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REVIEW

Xylose fermentation as a challenge for commercialization of lignocellulosic fuels and chemicals

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Abstract Fuel ethanol production from lignocellulosic materials is at a level where commercial biofuel production is becoming a reality. The solubilization of the hemicellulose fraction in lignocellulosic-based feedstocks results in a large variety of sugar mixtures including xylose. However, allowing xylose fermentation in yeast that normally is used for fuel ethanol production requires genetic engineering. Moreover, the efficiency of lignocellulosic pretreatment, together with the release and generation of inhibitory compounds in this step, are some of the new challenges faced during second generation ethanol production. Successful advances in all these aspects will improve ethanol yield, productivity and titer, which will reduce the impact on capital and operating costs, leading to the consolidation of the fermentation of lignocellulosic biomass as an economically feasible option for the production of renewable fuels. Therefore the development of yeast strains capable of fermenting a wide variety of sugars in a highly inhibitory environment, while maintaining a high ethanol yield and production rate, is required. This review provides an overview of the current status in the use of xylose-engineered yeast strains and describes the remaining challenges to achieve an efficient deployment of lignocellulosic-based ethanol production.

Keywords Biofuel · Ethanol · Lignocellulosic ethanol · Metabolic engineering · *Saccharomyces cerevisiae* · Xylose fermentation · Yeast

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Introduction

Challenges in commercial lignocellulosic ethanol production

Among the new renewable liquid fuel alternatives, ethanol production is the one closest to commercialization. Globally, more than ten demonstration scale and at least four commercial scale facilities are near completion (Cellulosic biofuels. Industry progress report 2012–2013). Therefore, the lessons learned when developing an economically-viable bioethanol facility will function as pilot case for the commercialization of other more advanced biofuels or biochemicals in near future, since similar technical challenges will possibly appear in all lignocellulosic-based microbial production processes.

Today fuel ethanol is produced almost exclusively using corn or sugar cane. This so-called first generation bioethanol made from starch- or sucrose-rich biomass is a well-established industrial process. Second generation bioethanol instead uses lignocellulosic-based

feedstocks, such as agricultural crop residues, wood and paper mill discards, or energy crops. Both 1st and 2nd generation processes have two key steps: generation of fermentable sugars from biomass, and fermentation of these sugars into ethanol. However, the process development from the starch- and sucrose-based bioethanol production cannot be directly transferred to production of 2nd generation ethanol, since novel challenges are faced in lignocellulosic bioethanol production.

The major new challenges encountered are that (1) hydrolysis of the lignocellulosic biomass requires use of different procedures and enzymes than in starch-based processes; (2) lignocellulosic biomass contain pentose sugars that cannot be fermented by yeast; and (3) higher level of toxic and acidic compounds are released during the treatment of lignocellulose. All these challenges have to be dealt with to generate an efficient production process.

Process variables

Lignocellulosic ethanol production involves a large number of variables that will contribute to the outcome of the process performance. The choice of feedstock—mostly based on availability—is the first factor, as the diversity in polymer content from one species to another results in variety of final sugar composition. Secondly, all feedstocks need to be hydrolyzed to generate a stream containing fermentable sugars. Numerous different methods exist for the pre-treatment, which aims to open up the structure of the fibers and dissolve fractions of the biomass (Galbe and Zacchi 2012; Viikari et al. 2012). Among them, steam explosion and dilute acid pre-treatment are the most common (Alvira et al. 2010). Following pre-treatment, a hydrolysis step is performed, usually by an enzymatic process with commercial enzyme mixtures containing cellulases and hemicellulases. In addition, the amounts and types of toxic compounds generated depend on the combination of different types of feedstock and pretreatment. These compounds may have a strong inhibitory effect on the fermenting microorganism.

In addition to these factors, the process can be run in different configurations. If the resulting stream from the hydrolysis step is subjected to fermentation, the process configuration is known as SHF (separate hydrolysis and fermentation). If hydrolysis is performed enzymatically, SSF (simultaneous saccharification and fermentation) can also be used by combining the hydrolysis and fermentation steps. The multiple combinations of process configurations, choice of feedstock and pre-treatment generate a diversity of lignocellulosic hydrolysate compositions, which influence the subsequent fermentation step.

Xylose fermentation

The major components of lignocellulose-based raw materials are cellulose, hemicellulose and lignin, and their relative composition in each raw material varies. In most commonly used feedstocks, glucan is the major polysaccharide constituting 33–51 % of dry matter (DM). The fraction of xylan has a greater variation depending on the type of feedstock, from low xylan levels in softwoods (often below 10 % DM) to up to one-fourth of the biomass composition found among hardwoods, agricultural residues and energy crops (Fig. 1a). Therefore, fermentation of the hydrolyzed xylan fraction can significantly increase the total ethanol yield, being in the case of agricultural residues an increment of around 50 % (Fig. 1b).

The most common microorganism for fermentation in most ethanol production facilities is *Saccharomyces cerevisiae* due to its high tolerance to sugar and ethanol (Gírio et al. 2010), although increasing effort has been recently put into evaluating naturally xylose-consuming yeasts (Long et al. 2012). The hexose sugars present in lignocellulosic hydrolysates, namely glucose, galactose and mannose, are efficiently metabolized and fermented by most *S. cerevisiae* strains. On the contrary, the pentose sugars, xylose and arabinose, are not naturally utilized. Therefore, genetic engineering is needed to enable pentose metabolism in yeast.

Initial xylose pathways

Two different xylose consumption pathways have been successfully introduced into *S. cerevisiae* by heterologous expression: the xylose isomerase (XI) pathway which catalyzes the isomerization of D-xylose to D-xylulose, and the XR/XDH pathway consisting of a xylose reductase (XR) and a xylitol dehydrogenase (XDH) enzymes. The latter is a two-step reaction, commonly found in yeasts and fungi,

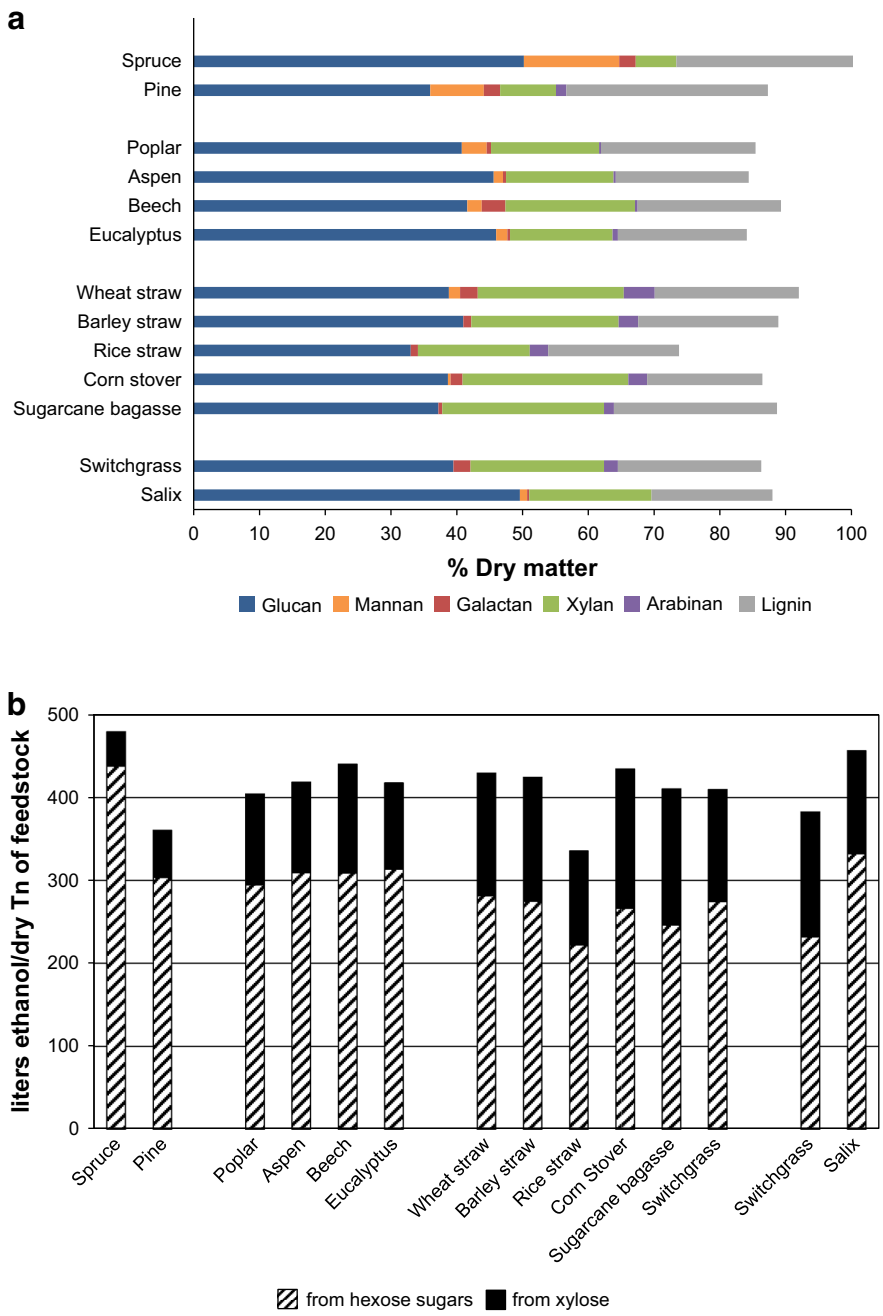

Fig. 1 a Composition of various lignocellulosic materials. Total values below 100 % depend on limitations in carbohydrate analysis. b Ethanol yields from sugar cane bagasse, corn stover and wheat straw considering hexose fraction (*striped bars*) or hexose and xylose fraction (*black bars*). For all cases, saccharification and fermentation yields were considered 95 % of the theoretical values (theoretical C6 saccharification yield = 1.11 g C6 sugar/g polymeric sugar; theoretical C5 saccharification yield = 1.136 g C5 sugar/g polymeric sugar; theoretical ethanol yield = 0.51 g ethanol/g sugar). References, Spruce (Wiman et al. 2012); Pine (Rana et al. 2014); Poplar (Wang et al. 2012); Aspen (Wang et al. 2012); Beech (Viell et al. 2013); Eucalyptus (Martin-Sampedro et al. 2014); Wheat Straw (Erdei et al. 2010); Barley straw (Nghiem et al. 2013); Rice straw (Gu et al. 2013); Corn stover (Rana et al. 2014); Sugarcane bagasse (Zhang and Wu 2014); Switchgrass (Li et al. 2010b); Salix (Han et al. 2013)

where D-xylose is first reduced to D-xylitol, which is then oxidized to D-xylulose.

The introduction of *Schefferomyces stipitis* XR and XDH enabling *S. cerevisiae* to utilize xylose was first reported in 1993 by two independent groups (Kötter and Ciriacy 1993; Tantirungkij et al. 1993). Later, the first recombinant *S. cerevisiae* strain harboring a functional XI was obtained by expression of a bacterial XI. Although xylose was utilized, the ethanol yield was low (Walfridsson et al. 1996). Since the first reported strains were only able to grow under aerobic conditions, anaerobic growth on xylose was considered an essential trait to obtain efficient ethanolic fermentation. Anaerobic growth on xylose was first

demonstrated in a strain expressing the XR and XDH encoding genes from *Sch. stipitis* obtained through evolutionary engineering (Sonderegger and Sauer 2003). In the same year, the introduction of the *XI* gene from the anaerobic fungus *Piromyces* resulted in the first XI carrying strain able to grow anaerobically on xylose (Kuyper et al. 2003). Later, other XI encoding genes from bacteria and fungi have also been successfully expressed in *S. cerevisiae* (Brat et al. 2009; Madhavan et al. 2009). Indeed, it has been shown that anaerobic growth rate on xylose correlates well with the ethanol production rate under laboratory conditions (Almeida et al. 2011; Klimacek et al. 2014).

Strain development for improved xylose metabolism

Intense research over the last two decades has been focused on the improvement of xylose fermenting yeast strains through rational engineering approaches. Recombinant *S. cerevisiae* strains harboring an XR/XDH pathway secrete xylitol during anaerobic xylose utilization in mineral laboratory medium, since XR prefers NADPH, while XDH almost exclusively uses NAD^+. This unmatching co-factor preference has been significantly alleviated through enzyme engineering strategies by generating NADH-preferring XR mutants (Runquist et al. 2010), an $NADP^+$-specific XDH mutant (Watanabe et al. 2007) or a combination of strictly NADH-dependent XR mutant together with an $NADP^+$-dependent XDH mutant (Khattab et al. 2013). In the case of strains harboring XI pathway, codon optimization (Brat et al. 2009) or mutagenesis (Lee et al. 2012) of the heterologous gene is often essential to obtain an efficient expression in *S. cerevisiae,* as well as evolution procedures to increase the copy number of the gene (Zhou et al. 2012). As even strains carrying XI tend to secrete xylitol, XI expression is often combined with deletion of the *GRE3* aldose reductase (Lee et al. 2012) or expression of XDH (Jordan et al. 2012).

Regardless of choice of xylose pathway, re-configuration of central metabolic pathways in *S. cerevisiae* is necessary to increase pentose utilization. Xylose fermentation was significantly improved by directing xylulose into the pentose phosphate pathway (PPP) via overexpression of the endogenous xylulokinase gene (Chang and Ho 1988). Also optimizing expression levels between the enzymes of the xylose pathway further minimizes xylitol accumulation (Eliasson et al. 2001). Additional overexpression of the non-oxidative part of the PPP, namely *TAL1*, *TKL1*, *RKI1* and *RPE1*, improved growth on xylose (Karhumaa et al. 2005). These PPP modifications are used in most of the published industrial xylose fermenting strains, both in combination with XI and with XR/XDH pathways.

Xylose enters the yeast cell via hexose transporters, as it does not have specific pentose transporters. The poor affinity of hexose transporters for xylose has been suggested to be a limiting factor in xylose utilization (Eliasson et al. 2000) along with the fact that strong regulation by glucose affects their expression (Sedlak and Ho 2004). Although overexpression of individual hexose transporters did not increase xylose uptake rate in the early engineered strains (Hamacher et al. 2002), expression of heterologous xylose/hexose transporters has been reported to improve xylose uptake rate (reviewed by Kim et al. 2012). Also, favorable mutants of hexose transporters have been reported (Farwick et al. 2014). Some of the additional genetic engineering undertaken include deletion of *p*-nitrophenylphosphatase *PHO13* (Van Vleet et al. 2008) and deletion of aquaglyceroporin *FPS1* that has a role in xylitol transport (Wei et al. 2013). The recent progress in yeast strain development for improved xylose fermentation has been recently reviewed by Kim and co-workers (2013). In addition to rational engineering, several yeast strains with improved xylose fermentation have been successfully developed through evolutionary engineering strategies (reviewed by Çakar et al. (2012), Dragosits and Mattanovich (2013)).

Industrial application of xylose fermenting yeasts

Strain development for industrial xylose fermentation has been working towards a moving target, since 2nd generation ethanol production is still under development to become a fully deployed industrial process, and the development of industrial strains to be used in commercial-scale facilities has been carried out in parallel to the process optimization. It is conceivable that different process configurations and lignocellulosic hydrolysate compositions require different characteristics from the yeast strain to reach optimal performance. Therefore, selection of the background

strain is critical for successful engineering process since traits already present in the host, such as tolerance to inhibitors, temperature or fermentation products can represent a competitive advantage in the resultant engineered strain. Significant natural variation in inhibitor tolerance can be found among *S. cerevisiae* strains which perform very differently in different hydrolysates (Albers and Larsson 2009; Almeida et al. 2009b; Brandberg et al. 2004).

The commonly acquired knowledge on xylose utilization from several years of research, although mostly done in laboratory strains, has become the basis of the industrial strain development. Several companies (such as DSM, Mascoma, Terranol) have reported development of XI-based industrial strains whereas others (such as C5LT, Iogen) have developed XR/XDH-based industrial strains. Almost all reported industrial strains have overexpressed genes from the pentose phosphate pathway and some of them may have other changes. More recently, several patent applications have reported on utilizing the uptake of acetate for increased tolerance in lignocellulose hydrolysates (Klaassen et al. 2014; Zelle et al. 2014). Moreover, unique features may be required to optimize the fermentation performance in a given application process. Further strain development has been achieved using evolutionary engineering strategies (Demeke et al. 2013) or adaptation to given hydrolysate (Koppram et al. 2012). However, evolutionary engineering demands time and resources, and may result in loss of advantageous characteristics present in the parental strain. Therefore rational genetic engineering with minimal genetic changes is preferred to generate functional industrial strains for xylose fermentation. Regardless of yeast engineering strategy, it is essential to develop industrial strains with well-chosen strain background, aiming at highly efficient fermentation performance under industrial-relevant conditions.

Industrial substrates

The presence of inhibitors in lignocellulosic hydrolysates further hinders the fermentation process. Acetic acid is generated by the de-acetylation of the hemicellulose fraction when lignocellulosic biomass is hydrolyzed. Several sugar degradation compounds are also formed during this process. At high temperature and pressure, dehydration of hexose sugars generates hydroxymethyl-2-furaldehyde (HMF), and xylose is degraded to 2-furaldehyde (furfural). Moreover, further degradation of HMF leads to the formation of levulinic and formic acids. Furfural, in turn, can be converted into formic acid during prolonged treatment. In addition, several phenolic compounds are released as a result of lignin degradation. All these inhibitors affect the yeast metabolism, often decreasing the fermentation performance.

The physiological effects of aldehydes, especially HMF and furfural, have been extensively studied (Almeida et al. 2009a; Taylor et al. 2012). A decrease in growth rate and ethanol production in the presence of furfural and HMF has been reported under both aerobic and anaerobic conditions (Heer and Sauer 2008; Klinke et al. 2004). In addition, the presence of aldehydes leads to a lag phase where the yeast viability decreases. Perhaps a more significant inhibitory effect results from the acids present in the lignocellulosic hydrolysates, even when pH is maintained at the optimal range for yeast. Although the presence of small concentrations of acid can lead to an increase in ethanol production rate (Graves et al. 2006; Pampulha and Loureiro-Dias 2000), a reduction on biomass yield and especially xylose consumption rate are generally observed in the presence of acid concentrations typical for lignocellulosic hydrolysates. Acetic acid is the most prevalent acid, but in some cases also formic and levulinic acids present a significant inhibitory effect.

Genetic engineering can be used for improving not only the metabolic pathways, but also the tolerance towards inhibitors. Many of the recent results on genetic engineering for improved tolerance relate to co-factor availability (Hasunuma et al. 2011, 2014; Laadan et al. 2008; Li et al. 2014), but also studies on genes related to stress responses have been reported (Fujitomi et al. 2012; Sakihama et al. 2014; Tanaka et al. 2012). Tolerance is also an easy target for improvement by evolutionary engineering (Koppram et al. 2012; Wallace-Salinas and Gorwa-Grauslund 2013; Wright et al. 2011). The effect of lignocellulosic inhibitors in yeast metabolism, and metabolic engineering strategies to alleviate their influence during pentose fermentation have been recently reviewed (Almeida et al. 2011; Laluce et al. 2012).

Xylose fermentation in commercial lignocellulosic ethanol production

Although there is no publically available data from fermentation performance of the commercially

available industrial strains, many studies from research laboratories have been published demonstrating high xylose-ethanol conversion during fermentation of lignocellulosic feedstocks. Table 1 summarizes selection of reported results on fermentation of lignocellulosic feedstocks with genetically-engineered *S. cerevisiae* strains from the last five years. It is almost impossible to make a fair comparison for fermentation efficiency, since the raw materials, sugar contents, yeast concentrations, fermentation set-ups and experimental conditions vary widely. For industrial applications, information on ethanol yield based on all available sugars is necessary to evaluate the overall process performance. Unfortunately, in some reported cases only metabolic ethanol yield based on consumed sugars is presented, although it still serves as a good measure to assess the fermentation efficiency of the introduced pathway. Most fermentation tests in lignocellulosic hydrolysates have been made with XR-XDH carrying strains, and little published information is available on the performance of XI based strains. However, it is clear that although xylose fermentation now is approaching industrially relevant levels, it is not as efficient as glucose fermentation, and it also seems to be more sensitive to inhibitors in the lignocellulosic hydrolysates.

High sugar concentrations result in high ethanol concentrations, and sugar concentrations largely depend on the efficiency of the pretreatment and hydrolysis steps. Therefore final ethanol concentrations presented in Table 1 describe the efficiency of the combination of pretreatment and fermentation. Ethanol concentrations of at least 50 g/l would also contribute to the reduction of the ethanol production cost, since the energy demand for distillation is strongly dependent on the ethanol concentration fed into the distillation column (Galbe et al. 2007). Among the latest reported lignocellulosic fermentations (Table 1), only two studies have reported ethanol concentrations over 50 g/l (Erdei et al. 2013b; Jin et al. 2013). Although there still is room for improvement, the first commercial yeast preparations are on the market (GranBio. Press release 24 September 2014; http://www.leaftechnologies.com). Unfortunately no clear data on the performance of the commercial preparations are available, although 40 % increase in total ethanol yield from wheat straw was reported by Dong Energy and DSM in demonstration scale (Inbicon, press release 4 December 2013).

Over the last year, the first three commercial-scale facilities for lignocellulosic ethanol production have been inaugurated. In October 2013, the Crescentino Biorefinery started its activities in Italy. It has a capacity to produce 7.5×10^6 l ethanol/year, and a mix of bagasse, wheat and rice straw, arundo donax and poplar is used as feedstock (www.betarenewables.com). In September 2014, two more commercial-scale facilities were inaugurated. In USA, Project Liberty, with a current capacity of 7.7×10^6 l ethanol/year (planned to increase to 9.5×10^6 l ethanol/year) uses corn crop residues such as baled corn cobs, leaves, husk and stalk as feedstock (www.poet-dsm.com). Bioflex I, located in Brazil, uses instead sugarcane straw and bagasse as feedstock and has a capacity of 8.2×10^6 l ethanol/year (www.granbio.com.br). However, there is yet no information on ethanol production from these plants.

It will take several years before production of second generation ethanol can be considered mature from a technical point of view. Nevertheless, the recently initiated production at these commercial-scale sites will pave the way for the construction of new facilities, despite that each new feedstock will require specific optimization.

Remaining challenges

The remaining two important challenges for lignocellulosic ethanol production are enzyme usage for hydrolysis, and fermentation efficiency. The pretreatment step has a major impact in all the other steps in the process, and a much harsher pre-treatment than the one required for starch-based biomass is needed because of recalcitrance of lignocellulose. There is, however, need for a compromise between sugar recovery yield and inhibitor formation, since the generation of degradation products from carbohydrates and lignin can severely inhibit the hydrolysis and fermentation steps. The wide variety of feedstock compositions and pretreatment technologies requires thorough optimization of the enzyme cocktail and its loading, to not to compromise the fermentation performance (Gao et al. 2011). To completely degrade the cellulose and hemicellulose fractions, it is estimated that up to ten different enzymes are required, each of which should be efficient in terms of specific activity, stability, binding and end-product inhibition (recently reviewed by Viikari et al. (2012)).

Table 1 Recent reported results of fermentation of lignocellulosic feedstocks with xylose engineered *S. cerevisiae* strains

Feedstock	Pretreatment and fermentation conditions	Yeast concentration	Strain	Xylose pathway	Additional metabolic modifications	Ethanol yield (%)	Metabolic ethanol yield (%)	Consumed (%)/residual (g/l) xylose	Final ethanol titer (g/l)	Ref
Corncobs	Steam PT, SO_2 cat. (185 °C, 5 min). Fed-batch SSCF (PDU scale 30L). Final WIS 10 %	–	KE6-12	XR/XDH	Ran. Mut. and Ev. Eng.	69	–	55 %	47	Koppram et al. (2013)
Corncobs	Steam PT, SO_2 cat. (185 °C, 5 min). Fed-batch SSCF (demo scale 10 m^3). Final WIS 10.5 %	–	KE6-12	XR/XDH	Ran. Mut. and Ev. Eng.	58	–	58 %	39.8	Koppram et al. (2013)
Corncobs	Steam PT, SO_2 cat. (185 °C, 5 min). SSCF with hydrolysed liquid fraction feeding (PDU scale 30L)	–	KE6-12	XR/XDH	Ran. Mut. and Ev. Eng.	77	–	–	32	Koppram et al. (2013)
Corn stover	AFEX PT (100 °C, 30 min). SSCF with 9 % glucan loading (shake flask)	–	GLBRCY35	XR/XDH	Mating	–	71.7	–	51.3	Jin et al. (2013)
Corn stover	Alkaline PT (80 °C, 2 % NaOH, 75 min). SHF (shake flask)	–	ZU-10	XR/XDH	–	82	–	88.5 %	41.2	Zhao and Xia (2010)
Corn stover	AFEX PT (140 °C, 15 min). SHF (shake flask)	–	424A(LNH-ST)	XR/XDH	–	–	98	84 %	41	Uppugundla et al. (2014)
Corn stover	AFEX PT (140 °C, 15 min). SSCF with 6 % glucan loading (6 h pre-hydrolysis) (shake flask)	–	424A(LNH-ST)	XR/XDH	–	–	88	3.2 g/l	39.9	Jin et al. (2012)
Corn stover	Alkaline PT (30 °C, 0.125 g H_2O_2/g biomass, 24 h min). SHF (shake flask)	–	GLBRCY87	XR/XDH	Ev. Eng.	68	–	90 %	39	Sato et al. (2014)
Corn stover	Commercial scale preparation. SHF.	1 g DW/l	C5LTe1032	XR/XDH	–	86	–	100 %	35	(Karhumaa and Sànchez i Nogué)
Corn stover	Acid PT, 1.5 % H_2SO_4 cat. (108 °C, 6 h). SHF (shake flask)	–	ZU-910	XR/XDH	–	69	–	90.2 %	27	Gao and Xia (2012)
Rice straw	Hot water treatment. SHF (shake flask)	–	MT8-1X/TAL-ADH	XR/XDH	–	96	–	82 %	13.4	Hasunuma et al. (2014)
Spruce	Steam PT. SSF with 10 % WIS.	4 g DW/l	GS1.11-26	XI	Ran. Mut., Mating and Ev. Eng.	63.9	–	0.4 g/l	35.3	Demeke et al. (2013)

Table 1 continued

Feedstock	Pretreatment and fermentation conditions	Yeast concentration	Strain	Xylose pathway	Additional metabolic modifications	Ethanol yield (%)	Metabolic ethanol yield (%)	Consumed (%)/residual (g/l) xylose	Final ethanol titer (g/l)	Ref
Spruce	Steam PT, dilute SO_2 catalyst (140 °C, 5 min). SSCF.	3 g DW/l	TMB3400	XR/XDH	Ran. Mut. and Ev. Eng.	78		85 %	35.2	Olofsson et al. (2010)
Sweet sorghum bagasse	AFEX PT (140 °C, 30 min) and enzymatic hydrolysis 6 % glucan loading. Batch fermentation (shake flask)	–	424A(LNH-ST)	XR/XDH	–	–	69.9	55.6 %	42.3	Li et al. (2010a)
Sweet sorghum bagasse	Steam PT (190 °C, 5 min). Fed-batch SSF.	5 g wet wt/l (final)	$D5A^{+H}$	XI	Ran. Mut. and Ev. Eng.	50	–	11.6 g/l	19.2	Smith et al. (2014)
Wheat straw	Steam PT, dilute H_2SO_4 cat. (190 °C, 10 min). Fed-batch (with low-rate saccharified wheat meal feed)	–	KE6-12	XR/XDH	Ran. Mut. and Ev. Eng.	76	–	31 %	60	Erdei et al. (2013b)
Wheat straw	Steam PT, dilute H_2SO_4 cat. (190 °C, 10 min). Fed-batch (with hydrolyzed wheat meal feed).	5 g/l	TMB3400	XR/XDH	Ran. Mut. and Ev. Eng.	95	–	100 %	43	(Erdei et al. 2012)
Wheat straw	Steam PT, dilute H_2SO_4 cat. (190 °C, 10 min). Fed-batch 1.5X liquid fraction with hydrolysate feeding	–	KE6-13i	XR/XDH	Ran. Mut. and Ev. Eng.	86	–	–	33	Erdei et al. (2013a)
Wheat straw	Steam PT, dilute H_2SO_4 cat. (190 °C, 10 min). SSCF with 7 % WIS content	–	TMB3425	XR/XDH	–	76	–	84 %	25.9	Olofsson et al. (2011)
Whole corn plants	AFEX PT (90 °C, 5 min). SHF (shake flask)	–	424A(LNH-ST)	XR/XDH	–	–	89.2	79.5 %	29.8	Shao et al. (2010)

PT Pretreatment, *Cat* catalyst, *Ran. Mut.* random mutagenesis, *Ev. Eng.* evolutionary engineering

Ethanol yield % based on the theoretical yield from available sugars

Metabolic yield % based on the theoretical yield from consumed sugars

In fermentation, the complexity of the substrate plays an important role in the overall process performance. Some sugars are consumed sequentially due to metabolic regulation and competition for transporters (Madhavan et al. 2012; Subtil and Boles 2012). In the case of xylose, the fermentation rate still remains lower than for glucose despite several years of strain development. Experiments in lignocellulosic hydrolysates often generate unexpected results due to the presence of inhibitors, since these vary widely in composition. Furthermore, use of co-factors in the detoxification by the cells may interfere with rest of the metabolism. In some cases the presence of inhibitors even improves fermentation, as is the case for the reduced xylitol production observed in hydrolysates with XR-XDH carrying strains (Hahn-Hägerdal et al. 2005; Karhumaa et al. 2007; Lu et al. 2009). Moreover, efficient fermentation becomes more difficult in hydrolysates with high xylose/glucose ratios since detoxification of inhibitory compounds and ATP-demanding tolerance mechanisms are presumably more efficient during glucose fermentation (Bellissimi et al. 2009). Therefore, development of yeast strains able to ferment all hexose and pentose sugars in highly inhibitory environments as efficiently as glucose remains a major challenge for the development of 2nd generation bioethanol processes.

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