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ORIGINAL RESEARCH PAPER

Discovery and characterization of a xylose reductase from *Zymomonas mobilis* ZM4

Manoj Agrawal · Rachel Ruizhen Chen

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Abstract Formation of xylitol, a byproduct from xylose fermentation, is a major limiting factor in ethanol production from xylose in engineered *Zymomonas* strains, yet the postulated xylose reductase remains elusive. We report here the discovery of xylose reductase in *Zymomonas mobilis* and, for the first time, to associate the enzyme function with its gene. Besides xylose and xylulose, the enzyme was active towards benzaldehyde, furfural, 5-hydroxymethyl furfural, and acetaldehyde, exhibiting nearly 150-times higher affinity with benzaldehyde than xylose. The discovery of xylose reductase paves the way for further improvement of xylose fermentation in *Z. mobilis*. The enzyme may also be used to mitigate toxicity of furfural and other inhibitors from plant biomass.

Keywords Cellulosic ethanol · Xylitol · Xylose fermentation · Xylose reductase · *Zymomonas mobilis*

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M. Agrawal · R. R. Chen (✉)
School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, 311 Ferst Drive, Atlanta, GA 30332-0100, USA
e-mail: rchen@chbe.gatech.edu

M. Agrawal
e-mail: pardesi.manoji@gmail.com

Introduction

Zymomonas mobilis, owing to its superior glucose fermenting capabilities, has received considerable scientific and commercial interests as a potential microbe for converting lignocellulose to ethanol (Swings and De Ley 1977; Panesar et al. 2006). However, wild-type *Z. mobilis* strains have a narrow range of substrates and can only utilize glucose, fructose and sucrose. As xylose is the second major component in lignocellulose (Jeffries and Jin 2000), it is necessary to engineer microbial catalysts to use xylose. Over a decade ago, Zhang et al. (1995) successfully constructed xylose-fermenting strains of *Z. mobilis*. These strains were improved by integrating the xylose-metabolic genes into chromosome (Zhang and Chou 2008). However, despite intensive efforts, xylose fermentation in engineered strains still lags far behind glucose utilization with the formation of xylitol during xylose fermentation being recognized as one of the main reasons (Feldmann et al. 1992; Kim et al. 2000). At least two mechanisms are responsible for the formation of xylitol from xylose. First, glucose-fructose oxidoreductase (GFOR) accepts xylose as an electron donor and reduces xylulose to xylitol (Zachariou and Scopes 1986; Viitanen et al. 2008). Additionally, a xylose reductase (XR) was postulated to be responsible for the conversion of xylose to xylitol, based on the cell extract activity using NADPH as electron donor (Feldmann et al. 1992). However, the enzyme was never isolated and

the gene association was not identified. Thus the XR remains elusive (Viitanen et al. 2008; Zhang et al. 2009).

We report here the discovery and characterization of a xylose reductase from *Z. mobilis* ZM4. In the process of improving engineered *Zymomonas* strains for xylose fermentation, we isolated an adapted strain that produced much less xylitol than the wild type strain. The reduced xylitol production coincided with a much decreased XR activity in cell extract. Subsequent sequencing analysis showed a single base mutation within the gene ZMO0976, annotated as a putative aldose reductase. Further studies with the recombinant ZMO0976 confirmed its xylose reductase activity. Besides xylose, the enzyme ZMO0976 is active towards benzaldehyde, furfural, acetaldehyde, 5-hydroxymethylfurfural (HMF) and xylulose, but not towards glucose and fructose.

Materials and methods

Strains

Zymomonas mobilis ZM4 and A3 were used. A3 was obtained by adaptation of a metabolically engineered *Z. mobilis* ZM4/pZMETX on 5% (w/v) xylose. Details on adaptation were described in Agrawal et al. (2010). *Escherichia coli* UT5600 (a K-12 strain) was used as host strain for expressing recombinant xylose reductase.

Cloning and heterologous expression of ZMO0976

ZMO0976 gene and its mutated form (designated mZMO0976) were cloned from *Z. mobilis* ZM4 and an adapted strain A3, respectively. The 5′-primer (5′-GCTATTGAC***GGTACC***ATGAACACTTCTACG CAAAAACC-3′) contains a KpnI site (in bold italics) and a start codon (underlined). The 3′-primer (5′-TAT TCGTACT***AAGCTT***TTATTTATCGCGTGGCGGGG GTG-3′) contains a *Hin*dIII site (in bold italics) and a stop codon (underlined). The cloned gene was ligated into the commercially available high copy number plasmid pQE80L (QIAGEN) at restriction sites KpnI and HindIII. Resultant plasmids were designated as pQEZM976 (containing ZMO0976) and pQEmZM 976* (containing mZMO0976). Both plasmids contained in-frame *N*-terminal histidine $(His)_6$-tag before the start codon of the gene and IPTG-inducible T5 promoter for transcription of the gene. These plasmids were transformed into *E. coli* UT5600 to construct UT5600/pQEZM976 and UT5600/pQEmZM976*, respectively. A control strain UT5600/pQE80L was constructed by transforming UT5600 with the empty vector.

Culture conditions

E. coli was grown with Luria–Bertani (LB) medium supplemented with 100 μg ampicillin/ml at 37°C in culture tube or shake-flask 250 rpm. Cells were induced with 0.5 mM IPTG at an OD_{600} of 0.4–0.6. Cells were then incubated for 4 h OD_{600} at 30°C.

Zymomonas mobilis was grown at 30°C in RM medium (1% w/v yeast extract, 0.2% KH_2PO_4) containing different amounts of glucose or xylose (as indicated) as carbon source. Plasmid containing cells were grown in RM medium supplemented with 100 μg chloramphenicol/ml.

Cell culture conditions are described in Agrawal et al. (2010). Briefly, pre-seed culture (PSC) and seed culture (SC) of *Z. mobilis* were cultivated in 15 ml centrifuge tube and 100 ml Pyrex screw-cap bottle, respectively, filled to 60% volume and shaken at 250 rpm. PSC was prepared by inoculating a single colony from agar plate into the liquid medium containing 2.5% (w.v) glucose and 2.5% (w/v) xylose. PSC was grown to the stationary phase. SC was prepared by inoculating it to an OD_{600} of 0.1 using the stationary phase PSC. SC and fermentation media in bioreactors contained 5% glucose and 5% (w/v) xylose (5%G-5%X). Appropriate amount of cells were harvested from SC at mid-growth phase, resuspended in fresh medium and were used to inoculate the bioreactor to get a starting OD_{600} of 0.1. The fermenter (Infors HT Multifors, Bottmingen, Switzerland) was stirred at 300 rpm, with minimum pH held at 5.75 by automatic addition of 1.7 M KOH. N_2 purging was not done. To maintain anaerobic conditions, the exhaust tube was immersed in a water column to prevent atmospheric oxygen from diffusing into the fermenter vessels. Dissolved O_2 was monitored; it remained close to 0% throughout the fermentation.

Three replicates were done for each experiment starting from three colonies on an agar plate.

Preparation of cell-free extract and protein purification

E. coli cells were prepared according to a published procedure (Akinterinwa and Cirino 2009). Briefly, cells were harvested by centrifugation at 5,000×*g* at 4°C for 30 min. Cells were washed once with extraction buffer [10 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 1 mM PMSF]. Washed cell pellets were stored at −20°C until use. Cell pellets were resuspended to an OD_{600} of 50 in the equilibration buffer (50 mM Na_2HPO_4, 300 mM NaCl and 10 mM imidazole at pH 8) supplemented with 1 mM DTT and 1 mM PMSF and sonicated (8 cycles of 10 s with 30 s cooling period). Cell debris was discarded after centrifugation at 16,000×*g* for 20 min at 4°C. Supernatant was used as cell-free extract (CFE) for enzymatic assays. Bradford assay was used for estimation of protein concentration in the extract.

His-tagged protein in the cell-free extract was purified at 4°C using HIS-Select HF nickel affinity gel (Sigma-Aldrich) which employs immobilized metal-ion affinity chromatography (IMAC). Manufacturer's protocol for large scale purification was followed. Briefly, 10 ml cell-free extract was added to 1 ml affinity gel in equilibration buffer. After overnight incubation, the gel was washed five times with equilibration buffer. The bound his-tagged protein was then eluted using 2 ml elution buffer (50 mM Na_2HPO_4, 300 mM NaCl and 250 mM imidazole at pH 8). Imidazole was removed by overnight dialysis at 4°C using extraction buffer as the dialysis solution.

Cell-free extract for *Z. mobilis* was prepared as above except that extraction buffer was used for sonication and after which, cell debris were spun down at higher centrifugal force (53,300×*g*).

Xylose reductase assay

Enzymatic kinetic studies were carried out in 200 µl in a microplate. NAD(P)H absorbance was measured at 340 nm using Spectramax M5 Plus spectrophotometer (Molecular devices, Sunnyvale, CA).

Xylose reductase was assayed in McIlvaine buffer at pH 7.2 (prepared by adding 16.5 ml 0.2 M Na_2HPO_4 to 3.5 ml 0.1 M citric acid) containing 0.35 mM NADPH or NADH (as indicated) and an appropriate volume of sample (cell-free extract or purified protein) (Viikari and Korhola 1986). Xylose concentration in the assay mixture is indicated in the text. For measuring the catalytic activity of xylose reductase towards other substrates, similar assay conditions were used. K_m and V_{max} were determined by varying the substrate concentrations (benzaldehyde: 0.25–2.00 mM, furfural: 0.5–10 mM, xylose: 0.1–0.26 M) under constant concentrations of NADPH (0.35 mM) and purified protein (2 µg/200 µl reaction) in the assay mixture and fitting the data to Lineweaver–Burk equation: $1/v = (K_m/V_{max})[S] + 1/V_{max}$.

For product identification, enzymatic reactions were carried out in a final volume of 400 µl in an eppendorf tube incubated in a thermomixer. The reaction conditions were similar to those for microplate assay except that a high concentration of NADPH (15 mM) was used to produce sufficient amount of xylitol for identification using HPLC. Trichloroacetic acid (TCA) was added to 10% (w/v) to stop the reaction.

Analytical methods

Cell growth was determined from the OD_{600} measurements. The formation of xylitol by xylose reductase was confirmed by HPLC equipped with an Aminex HPX-87H column (Bio-Rad) with 5 mM H_2SO_4 at 0.4 ml/min as mobile phase. SDS-PAGE and Coommassie Blue staining were used to confirm the expression of ZMO0976 and mZMO0976 proteins. 15 µg of cell-free extract protein and 1 µg of purified protein were loaded on a 12% Tris/HCl gel (Bio-Rad).

Results

Discovery of xylose reductase in *Zymomonas mobilis* ZM4

Adaptation by serial dilution was used to improve xylose fermentation in metabolically-engineered strains of *Z. mobilis* (Agrawal et al. 2010). As a result, a strain, referred as A3, with markedly improved xylose metabolism was obtained. A3 produced less xylitol than the control strain ZM4/pSTVZM27 (wild type ZM4 carrying an empty

plasmid) when fermenting a 5% (w/v) glucose–5% (w/v) xylose mixture (Agrawal et al. 2010). Enzymatic assay for xylose reductase (XR) in cell-free extracts gave an activity of 22 mU/mg protein for the control strain, comparable to a previous report by Feldmann et al. (1992) for *Z. mobilis* strain CP4, whereas no activity could be detected in the cell extract of A3. Thus, the absence of XR activity in the adapted strain A3 suggested that the xylose reductase(s) may have been mutated.

Using the NCBI's sequence analysis program, TBLASTN, the amino acid sequences of characterized and putative XRs from several fungi (including *Aspergillus niger* (Prathumpai et al. 2005), *Candida guilliermondii* (Handumrongkul et al. 1998), *Kluyveromyces lactis* (Billard et al. 1995)) were aligned against the *Z. mobilis* ZM4 genome. One gene, ZMO0976 annotated as aldose reductase, showed significant sequence identity (22–31%) to these fungal XRs. An example of protein sequence alignment of ZMO0976 to a characterized fungal xylose reductase is shown in Supplementary Fig. 1. Subsequent cloning and sequencing of the ZMO0976 gene from both the wild-type strain ZM4 and adapted strain A3 were carried out. A comparison of sequence showed a single base pair mutation, C874T, for the gene cloned from A3, which resulted in a single mutation from arginine to cysteine.

To facilitate further studies of the function of ZMO0976 and to assess the effect of the single amino acid mutation, both wild type ZMO0976 gene and the mutated form, mZMO0976 (GenBank Accession #HQ890327), were expressed in *Escherichia coli* UT5600. Cell-free extracts of both strains—UT5600/pQEZM976 and UT5600/pQEmZM976*—along with the control strain UT5600/pQE80L, were tested for NADPH-dependent xylose reductase activity. As shown in Table 1, the XR activity in the CFE for cells expressing ZMO0976 was high, 460 mU/mg protein, whereas cells expressing mZMO0976 exhibited a much diminished activity of 8 mU/mg protein. As expected, no activity was detected from cell extract of the control, UT5600/pQE80L.

The native and mutated forms of the recombinant enzyme were purified based on the *N*-terminal His-tag. The purified proteins ZMO0976 and mZMO0976 had an expected molecular weight of ~38 kDa as observed on SDS-PAGE (figure not shown). XR activities of the purified proteins are 3400 and 140 mU/mg for ZMO0976 and mZMO0976, respectively. Thus, the single mutation in mZMO0976 caused a significant reduction of XR activity.

Table 1 Xylose reductase activity of purified protein and cell-free extract (CFE)

Samples	Activity (mU/mg protein)
UT5600 expressing ZMO0976 (CFE)	460 ± 50
UT5600 expressing mZMO0976 (CFE)	8 ± 2
Purified recombinant ZMO0976	3400 ± 200
Purified recombinant mZMO0976	140 ± 50

Xylose concentration in the assay mixture was 260 mM. Data shown are averages of at least three replicates

To confirm xylitol as the product from xylose in a XR-catalyzed reaction, reaction mixtures were analyzed by an HPLC method. As shown in Fig. 1, a new peak emerged at 2 h at a retention time coinciding with the xylitol standard, indicating the product from the enzymatic reaction is indeed xylitol. The xylitol peak is relatively small as compared to xylose. This is

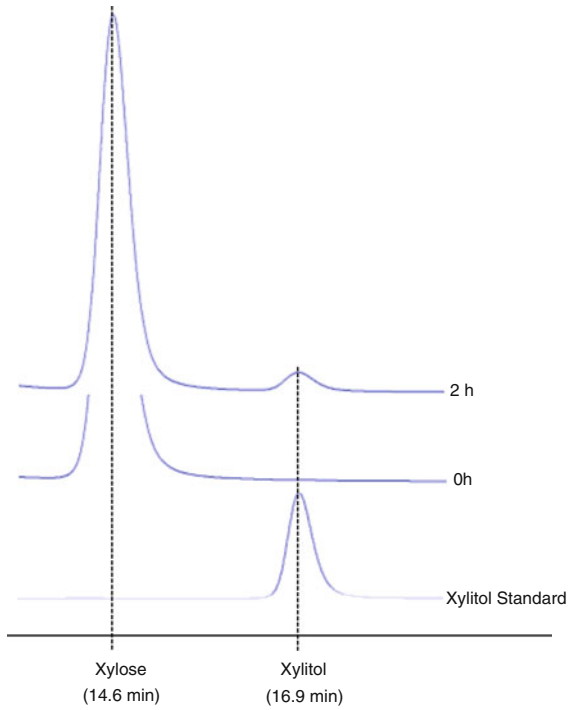

Fig. 1 HPLC chromatograms of a reaction containing 260 mM xylose catalyzed by purified ZMO0976. From top to bottom—chromatograms for samples taken at 2 h, 0 h and xylitol standard

due to the low cofactor NADPH concentration used (15 mM). Xylitol concentration from purified XR was estimated to be 0.18% (or 11.8 mM) at the end of a 2-h reaction. Similarly, whole-cell catalyzed reactions using transformant (UT5600/pQEZM976) were also analyzed by HPLC. Xylitol was detected at the same retention time as the standard whereas cells containing only the empty plasmid did not generate a xylitol peak (data not shown).

Altogether, these data indicate that ZMO0976 encodes the elusive xylose reductase that contributes to xylitol formation in *Zymomonas mobilis*.

Cofactor requirement and substrate specificity of ZMO0976

Feldmann et al. (1992) suggested XR activity was NADPH dependent. In our initial experiments, we therefore used NADPH as the cofactor. Subsequently, NADH was tested with purified ZMO0976 and 260 mM xylose. The results showed the enzyme could accept NADH as cofactor but with reduced efficiency. The activity measured with NADH was at least one order of magnitude lower than that with NADPH. For this reason, all subsequent studies and thus all data presented here used NADPH as cofactor.

Early in our study, we observed that high concentration of xylose (such as 260 mM) was necessary for the detection of XR activity. This prompted us to test other sugars and other molecules as possible substrates for the enzyme. Of all the substrates tested, the highest affinity was found with benzaldehyde. Activity was obvious with only 2 mM benzaldehyde whereas with xylose, it was evident only at concentration as high as 260 mM, suggesting that benzaldehyde was a much better substrate than xylose. The enzyme was also active toward acetaldehyde but required a significantly higher concentration, 260 mM, compared to 2 mM with benzaldehyde. No activity toward glucose and fructose was observed even when similarly high concentration (260 mM) was used. Interestingly, furfural and 5-hydroxymethylfurfural (HMF) were both good substrates for the enzyme. A comparison of activities on various substrates, relative to benzaldehyde, is shown in Table 2.

To evaluate the single amino acid mutation on the enzyme activity toward substrates other than xylose, the mutated form of ZMO0976 was also purified and tested against furfural, benzaldehyde and acetaldehyde. As shown in Table 3, activity of mZMO0976 is only 1–7% of that of the wild type, indicating, as with xylose, a drastic impact of the single mutation on the activity. This effect was apparently independent of the substrates.

Table 2 Aldo-keto reductase activities of recombinant ZMO0976 with different substrates

Substrate	Concentration (mM)[a]	Relative activity
Benzaldehyde	2	1
Acetaldehyde	260	0.62
Furfural	5.2	0.81
HMF	10	0.11
Xylose	260	0.83
Xylulose	260	0.49
Glucose	260	0
Fructose	260	0

[a] Concentration of the substrate in assay mixture

Enzymatic activity of with 2 mM benzaldehyde (4030 ± 250 mU/mg protein) was used as reference for relative activity calculations

For three representative substrates, xylose, benzaldehyde, and furfural, respective Michaelis–Menten kinetics parameters were determined. For xylose, K_m and V_{max} were 258 mM and 6.9 U/mg protein, respectively. The high K_m value is consistent with the early observation that detectable activity requires a high concentration of xylose. The K_m for benzaldehyde was 1.8 mM, about 150-fold lower than xylose. The K_m for furfural, 4.2 mM, was also much lower than that of xylose (Table 4). These data indicate that ZMO0976 is more active on benzaldehyde and furfural than xylose.

Discussion

The existence of a xylose reductase in *Zymomonas mobilis* was suggested as early as 1992 by Feldmann et al. (1992). NADPH-dependent XR activities in the cell extract were shown as the indication of the activity that correlated with xylitol formation during xylose fermentation (Feldmann et al. 1992). However, the gene encoding for the enzyme was never found. In this study, the phenotypic difference exhibited by an adapted strain A3 and its parental

Table 3 Specific aldo–keto reductase activities (mU/mg protein) of ZMO0976 and mZMO0976

	Furfural (10 mM)	Benzaldehyde (2 mM)	Acetaldehyde (520 mM)
ZMO0976	5470 ± 60	4030 ± 250	2500 ± 400
mZMO0976	180 ± 30	40 ± 10	170 ± 30

Substrate concentration in assay mixture is shown in parentheses. Data shown are averages of at least three replicates

Table 4 Apparent K_m and V_{max} of ZMO0976 for benzaldehyde, furfural and xylose in presence of 0.35 mM NADPH

	Benzaldehyde	Furfural	Xylose
K_m (mM)	1.77 ± 0.11	4.15 ± 0.18	258 ± 43
V_{max} (mU/mg protein)	7200 ± 200	5000 ± 1300	6900 ± 1200

Data shown are averages of at least three replicates

strain ZM4 in xylitol formation was used to identify the elusive xylose reductase. The sequenced *Z. mobilis* genome (Seo et al. 2004) was searched for candidates based on homology to known eukaryotic XRs. Sequencing analysis of the gene isolated from A3 indicated one single amino acid substitution in ZMO0976, which apparently was responsible for the reduction of cell extract XR activity and about 3-fold reduction in xylitol formation. Subsequent characterization after recombinant expression provided further evidence supporting the notion that the ZMO0976 indeed encodes a xylose reductase.

Besides xylose and xylulose, ZMO0976 readily reduces aromatic aldehydes. In fact, benzaldehyde and furfural are much better substrates than xylose. The affinity to these two aromatic aldehydes is one to two orders of magnitude higher than that of xylose. Intriguingly, neither glucose nor fructose, the two sugars that the microorganism ferments naturally, is a substrate for the enzyme. This raises an interesting question about its true physiological substrate as the microorganism is not known to use xylose or aromatic aldehydes. There has been only one report of a bacterial furfural reductase but it does not have any activity toward xylose (Gutierrez et al. 2006). Therefore the enzyme discovered from this work appears to be novel.

As shown in this study, cells with XR mutant produced less xylitol, suggesting XR is one of the major routes of xylitol synthesis. It is conceivable that xylose metabolism may be benefited by eliminating the XR activity in its entirety. This work, by identifying the XR gene, paves the way for further improvement of xylose fermentation in *Zymomonas* through metabolic engineering. For example, by deleting the XR gene, along with the GFOR gene (Viitanen et al. 2008), xylitol formation could be reduced further and xylose fermentation could be further improved without the impedance of the toxic byproduct. In addition, the increased availability of NADPH cofactor may result in higher biosynthetic activity promoting faster cell growth (Miller et al. 2010).

The finding that ZMO0976 reduced furfural and HMF is of significance from a very different perspective. The enzyme potentially provides a detoxification mechanism for cells fermenting lignocellulose for production of ethanol and other products. Furfural and HMF are produced during hydrolysis of lignocellulosic biomass and are potent inhibitors of microbial growth (Gutierrez et al. 2006). A pre-fermentation step employing recombinant ZMO0976 could be envisioned to reduce both furfural and HMF to concentrations tolerable to a fermenting microorganism in the subsequent process. Alternatively, a microbial strain (not necessarily *Z. mobilis*) could be engineered to overexpress the ZMO0976, thus endowing cells the ability to better tolerate these two biomass derived inhibitors. The activity with benzaldehyde may also be of interest as the enzyme was suggested to be involved in lignin degradation (Muheim et al. 1991).

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