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Research review paper

Data mining of *Saccharomyces cerevisiae* mutants engineered for increased tolerance towards inhibitors in lignocellulosic hydrolysates

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ABSTRACT

The use of renewable plant biomass, lignocellulose, to produce biofuels and biochemicals using microbial cell factories plays a fundamental role in the future bioeconomy. The development of cell factories capable of efficiently fermenting complex biomass streams will improve the cost-effectiveness of microbial conversion processes. At present, inhibitory compounds found in hydrolysates of lignocellulosic biomass substantially influence the performance of a cell factory and the economic feasibility of lignocellulosic biofuels and chemicals.

Here, we present and statistically analyze data on *Saccharomyces cerevisiae* mutants engineered for altered tolerance towards the most common inhibitors found in lignocellulosic hydrolysates: acetic acid, formic acid, furans, and phenolic compounds. We collected data from 7971 experiments including single overexpression or deletion of 3955 unique genes. The mutants included in the analysis had been shown to display increased or decreased tolerance to individual inhibitors or combinations of inhibitors found in lignocellulosic hydrolysates. Moreover, the data included mutants grown on synthetic hydrolysates, in which inhibitors were added at concentrations that mimicked those of lignocellulosic hydrolysates. Genetic engineering aimed at improving inhibitor or hydrolysate tolerance was shown to alter the specific growth rate or length of the lag phase, cell viability, and vitality, block fermentation, and decrease product yield. Different aspects of strain engineering aimed at improving hydrolysate tolerance, such as choice of strain and experimental set-up are discussed and put in relation to their biological relevance. While successful genetic engineering is often strain and condition dependent, we highlight the conserved role of regulators, transporters, and detoxifying enzymes in inhibitor tolerance. The compiled meta-analysis can guide future engineering attempts and aid the development of more efficient cell factories for the conversion of lignocellulosic biomass.

1. Introduction

Society faces large challenges on the road to a fossil-free future. One important technological milestone towards this goal is the production of biofuels and biochemicals from renewable raw materials via microbial fermentation. Plant biomass such as lignocellulose is relatively cheap and abundant but requires cell factories capable of efficiently fermenting streams containing mixed sugars and various inhibitory compounds. In the case of yeast-based processing, the lignocellulosic biomass needs to be pretreated and enzymatically hydrolyzed. Pretreatment increases accessibility of the substrate to microorganisms and solubilizes part of the hemicellulosic sugars. However, pretreatment causes also the release of inhibitory compounds that can be grouped into furans, weak acids, and phenolics. In the present review, we applied a data mining approach to comprehensively analyze existing efforts for improved conversion of lignocellulosic biomass by the yeast *Saccharomyces cerevisiae*. This species is central for future biorefinery development as it is already widely used for the production of bioethanol and several other bio-based compounds (Cunha et al., 2020; Baptista et al., 2021).

Lignocellulosic biomass can be roughly divided into hardwood,

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softwood, and annual plants. The major building blocks of lignocellulosic biomass are cellulose, hemicelluloses, and lignin (Table 1), whose relative content depends on the source of the material. The level of inhibitors in the streams after pretreatment and enzymatic hydrolysis of cellulose (and the remaining hemicellulose in polymeric or oligomeric form) varies substantially depending on the consistency and source of raw material and pretreatment methodology. Typically, inhibitors found in lignocellulosic hydrolysates range from 1 to 10 g/l for weak acids and furans; whereas phenolics are found at mg/l levels. Acetic and formic acid are the most abundant weak acids; while levulinic acid is found in small amounts only. The response to different weak acids has been determined to be similar (Guo and Olsson, 2016; Guo et al., 2018). As reviewed by Koppram et al. (2014) and Cunha et al. (2019), inhibitory compounds influence substantially the performance of cell factories, strongly limiting the economic feasibility of lignocellulosic biofuels and bio-based products.

The effects of inhibitory compounds on fermentation performance range from decreased specific growth rate, cell viability, vitality, and product yield to increased lag phase and impaired fermentation. The inhibitory effect depends on the presence of individual inhibitors and is compounded by environmental factors, including pH, temperature, and nutrient availability (Xiros and Olsson, 2014), as well as growth phase and previous growth conditions (Narayanan et al., 2017). At a cellular level, lignocellulosic inhibitors trigger various stress responses that influence cell growth (Fig. 1) (Cunha et al., 2019). Weak acids diffuse through the cell membrane in an undissociated form, but dissociate into ions and protons inside the cell, causing intracellular acidification. This promotes ATP consumption as cells strive to regain and maintain pH homeostasis and activates signaling pathways that promote metabolic changes and apoptosis (as reviewed by Guaragnella and Bettiga, 2021).

Cellular detoxification of furans and phenolic compounds involves redox mechanisms and leads to intracellular redox imbalance, whose downstream effect is decreased cellular performance (Ask et al., 2013). Detoxification or efflux of inhibitory compounds by active transport may pose an additional energetic burden on cells, while their concomitant intracellular accumulation triggers the formation of reactive oxygen species (ROS) (Guaragnella and Bettiga, 2021). Typical responses to oxidative stress in yeast include the production of ROS scavengers and the activation of pro-survival pathways, including mitophagy and programmed cell death. Lignocellulosic hydrolysates can trigger osmotic stress, while product inhibition at the end of the production process (e.g., ethanol) can exert a synergistic stressful effect (Wang et al., 2013). A negative additive effect originates also from the combination of several inhibitors (Jönsson et al., 2013), which explains why lignocellulosic hydrolysates present more challenges to fermentation than would be expected from individual inhibitors.

Given the large interest in fermentation of lignocellulosic hydrolysates, many studies have sought to improve the fermentative performance of microbial cell factories and understand the impact of lignocellulose streams. Given the extent of condition-specific information, it is difficult to guide strain improvement. Therefore, the overall aim of this review was to compile and create a comprehensive meta-analysis of published experimental studies reporting genetic alterations (gene deletion or overexpression) affecting tolerance of *S. cerevisiae* towards individual inhibitors or combinations of inhibitors found in lignocellulosic hydrolysates. By collecting and statistically analyzing available data, we were able to draw a general picture, frame it in relation to the biological relevance of the findings, and address the complex issue of how to engineer yeast to improve growth in lignocellulosic hydrolysates. The compiled database is available from the GitHub repository and can be used by the interested reader to design their own strains or perform their own data analysis.

Table 1
Simple overview of the composition of lignocellulosic materials and inhibitors produced and/or released during pretreatment.

	Cellulose	Hemicellulose	Lignin
Structural features	Linear chains of glucose that form fibers of several cellulose chains	Branched polymers	Large network of aromatic monomers
Building blocks	Glucose	Hexoses, pentoses, uronic acids and decorated sugars	*p*-coumaryl, conferyl and sinapyl alcohols
Inhibitors	Hydroxymethyl-furfural, levulinic acid and formic acid	Hydroxymethyl-furfural, furfural, levulinic acid, formic acid and acetic acid	Low molecular phenolic compounds, alcohols, acids and aldehydes

2. Systematic collection of data

2.1. Data compilation

To gain in-depth understanding of metabolic traits beneficial for the conversion of lignocellulosic biomass, we compiled a database, which lists phenotypic changes achieved through overexpression or deletion of genes in *S. cerevisiae*. The resulting database, scripts used for data mining, as well as additional analyses, a table describing the composition of the hydrolysates mentioned in the study, and supplementary figures are available at the GitHub repository https://github.com/JanZrimec/Hydrolysate_inhibitor_review.

Mutants included in our database shared the following features:

1. They were derived from *S. cerevisiae* strains of laboratory or industrial origin as well as from wild isolates.
2. They all had a single gene either overexpressed or deleted and displayed a significant change in at least one growth parameter in comparison to the non-modified parental strain in the presence of lignocellulosic inhibitors or hydrolysates. The growth parameters considered were specific growth rate, length of lag phase, final biomass yield, cell viability, and vitality.
3. They displayed increased or decreased tolerance towards lignocellulosic hydrolysate, acetic acid, furfural, hydroxymethylfurfural (HMF), formic acid or phenolic compounds (coniferyl aldehyde, vanillin, cinnamic acid, ferulic acid, 4-hydroxybenzoic acid, phenol or syringaldehyde).

A total of 7971 experiments including 3955 unique mutants, were assessed under various growth conditions (Table 2: e.g. on different liquid or solid media containing varying amounts of inhibitory compounds). The set-ups ranged from small-scale experiments to high-throughput screenings and controlled bioreactor cultivations. To transform such highly heterogeneous measurements into a common format suitable for comparison, we reduced the reported phenotypes to a binary read-out: + for increased tolerance and – for increased sensitivity to an inhibitor or hydrolysate. The analysis excluded conditional regulation or quantitative parameters such as the level of gene upregulation. Mutants whose gene expression was downregulated or mutants where the expression was conditional were not included in the database. Recent large-scale screenings of such mutants are discussed in Section 5.2.

A total of 34 parental strains were employed in the different studies: 20 laboratory strains, 11 industrial strains, and 3 wild-type isolates (Fig. 2). Tolerant and sensitive mutants were most often created via conventional genetic engineering rather than CRISPR/Cas9-based methods, and they frequently relied on antibiotic or auxotrophic selection markers. Some mutants carried plasmids for gene overexpression. Several different lignocellulosic materials were employed: corn stover, *Eucalyptus globulus* wood, hardwood, Miscanthus (silvergrass), rice straw, spruce, sugarcane bagasse and molasses, switchgrass, and wheat straw hydrolysate. Studies with synthetic hydrolysates comprising mixtures of inhibitors were also included. As a result, the database consisted of a large number of sensitive and tolerant mutants identified

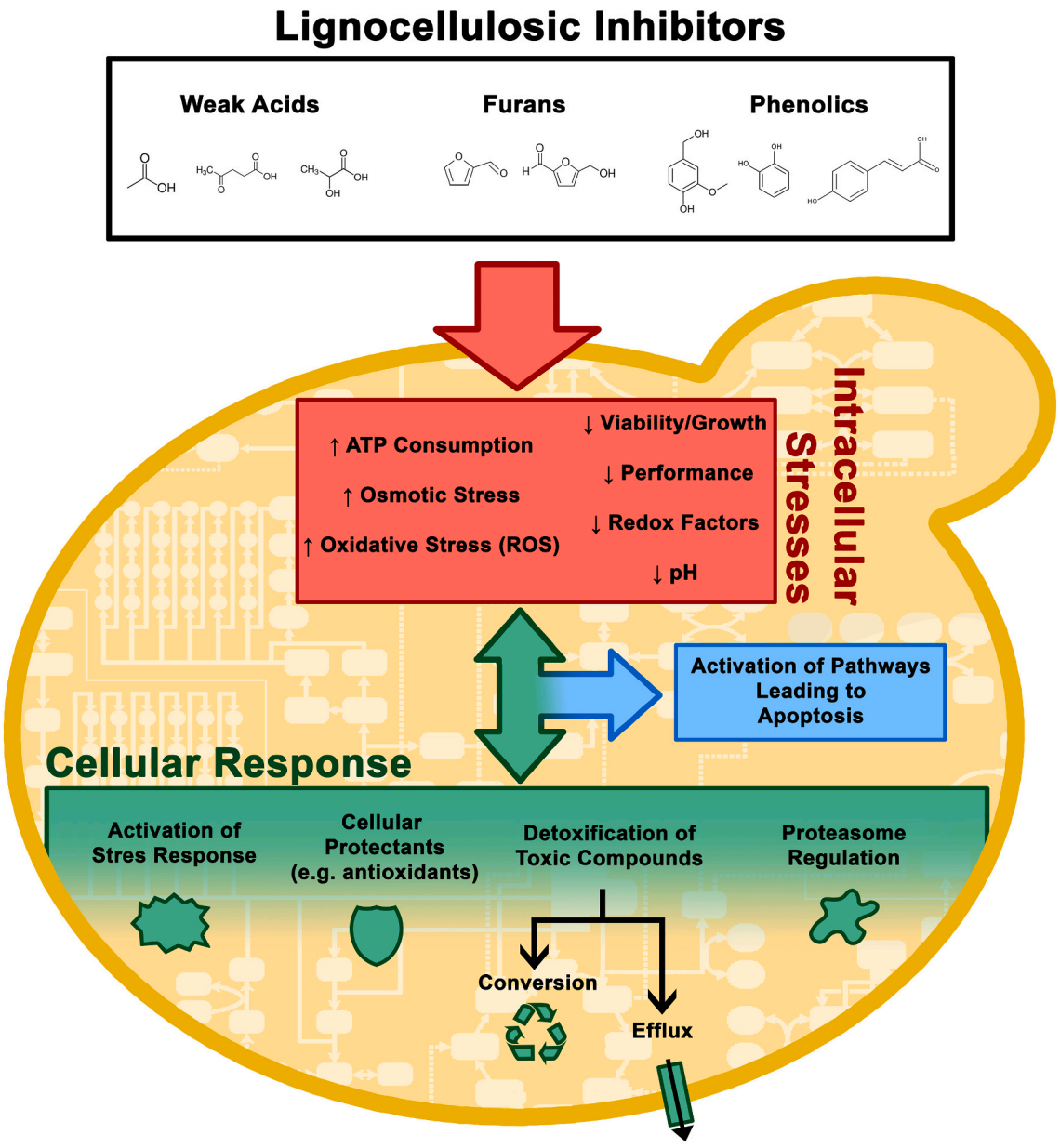

Fig. 1. Overview of stresses that yeasts are subjected to when grown in lignocellulosic hydrolysates and the cellular stress responses.

Table 2
Database overview.

Inhibiting compound(s)	Concentration range	Number of experiments	Number of studies
Acetic acid	33–400 mM	3615	39
Formic acid	30–175 mM	219	3
Furfural	5–60 mM	59	18
HMF	12–60 mM	38	9
Phenolics	1–35 mM	209	13
Diverse, as components of hydrolysates	variable	3831	21
Total:		7971	103

in a diverse set of strains and experimental conditions.

2.2. Overview and presentation of the collected data

Most studies included in the database involve mutants exposed to single inhibitors or hydrolysates (Fig. 3a, non-connected dots). The majority of mutants come from studies on acetate (3067 unique genes, Fig. 3a) or hydrolysate (2613 unique genes) tolerance, arising from genome-wide studies of deletion (Kawahata et al., 2006; Mira et al., 2010; Pereira et al., 2014; Serate et al., 2015; Skerker et al., 2013; Sousa et al., 2013) and overexpression libraries (Sardi et al., 2016). UpSet plots (Lex et al., 2014; Conway et al., 2017) were used to visualize the intersection of different datasets. UpSet plots are used for quantitative analysis of information from many different datasets and facilitate the understanding of relationships among data. In this study, datasets pertaining to mutants analyzed for a particular inhibitor or hydrolysates are shown as horizontal bars. The UpSet plots display the intersection of the defined sets as a matrix, whereby vertical bars describe the size of an intersection represented by connected dots.

Overall, most deletion mutants displayed increased sensitivity to the tested inhibitor, with the exception of acetate (Fig. 3b, c). The increased tolerance of mutants towards acetate is documented by Sousa et al. (2013), who reported 2159 mutants from the deletion collection as more resistant to acetic acid-induced programmed cell death than the wild-type strain based on propidium iodide staining. Sardi et al. (2016)

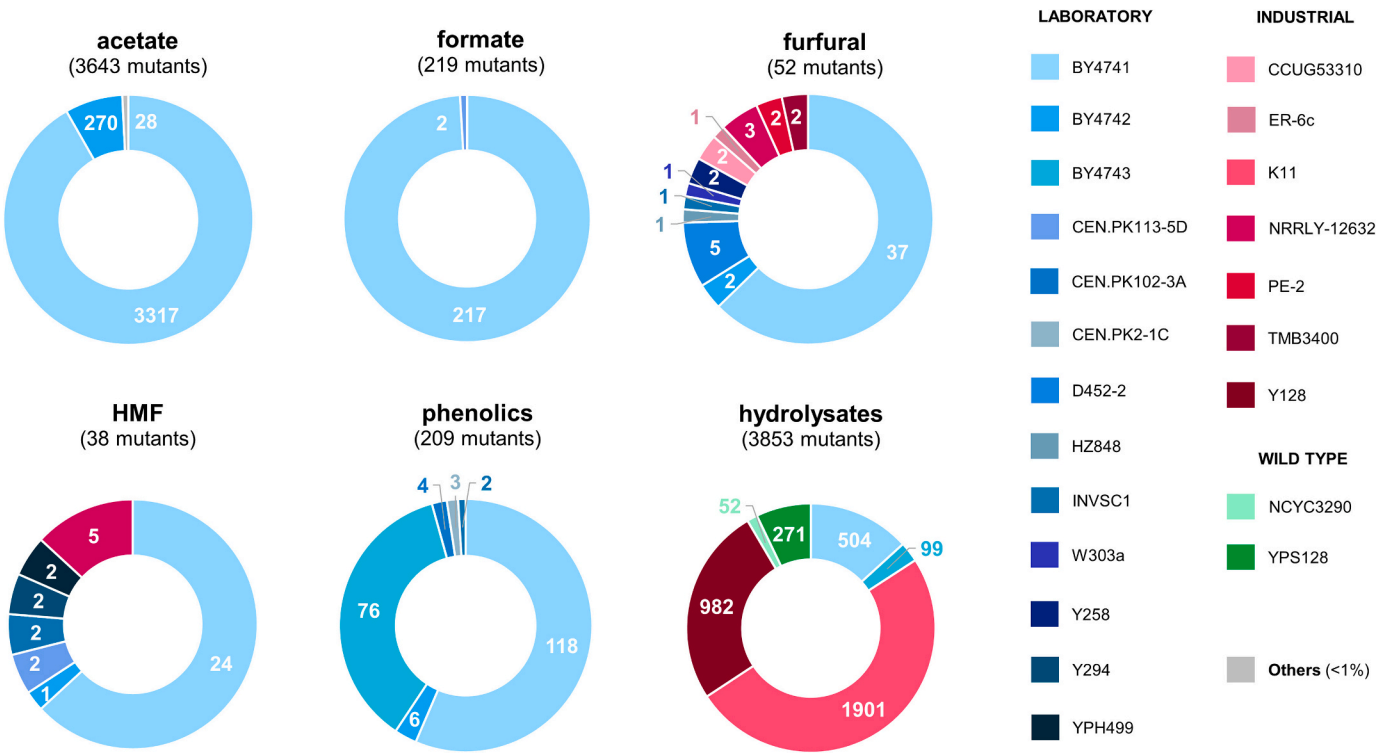

Fig. 2. Doughnut representation of the number of mutants comprising each inhibitor dataset color-coded by parental strain. Classification of the strains according to the inhibitor in which they have been tested. Laboratory (blue), industrial (red) and wild type (green) strains are represented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

screened a library of overexpression mutants for growth in synthetic hydrolysate and identified 1974 genes that, when overexpressed, resulted in a more sensitive phenotype (Fig. 3d), 124 that led to improved growth, and 29 that did so in the presence of specific inhibitors (Fig. 3e).

3. Data-driven profiling of inhibitor-related genes

The challenging composition of lignocellulosic hydrolysates requires the use of strains showing robustness towards different inhibitors. Strains sharing the same genetic modification, but a different background tend to perform differently depending on the hydrolysate employed (Costa et al., 2017). Screening strains directly in hydrolysates is often challenging due to the viscosity and color of the medium, which hinders high-throughput assays.

The generated database does not include any records of genes tested for tolerance towards all inhibitors when added individually to the growth medium. However, the database contains a small set of mutants, which were assessed for tolerance towards all the inhibitors except formic acid. Various deletion and overexpression mutants, tolerant or sensitive to a specific inhibitor, were found to display an opposite behavior when grown in the presence of a different inhibitor or hydrolysate (Fig. 4a). Therefore, analysis was carried out through pairwise comparisons and detailed assessment of genes tested against several inhibitors.

3.1. Pairwise comparison of inhibitors and mutants identifies genes responsible for increased sensitivity to two or more inhibitors

To spot the most relevant genetic alterations and identify similar trends across different inhibitors, a pairwise comparison between inhibitors was performed (Fig. 4b). As most of the mutants included in our database were tested in medium supplemented with acetic acid or in hydrolysates (Fig. 3a), the main subset of mutants corresponded to acetate-hydrolysates screenings (Fig. 4b). However, when grouping the genes of the tested mutants according to the resulting phenotype (Fig. 4a), only half of the correlations were statistically significant (hypergeometric test, p-value <0.05), meaning that genes included in those groups were overrepresented.

Most mutants displaying altered growth in the presence of two or more inhibitors were identified as sensitive (Fig. 3 and Fig. 4a). Mutants tolerant or sensitive towards furfural and HMF, HMF and hydrolysates, or phenolics and hydrolysates shared more than 90% of the deleted or overexpressed genes (Fig. 4a). These comparisons suggest that, when aiming for increased tolerance towards hydrolysates through screening in synthetic media, it may be better to assess growth in the presence of HMF or phenolics rather than acetate, as in the latter only 52% of genes identified in sensitive or tolerant mutants were common to acetate and hydrolysate studies (Fig. 4a).

3.2. Multiple GO terms are enriched for genes altered in sensitive mutants, but less so in tolerant mutants

To identify common gene ontologies (GOs), we performed GO enrichment analysis of sensitive or tolerant mutants that were tested for at least two inhibitors or an inhibitor and a hydrolysate. Notably, based on pairwise comparisons, only a few genes related to biological processes were assigned to tolerant phenotypes. The GO networks of biological processes comprising genes of mutants identified as sensitive or tolerant to at least two inhibitors or an inhibitor and a hydrolysate are deposited in the public repository NDEx (https://ndexbio.org/#/networkset/64ce2763-f6f3-11eb-b666-0ac135e8bacf). Additional figures illustrating GO analysis are available from the GitHub repository.

A comparison of mutants tolerant towards formate and phenolics, revealed three common annotated genes (*ERG3*, *4*, *5*) associated with ergosterol biosynthetic processes (GO:0006696). These and other ergosterol-related genes were also found in pairwise comparisons of acetate – formate, acetate – phenolics, and phenolics – hydrolysates.

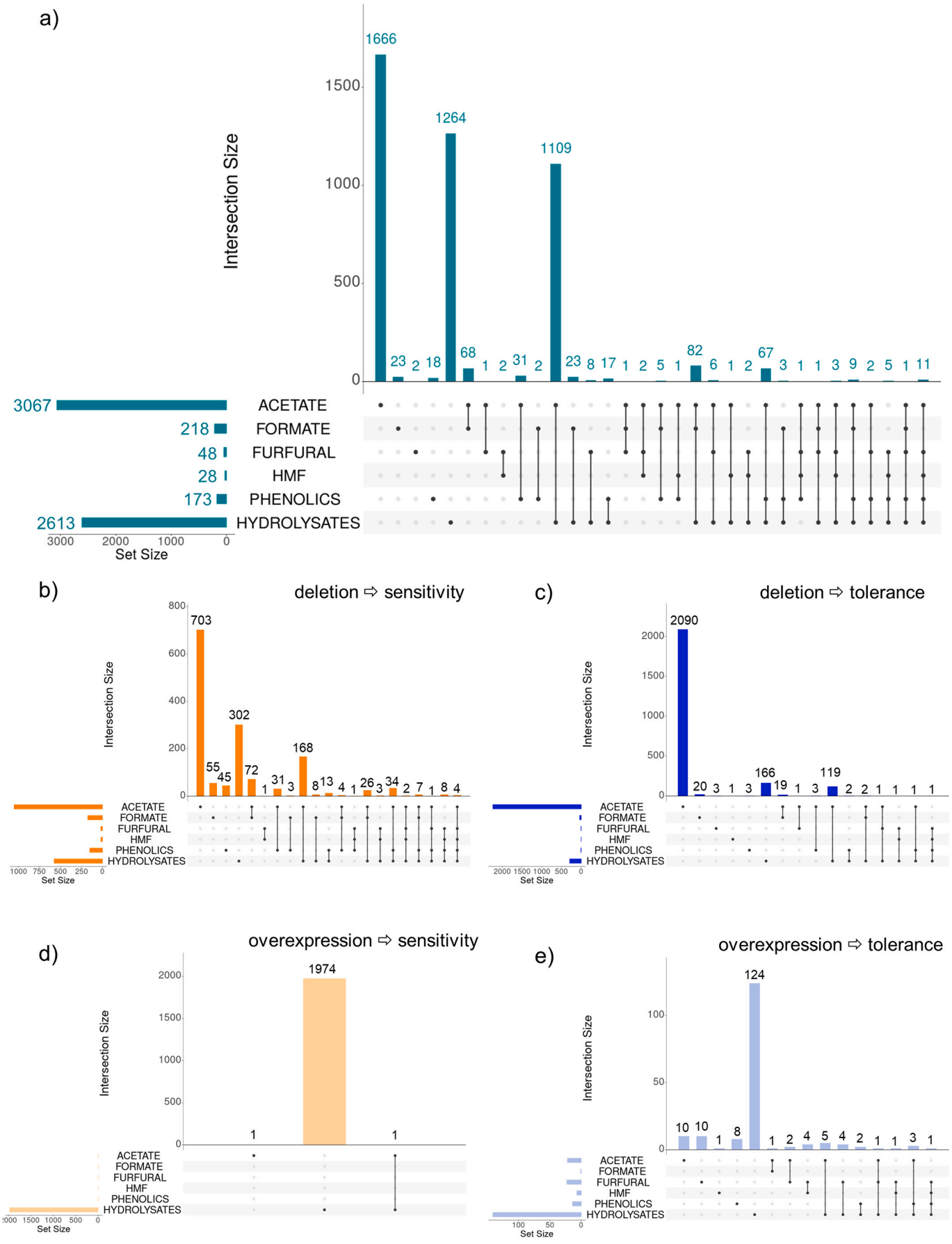

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Fig. 3. UpSet plots representing the number of mutants with altered expression (deletion or overexpression) of a specific gene, characterized in medium supplemented with an inhibitor or hydrolysate. a) Global profiling of the number of genes identified to result in a phenotypic change of a mutant (sensitivity or tolerance towards the inhibitor), in each condition and the intersection among different sets. b-c) Number of deleted genes that resulted in more sensitive (b) or tolerant (c) mutants. d-e) Number of overexpressed genes that resulted in more tolerant (d) or sensitive (e) mutants. In horizontal bars (set size), total number of genes tested for each inhibitor. Vertical bars summarize the number of genes for each unique or overlapping combination. Connected black dots indicate common genes tested in different inhibitors.

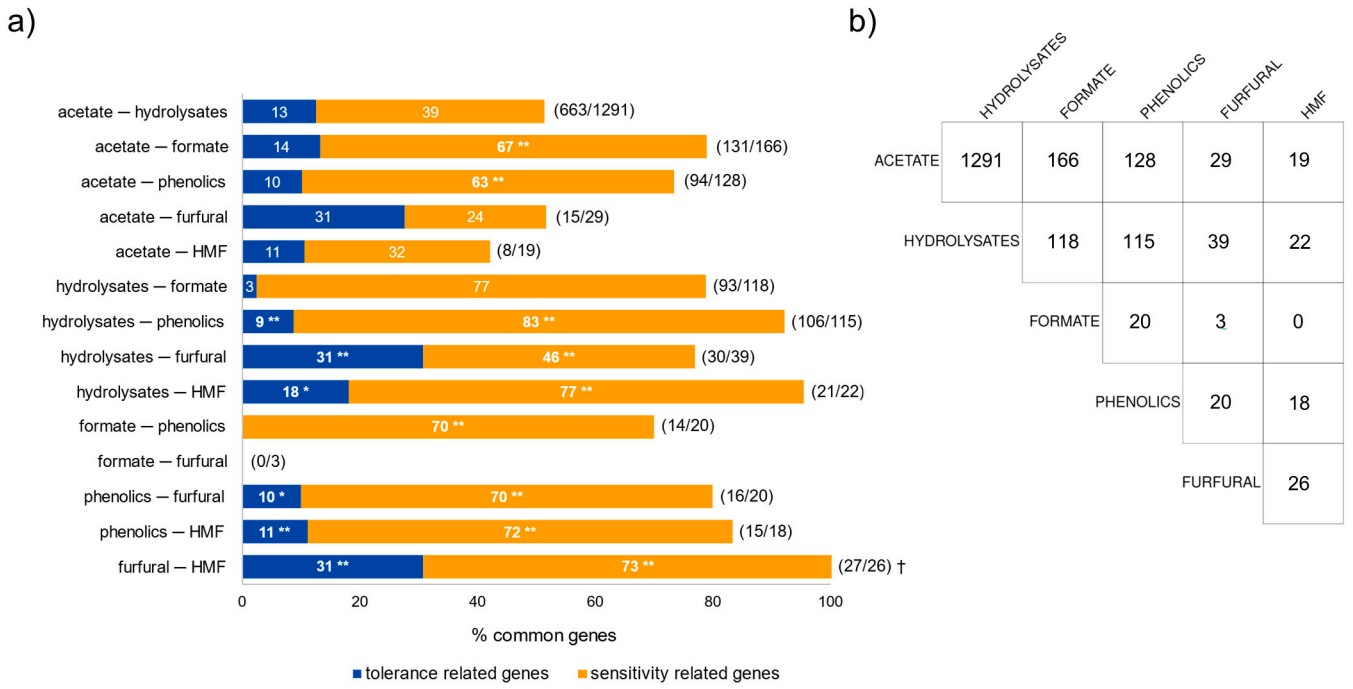

Fig. 4. Overview of the number of genes in mutants displaying a similar phenotype in the presence of different inhibitors or hydrolysates. a) Number of common genes resulting in a similar phenotype (tolerance or sensitivity) in different pairs of inhibitors. The percentage of common genes for each phenotype is indicated inside the bars whereas the number of shared genes causing the same phenotype out of the total number of shared genes is noted in the parentheses. Statistical significance represented as "*" for $p < 0.05$ and "**" for $p < 0.01$ (Hypergeometric test). † Deletion or overexpression of *YAP1* was in different studies reported to cause decreased and increased tolerance, respectively, both in the presence of furfural vs. HMF, therefore *YAP1* was calculated twice in this analysis. Pairwise comparisons of HMF-formate, and furfural-formate did not show any overlap among the mutants listed. b) Correlation map representing the fraction of overlapping genes of the mutants tested in different inhibitors.

Endo et al. (2008) reported that genes for ergosterol biosynthesis were required for tolerance to furans, weak acids, and phenolic compounds, suggesting that ergosterol was a key determinant of tolerance towards various lignocellulosic inhibitors. The comparison phenolics – hydrolysate revealed enrichment of the GO term IMP biosynthetic processes (GO:0006188), highlighting the role of *ADE1*, *ADE13*, and *ADE17*, which encode proteins involved in purine synthesis. Overexpression of these three genes resulted in a significantly higher γ-aminobutyric acid titer and improved performance of yeast in media supplemented with acetic acid, phenolics or hydrolysates (Zhang et al., 2019).

After merging redundant groups, 69% of the genes common to acetate and formate sensitive mutants were found to be enriched in the categories ATP transport (GO:0015867), autophagy (GO:0006914), and negative regulation of transcription (GO:0045892). When analyzing mutants sensitive to both acetate and phenolics, 58 of 77 common genes were assigned to 36 enriched GO terms, among them vesicle-mediated transport (GO:0016192), vacuolar transport (GO:0007034), Golgi vesicle transport (GO:0048193), and macroautophagy (GO:0016236). The GO term vesicle-mediated transport (GO:0016192) was enriched also in 15% of genes belonging to mutants sensitive to acetate and formate. Genes participating in autophagy and transcription regulatory pathways have previously been reported as differentially expressed in *S. cerevisiae* treated with acetic acid, presumably as a strategy to promote cell death processes (Dong et al., 2017). In line with this observation, earlier screenings of the deletion collection (Pereira et al., 2014; Sousa et al., 2013) and a more recent study using CRISPRi strains (Mukherjee et al., 2021) have reported that deletion or suppression of genes encoding vacuole-related proteins could increase the sensitivity to acetic acid.

In pairwise comparisons between furfural and another inhibitor, most GO enriched terms were common among genes engineered in mutants sensitive to furfural – HMF, furfural – hydrolysates, and furfural – phenolics. In these datasets, more than 75% of genes were related to transcription regulation processes, with the GO term DNA-binding transcription factor activity (GO:0003700) representing the largest group. GO enrichment analysis of genes of mutants sensitive to HMF – hydrolysates, HMF – phenolics or phenolics-hydrolysates showed clear enrichment of transcription-related terms. We discuss successful engineering of transcription factors for increased inhibitor tolerance in Section 4.7.

To summarize, pairwise enrichment analysis indicated that the response towards inhibitors or hydrolysates involved various shared GO categories. On the one hand, deficiency in several metabolic pathways, vesicle formation, and transport, as well as transcription-related processes implied sensitivity to many inhibitors and hydrolysates. On the other hand, mutants presenting deletion or overexpression of genes involved in central biosynthesis pathways were enriched among tolerant strains, indicating that re-wiring primary metabolism could promote hydrolysate tolerance.

3.3. Only a few mutants are identified as tolerant towards multiple inhibitors

A large number of deletion mutants (affecting one of 22 genes) were characterized as being sensitive to four different inhibitors or hydrolysates. Deletion of *MSN2*, encoding a transcription factor involved in the yeast stress response (Martínez-Pastor et al., 1996) resulted in increased tolerance towards furfural, HMF, phenolics, and hydrolysates (Wu et al., 2017). Overexpression of *YAP1*, encoding a transcription factor involved in the response to oxidative stress (Kuge et al., 1997), has been shown to improve the performance of several laboratory and industrial strains when tested in furfural, HMF, coniferyl aldehyde, and spruce hydrolysates (Alriksson et al., 2010; Kim and Hahn, 2013; Wallace-Salinas et al., 2014; Wu et al., 2017).

Data mining identified six strains with altered expression of *ZWF1*, which encodes a glucose-6-phosphate dehydrogenase that catalyzes the first step of the pentose phosphate pathway (Nogae and Johnston, 1990). These mutants were assessed for tolerance towards various inhibitors, except HMF. Nonetheless, strains where *ZWF1* was deleted or overexpressed have been evaluated in hydrolysates containing HMF (Cunha et al., 2015; Pereira et al., 2014; Serate et al., 2015; Skerker et al., 2013). Deletion of *ZWF1* resulted in increased sensitivity towards acetate (Kawahata et al., 2006), formate (Henriques et al., 2017), and coniferyl aldehyde (Fletcher et al., 2019). Overexpression of *ZWF1* improved tolerance of the laboratory strain BY4741 towards furfural (Liu et al., 2020), Miscanthus (Skerker et al., 2013), wheat straw, and synthetic hydrolysates (Pereira et al., 2014); whereas in two different industrial strains, it resulted in an increased sensitivity to *E. globulus* and corn cob hydrolysate (Cunha et al., 2015).

4. Engineering tolerant strains capable of improved growth in lignocellulosic hydrolysates

Besides data-driven analysis, we sought to identify and discuss mutants tolerant towards one or more inhibitors. The mutants and studies presented in this section were selected based on known biological relevance in terms of tolerance to lignocellulosic hydrolysates and availability of data supporting the conclusions drawn in the corresponding studies. In addition, mutants that have been studied for several inhibitors or in several strain backgrounds are reported.

4.1. Increased tolerance towards acetic and formic acid following engineered expression of regulator or transporter proteins

Substantial work has been done to improve the tolerance of *S. cerevisiae* towards acetic or formic acid through deletion or overexpression of single genes. So far, only a few of these mutations have been verified in several strains or conditions (Table 2, Fig. 3). Single deletion of *WHI2*, *HAA1*, and *PRS3* causes sensitivity to both acetic (Chen et al., 2016b; Kawahata et al., 2006; Mira et al., 2010) and formic acid (Henriques et al., 2017). *WHI2* encodes a phosphatase activator involved in the general stress response (Kaida et al., 2002). A *WHI2*-overexpressing mutant attained five-times higher specific ethanol productivity than the control strain in medium supplemented with acetic acid, as well as better fermentation of corn stover hydrolysates (Chen et al., 2016b). Notably, overexpression of *WHI2* (Ding et al., 2015), *HAA1* (Cunha et al., 2018; Inaba et al., 2013; Swinnen et al., 2017), and *PRS3* (Cunha et al., 2018) has been shown to increase resistance to acetic acid in laboratory strains. *HAA1* or *PRS3* overexpression has been verified also in industrial strains (Cunha et al., 2018; Inaba et al., 2013). Overexpression of *HAA1* or *PRS3* in the industrial strain PE-2 favored cell growth and sugar consumption in medium supplemented with acetic acid or hardwood hydrolysate containing 97 mM acetic acid (Cunha et al., 2018). Moreover, simultaneous overexpression of the two genes had an additive effect, leading to fewer cell wall defects upon acetic acid exposure. Prs3 is responsible for the synthesis of phosphoribosyl pyrophosphate, which is a precursor for nucleotides, histidine, and tryptophan, and is required for cell integrity (Wang et al., 2004). *HAA1* encodes a transcription factor driving a network of genes involved in acid stress. An Haa1 mutant capable of significantly increasing acetic acid tolerance has been identified (Swinnen et al., 2017).Overexpression of *HAA1* in the industrial bioethanol strain Ethanol Red led to increased ethanol production from sugarcane molasses containing 83 mM acetate (Inaba et al., 2013).

Deletion of *JJJ1* or *PDR12* has been shown to ameliorate acetic acid tolerance in both industrial and laboratory strains grown on different media and/or acid concentrations (Nygård et al., 2014; Sousa et al., 2013; van Dijk et al., 2020b; Wu et al., 2016). *JJJ1* encodes an ATPase activator involved in rRNA processing, biogenesis of the large ribosomal subunit, and nuclear export. Wu et al. (2016) found that deletion of *JJJ1* resulted in decreased intracellular acetic acid accumulation, changes in cell membrane fatty acid composition, as well as increased trehalose accumulation and catalase activity upon acetic acid stress. Deletion of *PDR12* resulted in shorter lag times or faster growth of a CEN.PK laboratory strain in synthetic medium supplemented with 25 mM formic acid or 100 mM acetic acid (Nygård et al., 2014). When grown on wheat straw hydrolysate, biomass output and fermentation capacity of Δ*Pdr12* mutants was highly dependent on strain background (van Dijk et al., 2020b). Pdr12 is an ATP-binding cassette transporter located in the plasma membrane. Altering the expression of *PDR12* may indirectly affect tolerance towards acids through changes in the composition of the cell membrane (Nygård et al., 2014). Ergosterol content in the cell membrane of *S. cerevisiae* has been reported to change considerably under acid stress (Guo et al., 2018), and deletion of one of several ergosterol synthesis genes (*ERG2–6*, *24*, *28*) was shown to alter acetic and formic acid tolerance. Overexpression of *ACE2,* encoding a transcription factor involved in regulation of a large set of genes, including six genes participating in ergosterol biosynthesis (Reimand et al., 2010), allowed yeast to tolerate up to 33 mM acetic acid (Chen et al., 2016b). In contrast, *ACE2* deletion was detrimental for the cells (Mira et al., 2010).

Deletion of *AZR1,* encoding a multidrug transporter, was found to severely affect acetic acid tolerance in W303 wild-type yeast cells (Tenreiro et al., 2000); whereas screening of the deletion collection (strain BY4741) identified the Δ*azr1* mutant as acetic acid tolerant (Sousa et al., 2013). Tenreiro et al. (2000) showed that overexpression of *AZR1* did not alter intracellular acetic acid accumulation. Even though several transporters, including Tpo2, Tpo3, and Aqr1 (Palma et al., 2018), become activated under acetic acid stress and are presumably involved in exporting acetate ions, acetic acid export remains poorly understood. Acetic acid stress has been shown to affect yeast membrane integrity (Zheng et al., 2011), but attempts at engineering the cell membrane for increased acetic acid tolerance have proven challenging (Lindahl et al., 2017; Maertens et al., 2021).

4.2. Counteracting oxidative stress and intracellular acidification as strategies for increased acid tolerance

Acetate has been shown to promote oxidative stress (Semchyshyn et al., 2011). Overexpression of *GND1*, encoding a 6-phosphogluconate dehydrogenase required for oxidative stress tolerance, was shown to increase tolerance towards acetic acid (Lee et al., 2015); whereas deletion of *GND1* increased acetic acid sensitivity (Kawahata et al., 2006). Overexpression of *SET5* was shown to increase acetate tolerance and decrease intracellular accumulation of ROS (Zhang et al., 2015); whereas deletion of *SET5* increased acetate sensitivity (Mira et al., 2010). *PEP3* and *PEP5* encode vacuolar proteins; the overexpression of *PEP3* was suggested to provide protection from acid stress by increasing the vacuolar surface area, V-ATPase activity, and proton-sequestering capacity (Ding et al., 2015). Several deletions of the vacuolar membrane ATPase complex (*VMA2–8*, *13*, *16*, *21*, *22*) or genes encoding proteins involved in vacuolar sorting (32 *VPS* mutants are listed in our database) have been shown to decrease tolerance to acetic or formic

acid. At present, no clear evidence points to a cell membrane transporter responsible for acetic or formic acid export; whereas cytosolic acidification caused by acetate accumulation has been well documented and several mechanisms for proton sequestration have been described. Proton transport and pH homeostasis in fungi have been thoroughly reviewed by Kane (2016).

4.3. HMF and furfural converting reductases as targets for increased tolerance towards furans

Yeast can adapt to growth on furans after a prolonged lag phase, during which these inhibitory compounds are converted into less toxic products such as alcohols (Heer and Sauer, 2008). Therefore, many attempts at increasing tolerance to furans have focused on finding endogenous HMF- and furfural-converting reductases. Overexpression of *ADH6*, which encodes a broad-spectrum alcohol dehydrogenase, was shown to ameliorate growth and ethanol productivity in the presence of both furfural and HMF (Almeida et al., 2008; Park et al., 2011). The positive effect of *ADH6* overexpression on HMF tolerance was confirmed in an industrial strain, NRRLY-12632 (Lewis Liu et al., 2008). Overexpression of another alcohol dehydrogenase-encoding gene, *ADH7*, has been shown to increase growth in the presence of HMF (Lewis Liu et al., 2008) or furfural (Heer et al., 2009). Growth in medium supplemented with either HMF or furfural was improved also upon overexpression of aldehyde reductase-encoding genes *ALD6* (Park et al., 2011), *ARI1* (Liu and Moon, 2009), *YDR541C* or *YGL039W* (Moon and Liu, 2015), *YNL134C* (Zhao et al., 2015), and *YKL071W* (Heer et al., 2009). Moreover, resistance to furfural was increased following overexpression of the aldose reductase-encoding gene *GRE3* (Park et al., 2011) and the isocitrate dehydrogenase-encoding gene *IDH1* (Unrean, 2017). These findings further indicate that increased furfural detoxification through non-specific reductases is an attractive bioengineering strategy for increasing tolerance towards furans. Gre2, another aldehyde reductase, has been shown to detoxify HMF and furfural by accelerating their reduction (Moon and Liu, 2012; Lam et al., 2021). Overexpression of endogenous *GRE2* (Jayakody et al., 2018; Lam et al., 2021) or the *GRE2* homologue from *Scheffersomyces stipites* increased the tolerance of *S. cerevisiae* towards furfural, HMF (Wang et al., 2016; Lam et al., 2021), glycolaldehyde, and other inhibitors found in lignocellulosic hydrolysates (Jayakody et al., 2018). Finally, other mutants overexpressing genes that encode proteins with antioxidant or redox-balance functions, and thus providing increased resistance to furfural, include *GSH1*, *GLR1*, *OYE1, ZWF1, POS5*, and *IDP1* (Kim and Hahn, 2013; Liu et al., 2020).

4.4. The complexity and varying toxicity of phenolic compounds hampers attempts at improving tolerance

The cytotoxic effect of phenolic compounds must be taken into account for the conversion of lignocellulosic substrates to bioethanol and other biochemicals (Fletcher and Baetz, 2020). Most studies have sought to engineer strains tolerant towards a few or a single phenolic compound. Screening of the yeast deletion collection revealed mutants resistant to coniferyl aldehyde, ferulic acid, 4-hydroxybenzoic acid (Fletcher et al., 2019), and vanillin (Endo et al., 2008). Even though the tested phenolic inhibitors were similar in structure, Fletcher et al. (2019) indicated that yeast employed distinct pathways to tolerate these. A total of 17 deletion mutants hypersensitive to both ferulic acid and vanillin have been identified thereafter (Fletcher and Baetz, 2020); whereas only a few deletions (*CHO1*, *DRS2*, and *ERG4*) have been found to confer sensitivity to both ferulic acid and 4-hydroxybenzoic acid (Fletcher et al., 2019). As with acetic and formic acid, deletion of several ergosterol synthesis genes (*ERG2–6*, *24*) or genes encoding vacuolar sorting proteins (14 *VPS* mutants), and vacuolar ATPases (*VMA2*, *8*) decreased resistance to phenolic compounds. Increased levels of ergosterol have been measured in vanillin-tolerant yeasts (Endo et al., 2008; Zheng et al., 2017), suggesting that fine-tuning ergosterol levels and cellular localization to specific organelles may counteract toxicity of phenolic compounds (Fletcher and Baetz, 2020).

Zheng et al. (2017) documented the bioconversion of vanillin, presumably by non-specific dehydrogenases, to less toxic compounds, namely vanillyl alcohol and vanillic acid, in strains with increased vanillin tolerance. In line with this observation, combined overexpression of genes encoding enzymes capable of catabolizing phenolic compounds, such as *ALD5*, *PAD1*, *ATF1*, and *ATF2* (Adeboye et al., 2017), or overexpression of *BDH2* (Ishida et al., 2016), have been shown to increase substrate conversion and tolerance. The increased tolerance towards different phenolic compounds by overexpression of *PAD1* has been reported in several strains (Adeboye et al., 2017; Larsson et al., 2001; Richard et al., 2015). Overexpression of *ATR1* or *FLR1*, both encoding transporter proteins, has been shown to increase resistance to coniferyl aldehyde (both genes) and hydroxymethyl-2-furaldehyde (*FLR1*) (Alriksson et al., 2010).

4.5. Synergies between inhibitors and other compounds affect cell physiology during growth in hydrolysates

Engineering yeast strains capable of growth on lignocellulosic hydrolysates has often originated from attempts at addressing tolerance to individual inhibitors, thus disregarding any complications derived from synergies between these compounds. Pereira et al. (2014) screened the yeast deletion collection for growth in a wheat straw hydrolysate and in a synthetic hydrolysate whose inhibitor concentrations mimicked the former. Of the 297 mutants found to be sensitive to either wheat straw or synthetic hydrolysate, 159 were common to both media. This finding indicated the presence of compounds other than the main inhibitors, which contributed to hydrolysate toxicity. Based on the observation that some genes required for vitamin biosynthesis were essential for growth in wheat straw hydrolysate and, less significantly, in synthetic hydrolysate, Pereira et al. (2014) suggested that wheat straw hydrolysate lacked key nutrients. Following on this hypothesis, van Dijk et al. (2020a) showed that adding a mixture of pyridoxine, thiamine, and biotin to cells during propagation improved cell growth and ethanol yields upon lignocellulose fermentation. Genes encoding enzymes involved in thiamine and biotin biosynthesis have been reported to be upregulated during cell propagation in wheat straw hydrolysate (van Dijk et al., 2021), and deletion of several thiamine biosynthesis genes (*THI2, 3, 6, 12, 20, 72*) lowered resistance to wheat straw hydrolysate (Pereira et al., 2014; Serate et al., 2015) or synthetic hydrolysates (Pereira et al., 2014; Sardi et al., 2016). In contrast, overexpression of *THI7*, *THI12, THI20* or *THI80* was shown to decrease tolerance to a synthetic hydrolysate in a strain-specific manner (Sardi et al., 2016).

Only 28 of the genes (32%) that increased fitness in a synthetic hydrolysate were shared by all three strains tested (see Section 6.1 for a discussion on strain background). In fact, numerous genetic manipulations listed here yielded opposite phenotypes when implemented in a different strain, or when the mutant was grown in different conditions. This discrepancy is particularly apparent for different *VMA* or *VPS* mutants. Nevertheless, deletion of several vacuole-related genes, namely *APM2*, *VMA1, VMA8*, *VMA22, VPS5, VPS16, VPS24, VPS27, VPS34, VPS36, VPS41*, *VPS52*, and *VPS61* (Pereira et al., 2014; Sardi et al., Serate et al., 2015; Skerker et al., 2013), improves hydrolysate tolerance across strains.

4.6. Modifications of amino acid synthesis genes improve tolerance towards inhibitors

The arginine biosynthesis pathway may be an interesting target for improving hydrolysate tolerance, as deletion or overexpression of multiple *ARG* genes has been shown to facilitate growth in various hydrolysates (Sardi et al., 2016; Serate et al., 2015; Skerker et al., 2013) or acetic acid (Sousa et al., 2013). Cheng et al. (2016) showed that arginine could protect yeast cells from damage caused by the presence of ethanol,

presumably by maintaining the integrity of the cell wall and cellular membranes. Thus, arginine would stabilize the morphology and function of organelles by preventing ROS-induced damage. As exposure to acetate, furfural or HMF induces ROS accumulation and membrane damage in *S. cerevisiae* (Allen et al., 2010; Kim and Hahn, 2013; Zhang et al., 2017), arginine may play a role in hydrolysate tolerance.

Collectively, deletion of genes important for aromatic amino acid synthesis has been shown to increase sensitivity to acids (*ARO1–4, 8–10, TYR1, TRP1, 2, 4, 5*) or hydrolysates (*ARO1, 2, 7, 9, 80*); although the opposite effect has also been reported. Similarly, many autophagy related genes have been associated with stress caused by acetic acid (*ATG1, 2, 5, 11–19, 22, 23, 26, 29, 31, 32*) or hydrolysate (*ATG4, 8 10, 11, 14, 19, 21, 22, 26, 27, 32, 33, 36, 38, 39*), and *S. cerevisiae* has been reported to commit to programmed cell death in response to acetic acid (Dong et al., 2017; Giannattasio et al., 2005; Ludovico et al., 2001). Overexpression of *ATG22* was shown to enhance cell death induced by acetic acid; whereas its deletion promoted intracellular methionine levels (Hu et al., 2019). Interestingly, the longevity of methionine-limited yeast (Δ*met15*) was linked to reduced acetic acid accumulation (Johnson and Johnson, 2014). In line with this finding, we found studies in which deletion of one of 11 different methionine requiring (*MET*) genes altered (mostly improved) acetic acid tolerance. Deletion of *MET5* or *MET12* has been demonstrated to augment tolerance towards switchgrass or corn stover hydrolysates (Serate et al., 2015).

4.7. Overexpression of transcription factors can lead to increased tolerance

Increased hydrolysate or inhibitor tolerance has been reported following overexpression of various transcription factors (Table 3). Overexpression of *YAP1* (Rodrigues-Pousada et al., 2010) has been shown to increase tolerance to coniferyl aldehyde, HMF, and furfural in several different laboratory strains (Alriksson et al., 2010; Kim and Hahn, 2013). Alriksson et al. (2010) argued that overexpression of genes encoding master transcription regulators of the stress response offered an attractive alternative to overexpression of single genes. Wu et al. (2017) investigated the role of different stress-related transcription factors in tolerance towards HMF and furfural: whereas individual deletions decreased the growth of BY4741 cells, deletion of *MSN2* or *LEU3* promoted it. Intriguingly, the opposite has been reported in the industrial strain ER-6c, whereby overexpression of *MSN2* favored furfural resistance and fermentation rate (Sasano et al., 2012). It should be noted that regulation by transcription factors is often based not (only) on the expression level of the transcription factor itself but on its post-translational modifications and cellular translocation in response to stimuli. Moreover, many transcription factors have overlapping functions, which is particularly true for the general stress response-governing transcription factors Msn2 and Msn4 (Görner et al., 2002).

5. Massive mutant libraries and high-throughput screenings generate a large amount of data

As described in Section 2.2., the data collected and reviewed is heavily influenced by high-throughput screenings performed with the EUROSCARF deletion collection, which covers about 5100 genes (Giaever and Nislow, 2014). Recently, CRISPR-Cas9 has become the preferred method for constructing not only single mutants but also massive strain libraries. While our database does not contain studies, in which gene expression was altered via CRISPR interference/activation (CRISPRi/a), this technology has already been used in a handful of large-scale screenings for genes that improved tolerance towards inhibitors or lignocellulosic hydrolysates (see Section 5.2).

Table 3
Overexpression of transcription factors (TFs) demonstrated to increase tolerance to individual inhibitors or hydrolysates.

TF	Regulation of genes involved in	Inhibitor	Strain	Reference
Ace2	Cytokinesis	Acetate	D452–2	Chen et al. (2016a)
Azf1	Diauxic shift, carbon metabolism, cell wall organization	Hydrolysate	K11, NCYC3290	Sardi et al. (2016)
Gzf3	Nitrogen metabolism	Hydrolysate	BY4741	Wu et al. (2017)
Haa1	Weak acid stress	Acetate, hydrolysate	Ethanol Red CEN:PK 113-7D PE-2	Cunha et al. (2018) Inaba et al. (2013) Swinnen et al. (2017)
Msn2	Environmental stress	Furfural	ER-6c	Sasano et al. (2012)
Pdr8	Pleiotropic drug resistance	Hydrolysate	BY4741	Sardi et al. (2016)
Ppr1	Pyrimidine synthesis	Acetate, hydrolysate	BY4741	Zhang et al. (2015)
Put3	Proline utilization	Hydrolysate	BY4741	Wu et al. (2017)
Rpn4	Proteasomal genes	Hydrolysate	BY4741	Sardi et al. (2016)
Sfp1	Ribosomal genes and biogenesis response to nutrients and stress	Acetate	D452–2	Chen et al. (2016a)
Stb5	Multidrug resistance and oxidative stress	Hydrolysate	BY4741	Wu et al. (2017)
Stp2	Transcription of amino acid permease genes	Hydrolysate	YPS128	Sardi et al. (2016)
Yap1	Environmental stress, oxidative stress	Furfural, HMF, hydrolysate	BY4741, Y294, INVSC1, GSE16	Alriksson et al. (2010) Kim and Hahn (2013) Wallace-Salinas et al. (2014) Wu et al. (2017)
War1	Weak acid stress	Hydrolysate	BY4741	Wu et al. (2017)

5.1. Results from screenings using the EUROSCARF deletion collection are often context dependent

The EUROSCARF deletion collection mutants have been screened individually for inhibitor tolerance in liquid (Sousa et al., 2013) or solid (Henriques et al., 2017; Kawahata et al., 2006; Mira et al., 2010; Pereira et al., 2014) medium. More than half of non-essential genes seem to participate in acetic or formic acid tolerance and sensitivity, at least under certain conditions and in the BY4741 or BY4742 background. As reviewed by Giaever and Nislow (2014), the results of screenings often vary between studies. This is confirmed by data collected in the present review and highlights how data generated from a genome-wide screening need individual validation across platforms.

When comparing the complete deletion collection screening for either acetic (Kawahata et al., 2006; Mira et al., 2010; Sousa et al., 2013) or formic (Henriques et al., 2017) acid tolerance, large differences between the outcomes emerge (Fig. 5a). Only two of the mutants from the deletion collection, Δ*ost4* and Δ*tps2,* were found to be sensitive both to acetic (in all three studies) and formic acid; whereas no mutants showed increased resistance to both acids in all screenings (Fig. 5). *OST4* encodes a subunit of the oligosaccharyltransferase complex located in the lumen of the endoplasmic reticulum, with Ost4 reportedly stabilizing interactions between subunits of the assembled OST complex (Dumax-Vorzet et al., 2013). The OST complex is responsible for N-glycosylation

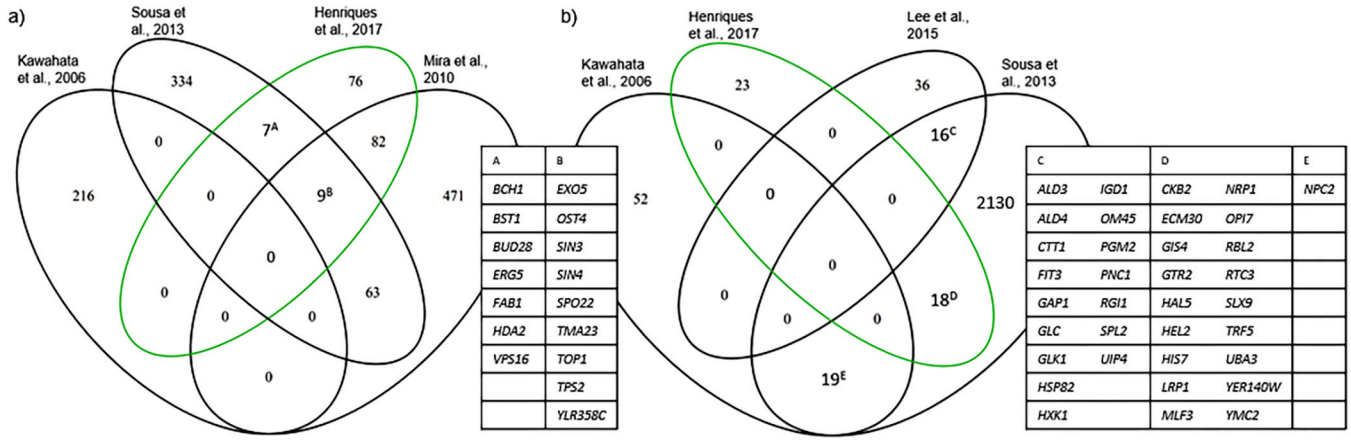

Fig. 5. Schematic representation of the number of mutants that displayed a) sensitivity or b) resistance to acetic (in black) or formic acid (in green) in screens including the whole EUROSCARF deletion collection (Henriques et al., 2017; Kawahata et al., 2006; Mira et al., 2010; Sousa et al., 2013) or 54 mutants of this collection, selected based on transcriptomic analysis of mutants stressed with acetic acid (Lee et al., 2015). Genes of mutants identified in several of the studies (intersections A-E) are listed in the insert tables. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and defects in N-glycosylation have been shown to induce apoptosis (Hauptmann et al., 2006), which is also caused by acetic acid stress (Ludovico et al., 2001). *TPS2* encodes a phosphatase subunit of the trehalose-6-P synthase/phosphatase complex. Trehalose has been shown to accumulate in cells upon acetic and formic acid exposure and, in this way, protect them from oxidative damage (Guo and Olsson, 2016). Overexpression of *TPS1*, another subunit of the trehalose-6-P synthase/phosphatase complex, was shown to increase the accumulation of trehalose and improve ethanol tolerance and ethanol yield when cells were fed with more than 15% glucose (Divate et al., 2016). Conversely, when *TPS1 or TPS2* were overexpressed, no apparent fitness benefit was observed in a synthetic hydrolysate containing a set of weak acids (Serate et al., 2016). In that study, a multicopy plasmid library containing 95% of all *S. cerevisiae* open reading frames (the MoBY-ORF 2.0 library) was introduced into three different strain backgrounds and screened through competitive growth. The authors concluded that strain background significantly influenced gene overexpression results, as indicated by only 32% of beneficial genes being common to all three strain backgrounds.

No overlap has been reported among sensitive strains identified in one of the whole deletion collection screenings and a screening that included 54 mutants from the deletion collection selected based on transcriptomics of mutants stressed with acetic acid (Lee et al., 2015). Sousa et al. (2013) highlighted that screenings using the deletion collections have been carried out with cells in different growth phases and conditions, which may explain why a large number of mutants with common gene alterations have exhibited contrasting phenotypes.

5.2. *CRISPR interference enables screening of essential genes involved in inhibitor tolerance*

As we limited our database to overexpression and deletion mutants, very few essential genes appear involved in inhibitor tolerance. CRISPRi technology, whereby an RNA-guided, endonuclease-dead Cas9 (dCas9) controls downregulation of genes by directing dCas9-fusions to their promoter region (Qi et al., 2013), provides a powerful tool for assessing the contribution of essential genes to a specific phenotype. The possibility to alter gene expression through CRISPRi opens new avenues for fine-tuning stress responses. Accordingly, fine-tuning gene expression may improve a phenotype, while deletion or overexpression may be harmful for the cell. As an example, repression of *SSK2* in *S. cerevisiae* KE6–12 improved growth in hydrolysate, but deletion of this gene led to impaired growth (Cámara et al., 2020).

Mukherjee et al. (2021) performed a large screening to determine the involvement of essential genes and those responsible for respiratory growth in acetic acid tolerance. Notably, many of the genes that upon repression lead to acetic acid tolerance encode subunits of the 19S regulatory particle of the 26S proteasome, demonstrating the importance of the proteasome in acetic acid tolerance (Mukherjee et al., 2021). When a genome-wide CRISPRi library was screened in spruce hydrolysate and in the presence of lignocellulosic inhibitors, various genes known to promote tolerance, such as *YAP1* or *HAA1*, as well as new ones, were identified (Gutmann et al., 2021). The authors proposed that overexpression of *CDC15* or *UGA3*, downregulation of *BUB1*, *DOT6* or *SKO1*, and repression of *PBS2* or *HOG1* offered potential engineering strategies for increased hydrolysate tolerance. In another study, the collection of so-called CRISPR-AID mutants comprising gRNAs capable of upregulating, downregulating or deleting all the genes in the yeast genome was screened for tolerance towards furfural (Lian et al., 2019). A couple of previously reported single gene alterations (*SIZ1*i and *SAP30* deletion) were confirmed to improve furfural tolerance. By integrating the *SIZ1*i expression cassette into the genome of the parental strain and performing another round of strain generation, many novel gene targets were identified and, together with downregulation of *SIZ1,* they increased tolerance towards furfural (Lian et al., 2019). The most prominent increase in furfural tolerance (up to 17.5 mM) accompanied by a similar ethanol output as under furfural-free conditions, was achieved by simultaneously repressing *SIZ1* and *PDR1*, while overexpressing *NAT1* (Lian et al., 2019). This finding highlights the importance of the interplay between genes and expression levels in determining and altering phenotypes.

6. *S. cerevisiae* strains engineered for increased tolerance display great diversity

The genetic alterations listed in our database were found through screenings aimed at understanding how particular inhibitors or mixtures of inhibitors affected cell phenotypes, or studies aimed at developing mutants that could cope better with stress than the parental strain. Moreover, the screenings were performed in a number of yeast strains with different genetic set-ups. For centuries, humans have subjected *S. cerevisiae* to multiple rounds of independent domestication and thousands of generations of artificial selection during both small- and industrial-scale production of wine, sake, beer, and bread. In parallel, *S. cerevisiae* has long served as a model organism for eukaryotic cells. For this purpose, laboratory strains have been developed by selecting easy-

to-cultivate mutants that grew as single non-flocculating cells. Consequently, this species comprises a diverse collection of wild, natural isolates, domesticated industrial and laboratory strains, as well as mosaic strains produced through sporulation and mating of different lineages. Through extensive genome sequencing of thousands of *S. cerevisiae* strains, a detailed picture of the high level of genetic diversity in this species has emerged (Duan et al., 2018; Giannakou et al., 2020; Peter et al., 2018).

6.1. The phenotype of a mutant may be strain specific

The importance of genetic background is exemplified by multiple gene deletions or overexpressions that result in highly strain-specific phenotypes (Cunha et al., 2015; Sardi et al., 2018). However, for the vast majority of genes identified in our dataset, the influence of genetic background has not been determined. It is likely that the inherent stress tolerance of the parental strain influences screening results, as the same concentration of a specific inhibitor can result in different base levels of stress and thus reveal or hide the influence of specific genes. Although the importance of studying *S. cerevisiae* strains suitable for the intended industrial purpose is widely recognized, academia tends to use laboratory strains to elucidate the genetic determinants for tolerance to individual inhibitors (Fig. 2). This may be due to old habits and convenience, availability of strains and high-throughput tools for their modification, as well as the ease of retrieving and generating genetic information.

Laboratory strains are often stable haploids due to deletion of the HO gene, which blocks mating-type switching and diploidization, thus facilitating straightforward genomic and phenomic studies. In addition, they are commonly auxotrophic, which allows efficient selection of prototrophic mutants. The most frequently applied auxotrophic markers are the *HIS3*, *LEU2*, *TRP1*, and *MET15* genes that encode essential enzymes for de novo synthesis of L-histidine, L-leucine, L-tryptophan, and L-methionine, respectively. Another commonly deleted marker gene is *URA3*, which encodes orotidine-5′-phosphate decarboxylase, an essential enzyme in pyrimidine biosynthesis (Pronk, 2002). When using such strains, it is important to note that cellular metabolism is significantly altered in auxotrophs compared to prototrophs (Alam et al., 2016) and may impact stress tolerance (Swinnen et al., 2015). For example, when studying acid tolerance in auxotrophic mutants, the need to import specific amino acids and their availability in the medium should be considered, as they may affect tolerance towards the tested inhibitors (Ding et al., 2013). Indeed, several mutants with altered amino acid synthesis display increased tolerance towards inhibitors (see Section 4.6).

Our dataset contains several strains of laboratory origin. The reference strain, S288C, is used extensively for genetic studies and represents the default sequence in the *Saccharomyces* Genome Database. After a number of genomic alterations to repair detrimental mutations and introduce auxotrophic markers, the S288C strain gave rise to the BY series of strains, from which the EUROSCARF genome-wide deletion collection was derived. In the acetate screenings included in our dataset, a total of 3615 mutants with altered growth characteristics were identified, of which 99.9% were laboratory strains and 92% of these were of BY4741 origin. Likewise, all the single modifications tested via genome-wide studies in formic acid and phenolics-containing media were carried out in laboratory strains, mainly in BY4741 (Fig. 2). The CEN.PK-family of laboratory strains has become a popular choice for physiology and industrial biotechnology studies (Daran-Lapujade et al., 2003). In our dataset, CEN.PK mutants have been used mainly in studies on HMF, furfural, and phenolic tolerance. CEN.PK113-5D possesses a rather high tolerance towards inhibitors present in lignocellulosic hydrolysates, comparable or even superior to that of certain industrial strains (Martin and Jönsson, 2003).

6.2. Industrial strains are often tolerant towards industrial stress factors

Contrary to screenings of individual inhibitors using predominantly laboratory strains, those performed in lignocellulosic hydrolysates often seek to develop more efficient industrial processes and use preferentially strains of industrial background. As the name implies, these strains should be suitable for industrial processes, and many have been isolated following continuous fermentation of sugar-rich starting materials (grape must, sake, beer malt, and starch). As a result, they have already developed tolerance towards industrial stress factors, such as low pH, elevated sugar content, and rapid fermentation conditions, and are more robust than laboratory strains, but have seldom been exposed to lignocellulosic inhibitors (Giannakou et al., 2020). Industrial strains are generally less characterized than their laboratory counterparts, both in terms of physiology and genetics. They range from haploids to diploids, tetraploids, and even euploids. While this makes strain construction more challenging, CRISPR/Cas9 technology has significantly facilitated genome editing of industrial strains (Stovicek et al., 2015).

Industrial strains provide the background for 70% of mutants included in our database. Most of these mutants were included in the global studies performed by Sardi et al. (2016) and Serate et al. (2015), with the ethanol-tolerant sake strain K11 and the hydrolysate-tolerant strain Y128 used as parental strains. Other mutants with industrial strain background include derivatives of Ethanol Red, which is widely used for 1st generation bioethanol production (Demeke et al., 2013), or TMB3400, a tolerant xylose-fermenting strain (Wahlbom et al., 2003). In screenings of furfural and HMF tolerance, around 15% of mutants were derived from industrial strains, most frequently NRRLY-12632 (Fig. 2), which was originally isolated from a brewery (Lewis Liu et al., 2008). It is evident that the different origins and evolutionary paths add to the heterogeneity of industrial strains.

6.3. Wild strains are largely unexplored but have high potential for inhibitor tolerance

In contrast to domesticated laboratory and industrial strains, many wild yeasts have been collected from harsh environments and have likely evolved under diverse selection pressure constraints. Some of these wild strains display superior stress tolerance to harsh environments compared to domesticated strains and may, therefore, serve as excellent starting points for the development of inhibitor-tolerant cell factories (Sardi et al., 2016). Wild strains remain severely underrepresented in genetic screenings for tolerance towards lignocellulosic inhibitors. Our dataset includes only three wild strains that have been engineered and tested for tolerance to hydrolysates: the inhibitor-tolerant YPS128 strain isolated from a North American oak tree, the inhibitor-sensitive mosaic strain YJM1444, and the West African strain NCYC3290 isolated from bili wine (Sardi et al., 2016) (Fig. 2). Nonetheless, the rapid development of high-throughput pipelines for strain engineering, as well as genotyping and phenotyping of microorganisms hold promise for more efficient identification and exploitation of wild strains.

7. Conclusions and perspectives

Considerable efforts have been made to develop and screen yeast mutants with a high tolerance to lignocellulosic hydrolysates or their constituent inhibitors. Lignocellulosic hydrolysates vary in composition and tolerance to them diverges greatly between yeast strains, adding to the challenge of engineering robust production hosts. Therefore, we collected and systematically analyzed engineering attempts to obtain a holistic view that would point to general trends and groups of genetic targets with proven increased tolerance towards lignocellulosic hydrolysates. The obtained data can form the basis for new information-driven strategies aimed at strain improvement. Furthermore, it can help translate gene alterations identified in laboratory strains into

industrially relevant strain backgrounds. The collected data suggest that it may not be worth optimizing strains for each inhibitor separately.

Our dataset contains tolerant and sensitive mutants identified through screenings in a wide variety of culture media and experimental set-ups, involving lignocellulosic hydrolysates as well as individual inhibitors, most commonly acetic acid. Moreover, many screenings were performed in solid medium with rather high concentrations of inhibitors, while a few correspond to controlled bioreactor studies. These factors likely explain some of the contradictory phenotypes we identified among the listed mutants. Extrapolating results from one study to draw more general guidelines is tricky. Indeed, our analysis shows that a phenotype identified as beneficial for a specific inhibitor does not necessarily translate into tolerance towards a collection of inhibitors, let alone hydrolysates. Nonetheless, GO analysis identified several ontologies that were significantly enriched among sensitive mutants, highlighting deletions of genes related to vesicle transport and transcription-related processes as leading to increased inhibitor or hydrolysate sensitivity. Similarly, ergosterol synthesis genes were identified across several studies as conferring resistance to many different inhibitors. This observation is supported by the findings of Endo et al. (2008), who suggested engineering the ergosterol biosynthesis pathway as a means towards multi-tolerant strains.

The difficulty to reproduce beneficial phenotypes obtained with one strain in other genetic backgrounds suggests that the strain should be carefully considered when addressing tolerance. Of note, laboratory strains with low general tolerance towards hydrolysates are over-represented in our collected data, as several screenings of the EUROSCARF deletion collection were included. As this is expected to impact meta-analysis of the data, the biological relevance of specific genetic alterations, as well as modifications of industrial strains are highlighted throughout this review. Furthermore, strain-overlapping omics analysis of the global response to stress factors will provide better understanding of how inhibitors present in lignocellulosic hydrolysates affect cell physiology.

With developments in automation, screening, and sequencing technologies, high-throughput assessment of massive libraries has become a popular approach to identify mutants that display higher-than-average tolerance to inhibitors (Fletcher et al., 2019; Gutmann et al., 2021; Lian et al., 2019; Mukherjee et al., 2021). Often, these libraries are screened through competitive growth assays, where the sensitive mutants are outcompeted by inherently more tolerant or improved counterparts. A number of CRISPRi screenings have been published in the past years, allowing the study of how essential genes impact tolerance towards lignocellulosic inhibitors, which was previously difficult to address. Recent developments in synthetic biology tools, such as CRISPR/Cas9-based genome editing (Stovicek et al., 2015) or expression tuning tools (Cámara et al., 2020), will facilitate the development of hydrolysate tolerance in industrially relevant production hosts. This will enable the re-assessment and synergistic combination of previously established alterations, as well as the fine-tuning of genes, all of which are less drastic strategies than gene deletion or promoter replacement. Our strain database and accompanying analysis may thus guide more sophisticated strain engineering ventures.

Availability of data and material

All relevant data generated or analyzed during this study is deposited in the GitHub repository; https://github.com/JanZrimec/Hydrolysate_inhibitor_review. The GO networks of mutants listed are deposited in the public repository NDEx https://ndexbio.org/#/networkset/64ce2763-f6f3-11eb-b666-0ac135e8bacf.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data collected is deposited at Github and NDex and links to data is given in the manuscript.

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