Article

A New Esterase from *Thermobifida halotolerans* Hydrolyses Polyethylene Terephthalate (PET) and Polylactic Acid (PLA)

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Abstract: A new esterase from *Thermobifida halotolerans* (Thh_Est) was cloned and expressed in *E. coli* and investigated for surface hydrolysis of polylactic acid (PLA) and polyethylene terephthalate (PET). Thh_Est is a member of the serine hydrolases superfamily containing the -GxSxG- motif with 85–87% homology to an esterase from *T. alba*, to an acetylxylan esterase from *T. fusca* and to various *Thermobifida* cutinases. Thh_Est hydrolyzed the PET model substrate bis(benzoyloxyethyl)terephthalate and PET releasing terephthalic acid and mono-(2-hydroxyethyl) terephthalate in comparable amounts (19.8 and 21.5 mmol/mol of enzyme) while no higher oligomers like bis-(2-hydroxyethyl) terephthalate were detected. Similarly, PLA was hydrolyzed as indicated by the release of lactic acid. Enzymatic surface hydrolysis of PET and PLA led to a strong hydrophilicity increase, as quantified with a WCA decrease from 90.8 ° and 75.5 °to 50.4 °and to a complete spread of the water drop on the surface, respectively.

1. Introduction

Enzymes have been used for a long time in many industrial processes [1–4] and consumer applications [5–7]. They allow simplification of complex chemical processes [8], synthesis of new molecules [9], or as additives they can increase the performance of the product in which they are formulated [10,11]. One of the fields in which enzymes are increasingly capturing the interest of researchers across the globe is material science. There are several interesting examples describing the functionalization of natural fibers using enzymes in order to increase antibacterial properties [12], antioxidant capacity [12–14], or to increase compatibility with synthetic materials [15]. Nevertheless, it is still a challenge to find suitable enzymes for the biotransformation of non-natural and polymeric substrates.

Recently, enzymes have been demonstrated to be suitable alternative tools for hydrophilization of polymer surfaces. Hydrophilization of polyesters is essential for numerous applications of PLA and PET, ranging from textiles to composites and medical devices [1,16]. Typically, chemical methods [17,18] or plasma technology are used to increase the hydrophilicity of polymers [19,20] or to create reactive groups on the surface of inert materials [21]. Enzyme-based approaches would avoid the use of harsh chemicals [22] and/or require considerable less energy [23].

Poly(alkyleneterephthalates) (PAT), and particularly polyethylene terephthalate (PET), are one of the most widely used synthetic polymers worldwide [24] which can be functionalized via limited enzymatic hydrolysis of the ester bond of the polymer backbone [25–29]. Cutinases [30–32], lipases [29,32], esterases [26,30,33] including paranitrobenzyl esterase [34] have been assessed for this purpose.

Among cutinases, representatives from *Aspergilllus oryzae*, *Humicola insolens* [35], *Penicillium citrinum* [36], *Fusarium solani* [30,37], *Thermobifida fusca* [29] and *Thermobifida cellulolysitica* [27] have been described to hydrolyze PET. Considerably fewer lipases have been described to hydrolyze PET, including enzymes from *Candida antarctica* [25], *Thermomyces lanuginosus* [32], *Burkholderia spp.* [38] and *Triticum aestivum* [39]. Esterases were believed to have less potential for surface hydrolysis of polyesters than cutinases or lipases [26,34].

Here we demonstrate for the first time that the esterase Thh_Est from *Thermobifida halotolerans* can efficiently hydrolyze PET and PLA thereby considerably increasing surface hydrophilicity of these materials.

2. Materials and Methods

2.1. Chemicals

Methanol and acetonitrile were purchased from Roth (Carl Roth, Karlsruhe, Germany). All other chemicals were of analytical grade from SIGMA (Germany) except the model substrate

bis(benzoyloxyethyl)terephthalate (3PET) which was synthesized in two steps as previously described [40].

2.2. Bacterial Strains, Plasmids and Culture Conditions

Thermobifida halotolerans DSM44931 was obtained from the German Resource Centre for Biological Material (DSMZ, Germany). The strain was maintained on LB/agar plates and cultivated in 500 mL shaking flasks (200 mL LB medium, 37 °C, 160 rpm, 24 h). Cells were harvested by centrifugation (3,200× g, 4 °C, 20 min). Vector pET26b(+) (Novagen, Germany) was used for expression of Thh_Est in *E. coli* BL21-Gold (DE3) (Stratagene, Germany).

2.3. General Recombinant DNA Techniques

All DNA manipulations described in this work were performed by standard methods [41]. The PCR was performed in a Gene Amp® PCR 2200 thermocycler (Applied Biosystems, USA). Digestion of DNA with restriction endonucleases (New England Biolabs, USA), dephosphorylation with alkaline phosphatase (Roche, Germany) and ligation with T4 DNA-ligase (Fermentas, Germany) were performed in accordance to the manufacturer's instructions. Plasmid Mini Kit from Qiagen (Germany) was used to prepare plasmid DNA. Plasmids and DNA fragments were purified by Qiagen DNA purification kits (Qiagen, Germany).

2.4. Cloning of Thh_est

The gene *Thh_est was* amplified from the respective genomic DNA of *T. halotolerans* DSM44931 by standard polymerase chain reaction (PCR). On the basis of the known sequences of genes coding for cutinases from *T. fusca* YX (Genbank accession numbers YP_288944 and YP_288943) two primers were designed, 5'-CCCCCGCTCATATGGCCAACCCCT ACGAGCG-3' (forward primer) and 5'-GTGTTCTAAGCTTCAGTGGTGGTGGTGGTGGTGCTCGAGTGCCAGGCACGAGA GTAGT-3' (reverse primer), facilitating amplification of the respective gene without signal peptide and introduction of the 6xHis-Tag at the C-terminus of the esterase. The designed primers included the restriction sites *Nde*I and *Hind*III for cloning the gene into the vector pET26b(+). The PCR was done in a volume of 50 μL with genomic DNA as template, 0.4 μM of each primer, 0.2 mM dNTP's, 5 units Phusion^{TM} DNA polymerase (Finnzymes) and 1×reaction buffer provided by the supplier. The DNA amplification was performed in 30 cycles according to the instructions for the Phusion^{TM} DNA polymerase. The amplified PCR-products were purified, digested with *Nde*I and *Hind*III, ligated to pET26b(+) and transformed into *E. coli* BL21-Gold(DE3). The nucleotide sequence was determined by DNA sequencing.

2.5. DNA Sequencing, Alignments and Deposition of Sequence Data

DNA was sequenced as custom service by Agowa (Germany). DNA analysis was performed with Vector NTI Suite 10 (Invitrogen, USA). BLAST search was performed using the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics, and sequences of related proteins were aligned using the Clustal W program (Swiss EMBnet node server). The

nucleotide sequence of *Thh_est* has been deposited in the GenBank database under submission JQ339742.

2.6. Expression and Purification

Thh_Est was expressed in shake flasks. Therefore the plasmid was freshly transformed in *E. coli* BL21-Gold(DE3). Transformants were used to inoculate 20 mL Luria-Bertani (LB) broth supplemented with 40 μg/mL kanamycin. The culture was grown overnight on a rotary shaker at 30 ℃ and 160 rpm. For inoculation of the main culture, 1 mL of the overnight culture was transferred to 200 mL of the same media and incubated at 30 ℃. At an optical density (600 nm) between 0.6 and 0.8 the culture was cooled down to 20 ℃ and induced by addition of 0.05 mM IPTG. After induction for 24 h the cells were harvested by centrifugation (3,200 g, 4 ℃, 20 min). Cell pellet was disrupted by sonification using three-times 30-s pulses under ice cooling (cell disruptor from BRANSON Ultrasonics). Purification of the enzyme was performed by affinity chromatography according to the manufacturer's instructions (IBA BioTAGnology, Germany). Afterwards the HisTag elution buffer was exchanged with 100 mM Tris HCl pH 7.0 by the use of PD-10 desalting columns (Amersham Biosciences).

2.7. Esterase Activity Assay

Esterase activity using *p*-nitrophenyl acetate (PNPA) and *p*-nitrophenyl butyrate (PNPB) as substrates was measured at 25 ℃. For the final assay mixture, 200 μL of the substrate solution was diluted in 50 mM Tris HCl buffer pH = 7 and mixed with 20 μL enzyme solution. Activity was determined by measuring the increase of the absorbance at 405 nm, which indicated an increase of *p*-nitrophenol (ε_{405}nm = 11.86 mmol^{-1}cm^{-1}) due to hydrolysis of PNPA or PNPB. A Spectromax Plus 384 plate reader (Molecular Devices) was used to follow the reaction. The activity was calculated in units, where 1 unit had been defined as being the amount of enzyme required to hydrolyze 1 μmol of substrate per minute under the given assay condition. A blank was measured using 20 μL buffer instead of sample.

2.8. PET and PLA Hydrolysis

Prior to the enzyme treatment, PET and PLA films were cut into pieces of 10×20 mm and washed in a three consecutive steps. In a first step, each PET piece was washed with a solution of Triton-X100, in a second step 100 mM Na_2CO_3 and finally deionized water was used. Each washing step lasted 30 min and was performed at 50 ℃ for PET and 37 ℃ for PLA. The final enzyme concentration was 100 mg/mL diluted in tris buffer (100 mM) pH = 7. Incubations were made at 130 rpm and 50 ℃ for PET and 37 ℃ for PLA. Hydrolysis products were measured by using high performance liquid chromatography - (HPLC) as described below. Controls were performed with heat-inactivated enzyme.

2.9. Hydrolysis of PET Model Substrate bis(benzoyloxyethyl)terephthalate

Hydrolysis of the PET model substrate bis(benzoyloxyethyl)terephthalate (3PET) was performed in 2 mL Eppendorf tubes as previously described [27]. 10 mg 3PET were incubated in 1000 μL of 100 mM

K_2HPO_4/KH_2PO_4 containing 100 μg/mL enzyme at 50 °C and pH 7.0 for 2 hours and 350 rpm. The released products were analyzed by using HPLC as described below.

2.10. Analysis of PET, 3PET and PLA Hydrolysis Released Products

PET and 3PET soluble released products were analyzed as previously described by Eberl *et al*. [32]. After enzymatic treatment proteins were precipitated using 1:1 (v/v) methanol abs. on ice. Samples were centrifuged (Hettich MIKRO 200 R, Tuttlingen, Germany) at 16,000 × g at 0 °C for 15 min. The supernatant for measurement was brought to an HPLC vial and acidified by adding 1 μL of HCl concentrated. The HPLC used was a DIONEX P-580 PUMP (Dionex Cooperation, Sunnyvale, USA) equipped with an ASI-100 automated sample injector and a PDA-100 photodiode array detector. Analysis of TA, benzoic acid (BA), 2-hydroxyethyl benzoate (HEB), mono-(2-hydroxyethyl) terephthalate (MHET) and bis-(2-hydroxyethyl) terephthalate (BHET) a reversed phase column RP-C18 (Discovery® HS-C18, 5 μm, 150 × 4.6 mm with precolumn, Supelco, Bellefonte, USA) was used.

Lactic Acid (LA) was also determined using the same conditions. Analysis was carried out with 20% acetonitrile, 20% 10 mM sulfuric acid and 60% (v/v) water as eluent. The flow rate was set to 1 mL min-1 and the column was maintained at a temperature of 25 °C. The injection volume was 10 μL. Detection of the analytes was performed with a photodiode array detector at the wavelength of 241 nm.

2.11. PET and PLA Hydrophilicity Measurements

Water contact angles of the PET and PLA films after enzymatic treatment were measured using a Contact Angle and Surface Tension Analyzer (Kruss GmbH, Hamburg, Germany). Deionized water was used as test liquid with a drop size of 3 μL and the speed of 300 μL/s. Contact angles were measured after 3 s and data are obtained from the averages of the measurements taken from at least 5 different points of the sample surface.

3. Results and Discussion

3.1. Cloning, Sequence Analysis and Heterologous Expression in E. coli of an Esterase from Thermobifida Halotolerans

Cutinases from *Thermobifida fusca*, *T. cellulosilytica* and *T. alba* have shown to hydrolyze PET-films [27,42]. However, for industrial processes, more efficient enzymes are still needed while their potential to surface hydrophylize PLA has been largely neglected. In 2008, *Thermobifida halotolerans* has been isolated from a salt mine sample collected from Yunnan province (Southwest China, [43]) and classified as a novel species of *Thermobifida*. In order to identify homologous cutinases from this novel species, primers were designed, based on the genome sequences of cutinases from *T. fusca* YX. By the use of the designed primers, the gene coding for Thh_Est was amplified from the genomic DNA of *T. halotolerans*. The open reading frame of the gene (789 bp) encodes for a protein comprising 262 amino acids and a calculated mass of 28.7 kDa. The gene comprised a GC content of 68% which is expected for the genus *Thermobifida*. Sequence analysis of Thh_Est
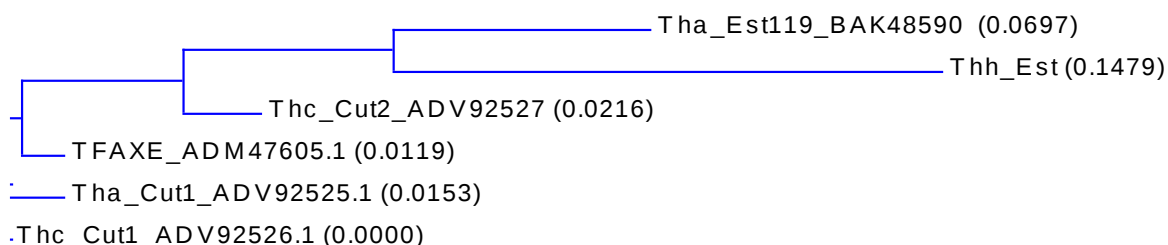
(Supporting Information File 1) revealed that the enzyme is a member of the serine hydrolases superfamily containing the –GxSxG- motif in which the catalytic Ser131 is embedded.

A BLAST search for homologous proteins listed an esterase from *T.alba* (Est119, Genbank BAK48590.1; [44]), two cutinases from *T.cellulosilytica* (Thc_Cut1 and Thc_Cut2, Genbank ADV92526 and ADV92527), the cutinase from *T.alba* (Tha_Cut1, Genbank ADV92526) as well as an acetylxylan esterase from *T.fusca* (TFAXE, Genbank ADM47605.1; [45]) as hits showing 85–87% similarity to Thh_Est (Table 1, Figure 1 and Appendix).

Table 1. Comparison of hydrolases from *Thermobifida* based on % homology of primary sequences.
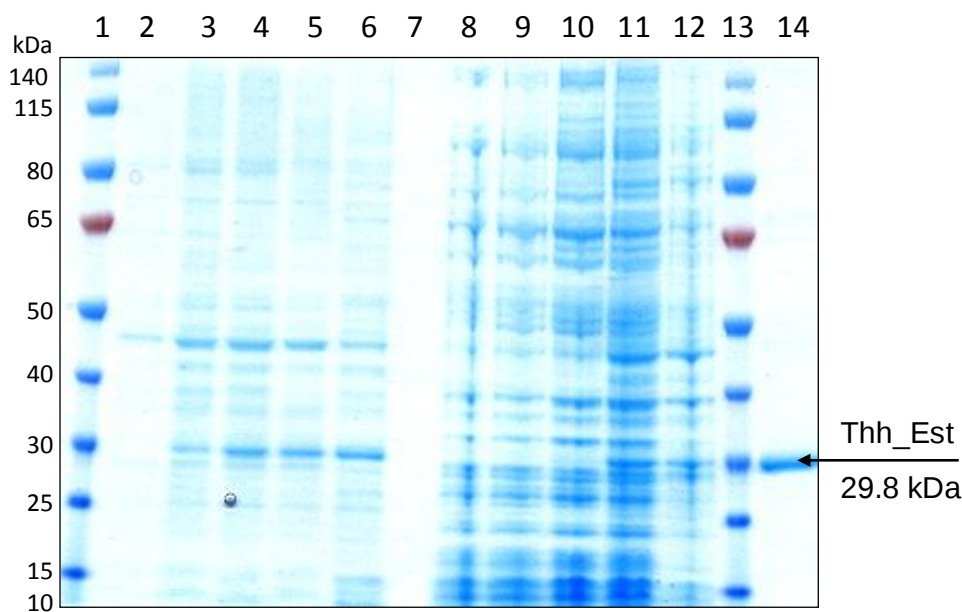
	Thh_Est	**Tha_Est119**	**Thc_Cut2**	**TFAXE**	**Tha_Cut1**	**Thc_Cut1**
Thh_Est	0	87%	87%	86%	85%	87%
Tha_Est119		0	93%	89%	88%	89%
Thc_Cut2			0	95%	94%	95%
TFAXE				0	98%	99%
Tha_Cut1					0	98%
Thc_Cut1						0

Figure 1. Phylogenetic tree based on amino sequences of the Thh_Est and other highly homologous hydrolases from *Thermobifida*. The calculation is based on a sequence distance method and utilizes the Neighbor Joining (NJ) algorithm of Saitou and Nei (1987). The tree displays the calculated distance values in parenthesis following the molecule name and the GenBank number on the tree.

The gene coding for Thh_Est was cloned through the *Nde*I/*Hind*III restriction sites into pET26b(+) for expression without the pelB leader sequence. The recombinant protein harboring a C-terminal hexahistidine tag (29.8 kDa) was expressed in *E. coli* BL21-Gold(DE3) at 20 °C by addition of 0.05 mM IPTG. The recombinant esterase was purified in one step by metal chelate affinity chromatography from the cell free *E. coli* extract to highest purity (Figure 2). Only low amounts of inclusion bodies were observed after 10h of induction (Figure 2, lane 11). At pH 7.0 and 25 °C, recombinant Thh_Est revealed specific activities of 500 U/mg with pNP butyrate and 550 U/mg with pNP acetate.

Figure 2. SDS-PAGE analysis of Thh_Est expressed in *E. coli* BL21-Gold (DE3). Samples were withdrawn at different times of induction, centrifuged, disrupted and centrifuged again. *Lane 1 and 13*: PageRuler Prestained Protein Ladder (Fermentas); *lane 2-6*: cleared lysates from induced cells after 0, 3, 6 and 10 h of induction with 0.05 mM IPTG; *lane 7*: empty; *lane 8-12*: insoluble cell fractions from induced cells after 0, 3, 6 and 10 h of induction; *lane 14*: purified Thh_Est.
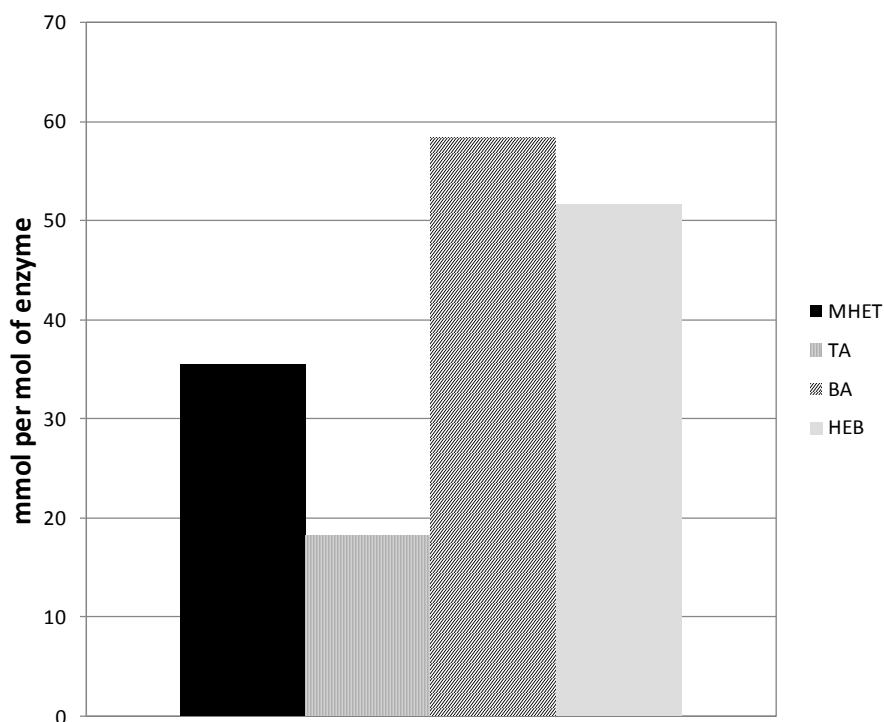

3.2. Hydrolysis of PLA Films

The ability of the Thh_Est to hydrolyze PLA films was assessed by analyzing the released products via HPLC-UV after hydrolysis and measurements of the hydrophilicity increase as WCA. The only released product found in the incubation solution was lactic acid in a concentration of 14.5 μM while no higher oligomers were detected. The degree of hydrophilization achieved was significant since it was not possible to measure a final value of the WCA due to the fact that the water drop was a complete spread on the polymer surface.

3.3. Hydrolysis of the PET Model Substrate bis(benzoyloxyethyl)terephthalate

Hydrolysis of the model substrate bis(benzoyloxyethyl)terephthalate (3PET) has been proven first to be valuable for the screening of enzymes capable of PET hydrolysis [40] and to provide important mechanistic information [27,32,34,42] about the type of hydrolysis mechanism. In the case of the new esterase Thh_Est, the hydrolysis product found in highest concentration was BA, followed by HEB, both in a comparable concentration higher than 50 mmol per mol of enzyme. In contrast, MHET and TA were detected in a lower amount, whereas no BHET was observed (Figure 3).

Figure 3. Hydrolysis of the PET model substrate 3PET with the esterase Thh_Est.

The 3PET hydrolysis product pattern of Thh_Est was similar to the profile previously reported for the cutinase 2 from *Thermobifida cellulosilytica* (Thc_Cut2). Other *Thermobifida fusca* cutinases [27,32] likewise released comparable amounts of BA and HEB whereas the ratio between MHET and TA was higher for Thh_Est. This might indicate a more exo-type hydrolysis mechanism of the Thh_Est esterase when compared to *Thermobifida* cutinases. In contrast, a *Humicola insolens* (Hi_Cut) cutinase released the terminal BA and MHET as major hydrolysis products [42]. A very similar hydrolysis profile was found for the *para*-nitrobenzylesterase from *Bacillus subtilis* (BsEstB) [34], although in this case the amount of release products was much lower than for Thh_Est or Hi_Cut. Interestingly, the overall amount of released products per mol of Thh_Est was significantly higher than the values reported for cutinases from *Thermobifida* species Thc_Cu1, Thc_Cut2 and Thf_42 [27].

3.4. Hydrolysis of PET Films

Upon incubation with PET, the new esterase from *T. halotolerans* only released MHET and TA while no BHET was detected. The amount of MHET and TA released were comparable with around 19.8 and 21.5 mmol/mol of enzyme, respectively. Previously, for a lipase from *T. lanuginosus* [29] a ten times higher release of MHET than TA from amorphous fibers and 3 times more from semi-crystalline PET fibers was reported. Interestingly, only in the case of amorphous fibers was BHET detected. Similarly, Eberl *et al*. reported higher amounts of MHET than of TA when the same lipase was used [32]. In the case of cutinase from *F. solani* again higher MHET to TA ratios were found and BHET was detected in non-significant amounts [25]. For cutinases from *Fusca sp*. comparable amounts of MHET and TA released from PET were reported, with no detection of BHET, when PET fabrics of around 40% crystallinity were used as substrate [32]. On the other hand,

cutinases from *Thermobifida fusca* DSM 44342 and cutinase 1 from *Thermobifida cellulolysitca* showed an opposite trend with higher amounts of TA than MHET. However, prolonged incubation together with the ability of the both enzymes to endo-wise hydrolyze the polyester may lead to this view [27].

3.5. Hydrophilicity Measurement of Enzymatically Treated PLA and PET

Thh_Est was able to hydrolyze films of both PLA and PET (Table 2). Provided a complete removal of the enzyme adhered in the polymer surface it is possible to compare the degree of hydrophilicity achieved by Water Contact Angle (WCA) measurement [29]. In the case of PLA films the hydrophilicity increase achieved was significantly higher than obtained for PET. The WCA in the case of PLA decreased from 75.5° to a complete spread of the water drop on the polymer surface after deposition. For PET it was possible to decrease the WCA from 90.8° to 50.4°.

Table 2. Hydrophilicity increase (quantified as Water contact angle WCA decrease) of PET and PLA upon surface hydrolysis with the esterase Thh_Est.

	Initial	**Control**	**Enzyme treated**
PET	90.8°±3.3°	74.8°±2.3°	50.4°±9.9°
PLA	75.5°±2.7°	68.4°±2.3°	<20°

Enzymatic hydrophilization of polyester surfaces has applications [1] ranging from improved coating with PVC [46] to more efficient textile finishing processes [29]. The advantage of enzymes derived from *Thermobifida species* is high thermal stability which allows working at temperatures below but close to the glass transition temperature of the polymer. These working conditions facilitate a high mobility of the polymer chains leading to higher accessibility of the polymer for enzymatic attack.

Concluding the results of this paper, the new esterase *Thermobifida* Thh_Est has a potential for both PET and PLA surface hydrolysis, similar to cutinases from the same genus. This is rather unexpected since cutinases were previously believed to be superior to esterases in terms of polyester hydrolysis [26,34].

Acknowledgements

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Appendix

Alignment of the primary sequence of Thh_Est, esterase from *T. alba* (Tha_Est119, Genbank Genbank BAK48590.1), cutinases from *T. cellulosilytica* (Thc_Cut1 and Thc_Cut2, Genbank ADV92526 and ADV92527), cutinase 1 from *T. alba* (Tha_Cut1, Genbank ADV92526) and acetylxylan esterase from *T. fusca* (TFAXE, Genbank ADM47605.1). Red: amino acids of the catalytic triade. Bold: conserved GxSxG motif.

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            ....|....| ....|....| ....|....| ....|....| ....|....|
                 10         20         30         40         50
Tha_Est119  MANPYERGPN PTESMLEARS GPFSVSEERA SRFGADGFGG GTIYYPRENN
TFAXE       MANPYERGPN PTDALLEARS GPFSVSEENV SRLSASGFGG GTIYYPRESN
Tha_Cut1    MANPYERGPN PTDALLEASS GPFSVSEENV SRLSASGFGG GTIYYPRENN
Thc_Cut1    MANPYERGPN PTDALLEASS GPFSVSEENV SRLSASGFGG GTIYYPRENN
Thc_Cut2    MANPYERGPN PTDALLEARS GPFSVSEERA SRFGADGFGG GTIYYPRENN
Thh_Est     MANPYERGPN PTNSSIEALR GPFRVDEERV SRLQARGFGG GTIYYPTDNN


            ....|....| ....|....| ....|....| ....|....| ....|....|
                 60         70         80         90        100
Tha_Est119  TYGAIAISPG YTGTQSSIAW LGERIASHGF VVIAIDTNTT LDQPDSRARQ
TFAXE       TYGAVAISPG YTGTEASIAW LGERIASHGF VVITIDTITT LDQPDSRAEQ
Tha_Cut1    TYGAVAISPG YTGTEASIAW LGGRIASHGF VVITIDTITT LDQPDSRAEQ
Thc_Cut1    TYGAVAISPG YTGTEASIAW LGERIASHGF VVITIDTITT LDQPDSRAEQ
Thc_Cut2    TYGAVAISPG YTGTQASVAW LGERIASHGF VVITIDTNTT LDQPDSRARQ
Thh_Est     TFGAVAISPG YTGTQSSISW LGERLASHGF VVMTIDTNTT LDQPDSRASQ


            ....|....| ....|....| ....|....| ....|....| ....|....|
                110        120        130        140        150
Tha_Est119  LNAALDYMLT DASSAVRNRI DASRLAVMGH SMGGGGTLRL ASQRPDLKAA
TFAXE       LNAALNHMIN RASSTVRSRI DSSRLAVMGH SMGGGGTLRL ASQRPDLKAA
Tha_Cut1    LNAALNHMIN RASSTVRSRI DSSRLAVMGH SMGGGGTPRL ASQRPDLKAA
Thc_Cut1    LNAALNHMIN RASSTVRSRI DSSRLAVMGH SMGGGGTLRL ASQRPDLKAA
Thc_Cut2    LNAALDYMIN DASSAVRSRI DSSRLAVMGH SMGGGGTLRL ASQRPDLKAA
Thh_Est     LDAALDYMVE DSSYSVRNRI DSSRLAAMGH SMGGGGTLRL AERRPDLQAA


            ....|....| ....|....| ....|....| ....|....| ....|....|
                160        170        180        190        200
Tha_Est119  IPLTPWHLNK SWRDITVPTL IIGAEYDTIA SVTLHSKPFY NSIPSPTDKA
TFAXE       IPLTPWHLNK NWSSVTVPTL IIGADLDTIA PVATHAKPFY NSLPSSISKA
Tha_Cut1    IPLTPWHLNK NRSSVTVPTL IIGADLDTIA PVATHAKPFY NSLPSSISKA
Thc_Cut1    IPLTPWHLNK NWSSVTVPTL IIGADLDTIA PVATHAKPFY NSLPSSISKA
Thc_Cut2    IPLTPWHLNK NWSSVRVPTL IIGADLDTIA PVLTHARPFY NSLPTSISKA
Thh_Est     IPLTPWHTDK TWGSVRVPTL IIGAENDTIA SVRSHSEPFY NSLPGSLDKA


            ....|....| ....|....| ....|....| ....|....| ....|....|
                210        220        230        240        250
Tha_Est119  YLELDGASHF APNITNKTIG MYSVAWLKRF VDEDTRYTQF LCPGPRTGLL
TFAXE       YLELDGATHF APNIPNKIIG KYSVAWLKWF VDNDTRYTQF LCPGPRDGLF
Tha_Cut1    YLELDGATHF APNIPNKIIG KYSVAWLKRF VDNDTRYTQF LCPGPRDGLF
Thc_Cut1    YLELDGATHF APNIPNKIIG KYSVAWLKRF VDNDTRYTQF LCPGPRDGLF
Thc_Cut2    YLELDGATHF APNIPNKIIG KYSVAWLKRF VDNDTRYTQF LCPGPRDGLF
Thh_Est     YLELDGASHF APNLSNTTIA KYSISWLKRF VDDDTRYTQF LCPGPSTGWG


            ....|....| ..
                260
Tha_Est119  SDVEEYRSTC PF
TFAXE       GEVEEYRSTC PF
Tha_Cut1    GEVEEYCSTC PF
Thc_Cut1    GEVEEYRSTC PF
Thc_Cut2    GEVEEYRSTC PF
Thh_Est     SDVEEYRSTC PF
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