THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

# CRISPRi/a for investigating yeast tolerance to inhibitors in lignocellulosic hydrolysates

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Cover: Schematic representation of CRISPRi/a mediated expression modulation of different stress response genes.

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*” Bihotzez ikasten dena ez da inoiz ahazten”*

- Porrotx

*” What’s wholeheartedly learnt is never forgotten”*

- Porrotx

# Preface

This dissertation serves as partial fulfilment of the requirements for obtaining the degree of Doctor of Philosophy at the Department of Life Sciences at Chalmers University of Technology. The work was funded by the Swedish Research council (Vetenskapsrådet), Novo Nordisk foundation and the Hasselblad foundation. The PhD studies were carried out between September 2019 and November 2023 under the supervision of Associate Prof. Yvonne Nygård and co-supervision of Research Prof. Verena Siewers and Assistant Prof. Florian David. The thesis was examined by Prof. Lisbeth Olsson.

The majority of the work in this thesis was carried out at the Division of Industrial Biotechnology (IndBio). Scan-o-matic screening was conducted at the Department of Chemistry and Molecular Biology at Gothenburg University by Dr. Vaskar Mukherjee and Dr. Ulrika Lind. Amplicon sequencing was performed at the National Genomics Infrastructure (NGI). Primary, sequencing analysis was conducted by CD Genomics. ChIP-exo assays were performed by Dr. Christoph Börlin at the Systems and Synthetic Biology division at Chalmers.

Ibai Lenitz Etxaburu,

October 2023

**CRISPRi/a for investigating yeast tolerance to inhibitors in lignocellulosic hydrolysates**

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## Abstract

*Saccharomyces cerevisiae* has immense potential as a cell factory in various biotechnological processes where biomass from agricultural industry residues is used as feedstock. Nonetheless, the inhibitors released during the pretreatment of the biomass makes lignocellulosic hydrolysates a challenging substrate for microorganisms. In this thesis, the CRISPR interference/activation (CRISPRi/a) technology was used in combination with high-throughput screening methods to improve tolerance of *S. cerevisiae* towards inhibitors found in lignocellulosic hydrolysates. The focus was on understanding the genetics behind formic and acetic acid tolerance, two abundant inhibitory compounds. The aims were to compare the responses to either acid and to explore how the results could be extrapolated to understand hydrolysate tolerance.

The CRISPRi/a technology was used to improve the hydrolysate tolerance of an industrial strain and to alter the expression of the transcription factor encoding genes *YAP1* and *PDR1*, leading to strains with altered tolerance to acetic acid. We performed ChIP-exo experiments, which demonstrated that both transcription factors showed increased binding of target genes in the presence of acetic acid. Notably, genes related to amino acid synthesis and cell membrane transporters were highly bound to Yap1 and Pdr1 in the presence of acetic acid. Furthermore, A CRISPRi strain library targeting the essential and respiratory essential genes in *S. cerevisiae* was studied for acetic and formic acid tolerance. The strains were screened by using various high-throughput methods such as competitive growth assays, fluorescence-activated cell sorting and screening for growth on solid media. Systematic analysis of the data highlighted genes encoding proteins with functions in intracellular vesicle transport, glycogen accumulation or chromatin regulation as important for tolerance towards acetic and formic acid. Interesting strains were further characterized individually in the presence of acetic or formic acid, in a synthetic hydrolysate medium or in the presence of oxidative stress causing agents.

To conclude, this research advances our knowledge on how the regulation of genes such as the ones related to chromatin remodeling can influence tolerance to weak acids as well as other inhibitors found in lignocellulosic hydrolysates. The results demonstrate the potential of CRISPRi/a technology to accelerate the development of more tolerant industrial yeast strains.

**Keywords**: Acetic acid; Formic acid; Lignocellulosic hydrolysates; CRISPRi/a; Competitive growth assay; Tolerance; ChIP-exo; Screening; Yeast; Biosensor

# List of publications

This thesis is based on the following papers:

I. Cámara, E., **Lenitz, I**., & Nygård, Y. (2020). A CRISPR activation and interference toolkit for industrial *Saccharomyces cerevisiae* strain KE6-12. *Scientific Reports 2020 10:1*, *10*(1), 1–13. DOI: 10.1038/s41598-020-71648-w

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. DOI: 10.1186/s12934-022-01938-7

III. Mukherjee, V.*, **Lenitz, I.***, Lind, U., Blomberg, A., & Nygård, Y. (2023). CRISPRi screen highlights chromatin regulation to be involved in formic acid tolerance in *Saccharomyces cerevisiae*. *Engineering Microbiology*, 100076. DOI: 10.1016/J.ENGMIC.2023.100076

IV. **Lenitz, I**., Mormino, M., Blomberg, A., Mukherjee, V., Nygård, Y. (2023) Pooled CRISPRi screen of essential genes of *Saccharomyces cerevisiae* reveals genes important for tolerance to acetic acid and formic acid. *Manuscript*

V. **Lenitz, I**., Börlin, C., Torello Pianale, L., Balachandran, D., Nielsen, J., David, F., Siewers, V., Nygård, Y. (2023) Chip-exo and CRISPRi/a reveal that Pdr1 and Yap1 play a part in acetic acid tolerance. *Manuscript*

*These authors contributed equally.

# Contribution summary

I. EC and I performed all the experiments and data analysis. EC drafted the manuscript and the rest of the authors contributed with discussion and interpretation of data as well as with writing and revision of the manuscript.

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

III. MM and UL performed the solid strain screen. I performed the liquid strain characterization. I did most data analysis and I drafted the manuscript with help from VM. The rest of authors contributed to the discussion and interpretation of the data, as well as with the writing and revision of the manuscript.

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

V. I performed the experiments with the help of CB and DB. I analyzed the data with the help of LT. 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 Contents

# Abbreviations

ABC: ATP-binding cassette

AI: Artificial intelligence

ATc: Anhydrotetracycline

ChIP: Chromatin immunoprecipitation

CP: Core particle

CRISPR: Clustered regularly interspaced short palindromic repeats

CRISPRa: CRISPR activation

CRISPRi: CRISPR interference

dCas9: dead Cas9

FACS: Fluorescence-activated cell sorting

FBA: Flux balance analysis

GO: Gene Ontology

HDR: Homology-directed repair

HMF: 5-hydroxymethylfurfural

HOG: High osmolarity glycerol

HTS: High-throughput screening

ML: Machine learning

NAD(P)H: Nicotinamide adenine dinucleotide (phosphate)

ROS: Reactive oxygen species

RP: Regulatory particle

sgRNA: guide RNA

(S)WSH: (Synthetic) wheat straw hydrolysate

UPR: Unfolded protein response

# Thesis outline and chapter focus

The unprecedent technological advancements of the past century have rested on excessive exploitation of fossil fuels and growing $CO_2$ emissions. To diminish our 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 a well-established workhorse in the production of biochemicals. As outlined below, the goal of this thesis was to provide new knowledge on the underlying tolerance mechanisms of *Saccharomyces cerevisiae* towards inhibitors found in lignocellulosic hydrolysates. Furthermore, high throughput screening tools were utilized to screen for tolerant strains that can unveil gene targets for the development of future cell factories.

**Chapter I**. Introduction to the role of yeast and lignocellulosic hydrolysates. The chapter ends with the Introduction of the research questions and aims of the thesis.

**Chapter II.** Introduction to lignocellulosic materials, biotechnological potential and composition. Then, the main characteristics of inhibitory compounds are described, as well as the synergistic effects of their combination.

**Chapter III.** Introduction of this thesis cell factory platform *Saccharomyces cerevisiae*. Past development and the main problems present with their use in fermentations with plant biomass as substrate. Strategies for the development of more tolerant yeast strains are described, with a special focus on CRISPR/Cas9 based rational engineering and CRISPRi/a-based gene expression regulation. Strain development for **Paper I-V** is discussed.

**Chapter IV.** Different high-throughput screening tools are described, focusing on both solid and liquid high throughput screening platforms, competitive growth assays and biosensor-based assays. The experimental designs used in the different **Papers I-V** are described and compared.

**Chapter V.** The responses of *S. cerevisiae* towards inhibitory substances found in lignocellulosic biomass are described. Tolerance related genes covered in **Paper I** and **Paper V** are described. The role of essential genes in inhibitor tolerance, covered by **Papers II-III-IV** is described and put in relation to other studies.

**Chapter VI.** The main conclusions of the thesis are summarized. The chapter describes future perspectives for yeast tolerance engineering for improvement of yeast cell factories.

# Chapter I: Introduction

Existing means of energy production based on fossil fuels, such as coal or petroleum, are facing increasing public criticism owing to their negative impact on the environment. In particular, the carbon dioxide ($CO_2$) released when burning these fuels is causing health issues and global warming (Costello et al., 2009; Lashof and Ahuja, 1990). The Paris Agreement from 2015 required that all 195 participating countries made plans to reduce $CO_2$ emissions, with the intent of preventing global mean temperatures from rising more than 1.5ºC compared to those before industrialization (United Nations Framework Convention on Climate Change, 2015). This has pushed governments and industries to shift towards cleaner and renewable energy sources. Sweden alone increased its share of renewable energy from 33% in 1990 to 60% in 2020 (Swedish Energy Agency, 2022).

Several studies have highlighted the potential of replacing fossil fuel-based processes with bio-based production of chemicals to reduce greenhouse gas emissions (Huang et al., 2021; Kätelhön et al., 2019; Khoo et al., 2016; Montazeri et al., 2016). In this context, microbial cell factories offer a promising alternative for the production of fuels, pharmaceuticals, food supplements or fine and bulk chemicals. Microbial cell factories include microorganisms engineered to produce chemicals from renewable carbon sources (Cho et al., 2022). *Escherichia coli* or *Saccharomyces cerevisiae* are some of the most widely applied cell factories, but also some of the best studied model microorganisms, which has led to the development of numerous molecular tools with which to engineer these strains (S. Y. Lee et al., 2019). To optimise production of specific compounds, other cell factories with superior metabolic properties have emerged, including *Corynebacterium glutamicum* (aminoacids), *Pichia pastoris* (heterologous proteins), *Pseudomonas putida* (isoprenoids), and *Yarrowia lipolytica* (fatty acids) (Gohil et al., 2021). Owing to its tolerance of varying environmental conditions, including temperature, pH, and salinity, along with its ability to produce proteins or metabolites at high titres, *S. cerevisiae* is particularly versatile and robust, making it the cell factory of choice for many applications (Nielsen 2019).

Microbial cell factories can convert renewable biomass into products of industrial interest. Nevertheless, it is often necessary to pretreat biomass so it meets the requirements of a specific microorganism. One of the feedstocks used in biorefinery industries is lignocellulosic biomass, which constitutes one of the most abundant sugary bioresources (Baruah et al., 2018;Hassan et al., 2019). Lignocellulosic biomass encompasses agricultural residues, energy crops, and virgin

biomass not used for food or feed (Yousuf et al., 2019). It is composed of cellulose, hemicellulose, and lignin at varying ratios. To open up its rigid structure and release its sugars, it needs to undergo physical or chemical pretreatment followed by enzymatic digestion (Zhao et al., 2022). However, pretreatment of hydrolysates often leads to the partial degradation of hemicellulose and lignin into toxic compounds, which can inhibit yeast growth (reviewed by Jönsson et al., 2013). These inhibitors can be classified into three main groups: furans, phenolic acids, and weak acids (Cámara et al., 2022; Jönsson et al., 2013; Vanmarcke et al., 2021). Additionally, cultivation conditions, such as temperature, pH, and nutrient availability, can have a bearing on the toxicity of inhibitors (Xiros and Olsson, 2014).

The presence of inhibitors in pretreated hydrolysates constitutes a bottleneck in the bioproduction of chemicals. They can impair yeast growth, lengthen the lag phase or affect product yield and fermentation efficiency (Cámara et al., 2022). Thus, efforts have been directed towards improving the fermentative capabilities and growth of yeast in the presence of lignocellulose-derived inhibitors, either via adapted evolution (Koppram et al., 2012a; Wright et al., 2011a) or rational genetic engineering (Adeboye et al., 2017; Brandt et al., 2021; Stovicek et al., 2022). The cellular response to a given inhibitor is multigenic, possibly triggering several signalling cascades with a high degree of cross-talk (Fuchs and Mylonakis, 2009). There are multiple ways in which the yeast cell counteracts inhibitor-derived stress, including inhibitor detoxification and efflux, energy conservation, and decrease in membrane permeability (Cámara et al., 2022). Consequently, the increase in cellular tolerance towards each inhibitor depends on multiple genes, which need to be upregulated or downregulated.

Over the last couple of decades, large-scale studies using the EUROSCARF deletion library (Winzeler et al., 1999) have systematically assayed the genome of *S. cerevisiae* for inhibitor tolerance (Henriques et al., 2017; Kawahata et al., 2006; Mira, Teixeira, et al., 2010; F. B. Pereira et al., 2014; Sousa et al., 2013; Vanacloig-Pedros et al., 2022). More recently, an efficient way of quickly making downregulation or overexpression mutants has emerged in the form of CRISPR/Cas9-derived CRIPSR interference (CRISPRi) and CRISPR activation (CRISPRa) technologies (L. S. Qi et al., 2013). CRISPRi/a employs a catalytically inactive Cas9 nuclease (dCas9) that can bind DNA with the help of a single guide RNA (sgRNA) (L. S. Qi et al., 2013); this interaction then alters the expression of target genes (Figure 3.1) (Momen-Roknabadi et al., 2020; J. D. Smith et al., 2016). CRSPRi/a-based strain libraries have gained popularity as the importance of fine-tuning gene expression has become apparent for tolerance screens (Mukherjee et al., 2021, 2023; Saeki et al., 2023; Sardi et al., 2016). Importantly, CRISPRi technology has enabled the inclusion of essential genes in downregulation screens. Previously, deletions of essential yeast genes had been tested

mainly by using heterozygous deletion mutants or strains carrying temperature-sensitive alleles (Franzosa et al., 2011; Z. Li et al., 2011; Okada et al., 2014). Such screenings do not always alter the gene function in a significant way, as essential genes are often haplosufficient (Giaever et al., 1999). Thus, CRISPRi presents an opportunity for testing various levels of downregulation of essential genes for their role in lignocellulose-derived inhibitor tolerance.

Understanding the harmful effects of individual inhibitors on yeast growth and the ensuing gene response is crucial for developing more tolerant industrial-use yeast strains. Given the complexity of inhibitors' toxic effects and the polygenic nature of the cell response to them, new ways of building mutant libraries and testing multiple mutants in a high-throughput manner are required. To address these issues, I studied the gene response of baker's yeast to single inhibitors and complex media. Specifically, the present thesis focuses on answering the following research questions:

- How are essential *S. cerevisiae* genes involved in tolerance towards lignocellulosic inhibitors?
- How can high-throughput strain screening methods be used to help identifying genes crucial for inhibitor tolerance in *S. cerevisiae*?
- Can strain fitness towards a single inhibitor be reproduced in more complex media such as hydrolysates?

To address the above questions, the following specific aims were set:

- To apply CRISPRi/a technology to rationally develop strains with altered expression of tolerance-related genes (**All papers**).
- To screen industrial (**Paper I**) and laboratory (**Paper V**) CRISPRi/a strains for tolerance.
- To identify gene targets for inhibitor tolerance engineering (**Papers II-V**) and compare the phenotypes of the engineered mutants in the presence of acetic and formic acid (**Papers I and III-IV**).
- To establish high-throughput solid (**Paper III**) and liquid (**Paper II and Paper IV**) media screening methods to assay tolerance to inhibitors present in lignocellulosic hydrolysates.

# Chapter II: Lignocellulosic biomass as substrate for *S. cerevisiae* cell factories

## Lignocellulosic hydrolysates: a renewable resource for the biorefinery industry

Plant biomass, also called lignocellulosic biomass, is composed of three main fractions: cellulose, hemicellulose, and lignin (Figure 2.1). Cellulose comprises a linear chain of several hundreds or thousands of D-glucose units; whereas hemicellulose is a heteropolymer containing both hexoses (mannose and galactose) and pentoses (xylose and arabinose). Lignin, instead, is composed of a heterogeneous group of polymers derived from phenylpropane (coniferyl alcohol, sinapyl alcohol, and paracoumaryl alcohol) bound together by different linkages. The combination of these three components makes the plant cell wall a highly stable and recalcitrant structure, which requires pretreatment and hydrolysis prior to utilisation in fermentation bioprocesses (Rowell, 2005). The main aim of the pretreatment step is to increase the amount of accessible cellulose, while solubilizing hemicelluloses and lignin (Xu and Huang, 2014). This procedure releases simple sugars, which can be fermented by microorganisms and turned into the final product.

Typically, lignocellulosic biomass used as feedstock in biorefineries undergoes pretreatment by a mixture of physical and chemical means, followed by enzymatic hydrolysis. While it is difficult to establish an ideal pretreatment for all source materials and downstream applications, all approaches aim for high sugar recovery and the lowest possible amount of inhibitors. Severe pretreatment conditions cause degradation of hemicellulose, resulting in the release of pentose and hexose sugars, acetyl decorations that will form acetic acid, and other weak acids (formic acid and levulinic acid) or furan aldehydes, including 5-hydroxymethylfurfural (HMF) and furfural (see Figure 2.1) (Galbe and Wallberg, 2019). After acidic pretreatment, lignin is recovered as a solid residue, while a small portion is degraded into phenolics and other aromatic compounds (Figure 2.1). Weak acids, phenolic compounds, and furans tend to inhibit microbial growth and lower the final fermentation yields (Jönsson et al., 2013).

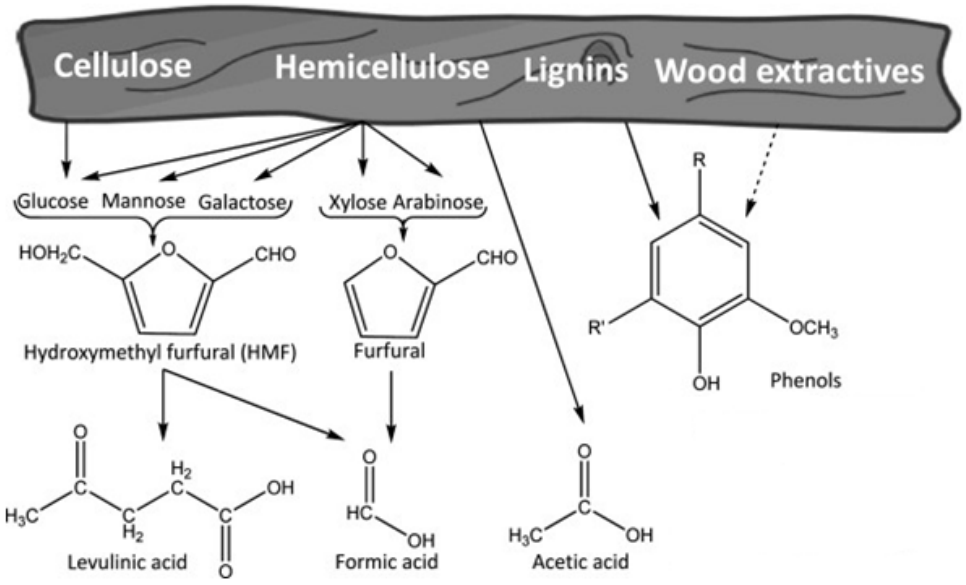


*Figure 2.1. Schematic representation of the principal inhibitors generated in lignocellulosic hydrolysates. Adapted from* Jönsson et al. (2013).

The profile of by-products arising from pretreatment of lignocellulosic biomass depends largely on the type of feedstock and the specific pretreatment method used (Table 2.1) (Olsson and Hahn-Hägerdal, 1996). Weak acids, such as acetic or formic acids, and furans are generally found at higher concentrations (1–10g/L) than phenolic compounds (0–1g/L) (Cámara et al., 2022; Vanmarcke et al., 2021) (Table 2.1). Inhibitors severely affect the productivity of microbial fermentation by stretching the lag phase, reducing the growth rate, lowering cell densities, and inhibiting product formation (J. R. M. Almeida et al., 2007).

*Table 2.1. Inhibitor composition of different lignocellulose hydrolysates. Adapted from* Vanmarcke et al. (2021). *Phenolic compounds are not included, due to limited studies describing their levels. N.D, not determined.*

| Hydrolysate | Acetic Acid (mM) | Formic Acid (mM) | Levulinic acid (mM) | Furfural (mM) | HMF (mM) |
|---|---|---|---|---|---|
| **Agave bagasse** | 129.9-138.2 | 43.5-78.2 | N.D | N.D | N.D |
| **Alder** | 151.5-186.5 | N.D | N.D | 2.1-14.6 | 20.6-35.7 |
| **Aspen hardwood** | 136.5-168.2 | 0 | 0 | 21.9-36.4 | 10.3-53.9 |
| **Bark** | 0-103.2 | N.D | N.D | 5.2-10.4 | 3.2-34.1 |
| **Barley straw** | N.D | N.D | N.D | 30.2 | 7.9 |
| **Biowaste** | 33.3 | N.D | N.D | 0 | N.D |
| **Birch hardwood** | 33.3-191.5 | 99.9 | 0 | 2.1-47.9 | 0.8-46 |
| **Cardboard** | 8.3 | N.D | N.D | 1.0 | N.D |
| **Coffee husks** | 48.3 | N.D | N.D | 0 | 2.4 |
| **Corn cob** | 99.9 | N.D | N.D | 4.2 | N.D |
| **Corn stover** | 0-36.6 | 0-147.7 | 0-18.9 | 6.2-114.5 | 0.8-42 |
| **Oil palm** | 83.3-149.9 | N.D | N.D | 10.4-12.5 | N.D |
| **Pine** | 0-61.6 | 0 | 0 | 7.3-71.8 | 7.9-68.2 |
| **Rice husks** | 30-35 | 54.3-58.7 | N.D | N.D | N.D |
| **Rice straw** | 38.3 | N.D | N.D | 1.0 | 2.4 |
| **Sorghum bagasse** | 16.7 | 3.5 | 1.7 | 0 | 12.7 |
| **Spruce** | 0-78.4 | 13-69.5 | 1.7-27.6 | 2.1-14.6 | 4-66.6 |
| **Sugar cane bagasse** | N.D-81.6 | N.D.-54.3 | N.D.-23.3 | 1-32.3 | 0.8-23.8 |
| **Wheat straw** | 50-116.6 | 0-28.2 | 0 | 4.2-14.6 | 0.8-2.4 |
| **Willow** | N.D | N.D | N.D | 3.1-33.3 | 4.8-30.9 |

*Phenolic compounds*

Phenolics are organic compounds containing aromatic rings that result from the degradation of lignin present in lignocellulosic hydrolysates. They form as a consequence of the acidic environment in the medium during or after pretreatment, although even non-acidic pretreatment methods (e.g. alkaline, ammonia fibre expansion, and liquid hot water) have been reported to cause lignin depolymerisation and release of phenolic compounds (Galbe and Wallberg, 2019). Soluble phenolic compounds resulting from pretreatment are structurally and chemically diverse, reflecting the type of biomass and treatment applied (Huang et al., 2019; Rasmussen et al., 2017). This, in turn, causes a wide range of inhibitory effects, which are difficult to trace to single compounds. Aldehydes and ketones are considered more toxic to *S. cerevisiae* than acids or alcohols (Klinke et al., 2003; Larsson et al., 2000). Small phenolic compounds have been associated with a strong inhibitory effect in yeast, probably due to their ability to penetrate cell membranes and damage internal structures (Jönsson and Martín, 2016; Klinke et al., 2004; Zhai et al., 2018). Phenolic inhibitors affect the fermentability of hydrolysates and directly impact ethanol production in yeast (Adeboye et al., 2014; Y. Kim et al., 2011; Klinke et al., 2004; Ko et al., 2015; Michelin et al., 2016; Qin et al., 2016). Nevertheless, there is no evidence pointing to a common mechanism of toxicity for phenolics. Fletcher and Baetz (2020) summarized several biological processes involved in tolerance to phenolics in a review, highlighting genes involved in oxidative stress response, oxidoreductase and mitochondrial activity, ergosterol biosynthesis, and membrane transport. Overall, they concluded that reactive oxygen species (ROS) scavenging, ergosterol synthesis, and efflux transporter were some of the key functions to engineer if one wanted to improve phenolic compound tolerance.

*Furan derivatives*

Furanic aldehydes, such as furfural and HMF, are formed by the dehydration of pentoses and hexoses, respectively (Larsson et al., 1999). These inhibitory compounds consist of a five-membered aromatic ring with aldehyde (furfural) or both aldehyde and alcohol (HMF) functional groups (Liu et al., 2004). Furans have been reported to negatively affect growth in *S. cerevisiae*, but not ethanol yield (Liu et al., 2004). This is notably due to the inhibitory effect of the attached functional groups rather than the furan ring (Liu et al., 2014). Consequently, the inhibitory effects reported for HMF and furfural are often similar to the ones caused by other phenolic compounds and include ROS accumulation, DNA degradation, cell wall damage, and loss of enzymatic activity (Allen et al., 2010; Khan and Hadi, 1994; Kim and Hahn, 2013; Modig et al., 2002; Sanchez and

Bautista, 1988). *S. cerevisiae* metabolises both furfural and HMF into less toxic derivatives (Diaz De Villegas et al., 1992; Taherzadeh et al., 2000) via NADPH- or NADH-dependent reducing enzymes (reviewed by Liu, 2021). Thus, genes expressing proteins of the pentose phosphate pathway could be potential targets for upregulation due to their role in cofactor generation (Lewis Liu et al., 2009).

### *Weak acids*

Carboxylic weak acids are a major group of inhibitors present in hydrolysates; the group includes acetic, formic, and levulinic acids (Palmqvist and Hahn-Hägerdal, 2000). Acetic acid, which is considered the most abundant acid in hydrolysates, is formed from the cleavage of acetyl groups in hemicellulose (Steinbach et al., 2017). Formic and levulinic acids are degradation products of both furfural and HMF, or solely HMF, respectively. Weak acids are employed as food preservatives due to their ability to inhibit microbial growth. In their undissociated form, these acids can passively diffuse through the membrane and cause a drop in cytosolic pH (Mira, Teixeira, et al., 2010). Furthermore, weak acids are involved in cytochrome C oxidase inhibition, ATP depletion, and ROS production (Du et al., 2008; Guaragnella and Bettiga, 2021). In biorefineries, elevated concentrations of weak acids in hydrolysates hamper yeast growth and lower ethanol yields (Palmqvist and Hahn-Hägerdal, 2000). Conversely, low concentrations of weak acids have been associated with increased alcoholic fermentation and ethanol production under anaerobic conditions, at the expense of lower biomass (Larsson et al., 1999; Verduyn et al., 1990). In lignocellulosic hydrolysates derived from hardwood, weak acid concentrations usually exceed the inhibitory threshold. This is particularly true for formic acid and acetic acid, with the former exerting a stronger inhibitory effect in spite of generally lower amounts (Larsson et al., 1999). In **Paper IV** of this thesis, similar concentrations of acids (120 mM formic acid and 125 mM acetic acid at pH 4.5) were chosen for competitive growth assays of a CRISPRi strain library. Even though both acids caused a similar growth reduction, formic acid resulted in greater strain depletion (disappearance after successive rounds of cultivation) and lower amount of enriched strains compared to acetic acid.

Several experiments in this thesis aimed to explore and compare some of the genes responsible for cell tolerance to acetic and formic acids. The mechanisms underlying the inhibitory action of these two compounds may depend on their specific size or $pK_a$ (Guo and Olsson, 2016; Zeng et al., 2022). In **Papers III and IV**, tolerance towards acetic and formic acid were compared for a CRISPRi downregulation strain library of essential and respiratory-essential genes. In **Paper III**, the set of

genes whose downregulation caused sensitivity to formic acid showed strong overlap (49%) with those identified in a previous acetic acid study (Mukherjee et al., 2021). Interestingly, no such overlap was found for the tolerant strains (10%). The results obtained in competitive growth assays of the pooled CRISPRi library in acetic and formic acid (**Paper IV**) were in line with those from **Paper III**. Both studies indicated that fine-tuning of genes involved in chromatin remodelling and condensation or genes involved in glycogen accumulation could contribute towards the development of more acid-tolerant strains.

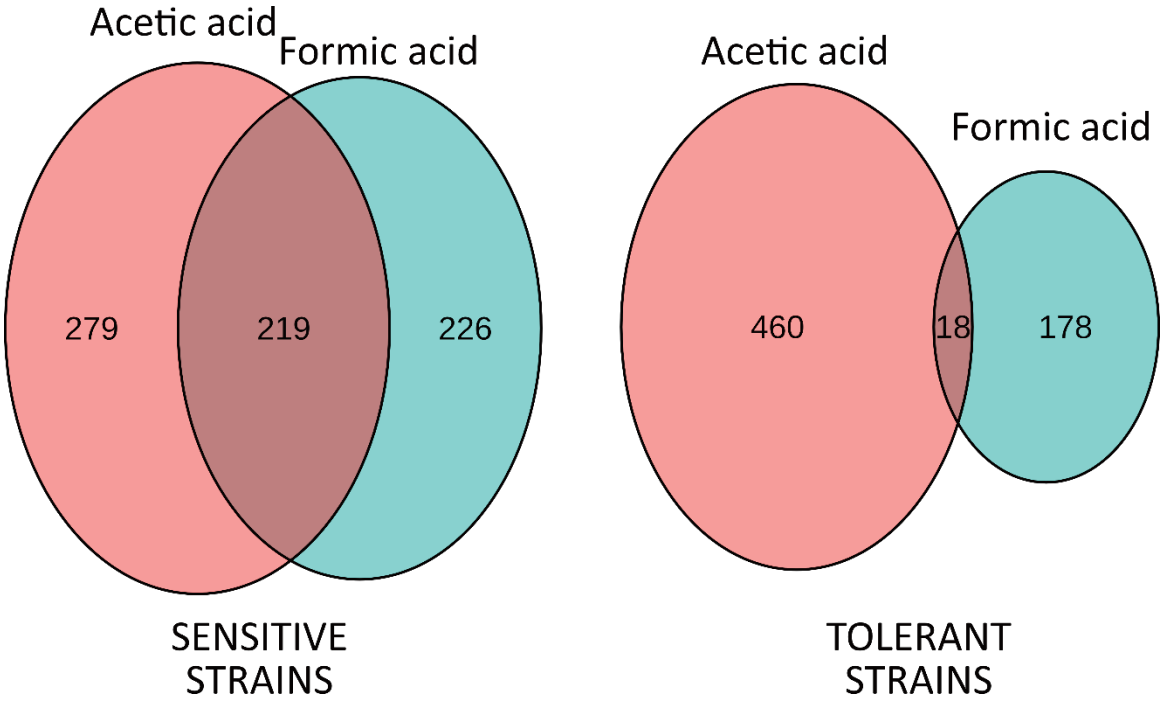


*Figure 2.2. Venn diagrams showing the overlap between formic acid (140 mM) (Red) and acetic acid (150 mM) (Green) sensitive, and tolerant strains identified through screening on solid medium (Paper III). Overlapping strains are displayed in the shaded region.*

### *Synergistic effects of combined inhibitors*

Synergism is the phenomenon, whereby the combined impact of multiple inhibitors surpasses the cumulative effect of individual inhibitors. Investigating synergistic effects between different stressors has been challenging due to the numerous combinations to be tested. As a result, most studies on lignocellulose-derived inhibitors have looked at them in isolation.

A study on the fermentation performance of second-generation bioethanol industrial yeast demonstrated that the synergistic effects of inhibitors exacerbated cell toxicity. The authors mixed

acetic acid, furfural, and p-hydroxybenzoic acid, and observed a drop in fermentation rates and biomass yield of *S. cerevisiae* and *Candida shehatae* (Palmqvist et al., 1999). More recently, Pereira et al. screened a yeast deletion collection in wheat straw hydrolysate (WSH), as well as in synthetic wheat straw hydrolysate (SWSH) that mimicked the natural inhibitor and sugar concentrations. The authors noted 50% overlap between strains sensitive to either medium, which could be explained by the presence of other compounds in WSH contributing to its toxicity. Notably, our findings from **Paper III** revealed that most strains targeting essential genes involved in the formic acid stress tolerance presented a similar profile when cells were grown in SWSH.

While the use of individual stressors is important for physiological studies and understanding stress mechanisms, the creation of resilient yeast strains requires the coordinated action of genes involved in various metabolic and cell responses (Cámara et al., 2022). Therefore, evaluating newly developed strains under conditions mirroring industrial settings is essential to better identify the factors that should be prioritised in the development of more tolerant strains.

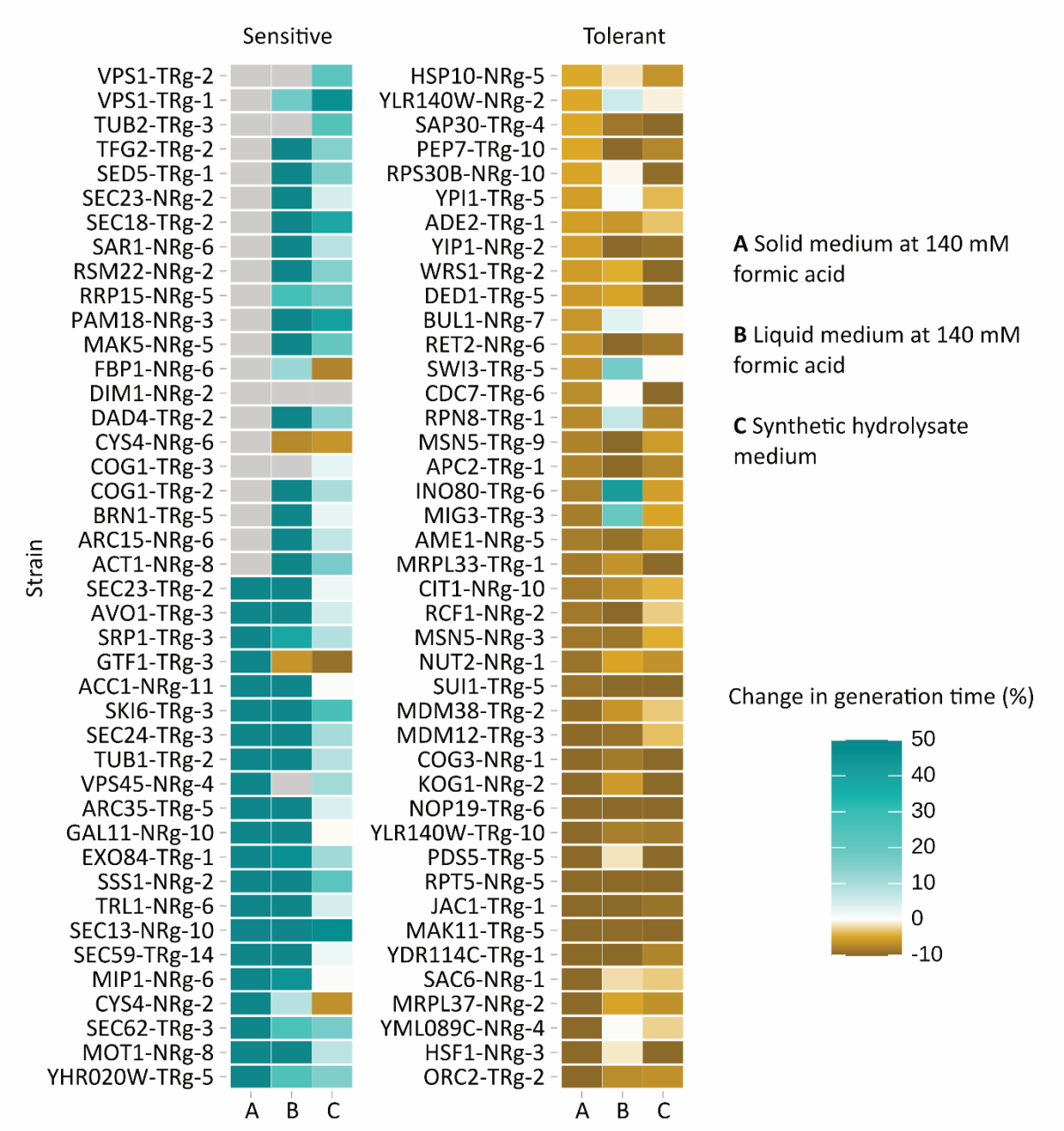


*Figure 2.3. Heat map displaying the change in mean generation time of 84 strains grown on solid (A, n = 6) or liquid (B, n = 3) medium at 140 mM formic acid or in liquid synthetic hydrolysate medium (C, n = 3). The CRISPRi strains in this heat map are listed in separate columns for sensitive and tolerant strains, and in increasing order of formic acid tolerance as determined by the screen on solid medium (A). Turquoise denotes inferior growth compared to the control strain; whereas golden yellow denotes better growth in the presence of formic acid or in the synthetic hydrolysate. Strains that grew similarly to the control strain are shown in white; those that did not grow in grey. The name of the gRNA (specifying the target gene) of each CRISPRi strain is shown on the left side of the heat map.*

# Chapter III: Development of more inhibitor-tolerant strains

## Saccharomyces cerevisiae as a cell factory

Yeasts such as *S. cerevisiae* have become prominent cell factory candidates to produce different biochemicals from renewable sources. *S. cerevisiae* has been widely used in fermentation to leaven bread or produce alcoholic beverages, such as beer, wine and sake. This explains its role as the most studied unicellular eukaryote (Botstein et al., 1997), with a very well understood metabolism and a variety of genetic tools to work with (Kavšček et al., 2015).

*S. cerevisiae* is one of the main tools in the production of first-generation bioethanol from feedstock rich in sucrose (sugarcane) or starch (corn and wheat) (Hong and Nielsen, 2012). These sugars can be broken down into hexoses, such as glucose and fructose, which are easily fermentable by yeast. In comparison, fermentation of lignocellulosic hydrolysates is more arduous, as the hemicellulose part consists primarily of pentoses (xylans and arabinoses), which cannot be utilised by *S. cerevisiae* (Moysés et al., 2016). To overcome this issue, metabolic engineering through introduction of D-xylose metabolising pathways (Borgström et al., 2019; Peng et al., 2012; Zhou et al., 2012) or expression of an L-arabinose transporter (Subtil & Boles, 2011) in industrially relevant *S. cerevisiae* strains has been attempted.

Another important factor to consider when choosing a suitable industrial strain is its tolerance towards production conditions (temperature, osmotic stress, inhibitor presence, etc.). As stress tolerance is a complex multigenic cellular response (Gasch et al., 2000), both industrial and basic research have favoured non-directed strain engineering strategies such as evolutionary engineering (Koppram et al., 2012; Wright et al., 2011). New targeted gene editing and synthetic biology tools have also yielded more tolerant laboratory and industrial strains (Heer et al., 2009; Inaba et al., 2013).

### *Discovery and development of industrial S. cerevisiae strains*

The simple genome and short doubling time of *S. cerevisiae*, along with its extensive use by humans, have made this organism a model for studies on ageing (Murakami and Kaeberlein, 2009), gene expression (Biddick and Young, 2009), cell cycle (Nasheuer et al., 2002), metabolism (Brocard-Masson and Dumas, 2006; López-Mirabal and Winther, 2008), signal transduction

(Hohmann et al., 2007), apoptosis (Owsianowski et al., 2008) or neurogenerative disorders (Miller-Fleming et al., 2008). More recently, yeast's natural fermenting ability has been exploited by the bioethanol industry, while its ease of manipulation has led to its use as a source of heterologous molecules, such as insulin, hepatitis vaccines or human papillomavirus vaccines (Hou et al., 2012).

Despite the numerous applications of industrial yeast strains, there is still substantial room for their improvement. Most yeast strains used in biofactories have been chosen based on their natural properties or ease of use and are thus often not suitable for delivering optimal performance when changing substrate or for producing a different product. *S. cerevisiae* shows hampered growth above moderate concentrations of butanol (Knoshaug and Zhang, 2009), vanillin (Hansen et al., 2009), benzaldehyde (Long and Ward, 1989) or organic acids (Fletcher et al., 2021; Narendranath et al., 2001; Nicolaï et al., 2021). These shortcomings make *S. cerevisiae* suboptimal for industrially producing weak acids, for which low pH conditions are preferred. However, the growing demands of the industry, the need to employ renewable feedstocks, and the drive for new bio-based products have sparked a search for more tolerant and productive strains.

There are several ways for obtaining more tolerant and productive yeast strains suitable for specific industrial settings. One of the most obvious ones would be to comb through existing natural variants capable of producing a compound of interest. For instance, some wild-type yeasts are well adapted to wine production(Lopes et al., 2007; Zagorc et al., 2001), while contaminating yeast strains are often well suited to fermentation conditions (Basso et al., 2008; Passoth et al., 2007). Unfortunately, most wild strains do not meet all the requirements of an ideal industrial strain, such as the ability to grow from cheap renewable feedstocks, high production yields, and fast growth rates. Nevertheless, even if most of these strains cannot be employed in industrial settings directly, some of their beneficial characteristics can be transferred to existing industrial strains and, therefore, exploited indirectly.

Yeast strains employed for large-scale production have been modified over the years by "domesticating" wild strains to industrial settings (Fay and Benavides, 2005; Sicard and Legras, 2011). The selection of optimal variants has prompted the appearance of naturally occurring hybrid species in beer making (Hutzler et al., 2023; Libkind et al., 2011). More recently, targeted mating or hybridising of yeast strains to maximise target properties has been used in biofuels production (Benjaphokee et al., 2012; Choi et al., 2010; Javadekar et al., 1995) or for bread and winemaking (Oda and Ouchi, 1990; Pérez-Través et al., 2012). Adaptive evolution of yeasts to industry conditions is another valuable method for obtaining strains with improved capabilities.

In adaptive evolution, yeast strains used in bioproduction are further adapted and refined through growth under industrial conditions (e.g. presence of multiple inhibitors, limited nutrients, and high/low operating conditions) for various generations using setups, such as long-term batch culturing in fermenters (Cadière et al., 2011) or long-term laboratory-scale fermentations (Cadière et al., 2012). One of the most studied *S. cerevisiae* industrial strains is Ethanol Red® (Le Saffre), a bioethanol-producing yeast evolved for both higher production and robustness. KE6-12 (Taurus Energy), which was used in **this thesis**, is another industrial yeast strain engineered for bioethanol production from lignocellulosic hydrolysates. This strain has been previously studied for its xylose fermenting capability (Novy et al., 2017), as well as for the influence of propagation in tolerance (Nielsen et al., 2015; Tomás-Pejó and Olsson, 2015; van Dijk et al., 2019). However, changes to tolerance-related gene expression have not been studied.

## Rational and evolutionary strain engineering

Use of microorganisms as factories for chemical production is a well-established practice with a long and rich history (Nielsen and Keasling, 2016). Bio-based manufacturing represents a promising alternative for achieving greater sustainability in the chemical industry (Saling, 2005). However, there are numerous challenges associated with transitioning from fossil fuel-based processes to those based on biomass utilisation, such as the inherent recalcitrance of lignocellulosic feedstock, suboptimal pretreatment, or the efficiency of hydrolytic enzymes (Kroukamp et al., 2018). A significant hindrance to strain and process development is the limited adaptation of host organisms to industrial conditions, even in cases involving well-studied bacterial or fungal hosts.

One strategy for engineering living cells is for nature itself to take charge of strain optimisation. Evolutionary engineering is one of the principal methods for improving strain phenotypes, owing to its simplicity and efficacy. The innate evolutionary capacity of microorganisms can be exploited to achieve phenotypical improvements that are hard to obtain by rational genome editing (Shepelin et al., 2018). This strategy involves continuous growth of the selected microorganism under selective pressure. Over time, mutations occurring during DNA replication can confer a fitter phenotype to some of the mutants. Additionally, this strategy is combined with different random mutagenesis approaches to further increase genetic diversity (Shepelin et al., 2018). Eventually, the fitter mutants become predominant due to their vastly superior fitness. DNA sequencing and omics technologies enable the assessment of evolved strains in a high-throughput manner, thereby identifying causative mutations (Good et al., 2017; Herring et al., 2006). The

obtained results provide information about the mechanisms involved in adaptation to the tested selective pressure. These mechanisms can then be modified with rational engineering. Evolutionary engineering has been successful in creating strains capable of metabolising a wider range of substrates (Cheng et al., 2014; Strucko et al., 2018) or with improved tolerance towards inhibitors and fermentation conditions (Almario et al., 2013; Caspeta et al., 2014; Mohamed et al., 2017).

The other main strategy employed to engineer microorganisms for industrial settings is rational strain design through metabolic engineering. In the past decade, advances in synthetic biology have led to more precise and reliable gene editing techniques, less costly DNA synthesis and sequencing, and the appearance of computational predictive technologies (Sun and Alper, 2016). Rational strain engineering employs synthetic biology tools to introduce new heterologous pathways or modify the expression of genes, thereby increasing the capabilities of the strain of interest. Its main use is the introduction of non-natural product pathways (reviewed in Wang et al. 2017) such as the different xylose-utilising pathways in *S. cerevisiae*, or to increase fermentation efficiency in lignocellulosic hydrolysates (Peng et al., 2012; Zhou et al., 2012). Rational strain engineering can fulfil a similar purpose as evolutionary engineering, namely obtaining a wider substrate range, improving metabolic capabilities, and increasing tolerance towards inhibitory compounds (Brandt et al., 2021; Cámara et al., 2020; Stovicek et al., 2022). In addition, metabolic engineering offers the possibility of inserting heterologous pathways into the genome of the target strain, allowing industrial production of exogenous proteins. Past rational strain engineering efforts have relied on insights or hypotheses published in previous studies on the functional effects of the target gene (Furusawa et al., 2013). However, it is still difficult to predict the exact outcome of the mutation from the conclusions of deletion or overexpression studies. For instance, several pathways involve key metabolites, such as ATP or NADH, or other precursors, which must be considered before designing the final strain. Besides, single gene expression modifications are often insufficient to confer the desired phenotype in complex environments (e.g. lignocellulosic hydrolysate). Fortunately, new developments in the construction of genome-scale models along with advances in bioinformatics have significantly improved prediction of the modifications required to obtain a desired phenotype (Cho et al., 2022). The experiments included in **this thesis** relied on metabolic engineering to further our understanding of tolerance towards lignocellulose-derived inhibitors.

## CRISPR/Cas9-mediated metabolic engineering

CRISPR/Cas9 is a molecular tool for targeted gene editing that has been adapted from a natural adaptive immune system in prokaryotes. It was first identified in 1993 in salt-tolerant archaea (Mojica et al., 1993). In 2003, Mojica matched a spacer sequence found in the CRISPR structure of an *E. coli* strain with the DNA sequence of a P1 phage the strain was resistant to (Mojica et al., 2005). This led to the conclusion that the CRISPR cassette was part of an immune response to viral infections, which allowed a nuclease related to the CRISPR system (Cas9) to cut the exogenous DNA and avoid infection.

The genetic engineering potential of CRISPR/Cas9 was not discovered until years later. In 2012, CRISPR/Cas9 was engineered to perform a targeted double-strand break in *E.coli* (Sapranauskas et al., 2011) as well as in vitro (Gasiunas et al., 2012; Jinek et al., 2012) and it quickly became the latest revolution in genetic engineering. In the next few years, the technology was rapidly adapted for many other organisms (Bortesi and Fischer, 2015; Dicarlo et al., 2013; Jiang et al., 2013). This opened the possibility of employing the technology for different applications, such as disease treatment (Cai et al., 2016) or industrial production of chemicals (Donohoue et al., 2018).

The most used CRISPR/Cas9 system is based on the Cas9 endonuclease and the sgRNA duplex, which can be easily programmed to precisely target and cleave a specific 20-bp site. The sgRNA is a fused oligonucleotide made of two components: CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). This oligo has the function of scaffolding the sgRNA-Cas9 duplex, while also guiding the complex to the target site by complementarity (Jinek et al., 2012). The nuclease can then recognise the protospacer adjacent motif (PAM) site following the DNA sequence targeted by the sgRNA and cleave DNA (Mojica et al., 2009). As a result, it is possible to perform deletions or substitutions via homology-directed repair by simply adding a suitable donor DNA. Furthermore, the target sequence may be switched by easily changing the 20-bp protospacer complementary site, with no need to alter any other parts.

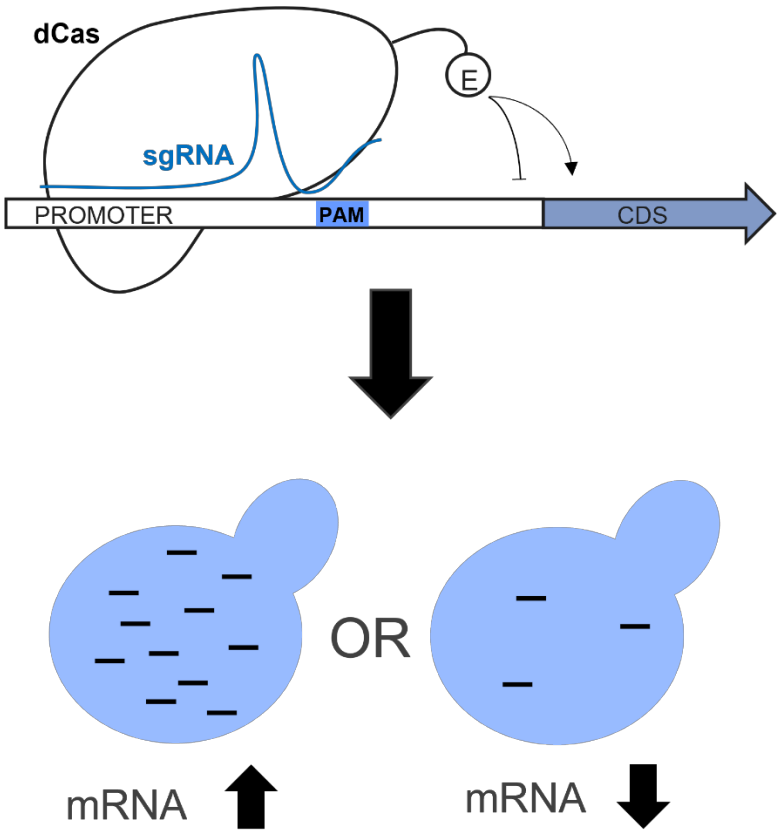


*Figure 3.1. The CRISPRi/a technology utilises a catalytically inactive Cas9 (dCas9) to modulate the expression of genes targeted by an sgRNA (in blue). The regulatory effect can be amplified by fusing an activation or repressor domain (E) to dCas9. PAM: Protospacer adjacent motif, CDS: Coding sequence.*

### *CRISPR interference and activation for strain engineering*

Shortly after CRISPR/Cas9 was established, approaches exploiting this technology to fine-tune gene expression were published (Gilbert et al., 2013; La Russa & Qi, 2015). The objective was to regulate the expression of genes in a reversible manner, without altering the gene sequence. Accordingly, the Cas9 nuclease was mutated to be catalytically inactive, but still able of binding DNA (L. S. Qi et al., 2013). The two resulting variants were named CRISPRi (for gene downregulation) and CRISPRa (for gene upregulation). The CRISPRi/a strategy couples DNA effectors with activating/repressing function to the Cas9 nuclease, resulting in accurate recruitment of these domains to specific target sites (La Russa & Qi, 2015). The effector domains, which may even be combined together to achieve a greater effect, are then able of recruiting or repelling the transcriptional complex depending on the desired objective (Figure 3.1).

Additionally, variable expression of the target gene may also be achieved depending on the localization of the sgRNA along the promoter site, making CRISPRi/a truly tunable. However, there are risks of off target effects when using CRISPR/Cas9 derived technologies, especially since the system can tolerate some mismatches between the sgRNA sequence and the target that results in unintended binding (Fu et al., 2013; Hsu et al., 2013; Mali et al., 2013; Tsai et al., 2015). Moreover, Cas9 has been reported to be toxic for yeast (Ryan & Cate, 2014, Paper I) as well as bacteria (Zhang & Voigt, 2018). Consequently, it is truly crucial to use proper controls (e.g strains expressing the CRISPRi/a system with a dummy sgRNA non-homologous to the genome) and later confirm the gene expression levels of the CRISPRi/a strains with qPCR or fluorescent assays (**Paper I**).

CRISPRi/a technology is the backbone of **this thesis**. The ability to easily target any given gene sequence in the yeast genome and modulate its expression represents a powerful tool for industrial yeast strain development. Furthermore, a gradient of expression for the target gene can be achieved by testing multiple sgRNAs along the promoter's sequence.

In **this thesis**, metabolic engineering was employed to modulate the expression of various genes in *S. cerevisiae* to expand our understanding of the tolerance towards inhibitors found in lignocellulosic hydrolysates. CRISPRi/a technology, which allows to modulate target gene expression at different levels, offered an efficient way of creating mutant strains to screen for inhibitor tolerance (**Papers I–V**). In **Paper I**, we transformed CRISPRi/a encoding plasmids into the bioethanol-producing KE6-12 industrial yeast strain. As the changes made with CRISPRi/a are generally transient and based on either inducible or plasmid systems, the CRISPRi/a strains with high tolerance can then be permanently edited. For instance, CRISPRi strains with sgRNAs targeting *SSK2*, MAP kinase involved in furfural tolerance (H. S. Kim et al., 2012), displayed a more fit phenotype in hydrolysate. In comparison, *SSK2* deletion worsened the fitness of KE6-12 towards WS hydrolysate. Consequently, one could exchange the natural promoter for one with weaker expression levels. Similarly, work in **Paper V** aimed to modulate the expression of transcription factors Pdr1 and Yap1, based on a previous ChIP-seq experiment results that pointed to their activation in acetic acid. Furthermore, in **Papers II–IV** CRISPRi/a mutant libraries of essential and respiratory-essential genes were coupled with high-throughput screening to reveal novel genes related to weak acid tolerance.

# Chapter IV: Screening methods for the development of novel yeast strains

## High-throughput screening

A variety of high-throughput screening methods suitable for strain engineering exist by now. Among the aims of **this thesis**, was the identification of novel target genes related to inhibitor tolerance, along with the establishment of related high-throughput screening methodologies in yeast. Specifically, I employed CRISPRi/a to generate multiple yeast strains with minor changes in gene expression and screened them for growth under challenging conditions. The growth rate and lag phase of strains were measured in a reproducible way by various high-throughput methods. These included liquid medium screening platforms, such as the Growth profiler and Biolector (**all papers**), solid medium screening platform, such as the Scan-o-Matic (**Paper III**), and fluorescence-activated cell sorting (**Papers II and IV**), as well as competitive growth assays (**Paper IV**).

Screening methods have typically combined microtiter plates for culturing and spectroscopic measurements for determining product titres. This approach has limitations, particularly in terms of library size and the types of products that can be screened, as it works solely for substances that can be detected via absorbance or fluorescence. To improve accuracy, cells are now cultivated at the nano-litre and pico-litre scale. At the same time, new detection techniques enable product titres to be red as fluorescence signals and identified through mass spectrometry. The latter can now be applied directly to a cell broth, reducing screening time while providing precise information on product type and concentration. Nevertheless, the real benefits will derive from identifying small-scale culture conditions that can predict performance at an industrial scale (reviewed by Leavell et al., 2020).

A functioning cell factory often requires precise changes to gene expression. These changes may be either targeted (directed mutagenesis) or random (random mutagenesis). By performing only a few changes in the genome without previous knowledge of the role of a gene, the probability of obtaining an improved strain is low. Hence, the use of mutant libraries that allow screening of large sets of genes has become critical for developing new industrial strains. In the last decade, rapid advances in synthetic biology, such as genetic engineering and DNA assembly, have revolutionised the field of biotechnology and facilitated the creation of large strain libraries (Boyle

and Gill, 2012). Given the growing need to assess mutant libraries efficiently and rapidly, high-throughput screening technologies have been developed, thereby significantly reducing manual work and error. High-throughput screening enables the simultaneous assessment of hundreds of thousands of experimental samples under predetermined conditions. The sample itself might consist of chemical compounds, amino acids or living cells. With high-throughput screening, one can quickly and effectively generate large amounts of data due to automated sample preparation, handling, and data processing. High-throughput screening can be applied for drug discovery or to identify genes and proteins that affect a certain biological pathway. It is gradually being adopted across different fields, including metabolic engineering, pharmacology, human cytogenetics, environmental engineering, and metagenomics (Sarnaik et al., 2020).

### *Liquid and solid screening methods*

In **Paper III**, high-throughput screening of multiple strains from the CRISPRi library was performed using the Scan-o-Matic solid medium system in the presence of formic acid. The Scan-o-Matic system, created at the University of Gothenburg, allows the simultaneous screening of up to 92,000 strains (Zackrisson et al., 2016). It is based on continuously scanning agar plates to capture images of yeast colonies and extract growth data based on colony size. The main challenge posed by this type of platform is spatial bias within the plates. This includes the effect of colony growth on neighbouring colonies, as well as environmental changes linked to colony position, such as improved aeration of fewer competing colonies at the edge of plates. To correct this shortcomings, the Scan-o-Matic is capable of accurately estimating population cell densities, while accounting for spatial bias (Zackrisson et al., 2016). The results obtained in the Scan-o-Matic must be further validated in liquid setups, as differences in growth platforms (solid vs. liquid media) can cause different growth responses (Zackrisson et al., 2016). In **Paper III**, 42 tolerant and 42 sensitive strains from the Scan-o-Matic screen were selected for further validation in liquid media (Figure 2.3). A correlation coefficient ($R^2$) of 0.66 between generation time and either solid or liquid medium was obtained (Figure 4.1), indicating good agreement between the two media types. Tolerant strains retained similar relative generation times compared to the controls; whereas sensitive strains often increased their sensitivity to formic acid when switched to liquid medium (Figure 2.3). These results highlight the value of the Scan-o-Matic platform as a first screening method for large libraries in tolerance assays.

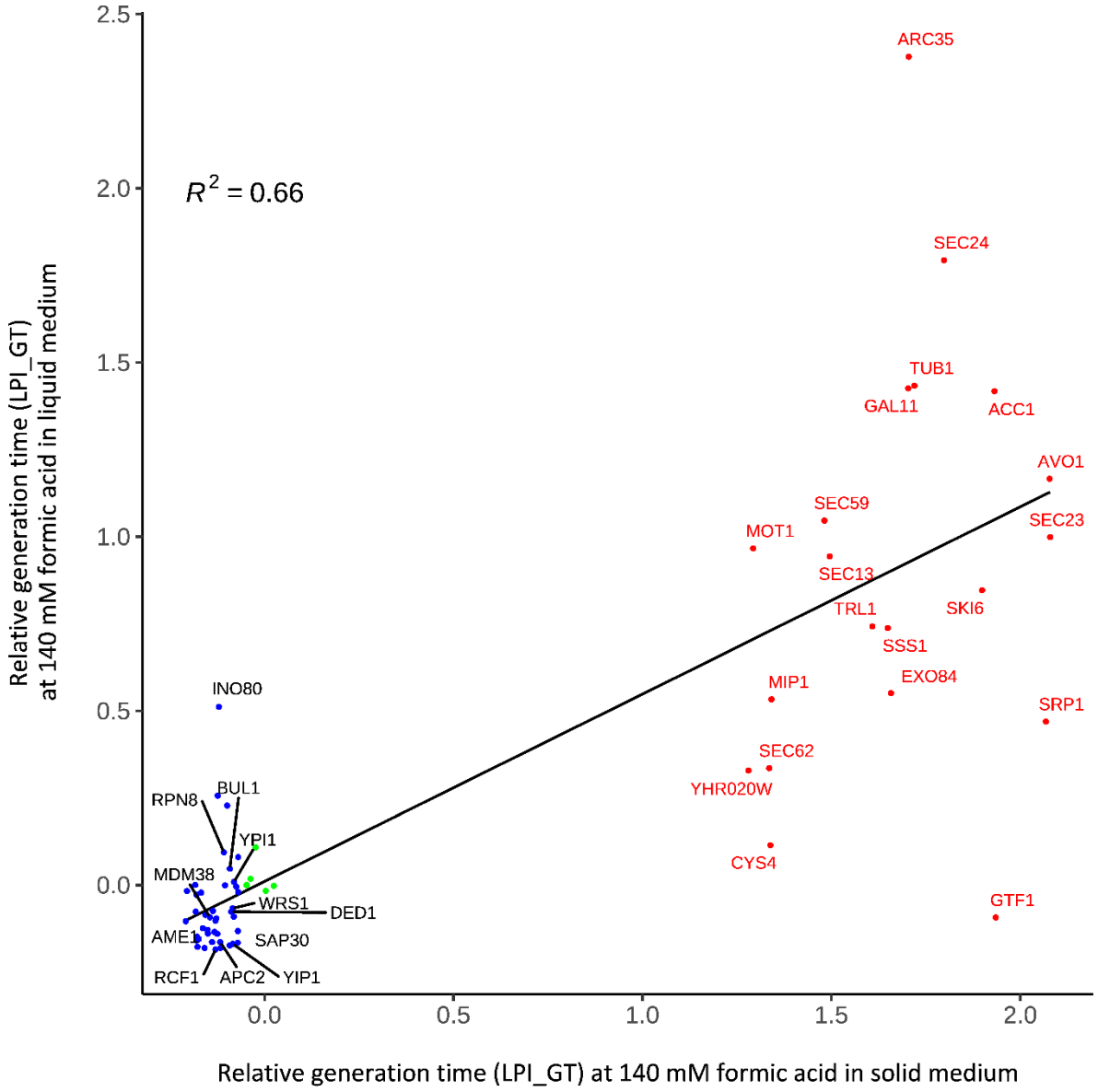


*Figure 4.1. Scatterplot for the relative generation time of yeast mutants grown in liquid and solid medium containign 140 mM formic acid (Paper III). The black line denotes linear regression of the data. The means from three replicates for each strain are plotted, with control strains in green, formic acid-sensitive strains in red, and formic acid-tolerant strains in blue. Genes repressed in the sensitive strains are indicated in red, whereas those repressed in the tolerant strains under both formic and acetic acid are indicated in black.*

The method used most in this thesis was liquid media screening in microtiter plates. While such method allows for screening of only 24, 48 or 96 wells, it employs liquid medium, which is closer to industrial settings. Moreover, it allows measurement of individual strains without any competition from neighbouring colonies, thereby offering an unbiased assessment of strain performance under challenging conditions. Thus, the Growth Profiler 960 (System Duetz,

EnzyScreen) was used for **all Papers** in this thesis, while the Biolector (Beckman Coulter / m2p-labs) microbioreactor system was used for screening in hydrolysates in **Paper I** and measure fluorescence in **Paper II**. The Growth Profiler 960 is a shaking device that monitors biomass formation in a quasi-continuous way. It takes pictures of each well at designated intervals and uses image processing to calculate culture density. If a calibration curve is available, the proprietary software translates picture analysis to optical density (OD) equivalents (green values) and enables culture density estimation up to OD = 60. This is particularly helpful when determining the length of the exponential growth phase or the highest population density achievable under the tested conditions. With 10 plate positions and the ability to employ different microtiter plates, this system enables high-throughput growth of up to 960 wells at once. As a downside, the Growth Profiler is not suitable for cultivation in dark media such as hydrolysates, as biomass measurement becomes inaccurate. To circumvent this issue, I used the Biolector. The latter could measure up to 192 strains simultaneously in microtiter plates. Its main advantage is its flexibility, as it can quantify biomass, fluorescent proteins, medium pH, and oxygen levels. In addition, it enables the configuration of shaking speed, measurement interval, temperature, and humidity. The Biolector measures biomass via scattered light, which allows screening for growth in lignocellulosic hydrolysates (van Dijk et al., 2020). In **Paper I**, CRISPRi/a-mediated modulation of four genes related to inhibitor tolerance (*BDH2*, *SSK2*, *HRK1*, and *ISC1*) was tested in WSH using the Biolector.

### *Competitive growth assays*

Competitive growth assays are employed for high-throughput selection in liquid media. Individual strains in a library are tagged with unique genomic barcodes and then pooled together in a single culture. When making competitive growth assays, the cells in the pooled culture are subjected to selective pressure such as elevated inhibitor content. By performing subsequent rounds of growth assays and reinoculations, the strains with the highest phenotypic advantage (e.g. faster growth rate and shorter lag phase) become progressively enriched within the culture. As a result, the final culture contains a reduced number of strains enriched over successive cultivations. For **Paper IV**, the CRISPRi essential and respiratory essential strain library was screened for formic and acetic acid tolerance using competitive growth assays (Figure 4.2). Each strain of the CRISPRi library had a unique sgRNA sequence that could be used as a barcode to identify the strain. Then, next-generation sequencing can be employed to identify the most overrepresented barcodes (Smith et al., 2012), as well as the strains that were depleted from the initial pool. The overrepresented strains are likely growing faster or have a shorter lag phase. Instead, the depleted strains are likely

more sensitive and, hence, are of interest when investigating how a specific genetic manipulation can trigger sensitivity to certain compounds. Downsides of this method include the loss of strain coverage in the pooling process, as well as small discrepancies in the initial abundance of different strains. Still, this bias may be addressed by normalizing samples to the initial sample taken before starting the assay (t = 0). In **Paper IV**, competitive growth assays revealed genes putatively involved in tolerance to acetic or formic acid. The results of the assay were largely comparable to those obtained with the Scan-o-Matic. Thus, competitive growth assays represent an effective method for high-throughput target gene identification in tolerance assays, especially when using liquid media. Additionally, the results can complement those obtained with other screening methodologies such as the Scan-o-Matic or fluorescence-activated cell sorting.

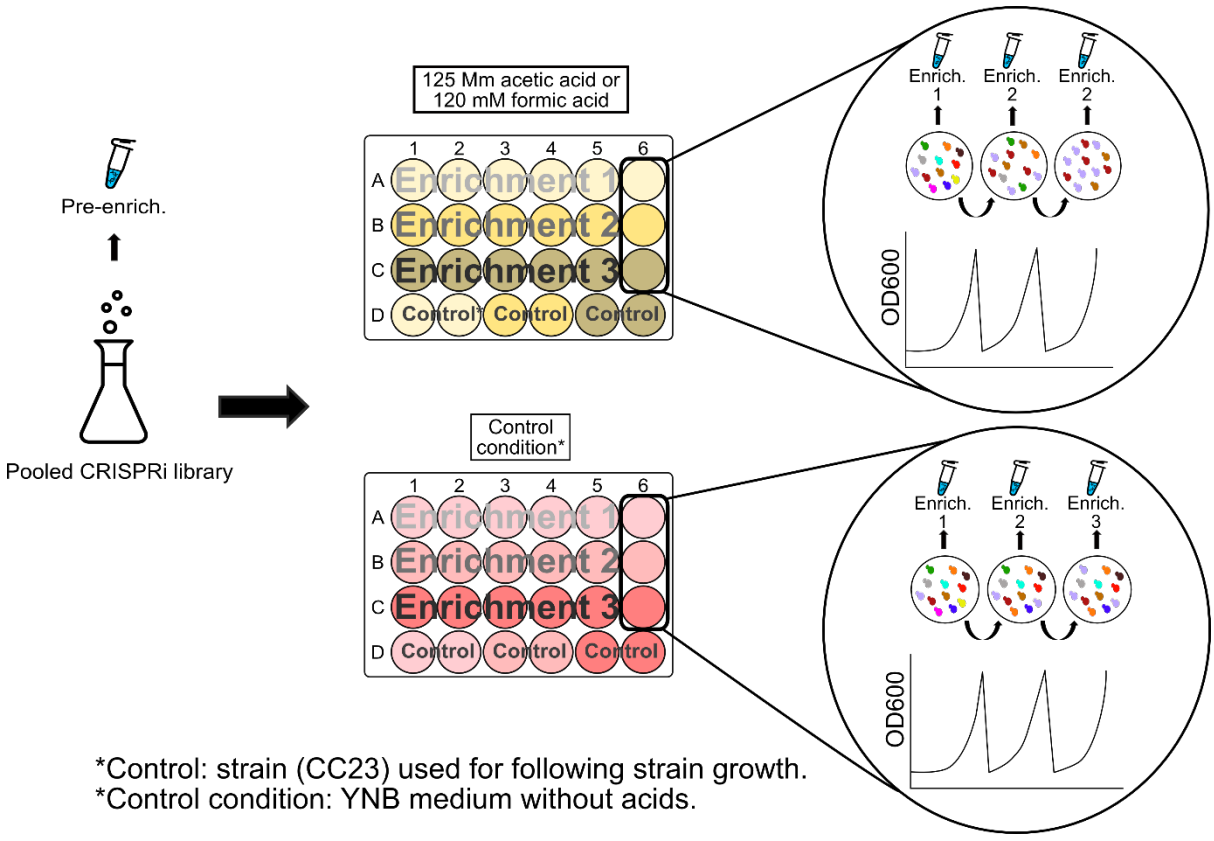


*Figure 4.2. Overview of the experimental setup for the competitive growth assays used in Paper IV. Strain composition of the initial pooled CRISPRi library (Pre-enrich.) and of the library after three sequential enrichments (Enrich. 1, Enrich. 2, and Enrich. 3) was identified via barcode sequencing.*

Table 4.1. Main characteristics of high-throughput screening methods used in this thesis.

| | **Scan-o-Matic** | **Competitive growth assays** | **Liquid screening in microtiter plates** | **Fluorescence-activated cell sorting** |
|---|---|---|---|---|
| Medium type | Solid | Liquid | Liquid | Liquid |
| Advantages | • Very high-throughput (up to 92,000 at once) (Zackrisson et al., 2016)<br>• Individual strain screening<br>• Cheap (normal scanners used) | • Very high-throughput<br>• Easy setup | • Cheap setup<br>• Individual strain screening in liquid medium<br>• Aerobic/semi-aerobic | • Isolation of single cells<br>• Very high-throughput<br>• Well established protocols |
| Disadvantages | • Complex setup<br>• Requires spatial bias normalisation<br>• Phenotype may not be reproducible in liquid medium | • Expensive (requires next-generation sequencing)<br>• Cells with shorter lag phase have an advantage (vs. higher yield) | • Less throughput<br>• Time consuming plate preparation | • Limited by biosensor (dynamic range)<br>• False positives |

*Biosensor screens*

Biosensors are genetic tools that allow to monitor parameters such as pH (as demonstrated by Zhu et al., 2019), cell density, stress responses or metabolite concentrations by generating a signal proportional to these stimuli (Kaczmarek and Prather, 2021). They are often based on transcription factors, and several have been developed for yeast in recent years (Bovee et al., 2007; Chou and Keasling, 2013; Feng et al., 2015; Mormino et al., 2021). Biosensors enable dynamic control of biochemical pathways, environmental monitoring, or the detection of fluctuating metabolite levels without the need for direct assessment(Kaczmarek and Prather, 2021). Biosensor screening benefits from high specificity, but often requires precise adjustments to ensure it is effective for a specific pathway. These adjustments can involve fine-tuning of screening conditions, altering the screening methodology or modifying the transcription factor itself (as demonstrated by Mormino et al., 2021). Vast efforts have been dedicated to developing biosensors that facilitate high-throughput library screening. Such advancements have played a pivotal role in testing and screening microbial libraries, significantly expanding the range of searchable compounds by enabling the detection of small molecules. Transcription factor biosensors modulate their output through transcriptional regulation upon detection of a target molecule (Schallmey et al., 2014).

Fluorescence-activated cell sorting (FACS) is one of the most used techniques for high-throughput screening in combination with biosensors. It allows to screen large mutant libraries at the single-cell level, thereby isolating cells exhibiting the highest levels of fluorescence within a pooled culture. Crucially, it enables the screening of libraries of up to a million mutants. Given its widespread adoption and well-established instrumentation, the technique benefits from standardised screening protocols (Binder et al., 2012). However, single-cell variation and the diffusion of products from one cell to another may limit fluorescence-activated cell sorting accuracy, potentially yielding false positive results (Kaczmarek and Prather, 2021). Nevertheless, its combination with biosensors has proven instrumental in yeast strain development, especially for enhanced production of chemicals of interest (Dabirian et al., 2019; Li et al., 2015; Wang et al., 2020).

In **Papers II and IV**, an Haa1-based biosensor (Mormino et al., 2021) was introduced in the CRISPRi strain library and used for monitoring intracellular acetic acid concentration. Additionally, a sfpHluorin-based pH sensor was employed to monitor the cytosolic pH (Reifenrath and Boles, 2018; Torello Pianale et al., 2022). Both fluorescence signals were monitored using a Biolector. The strains with the highest fluorescence signal in the presence of 50 mM acetic acid were sorted

by fluorescence-activated cell sorting and then identified through sgRNA region sequencing. Seven of these strains were further characterised in liquid medium supplemented with acetic acid, as described in **Paper II**. Six of the seven strains targeted genes that had been previously associated with acid-related stress tolerance (*QRC8*, *TIF34*, *MSN5*, *COX10*, *TRA1*, and *UBA2*). Surprisingly, cytosolic pH of the selected strains showed a strong positive correlation with the acetic acid biosensor ($R^2$ 0.78). Acetic acid tolerance has been linked to increased proton efflux from the cytosol, resulting in a stable neutral intracellular pH (Carmelo et al., 1996). Thus, one would expect a drop in cytosolic pH when the intracellular acetic acid concentration was high. Instead, the sfpHluorin biosensor indicated a higher intracellular pH for the selected strains, which could be explained by a higher buffering capacity of the cells. In highly fluorescent cells, the acetic acid biosensor response correlated strongly with the measured intracellular acetic acid ($R^2$ 0.82), in line with results by Mormino et al. (2021). This finding proved the usefulness of the tested biosensor in estimating intracellular acetic acid content. A pool of the top 5% most fluorescent cells under 50 mM acetic acid (after two consecutive sortings) was analysed in **Paper IV**. As with competitive growth assay enrichments, the sgRNA barcode regions of this fluorescent pool of cells were sequenced and the strains identified. Unsurprisingly, all individually sorted cells were also enriched and identified in the pool, as indicated by sgRNAs targeting of *COX10*, *UBA2*, and *MSN5*.

# Chapter V: The genetic response of *S. cerevisiae* to inhibitors

## Inhibitor tolerance related genes in *S. cerevisiae*

Differences among hydrolysates and their inhibitor content cause a varied response and adaptation by the cells. As the toxicity mechanisms triggered by different inhibitors (e.g. aliphatic acids, furans, and phenols) are very diverse, *S. cerevisiae* employs several different strategies to counteract those stresses. Moreover, as discussed in Chapter II, even a single inhibitor causes a number of responses. For instance, acetic acid has been reported to lower cytoplasmic pH, leading to ROS formation, protein misfolding and aggregation, membrane disruption, and inhibition of the pentose phosphate pathway (Brandt et al., 2019). The toxic effects caused by phenolic compounds have been difficult to elucidate due to the many pathways involved (Adeboye et al., 2014; Fletcher et al., 2019). Only a handful of single genes (*CHO1*, *DRS2*, and *ERG4*) have been found to confer sensitivity to more than one phenolic compound of the three tested (Fletcher et al., 2019). In this chapter, the genes of *S. cerevisiae* involved in tolerance mechanisms against all these different stressors are summarised according to the review from Cámara et al. (2022), who classified them into four categories: stress response activation/signalling, cellular protectants, inhibitor detoxification, and proteasome regulation (Figure 5.1). I will first focus on tolerance-related genes targeted in **Paper I** (*BDH2*, *ISC1*, *HRK1*, and *SSK2*), proceed to the most important essential and respiratory essential genes related to weak acid tolerance (**Papers II–IV**), and finally discuss the role of transcription factors driving the activation of multidrug resistance genes in the presence of acetic acid (**Paper V**).

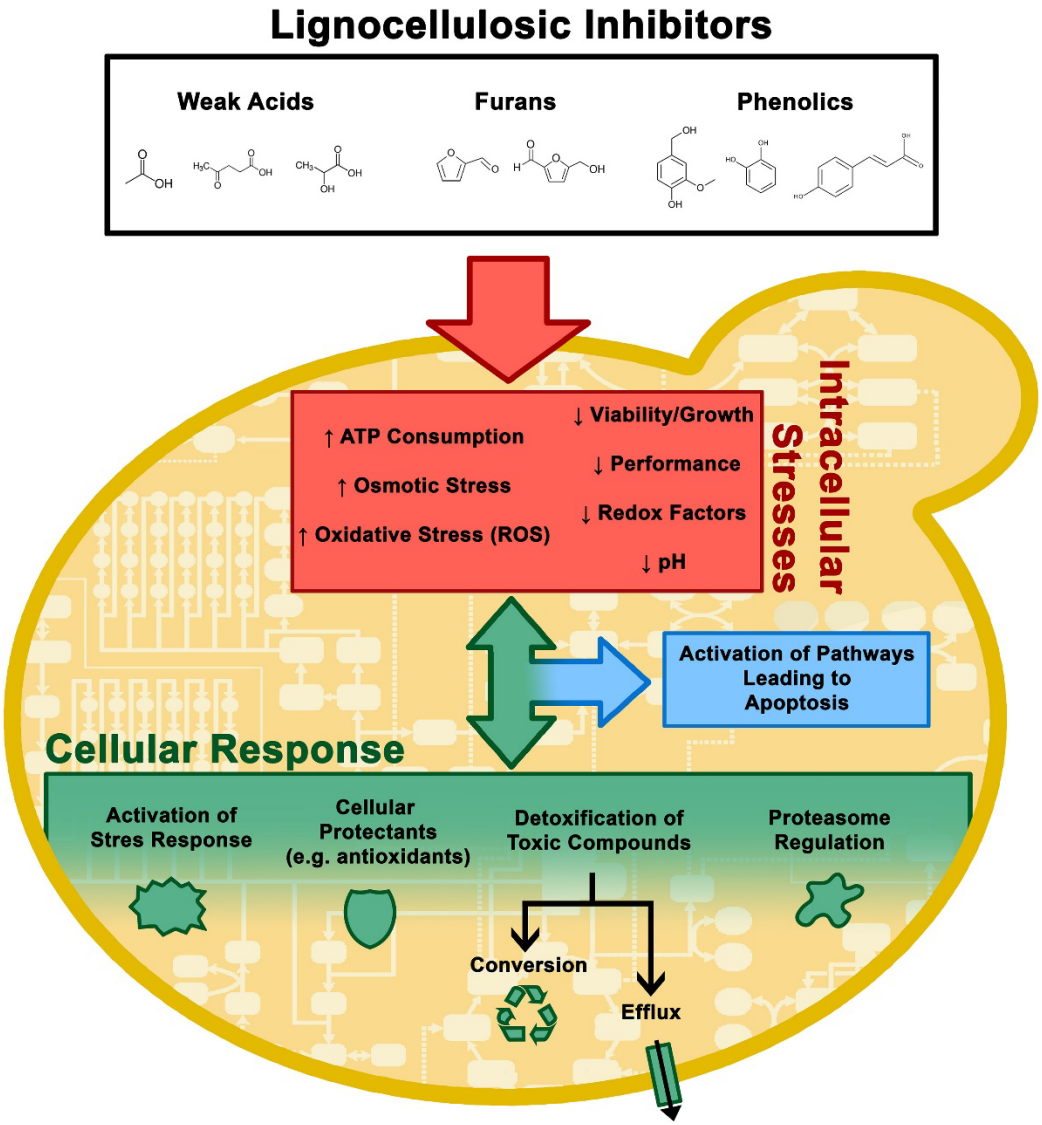


*Figure 5.1. Overview of the various forms of stress yeast cells are subjected to when grown in lignocellulosic hydrolysates and the corresponding responses* (Cámara et al., 2022).

*Stress response activation*

The cell wall and membrane are the main barriers responsible for keeping inhibitors outside the cell (Y. Qi et al., 2019; Udom et al., 2019). As such, modulating cell wall integrity and membrane permeability towards stressors may result in more tolerant yeast strains. Some 30%–60% of the cell wall is composed of β-glucans, whereas the rest is made up of cell wall proteins and chitin (Lipke and Ovalle, 1998). Upon encountering a toxic compound, the cell wall can reorganise itself and trigger a phosphorylation cascade that prompts the cell to adapt to the stress condition (Kock et al., 2016; Sanz et al., 2018).

To successfully counteract different stresses, yeast cells trigger multiple signalling cascades, including the high osmolarity glycerol (HOG), target of rapamycin, cAMP-protein kinase-A or cell wall integrity pathways. Many of these can be activated simultaneously and maintain a high degree of cross-talk (Fuchs and Mylonakis, 2009). The HOG pathway is vital for yeast survival under high osmolarity or heat, and is activated by two different membrane regulators, Sln1 and Sho1. Sln1 regulates the downstream cascade involving Ssk2, which mediates ethanol and heat stress tolerance (Kim et al., 2011). Nevertheless, two of its prime components (*HOG1* and *SKO1*) have also been associated with lignocellulosic hydrolysate tolerance (Gutmann et al., 2021).

In 2012, a transposon library screening for furfural-tolerant *S. cerevisiae* detected increased tolerance in a strain with downregulated *SSK2* (Kim et al., 2012). This result was confirmed in **Paper I**, where the promoter of *SSK2* was targeted by CRISPRi in the industrial KE6-12 strain growing in minimal medium containing 20 mM furfural and in 64% WSH. Three different strains with sgRNAs downregulating the *SSK2* promoter improved generation time and biomass yield in both media. However, disruption of the *SSK2* locus conferred a more sensitive phenotype compared to the wild-type in WSH (**Paper I**). Kim et al. (2012) suggested that Ssk2 might negatively regulate a pathway involved in furfural stress relief, possibly through scavenging of ROS or furfural detoxification. However, Ssk2 also seems to play a crucial role in the activation of Hog1 in response to ROS-generating agents, such as $H_2O_2$ and diamide (Y. M. Lee et al., 2017). Thus, a decrease in *SSK2* transcription may benefit cell fitness by attenuating the inhibition of furfural detoxifying pathways. Instead, *SSK2* knockdown may delay the activation of the Hog1-dependent oxidative stress response pathway and confer a sensitive phenotype in ROS-rich media such as hydrolysates.

### *Cellular protectants*

Mitochondria are key players in the response to stress, particularly oxidative stress (Grant et al., 1997). These organelles have a major role in generating energy within the cell by synthesizing ATP via the tricarboxylic acid cycle and oxidative phosphorylation. Some of this energy is needed by the cell to extrude protons released by weak acids. Furthermore, mitochondria play an important role in cellular ageing, ROS production, and programmed cell death in yeast (Perrone et al., 2008). Elevated acetic acid levels trigger ROS production and acetic acid-induced programmed cell death in yeast cells (Eisenberg et al., 2007; Guaragnella et al., 2012; C. Pereira et al., 2008).

*ISC1* ensures normal mitochondrial function (Kitagaki et al., 2007). It encodes for inositol phosphosphingolipid phospholipase C in yeast, an enzyme that hydrolyses inositol

phosphosphingolipids into ceramides (Sawai et al., 2000). This gene is an orthologue of mammalian neutral sphingomyelinases, which are involved in the formation of ceramides that regulate the cellular response to extracellular signals (Hannun and Obeid, 2002). *ISC1* deletion cells showed enhanced resistance to acetic acid-induced cell death, possibly by delaying mitochondrial outer membrane permeabilization and liberation of cytochrome-c in response to acetic acid (Rego et al., 2012). However, deletion of *ISC1* has also been reported to boost yeast susceptibility to hydrogen peroxide, possibly by favouring cellular iron uptake while lowering ATP production (T. Almeida et al., 2008). *ISC1* was targeted for downregulation with three different sgRNAs in **Paper I**. In minimal medium containing 100 mM acetic acid, the strains targeting *ISC1* displayed a longer lag phase and slower growth rate. This can be explained by lower ceramide content in mitochondria, which might impair mitochondrial function and ultimately affect cellular ATP levels. It is to be noted that Rego et al. (2012) employed very harsh conditions (180 mM acetic acid at pH 3); whereas those in **Paper I** (100 mM acetic acid, pH 4.5) allowed the cells to grow, even if stressed. Thus, *ISC1* expression could influence the ability of yeast cells to grow in the presence of acetic acid in a concentration-dependent manner.

Some lignocellulosic hydrolysate inhibitors, such as acetic and formic acid, can passively diffuse into the cytoplasm, thereby eliciting a wide range of damaging reactions. One strategy to overcome weak acid sensitivity may be to target and modify genes involved in plasma membrane composition and permeability. Membrane engineering for increased acetic acid tolerance has been attempted in a few studies, albeit obtaining a strain with an improved phenotype remains challenging (Lindahl et al., 2017; Maertens et al., 2021).

Ergosterol has also been reported to play a key role in maintaining cell membrane stability in the presence of weak acids (Godinho et al., 2018b; Guo et al., 2018; Mira et al., 2010a). Ergosterol accumulation protects against acid stress by maintaining membrane thickness and preventing interdigitation (Vanegas et al., 2012). Genes involved in ergosterol biosynthesis (*ERG5*, *ERG28*, *ERG7*, and *HMG1*) are upregulated also in the presence of vanillin (Endo et al., 2009), whereas their deletion hampers growth in hydrolysate and phenolics-containing media (Endo et al., 2009; Skerker et al., 2013). In **Paper V**, a ChIP-exo study was performed to reveal genes bound by transcription factors Pdr1 and Yap1 in the presence of acetic acid. *ERG5* and *ERG25* were among the most enriched for both transcription factors when comparing target gene binding with the control condition. From these results, one could conclude that Pdr1 and Yap1 may contribute to increased membrane stability and thickness in the presence of acetic acid through targeting and activation of the ergosterol biosynthesis pathway. It is noteworthy that genes linked to fatty acid metabolism (*TES1*), ergosterol synthesis (*ERG5*, *ERG7*, *ERG26*, *HMG1*, and *ERG28*), and proteins

associated with the cell membrane (*HES1* and *PUN1*) were also highly expressed during growth in the presence of phenolic compounds (Endo et al., 2009; Sardi et al., 2016; Thompson et al., 2016). The findings of **Paper V** coupled with previous results point to the importance of genes involved in ergosterol biosynthesis for achieving inhibitor tolerance. This, in turn, makes Pdr1, Yap1, and their gene targets of great interest for future strain development.

### *Inhibitor detoxification and efflux*

A major response mechanism underlying yeast tolerance involves the excretion of inhibitory compounds by plasma membrane exporters. These multidrug resistance efflux pumps belonging to ATP-binding cassette and major facilitator superfamilies include most of the plasma membrane inhibitor exporters in yeast (Buechel and Pinkett, 2020; dos Santos et al., 2014). Pdr1 is one of the main transcription factors responsible for controlling membrane efflux transporters. Indeed, it was discovered when screening mutants tolerant to coniferyl aldehyde (Wu et al., 2017). Yap1 similarly activates the transcription of various drug transporters in yeast (Berra et al., 2014; Oskouian and Saba, 1999; Wemmie et al., 1994). Yap1 and Pdr1 have been previously associated with oxidative stress and multidrug resistance, respectively. Here, a ChIP-exo experiment in **Paper V** revealed that binding of both transcription factors to target genes increased under acetic acid compared to control (basal) conditions. Membrane transporters with the greatest binding to Yap1 or Pdr1 included *SNQ2*, *PDR5*, and *PDR15*. These efflux pumps may be targeted by Pdr1 and Yap1 as part of a general activation response and, thus, may be unrelated to acetic acid stress. In fact, deletion studies targeting *SNQ2* (Godinho et al., 2018a; Sousa et al., 2013) and *PDR5* (Mira et al., 2010) have shown no effect on acetic acid tolerance in yeast; whereas *PDR15* was upregulated under acetic acid stress (Wolfgert et al., 2004). Instead, plasma membrane multidrug transporters have a prominent role in tolerance towards phenolic compounds. Genes involved in membrane transport were overexpressed when cells were exposed to vanillin, indicating the cell's attempt to prevent the buildup of phenolic compounds (Thompson et al., 2016; Wang et al., 2017). Furthermore, deletions of yeast ABC membrane transporters *PDR5*, *SNQ2*, and *YOR1* confer sensitivity to coniferyl aldehyde (Haclsalihoglu et al., 2019).

Another gene related to membrane efflux exporters is *HRK1,* which encodes a serine/threonine kinase belonging to the Npr1-kinase family. Hrk1 was thought to be involved mainly in activation of the Pma1 plasma membrane proton pump during glucose metabolism (Goossens et al., 2000). However, it was later found to play also a prominent role in tolerance to acetic acid (Mira et al., 2010c). According to that study, Δhrk1 cells grown in medium supplemented with acetic acid

showed higher intracellular accumulation of acetic acid along with higher susceptibility to it. This suggests that Hrk1 could be involved in acetic acid efflux via activation of a plasma membrane transporter. Hrk1 was shown to mediate the phosphorylation of about 40% of membrane-associated acetic-acid responsive proteins (Guerreiro et al., 2017), suggesting it is another potential candidate for overexpression. Accordingly, in **Paper I**, we targeted the *HRK1* promoter site with three sgRNAs coupled to a CRISPRa system based on the VPR transcriptional activator (Jensen, 2018). The aim was to determine the role of Hrk1 in acetic acid tolerance by screening the resulting strains. Of the three strains with sgRNAs targeting *HRK1*, two showed a higher tolerance compared to the control strain containing a non-homologous sgRNA. As explained in Chapter III, transcriptional activation by CRISPRa is highly dependent on the location targeted by sgRNA. Consequently, it is likely that one of the CRISPRa strains did not overexpress *HRK1*, possibly due to the complex being unable to bind the promoter site.

Even though weak acids can be directly expelled from the cells, other inhibitors, such as phenolic compounds or furans, may require prior detoxification or conversion into less toxic derivatives. *S. cerevisiae* is naturally able to detoxify furans and phenolic compounds, provided that the concentrations are not lethal to the cell. Furans such as furfural and HMF are first reduced into furan alcohols by alcohol dehydrogenases (Lewis Liu et al., 2008), and then excreted from the cell. Similarly, phenolic compounds such as coniferyl aldehyde, ferulic acid, cinnamic acid, and vanillin can also be reduced by yeast into less toxic forms through reactions driven by various oxidoreductases (Adeboye et al., 2017; Clausen et al., 1994; Shen et al., 2014; Wang et al., 2016). *BDH2* is thought to encode a putative medium-chain alcohol dehydrogenase participating in vanillin detoxification. An earlier study focused on understanding the transcriptome of vanillin-resistant mutants showed that *BDH2* was upregulated 4.6-fold compared to their parental strain (Shen et al., 2014). Conversely, the Δbdh2 mutant showed a hyper-sensitive phenotype characterised by vanillin accumulation in the cell (Ishida et al., 2016). These results clearly pointed out the importance of *BHD2* for vanillin detoxification. As with *HRK1*, CRISPRa was used to construct three strains with different sgRNAs targeting the *BDH2* promoter in **Paper I**. In this case, none of the CRISPRa strains was more fit than the control when screened in minimal medium containing 12 mM vanillin. One reason could be the difficulty of enhancing tolerance for inherently tolerant industrial strains. Alternatively, it could be that none of the tested sgRNAs altered *BDH2* expression. Hence, *BDH2* remains a possible target for enhancing tolerance towards vanillin.

*Proteasomal complex and unfolded protein degradation*

The function of any living cell is determined by the concerted action of its proteins. Whether for structural purposes, cell motility, energy and molecule synthesis or signalling, proteins drive cell growth and metabolite production. This is why the rate of protein degradation is key for maintaining good cellular homeostasis. For instance, structural proteins may remain in the cell for longer than transcription factors (Kallio et al., 1999). Additionally, incorrectly synthesized or damaged proteins need to be removed from the cytoplasm via specific proteases and the 26S ubiquitin-proteasome system (Finley et al., 2012). The proteasome is a large protease complex known for degrading unfolded and aggregated proteins in an ATP-dependent manner (Raynes et al., 2016). It is composed of two 19S regulatory particles (19S RP) and a single 20S core particle (20S CP) responsible for the degradation of oxidised proteins (Raynes et al., 2016). Endoplasmic reticulum stress and protein oxidation are the most common outcomes of exposing yeast to inhibitors derived from lignocellulosic hydrolysates (Ding et al., 2012; Kawazoe et al., 2017). The unfolded protein response is triggered by the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum. It causes the activation of various genes that contribute to the elimination of misfolded proteins, including molecular chaperones and endoplasmic reticulum-associated degradation proteins (Travers et al., 2000). Excessive misfolded protein accumulation has been reported to cause apoptosis in yeast (Guérin et al., 2008; Wu et al., 2014). Thus, strategies to make unfolded protein degradation more efficient could improve the phenotype of industrial strains.

A study by Mukherjee et al. (2021) assessing a library of downregulated essential genes revealed that repression of genes encoding proteins of the 19S RP increased the tolerance of *S. cerevisiae* towards acetic acid. In contrast, strains with sgRNAs targeting the 20S CP displayed severe growth defects. The authors speculated that targeting genes encoding for proteins forming the 19S RP of the proteasome could free 20S CP units. The ability of the 20S CP to degrade mildly oxidised proteins in an ATP- and ubiquitin-independent manner (Raynes et al., 2016) could help conserve energy in the cell and thus increase survival under acetic acid stress. As mentioned in Chapter II, acetic acid can enter the cell through passive diffusion and transporters, subsequently becoming deprotonated and causing acidification of the cytosol (Palma et al., 2018). To restore cytosolic pH and limit cellular damage, cells activate the ATPases present in the plasma membrane and vacuoles and start pumping protons out of the cytosol (Palma et al., 2018). As the cells' energy requirements are very high during stress, the downregulation of genes encoding for proteins of the 19S RP could be beneficial for the cell as it would increase the availability of ATP. In **Paper III**, the essential gene downregulation library was screened to study the effects of formic acid in *S.*

*cerevisiae*. That experiment also pointed out to some CRISPRi-targeted genes of the 19S RP (*RPT5* and *RPN8*), which resulted in fitter cells under formic acid stress. However, it identified also genes of the 19S RP that conferred a sensitive phenotype when targeted, namely *RPT3*, *RPT4*, *RPN7*, and *RPN12*. As discussed in Chapter II, formic acid is considered more toxic to yeast cells than acetic acid. Besides, the two acids may elicit a different response in yeast. Supporting this idea, comparisons between formic and acetic acid in **Papers III and IV** showed few overlapping genes, whose downregulation enhanced tolerance to both acids.

## Transcription factors involved in weak acid tolerance

*S. cerevisiae* has evolved various mechanisms to detect and respond to the different stressors present in the environment (Gasch et al., 2000). As mentioned previously in this chapter, yeast cells use different membrane and cell wall receptors to detect each kind of stress and activate a signalling cascade that translates in a cell response (reviewed in Ruis and Schüller, 1995). The main drivers of this activation are transcription factors, which regulate the expression of most tolerance-related genes (Estruch, 2000). Several transcription factors are crucial for cell survival against inhibitors found in lignocellulosic hydrolysate. Haa1 is one of the most important transcription factors involved in the response to acetic acid (Fernandes et al., 2005; Sugiyama et al., 2014; Tanaka et al., 2012), although it has been demonstrated to bind also to propionic and lactic acid (Fernandes et al., 2005). After binding to the acid, it relocates to the nucleus where it activates its gene targets (Collins et al., 2017). Haa1 drives the expression of *TPO2*, *TPO3* or *HRK1*, which encode proteins involved in proton efflux through plasma membrane ATPases, as well as *YGP1*, a gene encoding a glycoprotein important for cell wall hydrophobicity (Destruelle et al., 1994; Kahar et al., 2022). Another important transcription factor involved in carboxylic acid tolerance, *WAR1*, was reported to bind more lipophilic acids (e.g. sorbate and benzoate) and activate yeast genes in charge of responding to weak acid stress through regulation of the ABC transporter-encoding gene *PDR12* (Kim et al., 2019; Kren et al., 2003).

To better understand the response to acetic acid, **this work** focused on the transcription factors Pdr1 and Yap1. Pdr1 is the principal regulator of the pleiotropic drug response towards different cytotoxic compounds (Fardeau et al., 2007); whereas Yap1 is responsible for the activation of genes involved in antioxidant defence, ROS scavenging, and redox balance (Nguyên et al., 2001). A high-throughput study by Sousa et al. (2013) showed that deletion of *PDR1* improved cell fitness in the presence of acetic acid. Conversely, the *S. cerevisiae* Δ*YAP1* mutant exhibited lower survival in the presence of acetic acid (Semchyshyn et al., 2011). Therefore, the exact role of both

regulators during acetic acid stress remains to be determined. To fill this gap, **Paper V** included ChIP-exo analysis of target gene binding for both transcription factors in the presence/absence of acetic acid. Both Pdr1 and Yap1 targeted more genes and bound more strongly to them when cells were exposed to 120 mM acetic acid compared to the control, confirming their participation in the acetic acid response. As indicated earlier in the chapter, genes encoding membrane proteins were among the most targeted by both factors, hinting at potential acid efflux functions for Pdr15, Snq2 or Pdr5. To ascertain whether differential expression of *PDR1* and *YAP1* affected strain fitness in medium supplemented with acetic acid, CRISPRi/a-mediated modulation of both genes was tested. *YAP1* expression was upregulated by targeting its promoter with eight different sgRNAs; whereas *PDR1* was both upregulated and downregulated with eight sgRNAs. Hence, upregulation of *YAP1* and downregulation of *PDR1* could potentially increase yeast tolerance towards acetic acid. *YAP1* overexpression has been previously reported to increase tolerance towards coniferyl aldehyde, HMF, and furfural even in different background strains (Alriksson et al., 2010; Kim and Hahn, 2013), which makes it an ideal gene target for the development of lignocellulose-tolerant industrial strains.

## Role of essential genes in yeast inhibitor tolerance

In this thesis, **Papers II–IV** explored the role of essential and respiratory essential genes of *S. cerevisiae* in tolerance towards lignocellulose-derived inhibitors. Specifically, these studies broadened our understanding of formic and acetic acid tolerance mediated by essential genes. To this end, a CRISPRi strain library constructed by Smith et al. (2012) was used. The library targeted more than 98% of all essential and respiratory essential genes in *S. cerevisiae*, with 3–17 sgRNAs coupled to a dCas9-Mxi1 repressor per target gene. A first screening of the library was performed with the Scan-o-Matic platform using solid medium containing 150 mM acetic acid. This was followed by characterisation in liquid medium containing 125 mM acetic acid to identify the most tolerant and most sensitive strains (Mukherjee et al., 2021). As explained earlier in the chapter, targeting of 19S RP-encoding genes conferred enhanced acetic acid tolerance, as opposed to targeting of the 20S CP, which resulted in highly sensitive strains. Additionally, this study highlighted the importance of intracellular vesicle transport in the maintenance of cell fitness, as many strains with sgRNAs targeting vesicle transport-related genes showed severe growth defects under acid stress. This result was in line with other large-scale studies on acetic acid tolerance (Mira et al., 2010a; Sousa et al., 2013). Many sensitive strains targeted genes encoding subunits of COPI and COPIII vesicles, involved in protein transport between the endoplasmic reticulum and

Golgi apparatus. As acetic acid has been reported to cause misfolded protein accumulation in the endoplasmic reticulum, the function of these transporter complexes may be vital to relieve such stress (Mukherjee et al., 2021).

In **Paper III**, the CRISPRi library was screened in solid and liquid medium supplemented with 140 mM formic acid. The results allowed to compare the stress response of essential genes in the presence of two of the most abundant weak acids found in hydrolysates. While overlap accounted for 50% of total formic acid-sensitive strains, only 10% of total formic acid tolerant strains showed overlap (Figure 2.2). Among tolerant strains, those with sgRNAs that targeted genes involved in chromatin remodelling showed some of the biggest improvements in generation time. Interestingly, genes encoding subunits of the SWI/SNF chromatin remodelling complex (*SNF2*, *SNF6*, and *SWI3*) or INO80 chromatin structure remodelling complex (*INO80*, *RVB1*, and *RVB2*) showed greater fitness when targeted for downregulation. Chromatin remodelling is crucial for the control of cellular gene expression, as it allows the transcriptional machinery to access the DNA. Thus, downregulation of genes encoding complexes involved in chromatin remodelling or reorganization may promote expression of other tolerance-conferring genes.

In **Paper II**, an Haa1-based fluorescent biosensor (Mormino et al., 2021) was transformed into the CRISPRi strain library and then subjected to fluorescence-activated cell sorting. Out of the 41 most fluorescent strains, seven were selected for further growth and fluorescence measurements in liquid medium. Interestingly, except for *PAP1*, all targeted genes were previously reported to be involved in acetic acid stress. *PAP1* encodes a Poly(A) polymerase that may be important for stabilising the mRNA transcripts of some acetic acid stress response genes, but further studies will be needed to elucidate its role in acetic acid tolerance. Additionally, strains targeting *TIF34*, *MSN5*, *PAP1*, *COX10*, and *TRA1* showed higher sensitivity at 150 mM acetic acid. In line with this results, strains with sgRNAs targeting *COX10*, *UBA2*, and *MSN5* were among the most enriched within the sorted fluorescent pool in **Paper IV**. *MSN5*, in particular, was targeted by four unique sgRNAs after the second sorting. This gene encodes for a nuclear exportin involved in the relocation of several transcription factors, including Haa1 (Bose et al., 2005; DeVit and Johnston, 1999; Kim et al., 2019). *MSN5* deletion has been associated with an increased nuclear localisation of Haa1, which could explain the increased biosensor response (Bose et al., 2005; Kim et al., 2019; Sugiyama et al., 2014). Downregulation of *MSN5* was observed to be detrimental for growth in acetic acid in **Paper II**, likely due to the increased instability of Haa1 in the nucleus (Kim et al., 2019). Thus, fine tuning the expression of *MSN5* may be crucial for optimal localisation of Haa1 in the nucleus.

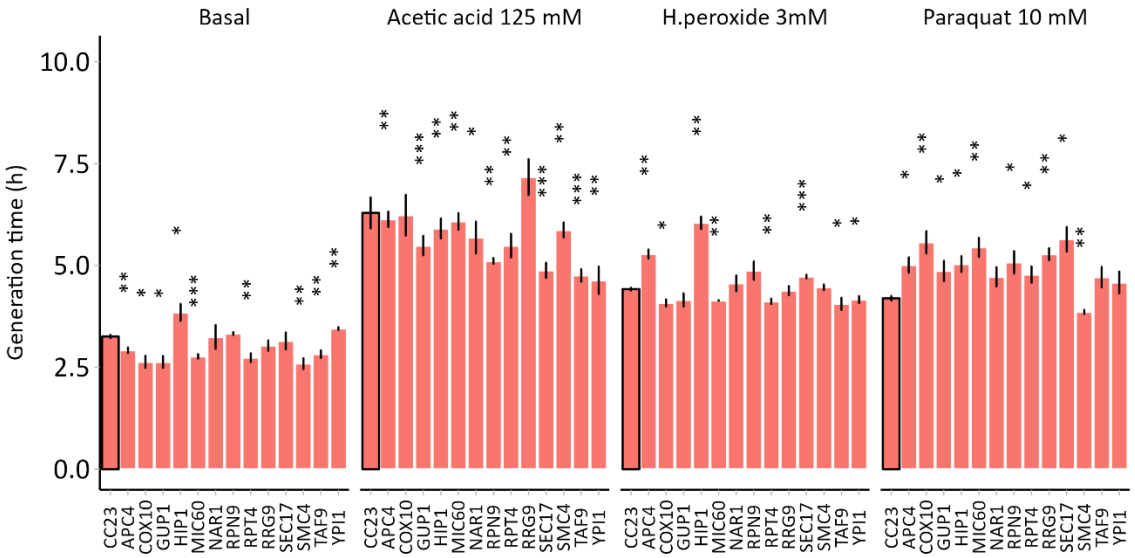


*Figure 5.2. Barplot displaying the generation time (h) in liquid media of the most enriched strains from the competitive growth assay in acetic acid (SMC4, RPT4, SEC17, TAF9, RPN9, MIC60, COX10 and YPI1) or formic acid (NAR1, HIP1, APC4, GUP1, and RRG9). Liquid media conditions are displayed above. The control strain (CC23) is outlined in black. The name of the gene targeted by each strain is displayed on the X-axis. Symbols indicating statistical significance (*: p <= 0.05; **: p <= 0.01; ***: p <= 0.001)*

In **Paper IV**, the strain library was pooled together and then subjected to a competitive growth assay in medium containing either acetic acid (125 mM) or formic acid (120 mM). Such setup forced the more than 9000 strains present in the library to compete for nutrients and favoured enrichment of the fastest growing strains (Figure 4.2). At the same time, the sgRNA region of the most fluorescent strains was also extracted and sequenced, allowing us to confirm and compare the strains discussed in **Paper II**. In agreement with the results from **Paper III**, sensitive strains from the assay (**Paper IV**) largely overlapped in both acidic conditions, whereas tolerant strains showed little overlap. Among enriched strains some targeted chromatin remodelling or the 19S RP of the proteasomal complex (*RPT* and *RPN* genes), in line with the findings from solid medium (**Paper III**). Specifically, 53 strains with sgRNAs targeting the 19S RP were enriched in acetic acid and seven in formic acid. New gene targets previously not reported to be involved in weak acid tolerance were also strongly enriched in the competitive assay (e.g. *SMC4* and *HIP1)*, which confirmed the complementarity of different screening methods to unveil novel tolerance-related gene targets. *SMC4* encodes a condensin involved in chromatin reorganisation, which adds to the importance of chromatin remodelling genes in tolerance to weak acid stress in yeast (Stray and

Lindsley, 2003). *HIP1* encodes a histidine permease involved in the export of manganese from the cytosol (Farcasanu et al., 1998; Tanaka and Fink, 1985). This could be explained by the role of manganese in forming antioxidant complexes that remove ROS.

Following the competitive growth assay, the strains with the highest enrichment values for acetic and formic acid were individually characterised in liquid medium. The screening conditions included medium with acetic acid, as well as media containing hydrogen peroxide and paraquat, two generators of ROS (Figure 5.2). Acetic acid has been shown to generate ROS inside the cell (Chapter II), whose accumulation depletes cellular ATP through activation of ATP-dependent proton pumps and increased NADPH production for ROS detoxification (Palma et al., 2018; Reichmann et al., 2018). Thus, studying the gene response to ROS-generating agents can help understand their role in acetic acid-induced stress. Expectedly, the majority (11 out of 13) of enriched strains displayed shorter generation times and higher growth rates than the control in the presence of acetic acid. The biggest improvements were for strains targeting *YPI1* (-26%), *TAF9* (-24%), and *SEC17* (-22%). Generation time was shorter for only 5 out of 13 strains in the presence of hydrogen peroxide (targeting *TAF9*, *COX10*, *RPT4*, *MIC60*, and *YPI1*) and 1 out of 13 in the presence of paraquat (targeting *SMC4*). The strains growing faster in hydrogen peroxide (*TAF9*) and paraquat (*SMC4*) had only 8% shorter generation times. In conclusion, the strains enriched under formic and acetic acid stress showed increased fitness in the presence of acetic acid, but not ROS-generating agents. Hence, it is likely that the genes targeted by these strains are involved in other stress responses triggered by weak acids, such as membrane reorganisation, unfolded protein response or activation of multidrug resistance transporters.

Results from **Papers II–IV** demonstrate that essential genes play a key role in the development of more inhibitor-tolerant industrial yeast strains. Furthermore, the fine-tuning of target genes is key to obtaining the desired phenotype, especially in the case of essential genes. The various genes found to be involved in acetic and formic acid tolerance in these three studies should be further investigated to elucidate their role in acid tolerance.

# Chapter VI: Summary and Conclusions

In this thesis, I present research aimed at furthering our understanding of the tolerance manifested by *S. cerevisiae* towards inhibitors present in lignocellulosic hydrolysates. Differences in inhibitor toxicity as well as the corresponding tolerance related genes has been presented and discussed. Furthermore, different methods for industrial strain development and screening described in the five papers included in the thesis has been summarised.

One aim of this thesis was to develop mutant strains with varying expression of genes involved in the response to lignocellulosic inhibitors. To achieve this, CRISPRi/a technology was chosen and applied in both laboratory and industrial strains. CRISPR/Cas9-based genetic manipulation forms the backbone of this thesis. In **Paper V**, plasmids containing either dCas9-Mxi1 or dCas9-VPR were transformed into the laboratory yeast CEN.PK 113-5D strain. The Pdr1 and Yap1 transcription factors involved in pleiotropic drug response and oxidative stress, respectively, were targeted each with eight sgRNAs to test whether their up- or downregulation affected the yeast's phenotype in the presence of 120 mM acetic acid (pH 4.5). This experiment was motivated by the results obtained via ChIP-seq, which determined a 2.2- and 4.5-fold increase in the number of bound genes for Pdr1 and Yap1, respectively, under acetic acid stress. Based on this evidence, the promoters of the two transcription factors were targeted with CRISPRa and then screened for growth in the presence of acetic acid. Additionally, given earlier suggestions that *PDR1* deletion could increase acetic acid tolerance (Sousa et al., 2013), this gene was targeted also with CRISPRi for downregulation. Upregulation of *YAP1* with CRISPRa resulted in a higher growth rate in the presence of acetic acid in two out of eight strains; whereas *PDR1* downregulation improved growth rate in seven out of eight strains. Hence, CRISPRi/a can be an optimal technology for rapid fine-tuning of yeast gene expression in tolerance assays. Notably, in **Paper I**, CRISPRi/a technology was shown for the first time to modulate gene expression of an industrial polyploid *S. cerevisiae* strain. The genetic makeup of industrial strains is rather diverse, as they typically result from hybridization between different species, followed by adaptive evolution in industrial conditions (Querol and Bond, 2009). This adaptation stabilises the genome of industrial strains, but hinders genetic engineering with synthetic biology tools (Le Borgne, 2012). Given that modifications in laboratory strains cannot be always extrapolated to industrial strains, an alternative approach involves working directly with the latter (Deparis et al., 2017). In **Paper I**, dCas9 and dCas9-VPR CRISPRi/a plasmids were transformed into KE6-12, an ethanol-producing strain, to modulate four

genes related to inhibitor tolerance. *SSK2*, *BDH2*, *HRK1*, and *ISC1* were selected as target genes due to their reported involvement in tolerance towards furfural, vanillin, and acetic acid. Downregulation of *SSK2* by dCas9-mediated targeting yielded more furfural-tolerant strains compared to the control, confirming the potential applicability of CRISPRi/a to select new targets in industrial strains. Nevertheless, more permanent changes in the expression of the targeted genes via promoter replacement or gene knockout need to be performed in the future to confirm the obtained phenotypes.

Another aim of this thesis was to identify and confirm gene targets for lignocellulosic hydrolysate inhibitor tolerance in yeast, as well as to compare the fitness of cells in which the expression of these genes was altered. Based on the involvement of essential and respiratory growth essential genes in weak acid tolerance (**Papers II–IV**), several downregulated targets capable of conferring a fitter phenotype to either formic or acetic acid were identified. For instance, the acetic acid biosensor screen showed that downregulation of *PAP1* increased intracellular acetic acid content and slowed yeast growth. Pap1 has not been reported previously to participate in weak acid tolerance, but its function may be crucial in the stabilization of some acid stress response gene transcripts. Moreover, tuning the expression of other genes highlighted in the biosensor screen such as *COX10*, *TIF34* or *TRA1* could also lead to improved acetic acid tolerance. **Papers III and IV** compared the stress response towards formic and acetic acid using the CRISPRi strain library. Sensitive strains were largely overlapping whereas tolerant strains were mostly unique to each acid. Moreover, the phenotype (sensitive/tolerant) displayed by sensitive or tolerant strains in solid medium supplemented with formic acid could be replicated in both liquid medium with formic acid and in synthetic hydrolysate. Tolerant strains contained sgRNAs targeting genes involved in glycogen accumulation (*YPI1*), chromatin remodelling (*SMC4*, RSC complex, SWI/SNF complex, and SAGA complex) or the 19S proteasome particle (*RPT* and *RPN* genes). Instead, sensitive strains targeted genes involved mostly in intracellular vesicle trafficking (COPII vesicle transport) or translation and translation initiation (*RPL* and *TIF* genes). Interestingly, most strains enriched in the competitive growth assay displayed an increased growth rate in the presence of acetic acid, but not when challenged with ROS-producing agents (hydrogen peroxide and paraquat), indicating that their role in acetic acid tolerance was not related to oxidative stress (Figure 5.2) . The ChIP-seq experiment using transcription factors Pdr1 and Yap1 unveiled that genes belonging to the “Small molecule biosynthetic process” Gene Ontology term were significantly enriched upon acid exposure. In particular, this included genes encoding biosynthetic proteins (*ERG5* and *ERG25*) or multidrug transporters (*SNQ2*, *PDR5*, and *PDR15*). Overall, the results show that the response towards lignocellulosic hydrolysate inhibitors is highly complex and

triggers the activation of several genes with diverse functions. The work in this thesis has shown that tolerance towards a single inhibitor such as acetic acid can be altered by regulating the expression of genes involved in proton efflux, chromatin remodelling, unfolded protein degradation or pleiotropic drug stress. This, added to the possible synergistic effects caused by the combination of several inhibitors at once, makes industrial strain development a challenging task. The work described herein has unveiled putative targets to screen in the pursuit of a more tolerant industrial yeast strain. Thus, further work should be done to find suitable combinations of genes, whose change in expressions may result in a tolerant phenotype against lignocellulosic hydrolysates.

The final aim of the thesis was to test diverse screening methods, with which to assess inhibitor tolerance in *S. cerevisiae*. Inhibitor tolerance assays were performed using microtiter plate-based liquid screening platforms, the high-throughput solid medium Scan-o-Matic platform, competitive growth assays, and biosensor-based strain sorting. Assessing strain performance in challenging industrial growth conditions may be difficult to reproduce at laboratory scale. Microtiter plate-based screening is the most typical and reliable method to measure growth parameters, but it has low output. When evaluating strain tolerance, it is common to screen large strain libraries and find as many gene targets as possible. Thus, a high-throughput screening method combined with microtiter-based liquid characterisation of the most tolerant or sensitive strains represents an efficient way to assess tolerance. Still, different screening methods may yield different tolerant and sensitive strains, based on differences in setup (solid vs. liquid), competition for nutrients or parameter measured (growth rate, lag phase or biosensor output). In conclusion, a more complete overview of tolerance towards an inhibitor will be achieved if different high-throughput methods were combined with a final characterisation step in liquid medium.

Collectively, this thesis improves our understanding of the gene responses triggered in *S. cerevisiae* towards inhibitors found in lignocellulosic hydrolysates. I used CRISPRi/a throughout the thesis to easily alter the expression of tolerance-related genes, including in an industrial yeast strain. Differences in gene response towards formic vs. acetic acid were assessed after screening an essential and respiratory growth essential CRISPRi library. Finally, multiple high-throughput screening methods were tested and compared in this thesis, proving their usefulness as complementary methodologies to study tolerance.

## Advancing the development of tolerant strains – an outlook

Driven by the depletion of fossil fuels and the need to reduce carbon emissions, bio-based production of chemicals through microbial cell factories is poised to grow in the coming years. Within such context, baker's yeast has emerged as one of the principal cell factories capable of utilising plant biomass to produce chemicals with high added-value for food, feed, pharmaceuticals, and agricultural companies. Studying and understanding the mechanisms of inhibitor tolerance will accelerate the development of future industrial strains.

With the tools provided by synthetic biology, including CRISPR/Cas9 genome editing, multi-part gene assembly, genome-scale models, and high-throughput strain screening, rational engineering has become a promising avenue in cell factory development. **This thesis** has exemplified how high-throughput screening and library-generating technologies can accelerate the development of tolerant strains. Still, testing thousands of strains or combinations of mutants requires complex and time-consuming experiments. Nowadays, synthetic biology tools are implemented iteratively and often manually (Gurdo et al., 2023). Such manual labour results in an elevated error rate, increased resource consumption, inaccurate data analysis, and development of unscalable techniques (Appleton et al., 2017). Automation of strain development, backed by robotics and artificial intelligence, could overcome the above shortcomings. In practice, this would mean the implementation of robotic stations equipped with liquid handling arms and pinning pads that allow to speed-up current workflows.

To keep pace with progress in high-throughput technologies, new analytical and predictive methods based on the generated data are emerging. Both modelling and machine learning have proven successful in this regard. For instance, genome-scale metabolic models constructed using whole-genome information allow the estimation of metabolic fluxes via flux balance analysis. The latter can maximise cell growth and product yield for a particular condition. By using an adequate genome-scale model and one parameter to maximise, flux balance analysis predicts the link between the sought phenotype, environmental conditions, and cell genotype (Furusawa et al., 2013). Furthermore, advances in machine learning and artificial intelligence have improved our ability to predict pathways, whose upregulation or downregulation will lead to the desired phenotype. I believe that the future of tolerant strain development lies in combining machine learning predictive models with metabolic engineering and adaptive evolution. This will allow us to predict the most efficient combinations of mutations to achieve a fit phenotype and reduce the amount of screening. Recently, some examples of automation have been successfully implemented in protein (Chory et al., 2021), lycopene (HamediRad et al., 2019) or dodecanol

(Opgenorth et al., 2019) production. Overall, these advancements are bringing us closer to the ideal high-throughput screening technology, one that matches the output required for large-scale production.

It was shown in **this thesis** that fine-tuning the expression of tolerance-related genes was often preferrable to silencing or overexpression. Still, the system's unpredictability and toxicity is not always optimal (Schultenkämper et al., 2020). Fortunately, substantial research is focusing on improving the existing CRISPRi/a technology. For instance, the development of new bioinformatic tools, such as E-CRISP (Heigwer et al., 2014), CHOPCHOP (Labun et al., 2019) or CRISPOR (Concordet and Haeussler, 2018) has allowed to reduce off targets. Furthermore, next-generation CRISPRi/a libraries with new effector molecules (Replogle et al., 2022) are being developed. On another front, ongoing efforts to reduce background expression of Cas9 nuclease (Koopal et al., 2019) will lower its toxicity and expand CRISPRi/a to new organisms. Finally, work is being done to widen the recognition of target sequences by the nuclease, either by engineering Cas9 to recognize more PAM sequences (Nishimasu et al., 2018) or by substituting Cas9 for other systems such as dCas12a (Specht et al., 2020).

Studying yeast tolerance mechanisms towards inhibitors found in lignocellulosic hydrolysates is a complex and challenging process. **This thesis** provides greater understanding of the underlying physiology, what makes a yeast cell tolerant, and how the corresponding genes are regulated. The obtained knowledge can be used to develop novel biochemical production hosts, which are crucial in the transition towards a circular economy. They can also contribute to future models of tolerance mechanisms. Finally, understanding gene regulation in yeast is the door to comprehend its workings in any eukaryotic cell. This, in turn, is important for understanding stress conditions and faulty regulation of gene expression, a common cause of disease in humans.

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Azkanik, eskerrik asko etxekuai. Mertzi beti galdetzearren, beti animuk emotearren laneako. Asko kostata, baine azkanin lorturot. Muso pillo bat danoi ta pillo bat matxe matxe. Mertzi lagunai be, nax eta neu desastre bat ixan mezu bat bixaltzearren ta bixitxan etortearren.

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