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Cloning, Overexpression in *Escherichia coli*, and Characterization of a Thermostable Fungal Acetylxylan Esterase from *Talaromyces emersonii*

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The gene encoding an acetylxylan esterase (AXE1) from the thermophilic ascomycete *Talaromyces emersonii* was cloned, expressed in *Escherichia coli*, and characterized. This form of AXE1, rTeAXE1, exhibits increased thermostability and activity at a higher temperature than other known fungal acetyl esterases, thus having huge potential application in biomass bioconversion to high value chemicals or biofuels.

Xylan is the hemicellulose constituent of plants and the most abundant renewable polysaccharide in nature after cellulose. Xylan and its hydrolysis products are potential resources for nutraceuticals, cosmetics, foods, bioalcohol, and industrial fine chemical production (18, 22). Due to its highly heterogeneous nature, synergistic enzymatic action is required for complete xylan degradation. Hardwood and cereal xylans contain a particularly large proportion (~70%) of acetyl substitutions (5).

Acetylxylan esterases (EC 3.1.1.72) release acetate from xylan and xylooligosaccharides and under anhydrous conditions catalyze acetyl group transfer (3). Acetylxylan esterases have been classified as belonging to the families CE1 to -7, -12, and -16 (http://www.cazy.org/CE1_eukaryota.html) in the Carbohydrate-Active Enzymes (CAZy) database. To date, most characterized acetylxylan esterases (AXE) are mesophilic, with several characterized fungal acetyl esterases having been reported (7), and one thermophilic actinomycete AXE was recently reported as well (13). Thermophilic microorganisms are increasingly important industrially as sources of inherently thermostable enzymes.

Talaromyces emersonii is a thermophilic filamentous ascomycete and a known producer of thermostable (hemi-)cellulolytic enzymes with excellent long-term storage properties (8, 10, 16, 17, 23). We report the cloning and physicochemical properties of *T. emersonii* acetylxylan esterase (TeAXE1) and its expression in *Escherichia coli*.

T. emersonii IMI 392299 was grown as previously reported (16), and TeAXE was induced by medium containing 1% (wt/vol) of various carbon sources. Fungal mycelia were harvested and RNA (1% birchwood xylan induced) extracted according to Chomczynski and Sacchi (6). Northern blotting confirmed TeAXE expression (21), and genomic DNA was extracted (20). An *axe1* genomic gene fragment (285 bp) was amplified by PCR with HotStarTaq DNA polymerase (Qiagen), using degenerate *axe* forward and reverse primers (Table 1). The 5′ and 3′ flanking regions were amplified using an mRNA template RACE (rapid amplification of cDNA ends) method (19) with gene-specific primers (Table 1). A full-length *axe1* cDNA [903-bp open reading frame (ORF), 168-bp 3′ untranslated region (UTR) with a poly(A) tail], and genomic DNA (1,049 bp with two 73-bp intronic positions; 196 to 269 bp and 524 to 597 bp) were amplified using *axe* start and stop primers (Table 1). PCR and RACE products were electrophoresed, purified, and sequenced (PE Biosystems 877 robotic system). Homology searches of the TeAXE1 sequence were performed with BLASTp (1, 2) and multiple alignment with ClustalW (11), showing ≤83% homology with mesophilic fungal acetyl esterase CE1 members (Fig. 1). The gene encoding mature TeAXE1 (GenBank accession no. ADX07526.1) was amplified with ThermalAce polymerase (Invitrogen) using rAXE forward and reverse primers.

TABLE 1 Oligonucleotide primers used for amplification of *axe1*

Amplification reaction	Primer	Sequence (5′–3′)[a]
Degenerate PCR	*axe* forward	AACGTGATGGCSGCSACSTAC
	axe reverse	GTASGTSGCSGCCATCACGTT
RACE PCR	RACE outer	TGCGGAGTGTCGATGACCT
	RACE inner	TTCGTGGACGAGACGAAGCAT
	RACE outer	ATCGACACTCCGCAGGTCTGG
	RACE inner	AGCATGTACCCGGGGCTACAA
Full-length amplification	*axe* start	ATGGCACGCTTTTCAATTCTTTCTA
	axe stop	CTACGCAAACCCAAACCACTCCAT
pET100/D-TOPO expression	rAXE forward	CACCCAGGTGCCTCAGGGCTCGCTTC
	rAXE reverse	CTACGCAAACCCAAACCACTCCAT

[a] S = C or G.

Figure 1 shows the multiple alignment of the TeAXE1 putative protein sequence with other fungal AXEs. The existence of a secretion sequence at positions 1 to 22 is suggested. The TeAXE secondary structure is composed of 39.0% α-helices, 22.3% β-strands, and 38.7% loops (Predict Protein Server), consistent with other fungal AXEs. A single putative N-glycosylation site was found at Asn189, and the serine-catalytic triad, conserved in different hydroxylases (esterases, protease, lipase, and cutinase), is also present.

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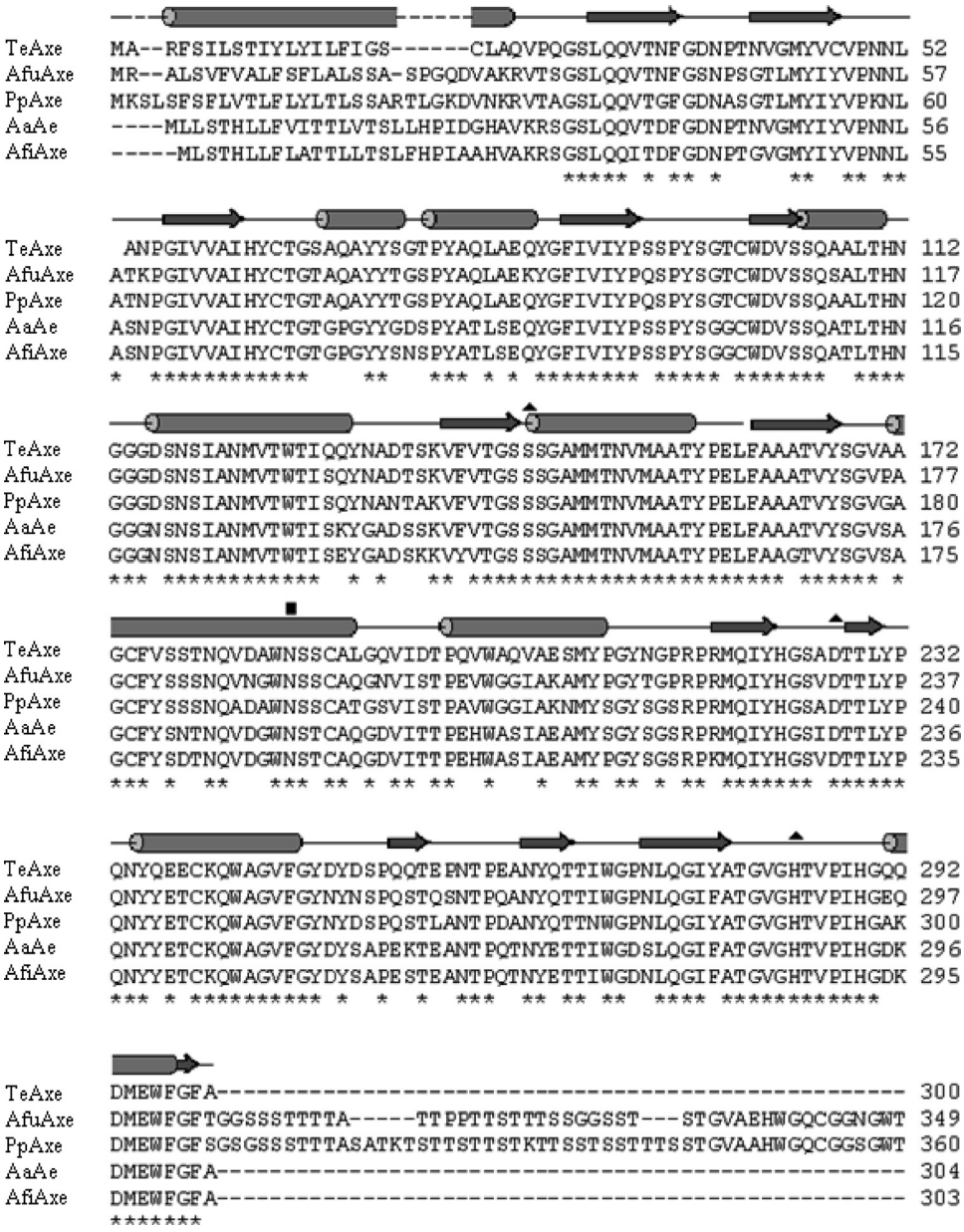

FIG 1 Multiple alignment of the TeAXE1 protein sequence and homologous enzymes. Abbreviations: TeAxe, AXE-1 from *T. emersonii* (GenBank accession number ADX07526.1); AfuAXE, AXE from *Aspergillus fumigatus* (accession number XM_742365.1); PpAXE, AXE-I from *Penicillium purpurogenum* (accession number Q8NJP6); AaAE, acetyl esterase from *Aspergillus awamori* (accession number Q92194); AfiAXE, AXE from *Aspergillus ficuum* (accession number Q96W96). Identical residues are indicated by asterisks. The catalytic serine triad and sole *N*-glycosylation site are indicated by closed triangles and a closed square, respectively. Helices are represented by cylinders, β-strands by arrows, and coils by lines. A dashed line represents gaps in the amino acid sequence alignment.

E. coli was used to express recombinant TeAXE1 (rTeAXE1) with directional ligation in the pET100/D-TOPO vector overexpressed by BL21-Star DE3 cells, as per the user manual (Invitrogen). A protein band was observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with increasing intensity over the 6-h induction time course. To confirm rTeAXE1 production, the terminal amino acid sequence of the protein was analyzed (Procise 494 protein sequencer; Perkin Elmer) and identified as rTeAXE1. The primary N-terminal sequence showed that the recombinant rTeAXE1 lacks a portion of the fused N-terminal His tag (25 to 27 residues), presumably because of differential processing by proteases from the *E. coli* host. Consistent with the deletion of the N-terminal His tag, the Ni column could not purify this protein. Instead, the bacterial cells were collected by centrifugation, and the pellets were frozen. The cells were thawed, suspended in 10 mM citric acid buffer (pH 6.0), and disrupted by sonication at 20 kHz (Minolta sonicator) for three 1-min cycles with 1-min rest intervals. The sample was salted using 50 mM McIlvaine's buffer (pH 4.7) containing 30% (wt/vol) ammonium sulfate. After ultracentrifugation at 40,000 rpm for 30 min under vacuum at 4°C, the supernatant showing hydrolytic activities of *p*-nitrophenyl acetate (*p*-NA) and α-naphthyl acetate (α-NA) was

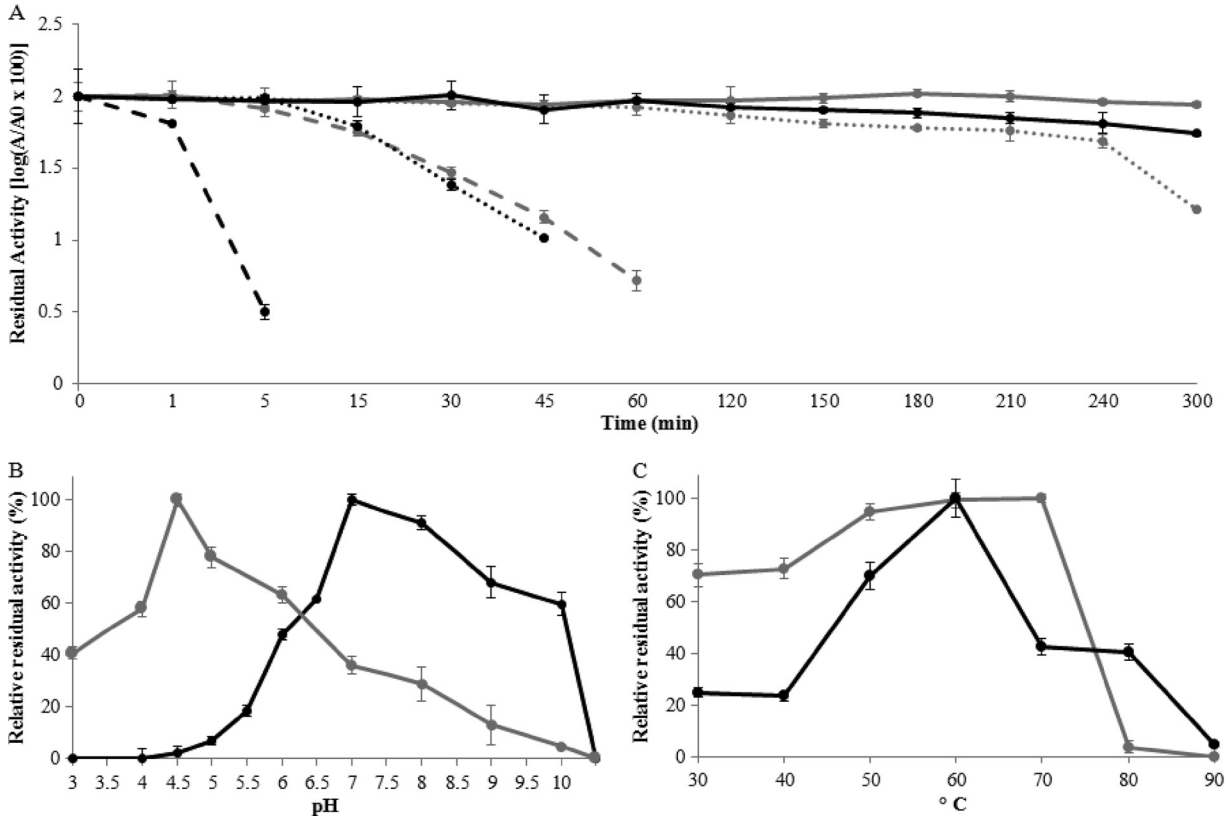

FIG 2 (A) Thermal stability of rTeAXE1 using α-NA and *p*-NA as assay substrates to detect residual enzyme activity at 60°C (solid gray lines, α-NA; solid black lines, *p*-NA), 65°C (dotted gray lines, α-NA; dotted black lines, *p*-NA), and 70°C (dashed gray lines, α-NA; dashed black lines, *p*-NA); (B and C) the effect of pH (B) and temperature (C) on rTeAXE1 hydrolysis of α-NA (black circles) and *p*-NA (gray circles) over a 15-min incubation period. Data are means ± standard deviations ($n = 3$) and are reported as percentages of initial activity.

collected and applied to a butyl-Sepharose hydrophobic interaction chromatography column (XK 26/20; GE Healthcare, United Kingdom). Separation was obtained using a linear gradient of 0.6 to 0 M ammonium sulfate in 50 mM citric acid buffer (pH 5.0). Fractions (5 ml) with absorbance at 280 nm were assayed using *p*-NA and α-NA, as described below. The active fractions were concentrated with ultrafiltration using a 5-kDa-cutoff membrane (Millipore) and stored (−80°C). The final yield and purification were 23% and 2-fold, respectively, based on *p*-NA specific activity, while activity on α-NA resulted in a 1.6-fold purification.

The molecular mass of rTeAXE was calculated as 32 kDa (12% SDS-PAGE gel), and its pI was at pH 3.94, as estimated by isoelectric focusing (5% polyacrylamide gel with electrolytes over a pH gradient from 2.4 to 5.5). Protein gels were stained with Coomassie brilliant blue, and the concentration was determined by the Bradford method (4). Experimentally obtained physicochemical values differ from the theoretical ones (34.4 kDa and pH 4.72, respectively), which is explained by the missing N-terminal His tag (Fig. 2).

To determine activity at various pHs and temperatures, hydrolytic activities for 1 mM *p*-NA and α-NA were measured over 15 min each in 50 mM McIlvaine's buffer (pH 3.0 to 7.5,) (9) or 50 mM universal buffer (pH 6.5 to 10.5) and at various temperatures (30 to 90°C) at pH 5.0. Hydrolysis was determined at 410 nm (*p*-NA) or 310 nm (α-NA), using the extinction coefficients for *p*-nitrophenol and α-naphthol (14, 15). One unit of activity was defined as the amount of rTeAXE hydrolyzing 1 μmol of substrate per min (Fig. 2). Steady-state kinetic constants (K_m and V_{max}) were found using Michaelis-Menten equations and Lineweaver-Burk double-reciprocal plots (Table 2).

Table 2 shows the kinetic constants for hydrolysis of α-NA and *p*-NA by rTeAXE1 and the enzyme inactivation constant (k) and half-life ($t_{1/2}$). The catalytic efficiency for α-NA is 57-fold higher than that for *p*-NA, which may be due to enzyme inhibition by *p*-nitrophenol. Substrate specificity is similar to that of other CE 1 AXEs. Inhibition by *p*-nitrophenol is noteworthy, since various *p*-nitrophenol ester substrates are frequently used for the assay of esterase, lipase, and protease activities (12). The specific activities for the hydrolysis of α-NA and *p*-NA are 28 and 1.4 IU/mg, respectively.

In conclusion, the first acetyl esterase gene was isolated from *T. emersonii* and actively expressed in *E. coli*. It has specificity for small acetylated substrates and is classified in CAZy family CE1. Its

TABLE 2 Steady-state kinetic constants for rTeAXE1

Substrate	K_m (mM)[a]	V_{max} (U/mg)[a]	Catalytic efficiency (V_{max}/K_m)	Inactivation constant (k) 70°C (min^{-1})	$t_{1/2}$ (ln2/k) (min)
p-NA	11 ± 1	0.6 ± 0.1	0.05	0.017	40.8
α-NA	7 ± 1	40 ± 1	5.70	0.013	53.3

[a] Values are means ± standard deviations ($n = 3$).

identity with fungal AXEs, known to deacetylate xylans (7), suggests that this esterase has the ability to liberate acetate from acetylated xylans. The commonly found CAZy CE1 serine catalytic triad is also found in rTeAXE1. Structure-function analysis of this enzyme would provide further information about the catalytic mechanism and thermostability of rTeAXE. This is the first heterologously overexpressed AXE from a thermophilic fungal species, and our findings will facilitate the further application of such enzymes in health, food, and nonfood processes.

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