



**PROGRAMA INTEGRADO (UNESP, USP E UNICAMP) DE PÓS-GRADUAÇÃO
EM BIOENERGIA**

**GENETIC PATHWAYS TOWARD EFFICIENCY: ADAPTIVE LABORATORY EVOLUTION
AND GENE EDITING STRATEGIES IN THE FERMENTATION OF LIGNOCELLULOSIC
HYDROLYSATE BY *Saccharomyces cerevisiae***

Yasmine Alves Menegon

**Rio Claro - SP
2024**



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE PESQUISA EM BIOENERGIA



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Yasmine Alves Menegon

Tese apresentada ao Instituto de Pesquisa em Bioenergia de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências.

Orientadora: Dr. Ana Paula Jacobus

Co-orientador: Dr. Jeferson Gross

**Rio Claro - SP
2024**

M541g

Menegon, Yasmine Alves

Genetic pathways toward efficiency: : adaptive laboratory evolution and gene editing strategies in the fermentation of lignocellulosic hydrolysate by *Saccharomyces cerevisiae* / Yasmine Alves Menegon. -- Rio Claro, 2024
266 p. : il., tabs., fotos

Tese (doutorado) - Universidade Estadual Paulista (UNESP), Instituto de Pesquisa em Bioenergia, Rio Claro

Orientadora: Ana Paula Jacobus

Coorientador: Jeferson Gross

1. Biotechnology. 2. Bioethanol. 3. Molecular biology. 4. Metabolomics. 5. Proteomics. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Universidade Estadual Paulista (UNESP), Instituto de Pesquisa em Bioenergia, Rio Claro. Dados fornecidos pelo autor(a).

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TÍTULO DA TESE: GENETIC PATHWAYS TOWARD EFFICIENCY: ADAPTIVE LABORATORY EVOLUTION AND GENE EDITING STRATEGIES IN THE FERMENTATION OF LIGNOCELLULOSIC HYDROLYSATE BY *Saccharomyces cerevisiae*

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Rio Claro, 27 de março de 2024

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Dedico este trabalho à minha mãe, fonte constante de apoio e inspiração.

AKNOWLEDGMENTS

Querida mãe, te agradeço por todo o apoio que tu me deste ao longo desses 10 anos de jornada acadêmica. Tua confiança e amor foram a base que me sustentou nos momentos mais difíceis. Tu és minha fonte de inspiração, obrigada por acreditar em mim desde o início. Essa conquista é nossa e te dedico essa tese com todo o meu coração.

Agradeço à minha irmã, Raphaela, e ao meu sobrinho, Bernardo, por preciosos momentos de descontração e cumplicidade que compartilhamos ao longo dos anos.

À minha tia Dileusa, à minha avó Sueli e ao meu primo João Pedro por sua constante amizade, motivação e apoio. Também desejo estender meu sincero agradecimento ao meu tio Pedro, à minha tia Lilian e meus primos Pyetra e Lucas pela amizade e companheirismo que sempre demonstraram.

À Prof. Caroline Blass, que em 2013 abriu as portas da escrita para mim. Minha profunda gratidão pela bondade e apoio. Nunca esquecerei o impacto positivo que tivestes na minha vida. Muito obrigada!

Ao Carlos Valério, que me proporcionou condições ótimas para eu concluir meus estudos escolares. Tua generosidade, lá em 2013, fez toda a diferença. Obrigada!

Ao meu orientador de graduação, professor Fábio Leivas Leite, por seu papel fundamental em minha formação acadêmica. Tu não apenas me ensinou os princípios da pesquisa e a importância da sustentabilidade, mas também moldaste meu pensamento crítico. Tua bondade e incentivo constante foram essenciais para meu crescimento. Sou profundamente grata pela tua orientação e apoio ao longo dos primeiros 5 anos deste trajeto.

Ao Santander Universidades, pelo fomento do intercâmbio acadêmico em 2017-2018, em Portugal.

À FAPESP pelo fomento desta pesquisa de doutorado e toda a contribuição para tal desenvolvimento, no processo no 2019/19168-5, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

À minha orientadora de doutorado, Dr. Ana Paula Jacobus, pelo apoio e direcionamento. Obrigada pelas oportunidades oferecidas ao longo destes 4 anos. Ao meu co-orientador, Dr. Jeferson Gross, pelos insights ao longo do trabalho e pela análise dos genomas.

À Universidade Estadual Paulista (UNESP) pela excelente oportunidade de formação acadêmica.

À CAPES, por tornar o intercâmbio para a Austrália possível. O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

To Professor Esteban Marcellin for his exceptional guidance and unwavering support during my exchange program. Additionally, I am deeply thankful to Dr. Axa for his generosity and invaluable guidance in the laboratory. The encouragement and recognition from both of you played a more than significant role in my academic and personal growth.

To my friends at AIBN, I offer my sincere appreciation for your support and friendship throughout this journey. Your presence has made this offshore experience truly memorable.

Às minhas duas companheiras de 4 patas, Cacau e Avelã, que estiveram comigo desde o início do doutorado. Agradeço por elas transformarem minha casa em um lar sempre alegre e reconfortante. Não poderia ser mais grata pela companhia fiel e amor incondicional.

Ao André, por cuidar não só de mim, mas também das nossas meninas, Cacau e Avelã. Não tenho palavras para agradecer por ser meu porto seguro em meio às tempestades acadêmicas. Tua paciência, apoio e amor foram fundamentais para me manter firme.

À todas as pessoas que, de alguma forma, contribuíram para a realização deste trabalho e para o meu crescimento pessoal e profissional, os meus sinceros agradecimentos.

Por fim, agradeço a banca examinadora pelo tempo disposto a ler e opinar sobre as melhorias desta tese.

“Never mistake knowledge for wisdom.
One helps you make a living; the other helps you make a life.”
(Sandra Carey)

RESUMO

Esta Tese explora as respostas adaptativas de cepas de *Saccharomyces cerevisiae* que foram submetidas a protocolos de Evolução Laboratorial Adaptativa (ALE) em hidrolisado lignocelulósico. Duas abordagens distintas, transferência serial (ALE1, ALE2, ALE3) e o choque/recuperação (ALE4), foram empregados, desenvolvendo tolerância significativa em cepas haplóides (ALE2 e ALE4) e diplóides de CEN.PK113-7D (ALE3), além de cepas diplóides de PE-2_H4 (ALE1). Pós-evolução, foram elaboradas competições de crescimento contra a cepa parental, que demonstrou quais cepas eram superiores. Além disso, as mutações genéticas foram identificadas através do sequenciamento do genoma completo (WGS) e demonstraram a importância do paralelismo das mutações. As cepas evoluídas e cepas de engenharia reversa foram analisadas sob condições de fermentação controlada, indicando as linhagens CHG, CHH (ALE2) e S_CHN (ALE4) com ótimo desempenho, indicando fases lag mais curtas, taxas de crescimento mais altas e maior produção de etanol/CO₂. Nas linhagens de engenharia reversa, *RET1SIZ1Δ* e *PMA1Δ* mostraram melhor crescimento em hidrolisado. Além disso, essa Tese investiga o desenvolvimento e a caracterização da cepa PE2_H4_GN1, revelando as sobreposições genética com populações evoluídas que a originaram (ALE1). As análises de fermentação demonstraram taxas de crescimento aumentadas no hidrolisado, com a metabolômica enfatizando a produção de etanol, desintoxicação de inibidores, produção de glicerol, variações de produção de aminoácidos, poliaminas e metabolismo do carbono central. Dados proteômicos fecharam o estudo, identificando termos enriquecidos de Gene Ontology, sugerindo mudanças adaptativas na síntese de ATP, biossíntese de aminoácidos e organização do citoesqueleto. Essas descobertas elucidaram as estratégias e vias metabólicas das linhagens de ALE. E a pesquisa em geral contribui com informações valiosas para os campos da evolução laboratorial, etanol de segunda geração e biotecnologia.

Palavras-chave: Etanol 2G; Fermentação alcoólica; CRISPR/Cas9; Metabolômica; Proteômica.

ABSTRACT

This Thesis explores the adaptive responses of *Saccharomyces cerevisiae* strains that were subjected to Adaptive Laboratory Evolution (ALE) protocols in the lignocellulosic hydrolysate. Two distinct approaches, serial transfer (ALE1, ALE2, ALE3) and shock/recovery (ALE4), were employed, developing significant tolerance in haploid (ALE2 and ALE4) and diploid CEN.PK113-7D (ALE3) strains, in addition to diploid strains of PE-2_H4 (ALE1). Post-evolution, growth competitions versus the parental strain were designed and demonstrated which strains were superior. Furthermore, genetic mutations were identified through whole genome sequencing (WGS) and demonstrated the importance of mutation parallelism. The evolved strains and reverse-engineered strains were analyzed under controlled fermentation conditions, indicating strains CHG, CHH (ALE2), and S_CHN (ALE4) with excellent performance, indicating shorter lag phases, higher growth rates, and greater ethanol production /CO₂. In the reverse-engineered lines, *RET1SIZ1Δ* and *PMA1Δ* showed better growth in hydrolysate. Furthermore, this Thesis investigates the development and characterization of the PE2_H4_GN1 strain, revealing the genetic overlaps with the evolved populations that originated it (ALE1). Fermentation analyses demonstrated increased growth rates in the hydrolysate, with metabolomics emphasizing ethanol production, inhibitor detoxification, glycerol production, variations in amino acid production, polyamines, and central carbon metabolism. Proteomic data ended the study, identifying enriched Gene Ontology terms, suggesting adaptive changes in ATP synthesis, amino acid biosynthesis, and cytoskeletal organization. These findings elucidated the metabolic strategies and pathways of ALE lineages, and the research in general contributes with valuable information to the fields of laboratory evolution, second-generation ethanol, and biotechnology.

Keywords: 2G Ethanol; Alcoholic fermentation; CRISPR/Cas9; Metabolomics; Proteomics.

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LIST OF ABBREVIATIONS

AFEX	- Ammonia Freeze Explosion
ALE	- Adaptive Laboratory Evolution
CO ₂	- Carbon Dioxide
DFV	- Dual-Fuel Vehicle
QP	Global Ethanol Volumetric Productivity
IEA	- International Energy Agency
LCH	- Lignocellulosic hydrolysate
MVB	- Multivesicular body
OD	- Optical Density
PCR	- Polymerase Chain Reaction
SNP	- Single-nucleotide polymorphism
STEX	- Steam explosion
S_LCH	- Synthetic lignocellulosic hydrolysate
TF	- Transcriptional factor
VERT	- Real-time evolution visualization
WGS	- Whole Genome Sequencing
WO	- Wet oxidation
WT	- Wild-type
YPD	- Yeast extract, Peptone and Dextrose Media

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CHAPTER 1 - GENERAL CONSIDERATIONS

1 GENERAL INTRODUCTION

To reduce the uncontrolled emission of carbon dioxide (CO₂) and the resulting climate change, replacing oil-based fuels with products derived from renewable resources, such as bioethanol, is required. However, a large amount of sugars produced by plants are insoluble, being in the form of cellulosic fiber. The dry biomass, also called lignocellulosic biomass, mainly comprises cellulose, hemicellulose, and lignin (aromatic polymer). Generally, the lignocellulosic biomass needs to be pretreated and hydrolyzed to convert its cellulose and hemicellulose into simple sugars for fermentation (ALMARIO; REYES; KAO, 2013). The production of inhibitor compounds during the pre-treatment step contains a substantial obstacle to the production of bioethanol during the fermentation stage. Those chemicals negatively impact the metabolism of the microorganism (COSTA; TEODORO; FILHO, 2015). *Saccharomyces cerevisiae* is the preferred microorganism for large-scale ethanol production because of its high ethanol yield, productivity, and general robustness. However, as strategies are implemented to generate larger and cheaper cellulosic ethanol volumes worldwide, *S. cerevisiae* is further challenged by new process requirements (PALMQVIST; HAHN-HÄGERDAL, 2000; WALLACE-SALINAS; GORWA-GRAUSLUND, 2013). One of the main bottlenecks for converting lignocellulose into ethanol is the related production processes. These processes must be economically competitive and overcome the yeast productivity gap as high inhibitor concentrations and harmful process conditions occur. Since extra process conditioning operations are economically and energetically expensive (CASPETA *et al.*, 2014; CASPETA; BUIJS; NIELSEN, 2013). To cope with these stressful conditions, adapted yeasts develop several mechanisms to respond to the lignocellulosic hydrolysate (LCH) environment that contains inhibitors such as furaldehydes, weak organic acids, and phenolic compounds (ALMEIDA *et al.*, 2008).

One way to create conditions for the yeast to find genetic ways to manage stressful conditions is through evolution and adaptation, in this way, Adaptive Laboratory Evolution (ALE) stands out as a process in which microorganisms, including yeast, are cultivated under controlled laboratory conditions for an extended period to select desirable traits. Studies of long-term adaptation experiments focused on improving the tolerance of *S. cerevisiae* to a specific inhibitor or a combination of

inhibitors derived from hydrolysates have already been reported (ALMARIO; REYES; KAO, 2013; GONZÁLEZ-RAMOS *et al.*, 2016; LIU; SLININGER; GORSICH, 2005; SMITH; VAN RENSBURG; GÖRGENS, 2014). After the ALE protocol, genomic sequencing, reverse engineering, proteomics, metabolomics, and transcriptomics have helped uncover some of the evolutionary mechanisms responsible for the superior traits in the evolved strains (GONZÁLEZ-RAMOS *et al.*, 2016; LIU *et al.*, 2019; LIU; MA, 2020; PEREIRA *et al.*, 2020; SALAS-NAVARRETE *et al.*, 2022; THOMPSON *et al.*, 2016).

By representing evolution, the ALE protocol already provides a direct path to improving the yeast phenotype. However, the utility of ALE as a pipeline for allele discovery offers a possibility to renew the robustness of yeast for 2G processes. In this study, the aim is to develop strong ALE protocols, combining different approaches, such as the use of different strains, and different ploidies. In addition to puts forward diploid lineages to genome shuffling under selective pressure, which helps to compile the main adaptive alleles related to tolerance to LCH. The populations and clones from these protocols were subjected to genomic sequencing, fermentative profile analysis in bioreactors, and proteomic and metabolomic profile analysis. Based on this information, this study further validated the adaptive variants using the CRISPR/Cas9 genome editing. Thus, this work offers a valuable contribution to improving the production of second-generation bioethanol, aligning with the urgent need to reduce carbon dioxide (CO₂) emissions.

1.1 Fuels: Environmental Concerns and Alternatives

Important environmental agreements that have made petroleum-based fuels the target of socio-environmental criticism by international media have emerged (SILVA *et al.*, 2017). Criticism focuses mainly on using non-renewable resources and releasing pollutants (SANTOS *et al.*, 2017; SZULEJKO *et al.*, 2017). CO₂ is one of the combustion-derived gases that can impact Earth's climate; its elevated atmospheric concentration is thought to play a significant role in climate change and global warming (YANG; WANG, 2015). This results in a well-known and unfavorable occurrence that raises the world's average temperature and harms human life (KARTHICKEYAN; BALAMURUGAN; SENTHIL, 2017). Interest in finding sustainable alternative energy sources has increased since the Kyoto Protocol was signed in 1997 (GRUBB *et al.*, 1999).

1.1.1 Biofuels: The Path to Sustainable Energy

Biofuels are fuels derived from renewable biomass sources such as plant matter or animal waste. They have gained increasing attention as alternatives to fossil fuels because of their potential to reduce greenhouse gas emissions and the current dependence on non-renewable resources (Jeswani *et al.*, 2020). There is a rapid expansion in the production and consumption of biofuels, which continually influences the field of a sustainable economy, ultimately maximizing social, economic, and environmental benefits (AMIGUN; MUSANGO; STAFFORD, 2011; CORREA *et al.*, 2019). Certain underutilized farmlands that are unsuitable for growing food crops are employed in the manufacture of biofuels. Farmers can increase their financial stability, profitability, and independence from traditional markets by generating biofuels (ALAZAIZA *et al.*, 2022; RATHMANN; SZKLO; SCHAEFFER, 2010).

However, there are obstacles in the way of creating sustainable biofuel economies, particularly in less developed nations where the uptake and integration of biofuels are trailing behind (HASAN *et al.*, 2023). Ensuring that biofuels can be affordably and economically utilized in all locations is crucial for the attainment of truly sustainable biofuel economies. The broad acceptance and worldwide influence of biofuels depend on this inclusion. Large-scale expansion is crucial in this situation, highlighting the necessity for substantial advancements in green technology innovation in the biofuel manufacturing industry (HASAN *et al.*, 2023). Adopting cutting-edge technologies is essential to improve biofuel production efficiency and significantly lowering the world economy's reliance on non-renewable fuels.

1.2 Bioethanol

Bioethanol is one of the most popular renewable energy sources for minimizing reliance on fossil fuels (BECERRA-RUIZ *et al.*, 2019). In the 1920s, Brazil began using bioethanol as fuel, primarily for transportation. Brazil's bioethanol program was driven by its need to reduce its dependence on imported oil. In the 1970s, the oil crisis increased interest in bioethanol as a renewable alternative to fossil fuels, and Brazil increased its efforts to promote alcohol usage (REIJNDERS; HUIJBREGTS, 2008). In the 1990s, Brazil's bioethanol program underwent a major expansion, with the government compelling the use of bioethanol in all gasoline sold

in the country (GOLDEMBERG, 2008). In the United States, ethanol production and consumption have increased dramatically in recent years due to government incentives and regulations (LUO; MILLER, 2013). In Brazil, a new regulatory law called RenovaBio was approved at the end of 2017, to improve domestic biofuel production and utilize the nation's capacity to produce renewable energy. The dual-fuel “flex” vehicle (DFV) also held an 88.6% market share in the Brazilian auto industry in 2017 (MARTINELLI *et al.*, 2022). Bioethanol is the main source of biofuel, with 27 billion gallons being generated globally in 2021.

In terms of output, the United States was the world's largest ethanol fuel producer in 2023, accounting for 53% of the global output (~15,6 million gallons), reflecting a significant increase in production since 2007. Second on the list, Brazil produces 28% (or ~8,3 million gallons) of the world's ethanol from sugar cane. The two nations account for approximately 81% of the ethanol produced worldwide. The European Union and India contributed 5% of global production each. China (3%), and Canada (2%) also had some significance, demonstrating the nation's desire to use ethanol as a fuel additive (RENEWABLE FUELS ASSOCIATION, 2024). First-generation ethanol is mostly produced from feedstocks high in carbohydrates for human or animal foods, such as sugarcane, corn, beetroots, and wheat. The conversion of sugars and/or starches to alcohol is necessary for the manufacturing of ethanol, and the well-known processes employed in this conversion include fermentation and distillation (JESWANI; CHILVERS; AZAPAGIC, 2020; RAY; RAMACHANDRAN, 2018).

1.2.1 Second-Generation Bioethanol: Transforming Biomass into Sustainable Energy

Second-generation biofuels are defined by the International Energy Agency (IEA) as biofuels made from lignocellulosic biomass, or feedstock consisting of cellulose, hemicellulose, and lignin (BACOVSKY, 2010). To facilitate the synthesis of 2G ethanol, hemicellulosic and cellulosic polymeric chains are broken down into fermentable sugars using pretreatment and hydrolysis processes. Microorganisms subsequently ferment the released sugars to produce ethanol (ROBAK; BALCEREK, 2020).

Biomass includes leftovers from processing grains, such as corn and rice; wood chips from forests, such as soft and hard wood; and agricultural leftovers, such as grass and alfalfa (KARAGOZ; BILL; OZKAN, 2019). Sugarcane bagasse, a

byproduct of the first-generation ethanol industry and food sector, is a promising lignocellulosic feedstock because of its high sugar content, excellent availability, and lack of competition with the food industry (DIAS *et al.*, 2015; UNGUREANU; VLĂDUȚ; BIRIȘ, 2022).

Depending on the pretreatment used, hemicellulosic hydrolysates prepared from sugarcane bagasse contain 38-50% cellulose (crystalline structure), 23-32 % hemicellulose (amorphous structure with xylose, glucose, etc.), and 15-25 % lignin (SOARES *et al.*, 2022; SUN *et al.*, 2004). A linear polymer of D-glucose units connected by 1-4 glycosidic linkages constitutes cellulose. Hexoses and pentoses with short ramifications, such as heteropolymers of xylan, galactomannan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan, make up the majority of the heteropolymers known as hemicellulose. Lignin is a complex polymer composed of three main building blocks: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These building blocks are linked by chemical bonds, including ether, carbon-carbon, and carbon-oxygen bonds (CHANDEL *et al.*, 2020; KUMAR *et al.*, 2020; PALMQVIST; HAHN-HÄGERDAL, 2000).

1.2.1.1 From Biomass to Bioethanol: Second-Generation Production Process

Second-generation bioethanol production from sugarcane involves the use of lignocellulosic biomass for ethanol production. Although several processes have been developed, a widely used method involves several steps, including pretreatment, hydrolysis, fermentation, and distillation (CARDONA; QUINTERO; PAZ, 2010; PARAWIRA; TEKERE, 2011). First, the material is chopped into pieces. Then a pre-treatment step consists mainly of a physical-chemical treatment that removes some of the structural lignin as phenolic compounds, decreases the crystallinity of cellulose, increases its porosity, and partially releases monomeric/oligomeric sugars from hemicelluloses for microbial conversion to ethanol. Several pretreatment methods are available, including physical, chemical, and biological methods. Several pre-treatment processes are available, including acid or basic hydrolysis, steaming/steam explosion (STEX), ammonia freeze explosion (AFEX), and wet oxidation (WO) (KUMAR *et al.*, 2009; MCINTOSH *et al.*, 2016; NAKANISHI *et al.*, 2018; ROQUE *et al.*, 2019; TOPALOĞLU *et al.*, 2023). At this stage, inhibitors of the yeast fermentative phase are generated, and these

compounds can be divided into three major groups; furaldehydes, weak acids, and phenolics.

The furaldehydes usually include 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF). The weak acids include acetic acid, formic acid, and levulinic acid; and phenolic compounds include vanillin, syringaldehyde, ferulic acid, vanillic acid, and 4-hydroxybenzaldehyde (MUSSATTO; ROBERTO, 2004; PALMQVIST; HAHN-HÄGERDAL, 2000). Although a milder pre-treatment could be used to achieve a lower inhibitor concentration, a significant amount of cellulolytic enzyme mixtures will still be required to convert cellulose and hemicellulose into monomeric sugar. This presents significant economic and logistical hurdles to the sector (HIMMEL *et al.*, 2007).

Saccharomyces cerevisiae is the most commonly used yeast in second-generation bioethanol production. However, *S. cerevisiae* is sensitive to the inhibitors present in the hydrolysate (CARDONA; QUINTERO; PAZ, 2010). Several strategies have been developed to overcome this problem, including the use of genetically modified yeast strains (CADETE *et al.*, 2016; JANSEN *et al.*, 2017; LI *et al.*, 2015b; SÀNCHEZ NOGUÉ; KARHUMAA, 2015; ZHANG *et al.*, 2016) or even co-cultivate with other microorganisms that are more tolerant or that can ferment pentose sugars, such as *Scheffersomyces stipitis*, *Scheffersomyces shehatae*, and *Spathaspora passalidarum* (BIAZI *et al.*, 2020; BONAN *et al.*, 2022; MOHD AZHAR *et al.*, 2017).

Thus, second-generation bioethanol production faces several challenges, including the high cost of pretreatment, low sugar yields, and the need for more efficient and robust microorganisms for fermentation. Research is ongoing to overcome these challenges and improve the efficiency and sustainability of second-generation bioethanol production from sugarcane.

1.3 LCH Inhibitors

1.3.1 Unveiling the Origins: Inhibitors in Lignocellulosic Hydrolysates

Inhibitors, such as HMF and furfural, are derived, respectively, from the dehydration of hexoses (glucose, mannose, and galactose) and pentoses (xylose and arabinose), under high-temperature and pressure conditions (LARSSON *et al.*, 1999b; LEWKOWSKI, 2003; PALMQVIST; HAHN-HÄGERDAL, 2000; PERNA *et al.*, 2018; ZHANG *et al.*, 2019). In addition, the increase in temperature and

pre-treatment time also favors the formation of furfural and HMF, which are often used as indicators of pretreatment severity (JÖNSSON; MARTÍN, 2016; LI *et al.*, 2015a).

Acetic acid, the most abundant weak acid in lignocellulosic hydrolysates, is generated from the deacetylation of acetyl groups attached to the main chain of hemicelluloses. It may also be produced as a fermentation byproduct of ethanol (KUMAR *et al.*, 2020; ZHANG *et al.*, 2019). Notably, lactic acid bacteria, which are known to be common contaminants and produce acetic acid in a problematic way, are another obstacle for yeasts in ethanol production (BREXÓ; SANT'ANA, 2017; RICH *et al.*, 2015).

Formic acid and levulinic acid can be present in hydrolysates as a result of HMF degradation (CUNHA *et al.*, 2019), and furfural further degrades into formic acid. Phenolic compounds such as vanillin, coumaric acid, ferulic acid, 4-hydroxybenzoic acid, and catechol, among others, are released by the depolymerization of lignin (CUNHA *et al.*, 2019; KUMAR *et al.*, 2020), and the lower the molecular mass of phenolic compounds, higher the toxicity (ZHANG *et al.*, 2019).

The inhibitor content in different LCHs is highly variable according to the type of feedstock and in each newly produced LCH, and as a result of the choice of pretreatment and the selected operational conditions (CUNHA *et al.*, 2019; PALMQVIST; HAHN-HÄGERDAL, 2000). Consequently, it is important to consider a specific biomass source and processing method when attempting to understand the tolerance of *S. cerevisiae* to LCH inhibitors.

1.3.2 Inhibitors Toxicity

Inhibitors in LCH liquors usually have synergistic effects that are detrimental to yeast fermentation, causing prolonged lag phases, slower growth rates, reduced biomass formation, and ethanol yield (CUNHA *et al.*, 2019; PALMQVIST; HAHN-HÄGERDAL, 2000). The underlying inhibition mechanisms differ and depend on the chemical structure of the inhibitor. For example, the harmfulness of acetic, formic, and levulinic acids depends on the extracellular and intracellular pH (worsens in external pH below 4.8), membrane permeability, and toxicity of their anionic forms (CASAL; CARDOSO; LEO, 1996; MIRA; BECKER; SÁ-CORREIA, 2010; PALMQVIST; HAHN-HÄGERDAL, 2000; VAN MARIS *et al.*, 2004). Acetic acid can diffuse undissociated across cell membranes and scatter inside cells, releasing

protons and lowering the cell's pH level (KŁOSOWSKI; MIKULSKI, 2021; PROCÓPIO *et al.*, 2023).

HMF and furfural induce changes in chromatin, DNA damage, and reduced translation in addition to acting in the inactivation of several glycolytic enzymes (ALLEN *et al.*, 2010; ASK *et al.*, 2013). Exposure of *S. cerevisiae* to these chemicals suggests that yeast growth is more sensitive to furfural than to HMF or high ethanol titers (TAHERZADEH *et al.*, 1999) because HMF has lower permeability and the conversion is less efficient than furfural (LARSSON *et al.*, 1999a). In addition, the accumulation of reactive oxygen species induced by furfural can damage the mitochondria and vacuoles of *S. cerevisiae* (ALLEN *et al.*, 2010). Both compartments regulate the redox balance of the cytosol, and the loss of their function can reduce glucose consumption rates (CASPETA; CASTILLO; NIELSEN, 2015). When mixtures of acetic acid and furfural are present during fermentation, the specific growth rate decreases by more than the sum of the individual effects (PALMQVIST *et al.*, 1999). Thus, many inhibitors commonly found in hydrolysates act synergistically (LARSSON *et al.*, 1999b). Phenols and furans exhibited potent inhibitory effects at 1 g/L. Organic acids had insignificant effects at concentrations of up to 2 g/L (CASPETA; CASTILLO; NIELSEN, 2015).

On the other hand, the morphological analysis showed that phenolic acids caused the wild-type strain (WT) to generate many cytoplasmic membrane invaginations with a crack at the top of these sites and some mitochondria gathered around (GU *et al.*, 2019), as well as causing membrane swelling (ADEBOYE; BETTIGA; OLSSON, 2014). The inhibition mechanism of phenolic acid might be attributed to the combined effect of the change in membrane permeability (CAMPOS *et al.*, 2009), disaggregation of the cytoplasmic membrane (GU; ZHANG; BAO, 2015) caused by its hydrophobic aromatic ring, and further intracellular acidification, induced by dissociation of its carboxylic acid group (GU *et al.*, 2019). In addition to DNA mutagenesis, translation inhibition, ATP depletion, protein denaturation, oxidative stress, and triggering changes in the protein-to-lipid ratio have been reported (FLETCHER; BAETZ, 2020; KEWELOH; WEYRAUCH; REHM, 1990; PIOTROWSKI *et al.*, 2014). Hence, these compounds affect cellular functions such as sorting and signaling (ADEBOYE; BETTIGA; OLSSON, 2014).

1.3.3 Cell Tolerance Physiology

To stabilize the yeast cell periphery and reduce its permeability to inhibitors, cellular responses to weak acids (and phenolics) entail the modification of the cell wall structure and membrane lipid composition (PALMA; GUERREIRO; SÁ-CORREIA, 2018). According to Mira, Becker, and Sá-Correia (2010), the transcription factor Haa1p, a master regulator of around 80% of genes responsive to acetic acid, mediates reactions in the case of weak acids. Another mechanism is that excessive accumulation of protons is pumped out of cells through proton translocation with plasma membrane H⁺-ATPase (mediated by ATP hydrolysis). (HOLYOAK *et al.*, 1996; PALMA; GUERREIRO; SÁ-CORREIA, 2018).

Yeast can metabolize furfural and HMF to their less toxic alcohols via a battery of oxidoreductase enzymes (e.g., Adh6p, Adh7p, Gre2p, and Oye3p) using NAD(P)H as cofactor, a metabolic process that occurs at high rates (ASK *et al.*, 2013; JAYAKODY; JIN, 2021; MA; LIU, 2010). Functional proteomic and transcriptomic analyses showed that genes involved in redox balance, oxidative stress, and the TFs, *MSN2/MSN4*, and *YAP1*, were mostly involved in stress responses and were related to the increase in yeast tolerance against furfural and HMF (LIN; QIAO; YUAN, 2009; SASANO *et al.*, 2012). Furthermore, Stb5p is an essential transcription factor for initiating the pentose phosphate pathway (PPP), necessary for NAD(P)H regeneration (WU; XU; JÖNSSON, 2017), while Yap1p controls the responses to oxidative stress (KIM; HAHN, 2013). Pleiotropic drug resistance (PDR) efflux pumps detoxify furfurals through cellular extrusion (MA; LIU, 2010).

In agreement with the transcriptional analysis (DICKSON *et al.*, 1997), the proteomic analysis also showed that redox and energy metabolism are significantly affected by the stress response in yeast exposed to hydrolysates containing furans, aldehydes, acids, and/or phenolics (LIN; QIAO; YUAN, 2009; LV *et al.*, 2014).

In a study by Gu *et al.* (2019), the adapted strain to phenolic acids presented a thicker cell wall and membrane, and a smaller cell size than those of the wild-type strain, the authors observed differential expression of approximately 200 genes, a similar number with 103 and 227 differentially expressed genes observed from yeast exposed to furfural and acetate, respectively (GU *et al.*, 2019; LI; YUAN, 2010). Moreover, the essential processes that respond to phenolic compounds have been identified by functional genomics and genetic methods (FLETCHER; BAETZ, 2020).

These include Yap1p-mediated responses to oxidative stress and enhanced Pentose phosphate pathway (PPP) flux, which supports redox power for glutathione replenishment and phenol detoxification via oxidoreductase enzymes.

The activation of membrane transporters, such as Pdr5p, Yor1p, and Snq2p, promotes the efflux of phenolics at the expense of ATP, whereas ergosterol production is triggered to stabilize the plasma membrane. Mitochondrial respiration increases to compensate for this energy loss (FLETCHER; BAETZ, 2020). More resistant yeasts were created through genetic engineering techniques because of the knowledge about the endogenous mechanisms of tolerance to inhibitors and adaptive laboratory evolution data. One of these studies was based on an ALE methodology that addresses the issue of yeast evolution under continuous exposure to acetic acid (WRIGHT *et al.*, 2011), or to make this tolerance permanent, (GONZÁLEZ-RAMOS *et al.*, 2016). In this last study, following approximately 50 batch transfers and UV mutagenesis, clones obtained from five populations were able to grow on up to 15 g/L acetic acid without pre-induction. After five days, the ancestral strain, CEN.PK113-7D was no longer viable at concentrations higher than 10 g/L. Mutations in the *ASG1*, *ADH3*, *SKS1*, and *GIS4* genes linked to acetic acid tolerance were discovered after whole-genome sequencing of the six selected clones. Backcrossing these clones with the parental strain revealed that these mutations led to acquired tolerance.

Reverse engineering verified the individual contribution of the discovered alleles to acetic acid tolerance. In media containing 5-9 g/L acetic acid, the reverse-engineered strain had a significantly shorter latency phase than the wild-type CEN.PK113-7D (GONZÁLEZ-RAMOS *et al.*, 2016). Other genetic modifications involving tolerance to vanillin, coumaric and ferulic acid have also been developed (PEREIRA *et al.*, 2020; WANG *et al.*, 2017).

1.4 Adaptive Laboratory Evolution

1.4.1 Navigating the ALE Principles

Adaptive Laboratory Evolution (ALE) is an increasingly popular technique to improve microbial phenotypes and to investigate their genotypes. This terminology has not been used in the last century, making the identification of pioneer publications challenging. One of the first reports was published in 1998 when strains of *Saccharomyces* spp. evolved to tolerate softwood pre-hydrolysate (KELLER *et al.*,

1998). In short, the ALE protocols consist of prolonged cultivation of cells in an environment chosen (selective pressure) for a selection of those that acquire beneficial/adaptive mutations. Given how quickly advantageous mutations can arise and spread, the use of ALE experiments for specific components is well-designed. In addition, the increasing use of ALE has been driven by decreasing the cost of high-throughput DNA sequencing (SHENDURE *et al.*, 2017), which, in conjunction with appropriate bioinformatics tools, facilitates the critical step of mutation identification in the ALE process. Genome engineering is an essential complementary technology (LIU *et al.*, 2015) that introduces desired mutations into strains of interest for causal determination or phenotypic modification.

Because evolution relies on natural selection, to enrich advanced mutants, lineage optimization can be accomplished without knowing a priori what genetic changes are necessary to drive these changes. Once a desired growth environment has been carefully selected, an ALE experiment allows the generation of an entire lineage of mutant strains, which performs better than the ancestral strain (BARRICK; LENSKE, 2013).

Selective variants typically occur because of the unique experiences that happen in the population. However, cultures also have some genetic diversity in which competition within the population can lead to clonal interference (MADDAMSETTI; LENSKE; BARRICK, 2015). Asexual reproduction also leads to clonal interference, which can prevent beneficial mutations from being fixed in evolving populations (ROZEN; DE VISSER; GERRISH, 2002). On this basis, modifications in the ALE methods were investigated to accelerate adaptation, increase mutation rate, or provide fixation of beneficial mutations (GONZÁLEZ-RAMOS *et al.*, 2016). Another important feature to be analyzed is that under conditions of strong selection and improved population fitness, can occur hypermutability, which leads to a steady decline in the genome and can cause a 'genomic drift' in which deleterious mutations hitchhike and cannot be expunged from the population (COUCE *et al.*, 2017). Although hypermutability can increase the speed and magnitude of fitness events, it has drawbacks regarding genome stability and adaptive potential if the mutation rate is excessive (Sprouffske *et al.*, 2018).

After ALE, genetic characterization enables the detection of adaptive mutations. To help the understanding, independent replicates from the same ancestral lineage often evolve simultaneously to facilitate the identification of

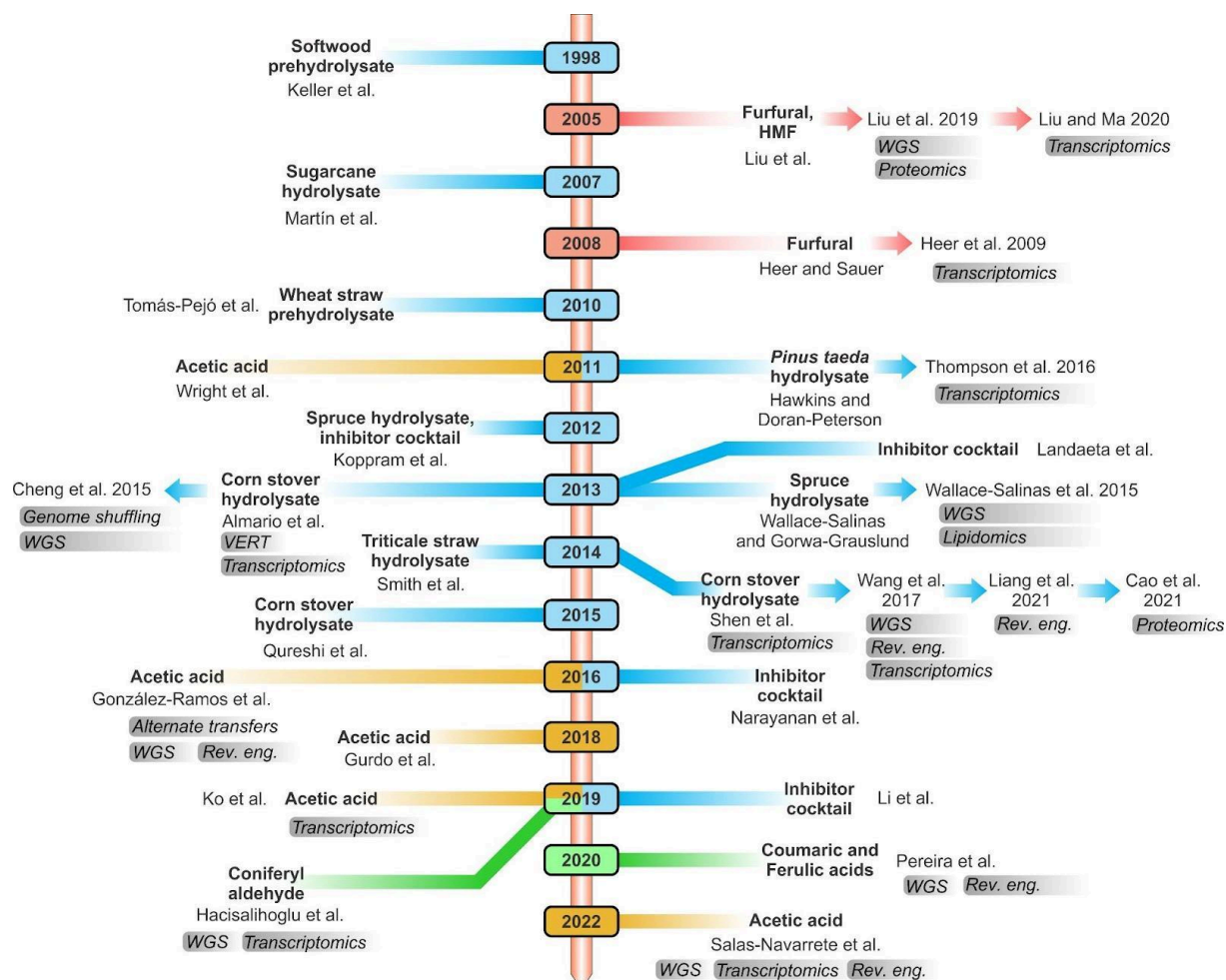
adaptive genes. Therefore, genes or genetic regions affected by independent replicates are most likely to be adaptive (BAILEY; RODRIGUE; KASSEN, 2015). Several studies have investigated the evolution of organisms in response to a particular stressor. In this way, ALE can be accompanied by genetic engineering to reconstruct metabolic pathways or to determine causation between mutations and phenotypic changes, as assessed by Whole Genome Sequencing (WGS) of evolutionary strains (SCHWENTNER *et al.*, 2018). For example, the effects of overexpression or knockout of identified genes can be analyzed, in which the phenotype of the mutated strain can be compared to the parental or evolved strain. ALE-enhanced phenotypes have become a powerful engineering tool, and studying the genetic and metabolic mechanisms underlying these phenotypic changes can provide valuable biological insights. In addition, modifications to the selected protocol and evolutionary environment can be readily exploited using ALE, allowing the investigation of methods to facilitate adaptation (SANDBERG *et al.*, 2019).

1.4.2 Engineering Tolerance via ALE

When cells are exposed to stress, they respond by activating stress responses that require redistribution of cellular resources to function, thus inhibiting growth potential. Cellular stress can be caused by a complex growth environment that induces metabolic dysfunction or exposure to specific inhibitory chemicals. Lignocellulosic hydrolysate introduces a diverse range of challenges that are overcome by stress response mechanisms, and ALE is an effective tool for overcoming these challenges (MENEGON; GROSS; JACOBUS, 2022), being focused on the hydrolysate as a whole (ALMARIO; REYES; KAO, 2013; HAWKINS; DORAN-PETERSON, 2011; KELLER *et al.*, 1998; KOPPRAM; ALBERS; OLSSON, 2012; LANDAETA *et al.*, 2013; LI *et al.*, 2019; MARTÍN *et al.*, 2007; NARAYANAN *et al.*, 2016; QURESHI; ZHANG; BAO, 2015; SHEN *et al.*, 2014; SMITH; VAN RENSBURG; GÖRGENS, 2014; TOMÁS-PEJÓ *et al.*, 2010; WALLACE-SALINAS; GORWA-GRAUSLUND, 2013) or specific inhibitors like furans (HEER; SAUER, 2008; LIU; SLININGER; GORSICH, 2005), acetic acid (GONZÁLEZ-RAMOS *et al.*, 2016; GURDO *et al.*, 2018; KO *et al.*, 2020; SALAS-NAVARRETE *et al.*, 2022; WRIGHT *et al.*, 2011) and phenols (HACISALIHOĞLU *et al.*, 2019; PEREIRA *et al.*, 2020).

WT cells are rarely well-equipped to handle the stresses imposed by growth environments with high concentrations of atypical components. Rational engineering approaches may fail because of a lack of knowledge, and attempts to rationally design strains for a given phenotype often result in fitness defects (SANDBERG *et al.*, 2019). Although specific tolerance mechanisms can be rationally modified in a strain, determining the required level of gene expression may be challenging. In contrast, ALE can identify the mechanisms underlying fitness defects and the gene expression levels required for a functional and robust phenotype, making it possible to use the results from ALE experiments to engineer strains. Importantly, when strain stress is reduced via ALE, it usually leads to an increase in growth rate and phenotypic improvements in industrially relevant traits such as production or degradation performance. A timeline with ALE studies using yeast and cellulosic hydrolysate components can be seen in Figure 1.1.

Figure 1.1- Timeline of ALE experiments with LCHs or individual inhibitors.



Evolution experiments with hydrolysate liquors or inhibitor cocktails are represented in blue. Evolution experiments with individual inhibitors are colored orange (acetic acid), green (phenols), and red (furan aldehydes). Arrows indicate follow-up studies with the evolved strains. Genomics, reverse engineering (Rev. eng.), and ALE approaches associated with the studies are marked in grey (MENEGON; GROSS; JACOBUS, 2022).

The use of ALE to improve hydrolysate tolerance is a potential application in metabolic engineering and industrial biotechnology. However, the industrial application of ALE techniques remains relatively limited (LEE; KIM, 2020).

1.5 Genomic Insights into Lignocellulosic Hydrolysate Resistance

1.5.1 Genome-Wide Discoveries

Ethanol concentration during industrial bioethanol production is one of the most stressful effects for yeast cells. Pooled-segregant WGS analysis revealed that *MKT1*, *SWS2*, and *APJ1* genes are associated with ethanol tolerance in *S. cerevisiae* (SWINNEN *et al.*, 2012). Another study reported the successful improvement of ethanol tolerance (up to 14%) and yield from sugarcane molasses in an industrial ethanol strain of *S. cerevisiae*, CAT-1. This result was obtained by overexpressing a truncated form of the *MSN2* gene (VARIZE *et al.*, 2022). In another study, ethanol-evolved clones demonstrated an increase in Msn2/4-mediated stress responses (JACOBUS *et al.*, 2024).

Transcriptome alterations linked to the transcription factors *Yap1*, *Met4*, *Msn2/4*, and *Pdr1/3* were found to be responsible for resistance to furfural and HMF (LIU; MA, 2020). Certain mutations affecting the *PDR1*, *GLN3*, and *CRZ1* genes cause resistance to coniferyl aldehyde (HACISALIHOĞLU *et al.*, 2019).

Under heat stress (39 °C), mutations in the *MTL1*, *FLO9/FLO11*, and *CYC3* genes were used to impart tolerance to furfural and HMF (WALLACE-SALINAS *et al.*, 2015; WALLACE-SALINAS; GORWA-GRAUSLUND, 2013).

1.5.2 Genes Validated via Genetic Engineering

Tolerance to furfural can also be increased by overexpression of *ADH7*, *YKL071W*, and *ARI1*, which encode reductases involved in furfural reduction (HEER; HEINE; SAUER, 2009; SEHNEM *et al.*, 2013). Furfural tolerance in *S. cerevisiae* is

also associated with the PPP genes *ZWF1*, *GND1*, *RPE1*, and *TKL1*, and by overexpressing the *ZWF1* gene, according to another study on the subject (GORSICH *et al.*, 2006).

With other inhibitors, improved vanillin tolerance in *S. cerevisiae* mutants has been correlated with enhanced vanillin-reducing activity (SHEN *et al.*, 2014). Overexpression of *ADH6* increases not only furfural and HMF tolerance, but also vanillin tolerance (WANG *et al.*, 2016), whereas *AST2* increased both furfural and HMF tolerance, but had minimal effect on vanillin tolerance (VANMARCKE *et al.*, 2021).

The *ADY2* gene, which codes for an acetate transporter, was removed to produce acetic acid-resistant *S. cerevisiae*. This led to a 14.7% increase in ethanol output when 3.6 g/L of acetic acid was present (ZHANG *et al.*, 2017). Gene mutations affecting *RAS2* and *HSF1* were implemented to provide resistance to 12 g/L acetate at pH 4 and 30 °C (SALAS-NAVARRETE *et al.*, 2022).

Considering other important factors present in ethanol production with lignocellulosic hydrolysate, in an environment with 8% ethanol, the specific growth rate increased significantly from 0.029 h⁻¹ to 0.32 h⁻¹ as a result of the introduction of mutations in the *SSD1* and *UTH1* genes (AVRAHAMI-MOYAL *et al.*, 2012). In addition, strains with the modified *ras2::MX*, *mds3::MX*, and *cyr1*^{A1474T} alleles exhibited greater fitness than the parental strain to tolerate ethanol shock (JACOBUS *et al.*, 2024).

1.6 Objectives

1.6.1 General Objective

Use ALE to develop and improve *Saccharomyces cerevisiae* CEN.PK113-7D and PE2_H4 tolerance to inhibitors from sugarcane lignocellulosic hydrolysate, aiming to optimize ethanol production.

1.6.2 Specific Objectives

- Utilizing Serial Transfer and Shock protocols, carry out Adaptive Laboratory Evolution (ALE) in *S. cerevisiae* CEN.PK113-7D and PE2_H4 strains to generate mutations that lead to a phenotype that tolerates the toxicity of the inhibitors present in the lignocellulosic hydrolysate;

- Identifying genetic variations and relevant adaptations that contribute to tolerance to lignocellulosic hydrolysate, through genomic sequencing of populations and clones obtained by ALE;
- Conduct reverse engineering to validate candidate genes using innovative CRISPR/Cas9 approaches and be able to understand their role in hydrolysate stress responses;
- Implement competition assays between evolved/genetically modified strains and parental strains, using flow cytometry, to estimate the change in fitness resulting from evolution or punctual mutations;
- Promote crosses between triplicates of diploid PE2_H4 populations, consolidating advantageous mutations and selecting a colony (PE2_H4_GN1) with higher tolerance and productivity.
- Investigate the fermentative processes in bioreactors, subjecting the evolved strains, modified strains, and the PE2_H4_GN1 strain to a cocktail with the 6 relatively most harmful inhibitors present in the hydrolysate;
- Analyze the metabolomics and proteomics of the PE2_H4_GN1 to elucidate the molecular mechanisms underlying the tolerance to lignocellulosic hydrolysate;

References

ADEBOYE, P. T.; BETTIGA, M.; OLSSON, L. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates. **AMB Express**, v. 4, p. 46, 2014.

ALAZAIZA, M. Y. D.; ALBAHNASAWI, A.; AHMAD, Z.; BASHIR, M. J. K.; AL-WAHAIBI, T.; ABUJAZAR, M. S. S.; ABU AMR, S. S.; NASSANI, D. E. Potential use of algae for the bioremediation of different types of wastewater and contaminants: Production of bioproducts and biofuel for green circular economy. **Journal of environmental management**, v. 324, p. 116415, 2022.

ALLEN, S. A.; CLARK, W.; MCCAFFERY, J. M.; CAI, Z.; LANCTOT, A.; SLININGER, P. J.; LIU, Z. L.; GORSICH, S. W. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. **Biotechnology for biofuels**, v. 3, p. 2, 2010.

ALMARIO, M. P.; REYES, L. H.; KAO, K. C. Evolutionary engineering of *Saccharomyces cerevisiae* for enhanced tolerance to hydrolysates of lignocellulosic biomass. **Biotechnology and bioengineering**, v. 110, n. 10, p. 2616–2623, 2013.

ALMEIDA, J. R. M.; RÖDER, A.; MODIG, T.; LAADAN, B.; LIDÉN, G.; GORWA-GRAUSLUND, M.-F. NADH- vs NADPH-coupled reduction of 5-hydroxymethyl furfural (HMF) and its implications on product distribution in *Saccharomyces cerevisiae*. **Applied microbiology and biotechnology**, v. 78, n. 6, p. 939–945, 2008.

AMIGUN, B.; MUSANGO, J. K.; STAFFORD, W. Biofuels and sustainability in Africa. **Renewable & Sustainable Energy Rev.**, v. 15, n. 2, p. 1360–1372, 2011.

ASK, M.; BETTIGA, M.; DURAISWAMY, V. R.; OLSSON, L. Pulsed addition of HMF and furfural to batch-grown xylose-utilizing *Saccharomyces cerevisiae* results in different physiological responses in glucose and xylose consumption phase. **Biotechnology for biofuels**, v. 6, n. 1, p. 181, 2013.

AVRAHAM-MOYAL, L.; ENGELBERG, D.; WENGER, J. W.; SHERLOCK, G.; BRAUN, S. Turbidostat culture of *Saccharomyces cerevisiae* W303-1A under selective pressure elicited by ethanol selects for mutations in SSD1 and UTH1. **FEMS yeast research**, v. 12, n. 5, p. 521–533, 2012.

BACOVSKY, D. **How close are second-generation biofuels?** [S. l.: s. n.] Disponível em: <https://doi.org/10.1002/bbb.222>

BAILEY, S. F.; RODRIGUE, N.; KASSEN, R. The effect of selection environment on the probability of parallel evolution. **Molecular biology and evolution**, v. 32, n. 6, p. 1436–1448, 2015.

BARRICK, J. E.; LENSKE, R. E. Genome dynamics during experimental evolution. **Nature reviews. Genetics**, v. 14, n. 12, p. 827–839, 2013.

BECERRA-RUIZ, J. D.; GONZALEZ-HUERTA, R. G.; GRACIDA, J.; AMARO-REYES, A.; MACIAS-BOBADILLA, G. Using green-hydrogen and bioethanol fuels in internal combustion engines to reduce emissions. **International journal of hydrogen energy**, v. 44, n. 24, p. 12324–12332, 2019.

BIAZI, L. E.; MARTÍNEZ-JIMENEZ, F. D.; BONAN, C. I. D. G.; SOARES, L. B.; MORAIS, E. R.; IENCZAK, J. L.; COSTA, A. C. A differential evolution approach to estimate parameters in a temperature-dependent kinetic model for second generation ethanol production under high cell density with *Spathaspora passalidarum*. **Biochemical engineering journal**, v. 161, n. 107586, p. 107586, 2020.

BONAN, C. I. D. G.; TRAMONTINA, R.; DOS SANTOS, M. W.; BIAZI, L. E.; SOARES, L. B.; PEREIRA, I. O.; HOFFMANN, Z. B.; COUTOUNÉ, N.; SQUINA, F. M.; ROBL, D.; IENCZAK, J. L. Biorefinery platform for *Spathaspora passalidarum* NRRL Y-27907 in the production of ethanol, xylitol, and single cell protein from sugarcane bagasse. **Bioenergy research**, v. 15, n. 2, p. 1169–1181, 2022.

BREXÓ, R. P.; SANT'ANA, A. S. Impact and significance of microbial contamination during fermentation for bioethanol production. **Renewable & Sustainable Energy Rev.**, v. 73, p. 423–434, 2017.

CADETE, R. M.; DE LAS HERAS, A. M.; SANDSTRÖM, A. G.; FERREIRA, C.; GÍRIO, F.; GORWA-GRAUSLUND, M.-F.; ROSA, C. A.; FONSECA, C. Exploring xylose metabolism in *Spathaspora* species: XYL1.2 from *Spathaspora passalidarum* as the key for efficient anaerobic xylose fermentation in metabolic engineered *Saccharomyces cerevisiae*. **Biotechnology for biofuels**, v. 9, p. 167, 2016.

CAMPOS, F. M.; COUTO, J. A.; FIGUEIREDO, A. R.; TÓTH, I. V.; RANGEL, A. O. S. S.; HOGG, T. A. Cell membrane damage induced by phenolic acids on wine lactic acid bacteria. **International journal of food microbiology**, v. 135, n. 2, p. 144–151, 2009.

CARDONA, C. A.; QUINTERO, J. A.; PAZ, I. C. Production of bioethanol from sugarcane bagasse: Status and perspectives. **Bioresource technology**, v. 101, n. 13, p. 4754–4766, 2010.

CASAL, M.; CARDOSO, H.; LEAO, C. Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae*. **Microbiology**, v. 142 (Pt 6), p. 1385–1390, 1996.

CASPETA, L.; BUIJS, N. A. A.; NIELSEN, J. **The role of biofuels in the future energy supply**. [S. l.: s. n.] Disponível em: <https://doi.org/10.1039/c3ee24403b>

CASPETA, L.; CARO-BERMÚDEZ, M. A.; PONCE-NOYOLA, T.; MARTINEZ, A. **Enzymatic hydrolysis at high-solids loadings for the conversion of agave bagasse to fuel ethanol**. [S. l.: s. n.] Disponível em: <https://doi.org/10.1016/j.apenergy.2013.07.036>

CASPETA, L.; CASTILLO, T.; NIELSEN, J. Modifying Yeast Tolerance to Inhibitory Conditions of Ethanol Production Processes. **Frontiers in bioengineering and biotechnology**, v. 3, p. 184, 2015.

CHANDEL, A. K.; GARLAPATI, V. K.; JEEVAN KUMAR, S. P.; HANS, M.; SINGH, A. K.; KUMAR, S. The role of renewable chemicals and biofuels in building a bioeconomy. **Biofuels, Bioproducts & Biorefining**, v. 14, n. 4, p. 830–844, 2020.

CORREA, D. F.; BEYER, H. L.; FARGIONE, J. E.; HILL, J. D.; POSSINGHAM, H. P.; THOMAS-HALL, S. R.; SCHENK, P. M. Towards the implementation of sustainable biofuel production systems. **Renewable & Sustainable Energy Rev.**, v. 107, p. 250–263, 2019.

COSTA, A. K. B.; TEODORO, J. C.; FILHO, R. M. **Fermentação de hidrolisado lignocelulósico por *Spathaspora passalidarum* UFMG-HMD 14.1**. [S. l.: s. n.] Disponível em: <https://doi.org/10.17648/sinaferm-2015-33396>

COUCE, A.; CAUDWELL, L. V.; FEINAUER, C.; HINDRÉ, T.; FEUGEAS, J.-P.; WEIGT, M.; LENSKE, R. E.; SCHNEIDER, D.; TENAILLON, O. Mutator genomes decay, despite sustained fitness gains, in a long-term experiment with bacteria. **Proceedings of the National Academy of Sciences of the United States of America**, v. 114, n. 43, p. E9026–E9035, 2017.

CUNHA, J. T.; ROMANÍ, A.; COSTA, C. E.; SÁ-CORREIA, I.; DOMINGUES, L. Molecular and physiological basis of *Saccharomyces cerevisiae* tolerance to adverse lignocellulose-based process conditions. **Applied microbiology and biotechnology**, v. 103, n. 1, p. 159–175, 2019.

DIAS, M. O. de S.; MACIEL FILHO, R.; MANTELATTO, P. E.; CAVALETTI, O.; ROSSELL, C. E. V.; BONOMI, A.; LEAL, M. R. L. V. Sugarcane processing for ethanol and sugar in Brazil. **Environmental development**, v. 15, p. 35–51, 2015.

DICKSON, R. C.; NAGIEC, E. E.; SKRZYPEK, M.; TILLMAN, P.; WELLS, G. B.; LESTER, R. L. **Sphingolipids Are Potential Heat Stress Signals in *Saccharomyces***. [S. l.: s. n.] Disponível em: <https://doi.org/10.1074/jbc.272.48.30196>

FLETCHER, E.; BAETZ, K. Multi-Faceted Systems Biology Approaches Present a Cellular Landscape of Phenolic Compound Inhibition in. **Frontiers in bioengineering and biotechnology**, v. 8, p. 539902, 2020.

GOLDEMBERG, J. The Brazilian biofuels industry. **Biotechnology for biofuels**, v. 1, n. 1, p. 6, 2008.

GONZÁLEZ-RAMOS, D.; GORTER DE VRIES, A. R.; GRIJSEELS, S. S.; VAN BERKUM, M. C.; SWINNEN, S.; VAN DEN BROEK, M.; NEVOIGT, E.; DARAN, J.-M. G.; PRONK, J. T.; VAN MARIS, A. J. A. A new laboratory evolution approach to select for constitutive acetic acid tolerance in *Saccharomyces cerevisiae* and identification of causal mutations. **Biotechnology for biofuels**, v. 9, p. 173, 2016.

GORSICH, S. W.; DIEN, B. S.; NICHOLS, N. N.; SLININGER, P. J.; LIU, Z. L.; SKORY, C. D. Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes ZWF1, GND1, RPE1, and TKL1 in *Saccharomyces cerevisiae*. **Applied microbiology and biotechnology**, v. 71, n. 3, p. 339–349, 2006.

GRUBB, M.; VROLIJK, C.; BRACK, D.; ENERGY AND ENVIRONMENTAL PROGRAMME (ROYAL INSTITUTE OF INTERNATIONAL AFFAIRS). **The Kyoto Protocol: A Guide and Assessment**. [S. l.]: Earthscan, 1999.

GU, H.; ZHANG, J.; BAO, J. High tolerance and physiological mechanism of *Zymomonas mobilis* to phenolic inhibitors in ethanol fermentation of corncob residue. **Biotechnology and bioengineering**, v. 112, n. 9, p. 1770–1782, 2015.

GU, H.; ZHU, Y.; PENG, Y.; LIANG, X.; LIU, X.; SHAO, L.; XU, Y.; XU, Z.; LIU, R.; LI, J. **Physiological mechanism of improved tolerance of *Saccharomyces cerevisiae* to lignin-derived phenolic acids in lignocellulosic ethanol fermentation by short-term adaptation**. [S. l.: s. n.] Disponível em: <https://doi.org/10.1186/s13068-019-1610-9>

GURDO, N.; NOVELLI POISSON, G. F.; JUÁREZ, Á. B.; RIOS DE MOLINA, M. C.; GALVAGNO, M. A. Improved robustness of an ethanologenic yeast strain through adaptive evolution in acetic acid is associated with its enzymatic antioxidant ability. **Journal of applied microbiology**, v. 125, n. 3, p. 766–776, 2018.

HACISALIHOĞLU, B.; HOLYAVKIN, C.; TOPALOĞLU, A.; KISAKESEN, H. İ.;

ÇAKAR, Z. P. Genomic and transcriptomic analysis of a coniferyl aldehyde-resistant *Saccharomyces cerevisiae* strain obtained by evolutionary engineering. **FEMS yeast research**, v. 19, n. 3, 2019. Disponível em: <https://doi.org/10.1093/femsyr/foz021>

HASAN, M.; ABEDIN, M. Z.; AMIN, M. B.; NEKMAHMUD, M.; OLÁH, J. Sustainable biofuel economy: A mapping through bibliometric research. **Journal of environmental management**, v. 336, p. 117644, 2023.

HAWKINS, G. M.; DORAN-PETERSON, J. A strain of *Saccharomyces cerevisiae* evolved for fermentation of lignocellulosic biomass displays improved growth and fermentative ability in high solids concentrations and in the presence of inhibitory compounds. **Biotechnology for biofuels**, v. 4, n. 1, p. 49, 2011.

HEER, D.; HEINE, D.; SAUER, U. Resistance of *Saccharomyces cerevisiae* to high concentrations of furfural is based on NADPH-dependent reduction by at least two oxireductases. **Applied and environmental microbiology**, v. 75, n. 24, p. 7631–7638, 2009.

HEER, D.; SAUER, U. Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain. **Microbial biotechnology**, v. 1, n. 6, p. 497–506, 2008.

HIMMEL, M. E.; DING, S.-Y.; JOHNSON, D. K.; ADNEY, W. S.; NIMLOS, M. R.; BRADY, J. W.; FOUST, T. D. Biomass recalcitrance: engineering plants and enzymes for biofuels production. **Science**, v. 315, n. 5813, p. 804–807, 2007.

HOLYOAK, C. D.; STRATFORD, M.; MCMULLIN, Z.; COLE, M. B.; CRIMMINS, K.; BROWN, A. J.; COOTE, P. J. Activity of the plasma membrane H(+)-ATPase and optimal glycolytic flux are required for rapid adaptation and growth of *Saccharomyces cerevisiae* in the presence of the weak-acid preservative sorbic acid. **Applied and environmental microbiology**, v. 62, n. 9, p. 3158–3164, 1996.

JACOBUS, A. P.; CAVASSANA, S. D.; OLIVEIRA, I. I.; BARRETO, J. A.; ROHWEDDER, E.; FRAZZON, J.; BASSO, T. P.; BASSO, L. C.; GROSS, J. **Optimal trade-off between boosted tolerance and growth fitness during adaptive evolution of yeast to ethanol shocks**. 2024. Disponível em: <https://doi.org/10.1101/2024.01.03.574056>

JANSEN, M. L. A.; BRACHER, J. M.; PAPAPETRIDIS, I.; VERHOEVEN, M. D.; DE BRUIJN, H.; DE WAAL, P. P.; VAN MARIS, A. J. A.; KLAASSEN, P.; PRONK, J. T. *Saccharomyces cerevisiae* strains for second-generation ethanol production: from academic exploration to industrial implementation. **FEMS yeast research**, v. 17, n. 5, 2017. Disponível em: <https://doi.org/10.1093/femsyr/fox044>

JAYAKODY, L. N.; JIN, Y.-S. In-depth understanding of molecular mechanisms of aldehyde toxicity to engineer robust *Saccharomyces cerevisiae*. **Applied microbiology and biotechnology**, v. 105, n. 7, p. 2675–2692, 2021.

JESWANI, H. K.; CHILVERS, A.; AZAPAGIC, A. Environmental sustainability of biofuels: a review. **Proceedings. Mathematical, physical, and engineering sciences / the Royal Society**, v. 476, n. 2243, p. 20200351, 2020.

JÖNSSON, L. J.; MARTÍN, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. **Bioresource technology**, v. 199, p. 103–112, 2016.

KARAGOZ, P.; BILL, R. M.; OZKAN, M. Lignocellulosic ethanol production: Evaluation of new approaches, cell immobilization and reactor configurations. **Renewable Energy**, v. 143, p. 741–752, 2019.

KARTHICKEYAN, V.; BALAMURUGAN, P.; SENTHIL, R. Comparative studies on emission reduction in thermal barrier coated engine using single blend ratio of various non-edible oils. **Journal of the Brazilian Society of Mechanical Sciences and Engineering**, v. 39, n. 5, p. 1823–1833, 2017.

KELLER, F. A.; BATES, D.; RUIZ, R.; NGUYEN, Q. Yeast adaptation on softwood prehydrolysate. **Applied biochemistry and biotechnology**, v. 70-72, p. 137–148, 1998.

KEWELOH, H.; WEYRAUCH, G.; REHM, H. J. Phenol-induced membrane changes in free and immobilized *Escherichia coli*. **Applied microbiology and biotechnology**, v. 33, n. 1, p. 66–71, 1990.

KIM, D.; HAHN, J.-S. Roles of the Yap1 transcription factor and antioxidants in *Saccharomyces cerevisiae*'s tolerance to furfural and 5-hydroxymethylfurfural, which function as thiol-reactive electrophiles generating oxidative stress. **Applied and environmental microbiology**, v. 79, n. 16, p. 5069–5077, 2013.

KŁOSOWSKI, G.; MIKULSKI, D. Impact of Lignocellulose Pretreatment By-Products on Strain Ethanol Red Metabolism during Aerobic and An-aerobic Growth. **Molecules**, v. 26, n. 4, 2021. Disponível em: <https://doi.org/10.3390/molecules26040806>

KO, J. K.; ENKH-AMGALAN, T.; GONG, G.; UM, Y.; LEE, S. **Improved bioconversion of lignocellulosic biomass by *Saccharomyces cerevisiae* engineered for tolerance to acetic acid**. [S. l.: s. n.] Disponível em: <https://doi.org/10.1111/gcbb.12656>

KOPPRAM, R.; ALBERS, E.; OLSSON, L. Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass. **Biotechnology for biofuels**, v. 5, n. 1, p. 32, 2012.

KUMAR, P.; BARRETT, D. M.; DELWICHE, M. J.; STROEVE, P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. **Industrial & engineering chemistry research**, v. 48, n. 8, p. 3713–3729, 2009.

KUMAR, V.; YADAV, S. K.; KUMAR, J.; AHLUWALIA, V. A critical review on current strategies and trends employed for removal of inhibitors and toxic materials generated during biomass pretreatment. **Bioresource technology**, v. 299, p. 122633, 2020.

LANDAETA, R.; AROCA, G.; ACEVEDO, F.; TEIXEIRA, J. A.; MUSSATTO, S. I. Adaptation of a flocculent *Saccharomyces cerevisiae* strain to lignocellulosic inhibitors by cell recycle batch fermentation. **Applied energy**, v. 102, p. 124–130, 2013.

LARSSON, S.; PALMQVIST, E.; HAHN-HÄGERDAL, B.; TENGBORG, C.; STENBERG, K.; ZACCHI, G.; NILVEBRANT, N.-O. **The generation of fermentation inhibitors during dilute acid hydrolysis of softwood**. [S. l.: s. n.] Disponível em: [https://doi.org/10.1016/s0141-0229\(98\)00101-x](https://doi.org/10.1016/s0141-0229(98)00101-x)

LARSSON, S.; REIMANN, A.; NILVEBRANT, N.-O.; JÖNSSON, L. J. **Comparison of Different Methods for the Detoxification of Lignocellulose Hydrolyzates of Spruce**. [S. l.: s. n.] Disponível em: https://doi.org/10.1007/978-1-4612-1604-9_9

LEE, S.; KIM, P. Current Status and Applications of Adaptive Laboratory Evolution in Industrial Microorganisms. **Journal of microbiology and biotechnology**, v. 30, n. 6, p. 793–803, 2020.

LEWKOWSKI, J. **Synthesis, Chemistry and Applications of 5-Hydroxymethyl-furfural and Its Derivatives**. [S. l.: s. n.] Disponível em: <https://doi.org/10.1002/chin.200302269>

LI, B.-Z.; YUAN, Y.-J. Transcriptome shifts in response to furfural and acetic acid in *Saccharomyces cerevisiae*. **Applied microbiology and biotechnology**, v. 86, n. 6, p. 1915–1924, 2010.

LI, H.; CHEN, X.; REN, J.; DENG, H.; PENG, F.; SUN, R. Functional relationship of furfural yields and the hemicellulose-derived sugars in the hydrolysates from corncob by microwave-assisted hydrothermal pretreatment. **Biotechnology for biofuels**, v. 8, p. 127, 2015 a.

LIN, F.-M.; QIAO, B.; YUAN, Y.-J. **Comparative Proteomic Analysis of Tolerance and Adaptation of Ethanologenic *Saccharomyces cerevisiae* to Furfural, a Lignocellulosic Inhibitory Compound.** [S. l.: s. n.] Disponível em: <https://doi.org/10.1128/aem.02594-08>

LIU, R.; BASSALO, M. C.; ZEITOUN, R. I.; GILL, R. T. Genome scale engineering techniques for metabolic engineering. **Metabolic engineering**, v. 32, p. 143–154, 2015.

LIU, Z. L.; HUANG, X.; ZHOU, Q.; XU, J. Protein expression analysis revealed a fine-tuned mechanism of in situ detoxification pathway for the tolerant industrial yeast *Saccharomyces cerevisiae*. **Applied microbiology and biotechnology**, v. 103, n. 14, p. 5781–5796, 2019.

LIU, Z. L.; MA, M. Pathway-based signature transcriptional profiles as tolerance phenotypes for the adapted industrial yeast *Saccharomyces cerevisiae* resistant to furfural and HMF. **Applied microbiology and biotechnology**, v. 104, n. 8, p. 3473–3492, 2020.

LIU, Z. L.; SLININGER, P. J.; GORSICH, S. W. Enhanced biotransformation of furfural and hydroxymethylfurfural by newly developed ethanologenic yeast strains. **Applied biochemistry and biotechnology**, v. 121-124, p. 451–460, 2005.

LI, W.-C.; ZHU, J.-Q.; ZHAO, X.; QIN, L.; XU, T.; ZHOU, X.; LI, X.; LI, B.-Z.; YUAN, Y.-J. Improving co-fermentation of glucose and xylose by adaptive evolution of engineering xylose-fermenting *Saccharomyces cerevisiae* and different fermentation strategies. **Renewable Energy**, v. 139, p. 1176–1183, 2019.

LI, X. *et al.* Expanding xylose metabolism in yeast for plant cell wall conversion to biofuels. **eLife**, v. 4, 2015 b. Disponível em: <https://doi.org/10.7554/eLife.05896>

LUO, Y.; MILLER, S. A game theory analysis of market incentives for US switchgrass ethanol. **Ecological economics: the journal of the International Society for Ecological Economics**, v. 93, p. 42–56, 2013.

LV, Y.-J.; WANG, X.; MA, Q.; BAI, X.; LI, B.-Z.; ZHANG, W.; YUAN, Y.-J. **Proteomic analysis reveals complex metabolic regulation in *Saccharomyces cerevisiae* cells against multiple inhibitors stress.** [S. l.: s. n.] Disponível em: <https://doi.org/10.1007/s00253-014-5519-8>

MADDAMSETTI, R.; LENSKE, R. E.; BARRICK, J. E. Adaptation, Clonal Interference, and Frequency-Dependent Interactions in a Long-Term Evolution Experiment with *Escherichia coli*. **Genetics**, v. 200, n. 2, p. 619–631, 2015.

MA, M.; LIU, Z. L. Comparative transcriptome profiling analyses during the lag phase uncover YAP1, PDR1, PDR3, RPN4, and HSF1 as key regulatory genes in genomic adaptation to the lignocellulose derived inhibitor HMF for *Saccharomyces cerevisiae*. **BMC genomics**, v. 11, p. 660, 2010.

MARTÍN, C.; MARCET, M.; ALMAZÁN, O.; JÖNSSON, L. J. Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. **Bioresource technology**, v. 98, n. 9, p. 1767–1773, 2007.

MARTINELLI, F. S.; BIBER-FREUDENBERGER, L.; STEIN, G.; BÖRNER, J. Will Brazil's push for low-carbon biofuels contribute to achieving the SDGs? A systematic expert-based assessment. **Cleaner Environmental Systems**, v. 5, n. 100075, p. 100075, 2022.

MCINTOSH, S.; ZHANG, Z.; PALMER, J.; WONG, H.-H.; DOHERTY, W. O. S.; VANCOV, T. Pilot-scale cellulosic ethanol production using eucalyptus biomass pre-treated by dilute acid and steam explosion. **Biofuels, Bioproducts & Biorefining**, v. 10, n. 4, p. 346–358, 2016.

MENEGON, Y. A.; GROSS, J.; JACOBUS, A. P. How adaptive laboratory evolution can boost yeast tolerance to lignocellulosic hydrolyses. **Current genetics**, v. 68, n. 3-4, p. 319–342, 2022.

MIRA, N. P.; BECKER, J. D.; SÁ-CORREIA, I. Genomic expression program involving the Haa1p-regulon in *Saccharomyces cerevisiae* response to acetic acid. **Omics: a journal of integrative biology**, v. 14, n. 5, p. 587–601, 2010.

MOHD AZHAR, S. H.; ABDULLA, R.; JAMBO, S. A.; MARBAWI, H.; GANSAU, J. A.; MOHD FAIK, A. A.; RODRIGUES, K. F. Yeasts in sustainable bioethanol production: A review. **Biochemistry and biophysics reports**, v. 10, p. 52–61, 2017.

MUSSATTO, S. I.; ROBERTO, I. C. Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. **Bioresource technology**, v. 93, n. 1, p. 1–10, 2004.

NAKANISHI, S. C.; NASCIMENTO, V. M.; RABELO, S. C.; SAMPAIO, I. L. M.; JUNQUEIRA, T. L.; ROCHA, G. J. M. Comparative material balances and preliminary technical analysis of the pilot scale sugarcane bagasse alkaline pretreatment to 2G ethanol production. **Industrial crops and products**, v. 120, p. 187–197, 2018.

NARAYANAN, V.; SÀNCHEZ I NOGUÉ, V.; VAN NIEL, E. W. J.; GORWA-GRAUSLUND, M. F. Adaptation to low pH and lignocellulosic inhibitors resulting in ethanolic fermentation and growth of *Saccharomyces cerevisiae*. **AMB**

Express, v. 6, n. 1, p. 59, 2016.

PALMA, M.; GUERREIRO, J. F.; SÁ-CORREIA, I. Adaptive Response and Tolerance to Acetic Acid in and : A Physiological Genomics Perspective. **Frontiers in microbiology**, v. 9, p. 274, 2018.

PALMQVIST, E.; GRAGE, H.; MEINANDER, N. Q.; HAHN-HÄGERDAL, B. Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. **Biotechnology and bioengineering**, v. 63, n. 1, p. 46–55, 1999.

PALMQVIST, E.; HAHN-HÄGERDAL, B. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. **Bioresource technology**, v. 74, n. 1, p. 25–33, 2000.

PARAWIRA, W.; TEKERE, M. Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. **Critical reviews in biotechnology**, v. 31, n. 1, p. 20–31, 2011.

PEREIRA, R.; MOHAMED, E. T.; RADI, M. S.; HERRGÅRD, M. J.; FEIST, A. M.; NIELSEN, J.; CHEN, Y. Elucidating aromatic acid tolerance at low pH in using adaptive laboratory evolution. **Proceedings of the National Academy of Sciences of the United States of America**, v. 117, n. 45, p. 27954–27961, 2020.

PERNA, M. dos S. C.; DOS SANTOS CORDEIRO PERNA, M.; BASTOS, R. G.; CECCATO-ANTONINI, S. R. **Single and combined effects of acetic acid, furfural, and sugars on the growth of the pentose-fermenting yeast *Meyerozyma guilliermondii***. [S. l.: s. n.] Disponível em: <https://doi.org/10.1007/s13205-018-1143-0>

PIOTROWSKI, J. S.; ZHANG, Y.; BATES, D. M.; KEATING, D. H.; SATO, T. K.; ONG, I. M.; LANDICK, R. Death by a thousand cuts: the challenges and diverse landscape of lignocellulosic hydrolysate inhibitors. **Frontiers in microbiology**, v. 5, p. 90, 2014.

PROCÓPIO, D. P. *et al.* Metabolic engineering of *Saccharomyces cerevisiae* for second-generation ethanol production from xylo-oligosaccharides and acetate. **Scientific reports**, v. 13, 2023. Disponível em: <https://doi.org/10.1038/s41598-023-46293-8>. Acesso em: 11 jan. 2024.

QURESHI, A. S.; ZHANG, J.; BAO, J. High ethanol fermentation performance of the dry dilute acid pretreated corn stover by an evolutionarily adapted *Saccharomyces cerevisiae* strain. **Bioresource technology**, v. 189, p. 399–404, 2015.

RATHMANN, R.; SZKLO, A.; SCHAEFFER, R. Land use competition for production of food and liquid biofuels: An analysis of the arguments in the current debate. **Renewable Energy**, v. 35, n. 1, p. 14–22, 2010.

RAY, R. C.; RAMACHANDRAN, S. **Bioethanol Production from Food Crops: Sustainable Sources, Interventions, and Challenges**. [S. l.]: Academic Press, 2018.

REIJNDERS, L.; HUIJBREGTS, M. **Biofuels for Road Transport: A Seed to Wheel Perspective**. [S. l.]: Springer Science & Business Media, 2008.

RENEWABLE FUELS ASSOCIATION. **Annual Ethanol Production**. [s. l.], 2024. Disponível em: <https://ethanolrfa.org/markets-and-statistics/annual-ethanol-production>. Acesso em: 13 mar. 2024.

RICH, J. O.; LEATHERS, T. D.; BISCHOFF, K. M.; ANDERSON, A. M.; NUNNALLY, M. S. Biofilm formation and ethanol inhibition by bacterial contaminants of biofuel fermentation. **Bioresource technology**, v. 196, p. 347–354, 2015.

ROBAK, K.; BALCEREK, M. Current state-of-the-art in ethanol production from lignocellulosic feedstocks. **Microbiological research**, v. 240, p. 126534, 2020.

ROQUE, L. R.; MORGADO, G. P.; NASCIMENTO, V. M.; IENCZAK, J. L.; RABELO, S. C. Liquid-liquid extraction: A promising alternative for inhibitors removing of pentoses fermentation. **Fuel**, v. 242, p. 775–787, 2019.

ROZEN, D. E.; DE VISSER, J. A. G. M.; GERRISH, P. J. Fitness effects of fixed beneficial mutations in microbial populations. **Current biology: CB**, v. 12, n. 12, p. 1040–1045, 2002.

SALAS-NAVARRETE, P. C.; DE OCA MIRANDA, A. I. M.; MARTÍNEZ, A.; CASPETA, L. Evolutionary and reverse engineering to increase *Saccharomyces cerevisiae* tolerance to acetic acid, acidic pH, and high temperature. **Applied microbiology and biotechnology**, v. 106, n. 1, p. 383–399, 2022.

SÀNCHEZ NOGUÉ, V.; KARHUMAA, K. Xylose fermentation as a challenge for commercialization of lignocellulosic fuels and chemicals. **Biotechnology letters**, v. 37, n. 4, p. 761–772, 2015.

SANDBERG, T. E.; SALAZAR, M. J.; WENG, L. L.; PALSSON, B. O.; FEIST, A. M. The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. **Metabolic engineering**, v. 56, p. 1–16, 2019.

SANTOS, T. B.; FERREIRA, V. P.; TORRES, E. A.; DA SILVA, J. A. M.; ORDONEZ, J. C. Energy analysis and exhaust emissions of a stationary engine fueled with diesel–biodiesel blends at variable loads. **Journal of the Brazilian Society of Mechanical Sciences and Engineering**, v. 39, n. 8, p. 3237–3247, 2017.

SASANO, Y.; WATANABE, D.; UKIBE, K.; INAI, T.; OHTSU, I.; SHIMOI, H.; TAKAGI, H. **Overexpression of the yeast transcription activator Msn2 confers furfural resistance and increases the initial fermentation rate in ethanol production.** [S. l.: s. n.] Disponível em: <https://doi.org/10.1016/j.jbiosc.2011.11.017>

SCHWENTNER, A.; FEITH, A.; MÜNCH, E.; BUSCHE, T.; RÜCKERT, C.; KALINOWSKI, J.; TAKORS, R.; BLOMBACH, B. Metabolic engineering to guide evolution - Creating a novel mode for L-valine production with *Corynebacterium glutamicum*. **Metabolic engineering**, v. 47, p. 31–41, 2018.

SEHNEM, N. T.; MACHADO, A. da S.; LEITE, F. C. B.; PITA, W. de B.; DE MORAIS, M. A., Jr; AYUB, M. A. Z. 5-Hydroxymethylfurfural induces ADH7 and ARI1 expression in tolerant industrial *Saccharomyces cerevisiae* strain P6H9 during bioethanol production. **Bioresource technology**, v. 133, p. 190–196, 2013.

SHENDURE, J.; BALASUBRAMANIAN, S.; CHURCH, G. M.; GILBERT, W.; ROGERS, J.; SCHLOSS, J. A.; WATERSTON, R. H. DNA sequencing at 40: past, present and future. **Nature**, v. 550, n. 7676, p. 345–353, 2017.

SHEN, Y.; LI, H.; WANG, X.; ZHANG, X.; HOU, J.; WANG, L.; GAO, N.; BAO, X. High vanillin tolerance of an evolved *Saccharomyces cerevisiae* strain owing to its enhanced vanillin reduction and antioxidative capacity. **Journal of industrial microbiology & biotechnology**, v. 41, n. 11, p. 1637–1645, 2014.

SILVA, T. R. V.; BAETA, J. G. C.; NETO, N. A. D.; MALAQUIAS, A. C. T.; CARVALHO, M. G. F.; FILHO, F. R. Effects of internal EGR on the downsized ethanol SIDI engine performance and emission. In: 26TH SAE BRASIL INTERNATIONAL CONGRESS AND DISPLAY, 2017-36-0264., 2017, 400 Commonwealth Drive, Warrendale, PA, United States. **SAE Technical Paper Series**. 400 Commonwealth Drive, Warrendale, PA, United States: SAE International, 2017. Disponível em: <https://doi.org/10.4271/2017-36-0264>

SMITH, J.; VAN RENSBURG, E.; GÖRGENS, J. F. Simultaneously improving xylose fermentation and tolerance to lignocellulosic inhibitors through evolutionary engineering of recombinant *Saccharomyces cerevisiae* harbouring xylose isomerase. **BMC biotechnology**, v. 14, p. 41, 2014.

SOARES, L. B.; DA SILVEIRA, J. M.; BIAZI, L. E.; LONGO, L.; DE OLIVEIRA, D.; FURIGO JÚNIOR, A.; IENCZAK, J. L. An overview on fermentation strategies to

overcome lignocellulosic inhibitors in second-generation ethanol production using cell immobilization. **Critical reviews in biotechnology**, p. 1–22, 2022.

SUN, J. X.; SUN, X. F.; ZHAO, H.; SUN, R. C. Isolation and characterization of cellulose from sugarcane bagasse. **Polymer degradation and stability**, v. 84, n. 2, p. 331–339, 2004.

SWINNEN, S. *et al.* Identification of novel causative genes determining the complex trait of high ethanol tolerance in yeast using pooled-segregant whole-genome sequence analysis. **Genome research**, v. 22, n. 5, p. 975–984, 2012.

SZULEJKO, J. E.; KUMAR, P.; DEEP, A.; KIM, K.-H. Global warming projections to 2100 using simple CO₂ greenhouse gas modeling and comments on CO₂ climate sensitivity factor. **Atmospheric pollution research**, v. 8, n. 1, p. 136–140, 2017.

TAHERZADEH, M. J.; GUSTAFSSON, L.; NIKLASSON, C.; LIDÉN, G. **Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae***. [*S. l.: s. n.*] Disponível em: [https://doi.org/10.1016/s1389-1723\(99\)89007-0](https://doi.org/10.1016/s1389-1723(99)89007-0)

THOMPSON, O. A.; HAWKINS, G. M.; GORSICH, S. W.; DORAN-PETERSON, J. Phenotypic characterization and comparative transcriptomics of evolved *Saccharomyces cerevisiae* strains with improved tolerance to lignocellulosic derived inhibitors. **Biotechnology for biofuels**, v. 9, n. 1, p. 200, 2016.

TOMÁS-PEJÓ, E.; BALLESTEROS, M.; OLIVA, J. M.; OLSSON, L. Adaptation of the xylose fermenting yeast *Saccharomyces cerevisiae* F12 for improving ethanol production in different fed-batch SSF processes. **Journal of industrial microbiology & biotechnology**, v. 37, n. 11, p. 1211–1220, 2010.

TOPALOĞLU, A.; ESEN, Ö.; TURANLI-YILDIZ, B.; ARSLAN, M.; ÇAKAR, Z. P. From *Saccharomyces cerevisiae* to Ethanol: Unlocking the Power of Evolutionary Engineering in Metabolic Engineering Applications. **Journal of Fungi**, v. 9, n. 10, 2023. Disponível em: <https://doi.org/10.3390/jof9100984>. Acesso em: 12 jan. 2024.

UNGUREANU, N.; VLĂDUȚ, V.; BIRIȘ, S.-Ștefan. Sustainable valorization of waste and by-products from sugarcane processing. **Sustainability: Science Practice and Policy**, v. 14, n. 17, p. 11089, 2022.

VANMARCKE, G.; DEPARIS, Q.; VANTHIENEN, W.; PEETERMANS, A.; FOULQUIÉ-MORENO, M. R.; THEVELEIN, J. M. A novel AST2 mutation generated upon whole-genome transformation of *Saccharomyces cerevisiae* confers high tolerance to 5-Hydroxymethylfurfural (HMF) and other inhibitors. **PLoS genetics**, v. 17, n. 10, p. e1009826, 2021.

VAN MARIS, A. J. A.; KONINGS, W. N.; VAN DIJKEN, J. P.; PRONK, J. T. Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes. **Metabolic engineering**, v. 6, n. 4, p. 245–255, 2004.

VARIZE, C. S.; BÜCKER, A.; LOPES, L. D.; CHRISTOFOLETI-FURLAN, R. M.; RAPOSO, M. S.; BASSO, L. C.; STAMBUK, B. U. Increasing ethanol tolerance and ethanol production in an industrial fuel ethanol *Saccharomyces cerevisiae* strain. **Fermentation**, v. 8, n. 10, p. 470, 2022.

WALLACE-SALINAS, V.; BRINK, D. P.; AHRÉN, D.; GORWA-GRAUSLUND, M. F. Cell periphery-related proteins as major genomic targets behind the adaptive evolution of an industrial *Saccharomyces cerevisiae* strain to combined heat and hydrolysate stress. **BMC genomics**, v. 16, n. 1, p. 514, 2015.

WALLACE-SALINAS, V.; GORWA-GRAUSLUND, M. F. Adaptive evolution of an industrial strain of *Saccharomyces cerevisiae* for combined tolerance to inhibitors and temperature. **Biotechnology for biofuels**, v. 6, n. 1, p. 151, 2013.

WANG, X.; LIANG, Z.; HOU, J.; BAO, X.; SHEN, Y. Identification and functional evaluation of the reductases and dehydrogenases from *Saccharomyces cerevisiae* involved in vanillin resistance. **BMC biotechnology**, v. 16, p. 31, 2016.

WANG, X.; LIANG, Z.; HOU, J.; SHEN, Y.; BAO, X. The Absence of the Transcription Factor Yrr1p, Identified from Comparative Genome Profiling, Increased Vanillin Tolerance Due to Enhancements of ABC Transporters Expressing, rRNA Processing and Ribosome Biogenesis in. **Frontiers in microbiology**, v. 8, p. 367, 2017.

WRIGHT, J.; BELLISSIMI, E.; DE HULSTER, E.; WAGNER, A.; PRONK, J. T.; VAN MARIS, A. J. A. Batch and continuous culture-based selection strategies for acetic acid tolerance in xylose-fermenting *Saccharomyces cerevisiae*. **FEMS yeast research**, v. 11, n. 3, p. 299–306, 2011.

WU, G.; XU, Z.; JÖNSSON, L. J. Profiling of *Saccharomyces cerevisiae* transcription factors for engineering the resistance of yeast to lignocellulose-derived inhibitors in biomass conversion. **Microbial cell factories**, v. 16, n. 1, p. 199, 2017.

YANG, N.; WANG, R. Sustainable technologies for the reclamation of greenhouse gas CO₂. **Journal of cleaner production**, v. 103, p. 784–792, 2015.

ZHANG, G.-C.; KONG, I. I.; WEI, N.; PENG, D.; TURNER, T. L.; SUNG, B. H.; SOHN, J.-H.; JIN, Y.-S. Optimization of an acetate reduction pathway for producing cellulosic ethanol by engineered yeast. **Biotechnology and bioengineering**, v. 113, n. 12, p. 2587–2596, 2016.

ZHANG, M.-M.; CHEN, H.-Q.; YE, P.-L.; WATTANACHAISAEEREEKUL, S.; BAI, F.-W.; ZHAO, X.-Q. Development of Robust Yeast Strains for Lignocellulosic Biorefineries Based on Genome-Wide Studies. **Progress in molecular and subcellular biology**, v. 58, p. 61–83, 2019.

ZHANG, M.; ZHANG, K.; MEHMOOD, M. A.; ZHAO, Z. K.; BAI, F.; ZHAO, X. Deletion of acetate transporter gene ADY2 improved tolerance of *Saccharomyces cerevisiae* against multiple stresses and enhanced ethanol production in the presence of acetic acid. **Bioresource technology**, v. 245, n. Pt B, p. 1461–1468, 2017.

CHAPTER 2 - HOW ADAPTIVE LABORATORY EVOLUTION CAN BOOST YEAST TOLERANCE TO LIGNOCELLULOSIC HYDROLYSES

Chapter developed based on the published manuscript: Menegon, Y. A. et al. "How adaptive laboratory evolution can boost yeast tolerance to lignocellulosic hydrolyses." *Current Genetics* (2022) 68:319–342. <https://doi.org/10.1007/s00294-022-01237-z>

REVIEW

How adaptive laboratory evolution can boost yeast tolerance to lignocellulosic hydrolyses

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Received: 13 December 2021 / Revised: 1 March 2022 / Accepted: 6 March 2022 / Published online: 1 April 2022

Abstract

The yeast *Saccharomyces cerevisiae* is an excellent candidate for establishing cell factories to convert lignocellulosic biomass into chemicals and fuels. To enable this technology, yeast robustness must be improved to withstand the fermentation inhibitors (e.g., weak organic acids, phenols, and furan aldehydes) resulting from biomass pretreatment and hydrolysis. Here, we discuss how evolution experiments performed in the lab, a method commonly known as adaptive laboratory evolution (ALE), may contribute to lifting yeast tolerance against the inhibitors of lignocellulosic hydrolysates (LCHs). The key is that, through the combination of whole-genome sequencing and reverse engineering, ALE provides a robust platform for discovering and testing adaptive alleles, allowing to explore the genetic underpinnings of yeast responses to LCHs. We review the insights gained from past evolution experiments with *S. cerevisiae* in LCH inhibitors and propose experimental designs to optimise the discovery of genetic variants adaptive to biomass toxicity. The knowledge gathered

through ALE projects is envisaged as a roadmap to engineer superior yeast strains for biomass-based bioprocesses.

Introduction

For reducing the unbridled emission of carbon dioxide (CO₂) and its impact on climate change, it is essential to replace oil-based chemicals with renewable materials. To this goal, first-generation (1G) microbial fermentation technologies have been successfully established for converting crop plant sugars (e.g., sugarcane sucrose and corn starch-derived glucose) into ethanol. Another key advance would be the development of second-generation (2G) fermentation platforms, i.e., when the plant biomass (i.e., the lignocellulosic material) is converted into fuels and high-value products via microbial fermentation (Jacobus et al. 2021a). The problem of the 2G fermentation technology is that sugars trapped into the plant cell wall as lignocellulosic polymers are inaccessible for the microbial metabolism (Palmqvist and Hahn-Hagerdal 2000). To tap into this rich resource, biomass must be processed (via pretreatment and hydrolysis) to break down cellulose and hemicellulose into simple hexose and pentose sugars (Kumar et al. 2009). The downside of this technical process is that the resulting liquor, the so-called lignocellulosic hydrolysate (LCH), also carries toxic substances to microorganisms, i.e., the LCH inhibitors (Jonsson et al. 2013).

Despite the need for heterologous pathways for the pentose (xylose and arabinose) metabolism, the practical use of *S. cerevisiae* in 2G processes also depends on an enhanced tolerance to these lignocellulosic byproducts (Jacobus et al. 2021a). One efficient (though poorly explored) possibility to boost yeast resilience to LCH inhibitors is the use of adaptive laboratory evolution (ALE). This approach mimics the evolution by natural selection through the application of restricting conditions for microbial propagations in the lab. Differential survival and proliferation of more adapted genotypes result in a population of microorganisms expressing a phenotype that is more fit to the challenging environment (Barrick and Lenski 2013). In this way, ALE has improved traits in many microorganisms subjected to diverse industrial stresses (Dragosits and Mattanovich 2013). Focussing on *S. cerevisiae*, we review studies that successfully applied the evolution approach to enhance yeast tolerance to a single or combined inhibitors present in LCHs. We discuss the prospects of diversifying ALE experimental designs to maximise adaptation and

genetic diversity. Combined with genomics approaches, such protocols may generate catalogues of genetic targets for engineering via modern CRISPR/Cas9 technology of LCH-resistant strains suited for the biomass-based industry.

The problem of LCH inhibitors for yeast fermentation

The nature and origin of LCH inhibitors

The polymers comprising the lignocellulose fraction of plants are represented by cellulose ($\beta(1 \rightarrow 4)$ linked D-glucose units), hemicellulose (heteropolymers of xylan, galactomannan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan), and lignin (formed by the phenylpropanoid units *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) (Kumar et al. 2020; Palmqvist and Hahn-Hagerdal 2000). Since *S. cerevisiae* cannot decompose cellulose and hemicellulose, the availability of fermentable sugars must rely on a chemical/physical pretreatment of lignocellulose, followed by chemical or enzymatic hydrolysis (Kumar et al. 2020). Through this process, cellulose is broken down into glucose units, while hemicellulose is decomposed into C5 (xylose and arabinose) and C6 (galactose, mannose, rhamnose, and glucose) monomeric sugars (Palmqvist and Hahn-Hagerdal 2000). Unfortunately, along with fermentable sugars, pretreatment and hydrolysis generate many undesirable toxic byproducts. At high temperature and pressure, degradation of xylose and arabinose results in furfural, while 5-hydroxy methylfurfural (HMF) is similarly derived from glucose, mannose, and galactose (Palmqvist and Hahn-Hagerdal 2000). Furfural further degrades into formic acid, while HMF can be broken down into formic and levulinic acids. Acetic acid is released upon hydrolysis of acetyl groups of hemicellulose and is widely present in all kinds of LCHs (Kumar et al. 2020). Finally, pretreatment releases phenolic compounds from lignin, such as 4-hydroxybenzoic acid, vanillin, catechol, ferulic acid, and coumaric acid, among others (Kumar et al. 2020). It should be noted that the complete list of inhibitors includes other less abundant compounds. In addition, the inhibitor content in different LCHs is highly variable according to the different types of feedstocks, such as hardwoods (e.g., oak and aspen), softwoods (e.g., pine and spruce), or agriculture residues (e.g., corn cob and sugarcane bagasse) (Cunha et al., 2019; Palmqvist and Hahn-Hagerdal 2000). Another factor influencing the content of inhibitors in each newly produced LCH is the choice of the pretreatment (e.g., diluted acid, alkali, or

hydrothermal treatment) and the selected operational conditions (e.g., treatment duration, catalyst concentration, and temperature) (Cunha et al., 2019; Kumar et al. 2020; Palmqvist and Hahn-Hagerdal 2000). Therefore, *S. cerevisiae* tolerance to LCH inhibitors should be ultimately understood in the context of a particular biomass source and its processing method.

Yeast responses to LCH toxicity

Inhibitors in LCH liquors usually have synergistic effects that are detrimental to yeast fermentation, causing prolonged lag phases, slower growth rates, and reducing biomass formation and ethanol yields (Cunha et al. 2019; Palmqvist and Hahn-Hagerdal 2000). Yeast responses to LCH stress (Fig. 1) generally involve ATP and NAD(P)H expending processes to detoxify inhibitors, repair their damage, and counteract the formation of reactive oxygen species (ROS) (Cunha et al. 2019; Piotrowski et al. 2014). Consequently, mechanisms of redox and energetic homeostasis are activated. For example, NAD(P)H regeneration is supported by upregulating the pentose phosphate pathway (PPP), while metabolic fluxes are balanced through glycolytic and respiratory pathways to optimise ATP generation (Guo and Olsson 2014). This may be promoted by Mns2/4p transcriptional factors (Kuang et al. 2017), which are key activators of the general stress response involving multiple adaptations, such as the synthesis of trehalose and heat shock proteins, important to withstand the damage caused by LCH inhibitors (Cunha et al. 2019).

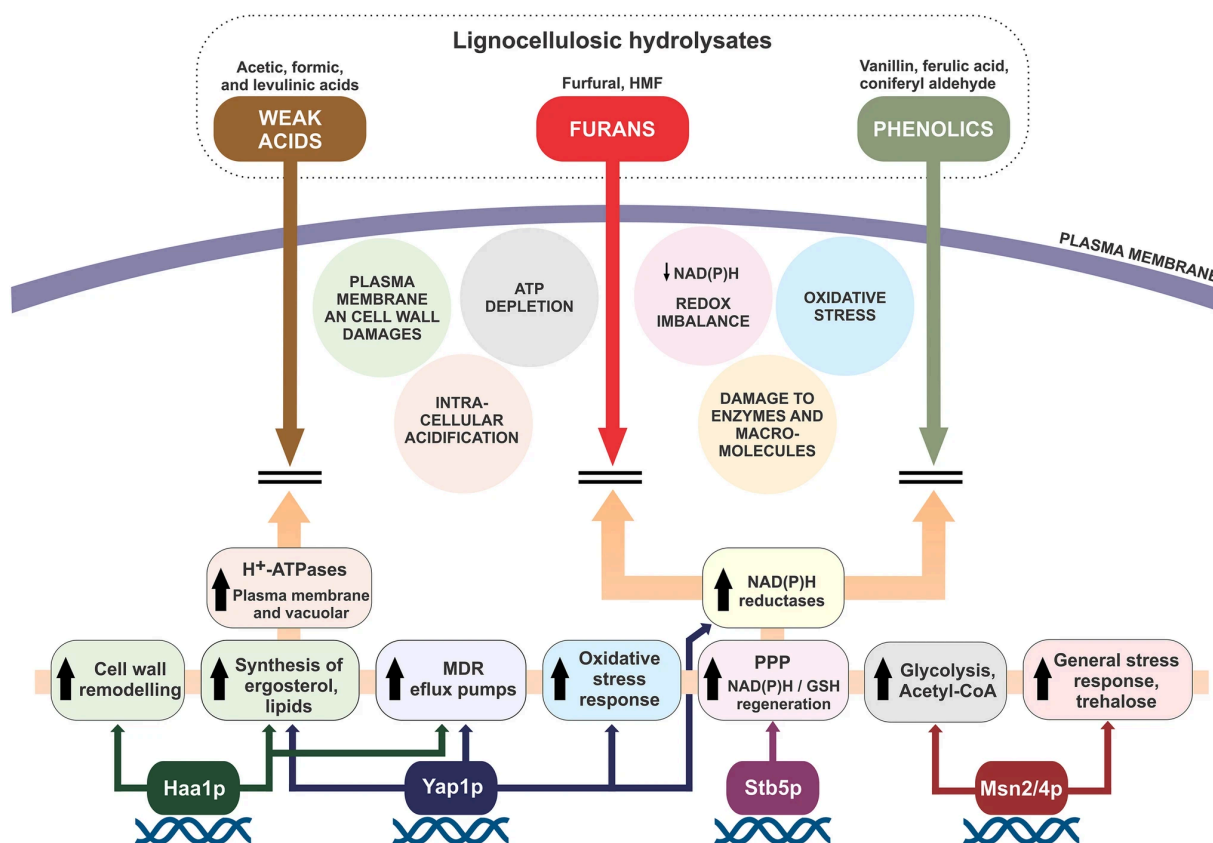


Figure 1: An overview of yeast responses against the toxic effects of LCH inhibitors. Weak acids, furan aldehydes, and phenolics are the main classes of LCHs inhibitors, and their most common toxic effects on the cell are represented within circles. Enhanced global cellular responses to the inhibitors are depicted in rectangles. Key transcriptional factors that regulate these responses are displayed at the bottom. *MDR* multidrug resistance, *PPP* pentose phosphate pathway, *GSH* reduced glutathione, *NAD(P)H* reduced nicotinamide adenine dinucleotide phosphate

Mechanisms of homeostatic balance (Fig. 1) are illustrated by typical yeast responses to weak acids, such as acetic, formic, and levulinic acids. At low pH resulting from biomass pretreatment and hydrolysis, weak acids shift to a protonated form, which easily permeates cell membranes. Inside the near-neutral cytosolic pH, acids dissociate and decrease intracellular pH, triggering the actions of the plasma membrane and vacuolar ATPases to clear protons from the cytosol (Palma et al. 2018). The anionic form of weak acids is also toxic by increasing intracellular osmolarity and ROS formation. As a countermeasure, multidrug resistance (MDR) transporters Tpo2p, Tpo3p, and Aqr1p promote the extrusion of anion acids (Mira et

al. 2010). Cellular responses to weak acids (and phenolics) also involve remodelling of the cell wall structure and membrane lipid composition to stabilise the cell periphery and decrease its permeability to the inhibitors (Palma et al. 2018). In the case of weak acids, such responses are mediated by the transcription factor Haa1p, a master regulator for ~ 80% of the genes responsive to acetic acid (Mira et al. 2010).

The toxicity of furan aldehydes is primarily exerted over enzymes controlling the central carbon and energy metabolism and through depletion of NAD(P)H pools during inhibitor detoxification via a battery of oxidoreductase enzymes (e.g., Adh6p, Adh7p, Gre2p, Oye3p) (Jayakody and Jin 2021; Ma and Liu 2010). Such redox reactions may also account for ROS formation as byproducts (Kim and Hahn 2013). The Yap1p transcription factor regulates the responses to oxidative stress (Kim and Hahn 2013), while Stb5p is a key transcription factor for inducing the PPP required for NAD(P)H regeneration (Wu et al. 2017). Detoxification of furfurals also occurs via cellular extrusion mediated by pleiotropic drug resistance (PDR) efflux pumps (e.g., Pdr5p, Pdr12p, Pdr15p, Snq2p, Yor1p) (Ma and Liu 2010).

The lipophilic nature of phenolic compounds accounts for a primary toxic effect at the plasma membrane, although phenols may cause protein denaturation, oxidative stress, decrease in intracellular pH, ATP depletion, translation inhibition, and DNA mutagenesis (Fletcher and Baetz 2020; Piotrowski et al. 2014). Functional genomics and genetic approaches have identified key functions induced as a response to phenolics (Fletcher and Baetz 2020). These include Yap1p-mediated oxidative stress responses, combined with increased flux through the PPP, providing redox power for glutathione regeneration and detoxification of phenols via oxidoreductases enzymes (e.g., Adh7p, Adh4p, and Ald6p). Ergosterol biosynthesis is also activated to stabilise the plasma membrane (e.g., Erg5p, Erg26p, Erg7p, Hmg1p, Erg28p), while the expression of membrane transporters (e.g., Pdr5p, Yor1p, and Snq2p) promote the efflux of phenolics at the expense of ATP. This energetic loss is compensated with enhanced mitochondrial respiration (Fletcher and Baetz 2020).

Overall, the knowledge gathered about endogenous tolerance mechanisms to phenolics and other inhibitors has been pivotal for developing more robust yeasts via genetic engineering approaches.

Genetic screens and approaches for engineering LCH tolerance

Transcriptomics and chemogenomic screens

Several chemical, physical, and biological methods to detoxify LCHs before fermentations have been developed (Kumar et al. 2020). However, pre-detoxification involves additional operational steps and increased costs with equipment, materials, and/or chemicals. In contrast, genetic engineering of yeast robustness dispenses inhibitor removal, representing a cheaper alternative for running 2G bioprocesses (Cunha et al. 2019; Jonsson et al. 2013). The key in a genetic engineering project is the choice of a gene or trait to be modified. For that purpose, several analytical methods are helpful to discern which genes and biological processes are involved in yeast responses to LCHs. One traditional approach is transcriptomic analysis, usually based on microarrays, or more recently, via high-throughput sequencing of the cell mRNA content (RNA-seq) (Fletcher and Baetz 2020; Wang et al. 2017). Global transcription patterns in yeasts treated with inhibitors or LCH liquors can be directly compared with untreated controls, unveiling which genes and pathways are differentially expressed in the stress condition (Ma and Liu 2010). Alternatively, transcriptomics of genetically modified or mutagenised strains resistant to LCH inhibitors can be directly compared to the sensitive parental, highlighting specific responses elicited only in the genetically altered background (Liu and Ma 2020; Wang et al. 2017).

Another practical approach to identifying genes implicated in LCH tolerance is through chemogenomic screens (Fletcher and Baetz 2020; Palma et al. 2018). The availability of yeast collections in which isogenic strains differ by single ORF deletions allows for interrogation of gene knockouts by spotting the deleted strains onto a solid medium containing a sub-lethal dose of the inhibitor. Any growth impairment of the knockout strain compared to its parental indicates that the missing gene may somehow mediate a tolerance response (Fletcher and Baetz 2020; Palma et al. 2018). Chemogenomic screens can also be performed with strains in which barcoded plasmid libraries promote the overexpression of most yeast ORFs. Therefore, a positive correlation between gene overexpression and LCH resistance can be established (Sardi et al. 2016).

Genetic engineering of LCH tolerance

Chemogenomic screens and transcriptomic analyses have found many genes in yeasts associated with LCH responses. Based on this knowledge, key candidates have been overexpressed for enhancing tolerance to individual inhibitors or hydrolysates from different feedstocks (see Table 1 for a comprehensive list). This is the case of the transcriptional factors *HAA1*, *YAP1*, and *STB5* (Fig. 1), whose boosted expression lifted global adaptive responses to weak acids, oxidative stress, and redox imbalance, respectively (Cunha et al. 2018; Wu et al. 2017). Overexpression of genes acting downstream to these transcriptional regulators has also been implemented, generally coffering tolerance to LCHs by (1) stimulating NAD(P)H regeneration via the PPP (e.g., *ZWF1*, *TAL1*, and *TKL1*) (Cunha et al. 2015; Hasunuma et al. 2011a, b); (2) strengthening oxidative stress responses (e.g., *GSH1*, *CYS3*, *GLR1*) (Ask et al. 2013); (3) converting inhibitors into less harmful compounds (e.g., *YKL071W*, *ADH7*) (Heer et al. 2009); or finally by (4) extrusion of LCH inhibitors from the cell via MDR transporters (e.g., *PDR5*, *YOR1*, *SNQ2*) (Wang et al. 2017).

Table 1: Genes that has been overexpressed in yeast to improve tolerance to LCH inhibitors

Function	Genes	Hydrolysate/ inhibitor tolerance	References
Transcription factors and regulators	<i>YAP1</i>	Coniferyl aldehyde, HMF, furfural, spruce LCH	Alriksson et al. (2010); Kim and Hahn (2013); Wallace-Salinas et al. (2014)
	<i>YAP1, STB5, WAR1, CAT8, PDR8, PUT3, GZF3</i>	Spruce and sugarcane bagasse LCH	Wu et al. (2017)
	<i>MSN2</i>	Furfural	Sasano et al. (2012)
	<i>HAA1</i>	Acetic acid	Cunha et al. (2018); Inaba et al. (2013); Sakihama et al. (2015); Swinnen et al. (2017); Tanaka et al. 2012)
	<i>HAA1</i>	Vanillin	Cao et al. (2021)

	<i>HAA1, TYE7, HAP4, YOX1, MGA1, SFP1, ACE2</i>	Acetic acid, furfural	Chen et al. (2016a); Li et al. (2021)
	<i>SAP30, ZNF1</i>	Acetic acid	Mira et al. (2010); Songdech et al. (2020)
	<i>SET5, PPR1</i>	Acetic acid, corn stover LCH	Zhang et al. (2015)
	<i>RPB4</i>	Acetic acid, furfural, HMF	Cunha et al. (2015)
	<i>MBF1</i>	Vanillin	Liang et al. (2021)
	<i>HTA2</i>	Furfural and HMF	Kim et al. (2015)
	<i>IrrE (D. radiodurans)</i>	Furfural, acetic acid, phenol	Wang et al. (2020)
	<i>UME6, eIF5A</i>	Acetic acid	Cheng et al. (2021)
Post-transcriptional and post-translational	<i>ESBP6</i>	Coumaric and ferulic acid	Pereira et al. (2020)
	<i>RTC3, ANB1</i>	Acetic acid, formic acid	Hasunuma et al. (2016)

onal regulators	<i>WHI2, PSR1, HRK1, RCK1, ASC1, COX20, PAB1</i>	Acetic acid	Chen et al. (2016b); Kumar et al. (2015); Lee et al. (2015); Martani et al. (2015); Mira et al. 2010; Oh et al. 2019)
	<i>MDJ1</i>	Synthetic AFEX corn stover hydrolysate	Sardi et al. (2016)
	<i>DBP2, TMA17</i>	Vanillin	Wang et al. (2017); Cao et al. (2021)
Reductases, dehydrogenases, and redox enzymes	<i>ADH1, ADH6, GRE2</i>	Glycolaldehyde, methylglyoxal, HMF, furfural, acetic acid, vanillin	Almeida et al. (2008); Jayakody et al. (2013, 2018; Petersson et al. 2006)
	<i>ADH6, ADH7, YKL071W</i>	HMF, furfural	Heer et al. (2009); Liu et al. (2008)
	<i>TAL1, ADH1, GLR1, OYE2, ZWF1, IDP1, POS5</i>	Furfural	Hasunuma et al. (2014); Liu et al. (2020)

	<i>ZWF1</i>	Furfural, HMF, acetic acid	Cunha et al. (2015); Gorsich et al. (2006)
	<i>ZWF1, ALD6, YNL134C, YJR096W, GCY1, YPR1</i>	Vanillin	Liang et al. (2021); Wang et al. (2016)
	<i>GND1, CTT1</i>	Acetic acid	Guaragnella et al. (2008); Lee et al. (2015)
	<i>FDH1</i>	formic acid	Hasunuma et al. (2011b)
	<i>TAL1, FDH1, ARI1, ADH6, PAD1, ICT1</i>	Inhibitor cocktail, sugarcane bagasse LCH and hardwood spent sulphite LCH	Brandt et al. (2021); Sanda et al. (2011)
	<i>YMR152W</i>	Acetaldehyde, furfural, glycolaldehyde	Ouyang et al. (2021)
	<i>gshF</i> (<i>S. thermophilus</i>), <i>gshF</i> (<i>L. monocytogenes</i>)	Steam pretreated spruce LCH	Raghavendran et al. (2020)

	<i>GSH1, CYS3, GLR1</i>	Pretreated spruce biomass	Ask et al. (2013)
	<i>TRX1</i>	Acetic acid, furfural	Unrean et al. (2018)
	<i>BDH1, BDH2</i>	Vanillin, acetaldehyde and glycolaldehyde	Ishida et al. (2016); Kuang et al. (2020)
	<i>ALD6</i>	Vanillin, furfural, and HMF	Ishida et al. (2017); Park et al. (2011)
	<i>YCR102C</i>	Acetic acid, inhibitor mixture	Chen et al. (2019)
	<i>MCR1</i>	Spruce LCH	Wallace-Salinas et al. (2014)
	<i>TPX1, TRX, TRXR</i> from <i>K. marxianus</i>	Formic acid, acetic acid, furfural	Gao et al. (2017a, b)
	<i>adhE</i> (<i>E. coli</i>), mutant <i>acs</i> (<i>S. entérica</i>)	Acetic acid	Zhang et al. (2016)
	<i>crtE, crtI, crtB</i>	Acetic acid, phenol, furfural	Pan et al. (2018)

	<i>CALDH</i> (<i>Pseudomonas</i> <i>HR199</i>)	Coniferyl aldehyde	Adeboye et al. (2016)
Efflux pumps	<i>ATR1, FLR1, PDR5,</i> <i>YOR1, SNQ2,</i> <i>PDR12</i>	Coniferyl aldehyde, HMF, vanillin, sorbic, propionic and levulinic acids	Alriksson et al. (2010); Wang et al. (2017) Nygard et al. (2014)
Metabolic and detoxification enzymes	<i>PRS3</i>	Eucalyptus globulus wood LCH, corn cob LCH, acetic acid, furfural, HMF	Cunha et al. (2015, 2018)
	<i>SPE3 + (ΔOAZ1, ΔTPO1), ADE17, PIR3</i>	Furfural, HMF, acetic acid	Kim et al. (2015)
	<i>ACS2</i>	Acetic acid	Ding et al. (2015b)
	<i>TAL1, TKL1</i>	Formic, acetic, and levulinic acids	Hasunuma et al. (2011a); Li et al. (2014)
	<i>PAD1, FDC1</i>	Ferulic/cinnamic/p-coumaric/caffein ic acids, spruce LCH	Larsson et al. (2001b); Richard et al. (2015)

	<i>lcc2</i> (<i>T. versicolor</i>), SSO2	Coniferyl aldehyde, spruce LCH	Larsson et al. (2001a)
	<i>MET14</i> , <i>ERG26</i>	Synthetic AFEX corn stover hydrolysate	Sardi et al. (2016)
	<i>PRO1</i> , <i>INO1</i>	Furfural, acetic acid, phenol	Wang et al. (2015)
	<i>ADE1</i> , <i>ADE13</i> , <i>ADE17</i>	Acid acetic, synthetic LCH, corn stover LCH	Zhang et al. (2019a, b)
	<i>ALD5</i> , <i>PAD1</i> , <i>ATF1</i> , <i>ATF2</i>	Coniferyl aldehyde, ferulic/p-coumaric acids	Adeboye et al. (2017)
Vacuole, peroxisome	<i>PEX5</i> , <i>PEP3</i>	Vanillin, acetic acid	Ding et al. (2015a); Liang et al. (2021)

Table 2: Gene deletions or genes with SNPs that increase yeast tolerance to LCH inhibitors

Mutation	Genes/deletions	Hydrolysate/Inhibitor tolerance	References
Single deletions	<i>ATG22Δ</i>	Acetic acid	Hu et al. (2019)
	<i>PHO13Δ</i>	Weak acids	Fujitomi et al. (2012); Li et al. (2014; Sakihama et al. (2015)
	<i>BNA7Δ</i>	Ferulic acid	Fletcher et al. (2019)
	<i>YRR1Δ</i>	Vanillin	Cao et al. (2021); Wang et al. (2017)
	<i>FPS1Δ</i>	Acetic acid	Brandt et al. (2021); Wei et al. (2013); Zhang et al. (2011)
	<i>MIG1Δ</i>	Acetic, formic and levulinic acids	Balderas-Hernandez et al. (2018)
	<i>JJJ1Δ</i>	Acetic acid	Wu et al. (2016)
	<i>QDR3Δ, IKS1Δ</i>	Acetic acid	Ma et al. (2015)

	<i>CMK1Δ</i>	Propionic, levulinic, sorbic, and benzoic acids	Holyoak et al. (2000); Nygard et al. (2014)
	<i>RTT109Δ</i>	Acetic acid	Cheng et al. (2016)
	<i>HAL3, 5Δ, SAT4Δ, TRK1Δ, CYC3Δ, ARN2Δ</i>	Formic acid	Henriques et al. (2017)
	<i>ADY2Δ</i>	Acetic acid	Zhang et al. (2017)
	<i>DOT6Δ, SKO1Δ</i>	Spruce LCH	Gutmann et al. (2021)
	<i>NTH1Δ, NTH2Δ</i>	Acetic acid	Yoshiyama et al. (2015)
	<i>EEB1Δ, SSH4Δ, VAM6Δ</i>	Synthetic hydrolysate	Xue et al. (2018)
	<i>SIZ1Δ</i>	Furfural	Xiao and Zhao (2014)
	<i>SIZ1Δ plus LCB3Δ</i>	Furfural	Bao et al. (2018)
	<i>SIZ1i plus NAT1a and PDR1i</i>	Furfural	Lian et al. (2019)

Screening of yeast knockout collections	<i>PCL1Δ, RPS22AΔ, TOS6Δ, RPL8BΔ, RPL22AΔ, OPI11Δ, RPS17BΔ, RPL13AΔ, FPS1Δ, DUR1/2Δ</i>	Acetic acid	An et al. (2015)
	54 deletion mutants	Acetic acid	Lee et al. (2015)
	2159 deletion mutants	Acetic acid	Sousa et al. (2013)
	52 deletion mutants	Acetic acid, lactic acid	Kawahata et al. (2006)
	172 deletion mutants	Formic acid	Henriques et al. (2017)
<i>Genes with SNP variants</i>	<i>NRG1, GSH1, GDH1, UBP7</i>	Hardwood spent sulphite liquor	Biot-Pelletier et al. (2018); Pinel et al. (2015)
	<i>ASG1, ADH3, SKS1, GIS4</i>	Acetic acid	Gonzalez-Ramos et al. (2016)

	<i>PAB1, HAA1</i>	Acetic acid	Martani et al. (2015); Swinnen et al. (2017)
	<i>SFA1, HAP1</i>	Miscanthus × giganteus plant LCH	Maurer et al. (2017)
	<i>GCY1, MBF1</i>	Vanillin	Liang et al. (2021)
	<i>Histone H3</i>	Acetic acid	Liu et al. (2014)
	<i>GRE2</i>	HMF	Moon and Liu (2012)
	<i>ADH1</i>	HMF	Laadan et al. (2008)
	<i>SPT15</i>	Acetic acid	An et al. (2015)
	<i>HAA1, VMA7, GLO1, DOT5, CUP2</i>	Acetic acid	Meijnen et al. (2016)
	<i>SNF4, TRT2, MET4, IRA2, RTG1, MSH2 + HAL9</i>	Acetic acid	Stojiljkovic et al. (2020)
	<i>YGL176C</i>	Inhibitor mixture	de Witt et al. (2019)

	<i>MET22, ESP1</i>	Acetic acid	Fernandez-Nino et al. (2018)
	<i>ARO80, NRG1</i>	Coumaric acid, ferulic acid	Pereira et al. (2020)
	<i>SIZ1</i>	Furfural	Bao et al. (2018)
	<i>RAS2, HSF1, SUM1</i>	Acetic acid, low pH, temperature	Salas-Navarrete et al. (2022)

Screens of yeast deletion collections have also identified gene knockouts that enhance tolerance to biomass-derived inhibitors (Table 2). Some of these suppressor genes were found to encode key transcriptional factors, such as *YRR1*, *MIG1*, and *RTT109* (Balderas-Hernandez et al. 2018; Cheng et al. 2016; Wang et al. 2017). Barcoded yeast deletion libraries allow the selection of adaptive knockouts in the presence of inhibitors, followed by allele identification via whole-genome sequencing (WGS). In such a manner, deletions of *EEB1* (fatty acid metabolism), *SSH4*, and *VAM6* (vesicle trafficking) were associated with increased tolerance to phenolics (Xue et al. 2018). Similarly, mass selection in LCH coupled to genome shuffling steps highlighted non-synonymous mutations in *NRG1*, *GSH1*, *GDH1*, and *UBP7* (Biot-Pelletier et al. 2018; Pinel et al. 2015). In contrast to ORF deletions, single nucleotide polymorphisms (SNPs) may result in partially defective genes or even in gain-of-function alleles conferring resistance to inhibitors (Table 2). Adaptive SNPs can be identified in wild or industrial yeast populations via quantitative trait loci (QTL) mapping. It is possible to map SNPs associated with an inhibitor-tolerant yeast through the screening and WGS of inhibitor-resistant segregants that result from a crossing between the tolerant parental with a sensitive one (Meijnen et al. 2016; Stojiljkovic et al. 2020). QTL mapping has facilitated the identification of polymorphisms related to acetic acid tolerance in the genes *HAA1*, *VMA7*, *GLO1*, *DOT5*, and *CUP2* (Meijnen et al. 2016), *SNF4*, *TRT2*, *MET4*, *IRA2*, *RTG1*, *MSH2*, and *HAL9* (Stojiljkovic et al. 2020). Similarly, QTL related to the *SFA1* promoter and the *HAP1* gene were introduced into the S288c strain, improving its tolerance to the dilute-acid pretreated *Miscanthus* × *giganteus* hydrolysate (Maurer et al. 2017).

Global genome and transcriptome engineering approaches

Other modern genetic tools for developing stress-tolerant phenotypes are based on genome-wide approaches. Global transcriptional machinery engineering (gTME) through expressing mutant variants of *SPT15* (encoding a TATA-binding transcriptional factor that functions at the core of the DNA-binding general transcription factor complex TFIID) and *HAA1* (encoding the key regulator of weak acids stress responses) led to the isolation of strains with transcriptional perturbations favouring acetic acid tolerance (An et al. 2015; Swinnen et al. 2017). More recently, CRISPR interference (CRISPRi), relying on the nuclease-deactivated (d)Cas9 protein fused to the Mxi1 repressor domain, has been used to screen yeasts

for LCH tolerance. This approach revealed that downregulation of *DOT6*, *SKO1*, and *BUB1* confers spruce hydrolysate resistance (Gutmann et al. 2021) while preventing the expression of the 19S regulatory particle of the 26S proteasome results in acetic acid tolerance (Mukherjee et al. 2021). Genome engineering via CRISPR/Cas9 has also been implemented in yeast through the expression of plasmid libraries encoding Cas9, gRNAs, and donors to generate genome-wide ORF deletions (Bao et al. 2018). Library selection in the presence of furfural highlighted the epistatic effect of double *SIZ1* and *LCB3* deletions for inhibitor tolerance. In addition, editing specific amino acids in Siz1p improved yeast furfural resistance (Bao et al. 2018). Such a role of *SIZ1* knockdown and deletion in furfural tolerance has been previously established by an RNAi screen in *S. cerevisiae* (Xiao and Zhao 2014) and was further confirmed in a multi-functional genome-wide CRISPR (MAGIC) approach combining CRISPR interference, activation, and deletion (Lian et al. 2019). Genome replication engineering-assisted continuous evolution (GREACE) accelerates mutation rates through the expression of a DNA polymerase with defective proofreading functions (Xu et al. 2018). This replication-based mutagenesis was coupled with continuous selection in the presence of acetic acid, leading to inhibitor adaptation. Automated multiplex genome-scale engineering in yeast has been carried out by CRISPR/Cas9-assisted genome integration of cDNA libraries expressing sense and antisense transcripts (Si et al. 2017). As the transformed yeast population was previously engineered with the RNA interference machinery, the expression of sense and antisense mRNAs led to transcriptome perturbations, including different levels of mRNA overexpression or suppression. Selection of the transformed population in the presence of acetic acid resulted in an enhanced inhibitor tolerance (Si et al. 2017).

Overall, these modern genome- and transcriptome-wide engineering approaches represent elegant strategies to accelerate strain optimisation. They rely on selection schemes for the enrichment of adapted genotypes. The systemic use of selection to favour randomly arising beneficial mutations is, nevertheless, the *modus operandi* of a further formidable biotechnological tool known as adaptive laboratory evolution.

Adaptive laboratory evolution of yeast tolerance to LCHs

ALE as an approach for phenotype improvement and allele discovery

By exploiting the creative forces of evolution driven by selection, ALE affords a unique experimental platform for engineering yeasts with superior biotechnological traits (Mans et al. 2018). The key is that, in an evolution process, the genetic basis of phenotype improvement is usually complex, often entailing unpredictable changes in cellular regulatory networks caused by combinations of adaptive alleles (Barrick and Lenski 2013). This underlying complexity highlights the value of ALE as an excellent research tool for studying LCH adaptation under a systems biology perspective (Liu and Ma 2020; Wang et al. 2017). Basically, ALE mimics a “Darwinian” evolution in which a restrictive condition (i.e., a selective pressure) for yeast propagation is embedded within the experimental design. During such evolution, randomly occurring adaptive mutations increase microorganism survival. By leaving more descendants, better fit mutants proliferate and eventually dominate the population, resulting in a superior phenotype (Barrick and Lenski 2013; Mans et al. 2018). In ALE experiments to enhance LCH tolerance, restricting conditions are usually provided by propagating yeasts in a liquid medium containing increasingly toxic concentrations of inhibitors (Fig. 2a) (Almario et al. 2013; Gonzalez-Ramos et al. 2016). Under selecting pressures, beneficial alleles are enriched within the evolving populations. Mutations underpinning superior phenotypes can be discovered through high-throughput genome sequencing technologies (Fig. 2b) (Gonzalez-Ramos et al. 2016; Wang et al. 2017). They may correspond to point mutations (SNPs), insertions or deletions (INDELs), gene copy number variations, or even chromosomal large-scale rearrangements or aneuploidies. Moreover, modern genetic tools, such as CRISPR/Cas9, allow the reverse engineering of ALE mutations into the parental strain, providing a way to study the adaptive effects of individual alleles or combined mutations (Gonzalez-Ramos et al. 2016; Pereira et al. 2020). This evolving-and-resequencing approach, followed by reverse engineering, has been successfully applied to improve yeast traits relevant for industrial fermentations, such as tolerance to the high alcohol content (Voordeckers et al. 2015), thermotolerance (Caspeta et al. 2014), free fatty acids biosynthesis (Yu et al. 2018), methanol assimilation (Espinosa et al. 2020), and xylose catabolism (Dos Santos et al. 2016). Despite its promising potential, using ALE with yeasts under lignocellulosic toxicity has been largely restricted to phenotype improvement (Fig. 3), while the discovery of adaptive mutations via WGS and allele validation through reverse engineering have

been often neglected. That leaves an open avenue for future molecular genetics applications aiming at cataloguing key adaptive alleles.

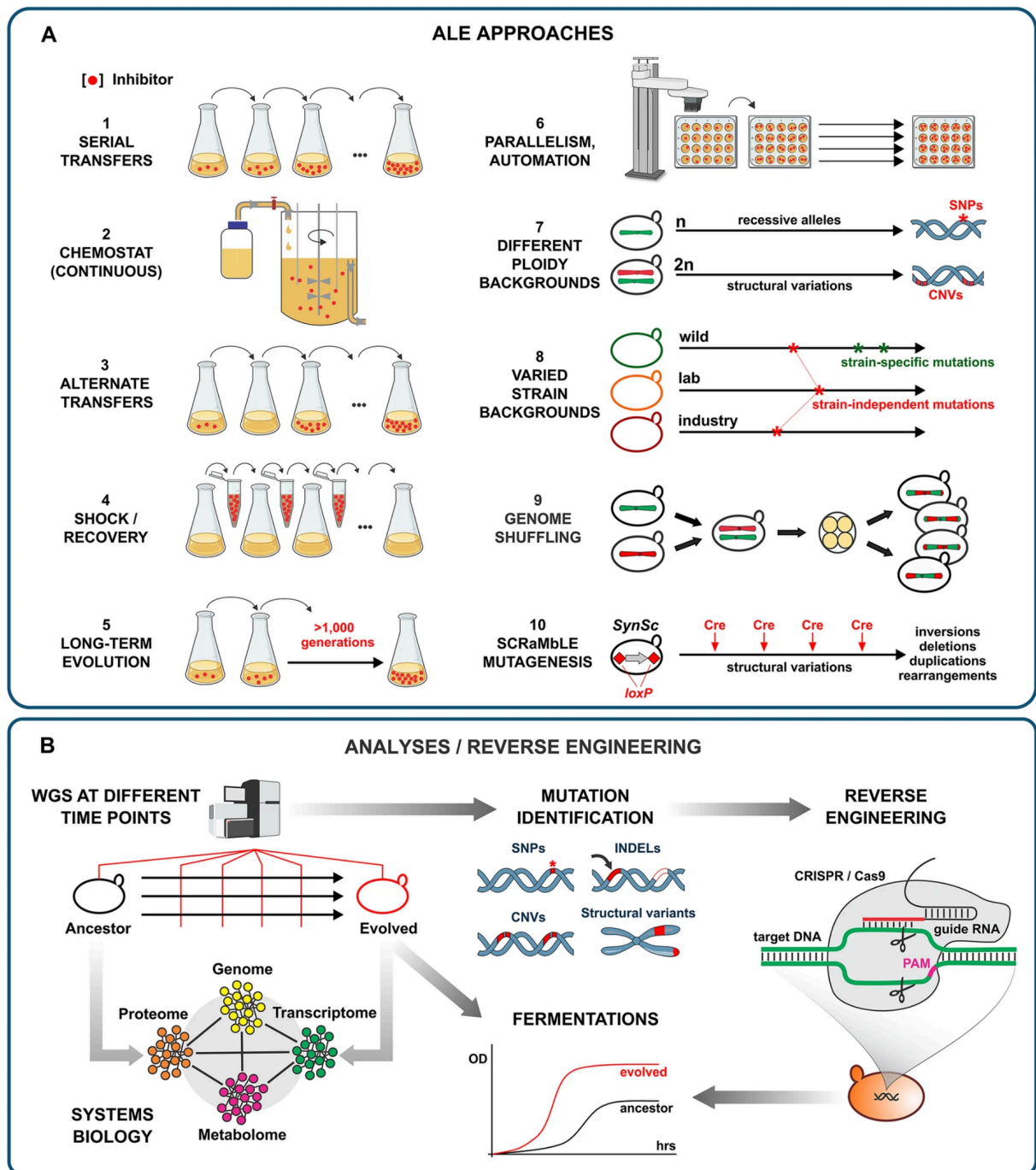


Figure 2: Modes of adaptive laboratory evolution with yeasts. **a** Ten different regimes of yeast propagation and selection (ALE approaches) are depicted. Advantages associated with each one of them are discussed in the text. The SCRaMbLE mutagenesis is performed on a synthetic *S. cerevisiae* (SynSc) engineered to have *loxP* sites nearby non-essential genes. The Cre recombinase is periodically induced driving structural variations on the genome. **b** Workflow of possible analyses to

understand the phenotypes and molecular mechanisms associated with the evolution process. WGS of evolved populations or clones compared to the ancestor reveals the mutations that occurred during the evolution, including single nucleotide polymorphisms (SNPs), insertions/deletions (INDELS), copy number variations (CNVs), and chromosome structural variations. Evolved alleles discovered through ALE are studied via reverse engineering of parental strains by the use of modern genetic tools, such as the CRISPR/Cas9. Comparison of ancestral, evolved, and reverse engineered strains are commonly performed through fermentation assays. Functional genomics approaches, such as transcriptomics, proteomics and metabolomics, are also useful to understand the impact of adaptive evolution under a systems biology perspective

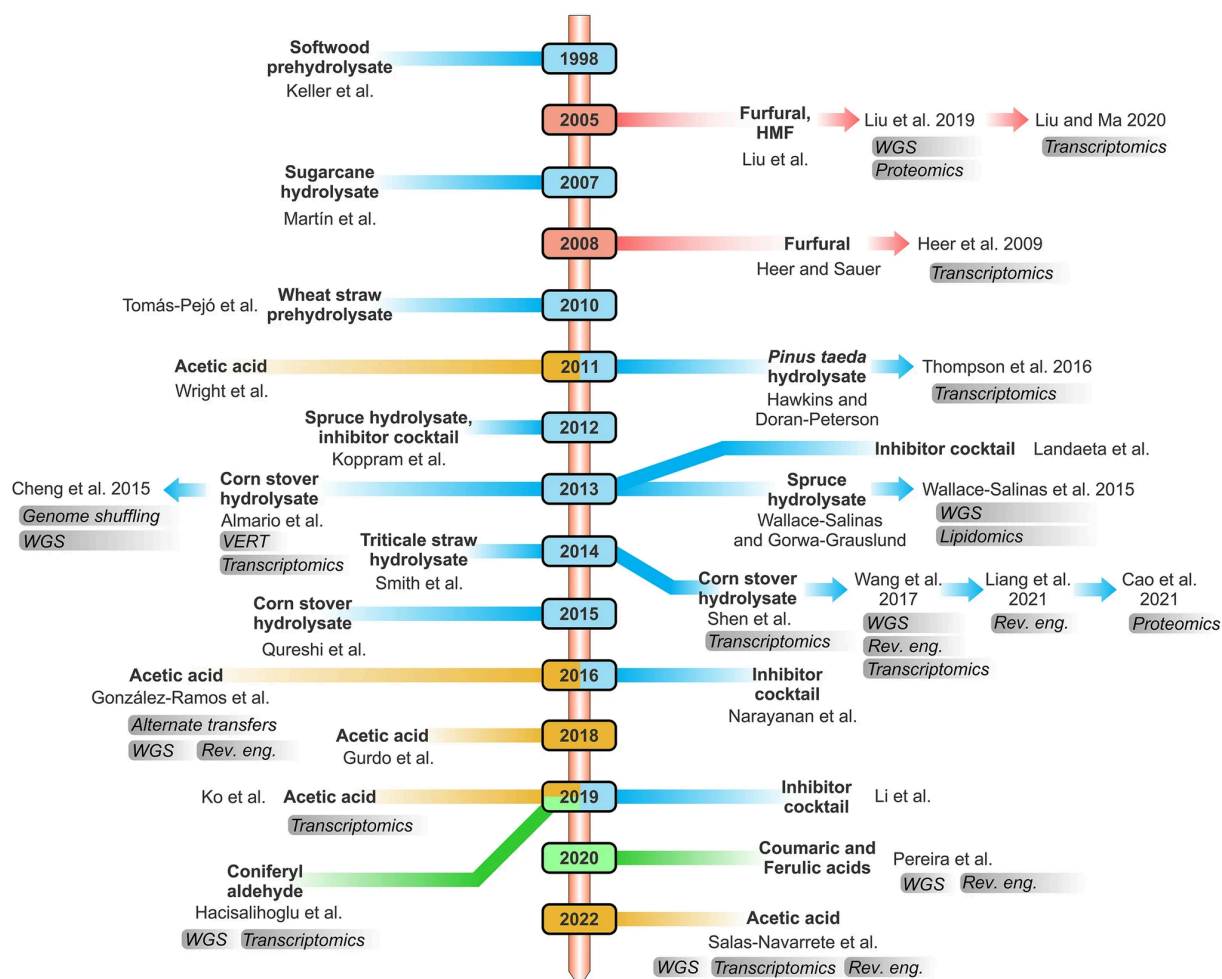


Figure 3: Timeline of ALE experiments with LCHs or individual inhibitors.

Evolution experiments with hydrolysate liquors or inhibitor cocktails are represented in blue. Evolution experiments with individual inhibitors are coloured in orange (acetic

acid), green (phenols), and red (furan aldehydes). Arrows indicate follow-up studies with the evolved strains. Genomics, reverse engineering (Rev. eng.), and ALE approaches associated with the studies are marked in grey background

Diverse ALE protocols illustrate yeast adaptability to LCHs

Several studies have used ALE to improve yeast fermentation of hydrolysates derived from different feedstocks or upon exposure to single inhibitors (see the timeline on Fig. 3). Those evolution experiments were conducted with *S. cerevisiae* from varied sources, including lab (Gonzalez-Ramos et al. 2016), industry (Shen et al. 2014), or genetically engineered strains for xylose consumption (Ko et al. 2020). Propagation schemes (Fig. 2a) were based on either batch or continuous cultures in fermenters (Wright et al. 2011) or serial dilutions in shake flasks (Almario et al. 2013). Toxic doses of LCH liquors (or inhibitors) were applied over repeated serial transfers or hundreds of cells generations, resulting in strains outperforming their respective ancestors in fermentations with LCH inhibitors (Heer and Sauer 2008; Liu et al. 2005; Pereira et al. 2020; Smith et al. 2014). Such improvements of the evolved strains were generally demonstrated in LCH fermentations through shorter lag phases, faster growth rates, increased biomass formation, and efficient sugar consumption coupled to higher ethanol yields. Some adapted strains also displayed better growth performance when tested in solid medium containing inhibitors (Hacisalihoglu et al. 2019; Shen et al. 2014). Taken together, ALE studies with hydrolysates or inhibitors illustrate the principle that propagation under selection has the power to engineer yeast strains with increased robustness against LCH toxicity.

Such evolutionary dynamics was elegantly demonstrated in an ALE approach named VERT (visualising evolution in real-time). For VERT, three *S. cerevisiae* isogenic strains, respectively tagged with yellow (YFP), red (RFP), or green (GFP) fluorescence proteins, were mixed in nearly equal proportions and left to compete through ~463 generations in a culture regime entailing increments of corn stover hydrolysate (Almario et al. 2013). During ALE, periodic fluorescence quantification via flow cytometry allowed real-time observation of rapid expansion events related to certain fluorescence-tagged subpopulations (Almario et al. 2013). This implies that adaptive mutations accumulated in particular clonal lineages, enabling their quick-spreading within the population (i.e., a selective sweep), while less fit clones became concomitantly underrepresented and eventually went extinct. Such

evolutionary race corresponds to a clonal interference mode of competition between subpopulations carrying different adaptive alleles, as typically observed in ALE protocols involving asexual propagation of microorganisms (Barrick and Lenski 2013). The VERT approach allowed a rational scheme for selecting clones related to subpopulation expansion events. When compared to the parental strains, the selected clones demonstrated improved growth rates of up to 57%, 24%, 22%, and 12% in hydrolysates, furfural, HMF and acetic acid, respectively (Almario et al. 2013).

Transcriptomics of evolved yeasts uncovers key responses to LCHs

Global gene expression analysis (transcriptomics) has been often applied to ALE-derived strains, providing valuable insights on the molecular basis of LCH adaptation. For example, the observation across different tolerant strains that reductive enzymatic systems are upregulated underscores a general mechanism for cellular detoxification of furan aldehydes, i.e., the in situ bioconversion of furfural and HMF into less harmful compounds (Fig. 4). Typically, the toxicity of furan aldehydes in yeast fermentations accounts for an extended lag phase and decrease in cell viability (Heer and Sauer 2008). This inhibition is relieved via chemical detoxification when reductive power is derived from NAD(P)H (or NADH) to convert furfural into 2-furanmethanol and 5-HMF into furan-2,5-dimethanol (Jayakody and Jin 2021). Transcriptomics profiles (microarray) of inhibitor-tolerant VERT clones under HMF and/or furfural showed upregulation of the aldehyde reductases/dehydrogenases encoding genes *ADH6*, *ALD4*, and *GRE3* (Almario et al. 2013). Similarly, the evolved TMB3400-FT30-3 strain, selected through ~ 300 generations under increasing furfural concentrations (Heer and Sauer 2008), boosted responses to the inhibitor that included upregulation of *ADH7* (a NAD(P)H-dependent alcohol dehydrogenase), as well as *YKL071W*, *GRE2*, *AAD4*, *YDL124W*, and *YLL056C* (encoding NAD(P)H- or NADH-dependent oxidoreductases). The overexpression of *ADH7* and *YKL071W* in the parental background reduced its lag phase by 30% in the presence of 11 mM furfural (Heer et al. 2009). In another example, the NRRL Y-50049 strain evolved under furfural and HMF selection (Liu et al. 2005, 2009). This mutant can perform fermentations at 20 mM of combined furfural and HMF and is capable of fully detoxifying these inhibitors after 65 h, while its parental strain cannot establish a viable culture under the same condition (Liu et al. 2009). Transcriptomic analyses of NRRL Y-50049 showed upregulation of the reductases/dehydrogenases *ADH1*, *ADH6*,

ADH7, *ALD4*, *SFA1*, *ARI1*, *GRE2*, and *GRE3* under exposure to furan aldehydes (Liu and Ma 2020; Liu et al. 2009). In addition, genes related to the PPP, including *ZWF1*, *SOL3*, *GND1*, *GND2*, *NQM1*, and *RBK1*, were also upregulated in this strain, supporting that adaptive evolution remodelled metabolic fluxes to optimally replenish the NAD(P)H pool for sustained inhibitor detoxification (Liu et al. 2019; Liu and Ma 2020).

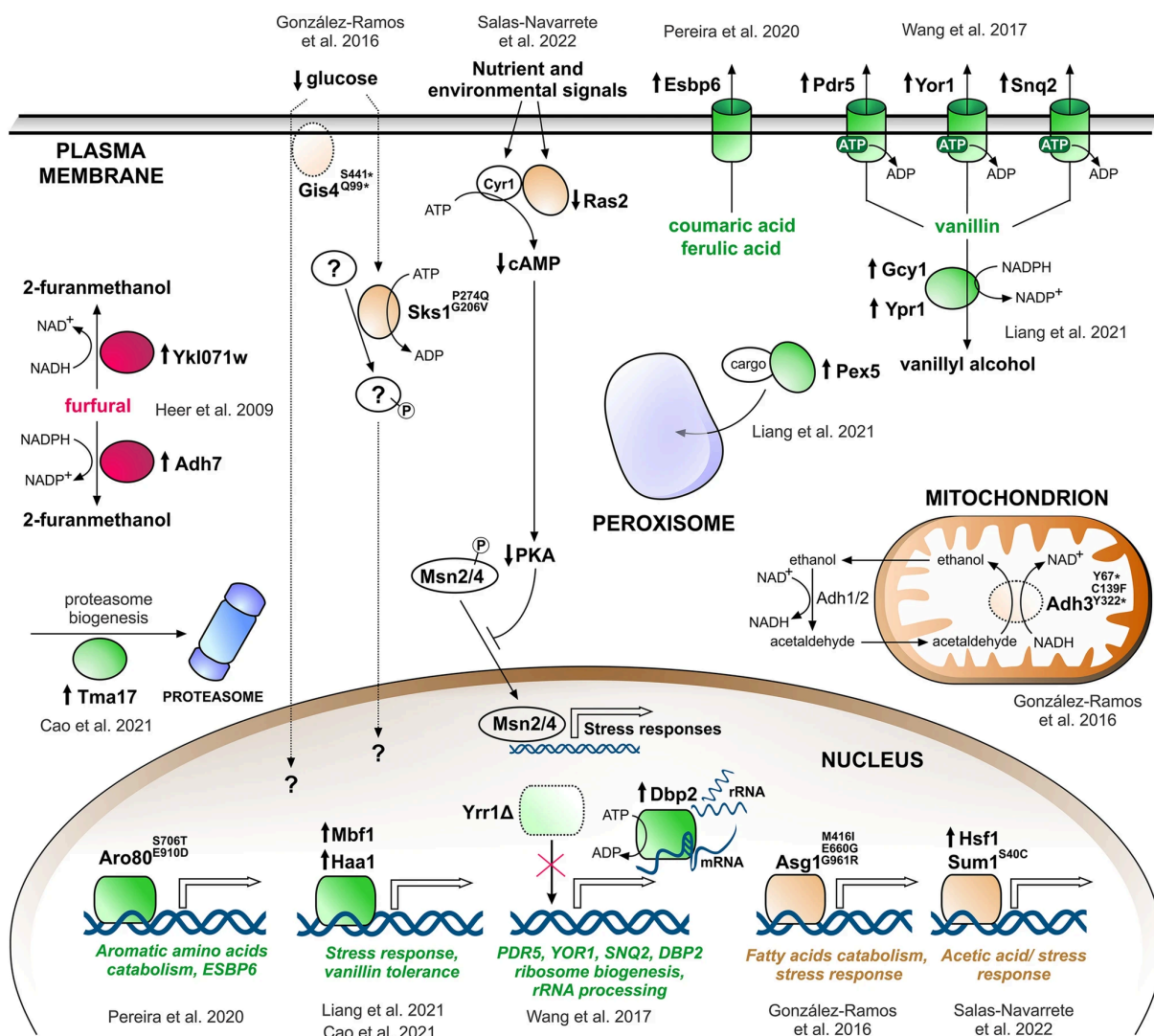


Figure 4: Putative cellular functions of proteins corresponding to alleles or overexpressed proteins found during yeast ALE to LCHs. Depicted are only molecular components that have been reverse engineered and positively impacted the tolerance to the inhibitors. The corresponding proteins are represented in the colour green (phenols), orange (acetic acid) or red (furfurals), according to the kind of inhibitor to which they confer tolerance. Protein names are written indicating the resulting amino acid change when the protein derives from point mutations. The

overexpression of proteins is represented by an upward arrow and downregulated proteins by a downward arrow. Molecular components missing as a consequence of gene knockouts or defective alleles are depicted in dotted lines and faint colours. DNA binding transcriptional factors (Aro80, Haa1, Yrr1, Asg1, Hsf1), transcriptional coactivator (Mbf1) or repressor (Sum1), and a DNA helicase (Dbp2) are represented as rectangles in the nucleus. Circular forms represent cytoplasmic components, while cylinders represent membrane transporters. Molecular mechanisms are putative. In some cases, downstream effectors are not known (?) and signal transduction cascades (dotted lines) are speculative (For example, Sks1 is a protein kinase and Gis4 is a membrane associated protein that are somehow involved in different signal transduction pathways responsive to the glucose status). Salas-Navarrete and collaborators (2022) demonstrated that the synergic effect of three alleles, comprising *SUM1* (S40C) and mutations at the 3' UTR of *RAS2* and *HSF1*, conferred combined tolerance to acetic acid, low pH and high temperature. Mechanistic aspects and tolerance effects of the reverse-engineered components are further discussed in the published studies (indicated) and in the main text of the manuscript.

Improved consumption (i.e., detoxification) of acetic acid and phenols was also observed in several ALE-derived yeasts (Almario et al. 2013; Hawkins and Doran-Peterson 2011; Li et al. 2019; Martin et al. 2007; Smith et al. 2014). In addition, transcriptomic of evolved strains corroborate that upregulation of NAD(P)H reductases and plasma membrane efflux pumps comprise general defence mechanisms for detoxification of phenolics (Hacisalihoglu et al. 2019; Shen et al. 2014) (Fig. 1). Neutralisation of ROS resulting from LCH stress is equally important and is usually achieved through a battery of antioxidative responses coordinated via Yap1p (Almario et al. 2013; Liu and Ma 2020) (Fig. 1). For example, a microarray analysis of strain NRRL Y-50049 was conducted with samples collected during the lag phase induced by 20 mM furfural/HMF. It was observed the higher transcription of 27 genes related to oxidative stress control, involving catalases (*CTA1*, *CTT1*), superoxide dismutases (*SOD1/2*), thioredoxins (*TRR1*, *TRX2*), glutathione metabolic genes (*GLR1*, *GPX1*, *GRX3*, *GTO1*, and *GTT2*), and also 22 genes related to the superpathway of sulphur amino acids biosynthesis (Liu and Ma 2020).

Upon exposure to multiple inhibitors, induction of genes supporting ROS detoxification (e.g., *CTT1* and *GTT1*) was also observed in an RNA-seq analysis of ALE clones tolerant to 17.5% dw/v pretreated pine (3% w/v SO₂ at 210 °C for 10

min) (Hawkins and Doran-Peterson 2011; Thompson et al. 2016). Transcriptional perturbations also included upregulation of genes encoding proteins involved in multidrug efflux (e.g., *PDR10* and *SGE1*), aldehyde metabolism (e.g., *ALD2/3*, *ADH1*, and *FDH1*), redox cofactor regeneration (e.g., *TDH3* and *TKL1/2*), and cell wall/plasma membrane functions (e.g., *HES1* and *PIR1*) (Thompson et al. 2016). Remodelling of the cell periphery was further highlighted through the analysis of ISO12, a strain derived after 280 generations in the presence of 50% spruce hydrolysate and under thermal stress (up to 39 °C) (Wallace-Salinas and Gorwa-Grauslund 2013). Under this condition the parental Ethanol Red strain is not capable of growth even after 50 h of incubation. WGS of ISO12 allowed estimation of positive selection for mutations in 11 genes, some of them encoding plasma membrane proteins (e.g., *MTL1*, *HXT13*, *CYC3*, and *SED4*). In addition, a complementary lipidomic analysis indicated that nine lipid types were altered in ISO12 in comparison to the parental strain (Wallace-Salinas et al. 2015). Finally, in response to 20 mM furfural/HMF, the ALE NRRL Y-50049 strain also showed differential upregulation of genes encoding plasma membrane and the cell wall proteins, corroborating that the cell periphery is an important target for adaptive evolution (Liu and Ma 2020).

WGS and reverse engineering: harnessing the power of adaptive evolution

Although transcriptomics has been emphasised in many ALE studies, phenotype improvement during evolution primarily emanates from DNA mutations. For this reason, WGS via high-throughput DNA sequencing platforms is nowadays indispensable in ALE studies (Mans et al. 2018; McDonald 2019). Fundamentally, charting DNA mutations is a requirement for reverse genetic engineering of the most important ALE variants (Mans et al. 2018; McDonald 2019). By introducing newly found polymorphisms into the ancestral background, it is possible to evaluate the individual contribution of the evolved alleles or their cumulative effects for LCH tolerance (Barrick and Lenski 2013; Mans et al. 2018). Fitness, defined as the propensity to leave descents in a given condition, can be reliably measured for reverse-engineered strains in direct competition with a reference, which is usually a parental strain expressing a phenotypic mark (e.g., a GFP reporter) (McDonald 2019). In a fitness assay, the competitors are mixed in equal proportions and let

propagate in the environment used for the ALE experiment (e.g., under LCH pressure). In the end, the proportion is once again measured (e.g., by flow cytometry), providing a way to quantify how better (or not) the reverse-engineered strain grew in comparison to the reference (McDonald 2019). Further evaluations of ancestor versus reverse-engineered strains, considering parameters such as tolerance to specific inhibitors, specific growth rates, fermentation performances, biomass yield, and even cell viability, are highly useful to gauge the contribution of evolved alleles to yeast adaptation (Dragosits and Mattanovich 2013). Reverse engineering and phenotyping are also important for identifying the occurrence of trade-offs, in which alleles beneficial in a given environment (e.g., LCH challenge) are detrimental to the fitness under different conditions or in a non-stress circumstance (e.g., growth in rich medium). Careful consideration of possible trade-offs effects in reverse-engineered yeasts is especially relevant to ensure that tolerance to LCHs does not come at the expense of fermentation efficiency (Dragosits and Mattanovich 2013; Mans et al. 2018). Finally, systems biology analysis, such as transcriptomics or proteomics of the reverse-engineered strains, offers a powerful complement to understanding the global impact of newly evolved traits (Espinosa et al. 2020; Wang et al. 2017).

From evolved genotypes to engineered tolerant phenotypes

Given its importance, WGS has been applied to strains selected to withstand LCH liquors or inhibitors (Cheng et al. 2015; Hacisalihoglu et al. 2019; Wallace-Salinas et al. 2015). However, in only a few cases, the identified mutations were validated through reverse engineering. One of these studies was based on an innovative ALE protocol designed to overcome the problem of evolving yeasts under continuous exposure to acetic acid. Such selection mode often results in a transient tolerant phenotype that is lost when the strain is cultured in a medium without acetic acid (Wright et al. 2011). However, by alternating propagation in the presence or not of acetic acid, it was possible to obtain evolved yeast populations presenting a permanent tolerance to the inhibitor (Gonzalez-Ramos et al. 2016). Clones isolated from five populations after ~50 alternate batch transfers and one UV-mutagenised strain were able to grow on up to 15 g/L acetic acid without pre-induction with the inhibitor, while the parental CEN.PK113-7D was not viable at concentrations higher than 10 g/L after five days of incubation. Six selected clones were subjected to WGS.

Mutations related to four genes, *ASG1*, *ADH3*, *SKS1* and *GIS4* (Fig. 4), were linked to acetic acid tolerance after backcrossing evolved clones with the sensitive parental strain. Reverse engineering of the identified alleles showed their individual contribution to the acquired acetic acid tolerance. In culture media containing 5–9 g/L acetic acid, reverse engineered strains displayed a latency phase significantly shorter than the parental CEN.PK113-7D. This reduction was even more pronounced when different mutations were combined in a single strain (Gonzalez-Ramos et al. 2016).

In another case study, the EMV-8 strain adapted to corn stover LCH displayed high tolerance to the phenol-type compound vanillin (Shen et al. 2014). In a culture medium with 2 g/L vanillin EMV-8 presented a maximum specific growth rate of $0.104 \pm 0.002 \text{ h}^{-1}$, compared to only $0.038 \pm 0.000 \text{ h}^{-1}$ displayed by the ancestor. WGS of EMV-8 and its parental strain captured 450 SNPs and 44 INDELs exclusive to EMV-8 (Wang et al. 2017). Reverse engineering revealed that *YRR1*-deleted strains had 142% increase in the maximum specific growth rate compared to the sensitive parental in the presence of 6 mM vanillin (Wang et al. 2017). *YRR1* encodes a Zn²-Cys₆ zinc-finger transcription factor whose deletion in the presence of vanillin led to upregulation of *PDR5*, *YOR1*, and *SNQ2* (Wang et al. 2017) and accumulation of the proteins Haa1p, Tma17p, and Mbf1p (Fig. 4) (Cao et al. 2021). Accordingly, in the presence of 6 mM vanillin the lag phase was reduced by the overexpression of *PDR5*, *YOR1*, and *SNQ2* (Wang et al. 2017), while overexpressing *HAA1*, *TMA17*, and *MBF1* increased the specific growth rates by 18%, 33%, and 15% compared to the control, respectively (Cao et al. 2021). Transcriptomic changes in the *YRR1*Δ strain also included upregulation of ribosome structural and rRNA processing genes. In accordance with this result, the overexpression of *DBP2* (encoding an RNA helicase involved in mRNA decay and rRNA processing) improved growth rates by 59% compared to the control in the presence of 6 mM vanillin (Wang et al. 2017).

The WGS of EMV-8 also uncovered non-synonymous mutations in *GCY1* and *MBF1* (Liang et al. 2021). The overexpression of evolved alleles *GCY1*^{A181E} and *MBF1*^{P148L}, the corresponding wild-types genes, and also the *GCY1* paralog *YPR1* were all beneficial to yeast growth in the presence of vanillin (Fig. 4) (Liang et al. 2021). Interestingly, the expression of Mbf1p and Gcy1p catalytic inactive forms similarly yielded vanillin resistance, indicating that inhibitor tolerance may be due to alternative functions associated with these proteins (Liang et al. 2021). In fact, the

glycerol dehydrogenase Gcy1p also acts as an mRNA-binding protein interacting with the *PEX5* mRNA. Pex5p functions as a peroxisomal signal receptor for protein import, and its enhanced expression positively affects vanillin tolerance (Liang et al. 2021). Overall, the overexpression of *GCY1*, *MBF1*, *YPR1*, and *PEX5* resulted in improvement of the specific growth rates by 49%, 48%, 41%, 44%, respectively, in the presence of 6 mM vanillin.

A final example of a well-designed ALE approach is provided by the evolution of yeast resistance to the phenolic compounds coumaric and ferulic acids (Pereira et al. 2020). In a robot-assisted protocol, five populations for each inhibitor were selected through several batch transfers with inhibitor concentrations increasing from 0.2 g/L to 1.0 g/L (90 passages for the coumaric acid populations and 69 for the ferulic acid subgroup). Three high-tolerant clones from each population (30 in total) were targeted for WGS. Non-synonymous mutations affecting *ARO80*, encoding a transcriptional regulator of aromatic amino acids catabolism, were found in all populations. The variants *ARO80*^{S706T} and *ARO80*^{E910D} were reverse-engineered into the ancestor background (Fig. 4), resulting in strains with improved growth rates under exposure to 0.8 g/L coumaric and 0.4 g/L ferulic acids, which are non-permissive concentrations for the parental strain propagation (Pereira et al. 2020). The notion that Aro80p is a regulatory protein allowed to identify one of its transcriptional targets, *ESBP6*, within a segmental amplification on the Chr. XIV shared among most evolved populations. Overexpression of *ESBP6* increased growth rates to 0.36 h⁻¹ for both coumaric (0.8 g/L) and ferulic (0.4 g/L) acids, a number higher than the growth rates observed for the ALE-derived clones. The product of *ESBP6* is a putative permease at the plasma membrane that presumably confers adaptation by excreting coumaric and ferulic acids out of the cell (Pereira et al. 2020).

These evolution experiments for yeast tolerance to phenolics exemplify how ALE provides a powerful approach for deciphering the nature and action of causative alleles. That includes testing variants, paralogs, and the analysis of molecular components whose expression/function is modulated by the primary mutations. Those downstream players usually are the actual effectors leading to cellular adaptation. Therefore, ALE provides a comprehensive testbed towards the development of superior 2G yeasts.

How to diversify ALE protocols for yeast LCH adaptation?

To date, only a few ALE experiments for improving *S. cerevisiae* tolerance to LCH inhibitors have been reported (Fig. 3). Most of these relied on small experimental settings with a single population or a limited number of replicates propagated for a short period. In some cases, WGS and/or transcriptomics analyses have been useful to understand LCH adaptation (Fig. 3). However, to our knowledge, the validation of evolved alleles via their reverse engineering into the parental strains, a golden standard for modern-day ALE, has been undertaken in a scarce number of experiments with LCH inhibitors (Gonzalez-Ramos et al. 2016; Liang et al. 2021; Pereira et al. 2020; Wang et al. 2017). Therefore, there are plenty of possibilities still open for using adaptive evolution in the context of 2G bioprocesses. However, what can effectively be done to retrieve different results? One critical question is how to diversify the ALE procedure for maximising the emergence of novel genetic variants, allowing a richer understanding of yeast responses to LCH toxicity (Fig. 2).

Unconventional ALE protocols: alternating treatments/ drastic selections

ALE protocols conventionally apply propagations schemes based on either serial batch transfers into a liquid medium or controlled growth conditions in chemostats (Dragosits and Mattanovich 2013). In both modes, there is a steady exposure to the stressor. By contrast, Gonzalez-Ramos and collaborators (2016) demonstrated the utility of alternating yeast propagation in a culture medium with and without the inhibitor to favour a constitutive acetic acid tolerance. This simple innovation allowed the selection of a different mutational landscape underpinning the desired phenotype (Gonzalez-Ramos et al. 2016). We suggest that the application of varying ALE regimes may promote the discovery of novel adaptive alleles. Another unconventional selection approach is using harsh treatments, such as a nearly-lethal dose of the inhibitor for a few hours (Fig. 2a). If surviving cells are recuperated in a rich medium without the inhibitor, then further rounds of shock/recovery can be applied. With high mortality rates, adaptive alleles can quickly sweep into dominance in a population, speeding up evolution (Barrick and Lenski 2013). However, evidence has suggested that selection through acute treatments may lead to undesirable trade-offs. For example, yeasts selected through high ethanol exposures actually displayed inferior fermentation performances (Snoek et al. 2015). Nonetheless, the genetic basis of adaptation to stressful shocks is still poorly understood and scarcely

investigated through ALE, an approach that could clarify whether alleles selected to survive toxic doses may benefit growth under moderate inhibitor concentrations. Anyway, contrasting continuous inhibitor exposure *versus* acute treatments in ALE projects may add to our understanding of yeast LCH adaptation.

Long-term evolution experiments

Some evolution experiments have been maintained for over a thousand cell generations (Fig. 2a). These enduring ALE protocols are epitomised by Richard Lenski and colleagues' Long-Term Evolution Experiment (LTEE), in which 12 *E. coli* populations have been cultured for over 75,000 generations in a glucose-limited medium (Good et al. 2017). Large-scale experiments with yeast propagating in YPD or synthetic complex medium were also conducted for 4000 (Fisher et al. 2018) and 10,000 generations (Johnson et al. 2021). One recurrent pattern in those long-term experiments in stable environments is that molecular evolution tends to be steady over time (Johnson et al. 2021). New mutations constantly arise even after decades of the *E. coli* LTEE (Good et al. 2017). In contrast, fitness increments contributed by each new mutation become lower over time (Barrick and Lenski 2013). Mutations fixed early have larger fitness effects than mutations dominating by the end of an ALE (Barrick and Lenski 2013; Good et al. 2017). Despite such diminishing returns in adaptability, maintaining protocols for over a thousand generations can maximise the discovery of possible beneficial mutations (Good et al. 2017; Johnson et al. 2021). Long-term experiments may be particularly informative when selective pressures are raised over time, as in the case of most ALE experiments on LCH adaptation. The genetic basis for tolerance at the beginning of an ALE (when stresses are mild) may differ from adaptive mechanisms to concentrated LCHs found at the evolution end. In this case, adaptive alleles fixed later in an ALE may better suit the needs for harsh industrial fermentations. This was observed for mutated alleles of *PRT1*, *VPS70* and *MEX67* that were isolated at the final stage of an ALE for alcohol tolerance, supporting yeast growth under high ethanol content (Voordeckers et al. 2015).

WGS of ALE populations at intermediate time points

WGS of populations or clones sampled at the ALE end-point provides only static information about the evolution outcome. By contrast, WGS at intermediate time points (Fig. 2b) allows to assess the evolutionary history and reconstruct

adaptation trajectories (Good et al. 2017; Johnson et al. 2021). This is important for an initial evaluation of which mutations may be relevant for adaptation. As mentioned, alleles providing higher fitness contributions are fixed early (Barrick and Lenski 2013), whereas mutations contributing to a high-stress tolerance emerge late if incremental selection pressure is applied (Voordeckers et al. 2015). WGS of populations at intermediate points may also discern patterns of clonal interference, in which lineages having mutations of medium or low fitness effect may be driven to extinction if a higher fitness subpopulation sweeps into fixation (Good et al. 2017; Johnson et al. 2021). In addition, mapping allele frequencies over time in a population permits the identification of neutral or deleterious hitchhiker mutations, which frequently accumulate in asexual ALE populations by genetic linkage to adaptive driver alleles (Good et al. 2017; Johnson et al. 2021). Finally, knowing when evolved alleles appeared during ALE helps to identify patterns of epistasis, in which an early fixed mutation modulates the fitness effect of a subsequent one or even facilitates its emergence (Barrick and Lenski 2013). Such historical contingency is perfectly exemplified by the appearance after 30,000 generations of a citrate consuming phenotype in a unique *E. coli* LTEE population, only possible after previous potentiating mutations (Blount et al. 2012).

Parallel experimental populations

By serial passages conducted in 96-well plate format, yeast evolution experiments have been successfully performed with tens or hundreds of populations in parallel for thousands of generations (Fig. 2a) (Fisher et al. 2018; Johnson et al. 2021). There are some key advantages in propagating replicate experimental lines. Fixation of adaptive alleles in ALE is a function of time. Thus, only a few mutations can sweep into dominance in a population during a short-term evolution (McDonald 2019). However, when many parallel populations are propagated, there is a proportional increase in the number of fixed mutations, including variants related to the same gene (McDonald 2019). When a gene is hit multiple times by mutations in different populations, this represents a signature of parallel adaptive evolution (Good et al. 2017; Johnson et al. 2021). The same reasoning is valid for recurrent mutations associated with genes of the same metabolic or signal transduction pathway. Such events of parallel evolution can be evaluated by statistical approaches to estimate the probability of recurrent mutations happening by chance within a targeted gene in

different populations (Good et al. 2017; Johnson et al. 2021). This is important, as parallelism may provide the best guidance for identifying key ALE causal alleles. For example, WGS of evolved clones from five different acetic acid-tolerant populations revealed different evolved alleles related to *ASG1*, *ADH3*, *SKS1*, and *GIS4* (Gonzalez-Ramos et al. 2016). Similarly, alternative *ARO80* mutations were recovered from 10/10 populations propagated in the presence of either coumaric or ferulic acids (Pereira et al. 2020). Reverse engineering confirmed the strong adaptive effects associated with the selected alleles (Gonzalez-Ramos et al. 2016; Pereira et al. 2020).

Different ploidy backgrounds

Most wild *S. cerevisiae* and industrial strains have a diploid or sometimes polyploid genome content, supporting that evolution frequently selects more than one copy of the yeast genome (Peter et al. 2018). In fact, the diploid state is typically associated with increased robustness to stressors. Diploids have a 3.6% growth advantage in direct competition with haploids in YPD medium (Fisher et al. 2018). Therefore, there is no surprise that haploid populations frequently undergo auto-diploidisation in evolution experiments (Fisher et al. 2018; Voordeckers et al. 2015). This is relevant considering that diploid populations follow a different path of molecular evolution than haploids. Newly arising adaptive recessive mutations tend to be expressed in haploid genomes. In contrast, they are buffered in a heterozygous state in a diploid genome unless a recombination event brings the mutation into a homozygous state (i.e., a loss-of-heterozygosity) (Fisher et al. 2018). Direct accessibility to beneficial recessive mutations is probably the reason why haploid populations tend to evolve faster than diploids in laboratory experiments (Fisher et al. 2018; Johnson et al. 2021). Diploid genomes favour fixation of dominant or semi-dominant mutations and are also permissive to the accumulation of recessive deleterious alleles in a heterozygous state (Fisher et al. 2018). Structural variants, copy number variations (CNVs), deletions, and chromosomal aneuploidies represent the selection targets that are more frequently observed in diploids than in haploids (Fisher et al. 2018). Therefore, contrasting diploid and haploid populations in ALE experiments may be advantageous to assess ploidy-specific mutations, diversifying the discovery of beneficial variants (Fig. 2a).

Contrasting strain backgrounds

Different *S. cerevisiae* isolates from industry, lab, or natural environments represent a source of genetic diversity (Jacobus et al. 2021b; Peter et al. 2018). Physiological responses, transcriptional patterns, and fitness to different stresses vary widely according to the yeast genetic background (Li et al. 2015; Peter et al. 2018). For example, 79 different isolates from distinct environmental or geographic provenances displayed a wide spectrum of tolerance/sensitivity profiles in synthetic hydrolysates (Sardi et al. 2016). Global gene expression in the presence or absence of inhibitors also significantly varies depending on the strain (Sardi et al. 2016). Genetic background-specific effects are as well observed in selection screens or when genes or metabolic pathways are engineered in different yeasts (Matsushika et al. 2009; Sardi et al. 2016). Given such strain-specific effects, ALE experiments may benefit from contrasting yeasts derived from different backgrounds, such as lab, industry, or natural isolates (Fig. 2a). This approach may favour a broad understanding of general aspects of LCH tolerance in *S. cerevisiae*, including identifying adaptive alleles shared between strains (i.e., background-independent variants) (Sardi et al. 2016). On the other hand, yeast engineering for 2G processes may profit from using industrial-derived chassis, naturally adapted to harsh conditions (Cunha et al. 2019; Li et al. 2015). In this case, it may be advantageous to target for ALE the same industrial yeast chosen for metabolic engineering, thereby allowing the identification of strain-specific alleles for LCH tolerance.

Genome shuffling: combining adaptive alleles, decoupling deleterious mutations

Genome shuffling refers to a strain development approach that relies on meiotic recombination between parental strains to segregate their respective adaptive alleles into a daughter cell (Snoek et al. 2015). In ALE protocols with yeasts, genome shuffling can be simply attained through mating and sporulation events (i.e., by eliciting the sexual cell cycle, Fig. 2a) (Cheng et al. 2015; McDonald et al. 2016). In the context of ALE, genome shuffling is useful for breaking clonal interference patterns and accelerating evolution, as competing genotypes are recombined and their beneficial mutations joined into one segregating genome (McDonald et al. 2016). On the other hand, recombination events between different cell lineages can dissociate deleterious hitchhiker mutations from adaptive driver alleles, promoting a

cleansing effect on genomes (McDonald et al. 2016). Genome shuffling has been applied to recombine clones selected in corn stover hydrolysate, resulting in a recombinant with 100% fitness increase under the challenge of HMF (Cheng et al. 2015). This significant improvement illustrates the usefulness of including sexual cycles in ALE protocols with yeasts to accelerate strain evolution.

Adaptive evolution via SCRaMbLE

Chemical and UV mutagenesis have been applied in ALE protocols aiming at selecting tolerance to corn stover LCH (Shen et al. 2014) and acetic acid (Gonzalez-Ramos et al. 2016), respectively. Mutagenesis may accelerate the evolution process when selection pressure is applied to enrich the newly arising adaptive mutations in a population. Recently, a new kind of mutagenesis technology was elegantly embedded within the synthetic *S. cerevisiae* (Sc2.0) genome (Richardson et al. 2017). Synthetic chromosome recombination and modification by LoxP-mediated evolution (SCRaMbLE) relies on the strategic placement of 4,000 loxP sites adjacent to non-essential genes in the Sc2.0 genome (Richardson et al. 2017). Induction of the Cre recombinase on-demand results in chromosome-wide rearrangements, generating a population of yeasts carrying structural variants that evolution can select upon. SCRaMbLE have been used for selecting yeasts with enhanced growth on xylose (Blount et al. 2018) and higher tolerance to heat, ethanol, and acetic acid (Luo et al. 2018). The use of SCRaMbLE in ALE to enhance yeast resistance to LCHs is not only attractive by affording a quick adaptation to the stress but also for selecting different mutation types (i.e., DNA deletions, inversions, and duplications, Fig. 2a), contrasting with SNPs prevalent in standard ALE protocols (Blount et al. 2018; Luo et al. 2018).

Complementary systems biology approaches

As previously exemplified, functional genomics approaches have often been used to complement ALE experiments with LCHs (Fig. 2b) (Almario et al. 2013; Shen et al. 2014). Transcriptomics (Liu and Ma 2020), proteomics (Liu et al. 2019), and metabolomics (Wallace-Salinas et al. 2015) have provided insights into the physiology of LCH adaptation and helped to identify molecular effectors and mechanisms involved in the expression of an adapted phenotype. This is important, as mutations related to general regulators (e.g., transcriptional factors) are commonly

found in ALE populations, implying that adaptation involves systemic changes in the cell (Wang et al. 2017). Knowing which downstream components are regulated by the primary mutations and how metabolic fluxes and physiological responses are modulated to support an adapted phenotype provides much more options to strategize how to implement targeted genetic modifications for obtaining robust 2G yeasts (Fletcher and Baetz 2020; Wang et al. 2017).

Perspectives: a genetic catalogue to redesign yeast LCH tolerance

By simply playing out evolution, ALE already provides a direct way for improving the yeast phenotype. However, the utility of ALE as a pipeline for allele discovery is what offers a promising possibility to revamp yeast robustness for 2G processes. We envisage that the implementation of technically sound ALE projects, by combining several useful approaches (Fig. 2a, b), may lead to a compilation of key adaptive alleles related to LCH tolerance. This resource complements molecular strategies validated in previous reports (Tables 1 and 2). Based on this information, the combinatorial introduction of adaptive variants and the overexpression of genes and pathways can be tested aiming at enhancing yeast adaptation to biomass toxicity. The CRISPR/Cas9 system provides a formidable tool for performing such an engineering endeavour. CRISPR/Cas9 is facilitating the introduction and/or optimisation in yeast of different biotechnological relevant traits, such as fatty acid accumulation (Zhang et al. 2019a, b), monoterpenes biosynthesis (Yee et al. 2019), and 2-phenylethanol production (Hassing et al. 2019). CRISPR/Cas9 can promote pathway engineering via multiple insertions at different loci, allowing the introduction of the β -carotene biosynthetic route (Ronda et al. 2015) and establishment of a strain combining xylose consumption and (R,R)-2,3-butanediol production (Shi et al. 2016). Increased tolerance to the inhibitors coumaric and ferulic acids has been recently achieved through reverse engineering of *ARO80* alleles facilitated by CRISPR/Cas9 (Pereira et al. 2020). Based on a dual-guide CRISPR/Cas9 approach, multikilobase loci harbouring QTL for LCH tolerance were introduced into the S288C background, resulting in a superior strain for LCH fermentation (Maurer et al. 2017). Those examples illustrate how CRISPR/Cas9 can be instrumental for translating knowledge gathered through ALE studies into applications for 2G processes. Currently, several commercial *S. cerevisiae* strains, engineered for the utilisation of biomass-derived

xylose, have been used at 2G ethanol units worldwide (Jacobus et al. 2021a). Despite this achievement, 2G strains still can be further improved with genetic boosts for enhancing LCH inhibitor tolerance. Contributing to this technological upgrade is an exciting perspective motivating innovative research approaches.

References

Adeboye PT, Olsson L, Bettiga M (2016) A coniferyl aldehyde dehydrogenase gene from *Pseudomonas* sp. strain HR199 enhances the conversion of coniferyl aldehyde by *Saccharomyces cerevisiae*. *Bioresour Technol* 212:11–19. <https://doi.org/10.1016/j.biortech.2016.04.003>

Article CAS PubMed Google Scholar

Adeboye PT, Bettiga M, Olsson L (2017) ALD5, PAD1, ATF1 and ATF2 facilitate the catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid in *Saccharomyces cerevisiae*. *Sci Rep* 7:42635. <https://doi.org/10.1038/srep42635>

Article CAS PubMed PubMed Central Google Scholar

Almario MP, Reyes LH, Kao KC (2013) Evolutionary engineering of *Saccharomyces cerevisiae* for enhanced tolerance to hydrolysates of lignocellulosic biomass. *Biotechnol Bioeng* 110:2616–2623. <https://doi.org/10.1002/bit.24938>

Article CAS PubMed Google Scholar

Almeida JR, Roder A, Modig T, Laadan B, Liden G, Gorwa-Grauslund MF (2008) NADH- vs NADPH-coupled reduction of 5-hydroxymethyl furfural (HMF) and its implications on product distribution in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 78:939–945. <https://doi.org/10.1007/s00253-008-1364-y>

Article CAS PubMed Google Scholar

Alriksson B, Horváth IS, Jonsson LJ (2010) Overexpression of *Saccharomyces cerevisiae* transcription factor and multidrug resistance genes conveys enhanced resistance to lignocellulose-derived fermentation inhibitors. *Process Biochem* 45:264–271. <https://doi.org/10.1016/j.procbio.2009.09.016>

Article CAS Google Scholar

An J, Kwon H, Kim E, Lee YM, Ko HJ, Park H, Choi IG, Kim S, Kim KH, Kim W, Choi W (2015) Tolerance to acetic acid is improved by mutations of the TATA-binding protein gene. *Environ Microbiol* 17:656–669. <https://doi.org/10.1111/1462-2920.12489>

Article CAS PubMed Google Scholar

Ask M, Mapelli V, Hock H, Olsson L, Bettiga M (2013) Engineering glutathione biosynthesis of *Saccharomyces cerevisiae* increases robustness to inhibitors in pretreated lignocellulosic materials. *Microb Cell Fact* 12:87. <https://doi.org/10.1186/1475-2859-12-87>

Article CAS PubMed PubMed Central Google Scholar

Balderas-Hernandez VE, Correia K, Mahadevan R (2018) Inactivation of the transcription factor mig1 (YGL035C) in *Saccharomyces cerevisiae* improves tolerance towards monocarboxylic weak acids: acetic, formic and levulinic acid. *J Ind Microbiol Biotechnol* 45:735–751. <https://doi.org/10.1007/s10295-018-2053-1>

Article CAS PubMed Google Scholar

Bao Z, Hamedirad M, Xue P, Xiao H, Tasan I, Chao R, Liang J, Zhao H (2018) Genome-scale engineering of *Saccharomyces cerevisiae* with single-nucleotide precision. *Nat Biotechnol* 36:505–508. <https://doi.org/10.1038/nbt.4132>

Article CAS PubMed Google Scholar

Barrick JE, Lenski RE (2013) Genome dynamics during experimental evolution. *Nat Rev Genet* 14:827–839. <https://doi.org/10.1038/nrg3564>

Article CAS PubMed PubMed Central Google Scholar

Biot-Pelletier D, Pinel D, Larue K, Martin VJJ (2018) Determinants of selection in yeast evolved by genome shuffling. *Biotechnol Biofuels* 11:282. <https://doi.org/10.1186/s13068-018-1283-9>

Article CAS PubMed PubMed Central Google Scholar

Blount ZD, Barrick JE, Davidson CJ, Lenski RE (2012) Genomic analysis of a key innovation in an experimental *Escherichia coli* population. *Nature* 489:513–518. <https://doi.org/10.1038/nature11514>

Article CAS PubMed PubMed Central Google Scholar

Blount BA, Gowers GF, Ho JCH, Ledesma-Amaro R, Jovicevic D, McKiernan RM, Xie ZX, Li BZ, Yuan YJ, Ellis T (2018) Rapid host strain improvement by in vivo rearrangement of a synthetic yeast chromosome. *Nat Commun* 9:1932. <https://doi.org/10.1038/s41467-018-03143-w>

Article CAS PubMed PubMed Central Google Scholar

Brandt BA, Garcia-Aparicio MDP, Gorgens JF, van Zyl WH (2021) Rational engineering of *Saccharomyces cerevisiae* towards improved tolerance to multiple inhibitors in lignocellulose fermentations. *Biotechnol Biofuels* 14:173. <https://doi.org/10.1186/s13068-021-02021-w>

Article CAS PubMed PubMed Central Google Scholar

Cao W, Zhao W, Yang B, Wang X, Shen Y, Wei T, Qin W, Li Z, Bao X (2021) Proteomic analysis revealed the roles of YRR1 deletion in enhancing the vanillin resistance of *Saccharomyces cerevisiae*. *Microb Cell Fact* 20:142. <https://doi.org/10.1186/s12934-021-01633-z>

Article CAS PubMed PubMed Central Google Scholar

Caspeta L, Chen Y, Ghiaci P, Feizi A, Buskov S, Hallstrom BM, Petranovic D, Nielsen J (2014) Biofuels. Altered sterol composition renders yeast thermotolerant. *Science* 346:75–78. <https://doi.org/10.1126/science.1258137>

Article CAS PubMed Google Scholar

Chen Y, Sheng J, Jiang T, Stevens J, Feng X, Wei N (2016a) Transcriptional profiling reveals molecular basis and novel genetic targets for improved resistance to multiple fermentation inhibitors in *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 9:9. <https://doi.org/10.1186/s13068-015-0418-5>

Article CAS PubMed PubMed Central Google Scholar

Chen Y, Stabryla L, Wei N (2016b) Improved acetic acid resistance in *Saccharomyces cerevisiae* by overexpression of the WHI2 gene identified through inverse metabolic engineering. *Appl Environ Microbiol* 82:2156–2166. <https://doi.org/10.1128/AEM.03718-15>

Article CAS PubMed PubMed Central Google Scholar

Chen H, Li J, Wan C, Fang Q, Bai F, Zhao X (2019) Improvement of inhibitor tolerance in *Saccharomyces cerevisiae* by overexpression of the quinone

oxidoreductase family gene YCR102C. FEMS Yeast Res.
<https://doi.org/10.1093/femsyr/foz055>

Article PubMed PubMed Central Google Scholar

Cheng C, Almario MP, Kao KC (2015) Genome shuffling to generate recombinant yeasts for tolerance to inhibitors present in lignocellulosic hydrolysates. Biotechnol Lett 37:2193–2200. <https://doi.org/10.1007/s10529-015-1895-0>

Article CAS PubMed Google Scholar

Cheng C, Zhao X, Zhang M, Bai F (2016) Absence of Rtt109p, a fungal-specific histone acetyltransferase, results in improved acetic acid tolerance of *Saccharomyces cerevisiae*. FEMS Yeast Res. <https://doi.org/10.1093/femsyr/fow010>

Article PubMed Google Scholar

Cheng Y, Zhu H, Du Z, Guo X, Zhou C, Wang Z, He X (2021) Eukaryotic translation factor eIF5A contributes to acetic acid tolerance in *Saccharomyces cerevisiae* via transcriptional factor Ume6p. Biotechnol Biofuels 14:38. <https://doi.org/10.1186/s13068-021-01885-2>

Article CAS PubMed PubMed Central Google Scholar

Cunha JT, Aguiar TQ, Romani A, Oliveira C, Domingues L (2015) Contribution of PRS3, RPB4 and ZWF1 to the resistance of industrial *Saccharomyces cerevisiae* CCUG53310 and PE-2 strains to lignocellulosic hydrolysate-derived inhibitors. Bioresour Technol 191:7–16. <https://doi.org/10.1016/j.biortech.2015.05.006>

Article CAS PubMed Google Scholar

Cunha JT, Costa CE, Ferraz L, Romani A, Johansson B, Sa-Correia I, Domingues L (2018) HAA1 and PRS3 overexpression boosts yeast tolerance towards acetic acid improving xylose or glucose consumption: unravelling the underlying mechanisms. Appl Microbiol Biotechnol 102:4589–4600. <https://doi.org/10.1007/s00253-018-8955-z>

Article CAS PubMed Google Scholar

Cunha JT, Romani A, Costa CE, Sa-Correia I, Domingues L (2019) Molecular and physiological basis of *Saccharomyces cerevisiae* tolerance to adverse lignocellulose-based process conditions. Appl Microbiol Biotechnol 103:159–175. <https://doi.org/10.1007/s00253-018-9478-3>

Article CAS PubMed Google Scholar

de Witt RN, Kroukamp H, Van Zyl WH, Paulsen IT, Volschenk H (2019) QTL analysis of natural *Saccharomyces cerevisiae* isolates reveals unique alleles involved in lignocellulosic inhibitor tolerance. FEMS Yeast Res. <https://doi.org/10.1093/femsyr/foz047>

Article PubMed Google Scholar

Ding J, Holzwarth G, Bradford CS, Cooley B, Yoshinaga AS, Patton-Vogt J, Abeliovich H, Penner MH, Bakalinsky AT (2015a) PEP3 overexpression shortens lag phase but does not alter growth rate in *Saccharomyces cerevisiae* exposed to acetic acid stress. Appl Microbiol Biotechnol 99:8667–8680. <https://doi.org/10.1007/s00253-015-6708-9>

Article CAS PubMed PubMed Central Google Scholar

Ding J, Holzwarth G, Penner MH, Patton-Vogt J, Bakalinsky AT (2015b) Overexpression of acetyl-CoA synthetase in *Saccharomyces cerevisiae* increases acetic acid tolerance. *Fems Microbiol Lett* 362:1–7. <https://doi.org/10.1093/femsle/fnu042>

Article CAS PubMed PubMed Central Google Scholar

Dos Santos LV, Carazzolle MF, Nagamatsu ST, Sampaio NM, Almeida LD, Pirolla RA, Borelli G, Correa TL, Argueso JL, Pereira GA (2016) Unraveling the genetic basis of xylose consumption in engineered *Saccharomyces cerevisiae* strains. *Sci Rep* 6:38676. <https://doi.org/10.1038/srep38676>

Article CAS PubMed PubMed Central Google Scholar

Dragosits M, Mattanovich D (2013) Adaptive laboratory evolution—principles and applications for biotechnology. *Microb Cell Fact* 12:64. <https://doi.org/10.1186/1475-2859-12-64>

Article PubMed PubMed Central Google Scholar

Espinosa MI, Gonzalez-Garcia RA, Valgepea K, Plan MR, Scott C, Pretorius IS, Marcellin E, Paulsen IT, Williams TC (2020) Adaptive laboratory evolution of native methanol assimilation in *Saccharomyces cerevisiae*. *Nat Commun* 11:5564. <https://doi.org/10.1038/s41467-020-19390-9>

Article CAS PubMed PubMed Central Google Scholar

Fernandez-Nino M, Pulido S, Stefanoska D, Perez C, Gonzalez-Ramos D, van Maris AJA, Marchal K, Nevoigt E, Swinnen S (2018) Identification of novel genes involved in acetic acid tolerance of *Saccharomyces cerevisiae* using pooled-segregant RNA sequencing. *FEMS Yeast Res*. <https://doi.org/10.1093/femsyr/foy100>

Article PubMed Google Scholar

Fisher KJ, Buskirk SW, Vignogna RC, Marad DA, Lang GI (2018) Adaptive genome duplication affects patterns of molecular evolution in *Saccharomyces cerevisiae*. *PLoS Genet* 14:e1007396. <https://doi.org/10.1371/journal.pgen.1007396>

Article CAS PubMed PubMed Central Google Scholar

Fletcher E, Baetz K (2020) Multi-faceted systems biology approaches present a cellular landscape of phenolic compound inhibition in *Saccharomyces cerevisiae*. *Front Bioeng Biotechnol* 8:539902. <https://doi.org/10.3389/fbioe.2020.539902>

Article PubMed PubMed Central Google Scholar

Fletcher E, Gao K, Mercurio K, Ali M, Baetz K (2019) Yeast chemogenomic screen identifies distinct metabolic pathways required to tolerate exposure to phenolic fermentation inhibitors ferulic acid, 4-hydroxybenzoic acid and coniferyl aldehyde. *Metab Eng* 52:98–109. <https://doi.org/10.1016/j.ymben.2018.11.010>

Article CAS PubMed Google Scholar

Fujitomi K, Sanda T, Hasunuma T, Kondo A (2012) Deletion of the PHO13 gene in *Saccharomyces cerevisiae* improves ethanol production from lignocellulosic hydrolysate in the presence of acetic and formic acids, and furfural. *Bioresour Technol* 111:161–166. <https://doi.org/10.1016/j.biortech.2012.01.161>

Article CAS PubMed Google Scholar

Gao J, Feng H, Yuan W, Li Y, Hou S, Zhong S, Bai F (2017a) Enhanced fermentative performance under stresses of multiple lignocellulose-derived inhibitors by overexpression of a typical 2-Cys peroxiredoxin from *Kluyveromyces marxianus*. *Biotechnol Biofuels* 10:79. <https://doi.org/10.1186/s13068-017-0766-4>

Article CAS PubMed PubMed Central Google Scholar

Gao J, Yuan W, Li Y, Bai F, Jiang Y (2017b) Synergistic effect of thioredoxin and its reductase from *Kluyveromyces marxianus* on enhanced tolerance to multiple lignocellulose-derived inhibitors. *Microb Cell Fact* 16:181. <https://doi.org/10.1186/s12934-017-0795-5>

Article CAS PubMed PubMed Central Google Scholar

Gonzalez-Ramos D, Gorter de Vries AR, Grijseels SS, van Berkum MC, Swinnen S, van den Broek M, Nevoigt E, Daran JM, Pronk JT, van Maris AJ (2016) A new laboratory evolution approach to select for constitutive acetic acid tolerance in *Saccharomyces cerevisiae* and identification of causal mutations. *Biotechnol Biofuels* 9:173. <https://doi.org/10.1186/s13068-016-0583-1>

Article CAS PubMed PubMed Central Google Scholar

Good BH, McDonald MJ, Barrick JE, Lenski RE, Desai MM (2017) The dynamics of molecular evolution over 60,000 generations. *Nature* 551:45–50. <https://doi.org/10.1038/nature24287>

Article CAS PubMed PubMed Central Google Scholar

Gorsich SW, Dien BS, Nichols NN, Slininger PJ, Liu ZL, Skory CD (2006) Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes ZWF1, GND1, RPE1, and TKL1 in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 71:339–349. <https://doi.org/10.1007/s00253-005-0142-3>

Article CAS PubMed Google Scholar

Guaragnella N, Antonacci L, Giannattasio S, Marra E, Passarella S (2008) Catalase T and Cu, Zn-superoxide dismutase in the acetic acid-induced programmed cell death in *Saccharomyces cerevisiae*. *FEBS Lett* 582:210–214. <https://doi.org/10.1016/j.febslet.2007.12.007>

Article CAS PubMed Google Scholar

Gurdo N, Novelli Poisson GF, Juarez AB, Rios de Molina MC, Galvagno MA (2018) Improved robustness of an ethanologenic yeast strain through adaptive evolution in acetic acid is associated with its enzymatic antioxidant ability. *J Appl Microbiol* 125:766–776. <https://doi.org/10.1111/jam.13917>

Article CAS PubMed Google Scholar

Gutmann F, Jann C, Pereira F, Johansson A, Steinmetz LM, Patil KR (2021) CRISPRi screens reveal genes modulating yeast growth in lignocellulose hydrolysate. *Biotechnol Biofuels* 14:41. <https://doi.org/10.1186/s13068-021-01880-7>

Article CAS PubMed PubMed Central Google Scholar

Hacisalihoglu B, Holyavkin C, Topaloglu A, Kisakesen HI, Cakar ZP (2019) Genomic and transcriptomic analysis of a coniferyl aldehyde-resistant *Saccharomyces*

cerevisiae strain obtained by evolutionary engineering. FEMS Yeast Res. <https://doi.org/10.1093/femsyr/foz021>

Article PubMed Google Scholar

Hassing EJ, de Groot PA, Marquenie VR, Pronk JT, Daran JG (2019) Connecting central carbon and aromatic amino acid metabolisms to improve de novo 2-phenylethanol production in *Saccharomyces cerevisiae*. Metab Eng 56:165–180. <https://doi.org/10.1016/j.ymben.2019.09.011>

Article CAS PubMed Google Scholar

Hasunuma T, Sanda T, Yamada R, Yoshimura K, Ishii J, Kondo A (2011a) Metabolic pathway engineering based on metabolomics confers acetic and formic acid tolerance to a recombinant xylose-fermenting strain of *Saccharomyces cerevisiae*. Microb Cell Fact 10:2. <https://doi.org/10.1186/1475-2859-10-2>

Article CAS PubMed PubMed Central Google Scholar

Hasunuma T, Sung KM, Sanda T, Yoshimura K, Matsuda F, Kondo A (2011b) Efficient fermentation of xylose to ethanol at high formic acid concentrations by metabolically engineered *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 90:997–1004. <https://doi.org/10.1007/s00253-011-3085-x>

Article CAS PubMed Google Scholar

Hasunuma T, Ismail KSK, Nambu Y, Kondo A (2014) Co-expression of TAL1 and ADH1 in recombinant xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production from lignocellulosic hydrolysates in the presence of furfural. J Biosci Bioeng 117:165–169. <https://doi.org/10.1016/j.jbiosc.2013.07.007>

Article CAS PubMed Google Scholar

Hasunuma T, Sakamoto T, Kondo A (2016) Inverse metabolic engineering based on transient acclimation of yeast improves acid-containing xylose fermentation and tolerance to formic and acetic acids. Appl Microbiol Biotechnol 100:1027–1038. <https://doi.org/10.1007/s00253-015-7094-z>

Article CAS PubMed Google Scholar

Hawkins GM, Doran-Peterson J (2011) A strain of *Saccharomyces cerevisiae* evolved for fermentation of lignocellulosic biomass displays improved growth and fermentative ability in high solids concentrations and in the presence of inhibitory compounds. Biotechnol Biofuels 4:49. <https://doi.org/10.1186/1754-6834-4-49>

Article CAS PubMed PubMed Central Google Scholar

Heer D, Sauer U (2008) Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain. Microb Biotechnol 1:497–506. <https://doi.org/10.1111/j.1751-7915.2008.00050.x>

Article CAS PubMed PubMed Central Google Scholar

Heer D, Heine D, Sauer U (2009) Resistance of *Saccharomyces cerevisiae* to high concentrations of furfural is based on NADPH-dependent reduction by at least two oxireductases. Appl Environ Microbiol 75:7631–7638. <https://doi.org/10.1128/AEM.01649-09>

Article CAS PubMed PubMed Central Google Scholar

Henriques SF, Mira NP, Sa-Correia I (2017) Genome-wide search for candidate genes for yeast robustness improvement against formic acid reveals novel susceptibility (Trk1 and positive regulators) and resistance (Haa1-regulon) determinants. *Biotechnol Biofuels* 10:96. <https://doi.org/10.1186/s13068-017-0781-5>

Article CAS PubMed PubMed Central Google Scholar

Holyoak CD, Thompson S, Ortiz Calderon C, Hatzixanthis K, Bauer B, Kuchler K, Piper PW, Coote PJ (2000) Loss of Cmk1 Ca(2+)-calmodulin-dependent protein kinase in yeast results in constitutive weak organic acid resistance, associated with a post-transcriptional activation of the Pdr12 ATP-binding cassette transporter. *Mol Microbiol* 37:595–605. <https://doi.org/10.1046/j.1365-2958.2000.02017.x>

Article CAS PubMed Google Scholar

Hu J, Dong Y, Wang W, Zhang W, Lou H, Chen Q (2019) Deletion of Atg22 gene contributes to reduce programmed cell death induced by acetic acid stress in *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 12:298. <https://doi.org/10.1186/s13068-019-1638-x>

Article CAS PubMed PubMed Central Google Scholar

Inaba T, Watanabe D, Yoshiyama Y, Tanaka K, Ogawa J, Takagi H, Shimoi H, Shima J (2013) An organic acid-tolerant HAA1-overexpression mutant of an industrial bioethanol strain of *Saccharomyces cerevisiae* and its application to the production of bioethanol from sugarcane molasses. *AMB Express* 3:74. <https://doi.org/10.1186/2191-0855-3-74>

Article CAS PubMed PubMed Central Google Scholar

Ishida Y, Nguyen TT, Kitajima S, Izawa S (2016) Prioritized expression of BDH2 under bulk translational repression and Its contribution to tolerance to severe vanillin stress in *Saccharomyces cerevisiae*. *Front Microbiol* 7:1059. <https://doi.org/10.3389/fmicb.2016.01059>

Article PubMed PubMed Central Google Scholar

Ishida Y, Nguyen TTM, Izawa S (2017) The yeast ADH7 promoter enables gene expression under pronounced translation repression caused by the combined stress of vanillin, furfural, and 5-hydroxymethylfurfural. *J Biotechnol* 252:65–72. <https://doi.org/10.1016/j.jbiotec.2017.04.024>

Article CAS PubMed Google Scholar

Jacobus AP, Gross J, Evans JH, Ceccato-Antonini SR, Gombert AK (2021a) *Saccharomyces cerevisiae* strains used industrially for bioethanol production. *Essays Biochem*. <https://doi.org/10.1042/EBC20200160>

Article PubMed Google Scholar

Jacobus AP, Stephens TG, Youssef P, Gonzalez-Pech R, Ciccotosto-Camp MM, Dougan KE, Chen Y, Basso LC, Frazzon J, Chan CX, Gross J (2021b) Comparative genomics supports that Brazilian bioethanol *Saccharomyces cerevisiae* comprise a unified group of domesticated strains related to cachaca spirit yeasts. *Front Microbiol* 12:644089. <https://doi.org/10.3389/fmicb.2021.644089>

Article PubMed PubMed Central Google Scholar

Jayakody LN, Jin YS (2021) In-depth understanding of molecular mechanisms of aldehyde toxicity to engineer robust *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 105:2675–2692. <https://doi.org/10.1007/s00253-021-11213-1>

Article CAS PubMed Google Scholar

Jayakody LN, Horie K, Hayashi N, Kitagaki H (2013) Engineering redox cofactor utilization for detoxification of glycolaldehyde, a key inhibitor of bioethanol production, in yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 97:6589–6600. <https://doi.org/10.1007/s00253-013-4997-4>

Article CAS PubMed Google Scholar

Jayakody LN, Turner TL, Yun EJ, Kong II, Liu JJ, Jin YS (2018) Expression of Gre2p improves tolerance of engineered xylose-fermenting *Saccharomyces cerevisiae* to glycolaldehyde under xylose metabolism. Appl Microbiol Biotechnol 102:8121–8133. <https://doi.org/10.1007/s00253-018-9216-x>

Article CAS PubMed Google Scholar

Johnson MS, Gopalakrishnan S, Goyal J, Dillingham ME, Bakerlee CW, Humphrey PT, Jagdish T, Jerison ER, Kosheleva K, Lawrence KR, Min J, Moulana A, Phillips AM, Piper JC, Purkanti R, Rego-Costa A, McDonald MJ, Nguyen Ba AN, Desai MM (2021) Phenotypic and molecular evolution across 10,000 generations in laboratory budding yeast populations. Elife. <https://doi.org/10.7554/eLife.63910>

Article PubMed PubMed Central Google Scholar

Jonsson LJ, Alriksson B, Nilvebrant NO (2013) Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol Biofuels 6:16. <https://doi.org/10.1186/1754-6834-6-16>

Article CAS PubMed PubMed Central Google Scholar

Kawahata M, Masaki K, Fujii T, Iefuji H (2006) Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. FEMS Yeast Res 6:924–936. <https://doi.org/10.1111/j.1567-1364.2006.00089.x>

Article CAS PubMed Google Scholar

Keller FA, Bates D, Ruiz R, Nguyen Q (1998) Yeast adaptation on softwood prehydrolysate. Appl Biochem Biotechnol 70–72:137–148. <https://doi.org/10.1007/BF02920131>

Article PubMed Google Scholar

Kim D, Hahn JS (2013) Roles of the Yap1 transcription factor and antioxidants in *Saccharomyces cerevisiae*'s tolerance to furfural and 5-hydroxymethylfurfural, which function as thiol-reactive electrophiles generating oxidative stress. Appl Environ Microbiol 79:5069–5077. <https://doi.org/10.1128/AEM.00643-13>

Article CAS PubMed PubMed Central Google Scholar

Kim SK, Jin YS, Choi IG, Park YC, Seo JH (2015) Enhanced tolerance of *Saccharomyces cerevisiae* to multiple lignocellulose-derived inhibitors through

modulation of spermidine contents. *Metab Eng* 29:46–55.
<https://doi.org/10.1016/j.ymben.2015.02.004>

Article CAS PubMed Google Scholar

Ko JK, Enkh-Amgalan T, Gong G, Um Y, Lee S (2020) Improved bioconversion of lignocellulosic biomass by *Saccharomyces cerevisiae* engineered for tolerance to acetic acid. *GCB Bioenergy* 12:90–100

Article CAS Google Scholar

Koppram R, Albers E, Olsson L (2012) Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass. *Biotechnol Biofuels* 5:32. <https://doi.org/10.1186/1754-6834-5-32>

Article CAS PubMed PubMed Central Google Scholar

Kuang Z, Pinglay S, Ji H, Boeke JD (2017) Msn2/4 regulate expression of glycolytic enzymes and control transition from quiescence to growth. *Elife*.
<https://doi.org/10.7554/eLife.29938>

Article PubMed PubMed Central Google Scholar

Kuang X, Ouyang Y, Guo Y, Li Q, Wang H, Abrha GT, Ayepa E, Gu Y, Li X, Chen Q, Ma M (2020) New insights into two yeast BDHs from the PDH subfamily as aldehyde reductases in context of detoxification of lignocellulosic aldehyde inhibitors. *Appl Microbiol Biotechnol* 104:6679–6692. <https://doi.org/10.1007/s00253-020-10722-9>

Article CAS PubMed Google Scholar

Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind Eng Chem Res* 48:3713–3729. <https://doi.org/10.1021/ie801542g>

Article CAS Google Scholar

Kumar V, Hart AJ, Keerthiraju ER, Waldron PR, Tucker GA, Greetham D (2015) Expression of mitochondrial cytochrome C oxidase chaperone gene (COX20) improves tolerance to weak acid and oxidative stress during yeast fermentation. *PLoS ONE* 10:e0139129. <https://doi.org/10.1371/journal.pone.0139129>

Article CAS PubMed PubMed Central Google Scholar

Kumar V, Yadav SK, Kumar J, Ahluwalia V (2020) A critical review on current strategies and trends employed for removal of inhibitors and toxic materials generated during biomass pretreatment. *Bioresour Technol* 299:122633. <https://doi.org/10.1016/j.biortech.2019.122633>

Article CAS PubMed Google Scholar

Laadan B, Almeida JR, Radstrom P, Hahn-Hagerdal B, Gorwa-Grauslund M (2008) Identification of an NADH-dependent 5-hydroxymethylfurfural-reducing alcohol dehydrogenase in *Saccharomyces cerevisiae*. *Yeast* 25:191–198.
<https://doi.org/10.1002/yea.1578>

Article CAS PubMed Google Scholar

Landaeta R, Aroca G, Acevedo F, Teixeira JA, Mussatto SI (2013) Adaptation of a flocculent *Saccharomyces cerevisiae* strain to lignocellulosic inhibitors by cell recycle

batch fermentation. *Appl Energy* 102:124–130.
<https://doi.org/10.1016/j.apenergy.2012.06.048>

Article CAS Google Scholar

Larsson S, Cassland P, Jonsson LJ (2001a) Development of a *Saccharomyces cerevisiae* strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase. *Appl Environ Microbiol* 67:1163–1170. <https://doi.org/10.1128/AEM.67.3.1163-1170.2001>

Article CAS PubMed PubMed Central Google Scholar

Larsson S, Nilvebrant NO, Jonsson LJ (2001b) Effect of overexpression of *Saccharomyces cerevisiae* Pad1p on the resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen-limited conditions. *Appl Microbiol Biotechnol* 57:167–174. <https://doi.org/10.1007/s002530100742>

Article CAS PubMed Google Scholar

Lee Y, Nasution O, Choi E, Choi IG, Kim W, Choi W (2015) Transcriptome analysis of acetic-acid-treated yeast cells identifies a large set of genes whose overexpression or deletion enhances acetic acid tolerance. *Appl Microbiol Biotechnol* 99:6391–6403. <https://doi.org/10.1007/s00253-015-6706-y>

Article CAS PubMed Google Scholar

Li YC, Gou ZX, Liu ZS, Tang YQ, Akamatsu T, Kida K (2014) Synergistic effects of TAL1 over-expression and PHO13 deletion on the weak acid inhibition of xylose fermentation by industrial *Saccharomyces cerevisiae* strain. *Biotechnol Lett* 36:2011–2021. <https://doi.org/10.1007/s10529-014-1581-7>

Article CAS PubMed Google Scholar

Li H, Wu M, Xu L, Hou J, Guo T, Bao X, Shen Y (2015) Evaluation of industrial *Saccharomyces cerevisiae* strains as the chassis cell for second-generation bioethanol production. *Microb Biotechnol* 8:266–274. <https://doi.org/10.1111/1751-7915.12245>

Article CAS PubMed PubMed Central Google Scholar

Li W-C, Zhu J-Q, Zhao X, Qin L, Xu T, Zhou X, Li X, Li B-Z, Yuan Y-J (2019) Improving co-fermentation of glucose and xylose by adaptive evolution of engineering xylose-fermenting *Saccharomyces cerevisiae* and different fermentation strategies. *Renewable Energy* 139:1176–1183. <https://doi.org/10.1016/j.renene.2019.03.028>

Article CAS Google Scholar

Li B, Wang L, Wu YJ, Xia ZY, Yang BX, Tang YQ (2021) Improving acetic acid and furfural resistance of xylose-fermenting *Saccharomyces cerevisiae* by regulating novel transcription factors revealed via comparative transcriptome. *Appl Environ Microbiol*. <https://doi.org/10.1128/AEM.00158-21>

Article PubMed PubMed Central Google Scholar

Lian J, Schultz C, Cao M, Hamedirad M, Zhao H (2019) Multi-functional genome-wide CRISPR system for high throughput genotype-phenotype mapping. *Nat Commun* 10:5794. <https://doi.org/10.1038/s41467-019-13621-4>

Article CAS PubMed PubMed Central Google Scholar

Liang Z, Wang X, Bao X, Wei T, Hou J, Liu W, Shen Y (2021) Newly identified genes contribute to vanillin tolerance in *Saccharomyces cerevisiae*. Microb Biotechnol 14:503–516. <https://doi.org/10.1111/1751-7915.13643>

Article CAS PubMed Google Scholar

Liu ZL, Ma M (2020) Pathway-based signature transcriptional profiles as tolerance phenotypes for the adapted industrial yeast *Saccharomyces cerevisiae* resistant to furfural and HMF. Appl Microbiol Biotechnol 104:3473–3492. <https://doi.org/10.1007/s00253-020-10434-0>

Article CAS PubMed Google Scholar

Liu ZL, Slininger PJ, Gorsich SW (2005) Enhanced biotransformation of furfural and hydroxymethylfurfural by newly developed ethanologenic yeast strains. Appl Biochem Biotechnol 121–124:451–460

Article Google Scholar

Liu ZL, Moon J, Andersh BJ, Slininger PJ, Weber S (2008) Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 81:743–753. <https://doi.org/10.1007/s00253-008-1702-0>

Article CAS PubMed Google Scholar

Liu ZL, Ma M, Song M (2009) Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways. Mol Genet Genomics 282:233–244. <https://doi.org/10.1007/s00438-009-0461-7>

Article CAS PubMed PubMed Central Google Scholar

Liu X, Zhang X, Zhang Z (2014) Point mutation of H3/H4 histones affects acetic acid tolerance in *Saccharomyces cerevisiae*. J Biotechnol 187:116–123. <https://doi.org/10.1016/j.jbiotec.2014.07.445>

Article CAS PubMed Google Scholar

Liu ZL, Huang X, Zhou Q, Xu J (2019) Protein expression analysis revealed a fine-tuned mechanism of in situ detoxification pathway for the tolerant industrial yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 103:5781–5796. <https://doi.org/10.1007/s00253-019-09906-9>

Article CAS PubMed Google Scholar

Liu CG, Li K, Li KY, Sakdaronnarong C, Mehmood MA, Zhao XQ, Bai FW (2020) Intracellular redox perturbation in *Saccharomyces cerevisiae* improved furfural tolerance and enhanced cellulosic bioethanol production. Front Bioeng Biotechnol 8:615. <https://doi.org/10.3389/fbioe.2020.00615>

Article PubMed PubMed Central Google Scholar

Luo Z, Wang L, Wang Y, Zhang W, Guo Y, Shen Y, Jiang L, Wu Q, Zhang C, Cai Y, Dai J (2018) Identifying and characterizing SCRaMbLEd synthetic yeast using ReSCuES. Nat Commun 9:1930. <https://doi.org/10.1038/s41467-017-00806-y>

Article CAS PubMed PubMed Central Google Scholar

Ma M, Liu ZL (2010) Comparative transcriptome profiling analyses during the lag phase uncover YAP1, PDR1, PDR3, RPN4, and HSF1 as key regulatory genes in genomic adaptation to the lignocellulose derived inhibitor HMF for *Saccharomyces cerevisiae*. BMC Genomics 11:660. <https://doi.org/10.1186/1471-2164-11-660>

Article CAS PubMed PubMed Central Google Scholar

Ma C, Wei X, Sun C, Zhang F, Xu J, Zhao X, Bai F (2015) Improvement of acetic acid tolerance of *Saccharomyces cerevisiae* using a zinc-finger-based artificial transcription factor and identification of novel genes involved in acetic acid tolerance. Appl Microbiol Biotechnol 99:2441–2449. <https://doi.org/10.1007/s00253-014-6343-x>

Article CAS PubMed Google Scholar

Mans R, Daran JG, Pronk JT (2018) Under pressure: evolutionary engineering of yeast strains for improved performance in fuels and chemicals production. Curr Opin Biotechnol 50:47–56. <https://doi.org/10.1016/j.copbio.2017.10.011>

Article CAS PubMed Google Scholar

Martani F, Marano F, Bertacchi S, Porro D, Branduardi P (2015) The *Saccharomyces cerevisiae* poly(A) binding protein Pab1 as a target for eliciting stress tolerant phenotypes. Sci Rep 5:18318. <https://doi.org/10.1038/srep18318>

Article CAS PubMed PubMed Central Google Scholar

Martin C, Marcet M, Almazan O, Jonsson LJ (2007) Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresour Technol 98:1767–1773. <https://doi.org/10.1016/j.biortech.2006.07.021>

Article CAS PubMed Google Scholar

Matsushika A, Inoue H, Murakami K, Takimura O, Sawayama S (2009) Bioethanol production performance of five recombinant strains of laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*. Bioresour Technol 100:2392–2398. <https://doi.org/10.1016/j.biortech.2008.11.047>

Article CAS PubMed Google Scholar

Maurer MJ, Sutardja L, Pinel D, Bauer S, Muehlbauer AL, Ames TD, Skerker JM, Arkin AP (2017) Quantitative trait loci (QTL)-guided metabolic engineering of a complex trait. ACS Synth Biol 6:566–581. <https://doi.org/10.1021/acssynbio.6b00264>

Article CAS PubMed Google Scholar

McDonald MJ (2019) Microbial experimental evolution—a proving ground for evolutionary theory and a tool for discovery. EMBO Rep 20:e46992. <https://doi.org/10.15252/embr.201846992>

Article CAS PubMed PubMed Central Google Scholar

McDonald MJ, Rice DP, Desai MM (2016) Sex speeds adaptation by altering the dynamics of molecular evolution. Nature 531:233–236. <https://doi.org/10.1038/nature17143>

Article CAS PubMed PubMed Central Google Scholar

Meijnen JP, Randazzo P, Foulquie-Moreno MR, van den Brink J, Vandecruys P, Stojiljkovic M, Dumortier F, Zalar P, Boekhout T, Gunde-Cimerman N, Kokosar J,

Stajdohar M, Curk T, Petrovic U, Thevelein JM (2016) Polygenic analysis and targeted improvement of the complex trait of high acetic acid tolerance in the yeast *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 9:5. <https://doi.org/10.1186/s13068-015-0421-x>

Article CAS PubMed PubMed Central Google Scholar

Mira NP, Becker JD, Sa-Correia I (2010) Genomic expression program involving the Haa1p-regulon in *Saccharomyces cerevisiae* response to acetic acid. *OMICS* 14:587–601. <https://doi.org/10.1089/omi.2010.0048>

Article CAS PubMed PubMed Central Google Scholar

Moon J, Liu ZL (2012) Engineered NADH-dependent GRE2 from *Saccharomyces cerevisiae* by directed enzyme evolution enhances HMF reduction using additional cofactor NADPH. *Enzyme Microb Technol* 50:115–120. <https://doi.org/10.1016/j.enzmictec.2011.10.007>

Article CAS PubMed Google Scholar

Mukherjee V, Lind U, St Onge RP, Blomberg A, Nygard Y (2021) A CRISPR interference screen of essential genes reveals that proteasome regulation dictates acetic acid tolerance in *Saccharomyces cerevisiae*. *Msystems*. <https://doi.org/10.1128/mSystems.00418-21>

Article PubMed PubMed Central Google Scholar

Narayanan V, Sanchez INV, van Niel EWJ, Gorwa-Grauslund MF (2016) Adaptation to low pH and lignocellulosic inhibitors resulting in ethanolic fermentation and growth of *Saccharomyces cerevisiae*. *AMB Express* 6:59. <https://doi.org/10.1186/s13568-016-0234-8>

Article CAS PubMed PubMed Central Google Scholar

Nygard Y, Mojzita D, Toivari M, Penttila M, Wiebe MG, Ruohonen L (2014) The diverse role of Pdr12 in resistance to weak organic acids. *Yeast* 31:219–232. <https://doi.org/10.1002/yea.3011>

Article CAS PubMed Google Scholar

Oh EJ, Wei N, Kwak S, Kim H, Jin YS (2019) Overexpression of RCK1 improves acetic acid tolerance in *Saccharomyces cerevisiae*. *J Biotechnol* 292:1–4. <https://doi.org/10.1016/j.jbiotec.2018.12.013>

Article CAS PubMed Google Scholar

Ouyang Y, Li Q, Kuang X, Wang H, Wu J, Ayepa E, Chen H, Abrha GT, Zhang Z, Li X, Ma M (2021) YMR152W from *Saccharomyces cerevisiae* encoding a novel aldehyde reductase for detoxification of aldehydes derived from lignocellulosic biomass. *J Biosci Bioeng* 131:39–46. <https://doi.org/10.1016/j.jbiosc.2020.09.004>

Article CAS PubMed Google Scholar

Palma M, Guerreiro JF, Sa-Correia I (2018) Adaptive response and tolerance to acetic acid in *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*: a physiological genomics perspective. *Front Microbiol* 9:274. <https://doi.org/10.3389/fmicb.2018.00274>

Article PubMed PubMed Central Google Scholar

Palmqvist E, Hahn-Hagerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Biores Technol* 74:25–33.
[https://doi.org/10.1016/S0960-8524\(99\)00161-3](https://doi.org/10.1016/S0960-8524(99)00161-3)

Article CAS Google Scholar

Pan S, Jia B, Liu H, Wang Z, Chai MZ, Ding MZ, Zhou X, Li X, Li C, Li BZ, Yuan YJ (2018) Endogenous lycopene improves ethanol production under acetic acid stress in *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 11:107.
<https://doi.org/10.1186/s13068-018-1107-y>

Article CAS PubMed PubMed Central Google Scholar

Park SE, Koo HM, Park YK, Park SM, Park JC, Lee OK, Park YC, Seo JH (2011) Expression of aldehyde dehydrogenase 6 reduces inhibitory effect of furan derivatives on cell growth and ethanol production in *Saccharomyces cerevisiae*. *Bioresour Technol* 102:6033–6038. <https://doi.org/10.1016/j.biortech.2011.02.101>

Article CAS PubMed Google Scholar

Pereira R, Mohamed ET, Radi MS, Herrgard MJ, Feist AM, Nielsen J, Chen Y (2020) Elucidating aromatic acid tolerance at low pH in *Saccharomyces cerevisiae* using adaptive laboratory evolution. *Proc Natl Acad Sci U S A* 117:27954–27961.
<https://doi.org/10.1073/pnas.2013044117>

Article CAS PubMed PubMed Central Google Scholar

Peter J, De Chiara M, Friedrich A, Yue JX, Pflieger D, Bergstrom A, Sigwalt A, Barre B, Freel K, Llored A, Cruaud C, Labadie K, Aury JM, Istace B, Lebrigand K, Barbry P, Engelen S, Lemainque A, Wincker P, Liti G, Schacherer J (2018) Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates. *Nature* 556:339–344.
<https://doi.org/10.1038/s41586-018-0030-5>

Article CAS PubMed PubMed Central Google Scholar

Petersson A, Almeida JR, Modig T, Karhumaa K, Hahn-Hagerdal B, Gorwa-Grauslund MF, Liden G (2006) A 5-hydroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae* ADH6 gene conveys HMF tolerance. *Yeast* 23:455–464. <https://doi.org/10.1002/yea.1370>

Article CAS PubMed Google Scholar

Pinel D, Colatriniano D, Jiang H, Lee H, Martin VJ (2015) Deconstructing the genetic basis of spent sulphite liquor tolerance using deep sequencing of genome-shuffled yeast. *Biotechnol Biofuels* 8:53. <https://doi.org/10.1186/s13068-015-0241-z>

Article CAS PubMed PubMed Central Google Scholar

Piotrowski JS, Zhang Y, Bates DM, Keating DH, Sato TK, Ong IM, Landick R (2014) Death by a thousand cuts: the challenges and diverse landscape of lignocellulosic hydrolysate inhibitors. *Front Microbiol* 5:90. <https://doi.org/10.3389/fmicb.2014.00090>

Article PubMed PubMed Central Google Scholar

Qureshi AS, Zhang J, Bao J (2015) High ethanol fermentation performance of the dry dilute acid pretreated corn stover by an evolutionarily adapted *Saccharomyces cerevisiae* strain. *Bioresour Technol* 189:399–404.
<https://doi.org/10.1016/j.biortech.2015.04.025>

Article CAS PubMed Google Scholar

Raghavendran V, Marx C, Olsson L, Bettiga M (2020) The protective role of intracellular glutathione in *Saccharomyces cerevisiae* during lignocellulosic ethanol production. *AMB Express* 10:219. <https://doi.org/10.1186/s13568-020-01148-7>

Article CAS PubMed PubMed Central Google Scholar

Richard P, Viljanen K, Penttilä M (2015) Overexpression of PAD1 and FDC1 results in significant cinnamic acid decarboxylase activity in *Saccharomyces cerevisiae*. *AMB Express* 5:12. <https://doi.org/10.1186/s13568-015-0103-x>

Article CAS PubMed PubMed Central Google Scholar

Richardson SM, Mitchell LA, Stracquadanio G, Yang K, Dymond JS, DiCarlo JE, Lee D, Huang CL, Chandrasegaran S, Cai Y, Boeke JD, Bader JS (2017) Design of a synthetic yeast genome. *Science* 355:1040–1044. <https://doi.org/10.1126/science.aaf4557>

Article CAS PubMed Google Scholar

Ronda C, Maury J, Jakociunas T, Jacobsen SA, Germann SM, Harrison SJ, Borodina I, Keasling JD, Jensen MK, Nielsen AT (2015) CrEdit: CRISPR mediated multi-loci gene integration in *Saccharomyces cerevisiae*. *Microb Cell Fact* 14:97. <https://doi.org/10.1186/s12934-015-0288-3>

Article CAS PubMed PubMed Central Google Scholar

Sakihama Y, Hasunuma T, Kondo A (2015) Improved ethanol production from xylose in the presence of acetic acid by the overexpression of the HAA1 gene in *Saccharomyces cerevisiae*. *J Biosci Bioeng* 119:297–302. <https://doi.org/10.1016/j.jbiosc.2014.09.004>

Article CAS PubMed Google Scholar

Salas-Navarrete PC, de Oca Miranda AIM, Martinez A, Caspeta L (2022) Evolutionary and reverse engineering to increase *Saccharomyces cerevisiae* tolerance to acetic acid, acidic pH, and high temperature. *Appl Microbiol Biotechnol* 106:383–399. <https://doi.org/10.1007/s00253-021-11730-z>

Article CAS PubMed Google Scholar

Sanda T, Hasunuma T, Matsuda F, Kondo A (2011) Repeated-batch fermentation of lignocellulosic hydrolysate to ethanol using a hybrid *Saccharomyces cerevisiae* strain metabolically engineered for tolerance to acetic and formic acids. *Bioresour Technol* 102:7917–7924. <https://doi.org/10.1016/j.biortech.2011.06.028>

Article CAS PubMed Google Scholar

Sardi M, Rovinskiy N, Zhang Y, Gasch AP (2016) Leveraging genetic-background effects in *Saccharomyces cerevisiae* to improve lignocellulosic hydrolysate tolerance. *Appl Environ Microbiol* 82:5838–5849. <https://doi.org/10.1128/AEM.01603-16>

Article CAS PubMed PubMed Central Google Scholar

Sasano Y, Watanabe D, Ukibe K, Inai T, Ohtsu I, Shimoi H, Takagi H (2012) Overexpression of the yeast transcription activator Msn2 confers furfural resistance and increases the initial fermentation rate in ethanol production. *J Biosci Bioeng* 113:451–455. <https://doi.org/10.1016/j.jbiosc.2011.11.017>

Article CAS PubMed Google Scholar

Shen Y, Li H, Wang X, Zhang X, Hou J, Wang L, Gao N, Bao X (2014) High vanillin tolerance of an evolved *Saccharomyces cerevisiae* strain owing to its enhanced vanillin reduction and antioxidative capacity. *J Ind Microbiol Biotechnol* 41:1637–1645. <https://doi.org/10.1007/s10295-014-1515-3>

Article CAS PubMed Google Scholar

Shi S, Liang Y, Zhang MM, Ang EL, Zhao H (2016) A highly efficient single-step, markerless strategy for multi-copy chromosomal integration of large biochemical pathways in *Saccharomyces cerevisiae*. *Metab Eng* 33:19–27. <https://doi.org/10.1016/j.ymben.2015.10.011>

Article CAS PubMed Google Scholar

Si T, Chao R, Min Y, Wu Y, Ren W, Zhao H (2017) Automated multiplex genome-scale engineering in yeast. *Nat Commun* 8:15187. <https://doi.org/10.1038/ncomms15187>

Article PubMed PubMed Central Google Scholar

Smith J, van Rensburg E, Gorgens JF (2014) Simultaneously improving xylose fermentation and tolerance to lignocellulosic inhibitors through evolutionary engineering of recombinant *Saccharomyces cerevisiae* harbouring xylose isomerase. *BMC Biotechnol* 14:41. <https://doi.org/10.1186/1472-6750-14-41>

Article CAS PubMed PubMed Central Google Scholar

Snoek T, Picca Nicolino M, Van den Brecht S, Mertens S, Saels V, Verplaetse A, Steensels J, Verstrepen KJ (2015) Large-scale robot-assisted genome shuffling yields industrial *Saccharomyces cerevisiae* yeasts with increased ethanol tolerance. *Biotechnol Biofuels* 8:32. <https://doi.org/10.1186/s13068-015-0216-0>

Article CAS PubMed PubMed Central Google Scholar

Songdech P, Ruchala J, Semkiv MV, Jensen LT, Sibirny A, Ratanakhanokchai K, Soontornngun N (2020) Overexpression of transcription factor ZNF1 of glycolysis improves bioethanol productivity under high glucose concentration and enhances acetic acid tolerance of *Saccharomyces cerevisiae*. *Biotechnol J* 15:e1900492. <https://doi.org/10.1002/biot.201900492>

Article CAS PubMed Google Scholar

Sousa M, Duarte AM, Fernandes TR, Chaves SR, Pacheco A, Leao C, Corte-Real M, Sousa MJ (2013) Genome-wide identification of genes involved in the positive and negative regulation of acetic acid-induced programmed cell death in *Saccharomyces cerevisiae*. *BMC Genomics* 14:838. <https://doi.org/10.1186/1471-2164-14-838>

Article CAS PubMed PubMed Central Google Scholar

Stojiljkovic M, Foulquie-Moreno MR, Thevelein JM (2020) Polygenic analysis of very high acetic acid tolerance in the yeast *Saccharomyces cerevisiae* reveals a complex genetic background and several new causative alleles. *Biotechnol Biofuels* 13:126. <https://doi.org/10.1186/s13068-020-01761-5>

Article CAS PubMed PubMed Central Google Scholar

Swinnen S, Henriques SF, Shrestha R, Ho PW, Sa-Correia I, Nevoigt E (2017) Improvement of yeast tolerance to acetic acid through Haa1 transcription factor

engineering: towards the underlying mechanisms. *Microb Cell Fact* 16:7.
<https://doi.org/10.1186/s12934-016-0621-5>

Article CAS PubMed PubMed Central Google Scholar

Tanaka K, Ishii Y, Ogawa J, Shima J (2012) Enhancement of acetic acid tolerance in *Saccharomyces cerevisiae* by overexpression of the HAA1 gene, encoding a transcriptional activator. *Appl Environ Microbiol* 78:8161–8163.
<https://doi.org/10.1128/AEM.02356-12>

Article CAS PubMed PubMed Central Google Scholar

Thompson OA, Hawkins GM, Gorsich SW, Doran-Peterson J (2016) Phenotypic characterization and comparative transcriptomics of evolved *Saccharomyces cerevisiae* strains with improved tolerance to lignocellulosic derived inhibitors. *Biotechnol Biofuels* 9:200. <https://doi.org/10.1186/s13068-016-0614-y>

Article CAS PubMed PubMed Central Google Scholar

Tomas-Pejo E, Ballesteros M, Oliva JM, Olsson L (2010) Adaptation of the xylose fermenting yeast *Saccharomyces cerevisiae* F12 for improving ethanol production in different fed-batch SSF processes. *J Ind Microbiol Biotechnol* 37:1211–1220.
<https://doi.org/10.1007/s10295-010-0768-8>

Article CAS PubMed Google Scholar

Unrean P, Gatgens J, Klein B, Noack S, Champreda V (2018) Elucidating cellular mechanisms of *Saccharomyces cerevisiae* tolerant to combined lignocellulosic-derived inhibitors using high-throughput phenotyping and multiomics analyses. *FEMS Yeast Res*. <https://doi.org/10.1093/femsyr/foy106>

Article PubMed Google Scholar

Voordeckers K, Kominek J, Das A, Espinosa-Cantu A, De Maeyer D, Arslan A, Van Pee M, van der Zande E, Meert W, Yang Y, Zhu B, Marchal K, DeLuna A, Van Noort V, Jelier R, Verstrepen KJ (2015) Adaptation to high ethanol reveals complex evolutionary pathways. *PLoS Genet* 11:e1005635.
<https://doi.org/10.1371/journal.pgen.1005635>

Article CAS PubMed PubMed Central Google Scholar

Wallace-Salinas V, Gorwa-Grauslund MF (2013) Adaptive evolution of an industrial strain of *Saccharomyces cerevisiae* for combined tolerance to inhibitors and temperature. *Biotechnol Biofuels* 6:151. <https://doi.org/10.1186/1754-6834-6-151>

Article CAS PubMed PubMed Central Google Scholar

Wallace-Salinas V, Signori L, Li YY, Ask M, Bettiga M, Porro D, Thevelein JM, Branduardi P, Foulquie-Moreno MR, Gorwa-Grauslund M (2014) Re-assessment of YAP1 and MCR1 contributions to inhibitor tolerance in robust engineered *Saccharomyces cerevisiae* fermenting undetoxified lignocellulosic hydrolysate. *AMB Express* 4:56. <https://doi.org/10.1186/s13568-014-0056-5>

Article CAS PubMed PubMed Central Google Scholar

Wallace-Salinas V, Brink DP, Ahren D, Gorwa-Grauslund MF (2015) Cell periphery-related proteins as major genomic targets behind the adaptive evolution of

an industrial *Saccharomyces cerevisiae* strain to combined heat and hydrolysate stress. BMC Genomics 16:514. <https://doi.org/10.1186/s12864-015-1737-4>

Article CAS PubMed PubMed Central Google Scholar

Wang X, Bai X, Chen DF, Chen FZ, Li BZ, Yuan YJ (2015) Increasing proline and myo-inositol improves tolerance of *Saccharomyces cerevisiae* to the mixture of multiple lignocellulose-derived inhibitors. Biotechnol Biofuels 8:142. <https://doi.org/10.1186/s13068-015-0329-5>

Article CAS PubMed PubMed Central Google Scholar

Wang X, Liang Z, Hou J, Bao X, Shen Y (2016) Identification and functional evaluation of the reductases and dehydrogenases from *Saccharomyces cerevisiae* involved in vanillin resistance. BMC Biotechnol 16:31. <https://doi.org/10.1186/s12896-016-0264-y>

Article CAS PubMed PubMed Central Google Scholar

Wang X, Liang Z, Hou J, Shen Y, Bao X (2017) The absence of the transcription factor Yrr1p, identified from comparative genome profiling, increased vanillin tolerance due to enhancements of ABC transporters expressing, rRNA processing and ribosome biogenesis in *Saccharomyces cerevisiae*. Front Microbiol 8:367. <https://doi.org/10.3389/fmicb.2017.00367>

Article PubMed PubMed Central Google Scholar

Wang L, Wang X, He ZQ, Zhou SJ, Xu L, Tan XY, Xu T, Li BZ, Yuan YJ (2020) Engineering prokaryotic regulator IrrE to enhance stress tolerance in budding yeast. Biotechnol Biofuels 13:193. <https://doi.org/10.1186/s13068-020-01833-6>

Article CAS PubMed PubMed Central Google Scholar

Wei N, Xu H, Kim SR, Jin YS (2013) Deletion of FPS1, encoding aquaglyceroporin Fps1p, improves xylose fermentation by engineered *Saccharomyces cerevisiae*. Appl Environ Microbiol 79:3193–3201. <https://doi.org/10.1128/AEM.00490-13>

Article CAS PubMed PubMed Central Google Scholar

Wright J, Bellissimi E, de Hulster E, Wagner A, Pronk JT, van Maris AJ (2011) Batch and continuous culture-based selection strategies for acetic acid tolerance in xylose-fermenting *Saccharomyces cerevisiae*. FEMS Yeast Res 11:299–306. <https://doi.org/10.1111/j.1567-1364.2011.00719.x>

Article CAS PubMed Google Scholar

Wu X, Zhang L, Jin X, Fang Y, Zhang K, Qi L, Zheng D (2016) Deletion of JJJ1 improves acetic acid tolerance and bioethanol fermentation performance of *Saccharomyces cerevisiae* strains. Biotechnol Lett 38:1097–1106. <https://doi.org/10.1007/s10529-016-2085-4>

Article CAS PubMed Google Scholar

Wu G, Xu Z, Jonsson LJ (2017) Profiling of *Saccharomyces cerevisiae* transcription factors for engineering the resistance of yeast to lignocellulose-derived inhibitors in biomass conversion. Microb Cell Fact 16:199. <https://doi.org/10.1186/s12934-017-0811-9>

Article CAS PubMed PubMed Central Google Scholar

Xiao H, Zhao H (2014) Genome-wide RNAi screen reveals the E3 SUMO-protein ligase gene *SLZ1* as a novel determinant of furfural tolerance in *Saccharomyces cerevisiae*. *Biotechnol Biofuels* 7:78. <https://doi.org/10.1186/1754-6834-7-78>

Article CAS PubMed PubMed Central Google Scholar

Xu X, Liu C, Niu C, Wang J, Zheng F, Li Y, Li Q (2018) Rationally designed perturbation factor drives evolution in *Saccharomyces cerevisiae* for industrial application. *J Ind Microbiol Biotechnol* 45:869–880. <https://doi.org/10.1007/s10295-018-2057-x>

Article CAS PubMed Google Scholar

Xue S, Jones AD, Sousa L, Piotrowski J, Jin M, Sarks C, Dale BE, Balan V (2018) Water-soluble phenolic compounds produced from extractive ammonia pretreatment exerted binary inhibitory effects on yeast fermentation using synthetic hydrolysate. *PLoS ONE* 13:e0194012. <https://doi.org/10.1371/journal.pone.0194012>

Article CAS PubMed PubMed Central Google Scholar

Yee DA, DeNicola AB, Billingsley JM, Creso JG, Subrahmanyam V, Tang Y (2019) Engineered mitochondrial production of monoterpenes in *Saccharomyces cerevisiae*. *Metab Eng* 55:76–84. <https://doi.org/10.1016/j.ymben.2019.06.004>

Article CAS PubMed PubMed Central Google Scholar

Yoshiyama Y, Tanaka K, Yoshiyama K, Hibi M, Ogawa J, Shima J (2015) Trehalose accumulation enhances tolerance of *Saccharomyces cerevisiae* to acetic acid. *J Biosci Bioeng* 119:172–175. <https://doi.org/10.1016/j.jbiosc.2014.06.021>

Article CAS PubMed Google Scholar

Yu T, Zhou YJ, Huang M, Liu Q, Pereira R, David F, Nielsen J (2018) Reprogramming yeast metabolism from alcoholic fermentation to lipogenesis. *Cell* 174(1549–1558):e1514. <https://doi.org/10.1016/j.cell.2018.07.013>

Article CAS Google Scholar

Zhang JG, Liu XY, He XP, Guo XN, Lu Y, Zhang BR (2011) Improvement of acetic acid tolerance and fermentation performance of *Saccharomyces cerevisiae* by disruption of the *FPS1* aquaglyceroporin gene. *Biotechnol Lett* 33:277–284. <https://doi.org/10.1007/s10529-010-0433-3>

Article CAS PubMed Google Scholar

Zhang MM, Zhao XQ, Cheng C, Bai FW (2015) Improved growth and ethanol fermentation of *Saccharomyces cerevisiae* in the presence of acetic acid by overexpression of *SET5* and *PPR1*. *Biotechnol J* 10:1903–1911. <https://doi.org/10.1002/biot.201500508>

Article CAS PubMed Google Scholar

Zhang GC, Kong II, Wei N, Peng D, Turner TL, Sung BH, Sohn JH, Jin YS (2016) Optimization of an acetate reduction pathway for producing cellulosic ethanol by engineered yeast. *Biotechnol Bioeng* 113:2587–2596. <https://doi.org/10.1002/bit.26021>

Article CAS PubMed Google Scholar

Zhang M, Zhang K, Mehmood MA, Zhao ZK, Bai F, Zhao X (2017) Deletion of acetate transporter gene ADY2 improved tolerance of *Saccharomyces cerevisiae* against multiple stresses and enhanced ethanol production in the presence of acetic acid. *Bioresour Technol* 245:1461–1468. <https://doi.org/10.1016/j.biortech.2017.05.191>

Article CAS PubMed Google Scholar

Zhang MM, Xiong L, Tang YJ, Mehmood MA, Zhao ZK, Bai FW, Zhao XQ (2019a) Enhanced acetic acid stress tolerance and ethanol production in *Saccharomyces cerevisiae* by modulating expression of the de novo purine biosynthesis genes. *Biotechnol Biofuels* 12:116. <https://doi.org/10.1186/s13068-019-1456-1>

Article PubMed PubMed Central Google Scholar

Zhang Y, Wang J, Wang Z, Zhang Y, Shi S, Nielsen J, Liu Z (2019b) A gRNA-tRNA array for CRISPR-Cas9 based rapid multiplexed genome editing in *Saccharomyces cerevisiae*. *Nat Commun* 10:1053. <https://doi.org/10.1038/s41467-019-09005-3>

Article CAS PubMed PubMed Central Google Scholar

Funding

Project FAPESP 2017/24453-5.

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Contributions

AJ had the idea of the article. YM, JG, AJ performed the literature search and wrote the manuscript. JG and AJ compiled the tables. JG and YM draw the figures. All authors critically revised the manuscript.

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Conflict of interest

The authors declare no competing interests.

CHAPTER 3 - ADAPTIVE LABORATORY EVOLUTION: PROTOCOLS AND PHENOTYPIC INSIGHTS IN *Saccharomyces cerevisiae*

3.1 Abstract

This chapter investigates the adaptive responses of *Saccharomyces cerevisiae* strains to sugarcane bagasse lignocellulosic hydrolysate (LCH), employing two distinct Adaptive Laboratory Evolution (ALE) protocols: the serial transfer approach (ALE1, ALE2, ALE3), which involves triplicates of diploid strains of *S. cerevisiae* PE-2, in addition to haploid and diploid strains of *Saccharomyces cerevisiae* CEN.PK113-7D; and the shock/recovery protocol (ALE4), which is subjecting CEN.PK113-7D haploid cells to highly concentrated LCH shocks, leading to an initial survival rate of 8.86% after a 48hr shock. Remarkably, after approximately 764.4 generations, the haploid CEN.PK113-7D (ALE 2) developed tolerance to 50% LCH, whereas the diploid PE-2_H4 (ALE1) demonstrated tolerance to 54% LCH. Post evolution, the populations were subjected to growth competition with the wild-type (WT) strain, these competitions were assisted by flow cytometry and provided information about the adaptation of the experimental populations to LCH. Additional characterization using microplate growth assays demonstrated the evolved populations' adaptability to LCH environments. With a detailed knowledge of evolutionary processes and possible applications in biotechnology and environmental adaptability, this comprehensive phenotypic research adds vital information about yeast evolution under lignocellulosic stress.

3.2 Introduction

One of the main barriers that yeast cells face while producing cellulosic ethanol is the presence of harmful inhibitors in lignocellulosic hydrolysates, produced during the pre-treatment (MENEGON; GROSS; JACOBUS, 2022; PIOTROWSKI *et al.*, 2014). These LCH inhibitors have synergistic effects and are harmful to yeast fermentation, from increasing the lag phase to decreasing growth rates and ethanol yields (CUNHA *et al.*, 2019). Evolutionary engineering proved to be a more appropriate and effective method than rational metabolic engineering for obtaining yeast cells with high resistance to various stress stimuli, as stress resistance is a multigenic and complex feature (CAKAR *et al.*, 2012). By incorporating a selective pressure, or limiting factor, into the experimental design, ALE mimics a "Darwinian" process of evolution. Random adaptive mutations throughout this evolutionary process boost the organisms' survival rate (CAKAR *et al.*, 2012). Better-suited mutants eventually take over the population by producing more offspring, which leads to a superior phenotype (MENEGON; GROSS; JACOBUS, 2022). Yeast cells can evolve and adapt to the challenging circumstances of producing bioethanol, raising process efficiency and lowering costs (TOPALOĞLU *et al.*, 2023).

It is possible to create genetically stable *S. cerevisiae* cells resistant to oxidative stress (KOCAEFE-ÖZŞEN *et al.*, 2022) and other stressors (CAKAR *et al.*, 2005) through evolutionary engineering. More specifically, there are reports of ALE where strains outperformed their ancestral counterparts in fermentations with LCH inhibitors. ALE protocols have varied by administering high doses of LCH (or inhibitors) over hundreds of cell generations or through repeated serial transfers (GONZÁLEZ-RAMOS *et al.*, 2016; HEER; SAUER, 2008; PEREIRA *et al.*, 2020; SMITH; VAN RENSBURG; GÖRGENS, 2014; WALLACE-SALINAS; GORWA-GRAUSLUND, 2013).

ALE strains have demonstrated their ability in LCH, exhibiting shorter lag phases, accelerated growth rates, increased biomass formation, and efficient sugar consumption, all while providing high ethanol yields (MENEGON; GROSS; JACOBUS, 2022; SHEN *et al.*, 2014). In a study that elegantly demonstrates this evolutionary dynamic, an ALE method known as VERT (real-time evolution visualization) was applied. In the VERT experiment, three strains of *S. cerevisiae* were combined in the same proportions and were able to compete in a cultivation regime involving successive increments of corn stover hydrolysate for approximately

463 generations (ALMARIO; REYES; KAO, 2013). The best clones showed enhanced growth rates of up to 57%, 24%, 22%, and 12% in hydrolysate, furfural, HMF, and acetic acid, respectively, when compared to the WT strain (ALMARIO; REYES; KAO, 2013).

To evaluate ploidy-specific mutations and broaden the scope of beneficial variant identification, it may be beneficial to compare diploid and haploid populations in ALE experiments (MENEGON; GROSS; JACOBUS, 2022). Furthermore, *S. cerevisiae* strains from industrial sectors, laboratories, or natural environments constitute a source of genetic diversity (JACOBUS *et al.*, 2021; PETER *et al.*, 2018). Physiological responses, transcription patterns, and fitness to different stresses may vary considerably according to the genetic profile of yeast (LI *et al.*, 2015; PETER *et al.*, 2018). Among the commercial strains of *S. cerevisiae* often found in Brazilian ethanol facilities, PE-2 stands out for its capacity to successfully combat indigenous contaminants and endure the difficulties associated with industrial fermentations. Remarkably, data from 2007 and 2008 show that PE-2 and CAT-1 were implemented in nearly 150 distilleries, accounting for almost 60% of Brazil's total ethanol output (BASSO *et al.*, 2008).

In this study, we decided to scrutinize diploid and haploid populations in ALE experiments to assess ploidy-specific mutations and broaden the scope of finding advantageous variations. We employed the haploid and diploid *S. cerevisiae* CEN.PK113-7D strain that has a laboratory background, being a model for modern industrial biotechnology (NIJKAMP *et al.*, 2012), and the diploid PE2_H4 strain, an isolate from the PE-2, a strain isolated from the Brazilian industrial sector (BASSO *et al.*, 2008). Serial transfers were performed on the three strains, each in triplicate, and the haploid CEN.PK113-7D strain also experienced a shock/recovery ALE protocol. By investigating changes brought about by different approaches, this method seeks to improve our knowledge of hydrolysate stress adaptation, protein patterns, and metabolic reactions.

3.3 Materials and Methods

3.3.1 Strains Development

The primers employed in developing the strains are documented in Table 1, located in Appendix A, Section 1.

3.3.1.1 *Saccharomyces cerevisiae* CEN.PK113-7D: Construction of haploid lineage

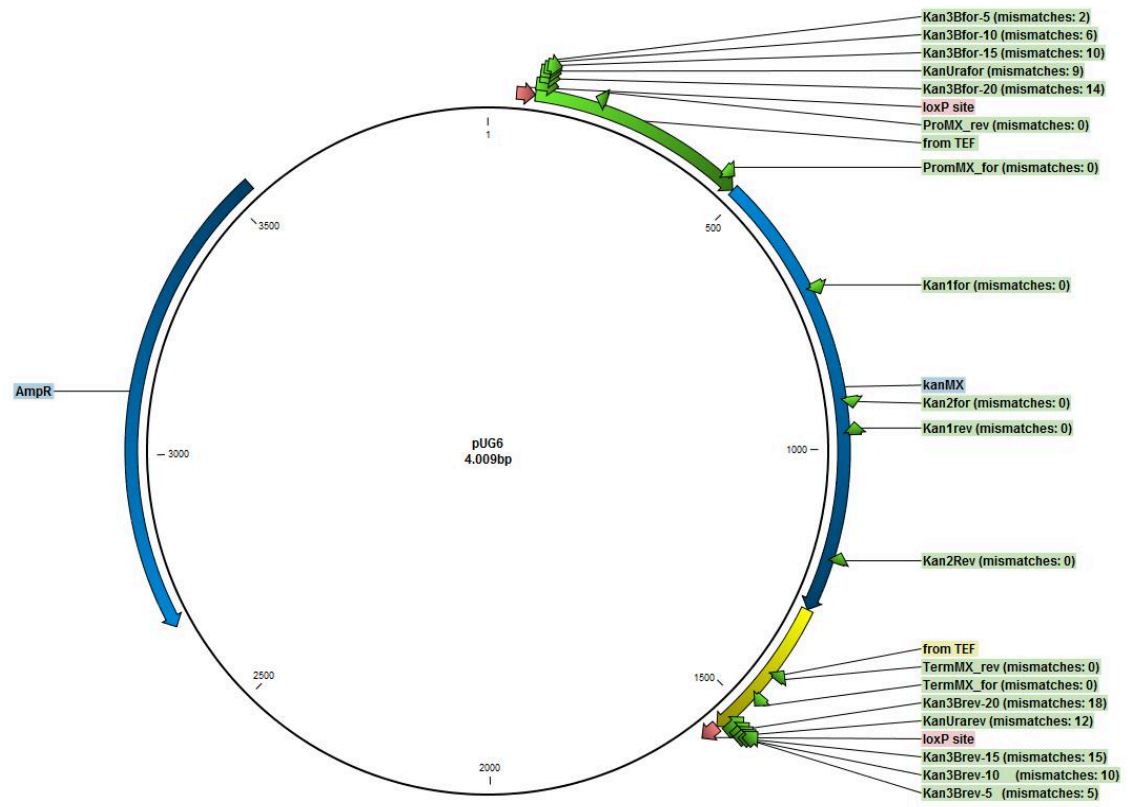
The yeast *Saccharomyces cerevisiae* CEN.PK113-7D was selected for the study because it is a popular laboratory strain for systems biology studies. This choice was made due to the greater sensitivity of the CEN.PK113-7D strain to lignocellulosic inhibitors, in addition to this strain being non-flocculant, which facilitates flow cytometry analysis. For the ALE purpose, three haploid populations (CEN.PK113-7D, MATa) were prepared by integrating, in each one, a different antibiotic resistance cassette: kanMX (geneticin resistance), hphMX (hygromycin) and natMX (nourseothricin) (GOLDSTEIN; MCCUSKER, 1999). The insertion site was the *FLO* locus, a gene that gives rise to a lectin-like protein involved in flocculation (Appendix A; Section 1; Figure 1).

3.3.1.1.1 *Saccharomyces cerevisiae* CEN.PK113-7D: Cassette amplification

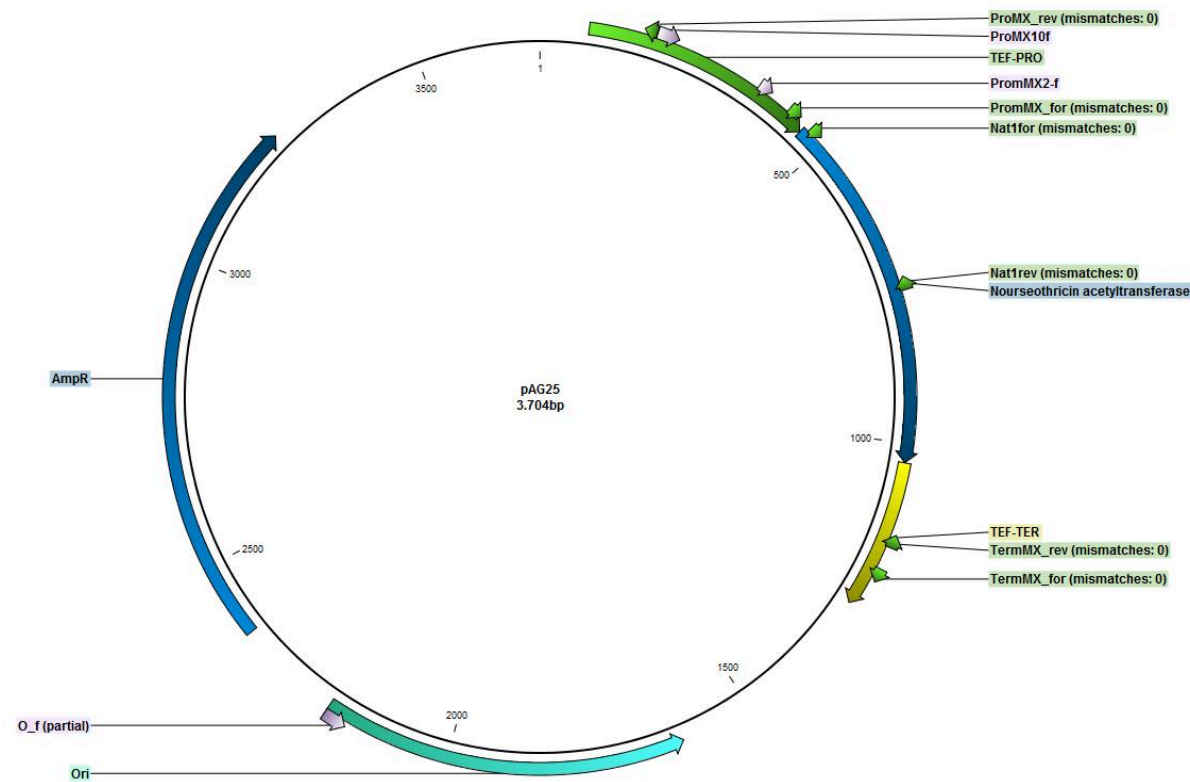
For the development of antibiotic-resistant strains, first, the kanMX cassette (1.672 pb) present in the pUG6 plasmid (Figure 3.1A) (GOLDSTEIN; MCCUSKER, 1999) was amplified with primers with homology to the *FLO* gene. The Polymerase Chain Reaction (PCR) technique was performed using Phusion® (high fidelity DNA polymerase-NEB) and the primers FLO8Kan_for and FLO8Kan_r. For amplification of the nourseothricin (1.031 pb) and hygromycin (1.487 pb) resistance cassette, plasmids pAG25 (Figure 3.1B) and pAG34 (Figure 3.1C) were used, respectively. The success of amplifications can be checked in Appendix A, Section 1, Figure 2. The primers used to amplify these fragments were promMX2F and terMXrev.

Figure 3.1 - Plasmids pUG6, pUG25, and pUG34.

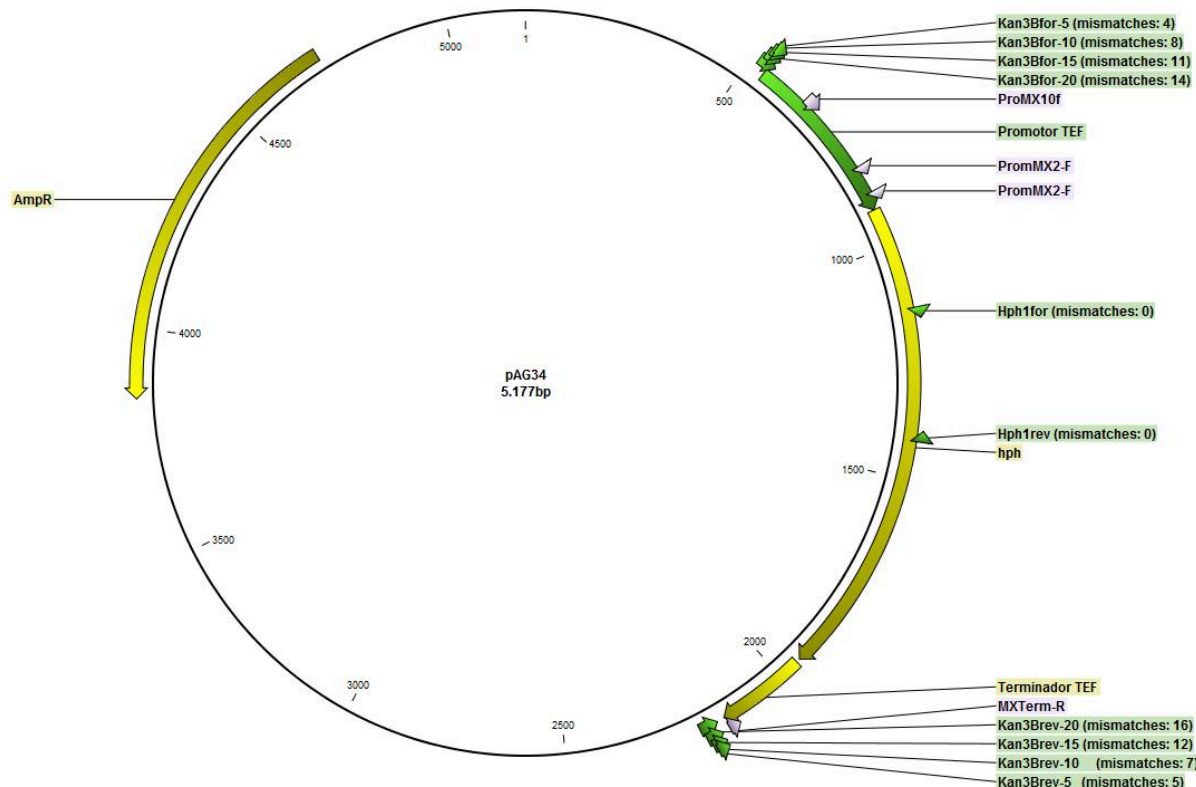
A



B



C



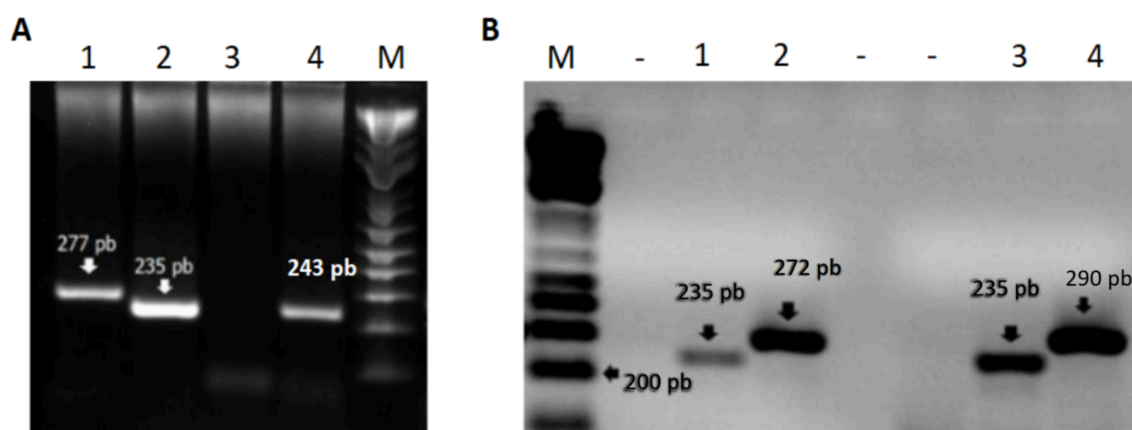
A- Plasmid pUG6, used to amplify the geneticin resistance cassette. **B-** pUG25, used to amplify the nourseothricin resistance cassette. **C-** pUG34, used to amplify the hygromycin resistance cassette.

3.3.1.2 Genetic transformation of *S. cerevisiae* CEN.PK113-7D FLO::kanMX, FLO::hphMX and FLO::natMx

The *S. cerevisiae* CEN.PK113-7D was transformed with the FLO::kanMX cassette from the addition of the DNA and the cell to be transformed into 600uL Polyethylene glycol (PEG 4000) 40% + LiAc 0.1M + TE 1x, incubated in a water bath at 42° C, 45 min, and subsequent incubation for 5 min on ice. Centrifuged for 3 min at 8,000 rpm and resuspended the pellet in 15 mL of 2% sucrose YPD medium. After this process, the cells were incubated in a Shaker at 28 °C, 200 rpm for 1 hour, and then centrifuged for 5 min at 8,000 rpm. Finally, resuspend, plated in YPD with geneticin (200ug/mL), and incubated at 28 °C for approximately 2 days (GIETZ; WOODS, 2006).

To confirm the insertion, PCR was performed in the internal region of the cassettes (Figure 3.2A). Transformation of the CEN.PK113-7D FLO::hphMX and FLO::natMX strains were performed using the cassette exchange technique. The inserted kanMX cassette is replaced through homologous recombination by other cassettes with homology in the promoter and terminator region. The DNA was the PCR product of the respective cassette transformed from plasmid pAG34 (hygromycin) and the pAG25 fragment (nourseothricin). The primers amplifying the resistance region to the respective antibiotics were used as internal controls (Figure 3.2B). The resulting populations CHG, CHH, and CHN are the progenitors of ALE2 and ALE4.

Figure 3.2 - Confirmation of antibiotic resistance cassette inserts.



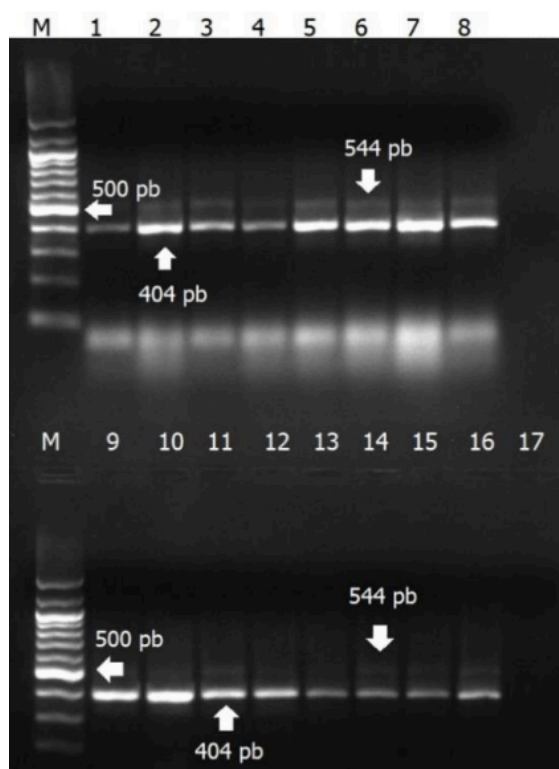
A- PCR amplification of kanMX cassette insert flanks. 1- The 2% agarose gel electrophoretic run profile of the 277 bp Flank 1 PCR product (ProMx_rev / ExL_FLO8) is shown. 2- Flank 2 amplification of 235 bp (TermMx_for / ExR_FLO8). 3- Absence of amplification of Externals (ExR_FLO8 / ExL_FLO8). Specifying kanMX cassette insertion. 4- 243bp positive control (ADH5 F2/ ADH5 R2). M- DNA Marker 1kb Plus. **B-** 2% agarose gel electrophoretic run profile of PCR products from CEN.PK113-7D FLO::HphMX and FLO::natMX. M - 1kb Plus DNA Marker. 1- FLO::HphMX flank of 235 bp (TermMx_for / ExR_FLO8), 2- Hygromycin antibiotic positive control of 272pb (Hph2_for / Mxterm_5r). 3- FLO::natMX flank 2 of 235 bp (TermMx_for / ExR_FLO8), 4- Internal positive control of the antibiotic nourseothricin of 290 bp (Nat 1F / Nat 1 R).

3.3.1.3 Construction of diploid *Saccharomyces cerevisiae* CEN.PK113-7D lineage

The diploid strains of CEN.PK113-7D were developed from the haploid populations of CEN.PK113-7D. For this, the CEN.PK113-7D CHH, resistant to hygromycin (haploid, FLO::hphMX) (*MATa*), was changed to *MATalfa* through the ectopic expression of endonuclease HO (HABER, 2012). After the transformation, 3 colonies were selected and placed in 1ml of YP + 2% galactose + zeocin (plasmid resistance marker) overnight at 28 ° C. Afterwards, they were plated in YPD + ampicillin, and individual colonies were tested for the mating type. The primer forward Mat Locus amplifies locus Mat with reverse primers *MATa* for mating type A (544 PB) and *MATalfa*, for mating type Alfa (404 PB) (Appendix A, Section 1, Figure 3).

The resulting strain (Matalpha, Flo::hphMX) was crossed with Haploid CHG to form the Diploid CDG (Flo::kanMX, Flo::HphMX) and with the Haploid CHN to originate the diploid CDN (FLO::natMX, FLO::hphMX). To achieve this, the *MATalfa* strain, Flo::hphMX was cultivated together or with the *MATa* strain Flo::kanMX or with the *MATa* strain Flo::natMX, in 125 ml Erlenmeyer bottle, with 20 ml of YPD 2% sucrose and inoculum of 50 uL of each cultivation, for 24 hours. After this period, 100ul of this co-cultivation was inoculated and propagated in a 125 ml Erlenmeyer bottle, with 20 ml of 2% sucrose YPD and their respective antibiotics, to select only the diploids of interest. The formation of diploid colonies was confirmed through the Mating type multiplex PCR of colonies (Figure 3.3).

Figure 3.3 - Multiplex PCR amplification of the mating type to identify diploids of CEN.PK113-7D.

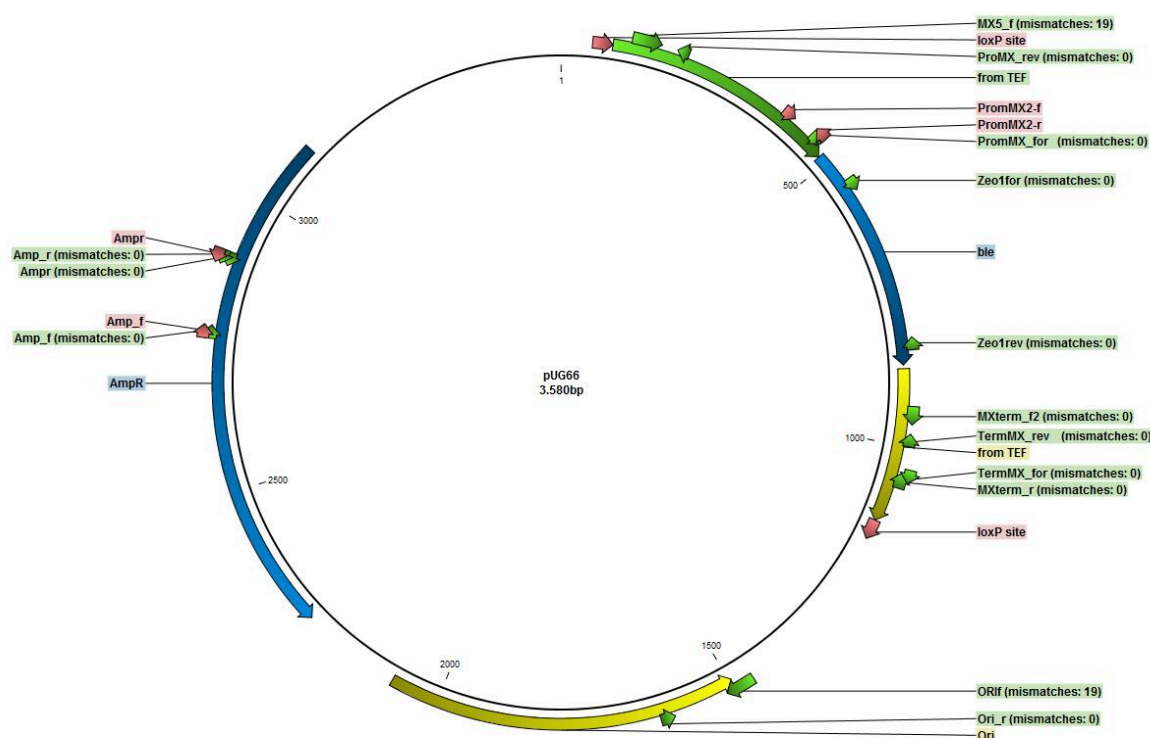


M - 1kb DNA marker Plus. 1/8 – The gel electrophoretic run profile is shown at 1.5% agarose from different colonies tested for mating type (FLO::kanMX, FLO::hphMX). 9/16 - Different colonies tested for mating type (FLO::natMX, FLO::hphMX). 17 - Negative control (PCR reaction without DNA).

3.3.1.4 Construction of the diploid *Saccharomyces cerevisiae* PE-2_H4 lineage

The construction of the H4 haploid parent (MATa), which was segregated from the diploid heterothallic yeast *S. cerevisiae* PE-2 (widely used Brazilian bioethanol strain) (BASSO *et al.*, 2008) and sequenced by our laboratory (JACOBUS *et al.*, 2020), allowed the subsequent production of diploid PE-2_H4 progenitors. Four haploid H4 (MATa) populations were prepared by integrating, in each one, a different antibiotic resistance cassette: kanMX (geneticin resistance), hphMX (hygromycin), natMX (nourseotricin), the same method used in the development of populations of CEN.PK113-7D, in addition to these, the zeoMX cassette (zeocin) was used, which was amplified from the pUG 66 plasmid (Figure 3.4) (GOLDSTEIN; MCCUSKER, 1999).

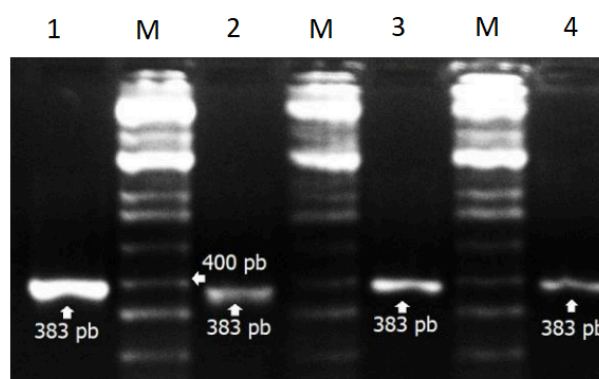
Figure 3.4 - Plasmid pUG66.



Plasmid is used to amplify the zeocin resistance cassette.

The insertion site was the HO locus, which is neutral in the *S. cerevisiae* PE-2 strain (Appendix A, Section 1, Figure 4). Confirmation PCR for these transformations amplifies the right flank of this insertion. The resulting haploid populations were labeled as HK (Haploid, HO::kanMX), HH (Haploid, HO::hphMX), HN (Haploid, HO::natMX), and HZ (Haploid, HO::zeoMX) (Figure 3.5).

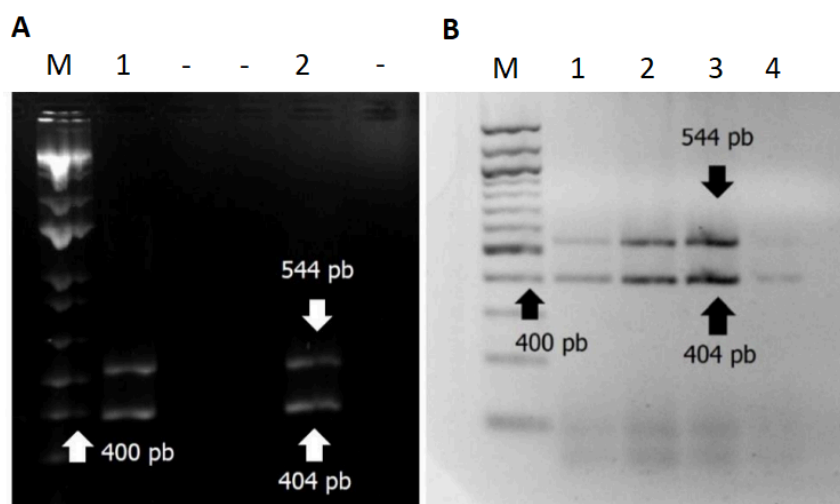
Figure 3.5 - Electrophoretic running profile of the insertion flanks kanMX, natMX, hphMX, and zeoMX cassettes in PE-2 H4.



1- Haploid, Ho::kanMX; B- Haploid, Ho::hphMX. C- Haploid, Ho::natMX. D- Haploid, Ho::zeoMX. M - DNA Marker 1KB Plus. Agarose gel 1.5%.

From haploid PE-2 strains, diploid PE-2 strains were developed. This was achieved through ectopic expression of the HO endonuclease as done with CEN.PK113-7D. The resulting strain (MAT α , HO: hphMX) was crossed with the HK haploid to form the PE-2_H4_HG diploid (HO::kanMX, HO::hphMX) and with the HN and HZ haploids to form the PE2_H4_HN (HO::natMX, HO::hphMX) and PE-2_H4_HZ diploid (HO::zeoMX, HO::hphMX), respectively. The strains were co-cultivated for the formation of diploids using the same protocol described in the previous section. The formation of diploid colonies was confirmed by mating type tests individually for the colonies (Figure 3.6). PE-2_H4_HG, PE-2_H4_HN, and PE-2_H4_HZ strains are the progenitors of ALE 1.

Figure 3.6 - Electrophoretic run profile of the mating type to identify PE-2_H4 diploids.



A- M- 1kb Plus DNA Marker. 1 - Diploid mating-type HO::hphMX, HO::kanMX is shown. 2 - Diploid HO::hphMX, HO::natMX. **B-** M - 1kb Plus DNA Marker. 1 to 4 - Different colonies tested for mating type Diploid HO::hphMX, HO::zeoMX. 1.5% agarose gel.

3.3.2 Sugarcane Bagasse Lignocellulosic Hydrolysate

3.3.2.1 Hydrolysate Production

To obtain the LCH, a pilot pre-treatment reactor was used, with a capacity of 350 L, built-in rodloy alloy 276 (Pope Scientific INC). The equipment belongs to the Pilot Plant for Process Development (PPDP) of the National Laboratory of Biorenewables (LNBR-CNPEM) and is equipped with a mechanical agitation system and heating through the injection of steam or thermal oil in the jacket, the experiment was carried out by the team of Dr. Sarita Rabelo.

The pretreatment reaction carried out using 160°C, 20 min, 0.75% (m/m) sulfuric acid, was carried out to potentiate the formation of fermentative inhibitory compounds, such as furan aldehydes (furfural, hydroxymethylfurfural (HMF)), organic acids (levulinic and formic acid) and phenolic compounds derived from lignin. Approximately 20 kg of sugarcane bagasse (dry basis) was loaded into the reactor, and then the sulfuric acid solution was fed, considering a final solids concentration of 10% (m/m). The reaction mixture was heated by thermal fluid in the jacket and maintained at the established temperature and time. After the reaction time, the reactor was gradually cooled until it reached a temperature of 100°C, depressurized,

and discharged into a Nutsche-type filter with a capacity of 100 L and made of 276-rod steel (Pope Scientific INC). The full hydrolysate production and characterization can be found in Appendix A, Section 2.

3.3.2.2 Hydrolysate Characterization

To obtain precise knowledge about the composition and quantities of each inhibitor present in the customized LCH, the LCH was subjected to standard HPLC detection techniques. Under the responsibility of Dr. Brienzo, the concentrations of furfural, HMF, and phenolic compounds were determined at the IPBEN facility by HPLC using a Waters instrument (USA) with a UV detector (at 276 nm) and using a Waters Resolve C18 column (5 μ m, 300 x 3.9 mm) at room temperature. Acetonitrile/water (1/8 with 1% acetic acid) served as the eluent at a flow rate of 0.8 mL/min and 20 μ L sample volume. Standard solutions of each compound were used for a reference curve at concentrations ranging from 0.001 - 0.5 g/L

3.3.2 Serial Transfer Protocols: ALE1, ALE2, and ALE3

The evolution of ALE1 populations: diploid PE-2 H4 (PE-2_H4_HG, PE-2_H4_HN, and PE-2_H4_HZ); ALE2: haploid CEN.PK113-7D (CHG, CHH and CHN); and ALE3: diploid CEN.PK113-7D (CDG and CDN) were challenged by propagation (serial transfers) at increasing concentrations of LCH. From insights gained from the pilot experiment (Appendix A, Section 3, Figure 5), the experiment started with a concentration of 25% hydrolysate for the diploid populations and 20% for haploid populations. The composition of what is called a “100% hydrolysate” can be found in Table 3.1.

The experiment volume was determined to be 5 mL (4,975 mL of hydrolysate) with a transfer of 25 μ L (1×10^8 cells/mL). Cultivations were carried out in 125 mL Erlenmeyer flasks at 28 °C and agitation at 120 rpm.

After each transfer, cells are allowed to grow to a stationary phase, after which 25 μ L are sampled and inoculated into 4,975 mL of fresh LCH (resulting in a 1:200 dilution) for a further round of propagation (approximately 7.644 generations per round). The approximate value of 7,644 generations per round is obtained through $\log_2 200$ ($\log_2 200 = 7.6438561897747$) (WISER; LENSKI, 2015).

Periodic increases in LCH concentrations were performed throughout the ALE when the experimental populations exhibited improved tolerance (growth in less

than 30h). The long-term ALE experiment was maintained for 100 transfers (approximately 764 generations).

Table 3.1 - Composition of the hemicellulose hydrolysate from the pre-treatment with dilute sulfuric acid.

Compounds	Concentration (g/L)
Glucose	5,62 ± 0,12
Xylose	10,78 ± 0,41
Arabinose	2,02 ± 0,080
Cellobiose	0,28 ± 0,021
Formic acid	1,25 ± 0,041
Acetic Acid	5,98 ± 0,062
HMF	0,150 ± 0,0020
Furfural	0,45 ± 0,0010
Total phenolic compounds	1,72 ± 0,12
Vanillic Acid	0,0980 ± 0,004
p-coumaric acid	0,579 ± 0,01
Syringic acid	0,190 ± 0,01
Xylooligosaccharides	0,00

3.3.3 Genome Shuffling Protocol for Diploid Populations ALE1 and ALE3: Streamlining for Efficiency

Sexual reproduction during ALEs (genome shuffling) is known to increase genetic diversity by recombining (via meiosis) adaptive alleles from different parental strains in the resulting offspring cells (CAO *et al.*, 2010; HOU, 2009; SHI; WANG; WANG, 2009; SNOEK *et al.*, 2015; ZHENG *et al.*, 2011). To take advantage of these

evolutionary strategies, after every 10 transfers (ALE1 and ALE3), 1 mL of diploid cells ($> 10^8$ cells) are sporulated in a nitrogen-poor medium (Potassium Acetate 10g/L, Yeast Extract 1 g/L and Sucrose 0.5 g/L) (Appendix A; Section 4; Figure 8). At the end of three days, spores are treated and germinated in 2% sucrose YPD medium overnight to form diploids again. Then, a new round of growth in 2% sucrose YPD containing the antibiotics of each population (HG: hygromycin and geneticin, HN: hygromycin and nourseothricin and HZ: hygromycin and zeocin; CDG: hygromycin and geneticin CDN: hygromycin and nourseothricin), will cause only diploid cells to survive. After each genome shuffling event, ALE1 and ALE3 populations were usually propagated in LCH for another 10 transfers when a new round of genome shuffling occurred.

3.3.4 Exploring the Impact of Highly Toxic LCH Shocks: ALE 4 Protocol

Haploid *S. cerevisiae* CEN.PK113-7D (the same used on ALE2) was challenged with shocks (acute treatments) of highly concentrated LCHs. The ALE4 population (triplicates: S_CHG, S_CHH, and S_CHN) was then initially cultured in YPD (2% sucrose) until the stationary phase was reached when 1 mL was pelleted, washed, and resuspended in a concentration of 100% LCH. Cells were maintained at this LCH concentration for 48 hours at 28° C, where the survival rate was ~8,86% (Appendix A, Section 3.2, Figure 7).

After the 48-hour shock, cells were transferred to a fresh 2% YPD medium (20 mL) for recovery and growth (2-4 days) until the stationary phase was reached, at which time 1 mL was used again for the next round of shock. The LCH concentration and survival rate, determined in a pilot study (Appendix A, Section 3.2, Figures 6 and 7) as 100% for 48h, meant that we could not increase the hydrolysate concentration or the exposure time to increase the challenge. Additionally, contamination control tests were performed during the 4 experiments (ALE1, ALE2, ALE3, and ALE4) and can be accessed in Appendix A, Section 4.2, Figure 9.

3.3.5 Flow cytometry-assisted Competition Assays

To assess fitness associated with evolved populations or evolved alleles, ALE protocols rely heavily on phenotypic analyses, such as competition assays in which at least one of the competitors is genetically transformed with a fluorescence marker, allowing differential cell counts and accurate measurements of strain

adaptation. For this purpose, a GFP reporter gene was inserted into the genome of the wild-type (WT) strains (*S. cerevisiae* PE-2_H4 and *S. cerevisiae* CEN.PK113-7D) by integration into the HO locus. The strains that express green fluorescent protein (GFP)—H4, HO::GFP, and CEN.PK113-7D, HO::GFP—serve as fluorescence-tagged standards for all evolved strains and will be competing with them.

3.3.6 Competition assays by flow cytometry: ALE1, ALE2, and ALE3

For the competition experiments, the evolved strain, and WT strain competed with the GFP-carrying WT strain. This is because it has already been proven that the insertion of GFP even in inactive genes (HO) leads to the production of new metabolites and the overexpression of others. This suggests that GFP integration is not neutral (BORDET *et al.*, 2022). In this way, in this experiment, the GFP's weight could be reduced, guaranteeing that competitions were always conducted indirectly. This allows for evaluating the competition's effects without interference from GFP, resulting in a controlled and insightful analysis of evolutionary dynamics. WT lines containing GFP (Green Fluorescent Protein) were developed as part of this study. Details on the production of these strains are available in Appendix A, Section 5, providing an additional source of information for reference.

Before the competition, yeasts were incubated until a stationary phase for pre-growth. Approximately 1×10^8 cells were incubated for two more 24-hour rounds in 15% LCH for acclimation. To compete (Evolved x WT GFP; WT x WT GFP), an initial mixture of each cell (in a 1:1 ratio) was subjected to the stress condition in LCH. The competition condition was the same as the serial transfer evolution, with 4,975 μ L of medium containing YPD-2x complemented with LCH and water at the desired concentrations in 25mL erlenmeyers and 25 μ L inoculum (approximately 5×10^8 cells), to complete 5mL. In this restrictive medium, the evolved cells (unlabeled) are expected to grow more efficiently than the sensitive cell line (expressing GFP), increasing the proportion of cells without fluorescence at the end of the experiment. After the competition (stationary phase), the total cells were quantified. The number of cells that do not express GFP corresponds to the number of cells in the evolved/WT lineage.

Each competition was performed in triplicate and different hydrolysate concentrations: 10%, 15%, and 25%, and control without hydrolysate (0%), totaling

36 competitions for each population. The results were read on the cytometer after 72 hours of incubation.

By knowing the number of evolved cells (EV) and the number of sensitive cells (GFP) at the initial (I) and final (F) points of the competition, it is possible to obtain the selection coefficient S (how much the evolved lineage is more “adapted” than the sensitive). This is called “fitness” and is calculated using the formula (DELUNA *et al.*, 2008):

$$S = \text{LN} (\text{evF} / \text{gfpF}) - \text{LN} (\text{evI} / \text{gfpl})$$

Data normalization for comparison purposes was carried out by generation calculation. To calculate the S/generation, the formula was used:

$$\text{S/generation} = S / \text{Log2} (\text{celF} / \text{cell})$$

Where “cel” is the total amount of cells (evolved and GFP together) expressed in numbers. It is essential to consider that the value indicated in the cytometer software is the value that was previously diluted, being under dilution factor 1: 100 (10^{-2}). Therefore, it is necessary to correct the dilution factor.

This experiment resulted in indirect competition. In this way, it is necessary to reduce the average generation of the parent from the S/generation so that the intrinsic result of the parent does not interfere with the analysis of the evolved ones. The normalized S/generation result was plotted in Fig 3.7 (in results section)

3.3.7 Microplate growth assays: ALE1, ALE2, and ALE3

For microplate LCH tolerance assays, WT and evolved yeasts were incubated up to the stationary phase for pre-growth. About 2×10^7 cells were inoculated for 24 hours in 15 ml of YPD. Cell number quantifications were performed by flow cytometry. The cells were diluted to a concentration of 2×10^5 cells per microliter. In each plate well, the same amount of YPD-2x and LCH is added so that the final hydrolysate concentration is 50% or YPD is added so that we have the growth in 0% hydrolysate. Thus, it was inoculated approximately 1×10^5 cells into each well and the growths are done at least in sextuplicate. In addition, at least two wells were used as a blank for each hydrolysate concentration. Plate conditions were 28°C and gentle

shaking every 3 minutes. Readings were taken every 15 min at OD 600 nm on the Tecan Sunrise© microplate reader. The microplate experiments were set up following the recommendations to avoid the biases of these types of experiments (AULD *et al.*, 2020).

The parameter we used to interpret the data is the Tmid (SPROUFFSKE; WAGNER, 2016) which refers to half the time required for the growth curve to reach the maximum OD during propagation. Thus, the Tmid is a parameter that relates the time of adaptation to the medium, the growth velocity during the log phase, and the maximum density of cells in the medium. This value is calculated in R with the ggplot2 package and growthcurver. One-way ANOVA and post-Bonferroni test statistics for multiple comparisons being the difference between evolved and WT populations at 50% different LCH with $p < 0.0001$ (****) and p of $p < 0.005$ (**).

3.4 Results

3.4.1 Constructed Strains: Recap from Previous Section

To improve the understanding of the developed populations of CEN.PK113-7D and PE-2 H4 and their names (Table 3.2), a figure was created (Figure 3.7).

Figure 3.7 - Representation of the construction of haploid and diploid experimental populations used in the LCH adaptive evolution experiments.

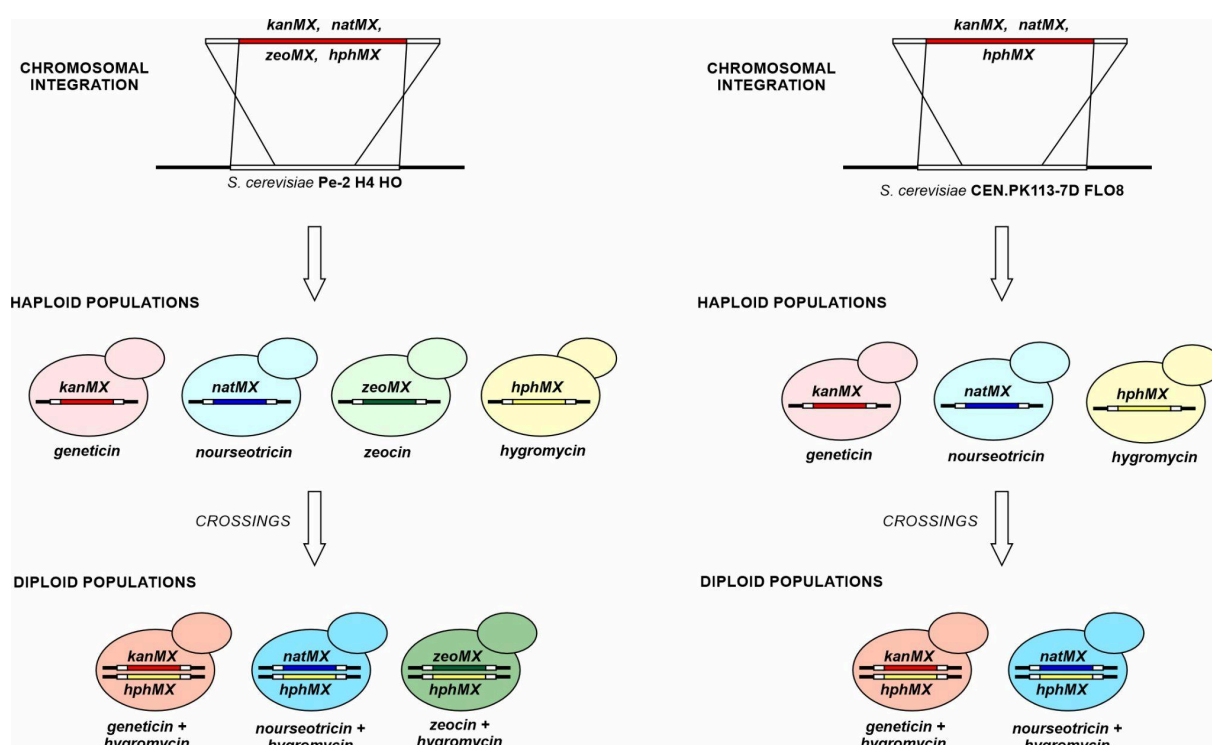


Table 3.2 - Overview of Developed Populations and Corresponding Names for CEN.PK113-7D and PE-2 H4.

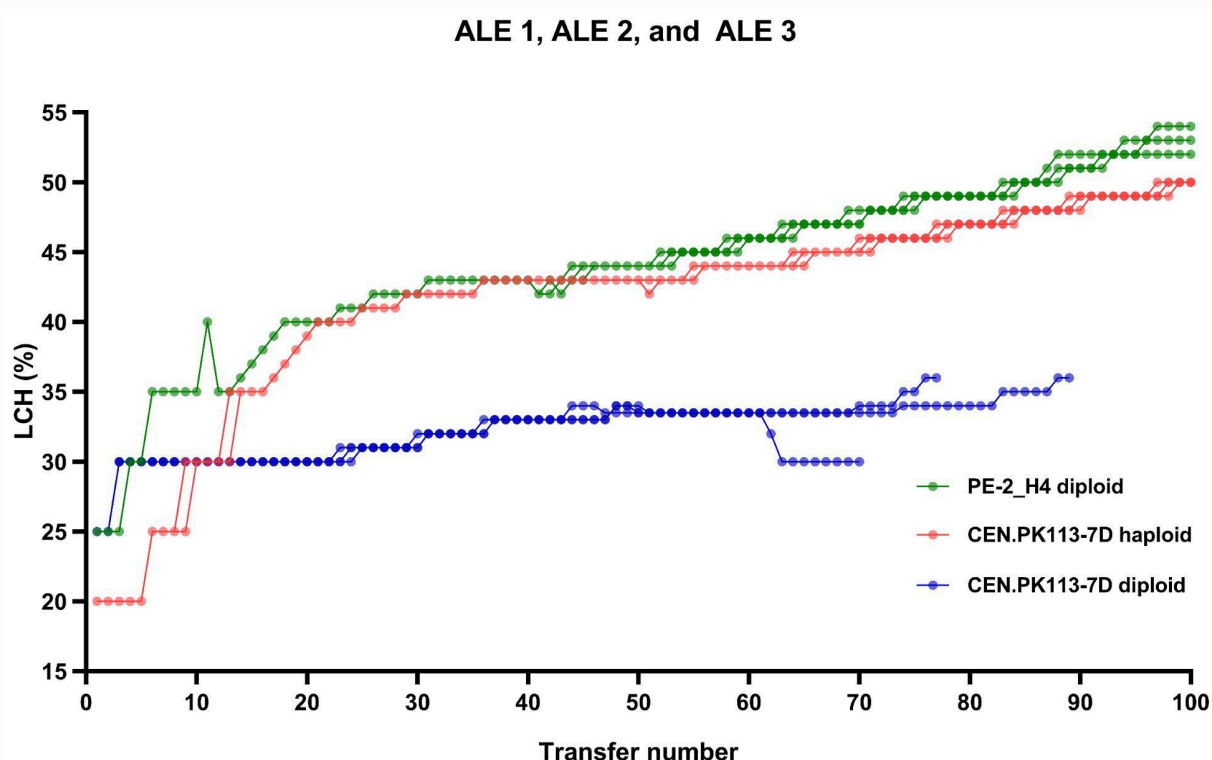
ALE	Protocol	Strain	Replicate Name
ALE 1	Serial Transfer	<i>Saccharomyces cerevisiae</i> PE-2 H4 diploid	PE-2_H4_HG
			PE-2_H4_HN
			PE-2_H4_HZ

ALE 2	Serial Transfer	<i>Saccharomyces cerevisiae</i> CEN.PK113-7D haploid	CHG
			CHH
			CHN
ALE 3	Serial Transfer	<i>Saccharomyces cerevisiae</i> CEN.PK113-7D diploid	CDG
			CDN
ALE 4	Shock/Recovery	<i>Saccharomyces cerevisiae</i> CEN.PK113-7D haploid	S_CHG
			S_CHH
			S_CHN

3.4.2 Serial Transfer Protocols: ALE1, ALE2, and ALE3

The triplicate of the diploid PE-2_H4 strain (Pe-2_H4_HG, Pe-2_H4_HN, and Pe-2_H4_HZ) reached 100 transfers after 1 year and 2 months of the experiment; The triplicate of the haploid CEN.PK113-7D strain (CHG, CHH, and CHN) reached 100 transfers after 10 months of the experiment; two diploid CEN.PK113-7D (CDG and CDN) ended the ALE with approximately ~580 generations, reaching 1 year of experimentation. While the diploid strains PE-2_H4 and haploid CEN.PK113-7D reached, respectively, tolerance to 54% and 50% of hydrolysate, the diploid CEN.PK113-7D reached tolerance to 36% (CDG) and 31% (CDN) of hydrolysate (Figure 3.8).

Figure 3.8 - Adaptive Laboratory Evolution (ALE) Serial Transfers experiments.



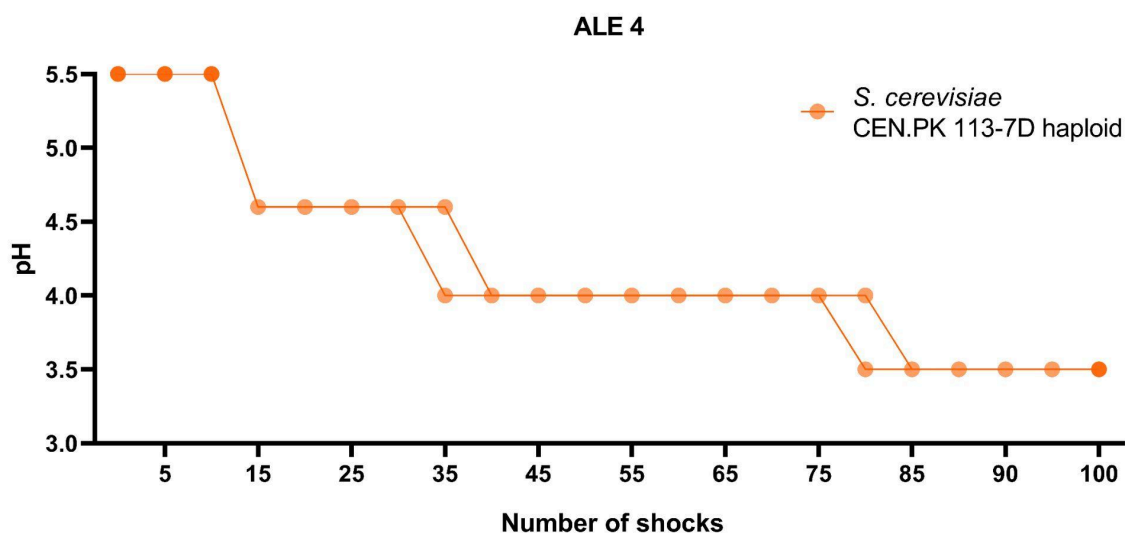
The graph displays the gradual increase in hydrolysate concentration as a function of the number of transfers. The diploid PE-2_H4 strains are represented in green, and the haploid and diploid strains CEN.PK113-7D in orange and blue, respectively.

3.4.3 Exploring the Impact of Highly Toxic LCH Shocks: ALE 4 Protocol

In treatments that eliminate the vast majority of cells in a population, surviving individuals (carriers of adapted alleles) rapidly repopulate the ALE experiment environment. This fast-changing evolutionary dynamic can be advantageous for accelerating the enrichment of adapted alleles during ALE experiments (COUCE *et al.*, 2017).

Early in the 48 h, 100% LCH shocks, the recovery time was reduced to 24 h during 3-4 successive shock/recovery rounds. This behavior was interpreted as a clear sign of adaptation. From that point on, to increase the challenge, the pH of the LCH progressively decreased each time we observed a consistent adaptation to it (Figure 3.9).

Figure 3.9: Adaptive Laboratory Evolution of LCH Shocks.

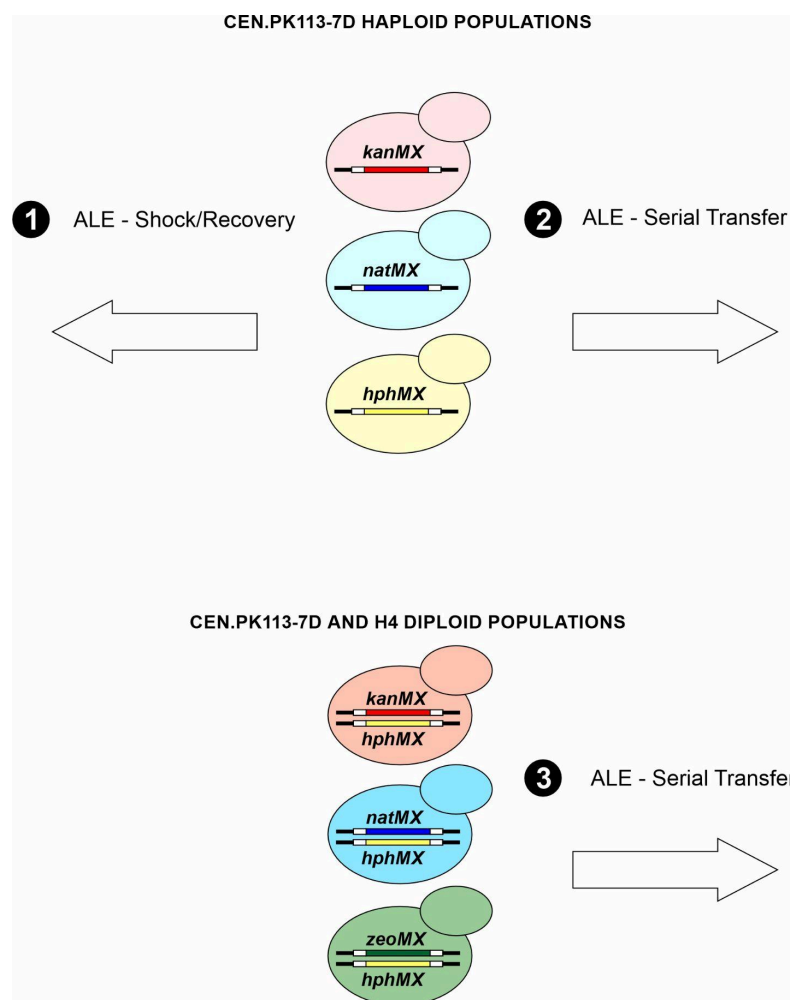


Repeated treatments (100 in total) of LCH with decreasing pH values (5.5-3.5) were applied, each cycle for 48 hr, followed by recovery on YPD 2% sucrose.

3.4.4 Protocol Overview: Summary of Methods Employed for Each Population

To enhance comprehension of the protocols employed for each population, there is an illustration (Figure 3.10).

Figure 3.10: Representation of ALE approaches in developed populations.



The figure represents the experimental design, where the haploid and diploid populations of CEN.PK113-7D, together with the diploid population of Pe-2_H4, were subjected to the serial transfer protocol. Furthermore, the Shock procedure was used for the haploid CEN.PK113-7D population.

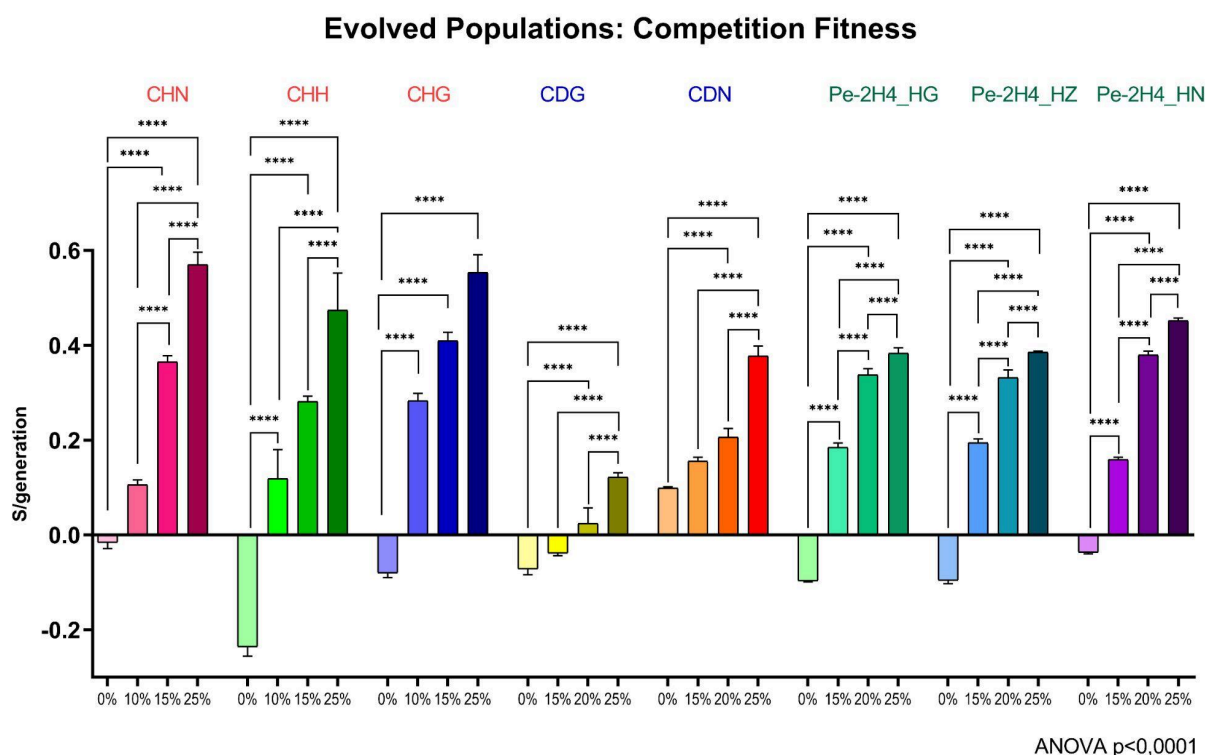
3.4.5 Competition assays by flow cytometry: ALE1, ALE2, and ALE3

The fitness of populations evolved at LCH was determined through flow cytometry-assisted growth competitions against GFP-tagged references, allowing the calculation of selection coefficients (S) per generation. The mean values of the triplicates were normalized by competition with the WT strains, and statistical significance was established at $p < 0.0001$ and was determined by one-way ANOVA with Bonferroni post-test for multiple comparisons.

Each triplicate of the strain was subjected to competition (in triplicate) in different concentrations of hydrolysate (0%, 10%, 15%, and 25% for haploid populations and 0%, 15%, 20% and 25% for diploid populations). Investigation of the adaptation phenotype of evolved populations to lignocellulosic hydrolysate (LCH) concentrations yielded valuable information about experimental lineages. At 0% hydrolysate concentration, all strains, except CDN, exhibited lower performance than the WT strain (Figure 3.11). This decline in fitness may be related to a trade-off phenomenon, which implies that, although they adapt to LCH, growth in the absence of hydrolysate or a rich medium is compromised. This trade-off phenomenon should be considered when understanding evolutionary dynamics.

Furthermore, as the hydrolysate concentration increased, a notable trend emerged; the fitness of the evolved populations surpassed that of the WT strain (Figure 3.7). This trend indicates that the phenotypes of the evolved strains were specifically shaped to confer tolerance to the hydrolysate.

Figure 3.11 - Fitness of the Evolved Populations on LCH.



Flow cytometry-assisted growth competitions of evolved populations vs. GFP-tagged references allowed assessment of selection coefficients (S) per generation. Average values from triplicates were normalized by competition with the WT strains. The

statistical method was the 2-way ANOVA with Bonferroni post-test for multiple comparisons. **** = $p < 0.0001$.

The improvement in fitness with increasing hydrolysate concentration suggests a robust adaptive response, showing the ability of these populations to adjust their phenotypes in response to environmental challenges.

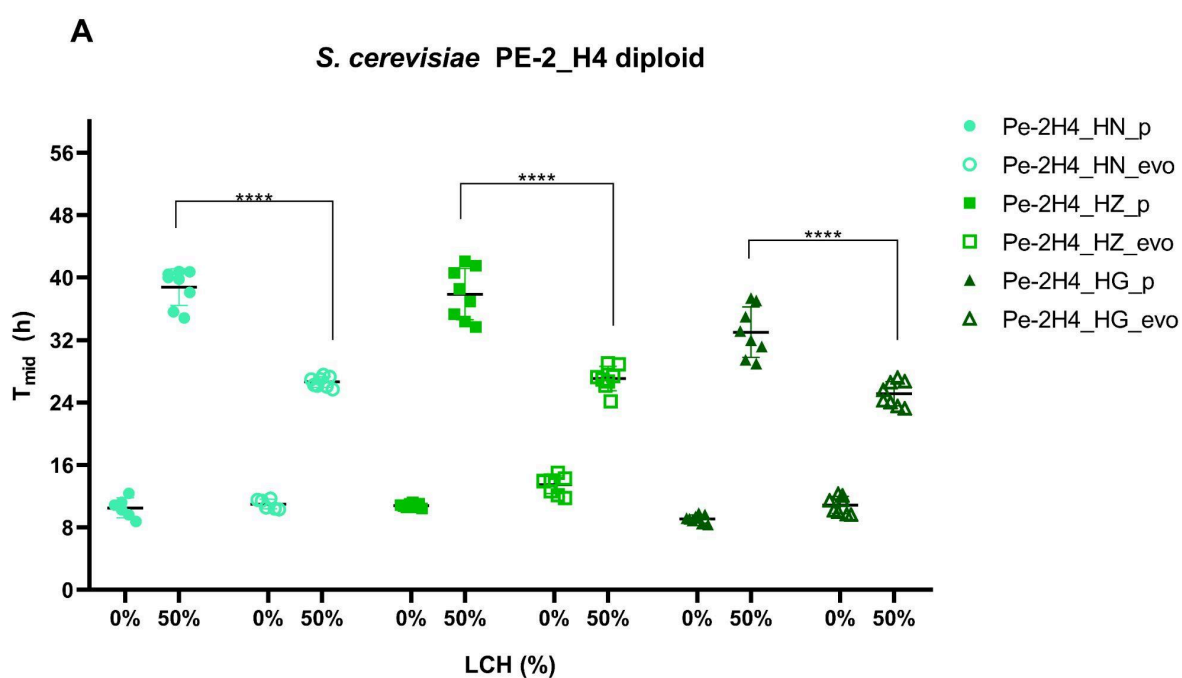
3.4.6 Microplate growth assays: ALE1, ALE2, and ALE3

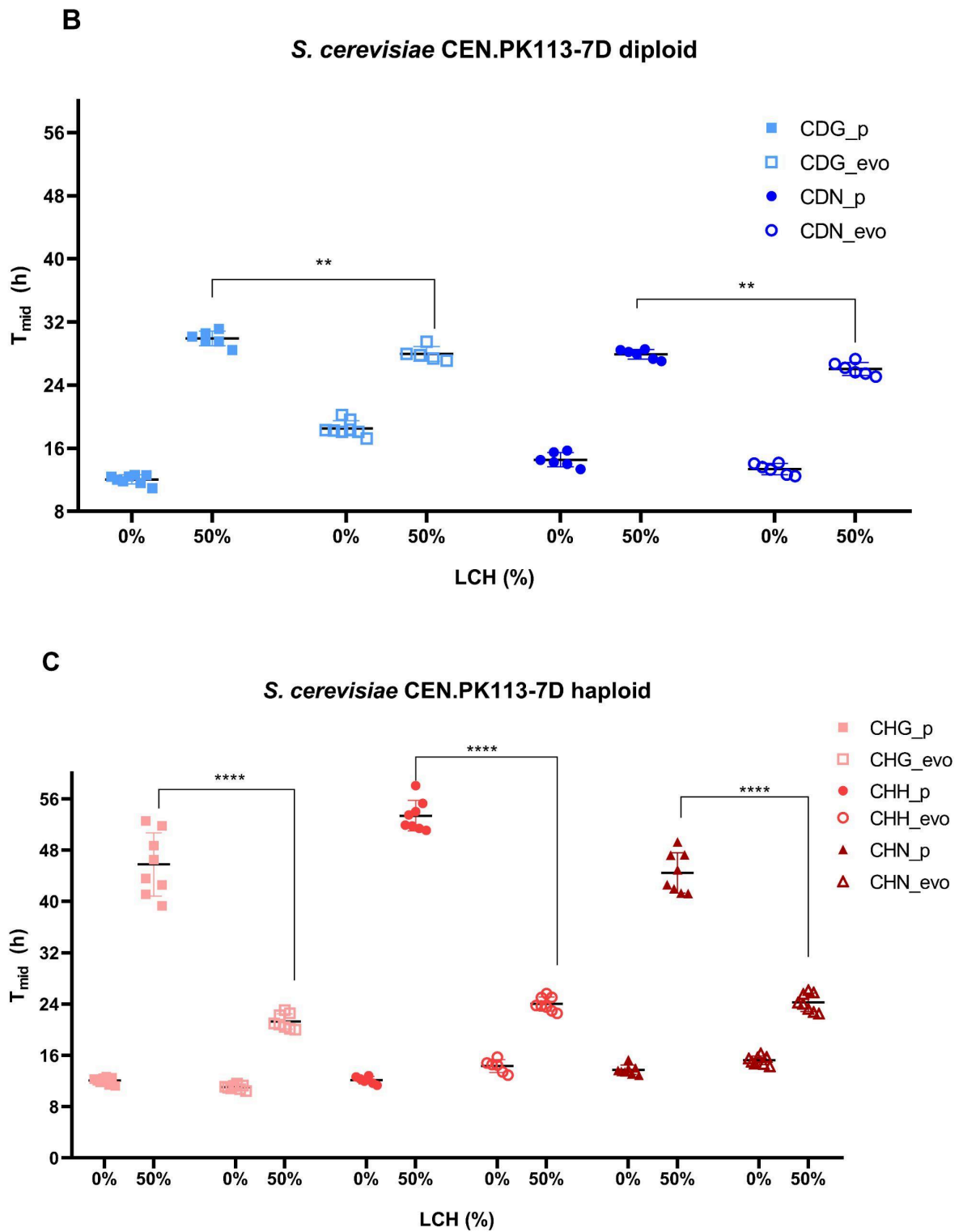
Microplate LCH tolerance assays, conducted as described in Materials and Methods, revealed insights into the adaptive responses of evolved yeast populations when compared to their WT counterparts. Graphs A, B, and C, represent the diploid PE-2_H4, diploid CEN.PK113-7D, and haploid CEN.PK113-7D populations, respectively, provide a visual representation of Tmid values at 0% and 50% LCH concentrations (Figure 3.12). Observation of Tmid values, which represent half the time required for the growth curve to reach maximum optical density during propagation, provides an understanding of the adaptive dynamics in response to lignocellulosic hydrolysate. Notably, lower Tmid values were consistently observed in the evolved populations, signifying an adaptation to the challenges posed by LCH.

In Plot A (diploid PE-2_H4), the evolved population exhibited substantially lower Tmid at 50% LCH concentration compared to the WT strain. This reduction in Tmid suggests the adaptability to LCH, emphasizing the success of the evolutionary process in shaping the growth dynamics of this diploid strain. Similarly, in Plot B (diploid CEN.PK113-7D), there is an observable trend of slightly lower Tmid values for the evolved population in both populations. It is noteworthy, however, that the difference in Tmid in the diploid population is relatively smaller compared to the haploid counterpart, indicating a potentially different adaptive strategy or a lower level of tolerance in the diploid strain. Examining Chart C (haploid CEN.PK113-7D), the haploid strain similarly exhibits lower Tmid values in the evolved populations, as do the Pe-2 H4 diploids. This reiteration of reduced Tmid values indicates a robust and versatile adaptive response. Notably, the extent of Tmid reduction in the haploid population is more pronounced when compared to the diploid population. These differences suggest that the haploid strain may possess a more effective adaptive strategy or greater tolerance to LCH than its diploid counterpart.

The significance of these results is highlighted by the statistical analyses performed, using one-way ANOVA and post-Bonferroni tests for multiple comparisons. The obtained p-values ($p < 0.0001$) indicate a highly significant difference between the evolved and WT populations at 50% LCH concentration.

Figure 3.12 - Characterization of evolved populations and progenitors strains in microplate growth assays (TECAN™).





A- PE-2_H4 diploid; **B-** CEN.PK113-7D diploid; **C-** CEN.PK113-7D haploid. The graph demonstrates T_{mid} values (half log phase time) with 0% and 50% of LCH concentration. Lower T_{mid} values for the evolved populations denote adaptation to the LCH.

In conclusion, results from microplate assays and Tmid analysis suggest successful adaptation of evolved yeast populations to lignocellulosic hydrolysate. The consistently lower Tmid values across different genetic backgrounds emphasize the universality and effectiveness of the observed evolutionary responses.

3.5 Discussion

ALE experiments, also known as adaptive laboratory evolution, are becoming increasingly used to enhance microbial phenotypes and study biological processes (SANDBERG *et al.*, 2019). Yeast adaptive laboratory evolution has proven successful in several cases for optimizing bioethanol production by increasing the lignocellulosic inhibitor and/or ethanol tolerance (MENEGON; GROSS; JACOBUS, 2022). The strategic application of rational metabolic engineering techniques can lead to changes in specific metabolic pathways, addressing limitations in metabolic rates. Consequently, such modifications impact predictions and growth rates of predicted strains (SANDBERG *et al.*, 2019). To overcome these challenges, adaptive laboratory evolution is commonly employed. This approach serves to increase the resilience of strains, further strengthening them against potential obstacles in achieving optimal bioethanol production. According to some reports, evolutionary engineering has evolved from "a simple black box strain improvement strategy" to an efficient instrument that can comprehend and build yeast cell factories in tandem with developments in yeast genome sequencing, analysis, and editing (MANS; DARAN; PRONK, 2018).

While exploring Adaptive Laboratory Evolution (ALE) strategies in different yeast populations, we noticed distinct adaptive responses influenced by the protocols used. In populations ALE1 (diploid PE-2 H4), ALE2 (haploid CEN.PK113-7D), and ALE3 (diploid CEN.PK113-7D), the serial transfer strategy showed specific nuances. The difference in initial lignocellulosic hydrolysate (LCH) concentrations between haploid and diploid populations may have played a role in adaptive responses. Adaptation over approximately 764 generations (ALE1 and ALE2) was assessed by growth under constant stress conditions, revealing notable variation in LCH tolerance.

One important step in ALE is the bottleneck, which plays a crucial role in population diversity (CHOI *et al.*, 2019; LEE *et al.*, 2011). It is essential to keep the cellular state constant during every cycle. Moreover, Lee and Kim (2020) suggest

that maintaining success requires optimizing the passing technique, which includes factors like progressively raising passing frequency and lowering pass size. They note that this becomes particularly important since extending culture causes growth to accelerate while decreasing fitness improvement (LEE; KIM, 2020). However, in our study, without intentional mutagenesis, when the cells began to expand more quickly and steadily, we continued by raising the hydrolysate concentration, lowering the growth rate, and estimating new adaptive mutations, while maintaining a consistent passage volume (1:200).

The evolutions were carried out in 25mL erlenmeyers, therefore, the population density can vary from bottle to bottle, as does the fluidity of the physical-chemical environment, including pH and dissolved oxygen, so it is difficult to keep these variables constant due to changes in the cell density that depends on the residual amount of nutrients (GRAF *et al.*, 2019; LEE; KIM, 2020). This can lead to erroneous evolutionary pressure, which causes an undesired phenotype and makes it difficult to identify the relationship between the genotype and the phenotype (HOSKISSON; HOBBS, 2005; LEE; KIM, 2020). A suitable explanation for the diploid population of CEN.PK113-7D to have had a less significant evolution than the haploid population can be explained by Gerrish and Lenski (1998) and Rozen *et al.* (2002) who suggested that, in an asexual population that experiences many mutations, large-effect beneficial mutations have greater probabilities of fixation than would be expected without clonal interference, as large-effect mutations are more likely to survive both genetic drift and to clonal interference (GERRISH; LENSKI, 1998; KIM; ALLEN ORR, 2005; ROZEN; DE VISSER; GERRISH, 2002). Kim and Allen's findings from 2005 further imply that clonal interference could not have a significant impact on the rate of adaptation. They demonstrate that whereas the average fitness of sexual reproductions often improves faster than that of asexual reproductions, the difference is negligible when advantageous mutations have very different effects (KIM; ALLEN ORR, 2005). In this way, we can understand how the haploid populations developed so well and why the diploid population had such a low adaptive rate in these protocols.

General ALE protocols usually employ propagation methods using sequential transfers in liquid media or controlled growth conditions in chemostats (DRAGOSITS; MATTANOVICH, 2013). In both cases, continuous exposure to the stressor. However, apart from this approach, another strategy was demonstrated by

Gonzalez-Ramos and colleagues (2016). They demonstrated an alternative protocol, where alternating propagation of yeast in culture medium with and without the inhibitor promotes constitutive tolerance to acetic acid. This simple innovation has proven functional in selecting a panel of distinct mutations to achieve the desired phenotype (GONZÁLEZ-RAMOS *et al.*, 2016). In this way, we developed the strategy employed in ALE4 (haploid CEN.PK113-7D), involving rounds of highly concentrated LCH shock following a round of recuperation, which added a layer of complexity to the discussion. Shock/recovery cycles can be repeated if live cells are recovered in an inhibitor-free, nutrient-rich media. Adaptive alleles can quickly take over the population in situations when death rates are high, which would hasten the evolutionary process (BARRICK; LENSKE, 2013). The survival rate of about 8.86% after a 48 hr exposure to 100% LCH emphasizes the selection pressure caused by our shock protocol. However as maximum concentration and exposure prevent the challenge from increasing further, it appears that the protocol reached a stress saturation point and was more focused mainly on tolerance to low pH.

In conclusion, the comparative study of different ALE procedures emphasizes the significance of taking into account not only the overall plan but also particular elements like ploidy and particular techniques used (MENEGON; GROSS; JACOBUS, 2022). These details directly affect the pursuit of more resilient and effective yeast strains for the production of cellulosic ethanol in complex conditions.

References

- ALMARIO, M. P.; REYES, L. H.; KAO, K. C. Evolutionary engineering of *Saccharomyces cerevisiae* for enhanced tolerance to hydrolysates of lignocellulosic biomass. **Biotechnology and bioengineering**, v. 110, n. 10, p. 2616–2623, 2013.
- AULD, D. S., Ph. . *et al.* Microplate Selection and Recommended Practices in High-throughput Screening and Quantitative Biology. *In*: MARKOSSIAN, S. *et al.* (org.). **Assay Guidance Manual**. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences, 2020.
- BARRICK, J. E.; LENSKI, R. E. Genome dynamics during experimental evolution. **Nature reviews. Genetics**, v. 14, n. 12, p. 827–839, 2013.
- BASSO, L. C.; DE AMORIM, H. V.; DE OLIVEIRA, A. J.; LOPES, M. L. Yeast selection for fuel ethanol production in Brazil. **FEMS yeast research**, v. 8, n. 7, p. 1155–1163, 2008.
- BORDET, F.; ROMANET, R.; EICHER, C.; GRANDVALET, C.; KLEIN, G.; GOUGEON, R.; JULIEN-ORTIZ, A.; ROULLIER-GALL, C.; ALEXANDRE, H. eGFP Gene Integration in HO: A Metabolomic Impact? **Microorganisms**, v. 10, n. 4, 2022. Disponível em: <https://doi.org/10.3390/microorganisms10040781>. Acesso em: 22 fev. 2024.
- CAKAR, Z. P.; SEKER, U. O. S.; TAMERLER, C.; SONDEREGGER, M.; SAUER, U. Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. **FEMS yeast research**, v. 5, n. 6-7, p. 569–578, 2005.
- CAKAR, Z. P.; TURANLI-YILDIZ, B.; ALKIM, C.; YILMAZ, U. Evolutionary engineering of *Saccharomyces cerevisiae* for improved industrially important properties. **FEMS yeast research**, v. 12, n. 2, p. 171–182, 2012.
- CAO, X.; HOU, L.; LU, M.; WANG, C.; ZENG, B. Genome shuffling of *Zygosaccharomyces rouxii* to accelerate and enhance the flavour formation of soy sauce. **Journal of the science of food and agriculture**, v. 90, n. 2, p. 281–285, 2010.
- CHOI, K. R.; JANG, W. D.; YANG, D.; CHO, J. S.; PARK, D.; LEE, S. Y. Systems Metabolic Engineering Strategies: Integrating Systems and Synthetic Biology with Metabolic Engineering. **Trends in biotechnology**, v. 37, n. 8, p. 817–837, 2019.
- COUCE, A.; CAUDWELL, L. V.; FEINAUER, C.; HINDRÉ, T.; FEUGEAS, J.-P.; WEIGT, M.; LENSKI, R. E.; SCHNEIDER, D.; TENAILLON, O. Mutator genomes

decay, despite sustained fitness gains, in a long-term experiment with bacteria. **Proceedings of the National Academy of Sciences of the United States of America**, v. 114, n. 43, p. E9026–E9035, 2017.

CUNHA, J. T.; ROMANÍ, A.; COSTA, C. E.; SÁ-CORREIA, I.; DOMINGUES, L. Molecular and physiological basis of *Saccharomyces cerevisiae* tolerance to adverse lignocellulose-based process conditions. **Applied microbiology and biotechnology**, v. 103, n. 1, p. 159–175, 2019.

DELUNA, A.; VETSIGIAN, K.; SHORESH, N.; HEGRENESS, M.; COLÓN-GONZÁLEZ, M.; CHAO, S.; KISHONY, R. Exposing the fitness contribution of duplicated genes. **Nature genetics**, v. 40, n. 5, p. 676–681, 2008.

DRAGOSITS, M.; MATTANOVICH, D. Adaptive laboratory evolution -- principles and applications for biotechnology. **Microbial cell factories**, v. 12, p. 64, 2013.

GERRISH, P. J.; LENSKE, R. E. The fate of competing beneficial mutations in an asexual population. **Genetica**, v. 102, n. 0, p. 127–144, 1998.

GIETZ, R. D.; WOODS, R. A. Yeast transformation by the LiAc/SS Carrier DNA/PEG method. **Methods in molecular biology**, v. 313, p. 107–120, 2006.

GOLDSTEIN, A. L.; MCCUSKER, J. H. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. **Yeast**, v. 15, n. 14, p. 1541–1553, 1999.

GONZÁLEZ-RAMOS, D.; GORTER DE VRIES, A. R.; GRIJSEELS, S. S.; VAN BERKUM, M. C.; SWINNEN, S.; VAN DEN BROEK, M.; NEVOIGT, E.; DARAN, J.-M. G.; PRONK, J. T.; VAN MARIS, A. J. A. A new laboratory evolution approach to select for constitutive acetic acid tolerance in *Saccharomyces cerevisiae* and identification of causal mutations. **Biotechnology for biofuels**, v. 9, p. 173, 2016.

GRAF, M.; HAAS, T.; MÜLLER, F.; BUCHMANN, A.; HARM-BEKBENBETOVA, J.; FREUND, A.; NIES, A.; PERSICKE, M.; KALINOWSKI, J.; BLOMBACH, B.; TAKORS, R. Continuous Adaptive Evolution of a Fast-Growing Strain Independent of Protocatechuate. **Frontiers in microbiology**, v. 10, p. 1648, 2019.

HABER, J. E. Mating-type genes and MAT switching in *Saccharomyces cerevisiae*. **Genetics**, v. 191, n. 1, p. 33–64, 2012.

HEER, D.; SAUER, U. Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain. **Microbial biotechnology**, v. 1, n. 6, p. 497–506, 2008.

HOSKISSON, P. A.; HOBBS, G. Continuous culture--making a comeback? **Microbiology**, v. 151, n. Pt 10, p. 3153–3159, 2005.

HOU, L. Novel methods of genome shuffling in *Saccharomyces cerevisiae*. **Biotechnology letters**, v. 31, n. 5, p. 671–677, 2009.

JACOBUS, A. P.; STEPHENS, T. G.; YOUSSEF, P.; GONZÁLEZ-PECH, R.; CHEN, Y.; BASSO, L. C.; FRAZZON, J.; CHAN, C. X.; GROSS, J. **Comparative genomics supports that Brazilian bioethanol *Saccharomyces cerevisiae* comprise a unified group of domesticated strains related to cachaça spirit yeasts.** [S. l.: s. n.] Disponível em: <https://doi.org/10.1101/2020.12.15.422965>

JACOBUS, A. P.; STEPHENS, T. G.; YOUSSEF, P.; GONZÁLEZ-PECH, R.; CICCOTOSTO-CAMP, M. M.; DOUGAN, K. E.; CHEN, Y.; BASSO, L. C.; FRAZZON, J.; CHAN, C. X.; GROSS, J. Comparative Genomics Supports That Brazilian Bioethanol Comprise a Unified Group of Domesticated Strains Related to Cachaça Spirit Yeasts. **Frontiers in microbiology**, v. 12, p. 644089, 2021.

KIM, Y.; ALLEN ORR, H. Adaptation in Sexuals vs. Asexuals: Clonal Interference and the Fisher-Muller Model. **Genetics**, v. 171, n. 3, p. 1377, 2005.

KOCAEFE-ÖZŞEN, N.; YILMAZ, B.; ALKIM, C.; ARSLAN, M.; TOPALOĞLU, A.; KISAKESEN, H. L. B.; GÜLSEV, E.; ÇAKAR, Z. P. Physiological and Molecular Characterization of an Oxidative Stress-Resistant Strain Obtained by Evolutionary Engineering. **Frontiers in microbiology**, v. 13, p. 822864, 2022.

LEE, D.-H.; FEIST, A. M.; BARRETT, C. L.; PALSSON, B. Ø. Cumulative number of cell divisions as a meaningful timescale for adaptive laboratory evolution of *Escherichia coli*. **PloS one**, v. 6, n. 10, p. e26172, 2011.

LEE, S.; KIM, P. Current Status and Applications of Adaptive Laboratory Evolution in Industrial Microorganisms. **Journal of microbiology and biotechnology**, v. 30, n. 6, p. 793, 2020.

LI, H.; WU, M.; XU, L.; HOU, J.; GUO, T.; BAO, X.; SHEN, Y. Evaluation of industrial *Saccharomyces cerevisiae* strains as the chassis cell for second-generation bioethanol production. **Microbial biotechnology**, v. 8, n. 2, p. 266–274, 2015.

MANS, R.; DARAN, J.-M. G.; PRONK, J. T. Under pressure: evolutionary engineering of yeast strains for improved performance in fuels and chemicals production. **Current opinion in biotechnology**, v. 50, p. 47–56, 2018.

MENEGON, Y. A.; GROSS, J.; JACOBUS, A. P. How adaptive laboratory evolution

can boost yeast tolerance to lignocellulosic hydrolyses. **Current genetics**, v. 68, n. 3-4, p. 319–342, 2022.

NIJKAMP, J. F. *et al.* De novo sequencing, assembly and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology. **Microbial cell factories**, v. 11, p. 36, 2012.

PEREIRA, R.; MOHAMED, E. T.; RADİ, M. S.; HERRGÅRD, M. J.; FEIST, A. M.; NIELSEN, J.; CHEN, Y. Elucidating aromatic acid tolerance at low pH in using adaptive laboratory evolution. **Proceedings of the National Academy of Sciences of the United States of America**, v. 117, n. 45, p. 27954–27961, 2020.

PETER, J. *et al.* Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates. **Nature**, v. 556, n. 7701, p. 339–344, 2018.

PIOTROWSKI, J. S.; ZHANG, Y.; BATES, D. M.; KEATING, D. H.; SATO, T. K.; ONG, I. M.; LANDICK, R. Death by a thousand cuts: the challenges and diverse landscape of lignocellulosic hydrolysate inhibitors. **Frontiers in microbiology**, v. 5, p. 90, 2014.

ROZEN, D. E.; DE VISSER, J. A. G. M.; GERRISH, P. J. Fitness effects of fixed beneficial mutations in microbial populations. **Current biology: CB**, v. 12, n. 12, p. 1040–1045, 2002.

SANDBERG, T. E.; SALAZAR, M. J.; WENG, L. L.; PALSSON, B. O.; FEIST, A. M. The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. **Metabolic engineering**, v. 56, p. 1, 2019.

SHEN, Y.; LI, H.; WANG, X.; ZHANG, X.; HOU, J.; WANG, L.; GAO, N.; BAO, X. High vanillin tolerance of an evolved *Saccharomyces cerevisiae* strain owing to its enhanced vanillin reduction and antioxidative capacity. **Journal of industrial microbiology & biotechnology**, v. 41, n. 11, p. 1637–1645, 2014.

SHI, D.-J.; WANG, C.-L.; WANG, K.-M. Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae*. **Journal of industrial microbiology & biotechnology**, v. 36, n. 1, p. 139–147, 2009.

SMITH, J.; VAN RENSBURG, E.; GÖRGENS, J. F. Simultaneously improving xylose fermentation and tolerance to lignocellulosic inhibitors through evolutionary engineering of recombinant *Saccharomyces cerevisiae* harbouring xylose isomerase. **BMC biotechnology**, v. 14, n. 1, p. 1–17, 2014.

SNOEK, T.; PICCA NICOLINO, M.; VAN DEN BREMT, S.; MERTENS, S.; SAELS, V.;

VERPLAETSE, A.; STEENSELS, J.; VERSTREPEN, K. J. Large-scale robot-assisted genome shuffling yields industrial *Saccharomyces cerevisiae* yeasts with increased ethanol tolerance. **Biotechnology for biofuels**, v. 8, p. 32, 2015.

SPROUFFSKE, K.; WAGNER, A. Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. **BMC bioinformatics**, v. 17, p. 172, 2016.

TOPALOĞLU, A.; ESEN, Ö.; TURANLI-YILDIZ, B.; ARSLAN, M.; ÇAKAR, Z. P. From *Saccharomyces cerevisiae* to Ethanol: Unlocking the Power of Evolutionary Engineering in Metabolic Engineering Applications. **Journal of Fungi**, v. 9, n. 10, 2023. Disponível em: <https://doi.org/10.3390/jof9100984>. Acesso em: 15 jan. 2024.

WALLACE-SALINAS, V.; GORWA-GRAUSLUND, M. F. Adaptive evolution of an industrial strain of *Saccharomyces cerevisiae* for combined tolerance to inhibitors and temperature. **Biotechnology for biofuels**, v. 6, n. 1, p. 151, 2013.

WISER, M. J.; LENSKI, R. E. A Comparison of Methods to Measure Fitness in *Escherichia coli*. **PloS one**, v. 10, n. 5, p. e0126210, 2015.

ZHENG, D.-Q.; WU, X.-C.; TAO, X.-L.; WANG, P.-M.; LI, P.; CHI, X.-Q.; LI, Y.-D.; YAN, Q.-F.; ZHAO, Y.-H. Screening and construction of *Saccharomyces cerevisiae* strains with improved multi-tolerance and bioethanol fermentation performance. **Bioresource technology**, v. 102, n. 3, p. 3020–3027, 2011.

CHAPTER 4 - EXPLORING ALE STRAINS: SEQUENCING, GENE VALIDATION, AND FERMENTATION PROFILING IN *Saccharomyces cerevisiae*

4.1 Abstract

This chapter discusses genetic mutations identified through whole-genome sequencing (WGS), highlighting the importance of parallelism. In addition to the control deletion of *SIZ1* Δ , the concomitant mutation of *RET1* with *SIZ1* Δ resulted in enhanced hydrolysate tolerance, as did the deletion of *CHD1* Δ , *MUK1* Δ , and *HRK1* Δ . Reverse-engineered strains along with clones of ALE2 and ALE4 were examined under different controlled fermentation conditions – rich medium (YPD) and synthetic hydrolysate (LCH). Optical density (OD) and carbon dioxide (CO₂) production were evaluated to determine strain patterns. The results revealed that strains CHG and CHH from ALE2, and S_CHN from ALE4, exhibited shorter latency phases in LCH, higher growth rates, and higher CO₂ production. Reverse-engineered strains that demonstrated reduced lag phases were *RET1SIZ1* Δ , *SIZ1* Δ , *CHD1* Δ , and *PMA1* Δ , suggesting the potential advantages of LCH. Between them, *RET1SIZ1* Δ and *PMA2* Δ had significantly higher growth rates than WT, suggesting the best adaptability to LCH. To better understand fermentation, extracellular metabolites focusing on ethanol production, glucose consumption, and glycerol production were assessed. CHG, CHH, CHN, and S_CHN strains showed improved ethanol yields in synthetic hydrolysate, emphasizing their metabolic capability under challenging conditions. The global volumetric ethanol productivity factor was analyzed, and the strains CHG, CHH, CHN, and S_CHN also had higher values than wild-type (WT) in LCH.

In summary, the analyses provided valuable information about the fermentative strategies adopted by the evolved strains, clarifying their growth patterns and ethanol production. Reverse engineering discoveries have contributed to a deeper understanding of strain adaptability. In this way, it was possible to select ALE strains that had better performance in producing ethanol from hydrolysate and to identify mutations that have direct links with this tolerance.

4.2 Introduction

This chapter describes an in-depth investigation of strains obtained through ALE in *Saccharomyces cerevisiae*. This approach incorporates sequencing techniques,

gene validation, and fermentation profiles, to offer substantial insights into the adaptive evolution of these strains in response to specific environmental conditions.

The first part of the chapter is dedicated to the exploration of evolutionary mutations through the sequencing of experimental populations and clones. Data analysis and interpretation, identification of potentially adaptive mutations, and their implications for the phenotype are also described. The second part of this chapter focuses on the reverse engineering of the haploid CEN.PK113-7D strain. The methodologies used for knockout and strategic introduction of Single Nucleotide Polymorphisms (SNPs) will be presented, in addition to the conduction of competition assays analyzed by flow cytometry. These techniques are crucial for validating genes and mutations, and offer a deeper understanding of their phenotypic impact. The third and final section of this chapter addresses the understanding of strain metabolism through fermentation profiles. The selected strains, culture media, and fermentation results are in detail. Extracellular metabolites analysis will contribute to the understanding of the physiological adaptations of evolved strains.

4.2.1 ALE Analysis

While ALE appears promising, most of the work done to build tolerance in yeast to lignocellulosic hydrolysate has focused on phenotypic improvement; adaptive mutations found using WGS and allele validation through reverse engineering have received less attention (MENEGON; GROSS; JACOBUS, 2022). Xue *et al.* (2018) found that greater tolerance to phenolics in *S. cerevisiae* is linked to deletions of *EEB1* (fatty acid metabolism), *SSH4* (Suppressor of *SHR3* deletion), and *VAM6* (vesicle trafficking) (XUE *et al.*, 2018). The *FLO* genes showed copy number changes in the experimental laboratory evolution strain ISO12, which were related to the multicellularity and invasive growth of the adapted strain (WALLACE-SALINAS *et al.*, 2015; WEITAO, 2009). Liu *et al.* (2019) found non-synonymous mutations at *RPL23A*, *RPS9B*, *YNL208W*, *ATP16*, *YMR315W*, and *FAS2* genes in the Furfural/HMF evolved *S. cerevisiae* (LIU *et al.*, 2019). Cheng *et al.* (2015), after corn stover hydrolysate ALE, found missense mutations including *ESC2* (SUMO-like protein), and *PKP1* (mitochondrial protein kinase) (CHENG; ALMARIO; KAO, 2015).

In 2017, Wang e collaborators reported that the deletion of *YRR1* can confer vanillin resistance to cells (WANG; HOU; BAO, 2017). In an acetic acid evolution experiment, mutations in four genes (*ASG1*, *ADH3*, *SKS1*, and *GIS4*) were identified

as causative factors for this tolerance (GONZÁLEZ-RAMOS *et al.*, 2016). Biot-Pelletier *et al.* (2018) and Pinel *et al.* (2015) reported that non-synonymous mutations in *NRG1*, *GSH1*, *GDH1*, and *UBP7* were revealed by mass selection in LCH in conjunction with genome shuffling processes (BIOT-PELLETIER *et al.*, 2018; PINEL *et al.*, 2015). In another study, after an ALE for tolerance to coniferyl aldehyde, the sequencing showed mutations in genes related to oxidoreductase activity (*AR11*), cell wall organization (*YPK1*, and *TRS65*), nutrient sensing (*GPR1*, *SNF4*, *IRA1*, and *IRA2*), besides genes involved in transcription regulation (*CRZ1*, *GLN3*, *RRN10*, and *PDR1*) (HACISALIHOĞLU *et al.*, 2019). Pereira *et al.* (2020) found that the upregulation of *ESBP6* was associated with nine different mutations in *ARO80* and the duplication of segments from chromosome XIV which included the *ESBP6* locus. They suggested that *ESBP6* overexpression can promote the export of acid anions from the cytosol and improve biomass yield, probably by reducing the stress burden imposed by excess aromatic acids inside the cell (PEREIRA *et al.*, 2020). Another study carried out in 2021, confirmed by reverse engineering the relevance of combined mutations in *RAS2*, *HSF1*, and *SUM1* for tolerance to acetic acid. However, the *RAS2* mutation alone results in tolerance to acetic acid (SALAS-NAVARRETE *et al.*, 2021).

Our work aims to deepen the understanding of the genes associated with the tolerance of *S. cerevisiae* to lignocellulosic hydrolysate thereby contributing to advances in the field of biotechnology. Furthermore, methodologies and strategies have been used to identify strains developed through ALE/genetic engineering that are resistant to the hydrolysate. Investigating these genetic and metabolic aspects is crucial for understanding the strains developed and optimizing the production of strains with industrial potential.

4.3 Materials and Methods

4.3.1 Evolutionary Diversity: Sequencing the ALEs for In-Depth Insights

4.3.1.1 Sequencing Methods

The DNA of ALE 1, ALE 2, and ALE 3 experiments were sequenced using 2x 150 PE sequencing on the Illumina NovaSeq6000 platform, with libraries prepared with the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina.

The ALE 4 population DNA, together with a clone from S_CHN, CHG, and CHN were sequenced using 2 x 151 PB sequencing on the Illumina NextSeq1000 platform, with libraries prepared with Illumina DNA Prep™ Kit for Illumina.

4.3.1.2 Data Analysis

To identify mutations (SNPs and InDels) reads from the evolved populations were filtered by size selection (>50 bases) and overall quality scores. Mapping to the *S. cerevisiae* CEN.PK113-7D and Pe-2_H4 reference genomes were performed by the reference mapping tools built into CLC Genomics Workbench 8.0. Identification of SNPS/InDels was performed directly from read mappings generated by the CLC Genomics Workbench using variant detection algorithms within the CLC package. A minimum coverage setting of 10x and variant frequency of at least 20% of reads were observed for calling variants from sequencing strains.

4.3.1.3 Selection of Potentially Adaptive Mutations

The approach used for the selection of mutations of interest was based on alleles that are affected by mutations in two or more populations (parallelism) and that are related to genes whose described function (for example, stress-related genes) can explain the adaptive phenotype (a deductive approach).

4.3.2 Reverse Engineered CEN.PK113-7D Haploid.

4.3.2.1 Reverse-Engineered Methods

Using the pEasyG3 approach that our group developed, the mutations were evaluated individually, inserting them punctually into the wild-type (WT) lineage (JACOBUS *et al.*, 2022). Using a single set of primers—gA, a forward primer that binds to the gRNA template, and gB, a reverse primer that binds to the SNR52 promoter (pSNR52), which is used for gRNA expression—, this method is based on CRISPR/Cas9 system, and amplifies fragments of the pEasyG3 plasmid (Figure 4.1). The 5' sequence of the gA and gB oligonucleotides specifies the 20-nucleotide spacer sequence for each Cas9 target, resulting in overlapping DNA areas that promote homologous recombination *in vivo*. It is important to highlight that there is no need for purification, making it possible to directly transform the PCR products. Due to the homology between the 20-nucleotide spacers, the amplicons produced from

pEasyG3-mic (1,888 bp) recombine in vivo with the PCR products pEasyG3-zeo/nat/hph (1,460 bp, 1,652 bp, 2,108 bp, respectively) (Figure 4.2).

Figure 4.1 - The pEasyG3 series. (A) Map of pEasyG3-zeo/nat/hph. (B) Map of pEasyG3-mic.

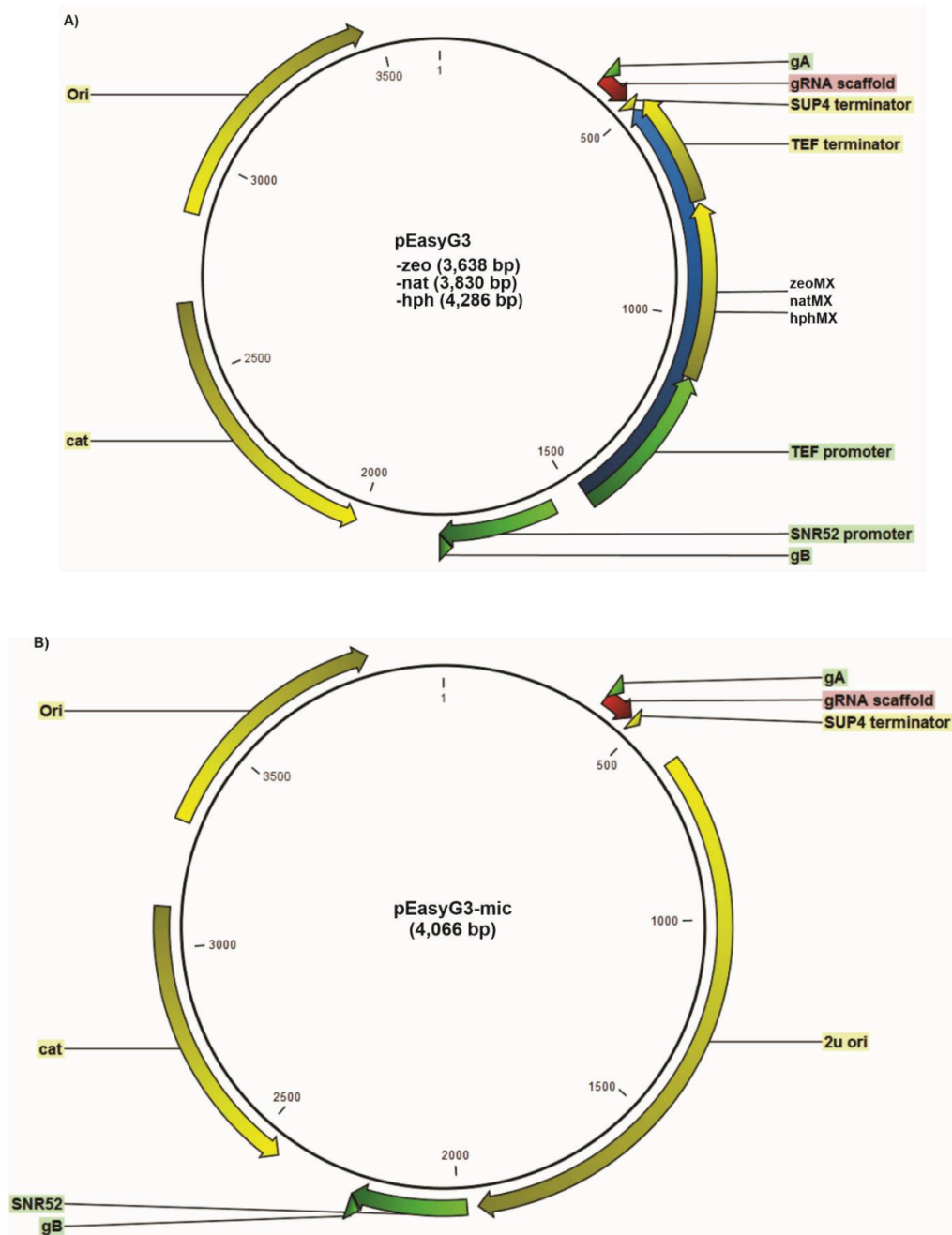
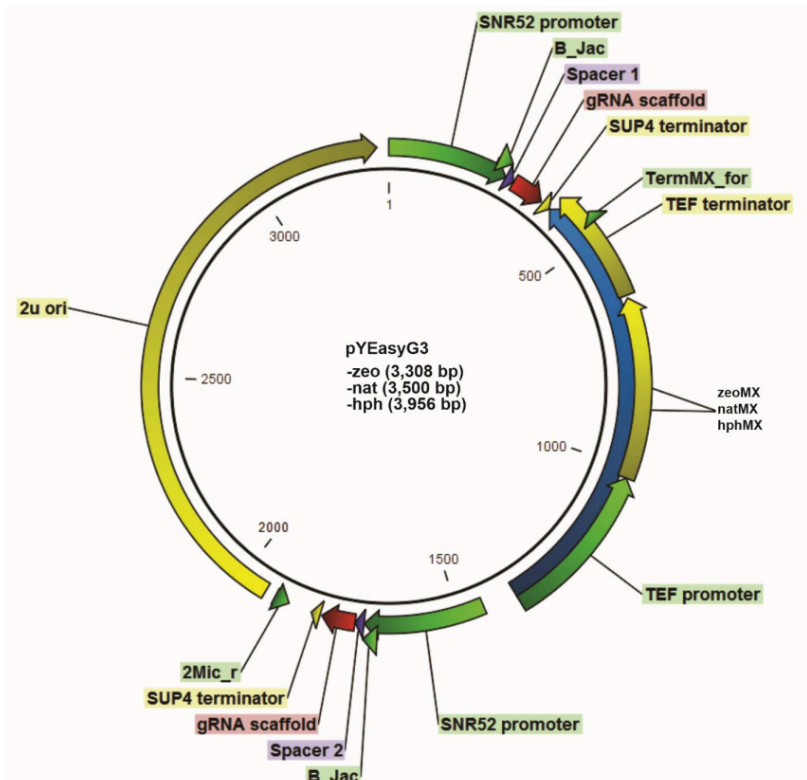


Figure 4.2- pYEasyG3 after in vivo assembly.



Plasmid resulting from recombination between the PCR products pEasyG3-zeo/nat/hph and pEasyG3_mic. Two gRNAs are assembled and can be confirmed by PCR with primer combinations B_JAC/2Mic_r (236 bp) and B_JAC /TermMX_for (232 bp) (JACOBUS *et al.*, 2022).

Table 4.1 provides an overview of the genetic alterations used in our investigation by displaying different mutations in important genes. The alterations consist of single nucleotide polymorphisms (SNPs) in *RET1* and *PMA1* and deletions (Δ) in *SIZ1* Δ , *MUK1* Δ , *CHD1* Δ , *HRK1* Δ , and *PDR3* Δ . These modifications were carefully selected to explore their functions in adaptive evolution, offering important new understandings of the genetic background of phenotypic changes we saw in our research.

Table 4.1 - Genetic Alterations in Selected Genes.

Gene	Mutation types
<i>SIZ1</i>	deletion (Δ)

<i>MUK1</i>	deletion (Δ)
<i>CHD1</i>	deletion (Δ)
<i>HRK1</i>	deletion (Δ)
<i>PDR3</i>	deletion (Δ)
<i>RET1SIZ1Δ</i>	SNP and Deletion (Δ)
<i>RET1</i>	SNP Gly783Cys
<i>PMA1</i>	SNP Ser158Gly

4.3.2.2 Deletions (Nonsense Mutation)

Among the mutations unveiled, those that indicated a loss of function (frameshift or stop codon) were reproduced as deletions (Δ). The gRNAs and donors were designed to generate a deletion after the action of CRISPR/Cas9, making the protein non-functional. Among the selected genes, *SIZ1* transcribes the SUMO E3 ligase protein, which promotes small ubiquitin-related juice modifier (Smt3p) binding to cytoplasmic proteins, a negative regulator of protein ubiquitination, and is involved in chromosome segregation. Genome-wide RNA interference screen identified this gene as a new furfural tolerance determinant (XIAO; ZHAO, 2014).

In this study, disruption of *SIZ1* function, through knockdown or deletion, significantly enhanced furfural tolerance, surpassing previous metabolic engineering strategies. The improved tolerance of *SIZ1 Δ* cells was accompanied by a rapid reduction of furfural to furfuryl alcohol, leading to higher ethanol productivity in the presence of furfural. Additionally, the *SIZ1 Δ* mutant demonstrated resistance to oxidative stress, indicating possible links between *SIZ1*-regulated SUMO and proteins involved in the response to oxidative stress (XIAO; ZHAO, 2014). The *SIZ1* mutations found in CHG, CHH, CHN, and Pe-2_H4_HN populations (Appendix A, Section 6, Tables 1, 2, 3, and 7) were Gln381*, Gln394Lys, Ser132*, and Asp345Gly, respectively. The goal of the deletion design was to eliminate the 34 bp fragment.

Our study examined the *MUK1* effect through genetic deletion in light of its critical function in the participation in the regulatory network directing vesicle trafficking and autophagy in *Saccharomyces cerevisiae* (HUH *et al.*, 2003;

SAMANTA; LIANG, 2003). This choice was made because, as other studies (CABRERA *et al.*, 2013; LI; WU; LIANG, 2019; PAULSEL; MERZ; NICKERSON, 2013) have demonstrated, *MUK1* gene is essential for golgi endosome trafficking, sorting through the multivesicular body (MVB). The *MUK1* alleles found in the CHG and CHN populations (Appendix A, Section 6, Tables 1 and 3) were Val232Asp and Asn489fs, respectively. The goal of the deletion design was to eliminate the 32 bp fragment.

In *Saccharomyces cerevisiae*, *CHD1* has a variety of roles in transcriptional control and chromatin dynamics. *CHD1*, found in SAGA and SLIK complexes, interacts with methylated histone H3 to identify transcriptionally active chromatin. *CHD1* interacts with elongation factors during transcription elongation and maintains chromatin integrity. Sister chromatid cohesion and chromosomal shape are impacted by *CHD1* deletion, which may be related to cohesin regulation (BOGINYA *et al.*, 2019; PRAY-GRANT *et al.*, 2005; SIMIC *et al.*, 2003; SMOLLE *et al.*, 2012; TRAN *et al.*, 2000). It was not known the role of the deletion of this gene in tolerance to LCH, but as triplicates of ALE2 presented this allele, it was decided to investigate in this study. The *CHD1* mutations found in the CHH and CHN populations (Appendix A, Section 6, Table 2 and 3), were Arg1071* and Tyr1459Cys, respectively. The goal of the deletion design was to eliminate the 29bp fragment.

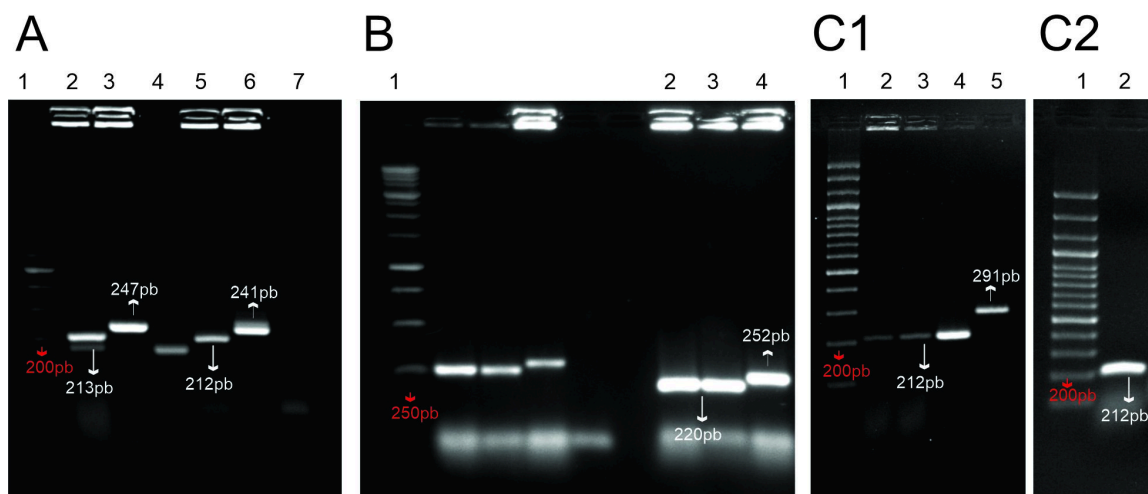
In *Saccharomyces cerevisiae*, the *HRK1* gene encodes a kinase involved in the activation of plasma H(+)-ATPase (Pma1) in response to glucose metabolism (GOOSSENS *et al.*, 2000; TKACH *et al.*, 2012). In a specific approach to acetic acid-induced stress, the kinase *HRK1* has emerged as a crucial component influencing proton and potassium homeostasis (ANTUNES *et al.*, 2023). We decided to delete this gene to investigate whether its absence would have a positive influence on the conditions studied whether its apparent deletion was linked to a synergistic interaction with another gene or whether it was just a hitchhiking gene. The *HRK1* mutations found in the CHN, CDHN, Pe-2H4_HN(2x), and Pe-2H4_HZ populations (Appendix A, Section 6, Tables 3, 5, and 7) were Pro154fs, Pro154fs, Asn725fs, Ile671fs, and Pro155fs. The goal of the deletion design was to eliminate the 79bp fragment and was performed in CENPK113-7D and Pe-2 H4.

The *PDR3* acts as a transcriptional activator in the drug resistance network, exerting control over the expression of ATP-binding cassette (ABC) transporters. In addition to playing a fundamental role in the response to drugs and organic solvents,

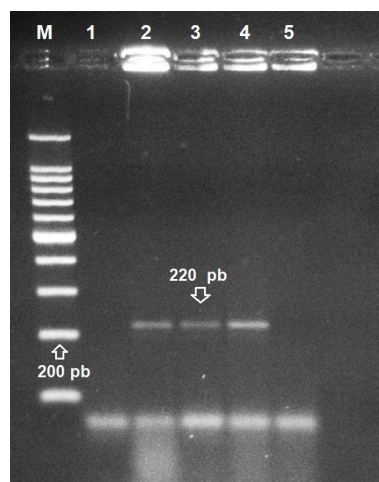
its post-translational positive regulation is evident in cells lacking a functional mitochondrial genome. This protein is also involved in the diauxic shift process and its relative nuclear distribution shows a notable increase during stress situations in DNA replication (BALZI; GOFFEAU, 1995; DELAVEAU *et al.*, 1994; DEVAUX *et al.*, 2002; TKACH *et al.*, 2012). When evaluating the CDG and CDN genome, amplifications were identified on chromosome II around the *PDR3* area. Therefore, it was decided to delete the *PDR3* gene. The found amplifications had a length of 23,112 bp and were approximately 2.993x in CDG and 1.727 in CDN. Knockout aims to explore the possible impact of *PDR3* on cellular dynamics. The goal of the deletion design was the insertion of the 492 bp TEF1 promoter.

The gels showing the amplifications of the guide and donor fragments can be seen in Appendix A, Section 6, Figure 13 and 14.. The confirmation of the deletion can be seen in Fig. 4.3 and 4.4.

Figure 4.3 - PCR amplification of deleted regions.



A- 1. 100pb promega marker, 2. CEN.PK113-7D::*SIZ1* Δ (*SIZ1*Check/*SIZ1*DonR - 213pb), 3. *SIZ1* WT (247pb), 4. White, 5. CEN.PK113-7D::*CHD1* Δ (*CHD1*CheckF/*CHD1*DonR - 212pb), 6. *CHD1* WT (241pb), 7. Blank *CHD1*. B- 1. 1kb marker, 2 and 3. CEN.PK113-7D::*MUK1* Δ (*MUK1*CheckF2/*MUK1*CheckR - 220pb), 4. *MUK1* WT (252pb). C1- 1. Marker 100pb , 2.3.4. CEN.PK113-7D::*HRK1* Δ (*HRK1*Check-f/*HRK1*Check-r - 212pb), 5. *HRK1* WT (291pb). C2- 100bp a marker, 2. Pe2_H4::*HRK1* Δ (212pb).

Figure 4.4 - PCR amplification of *PDR3* deleted region.

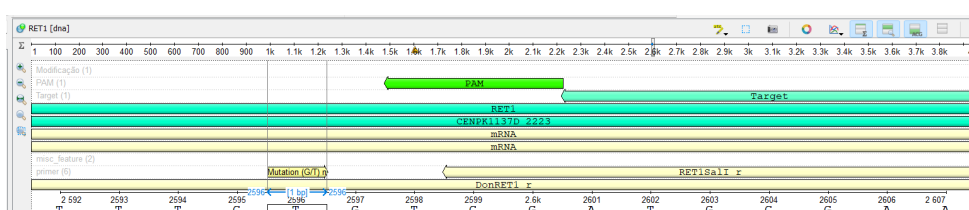
M- 100bp marker, 1. CEN.PK113-7D (pPDRf/tef1pro_r2), 2, 3, and 4- CEN.PK113-7D::*PDR3*Δ (pPDRf/tef1pro_r2 - 220pb), 5. Blank.

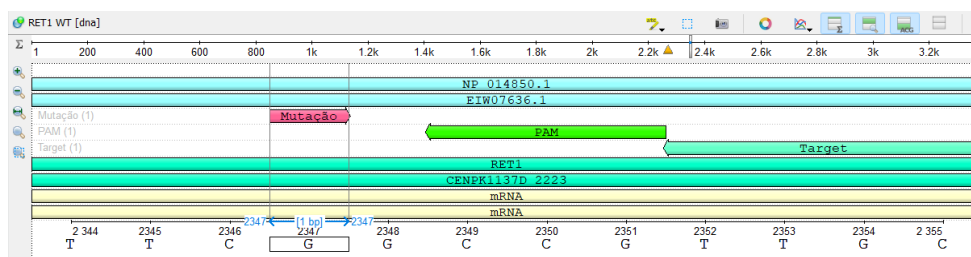
4.3.2.3 Missense SNPs

RET1 encodes the second-largest subunit of RNA polymerase III. RNA polymerase III is responsible for the transcription of genes related to transfer RNA (tRNA), 5S RNA, and several other low molecular weight RNAs (JAMES; HALL, 1990). The activity of RNA polymerase III, with its crucial contribution to this subunit, is vital for fundamental biological processes and cell survival. Therefore, it is not interesting to delete this gene, changing a single nucleotide is the best approach.

The SNPs found in the CHG and CHH populations may indicate a change in function (loss or gain) because, at positions 783 and 957, there is an exchange of lysine and glycine for asparagine and cysteine, respectively (Appendix A, Section 6, Tables 1 and 2). This way, we generated specific donor DNA for the SNP present in CHH, using the CRISPR/Cas9 artifact to reproduce the point mutation G>T (glycine x cysteine) at position 2347 (Figure 4.5). Gel proof of substitution can be seen in Figure 4.6, and Sanger comprovation in Figure 4.7.

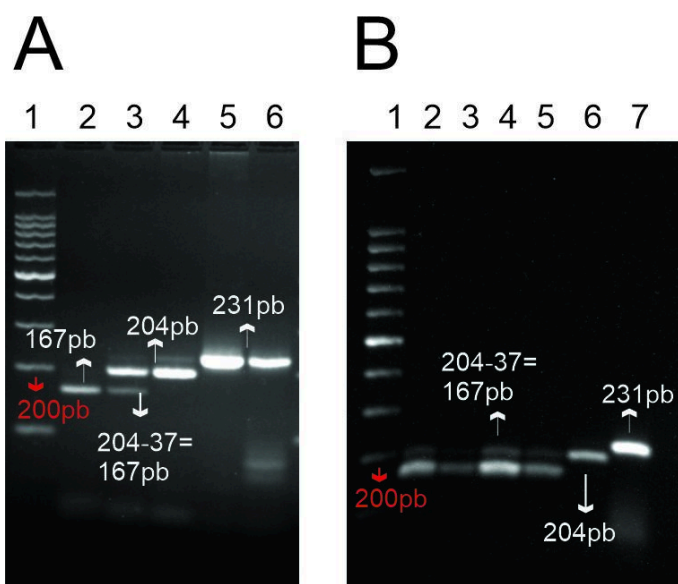
Figure 4.5 - Nucleotide Substitution.





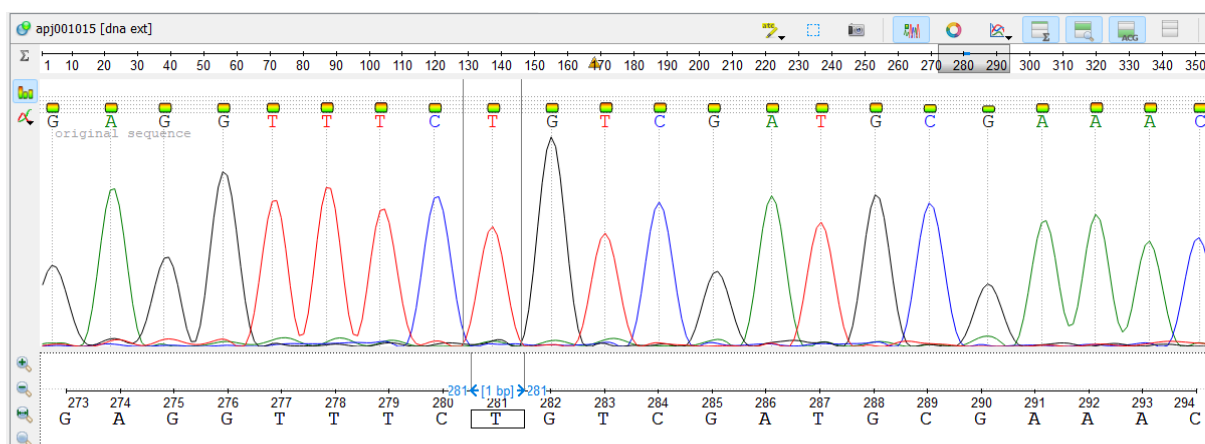
A Single Nucleotide Polymorphism (SNP) depicting the substitution of a Guanine (G) with Thymine (T) at position 2347.

Figure 4.6 - Digestion of the Sall 1 region of the strain CEN.PK113-7D::*RET1*G<T 2347 SNP (*RET1*Sall_f/*RET1*Sall_r).



A- 1. 100bp marker, 2. CEN.PK113-7D::*RET1*G<T with total digestion (buffer 3.1) 3. CEN.PK113-7D::*RET1*G<T 2347 with partial digestion (PCR reaction buffer), 4. CEN.PK113-7D WT, 5.6. CEN.PK113-7D::*RET1*G<T 2347 No cleaving. B- 1. 1kb promega marker, 2 to 5. CEN.PK113-7D::*SIZ1*Δ::*RET1*G<T 2347 with partial digestion, 6. CEN.PK113-7D WT, 7. CEN.PK113-7D No cleavage.

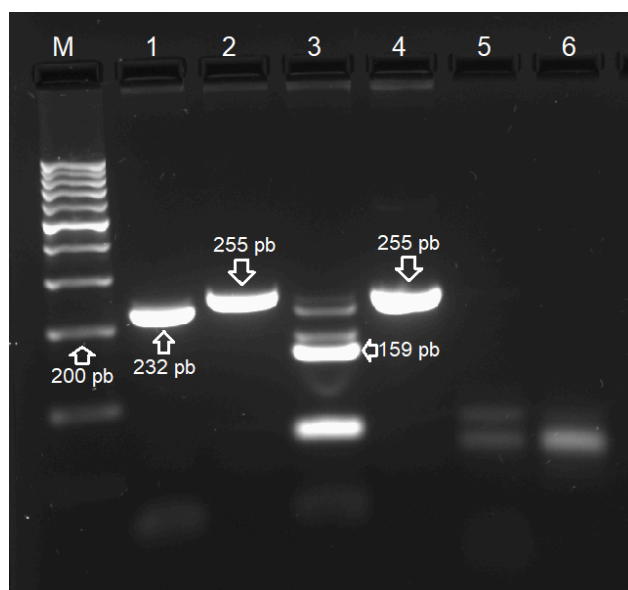
Figure 4.7 - Confirmation by SANGER sequencing of the nucleotide exchange in *RET1*.



It is possible to notice the exchange of nucleotide G for T (position 281). It is also possible to notice the change in the PAM sequence from CCG to TCG (position 283-285).

The same methodology was used for the single nucleotide substitution of the *PMA1* gene. *PMA1* codes for a P2-type H⁺-ATPase located in the plasma membrane, playing a fundamental role in regulating cytoplasmic pH and membrane potential. Young cells begin with low levels of Pma1p and during the aging process, Pma1p accumulates to high levels in mother cells. Regulation of Pma1p involves the participation of Hsp30p (HENDERSON; HUGHES; GOTTSCHLING, 2014; MEENA; THAKUR; CHAKRABARTI, 2011; SERRANO; KIELLAND-BRANDT; FINK, 1986). The SNPs found in PE-2_H4_HN and PE-2_H4_HZ were present at positions Ala639Leu and Gly158Ser, respectively (Tables 7 and 8). The substitution made is Ser158Gly. Proof of substitution can be seen in Fig. 4.8.

Figure 4.8 - Digestion with EcoRI on CEN.PK113-7D::*PMA1*(Ser158Gly) SNP (*PMA1Ecof*//*PMA1Ecor*).



M- 100pb marker, 1- CEN.PK113-7D::*PMA1* with total digestion (232 pb) 2- CEN.PK113-7D::*PMA1*(Ser158Gly) without digestion (255 pb) 3- CEN.PK113-7D WT with digestion (159 pb). 4- CEN.PK113-7D WT without digestion (255 pb). 5 and 6 - Blank.

All primers used are detailed in Appendix A, Section5, Table 14.

4.3.2.4 Competition assays by flow cytometry of engineered CEN.PK113-7D

A competition assay was conducted using the reverse-engineered strains compared to the WT strain. Each competition was performed in triplicate in rich medium (YPD) and 30% hydrolysate at pH 5.5. The methodology is described in detail in Chapter 3, section 3.3.5. The results were read on the flow cytometer after 72 hours.

4.3.3 Fermentation Insights: Understanding Strains Metabolisms

4.3.3.1 Bioreactor setup, strains, and media

64 Fermentations were carried out in an INFORS HT 0.75 L bioreactor with *Saccharomyces cerevisiae* CEN.PK113-7D clones from ALE2 and ALE4, besides the genetically engineered strains (Table 4.2). The strains were previously cultivated in sterile 50 ml tubes until they reached an optical density of 0.7. Inoculation took place in a 0.75-L bioreactor (useful volume of 0.18 – 0.5 L) with 250 ml of culture medium

(YPD) or synthetic hydrolysate (S_LCH) (Table 4.3), with an initial optical density of approximately 0.1. Air was injected into the upper part (gas mixture with 21% oxygen) at a flow rate of 30 ml/min via automatic control. Stirring was maintained at 100 revolutions per minute, the pH was stabilized at 5.5 with a controlled addition of 2N sodium hydroxide, and the temperature was controlled at 28 °C.

Table 4.2 - *Saccharomyces cerevisiae* CEN.PK113-7D Fermentations in INFORS HT 0.75 L bioreactor.

N°	Strain	Batches
1	<i>S. cerevisiae</i> CEN.PK113-7D CHG WT	2
2	<i>S. cerevisiae</i> CEN.PK113-7D CHH WT	2
3	<i>S. cerevisiae</i> CEN.PK113-7D CHN WT	2
4	<i>S. cerevisiae</i> CEN.PK113-7D CHG clone	2
5	<i>S. cerevisiae</i> CEN.PK113-7D CHH clone	2
6	<i>S. cerevisiae</i> CEN.PK113-7D CHN clone	2
7	<i>S. cerevisiae</i> CEN.PK113-7D S_CHG clone	2
8	<i>S. cerevisiae</i> CEN.PK113-7D S_CHH clone	2
9	<i>S. cerevisiae</i> CEN.PK113-7D S_CHN clone	2
10	<i>S. cerevisiae</i> CEN.PK113-7D HRK1Δ	2
11	<i>S. cerevisiae</i> CEN.PK113-7D CHD1Δ	2
12	<i>S. cerevisiae</i> CEN.PK113-7D MUK1Δ	2
13	<i>S. cerevisiae</i> CEN.PK113-7D PDR3Δ	2
14	<i>S. cerevisiae</i> CEN.PK113-7D SIZ1Δ	2
15	<i>S. cerevisiae</i> CEN.PK113-7D RET1SIZ1Δ	2

Table 4.3 - Components of the synthetic hydrolysate used for fermentations.

Component	Concentration mM	Concentration g/L
Formic acid	35.3	1,62
Acetic Acid	129,6	7,776
Vanillic Acid	0,757	0,1273
p-coumaric acid	4,581	0,7515
Furfural	10	0,9608
Hydroxymethylfurfural	10	1,2611
Glucose	111,02	20
Yeast extract	-	10
Bacto Peptone	-	20

4.3.3.2 Microplate growth assay, strains, and media

Strains CHG, CHH, CHN, and S_CHN were subjected to microplate growth assay. This experiment was carried out using the Thermo Scientific™ Varioskan™ LUX multimode microplate reader and the growths were made in sextuplicates. It was tested 4 concentrations of acetic acid (relatively 0%, 35%, 50% and 70%). The concentrations were worked considering 100% as the concentration present in the original hydrolysate (where the evolution experiment was carried out) (Table 4.4), and all concentrations were in a YPD medium.

Table 4.4 - Acetic Acid concentrations.

Acetic Acid Relative Concentration	Acetic Acid Concentration (g/L)	Acetic Acid Concentration (mM)
---------------------------------------	------------------------------------	-----------------------------------

0%	0	0
35%	2,094	34,810
50%	2,994	49,820
70%	4,191	69,748

4.3.3.3 Extracellular Metabolites

Supernatant samples were collected at crucial moments during the fermentations. Collections included samples at the beginning (time 0), the end of the experiment, and at specific points in the lag and exponential phases of microbial growth. For the detection of aromatics/phenols, such as protocatechuic acid, vanillin, p-coumaric acid, benzoate, 5-hydroxymethylfurfural, furfural, and 4-hydroxybenzoic acid, the method used was with two distinct mobile phases: Mobile Phase A, composed of 0.1 % formic acid in MilliQ water, and Mobile Phase B, consisting of 0.1% formic acid in acetonitrile. Analyzes were conducted using the Thermo Scientific DUO UHPLC system equipped with a DAD detector (HPLC Duo 1), operating exclusively in DAD mode. The column used was the Waters Acquity HSS T3, with dimensions of 100 x 2.1 mm and particles of 1.8 μm (Waters part number 186003539). The detector was adjusted to capture UV in the ranges of 280 nm, 320 nm, and 360 nm.

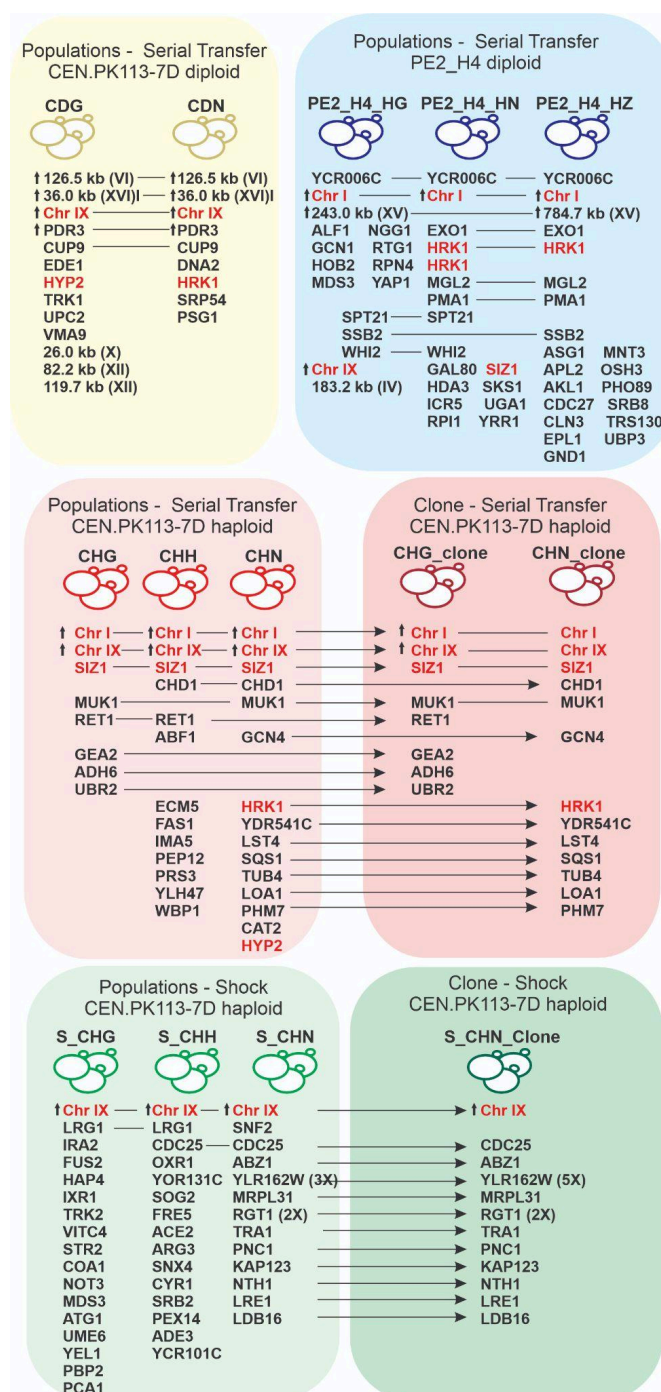
For the detection of organic acids such as maltose, glucose, succinate, lactate, formate, acetate, propionate, ethanol, and glycerol, the analytical method used the Thermo Vanquish Duo HPLC system, equipped with DAD and RI detectors. Two columns were used in the analyses, the first being an Agilent HiPlex H (H⁺ 8%, 300 x 7.7 mm, PN: PL1170-6830), and the second being a Phenomenex Rezex RHM Monosaccharide (H⁺ 8%, 300 x 7.8 mm, PN: 00H-0132-K0). The detectors included a Refractive Index Detector (RID) operating in positive polarity at 40°C. The experimental conditions were established with a column temperature of 65 °C, an injection volume of 25 μL , and a mobile phase composed of a 4 mM H₂SO₄ solution. The flow rate was maintained at 0.6 mL/min, and the run time was isocratic for 26 minutes.

4.4 Results

4.4.1 Unraveling Evolutionary Diversity: Sequencing the ALE for In-Depth Insights.

To improve the visualization of mutations, imaging schemes of mutations were developed in the four populations and some clones (Serial Transfer: CEN.PK113-7D diploid, Pe2_H4 diploid, CEN.PK113-7D haploid - CHG and CHN clones; Shock: CEN.PK113-7D haploid and S_CHN clone) (Figure 4.9).

Figure 4.9 - Summary of mutations found in each population/clone.



Intra-population mutations are identified by a black line connecting replicates; The Inter-background mutations are represented in red. Connections with arrows at the tip indicate mutations that were also identified in the best-selected clone

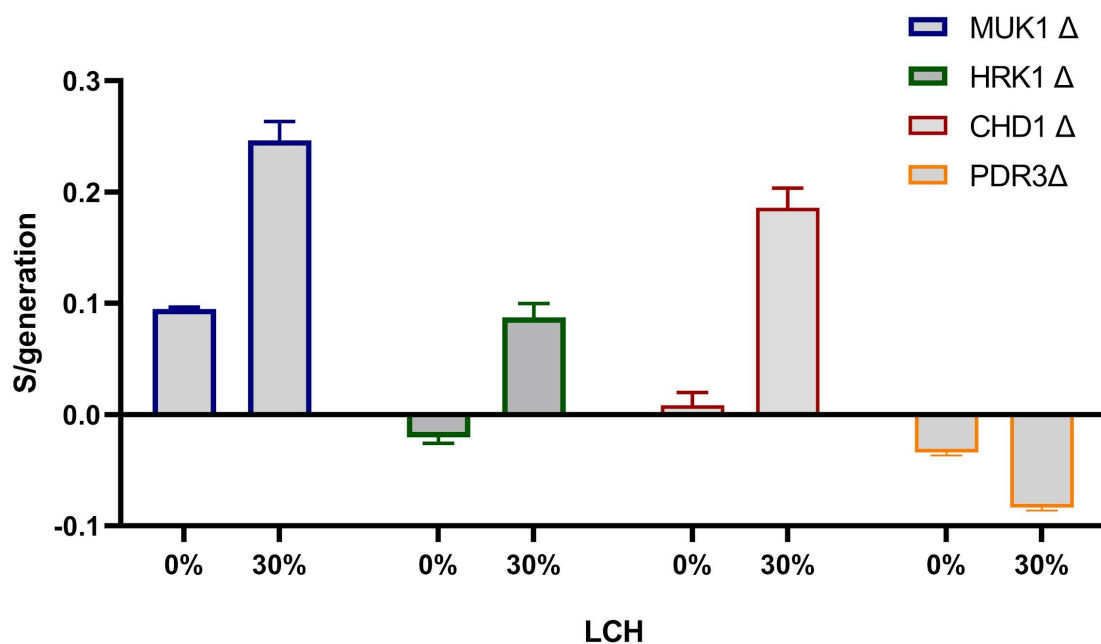
As a result, more than 100 SNPs were identified, with the possibility of identifying synonymous mutations, missense mutations, nonsense mutations, and, in some cases, causing stop codon or frameshift mutations (Appendix A, Section 6, Table 6 to 13).

4.4.2 Competition assays by flow cytometry of engineered versus WT (CEN.PK113-7D).

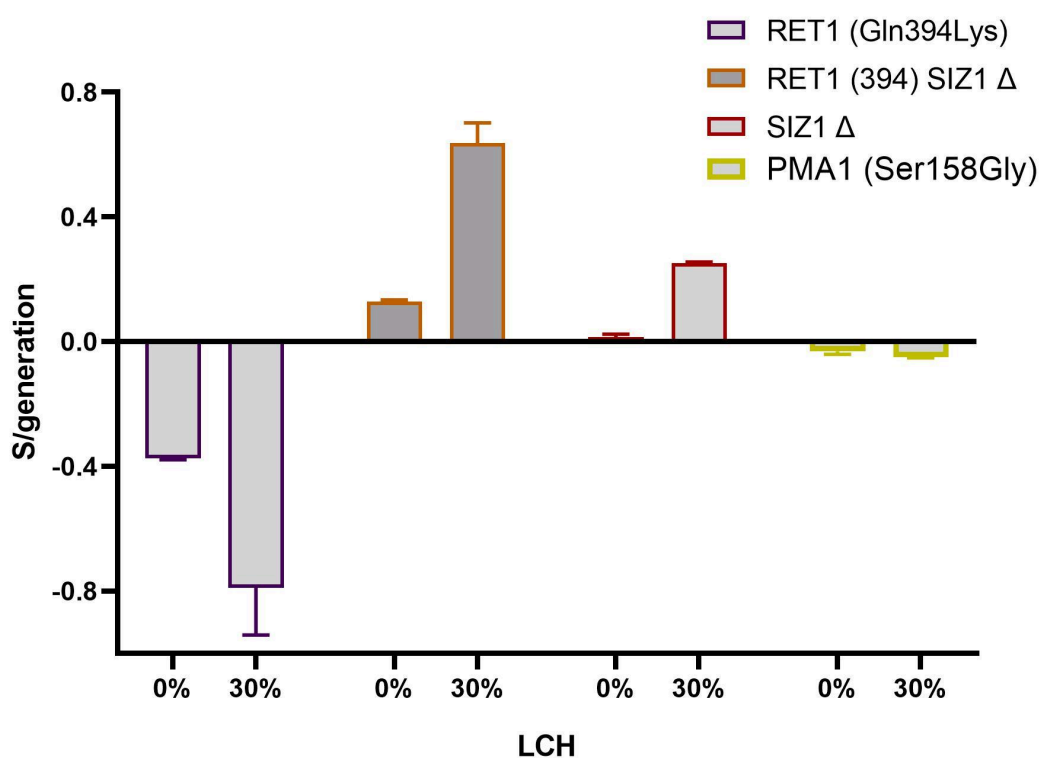
Disrupt mutations of *MUK1Δ*, *HRK1Δ*, *CHD1Δ*, and *PDR3Δ* confer significant fitness gains of ~25%, ~8,7%, ~18,6%, and loss of ~8,4% per doubling, respectively (Figure 4.10A). Deletion of *SIZ1Δ* conferred a gain of ~25%, and the SNP modification in *RET1*(2347G>T) conferred a fitness loss of ~7%. However, the substitution of *RET1*(2347G>T) into the *SIZ1Δ* strain conferred a fitness gain of ~63% (Figure 4.10B). This suggests that in the line of evolution, the mutation in the *SIZ1* gene occurred first, and only later the mutation in *RET1* occurred. Besides that, the point mutation on *PMA1*(Ser158Gly) conferred a fitness loss of ~5%.

Figure 4.10 - Fitness of engineered strains in the presence of LCH.

A



B



Flow cytometry-assisted growth competitions of engineered strains versus GFP-tagged references allowed assessment of selection coefficients (S) per generation. A- *MUK1Δ*, *HRK1Δ*, *CHD1Δ*, and *PDR3Δ*. B- *RET1I*, *RET1(2347G>T)*, *SIZ1Δ*, and *PMA1(Ser158Gly)*. Mean values of triplicates were normalized by

competition with wild-type (WT) lines. $p < 0.0001$ One-way ANOVA with Bonferroni post-test for multiple comparisons.

4.4.3 Fermentation Insights: Understanding Strains Metabolisms

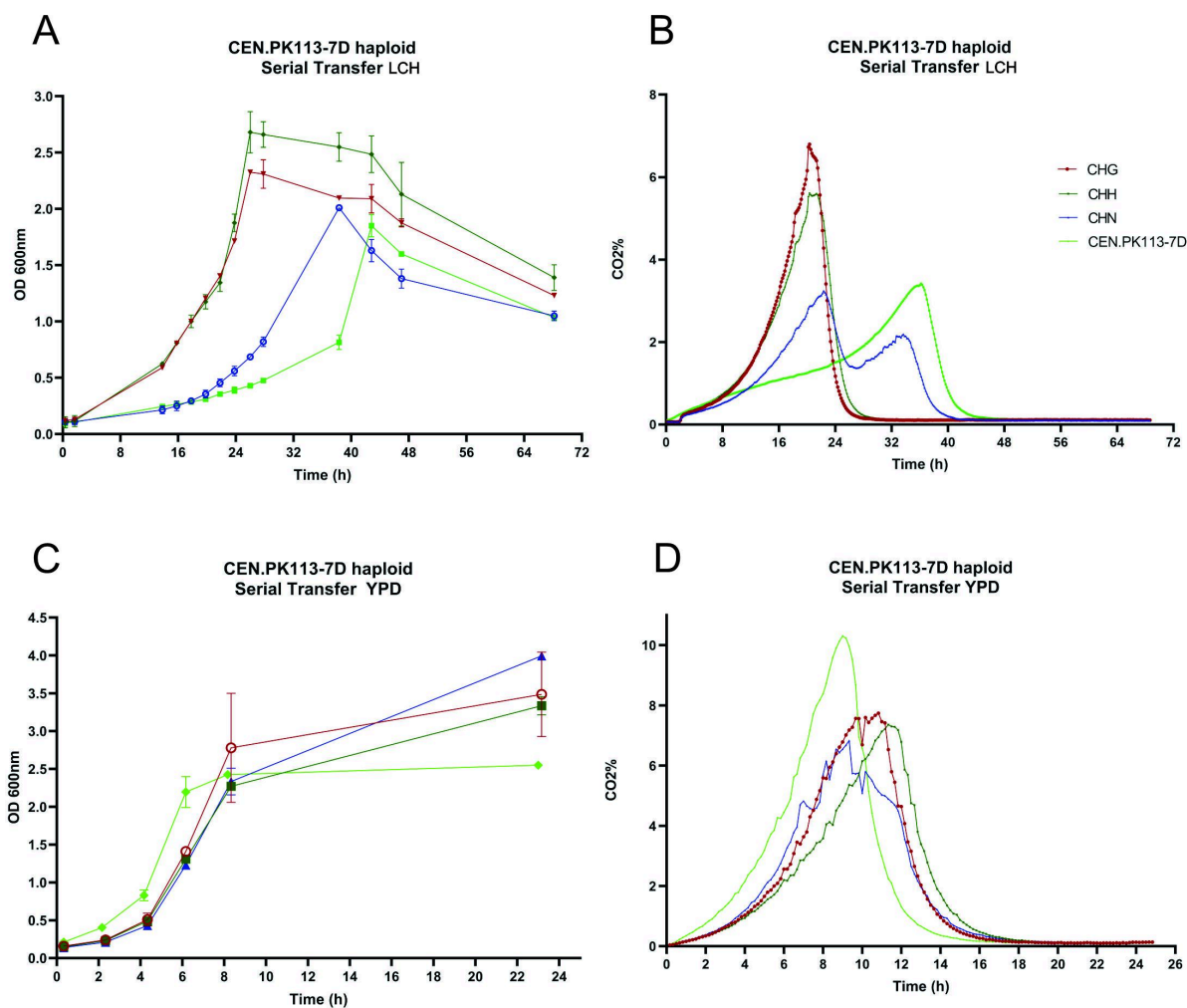
4.4.3.1 Bioreactor Outcome

The performance of the ALE2 (Figure 4.11), ALE4 (Figure 4.12), and reverse-engineered strains (Figure 4.14 and Figure 4.15) in comparison to the WT are revealed by the fermentation data. Rich medium (YPD) and synthetic S_LCH were the two distinct conditions under which sets of fermentations were conducted in duplicate.

Since CO₂ generation is indirectly connected to ethanol production, this investigation aimed to assess cell growth (optical density) and CO₂ production as important markers of fermentative metabolism. The acquired data provide light on the fermentative profile over different fermentation environments.

The graphical analysis below illustrates the OD and production of CO₂ in synthetic S_LCH and YPD of the resulting populations from the CEN.PK113-7D haploid of Serial Transfer Protocol (Figure 4.11).

Figure 4.11 - Optical Density (OD) and CO₂ production as a Function of Time in ALE2 strains.



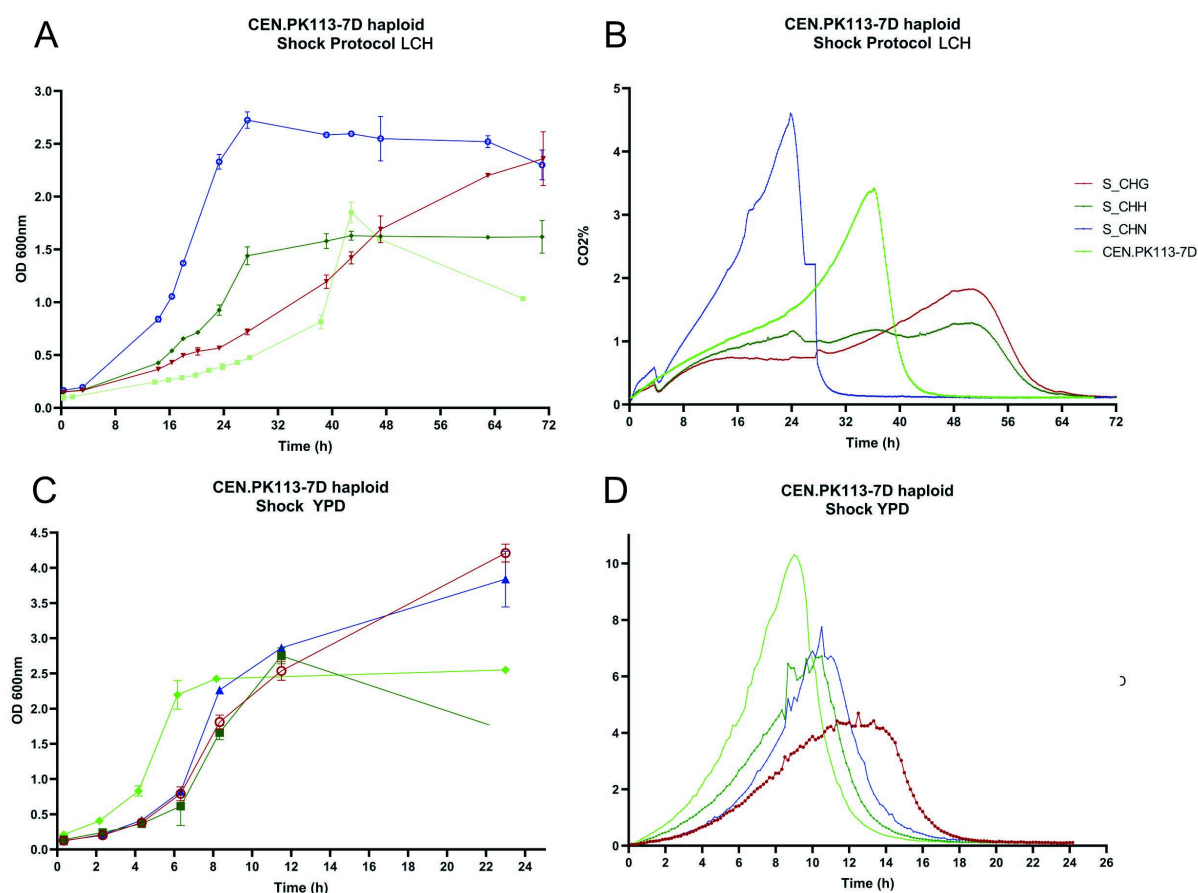
The tree populations in duplicate are represented (CHG, CHH, and CHN) and are compared to the CEN.PK113-7D WT (also in duplicate). A- OD in LCH media. B- CO₂ in LCH media. C- OD in YPD media. D- CO₂ in YPD media.

The graphs reveal distinct patterns of development over time, while in the rich medium, the strains have a short lag phase, in the hydrolysate the Lag phase tends to increase, more drastically in the WT, as expected. The production of CO₂ in the hydrolysate exhibits subtle variations at different times and amounts, indicating specific responses of the evolved in comparison to the WT, this last one had the lowest ethanol production over a longer time. It is worth mentioning that due to failure in data acquisition during a 10h period (from 23h to 33h), the CHN strain has an incorrect reading of CO₂ values. In YPD, the WT strain produced the most ethanol and showed to be also the fastest in growth, as expected (Figure 4.11)

The cell growth (OD) and carbon dioxide (CO₂) production curves over time in Shock populations (ALE 4) are illustrated below (Figure 4.12). These data allow an

assessment of these strains in continuous growth, which has not been performed until now. In the strains that evolved from the Shock protocol, in the S_LCH condition, the S_CHG, presented lower OD compared to the WT, including lower CO₂ production. This result may be attributed to the fact that these strains have not been specifically evolved for growth (like ALE2) or ethanol production, indicating that there may be better ways to test these strains. However, it is noteworthy that the S_CHN strain demonstrated a reduced Lag phase compared to the WT, achieving a higher cell density. This trend is also reflected in graph B, showing a greater and faster CO₂ production. Analyzing the performance of the strains in YPD, the WT strain presented a smaller Lag phase and a greater production of CO₂. These results corroborate our previous data that suggest the existence of a trade-off in experimentally evolved cells, where they lose fitness in a rich environment.

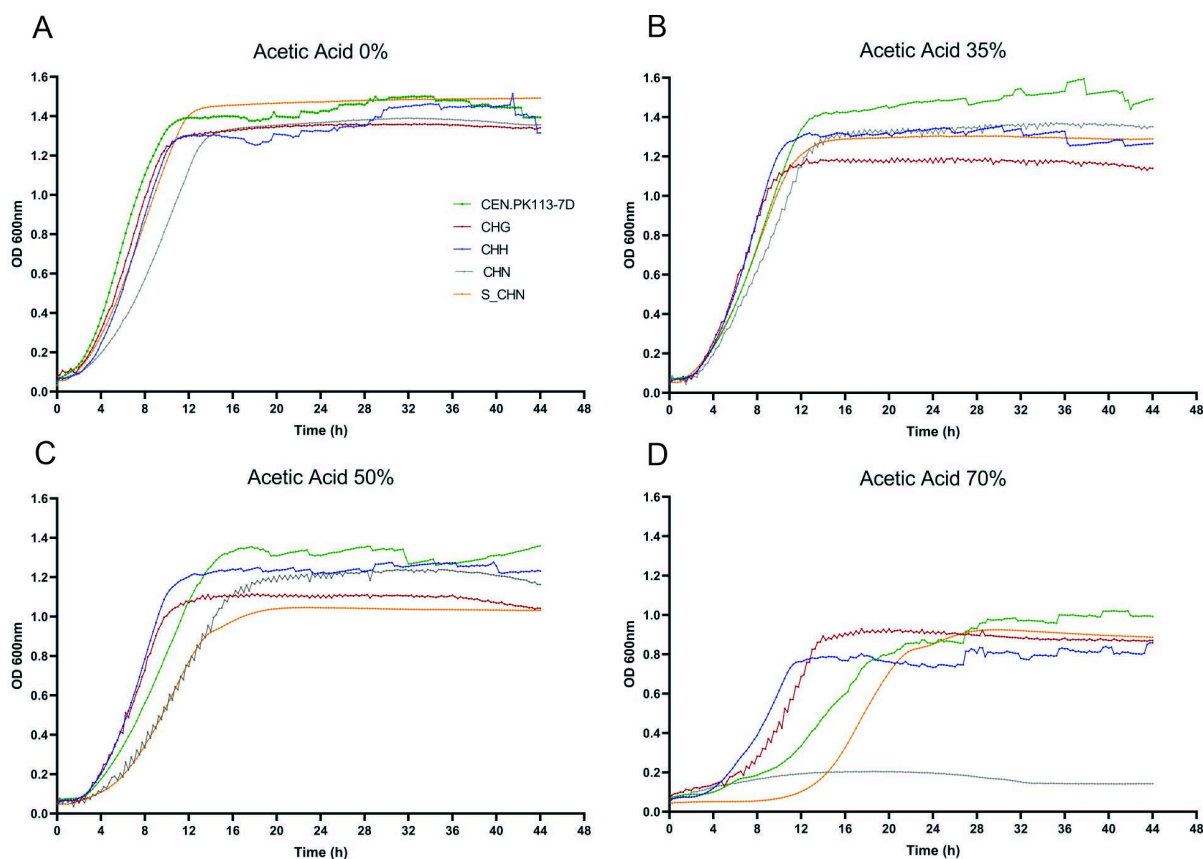
Figure 4.12 - Optical Density (OD) and CO₂ production as a Function of Time in ALE4 strains.



The tree populations in duplicate are represented (S_CHG, S_CHH, and S_CHN) and are compared to the CEN.PK113-7D WT (also in duplicate). A- OD in LCH media. B- CO₂ in LCH media. C- OD in YPD media. D- CO₂ in YPD media.

To explore more about the toxicity of the inhibitors, the ALE2 and S_CHN clones were challenged against only and exclusively acetic acid. In 0% and 35% acetic acid in original LCH, the WT had a similar lag phase, meanwhile, in 50% and 70% acetic acid found in LCH, CHG and CHH had a shorter lag phase than the WT. In all concentrations, the WT had a short lag phase when compared to the evolved CHN and S_CHN clones in the respective concentrations (Figure 4.13).

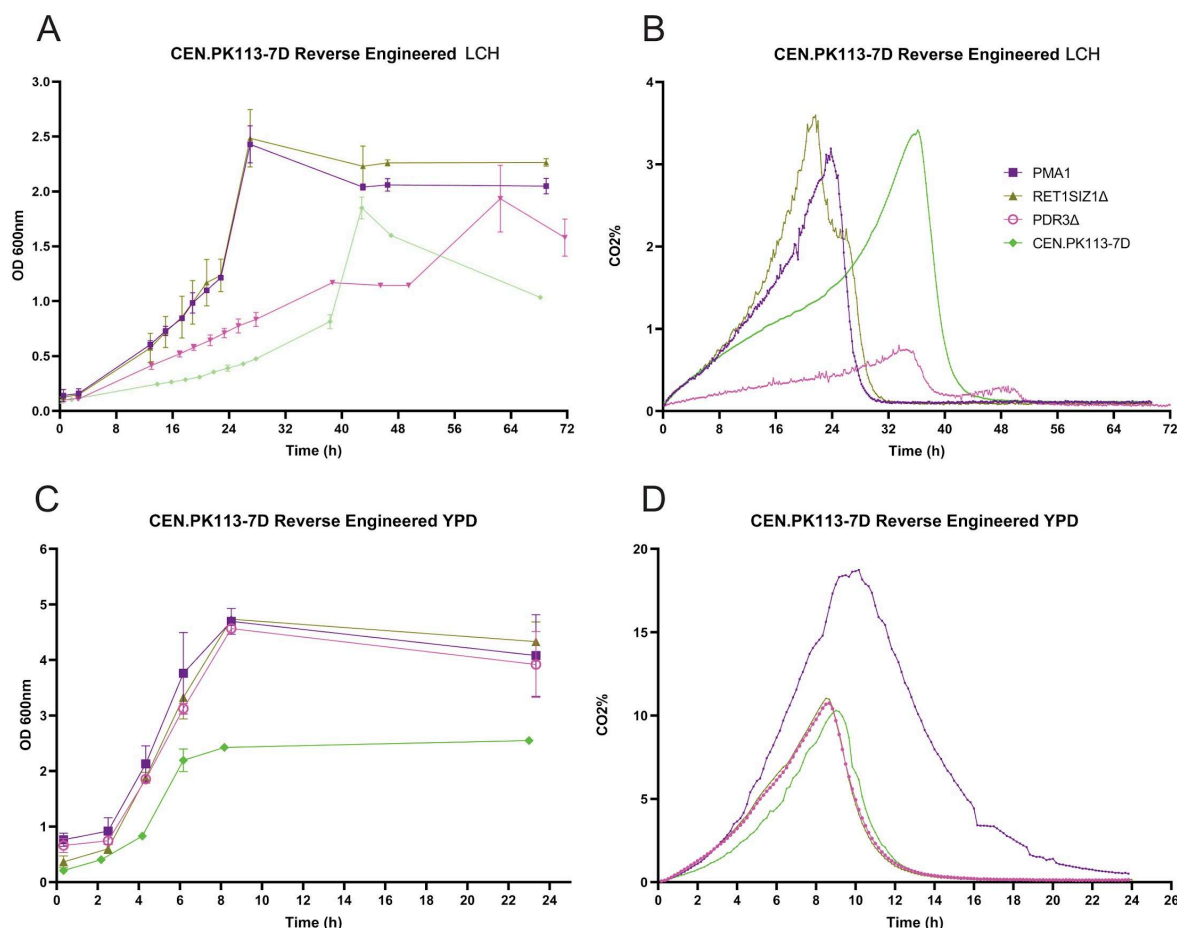
Figure 4.13 - Performance of Clones in Different Concentrations of Acetic Acid.



A. Under conditions of 0% and B. 35% acetic acid, the wild type (WT) demonstrated a similar lag phase. However, at concentrations of C. 50% and D. 70%, CHG and CHH clones exhibited a shorter lag phase compared to the WT, demonstrating their specific tolerance towards acetic acid. At all concentrations, the WT showed a shorter lag phase when compared to the evolved clones CHN and S_CHN. Demonstrating that these clones did not evolve to acetic acid tolerance exclusively.

Reverse-engineered strains (Figure 4.14) *PMA1* and *RET1SIZ1Δ* exhibited a reduced lag phase and reached a cell density higher than the WT in the S_LCH condition. Notably, CO₂ production in these strains was practically equivalent to that of the WT, suggesting similar metabolic efficiency. However, in the reverse-engineered strains, this pattern occurred approximately 10 hours earlier compared to the WT. In YPD fermentations, greater production of CO₂ was observed in the PMA1 strain, although a higher concentration influenced growth in OD in the inoculation. In this context, the need to calculate the cell growth rate as a more robust strategy to evaluate the performance of strains becomes evident, considering variables such as the initial inoculation.

Figure 4.14 - Optical Density (OD) and CO₂ production as a Function of Time in Reverse Engineered strains.

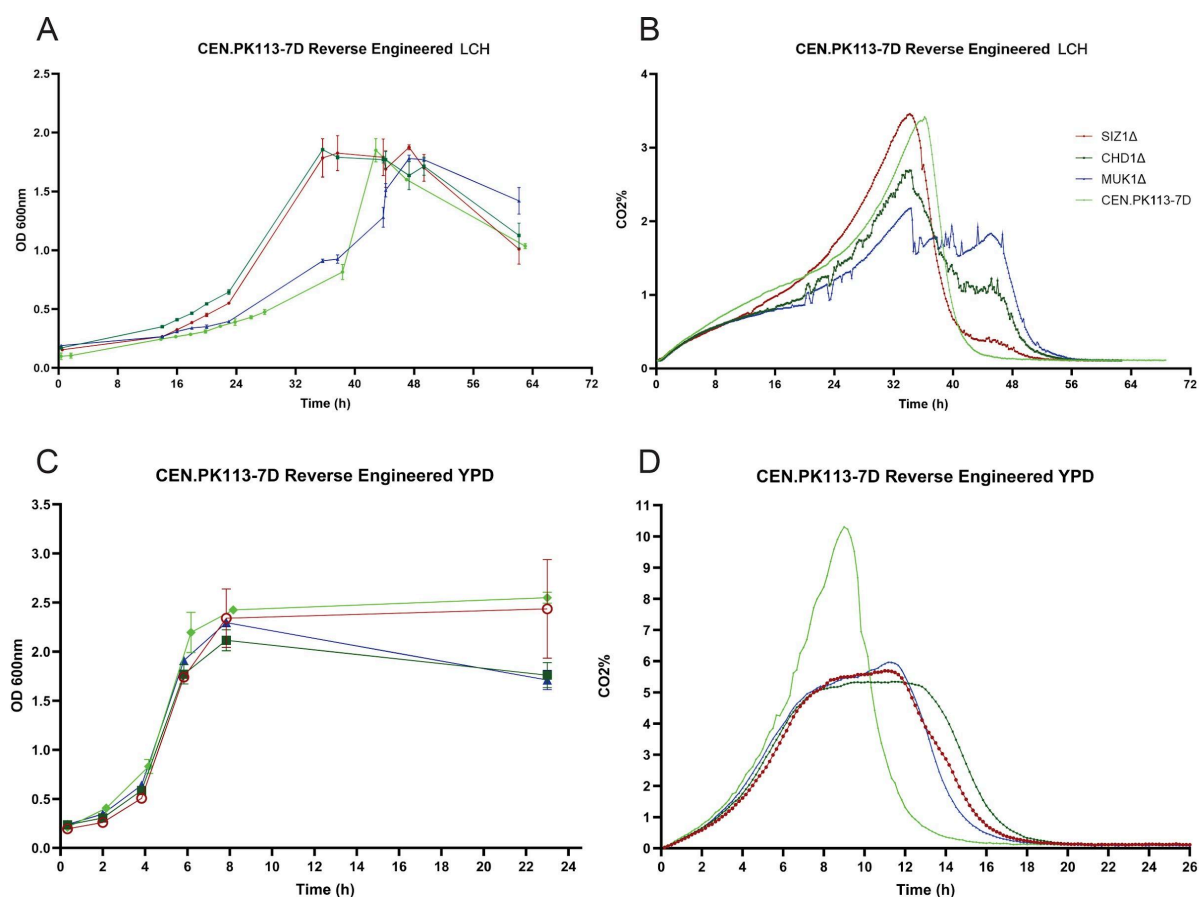


The tree clones in duplicate are represented (*PMA1*, *RET1SIZ1Δ*, and *PDR3Δ*) and are compared to the CEN.PK113-7D WT (also in duplicate). A- OD in LCH media. B- CO₂ in LCH media. C- OD in YPD media. D- CO₂ in YPD media.

In the other reverse-engineered strains (Figure 4.15), specifically the *SIZ1Δ* and *CHD1Δ* strains, a reduced lag phase was observed in the LCH treatment. However, the maximum cell density was similar to that of the *MUK1Δ* and WT strains. Regarding CO₂ production, the WT and the *SIZ1Δ* strains showed similar performance, while the *MUK1Δ* and *CHD1Δ* strains exhibited problems in detecting CO₂ values due to failures in obtaining the data by the equipment. In YPD fermentations, all strains showed similar behavior in cell growth. However, in CO₂ production, the WT strain demonstrated higher production compared to the modified

strains. This demonstrates that the trade-off not only influenced growth but also CO₂ production

Figure 4.15 - Optical Density (OD) and CO₂ production as a Function of Time in reverse-engineered strains.



The tree clones in duplicate are represented (*SIZ1Δ*, *CHD1Δ*, and *MUK1Δ*) and are compared to the CEN.PK113-7D WT (also in duplicate). A- OD in LCH media. B- CO₂ in LCH media. C- OD in YPD media. D- CO₂ in YPD media.

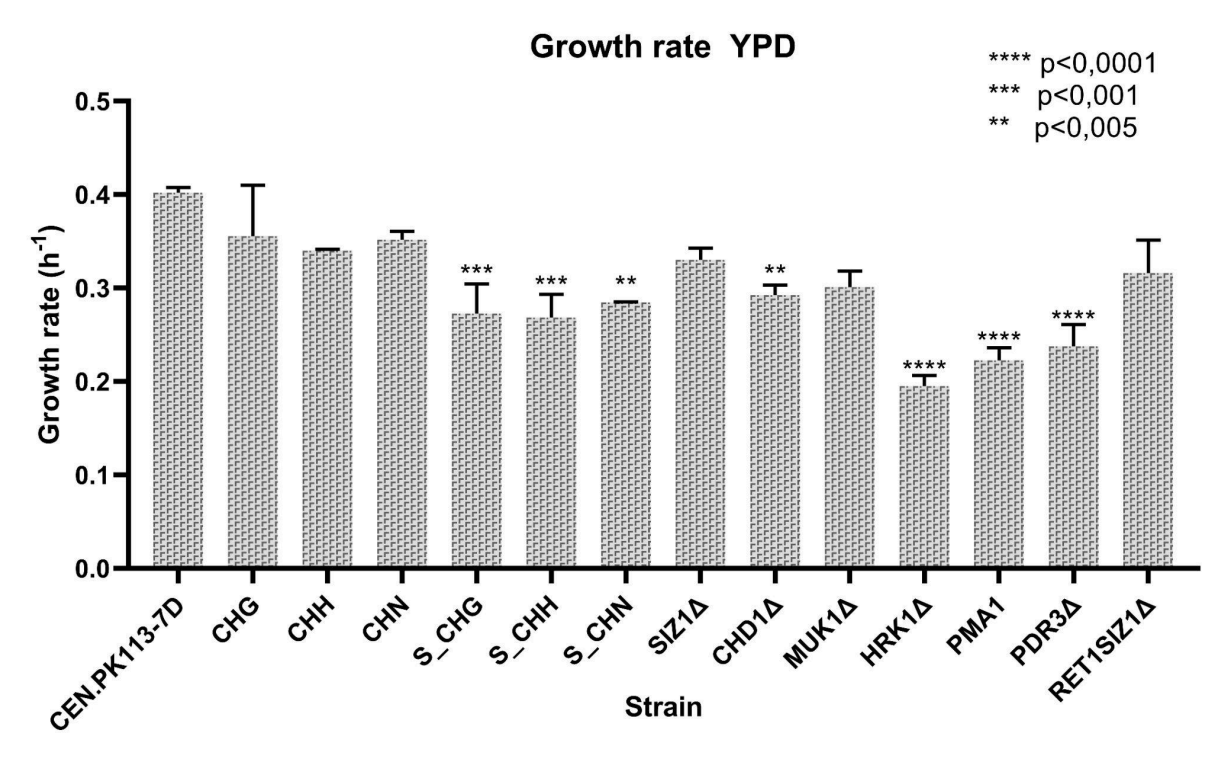
Nonetheless, minor fluctuations in the OD of the inoculum may affect the OD graph. In this manner, the growth rate of the strains in two distinct culture media—S_LCH and YPD—between time zero and the late exponential phase was determined. Growth rate is a key parameter in the analysis of microbial growth. It is calculated using the formula:

$$\text{Growth rate} = (\ln(\text{OD}_{\text{final}} / \text{OD}_{\text{start}})) / \Delta t$$

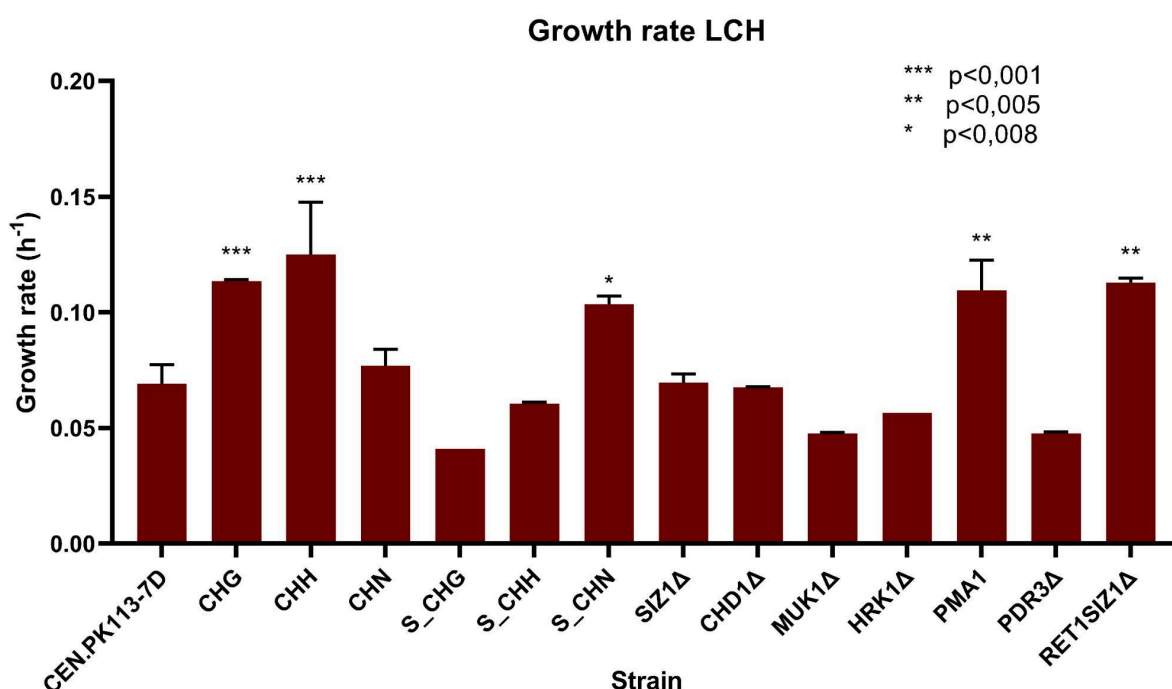
In this equation, OD_{final} is the optical density at the late exponential phase, OD_{start} represents the optical density at the time 0, and Δt is the time interval between measurements. This formula provides information about the rate of proliferation of microorganisms over a specific period, crucial information for understanding and optimizing microbial growth conditions. The corresponding graphs (Figure 4.16A for YPD and Figure 4.16B for S_LCH) showed the results.

Figure 4.16 - Growth rate.

A



B



A- Growth in YPD: The graph illustrates the growth rate of the strains compared to the WT (CEN.PK113-7D). Strains CHG, CHH, CHN, *CHD1Δ*, *HRK1Δ*, *PMA1*, and *PDR3Δ* exhibited a growth rate statistically significantly lower than the WT, indicating a general impairment of the strain or a possible trade-off. B- Growth in S_LCH: The graph highlights the growth rate of the strains in S_LCH, compared to the WT. Strains CHG, CHH, S_CHN, *PMA1*, and *RET1SIZ1Δ* demonstrated statistically significantly greater growth rates, demonstrating a greater hydrolysate growth capacity. One-way ANOVA test with Dunnetts post-test. * p<0,008, **p<0,005, ***p<0,001, and ****p<0,0001.

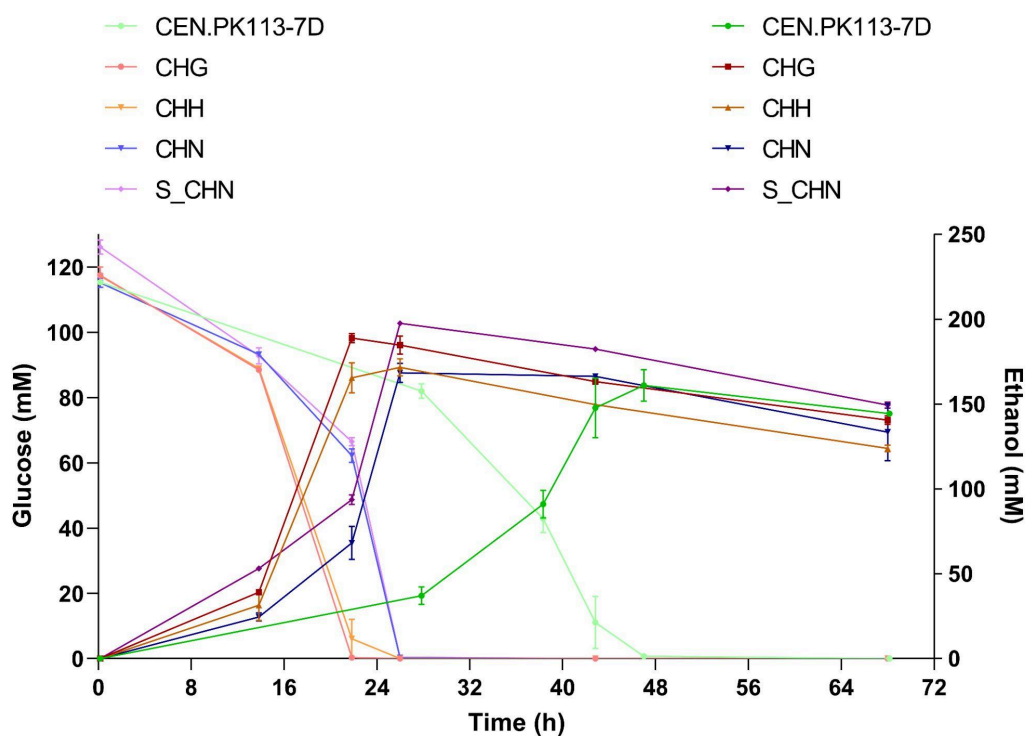
4.3.3.2 Extracellular Metabolites

The investigation of the extracellular metabolites of the CEN.PK13-7D strains derived from the serial transfer evolution experiment (CHG, CHH, and CHN), the S_CHN strain originating from the shock evolution experiment can be analyzed in the graphs below. The focus on the parameters of ethanol production and glucose consumption in the YPD medium and the hydrolysate aims to understand the fermentative strategies adopted by each strain (Figures 4.17A and B).

Figure 4.17 - Fermentation Extracellular Metabolites.

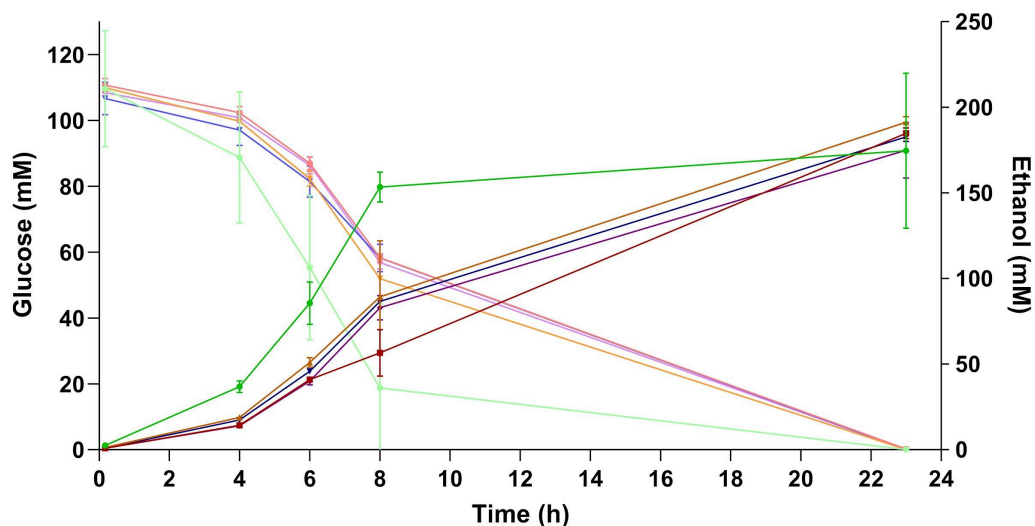
A

***S. cerevisiae* CEN.PK113-7D LCH**



B

***S. cerevisiae* CEN.PK113-7D YPD**



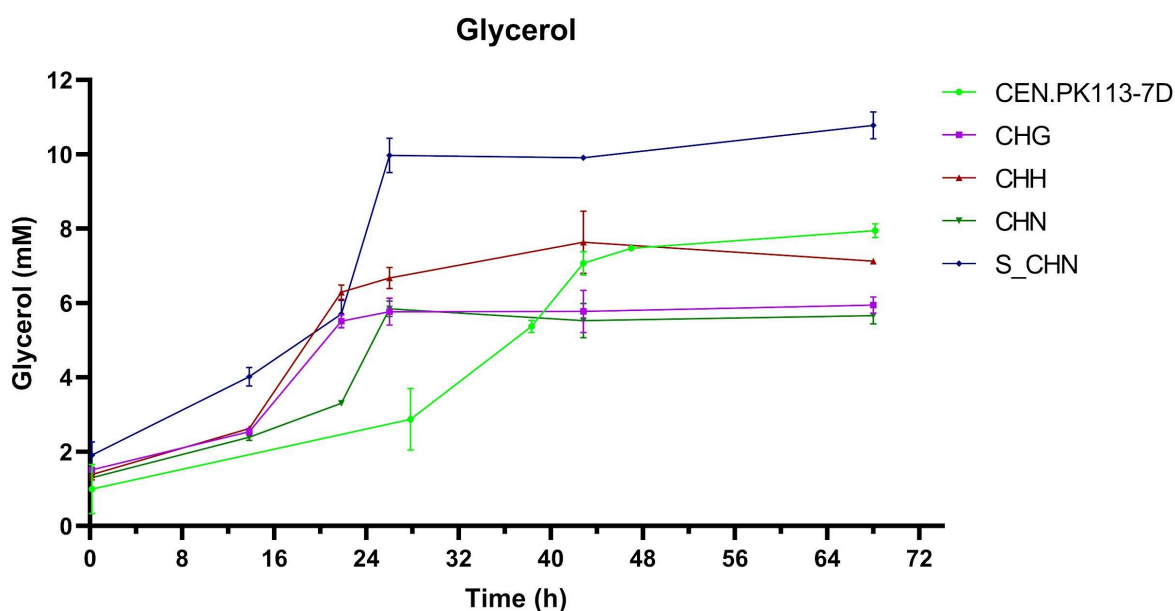
A- The graph reveals the distinctive performance of strains CHG, CHH, and S_CHN in S_LCH, where a higher OD not only translates into a faster consumption of glucose but also a notable ethanol production. Highlighting the CHN strain, which, although it did not have a higher growth performance than the WT, showed greater metabolic efficiency. B- On the other hand, in the YPD medium, all strains showed

reduced performance compared to the WT. The trade-off of these cells is demonstrated.

It was observed that the CHG, CHH, and S_CHN strains, which showed better performance in OD, also exhibited faster glucose consumption and higher ethanol production than the WT group. In addition, faster ethanol production and more efficient glucose consumption were observed in the CHN strain, regardless of whether it did not demonstrate higher growth. In the YPD medium, all four strains demonstrated lower performance than the WT group. This result suggests that the strains evolved to have advantages in specific hydrolysate conditions, but the YPD medium may not be ideal for maximizing their fermentative capabilities.

Another important feature to be analyzed is glycerol production as it acts as a natural redox neutralizer within the process (allowing regeneration of NAD⁺), in which, glycerol is produced from glucose and increases in response to osmotic stress (MUTTON *et al.*, 2019). The glycerol production over time can be analyzed in Figure 4.18.

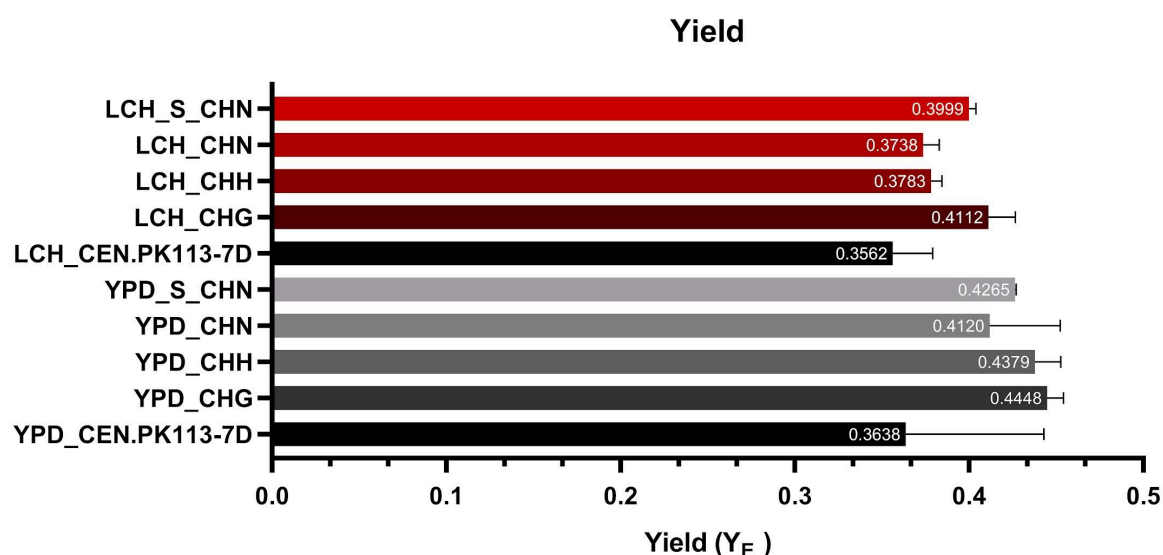
Figure 4.18 - Temporal analysis of glycerol production in different strains in LCH.



The synthesis of glycerol stands out mainly in the S_CHN and CHH strains. This variation demonstrates that the strains are dealing differently with the stress caused by S_LCH.

The analysis of the relative ethanol yield factor ($Y'_{ethanol/S}$) of the strains under study, both in YPD and S_LCH medium was calculated as the ratio between the ethanol produced (g/L) and the initial concentration of sugars (g/L) (PEREIRA *et al.*, 2021) (Figure 4.19). The maximum theoretical yield is 0.51 g ethanol per g hexose sugar. Industrial ethanol production operates at >90% of this theoretical yield (ANJOS *et al.*, 2013). This analysis aims to understand the yield considering small fluctuations in the initial glucose concentration.

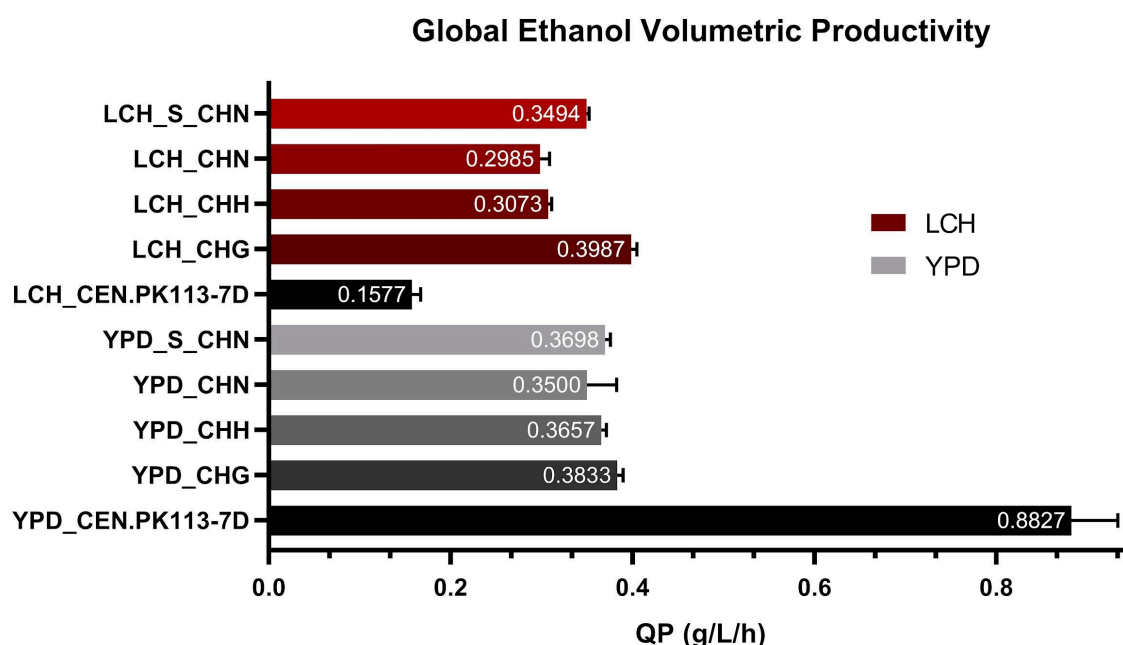
Figure 4.19 - Comparison of Yield values for different strains in different growth environments.



The results show the sugar-to-ethanol conversion efficiency for each strain under YPD and LCH growth conditions. Strains are identified according to their designation, and Yield values reflect the ability of each strain to optimize biomass yield under specific cultivation conditions. The WT is represented in black, strains that grow in YPD are represented in a scale of grey, and strains that grow in S_LCH are represented in a scale of red.

Global ethanol volumetric productivity (Q_P , g ethanol/L/h) was calculated as the ratio between the ethanol titer (g/L) and the respective fermentation time (h) (PEREIRA *et al.*, 2021). The evaluation of these conditions seeks to provide a deeper understanding of how the fermentative performance of each strain is reflected in the effective production of ethanol (Figure 4.20).

Figure 4.20 - Graphical representation of the comparison of Global ethanol volumetric productivity (QP) values for different strains in YPD and LCH environments.



Each strain is identified by its specific designation. The results reflect the overall efficiency of each strain in QP. The WT is represented in black, strains that grow in YPD are represented in a scale of grey, and strains that grow in S_LCH are represented in a scale of red.

When evaluating the results in YPD, we observed that the CHG_YPD and CHH_YPD strains showed higher yields (Yield) compared to the WT CEN.PK113-7D_YPD. However, the greater overall productivity of the WT strain indicates its greater efficiency in producing ethanol. These results suggest that the CHG_YPD and CHH_YPD strains can maintain a yield in YPD, but lose the WT in terms of productivity. When analyzing strains with fermentation in hydrolysate (LCH), it is notable that the evolved strains outperform WT CEN.PK113-7D_LCH in terms of yield and overall productivity.

On the other hand, these evolved strains showed an enhanced growth capacity and ethanol production in LCH compared to the WT. The significant increase in yield, with a focus on CHG and S_CHN, and overall productivity of these strains highlight the adaptability and efficacy in conditions with lignocellulosic inhibitors. To

enable the complete visualization of the data, Table 4.5 compiles Growth rate, Yield, and Global Productivity.

Table 4.5 - Growth rate, Yield, and Global productivity compilation

Strain	Growth rate	Yield	Global Productivity
WT_CEN.PK113-7D_YPD	0,402	0,3638	0,8827
WT_CEN.PK113-7D_LCH	0,0692	0,3562	0,1577
S_CHN_YPD	0,2845	0,4265	0,3698
S_CHN_LCH	0,1035	0,3999	0,3494
CHG_YPD	0,3555	0,4448	0,3833
CHG_LCH	0,1135	0,4112	0,3987
CHH_YPD	0,34	0,4379	0,3657
CHH_LCH	0,125	0,3783	0,3073
CHN_YPD	0,345	0,4120	0,3698
CHN_LCH	0,077	0,3738	0,2985

4.5 Discussion

This chapter employed schemes to visualize mutations observed in WGS in the 4 ALE (11 populations) and 3 clones (CHG_clone, CHN_clone, and S_CHN_chone) showing the evolutionary changes and which mutations are present in parallel (intra-population and inter-background). This helps to understand the importance of mutations. The identification of more than 100 single nucleotide polymorphisms (SNPs) is a significant finding. Categorization into synonymous, missense, nonsense, stop codon, or frameshift mutations (Tables 1 - 8) provides detailed insight into the nature and potential effects of these mutations. The methodology focused on the selection of mutations based on parallelism and their association with genes linked to the described functions is extremely useful and necessary for the first approach. However, it is worth highlighting that the exploration of orphan genes or the identification of new functions for existing genes in future studies is an approach that can lead to many discoveries (FAJARDO; SAINT JEAN; LYONS, 2023; VAKIRLIS; CARVUNIS; MCLYSAGHT, 2020). This additional approach can provide valuable information regarding adaptive phenotypes and improve our understanding of the genetic mechanisms generated during evolutionary processes. We carried out deletion of the already toxicity-related gene, *SIZ1*, to check its effect on the developed LCH and to corroborate our findings, since its correlation with tolerance is well known (XIAO; ZHAO, 2014). This experimental step confirms that *SIZ1Δ* is an important factor that enhances *Saccharomyces cerevisiae* resistance to LCH and suggests that our results are reliable.

The interplay between the mutations in *SIZ1Δ* and *RET1* is particularly interesting. The data suggest that the mutation in *SIZ1Δ* occurred first, followed by the mutation in *RET1* because the mutation in *RET1* alone is practically unsustainable for cell survival. Furthermore, it is possible to see a synergistic effect between the two, since the *RET1*(2347G>T) substitution in the *SIZ1Δ* strain conferred a greater gain in fitness (~63%) than the deletion of *SIZ1Δ* alone (~25%). In contrast, SNP with an amino acid change in *PMA1* (Ser158Gly) was associated with a loss of fitness of approximately 5%. However, this mutation resulted in a higher growth rate under microaeration, pH control, and higher levels of toxic compounds. In this way, it also contributes to improving the tolerance of strains to hydrolysate.

The *HRK1* gene plays a crucial role in the activation of Pma1 in response to glucose metabolism and proton homeostasis in stress situations (ANTUNES *et al.*, 2023;

GOOSSENS *et al.*, 2000). The deletion of this gene was investigated to assess its impact on the conditions studied. HRK1 Δ resulted in lower growth rates in rich media, indicating the negative effects of these mutations, as this mutation did not show better growth rates in LCH. Therefore, based on the results obtained, the importance of HRK1 is significant due to its fundamental role in regulating cellular homeostasis and responding to specific stresses (ANTUNES *et al.*, 2023).

Furthermore, our methodology predominantly focused on non-synonymous mutations. However, considering recent discoveries demonstrating that synonymous mutations in protein-coding genes have significant impacts on fitness, it may be pertinent that future investigations include an analysis of synonymous mutations. Contrary to the assumption of neutrality, experimental verification has shown that a large number of synonymous mutations leads to a significant reduction in fitness. Analysis of synonymous mutations could provide a more nuanced understanding of the genetic basis of adaptive phenotypes (SHEN *et al.*, 2022). Clonal interference is an additional topic for discussion. Clonal interference between subpopulations with different adapted alleles is commonly seen in ALE techniques involving the asexual growth of microorganisms (ALE 2 and ALE 4). The experiment performed by Almario and collaborators (2013) revealed an evolution that conforms to this interference (ALMARIO; REYES; KAO, 2013).

Fermentations conducted in a controlled environment evaluated the performance of triplicate clones of ALE2, ALE4, and reverse engineering experiments in comparison with the WT strain (CEN.PK113-7D) under different fermentation conditions (YPD and LCH). The graphical analysis in Figure 5 illustrates the dynamic changes in OD and CO₂ over time. The strains exhibited different patterns in the rich and hydrolysate media, and the lag phase tended to increase in the hydrolysate, proving that the synthetic environment was indeed challenging. On the other hand, the "trade-off" scenario of strains is one of the main obstacles to evolutionary engineering. It is described as the loss of a different trait throughout the strain's evolution toward a certain attribute (TOPALOĞLU *et al.*, 2023). The idea of a trade-off is prevalent in evolutionary biology and is acknowledged as a price for adaptation (CASPETA; NIELSEN, 2015). This is a crucial topic in evolutionary engineering research, particularly those conducted for commercial objectives. It is impossible to assess this trade-off beforehand, which might lead to a waste of time and money (WENGER *et al.*, 2011). Strains from the shock protocol (S_CHG,

S_CHH, S_CHN) showed a lower growth rate in YPD than in the WT, suggesting that the evolutionary adaptations of these strains may result in a competitive disadvantage in a richer environment such as YPD. However, only S_CHN achieved greater growth than the WT in LCH. Therefore, the shock protocol was not ideal for S_CHG and S_CHH to overcome the WT in the medium containing hydrolysate. Strains *CHD1Δ*, *HRK1Δ*, *PMA1*, and *PDR3Δ* also exhibited lower growth rates in YPD, suggesting negative effects of these mutations or trade-offs that compromise growth in rich media. Strains CHG, and CHH, from the ALE2 protocol, and strain S_CHN from the ALE4 protocol showed higher growth rates in LCH compared to the WT, which suggests that these strains have evolved to grow better under challenging conditions of LCH.

In addition, the strains obtained by reverse engineering (*PMA1*, *RET1SIZ1Δ*), help to understand which mutations are directly related to the best growth in a stressful environment such as the LCH. Furthermore, CHG, CHH, CHN, and S_CHN clones were tested specifically for their tolerance to acetic acid. It seems that CHG and CHH evolved in a way that suggests tolerance to acetic acid, while CHN and S_CHN were not able to perform better than the WT, even in 70% acetic acid. This suggests that their tolerance is toward other toxic compounds present in the hydrolysate. In summary, analyzing growth rates helps us to understand evolutionary adaptations and trade-offs among the strains studied. This information is valuable but requires additional data to corroborate the robustness of the results. For applications in ethanol production, the choice of strain should not focus on the growth rate, but rather on the ethanol yield and overall productivity. Some studies have even worked on reducing the biomass to increase ethanol yield, such as a proton-coupled transport system that was used in place of hexose transporters. With this strategy, biomass production was significantly reduced (44–47.6%) and ethanol yield increased (17.2%) (DE VALK *et al.*, 2022).

Thus it was necessary to investigate the metabolites produced during fermentation. Extracellular metabolites from the serial transfer evolution experiment (CHG, CHH, and CHN) and the S_CHN strain originating from the shock evolution experiment provide valuable insights into yield and productivity. The S_CHN and CHG strains, in addition to a higher yield (0.3999, and 0.4112, respectively), demonstrated a notable global Ethanol Volumetric Productivity (0.3494 g/L/h and 0.3987 g/L/h, respectively). Bermejo and collaborators (2021) affirmed that the

industrial ethanol yield on sugar is approximately 85% of the theoretical maximum value (0.511 g of ethanol per g of hexose-equivalent) (BERMEJO *et al.*, 2021), and not around 90% of this maximum threshold, as imagined (DELLA-BIANCA *et al.*, 2013; PEREIRA *et al.*, 2018). Thus, it may be worth it to revisit the formula applied and yields in our work. When looking at the glycerol production of these two strains, the behavior was different, with S_CHN reaching ~10mM of glycerol, while CHG reached ~6mM of glycerol. This difference can be attributed to the factors that influence glycerol formation, including temperature, inoculum concentration, pH, nutrients, and inhibitors (NORDSTRÖM, 1962; PANCHAL; STEWART, 1980). These variables demonstrate the complexity of fermentative processes, where many physical, chemical, and microbiological factors affect the efficiency of fermentation and the conversion of sugar into ethanol (DE ALMEIDA LIMA *et al.*, 2002). The production of glycerol is important for the metabolic balance of yeast, and it is involved in the regeneration of NAD (MUTTON *et al.*, 2019). However, in the industry, the formation of glycerol is undesirable, because it reduces fermentation efficiency (PANCHAL; STEWART, 1980). It is well known that under CO₂ (or N₂) conditions glycerol formation decreases and ethanol production increases compared to aerobic conditions (HAGMAN; PIŠKUR, 2015; TAN *et al.*, 2022). To maintain redox balance, the cell can reoxidize NADH by reducing acetate to ethanol (MEDINA *et al.*, 2010; TAN *et al.*, 2022). This helps to reduce acetate concentration and increase the ethanol yield. These observations highlight the complexity of metabolic regulation in response to specific conditions, such as the presence of CO₂, ethanol, and lignocellulosic inhibitors (TAN *et al.*, 2022).

In conclusion, a comprehensive analysis of competition assays, growth rates, ethanol yield, global ethanol volumetric productivity, and other extracellular metabolites contributes to the understanding of the mechanisms used by ALE strains. Additional studies could explore the molecular mechanisms underlying these behaviors observed in fermentations and identify other genes involved in tolerance.

References

- ALMARIO, M. P.; REYES, L. H.; KAO, K. C. Evolutionary engineering of *Saccharomyces cerevisiae* for enhanced tolerance to hydrolysates of lignocellulosic biomass. **Biotechnology and bioengineering**, v. 110, n. 10, p. 2616–2623, 2013.
- ANJOS, J.; DE SOUSA, H. R.; ROCA, C.; CÁSSIO, F.; LUTTIK, M.; PRONK, J. T.; SALEMA-OOM, M.; GONÇALVES, P. Fsy1, the sole hexose-proton transporter characterized in *Saccharomyces* yeasts, exhibits a variable fructose:H⁺ stoichiometry. **Biochimica et Biophysica Acta (BBA) - Biomembranes**, v. 1828, n. 2, p. 201–207, 2013.
- ANTUNES, M.; KALE, D.; SYCHROVÁ, H.; SÁ-CORREIA, I. The Hrk1 kinase is a determinant of acetic acid tolerance in yeast by modulating H and K homeostasis. **Microbial cell factories**, v. 10, n. 12, p. 261–276, 2023.
- BALZI, E.; GOFFEAU, A. Yeast multidrug resistance: the PDR network. **Journal of bioenergetics and biomembranes**, v. 27, n. 1, p. 71–76, 1995.
- BERMEJO, P. M.; BADINO, A.; ZAMBERLAN, L.; RAGHAVENDRAN, V.; BASSO, T. O.; GOMBERT, A. K. Ethanol yield calculations in biorefineries. **FEMS yeast research**, v. 21, n. 8, p. foab065, 2021.
- BIOT-PELLETIER, D.; PINEL, D.; LARUE, K.; MARTIN, V. J. J. Determinants of selection in yeast evolved by genome shuffling. **Biotechnology for biofuels**, v. 11, n. 1, p. 1–16, 2018.
- BOGINYA, A.; DETROJA, R.; MATITYAHU, A.; FRENKEL-MORGENSTERN, M.; ONN, I. The chromatin remodeler Chd1 regulates cohesin in budding yeast and humans. **Scientific reports**, v. 9, n. 1, p. 8929, 2019.
- CABRERA, M.; ARLT, H.; EPP, N.; LACHMANN, J.; GRIFFITH, J.; PERZ, A.; REGGIORI, F.; UNGERMANN, C. Functional separation of endosomal fusion factors and the class C core vacuole/endosome tethering (CORVET) complex in endosome biogenesis. **The Journal of biological chemistry**, v. 288, n. 7, p. 5166–5175, 2013.
- CASPETA, L.; NIELSEN, J. Thermotolerant Yeast Strains Adapted by Laboratory Evolution Show Trade-Off at Ancestral Temperatures and Preadaptation to Other Stresses. **mBio**, v. 6, n. 4, p. e00431, 2015.
- CHENG, C.; ALMARIO, M. P.; KAO, K. C. Genome shuffling to generate recombinant yeasts for tolerance to inhibitors present in lignocellulosic hydrolysates. **Biotechnology letters**, v. 37, n. 11, p. 2193–2200, 2015.

DE ALMEIDA LIMA, U.; AQUARONE, E.; SCHMIDELL, W.; BORZANI, W.
Biotechnologia industrial - vol. 3: processos fermentativos e enzimáticos. [S. l.]:
 Editora Blucher, 2002.

DELAVEAU, T.; DELAHODDE, A.; CARVAJAL, E.; SUBIK, J.; JACQ, C. PDR3, a new yeast regulatory gene, is homologous to PDR1 and controls the multidrug resistance phenomenon. **Molecular & general genetics: MGG**, v. 244, n. 5, p. 501–511, 1994.

DELLA-BIANCA, B. E.; BASSO, T. O.; STAMBUK, B. U.; BASSO, L. C.; GOMBERT, A. K. What do we know about the yeast strains from the Brazilian fuel ethanol industry? **Applied microbiology and biotechnology**, v. 97, n. 3, p. 979–991, 2013.

DE VALK, S. C.; BOUWMEESTER, S. E.; DE HULSTER, E.; MANS, R. Engineering proton-coupled hexose uptake in *Saccharomyces cerevisiae* for improved ethanol yield. **Biotechnology for biofuels and bioproducts**, v. 15, n. 1, p. 47, 2022.

DEVAUX, F.; CARVAJAL, E.; MOYE-ROWLEY, S.; JACQ, C. Genome-wide studies on the nuclear PDR3-controlled response to mitochondrial dysfunction in yeast. **FEBS letters**, v. 515, n. 1-3, p. 25–28, 2002.

FAJARDO, D.; SAINT JEAN, R.; LYONS, P. J. Acquisition of new function through gene duplication in the metallocarboxypeptidase family. **Scientific reports**, v. 13, n. 1, p. 2512, 2023.

GONZÁLEZ-RAMOS, D.; GORTER DE VRIES, A. R.; GRIJSEELS, S. S.; VAN BERKUM, M. C.; SWINNEN, S.; VAN DEN BROEK, M.; NEVOIGT, E.; DARAN, J.-M. G.; PRONK, J. T.; VAN MARIS, A. J. A. A new laboratory evolution approach to select for constitutive acetic acid tolerance in *Saccharomyces cerevisiae* and identification of causal mutations. **Biotechnology for biofuels**, v. 9, n. 1, p. 1–18, 2016.

GOOSSENS, A.; DE LA FUENTE, N.; FORMENT, J.; SERRANO, R.; PORTILLO, F. Regulation of yeast H(+)-ATPase by protein kinases belonging to a family dedicated to activation of plasma membrane transporters. **Molecular and cellular biology**, v. 20, n. 20, p. 7654–7661, 2000.

HACISALIHOĞLU, B.; HOLYAVKIN, C.; TOPALOĞLU, A.; KISAKESEN, H. İ.; ÇAKAR, Z. P. Genomic and transcriptomic analysis of a coniferyl aldehyde-resistant *Saccharomyces cerevisiae* strain obtained by evolutionary engineering. **FEMS yeast research**, v. 19, n. 3, p. foz021, 2019.

HAGMAN, A.; PIŠKUR, J. A Study on the Fundamental Mechanism and the Evolutionary Driving Forces behind Aerobic Fermentation in Yeast. **PloS one**, v. 10,

n. 1, p. e0116942, 2015.

HENDERSON, K. A.; HUGHES, A. L.; GOTTSCHLING, D. E. Mother-daughter asymmetry of pH underlies aging and rejuvenation in yeast. **eLife**, v. 3, p. e03504, 2014.

HUH, W.-K.; FALVO, J. V.; GERKE, L. C.; CARROLL, A. S.; HOWSON, R. W.; WEISSMAN, J. S.; O'SHEA, E. K. Global analysis of protein localization in budding yeast. **Nature**, v. 425, n. 6959, p. 686–691, 2003.

JACOBUS, A. P.; BARRETO, J. A.; DE BEM, L. S.; MENEGON, Y. A.; FIER, Í.; BUENO, J. G. R.; DOS SANTOS, L. V.; GROSS, J. EasyGuide Plasmids Support in Vivo Assembly of gRNAs for CRISPR/Cas9 Applications in. **ACS synthetic biology**, v. 11, n. 11, p. 3886–3891, 2022.

JAMES, P.; HALL, B. D. *ret1-1*, a yeast mutant affecting transcription termination by RNA polymerase III. **Genetics**, v. 125, n. 2, p. 293–303, 1990.

LIU, Z. L.; HUANG, X.; ZHOU, Q.; XU, J. Protein expression analysis revealed a fine-tuned mechanism of in situ detoxification pathway for the tolerant industrial yeast *Saccharomyces cerevisiae*. **Applied microbiology and biotechnology**, v. 103, n. 14, p. 5781–5796, 2019.

LI, W.; WU, Z.; LIANG, Y. Vrl1 relies on its VPS9-domain to play a role in autophagy in *Saccharomyces cerevisiae*. **Cell biology international**, v. 43, n. 8, p. 875–889, 2019.

MEDINA, V. G.; ALMERING, M. J. H.; VAN MARIS, A. J. A.; PRONK, J. T. Elimination of Glycerol Production in Anaerobic Cultures of a *Saccharomyces cerevisiae* Strain Engineered To Use Acetic Acid as an Electron Acceptor. **Applied and environmental microbiology**, v. 76, n. 1, p. 190, 2010.

MEENA, R. C.; THAKUR, S.; CHAKRABARTI, A. Regulation of *Saccharomyces cerevisiae* Plasma membrane H(+)-ATPase (Pma1) by Dextrose and Hsp30 during Exposure to Thermal Stress. **Indian journal of microbiology**, v. 51, n. 2, p. 153–158, 2011.

MENEGON, Y. A.; GROSS, J.; JACOBUS, A. P. How adaptive laboratory evolution can boost yeast tolerance to lignocellulosic hydrolyses. **Current genetics**, v. 68, n. 3-4, p. 319–342, 2022.

MUTTON, M. J. R.; FERRARI, F. C. S.; DE FREITA, L. A.; DE FREITA, C. M.; DA GRAÇA S. ANDRIETTA, M.; ANDRIETTA, S. R. Interaction between the production

of ethanol and glycerol in fed-batch bioreactors. **Brazilian journal of microbiology: [publication of the Brazilian Society for Microbiology]**, v. 50, n. 2, p. 389, 2019.

NORDSTRÖM, K. Formation of ethyl acetate in fermentation with brewer's yeast. Iii. Participation of coenzyme a. **Journal of the Institute of Brewing. Institute of Brewing**, v. 68, n. 5, p. 398–407, 1962.

PANCHAL, C. J.; STEWART, G. G. The effect of osmotic pressure on the production and excretion of ethanol and glycerol by a brewing yeast strain. **Journal of the Institute of Brewing. Institute of Brewing**, v. 86, n. 5, p. 207–210, 1980.

PAULSEL, A. L.; MERZ, A. J.; NICKERSON, D. P. Vps9 family protein Muk1 is the second Rab5 guanosine nucleotide exchange factor in budding yeast. **The Journal of biological chemistry**, v. 288, n. 25, p. 18162–18171, 2013.

PEREIRA, I. de O.; DOS SANTOS, Â. A.; GONÇALVES, D. L.; PURIFICAÇÃO, M.; GUIMARÃES, N. C.; TRAMONTINA, R.; COUTOUNÉ, N.; ZANELLA, E.; MATSUSHIKA, A.; STAMBUK, B. U.; IENCZAK, J. L. Comparison of *Spathaspora passalidarum* and recombinant *Saccharomyces cerevisiae* for integration of first- and second-generation ethanol production. **FEMS yeast research**, v. 21, n. 6, p. foab048, 2021.

PEREIRA, R. D.; RODRIGUES, K. C. S.; SONEGO, J. L. S.; CRUZ, A. J. G.; BADINO, A. C. A New Methodology to Calculate the Ethanol Fermentation Efficiency at Bench and Industrial Scales. **Industrial & engineering chemistry research**, 2018. Disponível em: <https://doi.org/10.1021/acs.iecr.8b03943>. Acesso em: 7 fev. 2024.

PEREIRA, R.; MOHAMED, E. T.; RADI, M. S.; HERRGÅRD, M. J.; FEIST, A. M.; NIELSEN, J.; CHEN, Y. Elucidating aromatic acid tolerance at low pH in using adaptive laboratory evolution. **Proceedings of the National Academy of Sciences of the United States of America**, v. 117, n. 45, p. 27954–27961, 2020.

PINEL, D.; COLATRIANO, D.; JIANG, H.; LEE, H.; MARTIN, V. J. Deconstructing the genetic basis of spent sulphite liquor tolerance using deep sequencing of genome-shuffled yeast. **Biotechnology for biofuels**, v. 8, p. 53, 2015.

PRAY-GRANT, M. G.; DANIEL, J. A.; SCHIELTZ, D.; YATES, J. R.; GRANT, P. A. Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. **Nature**, v. 433, n. 7024, 2005. Disponível em: <https://doi.org/10.1038/nature03242>. Acesso em: 29 jan. 2024.

SALAS-NAVARRETE, P. C.; DE OCA MIRANDA, A. I. M.; MARTÍNEZ, A.; CASPETA, L. Evolutionary and reverse engineering to increase *Saccharomyces cerevisiae*

tolerance to acetic acid, acidic pH, and high temperature. **Applied microbiology and biotechnology**, v. 106, n. 1, p. 383–399, 2021.

SAMANTA, M. P.; LIANG, S. Predicting protein functions from redundancies in large-scale protein interaction networks. **Proceedings of the National Academy of Sciences of the United States of America**, v. 100, n. 22, p. 12579–12583, 2003.

SERRANO, R.; KIELLAND-BRANDT, M. C.; FINK, G. R. Yeast plasma membrane ATPase is essential for growth and has homology with (Na⁺ + K⁺), K⁺- and Ca²⁺-ATPases. **Nature**, v. 319, n. 6055, p. 689–693, 1986.

SHEN, X.; SONG, S.; LI, C.; ZHANG, J. Synonymous mutations in representative yeast genes are mostly strongly non-neutral. **Nature**, v. 606, n. 7915, p. 725–731, 2022.

SIMIC, R.; LINDSTROM, D. L.; TRAN, H. G.; ROINICK, K. L.; COSTA, P. J.; JOHNSON, A. D.; HARTZOG, G. A.; ARNDT, K. M. Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. **The EMBO journal**, v. 22, n. 8, p. 1846–1856, 2003.

SMOLLE, M.; VENKATESH, S.; GOGOL, M. M.; LI, H.; ZHANG, Y.; FLORENS, L.; WASHBURN, M. P.; WORKMAN, J. L. Chromatin remodelers Isw1 and Chd1 maintain chromatin structure during transcription by preventing histone exchange. **Nature structural & molecular biology**, v. 19, n. 9, p. 884–892, 2012.

TAN, L.-R.; LIU, J.-J.; DEEWAN, A.; LEE, J. W.; XIA, P.-F.; RAO, C. V.; JIN, Y.-S.; WANG, S.-G. Genome-wide transcriptional regulation in *Saccharomyces cerevisiae* in response to carbon dioxide. **FEMS yeast research**, v. 22, n. 1, p. foac032, 2022.

TKACH, J. M. *et al.* Dissecting DNA damage response pathways by analysing protein localization and abundance changes during DNA replication stress. **Nature cell biology**, v. 14, n. 9, p. 966–976, 2012.

TOPALOĞLU, A.; ESEN, Ö.; TURANLI-YILDIZ, B.; ARSLAN, M.; ÇAKAR, Z. P. From *Saccharomyces cerevisiae* to Ethanol: Unlocking the Power of Evolutionary Engineering in Metabolic Engineering Applications. **Journal of Fungi**, v. 9, n. 10, 2023. Disponível em: <https://doi.org/10.3390/jof9100984>. Acesso em: 15 jan. 2024.

TRAN, H. G.; STEGER, D. J.; IYER, V. R.; JOHNSON, A. D. The chromo domain protein chd1p from budding yeast is an ATP-dependent chromatin-modifying factor. **The EMBO journal**, v. 19, n. 10, p. 2323–2331, 2000.

VAKIRLIS, N.; CARVUNIS, A.-R.; MCLYSAGHT, A. Synteny-based analyses indicate

that sequence divergence is not the main source of orphan genes. 2020. Disponível em: <https://doi.org/10.7554/eLife.53500>. Acesso em: 9 fev. 2024.

WALLACE-SALINAS, V.; BRINK, D. P.; AHRÉN, D.; GORWA-GRAUSLUND, M. F. Cell periphery-related proteins as major genomic targets behind the adaptive evolution of an industrial *Saccharomyces cerevisiae* strain to combined heat and hydrolysate stress. **BMC genomics**, v. 16, n. 1, p. 1–16, 2015.

WANG, X.; HOU, J.; BAO, X. The Absence of the Transcription Factor Yrr1p, Identified from Comparative Genome Profiling, Increased Vanillin Tolerance Due to Enhancements of ABC Transporters Expressing, rRNA Processing and Ribosome Biogenesis in *Saccharomyces cerevisiae*. **Frontiers in microbiology**, v. 8, p. 249679, 2017.

WEITAO, T. Multicellularity of a unicellular organism in response to DNA replication stress. **Research in microbiology**, v. 160, n. 1, p. 87–88, 2009.

WENGER, J. W.; PIOTROWSKI, J.; NAGARAJAN, S.; CHIOTTI, K.; SHERLOCK, G.; ROSENZWEIG, F. Hunger artists: yeast adapted to carbon limitation show trade-offs under carbon sufficiency. **PLoS genetics**, v. 7, n. 8, p. e1002202, 2011.

XIAO, H.; ZHAO, H. Genome-wide RNAi screen reveals the E3 SUMO-protein ligase gene SIZ1 as a novel determinant of furfural tolerance in *Saccharomyces cerevisiae*. **Biotechnology for biofuels**, v. 7, n. 1, p. 1–11, 2014.

XUE, S.; JONES, A. D.; SOUSA, L.; PIOTROWSKI, J.; JIN, M.; SARCS, C.; DALE, B. E.; BALAN, V. Water-soluble phenolic compounds produced from extractive ammonia pretreatment exerted binary inhibitory effects on yeast fermentation using synthetic hydrolysate. **PloS one**, v. 13, n. 3, p. e0194012, 2018.

CHAPTER 5 - UNVEILING THE POTENTIAL OF *Saccharomyces cerevisiae* PE2_H4_GN1: DEVELOPMENT, FERMENTATION PROFILE, METABOLOMICS, AND PROTEOMICS ANALYSIS

5.1 Abstract

Adaptive Laboratory Evolution (ALE) seeks to shape phenotypic characteristics in organisms through evolution and is becoming a great approach for improving strain phenotypes. This chapter explores the PE2_H4_GN1 strain, from its development to the unveiling of its genetic, fermentative, and molecular profiles. Employing population crosses, the development of PE2_H4_GN1 is detailed, revealing a genetic overlap with the evolved populations. Fermentation analyses demonstrated increased growth rates in the hydrolysate. Metabolomics reveals important extracellular and intracellular profiles, emphasizing ethanol production, furfural/HMF detoxification, glycerol production, variations in amino acids, polyamines, and central carbon metabolism. The proteomic data resulted in the analysis of enriched Gene Ontology (GO) terms, which suggest adaptive changes in ATP synthesis, amino acid biosynthesis, and cytoskeleton organization. Overall, these findings provide comprehensive information about the strategies and metabolic pathways utilized by strain PE-2H4_GN1 to adapt to the lignocellulosic hydrolysate optimally.

5.2 Introduction

Adaptive Laboratory Evolution (ALE) uses evolutionary pressure to create specific phenotypic characteristics in organisms. For example, ALE has been used to make *S. cerevisiae* more tolerant to furfural. This was achieved by converting furfural into less toxic compounds (HEER; HEINE; SAUER, 2009; LIN; QIAO; YUAN, 2009). In addition to this characteristic, the metabolome of evolved strains can help understand the tolerance mechanisms. In yeast that has evolved to tolerate LCH, metabolites such as alanine, GABA, and glycerol have been shown to play important roles (WANG *et al.*, 2013). However, it is important to remember that yeast metabolism is regulated at a global level, following the principles of mass and energy conservation (PATIL *et al.*, 2005). This means that when evaluating the effects of specific compounds, a bigger picture must be considered.

This chapter details the fundamental characteristics of the PE2_H4_GN1 strain, which resulted from crosses between the final evolved strains of the experiments from ALE1. This chapter aims to unveil the genetic and fermentative profile that characterizes its behavior under different conditions and conduct a comprehensive metabolomics and proteomics analysis. The first part of this chapter elucidates the development of the PE2_H4_GN1 strain, outlining the strategies used, such as population intercrossing and colony selection, culminating in the identification of PE2_H4_GN1 and subsequent analysis of its interaction with the XylA strain.

Next, the fermentative profile is understood, detailing the bioreactor configurations, strains, culture media, strain growth rate, and CO₂ production. In addition, extracellular metabolites contribute to unveiling ethanol yield, global ethanol productivity, HMF and Furfural detoxification, and other metabolites such as glycerol. When investigating the metabolism, amino acid profiles, polyamine production, and the dynamics of central carbon metabolism were explored, with an emphasis on the Pentose Phosphate Pathway (PPP), glycolysis, Tricarboxylic Acid Cycle (TCA), cofactors, and nucleotides.

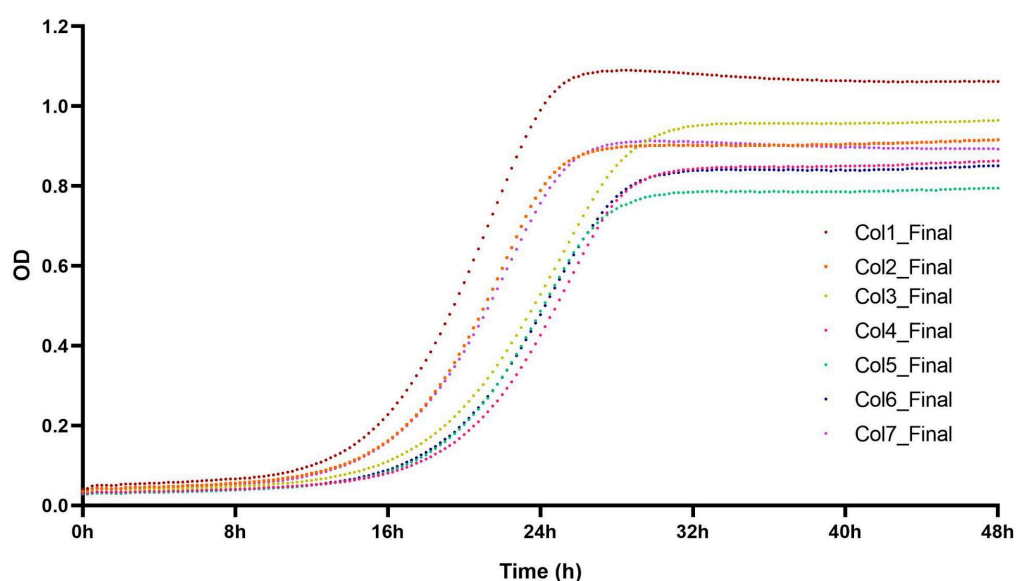
Proteomic analyses revealed differential expression of proteins, where it is possible to visualize gene ontology (GO) terms positively or negatively enriched in Biological Process, Molecular Function, and Cellular Component aspects. Each section of this chapter is structured to provide insights into the LCH tolerance that the

PE2_H4_GN1 strain exhibits and contributes significantly to the understanding of this promising strain.

From the PE2_H4_GNpop (population), we selected one colony with the best growth in 55% LCH. The population was plated on 60% LCH and 7 of the largest

colonies after 72h incubation were grown in 55% LCH to stationary phase for pre-growth. Then, about 2×10^7 cells were pre-inoculated for 24 hours in 15 ml of 55% LCH, for adaptation. Cell number counting was carried out by flow cytometry. The cells were diluted to a concentration of 40,000 cells/ul. When assembling the microplate, each well contained 200uL of 55% LCH or rich medium (YPD). The inoculum was approximately 200,000 cells per well and growths were runned in sextuplicate. Furthermore, two wells were used as blanks for the 55% hydrolysate and YPD. The cultivation conditions were 28°C and shaking every 3 minutes. Readings were taken every 15 minutes at OD 600 nm on the Tecan Sunrise® microplate reader. The experiments were set up to avoid biases (AULD *et al.*, 2004). The parameter we used to interpret the data was optical density as a function of time; the selected colony was number 1 (PE2_H4_GN1) (Fig. 5.2).

Figure 5.2 - Characterization of selected PE2_H4_GN strains.



The graph demonstrates the optical density values as a function of time. Colony 1 had the shortest Lag phase, with the best growth being selected for further analyses.

5.3.1.2.1 Sequencing Methods

The DNA of the PE2_H4_GN1 clone was sequenced using 2 x 151 PB sequencing on the Illumina NextSeq1000 platform, with libraries prepared with Illumina DNA Prep™ Kit for Illumina.

5.3.1.2.2 WGS Data Analysis

To identify mutations (SNPs and InDels) reads from the clone were filtered by size selection (>50 bases) and overall quality scores. Mapping to the PE2_H4 (or referred to as wild-type) reference genomes was performed by the reference mapping tools built into CLC Genomics Workbench 8.0. Identification of SNPs/InDels was performed directly from read mappings generated by the CLC Genomics Workbench using variant detection algorithms within the CLC package. A minimum coverage setting of 10x and variant frequency of at least 20% of reads were observed for calling variants from sequencing strains.

5.3.1.3 PE2_H4_GNpop x XylA Cross

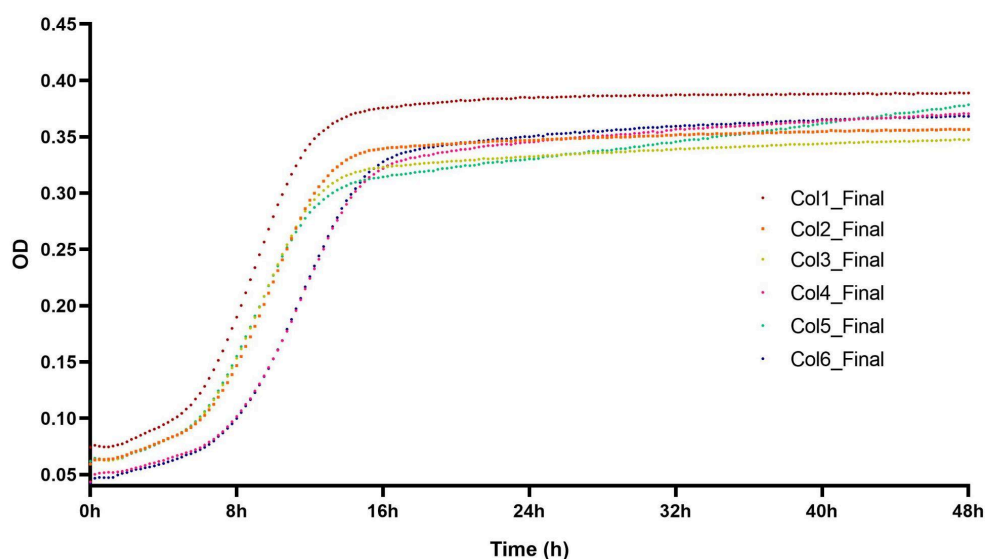
In addition to the obstacles posed by toxic compounds derived from lignocellulose, for 2G ethanol to operate on an industrial scale, other technical obstacles still need to be overcome. *S. cerevisiae* is unable to metabolize pentoses, which comprise an economically significant fraction of sugars released from biomass (BASSO *et al.*, 2013; DOS SANTOS *et al.*, 2016). Therefore, we crossed the PE2_H4_GNpop strain with XylA (resistant to hygromycin), an *S. cerevisiae* strain that contains xylose isomerase, allowing the strain to consume xylose.

For this, the two strains grew up to the exponential phase and were sporulated. Then, the populations germinated together, and each pair germinated in the combination of the two respective antibiotics: GH (geneticin/hygromycin), and NH (nourseothricin/hygromycin). After germination, 2 growth cycles (Yeast Nitrogen Base YNB + 3% xylose) occurred before putting it through the sporulation protocol. The next sporulation cycle occurred in a single vial containing the strains with the antibiotics marks, geneticin and nourseothricin, thus eliminating hygromycin. Two cycles of growth in a selective medium (YNB + 3% xylose) were carried out before starting a series of 10 transfers in 55% LCH (in YNB and sufficient quantity to reach 3% of xylose).

5.3.1.4 Selection of the best PE2_GN_XylA colony

The method used was the same as described in the previous section, 200,000 cells in 200uL of 55% LCH. The parameter used to interpret the data was the optical density as a function of time; and selected the best colony on this condition (colony number 1) (Fig. 5.3).

Figure 5.3 - Characterization of selected PE2_GN_XylA strains.



The graph demonstrates the optical density values as a function of time. Colony 1 had the shortest Lag phase, with the most significant growth selected for further analysis (PE2_GN_XylA1).

5.3.2 Fermentation Experiments

5.3.2.1 Bioreactor setup, strains, and media

Sixteen fermentations were carried out in an INFORS HT 0.75 L bioreactor with *Saccharomyces cerevisiae* PE2_H4_GN1 clone, PE2_H4_GNpop, and PE2_H4 (WT) (Table 5.1). The strains were previously cultivated in sterile 50 ml tubes until they reached an optical density of 0.7. Inoculation took place in a 0.75 L bioreactor (useful volume of 0.18–0.5 L) with 250 ml of culture medium (YPD) with or without synthetic hydrolysate (LCH) (Table 5.2), with an initial optical density of approximately 0.1. Air was injected into the upper part (gas mixture with 21% oxygen) at a flow rate of 30 ml/min via automatic control. Stirring was maintained at 100 rpm, the pH was stabilized at 5.5 with a controlled addition of 2N sodium hydroxide, and the temperature was controlled at 28 °C.

Table 5.1: *Saccharomyces cerevisiae* PE2_H4 Fermentations in INFORS HT 0.75 L bioreactor.

N°	Strain	Replicates
1	<i>S. cerevisiae</i> PE2_H4_GN1	3
2	<i>S. cerevisiae</i> PE2_H4_HphKan_MX	3
3	<i>S. cerevisiae</i> PE2_H4_GN_pop	2

Table 5.2: Components of the synthetic hydrolysate used for fermentations.

Component	Concentration mM	Concentration g/L
Formic acid	35.3	1,62
Acetic Acid	129,6	7,776
Vanillic Acid	0,757	0,1273
p-coumaric acid	4,581	0,7515
Furfural	20	1,9216
Hydroxymethylfurfural	20	2,5222
Glucose	111,02	20
Yeast extract	-	10
Bacto Peptone	-	20

5.3.2.2 Microplate Growth Assay in Acetic Acid

The PE2_H4_GN1 strain was subjected to microplate growth assay. This experiment was carried out using the Thermo Scientific™ Varioskan™ LUX multimode microplate reader and the growths were made in sextuplicates. It was tested 4 concentrations of Acetic Acid (0%, 50%, 70%, 100%, and 130% of original LCH) and started OD600 0.1. The concentrations were worked considering 100% of

the acetic acid concentration present in the original hydrolysate (where the evolution experiment was carried out) (Table 5.3), and all concentrations were in a YPD medium.

Table 5.3: Acetic Acid concentrations.

Acetic Acid Relative Concentration	Acetic Acid Concentration (g/L)	Acetic Acid Concentration (mM)
0%	0	0
50%	2,994	49,820
70%	4,191	69,748
100%	5,983	99,64mM
130%	7,778	129,52 mM

5.3.3 Extracellular Metabolites

Supernatant samples were collected at specific points during the fermentations. Collections included samples at the beginning (time 0), the end of the experiment, and at points in the lag and exponential phases of microbial growth. For the detection of aromatics/phenols, such as protocatechuic acid, vanillin, p-coumaric acid, benzoate, 5-hydroxymethylfurfural, furfural, and 4-hydroxybenzoic acid, the method used was with two distinct mobile phases: Mobile Phase A, composed of 0.1 % formic acid in MilliQ water, and Mobile Phase B, consisting of 0.1% formic acid in acetonitrile. Analyzes were conducted using the Thermo Scientific DUO UHPLC system equipped with a DAD detector (HPLC Duo 1), operating exclusively in DAD mode. The column used was the Waters Acquity HSS T3, with dimensions of 100 x 2.1 mm and particles of 1.8 μm (Waters part number 186003539). The detector was adjusted to capture UV in the ranges of 280 nm, 320 nm, and 360 nm.

For the detection of organic acids such as Maltose, Glucose, Succinate, Lactate, Formate, Acetate, Propionate, Ethanol, and Glycerol, the analytical method used the Thermo Vanquish Duo HPLC system, equipped with DAD and RI detectors. Two columns were used in the analyses, the first being an Agilent HiPlex H (H+ 8%,

300 x 7.7 mm, PN: PL1170-6830), and the second being a Phenomenex Rezex RHM Monosaccharide (H+ 8%, 300 x 7.8 mm, PN: 00H-0132-K0). The detectors included a Refractive Index Detector (RID) operating in positive polarity at 40°C. The experimental conditions were established with a column temperature of 65 °C, an injection volume of 25 µL, and a mobile phase composed of a 4 mM H₂SO₄ solution. The flow rate was maintained at 0.6 mL/min, and the run time was isocratic for 26 minutes.

5.3.4 Intracellular Metabolites

To measure intracellular metabolites, samples were collected during fermentations between OD₆₀₀ of 0.8 to 1. After quenching in methanol, followed by pellet resuspension and extraction, the metabolites were subjected to a biphasic separation with methyl tert-butyl ether (MTBE). The fractions were evaporated and reconstituted, undergoing a process of freezing, centrifugation, and freeze-drying. Specific methods were selected for quantifying amino acids, polyamines, and Central Carbon Metabolism (CCM), using the Thermo Scientific Vanquish Core U/HPLC system in conjunction with conditional methodologies for each of these categories. The HPLC was controlled by Chromeleon 6.80 software (ThermoFisher). The mass spectrometer was controlled by Analyst 1.6.3 software (AB Sciex). The collected data were processed using MultiQuant 2.1 (AB Sciex).

An Agilent Zorbax Extend C-18 column (3.5 µm, 4.6 x 150 mm) with a Phenomenex SecurityGuard Gemini C18 guard was used in the amino acid method. Two detectors were used: FLD 1 for amino acids derivatized with OPA and FLD 2 for derivatized with FMOC. The separation of the derivatized amino acids occurred on a mobile phase B gradient column over 27 minutes, at a temperature of 37 °C and a flow rate of 2 mL/min. Detection was performed in two distinct time windows: 340ex/450em nm for amino acids derivatized with OPA (1-18 min) and 266ex/305em nm for amino acids derivatized with FMOC (18-20 min). Amino acid quantification is based on standard curves derived from serial dilutions of a mixed standard, with limits of quantification between 1.95 and 500 µM.

In the method that uses LC-MS/MS for the analysis of putrescine and cadaverine, the Shimadzu shim-pack scepter PFPP-120 3µm, 2.1 x 150mm column was used with a temperature of 45 °C. The mobile phase consisted of 0.1% heptafluorobutyric acid and 0.05% formic acid in water as Solvent A and 0.1% formic

acid in acetonitrile as Solvent B. The gradient was varied from 2% to 98% B in 11 min and 300 $\mu\text{L}/\text{min}$. In MS, the precursor ions and products were set to putrescine (m/z 89.2) and cadaverine (m/z 103.1). The quantitative ions are 72.2 and 86.2, respectively. The auxiliary gas temperature was maintained at 300 $^{\circ}\text{C}$.

For the central carbon metabolism method, liquid chromatography was performed using a 50 min gradient, with a flow rate of 300 $\mu\text{L}/\text{min}$, on a Phenomenex Gemini-NX C18 column (150 $\times$ 2 mm, 3 μm , 110 Å), with guard (SecurityGuard Gemini-NX C18, 4 $\times$ 2 mm) and column temperature of 55 $^{\circ}\text{C}$. The mobile phases used were: 7.5 mM aqueous tributylamine (Sigma-Aldrich) with pH adjusted to 4.95 (± 0.05) using acetic acid (Labscan) for Solvent A and acetonitrile (Merck) for Solvent B. They were injected with 10 μL for analysis. Hardware parameters include: ion spray voltage -4500 V, ion source nebulizer (GS1), auxiliary ion source (GS2), curtain (CUR), and collision gases (CAD) were 60, 60, 20, and averages (arbitrary units), respectively, using an internal ultra-high purity liquid nitrogen (BOC) tank. The auxiliary gas temperature was maintained at 350 $^{\circ}\text{C}$. The quantities obtained for each detected metabolite were based on standard curves of serial dilutions of analytical standards, purchased from Sigma, where L1 = 200,000, L2 = 100,000, L3 = 50,000, L20 = 0.38 nM. This number of standard dilutions ensures that standard curves consist of at least five data points. The master mix and pooled samples were injected regularly throughout the run sequence for quality control.

5.3.4.1 Data analysis

The data were processed by normalizing concentration (nM) by biomass, then biological triplicates were averaged and ratios of averages were reported as Log2 fold changes (BAZURTO *et al.*, 2018). Fold changes greater than 2 ($\log_2 > 1$) or less than 0.5 ($\log_2 < -1$) are considered significantly different from 1 and in the graphs are identified by the dotted red line.

5.3.5 Proteomics

Triplicates of fermentations of strains PE2_H4_GN1 and PE2_H4 were subjected to proteomic analysis, in both YPD and synthetic hydrolysate culture media. This experimental design resulted in a total of 12 samples, representing a comprehensive approach to proteomic dynamics under the two cultivation conditions.

Additionally, two specific strains, PE2_GN_XylA1 (test) and Xyl_A (control), which were cultivated in real hydrolysate plus xylose, were included in the proteomic study. This experiment aimed to evaluate the differences between the xylose-consuming strain and the strain that, in addition to consuming xylose, also presents tolerance to the hydrolysate.

Protein samples were collected during fermentations with OD between 0.8 and 1. Cells were centrifuged, the pellet was stored, and the cells were washed and lysed. After reduction with DTT and heating, cysteines were alkylated with indole acetic acid (IAA). The acidified lysate was treated with phosphoric acid and S-Trap buffer, followed by centrifugation. After washing, enzymatic digestion was performed with trypsin. The peptides were eluted with acetonitrile and formic acid, dried, and resuspended for HPLC analysis. This process allows data to be obtained on the protein composition of the samples.

In the mass spectrometry analysis, filters were applied to ensure the quality of protein identifications, this included a cutoff of 0.2 for PEP (precursor-level posterior probability), 0.01 for Qvalue at Experiment Level, and 0.05 for Qvalue at Racing Level. Proteins were defined based on simplified sequences avoiding redundancies. Decoy proteins were generated to evaluate false discovery rates (FDR).

In this method, a target FDR of 0.01 was used for both mass spectrometry (MS1 and MS2) data. Furthermore, a minimum comparison threshold for templates was established at 0.25, contributing to a safe selection of models in the analysis.

Although ratios provide an intuitive measure of expression changes, they have the disadvantage of treating up- and down-regulated genes differently. Genes upregulated by a factor of 2 have an expression ratio of 2, while those downregulated by the same factor have an expression ratio of (-0.5) (QUACKENBUSH, 2002). The most widely used alternative ratio transformation is the base 2 logarithm, which has the advantage of producing a continuous spectrum of values and treating up-and-down-regulated genes similarly. The logarithms of the expression ratios, which are the format treated in this study, are also treated symmetrically so that a gene positively regulated by a factor of 2 has a $\log_2(\text{ratio})$ of 1, a gene negatively regulated by a factor of 2 has a $\log_2(\text{ratio})$ of -1 and a gene expressed at a constant level (with a ratio of 1) has a $\log_2(\text{ratio})$ of zero (QUACKENBUSH, 2002). In the remainder of this discussion, $\log_2(\text{ratio})$ will be used to represent expression levels.

5.3.5.1 Gene Ontology Enrichment

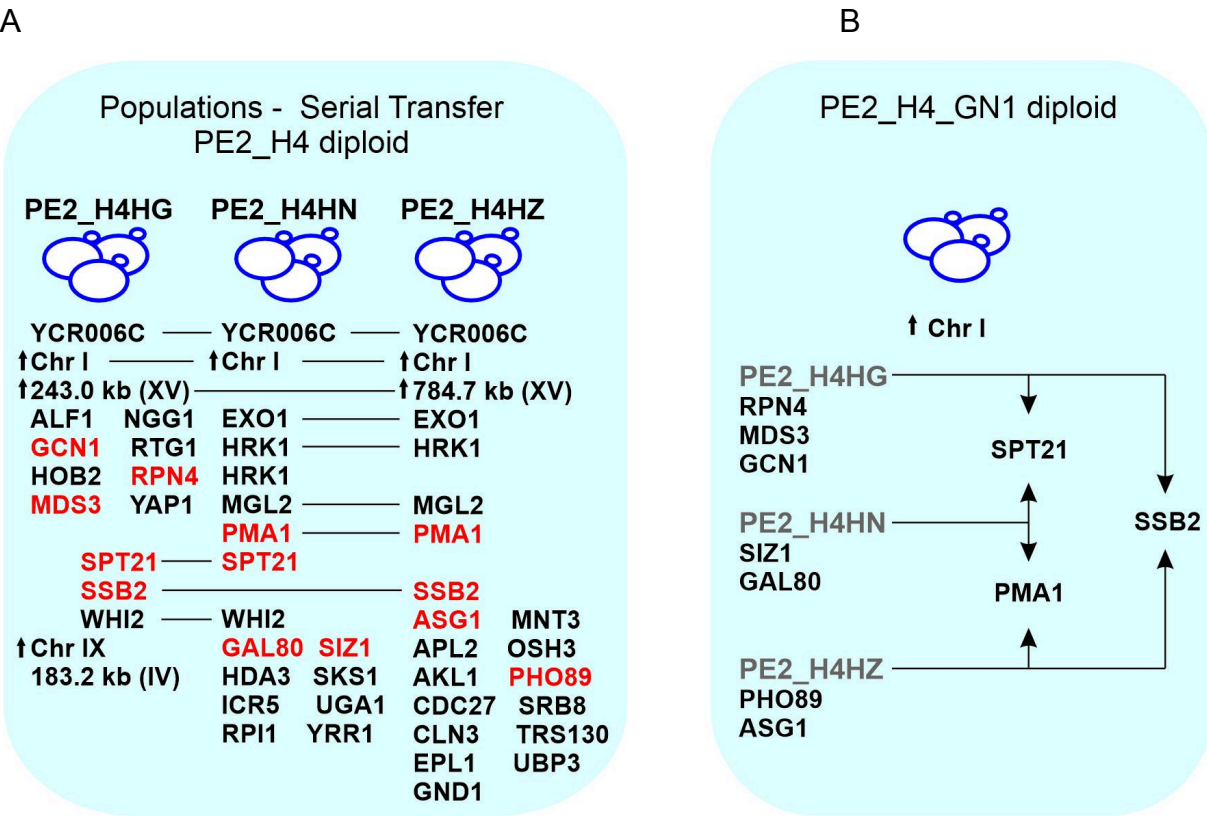
To perform GO enrichment the custom set of IDs itself was used to determine which IDs are orthologous to the genes in the annotation used for Yeast8 and then the set was mapped using the best bidirectional hits (LU *et al.*, 2019), the codes used were stored on GitHub (https://github.com/yasminealvesm/PE2_H4_GN1proteomics.git).

5.4 Results and Discussion

5.4.1 Unraveling Evolutionary Diversity: Sequencing the PE2_H4_GN1

During the sequencing analysis of the PE2_H4_GN1 clone, the genetic overlap of the three evolved lineages that gave rise to the formation of PE2_H4_GN1 was identified. Genes in common include *RPN4*, *MDS3*, *GCN1*, *SIZ1*, *GAL80*, *PHO89*, *ASG1*, *SPT21*, *PMA1*, and *SSB2* (Figure 5.4).

Figure 5.4 - Identified Mutations in ALE1 Populations and the Descendant, PE2_H4_GN1 Clone Strain.

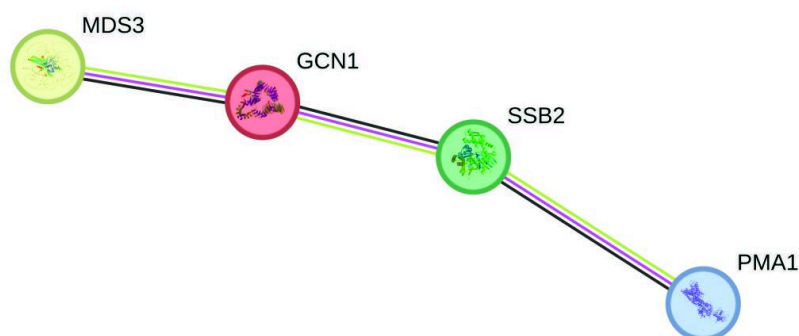


A. Modifications identified in whole genome sequencing (WGS) of the three distinct evolved populations. B. Shared mutations in the developed strain PE2_H4_GN1. PE2_H4_GN1 mutations shared among more than one population are connected by arrows. The mutations found in the clone PE2_H4_GN1 are highlighted in red in the populations panel (A), and connected with lines if present in more than one population.

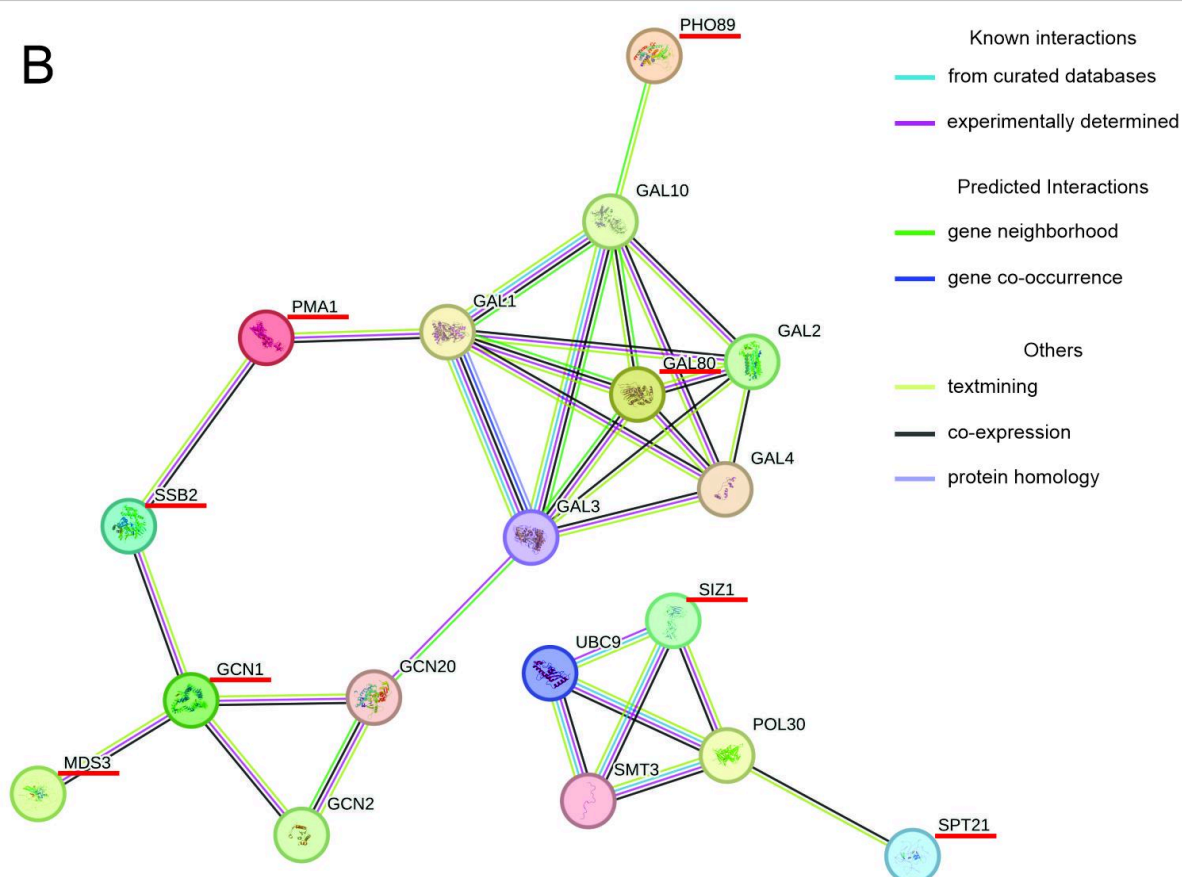
Computational analysis performed by the STRING software revealed a specific interaction between the *MDS3*, *PMA1*, *GCN1*, and *SSB2* genes (Figure 5.5A) (SZKLARCZYK *et al.*, 2023). These possible connections suggest shared functional links and signaling pathways between these genes. With a more comprehensive coverage, it was possible to make connections between other genes, which can be seen in Figure 5.5B.

Figure 5.5 - Networks of Protein Interactions Related to Genes Identified during Sequencing of the PE2_H4_GN1.

A



B



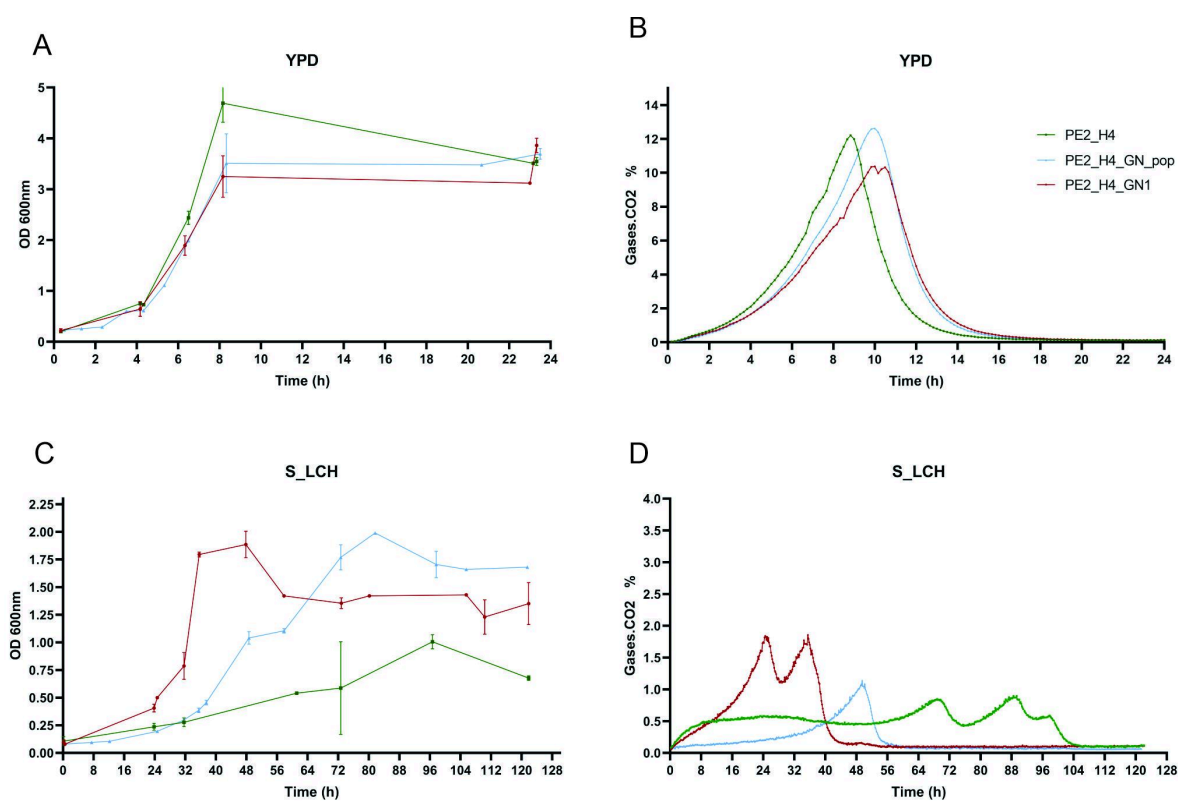
A. Four proteins demonstrated direct interactions as detected by computational analysis using STRING software: *MDS3*, *PMA1*, *GCN1*, and *SSB2*. B. To expand the understanding of the pathways, an increase in the network of connections is presented. To this extent, the figure includes other proteins associated with the genes initially mentioned, and others, visualizing potential communication and regulation pathways between them. Target proteins are underlined in red. Each connection color represents a type of relationship (SZKLARCZYK *et al.*, 2023).

5.4.2 Fermentation: Understanding Strains Metabolisms

5.4.2.1 Bioreactor Outcome

The performance of the PE2_H4_GN1 and PE2_H4_GNpop in comparison to the PE2_H4 WT is revealed by the fermentation data (Figure 5.6). Rich medium (YPD) and synthetic hydrolysate (LCH) were the two distinct conditions under which sets of fermentations were conducted in triplicate (PE2_H4_GN1 - red; PE2_H4 WT - green) or duplicate (PE2_H4_GNpop - blue).

Figure 5.6 - Dynamics of Growth and Metabolism in YPD and LCH Media.



A. Shows cell growth over time in YPD medium, measured by optical density (OD). B. Production of carbon dioxide (CO₂) during cultivation in YPD medium. C. Represents cell growth in LCH, measured by optical density (OD). D. CO₂ production during cultivation in LCH. The data are presented as the mean of triplicates (PE2_H4 and PE2_H4_GN1) or duplicates (PE2_H4_GNpop).

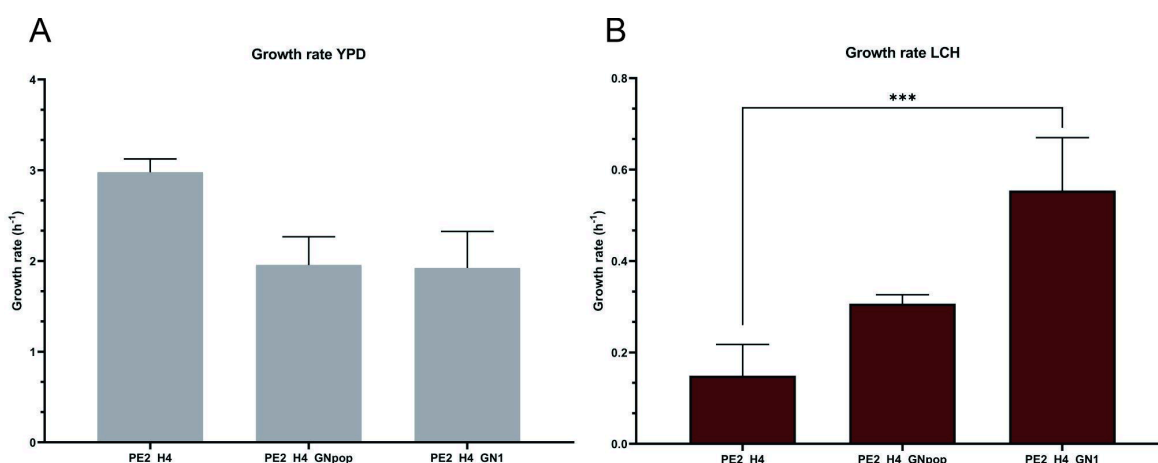
However, minor fluctuations in the optical density (OD) of the inoculum have affected the OD graph. Thus, the growth rate is a key parameter in the analysis of microbial growth and can be calculated using the formula:

$$\text{Growth rate} = (\ln(\text{OD}_{\text{final}} / \text{OD}_{\text{start}})) / \Delta t$$

More details about this approach can be found in Chapter 4.

The growth rate of the WT was better than the evolved (population and clone) strains in YPD, while the evolved were better than the WT in LCH, with the clone having better performance than the population (Figure 5.7)

Figure 5.7 - Comparison of Growth Rates between Strains PE2_H4_GN1, PE2_H4_GNpop, and PE2_H4 WT in Different Growth Conditions.



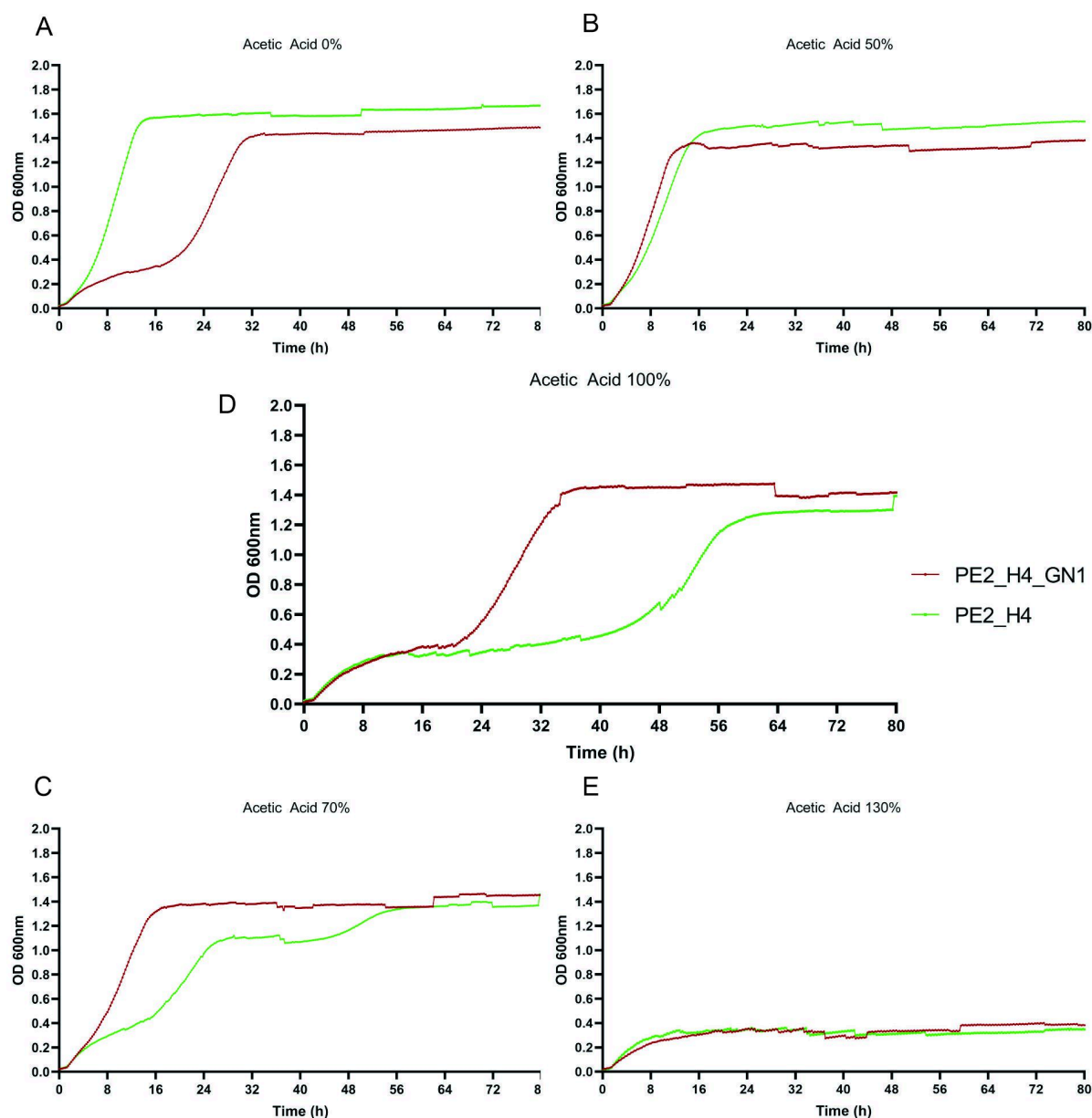
A. In the YPD medium, it is observed that the wild-type strain (WT) presents a no-significative higher growth rate than the evolved strains. B. On the other hand, in the hydrolysate, the growth rate of the PE2_H4_GN1 clone is higher than that of the wild-type strain (WT), indicating a change in the growth dynamics. The error bars represent the standard deviation, and the statistical analysis was conducted using One-way ANOVA, followed by Dunnett's test for multiple comparisons. *** p<0.005.

5.4.2.2 Microplate Growth Assay

To further explore the toxicity of the inhibitors, PE2_H4_GN1 was challenged with acetic acid only inhibitor (Figure 5.8). The results, with emphasis on the relative 100% acetic acid (Table 5.3), revealed the disparity in the growth curve between the

two strains, showing a notable advantage of approximately 20h in the lag phase. This observation suggests a marked ability of the PE2_H4_GN1 clone to adapt and grow more efficiently in an acetic acid-containing environment.

Figure 5.8 - PE2_H4_GN1 growth curves in Different Concentrations of Acetic Acid.



A. 0%. B. 50%. C. 70%. D. 100%. E. 130%. At low concentrations, PE2_H4_GN1 showed a trade-off, showing a worse growth than the WT. However, when the concentration was equal to or greater than 70%, the evolved strain demonstrated faster growth, with emphasis on the 100% concentration which shows a lag phase of

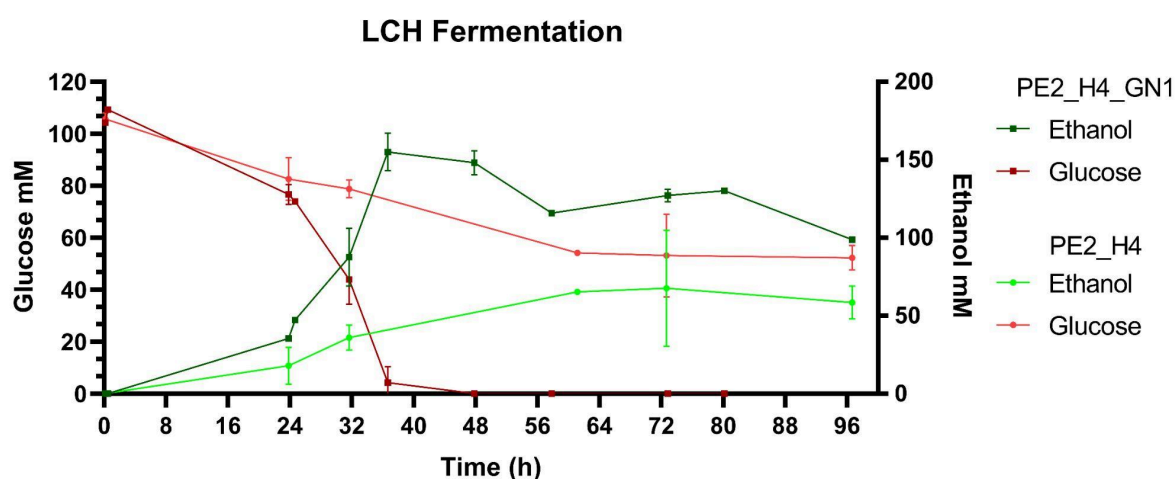
approximately 20h, while the WT is approximately 40h. At 130% Neither strain was able to grow. Green line is the WT strain and in red is PE2_H4_GN1.

5.4.3 Extracellular Metabolites

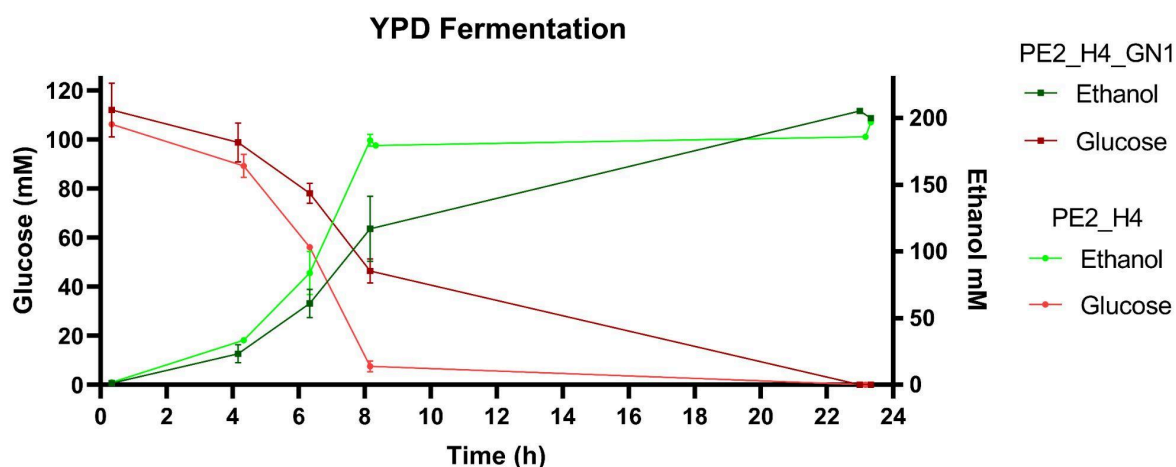
The investigation of the extracellular metabolites of the PE2_H4_GN1 strain can be analyzed in the graphs below. The focus on the parameters of ethanol production and glucose consumption in the YPD medium and the hydrolysate aims to understand the fermentative productivity adopted by the strain (Figure 5.9). The focus on the parameters of hydroxymethylfurfural (HMF), and furfural conversion in less toxic components aims to understand the PE2_H4_GN1 detoxification strategy.

Figure 5.9 - Glucose consumption and ethanol production of PE2_H4_GN1 and PE2_H4 WT strains in LCH and YPD.

A



B

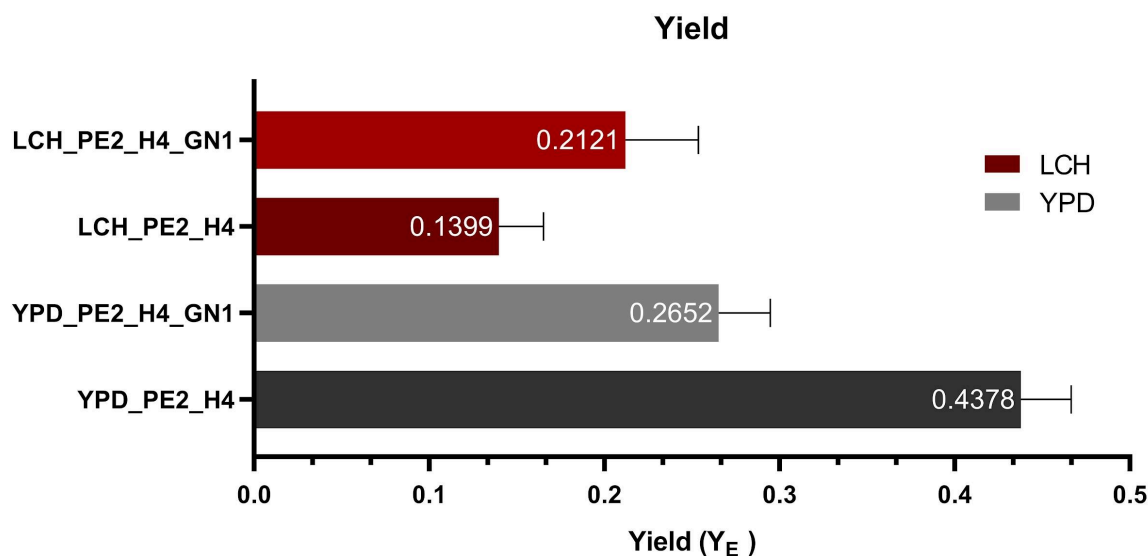


A. The performance of the evolved strain and the WT strain in LCH is observed. The PE2_H4_GN1 strain demonstrated remarkable efficiency, consuming all glucose and reaching the maximum ethanol concentration (~150mM) in less than 40 hours. Meanwhile, the WT strain maintained approximately half the glucose concentration until the end of cultivation (96 hours), reaching the ethanol peak at 72h (~67 mM). B. Fermentation in YPD. The fermentation profiles of the PE2_H4_GN1 and WT strains are illustrated, in this scenario, the evolved and WT strains showed comparable behaviors, but it is still possible to perceive the trade-off of the evolved strain.

It was observed that the PE2_H4_GN1 strain showed better performance in OD, faster glucose consumption, and higher ethanol production than the PE2_H4 WT in LCH media. In the YPD medium, PE2_H4_GN1 showed a lower growth rate, slower glucose consumption, and lower ethanol production when compared to the PE2_H4 WT. This result suggests that the strain evolved to have advantages in specific hydrolysate conditions, indicating a trade-off landscape.

The analysis of the relative ethanol yield factor ($Y'_{ethanol/S}$) of the PE2_H4_GN1 strain, both in YPD and LCH medium was calculated as the ratio between the ethanol produced (g/L) and the initial concentration of sugars (g/L) (PEREIRA *et al.*, 2021) (Figure 5.10).

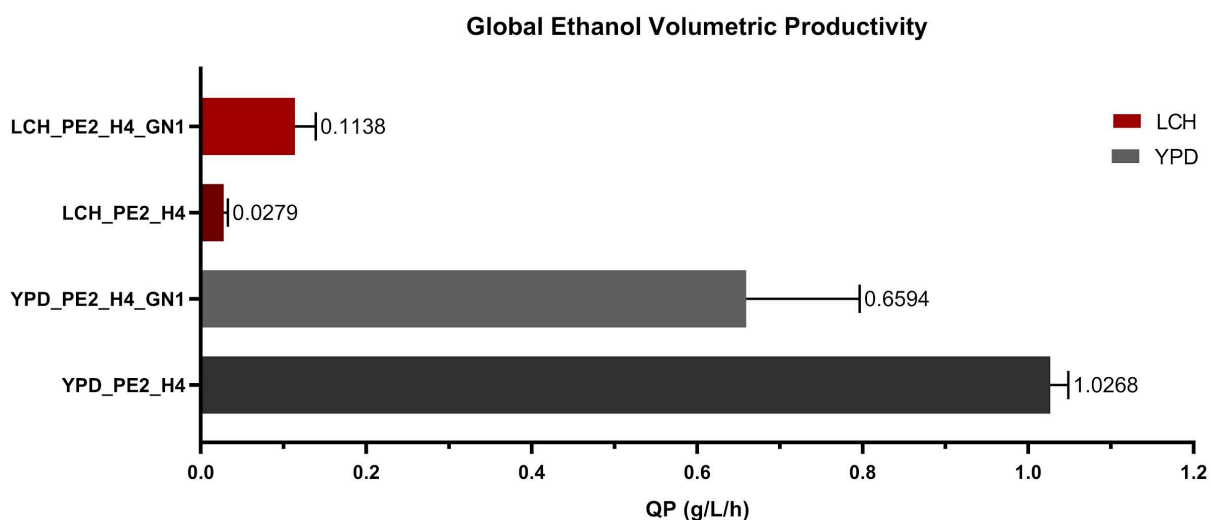
Figure 5.10 - Comparison of Yield in LCH and YPD Media.



The graph highlights the yield in the LCH medium (represented in red) and YPD medium (in gray). In LCH the yield of the evolved strain reached 0.2121, while in the WT was 0.1399. In YPD the yield of the evolved strain reached 0.2652, while the WT reached 0.4378. In this way, it is possible to see that the evolved strain presents a trade-off in YPD, but manages to achieve a higher yield at a high concentration of hydrolysate.

Global ethanol volumetric productivity (Q_P , g ethanol/L/h) was calculated as the ratio between the ethanol titer (g/L) and the respective fermentation time (h) (PEREIRA *et al.*, 2021). The evaluation of these conditions seeks to deeply understand the effects of inhibitors on fermentation, analyzing the ethanol production and the time spent on the fermentation (Figure 5.11).

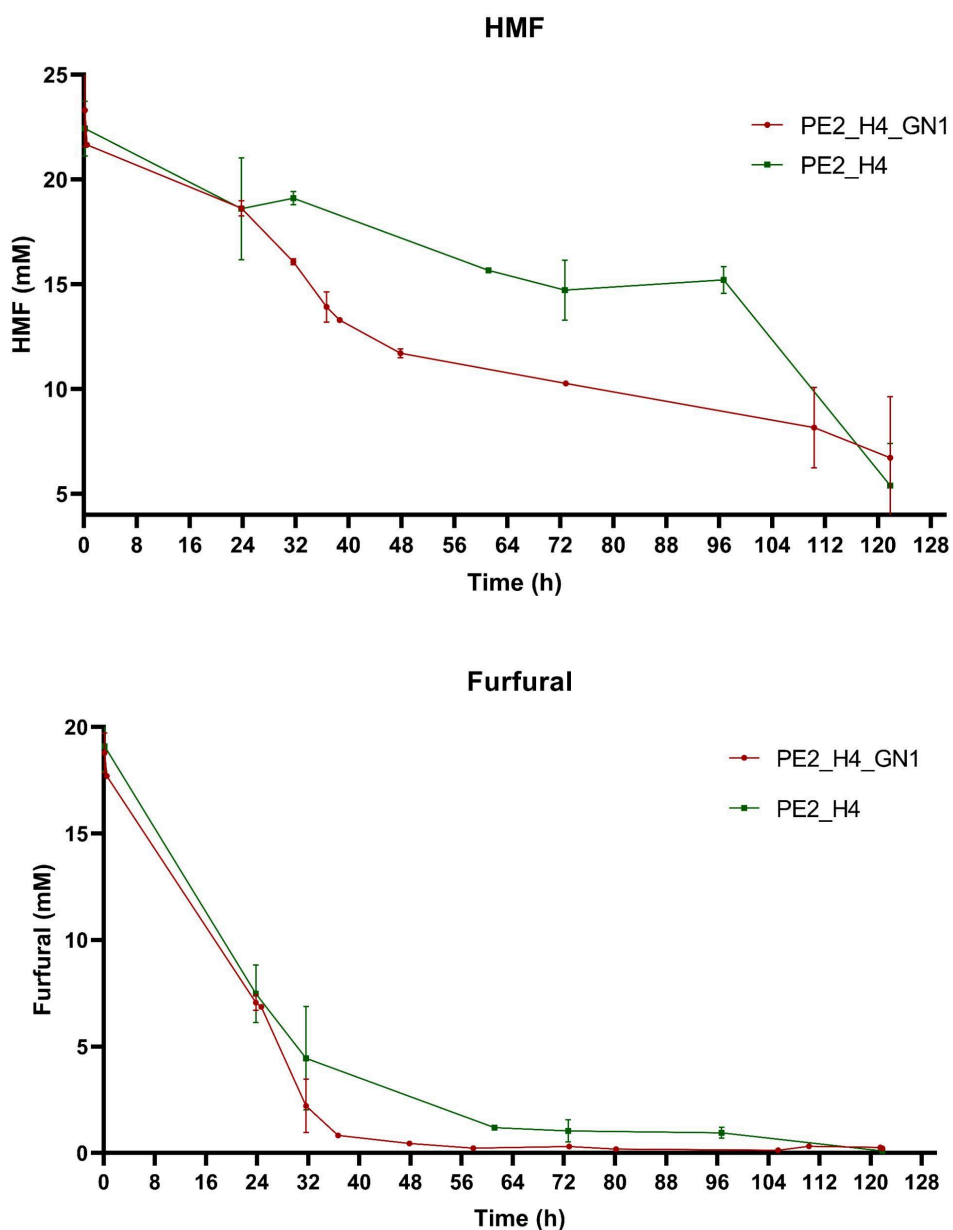
Figure 5.11 - Global Volumetric Ethanol Productivity.



The overall volumetric ethanol productivity (QP, g ethanol/L/h) in the synthetic hydrolysate was much lower than that in YPD, however, in LCH, the PE2_H4_GN1 yeast had a QP 4x higher than the WT. In red is the QP in LCH and grey is the QP in YPD.

In addition to these compounds, it is important to analyze the temporal dynamics of HMF and furfural concentrations during fermentation. This is because *S. cerevisiae* can convert these toxic compounds into less toxic derivatives (Figure 5.12). The detoxification of these inhibitors (furfural to furan methanol (FM), and HMF to furan di-methanol (FDM)) involves the reduction of aldehydes, a process that consumes NAD(P)H.

Figure 5.12 - Detoxification of HMF and Furfural in PE2_H4_GN1 and WT Strains during Fermentation.



B

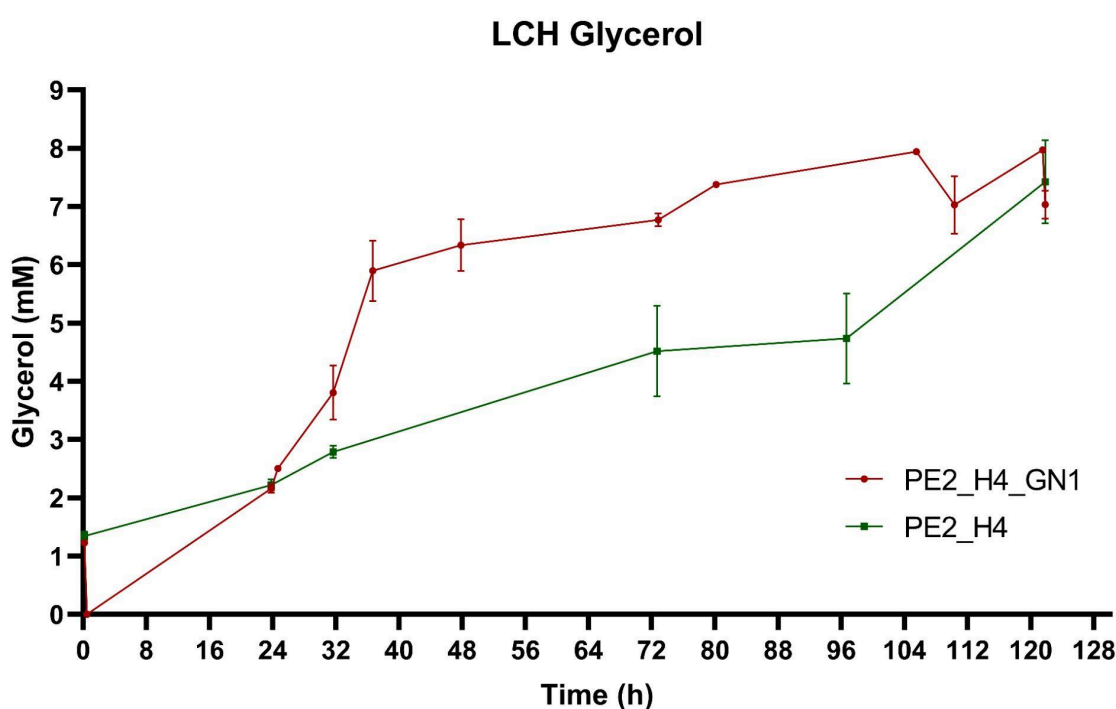
A. Detoxification of HMF. B. Detoxification of HMF.

It is worth mentioning that HMF detoxification was slightly slower in the WT, which remained above 15mM until 96h of fermentation, while the evolved strain dropped to 15mM in the first 40h. This analysis illustrates the ability of the strains to deal with different inhibitors during fermentation. In the Furfural analysis, both strains started detoxification efficiently, but after 50h the evolved strain managed to bring the concentration of Furfural to zero, whereas in the WT fermentation, the furfural was

still detectable until 96h.

Besides detoxification, the cell needs to maintain a healthy redox balance to ensure the proper functioning of its metabolic pathways. Analyzing glycerol production is crucial as it serves as a vital metabolite in the process, acting as a natural redox neutralizer. Glycerol allows the regeneration of NAD⁺ (Nicotinamide Adenine Dinucleotide) and is produced from glucose. Its levels increase in response to osmotic stress, contributing to the overall dynamics of the metabolic pathway (MUTTON *et al.*, 2019). The glycerol production over time can be analyzed in Figure 5.13.

Figure 5.13 - Glycerol accumulation. The graph displays the temporal accumulation of glycerol concentration during LCH fermentation.



The analysis of glycerol concentration helps to understand the metabolic response of cells to stressful conditions.

5.4.4 Intracellular Metabolites

The concentration of detected metabolites was normalized by the biomass present in the sample and represented accordingly (uM/ug or uM/g)

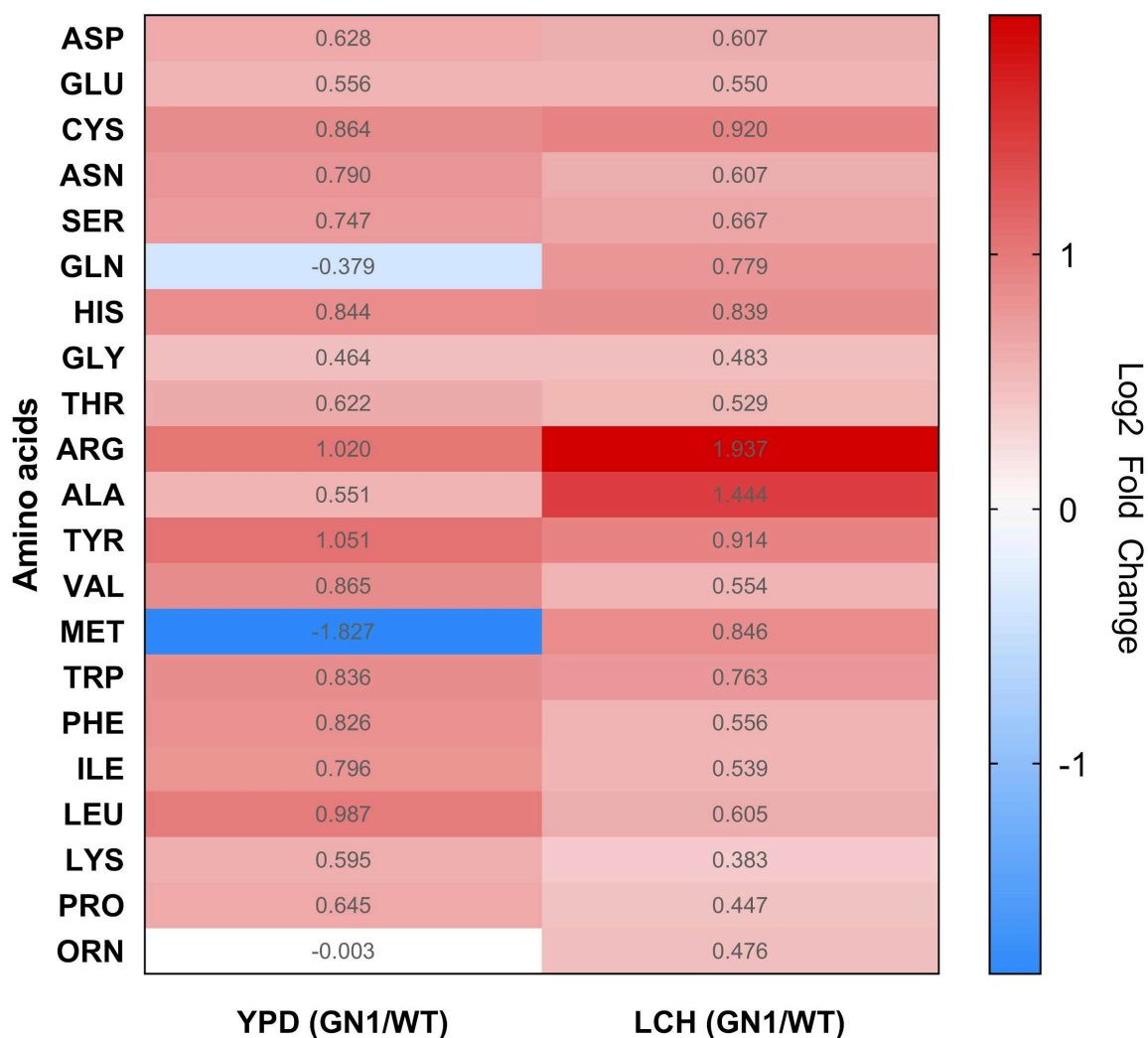
5.4.4.1 Amino acids

The amino acids heatmap for the PE2_H4_GN1 strain in YPD and LCH conditions displays normalized values relative to the control (ratio between the PE2_H4_GN1 values and the corresponding WT values).

This normalization approach provides a clear view of PE2_H4_GN1 strain-specific variations, eliminating the influence of basal protein expression present in the WT. The heatmap with individual values can be viewed in Appendix A, Section 7, Figure 15.

When looking at the overall heatmap of amino acid metabolism, some amino acids were lower in abundance under YPD conditions (glutamine and methionine). This corroborates with the results of fermentation in YPD, as methionine plays a fundamental role in promoting growth in *Saccharomyces cerevisiae* (SUTTER *et al.*, 2013; WALVEKAR *et al.*, 2018). Also, arginine and alanine are up represented in the experiment with LCH, suggesting some role in the mechanism of tolerance for these strains (Figure 5.14) (JUNG *et al.*, 2017).

Figure 5.14 - Intracellular amino acids in the PE2_H4_GN1 strain.

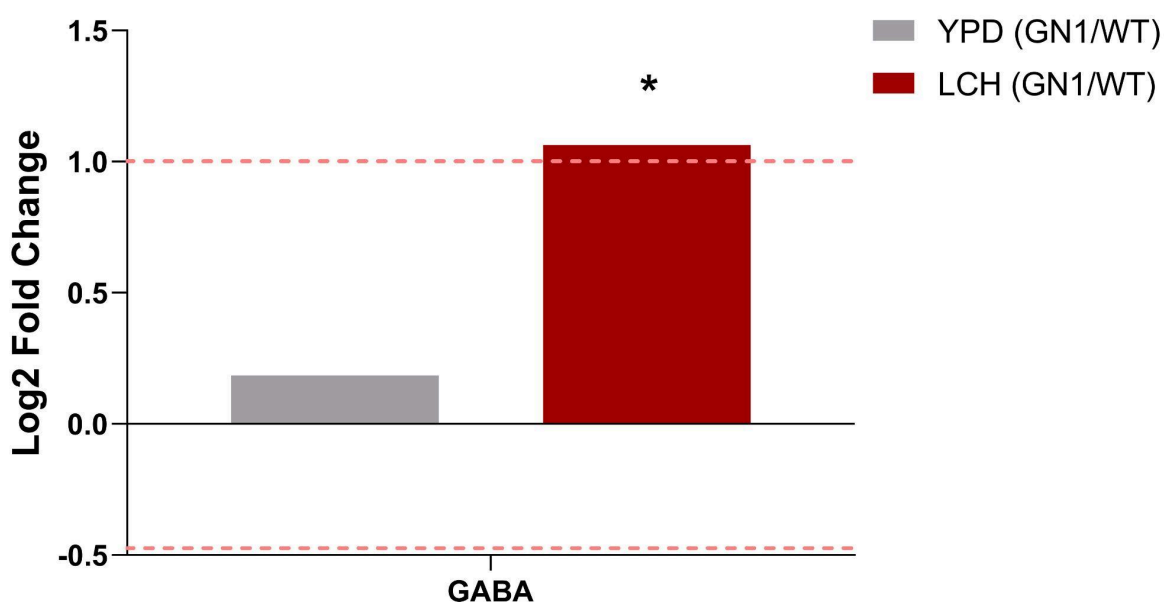


The visual representation, through heatmap, highlights the changes in amino acid concentration in the PE2_H4_GN1 strain, under growth in YPD and LCH. Each line of the heatmap corresponds to an amino acid, identified by its acronym. Normalization of values considers the Log2 Fold Change (GN1/H4). Aspartate (ASP), Glutamate (GLU), Cysteine (CYS), Asparagine (ASN), Serine (SER), Glutamine (GLN), Histidine (HIS), Glycine (GLY), Threonine (THR), Arginine (ARG), Alanine (ALA), GABA, Tyrosine (TYR), Valine (VAL), Methionine (MET), Tryptophan (TRP), Phenylalanine (PHE), Isoleucine (ILE), Leucine (LEU), Lysine (LYS), Proline (PRO), and Ornithine (ORN).

In a study by Hara and collaborators (2021), they described that amino acids related to the TCA cycle such as glutamate and arginine are increased and they are anaplerotic substrates that are converted to α -ketoglutarate (HARA *et al.*, 2021). Arginine can act to protect yeast cells from damage caused by ethanol, maintaining the integrity of the cell wall and cell membranes (CÁMARA *et al.*, 2022). Furthermore, it is possible to see an increase in alanine fold change which could be a strategy for cellular efficiency, possibly acting as reduction equivalents, maintaining redox homeostasis in yeast (CHICO; OLAVARRÍA; DE CASTRO, 1978).

It was proposed by Zhang and collaborators in 2019, that the increase in intracellular glutamate and GABA can have beneficial effects on acetic acid toxicity (ZHANG *et al.*, 2019). GABA concentrations of this study can be seen in Figure 5.15.

Figure 5.15 - GABA Concentration.



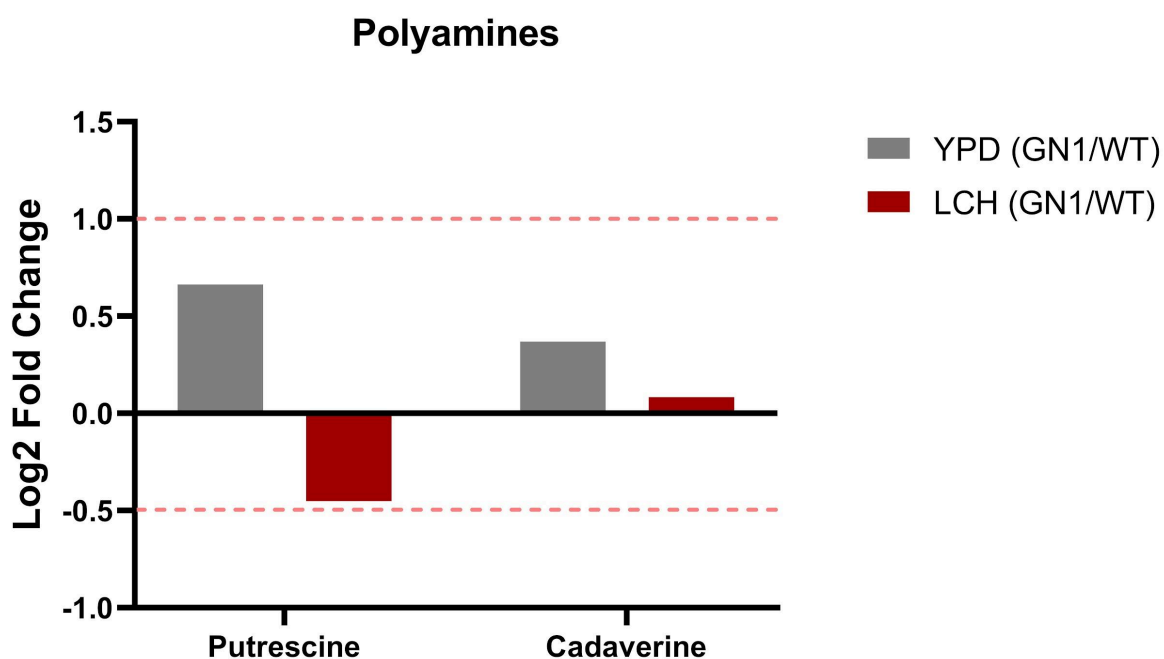
The figure shows the concentration of GABA (gamma-aminobutyric acid) in YPD (Grey) and in LCH (Red).

Analysis of the amount of gamma-aminobutyric acid in the evolved strain, when fermented in LCH, demonstrates a fold change larger than 1 ($\log_2(\text{fold change}) > 0.5$) increase. This finding suggests that the conditions provided by PE2_H4_GN1 in the LCH medium slightly favored GABA production or accumulation. However, more comprehensive results are needed to fully understand the mechanisms behind this slight increase.

5.4.4.2 Polyamines

The biosynthesis of polyamines results from the decarboxylation of ornithine in a reaction catalyzed by ornithine decarboxylase. Several studies have indicated that polyamines may be involved in protecting cells and cellular components against oxidative stress (DAS; MISRA, 2004; KHAN *et al.*, 1992; STEWART *et al.*, 2018; WALTERS; COWLEY, 1998). The quantitative analysis of cadaverine and putrescine production was analyzed in this study (Figure 5.16). The results suggest that hydrolysate conditions do not affect putrescine and cadaverine production, regardless of the evolution. Similar to what was found in a study using a medium with 12% ethanol, where the production was comparable in media with or without ethanol (WALTERS; COWLEY, 1998).

Figure 5.16 - Production of Cadaverine (A), and Putrescine (B).



The data highlights the consistency in the concentrations of both compounds, represented in red for LCH and in gray for YPD

5.4.4.3 Central Carbon Metabolism

Specific differences in the glycolytic, tricarboxylic acid cycle (TCA), and pentose phosphate pathway (PPP) pathways provide valuable clues about the metabolic

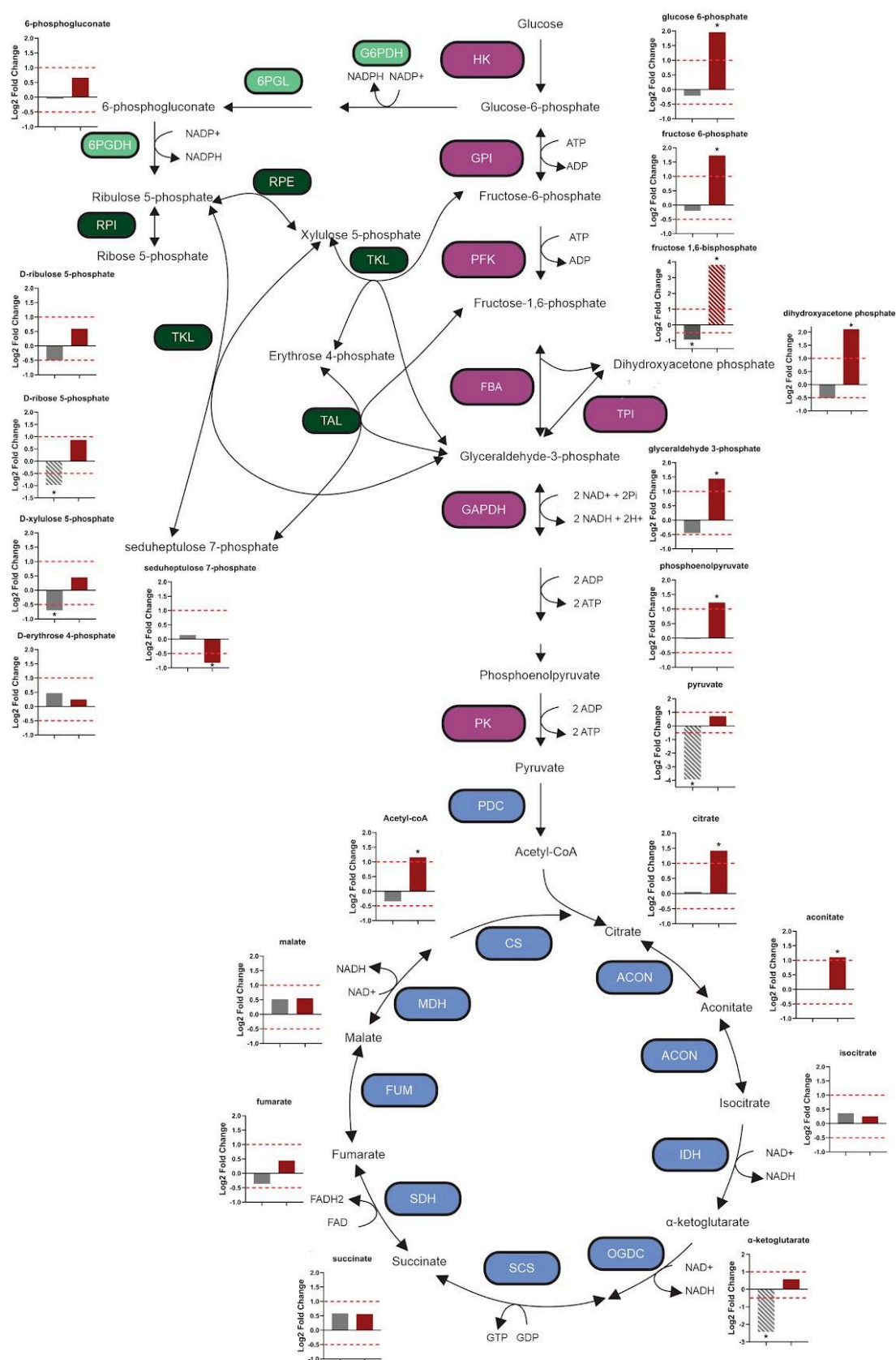
mechanisms underlying adaptation to LCH. In addition to understanding the quantification of nucleotides and cofactors, it is possible to understand more deeply the mechanisms of adaptation (JUNG *et al.*, 2017).

Analyzing the overall CCM, it is suggested that the glycolytic pathway is considerably more active in the PE2_H4_GN1 strain than the PE2_H4 WT strain in LCH (Figure 5.17). Data from the PE2_H4_GN1 strain suggest that this strain reprograms its metabolism to optimize energy production and the generation of biomolecule precursors essential for survival in adverse conditions. However, this intensification was not repeated in the pentose phosphate pathway (PPP), with the sedoheptulose-7-phosphate showing a decrease in the fold change (Figure 5.17).

Analyzing the CCM more deeply, the glycolysis pathway in LCH showed that fructose-1,6-phosphate was notably increased in the evolved strain (Figure 5.17). Other metabolites that were increased were glucose-6-phosphate, fructose-6-phosphate, dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, and phosphoenolpyruvate. While pyruvate maintained baseline levels. Another aspect to be observed is the trade-off in the YPD medium (Figure 5.17). In the absence of stressors, the changes in PE2_H4_GN1 result in a lower Fold Change of the glycolytic pathway, with emphasis on fructose-1,6-phosphate, and pyruvate (Figure 5.17).

In the TCA cycle, the fold change of acetyl-CoA, citrate, and aconitate were increased when fermented in LCH. In the absence of stressors, the changes in PE2_H4_GN1 result in a lower fold change of α -ketoglutarate.

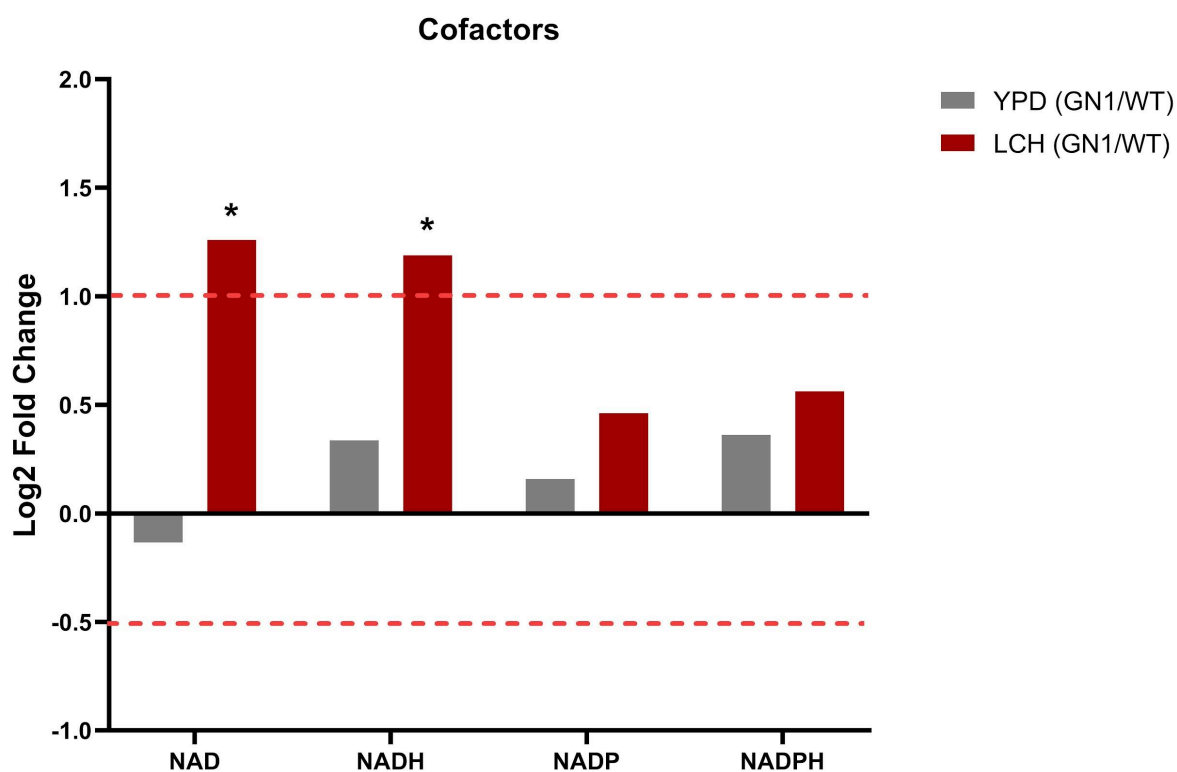
Figure 5.17 - Visual representation of the metabolic pathways PPP (Pentose Phosphate Pathway), Glycolysis, and TCA (Krebs Cycle).



Specific enzymes to each pathway are highlighted and colored according to their association with the respective pathway (PPP-green; glycolysis-purple; TCA-blue). Plots of metabolic intermediates are in the figure, values are normalized by PE2_H4 WT. The scales of the graphs are predominantly (2 to -1), but adjustments are applied for extreme values, and to better visualize the bars were marked with stripes. In red, are shown the relative metabolic levels of PE2_H4_GN1 in LCH, while in gray, are shown the relative concentrations of PE2_H4_GN1 in YPD.

According to these results, it has been reported in other studies that in furfural-evolved strains fluxes through glycolytic pathways are recovered or intensified, probably to generate adequate amounts of energy, cofactors, and other intermediate metabolites for the synthesis of aromatic amino acids and nucleotides (JUNG *et al.*, 2017). The evaluation of cofactors NAD⁺ and NADH in lignocellulosic hydrolysate fermentation is crucial due to their role in metabolic processes, especially in energy production. NAD⁺ and NADH are coenzymes that act in the transfer of electrons in biochemical reactions (CHANG *et al.*, 2018; HASUNUMA *et al.*, 2011; KUMAR; VENKATRAMAN; BRUHEIM, 2021). PE2_H4_GN1 demonstrated a higher amount of NAD⁺ and NADH, which suggests better performance in converting sugars into ethanol (Figure 5.18A). Therefore, the assessment of NAD⁺ and NADH is an important indicator of the efficiency and adaptation of the PE2_H4_GN1 strain. The lack of difference in the amount of NADP⁺/NADPH indicates that both strains maintained similar levels, regardless of the culture medium (YPD or LCH) (Figure 5.18B). Considering the degradation of HMF (Figure 5.12) and the greater production of glycerol (Figure 5.13), which uses reduced cofactors, it is possible to suggest an adaptive response of the PE2_H4_GN1 strain, since the fold change of the cofactors are not diminished (LIU *et al.*, 2008). In summary, the maintenance of these cofactors suggests an effective regeneration of redox cofactors.

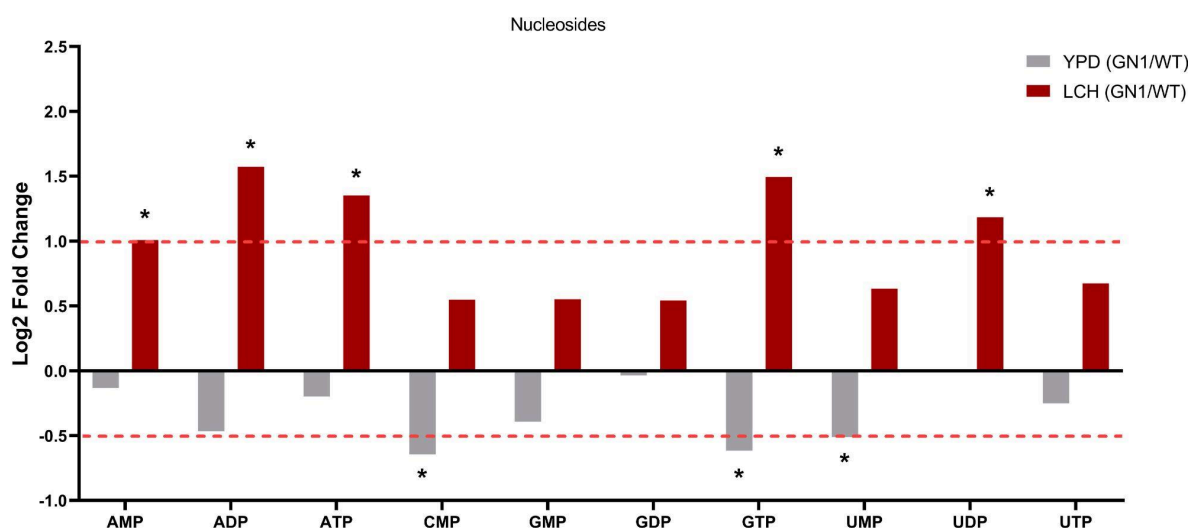
Figure 5.18 - Comparative Profile of Cofactors.



A. Levels of NAD⁺ and NADH, revealing a statistically significant difference, indicating that the evolved strain has a higher concentration of these cofactors compared to the WT. B. NADP⁺ and NADPH did not exhibit statistical differences between strains, suggesting stability in the levels of these cofactors.

Another analysis provided a nucleoside point of view. Our results show that LCH stress caused an increase in energy consumption in WT cells. Meanwhile, in the evolved strain this ATP concentration is restored to cope with those stressful conditions as well as ADP, AMP, and GTP. (Figure 5.19).

Figure 5.19 - Profile of Nucleoside Concentrations.



Intracellular concentrations of nucleosides, including ADP (adenosine diphosphate), AMP (adenosine monophosphate), ATP (adenosine triphosphate), CMP (cytidine monophosphate), CTP (cytidine triphosphate), GDP (guanosine diphosphate), GMP (guanosine monophosphate), GTP (guanosine triphosphate), UDP (uridine diphosphate), UDPNAG (uridine diphosphate N-acetylglucosamine), UMP (uridine monophosphate), and UTP (uridine triphosphate), are presented. In red is the Log2 Fold Change in LCH and in grey is the Log2 Fold Change in YPD.

The fact that the PE2_H4_GN1 strain has higher levels of ADP, AMP, ATP, and GTP compared to the PE2_H4 strain in LCH may be beneficial for metabolic processes that require rapid energy availability, such as the synthesis of compounds, or conversion of specific metabolites (GRAHAME HARDIE, 2011).

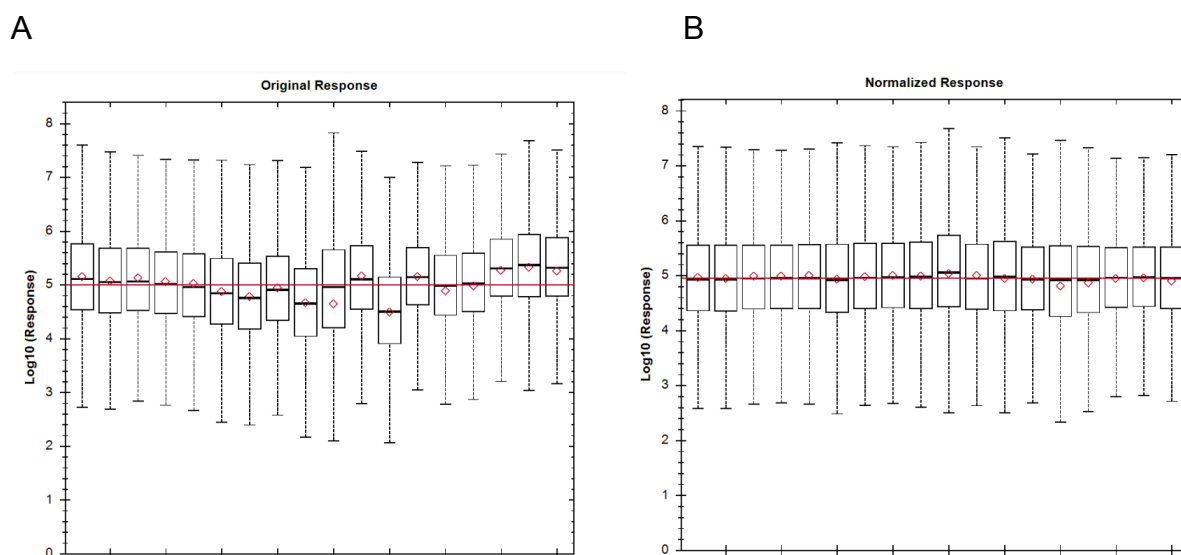
5.4.5 Proteomics

Proteomic analysis revealed an average of 37,242 profiles collected from a total of 62,819. Most profiles (62,375) contained valuable data (Sparse Profiles), while 6,680 were complete profiles. Quantification of these 62,375 profiles using the MaxLFQ method identified 50,034 proteotypic peptides out of a total of 52,087. The average number of peptide bonds not made was 0.4, indicating efficiency in sample preparation. The median coefficient of variation was 61.6%, with variations between experimental conditions. Peak capacity, representing the number of distinct peaks (or proteins) identified, averaged 106.2, indicating the breadth of protein identification.

The median width of charge index chromatograms (XIC) was 6.5 minutes, with variations under different experimental conditions. The average number of data points per peak, a measure of data quality for each identified protein, was 3.9, with small variations between experimental conditions contributing to data accuracy. The original data shown in Figure 5.20A illustrates proteomic expression before normalization, while Figure 5.20B presents the patterns after normalization. The effectiveness of normalization is crucial to ensuring data accuracy and reliability.

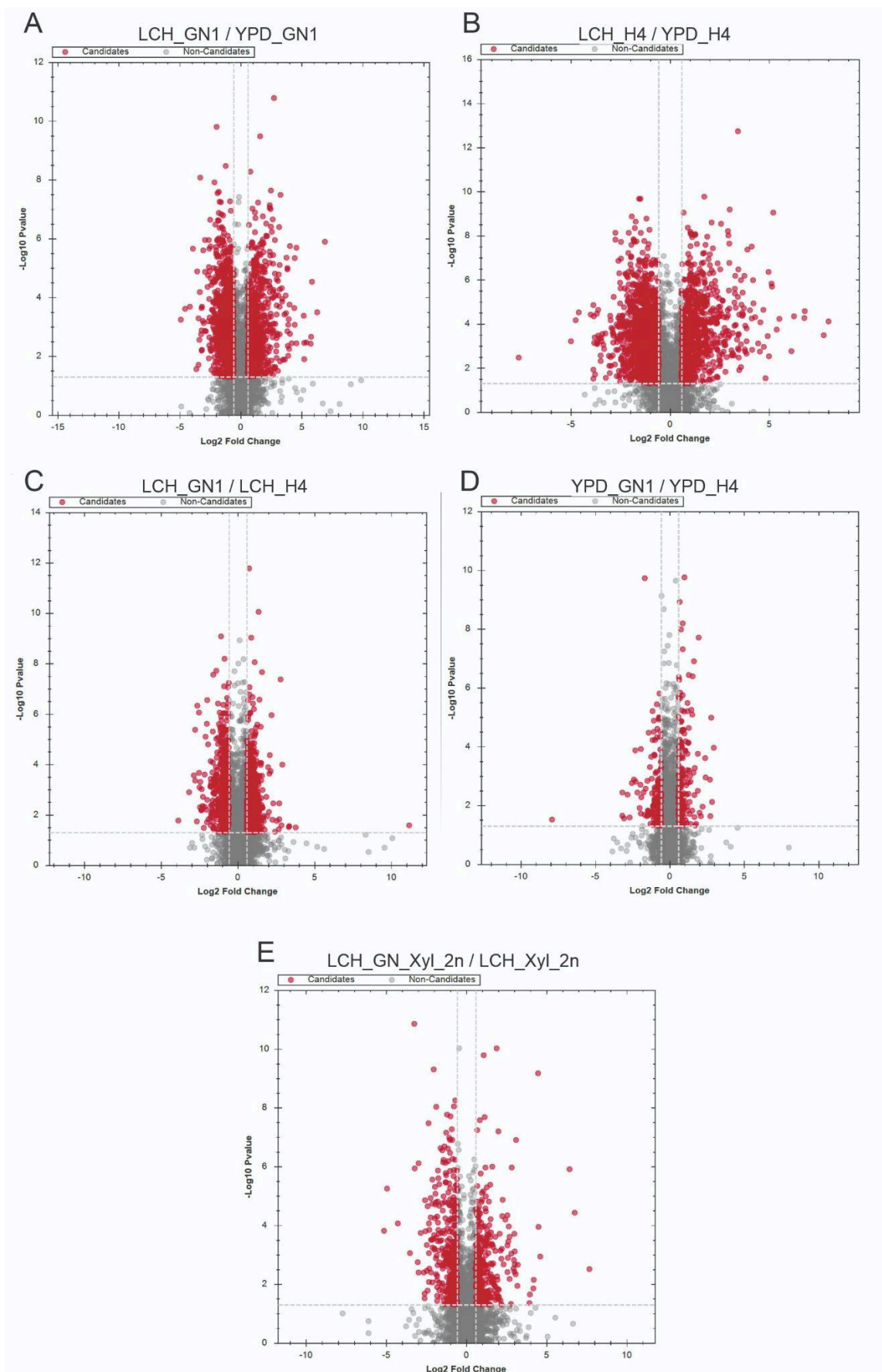
It is possible to have visual information about the difference in protein expression through Volcano Plots (Figure 5.21). Proteins with statistically significant differential expression are highlighted in red, indicating changes associated with different substrates or strains.

Figure 5.20 - Normalization in Proteomic Data.



A. Raw Data Before Normalization: Visual representation of raw proteomics data, highlighting subtle systematic variations before applying normalization techniques. B. Normalized Data: Proteomic profile after normalization, demonstrating the reduction of variations and improvement in the representation of real changes in proteins.

Figure 5.21 - Volcano Plot of Protein Expression Modulation.



A. Analysis of differentially expressed proteins between the PE2_H4_GN1 strain with fermentation in the hydrolysate, divided by the same strain with fermentation in YPD. B. Graphical representation of proteins differentially expressed between the PE2_H4 WT strain with fermentation in the hydrolysate, divided by the same strain with fermentation in YPD. C. Visual analysis of modulations in protein expression for the ratio between the evolved lineage PE2_H4_GN1 and the parental PE2_H4 WT with fermentation in hydrolysate. D. Visualization of protein expression with the ratio between the evolved lineage PE2_H4_GN1 and the parental PE2_H4 WT with fermentation in YPD. E. Statistical visualization using the Volcano Plot of strains PE2_GN_Xyl and PE2_Xyl growing in the hydrolysate. The graphs indicate proteins with statistically different protein expression in red color.

5.4.5.1 GO Enrichment

With the generation data, an enrichment analysis was carried out using Gene Ontology (GO), categorizing the results into Biological Process, Molecular Function, and Cellular Component. Over/underrepresentation relates to the number of genes associated with a specific term in the GO category that may be lower/higher than expected randomly (MI *et al.*, 2013). This does not directly imply the expression of the individual genes associated with the Term.

Log2-fold enrichment was used to assess enrichment in each category. Graphical analysis allowed clear identification of positive (above zero) and negative (below zero) enrichments. This detailed analytical approach provided valuable insights into the gene expression patterns associated with different GO categories (Figure). The code used for this analysis is available in the GitHub repository, ensuring transparency, and reproducibility and facilitating future investigations (gh repo clone yasminealvesm/PE2_H4_GN1proteomics, or https://github.com/yasminealvesm/PE2_H4_GN1proteomics.git).

In the YPD analysis with the GO terms PE2_H4_GN1/PE2_H4 in YPD (Figure 5.22A), the decrease in the GO term:0005935 may indicate that cell division is decreasing. Also, the decrease in the term GO:0032543 can indicate that the production of mitochondrial proteins is reduced. This can lead to a decrease in energy production and cellular respiration and may have some association with a decrease in TCA activity. Increased GO of Translation Elongation Activation (GO:0045901) and Translation Termination Activation (GO:0045905) may indicate an

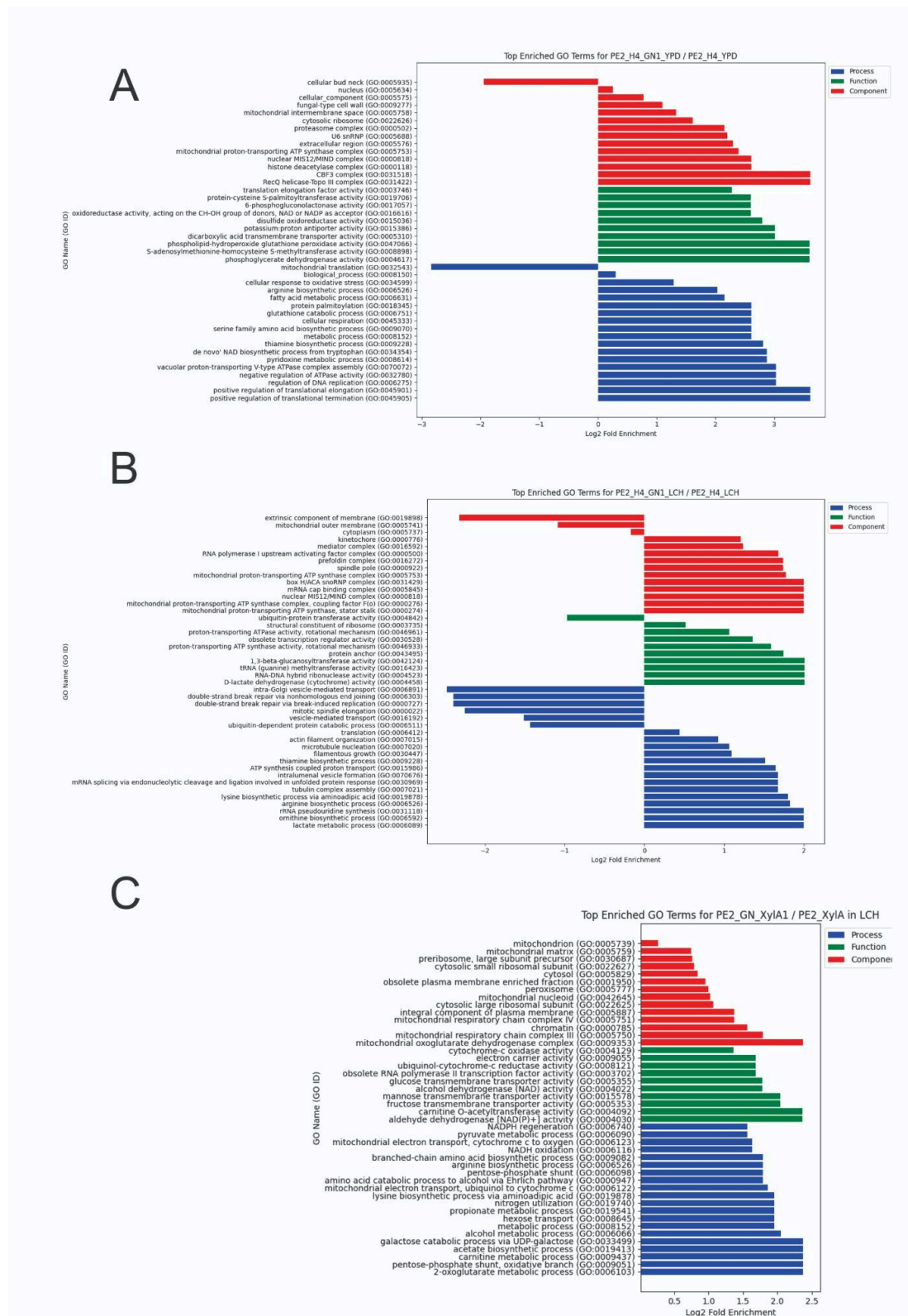
attempt by the cell to respond to unfavorable conditions by activating protein translation. However, the cell may be investing energy in processes that are not efficient for its survival or metabolic function, in YPD, being mechanisms that the organism developed to survive in the hydrolysate. (Figure 5.17).

In LCH growth, the decrease in GO:0019898 - Extrinsic Membrane Component may indicate a change in membrane composition, resulting in a different membrane configuration. Which could lead to implications for membrane permeability or cellular interactions. In the WGS of strain ISO12, generated by ALE in LCH, mutations were detected in 11 genes, some of them encoding plasma membrane proteins (for example, *MTL1*, *HXT13*, *CYC3*, and *SED4*) (WALLACE-SALINAS *et al.*, 2015). Furthermore, the ALE NRRL Y-50049 strain also showed differential regulation of genes encoding plasma membrane and cell wall proteins in response to furfural/HMF (LIU; MA, 2020), corroborating that the cell periphery is an important target for adaptive evolution. The increase in terms GO:0000274, GO:0000276, and GO:0005753 suggests adaptive changes in mitochondrial ATP synthase in response to the hydrolysate. These enriched GO terms can indicate structural optimization, subunit regulation, and global adjustments, reflecting efficiency in ATP production. This corroborates what was seen in ATP production (Figure 5.19). Furthermore, an increase in GOs Related to Amino Acid Biosynthesis, such as GO:0006592, GO:0006526, and GO:0019878, was also observed. These terms refer to the biosynthesis of ornithine, arginine, and lysine and indicate that PE2_H4_GN1 may have a greater capacity to synthesize these amino acids, which may contribute to its more efficient growth and ethanol production. Arginine and Lysine were identified as more concentrated, but ornithine was not, perhaps because it was already being metabolized (Figure 5.14). Another increased GO of interest is GO:0007021 - Assembly of the Tubulin Complex, which suggests influence on the organization of the cytoskeleton and cell division of the evolved strain.

The strain that resulted from the crossing of PE2_H4_GNpop and XylA was also subjected to GO analysis. This analysis indicated enrichment in mitochondrial components, such as an increase in the terms GO:0009353 (Mitochondrial Oxoglutarate Dehydrogenase Complex), GO:0005750 (Complex III of the Mitochondrial Respiratory Chain), GO:0005751 (Complex IV of the Mitochondrial Respiratory Chain) and GO:0042645 (Mitochondrial Nucleoid). These components suggest optimization of the efficiency of the mitochondrial respiratory chain. Leading

to a greater capacity to efficiently use available substrates (xylose) when in the hydrolysate.

Figure 5.22 - Differential enrichment of GO terms:



A. Log2 Fold Enrichment of the ratio of PE2_H4_GN1/PE2_H4 in YPD. B. Log2 Fold Enrichment of the ratio of PE2_H4_GN1/PE2_H4 in LCH. C. Log2 Fold Enrichment of the ratio of PE2_GN_XylA1/PE2_XylA in LCH. Terms in blue represent Biological Processes, in green are Molecular Functions, and in red are Cellular Components.

The increase in the activity of GO terms related to cofactors is also extremely important. GO:0004022 Alcohol Dehydrogenase (NAD⁺) suggests optimizing associated metabolic pathways. Additionally, increases in Aldehyde Dehydrogenase (NAD(P)⁺) (GO:0004030), NADPH Regeneration (GO:0006740), NADH Oxidation (GO:0006116), Oxidative Branch of the Pentose-Phosphate Pathway (GO:0009051) and Pentose-Phosphate Pathway (GO:0006098) indicate a specific positive regulation in metabolic pathways related to the cofactors NAD⁺ / NADH and NADP⁺ / NADPH. These adaptations suggest that the PE2_GN1_XylA strain has better redox balance, essential to face the stress caused by LCH. The increase in activities directly regulating these cofactors indicates an adaptive strategy to improve metabolic efficiency, favoring growth and ethanol production under challenging xylose hydrolysate conditions.

The difference in Gene Ontology (GO) enrichment of the same strain (PE2_H4_GN1 or PE2_H4) but in different media (LCH x YPD) can be found in Appendix A, Section 7, Figure 16.

5.5 Conclusão

Sequencing analysis of the PE2_H4_GN1 strain revealed genetic overlap with the three evolved lineages, demonstrating an efficient crossing strategy. The common genes identified suggest they are the alleles most related to adaptability. These genes are *RPN4*, *MDS3*, *GCN1*, *SIZ1*, *GAL80*, *PHO89*, *ASG1*, *SPT21*, *PMA1* and *SSB2* (Figure 5.4). Analysis using STRING software highlighted specific interactions between the *MDS3*, *PMA1*, *GCN1*, and *SSB2* genes, suggesting shared functional links and/or signaling pathways.

The strains PE2_H4_GN1 and PE2_H4_GNpop were subjected to fermentation (Figure 5.6) where PE2_H4_GN1 showed a better growth rate compared to the control. Furthermore, the PE2_H4_GN1 clone demonstrated faster growth in the presence of only acetic acid, in an experiment conducted in microplates (Figure 5.8).

The analysis of extracellular metabolites showed differences in the velocity of glucose consumption and ethanol production between strains PE2_H4_GN1 and PE2_H4 in LCH, demonstrating higher tolerance of the evolved strain to these challenging conditions of the hydrolysate (Figure 5.9). Analysis of intracellular metabolism revealed variations in the concentrations of amino acids, polyamines, and Central Carbon Metabolism. Understanding glycolytic pathways and the TCA cycle has provided insights into the metabolic mechanisms that drive adaptation to specific growth conditions in the hydrolysate. The analysis of GO terms enrichment also helped to elucidate the pathways chosen by the evolved strains, helping to understand the overall cell dynamics.

References

- AULD, D. S. *et al.* Microplate Selection and Recommended Practices in High-throughput Screening and Quantitative Biology. 2004. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/32520474/>. Acesso em: 15 mar. 2024.
- BASSO, T. P.; BASSO, T. O.; GALLO, C. R.; BASSO, L. C. **Towards the Production of Second Generation Ethanol from Sugarcane Bagasse in Brazil.** [*S. l.: s. n.*] Disponível em: <https://doi.org/10.5772/54179>
- BAZURTO, J. V.; DEARTH, S. P.; TAGUE, E. D.; CAMPAGNA, S. R.; DOWNS, D. M. Untargeted metabolomics confirms and extends the understanding of the impact of aminoimidazole carboxamide ribotide (AICAR) in the metabolic network of *Salmonella enterica*. **Microbial cell factories**, v. 5, n. 2, p. 74, 2018.
- CÁMARA, E.; OLSSON, L.; ZRIMEC, J.; ZELEZNIK, A.; GEIJER, C.; NYGÅRD., Y. Data mining of *Saccharomyces cerevisiae* mutants engineered for increased tolerance towards inhibitors in lignocellulosic hydrolysates. **Biotechnology advances**, v. 57, p. 107947, 2022.
- CHANG, D.; YU, Z.; UL ISLAM, Z.; FRENCH, W. T.; ZHANG, Y.; ZHANG, H. Proteomic and metabolomic analysis of the cellular biomarkers related to inhibitors tolerance in *Zymomonas mobilis* ZM4. **Biotechnology for biofuels**, v. 11, n. 1, p. 1–23, 2018.
- CHICO, E.; OLAVARRÍA, J. S.; DE CASTRO, I. N. L-Alanine as an end product of glycolysis in *Saccharomyces cerevisiae* growing under different hypoxic conditions. **Antonie van Leeuwenhoek**, v. 44, n. 2, p. 193–201, 1978.
- DAS, K. C.; MISRA, H. P. Hydroxyl radical scavenging and singlet oxygen quenching properties of polyamines. **Molecular and cellular biochemistry**, v. 262, n. 1-2, p. 127–133, 2004.
- DOS SANTOS, L. V.; CARAZZOLLE, M. F.; NAGAMATSU, S. T.; SAMPAIO, N. M. V.; ALMEIDA, L. D.; PIROLLA, R. A. S.; BORELLI, G.; CORRÊA, T. L. R.; ARGUESO, J. L.; PEREIRA, G. A. G. Unraveling the genetic basis of xylose consumption in engineered *Saccharomyces cerevisiae* strains. **Scientific reports**, v. 6, p. 38676, 2016.
- GRAHAME HARDIE, D. AMP-activated protein kinase—an energy sensor that regulates all aspects of cell function. **Genes & development**, v. 25, n. 18, p. 1895, 2011.
- HARA, Y.; KUME, S.; KATAOKA, Y.; WATANABE, N. Changes in TCA cycle and TCA

cycle-related metabolites in plasma upon citric acid administration in rats. **Heliyon**, v. 7, n. 12, 2021. Disponível em: <https://doi.org/10.1016/j.heliyon.2021.e08501>. Acesso em: 27 fev. 2024.

HASUNUMA, T.; SANDA, T.; YAMADA, R.; YOSHIMURA, K.; ISHII, J.; KONDO, A. Metabolic pathway engineering based on metabolomics confers acetic and formic acid tolerance to a recombinant xylose-fermenting strain of *Saccharomyces cerevisiae*. **Microbial cell factories**, v. 10, p. 2, 2011.

HEER, D.; HEINE, D.; SAUER, U. Resistance of *Saccharomyces cerevisiae* to high concentrations of furfural is based on NADPH-dependent reduction by at least two oxireductases. **Applied and environmental microbiology**, v. 75, n. 24, p. 7631–7638, 2009.

JUNG, Y. H.; KIM, S.; YANG, J.; SEO, J.; KIM, K. H. Intracellular metabolite profiling of *Saccharomyces cerevisiae* evolved under furfural. **Microbial biotechnology**, v. 10, n. 2, p. 395, 2017.

KHAN, A. U.; DI MASCIO, P.; MEDEIROS, M. H.; WILSON, T. Spermine and spermidine protection of plasmid DNA against single-strand breaks induced by singlet oxygen. **Proceedings of the National Academy of Sciences of the United States of America**, v. 89, n. 23, p. 11428–11430, 1992.

KUMAR, K.; VENKATRAMAN, V.; BRUHEIM, P. Adaptation of central metabolite pools to variations in growth rate and cultivation conditions in *Saccharomyces cerevisiae*. **Microbial cell factories**, v. 20, n. 1, p. 1–16, 2021.

LIN, F.-M.; QIAO, B.; YUAN, Y.-J. Comparative proteomic analysis of tolerance and adaptation of ethanologenic *Saccharomyces cerevisiae* to furfural, a lignocellulosic inhibitory compound. **Applied and environmental microbiology**, v. 75, n. 11, p. 3765–3776, 2009.

LIU, Z. L.; MA, M. Pathway-based signature transcriptional profiles as tolerance phenotypes for the adapted industrial yeast *Saccharomyces cerevisiae* resistant to furfural and HMF. **Applied microbiology and biotechnology**, v. 104, n. 8, p. 3473–3492, 2020.

LIU, Z. L.; MOON, J.; ANDERSH, B. J.; SLININGER, P. J.; WEBER, S. Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*. **Applied microbiology and biotechnology**, v. 81, n. 4, 2008. Disponível em: <https://doi.org/10.1007/s00253-008-1702-0>. Acesso em: 29 fev. 2024.

LU, H. *et al.* A consensus *S. cerevisiae* metabolic model Yeast8 and its ecosystem

for comprehensively probing cellular metabolism. **Nature communications**, v. 10, n. 1, p. 1–13, 2019.

MI, H.; MURUGANUJAN, A.; CASAGRANDE, J. T.; THOMAS, P. D. Large-scale gene function analysis with PANTHER Classification System. **Nature protocols**, v. 8, n. 8, p. 1551, 2013.

MUTTON, M. J. R.; FERRARI, F. C. S.; DE FREITA, L. A.; DE FREITA, C. M.; DA GRAÇA S. ANDRIETTA, M.; ANDRIETTA, S. R. Interaction between the production of ethanol and glycerol in fed-batch bioreactors. **Brazilian journal of microbiology: [publication of the Brazilian Society for Microbiology]**, v. 50, n. 2, p. 389, 2019.

PATIL, K. R.; ROCHA, I.; FÖRSTER, J.; NIELSEN, J. Evolutionary programming as a platform for in silico metabolic engineering. **BMC bioinformatics**, v. 6, p. 308, 2005.

PEREIRA, I. de O.; DOS SANTOS, Â. A.; GONÇALVES, D. L.; PURIFICAÇÃO, M.; GUIMARÃES, N. C.; TRAMONTINA, R.; COUTOUNÉ, N.; ZANELLA, E.; MATSUSHIKA, A.; STAMBUK, B. U.; IENCZAK, J. L. Comparison of *Spathaspora passalidarum* and recombinant *Saccharomyces cerevisiae* for integration of first- and second-generation ethanol production. **FEMS yeast research**, v. 21, n. 6, p. foab048, 2021.

QUACKENBUSH, J. Microarray data normalization and transformation. **Nature genetics**, v. 32 Suppl, p. 496–501, 2002.

STEWART, T. M.; DUNSTON, T. T.; WOSTER, P. M.; CASERO, R. A., Jr. Polyamine catabolism and oxidative damage. **The Journal of biological chemistry**, v. 293, n. 48, p. 18736, 2018.

SUTTER, B. M.; WU, X.; LAXMAN, S.; TU, B. P. Methionine inhibits autophagy and promotes growth by inducing the SAM-responsive methylation of PP2A. **Cell**, v. 154, n. 2, 2013. Disponível em: <https://doi.org/10.1016/j.cell.2013.06.041>. Acesso em: 11 mar. 2024.

SZKLARCZYK, D. *et al.* The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. **Nucleic acids research**, v. 51, n. D1, 2023. Disponível em: <https://doi.org/10.1093/nar/gkac1000>. Acesso em: 26 fev. 2024.

WALLACE-SALINAS, V.; BRINK, D. P.; AHRÉN, D.; GORWA-GRAUSLUND, M. F. Cell periphery-related proteins as major genomic targets behind the adaptive evolution of an industrial *Saccharomyces cerevisiae* strain to combined heat and hydrolysate stress. **BMC genomics**, v. 16, n. 1, 2015. Disponível em: <https://doi.org/10.1186/s12864-015-1737-4>. Acesso em: 1 mar. 2024.

WALTERS, D.; COWLEY, T. Polyamine metabolism in *Saccharomyces cerevisiae* exposed to ethanol. **Microbiological research**, v. 153, n. 2, p. 179–184, 1998.

WALVEKAR, A. S.; SRINIVASAN, R.; GUPTA, R.; LAXMAN, S. Methionine coordinates a hierarchically organized anabolic program enabling proliferation. **Molecular biology of the cell**, v. 29, n. 26, 2018. Disponível em: <https://doi.org/10.1091/mbc.E18-08-0515>. Acesso em: 11 mar. 2024.

WANG, X.; LI, B.-Z.; DING, M.-Z.; ZHANG, W.-W.; YUAN, Y.-J. Metabolomic analysis reveals key metabolites related to the rapid adaptation of *Saccharomyces cerevisiae* to multiple inhibitors of furfural, acetic acid, and phenol. **Omics: a journal of integrative biology**, v. 17, n. 3, p. 150–159, 2013.

ZHANG, M.-M.; XIONG, L.; TANG, Y.-J.; MEHMOOD, M. A.; ZHAO, Z. K.; BAI, F.-W.; ZHAO, X.-Q. Enhanced acetic acid stress tolerance and ethanol production in *Saccharomyces cerevisiae* by modulating expression of the de novo purine biosynthesis genes. **Biotechnology for biofuels**, v. 12, n. 1, p. 1–13, 2019.

CHAPTER 6 - CONCLUSION

6.1 Thesis Overview: Understanding and Enhancing Tolerance Mechanisms

The thesis opens by addressing the problems associated with oil-based fuels, followed by the explanations of biofuels and the challenges concerning lignocellulosic hydrolysate and its impact using *Saccharomyces cerevisiae*. Emphasis is placed on the significance of creating strains with increased tolerance to overcome inhibitory chemicals found in lignocellulosic hydrolysate.

- Evolutionary Engineering (ALE):

The first segment details the initial evolutionary engineering phase, focusing on the strains used—*Saccharomyces cerevisiae* CEN.PK113-7D and Pe2_H4. The strains were subjected to continuous cultivation, incorporating two protocols: serial transfer and shock evolution. Triplicates for each protocol were established, providing robust statistical significance to the results. A detailed development of the populations, their growth conditions, and the evolutionary selection pressures is provided.

- Genomic Sequencing of ALE Populations:

The ALE populations were sequenced to identify the genetic variants and adaptations that happened during the evolutionary process. The basis for comprehending the genetic landscape of evolving populations was established by the sequencing data.

- Genetic Modifications:

The next part is devoted to genetic changes to validate genes that may be associated with the developed tolerance. Modifications were made to certain genes, discovered by sequencing, such as *SIZ1*, *MUK1*, *CHD1*, *HRK1*, *PDR3*, *PMA1*, and *RET1*. Confirmation processes are also included in the thesis.

- Competition Assays:

The study next presents flow cytometry competition experiments to assess the fitness of the evolved strains/modified strains in relation to the WT strains. The fitness effects of punctual mutations and deletions were reevaluated, exposing notable gains/losses. These tests offer numerical information about the strain's growth performance.

- Fermentations in Bioreactors:

The fermentation phase is then examined in the thesis through 44 experiments conducted in an INFORS HT 0.75 L bioreactor setup. The evolved and modified *Saccharomyces cerevisiae* CEN.PK113-7D were exposed to a strong inhibitor

cocktail that included p-coumaric acid, vanillic acid, furfural, HMF, acetic acid, and formic acid. With an emphasis on total fermentation growth and, in some situations, ethanol yield, the experimental design sought to understand the strains' reactions to lignocellulosic hydrolysate stress.

- Interbreeding of *Saccharomyces cerevisiae* PE2_H4 Populations:

To consolidate beneficial mutations, PE2_H4 populations are interbred. The best-performing colony is chosen and the process for mixing mutations from several populations is described. *Saccharomyces cerevisiae* PE2_H4_GN1, the resultant clone strain, is described and made ready for genomic sequencing.

- Crossbreeding *Saccharomyces cerevisiae* PE2_H4_GNpop with Xylose Metabolism:

Saccharomyces cerevisiae PE2_H4_GNpop is crossed with *Saccharomyces cerevisiae* XylA to overcome more obstacles and allow the ingestion of xylose. The crossbreeding experiment's best-performing colonies were chosen through a thorough procedure that resulted in the identification and selection of the PE2_GN_XylA1 colony.

- *Saccharomyces cerevisiae* PE2_H4_GN1 Fermentation:

The *S. cerevisiae* PE2_H4_GN1 strain fermented in difficult circumstances, as evidenced by the same cocktail used previously but with increased levels of furfural and HMF. Under increased lignocellulosic hydrolysate stress, the study carefully examined PE2_H4_GN1 reactions, highlighting ethanol generation and overall fermentation efficiency.

- *Saccharomyces cerevisiae* PE2_H4_GN1 Metabolomics and Proteomics:

To learn more about the molecular processes driving the developed yeast strain, the research combines metabolomics and proteomics approaches. Production/consumption of inhibitors is examined through extracellular metabolites in multiple fermentation times. To comprehend the alterations in polyamine metabolism, amino acids, and central carbon metabolism, intracellular metabolites were examined. Target proteins involved in cell wall construction, ethanol generation, stress response, and nutrient metabolism are the main focus of proteomics. The cells subjected to proteomics were the WT, PE2_H4_GN, and PE2_GN_XylA1 strains. Methodologies for sample collection, extraction, and analysis are described in depth.

- Methodologies:

ALE with particular strains and triplicates, genomic sequencing of ALE populations, CRISPR/Cas9-assisted genetic modifications, Sanger sequencing confirmation of genetic modifications, flow cytometry competition assays, and population interbreeding are just a few of the methodologies used in this thesis. To fully characterize and comprehend the evolving strains, methods including fermentations, LC-MS/MS for metabolomics, and DIA proteomics are employed.

6.2 Research Summary

In this study, strains of *Saccharomyces cerevisiae* were subjected to Adaptive Laboratory Evolution (ALE) protocols in lignocellulosic hydrolysate. Two distinct approaches, serial transfer (ALE1, ALE2, ALE3) and shock/recovery (ALE4), were used. This generated significant tolerance in haploid (ALE2 and ALE4) and diploid CEN.PK113-7D (ALE3) strains, in addition to the diploid PE-2_H4 (ALE1) strain. After evolution, these strains were evaluated in several ways. Initially, phenotype analysis involved growth competitions versus the WT strain, in addition to individual growth analysis on microplates. These strains were also genetically analyzed, with mutations identified through whole genome sequencing (WGS). Mutations of interest were validated by reverse engineering, using the CRISPR/Cas9 technique.

Clones of evolved strains and reverse-engineered strains were tested under controlled conditions in a bioreactor using synthetic hydrolysate as a fermentation substrate. The CHG, CHH, and S_CHN strains stood out with reduced latency times, increased growth rates, and greater production of ethanol and carbon dioxide (CO₂). Among the reverse-engineered lines, *RET1SIZ1Δ* and *PMA1Δ* demonstrated better growth performance in the synthetic hydrolysate.

In addition to the analysis of the CEN.PK113-7D strain, a new strain, PE2_H4_GN1, was created and studied. It originated from the crossing of the three final populations of ALE1 (PE-2_H4). The resulting strain inherited mutations from all three populations, indicating that each of them possessed valuable unique mutations. Controlled fermentations revealed higher growth rates in hydrolysate, highlighting the strain's ability to produce ethanol, detoxify inhibitors, and generate glycerol.

Furthermore, metabolomics and proteomics studies have provided important information about evolutionary adaptations. In the strain PE2_H4_GN1, changes occurred in the amino acids glutamine and methionine, which decreased under rich medium conditions (YPD), while arginine and alanine increased under challenging

conditions (LCH). The stability of polyamine production suggested a consistent adaptive response, independent of evolution. In central carbon metabolism, the glycolytic pathway was intensified. This adaptation suggests that cells focus on producing energy and vital biomolecules to survive the challenging environment of LCH. A study of the cofactors NAD⁺ and NADH suggested efficient turnover of the redox cofactor. This is vital for the redox balance required for LCH adaptation.

In proteomics, a Gene Ontology (GO) enrichment analysis highlighted specific metabolic and cellular adaptations related to growth in LCH, including modulations in cell division, mitochondrial protein production, and mitochondrial ATP synthase activity. These adaptations reflect a complex response of the developed lineage to LCH conditions, with optimizations in metabolic and cellular processes essential for survival and ethanol production.

6.3 Future work

For future work, it is of interest to perform a detailed proteomic analysis to identify the enzymes of the metabolic pathways of interest in the PE2_H4_GN1 strain, allowing a deeper understanding of the mechanisms underlying the observed improvements. Furthermore, point genetic modification of the mutations found in the PE2_H4_GN1 clone is an option to evaluate the specific impact of these mutations on the strain's performance. This approach will enable the validation of key mutations and targeted optimization of desired traits. Furthermore, challenging the PE2_H4_GN1 strain with different inhibitors individually, in a similar way to what was done with acetic acid, to assess its tolerance to a variety of stressful conditions and identify possible areas for improvement is an option for future work.

6.4 Final Remarks

As we culminate in this thesis, the journey through the exploration of lignocellulosic hydrolysate stress adaptation has reached a significant juncture. Our comprehension of our evolved strains has been enhanced by the widespread use of ALE, phenotypic analysis, reverse engineering, fermentation, metabolomics, and proteomics approaches.

This understanding of the cellular responses to hydrolysate stress lays the groundwork for improving fermentation conditions and yielding more ethanol. The ultimate goal is to realize sustainable and efficient ethanol production, which will

close the gap between scientific studies and industrial applications as theoretical changes are applied. This circumstance not only signifies the end of a thesis but also the start of a path where information is translated into real advancements in the bioethanol manufacturing process.

APPENDIX

Section 1

Strain Development

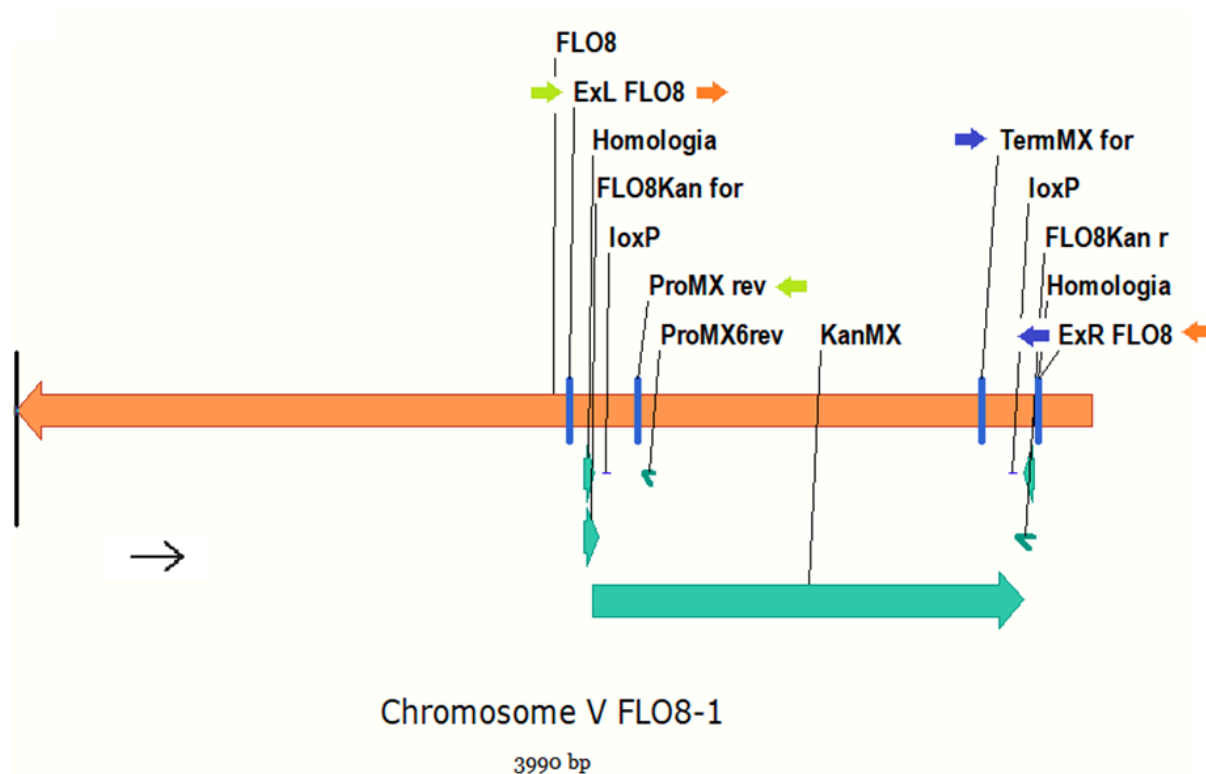


Figure 1: Chromosome V FLO8-1. Green arrows: Left flank. Blue arrows: Right flank. Orange arrows: External

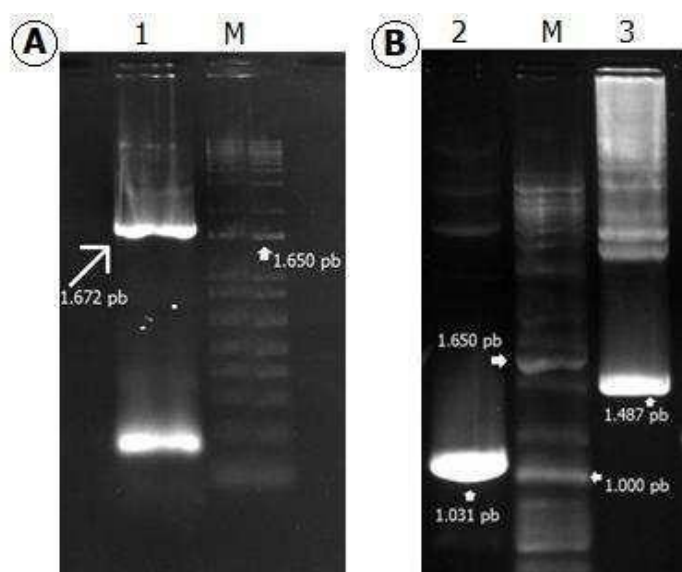


Figure 2: Electrophoretic profile of PCR kanMX, natMX, and higMX Insertion Cassettes for CEN.PK1137D populations. (A) PCR amplification of the kanMX insertion cassette: 1- The electrophoretic run profile on 1% agarose gel of the 1,672 bp PCR product specifying the kanMX cassette is shown. The lower band indicates primer dimers. M- 1kb Plus DNA Marker. (B) PCR amplification of the natMX and higMX cassette. 2- The profile and electrophoretic run on 1% agarose gel of the 1,031 bp PCR product specifying the natMX cassette are shown. M- 1kb Plus DNA Marker. 3- 1,487 bp PCR product specifying the higMX cassette.

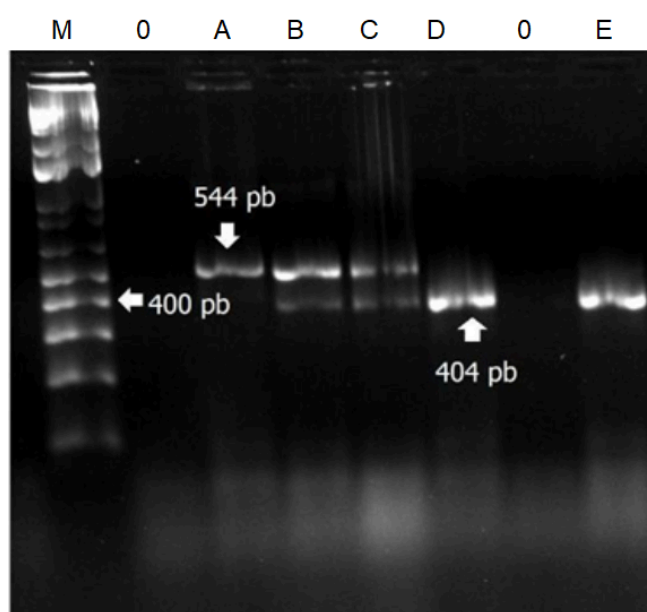


Figure 3: Electrophoretic profile of the mating type PCR for identification of

MATalpha, CEN.PK1137D FLO::HphMX. M - 1kb Plus DNA Marker. A – mating type a. D and E -mating type alpha. B and C - Diploids. 0- Wells without samples

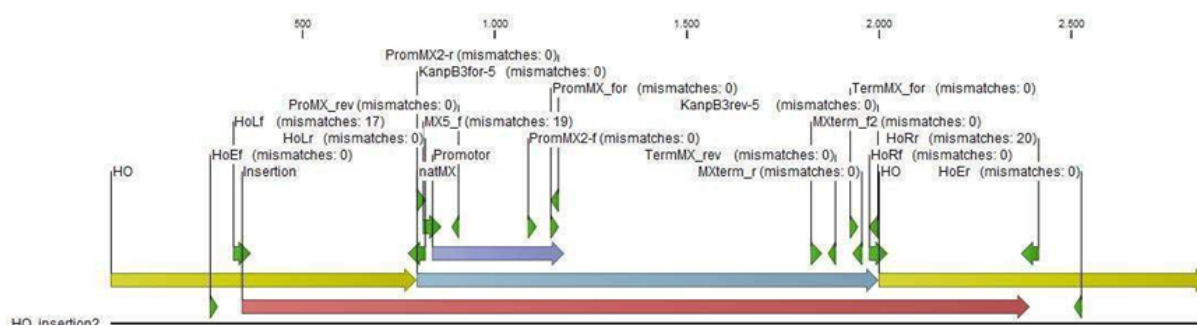


Figure 4: Insertion of the antibiotic tag cassette into the HO gene of haploid *Sacchamoryces cerevisiae* PE-2_H4.

Table 1: List of primers and sequences used in the strain development

Primer	Sequence
FLO8Kan_for	AGCAAAGGCTGCTGCAGTGTTTTTCAAAGACGACTTCG TACGCTGCAGGTCGACAACC
FLO8Kan_r	GGAAAAAATGAACTGCAAGAATACGCTCAATGAGTACAT AGGCCACTAGTGGATCTGATATCACC
promMX2F	GGTTAGGATTTGCCACTGAGGTT
termMXrev	CAGTATAGCGACCAGCATTACATAC
ProMx_rev	GGGGCATGATGTGACTGTCTG
TermMx_for	GTCAATCGTATGTGAATGCTGG
ExL_FLO8	TTTGGGTCCGTCGATTGGGTTTTTG
ExR_FLO8	GTGGATAAATCAACCTACGGCGG
ADH5 R2	GCGCCAACAATGTCTTTTTCTTCC

ADH5 F2	CCCAATCTTGTGTGCAGGTATCAC
Hph2_for	GGCTGTGTAGAAGTACTCGCCGATAGTGG
Nat 1F	ACTCTTGACGACACGGCTTAC
Nat 1R	GGAGTACGAGACGACCACGAA
Kan1F	CTGACGGAATTTATGCCTCTTCC
Kan1R	AATCACTCGCATCAACCAAACC
Zeo1F	TTCTATGAAAGGTTGGGCTTCGG
Zeo1R	GTTCTGTATCAGGCGCAGGAG
MatA	ACTCCACTTCAAGTAAGAGTTTG
Matalfa	GCACGGAATATGGGACTACTTCG
Mat locus	AGTCACATCAAGATCGTTTATGG

Section 2

Hydrolysate Development

The production of the hemicellulosic hydrolysate (LCH) was performed from LNBR/CNPEM, and the determination of the composition and amounts of each inhibitor present in the customized LCH was performed by standard HPLC detection. Dr. Sarita Cândida Rabelo and Dr. Michael Brienzo generated the data.

For this production, in-natura sugarcane bagasse was used, provided by Usina da Pedra, located in the municipality of Serrana/SP, referring to the 2018/2019 harvest. The material results from the last milling after extraction of the juice, whose cane is harvested mechanically, being then dried at room temperature and not going through any washing process or separating mineral impurities. To obtain the LCH, a pilot pre-treatment reactor was used, with a capacity of 350 L, built-in rodloy alloy 276 (Pope Scientific INC). The equipment belongs to the Pilot Plant for Process Development (PPDP) of the National Laboratory of Biorenewables (LNBR-CNPEM). It is equipped with a mechanical agitation system and heating through the injection of steam or thermal oil in the jacket.

The pretreatment reaction carried out using 160°C, 20 min, 0.75% (m/m) sulfuric acid, was carried out to potentiate the formation of fermentative inhibitory compounds, such as furan aldehydes (furfural, hydroxymethylfurfural (HMF)), organic acids (levulinic and formic acid) and phenolic compounds derived from lignin. Approximately 20 kg of sugarcane bagasse (dry basis) was loaded into the reactor, and then the sulfuric acid solution was fed, considering a final solids concentration of 10% (m/m). The reaction mixture was heated by thermal fluid in the jacket and maintained at the established temperature and time. After the reaction time, the reactor was gradually cooled until it reached a temperature of 100°C, depressurized, and discharged into a Nutsche-type filter with a capacity of 100 L and made of 276-rod steel (Pope Scientific INC).

2.1 Characterization of process streams

The chemical composition of the in-natura material and cellulignins was determined, and the hemicellulosic hydrolysate was analyzed by liquid chromatography (HPLC). A part of the material was used to quantify the monomeric sugars and production of pre-treatment solubilization degradation. In contrast, the other part was used to

quantify the total sugars (monomers and oligomers) after an acid hydrolysis step, according to the methodology described by (Jacobsen and Wyman, 2002).

2.2 Mass balance of the pre-treatment step

The mass balance of the dilute acid pretreatment process was determined by considering the reaction yield as a percentage of total solids recovered after pretreatment (dry basis). Polymers, oligomers, monomers, and degradation products such as HMF, furfural, formic acid, and levulinic acid were quantified and considered according to the degradation pathways to get as close as possible to 100% of the mass closure of the process. Glucose, cellobiose, formic acid, and HMF concentrations were used to calculate the cellulose content. In contrast, the hemicellulose content was calculated based on the concentrations of xylose, arabinose, acetic acid, and furfural. Their masses were divided by the dry weight of the starting material and multiplied by the hydrolysis factors, which were 0.9; 0.95; 1.29, and 3.52, respectively, for glucose, cellobiose, HMF and formic acid, and 0.88 for both xylose and arabinose, 0.72 for acetic acid and 1.37 for furfural. The soluble lignin contained in the liquid stream was quantified through absorbance measurements in a spectrophotometer, as described by (Gouveia *et al.*, 2009).

2.3 Dilute acid pretreatment

The chemical composition of sugarcane bagasse before and after the pretreatment step is shown in Table S2.

Table 2: Physical-chemical composition of in-natura sugarcane bagasse after pre-treatment with diluted acid.

Components (%)	In-natura	After pre-treatment 160°C, 20 min, 0,75% (m/m)
Cellulose	41,38 ± 0,50	60,25 ± 0,22
Arabinoxylan	25,06 ± 0,14	0,30 ± 0,13
Acetyl	3,20 ± 0,08	0,27 ± 0,01

Lignin	22,50 ± 0,43	31,98 ± 0,57
Ashes	4,62 ± 0,45	6,90 ± 0,62
Extractives	4,01 ± 0,06	-
Balance	100,77 ± 0,44	99,70 ± 0,50

Pretreatment with sulfuric acid resulted in a pretreatment yield of 54.5%. With the yield and composition data shown in Table S2, it was possible to calculate the solubilization of each macromolecular fraction. The pretreatment resulted in solubilization of 14.8%, 95.6%, and 8.6% cellulose, hemicelluloses, and lignin, respectively. Analyzing Table S2 shows that the acetyl group can be quantified as acetic acid through the solubilization/degradation of hemicelluloses (Palmqvist and Hahn-Hägerdal, 2000) and was almost wholly solubilized. The hemicellulosic fraction was preferentially solubilized due to the chemical characteristics of the pre-treatment. The results of the chemical composition of the hydrolysate are presented in Table S3. Analyzing the composition results of the hemicellulosic hydrolysate, it is possible to observe that the most prominent inhibitors in the medium are acetic acid and phenolic compounds.

Table 3: Composition of the hemicellulose hydrolysate from the pre-treatment with dilute sulfuric acid.

Compounds	Concentration (g/L)
Glucose	5,62 ± 0,12
Xylose	10,78 ± 0,41
Arabinose	2,02 ± 0,080
Cellobiose	0,28 ± 0,021
Formic acid	1,25 ± 0,041
Acetic Acid	5,98 ± 0,062

HMF	0,150 ± 0,0020
Furfural	0,45 ± 0,0010
Total phenolic compounds	1,72 ± 0,12
Vanillic Acid	0,0980 ± 0,004
p-coumaric acid	0,579 ± 0,01
Syringic acid	0,190 ± 0,01
Xylooligosaccharides	0,00

The LCH was prepared for use in the experiments, adjusting its pH to 5.5 and 4.6 with KOH and filtered through a 0.22µm filtering membrane. Partial data of the quantification of inhibitor compounds, before and after this process, is presented in Table S4:

Table 4: Concentrations of HMF, Furfural, and phenolic compounds present in hydrolysate samples

	Crude hydrolysate	Hydrolyzed pH 5.5	Hydrolyzed pH 4.6
Phenolic compounds			
Vanillic Acid	0,098	0,094	0,089
Coumaric Acid	0,579	0,543	0,540
Syringic Acid	0,190	0,157	0,149
HMF	0,150	0,170	0,180
Furfural	0,450	0,430	0,429

Section 3

Pilot experiments

1. Serial Transfer protocol - Pilot experiment

The pilot test evaluated yeast growth in 5 mL of hydrolyzate at a concentration of 25 and 30% for haploid CEN.PK113-7D and diploid H4, respectively, while the inoculum was tested in a volume of 25 μ L and 50 μ L (Figure 11), until stationary phase cultivation.

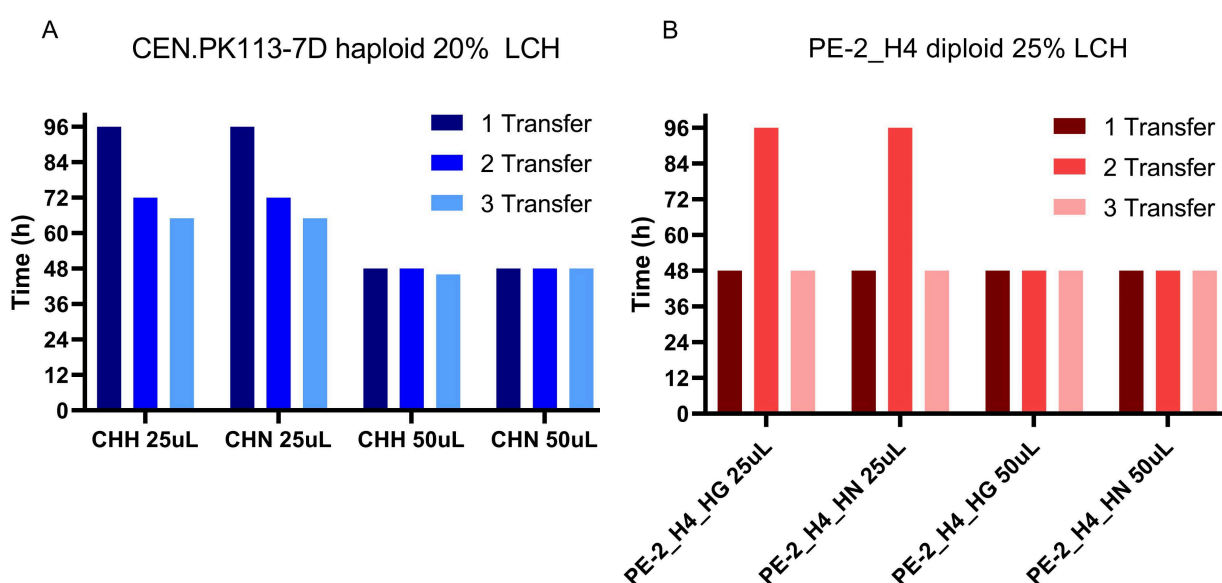


Figure 5: ALE Pilot experiment with different hydrolyzate concentrations and amounts of inoculum over 3 serial transfers. A- Observation of the growth of haploid *Saccharomyces cerevisiae* CEN.PK113-7D with 25uL and 50uL of transfer in duplicate (CHH and CHN). B- Observation of the growth of diploid *Saccharomyces cerevisiae* PE-2 H4 with 25uL and 50uL of transfer in duplicate (PE-2_H4_HG and PE-2_H4_HN)

2. Shock/Recovery protocol - Pilot experiment

A pilot test was conducted to evaluate the response of the haploid *S. cerevisiae* CEN.PK113-7D yeast under a period of exposure to 100% hydrolyzate for 24 hours. Selecting a 24-hour time frame provides initial insights into the viability and response of samples to the experimental stimulus.

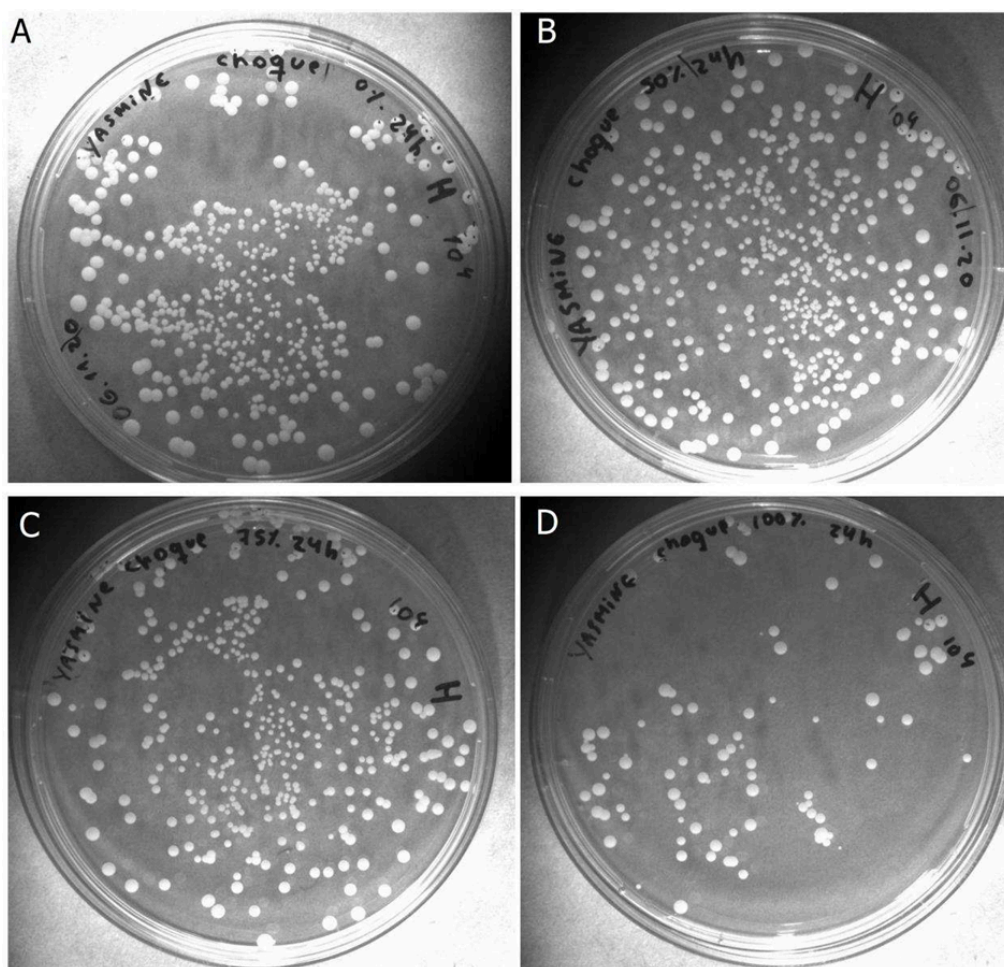


Figure 6: Pilot test of the 24-hour shock. A- 0% hydrolyzed (469 colonies). B- 50% hydrolyzed (432 colonies). C- 75% hydrolyzed (368 colonies). D- 100% hydrolyzed (84 colonies).

In the pilot test, the survival rate was evaluated by counting colonies (CFU) on agar plates with different concentrations of the hydrolyzate, and the survival rate for 100% in 24 was ~17.910%, using the following formula:

Surviving Rate (%) =

(number of surviving colonies/number of Initial colonies) x 100

17,91044776119403 =

(84/469) x 100

To achieve a rate below 10%, an additional pilot test was carried out, increasing the exposure time to 48 hours. The results obtained can be viewed below.

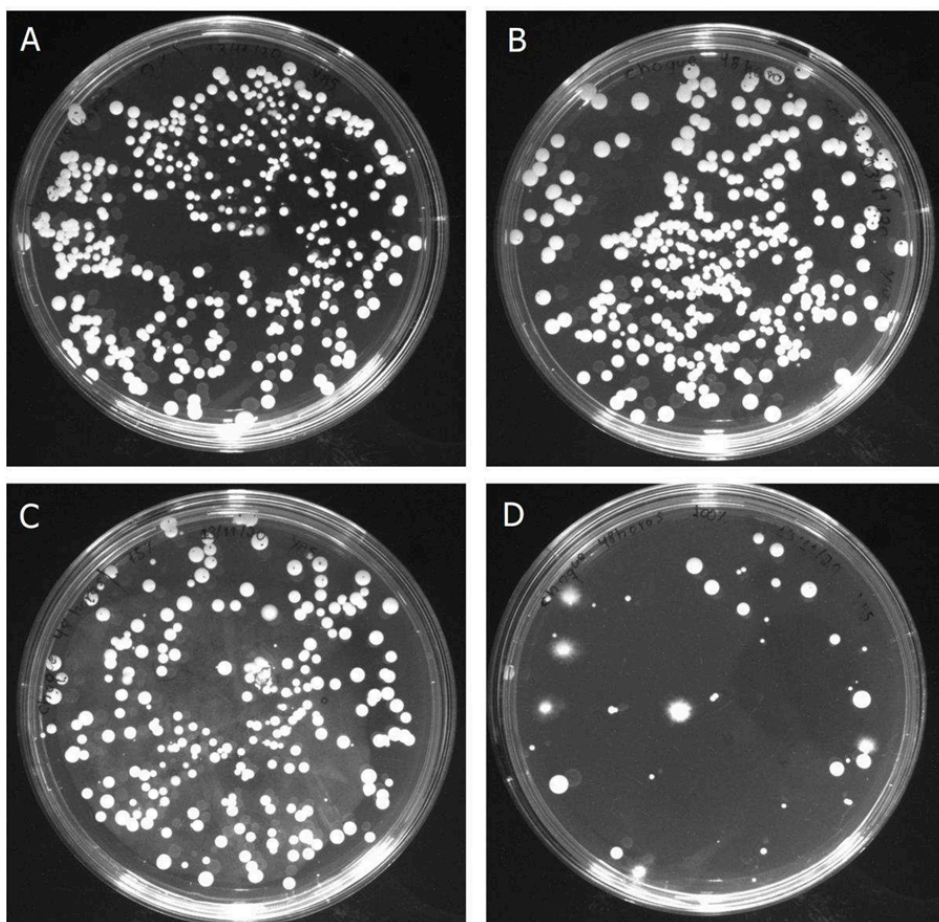


Figure 7: 48-hour shock pilot test. A- 0% hydrolyzed (395 colonies). B- 50% hydrolyzed (353 colonies). C- 75% hydrolyzed (225 colonies). D- 100% hydrolyzed (35 colonies).

The 48-hour test under 100% exposure demonstrated that the survival rate was ~8,86%. Given this result, the experiment continued under these conditions.

$$8,860759493670886 = \\ (35/395) \times 100$$

Section 4

Checking figures

1. Asci - genome shuffling protocol

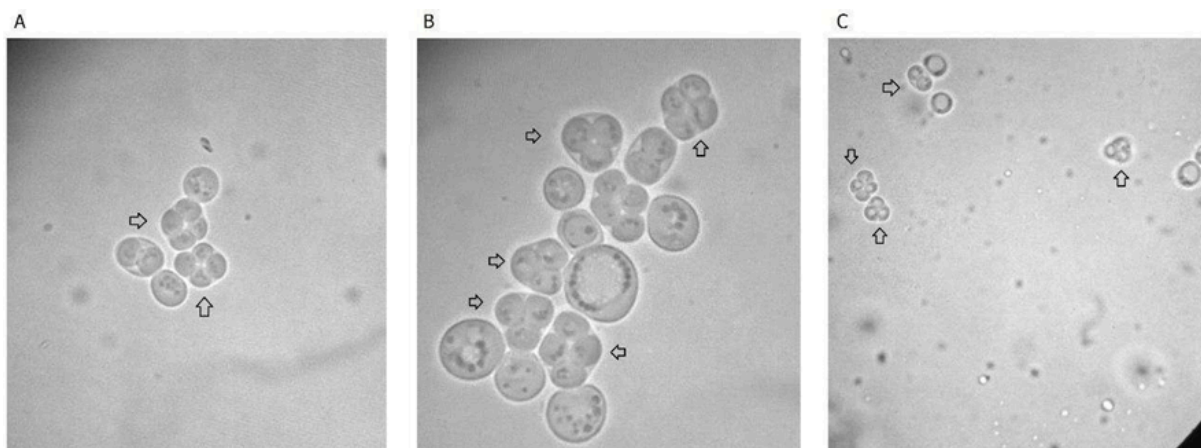


Figure 8: Asci resulting from sporulation of diploid PE-2_H4 populations. A-Pe-2H4_HG; B- Pe-2H4_HN; C-Pe-2H4_HZ. Arrows indicate asci with good visualization of spores.

2. Contamination Control

The periodic use of geneticin (CHG, CDG, Pe-2H4_HG, and S_CHG), nourseothricin (CHN, CDN, Pe-2H4_HN and S_CHN), hygromycin (CHH and S_CHH) and zeocin (Pe-2H4_HZ) prevent external and cross-contamination of populations, while hygromycin is also used to select for the diploid state (CDG, CDN, Pe-2H4_HG, Pe-2H4_HN and Pe-2H4_HZ). In this way, it is ensured that only successful mating and diploidization events are propagated after episodes of genome shuffling.

Furthermore, every 30 days the cells are tested against all antibiotics, to eliminate the possibility of any cross-contamination between strains and populations (Figure S. 13).

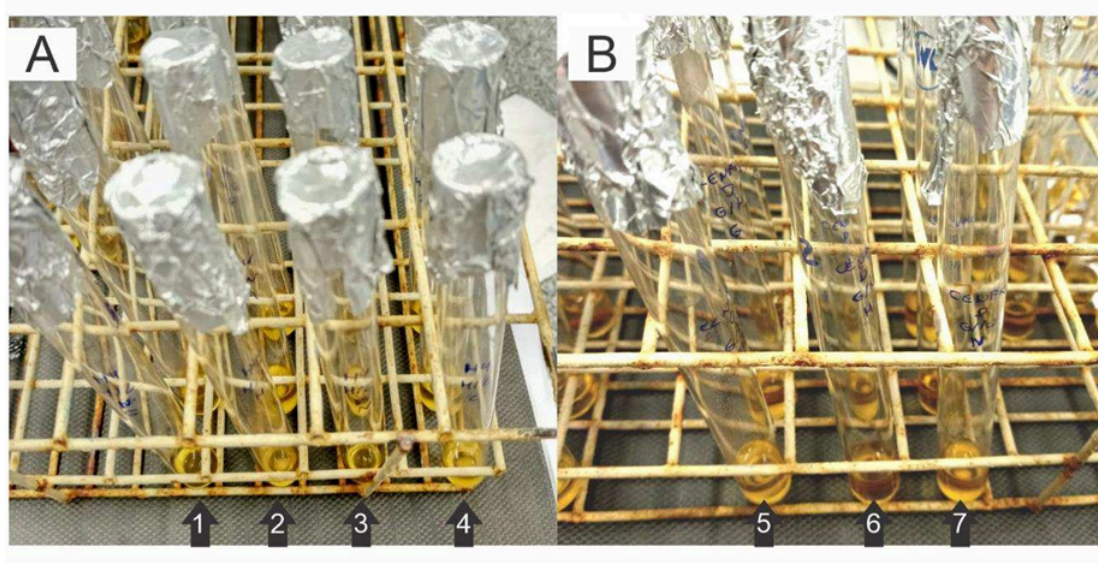


Figure 9: Contamination test carried out periodically (every 30 days). A- PE-2_H4 Diploid. 1- Pe-2H4_HZ in the antibiotic geneticin (-). 2- Pe-2H4_HZ in the antibiotic hygromycin (+) 3- Pe-2H4_HZ in the antibiotic nourseothricin (-). 4- Pe-2H4_HZ in the antibiotic zeocin (+). B- CEN.PK113-7D Diploid. 5- CDG in the antibiotic geneticin (+). 6- CDG in the antibiotic nourseothricin (-). 7- CDG in the antibiotic hygromycin (+). The sign (+) indicates that growth has been identified. The (-) sign indicates that no growth was identified.

Section 5

GFP-transformed strains

The CRISPR/Cas9 technique helped to introduce the GFP gene into the HO locus of CEN.PK113-7D (FLO8:NatMX) and Pe-2 H4 (Figure 10).

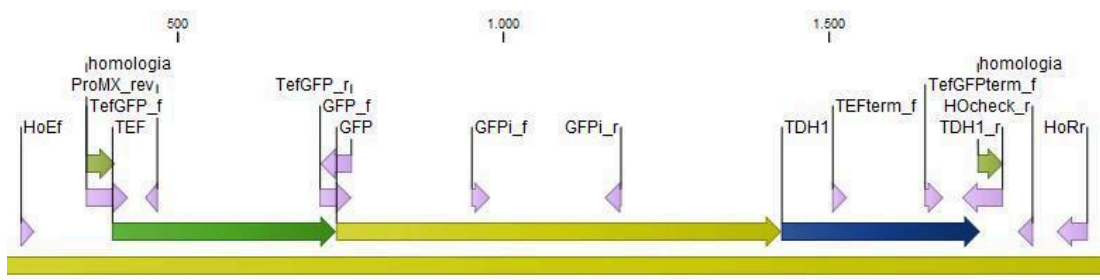


Figure 10: Graphical representation of the GFP construct inserted into the HO locus. The GFP Construct is shown inserted into the HO locus (yellow bar below). It contains the pTEF promoter (green), the GFP coding sequence (yellow), and the tTDH1 terminator (blue). Regions of homology to the HO locus are shown in lime green. Primers for the PCRs are indicated in lilac.

PCR reactions for cloning were performed with Phusion® High-Fidelity DNA Polymerase (New England BioLabs). Check-in PCRs were performed with Taq DNA polymerase (ThermoFisher Scientific). The oligonucleotides used as primers in the PCR reactions are indicated in Table 5. The confirmation PCR can be seen in Figure 11.

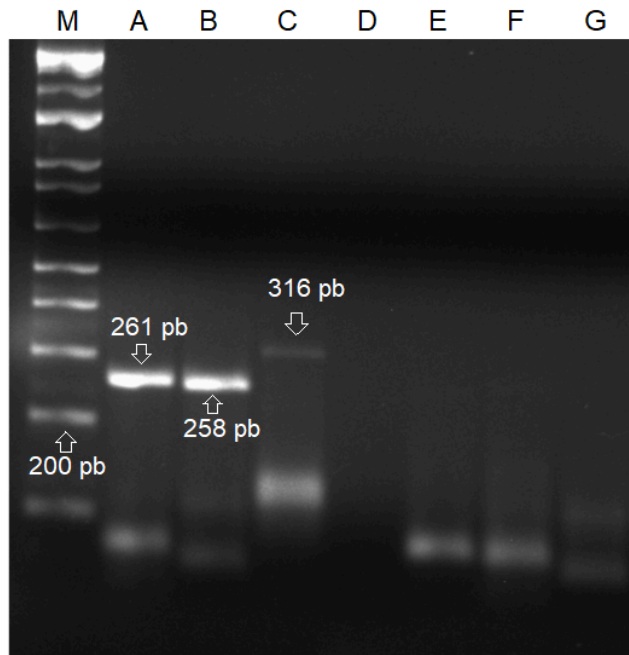


Figure 11: Electrophoretic run profile (2% agarose gel) of the confirmatory PCR of the insertion of the GFP construct in the HO locus of the CEN.PK113-7D lineage (FLO8:NatMX). M- Molecular weight marker: 1 Kb Plus DNA Ladder (Invitrogen). A- PCR products with ProMxRev/Ho.Tef2F (261pb) B- GFP.tef F / R (258pb) primers. C- PCR with GFP.int F / R primers (316pb) D- Empty E- Blank ProMxRev/Ho.Tef2F F- Blank GFP.tef F / R (258pb). G- White GFP.int F/R (316pb).

From haploid populations of PE-2_H4 with GFP and CEN.PK113-7D (FLO8:NatMX; HO::GFP), diploid strains were constructed (Fig. 12). This was done through the ectopic expression of the endonuclease HO method, previously reported.

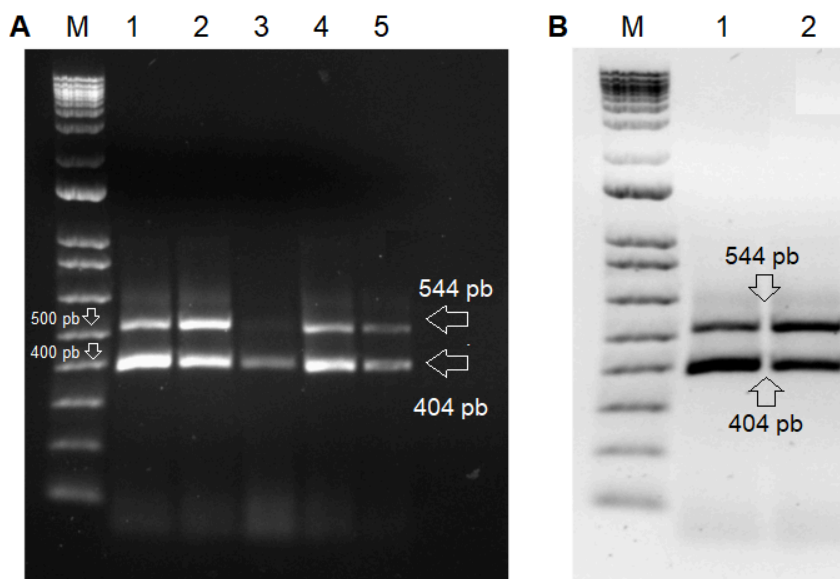


Figure 12: Electrophoretic run profile on 1.5% agarose gel of different colonies tested for mating type. A. Identification of PE-2_H4 (HO:GFP) diploids, *mating type a* - 544 pb; *mating type alfa* - 404 pb; diploid - both *mating types*. B. Identification of diploids of CEN.PK113-7D (FLO8:NatMX; HO:GFP). M - Molecular weight marker: DNA Ladder 1kb Plus Invitrogen.

Table 5: Oligonucleotides used in the development of strains with GFP.

Primers	Sequence
JacA_HO1	CGCTGCGTGCCAAACATGTAGTTTTAGAGCTAGAAATAG CAAGTTAAAATAAGG
JacB_HO1	TACATGTTTGGCACGCAGCGGATCATTATCTTTCACTG CGGAGAAGTTTCGAACGCC
JacA_HO2	GCCGGCTTGATCGACTCAGAGTTTTAGAGCTAGAAATAG CAAGTTAAAATAAGG
JacB_HO2	TCTGAGTCGATCAAGCCGGCGATCATTATCTTTCACTG CGGAGAAGTTTCGAACGCC
TefGFP_f	TTGATGGTAGAATAATAATAATTCCAAAAAACCATCATAAG ACATGGAGGCCCAAGAATACCCTCCTTG
TefGFP_r	CATTTCTTCTTTGATGACACTGACCATGGTTGTTTATGTT CGGATGTGATGTGAG
GFP_f	CTCACATCACATCCGAACATAAACAACCATGGTCAGTGT CATCAAAGAAGAA
TDH1_r	CAATTCCGTCCATAATGGATGAATAAACAGTTTGAATTGC AAAGGCAGCAATATATTGTTTACTTAT
GFPI_f	CTAAGTACCCAGCCGATATCCCAGATTAC
GFPI_r	TCATTTCCGGTGGATGGTTCCCACTTC
HoEf	TTGTCAGTCAGGGTCCCTACC
HoRr	AAAGTTGTTCCCCCAGCAACATTACAGTCGTATGTAAAT TGACATTGG
ProMX_r	CGACAGTCACATCATGCCCC
TEFterm_f	AGACGTAAATAAGTACATATTAC
HOcheck_r	CTGGTCGTCACAGTAGCTGACATA
TefGFPterm	GAATGTGGTGAATTGAAGTTCTTGGATGG

Section 6

Table 6 - Mutations found in the population CEN.PK 113-7D CHG population

CHG			
Chromosome	Frequency (%)	Gene	SNP
4	99,04	<i>SIZ1</i>	Gln381*
5	100	<i>GEA2</i>	mutação sinônima
12	100	<i>UBR2</i>	*1283Ser
13	100	<i>ADH6</i>	Ser131Leu
15	100	<i>RET1</i>	Lys957Asn
16	100	<i>MUK1</i>	Val232Asp

Table 7 - Mutations found in the CEN.PK 113-7D CHH population

CHH			
Chromosome	Frequency (%)	Gene	SNP
4	100	<i>SIZ1</i>	Gln394Lys
5	100	<i>CHD1</i>	Arg1071*
5	99,48	<i>WPB1</i>	mutação sinônima
8	35,16	<i>PRS3</i>	mutação sinônima
10	78,41	<i>IMA5</i>	Met521fs
11	100	<i>ABF1</i>	Pro100Leu
11	100	<i>FAS1</i>	Asp2028His

13	100	<i>ECM5</i>	mutação sinônima
15	100	<i>RET1</i>	Gly783Cys
15	100	<i>PEP12</i>	Glu163*
16	51,28	<i>YLH47</i>	Met85Thr

Table 8 - Mutations found in the CEN.PK 113-7D CHN population

CHN			
Chromosome	Frequency (%)	Gene	SNP
4	100	<i>YDR541C</i>	Lys257Asn
4	100	<i>SIZ1</i>	Ser132*
5	69,61	<i>GCN4</i>	Glu95*
5	26,94	<i>GCN4</i>	Leu84fs
5	100	<i>CHD1</i>	Tyr1459Cys
11	62,41	<i>LST4</i>	Phe15Leu
12	100	<i>TUB4</i>	Asp395Asn
14	100	<i>SQS1</i>	Glu709Val
15	63,91	<i>PHM7</i>	Leu300Ser
15	81,75	<i>HRK1</i>	Pro154fs
16	100	<i>LOA1</i>	mutação sinônima
16	84,8	<i>MUK1</i>	Asn489fs

Table 9 - Mutations found in the population CEN.PK 113-7D CDG

CDG			
Chromosome	Frequency (%)	Gene	SNP
2	100	<i>EDE1</i>	Leu333Phe
4	100	<i>UPC2</i>	Lys475Glu
5	100	<i>VMA8</i>	Glu95*
5	100	<i>HYP2</i>	Lys110Asn
10	99,05	<i>TRK1</i>	Thr995Lys
16	100	<i>CUP9</i>	[Asn239Thr
16	84,8	<i>MUK1</i>	Asn489fs

Table 10 - Mutations found in the CEN.PK 113-7D CDN population

CDN			
Chromosome	Frequency (%)	Gene	SNP
8	19,49	<i>DNA2</i>	Asn1169Lys
11	21,77	<i>YKL077W</i>	Val319Phe
15	27,78	<i>HRK1</i>	Pro154fs
16	53,77	<i>CUP9</i>	Asn239Thr
16	22,02	<i>SRP54</i>	Met364Arg

Table 11 - Mutations found in the PE-2_H4_HG population

PE-2_H4_HG			
Chromosome	Frequency (%)	Gene	SNP
3	57,79	<i>EO220_05445</i>	
4	29,9	<i>RPN4</i>	Leu319fs
4	25,96	<i>NGG1</i>	Tyr261fs
6	62,39	<i>MDS3</i>	Gln938*
6	85,83	<i>GCN1</i>	Leu1711His
13	28,95	<i>YAP1</i>	Asn33fs
13	85,05	<i>SPT21</i>	Glu144Asp
14	61,24	<i>SSB2</i>	Arg545fs
14	55,24	<i>ALF1</i>	Ala62fs
15	21,71	<i>RTG1</i>	Tyr170*
15	43,64	<i>WHI2</i>	Leu76fs
16	81,95	<i>HOB2</i>	Leu1028His

Table 12 - Mutations found in the PE-2_H4_HN population

PE-2_H4_HG			
Chromosome	Frequency (%)	Gene	SNP
3	53,52	<i>EO220_05445</i>	
4	35,25	<i>SIZ1</i>	Asp345Gly
5	14,79	<i>HPA3</i>	Ser13fs
7	45,45	<i>PMA1</i>	Ala639Leu
8	16,79	<i>PRP8</i>	Asp765Val
9	21,43	<i>RPI1</i>	Thr214fs
10	16,67	<i>ARP4</i>	Val408Ile
13	41,91	<i>GAL80</i>	Glu348*
13	58,71	<i>SPT21</i>	Glu144Asp
13	.80	<i>EO220_42330</i>	Glu49fs
15	31,14	<i>EXO1</i>	Asp502Tyr
15	47,37	<i>WHI2</i>	Asn275fs
15	25,85	<i>YRR1</i>	Asn125fs
15	54,2	<i>HRK1</i>	Asn725fs
15	32,59	<i>HRK1</i>	Ile671fs
16	13,48	<i>EO220_52920</i>	Cys389Arg
16	39,86	<i>SKS1</i>	Lys82fs
16	27,74	<i>HDA3</i>	Lys227Asn

Table 13 - Mutations found in the PE-2_H4_HZ population

PE-2_H4_HZ			
Chromosome	Frequency (%)	Gene	SNP
1	80,3	<i>CLN3</i>	Ser468Pro
2	31,76	<i>CDC27</i>	Ile382insAsn
2	34,88	<i>AKL1</i>	Val95Ala
2	88,24	<i>PHO89</i>	Asn563Lys
3	57,98	<i>EO220_05445</i>	
3	35,51	<i>SRB8</i>	Lys1304Asn
5	38,81	<i>UBP3</i>	Leu443fs
6	34,25	<i>EPL1</i>	Asn515fs
7	61,33	<i>PMA1</i>	Gly158Ser
8	22,06	<i>OSH3</i>	Asp925Gly
8	30,81	<i>GND1</i>	Met360del
9	38,14	<i>ASG1</i>	Lys661Arg
9	29,84	<i>MNT3</i>	Ser398Leu
11	36,13	<i>APL2</i>	
13	25,68	<i>EO220_42330</i>	Glu49fs
13	41,8	<i>TRS130</i>	Asn789Lys
14	71,31	<i>SSB2</i>	Val492Gly
15	69,71	<i>EXO1</i>	Asp502Tyr
15	73,33	<i>HRK1</i>	Pro155fs

Table 14 - Oligonucleotides used in the development of reverse engineering strains

Primers	Sequence
gA_SIZ1	AGTGGGAGACTGCCTAATAAGTTTTAGAGCTAGAAATAG CAAGTT
gB_SIZ1	TTATTAGGCAGTCTCCCACTGATCATTTATCTTTCACTGC GGAGAAG
SIZ1DonF	TCTATGGAGGGGCCTCCACGGTGCAGCAGCAATCACC TTAACGTCTACCACATCTTCCACATC
SIZ1DonR	CATCACTAATTCAGGGATTAGCC
SIZ1CheckF2	GATAGAACGGATCAGAAAGAATGAA
SIZ1CheckR2	GATGATGAAGAAGAAGAGGCATCTGG
gA_CDH1	CGCCTCAGCCCATATAGTTCGTTTTAGAGCTAGAAATAG CAAGTT
gB_CDH1	GAACTATATGGGCTGAGGCGGATCATTTATCTTTCACTG CGGAGAAG
CDH1DonF	ATGGCAGCCAAGGATATTTCTACTGAAGTGCTGCAAAT TAGAGCTGCTGCGCATCAACAAAAC
CDH1DonR	TCAAATTCGTCTTCGTCTCTTC
CHD1CheckF	CCTATACCCAATTCAAAGCAGAACC
gA_MUK1	GTTCTTGATGATTCCCTGAGTTTTAGAGCTAGAAATAGC AAGTT
gB_MUK1	TCAGGGAATCATACAAGAACGATCATTTATCTTTCACTGC GGAGAAG
MUK1DonF	ACTCCTCCGATAACCAATCCAAGGTTTGACCCTAATCAA TGGCATGCAATTTCAACAAAATCTACAT
MUK1DonR2	GGAGGTAAATTGGTGACAGTGTTCAACGC
MUK1CheckR	CGTACTTTGGCTGCTTTAGGTCATC
MUK1CheckF2	ACAATTATTCACTCCTCCGATAACC
MUK1CheckF3	CCATCAGGGAATCATACAAGAACAC
gA_RET1	TCTTTCTACGTGTTTCGCAAGTTTTAGAGCTAGAAATAGC AAGTT

gB_RET1	TTGCGAAACACGTAGAAAGAGATCATTATCTTTCACTG CGGAGAAG
DonRET1_f	CATAACCAATCCCCTCGTAACACATATC
DonRET1_r	ATCTTTTCAAACAGTGGTTGTCTTTCTACGTGTTTCGC AtCGaCaGAAACCTCTATC
RET1Sall_f	AAGAGAATAGATACATTATTATACTTGtcGACTTATCCACA ACAGCCAA
RET1Sall_r	CAAACAGTGGTTGTCTTTCTACGTGTTTCGCgtCG
Ret1seq_f	CAATTTTAGGTGCTGTTGCTGGTT
Ret1seq_r	TCATACCACCGATGATATCTTGG
gA_HRK1	GTTCTTGTGAGACCAACTGAGTTTTAGAGCTAGAAATAG CAAGTT
gB_HRK1	TCAGTTGGTCTCACAAGAACGATCATTATCTTTCACTG CGGAGAAG
HRK1Donf	AATACGGGAAATTGGGTAAGTTATTGGGTTCGGTGCC GGGAGAGTGTG
HRK1Donr	ATTCTGCGGTGCACTTCTTGGCATATTCTTTCACACTCT CCCGGCACCG
HRKCheck-f	TCACGATGATTGTATTCTGGCTC
HRKCheck-r	ATCGGACAGTACTCCATGACTTCA
gA_PMA1	TTAATGTTAAACGCTGGTGTGTTTTAGAGCTAGAAATAGC AAGTT
gB_PMA1	ACACCAGCGTTTAACATTAAGATCATTATCTTTCACTGC GGAGAAG
DonPma1f	CGGTGTTATCTGTGGTTTGTTAATGTTAAACGCTGGTGT TaGTTTCGTTCAAGAgTT
DonPma1r	AAGTCTTTTTCAATTCATCGACAATAGAACCAGCTTGGA AcTCTTGAACGAAAcA
Pma1Ecof	GAATCAAATGGCTGACGAAAAAGAATtCTTTAGTCG
Pma1Ecor	GACCGTCTCTGATAACAACAGCAGTG
Pam1Seq	GGTCCAATTCAATTCGTTATGGA
gA_PDR3	CAGTTGTCGCCTAAGACCTAGTTTTAGAGCTAGAAATAG CAAGTT

gB_PDR3	TAGGTCTTAGGCGACAACCTGGATCATTTATCTTTCCTG CGGAGAAG
TefPDRf	TATTTCTGTGTTTGTGTCTTCAGGAGCCTTCCAAAGCA AATAGCTTCAAAATGTTTCTACTCCT
TefPDRr	CTGTCGAAACTTTTGATCTAGTTGATTTCTTCACTTTCAT TTTGTAATTAAACTTAGATTAGA
pPDRf	GAACTATCACAGTGAGTCTTAGAA
PDR3r	AACAGCTGTAGTAGGCAAAGACTGG
TEF1pro_r2	GAGGGGAAATTTAGTATGCTGTGCTTGG
TEF1pro_f1	GAAAGCATAGCAATCTAATCTAAG

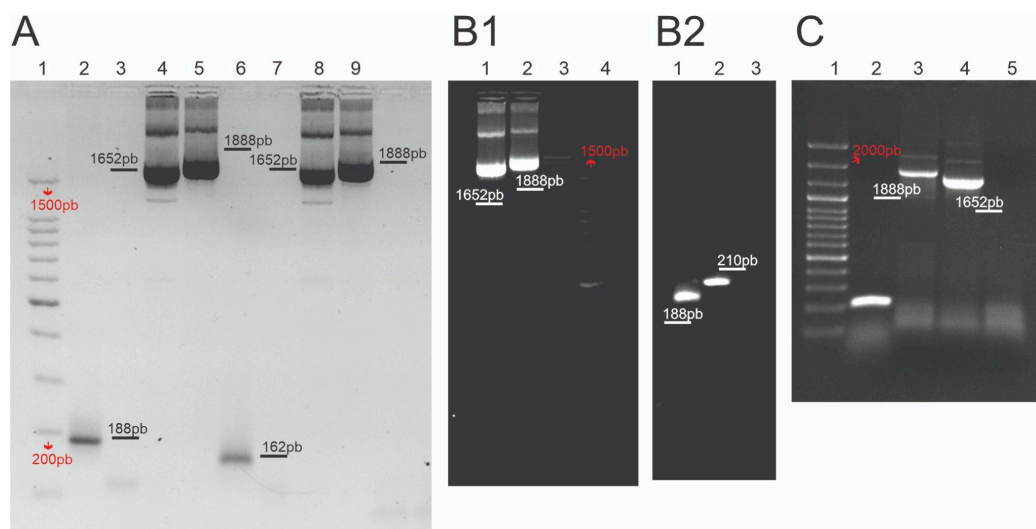


Figure 13: Amplifications of guides and donor fragments. A-1.1kb promega marker, 2. Donor SIZ1 (188bp), 4. pEasyG3-nat (1,652 bp) for SIZ1, 5. pEasyG3-mic (1,888 base pairs) for SIZ1. 6. Donor CHD1 (162bp), 8. pEasyG3-nat (1,652 bp) for CHD1, 9. pEasyG3-mic (1,888 base pairs) for CHD1. B1-1. pEasyG3-nat, for MUK1, 2.pEasyG3-mic, for MUK1. 4. 1kb marker promega. B2-1. 188bp marker, 2. Donor MUK1 (210bp). C-1. 100bp ladder promega, 2. Donor MUK1 (210 bp) run again, 3. pEasyG3-na, for HRK1 4. pEasyG3-nat, for HRK1.

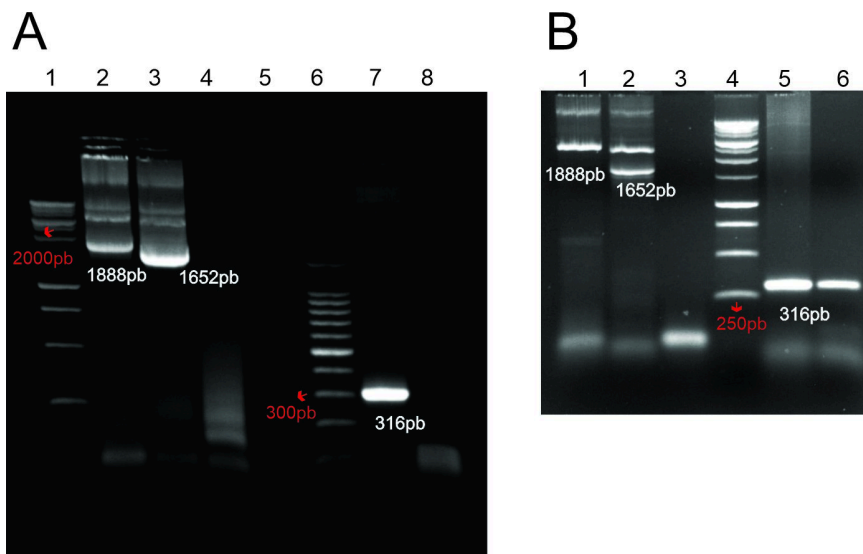


Figure 14: Amplifications of guides and donor fragments for RET1 transformation. A-1.1kb plus ladder marker, 2. pEasyG3-mic (1,888 base pairs) for RET1, 3. pEasyG3-nat (1,652 bp) for RET1. 6. 100bp ladder promega marker, 7. Donor RET1 (316bp). B-1. pEasyG3-mic (1,888 base pairs) for RET1 in SIZ1 Δ , 2. pEasyG3-nat (1,652 bp) for RET1 in SIZ1 Δ . 4. Promega 1kb DNA ladder marker, 5. Donor RET1 (316bp).

Section 7

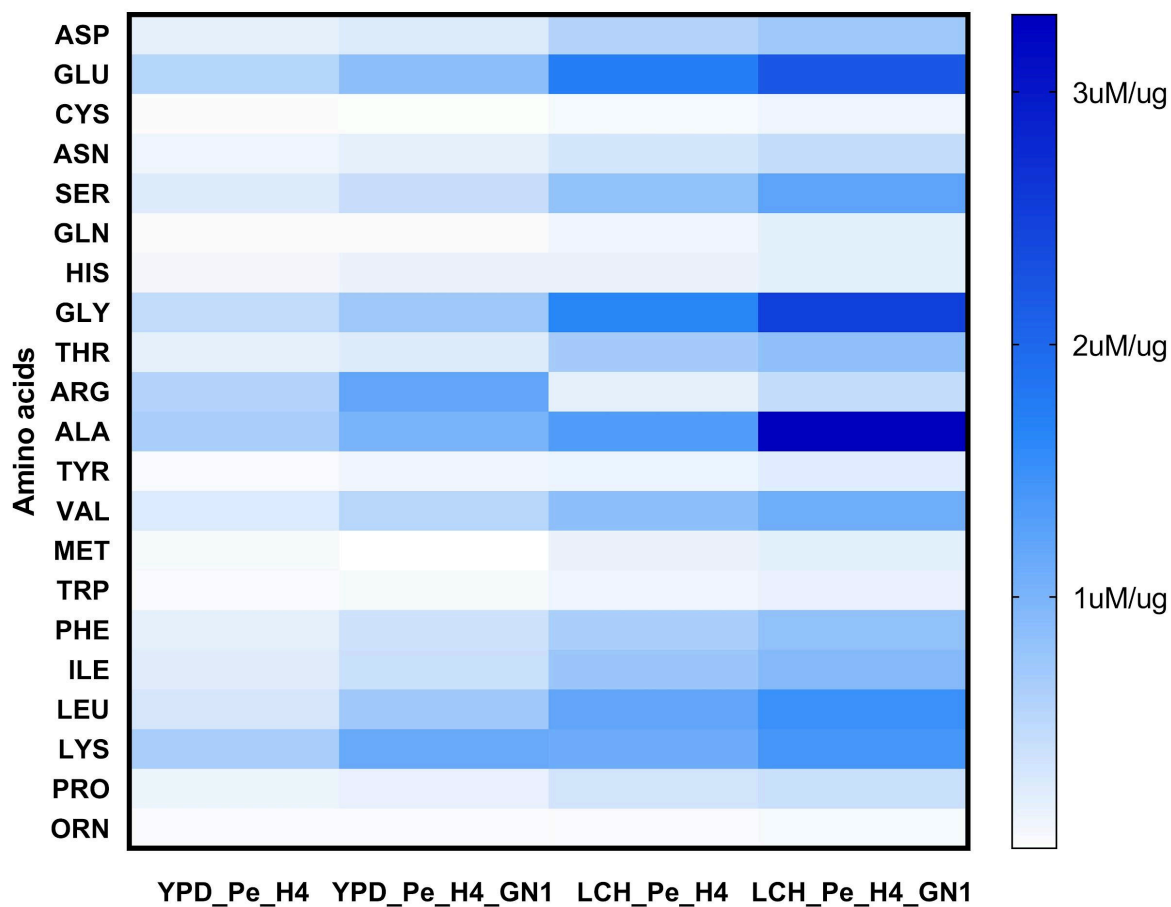
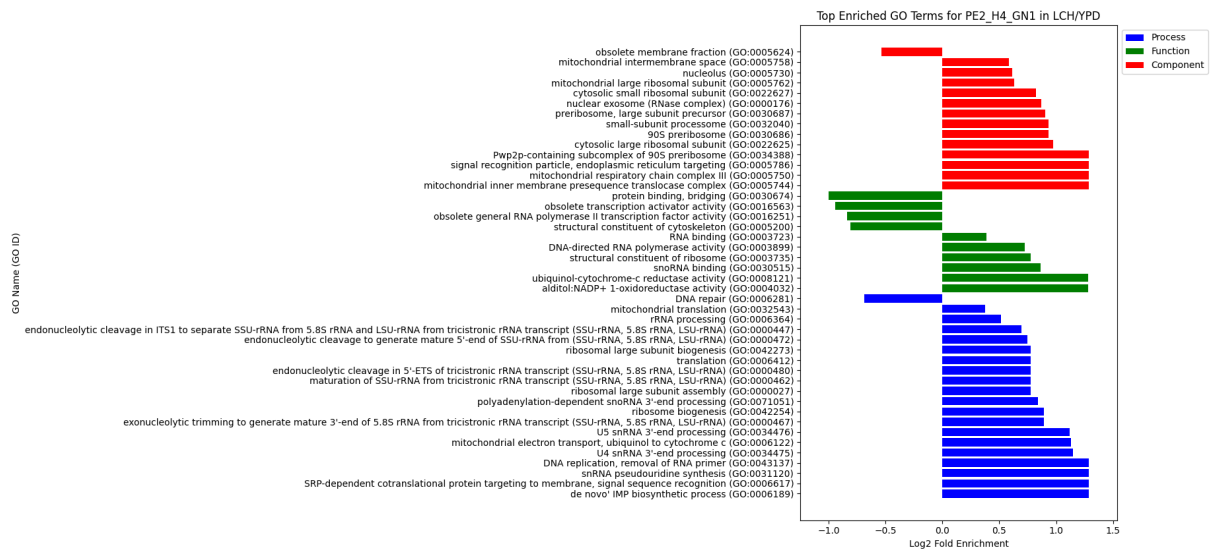


Figure 15:: Intracellular amino acids in the PE2_H4_GN1, and PE2_H4 (WT) strain. The visual representation, through heatmap, highlights the changes in amino acid concentration. Each line of the heatmap corresponds to an amino acid, identified by its acronym. Aspartate (ASP), Glutamate (GLU), Cysteine (CYS), Asparagine (ASN), Serine (SER), Glutamine (GLN), Histidine (HIS), Glycine (GLY), Threonine (THR), Arginine (ARG), Alanine (ALA), GABA, Tyrosine (TYR), Valine (VAL), Methionine (MET), Tryptophan (TRP), Phenylalanine (PHE), Isoleucine (ILE), Ornithine (ORN), Leucine (LEU), Lysine (LYS), Proline (PRO), and Ornithine (ORN).

A



B

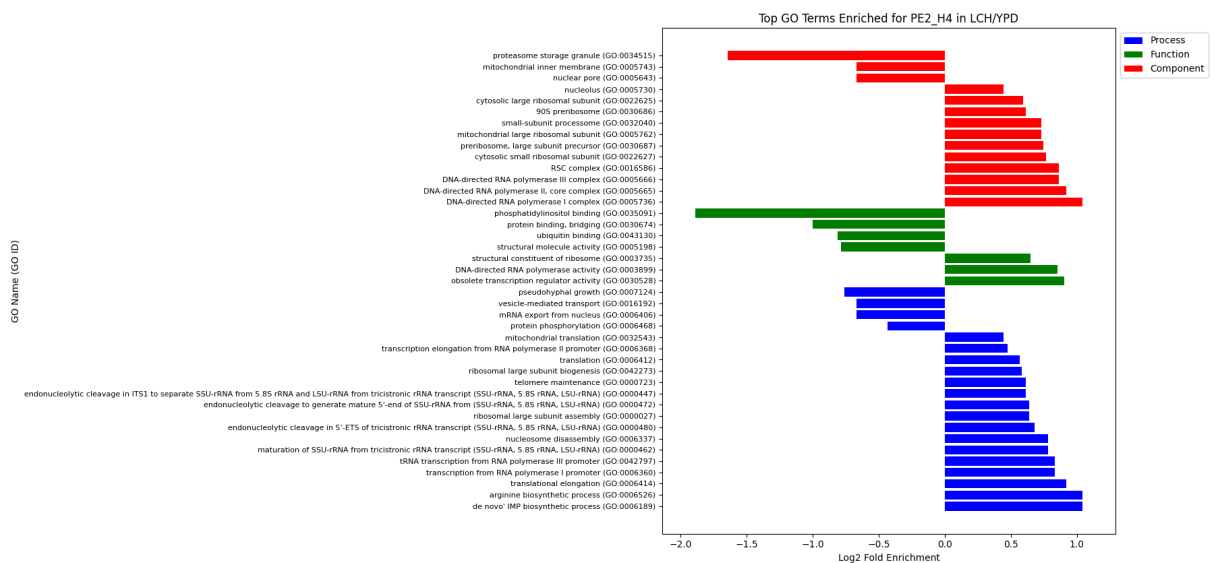


Figure 16: Differential enrichment of GO terms: A. Log2 Fold Enrichment of the ratio of PE2_H4_GN1 in LCH x in YPD. B. Log2 Fold Enrichment of the ratio of PE2_H4 in LCH x YPD. Terms in blue represent Biological Processes, in green are Molecular Functions, and in red are Cellular Components.