An atlas of rational genetic engineering strategies for improved xylose metabolism in *Saccharomyces cerevisiae*

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Xylose is the second most abundant carbohydrate in nature, mostly present in lignocellulosic material, and representing an appealing feedstock for molecule manufacturing through biotechnological routes. However, *Saccharomyces cerevisiae* – microbial cell widely used in the industry for ethanol production – is unable to assimilate this sugar. Hence, in a world with raising environmental awareness, the efficient fermentation of pentoses is a crucial bottleneck to producing biofuels from renewable biomass resources. In this context, advances in the genetic mapping of *S. cerevisiae* have contributed to noteworthy progress in the understanding of xylose metabolism in yeast, as well as the identification of gene targets that enables the development of tailored strains for cellulosic ethanol production. Accordingly, this review focuses on the main strategies addressed to understand the network of genes that are directly or indirectly related to this phenotype, and their respective contributions to xylose consumption in *S. cerevisiae*, especially for ethanol production. Altogether, the information in this work summarizes the recent most relevant results from scientific investigations that endowed *S. cerevisiae* with an outstanding performance for commercial ethanol production from xylose.

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ABSTRACT

Xylose is the second most abundant carbohydrate in nature, mostly present in lignocellulosic material, and representing an appealing feedstock for molecule manufacturing through biotechnological routes. However, *Saccharomyces cerevisiae* – a microbial cell widely used industrially for ethanol production – is unable to assimilate this sugar. Hence, in a world with raising environmental awareness, the efficient fermentation of pentoses is a crucial bottleneck to producing biofuels from renewable biomass resources. In this context, advances in the genetic mapping of *S. cerevisiae* have contributed to noteworthy progress in the understanding of xylose metabolism in yeast, as well as the identification of gene targets that enable the development of tailored strains for cellulosic ethanol production. Accordingly, this review focuses on the main strategies employed to understand the network of genes that are directly or indirectly related to this phenotype, and their respective contributions to xylose consumption in *S. cerevisiae*, especially for ethanol production. Altogether, the information in this work summarizes the most recent and relevant results from scientific investigations that endowed *S. cerevisiae* with an outstanding capability for commercial ethanol production from xylose.

INTRODUCTION

Modern globalization has been historically structured on the use of energy provided by fossil sources; however, due to the high industrialization rates and a continual increase in world energy demand, a climate emergency and fuel crisis seem to be the main issues that humanity will face in the future if alternative and renewable energy sources are not fully explored. Within this context, biorefineries – which use lignocellulosic biomass feedstock to produce a variety of molecules, such as ethanol –are important vectors for the generation of sustainable biofuels, envisioning the total or partial replacement of fossil-based fuels. Ethanol is the most used biofuel in the world; in 2021, 103.4 million liters were commercialized worldwide (“Renewable Fuels Association (2022) Annual fuel ethanol production,” 2022), and, due to the growing concern for environmental preservation in recent years, more investments are being made in order to develop new technologies that economically warrant the renewable energy industry.

For first generation (1G) ethanol production, the yeast *Saccharomyces cerevisiae* is used for the fermentation of hexoses, such as glucose, available from the main product of designated crops. From this process, lignocellulosic residues are generated, comprising a material with neglected sugar content that can be further exploited to produce second-generation (2G) ethanol (Santos et al., 2015). The concentration of such carbohydrates varies depending on the crop used; however, one of the main monomers found in the hemicellulose fraction is xylose (Chandel et al., 2021). The use of a microorganism with the ability to consume both hexoses (glucose) and pentoses (xylose) would be ideal to explore all the energy potential from such biomass. However, natural xylose-fermenting yeasts, such as *Scheffersomyces stipitis* and *Spathaspora passalidarum,* do not have the same fermentative capacity, tolerance to high levels of ethanol, or robustness shown by *S. cerevisiae* (Balat, 2011; Cadete et al., 2016). Thus, one of the main challenges for the efficient production of 2G ethanol is the insertion of xylose assimilation pathways into *S. cerevisiae*, since it does not consume this pentose naturally (Wang & Schneider, 1980).

There are two known pathways for xylose metabolism, each from distinct evolutionary origins and harboring different biochemical properties, that can be used for heterologous expression in *S. cerevisiae*: the oxidoreductive (XR-XDH) (Ho, Chen & Brainard, 1998) and the xylose isomerase (XI) (Brat, Boles & Wiedemann, 2009) pathways (**Figure 1**). However, the insertion of these pathways alone does not guarantee an optimal xylose fermentation, as several works have already stated (Sarthy et al., 1987; Amore, Wilhelm & Hollenberg, 1989; Moes, Pretorius & Zyl, 1996; Gárdonyi & Hahn-Hägerdal, 2003). In this context, efforts have been made to endow superior xylose-fermenting ability in *S. cerevisiae*, aiming at the efficient expression of the genetic architecture related to this phenotype. Different genetic mapping strategies allow the understanding of the gene network underlying such traits, and genetic engineering enables the development of yeast strains that can be used in the industry by increasing the productivity of lignocellulosic ethanol.

In this context, this review focuses on the main approaches used to unravel the genomic structure that is related to this phenotype and the contribution of such genotypes

to enhancing xylose metabolism and ethanol production in *S. cerevisiae*. The main biotechnological strategies used will be addressed, such as deletion of genes that hinder xylose metabolism; overexpression of genes that increase xylose metabolism; gene expression fine tuning for optimized pentose metabolism; improvement in cofactor availability in the oxidoreductive pathway; and expression of optimized transporters to increase xylose assimilation. An overview of the xylose assimilation pathways and the main challenges in the heterologous expression of each of them will also be discussed. Although most results presented here were developed on a lab scale bearing in mind commercial applications, minimum industrial settings have been directly applied in published research, and therefore will not be the focal point in this work.

SURVEY METHODOLOGY

Articles were identified in Google Scholar and in National Center for Biotechnology Information (NCBI) databases for a broader analysis of the results, using the terms: *xylose consumption genes*, *new xylose isomerases, new xylose reductase genes, xylose metabolism, xylose reductase, xylitol dehydrogenase, genetic modifications, metabolic engineering, cofactor preference, targets for deletion, targets for overexpression, xylose transporters, Saccharomyces cerevisiae, Pichia stipitis* and *ethanol production*. After a thorough reading of the articles, those referring to xylose consumption and ethanol production by *Saccharomyces cerevisiae* were selected. In the process of choosing relevant works, the most recent ones with outstanding results and other pertinent studies in the area were chosen. We tracked articles referring to the first mention in the literature of a certain genetic target and we identified papers that investigated such genes. The analysis allowed us to identify the experimental articles with the highest citations, which were also sorted out. A total of 160 papers were chosen.

ENABLING XYLOSE METABOLISM IN *S. cerevisiae*

The oxidoreductive pathway

The oxidoreductive pathway for xylose consumption is found in fermenting yeast and fungal species, and presents two steps catalyzed by the enzymes xylose reductase (XR) and xylitol dehydrogenase (XDH) (Jeffries, 1983; Ho, Chen & Brainard, 1998). In the

first reaction, XR reduces xylose to xylitol, preferentially using NADPH over NADH as a cofactor, in most cases. The xylitol produced is oxidized to xylulose by the enzyme XDH, which naturally uses only NAD^+ as a cofactor. The difference in the cofactor preference between the XR and XDH enzymes causes an imbalance that generates xylitol accumulation and consequently reduces ethanol production (Jeffries, 2006). Some studies have also indicated that, although the oxidoreductive pathway can present an imbalance of enzyme cofactor preference, and consequently the accumulation and production of xylitol, it is thermodynamically more favorable than the isomerase pathway, performing faster xylose assimilation in genetically modified strains (Karhumaa et al., 2007b; Bettiga, Hahn-Hägerdal & Gorwa-Grauslund, 2008; Li et al., 2016). **Table 1** summarizes the main work that has expressed this pathway in *S. cerevisiae*.

The heterologous genes *XYL1* and *XYL2* from *S. stipitis*, which encode the enzymes XR and XDH, respectively, have already been used in the construction of a recombinant *S. cerevisiae* strain expressing the xylose oxidoreductive assimilation pathway (Kötter et al., 1990; Walfridsson et al., 1995; Ho, Chen & Brainard, 1998; Eliasson et al., 2000). Therefore, Kötter *et al.* (1990) (Kötter et al., 1990) obtained a theoretical ethanol yield of 10%; later, in a study developed by Ho *et al.* (1998) (Ho, Chen & Brainard, 1998), a theoretical ethanol yield of 88% was obtained. Two genes encoding XR (*XYL1.1* and *XYL1.2*) have also been identified in the genome of *Spathaspora passalidarum*. The XR encoded by the *XYL1.2* gene was cloned and expressed in *S. cerevisiae*, and the obtained strain presented a higher activity of XR with NADH. Such a feature allowed an efficient consumption of xylose resulting in an ethanol yield of 78%, generating an improvement in ethanol production, as well as a lower xylitol production (Cadete et al., 2016).

Other studies have also used metabolic engineering strategies to reduce the accumulation of xylitol in the xylose metabolization process. Such accumulation is attributed to the excessive increase of NADH unable to be recycled by respiration under oxygen-limited conditions. This condition is related to the difference in cofactor preferences of XR (greater affinity for NADPH than for NADH - converting xylose into xylitol) and XDH (preferably NAD^+ - converting xylitol into xylulose) and expression levels

of these heterologous enzymes in *S. cerevisiae* (Karhumaa et al., 2007a; Hou et al., 2007; Matsushika & Sawayama, 2008). By changing cofactor affinity through specific amino acids changes in the binding domain of such enzymes, it was possible to reduce unfavorable xylitol excretion during xylose fermentation and optimize the XR-XDH relationship (Watanabe, Kodaki & Makino, 2005; Watanabe et al., 2007a; Hou et al., 2007; Matsushika et al., 2008). In addition, other strategies have relied on modifications to the redox environment in yeast metabolism in order to yield higher cofactor availability and consequently higher rates of ethanol production (Verho et al., 2003; Bro et al., 2006). Strategies for cofactor manipulation will be further discussed in this paper.

The isomerase pathway

The isomerase pathway is mainly found in bacteria, and represents a single-step conversion of xylose to xylulose, catalyzed by the metal ion-dependent enzyme xylose isomerase (XI) (Sarthy et al., 1987; Zhou et al., 2012; Kwak & Jin, 2017). This reaction does not require cofactors, and thus does not exhibit the redox imbalance observed in the oxidoreductive pathway when expressed in *S. cerevisiae*, also eliminating xylitol overproduction (Sarthy et al., 1987; Kwak & Jin, 2017). On the other hand, xylose isomerase genes (*XYLA*) are often not functional in *S. cerevisiae.* Previous efforts to express XI from *Escherichia coli* (Sarthy et al., 1987), *Bacillus subtilis* (Amore, Wilhelm & Hollenberg, 1989), *Actinoplanes missouriensis* (Amore, Wilhelm & Hollenberg, 1989), *Thermoanaerobacterium thermosulfurigenes* (Moes, Pretorius & Zyl, 1996) and *Streptomyces rubiginosus* (Gárdonyi & Hahn-Hägerdal, 2003) in *S. cerevisiae* have not been successful. Nevertheless, the possibility to functionally express heterologous XI (usually with codon-optimization) associated with metabolic flux optimizations and evolutionary engineering in *S. cerevisiae* has enabled the projection of strains with the ability to ferment xylose at an industrial scale (Demeke et al., 2013). The main XI expressed in *S. cerevisiae* with confirmed activity in xylose consumption are described in **Table 1**.

The first functional XI was identified in the bacterium *Thermus thermophilus* back in 1996: when episomally expressed in *S. cerevisiae* using the yeast *PGK1* promoter and terminator, the recombinant strain was able to produce ethanol equivalent to 24.6% of the theoretical yield. Low ethanol productivity in this strain is linked to the thermophilic enzyme's low activity at 30 ºC (Walfridsson et al., 1996). Other functional bacterial *XYLA* were found in *Clostridium phytofermentans* (Brat, Boles & Wiedemann, 2009), *Bacteroides stercoris* (Ha et al., 2011) and *Prevotella ruminicola* (Hector et al., 2013). For the first two sequences, strong and constitutive promoters were used for the construction of an expression cassette: *HXT7* and *TEF1*, respectively. *C. phytofermentans*'s XI optimized for expression in *S. cerevisiae* allowed an ethanol production corresponding to 84% of the maximum theoretical yield (Brat, Boles & Wiedemann, 2009), while the *B. stercoris* allele without codon optimization enabled 66% of this value in the engineered strain (Ha et al., 2011). For *P.* ruminicola, *XYLA* was codon-optimized and expressed in a high copy plasmid, allowing 68.6% of the ethanol theoretical yield. After an adaptive laboratory evolution (ALE) using serial batch cultures of the transformed strain in a medium with xylose, an increase of 14% in ethanol yield was observed (Hector et al., 2013).

Anaerobic fungi are also source microorganisms for functional XI in *S. cerevisiae*. Initially, an effective *XYLA* was identified in *Piromyces sp. E2* (ATCC 76762) – isolated from the feces of an Indian elephant (Kuyper et al., 2003). For the expression of this XI, Kuyper *et al.* (2003) (Kuyper et al., 2003) used a vector carrying the *XYLA* gene from this fungus without codon optimization induced by the constitutive promoter *TPI1* (Kuyper et al., 2003, 2004). Subsequently, the yeast underwent genetic modifications combined with ALE in xylose to optimize sugar consumption (Kuyper et al., 2004, 2005); the evolved strain showed a high rate of ethanol production from xylose (84.5% of the theoretical yield), without xylitol accumulation. Zhou *et al.*(2012) (Zhou et al., 2012) engineered a *S. cerevisiae* strain overexpressing a codon-optimized *Piromyces sp. E2*'s XI under *TDH3* promoter, via a multiple copy plasmid. Further genetic modifications and evolutionary engineering rendered ethanol production equivalent to 81% of the theoretical yield. The authors suggested that the high expression level of *XYLA* was caused by multiple copy

genomic integration in the evolved recombinant strain, which contributed to more efficient xylose assimilation.

Orpinomyces sp. – another anaerobic fungus, isolated from bovine rumen fluid – was found to also express the xylose isomerase enzyme, bearing 94% of amino acid sequence identity to *Piromyces*' *XYLA*, and similar specific enzyme activity (Madhavan et al., 2009). In the construction of a recombinant *S. cerevisiae* strain expressing XI from *Orpinomyces sp.*, the gene was cloned in a high copy vector under the control of *GAPDH* promoter for episomal expression, and other genetic modifications were introduced to enhance xylose conversion. In this work, Madhavan *et al.* (2009) reported an ethanol yield equivalent to 78% of the maximum theoretical (Madhavan et al., 2009).

Metagenomics approaches have boosted the identification of new enzymes with xylose isomerase activity. Parachin & Gorwa-Grauslund (2011) (Parachin & Gorwa-Grauslund, 2011), reported two new genes encoding functional XI in *S. cerevisiae* that were isolated from a soil metagenomic library (Parachin & Gorwa-Grauslund, 2011). Degenerated primers and a protein sequence similarity-based screening were applied to identify such genetic information. However, despite being functionally expressed, the aerobic growth rate in xylose of recombinant *S. cerevisiae* strains carrying multiple copy plasmids expressing such *XYLA* under the same promoter (*TEF1*) was much lower compared to the growth of yeast expressing the *Piromyces*'s XI under the same conditions (Parachin & Gorwa-Grauslund, 2011). In this study, yeasts containing the new XI were able to grow at a rate of 0.02 $hour^{-1}$ in xylose, while the strain expressing *Piromyces's XYLA* grew at 0.07 $hour^{-1}$. Ethanol production was not assessed for the newly identified XI.

Two other functional XIs were prospected in a metagenomics library from bovine rumen contents (Hou et al., 2016) and from a cDNA library of the protists residing in the hindgut of the termite *Reticulitermes speratus* (Katahira et al., 2017). The XI obtained from *R. speratus* was evaluated through episomal expression in *S. cerevisiae* using the *GAP1* promoter, resulting in an ethanol theoretical yield of 77% (Katahira et al., 2017). More recently, metagenomic data derived from resident microorganisms in the gut of the woody beetle *Odontotaenius disjunctus* revealed a new functional XI (Silva et al., 2021).

For that, a methodology that associates direct metagenome reconstruction combined with *in vitro* gene optimization and synthesis was used. The expression of this new XI in *S. cerevisiae* resulted in a 50% faster aerobic growth compared to XI from *Piromyces sp.* on xylose media, while no ethanol production was observed (Silva et al., 2021).

Endogenous xylose metabolism in *S. cerevisiae*

While wild-type *S. cerevisiae* strains are not recognized for their xylose-fermenting ability – which foments research on the expression of heterologous pathways –, the ability to grow in small concentrations of pentose has been reported (Toivari et al., 2004; Attfield & Bell, 2006; Wenger, Schwartz & Sherlock, 2010), suggesting the presence of a complete native xylose metabolization pathway. Studies suggest that this phenomenon is possible due to the presence of endogenous genes encoding putative enzymes of the oxidoreductive pathway (XR and XDH). In the genome of laboratory strain S288c, several genes encoding putative enzymes of the xylose pathway or showing a correlative contribution to the xylose consumption phenotype were identified. Genes *GRE3*, *GCY1*, *YPR1*, *YDL124W* and *YJR096W* encode putative XR enzymes while *XYL2*, *SOR1* and *SOR2* express enzymes with activity homologous to XDH (Wenger, Schwartz & Sherlock, 2010). However, the specific activity of these enzymes is much lower in *S. cerevisiae* when compared to other xylose-fermenting yeasts (Batt et al., 1986). Therefore, many efforts have been made to understand the role of these enzymes in *S. cerevisiae* or to identify other genes linked to xylose consumption.

In this sense, Träff-Bjerre *et al.* (2004) (Traäff-Bjerre et al., 2004) performed both deletion and overexpression of the endogenous *GRE3* to evaluate its contribution to xylose consumption. The gene knockout led to decreased xylitol formation by 49%, while its overexpression under a *PGK1* promoter and terminator generated an increment in ethanol production by 116% in a recombinant strain expressing XDH from S*. stipitis* (Traäff-Bjerre et al., 2004). Toivari *et al.* (2004) (Toivari et al., 2004) enabled growth of *S. cerevisiae* in xylose in a medium containing glucose in the presence of oxygen by overexpressing endogenous *GRE3* and *XYL2*. However, the mutant strains presented slower growth and greater xylitol accumulation compared to a recombinant *S. cerevisiae* strain expressing XR and XDH from *S. stipitis*.

Wenger, Schwartz & Sherlock (2010) (Wenger, Schwartz & Sherlock, 2010) described the endogenous gene *XDH1*, encoding a putative XDH, as responsible for enabling xylose consumption in an *S. cerevisiae* wine strain. Through mass segregation analysis (BSA) and yeast tiling arrays using the xylose-consuming wine strain and a non-consuming laboratory strain (S288C), the authors identified that the positive phenotype for xylose consumption is linked to a unique, dominant locus, located in a subtelomeric region on the right-end of chromosome XV - not present in the genome of S288C. Confirmation of the contribution of the *XDH1* gene to the xylose consumption phenotype was accomplished by its deletion in the *S. cerevisiae* wine strain, after which the phenotype was nullified. The gene was also cloned and expressed episomally in the laboratory yeast S288C, endowing this yeast with xylose consumption ability, the phenotype being lost upon plasmid removal.

Furthermore, in the same study (Wenger, Schwartz & Sherlock, 2010), other genes and their correlation with the positive xylose metabolism phenotype in *S. cerevisiae* were analyzed by performing different knockout combinations. By deleting each XDH (*sor1*Δ*, sor2*Δ*, xyl2*Δ) separately, an improvement in xylose consumption was observed, while deleting the three genes at the same time resulted in an enhanced phenotype, suggesting that endogenous XDHs may limit the xylose-consuming ability of non-recombinant *S. cerevisiae*. Wenger, Schwartz & Sherlock (2010) also confirmed the contribution of two putative XR genes (*GRE3* and *YPR1*) to growth on xylose: the two genes were the only ones that contributed significantly to the ability to utilize xylose in the used background, *GRE3* being the one that most affected the phenotype. A mutant presenting a *gre3*Δ *ypr1*Δ genotype had its xylose consumption phenotype almost completely removed, indicating that the presence of these genes allows the metabolism of this pentose in *S. cerevisiae*.

REWIRING METABOLIC PATHWAYS

Even though genes encoding enzymes of the oxidoreductive pathway, as well as an active xylitol dehydrogenase, are found in *S. cerevisiae*, this yeast does not efficiently consume xylose. Therefore, overexpression of endogenous genes and/or insertion of heterologous enzymes (*i.e.* XR, XDH or XI) are common strategies applied to use this microbe as a platform for xylose assimilation, as previously discussed. Nevertheless,

277 additional modifications are required to optimize the metabolic flux of this sugar,
278 especially for commercial purposes. In this manner, different approaches have been used
279 to optimize the metabolic pathways (**Figure 1**) to increase cellulosic ethanol yield. The
280 main strategies are: (I) knock-out of genes that hinder the flux of xylose metabolism; (II)
281 overexpression of genes that can increase xylose metabolism; (III) use of specific
282 promoters and terminators for gene expression fine-tuning; (IV) improvement of cofactor
283 availability for the XR-XDH pathway; and (V) expression of transporters with higher affinity
284 towards xylose to increase sugar assimilation. These different strategies will be discussed
285 in detail in the following sections.

286 **Deletions**

287 Several approaches have been explored to identify genes related to xylose
288 consumption in *S. cerevisiae*, such as (I) reverse engineering (Bengtsson et al., 2008;
289 Verhoeven et al., 2017; Tran Nguyen Hoang et al., 2018); (II) genome-wide synthetic
290 genetic array (SGA) screens (Usher et al., 2011); (III) transposon mutagenesis (Ni,
291 Laplaza & Jeffries, 2007); and (IV) omics approaches for comparative analysis of mutated
292 or evolved xylose-fermenting strains and their respective parents (Kim et al., 2013; Sato
293 et al., 2016; dos Santos et al., 2016; Palermo et al., 2021). Within these studies, several
294 genes were suggested as knockout targets that either directly contribute to xylose
295 metabolism or that, associated with other deletion/superexpression gene targets,
296 enhance this phenotype in *S. cerevisiae*. Following, we present the main gene deletions
297 described in the literature that are beneficial for ethanol production from xylose in *S.*
298 *cerevisiae*. This information is summarized in **Table 2**.

299 The *GRE3* gene encodes a non-specific aldose reductase that functions as an
300 NADPH-dependent XR and consequently contributes to the formation of xylitol (Traäff-
301 Bjerre et al., 2004). Therefore, the deletion of this gene is paramount for improved ethanol
302 yield from xylose when using the isomerase pathway (Träff et al., 2001; Lönn et al., 2003;
303 Karhumaa, Hahn-Hägerdal & Gorwa-Grauslund, 2005). For instance, compared to a
304 *GRE3* strain, *gre3*Δ were able to reduce xylitol production by 50%, boosting ethanol yield.
305 In addition, most of the XIs expressed in *S. cerevisiae* strains were sensitive to the
306 presence of xylitol – indicating that this metabolite can act as a potent inhibitor of these

enzymes (Yamanaka, 1969; Lönn et al., 2003). However, it is noteworthy that *GRE3* knockout was also related to reduction in biomass production, suggesting that the fine-tuning of gene expression would be preferable to deletion (Traäff-Bjerre et al., 2004).

In order to identify new gene targets for improved xylose metabolism, Bengtsson *et al.* (2008) (Bengtsson et al., 2008) compared strains with varying degrees of this phenotype using a genome-wide transcription analysis, and further reverse genetic engineering. Strains with null *NFG1* (Negative regulator of the Filamentous Growth MAPK pathway); *MNI1* (methyltransferase), or *RPA49* (RNA polymerase) showed growth on xylose 173%, 62% and 90% times better, respectively, compared to the reference strains. These results suggested that *NFG1*, *MNI1,* and *RPA49* could be involved in central carbon metabolism and xylose utilization in *S. cerevisiae* (Bengtsson et al., 2008). Later, the positive effect of *NFG1* deletion on xylose fermentation was also confirmed in another study (Parachin et al., 2010). The phenotype for *nfg1*Δ cells included different assimilation of other sugars and increased xylitol production, suggesting that *NFG1* is related to sugar transport or signaling. In general, strains with an *NFG1* knockout genotype were able to consume 27.1% of available xylose, while the reference yeast consumed only 18% of the sugar (Parachin et al., 2010).

Meanwhile, Usher *et al.* (2011) (Usher et al., 2011) used a genome-wide synthetic genetic array (SGA) screening methodology to identify deletion mutants and evaluate the contribution of non-essential genes to xylose utilization in a recombinant *S. cerevisiae* (strain expressing *xylA* from *Piromyces sp. E2)*. Four deletion mutants were identified: *BUD21* (component of the small ribosomal subunit, SSU, processome), *ALP1* (arginine transporter), *ISC1* (inositol phospho-sphingolipid phospholipase C) and *RPL20B* (component of the large ribosomal subunit, 60S). In order to evaluate the influence of each gene on the phenotype, they were individually knocked out, confirming that all contribute positively to xylose consumption. Xylose consumption improved 27.6%, 15.5%, 22.4%, and 12.1%, respectively, for each deleted gene compared to the reference strain. The authors suggested that such genes are xylose metabolic suppressors and could be regulators at the transcriptional or translational level. *BUD21* is of particular interest, as its exclusion allows certain aspects of the stress response not to be activated,

making it possible to circumvent some of the initial stress conditions that occur during xylose fermentation in *S. cerevisiae*. Despite the improvement obtained in the consumption of xylose, such genes may have a synergistic relationship with the response to stress, indicating the need for further study to assess the impact on the robustness of yeasts used in the fermentation of lignocellulosic hydrolysates.

The mutations G249V and G1161A in *PMR1*, a gene responsible for encoding a Golgi Ca_2^+/Mg_2^+ ATPase, was identified by Verhoeven *et al*. (2017) (Verhoeven et al., 2017) in an *S. cerevisiae* strain expressing *Piromyces* E2' XI and other additional modifications (overexpression of *XKS1*, *RKI1*, *RPE1*, *TKL1*, *TKL2*, *TAL1, NQM1 and gre3Δ*) after ALE in an anaerobic culture (Verhoeven et al., 2017). In parallel, Tran Nguyen Hoang *et al.* (2018) (Tran Nguyen Hoang et al., 2018) reported another mutation (G681A) in *PMR1,* found in an evolved recombinant *S. cerevisiae* strain harboring a mutant xylose isomerase gene from *Piromyces sp*. (*XYLA**3) and other additional metabolic alterations (overexpression of *XKS1* and *TAL1* and, *gre3*Δ and *pho13*Δ). To understand whether both mutations were accompanied by loss of function, a *PMR1* deletion was performed by both authors, which allowed phenotype improvement regarding ethanol production from xylose. The authors suggested that negative regulation of *PMR1* expression leads to the accumulation of manganese ions inside the cell, which would be available for ion-dependent enzymes such as xylose isomerases (Tran Nguyen Hoang et al., 2018). In general, there was an improvement of 114.8% in consumed xylose and 195.9% in ethanol production, in relation to the strain containing the original gene.

In that same study, Tran Nguyen Hoang *et al.* (2018) (Tran Nguyen Hoang et al., 2018) also described a mutation (Q237*) in *ASC1*, encoding the beta subunit of the G protein and the guanine dissociation inhibitor for Gpa2p. This gene is known as a negative regulator of several metabolic and signal transduction pathways. When the mutated gene was deleted, as well as when the mutation was expressed in knockout strains, a significant improvement of 59.6% in xylose consumption and 104.4% in ethanol production was observed. Therefore, the authors concluded that the Q237* mutation in *ASC1* is correlated with the loss of function of that gene (Tran Nguyen Hoang et al., 2018). *ASC1* had already been associated with cell growth in oxygen-limited conditions and,

when deleted, with the overexpression of genes correlated with xylose metabolism, being a negative regulator of metabolic pathways and of signal transduction. In particular, *ASC1* acts on the repression of the transcription factor (TF) *GCN4*, responsible for the regulation of genes linked to xylose metabolism in strains that have a high fermentative profile (Tran Nguyen Hoang et al., 2018).

Sato *et al.* (2016) (Sato et al., 2016) described null genotypes – including epistatic interactions – that alter the metabolic regulation of *S. cerevisiae* and enhance anaerobic xylose consumption, when analyzing the genome sequencing of a strain (genotype: *TAL1* overexpression, *XYL3* from *S. stipitis* and *XYLA* from *C. phytofermentans*) that underwent ALE in lignocellulosic hydrolyzate (Parreiras et al., 2014). Mutations G136A, A844del, G8782T, C412T were observed in the genes *GRE3*, *HOG1* (a component of MAP kinase, MAPK, signaling), *IRA2* (a GTPase activating protein) and *ISU1* (a scaffolding protein involved in mitochondrial iron-sulfur cluster assembly), respectively. For validation, the four genes underwent deletion in a combined manner in different yeast strains, resulting in faster anaerobic xylose consumption regardless of the background. It was suggested that the loss of function of *ISU1* is indispensable for the anaerobic fermentation of xylose, as well as epistatic interactions with mutations in *IRA2*, *HOG1* and *GRE3*. Null *ISU1* mutants were able to consume about 75% more xylose under aerobic conditions, and combined with the deletions of *IRA2*, *HOG1* and *GRE3* resulted in increased xylose-specific consumption and ethanol production rates comparable to the phenotype of the evolved strain. The authors claim that these deleterious genetic alterations influenced different metabolic pathways, such as xylose catabolism, the pentose phosphate pathway (PPP), the glycolytic pathway and aerobic respiration. Such changes together resulted in increased aerobic consumption and anaerobic fermentation of xylose (Sato et al., 2016).

In the work conducted by dos Santos *et al.* (2016) (dos Santos et al., 2016), two mutations that improve xylose consumption were identified. In this study, an industrial *S. cerevisiae* strain was modified (*gre3*Δ, *Orpinomyces sp* XI and overexpression of *XKS1*, *RKI1*, *RPE1*, *TKL1*, *TKL2* and *TAL1*) for pentose metabolism, associated with ALE in xylose. Genetic mapping of the evolved strains revealed that *ISU1* harbors mutations in some isolates, whereas *SSK2* (a member of the MAPKKK signaling pathway) presented

polymorphisms in others. For phenotype validation, the authors created knockout strains for both genes, resulting in an improvement in xylose metabolization compared to the wild-type strain. *SSK2* deletion in the non-evolved parental strain resulted in an 80% increase in fermentation efficiency. The deletion of *ISU1* allowed a reduction in fermentation time, from 80 to 40 hours in the evolved lineage (representing an upgrade in fermentation efficiency of 86% for *isu1*Δ cells). This is similar to that found in the strains where the mutations were identified, indicating that these genetic changes led to gene inactivation (dos Santos et al., 2016).

One gene that has been extensively investigated is *PHO13*, encoding a phosphatase with specific dephosphorylating activity on two side-products of central carbohydrate metabolism. This gene has been the deletion target of different inquiries, in order to understand its influence on xylose metabolism in *S. cerevisiae* (Van Vleet, Jeffries & Olsson, 2008; Fujitomi et al., 2012; Li et al., 2014; Lee, Jellison & Alper, 2014; Bamba, Hasunuma & Kondo, 2016). Loss of function mutations in *PHO13* in recombinant strains have been identified in different studies, regardless of the initial xylose uptake pathway (Ni, Laplaza & Jeffries, 2007; Kim et al., 2013). Insertional transposon mutagenesis was used to identify that *PHO13* deletion increased transcripts for *TAL1*, indicating that overexpression of transcripts for downstream enzymes of the xylose pathway may improve the assimilation of this sugar (Ni, Laplaza & Jeffries, 2007). Through a metabolomic analysis, it was revealed that the positive regulation of *TAL1*, which prevents sedoheptulose accumulation, is the critical point for improved xylose metabolism in *pho13*Δ mutant *S. cerevisiae* strains (Xu et al., 2016). In the same fashion, it was suggested that knockout of *PHO13* results in transcriptional and metabolic changes favorable for xylose fermentation, in particular, transcriptional activation of PPP genes and NADPH-producing enzymes as part of an oxidative stress response mediated by Stb5 activation (Kim et al., 2015). In another study, it was indicated that loss of *PHO13* function, acquired after ALE in xylose, plays an important role in improving xylose consumption rates and ethanol yields (Kim et al., 2013).

On the other hand, a recent study by Shin *et al.* (2021) (Shin et al., 2021) reported that the phenotype for xylose metabolization had not been affected by *PHO13*

deactivation in *S. cerevisiae* strains. Through resequencing of the *pho13*Δ strains, a loss-of-function Glu204* mutation in *GCR2* was identified and indicated as responsible for the improvement in the xylose consumption phenotype. *GCR2* is a global TF correlated with glucose metabolism. Deletion of *GCR2* led to positive regulation of the PPP genes, as well as negative regulation of glycolytic genes, with the changes being more significant under xylose conditions than in the presence of glucose. Although no synergistic effect was found between the deletion of *PHO13* and *GCR2* in improving xylose fermentation, *GCR2* was indicated as a knockout target to enhance ethanol production.

Many other genes were also identified and suggested as deletion targets to improve xylose fermentation in *S. cerevisiae*, directly or indirectly, alone or associated with other modifications (*i.e.,* combined deletions or overexpression). Deletion of *ALD6* – encoding a NADPH-dependent aldehyde dehydrogenase, part of the central carbon metabolism – yielded an improvement in xylose fermentation efficiency (Kim et al., 2013). In 2018, Wei and colleagues (Wei et al., 2018) suggested that deletion of the TF *THI2* (activator of thiamine biosynthetic genes) enables the co-fermentation of glucose and xylose by increasing ribosome synthesis, generating an increase in the specific utilization rate of xylose by 26.8%. Palermo *et al.* (2021), meanwhile, analyzed the effect of metal homeostasis under xylose fermentation and suggested two new deletion targets for metabolic engineering of *S. cerevisiae*: *CCC1* (vacuolar Fe_2^+/Mn_2^+ transporter) and *BSD2* (protein involved in heavy metal ion homeostasis) (Palermo et al., 2021). More recently, interruption of transcription factors of xylose catabolism (*ZNF1, SIP4, ADR1, TUP1* and *HAP4*) were evaluated in a xylose-fermenting *S. cerevisiae* strain; however only deletion of *hap4*Δ (global regulator of respiratory gene expression) generated an increase in ethanol production from xylose compared to the parental strain (Dzanaeva et al., 2021).

Overexpression

Among the rational genetic modifications performed in yeast to improve xylose fermentation, gene overexpression has become a prominent strategy, because it directly contributes to accelerating the uptake of this pentose and increases xylose metabolism flux in genetically modified *S. cerevisiae* strains (Nevoigt, 2008). Therefore, in this section

456 the main overexpression targets in *S. cerevisiae* aiming at an optimized xylose
457 consumption will be highlighted. The summarized information can be found in **Table 3**.

458 In both xylose assimilation pathways, xylulose is converted to xylulose-5-
459 phosphate by an endogenous xylulokinase (XK) encoded by *XKS1*, driving carbon flux to
460 the PPP (**Figure 1**). Because XK presents a low activity level, it may limit xylose
461 fermentation, making *XKS1* a major target for overexpression. Many studies have
462 evaluated endogenous and exogenous overexpression of *XKS1*, suggesting that this
463 genetic modification is responsible for a remarkable improvement in xylose fermentation
464 (Deng & Ho, 1990; Ho, Chen & Brainard, 1998; Kim et al., 2013). The first recombinant
465 *S. cerevisiae* strain overexpressing *XKS1* with *Pichia stipitis* XR and XDH resulted in
466 increased ethanol production and reduced xylitol excretion (Ho, Chen & Brainard, 1998).
467 Meanwhile, some studies have indicated that high XK activity can be harmful to xylose
468 metabolism, inhibiting or reducing xylose consumption – even in cases where
469 improvement in ethanol yield was achieved (Johansson et al., 2001; Jin et al., 2003).
470 Rodriguez-Peña *et al.* (1998) (Rodriguez-Peña et al., 1998) and Johansson *et al.* (2001)
471 (Johansson et al., 2001) even associated a deleterious effect in strains with uncontrolled
472 overexpression of *XKS1*. Despite the controversies, there is agreement on the need for
473 modulated XK expression to obtain efficient xylose fermentation in *S. Cerevisiae*,
474 especially considering the intrinsic characteristics of the host strain (Jin et al., 2003).

475 Other overexpression targets that have been described to improve xylose
476 metabolism are the genes responsible for encoding enzymes of the non-oxidative PPP in
477 *S. cerevisiae* – *RPE1*, *RKI1*, *TAL1* and *TKL1* (**Figure 1**). Studies analyzing the
478 bottlenecks for xylose consumption argue that the expression levels of such enzymes are
479 preeminent in the xylose utilization rate in fermenting yeast (Matsushika et al., 2012;
480 Bamba, Hasunuma & Kondo, 2016). Overexpression of *TAL1* alone is correlated to an
481 improved xylose assimilation rate in *S. cerevisiae* (Ni, Laplaza & Jeffries, 2007). In other
482 studies, all genes participating in the non-oxidative PPP, including *XKS1*, were
483 overexpressed simultaneously, which resulted in improved ethanol production in
484 recombinant *S. cerevisiae* (Kuyper et al., 2005; Karhumaa et al., 2007a). In a
485 characterization study of the enzymes of the non-oxidative PPP, the effects of the *TAL1*

and *TKL1* genes were analyzed by deletion. It was suggested that the enzymatic activities of the transaldolase and transketolase encoded by these genes, respectively, are limiting for efficient xylose utilization. Furthermore, their overexpression is responsible for an increased flux from the PPP to the glycolytic pathway in recombinant *S. cerevisiae* (Matsushika et al., 2012).

A molecular analysis of a recombinant xylose-consuming *S. cerevisiae* strain (expressing the XR-XDH pathway) and its mutant obtained through chemical mutagenesis with ethyl methanesulfonate to improve the ability to metabolize xylose, allowed the identification of different gene targets for overexpression (Wahlbom et al., 2003). Besides those already described, *XKS1*, *TAL1* and *TKL1*, *SOL3* (6-phosphogluconolactonase) and *GND1* (6-phosphogluconate dehydrogenase) were also evaluated. The authors reported an improvement in growth rate and xylose uptake when *SOL3* and *GND1* are overexpressed, attributing it to the altered expression of one or more transcriptional regulators that influence these genes (Wahlbom et al., 2003). Bengtsson *et al.* (2008) (Bengtsson et al., 2008), in a similar study, performed a transcriptome analysis of *S. cerevisiae* strains (expressing *S. stipitis* XR-XDH) with increased xylose consumption phenotype, in order to identify new targets for metabolic engineering. The authors validated the overexpression of *SOL3* and *TAL1*, in multicopy plasmids, resulting in 19% and 24% in growth improvement, respectively.

In a recent study focusing on the simultaneous co-fermentation of glucose and xylose, the *RPE1* gene (responsible for catalyzing a reaction in the non-oxidative part of the PPP) was selected as a target for overexpression, leading to an increased xylose consumption and ethanol production rate. Such results were attributed to a possible metabolic rearrangement of the xylose pathway, due to a cofactor-neutral xylose isomerase mutant present in this recombinant yeast (Hoang Nguyen Tran et al., 2020).

Wei *et al.* (2018) (Wei et al., 2018) reported a beneficial effect on xylose metabolism through overexpression of *NRM1* (Transcriptional co-repressor of MBF-regulated gene expression) and/or *YHP1* (Homeobox transcriptional repressor) in *S. cerevisiae*. *NRM1* overexpression increased the specific xylose use rate by 30.0%, while *YHP1* increased the volumetric xylose use rate by 5.6%. The authors suggested that

these modifications induced an acceleration in the yeast cell cycles, however, it is still unclear how such factors are affecting xylose metabolism.

In another study by Wei *et al.* (2019) (Wei et al., 2019), in order to assess how TF *Thi2* affects xylose metabolism, a transcriptomic analysis between a *thi2*Δ strain and its parent in the glucose depletion and glucose-xylose co-fermentation steps was performed, allowing the identification of new genes correlated with xylose metabolism. Through overexpression, it was indicated that the TFs *STT4* (Phosphatidylinositol-4-kinase), *RGI2* (respiratory growth induced, function unknown) and *TFC3* (subunit of RNA polymerase III transcription initiation factor complex) allowed an increase of specific xylose uptake rate in the strains by 36.9%, 29.7%, and 42.8%, respectively, in the glucose depletion step, allowing glucose-xylose co-fermentation in *S. cerevisiae*.

In addition to the endogenous overexpression targets, elevated expression of the initial genes of the xylose assimilation pathways – *XYL1*, *XYL2*, and/or *XYLA* – , also contributes to more efficient metabolism of this sugar, and has been described as paramount for efficient xylose fermentation in *S. cerevisiae* (Kim et al., 2012; Zhou et al., 2012). Overexpression of sugar transporters is another interesting approach to improve the performance of xylose-consuming strains, enabling even more effective xylose transport in recombinant *S. cerevisiae* strains (Tanino et al., 2012). The topic of sugar transporters will be further discussed in this review.

Regulation Fine Tuning

Metabolic engineering approaches often require fine-tuning gene expression to optimize the activity of certain enzymes and regulatory proteins. The modulation of gene transcription levels is of prime importance to balance metabolic fluxes and increase the production of metabolites of interest (Xu et al., 2021). In *S. cerevisiae*, promoters are responsible for controlling gene expression programs in response to a variety of circumstances (Maya et al., 2008). However, genes participating in the same metabolic pathway might present different levels of expression, as well as different catabolic intermediates. In this context, a widely used strategy in optimizing metabolic flux is

assembling expression cassettes using promoters with different activity levels to fine-tune the metabolic pathway in question (Hubmann, Thevelein & Nevoigt, 2014).

Endogenous *S. cerevisiae* promoters differ by strength magnitudes (*i.e.,* rates of transcription initiation) and regulation, and are classified as constitutive or inducible. Constitutive promoters have stable expression rates and are constantly active in the cell (Da Silva & Srikrishnan, 2012; Tang et al., 2020). Inducible promoters, on the other hand, are activated in response to different stimuli (Li et al., 2006; Weinhandl et al., 2014). Promoters can be obtained by characterizing gene expression or with targeted modifications in the sequence of already known promoters. The latter can be performed by either increasing transcriptional activation with the addition of upstream activating sequences (UASs) or by altering sequences using random mutations, deletions, nucleosome removal or intron insertion. Error-prone PCR (Feng & Marchisio, 2021), for instance, is a strategy used to obtain promoters with different activity degrees, due to mutations added to it during amplification (Alper et al., 2005).

Jeppsson *et al.* (2002) (Jeppsson et al., 2002), in an attempt to equilibrate cofactor imbalance in a strain expressing the XR-XDH pathway, indicated that interruption of the *ZWF1* gene (Glucose-6-phosphate dehydrogenase, G6PDH) increased ethanol and decreased xylitol yields (more on cofactor engineering will be reviewed in the next section). However, the strain showed a significant reduction in the xylose consumption rate, indicating the need for fine adjustment of this gene expression. In a new investigation, Jeppsson *et al.* (2003) (Jeppsson et al., 2003) used a synthetic promoter library to study the influence of different levels of G6PDH activity on xylose fermentation. Downregulation of *ZWF1* using the synthetic promoter *YRP13* resulted in the lowest G6PDH activity, which enabled a xylose consumption rate five times faster than the *zwf1*Δ strain, accompanied by higher ethanol and lower xylitol yields.

In order to optimize xylose fermentation, Lu & Jeffries (2007) (Lu & Jeffries, 2007) developed a multiple-gene-promoter shuffling (MGPS) technique to identify optimal expression levels of genes of interest induced by different promoters in *S. cerevisiae*. In this study, the genes *TAL1*, *TKL1* and *PYK1* (pyruvate kinase) were overexpressed in a recombinant xylose-fermenting *S. cerevisiae*, expressing the oxidoreductive pathway,

under control of the weak *GND2* and *HXK2* promoters. Such promoters were selected to avoid systemic saturation and obtain a balanced flux of metabolites. The authors describe that the optimum scenario for metabolic engineering was the combination of the *GND2* promoter overexpressing *TAL1* and the *HXK2* promoter overexpressing *TKL1* and *PYK1.* Overall, the study states that balanced overexpression of such genes optimized ethanol production from xylose in *S. cerevisiae.*

Zha *et al.* (2012) (Zha et al., 2012) reported a combined strategy of chassis selection and fine-tuning in the expression of *XYL1* and mutated *XYL2* to obtain efficient *S. cerevisiae* strains for xylose fermentation. In the engineered strain, promoters *PGK1*, *ADH1* and truncated *ADH1* were used to modulate the expression levels of *XYL1*, while *XYL2* was overexpressed under promoter *PGK1* in a multicopy plasmid. The authors concluded that only the strongest promoter (*PGK1*) was able to improve XR activity, increasing by a factor of 1.7 the ability to assimilate and metabolize xylose. Overexpression of *XYL2* allowed for 21% lower xylitol production and 35–40% higher ethanol production.

More recently, Hector & Mertens (2017) (Hector & Mertens, 2017) suggested the need for regulation at the transcriptional and post-translational levels in *S. cerevisiae* strains engineered to metabolize xylose. In this study, xylose-regulated synthetic hybrid promoters were developed from the *Ashbya gossypii TEF* constitutive promoter, a mutation being inserted in the second TATA sequence present at position -63. Furthermore, to control transcription in *S. cerevisiae*, the xylose-dependent DNA repressor obtained from *Caulobacter crescentus* was also used. The *TEF-xylO2-1* promoter in the presence of xylose showed activity comparable to other known *S. cerevisiae* promoters, with an increase in activity of up to 25 times in the presence of xylose, revealing an important strategy for further metabolic engineering.

Nambu-Nishida *et al.* (2017) (Nambu-Nishida et al., 2018), evaluated 30 *S. cerevisiae* promoters showing different expression levels, selected through microarray data, in a xylose-metabolizing yeast strain (expressing the XR-XDH pathway and *XKS1* overexpression). In the study, it was suggested that *TDH3*, *FBA1* and *TDH1* promoters showed high expression in aerobic culture and moderate expression in microaerobic

fermentation, while promoters *SED1*, *HXT7*, *PDC1*, *TEF1*, *TPI1* and *PGK1* had medium-high expression in the same conditions.

The activities of different native promoters and the synthetic hybrid promoter *p3xC-TEF1* (based on the *TEF1* promoter core with insertion of three tandem upstream activation sequences of the *CLB2* promoter) were evaluated in a *S. cerevisiae* strain expressing the XR-XDH pathway through a fluorescent reporter protein in the presence of xylose (Xiong et al., 2018). The *TDH3* promoter showed the highest activity in the presence of xylose as the only carbon source, followed by the synthetic hybrid (*p3xC-tef1*) and the *TEF1* promoter. In another study, the *TDH3* promoter and the *CYC1* terminator were used to control expression of the PPP genes (*TAL1*, *TKL1*, *RKI1* and *RPE1*), and as a result, improved xylose metabolism was obtained (Kobayashi et al., 2018).

Studies have also focused on the role of terminators and how their transcription regulation interferes with an enhanced metabolic flux (Curran et al., 2013; Matsuyama, 2019). There are two events related to gene expression termination: I) transcriptional termination and II) post-transcriptional regulation. In the first event, the terminator is responsible for determining where the mRNA will be cleaved for the addition of the poly(A) tail; while the second determines the stability, translation efficiency and position of the mRNA(Guo & Sherman, 1996; Tuller, Ruppin & Kupiec, 2009; Mischo & Proudfoot, 2013; Yamanishi et al., 2013; Curran et al., 2015). Curran *et al.* (2013) (Curran et al., 2013) analyzed 30 gene terminators and obtained *S. cerevisiae* strains presenting better growth on xylose when the *XYLA* gene was combined with the *TDH3* promoter and *CPS1* terminator. The authors indicated that there was an increase in transcriptional levels and, consequently, an increase in the xylose growth rate. This scenario suggested that a strong promoter combined with a weak terminator can increase metabolic flux, with terminators also being responsible for modulating protein expression. Finally, it was suggested that a high-expression terminator combined with a weaker promoter could achieve results equivalent to those obtained by strong promoters paired with standard terminators.

COFACTORS

As previously stated, although xylose fermentation by *S. cerevisiae* expressing heterologous XR and XDH is possible, the resulting strains present low ethanol productivity while accumulating a considerable amount of xylitol. Xylitol production is mainly attributed to the cofactor imbalance between the conversion steps. XR normally has a higher affinity for NADPH than for NADH, whereas XDH uses only NAD^+, which leads to an excessive accumulation of NADH and a shortage of NAD^+ necessary for the XDH reaction, as shown in **Figure 1**. Xylitol is formed to re-oxidize the NADH surplus resulting from those reactions, impairing ethanol yield. In this context, a plethora of strategies have been outlined to minimize xylitol formation and to improve cofactor availability, thus increasing ethanol yield. Protein engineering or mutagenesis techniques have been applied for that purpose, where coenzyme preference is altered - either of XR, by changing its preference from NADPH to NADH, or of XDH, from NAD^+ to $NADP^+$. Also, metabolic engineering has proven to be an alternative to disturb cofactor availability in yeast to favor ethanol formation from xylose. For that, strategies usually rely on tuning the activity of endogenous cofactor-dependent enzymes, or the introduction of exogenous cofactor-producing ones, to favor the redox environment for the oxidoreductive xylose pathway in *S. cerevisiae*. However, it is relevant to note that xylitol is an important by-product in xylose metabolism, and its formation could be advantageous in some scenarios.

For instance, Jeppsson *et al.* (2006) (Jeppsson et al., 2006) expressed a mutant XR (K270M) from *S. stipitis* (Kostrzynska, Sopher & Lee, 1998) with increased affinity for NADH in a recombinant *S. cerevisiae* harboring *S. stipitis* XDH and overexpression of endogenous *XKS1*: higher ethanol yield and reduced xylitol formation were obtained. Other mutant *S. stipitis* XR (K270R (Watanabe et al., 2007a), K270G (Watanabe et al., 2007a), R276H (Watanabe et al., 2007c), N272D (Watanabe et al., 2007a), K270R/N272D (Watanabe et al., 2007c), N272D/P275Q (Runquist, Hahn-Hägerdal & Bettiga, 2010), and K270R/R276H (Watanabe et al., 2007c)) with NADH preference were obtained and expressed in *S. cerevisiae*, showing the same results on ethanol and xylitol yields. Mutant XR has also been obtained from *Candida tenuis* (Kavanagh et al., 2002,

2003; Petschacher et al., 2005; Leitgeb et al., 2005; Petschacher & Nidetzky, 2005), which could be used to balance the redox environment in xylose consuming *S. cerevisiae.* Petschacher & Nidetzky (2008) (Petschacher & Nidetzky, 2008) expressed a double mutant XR (K274R-N276D CtXR) in a recombinant *S. cerevisiae* and the resulting strain showed an increase in NADH utilization, which improved ethanol production and decreased xylitol secretion.

Endeavors in changing the specificity of the coenzyme of XDH, from NAD^+ to $NADP^+$, have also been described. Watanabe, Kodaki & Makino (2005) (Watanabe, Kodaki & Makino, 2005) obtained the quadruple mutant ARSdR (D207A/I208R/F209S/N211R) that showed 4500-fold higher catalytic efficiency (kcat/Km) with $NADP^+$ than wild-type *S. stipitis* XDH. The ARSdR mutant was expressed in recombinant *S. cerevisiae* strains under the control of a strong constitutive promoter (*PGK1*), together with *S. stipitis* XR, achieving increased ethanol yield (41%) and lower xylitol production (86%) (Watanabe et al., 2007b).

Because the shortage of NADPH results in less xylitol formation, reducing flux through the oxidative PPP – where this cofactor is normally generated, and wasteful CO_2 is produced – is another approach for improved ethanol yield from xylose. The deletion of the *ZWF1* gene, which encodes G6PDH and is responsible for the regeneration of this cofactor, and the deletion of *GND1* - one of the isogenes of 6-phosphogluconate dehydrogenase - were evaluated in strains containing the *XYL1/XYL2* genes. Deletion of the *GND1* gene resulted in an improvement in ethanol yield by 24% and a decrease in xylitol production. A *ZWF1* null genotype, however, showed a significant increase in ethanol yield and a reduction in xylitol production. Although blocking the NADPH-producing PPP lowered xylitol formation, xylose fermentation was also reduced because XR reaction was mediated only by NADH (Jeppsson et al., 2002). To overcome this issue, overexpression of the fungal *GDP1* gene encoding an $NADP^+$ dependent *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) – not linked to CO_2 production - along with *ZWF1* deletion resulted in an improvement of ethanol yield of approximately 50% (Verho et al., 2003).

Metabolic engineering of ammonium assimilation has also been suggested as an alternative procedure to modulate redox metabolism and favor xylose fermentation in *S. cerevisiae*. Ammonium, often used as a nitrogen source in industrial fermentations with *S. cerevisiae*, is converted to glutamate by reaction with 2-oxoglutarate, catalyzed by an endogenous NADPH-dependent glutamate dehydrogenase encoded by *GDH1* (Moye et al., 1985). Therefore, deletion of *GDH1* and overexpression of *GDH2*, a NADH-dependent glutamate dehydrogenase, is expected to shift ammonia assimilation from being NADPH to NADH dependent, alleviating NADPH shortage for XR. Bearing that in mind, Roca, Nielsen & Olsson (2003) (Roca, Nielsen & Olsson, 2003) performed such metabolic engineering in a strain expressing *XYL1*, *XYL2* and overexpression of endogenous *XKS1*. The final strain presented an increased ethanol yield and a 44% reduction of xylitol excretion. The same group tested the overexpression of the GS-GOAT complex (*GLT1* and *GLN1*, participating in ammonium assimilation using NADH as cofactor) in the *gdh1* deleted strain, which also resulted in an increased ethanol yield. Later on, comparative metabolic flux analysis revealed that, in a mutant strain with deleted *GDH1* and overexpression of *GDH2*, a shift in the specific xylose reductase activity towards the use of NADH as a cofactor could explain the improved ethanol yield due to its benefit to cofactor imbalance (Grotkjær et al., 2005).

Meanwhile, the expression of heterologous enzymes that prevent cofactor imbalance has also been tested. Through a genome-scale metabolic modeling approach, Bro *et. al* (2006) (Bro et al., 2006) found that the heterologous expression of *GAPN* gene, encoding a *Streptococcus mutants* non-phosphorylating $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase, reduced the formation of xylitol by 33%, while increasing the production of ethanol by 24%. While glyceraldehyde-3-phosphate is converted to 3-phosphoglycerate in wild-type *S. cerevisiae* in a two-step NAD^+-dependent reaction, *S. mutants GAPN* allows the same reaction avoiding competition for the cofactor used by XDH. Overexpression of *NOXE*, encoding a water-forming NADH oxidase from *Lactococcus lactis*, in a XR/XDH *S. cerevisiae* led to decreased xylitol formation and increased ethanol production during xylose fermentation (Zhang, Liu & Ding, 2012). *NOXE* provides an extra route for the oxidization of NADH resulting from the XDH reaction, thus rebalancing the cofactor environment to favor xylitol reduction.

TRANSPORTERS

In the production of any metabolite from a cell, the first step is substrate assimilation. The efficient incorporation of substrate molecules into yeast cells is suggested as a critical factor for obtaining efficient biofactories (Hara et al., 2017). In yeast, sugar entry is facilitated by a family of sugar porters known as the major facilitator superfamily (MFS), present in different species in all kingdoms of nature (Marger & Saier, 1993; Rédei, 2008; Quistgaard et al., 2016). This transport of sugars occurs mostly by facilitated diffusion, being a passive transport mechanism of substances across the cell membrane (Jeffries, 1983). In *S. cerevisiae*, at least 18 genes encoding hexose transporters (*HXT1-17*) and galactose permease (*GAL2*) are found endogenously, however only *HXT1-7* and *GAL2* show active expression, with *HXT*8-*HXT17* being inactive (not transcribed) or expressed at very low levels (Özcan & Johnston, 1999; Hamacher et al., 2002; Sedlak & Ho, 2004).

Although native hexose transporters also have the ability to import pentoses, xylose-specific transporters are not found in *S. cerevisiae*, in such a way that their transport occurs inefficiently due to the lower affinity of such a transport system for this sugar (Sedlak & Ho, 2004; Subtil & Boles, 2012). Moreover, transporters that perform xylose assimilation suffer a strong inhibition in the presence of other sugars, especially glucose, and this repression is considered a limiting factor in mixed sugar fermentation, as most recombinant *S. cerevisiae* yeasts are unable to initiate xylose assimilation before glucose depletion (Bertilsson, Andersson & Lidén, 2008; Subtil & Boles, 2012; Farwick et al., 2014), causing a negative impact on the fermentation time of lignocellulosic biomass. Therefore, many studies have sought to design xylose-specific and/or glucose-insensitive transporters in order to obtain more efficient *S. cerevisiae* platforms for mixed sugar fermentation. In this context, cell platforms for sugar transporter characterization are obtained by deletion of native hexose transporters *(hxt* null), avoiding the interference of their effect on sugar transport analyses (Wieczorke et al., 1999; Boles & Oreb, 2018; Wijsman et al., 2019). *hxt* null strains do not exhibit the ability to grow on glucose as the sole carbon source, and have been used to characterize various endogenous hexose transporters, as well as those from different origins (Wieczorke et al., 1999; Hamacher et

al., 2002; Young et al., 2011; Hara et al., 2017; Boles & Oreb, 2018). Information on the heterologous expression of transporters for xylose uptake and modification of endogenous transporters to improve affinity for this sugar in *S. cerevisiae* are summarized in **Table 4**.

One compelling approach to optimizing xylose uptake in *S. cerevisiae* is the insertion of heterologous specific xylose transporters from bacteria, fungi, yeasts or plants (Nijland & Driessen, 2020). In this context, many studies have focused on identifying those proteins in different species, especially from other xylose-fermenting yeasts such as *Candida intermedia*, *S. stipitis* and *Meyerozyma guilliermondii.* However, although expression of such transporters allowed growth on xylose in *S. cerevisiae*, glucose inhibition was still observed (Leandro, Gonçalves & Spencer-Martins, 2006; Runquist et al., 2009; Tanino et al., 2012). In parallel to heterologous expression, mutagenesis in native sugar transporters allowed enhanced xylose transport kinetics in the presence of glucose, as well as the co-utilization of both sugars (Li, Schmitz & Alper, 2016).

The high-capacity, low-affinity glucose/xylose facilitated diffusion transporter (*GXF1*), obtained from *C. intermedia*, showed a threefold improvement in transport kinetics and xylose utilization when expressed in *S. cerevisiae*; however, *GXF1* improvements in xylose transport were only observed at low concentrations of this sugar. No changes in uptake rates at high concentrations of xylose were detected, suggesting that the expression of this specific transporter in *S. cerevisiae* would be beneficial only when the xylose concentration is not excessive (Runquist et al., 2009; Fonseca et al., 2011; Tanino et al., 2012). *GXS1* is another sugar transporter identified in *C. intermedia*, where a F40 point mutation was located (Young et al., 2012), indicating that substitutions in F40 have a relationship with sugar transport dynamics and consequently can produce different phenotypes, including improved xylose transport.

Young *et al.* (2014) (Young et al., 2014) evaluated the sequence similarity of different heterologous transporters expressed in *S. cerevisiae* and reported a conserved amino acid motif (G-G/F-XXX-G) as responsible for monosaccharide selectivity in sugar transporters. An improved *C. intermedia GXS1* was obtained by adding $Phe^{38}Ile^{39}Met^{40}$

783 mutations, resulting in a pentose transporter with a slight increase in xylose uptake rate;
784 nevertheless, transportation remained inhibited by glucose.

785 *S. stipitis* has also been widely used to prospect xylose transporters due to its
786 natural ability to ferment this sugar. Many transporters from this species were analyzed
787 and expressed in *hxt* null *S. cerevisiae* mutants, among them *SUT1*, *SUT2* and *SUT3*
788 (Weierstall, Hollenberg & Boles, 1999); *XUT1* and *XUT3* (Young et al., 2011). The *SUT1*
789 transporter, when expressed in a strain of *S. cerevisiae*, showed improvement in xylose
790 transport and ethanol productivity in fermentation (Katahira et al., 2008). The *XUT3*
791 transporter, on the other hand, had an average efficiency in transporting sugars, but with
792 a greater preference for xylose (Young et al., 2011). Young *et al.* (2012) (Young et al.,
793 2012) suggested that the E538K mutation in *XUT3* is responsible for improved xylose
794 affinity, in addition to improved growth at low xylose rates.

795 Other fungi have also been a source of efficient xylose transporters when
796 expressed in recombinant *S. cerevisiae.* Bueno *et al.* (2020) (Bueno et al., 2020) used an
797 evolutionary approach combined with analysis of diverse microbiomes to identify new
798 xylose transporter candidates. In the study, the *CS4130* transporter from *Candida sojae*
799 was identified and showed functional expression in *S. cerevisiae* at high xylose
800 concentrations, revealing an appealing alternative for industrial fermentation of that
801 pentose. The *MGT05196P* transporter identified in *M. guilliermondii* also showed elevated
802 xylose transport activity in S. *cerevisiae*, and mutant N360F was able to transport xylose
803 without any glucose inhibition (Wang et al., 2015). From the xylose-consuming
804 filamentous fungus *Trichoderma reesei*, the *XITR1P* was reported as a xylose transporter
805 with better efficiency than the endogenous *S. cerevisiae* transporter *GAL2*. Through site-
806 directed mutagenesis it was indicated that the N326F amino acid mutation is highly
807 correlated to xylose-uptake activity, and its expression in *S. cerevisiae* conferred high
808 efficiency in transporting this sugar, while being insensitive to glucose (Jiang et al., 2020).
809 Many other transporters have been identified in different origins: *HXTB* and *XTRD*
810 (*Aspergillus nidulans)* are two such examples (Colabardini et al., 2014; Dos Reis et al.,
811 2016).

In *Arabidopsis thaliana*, genes encoding sugar transporters *AT5G17010* and *At5g59250* were expressed in recombinant *S. cerevisiae* containing the genetic modifications for xylose consumption, and the consumption of this pentose was analyzed in fermentations. Strains expressing the *AT5G17010* and *AT5G59250* transporters consumed 25% and 40% more xylose, respectively, than the control strain (Hector et al., 2008). However, in another study using different concentrations of xylose, no significant values were obtained in the transport of the xylose transporter *AT5G59250* compared to the control strain (Runquist, Hahn-Hägerdal & Rådström, 2010).

Although many studies have focused on the expression of heterologous xylose transporters in *S. cerevisiae*, the low activity and stability of such exogenous proteins, as well as the fact that most of these transporters exhibit competitive inhibition by glucose, limits their use in fermentations with co-consumption of sugars (Hou et al., 2017). Thus, another widely used strategy is the expression of endogenous transporters modified to reconnect sugar affinity. Although recombinant strains exhibit the ability to ferment xylose as the sole carbon source, when mixed glucose and xylose fermentations are performed, xylose is consumed only after glucose depletion because the affinity of endogenous transporters for glucose is much higher than that of xylose, leading to slow metabolization of xylose in the presence of this hexose, even at low concentrations of this sugar (Subtil & Boles, 2012; Hou et al., 2017). Several studies have sought to improve the ability of simultaneous sugar metabolization in recombinant strains, requiring a reduction in the affinity of hexose transporters for glucose, as well as an increase in their affinity for xylose (Farwick et al., 2014).

In *S. cerevisiae*, endogenous *HXT1-7* transporters along with *GAL2*, are responsible for the facilitated diffusion of xylose monosaccharides (Sedlak & Ho, 2004). Many studies have used different methodologies aiming to improve the ability of xylose/glucose co-metabolism by increasing the affinity of hexose transporters to xylose in modified strains. Among the strategies used for this purpose are random mutagenesis, genetic shuffling, evolutionary engineering, and overexpression, which have identified several mutant xylose transporters that do not undergo strong inhibition by glucose (Farwick et al., 2014; Young et al., 2014; Shin et al., 2015; Li, Schmitz & Alper, 2016).

In this context, using an ALE strategy, a platform for the evaluation of xylose transporters that lack inhibition by glucose was developed. Through this approach and error-prone PCR-based mutagenesis, two glucose-insensitive mutant xylose transporters, *HXT7* (N370S) and *GAL2* (N376F), have been identified (Farwick et al., 2014). In another study, an endogenous chimeric transporter (*HXT36*) was constructed using the endogenous transporters *HXT3* and *HXT6*. After the evolutionary engineering of a strain expressing the synthetic *HXT36* transporter, an N367A mutation was identified that generated increased affinity for xylose (Nijland et al., 2014). In another evolutionary engineering study, an F79S mutation in *HXT7* resulted in improved D-xylose uptake (Apel et al., 2016). Shin *et al.* (2015) (Shin et al., 2015) identified a mutation on residue N366 in *HXT11* in a recombinant *S. cerevisiae* with gene knockouts *HXT1-7* and *GAL2* that altered the specificity of the glucose transporter for xylose and enabled improved co-fermentation of these sugars. Another mutation identified was C505P which resulted in a 3-fold improvement in the xylose affinity of *HXT2* (Nijland et al., 2018).

Although many mutations have been identified as contributors to the affinity change in hexose transporters, a conserved asparagine residue has been identified in several studies at positions 360, 366, 367, 370 and 376 in *Meyerozyma guilliermondii MGT05196P* (Wang et al., 2015), and endogenous *HXT11* (Shin et al., 2015), *HXT36* (Nijland et al., 2014), *HXT7* and *GAL2* (Farwick et al., 2014), respectively. This asparagine residue was mutated to different amino acids, causing a decreased affinity for glucose and, in some cases, an increased affinity for xylose, indicating this as an important target for mutagenesis. Later a, *GAL2* N376Y/M435I double mutant was obtained, reported to be completely insensitive to competitive inhibition by glucose, and presented an improved ability to transport xylose upon expression in recombinant *S. cerevisiae* (Rojas et al., 2021). Another mutation identified in *GAL2* was threonine at position 386 (T386A), allowing for increased xylose transport and reduced glucose sensitivity, as well as co-consumption at reduced substrate concentrations (Reznicek et al., 2015).

Overexpression of hexose transporters has also been shown as another compelling approach to improve xylose uptake. Different studies have proven that

overexpression of the endogenous hexose transporters, *HXT* and *GAL2*, can also provide improvements in the rate of xylose uptake in recombinant *S. cerevisiae* (Tanino et al., 2012; Gonçalves et al., 2014).

CONCLUSIONS

Metabolic engineering has been used to optimize microorganisms through targeted alteration in simple cellular characteristics. The genetic alterations listed in this document could be rationally introduced in yeast cells for improved xylose metabolism. In *S. cerevisiae*, such interventions have contributed to increased growth rates and xylose assimilation, ultimately leading to better fermentation performance. However, the need to upgrade this phenotype foments other engineering approaches that could result in highly efficient strains. Evolutionary engineering, associated with chemical mutagenesis techniques, genome shuffling, genomic library screenings or transposon mutagenesis, are feasible approaches to develop mutant strains with enhanced xylose consumption and increased ethanol production rates. Other complex approaches – such as the omics: genomics, transcriptomics, metabolomics and fluxomics – directly contribute to advancing the understanding of different phenotypes at the molecular level through the identification of new genetic targets responsible for the enhancement of phenotypes.

However, despite the success in approaches used to obtain xylose assimilating *S. cerevisiae*, the understanding of the metabolism, regulation and signaling pathways involved in xylose consumption is still limited. There are hidden features of xylose metabolism that need to be identified to optimize fermentation processes. New approaches should be sought to identify non-obvious gene targets and to analyze the role of essential genes for the xylose consumption phenotype, as well as to evaluate the optimal expression level of genes directly and indirectly involved in xylose metabolism. Ultimately, advances in pentose metabolism in *S. cerevisiae* are expected to boost biotechnological routes for the full exploration of lignocellulosic biomass in a low-carbon economy.

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REFERENCES

Alper H, Fischer C, Nevoigt E, Stephanopoulos G. 2005. *Tuning genetic control through promoter engineering*. DOI: 10.1073/pnas.0504604102.

Amore R, Wilhelm M, Hollenberg CP. 1989. Applied AFwrobiology Biotechnology The fermentation of xylose -- an analysis of the expression of Bacillus and Actinoplanes xylose isomerase genes in yeast *. 75:351–357.

Apel AR, Ouellet M, Szmidt-Middleton H, Keasling JD, Mukhopadhyay A. 2016. Evolved hexose transporter enhances xylose uptake and glucose/xylose co-utilization in Saccharomyces cerevisiae. *Scientific Reports* 6. DOI: 10.1038/srep19512.

Attfield P V., Bell PJL. 2006. Use of population genetics to derive nonrecombinant Saccharomyces cerevisiae strains that grow using xylose as a sole carbon source. *FEMS Yeast Research* 6:862–868. DOI: 10.1111/j.1567-1364.2006.00098.x.

Balat M. 2011. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. *Energy Conversion and Management* 52:858–875. DOI: 10.1016/j.enconman.2010.08.013.

Bamba T, Hasunuma T, Kondo A. 2016. Disruption of PHO13 improves ethanol production via the xylose isomerase pathway. *AMB Express* 6:1–10. DOI: 10.1186/s13568-015-0175-7.

Batt CA, Caryallo S, Easson DD, Akedo M, Sinskey AJ. 1986. *Direct evidence for a xylose metabolic pathway in Saccharomyces cerevisiae*. DOI: 10.1002/bit.260280411.

Bengtsson O, Jeppsson M, Sonderegger M, Parachin NS, Sauer U, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2008. Identification of common traits in improved xylose-growing Saccharomyces cerevisiae for inverse metabolic engineering. *Yeast* 25:835–847. DOI: 10.1002/yea.1638.

Bertilsson M, Andersson J, Lidén G. 2008. Modeling simultaneous glucose and xylose uptake in Saccharomyces cerevisiae from kinetics and gene expression of sugar transporters. *Bioprocess and Biosystems Engineering* 31:369–377. DOI: 10.1007/s00449-007-0169-1.

Bettiga M, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2008. Comparing the xylose reductase/xylitol dehydrogenase and xylose isomerase pathways in arabinose and xylose fermenting Saccharomyces cerevisiae strains. *Biotechnology for Biofuels* 1. DOI: 10.1186/1754-6834-1-16.

931 Boles E, Oreb M. 2018. A growth-based screening system for hexose transporters in yeast. In: *Methods in*
932 *Molecular Biology*. Humana Press Inc., 123–135. DOI: 10.1007/978-1-4939-7507-5_10.

933 Brat D, Boles E, Wiedemann B. 2009. Functional expression of a bacterial xylose isomerase in
934 Saccharomyces cerevisiae. *Applied and Environmental Microbiology* 75:2304–2311. DOI:
935 10.1128/AEM.02522-08.

936 Bro C, Regenberg B, Förster J, Nielsen J. 2006. In silico aided metabolic engineering of Saccharomyces
937 cerevisiae for improved bioethanol production. *Metabolic Engineering* 8:102–111. DOI:
938 10.1016/j.ymben.2005.09.007.

939 Bueno JGR, Borelli G, Corrêa TLR, Fiamenghi MB, José J, De Carvalho M, De Oliveira LC, Pereira GAG,
940 Dos Santos LV. 2020. Novel xylose transporter Cs4130 expands the sugar uptake repertoire in
941 recombinant Saccharomyces cerevisiae strains at high xylose concentrations. *Biotechnology for*
942 *Biofuels* 13. DOI: 10.1186/s13068-020-01782-0.

943 Cadete RM, De Las Heras AM, Sandström AG, Ferreira C, Gírio F, Gorwa-Grauslund MF, Rosa CA,
944 Fonseca C. 2016. Exploring xylose metabolism in Spathaspora species: XYL1.2 from Spathaspora
945 passalidarum as the key for efficient anaerobic xylose fermentation in metabolic engineered
946 Saccharomyces cerevisiae. *Biotechnology for Biofuels* 9:1–14. DOI: 10.1186/s13068-016-0570-6.

947 Chandel AK, Forte MBS, Gonçalves IS, Milessi TS, Arruda P V., Carvalho W, Mussatto SI. 2021. Brazilian
948 biorefineries from second generation biomass: critical insights from industry and future perspectives.
949 *Biofuels, Bioproducts and Biorefining* 15:1190–1208. DOI: 10.1002/bbb.2234.

950 Colabardini AC, Ries LNA, Brown NA, Dos Reis TF, Savoldi M, Goldman MHS, Menino JF, Rodrigues F,
951 Goldman GH. 2014. Functional characterization of a xylose transporter in Aspergillus nidulans.
952 *Biotechnology for Biofuels* 7. DOI: 10.1186/1754-6834-7-46.

953 Curran KA, Karim AS, Gupta A, Alper HS. 2013. Use of expression-enhancing terminators in
954 Saccharomyces cerevisiae to increase mRNA half-life and improve gene expression control for
955 metabolic engineering applications. *Metabolic Engineering* 19:88–97. DOI:
956 10.1016/j.ymben.2013.07.001.

957 Curran KA, Morse NJ, Markham KA, Wagman AM, Gupta A, Alper HS. 2015. Short Synthetic Terminators
958 for Improved Heterologous Gene Expression in Yeast. *ACS Synthetic Biology* 4:824–832. DOI:
959 10.1021/sb5003357.

960 Demeke MM, Dietz H, Li Y, Foulquié-Moreno MR, Mutturi S, Deprez S, Den Abt T, Bonini BM, Liden G,
961 Dumortier F, Verplaetse A, Boles E, Thevelein JM. 2013. Development of a D-xylose fermenting and
962 inhibitor tolerant industrial Saccharomyces cerevisiae strain with high performance in lignocellulose

963 hydrolysates using metabolic and evolutionary engineering. *Biotechnology for Biofuels* 6. DOI:
964 10.1186/1754-6834-6-89.

965 Deng XX, Ho NWY. 1990. Xylulokinase activity in various yeasts including Saccharomyces cerevisiae
966 containing the cloned xylulokinase gene - Scientific note. *Applied Biochemistry and Biotechnology*
967 24–25:193–199. DOI: 10.1007/BF02920245.

968 Dzanaeva L, Kruk B, Ruchala J, Sibirny A, Dmytruk K. 2021. The impact of transcription factors Znf1, Sip4,
969 Adr1, Tup1, and Hap4 on xylose alcoholic fermentation in the engineered yeast Saccharomyces
970 cerevisiae. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*
971 114:1373–1385. DOI: 10.1007/s10482-021-01607-6.

972 Eliasson A, Christensson C, Wahlbom CF, Hahn-Hagerdal B. 2000. *Anaerobic xylose fermentation by*
973 *recombinant Saccharomyces cerevisiae carrying XYL1, XYL2, and XKS1 in mineral medium*
974 *chemostat cultures*. DOI: 10.1128/AEM.66.8.3381-3386.2000.

975 Farwick A, Bruder S, Schadeweg V, Oreb M, Boles E. 2014. Engineering of yeast hexose transporters to
976 transport D-xylose without inhibition by D-glucose. *Proceedings of the National Academy of Sciences*
977 *of the United States of America* 111:5159–5164. DOI: 10.1073/pnas.1323464111.

978 Feng X, Marchisio MA. 2021. Saccharomyces cerevisiae promoter engineering before and during the
979 synthetic biology era. *Biology* 10. DOI: 10.3390/biology10060504.

980 Fonseca C, Olofsson K, Ferreira C, Runquist D, Fonseca LL, Hahn-Hägerdal B, Lidén G. 2011. The
981 glucose/xylose facilitator Gxf1 from Candida intermedia expressed in a xylose-fermenting industrial
982 strain of Saccharomyces cerevisiae increases xylose uptake in SSCF of wheat straw. *Enzyme and*
983 *Microbial Technology* 48:518–525. DOI: 10.1016/j.enzmictec.2011.02.010.

984 Fujitomi K, Sanda T, Hasunuma T, Kondo A. 2012. Deletion of the PHO13 gene in Saccharomyces
985 cerevisiae improves ethanol production from lignocellulosic hydrolysate in the presence of acetic and
986 formic acids, and furfural. *Bioresource Technology* 111:161–166. DOI:
987 10.1016/j.biortech.2012.01.161.

988 Gárdonyi M, Hahn-Hägerdal B. 2003. *The Streptomyces rubiginosus xylose isomerase is misfolded when*
989 *expressed in Saccharomyces cerevisiae*. DOI: 10.1016/S0141-0229(02)00285-5.

990 Gonçalves DL, Matsushika A, de Sales BB, Goshima T, Bon EPS, Stambuk BU. 2014. Xylose and
991 xylose/glucose co-fermentation by recombinant Saccharomyces cerevisiae strains expressing
992 individual hexose transporters. *Enzyme and Microbial Technology* 63:13–20. DOI:
993 10.1016/j.enzmictec.2014.05.003.

994 Grotkjær T, Christakopoulos P, Nielsen J, Olsson L. 2005. Comparative metabolic network analysis of two

xylose fermenting recombinant Saccharomyces cerevisiae strains. *Metabolic Engineering* 7:437–444. DOI: 10.1016/j.ymben.2005.07.003.

Guo Z, Sherman F. 1996. *Signals sufficient for 3'-end formation of yeast mRNA*. DOI: 10.1128/mcb.16.6.2772.

Ha SJ, Kim SR, Choi JH, Park MS, Jin YS. 2011. Xylitol does not inhibit xylose fermentation by engineered Saccharomyces cerevisiae expressing xylA as severely as it inhibits xylose isomerase reaction in vitro. *Applied Microbiology and Biotechnology* 92:77–84. DOI: 10.1007/s00253-011-3345-9.

Hamacher T, Becker J, Gárdonyi M, Hahn-Hägerdal B, Boles E. 2002. *Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization*. DOI: 10.1099/00221287-148-9-2783.

Hara KY, Kobayashi J, Yamada R, Sasaki D, Kuriya Y, Hirono-Hara Y, Ishii J, Araki M, Kondo A. 2017. Transporter engineering in biomass utilization by yeast. *FEMS Yeast Research* 17. DOI: 10.1093/femsyr/fox061.

Hector RE, Dien BS, Cotta MA, Mertens JA. 2013. Growth and fermentation of D-xylose by Saccharomyces cerevisiae expressing a novel D-xylose isomerase originating from the bacterium Prevotella ruminicola TC2-24. *Biotechnology for Biofuels* 6. DOI: 10.1186/1754-6834-6-84.

Hector RE, Mertens JA. 2017. A Synthetic Hybrid Promoter for Xylose-Regulated Control of Gene Expression in Saccharomyces Yeasts. *Molecular Biotechnology* 59:24–33. DOI: 10.1007/s12033-016-9991-5.

Hector RE, Qureshi N, Hughes SR, Cotta MA. 2008. Expression of a heterologous xylose transporter in a Saccharomyces cerevisiae strain engineered to utilize xylose improves aerobic xylose consumption. *Applied Microbiology and Biotechnology* 80:675–684. DOI: 10.1007/s00253-008-1583-2.

Ho NWY, Chen Z, Brainard AP. 1998. *Genetically engineered Saccharomyces yeast capable of effective cofermentation of glucose and xylose*. DOI: 10.1128/aem.64.5.1852-1859.1998.

Hoang Nguyen Tran P, Ko JK, Gong G, Um Y, Lee SM. 2020. Improved simultaneous co-fermentation of glucose and xylose by Saccharomyces cerevisiae for efficient lignocellulosic biorefinery. *Biotechnology for Biofuels* 13:1–14. DOI: 10.1186/s13068-019-1641-2.

Hou J, Qiu C, Shen Y, Li H, Bao X. 2017. Engineering of Saccharomyces cerevisiae for the efficient co-utilization of glucose and xylose. *FEMS Yeast Research* 17. DOI: 10.1093/femsyr/fox034.

Hou J, Shen Y, Jiao C, Ge R, Zhang X, Bao X. 2016. Characterization and evolution of xylose isomerase screened from the bovine rumen metagenome in Saccharomyces cerevisiae. *Journal of Bioscience*

1026 *and Bioengineering* 121:160–165. DOI: 10.1016/j.jbiosc.2015.05.014.

1027 Hou J, Shen Y, Li XP, Bao XM. 2007. Effect of the reversal of coenzyme specificity by expression of mutated
1028 Pichia stipitis xylitol dehydrogenase in recombinant Saccharomyces cerevisiae. *Letters in Applied*
1029 *Microbiology* 45:184–189. DOI: 10.1111/j.1472-765X.2007.02165.x.

1030 Hubmann G, Thevelein JM, Nevoigt E. 2014. Natural and modifi ed promoters for tailored metabolic
1031 engineering of the yeast saccharomyces cerevisiae. *Methods in Molecular Biology* 1152:17–42. DOI:
1032 10.1007/978-1-4939-0563-8_2.

1033 Jeffries TW. 1983. *Utilization of xylose by bacteria, yeasts, and fungi.* DOI: 10.1007/bfb0009101.

1034 Jeffries TW. 2006. Engineering yeasts for xylose metabolism. *Current Opinion in Biotechnology* 17:320–
1035 326. DOI: 10.1016/j.copbio.2006.05.008.

1036 Jeppsson M, Bengtsson O, Franke K, Lee H, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2006. The
1037 expression of a Pichia stipitis xylose reductase mutant with higher KM for NADPH increases ethanol
1038 production from xylose in recombinant Saccharomyces cerevisiae. *Biotechnology and Bioengineering*
1039 93:665–673. DOI: 10.1002/bit.20737.

1040 Jeppsson M, Johansson B, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2002. Reduced oxidative pentose
1041 phosphate pathway flux in recombinant xylose-utilizing Saccharomyces cerevisiae strains improves
1042 the ethanol yield from xylose. *Applied and Environmental Microbiology* 68:1604–1609. DOI:
1043 10.1128/AEM.68.4.1604-1609.2002.

1044 Jeppsson M, Johansson B, Jensen PR, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2003. The level of
1045 glucose-6-phosphate dehydrogenase activity strongly influences xylose fermentation and inhibitor
1046 sensitivity in recombinant Saccharomyces cerevisiae strains. *Yeast* 20:1263–1272. DOI:
1047 10.1002/yea.1043.

1048 Jiang Y, Shen Y, Gu L, Wang Z, Su N, Niu K, Guo W, Hou S, Bao X, Tian C, Fang X. 2020. Identification
1049 and Characterization of an Efficient d -Xylose Transporter in Saccharomyces cerevisiae. *Journal of*
1050 *Agricultural and Food Chemistry* 68:2702–2710. DOI: 10.1021/acs.jafc.9b07113.

1051 Jin YS, Ni H, Laplaza JM, Jeffries TW. 2003. Optimal growth and ethanol production from xylose by
1052 recombinant Saccharomyces cerevisiae require moderate D-xylulokinase activity. *Applied and*
1053 *Environmental Microbiology* 69:495–503. DOI: 10.1128/AEM.69.1.495-503.2003.

1054 Johansson B, Christensson C, Hobley T, Hahn-Hägerdal B. 2001. Xylulokinase Overexpression in Two
1055 Strains of Saccharomyces cerevisiae Also Expressing Xylose Reductase and Xylitol Dehydrogenase
1056 and Its Effect on Fermentation of Xylose and Lignocellulosic Hydrolysate. *Applied and Environmental*
1057 *Microbiology* 67:4249–4255. DOI: 10.1128/AEM.67.9.4249-4255.2001.

1058 Karhumaa K, Fromanger R, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2007a. High activity of xylose
1059 reductase and xylitol dehydrogenase improves xylose fermentation by recombinant Saccharomyces
1060 cerevisiae. *Applied Microbiology and Biotechnology* 73:1039–1046. DOI: 10.1007/s00253-006-0575-
1061 3.

1062 Karhumaa K, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2005. Investigation of limiting metabolic steps in
1063 the utilization of xylose by recombinant Saccharomyces cerevisiae using metabolic engineering.
1064 *Yeast* 22:359–368. DOI: 10.1002/yea.1216.

1065 Karhumaa K, Sanchez RG, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2007b. Comparison of the xylose
1066 reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by
1067 recombinant Saccharomyces cerevisiae. *Microbial Cell Factories* 6. DOI: 10.1186/1475-2859-6-5.

1068 Katahira S, Ito M, Takema H, Fujita Y, Tanino T, Tanaka T, Fukuda H, Kondo A. 2008. Improvement of
1069 ethanol productivity during xylose and glucose co-fermentation by xylose-assimilating S. cerevisiae
1070 via expression of glucose transporter Sut1. *Enzyme and Microbial Technology* 43:115–119. DOI:
1071 10.1016/j.enzmictec.2008.03.001.

1072 Katahira S, Muramoto N, Moriya S, Nagura R, Tada N, Yasutani N, Ohkuma M, Onishi T, Tokuhiro K. 2017.
1073 Screening and evolution of a novel protist xylose isomerase from the termite Reticulitermes speratus
1074 for efficient xylose fermentation in Saccharomyces cerevisiae. *Biotechnology for Biofuels* 10. DOI:
1075 10.1186/s13068-017-0890-1.

1076 Kavanagh KL, Klimacek M, Nidetzky B, Wilson DK. 2002. The structure of apo and holo forms of xylose
1077 reductase, a dimeric aldo-keto reductase from Candida tenuis. *Biochemistry* 41:8785–8795. DOI:
1078 10.1021/bi025786n.

1079 Kavanagh KL, Klimacek M, Nidetzky B, Wilson DK. 2003. *Structure of xylose reductase bound to NAD+*
1080 *and the basis for single and dual co-substrate specificity in family 2 aldo-keto reductases*. DOI:
1081 10.1042/BJ20030286.

1082 Kim SR, Ha SJ, Kong II, Jin YS. 2012. High expression of XYL2 coding for xylitol dehydrogenase is
1083 necessary for efficient xylose fermentation by engineered Saccharomyces cerevisiae. *Metabolic*
1084 *Engineering* 14:336–343. DOI: 10.1016/j.ymben.2012.04.001.

1085 Kim SR, Skerker JM, Kang W, Lesmana A, Wei N, Arkin AP, Jin YS. 2013. Rational and Evolutionary
1086 Engineering Approaches Uncover a Small Set of Genetic Changes Efficient for Rapid Xylose
1087 Fermentation in Saccharomyces cerevisiae. *PLoS ONE* 8. DOI: 10.1371/journal.pone.0057048.

1088 Kim SR, Xu H, Lesmana A, Kuzmanovic U, Au M, Florencia C, Oh EJ, Zhang G, Kim KH, Jin YS. 2015.
1089 Deletion of PHO13, encoding haloacid dehalogenase type IIA phosphatase, results in upregulation of

1090 the pentose phosphate pathway in Saccharomyces cerevisiae. *Applied and Environmental*
1091 *Microbiology* 81:1601–1609. DOI: 10.1128/AEM.03474-14.

1092 Kobayashi Y, Sahara T, Ohgiya S, Kamagata Y, Fujimori KE. 2018. Systematic optimization of gene
1093 expression of pentose phosphate pathway enhances ethanol production from a glucose/xylose mixed
1094 medium in a recombinant Saccharomyces cerevisiae. *AMB Express* 8. DOI: 10.1186/s13568-018-
1095 0670-8.

1096 Kostrzynska M, Sopher CR, Lee H. 1998. Mutational analysis of the role of the conserved lysine-270 in the
1097 Pichia stipitis xylose reductase . *FEMS Microbiology Letters* 159:107–112. DOI: 10.1111/j.1574-
1098 6968.1998.tb12848.x.

1099 Kötter P, Amore R, Hollenberg CP, Ciriacy M. 1990. Isolation and characterization of the Pichia stipitis
1100 xylitol dehydrogenase gene, XYL2, and construction of a xylose-utilizing Saccharomyces cerevisiae
1101 transformant. *Current Genetics* 18:493–500. DOI: 10.1007/BF00327019.

1102 Kuyper M, Harhangi HR, Stave AK, Winkler A a., Jetten MSM, De Laat WT a M, Den Ridder JJJ, Op Den
1103 Camp HJM, Van Dijken JP, Pronk JT. 2003. High-level functional expression of a fungal xylose
1104 isomerase: The key to efficient ethanolic fermentation of xylose by Saccharomyces cerevisiae? *FEMS*
1105 *Yeast Research* 4:69–78. DOI: 10.1016/S1567-1356(03)00141-7.

1106 Kuyper M, Hartog MMP, Toirkens MJ, Almering MJH, Winkler A a., Van Dijken JP, Pronk JT. 2005.
1107 Metabolic engineering of a xylose-isomerase-expressing Saccharomyces cerevisiae strain for rapid
1108 anaerobic xylose fermentation. *FEMS Yeast Research* 5:399–409. DOI:
1109 10.1016/j.femsyr.2004.09.010.

1110 Kuyper M, Winkler AA, Van Dijken JP, Pronk JT. 2004. Minimal metabolic engineering of Saccharomyces
1111 cerevisiae for efficient anaerobic xylose fermentation: A proof of principle. *FEMS Yeast Research*
1112 4:655–664. DOI: 10.1016/j.femsyr.2004.01.003.

1113 Kwak S, Jin YS. 2017. Production of fuels and chemicals from xylose by engineered Saccharomyces
1114 cerevisiae: A review and perspective. *Microbial Cell Factories* 16. DOI: 10.1186/s12934-017-0694-9.

1115 Leandro MJ, Gonçalves P, Spencer-Martins I. 2006. Two glucose/xylose transporter genes from the yeast
1116 Candida intermedia: First molecular characterization of a yeast xylose-H+ symporter. *Biochemical*
1117 *Journal* 395:543–549. DOI: 10.1042/BJ20051465.

1118 Lee SM, Jellison T, Alper HS. 2014. Systematic and evolutionary engineering of a xylose isomerase-based
1119 pathway in Saccharomyces cerevisiae for efficient conversion yields. *Biotechnology for Biofuels* 7.
1120 DOI: 10.1186/s13068-014-0122-x.

1121 Leitgeb S, Petschacher B, Wilson DK, Nidetzky B. 2005. Fine tuning of coenzyme specificity in family 2

aldo-keto reductases revealed by crystal structures of the Lys-274 → Arg mutant of Candida tenuis xylose reductase (AKR2B5) bound to NAD+ and NADP+. *FEBS Letters* 579:763–767. DOI: 10.1016/j.febslet.2004.12.063.

Li YC, Gou ZX, Liu ZS, Tang YQ, Akamatsu T, Kida K. 2014. Synergistic effects of TAL1 over-expression and PHO13 deletion on the weak acid inhibition of xylose fermentation by industrial Saccharomyces cerevisiae strain. *Biotechnology Letters* 36:2011–2021. DOI: 10.1007/s10529-014-1581-7.

Li X, Park A, Estrela R, Kim SR, Jin YS, Cate JHD. 2016. Comparison of xylose fermentation by two high-performance engineered strains of Saccharomyces cerevisiae. *Biotechnology Reports* 9:53–56. DOI: 10.1016/j.btre.2016.01.003.

Li H, Schmitz O, Alper HS. 2016. Enabling glucose/xylose co-transport in yeast through the directed evolution of a sugar transporter. *Applied Microbiology and Biotechnology* 100:10215–10223. DOI: 10.1007/s00253-016-7879-8.

Li N, Zhang LM, Zhang KQ, Deng JS, Prändl R, Schöffl F. 2006. Effects of heat stress on yeast heat shock factor-promoter binding in vivo. *Acta Biochimica et Biophysica Sinica* 38:356–362. DOI: 10.1111/j.1745-7270.2006.00170.x.

Lönn A, Träff-Bjerre KL, Cordero Otero RR, Van Zyl WH, Hahn-Hägerdal B. 2003. Xylose isomerase activity influences xylose fermentation with recombinant Saccharomyces cerevisiae strains expressing mutated xylA from Thermus thermophilus. *Enzyme and Microbial Technology* 32:567–573. DOI: 10.1016/S0141-0229(03)00024-3.

Lu C, Jeffries T. 2007. Shuffling of promoters for multiple genes to optimize xylose fermentation in an engineered Saccharomyces cerevisiae strain. *Applied and Environmental Microbiology* 73:6072–6077. DOI: 10.1128/AEM.00955-07.

Madhavan A, Tamalampudi S, Ushida K, Kanai D, Katahira S, Srivastava A, Fukuda H, Bisaria VS, Kondo A. 2009. Xylose isomerase from polycentric fungus Orpinomyces: Gene sequencing, cloning, and expression in Saccharomyces cerevisiae for bioconversion of xylose to ethanol. *Applied Microbiology and Biotechnology* 82:1067–1078. DOI: 10.1007/s00253-008-1794-6.

Marger MD, Saier MH. 1993. *A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport*. DOI: 10.1016/0968-0004(93)90081-W.

Matsushika A, Goshima T, Fujii T, Inoue H, Sawayama S, Yano S. 2012. Characterization of non-oxidative transaldolase and transketolase enzymes in the pentose phosphate pathway with regard to xylose utilization by recombinant Saccharomyces cerevisiae. *Enzyme and Microbial Technology* 51:16–25. DOI: 10.1016/j.enzmictec.2012.03.008.

1154 Matsushika A, Sawayama S. 2008. Efficient bioethanol production from xylose by recombinant
1155 saccharomyces cerevisiae requires high activity of xylose reductase and moderate xylulokinase
1156 activity. *Journal of Bioscience and Bioengineering* 106:306–309. DOI: 10.1263/jbb.106.306.

1157 Matsushika A, Watanabe S, Kodaki T, Makino K, Sawayama S. 2008. Bioethanol production from xylose
1158 by recombinant Saccharomyces cerevisiae expressing xylose reductase, NADP+-dependent xylitol
1159 dehydrogenase, and xylulokinase. *Journal of Bioscience and Bioengineering* 105:296–299. DOI:
1160 10.1263/jbb.105.296.

1161 Matsuyama T. 2019. Recent developments in terminator technology in Saccharomyces cerevisiae. *Journal*
1162 *of Bioscience and Bioengineering* 128:655–661. DOI: 10.1016/j.jbiosc.2019.06.006.

1163 Maya D, Quintero MJ, De La Cruz Muñoz-Centeno M, Chávez S. 2008. Systems for applied gene control
1164 in Saccharomyces cerevisiae. *Biotechnology Letters* 30:979–987. DOI: 10.1007/s10529-008-9647-z.

1165 Mischo HE, Proudfoot NJ. 2013. Disengaging polymerase: Terminating RNA polymerase II transcription in
1166 budding yeast. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms* 1829:174–185. DOI:
1167 10.1016/j.bbagrm.2012.10.003.

1168 Moes CJ, Pretorius IS, Zyl WH Van. 1996. *Cloning and Expression of the Clostridium Thermosulfurogenes*.

1169 Moye WS, Amuro N, Rao JKM, Zalkin H. 1985. Nucleotide sequence of yeast GDH1 encoding nicotinamide
1170 adenine dinucleotide phosphate-dependent glutamate dehydrogenase. *Journal of Biological*
1171 *Chemistry* 260:8502–8508. DOI: 10.1016/s0021-9258(17)39500-5.

1172 Nambu-Nishida Y, Sakihama Y, Ishii J, Hasunuma T, Kondo A. 2018. Selection of yeast Saccharomyces
1173 cerevisiae promoters available for xylose cultivation and fermentation. *Journal of Bioscience and*
1174 *Bioengineering* 125:76–86. DOI: 10.1016/j.jbiosc.2017.08.001.

1175 Nevoigt E. 2008. Progress in metabolic engineering of Saccharomyces cerevisiae. *Microbiology and*
1176 *molecular biology reviews : MMBR* 72:379–412. DOI: 10.1128/MMBR.00025-07.

1177 Ni H, Laplaza JM, Jeffries TW. 2007. Transposon mutagenesis to improve the growth of recombinant
1178 Saccharomyces cerevisiae on D-xylose. *Applied and Environmental Microbiology* 73:2061–2066.
1179 DOI: 10.1128/AEM.02564-06.

1180 Nijland JG, Driessen AJM. 2020. Engineering of Pentose Transport in Saccharomyces cerevisiae for
1181 Biotechnological Applications. *Frontiers in Bioengineering and Biotechnology* 7. DOI:
1182 10.3389/fbioe.2019.00464.

1183 Nijland JG, Shin HY, De Jong RM, De Waal PP, Klaassen P, Driessen AJM. 2014. Engineering of an
1184 endogenous hexose transporter into a specific D-xylose transporter facilitates glucose-xylose co-

consumption in Saccharomyces cerevisiae. *Biotechnology for Biofuels* 7. DOI: 10.1186/s13068-014-0168-9.

Nijland JG, Shin HY, de Waal PP, Klaassen P, Driessen AJM. 2018. Increased xylose affinity of Hxt2 through gene shuffling of hexose transporters in Saccharomyces cerevisiae. *Journal of Applied Microbiology* 124:503–510. DOI: 10.1111/jam.13670.

Özcan S, Johnston M. 1999. *Function and Regulation of Yeast Hexose Transporters*. DOI: 10.1128/mmbr.63.3.554-569.1999.

Palermo GC de L, Coutouné N, Bueno JGR, Maciel LF, dos Santos LV. 2021. Exploring metal ion metabolisms to improve xylose fermentation in Saccharomyces cerevisiae. *Microbial Biotechnology* 14:2101–2115. DOI: 10.1111/1751-7915.13887.

Parachin NS, Bengtsson O, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2010. The deletion of YLR042c improves ethanolic xylose fermentation by recombinant Saccharomyces cerevisiae. *Yeast* 27:741–751. DOI: 10.1002/yea.1777.

Parachin NS, Gorwa-Grauslund MF. 2011. Isolation of xylose isomerases by sequence- and function-based screening from a soil metagenomic library. *Biotechnology for Biofuels* 4. DOI: 10.1186/1754-6834-4-9.

Parreiras LS, Breuer RJ, Narasimhan RA, Higbee AJ, La A, Tremaine M, Qin L, Willis LB, Bice BD, Bonfert BL, Pinhancos RC, Balloon AJ, Uppugundla N, Liu T, Li C, Tanjore D, Ong IM, Li H, Pohlmann EL, Serate J, Withers ST, Simmons B a, Hodge DB, Westphall MS, Coon JJ, Dale BE, Balan V, Keating DH, Zhang Y, Landick R, Gasch AP. 2014. Engineering and Two-Stage Evolution of a Lignocellulosic Hydrolysate-Tolerant Saccharomyces cerevisiae Strain for Anaerobic Fermentation of Xylose from AFEX Pretreated Corn Stover. 9. DOI: 10.1371/journal.pone.0107499.

Petschacher B, Leitgeb S, Kavanagh KL, Wilson DK, Nidetzky B. 2005. *The coenzyme specificity of Candida tenuis xylose reductase (AKR2B5) explored by site-directed mutagenesis and X-ray crystallography*. DOI: 10.1042/BJ20040363.

Petschacher B, Nidetzky B. 2005. Engineering Candida tenuis xylose reductase for improved utilization of NADH: Antagonistic effects of multiple side chain replacements and performance of site-directed mutants under simulated in vivo conditions. *Applied and Environmental Microbiology* 71:6390–6393. DOI: 10.1128/AEM.71.10.6390-6393.2005.

Petschacher B, Nidetzky B. 2008. Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of Saccharomyces cerevisiae. *Microbial Cell Factories* 7. DOI: 10.1186/1475-2859-7-9.

1217 Quistgaard EM, Löw C, Guettou F, Nordlund P. 2016. Understanding transport by the major facilitator
1218 superfamily (MFS): Structures pave the way. *Nature Reviews Molecular Cell Biology* 17:123–132.
1219 DOI: 10.1038/nrm.2015.25.

1220 Rédei GP. 2008. *Major Facilitator Superfamily (MFS)*. DOI: 10.1007/978-1-4020-6754-9_9778.

1221 Dos Reis TF, De Lima PBA, Parachin NS, Mingossi FB, De Castro Oliveira JV, Ries LNA, Goldman GH.
1222 2016. Identification and characterization of putative xylose and cellobiose transporters in Aspergillus
1223 nidulans. *Biotechnology for Biofuels* 9:1–19. DOI: 10.1186/s13068-016-0611-1.

1224 Renewable Fuels Association (2022) Annual fuel ethanol production. 2022.

1225 Reznicek O, Facey SJ, de Waal PP, Teunissen AWRH, de Bont JAM, Nijland JG, Driessen AJM, Hauer B.
1226 2015. Improved xylose uptake in Saccharomyces cerevisiae due to directed evolution of galactose
1227 permease Gal2 for sugar co-consumption. *Journal of Applied Microbiology* 119:99–111. DOI:
1228 10.1111/jam.12825.

1229 Roca C, Nielsen J, Olsson L. 2003. Metabolic engineering of ammonium assimilation in xylose-fermenting
1230 Saccharomyces cerevisiae improves ethanol production. *Applied and Environmental Microbiology*
1231 69:4732–4736. DOI: 10.1128/AEM.69.8.4732-4736.2003.

1232 Rodriguez-Peña JM, Cid VJ, Arroyo J, Nombela C. 1998. The YGR194c (XKS1) gene encodes the
1233 xylulokinase from the budding yeast Saccharomyces cerevisiae. *FEMS Microbiology Letters* 162:155–
1234 160. DOI: 10.1016/S0378-1097(98)00118-9.

1235 Rojas SAT, Schadeweg V, Kirchner F, Boles E, Oreb M. 2021. Identification of a glucose-insensitive variant
1236 of Gal2 from Saccharomyces cerevisiae exhibiting a high pentose transport capacity. *Scientific*
1237 *Reports* 11. DOI: 10.1038/s41598-021-03822-7.

1238 Runquist D, Fonseca C, Rådström P, Spencer-Martins I, Hahn-Hägerdal B. 2009. Expression of the Gxf1
1239 transporter from Candida intermedia improves fermentation performance in recombinant xylose-
1240 utilizing Saccharomyces cerevisiae. *Applied Microbiology and Biotechnology* 82:123–130. DOI:
1241 10.1007/s00253-008-1773-y.

1242 Runquist D, Hahn-Hägerdal B, Bettiga M. 2010. Increased ethanol productivity in xylose-utilizing
1243 Saccharomyces cerevisiae via a randomly mutagenized xylose reductase. *Applied and Environmental*
1244 *Microbiology* 76:7796–7802. DOI: 10.1128/AEM.01505-10.

1245 Runquist D, Hahn-Hägerdal B, Rådström P. 2010. *Comparison of heterologous xylose transporters in*
1246 *recombinant Saccharomyces cerevisiae*. DOI: 10.1186/1754-6834-3-5.

1247 dos Santos LV, Carazzolle MF, Nagamatsu ST, Sampaio NMV, Almeida LD, Pirolla RAS, Borelli G, Corrêa

TLR, Argueso JL, Pereira GAG. 2016. Unraveling the genetic basis of xylose consumption in engineered Saccharomyces cerevisiae strains. *Scientific Reports* 6:38676. DOI: 10.1038/srep38676.

Santos LV, Graci MCB, Gallardo JCM, Pirolla RAS, Calderón LL, Carvalho-Neto O V, Parreira L, Camargo ELO, Drezza AL, Missawa SK, Teixeira GS, Lunardi I, Bressiani J, Pereira GAG. 2015. Second-Generation Ethanol: The Need is Becoming a Reality. *Industrial Biotechnoly*:40–57. DOI: 10.1089/ind.2015.0017.

Sarthy A V, McConaughy BL, Lobo Z, Sundstrom JA, Furlong CE, Hall BD. 1987. Expression of the Escherichia coli Xylose Isomerase Gene in Saccharomyces cerevisiae. *Applied and Environmental Microbiology* 53:1996–2000.

Sato TK, Tremaine M, Parreiras LS, Hebert AS, Myers KS, Higbee AJ, Sardi M, McIlwain SJ, Ong IM, Breuer RJ, Avanasi Narasimhan R, McGee MA, Dickinson Q, La Reau A, Xie D, Tian M, Reed JL, Zhang Y, Coon JJ, Hittinger CT, Gasch AP, Landick R. 2016. Directed Evolution Reveals Unexpected Epistatic Interactions That Alter Metabolic Regulation and Enable Anaerobic Xylose Use by Saccharomyces cerevisiae. *PLoS Genetics* 12. DOI: 10.1371/journal.pgen.1006372.

Sedlak M, Ho NWY. 2004. Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant Saccharomyces yeast. *Yeast* 21:671–684. DOI: 10.1002/yea.1060.

Shin HY, Nijland JG, De Waal PP, De Jong RM, Klaassen P, Driessen AJM. 2015. An engineered cryptic Hxt11 sugar transporter facilitates glucose-xylose co-consumption in Saccharomyces cerevisiae. *Biotechnology for Biofuels* 8. DOI: 10.1186/s13068-015-0360-6.

Shin M, Park H, Kim S, Oh EJ, Jeong D, Florencia C, Kim KH, Jin YS, Kim SR. 2021. Transcriptomic Changes Induced by Deletion of Transcriptional Regulator GCR2 on Pentose Sugar Metabolism in Saccharomyces cerevisiae. *Frontiers in Bioengineering and Biotechnology* 9. DOI: 10.3389/fbioe.2021.654177.

Silva PC, Ceja-Navarro JA, Azevedo F, Karaoz U, Brodie EL, Johansson B. 2021. A novel d-xylose isomerase from the gut of the wood feeding beetle Odontotaenius disjunctus efficiently expressed in Saccharomyces cerevisiae. *Scientific Reports* 11. DOI: 10.1038/s41598-021-83937-z.

Da Silva NA, Srikrishnan S. 2012. Introduction and expression of genes for metabolic engineering applications in Saccharomyces cerevisiae. *FEMS Yeast Research* 12:197–214. DOI: 10.1111/j.1567-1364.2011.00769.x.

Subtil T, Boles E. 2012. Competition between pentoses and glucose during uptake and catabolism in recombinant Saccharomyces cerevisiae. *Biotechnology for Biofuels* 5. DOI: 10.1186/1754-6834-5-14.

1280 Tang H, Wu Y, Deng J, Chen N, Zheng Z, Wei Y, Luo X, Keasling JD. 2020. Promoter architecture and
1281 promoter engineering in saccharomyces cerevisiae. *Metabolites* 10:1–20. DOI:
1282 10.3390/metabo10080320.

1283 Tanino T, Ito T, Ogino C, Ohmura N, Ohshima T, Kondo A. 2012. Sugar consumption and ethanol
1284 fermentation by transporter-overexpressed xylose-metabolizing Saccharomyces cerevisiae harboring
1285 a xyloseisomerase pathway. *Journal of Bioscience and Bioengineering* 114:209–211. DOI:
1286 10.1016/j.jbiosc.2012.03.004.

1287 Toivari MH, Salusjärvi L, Ruohonen L, Penttilä M. 2004. Endogenous xylose pathway in Saccharomyces
1288 cerevisiae. *Applied and Environmental Microbiology* 70:3681–3686. DOI: 10.1128/AEM.70.6.3681-
1289 3686.2004.

1290 Traäff-Bjerre KL, Jeppsson M, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2004. Endogenous NADPH-
1291 dependent aldose reductase activity influences product formation during xylose consumption in
1292 recombinant Saccharomyces cerevisiae. *Yeast* 21:141–150. DOI: 10.1002/yea.1072.

1293 Träff KL, Cordero RRO, Van Zyl WH, Hahn-Hägerdal B. 2001. Deletion of the GRE3 Aldose Reductase
1294 Gene and Its Influence on Xylose Metabolism in Recombinant Strains of Saccharomyces cerevisiae
1295 Expressing the xylA and XKS1 Genes. *Applied and Environmental Microbiology* 67:5668–5674. DOI:
1296 10.1128/AEM.67.12.5668-5674.2001.

1297 Tran Nguyen Hoang P, Ko JK, Gong G, Um Y, Lee SM. 2018. Genomic and phenotypic characterization of
1298 a refactored xylose-utilizing Saccharomyces cerevisiae strain for lignocellulosic biofuel production.
1299 *Biotechnology for Biofuels* 11. DOI: 10.1186/s13068-018-1269-7.

1300 Tuller T, Ruppin E, Kupiec M. 2009. Properties of untranslated regions of the S. cerevisiae genome. *BMC*
1301 *Genomics* 10. DOI: 10.1186/1471-2164-10-391.

1302 Usher J, Balderas-Hernandez V, Quon P, Gold ND, Martin VJJ, Mahadevan R, Baetz K. 2011. Chemical
1303 and synthetic genetic array analysis identifies genes that suppress xylose utilization and fermentation
1304 in saccharomyces cerevisiae. *G3: Genes, Genomes, Genetics* 1:247–258. DOI:
1305 10.1534/g3.111.000695.

1306 Verho R, Londesborough J, Penttilä M, Richard P. 2003. Engineering Redox Cofactor Regeneration for
1307 Improved Pentose Fermentation in Saccharomyces cerevisiae. *Applied and Environmental*
1308 *Microbiology* 69:5892–5897. DOI: 10.1128/AEM.69.10.5892-5897.2003.

1309 Verhoeven MD, Lee M, Kamoen L, Van Den Broek M, Janssen DB, Daran JMG, Van Maris AJA, Pronk JT.
1310 2017. Mutations in PMR1 stimulate xylose isomerase activity and anaerobic growth on xylose of
1311 engineered Saccharomyces cerevisiae by influencing manganese homeostasis. *Scientific Reports* 7.

DOI: 10.1038/srep46155.

Van Vleet JH, Jeffries TW, Olsson L. 2008. Deleting the para-nitrophenyl phosphatase (pNPPase), PHO13, in recombinant Saccharomyces cerevisiae improves growth and ethanol production on d-xylose. *Metabolic Engineering* 10:360–369. DOI: 10.1016/j.ymben.2007.12.002.

Wahlbom CF, Cordero Otero RR, Van Zyl WH, Hahn-Hägerdal B, Jönsson LJ. 2003. Molecular analysis of a Saccharomyces cerevisiae mutant with improved ability to utilize xylose shows enhanced expression of proteins involved in transport, initial xylose metabolism, and the pentose phosphate pathway. *Applied and Environmental Microbiology* 69:740–746. DOI: 10.1128/AEM.69.2.740-746.2003.

Walfridsson M, Bao X, Anderlund M, Lilius G, Bülow L, Hahn-Hägerdal B. 1996. *Ethanolic fermentation of xylose with Saccharomyces cerevisiae harboring the Thermus thermophilus xylA gene, which expresses an active xylose (glucose) isomerase*. DOI: 10.1128/aem.62.12.4648-4651.1996.

Walfridsson M, Hallborn J, Penttila M, Keranen S, Hahn-Hagerdal B. 1995. Xylose-metabolizing Saccharomyces cerevisiae strains overexpressing the TKL1 and TAL1 genes encoding the pentose phosphate pathway enzymes transketolase and transaldolase. *Applied and Environmental Microbiology* 61:4184–4190.

Wang C, Bao X, Li Y, Jiao C, Hou J, Zhang Q, Zhang W, Liu W, Shen Y. 2015. Cloning and characterization of heterologous transporters in Saccharomyces cerevisiae and identification of important amino acids for xylose utilization. *Metabolic Engineering* 30:79–88. DOI: 10.1016/j.ymben.2015.04.007.

Wang PY, Schneider H. 1980. *Growth of yeast on D-xylulose*. DOI: 10.1139/m80-193.

Watanabe S, Kodaki T, Makino K. 2005. Complete reversal of coenzyme specificity of xylitol dehydrogenase and increase of thermostability by the introduction of structural zinc. *Journal of Biological Chemistry* 280:10340–10349. DOI: 10.1074/jbc.M409443200.

Watanabe S, Pack SP, Saleh AA, Annaluru N, Kodaki T, Makino K. 2007a. The positive effect of the decreased NADPH-preferring activity of xylose reductase from Pichia stipitis on ethanol production using xylose-fermenting recombinant Saccharomyces cerevisiae. *Bioscience, Biotechnology and Biochemistry* 71:1365–1369. DOI: 10.1271/bbb.70104.

Watanabe S, Saleh AA, Pack SP, Annaluru N, Kodaki T, Makino K. 2007b. Ethanol production from xylose by recombinant Saccharomyces cerevisiae expressing protein engineered NADP+-dependent xylitol dehydrogenase. *Journal of Biotechnology* 130:316–319. DOI: 10.1016/j.jbiotec.2007.04.019.

Watanabe S, Saleh AA, Pack SP, Annaluru N, Kodaki T, Makino K. 2007c. Ethanol production from xylose by recombinant Saccharomyces cerevisiae expressing protein-engineered NADH-preferring xylose

reductase from Pichia stipitis. *Microbiology* 153:3044–3054. DOI: 10.1099/mic.0.2007/007856-0.

Wei S, Bai P, Liu Y, Yang M, Ma J, Hou J, Liu W, Bao X, Shen Y. 2019. A Thi2p Regulatory Network Controls the Post-glucose Effect of Xylose Utilization in Saccharomyces cerevisiae. *Frontiers in Microbiology* 10. DOI: 10.3389/fmicb.2019.01649.

Wei S, Liu Y, Wu M, Ma T, Bai X, Hou J, Shen Y, Bao X. 2018. Disruption of the transcription factors Thi2p and Nrm1p alleviates the post-glucose effect on xylose utilization in Saccharomyces cerevisiae. *Biotechnology for Biofuels* 11. DOI: 10.1186/s13068-018-1112-1.

Weierstall T, Hollenberg CP, Boles E. 1999. *Cloning and characterization of three genes (SUT1-3) encoding glucose transporters of the yeast Pichia stipitis*. DOI: 10.1046/j.1365-2958.1999.01224.x.

Weinhandl K, Winkler M, Glieder A, Camattari A. 2014. *Carbon source dependent promoters in yeasts*. DOI: 10.1186/1475-2859-13-5.

Wenger JW, Schwartz K, Sherlock G. 2010. Bulk segregant analysis by high-throughput sequencing reveals a novel xylose utilization gene from Saccharomyces cerevisiae. *PLoS genetics* 6:e1000942. DOI: 10.1371/journal.pgen.1000942.

Wieczorke R, Krampe S, Weierstall T, Freidel K, Hollenberg CP, Boles E. 1999. *Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in Saccharomyces cerevisiae*. DOI: 10.1016/S0014-5793(99)01698-1.

Wijsman M, Swiat MA, Marques WL, Hettinga JK, Van Den Broek M, Torre Cortés PD La, Mans R, Pronk JT, Daran JM, Daran-Lapujade P. 2019. A toolkit for rapid CRISPR-SpCas9 assisted construction of hexose-transport-deficient Saccharomyces cerevisiae strains. *FEMS Yeast Research* 19. DOI: 10.1093/femsyr/foy107.

Xiong L, Zeng Y, Tang RQ, Alper HS, Bai FW, Zhao XQ. 2018. Condition-specific promoter activities in Saccharomyces cerevisiae. *Microbial Cell Factories* 17. DOI: 10.1186/s12934-018-0899-6.

Xu H, Kim S, Sorek H, Lee Y, Jeong D, Kim J, Oh EJ, Yun EJ, Wemmer DE, Kim KH, Kim SR, Jin YS. 2016. PHO13 deletion-induced transcriptional activation prevents sedoheptulose accumulation during xylose metabolism in engineered Saccharomyces cerevisiae. *Metabolic Engineering* 34:88–96. DOI: 10.1016/j.ymben.2015.12.007.

Xu L, Liu P, Dai Z, Fan F, Zhang X. 2021. Fine-tuning the expression of pathway gene in yeast using a regulatory library formed by fusing a synthetic minimal promoter with different Kozak variants. *Microbial Cell Factories* 20. DOI: 10.1186/s12934-021-01641-z.

Yamanaka K. 1969. *Inhibition of d-xylose isomerase by pentitols and d-lyxose*. DOI: 10.1016/0003-

9861(69)90422-6.

Yamanishi M, Ito Y, Kintaka R, Imamura C, Katahira S, Ikeuchi A, Moriya H, Matsuyama T. 2013. A genome-wide activity assessment of terminator regions in saccharomyces cerevisiae provides a "terminatome" toolbox. *ACS Synthetic Biology* 2:337–347. DOI: 10.1021/sb300116y.

Young EM, Comer AD, Huang H, Alper HS. 2012. A molecular transporter engineering approach to improving xylose catabolism in Saccharomyces cerevisiae. *Metabolic Engineering* 14:401–411. DOI: 10.1016/j.ymben.2012.03.004.

Young E, Poucher A, Comer A, Bailey A, Alper H. 2011. Functional survey for heterologous sugar transport proteins, using Saccharomyces cerevisiae as a host. *Applied and Environmental Microbiology* 77:3311–3319. DOI: 10.1128/AEM.02651-10.

Young EM, Tong A, Bui H, Spofford C, Alper HS. 2014. Rewiring yeast sugar transporter preference through modifying a conserved protein motif. *Proceedings of the National Academy of Sciences of the United States of America* 111:131–136. DOI: 10.1073/pnas.1311970111.

Zha J, Hu ML, Shen MH, Li BZ, Wang JY, Yuan YJ. 2012. Balance of XYL1 and XYL2 expression in different yeast chassis for improved xylose fermentation. *Frontiers in Microbiology* 3. DOI: 10.3389/fmicb.2012.00355.

Zhang GC, Liu JJ, Ding WT. 2012. Decreased xylitol formation during xylose fermentation in saccharomyces cerevisiae due to overexpression of water-forming NADH oxidase. *Applied and Environmental Microbiology* 78:1081–1086. DOI: 10.1128/AEM.06635-11.

Zhou H, Cheng J sheng, Wang BL, Fink GR, Stephanopoulos G. 2012. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by Saccharomyces cerevisiae. *Metabolic Engineering* 14:611–622. DOI: 10.1016/j.ymben.2012.07.011.

Figure 1

Xylose metabolism in *S. cerevisiae*

The metabolic pathways for glucose and xylose metabolism in *Saccharomyces cerevisiae*. Metabolic pathways for xylose uptake are indicated, including glycolysis and gluconeogenesis, Oxidative and Non-Oxidative Pentose Phosphate Pathway and PK-PTA-AADH Pathway, and the main genes involved in xylose metabolism. Colored boxes represent different paths; names in black represent consumed/produced molecules; the names in gray are the genes that encode the enzymes that participate in each reaction; red are the cofactors; in bold the heterologous genes responsible for key enzymes in xylose metabolism in *S. cerevisiae*. (XYL1 = xylose reductase), (XYL2 = xylitol dehydrogenase) and (XYLA= xylose isomerase)

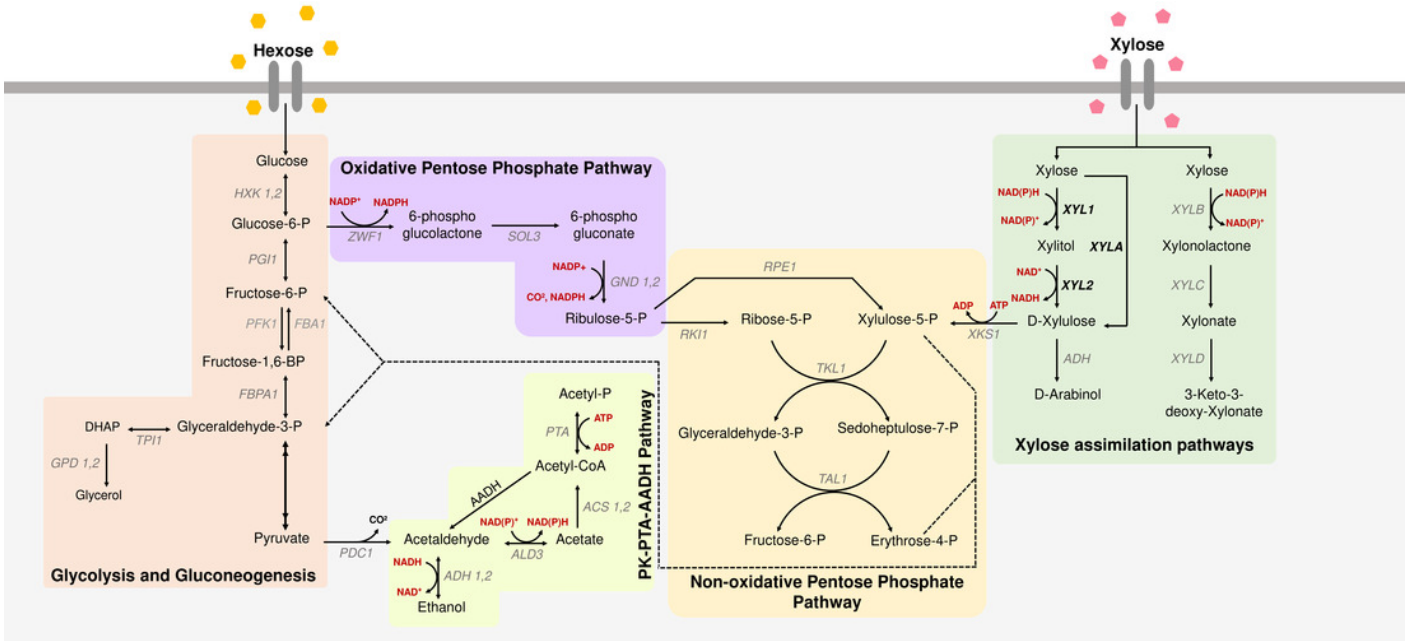

Table 1(on next page)

Heterologous pathways for xylose fermentation in *S. cerevisiae*

Heterologous pathways for xylose fermentation in *S. cerevisiae*: the main genes used for the oxidoreductive (XR/XDH) and xylose isomerase (XI) pathways

Table 1. Heterologous pathways for xylose fermentation in *S. cerevisiae*: the main genes used for the oxidoreductive (XR/XDH) and xylose isomerase (XI) pathways.

Pathway	Gene	Source Microorganism	Codon otimization	Xylose consumed/Ethanol produced	Yield in g of ethanol per g of substrate	Ethanol yield	Reference
XR/XDH	*XYL1/XYL2*	*Scheffersomyces stipitis*	Y	14 g/L / 0.73 g/L	0.052	10%	(Kötter et al., 1990)
	XYL1/XYL2	*Scheffersomyces stipitis*	N	34 g/L / NP	-	-	(Walfridsson et al., 1995)
	XYL1/XYL2	*Scheffersomyces stipitis*	Y	50 g/L / ~22.5 g/L	0.45	88%	(Ho, Chen & Brainard, 1998)
	XYL1.2	*Spathaspora passalidarum*	N	50 g/L / 20 g/L	0.4	78%	(Cadete et al., 2016)
XI	*XYLA*	*Thermus thermophilus*	Y	10.4 g/L / 1.3 g/L	0.125	24.4%	(Walfridsson et al., 1996)
	XYLA	*Clostridium phytofermentans*	Y	~18 g/L / ~7.74 g/L	0.43	84%	(Brat, Boles & Wiedemann, 2009)
	XYLA	*Bacteroides*	N	15.7 g/L / 4.9	0.312	66%	(Ha et al.,

		stercoris		g/L			2011)
	XYLA	*Prevotella ruminicola*	Y	32.1 g/L / 13.6 g/L	0.41	82.9%	(Hector et al., 2013)
	XYLA	*Piromyces sp. E2*	N	20 g/L / 8.68	0.43	84.5%	(Kuyper et al., 2003, 2004, 2005)
	XYLA	*Piromyces sp. E2*	Y	40 g/L / 16.8 g/L	0.41	81%	(Zhou et al., 2012)
	XYLA	*Orpinomyces sp.*	N	15.55 g/L / 6.05 g/L	0.39	78%	(Madhavan et al., 2009)
	XYLA	*xym1* and xym2 *(soil metagenomic library)*	N	NM	NM	NM	(Parachin & Gorwa-Grauslund, 2011)
	XYLA (K11T/D220V)	*Bovine rumen*	N	~18 g/L / ~7.5 g/L	0.06	80%	(Hou et al., 2016)
	XYLA	*Reticulitermes speratus*	Y	51 g/L / 20 g/L	0.39	77%	(Katahira et al., 2017)
	XYLA	*Odontotaenius disjunctus*	Y	NM	NM	NM	(Silva et al., 2021)

3 NP* Not produced.

NM* the value was not measured.

Table 2(on next page)

Deletion targets

Deletion targets that contribute to improved xylose metabolism in *S. cerevisiae*

1 **Table 2.** Deletion targets that contribute to improved xylose metabolism in *S. cerevisiae*.

Deletion targets	Relevant genetic Background	Reported Phenotype improvement	Reference
GRE3	*Thermus thermophilus XYLA / XKS1*	Xylitol formation decreased two-fold, and which produced ethanol from xylose with a yield of 0.28 mmol	(Träff et al., 2001)
	Thermus thermophilus mutated XYLA / XKS1	Deletion of *GRE3* was crucial for ethanol production as reduction of xylitol formation was observed	(Lönn et al., 2003)
NFG1 / MNI1 / RPA49	*Scheffersomyces stipitis XYL1* and *XYL2 / XKS1*	Improved growth rates on xylose in aerobiosis compared to the reference strain: 173% (*nfg1*Δ), 62% (*mni1*Δ) and 90% (*rpa49*Δ) faster	(Bengtsson et al., 2008)
NFG1	*XYL1 / XYL2 / XKS1*	Improvement of xylose consumption at low concentrations and in co-fermentation of glucose and xylose; deletion of *NFG1* improved aerobic growth on xylose	(Parachin et al., 2010)
BUD21 / ALP1 / ISC1 / RPL20B	*XYLA / XKS1*	Individual deletion of the 4 genes improved xylose assimilation in 27.6% (*bud21*Δ), 15.5% (*alp1*Δ), 22.4% (*isc1*Δ) and 12.1% (*rpl20b*Δ); production of ethanol in *bud21*Δ cells even without the presence *XYLA*	(Usher et al., 2011)
PMR1	*Piromyces sp. XYLA / XKS1 / RKI1 / RPE1 / TKL1 / TKL2 /*	Deletion of *PMR1* allowed anaerobic growth on xylose	(Verhoeven et al., 2017)

	*TAL1 / NQM1 / gre3*Δ		
PMR1 / ASC1	*Piromyces mutated XYLA3* / TAL1 / XKS1 / gre3*Δ *// pho13*Δ	Mutated *PMR1* and *ASC1* consumed 114.8% and 59.6% more xylose in relation to the control, respectively	(Tran Nguyen Hoang et al., 2018)
GRE3 / HOG1 / IRA2 / ISU1	*Clostridium phytofermentans XYLA / TAL1 / S. stipitis XYL3*	The mutation in *IRA2* only affects anaerobic xylose consumption; loss of *ISU1* function is indispensable for anaerobic xylose fermentation; Faster conversion of xylose obtained by deleting the *gre3*Δ, *hog1*Δ, *ira2*Δ and *isu1*Δ genes simultaneously	(Sato et al., 2016)
ISU1 / SSK2	*Orpinomyces sp. XYLA / XKS1 / RKI1 / RPE1 / TKL1 / TKL2 / TAL1 / gre3*Δ	*ISU1* or *SSK2* null strains showed improvement in xylose metabolism in unevolved yeast cells	(Dos Santos et al., 2016)
PHO13	*Scheffersomyces stipitis XYL1* and *XYL2*	Improvement in xylose assimilation	(Ni, Laplaza & Jeffries, 2007)
	Scheffersomyces stipitis XYL1, XYL2 and *XYL3*	Upregulation of the enzymes from PPP and NADPH-producing enzymes; improved xylose metabolism	(Kim et al., 2015)
	Scheffersomyces stipitis XYL1, XYL2 and *XYL3*	Transcriptional activation of genes from PPP; 98% reduction of sedoheptulose by upregulation of *tal1* in mutant strains (*pho13*Δ)	(Xu et al., 2016)
PHO13 / ALD6	*Scheffersomyces stipitis XYL1* and *XYL2*	*pho13*Δ strains presented a shorter lag time using xylose as carbon source and	(Kim et al., 2013)

		showed an improved xylose fermentation / *ald6*Δ strains showed improvement in the efficiency of xylose fermentation and prevention of acetate accumulation	
GCR2	*Scheffersomyces stipitis XYL1, XYL2* and *XYL3*	*gcr2*Δ cells with better xylose utilization and ethanol production.	(Shin et al., 2021)
THI2	Ru-*XYLA* (where Ru represents the rumen bovine) / *XKS1 / RKI1 / RPE1 / TKL1 / TKL2 / TAL1 / cox4*Δ */ gre3*Δ	Deletion increases 17.4% in growth rate, increase of 26.8% in specific xylose utilization rate and 32.4% increase in specific ethanol production rate in co-fermentation of glucose and xylose	(Wei et al., 2018)
CCC1 / BSD2	*Orpinomyces sp. XYLA / XKS1 / RKI1 / RPE1 / TKL1 / TKL2 / TAL1 / gre3*Δ	*ccc1*Δ and *bsd2*Δ strains had a 9-fold and 2.3-fold increase in xylose consumption	(Palermo et al., 2021)
HAP4	*Scheffersomyces stipitis XYL1, XYL2* and *XYL3*	*hap4*Δ strain: 1.8-fold increase in ethanol production from xylose; production of 10.38 g/L of ethanol; ethanol yield of 0.41 g/g of xylose	(Dzanaeva et al., 2021)

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Table 3(on next page)

Overexpression targets

Overexpression targets that contribute to improved xylose metabolism in *S. cerevisiae*

Table 3. Overexpression targets that contribute to improved xylose metabolism in *S. cerevisiae*.

Overexpression targets	Relevant genetic Background	Reported Phenotype Improvement	Reference
XKS1	*Scheffersomyces stipitis XYL1, XYL2*	Fermentation at high xylose concentrations and reduced xylitol production	(Ho, Chen & Brainard, 1998)
	Scheffersomyces stipitis XYL1, XYL2 and *XYL3 / pho13*Δ	Overexpression of XK genes (XYL3 or XKS1) increases the rate of xylose assimilation and maintain ATP levels inside cells	(Kim et al., 2013)
	LSK1 - xylulokinase mutant	Increased enzyme activity improved xylulose conversion and accelerated ethanol production by 30-130%	(Deng & Ho, 1990)
	Scheffersomyces stipitis XYL1, XYL2	Deleterious effect associated with uncontrolled overexpression of XKS1 / xylulose-5-phosphate accumulation and ATP depletion	(Johansson et al., 2001)
	FY1679 *(ura3- 52/ura3-52; his3v200/his3; leu2v1/leu2; trp1v63/ trp1; gal2/gal2)*	High levels of expression of this gene have	(Rodriguez-Peña et al., 1998)

		a deleterious effect	
XKS1 / Scheffersomyces stipitis XYL3	*Scheffersomyces stipitis XYL1, XYL2*	Growth inhibition on xylose / expression levels should consider the metabolic capacity of the strain	(Jin et al., 2003)
RPE1 / RKI1 / TAL1 / TKL1	*Piromyces sp*. E2 *XYLA / gre3*Δ	Specific xylose consumption rate of 1.1 g g^{-1} h^{-1}	(Kuyper et al., 2005)
	Scheffersomyces stipitis XYL1 and *XYL2 / XKS1 / gre3*Δ	Increased rate of xylose consumption	(Karhumaa et al., 2007a)
TAL1	*Scheffersomyces stipitis XYL1, XYL2 and XYL3 / gre3*Δ	Improvement in xylose assimilation	(Ni, Laplaza & Jeffries, 2007)
TAL1 / TKL1	*Scheffersomyces stipitis XYL1* and *XYL2 / XKS1*	Important role in xylose consumption and fermentation	(Matsushika et al., 2012)
XKS1 / TAL1/ TKL1 / SOL3 / GND1	*Scheffersomyces stipitis XYL1* and *XYL2 / XKS1*	Increased consumption of xylose by 31%	(Wahlbom et al., 2003)
GND1 / SOL3 / TAL1 / RKI1 / TKL1	*Orpinomyces sp. XYLA / XKS1 / gre3*Δ */ pho13*Δ	Improves xylose consumption rate	(Bamba, Hasunuma & Kondo, 2016)
SOL3 / TAL1	*Scheffersomyces stipitis XYL1* and *XYL2 / XKS1*	Fastest growth on xylose by 19% (SOL3) and 24% (TAL1)	(Bengtsson et al., 2008)
RPE1	*Piromyces sp*. *XYLA**3 / *pho13*Δ / *gre3*Δ */ asc1*Δ	Significantly improved xylose utilization	(Hoang Nguyen Tran et al., 2020)

NRM1/YHP1	Ru-*XYLA* / *XKS1 / RKI1 / RPE1 / TKL1 / TKL2 / TAL1 / cox4Δ / gre3Δ*	NRM1 increased the xylose utilization rate by 30%. YHP1 increased the volumetric xylose utilization rate by 5.6%	(Wei et al., 2018)
STT4 / RGI2 / TFC3	Ru-*XYLA / XKS1 / RKI1 / RPE1 / TKL1 / TKL2 / TAL1 / cox4Δ /gre3Δ*	Increased xylose specific utilization rates: STT4 (36.9%), RGI2 (29.7%) and TFC3 (42.8%)	(Wei et al., 2019)
Piromyces sp. E2 *XYLA*	*Scheffersomyces stipitis XYL3* and *TAL1 / RPE1 / RKI1 / TKL1*	Xylose consumption rate of 1.866 g g^{-1} h^{-1}	(Zhou et al., 2012)
XYL2	*Scheffersomyces stipitis XYL1* and *XYL3*	Increased ethanol yields and decrease in xylitol production	(Kim et al., 2012)

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Table 4(on next page)

Sugar transporters

Sugar transport modifications for improved xylose consumption in *S. cerevisiae* using heterologous expression and endogenous modification strategies

1 **Table 4.** Sugar transport modifications for improved xylose consumption in *S. cerevisiae*
2 using heterologous expression and endogenous modification strategies.

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Strategy	Transporter Genes	Mutation	Relevant genetic Background	Reported Phenotype Improvement	Reference
Heterologous expression	*GXF1* (*Candida intermedia*)	-	MT8-1 - XKδXI	Enhanced xylose consumption and ethanol production	(Tanino et al., 2012)
		-	TMB 3043 - *Scheffersomyces stipitis XYL1* and *XYL2* / *XKS1* / *RKI1* / *RPE1* / *TKL1* / *TKL2* / *TAL1* / *gre3*Δ	Under anaerobic conditions, increased xylose uptake and ethanol formation at low xylose concentrations	(Runquist et al., 2009)
		-	*Scheffersomyces stipitis XYL1* and *XYL2* / *XKS1*	2 times higher affinity for xylose	(Fonseca et al., 2011)
	GXS1 (*Candida intermedia*)	Phe[38]Ile[39]Met[40]	EX.12 - *Scheffersomyces stipitis XYL1*, *XYL2* and *XYL3* / *hxt1-17*Δ / *gal2*Δ	Growth on xylose but does not assimilate glucose	(Young et al., 2014)
	GXS1 (*Candida intermedia*)	F40	EX.12 - *Scheffersomyces stipitis XYL1*, *XYL2* and *XYL3* / *hxt1-17*Δ / *gal2*Δ	Increased affinity for xylose	(Young et al., 2012)
	XUT3 (*Scheffersomyces stipitis*)	E538K	EX.12 - *Scheffersomyces stipitis XYL1*, *XYL2* and *XYL3* / *hxt1-17*Δ / *gal2*Δ	Increased xylose uptake	(Young et al., 2012)
	SUT1 (*Scheffersomyces stipitis*)	-	*Scheffersomyces stipitis XYL1* and *XYL2* / *XKS1*	Increased xylose absorption capacity and ethanol productivity in	(Katahira et al., 2008)

				fermentation	
	XUT1 (Scheffersomyces stipitis)	-	EBY.VW4000 - *Scheffersomyces stipitis XYL1* and *XYL2 / hxt1-17Δ / gal2Δ*	Greater preference for xylose over glucose	(Young et al., 2011)
	CS4130 (Candida sojae)	-	EBY.VW4000 - *Scheffersomyces stipitis XYL1* and *XYL2* / XKS1 / *hxt1-17Δ / gal2Δ*	Xylose absorption at high substrate concentrations	(Bueno et al., 2020)
	MGT05196P (Meyerozyma guilliermondii)	N360F	EBY.VW4000 - *Scheffersomyces stipitis XYL1* and *XYL2* / XKS1 / *hxt1-17Δ / gal2Δ*	Xylose transport without inhibition by glucose	(Wang et al., 2015)
	XITR1P (Trichoderma reesei)	N326F	EBY.VW4000 - *hxt1-17Δ / gal2Δ*	High xylose transport activity / low growth in glucose	(Jiang et al., 2020)
	HXTB (*Aspergillus nidulans*)	-	EBY.VW4000 - *Scheffersomyces stipitis XYL1* and *XYL2* / XKS1 / *hxt1-17Δ / gal2Δ*	Higher xylose growth and ethanol production	(Dos Reis et al., 2016)
	XTRD (*Aspergillus nidulans*)	-	EBY.VW4000 - *Scheffersomyces stipitis XYL1* and *XYL2 / XKS1 / hxt1-17Δ / gal2Δ*	Higher affinity for xylose	(Colabardini et al., 2014)
	AT5G17010 (Arabidopsis thaliana)	-	BY4727 - *Scheffersomyces stipitis XYL1* and *XYL2 / XKS1*	25% and 40% increase in xylose consumption	(Hector et al., 2008)
	AT5G59250 (Arabidopsis thaliana)	-			
	AT5G59250 (Arabidopsis thaliana)	-	*Scheffersomyces stipitis XYL1* (K270R) and *XYL2 / XKS1 / TAI1 / TKL1 / RPK1 / RPE1 / gre3Δ*	Did not present significant results in the kinetics of xylose absorption	(Runquist, Hahn-Hägerdal & Rådström, 2010)

Endogenous modification	*HXT7*	F79S	BY4742 - *Piromyces sp. XYLA* / *XKS1* / *gre3*Δ	Improved xylose absorption rates	(Apel et al., 2016)
	HXT7	N370S	AFY10X - *Clostridium phytofermentans XYLA* / *TAL1* / *TKL1* / *RPI1* / *RKI1* / *XKS1* / *hxk1*Δ / *hxk2*Δ / *glk1*Δ / *gal1*Δ	Decreased inhibition by the presence of glucose	(Farwick et al., 2014)
	GAL2	N376F	AFY10X – *Clostridium phytofermentans XYLA* / *TAL1* / *TKL1* / *RPI1* / *RKI1* / *XKS1* / *hxk1*Δ / *hxk2*Δ / *glk1*Δ / *gal1*Δ	Lost the ability to transport hexoses and has a greater affinity for xylose	
		N376Y / M435I	SRY027 - *XYLA* / *TAL1* / *TKL1* / *RPE1* / *RKI1* / *XKS1* / *HXT2* / *HXT7* / *HXT9* / *GAL2*	Reduction of xylose consumption time by approximately 40%	(Rojas et al., 2021)
		T386A	DS69473 - *Clostridium phytofermentans XYLA* / *TAL1* / *TKL1* / *RPE1* / *RKI1* / *XKS1* / *gre3*Δ / *hxt1-7*Δ / *gal2*Δ / *hxk1*Δ, *hxk2*Δ, *glk1*Δ, *gal1*Δ	Increased xylose transport and reduced affinity for glucose	(Reznicek et al., 2015)
	HXT36 (Chimeric)	N367A	DS71054 - *XYLA* / *TAL1* / *TKL1* / *RPE1* / *RKI1* / *XKS1* / *gre3*Δ / *hxt1-7*Δ / *gal2*Δ / *glk1*Δ / *hxk1*Δ / *hxk2*Δ / *gal1*Δ	Xylose transport at high rates / efficient co-consumption of glucose and xylose	(Nijland et al., 2014)
	HXT11	N366	DS68625 - *XYLA* / *TAL1* / *TKL1* / *RPE1* / *RKI1* / *XKS1* / *hxt1-7*Δ / *gre3*Δ / *gal2*Δ	Increased affinity for xylose compared to glucose / high transport rates / efficient co-fermentation of xylose and glucose	(Shin et al., 2015)

	HXT2	C505P	DS68625 - *XYLA* / *TAL1* / *TKL1* / *RPE1* / *RKI1* / *XKS1* / *hxt1-7*Δ / *gre3*Δ / *gal2*Δ	Increased affinity and xylose transport flux at low concentrations of this substrate	(Nijland et al., 2018)

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