Appl Microbiol Biotechnol (2010) 86:1647–1658
DOI 10.1007/s00253-010-2538-y

MINI-REVIEW

Properties and applications of microbial β-D-xylosidases featuring the catalytically efficient enzyme from *Selenomonas ruminantium*

Douglas B. Jordan · Kurt Wagschal

Received: 26 January 2010 / Revised: 2 March 2010 / Accepted: 3 March 2010 / Published online: 30 March 2010

Abstract Xylan 1,4-β-D-xylosidase catalyzes hydrolysis of non-reducing end xylose residues from xylooligosaccharides. The enzyme is currently used in combination with β-xylanases in several large-scale processes for improving baking properties of bread dough, improving digestibility of animal feed, production of D-xylose for xylitol manufacture, and deinking of recycled paper. On a grander scale, the enzyme could find employment alongside cellulases and other hemicellulases in hydrolyzing lignocellulosic biomass so that reaction product monosaccharides can be fermented to biofuels such as ethanol and butanol. Catalytically efficient enzyme, performing under saccharification reactor conditions, is critical to the feasibility of enzymatic saccharification processes. This is particularly important for β-xylosidase which would catalyze breakage of more glycosidic bonds of hemicellulose than any other hemicellulase. In this paper, we review applications and properties of the enzyme with emphasis on the catalytically efficient β-D-xylosidase from *Selenomonas ruminantium* and its potential use in saccharification of lignocellulosic biomass for producing biofuels.

Keywords β-Xylosidase · Protein engineering · Glycoside hydrolase · Bioenergy · GH43 · Performance

D. B. Jordan (✉)
USDA Agricultural Research Service,
National Center for Agricultural Utilization Research,
Peoria, IL 61604, USA
e-mail: douglas.jordan@ars.usda.gov

K. Wagschal (✉)
USDA Agricultural Research Service,
Western Regional Research Center,
Albany, CA 94710, USA
e-mail: kurt.wagschal@ars.usda.gov

Introduction

This mini review concerns properties and practical aspects involving xylan 1,4-β-D-xylosidase (EC 3.2.1.37), hereafter referred to as β-xylosidase, that are subjects of investigation in the authors' laboratories, including discovery and improvement of catalytically efficient enzyme to fully deconstruct 1,4-β-xylooligosaccharides to D-xylose monosaccharides at the industrial level for subsequent processes such as fermentation to biofuels and other bioproducts. Therefore, properties and applications will not be addressed for α-D-xylosidase (EC 3.2.1.-; Lovering et al. 2005; Okuyama et al. 2006; Yoshikawa et al. 1994), xylan 1,3-β-xylosidase (EC 3.2.1.72; Umemoto et al. 2008), oligosaccharide reducing-end xylanase (EC 3.2.1.156; Honda and Kitaoka 2004; Fushinobu et al. 2005), and xylose-utilizing glycosynthase (Ducros et al. 2003; Kim et al. 2005; Shaikh and Withers 2008).

Currently, β-xylosidase is used on the industrial scale when it is included in xylanase cocktails for deinking recycled paper (Marques et al. 2003), processing wood pulp (Tsujibo et al. 2001), improving bread dough baking and nutritional quality (Dornez et al. 2007), hydrolysis of bitter xylosylated compounds from grape juice during extraction and liberation of aroma derived from xylosylated compounds of grapes during wine making (Manzanares et al. 1999), and hydrolysis of xylan to D-xylose residues for subsequent reduction to xylitol (Polizeli et al. 2005). For these applications, large quantities of the enzymes are prepared from fungal and bacterial sources such as *Aspergillus niger*, *Trichoderma reesei*, and *Bacillus* sp. among others (Polizeli et al. 2005). Whereas the latter processes can be large in scale, they are dwarfed by the potential use of β-xylosidase, other hemicellulases, and cellulases in processes that hydrolyze lignocellulosic

biomass to monosaccharides for fermentation to biofuels as well as conversion to other value-added products (Werpy and Petersen 2004). Therefore, a major concern is the discovery of cost-efficient β-xylosidase (and other enzymes) which includes positive enzyme attributes such as high k_{cat} and k_{cat}/K_m, low affinity for monosaccharide inhibitors, good stabilities to pH and temperature, low levels of adsorption/inactivation by biomass feedstocks, and low cost of enzyme production.

Fundamental properties of β-xylosidase

By definition of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (http://www.chem.qmul.ac.uk/iubmb/), xylan 1,4-β-D-xylosidase catalyzes hydrolysis of single xylosyl residues from the non-reducing end of 1,4-β-D-xylooligosaccharides, including 1,4-β-D-xylobiose. According to the CAZy database (http://www.cazy.org/; Cantarel et al. 2009), β-xylosidase is currently grouped into six glycoside hydrolase families (GH3, GH30, GH39, GH43, GH52, and GH54) based on amino acid sequence similarities (Table 1). Based on structural folds, GH30 and GH39 are grouped into clan GH-A, having fold $(\alpha/\beta)_8$, GH43 is in clan GH-F, having fold fivefold β-propeller, and the other families are unassigned. The rapid rate of three-dimensional structure determinations by X-ray crystallography will undoubtedly fill this knowledge gap with time. High-quality crystals that diffract beyond 2 Å are often obtained with glycoside hydrolases, including β-xylosidase (Brunzelle et al. 2008), under simple conditions (e.g., polyethylene glycol as precipitant). Site-directed mutagenesis has been used to create inactive enzyme for the preparation of crystals of β-xylosidase in complex with substrate (Brüx et al. 2006), which provided structural models that are most useful for understanding the organization of the active site. As an alternative to substrates, β-xylosidase has been co-crystallized with an amino alcohol inhibitor which binds in the active site binding pocket (Brunzelle et al. 2008). For many glycoside hydrolases including GH43 β-xylosidase, small positional changes occur upon binding substrate according to structural overlays (Brunzelle et al. 2008). This constancy portends well for obtaining good homology models for newly discovered β-xylosidase. Conveniently, such three-dimensional models can be readily obtained by submittal of an amino acid sequence to automated services such as the Swiss-Model Workspace (Arnold et al. 2006).

Thorough examination of glycoside hydrolase reactions and experimental means for their study are available (Sinnott 2007; Rempel and Withers 2008; Yip and Withers 2006; Withers 2001; Rye and Withers 2000). Importantly, for certain mechanistic and enzyme performance considerations, β-xylosidases (and most other glycosidases) catalyze hydrolysis of substrates by either of two canonical reaction mechanisms (Koshland 1953). Both reaction types employ two amino acid side chains containing carboxyl groups (Asp or Glu). In the inversion mechanism (or single displacement mechanism), the β-anomeric configuration of the substrate's non-reducing end xylosyl glycone residue is, upon hydrolysis, released as α-xylose. This hydrolysis mechanism entails a single transition state on the enzyme, implicating addition of the nucleophilic water molecule (activated by general base Asp) to C1 of the glycone in concert with proton delivery (by general acid Glu) to the aglycone alkoxide leaving group, facilitating its departure from C1 of the glycone (Fig. 1a). In the retaining mechanism (or double displacement mechanism), the substrate's nonreducing end xylosyl glycone residue is, upon hydrolysis, released as β-xylose. This β-xylosidase mechanism comprises two transition states (Fig. 1b), one TS for the xylosylation step forming an ester bond between the enzyme nucleophile (Asp or Glu) and C1 of the glycone with simultaneous proton delivery (by general acid Glu) to the aglycone alkoxide leaving group, promoting its departure and one TS for the dexylosylation step in which the ester bond is hydrolyzed.

Unlike the rapidly interconverting anomeric conformations of arabinofuranose, for example, D-xylose has the relatively lengthy half-life for mutarotation of 1 h at 25°C (Jordan et al. 2007b), and this makes ^{1}H NMR the method of choice for the determination of the anomeric conformation of the hydrolysis product D-xylose. In addition, two other methods have been reported: HPLC separation of the

Table 1 CAZy classification of 1,4-β-xylosidase

Family	Clan, fold	Mechanism	Catalytic base or nucleophile	Catalytic acid or acid/base
GH3	None	Retaining	Asp	Glu
GH30	GH-A, $(\alpha/\beta)_8$	Retaining	Glu	Glu
GH39	GH-A, $(\alpha/\beta)_8$	Retaining	Glu	Glu
GH43	GH-F, 5-fold β-propeller	Inverting	Asp	Glu
GH52	None	Retaining	Glu	Asp
GH54	None	Retaining	ND	ND

ND not determined

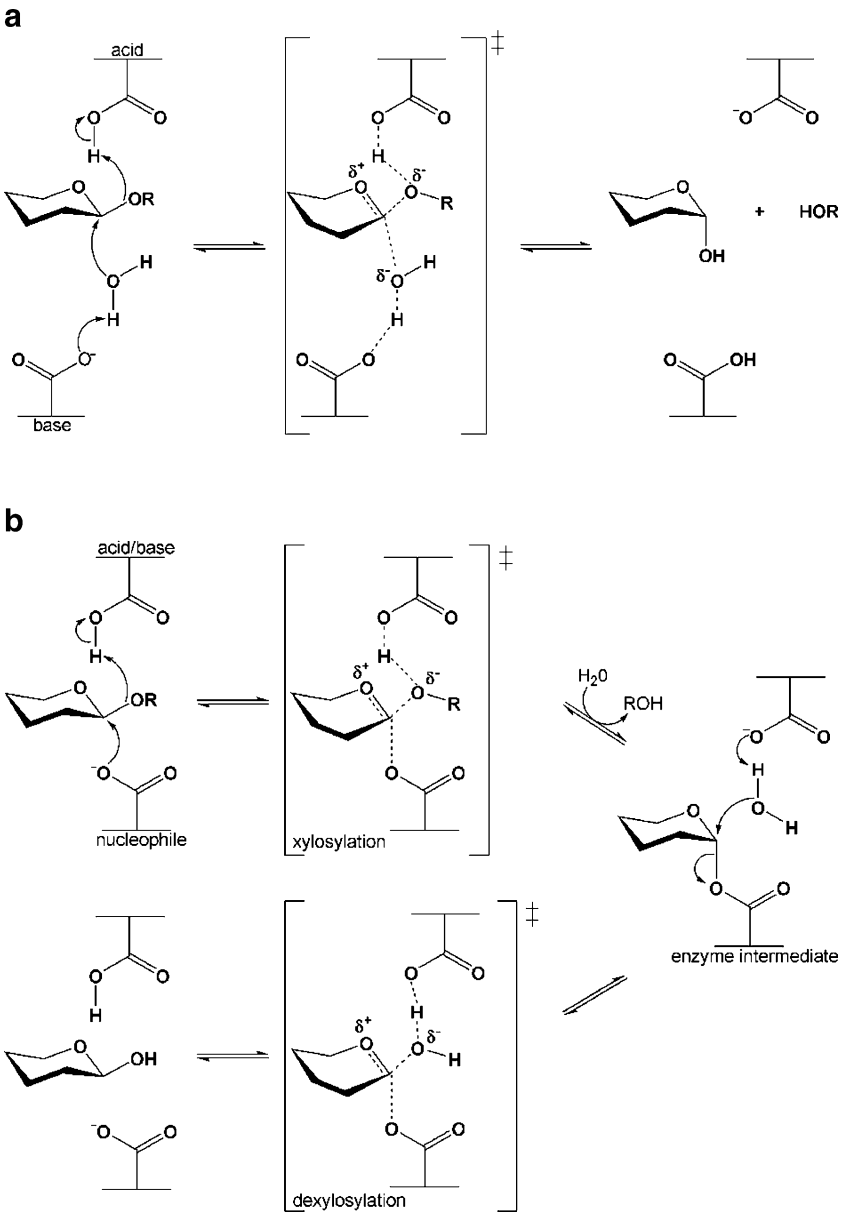

Fig. 1 Reaction mechanisms of β-xylosidase. **a** Inverting. **b** Retaining

two anomers of D-xylose (Braun et al. 1993) and a coupled enzyme system employing α-stereospecific D-xylose isomerase and sorbitol dehydrogenase (Kersters-Hilderson et al. 1976).

Identification of the catalytic base and catalytic acid for β-xylosidase operating through an inverting mechanism (GH43) has relied heavily on X-ray structural information, which implicates two of the three Asp and Glu residues of the active site (Brüx et al. 2006; Brunzelle et al. 2008). Mutation of the three highly conserved residues to nonreactive residues (Gly or Ala) produces inactive (>99.99%) enzyme (Shallom et al. 2005; Jordan et al. 2007b, 2009). However, one of these, an Asp, is in a poor position for serving as either catalytic base or acid; it is considered to aid catalysis by raising the pK_a of the catalytic acid and in stabilizing the position of the glycone moiety of substrate. The X-ray structures implicate the other active site Asp residue as the catalytic base and the active site Glu as the catalytic acid. Biochemical evidence supports the identities of the catalytic acid and base. Mutation of the catalytic acid to either Gly or Ala changes the k_{cat}/K_m pH profile so that the pK_a 7 attributed to the catalytic acid is lost (Shallom et al. 2005; Jordan and Braker 2010). Loss of activity by mutation of the catalytic base from Asp to Gly can be partially reversed by including azide in reaction mixtures; presumably, the azide serves as a nucleophile adding to substrate C1 or it serves as a catalytic base to activate an active site water to serve as the nucleophile (Shallom et al. 2005).

Identification of the enzyme nucleophile and catalytic acid/base of retaining β-xylosidase has depended largely on X-ray structures. In the case of a retaining GH39 β-xylosidase,

substantiating biochemical evidence supporting the identity of the enzyme nucleophile has been accomplished by using a suicide substrate, containing a fluorinated xylosyl residue that can xylosylate the enzyme but has a very slow rate of dexylosylation (Vocadlo et al. 1998). The xylosylated amino acid residue was identified using mass spectrometry. Identification of the catalytic acid/base residue of a retaining GH39 β-xylosidase was secured by mutagenesis of the likely candidate Glu to Ala (Bravman et al. 2001). The inactivated mutant enzyme was confirmed as the general acid/base by experiments demonstrating burst kinetics, pH shift, and azide rescue.

Retaining glycosidases exhibit, to varying extents, transglycosylation activity in which a nucleophile (e.g., sugar), in competition with water, attacks C1 of the glycosyl group of the glycosylated enzyme intermediate, whereas inverting glycosidases lack this activity. This is an important distinction since transglycosylation could seriously diminish the performance efficiency of retaining β-xylosidase in its function of catalyzing hydrolysis reactions. Among β-xylosidase families, only GH43 operates via an inverting mechanism (Table 1). It is likely that characterization of new β-xylosidases will uncover inverting enzymes that belong to existing GH families or define new ones.

Practical considerations: discovery of efficient β-xylosidase from nature

While β-xylosidase broadly impacts society in its numerous applications that include glycosynthase activity, our chief concern is the use of β-xylosidase for complete hydrolysis of xylooligosaccharides to D-xylose monosaccharide for the multiple uses mentioned or referred to in the "Introduction." For hydrolysis, highly active enzyme acting on xylooligosaccharides (k_{cat}, k_{cat}/K_m) is desired. Also, it is desirable to identify highly active β-xylosidases for operating in different niches of practice which encompass enzymes with diverse properties including tolerance of pH, temperature, and other factors.

To begin with, we survey the literature for β-xylosidase acting on xylobiose and xylooligosaccharides (Table 2) since these are the enzyme's natural substrates and the most relevant from a commercial application perspective. Despite its importance to the oeuvre, immediately obvious is the shortness of the list of β-xylosidase with kinetic parameters reported for substrate 1,4-β-D-xylobiose (X2); all but three of these were determined in recent years by the authors' laboratories who will continue to add to the list. There are dozens of enzymes with kinetic parameters reported for the artificial, relatively labile substrate 4-nitrophenyl-β-D-xylopyranoside (4NPX) without kinetic parameters for X2, and three of these are listed in Table 2. Of the latter, two have qualitative experimental results (TLC) indicating that they act on X2 (Smaali et al. 2006), demonstrating that the enzyme fulfills a major requirement for being categorized as β-xylosidase (EC 3.2.1.37). The other enzyme acts on 4NPX, but not X2; it has been designated as an arabinofuranosidase (EC 3.2.1.55; Wagschal et al. 2007).

Among those with kinetic parameters listed for acting on 4NPX and X2 (1,4-β-D-xylobiose), there is the qualitative correlation that all enzymes that act on X2, act on 4NPX.

Table 2 Kinetic parameters of β-xylosidase acting on xylobiose and 4NPX

Biological source	GH no.	k_{cat}^{X2} (s^{-1})	K_m^{X2} (mM)	k_{cat}^{4NPX} (s^{-1})	K_m^{4NPX} (mM)	Reaction pH/°C	Reference
Selenomonas ruminantium	43	185	2.06	32.1	0.716	5.3/25	Jordan (2008)
Bacillus pumilus 12	43	18.1	2.9	7.6	1.5	7.15/25	Van Doorslaer et al. (1985)
Bacillus pumilus IPO	43	8.7	8.9	76	3.9	7.0/37	Xu et al. (1991)
Thermoanaerobacterium sp.	39	2.7	3.3	4.2	0.090	6.0/35	Wagschal et al. (2005)
Geobacillus thermoleovorans IT-08	43	0.065	12.8	0.18	0.55	6.2/40	Wagschal et al. (2009a)
Uncultured bacterium	43	0.047	15.9	1.34	1.53	6.2/40	Wagschal et al. (2009b)
Trichoderma reesei	3	0.0044	0.40	0.0147	0.80	4.0/40	Rasmussen et al. (2006)
Talaromyces emersonii	3	0.002	0.19	0.017	0.060	4.0/40	Rasmussen et al. (2006)
Bacillus halodurans C-125	43	Yes (TLC)[a]	Yes (TLC)	12.1	4.40	8.0/45	Smaali et al. (2006)
Bacillus halodurans C-125	39	Yes (TLC)	Yes (TLC)	5.26	8.61	7.5/45	Smaali et al. (2006); Wagschal et al. (2008)
Uncultured bacterium	43	No activity	No activity	0.132	0.96	8.0/45	Wagschal et al. (2007)

Enzymes are listed according to k_{cat}^{X2}

[b] Qualitative determination using thin-layer chromatography

Quantitatively, it is a different matter as there is only slight correlation between k_{cat} and k_{cat}/K_m values of β-xylosidase acting on X2 and 4NPX: ratios of $k_{cat}^{X2}/k_{cat}^{4NPX}$ vary from 0.035 to 5.8. Ratios of $(k_{cat}/K_m)^{X2}/(k_{cat}/K_m)^{4NPX}$ vary from 0.0034 to 2.0.

β-xylosidase from *Selenomonas ruminantium* (hereafter referred to as SXA) is the most active β-xylosidase acting on X2 currently reported (Table 2). Kinetic parameters k_{cat} and $1/K_m$ of SXA acting on X2 and 4NPX have different patterns of dependence on pH and temperature (Fig. 2a), whereas the kinetic parameter k_{cat}/K_m, which reports free enzyme, has similar patterns (Fig. 2a). Relative kinetic parameters of $k_{cat}^{X2}/k_{cat}^{4NPX}$, $(k_{cat}/K_m)^{X2}/(k_{cat}/K_m)^{4NPX}$ and $(1/K_m)^{X2}/(1/K_m)^{4NPX}$ vary considerably with temperature and pH (Fig. 2b); the dependence of $(k_{cat}/K_m)^{X2}/(k_{cat}/K_m)^{4NPX}$ on pH changes the least with pH because it reports mainly free enzyme. Therefore, 4NPX is a poor surrogate for X2 in predicting catalytic activities of multiple β-xylosidases acting on X2. Similarly, 4NPX is a poor surrogate for predicting X2 activity of β-xylosidase under changing conditions of pH and temperature.

Clearly, there is a need to extend the list of Table 2 to include kinetic parameters of additional β-xylosidases acting on X2 and xylooligosaccharides. Facilitating this goal, there are five convenient, quantitative methods for determining D-xylose produced from β-xylosidase acting on X2 and larger xylooligosaccharides that have been reported. X2, 1,4-β-D-xylotriose (X3), 1,4-β-D-xylotetraose (X4), 1,4-β-D-xylopentaose (X5), 1,4-β-D-xylohexaose (X6), and the other reagents and equipment needed for the determinations are available commercially. Unlike 4NPX in which reaction progress can be monitored continuously and discontinuously, reaction progress of β-xylosidase acting on xylooligosaccharides is currently limited to discontinuous monitoring. Historically, the first of these methods for D-xylose quantification is the use of D-xylose isomerase and sorbitol reductase as coupling enzymes (Van Doorslaer et al. 1985). The second method relies on pyranose oxidase and peroxidase as the coupling enzymes (Wagschal et al. 2005). The third method uses colorimetric detection of reducing sugars (Rasmussen et al. 2006). The fourth method depends on HPLC separation of reaction product D-xylose which is monitored using pulsed amperometry and electrochemical detection (Jordan 2008). The fifth method uses xylose mutarotase and β-D-xylose dehydrogenase as coupling enzymes (Jordan and Braker 2009).

An example of performance considerations

Hydrolysis of lignocellulose to substituent monosaccharides can be achieved by chemical means. However, high yields of monosaccharides are withheld, in part, due to dehydration reactions that generate furfural from D-xylose and hydroxymethylfurfural from D-glucose (Larsson et al. 1999; Luo et al. 2002). The aldehydes and other side products are toxic to the fermenting organisms (Palmqvist et al. 1999; Klinke et al. 2004), preventing efficient ethanol production; furthermore, they are corrosive to the reactors. For these and other considerations, it is desirable to employ milder chemical methods for pretreatment of the biomass that produce lower levels of the toxins, but are sufficient to expose cellulose and hemicellulose fibers for subsequent saccharification by enzymatic means. Although the literature is replete with daunting statements on the requirement of many enzyme functions for achieving complete hydrolysis of xylan to free its constituent monosaccharides, it is clear that the requirements depend on the lignocellulosic materials and on the pretreatment method used prior to enzymatic hydrolysis. For sparsely substituted arabinoxylan, depleted of ester substituents following pretreatment, four enzymes (two xylanases, a β-xylosidase and an arabinofuranosidase) are sufficient to completely liberate the D-xylose content (Adelsberger et al. 2004). For such material, rather than being just one hemicellulase of many, β-xylosidase would in fact carry the greatest work load in terms of the number of glycosidic bonds cleaved, as well as relieving product inhibition of xylanase activities, making its performance of primary importance.

Inspection of Table 2 where k_{cat} values are presented in descending order reveals that SXA is the best catalyst known for hydrolysis of X2, and β-xylosidase from *Bacillus pumilus* is second best. Likewise, larger xylooligosaccharides are accepted as substrate better by SXA than by the *B. pumilus* β-xylosidase. Thus, from k_{cat} and k_{cat}/K_m values determined for SXA at pH5.3 and 25°C (Jordan 2008) and k_{cat} and k_{cat}/K_m values determined for the *B. pumilus* β-xylosidase at its pH optimum of pH7.15 at 25°C (Van Doorslaer et al. 1985), kinetic parameters are higher for SXA than the *B. pumilus* β-xylosidase by factors of 10 (X2), 32 (X3), 31 (X4), 20 (X5), and 27 (X6) for k_{cat} and factors of 14 (X2), 16 (X3), 15 (X4), 10 (X5), and 13 (X6) for k_{cat}/K_m. k_{cat}^{X2} for SXA acting on xylobiose is 185 s^{-1} at 25°C (Table 2) and 390 s^{-1} at 35°C (Jordan and Braker 2009). This puts SXA in the company of other highly active glycosidases acting on their natural substrates with k_{cat} values of 1,000 s^{-1} (Lairson and Withers 2004; Konstantinidis et al. 1993). However, SXA is clearly slower than certain glycosidases such as the *Bacillus cereus* β-amylase acting on maltohexaose (k_{cat}=1,200 s^{-1} at 25°C; Yamaguchi et al. 1996), suggesting that there may be room for improvement of SXA k_{cat}. Suitable for serving roles in biomass saccharification processes, SXA is stable at pH5 and above and at temperatures of 50°C and below, and it is only weakly inhibited by ethanol (Jordan et al. 2007a).

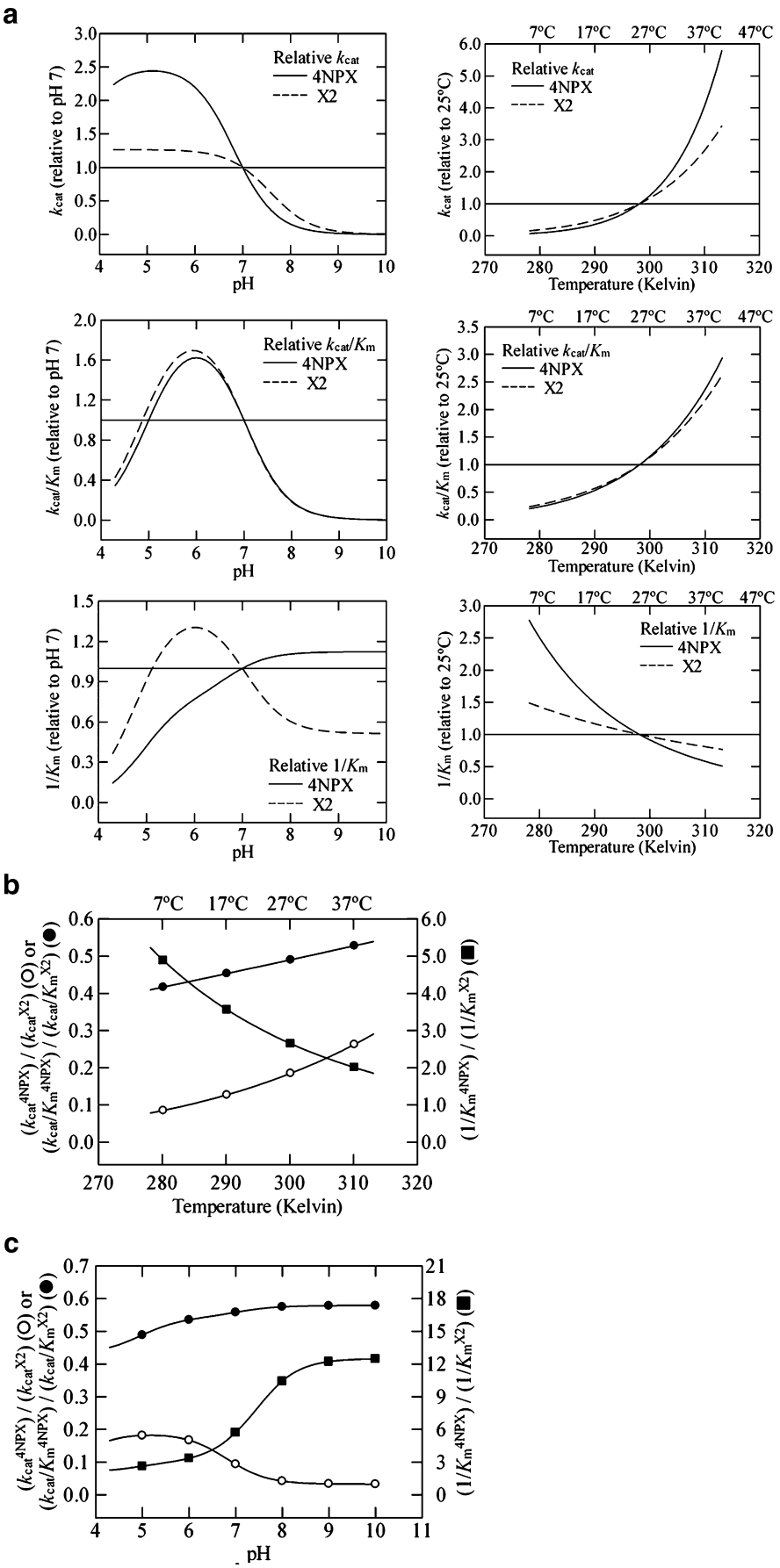

Fig. 2 pH and temperature dependence of kinetic parameters of SXA acting on 4NPX and X2. **a** Kinetic parameters were determined at 25°C and varied pH (Jordan et al. 2007b, 2009; Jordan and Braker 2007) or at pH5.3 and varied temperature (Jordan and Braker 2009). Values of pH-dependent parameters are relative to the value at pH7.0 and 25°C: $k_{cat}^{4NPX} = 12.7\,s^{-1}$, $k_{cat}/K_m^{4NPX} = 34.7\,mM^{-1}\,s^{-1}$, and $1/K_m^{4NPX} = 2.65\,mM^{-1}$ (Jordan et al. 2007b, 2009) and $k_{cat}^{X2} = 135\,s^{-1}$, $k_{cat}/K_m^{X2} = 62.1\,mM^{-1}\,s^{-1}$, and $1/K_m^{X2} = 0.464\,mM^{-1}$ (Jordan 2008). Values for the temperature-dependent parameters are relative to the value at 25°C and pH5.3: $k_{cat}^{4NPX} = 32.4\,s^{-1}$, $k_{cat}/K_m^{4NPX} = 42.2\,mM^{-1}\,s^{-1}$, and $1/K_m^{4NPX} = 1.3\,mM^{-1}$ (Jordan et al. 2007b, 2009) and $k_{cat}^{X2} = 188\,s^{-1}$, $k_{cat}/K_m^{X2} = 87.3\,mM^{-1}\,s^{-1}$, and $1/K_m^{X2} = 0.463\,mM^{-1}$ (Jordan 2008). **b** Ratios of kinetic parameters (4NPX/X2) are plotted versus temperature. Parameters were determined at pH5.3. **c** Ratios of kinetic parameters (4NPX/X2) are plotted versus pH. Parameters were determined at 25°C

Early on, it was recognized that a potential Achilles' heel of SXA performance in saccharification processes is its relatively high affinities for D-xylose (pH-independent K_i= 3.5 mM) and for D-glucose (pH-independent K_i=17.7 mM), which could accumulate to high levels in saccharification reactions (approaching 1 M) and inhibit SXA catalysis (Jordan and Braker 2007). Xylanase and β-xylosidase can operate synergistically in depolymerizing xylan to D-xylose (La Grange et al. 2001), presumably through a mechanism where xylanase releases small xylooligosaccharides from xylan, which serve as good substrates for β-xylosidase, and β-xylosidase catalyzes their conversion to D-xylose. Xylanase is inhibited by xylobiose (K_i=4.8 mM; Williams et al. 2000), so inhibition of the β-xylosidase by D-xylose accumulation could lead to the accumulation of xylobiose and inhibition of xylanase. Other relevant enzymes such as cellulase and β-glucosidase (Xiao et al. 2004) exhibit inhibition by glucose, making this a general problem affecting multiple steps of the saccharification process. SXA and other lignocellulosic enzymes may function effectively in simultaneous saccharification and fermentation (SSF) processes in which saccharification enzymes and the fermenting organism operate simultaneously in a single reactor since potentially inhibiting monosaccharides are consumed by the fermenting organism as they are generated by the saccharification enzymes. However, even in the SSF process, performance of SXA could benefit from lower affinities to monosaccharides.

In addressing the perceived problem, a series of experiments were carried out to define the binding properties of D-xylose, D-glucose, and other monosaccharides to the active site of SXA (Jordan and Braker 2007). Assisting the analysis of monosaccharide binding, SXA has the simplest active site possible for an efficient glycoside hydrolase by comprising two subsites: subsite −1 for binding substrate glycone, subsite +1 for binding substrate aglycone, and for substrates larger than X2, the reducing end extends into solvent (Jordan et al. 2007b; Brunzelle et al. 2008).

In the resulting model of Fig. 3 (Jordan and Braker 2007; Jordan et al. 2009), catalysis (Jordan et al. 2007b) and binding of monosaccharides is governed by pK_a 5 and pK_a 7, the former for the catalytic base (D14) and the latter for the catalytic acid (E186). When both carboxylate groups are protonated ($D14^HE186^H$), SXA is catalytically inactive and binds monosaccharides weakly. In its monoprotonated form ($D14^-E186^H$), SXA is catalytically active and can bind certain monosaccharides (e.g., D-glucose and D-xylose) to either subsite −1 or subsite +1, providing single occupation, and certain monosaccharides (e.g., L-arabinose and D-ribose) can bind to both subsite −1 and subsite +1, providing double occupation. Also, in its monoprotonated state, certain monosaccharides (e.g., D-xylose and D-ribose, but not D-glucose) can bind in subsite −1 simultaneously

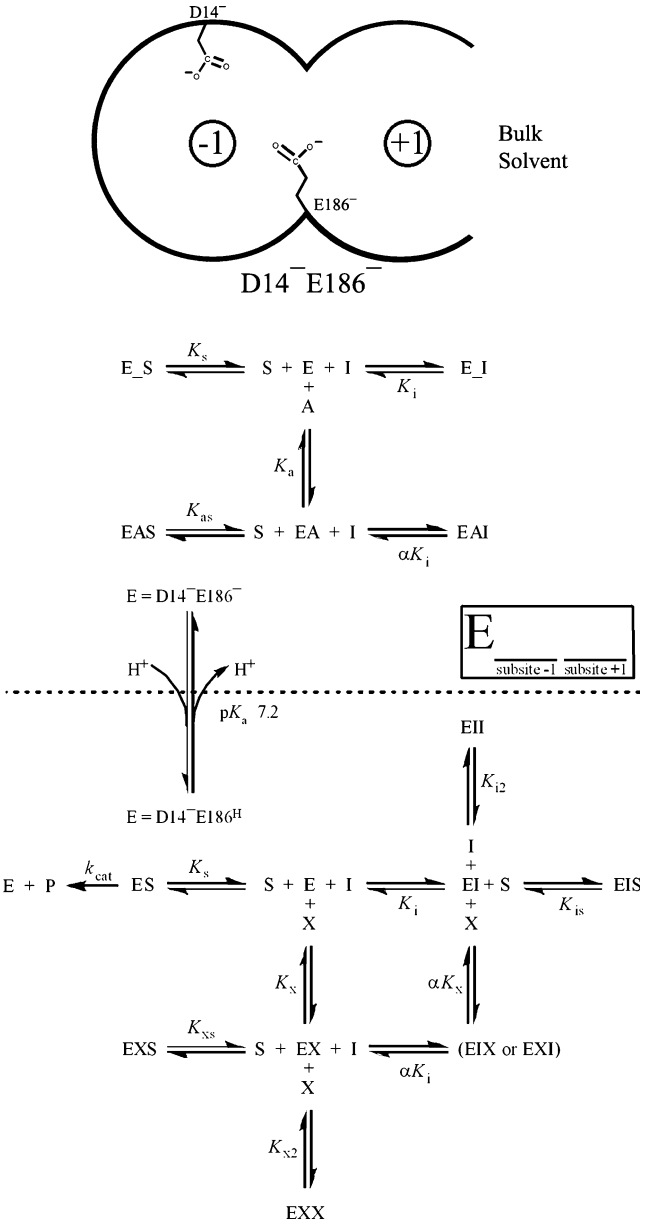

Fig. 3 Binding of monosaccharide inhibitors (*I* and *X*), amino alcohols (*A*), and substrate (*S*) to SXA (*E*). At the *top*, a model of the two-subsite active site of SXA in its dianionic state ($D14^-E186^-$) is shown. In the equilibrium model, *E* is shown in two pH-dependent forms: catalytically active, monoanionic form $D14^-E186^H$ and catalytically inactive, dianionic form $D14^-E186^-$. Amino alcohols bind only to the $D14^-E186^-$ form of enzyme and only to subsite −1. E_S and E_I designations indicate that the substrate and inhibitor bind in subsite +1 and not in subsite −1 of the $D14^-E186^-$ enzyme, whereas both subsites +1 and −1 can be occupied by *I*, *X*, and *S* in the $D14^-E186^H$ enzyme. Ligands do not bind to the diprotonated form of enzyme ($D14^HE186^H$). The model is based mainly on Jordan and Braker (2007) and Jordan et al. (2009)

with occupation of subsite +1 by the xylosyl moiety of 4NPX (and presumably that of X2, but not the arabinofuranosyl moiety of 4-nitrophenyl-α-L-arabinofuranoside (4NPA). In its doubly deprotonated state ($D14^-E186^-$),

SXA is catalytically inactive. However, its active site binds monosaccharides (e.g., D-glucose, D-xylose, L-arabinose, and D-ribose) and the glycone moieties of substrates 4NPX and X2, but not 4NPA within subsite +1. 4NPA does not bind to subsite −1 or subsite +1 of $D14^{-}E186^{-}$ SXA.

The model of Fig. 3 includes the binding of amino alcohols (e.g., ethanolamine, Tris, and triethanolamine) to the active site of SXA. The cationic form of the amines has affinity only for subsite −1 of the doubly deprotonated active site ($D14^{-}E186^{-}$). The neutral amine binds 1,000-fold more weakly. Small amino alcohols such as ethanolamine (but not the larger triethanolamine) bind to subsite −1 and accommodate simultaneous binding of monosaccharides in subsite +1. Thus, by saturating subsite −1 with a small amino alcohol, binding affinity of a monosaccharide to subsite +1 can be determined. Even though amino alcohols bind in subsite −1 of the SXA active site, inhibition patterns can appear competitive, noncompetitive, or uncompetitive, depending on the substrate and size of the amino alcohol (Jordan et al. 2009). Amino alcohols have been known as active site inhibitors of glycoside hydrolases for over half a century, and they provide a powerful means for determining the binding modes of monosaccharides, particularly within the relatively simple active site of SXA and its mutants (see below). As an aside, there are many examples in the recent literature where amino alcohols (e.g., Tris) have been used, unwarily, as pH buffers in determination of kinetic and equilibrium binding parameters of glycosidases (including β-xylosidase), and this has seriously diminished the value of the work.

Another method that has been developed for the analysis of SXA that applies specifically to β-xylosidases is comparison of kinetic parameters (particularly k_{cat}) for β-xylosidase acting on 4NPX and 4NPA (Jordan and Li 2007; Fan et al. 2010; Jordan and Braker 2010). Many β-xylosidases, particularly those of GH43, catalyze hydrolysis of 4NPA, 4NPX, and xylooligosaccharides, owing to the three-dimensional structural similarity of their glycones (Jordan and Li 2007) and the tenfold faster spontaneous hydrolysis rate of 4NPA over that of 4NPX (Jordan and Braker 2009). The energy required to flatten a portion of pyranose rings to their reactive conformation exceeds that needed for furanose rings by about 7 kcal/mol (Durette and Horton 1971). Therefore, mutants of SXA that cause a decrease in the ratio $k_{cat}^{4NPX}/k_{cat}^{4NPA}$ can assign the native residue as positively influencing the distortion state of the xylosyl glycone toward hydrolysis. SXA mutations that increase the ratio $k_{cat}^{4NPX}/k_{cat}^{4NPA}$ can assign the native SXA residue as positively influencing other factors of catalysis such as C1 migration, nucleophilic water activation, and protonation of the leaving group. This provides an independent method for determining whether an SXA mutation influences subsite −1 or subsite +1 of the active site.

Having some understanding of monosaccharide binding to SXA and experimental tools for their study in place, but no a priori conceptions on engineering lower inhibitor affinity, we set out to prepare mutants of SXA by error-prone PCR in a directed evolution effort (Yuan et al. 2005) and search for SXA variants with lower affinities for D-xylose and D-glucose (Fan et al. 2010).

Modification of efficient β-xylosidase for improved performance

Directed evolution selection of SXA with improved K_i values for D-xylose was achieved via a two-tier high-throughput screen where the active/inactive primary screen comprises an *Escherichia coli* transformant, whole cell spectrofluorometric assay of general utility that identifies active β-xylosidases capable of hydrolyzing 4-methylumbelliferyl xylopyranoside or the arabinosyl cogener. The cell-free secondary screen selects for improved 4NPX hydrolysis activity in the presence of D-xylose; the A_0 (activity without inhibitor)/A_i (activity with inhibitor) of normalized primary screen selectants provides the selection data (Fan et al. 2010). Using this strategy, the single-site mutation of SXA (W145G) was selected for characterization. For the SXA variant, $K_i^{D-xylose}$ is improved threefold from the wild-type 6.5 to 22 mM for W145G. $K_i^{D-glucose}$ was raised twofold from the wild-type 49 to 91 mM for W145G. Counteracting the performance improvement with respect to inhibitors are the similar values for $k_{cat}^{D-xylobiose}$ and the threefold larger value of $K_m^{D-xylobiose}$ of W145G-SXA ($k_{cat}^{D-xylobiose}$ 155 s^{-1}, $K_m^{D-xylobiose}$ 4.5 mM) compared with that of wild-type SXA ($k_{cat}^{D-xylobiose}$ 174 s^{-1}, $K_m^{D-xylobiose}$ 1.6 mM). Thus, the mutation offers no overall performance advantage for SXA acting on xylooligosaccharides in the presence of D-xylose and D-glucose. Interestingly, W145 resides 6 Å from the active site and has contacts with two residues of subsite +1 and one of subsite −1. By using methods described in the previous section, it was found that wild-type SXA and W145G have similar K_d values for triethanolamine (specific ligand for subsite −1), W145G has a threefold larger K_i for D-xylose than wild-type SXA in the presence of saturating triethanolamine (so D-xylose can only bind in subsite +1), and the W145G mutation causes a twofold increase in the ratio $k_{cat}^{4NPX}/k_{cat}^{4NPA}$. All three of these effects of W145G point to influences on subsite + 1 (and not subsite −1) in lowering the affinity for D-xylose (and by inference D-glucose). As this is the first report to date on engineering β-xylosidase in the laboratory for improved hydrolase performance, definition of process and biomass-specific selection targets such as improved k_{cat} and thermal stability, coupled with the availability of a straightforward primary screen, should

facilitate the execution of future β-xylosidase engineering campaigns.

β-Xylosidase has more commonly been the subject of "synergy engineering" which involves optimizing a mixture of several GH's with differing linkage hydrolysis specificities, as well as differing heteromeric enzyme quaternary structures (Wen et al. 2009). A high throughput method for lignocellulosic digestibility (Chundawat et al. 2008) has recently been used to optimize six core GH activities including β-xylosidase for hydrolysis of ammonia fiber expansion pretreated corn stover (Gao et al. 2010), and a minimal enzyme cocktail including β-xylosidase has been reported for wheat arabinoxylan (Sørensen et al. 2007). In efforts to create single polypeptides containing GHs with multiple linkage hydrolysis specificities, chimeric β-xylosidase fusion proteins have been created, including bifunctional enzymes having xylanase and either α-arabinofuranosidase activity or β-xylosidase activity (Fan et al. 2008), trifunctional enzymes with and without carbohydrate binding domains having xylanase, α-arabinofuranosidase, and β-xylosidase activities (Fan et al. 2009a), and a chimera possessing xylanase, endoglucanase, α-arabinofuranosidase, and β-xylosidase activities (Fan et al. 2009b). Such chimeras were shown to maintain parental levels of catalytic activities and synergistic properties, and they may be particularly useful for GH expression *in-plant* (recently reviewed by Taylor et al. 2008) since a single gene can encode multiple catalytic and binding domains of known stoichiometry. In a somewhat removed area of study, an example of anthropogenic selective evolution and potential refining of function in response to the advent of agriculture has recently been hypothesized for a β-xylosidase of a host-specialized plant pathogen (Brunner et al. 2009).

Examples of other GH enzymes that have been recent subjects of protein engineering efforts for practical applications include a high-activity barley α-amylase (Wong et al. 2004), an α-amylase active on raw starch (Wong et al. 2003), several examples for shifting the pH optima and pH stability of xylanases (Umemoto et al. 2009; Chen et al. 2001; De Lemos et al. 2005; Fenel et al. 2006; Liu et al. 2009), decreased affinity of an endoxylanase for inhibitory cereal proteins (Bourgois et al. 2007), β-glucosidase thermostability enhancement (Lopez-Camacho et al. 1996), low-temperature catalysis for a β-glucosidase (Lebbink et al. 2000), creation of a thermostable fungal class II cellobiohydrolases (Heinzelman et al. 2009), and improved catalytic efficiency of an endo-β-1,4-glucanase (Lin et al. 2009).

Conclusions

β-xylosidase is a critical component of industrial enzyme-based processes for the utilization of renewable biomass feedstocks, yet at present, few examples exist of the characterization of the enzyme acting on xylooligosaccharides, its natural substrates. With an avalanche of DNA sequence information in hand and more deposited regularly, there are many hundreds to thousands of putative β-xylosidase genes known with larger numbers sure to follow. Particularly intriguing are putative β-xylosidases arising from genome sequencing projects of organisms inhabiting diverse environments such as extremophiles. However, in order for us to tap this biotic wealth of genetic information, it is critical going forward that widespread β-xylosidase discovery efforts include the determination of kinetic parameters for the enzyme acting on its natural substrates (perhaps by using the currently available methods referenced above) so that the breadth of β-xylosidase catalytic capability generated by natural processes is genuinely sussed out. In addition to finding β-xylosidases with high k_{cat} and k_{cat}/K_m values acting on xylooligosaccharides, β-xylosidases with lower affinities for D-xylose and D-glucose and better stabilities versus extremes of pH and temperature could be uncovered. For example, the β-xylosidase from *Scytalidium thermophilum* is reported to be uninhibited by 200 mM D-xylose (Zanoelo et al. 2004), though there is currently no information on its k_{cat} and k_{cat}/K_m values when acting on xylooligosaccharides. In executing more extensive surveys of β-xylosidases, it is anticipated that in addition to the potential discovery of commercially relevant β-xylosidases straightaway, natural substrate catalytic parameters coupled with three-dimensional structures and amino acid sequence information will enable and inform future efforts in the area of single-activity engineering of β-xylosidase, which curiously is yet in its infancy for this key enzyme needed for enzymatic deconstruction of biomass.

Acknowledgments This work was supported by United States Department of Agriculture CRIS 5325-41000-046-00 (K.W.) and CRIS 3620-41000-118-00D (D.B.J.). The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture over other firms or similar products not mentioned.

References

Adelsberger H, Hertel C, Glawischnig E, Zverlov VV, Schwarz WH (2004) Enzyme system of *Clostridium stercorarium* for hydrolysis of arabinoxylan: reconstitution of the in vivo system from recombinant enzymes. Microbiology 150:2257–2266

Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22:195–201

Bourgois TM, Nguyen DV, Sansen S, Rombouts S, Beliën T, Fierens K, Raedschelders G, Rabijns A, Courtin CM, Delcour JA, Van Campenhout S, Volckaert G (2007) Targeted molecular engineering

of a family 11 endoxylanase to decrease its sensitivity towards *Triticum aestivum* endoxylanase inhibitor types. J Biotechnol 130:95–105

Braun C, Meinke A, Ziser L, Withers SG (1993) Simultaneous high-performance liquid chromatographic determination of both the cleavage pattern and the stereochemical outcome of the hydrolysis reactions catalyzed by various glycosidases. Anal Biochem 212:259–262

Bravman T, Mechaly A, Shulami S, Belakhov V, Baasov T, Shoham G, Shoham Y (2001) Glutamic acid 160 is the acid-base catalyst of β-xylosidase from *Bacillus stearothermophilus* T-6: a family 39 glycoside hydrolase. FEBS Lett 495:115–119

Brunner PC, Keller N, McDonald BA (2009) Wheat domestication accelerated evolution and triggered positive selection in the β-xylosidase enzyme of *Mycosphaerella graminicola*. PLoS ONE 4:e7884

Brunzelle JS, Jordan DB, McCaslin DR, Olczak A, Wawrzak Z (2008) Structure of the two-subsite β-D-xylosidase from *Selenomonas ruminantium* in complex with 1,3-bis[tris(hydroxymethyl)methylamino]propane. Arch Biochem Biophys 474:157–166

Brüx C, Ben-David A, Shallom-Shezifi D, Leon M, Niefind K, Shoham G, Shoham Y, Schomburg D (2006) The structure of an inverting GH43 β-xylosidase from *Geobacillus stearothermophilus* with its substrate reveals the role of the three catalytic residues. J Mol Biol 359:97–109

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Res 37:D233–D238

Chen Y-L, Tang T-Y, Cheng K-J (2001) Directed evolution to produce an alkalophilic variant from *Neocallimastix patriciarum* xylanase. Can J Microbiol 47:1088–1094

Chundawat SPS, Balan V, Dale BE (2008) High-throughput microplate technique for enzymatic hydrolysis of lignocellulosic biomass. Biotechnol Bioeng 99:1281–1294

De Lemos Esteves F, Gouders T, Lamotte-Brasseur J, Rigali S, Frère J-M (2005) Improving the alkalophilic performances of the Xy11 xylanase from *Streptomyces* sp. S38: structural comparison and mutational analysis. Protein Sci 14:292–302

Dornez E, Gebruers K, Cuyvers S, Delcour JA, Courtin CM (2007) Impact of wheat flour-associated endoxylanases on arabinoxylan in dough after mixing and resting. J Agric Food Chem 55:7149–7155

Ducros VM, Tarling CA, Zechel DL, Brzozowski AM, Frandsen TP, von Ossowski I, Schülein M, Withers SG, Davies GJ (2003) Anatomy of glycosynthesis: structure and kinetics of the *Humicola insolens* Cel7B E197A and E197S glycosynthase mutants. Chem Biol 10:619–628

Durette PL, Horton D (1971) Conformational analysis of sugars and their derivatives. Adv Carbohydr Chem Biochem 26:49–125

Fan Z, Wagschal K, Lee CC, Kong Q, Shen KA, Maiti IB, Yuan L (2008) The construction and characterization of two xylan-degrading chimeric enzymes. Biotechnol Bioeng 102:684–692

Fan Z, Wagschal K, Chen W, Montross MD, Lee CC, Yuan L (2009a) Multimeric hemicellulases facilitate biomass conversion. Appl Environ Microbiol 75:1754–1757

Fan Z, Werkman JR, Yuan L (2009b) Engineering of a multifunctional hemicellulase. Biotechnol Lett 31:751–757

Fan Z, Yuan L, Jordan DB, Wagschal K, Heng C, Braker JD (2010) Engineering lower inhibitor affinities in β-D-xylosidase. Appl Microbiol Biotechnol. doi:10.1007/s00253-009-2335-7

Fenel F, Zitting A-J, Kantelinen A (2006) Increased alkali stability in *Trichoderma reesei* endo-1,4-β-xylanase II by site-directed mutagenesis. J Biotechnol 121:102–107

Fushinobu S, Hidaka M, Honda Y, Wakagi T, Shoun H, Kitaoka M (2005) Structural basis for the specificity of the reducing end xylose-releasing exo-oligoxylanase from *Bacillus halodurans* C-125. J Biol Chem 280:17180–17186

Gao D, Chundawat SPS, Krishnan C, Balan W, Dale BE (2010) Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover. Bioresource Technol 101:2770–2781

Heinzelman P, Snow CD, Wu I, Nguyen C, Villalobos A, Govindarajan S, Minshull J, Arnold FH (2009) A family of thermostable fungal cellulases created by structure-guided recombination. Proc Nat Acad Sci 106:5610–5615

Honda Y, Kitaoka M (2004) A family 8 glycoside hydrolase from *Bacillus halodurans* C-125 (BH2105) is a reducing end xylose-releasing exo-oligoxylanase. J Biol Chem 279:55097–55103

Jordan DB (2008) β-D-Xylosidase from *Selenomonas ruminantium*: catalyzed reactions with natural and artificial substrates. Appl Biochem Biotechnol 146:137–149

Jordan DB, Braker JD (2007) Inhibition of the two-subsite β-D-xylosidase from *Selenomonas ruminantium* by sugars: competitive, noncompetitive, double binding, and slow binding modes. Arch Biochem Biophys 465:231–246

Jordan DB, Braker JD (2009) β-D-Xylosidase from *Selenomonas ruminantium*: thermodynamics of enzyme-catalyzed and non-catalyzed reactions. Appl Biochem Biotechnol 155:330–346

Jordan DB, Braker JD (2010) β-D-Xylosidase from *Selenomonas ruminantium*: role of glutamate 186 in catalysis revealed by site-directed mutagenesis, alternate substrates, and active-site inhibitor. Appl Biochem Biotechnol 161:395–410. doi:10.1007/s12010-009-8874-7

Jordan DB, Li X-L (2007) Variation in relative substrate specificity of bifunctional β-D-xylosidase/α-L-arabinofuranosidase by single-site mutations: roles of substrate distortion and recognition. Biochim Biophys Acta 1774:1192–1198

Jordan DB, Li X-L, Dunlap CA, Whitehead TR, Cotta MA (2007a) β-D-Xylosidase from *Selenomonas ruminantium* of glycoside hydrolase family 43. Appl Biochem Biotechnol 136–140:93–104

Jordan DB, Li X-L, Dunlap CA, Whitehead TR, Cotta MA (2007b) Structure–function relationships of a catalytically efficient β-D-xylosidase. Appl Biochem Biotechnol 141:51–76

Jordan DB, Mertens JA, Braker JD (2009) Aminoalcohols as probes of the two-subsite active site of β-D-xylosidase from *Selenomonas ruminantium*. Biochim Biophys Acta 1794:144–158

Kersters-Hilderson H, Claeyssens M, Doorslaer EV, De Bruyne CK (1976) Determination of the anomeric configuration of D-xylose with D-xylose isomerases. Carbohydr Res 140:342–346

Kim YW, Chen H, Withers SG (2005) Enzymatic transglycosylation of xylose using a glycosynthase. Carbohydr Res 340:2735–2741

Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66:10–26

Konstantinidis AK, Marsden I, Sinnott ML (1993) Hydrolyses of α- and β-cellobiosyl fluorides by cellobiohydrolases of *Trichoderma reesei*. Biochem J 291:883–888

Koshland DE (1953) Stereochemistry and mechanism of enzyme reactions. Biol Rev 28:416–436

La Grange DC, Pretorius IS, Claeyssens M, van Zyl WH (2001) Degradation of xylan to D-xylose by recombinant *Saccharomyces cerevisiae* coexpressing the *Aspergillus niger* β-xylosidase (*xlnD*) and the *Trichoderma reesei* xylanase II (*xyn2*) genes. Appl Environ Microbiol 67:5512–5519

Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant NO (1999) The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb Technol 24:151–159

Lairson LL, Withers SG (2004) Mechanistic analogies amongst carbohydrate modifying enzymes. Chem Commun 2:2243–2248

Lebbink JHG, Kaper T, Bron P, van der Oost J, de Vos WM (2000) Improving low-temperature catalysis in the hyperthermostable *Pyrococcus furiosus* β-glucosidase CelB by directed evolution. Biochemistry 39:3656–3665

Lin L, Meng X, Liu P, Hong Y, Wu G, Huang X, Li C, Dong J, Xiao L, Liu Z (2009) Improved catalytic efficiency of endo-β-1,4-glucanase from *Bacillus subtilis* BME-15 by directed evolution. Appl Microbiol Biotechnol 82:671–679

Liu L, Wang B, Chen H, Wang S, Wang M, Zhang S, Song A, Shen J, Wu K, Jia X (2009) Rational pH-engineering of the thermostable xylanase based on computational model. Process Biochem 44:912–915

Lopez-Camacho C, Salgado J, Lequerica JL, Madarro A, Ballestar E, Franco L, Polaina J (1996) Amino acid substitutions enhancing the thermostability of *Bacillus polymyxa* β-glucosidase A. Biochem J 314:833–838

Lovering AL, Lee SS, Kim Y-W, Withers SG, Strynadka NCJ (2005) Mechanistic and structural analysis of a family 31 α-glycosidase and its glycosyl-enzyme intermediate. J Biol Chem 280:2105–2115

Luo CD, Brink DL, Blanch HW (2002) Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol. Biomass Bioenergy 22:125–138

Manzanares P, Ramon D, Querol A (1999) Screening of non-*Saccharomyces* wine yeasts for production of β-xylosidase activity. Int J Food Micro 46:105–112

Marques S, Pala H, Alves L, Amaral-Collaço MT, Gama FM, Gírio FM (2003) Characterisation and application of glycanases secreted by *Aspergillus terreus* CCMI 498 and *Trichoderma viride* CCMI 84 for enzymatic deinking of mixed office wastepaper. J Biotechnol 100:209–219

Okuyama M, Kaneko A, Mori H, Chiba S, Kimura A (2006) Structural elements to convert *Escherichia coli* α-xylosidase (YicI) into α-glucosidase. FEBS Lett 580:2707–2711

Palmqvist E, Almeida JS, Hahn-Hägerdal B (1999) Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. Biotechnol Bioeng 62:447–454

Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577–591

Rasmussen LE, Sørensen HR, Vind J, Viksø-Nielsen A (2006) Mode of action and properties of the β-xylosidases from *Talaromyces emersonii* and *Trichoderma reesi*. Biotechnol Bioengineer 94:869–876

Rempel BP, Withers SG (2008) Covalent inhibitors of glycosidases and their applications in biochemistry and biology. Glycobiology 18:570–586

Rye CS, Withers SG (2000) Glycosidase mechanisms. Curr Opin Chem Biol 4:573–580

Shaikh FA, Withers SG (2008) Teaching old enzymes new tricks: engineering and evolution of glycosidases and glycosyl transferases for improved glycoside synthesis. Biochem Cell Biol 86:169–177

Shallom D, Leon M, Bravman T, Ben-David A, Zaide G, Belakhov V, Shoham G, Schomburg D, Baasov T, Shoham Y (2005) Biochemical characterization and identification of the catalytic residues of a family 43 β-D-xylosidase from *Geobacillus stearothermophilus* T-6. Biochemistry 44:387–397

Sinnott ML (2007) Carbohydrate chemistry and biochemistry. Structure and mechanism. The Royal Society of Chemistry, Cambridge

Sørensen HR, Pedersen S, Jørgensen CT, Meyer AS (2007) Enzymatic hydrolysis of wheat arabinoxylan by a recombinant "minimal" enzyme cocktail containing β-xylosidase and novel endo-1,4-β-xylanase and α-L-arabinofuranosidase activities. Biotechnol Prog 23:100–107

Smaali I, Rémond C, O'Donohue MJ (2006) Expression in *Escherichia coli* and characterization of β-xylosidases GH39 and GH-43 from *Bacillus halodurans* C-125. Appl Microbiol Biotechnol 73:582–590

Taylor LE, Dai Z, Decker SR, Brunecky R, Adney WS, Ding S-Y, Himmel ME (2008) Heterologous expression of glycosyl hydrolases in planta: a new departure for biofuels. Trends in Biotechnol 26:413–424

Tsujibo H, Takada A, Kosaka M, Miyamoto K, Inamori Y (2001) Cloning, sequencing, and expression of the gene encoding an intracellular β-D-xylosidase from *Streptomyces thermoviolaceus* OPC-520. Biosci Biotech Biochem 65:1824–1831

Umemoto Y, Onishi R, Araki T (2008) Cloning of a novel gene encoding β-1,3-xylosidase from a marine bacterium, *Vibrio* sp. strain XY-214, and characterization of the gene product. Appl Environ Microbiol 74:305–308

Umemoto H, Ihsanawati IM, Yatsunami R, Fukui T, Kumasaka T, Tanaka N, Nakamura S (2009) Improvement of alkaliphily of *Bacillus* alkaline xylanase by introducing amino acid substitutions both on catalytic cleft and protein surface. Biosci Biotechnol Biochem 73:965–967

Van Doorslaer E, Kersters-Hilderson H, De Bruyne CK (1985) Hydrolysis of β-D-xylo-oligosaccharides by β-D-xylosidase from *Bacillus pumilus*. Carbohydr Res 140:342–346

Vocadlo DJ, Mackenzie LF, He S, Zeikus GJ, Withers SG (1998) Identification of Glu-277 as the catalytic nucleophile of *Thermoanaerobacterium saccharolyticum* β-xylosidase using electrospray MS. Biochem J 335:449–455

Wagschal K, Franqui-Espiet D, Lee CC, Robertson GH, Wong DW (2005) Enzyme-coupled assay for β-xylosidase hydrolysis of natural substrates. Appl Environ Microbiol 71:5318–5323

Wagschal K, Franqui-Espiet C, Lee CC, Kibblewhite-Accinelli RE, Robertson GH, Wong DW (2007) Genetic and biochemical characterization of an α-L-arabinofuranosidase isolated from a compost starter mixture. Enz Microbial Technol 40:747–753

Wagschal K, Franqui-Espiet D, Lee CC, Robertson GH, Wong DW (2008) Cloning, expression and characterization of a glycoside hydrolase family 39 xylosidase from *Bacillus halodurans* C-125. Appl Biochem Biotechnol 146:69–78

Wagschal K, Heng C, Lee CC, Robertson GH, Orts WJ, Wong DW (2009a) Purification and characterization of a glycoside hydrolase family 43 β-xylosidase from *Geobacillus thermoleovorans* IT-08. Appl Biochem Biotech 155:304–313

Wagschal K, Heng C, Lee CC, Wong DW (2009b) Biochemical characterization of a novel dual-function arabinofuranosidase/xylosidase isolated from a compost starter mixture. Appl Microbiol Biotechnol 81:855–863

Wen F, Nair NU, Zhao H (2009) Protein engineering in designing tailored enzymes and microorganisms for biofuels production. Curr Opin Biotechnol 20:412–419

Werpy T, Petersen G (2004) Top value added chemicals from biomass, volume 1-results of screening for potential candidates from sugars and synthesis gas. US Department of Energy, Washington. DC. http://www.pnl.gov/main/publications/external/technicalreports/PNNL-14808.pdf

Williams JS, Hoos R, Withers SG (2000) Nanomolar versus millimolar inhibition by xylobiose-derived azasugars: significant differences between two structurally distinct xylanases. J Am Chem Soc 122:2223–2234

Withers SG (2001) Enzymatic cleavage of glycosides: how does it happen? Pure Appl Chem 67:1673–1682

Wong DWS, Batt SB, Lee CC, Robertson GH (2003) Direct screening of libraries of yeast clones for α-amylase activity on raw starch hydrolysis. Protein Pept Lett 10:459–468

Wong DWS, Batt SB, Lee CC, Robertson GH (2004) High-activity barley α-amylase by directed evolution. Protein J 23:453–460

Xiao Z, Zhang X, Gregg DJ, Saddler JN (2004) Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. Appl Microbiol Biotechnol 115:1115–1126

Xu W-W, Shima Y, Negoro S, Urabe I (1991) Sequence and properties of β-xylosidase from *Bacillus pumilus* IPO. Contradiction of the previous nucleotide sequence. Eur J Biochem 202:1197–1203

Yamaguchi T, Matsumoto Y, Shirakawa M, Kibe M, Hibino T, Kozaki S, Takasaki Y, Nitta Y (1996) Cloning, sequencing, and expression of a β-amylase gene from *Bacillus cereus* var. *mycoides* and characterization of its products. Biosci Biotechnol Biochem 60:1255–1259

Yip VL, Withers SG (2006) Breakdown of oligosaccharides by the process of elimination. Curr Opin Chem Biol 10:147–155

Yoshikawa K, Yamamoto K, Okada S (1994) Transfer action of α-D-xylosidases from *Aspergillus flavus* MO-5 on *p*-nitrophenyl-α-D-xylopyranoside. Biosci Biotechnol Biochem 58:121–125

Yuan L, Kurek I, English J, Keenan R (2005) Laboratory-directed protein evolution. Microbiol Mol Biol Rev 69:373–392

Zanoelo FF, Polizeli MLTM, Terenzi HF, Jorge JA (2004) Purification and biochemical properties of a thermostable xylose-tolerant β-D-xylosidase from *Scytalidium thermophilum*. J Ind Microbiol Biotech 31:170–176