Review

Stress-related challenges in pentose fermentation to ethanol by the yeast *Saccharomyces cerevisiae*

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Conversion of agricultural residues, energy crops and forest residues into bioethanol requires hydrolysis of the biomass and fermentation of the released sugars. During the hydrolysis of the hemicellulose fraction, substantial amounts of pentose sugars, in particular xylose, are released. Fermentation of these pentose sugars to ethanol by engineered *Saccharomyces cerevisiae* under industrial process conditions is the subject of this review. First, fermentation challenges originating from the main steps of ethanol production from lignocellulosic feedstocks are discussed, followed by genetic modifications that have been implemented in *S. cerevisiae* to obtain xylose and arabinose fermenting capacity per se. Finally, the fermentation of a real lignocellulosic medium is discussed in terms of inhibitory effects of furaldehydes, phenolics and weak acids and the presence of contaminating microbiota.

Keywords: Contamination · Ethanol · Inhibitors · Pentose fermentation · *Saccharomyces cerevisiae*

1 Introduction

The hemicellulose fractions of many non-food biomass resources that can be used for sustainable biofuel production – including major agricultural residues such as corn stover, wheat straw and sugar cane bagasse – are pentose-rich (Table 1). Conversion of pentoses after hydrolysis can be achieved using a variety of either natural fermenting or engineered microorganisms such as engineered *Escherichia coli* [1], *Zymomonas mobilis* [2], thermophilic bacteria such as *Thermoanaerobacterium ethanolicus* [3] or *Th. saccharolyticum* [4], fungi such as *Rhizopus oryzae* [5] and yeasts including both natural pentose fermenting yeasts such as *Pichia stipitis*, *Candida shehatae* or *Pachysolen tannophilus* [6] or metabolically engineered *Saccharomyces cerevisiae* (recently reviewed by [7, 8]). This review discusses stress challenges in pentose fermentation of hydrolysates using recombinant *S. cerevisiae*.

2 Challenges in lignocellulosic fermentation

The biological conversion route of lignocellulose to ethanol consists of a pretreatment step, an enzymatic hydrolysis step, a fermentation step and a final recovery of the formed ethanol [9]. A summary of the challenges faced by the yeast *S. cerevisiae* during the production of ethanol from lignocellulosic feedstocks is shown in Fig 1. The objective of the pretreatment – normally a high temperature...
treatment at 180–210°C for some minutes – is to facilitate the enzymatic hydrolysis by making the cellulose more accessible for enzymatic degradation. Acid catalysts (e.g. sulphuric acid, SO₂ or carboxylic acids) improve the hydrolysis of hemicellulose, whereas base catalysts (NaOH, ammonia, lime), organic solvents or oxygen facilitate partial solubilization of lignin [10]. The pH value must be adjusted after pretreatment, and this will lead to the formation of salt, which can cause osmotic stress for the yeast. The osmotic stress will trigger glycerol formation, affect membrane transport processes and slow down growth [11]. Acetyl groups cleaved off from the hemicelluloses will be found in the hydrolysate as acetate/acetic acid. The acetyl content is normally higher in pentose-rich materials than in materials with a low pentose content (such as softwoods). In particular glucuronoxylans in hardwoods are acetylated to a higher degree than the galactoglucomannans typical of softwoods. The stress caused by acetic acid is thus more important in xylose-rich hydrolysates. Furaldehydes, primarily 2-furaldehyde from pentoses and 5-hydroxymethyl-2-furaldehyde (HMF) from hex-

Table 1. Reported composition (g/100 g dry matter) of some pentose-rich lignocellulosic feedstocks

<table>
<thead>
<tr>
<th>Material</th>
<th>Glucan</th>
<th>Mannan</th>
<th>Galactan</th>
<th>Xylan</th>
<th>Arabinan</th>
<th>Lignin</th>
<th>Acetyl</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn stover (American)</td>
<td>36.1</td>
<td>1.8</td>
<td>2.5</td>
<td>21.4</td>
<td>3.5</td>
<td>17.2</td>
<td>3.2</td>
<td>[108]</td>
</tr>
<tr>
<td>Corn stover (Italian)</td>
<td>36.8</td>
<td>0.3</td>
<td>2.9</td>
<td>22.2</td>
<td>5.5</td>
<td>21.2</td>
<td>1.7</td>
<td>[108]</td>
</tr>
<tr>
<td>Sugar cane bagasse</td>
<td>43.3</td>
<td>NR</td>
<td>NR</td>
<td>24.3</td>
<td>2.0</td>
<td>22.8</td>
<td>2.0</td>
<td>[109]</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>41.2</td>
<td>NR</td>
<td>NR</td>
<td>26.1a</td>
<td>–</td>
<td>19.1</td>
<td>4.2</td>
<td>[110]</td>
</tr>
<tr>
<td>Barley straw</td>
<td>36.8</td>
<td>NR</td>
<td>2.2</td>
<td>17.2</td>
<td>5.3</td>
<td>14.3</td>
<td>NR</td>
<td>[112]</td>
</tr>
<tr>
<td>Rice straw</td>
<td>34.2</td>
<td>NR</td>
<td>NR</td>
<td>24.5</td>
<td>NR</td>
<td>11.9</td>
<td>NR</td>
<td>[113]</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>36.6</td>
<td>NR</td>
<td>NR</td>
<td>16.1</td>
<td>NR</td>
<td>14.9</td>
<td>NR</td>
<td>[114]</td>
</tr>
<tr>
<td>Switch grass</td>
<td>34.2</td>
<td>0.5</td>
<td>1.5</td>
<td>23.3</td>
<td>2.0</td>
<td>19.9</td>
<td>2.4</td>
<td>[115]</td>
</tr>
<tr>
<td>Salix</td>
<td>41.5</td>
<td>3.0</td>
<td>2.1</td>
<td>15.0</td>
<td>1.8</td>
<td>25.2</td>
<td>NR</td>
<td>[116]</td>
</tr>
</tbody>
</table>

a) Including arabinan.
NR, not reported.

Figure 1. Challenges faced by the yeast *S. cerevisiae* during the production of ethanol from lignocellulosic feedstocks.
oses, are formed by dehydration of the liberated sugar monomers at high temperatures and acidic conditions, and a fraction of the furaldehydes is also likely to be further degraded to other organic acids – e.g. levulinic acid and formic acid (as reviewed in [12]). The lignin fraction of the biomass gives rise to different phenolics after pretreatment, e.g. phenylpropanoid derivatives, such as p-coumaric and ferulic acid, or phenolics without the propanoid side chain [13]. The hydrolysis of the pretreated material may take place together with the fermentation, i.e. the so-called simultaneous saccharification and fermentation (SSF) [14]. Furthermore, the liquid fraction after pretreatment may either be separated before the hydrolysis, or treated together with the solid fraction. If the enzymatic hydrolysis takes place as a separate step, a temperature of 40–50°C is used, in agreement with the optimum temperature for the currently available cellulases. However, a lower temperature must be used in the SSF to stay within the allowable temperature for the yeast. An improved yeast thermal tolerance would thus be beneficial from a process point of view.

Also the end-product of the fermentation, ethanol, is in itself inhibitory at high levels (see [15] for a recent review), but in its present stage of development, ethanol concentrations in lignocellulose-based processes using pentoses seldom exceed 5% by weight, and inhibition by ethanol is therefore minor compared to that of many other compounds.

As described above, almost each process step gives rise to environmental stress factors that may impact the ethanol yield or productivity in the fermentation. In addition, the presence of highly contaminated feedstock and non-sterile conditions (see e.g. [16]) introduce an additional challenge of competition with contaminating microorganisms for the available sugars. Finally, the metabolic engineering of the yeast needed to convey pentose-utilising capacity adds genetic factors. The combination of the environmental and genetic factors result in the physiological changes shown in Figure 1.

3 Metabolic engineering for efficient fermentation and anaerobic growth on pentose sugars

3.1 Xylose utilization

Utilization of the pentose sugar xylose has been enabled in *S. cerevisiae* by expression of the heterologous enzymes xylose reductase (XR) and xylitol dehydrogenase (XDH) or xylose isomerase (XI) [7, 17] (Fig. 2). Both non-targeted and targeted metabolic engineering approaches have then been
followed to further improve xylose utilization, as reviewed below.

### 3.1.1 Improvement of xylose utilization by non-target approaches

Random mutagenesis and evolutionary engineering has been successfully applied to increase xylose utilization in a number of recombinant *S. cerevisiae* strains. The strains H2490-4 [18], TMB3400 [19] and C1 [20] were constructed based on the oxido-reductive XR/XDH pathway (Fig. 2) and were selected during prolonged chemostat cultivation following chemical mutagenesis (TMB3400 and C1) (Fig. 3A). The strain RWB218 was constructed using the XI pathway (Fig. 2) and was selected in chemostat followed by sequential batch cultivation (Fig. 3A) [21]. All strains had significantly improved ethanol productivity and growth rate on xylose, compared to their parental strains. Enzymatic analysis revealed increased activity of the heterologous enzymes XR and XDH in the strains C1 and TMB3400, whereas the activity of several enzymes in the pentose phosphate pathway (PPP) (Fig. 2) was increased in the strain H2490-4 [18, 22]. These observations were further corroborated by proteome analysis, which identified higher expression of XR, XDH and the non-oxidative PPP in TMB3400 compared to the parental strain [23]. Transcriptome analyses of the strains TMB3400 and C1 were, however, unsuccessful in identifying alterations at a single control point, but rather suggested an additive effect of several small mutations in the genome [24]. The strain RWB218 was not characterized at the transcriptome level, however increased transport affinity was detected in kinetic studies, which possibly resulted from increased expression of high-affinity native transporters [21].

### 3.1.2 Improvement of xylose utilization by rational pathway design

Rational design of the xylose utilization pathway in *S. cerevisiae* is based on identifying, altering and ultimately moving metabolic control points. A major controlling step in xylose utilization is the expression level of the heterologous enzymes XR/XDH and XI. Specifically, the low enzyme activity and substrate affinity of XR and XI requires high intracellular xylose concentration to push the rate of the reaction, which leads to decreased ethanol productivity as substrate is consumed [25]. Overexpression of XR somewhat alleviated this problem and increased the rate of xylose consumption substantially [26]. Similarly, very high expression level of XI encoding gene from episomal plasmids was required for anaerobic growth on xylose by the strain RWB202-AFX (Fig. 3A) [27].

<table>
<thead>
<tr>
<th>Notation</th>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Additional step(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>C1</td>
<td>XYL1, XYL2, XKS1</td>
<td>Chemical mutagenesis</td>
<td>[20]</td>
</tr>
<tr>
<td>2</td>
<td>TMB 3415</td>
<td>XYL1 (K270R), XYL2, XKS1, PPP</td>
<td>–</td>
<td>[46]</td>
</tr>
<tr>
<td>3</td>
<td>RWB202-AFX</td>
<td>XI multicopy</td>
<td>–</td>
<td>[27]</td>
</tr>
<tr>
<td>4</td>
<td>TMB3421</td>
<td>XYL1 (N272D P275Q), XYL2, XKS1, PPP</td>
<td>–</td>
<td>[121]</td>
</tr>
<tr>
<td>5</td>
<td>TMB 3420</td>
<td>XYL1 (N272D P275Q), XYL2, XKS1, PPP</td>
<td>Sequential batch selection</td>
<td>[121]</td>
</tr>
<tr>
<td>6</td>
<td>RWB 217</td>
<td>XI multicopy, XKS1, PPP</td>
<td>–</td>
<td>[36]</td>
</tr>
<tr>
<td>7</td>
<td>RWB 218</td>
<td>XI multicopy, XKS1, PPP</td>
<td>Batch and chemostat selection</td>
<td>[21]</td>
</tr>
</tbody>
</table>

**Figure 3.** (A) Engineered *S. cerevisiae* strains capable of anaerobic growth on xylose. (B) Relationship between the ethanol productivity ($r_e$) and the anaerobic growth rate on xylose.
Additional control points in xylose metabolism are the endogenous phosphorylating enzyme xylulokinase (XK), and the PPP, respectively (Fig. 2). Since XK uses ATP as a cofactor, the expression level of this enzyme needs to be carefully optimized [28–30]. Overexpression of XK improved the conversion of xylulose [31]. However, without concurrent overexpression of XR, xylose utilization was decreased [28, 30]. The non-oxidative PPP follows after XK in the conversion of xylulose to glycolytic intermediates (Fig. 2). Compared to glycolysis, the PPP is kinetically constrained since the PPP reactions are less thermodynamically favourable and operate close to the equilibrium [32]. Fermentation of xylulose to ethanol in native S. cerevisiae is thus orders of magnitude slower than that of glucose [31, 33]. When all genes of the non-oxidative PPP were overexpressed, the xylulose utilization increased, but xylulose conversion was not improved unless XR and XDH activities were raised [34, 35]. A considerably higher ethanol productivity was also observed when high XI level was combined with high expression of XK and non-oxidative PPP encoding genes in strains RWB 217 and RWB 218 (Fig. 3A) [27, 36].

In S. cerevisiae, xylose is transported via the hexose transporters, however with a much lower affinity, which limits its utilization in the presence of glucose [37]. On xylose medium however, it was initially shown that xylose transport did not control the flux of the pathway in a recombinant S. cerevisiae strain [25]. However, when XR was overexpressed the control coefficient of transport increased [38], and in a strain where the PPP was overexpressed, increased transport improved xylose utilization at least when the substrate concentration was below 4 g/L [39].

For S. cerevisiae strains employing the oxido-reductive xylose pathway, an additional control point is the imbalanced co-factor preference of the two recombinant enzymes XR and XDH. Since XR prefers NADPH and XDH is strictly NAD+ dependent, the cell creates an excess of NAPD+ and a deficiency in NADH which leads to the production of xylitol as a byproduct (Fig. 2) and lower ethanol yield [25, 40]. The cofactor specificity of XR and/or XDH has thus been modified by protein engineering for balanced cofactor usage of the two enzymes [41–44]. In this way the ethanol productivity, and consequently the anaerobic growth rate, was drastically improved for strains TMB 3415, TMB 3420 and TMB 3421 utilizing mutant XR (Fig. 3) [45, 46].

3.2 Arabinose utilization

Arabinose utilization has been investigated to less extent than xylose utilization due to the lower abundance of arabinose in lignocellulosic biomass (Table 1). Arabinose can be utilized by S. cerevisiae via the introduction of either an isomerization pathway consisting of L-arabinose isomerase (AI), L-ribulokinase (RK) and L-ribulose-5-P 4-epimerase (RE) or a reduction/oxidation-based pathway consisting of XR, L-arabinitol 4-dehydrogenase (LAD), L-xylulose reductase (LXR) and XDH (Fig. 2) [7]. Although arabinose utilization is considerably less efficient than xylose, it shares several control points with xylose utilization since both heterologous arabinose-catabolizing enzymes [47], the endogenous XK, the non-oxidative PPP as well as high affinity native transporters [48].

In practice, the fermentation of the arabinose fraction in lignocellulosic hydrolysates cannot be disconnected from the fermentation of xylose, which raises a set of new challenges. For instance, XR displays a similar affinity for xylose and arabinose [49, 50]. Thus, in S. cerevisiae strains combining the oxidation/reduction pathway for xylose and the isomerization pathway for arabinose-utilization, almost all consumed arabinose was converted to arabitol (arabinitol) by XR (Fig. 2) [51–53]. Arabitol formation can be avoided by using the XI-based pathway for xylose utilization, which does not include XR. Nevertheless this approach proved difficult since an elaborate selection procedure was required to enable arabinose utilization without lowering the capacity of the same strain for xylose utilization [54, 55]. Arabitol formation can also be reduced by employing the oxidation/reduction pathways for both xylose- and arabinose-utilization (Fig. 2), so that arabitol can be further metabolized to xylitol and ethanol. An oxidation/reduction pathway for arabinose metabolism has thus been described [56, 57] and its performance was recently improved using a suitable combination of enzymes [58].

4 Pentose fermentation in hydrolysates

In reported studies in which pentose-fermenting strains of S. cerevisiae have been evaluated in undetoxified pentose-rich lignocellulosic hydrolysates (Table 2), the maximum ethanol concentrations obtained did not exceed 45 g/L and in many cases, the xylose conversion was not complete even after
Table 2. Some studies in which pentose fermenting *S. cerevisiae* has been used for the conversion of lignocellulosic hydrolysates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Raw material</th>
<th>Pretreatment and fermentation conditions</th>
<th>Ethanol concentration and xylose conversion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>424A(LNH-ST) (XR/XDH/XK)</td>
<td>Sweet sorghum bagasse</td>
<td>AFEX pretreatment (2:1 ammonia to biomass loading, 140°C, 30 min) followed by enzymatic hydrolysis. Approximate liquid concentrations: glucose 65 g/L, xylose 33 g/L. Initial yeast concentration 0.3 g/L</td>
<td>Highest ethanol concentration reached 42.3 g/L, with a xylose conversion of 55.6% (after about 75 h). Very little xylitol formation.</td>
<td>[117]</td>
</tr>
<tr>
<td>424A(LNH-ST) (XR/XDH/XK)</td>
<td>Poplar</td>
<td>Comparison of many different pretreatments including AFEX, oxidative alkali, SO2 catalysed steam pretreatment, followed by enzymatic hydrolysis. Initial liquid concentrations (AFEX): glucose 62.3 g/L, xylose 16.2 g/L, acetic acid 3.5 g/L. Initial liquid concentrations (steam pretreatment SO2) glucose 33.2 g/L, xylose 25.8 g/L, acetic acid 6.2 g/L. Initial yeast concentration about 5 g/L</td>
<td>Highest ethanol concentration reached after 50 h: 40 g/L (lime pretreatment) with about 90% conversion xylose; SO2 steam explosion yielded 26 g/L ethanol, about 90% xylose conversion.</td>
<td>[84]</td>
</tr>
<tr>
<td>TMB3400 (XR/XDH/XK + mutagenesis and selection)</td>
<td>Corn stover</td>
<td>SSF Steam pretreatment at 200°C, 5 min, SO2 catalysed. Liquid composition: glucose 7.2, xylose 36, acetic acid, 2.2 g/L, HMF 0.2 g/L, furfural 1.5 g/L. Substantial amounts of xylan remaining in the solid material (6.6–9.2%), which was degraded during SSF. Initial yeast concentration: 5 g/L.</td>
<td>Ethanol concentration of 36.8 g/L reached at 11% WIS using fed-batch addition. Residual xylose concentration in liquid about 10 g/L after 96 h</td>
<td>[78]</td>
</tr>
<tr>
<td>TMB3400 (XR/XDH/XK)</td>
<td>Wheat straw</td>
<td>SSF Steam pretreatment, dilute H2SO4 catalyst, 210°C, 2.5 min. Liquid composition after pretreatment: glucose 10.9 g/L, xylose 51.2 g/L, acetic acid 5 g/L, HMF 0.6 g/L, furfural, 1.7 g/L. Xylan content in fibre about 3.5%. Fed-batch fermentation at a total solids loading of 9%WIS. Initial yeast concentration 4 g/L.</td>
<td>A final ethanol concentration of 38 g/L and a xylose conversion about 40% in fed-batch up to 9% WIS. Corresponding for fed-batch at 7%: ethanol concentration of 34 g/L and a xylose conversion of 74%.</td>
<td>[118]</td>
</tr>
<tr>
<td>TMB3400 (XR/XDH/XK)</td>
<td>Sugar cane bagasse</td>
<td>SSF Steam pretreatment at 190°C, 5 min, SO2 catalysed. Liquid composition after pretreatment: glucose 4.6 g/L, xylose 39.5 g/L, arabinose 2.7 g/L, furfural 0.9 g/L, acetic acid 4.2 g/L. Yeast concentration 4 g/L.</td>
<td>Ethanol concentration 26 g/L. Conversion of xylose about 40% at 7.5%WIS (Ethanol concentration 20 g/L, and xylose conversion 88% at 5%WIS).</td>
<td>[119]</td>
</tr>
<tr>
<td>TMB3400 (XR/XDH/XK)</td>
<td>Sugar cane bagasse</td>
<td>Steam pretreatment at 190°C, 5 min, SO2 catalysed. Liquid concentrations: 15 g/L xylose, 4 g/L glucose. Initial yeast concentration 5 g/L.</td>
<td>Only hemicellulose part fermented. Highest xylose conversion for liquid from pretreatment 65% after 20 h.</td>
<td>[109]</td>
</tr>
</tbody>
</table>
a long period of time. The specific xylose consumption rates obtained in hydrolysates were also clearly lower than those on synthetic media.

4.1 Effect of aldehyde inhibitors on xylose fermentation

The toxicity of lignocellulosic hydrolysates has been correlated with the presence and concentration of various aldehydes, such as furfural [59–61]. Among 60 identified phenolic compounds in lignocellulosic hydrolysates, the phenylaldehydes were found to be more toxic than the phenolic acids and alcohols [62, 63] underlining the importance of the aldehydes as inhibitors.

Aldehydes can interact with cellular structures, generate reactive oxygen species and inhibit enzymes in the central carbon metabolism [64–67]. This results in reduction of fermentation rate, cessation of growth, extension of lag-phase of yeast, and reduction of ethanol productivity and/or yield [12, 63]. Understanding and improving the mechanisms by which microorganisms respond to aldehydes is clearly important for the development of strains with increased tolerance towards lignocellulosic hydrolysates [12, 68].

4.1.1 Metabolically engineered microorganisms for aldehyde reduction

A fundamental observation is that as long as certain aldehydes – e.g. furfural, HMF, vanillin, veratraldehyde – are present in the medium, yeasts are not able to grow and show reduced metabolic activity [61, 69–71]. However, at permissive levels, the yeast can usually slowly convert the aldehydes to less toxic compounds and growth and metabolic activity are restored [61, 72–74].

S. cerevisiae, for instance, can reduce furfural and HMF to their corresponding alcohols; 2-furan methanol (FM or furfuryl alcohol) and furan 2,5-dimethanol (FDM or HMF alcohol). A few alcohol dehydrogenases, i.e. Adh6, Adh7 and a mutated Adh1 enzyme, as well as the \textit{P. stipitis} XR (Ps-XR) and the \textit{YKL071w} gene product have been identified as catalyzing furaldehyde reduction, and their positive effect upon overexpression of the corresponding gene has been confirmed [68, 75]. Overexpression of the transcription factor encoding gene \textit{YAP1}, known to be involved in oxidative stress response, was also shown to give an increased tolerance to hydrolysate and specific model inhibitors – and an increased reduction capacity of furaldehydes [76].
More recently an isolate of the gram positive bacteria Cupriavidus basilensis (HMF14) could aerobically completely metabolize HMF and furfural [77], which may open new possibilities for the microbial detoxification of hydrolysates.

4.1.2 Interactions between xylose and furaldehyde pathways

*S. cerevisiae* keeps its redox balance tightly regulated, essentially by regulating the activity of glycolytic and fermentative enzymes. The anaerobic glucose catabolism (when not considering draining the pathway of anabolic precursors), is balanced in the glycolysis and fermentation pathways with respect to generation/consumption of the co-factor pairs NADH/NAD⁺ (and NADPH/NADP⁺). However, during the fermentation of lignocellulosic hydrolysates there are additional compounds which will be reduced and/or oxidized: (i) the conversion of xylose by the XR/XDH pathway from *P. stipitis* is based on the use of NADPH and NAD⁺ (Fig. 2); (ii) the detoxification of aldehydes is based on reduction reactions using NADH and NADPH. Consequently, both xylose metabolism and detoxification reactions will affect the redox (or more correctly the co-factor) balance of the cell.

Ethanolic fermentation with *S. cerevisiae* strains overexpressing the XR/XDH pathway in defined mineral medium containing xylose often gives substantial yields of xylitol as a result of the co-factor imbalance between the NADPH-preferring XR and the NAD⁺-dependent XDH (cf. Fig. 2). However, fermentation of xylose in lignocellulosic hydrolysates by engineered *S. cerevisiae* strains showed considerably lower xylitol yields than during the fermentation of xylose in defined mineral medium [78, 79]. Most likely this is due to aldehydes and other compounds in the lignocellulosic hydrolysate which act as redox sinks [80, 81]. For instance, an NADH-dependent reduction of furfural regenerates NAD⁺ necessary for xylitol oxidation by XDH. Thereby glycerol and/or xylitol yields may decrease [81].

The simultaneous conversion of aldehyde compounds and xylose was investigated in an XR/XDH strain overexpressing either the NADH-dependent ADH1-S110P-Y295C encoding gene or the NADPH-dependent Adh6 encoding gene [82]. NADPH usage during HMF reduction slightly reduced xylitol formation, whereas the usage of NADH in HMF reduction considerably decreased both xylitol and glycerol production and increased biomass yield [82]. A metabolic flux analysis indicated that the reduction of HMF replaced the regeneration of NAD⁺ in the glycerol pathway and provided NAD⁺ for the oxidation of xylitol so that more carbon became available for biomass production.

4.2 Pentose fermentation in the presence of weak acids

Ethanol fermentation from lignocellulosic feedstocks is also challenged by the presence of weak acids in the medium, at concentrations that can be inhibitory to *S. cerevisiae* metabolism. Acetic acid originates from the deacetylation of the hemicellulose fraction, whereas formic and levulinic acids are formed during the breakdown of HMF and furfural [83]. The innate levels of acetic acid are highly dependent on the raw material. Reported acetyl contents in some pentose rich materials are in the range 1.7–4.2 g/100 g DM (Table 1). The concentrations of weak acids obtained in the fermentation broth will vary even more widely depending on both the method of pretreatment as well as the solids loading used in the pretreatment. It can be estimated that the concentration of acetic acid may easily be above 10 g/L after a typical steam pretreatment of poplar [84].

Weak acids are also produced during the fermentation stage, since acetic acid is a minor by-product of yeast fermentation. However, acetic and lactic acids are mainly generated from the contamination by lactic acid bacteria (LAB) and other bacteria (see also Section 4.3). For example, acetic and lactic acid levels above 12 and 16 g/L, respectively, have been reported in a pilot-scale study using corn fibre as substrate (LAB) [16, 85].

4.2.1 Effect of weak acids on yeast metabolism

Both acetic and lactic acid are known to increase the lag phase and reduce the specific growth rate of *S. cerevisiae*, thereby affecting ethanol productivity (see e.g. [86–89]). Acetic acid is more inhibitory than lactic acid, with a reported minimal inhibitory concentration for growth of 100 mM (6 g/L), as compared to 278 mM (25 g/L) for lactic acid in non-buffered defined medium [86].

The effects of weak acids are strongly pH dependent. At pH value below the pKₐ-value of the acid, the undissociated form of weak acids predominates. It is this form which enters the cell and once inside it dissociates (due to the higher intracellular pH), thereby releasing protons. In order to maintain a proper intracellular pH, protons must be transported out of the cells with the help of the plasma membrane ATPase and at the expense of ATP (Fig. 2). Whereas low levels of acids activate the glycolytic rate by stimulating ATP production [89], higher levels become inhibitory due to the acidification of the cytosol after depletion of the
available ATP. Also, as less ATP becomes available for biomass formation, ethanol productivity decreases. In addition, high levels of weak acids may result in the intracellular accumulation of anions (Fig. 2) [90], which has been shown to be inhibitory to several glycolytic enzymes [91]. ATP is also needed for the active efflux of the accumulating dissociated acids, which has been suggested to occur via Pdr12p [92].

4.2.2 Xylose fermentation in the presence of weak acids

The effect of acetic acid on xylose fermentation has been investigated in strains carrying either the XR-XDH or the XI pathway [84, 93–96]. A high level of acetic acid with a low pH caused dramatic reduction of xylose consumption rate for an XR-XDH strain, whereas a change in pH alone showed little effects on the xylose consumption [84]. Growth and ethanol production from xylose were also impaired in a recombinant XI-expressing strain at low pH (3.5) and in the presence of 3 g/L acetic acid [93], whereas the glucose consumption rate was only slightly affected under similar conditions.

The lower rate of xylose consumption, typically three to sixfold lower than for glucose in engineered S. cerevisiae [93, 94] affects the maximum specific ATP production rate, which explains why weak acids have a more dramatic effect on yeast growth on xylose than on glucose [93–95]. Indeed xylose fermentation could be restored in a recombinant XI-strain at pH 3.5 and in the presence of 3 g/L acetic acid by applying a continuous low feeding of glucose to the medium [93].

Acetic acid decreases ethanol productivity but its effect on ethanol yield is less clear. In some reports, the ethanol yield was shown to increase as the metabolism was redirected from anabolic (biomass) to dissimilatory (ethanol) pathway in the presence of acetic acid [93, 94]. In contrast, a lower ethanol yield from glucose and xylose was also reported in the presence of a more complex medium such as spent sulphite liquor (SSL) [96]. However, it is unclear whether the reported yield relates to the consumed or the initial sugar levels, since the tested XR and XDH-engineered strains displayed low xylitol and unchanged glycerol yields in acetic acid rich-hydrolysate media [84, 96].

4.2.3 Engineering approaches to overcome the weak acid inhibition

One straightforward strategy to overcome or limit the inhibition by acids is to run fermentations at a pH value sufficiently high to keep the undissociated acids at low levels. In practice however, it may be difficult to implement this strategy as fermentation of lignocellulosic hydrolysates are preferably run at a low pH to limit bacterial contaminations (Section 4.3).

Recently, S. cerevisiae was metabolically engineered allowing acetate to replace dihydroxyacetone phosphate (DHAP) as a redox sink during anaerobic growth in a glycerol mutant strain. This was achieved by introducing a bacterial NAD+-dependent acetylating acetaldehyde dehydrogenase, by which acetyl-CoA may be reduced to acetaldehyde (and CoA). The engineered strain was able to grow anaerobically and acetate was reduced to ethanol in the process [97]. Such approach may pave the way to other strategies enabling the conversion of the inhibitory acetate during fermentation.

4.3 The contamination issue

Large scale plants for the production of ethanol from lignocellulosic biomass are not yet available. However, one can anticipate that microbial competition will become an important problem, due to the presence of a large amount of pentose sugars that are still not, or only poorly, utilized by S. cerevisiae.

4.3.1 Microbial contaminants in lignocellulosic hydrolysates

Systematic and acute bacterial infections are recurrently observed in large scale facilities during the ethanol production from hexose sugars, with sugar cane in Brazil [98] and corn in United States of America [99]. However, little is known on the contaminant microbiota in lignocellulosic ethanol pilot plants. The most studied cases of contamination are in SSL, which is the waste stream of cellulose pulp that has been obtained by treating the wood mechanically and chemically. The SSL has a high sugar content which can be used as feedstock for various microorganisms.

Both yeast and bacterial contaminants have been isolated and identified in SSL plants, including the xylose-utilising yeast Pichia membranaefaciens, the acetic acid bacteria Acetobacter tropicalis and A. syzygii, the homofermentative bacteria Lactobacillus plantarum and L. pentosus as well as the heterofermentative L. buchneri [100] (C. Larsson and E. Albers, personal communication; V. Sánchez et al., unpublished). Lactobacillus contaminants have also been identified in a pilot-plant where corn fibres were used to produce ethanol [16]. During episodes of contamination in sugar cane ethanol plants, non-S. cerevisiae strains were also found as main contaminants [101].
4.3.2 Effects of microbial contamination on yeast fermentation

LAB compete with yeast for sugar utilization. Homofermentative bacteria are able to consume hexose and pentose sugars to produce lactic acid, whereas heterofermentative bacteria produce acetic acid in addition to lactic acid [102]. Finally, acetic acid bacteria produce acetic acid from ethanol [103]. An immediate consequence of the contamination is that less sugar and essential growth factors become available for \(S.\ cerevisiae\) and the ethanol yield decreases (see e.g. [104]). In addition, the production of considerable amount of acids decreases the pH which inhibits yeast fermentation by reducing biomass formation and ethanol yield.

4.3.3 Strategies to control a contamination

In the sugar cane ethanol industry, acid wash treatment is used as a common strategy for reducing acute episodes of contamination [105]. Pulses of sulphuric acid are made, leading to a temporary pH drop on the system. The bacterial population is then reduced due to its high sensitivity for low pH [106].

Various pasteurization procedures and the addition of biocides, such as penicillin, virginiamycin and nisin, have also been tested in a series of batch and continuous fermentation runs in a corn fibre based pilot plant. All these methods reduce temporarily the level of contamination but none has a permanent effect [16]. Also, the usage of biocides to control contamination is not economically feasible and not advisable from an environmental point of view.

Recent studies demonstrated that the addition of NaCl and ethanol on softwood hydrolysate could inhibit the growth of LAB whereas baker’s yeast was still able to grow (C. Larsson and E. Albers, personal communication).

The use of hops extract to control bacterial contamination in lignocellulosic fermentation plants is also discussed. Hop that gives a bitter taste and acts as antiseptic agent, is well-known in beer production. Hop cones have a high content of \(\alpha\)-acids, for which Gram-positive bacteria are sensitive to [106, 107].

In the near future, the development of \(S.\ cerevisiae\) strains with xylose consumption rates that are sufficiently high to compete with LAB for the utilization of all sugars present in the lignocellulosic hydrolysates, may provide another solution to the bacterial contamination issue.

5 Concluding remarks

Both targeted and non-targeted approaches have been applied to generate efficient pentose-fermenting \(S.\ cerevisiae\) strains. The strength of the non-targeted approach is that it requires less a priori knowledge of the pathway and is generally straightforward and efficient. The targeted approach on the other hand may require tedious identification of specific traits that enhance metabolism, however once identified these traits can be directly transferred between strains. Both approaches have been successful in the generation of \(S.\ cerevisiae\) strain that can grow anaerobically on xylose, a trait which is shown to be linearly correlated to ethanol productivity (Fig. 3B). Still the xylose consumption rate remains three to sixfolds lower than the glucose consumption rate. Generating tolerant \(S.\ cerevisiae\) with even higher xylose consumption rate would of course benefit ethanol productivity, but it would also enable the cells to better cope with the ATP-demanding acid stress and outcompete any pentose-utilizing LAB-contaminant in the process.

A central issue in converting pentoses from hydrolysates is also the presence of aldehydes – not least the furaldehydes. Recent identification of enzymes with aldehyde reduction activity has enabled targeted approaches for improving the in situ reduction capacity of \(S.\ cerevisiae\) strains. Similarly genetic engineering approaches are emerging to cope with the problem of weak acid inhibition. In both cases, however, it is important to understand how these modifications impact pentose metabolism.

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