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Evolution in Reverse: Engineering a D-Xylose-Specific Xylose Reductase

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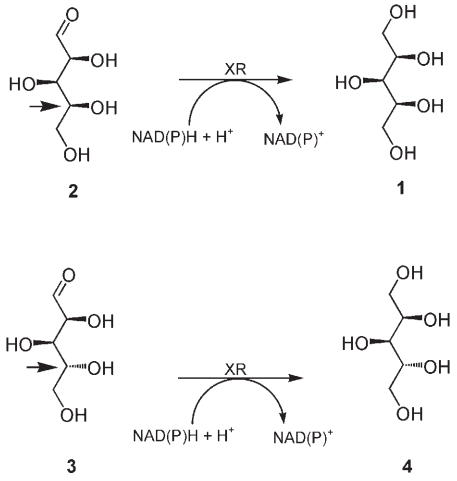
Xylitol (**1**) is a pentitol and is used not only as a sweetener but also as a platform chemical for the production of industrially important chemicals.[1] As a sweetener, it has been shown to possess several favorable properties in comparison to other sugar substitutes, such as anticariogenicity,[2] good gastrointestinal tolerance, low caloric content, and minimal insulin dependence for metabolism. As an alternative to direct chemical reduction with gaseous hydrogen over Raney nickel catalyst, safer and environmentally-friendly biosynthetic routes of producing xylitol by fermentation and enzymatic reduction of D-xylose (**2**) into xylitol by using xylose reductases (XR) have also been studied extensively (Scheme 1).[3,4] However, the unspecific nature of chemical reduction has not been addressed by the use of XRs. These enzymes have evolved to act as promiscuous aldose reductases and can reduce a number of pentoses and hexoses efficiently, of which L-arabinose (**3**) is of particular importance. L-Arabinose, which occurs in abundance with its epimer D-xylose in plant hemicellulose, is difficult to remove,[5] and if left unpurified, will be reduced to L-arabinitol (**4**)—an unwanted byproduct. To the best of our knowledge, no one has attempted to implement methods to alleviate this issue, which remains one of the primary obstacles in the economical production of xylitol. We propose a "kinetic resolution" of the two reacting epimers by selective reduction of xylose from a mixture of sugars. Here, we present an engineered XR with partially reversed promiscuity, which results in increased preference of the enzyme for D-xylose over L-arabinose. Very few examples exist in the literature of enzymes engineered to have narrowed substrate acceptance, and none for highly promiscuous sugar-utilizing enzymes.



Scheme 1. Reduction of D-xylose (**2**) to xylitol (**1**), and L-arabinose (**3**) to L-arabinitol (**4**) by xylose reductase (XR); the epimeric carbons are indicated with arrows.

XRs from the yeasts *Pichia stipitis* and *Candida tenuis* are most popular for xylitol production; however, these enzymes have higher catalytic efficiencies toward L-arabinose than D-xylose (Table 1).[6,7] Consequently, we decided that the recently isolated fungal XR from *Neurospora crassa* (NcXR) was a better choice for engineering due to its innate 2.4-fold preference for D-xylose, high activity, and high expression level in *E. coli*.[8] Semirational-design approaches, targeted site-saturation mutagenesis (TSSM), and combinatorial active-site saturation testing (CASTing) have been successfully applied to shift the substrate specificity of the human estrogen receptor α LBD,[9] and to alter enantioselectivity or substrate scope of lipases, respectively.[10,11] Thus, we sought to use a similar method to engineer a D-xylose-specific XR. In addition, random mutagenesis by error-prone polymerase chain reaction (epPCR) was used to recognize possible contributions by distant residues through allosteric interactions.

Table 1. Selectivities of two popular yeast XRs and that from *N. crassa*.

Organism	Selectivity[a]
Neurospora crassa[8]	2.4
Pichia stipitis[7]	0.625
Candida tenuis[6]	0.5

[a] Selectivity $= (k_{cat}/K_M)_{xylose}/(k_{cat}/K_M)_{arabinose}$.

For TSSM, first-shell residues within interacting distance (extended from a standard 4–5 to 8 Å for NcXR with a wide, solvent-accessible active site) were identified by docking and energy minimizing D-xylose and L-arabinose into a homology model.[8] Of the thirteen residues identified, two catalytic residues (Y49 and K78) were not mutated.[8] D48, F112, and N307 were noted to be particularly important due to their proximity to C4 of the two sugars **2** and **3**. Mutant S (F112S) was identified after screening as one with maximum increase in substrate specificity (Table 2). A second round of TSSM and screening on the remaining ten residues in the mutant S background did not, however, result in the identification of any improved mutants (see the Experimental Section for details of screening). Mutagenesis by epPCR with this template followed by selec-

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Table 2. Kinetic constants for NcXR mutants in 50 mM MOPS buffer (pH 6.3) at 25 °C.

Mutant	K_M [mM]	k_{cat} [min^{-1}]	Relative catalytic efficiency[a]	Selectivity[b]
D-xylose				
wt[c]	34 ± 4	3600 ± 200	1.0	2.4
S	450 ± 41	3380 ± 120	0.071	9.0
Q	82 ± 10	2860 ± 100	0.33	8.9
QC	100 ± 14	3330 ± 150	0.31	10.8
MQC	160 ± 15	4020 ± 125	0.24	11.7
MQCI	190 ± 20	2620 ± 100	0.13	16.1
VMQCI	430 ± 66	5160 ± 380	0.11	16.5
L-arabinose				
wt[c]	40 ± 10	1800 ± 100	0.41	
S	> 2000[d]	> 700[d]	0.0079	
Q	530 ± 52	2070 ± 90	0.037	
QC	530 ± 82	1640 ± 120	0.029	
MQC	990 ± 210	2130 ± 210	0.020	
MQCI	1510 ± 210	1290 ± 100	0.0081	
VMQCI	> 2000[d]	> 1000[d]	0.0079	

[a] Relative catalytic efficiency = $(k_{cat}/K_M)_{xylose,\,mutant}/(k_{cat}/K_M)_{xylose,\,wt}$. [b] Selectivity = $(k_{cat}/K_M)_{xylose}/(k_{cat}/K_M)_{arabinose}$. [c] Parameters for wild-type enzyme from published data.[8] [d] Values indeterminable as L-arabinose is not readily soluble at > 2000 mM.

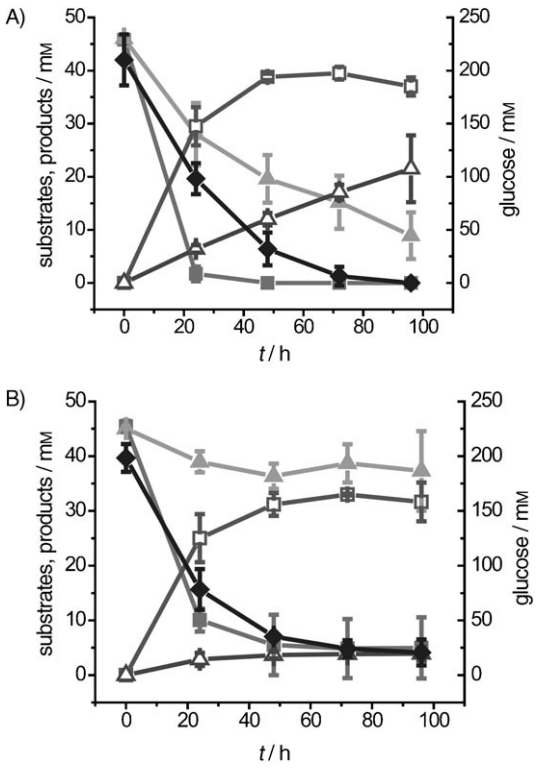

Figure 1. Conversion of D-xylose (■) to xylitol (□), and L-arabinose (▲) to L-arabinitol (△) by resting cells (OD_{600} = 15) at 30 °C in minimal medium without a nitrogen source by using: A) wild type (wt), and B) VMQCI mutant NcXR. Reduction of L-arabinose by the mutant enzyme stalls even before complete depletion of glucose (◆).

tion also failed to identify mutants with improved selectivity. With the inability to find improved specificity relative to mutant S, epPCR was performed with wild-type NcXR. After applying selective pressure to the library for improved D-xylose preference (see the Experimental Section and Supporting Information for details), we identified mutant Q (L109Q), which had 8.9-fold preference for D-xylose (Table 2).

In some previous examples of directed evolution, mutations of different mutants have been known to be collectively additive in improving the desired property,[12,13] but similar attempts to combine these two positions by simultaneous-saturation mutagenesis did not yield any improved mutants. Mapping of mutants Q and S on the homology model revealed their position on β-strand 4 of the proposed $(\beta/\alpha)_8$ barrel structure of NcXR. We speculated that this β strand might play an important role in determining substrate specificity, and mutagenesis was thereafter concentrated on residues in this secondary structure and those that flank it (L102 to Y118). Iterative rounds of TSSM identified additional mutations I110C, L107M, V114I, and L102V, and finally yielded mutant VMQCI. Each subsequent mutation increased the substrate preference for D-xylose, although it was accompanied by loss in overall catalytic efficiency (Table 2). Loss in affinity toward D-xylose is acceptable to a certain degree if it does not have a significant impact on the overall productivity.

To test the mutant in a mixed-sugar experiment, resting cell studies were performed by using *E. coli* strain HZ348 that expressed either wild-type (wt) or mutant VMQCI XR (Figure 1). Arabinitol was produced more slowly compared to xylitol in both cases, and the mutant produced significantly lower amounts of arabinitol (~5.5-fold), but only slightly decreased amounts of xylitol (~1.2-fold) over four days compared to wild-type XR. These data are consistent with in vitro kinetic parameters of the enzymes, and corroborate the idea that a D-xylose-specific XR can significantly reduce the amount of byproducts.

We have, for the first time, successfully shown that a mutant XR engineered to prefer D-xylose over L-arabinose is a viable method for circumventing purification issues during biosynthesis of xylitol. We have also shown that β-strand 4 in the $(\beta/\alpha)_8$ structure of XR plays an important role in discriminating sugar substrates. While it might be possible to create a mutant with further improved specificity, we feel that the loss in activity toward D-xylose could be too detrimental for productive xylitol synthesis. As an alternative, D-xylose and L-arabinose transporters can be targeted to modulate intracellular concentrations of sugars; this would amplify the effect of mutant VMQCI further.

Experimental Section

Library creation and selection: TSSM libraries were created by overlap-extension PCR by using primers that contained a degenerate NNS codon (N: any nucleotide; S: either cytosine or guanine), and high fidelity Phusion DNA polymerase. For the epPCR experiments, Taq polymerase reaction mixture supplemented with $MnCl_2$ (0.2 mM) was used to introduce mutations. Created libraries were ligated into pACYCDuet-1 (Novagen) between EcoRI and BglII restriction sites; this resulted in fusion to an N-terminal His_6-tag. TSSM libraries were electroporated into *E. coli* BL21(DE3) and selected for chloramphenicol resistance. The epPCR libraries were electroporated into *E. coli* HZ349 and subjected to selection pres-

sure. The largest colonies were further screened to test for improved specificity.

Strain construction and selection: *E. coli* HZ348 (*ΔxylA Δ(araBAD)567 ΔlacZ4787 rrnB-3 lacI^q Δ(rhaBAD)568 hsdR514 ΔphoBR580 galU95 recA ΔendA9* (DE3) *uidA*(ΔMluI)::*pir*(wt)) were created by inactivation of *xylA* as described elsewhere.[14] Gluconobacter oxydans ATCC 621 xylitol dehydrogenase (*xdh*) and *E. coli* DH5α L-ribulokinase (*araB*) were amplified from genomic DNA, spliced, and cloned into pTKXb for constitutive expression.[15] When transformed with this construct HZ348 gave the selection strain HZ349. Positive selective pressure over D-xylose resulted in an active XR to complement the *xdh* in its assimilation; this resulted in cell growth. A negative selective pressure by L-arabinose against promiscuous XR resulted in the accumulation of toxic arabinitol phosphate and led to growth inhibition.

Library screening and kinetic characterization: Lysates of mutant XR expressing cultures were screened by adapting the protocol previously described for a 96-well plate format.[8] For the best candidates, His_6-tagged enzymes were isolated by Co^{II} affinity purification and were characterized at 25 °C in 3-(*N*-morpholino)propanesulfonic acid buffer (MOPS; 50 mM), pH 6.3, also as previously described.[8] All kinetic parameters were averages of two or more independent experiments.

Resting-cell studies: Studies were performed as described elsewhere,[16] with slight variations. Cells were grown in LB medium to mid-log phase and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; 500 μM), and D-xylose and L-arabinose (1% of each) at 30 °C, overnight. Cells were then washed thoroughly and resuspended to give $OD_{600} = 15$ in minimal medium with glucose (190 mM), D-xylose (46 mM), and L-arabinose (46 mM), but no nitrogen source. Flasks were incubated at 30 °C and 250 rpm, and supernatants were analyzed for various sugars by using a Bio-Rad HPX-87C column on a Shimadzu HPLC with ELSD-LT detector according to the manufacturer's recommendations. Data were averaged from two independent experiments.

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Keywords: directed evolution · enzyme catalysis · protein engineering · substrate specificity · xylitol

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