FEMS Yeast Research, 17, 2017, fox044

doi: 10.1093/femsyr/fox044
Advance Access Publication Date: 16 June 2017
Minireview

MINIREVIEW

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

Mickel L. A. Jansen[1], Jasmine M. Bracher[2], Ioannis Papapetridis[2], Maarten D. Verhoeven[2], Hans de Bruijn[1], Paul P. de Waal[1], Antonius J. A. van Maris[2,†], Paul Klaassen[1] and Jack T. Pronk[2,*]

[1]DSM Biotechnology Centre, Alexander Fleminglaan 1, 2613 AX Delft, The Netherlands and [2]Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands

*Corresponding author: Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands. Tel: +31-15-2782416; E-mail j.t.pronk@tudelft.nl
†Current address: Division of Industrial Biotechnology, School of Biotechnology, KTH Royal Institute of Technology, AlbaNova University Center, SE 106 91, Stockholm, Sweden.
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.
Editor: Irina Borodina

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 ($(CH_2O)_n \rightarrow \frac{1}{3}n\ C_2H_6O + \frac{1}{3}n\ CO_2$). 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

Received: 13 April 2017; **Accepted:** 15 June 2017

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^{-1}
DuPont Cellulosic Ethanol LLC—Nevada	USA (IA)	Corn stover	113.6
Poet-DSM Advanced Biofuels LLC—Project Liberty[a]	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	*Arundo donax*, rice straw, wheat straw	75.0
Borregaard Industries AS—ChemCell Ethanol	Norway	Wood pulping residues	20.0

[a]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)$^{-1}$), ethanol titers of up to 21% (w/w), and volumetric productivities of 2–3 kg·m^{-3}·h^{-1} (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

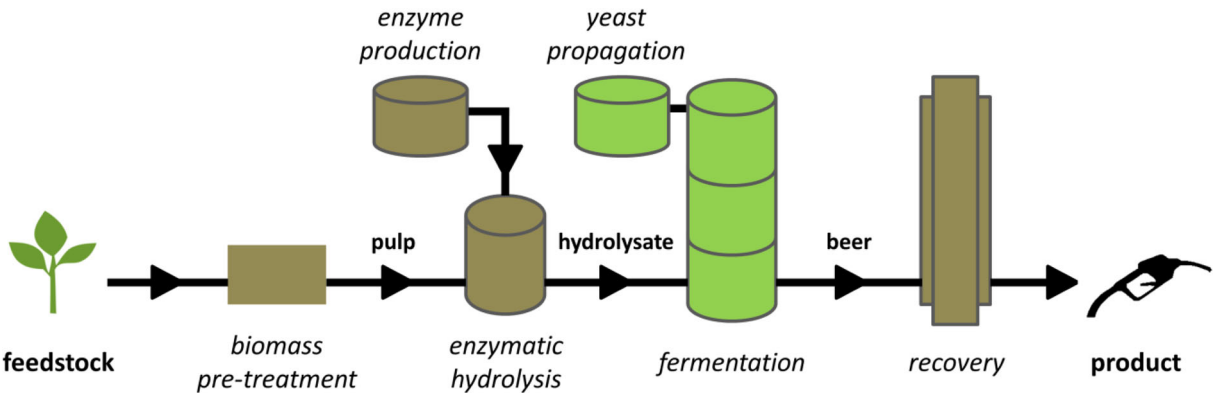

Figure 1. Schematic process-flow diagram for ethanol production from lignocellulose, based on physically separated processes for pre-treatment, hydrolysis and fermentation, combined with on-site cultivation of filamentous fungi for production of cellulolytic enzymes and on-site propagation of engineered 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; Pampulha 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 $g \cdot l^{-1}$, 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°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; Karhumaa *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^{2+}, 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; Almario, 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)

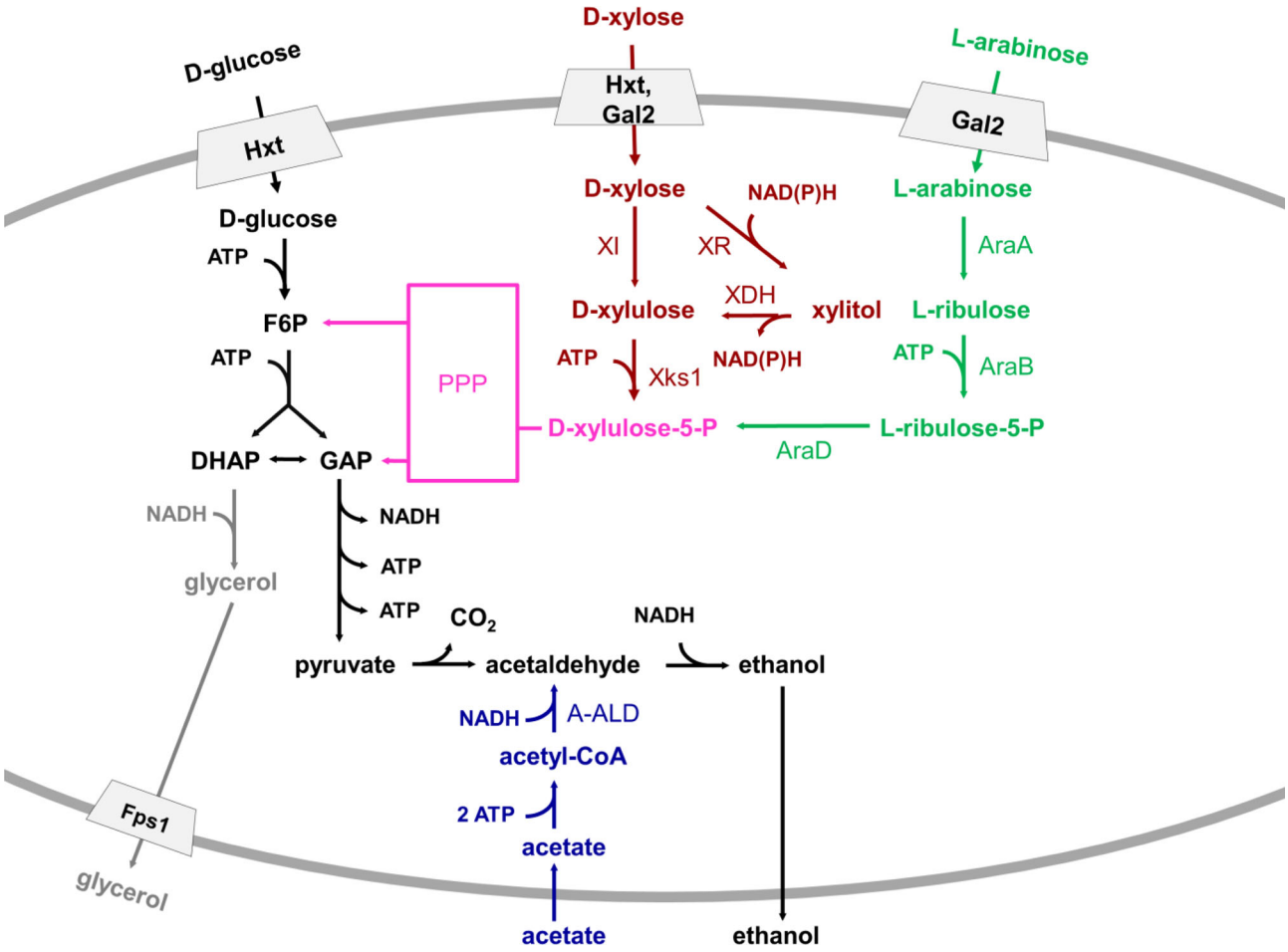

Figure 2. Key strategies for engineering carbon and redox metabolism in *S. cerevisiae* strains for alcoholic fermentation of lignocellulosic feedstocks. Colors indicate the following pathways and processes: black, native *S. cerevisiae* enzymes of glycolysis and alcoholic fermentation; magenta, native enzymes of the non-oxidative pentose-phosphate pathway (PPP), overexpressed in pentose-fermenting strains; red, conversion of D-xylose into D-xylulose-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-xylulose-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 (Wisselink *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; Wisselink *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 (Wisselink *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, Regelmann 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 $NAD(P)^+$-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_{xylose}, $q_{ethanol}$, expressed in $g \cdot (g\ biomass)^{-1} \cdot h^{-1}$) 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_2 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)$^{-1}$) 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)$^{-1}$·h^{-1}) in cultures of *S. cerevisiae* strains engineered for pentose fermentation, grown in synthetic media. Asterisks (*) indicate values estimated from graphs in the cited reference.

S. cerevisiae strain	Pentose fermentation strategy	Key genetic modifications	Fermentation conditions	$Y_{E/S}$ (g·g^{-1})	$q_{ethanol}$ (g·g^{-1}·h^{-1})	q_{xylose} (g·g·h^{-1})	$q_{arabinose}$ (g·g·h^{-1})	Reference
TMB3400	XR/XDH (*S. stipitis 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 (*S. stipitis 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 (*S. stipitis XYL1, XYL2*)	*SsXYL1*,*Ss XYL2*, *Ss XYL3*, *ald6*Δ, evolved on xylose	Anaerobic batch (reactor), 4% xylose	0.39	0.25	0.64		Wei *et al.* (2013)
TMB3421	XR/XDH (*S. stipitis XYL1, XYL2*)	*S. stipitis XYL1*$^{N272D/P275Q}$, *XYL2* + *XKS1*↑ *TAL1*↑ *TKL1*↑ *RPE1*↑ *RKI1*↑ *gre3*Δ, evolved on xylose	Anaerobic batch (reactor), 6% xylose	0.35	0.20	0.57		Runquist, Hahn-Hägerdal and Bettiga (2010a)
RWB 217	XI (*Piromyces XylA*)	*Piromyces XylA* + *XKS1*↑ *TAL1*↑ *TKL1*↑ *RPE1*↑ *RKI1*↑, *gre3*Δ	Anaerobic batch (reactor), 2% xylose	0.43	0.46	1.06		Kuyper *et al.* (2005a)
RWB 218	XI (*Piromyces XylA*)	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^{CS}	XI (*Piromyces XylA*)	*XylA*, *Xyl3*, *XKS1*↑ *TAL1*↑ *TKL1*↑ *RPE1*↑ *RKI1*↑, *gre3*Δ, evolved on xylose	Anaerobic batch (reactor), 4% xylose	0.43	0.76	1.9		Zhou *et al.* (2012)
IMS0010	XI/AraABD (*Piromyces XylA*, *L. plantarum AraA*, *B*, *D*)	*XylA*; *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 (*Piromyces XylA*, *L. plantarum AraA*, *B*, *D*, *K. lactis ARAT*)	*XylA*, *XKS1*↑ *TAL1*↑ *TKL1*↑ *RPE1*↑ *RKI1*↑ *XylA 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^{-1} 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; Sànchez 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)$^{-1}$) and biomass-specific rates of glucose and xylose consumption and ethanol production ($q_{glucose}$, q_{xylose} and $q_{ethanol}$, respectively, g·(g biomass)$^{-1}$·h^{-1}) 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[a]	$Y_{E/S}$ (g·g^{-1})	$q_{glucose}$ (g·g·h^{-1})	$q_{ethanol}$ (g·g·h^{-1})	q_{xylose} (g·g·h^{-1})	Reference
TMB3400	XR/XDH *S. stipitis XYL1* and XYL2; XKS1↑	Spruce, two-step dilute acid hydrolysis, 1.6% glucose, 0.4% xylose, 1% mannose, 1% galactose	Anaerobic batch (flasks), $(NH_4)_2HPO_4$ NaH_2PO_4 $MgSO_4$, YE	0.41	0.021	0.005	0.005	Karhumaa *et al.* (2007)
GLBRCY87	XR/XDH *S. stipitis 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 *S. stipitis 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 *S. stipitis* *XYL1*$^{(N272D/P275Q)}$ 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, $K_2O_5S_2$	0.3		0.12†,*	0.25†	Costa *et al.* (2017)
RWB 218	XI *Piromyces XylA*, *XKS1*↑ *TAL1*↑ *TKL1*↑ *RPE1*↑ *RKI1*↑, *gre3*Δ, evolved on glucose/xylose mixed substrate	Wheat straw hydrolysate, steam explosion, 5% glucose, 2% xylose	Anaerobic batch (reactor), $(NH_4)_2PO_4$	0.47	1.58†	1.0†	0.32†	Van Maris *et al.* (2007)
GS1.11-26	XI, AraABD *Piromyces XylA*, *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, $(NH_4)_2SO_4$, amino acids added	0.43	2.46†	0.3†	0.11†	Demeke *et al.* (2013a)
XH7	Multiple integrations of *RuXylA*; *XKS1*↑ *TAL1*↑ *TKL1*↑ *RPE1*↑ *RKI1*↑ *pho13*Δ *gre3*Δ, 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)

[a]Abbreviations of supplements: YE, yeast extract; YNB, yeast nitrogen base; YP, yeast extract and peptone.

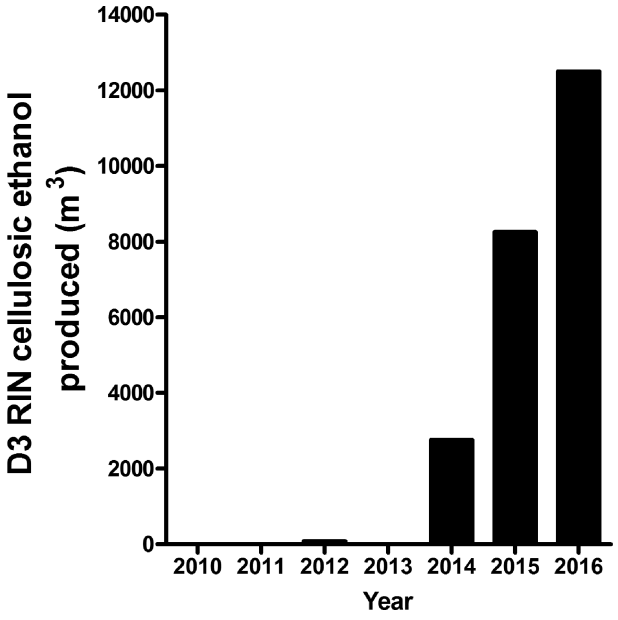

Figure 3. Annual production volumes of cellulosic ethanol in the USA from 2010 until November 2016. Numbers are based on RIN D code 3 RIN (renewable identification number) credits generated (accounted as cellulosic ethanol; United States Environmental Protection Agency 2017).

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. Moreover, 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

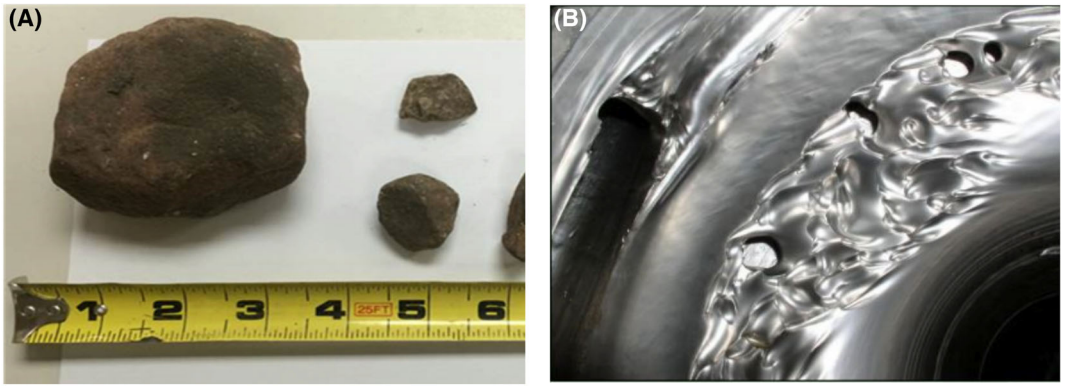

Figure 4. Problems not encountered in shake flask cultures: non-yeast-related challenges in large-scale processing of lignocellulosic biomass. (**A**) Small rocks collected from corn stover (picture courtesy of POET-DSM Liberty). (**B**) Example of severely eroded equipment (picture courtesy of Iogen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

ACKNOWLEDGEMENTS

We gratefully acknowledge our current and former colleagues and students at DSM and TU Delft for their contributions to our research collaboration. We thank Jim Lane from BiofuelsDigest and POET-DSM Advanced Biofuels for their kind permission to reproduce the photographs shown in Fig. 4 and in the Graphical Abstract.

FUNDING

Our joint research on second generation ethanol production is performed within the BE-Basic Research and Development Program (http://www.be-basic.org/), which is financially supported by an EOS Long Term grant from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I). The PhD project of IP is funded by DSM Bio-based Products & Services B.V. (Delft, The Netherlands).

Conflict of interest. Royal DSM owns intellectual property rights and commercializes aspects of the technology discussed in this paper.

REFERENCES

Abbott DA, Knijnenburg TA, De Poorter LMI *et al.* Generic and specific transcriptional responses to different weak organic acids in anaerobic chemostat cultures of *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2007;**7**:819–33.

Adeboye PT, Bettiga M, Olsson L. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates. *AMB Express* 2014;**4**:46.

Adeboye PT, Bettiga M, Olsson L. *ALD5*, *PAD1*, *ATF1* and *ATF2* facilitate the catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid in *Saccharomyces cerevisiae*. *Sci Rep* 2017;**7**:42635.

Albers E, Johansson E, Franzén CJ *et al.* Selective suppression of bacterial contaminants by process conditions during lignocellulose based yeast fermentations. *Biotechnol Biofuels* 2011;**4**:59.

Alkasrawi M, Rudolf A, Lidén G *et al.* Influence of strain and cultivation procedure on the performance of simultaneous saccharification and fermentation of steam pretreated spruce. *Enzyme Microb Technol* 2006;**38**:279–86.

Almario MP, Reyes LH, Kao KC. Evolutionary engineering of *Saccharomyces cerevisiae* for enhanced tolerance to hydrolysates of lignocellulosic biomass. *Biotechnol Bioeng* 2013;**110**:2616–23.

Almeida JRM, Bertilsson M, Hahn-Hägerdal B *et al.* Carbon fluxes of xylose-consuming *Saccharomyces cerevisiae* strains are affected differently by NADH and NADPH usage in HMF reduction. *Appl Microbiol Biotechnol* 2009;**84**:751–61.

Almeida JRM, Modig T, Petersson A *et al.* Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *J Chem Technol Biotechnol* 2007;**82**:340–9.

Ask M, Bettiga M, Duraiswamy VR *et al.* Pulsed addition of HMF and furfural to batch-grown xylose-utilizing *Saccharomyces cerevisiae* results in different physiological responses in glucose and xylose consumption phase. *Biotechnol Biofuels* 2013;**6**:181.

Axe DD, Bailey JE. Transport of lactate and acetate through the energized cytoplasmic membrane of *Escherichia coli*. *Biotechnol Bioeng* 1995;**47**:8–19.

Bailey J. Toward a science of metabolic engineering. *Science* 1991;**252**:1668–75.

Bamba T, Hasunuma T, Kondo A. Disruption of *PHO13* improves ethanol production via the xylose isomerase pathway. *Amb Express* 2016;**6**:4.

Banerjee N, Bhatnagar R, Viswanathan L. Inhibition of glycolysis by furfural in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 1981;**11**:226–8.

Basso LC, De Amorim HV, De Oliveira AJ *et al.* Yeast selection for fuel ethanol production in Brazil. *FEMS Yeast Res* 2008;**8**:1155–63.

Becker J, Boles E. A modified *Saccharomyces cerevisiae* strain that consumes L-arabinose and produces ethanol. *Appl Environ Microbiol* 2003;**69**:4144–50.

Beckner M, Ivey ML, Phister TG. Microbial contamination of fuel ethanol fermentations. *Lett Appl Microbiol* 2011;**53**:387–94.

Bellissimi E, Van Dijken JP, Pronk JT *et al.* Effects of acetic acid on the kinetics of xylose fermentation by an engineered, xylose-isomerase-based *Saccharomyces cerevisiae* strain. *FEMS Yeast Res* 2009;**9**:358–64.

Bernton H, Kovarik B, Sklar S. *The Forbidden Fuel: Power Alcohol in the Twentieth Century*. New York: Caroline House Pubns, 1982.

Bettiga M, Hahn-Hägerdal B, Gorwa-Grauslund MF. Comparing the xylose reductase/xylitol dehydrogenase and xylose isomerase pathways in arabinose and xylose fermenting *Saccharomyces cerevisiae* strains. *Biotechnol Biofuels* 2008;**1**:1.

Bischoff KM, Liu S, Leathers TD *et al.* Modeling bacterial contamination of fuel ethanol fermentation. *Biotechnol Bioeng* 2009;**103**:117–22.

Björkqvist S, Ansell R, Adler L *et al.* Physiological response to anaerobicity of glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 1997;**63**:128–32.

Brat D, Boles E, Wiedemann B. Functional expression of a bacterial xylose isomerase in Saccharomyces cerevisiae. *Appl Environ Microbiol* 2009;**75**:2304–11.

Bruinenberg PM, Bot PH, Dijken JP *et al.* The role of redox balances in the anaerobic fermentation of xylose by yeasts. *Appl Microbiol Biotechnol* 1983;**18**:287–92.

Canilha L, Chandel AK, Suzane Dos Santos Milessi T *et al.* Bioconversion of sugarcane biomass into ethanol: An overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation. *J Biomed Biotechnol* 2012;**2012**:989572.

Casey E, Mosier NS, Adamec J *et al.* Effect of salts on the cofermentation of glucose and xylose by a genetically engineered strain of *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 2013;**6**:83.

Caspeta L, Chen Y, Ghiaci P *et al.* Altered sterol composition renders yeast thermotolerant. *Science* 2014;**346**:75–8.

Chen Y, Sheng J, Jiang T *et al.* Transcriptional profiling reveals molecular basis and novel genetic targets for improved resistance to multiple fermentation inhibitors in *Saccharomyces cerevisiae*. Biotechnol Biofuels 2016;**9**:9.

Clomburg JM, Gonzalez R. Anaerobic fermentation of glycerol: a platform for renewable fuels and chemicals. *Trends Biotechnol* 2013;**31**:20–8.

Colabardini AC, Ries LNA, Brown NA *et al.* Functional characterization of a xylose transporter in *Aspergillus nidulans*. *Biotechnol Biofuels* 2014;**7**:1.

Costa CE, Romaní A, Cunha JT *et al.* Integrated approach for selecting efficient *Saccharomyces cerevisiae* for industrial lignocellulosic fermentations: Importance of yeast chassis linked to process conditions. *Bioresour Technol* 2017;**227**:24–34.

Crook N, Abatemarco J, Sun J *et al.* *In vivo* continuous evolution of genes and pathways in yeast. *Nat Commun* 2016;**7**:13051.

D3MAX. Advantages of D3MAX. 2017. https://www.d3maxllc.com/ (24 March 2017, date last accessed).

De Bont JAM, Teunissen AWRH, Klaassen P *et al.* Yeast strains engineered to produce ethanol from acetic acid and glycerol. US Patent 20150176032 A1. 2012.

Della-Bianca BE, Basso TO, Stambuk BU *et al.* What do we know about the yeast strains from the Brazilian fuel ethanol industry? *Appl Microbiol Biotechnol* 2013;**97**:979–91.

Della-Bianca BE, de Hulster E, Pronk JT *et al.* Physiology of the fuel ethanol strain *Saccharomyces cerevisiae* PE-2 at low pH indicates a context-dependent performance relevant for industrial applications. *FEMS Yeast Res* 2014;**14**:1196–205.

Demeke MM, Dietz H, Li Y *et al.* Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. *Biotechnol Biofuels* 2013a;**6**:89.

Demeke MM, Dumortier F, Li Y *et al.* Combining inhibitor tolerance and D-xylose fermentation in industrial *Saccharomyces*

cerevisiae for efficient lignocellulose-based bioethanol production. *Biotechnol Biofuels* 2013b;**6**:120.

Demeke MM, Foulquié-Moreno MR, Dumortier F *et al.* Rapid evolution of recombinant *Saccharomyces cerevisiae* for xylose fermentation through formation of extra-chromosomal circular DNA. *PLoS Genet* 2015;**11**:e1005010.

Den Haan R, Van Rensburg E, Rose SH *et al.* Progress and challenges in the engineering of non-cellulolytic microorganisms for consolidated bioprocessing. *Curr Opin Biotechnol* 2015;**33**:32–8.

DiCarlo JE, Norville JE, Mali P *et al.* Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Res* 2013;**41**:4336–43.

Diderich JA, Schepper M, van Hoek P *et al.* Glucose uptake kinetics and transcription of *HXT* genes in chemostat cultures of *Saccharomyces cerevisiae*. *J Biol Chem* 1999;**274**:15350–9.

Dos Santos LV, Carazzolle MF, Nagamatsu ST *et al.* Unraveling the genetic basis of xylose consumption in engineered *Saccharomyces cerevisiae* strains. *Sci Rep* 2016;**6**:38676.

Du J, Li S, Zhao H. Discovery and characterization of novel D-xylose-specific transporters from *Neurospora crassa* and *Pichia stipitis*. *Mol Biosyst* 2010;**6**:2150–6.

Dun B, Wang Z, Ye K *et al.* Functional expression of *Arabidopsis thaliana* xylose isomerase in *Saccharomyces cerevisiae*. *Xinjiang Agric Sci* 2012;**49**:681–6.

Dunlop AP. Furfural formation and behavior. *Ind Eng Chem* 1948;**40**:204–9.

Ethanol Producer Magazine. U.S. ethanol plants. 2017. http://www.ethanolproducer.com/plants/listplants/US/All/Cellulosic/ (14 February 2017, date last accessed).

Farrell AE, Plevin RJ, Turner BT *et al.* Ethanol can contribute to energy and environmental goals. *Science* 2006;**311**:506.

Farwick A, Bruder S, Schadeweg V *et al.* Engineering of yeast hexose transporters to transport D-xylose without inhibition by D-glucose. *Proc Natl Acad Sci U S A* 2014;**111**:5159–64.

Favaro L, Basaglia M, Trento A *et al.* Exploring grape marc as trove for new thermotolerant and inhibitor-tolerant *Saccharomyces cerevisiae* strains for second-generation bioethanol production. *Biotechnol Biofuels* 2013;**6**:168.

Ferreira D, Nobre A, Silva ML *et al.* *XYLH* encodes a xylose/H^+ symporter from the highly related yeast species *Debaryomyces fabryi* and *Debaryomyces hansenii*. *FEMS Yeast Res* 2013;**13**:585–96.

Field SJ, Ryden P, Wilson D *et al.* Identification of furfural resistant strains of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* from a collection of environmental and industrial isolates. *Biotechnol Biofuels* 2015;**8**:33.

Fletcher E, Feizi A, Bisschops MMM *et al.* Evolutionary engineering reveals divergent paths when yeast is adapted to different acidic environments. *Metab Eng* 2017;**39**:19–28.

Fonseca C, Olofsson K, Ferreira C *et al.* The glucose/xylose facilitator Gxf1 from *Candida intermedia* expressed in a xylose-fermenting industrial strain of *Saccharomyces cerevisiae* increases xylose uptake in SSCF of wheat straw. *Enzyme Microb Technol* 2011;**48**:518–25.

Galazka JM, Tian C, Beeson WT *et al.* Cellodextrin transport in yeast for improved biofuel production. *Science* 2010;**330**:84–6.

Garcia Sanchez R, Karhumaa K, Fonseca C *et al.* Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. *Biotechnol Biofuels* 2010;**3**:13.

González-Ramos D, Gorter de Vries AR, Grijseels SS *et al.* A new laboratory evolution approach to select for constitutive acetic acid tolerance in *Saccharomyces cerevisiae* and identification of causal mutations. *Biotechnol Biofuels* 2016;**9**:1.

Gorter De Vries AR, Pronk JT, Daran JM. Industrial relevance of chromosomal copy number variation in *Saccharomyces* yeasts. *Appl Environ Microbiol* 2017;**83**:e03206–16.

Goshima T, Tsuji M, Inoue H *et al.* Bioethanol production from lignocellulosic biomass by a novel *Kluyveromyces marxianus* strain. *Biosci Biotechnol Biochem* 2013;**77**:1505–10.

Grohmann K, Bothast R. Pectin-rich residues generated by processing of citrus fruits, apples, and sugar beets. In: Himmel ME, Baker JO, Overend RP (eds). *Enzymatic Conversion of Biomass for Fuels Production*. Washington: ACS Publications, 1994, 372–90.

Grohmann K, Bothast RJ. Saccharification of corn fibre by combined treatment with dilute sulphuric acid and enzymes. *Process Biochem* 1997;**32**:405–15.

Guadalupe-Medina V, Almering MJH, van Maris AJA *et al.* Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. *Appl Environ Microbiol* 2010;**76**:190–5.

Guo Z, Olsson L. Physiological response of *Saccharomyces cerevisiae* to weak acids present in lignocellulosic hydrolysate. *FEMS Yeast Res* 2014;**14**:1234–48.

Guo Z, Zhang L, Ding Z *et al.* Minimization of glycerol synthesis in industrial ethanol yeast without influencing its fermentation performance. *Metab Eng* 2011;**13**:49–59.

Ha S, Galazka JM, Rin Kim S *et al.* Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. *Proc Natl Acad Sci U S A* 2011;**108**:504–9.

Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF *et al.* Bioethanol – the fuel of tomorrow from the residues of today. *Trends Biotechnol* 2006;**24**:549–56.

Hahn-Hägerdal B, Karhumaa K, Fonseca C *et al.* Towards industrial pentose-fermenting yeast strains. *Appl Microbiol Biotechnol* 2007;**74**:937–53.

Hahn-Hägerdal B, Wahlbom CF, Gárdonyi M *et al.* Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. *Adv Biochem Eng Biotechnol* 2001;**73**:53–84.

Hamacher T, Becker J, Gárdonyi M *et al.* Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. *Microbiology* 2002;**148**:2783–8.

Harhangi HR, Akhmanova AS, Emmens R *et al.* Xylose metabolism in the anaerobic fungus *Piromyces sp.* strain E2 follows the bacterial pathway. *Arch Microbiol* 2003;**180**:134–41.

Hector RE, Dien BS, Cotta MA *et al.* Growth and fermentation of D-xylose by *Saccharomyces cerevisiae* expressing a novel D-xylose isomerase originating from the bacterium *Prevotella ruminicola* TC2-24. *Biotechnol Biofuels* 2013;**6**:84.

Hendriks ATWM, Zeeman G. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour Technol* 2009;**100**:10–8.

Henningsen BM, Hon S, Covalla SF *et al.* Increasing anaerobic acetate consumption and ethanol yields in *Saccharomyces cerevisiae* with NADPH-specific alcohol dehydrogenase. *Appl Environ Microbiol* 2015;**81**:8108–17.

Horak J, Regelmann J, Wolf DH. Two distinct proteolytic systems responsible for glucose-induced degradation of fructose-1,6-bisphosphatase and the Gal2p transporter in the yeast *Saccharomyces cerevisiae* share the same protein components of the glucose signaling pathway. *J Biol Chem* 2002;**277**:8248–54.

Horak J, Wolf DH. Catabolite inactivation of the galactose transporter in the yeast *Saccharomyces cerevisiae*: ubiquitination,

endocytosis, and degradation in the vacuole. *J Bacteriol* 1997;**179**:1541–9.

Hou J, Jiao C, Peng B *et al.* Mutation of a regulator Ask10p improves xylose isomerase activity through up-regulation of molecular chaperones in *Saccharomyces cerevisiae*. *Metab Eng* 2016a;**38**:241–50.

Hou J, Shen Y, Jiao C *et al.* Characterization and evolution of xylose isomerase screened from the bovine rumen metagenome in *Saccharomyces cerevisiae*. *J Biosci Bioeng* 2016b;**121**:160–5.

Hu ML, Zha J, He LW *et al.* Enhanced bioconversion of cellobiose by industrial *Saccharomyces cerevisiae* used for cellulose utilization. *Front Microbiol* 2016;**7**:241.

Hubmann G, Guillouet S, Nevoigt E. Gpd1 and Gpd2 fine-tuning for sustainable reduction of glycerol formation in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 2011;**77**:5857–67.

Hubmann G, Mathé L, Foulquié-Moreno MR *et al.* Identification of multiple interacting alleles conferring low glycerol and high ethanol yield in *Saccharomyces cerevisiae* ethanolic fermentation. *Biotechnol Biofuels* 2013;**6**:87.

ICM. Generation 1.5: grain fiber to cellulosic ethanol technology. 2012. http://icminc.com/products/generation-1-5.html (24 March 2017, date last accessed).

Ilmén M, den Haan R, Brevnova E *et al.* High level secretion of cellobiohydrolases by *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 2011;**4**:30.

Jacques KA, Lyons TP, Kelsall DR. *The Alcohol Textbook: A Reference for the Beverage, Fuel and Industrial Alcohol Industries*. Nottingham: Nottingham University Press, 2003.

Jeffries TW. Engineering yeasts for xylose metabolism. *Curr Opin Biotechnol* 2006;**17**:320–6.

Jeffries TW, Jin Y. Metabolic engineering for improved fermentation of pentoses by yeasts. *Appl Microbiol Biotechnol* 2004;**63**:495–509.

Jeppsson M, Johansson B, Jensen PR *et al.* The level of glucose-6-phosphate dehydrogenase activity strongly influences xylose fermentation and inhibitor sensitivity in recombinant *Saccharomyces cerevisiae* strains. *Yeast* 2003;**20**:1263–72.

Jönsson LJ, Alriksson B, Nilvebrant NO. Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnol Biofuels* 2013;**6**:16.

Karhumaa K, Sanchez RG, Hahn-Hägerdal B *et al.* Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Microb Cell Fact* 2007;**6**:5.

Karim AS, Curran KA, Alper HS. Characterization of plasmid burden and copy number in *Saccharomyces cerevisiae* for optimization of metabolic engineering applications. *FEMS Yeast Res* 2013;**13**:107–16.

Katahira S, Fujita Y, Mizuike A *et al.* Construction of a xylan-fermenting yeast strain through codisplay of xylanolytic enzymes on the surface of xylose-utilizing *Saccharomyces cerevisiae* cells. *Appl Environ Microbiol* 2004;**70**:5407–14.

Katahira S, Ito M, Takema H *et al.* Improvement of ethanol productivity during xylose and glucose co-fermentation by xylose-assimilating *S. cerevisiae* via expression of glucose transporter Sut1. *Enzyme Microb Technol* 2008; **43**:115–9.

Katahira S, Mizuike A, Fukuda H *et al.* Ethanol fermentation from lignocellulosic hydrolysate by a recombinant xylose- and cellooligosaccharide-assimilating yeast strain. *Appl Microbiol Biotechnol* 2006;**72**:1136–43.

Kenney KL, Smith WA, Gresham GL *et al.* Understanding biomass feedstock variability. *Biofuels* 2013;**4**:111–27.

Khoo HH. Review of bio-conversion pathways of lignocellulose-to-ethanol: Sustainability assessment based on land footprint projections. *Renew Sustainable Energy Rev* 2015;**46**:100–19.

Kim SR, Ha S-J, Wei N *et al.* Simultaneous co-fermentation of mixed sugars: a promising strategy for producing cellulosic ethanol. *Trends Biotechnol* 2012;**30**:274–82.

Kim SR, Skerker JM, Kang W *et al.* Rational and evolutionary engineering approaches uncover a small set of genetic changes efficient for rapid xylose fermentation in *Saccharomyces cerevisiae*. PLoS One 2013;**8**:e57048.

Kim Y, Mosier NS, Hendrickson R *et al.* Composition of corn dry-grind ethanol by-products: DDGS, wet cake, and thin stillage. *Bioresour Technol* 2008;**99**:5165–76.

Klaassen P, Hartman WWA. Glycerol and acetic acid converting yeast cells with improved acetic acid conversion. US Patent 20160208291 A1. 2014.

Kleinschmidt J. *Biofueling Rural Development: Making the Case for Linking Biofuel Production to Rural Revitalization*. Durham, NH: Carsey Institute, 2007. http://scholars.unh.edu/cgi/viewcontent.cgi?article=1019&context=carsey. (24 March 2017, date last accessed).

Klinke HB, Ahring BK, Schmidt AS *et al.* Characterization of degradation products from alkaline wet oxidation of wheat straw. *Bioresour Technol* 2002;**82**:15–26.

Klinke HB, Thomsen AB, Ahring BK. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Appl Microbiol Biotechnol* 2004;**66**:10–26.

Knoll JE, Anderson WF, Richard EP *et al.* Harvest date effects on biomass quality and ethanol yield of new energycane (*Saccharum* hyb.) genotypes in the Southeast USA. *Biomass Bioenergy* 2013;**56**:147–56.

Knoshaug EP, Vidgren V, Magalhães F *et al.* Novel transporters from *Kluyveromyces marxianus* and *Pichia guilliermondii* expressed in *Saccharomyces cerevisiae* enable growth on L-arabinose and D-xylose. *Yeast* 2015;**32**:615–28.

Koppram R, Albers E, Olsson L. Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass. *Biotechnol Biofuels* 2012;**5**:32.

Koppram R, Mapelli V, Albers E *et al.* The presence of pre-treated lignocellulosic solids from birch during *Saccharomyces cerevisiae* fermentations leads to increased tolerance to inhibitors – a proteomic study of the effects. *PLoS One* 2016;**11**:e0148635.

Kötter P, Amore R, Hollenberg CP *et al.* Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene, *XYL2*, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant. *Curr Genet* 1990;**18**:493–500.

Kötter P, Ciriacy M. Xylose fermentation by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 1993;**38**:776–83.

Kou S, Christensen MS, Cirillo VP. Galactose transport in *Saccharomyces cerevisiae* II. Characteristics of galactose uptake and exchange in galactokinaseless cells. *J Bacteriol* 1970;**103**: 671–8.

Krahulec S, Klimacek M, Nidetzky B. Engineering of a matched pair of xylose reductase and xylitol dehydrogenase for xylose fermentation by *Saccharomyces cerevisiae*. *Biotechnol J* 2009;**4**:684–94.

Kumar D, Singh V. Dry-grind processing using amylase corn and superior yeast to reduce the exogenous enzyme requirements in bioethanol production. *Biotechnol Biofuels* 2016;**9**:228.

Kumar R, Mago G, Balan V *et al*. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. *Bioresour Technol* 2009;**100**:3948–62.

Kuyper M, Hartog MM, Toirkens MJ *et al*. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res* 2005a;**5**:399–409.

Kuyper M, Toirkens MJ, Diderich JA *et al*. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *FEMS Yeast Res* 2005b;**5**:925–34.

Kuyper M, Winkler AA, van Dijken JP *et al*. Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. *FEMS Yeast Res* 2006;**4**:655–64.

La Grange DC, Pretorius IS, Claeyssens M *et al*. Degradation of xylan to D-xylose by recombinant *Saccharomyces cerevisiae* coexpressing the *Aspergillus niger* β-xylosidase (*xlnD*) and the *Trichoderma reesei* xylanase II (*xyn2*) genes. Appl Environ Microbiol 2001;**67**:5512–9.

Lane J. Editor's Sketchbook: Beta Renewables cellulosic ethanol project, Crescentino, Italy. *Biofuels Digest* 2014. http://www.biofuelsdigest.com/bdigest/2014/09/25/editors-sketchbook-beta-renewables-cellulosic-ethanol-project-crescentino-italy/ (24 March 2017, date last accessed).

Lane J. Cellulosic ethanol: Where are the gallons? Answers for your questions. *Biofuels Digest* 2015. http://www.biofuelsdigest.com/bdigest/2015/07/01/cellulosic-ethanol-where-are-the-gallons-answers-for-your-questions/ (24 March 2017, date last accessed).

Lane J. What's after Gen 1? The Digest's 2016 Multi-Slide Guide to ICM's Gen 1.5 cellulosic technology. *Biofuels Digest* 2016a. http://www.biofuelsdigest.com/bdigest/2016/12/14/whats-after-gen-1-the-digests-2016-multi-slide-guide-to-icms-gen-1-5-cellulosic-technology/ (24 March 2017, date last accessed).

Lane J. Getting it right: The Digest's Multi-Slide Guide to Iogen. *Biofuels Digest* 2016b. http://www.biofuelsdigest.com/bdigest/2016/05/30/getting-it-right-the-digests-multi-slide-guide-to-iogen/ (24 March 2017, date last accessed).

Larsson S, Quintana-Sainz A, Reimann A *et al*. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by *Saccharomyces cerevisiae*. *Appl Biochem Biotechnol* 2000;**84–86**:617–32.

Lawther JM, Sun R, Banks WB. Fractional characterization of alkali-labile lignin and alkali-insoluble lignin from wheat straw. *Ind Crops Prod* 1996;**5**:291–300.

Leandro MJ, Gonçalves P, Spencer-Martins I. Two glucose/xylose transporter genes from the yeast *Candida intermedia*: first molecular characterization of a yeast xylose-H^+ symporter. *Biochem J* 2006;**395**:543–9.

Lee JH, Heo SY, Lee JW *et al*. Thermostability and xylan-hydrolyzing property of endoxylanase expressed in yeast *Saccharomyces cerevisiae*. *Biotechnol Bioprocess Eng* 2009;**14**:639–44.

Lee N, Gielow W, Martin R *et al*. The organization of the *araBAD* operon of *Escherichia coli*. *Gene* 1986;**47**:231–44.

Lee SM, Jellison T, Alper HS. Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 2012;**78**:5708–16.

Lee SM, Jellison T, Alper HS. Systematic and evolutionary engineering of a xylose isomerase-based pathway in *Saccharomyces cerevisiae* for efficient conversion yields. *Biotechnol Biofuels* 2014;**7**:122.

Lee W, Kim M, Ryu Y *et al*. Kinetic studies on glucose and xylose transport in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 2002;**60**:186–91.

Lewis Liu Z, Moon J, Andersh BJ *et al*. Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of *in situ* detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 2008;**81**:743–53.

Li BZ, Yuan YJ. Transcriptome shifts in response to furfural and acetic acid in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 2010;**86**:1915–24.

Li C, Aston JE, Lacey JA *et al*. Impact of feedstock quality and variation on biochemical and thermochemical conversion. *Renew Sust Energ Rev* 2016a;**65**:525–36.

Li H, Schmitz O, Alper HS. Enabling glucose/xylose co-transport in yeast through the directed evolution of a sugar transporter. *Appl Microbiol Biotechnol* 2016b;**100**:10215–23.

Li H, Shen Y, Wu M *et al*. Engineering a wild-type diploid *Saccharomyces cerevisiae* strain for second-generation bioethanol production. *Bioresour Bioprocess* 2016c;**3**:51.

Li J, Xu J, Cai P *et al*. Functional analysis of two l-arabinose transporters from filamentous fungi reveals promising characteristics for improved pentose utilization in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 2015;**81**:4062–70.

Lin Y, Tanaka S. Ethanol fermentation from biomass resources: current state and prospects. *Appl Microbiol Biotechnol* 2006;**69**:627–42.

Liti G, Louis EJ. Advances in quantitative trait analysis in yeast. *PLoS Genet* 2012;**8**:e1002912.

Liu ZL. Molecular mechanisms of yeast tolerance and *in situ* detoxification of lignocellulose hydrolysates. *Appl Microbiol Biotechnol* 2011;**90**:809–25.

Liu ZL, Slininger PJ, Dien BS *et al*. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. *J Ind Microbiol Biotechnol* 2004;**31**:345–52.

Lopes ML, de Lima Paulillo SCdL, Godoy A *et al*. Ethanol production in Brazil: a bridge between science and industry. *Braz J Microbiol* 2016;**47**:64–76.

Lynd LR. Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment, and policy. *Annu Rev Energy Env* 1996;**21**:403–65.

Lynd LR, van Zyl WH, McBride JE *et al*. Consolidated bioprocessing of cellulosic biomass: an update. *Curr Opin Biotechnol* 2005;**16**:577–83.

Lynd LR, Weimer PJ, van Zyl WH *et al*. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol Mol Biol Rev* 2002;**66**:506–77.

Madhavan A, Tamalampudi S, Ushida K *et al*. Xylose isomerase from polycentric fungus *Orpinomyces*: gene sequencing, cloning, and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol. *Appl Microbiol Biotechnol* 2009;**82**:1067–78.

Mans R, van Rossum HM, Wijsman M *et al*. CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2015;**15**:fov004.

Marcheschi RJ, Gronenberg LS, Liao JC. Protein engineering for metabolic engineering: current and next-generation tools. *Biotechnol J* 2013;**8**:545–55.

Meijnen JP, Randazzo P, Foulquié-Moreno MR *et al*. Polygenic analysis and targeted improvement of the complex trait of

high acetic acid tolerance in the yeast *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 2016;**9**:1.
Mira NP, Palma M, Guerreiro JF *et al.* Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. *Microb Cell Fact* 2010;**9**:79.
Modig T, Lidén G, Taherzadeh MJ. Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. *Biochem J* 2002;**363**:769–76.
Moniruzzaman M, Dien B, Skory C *et al.* Fermentation of corn fibre sugars by an engineered xylose utilizing *Saccharomyces* yeast strain. *World J Microbiol Biotechnol* 1997;**13**:341–6.
Moon J, Liu ZL. Engineered NADH-dependent *GRE2* from *Saccharomyces cerevisiae* by directed enzyme evolution enhances HMF reduction using additional cofactor NADPH. *Enzyme Microb Technol* 2012;**50**:115–20.
Moysés DN, Reis, de Almeida JRMM VCB, Lidia Maria Pepe de *et al.* Xylose fermentation by *Saccharomyces cerevisiae*: challenges and prospects. *Int J Mol Sci* 2016;**17**:207.
Muthaiyan A, Limayem A, Ricke S. Antimicrobial strategies for limiting bacterial contaminants in fuel bioethanol fermentations. *Prog Energy Combust Sci* 2011;**37**:351–70.
Narendranath NV, Lewis SM. Systems and methods for yeast propagation. US Patent 9034631 B2. 2013.
Narron RH, Kim H, Chang HM *et al.* Biomass pretreatments capable of enabling lignin valorization in a biorefinery process. *Curr Opin Biotechnol* 2016;**38**:39–46.
Ni H, Laplaza JM, Jeffries TW. Transposon mutagenesis to improve the growth of recombinant *Saccharomyces cerevisiae* on D-xylose. *Appl Environ Microbiol* 2007;**73**:2061–6.
Nielsen F, Tomás-Pejó E, Olsson L *et al.* Short-term adaptation during propagation improves the performance of xylose-fermenting *Saccharomyces cerevisiae* in simultaneous saccharification and co-fermentation. *Biotechnol Biofuels* 2015;**8**:1.
Nielsen J, Larsson C, van Maris A *et al.* Metabolic engineering of yeast for production of fuels and chemicals. *Curr Opin Biotechnol* 2013;**24**:398–404.
Nijland JG, Shin HY, de Jong RM *et al.* Engineering of an endogenous hexose transporter into a specific D-xylose transporter facilitates glucose-xylose co-consumption in *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 2014;**7**:168.
Nijland JG, Vos E, Shin HY *et al.* Improving pentose fermentation by preventing ubiquitination of hexose transporters in *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 2016;**9**:158.
Nissen TL, Kielland-Brandt MC, Nielsen J *et al.* Optimization of ethanol production in *Saccharomyces cerevisiae* by metabolic engineering of the ammonium assimilation. *Metab Eng* 2000;**2**:69–77.
Noorman H. An industrial perspective on bioreactor scale-down: What we can learn from combined large-scale bioprocess and model fluid studies. *Biotechnol J* 2011;**6**:934–43.
Nordhoff S. Editorial: Food vs fuel – the role of biotechnology. *Biotechnol J* 2007;**2**:1451.
Olson DG, McBride JE, Joe Shaw A *et al.* Recent progress in consolidated bioprocessing. *Curr Opin Biotechnol* 2012;**23**:396–405.
Ota M, Sakuragi H, Morisaka H *et al.* Display of *Clostridium cellulovorans* xylose isomerase on the cell surface of *Saccharomyces cerevisiae* and its direct application to xylose fermentation. *Biotechnol Prog* 2013;**29**:346–51.
Otero JM, Panagiotou G, Olsson L. Fueling industrial biotechnology growth with bioethanol. *Adv Biochem Eng Biotechnol* 2007;**108**:1–40.
Oud B, van Maris AJ, Daran J-M *et al.* Genome-wide analytical approaches for reverse metabolic engineering of industrially relevant phenotypes in yeast. *FEMS Yeast Res* 2012;**12**:183–96.
Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. *Bioresour Technol* 2000a;**74**:17–24.
Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresour Technol* 2000b;**74**:25–33.
Pampulha ME, Loureiro-Dias MC. Energetics of the effect of acetic acid on growth of *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* 2000;**184**:69–72.
Papapetridis I, van Dijk M, Dobbe AP *et al.* Improving ethanol yield in acetate-reducing *Saccharomyces cerevisiae* by cofactor engineering of 6-phosphogluconate dehydrogenase and deletion of *ALD6*. *Microb Cell Fact* 2016;**15**:67.
Pereira LG, Dias MOS, Mariano AP *et al.* Economic and environmental assessment of n-butanol production in an integrated first and second generation sugarcane biorefinery: Fermentative versus catalytic routes. *Appl Energy* 2015;**160**:120–31.
Petschacher B, Leitgeb S, Kavanagh Kathryn L *et al.* The coenzyme specificity of *Candida tenuis* xylose reductase (*AKR2B5*) explored by site-directed mutagenesis and X-ray crystallography. *Biochem J* 2005;**385**:75–83.
Petschacher B, Nidetzky B. Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*. *Microb Cell Fact* 2008;**7**:9.
Pinel D, Colatriano D, Jiang H *et al.* Deconstructing the genetic basis of spent sulphite liquor tolerance using deep sequencing of genome-shuffled yeast. *Biotechnol Biofuels* 2015;**8**:53.
Pronk JT. Auxotrophic yeast strains in fundamental and applied research. *Appl Environ Microbiol* 2002;**68**:2095–100.
Pronk JT, Lee SY, Lievense J *et al.* How to set up collaborations between academia and industrial biotech companies. *Nat Biotech* 2015;**33**:237–40.
Radecka D, Mukherjee V, Mateo RQ *et al.* Looking beyond *Saccharomyces*: the potential of non-conventional yeast species for desirable traits in bioethanol fermentation. *FEMS Yeast Res* 2015;**15**:fov053.
Reifenberger E, Boles E, Ciriacy M. Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. *Eur J Biochem* 1997;**245**:324–33.
Reis TF, Lima PBA, Parachin NS *et al.* Identification and characterization of putative xylose and cellobiose transporters in *Aspergillus nidulans*. *Biotechnol Biofuels* 2016;**9**:204.
Renewable Fuels Association. World fuel ethanol production. 2016. http://ethanolrfa.org/resources/industry/statistics/ (24 March 2017, date last accessed).
Reznicek O, Facey SJ, de Waal PP *et al.* Improved xylose uptake in Saccharomyces cerevisiae due to directed evolution of galactose permease Gal2 for sugar co-consumption. *J Appl Microbiol* 2015;**119**:99–111.
Roca C, Nielsen J, Olsson L. Metabolic engineering of ammonium assimilation in xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production. *Appl Environ Microbiol* 2003;**69**:4732–6.
Rude MA, Schirmer A. New microbial fuels: a biotech perspective. *Curr Opin Microbiol* 2009;**12**:274–81.
Runquist D, Hahn-Hägerdal B, Bettiga M. Increased ethanol productivity in xylose-utilizing *Saccharomyces cerevisiae* via a randomly mutagenized xylose reductase. *Appl Environ Microbiol* 2010a;**76**:7796–802.

Runquist D, Hahn-Hägerdal B, Rådström P. Comparison of heterologous xylose transporters in recombinant *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 2010b;**3**:1.

Russel JB. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. *J Appl Microbiol* 1992;**73**:363–70.

Ryabova OB, Chmil OM, Sibirny AA. Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast *Hansenula polymorpha*. *FEMS Yeast Res* 2003;**4**:157–64.

Sadie CJ, Rose SH, den Haan R *et al*. Co-expression of a cellobiose phosphorylase and lactose permease enables intracellular cellobiose utilisation by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 2011;**90**:1373–80.

Saloheimo A, Rauta J, Stasyk V *et al*. Xylose transport studies with xylose-utilizing *Saccharomyces cerevisiae* strains expressing heterologous and homologous permeases. *Appl Microbiol Biotechnol* 2007;**74**:1041–52.

Sànchez i Nogué V, Narayanan V, Gorwa-Grauslund MF. Short-term adaptation improves the fermentation performance of *Saccharomyces cerevisiae* in the presence of acetic acid at low pH. *Appl Microbiol Biotechnol* 2013;**97**:7517–25.

Sanchez RG, Karhumaa K, Fonseca C *et al*. Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. *Biotechnol Biofuels* 2010;**3**:1.

Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotech* 2014;**32**:347–55.

Sárvári Horváth I, Franzén CJ, Taherzadeh MJ *et al*. Effects of furfural on the respiratory metabolism of *Saccharomyces cerevisiae* in glucose-limited chemostats. *Appl Environ Microbiol* 2003;**69**:4076–86.

Sato TK, Tremaine M, Parreiras LS *et al*. Directed evolution reveals unexpected epistatic interactions that alter metabolic regulation and enable anaerobic xylose use by *Saccharomyces cerevisiae*. *PLoS Genet* 2016;**12**:e1006372.

Sauer U. Evolutionary engineering of industrially important microbial phenotypes. *Adv Biochem Eng Biotechnol* 2001;**73**:129–69.

Scully S, Orlygsson J. Recent advances in second generation ethanol production by thermophilic bacteria. *Energies* 2015;**8**:1.

Sedlak M, Ho NW. Expression of E. coli *araBAD* operon encoding enzymes for metabolizing L-arabinose in *Saccharomyces cerevisiae*. *Enzyme Microb Technol* 2001;**28**:16–24.

Sedlak M, Ho NW. Production of ethanol from cellulosic biomass hydrolysates using genetically engineered *Saccharomyces* yeast capable of cofermenting glucose and xylose. *Appl Biochem Biotechnol* 2004a;**114**:403–16.

Sedlak M, Ho NW. Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant *Saccharomyces* yeast. *Yeast* 2004b;**21**:671–84.

Shi S, Liang Y, Zhang MM *et al*. A highly efficient single-step, markerless strategy for multi-copy chromosomal integration of large biochemical pathways in *Saccharomyces cerevisiae*. *Metab Eng* 2016;**33**:19–27.

Shin H, Nijland J, de Waal P *et al*. An engineered cryptic Hxt11 sugar transporter facilitates glucose-xylose co-consumption in *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 2015;**8**:176.

Silveira MHL, Morais ARC, Da Costa Lopes AM *et al*. Current pretreatment technologies for the development of cellulosic ethanol and biorefineries. *Chem Sus Chem* 2015;**8**:3366–90.

Sims-Borre P. The economics of enzyme production. *Ethanol Producer Magazine* 2010. http://www.ethanolproducer.com/articles/7048/ (24 March 2017, date last accessed).

Sinha H, Nicholson BP, Steinmetz LM *et al*. Complex genetic interactions in a quantitative trait locus. *PLoS Genet* 2006;**2**:e13.

Sivers MV, Zacchi G, Olsson L *et al*. Cost analysis of ethanol production from willow using recombinant *Escherichia coli*. *Biotechnol Prog* 1994;**10**:555–60.

Smith J, van Rensburg E, Görgens JF. Simultaneously improving xylose fermentation and tolerance to lignocellulosic inhibitors through evolutionary engineering of recombinant *Saccharomyces cerevisiae* harbouring xylose isomerase. *BMC Biotechnol* 2014;**14**:41.

Snoek T, Nicolino MP, van den Bremt S *et al*. Large-scale robot-assisted genome shuffling yields industrial *Saccharomyces cerevisiae* yeasts with increased ethanol tolerance. *Biotechnol Biofuels* 2015;**8**:32.

Sonderegger M, Sauer U. Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose. *Appl Environ Microbiol* 2003;**69**:1990–8.

Steiner G. Use of ethanol plant by-products for yeast propagation. US Patent 8183022 B2. 2008.

Stovicek V, Borodina I, Forster J. CRISPR–Cas system enables fast and simple genome editing of industrial *Saccharomyces cerevisiae* strains. *Metab Eng Commun* 2015;**2**:13–22.

Subtil T, Boles E. Improving L-arabinose utilization of pentose fermenting *Saccharomyces cerevisiae* cells by heterologous expression of L-arabinose transporting sugar transporters. *Biotechnol Biofuels* 2011;**4**:38.

Subtil T, Boles E. Competition between pentoses and glucose during uptake and catabolism in recombinant *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 2012;**5**:1.

Swinnen S, Fernández-Niño M, González-Ramos D *et al*. The fraction of cells that resume growth after acetic acid addition is a strain-dependent parameter of acetic acid tolerance in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2014;**14**:642–53.

Swinnen S, Schaerlaekens K, Pais T *et al*. Identification of novel causative genes determining the complex trait of high ethanol tolerance in yeast using pooled-segregant whole-genome sequence analysis. *Genome Res* 2012;**22**:975–84.

Taherzadeh MJ, Eklund R, Gustafsson L *et al*. Characterization and fermentation of dilute-acid hydrolyzates from wood. *Ind Eng Chem Res* 1997;**36**:4659–65.

Taherzadeh MJ, Gustafsson L, Niklasson C *et al*. Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae*. *J Biosci Bioeng* 1999;**87**:169–74.

Tantirungkij M, Nakashima N, Seki T *et al*. Construction of xylose-assimilating *Saccharomyces cerevisiae*. *J Ferment Bioeng* 1993;**75**:83–8.

Tenenbaum DJ. Food vs. fuel: Diversion of crops could cause more hunger. *Environ Health Perspect* 2008;**116**:A254–7.

Thomas K, Ingledew W. Production of 21% (v/v) ethanol by fermentation of very high gravity (VHG) wheat mashes. *J Ind Microbiol Biotechnol* 1992;**10**:61–8.

Thompson OA, Hawkins GM, Gorsich SW *et al*. Phenotypic characterization and comparative transcriptomics of evolved *Saccharomyces cerevisiae* strains with improved tolerance to lignocellulosic derived inhibitors. *Biotechnol Biofuels* 2016;**9**:200.

Tsai C-S, Kong, II, Lesmana A *et al*. Rapid and marker-free refactoring of xylose-fermenting yeast strains with Cas9/CRISPR. *Biotechnol Bioeng* 2015;**112**:2406–11.

Ulbricht R, Northup S, Thomas J. A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions. *Fundam Appl Toxicol* 1984;**4**:843–53.

Ullah A, Chandrasekaran G, Brul S *et al.* Yeast adaptation to weak acids prevents futile energy expenditure. *Front Microbiol* 2013;**4**:142.

UNCTAD. *Second Generation Biofuel Markets: State of Play, Trade and Developing Country Perspectives*. Geneva: United Nations, 2016.

United States Environmental Protection Agency. Public data for the Renewable Fuel Standard 2017. https://www.epa.gov/fuels-registration-reporting-and-compliance-help/public-data-renewable-fuel-standard (4 Jan 2017, date last accessed).

Van den Brink J, de Vries RP. Fungal enzyme sets for plant polysaccharide degradation. *Appl Microbiol Biotechnol* 2011;**91**:1477.

Van Dijken JP, Scheffers WA. Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol Lett* 1986;**32**:199–224.

Van Hazendonk JM, Reinerik EJM, de Waard P *et al.* Structural analysis of acetylated hemicellulose polysaccharides from fibre flax (*Linum usitatissimum L.*). *Carbohydr Res* 1996;**291**:141–54.

Van Maris AJA, Abbott DA, Bellissimi E *et al.* Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. *Antonie Van Leeuwenhoek* 2006;**90**:391–418.

Van Maris AJA, Winkler AA, Kuyper M *et al.* Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose isomerase as a key component. *Adv Biochem Eng Biotechnol* 2007;**108**:179–204.

Van Rossum HM, Kozak BU, Niemeijer MS *et al.* Requirements for carnitine shuttle-mediated translocation of mitochondrial acetyl moieties to the yeast cytosol. *Mbio* 2016;**7**:e00520–16.

Van Vleet JH, Jeffries TW, Olsson L. Deleting the para-nitrophenyl phosphatase (pNPPase), *PHO13*, in recombinant *Saccharomyces cerevisiae* improves growth and ethanol production on D-xylose. *Metab Eng* 2008;**10**:360–9.

Verduyn C, Postma E, Scheffers WA *et al.* Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* 1992;**8**:501–17.

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

Villadsen J, Noorman H. Scale-up and scale-down. In: Villadsen J (ed.) *Fundamental Bioengineering*. Weinheim: Wiley-VCH, 2016.

Vohra M, Manwar J, Manmode R *et al.* Bioethanol production: Feedstock and current technologies. *J Environ Chem Eng* 2014;**2**:573–84.

Wahlbom CF, Hahn-Hägerdal B. Furfural, 5-hydroxymethyl furfural, and acetoin act as external electron acceptors during anaerobic fermentation of xylose in recombinant *Saccharomyces cerevisiae*. Biotechnol Bioeng 2002;**78**:172–8.

Wang BL, Ghaderi A, Zhou H *et al.* Microfluidic high-throughput culturing of single cells for selection based on extracellular metabolite production or consumption. *Nat Biotechnol* 2014;**32**:473–8.

Wang C, Bao X, Li Y *et al.* Cloning and characterization of heterologous transporters in *Saccharomyces cerevisiae* and identification of important amino acids for xylose utilization. *Metab Eng* 2015a;**30**:79–88.

Wang G, Tang W, Xia J *et al.* Integration of microbial kinetics and fluid dynamics toward model-driven scale-up of industrial bioprocesses. *Eng Life Sci* 2015b;**15**:20–9.

Wang M, Yu C, Zhao H. Directed evolution of xylose specific transporters to facilitate glucose-xylose co-utilization. *Biotechnol Bioeng* 2016;**113**:484–91.

Watanabe S, Abu Saleh A, Pack SP *et al.* Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein-engineered NADH-preferring xylose reductase from *Pichia stipitis*. *Microbiology* 2007;**153**:3044–54.

Watanabe S, Kodaki T, Makino K. Complete reversal of coenzyme specificity of xylitol dehydrogenase and increase of thermostability by the introduction of structural zinc. *J Biol Chem* 2005;**280**:10340–9.

Wei N, Quarterman J, Kim SR *et al.* Enhanced biofuel production through coupled acetic acid and xylose consumption by engineered yeast. *Nat Commun* 2013;**4**:2580.

Weierstall T, Hollenberg CP, Boles E. Cloning and characterization of three genes (*SUT1–3*) encoding glucose transporters of the yeast *Pichia stipitis*. *Mol Microbiol* 1999;**31**:871–83.

Wiedemann B, Boles E. Codon-optimized bacterial genes improve L-arabinose fermentation in recombinant *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 2008;**74**:2043–50.

Wilkening S, Lin G, Fritsch ES *et al.* An evaluation of high-throughput approaches to QTL mapping in *Saccharomyces cerevisiae*. *Genetics* 2014;**196**:853–65.

Wimalasena TT, Greetham D, Marvin ME *et al.* Phenotypic characterisation of *Saccharomyces spp*. yeast for tolerance to stresses encountered during fermentation of lignocellulosic residues to produce bioethanol. *Microb Cell Fact* 2014;**13**:47.

Wisselink HW, Cipollina C, Oud B *et al.* Metabolome, transcriptome and metabolic flux analysis of arabinose fermentation by engineered *Saccharomyces cerevisiae*. *Metab Eng* 2010;**12**:537–51.

Wisselink HW, Toirkens MJ, del Rosario Franco Berriel M *et al.* Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. *Appl Environ Microbiol* 2007;**73**:4881–91.

Wisselink HW, Toirkens MJ, Wu Q *et al.* Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. *Appl Environ Microbiol* 2009;**75**:907–14.

Wisselink HW, Van Maris AJA, Pronk JT *et al.* Polypeptides with permease activity. US Patent 9034608 B2. 2015.

Wright J, Bellissimi E, de Hulster E *et al.* Batch and continuous culture-based selection strategies for acetic acid tolerance in xylose-fermenting *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2011;**11**:299–306.

Xia PF, Zhang GC, Liu JJ *et al.* GroE chaperonins assisted functional expression of bacterial enzymes in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 2016;**113**:2149–55.

Xu H, Kim S, Sorek H *et al.* PHO13 deletion-induced transcriptional activation prevents sedoheptulose accumulation during xylose metabolism in engineered *Saccharomyces cerevisiae*. *Metab Eng* 2016;**34**:88–96.

Yan X, Inderwildi OR, King DA. Biofuels and synthetic fuels in the US and China: A review of Well-to-Wheel energy use and greenhouse gas emissions with the impact of land-use change. *Energy Environ Sci* 2010;**3**:190–7.

Yang F, Hanna MA, Sun R. Value-added uses for crude glycerol—a byproduct of biodiesel production. *Biotechnol Biofuels* 2012;**5**:13.

Young EM, Comer AD, Huang H *et al.* A molecular transporter engineering approach to improving xylose catabolism in *Saccharomyces cerevisiae*. *Metab Eng* 2012;**14**:401–11.

Young EM, Tong A, Bui H *et al*. Rewiring yeast sugar transporter preference through modifying a conserved protein motif. *Proc Natl Acad Sci U S A* 2014;**111**:131–6.

Yu KO, Kim SW, Han SO. Engineering of glycerol utilization pathway for ethanol production by *Saccharomyces cerevisiae*. *Bioresour Technol* 2010;**101**:4157–61.

Yuan WJ, Chang BL, Ren JG *et al*. Consolidated bioprocessing strategy for ethanol production from Jerusalem artichoke tubers by *Kluyveromyces marxianus* under high gravity conditions. *J Appl Microbiol* 2012;**112**:38–44.

Zhang GC, Kong, II, Wei N *et al*. Optimization of an acetate reduction pathway for producing cellulosic ethanol by engineered yeast. *Biotechnol Bioeng* 2016a;**113**:2587–96.

Zhang K, Zhang L-J, Fang Y-H *et al*. Genomic structural variation contributes to phenotypic change of industrial bioethanol yeast *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2016b;**16**:fov118.

Zhou H, Cheng J-S, Wang BL *et al*. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. *Metab Eng* 2012;**14**:611–22.