THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Exploring *Saccharomyces cerevisiae*'s responses to acetic acid and other inhibitors found in lignocellulosic hydrolysates

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“Happiness only real when shared”

Alexander Supertramp

Exploring *Saccharomyces cerevisiae*'s responses to acetic acid and other inhibitors found in lignocellulosic hydrolysates.

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Abstract

The limited tolerance of the budding yeast *Saccharomyces cerevisiae* to lignocellulosic hydrolysate inhibitors is a key challenge to its use in biorefinery cell factories. Considerable resources have been invested in the isolation of yeast strains with better tolerance towards the inhibitors released during lignocellulose hydrolysis, such as acetic acid. The goal of this thesis was twofold: characterize the transcriptional response of *S. cerevisiae* to wheat straw hydrolysate and explore the role of essential *S. cerevisiae* genes in acetic and formic acid tolerance, using a new biosensor and competitive growth assays.

The transcriptomes of one laboratory strain, two industrial strains, and two wild-type isolates grown in wheat straw hydrolysate were profiled. Despite similar growth, the isolates showed different expression of genes encoding proteins involved in oxidative stress response, lipid accumulation, and transport, suggesting different genetic strategies for tolerance. The new acetic acid biosensor was based on two transcription factors, Haa1 from *S. cerevisiae* and BM3R1 from *Bacillus megaterium*. Biosensor and competitive growth were used in parallel to screen a *S. cerevisiae* CRISPR interference strain library. While fluorescence-activated cell sorting led to the isolation of cells with higher acetic acid retention and sensitivity, competitive growth assays allowed the identification of cells with higher acid tolerance. The results confirmed the role in acid stress response of genes involved in glycogen accumulation, chromatin modification, and mitochondrial or proteasomal functions. Two novel targets for improving tolerance were also identified: *PAP1* and *HIP1*.

Altogether, this thesis provides mechanistic insight into the stress response to lignocellulosic hydrolysates or weak acid inhibitors that limit yeast-mediated conversion of lignocellulosic biomass into biochemicals. Additionally, it offers new tools for the identification of strains with altered acetic acid tolerance.

Keywords: *Saccharomyces cerevisiae*, lignocellulosic inhibitor tolerance, differential gene expression, acetic acid biosensor, acetic acid, formic acid, CRISPRi screening

Preface

This dissertation serves as partial fulfilment of the requirements to obtain the degree of Doctor of Philosophy at the Department of Life Sciences at Chalmers University of Technology. The PhD studies were carried out between November 2018 and May 2023 at the Division of Industrial Biotechnology, under the supervision of Yvonne Nygård and the co-supervision of Verena Siewers. The thesis was examined by Lisbeth Olsson. The research was founded by the Swedish Research Council Formas, the Hasselblad Foundation, Ollie och Elof Ericssons Stiftelser, the Swedish Energy Agency and the Adlerbertska Research Foundation.

Maurizio Mormino

April 2023

List of publications

I. **Mormino M.**, Lenitz I., Siewers V., Nygård Y. (2021). "Development of an Haa1-based biosensor for acetic acid sensing in *Saccharomyces cerevisiae*", *FEMS Yeast Research*, 21(6), 49. https://doi.org/10.1093/femsyr/foab049

II. **Mormino M.**, Lenitz I., Siewers V., Nygård Y. (2022). "Identification of acetic acid sensitive strains through biosensor-based screening of a *Saccharomyces cerevisiae* CRISPRi library". *Microbial Cell Factories* 21(1), 214. https://doi.org/10.1186/s12934-022-01938-7

III. Lenitz I., **Mormino M.**, Blomberg A., Mukherjee V., Nygård Y. "Pooled CRISPRi screen of essential genes of *Saccharomyces cerevisiae* reveals genes important for tolerance to acetic acid and formic acid". (Manuscript)

IV. Cámara E.*, **Mormino M.***, Siewers V., Nygård Y. "*Saccharomyces cerevisiae* strains performing similarly during fermentation of lignocellulosic hydrolysates show great differences in transcriptional stress responses" (Manuscript)

*Authors contributed equally

Contribution summary

Paper I:

YN conceived the study. I performed all the experiments and data analysis. I drafted the manuscript and all authors contributed with discussion and interpretation of data, as well as with writing and revision of the manuscript.

Paper II:

YN conceived the study. I performed most of the experiments and data analysis. IL performed the screening of selected strains. I drafted the manuscript together with IL, and all authors contributed with discussion and interpretation of data, as well as with writing and revision of the manuscript.

Paper III:

YN, AB, and VM conceived the study. IL performed the competitive growth assay, analyzed the corresponding data, as well as part of the data from fluorescence-activated cell sorting. I carried out fluorescence-activated cell sorting and analyzed part of the resulting data. IL drafted the manuscript together with me, and all authors contributed with discussion and interpretation of data, as well as with writing and revision of the manuscript.

Paper IV:

YN and EC conceived the study. EC performed all the experiments and HPLC data analysis. I analyzed the growth and transcriptome data. I drafted the manuscript, and all authors contributed with discussion and interpretation of data, as well as with writing and revision of the manuscript.

Table of content

Abbreviations

ABC	ATP-binding cassette
AI	Artificial intelligence
ATP	Adenosine triphosphate
Cas9	CRISPR associated protein 9
CFU	Colony forming unit
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
dCas9	Dead Cas9
DNA	Deoxyribonucleic acid
FACS	Fluorescence-activated cell sorting
FRET	Förster resonance energy transfer
gRNA	Guide RNA
HMF	5-hydroxymethylfurfural
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
TCA	Tricarboxylic acid

Thesis outline and chapter focus

The unprecedent technological advancements of the past century have rested on an excessive exploitation of fossil fuels and growing CO_2 emissions. To diminish our environmental impact and reliance on fossil fuels, various commodities can be produced by microbial cell factories. However, to make this process viable, microbial performance requires further improvement.

The budding yeast *Saccharomyces cerevisiae* is considered a workhorse in the production of biochemicals. As outlined below, the goal of this thesis was to provide new knowledge, as well as tools to increase the efficiency of *S. cerevisiae* as a cell factory.

Chapter I. Introduction to the role of yeast and lignocellulosic hydrolysates. Research questions and goals of the thesis are presented at the end of the chapter.

Chapter II. Strategies to tackle and investigate the problems caused by inhibitors present in lignocellulosic hydrolysates are described. The main results of **Paper IV**, which summarizes transcriptomic analysis of different strains grown in wheat straw hydrolysate, are presented and related to previous studies. The current understanding of the stress response in yeast is outlined.

Chapter III. Different high-throughput screening tools are described, focusing in particular on genetically encoded biosensors in yeast, their applications, and types. The design, characterization, and validation of the transcription factor-based biosensor developed in **Paper I** and improved in **Paper II** are described.

Chapter IV. The challenges posed by acetic and formic acids in yeast-based biorefineries are illustrated. The main results relative to acetic and formic acid tolerance screenings performed in **Paper II** and **Paper III** are presented and related to previous studies, as well as to transcriptomic results from **Paper IV**. Potential and limitations of biosensors or competitive growth as screening methods are discussed.

Chapter V. The main conclusions of the thesis are summarized.

Chapter VI. The chapter describes future perspectives for biosensor exploitation and yeast improvement for cell factory use.

I. Introduction

Industrial development and growth have relied heavily on fossil fuels, which is now presenting us with an alarming price tag in the form of pollution, deforestation, the spread of diseases, and climate change driven by excessive CO_2 emissions. While politicians are trying to establish international agreements such as the Kyoto protocol, the scientific community has been tasked with finding solutions that can help the transition to a fossil-free society. The possibility of exploiting microorganisms to produce a vast range of products, including biofuels, bulk and fine chemicals, nutraceuticals, and pharmaceuticals, is attaining growing interest. Such microorganisms are known as cell factories, a concept that was established in the '80s (Lee et al., 2012; Marston, 1986). Compared to the first generation of cell factories, which focused on initial *Escherichia coli* or *Saccharomyces cerevisiae* strains, recent advancements in metabolic engineering and industrial biotechnology have led to the development of mutants with increased performances and widened the range of products (Navarrete et al., 2020). Some examples of *S. cerevisiae* industrial applications are listed in Table 1.1.

Through the years, *S. cerevisiae* and yeast in general have emerged as versatile platforms for cell factory development. Several key cellular processes, signaling pathways, and genes are highly conserved between yeast and human cells. This has made *S. cerevisiae* one of the most studied model organisms (Nielsen, 2019) and generated a massive amount of data on yeast genetics and physiology, including sequencing of its genome (Goffeau et al., 1996). Several tools have been developed to genetically engineer yeast and monitor its metabolism (Chae et al., 2017). Given such extensive knowledge and tools, as well as the ability of yeast to produce many different chemicals, *S. cerevisiae* has emerged as one of the preferred platforms to develop cell factories (Jiao et al., 2022).

Microbial cell factories need suitable nutrients to produce the desired bio-products. While edible feedstock such as glucose may pose issues in terms of agricultural land exploitation and food security (Gustavsson & Lee, 2016), non-edible lignocellulosic materials, such as wood, grass, forestry waste, agricultural residues, and municipal solid waste, constitute abundant and renewable energy sources. The production of commodities from lignocellulosic hydrolysates made of biomass unsuitable for food and feed could start a virtuous bio-based industrial cycle (Navarrete et al., 2020) (Figure 1.1). This is particularly relevant in a country such as Sweden, which is the world's third largest exporter of forest-based products

(https://www.forestplatform.org retrieved on April 9, 2023) and has 68.7% of its territory covered by forests (https://data.worldbank.org retrieved on April 9, 2023). Lignocellulosic biomass is composed of lignin, cellulose, and hemicellulose. While cellulose is a high-molecular weight linear polymer of β-1,4-linked D-glucose units; hemicellulose is a branched polysaccharide consisting of pentoses, such as D-xylose and L-arabinose, as well as hexoses, such as D-mannose, D-glucose, D-galactose, and uronic acids (Palmqvist & Hahn-Hägerdal, 2000a). None of these polymers are naturally fermented by *S. cerevisiae*; however, cellulose and hemicellulose polymers can be first degraded to their building blocks, such as glucose, and then fed to yeast. Lignocellulosic biomass is typically pretreated to solubilize recalcitrant structures using mechanical, chemical, biological or physicochemical means (Zhao et al., 2022). Various environmentally friendly pretreatment methods have emerged recently, including biochemical, ionic liquid, deep eutectic solvent, and supercritical fluid pretreatments (Zhao et al., 2022). The pretreatment results in a biomass composed by a liquid and a solid fraction (Zhao et al., 2022). The liquid fraction is rich in monomeric and oligomeric sugars derived from hemicellulose degradation, while the solid fraction consists mostly of cellulose polymers. The solid fraction can be further processed by enzymatic hydrolysis to obtain a liquid rich in glucose (Zhao et al., 2022).

Table 1.1: Examples of *S. cerevisiae* industrial application as cell factories

Company	Feedstock	Product	Source
Novo Nordisk	Glucose	Human insulin	(a)
Amyris	Sugarcane, corn, cellulosic biomass, waste glycerol	Pharmaceuticals, cosmetic ingredients, fine chemicals, biofuels	(b)
Novozymes	Corn, soybeans, barley, canola, cellulosic biomass	Enzymes for various industrial applications, such as biofuels and detergents	(c)
Avansya (DSM/Cargill)	Crude sugars *	Steviol glycosides	(d) (e)
Biocon	*	Human insulin	(f)
Genentech	Glucose, galactose, maltose	Monoclonal antibodies for cancer treatment	(g)

* No further details disclosed by the company
(a) https://www.novonordisk.com/
(b) https://amyris.com/ingredients
(c) https://www.novozymes.com/en
(d) https://www.dsm.com/corporate/home.html
(e) https://www.cargill.com/home
(f) https://archive.biocon.com/
(g) https://www.gene.com/
All information from websites was retrieved on April 9, 2023.

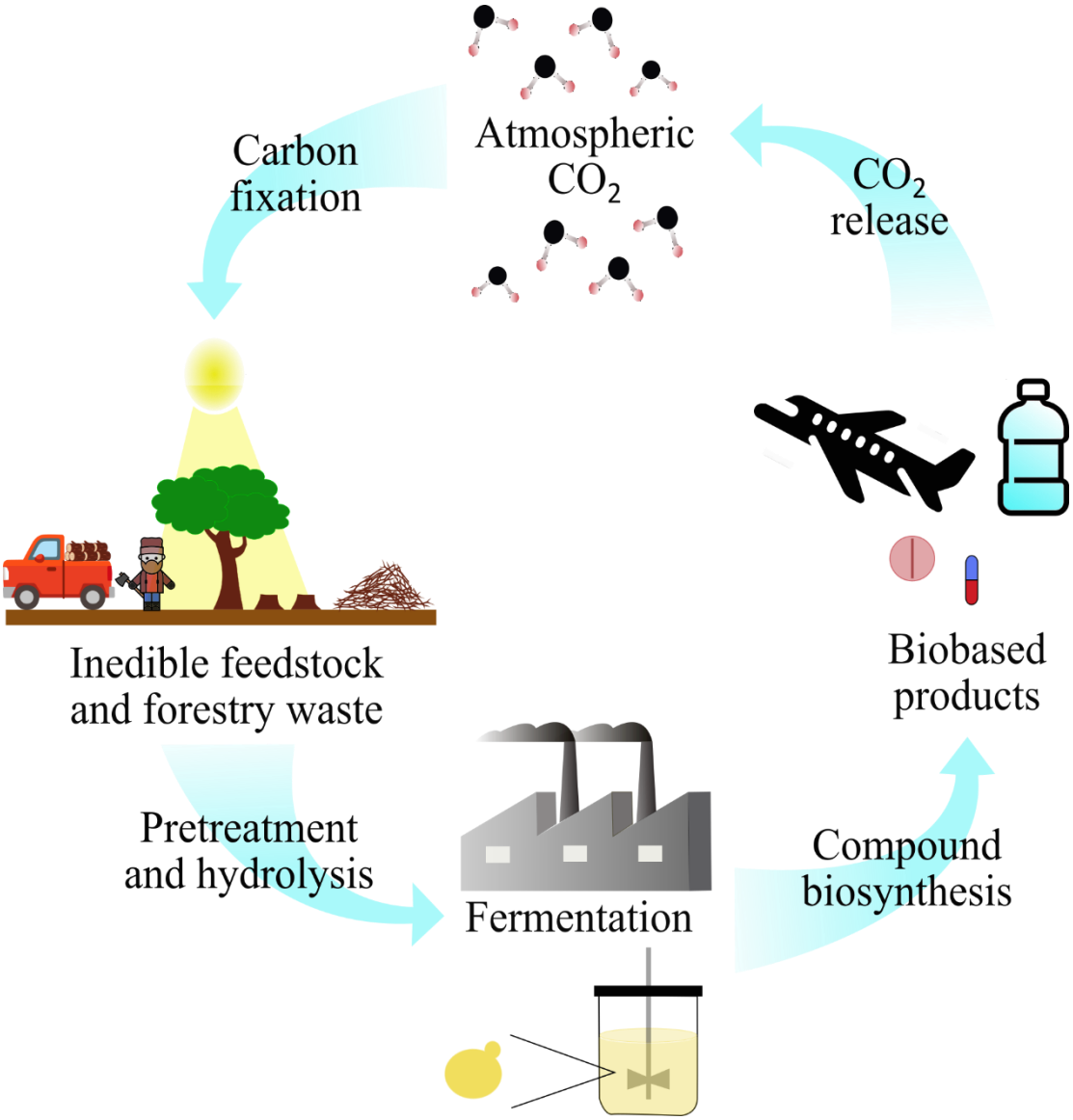

Figure 1.1: The industrial cycle for yeast second generation biorefineries. Industrial production combining yeast with lignocellulosic biomass can initiate a positive loop, whereby non-edible feedstock is subjected to different treatments and fed to yeast for fermentation and biosynthesis of specific compounds. Modification, exploitation, and distribution of compounds produced from the fermentation of hydrolysates may lead to the release of CO_2. The cycle is completed when the atmospheric CO_2 is fixed by plants through photosynthesis.

The steps required to obtain a lignocellulosic hydrolysate rich in monosaccharides lead also to the release of several compounds, which are inhibitory for microorganisms (Branco et al., 2019; Brandt et al., 2019), as well as hydrolytic enzymes (Mhlongo et al., 2015). The inhibitors can be classified into furans, weak acids, and phenolic compounds; their inhibitory mechanisms depend on their chemical nature (Adeboye et al., 2014). In furans, the main source of toxicity is a reactive aldehyde group attached to the furan ring (Brandt et al., 2019). The detoxification of furans implies depletion of the NAD(P)H pool required by oxidoreductases to catalyze

various redox reactions (Menegon et al., 2022). If unbalanced, the latter may result in the release of reactive oxygen species (ROS), which can lower glucose consumption, promote oxidative stress (Allen et al., 2010), and favor damage to DNA, proteins, and organelles such as mitochondria and vacuoles (Brandt et al., 2019).

The main inhibitory mechanism related to lipophilic weak acids, such as acetic and formic acids, involves their undissociated form, which can diffuse through the plasma membrane. Once in the cytoplasm, weak acids tend to dissociate due to a higher intracellular pH. Consequently, protons accumulate inside the cell causing acidification of the cytosol, inhibition of the pentose phosphate pathway, oxidative stress, turgor pressure, protein aggregation, lipid peroxidation, disruption of the plasma and vacuolar membranes, inhibition of membrane trafficking, and ultimately cell death (Brandt et al., 2019).

The toxicity of phenolic compounds is attributed mainly to the various reactive groups, such as hydroxyl, aliphatic, carboxylic, aldehyde and acyl groups, linked to the aromatic rings (Brandt et al., 2019), which exert inhibitory effects similar to those described for furans and weak acids (Fletcher et al., 2019). Moreover, the magnitude of this toxicity depends on environmental factors, such as pH, temperature, and nutrient availability (Xiros & Olsson, 2014), as well as growth phase and previous growth conditions (Narayanan et al., 2017). Furthermore, combinations of different inhibitors may result in synergistic toxic effects (Cámara et al., 2022).

Despite decades of research trying to uncover the mechanisms underlying the response of yeast to the inhibitors contained in lignocellulosic hydrolysates, several aspects remain unclear (Cámara et al., 2022). In fact, understanding and overcoming the stress caused by inhibitors remains a crucial challenge in the development of more efficient cell factories and second-generation biorefineries. Equally important, is to improve the set of tools and techniques required to identify strains with greater tolerance towards lignocellulosic hydrolysates and the inhibitors therein. To help solve these issues, the work described in this thesis focused on the response of yeast to inhibitors found in lignocellulosic hydrolysates. Specifically, this thesis sought to address the following research questions:

(1) Which genes are important for the tolerance of yeast towards lignocellulosic hydrolysates and the inhibitors therein?

(2) How can high-throughput screening be used to identify and develop strains that are more tolerant towards the inhibitors present in lignocellulosic hydrolysates?

To answer these questions, the following objectives were set:

- identify strain-dependent transcriptional responses of *S. cerevisiae* grown in wheat straw hydrolysate (**Paper IV**);
- develop a transcription factor-based biosensor for acetic acid sensing (**Paper I** and **Paper II**);

- explore the role of essential and respiratory growth essential genes of *S. cerevisiae* in the response to acetic acid and formic acid stress, using a biosensor and competitive growth assays (**Paper II** and **Paper III**).

In the next chapter (Chapter II), the transcriptomic analysis of different *S. cerevisiae* strains grown in wheat straw hydrolysate (**Paper IV**) is presented. Following construction of the acetic acid biosensor (**Paper I** and **Paper II**) as described in Chapter III, its use to screen for acetic acid and formic acid tolerance in a strain library (**Paper II** and **Paper III**), along with competitive growth assays, is presented in Chapter IV.

II. Yeast response to lignocellulosic hydrolysates stress

Exploitation of lignocellulosic hydrolysates as a carbon source for microorganisms presents numerous challenges (Chapter I). The inhibitors contained in hydrolysates cause multiple forms of stress in yeast cells, hampering growth and metabolic pathways. Given such detrimental effects, it is crucial to understand the mechanisms underlying the response of yeast cells to stress. Metabolic adaptations enabling tolerance to inhibitors may be a key to successful conversion of lignocellulosic hydrolysates. To overcome the challenges posed by inhibitors, some methods aim for a lower presence of inhibitors in the hydrolysates, while others focus on improving tolerance. Conspicuous resources have been employed on the second approach, resulting in various means of assessing the metabolic strategies that help cells counteract the toxic effect of lignocellulosic hydrolysate inhibitors. **Paper IV** contributes to this this area by exploiting transcriptomic analysis to investigate the strain-dependent response of *S. cerevisiae* to lignocellulosic hydrolysate inhibitors. The accrued knowledge could be utilized to design new strategies and create strains with improved performance.

Lignocellulosic hydrolysates detoxification and strain improvement

One of the approaches to offset lignocellulosic hydrolysate inhibitors is medium detoxification prior to fermentation (Chandel et al., 2013). Physical, chemical, and biological detoxification techniques have been proposed (Figure 2.1a). Physical methods include evaporation, which can remove volatile compounds such as acetic acid (Palmqvist et al., 1996), and membrane-mediated detoxification, which focuses on depleting cell wall-derived inhibitors (Wickramasinghe & Grzenia, 2008). Chemical detoxification encompasses alkali treatment with calcium hydroxide (over-liming), which neutralizes the acidic pH of sulfuric acid-pretreated hydrolysates while precipitating the inhibitors (Chandel et al., 2011); activated charcoal columns (Canilha et al., 2008) and ion-exchange resins (Villarreal et al., 2006), which adsorb or separate inhibitors from the medium, respectively; and ethyl acetate, which helps extract inhibitors from the hydrolysate (Cruz et al., 1999). Finally, biological detoxification relies on enzymes, such as laccases and peroxidases (Jönsson et al., 1998), or microorganisms, such as *Trichoderma reesei* (Palmqvist et al., 1997) and *Acinetobacter baylyi* (Kannisto et al., 2015), which degrade the inhibitors prior to yeast fermentation. Alternatively, co-culturing systems allow simultaneous medium detoxification/saccharification and yeast fermentation. Zhu et al. (2016) described how the co-cultivation of a xylose-fermenting and an inhibitor-

tolerant *S. cerevisiae* strain augmented bioethanol production in corn stover hydrolysates. All the above techniques and their combinations have been proven to remove inhibitors with varying degrees of success; however, they require additional dedicated steps, which increase costs (Sivers et al., 1994). Biological approaches can be particularly challenging due to the high cost of enzymes (Kudanga & Le Roes-Hill, 2014) and significant loss of fermentable sugars via microbial metabolism (Larsson et al., 1999).

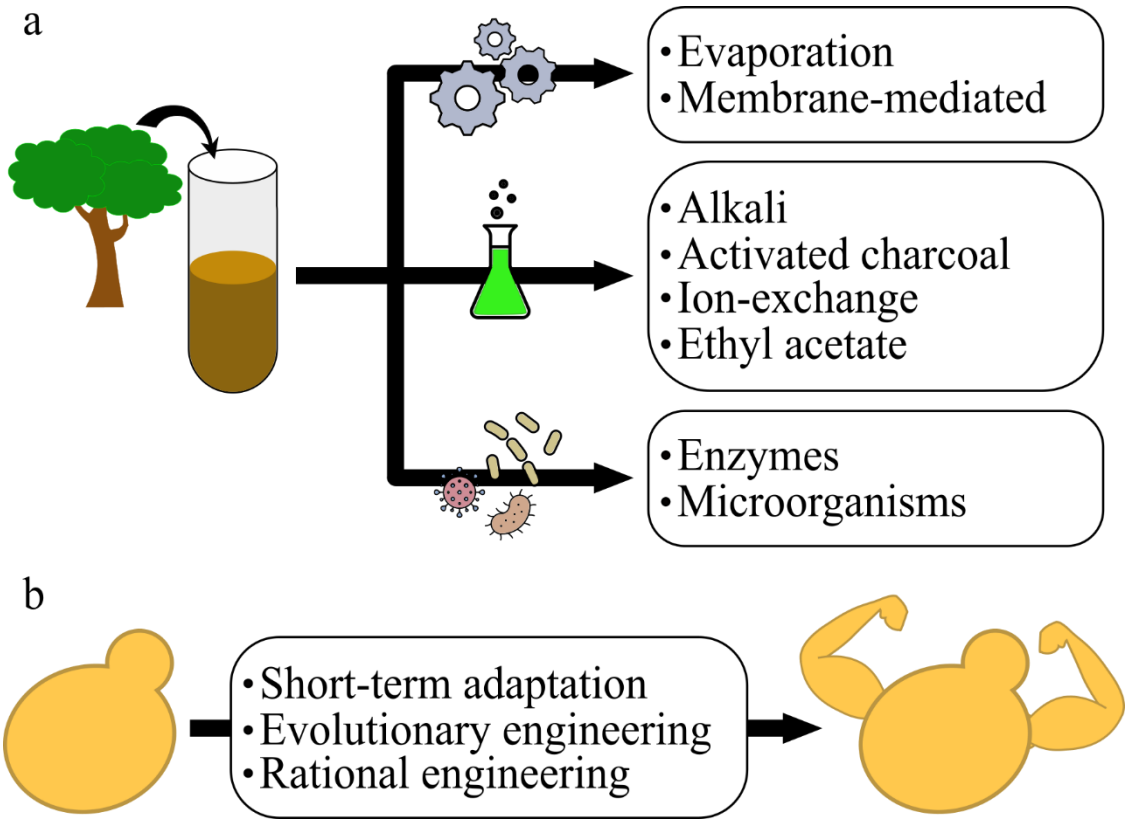

Figure 2.1: Different approaches to contrast the toxic effect of inhibitors found in lignocellulosic hydrolysates. (a) Physical (gears), chemical (flask) and biological (microorganisms) techniques to detoxify the lignocellulosic hydrolysates. (b) Different methods to improve yeast tolerance and robustness to lignocellulosic hydrolysate inhibitors.

Another approach to contrast the inhibitory effects of lignocellulosic hydrolysates is to improve tolerance of the exploited strain (Figure 2.1b). One way to achieve this is through short-term adaptation. By supplementing sub-lethal doses of hydrolysates to the propagation culture it is possible to increase the cells' robustness and stress tolerance during fermentation. The fermentation of two *S. cerevisiae* industrial strains in wheat straw hydrolysate following short-term adaptation in the same hydrolysate resulted in improved biomass yield (for one strain), viability and fermentation capacity (van Dijk et al., 2019). Short-term adaptation also led to improved ethanol yield and increased tolerance to inhibitors in a xylose-fermenting *S. cerevisiae* strain during simultaneous saccharification and co-fermentation (Nielsen et al.,

2015). A recent study revealed substantial changes, during the propagation step, in genes involved in oxidative stress response, detoxification, multidrug transport, biotin and thiamine metabolism following short-term adaptation to wheat straw hydrolysate (van Dijk et al., 2021). Nevertheless, the cellular mechanisms regulating short-term adaptation remain poorly known.

Yeast tolerance to inhibitors can be improved also through evolutionary engineering, whereby the cells are subjected to mutations and/or cultivated under conditions that mimic the natural selective pressure to improve one or more traits (Brandt et al., 2019). In such settings, the choice of suitable screening conditions is crucial to achieve the desired improvement. Multiple *S. cerevisiae* strains with increased tolerance towards inhibitors have been developed following this approach. For example, a furan-resistant *S. cerevisiae* strain, with shorter lag phase compared to its parental strain, was developed by long-term adaptation to progressively higher concentrations of furfural and 5-hydroxymethylfurfural (HMF) (Heer & Sauer, 2008). Similarly, sequential cultivations of a *S. cerevisiae* strain in the presence of increasing concentrations of coniferyl aldehyde, led to increased tolerance to coniferyl aldehyde but also ferulic acid, vanillin, and 4-hydroxybenzaldehyde (Hacısalihoglu et al., 2019). Due to its effectiveness, evolutionary engineering is widely utilized in industrial strain development (Winkler & Kao, 2014). However, the unpredictability and length of experiments are still important limitations affecting this technique. Furthermore, its broad configuration makes it difficult to manipulate traits other than growth and substrate consumption in the presence of specific conditions.

Rational engineering has the potential to drastically reduce the uncertainty and duration of experiments by targeting specific genes or sets of genes to generate desirable phenotypes. Rational engineering exploits genome editing to insert, delete, replace or modify the DNA of one or multiple genes simultaneously. This can be achieved with different techniques, such as plasmid expression, homology-dependent recombination, restriction enzymes (meganucleases), zinc finger nucleases, transcription activator-like effector-based nucleases, and the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system (Adebami et al., 2022). Owing to its effectiveness, accuracy, simplicity, and low cost, CRISPR/Cas9 has become the method of choice for genome editing. With rational engineering, it is possible to directly modify the coding sequence (protein engineering), regulate expression (promoter engineering) or fully add/delete one or more genes (genetic engineering) (Adebami et al., 2022). While yeast protein engineering has been mainly employed to improve the uptake of pentoses or hexose transport (Jansen et al., 2017), promoter as well as genetic engineering have been successfully utilized to develop strains with enhanced tolerance towards different stressors (Table 2.1). While rational engineering effectively improves yeast tolerance to lignocellulosic hydrolysate inhibitors, it requires that the genetic determinants of the desired phenotype are known. In the next section, common methods to investigate the role of specific genes in the stress response are presented.

Table 2.1: Examples of genes the deletion, overexpression or integration of which led to altered tolerance towards inhibitors.

Genes	Mutation	Condition	Phenotype	Study
HAA1	Deletion	MM4 + 50-60 mM acetic acid	Higher acetic acid sensitivity	(Fernandes et al., 2005)
	Overexpression	YPD + 0.5% acetic acid	Higher acetic acid tolerance	(Inaba et al., 2013; Tanaka et al., 2012)
YAP1	Deletion	SC + 15 mM HMF	Higher HMF sensitivity	(Ma & Liu, 2010)
	Overexpression	SC + 20-40 mM furfural or 30-40 mM HMF	Higher furfural and HMF tolerance	(Kim & Hahn, 2013)
IrrE	Integration	SC-URA + 0.8 g L-1 furfural, 3.0 g L-1 acetic acid and 0.3 g L-1 phenol	Higher tolerance to multiple stress	(Wang et al., 2020b)
HAA1	Overexpression	YPX or YPD + 4 g L-1 acetic acid	Higher acetic acid tolerance and sugar consumption	(Cunha et al., 2018)
PRS3	Overexpression			
TPS1	Overexpression	YPD + 0-18% ethanol, 10 mM furfural or 30 mM HMF	Higher ethanol, furfural and HMF tolerance	(Divate et al., 2017)
ARI1	Overexpression			
NTH1	Deletion			

Investigating the role of specific genes in the stress response

A classic approach for exploring the role of a specific gene in response to a certain stress is to delete and/or overexpress the target gene, test the resulting strain in the presence of the stressor, and analyze the observed phenotype (Table 2.1). In 1999, the EUROSCARF library harboring deletion strains for each annotated yeast gene was launched (Winzeler et al., 1999). Later on, an overexpression library covering 97% of the yeast genome was produced (Jones et al., 2008). Mutants generated in both deletion or overexpression libraries have been used in genome-wide or gene-specific investigations (Ding et al., 2015; Mira et al., 2010a; Sousa et al., 2013). Different studies employing these libraries to assess acetic acid and formic acid tolerance will be presented in Chapter IV.

Investigating the function of genes by producing deletion mutant is a useful technique, but it cannot be employed to study the role of essential genes in stress response of haploid strains, as such mutants are often not viable. Haploid cell lines are usually preferred for genetic investigations as they facilitate the isolation of mutants and interpretation of the resulting phenotypes. While it is not possible to produce viable mutants in haploid cell-lines when deleting essential genes, it is possible to replace the promoter of the target gene with a weaker one to mitigate its expression. Following this approach, a library of mutants, each expressing a

different essential gene downregulated by a $tetO_7$-promoter was generated (Mnaimneh et al., 2004). Up- or downregulation of essential genes can be achieved also using CRISPR activation (CRISPRa) or CRISPR interference (CRISPRi), respectively (Qi et al., 2013). CRISPRa/i are extensions of the CRISPR/Cas9 techniques, whereby the endonuclease activity of Cas9 driven by a guide RNA (gRNA) cuts a DNA sequence at a specific genetic location and achieves precise genome editing through homology-dependent recombination (Meng et al., 2020) (Figure 2.2). Unlike the classic CRISPR/Cas9 system, CRISPRa/i rely on an endonuclease-deficient Cas9 (dCas9) often fused to a transcriptional activator (CRISPRa) or repressor (CRISPRi) capable of promoting or blocking the recruitment of the transcriptional machinery to the target gene (Qi et al., 2013) (Figure 2.2). The level of induction or repression depends on the promoter's region targeted by the gRNA (Smith et al., 2016). The considerable interest raised by such systems is testified by several CRISPRi/a libraries generated in the past years, targeting from a few to all the genes of *S. cerevisiae* (Gilbert et al., 2014; Gutmann et al., 2021; Jaffe et al., 2019; Lian et al., 2019; Momen-Roknabadi et al., 2020; Smith et al., 2017). Only a few screenings have relied on the CRISPRa system alone, likely due to the unpredictable steric repression that the dCas9-activator complex can sometimes exert by binding to different regions on the target gene (Dong et al., 2020). Instead, numerous tolerance screenings have exploited the CRISPRi system. Accordingly, Cámara et al. (2020) revealed that downregulation of *SSK2* in an industrial *S. cerevisiae* strain improved tolerance to wheat straw hydrolysate. In another study, a CRISPRi screening confirmed the pivotal role of known genes, such as *HAA1*, *YAP1* or *STB5*, in growth on spruce hydrolysates (Gutmann et al., 2021). The same study also uncovered novel genes, whose downregulation either improved (*BUB1*, *DOT6*, *SKO1*) or lowered (*CDC15*, *UGA3*) tolerance towards the hydrolysate. A *S. cerevisiae* CRISPRi strain library, in which 98% of all essential and respiratory growth genes are singularly targeted in each strain, has been screened via the Scan-o-matic phenomics platform (Zackrisson et al., 2016) for tolerance towards acetic (Mukherjee et al., 2021) and formic acid (Mukherjee et al., 2023). The screenings revealed an important role for *GLC7*, *YPI1*, and *RPN9* in response to acetic acid stress, and *TIF34* and *HSF1* to formic acid. In **Paper II** and **Paper III**, the same CRISPRi library was screened to investigate the genes involved in stress response to acetic and formic acid utilizing a transcription factor-based biosensor or competitive growth assay, followed by barcode sequencing (see Chapter IV).

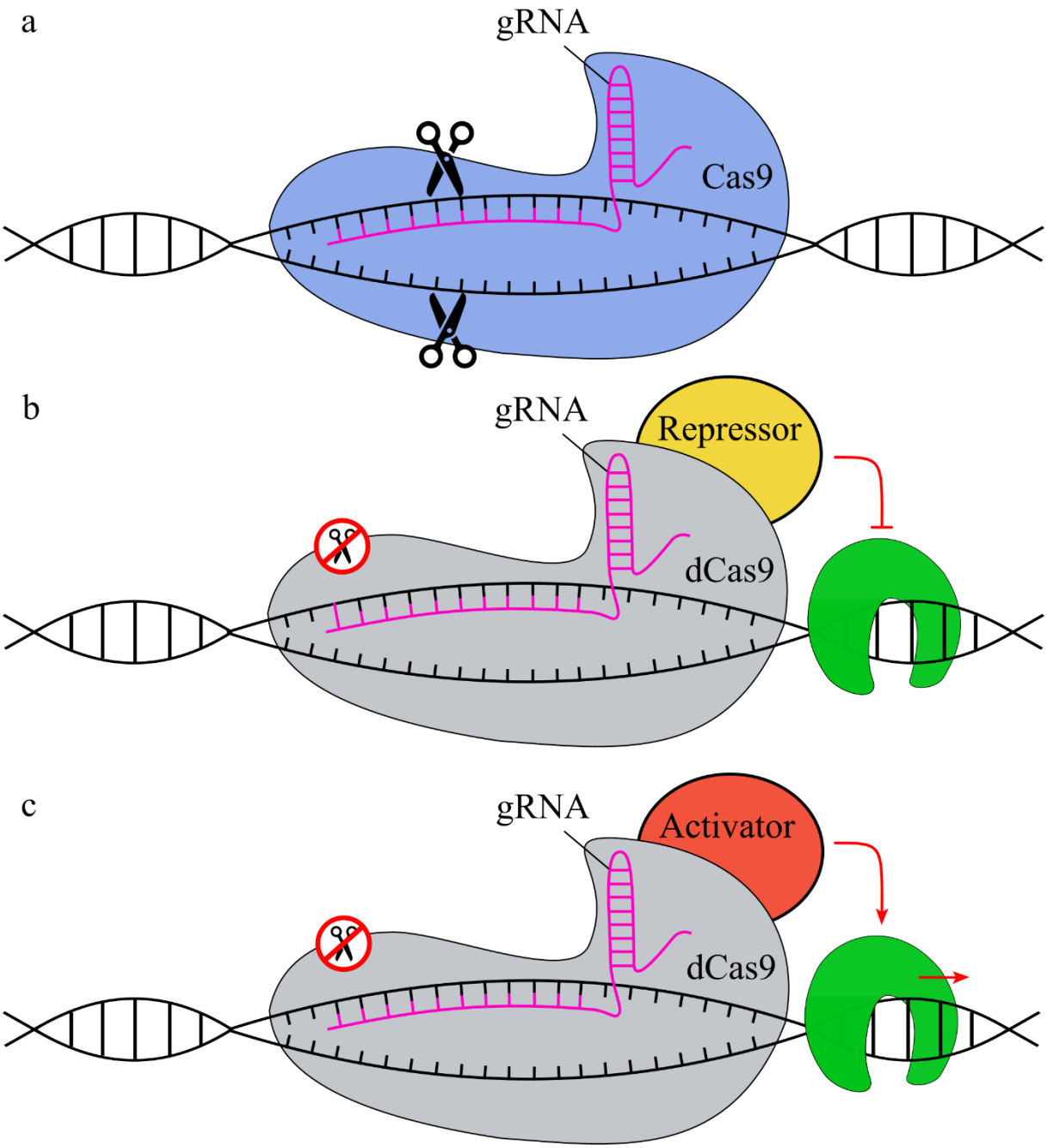

Figure 2.2: Schematic representation of CRISPR/Cas9 technologies. a) Classic CRISPR/Cas9, with gRNA driving the Cas9 endonuclease to the target sequence, where it can cleave the DNA. b) CRISPRi and c) CRISPRa mechanisms for gene repression or induction, respectively. Cas9 is deprived of its endonuclease activity (dCas9) and fused to a repressor/activator to prevent or facilitate the recruitment of the transcription machinery (here represented in green) and expression of the target gene.

While tuning the expression of a single gene may provide information over its role in the tolerance towards a certain stressor, it is harder to draw conclusions in relation to the general mechanisms regulating the stress response. For the latter, omics studies are preferable, as they offer insights on the overall status of the cell by providing a comprehensive overview of DNA

and its binding proteins (genomics and epigenomics), RNA (transcriptomics), proteins (proteomics), and metabolites (metabolomics) (Yamada et al., 2021). Various omics studies have uncovered the key components involved in yeast tolerance towards different stress factors. The metabolic profile of a *S. cerevisiae* strain tolerant to a mixture of inhibitors found in lignocellulosic hydrolysate was analyzed and compared to the parental strain, revealing a different concentration of amino acids, inositol, phenethylamine, and pyrimidines between the two strains (Ding et al., 2012). Genomic comparison between *S. cerevisiae* strains obtained through laboratory evolution and the native strain identified the genes *ASG1*, *ADH3*, *SKS1*, and *GIS4* to be responsible for acetic acid tolerance (González-Ramos et al., 2016). A proteomic study comparing two *S. cerevisiae* isolates exhibiting medium and high tolerance towards lignocellulosic hydrolysate inhibitors revealed increased expression of proteins related to energy management in the most tolerant strain (De Witt et al., 2019). The authors suggested a detoxification mechanism based on enhanced cofactor supply and energetic management of cellular processes. Transcriptomic studies, alone or combined with other omics approaches, may help improve yeast tolerance towards lignocellulosic inhibitors. While RNAseq is a powerful transcriptomic technique, the expression of a gene does not necessarily correspond to a proportional change in the intracellular content of its protein product (Liu et al., 2016). In fact, the latter depends both on its synthesis (regulated at a transcriptional, translational, and post-translational level) as well as its degradation. Nevertheless, several transcriptomic studies have been proven useful for the identification of target genes with which to engineer stress-resistant strains. A strain of *S. cerevisiae* tolerant to multiple stressors, such as weak acids, ROS, and ethanol, was created by overexpressing the H^+-ATPase-encoding gene *PMA1* (Lee et al., 2017), following the results of a previous transcriptomic analysis (Lee et al., 2015). The same transcriptomic study led to the development of two acetic acid tolerant strains through the overexpression of *ASC1* and *GND1* (Lee et al., 2015). Another transcriptomic investigation uncovered the role of *YAP1* in *S. cerevisiae* adaptation to HMF (Ma & Liu, 2010). These results contributed to the development of a furfural and HMF tolerant strain, overexpressing *YAP1* and two of its target genes (*CTA1* and *CTT1*) (Kim & Hahn, 2013).

Bioethanol production, along with other biotechnological production processes, is typically run anaerobically. However, most transcriptomic studies during stress with hydrolysates relevant for bioprocesses have been conducted under aerobic conditions, which require less advanced instrumentation. A further complication arises from strain-dependent variability in responding to lignocellulosic hydrolysates (Cámara et al., 2022). The transcriptome of five *S. cerevisiae* strains, one laboratory strain, two industrial strains, and two wild-type isolates, was investigated in **Paper IV** under anaerobic conditions using wheat straw hydrolysate as feedstock. In the following section, the pathways and specific genes involved in tolerance to lignocellulosic hydrolysates will be discussed based on the findings from **Paper IV**.

Strategies leading to yeast tolerance to lignocellulosic hydrolysates

Adaptation of *S. cerevisiae* to growth on lignocellulosic hydrolysates is a complex process that involves several groups of genes and molecules. Most studies on this topic have focused on

elucidating the effects of single inhibitors, such as acetic acid, formic acid, HMF, furfural, and phenols; whereas only a few have tackled the synergistic effects of different inhibitors (Cunha et al., 2019). Other evidence points to dependence on the genetic background of the strains (Cámara et al., 2022). Collectively, these studies have identified common strategies regulating the response of yeast cells to hydrolysates, including the control of pH homeostasis through ATP-dependent proton extrusion, neutralization of oxidative stress through cofactor renewal, cell wall and plasma membrane remodeling, and regulation of transporters. The transcriptomic analysis performed in **Paper IV** on five different strains of *S. cerevisiae* cultivated on wheat straw hydrolysate further clarified these strategies by identifying strain-dependent differences in gene expression. The strains included a commonly used laboratory strain of the CEN.PK linage, two industrial strains used for bioethanol production (KE6-12 and Ethanol red) and two strains isolated from cachaça distilleries (LBCM31 and LBCM109) (**Paper IV: Table 1**).

Proton pumps, pH homeostasis and ATP renewal

The importance of the H^+-ATPase in counteracting the stress elicited by hydrolysate inhibitors has been reviewed by Guaragnella & Bettiga (2021). Cytosolic acidification resulting from weak acids, such as acetic or formic acid (see Chapter I), is countered by the extrusion of excess protons. The protons are pumped out of the cytosol through plasma membrane and vacuolar H^+-ATPases, thereby creating an electrochemical proton gradient across cell membranes (Palma et al., 2018). Strains overexpressing the genes *PMA1* (Lee et al., 2017) or *VMA3* (Konarzewska et al., 2017), encoding a plasma membrane and a vacuolar H^+-ATPase, respectively, displayed enhanced tolerance to acetic acid. In contrast, mutants harboring deletions of several genes encoding subunits of the vacuolar ATPase complex (namely, *VMA2*, *VMA3*, *VMA4*, *VMA5*, *VMA8*, *VMA13*, *VMA16*, *VMA21*, and *VMA22*) were found to be sensitive to acetic acid (Mira et al., 2010a; Sousa et al., 2013). A similar sensitivity to acetic acid and/or formic acid was recorded also with CRISPRi-mediated downregulation of *VMA3*, *VMA4*, *VMA7*, and *VMA11* (Mukherjee et al., 2021; 2023). As revealed in **Paper IV** (**Figure 4**), *PMP1*, *QDR2*, and *SMF1* were the most upregulated genes in acid-tolerant wild-type LBCM109 and LBCM31 strains grown on wheat straw hydrolysate compared to the industrial Ethanol Red strain. Pmp1 is a regulator of Pma1 (Ambesi et al., 2000), while Qdr2 and Smf1 are both plasma membrane transporters involved in cation efflux. The strong induction of *PMP1*, *QDR2*, and *SMF1* in LBCM strains was in line with the increased demand for proton efflux imposed by the weak acids contained in the hydrolysate (**Paper IV: Table 2**).

The H^+-ATPase activity is key for maintaining the intracellular physiological pH upon weak acid stress. This is particularly relevant during acetic acid stress, whereby the cell's ability to restore the cytosolic pH is a major cause of growth inhibition (Ullah et al., 2012). Proton pumping consumes up to 20% of the cellular ATP for cells actively growing on glucose (Morsomme et al., 2000). Adequate energy regulation during acid stress, when H^+-ATPase activity is essential, is crucial to ensure sufficient ATP for detoxification, as well as for its physiological activities. Not surprisingly, central carbon metabolism is rewired in response to different inhibitors, such as organic acids (Guo & Olsson, 2014; Li et al., 2020; Li & Yuan,

2010) and furfural (Chen et al., 2016a). Furthermore, a previous study found that *S. cerevisiae* appeared to regulate the metabolism of specific amino acids to facilitate a short path towards the tricarboxylic acid (TCA) cycle in response to HMF (Ma & Liu, 2010). In particular, the authors speculated that by enhancing proline, serine, and alanine catabolism, as well as downregulating arginine biosynthesis, cells were likely to boost ATP and NAD(P)H regeneration via the TCA cycle. Altogether, these studies suggest that the regulation of specific pathways of central carbon metabolism may be one of the strategies through which yeast adapts to different inhibitors, ensuring the production of intermediate substrates for adequate energy and NAD(P)H regeneration.

Cofactor renewal and oxidative stress

Regulation of central carbon metabolism during stress plays an important function in the renewal of NAD(P)H cofactors via the TCA cycle or the pentose phosphate pathway. NAD(P)H has been proposed to play a role in the response to acetic acid-induced acidification of the cell (Dong et al., 2017). Furthermore, NAD(P)H acts against oxidative stress by detoxifying the ROS released from HMF, furfural, and inhibitors (Almeida et al., 2007; Perrone et al., 2008). The activity of functional reductases and other enzymes, such as Adh6, Adh7, Ald4, Ari1, Ari2, Ari3, Oye3, Gre2, and Gre3, is linked to their capability to reduce inhibitors found in lignocellulosic hydrolysates while promoting NAD(P)H oxidation (Alriksson et al., 2010; Heer et al., 2009; Lewis Liu et al., 2008; Liu & Moon, 2009; Nilsson et al., 2005; Petersson et al., 2006).

Genes involved in counteracting oxidative stress were significantly differentially regulated in the transcriptomic analysis described in **Paper IV**. The "Oxidation-reduction processes" GO term (GO:0055114) was always enriched in wild-type LBCM strains when compared to the other strains used in **Paper IV** (CEN.PK113-7D, Ethanol red and KE6-12) (**Paper IV: Table S2**). Out of the 290 genes belonging to this GO term, 124 were significantly upregulated in LBCM109 compared to LBCM31; whereas 61 were significantly downregulated (**Paper IV: Figure 5**), suggesting a disparity between the two LBCM strains. The most upregulated genes in LBCM109 were *BNA6*, *DOG2*, and *YHB1* (Figure 2.3a), which encode proteins involved in the oxidative stress response. *BNA6* encodes a quinolinate phosphoribosyl transferase, required for the biosynthesis of NAD from tryptophane (Panozzo et al., 2002). The deletion of *BNA6* boosts growth in medium supplemented with a hydrolysate-inhibitor cocktail, while its overexpression has the opposite effect (Sardi et al., 2018). *DOG2* encodes a phosphatase, which is induced during oxidative and osmotic stress (Tsujimoto et al., 2000). Deletion of *DOG2* has been shown to significantly affect the cytosolic redox status under steady-state conditions (Ayer et al., 2012). *YHB1* encodes a flavohemoglobin oxidoreductase targeting nitric oxide (Zhao et al., 1996). Overexpression of *YHB1* has been reported to result in enhanced ethanol tolerance and productivity in medium containing 5 g L^{-1} acetic acid (Lamour et al., 2019). *YHB1*, *DOG2*, and *BNA6* are all regulated by Sfp1 and Yap1. As shown in **Paper IV**, *SFP1* was upregulated in LBCM109 compared to LBCM31, while *YAP1* was slightly downregulated. Sfp1 is a transcription factor controlling several functions, including the response to nutrients and stress

(Marion et al., 2004), but also transcription of ribosomal proteins (Zencir et al., 2020). *SFP1* overexpression was found to improve ethanol production of an industrial strain in the presence of acetic acid and furfural (Chen et al. 2016a). Furthermore, deletion of *SFP1* has been shown to result in increased susceptibility to wheat straw hydrolysate (Pereira et al. 2014). Yap1 is the main transcription factor regulating oxidative stress responses (Delaunay et al., 2000) and its mild downregulation observed in **Paper IV** in LBCM109 compared to LBCM31 was rather surprising in light of *YHB1*, *DOG2*, and *BNA6* induction. However, it should be noted that Yap1 regulation is based chiefly on nuclear re-localization upon oxidative stress, rather than its absolute levels (Kuge et al., 1997). Moreover, when van Dijk et al. (2021) compared the transcriptomes of *S. cerevisiae* adapting or not to wheat straw hydrolysate, they found that many genes encoding transcription factors relevant for stress responses were downregulated in the adapted cultures.

Transcriptomic analysis in **Paper IV** revealed that *GTT1* was expressed at a higher level in the two tolerant LBCM strains than in the CEN.PK isolate (Figure 2.3). *GTT1* encodes a glutathione S-transferase associated to the endoplasmic reticulum (Choi et al., 1998) and its upregulation (Thompson et al., 2016) or downregulation (Ask et al., 2013) has been previously reported upon exposure to a mixture of inhibitors found in lignocellulosic hydrolysates. Glutathione functions mainly as an antioxidant agent (Carmel-harel & Storz, 2000; Grant et al., 1998a). In **Paper IV**, the analysis of genes involved in glutathione metabolism revealed that, besides *GTT1*, other genes (*URE2*, *GLR1*, *IDP3*, *IDP2*, *GND2*, *ZWF1*, *PRX1*) were upregulated in both LBCM strains compared to CEN.PK (Figure 2.3). These genes encode proteins involved in fundamental steps of glutathione metabolism, such as glutathione recycling (*GLR1*) (Collinson & Dawes, 1995), glutathione-mediated reduction of oxidized molecules (*URE2*, *PRX1*) (Bai et al., 2004; Darren Greetham & Grant, 2009), and NADPH renewal (*IDP3*, *IDP2*, *GND2*, *ZWF1*) (Henke et al., 1998; Loftus et al., 1994; Nogae & Johnston, 1990; Sinha & Maitra, 1992). At the same time, the expression of *GSH2*, which encodes an ATP-dependent glutathione synthase (Inoue et al., 1998), was repressed in both wild-type strains compared to CEN.PK (Figure 2.3). While *GSH2* repression may lead to a lower ATP consumption, the induction of different genes involved in glutathione metabolism may result in increased glutathione-dependent antioxidant activity accompanied by greater NADPH renewal.

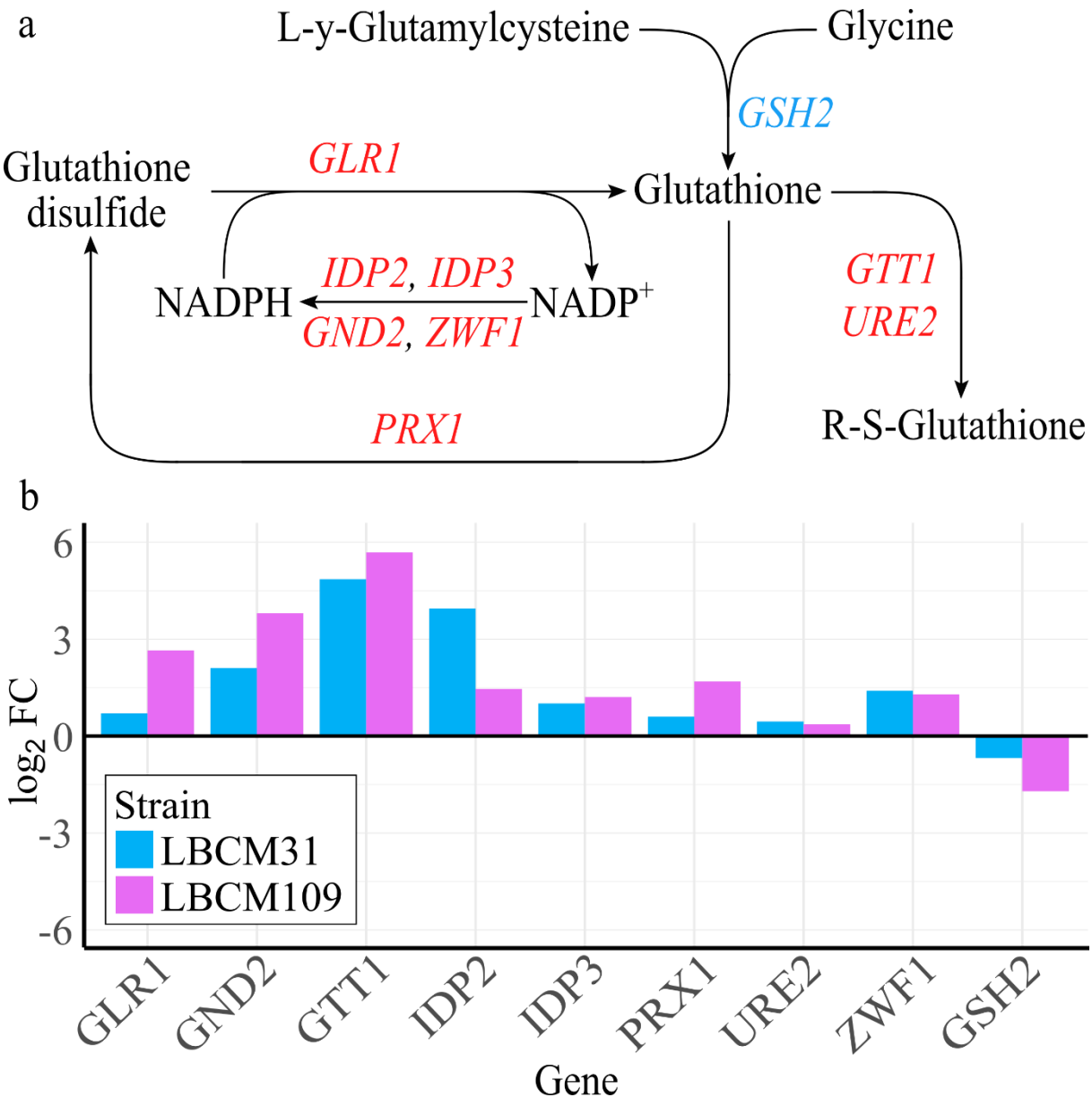

Figure 2.3: Expression of genes related to glutathione metabolism, as observed in Paper IV. a) Schematic map depicting the metabolic pathway of glutathione. Elements in red and blue represent genes significantly (adjusted p value < 0.01) upregulated and downregulated in both LBCM strains compared to CEN.PK113-7D. b) Differential expression of genes related to glutathione metabolism in LBCM31 (blue bars) and LBCM109 (purple bars) compared to CEN.PK113-7D. The values were recorded at the end of anaerobic cultivation in medium supplemented with wheat straw hydrolysate (Paper IV). The level of each gene is visualized as $\log_2$ of the fold change ($\log_2$ FC). Significance was defined as adjusted p value < 0.01 and fold change ≥ 2. Data were obtained from four biological replicates.

Cell wall and plasma membrane remodeling

The cell wall constitutes the outermost barrier separating the yeast cell from the outside environment. An intact cell wall is crucial to avoid external stressors (Li et al., 2022). The cell wall of *S. cerevisiae* is composed of four main macromolecules: proteins, 1,6-beta-glucan, 1,3-beta-glucan, and chitin (Orlean, 2012). The expression of genes responsible for the biosynthesis of cell wall components varies according to environmental conditions (Liu & Ma, 2020). Upon stress, *S. cerevisiae* promotes the remodeling of the cell wall by regulating protein activity and gene transcription (Levin, 2011). The cell wall integrity pathway is activated in response to various inhibitors found in lignocellulosic hydrolysates, including furfural and HMF (Liu et al., 2018; Zhou et al., 2014). As polysaccharides account for approximately 90% of the cell wall (Latgé, 2007), carbohydrate metabolism is likely to influence cell wall composition. In **Paper IV**, the "Carbohydrate metabolic process" GO term (GO:0005975) was enriched in LBCM109 but not LBCM31. Three of the 10 most upregulated genes in this GO term, namely *SCW4*, *GAS2*, and *GAS5*, are related to the cell wall. *SCW4* encodes a cell wall protein (Cappellaro et al., 1998), whereas *GAS2* and *GAS5* encode 1,3-beta-glucanosyltransferases (Ragni et al., 2008). Mutants harboring deletions of *SCW4* or *GAS5* exhibit altered cell wall properties (Yin et al., 2005). Transcriptomic analysis performed in **Paper IV** revealed that, besides *GAS2* and *GAS5*, other genes involved in 1,3-beta-glucan synthesis (*GSC2*, *FKS1*) or regulation (*GAS1*, *GAS4*, *WSC3*) were significantly induced in LBCM109 compared to LBCM31 (Figure 2.4a). Three of the genes involved in 1,6-beta-glucan synthesis were differentially regulated between the wild-types in **Paper IV**: *SKN1* and *PSK1* were induced, whereas *KEG1* was repressed in LBCM109 (Figure 2.4a). The chitin synthase-encoding gene *CHS1* was upregulated in LBCM109 compared to LBCM31 (**Paper IV**), along with other genes involved in chitin metabolism (*CHS5*, *CTS1*, *BGL2*), except *CHS7* and *CTS2* (Figure 2.4a). The differential expression of genes involved in the metabolisms of cell wall polysaccharides observed in **Paper IV** might be part of a different adaptive strategy to inhibitors. In particular, the upregulation in LBCM109 of different genes responsible for the synthesis and elongation of glucans may correlate with greater incorporation of these molecules in the cell wall. In line with that, a recent study observed a higher glucan content in the cell wall and an increased stiffness of cells exposed to sub-lethal concentrations of acetic acid (Ribeiro et al., 2021).

The cell wall detects and communicates cell surface stress through five sensors (Wsc1, Wsc2, Wsc3, Mid2, and Mtl1), which reach into the plasma membrane and may induce its remodeling in response to a specific stress (Kock et al., 2015). Furfural, for instance, has been reported to alter cell membrane permeability of *S. cerevisiae*, causing the leakage of intracellular content and ultimately impairing growth (Zeng et al., 2022). In yeast, trehalose accumulation has been associated with remodeling and integrity of the plasma membrane during heat (Mensonides et al., 2014), oxidative (Alvarez-Peral et al., 2002), and weak acid stress (Guo & Olsson, 2014). Additionally, yeast can adapt to stress by rearranging the lipid composition of the plasma membrane (Lindberg et al., 2013). In **Paper IV**, a comparison between LBCM109 and LBCM31 revealed that 28 of the 50 genes comprised in the GO term "Lipid metabolic process" (GO:0006629) were differentially regulated between the two isolates (**Paper IV: Figure 5**). Those genes encoded proteins regulating the metabolism and transport of a variety of lipids,

including fatty acids (*OLE1*, *CAT2*, *YAT1*, *YAT2*), sphingolipids (*FAA4*, *FAA1*, *NCR1*, *LCB4*, *LAC1*, *LIP1*, *LAG1*, *LCB3*), acylglycerols (*PLC1*, *TGL3*, *DGA1*), phospholipids (*TGL4*, *SFK1*, *TGL5*, *NTE1*, *PGC1*, *GDE1*, *ARV1*, *DCI1*), and sterols (*YEH1*, *YEH2*) (**Paper IV: Figure 6b**). Several of the above genes encoding for proteins promoting the accumulation of sphingolipids (*FAA4*, *FAA1*, *NCR1*, *LCB4*, *LAC1*, *LIP1*, *LCB3*) or phospholipids (*TGL4*, *SFK1*, *TGL5*, *NTE1*, *PGC1*) were upregulated in LBCM109. Sphingolipids and phospholipids are important components of cellular membranes (Hannun & Obeid, 2018). High levels of complex sphingolipids have been associated with natural acetic acid tolerance of the yeast *Zygosaccharomyces bailii* (Guerreiro et al., 2016; Lindahl et al., 2016; Lindberg et al., 2013). Furthermore, *S. cerevisiae* exposed to weak acid stress has been reported to contain more very-long-chain fatty acids, which are the precursors of sphingolipids (Guo et al., 2018). The same study also noted that acid stress resulted in a different phospholipid composition, and that overexpression of *OLE1* led to an increased ratio of unsaturated fatty acids in the plasma membrane, along with higher tolerance to acetic, formic, and levulinic acid. Ole1 is the only Δ-9 fatty acid desaturase in *S. cerevisiae* and is required for the production of monounsaturated fatty acids. In **Paper IV**, *OLE1* was the most upregulated gene under the GO term "Lipid metabolism" in LBCM109 compared to LBCM31. Altogether, distinct expression of genes involved in fatty acid, sphingolipid, phospholipid, and sterol metabolisms may hint at different strategies between the two isolates with respect to remodeling of lipids in the cell membrane during growth on lignocellulosic hydrolysates.

Lastly, ergosterol biosynthesis has been reported to affect plasma membrane function. Guo et al. (2018), revealed a change in the ergosterol content of *S. cerevisiae* upon organic acid stress. Furthermore, deletion of genes involved in ergosterol synthesis was shown to have an impact on acetic and formic acid tolerance by *S. cerevisiae* (Cámara et al., 2022). In **Paper IV** several genes involved in ergosterol biosynthesis were significantly induced in LBCM109 compared to LBCM31. These genes included *ERG27*, *ERG4*, *ERG28*, *ERG26*, *ERG9*, *ERG20*, *ERG1*, and *ERG29* (Figure 2.4b). Collectively, the transcriptomic differences between the two LBCM strains in relation to lipid and ergosterol metabolism may suggest a different membrane composition for the two isolates. However, further data are required to confirm this hypothesis.

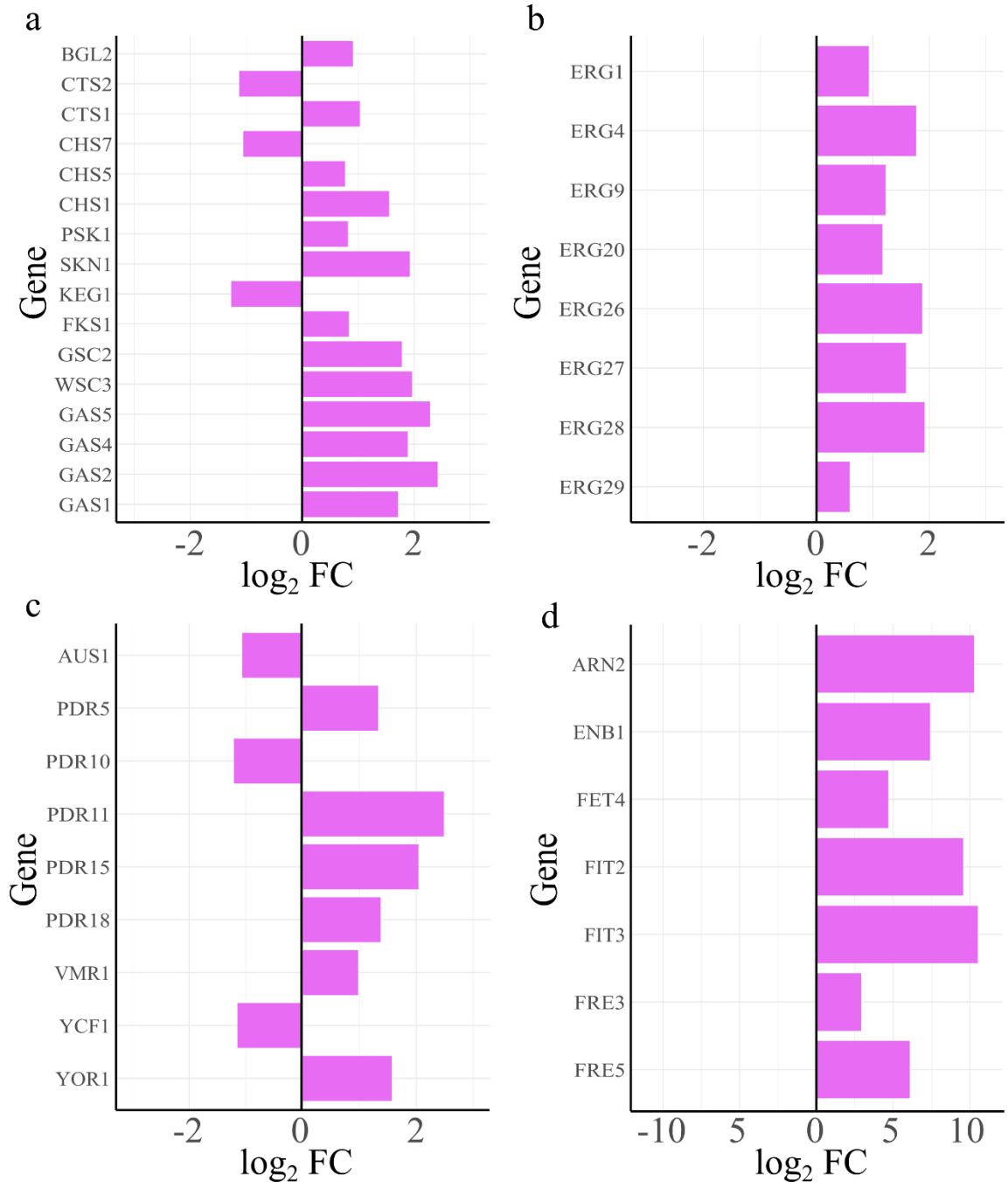

Figure 2.4: Log$_2$ of the fold change (log$_2$ FC) for significantly up- or downregulated genes in LBCM109 compared to LBCM31, as observed in Paper IV. a) Genes involved in the metabolisms of cell wall 1,3-beta-glucan (*GAS1*, *GAS2*, *GAS4*, *GAS5*, *WSC3*, *GSC2*, *FKS1*), 1,6-beta-glucan (*KEG1*, *SKN1*, *PSK1*), and chitin (*CHS1*, *CHS5*, *CHS7*, *CTS1*, *CTS2*, *BGL2*). b) Genes involved in ergosterol biosynthesis. c) Genes encoding ABC transporters, involved in multidrug resistance (*YOR1*, *PDR5*, *PDR10*, *PDR15*), sterol import (*AUS1*, *PDR11*), lipid trafficking (*YOR1*, *PDR5*, *PDR15*, *PDR18*), metal stress (*YCF1*, *VMR1*), and oxidative stress (*YCF1*, *PDR5*). d) Genes involved in iron transport (*ARN2*, *ENB1*, *FET4*) and homeostasis (*FIT2*, *FIT3*, *FRE3*, *FRE5*). Significance was defined as adjusted p value < 0.01 and fold change ≥ 2. Data were obtained from four biological replicates.

Regulation of transporters

Godinho et al. (2018) found that, besides the genes for ergosterol biosynthesis, acetic acid inhibition stimulated also the expression of *PDR18*. Pdr18 is an ATP-binding cassette (ABC) transporter (Cabrito et al., 2011), which mediates the transport of ergosterol and regulates the distribution of lipids in the plasma membrane, as well as its non-specific permeability (Godinho et al., 2018). Yeast ABC transporters play crucial roles in various cellular processes, including tolerance to the inhibitors found in lignocellulosic hydrolysates (Buechel & Pinkett, 2020). Different genes encoding ABC transporters have been associated with the response to furfural and HMF (Kim & Hahn, 2013; Liu, 2011; Ma & Liu, 2010), sake mash (Watanabe et al., 2000), lignocellulosic hydrolysates (Serate et al., 2015; van Dijk et al., 2020), weak acids (Nygård et al., 2014), and ethanol (Teixeira et al., 2012). In **Paper IV**, several genes encoding ABC transporters were differentially regulated between the two LBCM isolates, namely *PDR5*, *PDR10*, *PDR11*, *PDR15*, *PDR18*, *YOR1*, *AUS1*, *YCF1*, and *VMR1*. Of these, *PDR10*, *AUS1*, and *YCF1* were downregulated in LBCM109 compared to LBCM31; while the rest were upregulated (Figure 2.4c). ABC transporters-encoding genes differentially regulated between LBCM strains participate in multidrug resistance, sterol import, lipid trafficking, metal stress, and oxidative stress (Buechel & Pinkett, 2020). Interestingly, all known genes encoding ABC transporters involved in lipid trafficking (*PDR5*, *PDR15*, *PDR18*, *YOR1*) were significantly upregulated in LBCM109 compared to LBCM31 (Buechel & Pinkett, 2020) in **Paper IV** (Figure 2.4c). In the previous section, the pivotal role of lipid regulation in yeast tolerance to different stressors was discussed. *PDR5*, *PDR15*, *PDR18*, and *YOR1* have all been reported to mediate the response to inhibitors found in lignocellulosic hydrolysates. Deletion of *PDR15* or *YOR1* was associated with improved tolerance under acetic acid stress (Sousa et al., 2013); whereas deletion of *PDR18* was shown to enhance sensitivity towards acetic acid (Godinho et al., 2018). Overexpression of *PDR5* or *YOR1* (Wang et al., 2017) improved growth upon phenolic exposure. The two genes were also overexpressed in a highly resistant sake yeast mutant when grown in sake mash; whereas their deletion dramatically reduced fermentation efficiency (Watanabe et al., 2000). The expression of *PDR5*, *PDR15*, and *YOR1* was reported to be upregulated in *S. cerevisiae* upon HMF exposure (Liu, 2011; Ma & Liu, 2010). Deletion of *YOR1* resulted in enhanced sensitivity towards switchgrass hydrolysate (Serate et al., 2015). While the role of *PDR5*, *PDR15*, *PDR18*, and *YOR1* in yeast tolerance against inhibitors found in lignocellulosic hydrolysates is still unclear, their regulation appears to be important for the response to various stressors. The differential regulation of ABC transporters involved in lipid trafficking between the LBCM strains in **Paper IV** is in line with the aforementioned differences between isolates in terms of lipid metabolism. In particular, the induction of *PDR5*, *PDR15*, *PDR18*, and *YOR1* in LBCM109 compared to LBCM31, may suggest a diverse rate of lipid transport in the two wild-type strains, supporting the hypothesis of two different regulations of the lipid metabolism.

Besides ABC transporters, several other transporter-encoding genes have been associated with the response to inhibitors present in lignocellulosic hydrolysates (Cámara et al., 2022). Transporters exert an important detoxifying function by allowing the transfer of compounds

between compartments and in-/outside the cell. Multidrug resistance transporters of the major facilitator superfamily Tpo2 and Tpo3 have been found to enhance yeast tolerance towards acetic, propionic, benzoic, and octanoic acids, likely through the active efflux of the anionic form of the acid (Fernandes et al., 2005). Instead, the high-affinity potassium transporter Trk1 was associated with yeast sensitivity to formic acid, presumably by favoring the uptake of acid from the medium (Henriques et al., 2017). Transporters can also influence the yeast response to stress by regulating the intracellular localization of certain transcription factors. A clear example of this will be discussed in Chapter IV, in relation to the nuclear exportin Msn5, which regulates the intracellular localization of major regulators of weak acid response, such as Haa1 and Msn2/4 (Bose et al., 2005; DeVit & Johnston, 1999; Kim et al., 2018). Transporters can further improve performance by favoring or reducing the uptake of nutrients and other metabolites. Amino acid supplementation has been reported to help yeast cope with different stresses (including ethanol and osmotic stress) during very high-gravity fermentation, suggesting a role for amino acid transporters in yeast tolerance to these inhibitors (Zhao & Bai, 2009). The intake and outtake of different metal ions dissolved in lignocellulosic hydrolysates, such as iron, manganese, zinc and copper, are crucial for maintaining the number of enzymatic cofactors within acceptable toxicity limits (De Freitas et al., 2003). In this regard, the role of manganese and its Hip1-mediated export during oxidative stress will be discussed in Chapter IV. Sugar transporters are fundamental to ensure the uptake of glucose and other fermentable polysaccharides necessary to sustain cell metabolism.

In **Paper IV**, the GO terms "transport" (GO:0006810) and "transmembrane transport" (GO:0055085) were both enriched in the comparison between LBCM109 and LBCM31. (**Paper IV: Figure 5**). Several genes encoding hexose (*HXT2*, *HXT3*, *HXT5*, *HXT7*, *HXT13*, *GAL2*), trehalose (*MAL11*), and maltose (*MAL31*) transporters were among the most differentially regulated genes between the two LBCM isolates (**Paper IV: Figure 6d**). In particular, the *HXT* genes and *MAL11* were induced in LBCM31 compared to LBCM109; whereas *GAL2* and *MAL31* were repressed. Overexpression of hexose transporters in *S. cerevisiae* boosts the rate of glucose (Kim et al., 2015) and xylose (Sharma et al., 2017) uptake, resulting in faster growth. A higher glucose flux into the cell may lead to faster regeneration of energy and cofactors, which could then counteract the toxic effects of inhibitors (Qiao et al., 2021). In addition, faster depletion of the glucose dissolved in the hydrolysate may induce cells to utilize alternative carbon sources such as acetic acid earlier during fermentation, speeding up medium detoxification. This idea is supported by lower final acetic acid levels in LBCM31 compared to LBCM109 cultures (**Paper IV: Table 2**). Thus, the induction of hexose transporters in LBCM31 could be part of its response to the inhibitors present in wheat straw hydrolysates.

Transcriptomic analysis performed in **Paper IV** revealed that *FET4*, *ARN2*, and *ENB1* were among the most upregulated transporter genes in LBCM109 compared to LBCM31 (Figure 2.4d). All three genes are involved in iron transport, encoding a plasma membrane iron transporter (Dix et al., 1994), a siderophore-iron chelate transporter (Yun et al., 2000), and a ferric enterobactin transmembrane transporter (Heymann et al., 2000), respectively. Analysis of the "Transporter" GO term performed in **Paper IV** showed that other genes involved in iron

homeostasis and transport were highly upregulated in LBCM109 compared to LBCM31. They include *FIT2* and *FIT3*, which encode mannoproteins involved in the retention of siderophore-iron in the cell wall (Protchenko et al., 2001), as well as *FRE3* and *FRE5*, which encode ferric reductases mediating the reduction of siderophore-bound iron (Yun et al., 2001) (Figure 2.4d). These four genes, as well as the three iron transporters Fet4, Arn2, and Enb1, were previously reported to be involved in the yeast response to inhibitors found in lignocellulosic hydrolysates. Induction of *FIT2* and *FIT3* upon exposure to furfural in two *S. cerevisiae* strains (Heer et al., 2009) was correlated with an increased demand for iron imposed by the inhibitors. Indeed, a higher uptake of iron could be beneficial during iron starvation, as iron is involved in fundamental electron transfer reactions and is a cofactor of many metabolic enzymes (De Freitas et al., 2003). However, an excessive intracellular concentration of iron could also be detrimental for the cell, as iron ions can favor the formation of ROS (De Freitas et al., 2003). While present transcriptomic data suggest that regulation of iron homeostasis constitutes a different adaptive strategy between LBCM109 and LBCM31, further evidence is needed to confirm this hypothesis.

In summary, yeast adapts its gene expression profile to the inhibitors present in lignocellulosic hydrolysates through multiple strategies and cellular components. The availability of molecules such as ATP, NAD(P)H, and glutathione is crucial to maintain the pH and oxidative balance inside the cell during inhibitors-induced stress. The cell wall and cell membrane, representing the first barriers towards the extracellular space and play a pivotal role in the yeast response to stress. Remodeling of the cell wall and membrane structures represents an important strategy to maintain their integrity. **Paper IV** showed that the regulation of genes involved in lipid and ergosterol metabolism might help mitigate the toxicity of lignocellulosic hydrolysate inhibitors. Cell membrane composition dictates also the activity of transporters harbored in it. Multiple studies, including **Paper IV**, highlight the link between the tolerance of certain strains to the regulation of specific transporters. By changing the content of transporters in *S. cerevisiae*, it may be possible to alter the flux of specific compounds across compartments or in-/outside the cell, possibly contributing to increased adaptation of the cell. Altogether, the data in this section summarize our current understanding of stress response in yeast, with **Paper IV** highlighting strain-specific mechanisms regulating lignocellulosic hydrolysate tolerance. This knowledge can be applied to engineer *S. cerevisiae* and improve its stress tolerance in biorefinery applications. In this respect, the development of advanced screening methods enabling high-throughput selection of tolerant yeast might facilitate the identification of improved strains.

III. Biosensors for high-throughput screening

Biotechnological development proceeds through the design-build-test-learn cycle. This loop is a metabolic engineering framework that offers a systematic and efficient approach to strain development according to desired specifications. Technologies such as error-prone PCR and CRISPR/Cas9 genome editing have considerably hastened the construction of yeast variants in the 'design' and 'build' steps. However, the limited availability of high-throughput screening tools has resulted in an imbalance between the number of mutants produced and the number that can be analyzed in the 'test' and 'learn' part of the cycle. While different more or less high-throughput screening techniques have become available, more tools to monitor inhibitory compounds or metabolites are required to advance the development of industrial strains. Genetically encoded biosensors are biological tools sensing molecule of interest that can be used for strain evaluation and high-throughput screening, thereby accelerating the analytical part of the above cycle. In particular, transcription factor-based biosensors have emerged as widely popular to track molecules or control metabolic pathways. Here, the design, characterization, and validation of a biosensor based on the *S. cerevisiae* transcription factor Haa1 and BM3R1 from *Bacillus megaterium* is described. The study presented in this chapter (**Paper I** and **Paper II**), proved the capability of the developed biosensor to monitor acetic acid production in *S. cerevisiae* and its potential for high-throughput screening.

High-throughput screening tools in yeast

The development of cell factories suitable for industrial production can be challenging and often requires many attempts before an optimal combination of genetic elements is constructed. While the development of recombination-mediated genetic engineering (recombineering) and CRISPR/Cas9 genome editing hastened tremendously the construction of new strains (Yilmaz et al., 2022), screening techniques to evaluate the phenotypes of these mutants have not evolved at the same pace (Zeng et al., 2020). Current methods allow for screening of individual strains using agar or microtiter plates, as well as pooled cultures through competitive growth assays or fluorescence-based sorting (Figure 3.1).

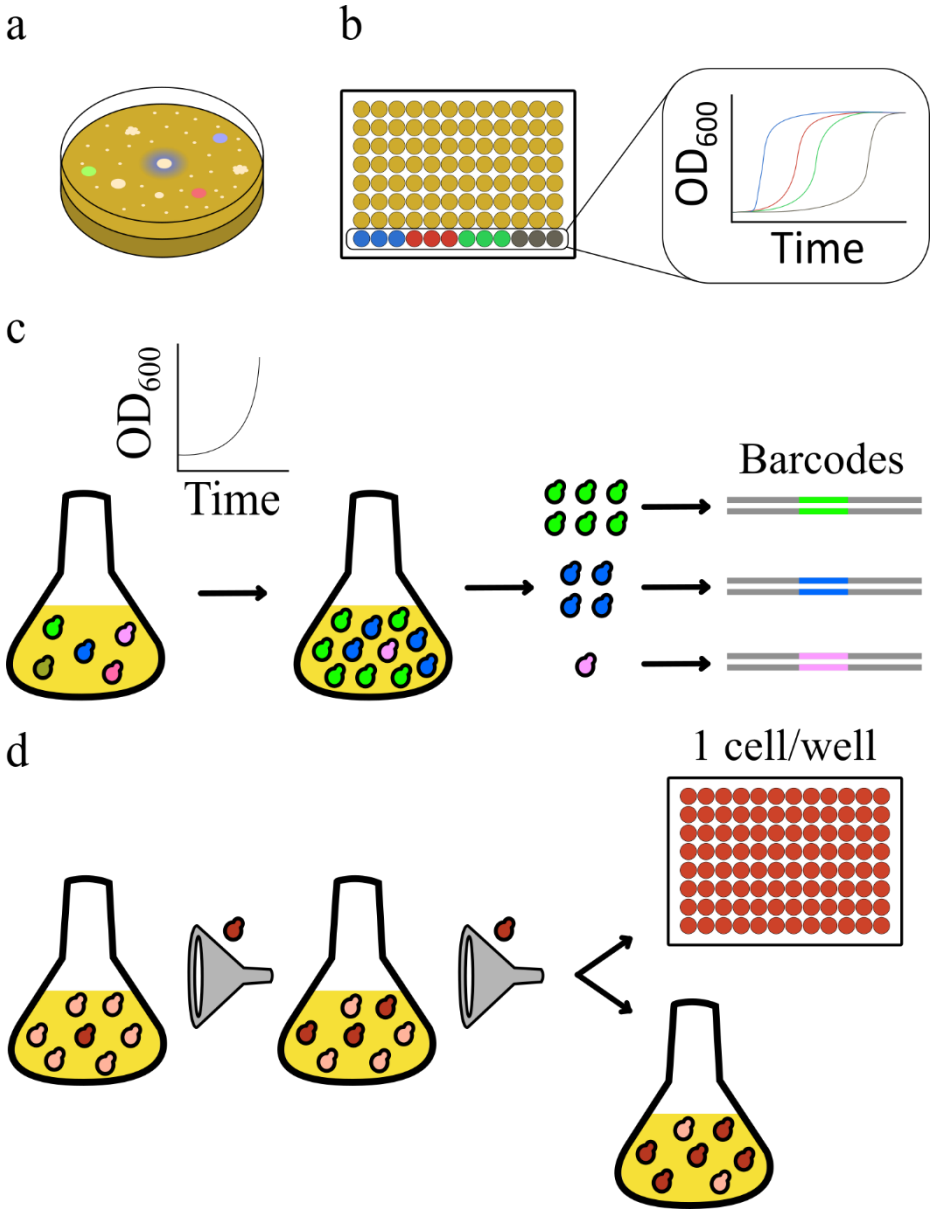

Figure 3.1. Examples of screening techniques for yeast. Schematic representation of (a) screening in an agar plate based on colony size, morphology, color or surrounding; (b) screening in a 96-well plate, where the growth of strains is monitored throughout the cultivation; (c) competitive growth assay, where cells trains with better fit for the conditions are enriched, followed by strain identification performed through genetic barcodes; (d) FACS-based high-throughput screening, where cells are sequentially selected based on their fluorescence and finally sorted in a pooled culture or in a 96-well plate, containing one cell per well.

Techniques based on agar plates usually exploit size, morphology or colorimetric changes in the colonies or their surroundings to evaluate the tested phenotypes (Zhou & Alper, 2019). A widely utilized methodology, especially in tolerance/sensitivity studies, is the spot assay, which helps evaluate the effect of genotypes and experimental conditions on cell growth and survival (Mira et al., 2010a). In spot assays, a micropipette or pins are used to spot yeast cells onto solid

medium. Cell growth and/or survival is then measured by evaluating the cell density in spots of the same size (Figure 3.1a). This method allows for testing of different strains and/or experimental conditions to assess tolerance or sensitivity (Chen et al., 2016b; Mira et al., 2010a).

Classic microtiter/well plate systems provide information on yeast growth and survival under specific conditions. While this approach is typically performed in 96-, 48-, or 24-well plates and is not ideal for very high-throughput rapid screenings (Figure 3.1b), new platforms allowing for more strains to be tested in one single experiment are emerging. One example is the Scan-o-matic microbial phenomics platform (Zackrisson et al., 2016), whereby 96, 384 or 1536 samples can be pinned on solid medium in a fully automated manner, and scanners can capture the pixel intensity of each spot over time. In this way, Scan-o-matic can be used to produce growth curves for large numbers of strains.

During competitive growth assays, strains are pooled together in a culture comprising the whole library. The proportion of strains better fit for the tested conditions will increase over time (Figure 3.1c). Competitive growth assays are often performed sequentially to allow for enrichment of the best performing strains (**Paper III**). The genetic background of the enriched strains is evaluated by DNA sequencing. Traditionally, strains may be identified by plating the culture onto solid medium and selecting random colony-forming units (CFUs) for DNA sequencing (Ding et al., 2013, 2015). In the past years, the use of barcodes (i.e., short DNA sequence markers) in strain libraries has streamlined species identification (Hebert et al., 2003), making it cheaper and overcoming the need for whole-genome sequencing to identify the mutation resulting in the phenotype of interest (**Paper III**).

Another alternative to high-throughput screening is fluorescence-activated cell sorting (FACS), which identifies cells based on fluorescence or size (**Paper II** and **III**) (Figure 3.1d). Combining FACS with immunostaining enables the identification of genetic determinants for complex phenotypes. Immunostaining exploits modified antibodies and is widely used to detect surface proteins. In its basic version, the cells are exposed to an antibody that can bind the protein of interest. When combined with FACS, the antibody is tagged with a fluorophore, implying a higher signal if more target proteins are exhibited by the cell (Schaffer & Willerth, 2017). Lian et al. (2019) utilized this approach to screen a strain library. Following sorting, the genetic background of the isolated strains can be assessed via sequencing, as described for competitive growth assays.

The screening of large yeast collections represents a bottleneck, especially when the desired outcome (e.g., growth) does not result in an easy-to-monitor phenotype (Qiu et al., 2019). Genetically encoded biosensors, including transcription factor-based biosensors, are powerful tools for rapid strain evaluation and hold great potential in high-throughput screening (Qiu et al., 2019).

Genetically encoded biosensors

Genetic biosensors can be defined as molecular devices that sense a molecule or compound of interest in-/outside the cell and generate a detectable output signal in response (Williams et al., 2016). Living organisms express endogenous genetic biosensors to sustain a highly dynamic yet also tightly regulated cellular metabolism. Signaling and regulatory systems sensitive to environmental conditions as well as to specific extra-/intra-cellular compounds are examples of natural biosensors. They have inspired the development of genetically encoded biosensors, exploitable for different metabolic engineering purposes (Qiu et al., 2019). The applicability of a biosensor to a study is tightly linked to the characteristics of said biosensor. On the one hand, biosensors relying on an on/off mechanism are suitable for dynamic pathway regulation. On the other hand, high-throughput screenings that aim to distinguish between cells with high and low amounts of a target compound, likely require biosensors expressing a gradient of outputs in response to the concentration of target compound. Plotting the biosensor output against the relative compound concentration generates a response curve, which is used to validate and describe the performance of a biosensor (Ang et al., 2013) (Figure 3.2a).

The main parameters associated with the response curve are the dynamic and operational range, which dictate the shape of the curve (Figure 3.2a). The dynamic range describes the degree of induction of the biosensor, and corresponds to the difference between maximal and minimal activation (Chen et al., 2018). The operational range denotes the concentration of the target molecule for which the biosensor shows a change in output signal. A small dynamic range might reduce the operational range, as it might be difficult to distinguish between the activation signal induced by the target compound and background noise (Figure 3.2b). In contrast, a wide dynamic range does not necessarily entail a broad operational range. Depending on the sensitivity of the biosensor, the output signal could shift from minimum to maximum with relatively small changes in the concentration of the target compound. The sensitivity of the biosensor is visually represented by the slope of the response curve (Figure 3.2a): a steep slope describes an on/off system, while a gentle slope implies a gradual increase of the output signal.

Another important biosensor feature is the specificity of the system, which describes how and if the sensor can be activated by a predefined set of potential ligand molecules. If none of the potential ligand molecules were present in the host, a biosensor could be applied even when its specificity is low (De Paepe et al., 2017). Lastly, orthogonality is another property that needs to be taken into account, as it determines the applicability of a biosensor. Orthogonality conveys the impact of the biosensor on the host metabolism and vice-versa, including eventual crosstalk or interactions with regulatory molecules other than the target compounds. Moreover, it describes whether and to what extent the biosensor can be functional in different hosts (strains or species). Poor orthogonality hinders robust and predictive output signals and can prevent the utilization of the biosensor in a host.

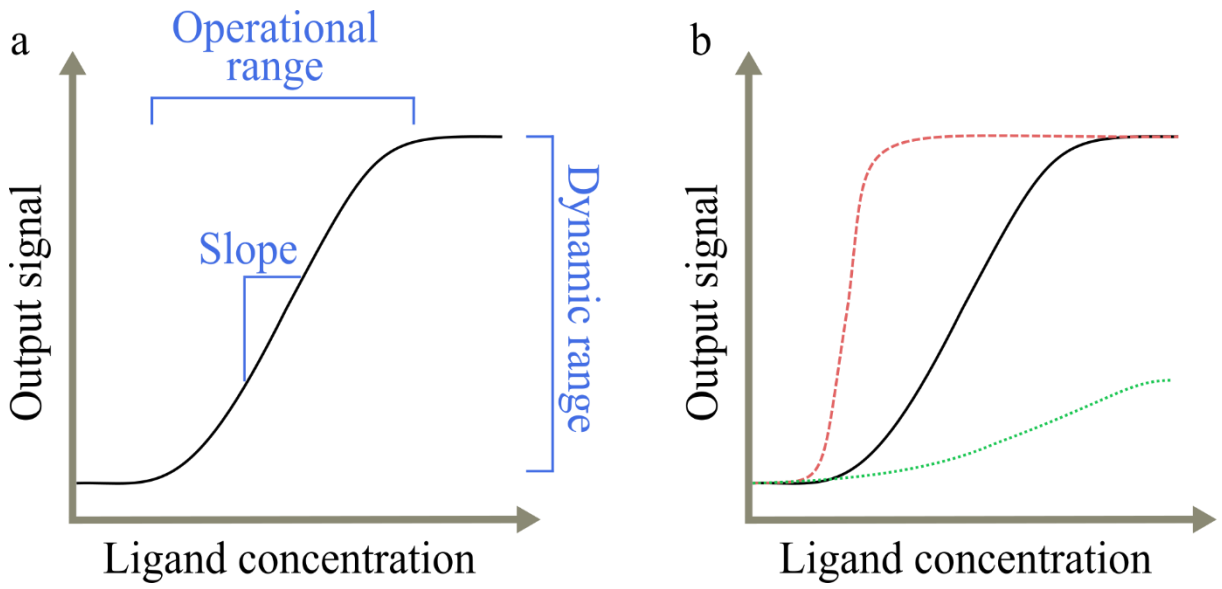

Figure 3.2: Biosensor response curve. (a) Representation of a general biosensor response curve, highlighting its main features: dynamic range, operational range, and biosensor slope. (b) Examples of different response curves, displaying a reduced dynamic range (green dotted line) or operational range (red dashed line) compared to the first general example.

Different types of genetically encoded biosensors

Genetically encoded biosensors can be employed to detect and monitor environmental changes, as well as extra-/intracellular molecules. Those inputs are translated into a measurable phenotype, such as improved fitness or fluorescence, which could be used to select traits of interest. Biosensors have proven very useful in screening large libraries (Sarnaik et al., 2020), which is important for isolating strains of interest, as well as to identify genetic determinants of desired phenotypes (**Paper II** and **Paper III**). With such approach, it was possible to select strains with altered production of different compounds, such as acyl-CoA (Dabirian et al., 2019) and cis, cis-muconic acid (Wang et al., 2020a), but also to assess yeast tolerance towards acetic acid (**Paper II** and **Paper III**). Alternatively, biosensors could be exploited for dynamic pathway regulation, based on any compound of interest. One such case involves a genetically encoded biosensor capable of monitoring the glycolytic flux by tracking the intracellular concentration of one of its main metabolites, fructose-1,6-bisphosphate (Ortega et al., 2021). Furthermore, different biosensors could be co-expressed in the same host to monitor diverse parameters of the cell, such as intracellular pH, ATP levels, oxidative stress, glycolytic flux, and ribosome production (Torello Pianale et al., 2022).

Two-hybrid system biosensors

Yeast two-hybrid system biosensors are used to assess protein-protein interactions *in vivo*. Those systems typically rely on the Gal4 protein, which induces transcription in the presence

of galactose (Ptashne, 1988). When Gal4 C- and N-terminal domains (containing the activation and DNA-binding domain, respectively) are expressed separately, they can still exert their activating function if they interact and bind covalently. The expression of these domains fused with two proteins of interest (referred to as “bait” and “prey”), may restore the activation function of Gal4 upon interaction of the two proteins of interest. To verify the interaction, a reporter gene is placed under the control of a promoter induced by Gal4 (Dhakal & Macreadie, 2022) (Figure 3.3a). Two-hybrid system biosensors are particularly useful for studying the protein interaction space or interactome in yeast (Ito et al., 2001; Uetz et al., 2000), as well as for screening libraries of prey proteins likely to interact with the bait protein (Dhakal & Macreadie, 2022). Additionally, these biosensors can be used as part of drug discovery platforms (Scott et al., 2022) or to monitor metabolites and small molecules when the protein-protein interaction depends on them (Zimran et al., 2022).

FRET-based biosensors

The electromagnetic phenomenon known as Förster resonance energy transfer (FRET) consists of the non-radiative transfer of energy from a light-excited fluorophore molecule (FRET donor) to another molecule (FRET acceptor) situated in short proximity (typically less than 10 nm) from the former (Skruzny et al., 2019). When the FRET acceptor is another fluorophore, a FRET event manifests as lower fluorescence emission by the FRET donor, while the FRET acceptor becomes excited and emits fluorescence. Such FRET events can be exploited to develop biosensors for the analysis of biochemical and biophysical interactions. In a FRET-based biosensor, the FRET donor-acceptor pair is used to report a specific molecular event by changing their proximity or orientation and inducing a FRET event (Dhakal & Macreadie, 2022). Furthermore, the donor and acceptor parts of a FRET biosensor can be expressed separately or as part of a single macromolecule (Figure 3.3b). In the latter case, the final readout is easier to interpret due to always identical concentrations and subcellular localizations of the donor/acceptor pair (Skruzny et al., 2019). While their development is often laborious (Marx, 2017), FRET biosensors are very versatile in yeast. They have been employed to track metabolites (Bermejo et al., 2013), follow ion fluxes (Choi et al., 2018), assess medically related prion aggregation (Khan et al., 2018), monitor cellular processes (Conlon et al., 2016) or even investigate their biophysical properties (Suzuki et al., 2016).

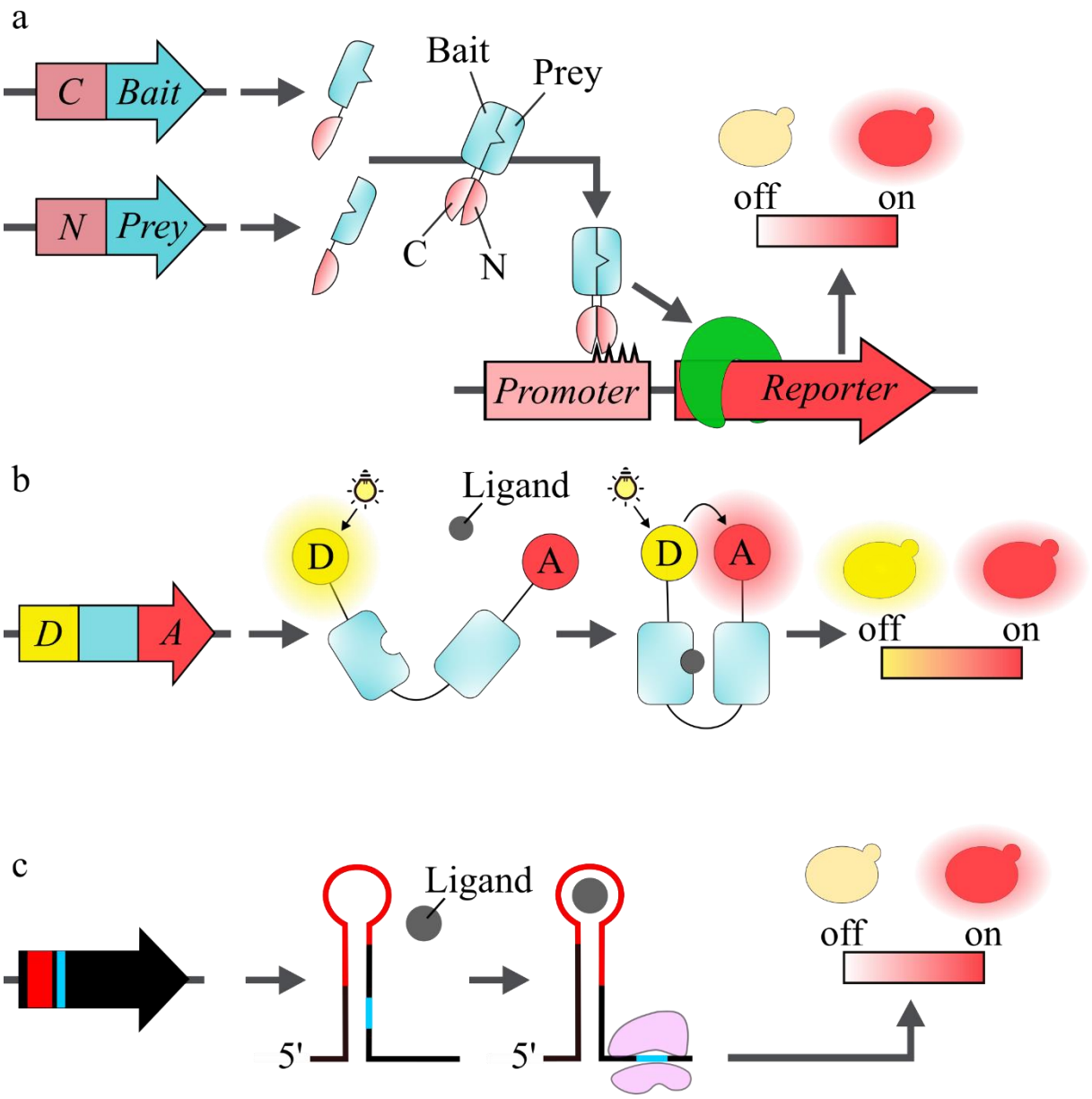

Figure 3.3: Schematic of different types of genetically encoded biosensors. a) Example of two-hybrid system biosensor where the bait protein and the prey protein are fused to the C-terminal domain (C) or the N-terminal domain (N) of Gal4. If prey and bait interact, Gal4 functionality is restored and it can recruit the transcription machinery (here in green) activating the reporter expression. b) Example of FRET-based biosensor where donor (D) and acceptor (A) are expressed in the same protein. Upon binding to the target molecule, the protein changes its conformation, approaching the light-excited donor to the acceptor. The acceptor gets excited by the donor generating a fluorescence emission. c) Example of RNA-based biosensor where the aptamer riboswitch (here the red lines), upon binding to the target molecule, induces a conformational change of the mRNA structure. The conformational change allows the ribosome (here in pink) to recognize the ribosome binding site (here the cyan lines) and start the translation of the reporter.

RNA-based biosensors

RNA-based biosensors usually rely on riboswitches, which are regulatory segments located in the mRNA 5'-UTR capable of recognizing and binding specific metabolites via aptamer domains (Qiu et al., 2019). The bond between the ligand and the riboswitch alters the conformation of the mRNA, with consequent regulation of transcription termination, translation initiation or mRNA decay (Link & Breaker, 2009) (Figure 3.3c). While RNA-based biosensors are naturally present in different prokaryotes, the development of riboswitches in eukaryotic cells can be difficult due to numerous translation steps (Qiu et al., 2019). Nevertheless, they have been applied also in eukaryotes, including yeast. In ribozyme-based riboswitches, the bond with the target molecule triggers a self-cleavage activity, which destabilizes the mRNA, thereby regulating its translation (Qiu et al., 2019). Previous studies have employed RNA-based biosensors in yeast to screen a caffeine demethylase library (Michener & Smolke, 2012) or develop a glucosamine 6-phosphate-producing strain via evolutionary engineering (Lee & Oh, 2015).

Transcription factor-based biosensors

Transcription factor-based biosensors comprise two main components: the sensor, made of the metabolite-responsive transcription factor and the corresponding promoter, and the reporter gene (Figure 3.4). Transcription factors are regulatory proteins, whose function is to bind specific sequences of DNA and thereby tune the expression of target genes. Besides the DNA-binding domain, metabolite-responsive transcription factors contain also an effector-binding domain, which is essential for recognizing the target ligand (Hahn & Young, 2011). While effector-binding domains may differ from protein to protein, DNA-binding domains tend to be more conserved and can be classified into structural groups such as the zinc-fingers type (Yao et al., 2022). Yeast biosensors often use heterologous, bacterial transcription factors as a starting point (Qiu et al., 2019), although yeast biosensors based on eukaryotic transcription factors have also been reported (Bovee et al., 2007; Chou & Keasling, 2013; Feng et al., 2015). Endogenous yeast transcription factors are known to bind specific ligands and regulate target genes in response to changing conditions. While some sensor systems exist in nature, they often require a few engineering steps to improve their dynamic and operational ranges prior to their use as a biosensor (D'Ambrosio & Jensen, 2017). This can be achieved by engineering the different components of the biosensor. Fine-tuning the expression of the transcription factor by changing its promoter can increase the dynamic and operational range (Qiu et al., 2019). Changing the transcription factor's DNA-binding domain or altering the number of binding sites in the reporter's promoter have been employed to optimize transcription factor-based biosensors (Qiu et al., 2019). Changing the transcription factor's effector-binding domain is a common strategy for changing the specificity of the biosensor (Qiu et al., 2019). Selecting a reporter more suitable for the applied screening conditions (e.g., instrument, pH, and temperature) can also improve biosensor performance (**Paper I**).

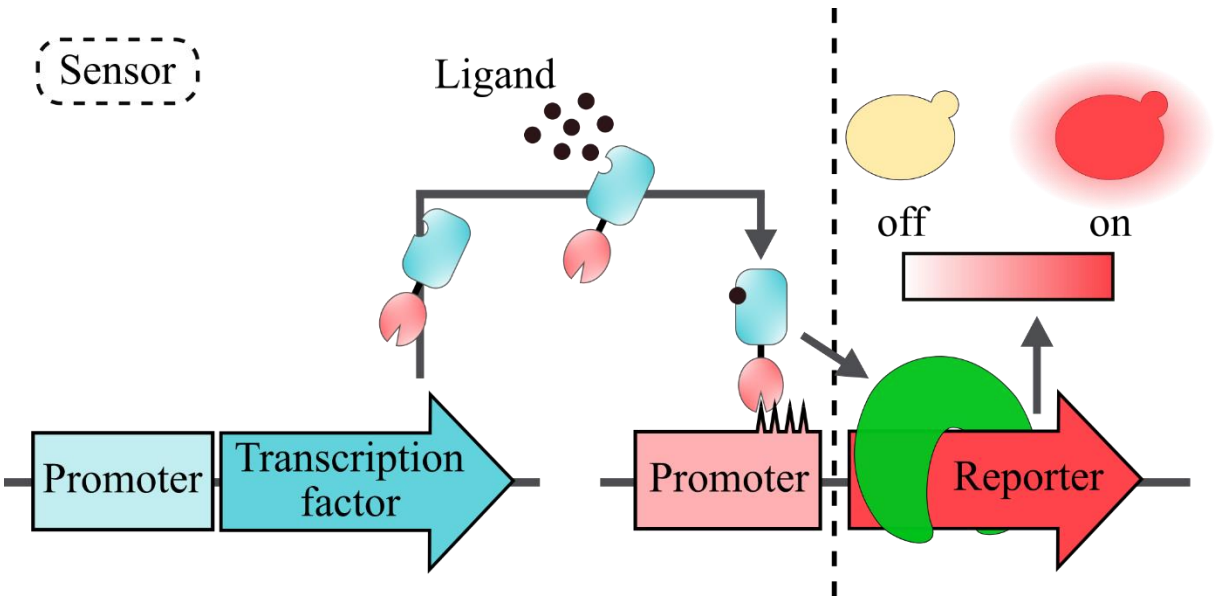

Figure 3.4: Schematic representation of a transcription factor-based biosensor mechanism. A transcription factor capable of recognizing the target molecule is expressed in the host. Upon binding to the target ligand, the transcription factor recognizes the binding sites in the reporter's promoter and recruits the transcription machinery (here represented in green) to express the reporter. The magnitude of the output signal generated by the reporter depends on how many transcription factor units bind the promoter. The sensor part of the biosensor, including the transcription factor and the promoter driving the expression of the reporter, is displayed on the left of the dashed line. On the right, the reporter gene is shown.

Promoter activity depends on different factors, such as the DNA sequence, nucleosomes, and chromatin remodeling (Kanhere & Bansal, 2005; Rando & Winston, 2012). The key elements of eukaryotic promoters are the core promoter and the upstream region (Ottoz & Rudolf, 2018). The core promoter harbors TATA/TATA-like sequences, which serve to recruit the transcription preinitiation complex and initiate transcription (Ottoz & Rudolf, 2018). Upstream regions can be recognized by gene-specific transcription factors which then regulate the expression of reporter genes (Ottoz & Rudolf, 2018). In transcription factor-based biosensors, a reporter gene is placed under the control of the promoter recognized by the transcription factor of the system (Qiu et al., 2019). The reporter must produce an easily measurable output in response to how much metabolite is sensed by the transcription factor. For this reason, reporter genes typically encode fluorescent proteins or proteins conferring auxotrophy or antibiotic resistance, measurable as altered growth under selective pressure. While screenings based on changes in growth are often cheaper as they require less instrumentation, fluorescence-based screenings usually provide higher resolution and enable easier selection of positive or negative cells (Dietrich et al., 2010).

The limited availability of high-throughput screening procedures to evaluate potential yeast-based cell factories has led to the design of intracellular metabolic biosensors that allow real-

time monitoring of either end products or metabolic intermediates. The utilization of transcription factor-based biosensors has been applied for the dynamic control of synthesis pathways, high-throughput screenings, and adaptive laboratory evolution. David et al. (2016) established a transcription factor-based biosensor for malonyl-CoA with a green fluorescent protein as reporter. The optimized biosensor was used in a hierarchical system to control the production of 3-hydroxypropionic acid, improving the final titer from 0.4 to 1.0 g L^{-1}. Dabirian et al. (2019) applied a biosensor based on the bacterial transcription factor FadR to screen an overexpression library for genes improving fatty acyl-CoA production. The screening singled out *RTC3*, *GGA2*, and *LPP1*, whose overexpression resulted in approximately 80% higher fatty alcohol levels. Leavitt et al. (2017) coupled a transcription factor-based biosensor with adaptive laboratory evolution to improve the muconic acid titer of a *S. cerevisiae* strain. In the following section, the development of an acetic acid-responsive biosensor is presented as a case in point describing the design and optimization of a transcription factor-based biosensor in yeast.

Development of an acetic acid-responsive biosensor in S. cerevisiae

Acetic acid is an acetyl-CoA precursor with an important role in central carbon metabolism (Jell et al., 2007; Jeukendrup, 2002). It is also one of the main inhibitors found in lignocellulosic hydrolysates, whose effects are detailed in Chapter IV. Thus, monitoring acetic acid levels can be particularly useful when developing industrial strains.

The *S. cerevisiae* zinc-finger transcription factor Haa1, a major regulator of yeast response to weak acids (Collins et al., 2017), has been reported to directly bind acetate ions in the cytosol (Kim et al., 2018). Upon binding to acetate, Haa1 relocates to the nucleus where it activates numerous genes (Collins et al., 2017). Owing to its natural ability to recognize acetic acid, Haa1 was selected to design the transcription factor-based biosensor for acetic acid outlined in **Paper I** and **Paper II**.

To test the level of acetic acid-induced activation of an Haa1-based biosensor in *S. cerevisiae*, in **Paper I**, Haa1 was coupled to a promoter/reporter pair comprising a native target promoter of Haa1 (*YGP1* promoter). A cassette harboring *HAA1* fused to *mTurquoise2* expressed under the native *HAA1* promoter and the reporter gene *mRuby2* expressed under the control of the *YGP1* promoter was integrated into *S. cerevisiae* (Figure 3.5). The nuclear localization of the Haa1-mTurquoise2 construct upon exposure to acetic acid was confirmed through fluorescence microscopy (**Paper I: Figure 2**). Furthermore, the strain harboring this first version of the biosensor was cultivated in the presence or absence of 50 mM acetic acid, displaying weak acetic acid-induced reporter expression (**Paper I: Figure S2**).

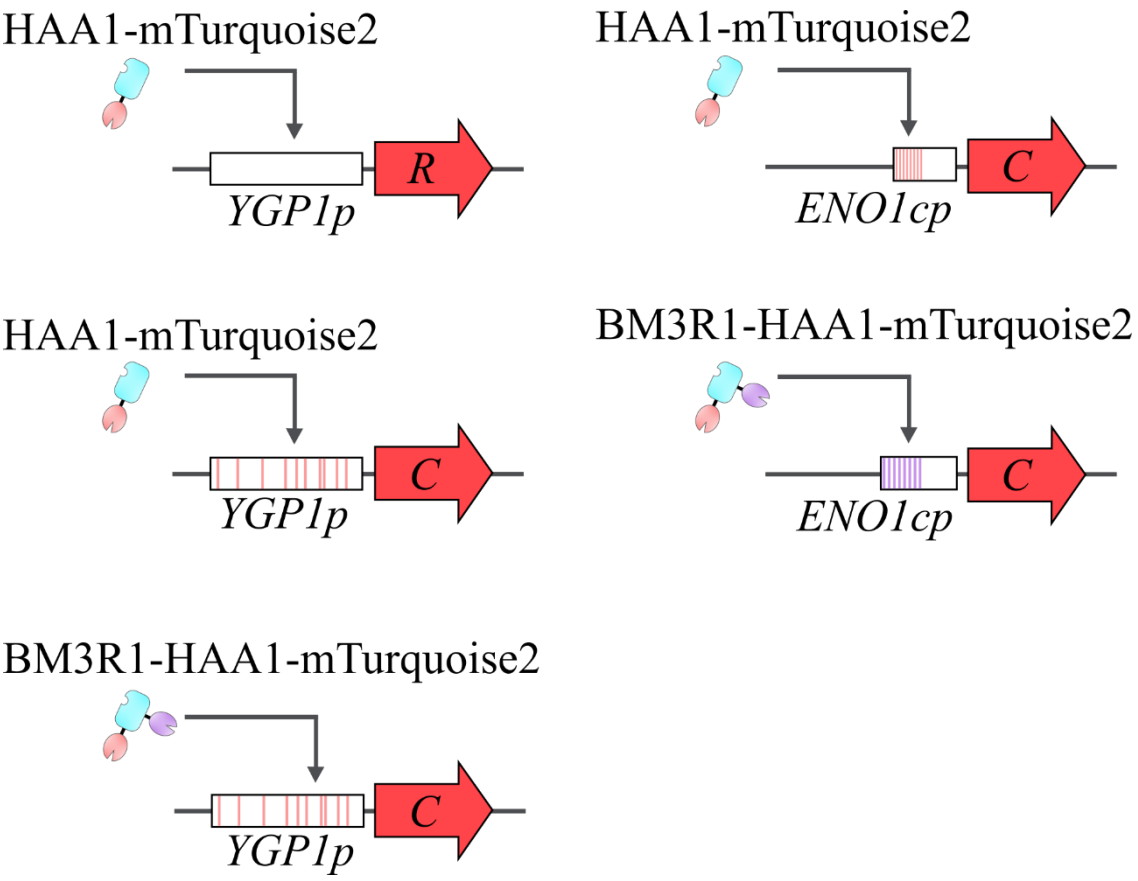

Figure 3.5: Schematic representation of the biosensor constructs developed in Paper I. Haa1 with fusion proteins is expected to relocate to the nucleus upon acetic acid exposure. The biosensor output reflects the expression of the reporter (mRuby2 or mCherry) under various synthetic promoters, based on the promoter of *YGP1* (*YGP1p*) or on the *ENO1* core promoter (*ENO1cp*). Sizes of promoters and genes are scaled to represent their actual length. Additional binding sites for Haa1 or for BM3R1 are indicated with red or violet bars inside the promoters, respectively. Red arrows labeled with R and C refer to the mRuby2 or mCherry reporter genes.

Following verification of biosensor activation upon acetic acid induction, the system had to be improved to allow its application in screening studies. First, to obtain a stronger output signal, mRuby2 was replaced with the more pH-resistant reporter protein mCherry (**Paper I**). Afterwards, new integration cassettes were built harboring different combinations of transcription factor and reporter's promoter (Figure 3.4). Besides Haa1 fused to mTurquoise2, a second synthetic transcription factor was tested, consisting of the transcription factor BM3R1 from *B. megaterium* (Ramos et al., 2005) fused to the N-terminus of Haa1-mTurquoise2. The new reporter's promoters were the *YGP1* promoter modified to include 10 additional Haa1 binding sites and two synthetic promoters containing the *ENO1* core promoter preceded by either eight Haa1 binding sites or eight BM3R1 binding sites. Use of the BM3R1 transcription factor and the *ENO1* core promoter with BM3R1 binding sites upstream was inspired by a previous study by Rantasalo et al. (2018). The biosensor coupling the synthetic transcription factor BM3R1-Haa1-mTurquoise2 with the *ENO1* core promoter preceded by eight BM3R1

binding sites yielded the highest reporter induction upon exposure to 50 mM acetic acid (**Paper I: Figure 3**). This version of the biosensor was characterized in **Paper I**. It displayed an operational range spanning 10–60 mM acetic acid, and a dynamic range reaching up to approximately 6-fold the basal induction (**Paper I: Figure 4**). Both these parameters were in line with previous studies describing biosensor development and applications in yeast (Dabirian et al., 2019; Hahne et al., 2021; Li et al., 2015; Liu et al., 2017; Wang et al., 2013; Wu et al., 2020). The biosensor also showed good orthogonality, as it did not affect host growth under the tested conditions (**Paper I: Figure S3**). Furthermore, the synthetic transcription factor BM3R1-Haa1-mTurquoise2 could not regulate the expression of genes under the control of a promoter containing Haa1 binding sites (**Paper I: Figure 3**). This suggested that the biosensor should not interfere with the endogenous Haa1 regulatory network, nor should it have noticeable effects on the physiology of the cells.

The biosensor cassette was integrated in a small collection of strains accumulating different levels of acetic acid to monitor acid production and validate the biosensor. The analysis performed on this small strain collection revealed strong correlation between the biosensor reporter output and exogenous acetic acid (**Paper I: Figure 6**). On the contrary, no correlation was observed between reporter output and intracellular acetic acid. Two factors could have contributed to this discrepancy. On the one hand, glucose depletion differed between the strains (**Paper I: Figure 6**). Glucose-repressed cells are less permeable to acetate (Cassio et al., 1987), leading to a higher intracellular acetic acid retention when there is no glucose in the medium. On the other hand, the strains started producing acetic acid at different time points (**Paper I: Figure 6**), and biosensor activity was influenced by the time of acetic acid injection (**Paper I: Figure 5**).

The acetic acid biosensor developed in **Paper I** was further optimized in **Paper II**. While in **Paper I** the chosen approach focused on modifying the transcription factor, as well as the reporter gene and its promoter; in **Paper II**, different promoters driving the expression of the synthetic transcription factor BM3R1-Haa1-mTurquoise2 were evaluated. These promoters allowed us to test a wide range of BM3R1-Haa1-mTurquoise2 expression levels. Indeed, a 20% higher dynamic range was obtained after replacing the native *HAA1* promoter with the medium-weak constitutive promoter of *RET2* (**Paper II: Figure 1**).

This last version of the biosensor was then successfully employed in **Paper II** and **Paper III** to screen a yeast library for acetic acid sensitivity. When transferred in the new host, the biosensor maintained similar features compared to the original strain in which it was developed, with dynamic range reaching approximately 3.5-fold of induction (**Paper II: Figure S2**) and the operational range spanning 0–50 mM acetic acid (**Paper II: Figure 2**). The main findings of **Paper II** and **Paper III**, including the results relative to biosensor-based screenings, are presented in the following chapter.

IV. Yeast response to acetic and formic acid stress

In the previous chapter, different high-throughput screening methods were presented, along with genetically encoded biosensors such as the one developed in **Paper I** and **Paper II** to sense acetic acid. The latter is a major inhibitor in lignocellulosic hydrolysates and understanding how yeast responds to acetic acid stress is crucial for developing robust cell factories. A similar challenge is posed by formic acid. Several attempts to improve tolerance of *S. cerevisiae* towards acetic and formic acid have been made. Here, the acetic acid biosensor described in Chapter III was used together with competitive growth assays to screen a *S. cerevisiae* library for acetic acid or formic acid tolerance. This screening identified new target genes capable of enhancing resistance in newly engineered strains, while also confirming the role of genes already known for their involvement in the response to acid stress. Side by side use of the acetic acid biosensor and competitive growth assay led also to some general reflections on the use of different methods to screen yeast libraries for tolerance towards inhibitors.

Acetic acid and formic acid challenges in yeast-based bioeconomy

As discussed in Chapter I, organic acids are among the main yeast inhibitors found in lignocellulosic hydrolysates. Among them, acetic acid and formic acid are of particular concern due to their detrimental effect and abundance in hydrolysates. Weak undissociated acids can diffuse freely through the plasma membrane. Once inside the cell, acetic or formic acids dissociate into acetate or formate plus protons due to a more alkaline environment (Figure 4.1). Proton accumulation and the resulting acidification of the cytosol lead to a series of metabolic responses and toxic effects (Figure 4.1), which can hinder cell growth and/or productivity.

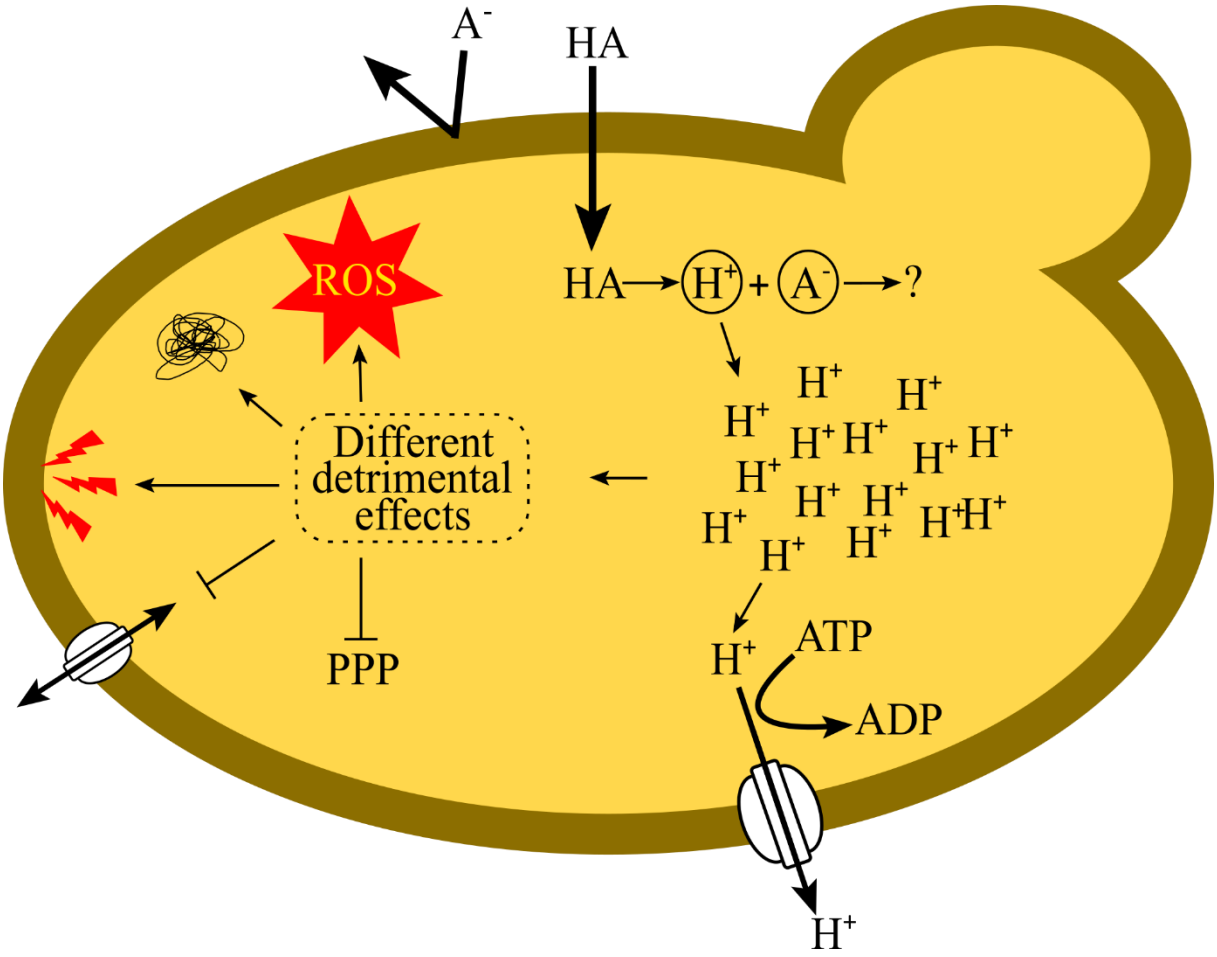

Figure 4.1: Inhibitory mechanism of acetic and formic acids. In their undissociated form (HA), acetic acid and formic acid can freely enter the cell and, once in the cytosol, they dissociate into protons (H^+) and anions (A^-). While any toxic effect of the anionic form remains unclear, intracellular proton accumulation triggers the activity of ATP-dependent proton pumps, which maintain the physiological pH in the cytosol. The intracellular accumulation of protons causes different cytotoxic effects, including acidification of the cytosol, ATP depletion, ROS accumulation, protein aggregation, disruption of the cell membranes, inhibition of membrane trafficking, and inhibition of the pentose phosphate pathway (PPP).

Acetic and formic acid are short-chain carboxylic acids. Formic acid is the simplest monocarboxylic acid in nature. Under physiological conditions, yeast can utilize acetic acid as an alternative carbon source to overcome the absence of glucose (Guaragnella & Bettiga, 2021). Under aerobic and glucose-limited (7.5 g L^{-1}) conditions, *S. cerevisiae* can co-utilize formic acid and glucose to generate NADH through via the two formate dehydrogenases Fdh1 and Fdh2 (Babel et al., 1993; Overkamp et al., 2002). However, at higher concentrations, acetic and formic acids can become toxic for yeast and even lead to cell death. Their toxicity depends on time of exposure, strain, medium composition, extracellular pH, and acid concentration (Guaragnella & Bettiga, 2021), and is exerted mainly through acidification of the cytosol. In contrast, the anionic forms are not particularly toxic to yeast cells (Figure 4.1). Deletion of

PDR12, which encodes the main transporter responsible for acetate extrusion, resulted in no significant changes (Ullah et al., 2012) or improved tolerance (Nygård et al., 2014) to acetic acid in two different *S. cerevisiae* strains, but it improved tolerance to formic acid. While the responses of *S. cerevisiae* towards acetic acid and formic acid show some similarities (Mukherjee et al., 2023), the latter is more toxic than the former. Larsson et al. (1998) found that, at concentrations above 100 mM, formic acid had a more negative impact on ethanol yield than acetic acid, which could be explained by a lower pKa (3.74) compared to the latter (4.76). A lower pKa means greater dissociation and, consequently, higher potential to acidify the cytosol. This, in turn, explains the inhibitory effects described in Chapter I. Acetic and formic acid concentration is crucial in yeast-based industrial fermentation, as they are two of the main by-products of such processes. Depending on feedstock and treatment, approximately 17–250 mM acetic acid (Klinke et al., 2004; Palmqvist & Hahn-Hägerdal, 2000b) or 5–100 mM formic acid (Greetham et al., 2014) may be formed from the hydrolysis of raw lignocellulosic biomass. Yeast cells cultured under aerobic conditions at pH 5 displayed a longer lag phase and slower growth rate when grown in the presence of 95–220 mM acetic or formic acid compared to the control condition (Guo & Olsson, 2014)

The abundance and inhibitory effects reported for acetic acid and formic acid explain the strong interest for finding strains capable of fermenting biomass in the presence of these compounds. In the past decades, a combination of omics studies, metabolic engineering, and synthetic biology has enabled the engineering and/or identification of improved yeast strains for the production of biofuels and added-value chemicals. In the next section, different screening studies aimed at the identification of strains with increased resistance to acetic and formic acid are presented.

Strain-library screenings for acetic acid or formic acid tolerance in yeast

Over the past decades, the need to find more robust strains for yeast-based industrial fermentation processes has led to the creation of different mutant libraries. Several of those libraries have been screened in the presence of acetic and/or formic acid to identify strains with increased tolerance, or simply attain a better understanding of the mechanisms underlying the response to such stressors. Examples of the above libraries include: the EUROSCARF collection of deletion strains already mentioned in Chapter II (Winzeler et al., 1999), an overexpression strain library covering 97% of the yeast genome (Jones et al., 2008), a CRISPRi strain library targeting all yeast essential and respiratory growth genes (Smith et al., 2017), a genome-wide library for inverse genomic engineering (Chen et al., 2016b), the TATA-binding protein encoding gene *SPT15* random mutation library (An et al., 2015), and a zinc-finger protein transcription factor strain library (Park et al., 2003). As summarized in Table 4.1, while multiple studies have been conducted to enhance acetic acid resistance, equivalent screenings on formic acid are scarce.

The EUROSCARF collection of deletion strains has been screened for both acetic acid and formic acid tolerance. Some of these screenings were performed using the spot assay technique

(Henriques et al., 2017; Kawahata et al., 2006; Mira et al., 2010a), while others relied on a phenotypic microarray (Sousa et al., 2013) or a competitive growth approach (Ding et al., 2013). Different screening techniques and experimental conditions, led to the identification of several determinants of yeast tolerance to the two weak acids. They include the regulation of mitochondrial activity, carbohydrate and amino acid metabolic pathways (Sousa et al., 2013), cell wall architecture (Kawahata et al., 2006; Mira et al., 2010a), vacuolar ATPases and HOG-MAPK proteins (Kawahata et al., 2006), transcription factors, as well as iron and potassium transporters (Mira et al., 2010a). A spot assay screening performed in formic acid (Henriques et al., 2017) revealed that Haa1 and the Haa1-regulon contributed to yeast resistance to this acid. Haa1 was central in the development of the acetic acid biosensor described in chapter III (**Paper I** and **Paper II**) and its role in regulating the *S. cerevisiae* response to acetic acid stress is discussed in the following section.

The yeast libraries developed by Jones et al. (2008) and Chen et al. (2016b) overexpressed single genes or portions of the genome. Both were screened in acetic acid; the first one using a competitive growth assay (Ding et al., 2015), and the second one using a spot assay (Chen et al., 2016b). Those studies led to the identification of two acetic acid-tolerant strains overexpressing either *PEP3* (Ding et al., 2015) or *WHI2* (Chen et al., 2016b).

The *SPT15* random mutation library (An et al., 2015) and the zinc-finger protein transcription factor strain library (Park et al., 2003) were both screened using spot assays on medium containing acetic acid. Unlike the strain collections described above, in which genes were either upregulated or deleted, these two libraries allow the induction or repression of genes. However, as they do not permit prior selection of the genetic targets for each mutant, the screening should be followed by downstream analysis to identify the regulated genes. The screening for acetic acid tolerance performed on these two libraries led to the identification of three strains with improved tolerance to acetic acid: MRRC3252 and MRRC3253 from An's collection (An et al., 2015) and ATCC4126 from Park's collection (Ma et al., 2015).

Unlike An's and Park's libraries, the CRISPRi library (Smith et al., 2017) enables the regulated expression of selected genes. Smith's library targeted all yeast essential and respiratory growth genes, aiming to downregulate each one of them. This library was screened for acetic acid (Mukherjee et al., 2021) or formic acid tolerance (Mukherjee et al., 2023), exploiting the Scan-o-matic phenotypic microarray described in Chapter III. These two studies revealed that *S. cerevisiae* strains were strongly inhibited by the two weak acids when the gRNAs targeted genes encoding intracellular vesicle transport and chromatin remodeling proteins. Furthermore, acetic acid screening revealed that genes involved in glycogen accumulation (such as *GLC7* or *YPI1*) as well as proteasomal degradation of oxidized proteins might influence acetic acid tolerance.

To obtain further insights on the response to the above two organic acids, the CRISPRi library was screened again both in acetic and formic acid, exploiting different approaches. In the following section, the results relative to the biosensor-based acetic acid screenings and the competitive growth assays performed in **Paper II** and **Paper III** on the Smith's CRISPRi library are presented.

Table 4.1: Examples of studies, in which *S. cerevisiae* libraries were screened for acetic acid or formic acid tolerance

Acid	Screening method	Study	Media	Library
Acetic acid	Spot assay	Kawahata et al. 2006	YPD + 66.7-83.3 mM acetic acid	EUROSCARF deletion library of all non-essential ORFs (Winzeler et al., 1999)
		Mira et al. 2010a	MM4 + 70-90 mM acetic acid	EUROSCARF deletion library of all non-essential ORFs (Winzeler et al., 1999)
		An et al. 2015	SC + 100-150 mM acetic acid	SPT15 random mutation library (An et al., 2015)
		Ma et al. 2015	YPD + 83.3 mM acetic acid	Zinc-Finger Protein Transcription Factor library (Park et al., 2013)
		Chen et al. 2016b	SC + 33.3-58.3 mM acetic acid	Genome-wide library for inverse genomic engineering (Chen et al., 2016b)
	Phenotypic microarray	Mukherjee et al. 2021	YNB + 150 mM acetic acid	CRISPRi library targeting all essential and respiratory growth essential genes (Smith et al., 2017)
		Sousa et al. 2013	YPD + 400 mM acetic acid	EUROSCARF deletion library of all non-essential ORFs (Winzeler et al., 1999)
	Competitive growth	Ding et al. 2013	YNB + 112.5 mM acetic acid	EUROSCARF deletion library of all non-essential ORFs (Winzeler et al., 1999)
		Ding et al. 2015	YNB + 140 mM acetic acid	Overexpression library covering 97 % of the yeast genome (Jones et al., 2008)
Formic acid	Spot assay	Henriques et al. 2017	MM4 + 60-80 mM formic acid	EUROSCARF deletion library of all non-essential ORFs (Winzeler et al., 1999)
	Phenotypic microarray	Mukherjee et al. 2023	YNB + 140 mM formic acid	CRISPRi library targeting all essential and respiratory growth essential genes (Smith et al., 2017)

Use of competitive growth and biosensor to screen a CRISPRi strain library

The acetic acid biosensor developed in **Paper I** and improved in **Paper II** was integrated in the CRISPRi library created by Smith et al. (2017). The resulting biosensor-integrated library was screened under two different modalities. In **Paper II**, the acetic acid biosensor was used to isolate single cells displaying higher reporter signal in medium supplemented with acetic acid. In **Paper III**, the acetic acid biosensor helped sort a population of approximately 50 000 cells exhibiting higher reporter signal when cultivated in acetic acid. At the same time, the biosensor-free pooled library was screened using competitive growth assays in medium supplemented with acetic or formic acid. The strains isolated in **Paper II**, as well as the composition of the cultures enriched in **Paper III**, were determined by sequencing the cells' gRNAs. The main findings from **Paper II** and **Paper III** in relation to acetic acid and formic acid stress response in yeast are reported and discussed hereafter.

Glycogen accumulation contributes to acetic acid tolerance

After three rounds of competitive growth in acetic acid (**Paper III**), three of the most enriched strains were found to harbor gRNA targeting *YPI1* (**Paper III: Figure 2a**). Ypi1 is a regulatory subunit of the protein phosphatase Glc7, which is involved in glycogen accumulation (Cannon et al., 1994). Yeast stores energy in the form of glycogen during stress conditions (Guo & Olsson, 2014; Kitanovic et al., 2012). In the Scan-o-matic screening previously performed on this CRISPRi library, *YPI1* was the gRNA target of several strains with improved tolerance towards both acetic acid (Mukherjee et al., 2021) and formic acid (Mukherjee et al., 2023). This result is consistent with induction of *YPI1* upon acetic acid exposure (Dong et al., 2017). *YPI1* was the gRNA target of four strains significantly enriched after the two rounds of acetic acid biosensor-based sorting performed in **Paper III**. These cells were characterized by greater intracellular accumulation of acetic acid, as indicated by FACS, which identified cells with higher acetic acid biosensor output. A strain harboring a gRNA targeting *GLC7* was also among the most enriched ones after the last round of biosensor-based sorting. In line with that, a previous screening by Mukherjee et al. (2021) revealed that repression of *GLC7* resulted in higher acetic acid sensitivity. Four or five strains with gRNAs targeting *GLC7* were either depleted or less frequent after the competitive growth assays performed on formic or acetic acid (**Paper III**). Glc7 contributes to the dephosphorylation and subsequent activation of glycogen synthase; hence, its dysregulation may result in altered intracellular glycogen content. Therefore, controlling the expression of *GLC7* may alter the tolerance to acetic acid.

Intracellular manganese might support a more energy efficient ROS detoxification

Two of the nine strains enriched in **Paper III** after each round of competitive growth harbored gRNA targeting the high-affinity histidine permease *HIP1* (Tanaka & Fink, 1985) (**Paper III: Figure 2b**). The competitive growth assay performed in acetic acid also enriched two strains with gRNA targeting *HIP1*. Besides its histidine permease activity, Hip1 has also been reported to function as a manganese exporter (Farcasanu et al., 1998). Manganese is involved in the non-enzymatic defense against intracellular ROS by forming antioxidant complexes (Culotta & Daly, 2013). Both acetic acid and formic acid promote oxidative stress and ROS release (Du et al., 2008; Guaragnella & Bettiga, 2021). Thus, lower manganese extrusion caused by downregulation of *HIP1* may promote tolerance towards weak organic acids by favoring intracellular ROS scavenging. Chapter II and **Paper IV** discuss the regulation of iron homeostasis in relation to the response to stress. Metal ions play a dual role during oxidative stress in yeast. On the one hand, iron, copper, and manganese favor the formation of •OH by reacting with superoxide radicals and H_2O_2 in Fenton or Fenton-like reactions (Cao et al., 2021). On the other hand, these metals are involved in ROS detoxification by serving as cofactors for superoxide dismutase or various transcription factors (Herrero et al., 2008). Thus, careful regulation of the transporters controlling the intracellular content of metal ions is critical for the response to oxidative stress in yeast. In particular, ATP-independent ROS removal, favored by

higher levels of intracellular manganese ions, is crucial for the cell during oxidative stress. ROS accumulation reduces ATP availability due to higher activity of ATP-dependent proton pumps to maintain intracellular pH homeostasis and sustain NADPH production (Palma et al., 2018; Reichmann et al., 2018). Indeed, increased tolerance of strains exhibiting downregulation of the 19S proteosome subunit, observed by Mukherjee et al. (2021) when screening the CRISPRi library, suggested that repression of genes encoding the 19S subunit might improve ATP-dependent turnover of misfolded proteins accumulated during oxidative stress.

Genes encoding the 19S proteosome particle are potential targets for improved acid tolerance

The importance of the proteasome in the response to acetic acid was noted in **Paper III** by enrichment of the GO term "Proteasome regulatory particle" after competitive growth in acetic acid (**Paper III: Table 2**). The same screening confirmed the enrichment of five strains harboring gRNAs targeting *RPT1*, *RPN9* or *RPT4*. *RPN* and *RPT* genes encode subunits of the proteasome (Groll et al., 1997). Fifty-three strains with gRNAs targeting *RPN* and *RPT* genes were enriched at the end of competitive growth in acetic acid and seven upon growth in formic acid. In particular, four strains harboring gRNAs targeting *RPT4*, which encodes one of the six ATPases in the 19S subunit, were highly enriched at the end of competitive growth assays in either of the two acids. At the same time, FACS-based sorting of the biosensor-integrated CRISPRi library performed in acetic acid (**Paper III**), showed that 31 and 30 strains with gRNA targeting *RPN* and *RPT* genes were enriched after the first and second round of sorting, respectively. Among those, two of the most enriched strains harbored gRNAs targeting *RPN2* or *RPN6*, which encode subunits of the 26S proteasome (**Paper III: Table 3**). The results presented in Chapter III relative to biosensor improvement (**Paper II**), showed that acetic acid biosensor-integrated cells displaying a higher reporter signal contained also more acetic acid (**Paper II: Figure 5**). Accordingly, cells enriched through FACS in **Paper II** and **Paper III** were expected to accumulate more acetic acid. It should be noted that the acetic acid concentration utilized for the FACS-based selection (50 mM, compared to 125 mM in competitive growth assays) may not be sufficient to trigger oxidative stress. Moreover, the experimental set-up revealed only absolute enrichment, rather than the relative enrichment measured in competitive growth assays. Nevertheless, biosensor-based sorting led to the enrichment of several strains of interest for future studies. In this context, the identification of multiple strains with different gRNAs targeting the same gene reinforces the role of such genes in relation to the observed phenotypes (Smith et al., 2017).

SAGA complex-mediated chromatin modification may influence the response to acid stress

In **Paper III**, two of the most enriched strains throughout the competitive growth assay in acetic or formic acid harbored gRNA targeting *SMC4*. *SMC4* encodes a subunit of the condensing complex that participates in chromatin reorganization (Stray & Lindsley, 2003). Previous studies already highlighted the role of chromatin remodeling in the response to acid stress in

yeast (Mukherjee et al., 2023; Sousa et al., 2013; Vanacloig-Pedros et al., 2022). In the Scan-o-matic screening of the CRISPRi library performed by Mukherjee et al. (2023), chromatin-related genes were found to participate in formic acid tolerance. Screening of the EUROSCARF deletion collection revealed enrichment of the GO term "Chromatin remodeling" upon exposure to acetic acid (Sousa et al., 2013) or various inhibitors present in lignocellulosic hydrolysates (Vanacloig-Pedros et al., 2022). In line with this, strains harboring gRNAs targeting *TAF6* and *TAF9*, which encode proteins involved in chromatin remodeling and histone acetylation (Grant et al., 1998b), were among the most enriched after competitive growth in acetic acid (**Paper III: Table 1 and Table S5**). Taf6 and Taf9 are also subunits of the SAGA complex. Biosensor-based sorting performed in **Paper II** led to the isolation of an acetic acid-sensitive strain expressing a gRNA targeting *TRA1* (**Paper II: Figure 6**), which encodes for another subunit of the SAGA complex, as well as NuA4 histone acetyltransferase. Tra1 has been reported to interact with transcription factors, leading to transcription activation (Grant et al., 1998c). In **Paper III**, two strains with gRNA targeting *TRA1* were enriched after two rounds of FACS in acetic acid, while one strain was depleted after competitive growth in formic acid. Mutations in the *TRA1* gene have been associated with increased temperature sensitivity and reduced growth in various media (Mutiu et al., 2007). Different studies have previously reported the involvement of the SAGA and NuA4 complexes in the yeast response to acetic acid. Strains harboring deletion or overexpression of *ADA2*, *SGF29*, and *TAF9*, which are important for histone acetylation/deacetylation, displayed increased cell death upon acetic acid exposure (Dong et al., 2017). The deletion of several SAGA components, such as *NGG1*, *SPT3*, *SPT7*, *SPT8*, and *SPT20*, in EUROSCARF strains resulted in hampered growth in acetic acid-supplemented medium (Mira et al., 2010a; Sousa et al., 2013). Furthermore, the Scan-o-matic screening performed on acetic acid revealed that repression of *ADA2* and *TAF12* from the SAGA complex or *EPL1* from the NuA4 complex led to acetic acid sensitivity (Mukherjee et al., 2021). Not surprisingly, biosensor-based sorting in acetic acid (**Paper III**) led to enrichment of strains harboring gRNAs targeting the SAGA components-encoding genes *NGG1*, *TAF5*, *TAF6*, and *ADA2*. Additionally, two strains with gRNAs targeting *TAF12* were depleted after competitive growth assays in acetic acid and two after doing so in formic acid. Furthermore, a strain harboring the gRNA targeting *ADA2* was significantly de-enriched at the end of competitive growth in formic acid. Altogether, results from **Paper II** and **Paper III** suggest that an imbalance in acetylation plays a role in acid sensitivity. Fine-tuning the expression of *TRA1* as well as that of other genes of the TAF (*TAF5*, *TAF6*, *TAF9*, *TAF10*, *TAF12*) or acetylase (*ADA2*, *GNC5*, *SGF29*, *NGG1*) modules from the SAGA complex may regulate many of the cellular responses towards acetic and formic acid, leading to more robust and efficient strains for second-generation biorefineries.

The ambiguous role of COX genes in the response to acetic acid

Eight strains harboring gRNAs targeting a total of six *COX* genes were enriched after acetic acid biosensor-based sorting in **Paper III**. *COX* genes encode mitochondrial proteins involved in oxidative phosphorylation. *COX10*, which was the gRNA target gene of the most enriched

strain at the end of biosensor-based sorting (**Paper III: Figure 5 and Table 3**), encodes a heme A farnesyltransferase required for cytochrome c oxidase activity (Glerum & Tzagoloff, 1994). Cytochrome c oxidase is a crucial mitochondrial respiratory chain component catalyzing the transfer of electrons from reduced cytochrome c to molecular oxygen (Shoubridge, 2001). The strain with the highest enrichment after FACS in **Paper III** (COX10-NRg-3), was also one of the isolates in **Paper II** displaying increased sensitivity to acetic acid (**Paper II: Figure 6**). Evidence that mitochondrial dysfunction might lead to acetic acid sensitivity, emerged also from Sousa et al. (2013), even though in that study deletion of *COX10* led to increased tolerance during growth at higher acetic acid concentrations (400 mM). Similarly, COX10-NRg-3 was one of the most enriched strains at the end of competitive growth in acetic acid (**Paper III: Table 1**). Instead, at lower concentrations of acetic acid (70–90 mM), deletion of *COX10* resulted in increased sensitivity (Mira et al., 2010a). These data may indicate a condition-dependent involvement of *COX10* in the yeast response to acetic acid stress. When the acetic acid biosensor was expressed in the *cox10Δ* mutant, the reporter signal was lower compared to the one detected in the strain with gRNA targeting *COX10* (**Paper II: Figure 4**), indicating lower acetic acid accumulation in *cox10Δ* cells. Earlier screens of the deletion collection (Mira et al., 2010a; Sousa et al., 2013) or the CRISPRi collection (Mukherjee et al., 2021) identified *COX* mutants among both sensitive strains and tolerant strains. Many *COX* genes seem to be involved in the response to acetic acid, suggesting that the regulation of cytochrome c oxidase is important for this function in yeast, although their exact role requires further elucidation.

TIF34, MSN5 and PAP1 are potential target genes for improved acetic acid tolerance

Five of the seven strains isolated through FACS in **Paper II** showed increased acetic acid sensitivity (**Paper II: Figure 6**). Two of these strains, with gRNAs targeting *TRA1* and *COX10*, have been mentioned above. The gRNA targets of the other three strains were *MSN5*, *TIF34*, and *PAP1*. Confirming our data, Scan-o-matic screening of the CRISPRi collection performed by Mukherjee et al. (2021) identified an acetic acid-sensitive strain harboring a gRNA targeting *MSN5*. *MSN5* encodes a nuclear exportin involved in the relocation and regulation of several transcription factors (Bose et al., 2005; DeVit & Johnston, 1999; Kim et al., 2018). *MSN5* was targeted in three or four unique strains after the first and second FACS, respectively (**Paper III: Table S11 and S12**). Deletion of *MSN5* results in the constitutive nuclear localization of different phosphorylated transcription factors, including Haa1 (Bose et al., 2005; Kim et al., 2018; Sugiyama et al., 2014). Haa1 regulates a network of genes involved in acetic acid stress responses (Mira et al., 2011), and the binding of acetate allows Haa1 to bind to DNA (Kim et al., 2018). Haa1 is then destabilized upon relocation to the nucleus (Kim et al., 2018). Deletion of *MSN5* has also been associated with decreased levels of Msn2 (Durchschlag et al., 2004), which is another transcription factor involved in the acetic acid stress response in yeast (Mira et al., 2010b). In light of these findings, repression of *MSN5* might favor the nuclear localization of transcription factors important for acetic acid tolerance; whereas *MSN5* downregulation may destabilize these transcription factors, thereby interfering with the acetic acid stress response. This hypothesis was supported by the level of biosensor reporter induction observed in the strain

with gRNA targeting *MSN5* (~1.7-fold) compared to the control pool (~3.7-fold) upon exposure to 0 and 50 mM acetic acid (**Paper II: Figure 4**). However, it should be noted that, being Haa1 the core component of the acetic acid biosensor, repression of *MSN5* might promote nuclear retention of BM3R1-Haa1-mTurquoise2. Prolonged presence of the BM3R1-Haa1-mTurquoise2 complex inside the nucleus might lead to higher biosensor expression due to binding of BM3R1 to its binding sites in the *ENO1* core promoter. Supporting this rationale, reporter expression was significantly higher in the strain with gRNA targeting *MSN5* or in the biosensor-integrated *msn5Δ* mutant than in the control pool (**Paper II: Figure 4**).

TIF34 and *PAP1* are essential genes required for gene expression (**Paper II: Table 1**) (Naranda et al., 1997; Preker et al., 1997). *TIF34* encodes a subunit of the eIF3 core complex and becomes upregulated following acetic acid exposure (Silva et al., 2013). Scan-o-matic screening of the CRISPRi collection identified a strain with gRNA targeting *TIF34* as sensitive to acetic acid (Mukherjee et al., 2021). In the same study, another strain expressing a gRNA targeting *SUI1* was among the sensitive strains. Sui1 is a translation initiation factor, which interacts directly with eIF3 and eIF5 (Jansen et al., 1995). Another translation initiation factor (eIF5A) was reported to regulate the response to acetic acid stress through the eIF5A-Ume6 switch (Cheng et al., 2021). Isolation of eIF3 revealed that the core of the complex (composed by the five subunits Tif32, Prt1, Nip1, Tif35, and Tif34) was associated with eIF5 (Phan et al., 1998). Accordingly, the eIF3 complex could be involved in modulating acetic acid tolerance in yeast. Therefore, repression of *TIF34*, which is a subunit of the eIF3 core complex, may result in dysregulation and higher sensitivity to acetic acid.

PAP1 encodes a poly(A) polymerase important for mRNA export, whose participation in the acetic acid stress response has not been reported prior to the findings in **Paper II**. The sixth most enriched strain following biosensor-based sorting in **Paper III** was a strain with gRNA targeting *PAP1* (**Paper III: Table S12**). Polyadenylation by Pap1 could boost the stability of transcripts related to acetic acid resistance. Thus, a fully functional transcriptional system may be crucial to ensure mRNA maturation of all components leading to acetic acid tolerance.

Echoing the results in **Paper II**, all seven strains isolated from that screening were also significantly enriched after biosensor-based sorting (**Paper III: Table S12**). This was expected, considering the similar experimental settings between the two studies. Three of those strains, namely COX10-NRg-3, UBA2-NRg-6, and MSN5-NRg-1, were highly enriched after FACS in **Paper III**. Conversely, QCR8-TRg-4, PAP1-TRg-1, TIF34-NRg-4, and TRA1-NRg-2 displayed lower enrichment in **Paper III**, despite them showing the highest biosensor signal (**Paper II: Figure 4**). While biosensor-based sorting presumably selected for acetic acid-sensitive strains; cultivation of the strains prior to sequence identification (**Paper III**) may have enriched cells with a shorter lag phase, explaining this discrepancy. Nevertheless, biosensor-based enrichment in **Paper III** of strains harboring gRNAs targeting the same genes identified in **Paper II** supports the role of those genes in the response to acetic acid stress. Further investigation of their role may advance the design of strains tolerant to inhibitors of lignocellulosic hydrolysates.

Comparing biosensor-based and competitive growth screening techniques

While screening large strain libraries can be particularly useful for identifying global responses to specific conditions, as well as single genes involved in tolerance or sensitivity, the experimental set-up will also influence the outcome. Similar conditions, such as those employed in the biosensor-based screenings of **Paper II** and **Paper III**, led to the identification of similar sets of strains. Conversely, when the screening method varied, as occurred in **Paper III** when competitive growth assays and a higher acetic acid concentration were used in parallel with biosensor-based screening, the collected data displayed some substantial differences. This observation highlights the role of the different screening techniques presented in this thesis.

Pooling a whole library into a single culture for a competitive growth assay allows for screening of larger strain collections, using relatively few and cheap instruments. This method enriches cells with the fastest growth under the given experimental conditions and time frame (Gutmann et al., 2021; Lian et al., 2019; Momen-Roknabadi et al., 2020; Smith et al., 2017). However, competitive growth assays are intrinsically biased towards strains with a shorter lag phase; whereas cells with a longer lag phase but potentially higher growth rates might be lost due to previous depletion of nutrients (Mukherjee et al., 2021). This could pose a bias during data interpretation, as depletion or de-enrichment of a strain after competitive growth does not necessarily imply an overall lower tolerance compared to enriched cells. In fact, it only indicates that the depleted/de-enriched strain requires an extended lag phase to adapt to the given experimental conditions. Furthermore, pooling together a library into a single culture may pose a problem in terms of even representation of each strain in the initial pooled culture. This was observed in **Paper III**, whereby the pooled CRISPRi library harbored only 7895 (acetic acid) or 7853 (formic acid) strains prior to the competitive growth assays, compared to the 9078 strains contained in the original CRISPRi library. The cells may also behave differently in a mixed culture as opposed to a pure culture due to peculiar strain-to-strain interactions. Lastly, while single strain characterization provides beforehand knowledge of the genetic background of each strain (Mukherjee et al., 2021; Mukherjee et al., 2023), competitive growth requires an additional identification step at the end of the screening to determine which mutant corresponds to the phenotype of interest.

Even though biosensors can be exploited for the characterization of single strain cultures grown in parallel, they provide a much higher throughput power when used to screen pooled cultures. In **Paper II** and **Paper III**, fluorescence-based biosensors were combined with FACS. However, as with the competitive growth assay, the strains isolated or enriched from the pooled culture need to be identified to associate the observed phenotype to a particular mutant. Representation of each strain in the initial pooled culture may be an issue, too. Some strains may be lost during biosensor transformation, as documented in **Paper III**, where the pooled biosensor-integrated CRISPRi library harbored only 8702 of the 9078 strains contained in the original collection. Thus, to ensure good coverage, transformation of the library with the biosensor should produce enough transformants. For instance, the biosensor-integrated CRISPRi library created in **Paper II** had an 11× coverage compared to the original library. Dabirian et al. (2019) and Wang et al. (2019) successfully screened two different strain

collections using biosensor libraries that were approximately six times and three times the original ones, respectively. It should be noted that transforming a biosensor into a strain library can be problematic in terms of number of transformants obtained in a single experiment, diminishing the throughput of the method. Nevertheless, biosensor-based screening of yeast strain libraries has been proven suitable for identifying strains of interest from a large pool (Dabirian et al., 2019; Li et al., 2015) (**Paper II**, **Paper III**). Compared to competitive growth assays, biosensor screening enables the enrichment of cells with specific phenotypes rather than just those with better fitness. By doing so, biosensors can reduce the bias favoring strains with a shorter lag phase. Furthermore, modern FACS enables the sorting of hundreds of thousands of cells down to single cells, amplifying the range of traits to be screened.

In summary, screening a library by pooling it into a single culture may lead to an imperfect representation of the original collection, as shown in **Paper II** and **Paper III**. However, competitive growth assays, as well as biosensor-based screenings have the potential to dramatically speed up the identification of strains of interest due to their extreme processivity (Yilmaz et al., 2022). The choice of screening technique based on the desired phenotype is important when designing a study. While competitive growth assays are powerful tools to isolate cells based on growth, biosensor-based screening may be employed to select for traits unrelated to growth such as improved production rate of specific compounds.

V. Conclusions

The work performed in my thesis sought to answer the two research questions presented in Chapter I: **1)** *which genes are important for the tolerance of yeast towards lignocellulosic hydrolysates and the inhibitors therein?* **2)** *How can high-throughput screening be used to identify and develop strains that are more tolerant towards the inhibitors present in lignocellulosic hydrolysates?* Addressing the questions should contribute to elucidating the mechanisms through which *S. cerevisiae* adapted to stress, in an attempt to increase the overall knowledge of this organism and find ways to improve its performance as a cell factory. Three main goals were set to address the research questions of the thesis:

- To identify strain-dependent transcriptional responses of *S. cerevisiae* grown in wheat straw hydrolysate (**Paper IV**);
- To develop a transcription factor-based biosensor for acetic acid sensing (**Paper I** and **Paper II**);
- To explore the role of essential and respiratory growth essential genes of *S. cerevisiae* in the response to acetic acid and formic acid stress, using a biosensor and competitive growth assays (**Paper II** and **Paper III**).

Stress responses are complex events, which require extensive datasets to draw any conclusions about their workings. Given the power of different omics techniques in uncovering whole-cell responses, a transcriptomic analysis was performed on five *S. cerevisiae* strains. The analysis led to the identification of common, as well as strain-specific genetic strategies supporting tolerance to growth on lignocellulosic hydrolysates (**Paper IV**). The two tolerant wild-type strains, LBCM31 and LBCM109, exhibited similar regulation of various genes involved in glutathione metabolism, compared to the CEN.PK strain. The regulation of those genes may result in more efficient glutathione and NADPH regeneration, which could help counteract the oxidative stress induced by inhibitors present in lignocellulosic hydrolysates. At the same time, different sugar and iron transporters were upregulated in LBCM31 compared to LBCM109, which may favor energy regeneration and medium detoxification. Controlling the intracellular accumulation of iron is crucial for obtaining cofactors for different metabolic reactions and prevent the formation of ROS. Compared to LBCM31, the LBCM109 transcriptome showed significant induction of several genes involved in lipid metabolism and transport, suggesting a different remodeling of the cell membrane in response to wheat straw hydrolysate. In particular,

a remarkable induction was observed for genes that promoted the accumulation of ergosterol, possibly reflecting their importance in determining the cells' tolerance towards lignocellulosic hydrolysates. Collectively, the transcriptomic response of LBCM strains to WSH highlighted the induction of some genes involved in the oxidative stress response as a possible common strategy to develop tolerance. At the same time, the regulation of genes controlling the composition of the membrane layout, including genes regulating transporters and lipid metabolism, may be part of a strain-dependent adaptation.

During pretreatment of lignocellulosic biomass, acetic and formic acid are released in amounts that significantly influence cellular performance. Different studies have sought to identify strains with improved tolerance towards these two acids; however, only limited tools exist for high-throughput screening of yeast libraries. The biosensor developed and described in **Paper I** and **Paper II** enables biosensor-based detection of intracellular acetic acid in *S. cerevisiae*. The dynamic range and orthogonality of the biosensor were progressively improved by changing its genetic components. The acetic acid biosensor provides a new tool for the identification of strains based on their production or intracellular accumulation of acetic acid. As demonstrated in **Paper II** and **Paper III**, the biosensor was successfully validated for high-throughput screenings to isolate strains with altered tolerance towards acetic acid.

The competitive growth and biosensor-based screenings performed on the CRISPRi library in **Paper II** and **Paper III** associated different genes encoding cytochrome c oxidase or proteosome subunits with tolerance towards acetic and formic acid. These findings suggested a role for these complexes in the adaptation of yeast cells to acid stress. The downregulation of different genes involved in histone acetylation affected tolerance to acetic and formic acid (**Paper II** and **Paper III**). In particular, several of these genes encoded subunits of the SAGA complex, implicating histone acetylation in the regulation of cellular responses to acid stress. Lastly, *PAP1* and *HIP1* emerged as new potential targets for the design of strains with improved tolerance to weak organic acids. The regulation of *PAP1* may affect the genes involved in acid stress response by altering the polyadenylation and stability of their mRNAs; whereas *HIP1* could strengthen yeast defenses against ROS and oxidative stress by influencing the intracellular concentration of manganese.

Altogether, my thesis enriches our knowledge of strain-dependent responses set in place by *S. cerevisiae* to withstand growth in lignocellulosic hydrolysates, as well as the mechanisms establishing tolerance towards acetic and formic acid. Additionally, the acetic acid biosensor developed in my thesis was proven to identify strains based on their production or intracellular accumulation of acetic acid, enabling high-throughput screening to isolate strains with altered tolerance towards acetic acid. Importantly, the acetic acid biosensor has the potential to be employed to monitor the impact of genetic changes on the acetic acid accumulation in the strains. Furthermore, the acetic acid biosensor represents a new powerful tool for the generation of improved strains for biorefinery applications.

VI. Outlook and future perspectives

The growing need for alternatives to fossil fuel-based production is pushing the implementation of industrial bioprocesses. *S. cerevisiae* is one of the main platforms exploited in second-generation biorefineries; therefore, its optimization as a cell factory is central for the transition to a fossil-free society. For this reason, exploring the mechanisms underlying the response of yeast cells to the inhibitors contained in lignocellulosic hydrolysates will likely remain a pivotal topic in the coming years.

In the second chapter of my thesis, several genes encoding different transporters or proteins involved in oxidative stress response and membrane remodeling were shown to be important for the adaptation of *S. cerevisiae* to growth in lignocellulosic hydrolysates (**Paper IV**). Analyzing the plasma membrane composition of LBCM strains cultivated in lignocellulosic hydrolysates may clarify whether changes in the expression of genes favoring ergosterol accumulation translate into a different lipidic layout of the membrane. Monitoring the iron concentration inside and outside LBCM strains could help explain the induction of iron transporter genes observed in LBCM31, providing new insight on the role of iron metabolism in *S. cerevisiae* adaptation to lignocellulosic hydrolysates. Altering simultaneously the expression of different genes involved in ergosterol metabolism, glutathione metabolism, as well as ABC, hexose or iron transporters using knockout, overexpression or CRISPRa/i libraries could disclose the synergistic effect between these groups of genes in mediating tolerance towards lignocellulosic hydrolysates and the inhibitors therein.

In the third chapter of my thesis, different approaches for designing and optimizing the acetic acid biosensor were presented (**Paper I** and **Paper II**). Further improvements in terms of dynamic and operational range are still possible. For instance, different core promoters driving the expression of the reporter gene could be tested. Additionally, varying the number of BM3R1 binding sites in the reporter's promoter could alter the ability of the transcription factor to bind the promoter and recruit the transcription machinery, possibly improving the dynamic and operational range. Creating and screening a library of randomly mutated BM3R1-Haa1-mTurquoise2 may lead to the identification of alternative versions of the current biosensor, in which the transcription factor has a different affinity for acetic acid or the binding sites on the promoter. Furthermore, random mutagenesis and rational engineering on the effector binding domain of Haa1 could modify the specificity of the biosensor towards other valuable molecules,

such as lactic acid. Additionally, the acetic acid biosensor could be employed to verify how the altered expression of specific target genes, such as the ones identified in **Paper III** and **Paper IV**, affects the acetic acid accumulation and growth of *S. cerevisiae*. The biosensor could be also utilized for studies beyond strain library screenings, such as online monitoring of acetic acid production in bioreactors or to assess the level of acetic acid in lignocellulosic hydrolysates following different processing steps.

In the fourth chapter of my thesis, the screenings performed on the CRISPRi library led to identification of multiple genes involved in the yeast response to acetic and formic acid (**Paper II** and **Paper III**). *PAP1* and *HIP1* emerged as interesting novel target genes for improving acetic or formic acid tolerance. Overexpressing *PAP1* could represent a relatively fast and easy way to evaluate its potential in enhancing tolerance towards weak organic acids. Transcriptomic and/or proteomic analyses of cells grown in acetic or formic acid could be performed using strains with altered expression of *PAP1*, thereby revealing the metabolic networks influenced by this gene during stress. Evaluating the correlation between the intracellular concentration of manganese and tolerance to acetic or formic acid in strains with altered expression of *HIP1* could elucidate the role of this transporter in yeast resistance to acid stress. The level of DNA acetylation upon acid exposure in the identified strains harboring gRNAs targeting genes encoding for subunits of the SAGA complex, such as *SMC4* or *TRA1*, could be investigated. Such a study may provide new insights on the role of SAGA-mediated DNA acetylation in yeast tolerance to acetic acid and formic acid. Additionally, the transcriptome of the identified strains with gRNAs targeting genes involved in the SAGA complex could be analyzed to determine the changes in their global gene expression and put those changes in relation with the acetic acid or formic acid tolerance of the strains.

Finally, the information collected in my thesis could be used for an AI approach to predict and optimize yeast strategies leading to tolerance towards lignocellulosic hydrolysates and the inhibitors therein. For instance, predictive modeling and machine learning algorithms could be used to model the target genes of the lipid metabolic pathways identified in my thesis. Modeling the lipid metabolic pathways could lead to predict connections between the expression of the genes involved in those pathways and the cell membrane composition. Comparing the predictions from the model with the actual changes observed in the lipid metabolism of strains cultivated in presence of lignocellulosic hydrolysates could provide clues on how membrane remodeling contributes to the development of tolerance towards hydrolysates-induced stress. Moreover, such an approach would allow for the identification of additional promising targets for genetic engineering to improve yeast resistance to lignocellulosic hydrolysates.

Uncovering the different strategies evolved by yeast strains to adapt to the inhibitors present in lignocellulosic hydrolysates will expand our command of the complex network regulating the response to these stressors. Understanding the underlying mechanisms is crucial to optimize cell factories for second-generation biorefineries. Every new snippet of information adds a new piece to the puzzle, eventually enabling metabolic engineering strategies capable of improving the performance of yeast cell factories.

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