

Genetic improvement of native xylose-fermenting yeasts for ethanol production

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Abstract Lignocellulosic substrates are the largest source of fermentable sugars for bioconversion to fuel ethanol and other valuable compounds. To improve the economics of biomass conversion, it is essential that all sugars in potential hydrolysates be converted efficiently into the desired product(s). While hexoses are fermented into ethanol and some high-value chemicals, the bioconversion of pentoses in hydrolysates remains inefficient. This remains one of the key challenges in lignocellulosic biomass conversion. Native pentose-fermenting yeasts can ferment both glucose and xylose in lignocellulosic biomass to ethanol. However, they perform poorly in the presence of hydrolysate inhibitors, exhibit low ethanol tolerance and glucose repression, and ferment pentoses less efficiently than the main hexoses glucose and mannose. This paper reviews classical and molecular strain improvement strategies applied to native pentose-fermenting yeasts for improved ethanol production from xylose and lignocellulosic substrates. We focus on *Pachysolen tannophilus*, *Scheffersomyces (Candida) shehatae*, *Scheffersomyces (Pichia) stipitis*, and *Spathaspora*

passalidarum which are good ethanol producers among the native xylose-fermenting yeasts. Strains obtained thus far are not robust enough for efficient ethanol production from lignocellulosic hydrolysates and can benefit from further improvements.

Keywords Adaptation · Cofactor imbalance · Genome shuffling · Metabolic engineering · Protoplast fusion · Random mutagenesis · Xylose reductase

Introduction

Efficient utilization of pentose sugars in conjunction with the overall utilization of lignocellulose is important in the bioconversion of plant biomass for the production of chemicals and liquid fuels [17, 43, 71]. Potential substrates envisioned for the application of pentose-fermenting technologies include hydrolysates of plant biomass or waste liquors of pulp and paper processing industries. However, xylose is not as readily utilized as glucose by yeasts. Native strains of the well-known fermentative yeast, *Saccharomyces cerevisiae*, are unable to utilize xylose as a sole carbon source for aerobic growth [44] despite the existence of activities for xylose transport, xylose reductase (XR), xylulokinase, and subsequent enzymes needed for a full xylose metabolic pathway [7]. Prior to 1981, xylose was thought to be unfermentable by yeasts and schemes to ferment xylose typically involved two stages: (1) xylose was transformed to xylulose in vitro using glucose isomerase; and (2) fermentation of xylulose to ethanol [87]. This picture changed with the discovery of pentose-fermenting yeasts in 1981–1982 [19, 28, 74, 77]. This discovery was considered a significant milestone due to the recognized need for complete utilization and

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conversion of all sugars in potential lignocellulosic substrates to improve process economics.

Since 1981, numerous yeasts able to produce some ethanol from xylose have been identified. Among these, *Pachysolen tannophilus*, *Scheffersomyces (Candida) shehatae*, and *Scheffersomyces (Pichia) stipitis* were notable for their ability to produce relatively high ethanol yields from xylose. Considerable research was undertaken on the physiology and biochemistry of these yeasts [33]. The results showed that the rate and yield of ethanol produced from xylose by these yeasts were lower than those for glucose fermentation [30]. This low ethanol yield was partially attributed to concurrent utilization of ethanol in the presence of appreciable concentrations of xylose [58] and to formation of by-products such as xylitol, ribitol, arabitol, and acetic acid [73].

Yeasts that convert xylose efficiently in defined media often perform poorly in pretreated biomass hydrolysates or waste liquors of lignocellulosic materials. A central problem is the presence of hexoses (mainly glucose and mannose) that compete with or inhibit xylose utilization [9, 63]. Pentose-fermenting yeasts can convert glucose or xylose individually to ethanol. The fermentation of xylose present in a mixture with glucose, however, does not proceed efficiently. This is because the fermentation of glucose precedes that of xylose and pentose-fermenting yeasts are generally not sufficiently tolerant of ethanol to complete the second process. Another problem is the occurrence of cofactor imbalance in the first two enzymes of xylose metabolism, XR that has a higher affinity for NADPH and xylitol dehydrogenase (XDH) that is only active with NAD [11]. During xylose metabolism, the different coenzymes preferred by XR and XDH result in an accumulation of NADP and NADH from the first and second reactions, respectively. This cofactor imbalance inhibits further metabolism along this pathway and regeneration of the cofactors by oxygen is necessary for xylose metabolism to continue. This led Bruinenberg et al. [12] to propose that having an NADH-preferring XR would obviate the need for cofactor regeneration and this may enable xylose to be fermented anaerobically. The requirement of oxygen for xylose utilization by *P. tannophilus* was observed by Neirinck et al. [61], and this was recognized as one of the reasons for inefficient xylose fermentation. Moreover, the presence of oxygen during xylose fermentation contributes to the concurrent production and utilization of ethanol while considerable amount of xylose remains in the medium [56]. Another problem is the presence of various pretreatment-derived inhibitors that adversely affect yeast growth and fermentation. Both physical and chemical pretreatments yield a complex mixture of inhibitors that adversely affect yeast growth, viability and fermentation [40, 53, 70, 85]. Different upstream processing conditions

are needed for different substrates and these factors may result in the production of varying concentrations of different inhibitors.

The above challenges have led to efforts to address these problems to improve the performance of native pentose-fermenting yeasts in lignocellulosic hydrolysates. This review examines research to genetically improve xylose-fermenting yeasts, with a focus on the improvement of *P. tannophilus*, *S. shehatae*, *S. stipitis* and *S. passalidarum* for ethanol production from xylose and lignocellulosic hydrolysates.

Genetic improvement of native pentose-fermenting yeasts

Improved tolerance to pretreatment-derived inhibitors

Adaptation The complex and variable nature of the mixture of toxic compounds in lignocellulosic hydrolysates and difficulties in identifying them limit our understanding of how each may contribute to overall cellular toxicity. Furthermore, various inhibitory compounds may act synergistically to exert toxicity; thus even compounds present at low concentrations may contribute to the overall toxicity. Due to this lack of knowledge on the mechanisms involved in inhibitor toxicity and tolerance, adaptation has been hypothesized as the best approach to address their combined toxic effect. A number of studies have used adaptation to improve the performance of pentose-fermenting yeasts, mostly *S. stipitis* (Table 1). Adaptation involves repeated sub-culturing or recycling of yeast cells to increasing concentrations of the inhibitor(s) in lignocellulosic hydrolysates. One advantage of adaptation is that strains adapted to tolerate one hydrolysate may exhibit cross-tolerance to other hydrolysates. This is not surprising considering that different lignocellulosic hydrolysates contain similar kinds of inhibitors but with different compositions and concentrations. However, very few studies assessed this possible cross-tolerance in the adapted strains. Initial screens for adaptation are typically growth-based, but another key improvement sought is fermentation, which cannot be selected for on the basis of growth alone. Thus, adapted strains selected for improved growth have to be assessed also for the ability to ferment the sugars in the presence of inhibitors in lignocellulosic hydrolysates. The availability of yeast strains with the ability to tolerate inhibitory compounds in lignocellulose hydrolysates would reduce the need for detoxification procedures, and this can decrease the overall production cost.

Adaptation has been done to individual inhibitor(s) as well as to a mixture of inhibitors, with the latter approach being the most common. A limitation to adaptation to individual inhibitors is that the adapted strain(s) may not exhibit improved growth and fermentative ability in the complex mixture of inhibitors found in lignocellulosic hydrolysates. In one study of this kind, Liu et al. [52] adapted *S. stipitis*

Table 1 Selected native pentose-fermenting yeasts improved by adaptation and recycling

Yeast	Strain	Substrate	pH	Adapted or recycled	Initial sugar utilized (%)	Max [EtOH] (g/L)	Yield (g EtOH/g sugar)	References
<i>S. stipitis</i>	BCRC21777	Rice straw hydrolysate	5.0	WT	ND	9.39	0.40	[25]
				Adapted to increasing concentration	ND	10.27	0.44	
	CBS 6054	Corn cob hemicellulose acid hydrolysate	ND	WT	18	1.5	0.21	[1]
				Adapted to increasing concentration	90	13.3	0.41	
	CBS 6054	Corn stalk hydrolysate	5.0	WT	ND	0	0	[96]
				Adapted to increasing concentration	ND	43.42	0.47	
	NBRC1687	Ethanol (50–70 g/L)	ND	WT	ND	38	ND	[90]
				Adapted to increasing concentration	ND	44	ND	
	NRRL Y-7124	Read oak hydrolysate (30 % v/v)	5.0	WT	80	9.8	0.25	[62]
				Adapted to increasing concentration	93.5	14.5	0.40	
NRRL Y-7124	Red oak hydrolysate (60 % v/v)	5.0	WT	0	0	0		
			Adapted to increasing concentration	66.94	8.3	0.31		
NRRL Y-7124	Synthetic prehydrolysate (with 5 g/L acetic acid)	5.0	WT	15.6	0.8	0.002		
			Adapted to increasing concentration	90	13.3	0.35		
NRRL Y-7124	HMF	ND	WT	ND	0	ND	[52]	
			Adapted to increasing concentration	ND	10	ND		
NRRL Y-7124	Sugar maple hemicellulose hydrolysate (75 % v/v)	5.5	WT	ND	0	ND	[80]	
				Adapted to increasing concentration	ND	9.6	ND	

ND no data available

NRRL Y-7124 cells to increasing concentrations of hydroxymethyl furfural (HMF). After 100 cycles, the adapted strain, designated 307-10H60, showed an enhanced ability to transform HMF to 2,5-bis-hydroxymethylfuran compared to the wild-type (WT) strain. In defined medium containing 60 mM HMF, strain 307-10H60 converted 60 % of HMF and utilized glucose in 20 h to produce 10 g/L ethanol. In comparison, the WT did not ferment glucose at this HMF concentration [52]. Unfortunately, the adapted strain was not tested for xylose fermentation or in a mixture of inhibitors typically found in lignocellulosic hydrolysates. Thus, it is not known if tolerance to HMF would improve the performance of this xylose-fermenting strain in industrially relevant hydrolysates.

More commonly, adaptation was performed by sequential transfers or recycling of cultures to increasing concentrations of lignocellulosic hydrolysates. Several groups have independently obtained *S. stipitis* strains adapted to different hydrolysates (Table 1). These studies demonstrate the potential for using adaptation to obtain yeast strains

more tolerant to pretreatment-derived inhibitors, thereby producing higher fermentation rates and yields compared to the WT strains. Possibly, adaptation resulted in selection of mutant strains, although this has not been explicitly stated and the nature of possible mutations was not examined. A drawback of these studies was that adaptation and subsequent testing were done in the presence of yeast extract and peptone. These adapted strains would not perform as well in hydrolysates without these added nutrients; thus the utility of these adapted strains under industrial conditions remains uncertain. Furthermore, the long-term stability of the adapted strains was not assessed in these studies.

Most native xylose-fermenting yeasts are subject to glucose repression [9, 13, 39] and glucose inactivation [47]. In repression, glucose inhibits the synthesis of xylose-metabolizing enzymes at the transcriptional level. On glucose depletion, xylose-metabolizing enzymes are synthesized to enable xylose utilization, i.e., de novo protein synthesis is required. In inactivation, glucose inhibits the activities of xylose transport and/or other xylose-metabolizing

enzymes. Xylose consumption may resume before glucose is completely consumed, and this resumption does not require de novo protein synthesis. As a result, in a glucose-xylose mixture, glucose is fermented before xylose and the yeast is often not sufficiently tolerant of ethanol [5] to complete the xylose fermentation. One exception to the above scenario is *S. passalidarum* which utilizes xylose and glucose simultaneously and ferments glucose, xylose and cellobiose simultaneously under oxygen-limiting conditions [54]. To improve the ability of *S. passalidarum* NRRL Y-27907 to ferment the sugars in hydrolysates, this strain was adapted to YPX-Cb medium (containing 10 g/L yeast extract, 10 g/L peptone, 45 g/L xylose and 25 g/L cellobiose) followed by cultivation in corn stover hydrolysate for 2 months (54 doublings) under oxygen-limiting conditions [54]. Fermentation of the adapted strain E7 on maple hemicellulose hydrolysate (MHH) was compared to that on a sugar mixture (SM) designed to mimic the hydrolysate (65 g/L xylose and 35 g/L glucose). Glucose and xylose were co-utilized in the MHH, while xylose utilization was delayed by 12 h in the SM. E7 produced a peak ethanol concentration (40 g/L) more rapidly in SM (38 h) than in MHH (38 g/L and 59 h, respectively). However, the fermentation performance was not compared to the WT strain under the same conditions; thus, the extent of the improvement for the adapted strain is not known. The inherent ability of *S. passalidarum* to ferment xylose faster than glucose and to simultaneously ferment glucose, xylose and cellobiose are desirable characteristics for use in lignocellulosic biomass conversion.

Random mutagenesis

The adaptation approach, while useful, can be slow and time-consuming. Industrial strain improvement typically involves randomly generated mutants from which a few of the top performers are selected for further mutagenesis and screening. An effective and sensitive screen is necessary to select mutants of interest.

Despite the attractiveness of random mutagenesis, only a few studies have used this approach to obtain mutants of xylose-fermenting yeasts with improved tolerance to inhibitors in lignocellulosic hydrolysates. In one study, successive rounds of UV mutagenesis followed by screening on a gradient plate of hardwood spent sulfite liquor (HW SSL) was used to successfully obtain mutants of *S. stipitis* NRRL Y-7124 with improved tolerance to HW SSL [5]. Growth assessment indicated the WT grew in 65 % (v/v) HW SSL while mutants (PS301 and PS302) selected after three rounds of UV mutagenesis and screening grew in 75 % (v/v) HW SSL and survived in 80 % (v/v) HW SSL. In addition, these mutants were more tolerant of HMF than the WT and grew faster with a shorter lag phase in

5 g/L HMF. The WT did not produce ethanol in 60 % (v/v) HW SSL whereas mutants produced peak ethanol ranging from 3.3 to 6.4 g/L. These mutants also showed slightly improved tolerance to acetic acid (2.8–3.0 g/L) compared to the WT (2.5 g/L).

Using a similar approach, Harner et al. [21] obtained mutants of *P. tannophilus* NRRL Y-2460 with enhanced tolerance to HW SSL and acetic acid in two separate selection lines. The WT strain grew in 50 % (v/v) HW SSL while third round HW SSL mutants (UHW301, UHW302 and UHW303) grew in 60 % (v/v) HW SSL with two of these isolates (UHW302 and UHW303) being viable and growing, respectively, in 70 % (v/v) HW SSL. In defined liquid media containing acetic acid, the WT strain grew in 7 g/L acetic acid, while third round acetic acid mutants (UAA301, UAA302 and UAA303) grew in 8 g/L acetic acid with one isolate (UAA302) growing in 9 g/L acetic acid. Cross-tolerance of HW SSL-tolerant mutants to acetic acid and vice versa was observed. The fermentative ability of UAA302 in defined media was similar to that of the WT, while UHW303 fermented slightly less well than the WT [21].

Genome shuffling

Genome shuffling combines classical mutagenesis with the possibility of recombination to allow for further strain improvement. This method uses recursive recombination to enhance the genetic diversity of mutant populations with improved phenotypes, allowing for the creation of new mutant combinations and strains with superior characteristics [10, 66]. Whole genome recombination accelerates the accumulation of multiple beneficial mutations in strains while having the potential to remove deleterious mutations, thereby allowing for the engineering of complex phenotypic traits with less time and effort [64, 100]. As with random mutagenesis, the key criterion for successful genome shuffling is an effective and sensitive screen to select the improved mutants.

In genome shuffling, recombination can be carried out using recursive protoplast fusion or cross-mating. Most reports on genome shuffling to improve inhibitor tolerance or ethanol productivity of yeasts are based on protoplast fusion [22, 94]. Genome shuffling carried out by protoplast fusion, however, may yield unstable hybrids [18].

A strategy using yeast cross-mating instead of protoplast fusion was performed to improve the tolerance of *S. stipitis* to HW SSL [4]. Six UV-induced mutants of *S. stipitis* NRRL Y-7124 were subjected to four rounds of genome shuffling. Two mutants from the third round (GS301 and GS302) grew in 85 % (v/v) HW SSL, with GS301 being viable in 90 % (v/v) HW SSL. The WT *S. stipitis* was unable to grow in HW SSL unless it was diluted to 65 % (v/v)

or lower [4]. In fermentation tests, the WT did not utilize any sugars in undiluted HW SSL (pH 5.5), while GS301 and GS302 utilized glucose faster than PS302, the best UV-induced mutant [5]. The WT and two genome shuffled *S. stipitis* strains (GS301 and GS302) were tested for growth and fermentation performance in three wood hydrolysates: steam-pretreated enzymatically hydrolyzed poplar hydrolysate, steam-pretreated poplar hydrolysate and mixed hardwood pre-hydrolysate [3]. All strains grew in the steam-pretreated enzymatically hydrolyzed poplar hydrolysate, GS301 and GS302 grew in the mixed hardwood pre-hydrolysate, but none of the strains grew in the steam pretreated poplar hydrolysate. In fermentation tests, GS301 and GS302 completely utilized glucose and xylose in each hydrolysate and produced between 3.9 and 14 g/L ethanol while the WT did not utilize, or poorly utilized, the sugars, producing low concentrations of ethanol. These results demonstrated cross-tolerance of the mutants to inhibitors in different wood hydrolysates [3].

One advantage of using a cross-mating-based genome shuffling approach for industrial yeast strain improvement is that the genetically stable haploid cells obtained are amenable to further improvement by mutagenesis and mating. In contrast, after several rounds of protoplast fusion, the resulting population may have uncertain ploidy levels or may be aneuploid, some of which may be unstable or not amenable for further improvement. Bajwa et al. [2] have provided a detailed protocol for genome shuffling based on recursive cross-mating in *S. stipitis*.

Improved ethanol tolerance and production

Random mutagenesis

Native pentose-fermenting yeasts are known to be less tolerant of ethanol than the fermentative yeast *S. cerevisiae*. The basis for this is not known and there has always been a desire to improve the ethanol tolerance of pentose-fermenting yeasts. Ethanol tolerance is thought to be a multi-genic trait and different approaches have been used to identify potential genetic elements contributing to this phenotype [16, 55, 81, 84]. The complex nature makes genetic studies and improvement of ethanol tolerance difficult. Ethanol tolerant strains have been obtained primarily using random mutation approaches.

Watanabe et al. [90] used UV mutagenesis followed by screening on a solid medium containing 50 g/L ethanol (supplemented with 50 g/L xylose, 1.7 g/L YNB without amino acids, 2.0 g/L ammonium sulfate and 5.0 g/L peptone) to select mutants of *S. stipitis* NBRC1687 with improved tolerance to ethanol. Three mutants (PXF4, PXF36 and PXF58) producing larger colonies compared to the WT on plates were selected. During xylose

fermentation in the same medium above, the WT produced less ethanol (16.4 g/L) compared to PXF4 (17.4 g/L), PXF36 (17.3 g/L) and PXF58 (19.4 g/L). In a medium containing xylose (110 g/L), glucose (120 g/L) or fructose (120 g/L) supplemented with peptone, the WT produced about 30 g/L ethanol while PXF58 produced about 40 g/L ethanol. To further improve its ethanol tolerance, PXF58 was subjected to 20 cycles of adaptation in increasing ethanol concentrations (50–70 g/L). This resulted in the isolation of a mutant (PET41) which produced 44 g/L ethanol compared to 40 g/L by PXF58 and 38 g/L by the WT strain after fermentation (96 h) in peptone-supplemented media containing 45 g/L xylose with glucose added to final concentrations of 40 and 60 g/L after 36 and 60 h, respectively [90]. The performance of the mutants, however, was not tested in a defined medium without peptone supplementation; thus, it is uncertain how these strains might perform in the absence of rich nutrient components.

Protoplast fusion

Some researchers have used cross-species protoplast fusion to combine the desirable traits of *S. cerevisiae*, such as greater tolerance to ethanol and inhibitors with xylose-utilizing ability of pentose-fermenting yeasts, to generate hybrids with improved properties. In an earlier study, Yoon et al. [97] combined *S. cerevisiae* STV 89 and *S. stipitis* CBS 5776 by protoplast fusion. Based on its fermentation performance in xylose and sucrose media, one fusant (designated F5) was selected for further study. During fermentation of 100 g/L xylose (medium also contained 5 g/L yeast extract, 5 g/L peptone and mineral salts), F5 produced more ethanol with a higher yield (44 g/L and 0.44 g ethanol/g xylose consumed, respectively) compared to the *S. stipitis* parental strain (38.8 g/L and 0.39 g/g, respectively). However, the fusants were unstable. After six rounds of sub-culturing all fusants, including F5, dissociated into segregants which resembled *S. stipitis* based on fermentation performance.

In another study, *S. cerevisiae* 2.0251 was fused with *P. tannophilus* ATCC 2.1662 [95]. Fermentation in media containing 10 g/L glucose, 10 g/L peptone and 5 g/L yeast extract (YEPA) supplemented with xylose (5.52 g/L) was carried out with 20 putative fusants. Based on fermentation performance, the best isolate (Fusant 1) utilized more xylose (72 %) and produced more ethanol (3.4 g/L) than the *P. tannophilus* parental strain (32 % and 1.2 g/L). During fermentation of mixed sugars (30 g/L glucose and 20 g/L xylose), Fusant 1 produced more ethanol (11.76 g/L) compared to either the *S. cerevisiae* (11.2 g/L) or *P. tannophilus* (8.8 g/L) parental strains. Maximum ethanol concentrations and complete glucose utilization occurred after 24 h for all strains. Fusant 1 was capable of simultaneous sugar

utilization and consumed 20 % of the initial xylose within the first 24 h. In contrast, the *P. tannophilus* parental strain began to utilize xylose after 24 h, which may explain the unusually low peak ethanol concentration produced by this strain. Fusant 1 was tolerant to an ethanol concentration (63.1 g/L) which was intermediate between that of *S. cerevisiae* (78.9 g/L) and *P. tannophilus* (39.45 g/L) and exhibited better growth in softwood acid hydrolysate (pH 5.5) compared to the parental strains. The average DNA content of Fusant 1 (0.80×10^7 $\mu\text{g/mL}$) suggested that most of its DNA came from the *P. tannophilus* parental strain (DNA content = 0.78×10^7 $\mu\text{g/mL}$) and only a small amount from the *S. cerevisiae* parental strain (DNA content = 1.74×10^7 $\mu\text{g/mL}$). Fusant 1 appeared to be stable based on mixed sugar fermentation performance assessed periodically over 15 generations. Its long-term stability or ploidy level is not known.

Kumari and Pramanik [45] fused *S. cerevisiae* NCIM-3090 individually with different pentose-fermenting yeasts including *S. shehatae* NCIM-3500, *P. tannophilus* NCIM-3502 and *S. stipitis* NCIM-3507 to yield hybrids capable of converting hexose and pentose sugars. The fermentation performance of the fusants was first evaluated in a glucose (150 g/L) and xylose (50 g/L) mixture (containing 2 g/L yeast extract and minerals). Three fusant strains produced more ethanol with a greater yield and higher volumetric productivity than the *S. cerevisiae* parental strain (69.1 g/L, 0.436 g ethanol/g sugar consumed and 0.96 g/L/h, respectively). A *S. cerevisiae* and *P. tannophilus* fusant (RPR39) was found to be the most efficient strain giving high-peak ethanol concentration (76.8 g/L), volumetric productivity (1.06 g/L/h) and ethanol yield (0.458 g/g). Comparable fermentation efficiencies were also observed with another *S. cerevisiae* and *P. tannophilus* fusant (RPR51) and a *S. cerevisiae* and *S. stipitis* fusant (RPR16) producing ethanol yields of 0.454 and 0.437 g/g, respectively. The percentage of the DNA originating from each parental strain was not determined. The DNA content in fusants was analyzed using flow cytometry, and fusant DNA content was found to be doubled that of individual parental strains. Fusants were considered stable if they retained their characteristic mixed sugar utilization and ethanol production profiles for 9 months. The long-term stability of RPR39 was not investigated. The fusant RPR51 was found to be unstable while RPR39 and RPR16 were stable after 9 months.

To further improve the fermentation properties of RPR39, Kumari and Pramanik [46] subjected it to combinations of mutagens (ethyl methane sulphonate, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, near UV or far UV radiation) followed by screening under various stress conditions to obtain putative mutants more tolerant to multiple fermentation stressors. Among the eight mutants, RPRT90 was the best in terms of genetic stability and tolerance to ethanol,

temperature and inhibitory compounds. The combined effect of these stress factors was studied by subjecting RPRT90 to various conditions simultaneously during fermentation of a glucose (187.5 g/L) and xylose (62.5 g/L) mixture (containing 2 g/L yeast extract and minerals). When subjected to the combined effect of temperature (39 °C), ethanol (55.2 g/L) and inhibitors (0.25 g/L vanillin, 0.5 g/L furfural and 4 g/L acetic acid), RPRT90 converted 64.8 % of the sugars, producing an additional 47.9 g/L ethanol with a yield of 0.295 g/g and volumetric productivity of 0.57 g/L/h. Under the same conditions, fusant RPR39 converted 40.3 % of the sugars, producing an additional 25 g/L ethanol, with an ethanol yield of 0.21 g/g and volumetric productivity of 0.42 g/L/h. At the end of the 96-h fermentation, more glucose was utilized (about 45 and 55 g/L remaining) compared to xylose (about 45 g/L remaining) by both RPRT90 and RPR39 strains [46].

In a slightly different approach, a combination of UV mutagenesis and protoplast fusion was used to improve the inhibitor tolerance and fermentative ability of *S. passalidarum* MYA-4345 in wheat straw hydrolysate [24]. Furfural-tolerant mutants were isolated by plating UV mutagenized cells on a rich medium containing 10 g/L yeast extract, 20 g/L peptone, 30 g/L xylose and 2.5 g/L furfural (YPXF). Two selected mutants (M5 and M7) showed improved xylose consumption, ethanol yield, final ethanol concentration achieved and higher furfural reduction rates during fermentation in YPX compared to the WT. Cross-species protoplast fusion was carried out between M7 and *S. cerevisiae* ATCC 96581. Putative fusants were plated on agar prepared with 75 % wheat straw hydrolysate (containing 1.72 g/L glucose, 2.29 g/L xylose, 0.54 g/L furfural, 0.29 g/L HMF and 3.39 g/L acetic acid) supplemented with 30 g/L xylose to select for hybrids. Repeated transfers between 75 % wheat straw hydrolysate and medium containing 10 g/L yeast extract, 20 g/L peptone and 30 g/L xylose (YPX) were done to maintain the hybrids' tolerance character. This selection regime was based on the knowledge that unfused parents could not grow on the plate, as *S. cerevisiae* could not utilize xylose as the sole carbon source and M7 could not tolerate the high inhibitor concentration. Among the 24 hybrids selected, FS22 was the best isolate during anaerobic fermentation in 75 % wheat straw hydrolysate supplemented with 30 g/L xylose. Hybrid FS22 showed good xylose consumption (39.4 %) and ethanol yield (0.40 g ethanol/g xylose consumed) compared to M7 which was unable to survive or ferment at this concentration of hydrolysate. Based on the restriction patterns of the PCR fragments, it was determined that *S. passalidarum* M7 was the dominant contributor of DNA to the hybrid.

Interestingly, none of these inter-genera hybridization studies provided the percentage of the DNA in hybrids from each parental strain. Based on DNA content comparisons,

phenotypic characterization or partial molecular characterization it is difficult to determine whether these are true hybrids. Whole genome sequencing would be an effective strategy to better characterize these hybrid strains and perhaps identify the genetic factors from each parental strain conferring the improved phenotypes in the hybrids as well as to confirm hybridization of parental strains.

Genome shuffling

Zhang and Geng [99] used recursive recombination of the whole genomes of *S. stipitis* CBS 6054 and *S. cerevisiae* ATCC 24860 to construct strains with enhanced ethanol production, tolerance to ethanol and faster rates of xylose utilization. Genomic DNA of *S. stipitis* was extracted and transferred into the *S. cerevisiae* strain by electroporation to allow genetic recombination. Selection of first round strains was carried out on plates containing 6.7 g/L YNB and 50 g/L xylose followed by fermentation of 150 g/L xylose in a rich medium (containing 7 g/L yeast extract and 2 g/L peptone). The best ethanol producing strain (F1-8) was used for the next round of genome shuffling in which the whole genome of *S. cerevisiae* was extracted and transferred into F1-8 by electroporation. Second round strains were screened on plates containing 6.7 g/L YNB, 50 g/L xylose and 50 g/L ethanol. One obtained strain (ScF2) produced 47 g/L of ethanol in 6 days from 250 g/L xylose in a medium supplemented with 7 g/L yeast extract, 2 g/L peptone and minerals. In comparison, the *S. stipitis* parental strain produced 20 g/L ethanol on day 7 under the same conditions. During mixed sugar (50 g/L glucose and 50 g/L xylose) fermentation, *S. cerevisiae* only utilized glucose and produced a peak ethanol concentration of 22 g/L in about 24 h. In contrast, *S. stipitis* and ScF2 utilized both sugars, producing peak ethanol concentrations of 31 g/L at 96 h and 40 g/L at 144 h, respectively. Randomly amplified polymorphic DNA (RAPD) analysis showed different banding patterns between ScF2 and its parental strains, suggesting that ScF2 is genetically different from the parental strains. This genome shuffling approach successfully combined features of parents, *S. cerevisiae* (ethanol and sugar tolerance) and *S. stipitis* (xylose utilization), in ScF2. Its performance in defined media and industrially relevant hydrolysates remains to be assessed.

Glucose derepression

Random mutagenesis

As mentioned before, native xylose-fermenting yeasts typically suffer from glucose repression [9] and glucose inactivation [47]. As a result, in a glucose-xylose mixture, glucose is fermented before xylose and these yeasts are

often not sufficiently tolerant of ethanol [6] to complete the xylose fermentation.

Some researchers have selected for glucose derepressed cells using the glucose analog 2-deoxyglucose (2-DG) which cannot be metabolized by yeasts beyond the initial phosphorylation step [79]. In an earlier study, glucose derepressed mutants of *P. tannophilus* NRRL Y-2460 were obtained by isolating UV mutants resistant to 2-DG followed by replica-plating on solid media containing hexose sugars to select for strains showing reduced growth [92]. During growth on mixed sugars, mutant P509-1B exhibited reduced glucose repression and was able to co-utilize glucose and xylose compared to the WT. Enzyme studies showed this strain to be deficient in hexose-phosphorylating activity (possessing only 6 % of the WT activity) [92]. Wedlock and Thornton [93] further examined the role of the two hexokinases in glucose repression in *P. tannophilus*. Three mutants were constructed and grown under mixed or individual sugars followed by measurement of XR and XDH activities as an indicator of glucose repression. Mutant P510-5A had a defect in glucokinase (encoded by *glk*), P509-3C had a defect in hexokinase (encoded by *hvk-a*) and P509-1B had defects in both *glk* and *hvk-a* [92]. When grown in a mixture of glucose (20 g/L) and xylose (20 g/L), reduced XR and XDH activities were seen in P510-5A, but not P509-3C and P509-1B. The results indicate the involvement of *hvk-a*, but not *glk*, in glucose repression in *P. tannophilus* [93]. Unfortunately, the fermentation performance of P510-5A in mixed sugars was not tested to confirm the glucose derepressed phenotype.

In another study, Sreenath and Jeffries [79] used chemical mutagenesis (ethyl methane sulphonate) followed by selection on 2-DG to obtain mutants of *S. stipitis* FPL-061 with improved xylose utilization in the presence of glucose. Of the 2-DG resistant strains selected, FPL-DX26 was the best in fermentation. When grown in a mixture of 40 g/L glucose and 40 g/L xylose, this strain consumed the sugars simultaneously after 20 h and all the sugars were consumed by 45 h. In comparison, the WT utilized xylose only after glucose was consumed and complete utilization of these sugars required 120 h [79].

Miscellaneous attempts to improve xylose utilization and fermentation

Random mutagenesis

Approaches, ranging from random to semi-rational, have been used to improve xylose utilization in native xylose-fermenting yeasts. Some of the successful attempts are described below. Mutants able to utilize xylose faster typically exhibit better xylose fermentation rate.

In an effort to isolate strains with improved mixed sugar fermentation, Sreenath and Jeffries [78] selected for rapidly growing mutants of *S. stipitis* CBS 6054 on L-xylose in the presence of respiratory inhibitors antimycin A and salicyl hydroxamic acid (SHAM). The ability to grow on L-xylose was used to select for mutants whose aldose reductase may be derepressed. To ensure that selected strains retained fermentative ability, mutants exhibiting lower respiratory activity were selected via resistance to respiratory inhibitors. *S. stipitis* possesses both antimycin-sensitive and SHAM-sensitive respiratory pathways and inhibitors for both pathways were used to inhibit growth, as blockage of either pathway alone is insufficient to inhibit growth [78]. One mutant isolated (FPL-061) produced higher ethanol concentration (29.4 g/L) and yield (0.42 g ethanol/g sugar consumed) compared to the WT (25.7 g/L and 0.35 g/g, respectively) in a 1:1 mixture of glucose and xylose (having final sugar concentrations of 60–80 g/L) supplemented with salts and some peptone [78].

In another study, Jeffries [29] used UV mutagenesis followed by selection for rapid growth in nitrate-xylytol broth to obtain mutants of *P. tannophilus* NRRL Y-2460 with enhanced rates of xylose fermentation. Nitrate utilization requires an active pentose phosphate pathway, which provides NADPH for nitrate reduction. The rationale was that strains capable of rapid growth on nitrate should possess higher levels of pentose phosphate pathway enzymes, consequently having faster xylose fermentation rates. One mutant (NO3–NO3-4) fermented xylose more rapidly, produced ethanol twice as fast and had a 32 % higher ethanol yield compared to the WT (0.25 g ethanol/g xylose consumed compared to 0.19 g/g) [29].

Some pentose-fermenting yeasts are known to produce and consume ethanol concurrently during xylose fermentation, and this has been suggested as one of the reasons for low ethanol yield from xylose [58]. In an effort to minimize such loss, UV-induced mutants of *P. tannophilus* NRRL Y-2460 with diminished ability to grow on ethanol while retaining the ability to utilize xylose were isolated [49]. Several ethanol-defective mutants that produced more ethanol than the WT were obtained. One mutant, designated *eth 2-1*, showed considerably improved ethanol production from xylose than the WT. During fermentation in 40 g/L xylose, the maximum ethanol concentration (10 g/L) and ethanol yield (0.29 g ethanol/g xylose consumed) produced by *eth 2-1* exceeded those (6.5 g/L and 0.16 g/g, respectively) produced by the WT [49]. In addition, *eth 2-1* produced less xylitol than the WT. Biochemical studies showed the *eth 2-1* mutant to be deficient in malate dehydrogenase, an enzyme in the tricarboxylic acid cycle [49].

To further improve their xylose-fermenting ability, mutant *eth 2-1* [49] was cross-mated with mutant NO3–NO3-4 [29] described above. This led to the isolation of

P. tannophilus recombinants that possessed the desired characteristics of both strains [14]. Four hybrid strains (P587-4A, P587-7B, P587-10D and P587-11B) produced as much ethanol as strain NO3–NO3-4. The incorporation of the *eth 2-1* mutation was confirmed by decreased ethanol consumption under aerobic conditions by the hybrids [14].

In other research to minimize aerobic ethanol loss, Hughes et al. [26] selected for UV-generated mutants of *S. stipitis* NRRL Y-7124 able to grow anaerobically on xylose plates for 5 months. Five strains (WT-1-11, WT-2-1, 14-2-6, 22-1-1 and 22-1-12) were isolated and tested for fermentation of mixed sugars (20 g/L xylose, 20 g/L glucose; supplemented with 10 g/L yeast extract and 20 g/L peptone) under microaerophilic conditions. Sugar utilization and ethanol yields obtained for the mutants (ranging from 0.23 to 0.24 g ethanol/g sugar consumed) were slightly lower than those for the WT (0.27 g/g). Mixed sugar fermentation under strictly anaerobic conditions was also tested using three of the mutants (WT-1-11, 14-2-6 and 22-1-12) and *S. cerevisiae* NRRL Y-2034. *S. cerevisiae* achieved a maximum ethanol concentration (9.5 g/L) and yield (0.24 g/g) within 12 h, but no further increase in ethanol concentration occurred after depletion of glucose. The mutants produced a similar concentration of ethanol as that by *S. cerevisiae* in 80–180 h after which ethanol continued to be produced from xylose. Mutant 22-1-12 produced the highest peak ethanol (13 g/L) and yield (0.33 g/g) in 490 h. Ethanol production by the *S. stipitis* WT was not evaluated because the strain could not grow under strictly anaerobic conditions [26]. The researchers succeeded in obtaining mutants of *S. stipitis* able to ferment glucose and xylose under anaerobic conditions, though at exceedingly low rate(s).

Recently, Li et al. [51] isolated a high ethanol yielding UV mutant of *S. shehatae* ATCC 22984 that also produces less xylitol. Two rounds of mutagenesis and screening on rich xylose-containing medium (3 g/L yeast extract, 3 g/L peptone, 3 g/L malt extract and 10–20 g/L xylose; YPM-X) supplemented with 0.05 g/L antimycin and 0.05 g/L of 2-3-5-triphenyltetrazolium chloride (TTC) were conducted. Antimycin A is a respiratory inhibitor and TTC is reduced under anaerobic conditions by dehydrogenases to the red compound formazan. The selected mutant Cs3512 showed better fermentation of xylose (initial concentration 121.3 g/L) and of sugar mixtures containing xylose (21.2 g/L) and glucose (52.9 g/L) in rich media, while producing more ethanol (44.4 and 31.0 g/L, respectively) compared to the WT (39 and 28.7 g/L, respectively) and less xylitol (10.2 and 1.81 g/L, respectively) compared to the WT (16.3 and 2.86 g/L, respectively). Fermentation was also assessed in rice straw hydrolysate (pH 6.5). After 72 h of fermentation CS3512 produced 21.3 g/L of ethanol, which is about 77 % of the theoretical yield. The

fermentation performance of the WT was not assessed; thus the extent of improvement in the fermentation performance of CS3512 in rice straw hydrolysate is not known.

Protoplast fusion

Protoplast fusion has been used to generate polyploidy fusants, some of which exhibit improved xylose fermentation, as summarized in Table 2. Maleszka et al. [57] tested the fermentation performance of several polyploidy strains of *P. tannophilus* NRRL Y-2460. They observed a correlation between increasing the chromosome number above the haploid level to increases in ethanol yield from xylose. A tetraploid strain (P45-#14) gave the highest ethanol yield from 20 g/L xylose (83.3 % of theoretical). A triploid strain (P56-#8) also produced high ethanol yield from 40 g/L xylose (70 % of theoretical). These values were 14.2 and 11 % higher than those of the haploid WT strain on 20 and 40 g/L xylose, respectively. Interestingly, an aneuploid strain P45-#9 gave a high rate of ethanol production from xylose (1.87 times that of the haploid WT), followed by the tetraploid strain P45-#14 (1.83 times the WT rate). Thus, using polyploids could be a useful approach to obtain strains that produce ethanol more

efficiently. It is not known, however, how stable the polyploids are, considering these are strains of *P. tannophilus* which prefer the haploid state. A similar trend was seen using diploid, triploid and tetraploid strains of *S. shehatae* CBS2779 [35] in that an increase in ploidy levels led to enhanced rate, but not the final yield of ethanol produced from xylose. The increase in fermentative ability was attributed to the increased gene pool. However, many of the presumptive diploid, triploid and tetraploid strains isolated were unstable and readily segregated into cells of lower ploidy level [35].

In contrast to the reported enhanced rates and yields of ethanol production from xylose with increasing chromosome number in *P. tannophilus*, polyploid strains of *S. stipitis* CSIR Y633 produced by protoplast fusion did not show improved ethanol productivity with increasing ploidy levels [20]. Of the four fusant strains showing an approximate two-fold increase in cellular DNA content, only strains P2-2 and P2-3 produced slightly higher yields (0.47 g ethanol/g xylose consumed) compared to the WT (0.45 g/g) in a slightly shorter fermentation period (44, 45 and 47 h, respectively). The reason for the variable fermentation performance of polyploids from different species of pentose-fermenting yeasts is not known.

Table 2 Ethanol production by selected hybridized native pentose-fermenting yeasts

Yeast/fusant	Cell DNA content	Volumetric ethanol productivity (g/L/h)	Yield (g ethanol/g sugar)	Max [ethanol] (g/L)	Time to maximum ethanol (h)	References
<i>P. tannophilus</i> NRRL Y-2460	<i>n</i>	0.46	0.35	ND	ND	[57]
P56-#3-3B	<2 <i>n</i>	0.42	0.40	ND	ND	
NRRL Y-2460-#2	2 <i>n</i>	0.49	0.41	ND	ND	
P56-#8	3 <i>n</i>	0.62	0.42	ND	ND	
P45-#14	4 <i>n</i>	0.84	0.42	ND	ND	
P45#9	<4	0.86	0.42	ND	ND	
<i>S. stipitis</i> CBS 5776	ND	0.54	0.39	38.8	72	[97]
<i>S. cerevisiae</i> / <i>S. stipitis</i> Fusant F5	ND	0.46	0.44	44	96	
<i>S. cerevisiae</i> NCIM-3090	2 <i>n</i>	0.96	0.436	69.1	60	[45]
<i>S. cerevisiae</i> / <i>S. shehatae</i> NCIM-3500						
RPR119	4 <i>n</i>	0.88	0.432	63.4	ND	
<i>S. cerevisiae</i> / <i>P. tannophilus</i> NCIM-3502						
RPR39	4 <i>n</i>	1.06	0.458	76.8	72	
RPR51	4 <i>n</i>	1.02	0.454	73.9	ND	
RPR87	4 <i>n</i>	0.95	0.440	68.4	ND	
<i>S. cerevisiae</i> / <i>S. stipitis</i> NCIM-3507						
RPR16	4 <i>n</i>	0.98	0.437	71	ND	
RPR48	4 <i>n</i>	0.96	0.443	69.6	ND	
<i>S. stipitis</i> CSIR Y633	24.35 fg/cell	0.90	0.45	21.10	47	[20]
P2-2	44.10 fg/cell	1.12	0.47	21.53	44	
P2-3	44.86 fg/cell	1.00	0.47	21.30	45	

ND no data available

Metabolic engineering

Classical strain improvement approaches may result in random changes throughout the genome, some of which may be undesirable. In contrast, metabolic engineering approaches allow precise modifications of specific pathways [31, 32]. The difficulty with the latter methods is that there is limited knowledge of what changes are required to produce a desired phenotypic effect.

Previous studies have shown that some respiratory deficient yeast strains can accumulate higher ethanol concentrations, although the mechanisms involved are not well understood [27, 75, 76]. It is known that in addition to the cytochrome-based respiratory system, *S. stipitis* possesses a secondary SHAM-sensitive respiratory system. To examine the contribution of the SHAM-sensitive system towards xylose fermentation without influence of the cytochrome system, Shi et al. [76] created a strain with a nonfunctional cytochrome pathway by disrupting the heme protein cytochrome *c*. Interestingly, this mutant produced more ethanol (36.8 g/L) than the WT (27.2 g/L) from xylose (80 g/L) in a rich peptone-containing medium. The authors postulated that the limited energy produced by the secondary SHAM respiratory system was sufficient for cell growth and ethanol production by the mutant [76].

Efforts to address cofactor imbalance during xylose metabolism

Yeasts metabolize xylose through an inducible pathway where it is first reduced by an NAD(P)H-dependent XR to xylitol. Xylitol is then oxidized by an NAD-dependent XDH to xylulose which can enter the pentose phosphate, Embden-Meyerhof or phosphoketolase pathways [91]. Some yeast XRs are strictly NADPH-dependent [36], while most function with either NADH or NADPH, with a preference for NADPH [48]. Two exceptions are the XRs from *Candida parapsilosis* [50] and *S. passalidarum* [23, 54] that prefer NADH to NADPH. During xylose metabolism, the different coenzymes required by XR and XDH cause a cofactor imbalance resulting in an accumulation of NADP and NADH from the first and second reactions, respectively. In the absence of a transhydrogenase, oxygen is required for the regeneration of NAD. It is postulated that the ability of some yeasts to ferment xylose anaerobically is due to the NADH-dependent XR activity [12]. Consistent with this postulate, a *P. tannophilus* mutant deficient in NADPH-dependent XR activity, but expressing normal levels of NADH-dependent XR activity, exhibited a xylose utilization rate similar to the WT under anoxic conditions [72]. More recently, the yeast *S. passalidarum*, which possesses NADH-preferring XR, was shown to ferment xylose efficiently under oxygen-limited conditions [23, 54].

One approach to address the cofactor imbalance in pentose-fermenting yeasts is to change the cofactor preferences of XR or XDH. Many studies have attempted to alter the cofactor preferences of these two enzymes in an effort to improve xylose flux and fermentation (Table 3). Addressing the cofactor imbalance in these enzymes can theoretically enable anaerobic xylose fermentation. Two approaches have been used to alter the cofactor dependence of XRs or XDHs. The first is to construct NADH-preferring XR (to be coupled with NAD-dependent XDH) and the second is to construct NADP-preferring XDH (to be coupled with NADPH-dependent XR). Successes have been reported with both approaches and selected examples are described below. The physiological effects of some of the XR and XDH variants have been tested mostly in *S. cerevisiae*, and some in *Candida utilis* and *Hansenula polymorpha* (Table 4).

Modifying XRs for increased NADH preference and reduced NADPH preference

Yeast XRs, members of the aldo-keto reductase (AKR) superfamily, typically contain a conserved IPKS (Ile-Pro-Lys-Ser) motif. The Lys residue within this motif interacts with the 2'-phosphate group of NADPH [48]. Removing this stabilizing interaction should reduce the affinity for NADPH without affecting the affinity for NADH, which lacks the 2'-phosphate group. This was observed in a K270 M variant of the *S. stipitis* XR which showed a 16.7-fold decrease in affinity towards NADPH, while the affinity towards NADH was unaffected [42]. Unexpectedly, the K270 M variant showed a 14-fold reduced affinity for xylose compared to the WT ($K_m = 43.5$ mM) [42]. When two copies of the *SsXR/K270M* gene were expressed in *S. cerevisiae* TMB3001 (the parental strain) along with the genes encoding the *S. stipitis* XDH and *S. cerevisiae* XK, the resultant TMB3271 strain exhibited considerably higher XR activities ($\text{XR}_{\text{NADH activity}} = 4.7$ U/mg and $\text{XR}_{\text{NADPH activity}} = 11.0$ U/mg) compared to TMB3001 ($\text{XR}_{\text{NADH activity}} = 0.1$ U/mg and $\text{XR}_{\text{NADPH activity}} = 0.3$ U/mg) in cell extracts. The ethanol yield from 50 g/L xylose after 70 h in a defined medium was 0.31 g ethanol/g of xylose [34].

In a subsequent study, Bengtsson et al. [8] prepared a K270R variant of the *SsXR* and tested the physiological effect of this mutation on xylose fermentation in *S. cerevisiae*. Two *S. cerevisiae* strains, with the native XR gene deleted, were constructed: TMB 3321 (carrying the native *SsXR*) and TMB 3323 (carrying the *SsXRm/K270R*). In anaerobic batch fermentation of glucose (20 g/L) and xylose (50 g/L) in defined media, TMB 3323 had a slightly higher xylose consumption rate (0.39 g/L/h) but a lower xylitol yield (0.09 g xylitol/g sugar consumed) compared

Table 3 Selected XR and XDH variants constructed to address cofactor imbalance

Yeast	Mutation	Purpose	Specific activity (U/mg)				K_m (μM)				K_{cat} (min^{-1})				Catalytic efficiency (k_{cat}/K_m) $\mu\text{M}^{-1} \text{min}^{-1}$				References
			NADP/		NADPH		NADH/		ADPH/		ADH/		NADPH/		NADH/		NADPH/		
			NAD/	NADP/	NADH/	NADPH	NADH/	NADPH/	NAD/	ADPH/	NADH/	ADH/	NADPH/	NADH/	NADPH/	NAD/	NADPH/		
XR mutants																			
<i>C. tenuis</i>																			
NA		Reference	ND	ND	38	3	660	780	17.4	260.0	[67]								
K274M		NADPH to NADH dependence	ND	ND	380	75	1,140	2,160	3.0	28.8									
K274R		NADPH to NADH dependence	ND	ND	21	5	600	480	28.6	96.0									
S275A		NADPH to NADH dependence	ND	ND	75	9	1,440	1,740	19.2	193.3									
N276D		NADPH to NADH dependence	ND	ND	26	17	840	2,220	32.3	130.6									
R280H		NADPH to NADH dependence	ND	ND	15	4	1,260	900	84.0	225.0									
RD (K274R/N276D)		NADPH to NADH dependence	ND	ND	41	128	720	1,800	17.6	14.1	[68]								
RH (K274R/R280H)		NADPH to NADH dependence	ND	ND	12	2	840	300	70.0	150.0									
RHD (K274R/N276D/R280H)		NADPH to NADH dependence	ND	ND	65	38	1,080	780	16.6	20.5									
<i>H. polymorpha</i>																			
NA		Reference	0.012	0.034	85.0	7.0	ND	ND	ND	ND	[15]								
K341R/N343D		NADPH to NADH dependence	0.014	0.01	112	152	ND	ND	ND	ND									
NA		Reference	10	14.2	30.6	16.2	449	622	14.7	38.4	[38]								
E223A		Strict NADPH dependence	0	8	ND	35.2	ND	349	ND	9.9									
E223D		Strict NADPH dependence	2	7	305	55.4	169	314	0.6	5.7									
S271A		Strict NADPH dependence	8.5	18	53.3	30.1	480	874	9.0	29.0									
AA (E223A/S271A)		Strict NADPH dependence	0	15	ND	17.5	ND	567	ND	32.4									
DA (E223D/S271A)		Strict NADPH dependence	1.3	18	391	20.4	156	733	0.4	35.9									
NA		Reference	6.61	20.3	21	25	ND	ND	ND	ND	[98]								
K21A		NADPH to NADH dependence	2.37	ND	32	ND	ND	ND	ND	ND									
NA		Reference	ND	ND	ND	11.1	ND	ND	ND	ND	[42]								
K270M		NADPH to NADH dependence	ND	ND	ND	185.3	ND	ND	ND	ND									
XDH mutants																			
<i>S. stipitis</i>																			
NA		Reference	1,110	1.2	381	170,000	1,050	110	2.8	6.5×10^{-4}	[89]								
I208R		NAD to NADP dependence	1,630	32.2	498	21,100	1,430	590	2.9	2.8×10^{-2}									
F209S		NAD to NADP dependence	1,330	25.3	848	28,900	1,220	590	1.4	2.0×10^{-2}									
N211R		NAD to NADP dependence	1,650	9.03	538	56,500	2,550	280	4.7	5.0×10^{-3}									
AR (D207A/I208R)		NAD to NADP dependence	640	223	568	11,300	620	1,680	1.1	0.1									
AI5 (D207A/F209S)		NAD to NADP dependence	210	491	162	9,960	200	480	1.2	4.8×10^{-2}									

Table 3 continued

Yeast	Mutation	Purpose	Specific activity (U/mg)		K_m (μM)		K_{cat} (min^{-1})		Catalytic efficiency (k_{cat}/K_m) $\mu\text{M}^{-1} \text{min}^{-1}$		References
			NAD/ NADH	NADP/ NADPH	NADH/ NAD	ADPH/ NADP	ADH/ NAD	NADPH/ NADP	NADH/ NAD	NADPH/ NADP	
	ARS (D207A/I208R/ F209S)	NAD to NADP dependence	320	2,350	1,300	897	240	2,500	0.2	2.8	
	ART (D207A/I208R/ F209T)	Check functionality of F209	280	1,900	265	638	310	1,970	1.2	3.1	
	ARSdR (D207A/ I208R/F209S/ N211R)	NAD to NADP dependence	259	2,570	17,300	1,380	1,430	3,840	0.1	2.8	
	C4/ARS (S96C/S99C/ Y102C/D207A/ I208R/F20)	NADP dependence and increase thermostability	160	8,010	23,500	1,180	1,770	12,600	0.1	10.7	
	C4/ARSdR (S96C/ S99C/Y102C/ D207A/I208R/ F209S/N211R)	NADP dependence and increase thermostability	200	7,210	7,600	1,040	790	11,000	0.1	10.6	
	NA	Reference	ND	ND	227	2,500	ND	ND	ND	ND	[60]
	D207G	NADP dependence	ND	ND	2,000	2,500	ND	ND	ND	ND	
	D207G/D210G	NADP dependence	ND	ND	2,000	2,500	ND	ND	ND	ND	
	D207G/I208S/F209R/ N211V/K212C	NADP dependence	ND	ND	2,500	2,500	ND	ND	ND	ND	

ND no data available

to TMB 3321 (0.26 g/L/h and 0.26 g/g, respectively). As a result, the peak ethanol produced for TMB 3323 was higher (25.3 g/L) compared to TMB 3321 (16.7 g/L) [8].

In addition to the IPKS motif, other amino acid residues are also involved in interaction with the cofactor in yeast XRs. Based on sequence information of the AKR family enzymes and the structure of the *Candida tenuis* XR (*CtXR*), several residues thought to interact with different functional groups of the cofactors were mutated in combination. Two double mutants [RH (K274R/R280H) and RD (K274R/N276D)] of the *C. tenuis* XR were constructed in an effort to increase affinity towards NADH [67, 68]. Of these, the RH variant ($K_{m, \text{NADH}} = 12 \mu\text{M}$, $K_{m, \text{NADPH}} = 2 \mu\text{M}$) can bind to both cofactors slightly better than the WT ($K_{m, \text{NADH}} = 38 \mu\text{M}$, $K_{m, \text{NADPH}} = 3 \mu\text{M}$), while the RD variant had similar NADH binding affinity ($K_{m, \text{NADH}} = 41 \mu\text{M}$), but poorer NADPH binding ($K_{m, \text{NADPH}} = 128 \mu\text{M}$) compared to the WT [67, 68].

In a follow-up study, Petschacher and Nidetzky [69] expressed the RD variant of the *C. tenuis* XR along with XDH from *Galactocandida mastotermitis* (*GmXDH*) and an extra copy of the endogenous *ScXK* gene in *S. cerevisiae*. The recombinant strain (BP10001) was tested for anaerobic fermentation of 20 g/L xylose in defined medium and compared to the parent strain (BP000) that expressed the WT *CtXR*, *GmXDH* and endogenous *ScXK*. BP10001 showed elevated NADH-dependent XR activity (0.26 U/mg) compared to BP000 (0.15 U/mg). The NADPH-dependent XR activity for BP10001 was 0.33 U/mg compared to 0.18 U/mg for BP000. BP10001 had a 42 % increase in ethanol yield (0.34 g ethanol/g xylose consumed), a 52 % decrease in xylitol yield (0.19 g/g) and a 57 % decrease in glycerol yield (0.02 g/g) during anaerobic fermentation compared to BP000 [69].

Based on results from Petschacher et al. [67] and Petschacher and Nidetzky [68], Dmytruk et al. [15] introduced two point mutations K341R and N343D in the *H. polymorpha* XR. The XR variant displayed reduced affinity towards NADPH ($K_m = 152 \mu\text{M}$) compared to the WT enzyme ($K_m = 7 \mu\text{M}$), while the affinity towards NADH was unaffected. The physiological effect of this XR variant was assessed in fermentation tests using the xylose-utilizing, but non-fermenting *H. polymorpha*. The results showed insignificant ethanol productivity [15].

Candida utilis is another xylose-utilizing, but non-fermenting, yeast which can be imparted with the ability to ferment xylose to ethanol by heterologous expression of XR, XDH and XK from other xylose-fermenting yeasts [80]. In this study, a NADH-preferring K275R/N277D XR variant and WT XDH from *S. shehatae* along with the WT XK from *S. stipitis* were expressed in *C. utilis* to yield the recombinant strain TMS174. The amino acid modifications in the XR were selected based on greater preference

towards NADH seen in the *SsXR* bearing the same corresponding mutations from a previous study [8]. The recombinant strain TMS174 had elevated NADH-dependent XR activity (0.75 U/mg) compared to a parental strain TMS170 expressing native *SsheXR*, *SsheXDH* and *SsXK* (0.41 U/mg) and lower NADPH-dependent XR activity (0.25 U/mg) compared to TMS170 (0.73 U/mg). In a peptone-based fermentation containing 50 g/L xylose, TMS174 produced 17.4 g/L ethanol in 20 h. This ethanol yield was much higher than the parental strain which produced 2.1 g/L ethanol in 30 h [82]. To further improve ethanol yield, the same group constructed a strain by transforming different ratios of the XR variant and WT XDH from *S. shehatae*, along with the WT XK from *S. stipitis* into *C. utilis*. Following screening, a recombinant strain (CI35) with an integrated copy number ratio of 4.9:7.2:7.4 in *SsheXR*(K275R/N277D):*SsheXDH*:*SsXK* was found to give the best ethanol yield. During fermentation of 80 g/L xylose in a peptone-containing medium, strain CI35 produced peak ethanol of 29.2 g/L with a yield of 0.402 g/g sugar within 70 h. This was a considerable improvement over the performance of TMS174 (peak ethanol of 21.3 g/L; ethanol yield of 0.283 g/g sugar; 142 h) [83]. This research suggested that adjusting the ratios of enzymes involved in xylose metabolism might further improve xylose fermentation.

Wang et al. [86] suggested that in the *S. stipitis* XR, Lys21 forms H-bond with NADPH. A K21A variant of the *S. stipitis* XR was constructed to disrupt this H-bond. The XR variant had no detectable NADPH-dependent activity, but exhibited similar NADH-dependent activity ($K_{m, \text{NADH}} = 32 \mu\text{M}$) compared to the WT ($K_{m, \text{NADH}} = 21 \mu\text{M}$) [98]. This study suggests that a single amino acid substitution may be able to completely eliminate NADPH-dependent activity in the *S. stipitis* XR. The reason for the complete loss of NADPH-dependent activity is not entirely clear, as many residues are involved collectively in cofactor interactions. It would be useful to verify and test such a substitution in other yeast XRs.

Modifying XDHs for increased NADP preference and reduced NAD preference

In addition to modifying the XR in pentose-fermenting yeasts, the cofactor preference of XDH can also be modified to address the cofactor imbalance. XDH shares a similar coenzyme-binding motif with the medium-chain dehydrogenase/reductase (MDR) superfamily of enzymes. For most NAD-dependent enzymes, amino acids from this conserved coenzyme-binding region are responsible for interacting with the 2'- and 3'-OH groups of NAD. Generally, changing the charged amino acid residues to uncharged residues can alter cofactor preference by reducing interactions with -OH groups of NAD [32, 41, 65].

Table 4 Enzyme activities of XR and XDH variants in *C. utilis*, *H. polymorpha* and *S. cerevisiae*

Recombi- nant hosts	Strain	Recombinant trait	Purpose	XR		XDH		XK		K_m (μ M)	References	
				Specific activity (U/mg)		Specific activity (U/mg)		Specific activity (U/mg)				
				NAD/ NADH	NADP/ NADPH	NAD/ NADH	NADP/ NADPH	NAD/ NADH	NADP/ NADPH			
Modified XR												
<i>H. poly- morpha</i>	XRn	<i>Hp</i> XR- <i>Hp</i> XDH- <i>Hp</i> XK	Reference	ND	ND	0.551	0.551	ND	ND	0.02	0.09	[15]
	XRm	<i>Hp</i> XR(K341R/N343D)- <i>Hp</i> XDH- <i>Hp</i> XK	NADPH to NADH dependence	ND	ND	0.504	0.504	ND	ND	0.01	0.01	[82]
<i>C. utilis</i>	TMS170	<i>Cshe</i> XR- <i>Cshe</i> XDH- <i>Ss</i> XK	Reference	0.41	0.73	2.26	2.26	0.04	0.32	ND	ND	[82]
	TMS174	<i>Cshe</i> XR (K275R/N277D)- <i>Cshe</i> XDH- <i>Ss</i> XK	NADH preferring	0.75	0.25	2.31	2.31	0.04	0.32	ND	ND	
	CIS18	<i>Cshe</i> XR (K275R/N277D)- <i>Cshe</i> XDH- <i>Ss</i> XK	NADH preferring	0.12	1.59	1.59	1.59	0.36	0.36	ND	ND	[83]
	CIS35	<i>Cshe</i> XR (K275R/N277D)- <i>Cshe</i> XDH- <i>Ss</i> XK	NADH preferring	0.085	2.14	2.14	2.14	0.57	0.57	ND	ND	
<i>S. cerevi- siae</i>	BP000	<i>Ct</i> XR- <i>Gm</i> XDH- <i>Ss</i> XK	Reference	0.15	0.18	1.1	1.1	1.7 \pm 0.2	1.7 \pm 0.2	ND	ND	[69]
	BP10001	<i>Ct</i> XR [RD (K274R/N276D)]- <i>Gm</i> XDH- <i>Ss</i> XK	NADPH to NADH dependence	0.26	0.33	1.3	1.3	2.4 \pm 0.1	2.4 \pm 0.1	ND	ND	
	SK-N1	<i>Ss</i> XR- <i>Ss</i> XDH(ARSDR)- <i>Ss</i> XK	Reference	0.66	1.41	0.97	0.97	14.11	14.11	ND	ND	[37]
	SK-N2	<i>Ss</i> XR [AA (E223A/S271A)]- <i>Ss</i> XDH(ARSDR)- <i>Ss</i> XK	Strict NADPH dependence	0.09	1.50	1.04	1.04	14.88	14.88	ND	ND	
	SK-N3	<i>Ss</i> XR [DA (E223D/S271A)]- <i>Ss</i> XDH(ARSDR)- <i>Ss</i> XK	Strict NADPH dependence	0.27	2.52	0.65	0.65	14.81	14.81	ND	ND	
	SK-N4	<i>Ss</i> XR [GA (E223G/S271A)]- <i>Ss</i> XDH(ARSDR)- <i>Ss</i> XK	Strict NADPH dependence	0.06	1.11	0.63	0.63	15.29	15.29	ND	ND	
	TMB3001	<i>Ss</i> XR- <i>Ss</i> XDH- <i>Ss</i> XK	Reference	0.13	0.25	ND	ND	ND	ND	ND	ND	[34]
	TMB3271	<i>Ss</i> XR (K270 M)- <i>Ss</i> XDH- <i>Ss</i> XK	Strict NADPH dependence	4.72	11.03	ND	ND	ND	ND	ND	ND	
	TMB 3321	<i>Ss</i> XR- <i>Ss</i> XDH- <i>Ss</i> XK	Reference	0.10	0.23	ND	ND	ND	ND	28.7	1.0	[8]
	TMB 3322	<i>Ss</i> XR (K270 M)- <i>Ss</i> XDH- <i>Ss</i> XK	NADPH to NADH dependence	0.04	0.08	ND	ND	ND	ND	ND	290	
TMB 3323	<i>Ss</i> XR (K270R)- <i>Ss</i> XDH- <i>Ss</i> XK	NADPH to NADH dependence	0.32	0.54	ND	ND	ND	ND	62.8	25.8		

Table 4 continued

Recombi- nant hosts	Strain	Recombinant trait	Purpose	XR		XDH		XK	K_m (μ M)	References
				Specific activity (U/mg)		Specific activity (U/mg)				
				NAD/ NADH	NADP/ NADPH	NAD/ NADH	NADP/ NADPH	NAD/ NADH	NADP/ NADPH	
Modified XDH										
<i>S. cerevi- siae</i>	Y-WT	S ₅ XR-S ₅ XDH	Reference	0.04		0.07	0.03	ND	ND	[88]
	Y-ARS	S ₅ XR-S ₅ XDH [ARS(D207A/I208R/ F209S)]	NADP dependence	5		0.05	0.26	ND	ND	
	Y-ARSdR	S ₅ XR-S ₅ XDH [ARSdR(D207A/I208R/ F209S/N211R)]	NADP dependence	0.05		0.05	0.43	ND	ND	
	Y-C4/ARS	S ₅ XR-S ₅ XDH [C4/ARS(S96C/S99C/ Y102C/D207A/I208R/F209S)]	NADP dependence	0.05		0.05	0.71	ND	ND	
	Y-C4/ ARSdR	S ₅ XR-S ₅ XDH [C4/ARSdR(S96C/ S99C/Y102C/D207A/I208R/F209S/ N211R)]	NADP dependence	0.04		0.05	0.35	ND	ND	

ND no data available

Several groups have altered XDH to reduce its affinity towards NAD. Metzger and Hollenberg [60] constructed single (D207G) and double (D207G/D210G) mutants of the *S. stipitis* XDH. The mutations were designed to eliminate H-bonding interaction with the 2'-OH group of NAD and introduce bonding with 2'-phosphate group of NADP. These mutants exhibited no change in affinity towards NADP, but an 11-fold decrease in NAD affinity ($K_{m, \text{NAD}} = 2.5 \text{ mM}$) compared to the WT enzyme ($K_{m, \text{NAD}} = 0.227 \text{ mM}$).

In an experiment to increase the affinity of *Ss*XDH towards NADP, Watanabe et al. [89] replaced Asp207 with a small and uncharged residue and changed an adjacent residue to Arg to form a binding site for NADP. Other combinations of mutations were made also to active site residues based on information from the strictly NADP-dependent *Bemisia argentifolii* sorbitol dehydrogenase. Of the ten mutants constructed, the triple mutant ARS (D207A/I208R/F209S) and quadruple mutant ARSdR (D207A/I208R/F209S/N211R) were the best in terms of improved NADP-dependent activity and affinity. The NAD-dependent specific activities for the ARS and ARSdR variants were 320 and 259 $\mu\text{mol cofactor min}^{-1}$ compared to 1,110 $\mu\text{mol cofactor min}^{-1}$ for the WT enzyme; their NADP-dependent specific activities were 2,350 and 2,570 $\mu\text{mol cofactor min}^{-1}$ compared to 1.2 $\mu\text{mol cofactor min}^{-1}$ for the WT enzyme. The K_m values for NAD were 1.3 and 17.3 mM for the ARS and ARSdR variants, respectively, and those for NADP were 0.90 and 1.38 mM, respectively. In comparison, the K_m values of the WT enzyme were 0.381 and 170 mM for NAD and NADP, respectively. Thus, both mutants showed increased activities and affinity towards NADP, accompanied by decreased activities and affinity towards NAD. To assess the physiological performance of the NADP-preferring XDH variants, a recombinant *S. cerevisiae* strain [*Ss*XR/*Ss*(ARSdR)XDH/*Sc*XK] was constructed [88]. Enzymatic activities were measured in cell extract after growth of the transformant in rich peptone media and compared against a *S. cerevisiae* strain (Y-WT) harboring *Ss*XR, *Ss*XDH and *Sc*XK. The recombinant strain D-XR/ARSdR/XK produced NAD-dependent XDH activity of 0.048 U/mg (two-fold higher than the WT) and NADP-dependent XDH activity of 0.426 U/mg (13-fold higher than the WT). During oxygen-limited fermentation at 30 °C in YNB medium containing glucose (5 g/L) and xylose (15 g/L) supplemented with essential amino acids, strain D-XR/ARSdR/XK consumed all the glucose within 15 h and 68 % of the xylose in 72 h. Under the same conditions, the WT also consumed glucose in the first 15 h, but only 58 % of xylose was consumed in 72 h. Strain D-XR/ARSdR/XK produced a peak ethanol concentration of 7.02 g/L and a yield of 0.46 g ethanol/g sugar consumed, both values being 1.4-fold higher than those of Y-WT [88].

Modifying both XR and XDH

Some researchers have attempted to address the cofactor imbalance during xylose fermentation by reducing the NADH preference of XRs to make them more NADPH-dependent and coupling this with a NADP-preferring XDH. Khattab et al. [38] constructed two double mutants [E223D/S271A (DA) and E223A/S271A (AA)] of the *S. stipitis* XR (*Ss*XR) with increased affinity towards NADPH. Both variants exhibited slightly increased activity with NADPH, but much reduced or no activity towards NADH. The DA variant showed similar affinity for NADPH ($K_m = 20.4 \mu\text{M}$) compared to the WT XR ($K_m = 16.2 \mu\text{M}$), but a 7.3-fold decrease in affinity for NADH ($K_m = 391 \mu\text{M}$) compared to the WT ($K_m = 30.6 \mu\text{M}$). The affinity of the AA variant towards NADPH ($K_m = 17.5 \mu\text{M}$) was also similar to the WT XR, but it had non-detectable activity towards NADH [38]. In a follow-up study, Khattab et al. [37] tested the physiological performance of the NADP-preferring XR [38] and XDH variants described earlier [89] in *S. cerevisiae*. The control *S. cerevisiae* strain, SK-N1, carried the *Ss*XR(WT)-*Ss*XDH(ARSdR)-*Sc*XK construct while the test strain SK-N2 carried the construct *Ss*XR(AA)-*Ss*XDH(ARSdR)-*Sc*XK. During mixed sugar (55 g/L xylose and 20 g/L glucose) semi-aerobic fermentation in peptone-rich media, strain SK-N2 produced a peak ethanol concentration of 30.1 g/L which was 20 % higher than that of the control strain SK-N1 (25.1 g/L peak ethanol) [37]. It remains to be seen how strain SK-N2 performs under anaerobic conditions in a chemically defined medium.

Testing with native NADH-preferring XRs

While most yeast XRs are either strictly NADPH-dependent or prefer NADPH over NADH, there are two known exceptions. They are the XR from *C. parapsilosis* ($K_{m, \text{NADH}} = 3.3 \mu\text{M}$; $K_{m, \text{NADPH}} = 36.5 \mu\text{M}$) [50] and the mixed XRs from *S. passalidarum* ($K_{m, \text{NADH}} = 17.3 \pm 1.8 \mu\text{M}$; $K_{m, \text{NADPH}} = 31.7 \pm 2.6 \mu\text{M}$) [23, 54] that exhibit a higher affinity towards NADH than NADPH. Since *S. passalidarum* contains two genes for XR, it was not known which gene product contributed more to the cofactor affinities measured in crude homogenates [54]. These unique NADH-preferring XRs have been used to alleviate cofactor imbalance during xylose metabolism when expressed heterologously. Lee et al. [50] expressed the *xyII* gene encoding the *C. parapsilosis* XR in *C. tropicalis* to yield strain BN-1 which was subjected to micro-aerobic fed-batch fermentation (glucose 45 g/L first and subsequently 300 g/L xylose supplied at rates of 5–40 mL/h). For the WT, ethanol production declined after complete consumption of glucose, and the peak ethanol produced was 15 g/L. Strain BN-1 produced the same amount of ethanol from glucose, but after depletion

of glucose, ethanol continued to be produced up to 21 g/L when all the xylose was consumed. The *C. tropicalis* strain used is a natural xylitol producer. Expression of CpXR in strain BN-1 had no apparent effect on xylitol productivity, and this strain produced a similar amount of xylitol (250 g/L) compared to WT. Given that a NADH-preferring XR should enable anaerobic xylose utilization, it would be interesting to test how a recombinant ethanol-producing strain with NADH-preferring XR might perform under anaerobic conditions. Equally interesting would be to examine the effect of expressing the NADH-preferring *C. parapsilosis* XRs in a xylose-fermenting yeast such as *S. stipitis* which does not produce much xylitol.

The gene encoding the NADH-preferring XR from *S. passalidarum* was cloned and expressed in *S. cerevisiae* [59]. However, xylose fermentation in this case was not tested.

The studies described in this section showed that the cofactor preference of either XR or XDH can be altered successfully. While some of the XR or XDH variants have been tested in vivo, more testing needs to be done preferably in pentose-fermenting yeasts to better assess the potential for improved xylose fermentation under anaerobic conditions. While changing the cofactor preference for XR and XDH is a prerequisite for addressing the cofactor imbalance, the work of Tamakawa et al. [83] suggests that the activity ratios of these two enzymes should be balanced also to further improve the overall xylose flux.

Concluding remarks

Lignocellulosic plant biomass represents one of the most attractive feedstocks for the production of fuel ethanol and chemicals. To improve the economic prospect of such a process, it is essential that potential biocatalysts be able to efficiently convert both the hexoses and pentoses found in industrially relevant lignocellulosic hydrolysates into the desired end product(s). Native xylose-fermenting yeasts possess the ability to ferment the major sugars in lignocellulose hydrolysates into ethanol, and they have been the subject of intense interest ever since their discovery in the early 1980s. However, these yeasts ferment xylose less efficiently and with considerably lower yield than glucose fermentation. Moreover, they suffer from glucose repression, low ethanol tolerance and are susceptible to various pretreatment-derived inhibitors in industrially relevant hydrolysates. These problems have limited their biotechnological potential and have prompted efforts to improve their properties over the past 30 years using a blend of classical and molecular methods. Although improved strains have been developed, ethanol yield and productivity from xylose have not yet matched the performance of the traditional

fermentative yeast *S. cerevisiae* on glucose. Furthermore, the performance of these yeasts in pretreated industrially relevant hydrolysates remains poor.

Genetic improvement of microorganisms, including native pentose-fermenting yeasts, will continue to be essential for the development of competent strain(s) for lignocellulosic biomass conversion. With the advent of high-throughput screening and various “omics” approaches, strain development is poised for significant advancement. In particular, the new approaches, applied to pentose-fermenting yeasts, can potentially uncover new vistas on some of the biological factors and metabolic constraints that limit efficient fermentation of pentose sugars in lignocellulosic hydrolysates. These new insights hold considerable promise towards the development of robust strains that can ferment all the sugars in lignocellulosic hydrolysates to ethanol.

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