University of Groningen

Xylose isomerase from *Piromyces*

Lee, Misun

DOI:
[10.33612/diss.132961783](https://doi.org/10.33612/diss.132961783)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Lee, M. (2020). *Xylose isomerase from *Piromyces*: Characterization and engineering for improving *S. cerevisiae*-based lignocellulosic bioethanol production*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen. <https://doi.org/10.33612/diss.132961783>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Xylose isomerase from *Piromyces*

Characterization and engineering for improving
S. cerevisiae-based lignocellulosic bioethanol production

Misun Lee

Cover design by Misun Lee

Cover and thesis layout by Douwe Oppewal

Printed by Ipskamp Drukkers B.V.

The research described in the thesis was carried out at the Groningen Biotechnology and Biomolecular Sciences Institute of the University of Groningen and was financially supported by the BE-Basic R&D Program, for which an FES subsidy was granted from the Dutch Ministry of Economic Affairs, Agriculture, and Innovation (EL&I)

rijksuniversiteit
 groningen

Xylose isomerase from *Piromyces*

**Characterization and engineering for
improving *S. cerevisiae*-based lignocellulosic
bioethanol production**

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Friday 2 October 2020 at 9.00 a.m.

by

Misun Lee

born on 22 August 1984
in Daegu, Republic of Korea

Supervisors

Prof. D.B. Janssen

Prof. R.A.L. Bovenberg

Assessment Committee

Prof. A.J.M. Driessen

Prof. M.J.E.C. van der Maarel

Prof. H.J.M. op den Camp

TABLE OF CONTENTS

Chapter 1. General introduction and scope of the thesis	7
Chapter 2. Metal dependence of xylose isomerase from <i>Piromyces</i> sp. E2 explored by activity profiling and protein crystallography	37
Chapter 3. Rational engineering of xylose isomerase from <i>Piromyces</i> sp. E2 for improved xylose metabolism by <i>S. cerevisiae</i>	75
Chapter 4. Mutations in <i>PMR1</i> stimulate xylose isomerase activity and anaerobic growth on xylose of engineered <i>Saccharomyces cerevisiae</i> by influencing manganese homeostasis	111
Chapter 5. Structure-based directed evolution improves <i>S. cerevisiae</i> growth on xylose by influencing in vivo enzyme performance	147
Summary and concluding remarks	181
Nederlandse samenvatting	190
Curriculum vitae	194
List of publications	195
Acknowledgements	196

CHAPTER 1

General introduction and scope of the thesis

Misun Lee and Dick B. Janssen

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute
(GBB), University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

ABSTRACT

Xylose isomerases are tetrameric metalloenzymes that catalyze aldose-ketose interconversion. Their activity is widely used in industrial biocatalysis, especially for the preparation of high-fructose syrups from glucose-containing starch hydrolysates. The activity with xylose is industrially relevant because it converts xylose that cannot be fermented by yeast to xylulose, which is accepted as a substrate for alcoholic fermentation. Several crystal structures of xylose isomerases have been solved and the catalytic mechanism has been investigated, opening the way to engineering more efficient variants of these enzymes. The properties of xylose isomerases and the potential of protein engineering to improve xylose metabolism by yeast are discussed here.

PART 1. GENERAL PROPERTIES OF XYLOSE ISOMERASES

Figure 1. Word map of xylose isomerase.

The map displaying xylose isomerase-related words was obtained from the BRENDA enzyme database. Through text mining algorithms, the 50 most appearing and relevant words based on the titles and abstracts of literatures available in PubMed are selected for the word map generation [1]. The words are ranked by font size based on the relevance as well as categorized (ex. organisms and application etc.) and color-coded accordingly. The two industrial applications most often associated with xylose isomerase are enzymatic production of high-fructose corn syrup and fermentative production of lignocellulosic bioethanol. The most studied xylose isomerase is an enzyme from *Streptomyces*. The xylose isomerase from *Piromyces* has received much attention because of its high functional expression in *S. cerevisiae* and thereby its role in bioethanol production.

Industrial application of xylose isomerase

Xylose isomerases (EC 5.3.1.5) are enzymes that catalyze interconversion of aldoses and ketoses. Since their first discovery in the early 1950s [2], xylose isomerases have become one of the most important classes of industrial enzymes. In the 1960s, xylose isomerases were usually referred to as glucose isomerases in view of the ability to catalyze interconversion of D-glucose and D-fructose. Enzymatic glucose to fructose isomerization is widely used to produce HFCS (high fructose corn syrup) from corn starch hydrolysates. HFCS is an important sweetener for the food and beverage industry. Often, the isomerization reactions are carried out at elevated temperature (e.g. 60°C) and at very high substrate and product concentrations (up to 42% glucose of which 50% is isomerized). Later, it was realized that these xylose isomerases can also convert D-xylose to D-xylulose (Figure 2), and in most cases xylose isomerization is catalyzed much more efficiently than glucose isomerization owing to the much lower K_M for the former [3–7]. Some xylose isomerases were shown to isomerize other sugars such as

D-ribose, L-arabinose or D-lyxose as well but these reactions and their application are much less explored [8–10]. Some xylose isomerases were engineered to steer the activity towards non-natural substrates [11,12]. The rising awareness of the urgency to develop renewable energy sources and the possible application of xylose isomerase in second-generation bioethanol production revived a strong interest in these enzymes.

Figure 2. Reactions catalyzed by xylose isomerase (XI).

Currently, most of the industrial bioethanol is produced by fermentation of sucrose obtained from sugar cane or sugar beet and of glucose and maltose derived from starch. The starch is obtained from corn or wheat (<https://www.epure.org/resources/statistics/>). The fermentation processes are well-established and ethanol yields obtained with the common yeast *Saccharomyces cerevisiae* are high. However, there are serious concerns about the use of starch- and sugar-based carbohydrates as feedstock for biofuel production since starch is the main component of staple foods world-wide. A higher demand for starch will increase food prices, either directly in case of starches from edible plants or indirectly due to competition for cultivatable land. Thus, large-scale use of these so-called ‘first-generation’ bioethanol processes is undesirable [13]. The food-fuel problem increased interest in the use of cellulose, hemicellulose and lignocellulose as feedstock. These are abundantly available as agricultural waste material or side product in the food industry and used in the so called ‘second generation’ bioethanol processes. Possible sources of lignocellulose include wheat straw and corn stover. However, due to the recalcitrant structural properties of these plant materials in comparison to starch, the second-generation bioethanol processes are more laborious. Cellulose and lignocellulose are not accepted as fermentable carbon sources by yeast, and their use requires careful design and optimization of pre-treatment steps in the production process [14].

To release fermentable sugars from (ligno)cellulosic biomass, it is necessary that during pre-treatment the plant cell wall structure is degraded, releasing cellulose and hemicellulose from lignin (Figure 3). Subsequent treatment with hydrolytic enzymes releases monosaccharides such as D-glucose, D-xylose and L-arabinose, which subsequently must be fermented to ethanol. Since biomass represents one of the major cost factors, maximizing the efficiency of its use

is essential for attractive process economics. Ideally, all sugars present in the hydrolysate should be converted to ethanol. However, *S. cerevisiae*, which is the most commonly employed microorganism for the fermentation process, is unable to utilize 5-carbon sugars like arabinose and xylose. The robustness of this yeast and the widely available genetic engineering tools have triggered research aimed at improving its suitability for fermentation of hydrolyzed hemi- and lignocellulose [15,16]. For example, it is crucial to develop a yeast strain that can tolerate inhibiting or toxic compounds such as acetic acid, hydroxymethylfurfural (HMF) and furfural which are produced during biomass pre-treatment [17–19]. To overcome the limitations of the *S. cerevisiae* sugar transport system with respect to uptake of mixed sugars, more efficient transporters have been engineered [20–22].

Figure 3. Simplified scheme of the second-generation bioethanol production.

The pre-treatment can be done with biological, mechanical, chemical or physicochemical methods [14,23]. It should open up the recalcitrant lignin-containing cell wall structure, exposing cellulose and hemicellulose, which can be hydrolyzed enzymatically using enzyme cocktails typically composed of endoglucanases, exoglucanases, β -glucosidases and xylanases, or by acid treatment [24]. Glucose and xylose are the most abundant sugar monomers formed by hemicellulose hydrolysis and are converted to ethanol through fermentation by *S. cerevisiae*.

The composition of the sugar mixture obtained by pre-treatment depends on the source of the biomass but in general D-glucose is most abundant followed by D-xylose which takes up around 30% [25]. It is therefore important to achieve xylose to ethanol conversion, which is not possible with natural isolates of *S. cerevisiae* [16]. Consequently, one of the main challenges in the development of second-generation bioethanol processes is to engineer *S. cerevisiae* strains capable of efficient xylose fermentation. Fortunately, this yeast can metabolize D-xylulose, the ketose isomer of D-xylose [26,27]. Introducing a path to convert xylose into xylulose indeed enables xylose fermentation of *S. cerevisiae* [28–30]. This aldose to ketose interconversion can be catalyzed by a xylose isomerase heterologously expressed in *S. cerevisiae* (Figure 4A). In addition to this one-enzyme conversion, xylose can also be converted to xylulose in two steps by xylose reductase (XR) and xylitol dehydrogenase (XDH). Xylose is then first

reduced to xylitol by XR using NADPH and xylitol is subsequently oxidized to xylulose by XDH using NAD⁺ as the cofactor. When these enzymes are expressed together in *S. cerevisiae* (Figure 4A), the recombinant yeast can convert xylose but the cofactor imbalance between NAD⁺ and NADPH causes accumulation of the intermediate xylitol, which has a negative impact on the final ethanol yield. *S. cerevisiae* using xylose isomerase for xylose to xylulose conversion produces much less xylitol, leading to higher ethanol yields and making this the preferred pathway [31,32].

Expression of xylose isomerase in *S. cerevisiae*

Since the first attempt to express xylose isomerase (XI) from *E. coli* in yeast [26], there have been many efforts to find suitable xylose isomerases, including enzymes that are better expressed and give higher isomerization activities. However, several XIs could not be produced in yeast or were expressed in some inactive form [33–36]. The causes of poor functional expression of some xylose isomerases are not clear but the use of non-codon-optimized genes may possibly have led to this unfortunate result in some cases, especially for the bacterial XI genes [26,34,35]; it is only recent that we are privileged of having access to cost efficient gene synthesis services [37]. The first xylose isomerase expressed in *S. cerevisiae* with a detectable activity originated from the thermophilic bacterium *Thermus thermophilus*. However, due to the significant drop in activity at lower temperatures, further engineering of this thermophilic xylose isomerase was necessary for in vivo function of the enzyme at the typical fermentation temperature of 30 – 40 °C [38,39]. More recently, the successful expression of a putative xylose isomerase gene from the fungus *Piromyces* E2 (PirXI) paved the way for achieving effective xylose isomerization and xylose to ethanol fermentation in *S. cerevisiae* [30]. Engineered *S. cerevisiae* (Figure 4B) expressing the PirXI gene as well as the native xylulose kinase gene and genes coding for enzymes involved in the non-oxidative pentose phosphate pathway showed effective xylose to ethanol fermentation [30,40,41]. Furthermore, deletion of the aldose reductase encoding gene *GRE3* (Figure 4B) greatly reduced formation of the side product xylitol, which resulted in higher ethanol yields [40,42].

After discovery of PirXI, several other xylose isomerases which could be functionally expressed in *S. cerevisiae* were identified. Figure 5 and table 1 show xylose isomerases which were expressed well or were attempted but failed to be expressed in *S. cerevisiae*. Since the XI pathway of xylose metabolism is known to be utilized mainly by bacterial phyla such as *Bacteriotes* and *Firmicutes*, it is not surprising that most of the XIs studied originate from bacteria [43,44]. Most fungal strains that have been examined use the reductase/dehydrogenase reactions for xylose metabolism; in this respect organisms such as *Piromyces*

and *Orpinomyces* [45] seem to be exceptional. The anaerobic fungus *Piromyces* sp. E2 isolated from the feces of an Indian elephant showed xylose isomerase activity and cDNA encoding XI could be obtained [30,44]. Xylose isomerase from *Orpinomyces*, an anaerobic fungus isolated from cattle rumen fluid show a very high sequence similarity (94.5 %) to PirXI [45]. Both fungal xylose isomerases showed functional expression in *S. cerevisiae*, enabling growth on xylose [30,45]. Interestingly, the XIs from these fungi share a high amino acid sequence identity with xylose isomerases from the *Bacteroidetes* phylum of gram-negative bacteria (Figure 5) leading to the suggestion that their XI genes were acquired through horizontal gene transfer [46].

Figure 4. Engineered xylose metabolic pathway in *S. cerevisiae*.

Xylose to xylulose conversion can be accomplished (A) by a combination of a xylose reductase (XR) and xylose dehydrogenase (XDH) or (B) by incorporating xylose isomerase (e.g. PirXI) in *S. cerevisiae*. Genes encoding heterologously expressed enzymes are indicated with red letters. The green upwards arrows show overexpression of native *S. cerevisiae* genes. A red cross indicates deletion to avoid misrouting.

To date, PirXI seems to be one of the best xylose isomerases for introducing xylose fermentation in *S. cerevisiae*. However, the xylose fermentation properties of yeast strains expressing such a heterologous XI still suffer from limitations and improvement of several aspects is desired, such as a more efficient pentose transport system, optimization of the regulation of central metabolism, increased

cellular robustness, and better kinetic properties of the xylose isomerase itself [47,48]. Insufficient in vivo performance of PirXI is reflected in the high copy number of the *XylA* gene that is required for growth of *S. cerevisiae* on xylose [30,31]. Expression of a recombinant enzyme to very high levels will be a burden to the cells with a negative effect on growth and product yield [49,50]. Therefore, engineering xylose isomerase variants for improved in vivo performance will help to optimize xylose fermentation by *S. cerevisiae*.

Figure 5. Neighbor-joining tree based on amino acid sequences of xylose isomerases expressed in *S. cerevisiae*. The xylose isomerases from organisms indicated in red failed to express, while the ones in blue showed expression of the protein but no activity. *E. coli* XI was initially not expressed at all [26] and in a later study expressed as a non-active protein [33]. The other XIs, indicated in black, were functionally expressed in *S. cerevisiae*.

* Very low activity at the relevant fermentation temperature, 30 - 40 °C

** Expression on the cell surface

*** Co-expression with a chaperonin complex GroEL-GroES

Table 1. Xylose isomerases expressed in *S. cerevisiae*.

	Group	% Sequence identity to PirXI	Expres- sion	Func- tional	Class	Codon optimiza- tion	Ref.
<i>Piromyces</i> sp. E2	Fungi		Y	Y	II	No	[30,44]
<i>Escherichia coli</i> K12	Proteobacteria	47.8	N	N	II	No	[26]
<i>Escherichia coli</i> K12	Proteobacteria	47.8	Y	N	II	No	[33]
<i>Actinoplanes missouriensis</i>	Actinobacteria	21.4	N	N	I	No	[35]
<i>Bacillus subtilis</i>	Firmicutes	47.1	Y	N	II	No	[35]
<i>Lactobacillus pentosus</i>	Firmicutes	44.7	N	N	II	No	[51]
<i>Thermoanarobacterium thermosulfurogenes</i>	Firmicutes	49.4	N	N	II	No	[34]
<i>Thermus thermophilus</i>	Deinococcus- Thermus	24.6	Y	Y	I	No	[39]
<i>Streptomyces rubiginosus</i>	Actinobacteria	25.4	Y	N	I	No	[36]
<i>Clostridium phytofermentans</i>	Firmicutes	53	Y	Y	II	Yes	[28]
<i>Orpinomyces</i>	Fungi	94.5	Y	Y	II	No	[45]
<i>Bacteroides stercoris</i> HJ-15	Bacteroidetes	80.6	Y	Y	II	No	[52]
xym1 (soil metagenome)	Bacteria (Unspecified)	61.4	Y	Y	II	No	[53]
xym2 (soil metagenome)	Bacteria (Unspecified)	59.5	Y	Y	II	No	[53]
<i>Ruminococcus flavefaciens</i>	Firmicutes	49.2	Y	Y	II	Yes	[54]
<i>Prevotella ruminicola</i>	Bacteroidetes	77.5	Y	Y	II	Yes	[55]
<i>Burkholderia cenocepacia</i>	Proteobacteria	50.2	Y	Y	II	No	[29]
<i>Lactococcus lactis</i>	Firmicutes	46.8	Y	Y	II	Yes	[56,57]
Protists of <i>R. speratus</i>	Protist	49.7	Y	Y	II	Yes	[57]
<i>Propionibacterium acidipropionici</i>	Actinobacteria	45.3	Y	Y	II	Yes	[58]
<i>Bacteroides uniformis</i> ATCC8492	Bacteroidetes	81.2	Y	Y	II	Yes	[59]
<i>Bacteroides vulgatus</i>	Bacteroidetes	81.5	Y	Y	II	No	[60]
<i>Tannerella</i> sp. 6_1_58FAA_ CT1	Bacteroidetes	81	Y	Y	II	No	[60]
<i>Alistipes</i> sp. HGB5	Bacteroidetes	76.5	Y	Y	II	No	[60]
<i>Paraprevotella xylaniphila</i>	Bacteroidetes	81.9	Y	Y	II	No	[60]
<i>Bacteroides thetaiotaomicron</i>	Bacteroidetes	83.6	Y	Y	II	Yes	[61]
<i>Clostridium cellulovorans</i>	Firmicutes	50	Y	Y	II	No	[62]
Bovin rumen metagenome	Bacteroidetes*	77.6	Y	Y	II	No	[63]

* Deduced from the high sequence identity shared with *Prevotella ruminicola*.

Structure and mechanism of xylose isomerase

Contemporary enzyme engineering is guided by structural and biochemical information of the target enzyme and its close homologs. As xylose isomerases have been studied for many years, there is a substantial amount of literature and structural data as well as sequence information available. More than 160 structures of xylose isomerases from 16 different strains have been deposited in the protein structure database (<https://www.rcsb.org/>). In general, xylose isomerases are homotetramers with each monomer comprising a TIM-barrel motif with an active site. Xylose isomerase is a metalloenzyme which requires two metals for its activity [8,64–67]. The highly conserved active sites include several metal-binding residues as well as 2-3 conserved tryptophan groups involved in substrate binding (Chapter 2, Figure 4).

Xylose isomerases can structurally be divided in class I and class II enzymes. The sequence of PirXI shows that it belongs to the class II enzymes. Overall structures of enzymes from these classes are very similar but the class II XIs isomerases typically contain an additional 30-45 amino acid segment at the N-terminus of which the role is not clear (Figure 6). Interestingly, most of the enzymes reported to be functionally expressed in yeast are the longer class II XIs (Figure 5 and Table 1). Whether the extension plays a role in expression is unclear; it is also possible that homologs of the class II PirXI, which was the first one to be well produced in yeast, have been more often examined for the expression.

Most of the structures deposited in the protein databank are from class I isomerases, including several crystal structures from the *Streptomyces rubiginosus* enzyme. Only a few class II XI structures were available until recently, all without substrate bound [68]. The structure of PirXI was also not available at the start of the work described in this thesis. Obtaining structures of PirXI is important both for understanding biochemical differences that the two classes of XI may possess and for structure-guided enzyme engineering. Furthermore, a structure of a class II XI that is functionally expressed in *S. cerevisiae* may be used as a reference for comparing other xylose isomerases to improve their performance in yeast. Therefore, we have solved the X-ray structures of PirXI, obtaining 12 structures with different combinations of ligands, as discussed in Chapter 2.

The catalytic mechanism of xylose isomerase has been studied in detail. The reaction was initially thought to follow the triose-phosphate isomerase-like proton transfer mechanism which involves an enediol intermediate [69,70] but later proven to occur via a metal-mediated hydride shift mechanism [66,71–74] (Figure 7). This conclusion is based on the observation with deuterium isotopes that the hydrogen transferred to the carbonyl carbon of the substrate originates from the neighboring carbon atom instead of from the solvent. The extremely low isotope

Figure 6. Major structural differences of class I and class II XIs.

(A) Tetrameric structures of a class I XI (5XIN). (B) Tetrameric structure of class II XI (5NH7). Each monomer is depicted by different colors and the longer N-terminal tails in class II XIs are depicted as darker shades. (C) Aligned structures of monomers of class I and class II XIs. Class II XIs exhibit an extended N-terminal tail and a longer $\alpha 1$ helix compared to class I XIs which is shown in darker orange color. The metals and xylose are depicted as green colored balls and yellow sticks, respectively.

exchange with the solvent invalidated the proton transfer mechanism for the xylose isomerase reaction [75]. The difference in reaction mechanism is maybe reflected in the very different catalytic rates of these two classes of enzymes. Compared to triosephosphate isomerase which is known to be one of the fastest enzymes (typically over several thousand of turnovers per s), xylose isomerase is rather slow (less than 10 s^{-1} at 30°C). The xylose isomerase mechanism involves two divalent cations – each facilitating substrate binding and hydride shift. Apart

from the fact that the metal mediated isomerization of xylose involves more steps in the catalytic cycle such as metal binding and ring opening/closure of substrate/product, the required movement of the catalytic metal prior to the hydride transfer may cause such a difference in the reaction rate compared to triosephosphate isomerase [66,76,77]. In Chapter 2, we describe a step-wise reaction mechanism of PirXI based on previous studies and possible role of the two metals which is supported by X-ray structures with different ligand combinations.

To date, many xylose isomerases are characterized and the information is available via the enzyme database BRENDA (www.brenda-enzymes.org). Extensive enzymatic studies were performed on xylose isomerases from several organisms such as *Streptomyces rubiginosus*, *Streptomyces violaceoruber*, *Actinoplanes missouriensis* and *Arthrobacter sp.* [4,10,78–81]. Since XI is a metalloenzyme, many of these studies were focused on the metal dependent activities. XIs seem to be rather promiscuous with respect to the types of metal that can activate the enzyme. The native metal binding status of xylose isomerases, i.e. the in vivo metal occupancy in the native organism, is mostly unknown. Activity assays of heterologously expressed XIs are commonly performed in vitro in the presence of different added metals, with Mg^{2+} used most often in the standard assay conditions. Metals reported to activate xylose isomerase include Mg^{2+} , Mn^{2+} , and Co^{2+} . Although there is an exception [38,82], class I XIs seem to be better activated by Mg^{2+} whereas class II enzyme prefer Mn^{2+} for activity [4,10,67,83].

Figure 7. Possible mechanisms of aldose-ketose interconversion [70,72,73].

PART 2. ENGINEERING OF XYLOSE ISOMERASE

Rational enzyme engineering

Molecular enzyme engineering uses biochemical, sequence and structural information for mutant design. The design strategy is usually inspired by comparison of homologous enzymes and detailed analysis of the reaction mechanism and enzyme-substrate interactions. Challenges in rational enzyme engineering come from the limited knowledge on the role of protein dynamics and quantitative contribution of specific interactions to stability and catalysis. Rational engineering usually focuses on a local area of a protein such as an enzyme active site. This can be very effective since it reduces space for beneficial mutations. On the other hand, it may result in no improvement or even loss of activity as it overlooks global interactions and their effect on dynamics [84]. Nevertheless, the increasing amount of sequence information in protein databases and the rapidly developing computational tools continue to make enzyme engineering more efficient and there are numerous successful examples in the literature. Through rational engineering,

it was possible to improve enzyme properties such as the catalytic rate [85], the substrate scope [86–89] or solvent- and thermostability [90,91].

Rational engineering of xylose isomerase has been focused on the robustness of the enzyme for industrial HFCS production, ultimately to achieve more cost-efficient use of the biocatalyst. For instance, since the thermodynamic equilibrium of glucose to fructose isomerization increases towards fructose with increasing temperature, xylose isomerase variants with high thermal resistance are desirable. Besides searching for a thermostable natural enzyme with a reasonable activity [92,93], it is also possible to increase the thermostability of an enzyme that already has high activity [94–96]. In order to prevent enzyme denaturation induced by glycation (covalent linkage formation between lysine residues and the substrate glucose) as well as encouraged by the notion that the Arg/Lys ratio is higher in thermostable proteins, Mrabet et al. substituted some lysines to arginines in XI from *Actinoplanes missouriensis* [95]. Indeed, the engineered XI showed improved thermostability both in the presence and absence of glucose. Substitution of a glycine to proline at the turn of a flexible loop located nearby the active site increased the thermostability of xylose isomerase from *Streptomyces diastaticus* [96]. A proline is present at the corresponding position in xylose isomerases from thermostable organisms such as *Thermus thermophilus*.

Another engineering target to improve applicability of xylose isomerase in HFCS production is to increase the activity of the enzyme at lower pH. This prevents formation of impurities and the “browning effect” that occurs at alkaline pH. With a few exceptions of xylose isomerases which show optimum activity at slightly acidic pH [5,7,97,98], most xylose isomerases characterized so far, including PirXI, show a neutral to slightly basic pH optimum. The pH optimum of an enzyme can be changed through rational engineering. Several rational engineering studies have shown that removing a negative charge at a single position close to the active site can lower the pH optimum of the enzyme – mutations E186Q and D255N of XI from *A. missouriensis* lowered the pH optimum by more than 1 pH unit and D56N and E221A of XI from *S. rubiginosus* showed a pH optimum lowered by more than 0.5 unit [4,78,99].

The substrate specificity of xylose isomerase can also be changed through rational engineering. Substitutions at two active site residues (W137F/Y, V186T) of xylose isomerase from *Clostridium thermosulfurogenes* (positions Trp140 and Val187 in PirXI) created more space in the active site and provided additional hydrogen bonding and thereby switched the substrate preference from xylose to glucose, which is advantageous for HFCS production [100]. Mutations may be at some distance from the active site. The distant Q256D mutation (position Thr309 in PirXI) in *A. missouriensis* XI caused an increased activity on L-arabinose while maintaining activity on glucose [12].

Besides engineering the enzyme to change a certain property for application purposes, site-directed mutagenesis was performed in order to elucidate the mechanism of the enzyme and the role of specific residues, including the metal-binding groups [69,101–103]. The important roles of active site residues His105 and His272 (numbering in PirXI) for pyranose or furanose ring-opening and for metal-mediated hydride transfer, respectively were identified through mutagenesis study on *E. coli* and *Streptomyces rubiginosus* XI [69,101]. Through mutagenesis of the metal-binding residues of *Streptomyces olivochromogenes* XI, the role of each metal could be elucidated. The hexacoordinated metal M1 is mainly involved in proper substrate binding and ring-opening, whereas the tetracoordinated metal M2, which is absent in a Glu180Lys mutant, catalyzes the hydride transfer [102]. Other near active site residues critical for the enzyme activity were probed in *S. rubiginosus* D-xylose isomerase. The ring-opened substrate is positioned in a pocket formed by Trp16 (PirXI Trp50), Trp137 (PirXI Trp189), Phe26 (PirXI Phe61), Phe94 (PirXI Phe146) and His54 (His102 in PirXI). His54 (PirXI His102) interacts with the xylose O5. His220 (PirXI His272) is located on the opposite side of the active site pocket and also seems involved in stabilizing the acyclic xylose, in agreement with the loss of activity upon replacement by site-directed mutagenesis [103,104].

Rational engineering of xylose isomerase for improved in vivo application is less straightforward compared to engineering enzyme variants for better performance in cell-free biocatalytic processes. As described in Chapter 2, xylose isomerase from *Piromyces* is well-expressed in yeast. It is possible to estimate the theoretical (expected) in vivo activity of the enzyme from the in vitro kinetic parameters and the cellular expression level. Such a calculation suggests that expressed PirXI should provide *S. cerevisiae* with sufficient activity to allow rapid growth on xylose. We have observed that the enzyme is also fairly stable showing an apparent T_m between 50 – 70 °C (depending on the metal availability) and retaining the activity for prolonged periods at the fermentation temperature of 30 °C (unpublished data). Yet, the in vivo performance of the enzyme seems to be a limiting factor for xylose-supported growth of yeast cells, as indicated by the high expression level of the enzyme required, especially for growth under anoxic conditions [105,106]. The true in vivo activity is difficult to measure and predict due to the complexity of the cytoplasmic matrix. Furthermore, xylose isomerase has two metal binding sites which may bind various metals in vivo, with drastic effects on activity [77,105,107]. These issues make it difficult to define the kinetic and biochemical properties required for a certain growth rate.

This complexity suggests that testing in vivo performance should be an early step in strategies aimed at improving xylose isomerase for application in ethanol fermentation. Although in directed evolution protocols both mutations near and

distant from the active site can improve activity of an enzyme, the design and use of small focused mutant libraries will benefit from reducing the search space to residues near the active site, since the chance that mutations have a positive effect is larger and the type of effect can be better predicted [108]. Consequently, fewer screening will be required to discover improved enzymes. However, the active site of xylose isomerase is highly conserved and sequence comparisons do not suggest obvious mutations (Figure 8). Most of the conserved residues near the active site are directly or indirectly involved in metal and substrate binding and the possibilities for introducing mutations at these residues are meagre. Instead, altering residues surrounding these conserved amino acids can possibly change the activity of the enzyme through subtle effects. Interestingly, class I and class II xylose isomerases show differences in conserved residues (Figure 8 and Chapter 3, Figure 1) near the active site, which may suggest targets for mutagenesis based on class-specific enzyme properties. Changing the residues that are further away from the active site can also influence the activity of an enzyme by subtle structural changes or long-range interactions [108–110]. The distant mutations K355A and V144A improved the activity of xylose isomerase from thermophilic bacterium *Thermus thermophilus* at low temperature, making the enzyme a better candidate for in vivo application [111]. The mutations were designed by comparing the amino acid sequences of XI from thermophiles to mesophilic XIs and caused a decrease of the inter-subunit interactions. The reduced rigidity of the enzyme increased activity.

Figure 8. Conservation of the active site of xylose isomerases (PDB 5NH7).

The sequences around the active site of 124 xylose isomerases were analyzed. The amino acid residues that are within 12 Å away from the C1 atom of xylose are shown. The fully conserved amino acid residues, including the metal binding residues which are conserved throughout all xylose isomerases, are depicted in orange color. The turquoise-colored residues are conserved within class II enzymes. The non-conserved residues are shown in grey color. The substrate xylose and metal cofactor Mg²⁺ ions are shown in a yellow sticks and green spheres, respectively.

Decreasing the pH optimum or increasing the activity of xylose isomerase at lower pH may also improve in vivo performance since the physiological pH of yeast cytoplasm during fermentation is slightly acidic [112–114]. In a previous study, Waltman et al. have shown that mutations D57N and N215D designed by structural inspection improved the catalytic efficiency of XI from *S. rubiginosus* at lower pH by decreasing the K_M for the substrate [79]. Based on the hypothesis that the decrease in metal binding affinity at lower pH causes the low activity, the authors designed mutations around the metal binding residues in order to lower the pK_a of the metal binding residues so that the metal binding at lower pH improves. The pH optima of xylose isomerases from different organisms may vary even among enzymes that are close homologs. For example, while the xylose isomerase from *Streptomyces* sp. SK shows a pH optimum of 6.0 – 6.5, the enzymes from *S. flavogriseus* and *S. rubiginosus* show a neutral and a slightly basic pH optimum, respectively [78,97,98,115]. The differences in biochemical properties despite the high overall sequence similarities suggests that identifying subtle sequence differences between XIs can still be useful for engineering the pH optimum.

Besides the above-mentioned approaches, several strategies such as changing the metal specificity or improving the substrate binding affinity can possibly be used to improve in vivo performance of xylose isomerase. What is as important as engineering the enzyme itself, is investigating if any introduced changes indeed have the desired in vivo effect. Conditions in the cell are very different from laboratory assay conditions, and expression and compartmentalization play a role only in vivo. There are some examples of rational enzyme engineering for improved in vivo application. Matsushika et al. changed the cofactor specificity of NAD⁺ dependent xylitol dehydrogenase from *Pichia stipitis* towards NADP⁺ in order to reduce xylitol production which is caused by cofactor imbalance in *S. cerevisiae* expressing XR-XDH during xylose fermentation [116]. In another study, engineering cofactor specificity of a glyceraldehyde 3-phosphate dehydrogenase from NAD⁺ to NADP⁺ improved production of lysine by *Corynebacterium glutamicum* [117].

To our knowledge, there were no rationally designed mutants of xylose isomerase that had been tested for their effects on xylose fermentation before we examined the in vivo performance of PirXI variants in this work (Chapter 3). Since it is uncertain which properties of the enzyme determine in vivo performance, rational engineering of xylose isomerase for this purpose is complicated. Uncertainties are due to the requirement of two metals, the metal promiscuity of the enzyme and the varying in vivo metal availability. In view of these challenges, an early validation of the in vivo effects of mutations which influence in vitro enzyme properties will be essential for engineering xylose isomerases with improved applicability in bioethanol production.

Directed evolution

Directed evolution of an enzyme uses a natural selection-like strategy, where host cells expressing a library of randomly mutated enzyme-encoding genes are screened for the presence of variants with improved properties [118,119]. The advantages of directed evolution include that no structural information about the enzyme is required (although it may be used if available) and screening protocols can be adapted to ensure discovery of variants that perform better under specific conditions, including activity *in vivo*. After the initial idea of laboratory evolution was explored with RNA molecules [120], the concept of directed evolution has been applied to other biological molecules, including antibodies and enzymes [121]. The first practical success of evolving enzyme activity was presented by Chen et al. [122] where subtilisin E variants with a significantly improved activity in organic solvents were discovered.

Several molecular biology tools are available to generate genetic diversity. A classical method such as random mutagenesis by error-prone PCR is still frequently used. Other methods that can be considered are DNA shuffling and (combinatorial) site-saturation mutagenesis [123,124]. Advanced techniques in molecular biology may offer opportunities for even more creative methods for mutant library generation. Recently, Crook et al developed a continuous directed evolution method using the retrotransposon Ty1 replication system [125]. By integrating a gene of interest in the inducible Ty1 retrotransposon, taking advantage of the error-prone self-replication system, efficient *in vivo* mutant library generation and screening in *S. cerevisiae* could be performed simultaneously. Random mutagenesis methods require balancing between a high mutation frequency, which increases genetic diversity in the library, and a restricted mutation load, which is needed to avoid loss of library quality by introduction of lethal mutations.

If detailed information on the enzyme is available, so-called smart libraries can be created by constraining the mutational space to the most relevant positions of a protein and/or by limiting the diversity in the set of amino acids that is introduced at target positions. In order to incorporate only a subset of the 20 proteinogenic amino acids, partially undefined codons can be used when designing primers. Furthermore, mutations at several specific residues in different combinations can easily be incorporated using efficient cloning methods such as Golden-gate cloning or Gibson assembly [126,127]. The use of computational methods can further improve the quality of mutant libraries by removing potentially lethal mutations.

Screening of the library can be performed *in vitro* or *in vivo*. In the former case, the screening does not include effects of mutations on the fitness or viability of the host cells. Cells can be used as platforms to display or immobilize enzyme

variants such as with the yeast surface display method [128,129]. Very often, cells are used as an expression host and are lysed in the screening process, followed by activity measurement with cell-free extract or purified enzymes [130]. Directed evolution with in vivo screening resembles the process of Darwinian's evolution in the sense that variants with beneficial properties are enriched in subsequent cycles of mutagenesis and selection.

The most frequently used host organisms for directed evolution of enzymes are the prokaryote *E. coli* and the eukaryote *S. cerevisiae* [131]. Multiple tools for recombinant protein expression and molecular genetics are available for these hosts, and they can be used for industrial production of enzymes or metabolites as well. Efficient biosynthesis or metabolism of specific compounds often requires metabolic pathway engineering in combination with directed evolution of key enzymes in that pathway. Directed evolution and testing for improved in vivo performance can be performed either in two different organisms [132] or in the same organism. In the latter case, complications due to possible divergent effects of mutations in different host organisms are avoided, making the directed evolution process more efficient.

There are several successful examples of in vivo screening of enzyme libraries in organisms that are also used for the final application. For example, Umeno et al. improved the biosynthesis of previously undiscovered long chain carotenoid backbones in *E. coli* by directed evolution of a carotenoid synthase [133]. In another study, production of 1-propanol and 1-butanol in *E. coli* was improved by 9- to 22-fold through directed evolution of citramalate synthase from *Methanococcus jannashii* [134]. Screening of enzyme libraries using *S. cerevisiae* for improved ethanol production is also a good example. Through in vivo screening using *S. cerevisiae*, Li et al. discovered a sugar transporter variant with decreased inhibition of xylose uptake by glucose, thereby improving xylose/glucose co-transport [135].

Directed evolution has also been applied to xylose isomerase expressed in *S. cerevisiae* in order to optimize the xylose fermentation process. Screening a random mutant library generated by error-prone PCR revealed xylose isomerase variants from *Thermus thermophilus* with improved activity at lower temperatures [38]. This provided the possibility to use this thermophilic enzyme for xylose fermentation, which is performed at a temperature 30 – 40 °C. However, in this study mutants were identified by in vitro activity screening after expression in *E. coli*. Whether this improves xylose fermentation needs to be seen since in vitro assay condition used for selection might be irrelevant for in vivo enzyme performance.

From an application point of view, in vivo screening of enzyme libraries to discover variants that improve biofuel production is preferably carried out using

S. cerevisiae instead of *E. coli*. Since xylose isomerase activity is limiting the rate of growth of *S. cerevisiae* on xylose, differences in growth properties can be used for the selection of improved XI variants. Hou et al. discovered a variant of a xylose isomerase originating from the bovine rumen metagenome that improved xylose utilization by *S. cerevisiae* through growth-based screening of a randomly generated mutant library [63]. Some improved PirXI variants were also discovered through in vivo screening. By iterative rounds of library generation by error prone PCR and enrichment of fast-growing variants, Lee et al discovered a PirXI variant carrying two mutations (E15D+T142S) which significantly improved the growth rate of *S. cerevisiae* on xylose [42]. More recently, Katahira et al discovered that several mutations of residue Asn337 in the XI from a protists residing in the *Reticulitermes speratus* (Asn338 in the PirXI sequence), especially N338C, improved xylose consumption by *S. cerevisiae* [57]. This mutation was also effective when introduced in related xylose isomerases from different organisms, including XI from *Piromyces* [57]. This discovery was achieved by transforming *S. cerevisiae* with the protists XI library generated by error-prone PCR and selecting for fast-growing colonies on xylose medium. As described in Chapter 5, we explored similar in vivo screening approaches in order to discover improved PirXI variants. Instead of the randomized library generation method used in the above studies, we constructed focused libraries taking advantage of the structural and biochemical data we obtained.

Several studies including ours described in Chapter 5 [42,57,107,136] have shown that engineering XI can improve growth of *S. cerevisiae* on xylose, which proves the hypothesis that the in vivo performance of XI is a limiting factor for efficient xylose fermentation. The in vivo enzyme performance is indeed reflected in the growth rate and/or ethanol yield. It should be noted that among studies comparing the performance of different xylose isomerases or XI variants in a xylose fermentation system, factors such as strain background and growth conditions often are different. This may affect the outcome of such comparisons. For example, the strain used in our study includes more genetic modifications opted for xylose growth (overexpression of *TAL1*, *RKI1*, *RPE1*, *XKS1*) compared to the strain used by Lee et al [42]. Seike et al. pursued their study using rich media and found a higher growth rate [136], but nutrient-poor mineral medium was used in our work. Furthermore, inoculum densities of cultures and initial CO₂ concentrations can influence the lag phase in growth experiments, as well as the final cell density [137].

SCOPE OF THE THESIS

The work reviewed above indicates that yeast xylose metabolism can be improved by optimizing xylose isomerase activity, which can be achieved by directed evolution or by introducing a better performing enzyme from nature's biodiversity. Directed evolution seems to be a particularly useful tool for this purpose although it remains unclear which biochemical properties of xylose isomerase variants govern enhanced xylose fermentation rates observed in previous work. Furthermore, whereas error prone PCR and similar random mutagenesis methods can explore a wide diversity, structural information has not been used in attempts to improve xylose isomerase, in part because crystal structures are lacking for the *Piromyces* xylose isomerase, which so far is probably the most attractive enzyme for xylose isomerization. Another largely unexplored issue is the role of metal availability and incorporation on the xylose isomerase activity, which we assumed to be of considerable importance because metal loading of heterologously expressed xylose isomerases may well be incomplete or problematic due to misloading. The work reported in this thesis aims to address these issues by exploring enzyme properties and mutations around the metal binding sites that influence xylose metabolism by *S. cerevisiae*. Therefore, we investigated wild-type and engineered variants of the *Piromyces* xylose isomerase (PirXI), with a focus on metal-dependence of activity and the effect of near active site mutations.

First, we investigated the kinetic properties, metal-dependence and structural properties of the wild-type PirXI enzyme in order to enable structure-based engineering of the active site region. **Chapter 2** describes the purification of PirXI, as well as kinetic data and crystal structures of the protein with different metals bound. Through kinetic measurements, we explored the effect of different metals on activity and sought to understand how metal binding and selectivity influence in vivo performance of the enzyme. Crystal structures with various metals suggested that manganese gives the highest catalytic activity and shows the highest binding affinity.

Using the structural information, we aimed to explore the use of structure-based protein engineering to find PirXI mutants that improve growth of *S. cerevisiae* on xylose. Both structure-based engineering of the pH optimum and bioinformatics-inspired mutagenesis to enhance activity were explored. Accordingly, **Chapter 3** describes the design and construction PirXI mutants and the use of different screening strategies. High-throughput expression and purification methods using 96-well plate assays are used to analyze a large number of variants. This chapter further explores the effect of different mutant properties on growth of *S. cerevisiae* on xylose.

In **Chapter 4**, we describe the importance of in vivo metal composition on xylose isomerase performance and growth of *S. cerevisiae* cells. During laboratory evolution of yeast strains that more rapidly ferment xylose to ethanol, it was discovered by our collaborators at TU Delft that mutations that disturb metal homeostasis in *S. cerevisiae* by influencing a metal transporter have a positive effect on growth of on xylose. The properties of xylose isomerase that we isolated from such a mutant strain and its metal content indeed suggested an effect of metal loading on XI activity. The enhanced manganese content of the cells and of their PirXI caused improved xylose conversion kinetics.

In **Chapter 5**, a structure-based directed evolutionary approach for PirXI engineering is explored. Using the biochemical and structural information of the enzyme described in Chapter 2, we designed focused semi-rational libraries. Libraries were made in the laboratory by oligonucleotide-assisted semi-random localized mutagenesis. We explored two different in vivo screening methods, both based on growth of transformed *S. cerevisiae* on xylose. This work indeed resulted in the discovery of improved PirXI variants. The properties of the selected variants are presented, which indicated only very minor changes in kinetic properties. Which biochemical or physiological changes are responsible for the improved growth induced by these mutants remains enigmatic. However, growth experiments convincingly showed significant and reproducible positive effects on xylose metabolism.

Chapter 6 summarizes the progress in improving PirXI for xylose fermentation starting from enzyme characterization to engineering of the enzyme. Future directions on engineering of PirXI as well as other enzymes involved in xylose metabolism for optimizing ethanol production processes are suggested.

REFERENCES

- Schomburg I, Jeske L, Ulbrich M, Placzek S, Chang A, Schomburg D. The BRENDA enzyme information system—From a database to an expert system. *J Biotechnol.* 2017;261:194–206.
- Hochster RM, Watson RW. Enzymatic isomerization of D-xylose to D-xylulose. *Arch Biochem Biophys.* 1954;48:120–9.
- Yamanaka K. Purification, crystallization and properties of the D-xylose isomerase from *Lactobacillus brevis*. *Biochim Biophys Acta.* 1968;151:670–80.
- van Tilbeurgh H, Jenkins J, Chiadmi M, Janin J, Wodak SJ, Mrabet NT, et al. Protein engineering of xylose (glucose) isomerase from *Actinoplanes missouriensis*. 3. Changing metal specificity and the pH profile by site-directed mutagenesis. *Biochemistry.* 1992;31:5467–71.
- Staudigl P, Haltrich D, Peterbauer CK. L-arabinose isomerase and D-xylose isomerase from *Lactobacillus reuteri*: characterization, coexpression in the food grade host *Lactobacillus plantarum*, and application in the conversion of D-galactose and D-glucose. *J Agric Food Chem.* 2014;62:1617–24.
- Kawai Y, Konishi H, Horitsu H, Sakurai H, Takamizawa K, Suzuki T, et al. Purification and characterization of D-xylose isomerase from *Bifidobacterium adolescentis*. *Biosci Biotechnol Biochem.* 1994;58:691–4.
- Liu S ying, Wiegel J, Gherardini FC. Purification and cloning of a thermostable xylose (glucose) isomerase with an acidic pH optimum from *Thermoanaerobacterium* Strain JW/SL-YS 489. *J Bacteriol.* 1996;178:5938–45.
- Lehmacher A, Bisswanger H. Comparative kinetics of D-xylose and D-glucose isomerase. Activities of the D-xylose isomerase from *Thermus aquaticus* HB8. *Biol Chem Hoppe Seyler.* 1990;371:527–36.
- Santa H, Kammonen J, Lehtonen O, Karimäki J, Pastinen O, Leisola M, et al. Stochastic boundary molecular dynamics simulation of L-ribose in the active site of *Actinoplanes missouriensis* xylose isomerase and its Val135Asn mutant with improved reaction rate. *Biochim Biophys Acta.* 2005;1749:65–73.
- Smith CA, Rangarajan M, Hartley BS. D-Xylose (D-glucose) isomerase from *Arthrobacter* strain N.R.R.L B3728. *Biochem J.* 1991;277:255–61.
- Patel DH, Cho EJ, Kim HM, Choi IS, Bae HJ. Engineering of the catalytic site of xylose isomerase to enhance bioconversion of a non-preferential substrate. *Protein Eng Des Sel.* 2012;25:331–6.
- Karimäki J, Parkkinen T, Santa H, Pastinen O, Leisola M, Rouvinen J, et al. Engineering the substrate specificity of xylose isomerase. *Protein Eng Des Sel.* 2005;17:861–9.
- Tenenbaum DJ. Food vs. fuel: Diversion of crops could cause more hunger. *Env Heal Perspect.* 2008;116:A254–7.
- Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresour Technol.* 2010;101:4851–61.
- Lian J, Mishra S, Zhao H. Recent advances in metabolic engineering of *Saccharomyces cerevisiae*: New tools and their applications. *Metab Eng.* 2018;50:85–108.
- Mohd Azhar SH, Abdulla R, Jambo SA, Marbawi H, Gansau JA, Mohd Faik AA, et al. Yeasts in sustainable bioethanol production: A review. *Biochem Biophys Reports.* 2017;10:52–61.
- Banerjee N, Bhatnagar R, Viswanathan L. Inhibition of glycolysis by furfural in *Saccharomyces cerevisiae*. *Eur J Appl Microbiol Biotechnol.* 1981;11:226–8.
- Adeboye PT, Bettiga M, Olsson L. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates. *AMB Express.* 2014;4.
- Ullah A, Chandrasekaran G, Brul S, Smits GJ. Yeast adaptation to weak acids prevents futile energy expenditure. *Front Microbiol.* 2013;4:1–10.
- Hamacher T, Becker J, Gárdonyi M, Hahn-Hägerdal B, Boles E. Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. *Microbiology.* 2002;148:2783–8.
- Nijland JG, Shin HY, De Jong RM, De Waal PP, Klaassen P, Driessen AJM. Engineering of an endogenous hexose transporter into a specific D-xylose transporter facilitates glucose-xylose co-consumption in *Saccharomyces cerevisiae*. *Biotechnol Biofuels.* 2014;7:168.

22. Verhoeven MD, Bracher JM, Nijland JG, Bouwknegt J, Daran JMG, Driessen AJM, et al. Laboratory evolution of a glucose-phosphorylation-deficient, arabinose-fermenting *S. cerevisiae* strain reveals mutations in GAL2 that enable glucose-insensitive L-arabinose uptake. *FEMS Yeast Res.* 2018;18:1–15.
23. Chen H, Liu J, Chang X, Chen D, Xue Y, Liu P, et al. A review on the pretreatment of lignocellulose for high-value chemicals. *Fuel Process Technol.* 2017;160:196–206.
24. Aditiya HB, Mahlia TMI, Chong WT, Nur H, Sebayang AH. Second generation bioethanol production: A critical review. *Renew Sustain Energy Rev.* 2016;66:631–53.
25. Gray KA, Zhao L, Emptage M. Bioethanol. *Curr Opin Chem Biol.* 2006;10:141–6.
26. Briggs KA, Lancashire WE, Hartley BS. Molecular cloning, DNA structure and expression of the *Escherichia coli* D-xylose isomerase. *EMBO J.* 1984;3:611–6.
27. Wang PY, Schneider H. Growth of yeast on D-xylulose. *Can J Microbiol.* 1980;26:1165–8.
28. Brat D, Boles E, Wiedemann B. Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* 2009;75:2304–11.
29. de Figueiredo Vilela L, de Mello VM, Reis VCB, Bon EP da S, Gonçalves Torres FA, Neves BC, et al. Functional expression of *Burkholderia cenocepacia* xylose isomerase in yeast increases ethanol production from a glucose-xylose blend. *Bioresour Technol.* 2013;128:792–6.
30. Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MSM, de Laat WTAM, et al. High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Res.* 2003;4:69–78.
31. Zhou H, Cheng J sheng, Wang BL, Fink GR, Stephanopoulos G. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. *Metab Eng.* 2012;14:611–22.
32. Jansen MLA, Bracher JM, Papapetridis I, Verhoeven MD, de Bruijn H, de Waal PP, et al. *Saccharomyces cerevisiae* strains for second-generation ethanol production: from academic exploration to industrial implementation. *FEMS Yeast Res.* 2017;17:1–20.
33. Sarthy A V., McConaughy BL, Lobo Z, Sundstrom JA, Furlong CE, Hall BD. Expression of the *Escherichia coli* xylose isomerase gene in *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* 1987;53:1996–2000.
34. Moes CJ, Pretorius IS, van Zyl WH. Cloning and Expression of the *Clostridium thermosulfurogenes* D-xylose isomerase gene (*xylA*) in *Saccharomyces cerevisiae*. *Biotechnol Lett.* 1996;18:297–274.
35. Amore R, Wilhelm M, Hollenberg CP. The fermentation of xylose - an analysis of the expression of *Bacillus* and *Actinoplanes* xylose isomerase genes in yeast. *Appl Microbiol Biotechnol.* 1989;30:351–7.
36. Gárdonyi M, Hahn-Hägerdal B. The *Streptomyces rubiginosus* xylose isomerase is misfolded when expressed in *Saccharomyces cerevisiae*. *Enzyme Microb Technol.* 2003;32:252–9.
37. Carlson R. The changing economics of DNA synthesis. *Nat Biotechnol.* 2009;27:1091–4.
38. Lönn A, Gárdonyi M, van Zyl W, Hahn-Hägerdal B, Otero RC. Cold adaptation of xylose isomerase from *Thermus thermophilus* through random PCR mutagenesis. *Eur J Biochem.* 2002;269:157–63.
39. Walfridsson M, Bao X, Anderlund M, Lilius G, Bülow L, Hahn-Hägerdal B. Ethanolic fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus xylA* gene, which expresses an active xylose (glucose) isomerase. *Appl Environ Microbiol.* 1996;62:4648–51.
40. Kuyper M, Hartog MMP, Toirkens MJ, Almering MJH, Winkler AA, van Dijken JP, et al. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res.* 2005;5:399–409.
41. Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *FEMS Yeast Res.* 2005;5:925–34.
42. Lee SM, Jellison T, Alper HS. Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* 2012;78:5708–16.
43. Kim D, Woo HM. Deciphering bacterial xylose metabolism and metabolic engineering of industrial microorganisms for use as efficient microbial cell factories. *Appl Microbiol Biotechnol.* 2018;102:9471–80.
44. Harhangi HR, Akhmanova AS, Emmens R, van Der Drift C, de Laat WTAM, van Dijken JP, et al. Xylose metabolism in the anaerobic fungus *Piromyces* sp. strain E2 follows the bacterial pathway. *Arch Microbiol.* 2003;180:134–41.

45. Madhavan A, Tamalampudi S, Ushida K, Kanai D, Katahira S, Srivastava A, et al. Xylose isomerase from polycentric fungus *Orpinomyces*: gene sequencing, cloning, and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol. *Appl Microbiol Biotechnol*. 2009;82:1067–78.
46. van Maris AJA, Winkler AA, Kuyper M, de Laat WTAM, van Dijken JP, Pronk JT. Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose isomerase as a key component. *Adv Biochem Eng Biotechnol*. 2007;108:179–204.
47. Kobayashi Y, Sahara T, Ohgiya S, Kamagata Y, Fujimori KE. Systematic optimization of gene expression of pentose phosphate pathway enhances ethanol production from a glucose/xylose mixed medium in a recombinant *Saccharomyces cerevisiae*. *AMB Express*. 2018;8:139.
48. Kwak S, Jin YS. Production of fuels and chemicals from xylose by engineered *Saccharomyces cerevisiae*: a review and perspective. *Microb Cell Fact*. 2017;16:1–15.
49. Snoep JL, Yomano LP, Westerhoff H V., Ingram LO. Protein burden in *Zymomonas mobilis*: Negative flux and growth control due to overproduction of glycolytic enzymes. *Microbiology*. 1995;141:2329–37.
50. Karim AS, Curran kathleen A, Alper HS. Characterization of plasmid burden and copy number in *Saccharomyces cerevisiae* for optimization of metabolic engineering applications. *FEMS Yeast Res*. 2013;13:107–16.
51. Hallborn J. Metabolic engineering of *Saccharomyces cerevisiae* : expressions of genes involved in pentose metabolism. 1995.
52. Ha SJ, Kim SR, Choi JH, Park MS, Jin YS. Xylitol does not inhibit xylose fermentation by engineered *Saccharomyces cerevisiae* expressing *xylA* as severely as it inhibits xylose isomerase reaction in vitro. *Appl Microbiol Biotechnol*. 2011;92:77–84.
53. Parachin NS, Gorwa-Grauslund MF. Isolation of xylose isomerases by sequence- and function-based screening from a soil metagenomic library. *Biotechnol Biofuels*. 2011;4:9.
54. Aeling KA, Salmon KA, Laplaza JM, Li L, Headman JR, Hutagalung AH, et al. Co-fermentation of xylose and cellobiose by an engineered *Saccharomyces cerevisiae*. *J Ind Microbiol Biotechnol*. 2012;39:1597–604.
55. Hector RE, Dien BS, Cotta MA, Mertens JA. Growth and fermentation of D-xylose by *Saccharomyces cerevisiae* expressing a novel D-xylose isomerase originating from the bacterium *Prevotella ruminicola* TC2-24. *Biotechnol Biofuels*. 2013;6:84.
56. Rönnow B, Andersen TH, Sibbesen O. Microorganism expressing xylose isomerase. 2009. US 2011/0244525 A1
57. Katahira S, Muramoto N, Moriya S, Nagura R, Tada N, Yasutani N, et al. Screening and evolution of a novel protist xylose isomerase from the termite *Reticulitermes speratus* for efficient xylose fermentation in *Saccharomyces cerevisiae*. *Biotechnol Biofuels*. 2017;10:1–18.
58. Temer B, Santos LV, Negri VA, Galhardo JP, Magalhães PHM, José J, et al. Conversion of an inactive xylose isomerase into a functional enzyme by co-expression of GroEL-GroES chaperonins in *Saccharomyces cerevisiae*. *BMC Biotechnol*. 2017;17:1–13.
59. Klaassen P, Van der Laan JM, Gielesen BEM, Van Suylekom GP. A pentose sugar fermenting cell. 2009. WO 2009/109634 A1
60. Peng B, Huang S, Liu T, Geng A. Bacterial xylose isomerases from the mammal gut Bacteroidetes cluster function in *Saccharomyces cerevisiae* for effective xylose fermentation. *Microb Cell Fact*. 2015;14:1–14.
61. Mert MJ, la Grange DC, Rose SH, van Zyl WH. Engineering of *Saccharomyces cerevisiae* to utilize xylan as a sole carbohydrate source by co-expression of an endoxylanase, xylosidase and a bacterial xylose isomerase. *J Ind Microbiol Biotechnol*. 2016;43:431–40.
62. Ota M, Sakuragi H, Morisaka H, Kuroda K, Miyake H, Tamaru Y, et al. Display of *Clostridium cellulovorans* xylose isomerase on the cell surface of *Saccharomyces cerevisiae* and its direct application to xylose fermentation. *Biotechnol Prog*. 2013;29:346–51.
63. Hou J, Shen Y, Jiao C, Ge R, Zhang X, Bao X. Characterization and evolution of xylose isomerase screened from the bovine rumen metagenome in *Saccharomyces cerevisiae*. *J Biosci Bioeng*. 2015;121:160–5.
64. Carrell HL, Hoier H, Glusker JP. Modes of binding substrates and their analogues to the enzyme D-xylose isomerase. *Acta Crystallogr, Sect D Biol Crystallogr*. 1994;50:113–23.
65. Henrick K, Collyer CA, Blow DM. Structures of D-xylose isomerase from *Arthrobacter* strain B3728 containing the inhibitors xylitol and D-sorbitol at 2.5 Å and 2.3 Å resolution, respectively. *J Mol Biol*. 1989;208:129–57.

66. Kovalevsky AY, Hanson L, Fisher SZ, Mustyakimov M, Mason SA, Trevor Forsyth V, et al. Metal ion roles and the movement of hydrogen during reaction catalyzed by D-xylose isomerase: A joint X-ray and neutron diffraction study. *Structure*. 2010;18:688–99.
67. van Bastelaere P, Vangrype W, Kersters-Hilderson H. Kinetic studies of Mg²⁺-, Co²⁺- and Mn²⁺-activated D-xylose isomerases. *Biochem J*. 1991;278:285–92.
68. Han B, Bong SM, Cho J, Kim M, Kim SJ, Lee B II. Crystal structure of a class 2 D-xylose isomerase from the human intestinal tract microbe *Bacteroides thetaiotaomicron*. *Biodesign*. 2015;3:41–7.
69. Batt CA, Jamieson AC, Vandeyar MA. Identification of essential histidine residues in the active site of *Escherichia coli* xylose (glucose) isomerase. *Proc Natl Acad Sci*. 1990;87:618–22.
70. Rose IA. Mechanism of the aldose-ketose isomerase reactions. *Adv Enzymol Relat Areas Mol Biol*. 1975;43:491–517.
71. Schray K, Mildvan AS. Kinetic and magnetic resonance of the mechanism of D-xylose isomerase. *J Biol Chem*. 1972;247:2034–7.
72. Collyer CA, Blow DM. Observations of reaction intermediates and the mechanism of aldose-ketose interconversion by D-xylose isomerase. *Proc Natl Acad Sci*. 1990;87:1362–6.
73. Collyer CA, Henrick K, Blow DM. Mechanism for aldose-ketose interconversion by D-xylose isomerase involving ring opening followed by a 1,2-hydride shift. *J Mol Biol*. 1990;212:211–35.
74. Bhosale SH, Rao MB, Deshpande V V. Molecular and industrial aspects of glucose isomerase. *Microbiol Rev*. 1996;60:280–300.
75. Alper H, Stephanopoulos G. Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? *Nat Rev Microbiol*. 2009;7:715–23.
76. Fenn TD, Ringe D, Petsko GA. Xylose isomerase in substrate and inhibitor Michaelis states: Atomic resolution studies of a metal-mediated hydride shift. *Biochemistry*. 2004;43:6464–74.
77. Lee M, Rozeboom HJ, de Waal PP, de Jong RM, Dudek HM, Janssen DB. Metal dependence of the xylose (glucose) isomerase from *Pirromyces* sp. E2 explored by activity profiling and protein crystallography. *Biochemistry*. 2017;56:5991–6005.
78. Cha J, Batt C. Lowering the pH optimum of D-xylose isomerase: the effect of mutations of the negatively charged residues. *Mol Cells*. 1998;8:374.
79. Waltman MJ, Yang ZK, Langan P, Graham DE, Kovalevsky A. Engineering acidic *Streptomyces rubiginosus* D-xylose isomerase by rational enzyme design. *Protein Eng Des Sel*. 2014;27:59–64.
80. Jenkins J, Janin J, Rey F, Chiadmi M, van Tilbeurgh H, Lasters I, et al. Protein engineering of xylose (glucose) isomerase from *Actinoplanes missouriensis*. 1. Crystallography and site-directed mutagenesis of metal binding sites. *Biochemistry*. 1992;31:5449–58.
81. Callens M, Kersters-Hilderson H, Van Opstal O, De Bruyne CK. Catalytic properties of D-xylose isomerase from *Streptomyces violaceoruber*. *Enzyme Microb Technol*. 1986;8:696–700.
82. Umemoto Y, Shibata T, Araki T. D-Xylose Isomerase from a marine bacterium, *Vibrio* sp. Strain XY-214, and D-xylooligosaccharide production from β-1,3-xylan. *Mar Biotechnol*. 2012;14:10–20.
83. Cha J. Contribution of second metal binding site for metal specificity of D-xylose isomerase. *J Microbiol Biotechnol*. 1999;9:757–63.
84. Chen R. Enzyme engineering: rational redesign versus directed evolution. *Trends Biotechnol*. 2001;19:13–4.
85. Getzoff ED, Cabelli DE, Fisher CL, Parge HE, Viezzoli MS, Banci L, et al. Faster superoxide dismutase mutants designed by enhancing electrostatic guidance. *Nature*. 1992;358:347–51.
86. Ferrari AR, Lee M, Fraaije MW. Expanding the substrate scope of chitinooligosaccharide oxidase from *Fusarium graminearum* by structure-inspired mutagenesis. *Biotechnol Bioeng*. 2015;112:1074–80.
87. Liu L, Murphy P, Baker D, Lutz S. Computational design of orthogonal nucleoside kinases. *Chem Commun*. 2010;46:8803–5.
88. Landwehr M, Carbone M, Otey CR, Li Y, Arnold FH. Diversification of catalytic function in a synthetic family of chimeric cytochrome P450s. *Chem Biol*. 2007;14:269–78.
89. Seebeck FP, Guainazzi A, Amoreira C, Baldrige KK, Hilvert D. Stereoselectivity and expanded substrate scope of an engineered PLP-dependent aldolase. *Angew Chemie - Int Ed*. 2006;45:6824–6.
90. Lehmann M, Loch C, Middendorf A, Studer D, Lassen SF, Pasamontes L, et al. The consensus concept for thermostability engineering of proteins: further proof of concept. *Protein Eng*. 2002;15:403–11.

91. Eijlsink VGH, Bjørk A, Gåseidnes S, Sirevåg R, Synstad B, Burg B Van Den, et al. Rational engineering of enzyme stability. *J Biotechnol.* 2004;113:105–20.
92. Neifar S, Hlima H Ben, Mhiri S, Mezghani M, Bouacem K, Ibrahim AH, et al. A novel thermostable and efficient Class II glucose isomerase from the thermophilic *Caldicoprobacter algeriensis*: Biochemical characterization, molecular investigation, and application in High Fructose Syrup production. *Int J Biol Macromol.* 2019;129:31–40.
93. Jia DX, Zhou L, Zheng YG. Properties of a novel thermostable glucose isomerase mined from *Thermus oshimai* and its application to preparation of high fructose corn syrup. *Enzyme Microb Technol.* 2017;99:1–8.
94. Hartley BS, Hanlon N, Jackson RJ, Rangarajan M. Glucose isomerase: insights into protein engineering for increased thermostability. *Biochim Biophys Acta.* 2000;1543:294–335.
95. Mrabet NT, Van den Broeck A, Van den Brande I, Stanssens P, Laroche Y, Lambeir AM, et al. Arginine residues as stabilizing elements in proteins. *Biochemistry.* 1992;31:2239–53.
96. Zhu GP, Xu C, Teng MK, Tao LM, Zhu XY, Wu CJW, et al. Increasing the thermostability of D-xylose isomerase by introduction of a proline into the turn of a random coil. *Protein Eng.* 1999;12:635–8.
97. Ben Hlima H, Bejar S, Riquet J, Haser R, Aghajari N. Identification of critical residues for the activity and thermostability of *Streptomyces* sp. SK glucose isomerase. *Appl Microbiol Biotechnol.* 2013;97:9715–26.
98. Srih-Belghith K, Bejar S. A thermostable glucose isomerase having a relatively low optimum pH: study of activity and molecular cloning of the corresponding gene. *Biotechnol Lett.* 1998;20:553–6.
99. van Bastelaere PBM, Kersters-Hilderson HLM, Lambeir A-M. Wild-type and mutant D-xylose isomerase from *Actinoplanes missouriensis*: metal-ion dissociation constants, kinetic parameters of deuterated and non-deuterated substrates and solvent-isotope effects. *Biochem J.* 1995;307:135–42.
100. Meng M, Lee C, Bagdasarian M, Zeikus JG. Switching substrate preference of thermophilic xylose isomerase from D-xylose to D-glucose by redesigning the substrate binding pocket. *Proc Natl Acad Sci.* 1991;88:4015–9.
101. Cha J, Cho Y, Whitaker RD, Carrell HL, Glusker JP, Karplus PA, et al. Perturbing the metal site in D-xylose isomerase. *J Biol Chem.* 1994;269:2687–94.
102. Allen KN, Lavie A, Glasfeld A, Tanada TN, Gerrity DP, Carlson SC, et al. Role of the divalent metal ion in sugar binding, ring opening, and isomerization by D-xylose isomerase: replacement of a catalytic metal by an amino acid. *Biochemistry.* 1994;33:1488–94.
103. Whitaker RD, Cho Y, Cha J, Carrell HL, Glusker JP, Karplus PA, et al. Probing the roles of active site residues in D-xylose isomerase. *J Biol Chem.* 1995;270:22895–906.
104. Lambeir AM, Lauwereys M, Stanssens P, Mrabet NT, Snauwaert J, Van Tilbeurgh H, et al. Protein engineering of xylose (glucose) isomerase from *Actinoplanes missouriensis*. 2. Site-directed mutagenesis of the xylose binding site. *Biochemistry.* 1992;31:5459–66.
105. Verhoeven MD, Lee M, Kamoen L, van den Broek M, Janssen DB, Daran J-MG, et al. Mutations in PMR1 stimulate xylose isomerase activity and anaerobic growth on xylose of engineered *Saccharomyces cerevisiae* by influencing manganese homeostasis. *Sci Rep.* 2017;7:46155.
106. Papapetridis I, Verhoeven MD, Wiersma SJ, Goudriaan M, van Maris AJA, Pronk JT. Laboratory evolution for forced glucose-xylose co-consumption enables identification of mutations that improve mixed-sugar fermentation by xylose-fermenting *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 2018;18:1–17.
107. Lee M, Rozeboom HJ, Keuning E, De Waal P, Janssen DB. Structure-based directed evolution improves *S. cerevisiae* growth on xylose by influencing in vivo enzyme performance. *Biotechnol Biofuels.* 2020;13:1–18.
108. Morley KL, Kazlauskas RJ. Improving enzyme properties: When are closer mutations better? *Trends Biotechnol.* 2005;23:231–7.
109. Vakulenko SB, Taibi-Tronche P, Tóth M, Massova I, Lerner SA, Mobashery S. Effects on substrate profile by mutational substitutions at positions 164 and 179 of the class A TEM_{PUC19} β -lactamase from *Escherichia coli*. *J Biol Chem.* 1999;274:23052–60.
110. van den Heuvel RHH, van den Berg WAM, Rovida S, van Berkel WJH. Laboratory-evolved vanillyl-alcohol oxidase produces natural vanillin. *J Biol Chem.* 2004;279:33492–500.
111. Xu W, Yan M, Xu L, Ding L, Quyang P. Engineering the activity of thermophilic xylose isomerase by site-directed mutation at subunit interfaces. *Enzyme Microb Technol.* 2009;44:77–83.

112. Carmelo V, Santos H, Sá-Correia I. Effect of extracellular acidification on the activity of plasma membrane ATPase and on the cytosolic and vacuolar pH of *Saccharomyces cerevisiae*. *Biochim Biophys Acta*. 1997;1325:63–70.
113. Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates . II : Inhibitors and mechanisms of inhibition mechanisms of inhibition. *Bioresour Technol*. 2000;74:25–33.
114. Elstohy MM, Chauhan VM, Markus R, Kyyaly MA, Tendler SJB, Aylott JW. Real-time measurement of the intracellular pH of yeast cells during glucose metabolism using ratiometric fluorescent nanosensors. *Nanoscale*. 2017;9:5904–11.
115. Chen WP, Anderson AW. Purification, immobilization, and some properties of glucose isomerase from *Streptomyces flavogriseus*. *Appl Environ Microbiol*. 1979;38:1111–9.
116. Matsushika A, Watanabe S, Kodaki T, Makino K, Inoue H, Murakami K, et al. Expression of protein engineered NADP⁺-dependent xylitol dehydrogenase increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol*. 2008;81:243–55.
117. Bommareddy RR, Chen Z, Rappert S, Zeng AP. A de novo NADPH generation pathway for improving lysine production of *Corynebacterium glutamicum* by rational design of the coenzyme specificity of glyceraldehyde 3-phosphate dehydrogenase. *Metab Eng*. 2014;25:30–7.
118. Arnold FH. Directed evolution: Bringing new chemistry to life. *Angew Chemie - Int Ed*. 2018;57:4143–8.
119. Porter JL, Rusli RA, Ollis DL. Directed evolution of enzymes for industrial biocatalysis. *ChemBioChem*. 2016;17:197–203.
120. Milo R. What is the total number of protein molecules per cell volume? A call to rethink some published values. *BioEssays*. 2013;35:1050–5.
121. The royal Swedish academy of science. Scientific background on the Nobel Prize in Chemistry 2018. Directed evolution of enzymes and binding proteins. 2018.
122. Chen K, Arnold FH. Enzyme engineering for nonaqueous solvents: Random mutagenesis to enhance activity of subtilisin E in polar organic media. *Nat Biotechnol*. 1991;9:1073–7.
123. Stemmer WPC. DNA shuffling by random fragmentation and reassembly: In vitro recombination for molecular evolution. *Proceeding Natl Academy Sci*. 1994;91:10747–51.
124. Wong TS, Tee kang L, Hauer B, Schwaneberg U. Sequence saturation mutagenesis (SeSaM): a novel method for directed evolution. *Nucleic Acids Res*. 2004;32:e26.
125. Crook N, Abatemarco J, Sun J, Wagner JM, Schmitz A, Alper HS. In vivo continuous evolution of genes and pathways in yeast. *Nat Commun*. 2016;7:1–14.
126. Gibson DG, Young L, Chuang RY, Venter JG, Hutchison III CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*. 2009;6:343–5.
127. Engler C, Gruetzner R, Kandzia R, Marillonnet S. Golden gate shuffling: A one-pot DNA shuffling method based on type IIs restriction enzymes. *PLoS One*. 2009;4.
128. Gai SA, Wittrup KD. Yeast surface display for protein engineering and characterization. *Curr Opin Struct Biol*. 2007;17:467–73.
129. Boder ET, Wittrup KD. Yeast surface display for directed evolution of protein expression, affinity, and stability. *Methods Enzymol*. 2000;328:430–44.
130. Liang L, Zhang J, Lin Z. Altering coenzyme specificity of *Pichia stipitis* xylose reductase by the semi-rational approach CASTing. *Microb Cell Fact*. 2007;6:1–11.
131. Pourmir A, Johannes TW. Directed evolution: Selection of the host organism. *Comput Struct Biotechnol J*. 2012;2.
132. Alberstein M, Eisenstein M, Abeliovich H. Removing allosteric feedback inhibition of tomato 4-coumarate:CoA ligase by directed evolution. *Plant J*. 2012;69:57–69.
133. Umeno D, Arnold FH. Evolution of a pathway to novel long-chain carotenoids. *J Bacteriol*. 2004;186:1531–6.
134. Atsumi S, Liao JC. Directed evolution of *Methanococcus jannaschii* citramalate synthase for biosynthesis of 1-propanol and 1-butanol by *Escherichia coli*. *Appl Environ Microbiol*. 2008;74:7802–8.
135. Li H, Schmitz O, Alper HS. Enabling glucose/xylose co-transport in yeast through the directed evolution of a sugar transporter. *Appl Microbiol Biotechnol*. 2016;100:10215–23.

136. Seike T, Kobayashi Y, Sahara T, Ohgiya S, Kamagata Y. Molecular evolutionary engineering of xylose isomerase to improve its catalytic activity and performance of micro-aerobic glucose/xylose co-fermentation in *Saccharomyces cerevisiae*. *Biotechnol Biofuels*. 2019;12:1–16.
137. Bracher JM, Martinez-Rodriguez OA, Dekker WJC, Verhoeven MD, van Maris AJA, Pronk JT. Reassessment of requirements for anaerobic xylose fermentation by engineered, non-evolved *Saccharomyces cerevisiae* strains. *FEMS Yeast Res*. 2019;19.

CHAPTER 2

Metal dependence of xylose isomerase from *Piromyces* sp. E2 explored by activity profiling and protein crystallography

Misun Lee,¹ Henriëtte J. Rozeboom,¹ Paul de Waal,² Rene M. de Jong,² Hanna M. Dudek,^{1,3} and Dick B. Janssen^{1,*}

¹Biochemical Laboratory, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

²DSM Biotechnology Center, Alexander Fleminglaan 1, 2613 AX, Delft, The Netherlands

³Present address: c-LEcta GmbH, Perlickstr. 5, 04103 Leipzig, Germany

*Corresponding author

Published in *Biochemistry*. 2017; 56: 5991-6005
(<https://pubs.acs.org/doi/10.1021/acs.biochem.7b00777>)

Author's contribution

All authors designed the study together. ML and HMD performed experiments. HJR performed the crystallization and determined the structures. ML, HJR, HMD and DBJ wrote the manuscript.

ABSTRACT

Xylose isomerase from *Piromyces* sp. E2 (PirXI) can be used to equip *Saccharomyces cerevisiae* with the capacity to ferment xylose to ethanol. The biochemical properties and structure of the enzyme have not been described even though its metal content, catalytic parameters, and expression level are critical for rapid xylose utilization. We have isolated the enzyme after high-level expression in *Escherichia coli*, analyzed the metal dependence of its catalytic properties, and determined 12 crystal structures in the presence of different metals, substrates, and substrate analogues. The activity assays revealed that various bivalent metals can activate PirXI for xylose isomerization. Among these metals, Mn^{2+} is the most favorable for catalytic activity. Furthermore, the enzyme shows the highest affinity for Mn^{2+} , which was established by measuring the activation constants (K_{act}) for different metals. Metal analysis of the purified enzyme showed that in vivo the enzyme binds a mixture of metals that is determined by metal availability as well as affinity, indicating that the native metal composition can influence activity. The crystal structures show the presence of an active site similar to that of other xylose isomerases, with a D-xylose binding site containing two tryptophans and a catalytic histidine, as well as two metal binding sites that are formed by carboxylate groups of conserved aspartates and glutamates. The binding positions and conformations of the metal coordinating residues varied slightly for different metals, which is hypothesized to contribute to the observed metal-dependence of the isomerase activity.

INTRODUCTION

Cost-effective production of second-generation bioethanol requires maximal utilization of sugars present in cellulosic biomass, because raw materials account for about one-third of the overall production cost [1]. Besides D-glucose, the most abundant monosaccharide in lignocellulose and hemicellulose is D-xylose, and fermentation of xylose along with glucose would significantly increase the total ethanol yield [2]. For such simultaneous fermentation of glucose and xylose, *Saccharomyces cerevisiae* would be a particularly attractive organism as it is an established ethanol producer that is not very vulnerable to inhibitors present in cellulose hydrolysates and shows relatively high tolerance to extracellular ethanol [1]. However, natural strains of *S. cerevisiae* do not metabolize xylose, which is due to its inability to convert D-xylose to D-xylulose, an aldose to ketose isomerization reaction. Since D-xylulose can be metabolized, research has been devoted to engineer yeast variants that express a heterologous xylose isomerase for catalyzing this reaction [3–6]. Alternatively, xylose isomerization can be achieved by incorporating both a xylose reductase and a xylitol dehydrogenase [7] although this is not preferred since it could cause a cellular cofactor imbalance [8,9]. Because xylose isomerase only requires metal cofactors, it offers the most attractive solution. Furthermore, the use of an isomerase rather than dehydrogenases prevents the formation of xylitol, which may emerge as an unwanted side product [10].

Xylose isomerases and related glucose isomerases have been studied for decades because of their application as glucose isomerases in the production of high-fructose corn syrups from starch hydrolysates [11]. The use of the enzymes in ethanol producing yeasts has been more recent. Isomerases from different bacterial sources including *E. coli*, *A. missouriensis*, *S. rubiginosus*, *B. subtilis*, *C. thermosulfurogenes* have been tested but functional expression in *S. cerevisiae* appeared problematic [12–15]. Xylose isomerase from *Thermus thermophilus* was functionally expressed in *S. cerevisiae*, but the enzyme from this thermophilic organism showed very low activity at 30 °C [16].

The first eukaryotic XI gene that could be well expressed in active form in *S. cerevisiae* was obtained from the anaerobic fungus *Piromyces* sp. E2 (PirXI) and its expression in *S. cerevisiae* enabled xylose fermentation to ethanol [5,17,18]. Although later a few xylose isomerases from other sources were also expressed in *S. cerevisiae* [3,4,6,19,20], xylose-based growth rates and ethanol production levels do not exceed those found for the *S. cerevisiae* expressing XI from *Piromyces* sp. E2 [18]. A further improved *S. cerevisiae* strain equipped with PirXI indeed showed high ethanol production yields, reaching almost the theoretical level, and the use of xylose isomerase appears preferable over the combination

of a xylose reductase and a xylitol dehydrogenase allowing isomerization via xylitol [10,20].

In spite of these promising achievements, there is significant room for improving anaerobic metabolism of xylose. Engineering of the strain by overexpressing the non-oxidative pentose phosphate pathway enzymes may enhance the growth rate [20,21]. Evolutionary optimization can also improve the rate of xylose consumption [22,23]. In such improved *S. cerevisiae* strains, a very high expression level of PirXI is still required for growth on xylose, especially for anaerobic metabolism, and in such evolved strains PirXI may be the major intracellular protein (unpublished data). This must be an energetic burden to the cells, and also indicates that xylose isomerization still is the rate-limiting step in engineered strains that have lower expression levels of xylose isomerase. No detectable accumulation of the product xylulose in an anaerobic culture of an engineered and PirXI-expressing strain growing on xylose was found, further suggesting that PirXI activity is limiting xylose metabolism [21]. Therefore, engineering of PirXI to enhance its activity will further improve xylose utilization. A directed evolution study using error-prone PCR showed that an engineered xylose isomerase could enhance the growth of *S. cerevisiae* on xylose [24]. Further progress and more focused engineering of the enzyme are hampered by the lack of a description of the biochemical properties of PirXI and the absence of structural information.

Xylose isomerases are tetrameric enzymes which require two bivalent metals for catalyzing isomerization of the aldose D-xylose to the ketose D-xylulose (Figure 1). One of them (M_1) is often described as the structural metal as it is involved in the proper binding of the substrate and the other (M_2) as the catalytic metal as it is critical for the isomerization reaction [30]. The activity of xylose isomerase is reported to be influenced by the type of metal that is bound. For example, Mg^{2+} , Co^{2+} and Mn^{2+} are known to be activators and Ni^{2+} , Cu^{2+} , Zn^{2+} , Ca^{2+} and Fe^{2+} mostly act as inhibitors [26,29,31,32]. Although the active sites of xylose isomerases are highly conserved, the metal preferences of the enzymes vary. Some XIs seems to be most active with Mg^{2+} while others show the highest activity with Mn^{2+} [31,33,34]. The importance of metal dependence for in vivo conversion of D-xylose was recently discovered in a study showing that mutations influencing metal homeostasis through inactivation of the PMR1 gene increase the relative Mn^{2+} content of *S. cerevisiae* cells and of the expressed PirXI [35].

2

Figure 1. Proposed mechanism of D-xylose conversion to D-xylulose [25,26]. The close ring form of xylose is linearized by the His102/Asp105 pair with Nε2 acting as a hydrogen bond donor to O5 of the sugar ring. The proton abstraction at O1, which is required for the ring opening, is proposed to occur by the neutral form of His102 [27–29] or by a nearby lysin residue Lys342 [26]. Upon linearization of the sugar, the catalytic metal moves from position M_1 to position M_2 where it coordinates with O1 and O2 of the sugar. O1 is protonated by the M_2 -bound water molecule and O2 is deprotonated by the nearby Asp340 [26]. Subsequently, a hydride shifts from C2 to C1 and D-xylulose is formed by ring closure.

XIs can be divided into two structural types, i.e. Class I and Class II enzymes. The major difference between the two classes is an N-terminal extension in Class II xylose isomerases. From sequence analysis, the PirXI enzyme explored here appears to be a Class II xylose isomerase. Currently, only a few Class II

XI structures are available, [36] and these have no bound substrates, unlike structures for Class I XIs which have been obtained with different ligands.

In this study, we describe the catalytic properties of PirXI expressed in *E. coli*. We also examined the metal specificity of the enzyme by measuring catalytic activities and affinities with a range of different metals that might be present in cytosol of *S. cerevisiae* during the fermentation process. Furthermore, we report several structures of the Class II XI from *Piromyces*, determined by X-ray crystallography, with various combinations of metals (Mg^{2+} , Mn^{2+} , Co^{2+} , Fe^{2+} , Ca^{2+} , Ni^{2+} , Cd^{2+}) and substrate (xylose), product (xylulose) or inhibitor (xylitol, sorbitol). The results indicate that small differences in metal-protein interactions influence the position and reactivity of bound metals.

RESULTS

Expression of PirXI in *E. coli* and enzyme isolation

To obtain overexpression of the *Piromyces* sp. E2 xylose isomerase (PirXI) in *E. coli*, a codon-optimized synthetic *xylA* gene was cloned into the pBAD vector and transformed to *E. coli* Top10. Overnight cultivation at 37 °C with induction of expression of PirXI by 0.2 % arabinose resulted in high levels of soluble PirXI. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis showed that PirXI is the predominant protein in cell lysates, accounting for >50 % of the total soluble protein. This allowed purification by a single step of ion-exchange chromatography, which after desalting routinely yielded 300 – 500 mg purified xylose isomerase per liter of culture. From this material, apoenzyme was prepared by treatment with EDTA for studying the metal-dependence of the isomerase activity. SDS-PAGE showed that the size of the protein monomer is around 50 kDa, which is in good agreement with the calculated size of the protein, 49.5 kDa. Analytical size exclusion chromatography revealed a molecular weight of ~200 kDa for the native PirXI, showing that the enzyme is a tetramer.

Metal dependence of PirXI activity

Because xylose and glucose isomerases require bivalent metals for activity [37] and the type of metal that is bound may influence activity [31], we examined the effect of different metal ions on the activity of PirXI. Kinetic parameters of apo-PirXI in the presence of Mg^{2+} , Mn^{2+} , Co^{2+} or Ca^{2+} were measured using the SDH-coupled assay as described in the Materials and Methods. PirXI showed good activity in the presence of Mg^{2+} , Co^{2+} and Mn^{2+} (Table 1). Among these metals, the highest activity (k_{cat}) of the enzyme was obtained with Mn^{2+} , followed by Mg^{2+} and Co^{2+} . The substrate affinity with xylose (K_{M}) on the other hand was best in the presence of

Co^{2+} , which gave a K_M of 0.3 mM and also the highest catalytic efficiency (k_{cat}/K_M). In the presence of Mn^{2+} or Mg^{2+} the K_M of the enzyme was 15-20-fold higher. This metal preference of PirXI is similar to what was described for other Class II XIs, which were reported to be most active with Mn^{2+} , whereas the Class I enzymes are most active with Mg^{2+} [31,33,34]. When Ca^{2+} was added as the metal cofactor of PirXI, the enzyme showed activity although it was lower than with the other metals mentioned above. A striking observation was the extremely low xylose affinity ($K_M \sim 2 \text{ M}$) in the presence of Ca^{2+} , resulting in a poor catalytic efficiency ($k_{\text{cat}}/K_M = 0.2 \text{ s}^{-1} \cdot \text{M}^{-1}$). The low substrate affinity suggests that Ca^{2+} -containing enzyme will provide a negligible contribution to in vivo conversion of xylose by PirXI.

Further assays for determining the metal-dependence of PirXI activity were performed using an HPLC assay for measuring xylulose formation because the SDH-coupled assay is not compatible with the presence of Zn^{2+} , Fe^{2+} , Cd^{2+} , Ni^{2+} and Al^{3+} as these metals inhibit SDH activity. Furthermore, in the case of Fe^{2+} -dependent activity, the reactions were performed under reduced oxygen conditions to prevent oxidation of Fe^{2+} to Fe^{3+} . In these experiments, we measured initial conversion rates (units per milligram) of xylose that was added to a final concentration of 200 mM. PirXI showed activity in D-xylose isomerization in the presence of Zn^{2+} , Fe^{2+} and Cd^{2+} . The rate of xylose conversion with Zn^{2+} was almost half of that measured with Mg^{2+} . Furthermore, Fe^{2+} gave moderate activity compared to the other metals. Unexpectedly, the enzyme also showed some activity in the presence of Cd^{2+} which was reported to be an inhibitor of another xylose isomerase [26]. However, the activity (0.2 unit/mg) was very low. No detectable amount of xylulose ($< 50 \mu\text{M}$) was found with Ni^{2+} or Al^{3+} as the metal cofactor upon measurement after incubation of the reaction mixture for 2 h.

Metal affinity of PirXI

The above results show that PirXI can be activated by a range of different bivalent metal ions. Obviously, the activity in vivo will also be determined by metal affinity and metal availability in the cytoplasmic environment. Therefore, we investigated the activity of PirXI on xylose in the presence of different concentrations of divalent metals, and determined half-saturation constants for activation of the enzyme [K_{act} values, (Table 1)]. Of the metals tested (Mg^{2+} , Mn^{2+} , Co^{2+} , Ca^{2+}) the highest affinity was found with Mn^{2+} . The enzyme also showed a high affinity towards cobalt, but the affinities for Mg^{2+} and Ca^{2+} were 70- and 100-fold lower compared to that of Mn^{2+} , respectively.

Analysis by inductively-coupled plasma atomic emission spectroscopy showed that the main divalent metal in native PirXI isolated from *E. coli* is Mg^{2+} , which accounted for approximately half of the total metal content (Table 2). Ca^{2+} and Fe^{2+} were also detected, whereas the enzyme contained only a few percent (moles

per mole) of Mn^{2+} and Zn^{2+} . This indicates that Mg^{2+} would strongly influence the in vivo enzyme activity in *E. coli*, while Mn^{2+} ions which are most beneficial for activity would have little influence. In agreement with this, the native enzyme isolated from *E. coli* showed activity similar to that of enzyme reconstituted with Mg^{2+} or even slightly lower activity, probably due to the effect of Ca^{2+} and Fe^{2+} . The metal composition and the activity of the enzyme are slightly different from one batch of isolated enzyme to another (WT1 – 3). The data also suggest that intracellular metal availability influences the metal composition of the enzyme ($\text{Mg} > \text{Ca} > \text{Mn} > \text{Co}$) as much as the relative affinities ($\text{Mn} > \text{Co} > \text{Mg} > \text{Ca}$). Accordingly, in vivo, the enzyme may have suboptimal activity due to the level and type of metal binding. Recently, Verhoeven et al. [35] showed that the increase in the intracellular Mn^{2+} level improved growth of *S. cerevisiae* on xylose by enhancing PirXI activity.

pH dependence of PirXI activity

The cytosolic pH of *S. cerevisiae* during the fermentation process is below 7.0, possibly due to the influence of acidic lignocellulose hydrolysate [38,39]. We measured the activity of PirXI at different pH values ranging from 5.5 to 7.5 in the presence Mg^{2+} , Mn^{2+} , Ca^{2+} or Co^{2+} (Figure 2). Because of the high K_M of the enzyme in the presence of Ca^{2+} , the activity was measured at higher xylose concentration, 1 M instead of 150 mM. With Mg^{2+} , Mn^{2+} and Ca^{2+} the results show a similar broad pH profile, with the highest activity at pH 7.5, and a significantly reduced activity at pH 6.5. The lower activity at low pH was more pronounced for Mg^{2+} , the quantitatively most important cation in the non-reconstituted enzyme. The activity of the Co^{2+} -reconstituted PirXI was much less dependent on the pH in the range of 5.5 - 7.5.

Table 1. Metal-dependence of PirXI activity ^a.

substrate	Cation	V (200 mM) (s ⁻¹)	k_{cat} (s ⁻¹)	K_M (mM)	k_{cat}/K_M (s ⁻¹ ·M ⁻¹)	K_{act} ^c (μM)
xylose	Mg ²⁺	1.95 ± 0.12 ^b	2.0 ± 0.1	7.5 ± 1.2	270	98.9 ± 4.3
xylose	Mn ²⁺	4.4 ± 0.42 ^b	4.5 ± 0.3	4.3 ± 0.1	1100	1.45 ± 0.28
xylose	Ca ²⁺	0.075 ± 0.017 ^b	0.8 ± 0.1	1930 ± 70	0.2	159 ± 10
xylose	Co ²⁺	1.0 ± 0.14 ^b	1.0 ± 0.1	0.3 ± 0.04	3300	6.5 ± 1.21
glucose	Mg ²⁺	0.032 ± 0.005 ^b	0.1 ± 0.01	430 ± 10	0.2	-
xylose	Zn ²⁺	1.1 ± 0.1	-	-	-	-
xylose	Fe ²⁺	1.6 ± 0.2	-	-	-	-
xylose	Cd ²⁺	0.2 ± 0.01	-	-	-	-
xylose	Ni ²⁺	n.d. ^d	-	-	-	-
xylose	Al ³⁺	n.d. ^d	-	-	-	-

^a The values represent averages and the mean deviation calculated from two or three replicates.

^b Initial rates at 200 mM substrate were calculated from steady state parameters.

^c K_{act} is defined as the metal concentration at which the enzyme activity is half the V_{max} .

^d No activity detected with the HPLC assay described in the Materials and Methods.

Table 2. Metal content of PirXI purified from *E. coli* ^a.

	mol metal/mol PirXI					nXI activity	
	Ca	Fe	Mg	Zn	Co	Mn	k_{obs} (s ⁻¹)
WT1	0.59	0.35	1	0.03	< ^b	0.01	1.9 ± 0.1
WT2	0.7	0.4	0.93	0.03	< ^b	0.01	1.7 ± 0.03
WT3	0.56	0.36	1.28	0.02	< ^b	0.02	2.1 ± 0.06

^a Metal content of PirXI purified from *E. coli* was measured, and moles of metal per mole of the enzyme monomer were calculated. WT1 - WT3 represent the enzyme purified from three independent cultures. The activities of the holoenzymes were measured with 150 mM xylose. The indicated k_{obs} values are averages and mean deviations of duplicate measurements.

^b <, Below detection limit (< 0.002 mol/mol PirXI).

Figure 2. pH-activity profile of PirXI in the presence of different metal cofactors. Specific activities ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) of PirXI on xylose (150 mM for Mg^{2+} , Mn^{2+} and Co^{2+} and 1 M for Ca^{2+}) were measured at various pH in the presence of different metal ion cofactors. The error bars indicate the mean deviation of duplicate measurements.

Crystal structure of PirXI

PirXI isolated from *E. coli* readily crystallized from PEG3350 at pH 7. The structure was determined by using X-ray diffraction and molecular replacement, and was refined against 1.8 Å resolution diffraction data to an R-factor of 0.116 ($R_{\text{free}} = 0.148$) with good stereochemistry (Table 4, Figure 3A). The P1 unit cell contains four monomers that form a tetramer of $92 \times 77 \times 77$ Å, having non-crystallographic 222 symmetry. The four subunits have root mean square deviation values of 0.11 - 0.16 Å on Ca atoms. Each PirXI subunit comprises two structural domains, a larger catalytic domain (residues 1 to 377) with a distorted (β/α)8-barrel (TIM-barrel) fold, and a C-terminal domain (residues 378-437) forming an extended tail containing three α -helices.

With other Class II xylose isomerases having PDB codes 4XKM (*Bacteroides thetaiotaomicron*) [36], 1A0C (*Thermoanaerobacterium thermosulfurigenes*), 1A0D (*Bacillus stearothermophilus*) and 1A0E (*Thermotoga neapolitana*) rmsd values are 0.4 Å on 435 Ca atoms, 1.2 Å on 430 Ca atoms, 1.1 Å on 425 Ca atoms and 1.2 Å on 422 Ca atoms, respectively, while with the well-studied *Streptomyces rubiginosus* Class I enzyme the rmsd is 2.0 Å on 352 Ca atoms and 27% sequence identity. Major differences between the Class I *S. rubiginosus* XI and the Class II PirXI are the presence in the latter enzyme of an N-terminal extension of ~33 residues, an N-terminal addition of α -helix 1 and a larger loop before α -helix 2. Furthermore, the C-terminal regions deviate the most with low identity, except for the ultimate α -helix 12 (Figure S1).

A pair of subunits forms the 'yin-yang' dimer with rotational symmetry by contacts of the catalytic domain of subunit A interacting with the C-terminal tail of subunit B, while the core of subunit B is interacting with the C-terminal tail of

A (Figure 3B). The other dimer pairs are called the 'butterfly' dimer (Figure 3C) and the 'diagonal' dimer (Figure 3D). The tetramer has a surface area of $53920 \text{ \AA}^2$ of which $36840 \text{ \AA}^2$ is buried. Each subunit has a total solvent accessible area of $21100 \text{ \AA}^2$ of which $8110 \text{ \AA}^2$ is buried. The interactions in the yin-yang and diagonal dimers are mainly present between the N- and C-terminal tails of the subunits, while the butterfly dimer has interactions between the cores of the subunits.

The sizes of the interface areas are similar in other class II xylose isomerases of which the structure is determined (PDB entries 4XKM, 1A0C, 1A0D and 1A0E) (Table 3). However, the xylose isomerase from *S. rubiginosus*, the most studied Class I XI in the PDB database, has a larger interface for the yin-yang dimer ($4670 \text{ \AA}^2$) and a smaller interface for the diagonal dimer ($1460 \text{ \AA}^2$) as it is missing the N-terminal extension of the Class II enzymes. Class II enzymes, having larger dimer interfaces and interactions to the other subunit, should be regarded as tetramers unlike Class I enzymes which are usually described as a dimer of dimers.[36]

Figure 3. Overall crystal structure of PirXI. PirXI is displayed in cartoon format and subunits B - D are colored in pink, cyan and orange, respectively. The catalytic domain of subunit A is colored in green, the N-terminal extension in dark blue and the C-terminal domain in red. Ions in M1 position are depicted in orange. (A) Tetramer and possible dimer pairs in (B) yin-yang dimer (subunits A and B), (C) butterfly dimer (subunits A and C) and (D) diagonal dimer (subunits A and D).

Table 3. Subunit organization of PirXI.

	subunits	Interface area ($\text{\AA}^2$)	complexation significance score	no. of H-bonds/salt bridges
tetramer	ABCD			
yin-yang	AB and CD	4050	0.505	51 / 28
butterfly	AC and BD	1395	0.210	22 / 12
diagonal	AD and BC	1823	0.233	27 / 14

Active site structure

The active sites of the PirXI subunits are located on the C-terminal end of the (β/α)8-barrel of the core domain. The active sites are 36 Å in yin-yang, 31 Å in butterfly and 39 Å apart in diagonal dimers. Residues from the loops following the β -strands shape the active site. The active site pocket is lined by the side chain indole groups from Trp50 and Trp189 which are situated 8.8 Å away from each other forming a cassette for sugar binding (Figure 4A, B). Other hydrophobic residues surrounding the substrate binding site are Phe61 from another subunit of the butterfly dimer, Phe146 and Trp140.

In the crystals obtained with native PirXI isolated from *E. coli*, only the position of the structural metal (M_1) in the active site is occupied. Residues Glu233, Glu269, Asp297 and Asp340 bind M_1 with octahedral geometry (Figure 4A). The nature of the metal ion was unclear from the structure as a mixture of different metal may bind. The observed metal-donor distances were 2.1-2.2 Å. The optimal distance for Ca^{2+} is 2.36 Å, for Mg^{2+} 2.26 Å and for Fe^{2+} it is 2.01 Å to monodentate Asp or Glu [40]. An anomalous difference map showed a peak at the M_1 position indicating a bound Ca^{2+} or Fe^{2+} ion. Refining with a Ca^{2+} ion with full occupancy showed residual $F_o - F_c$ density and distances that were too short, while refining with 100% Fe^{2+} showed deficit density and distances that were too long. Therefore, M_1 was refined to occupancies of 0.4, 0.35 and 0.25 for Ca, Mg and Fe, respectively, which gave a flat F_o - F_c map after refinement. The ambiguous metal ion content has also been observed in native crystals of *S. rubiginosus* with 0.6 equiv of Mn, < 0.6 equiv of Mg and < 0.1 equiv of Co per monomer [41]. In this PirXI structure, a glycerol molecule from the cryoprotectant is bound in the active site to the metal ion with a Me-O2 distance of 2.3 Å and a Me-O3 distance of 2.4 Å. Glycerol, acting as a sugar mimic, is often found in active sites of carbohydrate-converting enzymes [42].

The crystal structure obtained with EDTA-treated XI is highly similar to the native structure with an rmsd of 0.1 Å. As expected, this apo enzyme does not contain any metals in the active site, but a glycerol molecule is bound as in the native enzyme. A water molecule is located at position M_1 ; consequently, O3 of the glycerol has shifted 1.4 Å toward His272 (Figure 4B). To determine if and how other metals bind to PirXI, crystal soaks were performed. Several structures of binary and ternary complexes of PirXI with metals, substrates and inhibitors were determined (Table 4 and Table S1).

Figure 4. Active site of native XI (A) and apo-XI (B). The residues forming the active site are shown as green sticks. The His102-Asp105 pair, F61' from another subunit and the glycerol molecule are colored salmon, cyan and yellow, respectively. Other molecules are colored as follow: water molecules - red, Ca^{2+} - green, Mg^{2+} - light green and Fe^{2+} - brown balls. Fe^{2+} is only slightly visible due to the overlapped position with Mg^{2+} .

Mg, xylose and xylitol binding

In electron density maps of crystals of apoenzyme soaked with magnesium and glycerol or with magnesium and the competitive inhibitor (or substrate analogue) xylitol [31], only a single Mg^{2+} ion at the M_1 site was observed (Figure 5A, B). There was no $2\text{Fo} - \text{Fc}$ density present for the Mg^{2+} ion at the M_2 position, despite the presence of Mg^{2+} in the soak solution at 10 mM (Figure S2). Glycerol was bound similar as in the native PirXI crystals. The position of xylitol was overlapping with that of the glycerol molecule in the Mg-glycerol structure, but the larger xylitol molecule bound deeper in the active site toward the putative M_2 position. Xylitol was bound with its O1 to NE2 of His272 and a water molecule, O2 to Glu233, Glu269, Asp340 and M_1 (Mg^{2+}), O3 to Asp340 and a water molecule, O4 to Glu233 (bidentate), Asp297, Asp340 and M_1 , and O5 to NE2 of His102 and a water molecule (Figure 5B). His102 is likely involved in pyranose ring opening [26,43].

Only when a soak was performed with magnesium and the substrate D-xylose the second metal site (M_2) was fully occupied with elongated (elliptical) electron density, showing two distinct positions for the metal, with approximately 50/50 occupancy, at distances of 3.8 Å (M_{2b}) and 5.5 Å (M_{2a}) from M_1 (Figure 5C). It has been elucidated by structural studies, including neutron diffraction, that the hydride shift of the isomerization reaction is mediated by the metal at M_2 [25–27,44].

In the Mg-xylose structure, the substrate is in its open form in the same conformation as xylitol, indicating ring opening has been completed. At the current resolution of the datasets (1.9 Å) it is not possible to distinguish between the linear forms of substrate and product by electron density, and probably a

mixture of both is bound. The open-chain forms are similar in shape, and differences exist at the C1 and C2 carbons, having sp^2 or sp^3 hybridization. Because all soaks were performed with a high concentration (2.2 M) of substrate and the rate-limiting step in the catalytic cycle was proposed to be the hydride-shift reaction [29,45], we have modeled the substrate used in the soak. The xylose stacks on the ring system of Trp189, which probably ensures a flat conformation of the substrate (Figure 5C). The M_2b metal has interactions with O1 and O2 of the xylose, NE2 of His272, Glu269 (bidentate) and a water molecule. The metal at M_2b is in the catalytically more favorable position as the interactions with O1 and O2 likely stabilize the transition state while hydride is shifting from C2 to C1. These interactions with xylose O1 and O2 are absent when the metal is at position M_2a . It was therefore suggested that the metal moves from the M_2a to the M_2b position during the reaction [25]. Mg^{2+} at M_2a is at 2.0 Å distance to M_2b and has interactions with NE2 of His272, OE2 of Glu269, OD1 of Asp310, Asp308 (bidentate) in one of its conformations, and the water molecule. In another conformation Asp308 and O1 of the xylose have interactions with NZ of Lys235, which has moved closer into the active site compared to the Mg-xylitol structure (Figure 5C). Glu238, having an H-bond with the backbone amide of Asp308, also has achieved a double conformation. Hence, its structural neighbor, the side chain of Phe61 from the adjacent subunit, shifts by 1.4 Å toward the substrate. The double side-chain conformations of Asp308 and Glu238, obtained upon binding of the catalytic Mg^{2+} ion and xylose, are also observed in *S. rubiginosus* XI with two Mg^{2+} atoms and xylitol/xylose in the active site as well as *Arthrobacter* XI [41,46]. The conformational changes of the residues appear to be the consequence of the movement of the metal from M_2b to M_2a as it causes the aspartate to lose its coordination with the metal and interact with the nearby glutamate.

Figure 5. Active site of Mg-GOL-XI (A), Mg-XYL-XI (B), and Mg-XLS-XI (C). The residues involved in metal binding are shown as green sticks. His102 and F61' from another subunit are colored salmon and magenta, respectively. The glycerol, xylitol and xylose molecules are colored yellow. Water molecules are shown as red spheres and Mg^{2+} ions as light green spheres.

XI-Cd and XI-Mn-sorbitol

In the Cd-xylose complex of PirXI, bound substrates are in a linear form (XLS) in subunit A and subunit B, while in subunit C and subunit D of the yin-yang dimer the substrates are in the pyranose ring form (Figure 6A, B). This mixture of open and closed structures was only observed for the PirXI-Cd structure. For PirXI structures soaked with other metals, the electron density map showed only the linear form of the substrate in the active site (Figure S2B, C, D). The active site structure of PirXI-Cd with the xylose in a linear form is very similar to the structure of Mg-xylose. Cd^{2+} at M_2 is observed at two distinct positions with 1.4 Å distance between M_{2a} and M_{2b} .

The electron density map in the active centers of subunits C and D shows α -D-xylopyranose (XYS) which is coordinated to M_1 with O3 and O4, whereas the endocyclic O5 is hydrogen bonded to His102. O4 of α -D-xylopyranose is also coordinated to M_{2b} at a distance of 2.7 Å (Figure 6B).

Figure 6. Active site of Cd-linear-XLS-XI (A), and Cd-cyclic-XLS-XI (B). The residues involved in the metal binding sites are shown as green sticks and His102 is colored salmon. The xylose molecules are colored yellow. Cd^{2+} ions are depicted as light orange spheres and interactions between the metal ions and the hydroxyl group of the xylose are shown as dashed lines. The difference electron density, $F_o - F_c$ contoured at the 2σ level for the sugars is shown.

The crystal structure of XI-Mn soaked with sorbitol, a C6 sugar analogue with the same stereo configuration as D-glucose, shows two singly occupied metal binding sites (Figure 7). The binding of the sorbitol is quite different from the binding of xylitol in the Mg-xylitol structure. O1 of sorbitol has interacts with His102 (2.6 Å) and a water molecule, and O2 interacts with Glu233 (bidentate), Asp297 and M_1 - Mn^{2+} . O3 is ligated by Glu233, Asp340 and M_1 , and O4 is ligated by Asp340 (bidentate) and Ne of Trp50. O5 and O6 have interactions with water molecules. Interactions with M_2 occur via water molecules. The binding mode of sorbitol is also quite different from that in class I XI from *A. missouriensis* complexed with Co^{2+} and sorbitol (Figure 7) and from *S. rubiginosus* complexed with Ni^{2+} and sorbitol.[47,48] In class II enzymes, the bulkier Trp140 replaces a Met (Figure S1) in the rear side of the active site. Therefore, steric hindrance was expected in the

active site of class II XI as the C6 atom of sorbitol would be too close to Trp140. In PirXI, the sorbitol molecule has shifted by 2.3 Å toward the entrance of the active site. Assuming that its C6 analogue binds in a similar manner, it indicates that glucose would bind to the active site in a less productive mode.

Figure 7. Overlay of the active sites of Mn-SOR-XI and XI from *A. missouriensis* complexed with Co^{2+} and sorbitol (PDB entry 2XIN). The residues involved in the metal binding sites are displayed as green sticks. His102 and the sorbitol molecule are colored salmon and yellow, respectively. The residues and sorbitol from 2XIN are shown in gray. Mn^{2+} and Co^{2+} ions are depicted as purple and pink spheres, respectively. Interactions between the metal ions and hydroxyls of the sorbitol are shown as dashed lines.

XI-Ni, XI-Fe and XI-Ca

The XI-Fe structure contains two iron atoms in the active site at 1.9 - 2.2 Å to the surrounding ligands (Figure S3A). No glycerol molecules could be observed in the electron density which could be caused by the lower resolution of the data set. The M_2 Fe^{2+} is positioned at the M_2b location at 2.4 Å from His272. The Fe^{2+} atoms are positioned at 3.8 Å distance from each other.

A soak with xylose of a Ni^{2+} grown XI crystal reveals xylose in its linear form in the active site (Figure S3B). Its position superimposes well on the Mg-xylose structure. The Ni^{2+} cation at the M_2 position has two distinct locations (M_2a and M_2b), as in the Mg-xylose structure. However, Asp308 does not have the double conformation as observed in the Mg-xylose structure.

A soak with Ca^{2+} resulted in both M_1 and M_2 occupied (Figure 8A). The M_2 is bound in a distorted octahedral geometry by His272 at 3.4 Å, Glu269 at 2.3 Å, Asp308 at 2.2 Å, Asp310 at 2.4 Å and two water molecules. To the best of our knowledge, in class I XIs the M_1 site was never occupied by a Ca^{2+} ion. At M_2 , other Class I XIs bound Ca^{2+} at the M_2a position, similar to what we observed in the PirXI- Ca^{2+} -xylose structure but the equivalent residue of Asp308 binds the M_2 in a

bidentate manner in other enzymes [49]. The large size of M_2 - Ca^{2+} shifts Asp308 and Asp310 slightly out and the O1 position of the linear xylose by 1.5 Å towards Phe'61. This leaves the substrate in an unfavorable position for isomerization.

Figure 8. Overlay of the active sites of Mn-XLS-XI and Ca-XLS-XI (A) and the active site of Co-XUL-XI (B). In panel A, the residues involved in calcium binding are shown as green sticks. The bound manganese is colored pink. The xylose molecule in the Ca^{2+} structure is colored forest green and in the Mn^{2+} structure magenta. In panel B, the residues involved in the metal binding sites are shown as green sticks. His102 is colored salmon. The xylose molecules are colored yellow. Co^{2+} ions are colored pink. Interactions between the metal ions and the ligand amino acids and hydroxyl of the xylose or xylulose are shown as dashed lines (green for Ca^{2+} and black for Mn^{2+} in panel A).

XI-Mn, XI-Co, and XI substrate/product

The PirXI structure with two manganese ions and the substrate xylose well represents the most active form of the enzyme (Figure 8A). The Mn^{2+} at M_1 has its usual octahedral coordination while M_2 has full occupancy at the catalytic M_2b position (no occupation of the M_2a site). The M_1 and M_2b metal binding sites are at 4.1 Å distance from each other. M_2b is coordinated by His272 at 2.4 Å, Glu269 at 2.3 and 2.9 Å, Asp308 at 2.0 Å, Asp310 at 3.2 Å, and O1 of xylose at 2.8 Å and O2 at 2.7 Å. The coordination is highly similar to that observed in the *A. missouriensis* XI (9XIM) mutant E186Q ternary complex with two Mn^{2+} atoms and a linear xylose [47]. It is interesting that in the PirXI structure with the highest activity, which is the Mn^{2+} -dependent activity (Table 1), only the M_1 and M_2b sites are occupied, not M_2a .

Xylulose was soaked in a Co^{2+} cocrystallized crystal and cryoprotected with 2 M proline. The structure showed that the M_2 - Co^{2+} ion occupies 2 positions (M_2a and M_2b) with 1.8 Å distance (Figure 8B). Furthermore, similar conformational changes for Asp308, Asp310 and Phe'61 are observed as in the Mg^{2+} -xylose structure. In this structure, we modeled D-xylulose as it was present at a concentration of ~ 0.25 M in the soaking solution. The position is similar to that of the xylulose found in the crystal structure of *S. rubiginosus* (1XII) [50] and in the neutron diffraction structure of *S. rubiginosus* (3XWH) [43].

DISCUSSION

An efficient xylose isomerase that is well expressed in active form in *S. cerevisiae* is important for the development of xylose-fermenting yeast strains. The *Piromyces* sp. E2 xylose isomerase has emerged as an attractive enzyme for this purpose [5]. Our recent work indicates that metal incorporation is critical for in vivo activity, as suggested by the effect of mutations influencing manganese homeostasis on XI activity and anaerobic xylose metabolism [35]. The current work corroborates this by exploring the metal-dependence of catalytic performance and by examining crystal structures of the enzyme soaked with different metals, substrates and substrate analogs.

Studying metal dependence of the catalytic activity, we observed that xylose isomerization of PirXI can be activated by various bivalent metals but the level of activities is dependent on the metals. The enzyme was not activated by trivalent metal such as Al^{3+} . Previous structural studies of XI from *Arthrobacter* showed that binding of the metal at M_2 and substrate binding were disturbed when the enzyme was soaked with Al^{3+} and xylose or fructose [27,51]. Such a dependence of activity on the type of the metal that is bound was also observed in previous studies [31,33,34]. It seems that the class I and the class II xylose isomerase have different metal preferences as they mostly show the best activity with Mg^{2+} and Mn^{2+} metal cofactor, respectively. As a class II xylose isomerase, PirXI indeed also showed the highest activity with Mn^{2+} . Here, we explore the metal dependence of the enzyme by analyzing the structural differences of PirXI reconstituted with different ligands (metals/substrate or analogues) and interpreting the effects in view of the proposed catalytic mechanism of xylose isomerases.

The isomerase mechanism includes at least three chemical steps, accompanied and enabled by proton transfer reactions: ring opening of the substrate, hydride transfer from C2 to C1 accompanied by proton transfer from O2 to O1, and ring closure (Figure 1). Structural analysis of xylose isomerase from *S. rubiginosus* showed that the reaction involves movement of one of the metals (M_2 , the catalytic metal) and rotation of some of the active site residues such as Asp255 and Lys289 which correspond to Asp308 and Lys342 in PirXI [26].

The His102-Asp105 pair, which is conserved throughout xylose isomerase sequences, is involved in ring-opening of the substrate which is bound to M_1 [26]. This ring-opening step seems to be dependent on the metals that are bound to the enzyme. In the structure of PirXI with Cd^{2+} and xylose, the substrate is found both in cyclic and linear form (Figure 6A, B). This indicates a slow ring-opening that led to the very low enzyme activity in the presence of Cd^{2+} . For many xylose isomerases Cd^{2+} is reported to inhibit the enzyme reaction, probably by preventing the ring-opening of the substrates as the structure with this metal shows only the cyclic form of the substrate [26].

After linearization of the substrate M_2a which is coordinated with three aspartates, a histidine and a water molecule shifts to M_2b where it now coordinates O1 and O2 of the sugar and must therefore be in the catalytically competent position [52]. The mobility of the M_2 metal is supposed to aid the reaction after ring-opening to facilitate isomerization in its linear form [26,33]. A structure of the catalytically most competent enzyme (Mn^{2+} and xylose bound) revealed that Mn^{2+} occupies only the M_2b position of the two conformations at the M_2 site (Figure 8A). This might indicate that the rapid or more complete shift of the metal from M_2a to M_2b contributed to the higher activity of the enzyme in the presence of Mn^{2+} compared to the other metals. The k_{cat} of PirXI- Mg^{2+} is half that of PirXI- Mn^{2+} and in the enzyme structure with Mg^{2+} the metal is observed in both the M_2a and M_2b positions. In the structure of PirXI- Ca^{2+} , the M_2 metal is found at only the M_2a position (Figure 8A). The large size of Ca^{2+} could hamper its movement which may be one of the causes of the low reaction rate of PirXI with this metal.

Variations in transition state stabilization of the actual hydride transfer steps among the different metals bound in the M_2b site may also contribute to the observed differences in activity. Movement of the metal at position M_2 does not necessarily mean that the metal can activate the enzyme. Although Ni^{2+} was found at both M_2 positions, the enzyme showed no detectable activity (Figure S3B). The presence of Ni^{2+} at two distinctive M_2 positions was also observed in the structure of *S. rubiginosus* XI which is also inactive with the metal [53]. After the ring opening of the substrate, Ni^{2+} may be incapable of inducing the polarization of the catalytic water necessary to proceed with isomerization. This inhibiting effect of Ni^{2+} has been explained by the preference for square planar coordination and its high electron affinity [26].

PirXI is also active with Fe^{2+} as the metal cofactor and the specific activity was ~70 % that of PirXI- Mg^{2+} . The PirXI- Fe^{2+} structure shows two iron ions in the active site (Figure S3A). Although the reaction of PirXI in the presence this metal was performed under an O_2 limited condition we cannot exclude the possibility of Fe^{2+} partially being oxidized to Fe^{3+} , which would influence the activity. Strong inhibition of a class II XI by iron has been described [54] and PirXI is one of a few xylose isomerases reported so far that shows activity on xylose with the Fe^{2+} cofactor [55,56].

Binding of substrate is also dependent on the type of metal cofactor bound to PirXI. As the ionic radius of Ca^{2+} is larger (1.14 Å) than that of other metals, the carbonyl and the 2-hydroxyl of the bound xylose are shifted away from the M_2 site, placing the substrate in a position that is not favorable for catalysis (Figure 8A). This could explain the extremely high K_M for xylose in the presence of Ca^{2+} whereas it is relatively low with Mg^{2+} or Mn^{2+} . PirXI- Co^{2+} shows the highest affinity for xylose which led it to have the highest catalytic efficiency. However, this may

be hardly relevant *in vivo* as cobalt is a less abundant metal and metal content analysis showed that almost no cobalt was bound to the purified native PirXI.

While the metal cofactors influence binding of substrate, the type of substrate/inhibitor seems to influence the binding of the second metal. The crystal structure of PirXI-Mg²⁺ with either xylitol or glycerol, only position M₁ is occupied by a metal. This indicates that the binding of the metal at M₂ position, in case of Mg²⁺, requires a proper binding of the substrate as observed in the PirXI-Mg²⁺-xylose structure. The absence of metal binding at M₂ position in the presence of an inhibitor such as xylitol and sorbitol has also been observed in structures of other XIs [41,46,47].

As shown in the structure of PirXI with sorbitol, binding of a C6 sugar is different between PirXI and Class I enzymes (Figure 7). Because of a bulky tryptophan at position 140, which is substituted to a Met in the class I enzymes, binding of the C6 sugar is sterically hindered in PirXI. In the structure of a class I XI from *Arthrobacter* strain B3728 it was shown that O6 of the D-sorbitol was pointing towards the methionine residue [57]. Indeed, the activity assays of PirXI show that the enzyme is barely active with glucose (very low k_{cat} and high K_M) whereas the class I enzymes have higher activity with glucose [34]. In a previous study of another class II XI from *C. thermosulfurogenes*, replacing the tryptophan with the smaller phenylalanine improved both K_M and k_{cat} of the enzyme for glucose, proving that the steric hindrance of the tryptophan was responsible for the poor activity of the enzyme towards C6 sugars. Nevertheless, this property of PirXI makes it more favorable as a specialized catalyst for lignocellulosic bioethanol production as the enzyme will be devoted to only converting xylose to xylulose.

As we have revealed the impact of various metals on the kinetic properties of PirXI, it is important to understand the *in vivo* metal binding of the enzyme and how it influences its activity. In this study, we have measured the metal content of PirXI isolated from *E. coli*. The enzyme was found to bind a mixture of several metals and the contents were different depending on the cultures that the enzyme was purified from. This indicates that the metal contents of the overexpressed enzyme can be influenced by the intracellular metal composition as well as the enzyme-metal affinity. Therefore, measurements of xylose isomerase activities with cell-free extracts or with enzyme samples of which the metal content is not strictly controlled by reconstitution of chelator-treated apoenzyme may give results with little significance due to variations in metal content, some of which may be introduced during sample preparation. Thus, medium composition, expression level, and growth phase of cultures may influence metal availability and apparent XI activity [54,55]. Previously, Hlima and colleagues found that the recombinant xylose isomerase from *Streptomyces* sp. SK expressed in *E. coli* has lower activity than the native enzyme which was explained by the lack of

posttranslational modification [58]. However, overexpression of the recombinant enzyme could have led to the different metal contents and, hence, the different activities. In a recent study, we have shown that intracellular metal contents play a critical role for the in vivo activity of PirXI expressed in *S. cerevisiae* as it influences metal loading of the enzyme and, thereby, the in vivo catalytic activity and the rate of xylose metabolism [35].

Although the total metal contents of the purified enzyme were close to the theoretical value (2 metal ions per a monomer of PirXI) it seems that the enzyme loses some of the metals during sample preparation for crystallization. The crystal structure of the native PirXI showed that only M_1 was occupied, which indicates a stronger metal binding at M_1 than at M_2 . Interestingly, His272, one of the M_2 metal-coordinating residues, was shown to be doubly protonated half of the time and this could cause weak binding of the metal [26,44], although this could be dependent on the type of the metal. The refinement of the structure with different metals at M_1 showed that the occupancy of metals decreases in the following order: $Ca^{2+} > Mg^{2+} > Fe^{2+}$. Since the metal content analysis showed that the enzyme bound mainly Mg^{2+} and lower levels of Ca^{2+} and Fe^{2+} , we speculate that more Mg^{2+} was bound at M_2 than at M_1 in the native PirXI. Different affinities of various metal ions for the M_1 and M_2 binding sites were also observed in previous studies and the metal preferences differ between the isomerases. For instance, XI from *Arthrobacter* B3728 showed high affinity for Mn^{2+} , Co^{2+} , Cd^{2+} and Pb^{2+} at M_2 and Mg^{2+} and Al^{3+} at M_1 while XI from *S. rubiginosus* prefers Mg^{2+} at M_1 position and other various metal ions at position M_2 [26,46,57].

In the current study we measured the activation constants (K_{act}) of different metals, which are probably determined by the binding site with lower affinity. PirXI has the highest affinity for Mn^{2+} which is also the superior metal for the enzyme activity. The affinity of the enzyme for different metals does not necessarily correlate with chemical reactivity of the metals for the isomerization reaction. Even though the K_{act} for Co^{2+} is much lower than that for Mg^{2+} , the enzyme is more active with Mg^{2+} . Interestingly, metal binding affinity of XI from a *Arthrobacter* strain is similar to that of PirXI ($Mn > Co > Mg$) but this enzyme shows the highest activity in the presence of Mg^{2+} [29]. As metal binding residues in xylose isomerases are highly conserved, it is likely that different chemical reactivity of a metal in different XIs is influenced by the overall protein environment including second shell residues and perhaps the residues that are further away from the active site.

The results reported here reveal clear differences between different metals with respect to binding mode, affinity and catalytic activity of PirXI. The various assays and crystallography experiments were performed with apoenzyme reconstituted with different metals. Several factors including metal dependent

PirXI activity, metal availability and metal affinity could affect the efficiency of the enzyme *in vivo*. It is possible that further exploration using mixtures of metals will be necessary to fully understand the *in vivo* activity of the enzyme. Previously, the activities of PirXI in the presence of metal mixtures that represented the *in vivo* metal composition of different *S. cerevisiae* strains showed that Mn^{2+} influenced the overall activity significantly, despite it being a minor component of the overall mixtures [35]. This suggests a higher enzyme affinity towards this metal, in agreement with the current half saturation constants (K_{act}). On the other hand, an inhibitory effect of Ca^{2+} was not pronounced at concentrations mimicking the *in vivo* levels, as the activities with the metal mixtures were as high as the activity with Mg^{2+} alone.

Besides metal content, the pH-dependence of XI may be important for the *in vivo* activity. PirXI shows an optimum pH of 7.5 or higher in the presence of Mg^{2+} , Mn^{2+} and Ca^{2+} . The enzyme activity decreases rapidly at pH lower than 6.5 and this decrease is thought to be related to the local pK_a of His272 which is involved in the metal (M_2) coordination or Lys342, which in turn interacts with the metal-binding residue Asp310 [26]. The pH-activity profile seems to be also dependent on the metals as PirXI- Co^{2+} showed a pH profile different from that of the enzyme with other metals. The activity of PirXI- Co^{2+} is unaffected by the pH, at least in the range of 5.5 to 7.5. The pH profiles of xylose isomerases of different biological origin may vary. Some show the best activity at acidic pH range [58–61], some are most active at neutral pH range [3,55,62] or at basic pH range [34,63]. Comparing the sequences and structures of these XIs with different pH optimum may be useful for engineering PirXI to optimize its activity for the cytosolic pH of the *S. cerevisiae*.

CONCLUSION

Xylose isomerase from *Piromyces* sp. E2, which catalyzes xylose isomerization in metabolically engineered *S. cerevisiae* strains for conversion of pentoses to ethanol, can accept various metals. The catalytic performance of the enzyme is dependent on the type of metal that is bound, with Mn^{2+} being the best metal, followed by Mg^{2+} . Ca^{2+} and Cd^{2+} poorly activate the reactions and Ni^{2+} and Al^{3+} are not activating. Since there is no clear correlation between the metal dependent activities and the affinity of the enzyme for metal ions, *in vivo* XI performance will be influenced by metal availability in the medium, uptake and distribution in cells, and incorporation in the enzyme.

Overall, the PirXI crystal structures are similar to other XI structures with the major differences being the N-terminal α -helix extension and the size of interface

area in the tetramers as compared to class I XIs. The active site of each PirXI subunit contains a substrate binding site and two metal binding sites formed by several aspartates and glutamates that are conserved throughout XIs. The results of soaking experiments confirmed that PirXI is highly promiscuous with respect to metal binding and suggest that differences in movement of the catalytic metal to a position required for hydride shift catalysis and associated conformational changes of metal binding side chains may be related to differences in catalytic performance.

Understanding the metal dependent catalytic properties of the enzyme through structural and biochemical studies as described in this study provides critical insights for the enzyme engineering to optimize the in vivo enzyme activity, thereby improving ethanol production from xylose.

MATERIALS AND METHODS

Expression of PirXI in *E. coli*

A synthetic *xylA* gene encoding XI from *Piromyces* sp. E2 (GenBank accession number AJ249909) was obtained from GenScript USA Inc. (Piscataway, NJ, USA). The sequence was codon-optimised for expression in *E. coli*. The *xylA* gene was cloned into a pBAD/myc-His-derived plasmid (Invitrogen). In this plasmid, the original NdeI sites were removed and the NcoI site was replaced with an NdeI site. The *xylA* gene was cloned using NdeI and HindIII sites with insertion of a stop codon at the end of the *xylA* coding sequence. The obtained pBAD-PirXI plasmid was transformed into *E. coli* TOP10 (Invitrogen) or NEB 10-beta (New England Biolabs). The sequence of the plasmid was verified by sequencing.

For expression of XI, *E. coli* TOP10 cells or NEB 10-beta cells harboring the pBAD-PirXI plasmid were cultivated in TB medium (per l: 12 g tryptone, 24 g yeast extract, 5 mL glycerol, 2.31 g KH_2PO_4 , 16.43 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$) supplemented with 50 $\mu\text{g} \cdot \text{ml}^{-1}$ ampicillin and 0.2% (w/v) L-arabinose for 16 h at 37 °C. The cells were harvested by centrifugation, washed with 20 mM Tris-HCl, pH 8.5, and directly used for enzyme purification or stored at -20 °C until further use.

Purification of XI overexpressed in *E. coli*

The cells were disrupted by sonication and cell debris was removed by centrifugation for 45 min at 31,000 × g, 4 °C. The supernatant was applied to a Q-sepharose Fast Flow resin (GE Healthcare) equilibrated with 20 mM Tris-HCl, pH 8.5 in a gravity flow column, and incubated for 30 min at 4 °C on a rotating format. The column was washed with 15 column volumes of 20 mM Tris-HCl, pH 8.5. Then xylose isomerase was eluted from the column using 20 mM Tris-HCl,

pH 8.5, containing 100 mM KCl. Fractions containing protein were pooled and desalted using an EconoPac 10-DG desalting column (Bio-Rad) pre-equilibrated with 10 mM MOPS, pH 7.0. Protein concentrations were determined using the theoretical extinction coefficient at 280 nm ($\epsilon_{280, \text{XI}} = 73,800 \text{ M}^{-1} \cdot \text{cm}^{-1}$) calculated by the ProtParam tool (<http://web.expasy.org/protparam/>). The protein was stable when stored at 4 °C for several weeks. For long-term storage, the protein was frozen in liquid nitrogen and stored at -80 °C.

Activity assays

Most assays were done using enzyme with controlled metal composition. For this, we first prepared apo-enzyme by buffer exchange to 10 mM EDTA in 10 mM Tris·HCl, pH 8.0, using either dialysis or EconoPac 10-DG desalting columns (Bio-rad). The samples were incubated with EDTA for 30 min. Subsequently, EDTA was removed by buffer exchange to 10 mM MOPS, pH 7.0. The activity of this apoXI assayed in the absence of added bivalent metal ions was less <0.02 U/mg.

In most cases, XI activities were measured by a coupled enzyme assay using D-sorbitol dehydrogenase (SDH)[64]. D-xylulose formed by XI activity is reduced by SDH to xylitol, which is monitored by following NADH oxidation spectrophotometrically at 340 nm. SDH was obtained from Roche Diagnostics GmbH (Mannheim, Germany). The standard reactions were performed at 30 °C and contained 20 mM MOPS, pH 7.0, 1 mM bivalent metal ions, 150 mM D-xylose, 0.15 mM NADH and 1 unit/ml SDH. Reactions were initiated by adding 0.05-0.2 μM apoXI. The amount of XI was adjusted so that the ratio of SDH activity to XI activity would be at least 30. In case of assays in the presence of Co^{2+} , the amount of SDH was doubled to balance the inhibitory effect of Co^{2+} ions on SDH activity. The absorbance at 340 nm was followed using a spectrophotometer (Jasco) or a Synergy Mx microtiter plate reader (BioTek Instruments, Inc.). D-xylose and D-fructose stocks (2 M) were made in MilliQ water and stored at -20 °C. The measurements were performed in duplicate. One unit of XI activity is defined as the amount of enzyme catalyzing conversion of 1 μmol of D-xylose per min under the assay conditions.

Kinetic parameters of XI for D-xylose were determined by measuring XI activity with varying D-xylose concentrations in the range of 0.04 to 1875 mM, depending on the metal cofactor used. For pH profiling of XI activities, different buffers (50 mM) were used to establish pH values: MES for pH 5.5 and 6.0, MOPS for pH 6.5, 7.0 and 7.5. Activities of native enzyme isolated from *E. coli* were measured in the presence of 1 mM MgCl_2 using the spectrophotometric assay described above. For determining the apparent metal affinities (K_{act} values) activity of apo-PirXI was measured in the presence of varying metal concentrations (1-4000 μM) using 150 mM xylose as the substrate for Mg^{2+} and Mn^{2+} , 1375 mM xylose for Ca^{2+} , and

at 15 mM xylose for Co^{2+} . The concentration of the enzyme used was in the range of 0.05 – 0.2 μM for the reactions with Mg^{2+} , Mn^{2+} and Co^{2+} and 0.5 – 1.0 μM for the reaction with Ca^{2+} .

For measuring the activities of PirXI with metal cofactors which are not compatible with the SDH-coupled assay, activities were examined by measuring the formation of D-xylulose from D-xylose by high-performance liquid chromatography (HPLC). Reaction mixtures containing the substrate, PirXI, and 1 mM metal ions in MOPS (pH 7.0) were incubated at 30 °C while being shaken. Reactions were stopped by acidification with HCl and addition of acetonitrile to 80 %. Samples were then separated on an XBridge BEH Amide column (4.6 mm $\times$ 250 mm, pore size 130 Å, particle size, 3.5 μm , Waters). Separation conditions were isocratic with 80 % acetonitrile, 20 % 20 mM MOPS (pH 7.0) and a flow rate of 1 mL/min, 80 °C. The ultraviolet (UV) absorbance at 210 nm was measured for D-xylulose quantification. For measuring XI activity with Fe^{2+} , oxygen-limited reaction mixtures were prepared by flushing the reaction components and the mixture with argon. The reaction mixtures were incubated in a 30 °C water bath. During the reaction the argon was flushed over the surface of the reaction mixture and for sampling a syringe was used. The activity of PirXI on glucose was also measured using the HPLC assay as described above. The activities with different concentrations of glucose ranging from 0.1 M to 1.5 M were measured in the presence of 1 mM MgCl_2 as the metal cofactor. A refractive index detector was employed for D-fructose quantification.

Metal analysis

XI purified from *E. coli* was lyophilized and analyzed for the presence of calcium, cobalt, copper, iron, magnesium, manganese and zinc using inductively-coupled plasma optical emission atomic spectroscopy on an Optima 7000DV ICP-OES apparatus (PerkinElmer). The measurements were performed in duplicate. Before lyophilization, a molybdenum standard (molybdenum ICP standard Certipur, Merck Millipore) was added to the sample to a final concentration of 5.0 $\mu\text{g/mL}$.

Crystallization and structure determination

Initial vapor-diffusion crystallization experiments were performed using a Mosquito crystallization robot (TTP Labtech). In a typical experiment, 0.1 μL screening solution was added to 0.1 μL protein solution on a 96-well MRC2 plate (Molecular Dimensions); reservoir wells contained 50 μL screening solution. The screening solutions used for the experiments were PACT and JCSG+ (Qiagen Systems). Crystallization conditions were optimized using hanging-drop setups with 13-15% (w/v) PEG3350 and 0.1 M ammonium sulfate in 0.1 M HEPES (pH 7.0) as precipitant, and drops containing 1 μL protein solution (6 mg mL^{-1} in 10 mM

triethanolamine, pH 7.6) and 1 μ l reservoir solution at 295 K. Native PirXI crystals belong to space group P1 with four monomers of 49 kDa in the asymmetric unit. The V_M is 2.3 $\text{\AA}^3/\text{Da}$ [65] with a solvent content of 45%. Apo crystals were grown under the same condition as native crystals, and binary complexes of apo-XI with metal cofactors were prepared by addition of the required metal in the crystallization drop. Before data collection, crystals were briefly soaked in a cryoprotectant solution, consisting of 20% glycerol, 15% (v/v) PEG 3350 and 0.1 M ammonium sulfate in 0.1 M HEPES buffer, pH 7.0. Ternary complexes with substrate or inhibitor were prepared from the binary complexes by stepwise addition of the sugars to the crystals, reaching a final sugar concentration of 2.2 M, which was high enough to serve as cryoprotectant [66]. For the structure of XI with xylulose, 2.5 M trans-4-hydroxy-L-proline was used as cryo-protectant as xylulose was available only as a 0.5 M solution.

X-ray diffraction data to 1.8 $\text{\AA}$ resolution were collected from single cryo-cooled crystals mounted on an in-house MarDTB Goniostat System using Cu-K α radiation from a Bruker MicrostarH rotating-anode generator equipped with HeliosMX mirrors. Intensity data were processed using iMosflm [67] and scaled using Aimless [68].

Phases were obtained by molecular replacement using the program Phaser [69]. The coordinates of three xylose isomerase structures [Protein Data Bank (PDB) entries 1A0C, 1A0D, and 1A0E], which have sequences that are ~50 % identical with PirXI, were used to construct a composite search model. The PirXI structure was built using the programs ArpWarp [70] and Coot [71] and refined using Refmac5 [72]. Water molecules were placed automatically in $F_o - F_c$ difference Fourier maps at a 3σ cutoff level, and validated to ensure correct coordination geometries using Coot. The first residues from the four XI subunits were not visible in the electron density, and therefore not included in the final model. In the binary and ternary complexes $2F_o - F_c$ and $F_o - F_c$ maps showed extra density in the active site. To confirm the metal ions, we calculated anomalous difference Fourier maps, with data collected at a wavelength of 1.54 $\text{\AA}$ (1.3 anomalous electrons for Ca, 2.8 for Mn, 3.2 for Fe, 3.7 for Co, 0.5 for Ni, 4.7 for Cd), using phases obtained from the model without any metal ions. Occupancies of the metal ions were calculated with Phenix [73]. Relevant statistics of the data collection and model refinement are given in Table 4. The stereochemical quality of the model was assessed with MolProbity [74]. Interface areas between the subunits were calculated with PDBePISA [75]. Figures were prepared with PyMOL (<http://www.pymol.org>) and ESPript [76]. Atomic coordinates and experimental structure factor amplitudes have been deposited in the Protein Data Bank (PDB) (Table 4).

Structure-based sequence alignment.

A structure-based alignment of Class II xylose isomerases from *Piromyces* sp. E2, *Bacteroides thetaiotaomicron* (PDB entry 4XKM)[36], *Thermoanaerobacterium thermosulfurigenes* (PDB entry 1A0C), *Thermotoga neapolitana* (PDB entry 1A0E), *Bacillus stearothermophilus* (PDB entry 1A0D) and a Class I xylose isomerase from *Streptomyces rubiginosus* (PDB entry 4W4Q)[49] was made with Promals3D [77]. The alignment figure was created with ESPript [78].

Table 4. Data collection and refinement statistics.

	Native	Apo	Mg-glycerol	Mg-xylitol	Mg-xylose	Ca-xylose
cryoprotectant	20% glycerol	20% glycerol	0.5 M xylose + 20% glycerol	2.2 M xylitol	2.2 M xylose	2.2 M xylose
unit cell dimensions						
a, b, c (Å), α, β, γ (°)	78.7, 79.4, 92.2 115.3, 90.3, 117.0	78.9, 79.4, 91.1 115.8, 89.4, 116.7	78.6, 79.4, 92.1 115.5, 90.2, 116.9	78.8, 79.3, 91.8 115.4, 90.2, 116.7	78.6, 79.3, 92.0 115.4, 89.9, 117.1	78.8, 79.3, 91.9 115.4, 89.9, 117.2
resolution (Å)	1.80	1.67	1.80	1.75	1.90	1.86
R _{sym} (%) ^a	4.5 (23.0)	7.8 (28.6)	8.9 (35.0)	6.6 (43.1)	9.3 (35.3)	9.0 (48.8)
completeness (%) ^a	92.3 (66.7)	92.4 (75.6)	93.4 (80.7)	93.3 (89.0)	92.0 (84.7)	93.7 (75.7)
I/σ (I) ^a	20.6 (4.9)	9.1 (3.7)	8.4 (2.6)	14.0 (2.9)	6.1 (2.0)	10.6 (2.1)
redundancy	3.9 (3.2)	4.0 (3.8)	4.0 (3.6)	4.0 (4.0)	2.0 (2.0)	4.0 (3.2)
refinement						
R / R _{free} (%)	12.1 / 14.8	18.4 / 21.0	19.4 / 22.9	14.0 / 16.1	15.3 / 17.6	17.6 / 20.1
ions for active site occupancy	4 (Ca ²⁺ , Mg ²⁺ , Fe ²⁺) 0.4, 0.35, 0.25	-	4 Mg ²⁺	4 Mg ²⁺	8 Mg ²⁺ in 2 conf.	8 Ca ²⁺
ligand active site	glycerol	glycerol	glycerol	xylitol	linear xylose	linear xylose
waters	2373	2317	1638	1836	1775	1620
other ligands				-	14 xyloses	8 xyloses
other atoms	20 GOL, 3 SO ₄ ²⁻	7 GOL, 4 SO ₄ ²⁻ , 2 acetate	11 GOL, 3 SO ₄ ²⁻	3 SO ₄ ²⁻	7 SO ₄ ²⁻	6 SO ₄ ²⁻ , 2Ca ²⁺
rms deviations						
bond lengths (Å)	0.013	0.009	0.014	0.010	0.010	0.009
bond angles (°)	1.49	1.31	1.48	1.33	1.32	1.24
PDB code	5NH5	5NHM	5NH4	5NH6	5NH7	5NH8

^a Values in parentheses are for the highest-resolution shell.

Mn-xylose	Mn-sorbitol	Fe-glycerol	Co-xylulose	Ni-xylose	Cd-xylose
2.2 M xylose	2.2 M sorbitol	20% glycerol	0.25 M xylulose + 2.5 M transPRO	2.2 M Xylose	2.2 M Xylose
78.7,79.4,92.1 115.4,90.0,117.1	79.0,79.4,91.3, 115.7,89.4,116.9	79.5,79.5,92.2 115.6,90.0,117.7	79.3,79.3,91.4, 115.7,89.4,116.9	78.2,79.0,92.01 15.1,90.4,17.0	78.6, 79.4,92.0 115.4,90.0,117.2
2.08	1.80	2.40	1.93	1.80	1.86
11.0 (43.5)	9.4 (48.2)	13.9 (49.0)	7.8 (29.3)	8.8 (49.1)	6.3 (38.6)
92.4 (59.7)	93.6 (79.6)	85.8 (81.1)	94.3 (79.3)	91.4 (87.3)	87.8 (55.9)
9.3 (2.7)	11.6 (2.1)	5.9 (2.1)	10.9 (2.9)	8.0 (1.9)	9.4 (2.0)
3.9 (2.9)	3.9 (3.2)	4.0 (4.0)	2.8 (2.4)	3.8 (3.8)	2.0 (1.8)
16.8 / 20.3	15.5/ 18.1	24.4 / 27.8	15.7 / 18.8	15.4 / 17.5	14.4 / 16.5
8 Mn ²⁺	8 Mn ²⁺	8 Fe ²⁺	8 Co ²⁺ in 2 conf.	8 Ni ²⁺ in 2 conf.	8 Cd ²⁺
linear xylose	sorbitol	-	xylulose	linear xylose	linear xylose or xylopyranose
1522	1984	477	2185	1751	1837
8 xyloses	-	-	4-hydroxyproline	15 xylose	13 xylose
3 SO ₄ ²⁻	4 SO ₄ ²⁻	3 SO ₄ ²⁻	-	5 SO ₄ ²⁻	8 SO ₄ ²⁻
0.012	0.012	0.012	0.011	0.011	0.011
1.37	1.48	1.42	1.38	1.37	1.42
5NH9	5NHA	5NHB	5NHC	5NHD	5NHE

SUPPLEMENTARY INFORMATION

Figure S1. Structure-based sequence alignment of xylose isomerases. Structure-based alignment of class II xylose isomerases from *Piromyces* sp. E2 (Pir), *Bacteroides thetaiotaomicron* (BaThet), *Thermoanaerobacterium thermosulfurigenes* (ThThe), *Thermotoga neapolitana* (ThNeap), *Bacillus stearothermophilus* (GsStea) and a Class I xylose isomerase from *Streptomyces rubiginosus* (StRubi). The secondary structure elements at the top of the sequence alignment are from the crystal structure of PirXI. Identical residues have a red background color, similar residues have a red color. Residues involved in metal binding have a cyan background and hydrophobic residues in the active have an orange background. Residues which are different in the active site from Class I have a green background.

Figure S2. Active site of Mg-xylitol-XI (A), Ca-linear-xylose-XI (B), Mn-linear-xylose-XI (C) and Co-linear-xylose-XI (D). The residues involved in the metal binding sites are displayed as sticks in green, His102 in salmon, and the xylose, xylulose and xylitol molecules in yellow. Mn²⁺ ions are depicted as purple balls, Ca²⁺ and Mg²⁺ as green balls, and Co²⁺ as salmon balls. 2Fo - Fc density is contoured at 10 σ level in panel A and omit electron density for the sugars is shown at 30 σ for panel B-D.

Figure S3. Active site of Fe-XI (A) and Ni-XLS-XI (B). The residues involved in the metal binding sites are displayed as sticks, in green, His102 in salmon, and the xylose molecule in yellow. Fe²⁺ ions are depicted as brown balls and Ni²⁺ as green balls. Interactions between the metal ions, the hydroxyl of the xylose and the ligand amino acids are shown as dashed lines.

Table S1. Distances of coordination to the metal ions

	Native	Mg-GOL	Mg-XYL	Mg-XLS	Ca-XLS
Metal 1	Ca ²⁺ , Mg ²⁺ , Fe ²⁺	Mg ²⁺	Mg ²⁺	Mg ²⁺	Ca ²⁺
B-fact./Occ.	26.8/24.5/20.3 (0.40/0.26/0.30)	21.6/1.0	13.7/1.0	9.8/1.0	21.8/1.0
Glu233OE1	-	3.19	-	-	-
Glu233OE2	2.20	2.05	2.07	2.11	2.28
Glu269OE1	2.12	2.11	2.05	1.97	2.19
Asp297OD2	2.07	2.02	1.97	2.08	2.18
Asp340OD2	2.14	2.02	2.04	2.09	2.22
O2	2.33	2.17	2.15	1.95	2.33
O4/O3	2.42	2.09	2.09	2.06	2.40
Metal 2	-	-	-	Mg ²⁺ 2a/2b	Ca ²⁺
B-fact./Occ.	-	-	-	8.6/5.9 (0.4/0.6)	37.4/1.0
Glu269OE1	-	-	-	-/2.97	-
Glu269OE2	-	-	-	2.54/2.17	2.26
His272NE2	-	-	-	2.99/2.34	3.41
Asp308OD1	-	-	-	1.66/-	-
Asp308OD2	-	-	-	3.18/- (2.95)	2.10
Asp310OD1	-	-	-	-/2.59	2.44
H ₂ O/OH ⁻	-	-	-	2.55/1.88	2.11
Metal 1-Metal 2	-	-	-	3.64 (1-2b) 1.91 (2a-2b)	5.2
Ligand	glycerol	glycerol	xylitol	linear xylose	linear xylose
O1	-	-	-	2.99/2.23	-
O2	-	-	-	-/2.06	-

Mn-XLS	Mn-SOR	Fe	Co-XUL	Ni-XLS	Cd-XLS	Cd-XYS
Mn ²⁺	Mn ²⁺	Fe ²⁺	Co ²⁺	Ni ²⁺	Cd ²⁺	Cd ²⁺
17.0/1.0	17.2/1.0	15.5/1.0	13.5/1.0	17.5/0.8	14.4/0.5	13.9/0.5
-	-	1.89	-	2.14	3.42	3.35
2.17	2.09	2.91	2.16	3.02	2.24	2.21
2.13	2.19	2.04	2.05	2.17	2.04	2.06
2.12	2.08	2.01	2.05	2.10	2.19	2.13
2.11	2.17	2.16	2.16	2.18	2.09	2.16
2.17	2.34	-	2.07	2.01	2.15	2.27 (O2)
2.23	2.27	-	2.20	2.23	2.34	2.31 (O4)
Mn ²⁺	Mn ²⁺	Fe ²⁺	Co ²⁺	Ni ²⁺ 2a/2b	Cd ²⁺ 2a/2b	Cd ²⁺ 2a/2b
30.7/1.0	24.4/1.0	16.6/1.0	20.0/1.0	13.1/12.5 (0.5/0.5)	12.4/34.9 (0.40/0.35)	13.2 20.9 (0.40/0.25)
2.90	3.06	2.92	2.97	-/2.67	3.19/2.94	3.17/3.00
2.31	1.99	2.17	2.28	2.04/2.43	1.97/2.75	2.63/1.9
3.14	2.65	2.27	2.22	2.74/2.07	2.79/2.30	2.75/2.22
-	2.15	-	-	2.26/-	2.62/-	2.63/-
1.95	2.60	2.90	2.83	2.01/-	2.20/2.57	2.27/2.43
3.21	2.47	4.01	-	2.25/-	2.47	2.63/-
2.19		-	2.05	2.50/1.85	-/2.63	2.35/2.04
4.10	4.70	3.75	3.75	3.66 (1-2b) 1.88 (2a-2b)		4.05 (1-2b) 1.31 (2a-2b)
linear xylose	sorbitol	-	xylulose	linear xylose	linear xylose	cyclic xylose
2.76	-		2.24	-/2.38	-	-/2.74 (O3)
2.67	4.10		2.35	-/2.26	2.57	

REFERENCES

1. Hahn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF. Towards industrial pentose-fermenting yeast strains. *Appl Microbiol Biotechnol*. 2007;74:937–53.
2. Sánchez Nogué V, Karhumaa K. Xylose fermentation as a challenge for commercialization of lignocellulosic fuels and chemicals. *Biotechnol Lett*. 2015;37:761–72.
3. Madhavan A, Tamalampudi S, Ushida K, Kanai D, Katahira S, Srivastava A, et al. Xylose isomerase from polycentric fungus *Orpinomyces*: gene sequencing, cloning, and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol. *Appl Microbiol Biotechnol*. 2009;82:1067–78.
4. Hector RE, Dien BS, Cotta MA, Mertens JA. Growth and fermentation of D-xylose by *Saccharomyces cerevisiae* expressing a novel D-xylose isomerase originating from the bacterium *Prevotella ruminicola* TC2-24. *Biotechnol Biofuels*. 2013;6:84.
5. Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MSM, de Laat WTAM, et al. High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Res*. 2003;4:69–78.
6. de Figueiredo Vilela L, de Mello VM, Reis VCB, da Silva Bon EP, Gonçalves Torres FA, Neves BC, et al. Functional expression of *Burkholderia cenocepacia* xylose isomerase in yeast increases ethanol production from a glucose-xylose blend. *Bioresour Technol*. 2012;128:792–6.
7. Matsushika A, Inoue H, Kodaki T, Sawayama S. Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: Current state and perspectives. *Appl Microbiol Biotechnol*. 2009;84:37–53.
8. Walfridsson M, Anderlund M, Bao X, Hahn-Hägerdal B. Expression of different levels of enzymes from the *Pichia stipitis* XYL1 and XYL2 genes in *Saccharomyces cerevisiae* and its effects on product formation during xylose utilisation. *Appl Microbiol Biotechnol*. 1997;48:218–24.
9. Young E, Lee S, Alper H. Optimizing pentose utilization in yeast: the need for novel tools and approaches. *Biotechnol Biofuels*. 2010;3:24.
10. Karhumaa K, Garcia Sanchez R, Hahn-Hägerdal B, Gorwa-Grauslund M-F. Comparison of the xylose reductase-xylytol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Microb Cell Fact*. 2007;6:5.
11. Bhosale SH, Rao MB, Deshpande V V. Molecular and industrial aspects of glucose isomerase. *Microbiol Rev*. 1996;60:280–300.
12. Amore R, Wilhelm M, Hollenberg CP. The fermentation of xylose - an analysis of the expression of *Bacillus* and *Actinoplanes* xylose isomerase genes in yeast. *Appl Microbiology Biotechnol*. 1989;30:351–7.
13. Moes C, Pretorius I, Zyl W. Cloning and expression of the *Clostridium thermosulfurogenes* D-xylose isomerase gene (*xylA*) in *Saccharomyces cerevisiae*. *Biotechnol Lett*. 1996;18:269–74.
14. Sarthiy A V, Mcconaughey BL, Lobo Z, Sundstrom JA, Furlong CE, Hall BD. Expression of the *Escherichia coli* xylose isomerase gene in *Saccharomyces cerevisiae*. *Appl Environ Microbiol*. 1987;53:1996–2000.
15. Gárdonyi M, Hahn-Hägerdal B. The *Streptomyces rubiginosus* xylose isomerase is misfolded when expressed in *Saccharomyces cerevisiae*. *Enzyme Microb Technol*. 2003;32:252–9.
16. Walfridsson M, Bao X, Anderlund M, Lilius G, Bülow L, Hahn-Hägerdal B. Ethanolic fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus* *xylA* gene, which expresses an active xylose (glucose) isomerase. *Appl Environ Microbiol*. 1996;62:4648–51.
17. Harhangi H, Akhmanova AS, Emmens R, van der Drift C, de Laat WTAM, van Dijken JP, et al. Xylose metabolism in the anaerobic fungus *Piromyces* sp. strain E2 follows the bacterial pathway. *Arch Microbiol*. 2003;180:134–141.
18. van Maris AJA, Winkler AA, Kuyper M, de Laat WTAM, van Dijken JP, Pronk JT. Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose isomerase as a key component. *Adv Biochem Eng Biotechnol*. 2007;108:179–204.
19. Brat D, Boles E, Wiedemann B. Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. *Appl Environ Microbiol*. 2009;75:2304–11.
20. Zhou H, Cheng J sheng, Wang BL, Fink GR, Stephanopoulos G. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. *Metab Eng*. 2012;14:611–22.
21. Kuyper M, Hartog MMP, Toirkens MJ, Almering MJH, Winkler AA, van Dijken JP, et al. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res*. 2005;5:399–409.
22. Wisselink HW, Toirkens MJ, Berriel MDRF, Winkler AA, Van Dijken JP, Pronk JT, et al. Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. *Appl Environ Microbiol*. 2007;73:4881–91.

23. Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *FEMS Yeast Res.* 2005;5:925–34.
24. Lee SM, Jellison T, Alper HS. Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* 2012;78:5708–16.
25. Fuxreiter M, Ödön F, Náray-Szabó Gábor. Molecular modelling of xylose isomerase catalysis: the role of electrostatics and charge transfer to metals. *Protein Eng.* 1995;8:925–33.
26. Kovalevsky AY, Hanson L, Fisher SZ, Mustyakimov M, Mason SA, Trevor Forsyth V, et al. Metal ion roles and the movement of hydrogen during reaction catalyzed by D-xylose isomerase: A joint x-ray and neutron diffraction study. *Structure.* 2010;18:688–99.
27. Collyer CA, Henrick K, Blow DM. Mechanism for aldose-ketose interconversion by D-xylose isomerase involving ring opening followed by a 1,2-hydride shift. *J Mol Biol.* 1990;212:211–35.
28. Blow DM, Collyer CA, Goldberg JD, Smart OS. Structure and mechanism of D-xylose Isomerase. 1992;67–73.
29. Rangarajan M, Hartley BS. Mechanism of D-fructose isomerization by *Arthrobacter* D-xylose isomerase. *Biochem J.* 1992;283:223–33.
30. Fuxreiter M, Böcskei Z, Szeibert A, Szabó E, Dallmann G, Náray-Szabó Gábor, et al. Role of electrostatics at the catalytic metal binding site in xylose isomerase action: Ca²⁺-inhibition and metal competence in the double mutant D254E/D256E. *Proteins Struct Funct Genet.* 1997;28:183–93.
31. van Bastelaere PBM, Vangrýsperre W, Kersters-Hildersont H. Kinetic studies of Mg²⁺-, Co²⁺- and Mn²⁺-activated D-xylose isomerases. *Biochem J.* 1991;278:285–92.
32. Callens M, Tomme P, Kersters-Hilderson H, Cornelis R, Vangrýsperre W, De Bruyne CK. Metal ion binding to D-xylose isomerase from *Streptomyces violaceoruber*. *Biochem J.* 1988;250:285–90.
33. van Tilbeurgh H, Jenkins J, Chiadmi M, Janin J, Wodak SJ, Mrabet NT, et al. Protein engineering of xylose (glucose) isomerase from *Actinoplanes missouriensis*. 3. Changing metal specificity and the pH profile by site-directed mutagenesis. *Biochemistry.* 1992;31:5467–71.
34. Smith CA, Rangarajan M, Hartley BS. D-Xylose (D-glucose) isomerase from *Arthrobacter* strain N.R.R.L B3728. *Biochem J.* 1991;277:255–61.
35. Verhoeven MD, Lee M, Kamoen L, van den Broek M, Janssen DB, Daran J-MG, et al. Mutations in PMR1 stimulate xylose isomerase activity and anaerobic growth on xylose of engineered *Saccharomyces cerevisiae* by influencing manganese homeostasis. *Sci Rep.* 2017;7:46155.
36. Han B, Bong SM, Cho J, Kim M, Kim SJ, Lee B II. Crystal structure of a class 2 D-xylose isomerase from the human intestinal tract microbe *Bacteroides thetaiotaomicron*. *Biodesign.* 2015;3:41–7.
37. Allen KN, Lavie A, Glasfeld A, Tanada TN, Gerrity DP, Carlson SC, et al. Role of the divalent metal ion in sugar binding, ring opening, and isomerization by D-xylose isomerase: replacement of a catalytic metal by an amino acid. *Biochemistry.* 1994;33:1488–94.
38. Valli M, Sauer M, Branduardi P, Borth N, Porro D, Mattanovich D. Intracellular pH distribution in *Saccharomyces cerevisiae* cell populations, analyzed by flow cytometry. *Appl Environ Microbiol.* 2005;71:1515–21.
39. Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates . II: Inhibitors and mechanisms of inhibition mechanisms of inhibition. *Bioresour Technol.* 2000;74:25–33.
40. Harding MM. Geometry of metal–ligand interactions in proteins. *Acta Crystallogr Sect D Biol Crystallogr.* 2001;57:401–11.
41. Whitlow M, Howard AJ, Finzel BC, Poulos TL, Winborne E, Gilliland GL. A metal-mediated hydride shift mechanism for xylose isomerase based on the 1.6 Å *Streptomyces rubiginosus* structure with xylitol and D-xylose. *Proteins Struct Funct Bioinforma.* 1991;9:153–73.
42. Rozeboom HJ, Yu S, Madrid S, Kalk KH, Zhang R, Dijkstra BW. Crystal structure of α-1,4-glucan lyase, a unique glycoside hydrolase family member with a novel catalytic mechanism. *J Biol Chem.* 2013;288:26764–74.
43. Kovalevsky AY, Katz AK, Carrell HL, Hanson L, Mustyakimov M, Fisher SZ, et al. Hydrogen location in stages of an enzyme-catalyzed reaction : Time-of-flight neutron structure of D-xylose isomerase with bound D-xylulose. *Biochemistry.* 2008;47:7595–7.
44. Katz AK, Li X, Carrell HL, Hanson BL, Langan P, Coates L, et al. Locating active-site hydrogen atoms in D-xylose isomerase: time-of-flight neutron diffraction. *Proc Natl Acad Sci.* 2006;103:8342–7.
45. Garcia-Viloca M, Alhambra C, Truhlar DG, Gao J. Hydride transfer catalyzed by xylose isomerase: mechanism and quantum effects. *J Comput Chem.* 2003;24:177–90.
46. Collyer CA, Blow DM. Observations of reaction intermediates and the mechanism of aldose-ketose interconversion by D-xylose isomerase. *Proc Natl Acad Sci U S A.* 1990;87:1362–6.

47. Jenkins J, Janin J, Rey F, Chiadmi M, van Tilbeurgh H, Lasters I, et al. Protein engineering of xylose (glucose) isomerase from *Actinoplanes missouriensis*. 1. Crystallography and site-directed mutagenesis of metal binding sites. *Biochemistry*. 1992;31:5449–58.
48. Kovalevsky A, Hanson BL, Mason SA, Forsyth VT, Fisher Z, Mustyakimov M, et al. Inhibition of D-xylose isomerase by polyols: atomic details by joint X-ray/neutron crystallography. *Acta Crystallogr D Biol Crystallogr*. 2012;68:1201–6.
49. Sugahara M, Mizohata E, Nango E, Suzuki M, Tanaka T, Masuda T, et al. Grease matrix as a versatile carrier of proteins for serial crystallography. *Nat Methods*. 2014;12:61–3.
50. Carrell HL, Hoier H, Glusker JP. Modes of binding substrates and their analogues to the enzyme D-xylose isomerase. *Acta Crystallogr, Sect D Biol Crystallogr*. 1994;50:113–23.
51. Gérczei T, Böcskei Z, Szabó E, Asbóth B, Náray-Szabó G. Structure determination and refinement of the Al³⁺ complex of the D254,256E mutant of *Arthrobacter* D-xylose isomerase at 2.40 Å resolution. Further evidence for inhibitor-induced metal ion movement. *Int J Biol Macro*. 1999;25:329–36.
52. Fenn TD, Ringe D, Petsko GA. Xylose isomerase in substrate and inhibitor michaelis states: atomic resolution studies of a metal-mediated hydride shift. *Biochemistry*. 2004;43:6464–74.
53. Kovalevsky AY, Hanson L, Fisher SZ, Mustyakimov M, Mason SA, Trevor Forsyth V, et al. Metal ion roles and the movement of hydrogen during reaction catalyzed by D-xylose isomerase: A joint x-ray and neutron diffraction study. *Structure*. 2010;18:688–99.
54. Karaoglu H, Yanmis D, Sal FA, Celik A, Canakci S, Belduz AO. Biochemical characterization of a novel glucose isomerase from *Anoxybacillus gonensis* G2T that displays a high level of activity and thermal stability. *J Mol Catal B Enzym*. 2013;97:215–24.
55. Lee CY, Zeikus JG. Purification and characterization of thermostable glucose isomerase from *Clostridium thermosulfurogenes* and *Thermoanaerobacter* strain B6A. *Biochem J*. 1991;273:565–71.
56. Callens M, Kersters-Hilderson H, Van Opstal O, De Bruyne CK. Catalytic properties of D-xylose isomerase from *Streptomyces violaceoruber*. *Enzyme Microb Technol*. 1986;8:696–700.
57. Henrick K, Collyer CA, Blow DM. Structures of D-xylose isomerase from *Arthrobacter* strain B3728 containing the inhibitors xylitol and D-sorbitol at 2.5 Å and 2.3 Å resolution, respectively. *J Mol Biol*. 1989;208:129–57.
58. Hlima H Ben, Ayadi D, Aghajari N, Bejar S. Differential properties of native and tagged or untagged recombinant glucose isomerases of *Streptomyces* sp. SK and possible implication of the glycosylation. *J Mol Catal B Enzym*. 2013;94:82–7.
59. Staudigl P, Haltrich D, Peterbauer CK. L-arabinose isomerase and D-xylose isomerase from *Lactobacillus reuteri*: characterization, coexpression in the food grade host *Lactobacillus plantarum*, and application in the conversion of D-galactose and D-glucose. *J Agric Food Chem*. 2014;62:1617–24.
60. Srih-Belghith K, Bejar S. A thermostable glucose isomerase having a relatively low optimum pH: study of activity and molecular cloning of the corresponding gene. *Biotechnol Lett*. 1998;20:553–6.
61. Liu S-Y, Wiegel J, Gherardini FC. Purification and cloning of a thermostable xylose (glucose) isomerase with an acidic pH optimum from *Thermoanaerobacterium* strain JW/SL-YS 489. *J Bacteriol*. 1996;178:5938–45.
62. van Bastelaere PBM, Kersters-Hilderson HLM, Anne-Marie L. Wild-type and mutant D-xylose isomerase from *Actinoplanes missouriensis*: metal-ion dissociation constants, kinetic parameters of deuterated and non-deuterated substrates and solvent-isotope effects. *Biochem J*. 1995;307:135–42.
63. Cha J, Batt C. Lowering the pH optimum of D-xylose isomerase: the effect of mutations of the negatively charged residues. *Mol Cells*. 1998;8:374.
64. Yamanaka K. Enzymatic assay of D-xylose isomerase with D-sorbitol dehydrogenase. *Agr Biol Chem*. 1974;38:2035–7.
65. Matthews BW. Solvent content of protein crystals. *J Mol Biol*. 1968;33:491–7.
66. Berejnov V, Husseini NS, Alsaied OA, Thorne RE. Effects of cryoprotectant concentration and cooling rate on vitrification of aqueous solutions. *J Appl Crystallogr*. 2006;39:244–51.
67. Battye TGG, Kontogiannis L, Johnson O, Powell HR, Leslie AGW. iMOSFLM: A new graphical interface for diffraction-image processing with MOSFLM. *Acta Crystallogr Sect D Biol Crystallogr*. 2011;67:271–81.
68. Evans PR, Murshudov GN. How good are my data and what is the resolution? *Acta Crystallogr Sect D Biol Crystallogr*. 2013;69:1204–14.
69. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. *J Appl Crystallogr*. 2007;40:658–74.
70. Langer GG, Cohen SX, Lamzin VS, Perrakis A. Automated macromolecular model building for X-ray crystallography using ARP/wARP version 7. *Nat Protoc*. 2008;3:1171–9.
71. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta Cryst*. 2010;66:486–501.

72. Murshudov GN, Skubák P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, et al. REFMAC5 for the refinement of macromolecular crystal structures. *Acta Cryst.* 2011;67:355–67.
73. Adams PD, Afonine P V., Bunkóczi G, Chen VB, Davis IW, Echols N, et al. PHENIX: A comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr Sect D Biol Crystallogr.* 2010;66:213–21.
74. Chen VB, Arendall WB, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, et al. MolProbity: All-atom structure validation for macromolecular crystallography. *Acta Crystallogr Sect D Biol Crystallogr.* 2010;66:12–21.
75. Krissinel E, Henrick K. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallogr Sect D Biol Crystallogr.* 2004;60:2256–68.
76. Robert X, Gouet P. Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res.* 2014;42:320–4.
77. Pei J, Kim B-H, Grishin N V. PROMALS3D: a tool for multiple protein sequence and structure alignments. *Nucleic Acids Res.* 2008;36:2295–300.
78. Gouet P, Courcelle E, Stuart D, Metz F. ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics.* 1999;15:305–8.

CHAPTER 3

Rational engineering of xylose isomerase from *Piromyces* sp. E2 for improved xylose metabolism by *S. cerevisiae*

3

Misun Lee¹, Daniël-Moráh Meijer¹, Elisa Rasca¹
and Dick B. Janssen^{1*}

¹Biochemical Laboratory, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

*Corresponding author

Author's contribution

ML and DBJ devised the project and wrote the manuscript. ML evaluated the data. ML, DMM and ER designed the mutations and performed the experiments.

ABSTRACT

Efficient xylose to xylulose conversion is of great interest for enabling xylose fermentation by engineered *S. cerevisiae*, which in turn is required for cost-efficient production of bioethanol from lignocellulosic biomass. Xylose isomerase from *Piromyces* sp. E2 (PirXI) is known to be one of the most effective enzymes for this conversion but still requires improvement since very high expression levels in *S. cerevisiae* are needed for rapid anaerobic xylose conversion. Inspired by the biochemical and structural properties of PirXI and comparison to homologous XIs, we exploited two rational enzyme engineering approaches. Through screening of designed mutant PirXIs by in vitro activity assays, we discovered variants with increased activity with Mg^{2+} as the metal cofactor and variants with increased activity increase at lower pH. Furthermore, we evaluated the effects of these mutants on the xylose-dependent growth of *S. cerevisiae* and discovered a disconnect between in vivo and in vitro performance of xylose isomerase, suggesting in vivo performance is influenced by as yet unidentified xylose isomerase properties.

INTRODUCTION

Incorporating xylose isomerase activity enables xylose to ethanol fermentation by *S. cerevisiae*, which is required for cost-effective second-generation bioethanol production. Previous studies on the expression of various xylose isomerases in *S. cerevisiae* showed that the enzyme from *Piromyces* is relatively well expressed in an active form [1–3]. However, a high PirXI gene copy number is required to achieve sufficient activity for rapid anaerobic xylose metabolism, suggesting in vivo activity is not optimal [4–6]. Therefore, the discovery and engineering of xylose isomerase variants with better in vivo catalytic performance is important for engineering yeast strains with enhanced fermentation properties.

Rational engineering of an enzyme requires structural and biochemical information. Xylose isomerases have mainly been investigated for their importance in industrial production of fructose syrups from starch. Sequences and crystal structures of multiple xylose isomerases are available and biochemical data have been reported [7,8]. Despite the attractive properties of PirXI for introducing the capacity to metabolize xylose in *S. cerevisiae*, no enzymological studies on the enzyme were available until we recently explored the properties of the purified enzyme and solved crystal structures. Because xylose isomerase are metalloenzymes that require two divalent metals [9], the study was focused on the metal-dependency of the enzyme (Chapter 2)

The global structures of xylose isomerases of different origin are similar. The enzymes are tetramers with subunits that are folded into a TIM-barrel motif. Two classes of xylose isomerases can be distinguished based on sequence comparison. Class II enzymes, to which PirXI belongs, generally show a 30 – 45 amino acid extension at the N-terminus as well as a longer α 1 helix as compared to the class I enzymes. Interconversion of xylose to xylulose by xylose isomerase is known to occur by metal-mediated hydride shift. Of the two divalent metal ions that are required, one assists in proper binding of the ring-opened substrate (M_1) and the other drives the hydride shift from C1 to C2 by polarizing a catalytic water molecule (M_2). The metal coordinating residues are Asp340 for M_1 and Glu269, His272, Asp308 and Asp310 for M_2 (the numbering refers to the PirXI sequence here and throughout the chapter unless otherwise stated); these are fully conserved throughout known xylose isomerases [9].

Besides the metal binding residues, several other amino acids lining the active site of xylose isomerase are highly conserved. Assuming these amino acids are involved in catalysis, it makes rational engineering of xylose isomerase for improving catalytic activity challenging. Besides the metal binding residues, ring-opening residues (His102 and Asp105) [10,11], active site hydrophobic residues (Phe61, Trp50 and Trp137) [12] and other catalytically important residues

(e.g. Lys235) [12,13] should not be mutated. Despite the similar active sites, the activity profiles of xylose isomerases are diverse [14–17], indicating that second shell residues and perhaps residues even further away from the active site are responsible for the differences. Furthermore, some residues near the active site are differentially conserved amongst class I and class II enzymes, which deserves further investigation because the functional implications are unclear.

Xylose isomerase can be activated with different types of metals giving different specific activities. The metals that are most frequently used for activity measurements are Mg^{2+} , Mn^{2+} and Co^{2+} whereas Ca^{2+} is often reported to be an inhibitor [18,19]. Other metals such as Fe^{2+} and Zn^{2+} were also found to activate some xylose isomerases [9,10,20]. Although there are a few exceptions, there seem to be differences in metal preference between class I and II enzymes. Generally, the former show the highest activity with Mg^{2+} while the latter prefer Mn^{2+} [9,14,21]. As a class II XI, PirXI shows the best activity with Mn^{2+} with k_{cat} and K_{M} values around 7 s^{-1} and 5 mM , respectively [9]. Compared to activities of enzymes involved in upper glycolysis, the activity level of PirXI does not seem to be a bottleneck for xylose metabolism [22,23]. However, due to the limited availability and low levels of intracellular Mn^{2+} , in vivo PirXI activity is expected to be much lower. Increasing activity of PirXI with a more abundant metal such as Mg^{2+} may therefore be considered as a strategy to enhance in vivo activity and improve growth on xylose.

In the current study, we explored two rational enzyme engineering approaches using available information on xylose isomerases in order to improve in vivo performance of PirXI. In the first approach, we sought to increase the catalytic activity by designing mutations using phylogenetic information. We analyzed sequences of homologous xylose isomerases and compared them to xylose isomerases with higher reported activities. We chose a class I xylose isomerase from *Actinoplanes missouriensis* (AmXI) as the reference because of its high activity especially with Mg^{2+} as the activating metal. The k_{cat} for xylose conversion of AmXI is about 4-fold higher than with PirXI while the K_{M} values are very similar [24]. In addition, the availability of extensive structural and biochemical information on AmXI makes it possible to search for structural explanations for differences in catalytic properties. The sequence and structural comparisons pointed at various residues as potential targets for engineering. These were located at varying distances from the catalytic site. Besides AmXI, we also examined XI from *Vibrio sp.* (ViXI) to support mutant design [16]. Even though ViXI belongs to the class II enzymes it shows higher activity with Mg^{2+} than with Mn^{2+} . The reported activity with Mg^{2+} is about 5-fold higher than that of PirXI. By comparing 124 (24 class I and 100 class II) XI sequences and inspecting previously solved PirXI structures using AmXI as the reference, we designed 36 mutant

PirXIs, which were constructed, expressed in *E. coli*, purified and characterized by activity assays.

Another approach we used for improving in vivo performance of PirXI was to optimize its pH-activity profile for physiological conditions. The intracellular pH of *S. cerevisiae* is known to be around 7.0 or slightly lower while the pH optimum of PirXI is above 7.5 [9,25,26]. Furthermore, since the fermentation process would be performed with an acidic hydrolysate of hemicellulose as the substrate, the in vivo pH of *S. cerevisiae* may be lower than 7.0. Therefore, we hypothesized that lowering the pH optimum of PirXI or increasing enzyme activity at lower pH can improve its in vivo performance and growth on xylose. Changing the pH optimum of xylose isomerase was explored previously for application in high-fructose corn syrup production, where acidic conditions are beneficial for the reaction equilibrium [27,28]. Xylose isomerases isolated from different organisms may show rather different pH optima. Interestingly, some enzymes sharing high sequence similarity show different pH optima, indicating that it may be feasible to change the pH optimum by replacing just a few amino acids [27,29–32].

In order to lower the pH optimum of an enzyme, the pK_a of active site residues of which the protonation state influences the reaction should be considered. If the deprotonated state has higher activity, the pK_a of such a group must be lowered. His272, together with three acidic residues (Glu269, Asp308 and Asp310), coordinates the catalytic metal (M_2) and its role was proven through several mutagenesis studies [24,33]. To serve as a ligand, the metal-coordinating N ϵ 2 atom of the histidine needs to be deprotonated [10]. A loss of metal-binding by protonated His272 would certainly influence the activity. Unlike the metal-coordinating acidic residues which are most likely always deprotonated at *S. cerevisiae* physiological pH, the N ϵ 2 atom with its higher pK_a value might become protonated when the pH is decreased to below the physiological value. Neutron diffraction studies on xylose isomerase from *S. rubiginosus* have shown that around 50% of the N ϵ 2 atoms are protonated, which indicates a pH-sensitive and vulnerable coordination between the metal and His272 [10,34]. Therefore, we hypothesized that lowering the pK_a of His272 can increase the activity of xylose isomerase at lower pH.

The pK_a of a titratable group in a protein is influenced by its electrostatic environment [35,36]. Change in electrostatic interactions of a residue can be achieved by modification of surrounding charges [36,37]. In particular, to lower the pK_a of a (de)protonatable side chain, the net positive charge within the affected area should be increased either by introducing positively charged residues or by removing negatively charged residues [38]. Accordingly, Cha et al. previously shifted the pH optimum of xylose isomerase from *S. rubiginosus* to a slightly lower value by removing negatively charged groups located near

active site histidines [27]. Considering this principle, we have designed mutations to introduce positively charged residues (arginine and lysine) or to remove negatively charged ones (glutamate and aspartate). Taking the location and predicted effect on stability of the mutations into consideration, 93 PirXI variants were constructed and tested for improved activity at lower pH.

The primary objective of this study is to rationally engineer PirXI to change its properties which ultimately can improve the *in vivo* functional activity of the enzyme. In this report, we describe rational mutant design inspired by phylogenetic and structural analysis. We also report the construction, screening and *in vitro* characterization of the mutants as well as the effect of promising mutations on growth of *S. cerevisiae* on xylose. Based on the results, we evaluate the use of rational engineering of PirXI for improving yeast xylose metabolism.

RESULTS AND DISCUSSION

1. Engineering of PirXI based on phylogenetic analysis

Mutant design and construction

Despite their highly conserved active sites, xylose isomerases show diverse activity profiles, indicating that interactions of second shell and more distant residues with catalytic and/or other active site residues influence the catalytic properties. Differences in electrostatic environment may have an effect on the strength of polarizing interactions with substrate and water and thereby the rate of hydride transfer or the rate of other chemical steps in the catalytic cycle. To examine the possibility to engineer the activity of PirXI by replacing residues that influence electrostatic interactions in the active site, we designed small sets of mutants using structural information of PirXI [9] as well as biochemical and sequence information on related XIs [16,24]. An important aim was activity improvement in the presence of Mg^{2+} as it is a relatively abundant divalent metal ion *in vivo* [39]. We used *in silico* methods including sequence and structural comparisons to identify promising mutations, paying special attention to the sequences of AmXI and ViXI, which are reported to have higher *in vitro* activity than PirXI [16,24]. They also have a metal preference for Mg^{2+} [16,40], unlike PirXI which prefers Mn^{2+} for activity. Some mutations introducing AmXI and ViXI residues in PirXI were expected to improve activity with Mg^{2+} .

For sequence comparison of class I and class II XIs, we generated consensus sequences for each class using Geneious. Structure-based sequence alignments of PirXI with AmXI, ViXI and the consensus sequences were used for selection of target positions (Figure 1). The direct metal-binding residues and other

Figure 1. Structure-based sequence alignment of xylose isomerases.

Fully conserved residues are shown with red background and similar residues are indicated with red-colored letters. The residues in green background are differently conserved between class I and class II enzymes. The cyan background indicates the metal binding residues. The red stars indicate the residues that were targeted for phylogenetic approach mutagenesis. A. m: *Actinoplanes missouriensis*

residues which are fully conserved among these proteins were excluded from the mutant library design. Of the selected residues, six (indicated in red in Table 1) show different distribution between the two XI classes. Other residues such as Ala55 or Gln60 of PirXI were selected as these amino acids are infrequent at the corresponding positions in other class II xylose isomerases.

According to this comparison, we decided to substitute PirXI amino acids at 24 selected positions by the corresponding residues in the aligned AmXI or ViXI sequence or by the most frequent residue in class I XIs. In the final set of 36 PirXI mutants most substitutions are towards the AmXI sequence and a few, such as V104T, G147S and H148N, are towards the ViXI sequence (Table 1). Considering possible interactions between neighboring residues, 6 double or triple mutants were also designed. For example, residue Asn271 is very close to Gly301 so that a G301Q substitution adopted from class I XIs might cause a steric clash. Since a glycine is present in class I enzymes at the position of Asn271, we designed the double mutant N271G-G301Q.

Library screening

For screening the designed 36 mutant library, we expressed and purified the mutants in 96-well plate format. All variants gave soluble protein and were isolated in good purity, similar to wild-type PirXI. Promising mutants were detected by measuring activities with Mg^{2+} , Mn^{2+} or Ca^{2+} at a high xylose concentration (200 mM, saturating for wild-type except for Ca^{2+} -bound enzyme) and at a lower near-in vivo concentration (5 mM). The effect of the mutations on the isomerase activities seemed to be metal-dependent. Most variants showed an activity change which was more pronounced for one of the metals (Figure 2). For example, mutations that improved activity with Mg^{2+} decreased or did not influence activity with Mn^{2+} . Similarly, mutants with improved activity with Mn^{2+} were less affected in Mg^{2+} -dependent activity. A few mutations improved the activity with Ca^{2+} , especially the triple mutant C54R-A55W-V104T had an 8-fold higher activity with this metal than wild-type XI.

Given the importance of Mg^{2+} for the in vivo activity of PirXI, as suggested by high in vivo availability of Mg^{2+} [39], we selected mutants that showed increased activity with Mg^{2+} for further verification. These are variants A55W, Q60A, Q60P, A55W-Q60A, A55W-Q60P, C54R-A55W-V104T, and A55W-E56G-N71L. In addition, we selected the PirXI mutants T248V and V270I since these showed increased activity with Mn^{2+} while maintaining activity with Mg^{2+} . The selected variants were biochemically characterized and their performance in *S. cerevisiae* was examined (see below).

The variants W140M, G301Q and T309N showed decreased activity with all metals indicating that the replaced residues are important for PirXI activity

Table 1. PirXI mutations designed based on phylogenetic information.

Mutations	PirXI	Class I*	AmXI	Class II*	ViXI	Remarks
A55W	A55	W, N, Y, V	W	W, A, G, M	W	At the potential substrate entrance
A58R	A58	R, A	R	A, G, T, S	A	At the butterfly dimer interface
Q60A Q60P	Q60	P, A	P	P, M, Q, I, V	I	At the butterfly dimer interface
V104D V104N	V104	D, N, E	D	V, A, R, T, R, I	T	On the second shell, next to the ring opening residue Asp105
E109F	E109	F, R	F	E, D, T	E	At the potential substrate entrance and near surface
W140M	W140	M	M	W	W	Near the substrate and metal M1
G147T G147S	G147	T, S	T	S, T, G, D, T, I, M	S	At the potential substrate entrance
H148N	H148	H, D, A	H	N, H	N	At the butterfly dimer interface
L231A	L231	A	A	L, Y	L	On the second shell
Q243I	Q243	I	I	Q	Q	On the second shell. Possible interaction with Lys235
T248I T248L T248V T248S	T248	V, A, I, L	A	V, A, T, S	T	On the second shell
V270T V270A V270I	V270	V, T, F	T	A, Q, V, C, T	A	Near the metal M2 binding site, in between conserved residues
N271G	N271	G, A	G	N, G	N	Near active site. Possible interaction with a metal binding residue Asp308
A273D	A273	E	E	A	A	On the second shell
T274Q T274W	T274	Q, T	Q	T, I, W	T	At the diagonal dimer interface
L275M	L275	M	M	L	L	On the second shell
G301Q	G301	Q	Q	G, N, R	G	At the diagonal dimer interface
T309Q T309N	T309	Q	Q	T, I, W	T	in between two Asp308 and Asp 310 residues which bind the metal M2
K342R	K342	K, H	K	K, H	K	Interaction with the metal M2 binding residue Asp310
T343P	T343	P, A	P	V, L, T, A	V	On the second shell
R344S	R344	P, S, L	S	R	R	Interaction with Lys342
C54R-A55W-V104T	C54			C, V, T, A, R, G, D	R	
A55W-E56G-N71L	E56 N71	Q, T, V, E -	Q -	N, P, E, D, T, G Q, F, D, N, I, H, E	G L	At the potential substrate entrance. Possible interaction between Glu56 and Asn71
A55W-Q60A A55W-Q60P						
N271G-G301Q						Interaction between N271 and G301 may be replaced by G271 and Q301

*Amino acids are ordered by abundance at each position of xylose isomerases within the classes. Residues indicated in red are differently conserved between class I and class II XIs.

independent of the nature of the bound metal. Most other mutations tested did not change the activity. Residue Trp140 is located in the vicinity of the active site near C5 of xylose. This Trp is fully conserved in class II enzymes while all class I XIs have a methionine at this position. An exception to this is a novel class I xylose isomerase from *Streptomyces* sp. F-1 that has an Ala at the corresponding position which contributes to the different catalytic properties of this ortholog XI [41]. The activity loss caused by the W140M mutation is not easily understood from an inspection of the structure. It is possible that the additional space created by the W140M substitution lower the activity. In an earlier study on a class II XI from *Clostridium thermosulfurogenes*, it was shown that substitutions of this Trp to a smaller aromatic residue (Phe or Tyr) decreased the enzyme activity on xylose drastically while improving the activity towards glucose especially by lowering the K_M [42]. This indicates that the Trp in class II XIs may play a role in proper xylose binding even though X-ray structures show no apparent interaction with the substrate such as ring stacking. The loss of activity of the G301Q enzyme is probably caused by clashing with Asn271. These residues are conserved in pairs: Asn271-Gly301 in class II enzymes and Gly219-Gln249 in class I XIs (residue numbered by AmXI sequence). In fact, mutating both residues (N271G-G301Q) recovered the activity of the wild type.

Characterization of selected variants

The 9 mutants mentioned above and V270A were expressed at larger scale (50 ml), purified and their kinetic parameters were measured in the presence of Mg^{2+} or Mn^{2+} (Table 2). Single mutations in PirXI at positions 55 and 60 (A55W, Q60A and Q60P) increased the k_{cat} in the presence of Mg^{2+} more than 2-fold. Interestingly, while A55W and Q60P maintained the k_{cat} with Mn^{2+} or showed a slight decrease, Q60A exhibited only half the activity of wild-type PirXI and a 3-fold higher K_M for xylose in the presence of Mn^{2+} . Combining the mutations at these positions (A55W-Q60A and A55W-Q60P) gave no synergistic effect on activity in the presence of Mg^{2+} ; the double mutants showed kinetic properties similar to the single mutants. Rather, the k_{cat} of both double mutants with Mn^{2+} decreased to half of that of single mutants Q60A and Q60P.

These results indicate that amino acids at positions 55 and 60 contribute to the difference in metal preference of PirXI compared to class I enzymes [14] and ViXI. Most xylose isomerases (81% of the class I and 65.7% of the class II enzymes) have a Trp at position 55, with varying amino acids at this position in other enzymes. Position 55 is at the substrate entrance, not far from the active site and the region seems to be spacious enough to accommodate a diverse size range of amino acids (Table 1 and Figure 3). AmXI and ViXI also have a Trp at position 55, but PirXI has an Ala, creating a more spacious active site entrance (Figure 3).

Figure 2. Activity changes of PirXI variants. Specific activities (U/mg) of purified PirXI variants were measured in the presence of 1 mM of Mg^{2+} , Mn^{2+} or Ca^{2+} . Fold changes of specific activities of the mutants compared to wild-type PirXI are plotted. The red dotted lines indicate no change. For Mg^{2+} - and Mn^{2+} -dependent activities, saturating (200 mM) and near K_M (5 mM) concentrations of xylose were used. Because of the high K_M (molar range, [9]) only the higher xylose concentration was used for measuring the activities with Ca^{2+} .

The smaller substrate entrance channel in the A55W mutant of PirXI may cause the slight increase in $K_{Mxylose}$. Since a hydrophobic environment is required for efficient hydride transfer [43,44], we speculate that the presence of the bulkier hydrophobic Trp at this position probably causes more efficient shielding of the hydride ion from solvent, resulting in increased activity.

At position 60, the most frequent amino acids are Pro and Ala in class I enzymes and Pro and Met in class II XIs. Only a few xylose isomerases, including PirXI, have a Gln. Residue 60 is located in a loop at the interface of the monomers that form the butterfly dimers and is between the conserved Asp59 (in class II XIs) and Phe61 (fully conserved in class I and class II enzymes)(Figure 4)[9]. It is possible that mutations at position 60 influence the activity by changing the interaction between the monomers even though a mechanistic explanation for such an effect is lacking. Mutations at either position of the substrate entrance (A55W and Q60A/Q60P) increased the K_M for xylose compared to the wild type, resulting in slightly lower catalytic efficiencies.

Phe61 residues from chain A and C are part of the active sites of chains B and D, respectively, and vice versa, possibly by interacting with Trp189 and providing hydrophobicity in the active site. While Asp59 and Phe61 are conserved, residue 60 is diverse. The Gln residues present at position 60 in PirXI between chains A and C and chains B and D are in very close proximity, forming a hydrogen bond. In the class I xylose isomerase AmXI, alanines are at this position with a distance of 4.5 Å between the carbons of the methyl groups. Here, a hydrogen bond between the chains of the butterfly dimer is formed by the nearby Arg23 residues which corresponds to Ala58 of PirXI (Figure 4). The loss of this inter-chain interaction in the Q60A and Q60P mutants might change the conformation at the substrate entrance, resulting in an increased $K_{Mxylose}$. The interactions that cause the increase in catalytic activity (k_{cat}), on the other hand, are difficult to identify rationally.

Table 2. Kinetic properties of selected PirXI variants.

Metal	Kinetic parameters					
	Mg^{2+}			Mn^{2+}		
Mutation	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1} \cdot M^{-1}$)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1} \cdot M^{-1}$)
WT	2.5 ± 0.1	3.5 ± 0.2	714	7.7 ± 0.1	4.3 ± 0.1	1791
A55W	5.3 ± 0.1	10 ± 0.8	530	5.6 ± 0.1	7.5 ± 0.4	747
Q60A	6.3 ± 0.1	12.7 ± 0.7	496	3.7 ± 0.1	15 ± 0.9	247
Q60P	5.9 ± 0.2	11 ± 1.1	536	6.2 ± 0.1	4.6 ± 0.3	1348
A55W-Q60P	5.3 ± 0.2	12.4 ± 1.3	427	3.1 ± 0.1	6.1 ± 0.4	500
A55W-Q60A	6.0 ± 0.1	16 ± 0.8	375	1.5 ± 0.1	16 ± 1.4	94
C54R-A55W-V104T	4.0 ± 0.1	6.5 ± 0.4	608	6.7 ± 0.1	8 ± 0.4	838
A55W-E56G-N71L	5.4 ± 0.2	11 ± 1.4	491	5.0 ± 0.1	9.1 ± 0.9	549
T248V	2.7 ± 0.1	4.3 ± 0.4	628	9.0 ± 0.2	4.9 ± 0.5	1818
V270A	3.7 ± 0.1	6 ± 0.5	617	5.4 ± 0.1	7.9 ± 0.4	684
V270I	2.8 ± 0.1	5 ± 0.4	560	9.5 ± 0.2	5.1 ± 0.3	1863

Figure 3. Structural view of the substrate entrance of PirXI (A) and AmXI (B). The structures are colored by CPK convention (C = grey, N = blue, O = red, S = yellow). Xylose molecules in the active site are shown as yellow sticks and magnesium ions are depicted as light green spheres. Ala55 of PirXI and the corresponding residue Trp20 of AmXI are shown as sticks and their positions are marked with dotted black circles.

Figure 4. Interfaces within the butterfly dimer of PirXI (A, PDB 5NH7) and AmXI (B, PDB 6XIM). The selected mutations and metal binding residues are shown in sticks. Xylose and Mg^{2+} are depicted as a yellow sticks and green spheres, respectively.

Variants with additional mutations in the vicinity of Trp55, generating C54R-A55W-V104T and A55W-E56G-N71L, maintained the activity of the single mutant A55W. Although the k_{cat} of variant C54R-A55W-V104T is slightly lower than that of A55W, its decreased K_{M} shows that the additional mutations compensate the negative effect of the single A55W mutation on substrate binding. The substitutions in the triple mutant introduce the same amino acids as present in ViXI. The mutations C54R and A55W seem to make the entrance site for the substrate smaller and the interaction between Thr104 and Arg54 might further reduce the size of the entrance site (Figure 5A, B). In mutant A55W-E56G-N71L, loss of the Glu-Asn hydrogen bond (Figure 5C, D) would make the loop on which Trp55 sits more flexible, bringing the residue closer to the active site. Whereas it apparently does not increase the positive effect of A55W on the catalytic rate, the structural changes could improve binding and thereby lower the K_{M} for xylose.

Mutations at positions 248 and 270 were tested to examine the role of hydrophobicity changes near the active site on activity. All class I XIs have hydrophobic amino acids (Ala196 in AmXI) at the position corresponding to Thr248 of PirXI. Class II XIs also predominantly have a hydrophobic amino acid at this position except a few, including PirXI and ViXI, which have a threonine and a serine, respectively. We substituted Thr248 of PirXI to either hydrophobic residues (Ile, Leu or Val) or to Ser (Table 1). The screening results showed that the T248S substitution did not change the activity and replacing Thr248 to Ile or Leu decreased activity with both Mg^{2+} and Mn^{2+} . Interestingly, introducing a Val, which is most frequent at this position, increased the activity with Mn^{2+} slightly while maintaining the activity with Mg^{2+} (Table 2)

The hydrophobicity of PirXI at position 270 which is located near the M2 binding site, also varies among xylose isomerases. We substituted the Val270 of PirXI to the polar residue Thr (found in AmXI), or to the hydrophobic residues Ala (present

in ViXI) or Ile. These substitutions did not affect the activity with Mg^{2+} . On the other hand, in the presence of Mn^{2+} , a slight increase in hydrophobicity (V270I) at position 270 improved the activity while mutations reducing the hydrophobicity (V270T and V270A) decreased the activity. Mutations that make the active site more hydrophobic thus differently change the contribution to catalysis of active site Mg^{2+} and Mn^{2+} .

Figure 5. Xylose isomerase structures at mutation sites. The left panels (A, C) and the right panels (B, D) show the structure of PirXI (5NH7) and the YASARA model of the structure of ViXI, respectively. The mutated residues of PirXI Thr103, Arg53 and Trp54 are shown in green sticks and the corresponding residues in ViXI model structure are shown in cyan sticks. Xylose and Mg^{2+} are depicted as yellow sticks and green spheres, respectively. The metal-binding residues are shown as grey sticks.

Effect of PirXI variants on growth of *S. cerevisiae* on xylose

In order to evaluate the influence of the mutations on in vivo enzyme performance and xylose metabolism, we evaluated the growth of *S. cerevisiae* carrying different PirXI variants. Three single mutants with improved Mg^{2+} -dependent activity (A55W, Q60A and Q60P) and two single mutants with improved activity with Mn^{2+} (T248V and V270I) were selected and transformed to yeast cells. The double and triple mutants were excluded because of the small activity differences compared to the single mutants. Although measuring the absolute growth rate in microtiter plate format is not very accurate due to volume changes and poorly controlled factors such as oxygen availability, the use of parallel cultures of independent clones and averaging their growth rates made a reliable comparison of different XI variants possible. Surprisingly, all five mutants did not exhibit a positive effect on growth on xylose (Figure 6).

Cells containing the PirXI variants A55W or T248V showed growth curves similar to cells expressing wild-type PirXI. The mutant PirXIs Q60A, Q60P and V270I seem to negatively affect growth on xylose as cells containing these variants grew slower than the wild type and visible growth started 10 – 20 h later. It is intriguing that variants with similar kinetic properties show different growth properties as indicated by the effect of A55W and Q60P as well as T248V and V270I. Overall, the improved in vitro activity of these mutants did not contribute to better growth of *S. cerevisiae* on xylose. One of the possible reasons for this discrepancy might be the slightly higher K_M of the mutants compared to the wild type, although calculated substrate concentrations at which the activity of the mutants would be lower than the wild type do not fully support this proposition. For example, based on the kinetic parameters of Mg^{2+} -dependent activity, Q60P and V270I would have lower activity than wild type at xylose concentrations lower than 2 mM and 9 mM, respectively. However, Q60P showed a larger negative impact on growth as cells harboring Q60P grew slower than the ones with V270I. Furthermore, cells containing A55W of which the activity would be lower than the wild type at 2.3 mM of xylose still showed a comparable growth profile to the wild-type cells. This indicates that other factors than the measured kinetic parameters of the enzymes are responsible for variations of in vivo performance.

Figure 6. Effect of PirXI variants on growth of *S. cerevisiae*.

S. cerevisiae containing the selected PirXI variants were grown on 2 % xylose and growth was monitored in a micro-titer plate reader for 120 h. Initial inocula of all cultures were adjusted to $OD_{600} \sim 0.02$. In all panels, the green and the red lines show the growth of the wild type and the mutant, respectively. Individual lines represent biological replicates.

2. Engineering the pH-activity profile of PirXI

Mutant design and construction

Considering the sub-optimal activity of PirXI at the physiological pH of *S. cerevisiae*, we pursued an engineering approach aimed increasing in vivo performance of PirXI by lowering its pH optimum. For this, we designed mutations that would reduce negative charge in the active site, allowing a decrease in pK_a of active site groups that must be deprotonated for activity and thus may downshift the lower limb of the pH-activity curve. We first performed in silico design of mutations which would cause an increased overall positive charge of the protein. Glutamates and aspartates were individually replaced by glutamine, asparagine or alanine. In addition, all negatively charged and neutral residues were individually substituted in silico to arginine and lysine. Based on the wild-type PirXI structure (5NH7), all these mutant structures were modeled using YASARA, and their folding energies were subsequently calculated by FoldX 4 [45]. We excluded potentially unstable mutants with calculated $\Delta\Delta G_{\text{fold}}$ of $> +5$ kJ mol⁻¹ from the initially designed mutants. In total 93 variants were selected based on structural inspection and predicted stability, giving priority to mutations that are closer to the active site. Metal binding residues and other residues important for activity of the enzyme were excluded. The 93 mutations were distributed over the structure of the protein (Figure 7).

Figure 7. Positions of mutated residues for pH-optimum change. Xylose (magenta) and the metal-binding residues are depicted as sticks (PDB 5NH7). Mg^{2+} ions are shown as green spheres. Residues which were mutated to introduce a positive charge or to remove a negative charge are shown as cyan- and red-colored spheres, respectively in a monomer of the tetramer structure.

pH-activity profiles of the selected variants

All 93 PirXI mutants were obtained by site-directed mutagenesis and 77 of them were expressed and could be purified at small scale using the 96-well plate format. The yields of other mutants (T52K, T142R, N144K, M210R, Q243K, D245K, D245R, T250K, H282K, G283K, G283N, D289K, D289R, E325K, E325R and T343R) were very low, either due to low expression or insolubility. SDS-PAGE analysis of randomly selected variants showed that the purity of isolated variants was as good as wild-type.

To identify mutants with decreased pH optimum or increased activity at low pH, activities of the PirXI mutants were measured at three different pH values (6.5, 7.0 and 7.5) (Figure 8). The variants were re-constituted with Mg^{2+} which is a relatively abundant metal in vivo [46] and gives moderate activity in vitro. Generally, each mutation had a similar effect (increasing or decreasing) on the activity at all three pH values, and no clear shift of the optimum pH was observed. Four mutations (E109A, E109N, E115Q and E184Q) improved activity, especially at lower pH (6.5 and 7.0). Interestingly, we noticed that charge differences at positions 115 and 184 may contribute to the differences in pH-optimum between homologous XIs. XI from *Streptomyces rubiginosus* (SrXI) shows a pH optimum of 8.5 – 8.8 [27] while XI from *Streptomyces* sp. SK (SskXI) shows a much lower pH optimum of 6.0 – 6.5 [30,47] even though they share over 90 % sequence identity. The amino acid at position 132 (corresponding to Glu184 of PirXI) is glutamate in SrXI but lysine in SskXI. Similarly, residue Glu114 (Glu115 in PirXI) of XI from *Thermoanaerobacterium thermosulfurigenes* (TtXI) is Lys114 in the related XI from *T. thermosulfurigenes* strain JW/SL-YS 489 (JwXI). These two enzymes share 97 % sequence identity but JwXI shows a slightly lower pH optimum than TtXI [29].

For further analysis, these mutant XIs were expressed at larger scale (50 ml) and purified. Activities of freshly purified wild-type PirXI and the mutants were measured at four different pH values ranging from 6 to 7.5 (Figure 9). In addition to activity measurements with Mg^{2+} , which we were performed in the initial assays, we also measured activities with Mn^{2+} to test the metal-dependence of changes in activity. Among the mutants, E109A performed the best in terms of the activities in the presence of Mg^{2+} as well as Mn^{2+} (Figure 9). The mutant showed up to 1.5 to 2-fold higher activity than wild-type at low pH with Mn^{2+} and Mg^{2+} , respectively. To a lesser extent, the activities of the other mutants were also increased at all pH values, except for mutant E184Q which had lowered activity with Mn^{2+} at the higher pH.

The results also show that the pH optimum of xylose isomerase variants can be influenced by the type of activating metal [9,14]. In the presence of Mg^{2+} as the activating metal, the pH optimum of the mutants E109A, E109N and E184Q

decreased slightly giving a maximum activity at pH 7.0 and 7.5, in contrast to the wild-type pH optimum of >7.5. Activity of all four PirXI variants and of the wild-type XI drastically decreased at pH 6. Although less drastic, the activity of wild-type PirXI with Mn^{2+} also decreased at lower pH, similar to our previous observations [9]. The mutant E115Q showed a similar pH-activity profile in the presence of Mn^{2+} as the wild type but with higher activity at all pH values. Interestingly, in the presence of Mn^{2+} variants E109A, E109N and E184Q did not show a significant decrease in activity at lower pH and they displayed the highest activity at pH 6.5. In contrast to its increased activity with Mg^{2+} at all pH values, mutant E109N showed decreased activity with Mn^{2+} over the whole pH range as compared to wild type, indicating the effect of a mutation on activity may be strongly metal-dependent. It also implies that metal specificity of xylose isomerase can be engineered by introducing mutations that influence active site electrostatics, confirming earlier work [15]. Overall, the activity with Mn^{2+} of all variants, including the wild-type PirXI, remained relatively high at pH 6.0. This means that if reduced activity at low pH is a detrimental for in vivo performance of PirXI, this is not primarily due to Mn^{2+} -bound enzyme. Considering all the results, mutant E109A seems to be the best variant, showing increased activities at all pH values measured in the presence of both Mg^{2+} and Mn^{2+} .

Figure 8. Screening of 93 PirXI mutants for decreased pH-optimum.

Activities of purified PirXI variants were measured with 1 mM Mg^{2+} at pH 6.5, 7.0 and 7.5. Measurements were performed in duplicate and the average values were compared to wild-type activities. Average activity values of two biological duplicates of the wild type were used for the comparison. The red dotted line indicates no change in specific activity compared to the wild type.

Figure 9. pH-activity profile of selected PirXI variants. Activities were measured with 200 mM xylose at 30 °C and pH 6.0, 6.5, 7.0 and 7.5 in the presence of 1 mM Mg^{2+} (left) or Mn^{2+} (right). Error bars represent the standard deviation of triplicate measurements.

For precise estimation of pH optima, activities at multiple pH values in the range of 5.5 to 8.0 were measured in the presence of Mg^{2+} (Figure 10, left). The pH optimum of variants E109A, E109N and E184Q was around 7.25, which is slightly lower than the wild-type pH optimum of around 7.5. For possible synergistic effects on lowering the pH optimum, we constructed a double mutant E109N-E184Q. Indeed, combining the single mutations seemed to have an additive effect since the double mutation further decreased the pH optimum of the enzyme to around 6.75. However, the overall activity of the double mutant was decreased compared to the single mutants. Interestingly, activities of the double mutant at pH below 6.75 remained higher than the activities of the wild-type XI.

Figure 10. Effect of combining single mutations (left) and effect of charge inversion (right) on PirXI pH-activity profile. Activities on 200 mM xylose were measured at 30 °C in the presence of 1 mM Mg^{2+} . The error bars represent the standard deviation of duplicate measurements.

The number of charges that is changed in the active site is expected to affect the magnitude of the pH optimum shift, provided that a mutation itself does not directly interfere with activity. As an example, we examined the influence of different mutations at position Glu184 on the pH optimum (Figure 10, right). In contrast to the insignificant effect on the pH optimum of removing negative charge (E184Q and E184A), introducing a positive charge (E184K) resulted in a more obvious change in pH optimum. Mutant E184K showed a pH optimum shift to around 7 and a greater activity increase at lower pH compared to E184Q and E184A. As mentioned above, the amino acid at the corresponding residue is Lys in SskXI whose pH optimum is more than 2 units lower compared to the very close homolog SrXI confirming the large impact of this residue on the pH optimum.

The screening results showed that there are several other potential mutants such as Q60R, V104R and G147R with a shifted pH optimum. From the activity measurements at 3 different pH values, we suppose that the pH optimum of the mutants was lowered because activity was mainly reduced at the higher pH. Although these mutants were not characterized further in view of the reduction in catalytic activity, it supports the principle that altering electrostatics by lowering the pK_a of nearby groups shifts the pH-activity profile to lower pH values in xylose isomerase. Previously, removing a negative charge at the residue corresponding to Val104 of PirXI in XI from *Streptomyces rubiginosus* (D54N) was shown to lower the pH optimum of the enzyme about by 0.5 unit [27]. Interestingly, residue Val104 in PirXI is located adjacent to Glu109, close to the active site, and removing negative charge at this position (E109A) lowered pH optimum while improving the overall activity of the enzyme as described above.

Figure 11. Distances between the selected mutated residues and His272.

Metal binding residues (grey) and substrate xylose (yellow) are shown in sticks and Mg^{2+} ions are depicted in green spheres. The distances from the side chain oxygen atoms to Ne2 are shown in yellow dotted lines.

Mutations close to the active site were expected to have a larger effect on the pK_a and thus more likely affect activity [38]. Interestingly, the pH optimum of PirXI was influenced more by mutations distant from the active site than by nearby mutations (Figure 11). The distance of the metal-ligand His272 N ϵ 2 to the side-chain oxygen atom of the nearest mutated Glu residue is around 20 Å. The distance to Glu115, which is located at the surface of the protein, is even larger, around 30 Å. A change in the active site pK_a by long range electrostatic interactions was shown in previous studies [48,49].

The screening results showed that some mutations significantly decreased activity (Figure 8) for some of which the loss of activity can be rationalized. Thr142 seems important for binding of xylose via a water molecule and perturbing these connections by the T142K mutation caused loss of activity. His51 and Asn269 interact with metal binding residues Asp340 and Asp308, respectively. Mutation of these residues to Lys or Arg may perturb the orientation of the connected metal-binding residues, disturbing metal binding. Residues Val270 and Asn271 are near the active site with the side chains pointing away from the active site, and substitution of these residues to a bulky Arg inactivated the enzyme probably due to difficulties in accommodating a bulky positively-charged group. The loss of activity in other inactive mutations seems to be due to disturbed interactions between monomers as all affected residues are located at interfaces.

Effect of net charge on the metal specificity

Previously, a study of AmXI showed that mutation E186Q near the active site lowered the pH-optimum and drastically decreased activity with Mg^{2+} while increasing activity with Mn^{2+} at low pH [15]. In order to see the effect of the mutation on PirXI, we produced mutant E238Q. Activity of the mutant was drastically decreased especially with Mg^{2+} as the cofactor (Figure 12A). The pH-optimum of the mutant enzyme decreased especially in the presence of Mn^{2+} , giving a maximum activity at pH 6.0. This mutant also showed a significant increase in activity with Ca^{2+} , a metal that barely activates the wild-type enzyme (Figure 12B). Apparently, perturbation of charges does not only influence the pH optimum of the enzyme but also influences metal dependence of activity. Specifically, removing a negative charge decreased PirXI activity with Mg^{2+} significantly.

To further investigate the effect of a negative charge removal on the metal dependent activity decrease, we constructed double mutants that either restore the removed negative charge (Q60E-E238Q), maintains the decreased negative charge (Q60A-E238Q) or further increases the unit change of the net charge (Q60K-E238Q). The results of activity assays showed that changing the net charge significantly affects the metal specificity of the enzyme even when two mutated

residues are far away (around 17.5 Å) from each other (Figure 12B). When the negative charge removed by E238Q was recovered by additional mutation of Q60E, producing Q60E-E238Q, the lost activities with Mg^{2+} and Mn^{2+} were restored. Furthermore, the activity with Mg^{2+} of this double mutant even slightly increased compared to the wild type. Interestingly, the increase in activity with Ca^{2+} caused by mutation E238Q vanished by addition of Q60E, the double mutant showing even lower activity with Ca^{2+} than wild type. This suggests a possibility that some potential mutants in the library with shifted pH-optimum were undiscovered due to a decreased activity with Mg^{2+} caused by removed negative charges. Lowering the pH optimum of PirXI while maintaining or increasing activity especially with Mg^{2+} seems to be almost incompatible. Therefore, to find a ‘sweet spot’ of the enzyme to decrease the pH optimum while not affecting the metal reactivity is challenging.

Figure 12. Effect of mutations influencing net charge change on the metal- and pH-dependence of PirXI activity. (A), Activities of variant E238Q were measured at a pH range between 5.5–8.0 in the presence of 1 mM Mg^{2+} or Mn^{2+} . (B), Metal specificity of E238Q and double mutants with different net charges were measured in the presence of 1 mM Mg^{2+} , Mn^{2+} or Ca^{2+} . All measurements were performed at 30 °C with 200 mM xylose (for Mg^{2+} - and Mn^{2+} -dependent activities) or 1 M xylose (for Ca^{2+} -dependent activity). Error bars represent standard deviations of duplicate measurements.

Effect of pH-optimum change of PirXI on growth of *S. cerevisiae*

Following the discovery of PirXI variants with increased activity at lower pH, we evaluated their in vivo performance. The growth of *S. cerevisiae* bearing the PirXI variants E109A, E109N or E115Q on xylose medium was tested (Figure 6). We chose these three variants because of their higher activity with Mg^{2+} at all pH values compared to the wild type. Unexpectedly, all three mutant PirXIs did not influence the growth of the cells on xylose, revealing no visible differences in growth performance compared to *S. cerevisiae* expressing wild-type PirXI. This indicates that even the clearly improved activity found with variant E109A, both

with Mg^{2+} and Mn^{2+} , did not contribute to faster xylose metabolism. This further implies that actual in vivo catalytic performance is poorly predicted by examining these mutations under in vitro conditions. In addition, the lower activity with Mn^{2+} of the E109N mutant compared to wild-type PirXI does not seem to seriously disturb the in vivo performance of the enzyme as reflected by xylose-supported growth.

CONCLUSION

We have employed two approaches for rational engineering of PirXI to detect structural features that influence in vitro catalytic activity and to ultimately obtain variants with better in vivo performance for growth of *S. cerevisiae* on xylose. To increase the chance of discovery of relevant mutations, we exploited computational screening of mutations at rationally selected positions to dismiss potentially unstable mutants and used medium-throughput 96-well plate methods for construction, purification and activity screening. Except for a few mutants which were poorly expressed, we obtained sufficient purified enzymes from 1 ml cultures of the desired mutants to allow initial activity screening.

Through the rational engineering and screening by in vitro activity assays, we discovered several improved PirXI variants with different desired kinetic properties – increased activity with Mg^{2+} and lowered pH optimum. Characterization of these new XI mutants can provide further insights in the structure – function relation of the enzyme. Even though the beneficial effect of mutations at residues such as Gln60 and Ala55 on the metal specificity of XI is not easily predicted or explained by inspection of structures, comparing enzymes which show different metal preferences did reveal them. We have also shown that charge-changing mutations at a location distant from the active site can effectively alter the pH optimum of XI, probably by long-range electrostatic interactions or unknown dynamic effects. When changing the pH optimum by doing so, careful inspection is required as the pH optimum change of XI is metal-dependent.

As opposed to our prediction that these mutants with enhanced activity would improve growth on xylose when introduced in *S. cerevisiae*, there appeared to be a disconnect between the in vivo performance and in vitro activity of the enzyme. Despite the improved activity, especially with Mg^{2+} , the mutant PirXIs did not really affect the growth of yeast on xylose. Mutants Q60A, Q60P and V270I even seem to have a slightly negative effect on growth. Several studies have shown that xylose isomerase is a limiting factor for optimal xylose utilization, which was indicated by the high copy number of XI gene and the subsequent over-expression of the enzyme [4,6,50] as well as by the improved xylose fermentation through

engineered XIs [51,52]. However, the insignificant effect of the engineered PirXI on growth shown in this study suggest that in vitro enzyme activities which are measured in defined conditions do not necessarily well reflect the in vivo enzyme activity which is affected by unpredictable factors such as metal availability and possible competition with other metalloenzyme. It is also possible that the over-expression of XI in the *S. cerevisiae* cultures with improved growth on xylose may not be directly related to the enzyme activity *per se* but could be due to a regulatory response to this unnatural carbon source. Metabolic flux and signaling pathway studies have shown an unexpected cellular response of *S. cerevisiae* on xylose which resembles that on carbon starvation [53,54]. An unknown factor in this response might have caused the high expression of the heterologous enzyme, even if not necessary for rapid growth.

We conclude that it is possible to improve the xylose isomerase activity through rational engineering. However, from an applicational point of view, we encountered some limitations to the two engineering approaches employed, especially concerning the in vivo performance of the selected mutants. To overcome these limitations, in vivo selection of the enzyme through a directed evolution approach can be performed and further characterization of selected enzymes may help understanding of the properties that limit the optimal growth of *S. cerevisiae* on xylose.

MATERIALS AND METHODS

Strains and plasmid constructs

For construction and expression of PirXI variants we used *E. coli* strain Neb10 β and a codon-optimized *xylA* gene cloned in a pBAD/myc-His-derived plasmid (Invitrogen), as described in a previous study [9]. To test in vivo performance of PirXI variants, the *S. cerevisiae* codon-optimized PirXI gene-expression cassette P_{TPH1}-*XylA*-T_{CYC1} was obtained from the group of Prof. Jack Pronk (TU Delft) and cloned into the 2 μ plasmid pRS426-URA using SacI and SalI restriction sites. Using this construct and constructs with relevant PirXI mutations, we expressed PirXI variants in CEN.PK derived *S. cerevisiae* strain DS75543.

Design of PirXI mutants

For mutant design based on the phylogenetic information, the sequence (accession P10654) and structure (PDB 5XIN) of xylose isomerase from *Actinoplanes missouriensis* (AmXI) were used as a reference. The sequence of xylose isomerase from *Vibrio* sp. (ViXI, accession C7G532) was also used for comparison. Since the structure of ViXI was not available, a homology model

was created using YASARA. A crystal structure of PirXI (5NH7) was used as the template. For consensus sequence analysis, a multiple sequence alignment of sequences from 100 class II and 24 class I xylose isomerases was performed.

Structure-based sequence alignment

Structure-based sequence alignments of PirXI (5NH7), AmXI (6XIM), ViXI (homology model), and class I and class II consensus sequences were performed using Promals3D [55]. The consensus sequences were generated based on 24 class I and 100 class II sequences using Geneious 8.1.5 (<https://www.geneious.com>). The alignment figure was created using ESPript [56].

Site-directed mutagenesis

All mutants were constructed by QuikChange site-directed mutagenesis. For PCR, PfuUltra II Hotstart master mix (Agilent) was used in 20 μl total reaction volumes following the manufacturer's instructions. Subsequently, 20 U of DpnI was added to the reaction mixture, which was incubated at 37 °C for 3 h. Next, 5 μl of the reaction mixture were used to transform 50 μl Neb10 β cells. The transformation of RbCl₂-competent Neb10 β cells was done using a heat-shock method. After the heat-shock, 500 μl of LB medium were added and the cells were incubated for 30 min at 37 °C. In view of the large number of mutants, the aforementioned steps were carried out in 96-well plate format. For plating transformed cells, 24-well plates containing LB agar with 50 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin were used. 50 μl of cell-suspension were added to each well followed by gentle swirling for equal distribution of the cells. The plates were dried and subsequently incubated overnight at 37 °C, after which individual colonies were picked.

Expression and purification of PirXI

We expressed PirXI by growing the transformed *E. coli* cells overnight under inducing conditions (0.2% arabinose added) in TB medium at 37°C. For screening enzyme activity, expression was performed at small scale using 96-well format plates. In a 96-well deep-well plate, 200 μl LB medium was inoculated with Neb10 β cells containing PirXI variants and the plates were incubated at 37°C for 8 h. Subsequently, 1 ml TB medium containing 50 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin and 0.2 % (w/v) arabinose were added and cells were cultivated overnight at 37 °C. Cells were harvested and washed with 20 mM Tris·HCl (pH 8.5) and subsequently resuspended with 400 μl of lysis buffer (20 mM Tris·HCl, pH 8.5, 5 mM MgCl₂, 1 mg·mL⁻¹ lysozyme, 0.05 mg·mL⁻¹ DNase) and incubated for 30 min at 30 °C. The cell suspension was then frozen and kept at -80 °C for 45 min and thawed in a 30 °C water bath (approximately 20 min). The cell lysates were spun down at 2250xg and 4 °C for 45 min. The cell-free extracts were incubated in a 96-well filter plate

(AcroPre, Advance by Pall Corporation) for 30 min with 100 μ l of anion exchange resin (Q-Sepharose Fast Flow, GE Healthcare Life Sciences, pre-equilibrated with 20 mM Tris-HCl pH 8.5). By centrifugation at 50xg for 2 min, unbound protein (flow-through fractions) was removed and the resins were washed with 1 ml of 20 mM Tris-HCl, pH 8.5. PirXI was eluted with 200 μ l of 20 mM Tris-HCl, pH 8.5, containing 100 mM KCl, by centrifugation at 50xg for 2 min. The yield of isolated enzyme varied between individual variants in the range of 0.02 to 0.3 mg per mL of culture.

For purification of PirXI expressed at larger scale (> 50 ml), the cells were lysed by sonication with a VibraCell VB 18 instrument (Sonics) for 3 min at 70% amplitude (2 s on, 3 s off). The cell lysates were then centrifuged at 17,000xg and 4 °C for 30 min. The purification of XI variants was performed in a gravity-flow ion-exchange chromatography column (Econo-pac by Bio-Rad) using the buffers mentioned above. One ml of resin was used per 50 ml culture and the protein was eluted with 2 column volumes of elution buffer. Purification of PirXI from > 50 ml culture generally yielded 300 – 500 mg protein per L culture. The single-step purification method using ion-exchange chromatography provided high level purity of the protein – no major contaminants were visible by SDS-PAGE analysis.

Preparation of apo-enzyme

In order to measure enzyme activity with controlled metal composition we used apo-enzyme reconstituted with the metal of choice. Apo-enzyme was prepared by incubating xylose isomerase with 10 mM EDTA overnight at 4 °C. Excess EDTA and EDTA-metal complexes were removed by exchanging the buffer to 20 mM MOPS, pH 7. For enzymes purified in 96-well format this was done using a 96-well desalting plate (PD MultiTrap G-25 by GE Healthcare) following the manufacturer's manual. Buffer exchange of enzyme purified at larger scale was performed using a centrifugal filter tube (Amicon Ultra Centrifuge Filter 30K by Millipore). The EDTA and the EDTA-metal complexes were diluted out at least 1500-fold by repeated addition of new buffer and centrifugation at ca. 3,000xg and 4 °C.

Activity assays

Activities of PirXI variants were measured using a sorbitol dehydrogenase (SDH)-coupled spectrophotometric assay as described in our previous report [9]. Unless otherwise stated, the reactions were performed at 30 °C, pH 7.0 (20 mM MOPS) with 1 mM divalent metal, 200 mM xylose, 0.15 mM NADH and 1 U/ml SDH and initiated by addition of 0.05-0.2 μ M apoXI. Kinetic parameters of XI for D-xylose were determined by measuring XI activity with varying D-xylose concentrations in the range of 0.04 to 1875 mM, depending on the metal cofactor used. For pH

profiling of PirXI activities, 100 mM of MES (for pH 5.5 and 6.0), MOPS (for pH 6.5, 7.0 and 7.5) and EPPS (for pH 8.0) were used. We tested the PirXI activity at pH 6.5 with both MES and MOPS and at 7.5 with both MOPS and EPPS. As we observed no significant effect of the type of buffer on PirXI activity, we used these buffers at the given pH values for further assays.

Transformation of *S. cerevisiae*

Prior to *S. cerevisiae* transformation, PirXI variants were constructed in the pRS426-URA vector, which was amplified in and isolated from *E. coli*. Transformation of *S. cerevisiae* strain DS75543 was done using the LiAc/SS carrier-DNA/PEG method, following a protocol established by Gietz et al. [57]. The duration of the heat shock optimized for strain DS75543 was approximately 1 h. The transformants were plated on solid Verduyn medium [58] containing 2 % glucose and incubated at 30 °C for 2 – 3 days.

Growth of yeast on xylose

Growth rates of *S. cerevisiae* DS75543 transformants containing PirXI variants were measured using a microtiter plate reader (Synergy H1, BioTek). Single colonies of transformed yeast cells were inoculated in 500 µl 2 % glucose Verduyn medium in 96-deep-well plates and grown overnight at 30 °C with shaking. The next morning, the cultures were normalized to an OD₆₀₀ of 0.5 and cultivated until the mid-exponential growth phase. The cultures were then washed with 1 ml of Verduyn medium (without sugars) and resuspended in Verduyn medium supplemented with 2 % xylose. The cultures were diluted into 200 µl 2 % xylose Verduyn medium in 96-well cell culture plates (Eppendorf) to an OD₆₀₀ of 0.02. Plates were covered with optically clear gas-permeable seals (Breathe-Easy, Diversified Biotech) and the cultures were grown in the plate reader (30 °C, continuous linear shaking at 731 cpm). Growth was monitored by measuring OD₆₀₀ in 30 min intervals.

REFERENCES

1. Brat D, Boles E, Wiedemann B. Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. Appl Environ Microbiol. 2009;75:2304–11.
2. Parachin NS, Gorwa-Grauslund MF. Isolation of xylose isomerases by sequence- and function-based screening from a soil metagenomic library. Biotechnol Biofuels. 2011;4:9.
3. van Maris AJA, Winkler AA, Kuyper M, de Laat WTAM, van Dijken JP, Pronk JT. Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose isomerase as a key component. Adv Biochem Eng Biotechnol. 2007;108:179–204.
4. Zhou H, Cheng J sheng, Wang BL, Fink GR, Stephanopoulos G. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. Metab Eng. 2012;14:611–22.
5. Kuyper M, Hartog MMP, Toirkens MJ, Almering MJH, Winkler AA, van Dijken JP, et al. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res. 2005;5:399–409.
6. Verhoeven MD, Lee M, Kamoen L, van den Broek M, Janssen DB, Daran J-MG, et al. Mutations in PMR1 stimulate xylose isomerase activity and anaerobic growth on xylose of engineered *Saccharomyces cerevisiae* by influencing manganese homeostasis. Sci Rep. 2017;7:46155.
7. Bhosale SH, Rao MB, Deshpande V V. Molecular and industrial aspects of glucose isomerase. Microbiol Rev. 1996;60:280–300.
8. Jansen MLA, Bracher JM, Papapetridis I, Verhoeven MD, de Bruijn H, de Waal PP, et al. *Saccharomyces cerevisiae* strains for second-generation ethanol production: from academic exploration to industrial implementation. FEMS Yeast Res. 2017;17:1–20.
9. Lee M, Rozeboom HJ, de Waal PP, de Jong RM, Dudek HM, Janssen DB. Metal dependence of the xylose isomerase from *Pyromyces* sp. E2 explored by activity profiling and protein crystallography. Biochemistry. 2017;56:5991–6005.
10. Kovalevsky AY, Hanson L, Fisher SZ, Mustyakimov M, Mason SA, Trevor Forsyth V, et al. Metal ion roles and the movement of hydrogen during reaction catalyzed by D-xylose isomerase: A joint x-ray and neutron diffraction study. Structure. 2010;18:688–99.
11. Collyer CA, Henrick K, Blow DM. Mechanism for aldose-ketose interconversion by D-xylose isomerase involving ring opening followed by a 1,2-hydride shift. J Mol Biol. 1990;212:211–35.
12. Lambeir AM, Lauwereys M, Stanssens P, Mrabet NT, Snauwaert J, Van Tilbeurgh H, et al. Protein engineering of xylose (glucose) isomerase from *Actinoplanes missouriensis*. 2. Site-directed mutagenesis of the xylose binding site. Biochemistry. 1992;31:5459–66.
13. Fuxreiter M, Ödön F, Náray-Szabó Gábor. Molecular modelling of xylose isomerase catalysis: the role of electrostatics and charge transfer to metals. Protein Eng. 1995;8:925–33.
14. van Bastelaere PBM, Vangrupsperre W, Kersters-Hildersont H. Kinetic studies of Mg²⁺, Co²⁺- and Mn²⁺-activated D-xylose isomerases. Biochem J. 1991;278:285–92.
15. van Tilbeurgh H, Jenkins J, Chiadmi M, Janin J, Wodak SJ, Mrabet NT, et al. Protein engineering of xylose (glucose) isomerase from *Actinoplanes missouriensis*. 3. Changing metal specificity and the pH profile by site-directed mutagenesis. Biochemistry. 1992;31:5467–71.
16. Umemoto Y, Shibata T, Araki T. D-Xylose Isomerase from a marine bacterium, *Vibrio* sp. Strain XY-214, and D-xylulose production from β-1,3-Xylan. Mar Biotechnol. 2012;14:10–20.
17. Smith CA, Rangarajan M, Hartley BS. D-Xylose (D-glucose) isomerase from *Arthrobacter* strain N.R.R.L B3728. Biochem J. 1991;277:255–61.
18. Hlima H Ben, Aghajari N, Ali M Ben, Haser R, Bejar S. Engineered glucose isomerase from *Streptomyces* sp. SK is resistant to Ca²⁺ inhibition and Co²⁺ independent. J Ind Microbiol Biotechnol. 2012;39:537–46.
19. Fuxreiter M, Böcskei Z, Szeibert A, Szabó E, Dallmann G, Náray-Szabó Gábor, et al. Role of electrostatics at the catalytic metal binding site in xylose isomerase action: Ca²⁺-inhibition and metal competence in the double mutant D254E/D256E. Proteins Struct Funct Genet. 1997;28:183–93.
20. Lehmacher A, Bisswanger H. Comparative kinetics of D-xylose and D-glucose isomerase. Activities of the D-xylose isomerase from *Thermus aquaticus* HB8. Biol Chem Hoppe Seyler. 1990;371:527–36.
21. Rangarajan M, Hartley BS. Mechanism of D-fructose isomerization by *Arthrobacter* D-xylose isomerase. Biochem J. 1992;283:223–33.
22. Daran-Lapujade P, Rossell S, van Gulik WM, Luttk MAH, de Groot MJL, Slijper M, et al. The fluxes through glycolytic enzymes in *Saccharomyces cerevisiae* are predominantly regulated at posttranscriptional levels. Proc Natl Acad Sci. 2007;104:15753–8.

23. van den Brink J, Canelas AB, van Gulik WM, Pronk JT, Heijnen JJ, de Winde JH, et al. Dynamics of glycolytic regulation during adaptation of *Saccharomyces cerevisiae* to fermentative metabolism. *Appl Environ Microbiol.* 2008;74:5710–23.
24. Jenkins J, Janin J, Rey F, Chiadmi M, van Tilbeurgh H, Lasters I, et al. Protein engineering of xylose (glucose) isomerase from *Actinoplanes missouriensis*. 1. Crystallography and site-directed mutagenesis of metal binding sites. *Biochemistry.* 1992;31:5449–58.
25. Elsutohy MM, Chauhan VM, Markus R, Kyyaly MA, Tendler SJB, Aylott JW. Real-time measurement of the intracellular pH of yeast cells during glucose metabolism using ratiometric fluorescent nanosensors. *Nanoscale.* 2017;9:5904–11.
26. Valli M, Sauer M, Branduardi P, Borth N, Porro D, Mattanovich D. Intracellular pH distribution in *Saccharomyces cerevisiae* cell populations, analyzed by flow cytometry. *Appl Environ Microbiol.* 2005;71:1515–21.
27. Cha J, Batt C. Lowering the pH optimum of D-xylose isomerase: the effect of mutations of the negatively charged residues. *Mol Cells.* 1998;8:374.
28. Siddiqui KS, Loviny-anderton T, Hartley BS. Arthrobacter D-xylose isomerase: chemical modification of carboxy and protein engineering of pH optimum groups. *Biochem J.* 1993;296:685–91.
29. Liu S-Y, Wiegel J, Gherardini FC. Purification and cloning of a thermostable xylose (glucose) isomerase with an acidic pH optimum from *Thermoanaerobacterium* strain JW/SL-YS 489. *J Bacteriol.* 1996;178:5938–45.
30. Srih-Belghith K, Bejar S. A thermostable glucose isomerase having a relatively low optimum pH: study of activity and molecular cloning of the corresponding gene. *Biotechnol Lett.* 1998;20:553–6.
31. Staudigl P, Haltrich D, Peterbauer CK. L-arabinose isomerase and D-xylose isomerase from *Lactobacillus reuteri*: characterization, coexpression in the food grade host *Lactobacillus plantarum*, and application in the conversion of D-galactose and D-glucose. *J Agric Food Chem.* 2014;62:1617–24.
32. Yamanaka K. Enzymatic assay of D-xylose isomerase with D-sorbitol dehydrogenase. *Agr Biol Chem.* 1974;38:2035–7.
33. Cha J, Cho Y, Whitaker RD, Carrell HL, Glusker JP, Karplus PA., et al. Perturbing the metal site in D-xylose isomerase. *J Biol Chem Biol Chem.* 1994;269:2687–94.
34. Katz AK, Li X, Carrell HL, Hanson BL, Langan P, Coates L, et al. Locating active-site hydrogen atoms in D-xylose isomerase: time-of-flight neutron diffraction. *Proc Natl Acad Sci.* 2006;103:8342–7.
35. Russell AJ. Protein engineering of the pH dependence of subtilisin BPN' [Doctorial assertion]. *Imp. Coll. Sci. Technol.* 1987.
36. Thomas PG, Russell AJ, Fersht AR. Tailoring the pH dependence of enzyme catalysis using protein engineering. *Nature.* 1985;318:375–6.
37. Russell AJ, Fersht AR. Rational modificatin of enzyme catalysis by engineering surface charge. *Nature.* 1987;328:496–500.
38. Tynan-connolly BM, Nielsen JE. Redesigning protein pK_a values. *Protein Sci.* 2007;16:239–49.
39. Davidi D, Noor E, Liebermeister W, Bar-Even A, Flamholz A, Tumbler K, et al. Global characterization of in vivo enzyme catalytic rates and their correspondence to in vitro k_{cat} measurements. *Proc Natl Acad Sci.* 2016;113:3401–6.
40. van Bastelaere PBM, Kersters-Hilderson HLM, Lambeir A-M. Wild-type and mutant D-xylose isomerase from *Actinoplanes missouriensis*: metal-ion dissociation constants, kinetic parameters of deuterated and non-deuterated substrates and solvent-isotope effects. *Biochem J.* 1995;307:135–42.
41. Miyamoto RY, de Sousa AS, Vieira PS, de Melo RR, Scarpassa JA, Ramos CHI, et al. Crystal structure of a novel xylose isomerase from *Streptomyces* sp. F-1 revealed the presence of unique features that differ from conventional classes. *Biochim Biophys Acta - Gen Subj.* 2020;1864.
42. Meng M, Lee C, Bagdasarian M, Zeikus JG. Switching substrate preference of thermophilic xylose isomerase from D-xylose to D-glucose by redesigning the substrate by redesigning the substrate binding pocket. *Proc Natl Acad Sci.* 1991;88:4015–9.
43. Garcia-Viloca M, Alhambra C, Truhlar DG, Gao J. Hydride transfer catalyzed by xylose isomerase: mechanism and quantum effects. *J Comput Chem.* 2003;24:177–90.
44. Swan MK, Graham Solomons JT, Beeson CC, Hansen T, Schönheit P, Davies C. Structural evidence for a hydride transfer mechanism of catalysis in phosphoglucose isomerase from *Pyrococcus furiosus*. *J Biol Chem.* 2003;278:47261–8.
45. Guerois R, Nielsen JE, Serrano L. Predicting changes in the stability of proteins and protein complexes: a study of more than 1000 mutations. *J Mol Biol.* 2002;320:369–87.
46. Eide DJ, Clark S, Nair TM, Gehl M, Gribskov M, Guerinet M Lou, et al. Characterization of the yeast ionome: a genome-wide analysis of nutrient mineral and trace element homeostasis in *Saccharomyces cerevisiae*. *Genome Biol.* 2005;6:R77.

47. Ben Hlima H, Bejar S, Riguet J, Haser R, Aghajari N. Identification of critical residues for the activity and thermostability of *Streptomyces* sp. SK glucose isomerase. *Appl Microbiol Biotechnol*. 2013;97:9715–26.
48. Sternberg MJE, Hayes FRF, Russell AJ, Thomas PG, Fersht AR. Prediction of electrostatic effects of engineering of protein charges. *Nature*. 1987;330:86–8.
49. Russell AJ, Thomas PG, Fersht AR. Electrostatic effects on modification of charges groups in the active site cleft of subtilisin by protein engineering. *J Mol Biol*. 1987;193:803–13.
50. Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MSM, de Laat WTAM, et al. High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Res*. 2003;4:69–78.
51. Lee SM, Jellison T, Alper HS. Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. *Appl Environ Microbiol*. 2012;78:5708–16.
52. Katahira S, Muramoto N, Moriya S, Nagura R, Tada N, Yasutani N, et al. Screening and evolution of a novel protist xylose isomerase from the termite *Reticulitermes speratus* for efficient xylose fermentation in *Saccharomyces cerevisiae*. *Biotechnol Biofuels*. 2017;10:1–18.
53. Matsushika A, Nagashima A, Goshima T, Hoshino T. Fermentation of xylose causes inefficient metabolic state due to carbon/energy starvation and reduced glycolytic flux in recombinant industrial *Saccharomyces cerevisiae*. *PLoS One*. 2013;8.
54. Osiro KO, Brink DP, Borgström C, Wasserstrom L, Carlquist M, Gorwa-Grauslund MF. Assessing the effect of D-xylose on the sugar signaling pathways of *Saccharomyces cerevisiae* in strains engineered for xylose transport and assimilation. *FEMS Yeast Res*. 2018;18:1–15.
55. Pei J, Kim B-H, Grishin N V. PROMALS3D: a tool for multiple protein sequence and structure alignments. *Nucleic Acids Res*. 2008;36:2295–300.
56. Gouet P, Courcelle E, Stuart D, Metoz F. ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics*. 1999;15:305–8.
57. Gietz RD, Schiestl RH. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc*. 2007;2:31–4.
58. Verduyn C, Postma E, Scheffers WA, van Dijken JP. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast*. 1992;8:501–17.

CHAPTER 4

Mutations in *PMR1* stimulate xylose isomerase activity and anaerobic growth on xylose of engineered *Saccharomyces cerevisiae* by influencing manganese homeostasis

4

Maarten D. Verhoeven^{1,+}, Misun Lee ^{2,+}, Lycka Kamoen ¹,
Marcel van den Broek ¹, Dick B. Janssen², Jean-Marc G. Daran¹,
Antonius J.A. van Maris^{1,†} & Jack T. Pronk^{1,*}

¹Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands

²Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

⁺These authors contributed equally to this work

[†]Current address: Division of Industrial Biotechnology, School of Biotechnology, KTH Royal Institute of Technology, AlbaNova University Center, SE 106 91, Stockholm, Sweden.

^{*}Corresponding author

Published in Nature Scientific Reports. 2017; 7: 46155
<https://www.nature.com/articles/srep46155>

Author contributions

M.D.V., J.M.D., A.J.A.v.M., M.L., D.B.J. and J.T.P. together designed this study; M.D.V., L.K. and M.L. designed and performed all wet-lab experiments (M.D.V. and L.K. designed and performed the molecular genetic experiments); M.v.d.B. performed all analyses of genome sequencing data; M.D.V., M.L., D.B.J., A.J.A.v.M and J.T.P. wrote the manuscript. All authors read and approved the submitted version of the manuscript.

ABSTRACT

Combined overexpression of xylulokinase, pentose-phosphate-pathway enzymes and a heterologous xylose isomerase (XI) is required but insufficient for anaerobic growth of *Saccharomyces cerevisiae* on D-xylose. Single-step Cas9-assisted implementation of these modifications yielded a yeast strain expressing *Piromyces* XI that showed fast aerobic growth on D-xylose. However, anaerobic growth required a 12-day adaptation period. Xylose-adapted cultures carried mutations in *PMR1*, encoding a Golgi $\text{Ca}^{2+}/\text{Mn}^{2+}$ ATPase. Deleting *PMR1* in the parental XI-expressing strain enabled instantaneous anaerobic growth on D-xylose. In *pmr1* strains, intracellular Mn^{2+} concentrations were much higher than in the parental strain. XI activity assays in cell extracts and reconstitution experiments with purified XI apoenzyme showed superior enzyme kinetics with Mn^{2+} relative to other divalent metal ions. This study indicates engineering of metal homeostasis as a relevant approach for optimization of metabolic pathways involving metal-dependent enzymes. Specifically, it identifies metal interactions of heterologous XIs as an underexplored aspect of engineering xylose metabolism in yeast.

INTRODUCTION

In conventional feedstocks for fermentative production of fuel ethanol, such as corn starch and cane sugar, carbohydrates predominantly occur as dimers or polymers of hexose sugars. These hexose sugars can be efficiently and rapidly fermented by *Saccharomyces cerevisiae*. Economically feasible ethanol production from non-food lignocellulosic feedstocks additionally requires efficient, anaerobic fermentation of D-xylose and L-arabinose [1,2]. Although wild-type *S. cerevisiae* strains cannot ferment these pentose sugars, they can slowly convert D-xylulose [3].

In yeast species that can grow on D-xylose such as *Scheffersomyces stipitis*, its metabolism is initiated by a two-step conversion into D-xylulose by the combined activity of a xylose reductase (XR) and a xylitol dehydrogenase (XDH) [4]. The different redox cofactor preferences of XR and XDH represent a challenge in their use for constructing D-xylose-fermenting *S. cerevisiae* strains. This redox problem causes the production of substantial amounts of xylitol by anaerobic cultures of such engineered strains [5–7]. Elegant engineering strategies in which cofactor specificities of XR and/or XDH were altered, have not yet completely eliminated the formation of this by-product [8,9]. In bacteria, D-xylose conversion is often initiated by its direct isomerization to D-xylulose, catalyzed by xylose isomerase (XI) (EC 5.3.1.5). Until 2003, attempts to express heterologous XI genes in *S. cerevisiae* yielded no or very low XI activities under physiologically relevant conditions [10–13]. Then, multi-copy expression of a newly discovered xylose isomerase gene (*xylA*) from the anaerobic fungus *Piromyces* sp. E2 [14] was shown to yield high XI activity in cell extracts of *S. cerevisiae* [15,16]. *Piromyces xylA* shows strong sequence similarity with *Bacteroides* XI genes, suggesting that the fungus acquired the gene by horizontal gene transfer. Indeed, expression of XI genes from *Bacteroides* species also yielded XI activity in *S. cerevisiae* [17,18]. Consistent with the slow growth of wild-type *S. cerevisiae* strains on D-xylulose [3], functional expression of *xylA* by itself only enabled very slow aerobic growth on xylose [15,16]. Kuyper *et al.* (2005a) [19] reported that expression of *xylA* combined with constitutive overexpression of the genes encoding the native *S. cerevisiae* xylulokinase (*XKS1*, EC 2.7.1.17), ribulose 5-phosphate epimerase (*RPE1*, EC 5.3.1.1), ribulose 5-phosphate isomerase (*RKI1*, EC 5.3.1.6), transketolase (*TKL1*, EC 2.2.1.1) and transaldolase (*TAL1*, EC 2.2.1.2) was sufficient to enable anaerobic growth on D-xylose at a specific growth rate of 0.07 h⁻¹. Several subsequent studies confirmed that overexpression of a heterologous XI, combined with overexpression of xylulokinase and the enzymes of the non-oxidative pentose-phosphate pathway, is required for fast anaerobic fermentation of D-xylose [20,21]. Laboratory evolution experiments designed to further improve the kinetics of

xylose fermentation revealed expression of the heterologous XI as a key factor, as reflected by amplification of the XI gene via formation of extra-chromosomal circular DNA [22] or increased numbers of XI genes on the yeast chromosomes [21]. Other studies demonstrated improved xylose fermentation in yeast strains in which XI expression was increased by random mutagenesis, codon optimization or by mutations influencing protein folding [23–25]. Additional mutations that improve pentose-fermentation kinetics, mainly identified by resequencing of laboratory-evolved strains, affected structural genes encoding native yeast hexose transporters [26–30] and in the ‘secondary’ transaldolase and transketolase isoenzymes *NQM1* and *TKL2* [20,31]. Over a decade of intensive research on D-xylose fermentation by XI-based, engineered *S. cerevisiae* strains yielded many important insights into their physiology. However, one important and industrially relevant aspect remains incompletely understood. While an initial study [19] reported that combined overexpression of *xylA*, xylulokinase and non-oxidative pentose-phosphate pathway enzymes was sufficient to enable anaerobic growth of *S. cerevisiae* on D-xylose subsequent reports indicated that anaerobic growth on xylose required additional, as yet unidentified mutations [21,23]. The aim of the present study was to investigate the molecular basis for anaerobic growth of engineered *xylA*-expressing, D-xylose-metabolizing *S. cerevisiae*. To this end, we used CRISPR-Cas9 mediated genome editing for single-step construction of an *S. cerevisiae* strain that grew aerobically on D-xylose as sole carbon source. After adaptation to anaerobic growth in xylose-grown bioreactor batch cultures, we showed that mutations in a single gene enabled anaerobic growth on xylose. Via a combination of physiological and enzymological analyses, we investigated how these mutations affected intracellular metal homeostasis and D-xylose metabolism.

RESULTS

One-step construction of a xylose-utilizing *Saccharomyces cerevisiae* strain.

To construct a xylose-metabolizing *S. cerevisiae* strain, nine copies of an expression cassette containing *Piromyces xylA*, as well as single expression cassettes for constitutive overexpression of the native yeast genes for xylulokinase (*XKS1*) and for the enzymes of the non-oxidative branch of the pentose-phosphate pathway (*RKI1*, *RPE1*, *TKL1*, *TKL2* and *TAL1*) were introduced in *S. cerevisiae* CEN.PK113-7D. Additionally, an expression cassette for *NQM1*, a paralog of *TAL1* whose duplication has been shown to enhance pentose fermentation by engineered *S. cerevisiae* [31], was introduced. Combination of in vivo assembly [32] and CRISPR/Cas9-mediated chromosomal integration [33]

enabled a one-step introduction of all expression cassettes in the *GRE3* locus, thereby inactivating *GRE3*, which encodes a non-specific aldose reductase that can reduce xylose to xylitol [34]. The nine copies of the *xyIA* cassette were introduced as tandem repeats to facilitate adaptation of the *xyIA* copy number by homologous recombination. Transformants obtained after plating on xylose synthetic medium (SMX) plates were restreaked thrice on the same medium. The genome of the resulting strain IMX696 (Table 1), in which correct integration of the cassettes was confirmed by diagnostic PCR using primers listed in Table S3, was sequenced to assess whether mutations had occurred during growth on SMX plates. No single-nucleotide polymorphisms (SNPs), insertion/deletions in coding regions or changes in chromosomal copy numbers were observed. However, read-depth analysis revealed the presence of 36 rather than 9 copies of the *xyIA* cassette. This amplification of *xyIA* is consistent with earlier reports that showed a positive impact of high *xyIA* copy numbers on xylose metabolism by engineered *S. cerevisiae* strains [21,22,25]. In aerobic shake-flask cultures on SMX, strain IMX696 exhibited a specific growth rate of 0.21 h^{-1} (Figure 1).

Anaerobic growth on xylose requires prolonged adaptation.

Anaerobic growth of the engineered xylose-fermenting strain IMX696 was investigated in nitrogen-sparged bioreactor cultures on SMX, supplemented with the anaerobic growth factors Tween-80 and ergosterol. In duplicate experiments, CO_2 production, which was continuously monitored in the off-gas of the bioreactors, was only observed after 12 days of incubation (Supplementary Figure S1). To investigate this slow adaptation to anaerobic growth on xylose in more detail, the experiment was repeated, with regular analysis of culture viability, metabolite concentrations and growth (Figure 2). Again, no significant xylose consumption occurred during the first 12 days of the experiment. A subsequent increase in biomass concentration coincided with the conversion of xylose to ethanol and glycerol. The specific growth rate after the onset of anaerobic growth was estimated at 0.11 h^{-1} based on biomass dry weight measurements during the mid-exponential growth phase. Biomass and ethanol yield on xylose were $0.086 \pm 0.01 \text{ g biomass (g xylose)}^{-1}$ and $0.382 \pm 0.01 \text{ g ethanol (g xylose)}^{-1}$, respectively (Figure 2a, Supplementary Figure S2a). The dynamics of adaptation to anaerobic growth were further investigated by plating culture samples on synthetic medium with either glucose (SMD) or xylose (SMX). Colony counts on these plates were determined after aerobic and anaerobic incubation (Figure 2b-e). On anaerobic SMX plates, colonies were first observed after 10 d, at which time they represented a fraction of only $1.8 \cdot 10^{-4}$ of the number of cells that were plated. Subsequently, consistent with the exponential growth observed by biomass dry weight measurements, the fraction of cells capable of anaerobic growth of

xylose rapidly increased (Figure 2b). When culture samples were plated on SMD, aerobic and anaerobic plates showed similar trends in colony counts (Figure 2d-e). Conversely, plating on SMX revealed a strong trade-off between the ability to grow aerobically and anaerobically on xylose. On aerobically incubated SMX plates cell counts did not increase, not even when exponential growth on xylose took off during the final days of the bioreactor experiments and strongly increasing colony counts were observed on anaerobically incubated SMX plates (Figure 2c).

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Relevant genotype/description	Reference
CEN.PK 113-7D	<i>MATa MAL2-8c SUC2</i>	(57)
IMX581	<i>MATa ura3-52 MAL2-8c SUC2 can1Δ::cas9-natNT2</i>	(33)
IMX696	<i>MATa ura3-52 MAL2-8c SUC2 CAN1::cas9-natNT2 gre3:: [pTDH3_RPE1-pPGK1_TKL1-pTEF1_TAL1-pPGI1_NQM-pTPI1_RKI1-PYK1_TKL2-(pTPI1_xylA_tCYC1)*36 pTEF1_XKS1] pUDE335</i>	This study
IMS0488	Single-cell line isolated after adaptation of IMX696 to anaerobic growth on xylose (reactor 1)	This study
IMS0489	Single-cell line isolated after adaptation of IMX696 to anaerobic growth on xylose (reactor 2)	This study
IMX906	<i>MATa ura3-52 MAL2-8c SUC2 CAN1::cas9-natNT2 gre3:: [pTDH3_RPE1-pPGK1_TKL1-pTEF1_TAL1-pPGI1_NQM1-pTPI1_RKI1-pPYK1_TKL2-(pTPI1_xylA_tCYC1)*36 pTEF1_XKS1] pmr1Δ::amdSYM pUDE335</i>	This study
IMX979	IMX906 with <i>PMR1</i> reintegrated at <i>PMR1</i> locus	This study
IMK692	<i>MATa MAL2-8c SUC2 pmr1Δ::amdSYM</i>	This study

Native gene terminator sequences were used for expression of *RPE1*, *TKL1*, *TAL1*, *NQM1*, *RKI1*, *TKL2* and *XKS1*.

Figure 1. Specific growth rates on xylose of *S. cerevisiae* strains with different *PMR1* alleles in aerobic cultures. Aerobic growth curves in shake-flask cultures grown on synthetic medium with 20 g l⁻¹ xylose. Symbols indicate the following *S. cerevisiae* strains: ●, IMX696 (*xylA*, *PPP*↑, *XKS1*↑), ■, IMX906 (*xylA*, *PPP*↑, *XKS1*↑, *pmr1*Δ), ○, IMX979 (*xylA*, *PPP*↑, *XKS1*↑, *PMR1*), ◆, IMS0488 (isolate from IMX696 culture adapted to anaerobic growth on xylose carrying *Pmr1*^{G249V} mutation) and □ IMS0489 (isolate from IMX696 culture adapted to anaerobic growth on xylose carrying *Pmr1*^{W387*} mutation). Data shown are from a single flask experiment for each strain. For all strains, data obtained from independent duplicate experiments differed by less than 5%.

Adaptation to anaerobic growth on xylose coincides with mutations in *PMR1*.

The dynamics of colony counts on SMX plates (Figure 2b) suggested that adaptation to anaerobic growth might have involved one or more mutations. To test this hypothesis, the genomes of strains IMS0488 and IMS0489, which were isolated from the independent anaerobic adaptation experiments shown in Figure 2 and Supplementary Figure S2, were sequenced. Read-depth analysis of both strains revealed a decrease of the *xylA* copy number to 24 and 25, respectively, as compared to 36 in the parental strain IMX696. No other changes in chromosomal copy numbers were observed. Strikingly, both strains carried non-synonymous SNPs in the coding region of *PMR1* (Table 2), which encodes a high-affinity Golgi Ca²⁺/Mn²⁺ P-type ATPase [35]. These mutations caused a single amino acid change (*Pmr1*^{G249V}) in strain IMS0488 and introduced a premature stop codon (*Pmr1*^{W387*}) in strain IMS0489.

Figure 2 Anaerobic growth of *S. cerevisiae* IMX696 (*xylA*, *PPP*[↑], *XKS1*[↑]) on xylose requires a prolonged adaptation period. (a) Growth, xylose consumption and product formation after inoculation of aerobically pre-grown cells in anaerobic bioreactors containing synthetic medium with xylose (20 g l⁻¹). Symbols: ●, xylose, ■, ethanol, ○, biomass, □, glycerol. (b) Colony-forming units (CFU) on anaerobically incubated xylose medium reflect adaptation to growth on xylose in the absence of oxygen. (c) CFU on aerobically incubated xylose medium reflect trade-off between aerobic and anaerobic growth on xylose. (d) and (e) CFU on anaerobically and aerobically incubated glucose medium, respectively, showing that oxygen sensitivity of cells adapted to anaerobic growth on xylose is not carbon-source dependent. Data shown in figure are from one of two independent replicates, the replicate experiment is shown in Supplementary Figure S2.

Mutations in *PMR1* enable anaerobic growth on xylose.

To investigate the role of the *PMR1* mutations in the adaptation to anaerobic growth on xylose, the gene was deleted in the parental strain IMX696. In replicate anaerobic bioreactor cultures on xylose, the resulting strain IMX906 grew within 24 h and completely consumed all sugar within 70 h (Figure 3). The specific growth rate of both cultures was 0.08 h⁻¹, while biomass and ethanol yields on xylose were 0.086 g ± 0.01 biomass (g xylose)⁻¹ and 0.40 g ± 0.01 ethanol (g

xylose)⁻¹, respectively. To further investigate the role of the *PMR1* deletion in the instantaneous anaerobic growth of strain IMX906 on xylose, the wild-type *PMR1* allele was reintegrated in this strain. The resulting strain IMX979 showed a lag phase of over 250 h in duplicate anaerobic bioreactor cultures on xylose (Supplementary Figure S1), thereby confirming the key role of *PMR1* inactivation in the ability of engineered, *XylA*-based *S. cerevisiae* to grow anaerobically on xylose. The plate count experiments during the anaerobic adaptation phase on xylose suggested a trade-off between aerobic and anaerobic growth on xylose (Figure 2b, c). This possible trade-off was further explored by growth experiments in aerobic shake flasks on SMX. In these experiments, strains in which *PMR1* was mutated or deleted consistently showed a lower specific growth rate than strains that carried a wild-type *PMR1* allele (0.10 h⁻¹ and 0.21 h⁻¹, respectively; Figure 1). Furthermore, aerobic xylose-grown shake-flask cultures of strains with mutated *PMR1* alleles accumulated ethanol to 3–4-fold higher concentrations than corresponding cultures of strains with wild-type *PMR1* alleles (Supplementary Figure S3). Consistent with previous results [36], aerobic shake flask cultures growing on glucose also revealed an approximately 50% reduced growth rate of the *pmr1Δ* strain IMK692(Supplementary Figure S4).

Table 2: Single-nucleotide mutations in engineered *S. cerevisiae* strains adapted to anaerobic growth on xylose.

Strain	Gene	Nucleotide change	Amino acid change	Change in codon
IMS0488	<i>PMR1</i>	G746T	G249V	gGt/gTt
IMS0489	<i>PMR1</i>	G1161A	W387*	tgG/tgA

Strains IMS0488 and IMS0489 were isolated from independent anaerobic batch cultures of strain IMX696 (*xylA*, *PPP*[†], *XKS1*[†]). The genome sequence of IMX696 was used as a reference.

* introduction of stop codon.

Mutations in *PMR1* affect intracellular metal concentrations in xylose-metabolizing *S. cerevisiae* strains.

Pmr1 is an ATP-dependent transporter that imports Ca²⁺ and Mn²⁺ into the Golgi complex [37]. Based on the observation that *pmr1* null mutants accumulate Ca²⁺ and Mn²⁺ intracellularly, *Pmr1* has also been implicated in secretion of divalent metal ions via the Golgi complex [38]. To explore a possible relation between metal homeostasis and anaerobic growth on xylose, we analyzed intracellular concentrations of Ca²⁺, Mn²⁺, Mg²⁺ and Fe²⁺ in biomass samples from anaerobic mid-exponential phase bioreactor cultures using inductively coupled plasma mass spectrometry. Contents of Mg²⁺, Ca²⁺ and Fe²⁺ were similar in all analyzed strains, with Mg²⁺ accounting for over 80% of the analyzed divalent metal ions, followed by Ca²⁺, and with Fe²⁺ accounting for less than 1% of the measured

metals. Conversely, large differences were observed for the Mn^{2+} content. While in strains with a wild-type *PMR1* allele, Mn^{2+} represented less than 0.2% of the measured metal ions, 12- to 29-fold higher Mn^{2+} contents were observed in strains with mutated *PMR1* alleles, irrespective of whether they were grown on xylose or glucose (Table 3). The observation that mutations in *PMR1* affected cellular contents of Mn^{2+} but not those of Ca^{2+} is consistent with a previous study [39].

Figure 3. Deletion of *PMR1* enables anaerobic growth on xylose of engineered *S. cerevisiae* without prior adaptation phase. Growth and product formation of *S. cerevisiae* strain IMX906 (*xylA*, *PPP*↑, *XKS1*↑, *pmr1*Δ) on xylose (20 g l⁻¹) in anaerobic bioreactors. Symbols: ●, xylose, ■, ethanol, ○, biomass, □, glycerol. The data shown are from one of two independent replicates.

Activity and metal content of xylose isomerase purified from *S. cerevisiae* strains.

Laboratory evolution studies have identified XI activity as a key factor in rapid fermentation of xylose to ethanol [16,21,22]. XI enzymes are known to be metal dependent, with pronounced differences in metal binding and impact of metal identity on enzyme kinetics [40]. To examine the impact of Mn^{2+} on XylA activity, XI activities were assayed in cell extracts of strains IMX906 (*pmr1*Δ) and its parental strain IMX696 after aerobic and anaerobic growth on SMD in shake-flask cultures (Supplementary Figure S5). Cell extracts from both strains exhibited similar activities in assays without added metal ions. These activities do not necessarily reflect in vivo metal binding as they may, for example, have been influenced by binding of metals released during preparation of cell extracts, e.g. by disruption of vacuoles. Addition of Mn^{2+} and, to a lesser extent, of Mg^{2+} to the XI assays yielded significantly higher XI activities than observed in the absence of added metals. Conversely, addition of Ca^{2+} led to lower activities.

Table 3. Impact of the deletion of *PMR1* on intracellular metal ion concentrations.

<i>S. cerevisiae</i> strain (relevant genotype)	Carbon source	$\mu\text{mol (g dry biomass)}^{-1}$				
		Mg ²⁺	Ca ²⁺	Fe ²⁺	Mn ²⁺	Total
IMX696 (<i>xylA</i> , <i>PPP</i> ↑, <i>XKS1</i> ↑)	glucose	60 ± 1	8.8 ± 1.6	0.56 ± 0.02	0.069 ± 0.001	69 ± 2
IMX906 (<i>xylA</i> , <i>PPP</i> ↑, <i>XKS1</i> ↑, <i>pmr1</i> Δ)	glucose	64 ± 1	12 ± 2	0.51 ± 0.02	1.2 ± 0.1	77 ± 2
IMX906 (<i>xylA</i> , <i>PPP</i> ↑, <i>XKS1</i> ↑, <i>pmr1</i> Δ)	xylose	50 ± 1	4.0 ± 0.7	0.36 ± 0.01	1.4 ± 0.1	56 ± 1
CEN.PK113-7D (reference strain)	glucose	61 ± 1	6.8 ± 1.3	0.44 ± 0.01	0.046 ± 0.001	68 ± 2
IMK692 (<i>pmr1</i> Δ)	glucose	72 ± 1	6.7 ± 1.2	0.46 ± 0.02	0.86 ± 0.06	80 ± 2

S. cerevisiae strains were grown in anaerobic bioreactors on xylose or glucose (20 g l⁻¹). Data represent average and mean deviation calculated from analyses on independent duplicate cultures.

Table 4. Impact of *PMR1* deletion on metal content and activity of XylA.

<i>S. cerevisiae</i> strain	Carbon source	mol metal/mol XylA monomer				Sp. activity (U/mg XylA protein)	
		Mg ²⁺	Ca ²⁺	Fe ²⁺	Mn ²⁺	No metal added	Mg ²⁺ added (1 mM)
IMX906	xylose	0.18 ± 0.01	0.54 ± 0.11	0.06 ± 0.01	0.38 ± 0.04	2.39 ± 0.61	2.68 ± 0.5
IMX906	glucose	0.20 ± 0.06	0.66 ± 0.07	0.064 ± 0.02	0.30 ± 0.03	1.35 ± 0.14	2.10 ± 0.1
IMX696	glucose	0.22 ± 0.06	0.93 ± 0.41	0.086 ± 0.04	0.017 ± 0.004	0.60 ± 0.24	1.68 ± 0.1

XylA protein was isolated from *S. cerevisiae* cultures grown on xylose or glucose (20 g l⁻¹) in anaerobic bioreactors. Data represent average and mean deviation of analyses on XylA isolated from independent duplicate cultures. The relative genotypes of the strains: IMX906 (*xylA*, *PPP*↑, *XKS1*↑, *pmr1*Δ) and IMX696 (*xylA*, *PPP*↑, *XKS1*↑)

For a further analysis of the effect of Mn²⁺ on XylA activity, we purified the enzyme from the controlled anaerobic bioreactor cultures that were also used to determine cellular metal contents (Supplementary Figure S6). Concentrations of Mg²⁺, Ca²⁺, Fe²⁺ and Mn²⁺ were measured in purified protein samples and the amount of each metal per enzyme active site was calculated (Table 4). These analyses showed that the isolated enzymes contained fewer than two metal ions per subunit, indicating that their metal binding sites were not fully occupied.

In independent replicate experiments, large and consistent differences were observed in the Mn^{2+} contents of XylA isolated from strains with wild-type and mutated *PMR1* alleles (0.017 and 0.30 mol Mn (mol XylA subunit) $^{-1}$, respectively, Table 4). The higher Mn^{2+} content of XylA isolated from xylose- or glucose-grown cells of the *pmr1* Δ strain coincided with a ca. 2-fold higher specific activity than measured with enzyme purified from the *PMR1* strain (Table 4). Although metal binding may have changed during cell disruption and enzyme purification, this correlation does indicate that Mn^{2+} -loaded XylA is a better catalyst than the Mg^{2+} -loaded enzyme. Addition of 1 mM MgCl_2 to purified enzyme preparations enhanced their XI activities, consistent with incomplete metal loading in the cell and/or metal loss during purification and activity assays.

Table 5. Kinetic parameters of XylA measured after reconstituting apo-XylA with different divalent metal ions.

Metal	Kinetic parameters		
	k_{cat} (s^{-1})	K_{M} (mM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1}\cdot\text{M}^{-1}$)
Mg^{2+}	2.8 ± 0.2	5.5 ± 0.4	500
Mn^{2+}	7.8 ± 0.1	3.9 ± 0.2	2000
Ca^{2+}	0.6 ± 0.06	420 ± 90	1.3

k_{cat} and K_{M} values represent average and mean deviation of independent duplicate experiments, calculated for each metal.

Mn^{2+} binding results in superior catalytic efficiency of XylA.

To accurately analyze the effect of different metals on catalytic properties of XylA, apoenzyme was prepared from XylA isolated from xylose-grown cultures of strain IMX906. Subsequently, XI activities were measured after reconstitution of apo-XylA with Mg^{2+} , Ca^{2+} or Mn^{2+} (Table 5). The activities of Mn^{2+} - and Mg^{2+} -reconstituted apo-XylA were higher than activities in non-metal-supplemented assays with XylA purified from yeast cultures (Table 4). The reconstituted enzyme showed the highest catalytic efficiency in the presence of Mn^{2+} , with a $k_{\text{cat}}/K_{\text{M}}$ ratio that was 4-fold and 1500-fold higher than with Mg^{2+} and Ca^{2+} , respectively. Both the highest k_{cat} and the lowest K_{M} were observed with Mn^{2+} and contribute to the superior catalytic efficiency with this metal cofactor (Table 5). When XylA apoenzyme was reconstituted with mixtures of divalent metals that resembled those observed in intracellular metal content analyses (Table 3) of strains IMX696 (*PMR1*) and IMX906 (*pmr1* Δ), an 80–90% increase of XI activity was observed as the fraction of Mn^{2+} was increased from 0.002 to 0.01 (Supplementary Table S1).

DISCUSSION

4

One-step, Cas9-assisted integration of a heterologous XI (*Piromyces* XylA) and overexpression of native yeast genes encoding xylulokinase and enzymes of the non-oxidative pentose-phosphate pathway (PPP) enabled fast aerobic growth on xylose by *S. cerevisiae*, thus illustrating the efficiency of Cas9-based genome editing in this yeast [33,41]. Nine copies of the *xylA* cassette were incorporated in tandem to facilitate expansion or compression of the *xylA* copy number by homologous recombination. This approach was validated by the four-fold higher *xylA* copy number in transformants isolated on xylose medium and its decrease in independent replicate cultures after subsequent adaptation to anaerobic growth. The observed amplification of *xylA* was consistent with the previously reported positive impact of high *xylA* copy numbers on xylose metabolism [20–22]. In line with earlier studies [21,23], this metabolic engineering strategy did not enable anaerobic growth on xylose. In principle, the engineered XI-based pathway should allow for efficient, redox-cofactor-balanced alcoholic fermentation on this sugar. However, anaerobic growth on xylose requires much higher fluxes through XI since the ATP yield of anaerobic, fermentative metabolism of this sugar is approximately eight-fold lower than that of its aerobic, respiratory dissimilation (assuming an in vivo P/O ratio of 1.0) [42].

In independent replicate cultures, anaerobic growth on xylose required a two-week adaptation, which was shown to reflect the accumulation of spontaneous mutants with single-nucleotide mutations in *PMR1*. The observation that single, easily acquired point mutations enabled this adaptation may explain an earlier report that overexpression of XylA, xylulokinase and PPP enzymes sufficed to enable anaerobic growth of *S. cerevisiae* on xylose [19]. Here, we demonstrate that inactivation of *PMR1* caused both a strongly elevated intracellular Mn^{2+} concentration and an increased loading of heterologously expressed XylA with Mn^{2+} . Moreover, in vitro studies showed that loading of XylA apoenzyme with Mn^{2+} led to higher enzyme activities than binding of other divalent metal ions present in the yeast cytosol.

Consistent with the conclusion that intracellular Mn^{2+} homeostasis affects anaerobic xylose metabolism through its impact on in vivo XylA activity, none of the five *S. cerevisiae* enzymes that subsequently convert D-xylulose into glycolytic intermediates (xylulokinase, ribulose-5-phosphate isomerase, ribulose-5-phosphate 3-epimerase, transaldolase and transketolase) have been documented to be Mn^{2+} dependent (BRENDA database [43]). Our results do not exclude the possibility that altered Mn^{2+} levels influenced pentose metabolism by mechanisms other than influencing XylA activity. However, a key role of XylA is consistent with the observation that acquisition of mutations in *PMR1* coincided

with a decrease of the *xylA* copy number from 36 to 25. This decrease, which occurred during the course of a single batch culture, suggests that mutations in *PMR1* may have affected a trade-off between the need for a high in vivo activity of XylA and the metabolic burden associated with its high-level synthesis. As demonstrated in a study on the energetic impacts of galactose-induced synthesis of the enzymes of the Leloir pathway [44], such a metabolic burden is much more pronounced in anaerobic cultures than in aerobic, respiring cultures due to the lower ATP yield from fermentative sugar dissimilation.

The mutations in *PMR1* that enabled anaerobic growth on xylose negatively affected aerobic growth. High intracellular Mn^{2+} concentrations have previously been implicated in impaired mitochondrial function [36], which is consistent with the increased accumulation of ethanol in aerobic shake-flask cultures of *pmr1* strains (Supplementary Figure 3). Moreover, TORC1 signaling, which is involved in regulation of mitochondrial respiratory functions, is inhibited by Mn^{2+} and *Pmr1* has been identified as a negative regulator of *TOR1*, which encodes a subunit of the TORC1 complex [45]. Additionally, Mn^{2+} -induced apoptosis mediated by Ndi1 [46,47], a mitochondrial NADH dehydrogenase, may have contributed to low colony counts observed when cultures adapted to anaerobic growth on xylose were plated under aerobic conditions (Figure 1c). The reduced growth rate in aerobic cultures of strains carrying *PMR1* mutations should be considered when their anaerobic industrial application is preceded by an aerobic biomass propagation phase.

Despite the pivotal role of the functional expression of a heterologous XI in *S. cerevisiae* [18], and the well documented role of metal ions in the active sites of XIs from taxonomically diverse organisms [48], the impact of metal loading on the performance of heterologously expressed XIs in *S. cerevisiae* has previously not been investigated. Similar to bacterial XIs [40], apo-XylA isolated from yeast could be activated with different metals. The results of this study suggest that metal loading can have a large effect on the in vivo catalytic performance of the enzyme.

The pronounced influence of cellular metal content on XI activity was in agreement with its promiscuity towards metal cofactors found in the in vitro analyses. However, while the fraction of the XI-bound Mn^{2+} increased by more than 10-fold in strains that carried mutations in *PMR1*, cellular contents of Mn^{2+} were still at least 40 times lower than the combined Mg^{2+} and Ca^{2+} contents (Tables 3 and 4). This observation suggests that the affinity of XylA for Mn^{2+} is higher than for the other divalent metal ions. Functional expression of heterologous XIs in *S. cerevisiae* initially represented a formidable challenge in engineering *S. cerevisiae* for anaerobic xylose fermentation [10–13]. After *Piromyces xyla* [14], XI genes from several eukaryotes and prokaryotes sources were shown to also

be functionally expressed in *S. cerevisiae*, including those from *Orpinomyces* sp [49], *Arabidopsis thaliana* [50], *Clostridium phytofermentans* [23], *Bacteroides thetaiotaomicron* [18], *Bacteroides stercoris*, *Prevotella ruminicola* TC2-24 [51] and *Sorangium cellosum* [52]. In view of the key catalytic role of metal ions in all known xylose isomerases, we expect that in vivo activity of these and other XIs in yeast cells will also be affected by engineering of metal homeostasis.

Mutations in *PMR1* have been identified in two previous studies on adaptive laboratory evolution of XylA-based, engineered *S. cerevisiae* strains. Klaassen *et al.* [53] identified a mutation in *PMR1* (Y38C) in a strain evolved for fermentation of L-arabinose and xylose to ethanol. Recently, Hou *et al.* [25], reported a mutation in *PMR1* (G698V) in a respiratory-deficient XylA based *S. cerevisiae* strain obtained by adaptive laboratory evolution on xylose medium. Our results strongly suggest that, in both studies, the mutations in *PMR1* may have contributed to the selected phenotypes. Additionally, the superior catalytic efficiency of Mn²⁺-loaded XylA may explain a recent report that MnSO₄ supplementation enhanced growth on xylose of acetate-stressed cultures of a XylA-based xylose-fermenting *S. cerevisiae* strain [54].

Our study demonstrates the importance of metal homeostasis and enzyme loading in XI-based yeast metabolic engineering strategies for anaerobic conversion of xylose-containing lignocellulosic feedstocks into fuels and chemicals. Inactivation of *PMR1*, combined with overexpression of PPP enzymes, xylulokinase and *xylA* was shown to be sufficient to enable anaerobic growth of *S. cerevisiae* on xylose. Beyond xylose utilization, engineering of metal homeostasis has the potential to improve in vivo performance of other metal-dependent heterologous enzymes or pathways.

MATERIALS AND METHODS

Strains and maintenance.

All *S. cerevisiae* strains used in this study (Table 1) originate from the CEN.PK lineage [55,56]. Frozen stock cultures were stored at -80 °C in 30% (vol/vol) glycerol.

Plasmid and strain construction.

Plasmids used in this study are presented in Supplementary Table S2. Expression cassettes required for xylose fermentation were introduced into the *GRE3* locus of *S. cerevisiae* strain IMX581 by simultaneous *in-vivo* assembly and integration [32]. Expression cassettes for *RPE1*, *RKI1*, *TAL1*, *NQM1*, *TKL1*, *TKL2* and *XKS1* were obtained by fusing constitutive promoter sequences, ORFs and

terminator sequences amplified from CEN.PK113-7D in a fusion-PCR [57] using the primers specified in Supplementary Table S3. Plasmid pYM-N18 [58] was used as a template for the *TEF1* promoter. The resulting fragments were cloned into pJET-1.2 blunt-end vectors. Correct assembly was verified by sequencing as described below. PCR amplification of expression cassettes and plasmids was performed using Phusion Hot Start II High Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA), according to the manufacturer's protocol. Integration in *GRE3* locus was mediated by a chimeric CRISPR/Cas9 editing system [33,41] with gRNA expressed from an episomal plasmid [33]. The plasmid backbone was PCR amplified from pMEL10 using primers 5792–5980 (Supplementary Table S3). A plasmid insert containing the 20 bp gRNA-targeting sequence was obtained by PCR amplification with primers 5978–5979 using pMEL10 as template. The resulting fragment was fused to the plasmid backbone with the Gibson Assembly Cloning kit (New England Biolabs, Ipswich, MA), yielding plasmid pUDE335. *E. coli* DH5a cells were transformed with 1 μ L of the Gibson-assembly mix using a Gene PulserXcell Electroporation System (Biorad, Hercules, CA). Plasmid DNA was isolated from *E. coli* cultures using a Sigma GenElute Plasmid kit (Sigma-Aldrich, St. Louis, MO). The presence of the *GRE3* cutting gRNA was confirmed by PCR-amplification using primer pair 2528–960 followed by digestion with FastDigest *Clal* (Thermo Scientific).

The coding region of the *Piromyces* sp. E2 xylose isomerase gene [Genbank: CAB76571.1] was codon optimized according to the codon preference of highly expressed glycolytic genes in *S. cerevisiae* [59]. The codon-optimized sequence, flanked by the constitutive *TPI1* promoter and *CYC1* terminator, was synthesized by GeneArt GmbH (Regensburg, Germany). After subsequent transformation of the pMK-RQ (GeneArt) based vector pUDR350 into *E. coli*, nine different expression cassettes of *xylA* were made, flanked by 60 bp synthetic recombinant sequences (Supplementary Figure S7). For XylA expression in *E. coli*, a codon-optimized synthetic *xylA* was cloned into pBAD/myc-His-derived plasmid.

Yeast transformation was performed using the lithium acetate protocol [60]. Strain IMX696 was obtained by adding 200 pmol of each of the 15 fragments combined with 500 ng of plasmid pUDE335. After one hour of incubation in synthetic medium with glucose (SMD) the cells were plated on SM plates with xylose as the carbon source (SMX). Correct assembly of all fragments in the *GRE3* locus was confirmed by diagnostic PCR (Dreamtaq, Thermo Scientific) using primers listed in Table S3. Deletion of *PMR1* in *S. cerevisiae* strains IMX696 and CEN.PK113-7D was done by integrating an *amdSYM*-based deletion cassette [61], which was derived by PCR amplification from pUG-*amdSYM* using primers 8638/8639 as template. After transformation, cells were plated on glucose synthetic medium with acetamide as the nitrogen source (SMD-Ac). Gene deletion was confirmed

by diagnostic PCR and the resulting strains were named IMX906 and IMK692, respectively. To reintegrate *PMR1*, the *PMR1* ORF was PCR-amplified from CEN. PK113-7D and transformed into strain IMX906. After overnight incubation in SMD-Ac, cells were plated on SMD plates supplemented with 2.3 g l⁻¹ fluoroacetamide (SMD-Fac). Correct integration of *PMR1* in the resulting strain, IMX969, was confirmed by diagnostic PCR.

Cultivation and media.

Shake-flask cultures were grown at 30 °C in an orbital shaker at 200 rpm, using 500-ml flasks containing 100 ml medium. Physiological characterization of aerobic growth was performed in shake flasks containing SMX or SMD with urea as sole nitrogen source to prevent acidification. Prior to filter sterilization, media were adjusted to pH 5.0 with 2 M KOH. For pre-cultures, SM adjusted to pH 6.0 was autoclaved at 12 °C for 20 min after which a 50 w/v % solution of sterile glucose or xylose was added to obtain a final sugar concentration of 20 g l⁻¹, together with filter-sterilized vitamin solution [62]. Glucose and xylose solutions were autoclaved separately (20 min at 110 °C). For plates, 2% agar was added to media prior to autoclaving. Frozen stocks (1 ml aliquots in 30% glycerol) were inoculated directly into pre-culture shake flasks. In late exponential phase an aliquot was transferred to a second pre-culture to obtain an initial OD₆₆₀ of 0.1. Flasks or anaerobic bioreactors used for characterization were inoculated from these cultures at an initial OD₆₆₀ of between 0.1 and 0.2. Anaerobic batch cultures were conducted in 2-l bioreactors (Applikon, Delft, The Netherlands) with a working volume of 1 l. Biomass for metal content analysis was grown in 3-l bioreactors (Applikon) with a working volume of 2 l were used. Bioreactor cultures were grown at 30 °C, pH 5.0, and stirred at 800 rpm. To ensure anaerobic conditions, bioreactors were equipped with Viton O-rings and Norprene tubing. During cultivation, nitrogen gas (< 10 ppm oxygen) was continuously sparged through the cultures at 0.5 l min⁻¹. After autoclaving, synthetic medium used for anaerobic cultivation was supplemented with 0.2 g l⁻¹ sterile antifoam C (Sigma-Aldrich), as well as Tween 80 (420 mg l⁻¹) and ergosterol (10 mg l⁻¹) dissolved in ethanol [63].

Analytical methods.

Cell dry weight (CDW) measurements were done using pre-weighed nitrocellulose filters (pore size, 0.45 µm; Gelman Laboratory, Ann Arbor, MI) to filter 10 ml of culture. Before weighing the sample, filters were washed with demineralized water and dried in a microwave oven (Bosch, Stuttgart, Germany) for 20 min at 360 W. Growth was monitored by optical density (OD) measurements at a wavelength of 660 nm using a Libra S11 spectrophotometer (Biochrom, Cambridge, United

Kingdom). A correlation between OD measurements and CDW was used to estimate CDW in samples for which no direct CDW measurements were taken. This correlation was based on at least six points during the exponential phase. CO₂ and O₂ concentrations in bioreactor exhaust gas were measured using an NGA 2000 analyzer (Rosemount Analytical, Orrville, OH) after the gas was cooled by a condenser (2 °C) and dried with a Permapure MD-110-48P-4 dryer (Permapure, Toms River, NJ). Metabolite levels in culture supernatants obtained by centrifugation were measured via high-performance liquid chromatography (HPLC) analysis on an Agilent 1260 HPLC (Agilent Technologies, Santa Clara, CA) fitted with a Bio-Rad HPX 87 H column (Bio-Rad, Hercules, CA). The column was eluted at 60 °C with 0.5 g l⁻¹ H₂SO₄ at a flow rate of 0.6 ml min⁻¹. Detection was by means of an Agilent refractive-index detector and an Agilent 1260 VWD detector. Correction for ethanol evaporation were done for all bioreactor experiments as described previously [64]. Viability of strain IMX696 during anaerobic cultivation was assessed by plating culture samples. The number of cells per ml was measured using a Z2 Coulter Counter (Beckman Coulter, Woerden, The Netherlands) after which dilutions were plated in duplicate on SMX and SMG agar plates and incubated at 30 °C. To limit exposure to oxygen, cells that were used to determine anaerobic viability measurements were sampled directly into a container flushed with argon and immediately transferred into an anaerobic chamber (5% H₂, 6% CO₂, and 89% N₂, Sheldon MFG Inc., Cornelius, OR) for plating and incubation. Colony-forming units (CFU) were counted after incubation at 30 °C for 4 days (aerobic growth) or 8 days (anaerobic growth).

DNA sequence analysis.

Genomic DNA of strains IMX696, IMS0488 and IMS0489 was isolated using the QIAGEN Blood & Cell Culture DNA Kit with 100/G Genomics-tips (QIAGEN, Valencia, CA) according to the manufacturer's protocol. From these DNA samples, 350-bp insert libraries were constructed using the Nextera XT DNA kit (Illumina, San Diego, CA). Paired-end sequencing (100-bp reads) of genomic or plasmid DNA was performed with an Illumina HiSeq 2500 sequencer (Baseclear BV, Leiden, The Netherlands). Data were mapped to the CEN.PK113-7D genome or to in silico-generated plasmid sequences using the Burrows-Wheeler alignment tool [65] and processed with Pilon [66]. Identified single-nucleotide differences were inspected with the Integrated Genomics Viewer [67] (IGV). The chromosomal copy number variance (CNV) was estimated using the Poisson mixture model based algorithm Magnolya [68]. The copy number of *xyIA* was estimated by comparing the read depth to the average read depth of all chromosomes. Raw sequence data of strains IMX696, IMS0488 and IMS0489 are deposited at the NCBI Sequence Read archive (www.ncbi.nlm.nih.gov/sra) under BioProject ID PRJNA349142.

Purification of xylose isomerase.

Cell pellets were resuspended in 10 mM MOPS, pH 7.0, containing protease inhibitors (cOmplete ULTRA tablets, Roche) and disrupted using a high-pressure homogenizer (Constant Systems Ltd, Low March, United Kingdom). Samples were passed through the apparatus twice at 39 kpsi and cell debris was removed by centrifugation at $35,000 \times g$ for 45 min at 4 °C. A single-step purification procedure based on anion-exchange chromatography was applied to minimize the loss of protein-bound metals. Cell-free extracts were loaded on a strong anion-exchange column (Resource Q, GE Healthcare, Chicago, IL) equilibrated with 10 mM MOPS, pH 7.0. A gradient elution was applied using 10 mM MOPS, pH 7.0, containing 0–200 mM KCl. XylA eluted at approximately 40 mM KCl. Protein concentrations were determined using the theoretical extinction coefficient at 280 nm (ϵ_{280} , $XI = 73,800 \text{ M}^{-1} \text{ cm}^{-1}$) calculated by the ProtParam tool (<http://web.expasy.org/protparam/>).

Metal content analysis.

Metal concentrations were analyzed with an inductively coupled plasma mass spectrometer (ICP-MS, Varian 820). All measurements were performed 5 times for each sample and yttrium was used as an internal standard. Purified protein samples were lyophilized and analyzed for contents of magnesium, calcium, iron and manganese. Prior to measurement, samples were dissolved in 1% nitric acid solution. All analyses were performed on protein samples isolated from two replicate cultures. For intracellular metal analysis, cells were prepared with a protocol adopted from Eide *et al.* [69]. The harvested cells were washed three times each with 1 μM EDTA solution and subsequently with deionized water (Milli-Q) and suspended in 1 ml 30% (w/v) nitric acid and incubated at 60 °C for 4 h. Cell lysates were centrifuged at $16,000 \times g$ and supernatants were collected. Pellets were washed with 1 ml deionized water and the supernatants were collected as before. The 2 ml of final sample solution containing approximately 15% (w/v) nitric acid were then subjected to the measurements. The metal content was determined with samples from two separate bioreactor batch cultures.

Preparation of cell extracts.

Cell extracts were prepared following a previously published procedure with minor modifications [16]. To limit loss of metals during preparation, no EDTA was added prior to sonication. Cells were washed and suspended in 10 mM MOPS buffer pH 7.0 to avoid precipitation of MnCl_2 and 10 mM DTT was added. After sonication (4 bursts of 30 s with 30 s intervals at 0 °C, amplitude 8 μm) using a Soniprep 150 sonicator (Beun de Ronde BV, Abcoude, The Netherlands), cell debris was removed by centrifugation (4 °C, 20 min at $48,000 \text{ g}$) and the clear supernatant was used for XylA assays.

Enzyme Kinetics

Activity of XylA was measured with a coupled enzyme assay using D-sorbitol dehydrogenase [70]. D-sorbitol dehydrogenase (SDH) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Reactions were performed at 30 °C and pH 7.0 (20 mM MOPS buffer). The decrease in absorbance at 340 nm was monitored in either a spectrophotometer (Jasco, Easton, MD) or a Synergy Mx microtiter plate reader (BioTek Instruments, Winooski, VT). Reaction mixtures included 5, 200- or 500-mM xylose, 250 μ M NADH and U ml⁻¹ of SDH. Addition of 0.03 to 1 μ M (depending on the substrate concentration and the metal added) XI or cell free extract into the mixture initiated the reaction. For measuring XylA activity in the presence of different metal cofactors, samples of apo-XylA were prepared by overnight incubation of the purified enzyme with 10 mM EDTA. Subsequently, EDTA was removed by buffer exchange to 20 mM MOPS, pH 7.0 and 1 mM of divalent metal solutions (MgCl₂, MnCl₂ or CaCl₂) were added in the reaction. For kinetic analyses, D-xylose was added at concentrations ranging from 0.5 mM to 1.50 M.

ACKNOWLEDGEMENTS

This work was performed within the BE-Basic R&D Program (<http://www.be-basic.org/>), which is financially supported by an EOS Long Term grant from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I). The authors thank Hanna Dudek (RUG), Jasmine M. Bracher (TUD), Paul de Waal (DSM), Hans de Bruijn (DSM) and Paul Klaassen (DSM) for their input in this project.

SUPPLEMENTARY INFORMATION

Table S1. Activity of XylA in the presence of different metal mixtures.

Xylose (mM)	XylA activities (U·(mg protein) ⁻¹)			
	Mg ²⁺	Mn ²⁺	Mix 1 Mg:Ca:Mn 0.85:0.15:0.001	Mix 2 Mg:Ca:Mn 0.84:0.14:0.02
5	1.73 ± 0.19	4.03 ± 0.44	1.87 ± 0.42	3.53 ± 0.73
200	3.13 ± 0.10	7.05 ± 0.67	3.35 ± 0.06	6.02 ± 0.45

XylA was expressed in *E. coli*, purified and EDTA-treated. Enzyme activities were measured in the presence different metal (mixtures), total concentration 1 mM. The molar fractions in metal mixes 1 and 2 represent the intracellular metal composition of IMX696 and IMX906, respectively (see Table 3 and main text). Reactions were performed at 30 °C and pH 7.0 with saturating concentration (200 mM) and near K_M (5 mM) of xylose. The values represent average and mean deviation of measurements with XylA isolated from independent duplicate *E. coli* cultures.

Table S2. Plasmids used in this study.

Plasmid	Characteristics	Origin
pMEL10	2 µm ori, <i>KIURA3</i> , p <i>SNR52</i> -gRNA.CAN1.Y-t <i>SUP4</i>	Mans <i>et al.</i> (2015) [33]
pJET1.2Blunt	Multi-purpose cloning vector	ThermoFisher
pUD344	pJET1.2Blunt + TagA_p <i>PGI1_NQM1</i> _TagB	This study
pUD345	pJET1.2Blunt + TagB_p <i>TPI1_RKI1</i> _Tagc	This study
pUD346	pJET1.2Blunt + TagC_p <i>PYK1_TKL2</i> _TagF	This study
pUD347	pJET1.2Blunt + TagG_p <i>TDH3_RPE1</i> _TagH	This study
pUD348	pJET1.2Blunt + TagH_p <i>PGK1_TKL1</i> _TagI	This study
pUD349	pJET1.2Blunt + TagI_p <i>TEF1_TAL1</i> _TagA	This study
pUD350	pMK-RQ_p <i>TPI1_xylA</i> _tcyc	This study
pUD353	pJET_Blunt_p <i>TEF1_XKS1</i> _t <i>XKS1</i>	This study
pUDE335	2 µm ori, <i>KIURA3</i> , p <i>SNR52</i> -gRNA. <i>GRE3</i> .Y-t <i>SUP4</i>	This study
pUG-AmdSYM	Template of AmdSYM cassette for <i>PMR1</i> deletion	Solis-Escalante <i>et al.</i> (2013) [63]

Native gene terminator sequences were used for expression of *RPE1*, *TKL1*, *TAL1*, *NQM1*, *RKI1*, *TKL2* and *XKS1*.

Table S3. Oligonucleotide primers used in this study.**Construction of cassettes containing constitutively expressed-pentose-phosphate-pathway and *XKS1* genes:**

Primer nr.:	Purpose:	Template:	Sequence 5' -> 3':
5924	<i>PGI1</i> promoter fragment	CEN.PK113-7D	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACAT-TCCGCCAGATCATCAATAGGCACCAGGGCACTACTTC-TACACATCAACG
5925	<i>PGI1</i> promoter fragment	CEN.PK113-7D	TTTtagGCTGGTATCTTGATTCTAAATCG
5926	<i>NQM1</i> ORF fragment	CEN.PK113-7D	TCGATTTAGAATCAAGATACCAGCCTAAAAATGTCA GAACCTTCAGAGAAAAAAC
5927	<i>NQM1</i> ORF fragment	CEN.PK113-7D	GTTGAACATTCTTAGGCTGGTCAATCATTTAGA- CACGGGCATCGTCTCTCGAAAGGTGGCCCAAGAG- GAT ATTAAGTACTAATGTGG
3847	fusion-PCR of p <i>PGI1</i> and <i>NQM1</i>	p <i>PGI1</i> + <i>NQM1</i> fragment	ACTATATGTGAAGGCATGGCTATGG
3276	fusion-PCR of p <i>PGI1</i> and <i>NQM1</i>	p <i>PGI1</i> + <i>NQM1</i> fragment	GTTGAACATTCTTAGGCTGGTCAATC
5928	<i>TPI1</i> promoter fragment	CEN.PK113-7D	CACCTTTCGAGAGGACGATGCCCGTGTCTAAATGAT- TCGACCAGCCTAAGAATGTTCAACGCGGCCGTGTTT AAAGATTAC
5929	<i>TPI1</i> promoter fragment	CEN.PK113-7D	CCGCGGAGTTTATGTATG
5930	<i>RKI1</i> ORF fragment	CEN.PK113-7D	CTTAAATCTATAACTACAAAAACACATA- CATAAACTCCGCGGATGGCTGCCGGTGTCCC
5931	<i>RKI1</i> ORF fragment	CEN.PK113-7D	CTAGCGTGTCTCGCATAGTTCTTAGATTGTGCG- TACGGCATATACGATCCGTGAGACGTATCATAGGT- GAGAAAGAGATGGAGAATGTAGTACTGC
4672	fusion-PCR of p <i>TPI1</i> and <i>RKI1</i>	p <i>TPI1</i> + <i>RKI1</i> fragment	CACCTTTCGAGAGGACGATG
3277	fusion-PCR of p <i>TPI1</i> and <i>RKI1</i>	p <i>TPI1</i> + <i>RKI1</i> fragment	CTAGCGTGTCTCGCATAGTTCTTAGATTG
5932	<i>PYK1</i> promoter fragment	CEN.PK113-7D	ACGTCTCACGGATCGTATATGCCGTAGCGA- CAATCTAAGAACTATGCGAGGACACGCTAGGGTAG- CGCCCTGGTCAAACCTCAGAAC
5933	<i>PYK1</i> promoter fragment	CEN.PK113-7D	TGTGATGATGTTTTATTTGTTTTGATTGGTGTG
5934	<i>TKL2</i> ORF fragment	CEN.PK113-7D	CACCAATCAAAACAAATAAAACATCATCACAATGG- CACAGTTCTCCGACATTGATAAACTTGC
5935	<i>TKL2</i> ORF fragment	CEN.PK113-7D	TGCCGAACTTTCCCTGTATGAAGCGATCTGAC- CAATCCTTTGCCGTAGTTTCAACGTATGGCAGCCCCAT- AACTCAAAGC
3283	fusion-PCR of p <i>PYK1</i> and <i>TKL2</i>	p <i>PYK1</i> + <i>TKL2</i> fragment	ACGTCTCACGGATCGTATATGC
3288	fusion-PCR of p <i>PYK1</i> and <i>TKL2</i>	p <i>PYK1</i> + <i>TKL2</i> fragment	TGCCGAACTTTCCCTGTATGAAGC
5912	<i>TDH3</i> promoter fragment	CEN.PK113-7D	GCCAGAGGTATAGACATAGCCAGACCTACCTAATTG- GTGCATCAGGTGGTCATGGCCCTTCCGGGAGTTTAT- CATTATCAATACTCG

5913	<i>TDH3</i> promoter fragment	CEN.PK113-7D	CCGTCGAAACTAAGTTCTTGG
5914	<i>RPE1</i> ORF fragment	CEN.PK113-7D	TTAGTTTTAAAAACCAAGAACTTAGTTTCGACGGAT- GGTCAAACCAATTATAGCTCCAGTATCC
5915	<i>RPE1</i> ORF fragment	CEN.PK113-7D	GTCACGGGTTCTCAGCAATTCGAGCTATTAC- CGATGATGGCTGAGGCGTTAGAGTAATCTCTTCTC- CGGCCTCCATCACCAC
4870	fusion-PCR of pTDH3 and <i>RPE1</i>	pTDH3 + <i>RPE1</i> fragment	GCCAGAGGTATAGACATAGCC
3290	fusion-PCR of pTDH3 and <i>RPE1</i>	pTDH3 + <i>RPE1</i> fragment	GTCACGGGTTCTCAGCAATTCTG
5916	<i>PGK1</i> promoter fragment	CEN.PK113-7D	AGATTACTCTAACGCCTCAGCCATCATCGGTAAT- AGCTCGAATTGCTGAGAACCCGTACTGCCCT- TATCTTGTGCAGTTAGAC
5917	<i>PGK1</i> promoter fragment	CEN.PK113-7D	TGTTTTATATTTGTTGTAAAAAGTAGATAATTACTTCC
5918	<i>TKL1</i> ORF fragment	CEN.PK113-7D	GGAAGTAATTATCTACTTTTTACAACAAATATAAAA- CAATGACTCAATTCAGTGACATTGATAAGC
5919	<i>TKL1</i> ORF fragment	CEN.PK113-7D	GCCTACGGTTCCCGAAGTATGCTGCTGATGTCTGGC- TATACCTATCCGTCTACGTGAATAATGAATGCGACCGA- TATTTTTGG
3291	fusion-PCR of pPGK1 and <i>TKL1</i>	pPGK1 + <i>TKL1</i> fragment	CTCTAACGCCTCAGCCATCATCG
4068	fusion-PCR of pPGK1 and <i>TKL1</i>	pPGK1 + <i>TKL1</i> fragment	GCCTACGGTTCCCGAAGTATGC
5920	<i>TEF1</i> promoter fragment	pYM-N18	TATTCACGTAGACGGATAGGTATAGCCAGACATCAG- CAGCATACTTCGGGAACCGTAGGCAGCTCATAGCT- TCAAAATGTTTCTACTCC
5921	<i>TEF1</i> promoter fragment	pYM-N18	AAAAGTTAGATTAGATTGCTATGCTTTCTTTCTAAT- GAGC
5922	<i>TAL1</i> ORF fragment	CEN.PK113-7D	GCTCATTAGAAAAGAAAGCATAGCAATCTAATCTAAGT- TTTATGTCTGAACAGCTCAAAAGAAACAAAAGG
5923	<i>TAL1</i> ORF fragment	CEN.PK113-7D	GTGCCTATTGATGATCTGGCGGAATGTCTGC- CGTGCCATAGCCATGCCTTACATATAGTCATTGT- GATCCTCCTATGTTGTAGTATAGTGC
3274	fusion-PCR of pTEF1 and <i>TAL1</i>	pTEF1+ <i>TAL1</i> fragment	TATTCACGTAGACGGATAGGTATAGC
3275	fusion-PCR of pTEF1 and <i>TAL1</i>	pTEF1+ <i>TAL1</i> fragment	GTGCCTATTGATGATCTGGCGGAATG
6278	<i>XKS1</i> ORF fragment	CEN.PK113-7D	GCAATGACAAATTCAAAAGAAGACGCCGACATAGAG- GAGAAGCATATGTACAATGAGCCGGTCCAGTGCTT CCACATC
6279	<i>XKS1</i> ORF fragment	CEN.PK113-7D	GCTCATTAGAAAAGAAAGCATAGCAATCTAATCTAAGT- TTTATGTTGTGTTCACTAATTCAGAGACAG

Primers used for amplification of integration fragments:

Primer nr.:	Purpose:	Template:	Sequence:
7133	fl_RPE1_H fragment fw	pUD347	TATAATATTTTCATTATCGGAACTCTAGATTCTAT- ACTTGTTTCCCAATTGTTGCTGGTAGGGCCCTTCCGG- GAGTTTATC
3290	fl_RPE1_H fragment rv	pUD347	GTCACGGGTTCTCAGCAATTTCG
3291	H_TKL1_I fragment fw	pUD348	CTCTAACGCCTCAGCCATCATCG
4068	H_TKL1_I fragment rv	pUD348	GCCTACGGTTCCTCGAAGTATGC
3274	I_TAL1_A fragment	pUD349	TATTCACGTAGACGGATAGGTATAGC
3275	I_TAL1_A fragment	pUD349	GTGCCTATTGATGATCTGGCGGAATG
3847	A_NQM1_B	pUD344	ACTATATGTGAAGGCATGGCTATGG
3276	A_NQM1_B	pUD344	GTTGAACATTCTTAGGCTGGTCAATC
4672	B_RK11_C fragment	pUD345	CACCTTTCGAGAGGACGATG
3277	B_RK11_C fragment	pUD345	CTAGCGTGTCTCGCATAGTTCTTAGATTG
3283	C_TKL2_F fragment	pUD346	ACGTCTCACGGATCGTATATGC
3288	C_TKL2_F fragment	pUD346	TGCCGAACCTTCCCTGTATGAAGC
7138	F_xy/A_P fragment	pUD350	CTGATAGTGCTGAAGTCGCCTCCATCTTAGCA- GAGCTGTCCCTGAATGCGTACTCGTGAGCGAT- ACCCTGCGATCTTC
7136	F_xy/A_P fragment	pUD350	CATACGTTGAACTACGGCAAAGGATTGGT- CAGATCGCTTCATACAGGGAAAGTTCGGCACGCG- CAGATTAGCGAAGC
7139	P_xy/A_Q fragment	pUD350	GAGCTGAATGTATATGCTGCGGGATCATTGCA- CAGCTCTGAGAGCCCTGCAACGCGATATGCGAT- ACCCTGCGATCTTC
7137	P_xy/A_Q fragment	pUD350	TCACGAGTACGCATTACAGGGACAGCTCTGCTAAGAT- GGAGGCGACTTACAGCACTATCAGCGCGCAGATTAG- CGAAGC
7142	Q_xy/A_E fragment	pUD350	AGCGATCTGCGAGACCGTATAGCCATGACGAGGTCG- CAATCTTGCGGACAGTGTAGCTCAGCGATACCCTG- CGATCTTC
7140	Q_xy/A_E fragment	pUD350	ATATCGCGTTGCAGGGCTCTCAGAGCTGTGCAAT- GATCCCGCAGCATATACATTAGCTCCGCGCAGATT- AGCGAAGC
7141	E_xy/A_G fragment	pUD350	TGAGCTACACTGTCCGCAAGATTGCGACCTCGTCAT- GGCTATACGGTCTCGCAGATCGCTCGCGCAGATTAG- CGAAGC
6285	E_xy/A_G fragment	pUD350	AAGGGCCATGACCACCTGATGCACCAATTAGGTAG- GTCTGGCTATGTCTATACCTCTGGCGCGATACCCTG- CGATCTTC
6273	G_xy/A_D fragment	pUD350	GCCAGAGGTATAGACATAGCCAGACCTACCTAATTG- GTGCATCAGGTGGTCATGGCCCTTCGCGCAGATTAG- CGAAGC

6284	G_xy/A_D fragment	pUD350	AATCACTCTCCATACAGGGTTTCATACATTTCTCCACG- GGACCCACAGTCGTAGATGCGTGCGATACCCTG- CGATCTTC
6283	D_xy/A_M fragment	pUD350	ACGCATCTACGACTGTGGGTCCCGTGGAGAAATG- TATGAAACCCTGTATGGAGAGTGATTGCGATACCCTG- CGATCTTC
6275	D_xy/A_M fragment	pUD350	ACGAGAGATGAAGGCTCACCGATGGACTTAGTAT- GATGCCATGCTGGAAGCTCCGGTCATCGCGCAGATT- AGCGAAGC
6287	M_xy/A_N fragment	pUD350	ATGACCGGAGCTTCCAGCATGGCATCATACTAAGTC- CATCGGTGAGCCTTCATCTCTCGTGCGATACCCTG- CGATCTTC
6276	M_xy/A_N fragment	pUD350	TTCTAGGCTTTGATGCAAGGTCCACATATCTTCGT- TAGGACTCAATCGTGGCTGCTGATCCGCGCAGATTAG- CGAAGC
6288	N_xy/A_O fragment	pUD350	GATCAGCAGCCACGATTGAGTCCTAACGAAGATATGT- GGACCTT GCATCAAAGCCTAGAAGCGATACCCTGCGATCTTC
6277	N_xy/A_O fragment	pUD350	ATACTCCCTGCACAGATGAGTCAAGCTATTGAACAC- CGAGAACGCGCTGAACGATCATTCCGCGCAGATTAG- CGAAGC
6289	O_xy/A_L fragment	pUD350	GAATGATCGTTTCAGCGGTTCTCGGTGTTCAAT- AGCTTGACTCATCTGTGCAGGGAGTATGCGAT- ACCCTGCGATCTTC
6627	O_xy/A_L fragment	pUD350	GCCGTAGCTTCCGCAAGTATGCCGTAGTTGAAGAG- CATTGCCGTCGGTTCAGGTATATCGCGCAGATTAG- CGAAGC
7135	L_XKS1_fl fragment	pUD353	ATATGACCTGAACCGACGGCAAATGCTCTTCAAC- TACGGCATACTTGCGGAAGCTACGGCCATAGCT- TCAAAATGTTTCTACTCC
7134	L_XKS1_fl fragment	pUD353	TGTGGCACCGCAATCATTACTATGGCTAGTGCTAT- CATTGCTGTTTGACGCACTGATGGGTCCAGTGCTTC- CACATCAATTTG
8638	<i>PMR1</i> KO cassette	pUG-AmdSYM	CAAGACGAAGCAAGGCCAGCACAGACGTAAGCT- TAAGTGTAAGTAAAGATAAGATAATTACAGCT- GAAGCTTCGTACGC
8639	<i>PMR1</i> KO cassette	pUG-AmdSYM	ACATATGTTCCCATTAATTAGTACATTTACCTCAATAG- GGTTGGGCGTCCTCATGAAAGAGCATAGGCCACTAGT- GGATCTG
8640	<i>PMR1</i> reintegration	CEN, PK113-7D	CCATGGCTACTGCTATTTTCG
8641	<i>PMR1</i> reintegration	CEN, PK113-7D	AGGGCGTTGATAGGATG

Primers used for construction of plasmid containing the gRNA cutting in *GRE3*:

Primer nr.:	Purpose:	Template:	
5792	pUDE335 backbone	pMEL10	GTTTTAGAGCTAGAAATAGCAAGTTAAATAAG
5980	pUDE335 backbone	pMEL10	CGACCGAGTTGCTCTTG
5978	pUDE335 gRNA	pMEL10	ATTTTAACTTGCTATTCTAGCTCTAAAACCTTTAT-GACGTATACGTTTACGATCATTTATCTTTCACGCGG
5979	pUDE335 gRNA	pMEL10	TATTGACGCCGGGCAAGAGC
2528	restriction analysis of gRNA	pUDE335	TCTTTCCTGCGTTATCCC
960	restriction analysis of gRNA	pUDE335	GTGGATGATGTGGTCTCTAC

Primers used for verifying integration of fragments:

Primer nr.:	Purpose:	Sequence:
6640	checking PPP integration	CTAGATGTGGTCAGCCATTC
976	checking PPP integration	CACCAGTGTCGGCAACAACG
6717	checking PPP integration	CTCATTAGAAAAGAAAGCATAGCAATC
5603	checking PPP integration	CGCAAGTTTATCAATGTCGG
4656	checking PPP integration	CCTTCCCATATGATGCTAGG
7056	checking <i>xylA</i> integration	AGAGGTGGTGGTTTCGTTAC
6632	checking <i>xylA</i> integration	AGCGTCGTAGTAGTGGAAGC
7370	checking <i>xylA</i> integration	TGCTGTAAGTCGCCTCCATC
3293	checking <i>xylA</i> integration	GAGCTGAATGTATATGCTGCGGGATC
7369	checking <i>xylA</i> integration	AGCGATCTGCGAGACCGTATAG
4692	checking <i>xylA</i> integration	AAGGGCCATGACCACCTG
5231	checking <i>xylA</i> integration	AATCACTCTCCATACAGGG
3354	checking <i>xylA</i> integration	ACGCATCTACGACTGTGGGTC
4184	checking <i>xylA</i> integration	ATGACCGGAGCTTCCAGCATG
3843	checking <i>xylA</i> integration	GATCAGCAGCCACGATTG
3837	checking <i>xylA</i> integration	GAATGATCGTTTCAGCGCG
6921	checking <i>xylA</i> integration	AGAGGTGGTGGTTTCGTTAC
3286	checking <i>xylA</i> integration	GCCGTAGCTTCCGCAAGTATG
8640	checking <i>PMR1</i> KO/integration	CCATGGCTACTGCTATTTTCG
8641	checking <i>PMR1</i> KO/integration	AGGGCGTTGATAGGATG
9	checking <i>PMR1</i> KO/integration	CGCACGTCAAGACTGTCAAG
10	checking <i>PMR1</i> KO/integration	TCGTATGTGAATGCTGGTCG
8792	checking <i>PMR1</i> KO/integration	GTTGGACTGTCTCTGTTAGG
8793	checking <i>PMR1</i> KO/integration	CTTCGTCCACGGATAAAG

Figure S1 Off-gas CO₂ profiles of anaerobic bioreactor cultures on synthetic medium with xylose (20 g l⁻¹). Black and grey lines indicate results from independent duplicate cultures of strain *S. cerevisiae* IMX696 (*xylA*, *PPP*↑, *XKS1*↑) and strain IMX979 (*xylA*, *PPP*↑, *XKS1*↑, *PMR1*), respectively.

Figure S2 Anaerobic growth of *S. cerevisiae* IMX696 (*xylA*, *PPP* $\uparrow$, *XKS1* $\uparrow$) on xylose, independent replicate of experiment shown in Figure 1. (a) Growth, xylose consumption and product formation after inoculation of aerobically pre-grown cells in anaerobic bioreactors containing synthetic medium with xylose (20 g l^{-1}). Symbols: $\bullet$, xylose, $\blacksquare$, ethanol, $\circ$, biomass, $\square$, glycerol. (b) Colony-forming units (CFU) on anaerobically incubated xylose medium reflect adaptation to growth on xylose in the absence of oxygen. (c) CFU on aerobically incubated xylose medium reflect trade-off between aerobic and anaerobic growth on xylose. (d) and (e) CFU on anaerobically and aerobically incubated glucose medium, respectively, showing that oxygen sensitivity of cells adapted to anaerobic growth on xylose is not carbon-source dependent. Data shown in this figure are from one of two independent replicates.

Figure S3. Growth of *S. cerevisiae* strains on xylose in aerobic shake-flask cultures.

(a) IMX696 (*xy1A*, *PPP1*, *XKS1*↑). (b) IMS0488 (isolated from culture of strain IMX696 adapted to anaerobic growth on xylose). Both strains were grown in aerobic shake flasks on synthetic medium containing 20 g l⁻¹ xylose. Symbols: ●, xylose, ■, ethanol and ○, biomass. The data shown in the figure are from a single shake-flask experiment of each strain. Data from duplicate experiments with each strain differed by less than 5%.

Figure S4. Impact of a *PMR1* deletion on aerobic growth of *S. cerevisiae* CEN.PK113-7D on glucose. Growth was monitored in aerobic shake-flask cultures grown on synthetic medium with 20 g l⁻¹ glucose. Symbols indicate the following *S. cerevisiae* strains: ●, CEN.PK113-7D and ■, IMK692 (*pmr1Δ*). Data shown are from a single flask experiment for each strain. For both strains, data obtained from independent duplicate experiments differed by less than 5%.

Figure S5. XylA activity measured in cell extracts and the effect of divalent metals. Xl activity measured in cell-free extracts prepared from exponentially growing shake-flask cultures of *S. cerevisiae* strains IMX696 (*xylA*, *PPP*↑, *XKS1*↑) and IMX906 (*xylA*, *PPP*↑, *XKS1*↑, *pmr1Δ*), pre-grown under aerobic and anaerobic conditions on glucose. 25–50 μl of the extract was used in a 1 ml reaction mixture containing 20 mM MOPS buffer pH 7.0, 0.25 mM NADH, 500 mM xylose, 2U sorbitol dehydrogenase and 10mM of the divalent metal ion indicated. The activity values represent average and mean deviation of independent duplicate experiments.

Figure S6. SDS-polyacrylamide gel electrophoresis of xylose isomerases isolated from different engineered *S. cerevisiae* strains. Lane 1: IMX906 (*xylA*, *PPP* $\uparrow$, *XKS1* $\uparrow$, *pmr1* Δ) grown on glucose; Lane 2: IMX696 (*xylA*, *PPP* $\uparrow$, *XKS1* $\uparrow$) grown on glucose; Lane 3: IMX906 grown on xylose; Lane 4: IMX696 grown on glucose; Lane 5: IMX696 grown on glucose; Lane 6: IMX906 grown on xylose.

Figure S7. Schematic overview of the integrated construct that enables xylose consumption in IMX696 (*xylA*, *PPP* $\uparrow$, *XKS1* $\uparrow$). The construct consists of 15 cassettes containing 60bp homologous sequences named A to Q. The fragments were transformed with pUD335 allowing for a Cas9-induced double-strand break in *GRE3*. Correct integration of all the fragments in *GRE3* was verified by diagnostic PCR.

REFERENCES

- Alper H, Stephanopoulos G. Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? *Nat Rev Microbiol*. 2009;7:715–23.
- van Maris AJA, Abbott DA, Bellissimi E, van den Brink J, Kuyper M, Luttik MAH, et al. Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. *Antonie Van Leeuwenhoek*. 2006;90:391–418.
- Wang PY, Schneider H. Growth of yeast on D-xylulose. *Can J Microbiol*. 1980;26:1165–8.
- Jeffries TW. Utilization of xylose by bacteria, yeasts, and fungi. *Adv Biochem Eng Biotechnol*. 1983;27:1–32.
- Jeffries TW. Engineering yeasts for xylose metabolism. *Curr Opin Biotechnol*. 2006;17:320–6.
- Hahn-Hägerdal B, Wahlbom CF, Gárdonyi M, van Zyl WH, Cordero Otero RR, Jönsson LJ. Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. *Adv Biochem Eng Biotechnol*. 2001;73:53–84.
- Kötter P, Ciriacy M. Xylose fermentation by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol*. 1993;38:776–83.
- Runquist D, Hahn-Hägerdal B, Bettiga M. Increased ethanol productivity in xylose-utilizing *Saccharomyces cerevisiae* via a randomly mutagenized xylose reductase. *Appl Environ Microbiol*. 2010;76:7796–802.
- Watanabe S, Saleh AA, Pack SP, Annaluru N, Kodaki T, Makino K. Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein-engineered NADH-preferring xylose reductase from *Pichia stipitis*. *Microbiology*. 2007;153:3044–54.
- Walfridsson M, Bao X, Anderlund M, Lilius G, Bülow L, Hahn-Hägerdal B. Ethanol fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus* *xylA* gene, which expresses an active xylose (glucose) isomerase. *Appl Environ Microbiol*. 1996;62:4648–51.
- Sarthiy A V, McConaughy BL, Lobo Z, Sundstrom JA, Furlong CE, Hall BD. Expression of the *Escherichia coli* xylose isomerase gene in *Saccharomyces cerevisiae*. *Appl Environ Microbiol*. 1987;53:1996–2000.
- Moes C, Pretorius I, Zyl W. Cloning and expression of the *Clostridium thermosulfurogenes* D-xylose isomerase gene (*xylA*) in *Saccharomyces cerevisiae*. *Biotechnol Lett*. 1996;18:269–74.
- Gárdonyi M, Hahn-Hägerdal B. The *Streptomyces rubiginosus* xylose isomerase is misfolded when expressed in *Saccharomyces cerevisiae*. *Enzyme Microb Technol*. 2003;32:252–9.
- Harhangi HR, Akhmanova AS, Emmens R, van Der Drift C, de Laat WTAM, van Dijken JP, et al. Xylose metabolism in the anaerobic fungus *Piromyces* sp. strain E2 follows the bacterial pathway. *Arch Microbiol*. 2003;180:134–41.
- Kuyper M, Winkler AA, Van Dijken JP, Pronk JT. Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. *FEMS Yeast Res*. 2004;4:655–64.
- Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MSM, de Laat WTAM, et al. High-level functional expression of a fungal xylose isomerase: the key to efficient ethanol fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Res*. 2003;4:69–78.
- Ha SJ, Kim SR, Choi JH, Park MS, Jin YS. Xylitol does not inhibit xylose fermentation by engineered *Saccharomyces cerevisiae* expressing *xylA* as severely as it inhibits xylose isomerase reaction in vitro. *Appl Microbiol Biotechnol*. 2011;92:77–84.
- van Maris AJ a, Winkler A a, Kuyper M, de Laat WT a M, van Dijken JP, Pronk JT. Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose isomerase as a key component. *Adv Biochem Eng Biotechnol*. 2007;108:179–204.
- Kuyper M, Hartog MMP, Toirkens MJ, Almering MJH, Winkler AA, van Dijken JP, et al. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res*. 2005;5:399–409.
- Demeke MM, Dietz H, Li Y, Foulquié-Moreno MR, Mutturi S, Deprez S, et al. Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. *Biotechnol Biofuels*. 2013;6:89.
- Zhou H, Cheng J sheng, Wang BL, Fink GR, Stephanopoulos G. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. *Metab Eng*. 2012;14:611–22.
- Demeke MM, Foulquié-Moreno MR, Dumortier F, Thevelein JM. Rapid evolution of recombinant *Saccharomyces cerevisiae* for xylose fermentation through formation of extra-chromosomal circular DNA. *PLoS Genet*. 2015;11:e1005010.
- Brat D, Boles E, Wiedemann B. Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. *Appl Environ Microbiol*. 2009;75:2304–11.

24. Lee SM, Jellison T, Alper HS. Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* 2012;78:5708–16.
25. Hou J, Jiao C, Peng B, Shen Y, Bao X. Mutation of a regulator Ask10p improves xylose isomerase activity through up-regulation of molecular chaperones in *Saccharomyces cerevisiae*. *Metab Eng.* 2016;38:241–50.
26. Farwick A, Bruder S, Schadeweg V, Oreb M, Boles E. Engineering of yeast hexose transporters to transport D-xylose without inhibition by D-glucose. *Proc Natl Acad Sci.* 2014;111:5159–64.
27. Nijland JG, Shin HY, De Jong RM, De Waal PP, Klaassen P, Driessen AJM. Engineering of an endogenous hexose transporter into a specific D-xylose transporter facilitates glucose-xylose co-consumption in *Saccharomyces cerevisiae*. *Biotechnol Biofuels.* 2014;7:168.
28. Shin HY, Nijland JG, De Waal PP, De Jong RM, Klaassen P, Driessen AJM. An engineered cryptic Hxt11 sugar transporter facilitates glucose-xylose co-consumption in *Saccharomyces cerevisiae*. *Biotechnol Biofuels.* 2015;8:176.
29. Apel AR, Ouellet M, Szmidszt-Middleton H, Keasling JD, Mukhopadhyay A. Evolved hexose transporter enhances xylose uptake and glucose/xylose co-utilization in *Saccharomyces cerevisiae*. *Sci Rep.* 2016;6:19512.
30. Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *FEMS Yeast Res.* 2005;5:925–34.
31. Wisselink HW, Cipollina C, Oud B, Crimi B, Heijnen JJ, Pronk JT, et al. Metabolome, transcriptome and metabolic flux analysis of arabinose fermentation by engineered *Saccharomyces cerevisiae*. *Metab Eng.* 2010;12:537–51.
32. Kuijpers NGA, Chroumpi S, Vos T, Solis-Escalante D, Bosman L, Pronk JT, et al. One-step assembly and targeted integration of multigene constructs assisted by the I-SceI meganuclease in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 2013;13:769–81.
33. Mans R, van Rossum HM, Wijsman M, Backx A, Kuijpers NGA, van den Broek M, et al. CRISPR/Cas9: a molecular swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 2015;15:fov004.
34. Träff KL, Cordero Otero RR, van Zyl WH, Hahn-Hägerdal B. Deletion of the *GRE3* aldose reductase gene and its influence on xylose metabolism in recombinant strains of *Saccharomyces cerevisiae* expressing the *xyIA* and *XKS1* genes. *Appl Env Microbiol.* 2001;67:5668–74.
35. Rudolph HK, Antebi A, Fink GR, Buckley CM, Dorman TE, LeVitre JA, et al. The yeast secretory pathway is perturbed by mutations in PMR1, a member of a Ca²⁺ ATPase family. *Cell.* 1989;58:133–45.
36. Lapinskas PJ, Cunningham KW, Liu XF, Fink GR, Culotta VC. Mutations in PMR1 suppress oxidative damage in yeast cells lacking superoxide dismutase. *Mol Cell Biol.* 1995;15:1382–8.
37. Sorin A, Rosas G, Rao R. PMR1, a Ca²⁺-ATPase in yeast golgi, has properties distinct from sarco/endoplasmic reticulum and plasma membrane calcium pumps. *J Biol Chem.* 1997;272:9895–901.
38. Culotta VC, Yang M, Hall MD. Manganese transport and trafficking: lessons learned from *Saccharomyces cerevisiae*. *Eukaryot Cell.* 2005;4:1159–65.
39. Yu D, Danku JMC, Baxter I, Kim S, Vatamaniuk OK, Vitek O, et al. High-resolution genome-wide scan of genes, gene-networks and cellular systems impacting the yeast ionome. *BMC Genomics.* 2012;13:623.
40. van Bastelaere PBM, Vangrysperre W, Kersters-Hildersont H. Kinetic studies of Mg²⁺, Co²⁺ and Mn²⁺-activated D-xylose isomerases. *Biochem J.* 1991;278:285–92.
41. Dicarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Res.* 2013;41:4336–43.
42. Bakker BM, Overkamp KM, Van Maris AJA, Kötter P, Luttik MAH, Van Dijken JP, et al. Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev.* 2001;25:15–37.
43. Chang A, Schomburg I, Placzek S, Jeske L, Ulbrich M, Xiao M, et al. BRENDA in 2015: Exciting developments in its 25th year of existence. *Nucleic Acids Res.* 2015;43:D439–46.
44. van den Brink J, Akeroyd M, van der Hoeven R, Ponk JT, de Winde JH, Daran-Lapujade P. Energetic limits to metabolic flexibility: responses of *Saccharomyces cerevisiae* to glucose-galactose transitions. *Microbiology.* 2009;155:1340–50.
45. Devasahayam G, Ritz D, Helliwell SB, Burke DJ, Sturgill TW. Pmr1, a golgi Ca²⁺/Mn²⁺-ATPase, is a regulator of the target of rapamycin (TOR) signaling pathway in yeast. *Proc Natl Acad Sci.* 2006;103:17840–5.
46. Cui Y, Zhao S, Wu Z, Dai P, Zhou B. Mitochondrial release of the NADH dehydrogenase Ndi1 induces apoptosis in yeast. *Mol Biol Cell.* 2012;23:4373–82.
47. Li W, Sun L, Liang Q, Wang J, Mo W, Zhou B. Yeast AMID homologue Ndi1p displays respiration-restricted apoptotic activity and is involved in chronological aging. *Mol Biol Cell.* 2006;17:1802–11.

48. Hartley BS, Hanlon N, Jackson RJ, Rangarajan M. Glucose isomerase: insights into protein engineering for increased thermostability. *Biochim Biophys Acta*. 2000;1543:294–335.
49. Madhavan A, Tamalampudi S, Ushida K, Kanai D, Katahira S, Srivastava A, et al. Xylose isomerase from polycentric fungus *Orpinomyces*: gene sequencing, cloning, and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol. *Appl Microbiol Biotechnol*. 2009;82:1067–78.
50. Dun B, Wang Z, Ye K, Zjang B, Li G, Lu M. Functional expression of Arabidopsis thaliana xylose isomerase in *Saccharomyces cerevisiae*. *Xinjiang Agric Sci*. 2012;49:681–6.
51. Hector RE, Dien BS, Cotta MA, Mertens JA. Growth and fermentation of D-xylose by *Saccharomyces cerevisiae* expressing a novel D-xylose isomerase originating from the bacterium *Prevotella ruminicola* TC2-24. *Biotechnol Biofuels*. 2013;6:84.
52. Hou J, Shen Y, Jiao C, Ge R, Zhang X, Bao X. Characterization and evolution of xylose isomerase screened from the bovine rumen metagenome in *Saccharomyces cerevisiae*. *J Biosci Bioeng*. 2015;121:160–5.
53. Klaassen P, Gielesen BEM, Van Suylekom GP, Sarantino-Poulos P, Heijne WHM, Greeve A. Yeast cell capable of converting sugars including arabinose and xylose. 2012. US 20140141473 A1
54. Ko JK, Um Y, Lee SM. Effect of manganese ions on ethanol fermentation by xylose isomerase expressing *Saccharomyces cerevisiae* under acetic acid stress. *Bioresour Technol*. 2016;222:422–30.
55. Entian KD, Kötter P. 25 Yeast genetic strain and plasmid collections. *Methods Microbiol*. 2007;36:629–66.
56. Nijkamp JF, van den Broek M, Datema E, de Kok S, Bosman L, Lutik MA, et al. De novo sequencing, assembly and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology. *Microb Cell Fact*. 2012;11:36.
57. Shevchuk NA, Bryksin A V, Nusinovich YA, Cabello FC, Sutherland M, Ladisch S. Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. *Nucleic Acids Res*. 2004;32:19e.
58. Janke C, Magiera MM, Rathfelder N, Taxis C, Reber S, Maekawa H, et al. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast*. 2004;21:947–62.
59. Wiedemann B, Boles E. Codon-optimized bacterial genes improve L-arabinose fermentation in recombinant *Saccharomyces cerevisiae*. *Appl Environ Microbiol*. 2008;74:2043–50.
60. Gietz RD, Woods RA. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol*. 2002;350:87–96.
61. Solis-Escalante D, Kuijpers NGA, Bongaerts N, Bolat I, Bosman L, Pronk JT, et al. *amdSYM*, a new dominant recyclable marker cassette for *Saccharomyces cerevisiae*. *FEMS Yeast Res*. 2013;13:126–39.
62. Verduyn C, Postma E, Scheffers WA, van Dijken JP. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous culture study on the regulation of respiration and alcoholic fermentation. *Yeast*. 1992;8:501–17.
63. Verduyn C, Postma E, Scheffers WA, van Dijken JP. Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. *J Gen Microbiol*. 1990;136:395–403.
64. Guadalupe MV, Almering MJH, van Maris AJA, Pronk JT. Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. *Appl Environ Microbiol*. 2010;76:190–5.
65. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25:1754–60.
66. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One*. 2014;9:e112963.
67. Thorvaldsdóttir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): High-performance genomics data visualization and exploration. *Brief Bioinform*. 2013;14:178–92.
68. Nijkamp JF, Van Den Broek MA, Geertman JMA, Reinders MJT, Daran JMG, de Ridder D. De novo detection of copy number variation by co-assembly. *Bioinformatics*. 2012;28:3195–202.
69. Eide DJ, Clark S, Nair TM, Gehl M, Gribskov M, Guerinot M Lou, et al. Characterization of the yeast ionome: a genome-wide analysis of nutrient mineral and trace element homeostasis in *Saccharomyces cerevisiae*. *Genome Biol*. 2005;6:R77.
70. Kersters-Hilderson H, Callens M, Van Opstal O, Vangrype W, De Bruyne CK. Kinetic characterization of D-xylose isomerases by enzymatic assays using D-sorbitol dehydrogenase. *Enzyme Microb Technol*. 1987;9:145–8.

CHAPTER 5

Structure-based directed evolution improves *S. cerevisiae* growth on xylose by influencing in vivo enzyme performance

5

Misun Lee¹, Henriëtte J. Rozeboom¹, Eline Keuning¹, Paul de Waal², and Dick B. Janssen^{1*}

¹ Biochemical Laboratory, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands.

² DSM Biotechnology Center, Alexander Fleminglaan 1, 2613 AX, Delft, The Netherlands.

* Corresponding author

Published in *Biotechnology for Biofuel*. 2020; 13: 5
(<https://doi.org/10.1186/s13068-019-1643-0>)

Authors' contributions

This study was designed by ML, PdW and DBJ. ML conducted all experiments except X-ray crystallography. HJR performed the crystallization and determined the structures. EK contributed to the design of PirXI libraries. The manuscript was written by ML, HJR and DBJ. All authors have read and approved the submitted manuscript.

ABSTRACT

Efficient bioethanol production from hemicellulose feedstocks by *Saccharomyces cerevisiae* requires xylose utilization. Whereas *S. cerevisiae* does not metabolize xylose, engineered strains that express xylose isomerase can metabolize xylose by converting it to xylulose. For this, the type II xylose isomerase from *Piromyces* (PirXI) is used but the in vivo activity is rather low and very high levels of the enzyme are needed for xylose metabolism. In this study, we explore the use of protein engineering and in vivo selection to improve the performance of PirXI. Recently solved crystal structures were used to focus mutagenesis efforts. We constructed focused mutant libraries of *Piromyces* xylose isomerase by substitution of second shell residues around the substrate- and metal-binding sites. Following library transfer to *S. cerevisiae* and selection for enhanced xylose-supported growth under aerobic and anaerobic conditions, two novel xylose isomerase mutants were obtained, which were purified and subjected to biochemical and structural analysis. Apart from a small difference in response to metal availability, neither the new mutants nor mutants described earlier showed significant changes in catalytic performance under various in vitro assay conditions. Yet, in vivo performance was clearly improved. The enzymes appeared to function sub-optimally in vivo due to enzyme loading with calcium, which gives poor xylose conversion kinetics. The results show that better in vivo enzyme performance is poorly reflected in kinetic parameters for xylose isomerization determined in vitro with a single type of added metal. This study shows that in vivo selection can identify xylose isomerase mutants with only minor changes in catalytic properties measured under standard conditions. Metal loading of xylose isomerase expressed in yeast is suboptimal and strongly influences kinetic properties. Metal uptake, distribution and binding to xylose isomerase are highly relevant for rapid xylose conversion and may be an important target for optimizing yeast xylose metabolism.

Keywords: Xylose isomerase, Metalloenzyme, Directed evolution, Bioethanol, Protein engineering

INTRODUCTION

Efficient xylose conversion is an important property when selecting or engineering yeast strains to be used in second-generation bioethanol production. Fermentation of lignocellulose-derived feedstocks, which contain up to 30 % D-xylose, is often carried out by *Saccharomyces cerevisiae*. Since this yeast does not metabolize the aldopentose D-xylose naturally, incorporation of either xylose isomerase or a combination of xylose reductase and xylitol dehydrogenase is necessary to convert D-xylose to the ketose D-xylulose, which can be metabolized [1–4]. The use of xylose isomerase has the advantage over the xylose reductase-xylitol dehydrogenase system that there is no intermediate production of xylitol and less formation of side products, but combining the pathways may also have certain benefits [5]. Several efforts to find suitable xylose isomerases have been reported [6–11]. A xylose isomerase discovered by Kuyper et al. [12,13] in the fungal strain *Piromyces* E2 (PirXI) through genome mining is an attractive candidate for xylose isomerization in engineered *S. cerevisiae* strains, and is used in several studies [14]. However, in vivo performance of the enzyme is modest, as indicated by the high copy number (up to 10) of the chromosomally inserted XI-encoding gene observed in evolved strains that are capable of anaerobic D-xylose fermentation [15]. A multi-copy plasmid leading to overproduction of the of the PirXI protein has also been used for enhanced xylose metabolism [16]. The engineering of yeast strains showing faster xylose metabolism is an important challenge in the pursuit of strain improvement for second-generation bioethanol production [17–20].

The observation that strains with multiple copies of PirXI genes evolve during prolonged adaptation suggests that in vivo enzyme activity in *S. cerevisiae* is limiting xylose turnover [21]. Mutations in different xylose isomerases can lead to accelerated xylose metabolism and protein engineering of xylose isomerase is receiving significant attention [11,14,19,20]. However, it is unclear which properties of the enzyme need to be tailored to improve its in vivo performance. A straightforward hypothesis is that the kinetic properties as reflected in catalytic rate (k_{cat}) and/or substrate affinity (K_M) at physiological conditions are not optimal for efficient xylose metabolism. On the other hand, metal affinity and in vivo metal content of xylose isomerase may also play an important role causing the enzyme to function sub-optimally. Xylose isomerase is a metalloenzyme that requires two divalent metals for activity, and the wild-type PirXI shows the best activity with Mn^{2+} [22]. However, metal content of the enzyme expressed in yeast may vary [21,22]. A yeast strain with a mutation in its PMR1 gene which influences manganese homeostasis and increases Mn^{2+} content of the PirXI protein showed an enhanced rate of xylose consumption [21]. The high expression levels of the

PirXI protein in selected xylose-metabolizing strains could be a burden for cell growth, and reduced expression of a more active enzyme would improve xylose consumption and growth rates. In view of the complexity of yeast cells, there may well be other factors that determine the performance of heterologously expressed enzymes, including compartmentalization, enzyme stability, and competition for metals with other cellular components.

Modern protein engineering tools enable tailoring of enzyme properties for specific applications. A particularly effective strategy is the use of directed evolution, i.e. the construction of mutant libraries and screening those for improved variants [23–25]. This approach does not require structural information. Here, one can take advantage of the fact that xylose isomerase activities limit the growth of *S. cerevisiae* on D-xylose and employ a random mutagenesis method with in vivo selection for improved growth [9,14]. This allowed the discovery of unexpected xylose isomerase mutations, some of which were far away from the active site. It is known that distant mutations can enhance activity, e.g., by influencing enzyme surface properties [26]. On the other hand, the lack of focus in random mutagenesis protocols yields libraries with a low abundance of beneficial mutations and very large numbers of mutants often must be screened to discover better enzymes. So-called smart libraries, which incorporate phylogenetic and structural information in the design, are assumed to better cover functional sequence space, increasing the chance of discovering useful mutations and reducing the need for extensive screening [27–29].

To support PirXI engineering and understand the effects of selected mutations, we have recently characterized the enzyme both structurally and biochemically, which is described in chapter 2 [22]. Even though the unidentified causes of the modest in vivo performance of PirXI and the complexity of the kinetic mechanism cause uncertainty about the types of mutations to introduce, the structures still provide useful information by revealing the residues that shape the substrate- and metal-binding sites. In the crystal structures, PirXI appeared as a homotetramer with each monomer (49.5 kDa, 437 aa) possessing an active site in which two divalent metal ions are bound. Soaking and cocrystallization studies showed that the ring-opened xylose binds in between two fully conserved tryptophan residues (Trp50 and Trp189) which play a role in the correct positioning of the substrate for catalysis [22,30]. Of the two active site metals, one (M_1) is responsible for substrate binding while the other (M_2) is essential for catalysis by polarizing the M_2 -bound catalytic water that protonates O1 of the substrate and consequently generates a carbocation on C1 promoting the C2 to C1 hydride shift [22,31]. The catalytic metal M_2 moves during the reaction from the M_{2a} to the M_{2b} position, which is also visible in structures with certain combination of ligands: 5NH7 (xylose and Mg^{2+}), 5NHC (xylulose and Co^{2+}), 5NHD (xylose and Ni^{2+}) and 5NHE (xylose and Cd^{2+}). (Figure 1) [22].

To examine if structure-inspired mutagenesis can contribute to obtaining improved xylose isomerase variants, and to investigate the possibility that such mutants may be useful to identify catalytic properties relevant for in vivo performance, we now designed focused mutant libraries with mutations surrounding the residues involved in metal and substrate binding. These were screened for enhanced growth after expression in yeast. Improved enzymes were indeed discovered and their biochemical properties were investigated, in comparison to previously reported PirXI variants. The results indicate that small changes in catalytic properties may be accompanied by significant effects on xylose-supported growth. Furthermore, in vivo selection may govern mutations that improve xylose metabolism without changing kinetic properties measured under standard conditions with Mg^{2+} as the activating metal.

RESULTS

Library design

To discover mutants of PirXI that enhance xylose metabolism, we designed and constructed small focused mutant libraries followed by in vivo screening for better variants. Recent structural and biochemical information was used to select target positions for mutagenesis, focusing on residues that surround the metal-binding sites. Replacing the second shell residues might have an effect on metal binding or reactivity and thereby influence the activity. The fully conserved metal-coordinating residues in PirXI are Glu233, Glu269, Asp297 and Asp340 for site M_1 and Glu269, His272, Asp308 and Asp310 for the catalytic metal M_2 . For the first library (LibM1), we targeted residues that are in close proximity of the M_2 site. Five residues were selected: three (Val270, Ala273 and Thr274) that lie on the same helix as the metal binding residues (His272 and Glu269), and two (Trp307 and Thr309) that are on a nearby loop (Figure 1). Amino acid diversity to be introduced at each position was selected based on phylogenetic diversity, in silico-predicted stabilities of the mutants, and visual inspection of the predicted mutant structures. For phylogenetic input, a multiple sequence alignment was performed on 22 different class I and 100 class II XI sequences. Considering conservation scores and similarities between amino acid properties, the library diversity was decided. For example, residue Ala270 is fully conserved throughout all class II enzymes and therefore the diversity at this position was restricted to alanine and glycine to avoid extreme modifications. Changes in free energy of folding ($\Delta\Delta G_{fold}$) of mutants relative to the wild-type enzyme were predicted using FoldX calculations [32]. Large decreases in predicted stability were used to dismiss mutations from the library design. The resulting LibM1 library included 1008 different variants (Table 1).

Figure 1. Structure of the PirXI active site and design of mutant library LibM1. The figure shows the active site structure of PirXI with xylose (yellow) and Mg^{2+} ions (green spheres) bound (PDB: 5NH7). The target residues (orange) are located near the active site. The catalytic metal (M_2) can occupy two positions (M_{2a} and M_{2b}). W307' is a residue from a neighboring subunit.

Library construction

To construct the LibM1 mutant library, at each target position a minimum number of partially undefined codons covering the selected set of amino acid substitutions was chosen using a spreadsheet implementing the CodonFinder routine [33]. The codons were selected in such a way that all the desired amino acids (including the wild-type residues) are incorporated at balanced coverage without introduction of undesired codons like stop codons. The 1008 LibM1 mutant library was covered by 8 partially undefined codons (Table 1).

Library DNA was obtained by generating gene fragments using PCR and subsequent cloning into *E. coli* – yeast shuttle expression vector (pRS426-URA) as described in Methods. Transformation of the library DNA to *E. coli* resulted in over 8000 clones, of which pooled plasmid DNA was transformed to *S. cerevisiae* strain DS75543, producing 6000 colonies. Considering the library size of 1008, these numbers are sufficient for near full library coverage [33]. Prior to yeast transformation, library diversity was confirmed by sequencing a mixture of plasmids isolated from the mixed collection of *E. coli* transformants. The sequencing results showed that all expected bases were incorporated at the correct positions, indicating sufficient library quality to proceed to screening.

Table 1. Design of PirXI library LibM1.

Residue	Amino acids	Codons	Freq. WT ^a
V270	V A G	GBT	0.33
A273	A G	GST	0.5
T274	T I K Q	AYC+MAA	0.25
W307	W F Y I S T N	WHT+TGG	0.14
T309	T S K R N Q	AVS+CAG	0.29

^a Codons at each position were selected in such a way that each desired amino acid, including the wild type, is included at a frequency of 1/(total diversity).

Screening for improved xylose utilization

Library LibM1 was screened by growth competition of *S. cerevisiae* strain DS75543 transformed with library plasmids DNA. The entire collection of yeast transformants was inoculated into xylose medium and cells were cultivated with serial transfers to fresh xylose medium. Faster growing cells, which over time became dominant, were assumed to harbor an improved PirXI. Screening was performed both under aerobic and anaerobic (oxygen-limited) conditions, each in duplicate, as different variants can be expected depending on the metabolic status of the cells. For anaerobic growth, the cultures were kept oxygen-limited as described in Methods. The effect of limited oxygen availability was reflected in the final cell densities (OD₆₀₀) of the cultures which were ~ 3 and > 20 for anaerobic and aerobic conditions, respectively. Anaerobic cultures initially required 8 – 9 days before growth occurred. A reduced lag time and/or increased growth rate was observed after multiple transfers with all four selection cultures, also in comparison to a control culture harboring only wild-type PirXI.

Both aerobic and anaerobic cultures were harvested after the 10th transfer and plasmids were isolated to evaluate the selected PirXI genes. Sequencing showed that all four cultures, i.e., both the aerobic duplicates and anaerobic duplicates, contained only one PirXI variant, which carried the mutations V270A and A273G.

Effect of V270A-A273G PirXI on xylose utilization

The consistent selection of the V270A-A273G variant from library LibM1 suggested that the screening method was reliable and sensitive. Nevertheless, it is possible that other events such as genomic mutations or variations in expression level are responsible for the improved growth of yeast carrying the V270A-A273G mutant PirXI. The replicon of the pRS416 vector used in this work is derived from yeast plasmid 2μ, and plasmid copy numbers can vary from culture to culture [34]. Furthermore, in laboratory evolution of *S. cerevisiae* for growth on xylose, cells may acquire diverse chromosomal mutations that cause improved growth [35,36]. To prove that in our case the selected mutations in the PirXI structural

gene caused improved growth on xylose, the mutations were re-constructed by site-directed mutagenesis in the original PirXI gene and cloned in a vector that was not subjected to previous selection. *S. cerevisiae* DS75543 cells were then transformed with the freshly prepared constructs and their growth performance in a 96-well plate was monitored. We have repeated this process several times and consistently observed that the cells containing the mutant PirXI grow on xylose better compared to those containing wild-type PirXI (Figure 2a). We have also observed that general growth performance of the cells slightly differs between experiments as well as between clones picked from a single transformation experiment. Figure 2B shows a high variability in growth between 30 independent transformants despite their identical genotype. Nevertheless, the results clearly indicate that the V270A-A273G mutant PirXI improves growth on xylose as compared to wild-type, especially in the earlier phases of growth (Figure 2b). On average, the mutant cultures started to grow earlier and more quickly reached their final density.

In 96-well plates, oxygen availability may not be well controlled and results could be influenced by evaporation. Therefore, a comparison between the wild-type and V270A-A273G XI variants was also performed using shake flask cultures with replicates inoculated with pre-cultures from independent transformants. The resulting growth curves (Figure 2c) confirm that the mutated PirXI is beneficial for growth on xylose. The specific growth rates (μ) were calculated from the exponential part of the curves, using the following equation for fitting: $\ln X = \ln X_0 + \mu (t - t_0)$, where X is the measured OD_{600} and μ is the rate. The average growth rates of the wild type and the mutants are 0.13 ± 0.01 and $0.18 \pm 0.01 \text{ h}^{-1}$, respectively. These results show that the observed improved growth is due to the V270A-A273G mutations in PirXI, not by unidentified mutations elsewhere on the plasmid or in the chromosome of the selected transformants.

Yeast cells selected for good growth on xylose show high overexpression of PirXI [12,15], which may be a metabolic burden for the cells and trigger selection of mutants with a higher activity: expression ratio in competition experiments. To examine if the PirXI mutations affected enzyme expression, we studied XI levels in cells grown on xylose. The specific activity with 100 mM xylose measured with the cell-free extracts were 0.94 U/mg and 0.54 U/mg for the wild type and the mutant, respectively. SDS-PAGE gels revealed that the expression levels for the wild-type and the mutant enzyme were similar (Figure 3).

Figure 2. PirXI V270A-A273G improves growth of *S. cerevisiae* strain DS75543 on xylose. The orange and green lines represent growth on xylose (20 g·l⁻¹) of yeast expressing the mutant and the wild-type PirXI, respectively. a, b Growth in 96-well plate with continuous measurement of optical density. Each line represents an individual clone selected from a transformation plate. c Growth in triplicate in batch culture with intermittent sampling and measurement of optical densities.

Figure 3. Expression level and cell-free extract activities of PirXI variants. Samples of 2 μ g of crude extract protein prepared from cells grown on xylose medium were loaded on an SDS gel. The dominant bands at ~50 kDa represent PirXI. The expression level calculated by measuring the intensity of the bands is about 25% for both samples. WT: wild-type PirXI VA: V270A-A273G PirXI.

The uncertainty of in vivo metal binding properties of PirXI and metal content of the yeast cytoplasm makes it difficult to define a metal composition for assays that gives results reporting on in vivo performance. When we measured the PirXI activity of extracts of *S. cerevisiae* DS75543 cells without metal addition, the results indicated that the activity of the wild-type enzyme was almost two-fold higher compared to the mutant. This unexpected observation could be caused by changes in PirXI metal composition during enzyme preparation and dilution, e.g., due to binding of metals released from organelles such as vacuoles or from changes in metal-protein interactions.

To examine if the individual mutations in the PirXI variant are both necessary for improved growth, we constructed the single mutants V270A and A273G and examined the effect on growth on xylose, particularly on the early growth phase. The growth curves indicate that the V270A mutation has a larger effect showing much earlier initiation of exponential growth (Figure 4), but also cells containing the A273G mutant PirXI showed a slight growth improvement compared to the cells expressing the wild-type enzyme.

Figure 4. Effect of the mutations V270A and A273G on growth. Growth in xylose medium of *S. cerevisiae* cells containing PirXI variants was followed. Wild type (green lines), V270A (blue lines), A273G (red lines) or V270A-A273G (orange lines). Measurements were performed in triplicate, shown as individual lines.

Kinetic properties of PirXI V270A-A273G

We expected that the positive effect of the V270A-A273G mutations on xylose-supported growth would be due to improved catalytic parameters, i.e., increased catalytic rate (k_{cat}) or a better substrate affinity (reduced K_{M}). Since PirXI can be activated by different divalent metals and activities depend on the type of metal that is bound [22], we measured XI activities with metals that the enzyme potentially binds in vivo as previously found by metal analysis (Mg^{2+} , Mn^{2+} or Ca^{2+}) [21]. With none of these metals, the in vitro activities revealed an increased k_{cat} or decreased K_{M} for the mutant enzyme in comparison to wild type. In contrast, the wild type performed better in the presence of all metals tested, showing slightly higher catalytic rates and substrate affinities (Table 2). Especially, with Mn^{2+} , the activity of the mutant decreased 50% compared to the wild type. This result indicates that an increase in specific activity with these metals, at least individually, is not responsible for the improved growth on xylose of yeast expressing V270A-A273G PirXI.

Table 2. Kinetic parameters of PirXI variants.

PirXI variants	Metal	K_{act} (μ M) ^c	Kinetic parameters ^a		
			k_{cat} (s^{-1})	K_M (mM)	k_{cat} / K_M ($s^{-1} \cdot M^{-1}$)
WT	Mg ²⁺	130 $\pm$ 7	2.2 $\pm$ 0.1	7.1 $\pm$ 0.5	310
	Mn ²⁺	0.44 $\pm$ 0.05	6.9 $\pm$ 0.2	6.2 $\pm$ 0.7	1110
	Ca ²⁺	230 $\pm$ 20	0.8 $\pm$ 0.1	1360 $\pm$ 160	0.4 ^b
V270A-A273G	Mg ²⁺	160 $\pm$ 14	1.8 $\pm$ 0.1	11.6 $\pm$ 1.6	155
	Mn ²⁺	0.39 $\pm$ 0.05	2.8 $\pm$ 0.1	9 $\pm$ 1	310
	Ca ²⁺	140 $\pm$ 12	0.28 $\pm$ 0.01	1760 $\pm$ 120	0.18 ^b
S141N-T142S-A143S-G174A	Mg ²⁺	126 $\pm$ 10	1.6 $\pm$ 0.2	27 $\pm$ 9	60
	Mn ²⁺	2.4 $\pm$ 0.5	4.9 $\pm$ 1	37 $\pm$ 1.5	132
	Ca ²⁺	96.5 $\pm$ 3.5	0.06 $\pm$ 0.01	1200 $\pm$ 300	0.03 ^b
E15D-T142S	Mg ²⁺	190 $\pm$ 10	1.9 $\pm$ 0.1	22 $\pm$ 2	86
	Mn ²⁺	2.1 $\pm$ 0.3	5 $\pm$ 0.2	53 $\pm$ 4	94
	Ca ²⁺	50 $\pm$ 10	> 0.02	N. A	0.018 ^b
N338C	Mg ²⁺	112 $\pm$ 7	4.2 $\pm$ 0.2	22 $\pm$ 2	191
	Mn ²⁺	1.3 $\pm$ 0.3	7.2 $\pm$ 0.2	29 $\pm$ 1.5	248
	Ca ²⁺	163 $\pm$ 6.5	0.78 $\pm$ 0.2	2800 $\pm$ 800	0.2 ^b

^a All assays were performed at 30°C in 20 mM MOPS buffer, pH 7. Values are averages from three (wild type, V270A-A273G PirXI) or two (S141N-T142S-A143S-G174A, E15D-T142S and N338C PirXI) independent measurements. The margins represent standard deviations.

^b Catalytic efficiencies obtained from slopes of Michaelis-Menten plots at $[S] \ll K_M$ because of the high K_M for xylose in the presence of Ca²⁺ as activating metal.

^c The K_{act} value is defined as the metal concentration giving half-maximal activity, as measured with a saturating concentration of xylose (100 mM in case of Mg⁺ and Mn²⁺, 400 mM in case of Ca²⁺).

Besides the metal-dependence of the isomerase, metal affinities were considered as a possible cause of improved in vivo enzyme performance. We estimated metal affinities of the wild-type and the mutant enzyme by measuring the activation constant (K_{act}) for each metal. This constant represents the metal concentration giving half-maximal enzyme activity. Since xylose isomerase requires two metals for activity, the value depends on the binding sites with the lowest affinity if both sites must be occupied. For measuring K_{act} with Mg²⁺ and Mn²⁺, 100 mM xylose was used as substrate. In case of Ca²⁺, 400 mM xylose was used since the $K_{M,xylose}$ of the PirXI-Ca²⁺ is very high (Table 2). The data showed that the V270A-A273G mutant showed slightly higher affinity for Ca²⁺ and Mn²⁺, whereas the wild-type enzyme has slightly higher affinity for Mg²⁺ (Table 2). However, the differences are small and do not indicate a shift in metal affinity as the cause of improved growth.

Metal affinity was also examined by measuring the effect of metal addition on PirXI thermostability since metal binding can stabilize metalloenzymes [37].

Effects on apparent melting temperatures were measured in the presence of different concentrations of metals using thermal shift assays (Figure 5). The results showed that the apo forms of wild-type and V270A-A273G PirXI have a similar thermostability. Interestingly, whereas $T_{m,app}$ of the wild-type PirXI increased with metal concentration according to a hyperbolic saturation-like curve, the $T_{m,app}$ of the mutant enzyme was constant up to ca. 200 μM of Mn^{2+} or Ca^{2+} added, with an increase at higher metal concentrations (Figure 5a, c). In contrast, when Mg^{2+} was added, the thermostability of the mutant enzyme was not increased even at concentrations that were saturating for enzyme activity (Figure 5b). The difference between the metal-concentration dependence of mid points of thermal shift assays and K_{act} values measured in the presence of substrate suggests that substrate influences metal binding, as also observed when examining X-ray structures of the enzyme with different combinations of ligands [22]. Only in the presence of the substrate xylose both metal binding sites in the crystal structures were occupied, whereas in xylitol- or glycerol-bound enzyme only the M_1 metal site was occupied with a metal ion.

The metal content of yeast cells is complex and consists of both free metal ions and metal ions bound to macromolecules [38]. In general, in vivo metal binding by metalloproteins is controlled by mechanisms such as intracellular metal homeostasis, localization of protein folding, and activities of metal transporters and metallochaperones [39]. In a previous study (Chapter 4), we showed that changes in intracellular metal composition affect metal composition of PirXI, which in turn influences catalytic performance [21]. PirXI isolated from yeast grown on xylose is mostly bound with Ca^{2+} , which barely activates the enzyme. Therefore, a large portion of PirXI does not contribute to in vivo conversion of xylose. In contrast, the smaller fraction of PirXI that is bound with the strongly activating Mn^{2+} contributes most to the in vivo enzyme activity [21].

Figure 5. Effect of metals on thermostability of PirXI. The graphs show the apparent melting temperatures of purified and EDTA-treated wild-type PirXI (green lines) and the V270A-A273G mutant PirXI (orange lines) in the presence of various concentrations of metals (a Mn^{2+} ; b Mg^{2+} ; and c Ca^{2+}). The T_m values are averages from two independent measurements. $[E] = 20 \mu M$. Error bars represent standard deviations.

In view of the complex metal composition of *S. cerevisiae*, we measured the activities of the wild type and the mutant in the presence of varying concentrations of Mn^{2+} and a fixed high concentration (1 mM) of Ca^{2+} (Figure 6). As expected, both variants showed higher activity with increasing concentration of Mn^{2+} . Interestingly, the degree to which Mn^{2+} influenced the activity was different between the wild-type and the mutant enzyme. At low concentrations of Mn^{2+} (10 – 100 μM) and in the presence of 1 mM Ca^{2+} the mutant enzyme showed slightly higher activity. This indicates that the activation of the mutant enzyme by Mn^{2+} in the presence of a high concentration of Ca^{2+} is improved. Even though the activity of the mutant is lower in the presence of Mn^{2+} or Ca^{2+} alone, at certain low Mn^{2+}/Ca^{2+} concentration ratios, the V270A-A273G mutant enzyme is better activated than the wild type. These results suggest that differences in in vivo metal activation may be responsible for the improved growth of yeast cells expressing the V270A-A273G mutant PirXI.

Figure 6. PirXI activity in the presence of Ca^{2+} and Mn^{2+} .

The activities of wild-type enzyme (green line) and the variant V270A-A273G (orange line) on 100 mM xylose were measured in the presence of a mixture of 1 mM Ca^{2+} and various concentrations of Mn^{2+} . The data represent the average values from duplicate measurements and the error bars represent standard deviations.

Crystal structures of PirXI wild type and V270A-A273G

To examine possible structural changes in the V270A-A273G PirXI, we solved and compared crystal structures of this variant and the wild-type enzyme purified from yeast (Figure 7). The overall structures of the wild type and mutant enzyme are very similar and confirmed the mutations. In the mutant structure, the side chain of Phe280 moves ~ 0.5 Å in the direction of Ala270. Due to the decrease in hydrophobicity and size the surrounding waters also shift towards Ala270. Mutation A273G shows no effect on the structure. There is no significant difference between the two structures to explain the improved in vivo performance of the mutant.

The enzyme crystals were prepared without removal or addition of metal ions so that only intrinsic metal ions are present. When the metal ions were refined as Mg^{2+} , the $\text{Fo} - \text{Fc}$ map showed unaccounted electron density at the metal positions suggesting the presence of heavier ions. In an anomalous electron density map a clear signal was observed at the M_1 and M_2 positions with levels of 4.8 and 3.5, respectively. Mg^{2+} ions do not have an anomalous signal at the in-house used wavelength of 1.54 Å. However, a comparison with anomalous maps of previously determined structures [22] shows similar peak heights in the Ca-xylose structure of PirXI (PDB entry 5NH8). Therefore, metal ions at the M_1 and M_2 positions were refined as Ca^{2+} with 100% occupancy resulting in a flat $\text{Fo} - \text{Fc}$ map in both the wild-type and the double-mutant structures. The temperature factors (B-factors) of the two Ca^{2+} ions are 11.9 and 16.4 for the wild type and 11.4 and 19.3 for the mutant, which are lower than those of the surrounding residues. The distances of the coordinating side chains to the M_1 ion in the wild type and the mutant enzyme isolated from yeast are similar to those in the wild-type Ca-xylose structure reported earlier (5NH8). These results indicate that most of the metal binding sites of PirXI isolated from yeast are occupied by poorly activating Ca^{2+} ions, both in the wild-type and in the PirXI V270A-A273G mutant enzyme. Other metal ions, such as Mg^{2+} , Fe^{2+} , Mn^{2+} or Co^{2+} , may be bound with low occupancy.

Figure 7. Structural alignment of active sites of wild-type and V270A-A273G PirXI.

The X-ray structures are of wild-type (grey) and V270A-A273G mutant (orange) PirXI, both isolated from yeast cells grown on xylose. Ligand colors: xylose (yellow – wild type, cyan – mutant); metals (light green – wild type, dark green – mutant).

Construction and screening of library LibM2

A second library design for discovery of better xylose isomerase mutants focused on mutations in a stretch of six residues flanking the substrate binding site (Table 3). In this case, to avoid the risk of improved growth by chromosomal mutations, we modified the screening procedure. After the yeast transformation with the library DNA, the growth of library colonies on solid medium containing xylose as sole carbon source was monitored by visual inspection. Plasmid DNA was isolated from suspected positive (larger) clones, retransformed and rescreened.

Library design again included selection of target positions and diversity to be introduced at each position (Figure 8). The residues at the six target positions (Ser141, Thr142, Ala143, Asn144, Val145 and Gly147) at the C5 side of the substrate interact with the substrate either directly or indirectly. Therefore, it was expected that modifying these residues can improve substrate binding and the catalytic rate. Residue Thr142 is fully conserved throughout all known xylose isomerase sequences. In the structure it is connected to O5 of the substrate via a water molecule. To keep this interaction, we limited the diversity at this position to Thr and Ser. In a previous study, the PirXI T142S mutation was discovered to improve the growth of yeast on xylose [14]. We preserved Phe146 as it is fully conserved and it plays an important role in keeping the active site hydrophobic. Together with Trp189 and other hydrophobic aromatic residues (Trp50 and Phe61) this promotes the hydride shift by shielding the hydride from solvent [40–42]. The resulting library consists of 3584 variants (Table 3).

Figure 8. Target residues for library LibM2.

Residues mutated in library M2 (magenta) surround the substrate binding site. Residue Thr142 and xylose (yellow) may interact (dashed lines) via a water molecule (red sphere). Magnesium ions and the coordinating residues are depicted as green spheres and gray sticks, respectively. The metal binding residues and conserved active site hydrophobic residues Phe146 and Trp189 are shown as gray sticks. PDB 5NH7.

5

The library was constructed using the same strategy as for library LibM1. The initial *E. coli* transformation yielded ca. 8000 colonies and the diversity was confirmed by sequencing a plasmid mixture obtained from pooled transformants. The subsequent transformation to *S. cerevisiae* DS75543 resulted in over 8000 colonies as well. For identification of clones showing improved xylose utilization, transformed cells were washed from glucose plates and spread on xylose plates (see Methods for details). Many cells did not grow at all or started to grow very slow, causing visible differences between individual colonies, also for wild type. The latter indicated that other factors than PirXI activity influenced colony growth, for example the physiological status of transformed cells at the moment of plating. After three rounds of screening and retransformation, 46 colonies which showed superior growth were selected. To identify the best variant, growth in xylose containing liquid medium was measured using 96-well plates and compared to wild type. Most of the 46 variants reproducibly showed improved growth. The xylose isomerase genes from the 24 best growing variants were sequenced, revealing 10 different variants, one of which was wild type. The sequences that appeared most frequent (4-8 times) were reconstructed in a clean background and the effects on growth on xylose were evaluated after transformation to fresh DS75543 cells. Among these mutants, variant S1 (S141N-T142S-A143S-G147A) consistently showed the biggest improvement on growth on xylose when several independent cultures were tested. As shown with variant V270A-A273G, the most significant effect of mutant S1 also appears to be on the earlier start of the growth while showing a slightly increased exponential growth rate (Figure 9).

Table 3. Library LibM2 variants and codons.

Residue	Variants	Codons	freq. WT
S141	S I V A T G N	RBC+AAC	0.14
T142	T S	ASC	0.5
A143	A T G S	RSC	0.25
N144	N L Q T	CWG+AMC	0.25
V145	V L I C	VTT+TGC	0.25
G147	G A T S	RSC	0.25

Kinetic properties of PirXI S1

The reconstructed PirXI mutant S1 was purified from *E. coli* and its activity was measured after reconstitution with different metals. As with variant V270A-A273G, the Michaelis-Menten kinetic parameters measured in the presence of Mg^{2+} , Mn^{2+} or Ca^{2+} revealed reduced k_{cat} values as compared to wild-type (Table 2). The K_{M} for xylose was also several fold higher in the presence of Mg^{2+} or Mn^{2+} compared to the wild type. Furthermore, the K_{act} values indicate that a shift in metal affinity does not promote better in vivo performance of the mutant enzyme as the affinity towards the most activating metal Mn^{2+} decreased, while the affinity towards Ca^{2+} , which poorly activates the enzyme, increased.

Performance of other xylose isomerase mutants

The results described above indicate that both libraries yielded PirXI mutants that caused accelerated growth on xylose. Their properties also exhibited a disconnection between in vivo performance and in vitro catalytic properties. We further explored the ambiguous relation between in vivo and in vitro enzyme properties by studying PirXI mutants discovered independently in previous studies, using different *S. cerevisiae* host strains [9,14]. Using directed evolution with random mutant libraries, Lee et al. discovered PirXI variant E15D-T142S which increased the growth rate on xylose from 0.01 h^{-1} up to 0.06 h^{-1} [14]. Activities of these enzymes have only been measured with cell lysates, making a comparison difficult, but K_{M} values appear high. Later, Katahira et al. discovered that mutations at position N338, especially substitution N338C, improved growth of yeast on xylose. This mutation was effective not only in PirXI but also in related XIs [9]. It was reported that yeast cells carrying the N338C variant of PirXI consumed xylose 3 – 4 times faster than cells carrying wild-type PirXI. The catalytic properties of the mutant enzymes from these studies have not been described. Very recently, when our study work was nearing completion, Seike et al. described mutations in XI from *Lachnoclostridium phytofermentans* (LpXI) that enhanced D-xylose metabolism [11]. The most effective mutations were T63I and V162A. The corresponding positions in PirXI are distant from the active site and the mutations were not examined here.

Figure 9. PirXI mutant S1 (S141N-T142S-A143S-G147A) improves growth on xylose. Growth of *S. cerevisiae* DS75543 cells harboring wild-type PirXI (green) or mutant PirXI S1 (magenta) on xylose (20 g·l⁻¹). Each line represents a biological replicate. The calculated average growth rates were 0.13 ± 0.01 h⁻¹ and 0.18 ± 0.01 h⁻¹ for the wild type and the mutant, respectively.

We constructed the two earlier PirXI mutants [9,14], E15D-T142S and N338C, and confirmed that the variants are beneficial for PirXI-mediated growth of strain DS75543 on D-xylose as well (Figure 10). Subsequently, we expressed the mutants in *E. coli*, purified the enzymes and measured kinetic parameters. For this, activities were determined with Mg²⁺, Mn²⁺ or Ca²⁺ added to the apoenzyme and the activation constants were also determined (Table 2). As with the new mutants described in the current paper, Michaelis-Menten parameters and metal affinities did not reflect the positive effect of the mutations on growth, with the exception of an increased k_{cat} of the N338C mutant in the presence of Mg²⁺ and Mn²⁺. However, this enzyme also has a higher K_M . This result shows that the disconnection between in vivo performance and in vitro properties of PirXI is not dependent on the screening strain or selection conditions.

Figure 10. Growth of *S. cerevisiae* expressing PirXI variants E15D-T142S or N338C. Growth of cells expressing the wild-type PirXI (green lines), a mutant PirXI E15D-T142S (red lines) or N338C (blue lines) on xylose (20 g·l⁻¹) medium were measured using a microtiter plate reader. Individual lines represent biological replicates.

DISCUSSION

Improving D-xylose metabolism is an ongoing challenge for optimizing second-generation bioethanol production by engineered strains of *S. cerevisiae*. Important improvements in yeast performance have been achieved by evolutionary and metabolic engineering, which can influence different steps in central metabolism [16,43], and by enhancing the xylose uptake system [44,45]. Introduction of efficient enzymes for initial isomerization of D-xylose to D-xylulose is an equally important target. The incorporation of *Piromyces* xylose isomerase in xylulose fermenting yeast strains allowed xylose utilization [12,13], but very high expression levels are needed for optimal performance, as indicated by gene amplification up to over 30 copies during adaptation [15,21], leading to production of xylose isomerase at up to 25% of the cellular protein (Figure 3). This suggested that the enzyme has poor in vivo kinetics and stimulated research aimed at discovering better xylose isomerase variants [9,14].

In view of the catalytic properties of PirXI, the need for such a high expression level is unexpected. At the observed xylose isomerase content of 25%, one would expect the isomerization reaction not to be growth-limiting. The relation between growth rate μ and xylose consumption V can be expressed as:

$$\mu = V \cdot Y, \text{ with}$$

$$V = [E] \cdot \frac{V_{\max} \cdot [S]}{K_M + [S]}$$

where $[E]$ is the enzyme content (ca. 0.1 g PirXI per g biomass estimated based on 25% of the total protein being XI, and a total protein content of 0.4 g per g biomass [46,47]), $V_{\max}$ (8.3 U/mg, from $k_{\text{cat}} = 6.9 \text{ s}^{-1}$ with Mn^{2+}) and K_M (6.2 mM) have their usual meaning. Y represents the yield on xylose (0.25 g cell dry weight/g xylose converted) estimated from a previous study performed with *S. cerevisiae* grown in a similar condition [48]. This predicts a PirXI activity at $[S] = 1 - 10 \text{ mM}$ (reasonable intracellular substrate concentration) [49,50] of 1 - 4.6 g xylose converted per g biomass per h, allowing a growth rate of $\mu = 0.25 - 1.15 \text{ h}^{-1}$. The experimentally observed growth was around 0.13 h^{-1} , suggesting that xylose isomerase should not be rate limiting if it was fully active. PirXI is also well expressed and folded in vivo, which may be troublesome with other XIs, as illustrated by the extreme case of the xylose isomerase from *Actinoplanes missouriensis*, which in vitro looks catalytically superior to PirXI but fails to function in *S. cerevisiae* [6,51]. In view of earlier work on the effect of metals on PirXI activity and the observation that mutations influencing manganese homeostasis can improve xylose metabolism, we initially suspected that the modest activity of the enzyme might be due to suboptimal metal loading and

that xylose utilization could be improved by engineering PirXI variants carrying mutations surrounding the metal binding sites.

The discovery and engineering of XI variants that improve xylose metabolism has been pursued by different groups [9,11,52]. However, the connection between in vitro kinetics of xylose isomerase and yeast growth on xylose remains rather unclear. Recently, Seike et al. [11] compared xylose isomerases from different organisms and found that an enzyme from *L. phytofermentans* (LpXI) and two mutants thereof gave the highest xylose consumption rate even though its activity measured in cell lysates were not better than those of cells expressing PirXI [7]. Mutants of LpXI that gave better xylose consumption were found. A higher activity ($V_{\max}$) and lower K_M were found with a double mutant of LpXI, but the experiments were done with whole cell lysates reconstituted with Mg^{2+} so a comparison of intrinsic kinetic parameters is difficult. Similarly, an XI from *Burkholderia cenocepacia* which gave higher in vitro activity of cell lysates compared to PirXI and LpXI [52] did not seem beneficial for xylose fermentation [11]. In the same context, a recently discovered XI from the gut bacterium *R. speratus* was found to be better for xylose fermentation than PirXI, but this could not be explained by differences in in vitro catalytic performance [9].

We initially expected that selection of faster growing yeast strains from libraries expressing mutants of PirXI would give variants with improved kinetic parameters (higher k_{cat} , lower K_M) with Mn^{2+} . Also changes in metal binding affinity or shifts in metal preference could be expected. Two focused libraries with good diversity at the target positions were constructed and improved mutants were indeed obtained by batch culture selection and plate screening for higher growth rates. From the first library (LibM1), the same V270A-A273G PirXI variant was repeatedly retrieved, both from aerobic and anaerobic duplicate cultures. This indicates that the features of PirXI which limit growth of yeast on xylose are not dependent on oxygen availability, and that aerobic screening is possible to discover mutations that contribute to anaerobic xylose metabolism as well. Examining a reconstructed V270A-A273G PirXI mutant demonstrated that the improved performance was due to the mutations in the PirXI structural gene. A second focused library (LibM2) was screened on solid xylose medium and led to the discovery of the 4-fold mutant S141N-T142S-A143S-G174A. Again, the contribution of chromosomal mutations was excluded. After discovery of these new mutants, we investigated the relation between enzyme kinetics and improved growth, an issue that is also still open for earlier mutants of PirXI which were obtained by enrichment after error-prone PCR [9,14]. With the reconstructed mutant genes expressed in a clean expression vector and host, we found that all four PirXI variants improved growth on xylose of *S. cerevisiae* DS75543, a strain different from the one used earlier by others [9,14].

Using purified proteins of the two new mutants (V270A-A273G and S141N-T142S-A143S-G174A) as well as of the earlier variants (E15D-T142S and N338C) [9,14], we measured activities and kinetic parameters under a variety of conditions. Activities at physiological pH (~7) and temperature (30 °C) initially did not reveal any obvious features of the mutant enzymes that account for faster growth on xylose. Measurements with enzyme variants that were reconstituted with a single type of metal indicated that the mutants under these conditions had no advantage over the wild type, but likely this incompletely reflects *in vivo* conditions where the enzyme binds mixtures of metals and needs to function in the complex environment of the cytoplasm. Metal binding in the cytosol of *S. cerevisiae* is dependent on metal availability as well as binding affinities, which differ among xylose isomerases and between the two binding sites [53,54]. The most pronounced yet small effect was the increased k_{cat} of the N338C mutant with Mn^{2+} and Mg^{2+} as activating metals, as well as a slightly improved activity of the V270A-A273G variant in the presence of a low concentration of Mn^{2+} and a high concentration of Ca^{2+} . Thus, at certain concentrations of these two metals, the mutant enzyme can be more active than the wild type. With all PirXI variants examined, Mn^{2+} gave much better activity than Ca^{2+} , emphasizing the importance of Mn^{2+} homeostasis for enzyme activity, which was also shown in our previous study where cellular manganese content was enhanced by mutations in a metal transporter [21].

We also found that the main metal in PirXI isolated from yeast is Ca^{2+} and only a small amount of Mn^{2+} is present [21]. This metal composition of the enzyme is far from optimal for catalysis as Ca^{2+} -bound PirXI shows very high K_{M} for xylose, over 200-fold higher than with the catalytically preferred Mn^{2+} (Table 2). A shift in metal preference thus can explain improved *in vivo* performance, although establishing a quantitative correlation is impossible since *in vivo* metal binding of the enzyme is difficult to predict and measure, especially because the enzyme has two metal binding sites with different affinities, with one site essential for catalysis yet probably only occupied when substrate is bound [22]. Indeed, the fact that the metal composition of PirXI does not strictly follow the apparent metal affinities (K_{act}) and intracellular metal composition of *S. cerevisiae* indicates a complex *in vivo* metal binding mechanism of the enzyme [21]. Changes in metal binding were also suggested by a different response of the wild-type and mutant PirXIs to metal titration followed by thermal shift assays. While the thermostability of wild type increases with increasing metal concentrations, the mutant V270A–A273G required high concentrations of the metals (>200 μM) for an effect on thermostability, illustrating that the typical increase in thermostability of a metalloenzyme upon metal binding is abolished by the mutations. It is possible that binding of metals is affected by the availability and binding of substrate [22,40].

Even though the mutations changed the metal specificity of the enzyme, increasing the affinity for both Mn^{2+} and Ca^{2+} slightly, crystal structures of mutated PirXI isolated from yeast showed that the enzyme still has Ca^{2+} ions occupying both metal binding sites. It is interesting that the structure of PirXI isolated from *E. coli* showed a metal composition quite different from that of the enzyme produced in yeast, with in case of the yeast enzyme only the M_1 binding site being occupied by Fe^{2+} (25 %), Ca^{2+} (40%) and Mg^{2+} (35%) as estimated from structural refinement and with the M_2 site left empty. These data confirm that in yeast most of the enzyme is loaded with the catalytically impractical Ca^{2+} ions, with a high impact on the in vivo performance of the enzyme. This indicates that conclusions about increased V_{max} values of evolved XIs should be considered with care, especially in case of measurements performed with cell-free extracts instead of purified enzyme and in the presence of an excess of added Mg^{2+} , which is routinely used in XI assays [11,14].

Other factors that might possibly influence in vivo enzyme performance include enzyme stability (lifetime), compartmentalization, and interaction with other cellular macromolecules. PirXI originates from a heterologous host causing the enzyme to be not evolutionarily optimized for functioning in *S. cerevisiae*. The distribution of metals over cellular compartments and the sequestration of metals by other macromolecules are likely to have a major impact on the metal availability for PirXI in vivo [55]. Such differences in enzyme properties beyond kinetic parameters may influence the performance of xylose isomerase variants. Differences between host strains, as well as variations in cultivation conditions and assay conditions make it difficult to compare the performance of xylose isomerases in yeast xylose metabolism.

CONCLUSION

As part of developing efficient second-generation bioethanol production, there have been efforts to engineer xylose isomerase variants that improve growth of *S. cerevisiae* on xylose. We found that design of focused libraries of *Piromyces* xylose isomerase, based on inspection of the crystal structure, followed by growth-based screening, gives mutants that improve the growth of yeast on xylose. The mutations differ from those found earlier in random mutant libraries constructed by error-prone PCR. The new mutants described here and the two mutants discovered earlier, did not show improved xylose isomerization kinetics when tested in vitro in the presence of an excess of single metals. Yet, metal occupation of the enzyme is of key importance as indicated by the low in vivo activities and the high calcium content found by metal analysis and X-ray

crystallography of xylose isomerase isolated from yeast. Small differences in relative metal affinities and activities can explain the improved growth caused by mutations in the second shell of metal coordination. Rational redesign of xylose isomerase for better in vivo performance would require a theoretical framework that describes xylose isomerase structure-activity relations as a function of metal incorporation and activation.

METHODS

Strain and plasmid construct

The *E. coli* strain used in this work is NEB 10 β (New England Biolab). *S. cerevisiae* strain DS75543 is derived from RWB217 (Kuyper et al., 2005) and was constructed for xylose fermentation by genetic engineering and further improved for growth rate on xylose by laboratory evolution. Plasmids bearing the *XKS1* and *PirXI* expression cassette were cured from the strain, and an *XKS1* overexpression cassette was re-introduced by integration in the yeast genome. The relevant genotype of DS75543 is the following: MATa, *ura3-52*, *leu2-112*, *gre3::loxP*, *loxP-Ptpi::TAL1*, *loxP-Ptpi::RKI1*, *loxP-Ptpi::TKL1*, *loxP-Ptpi::RPE1*, *TY1::Padh1XKS1+LEU2*.

A yeast codon-optimized xylose isomerase gene-expression cassette, P_{TPI1} -*XylA*_T_{CYC1'} was obtained from Prof. J.T. Pronk, TU Delft, and cloned into the 2 μ plasmid pRS426-URA using SacI and SalI restriction sites. For *E. coli* expression, a pBAD/myc-His-derived plasmid containing *E. coli* codon-optimized *XylA* was used as described in our previous study [22].

Library construction

The library construction strategy is schematically shown in Figure 11. A mutant *PirXI* library was created by cloning *XylA* fragments containing mutations into the pRS426-URA vector. The fragments and the vector were designed to contain > 20 bp overlaps for use of the Gibson assembly method for cloning. Appropriate partially undefined codons for generating the library were designed using a spreadsheet-based site-restricted library design tool called CoFinder [33]. Using primers containing the partially undefined codons and pRS426_pTPI_*XylA* as the template, PCR was performed to generate the library fragments. For creating the backbone, linearized pRS426_pTPI_*XylA* was used as the PCR template. The plasmid was linearized by using a restriction enzyme that cuts the DNA once outside of the backbone region. An AatII site was created for library LibM1 and the single existing BglII site was used for library LibM2. For both the fragment and the backbone generation, Phusion high-fidelity polymerase (ThermoFisher) was

used and the instructions of the manufacturer were followed for the PCR reactions. After PCR, template fragments were degraded by incubating the reaction mixtures with 1 μ l of DpnI at 37 °C for 3 h. For Gibson assembly of library fragments and backbone, a total of ~100 ng DNA was added at a 1:3 ratio of purified backbone to gene fragments. The library DNA fragments were mixed proportionally to the number of variants that is theoretically carried by each fragment. The assembly reactions were performed following the protocol established by Gibson et al. [56]. Subsequently, three aliquots of 100 μ l *E. coli* were transformed with 10 μ l reaction mixtures and transformants were selected on LB agar plates containing 50 μ g mL⁻¹ ampicillin. Plasmids from the entire *E. coli* transformant mixture were isolated, sequenced to confirm the diversity of the library and transformed to *S. cerevisiae*.

Figure 11. Library construction scheme.

In silico prediction of enzyme stability

The relative folding free energy differences $\Delta\Delta G^{\text{Fold}}$ between PirXI wild type and variants were predicted using FoldX calculations [32] based on the x-ray structure of the wild-type enzyme (5NH7) and in silico generated mutants.

Site-directed mutagenesis

Selected PirXI variants for expression in *E. coli* and *S. cerevisiae* were reconstructed by QuikChange site-directed mutagenesis. For PCR, PfuUltra II Hotstart master mix (Agilent) was used following the manufacturer's instructions. Subsequently, 1 μ l DpnI was added to the reaction mixture, which was incubated at 37 °C for 3 h. Next, 10 μ l of the reaction mixture were used to transform 100 μ l NEB 10 β cells.

Transformation

For *E. coli* transformation RhCl_2 -competent NEB 10 β cells were used and a standard heat-shock protocol was applied. *S. cerevisiae* transformation was performed using the LiAc/SS carrier-DNA/PEG method. The protocol established by Gietz et al. was followed [57], adjusting the duration of heat-shock to that works best for the DS75543 strain which was approximately 1 h.

Yeast cultivation

For all yeast growth experiments a defined medium prepared according to Verdyun et al. [58] was used, either supplemented with $20 \text{ g}\cdot\text{l}^{-1}$ glucose (glucose medium) or $20 \text{ g}\cdot\text{l}^{-1}$ xylose (xylose medium) unless stated otherwise. For solid medium 2% agar was added prior to autoclaving. Cells were pre-grown on glucose medium and leftover glucose was washed away with ddH_2O . The washed cells were resuspended in xylose medium and diluted appropriately. On-line growth measurements of *S. cerevisiae* were performed using a microplate reader (Synergy H1, BioTek). For this, $200 \mu\text{l}$ xylose medium was used in 96-well cell culture plates (Eppendorf) and samples from pre-cultures were added to an OD_{600} of 0.02. Plates were covered with an optical-clear gas-permeable seal (Breath-easy, Diversified Biotech). Cultures were grown at 30°C with continuous shaking (linear, 731 cpm, 2 mm shaking amplitude) and the OD_{600} was measured with 30 min intervals. For off-line growth measurements cells were cultivated at 30°C in 100-ml shake flasks containing 25 ml xylose medium and the optical densities at 600 nm was measured in a spectrophotometer using plastic cuvettes.

Library screening by competitive growth

After transformation, yeast cells were plated on solid glucose medium. All transformants were collected by gently scraping the colonies from the plate and divided for duplicates of aerobic and anaerobic screening. Prior to the screening, cells were pre-grown on glucose until the mid-exponential phase. For the aerobic screening, precultures prepared were diluted in 50 ml xylose medium to an OD_{600} of ~ 0.1 and grown at 30°C with shaking at 135 rpm. For anaerobic growth, cells prepared from pre-cultures were inoculated in 100 ml xylose medium supplemented with $420 \text{ mg}\cdot\text{l}^{-1}$ Tween 80 and $10 \text{ mg}\cdot\text{l}^{-1}$ ergosterol. To keep the conditions oxygen-limited, the cultures were grown in 100-ml Schott glass bottles which were kept air-tight with a rubber stopper and a glass airlock. Autoclaved medium was flushed with argon for at least 20 min prior to inoculation. The cultures were grown at 30°C without shaking, but occasionally stirred briefly to keep homogenous cell suspensions. For aerobic and anaerobic screening, transfers to fresh xylose medium were carried out when the cultures reached an OD_{600} of 8–10 and 2, respectively. After the 10th transfer, cultures were

harvested to investigate the evolved library diversity. Cells were pre-treated with zymolyase (Amsbio) and plasmids were isolated using a miniprep kit (Qiagen). The isolated plasmids were transformed to *E. coli* to produce sufficient plasmid DNA for sequencing. The plasmids were isolated from *E. coli* transformants using a miniprep kit and sequenced by GATC (Konstanz, Germany).

Library screening by comparative growth

All library transformants were collected from glucose medium plates, washed and diluted with sterile ddH₂O and plated on solid xylose medium. After 2–3 days of incubation, around 100 colonies that grew faster were selected by comparing colony sizes with cells expressing wild-type PirXI. Plasmids were isolated from selected transformants as described above and retransformed into fresh non-evolved yeast cells. The selection procedure was repeated twice and after the last round of yeast transformation and selection approximately 200 random colonies were picked and replicated both on glucose and xylose plates. Several colonies harboring wild-type PirXI were included as controls. Next, 46 colonies that grew faster than the controls on the xylose medium were selected and the corresponding colonies were picked from the glucose plate. The growth of the selected transformants in liquid xylose medium was measured in a 96-well plate using a microtiter plate reader. The growth experiments with these 46 transformants and wild-type PirXI carrying clones were performed in duplicate. The 24 best growing colonies were selected, plasmids were isolated and sequenced as described above.

Enzyme expression in *E. coli* and purification

For in vitro enzyme analysis, PirXI variants were expressed in *E. coli* and purified. NEB 10 β cells harboring PirXI variants were grown in TB medium (12 g/l tryptone, 24 g/l yeast extract, 5 ml/l glycerol, 2.31 g/l KH₂PO₄, and 16.43 g/l K₂HPO₄·3H₂O) containing 50 μ g·ml⁻¹ ampicillin at 37 °C. For inducing expression, 0.2% (w/v) L-arabinose was added and the cells were cultivated for 16 h at 37 °C. The cells were harvested by centrifugation and purification of overexpressed XI was done as described in previously [22].

In vitro enzyme activity and metal affinity

For measuring the XI activity in the presence of different metal cofactors, purified enzymes were first incubated overnight with 10 mM EDTA. Subsequently, any metal-EDTA complex and excess EDTA were removed by buffer exchange to 20 mM MOPS (pH 7.0) using EconoPac 10-DG desalting columns (Bio-Rad). All enzyme activities were measured with sorbitol dehydrogenase (SDH, Roche Diagnostics GmbH) – coupled assay at 30 °C and pH 7.0 (20 mM MOPS). The

reactions were followed either using a spectrophotometer (1 ml mixtures) (Jasco) or with a microplate reader in (200 μ l reactions) (Synergy H1, BioTek). All reactions contained 0.15 mM NADH, 1 mM divalent metal, 1.5 unit/ml SDH and D-xylose. The mixtures were incubated at 30 °C for 5 min and the reactions were initiated by addition of 0.05 – 0.2 μ M apo XI. For measuring the activation constant for each metal, activities of XI on 100 mM (for Mg^{2+} and Mn^{2+}) or 400 mM (for Ca^{2+}) D-xylose in the presence of various concentrations of metals were determined using a microplate reader. Various concentrations of metals and PirXI were used according to the level of metal-dependent activity of the enzyme.

Thermostability measurement

Thermostability of PirXI variants were determined by measuring an increase in fluorescence of Sypro Orange (Life Technologies, Carlsbad, CA, USA) during thermal unfolding [59]. The change of fluorescence emission at 575 nm was measured with CFX RT-PCR system (Biorad) while increasing the temperature from 20 to 90 °C at the rate of 0.5 °C $\cdot$ min⁻¹. In order to evaluate the metal affinity of PirXI variants, various concentrations of divalent metal ions ($MgCl_2$, $MnCl_2$ or $CaCl_2$) ranging from 0 to 1.28 mM were included in 25 μ l reaction mixtures containing 1 mg/ml apo PirXI and 2x of Sypro Orange dye.

Cell extract activity

S. cerevisiae cells grown on xylose were harvested, washed with sterile ddH₂O and resuspended with lysis buffer containing 20 mM MOPS (pH 7.0) and 100 U/g cells (w/w) zymolyase (Amsbio). The cell suspension was incubated at 30 °C with mild shaking at 50 rpm for 20 min. Next, the zymolyase-treated cells were disrupted by vortexing for 30 s with an equal volume of glass beads followed by cooling on ice for 1 min. The process was repeated five times. The cell lysate was spun down by centrifugation at 17,000 x g and the supernatant was collected as the cell-free extract (CFE). The total protein concentration of the CFE was measured by Bradford assay using bovine serum albumin (BSA) as the standard. Activity of the CFE on 100 mM xylose was measured by the SDS-coupled assay as described above without addition of any metals.

Enzyme expression levels

Crude extracts of *S. cerevisiae* expressing either wild-type or V270A-A273G PirXI grown on xylose were prepared by lysing the cells as described above. Total protein concentrations were determined by the Bradford assay and 2 μ g of protein from each cell extract was used for SDS-PAGE analysis. Along with the crude extract samples, 0.2, 0.4, 0.6, 0.8, 1 and 2 μ g of purified PirXI were loaded on the same gel as references. An image of the gel was taken and analyzed using

the image processing program Image J (<https://imagej.net>). The intensity of the bands corresponding to PirXI were quantified and the expression levels were calculated.

Purification of PirXI expressed in *S. cerevisiae* for crystallography

A single colony of DS75543 cells containing PirXI was inoculated in 5 ml glucose medium and grown overnight. Next, cultures were diluted into a 50 ml glucose medium to an OD₆₀₀ of 0.1 and cultivated at 30 °C until mid-exponential phase. The cells were washed and diluted into a 2 l xylose medium to a starting OD₆₀₀ of 0.1. The cultures were grown at 30 °C and harvested when the OD₆₀₀ reached 3 – 4. In order to minimize metal contamination and preserve in vivo enzyme-bound metals, we applied a gentle cell lysis method and a minimal purification step required as described below. Buffers used are as follows: A, 10 mM MOPS, pH 7.5; B, 10 mM MOPS, pH 7.5 + 5 mM DTT; C, 10 mM MOPS, pH 7.5 + 0.5 M sucrose; D, 10 mM MOPS, pH 7.5 + 0.5 M sucrose + 100 U/g cells (w.w) of zymolyase + EDTA-free protease inhibitor cocktail tablets (Roche); and E, 10 mM MOPS, pH 7.5 + 0.5 M KCl. The harvested cells were washed with buffer A and the pellets were resuspended in buffer B and subsequently incubated for 10 min on ice. The cells were centrifuged and washed with buffer C. The pellets were resuspended in 25 ml of buffer D and incubated at 30 degrees while gently shaken at 50 rpm for 1 h. The disruption of the cell walls of the yeast was monitored under a light microscope. Next, 25 ml of buffer A was added to the cell lysate mixtures and the cells were spun down at 1500 g for 5 min. The supernatants were carefully collected and centrifuged at 16,500 rpm for 30 min. PirXIs were purified from the CFE using an anion exchange column (Resource Q) by applying an ionic strength gradient using buffer A and buffer E. Prior to sample loading the CFEs were diluted appropriately to reduce the ionic strength of the samples to a similar level of buffer A. Most of PirXI eluted with 50 – 100 mM KCl and the fractions of highest purity were collected for crystallization.

Crystallization and structure determination

Wild-type xylose isomerase and XI mutant V270A-A273G were crystallized by the hanging-drop vapor diffusion method with 14-17% PEG3350, 0.08 M ammonium sulfate and 0.1 M HEPES, pH 7.0 [22]. For soaking experiments, the stabilizing solution was supplemented with 2 M xylose. Datasets were collected at the in-house source at 110 K [22]. Details and processing statistics are given in Table 4. Processing was done with XDS [60]. The structure of xylose isomerase (PDB code 5NH5), with all waters and ligands removed, was used as a starting model. Refinement was done with Refmac5 [61]. Sugar ligands and ions were manually placed in sigmaA-weighted 2Fo – Fc, Fo – Fc and anomalous electron density

maps [62] with the program Coot [63]. The atomic coordinates and the structure factors of the structures have been deposited in the Protein Data Bank (code 6T8E for native and 6T8F for V270A-A273G PirXI)

Table 4. Data collection and refinement statistics.

	Wild type	V270A-A273G
Resolution range (Å) ^a	46.6 – 1.86 (1.89 - 1.86)	46.6 – 2.0 (2.03 – 2.00)
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	78.5, 79.3, 91.9	78.6, 79.4, 92.0
α , β , γ (°)	115.5, 90.0, 117.1	115.5, 90.0, 117.1
Number of unique reflections	135735 (5669)	111563 (5325)
Completeness (%)	93.6 (79.1)	95.3 (92.1)
Overall <i>I</i> / σ (<i>I</i>)	7.1 (1.9)	5.3 (2.0)
<i>R</i> _{merge} (%)	6.7 (31.8)	6.8 (24.4)
<i>R</i> / <i>R</i> _{free} (%)	13.9 / 17.6	15.4 / 18.5
R.m.s. deviations from ideal values		
Bond lengths (Å)	0.009	0.010
Bond angles (°)	1.55	1.55
Protein residues		
Ca ²⁺ ions	4 x 2	4 x 2
Water molecules	1907	1745
Xylose molecules	22	18
Sulfate ions	7	6
PDB accession ID	6T8E	6T8F

^a Values in parentheses are for the highest resolution shell.

ACKNOWLEDGEMENTS

We acknowledge Jack Pronk (TU Delft) for providing the xylose isomerase gene expression cassette P_{TPI1}-XylA-T_{CYC1} and for his scientific advice. We thank Marcelo Masman for performing the FoldX calculations of PirXI mutants.

REFERENCES

- Briggs KA, Lancashire WE, Hartley BS. Molecular cloning, DNA structure and expression of the *Escherichia coli* D-xylose isomerase. *EMBO J.* 1984;3:611–6.
- Jansen MLA, Bracher JM, Papapetridis I, Verhoeven MD, de Bruijn H, de Waal PP, et al. *Saccharomyces cerevisiae* strains for second-generation ethanol production: from academic exploration to industrial implementation. *FEMS Yeast Res.* 2017;17:1–20.
- Walfridsson M, Anderlund M, Bao X, Hahn-Hägerdal B. Expression of different levels of enzymes from the *Pichia stipitis* *XYL1* and *XYL2* genes in *Saccharomyces cerevisiae* and its effects on product formation during xylose utilisation. *Appl Microbiol Biotechnol.* 1997;48:218–24.
- Kötter P, Ciriacy M. Xylose fermentation by *Saccharomyces cerevisiae*. *Appl microbiol biotechnol.* 1993;38:776–83.
- Cunha JT, Soares PO, Romani A, Thevelein JM, Domingues L. Xylose fermentation efficiency of industrial *Saccharomyces cerevisiae* yeast with separate or combined xylose reductase/xylitol dehydrogenase and xylose isomerase pathways. *Biotechnol Biofuels.* 2019;12:1–14.
- Amore R, Wilhelm M, Hollenberg CP. The fermentation of xylose - an analysis of the expression of *Bacillus* and *Actinoplanes* xylose isomerase genes in yeast. *Appl Microbiol Biotechnol.* 1989;30:351–7.
- Brat D, Boles E, Wiedemann B. Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* 2009;75:2304–11.
- de Figueiredo Vilela L, de Mello VM, Reis VCB, da Silva Bon EP, Gonçalves Torres FA, Neves BC, et al. Functional expression of *Burkholderia cenocepacia* xylose isomerase in yeast increases ethanol production from a glucose-xylose blend. *Bioresour Technol.* 2012;128:792–6.
- Katahira S, Muramoto N, Moriya S, Nagura R, Tada N, Yasutani N, et al. Screening and evolution of a novel protist xylose isomerase from the termite *Reticulitermes speratus* for efficient xylose fermentation in *Saccharomyces cerevisiae*. *Biotechnol Biofuels.* 2017;10:1–18.
- Hector RE, Dien BS, Cotta MA, Mertens JA. Growth and fermentation of D-xylose by *Saccharomyces cerevisiae* expressing a novel D-xylose isomerase originating from the bacterium *Prevotella ruminicola* TC2-24. *Biotechnol Biofuels.* 2013;6:84.
- Seike T, Kobayashi Y, Sahara T, Ohgiya S, Kamagata Y. Molecular evolutionary engineering of xylose isomerase to improve its catalytic activity and performance of micro-aerobic glucose/xylose co-fermentation in *Saccharomyces cerevisiae*. *Biotechnol Biofuels.* 2019;12:1–16.
- Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MSM, de Laat WTAM, et al. High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Res.* 2003;4:69–78.
- Harhangi HR, Akhmanova AS, Emmens R, van der Drift C, de Laat WTAM, van Dijken JP, et al. Xylose metabolism in the anaerobic fungus *Pyromyces* sp. strain E2 follows the bacterial pathway. *Arch Microbiol.* 2003;180:134–41.
- Lee SM, Jellison T, Alper HS. Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* 2012;78:5708–16.
- Zhou H, Cheng J sheng, Wang BL, Fink GR, Stephanopoulos G. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. *Metab Eng.* 2012;14:611–22.
- Kuyper M, Hartog MMP, Toirkens MJ, Almering MJH, Winkler AA, van Dijken JP, et al. Metabolic engineering of a xylose isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res.* 2005;5:399–409.
- Bracher JM, Martinez-Rodriguez OA, Dekker WJC, Verhoeven MD, van Maris AJA, Pronk JT. Reassessment of requirements for anaerobic xylose fermentation by engineered, non-evolved *Saccharomyces cerevisiae* strains. *FEMS Yeast Res.* 2019;19.
- Kwak S, Jin YS. Production of fuels and chemicals from xylose by engineered *Saccharomyces cerevisiae*: a review and perspective. *Microb Cell Fact.* 2017;16:1–15.
- Hoang PTN, Ko JK, Gong G, Um Y, Lee SM. Genomic and phenotypic characterization of a refactored xylose-utilizing *Saccharomyces cerevisiae* strain for lignocellulosic biofuel production. *Biotechnol Biofuels.* 2018;11:1–13.
- Young E, Lee S, Alper H. Optimizing pentose utilization in yeast: the need for novel tools and approaches. *Biotechnol Biofuels.* 2010;3:24.
- Verhoeven MD, Lee M, Kamoen L, Van Den Broek M, Janssen DB, Daran JMG, et al. Mutations in *PMR1* stimulate xylose isomerase activity and anaerobic growth on xylose of engineered *Saccharomyces cerevisiae* by influencing manganese homeostasis. *Sci Rep.* 2017;7:1–11.

22. Lee M, Rozeboom HJ, de Waal PP, de Jong RM, Dudek HM, Janssen DB. Metal dependence of the xylose isomerase from *Pyromyces* sp. E2 explored by activity profiling and protein crystallography. *Biochemistry*. 2017;56:5991–6005.
23. Cherry JR, Fidantsef AL. Directed evolution of industrial enzymes: an update. *Curr Opin Biotechnol*. 2003;14:438–43.
24. Packer MS, Liu DR. Methods for the directed evolution of proteins. *Nat Rev Genet*. 2015;16:379–94.
25. Kuchner O, Arnold FH. Directed evolution of enzyme catalysts. *Trends Biotechnol*. 1997;15:523–30.
26. de Kreijl A, van Burg B, Den, Venema G, Vriend G, Eijssink VGH, Nielsen JE. The effects of modifying the surface charge on the catalytic activity of a thermolysin-like protease. *J Biol Chem*. 2002;277:15432–8.
27. Tawfik DS, Goldsmith M. Directed enzyme evolution: beyond the low-hanging fruit. *Curr Opin Struct Biol*. 2012;22:406–12.
28. Lutz S. Beyond directed evolution - semi-rational protein engineering and design. *Curr Opin Biotechnol*. 2011;21:734–43.
29. van Leeuwen JGE, Wijma HJ, Floor RJ, van der Laan JM, Janssen DB. Directed evolution strategies for enantiocomplementary haloalkane dehalogenases: from chemical waste to enantiopure building blocks. *ChemBioChem*. 2012;13:137–48.
30. Lambeir AM, Lauwereys M, Stanssens P, Mrabet NT, Snauwaert J, Van Tilbeurgh H, et al. Protein engineering of xylose (glucose) isomerase from *Actinoplanes missouriensis*. 2. Site-directed mutagenesis of the xylose binding site. *Biochemistry*. 1992;31:5459–66.
31. Kovalevsky AY, Hanson L, Fisher SZ, Mustyakimov M, Mason SA, Trevor Forsyth V, et al. Metal ion roles and the movement of hydrogen during reaction catalyzed by D-xylose isomerase: A joint X-ray and neutron diffraction study. *Structure*. 2010;18:688–99.
32. Guerois R, Nielsen JE, Serrano L. Predicting changes in the stability of proteins and protein complexes: A study of more than 1000 mutations. *J Mol Biol*. 2002; 320:369–87.
33. van Leeuwen JGE. Library design and screening strategies for efficient enzyme evolution [Doctoral dissertation]. University of Groningen, Groningen; 2015.
34. Fitcher AB, Cox BS. Copy number and the stability of 2-micron circle-based artificial plasmids of *Saccharomyces cerevisiae*. *J Bacteriol*. 1984;157:283–90.
35. Kim SR, Skerker JM, Kang W, Lesmana A, Wei N, Arkin AP, et al. Rational and evolutionary engineering approaches uncover a small set of genetic changes efficient for rapid xylose fermentation in *Saccharomyces cerevisiae*. *PLoS One*. 2013;8:e57048.
36. Papapetridis I, Verhoeven MD, Wiersma SJ, Goudriaan M, van Maris AJA, Pronk JT. Laboratory evolution for forced glucose-xylose co-consumption enables identification of mutations that improve mixed-sugar fermentation by xylose-fermenting *Saccharomyces cerevisiae*. *FEMS Yeast Res*. 2018;18:1–17.
37. Coolbear T, Whittaker JM, Daniel RM. The effect of metal ions on the activity and thermostability of the extracellular proteinase from a thermophilic *Bacillus*, strain EA.1. *Biochem J*. 1992;287:367–74.
38. Dean KM, Qin Y, Palmer AE. Visualizing metal ions in cells: An overview of analytical techniques, approaches, and probes. *Biochim Biophys Acta*. 2012;1823:1406–15.
39. Foster AW, Osman D, Robinson NJ. Metal preferences and metallation. *J Biol Chem*. 2014;289:28095–103.
40. Rangarajan M, Hartley BS. Mechanism of D-fructose isomerization by *Arthrobacter* D-xylose isomerase. *Biochem J*. 1992;283:223–33.
41. Collyer CA, Blow DM. Observations of reaction intermediates and the mechanism of aldose-ketose interconversion by D-xylose isomerase. *Proc Natl Acad Sci*. 1990;87:1362–6.
42. Collyer CA, Henrick K, Blow DM. Mechanism for aldose-ketose interconversion by D-xylose isomerase involving ring opening followed by a 1,2-hydride shift. *J Mol Biol*. 1990;212:211–35.
43. Kuypers M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *FEMS Yeast Res*. 2005;5:925–34.
44. Nijland JG, Shin HY, Boender LGM, de Waal PP, Klaassen P, Driessen AJM. Improved xylose metabolism by a CYC8 mutant of *Saccharomyces cerevisiae*. *Appl Env Microbiol*. 2017;83:1–12.
45. Nijland JG, Shin HY, de Jong RM, de Waal PP, Klaassen P, Driessen AJM. Engineering of an endogenous hexose transporter into a specific D-xylose transporter facilitates glucose-xylose co-consumption in *Saccharomyces cerevisiae*. *Biotechnol Biofuels*. 2014;7:1–11.
46. Milo R, Phillips R. Cell biology by the numbers. 1st ed. Garl. Sci. 2015.
47. Huang M, Bao J, Hallström BM, Petranovic D, Nielsen J. Efficient protein production by yeast requires global tuning of metabolism. *Nat Commun*. 2017;8.

48. Osiro KO, Borgström C, Brink DP, Fjölnisdóttir BL, Grauslund MFG. Exploring the xylose paradox in *Saccharomyces cerevisiae* through in vivo sugar signalomics of targeted deletants. *Microb Cell Fact*. 2019;18:88.
49. Wasylenko TM, Stephanopoulos G. Metabolomic and ^{13}C -metabolic flux analysis of a xylose-consuming *Saccharomyces cerevisiae* strain expressing xylose isomerase. *Biotechnol Bioeng*. 2015;112:470–83.
50. Hasunuma T, Sanda T, Yamada R, Yoshimura K, Ishii J, Kondo A. Metabolic pathway engineering based on metabolomics confers acetic and formic acid tolerance to a recombinant xylose-fermenting strain of *Saccharomyces cerevisiae*. *Microb Cell Fact*. 2011;10.
51. van Bastelaere PBM, Kersters-hilderson HLM, Lambeirt A. Wild-type and mutant D-xylose isomerase from *Actinoplanes missouriensis*: metal-ion dissociation constants, kinetic parameters of deuterated and non-deuterated substrates and solvent-isotope effects. *Biochem J*. 1995;307:135–42.
52. Vieira IPV., Cordeiro GT, Gomes DEB, Melani RD, Vilela LF, Domont GB, et al. Understanding xylose isomerase from *Burkholderia cenocepacia*: insights into structure and functionality for ethanol production. *AMB Express*. 2019;9.
53. Callens M, Tomme P, Kersters-Hilderson H, Cornelis R, Vangrýsperre W, De Bruyne CK. Metal ion binding to D-xylose isomerase from *Streptomyces violaceoruber*. *Biochem J*. 1988;250:285–90.
54. van Bastelaere PBM, Vangrýsperre W, Kersters-Hilderson H. Kinetic studies of Mg^{2+} , Co^{2+} - and Mn^{2+} -activated D-xylose isomerases. *Biochem J*. 1991;278:285–92.
55. Dudev T, Lim C. Competition among metal ions for protein binding sites: Determinants of metal ion selectivity in proteins. *Chem Rev*. 2014;114:538–56.
56. Gibson DG, Young L, Chuang RY, Venter JG, Hutchison III CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*. 2009;6:343–5.
57. Gietz RD, Schiestl RH. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method R. *Nat Protoc*. 2007;2:31–4.
58. Verduyn C, Postma E, Scheffers WA, van Dijken JP. Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast*. 1992;8:501–17.
59. Ericsson UB, Hallberg BM, DeTitta GT, Dekker N, Nordlund P. Thermofluor-based high-throughput stability optimization of proteins for structural studies. *Anal Biochem*. 2006;357:289–98.
60. Kabsch W. XDS. *Acta Cryst*. 2010;66:125–32.
61. Murshudov GN, Skubák P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, et al. REFMAC5 for the refinement of macromolecular crystal structures. *Acta Cryst*. 2011;67:355–67.
62. Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, et al. Overview of the CCP4 suite and current developments. *Acta Cryst*. 2011;67:235–42.
63. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta Cryst*. 2010;66:486–501.

Summary and concluding remarks

Since many years, xylose isomerase (or glucose isomerase) is an important enzyme in the production of high-fructose syrups from starch. More recent interest is mainly related to its application in the conversion of D-xylose to D-xylulose. Whereas xylose is not fermented to ethanol by the common yeast *Saccharomyces cerevisiae*, its isomerization product xylulose can be metabolized, making this an important reaction for efficient production of second-generation bioethanol from xylose-rich hemicellulose biomass. The use of xylose isomerase for the classical glucose to fructose conversion is well established and highly optimized, e.g. by exploring various homologous enzymes, by improving enzyme stability via protein engineering, and through bioprocess design, including enzyme immobilization. As a result, the xylose isomerase-catalyzed production of high-fructose syrups is widely applied in the food and beverages industries with an annual production of over 15 million tons globally. On the other hand, efforts to optimize the enzyme for application in second-generation bioethanol production have started only recently.

Engineering of xylose isomerase for better ethanolic fermentation of xylose should be aimed at improving in vivo enzyme performance in engineered strains of *S. cerevisiae*. Lignocellulosic biomass is a much more attractive raw material for ethanol production than starch or sugars obtained from crops that need to be specially cultivated for this purpose. However, efficient use of xylose present in lignocellulose hydrolysates requires metabolically engineered yeast strains capable of rapid xylose isomerization. Since the kinetic properties of the xylose isomerase will be reflected in the efficiency and rate of xylose fermentation, discovery or engineering of xylose isomerases with better activities is highly important for developing improved yeast strains. In this PhD project, we have explored xylose isomerase from the fungus *Piromyces* (PirXI) and exploited different engineering approaches to improve its activity and in vivo performance. The high expression level of xylose isomerase in laboratory-evolved yeast strains and the modest activity of the enzyme recovered from yeast cells indicate poor in vivo performance of the enzyme.

To develop an effective strategy for structure-based enzyme engineering, characterization of the enzyme was necessary. No reports on the biochemical properties of isolated PirXI were available at the start of this work. Accordingly, we isolated the PirXI protein via expression in *E. coli* and performed a thorough analysis of the catalytic properties of the enzyme and solved several structures by X-ray crystallography. We focused on the metal dependence of the catalytic properties and how it might affect the in vivo activity of the enzyme. The results from isomerase activity assays, metal analysis of purified enzyme, and inspection of X-ray structures of native and metal-reconstituted variants all suggested that PirXI is promiscuous regarding metal acceptance. Among the metals tested,

Mn²⁺ was the best both in terms of activity and affinity. However, despite this high affinity, the low levels of Mn²⁺ present in yeast cells in combination with the high-level expression of PirXI caused in vivo enzyme activity to be far lower than expected. The metal analysis of the PirXI protein purified from yeast cells grown on xylose showed that most enzyme was metal-free or contained Ca²⁺ as metal, which was a poor activator. The more rapid growth on xylose of mutant yeast cells with an elevated intracellular Mn²⁺ content (Δ PMR1 strains) showed the importance of PirXI metal content for its in vivo catalytic performance and identified metal loading as a limiting factor for rapid xylose metabolism.

The structural and biochemical information gained in Chapter 2 and the finding that the intracellular metal composition of PirXI is important for xylose metabolism described in Chapter 4 allowed us to design enzyme engineering studies comprising both rational and semi-random approaches. Structural and phylogenetic information was used in a strategy aimed at optimizing the pH-activity profile and metal specificity in order to better meet the intracellular conditions of *S. cerevisiae*. In Chapter 3 we identified several mutations that gave the desired changes in catalytic properties – increased activity at lower pH or higher activity with Mg²⁺, a metal that is abundant in vivo. Through sequence- and structure comparison with xylose isomerases that show a higher activity with Mg²⁺ as the activating metal, we discovered mutations that increased activity with Mg²⁺, which would have been difficult to predict as they occur at positions that are quite distant from the active site. Altering electrostatic interactions by mutations replacing charged residues increased activity at lower pH, which should be advantageous for activity in the slightly acidic intracellular environment of yeast cells. Again, some of the mutations were far away from the active site. Interestingly, we discovered that the higher in vitro enzyme activity did not necessarily improve in vivo enzyme performance, which should have been reflected by more rapid growth on xylose.

For discovery of further enzyme variants with improved xylose isomerase activity, we exploited growth based-screening methods in combination with semi-random mutagenesis. In Chapter 5, using the biochemical and structural data established earlier, we designed focused libraries of PirXI with mutations around the metal-binding residues and by growth-based selection we discovered variants that gave improved growth of *S. cerevisiae* on xylose. These mutants, however, did not show any superior in vitro property that could explain the improved growth of the yeast cells.

In both engineering approaches we explored throughout the project, site-directed mutagenesis with in vitro screening (Chapter 3) and structure-based semi-random mutagenesis with in vivo screening (Chapter 5), we encountered for most mutants a discrepancy between the in vivo and the in vitro enzyme

activities. The consistent appearance of a disconnection between the in vitro activity of xylose isomerase and in vivo performance suggests that the enzyme has some distinct properties that have a major effect on the kinetics of xylose utilization by yeast but that are not detected in the common laboratory assays. Further studies are needed to uncover these properties, which would be of major importance for new strategies for mutant design. Below, we propose some possible causes of the observed disconnection and suggest studies that should be considered for further engineering of xylose isomerase.

First of all, in vitro activity assay conditions may not quite represent the in vivo environment where the enzyme acts in the complex cytoplasm of *S. cerevisiae*. Although activity assays were done while taking into account some physiological conditions (30°C, pH 7.0), other factors than only temperature and pH will influence in vivo activity as well. One major issue is the selection of appropriate metal compositions for in vitro assays which should accurately reflect in vivo availability of metals. For estimation of the latter, a reliable measurement of cytosolic metal composition is necessary. Yeast ionic data are available from several studies and we have also measured cellular metal composition of *S. cerevisiae* grown on xylose, but it should be noted that the values represent total or averaged metal content. Since *S. cerevisiae* sequesters metal ions in organelles such as the vacuole for regulation of intracellular metal concentration, such values may not be appropriate for estimating the availability for incorporation into xylose isomerase. Metal analysis of carefully isolated cytosolic fraction may provide a more realistic estimate of metal availability for in vivo activity of xylose isomerase, but still would not account for sub-cytoplasmic heterogeneity and the kinetics of metal incorporation. Part of the cytoplasmic Mn^{2+} and Mg^{2+} could be bound to other biomacromolecules.

Besides determining the metal composition of enzyme isolated from yeast cells, further studies on the kinetics and thermodynamics of metal binding could help to better understand the process of in vivo metal binding and elucidate how it influences activity. Although we learned that Mn^{2+} is the preferred metal for PirXI activity, the two metal binding sites of xylose isomerases may bind different metals with distinct affinities, which should be further investigated. For this, one can pursue the use of techniques such as stopped-flow spectroscopy, taking advantage of the three tryptophan residues that are near the substrate and metal binding sites. In trial stopped-flow assays we found that metal binding gives a clearly detectable change in intrinsic protein fluorescence and observed a pronounced effect of substrate on the observed rates. So far, fitting of the data to standard models for ligand binding failed, but more detailed measurements and analysis with different kinetic schemes may solve this problem and unravel details on the process of metal binding. It may be rather complicated in view

of the two binding sites, the tetrameric structure, the metal promiscuity of the enzyme, and a possible effect of the catalytic cycle on metal complexation.

Secondly, it is possible that other factors than enzyme catalytic activity *per se* are responsible for the suboptimal *in vivo* performance of the enzyme. In Chapter 3, we discovered incomplete metal binding of PirXI expressed in *S. cerevisiae* and found that most of the bound metal was Ca^{2+} , which is a poor activator because it enhances the K_M with xylose to a very high value. The low amount of isomerase protein that is appropriately activated by Mg^{2+} or Mn^{2+} could possibly cause overproduction of the enzyme in adapted strains to compensate for the abundance of inactive enzyme. Unknown interactions of the recombinantly expressed xylose isomerase with other enzymes could also affect xylose assimilation. During isolation of PirXI from *S. cerevisiae*, we often experienced difficulties to separate PirXI from a protein that co-elutes PirXI, which was discovered to be a triosephosphate isomerase (TPI) by mass spectrometry. TPI is an essential enzyme in glycolysis as well as in xylose metabolism, and co-elution of the enzyme with PirXI indicates a possible interaction. To examine such an interaction and establish if it interferes with activity, further investigation is required. Besides the catalytic properties of the individual enzymes, interactions with other macromolecules and the presence of low-molecular weight effectors may influence *in vivo* performance. Possibly only a fine balance between intrinsic activity, regulatory mechanisms, and the influence of yet unknown components in the crowded cytoplasm can result in optimal xylose isomerase activity. Mutations more distant from the active site, few of which were pursued in this work, may act via global effects on interactions beyond enzyme-substrate recognition.

Even though there are still many questions to be solved to obtain a complete understanding of the *in vivo* behavior of PirXI, we believe that the results of the extensive studies reported in this thesis brought us a step closer to obtaining an optimized enzyme for efficient bioethanol production. We reported the first 3D structures of a class II xylose isomerase with various combinations of ligands bound. These PirXI structures will be useful for further understanding the structural diversity of xylose isomerases and for unraveling structure-function relations of the enzyme. By comparing structures with combinations of metals, substrate, product and substrate analogs, we gained an understanding of the metal-dependence of the catalytic activity. The biochemical studies on PirXI provided information on the metal-dependence, which differed from that of most other xylose isomerases. The collaboration with our metabolic engineering colleagues in Delft led to the important discovery that the *in vivo* metal composition strongly influences PirXI activity and thereby metabolism of xylose by *S. cerevisiae*. Our enzyme engineering studies, both with rational and smart-library based approaches, brought us to the sobering yet important conclusion

that there is a disconnect between in vitro properties and in vivo performance of the enzyme.

From a practical enzyme engineering point of view, the semi-random approach in combination with in vivo selection seems to be more efficient than activity-based in vitro screening. The former will avoid complications due to uncertainty about interactions that obstruct the correlation between in vivo and in vitro performance, and the discovered variants can quickly be examined for fermentation properties. Insight from such selections may be obscured by mutations not in the PirXI gene but elsewhere in the yeast genome, but the identity and effects of such mutations can be established by sequencing and retransformation experiments. At the same time, rational engineering of the enzyme and further characterization of the behavior of mutants will expand our understanding of enzyme properties that govern in vivo behavior, which in turn can be used to improve mutant library design. Detailed analysis of the cellular behavior of selected variants should hopefully connect the missing dots between the in vitro properties and in vivo performance of xylose isomerases in xylose metabolism.

Nederlandse samenvatting

Curriculum vitae

List of publications

Acknowledgements

NEDERLANDSE SAMENVATTING

Translated by Titia R. Oppewal

Al heel lang is xylose-isomerase (of glucose-isomerase) een belangrijk enzym bij de productie van siropen met een hoog fructosegehalte. Recentelijk is er interesse ontstaan het enzym toe te passen bij de conversie van D-xylose naar D-xylulose. Xylose kan niet worden gefermenteerd tot ethanol door de gist *Saccharomyces cerevisiae*, maar het isomerisatieproduct xylulose wel, hetgeen deze reactie belangrijk maakt voor een efficiënte productie van tweede-generatie bio-ethanol uit xylose-rijke hemicellulosebiomassa. Als veelgebruikt enzym voor de klassieke omzetting van glucose in fructose is xylose-isomerase al sterk geoptimaliseerd, bijvoorbeeld door het vergelijken van verschillende homologe enzymen, door verbetering van de enzymstabiliteit via protein-engineering en door bioprocessontwerp, inclusief enzymimmobilisatie. Deze door xylose-isomerase gekatalyseerde productie van fructose-siropen wordt op grote schaal toegepast in de voedingsmiddelen- en drankenindustrie met een jaarlijkse omvang van meer dan 15 miljoen ton wereldwijd. Inspanningen om het enzym te optimaliseren voor toepassing bij de productie van tweede-generatie bio-ethanol zijn echter pas kort geleden begonnen.

Het sleutelen aan xylose-isomerase voor een betere productie van ethanol uit xylose moet gericht zijn op het optimaliseren van de prestaties van het enzym in genetisch gemodificeerde stammen van *S. cerevisiae*. Lignocellulose-houdende biomassa is een veel aantrekkelijker grondstof voor de productie van ethanol dan zetmeel of suikers die zijn verkregen uit gewassen die speciaal voor dit doel moeten worden geteeld. Efficiënt gebruik van de xylose die ruimschoots aanwezig is in lignocellulose-hydrolysaten vereist echter genetisch gemodificeerde giststammen die in staat zijn tot snelle xylose-isomerisatie. Omdat de kinetische eigenschappen van het xylose-isomerase hun weerslag hebben op de efficiëntie en snelheid van het fermentatieproces, is het ontwikkelen van xylose-isomerasen met een betere activiteit van groot belang. In dit promotieonderzoek hebben we xylose-isomerase uit de schimmel *Piromyces* (PirXI) onderzocht en verschillende benaderingen gebruikt om zowel de activiteit als de in vivo-prestaties te verbeteren.

Teneinde een effectieve strategie de verbetering van xylose isomerase te ontwikkelen, was allereerst een goede karakterisering van het enzym noodzakelijk. Aan het begin van ons onderzoek was geen informatie over de biochemische eigenschappen van PirXI beschikbaar. Wel was bekend dat xylose-isomerasen metaalionen nodig hebben voor activiteit. Om dit enzym te kinnen bestuderen isoleerden we het PirXI-eiwit via expressie in *E. coli*, voerden een grondige

analyse uit van de katalytische eigenschappen en losten verschillende structuren op met röntgenkristallografie. We hebben ons in die fase vooral gericht op de metaalafhankelijkheid van de katalytische eigenschappen en hoe deze de in vivo-activiteit van het enzym kan beïnvloeden (hoofdstuk 2). De resultaten van metingen van isomerase-activiteiten, metaalanalyse van gezuiverd enzym en inspectie van röntgenstructuren van natuurlijke en met metaal gereconstitueerde varianten suggereerden allemaal dat PirXI niet eenkennig is met betrekking tot metaalacceptatie. Wel zijn er belangrijke verschillen tussen het effect van verschillende metalen. Van de geteste metalen was Mn^{2+} de beste, zowel qua activiteit als affiniteit voor xylose. Ondanks dat was de in vivo-enzymactiviteit veel lager dan verwacht vanwege de lage concentraties van Mn^{2+} in gistcellen in combinatie met de hoge expressie van PirXI. Gistcellen bevatten meer Mg^{2+} en Ca^{2+} , maar helaas geven deze metalen minder goede activatie van het enzym.

Met de structurele en biochemische informatie verkregen in hoofdstuk 2 en de ontdekking dat de intracellulaire metaalsamenstelling van PirXI belangrijk is voor het xylose-metabolisme (hoofdstuk 4), slaagden we erin het proteïne-engineering onderzoek zo op te zetten dat het zowel rationele als semi-willekeurige benaderingen omvatte. We gebruikten structurele en fylogenetische informatie om het de pH-afhankelijkheid en de metaalspecificiteit te optimaliseren en zo beter te voldoen aan de intracellulaire condities van *S. cerevisiae*. In hoofdstuk 3 identificeerden we verschillende mutaties die de katalytische eigenschappen verbeterden, namelijk verhoogde activiteit bij lagere pH en hogere activiteit in aanwezigheid van Mg^{2+} – een metaal dat voldoende in de cel aanwezig is. Door sequentie- en structuurvergelijking met andere xylose-isomerasen ontdekten we mutaties die de activiteit van het enzym verhoogden in aanwezigheid van Mg^{2+} . Deze mutaties zouden anderszins lastig te voorspellen zijn geweest, omdat ze optreden op posities die ver van het actieve centrum verwijderd zijn. Tevens vonden we dat het wijzigen van elektrostatische interacties door het aanbrengen van mutaties die geladen aminozuurresiduen vervangen een positief effect had op de activiteit van het enzym bij lage pH, hetgeen gunstig zou moeten zijn voor activiteit in gistcellen met zijn licht zure omgeving. Opnieuw bleek dat sommige gunstige vrij ver weg lagen van het actieve centrum. Opvallend is dat een hogere enzymactiviteit in vitro niet noodzakelijkerwijs de in vivo-enzymprestaties verbeterde. Een enzym dat beter leek bij initiële testen gaf niet altijd een sneller metabolisme van xylose.

Voor de ontdekking van enzymvarianten die ook in vivo beter functioneerden hebben we op groei gebaseerde screeningsmethoden gebruikt, in combinatie met semi-willekeurige mutagenese. Op grond van de eerder vastgestelde biochemische en structurele gegevens ontwierpen we in hoofdstuk 5 gerichte bibliotheken van PirXI-varianten met mutaties rond de metaalbindende residuen.

Vervolgens ontdekten we met in vivo selectie varianten die verbeterde groei van *S. cerevisiae* op xylose gaven. Deze mutanten vertoonden echter geen superieure in vitro-eigenschappen die de snellere groei van de gistcellen konden verklaren.

Bij beide benaderingen die we gedurende het project hebben onderzocht, namelijk plaats-gerichte mutagenese met in vitro-screening (hoofdstuk 3) en op structuur gebaseerde mutagenese met in vivo-screening (hoofdstuk 5) vonden we voor de meeste mutanten een discrepantie tussen de in vivo- en de in vitro-enzymactiviteiten. Het consistent optreden van een verschil tussen de in vitro-activiteit van xylose-isomerase en de in vivo-prestaties doet vermoeden dat het enzym specifieke eigenschappen heeft die een groot effect hebben op de kinetiek van het gebruik van xylose door gist, maar die niet worden waargenomen in de gebruikelijke laboratoriumtests. Verdere studies zijn nodig om deze eigenschappen aan het licht te brengen aangezien deze van groot belang kunnen zijn voor de ontwikkeling van nieuwe strategieën voor mutantontwerp.

Hoewel er nog veel vragen moeten worden opgelost voor een volledig begrip van het in vivo-gedrag van PirXI, zijn we van mening dat de resultaten van het onderzoek die in dit proefschrift worden beschreven ons een stap dichterbij het verkrijgen van een geoptimaliseerd enzym voor efficiënte bio-ethanolproductie hebben gebracht. We rapporteren de eerste 3D-structuren van een klasse II xylose-isomerase met verschillende combinaties van gebonden liganden. Deze PirXI-structuren zijn nuttig voor een beter begrip van de structurele diversiteit van xylose-isomerasen en voor het ontrafelen van structuur-functierelaties van het enzym. Door structuren te vergelijken met combinaties van metalen, substraat, product en substraatanalogen kregen we inzicht in de metaalafhankelijkheid van de katalytische activiteit. Het biochemische onderzoek van PirXI leverde informatie op over de metaalafhankelijkheid, die verschilde van die van de meeste andere xylose-isomerasen. De samenwerking met onze Delftse collega's op het gebied van metabole engineering leidde tot de ontdekking dat de metaal-gehalte van de cel invloed heeft op metaalbinding door xylose-isomerase – en daarmee op het metabolisme van xylose door *S. cerevisiae*. Het protein-engineering onderzoek, met gebruik verschillende strategieën voor het construeren van enzym-varianten, bracht ons tot de enigszins ontnuchterende maar niet minder belangrijke conclusie dat de in vitro-eigenschappen van het enzym niet altijd direct verbonden zijn met zijn in vivo-prestaties.

Vanuit praktisch oogpunt van enzymontwerp lijkt de semi-willekeurige benadering in combinatie met in vivo-selectie efficiënter te zijn dan een op activiteit gebaseerde in vitro-screening. De eerste vermijdt de complicaties die samenhangen met de onzekerheid over de interacties die de overeenkomst tussen in vivo- en in vitro-prestaties verstoren. Bovendien kunnen de fermentatie-eigenschappen van de ontdekte varianten snel worden onderzocht. Inzichten

uit dergelijke selecties kunnen worden vertroebeld door mutaties die niet in het PirXI-gen voorkomen maar elders in het gistgenoom. De identiteit en effecten van dergelijke mutaties kunnen echter worden vastgesteld door middel van sequentiebepaling en hertransformatie-experimenten. Tegelijkertijd zullen het rationeel ontwerp van het enzym en de verdere karakterisering van het gedrag van mutanten ons begrip vergroten van enzymeigenschappen die het in vivo-gedrag bepalen. Dit kan op zijn beurt worden gebruikt om de strategieën voor het ontwerp van mutanten te verbeteren. Gedetailleerde analyse van het cellulair gedrag van geselecteerde varianten zal hopelijk de ontbrekende schakels tussen de in vitro-eigenschappen en in vivo-prestaties van xylose-isomerasen in het xylose-metabolisme ophelderen.

CURRICULUM VITAE

I started my career in science by pursuing my BSc. in Life Science and Biotechnology at Kyungpook National University in my hometown of Daegu, South Korea. During my undergraduate studies, my curiosity for the world had led me to spend a year in Australia and an exchange semester at the Bonn-Rhein-Sieg University of Applied Sciences, Germany.

Upon completion of my study, I moved to Dubai, U.A.E. working as a cabin crew at Emirates airline. After having enjoyed the privilege to travel the world for four and a half years I decided to continue my path in science in The Netherlands, starting my graduate studies in Molecular Biology and Biotechnology in 2012.

During my first project in Prof. Fraaije's group at the University of Groningen, I learned the tools and methods for enzyme engineering in order to expand the substrate scope of Chitooligosaccharide oxidase. In my second project at Dyadic in Wageningen, I helped to develop a fungal strain for biofuel production.

After finishing my MSc, I wanted to continue focusing on enzyme engineering and I joined Prof. Dick Janssen's group for my PhD project. This project, which was part of the BE-Basic R&D program, aimed to improve the efficiency of *S. cerevisiae*-based lignocellulosic bioethanol production. My goal, specifically, was to improve xylose isomerase for efficient in vivo conversion of xylose, applying a variety of enzyme engineering strategies and enzyme screening methods while collaborating with experts in the field of yeast biology.

LIST OF PUBLICATIONS

Ferrari AR, **Lee M**, Fraaije MW. Expanding the substrate scope of chitooligosaccharide oxidase from *Fusarium graminearum* by structure-inspired mutagenesis. *Biotechnol Bioeng*. **2015**; 112:1074–1080

Lee M, Rozeboom HJ, de Waal PP, de Jong RM, Dudek HM, Janssen DB. Metal dependence of the xylose isomerase from *Piromyces* sp. E2 explored by activity profiling and protein crystallography. *Biochemistry*. **2017**; 56:5991–6005.

Verhoeven MD, **Lee M**, Kamoen L, van den Broek M, Janssen DB, Daran J-MG, et al. Mutations in PMR1 stimulate xylose isomerase activity and anaerobic growth on xylose of engineered *Saccharomyces cerevisiae* by influencing manganese homeostasis. *Sci Rep*. **2017**; 7:46155.

Lee M, Rozeboom HJ, Keuning E, de Waal PP, Janssen DB. Structure-based directed evolution improves *S. cerevisiae* growth on xylose by influencing in vivo enzyme performance. *Biotechnol Biofuels*. **2020**; 13:5.

ACKNOWLEDGEMENTS

There are many people who were part of my past long years in and outside of the lab, contributing to the completion of this thesis. All of these people made my PhD experience great and my life far away from home easier.

Dear **Dick**, first of all, thank you for giving me the opportunity to go through this unique experience. I believe that I have learned a lot from you over the past few years – scientifically through many discussions we had, how to write a good scientific paper and how to think critically. Sometimes, there were challenging moments but I think the process of overcoming those made me grow personally. I have always appreciated your fair and critical feedback which has helped me to grow as an independent scientist. Thank you!

Dear **Marco**, you played a big part influencing me to pursue my PhD. During my Master's project in your group you assured me that I was doing OK. Although you probably do not remember, you told me "I think, you can be a good researcher" and that meant a lot to me – thank you.

Dear **Alex**, I am very glad that you were my first supervisor during my Master's study. Your patience and support helped me a lot to build up my confidence and to finish the project well. Thank you.

Dear **Hanna**, I was very lucky to have a mentor like you around in the beginning of my PhD. I have learned so much from you which helped me to start the project smoothly – thank you very much! Not only have I enjoyed our fruitful scientific discussions but also plenty of our social events.

Dear **Cyntia**, I have so many memories with you. I am very glad that you have been my best friend all these times. We had so much fun doing silly things together. To have you around at work was great, sharing many ideas – sometimes nonsense sometime serious. I appreciate that we can share almost anything – you are my family outside of Korea.

Dear **Marcelo**, it was great working with you. Thank you for all your computational input for the project which was really helpful. I enjoyed all the scientific and non-scientific discussions we had.

Dear **Willem**, I always enjoyed having conversations with you both in and outside of work as you are a good listener and fun to talk with. Thank you for introducing me to Sven, my drum teacher. It had a great impact on my life.

Dear **Antonija**, thank you for being a good colleague and a good friend. You are a very caring and mature person which I admire and appreciate. I enjoyed the many conversations, all the dinners and parties we shared.

Dear **Hugo**, it has been really nice working with you having you as a colleague for many years. Thank you for bringing me to the Taekwondo class, it was fun.

Dear **Hein**, thank you for your kind explanations whenever I had some silly questions about computational methods or chemistry. Also, thank you for joining the project meetings sometimes and sharing your ideas.

Dear **Henriette**, I was very lucky to work with you – you solved so many PirXI structures in no time! Thank you! I enjoyed our collaboration, all the meetings we had and the many train rides to Delft together.

Dear **Piet**, I would say that you were the MacGyver of the lab. It was amazing to have you in the lab – you could fix everything! Thank you for helping me with Acta, HPLC and most of all for fixing my pipette when I broke it – several times.

Dear **Sandra**, thank you for managing and organizing many lab events and all the administrative works. Thanks to you all of the scientists and students can actually focus on their work!

Dear **Christiaan**, thank you for taking care of lab safety, the equipment and all other lab issues. I am happy for you that you are taking the next step in your career. I wish you all the best!

Dear **Elvira** (Romero), I have always admired you for your hard work and devotion to science. You do not only focus on your own work but also always help others. Whenever I had trouble and issues to solve in the lab you were always willing to think with me – thank you. Also thank you for sharing many moments with me. I have always enjoyed hanging out with you.

Dear **Elisa**, thanks for introducing useful tools and protocols to the lab. Since you introduced the Gibson assembly, I have been using it very often and it always (almost) works! Thank you also for being a good friend outside of the lab – I enjoyed all the parties and trips we did together very much.

Dear **Hesam**, you are one of the greatest and strongest people I know. I always appreciated your positive attitude, which also gives good energy to others. I have never seen you complaining in the lab. And thank you for the pleasant chats about everything – I enjoy every visit.

Dear **Milos**, thank you for always answering my silly chemistry questions and helping me out in the lab with HPLC, LCMS and many other things. And thanks for bringing your amazing girls, Sofija and Jasna, into our social group – it is always fun to hang out with you guys.

Dear **Simone**, the Crown manager, thank you for all the fun Friday nights at the Crown! And thanks for accepting my kicks! You know that I only kick the people I like, right?

Dear **Cora**, our friendship started rather late but I am glad that we have managed to build it before you left. Thank you for being a good and caring friend.

We have done quite a few trips together and countless parties. They were always fun with you!

It is important to have nice office mates as you spend many hours per day at the office. I am thankful that I have always had good ones. Dear **Cate**, thank you for always caring. And thank you for contributing your organization skills to both social events and lab matters – that makes other people's lives easier. Dear **Lur**, I love that your keyboard does not make much sound! Thank you also for all the great parties and movie nights you and Nikola hosted. I am very happy that you will stick around in Groningen longer! Dear **Andrea**, my little sister, I was very happy that you came back to our lab since you left after your Master's project. Thank you for all the great times, for your silly jokes, our trips and fun nights.

Dear **Hemant**, I think I told you a few times, you are one of the few people I know who don't get upset or complain (at least I've never seen it). You were an amazing person to be around – thank you! And thank you for letting our band have our very first gig at your defense party. It was great fun!

Dear **Gautier**, the entertainer of the lab, thanks for making me laugh a lot in the lab! You make the lab atmosphere bright and cheerful although your choice of music can sometimes be difficult to handle.

Dear **Yapei**, a.k.a Yapapai, you are my jukebox for lab materials – thanks for always supplying me with media, plates and many other materials in case of emergency. And thanks for always offering your help – it's very sweet of you. Most importantly, thank you very much for all the delicious food you cooked for me!

Dear **JJ**, even though it was very short, I enjoyed the time when you were in Groningen. Thank you for keeping in touch even after you left. My visit to Bangkok and to see you there was awesome.

I would like to thank all the previous and current group members for the supportive work environment, coffee breaks and all the fun events. Dear **Marzena**, **Mohamed**, **Dana**, **Thai**, **Anna** and **Matt**, thank you for being such nice colleagues. All of you guys now have moved on and work in different places and I wish you all the very best. Dear **Jeroen**, **Estela**, **Qinglong**, **Yiming**, **Nikolas** and **Elvira** (Bombino), although it feels like you guys have started your PhD not long ago (probably because I have been around too long), in fact you are almost finishing! Thank you for being nice colleagues and I wish you good luck with finishing up! I would also like to thank some of our neighbor group members. Dear **Rudy**, **Titia**, **Linda**, **Clemens**, **Manuela**, **Ivana**, **Sambika** – thanks for all the good times we shared together. **Titia**, thanks again for your flawless Dutch summary! **Clemens**, thanks for your critical questions and discussion during our work discussion – I learned a lot. **Manuela**, **Ivana** and **Sambika**, thank you for all the fun trips! The project also involved hard work of many students. Dear **Elisa**, my very first

student, thanks for being such a good, motivated student. You have done a very good job, especially your lab journal was perfectly written which was very helpful for me. Dear **Daniël**, even though we spent quite some effort to understand each other in the beginning, I think it all worked out well in the end. Thank you for staying motivated and working in a persistent and accurate way all the way through. Dear **Eline**, thank you for the contribution of your smart ideas into the project which ended up in a nice publication! I would like to also thank the visiting PhD students, **Sandra** and **Megan**. Thank you for letting me help you on your projects. It was a great experience and through that I also learned a lot.

One of the great aspects of the project was the collaboration with several experts in various subjects - **Paul** (de Waal), **Paul** (Klassen) and **René** from DSM, **Maarten**, **Jasmin**, **Prof. Pronk** from TU Delft and **Jeroen**, **Hyun Yong** and **Prof. Driessen** from University of Groningen. I enjoyed working together with you, all the fruitful discussions and meetings very much. I think it was how an ideal research collaboration should be. Thank you for always showing sincere interest in my research, giving useful suggestions and critical feedback – I have learned a lot from all of you. I am also very happy and grateful that our collaboration work has resulted in several good publications.

Even though people say that PhD students don't have a life, I managed to do two other great things other than science. First of all, I got married. The reason I mention this here is to thank again all the people from Groningen and other parts of the world that came all the way to Korea to celebrate this great moment with me - **Cyntia**, **Joris**, **Max**, **Cora**, **Antonija**, **Eric**, **JJ**, **Joe**, **Sambika**, **Elisa**, **Malcolm**, **Elvira** and **Tian**. It was amazing to have all of you there!

Another thing is the band: I enjoyed every practice and all the gigs we had and would like to thank all the former and current band members for that – **Max**, **Friso**, **Ilias**, **Nikolas**, **Mel**, **Cesar** and **Aike**. Dear **Max**, thank you for being excited about the somewhat silly idea (at that time) of forming a band. Thank you also for all the fun parties, organizing BBQs and trips we did together. And it was an honor to be your paranymp – I enjoyed it and the gig at your party was so much fun. Dear **Friso**, our bass player, thank you for accepting my suggestion to start learning bass guitar. It turned out that you are a great bass player and I am glad that you are back. Thank you also for being a great office mate in the good old 'happy office'. I enjoyed all the scientific and non-scientific discussions we had. Dear **Ilias**, besides joining the band, thank you for bothering me to join the borrel every Friday! Dear **Sven**, my drum teacher, thanks to you my old dream to play drum finally became real. Thank you for your patience and the fun lessons!

Dear paranymphs, **Ale** and **Nikola**, thank you for being great paranymphs, helping me a lot with the mental and physical preparation of the defense and celebrations. Dear **Ale**, thank you for being a great friend who I can share my good and bad moments with. Chatting with you is always a fun part of my day. And I swear, I did not ignore you at the Vismarkt that day but I just did not see you. I hope you forgive me now. Dear **Nikola**, I have you, being in the group as long as I have, to share good old memories as well as new ones. Thank you for helping me whenever I need help both at and outside of work - you always have good advice. Also, thank you for initiating and organizing many group activities, BBQs and parties.

Liebe **Doris**, lieber **Klaus**, vielen Dank für eure Unterstützung und Liebe!. Being away from home, I feel that you are like my parents and it helps me get through difficult moments. Thank you!

사랑하는 **엄마 아빠**, 무엇보다도 항상 저를 믿어 주시고 응원해 주셔서 고맙습니다. 두바이에서 승무원 생활하는 것부터 시작해서 타지 생활을 한지 벌써 12년이 지났습니다. 항상 가까이 있어 드리지 못해서 죄송한 마음뿐입니다. 이제 부터라도 연락 자주 드릴 게요 - 늘 건강 하세요. 사랑하는 **내 동생 경민이 재홍이**, 비록 몸은 멀리 있지만 너희들이 있어서 항상 마음 한 편으로는 든든하다. 너희들이나마 부모님 곁에 있어서 고맙고, 착한 동생들이 되어 줘서 고마워.

Dear **Martin**, my husband, without you everything would have been much more difficult. Thank you for all your support and always being there for me. Even though you had to drive back and forth between Groningen and Nürnberg almost every week, you never complained - you are an amazing 남편. I am also very sorry for the many weekends we spent in the lab - my PhD degree is our coproduction work. 사랑한다!!