycosylation patterns and purification methods may contribute to the variations recorded.

This study has demonstrated the potential of *A. vadensis* as a host for recombinant protein expression. Not only *A. vadensis* does not acidify the culture medium but also it produces very low levels of extracellular proteases and so facilitates many downstream processes. To date, the use of His-tag affinity chromatography in fungal systems has not been either efficient or practical in fungal systems due to the difficulties experienced with degradation of the Histidine residues by extracellular proteases. Purification of recombinant enzymes from *A. vadensis* through His-tag affinity chromatography has worked well thus far, suggesting its potential as a versatile host in the fundamental research of proteins and for industrial enzyme production. With an initial expression yield of ~30 mg/L, *A. vadensis* shows positive signs of becoming an industrially significant contender but considerable improvements will be required to rival the secretion abilities of commercial production strains such as *A. niger* and *T. reesei*. Strategies for improving recombinant protein production in fungi, including the use of strong promoters and effective secretion signals or gene fusions to genes associated with well-expressed and secreted proteins have been implemented successfully to other systems in the past [32, 41]. Successes have also been obtained using a bioprocessing approach of optimising fungal morphology, mycelia immobilisation and culture conditions [1, 6]. It should be noted, however, that the *A. vadensis* strain used in these experiments is near wild type and has not undergone the extensive strain improvement strategies that have resulted in the commercial *A. niger* and *T. reesei* strains that secrete much higher levels of enzyme, suggesting room for improvement.

The results of this study demonstrate significant variations in biochemical characteristics in the different α-arabinofuranosidases, AnAbfA and AvAbfB but also in the orthologous *endo*-1,4-β-D-glucanases (EglA) when produced from *A. niger* and *A. vadensis*, despite these organisms being taxonomically very close. Despite AnAbfA (GH51) and AvAbfB (GH54) having just 22 % identity at an amino acid level (Supplemental Figure 1A) and being assigned to different CAZy families, the biochemical differences observed between these two enzymes are not significantly greater than those observed in the orthologous EglA comparison which have 95 % identity. In particular, the difference in temperature optima and stability for these orthologous enzymes is noteworthy and would directly impact the applications of these enzymes. Screening of

orthologs from related fungi may therefore be worthwhile for many industrial applications and suggests that it is not always necessary to go to distantly related species or enzyme classifications to get significant changes in biochemical properties. Detailed structural comparison of these orthologous enzymes may reveal insight into the molecular basis of this difference in stability and is worth pursuing in follow-up studies.

Conflict of interest The authors declare no commercial or financial conflict of interest.

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