Research review paper

Strain engineering of *Saccharomyces cerevisiae* for enhanced xylose metabolism

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ABSTRACT

Efficient and rapid fermentation of all sugars present in cellulosic hydrolysates is essential for economic conversion of renewable biomass into fuels and chemicals. Xylose is one of the most abundant sugars in cellulosic biomass but it cannot be utilized by wild type *Saccharomyces cerevisiae*, which has been used for industrial ethanol production. Therefore, numerous technologies for strain development have been employed to engineer *S. cerevisiae* capable of fermenting xylose rapidly and efficiently. These include i) optimization of xylose-assimilating pathways, ii) perturbation of gene targets for reconfiguring yeast metabolism, and iii) simultaneous co-fermentation of xylose and cellobiose. In addition, the genetic and physiological background of host strains is an important determinant to construct efficient and rapid xylose-fermenting *S. cerevisiae*. Vibrant and persistent researches in this field for the last two decades not only led to the development of engineered *S. cerevisiae* strains ready for industrial fermentation of cellulosic hydrolysates, but also deepened our understanding of operational principles underlying yeast metabolism.

Contents

1. Introduction

While corn or sugar-based ethanol is commercially produced in the US and Brazil, many limitations in terms of sustainability and ethics have been raised. Although cellulosic ethanol production can replace substantial portions of petroleum-based liquid fuels while substantially reducing greenhouse gas emissions (Searchinger et al.,

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2008), cellulosic ethanol is not commercially produced due to technical difficulties. Among the technical difficulties, incomplete and inefficient fermentation of carbohydrates present in cellulosic biomass is a serious problem hampering economic production of cellulosic ethanol.

Lignocellulosic materials such as energy crops, wood, and non-edible parts of food crops consist of cellulose, hemicellulose, and lignin (Mosier et al., 2005). Regardless of hydrolysis methods, glucose and xylose are the most abundant sugars in cellulosic hydrolysates. Typically, cellulosic hydrolysates consist of 60–70% glucose and 30–40% xylose (Mosier et al., 2005). Therefore, efficient and rapid utilization of xylose is a pre-requisite for producing biofuels and chemicals from renewable biomass sustainably and economically (Hahn-Hägerdal et al., 2006; Somerville, 2007). While xylose-fermenting microorganisms exist in nature (Jeffries et al., 2007), they cannot conduct xylose fermentation under industrially relevant fermentation conditions, such as high osmotic levels, strict anaerobic environment or with fermentation inhibitors that are inevitably present in cellulosic hydrolysates (Jeffries and Jin, 2004; Rudolf et al., 2008). Therefore, numerous metabolic engineering approaches to introduce xylose metabolic pathways into *Saccharomyces cerevisiae*, the most widely used microorganism for industrial production of ethanol from corn or sugarcane, have been practiced. This review will discuss metabolic engineering strategies to improve xylose fermentation capabilities of engineered *S. cerevisiae*. As many reviews have already been published on this theme, we will focus on recent progress in the areas of 1) optimization of heterologous xylose-assimilating metabolism, 2) metabolic reconfiguration of the native pathways for efficient xylose fermentation,

and 3) simultaneous co-fermentation of mixed sugars derived from cellulosic biomass. In addition, we aimed to provide a comprehensive and objective comparison of fermentation performances by engineered *S. cerevisiae* strains constructed by different strategies.

2. Engineering of xylose-assimilating pathways in *S. cerevisiae*

Two heterologous xylose-assimilating pathways are currently being used to engineer xylose-fermenting *S. cerevisiae* (Fig. 1): xylose isomerase (XI) (Brat et al., 2009; Ha et al., 2011b; Karhumaa et al., 2007b; Kuyper et al., 2005a; Madhavan et al., 2009; Walfridsson et al., 1996), and xylose reductase (XR) and xylitol dehydrogenase (XDH) (Ho et al., 1998; Jin et al., 2000; Kötter et al., 1990; Walfridsson et al., 1995). Both pathways require overexpression of xylulokinase (XK) which connects xylulose to the endogenous pentose phosphate pathway of *S. cerevisiae* (Jin et al., 2005; Johansson et al., 2001; Lee et al., 2003a; Toivari et al., 2001; Wahlbom et al., 2003a). Due to different biochemical properties and different origins of the pathways, there are various considerations for expressing them efficiently in *S. cerevisiae*. Studies for the optimization of the xylose metabolism are still ongoing; therefore, it is still difficult to determine which pathway is better for engineering *S. cerevisiae*.

2.1. Expression of xylose isomerase in S. cerevisiae

Xylose isomerase (XI) genes identified from bacteria such as *Thermus thermophiles* (Walfridsson et al., 1996), *Clostridium phytofermentans* (Brat
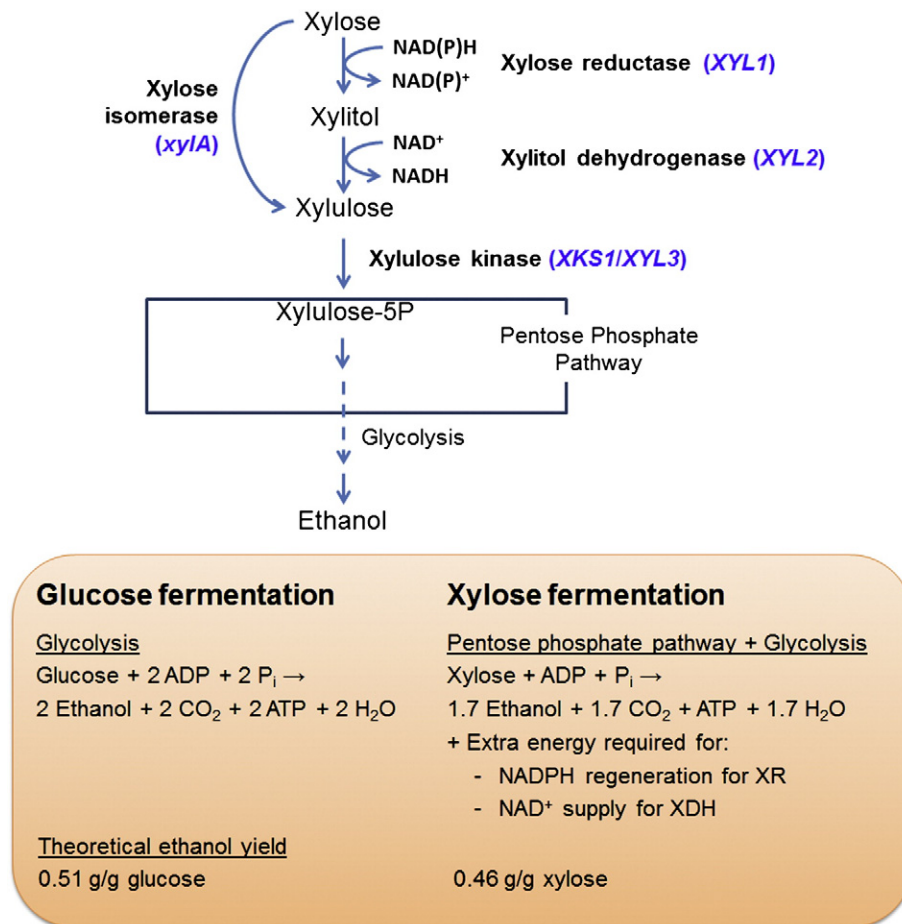
Fig. 1. Two xylose-assimilating pathways (top) and comparison of glucose and xylose fermentation (bottom). There are two types of xylose-assimilating pathways in nature: xylose isomerase (XI) and xylose reductase/xylitol dehydrogenase (XR/XDH) (the blue letters in parentheses represent the genes coding for the respective enzymes). Both the pathways require overexpression of endogenous xylulokinase (*XKS1*) or the introduction of heterologous xylulokinase (*XYL3*) for ethanol production from xylose in *S. cerevisiae*. Xylose fermentation is inferior to glucose fermentation due to a low ATP yield and unbalanced cofactor requirements of the XR/XDH pathway. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2009), and *Bacteroides stercoris* (Ha et al., 2011b) or anaerobic fungi such as *Piromyces* sp. E2 (Karhumaa et al., 2007b; Kuyper et al., 2005a) and *Orpinomyces* sp. (Madhavan et al., 2009) have been functionally expressed in *S. cerevisiae*. Although many bacterial xylose isomerase genes were identified, their functional expression in *S. cerevisiae* was not successful (Amore et al., 1989; Moes et al., 1996; Sarthy et al., 1987). Screening for a novel xylose isomerase was performed recently using *Escherichia coli* expressing a soil metagenomic library (Parachin and Gorwa-Grauslund, 2011). *S. cerevisiae* strains expressing newly isolated xylose isomerase genes (*xym1* and *xym2*), however, exhibited only 25% of the growth rate of a strain expressing *Piromyces* XylA. For the successful expression of bacterial xylose isomerase genes in *S. cerevisiae*, the gene search has to be done directly in *S. cerevisiae* or the gene synthesis for optimizing their codon usage might be required.

Codon optimization is performed by changing the original codons of the heterologous genes to those of highly expressed genes in *S. cerevisiae*, like glycolytic enzymes, through a commercial service for gene synthesis. Expression of prokaryotic genes in *S. cerevisiae* often requires their codon-optimization to achieve desired phenotypes (Wiedemann and Boles, 2008). Recently, the strain expressing the codon-optimized XI gene from *C. phytofermentans* showed a 46% improved specific growth rate on xylose in comparison to the strain expressing the original gene (Brat et al., 2009). More studies will be undertaken to test codon-optimized xylose isomerase genes by means of inexpensive gene synthesis services.

2.2. Expression of xylose reductase and xylitol dehydrogenase in S. cerevisiae

For the heterologous expression of XR and XDH in *S. cerevisiae*, a lot of studies focused on the *XYL1* and *XYL2* genes derived from *Scheffersomyces stipitis* (used to be *Pichia stipitis*) (Ho et al., 1998; Jin et al., 2000; Kötter et al., 1990; Walfridsson et al., 1995). Although the XR/XDH pathway has two intrinsic defects including xylitol secretion and unbalanced cofactor requirements, its thermodynamic advantage compared to the XI pathway yielded faster xylose assimilation and ethanol production (Karhumaa et al., 2007b). For the optimization of the XR/XDH pathway, protein engineering was attempted to modify cofactor specificities of XR and XDH (Bengtsson et al., 2009; Jeppsson et al., 2006; Khoury et al., 2009; Lee et al., 2012; Matsushika et al., 2008; Petschacher and Nidetzky, 2005; Watanabe et al., 2005, 2007a, 2007b). The expression levels of the *XYL1/XYL2/XYL3* gene also played an important role in the xylose metabolism (Eliasson et al., 2001; Jeppsson et al., 2003; Jin et al., 2003; Karhumaa et al., 2007a; Kim et al., 2012a; Matsushika and Sawayama, 2008, 2011; Parachin et al., 2011; Walfridsson et al., 1997). The details about the protein engineering and the optimized expression of XR and XDH are described below.

2.2.1. Cofactor specificity of xylose reductase and xylitol dehydrogenase

The dual cofactor preference of NADPH and NADH-dependent XR (encoded by *XYL1*) brought up two issues when it was coexpressed with NAD$^+$-specific XDH (*XYL2*): NAD$^+$ deficiency and NADPH regeneration. It is speculated that the cofactor imbalance problem is responsible for defects in xylose fermentation, primarily for xylitol accumulation (Krahulec et al., 2012). Genome-scale modeling of engineered *S. cerevisiae* metabolic pathways predicted that a balanced cofactor system of XR/XDH would increase ethanol yield by 25% and xylose consumption rate by 70% (Ghosh et al., 2011). Site-directed mutagenesis of cofactor-binding sites (Petschacher and Nidetzky, 2005; Watanabe et al., 2005) and computational redesign of proteins (Khoury et al., 2009) successfully altered the cofactor preference of XR or XDH to balance their requirements. Many studies demonstrated that the expression of an NADH-preferring XR mutant (K270M, K270R, K270R/N272D, N272D/P275Q, R276H) together with the wild type XDH specific for NAD$^+$ improved ethanol yield and productivity while decreasing xylitol yield (Bengtsson

et al., 2009; Lee et al., 2012; Watanabe et al., 2007a). Engineered *S. cerevisiae* strains expressing the wild type XR and an NADP$^+$-specific XDH mutant (D207A/I208R/F209S/N211R) also showed lower xylitol and higher ethanol yields while metabolizing xylose 32% faster than a recombinant yeast strain expressing the wild types of XR and XDH (Matsushika et al., 2008; Watanabe et al., 2007b). Coexpression of XR and XDH enzymes originated from other organisms also has the same redox imbalance issue. Engineering of recombinant *S. cerevisiae* expressing both a mutant XR from *Candida tenuis* (K274R/N276D) preferring NADH and a wild type XDH from *Galactocandida mastotermitis* improved ethanol production and reduced byproduct formation (Krahulec et al., 2010; Petschacher and Nidetzky, 2008). Although the beneficial effects of the cofactor match on xylitol and ethanol yields of the engineered *S. cerevisiae* strains were found in various studies, we still do not understand how *Sch. stipitis* metabolizes xylose efficiently with the native XR/XDH enzymes possessing different cofactor requirements (Gutiérrez-Rivera et al., 2012; Wahlbom et al., 2003b). Hence, excess supply of oxygen did not resolve the redox imbalance issue contrast to what flux balance analysis predicted (Jin and Jeffries, 2004). This may suggest that 1) cofactor imbalance is not a major factor hindering xylose fermentation or 2) another mechanism compensates the redox imbalance caused by the reaction of the native XR and XDH enzymes in *Sch. stipitis*.

2.2.2. Relative activities of xylose reductase and xylitol dehydrogenase

Although two studies (Jeppsson et al., 2003; Karhumaa et al., 2007a) reported that the relatively high enzymatic activity of XR compared to XDH decreased xylitol yields, there are more experimental results supporting the opposite. A kinetic model predicted that a low XR/XDH activity ratio (≤ 0.1) would minimize xylitol production (Eliasson et al., 2001). The prediction corresponded to the experimental results: a yeast strain with 0.067 of XR/XDH ratio produced 50% less xylitol (0.1 g/g xylose) than a yeast strain with 7.1 of XR/XDH ratio, while the former strain produced 63% more ethanol at a similar xylose consumption rate (approximately 0.4 g/l/h) (Eliasson et al., 2001). Another study reported a similar result that a recombinant *S. cerevisiae* strain with 0.059 of XR/XDH ratio consumed 3.25 g/l xylose and did not accumulate xylitol, while the same strain with 5 of XR/XDH ratio consumed 2.0 g/l xylose and accumulated xylitol at a yield of 0.58 (g/g xylose) during 50 h of fermentation (Walfridsson et al., 1997). Even if the XR activities of the two strains were identical, a yeast strain with 0.57 of XR/XDH ratio produced 50% lower xylitol than a yeast strain with 7.1 of XR/XDH ratio, when grown with approximately 20 g/l xylose during 180 h (Jin and Jeffries, 2003). Recently, low expression levels of the *XYL2* gene were identified as a major reason for xylitol accumulation in strains expressing *XYL2* and *XYL3* with either *XYL1* or *GRE3* (coding for aldose reductase) (Kim et al., 2012a; Kim et al., in press). These results suggested that the absolute and relative activities of XDH need to be high to minimize xylitol accumulation.

2.3. Optimal activities of xylulokinase

Overexpression of endogenous *S. cerevisiae* XK gene (*XKS1*) or the introduction of heterologous XK gene (*XYL3* from *Sch. stipitis*) is required to facilitate xylose metabolism through the pentose phosphate pathway (Eliasson et al., 2001; Jin et al., 2005; Johansson et al., 2001; Lee et al., 2003a; Matsushika and Sawayama, 2011; Parachin et al., 2011; Toivari et al., 2001; Träff-Bjerre et al., 2001; Wahlbom et al., 2003a). However, it was suggested that too strong expression of the XK genes could cause growth inhibition on xylose (Jin et al., 2003; Ni et al., 2007; Rodriguez-Peña et al., 1998) or reduction in a xylose consumption rate (Johansson et al., 2001). In a strain overexpressing *XYL1* and *XYL2*, moderate expression of *XKS1* led to basal xylitol accumulation and more ethanol production (Matsushika and Sawayama, 2008). The detrimental effect of the XK gene overexpression might be explained by "substrate-accelerated

death" as described previously (Teusink et al., 1998). Glycolysis has a regulatory mechanism to prevent overly rapid hexokinase activity for the later portions of glycolysis, where ATP is produced. Because the heterologous xylose-assimilating pathway does not lie under an elaborate regulatory system, an elevated ATP consumption rate by XK overexpression can exceed an ATP production rate, resulting in ATP depletion (Jin et al., 2003). On the other hand, two recent studies suggested that high expression of a XK gene was the most important factor for reduction of xylitol yield (Matsushika and Sawayama, 2011; Parachin et al., 2011). Kinetic modeling of xylose transport and the XR/XDH/XK pathway identified XK as a limiting step, and strains overexpressing either XR/XDH or XI had significant reduction in xylitol when additional XK gene was integrated (Parachin et al., 2011). These contradictions suggested that a high metabolic flux through the xylose-assimilating pathway consisting of XR/XDH/XK might be more important than activities of individual enzymes to minimize xylitol accumulation and to improve ethanol yield.

3. Metabolic reconfiguration of endogenous metabolic pathways in S. cerevisiae

Internal limitations of engineered S. cerevisiae metabolizing xylose have been identified regardless of the type of xylose-assimilating pathways (Kötter and Ciriacy, 1993) as summarized in Fig. 2. Numerous rational and inverse metabolic engineering approaches have been attempted to reconfigure the endogenous metabolism in S. cerevisiae strains expressing a heterologous xylose-assimilating pathway (Table 2). First, xylose transport phenomena were characterized in xylose-assimilating S. cerevisiae (Hamacher et al., 2002; Lee et al., 2002) and by introducing heterologous sugar transporters such as SUT1 and SUT2 from Sch. stipitis (Du et al., 2010; Hector et al., 2008; Katahira et al., 2008; Runquist et al., 2009; Saloheimo et al., 2007). Second, genes coding for enzymes in the pentose phosphate pathway were modified by gene knockout or overexpression to improve the rate of xylose fermentation in engineered S. cerevisiae (Jin et al., 2005; Johansson and Hahn-Hägerdal, 2002; Karhumaa et al., 2005; Lu and Jeffries, 2007; Meinander et al., 1999; Sonderegger et al., 2004; Walfridsson et al., 1995). Third, random mutagenesis (Liu and Hu, 2011; Ni et al., 2007; Thanvanthri Gururajan et al., 2007b; Wahlbom et al., 2003b) and laboratory evolution (Garcia Sanchez et al., 2010; Kuyper et al., 2005b; Liu and Hu, 2011; Peng et al., 2012; Sonderegger and Sauer, 2003; Wouter Wisselink et al., 2009) were also employed to improve xylose-fermenting ability. Recent progress in engineering sugar trans-porters can be found in our previous review (Kim et al., 2012b), and studies on optimizing pentose phosphate and other endogenous metabolic pathways are discussed below.

3.1. Perturbation of gene targets identified through rational approaches

In an engineered S. cerevisiae strain expressing XYL1 and XYL2, an analysis of intracellular metabolites from xylose provided that sedoheptulose-7-phosphate accumulated while fructose-1,6-bisphosphate did not (Kötter and Ciriacy, 1993). This finding suggested that the heterologous xylose-assimilating pathway consisting of XR/XDH/XK was not a limiting step, but the following non-oxidative pentose phosphate pathway (Fig. 3) was. Although the overexpression of transketolase (TKL1), the second enzyme in the pentose phosphate pathway, was quite debated, the overexpression of transaldolase (TAL1), the third enzyme, improved the rate of xylose assimilation (Lee et al., 2012; Matsushika et al., 2012; Walfridsson et al., 1995). In addition, the simultaneous overexpression of all of the enzymes in the non-oxidative pentose phosphate pathway (XKS1, RKI1, RPE1, TKL1, and TAL1) improved growth of recombinant yeast cells expressing an XI gene and growing on xylose (Karhumaa et al., 2005; Kuyper et al., 2005a). In contrast, a strain expressing XYL1 and XYL2 moderately, and overexpressing XKS1 did not show much improvement after overexpression of RKI1 coding for ribose 5-phosphate ketol isomerase and RPE1 for ribulose 5-phosphate epimerase, TKL1, and TAL1 (Bera et al., 2011; Johansson and Hahn-Hägerdal, 2002).

Since the oxidative pentose phosphate pathway (Fig. 3) is known to be a main route for NADPH generation, some studies attempted to decrease the activity of the enzymes engaged in the oxidative pentose phosphate pathway, which would facilitate the NADH-dependent XR reaction as well as low CO_2 production. By deleting ZWF1 coding for glucose-6-phosphate dehydrogenase and/or GND1 for 6-phosphogluconate dehydrogenase, XYL1/XYL2-expressing strains had a higher ethanol yield as expected. However, a decrease in xylose fermenting ability was possibly caused by reduction of the NADPH-dependent XR reaction (Jeppsson et al., 2002; Verho et al., 2003). As a solution to the problem, overexpression of a fungal $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene together with ZWF1 deletion improved both ethanol yield and productivity (Verho et al., 2003). Heterologous expression of an $NADP^+$-dependent GAPDH gene alone also improved ethanol yields
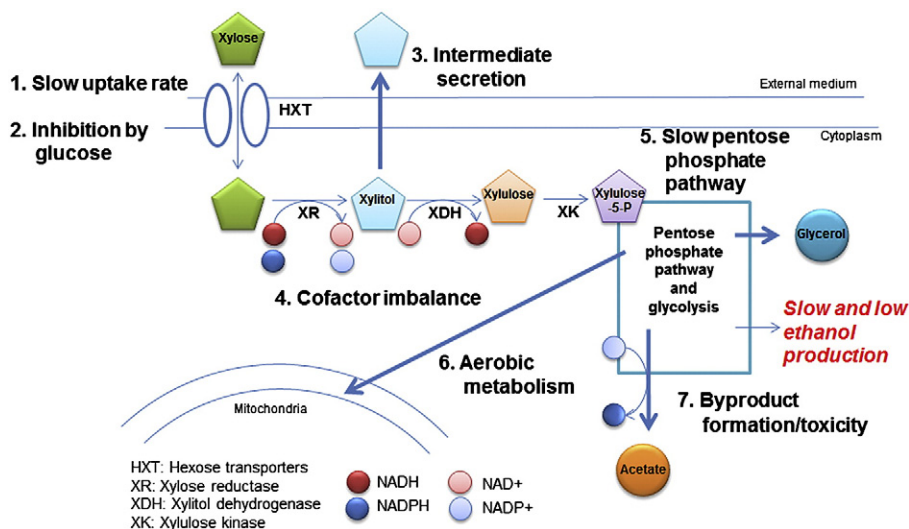

Fig. 2. Major limitations in xylose metabolism by engineered S. cerevisiae expressing a heterologous xylose-assimilating pathway consisting of xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK).
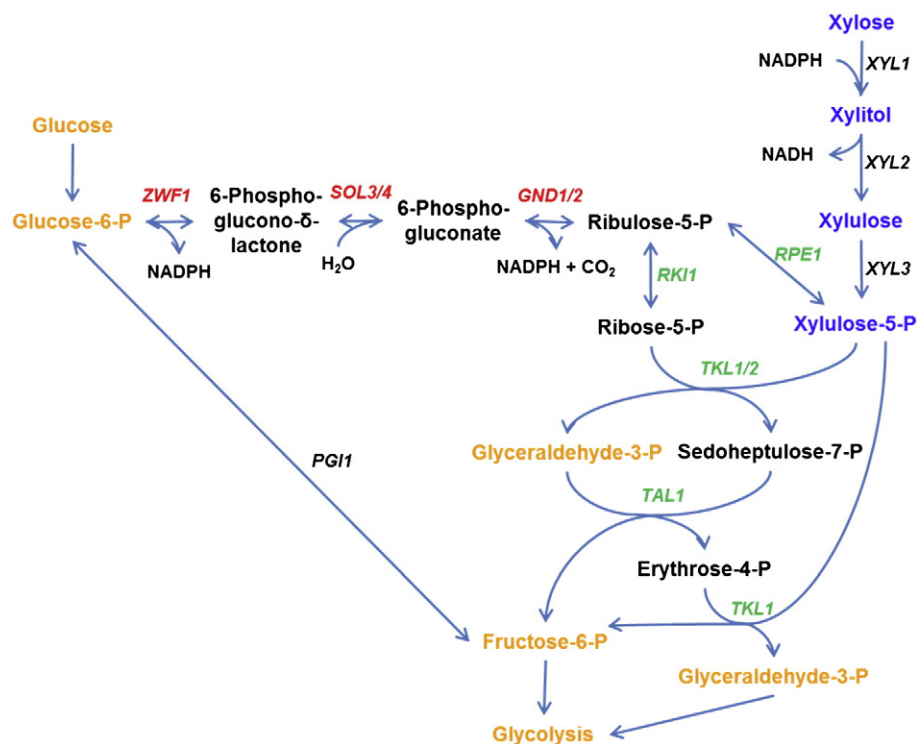
Fig. 3. Genes coding for the enzymes engaged in the oxidative (red) and non-oxidative (green) pentose phosphate pathway. Heterologous xylose-assimilating pathway is marked in blue. Metabolic intermediates of glycolysis are marked in orange. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

from xylose, as predicted by genome-scale modeling (Bro et al., 2006; Verho et al., 2003).

Alternatively, other pathways and genes were manipulated to improve xylose metabolism. Modification of the cellular redox balance by the deletion of *GDH1* (NADPH-dependent glutamate dehydrogenase gene) and the overexpression of *GDH2* (NADH-dependent glutamate dehydrogenase gene) in the ammonia assimilation pathway decreased xylitol yield and increased ethanol yield when an engineered *S. cerevisiae* strain fermented a mixture of glucose and xylose (Roca et al., 2003). In a follow-up study, a metabolic flux analysis confirmed that the engineered pathway was functional by presenting higher NADH-specific XR activities in the mutant strain (Grotkjar et al., 2005). The overexpression of the water-forming NADH oxidase gene (*noxE*) from *Lactococcus lactis* reduced the xylitol accumulation during xylose fermentation by engineered *S. cerevisiae* (Zhang et al., 2012). Lastly, the deletion of *ALD6* (NADP$^+$-dependent aldehyde dehydrogenase gene) in the acetate biosynthesis pathway improved xylose fermentation (Lee et al., 2012).

3.2. Perturbation of gene targets identified through combinatorial approaches

Concurrently with the above rational metabolic engineering approaches, various inverse metabolic engineering strategies have been explored to improve the xylose-fermenting capability of engineered *S. cerevisiae*. Among various traditional metabolic engineering approaches, adaptive evolution has a long history in industrial yeast processes such as wine and beer-making for avoiding any genetic modification with foreign genes (Cadièrea et al., 2011; Kutyna et al., 2012; Nakao et al., 2009; Piskur et al., 2006; Querol and Bond, 2009). Owing to the development of a multiplex genome sequencing technique, adaptive evolution has become popular these days for identifying unknown gene targets as well as improving strain phenotypes as desired (Hong et al., 2011; Kim et al., 2013; Oud et al., 2012). Various strategies for adaptive evolution have been explored to overcome inefficient xylose metabolism of engineered *S. cerevisiae*

strains (Garcia Sanchez et al., 2010; Ha et al., 2011a; Kim et al., 2013; Kuyper et al., 2005b; Liu and Hu, 2011; Peng et al., 2012; Sonderegger and Sauer, 2003; Wouter Wisselink et al., 2009; Zhou et al., 2012). During repeated batch cultures of strains expressing XR/XDH/XK using xylose as a sole carbon source, spontaneous mutations were induced, and the mutants that grew better on xylose were enriched and isolated (Ha et al., 2011a; Kim et al., 2013; Peng et al., 2012). Through long-term cultivation of a strain overexpressing XI during xylose-limited continuous fermentations, mutants with an improved xylose uptake rate were isolated (Kuyper et al., 2005b; Zhou et al., 2012). To improve fermentation rates with mixed sugars, one study performed continuous fermentations using media containing xylose and arabinose (Garcia Sanchez et al., 2010), while another study performed multiple cycles of consecutive batch fermentations using media containing glucose, xylose, and arabinose, separately (Wouter Wisselink et al., 2009). Random mutagenesis with ethyl methane sulfonate (EMS) successfully induced genetic variation in strains expressing XR/XDH/XK (Thanvanthri Gururajan et al., 2007b; Wahlbom et al., 2003b), and facilitated adaptive evolution processes (Liu and Hu, 2011; Sonderegger and Sauer, 2003). Recently, the genome of evolved xylose-fermenting strain was resequenced for the first time, and a mutation responsible for the improved phenotype was identified as described in Table 1 (Kim et al., 2013). Genome shuffling, another type of random approach, is recently applied for developing robust yeast strains exhibiting a higher ethanol yield from xylose (Jingping et al., 2012). The improvement of the phenotypes of engineered *S. cerevisiae* by random genome rearrangements suggests that unknown mechanisms may regulate its xylose metabolism (Jingping et al., 2012).

A genome-wide analysis through genetic array, transcriptomics, proteomics, and metabolomics was performed to understand the genetic and physiological states of xylose metabolism (Bergdahl et al., 2012; Jin et al., 2004; Salusjarvi et al., 2003, 2006; Wahlbom et al., 2003a) and to identify genetic perturbation targets for the improvement of xylose metabolism in engineered *S. cerevisiae*. From a recent genetic array analysis, *ALP1* (arginine transporter gene), *ISC1* (inositol phosphosphingolipid phospholipase C gene), *RPL20B*

Table 1
Various combinatorial approaches for identification of genetic perturbation targets to improve xylose metabolism.

Approaches	Genetic backgrounds	Overexpression targets	Deletion targets	References
Microarray	XYL1/XYL2/ XKS1	HXT5, XKS1, SOL3, GND1, TAL1, TKL1, PET18	TEC1, ARR1	Wahlbom et al., 2003a
	XYL1/XYL2/ XKS1	SOL3, TAL1	YLR042C, MNI1, RPA49	Bengtsson et al., 2008
Synthetic genetic array	Piromyces xylA, XKS1		ALP1, ISC1, RPL20B, BUD21	Usher et al., 2011
Genomic overexpression library	XYL1/XYL2	XYL3, TAL1		Jin et al., 2005
	XYL1/XYL2/ XYL3	XYL2		Kim et al., 2012a
Transposon mutagenesis			PHO13, TAL1$_P$	Ni et al., 2007
Adaptive evolution and genome resequencing	XYL1/XYL2/ XYL3		PHO13	Kim et al., 2013

HXT5, hexose transporter; *SOL3*, 6-phosphogluconolactonase; *GND1*, 6-phosphogluconate dehydrogenase; *TAL1*, transaldolase; *TKL1*, transketolase; *TEC1* and *ARR1*, transcription factor; *MNI1*, methyltransferase; *RPA49*, RNA polymerase; *ALP1*, arginine transporter; *ISC1*, inositol phosphosphingolipid phospholipase; *RPL20B*, ribosomal 60S subunit protein; *BUD21*, small ribosomal subunit; *PET18*, *YLR042C*, *PHO13*, uncharacterized; *TAL1$_P$*, the promoter region of *TAL1*.

(a protein component gene for the large 60S ribosomal subunit), and *BUD21* (coding for a component of small ribosomal subunit processosome) were chosen as gene deletion targets and their disruption improved the xylose metabolism in recombinant *S. cerevisiae* expressing *Piromyces xylA* and overexpressing *XKS1* (Usher et al., 2011). By comparing the genome-wide transcripts of a xylose-utilizing *S. cerevisiae* strain and its mutant possessing improved xylose-fermenting capability, *XKS1*, *SOL3* (6-phosphogluconolactonase), *GND1* (6-phosphogluconate dehydrogenase), *TAL1*, *TKL1*, *YCR020C*, *YBR083W*, and *YPR199C* were found as overexpression or deletion targets (Wahlbom et al., 2003a). Contrary to the expectation, the overexpression or deletion of *YCR020C* in the xylose-utilizing strain did not affect its xylose-fermenting capability. In a similar study, the global transcript analysis of the good and bad groups of xylose-assimilating *S. cerevisiae* strains proposed two gene overexpression targets of *SOL3* and *TAL1*, and three gene deletion targets of *YLR042C*, *MNI1* (methyltransferase), and *RPA49* (RNA polymerase) (Bengtsson et al., 2008). In a later study, the beneficial effect of *YLR042C* deletion on xylose fermentation was confirmed (Parachin et al., 2010).

Meanwhile, genome library screening (Jin et al., 2005; Kim et al., 2012a) and transposon mutagenesis (Ni et al., 2007) provided more gene targets, of which overexpression or disruption was responsible for improved xylose fermentation by engineered *S. cerevisiae*. To improve the xylose assimilation rate, a genome overexpression library of *Sch. stipitis* was constructed on the basis of engineered *S. cerevisiae* expressing *XYL1* and *XYL2*, and hence the *XYL3* and *TAL1* genes were identified as overexpression targets (Jin et al., 2005). The *PHO13* gene coding for an alkaline phosphatase and the upstream region of *TAL1*, which up-regulated *TAL1* expression, was found through transposon mutagenesis as a knockout target for improving the growth of engineered *S. cerevisiae* on xylose (Ni et al., 2007). It was confirmed that the deletion of *PHO13* in an engineered strain expressing *XYL1/XYL2/XYL3* improved both cell growth rate and ethanol productivity on xylose (Fujitomi et al., 2012; Van Vleet et al., 2008). The regulatory mechanisms of *YLR042C* and *PHO13* in xylose metabolism are not clear to date. Table 2.

4. Effects of genetic backgrounds of xylose-fermenting yeasts

Industrially adapted and isolated strains of *S. cerevisiae*, so called industrial strains, were often compared with laboratory strains in terms of sugar utilization efficiency (Hector et al., 2011; Karhumaa et al., 2006; Matsushika et al., 2009) and stress tolerance (Garay-Arroyo et al., 2004). To compare xylose-fermenting capability, two laboratory and three industrial strains of *S. cerevisiae* were used to construct the same xylose-assimilating pathway consisting of the XR/XDH/XK genes (Matsushika et al., 2009). All of the industrial strains had faster xylose consumption rates and higher ethanol productivities than the laboratory strains. In a recent study, an engineered laboratory strain expressing the *XYL1*, *XYL2*, and *XKS1* genes showed a similar xylose consumption rate compared with six industrial strains expressing the same genes when fermenting 50 g/l xylose (Hector et al., 2011). Therefore, not only the sources but also the individual backgrounds of *S. cerevisiae* host strains could affect their xylose-fermenting capability when engineered with a heterologous xylose-assimilating pathway.

5. Co-fermentation of mixed sugars

By the compositional nature of lignocellulosic biomass, cellulosic hydrolysates contain both glucose and xylose. Typically, a glucose fermentation rate is 3–10 fold faster than xylose fermentation rate in engineered *S. cerevisiae*. In addition, glucose and xylose are sequentially utilized (Casey et al., 2010; Kuyper et al., 2005b; Matsushika and Sawayama, 2010) (Table 3) because xylose metabolism is delayed when glucose is present. This sequential utilization of a glucose and xylose mixture results in low overall ethanol productivity, and becomes a significant barrier to rapid and efficient fermentation of cellulosic hydrolysates.

The native carbon catabolite repression mechanisms of *S. cerevisiae* by which glucose inhibits metabolism of non-glucose sugars have not been thoroughly studied for engineered xylose-metabolic pathways of *S. cerevisiae* (Jojima et al., 2010; Kim et al., 2010, 2012b). Some researchers hypothesized that glucose may repress xylose at the transport level because xylose is transported by native hexose transporters (Kim et al., 2012b; Subtil and Boles, 2012). On the other hand, it has been reported that a low amount of glucose (0.5 g/l) facilitated xylose transport and metabolism (Lee et al., 2003b; Meinander and Hahn-Hägerdal, 1997). In addition, the transcriptome and proteome studies indicated that engineered *S. cerevisiae* strains exhibited moderate levels of glucose-repressed signals during xylose metabolism (Belinchon and Gancedo, 2003; Salusjarvi et al., 2008). These results suggested that glucose-repressed conditions could, paradoxically, help xylose metabolism. Therefore, two directions of studies were established to attempt modifications of the regulatory mechanism of xylose metabolism: 1) derepression of glucose-repressive signals (Kahar et al., 2011) and 2) constitutive expression of the signals (Thanvanthri Gururajan et al., 2007a). However, the improvements in xylose metabolism were very marginal, and glucose and xylose could not be simultaneously consumed in either case. The detailed carbon catabolite repression mechanism and its metabolic engineering approaches can be found in our previous review (Kim et al., 2012b).

Two successful strategies to bypass the sequential utilization have been demonstrated. Both strategies were developed from the observation that cellobiose, an intermediate product of cellulose hydrolysis, does not repress xylose fermentation. Because *S. cerevisiae* cannot consume cellobiose, metabolic enzymes for utilization of either extracellular or intracellular cellobiose need to be introduced. The extracellular cellobiose utilization was achieved by displaying β-glucosidase on the surface of yeast host cells (Saitoh et al., 2010) while the intracellular cellobiose utilization was implemented by introduction of a cellobiose transporter and an

Table 2
Improvements achieved by overexpression or deletion of target genes in xylose-fermenting *S. cerevisiae*.

	Pathways	Target genes	Background strains	Improvements	References
Rational metabolic engineering approaches	Non-oxidative pentose phosphate pathway	TAL1	XYL1/XYL2	Enhanced growth on a xylose plate	Walfridsson et al., 1995
			XYL1/XYL2/XKS1	No effect on r_{Xyl} (5× higher μ on Xylulose)	Johansson and Hahn-Hägerdal, 2002
				No effect on r_{Xyl}	Matsushika et al., 2012
		TKL1	XYL1/XYL2	No effect on μ	Walfridsson et al., 1995
			XYL1/XYL2/XKS1	No effect on r_{Xyl}	Matsushika et al., 2012
		RKI1, RPE1, TKL1, TAL1	XYL1/XYL2/XKS1	No effect on r_{Xyl} (9× higher μ on Xylulose)	Johansson and Hahn-Hägerdal, 2002
				3× higher μ	Karhumaa et al., 2005
				40% lower Y_{Xylit}, slightly faster xylose consumption	Bera et al., 2011
	Oxidative pentose phosphate pathway	$\Delta zwf1$	XYL1/XYL2/XKS1	80% lower Y_{Xylit} (0.05), 30% higher Y_{EtOH} (0.41), 84% lower r_{Xyl}	Jeppsson et al., 2002
				50% lower Y_{Xylit}, 30% higher Y_{EtOH}, 10% lower r_{Xyl}, 11% more biomass	Verho et al., 2003
		$\Delta zwf1$, KcGAPDH	XYL1/XYL2/XKS1	40% lower Y_{Xylit}, 2× higher Y_{EtOH}, 50% lower r_{Xyl}, 2.2× higher P_{EtOH}	Verho et al., 2003
		$\Delta gnd1$	XYL1/XYL2/XKS1	60% lower Y_{Xylit}, 20% higher Y_{EtOH}, 40% lower r_{Xyl}	Jeppsson et al., 2002
		10% PGI1 activity	XYL1/XYL2/XKS1	30% lower Y_{Xylit}, 10% higher Y_{EtOH}, 40% lower r_{Xyl}	Jeppsson et al., 2002
	Ammonia assimilation	$\Delta gdh1$, GDH2	XYL1/XYL2/XKS1	40% lower Y_{Xylit}, 20% higher Y_{EtOH},	Roca et al., 2003
	Acetate biosynthesis	$\Delta ald6$	XYL1/XYL2/XKS1	Accumulate much less acetate, 50% higher r_{Xyl}^{*}	Sonderegger et al., 2004
	Others	KlGAPDH	XYL1/XYL2/XKS1	10% lower Y_{Xylit}, 30% higher Y_{EtOH}, 30% lower r_{Xyl}	Verho et al., 2003
				20% lower Y_{Xylit}, higher r_{Xyl}	Bera et al., 2011
		SmGAPDH	XYL1/XYL2	30% higher Y_{EtOH}	Bro et al., 2006
		noxE	XYL1/XYL2/XKS1	70% lower Y_{Xylit}, 40% higher Y_{EtOH}, 40%, lower r_{Xyl}	Zhang et al., 2012
Inverse metabolic engineering approaches		PET18	XYL1/XYL2/XKS1	No effect on μ_{max}	Wahlbom et al., 2003a
		$\Delta ylr042c$	XYL1/XYL2/XKS1/TAL1 /TKL1/RKI1/RPE1	40% improved r_{Xyl}^{*}	Parachin et al., 2010
		$\Delta pho13$	XYL1/XYL2/XYL3	2× higher r_{Xyl}	Van Vleet et al., 2008
				3× higher r_{Xyl}^{*}	Fujitomi et al., 2012
				5× higher r_{Xyl}, 2× higher Y_{EtOH},	Kim et al., 2013

μ, specific growth rate (1/h); r_{Xyl}, xylose consumption rate (g/l/h); r_{Xyl}^{*}, specific xylose consumption rate (g/g cell/h); Y_{Xylit}, xylitol yield (g xylitol/g xylose); Y_{EtOH}, ethanol yield (g ethanol/g sugars); P_{EtOH}, volumetric ethanol productivity (g/l/h); P_{EtOH}^{*}, specific ethanol productivity (g/g cell/h); *TAL1*, transaldolase; *TKL1*, transketolase; *RKI1*, ribose-5-phosphate ketol-isomerase; *RPE1*, D-ribulose-5-phosphate 3-epimerase; *ZWF1*, glucose-6-phosphate dehydrogenase; *KlGAPDH*, *Kluyveromyces lactis* glyceraldehyde-3-phosphate dehydrogenase; *GND1*, 6-phosphogluconate dehydrogenase; *PGI1*, phosphoglucose isomerase; *GDH1*, NADP^{+}-dependent glutamate dehydrogenase; *GDH2*, NAD^{+}-dependent glutamate dehydrogenase; *ALD6*, aldehyde dehydrogenase; *SmGAPDH*, *Streptococcus mutants* glyceraldehyde-3-phosphate dehydrogenase; *noxE*, *Lactococcus lactis* water-forming NADH oxidase; *PET18*, *YLR042C*, and *PHO13*, uncharacterized.

intracellular β-glucosidase (Aeling et al., 2012; Ha et al., 2011a; Li et al., 2010). Interestingly, xylose was consumed much faster during the co-fermentation with cellobiose than when xylose was used as a sole carbon source (Ha et al., 2011a) (Table 3), suggesting that there are synergistic effects from simultaneous co-fermentation of the two cellulosic sugars (cellobiose and xylose).

5.1. Simultaneous co-fermentation via extracellular hydrolysis of cellobiose

The first simultaneous co-fermentation of cellobiose and xylose was demonstrated through expressing β-glucosidase on the surface of an engineered *S. cerevisiae* capable of xylose fermentation. When a mixture of cellobiose and xylose was used, the engineered strain was able to simultaneously co-ferment cellobiose and xylose with a decent yield and productivity (0.36 g ethanol/g sugar and 0.50 g/l/h) (Nakamura et al., 2008). The rate of co-fermentation improved further through introducing the xylose fermenting pathway (XR, XDH, and XK) into an industrial yeast strain and optimizing β-glucosidase activity on the surface of the yeast cells (Saitoh et al., 2010). The resulting strain (OC2-ABGL4Xyl2) showed a specific xylose consumption rate of 0.38 g xylose/g cell·h during co-fermentation of cellobiose and xylose, which was much higher than 0.18 g xylose/g cell·h during co-fermentation of glucose and xylose. Expression levels of β-glucosidase on the surface of the yeast cells were found to be a critical parameter for efficient

co-fermentation. The β-glucosidase activity needs to be high enough to supply ample amounts of glucose to yeast cells, but too high β-glucosidase may result in overproduction of glucose and reduce xylose consumption.

5.2. Simultaneous co-fermentation via intracellular hydrolysis of cellobiose

The recent discovery of cellobiose transporters encoded by the *cdt-1* and *cdt-2* genes, and intracellular β-glucosidase by the *gh1-1* gene from *Neurospora crassa*, a model cellulolytic fungi made it possible to construct engineered yeast fermenting cellobiose via intracellular hydrolysis (Galazka et al., 2010). Because the cellobiose hydrolysis is carried out inside yeast cells, repression of xylose fermentation by extracellular glucose may be eliminated. Introduction of the intracellular cellobiose hydrolysis pathway into a xylose-fermenting *S. cerevisiae* indeed led to simultaneous co-fermentation of cellobiose and xylose (Ha et al., 2011a). The DA24-16BT3 strain harboring both the xylose fermenting pathway and the intracellular cellobiose fermenting pathway was able to ferment cellobiose and xylose simultaneously regardless of concentrations of cellobiose and xylose. Moreover, overall ethanol yields (~0.40 g ethanol/g sugar) and productivities (~1 g/l/h) from co-fermentations of cellobiose and xylose were high even when a laboratory host strain was used with a small inoculum size (~0.3 g/l). With a similar strategy, co-fermentation of cellobiose and galactose, which are abundant sugars

Table 3
Cofermentation of mixed sugars by engineered *S. cerevisiae*.

Sugar	Strains	Metabolic pathways		Fermentation conditions			Fermentation parameters						References
		Xylose	Cellobiose	Aeration	Initial biomass	Media	r_{Xyl}	r_{Xyl}^*	Y_{Xylit}	Y_{EtOH}	P_{EtOH}	P_{EtOH}^*	
Glucose Xylose	RWB218	*Pi*XI, *XKS1*, PPP, *gre3*Δ, Evolved	–	AN	0.2 g/l	D20 X20	0.83	–	0.07	0.40	0.66	–	Kuyper 2005b
	MA-R4	XYL	–	AN	1.6 g/l	D45 X45	0.94	–	0.10	0.40	0.74	–	Matsushika and Sawayama, 2010
	424A	XYL	–	AN	4.8 g/l	D60 X60	–	–	–	0.40	1.23	–	Casey et al., 2010
	DA24-16BT3	XYL, evolved	*Nc*CBT, *Nc*BGL	OL	3 g/l	D100 X60	0.44	0.07	0.03	0.42	0.91	0.15	Ha et al., 2011a
Cellobiose Xylose	DA24-16BT3	XYL, evolved	*Nc*CBT, *Nc*BGL	OL	3 g/l	C100 X60	0.89	0.14	0.08	0.41	1.06	0.17	Ha et al., 2011a
					0.3 g/l	C40 X40	0.84	0.21	0.06	0.39	0.65	0.16	
	SL01	XYL	*Nc*CBT, *Nc*BGL	OL	2%	C40 X50	0.68	–	–	0.39	0.49	–	Li et al., 2010
	OC2-ABGL4Xyl2	XYL	*Aa*BGL[a]	AN	OD 8	C90 X60	1.20	0.33	–	0.39	1.20	–	Saitoh et al., 2010
Glucose Cellobiose Xylose	DA24-16BT3	XYL, evolved	*Nc*CBT, *Nc*BGL	OL	0.3 g/l	D10 C80 X40	0.78	0.20	0.05	0.36	0.94	0.24	Ha et al., 2011a
	BF3645	*Rf*XI, *XKS1*, *GAL2*, *pho13*Δ, *gre3*Δ	*Nc*CBT, *Kl*LAC12, *Rf*CBP	AN	OD 0.6	D80 C20 X40	0.20	–	0.07	0.44	0.51	–	Aeling et al., 2012

r_{Xyl}, xylose consumption rate (g/l/h); r_{Xyl}^*, specific xylose consumption rate (g/g cell/h); Y_{Xylit}, xylitol yield (g xylitol/g xylose); Y_{EtOH}, ethanol yield (g ethanol/g sugars); P_{EtOH}, volumetric ethanol productivity (g/l/h); P_{EtOH}^*, specific ethanol productivity (g/g cell/h); AN, anaerobic; OL, oxygen-limited; *Pi*, *Piromyces* sp. E2; *Aa*, *Aspergillus aculeatus*; *Ss*, *Scheffersomyces stipites*; *Sc*, *Saccharomyces cerevisiae*; *Nc*, *Neurospora crassa*; *Kl*, *Kluyveromyces lactis*; *Rf*, *Ruminococcus flavefaciens*; XI, xylose isomerase; XYL, *SsXYL1*, *SsXYL1*, and *SsXYL3* or *ScXKS1*; PPP, overexpression of non-oxidative pentose phosphate pathway genes (*TAL1*, *TKL1*, *RPE1*, *RKI1*); CBP, cellobiose phosphorylase; CBT, cellobiose transporter; BGL, beta-glucosidase.

[a] Expression by cell-surface displaying.

in the hydrolysates of marine macroalgae, was also demonstrated (Ha et al., 2011c).

In addition to the intracellular cellobiose utilization pathway using β-glucosidase, an alternative one was recently reported (Ha et al., 2012a; Sadie et al., 2011). The glucosidic linkage of cellobiose can be cleaved by either β-glucosidase (hydrolytic) or a cellobiose phosphorylase (phosphorolytic) in yeast cells. The difference between the hydrolytic and phosphorolytic pathways is that the phosphorolytic one can produce more ATP because it yields one glucose molecule and one glucose-1-phosphate molecule from one cellobiose molecule whereas the hydrolytic one produces two glucose molecules. The energetic benefit of the phosphorolytic pathway can be significant, especially under anaerobic conditions, which is preferred for industrial fermentations. However, engineered yeast strains with the phosphorolytic pathway consisting of cellobiose transporter (*cdt-1*) and cellobiose phosphorylase (*Saccharophagus degradans* SdCBP) fermented cellobiose slower than the engineered strains with the hydrolytic pathway (*cdt-1* and *gh1-1*) (Ha et al., 2012a). It was because of the unfavorable thermodynamics of cellobiose phosphorolysis (ΔG° = +3.6 kJ/mol). Expression of the mutant cellobiose transporter exhibiting higher V_{max} than the wild type was required to compensate for inefficient cellobiose phosphorolysis and to achieve rapid cellobiose fermentation (Ha et al., 2012a).

It was recently reported that an engineered *S. cerevisiae* strain expressing *HXT2.4* coding for a putative hexose transporter from *Sch. stipitis* and *gh1-1* could ferment cellobiose (Ha et al., 2012b). The observation suggested that HXT2.4p might function as a cellobiose transporter when *HXT2.4* is overexpressed in *S. cerevisiae*. While the engineered yeast strain expressing *HXT2.4* and *gh1-1* fermented cellobiose much slower and less efficiently than the strain expressing *cdt-1* and *gh1-1*, single amino acid substitution of alanine (A291) into charged amino acids (A291D, A291E, A291K, and A291R) of Hxt2.4p improved cellobiose fermentation substantially. The mutant HXT2.4 (A291D) transporter exhibited a four-fold higher V_{max} when compared to the wild type HXT2.4, suggesting that the improved kinetic properties of the mutant HXT2.4 (A291D) might have resulted in rapid and efficient cellobiose fermentation (Ha et al., 2012b).

In addition to ethanol production by co-fermentation of cellobiose and xylose or cellobiose and galactose, the co-fermentation strategy can be applied for enhanced xylitol production from cellulosic hydrolysates. While xylitol can be produced using an engineered *S. cerevisiae* expressing *XYL1* with an almost theoretical yield (1 g xylitol/g xylose), supply of co-substrate for cell growth and re-generation of NADPH is necessary for efficient xylitol production from xylose (Kwon et al., 2006; Oh et al., 2007). Glucose has been used as a co-substrate, but glucose-limited fed-batch fermentations need to be optimized because glucose inhibits xylose transport in *S. cerevisiae* (Lee et al., 2000). Moreover, pure xylose, which is expensive, is necessary to produce xylitol using glucose feeding. These problems can be solved if xylitol is produced through simultaneous co-utilization of cellobiose and xylose which are abundant in inexpensive cellulosic hydrolysates. When the xylitol producing pathway (*XYL1*) and the cellobiose utilization pathway (*cdt-1* and *gh1-1*) were combined, the resulting D-10-BT strain produced xylitol constitutively via co-fermentation of cellobiose and xylose. Moreover, the D-10-BT exhibited higher xylitol production rates when a mixture of cellobiose and xylose was provided as compared to the case of a mixture of glucose and xylose (Oh et al., 2012). These results suggested that simultaneous co-fermentation of mixed sugars is a promising strategy for producing biofuels and chemicals from cellulosic hydrolysates which are inexpensive and available even in large quantities.

Simultaneous fermentation of cellobiose and other non-glucose sugars would be economically advantageous compared to sequential fermentation using monomeric sugars for the following reasons. First, it would reduce the cost of supplementing with β-glucosidase.

Most cellulases produced from fungal cultures exhibit weak β-glucosidase activity, so that supplementation of β-glucosidase is often required for efficient hydrolysis of cellulose. However, this expense would not be necessary for co-fermenting strains capable of utilizing cellobiose intracellularly. Second, hexose and pentose fermentations could be performed by single-step fermentation for producing cellulosic biofuels. This would contribute to substantial cost reductions in terms of both capital expenditures and operation costs by eliminating an extra fermentation step. Third, simultaneous co-fermentation would allow a continuous fermentation process, which is the most effective way to reduce capital expenditures. With these potential cost advantages, simultaneous co-fermentation of cellobiose and non-glucose sugars would be a quantum advance in commercial production of cellulosic biofuels.

6. Conclusion

As xylose is one of the most abundant sugars in cellulosic biomass, the efficient utilization of xylose is very critical in developing an economically viable process for production of biofuels and biochemicals from cellulosic biomass. The efficiency and rate of xylose utilization have been improved significantly over the decades by using various biotechnologies including metabolic engineering, protein engineering, and omic technologies. In recent studies, genetically engineered and evolved strains of S. cerevisiae were able to ferment xylose as a sole carbon source as fast as 0.2–0.8 g ethanol/g cells/h (Kim et al., 2013; Kuyper et al., 2005b; Runquist et al., 2010; Zhou et al., 2012). As genome resequencing of those evolved strains identified the genetic basis of the improved xylose-fermenting capabilities (Kim et al., 2013), new directions of research have been established to further improve the strains for industrial applications: 1) efficient xylose fermentation under strict anaerobic conditions (Cai et al., 2012), 2) resistance to phenolic compounds and organic acids in cellulosic hydrolysates (Liu, 2011; Parawira and Tekere, 2011), and 3) simultaneous fermentation of mixed sugars (Kim et al., 2012b). Global optimization of an engineered S. cerevisiae system will be necessary for metabolizing xylose both at a cellular level and at a process scale.

Acknowledgments

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