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Acetyl Xylan Esterase Axe1 (*T. reesei*, Carbohydrate Esterase Family 5) Supplemented to a (Hemi)cellulolytic Preparation Enhances Degradation of Recalcitrant Corn Silage Polysaccharides

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The increase in hydrolytic activity towards corn silage water unextractable solids by supplementation of acetyl xylan esterase 1 (*T. reesei, Tr*Axe1), belonging to carbohydrate esterase (CE) family 5, to an *A. niger / T. emersonii* enzyme preparation is presented. *Tr*Axe1 was cloned and expressed in *A niger*. The hydrolytic activity of *Tr*Axe1 was analysed with a *p*-nitrophenyl-acetate standard assay and by the measurement of the acetic acid released from neutral and acidic eucalyptus xylo-oligosaccharides. The activity obtained for *Tr*Axe1 was compared to acetyl xylan esterases belonging to CE families 1, 5 and 16 from *A. niger*. High activities were obtained for the CE5 classified acetyl xylan esterases from *T. reesei* and *A. niger*. The data obtained confirm the suggested increase in hydrolytic activity by supplementation of CE5 classified acetyl xylan esterases to the (hemi)cellulolytic *A. niger / T. emersonii* preparation (Neumüller et al. 2014b).

1. Introduction

Corn silage is a valuable renewable resource for the production of biofuels (Colussi et al. 2013). The enzymatic conversion of corn polysaccharides to fermentable sugars is limited by feedstock recalcitrance (Himmel et al. 2007). Presence of complex xylan substituents render corn arabinoxylan recalcitrant (Appeldoorn et al. 2010, 2013). Acetylation of the xylan backbone of corn silage and recalcitrant corn fractions is a main impediment for the hydrolysis by industrial enzyme preparations (Neumüller et al. 2013).

Various enzyme activities are necessary to degrade corn arabinoxylan. Beta-1,4-endo-xylanases and beta-1,4-xylosidases are required to degrade the xylan backbone. Furthermore, alpha-arabinofuranosidases, arabinoxylan arabinofuranohydrolases, alpha-glucuronidases, alpha-1,4-galactosidases, beta-1,4-galactosidases, feruloyl/p-coumaroyl esterases and acetyl xylan esterases are necessary accessory enzymes. Acetyl xylan esterases are classified in structurally related carbohydrate esterase (CE) families (Carbohydrate-active enzymes database, www.cazy.org). These accessory acetyl esterases are required for the conversion of acetylated substrates (Biely et al. 2012).

By screening for the enzymatic degradation of corn silage fractions, high levels of arabinoxylan conversion were obtained by supplementation of culture filtrates from *T. reesei* and *T. longibrachiatum* to an industrial *A. niger / T. emersonii* preparation (Neumüller et al. 2014a). Our previously obtained data suggest synergistic potential by an acetyl xylan esterase 1 (Axe1, CE5) rich fraction of a *T. longibrachiatum* preparation with the *A. niger / T. emersonii* preparation. Previously we found that Axe1 is capable of hydrolysing acetylated xylo-oligosaccharides that are not deacetylated by the acetyl xylan esterases present in the *A. niger / T. emersonii* preparation (Neumüller et al. 2014b).

In this work the acetyl xylan esterase from *T. reesei* belonging to CE family 5 (*Tr*Axe1, cloned and expressed in *A. niger*), was supplemented to a (hemi)cellulolytic enzyme preparation and the increase in

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the conversion of corn silage water unextractable solids (WUS) was monitored. The activity of *Tr*Axe1 on *p*-nitrophenyl acetate and neutral and acidic eucalyptus model oligosaccharides was determined and compared to activities obtained for acetyl xylan esterases from *A. niger* belonging to CE families 1, 5 and 16.

2. Materials and methods

2.1 Materials

The corn silage water unextractable solids (WUS) used have been described previously (Neumüller et al. 2014a). The composition of WUS is shown in Table 1. *Eucalyptus globulus* xylan hydrolysate was kindly donated by Prof. Dr. J.C. Parajo of the University of Vigo-Ourense, Spain (Gullón et al., 2008). Neutral, linear acetylated xylo-oligosaccharides (AcXOS) and acidic, acetylated 4-*O*-methyl glucuronic acid (MeGlcA)-substituted xylo-oligosaccharides (AcUXOS) fractions from the eucalyptus xylan hydrolysate and their composition have been described (Koutaniemi et al. 2013, Table 1).

Table 1: Composition of corn silage water unextractable solids (WUS, Neumüller et al. 2014a), linear acetylated xylo-oligosaccharides (AcXOS) and acetylated 4-O-methyl glucuronic acid (MeGlcA)-substituted xylo-oligosaccharides (AcUXOS; Koutaniemi et al. 2013).

sample	starch	non-starch Glc	Xyl	Ara	Gal	Man	Rha	ferulic acid	*p*-coumaric acid	acetic acid	MeGlcA
WUS*	40	305	236	43	12	6	2	9	12	39	n.d.
AcXOS*	n.d.	93	803	18	75	62	n.d.	n.d.	n.d.	117	not detected
AcUXOS*	n.d.	not detected	765	0	42	traces	n.d.	n.d.	n.d.	138	207

[n.d. (not determined)]

**) Results expressed as mg g^{-1} dry matter (DM).*

2.2 Enzymes

Acetyl xylan esterases from *A. niger* belonging to CE families 1, 5 and 16 (*An*CE1, *An*CE5 and *An*CE16) were from DSM (Heerlen, The Netherlands). An acetyl xylan esterase (CE1) was purified from an industrial *A. niger* culture filtrate [*An*CE1(AEC,SEC) Neumüller et al. 2014b]. Figure 1 shows the SDS-PAGE of *An*CE1, *An*CE5 and *An*CE16 and the purified *An*CE1(AEC,SEC).

The crude enzyme preparations (*A.niger, T. emersonii, T. longibrachiatum*) were described previously (Neumüller et al. 2014a).

Cloning and expression of TrAxe1

The protein sequence of *T. reesei* acetyl xylan esterase 1 (*Tr*Axe1, accession: jgi trire1 32857) was retrieved from the National Center for Biotechnology Information (Bethesda, MD, USA; www.ncbi.nlm.nih.gov). Codon pair optimization of the cDNA for the expression in *A. niger* ISO527 (DSM) was done at DSM BioIT (Delft, The Netherlands). The cDNA (*Tr*Axe1) was cloned in the AnGLA.bbn vector, containing PglaA/3'glaA flankings and a hygromycin B selection marker, by DNA2.0 (Menlo Park, CA, USA) and transformed in *A. niger* ISO527. Successfully transformed *A. niger* ISO527 cultures were selected on potato dextrose agar containing hygromycin B (60 µg mL^{-1}) and grown (glucose medium + 10 µM $CuSO_4$; DSM, Delft Biotechnology Center, The Netherlands) until the glucose was depleted, followed by centrifugation (4500 g, 10 min) and filtration [0.2 µm PES bottle top filter (Nalgene, Rochester, NY, USA)].

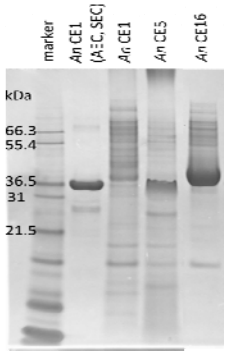

Figure 1: SDS-PAGE of the culture filtrates from A. niger ISO527 containing acetyl esterases belonging to carbohydrate esterase (CE) families 1, 5 and 16 and the purified acetyl xylan esterase [AnCE1(AEC, SEC)] from a crude A. niger preparation.

2.3 Protein concentration

The culture filtrates (270 µL) were incubated with 830 µL acetone (900 g kg^{-1}), trichloroacetic acid solution (0.7 mol L^{-1}) and stored on ice for 1 h. The tubes were centrifuged at 3200 g, 4 ºC for 30 min. The supernatant was discarded and the pellet was dried at room temperature (RT) for 1 h. The pellet was dissolved in 3 mL BioQuant protein reagent (Merck, Darmstadt, Germany). After addition of 1 mL of water to each sample, the samples were stored in the dark for 1 h at RT and the absorbance was measured at 546 nm with a Hitachi U2900 spectrophotometer (Hitachi, Tokyo, Japan). A calibration curve was prepared with bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, USA).

2.4 Enzyme assays

p-Nitrophenyl acetate (p-NP-Ac) assay

Enzyme assays with *p*-NP-Ac were carried out as described previously (Juturu et al. 2013) in a 96 well plate at 300 µL scale in sodium acetate buffer (10 mM, pH 5.0). The *p*-nitrophenol release was determined by continuous measurement for 15 min at 405 nm after addition of 0.01 mg enzyme using an Infinite M1000 PRO microplate reader (Tecan Group Ltd., Männedorf, Switzerland). A calibration curve was prepared with *p*-nitrophenol standard solution (Sigma-Aldrich).

Acetic acid released

AcXOS and AcUXOS (5 g L^{-1}) were incubated in sodium citrate buffer (50 mM, pH 5.0, containing 0.5 g kg^{-1} sodium azide) with acetyl xylan esterases (10 g kg^{-1} DM) for 24 h at 50 ºC and 700 rpm. The samples were heat inactivated (98 ºC, 5 min). The acetic acid released was determined with the K-ACET kit from Megazyme (Wicklow, Ireland).

Synergy assay

*Tr*Axe1 (2 g protein kg^{-1} substrate) was applied in combination with the *A. niger* / *T. emersonii* culture filtrates mixture (100 g protein kg^{-1} substrate) to incubate WUS. Higher concentrations of *the A. niger* / *T. emersonii* culture filtrates mixture did not result in increased monosaccharide release (data not shown). Incubations of WUS (1 % w/v) were done at 1 mL scale at 50 °C and 300 rpm for 48 h in sodium phosphate buffer (50 mM, pH 5.0, 0.5 g kg^{-1} sodium azide). After incubation, the samples were heat inactivated (98 °C for 10 min), centrifuged (20.000 g, 10 min, RT) and analyzed for the monosaccharides released by high performance anion-exchange chromatography (HPAEC) according to the procedure described previously (Neumüller et al. 2014a).

3. Results and discussion

3.1 Cloning and expression of *Tr*Axe1

In order to determine the presence and expression level of *Tr*Axe1 in the culture filtrates of two successfully transformed *A. niger* ISO527 cultures, SDS-PAGE analysis was performed. A protein band at 34 kDa was obtained for both culture filtrates, matching the reported molecular mass of *Tr*Axe1 (Sundberg and Poutanen 1991). A clearly higher secretion level of *Tr*Axe1 was observed in culture filtrate B (Figure 2). Hence, this culture filtrate was used for further experiments. The SDS-PAGE shows that presence of other proteins in the supernatant was low (9% of the total proteins present, culture filtrate B). The relative amounts (%) of *Tr*Axe1 in the culture filtrates obtained are shown in Figure 2.

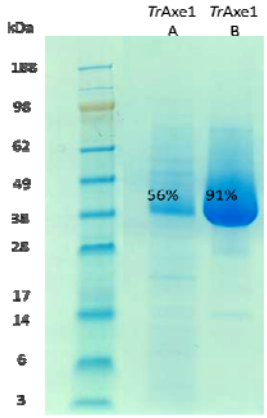

Figure 2: SDS-PAGE of TrAxe1 containing culture filtrates. The relative amounts of Tr*Axe1 are shown as % of the total proteins present.*

3.2 Hydrolysis of WUS by supplementation of *Tr*Axe1 to an industrial (hemi)cellulolytic preparation

The monosaccharides released by incubation of WUS with the *A. niger* / *T. emersonii* preparation, the *T. longibrachiatum* preparation and an Axe1 (CE5) rich fraction (*T. longibrachiatum*) supplemented to the *A. niger* / *T. emersonii* preparation have previously been determined and are shown in Figure 3 (Neumüller et al. 2014b). The observed increase in monosaccharides released by supplementation of Axe1 (*T. longibrachiatum*, CE5) to the *A. niger* / *T. emersonii* preparation suggests synergy by Axe1 (CE5) and the *A. niger* / *T. emersonii* mixture. We previously found that supplementation of purified *An*CE1 to the *A. niger* / *T. emersonii* preparation resulted in lower monosaccharide release compared to supplementation of the Axe1 rich fraction from *T. longibrachiatum* (data not shown). Since the Axe1 rich fraction (*T. longibrachiatum*) also contained other carbohydrases, the nearly pure *Tr*Axe1 (CE5), obtained by expression with *A. niger* ISO527, was supplemented to the *A. niger* / *T. emersonii* preparation. The increase in monosaccharides released for the hydrolysis of WUS compared to hydrolysis with the sole *A. niger* / *T. emersonii* preparation was determined. An increase in the monosacchrides released of 23, 26 and 2 mg g^{-1} for Xyl, Glc and Ara, respectively, was obtained for the hydrolysis of WUS by incubation with the TrAxe1 supplemented *A. niger* / *T. emersonii* preparation compared to levels obtained by sole application of the *A. niger* / *T. emersonii* preparation. The data obtained confirm the suggested synergistic potential by supplementation of the CE5 classified acetyl xylan esterase to the *A. niger* / *T. emersonii* culture filtrates mixture.

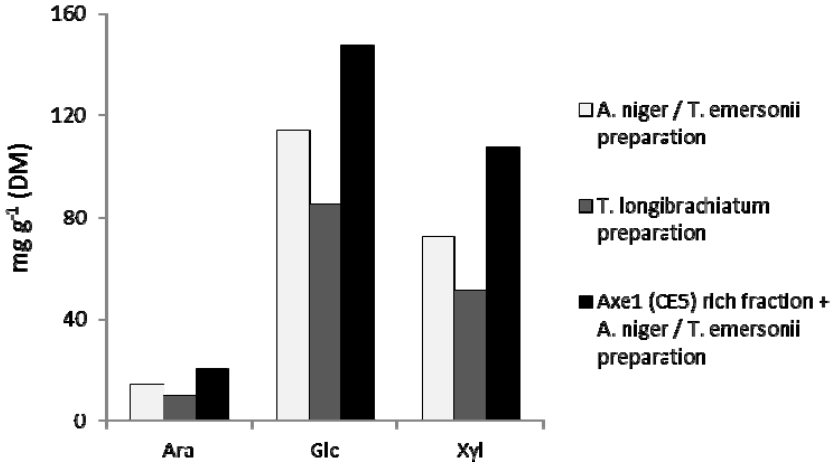

Figure 3: Monosaccharides released by incubation of corn silage water unextractable solids (WUS) with the A. niger / T. emersonii preparation, the T. longibrachiatum preparation and an Axe1 (CE5) rich fraction (T. longibrachiatum) supplemented to the A. niger / T. emersonii preparation (Neumüller et al. 2014b).

3.3 Activity of *Tr*Axe1 compared to *An*CE1, *An*CE5 and *An*CE16

Activity on p-nitrophenyl acetate (p-NP-Ac)

The activity of *Tr*Axe1 on *p*-NP-Ac was determined and compared to activities obtained for acetyl xylan esterases classified as belonging to CE families1, 5 and 16 from *A. niger*. Figure 4 shows the hydrolysis curve obtained by detection of *p*-nitrophenol released from incubations with *p*-NP-Ac. *Tr*Axe1 (CE5) and *An*CE5 showed high activities on *p*-NP-Ac.

It was reported that standard assays are not always suitable to accurately predict the hydrolysis of plant polysaccharides (Kabel et al. 2006). Therefore the acetic acid released by incubation of linear acetylated xylo-oligosaccharides (AcXOS) and acetylated 4-O-methyl glucuronic acid (MeGlcA)-substituted xylo-oligosaccharides (AcUXOS) has been determined.

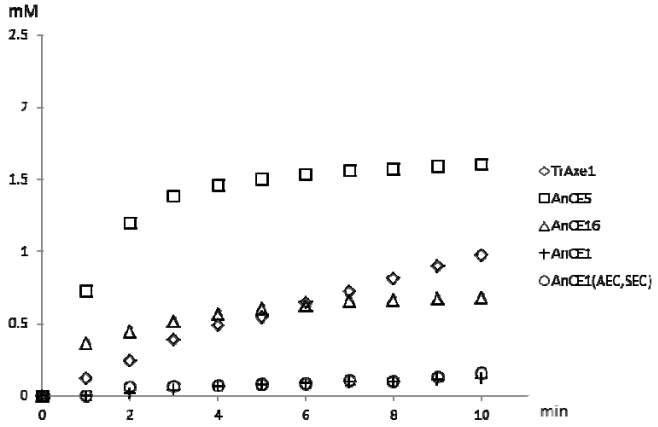

Figure 4: Hydrolysis of p-nitrophenyl acetate (mM p-nitrophenol released) by the different culture filtrates containing TrAxe1, AnCE1, AnCE5, AnCE16 and the purified acetyl xylan esterase [AnCE1(AEC, SEC)] from a crude A. niger preparation.

Acetic acid release

The acetic acid released by incubation of AcXOS and AcUXOS eucalyptus xylo-oligosaccharides with *Tr*Axe1 and acetyl xylan esterases from *A. niger* belonging to CE families 1, 5 and 16 was determined and is shown in Figure 5. The CE5 classified acetyl xylan esterases from *T. reesei* and *A. niger* showed high levels of acetic acid released. This is in agreement with the high activities observed for the CE5 classified enzymes by hydrolysis of *p*-NP-Ac as described above. The acetic acid released by the CE16 classified acetyl xylan esterase was higher than levels obtained with *An*CE1. CE16 classified enzymes have been reported to deacetylate *O*-4 acetylated xylopyranosyl units, which might be present in the eucalyptus xylo-oligosaccharides mixture due to acetyl migration (Biely et al. 2011). For a CE1 classified acetyl xylan esterase from *S. commune* a preference towards 2,3-diacetylated xylo-oligosaccharides was reported (Biely et al. 2013). 2,3-diacetylated xylopyranosyl units are present in eucalyptus heteroxylan (Evtuguin et al. 2002), but their amounts are indicated to be lower compared to *O*-2 or *O*-3 acetylated xylopyranosyl units. Differences in the deacetylation efficiency obtained by the hydrolysis of eucalyptus xylo-oligosaccharides might be due to the positional preference of the enzymes, their affinity to the substrate or hindrance by other xylan substituents.

No presence of monosaccharide releasing side activities was observed by incubation of WUS with the acetyl xylan esterases used (data not shown).

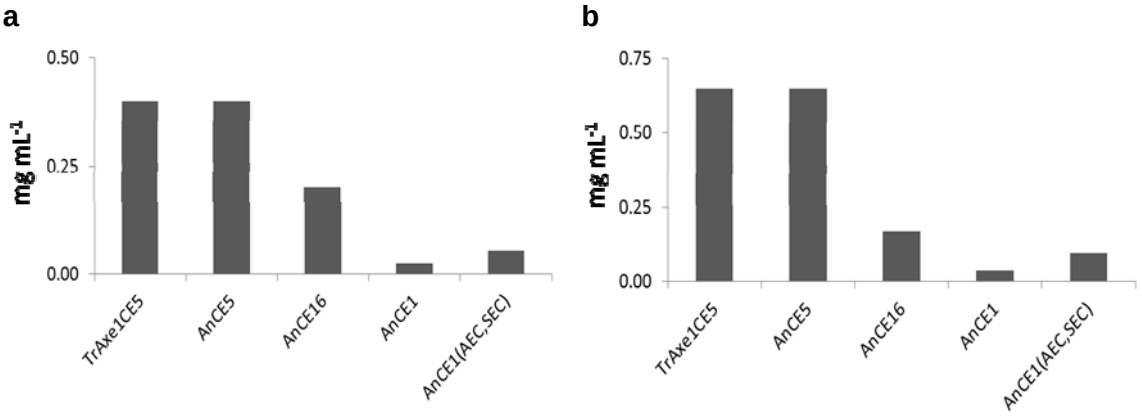

Figure 5: Acetic acid released by hydrolysis of (a) linear acetylated xylo-oligosaccharides (AcXOS) and (b) acetylated 4-O-methyl glucuronic acid (MeGlcA)-substituted xylo-oligosaccharides (AcUXOS) with TrAxe1(CE5) and A. niger acetyl esterases [AnCE1, AnCE1(AEC,SEC), AnCE5 and AnCE16].

4. Conclusions

A high secretion level of *Tr*Axe1 was observed in the culture supernatant of recombinant *A. niger* ISO527. The hydrolytic rate for CE family 5 classified *Tr*Axe1 and *An*CE5 for the hydrolysis of *p*-NP-Ac and the levels of acetic acid released from eucalyptus xylo-oligosaccharides were higher compared to *An*CE1 and *An*CE16. The hydrolytic gain for the hydrolysis of WUS by supplementation of the CE5 classified acetyl xylan esterase to a (hemi)cellulolytic enzyme mixture was confirmed by an increase in substrate conversion after addition of nearly pure *Tr*Axe1 (CE5) to the *A. niger* / *T. emersonii* preparation.

References

Appeldoorn M.M., Kabel M.A., Van Eylen D., Gruppen H., Schols H.A., 2010, Characterization of oligomeric xylan structures from corn fiber resistant to pretreatment and simultaneous saccharification and fermentation, J. Agric. Food Chem., 58, 11294-11301.

Appeldoorn M.M., de Waard P., Kabel M.A., Gruppen H., Schols H.A., 2013, Enzyme resistant feruloylated xylooligomer analogues from thermochemically treated corn fiber contain large side chains, ethyl glycosides and novel sites of acetylation, Carbohyd. Res., 381, 33-42.

Biely P., 2012, Microbial carbohydrate esterases deacetylating plant polysaccharides, Biotechnol. Adv., 30, 1575-88.

Biely P., Cziszárová M., Uhliariková I., Agger J.W., Li X.L., Eijsink V.G.H., Westereng B., 2013, Mode of action of acetylxylan esterases on acetylated gucuronoxylan and acetylated oligosaccharides generated by a GH 10 endoxylanase. Biochim. Biophy. Acta, 1830(11), 5075-86.

Biely P., Mastihubová M., Tenkanen M., Eyzaguirre J., Li X.L., Vrsanská M., 2011, Action of xylan deacetylating enzymes on monoacetyl derivatives of 4-nitrophenyl glycosides of β-D-xylopyranose and α-L-arabinofuranose, J. Biotechnol., 151(1), 137-42.

Colussi I., Cortesi A., Piccolo C.D., Callo V., Fernandez A.S.R., Vitanza R., 2013, Improvement of methane yield from maize silage by a two-stage anaerobic process, Chemical Engineering Transactions, 32, 151-156.

Evtuguin D.V., Tomás J.L., Silva A.M.S., Pascaol Neto C., 2002, Characterization of an acetylated heteroxylan from *Eucalyptus globulus* Labill, Carbohyd. Res., 338, 597-604.

Gullón P., González-Muñoz M.J., Domínguez H., Parajó J.C., 2008, Membrane processing of liquors from *Eucalyptus globulus* autohydrolysis, J. Food Eng., 87(2), 257-65.

Himmel M.E., Ding S.Y., Johnson D.K., Adney W.S., Nimlos M.R., Brady J.W., Foust T.D., 2007, Biomass recalcitrance: engineering plants and enzymes for biofuels production, Science, 315(5813), 804-7.

Juturu V., Aust C., Wu J.C., 2013, Heterologous expression and biochemical characterization of acetyl xylan esterase from Coprinopsis cinerea, World J. Microbiol. Biotechnol., 29(4), 597-605.

Koutaniemi S., van Gool M.P., Juvonen M., Jokela J., Hinz S.W., Schols H.A., Tenkanen M., 2013, Distinct roles of carbohydrate esterase family CE16 acetyl esterases and polymer-acting acetyl xylan esterases in xylan deacetylation. J. Biotechnol., pii: S0168-1656(13)00442-2.

Kabel M.A., van der Maarel M.J., Klip G., Voragen A.G., Schols H.A., 2006, Standard assays do not predict the efficiency of commercial cellulase preparations towards plant material, Biotechnol. Bioeng., 93(1), 56-63.

Neumüller K.G., Carvalho de Souza A., Van Rijn J., Appeldoorn M.M., Streekstra H., Schols H.A., Gruppen H., 2013, Fast and robust method to determine phenoyl and acetyl esters of polysaccharides by quantitative (1)h NMR, J. Agric. Food Chem., 61(26), 6282-87.

Neumüller K.G., Streekstra H., Schols H.A., Gruppen H., 2014a, Synergistic action of enzyme preparations towards recalcitrant corn silage polysaccharides, Biomass Bioenerg., 60, 88-97.

Neumüller K.G., Streekstra H., Schols H.A., Gruppen H., 2014b, Trichoderma longibrachiatum acetyl xylan esterase 1 enhances hemicellulolytic preparations to degrade corn silage polysaccharides, manuscript submitted for publication.

Sundberg M., Poutanen K., 1991, Purification and properties of two acetylxylan esterases of Trichoderma reesei, Biotechnol. Appl. Biochem., 13, 1-11.