Special Issue on

**Industrial Biotechnology-Made in Germany:**
The path from policies to sustainable energy, commodity and specialty products

Edited by:

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Biochemical Characterization of a Recombinant Xylanase from Thermus brockianus, Suitable for Biofuel Production

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Abstract
To discover new industrially relevant, thermoactive xylanases a gene library from Thermus brockianus was constructed. Function-based screening revealed a novel xylanase-encoding gene (xyn10) which was successfully expressed in E. coli BL21 (DE3). The resulting protein (38.7 kDa), a member of glycoside hydrolase family 10 was purified to homogeneity and biochemically characterized. Catalytic activity was detected up to 115 °C and highest activity was measured at 95 °C and pH 6.0. The protein was extremely thermostable and showed 80 % remaining activity after incubation at 50-70 °C for 24 h. HPLC analysis showed that Xyn10 hydrolyzes insoluble and soluble substrates, such as oat spelt xylan, xylan from beech- and birchwood forming xylobiose and xylose. Specific activity of the enzyme was 1119.5 U/mg for oat spelt xylan and 994.0 U/mg for beechwood xylan, respectively. The xylanase exhibited remarkable stability in the presence of various detergents and chaotropic agents, such as CHAPS, guanidine hydrochloride and urea.

This is the first report of the heterologous production, purification and characterization of a xylanase from Thermus sp.

ABBREVIATIONS
CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

INTRODUCTION
Lignocellulose which is the major component of the plant cell wall consists of 40-50 % cellulose, 20-30 % hemicelluloses and 20 % lignin. It represents one of the most abundant sustainable feed stocks which can be used as a resource for the production of high value chemicals and alcohols [1,2]. The hydrolysis of the β-1,4-glycosidic linkages of cellulose by endo-1,4-β-D-glucanases (EC 3.2.1.4), exo-1,4-β-D-glucanases (EC 3.2.1.91) and 1,4-β-D-glucosidases (EC 3.2.1.21) releases glucose [3]. Hemicellulose, the second abundant polymer of lignocellulose, shows a more complex structure that is composed of different pentoses, hexoses, uronic acids and acetyl residues. Xylan is the major component of hemicellulose and comprises a β-1,4-linked backbone of D-xylose. Due to the complex structure of xylan synergistic action of endo-1,4-β-D-xylanases (EC 3.2.1.8), 1,4-β-D-xylidoses (EC 3.2.1.37), esterases (EC 3.1.1-) and glucuronidases (EC 3.2.1.31) is required [4]. Traditional industrial applications for xylanases include bioleaching of pulp, enhancement of digestibility of feed or processing of food. Especially for the clarification of juices and the improvement of dough properties during baking xylanases are frequently employed [5]. The availability of efficient enzymes is necessary when bioethanol has to be produced on an industrial scale e. g. in biorefineries of the 2nd generation [6]. For this purpose more efficient yeast strains that are able to utilize both, glucose and xylose as carbon source also have to be developed [7]. Recent progress in metabolic engineering has resulted in xylose-fermenting yeast strains. This offers great opportunities to improve economic efficiency of existing biorefinery plants [8]. Therefore, the finding of novel, thermoactive xylanases is crucial since heat-stable enzymes can be directly added to the complex substrate during the hydrothermal treatment step [9]. Thermostable enzymes offer many advantages for industrial applications as they are active at high temperatures and resistant against different reagents. Furthermore, the substrate accessibility at elevated temperatures is enhanced and the risk of contaminations is reduced [10].

Bacteria of the genus Thermus are promising sources for thermostable enzymes since most of the species have been isolated from hydrothermal habitats with optimal growth temperatures between 53 and 86 °C [11-13]. During the last two decades several enzymes, such as proteases, catalases or DNA processing enzymes, have been characterized from these microorganisms [14].

Although xylanolytic Thermus thermophilus strains

were discovered, so far no recombinant xylanase has been characterized [15].

In this study we report on the identification of the first xylanase-encoding gene from *Thermus* sp. and the production, purification and biochemical characterization of the recombinant enzyme.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids and cultivation**

*Escherichia coli* EPI300 (Epicentre, Hessisch Oldendorf, Germany) and the bifunctional shuttle vector pCT3FK were used for the construction of a fosmid library [16]. *E. coli* strains Top10 and DH5α and the vector pCR-XL-TOPO (Invitrogen, Karlsruhe, Germany) were employed to construct a shotgun library. For cloning and expression the *E. coli* strains Nova Blue Single (Novagen, Darmstadt, Germany), BL21 Star<sup>TM</sup> (DE3) (Invitrogen, Karlsruhe, Germany) and the plasmids pJET (Thermo Scientific, Schwerte, Germany) and pQE-80L (Qiagen, Hilden, Germany) were used.

All *E. coli* strains were cultivated in LB medium at 37 °C and 160 rpm for 12-18 h. *Thermus brockianus* GE-1 was grown in *Thermus* 162 medium (DSMZ medium 878) at 70 °C and 160 rpm for 12-24 h.

**Genomic library construction and screening**

Genomic DNA from *T. brockianus* GE-1 was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Isolated genomic DNA was directly ligated into the vector pCT3FK [16]. Further steps for fosmid library construction were carried out according to the manufacturer’s instructions of the Copy Control<sup>TM</sup> Fosmid Library Production Kit (Epicentre, Hessisch Oldendorf, Germany).

To screen the fosmid library for clones with xylanase activity the cells were cultivated on LB agar plates (12.5 µg/mL chloramphenicol), overlayed with top agarose (50 mM sodium chloride, 25 mM AZCL-xylan, 1 % agarose) and incubated at 70 °C for 10-18 h.

A shotgun library was constructed from isolated fosmid DNA of a xylanase-positive clone resulting from the fosmid library screening. Isolated fosmid DNA was cut to 1-6 kb using *Sma*I and further purified from an agarose gel with the help of the GeneJET Gel Extraction Kit (Thermo Scientific, Schwerte, Germany). After the addition of a single 3'-deoxynucleosine the ligation into the pCR-XL-TOPO vector and the transformation of *E. coli* Top 10 cells was carried out according to the manufacturer’s instructions (TOPO XL PCR Cloning Kit, Invitrogen, Karlsruhe, Germany). Accordingly, isolated plasmid DNA was used to transform *E. coli* DH5α (Qiagen Plasmid Plus Midi Kit, Hilden, Germany). Subsequent screening was carried out as described above using AZCL-xylan containing top agarose.

**DNA sequencing and bioinformatic analysis**

Plasmids from xylanase-positive clones were isolated using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Schwerte, Germany). Sequencing of inserted DNA was done by Eurofins MWG Operon (Ebersberg, Germany). Open reading frames (ORFs) were identified with frameplot 4.0beta (http://nocardia.nih.go.jp/ftp/). BLAST (Basic Local Alignment Search Tool) was used for sequence identity analysis [17]. SignalP 4.0 was employed for potential signal peptide prediction [18]. Multiple sequence alignments were done using ClustalX 2.0 [19]. SWISS-MODEL was used for hypothetical protein modeling [20].

**Cloning of the xylanase-encoding gene**

The xylanase-encoding gene *xyn10* was amplified by PCR without signal peptide-encoding sequence using the following primers:

\[
\text{xyn10}_f \text{ BamHI: GGTACCCAGGTGACCTCGTAACCCAG (BamHI recognition site underlined)}
\]

\[
\text{xyn10}_r \text{ SalI: GTCGACCTACCTCCTTTFGGCGGCTAC (SalI recognition site underlined)}
\]

PCR was carried out with the Phusion High-Fidelity DNA polymerase (Thermo Scientific, Schwerte, Germany) according to the manufacturer’s instruction. The resulting PCR product was ligated into the vector pJET (Thermo Scientific, Schwerte, Germany) prior to transformation of competent *E. coli* Nova Blue. After the identification of a recombinant clone by colony PCR the plasmid was isolated and double digested with *Bam*HI and *Sal*I. To recover and purify the gene *xyn10* gel electrophoresis with further gel extraction (GeneJET Gel Extraction Kit, Thermo Scientific, Schwerte, Germany) was conducted. The xylanase-encoding gene was then ligated into the *Bam*HI and *Sal*I double digested vector pQE-80L and subsequently used for the transformation of *E. coli* BL21 Star<sup>TM</sup> (DE3) (Invitrogen, Karlsruhe, Germany).

**Heterologous expression and purification of the recombinant xylanase**

The recombinant clone *E. coli* BL21 Star<sup>TM</sup> (DE3)/pQE-80L::*xyn10* was cultivated in 700 mL LB (100 µg/mL ampicillin) until OD<sub>600</sub> of 0.5-0.7 and subsequently induced for 12 h at 30 °C with 0.5 mM IPTG. The cells were harvested by centrifugation at 7000 rpm for 20 min at 4 °C, resuspended in lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8) and disrupted using a French press (French<sup>®</sup> Pressure Cell Press, SLM-Aminco, Maryland, USA). The crude extract was obtained by subsequent centrifugation at 13,000 rpm for 20 min at 4 °C.

The crude extract was heat precipitated for 15 min at 70 °C and again centrifuged at 13,000 rpm for 20 min at 4 °C. The resulting supernatant was further purified through Ni<sup>2+</sup>-nitrilic acid (Ni-NTA) affinity chromatography using a 1.5 mL Ni-NTA superfion column (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Eluted fractions containing the purified xylanase were pooled, dialyzed against 50 mM maleate buffer pH 6.0 (50 mM maleic acid, 50 mM NaOH) and stored at 4 °C.

The purity of the recombinant xylanase was analyzed by SDS-PAGE (12 %) according to Laemmli [21]. Hydrolytic activity of the purified xylanase was verified with a zymogram. After SDS-PAGE
the gel was incubated for 1 h in 1 % Triton X-100 (v/v). Then it was incubated in a solution of 1 % beechwood xylan (w/v) for 1 h at 70 °C, stained with Congo red (2 %, w/v) for 30 min and destained in 1 M NaCl.

Protein concentration was determined by using bovine serum albumin as standard according to Bradford [22].

The molecular mass was determined by gel filtration applying the ÄKTA™ Fast Protein Liquid Chromatography system (GE Healthcare, München) with a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare, München) following the manufacturer’s instructions.

**Enzyme activity assays**

Enzymatic activity was determined by measuring the amount of reducing sugars released from xylan and cellulose employing the 3,5-dinitrosalicylic acid (DNS) assay according to Miller [23]. The standard reaction mixture of 500 µL contained 0.5 % substrate (w/v) in 50 mM maleate buffer, pH 6.0 (50 mM maleic acid, 50 mM NaOH) and 50 µL of the enzyme solution. The hydrolysis was conducted at 95 °C for 15 min. To stop enzymatic hydrolysis 500 µL of DNS reagent were added and the reaction was transferred on ice. After 5 min of incubation at 99 °C and subsequent centrifugation for 5 min at 4 °C the absorption was determined at 546 nm. Calibration curves were prepared for xylose and glucose (supplementary material, Figure S1). Mixtures for calibration contained 500 µL of DNS reagent and 500 µL of xylose or glucose, respectively (0-2.5 mM in 50 mM maleate buffer). After incubation for 5 min at 99 °C absorptions were measured at 546 nm. A time dependant activity profile was measured to confirm that the time of 15 min for enzymatic hydrolysis is suitable (supplementary material, Figure S2).

All measurements were done in triplicates in 3 independent measurements. One unit of enzymatic activity was defined as the amount of enzyme required for the release of 1 µmol of reducing sugars per minute.

To determine β-xylosidase activity the release of 4-nitrophenol from 4-nitrophenol-β-D-xylopyranoside was measured. The standard reaction mixture of 1 mL contained 2 mM 4-nitrophenol-β-D-xylopyranoside in 50 mM maleate buffer, pH 6.0 and 10 µL of enzyme solution. The reaction was carried out for 10 min at 95 °C before 0.1 mL of 0.1 M Na₂CO₃ was added to stop hydrolysis. The absorption was monitored at 410 nm. One unit of enzymatic activity was defined as the amount of enzyme required for the release of 1 µmol of 4-nitrophenol per minute.

**Determination of enzyme characteristics and hydrolysis products**

To investigate the influence of temperature on the enzyme relative activities against oat spelt xylan were measured in the range of 10-115 °C in 50 mM maleate buffer, pH 6.0. To study temperature stability the enzyme was preincubated in 50 mM maleate buffer, pH 6.0 for 3 and 24 h at 50-90 °C in presence of 0.5 mg/mL bovine serum albumin.

The influence of pH was examined by determining relative activity towards oat spelt xylan in 50 mM Britton-Robinson buffer in a range of pH 3.0-10.0 at 95 °C [24]. To examine pH-stability the enzyme was preincubated for 24 h at 4 °C and 70 °C in Britton-Robinson buffer pH 3.0-10.0 in presence of 0.5 mg/mL bovine serum albumin. Subsequently, relative activity against oat spelt xylan was measured for 15 min at 70 °C.

The effect of metal ions was studied by measuring relative activity towards oat spelt xylan in presence of 1 and 5 mM, and 10 mM AgNO₃, AlCl₃, CaCl₂, CoCl₂, CrCl₃, CuCl₂, FeCl₃, FeCl₄, KCl, MgCl₂, MnCl₂, NaCl, NiCl₂, RbCl, SrCl₂ and ZnCl₂ in 50 mM maleate buffer, pH 6.0 at 95°C. Furthermore, the influence of 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), cetlytrimethyl-ammonium bromide (CTAB), Triton X-100, Tween 20, Tween 80, guanidine hydrochloride, urea, dithiotreitol (DTT), β-mercaptoethanol, EDTA, iodacetic acid, sodium azide and Pefabloc was monitored. Relative activity was measured in presence of 5 mM additive in 50 mM maleate buffer, pH 6 at 95 °C against oat spelt xylan.

To determine substrate specificity of the xylanase enzymatic hydrolysis was performed for 15 min at 95 °C in 50 mM maleate buffer, pH 6.0 using 0.5 % beechwood-, birchwood-, oat spelt xylan, cellulose, carboxymethylcellulose and 2 mM 4-nitrophenol-β-D-xylopyranoside as substrates. Kinetic parameters were determined with oat spelt xylan.

To investigate hydrolysis products of beechwood-, birchwood- and oat spelt xylan (2 %, w/v) HPLC analysis was performed using a LaChrom system (Merck-Hitachi, Tokio, Japan) with following components: sampler L-7200, pump L-7100, oven L 7350, (RI)-detector L-7490. For sample preparation enzymatic hydrolysis was conducted for 1 h at 80 °C. After centrifugation of the reaction mixtures for 20 min at 4 ºC the supernatant was filtered (0.2 µm, Pall, Darmstadt, Germany). A volume of 20 µL of the filtered supernatant was applied to a HFX42A column (Bio-Rad, München, Germany). Filtered and helium flushed high purity water was used as solvent with a flow rate of 0.6 mL/min. Retention times in minutes were determined for the following standards (Megazyme, Ireland): xylose: 18.05, xylobiose: 16.33, xylotriose: 14.95, xylotetraose: 13.79, xylopentaose: 12.82.

**Nucleotide sequence accession number**

The nucleotide sequence of the xylanase-encoding gene from *Thermus brockianus* GE-1 has been deposited in GenBank under the accession number HG931726.

**RESULTS AND DISCUSSION**

Identification of the xylanase-encoding gene and sequence analysis.

Function-based screening of a fosmid library constructed from genomic DNA of *T. brockianus* resulted in two clones with xylanase activity. The respective fosmids were isolated and used to construct a shotgun library. Based on a functional screening using azurine-crosslinked xylan a positive clone was identified, carrying a plasmid with a 1.9 kb insert. Sequencing of the insert resulted in two clones with 12 nucleotides upstream of the start codon for ORF2 indicated an ooper-like structure.
The association of genes with similar functions in operons permits their simultaneous regulation allowing organisms to adapt faster to environmental changes [25]. Especially hydrolysis of complex substrates like hemicellulose requires synergistic action of different enzymes [26,27].

The deduced amino acid sequence of ORF1 exhibited 51 % identity to a characterized xylanase from Streptomyces thermocarboxydus HY-15 (AC64040.1) [28]. ORF2 comprises 752 bp and the respective protein showed identity of 43 % to a putative family 5 glycoside hydrolase from Kribbella flavida DSM 17836 (YP_003379978.1).

The xylanase-encoding gene xyn10 consists of 1077 bp and showed a GC-content of 51.2 %. Within the resulting 358 amino acids (aa) long sequence an N-terminal signal peptide of 25 aa was detected, indicating that the protein is secreted. Xylanases are often synthesized with signal peptides because hydrolysis of the heteropolymer xylan occurs extracellularly. Proteins showing sequence identity to Xyn10 also have signal peptides [29-31]. The conserved catalytic domain for family 10 glycoside hydrolases extends from aa 41 to 346 suggesting that the protein belongs to this family. Glycoside hydrolases are grouped in families according to amino acid sequence homologies [32]. Multiple sequence alignments resulted in the identification of two putative catalytic glutamate residues within the frequently conserved motifs VNNEA (aa 159-163) and TEBDV (aa 270-273). Hypothetical protein modelling indicated a characteristic TIM-barrel fold for Xyn10 as it is typical for members of glycoside hydrolase family 10 [33].

**Production and purification of Xyn10**

The gene xyn10 was amplified without signal peptide, cloned into pQE-80L and successfully overexpressed in E. coli BL21 (DE3). The expression of functional xylanase-encoding genes from thermophiles in E. coli BL21 (DE3) was reported recently [34,35]. E. coli was also shown to be a suitable host in this study. The recombinant thermostable xylanase Xyn10 could be detected and purified from the soluble fraction of the crude extract. Xyn10 was purified to homogeneity by heat precipitation and subsequent Ni-NTA affinity chromatography with a purification and purified from the soluble fraction of the crude extract.

**Biochemical properties of Xyn10**

Xyn10 exhibited catalytic activity in the range of 70-115 °C with the highest value at 95 °C (Figure 2A). The protein exhibited high thermal stability retaining 80-100 % activity after incubation for 24 h at 50-70 °C (supplementary material, Figure S3). At 80 °C the half life time was approximately 150 min (Figure 2B). Sequence analysis of Xyn10 showed some possible features for thermal stability described for other thermoactive proteins, such as elevated percentage of charged amino acids, higher arginine/lysine ratio and no cysteine residues [35-38]. Xyn10 showed higher thermal stability as well as optimal temperature compared to characterized glycoside hydrolases derived from *Thermus* species and recently characterized xylanases from *Geobacillus* sp. and *Bacillus halodurans* [39-42]. Operation temperature of several enzymatic processes in industry is limited to 50-60 °C because most commercially available xylanases are produced by *Aspergillus* and *Trichoderma* and are heat-labile. The extraordinary temperature range between 70 and 115 °C as well as the stability for more than 24 h at 70 °C makes Xyn10 a powerful candidate for pretreatment of lignocellulose at the elevated temperature of 70 °C in second generation biorefineries or paper industry.

Xyn10 displayed highest hydrolytic activity at pH 6.0 (Figure 3A), similar to other characterized glycoside hydrolases from *Thermusspp.* [39,40]. However, the enzyme showed high stability at a broad range of pH 4.0-10.0 with more than 80 % residual activity after incubation for 24 h at 4 °C at the mentioned pH values. Residual activities of more than 80 % were detected after incubation of 24 h at pH 6.0-8.0 and 70 °C. After 24 h of incubation at 70 °C in the range of pH 4.0-5.0 and 8.0-10.0 Xyn10 exhibited 30-50 % residual activity (Figure 3B). The decrease of stability of Xyn10 at acidic and alkaline pH was probably caused through the additional denaturing effect of the elevated temperature. The characterized xylanase from *Thermotoga maritima* showed lower stability because after incubation for 8 h at pH 5.1-10.1 residual activities of 10-40 % were detected [43].

The effect of metal ions on the catalytic activity of Xyn10 is shown in Table 2. The protein showed no detectable activity in presence of Cu²⁺ and was strongly inhibited in presence of 1 mM Al³⁺,Fe²⁺, Fe³⁺ and Zn²⁺. High sensitivity towards these cations has been described for other xylanases [28,30,41]. Especially for the inhibitory effect of Cu²⁺ on GH 10 xylanases numerous examples have been reported [35,44,45]. No metal ion caused enhancement of catalytic activity. Therefore, Xyn10 seemed to belong to the 61 % of hydrolases that do not require metal ions for catalytic action [46]. Enzymes from thermophilic microorganisms often display high stability in presence of several detergents and reagents which is advantageous for many industrial applications.

**Table 1: Purification of Xyn10.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein conc. [mg/mL]</th>
<th>Total activity [U]</th>
<th>Activity [U/mL]</th>
<th>Spec. activity [U/mg]</th>
<th>Yield [%]</th>
<th>PF</th>
</tr>
</thead>
<tbody>
<tr>
<td>GE</td>
<td>24.9</td>
<td>157,035.0</td>
<td>10,469.0</td>
<td>420.0</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>HP</td>
<td>13.0</td>
<td>111,468.0</td>
<td>9289.0</td>
<td>714.0</td>
<td>71.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>4.7</td>
<td>26,470.0</td>
<td>5294.0</td>
<td>1126.0</td>
<td>17.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Abbreviations: conc: concentration; spec: Specific; PF: Purification factor; GE: Crude extract; HP: Heat precipitation; Ni-NTA: Ni-NTA Affinity chromatography
Figure 1 Purification process of Xyn10 and zymogram analysis. Proteins were separated via SDS-PAGE (gel 12 %) and stained with Coomassie brilliant blue. For zymogram analysis the gel was incubated for 1 h at 70 °C in a solution of beechwood xylan (1 %, w/v), stained with Congo red and decolorized in 1 M NaCl. kDa: kilo Dalton, M: Unstained Protein Molecular Weight Marker (Fermentas), C: control (crude extract of E. coli BL21 (DE3)/pQE-80L), CE: crude extract of E. coli BL21 (DE3)/pQE-80L::xyn10, HP: heat precipitation, N: Ni-NTA affinity chromatography, Zym: zymogram.

Figure 2 Influence of temperature on the activity and stability of Xyn10. (A) Determination of relative activities in the range of 50-115 °C was performed in 50 mM maleate buffer pH 6.0 using 0.5 % oat spelt xylan (w/v) as substrate. Time for hydrolysis was set to 15 min. Relative activity at 95 °C was considered as 100 %.

(B) Thermal stability was examined by incubation of Xyn10 for 0-180 min at 80°C. Subsequently residual activity against 0.5 % oat spelt xylan (w/v) was measured at 95 °C in 50 mM maleate buffer, pH 6.0. Detected relative activity without preceding incubation was set to 100 %.

Figure 3 Influence of pH on the activity and stability of Xyn10. (A) Relative activity was measured at 95 °C in the range of pH 4.0-10.0 (50 mM Britton-Robinson buffer) for 15 min using 0.5 % oat spelt xylan (w/v) as substrate. The value at pH 6.0 was considered as 100 %.

(B) pH stability was investigated by determining residual activity after 24 h of incubation at pH 4.0-10.0 (50 mM Britton-Robinson buffer) at 4 °C and 70 °C. Subsequently, the activity assay was conducted at pH 6 using 0.5 % oat spelt xylan (w/v) as substrate, the hydrolysis-assay was done for 15 min at 70 °C. The measured values without incubation were considered as 100 %.

Therefore, the influence of different reagents on the activity of Xyn10 was examined. Remarkably high tolerance (relative activities of 80-100 %) was detected for several reagents, but hydrolytic activity decreased in the presence of Pefabloc and CTAB (Table 3). The inhibitory effect of the cationic surfactant CTAB could be due to its interaction with negatively charged (catalytic) residues of Xyn10. Slight increase of activity was observed in presence of DTT as described for several xylanases [28,45].

Xyn10 was able to hydrolyze oat spelt xylan (1119.5 ± 20.2 U/mg), beech- and birchwood xylan (944.1 ± 35.0 U/mg, 854.7 ± 27.1 U/mg) as well as 4-NP-β-D-xylopyranoside (6.4 ± 0.6 U/mg) but did not show side activity towards cellulose (Table 4). Xylanases from glycoside hydrolase family 10 often exhibit activity against a variety of branched substrates as it can be observed for the enzyme of T. brockianus [48]. The xylanase described here demonstrates enormous potential for application in second generation biorefineries due to higher specific activities towards soluble and insoluble xylan from different sources compared to commercially available enzymes (sigmaaldrich.com) or recently characterized proteins from thermophiles [34,35].

The kinetic parameters, such as $V_{max}$, $K_m$, $k_{cat}$ and the catalytic
xylobiose and xylose are the main hydrolysis products generated by Xyn10 ([supplementary material, Figure S4]). Due to often existing β-xylosidase side activity xylose is a common hydrolysis product of family 10 xylanases [49]. Especially for the application in biorefineries the direct production of xylose from xylan is an important advantage.

CONCLUSION

The utilization of lignocellulose for biofuel production offers chances to satisfy the growing demand for energy in a sustainable way. Moreover, food vs. fuel conflicts which are the most controversial aspects of 1st generation bioethanol are avoided [50]. However, for the competitive production of biofuel from lignocellulose highly efficient, thermostable enzymes are required. Especially xylanases play an essential role because additional fermentation of hemicellulose-derived xylose will contribute to higher yields and therefore improve efficiency of biorefineries [7,8].

The first xylanase-encoding gene from the genus *Thermus* was identified. The xylanolytic enzyme Xyn10 was successfully produced, purified and characterized. In comparison to commercially available xylanases the protein exhibits higher specific activities, reaction temperatures and better thermal stability.

ACKNOWLEDGEMENT

This work was financially supported by the German Federal Ministry of Education and Research (BMBF, funding code 0315559A) within the Cluster “Biorefinery2021”. We thank Angel Angelov (Technical University Munich, TUM, Germany) and Vera Haye for support.

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SUPPLEMENTARY FIGURES

**Figure S1** Calibration curves. (A) Calibration curve for xylose, (B) Calibration curve for glucose. 500 µL of xylose or glucose, respectively (0-2.5 mM in 50 mM maleate buffer, pH 6.0) were mixed with 500 µL of DNS reagent. After incubation for 5 min at 99 °C, absorptions were measured at 546 nm.

**Figure S2** Time dependent activity profile. Hydrolysis of 0.5 % oat spelt xylan (w/v) in 50 mM maleate buffer (pH 6.0) was conducted at 95 °C for 0-35 min. After the addition of 500 µL DNS reagent the reaction was transferred on ice and subsequently incubated at 99 °C for 5 min. Absorption was determined at 546 nm.
**Figure S3** Thermal stability of Xyn10.
After incubation of the protein at 50-70 °C for 3-24 h the residual activity was determined using 0.5 % oat spelt xylan (w/v) as substrate. Hydrolysis was conducted at 95 °C for 15 min in 50 mM maleate buffer, pH 6.0. Detected relative activity without preceding incubation was set to 100 %.

**Figure S4** HPLC analysis of hydrolysis products.
Hydrolysis of 2 % xylan (w/v) by Xyn10 was carried out for 1 h at 80 °C. Hydrolysis products were monitored by HPLC (LaChrom, RI-detector: L-7490, Merck-Hitachi, Tokio, Japan) using a HPX42A column and high purity water as solvent with a flow rate of 0.6 mL/min. The following retention times in minutes were determined: xylobiose: 16.23, xylose: 18.02.