Xylose reductase from the thermophilic fungus *Talaromyces emersonii*: cloning and heterologous expression of the native gene (*Texr*) and a double mutant (*Texr*$^{K271R + N273D}$) with altered coenzyme specificity

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Xylose reductase is involved in the first step of the fungal pentose catabolic pathway. The gene encoding xylose reductase (*Texr*) was isolated from the thermophilic fungus *Talaromyces emersonii*, expressed in *Escherichia coli* and purified to homogeneity. *Texr* encodes a 320 amino acid protein with a molecular weight of 36 kDa, which exhibited high sequence identity with other xylose reductase sequences and was shown to be a member of the aldoketoreductase (AKR) superfamily with a preference for reduced nicotinamide adenine dinucleotide phosphate (NADPH) as coenzyme. Given the potential application of xylose reductase enzymes that preferentially utilize the reduced form of nicotinamide adenine dinucleotide (NADH) rather than NADPH in the fermentation of five carbon sugars by genetically engineered microorganisms, the coenzyme selectivity of *Te*XR was altered by site-directed mutagenesis. The *Te*XR$^{K271R+N273D}$ double mutant displayed an altered coenzyme preference with a 16-fold improvement in NADH utilization relative to the wild type and therefore has the potential to reduce redox imbalance of xylose fermentation in recombinant *S. cerevisiae* strains. Expression of *Texr* was shown to be inducible by the same carbon sources responsible for the induction of genes encoding enzymes relevant to lignocellulose hydrolysis, suggesting a coordinated expression of intracellular and extracellular enzymes relevant to hydrolysis and metabolism of pentose sugars in *T. emersonii* in adaptation to its natural habitat. This indicates a potential advantage in survival and response to a nutrient-poor environment.

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1. Introduction

D-Xylose is the most abundant five carbon sugar in nature and is the major constituent monosaccharide in plant hemicellulose. In fungi, xylose catabolism involves a series of oxidation and reduction reactions to form D-xylulose, which then enters the pentose phosphate pathway after phosphorylation to D-xylulose-5-phosphate. The combined action of xylose reductase (EC 1.1.1.21) and xylitol dehydrogenase (EC 1.1.1.9) is required to convert D-xylose to D-xylulose and all enzymes of the D-xylose pathway can be used in the L-arabinose pathway, where arabitol is oxidized by NAD^+-dependent arabitol dehydrogenase (EC 1.1.1.12) producing L-xylulose. This is then converted to xylitol by NADPH-dependent L-xylulose reductase (EC 1.1.1.10).

Keywords. Xylose reductase; *Talaromyces emersonii*; thermophilic; co-enzyme specificity; transcriptional analysis

Abbreviations used: AKR, aldoketoreductase; BSA, bovine serum albumin; IPTG, *iso*propylthio-*β*-galactoside; LOOPP, Learning, Observing and Outputting Protein Patterns; NADH, reduced form of nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NCBI, National Centre for Biotechnological Information; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends

Xylose reductase, a pentose reductase and member of the aldoketoreductase family 2 (AKR2), catalyses the first step in five carbon metabolism by reducing xylose and arabinose to xylitol and arabitol. AKRs are a superfamily of enzymes comprising 15 separate families with approximately 120 identified members (Jez and Penning 2001). AKRs catalyse the reversible reduction of aldehydes and/or ketones to their corresponding alcohols, almost exclusively utilizing NADPH as a coenzyme. A few are capable of dual NADPH/NADH specificity but, to date, only one *E. coli* enzyme has been shown to be specific for NADH (Di Luccio *et al.* 2006).

Altering the coenzyme preference of pentose reductases towards NADH utilization is critical in the genetic engineering of microorganisms catalysing the bioconversion of xylose- and arabinose-rich wastes to ethanol. Redox fluxes through metabolic pathways are essential to improve the fermentative capabilities of recombinant *Saccharomyces* strains engineered for xylose metabolism (Hahn-Hägerdal *et al.* 2001). Different redox cofactors are used in the pentose pathway, which affects the cellular demand for oxygen. The imbalance of these redox cofactors means that pentoses are not fermented efficiently to ethanol (Hahn-Hägerdal *et al.* 2006; Hahn-Hägerdal *et al.* 2007). NADH-specific xylose reductase enzymes would enable efficient recycling of the coenzyme in the next step in xylose and arabinose metabolism, which involves conversion of xylitol to D-xylulose and arabitol to L-xylulose by NAD^+-specific dehydrogenases. Furthermore, NADH is more stable and intracellular concentrations are naturally much higher than those of NADPH (Liu *et al.* 2006).

Several xylose reductase genes have been identified from different sources (Amore *et al.* 1991; Billard *et al.* 1995; Handumrongkul *et al.* 1998), and all show a common specificity for NADPH. Site-directed mutagenesis and structural studies with *Candida tenuis* xylose reductase (Kratzer *et al.* 2006), a dual-specific enzyme with a preference for NADPH, revealed the main determinants involved in pentose-specific substrate-binding recognition, key residues involved in coenzyme interaction and suggested mechanisms by which certain AKRs can utilize both co-enzymes (Leitgeb *et al.* 2005; Petschacher and Nidetzky 2005; Petschacher *et al.* 2005; Di Luccio *et al.* 2006). A double mutant of the *C. tenuis* xylose reductase $K^{274}R$-$N^{276}D$ was shown to have undergone almost complete reversal of coenzyme preference from NADPH to NADH (Petschacher *et al.* 2005). Furthermore, improved fermentative capabilities in terms of ethanol yield were shown for a recombinant *S. cerevisiae* strain carrying a single copy of the *C. tenuis* xylose reductase double mutant when compared to *S. cerevisiae* harbouring the wild-type *C. tenuis* xylose reductase (Petschacher and Nidetzky 2008). A semi-rational and directed mutagenesis approach applied to xylose reductase from *Pichia stipitis,* also targeting amino acids involved in coenzyme interaction, resulted in mutant proteins with reversed coenzyme preference from NADPH to NADH in two independent studies (Liang *et al.* 2007; Watanabe *et al.* 2007).

T. emersonii is a thermophilic filamentous fungus which inhabits the soil, decaying masses of plant material, piles of agricultural and forestry products, and other accumulations of organic matter (Stolk 1972). Thermostable lignocellulolytic enzyme systems from this fungus have been described and characterized previously (Moloney *et al.* 1985; Brooks *et al.* 1992; Tuohy and Coughlan 1992; Tuohy *et al.* 1993; Murray *et al.* 2001; Tuohy *et al.* 2002; McCarthy *et al.* 2003; McCarthy *et al.* 2005). *T. emersonii* utilizes xylose and arabinose as the sole carbon sources; however, no studies to date have reported on the intracellular pathway enzymes involved in metabolism of these monosaccharide products of biomass degradation. The aim of the present study was to clone and characterize the xylose reductase gene product from the filamentous fungus *T. emersonii*, and investigate coenzyme specificity by site-directed mutagenesis.

2. Materials and methods

Unless otherwise stated, general reagents and chemicals (molecular biology grade) were purchased from Sigma-Aldrich (Dublin, Ireland).

2.1 *Microbial strains, cultivation conditions and vectors*

Mycelia harvested from cultures of *T. emersonii,* grown at 45ºC on Sabouraud dextrose agar, were used to inoculate liquid nutrient media, as described previously (Moloney *et al.* 1983). Liquid cultures were grown at 45ºC with shaking at 220 rpm. After appropriate timed intervals, mycelia were harvested by filtration through several layers of fine grade muslin, washed with 75 mM sodium citrate, pH 7.5, and frozen immediately under liquid nitrogen prior to isolation of nucleic acids. The pGEM-T-easy plasmid (Promega Gmbh, Mannheim, Germany) was used for subcloning and sequencing of polymerase chain reaction (PCR) products. The pET100/D-TOPO® plasmid (Invitrogen, Paisley, UK) was used for protein expression. *E. coli* strains JM109 (Promega Gmbh, Mannheim, Germany) and One Shot® Top 10 (Invitrogen, Paisley, UK) were used as plasmid hosts. *E. coli* BL21-Star DE3® strain (Invitrogen, Paisley, UK) was used for protein expression. *E. coli* strains were grown in Luria–Bertani medium, unless otherwise stated, according to the supplier's instructions.

2.2 *PCR amplification and cloning of a specific fragment of the* Texr *gene*

Genomic DNA was isolated from *T. emersonii* mycelia, cultivated for 24 h in mineral salt liquid medium containing

20 g l^{-1} glucose (Murray *et al.* 2003) by the method of Raeder and Broda (1985). Amplification of DNA encoding *Texr* gene fragments was performed using PCR, with degenerate primers designed from existing fungal xylose reductase sequences present in the database (table 1), and *T. emersonii* genomic DNA as template. Reaction conditions for PCR amplification consisted of an initial denaturation at 95°C for 30 s, 30 cycles of denaturation at 95°C for 10 s/30 s, annealing at 49–60°C (depending on the primers) for 30 s and extension at 72°C for 30–60 s/kb, with a final extension of 5 min at 72°C. HotStar Taq DNA polymerase (Qiagen, Crawley) was used in the PCR reactions. PCR products were separated by electrophoresis, purified using the QIAquick gel extraction kit (Qiagen, Sussex, UK) and cloned into the pGEM-T easy vector (Promega Gmbh, Mannheim, Germany), following the manufacturers' protocols. Plasmids were purified from *E. coli* cultures using the Qiaprep Spin Miniprep Kit (Qiagen, Sussex, UK) and sequenced.

2.3 *Rapid amplification of cDNA ends (RACE)*

Based on the sequence of the individual gene-specific PCR products, primers were designed for RACE. Total RNA, isolated by the method of Chomczynski and Sacchi (1987) from liquid cultures induced with mineral salt medium containing 20 g l^{-1} xylose for 20 h, was used as a template for RACE in a modification of the Ambion RACE protocol outlined previously (Murray *et al.* 2003). First-strand cDNA was prepared from the RNA using Thermo-X reverse transcriptase (Invitrogen, Paisley, UK) and the 3′ RACE adapter [5′-GCGAGCACAGAATTAATACGACT CACTATAGGT$_{20}$TVN-(V = A, C, or G) N (N = A, C, T, or G)-3′]. cDNA synthesis was performed at 55°C for 1 h. Gene-specific primers for *Texr* used for RACE are listed in table 1. Reaction conditions for RACE PCR and subsequent subcloning of products were as described earlier.

2.4 *Isolation of the full-length* Texr *gene*

The full-length *Texr* sequence was amplified from *T. emersonii* first-strand cDNA and genomic DNA by PCR with primers (table 1) corresponding to the regions containing the putative start and stop codons deduced from the 5′ and 3′ RACE PCR products. A proofreading Taq polymerase enzyme, Phusion DNA polymerase (Finnzymes, Espoo, Finland), was used to generate the blunt-end PCR products. To facilitate 'TA cloning' into pGEM-T Easy (Promega), these products were polyA-tailed according to the procedure outlined in the pGEM-T Easy handbook.

2.5 *Site-directed mutagenesis*

The full-length *Texr* gene was used as the template for site-directed mutagenesis. Mutation of Lysine^{271} to Aspartate and Asparagine^{273} to Arginine in *Texr* was carried out using the QuikChange system (Stratagene, California, USA) according to the manufacturer's instructions. Mutagenic oligonucleotide primers complementary to the coding and non-coding strands were used (table 1).

2.6 *Northern blot analysis*

RNA isolated from *T. emersonii* mycelia cultivated on various inducing substrates was separated electrophoretically on 1.2% formaldehyde–agarose gel and then transferred to SuperCharge nylon membranes (Schleicher and Schuell, New Hampshire, USA) by vacuum

Table 1. Primer sequences used in PCR reactions to amplify *Texr*. Note that I = A, T, G or C; S = C or G; Y = C or T and altered nucleotides are underlined.

Amplification reactions	Primer name	Sequence (5′ to 3′)
Degenerate PCR	Texr forward	GTSAAGCGYGAGGASCTCTTC
	Texr reverse	CAGATCGAGCACCACCCITAC
RACE	5′Race outer Texr	CAAGGTCGAGCCGATCGCGCGGAAG
	5′Race inner Texr	CATTGTGTCCAAGCTGTGGAACACG
	3′Race outer Texr	CATTGTGTCCAAGCTGTGGAACAC
	3′Race inner Texr	CAAGGTCGAGCCGATCGCGCGGAAG
Full-length PCR	Texr start	ATGGCCACGCCTACTATCAAGCTG
	Texr stop	GTACATCCCGATCTTCGCTTAG
Mutagenic PCR	Texr mutant forward	CGCCGTCATCCCC<u>CGC</u>AGC<u>GAC</u>AACCCGGGCCGCCTG
	Texr mutant reverse	CAGGCGGCCCGGGTT<u>GTC</u>GCT<u>GCG</u>GGGGATGACGGCG
Expression PCR	Texr forward	CACCATGGCCACGCCTACTATCAAGC
	Texr reverse	GTACATCCCGATCTTCGCTTAG

blotting (Bio-Rad, Munchen, Germany). Hybridization was carried out overnight at 60ºC in 7% SDS, 50% deionized formamide, 5× SSC, 50 mM sodium phosphate, pH 7.0, *N*-lauroylsarcosine and 2% blocking reagent. A concentration of 20 ng digoxygenin-labelled full-length *Texr* cDNA probe per millilitre of hybridization buffer was used in all cases. Detection was performed after incubation of the membrane with the conjugate antibody, using CDP-Star (Roche Molecular Biochemicals, Mannheim Germany) as the chemiluminescent substrate according to the manufacturer's instructions.

2.7 *Expression of histidine-tagged wild type (*Texr*) and mutant (*Texr$^{K271R + N273D}$*) in* E. coli *BL21-Star DE3®*

Primers containing CACC corresponding to the GTGG overhang in the TOPO® cloning vector (Invitrogen Ltd, Paisley, UK) were used to amplify wild-type *Texr* and the double mutant *Texr*$^{K271R + N273D}$ (table 1). Plasmid DNA was purified and transformed into BL21-Star® DE3 one-shot competent *E. coli* cells (Invitrogen Ltd., Paisley, UK), which were cultured to mid-log phase, and expression was induced by the addition of 1 mM *iso*propylthio-*β*-galactoside (IPTG) followed by a further growth period of 6 h at 37ºC. Samples were centrifuged at 6000 rpm for 10 min at 4ºC and cell pellets were then lysed using the CelLytic lysis kit (Sigma Aldrich, Dorset, UK). The overexpressed His_6-tagged recombinant wild-type and mutant *Te*XR proteins were purified by affinity chromatography using a Nickel-NTA purification column (Invitrogen Ltd, Paisley, UK) according to the manufacturer's instructions.

2.8 *Enzyme and protein assays*

Xylose reductase enzyme activity was determined by monitoring the consumption and formation of NAD(P)H at 340 nm ($\varepsilon_{NAD(P)H}$ = 6220 $M^{-1}.cm^{-1}$) in a Shimadzu UV1601 spectrophotometer. Apparent kinetic parameters of NAD(P)H-dependent xylose reduction were obtained from initial-rate measurements under conditions in which one reactant was varied and the other reactant was present at a constant and saturating concentration. Measurements were made by varying the substrate concentration to a maximum of 1 M with a constant concentration of either NADPH or NADH and by varying the cofactor concentration up to 300 μM in the presence of a constant substrate concentration in order to define suitable conditions for a full kinetic study. Reactions were started by the addition of coenzyme, and enzyme concentrations in the assays were chosen such that the observed rates were in the range 0.001–0.1 ΔA/min and constant for ≥1 min. Appropriate controls were recorded and the results represent the mean values of triplicate assays. Kinetic parameters were calculated from a Hanes–Woolf plot. Protein concentrations in enzyme and bovine serum albumin (BSA) standard samples were determined using the Bradford method (Bradford 1976). Optimum pH values were determined in 50 mM ammonium acetate buffer (pH 5–6), 50 mM HEPES buffer (pH 7–8) and 50 mM Tris-HCl (pH 9–10) at 37ºC. Optimum temperatures for activity were determined in 50 mM HEPES buffer (pH 7.0) over the temperature range of 20–60°C.

2.9 *Sequence analysis and structure prediction*

All sequencing was carried out in both directions to eliminate read errors. Sequencing reactions were conducted by AGOWA laboratories, Berlin, Germany. Database similarity searches were performed using the National Centre for Biotechnological Information (NCBI) online program BLAST (Altschul *et al.* 1990) against protein (BlastX) and nucleotide (BlastN) sequences stored in GenBank. Multiple sequence alignments were done using the CLUSTALW program (Higgins 1994). The PSIPRED V2.4 program in combination with NCBI Blast packages, the Learning, Observing and Outputting Protein Patterns (LOOPP) program (*http://www.tc.cornell.edu/CBI0/loopp_doc.html*) and the Predict Protein Server (Rost 1996) were used to predict secondary structure (Bairoch *et al.* 2005).

3. Results

3.1 *Isolation of full-length genomic and cDNA* Texr *clones and sequence analysis*

Using degenerate oligonucleotide primers based on existing fungal *xylose reductase* gene sequences in the database, a specific band of 120 bp was amplified from *T. emersonii* chromosomal DNA. Sequence analysis confirmed that the PCR product displayed high homology to other fungal *xylose reductase* gene family members. Using this PCR product sequence, 5′ and 3′ RACE primers were designed to amplify the ends of the *Texr* gene from cDNA generated from *T. emersonii* RNA induced with 20 g l^{-1} xylose. Specific bands corresponding to the 5′ (368 bp including 65 bp 5′UTR) and 3′ ends (769 bp including 199 bp of 3′ UTR with a poly-A tail) of *Texr* were amplified, purified and sequenced. Full-length genomic and cDNA *Texr* clones were amplified from chromosomal DNA and first-strand cDNA, respectively, using 5′and 3′ termini *Texr*-specific primers based on the sequences of RACE products. *Texr* consists of a 960 bp open reading frame, interrupted by 2 introns (79 and 71 bp) with consensus 5′ and 3′ intron splice sites that encode a 320 amino acid protein. The theoretical molecular weight of the deduced *Te*XR protein is 36 kDa, which is in

good agreement with the approximate molecular weight of other AKR family members (Jez and Penning 2001).

Comparison of the deduced amino acid sequence of *Texr* (GenBank ACR78268) with xylose reductase sequences from *Aspergillus niger* (GenBank AAF61912), *Magnaporthe grisea* (GenBank CAI67591), *Hypocrea jecorina* (GenBank AAM66765), *Candida tenuis* (GenBank AAC25601) and *Pichia stipitis* (GenBank CAA42072) revealed identity values (CLUSTALW) of 79%, 63%, 63%, 55% and 52%, respectively (figure 1). The canonical active site conformation of AKRs involves a tyrosine, lysine, aspartate and histidine tetrad (Jez *et al.* 1997). The positions of the tetrad residues are highly conserved within this family and suggest a common mechanism of catalysis among AKRs (Hyndman *et al.* 2003). Tyrosine acts as the general acid/base catalyst of the reaction with its pKa value being lowered through hydrogen bond formation with a lysine residue, which is salt-linked to aspartate (Schlegel *et al.* 1998). Histidine has been shown to facilitate the reaction through the precise positioning of the substrate carbonyl group at the active site in the *C. tenuis* xylose reductase (Kavanagh *et al.* 2003; Kratzer *et al.* 2004). This highly conserved canonical active-site tetrad is conserved in *Te*XR as Asp^{45}, Tyr^{50}, Lys^{79} and His^{115} (figure 1).

Site-directed mutagenesis studies with *C. tenuis* xylose reductase (Kratzer *et al.* 2006) revealed that Trp^{23}, Asp^{50} and Asn^{309} are the main determinants of pentose-specific substrate-binding and recognition; these residues are conserved in *Te*XR as Trp^{22}, Asp^{49} and Asn^{307} (figure 1). The conserved glutamate residue involved in cofactor recognition (Kavanagh *et al.* 2003) as well as the residues responsible for cosubstrate binding are all conserved in *Te*XR as Glu^{224}, Lys^{273}, Ser^{274}, Asn^{275} and Arg^{279} (figure 1).

Structure prediction of *Te*XR was based on the previously published 1.8 Å structure of xylose reductase from *C. tenuis* in complex with NADH (Kavanagh *et al.* 2003) (PDB File 1mi3A) and revealed a high sequence identity of 55%. The LOOPP program (*http://www.tc.cornell.edu/CBI0/loopp_doc.html*) predicts that alignment with 1mi3A shows a Z-score of 19.311 with high confidence (99.69%). The Predict Protein Server (Rost 1996) predicts *Te*XR to be a compact globular protein composed of 35.62% helices, 12.81% sheets and 51.56% loops (figure 1). The structure of *Te*XR has therefore been predicted to be a $(\beta/\alpha)_8$ barrel, the most common fold motif adopted by the AKR superfamily (Branden 1991).

3.2 *Expression of wild-type* TeXR *and* $TeXR^{K271R+N273D}$ *double mutant in* E. coli *and kinetic characterization*

A double mutant of the *C. tenuis* xylose reductase $K^{274}R$-$N^{276}D$ was shown to have undergone almost complete reversal of coenzyme preference from NADPH to NADH (Petschacher *et al.* 2005). A high degree of sequence homology exists between *Te*XR and *C. tenuis* xylose reductase, and the amino acid residues involved in coenzyme interaction, identified and extensively studied in *C. tenuis* xylose reductase (*Ct*XR) (Leitgeb *et al.* 2005; Petschacher and Nidetzky 2005; Petschacher *et al.* 2005; Di Luccio *et al.* 2006), are all conserved in *Te*XR. Furthermore, xylose reductase from *Candida parapsilosis* has a 100-fold higher catalytic efficiency with NADH than NADPH, and possesses a unique arginine (Jung-Kul *et al.* 2003) corresponding to $lysine^{271}$ in *Te*XR. Using this rationale, both wild-type *Te*XR and the double-mutant $TeXR^{K271R+N273D}$ proteins were heterologously expressed in *E. coli* BL21-Star™ DE3 under identical experimental conditions to investigate the coenzyme preference and kinetic characteristics of both wild-type and mutant proteins.

Wild-type and double-mutant histidine-tagged proteins were purified to apparent homogeneity as judged by SDS-PAGE following Nickel-NTA affinity purification (figure 2). Both proteins migrated with an expected molecular weight of 40 kDa, which was consistent with the molecular weights estimated from the deduced amino acid sequence.

Both wild-type and double-mutant proteins were active from pH 5 to pH 8 with maximal activity at pH 6.5. Both proteins displayed activity over a broad temperature range (30–60ºC) with optimum activity observed at 37ºC. The stability of *Te*XR was relatively high, as it retained 28% of activity at 60ºC. The purified wild-type and mutant enzymes were characterized for xylose reduction with NADH and NADPH as the respective coenzymes. The kinetic data calculated from Hanes–Woolf plots are summarized in table 2.

Wild-type *Te*XR showed dual coenzyme specificity but was preferentially NADPH-dependent, with affinity for NADPH being 1.1-fold higher than NADH and catalytic

Table 2. Kinetic parameters for xylose reduction by wild-type *Te*XR and double-mutant $TeXR^{K271R+N273D}$ proteins expressed in *E. coli*

Enzymes	$K_{m\ xylose}$ (NADH) [mM]	$K_{m\ xylose}$ (NADPH) [mM]	NADH K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$mM^{1}.s^{-1}$]	NADPH K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$mM^{-1}.s^{-1}$]
*Te*XR wild type	89.40 ± 6.82	24.56 ± 4.43	0.263 ± 0.132	15753.08 ± 665.36	59916.38 ± 2660.96	0.244 ± 0.020	323974.10 ± 62346.35	1466242.00 ± 131280.90
*Te*XR mutant	76.06 ± 13.96	76.50 ± 6.78	0.300 ± 0.008	25110.66 ± 2003.10	83751.60 ± 8745.10	0.747 ± 0.057	100943.10 ± 3909.00	135532.50 ± 6407.85

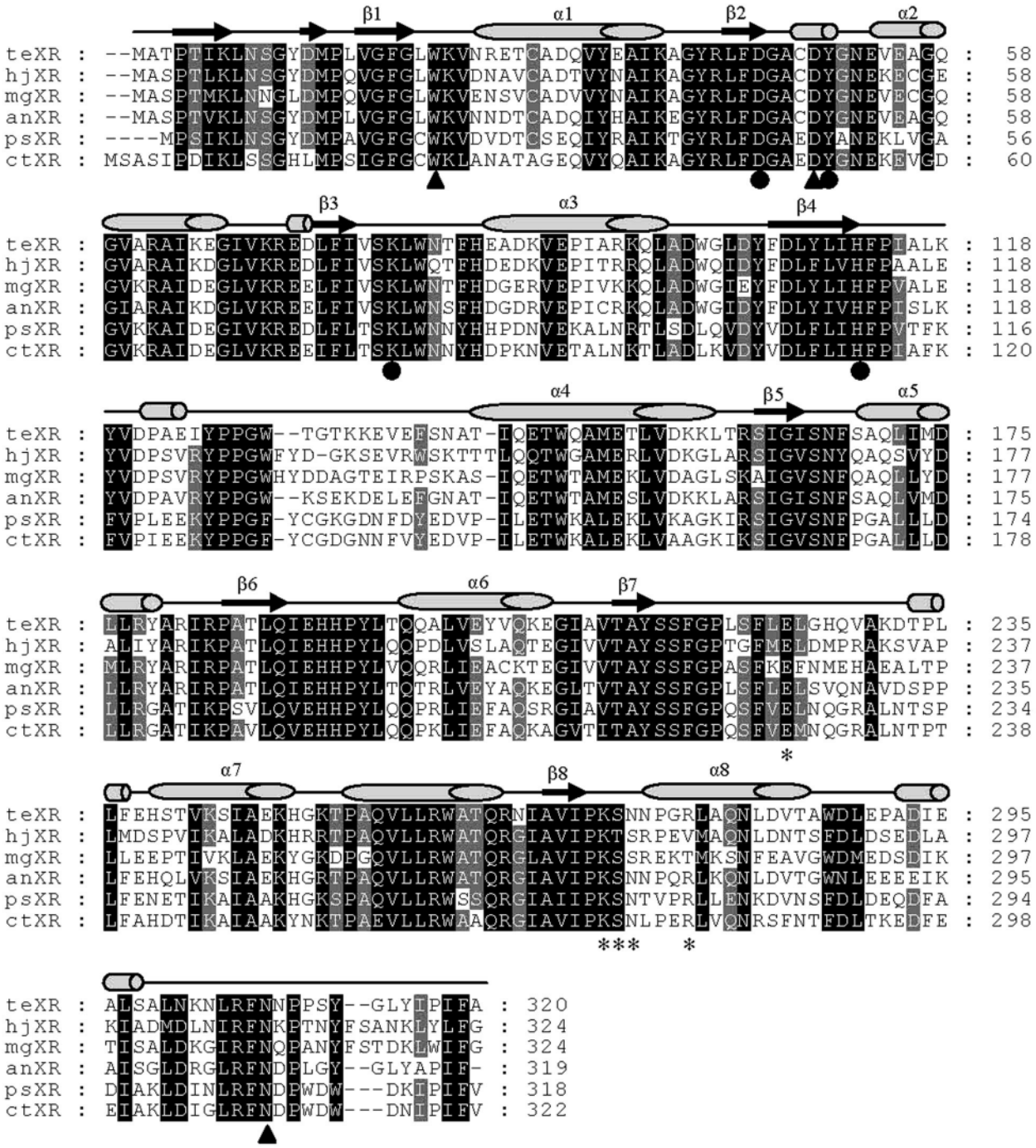

Figure 1. Genedoc alignment of *Te*XR with XRs from *A. niger* (anXR), *M. grisea* (mgXR), *H. jecorina* (hjXR), *C. tenuis* (ctXR) and *P. stipitis* (psXR). Single letter amino acid code is used. Residues identical or with a conservative substitution in five of the six sequences are printed in white on a grey background. Residues identical or with a conservative substitution in all sequences are printed in white on a black background. The active site tetrad (●), substrate-binding residues (▲), the glutamate residue critical for coenzyme utilization and other residues for cosubstrate binding (*) are shown. Helices are represented by cylinders and *β*-strands by arrows.

efficiency (k_{cat}/K_m) 24.5-fold higher with NADPH as coenzyme. It is interesting to note also that affinity for xylose was 3.6-fold higher with NADPH as coenzyme.

In the *Te*XR$^{K271R+N273D}$ double mutant, affinity for NADPH decreased 3.1-fold, while affinity for NADH remained relatively unchanged in comparison with the

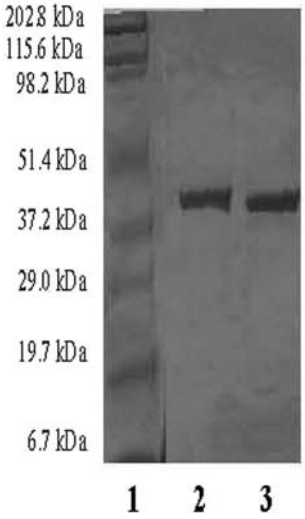

Figure 2. SDS-PAGE analysis of the purified *T. emersonii* wild-type and mutant enzymes. Lane 1: protein molecular weight marker; lane 2: wild-type His_6-*Te*XR; lane 3: double-mutant His_6-*Te*$XR^{K271R + N273D}$

wild-type enzyme. The turnover number (k_{cat}) increased 1.6-fold for the double mutant with NADH and decreased 3.2-fold with NADPH relative to the wild-type enzyme. As a consequence, the catalytic efficiency of the double mutant (k_{cat}/K_m) increased 1.4-fold with NADH and decreased 10.8-fold with NADPH relative to the wild-type enzyme. Using the specificity constant (k_{cat}/K_m^{NADH} / k_{cat}/K_m^{NADPH}) the coenzyme preference for NADH was improved 16-fold in the *Te*$XR^{K271R+N273D}$ double-mutant enzyme.

3.3 *Northern blot analysis of* Texr *expression*

The transcriptional regulation of *Texr* was studied by Northern analysis during growth on different carbon sources. Expression of *Texr* was detected on all carbon sources tested (figure 3), albeit at markedly different levels. Highest transcript levels of *Texr* were detected with xylose and to a lesser extent with arabinose as the sole carbon sources. *Texr* expression was also high when the pentitol products of the first step in five carbon metabolism, xylitol and arabitol, were used as the sole carbon sources. Sugar beet, which has a high pentose content (Fernandes *et al*. 2008), also induced *Texr* expression when used as sole inducing carbon source.

4. Discussion

T. emersonii is a filamentous fungus that inhabits the soil, decaying masses of plant material, and other accumulations of organic matter wherein the warm, humid and aerobic environment provides the basic conditions for its growth and development. This organism can also readily utilize

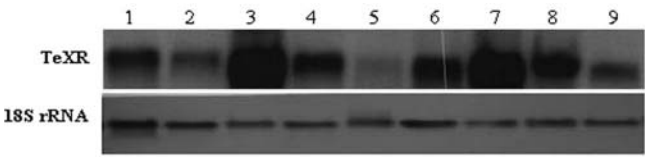

Figure 3. Northern blot analysis of *Texr* gene expression during cultivation of *T. emersonii* on various carbon sources included at 20 g l^{-1}. Lane 1: glycerol 10 h; lane 2: glucose 10 h; lane 3: D-xylose 10 h; lane 4: L-arabinose 10 h; lane 5: galactose 10 h; lane 6: xylitol 10 h; lane 7: L-arabitol 10 h; lane 8: galactitol 10 h; lane 9: sugar beet 36 h

pentose sugars such as xylose or arabinose as the sole carbon sources for growth, which makes this thermophilic fungus an attractive source organism for intracellular proteins with applications in biomass bioconversion strategies. Therefore, the isolation and characterization of genes involved in pentose sugar metabolism from *T. emersonii* may prove relevant in improving the biotransformation capabilities of *S. cerevisiae*.

The gene encoding xylose reductase, involved in the first step of pentose metabolism, was isolated and characterized from the thermoplilic fungus *T. emersonii*. *Te*XR is a monomeric $(\alpha/\beta)_8$-barrel protein 320 amino acids in length, which binds NAD(P)H to metabolize xylose and consequently is considered a member of the AKR superfamily. The residues involved in the canonical active site conformation of AKRs as well as the determinant residues of pentose-specific substrate-binding and recognition are conserved in *Te*XR. In addition, the conserved glutamate residue involved in cofactor recognition and the residues responsible for cosubstrate binding are all conserved in *Te*XR. The network of contacts between Glu^{227}, Lys^{274} and Asn^{276}, and the coenzyme 2′-phosphate group in a *C. tenuis* xylose reductase–$NADP^+$ complex have been identified as critical (Di Luccio *et al.* 2006). The presence of a carboxylate-containing amino acid, structurally homologous to Glu^{227} in *C. tenuis* xylose reductase located on a coil region instead of a helix, appears to confer ability among AKRs to utilize both NADH and NADPH as coenzymes (Petschacher and Nidetzky 2005). Increased mobility of this carboxylate residue appears to be critical for NADPH binding. Consequently, removal of steric hindrance that allows movement of the carboxylate moiety away from the 2′-phosphate of the coenzyme should allow a basis for structural engineering and reversal of coenzyme preference from NADPH to NADH for xylose reductases from other microbial sources (Di Luccio *et al.* 2006; Petschacher and Nidetzky 2008). As a consequence, a double mutant of the *C. tenuis* xylose reductase $K^{274}R$-$N^{276}D$ was shown to have increased preference for NADH (Petschacher *et al.* 2005).

Compared with the wild type, the *Te*$XR^{K271R+N273D}$ double mutant showed a 16-fold increase in the coenzyme preference

for NADH, showing that Lysine and Asparagine are the major determinants of NAD(P)H recognition. Mutational analysis of *Ps*XR has revealed that Lys^{270} is involved in both NADPH and D-xylose binding, and replacement of the conserved amino acid resulted in 80–90% loss of activity with xylose as substrate (Kostrzynska *et al.* 1998). However, only a slight difference was noted between the affinity for xylose with NADPH and NADH in *Te*$XR^{K271R+N273D}$ with the replacement of the amino acid Lysine.

Recently, *S. cerevisiae* strains harbouring the *Ct*$XR^{K274R+N276D}$ (Petschacher and Nidetzky 2008) and *Ps*$XR^{K270R+N272D}$ (Watanabe *et al.* 2007) mutant proteins have been shown to have improved fermentative ability for xylose. Since most of the AKRs have a strong preference for NADPH over NADH, the ability of a *Te*$XR^{K271R+N273D}$ mutant to use NADH preferentially to NADPH as a coenzyme in the first step of pentose metabolism could have dramatic effects on improving the xylose conversion process by reducing the redox imbalance. Production of ethanol by *S. cerevisiae*, genetically engineered with pentose metabolic pathway components, is not effective due to the excessive production of xylitol caused in part by the dual cofactor capacity of xylose reductase enzymes. Therefore, a highly stable NADH-dependent xylose reductase is of great importance in industrial pentose fermentation strategies for the elimination of redox imbalances in the initial steps of pentose metabolism.

To investigate the properties of *Te*XR that evolved in nutrient-poor environments, its transcriptional regulation was studied by Northern analysis. *Texr* expression is evident on glycerol and to a lesser extent on glucose, which is indicative of a lack of regulation by carbon-mediated catabolite repression and is consistent with the observed regulation of the *xylose reductase* gene from *A. niger* (Hasper *et al.* 2000). We have previously shown that expression of *T. emersonii* genes relevant to xylan degradation (*xylanase* and *β-xylosidase I*) were also upregulated when sugar beet was used as the sole carbon source (Fernandes *et al.* 2008). This finding suggests a coordinated regulation of genes encoding extracellular xylan-degrading enzymes and intracellular genes involved in D-xylose metabolism in *T. emersonii*. A similar coordinated regulation of xylan-degrading and xylose metabolic genes has also been demonstrated in *A. niger* (Hasper *et al.* 2000) and is directly controlled by the *A. niger* transcriptional regulator of xylanase and cellulase genes, XlnR (van Peij *et al.* 1998; van Kuyk *et al.* 2001). Expression of xylose reductase and the xylanolytic degradative enzyme system is coordinated through xylitol, which has been shown to induce xylan-degrading gene expression via XlnR (van Peij *et al.* 1998).

Similarly, the xylanase regulator Xyr1 in *Hypocrea jecorina* was shown to regulate the hydrolytic enzyme system and to take part in control of the fungal xylose pathway, in particular, the regulation of xylose reductase (Stricker *et al.* 2006). A homologue of *XlnR*/*Xyr1* has recently been cloned from *T. emersonii* (GenBank FJ349326) and it is therefore likely that a similar regulatory mechanism may occur in *T. emersonii* in response to monosaccharide products released from biomass substrates by the action of its extracellular polysaccharide hydrolysing enzyme systems. It is tempting to speculate that in adapting to nutrient-poor environments, *T. emersonii* developed a mechanism that would enable this fungus to live on decaying pentose-rich biomass and to adjust intracellular metabolic pathways relevant to pentose metabolism. These data also suggest that *Te*XR may be valuable in the production of xylitol and ethanol from renewable resources rich in pentose sugars.

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