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## MINIREVIEW

# Saccharomyces cerevisiae strains for second-generation ethanol production: from academic exploration to industrial implementation

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**One sentence summary:** This minireview discusses how academic and industrial research yielded the robust, engineered yeast strains that are now used in the first large-scale factories for fuel-ethanol production from non-food agricultural residues.

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## ABSTRACT

The recent start-up of several full-scale 'second generation' ethanol plants marks a major milestone in the development of *Saccharomyces cerevisiae* strains for fermentation of lignocellulosic hydrolysates of agricultural residues and energy crops. After a discussion of the challenges that these novel industrial contexts impose on yeast strains, this minireview describes key metabolic engineering strategies that have been developed to address these challenges. Additionally, it outlines how proof-of-concept studies, often developed in academic settings, can be used for the development of robust strain platforms that meet the requirements for industrial application. Fermentation performance of current engineered industrial *S. cerevisiae* strains is no longer a bottleneck in efforts to achieve the projected outputs of the first large-scale second-generation ethanol plants. Academic and industrial yeast research will continue to strengthen the economic value position of second-generation ethanol production by further improving fermentation kinetics, product yield and cellular robustness under process conditions.

**Keywords:** biofuels; metabolic engineering; strain improvement; industrial fermentation; yeast biotechnology; pentose fermentation; biomass hydrolysates

## INTRODUCTION

Alcoholic fermentation is a key catabolic process in most yeasts and in many fermentative bacteria that concentrates the heat of combustion of carbohydrates into two-thirds of their carbon atoms ( $(\text{CH}_2\text{O})_n \rightarrow \frac{1}{3}n \text{C}_2\text{H}_6\text{O} + \frac{1}{3}n \text{CO}_2$ ). Its product, ethanol, has

been used as an automotive fuel for over a century (Bernton, Kovarik and Sklar 1982). With an estimated global production of 100 Mton (Renewable Fuels Association 2016), ethanol is the largest-volume product in industrial biotechnology. Its production is, currently, mainly based on fermentation of cane sugar

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**Table 1.** Overview of operational commercial-scale (demonstration) plants for second-generation bioethanol production. Data for USA and Canada reflect status in May 2017 (source: Ethanol Producer Magazine 2017); data for other countries (source: UNCTAD 2016) reflect status in 2016.

Company/plant	Country (state)	Feedstock	Capacity ML·year <sup>-1</sup>
DuPont Cellulosic Ethanol LLC—Nevada	USA (IA)	Corn stover	113.6
Poet-DSM Advanced Biofuels LLC—Project Liberty <sup>a</sup>	USA (IA)	Corn cobs/corn stover	75.7
Quad County Cellulosic Ethanol Plant	USA (IA)	Corn fiber	7.6
Fiberight Demonstration Plant	USA (VA)	Waste stream	1.9
ICM Inc. Pilot integrated Cellulosic Biorefinery	USA (MO)	Biomass crops	1.2
American Process Inc.—Thomaston Biorefinery	USA (GA)	Other	1.1
ZeaChem Inc.—demonstration plant	USA (OR)	Biomass crops	1.0
Enerkem Alberta Biofuels LP	Canada (AB)	Sorted municipal solid waste	38.0
Enerkem Inc.—Westbury	Canada (QC)	Woody biomass	5.0
Iogen Corporation	Canada (ON)	Crop residue	2.0
Woodlands Biofuels Inc.—demonstration plant	Canada (ON)	Woody biomass	2.0
GranBio	Brazil	Bagasse	82.4
Raizen	Brazil	Sugarcane bagasse/straw	40.3
Longlive Bio-technology Co. Ltd—commercial demo	China	Corn cobs	63.4
Mussi Chemtex/Beta Renewables	Italy	<i>Arundo donax</i> , rice straw, wheat straw	75.0
Borregaard Industries AS—ChemCell Ethanol	Norway	Wood pulping residues	20.0

<sup>a</sup>With expansion of capacity to 94.6 ML per year.

or hydrolysed corn starch with the yeast *Saccharomyces cerevisiae*. Such 'first generation' bioethanol processes are characterized by high ethanol yields on fermentable sugars (>90% of the theoretical maximum yield of 0.51 g ethanol·(g hexose sugar)<sup>-1</sup>), ethanol titers of up to 21% (w/w), and volumetric productivities of 2–3 kg·m<sup>-3</sup>·h<sup>-1</sup> (Thomas and Ingledew 1992; Della-Bianca et al. 2013; Lopes et al. 2016).

Over the past two decades, a large international effort, involving researchers in academia, research institutes and industry, has aimed to access abundantly available agricultural and forestry residues, as well as fast-growing energy crops, as alternative feedstocks for fuel ethanol production (Rude and Schirmer 2009). Incentives for this effort, whose relative impact depends on geographical location and varies over time, include reduction of the carbon footprint of ethanol production (Otero, Panagiotou and Olsson 2007), prevention of competition with food production for arable land (Nordhoff 2007; Tenenbaum 2008), energy security in fossil-fuel importing countries (Farrell et al. 2006) and development of rural economies (Kleinschmidt 2007). Techno-economic forecasts of low-carbon scenarios for global energy supply almost invariably include liquid biofuels as a significant contributor (Yan, Inderwildi and King 2010). Moreover, successful implementation of economically and environmentally sustainable 'second generation' bioethanol processes can pave the way for similar processes to produce other biofuels and commodity chemicals (Pereira et al. 2015).

In contrast to starch, a plant storage carbohydrate that can be easily hydrolysed, the major carbohydrate polymers in lignocellulosic plant biomass (cellulose, hemicellulose and, in some cases, pectin) contribute to the structure and durability of stalks, leaves and roots (Hahn-Hägerdal et al. 2006). Consistent with these natural functions and with their chemical diversity and complexity, mobilization of these polymers by naturally occurring cellulose-degrading microorganisms requires complex arrays of hydrolytic enzymes (Lynd et al. 2002; Van den Brink and de Vries 2011).

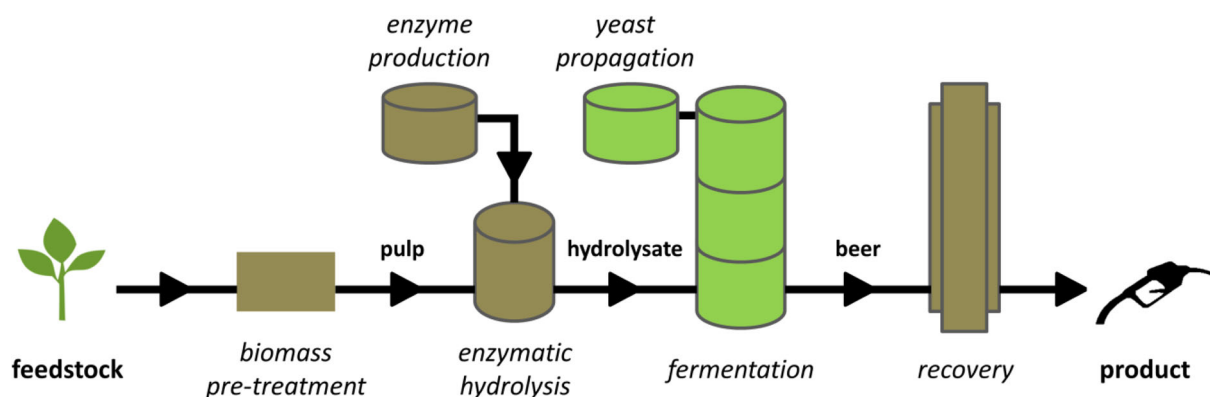
The second-generation ethanol processes that are now coming on line at demonstration- and full commercial scale (Table 1) are mostly based on fermentation of lignocellulosic biomass hydrolysates by engineered strains of *S. cerevisiae*.

While this yeast has a strong track record in first-generation bioethanol production and its amenability to genetic modifications is excellent, *S. cerevisiae* cannot hydrolyse cellulose or hemicellulose. Therefore, in conventional process configurations for second-generation bioethanol production, the fermentation step is preceded by chemical/physical pretreatment and enzyme-catalysed hydrolysis by cocktails of fungal hydrolases, which can either be produced on- or off-site (Fig. 1; Sims-Borre 2010). Alternative process configurations, including simultaneous saccharification and fermentation and consolidated bioprocessing by yeast cells expressing heterologous hydrolases are intensively investigated (Olson et al. 2012; Den Haan et al. 2015). However, the high temperature optima of fungal enzymes and low productivity of heterologously expressed hydrolases in *S. cerevisiae* have so far precluded large-scale implementation of these alternative strategies for lignocellulosic ethanol production (Vohra et al. 2014; Den Haan et al. 2015). This minireview will, therefore, focus on the development of yeast strains for conventional process designs.

Over the past decade, the authors have collaborated in developing metabolic engineering concepts for fermentation of lignocellulosic hydrolysates with engineered *S. cerevisiae* strains and in implementing these in advanced industrial strain platforms. Based on their joint academic-industrial vantage point, this paper reviews key conceptual developments and challenges in the development and industrial implementation of *S. cerevisiae* strains for second generation bioethanol production processes.

## FERMENTING LIGNOCELLULOSIC HYDROLYSATES: CHALLENGES FOR YEAST STRAIN DEVELOPMENT

A wide range of agricultural and forestry residues, as well as energy crops, are being considered as feedstocks for bioethanol production (Khoo 2015). Full-scale and demonstration plants using raw materials such as corn stover, sugar-cane bagasse, wheat straw, and switchgrass are now in operation (Table 1). These lignocellulosic feedstocks have different chemical



**Figure 1.** Schematic process-flow diagram for ethanol production from lignocellulose, based on physically separated processes for pre-treatment, hydrolysis and fermentation, combined with on-site cultivation of filamentous fungi for production of cellulolytic enzymes and on-site propagation of engineered pentose-fermenting yeast strains.

compositions, which further depend on factors such as seasonal variation, weather and climate, crop maturity, and storage conditions (Kenney et al. 2013). Despite this variability, common features of feedstock composition and biomass-deconstruction methods generate several generic challenges that have to be addressed in the development of yeast strains for second-generation bioethanol production.

### Pentose fermentation

For large-volume products such as ethanol, maximizing the product yield on feedstock and, therefore, efficient conversion of all potentially available substrate molecules in the feedstock is of paramount economic importance (Lin and Tanaka 2006). In addition to readily fermentable hexoses such as glucose and mannose, lignocellulosic biomass contains substantial amounts of D-xylose and L-arabinose. These pentoses, derived from hemicellulose and pectin polymers in plant biomass, cannot be fermented by wild-type *S. cerevisiae* strains. D-Xylose and L-arabinose typically account for 10–25% and 2–3%, respectively, of the carbohydrate content of lignocellulosic feedstocks (Lynd 1996). However, in some feedstocks, such as corn fiber hydrolysates and sugar beet pulp, the L-arabinose content can be up to 10-fold higher (Grohmann and Bothast 1994; Grohmann and Bothast 1997). Early studies already identified metabolic engineering of *S. cerevisiae* for efficient, complete pentose fermentation as a key prerequisite for its application in second-generation ethanol production (Bruinenberg et al. 1983; Kötter et al. 1990; Hahn-Hägerdal et al. 2001; Sedlak and Ho 2001).

### Acetic acid inhibition

Since hemicellulose is acetylated (Van Hazendonk et al. 1996), its complete hydrolysis inevitably results in the release of acetic acid. Bacterial contamination during biomass storage, pretreatment and/or fermentation may further increase the acetic acid concentrations to which yeasts are exposed in the fermentation process. First-generation bioethanol processes are typically run at pH values of 4–5 to counter contamination with lactic acid bacteria (Beckner, Ivey and Phister 2011). At these low pH values, undissociated acetic acid ( $pK_a = 4.76$ ) easily diffuses across the yeast plasma membrane. In the near-neutral pH environment of the yeast cytosol, the acid readily dissociates and releases a proton, which forces cells to expend ATP for proton export via the plasma membrane ATPase to prevent cytosolic

acidification (Verduyn et al. 1992; Axe and Bailey 1995; Pamulha and Loureiro-Dias 2000). The accompanying accumulation of the acetate anion in the cytosol can cause additional toxicity effects (Russel 1992; Palmqvist and Hahn-Hägerdal 2000b; Ullah et al. 2013). Acetic acid concentrations in some lignocellulosic hydrolysates exceed  $5 \text{ g l}^{-1}$ , which can cause strong inhibition of anaerobic growth and sugar fermentation by *S. cerevisiae* (Taherzadeh et al. 1997). Acetic acid tolerance at low culture pH is therefore a key target in yeast strain development for second-generation ethanol production.

### Inhibitors formed during biomass deconstruction

In biomass deconstruction, a trade-off exists between the key objective to release all fermentable sugars at minimal process costs and the need to minimize generation and release of compounds that compromise yeast performance. Biomass deconstruction generally encompasses three steps: (i) size reduction to increase surface area and reduce degree of polymerization, (ii) thermal pretreatment, often at low pH and high pressure, to disrupt the crystalline structure of cellulose while already (partly) solubilizing hemicellulose and/or lignin, and (iii) hydrolysis with cocktails of fungal cellulases and hemicellulases to release fermentable sugars (Hendriks and Zeeman 2009; Silveira et al. 2015; Narron et al. 2016). Several inhibitors of yeast performance are generated in chemical reactions that occur during biomass deconstruction and, especially, in high-temperature pretreatment. 5-Hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde (furfural) are formed when hexoses and pentoses, respectively, are exposed to high temperature and low pH (Dunlop 1948; Ulbricht, Northup and Thomas 1984; Palmqvist and Hahn-Hägerdal 2000b). These furan derivatives inhibit yeast glycolysis, alcoholic fermentation and the TCA cycle (Banerjee, Bhatnagar and Viswanathan 1981; Modig, Lidén and Taherzadeh 2002; Sárvári Horváth et al. 2003) while, additionally, depleting intracellular pools of NAD(P)H and ATP (Almeida et al. 2007). Their further degradation during biomass deconstruction yields formic acid and levulinic acid (Dunlop 1948; Ulbricht, Northup and Thomas 1984), whose inhibitory effects overlap with those of acetic acid (Palmqvist and Hahn-Hägerdal 2000b). Inhibitor profiles of hydrolysates depend on biomass structure and composition as well as on the type and intensity of the biomass deconstruction method used (Almeida et al. 2007; Kumar et al. 2009). During pressurized pretreatment at temperatures above  $160^\circ\text{C}$ , phenolic inhibitors are

generated by partial degradation of lignin. This diverse class of inhibitors includes aldehydes, ketones, alcohols and aromatic acids (Almeida et al. 2007). Ferulic acid, a phenolic compound that is an integral part of the lignin fraction of herbaceous plants (Lawther, Sun and Banks 1996; Klinke et al. 2002), is a potent inhibitor of *S. cerevisiae* fermentations (Larsson et al. 2000). The impact of phenolic inhibitors on membrane integrity and cellular functions depends on the identity and position of functional groups and carbon-carbon double bonds (Adeboye, Bettiga and Olsson 2014).

Concentrations of inorganic salts in hydrolysates vary depending on the feedstock used (Klinke, Thomsen and Ahring 2004). Moreover, high salt concentrations in hydrolysates can originate from pH adjustments during pretreatment (Jönsson, Alriksson and Nilvebrant 2013). Salt- and osmotolerance can therefore be important additional requirements in yeast strain development (Casey et al. 2013).

The inhibitors in lignocellulosic hydrolysates do not always act independently but can exhibit complex synergistic effects, both with each other and with ethanol (Taherzadeh et al. 1999; Palmqvist and Hahn-Hägerdal 2000b; Liu et al. 2004), while their impact can also be modulated by the presence of water-insoluble solids (Koppram et al. 2016). Furthermore, their absolute and relative impact can change over time due to variations in feedstock composition, process modifications, or malfunctions in biomass deconstruction. While process adaptations to detoxify hydrolysates have been intensively studied (Sivers et al. 1994; Palmqvist and Hahn-Hägerdal 2000a; Canilha et al. 2012; Jönsson, Alriksson and Nilvebrant 2013), the required additional unit operations typically result in a loss of fermentable sugar and are generally considered to be too expensive and complicated. Therefore, as research on optimization of biomass deconstruction processes continues, tolerance of the chemical environments generated by current methods is a key design criterion for yeast strain development.

## YEAST STRAIN DEVELOPMENT FOR SECOND-GENERATION ETHANOL PRODUCTION: KEY CONCEPTS

For almost three decades, yeast metabolic engineers have vigorously explored strategies to address the challenges outlined above. This quest benefited from rapid technological development in genomics, genome editing, evolutionary engineering and protein engineering. Box 1 lists key technologies and examples of their application in research on yeast strain development for second-generation ethanol production.

### Xylose fermentation

Efficiently linking D-xylose metabolism to glycolysis requires two key modifications of the *S. cerevisiae* metabolic network (Fig. 2) (Jeffries and Jin 2004; Van Maris et al. 2007): introduction of a heterologous pathway that converts D-xylose into D-xylulose and, simultaneously, alleviation of the limited capacity of the native *S. cerevisiae* xylulokinase and non-oxidative pentose-phosphate pathway (PPP). Two strategies for converting D-xylose into D-xylulose have been implemented in *S. cerevisiae*: (i) simultaneous expression of a heterologous xylose reductase (XR) and xylitol dehydrogenase (XDH) and (ii) expression of a heterologous xylose isomerase (XI).

The first *S. cerevisiae* strains engineered for xylose utilization were based on expression of XR and XDH from the

xylose-metabolizing yeast *Scheffersomyces stipitis* (Kötter and Ciriacy 1993). Due to the non-matching redox-cofactor preferences of these enzymes, these strains produced large amounts of the by-product D-xylitol (Kötter and Ciriacy 1993; Hahn-Hägerdal et al. 2001; Jeffries 2006). Modification of these cofactor preferences by protein engineering resulted in reduced xylitol formation under laboratory conditions (Watanabe et al. 2007; Runquist, Hahn-Hägerdal and Bettiga 2010a). A much lower xylitol formation by XR/XDH-based strains in lignocellulosic hydrolysates was attributed to NADH-dependent reduction of furfural, which may contribute to *in situ* detoxification of this inhibitor (Moniruzzaman et al. 1997; Wahlbom and Hahn-Hägerdal 2002; Sedlak and Ho 2004a; Katahira et al. 2006; Karhuma et al. 2007). A potential drawback of XR/XDH-based strains for application in large-scale anaerobic processes is that, even after prolonged laboratory evolution, their anaerobic growth rates are very low (Sonderegger and Sauer 2003).

Combined expression of a fungal XI (Harhangi et al. 2003) and overexpression of the native *S. cerevisiae* genes encoding xylulokinase and non-oxidative PPP enzymes enabled anaerobic growth of a laboratory strain on D-xylose. In anaerobic cultures of this strain, in which the aldose-reductase encoding GRE3 gene was deleted to eliminate xylitol formation, ethanol yields on D-xylose were the same as on glucose (Kuyper et al. 2005a). This metabolic engineering strategy, complemented with laboratory evolution under anaerobic conditions, has been successfully reproduced in different *S. cerevisiae* genetic backgrounds and/or with different XI genes (Brat, Boles and Wiedemann 2009; Madhavan et al. 2009; Ha et al. 2011; Dun et al. 2012; Hector et al. 2013; Hou et al. 2016b).

Laboratory evolution (Box 1) for faster D-xylose fermentation and analysis of evolved strains identified high-level expression of XI as a major contributing factor (Zhou et al. 2012; Demeke et al. 2015; Hou et al. 2016a). Multi-copy introduction of XI expression cassettes, optimization of their codon usage, and mutagenesis of their coding sequences have contributed to higher D-xylose fermentation rates (Brat, Boles and Wiedemann 2009; Lee, Jellison and Alper 2012; Crook et al. 2016). Whole-genome sequencing of evolved D-xylose-fast-fermenting strains expressing *Piromyces* XI identified mutations affecting intracellular homeostasis of Mn<sup>2+</sup>, a preferred metal ion for this XI (Verhoeven et al. 2017). Other mutations affected stress-response regulators and, thereby, increased expression of yeast chaperonins that assisted functional expression of XI (Hou et al. 2016a). Consistent with this observation, co-expression of the *Escherichia coli* GroEL and GroES chaperonins enabled *in vivo* activity of *E. coli* XI in *S. cerevisiae* (Xia et al. 2016). A positive effect of mutations in the *PHO13* phosphatase gene on xylose fermentation rates in XI- and XR/XDH-based strains has been attributed to transcriptional upregulation of PPP-related genes by an as yet unknown mechanism (Ni, Laplaza and Jeffries et al. 2007; Van Vleet, Jeffries and Olsson 2008; Bamba, Hasunuma and Kondo 2016; Xu et al. 2016). Additionally, Pho13 has been implicated in dephosphorylation of the PPP intermediate sedoheptulose-7-phosphate (Xu et al. 2016). For other mutations in evolved strains, e.g. in genes involved in iron-sulfur cluster assembly and in the mitogen-activated protein kinase signaling pathway (dos Santos et al. 2016; Sato et al. 2016), the mechanisms by which they affect D-xylose metabolism remain to be identified.

### Arabinose fermentation

The metabolic engineering strategy for constructing L-arabinose-fermenting *S. cerevisiae* is based on heterologous

**Box 1.** Overview of key technologies used for development of *Saccharomyces cerevisiae* strains for second-generation bioethanol production and examples of their application.

#### Metabolic engineering

Application of recombinant-DNA techniques for the improvement of catalytic and regulatory processes in living cells, to improve and extend their applications in industry (Bailey 1991).

Metabolic engineering of pentose-fermenting strains commenced with the functional expression of pathways for xylose reductase/xylitol dehydrogenase- (Kötter and Ciriacy 1993; Tantirungkij et al. 1993) or xylose isomerase-based (Kuyper et al. 2005a) xylose utilization and pathways for isomerase-based arabinose utilization (Becker and Boles 2003; Wisselink et al. 2007). Further research focused on improvement of pathway capacity (Kuyper et al. 2006; Wiedemann and Boles 2008), engineering of sugar transport (Fonseca et al. 2011; Subtil and Boles 2011; Nijland et al. 2014, 2016), redox engineering to decrease byproduct formation and increase ethanol yield (Roca, Nielsen and Olsson 2003; Sonderegger and Sauer 2003; Watanabe, Kodaki and Makino 2005; Wei et al. 2013; Guadalupe-Medina et al. 2010; Yu, Kim and Han 2010; Henningsen et al. 2015; Papapetridis et al. 2016; Zhang et al. 2016a) and expression of alternative pathway enzymes (Brat, Boles and Wiedemann 2009; Ota et al. 2013). Expression of heterologous hydrolases provided the first steps towards consolidated bioprocessing (Ha et al. 2011; Ilmén et al. 2011; Sadie et al. 2011; den Haan et al. 2015).

#### Evolutionary engineering

Application of laboratory evolution to select for industrially relevant traits (Sauer 2001). Also known as adaptive laboratory evolution (ALE).

Evolutionary engineering in repeated-batch and chemostat cultures has been intensively utilized to improve growth and fermentation kinetics on pentoses (e.g. Sonderegger and Sauer 2003; Kuyper et al. 2005b; Wisselink et al. 2009; Garcia Sanchez et al. 2010; Zhou et al. 2012; Demeke et al. 2013a; Kim et al. 2013; Lee, Jellison and Alper 2014) and inhibitor tolerance (Wright et al. 2011; Koppram, Albers and Olsson 2012; Almarino, Reyes and Kao 2013; Smith, van Rensburg and Görgens 2014; González-Ramos et al. 2016).

#### Whole genome (re)sequencing

Determination of the entire DNA sequence of an organism.

Availability of a high-quality reference genome sequence is essential for experimental design in metabolic engineering. When genomes of strains that have been obtained by non-targeted approaches (e.g. evolutionary engineering or mutagenesis) are (re)sequenced, the relevance of identified mutations can subsequently be tested by their reintroduction in naïve strains, non-evolved strains and/or by classical genetics (reverse engineering; Oud et al. 2012). This approach has been successfully applied to identify mutations contributing to fast pentose fermentation (Nijland et al. 2014; dos Santos et al. 2016; Hou et al. 2016a) and inhibitor tolerance (e.g. Pinel et al. 2015; González-Ramos et al. 2016).

#### Quantitative trait locus (QTL) analysis

QTL analysis identifies alleles that contribute to (complex) phenotypes based on their meiotic co-segregation with a trait of interest (Liti and Louis 2012; Wilkening et al. 2014). In contrast to whole-genome (re)sequencing alone, QTL analysis can identify epistatic interactions.

QTL analysis currently enables resolution to gene or even nucleotide level (Swinnen et al. 2012). It has been used to identify alleles contributing to high-temperature tolerance (Sinha et al. 2006), ethanol tolerance (Swinnen et al. 2012) and improved ethanol-to-glycerol product ratios (Hubmann et al. 2013). The requirement of QTL analysis for mating limits its applicability in aneuploidy and/or poorly sporulating industrial *S. cerevisiae* strains.

#### Protein engineering

Modification of the amino acid sequences of proteins with the aim to improve their catalytic properties, regulation and/or stability in industrial contexts (Marcheschi, Gronenberg and Liao 2013).

Protein engineering has been used to improve the pentose-uptake kinetics, reduce the glucose sensitivity and improve the stability of yeast hexose transporters (e.g. Farwick et al. 2014; Young et al. 2014; Wang et al. 2015a; Reznicek et al. 2015; Shin et al. 2015; Li et al. 2016b; Nijland et al. 2016). The approach has been utilized to improve the redox cofactor specificity of xylose reductase and/or xylitol dehydrogenase to decrease xylitol formation (Petschacher et al. 2005; Watanabe, Kodaki and Makino 2005; Watanabe et al. 2007; Petschacher and Nidetzky 2008; Krahulec, Klimacek and Nidetzky 2009). Directed evolution of xylose isomerase yielded xylose isomerase variants with increased enzymatic activity (Lee, Jellison and Alper 2012). Directed evolution of native yeast dehydrogenases has yielded strains with increased HMF tolerance (Moon and Liu 2012).

#### Genome editing

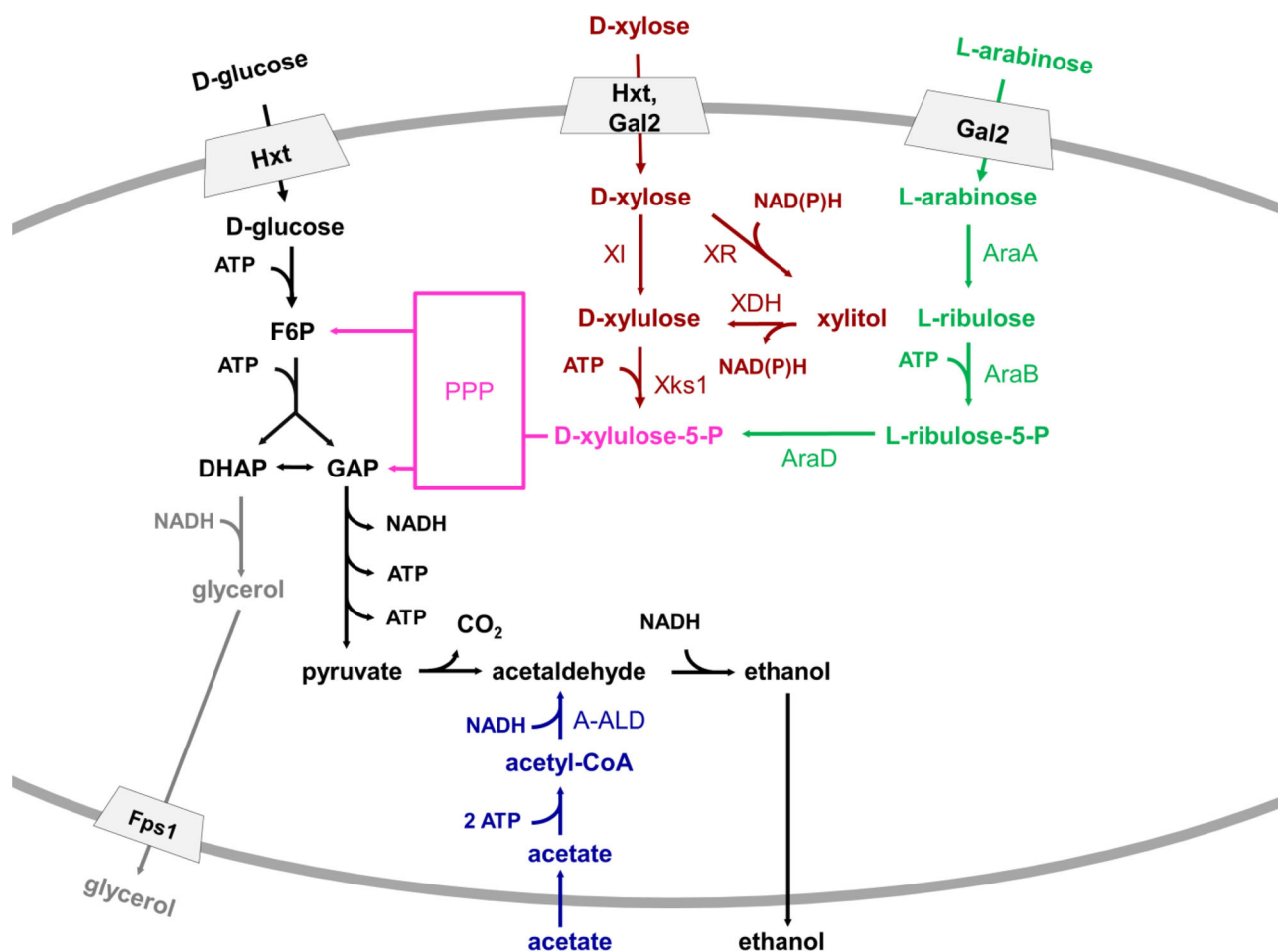
While 'classical' genetic engineering encompasses iterative, one-by-one introduction of genetic modifications, genome editing techniques enable simultaneous introduction of multiple (types of) modifications at different genomic loci (Sander and Joung 2014).

The combination of CRISPR-Cas9-based genome editing (DiCarlo et al. 2013; Mans et al. 2015) with *in vivo* assembly of DNA fragments has enabled the one-step introduction of all genetic modifications needed to enable *S. cerevisiae* to ferment xylose (Tsai et al. 2015; Shi et al. 2016; Verhoeven et al. 2017). Recent developments have enabled the application of the system in industrial backgrounds (Stovicek, Borodina and Forster 2015). CRISPR-Cas9 has been used in reverse engineering studies to rapidly introduce multiple single-nucleotide mutations observed in evolutionary engineering experiments in naïve strains (e.g. van Rossum et al. 2016).

expression of a bacterial pathway for conversion of L-arabinose into xylulose-5-phosphate, involving L-arabinose isomerase (AraA), L-ribulokinase (AraB), and L-ribulose-5-phosphate-4-epimerase (AraD) (Lee et al. 1986). Together with the non-oxidative PPP and glycolysis, these reactions enable

redox-cofactor-balanced alcoholic fermentation of L-arabinose (Fig. 2).

Combined expression of *Bacillus subtilis* or *B. licheniformis* *araA* and *E. coli* *araBD* (Becker and Boles 2003; Bettiga, Hahn-Hägerdal and Gorwa-Grauslund 2008; Wiedemann and Boles 2008)



**Figure 2.** Key strategies for engineering carbon and redox metabolism in *S. cerevisiae* strains for alcoholic fermentation of lignocellulosic feedstocks. Colors indicate the following pathways and processes: black, native *S. cerevisiae* enzymes of glycolysis and alcoholic fermentation; magenta, native enzymes of the non-oxidative pentose-phosphate pathway (PPP), overexpressed in pentose-fermenting strains; red, conversion of D-xylose into D-xylose-5-phosphate by heterologous expression of a xylose isomerase (XI) or combined expression of heterologous xylose reductase (XR) and xylitol dehydrogenase (XDH), together with the overexpression of (native) xylulokinase (Xks1); green, conversion of L-arabinose into D-xylose-5-phosphate by heterologous expression of a bacterial AraA/AraB/AraD pathway; blue, expression of a heterologous acetylating acetaldehyde dehydrogenase (A-ALD) for reduction of acetic acid to ethanol; gray, native glycerol pathway.

allowed aerobic growth of *S. cerevisiae* on L-arabinose. Anaerobic growth of *S. cerevisiae* on L-arabinose was first achieved by expressing the *Lactobacillus plantarum* *araA*, *B* and *D* genes in an XI-based xylose-fermenting strain that already overexpressed the enzymes of the non-oxidative PPP (Fig. 2), followed by evolutionary engineering under anaerobic conditions (Wiselink et al. 2007). Increased expression levels of *GAL2*, which encodes a galactose transporter that also transports L-arabinose (Kou, Christensen and Cirillo 1970), was essential for L-arabinose fermentation (Becker and Boles 2003; Wiselink et al. 2010; Subtil and Boles 2011; Subtil and Boles 2012). Increased expression of the transaldolase and transketolase isoenzymes *Nqm1* and *Tkl2* contributed to an increased rate of L-arabinose fermentation in strains evolved for fast L-arabinose fermentation (Wiselink et al. 2010). The set of L-arabinose isomerase genes that can be functionally expressed in *S. cerevisiae* was recently expanded by coexpression of *E. coli* *araA* with the *groEL* and *groES* chaperonins (Xia et al. 2016).

### Engineering of sugar transport and mixed-substrate fermentation

In early *S. cerevisiae* strains engineered for pentose fermentation, uptake of D-xylose and L-arabinose exclusively relied on

their native hexose transporters. While several of the 18 *S. cerevisiae* Hxt transporters (Hxt1–17 and Gal2) transport D-xylose, their  $K_m$  values for this pentose are one to two orders of magnitude higher than for glucose (Reifenberger, Boles and Ciriacy 1997; Hamacher et al. 2002; Lee et al. 2002; Saloheimo et al. 2007; Farwick et al. 2014). High-affinity glucose transporters, which are only expressed at low glucose concentrations (Diderich et al. 1999), display a lower  $K_m$  for D-xylose than low-affinity glucose transporters (Hamacher et al. 2002; Lee et al. 2002). The galactose transporter Gal2, which also catalyses high-affinity glucose transport (Reifenberger, Boles and Ciriacy 1997), also has a much higher  $K_m$  for L-arabinose than for glucose (Subtil and Boles 2011, 2012).

The higher affinities of Hxt transporters for glucose, combined with the transcriptional repression of Gal2 (Horak and Wolf 1997; Horak, Regelman and Wolf 2002) and other high-affinity Hxt transporters (Diderich et al. 1999; Sedlak and Ho 2004b) at high glucose concentrations, contribute to a sequential use of glucose and pentoses during mixed-substrate cultivation of engineered strains that depend on Hxt-mediated pentose uptake. Furthermore, the high  $K_m$  values of Hxt transporters for pentoses cause a deceleration of sugar fermentation during the pentose-fermentation phase. This 'tailing' effect is augmented by accumulation of ethanol and by the reduced inhibitor

tolerance of *S. cerevisiae* at low sugar fermentation rates (Bellissimi et al. 2009; Ask et al. 2013; Demeke et al. 2013b). Intensive efforts have been made to generate yeast strains that can either co-consume hexoses and pentose sugars or sequentially consume all sugars in hydrolysates in an economically acceptable time frame (Kim et al. 2012; Moysés et al. 2016).

Evolutionary engineering experiments played a major role in accelerating mixed-sugar utilization by engineered pentose-fermenting strains (Sonderegger and Sauer 2003; Kuyper et al. 2005b; Wisselink et al. 2009; Sanchez et al. 2010; Zhou et al. 2012). Repeated batch cultivation on a sugar mixture can favor selection of mutants that rapidly ferment one of the sugars, while showing deteriorated fermentation kinetics with other sugars in the mixture. In practice, such trade-off scenarios can increase rather than decrease the time required for complete conversion of sugar mixtures (Wisselink et al. 2009). A modified strategy for repeated batch cultivation, designed to equally distribute the number of generations of selective growth on each of the individual substrates in a mixture, enabled acceleration of the anaerobic conversion of glucose–xylose–arabinose mixtures by an engineered *S. cerevisiae* strain (Wisselink et al. 2009).

Recently constructed glucose-phosphorylation-negative, pentose-fermenting *S. cerevisiae* strains enabled evolutionary engineering experiments for *in vivo* directed evolution of Hxt variants that supported growth on D-xylose or L-arabinose in the presence of high glucose concentrations (Farwick et al. 2014; Nijland et al. 2014; Wisselink et al. 2015; Shin et al. 2015). Several of the evolved HXT alleles were confirmed to encode transporters whose D-xylose-transport kinetics were substantially less sensitive to glucose inhibition (Farwick et al. 2014; Nijland et al. 2014; Wisselink et al. 2015; Shin et al. 2015). Remarkably, independent evolutionary engineering studies aimed at selecting glucose-insensitive D-xylose and L-arabinose Hxt transporters yielded single-amino-acid substitutions at the exact corresponding positions in Hxt7 (N370), in Gal2 (N376), and in a chimera of Hxt3 and Hxt6 (N367) (Farwick et al. 2014; Nijland et al. 2014; Wisselink et al. 2015). Additional Hxt variants with improved relative affinities for pentoses and glucose were obtained by *in vitro* directed evolution and knowledge-based protein engineering (Farwick et al. 2014; Reznicek et al. 2015; Box 1).

Low-, moderate-, and high-affinity pentose transporters from pentose-metabolizing filamentous fungi or non-*Saccharomyces* yeasts have been functionally expressed in *S. cerevisiae* (Weierstall, Hollenberg and Boles 1999; Leandro, Gonçalves and Spencer-Martins 2006; Katahira et al. 2008; Du, Li and Zhao et al. 2010; Runquist, Hahn-Hägerdal and Rådström 2010b; Subtil and Boles 2011; Young et al. 2012; Ferreira et al. 2013; Colabardini et al. 2014; Knoshaug et al. 2015; Li et al. 2015; Reis et al. 2016). Expression and/or activity of several of these transporters were further improved by directed evolution (Young et al. 2012; Li et al. 2015; Li, Schmitz and Alper 2016b) or evolutionary engineering (Moysés et al. 2016; Wang, Yu and Zhao 2016). Such high-affinity transporters may be suited to 'mop-up' low concentrations of pentoses towards the end of a fermentation process. Since high-affinity sugar transporters are typically proton symporters, care should be taken to avoid scenarios in which their simultaneous expression with Hxt-like transporters, which mediate facilitated diffusion, causes futile cycles and negatively affects inhibitor tolerance.

### Inhibitor tolerance

Yeast enzymes involved in detoxification of specific inhibitors provide logical targets for metabolic engineering. For example,

overexpression of native NAD(P)<sup>+</sup>-dependent alcohol dehydrogenases stimulates conversion of furfural and HMF to the less toxic alcohols furanmethanol and furan-2,5-dimethanol, respectively (Jeppsson et al. 2003; Lewis Liu et al. 2008; Almeida et al. 2009). Similarly, combined overexpression of the aldehyde dehydrogenase Ald5, the decarboxylase Pad1, and the alcohol acetyltransferases Atf1 and Atf2 increased resistance to several phenolic inhibitors (Adeboye, Bettiga and Olsson 2017).

Genome-wide expression studies have revealed intricate, strain- and context-dependent stress-response networks as major key contributors to inhibitor tolerance (Abbott et al. 2007; Almeida et al. 2007; Li and Yuan 2010; Mira et al. 2010; Liu 2011; Ullah et al. 2013; Guo and Olsson 2014). An in-depth transcriptome analysis implicated SFP1 and ACE2, which encode transcriptional regulators involved in ribosomal biogenesis and septum destruction after cytokinesis, respectively, in the phenotype of an acetic acid and furfural-tolerant strain. Indeed, overexpression of these transcriptional regulators significantly enhanced ethanol productivity in the presence of these inhibitors (Chen et al. 2016).

Whole-genome resequencing of tolerant strains derived from evolutionary engineering, mutagenesis, and/or genome shuffling has yielded strains with increased tolerance whose causal mutations could be identified (Almario, Reyes and Kao 2013; Demeke et al. 2013a; Pinel et al. 2015; González-Ramos et al. 2016; Thompson et al. 2016). Physiological and evolutionary engineering experiments demonstrated the importance of high sugar fermentation rates for acetic acid tolerance (Bellissimi et al. 2009; Wright et al. 2011). When the acetic acid concentration in anaerobic, xylose-grown continuous cultures was continually increased over time, evolving cultures acquired the ability to grow at acetic acid concentrations that prevented growth of the non-evolved *S. cerevisiae* strain. However, after growth in the absence of acetic acid, full expression of their increased tolerance required pre-exposure to a lower acetic acid concentration. This observation indicated that the acquired tolerance was inducible rather than constitutive (Wright et al. 2011). Constitutive tolerance to acetic acid was shown to reflect the fraction of yeast populations able to initiate growth upon exposure to acetic acid stress (Swinnen et al. 2014). Based on this observation, an evolutionary engineering strategy that involved alternating batch cultivation cycles in the presence and absence of acetic acid was successfully applied to select for constitutive acetic acid tolerance (González-Ramos et al. 2016).

Exploration of the natural diversity of inhibitor tolerance among *S. cerevisiae* strains (Favaro et al. 2013; Wimalasena et al. 2014; Field et al. 2015) is increasingly used to identify genes and alleles that contribute to tolerance. In particular, combination of whole genome sequencing and classical genetics is a powerful approach to identify relevant genomic loci, genes, and even nucleotides (Liti and Louis 2012) (quantitative trait locus analysis; see Box 1). For example, Meijnen et al. (2016) used whole-genome sequencing of pooled tolerant and sensitive segregants from crosses between a highly acetic-acid-tolerant *S. cerevisiae* strain and a reference strain to identify mutations in five genes that contributed to tolerance.

### Reduction of acetic acid to ethanol: converting an inhibitor into a co-substrate

Even small improvements of the product yield on feedstock can substantially improve the economics of biotechnological processes for manufacturing large-volume products such as ethanol (Van Maris et al. 2006; Nielsen et al. 2013). In industrial,



anaerobic ethanol production processes, a significant amount of sugar is converted into the byproduct glycerol (Nissen *et al.* 2000). Glycerol formation, catalyzed by the two isoforms of glycerol-3-phosphate dehydrogenase (Gpd1 and Gpd2) and of glycerol-3-phosphate phosphatase (Gpp1 and Gpp2), is required during anaerobic growth of *S. cerevisiae* for reoxidation of NADH generated in biosynthetic reactions (Van Dijken and Scheffers 1986; Björkqvist *et al.* 1997). Metabolic engineering strategies to diminish glycerol formation focused on modification of intracellular redox reactions (Nissen *et al.* 2000; Guo *et al.* 2011) or modulation of GPD1 and GPD2 expression (Hubmann, Guillouet and Nevoigt 2011). Replacement of GPD1 and GPD2 with a heterologous gene encoding an acetylating acetaldehyde dehydrogenase (A-ALD) and supplementation of acetic acid eliminated glycerol formation in anaerobic *S. cerevisiae* cultures (Guadalupe-Medina *et al.* 2010). By enabling NADH-dependent reduction of acetic acid to ethanol (Fig. 2), this strategy resulted in a significant increase in the final ethanol yield, while consuming acetic acid. This engineering strategy has recently been extended by altering the redox-cofactor specificities of alcohol dehydrogenase (Henningsen *et al.* 2015) and 6-phosphogluconate dehydrogenase (Papapetridis *et al.* 2016). These further interventions increased the availability of cytosolic NADH for acetate reduction and should, upon implementation in industrial strains, further improve *in situ* detoxification of acetic acid. The A-ALD strategy was also shown to decrease xylitol formation in XR/XDH-based xylose-fermenting engineered strains by reoxidation of excess NADH formed in the XDH reaction (Wei *et al.* 2013; Zhang *et al.* 2016a).

## DEVELOPMENT OF INDUSTRIAL YEAST STRAINS AND PROCESSES

Much of the research discussed in the preceding paragraphs was based on laboratory yeast strains, grown in synthetic media whose composition can be different from that of industrial lignocellulosic hydrolysates. Table 2 provides examples of ethanol yields and biomass-specific conversion rates that have been obtained with engineered *S. cerevisiae* strains in synthetic media.

While data on the performance of current industrial strains on industrial feedstocks are proprietary, many scientific publications describe the fermentation of hydrolysates by D-xylose-fermenting strains (either XI- or XR-XDH-based, but so far without arabinose pathways). These studies cover a wide variety of feedstocks, biomass deconstruction and fermentation strategies (batch, fed-batch, simultaneous saccharification and fermentation), aeration regimes and nutritional supplementations (e.g. yeast extract, peptone, low-cost industrial supplements, trace elements, nitrogen sources). However, with few exceptions, these data are restricted to final ethanol yields and titers, and do not include quantitative information on the biomass-specific conversion rates ( $q_{\text{xylose}}$ ,  $q_{\text{ethanol}}$ , expressed in  $\text{g}(\text{g biomass})^{-1}\cdot\text{h}^{-1}$ ) that are essential for strain comparison and process design. Table 3 summarizes results of studies on fermentation of biomass hydrolysates that include or enable calculation of biomass-specific conversion rates and ethanol yields.

Despite the heterogeneity of the studies included in Tables 2 and 3, the available data clearly illustrate that, while even 'academic' strain platforms can exhibit high ethanol yields in hydrolysates, conversion rates under these conditions are much lower than in synthetic media. Improving kinetics and robustness in industrial hydrolysates is therefore the single most important objective in industrial yeast strain development platforms.

In the authors' experience, aspects such as spatial and temporal heterogeneity, hydrostatic pressure and CO<sub>2</sub> concentrations, which are highly important for down-scaling aerobic industrial fermentation processes (Noorman 2011), do not represent substantial challenges in down-scaling second-generation ethanol processes. Provided that anaerobic conditions can be maintained, strain performance can therefore be adequately assessed in small-scale systems. Access to hydrolysates whose composition and concentration are fully representative of the target industrial substrate(s) may be necessary for strain development. This requirement is not a trivial one due to feedstock variability, the plethora of pretreatment options and the limited scalability and continuous innovation in biomass deconstruction (Knoll *et al.* 2013; Li *et al.* 2016a).

Due to the complex, multigene nature of inhibitor tolerance, screening of natural and industrial *S. cerevisiae* strains is a logical first step in the development of industrial strain platforms. The power of this approach is illustrated by the Brazilian first-generation bioethanol strain PE-2. Stable maintenance of this strain in non-aseptically operated industrial reactors, over many production campaigns (Basso *et al.* 2008), was attributed to its innate tolerance to the sulfuric acid washing steps that are employed between fermentation cycles to combat bacterial contamination (Della-Bianca *et al.* 2014). In contrast to most laboratory strains, robust industrial strains of *S. cerevisiae* are heterozygous diploids or polyploids that, additionally, are prone to whole-chromosome or segmental aneuploidy (Zhang *et al.* 2016b; Gorter De Vries, Pronk and Daran 2017). Acquiring high-quality, well annotated genome sequences (Box 1) of these complex genomes is an important prerequisite for interpreting the results of strain improvement campaigns and for targeted genetic modification.

Episomal expression vectors carrying auxotrophic marker genes, which are commonly used in academic research, do not allow for stable replication and selection, respectively, in complex industrial media (Pronk 2002; Hahn-Hägerdal *et al.* 2007; Karim, Curran and Alper 2013). Instead, industrial strain development requires chromosomal integration of expression cassettes. Even basic academic designs of xylose- and arabinose-fermenting strains encompass the introduction of 10–12 different expression cassettes (Wisselink *et al.* 2007, 2010), some of which need to be present in multiple copies (e.g. for high-level expression of XI genes; Zhou *et al.* 2012; Wang *et al.* 2014; Demeke *et al.* 2015; Verhoeven *et al.* 2017). Additional genetic modifications, on multiple chromosomes in the case of diploid or polyploid strains, are required to reduce by-product formation, improve inhibitor tolerance and/or improve product yields. Genetic modification of complex industrial yeast genomes has now been strongly accelerated by novel, CRISPR-based genome editing tools (Box 1).

Non-targeted strategies for strain improvement (Box 1) including mutagenesis with chemical mutagens or irradiation, evolutionary engineering, recursive breeding and/or genome shuffling remain essential for industrial strain improvement. Down-scaling, automation and integration with high-throughput screening of the resulting strains in hydrolysates strongly increases the success rates of these approaches (e.g. for ethanol tolerance; Snoek *et al.* 2015). In non-targeted strain improvement campaigns, it is important to maintain selective pressure on all relevant aspects of strain performance, to avoid trade-offs between, for example, fermentation kinetics with different sugars (glucose, D-xylose and L-arabinose), and/or inhibitor tolerance (Wisselink *et al.* 2009; Demeke *et al.* 2013a; Smith, van Rensburg and Görgens 2014).

**Table 2.** Ethanol yields ( $Y_{E/S}$ , g ethanol·(g sugar)<sup>-1</sup>) and biomass-specific rates of D-xylose and/or L-arabinose consumption and ethanol production ( $q_{xylose}$ ,  $q_{arabinose}$  and  $q_{ethanol}$ , respectively, g(g biomass)<sup>-1</sup>·h<sup>-1</sup>) in cultures of *S. cerevisiae* strains engineered for pentose fermentation, grown in synthetic media. Asterisks (\*) indicate values estimated from graphs in the cited reference.

<i>S. cerevisiae</i> strain	Pentose fermentation strategy	Key genetic modifications	Fermentation conditions	$Y_{E/S}$ (g·g <sup>-1</sup> )	$q_{ethanol}$ (g·g <sup>-1</sup> ·h <sup>-1</sup> )	$q_{xylose}$ (g·g <sup>-1</sup> ·h <sup>-1</sup> )	$q_{arabinose}$ (g·g <sup>-1</sup> ·h <sup>-1</sup> )	Reference
TMB3400	XR/XDH ( <i>S. stipitis</i> XYL1, XYL2)	SsXYL1, SsXYL2 + XKS1↑, random mutagenesis	Anaerobic batch (bioreactor), 5% xylose	0.33	0.04	0.13		Karhumaa et al. (2007)
GLBRCY87	XR/XDH ( <i>S. stipitis</i> XYL1, XYL2)	SsXYL1, SsXYL2, SsXYL3, evolved on xylose and hydrolysate inhibitors	Semi-anaerobic batch (flask), 5% glucose and 5% xylose	0.34*	0.036*	0.13		Sato et al. (2016)
SR8	XR/XDH ( <i>S. stipitis</i> XYL1, XYL2)	SsXYL1, Ss XYL2, Ss XYL3, <i>ald6Δ</i> , evolved on xylose	Anaerobic batch (reactor), 4% xylose	0.39	0.25	0.64		Wei et al. (2013)
TMB3421	XR/XDH ( <i>S. stipitis</i> XYL1, XYL2)	<i>S. stipitis</i> XYL1 <sup>N272D/P275Q</sup> , XYL2 + XKS1↑ TAL1↑ TKL1↑ RPE1↑ RKI1↑ <i>gre3Δ</i> , evolved on xylose	Anaerobic batch (reactor), 6% xylose	0.35	0.20	0.57		Runquist, Hahn-Hägerdal and Bettiga (2010a)
RWB 217	XI ( <i>Piromyces XylA</i> )	<i>Piromyces XylA</i> + XKS1↑ TAL1↑ TKL1↑ RPE1↑ RKI1↑, <i>gre3Δ</i>	Anaerobic batch (reactor), 2% xylose	0.43	0.46	1.06		Kuyper et al. (2005a)
RWB 218	XI ( <i>Piromyces XylA</i> )	Derived from RWB 217 after evolution on glucose/xylose mixtures	Anaerobic batch (reactor) 2% xylose	0.41	0.49	1.2		Kuyper et al. (2005b)
H131-A3-AL <sup>CS</sup>	XI ( <i>Piromyces XylA</i> )	<i>XylA</i> , <i>Xyl3</i> , XKS1↑ TAL1↑ TKL1↑ RPE1↑ RKI1↑, <i>gre3Δ</i> , evolved on xylose	Anaerobic batch (reactor), 4% xylose	0.43	0.76	1.9		Zhou et al. (2012)
IMS0010	XI/AraABD ( <i>Piromyces XylA</i> , <i>L. plantarum</i> AraA, B, D)	<i>XylA</i> ; XKS1↑ TAL1↑ TKL1↑ RPE1↑ RKI1↑ AraT, AraA, AraB, AraD, evolved on glucose, xylose, arabinose mixtures	Anaerobic batch (reactor), 3% glucose, 1.5% xylose and 1.5% arabinose	0.43		0.35	0.53	Wisselink et al. (2009)
GS1.11-26	XI/AraABD ( <i>Piromyces XylA</i> , <i>L. plantarum</i> AraA, B, D, K, <i>lactis</i> ARAT)	<i>XylA</i> , XKS1↑ TAL1↑ TKL1↑ RPE1↑ RKI1↑ <i>XylA</i> HXT7↑ KlAraT, AraA, AraB, AraD, TAL2↑ TKL2↑, several rounds of mutagenesis and evolution on xylose	Semi-anaerobic batch (flask), synthetic medium, 3.5% xylose	0.46	0.48	1.1		Demeke et al. (2013a)

Even when kinetics of yeast growth and fermentation in hydrolysates are suboptimal (Table 2) due to the impact of inhibitors and/or strain characteristics, industrial fermentation processes need to achieve complete sugar conversion within acceptable time limits (typically 72 h or less). This can be accomplished by increasing the initial yeast biomass densities, which, in second generation processes, are typically 2- to 8-fold higher than the initial concentrations of 0.125–0.25 g·l<sup>-1</sup> that are used in first-generation processes without biomass recycling (Jacques, Lyons and Kelsall 2003). Several second-generation bioethanol plants therefore include on-site bioreactors for cost-effective generation of the required yeast biomass. Precultivation in the presence of mild concentrations of inhibitors can prime yeast cells for improved performance upon exposure to stressful conditions (Alkasrawi et al. 2006; Sánchez i Nogué, Narayanan and Gorwa-Grauslund 2013; Nielsen et al. 2015). Especially when biomass propagation uses non-lignocellulosic

feedstocks (Steiner 2008; Narendranath and Lewis 2013) and/or is operated aerobically to maximize biomass yields, yeast strain development must take the need to maintain pentose-fermentation kinetics and inhibitor tolerance during biomass propagation into account.

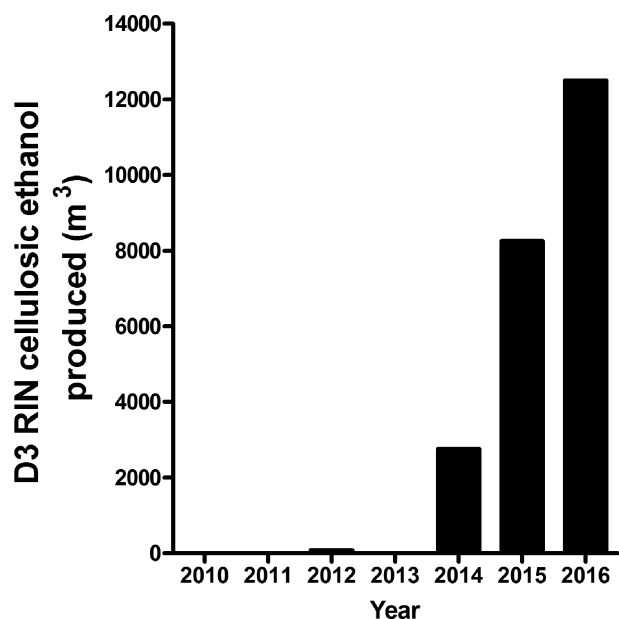
## FULL-SCALE IMPLEMENTATION: STATUS AND CHALLENGES

Vigorous lab-scale optimization of each of the unit operations in yeast-based ethanol production from lignocellulosic feedstocks enabled the design, construction and operation of processes at pilot scale. Recently, several industrial parties started or announced the first commercial-scale cellulosic ethanol plants, most of which rely on yeast for the fermentation step (Table 1). Actual cellulosic ethanol production volumes in the

**Table 3.** Ethanol yields on consumed sugar ( $Y_{E/S}$ , g ethanol:(g sugar)<sup>-1</sup>) and biomass-specific rates of glucose and xylose consumption and ethanol production ( $q_{\text{glucose}}$ ,  $q_{\text{xylose}}$  and  $q_{\text{ethanol}}$ , respectively, g:(g biomass)<sup>-1</sup>.h<sup>-1</sup>) in cultures of *S. cerevisiae* strains engineered for pentose fermentation, grown in lignocellulosic hydrolysates. Asterisks (\*) indicate specific conversion rates estimated from graphs in the cited reference; daggers (†) indicate crude estimates of biomass-specific rates calculated based on the assumption that biomass concentrations did not change after inoculation; these estimates probably overestimate actual biomass-specific conversion rates.

S. cerevisiae strain	Description	Feedstock, pretreatment conditions, hydrolysate sugar composition	Fermentation conditions, added nutrients <sup>a</sup>	$Y_{E/S}$ (g.g <sup>-1</sup> )	$q_{\text{glucose}}$ (g.g.h <sup>-1</sup> )	$q_{\text{ethanol}}$ (g.g.h <sup>-1</sup> )	$q_{\text{xylose}}$ (g.g.h <sup>-1</sup> )	Reference
TMB3400	XR/XDH <i>S. stipitis</i> XYL1 and XYL2; XKS1↑	Spruce, two-step dilute acid hydrolysis, 1.6% glucose, 0.4% xylose, 1% mannose, 1% galactose	Anaerobic batch (flasks), (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> NaH <sub>2</sub> PO <sub>4</sub> MgSO <sub>4</sub> , YE	0.41	0.021	0.005	0.005	Karhumaa et al. (2007)
GLBRCY87	XR/XDH <i>S. stipitis</i> XYL1, XYL2 and XYL3 evolved on xylose and hydrolysate inhibitors	Corn stover, ammonia fiber expansion, 8% glucose, 3.8% xylose	Semi-anaerobic batch (flasks), pH 5.5, urea, YNB	0.28	1.4*	0.27*	0.04	Sato et al. (2016)
GLBRCY87	XR/XDH <i>S. stipitis</i> XYL1, XYL2 and XYL3 evolved on xylose and hydrolysate inhibitors	Switchgrass, ammonia fiber expansion, 6.1% glucose, 3.9% xylose	Semi-anaerobic batch (flasks), urea, YNB	0.35	1.65*	0.28*	0.07	Sato et al. (2016)
MEC1122	XR/XDH, industrial host strain <i>S. stipitis</i> XYL1 <sup>(N272D/P275Q)</sup> and XYL2, XKS1↑ TAL1↑	Corn cobs, autohydrolysis (202°C), liquid fraction acid-treated. 0.3% glucose, 2.6% xylose	Oxygen limited batch (flasks), cheese whey, urea, YE, K <sub>2</sub> O <sub>5</sub> S <sub>2</sub>	0.3		0.12 <sup>†,*</sup>	0.25 <sup>†</sup>	Costa et al. (2017)
RWB 218	XI <i>Piromyces XylA</i> , XKS1↑ TAL1↑ TKL1↑ RPE1↑ RKI1↑, <i>gre3Δ</i> , evolved on glucose/xylose mixed substrate	Wheat straw hydrolysate, steam explosion, 5% glucose, 2% xylose	Anaerobic batch (reactor), (NH <sub>4</sub> ) <sub>2</sub> PO <sub>4</sub>	0.47	1.58 <sup>†</sup>	1.0 <sup>†</sup>	0.32 <sup>†</sup>	Van Maris et al. (2007)
GS1.11-26	XI, AraABD <i>Piromyces XylA</i> , XKS1↑ TAL1↑ TKL1↑ RPE1↑ RKI1↑ HXT7↑ AraT, AraA, AraB, AraD, TAL2↑ TKL2↑, several rounds of mutagenesis and evolution on xylose	Spruce (no hydrolysis), acid pre-treated, 6.2% glucose, 1.8% xylose, 1% mannose	Semi-anaerobic batch (flasks), YNB, (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , amino acids added	0.43	2.46 <sup>†</sup>	0.3 <sup>†</sup>	0.11 <sup>†</sup>	Demeke et al. (2013a)
XH7	Multiple integrations of RuXylA; XKS1↑ TAL1↑ TKL1↑ RPE1↑ RKI1↑ <i>pho13Δ gre3Δ</i> , evolved on xylose	Corn stover, steam explosion, 6.2% glucose, 1.8% xylose	Semi-anaerobic batch (flasks), urea	0.39	0.14	0.080	0.096	Li et al. (2016c)
LF1	Selection mutant of XH7 further evolved on xylose and hydrolysates with MGT transporter introduced	Corn stover, steam explosion, 8.7% glucose, 3.9% xylose	Semi-anaerobic batch (flasks), urea	0.41	0.57	0.34	0.23	Li et al. (2016c)

<sup>a</sup>Abbreviations of supplements: YE, yeast extract; YNB, yeast nitrogen base; YP, yeast extract and peptone.



**Figure 3.** Annual production volumes of cellulose ethanol in the USA from 2010 until November 2016. Numbers are based on RIN D code 3 RIN (renewable identification number) credits generated (accounted as cellulose ethanol; United States Environmental Protection Agency 2017).

USA, derived from registered renewable identification number (RIN) credits (United States Environmental Protection Agency 2017), indicate an increase in recent years (Fig. 3). However, based on these numbers and estimates for plants elsewhere in the world, the global production volume of cellulosic ethanol is still below 1% of that of first-generation processes. This places actual production volumes years behind earlier projections (Lane 2015) and indicates that currently installed commercial-scale plants still operate below their nominal capacity. For obvious reasons, industrial parties cannot always be fully transparent on factors that impede acceleration and intensification of cellulosic ethanol production. However, presentations at conferences and trade fairs enable a few general observations. Many aspects of full-scale plants can be assessed prior to commercialization by carefully down-scaling all process steps. Such down-scaling is crucial for optimal process development and equipment design (sizing, layout, mixing requirements, scheduling, etc.; Noorman 2011; Wang et al. 2015b; Villadsen and Noorman 2016). As indicated above, most aspects of the performance of engineered yeast strains in full-scale plants can be, and indeed have been, adequately predicted from such lab-scale studies. Other aspects, such as impacts of seasonal and regional variation of plant biomass and other in-process streams, are more difficult to predict. Additionally, continued optimization of upstream unit operations in commercial-scale plants requires continual ‘tuning’ of yeast strain characteristics to address impacts on the fermentation process.

An aspect that may have been underestimated in down-scaled experiments is bacterial contamination. Yield losses caused by contamination with lactic acid bacteria is a well-known problem in first-generation bioethanol production (Bischoff et al. 2009; Beckner, Ivey and Phister 2011). The longer pretreatment and fermentation times in current cellulosic ethanol processes, caused by inhibitors in the hydrolysates, allow lactic acid bacteria more time to compete with the engineered yeast strains than in first-generation processes. More-

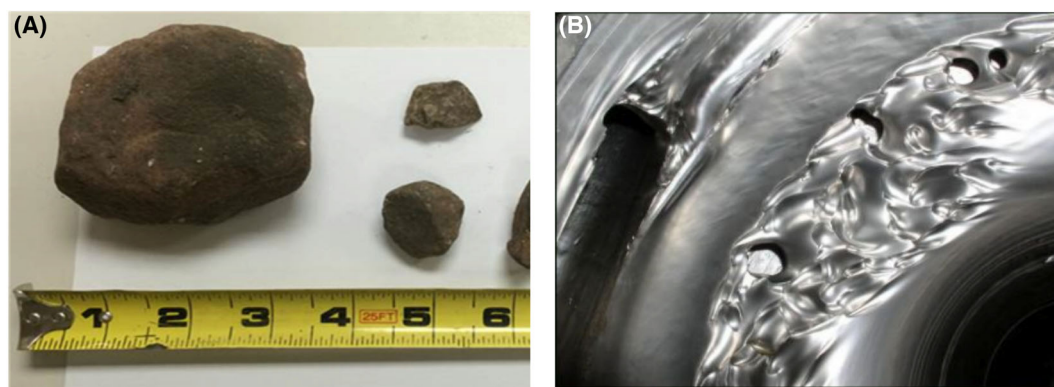
over, concentrations of ethanol, a potent inhibitor of lactic acid bacteria, are typically lower in second generation processes (Albers et al. 2011). While requiring constant attention, bacterial contamination is a manageable problem that can be addressed with currently available technology and without insurmountable additional costs. Strict attention to hygiene aspects in all aspects of plant design and operation, e.g. by avoiding dead legs, implementing full drainability and robust cleaning-in-place procedures, is crucial in this respect. For example, installing appropriate valves and filters should be an integral part of plant design and be combined with measures to minimize survival and propagation of bacterial contaminants that do make it into the process. As a last and sometimes inevitable resort, antibacterial compounds can be used to minimize bacterial load and impact (Muthaiyan, Limayem and Ricke 2011).

An important factor that appears to have escaped attention in most small-scale studies is that the agricultural residues entering a factory contain an abundance of non-plant solids. Rocks, sand and metal particles coming off agricultural fields and/or equipment can rapidly damage and erode expensive equipment (Fig. 4). In pilot- and commercial-scale plants, clogging of pipes and reactors during biomass handling and pretreatment remains a point of attention. These challenges, which can result in significant down-time of plants, can either be addressed by elimination of high-density solids during harvesting and storage of the biomass or by installing extra unit operations in factories. For example, Beta Renewables installed a biomass washing step at their Crescentino plant (Lane 2014). While these engineering solutions cannot be easily down-scaled and retrofitting of existing processes may be complicated and expensive, they are technologically surmountable.

## OUTLOOK

Second-generation bioethanol plants are complex, multi-step biorefineries for conversion of crude and variable feedstocks. Just as high-efficiency petrochemical refineries did not appear overnight, optimizing the performance of the current frontrunner plants requires significant process engineering efforts. As remaining challenges in biomass processing and deconstruction are conquered, yeast-based processes for second-generation biofuels should soon leave the demonstration phase, become fully economically viable, and expand the production volume. Such an expansion will generate new incentives for improving conversion yields, while reducing carbon footprints and overall costs. For example, the stillage fraction that remains after distillation is currently considered a waste stream and treated by anaerobic digestion. As proposed for first-generation processes (Clomburg and Gonzalez 2013), options may be explored to convert stillage fractions from second-generation plants into biogas or higher value products.

The yeast technology developed for conversion of second-generation feedstocks can also be applied to improve ethanol yields of first-generation bioethanol production processes and plants. For example, in current first-generation ethanol processes, corn fiber is separated from whole stillage as ‘wet-distillers’ grains’, mixed with the concentrated stillage liquid fraction (CDS, ‘condensed distillers’ solubles’) and dried to yield DDGS (‘dried distillers’ grains with solubles’), which is sold as cattle feed (Jacques, Lyons and Kelsall 2003; Kim et al. 2008). Processes that enable conversion of this fiber-based side stream, which is more easily hydrolysed than other cellulosic feedstocks, in the context of existing first-generation bioethanol



**Figure 4.** Problems not encountered in shake flask cultures: non-yeast-related challenges in large-scale processing of lignocellulosic biomass. (A) Small rocks collected from corn stover (picture courtesy of POET-DSM Liberty). (B) Example of severely eroded equipment (picture courtesy of Iogen Corporation; Lane 2016b).

facilities, are referred to as 'Gen 1.5' technology. Several Gen 1.5 processes are currently being implemented commercially and have the potential to increase the ethanol yield per bushel of corn by approximately 10% (ICM 2012; Lane 2016a; D3MAX 2017).

Metabolic engineering strategies to further improve yeast performance in second generation bioethanol processes are already being explored. For example, the option to implement the strategies discussed above in non-*Saccharomyces* yeasts with industrially interesting properties, such as high-temperature- and low-pH-tolerant strains is being investigated (Ryabova, Chmil and Sibirny 2003; Yuan et al. 2012; Goshima et al. 2013; Radecka et al. 2015). Other research focuses on the improvement of these characteristics in *S. cerevisiae* (Caspeta et al. 2014; Fletcher et al. 2017). Furthermore, as production volume increases, the economic relevance of the conversion of minor, potentially fermentable substrates such as uronic acids and deoxysugars into ethanol (Van Maris et al. 2006) will increase. Co-feeding of additional, low-value carbon sources can be explored as a strategy to further increase ethanol yield. For example, glycerol, derived from fermentation stills or biodiesel manufacturing (Yang, Hanna and Sun 2012) is considered as a potential co-substrate. Significant rates of glycerol utilization have already been achieved in *S. cerevisiae* strains by simultaneously (over-) expressing glycerol dehydrogenase (GCY1), dihydroxyacetone kinase (DAK1) and a heterologous glycerol transporter (Yu, Kim and Han 2010). These glycerol conversion pathways can be combined with the engineered pathways for acetic acid reduction discussed above to further optimize ethanol yields and process robustness (De Bont et al. 2012; Klaassen and Hartman 2014).

Consolidated bioprocessing, i.e. the full integration of pretreatment, hydrolysis and fermentation towards ethanol in a single microbial process step, remains a 'holy grail' in lignocellulosic ethanol production. Engineered starch-hydrolysing *S. cerevisiae* strains are already applied in first-generation processes (Kumar and Singh 2016). The first important steps towards efficient cellulose and xylan hydrolysis by *S. cerevisiae* have been made by functional expression of heterologous polysaccharide hydrolases (Olson et al. 2012; Den Haan et al. 2015). The resulting engineered strains often produce significant amounts of di- and/or trisaccharides (La Grange et al. 2001; Katahira et al. 2004; Lee et al. 2009). The ability to ferment cellobiose has been successfully introduced into *S. cerevisiae* by combined expression of a heterologous cellobiose transporter and  $\beta$ -glucosidase (Galazka et al. 2010; Hu et al. 2016).

Our confidence in yeast-based processes notwithstanding, it is relevant to look beyond yeasts. Fast progress is made in engineering thermophilic and cellulolytic bacteria for efficient ethanol production. High-temperature fermentation processes require less cooling and reduce contamination risks (Scully and Orlýgsson 2015). If, moreover, thermophilic consolidated bioprocessing can integrate a simple mechanical pretreatment with biomass deconstruction and fermentation by a single organism (Lynd et al. 2005; Olson et al. 2012), while matching the robustness of yeasts under industrial conditions, it could develop into a highly interesting approach for second-generation ethanol production.

Technological and scientific progress aside, development of yeast platforms for lignocellulosic ethanol production has provided a generation of academic and industrial researchers with a challenging common goal. We hope that this minireview not only informs readers about scientific and technological progress in this field, but also conveys our genuine conviction that combining and integrating academic and industrial research efforts (Pronk et al. 2015) is a stimulating, positively challenging way towards sustainable innovation.

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