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Cloning, Purification and Characterization of Acetyl Xylane Esterase from *Anoxybacillus flavithermus* DSM 2641^T with Activity on Low Molecular-Weight Acetates

Ayşenur Eminoğlu[1] · Serdar Ülker[1] · Cemal Sandallı[1]

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Abstract Family 4 carbohydrate esterases (CE-4) have deacetylate different forms of acetylated poly/oligosaccharides in nature. This family is recognized with a specific polysaccharide deacetylase domain assigned as NodB homology domain in their secondary structure. Most family 4 carbohydrate esterases have been structurally and biochemically characterized. However, this is the first study about the enzymological function of *pdaB*-like CE4s from thermophilic bacterium *Anoxybacillus flavithermus* DSM 2641^T. *A. flavithermus* WK1 genome harbors five putative CE4 family genes. One of them is 762 bp long and encodes a protein of 253 amino acids in length and it was used as reference sequence in this study. It was described as acetyl xylane esterase (AXE) in genome project and this *Af*AXE gene was amplified without signal sequence and cloned. The recombinant protein was expressed in *E. coli* BL21 (DE3), purified by nickel affinity chromatography and its purity was visualized on SDS-PAGE. The activity of the recombinant enzyme was shown by zymogram analysis with α-naphtyl acetate as a substrate. The enzyme was characterized spectrophotometrically using chromogenic *p*-nitrophenyl acetate. Optimum temperature and pH were determined as 50 °C and 7.5, respectively. K*m* and V*max* were determined as 0.43 mM and 3333.33 U/mg, respectively under optimum conditions. To our knowledge this is the first enzymological characterization of a *pdaB*-like family 4 carbohydrate esterase from the members of *Anoxybacillus* genus.

✉ Cemal Sandallı
cemal.sandalli@erdogan.edu.tr

[1] Department of Biology, Molecular Biology Research Laboratory, Recep Tayyip Erdogan University, 53100 Fener, Rize, Turkey

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Abbreviations

AXE	Acetyl xylan esterase
p-NPAc	*para*-Nitrophenyl acetate
p-NP	*para*-Nitrophenol
α-NAc	α-Naphtyl acetate
pdaA	peptidoglycan *N*-acetylmuramic acid deacetylase A
pdaB	A polysaccharide deacetylase Gene B
Af	*Anoxybacillus flavithermus*
CE4	Carbohydrate esterases 4
NodB	Nodulation protein B
MurNAc	*N*-acetylmuramic acid
GlcNAc	*N*-acetylglucosamine

1 Introduction

In order to overcome the acetylation barrier, some microorganisms produce enzymes that have been termed carbohydrate esterases (CEs) that enable the removal of acetyl groups from acetylated poly/oligosaccharides including chitin, acetylated xylan, peptidoglycan and rhizobial Nod factors [1]. There is a classification system for categorizing carbohydrate active enzymes based on the amino acid sequence similarities, protein folds and enzymatic mechanisms in the CAZy database [2, 3] and CEs are currently represented by 16 families. Family 4 is the largest group with over 1000 open reading frames [4] and they have various substrate specifity [5]. Although these family

members are diverse in biochemical properties, function and substrate specifity as well as in catalytic and the active site residues, they are recognized by five well conserved regions termed 'Polysaccharide homology domain-NodB homology domain' in their primary structure [5, 6] and conserved His–His–Asp-metal binding triad to accomplish acid/base catalysis [7]. However, pdaA-like CE4 superfamily members reveal differences in this catalytic triad. *B. subtilis* genome harbors six polysaccharide deacetylase homologue paralogs (pdaA, pdaB, pdaC, yheN, yxkH and ylxY), however, there has only been one study reported on *pdaB*-like CE4 [8]. The *pdaB* is highly conserved in spore-forming bacteria and it was reported that it is involved in cortex formation of spores in *B. subtilis*. Nevertheless, its enzymatic activity has been ambiguous.

Xylan is the prevalent carbohydrate in hemicellulose. Hemicellulose is a branched hetero-polysaccharide and its degradation to carbohydrate monomers needs a collective enzyme activity [9]. Acetyl xylane esterases (AXEs) [EC 3.1.1.72] catalyze the deacetylation of acetyl groups in positions 2 and/or 3 of the xylose units and assist the xylanase activity to complete degradation of xylane biomass with other accessory enzymes [1, 10]. In the present work, we describe a *pdaB*-like AXE from spore forming thermophilic bacteria *A. flavithermus* DSM 2641^T that exhibit significant deacetylase activity towards chromogenic artificial acetate substrates α-NAc and *p*-NPAc. To date, 19 species and numerous carbohydrate metabolism related enzymes have been reported from *Anoxybacillus* genus [11]. Our study is the first enzymological description of a *pdaB*-like family 4 carbohydrate esterase from this genus.

2 Materials and Methods

2.1 Cloning of *Af*AXE and Construction of Recombinant Expression Vector

A. flavithermus DSM 2641^T was obtained as type strain from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Its genomic DNA was obtained with Wizard Genomic DNA Purification Kit (Promega) and the *AXE* gene of *A. flavithermus* was cloned into the pET-15b expression. Briefly, the *AXE* gene was amplified by two primers; *Nde*I (Fw: 5′-GCATATGAGTGAGTTTGTATTTTCCACTGACC-3′ and Rw: 5′-CGGATCCTCATTTTATTTCTTTACTTTTTATATGAGC-3′). The PCR reaction was carried out in a 50 µl reaction volume containing 1× polymerase buffer, 5 ng of genomic DNA, 3 mM $MgCl_2$, 200 µM of each dNTP, 10 µM primers, and 1.5 U of Expand High Fidelity Taq DNA polymerase (Fermentas). Amplification was performed by PCR (Bio-Rad) using the following cycling parameters: 94 °C for 3 min (one cycle), denaturation 95 °C for 45 s, followed by annealing 52 °C 1 min and primer extension 72 °C for 1 min 20 s (32 cycles) and then the final extension at 72 °C for 10 min. After purification (GeneJET PCR Purification Kit; Fermentas), amplification products were cloned into pGEM-T Easy vector (Promega). Positive clones were selected on LB-ampicillin plates. Both the positive clone and the expression vector pET-15b (Novagen) were digested separately with 10 U of *NdeI* (Fermentas) in a 50 µl reaction volume at 37 °C for 2 h. The digested products were purified again and ligation reaction was carried out in a 20 µl reaction volume using 10 U of T4 DNA ligase (Fermentas) at 16 °C for 16 h. The ligation mixture was transformed into the *E. coli* DH5α (Novagen). Plasmids were purified from several colonies and positive clones were verified with enzymatic digestion and sequencing (Macrogen).

2.2 Overexpression and Purification

The recombinant vector designed as p*Af*AXE was transformed into *E.coli* BL21 (DE3) (Novagen) for overexpression. A single colony harboring the recombinant plasmid was chosen and cultured overnight in LB medium containing 50 µg/ml ampicillin. This culture was used to inoculate 200 ml LB-ampicillin medium and incubated at 37 °C with vigorous shaking until mid-log phase OD_{600} was approximately 0.6–0.8. Then expression was induced by 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and maintained for 3 h at 37 °C for the production of recombinant *Af*AXE. Then, cells were collected by centrifugation at 7000× rcf for 10 min at 4 °C. The pellet was resuspended in lysis buffer (20 mM Tris–HCl, 200 mM NaCl and 2 mg/ml lysozyme) and sonicated. Nonspecific proteins were removed with heat treatment at 55 °C [12]. After centrifugation, the supernatant containing N-terminally His-tagged protein was loaded onto the column including HisLink Protein Purification Resin (Promega) pre-equilibrated with 20 mM Tris–HCl (pH 7.5) and 200 mM NaCl. Following the washing step with 20 mM Tris–HCl buffer (pH 7.5), 200 mM NaCl and 20 mM imidazole, *Af*AXE was eluted with 500 mM imidazole in the same buffer. SDS-PAGE (12 %) was run to evaluate the purity. Purified *Af*AXE was dialyzed overnight in dialysis buffer (50 mM sodium phosphate pH 7.5, 50 mM NaCl, 1 mM dithiothreitol-DTT) at 4 °C. After dialysis, recombinant protein was concentrated with 10 K ultracentrifugation system (Amicon Ultra-4) and its concentrations were measured with NanoDrop Spectrophotometer 2000.

2.3 Activity Assays and Biochemical Characterization of *Af*AXE

To determine deacetylase activity, zymography staining was performed on SDS-PAGE (12 %) with 100 mM

α-naphthyl acetate (α-NAc) after renaturation in 100 mM Tris–HCl (pH 7.5) buffer containing fast red salt (Sigma, St. Louis, Mo) [12]. Acetyl xylan esterase activity, optimum pH and temperature were determined spectrophotometrically (Molecular Device, SpectraMax M5) at A_{405} by monitoring the *p*-nitrophenol released from 2 mM *p*-nitrophenyl acetate (*p*-NPAc) as substrate. The acetyl xylan esterase activity was assayed in 400 µl final volume with 5 mM sodium phosphate buffer (pH 7.5), 70 µl enzyme sample and 10 µl *p*-NPAc at 50 °C 90 s [13]. The reaction was terminated with 50 mM citric acid on ice. Due to spontaneous hydrolysis of substrate, a reaction mixture including post-dialyzed buffer instead of enzyme was used as blank under the same conditions. Xylanase activity was examined as described before [14] and after running SDS-PAGE (12 %) containing birch wood xylane (Sigma) 0.1 %, the gel was shaked in 2.5 % Triton X-100 at room temperature for 30 min and then incubated in 50 mM phosphate buffer (pH 7.5) for 30 min. After additional 15 min incubation in the same buffer at 50 °C, the gel was stained with Congo red 0.1 % for 15 min and washed with 1 M NaCl so that bands could become visible and then was soaked in 5 % (w/v) acetic acid until the formation of white bands on dark blue background. The optimum temperature was determined in 400 µl volume using 5 mM sodium phosphate buffer (pH 7.5), 20 µg of purified *Af*AXE and temperature in the range from 20 to 90 °C for 90 s. Optimum pH was determined in the following 5 mM buffers; sodium acetate at pH 5.0, sodium phosphate at pH 6.0–8.0 and sodium pyrophosphate at pH 8.5–9.0, respectively. The reactions were conducted in 96-well plate at 50 °C under standard conditions. Effect of EDTA on the activity of *Af*AXE was carried out in Na-Phosphate buffer (pH 7.5) with *p*-NPAc at 405 nm under optimum conditions [1]. The enzyme was incubated for 1 min in EDTA varied concentrations (from 0.1 to 10 mM). All assays were performed with three replicates and the maximum absorbance rates were assumed as optimum. The kinetic parameters of *Af*AXE were provided from double-reciprocal plots of Lineweaver and Burk [15] by quantifying the liberated *p*-NP in 90 s from *p*-NPAc, as described above containing varied substrate concentrations (0.5–7 mM) of enzyme-buffer mixture at optimum conditions.

2.4 Bioinformatic Analysis

The nucleotide and amino acid sequence of *Af*AXE were obtained from the genome project of *A. flavithermus* WK1 [16] (nucleotide sequence accession number: CP000922.1 and amino acid sequences accession number: ACJ32529.1) (http://www.ncbi.nlm.nih.gov/). The identification of amino acid resemblance was determined by DELTA-BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The signal peptide was estimated by SignalP 3.0 [17] and multiple amino acid sequence alignment was performed by clustal omega [18].

3 Results

3.1 Purification of *Af*AXE

Predicted molecular mass of *Af*AXE was calculated as 24.36 kDa from the deduced amino acid sequence without signal peptide. The recombinant *Af*AXE was purified in concentrations in the range of 0.015–1.2 mg/ml protein from 200 ml culture. Purified enzyme was subjected to 12 % SDS-PAGE analysis and shown as a distinct single band at the predicted position (Fig. 1a, column P).

3.2 Biochemical Characterization of *Af*AXE

Characteristic esterase activity of *Af*AXE was demonstrated by zymogram analysis using fast red staining and it was determined as a single band on SDS-PAGE (Fig. 1b, column 1). Optimum pH and temperature of *Af*AXE were measured as 7.5 and between 50–55 °C (Fig. 2), respectively. Considering non-enzymatic hydrolysis of substrates at high temperatures and in high alkaline conditions, optimum pH assays were set up with various buffers depending

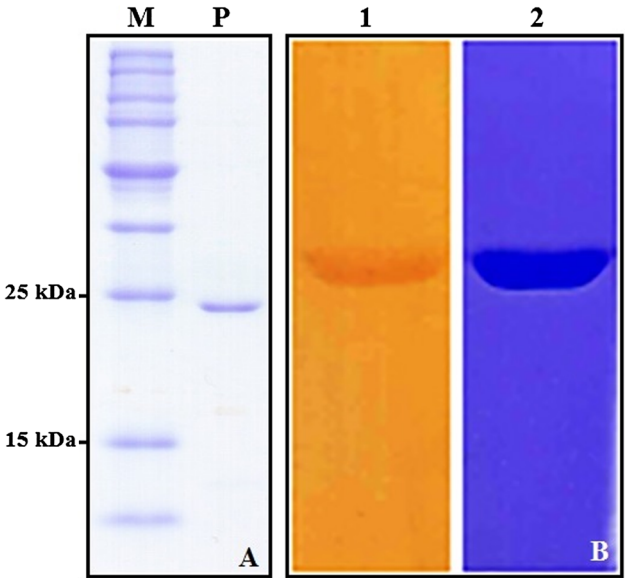

Fig. 1 **a**, SDS-PAGE (12 %) analysis of purified *Af*AXE protein. Column *M*, molecular weight marker (Promega, V8491) and column *P*, purified *Af*AXE. **b**, Zymogram analysis of purified *Af*AXE after separation by SDS-PAGE. In column (*1*), deacetylase activity band of *Af*AXE with fast red staining in 100 mM Tris–HCl (pH 7.5) buffer, in column (*2*) coomassie brilliant blue staining of *Af*AXE before renaturation

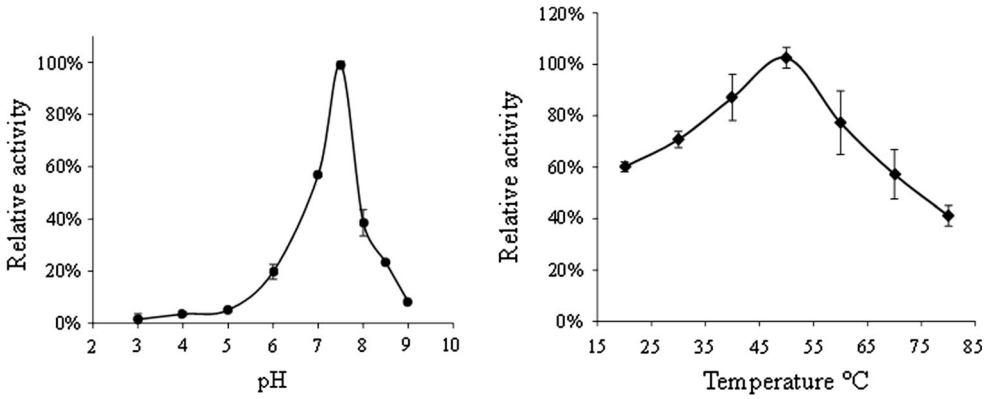

Fig. 2 Effect of pH and temperature on the activity of *Af*AXE. Optimum pH was determined in the following 5 mM buffers; sodium acetate at pH 5.0, sodium phosphate at pH 6.0–8.0 and sodium pyrophosphate at pH 8.5–9.0, respectively. Optimum temperature was specified in 5 mM sodium phosphate buffer (pH 7.5) in the range of 20–90 °C, in 90 s. All assays were performed with three replicates and the maximum absorbance rates were assumed as optimum

on the stability of *p*-NPAc. The kinetic constants K*m* and V*max* of *Af*AXE were found as 0.43 mM and 3333.33 U/mg, respectively. The *Af*AXE activity was not inhibited by EDTA even at 10 mM concentration.

3.3 Bioinformatic Analysis

According to the data obtained from NCBI, the gene of *Af*AXE was 762 bp in length and it encoded a protein in 253 amino acid length. Signal peptide sequence was predicted between positions 35–36 (QLA-SE). Based on the amino acid sequence alignment and conserved domain search, *Af*AXE has differences in active site residues and NodB domain compared to the other members of CE4s. With respect to DELTA-BLAST results, among the CE4s family members, *Af*AXE is the closest to *B. subtilis pdaB* (*Bs*PdaB) by 56 % homology. When the conserved domains of *Bs*PdaB and *Af*AXE were compared, it was seen that NodB domains of *Af*AXE are not completely identical with *Bs*PdaB. The putative active and catalytic sites are almost homologous with few residues exceptions. In NodB homology domain, there have been some differences in motif I and motif IV. However, both of *Af*AXE and *Bs*PdaB show variation from the consensus sequence in putative metal binding residues at their active sites. The classical His–His–Asp triad was not absent. In most members of CE4, this triad shows strict conservation excluding *pdaB*-like superfamily (Fig. 3).

4 Discussion

Acetyl xylan esterases contribute to the lignocellulosic fermentation process by enhancing the action of glycoside hydrolases on acetylated poly/oligosaccharides. For that reason, they have broad application in industry, especially in biomass conversion and biocatalysis [19]. AXEs of CE4 are mainly specific for acetyl xylan and do not act on artificial acetate substrates, which have low molecular mass [1, 20]. But the NodB domain of XynA of *Clostridium cellulovorans* ATCC 35296 has a rather high affinity for such acetates [21]. In this aspect, they are different from classical serine esterases [22].

According to Saw et al. [16] *A. flavithermus* genome harbors five putative CE4 family genes (GeneBank accession no: ACJ32529.1; ACJ34049.1; ACJ32570.1; ACJ33583.1; ACJ32713.1). In this study, a putative xylanase/chitin deacetylase (ACJ32529.1) gene was selected to elucidate its biochemical properties. This gene is predicted to have two important regions designated as CDA1 (a putative xylanase/chitin deacetylase) and pdaB (a polysaccharide deacetylase family sporulation protein). CDAs (chitin deacetylases) are metalloproteins that deacetylate *N*-acetylglucosamine units of chitin and chitosan to generate glucosamine and acetic acid [23]. Most CE4s are bifunctional enzymes [5, 21, 24, 25]. With respect to its putative activities, we investigated its xylanase activity and found that *Af*AXE did not have any xylanase activity according to zymography analysis (data not shown).

Based on the kinetic parameters, *Af*AXE has high affinity to *p*-NPAc. However, *Ct*CE4 and *Sl*AXE have no evaluable activity on artificial acetate substrates [21]. When compared to the other AXEs from different carbohydrate acetate families, *Af*AXE exhibits significantly higher deacetylase activity on *p*-NPAc. It could be suggested that this high affinity is due to the effect of aliphatic and aromatic residues in the putative active site of *Af*AXE, unlike the other CE4 family members, which bear polar residues that prefer to bind sugar based substrates [5].

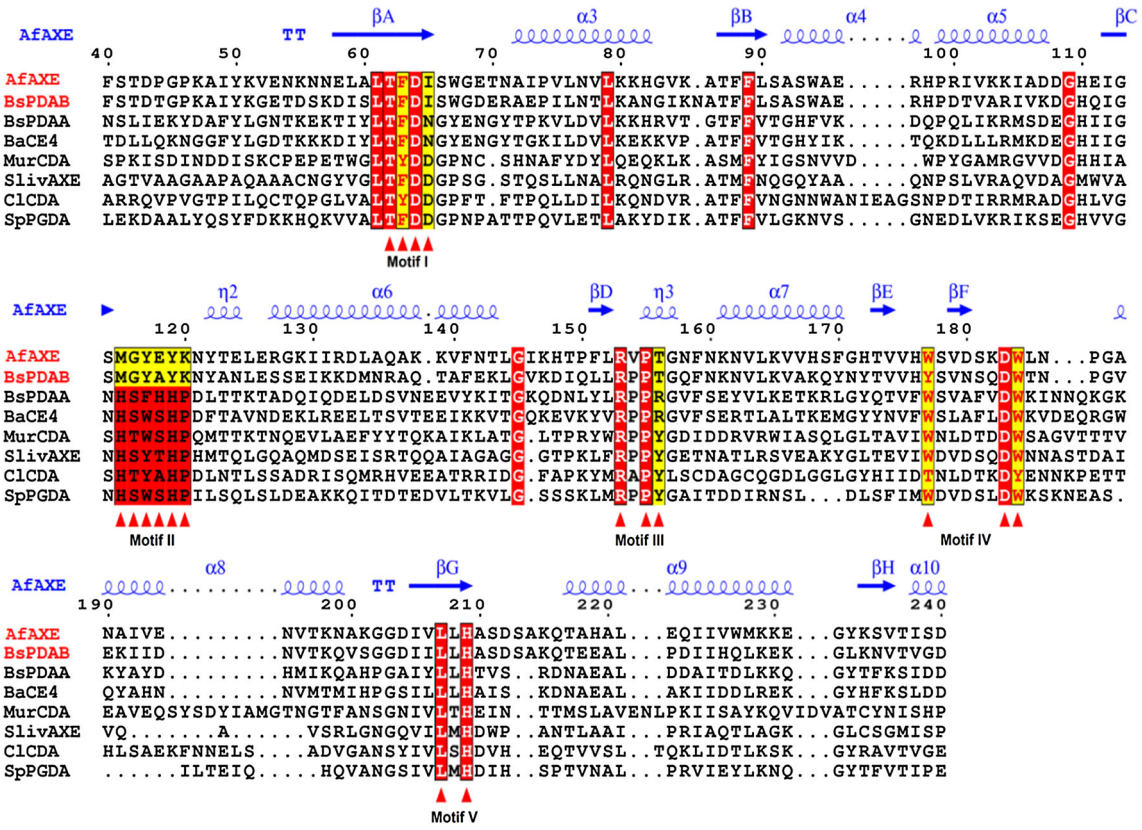

Fig. 3 Multiple sequence alignment of certain CE-4 family members from *Anoxybacillus flavithermus* WK1 acetyl xylan esterase (*Af*AXE), *Bacillus subtilis subsp. subtilis str.* probable polysaccharide deacetylase (*Bs*PDAB) and *N*-acetylmuramic acid deacetylase (*Bs*PDAA), *Bacillus anthracis str. Ames* carbohydrate esterase (*Ba*CE4), *Mucor rouxi* chitin deacetylase (*Mur*CDA), *Streptomyces lividans* 1326 acetyl xylan esterase (*Sliv*AXE), *Colletotrichum lindemuthianum* chitin deacetylase (*Cl*CDA) and *Streptococcus pneumoniae* R36A peptidoglycan GlcNAc deacetylase (*Sp*PGDA). *Triangles* indicate the NodB domain residues. Secondary structure elements are shown at the *top* of the sequences in *blue*. Strictly conserved residues were shaded in *red* with *white letters* (Color figure online)

In virtue of the spontaneous hydrolysis of the esters under higher alkali conditions at elevated temperatures, there is no significant activity observed above pH 9.5 with *p*-NPAc. CE4 families generally show maximum activity toward 7.5–9.0 pH [7, 21, 22, 26, 27] with the exception of *Mucor rouxii* CDA [28]. Established optimum temperature (50–55 °C) for *Af*AXE activity is slightly lower than other thermophilic enzymes. Most of CE4 family AXEs have optimum activity at about 60–70 °C [20, 26].

Most family CE4 activities are metal-depended, however, there have been members who display activity without any metal ion [29, 30]. *Af*AXE activity does not require the supplement of any divalent cations, like *Cl*CDA [29] and it was not inhibited by EDTA. However, there are some studies on CE4 family that indicate that EDTA could not abolish the activity even if the enzymes have been metal dependent as observed previously with the chitin deacetylase CDA from *Collectotrichum lindemuthianum* [31] and recently with the *E. coli* PgaB [32]. Moreover, in some CE4 family members, metal ion is only required with some substrates [33]. Independent metal activity and particular action on generic acetate substrates of *Af*AXE related to its putative active site bearing non-polar amino acids needs to be confirmed with comprehensive mutation analysis of relevant residues.

In the current study, contrary to common opinion, we demonstrated that *Af*AXE from CE4 family acts efficiently on generic acetate substrates such as *p*-nitrophenyl acetate and α-naphtyl acetate. The action of *Af*AXE on removing acetyl groups from low molecular-weight substrates is obvious. To reveal the *Af*AXEs putative chitin deacetylase activity on chitinous substrates and to demonstrate the reflection of overall structure on *Af*AXE function, further studies are needed. We assign as acetyl xylan esterase due to its NodB homology domain. Our findings suggest that thermophilic spore forming bacteria could harbor significant biocatalytic potential family CE4 with novel features that need to be discovered. *Af*AXE is the first CE4 family enzyme that exhibits significant activity on general acetate substrates. Although there is increasing research on this family of enzymes, to our knowledge this is the first enzymological characterization of a *pdaB*-like family 4 carbohydrate esterase from *Anoxybacillus* genus.

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