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Improving Stress Tolerance in Industrial Saccharomyces cerevisiae Strains for Ethanol Production from Lignocellulosic Biomass

Wallace, Valeria

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Improving stress tolerance in industrial *Saccharomyces cerevisiae* strains for ethanol production from lignocellulosic biomass

Valeria Wallace Salinas
Division of Applied Microbiology
Department of Chemistry
Faculty of Engineering
Lund University

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Title and subtitle **Improving stress tolerance in industrial *Saccharomyces cerevisiae* strains for ethanol production from lignocellulosic biomass**

Abstract

The present work was aimed at developing industrial *S. cerevisiae* strains with improved tolerance to two types of stressors encountered during the fermentation of lignocellulosic biomass that affect ethanol yield and productivity, namely hydrolysate-derived inhibitors and high temperature, and at understanding the response of yeast and mechanisms of adaptation to such stressors. In one part of the study, key amino acid substitutions that were responsible for the acquired ability of a mutated yeast enzyme to convert HMF, one of the lignocellulosic inhibitors (LI), into a less inhibitory compound were identified in the active site of the enzyme. The specific properties of the mutant were investigated. In the second part of the thesis, different strategies were applied to develop yeast strains with increased tolerance to combined stresses. In one approach, the effects of two targeted proteins that were previously shown to be involved in the response to oxidative stress in laboratory yeast strains were re-evaluated under process-mimicking conditions, i.e. using a robust industrial strain background and fermenting highly inhibitory spruce hydrolysate. The beneficial effects on tolerance to LI were confirmed in the industrial strain, but they were shown to be strain-dependent and limited to the fermentation of 6-carbon sugars (C6); unexpected negative interactions were also identified for one of the candidates in the fermentation of C5 sugars. The second approach focused on improving the tolerance to high temperature in the presence of LI. A strain with combined tolerance to both stressors was obtained by long-term adaptation. In contrast to its parental strain, the evolved strain was capable of growing and fermenting C6 in the presence of LI and at high temperature (39°C). Possible mechanisms behind the improved performance of this strain were investigated using genome-wide approaches. Significant differences were found in lipid composition, which correlated with changes at the genome level in different genes involved in lipid transport, synthesis, and other steps of lipid metabolism, thereby indicating that alterations in membrane composition may be behind the improved combined tolerance. Overall, the work performed for this thesis resulted in the development of several strains with improved characteristics that are suitable for fermentation of LI. The work has also contributed to a better understanding of the mechanisms of stress response in yeast.

Key words: *Saccharomyces cerevisiae*, lignocellulose, ethanol, inhibitors, temperature, stress tolerance

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Valeria Wallace Salinas
Division of Applied Microbiology

Division of Applied Microbiology
Department of Chemistry
Faculty of Engineering
P.O. Box 124
SE-221 00 Lund
Sweden

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To my family

Abstract

The present work was aimed at developing industrial *S. cerevisiae* strains with improved tolerance to two types of stressors encountered during the fermentation of lignocellulosic biomass that affect ethanol yield and productivity, namely hydrolysate-derived inhibitors and high temperature, and at understanding the response of yeast and mechanisms of adaptation to such stressors. In one part of the study, key amino acid substitutions that were responsible for the acquired ability of a mutated yeast enzyme to convert HMF, one of the lignocellulosic inhibitors (LI), into a less inhibitory compound were identified in the active site of the enzyme. The specific properties of the mutant were investigated. In the second part of the thesis, different strategies were applied to develop yeast strains with increased tolerance to combined stresses. In one approach, the effects of two targeted proteins that were previously shown to be involved in the response to oxidative stress in laboratory yeast strains were re-evaluated under process-mimicking conditions, i.e. using a robust industrial strain background and fermenting highly inhibitory spruce hydrolysate. The beneficial effects on tolerance to LI were confirmed in the industrial strain, but they were shown to be strain-dependent and limited to the fermentation of 6-carbon sugars (C6); unexpected negative interactions were also identified for one of the candidates in the fermentation of C5 sugars. The second approach focused on improving the tolerance to high temperature in the presence of LI. A strain with combined tolerance to both stressors was obtained by long-term adaptation. In contrast to its parental strain, the evolved strain was capable of growing and fermenting C6 in the presence of LI and at high temperature (39°C). Possible mechanisms behind the improved performance of this strain were investigated using genome-wide approaches. Significant differences were found in lipid composition, which correlated with changes at the genome level in different genes involved in lipid transport, synthesis, and other steps of lipid metabolism, thereby indicating that alterations in membrane composition may be behind the improved combined tolerance. Overall, the work performed for this thesis resulted in the development of several strains with improved characteristics that are suitable for fermentation of LI. The work has also contributed to a better understanding of the mechanisms of stress response in yeast.

Popular scientific summary

Oil resources are finite; and political and economic factors affecting oil production, together with the environmental damage associated with the increased use of oil by a rapidly growing population, have underscored the need for developing cleaner technologies based on sustainable resources. In this context, any lignocellulosic biomass that is not used in the food chain, such as woody crops and agricultural and forestry by-products, is an appealing raw material for the production of so-called "second-generation (2G) biofuels", and more specifically 2G ethanol (as opposed to the "first-generation (1G) ethanol", which is based on edible cellulosic biomass).

Saccharomyces cerevisiae, commonly known as baker's yeast (or even yeast) is the preferred microorganism for 1G ethanol production on a large scale, due to its capacity to convert the six-carbon (C6) sugars fast and efficiently. However due to the chemical properties of, and the wide sugar distribution in lignocellulosic biomass, there are specific challenges associated with 2G ethanol production in yeast. In particular, a pretreatment step is required to release all the sugar monomers from the complex lignocellulose chains and make them available for fermentation. During this step, inhibitory substances of different types are produced at the same time and their presence in the hydrolysate affects the fermentation performance of yeast. So the aim of the present work was to investigate the mechanisms of yeast tolerance to these inhibitors with or without other types of stressors encountered during the fermentation of lignocellulosic substrates, in order to develop efficient yeast biocatalysts for 2G ethanol production.

In one part of the study, a mutated yeast enzyme responsible for the conversion of furaldehyde compounds, which are one group of lignocellulosic inhibitors (LI), into a less inhibitory compound, was studied and key mutated amino acids responsible for this conversion were identified. In the second part of the thesis, different strategies were used to develop yeast strains with increased tolerance to combined stresses. Two targeted proteins that were involved in the response to oxidative stress under laboratory conditions were re-evaluated under process-mimicking conditions. The beneficial effect on tolerance to LI was confirmed, although it was limited to the fermentation of C6 sugars, and unexpected negative interactions were identified for one candidate in the fermentation of C5 sugars. A second approach concentrated on obtaining a yeast strain with combined tolerance to LI and high temperature, as increased thermotolerance reduces production costs. Using long-term evolution under selective selection pressure, so-called "evolutionary engineering", a strain capable of growing and fermenting in the presence of LI and at high temperature (39°C) was generated. Significant differences in the lipid composition of the evolved strain were found, which were confirmed by changes at the genome level in different genes involved in lipid transport, synthesis, and other steps of lipid metabolism, thereby

implicating alterations in the composition of the yeast membrane as being responsible for combined tolerance.

Overall, the work performed for this thesis resulted in the development of several strains with improved characteristics that were suitable for fermentation of LI. The work also contributed to a better understanding of the mechanisms of stress response in yeast.

List of papers

This thesis is based on the following research papers, which will be referred to in the text by their Roman numerals in the text. The papers can be found at the end of the thesis.

I. **Furaldehyde substrate specificity of *Saccharomyces cerevisiae* alcohol dehydrogenase 1 variants**
Boaz Laadan, Valeria Wallace-Salinas, Åsa Janfalk Carlsson, João R. M. Almeida, Peter Rådström, Marie F. Gorwa-Grauslund.
Submitted.

II. **Re-assessment of *YAP1* and *MCR1* contributions to inhibitor tolerance in robust engineered *Saccharomyces cerevisiae* fermenting undetoxified lignocellulosic hydrolysate**
Valeria Wallace-Salinas, Lorenzo Signori, Yingying Li, Magnus Ask, Maurizio Bettiga, Danilo Porro, Johan M. Thevelein, Paola Branduardi, María R. Foulquié-Moreno, Marie Gorwa-Grauslund.
Submitted.

III. **Adaptive evolution of an industrial strain of *Saccharomyces cerevisiae* for combined tolerance to inhibitors and temperature**
Valeria Wallace-Salinas and Marie F. Gorwa-Grauslund (2013). Biotechnology for Biofuels 6(1): 151.

IV. **Investigating the cellular response of *Saccharomyces cerevisiae* after adaptive evolution to high temperature in the presence of lignocellulosic hydrolysate**
Valeria Wallace-Salinas, Daniel Brink, Marie F. Gorwa-Grauslund
Manuscript.

My contribution to the papers

I. I performed the fermentations and analyzed the corresponding data. I contributed to writing of the manuscript.

II. I participated in the design of the experiments and in the construction of the strains. I performed the fermentations and the statistical analysis of the data in collaboration with Lorenzo Signori and Yingying Li. I performed the biological interpretation of the data and wrote the manuscript.

III. I participated in the design of the experiments and performed all of them. I analyzed the data and wrote the manuscript.

IV. I designed the study and produced the samples for lipid analysis. I supervised the master's student who performed the experiments with spruce hydrolysate and lyticase. I performed the biological interpretation of the data and wrote the manuscript.

Abbreviations

ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CBP	Consolidated bioprocessing
CWI	Cell wall integrity
ESR	Environmental stress response
HMF	Hydroxymethyl furfural
HSE	Heat shock elements
HSR	Heat shock response
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
SHF	Separate hydrolysis and fermentation
SNVs	Single nucleotide variations
INDELs	Insertions and deletions
SSF	Simultaneous saccharification and fermentation
STRE	Stress-responsive element

Contents

Introduction

With the drilling of the first oil wells on a commercial scale around 150 years ago, a new age of industrial and technological development had started. The immense supply of oil and its high energy density not only represented a more effective source of fuel for transportation and generation of electrical and heating power, but it also became the raw material for the production of a large number of commodities, and it led to the rapid expansion of agriculture and industry in general. Nowadays, oil-based products include countless ordinary articles such as chemicals, plastics, textiles, pharmaceuticals and computers. Oil, however, is finite and non-renewable, and estimations indicate that we are now consuming more oil than the oil that is being discovered (Sorrel S. et al. 2009). The finite nature of oil, its complexity in terms of geological, technical, economic and political factors affecting its production, and the environmental consequences of oil-driven developments in different societies have become growing causes for concern. All these components indicate that we need to reconsider our use of oil; and many countries have now implemented a series of policies to promote the development of alternative energy sources (Lefèvre, N. 2007).

The use of renewable sources of energy for the generation of electricity, heat, fuels, and other commodities on a scale large enough to substantially reduce our oil dependency undoubtedly means technological challenges. But it also represents great opportunities for the development of new technologies and for generation of new knowledge in many fields. An example of this has been the extensive research aimed at increasing our capacity to produce bioethanol for use as transportation fuel. Although production of first-generation bioethanol (e.g. ethanol obtained from the fermentation of starch and sucrose) is a well-established process, the volumes required to satisfy the growing worldwide demand for the fuel would jeopardize the production and prices of food commodities. Instead, production of ethanol from lignocellulosic biomass (also known as second-generation ethanol) would enable the use of a wide source of raw material that does not compete directly with food. Integration of these two levels of technology (i.e., first- and second-generation ethanol) is considered to be a viable alternative in order to satisfy the worldwide demand for biofuels in the near future (Sims, R. et al. 2008).

Conversion of lignocellulosic biomass to ethanol on a large scale does, however, mean that several different technical challenges must be overcome before it can become economically feasible. For the last 40 years, significant investment in research and development (from both private and public sectors) has been carried out in this area

to ensure not only the sustainable production of different biomass feedstock but also the development of viable technologies for their efficient conversion. In this regard, we now have a better understanding of crop feedstock management and chain supply logistics (Henry, R. J. 2010; Beringer, T. et al. 2011) and also improved methods for biomass pretreatment (recently reviewed in (Haghighi Mood, S. et al. 2013)). Furthermore, the requirement for more efficient enzymes for biomass hydrolysis has resulted in the identification of new sources of biocatalysts with improved characteristics and also in the development of new methods for their production (Merino, S. et al. 2007; Weiss, N. et al. 2013). Likewise, much effort has been put into the development of fermenting microorganisms (Dien, B. S. et al. 2003; Chang, T. et al. 2011; Laluce, C. et al. 2012).

In this context, the yeast *Saccharomyces cerevisiae* has been the subject of intensive research aimed at improving its fermentation capacity, which has resulted not only in the development of strains better adapted for lignocellulosic ethanol production but also in a better understanding of the biology of this model organism (Nevoigt, E. 2008; Matsushika, A. et al. 2009; Laluce, C. et al. 2012). However, important difficulties regarding the different factors that affect the performance of the yeast still need to be overcome.

The aim of the work presented in this thesis was to develop industrial *S. cerevisiae* strains with improved tolerance to hydrolysate-derived inhibitors and high temperature. These are two types of stressors encountered during the fermentation of lignocellulosic biomass that affect ethanol yield and productivity. A secondary aim was to understand the response of yeast and adaptation mechanisms to such stressors.

The thesis is based on the four papers presented at the end of the summary. In **paper I**, the effects that different amino acid substitutions (identified in a mutated variant of Adh1p) have on the enzyme's substrate specificity are reported. The *in vitro* and *in vivo* reduction activities in relation to the overall detoxification capacity of the cell are also compared. In **paper II**, we investigated the effects of overexpressing two genes, either alone or in combination, in an industrial strain of *S. cerevisiae* , and the results highlighted the relevance of the genetic background and the importance of integral assessments in the final result of the genetic engineering strategy. **Paper III** describes a successful long-term adaptation experiment through which a stable strain with combined tolerance to high temperature and hydrolysate-derived inhibitors was obtained. **Paper IV** deals with a comparison of the cellular response of the evolved strain developed in **paper III** to that of the parental strain. The results suggest that alterations in membranes and the development of a multicellular phenotype may account for the mechanisms of adaptation to the stressors.

As an introduction to the thesis, Chapter 1 gives a general description of the lignocellulosic feedstocks, the different steps of the ethanol production process, and some of the challenges associated with the fermentation of lignocellulosic biomass by *S. cerevisiae*. In Chapter 2, the effects that high temperature and hydrolysate-derived

inhibitors have on the physiology and performance of the yeast are presented, including some theoretical aspects and examples of the environmental stress response in *S. cerevisiae*. Based on the mechanisms of stress tolerance and the synergistic effects of stressors on *S. cerevisiae* described in Chapter 2, different strategies for development of industrial strains including rational metabolic engineering and evolutionary engineering are described in Chapter 3. As part of a more integrative view of the development of improved strains, Chapter 4 introduces the concept of reverse metabolic engineering, and highlights different genome-wide technologies used for analysis of the cellular biology at different levels. In the last section of the thesis I summarize the main conclusions of the research and discuss some ideas for future studies to address some of the unanswered questions.

"Nobody ever figures out what life is all about, and it doesn't matter. Explore the world. Nearly everything is really interesting if you go into it deeply enough."
Richard Feynman

1. Lignocellulosic biomass as a substrate for ethanol production

The worldwide capacity for production of ethanol could be significantly increased by using lignocellulosic biomass, i.e. plant dry matter, as raw material. Lignocellulosic biomass is highly abundant and diverse, and includes: trees, energy crops, and agricultural and forestry by-products such as corn stover, sugarcane bagasse, saw mill and paper mill, among others. While such abundance and diversity suggests that there is an almost unlimited supply of resources, the chemical composition of this type of biomass imposes different technical challenges that have delayed its use in large-scale ethanol plants. This section describes the general properties of lignocellulosic biomass and the steps necessary for its conversion to ethanol, including some of the challenges during the fermentation step when *S. cerevisisae* is used as biocatalyst.

1.1 Composition of lignocellulosic biomass

Biomass such as sugarcane, corn, molasses and sugar beet contains large amounts of starch or sucrose that can be processed by relatively simple methods to make the hexose sugars available for fermentation. In contrast, lignocellulosic biomass is composed of three different polymers: cellulose, hemicellulose, and lignin (Figure 1); their relative concentrations largely depend on the plant material used (Haghighi Mood, S. et al. 2013). Both the chemical properties of each polymer and the way they are assembled in the plant hinder access to and conversion of the polysaccharides to fermentable sugars.

Cellulose is a linear polymer formed from glucose units linked by β(1→4) glycosidic bonds, and it accounts for 25-55% of biomass dry matter (Sun, Y. et al. 2002). Despite the fact that it is a polar molecule with several hydroxyl groups, cellulose is insoluble in water (Lindman, B. et al. 2010). Cellulose exists in different crystalline structures with some more amorphous regions, and its properties vary in relation to the degree of polymerization (Pérez, J. et al. 2002).

Hemicellulose, which is formed from heteropolymers of hexoses (glucose, mannose, and galactose) and pentoses (xylose and arabinose) accounts for 25-

55% of dry matter (Sun, Y. et al. 2002). In contrast to cellulose, hemicellulose is highly branched and it has a lower degree of polymerization. Some of the hydroxyl groups of the sugar units in the side chains have been replaced by acetyl groups, so hydrolysis of hemicellulose will result in the release of both monomeric sugars and acetic acid (Pérez, J. et al. 2002). Hemicellulose is linked to cellulose and lignin by hydrogen bonds and covalent bonds, respectively (Jin, Z. et al. 2006).

Lignin is a complex aromatic polymer resulting from the polymerization of three major phenolic components: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Frei, M. 2013); it accounts for 10-30% of dry matter (Sun, Y. et al. 2002). Lignin has been described as the cellular glue that holds cellulose and hemicellulose fibers together; its high molecular weight, structural complexity, and insolubility in water make its degradation very challenging (Rubin, E. M. 2008).

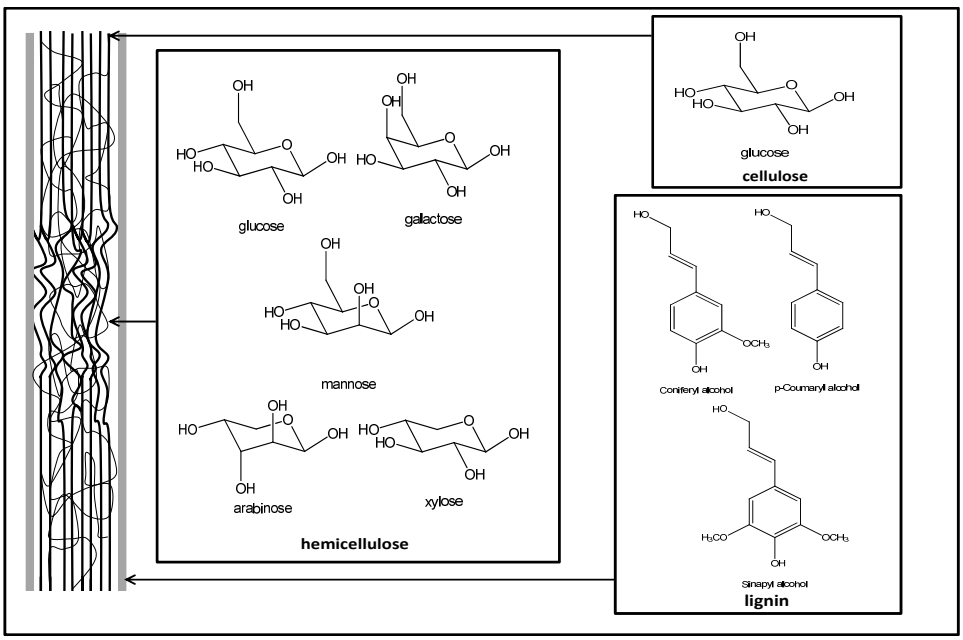

Figure 1. Schematic representation of the structure of lignocellulose and its major components (cellulose, hemicellulose, and lignin).

Lignocellulose is a natural barrier that not only gives rigidity and strength to the plant cell, but also protects it from attack by microorganisms and insects. As a substrate for ethanol production, however, depolymerization of the lignocellulosic structure is a technological challenge.

1.2 From lignocellulosic biomass to ethanol

Production of ethanol from lignocellulose biomass involves the following main operations: pretreatment, hydrolysis (usually with the help of enzymes), fermentation, and distillation, where ethanol is concentrated and separated from lignin residues (Galbe, M. et al. 2007) (Figure 2).

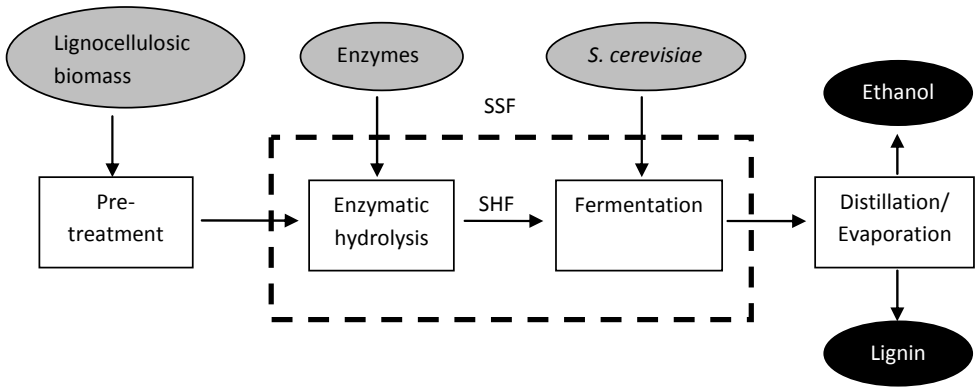

Figure 2. Schematic representation of the process of ethanol production from lignocellulosic biomass. (SHF: separate hydrolysis and fermentation; SSF: simultaneous saccharification and fermentation).

The ***pretreatment*** step is aimed at altering the physical and chemical structure of the biomass, so that further hydrolysis of the carbohydrates can proceed faster and with higher yields. It is considered to be the most critical step since its outcome has a large influence not only on the digestibility of cellulose but also on the generation of toxic compounds that can affect yeast fermentation performance, the requirements in energy demand (for stirring and downstream processing), and demands for wastewater treatment (Galbe, M. et al. 2007). The different pretreatment methods are generally classified as biological, physical, or chemical, with some methods involving more than one effect (Mosier, N. et al. 2005). Biological methods are based on the use of wood-degrading fungi, but at present they are still too inefficient to be considered economically attractive (Chandra, R. P. et al. 2007). Instead, the use of elevated temperature and high pressure, or the addition of chemicals such as NaOH, H_2SO_4, or ammonia have largely been assessed as pretreatment processes—with each method having inherent advantages and disadvantages, and with an effectiveness that is strongly substrate-dependent (Chandra, R. P. et al. 2007). In general, an effective pretreatment method should fulfill the following criteria: it should preserve the hemicellulose fraction, it should generate cellulose fibers with reduced crystallinity

and a reduced degree of polymerization, and it should minimize energy demands (Mosier, N. et al. 2005). In addition, the formation and release of inhibitors (such as furaldehydes, weak acids, and phenolic compounds) should be limited in order to facilitate ethanol production in the fermentation step (Palmqvist, E. et al. 2000b).

After the pretreatment step, two different fractions are obtained: (1) a liquid fraction consisting of both monomeric sugars and oligomeric sugars, mainly from the hemicellulose fraction, and also some inhibitors resulting from sugar degradation; and (2) a solid fraction mainly containing cellulose and lignin, with some hemicellulose if the pretreatment conditions were not severe (Galbe, M. et al. 2007).

Baker's yeast (*Saccharomyces cerevisiae*), which is the most commonly used microorganism for fermentation, cannot metabolize polymeric and oligomeric sugars. Thus, a step for ***hydrolysis*** of cellulose (and possibly hemicellulose) is required, usually using enzyme cocktails whose composition depends on the type of solid fraction (Merino, S. T. et al. 2007). Most commercial cellulase enzymes are produced by submerged fermentation of the mesophilic fungus *Trichoderma reesei* and include three enzyme classes: exogluconases, endogluconases, and β-glucosidases (Merino, S. T. et al. 2007). Hydrolysis of hemicellulose involves the use of a wider range of enzymes including endoxylanases, xylosidases, endomannanases, and mannosidase (Brink, J. et al. 2011).

Fermentation, i.e. the conversion of the released monomeric sugars into ethanol by microbial organisms, is the next step in the process. The two main goals at this stage are to obtain ethanol yields that are close as possible to the theoretical values (0.51 g ethanol per gram of monomeric sugar) and high specific productivities (preferably over 1.3 g ethanol per gram cell and hour) (Olofsson, K. et al. 2008). Although different microorganisms are being investigated for ethanol production from lignocellulosic biomass, the work for this thesis focused on the most-well studied one, the yeast *S. cerevisiae*. The different strategies that have been considered for optimization of the fermentation performance of *S. cerevisiae* are discussed in detail in the section.

As shown in Figure 2, enzymatic hydrolysis and fermentation can be carried out in separate processes (separate hydrolysis and fermentation, SHF) or simultaneously (simultaneous saccharification and fermentation, SSF). The characteristics of both types of process configurations are presented in Table 1.

Table 1. Comparison of hydrolysis and fermentation strategies (Taherzadeh, M. et al. 2007; Olofsson, K. et al. 2008)

Configuration	Advantages	Disadvantages
Separate hydrolysis and fermentation: SHF	Hydrolysis and fermentation can be carried out at their own optimum temperature and pH. Yeast cells can be recirculated.	Enzymes are inhibited by the sugars released (product inhibition). Enzymes may be inhibited by toxic compounds formed during the pretreatment.
Simultaneous saccharification and fermentation: SSF	Enzyme inhibition is reduced due to the rapid consumption of the sugars and conversion of the inhibitory substances by the fermenting microorganism. Lower risk of contamination due to the presence of ethanol. Both operations can be performed in one tank, reducing the capital costs of the process. Continuous release of glucose improves xylose utilization.	The compromise between the optimal temperature for enzymatic hydrolysis (~45-50°C) and fermentation by *S. cerevisiae* (~30-35°C) limits the enzymatic hydrolysis rate. Cellulase inhibition by ethanol is possible. Yeast recirculation is not possible.

Different modifications of the strategies described above have been studied. Although the convenience of one configuration over the other is largely determined by the raw material and pretreatment technology, SSF processes are generally considered to be a very promising option (Olofsson, K. et al. 2008; Tomás-Pejó, E. et al. 2008; Ask, M. et al. 2012).

A third option, referred to as consolidated bioprocessing (CBP), has also been investigated more recently. It is defined as "the combining of the four biological events required for (the) conversion process (production of saccharolytic enzymes, hydrolysis of the polysaccharides present in pretreated biomass, fermentation of hexose sugars, and fermentation of pentose sugars) in one reactor" (Zyl, W. et al.

2007). In the particular case of *S. cerevisiae*-based processes, it consists of having yeast strains that have been engineered to produce and excrete hydrolytic enzyme cocktails in the broth. Although it is an appealing strategy to reduce the costs of biomass processing, it still faces some challenges associated with genetic engineering of all the genes required to have efficient hydrolases in one single microorganism (Zyl, W. et al. 2007; Matano, Y. et al. 2012).

The main objectives of the downstream operations are the ***recovery of ethanol*** by distillation (or a combination of distillation and evaporation), recovery of valuable sub-products (e.g. lignin), and reduction of the need for wastewater treatment (Wingren, A. et al. 2008). As the recovery of ethanol is the operation with the highest energy demand in the process, strategies that have so far been investigated for reduction of the associated costs rely on increasing the dry matter content during fermentation (so that higher concentrations of ethanol can be achieved (Hoyer, K. et al. 2013)) and recirculation of the process streams (Alkasrawi, M. et al. 2002).

1.3 Old and new challenges with *Saccharomyces cerevisiae*

Many of the technical challenges associated with ethanol production from lignocellulose by *S. cerevisiae* are also encountered during ethanol production from starch and sucrose-based substrates. Optimization of the fermentation performance of *S. cerevisiae*, regardless of the type of substrate, includes increasing the microorganism's ***tolerance to high ethanol concentrations*** (Alper, H. et al. 2006) and ***high osmolarity*** associated with fermentations at very high sugar/solids content (so-called "very-high-gravity (VHG) fermentations") (Pereira, F. B. et al. 2011; Puligundla, P. et al. 2011), and also reducing the production of ***by-products*** such as glycerol (Medina, V. G. et al. 2010; Pagliardini, J. et al. 2013).

Since ethanol plants at large-scale facilities are not run under sterile conditions, ***contamination*** by both bacterial species (lactic acid bacteria) and yeast species (e.g. *Dekkera bruxellensis*, *Schizosaccharomyces pombe*) may affect ethanol production (Skinner, K. et al. 2004; de Souza Liberal, A. T. et al. 2005). Contaminants may reduce the ethanol yield and/or produce compounds that can inhibit *S. cerevisiae* (de Souza Liberal, A. T. et al. 2007; Basilio, A. C. M. et al. 2008; Beckner, M. et al. 2011), although the real effect depends on the specific bacterial and yeast species (Souza, R. et al. 2012). Improvement in the capacity of *S. cerevisiae* to ferment sugars at low pH is one of the strategies that have been studied to reduce the incidence of bacterial contamination (Kádár, Z. et al. 2007). Nevertheless, contamination of ethanol plants with wild-type *S. cerevisiae* strains may result in interesting candidates for starter cultures, due to their general robustness. In this respect, isolation of industrial strains from environments associated with ethanol production has resulted in already adapted strains that can be used directly as platform strains for further

improvements (Banat, I. M. et al. 1992; Lindén, T. et al. 1992; Basso, L. C. et al. 2008; Sànchez i Nogué, V. et al. 2012).

Incomplete fermentation during ethanol production from starch or sucrose-based raw material may occur as result of ***catabolite repression*** (D'Amore, T. et al. 1989), i.e. the repression caused by glucose in the assimilation of alternative sugars such as fructose, maltose, and maltotriose (Gancedo, J. M. 1998). Avoiding repression of galactose catabolism by glucose has been a particularly intensive area of research, since industrial processes that use galactose-based substrates (e.g. whey permeate, sugar beet) are also affected by the effect of catabolite repression in *S. cerevisiae* or other microorganisms (Gutierrez, N. et al. 1993; Rønnow B et al. 1999; Bro, C. et al. 2005; Garcia Sanchez, R. et al. 2010). Given the diversity of sugars in wood-based materials, the challenge of catabolite repression will continue to be the subject of research in lignocellulosic-based processes also.

An additional major challenge associated with lignocellulosic material is the ***fermentation of pentose sugars*** (xylose and arabinose). This does not occur naturally in *S. cerevisiae* (Hahn-Hägerdal, B. et al. 2006).

The use of pentose sugars has been established in *S. cerevisiae* by expressing various heterologous enzymes of fungal or bacterial origin (Karhumaa, K. et al. 2006; Wisselink, H. W. et al. 2007). Due to the fact that ***xylose*** is one of the most abundant carbohydrates in nature, xylose metabolism by *S. cerevisiae* has been heavily studied (Jeffries, T. W. 2006; Hahn-Hägerdal, B. et al. 2007). Xylose utilization was established in *S. cerevisiae* strains by expression of the fungal oxido-reductive pathway—consisting of the enzymes xylose reductase (XR) and xylose dehydrogenase (XDH) (Kötter, P. et al. 1993)—or the bacterial pathway, in which a xylose isomerase (XI) catalyzes the direct conversion of xylose to xylulose (Kuyper, M. et al. 2003) (see Figure 3). Later, arabinose utilization was made possible by overexpressing the bacterial arabinose pathway, and notably the AraA gene from *Bacillus subtilis*, together with the AraB and AraD genes from *Escherichia coli* (Becker, J. et al. 2003). Introduction of the five genes of the fungal pathway of *Trichoderma reesei* also resulted in some arabinose utilization (Richard, P. et al. 2002).

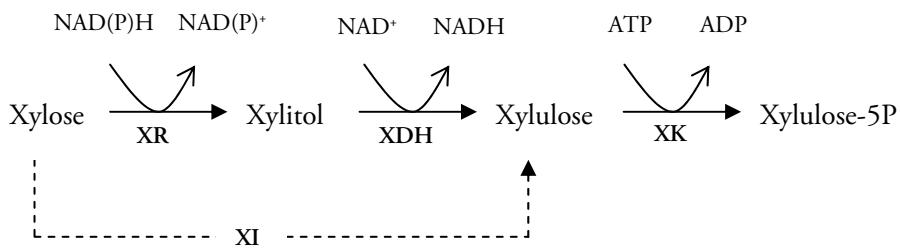

Figure 3. Xylose utilization pathways introduced in *S. cerevisiae*

However, efficient pentose utilization in *S. cerevisiae* requires additional genetic modifications. For example, an increased (but controlled) level of xylulokinase activity (XK) gives higher ethanol yields from xylose and arabinose (Johansson, B. et al. 2001; Karhumaa, K. et al. 2006). Also, limitations in the capacity of the non-oxidative part of the pentose phosphate pathway (PPP) to use xylose were overcome by overexpression of the corresponding genes (Johansson, B. et al. 2002). The redox imbalance that results from the difference in cofactor preference in the fungal xylose pathway and that causes accumulation of xylitol, was limited by changing the cofactor specificity of XR and XDH by protein engineering (Watanabe, S. et al. 2005; Jeppsson, M. et al. 2006; Runquist, D. et al. 2010a). Finally, the lack of specific pentose transport systems in *S. cerevisiae* has been addressed by overexpression of heterologous transporters from natural xylose-consuming microorganisms such as *Pichia stipitis* and *Candida intermedia* (Saloheimo, A. et al. 2007; Runquist, D. et al. 2009; Runquist, D. et al. 2010b). Overexpression of endogenous *GAL2*, encoding galactose permease, was also reported to increase the rate of uptake of arabinose (Becker, J. et al. 2003). Although there have been significant advances in our understanding of the limitations of pentose metabolism in *S. cerevisiae*, further development of strains is still required in order to achieve acceptable yields and productivity, and thereby make the processes profitable.

Other challenges that are specifically associated with the use of lignocellulosic material concern the negative effects of the ***hydrolysate-derived inhibitors*** on cell performance. This will be discussed in detail in the next chapter, which covers environmental stressors.

2. Environmental stressors for *S. cerevisiae* during fermentation of lignocellulosic substrates

The capacity of a cell to sense and respond to alterations in the environment determines its ability to survive. When an organism is exposed to chemical or physical conditions that negatively affect its growth capacity, a series of complex molecular mechanisms are activated. These mechanisms, known as stress response mechanisms, include activation of sensing and signal transduction networks that result in the adjustment of the gene expression program, metabolism, and other cellular processes in order to repair possible damage; adjustment of growth to the new conditions, and prevention of further damage occurring (Hohmann, S. et al. 2003). Stressors therefore refer to as any environmental conditions or factors that activate a stress response mechanism in a cell. Sustainable production of second-generation ethanol from lignocellulosic biomass requires that *S. cerevisiae* is able to respond fast and efficiently to a variety of stress-generating conditions. In this chapter, I give a short overview of the general stress response in *S. cerevisiae*, and discuss in more detail specific effects that hydrolysate-derived inhibitors and high temperature have on the performance of yeast.

2.1 General stress response in yeast

The optimal growth and cellular functioning of *S. cerevisiae* takes place under well-defined and controlled internal conditions, that property of an organism that is usually referred to as homeostasis. Stress-generating conditions such as depletion of a nutrient, changes in osmolarity, increase in temperature, presence of oxidative elements, changes in pH, etc. will jeopardize this internal homeostasis and therefore affect cell growth and viability. Some of the molecular changes that are activated during a stress response are stress-specific, but there are also common responses that are triggered regardless of the type of stressor; these are referred as the ***general stress response***.

At the genetic level, the general stress response in controlled by stress-responsive cis-elements (STREs) in the promoter region of a wide range of stress-induced genes (M

T Martínez-Pastor et al. 1996). Transcriptional changes at the genome-wide level have led us the concept of a common program of gene expression, referred as the "environmental stress response" (ESR) (Gasch, A. P. et al. 2000). This program accounts for the coordinated regulation of transient change in the expression level of around 900 genes (approximately 14% of the genes so far predicted to occur in the *S. cerevisiae* genome). Such genes are divided into two groups: those whose transcription level increases after being exposed to an environmental stressor, and genes whose transcription level decreases in response to the stress. In general, the biological functions represented by the group of downregulated genes are associated with protein synthesis and growth, including for example genes involved in ribosome synthesis, tRNA synthesis, and rRNA processing (Gasch, A. P. 2003). The functions associated with the upregulated genes are more diverse and include carbohydrate metabolism, respiration, defense against oxidative stress, and protein folding and degradation. And it has been observed that the transcription factors Msn2p and/or Msn4p modulate the expression of many of these genes (Gasch, A. P. et al. 2000; Causton, H. C. et al. 2001).

Other observations have indicated that the general stress response is regulated by additional mechanisms. For instance, only specific isozymes have been associated with the ESR, while their paralogs are not induced by the ESR but by a specific type of stress. One example of this is the induction of Ctt1p (cytosolic catalase) during ESR, while Cta1p (peroxisomal catalase) is specifically induced by high peroxisomal activity, suggesting that the response mechanism may vary according to subcellular location (Gasch, A. P. 2003). On the other hand, the induction of genes encoding "reverse" activities (such as synthesis and degradation of the same compound, such as trehalose) gives the cell the possibility of rapid regulation at the posttranscriptional level (Gasch, A. P. 2003).

It has been suggested that part of the purpose of the general stress response is to confer tolerance to the cell so that it will be prepared to withstand future stresses (Berry, D. B. et al. 2008). Indeed, at the phenotypic level the general stress response can be observed upon pre-exposure to stress (also called pre-adaptation or ***same-stress resistance***) (Berry, D. B. et al. 2008; Stanley, D. et al. 2010), in which exposing the cells to a mild stress condition from a particular stressor gives the cells the ability to survive a severe dose of the same stressor. For example, exponential cultures growing at 36°C were found to be less affected by a heat shock at 52°C than exponential cultures growing at 23°C (Fintan Walton, E. et al. 1980). The general stress response also accounts for the ***cross-protection*** effect; that is, exposure of the cells to a particular stressor will generally confer some ability to at least tolerate another type of stressor (Jenkins, D. E. et al. 1988). Probably the most familiar cross-protection effect is the high tolerance of cells in stationary phase to different types of stresses such as heat, NaCl, and H_2O_2 (Werner-Washburne, M. et al. 1993; Berry, D. B. et al. 2008). However, this effect is not universal, and in some cases it may be strain-dependent. For example, mild ethanol treatment of cells of the laboratory strain

S288C did not protect the culture from any other type of stressors, whereas acquired stress resistance after the same pretreatment was observed in other strains (Berry, D. B. et al. 2008).

Interestingly, the regulatory mechanisms behind cross-protection and same-stress resistance appear to be different. For instance, acquired resistance to high doses of H_2O_2 or NaCl after a mild heat shock was found to be dependent on Msn2p and Msn4p while resistance to severe heat shock after the same pretreatment was not (Berry, D. B. et al. 2008). Also, the mechanisms behind acquired tolerance of H_2O_2 have been shown to depend on the type of stressor used for the mild treatment, indicating that in fact both the actual stressor and the previous experience of the cells can influence the response mechanisms (Berry, D. B. et al. 2011).

The characteristics of the general stress response described above are just a small part of a much more elaborate response mechanism initiated by the cell to respond to a wide set of conditions that threaten its internal homeostasis. An additional part of the complexity involves the specificity of the response determined by the stressor. Since covering all these subjects is outside the scope of this work, the next parts of this chapter will deal with two main types of stressors that are present during ethanol production from lignocellulosic biomass, hydrolysate-derived inhibitors and temperature. Also, the synergistic effects of such stressors on the physiology of *S. cerevisiae* will be discussed.

2.2 Environmental stressors

In addition to the general stress response, cells possess specific responses to individual stressors. The type of stressor defines how the stress is sensed by the cell, what type of signal transduction is initiated, which transcriptional factor(s) control(s) the expression level of the genes to be induced or repressed, and so on.

Environmental factors can affect the tolerance of a microorganism during the fermentation process. For example, reductions in the costs of distillation and water treatment can be achieved by running very-high-gravity (VHG) fermentations, which means high solids content—from which the yeast is subjected to osmotic stress—and conditions of low water content and high ethanol concentration (Puligundla, P. et al. 2011). In recombinant *S. cerevisiae* also, the use of xylose as a carbon source triggers metabolic responses mimicking carbon starvation (Bergdahl, B. et al. 2012; Matsushika, A. et al. 2013), suggesting that xylose fermentation represents an additional type of stress encountered by the yeast during conversion of lignocellulosic biomass. In this chapter, I will focus on the two major types of stressors investigated: hydrolysate-derived inhibitors and high temperature.

2.2.1 Stress associated with hydrolysate-derived inhibitors

Inhibitors present in lignocellulosic hydrolysate

Toxic compounds are released and formed during the pretreatment of biomass; their amount and composition depend on many factors, including not only the type of raw material but also the method and conditions used for the pretreatment (Larsson, S. et al. 1999; Palmqvist, E. et al. 2000a).

The hydrolysate-derived inhibitors have been divided into 3 main groups: ***furaldehyde-derived compounds*** including 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde (furfural), which come from the degradation of hexose and pentose sugars, respectively; ***aliphatic acids*** such as acetic acid, originating from hydrolysis of the acetyl groups of hemicellulose, formic acid, which results from furfural and HMF degradation, and levulinic acid, which is a product of HMF degradation. The third group of inhibitors corresponds to ***phenolic compounds*** representing a very heterogenous group of chemical species originating from lignin. This includes vanillin, cinnamic acid, ferulic acid, catechol, coniferyl aldehyde, and phenol (Larsson, S. et al. 1999; Palmqvist, E. et al. 2000b; Jönsson, L. et al. 2013) (Figure 4).

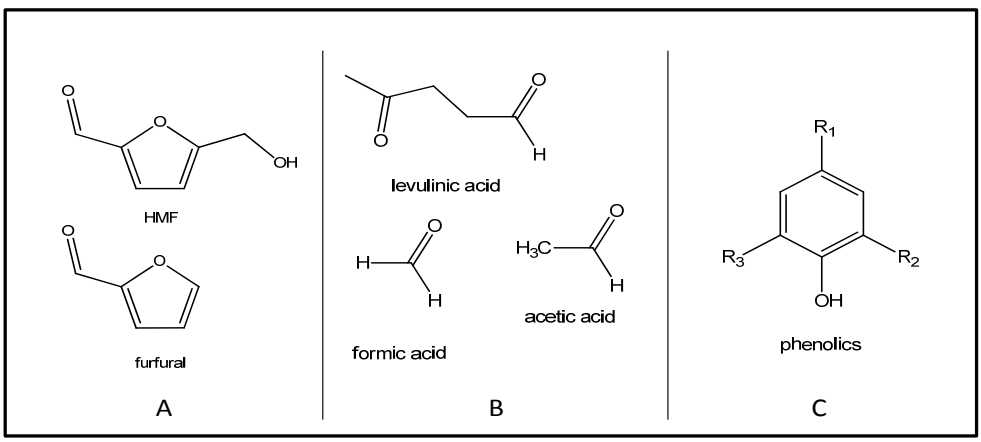

Figure 4. The most common hydrolysate-derived inhibitors (A, furaldehydes; B, aliphatic acids; and C, phenolics)

So far, most of the studies carried out to investigate the effect of inhibitors on the physiology of *S. cerevisiae* have been focused on HMF, furfural, and acetic acid. Furaldehyde-derived compounds are among the most potent inhibitors, affecting the growth and fermentation capacities of *S. cerevisiae* (Olsson, L. et al. 1996; Liu, Z. L. et al. 2004; Liu, Z. L. 2006; Almeida, J. R. M. et al. 2008; Ask, M. et al. 2013b; Glebes, T. Y. et al. 2014). Investigations of furfural and HMF conversion by *S.*

cerevisiae have included experiments related to cofactor utilization during the reduction of these compounds (Almeida, J. R. M. et al. 2009) and studies on amino acid substitutions in different reductases—either to enhance their conversion capacity (Moon, J. et al. 2012) or to understand the effect of mutations on cofactor preference (paper I).

As for acetic acid, it has been shown to have a particularly negative effect on xylose fermentation (Bellissimi, E. et al. 2009; Casey, E. et al. 2010; Almeida, J. R. M. et al. 2011; Wei, N. et al. 2013) due to the lower rate of production of ATP with this sugar.

However, there are many other hydrolysate inhibitors that have not been yet accounted for. This might explain the higher overall toxicity of "true" hydrolysates relative to the toxicities of designed synthetic mixtures. In particular, the large number of aromatic compounds together with their diversity has complicated their study (Jönsson, L. et al. 2013). Recently, methylglyoxal was identified as one additional inhibitor of miscanthus (*M. giganteus*) hydrolysate, and its significant contribution to the overall toxicity of the hydrolysate was revealed. (Skerker, J. M. et al. 2013). In fact, it was also found that a synthetic hydrolysate made with 37 different inhibitors did not have the toxic effect of the real hydrolysate, thus suggesting that important inhibitors were missing from the synthetic mixture (Skerker, J. M. et al. 2013).

Effect of inhibitors on the performance of S. cerevisiae

The impact of major hydrolysate-derived inhibitors on the performance of *S. cerevisiae* during ethanol production is presented in Table 2. It is important to keep in mind that the degree of inhibition in yeast depends on many factors, including the genetic background of the strain and the biomass cultivation procedure. Thus, laboratory strains are normally more affected than industrial strains (Lindén, T. et al. 1992; Modig, T. et al. 2008) (Paper II); also, pre-adaptation of the cells using the liquid fraction of pretreated hydrolysate results in better performance during SSF fermentations (Alkasrawi, M. et al. 2006). The initial cell density and age of the inoculum also affect the degree of inhibition; in this case, a larger inoculum responds better than a smaller one, and stationary cells are generally more tolerant than exponentially growing cells (Palmqvist, E. et al. 1999b; Zingaro, K. A. et al. 2013). The composition of the culture medium also influences the degree of inhibition. This has been clearly exemplified by the differences in the effect that inhibitors have on *S. cerevisiae* when fermenting xylose rather than glucose as carbon source (Bergdahl, B. et al. 2012; Ask, M. et al. 2013a) (Paper II) or when using rich medium rather than defined mineral medium (Zingaro, K. A. et al. 2013).

Table 2. Effects of major hydrolysate-derived inhibitors on the performance of *S. cerevisiae* strains during ethanol production

Inhibitor	Effect[1]	Example	References
Furfural and HMF	Lag phase increased (strain ATCC 211239)	30 mM furfural or HMF extended the lag phase 24 h and 16h respectively (initial OD_{620}= 0.15)	(Liu, Z. L. et al. 2004)
	Specific growth rate μ (h^{-1}) decreased (strain CEN.PK 113-5D)	1.5 g/L HMF reduced the specific growth rate by around 32% (initial biomass 0.8 g/L)	(Petersson, A. et al. 2006)
	Specific ethanol production rate (g/g . h) decreased (strain CBS 8066)	4 g/L HMF reduced the specific ethanol production rate by 40%	(Taherzadeh, M. J. et al. 2000)
Acetic acid	Biomass formation decreased (strain CBS 8066)	21% reduction in biomass yield in the presence of 9 g/L acetic acid (pH = 5.0; anaerobic batch)	(Taherzadeh, M. J. et al. 1997)
	Specific xylose consumption rate decreased (xylose-consuming strain 424A LNH-ST)	The initial specific xylose consumption rate (g_{xyl}/g dry cell . h) decreased by 52% in the presence of 7.5 g/L acetic acid (pH=5.5)	(Casey, E. et al. 2010)
Aromatic (Phenolics)	Volumetric ethanol productivity ($g_{ethanol}$/L . h) reduced (strain commercial baker's yeast)	The volumetric ethanol productivity was reduced 58% in the presence of 0.2g/L cinnamic acid (oxygen limited, batch; initial OD_{620} = 0.5)	(Larsson, S. et al. 2000)

Higher concentrations of an inhibitor generally translate into stronger negative effect on the performance of the cells, although the detoxification process can sometimes have a beneficial effect on yeast physiology. For example, if present at low concentrations, HMF and furfural can be reduced by *S. cerevisiae* to the less toxic alcohol forms (furan 2,5-dimethanol and 2-furanmethanol respectively) (Villa, G. P.

[1] Effect is in relation to control conditions, i.e. without the presence of the corresponding inhibitor.

et al. 1992; Liu, Z. L. et al. 2004) by the action of different oxido-reductases (Larroy, C. et al. 2002; Petersson, A. et al. 2006; Laadan, B. et al. 2008). The capacity of NAD^+ regeneration during furfural reduction has been correlated to lower glycerol production and higher ethanol yields than in the absence of furfural (Palmqvist, E. et al. 1999a). Based on the same principle, it has been proposed that small amounts of furfural can benefit the utilization of xylose since more NAD^+ can be made available for the conversion of xylitol by xylitol dehydrogenase (XDH) (Wahlbom, C. F. et al. 2002).

A positive effect was also observed with low amounts of acetic acid, which led to a higher ethanol yield than the fermentation without the acid (Taherzadeh, M. J. et al. 1997). This result—which was reported earlier (Verduyn, C. et al. 1990)—can be explained by the effect of the acid on the internal pH of the cell: as the dissociation of the acid inside the cell causes a decrease in pH, the protons are pumped out of the cell through the plasma membrane ATPase. This extra demand for ATP is satisfied by increasing the flux to ethanol production, with a reduction in the biomass yield (Verduyn, C. et al. 1990). The magnitude of the uncoupling effect exerted by the weak acids depends, however, on many variables such as the extracellular pH and cytosolic pH, the concentration of acid and the acid dissociation constant and liposolubility, among other factors (Verduyn, C. et al. 1990). The strong pH-dependent effect of weak acids is another element to be considered, since the xylose consumption rate in particular can be severely reduced at low pH (3.5) in the presence of acetic acid (3g/L) (Bellissimi, E. et al. 2009).

The synergistic effect between different inhibitors during ethanol production is another important factor to consider. For instance, Liu and co-workers (Liu, Z. L. et al. 2004) showed that furfural and HMF act synergistically to suppress *S. cerevisiae* cell growth suggesting that there is a different inhibitory and/or adaptation mechanism for each compound (Liu, Z. L. et al. 2004).

Cellular effects and response of S. cerevisiae to hydrolysate-derived inhibitors

The reduced performance of *S. cerevisiae* in the presence of the different types of inhibitors is related to the effects that each compound has on different cellular components (Figure 5).

Furfural has been shown to induce the accumulation of reactive oxygen species (ROS) and to damage mitochondrial and vacuole membranes, the actin cytoskeleton, and chromatin (Allen, S. A. et al. 2010). Also, furfural and HMF have been shown to be thiol-reactive electrophiles capable of generating oxidative stress (Kim, D. et al. 2013) through activation of the transcription factor *YAP1*, which mediates the response to oxidative stress in *S. cerevisiae* (Toone, W. M. et al. 1999). At the protein level, it was found that furfural reduced the activity of various glycolytic enzymes such as triose phosphate dehydrogenase and alcohol dehydrogenase (Banerjee, N. et al. 1981). More recently, Ask and co-workers reported a decrease in the catabolic and anabolic

reduction charges when *S. cerevisiae* was cultivated in the presence of furfural and HMF (Ask, M. et al. 2013b).

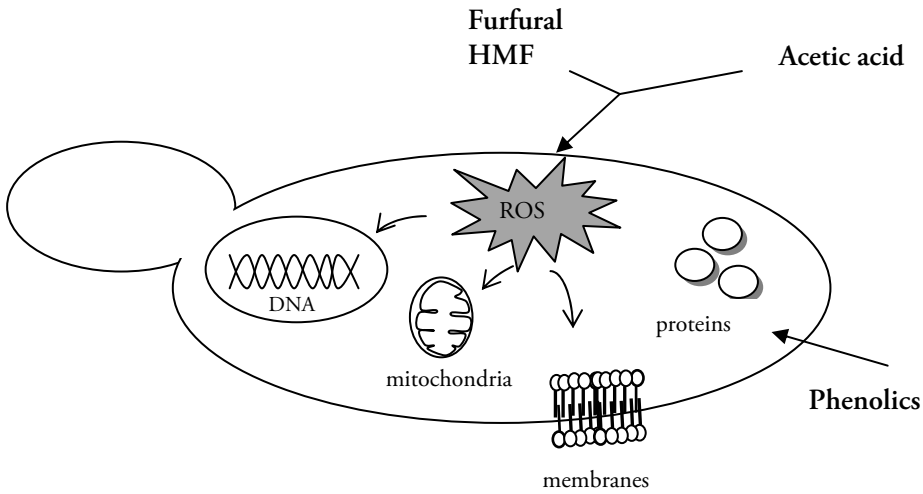

Figure 5. Simplified scheme showing macromolecules affected by hydrolysate-derived inhibitors

Acetic acid has also been shown to induce oxidative stress in *S. cerevisiae* (M, S. H. et al. 2011). As for phenolics, a possible involvement of these compounds in the precipitation of proteins was recently discussed (Jönsson, L. et al. 2013).

Peroxidation of lipids by ROS may be one of the reasons behind the damage to cellular membranes (Alic, N. et al. 2001) associated with hydrolysate inhibitors. The effect of the different inhibitors, including phenolic compounds, on membrane stability can be explained by the sensitivity of the lipids molecules to alterations in environmental conditions, which may lead to disruption of their physiological functions and of those of the proteins associated with them (Hazel, J. R. et al. 1990; Sikkema, J. et al. 1995).

Different response mechanisms to lignocellulosic inhibitors have been identified in *S. cerevisiae*. For instance, the efflux of inhibitory compounds by members of the ATP-binding cassette (ABC) transporter family is one molecular response behind tolerance to hydrolysate-derived inhibitors (Alriksson, B. et al. 2010; Ma, M. et al. 2010). Regarding acetic acid, the indirect degradation of the protein Fps1p aquaglyceroporin by Hog1p has also been observed as a tolerance mechanisms present in yeast (Piper, P. W. 2011). Other molecular mechanisms important for development of tolerance to hydrolysate inhibitors include biosynthesis of amino acids, activation of transcription factors, degradation of damaged proteins, and protein modifications

(Ma, M. et al. 2010; Tanaka, K. et al. 2012; Ask, M. et al. 2013b; Skerker, J. M. et al. 2013). Moreover, the capacity of organisms to re-structure their membranes by changing the lipid composition is one of the evolutionary events behind adaptation, as discussed in Paper IV. In fact, analysis of the lipid composition of tolerant microorganisms has offered some clues to explain traits regarding tolerance to different environmental conditions, as discussed in Chapter 5.

Consistent with all these observations, several genes and pathways involved in the oxidative stress response and regeneration of cofactors have proven to be an important part of the mechanisms of tolerance to hydrolysate inhibitors in *S. cerevisiae*. They have been manipulated through different metabolic engineering strategies for the development of more tolerant strains, as discussed in the next chapter.

2.2.2 Stress associated with high temperature

The use of fermentation temperatures higher than 35°C could lead to reductions in the costs associated with lower energy input between the different steps of the production process, i.e. by reducing cooling costs between pretreatment and fermentation (especially in tropical countries) and by reducing the energy input for ethanol separation (Banat, I. M. et al. 1998; Taylor, M. P. et al. 2009). In addition, the use of a higher fermentation temperature could improve the SSF processes since in the actual set-up, the temperature normally used (30 - 35°C) compromises the rate of saccharification by the cellulolytic enzymes (Olofsson, K. et al. 2008).

Effects of increased temperature on the fermentation performance of S. cerevisiae

S. cerevisiae is classified as a mesophilic yeast with an optimum growth temperature of between 30°C and 35°C. However, the temperature profile, i.e. the maximum and minimum temperature for growth and the optimum temperature for growth under given conditions, is strain-dependent (Van Uden, N. 1985).

The effect of changes in temperature during aerobic batch cultivation of *S. cerevisiae* (laboratory strain X2180-1A) on glucose was analyzed by varying the growth temperature from 28°C to 37, 39, 40, 41, 42, or 43°C (Mensonides, F. C. et al. 2002). This showed that small changes in temperature (even less than 1 degree) could make a difference between conditions of growth, non-growth and, death: at temperatures above 39°C, the specific growth rate was proportionally affected (around 18% reduction at 40°C and 60% reduction at 41°C), with no observable growth at 42°C. However, even at 42°C, the viability of the cells remained close to 90% while at 43°C they rapidly lost viability (Mensonides, F. C. et al. 2002). Also, the biomass yield—based on the glucose consumed—was inversely proportional to temperature, indicating higher maintenance requirements at high temperatures (Mensonides, F. C. et al. 2002).

In a different study performed using aerobic chemostat cultures, glucose was respired at temperatures ranging from 30°C to 37°C, while the cells showed a respire-fermentative metabolism and an increased flux through the glycolytic enzymes above 37°C (Postmus, J. et al. 2008). As the concentrations of intracellular glycolytic metabolites (i.e. substrates, products, and effectors) were affected at 38°C, it was postulated that the changes in flux were mainly caused by the effect that these new concentrations had on the glycolytic enzymes (Postmus, J. et al. 2008). The negative effect that high temperature has on mitochondria (see below) could also account for the differences seen in metabolism, as demonstrated in other investigations (Van Uden, N. 1985; Davidson, J. F. et al. 2001; Rikhvanov, E. G. et al. 2005).

Cellular effects and response of S. cerevisiae to higher temperatures

The effects of high temperatures on *S. cerevisiae* have been widely investigated through heat shock studies (see e.g. (Estruch, F. 2000; Hohmann, S. et al. 2003; Morano, K. A. et al. 2012)).

Heat stress affects several cellular processes, including a transient inhibition of cell division, which results in a brief accumulation of unbudded cells that can later recover and continue proliferating (Rowley, A. et al. 1993). Imbalance of protein homeostasis, caused by the unfolding and aggregation of proteins, is one of the most damaging effects of heat, and it is accompanied by defects in the structure of the cytoskeleton and organelles such as the endoplasmic reticulum (ER) and the Golgi system, with deleterious consequences for the trafficking of proteins (Richter, K. et al. 2010). ATP synthesis is another cellular process that is affected, since heat impairs coupling of oxidative phosphorylation (Patriarca, E. J. et al. 1990). Likewise, damage to membranes—related to changes in the organization and properties of the membrane lipids, and alterations in the activity of membrane-associated enzymes and transporters—has been reported to be a consequence of heat stress. In fact, different lines of evidence support the notion that heat stress induces a subsequent oxidative stress in *S. cerevisiae* from its effect on the mitochondrial electron transport chain (Morano, K. A. et al. 2012), and a high occurrence of petite mutants has been observed when cells are grown at high temperature (Van Uden, N. 1985). Furthermore, it has been shown that adaptation to mild heat stress is coupled to downregulation of mitochondrial functions (Sakaki, K. et al. 2003), and that anaerobic conditions result in a significant increase in thermotolerance compared to aerobic conditions (Davidson, J. F. et al. 2001).

Experimental data suggest that yeast cells are capable of sensing different levels of stress intensity and that the response is proportional to the strength of the stress, with a maximum activation achieved around 39–40°C (Santoro, N. et al. 1998).

A large amount of evidence has accumulated on the major role of the heat shock transcription factor 1 encoded by *HSF1*, an essential gene that acts as the main regulator of the heat shock response (HSR). Part of the HSR involves the repression

of protein biosynthesis and the induction of genes with protective functions (Morano, K. A. et al. 2012). Hsf1p regulates the expression of these genes by binding to heat shock elements (HSE) situated in the promoter regions of many Hsf1p-responsive genes (Figure 6).

Among these genes are those coding for chaperones. ***Chaperone systems,*** in both the cytoplasm and other organelles (including mitochondria), have been shown to assist correct protein folding or degradation of aberrant proteins (Verghese, J. et al. 2012). *S. cerevisiae* has two main chaperone systems. The Hsp70 system, which includes proteins found in the endoplasmic reticulum (ER), the mitochondria and the cytoplasm, plays a role in general folding, in assisting the translocation of nascent proteins, and in refolding of damaged proteins and degradation. The Hsp90 chaperone system participates in the final steps of protein maturation and interacts with a smaller number of proteins (Feder, M. E. et al. 1999).

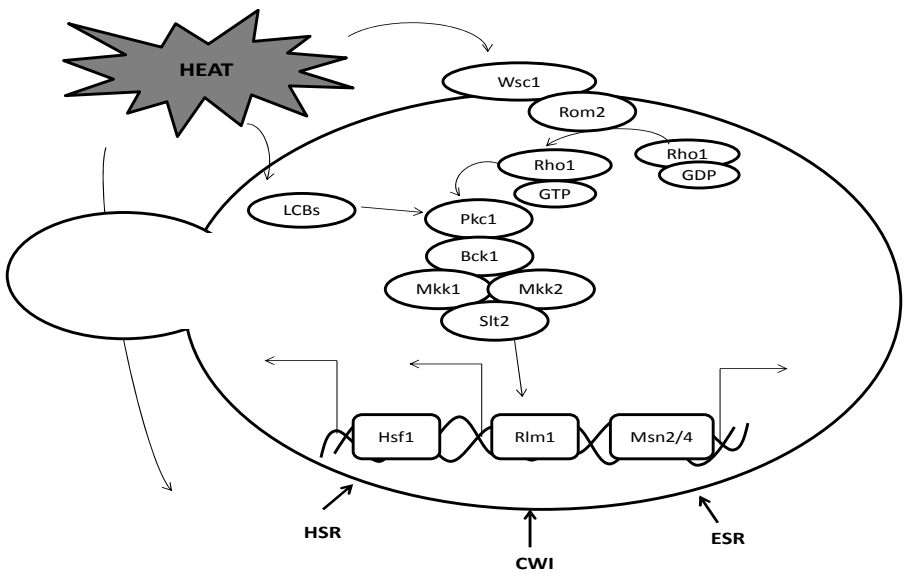

Figure 6. Simplified scheme for induction of the heat shock response (HSR), the environmental stress response (ESR) and the cell wall integrity (CWI) pathway by heat in *S. cerevisiae*.

In addition to the heat shock proteins (HSP), Hsf1p also participates in induction of the transcription of proteins involved in detoxification, energy generation, carbohydrate metabolism, cell-wall maintenance, and other cellular processes (Yamamoto, A. et al. 2005). Together with Hsf1, the transcription factors Msn2/4 contribute by controlling the changes in gene expression necessary to withstand exposure to lethal temperatures (reviewed in (Morano, K. A. et al. 2012)).

Apart from the heat shock response regulated by Hsf1 and the environmental stress response regulated by Msn2/4, the cell wall integrity (CWI) pathway is also induced by heat (Figure 6). It has been proposed that Wsc1, one of the proteins of the CWI pathway, is the protein that acts as a sensor of heat shock, and that it triggers a signaling cascade that results in phosphorylation of the terminal mitogen activated protein (MAP) kinase (MAPK) slt2/Mpk1 and subsequent activation of the transcription factor Rlm1(Levin, D. E. 2005). Rlm1p regulates the expression of at least 25 genes coding for cell wall proteins or with a function in cell wall biogenesis that are required for high-temperature growth (Levin, D. E. 2005). Activation of Wsc1 by heat would also result in the negative regulation of a target of RAS proteins in the cyclic AMP (cAMP) protein kinase A pathway (Fuchs, B. B. et al. 2009). The sphingolipids identified as long-chain bases (LCBs) such as dihydrosphingosine and phytosphingosine, have also been proposed as candidates in activation of the CWI pathway during heat stress (Dickson, R. 2010).

Finally, the disaccharide trehalose has been shown to contribute to yeast thermotolerance by its capacity to stabilize proteins and suppress the aggregation of misfolded proteins, among other effects (Verghese, J. et al. 2012). Likewise, changes in cellular make-up—such as alterations in the lipid composition of membranes—have been suggested as mechanisms for thermotolerance, allowing the cells to maintain proper fluidity and functionality of their membranes (Arthur, H. et al. 1976; Henderson, C. M. et al. 2013b). Rearrangements of the lipidome have also been observed for an *S. cerevisiae* strain after a long-term adaptation experiment combining high temperature and hydrolysate-derived inhibitors (paper IV), which suggests that changes in the membrane composition may be part of the cellular adaptation response to continuous heat stress.

2.2.3 Synergistic effects of stressors

The temperature-dependent growth profile of yeasts is largely influenced by the media composition. For instance, the inhibitory effect of ethanol is more severe at high temperatures, and the same pattern has been observed with other alcohols and acids (Van Uden, N. 1985; Ramos, M. T. et al. 1990). The same synergistic effect actually applies to hydrolysate-derived inhibitors and high temperature (Lu, Y. et al. 2012; Mutturi, S. et al. 2012). In fact, the results described in paper III indicate not only that different types of stressors have an additive effect on the internal homeostasis of the yeast, but also that some of the tolerance mechanisms evolved during the long-term adaptation experiment are specifically geared to the combination of stresses.

In the context of ethanol production, the synergistic effect of different types of stressors is of great relevance when considering strain development strategies. Even when the use of a single stressor is important for physiological studies and for

understanding the mechanisms of adaptation, it is clear that the development of tolerant phenotypes involves the coordinated action of genes acting in multiple metabolic and cellular responses (Nevoigt, E. 2008). From this point of view, the assessment of newly developed strains under conditions that reflect the process conditions would be expected to give a better indication of which factors should be emphasized during the development of more robust strains. The distinctive effect of *YAP1* overexpression during the glucose consumption phase in relation to the xylose consumption phase (paper II) underscores the complexity of the cellular processes and their interactions as well as adding evidence to the importance of integral assessments.

"Multiply, vary, let the strongest live and the weakest die"
Charles Darwin.

3. Improvement of the robustness of industrial strains of *S. cerevisiae*

In the context of this thesis, the concept of robustness is defined as the capacity of *S. cerevisiae* to maintain unaffected growth and fermentation performance despite changes in environmental conditions. In the case of ethanol production from lignocellulosic biomass, the development of robust strains involves enhancing the ability of *S. cerevisiae* to tolerate and respond to hydrolysate-derived inhibitors and high temperatures, among other stressors (cf. Chapter 2). In this chapter, some of the common approaches to improving stress tolerance in *S. cerevisiae* are described, with particular emphasis on improvement of industrial strains.

3.1 Laboratory strains vs. industrial strains

S. cerevisiae occupies an exclusive place in human history, not only because of its association with relevant human activities for millennia (e.g. baking, brewing, and wine making) but also for its central role as a model organism for the study of major eukaryotic cellular processes (Landry, C. R. et al. 2006; Legras, J.-L. et al. 2007). Different studies on population genomics of *S. cerevisiae* have generated a large amount of information that help to explain the enormous genetic variability within the species and also the evolutionary processes that have led to the adaptation of particular strains to specific environments (Fay, J. C. et al. 2005; Liti, G. et al. 2009). For the purposes of this thesis, however, this huge diversity of *S. cerevisiae* strains will be classified into two main groups: laboratory strains and industrial strains. The purpose of such a classification relies on physiological, phenotypic, and genetic differences between these groups that are of importance when improving relevant industrial phenotypes.

Laboratory strains can be defined as a set of reference strains, usually of defined ploidy and mating type, that are amenable to experimental procedures and that are usually used in physiological, genetic, and biochemical engineering research (van Dijken, J. P. et al. 2000). Some of the most commonly used *S. cerevisiae* laboratory strains are: *S. cerevisiae* S288C (Mortimer, R. K. et al. 1986), which was used as reference for the yeast genome sequence project (Goffeau A et al. 1996; Engel, S. R. et al. 2013); *S.*

cerevisiae BY4743, which is derived from S288C and was used in the "*Saccharomyces* Genome Deletion Project"; and the *S. cerevisiae* CEN.PK family, later selected as a platform for cell factory research (van Dijken, J. P. et al. 2000). The preference for a particular strain depends on the research interest. For example, CEN.PK laboratory strains are not considered to be optimal hosts for studying glucose sensing mechanisms due to mutations in the RAS-cAMP pathway (Vanhalewyn, M. et al. 1999).

In general, and in contrast to industrial strains, the laboratory strains are characterized by—and have been selected for—high sporulation efficiency, spore viability and mating efficiency, high transformation efficiency, heterothallic[2] behavior and homozygosity[3],and the availability of auxotrophic markers and isogenic strains (van Dijken, J. P. et al. 2000; Cebollero, E. et al. 2007). Because of these genetic and phenotypic traits, laboratory strains are more easily genetically engineered than industrial strains. However, laboratory strains are also characterized by a generally inferior robustness and reduced fermentation capacity under non-optimal conditions (Martín, C. et al. 2003; Modig, T. et al. 2008) (Paper II).

Industrial strains, on the other hand, are usually polyploid and/or aneuploid strains that are classified according to the production purpose this—as bread (baker's), wine, beer, sake, and bioethanol (biofuel) yeasts. Regarding industrial strains for ethanol production, and in particular in the case of the Brazilian ethanol industry (based on cane juice or diluted molasses), the *S. cerevisiae* strains currently used have been selected for their ability to keep high fermentative capacity and viability under stressful industrial conditions, including high ethanol concentrations (up to 17% (v/v; batch mode) (Casey, G. P. et al. 1986)), low nutrients and pH, high sugar concentrations, and process interruptions, amongst other factors (Stambuk, B. U. et al. 2009). Because of all these phenotypic traits, the strains used for first-generation ethanol production (such as the Brazilian strains) are amongst the favorite candidates for ethanol production from lignocellulosic materials.

So far, most of the research regarding surmounting of challenges imposed by lignocellulosic substrate for ethanol production has been carried out in laboratory strains and, in the particular case of pentose metabolism, the research has mainly been carried out under standard laboratory conditions (e.g. rich or mineral medium and well-controlled environmental conditions) (Chapter 2). However, the development of efficient industrial processes requires transfer of all relevant genetic modifications to the more robust background of industrial strains and to assess the changes under process conditions. In this context, it is worth noticing that in the last twenty years the toolbox for genetic engineering of industrial strains has expanded significantly;

[2] This means that the mating types reside in different cells. A cell cannot undergo switching of mating type; therefore, mother-daughter mating is not possible.

[3] This describes the genotype of a diploid organism in which both alleles of the same gene are identical.

and previous limitations associated with lack of markers or restrictions in the use of antibiotics have been overcome. Among the advantages of these genetic tools is the possibility of chromosomal integration so that the genetic stability of the construct is suitable for long-term cultivation. Moreover, the use of systems based on the Cre-LoxP cassette (Güldener, U. et al. 1996) and attB/attP mediated integration by øC31 integrase (Groth, A. C. et al. 2000) allows multiple rounds of gene introduction as well as the recycling and final removal of the marker (Gueldener, U. et al. 2002; Jensen, N. B. et al. 2013). Furthermore, optimization of transformation protocols for industrial strains has improved the chances of successful transformations (Gietz, R. D. et al. 2007).

In the following sections, the main strategies that have been used for improvement of the tolerance of *S. cerevisiae* to hydrolysate-derived inhibitors and/or temperature (the two main types of stressors considered in this thesis) will be presented.

3. 2 Approaches for development of *S. cerevisiae* strains with increased tolerance

The strategies commonly used for improving the tolerance of *S. cerevisiae* to environmental stressors associated with ethanol production can be classified into two main groups: (i) rational metabolic engineering approaches (or targeted genetic engineering) and (ii) non-targeted approaches. In the first case, also known as "forward metabolic engineering" (as opposed to "inverse metabolic engineering"; see Chapter 5), a deep understanding of the pathways and mechanisms behind the phenotype of interest is required, including knowledge of the genes involved in the traits that should be improved (Cebollero, E. et al. 2007; Oud, B. et al. 2012). This also implies that the control over the genetic changes to be introduced is relatively high; and in contrast to the second approach, there is no accumulation of unfavorable mutations (although cellular responses to the genetic change can vary, e.g. depending on epistatic interactions) (Fierst, J. L. et al. 2012). The non-targeted strategies, on the other hand, have usually been used when the background information about the biochemistry or genetics of the relevant phenotypes has been limited, or when the desired phenotype involved interactions between multiple metabolic pathways and regulatory factors, so that manipulation of the strain by targeted metabolic engineering approaches became too complex (Çakar, Z. P. et al. 2012) (Paper III).

3.2.1 Rational metabolic engineering

Bailey defined metabolic engineering as "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the

use of recombinant DNA technology" (Bailey, J. 1991). The information on the effects that the different stressors have on yeast physiology and our understanding of the innate response mechanisms of yeast (Chapter 2) have permitted the identification of several classes of protein associated with tolerance to inhibitors and heat stress, and have enabled their genetic manipulation in *S. cerevisiae* as discussed above.

Enzyme-driven detoxification

The heterologous expression in *S. cerevisiae* of *Trametes versicolor* laccase resulted in a strain with improved capacity to grow and ferment in the presence of phenolic inhibitors and spruce hydrolysate (Larsson, S. et al. 2001). This was explained by the enzyme's ability to convert the phenolic compounds to less inhibitory polymerization products (Larsson, S. et al. 2001). Also, overexpression of decarboxylases was shown to increase the resistance of *S. cerevisiae* to various phenolic compounds (Clausen, M. et al. 1994; Mukai, N. et al. 2010). Larsson et al. (Larsson S et al. 2001) showed that overexpression of the *PAD1* gene in *S. cerevisiae* improved the growth rate and ethanol productivity in the presence of phenolic acids and dilute-acid spruce hydrolysate.

However, most of the efforts have focused on the detoxification of aldehyde compounds, since microbial reduction of the furaldehydes furfural and HMF, and some phenolic compounds such as vanillin, to less inhibitory alcohols (i.e. furfuryl alcohol, furan-2, 5-dimerhanol and vanillin alcohol respectively) can be carried out by oxido-reductases (Wulf, O. et al. 1989; Gutiérrez, T. et al. 2002; Larroy, C. et al. 2002; Koopman, F. et al. 2010). Indeed, the overexpression of homologous and heterologous oxido-reductases in *S. cerevisiae* has been one of the most used and successful approaches for improvement of tolerance to aldehydes; and the new strains have shown better growth in synthetic media supplemented with one or several of the aldehyde inhibitors (Petersson, A. et al. 2006; Laadan, B. et al. 2008; Moon, J. et al. 2012) and also better performance when grown in hydrolysate (Almeida, J. R. M. et al. 2008). However, industrial strains *per se* have generally been found to have higher reductase capacities (Modig, T. et al. 2008). Also, expression of furaldehyde reductases was found to be upregulated during production of the inoculum to be used during SSF experiments (Alkasrawi, M. et al. 2006; Almeida, J. R. M. 2009). This suggests that the effect of further increasing the reductase pool by gene overexpression during large-scale fermentation may be more limited than under laboratory conditions; it also indicates that proper assessment of such genetic modifications should be done in the production host (see below).

Interestingly, reductases have also been shown to be involved in a different type of stress response: overexpression of the mitochondrial NADH-cytochrome b5 reductase encoding gene *MCR1* in *S. cerevisiae* led to a shorter lag phase and a faster growth rate than in the control strain in the presence of 12 g/L acetic acid. This indicates a

possible involvement of the enzyme in the response to the oxidative stress caused by the weak acid (Signori L, personal communication; paper II).

Tolerance to furaldehyde compounds was also achieved by overexpressing the *GSH1* gene, which encodes gamma glutamylcysteine synthetase, in a laboratory yeast strain during simultaneous saccharification and fermentation (Ask, M. et al. 2013c). This and other studies point to a possible depletion of reduced cofactors caused by HMF and furfural reduction (Gorsich, S. W. et al. 2006; Ask, M. et al. 2013b) and suggest that engineering of the availability of redox cofactors may be an important strategy to improve stress tolerance further (Ask, M. et al. 2013b).

Transporter-driven detoxification

Several genes involved in the transport of xenobiotic compounds or endogenously produced toxic compounds have been identified through transcriptome studies of *S. cerevisiae* in the presence of hydrolysate inhibitors, e.g *PDR5* and *PDR15* plasma membrane ATP-binding cassette (ABC) transporters. (Almeida, J. R. M. 2009; Ma, M. et al. 2010; Sundström, L. et al. 2010). The negative effect of acetic acid in the transport of nutrients inside the cell has also been reported (Ding, J. et al. 2013). An example of enhanced resistance to phenolic inhibitors and HMF by overexpression of multidrug resistance genes (*ATR1* and *FLR1*) in *S. cerevisiae* was reported by Alriksson et al. (Alriksson, B. et al. 2010).

Transcription factors and detoxification

Simultaneous regulation of the transcription of several genes that participate in stress response mechanisms can be done by manipulation of the level of transcription factors. Due to the variety of biological functions of the target genes, the effect of overexpressing a transcription factor would be expected to be broader than the single expression of a gene coding for an enzyme (Alriksson, B. et al. 2010). Overexpression of *YAP1*, a transcription factor involved in the oxidative stress response of *S. cerevisiae* (Toone, W. M. et al. 1999), has been found to increase the resistance of laboratory strains of *S. cerevisiae* to HMF, furfural, coniferyl aldehyde, and spruce hydrolysate (Alriksson, B. et al. 2010; Kim, D. et al. 2013). Also, overexpression of the transcriptional activator *HAA1* resulted in an enhancement of acetic acid tolerance (Tanaka, K. et al. 2012). More recently, the effect of *YAP1* and *MCR1* overexpression (either alone or in combination) was assessed in an already robust pentose-consuming industrial strain (*S. cerevisiae* GSE16 (Demeke, M. M. et al. 2013b)) during the fermentation of undetoxified lignocellulosic spruce hydrolysate (paper II). The results showed that overexpression of both genes was still relevant under the conditions being assessed and that there was no additive effect from the simultaneous overexpression of the genes. The results also revealed unexpected interactions between xylose utilization and *YAP1* overexpression in GSE16 that would require further investigation.

Thermotolerance

Thermotolerance was also improved in *S. cerevisiae* by rational engineering. For example, overexpression of *TPS1*, the gene coding for trehalose-6-phosphate synthase, led to higher concentrations of trehalose, which in turn resulted in higher fermentation efficiency at 38°C, and the critical growth of the new strain increased from 36°C to 42°C (An, M.-Z. et al. 2011). Improved thermotolerance was also achieved by overexpression of a mutated allele of *RSP5*, encoding an ubiquitin ligase enzyme (Shahsavarani, H. et al. 2012). The mutations, identified in a wild-type thermotolerant *S. cerevisiae* strain, correlated with higher transcription levels of the gene and with increased ubiquitination of the enzyme's target proteins, such as heat-denatured proteins, facilitating their degradation (Shahsavarani, H. et al. 2012). It is worth noticing that in both cases the evaluation of the phenotype was performed in rich medium; therefore, the synergistic effect observed between temperature and chemical inhibitors (such as hydrolysate-derived inhibitors) was not assessed. Additionally, modifications in the expression of the heat shock protein 104 (Hsp104) has also been proposed as a mechanism behind enhanced thermotolerance in *S. cerevisiae*, given its central role in promoting the resolubilization and reactivation of proteins after severe stress (Lindquist, S. et al. 1996). Likewise, there have been reports in which alterations in the expression of heat shock transcription factors of one plant species even resulted in increased thermotolerance in a different plant species (Li, Z. et al. 2013). However, no reports could be found on direct genetic engineering of *HSF1* or alteration of the expression of Hsp104 in *S. cerevisiae* strains with the aim of improving its thermotolerance during ethanol production from lignocellulose-based biomass. The high-temperature-growth phenotype (Htg) is a quantitative trait with complex interactions between the genes responsible for the phenotype (Steinmetz, L. M. et al. 2002), which may explain the relatively few reports of genetic engineering as a strategy for improving thermotolerance.

3.2.2 Non-targeted approaches

One main advantage of non-targeted approaches is that preliminary knowledge about the pathways and enzymes behind the phenotype of interest is not needed. Furthermore, these strategies allow us to address several traits simultaneously, which is highly desirable for *S. cerevisiae* strains used in ethanol production (Nevoigt, E. 2008).

Random mutagenesis and genome shuffling

Random mutagenesis by physical methods (ultraviolet light) or chemical methods (ethyl-methane sulfonate, EMS) has been widely used for the genetic improvement of yeast strains (Sridhar M et al. 2002; Cebollero, E. et al. 2007; Thammasittirong, S. N.-R. et al. 2013). But the fact that industrial strains are usually polyploid makes the selection of recessive mutations difficult (Cebollero, E. et al. 2007).

Improvement of phenotypes by genome shuffling (or protoplast fusion) includes the possibility of using more than two parental strains for hybridization. Genome shuffling between different species is also possible, which further increases the genetic diversity during strain improvement (Pina, A. et al. 1986). A common strategy during strain development is to use a combination of both approaches, i.e. the parental cells are first subjected to random mutagenesis followed by multiple rounds of genome shuffling. In both approaches (mutagenesis and genome shuffling), high-throughput screening systems are highly desirable (Nevoigt, E. 2008).

Thermotolerant and inhibitor-tolerant strains have been obtained by these two approaches (Kida, K. et al. 1992; Shi, D.-j. et al. 2009; Pinel, D. et al. 2011). For example, Lu and co-workers used chemical mutagenesis and several rounds of genome shuffling for the generation of sufficient genetic diversity; then they performed the screening with a combination of stresses (Lu, Y. et al. 2012). The approach resulted in the improvement of *S. cerevisiae* robustness under co-stress of heat and acetic acid and also under co-stress of heat and furfural. Another interesting example involved protoplast fusion between *S. cerevisiae* and *Candida shehatae*, which produced cells capable of growing at 42°C and that were able to utilize xylose (Pasha, C. et al. 2007).

Introduction of libraries

Another approach to strain improvement is based on the use of genetic libraries of relevant genes in which random mutations have been introduced. For instance, the use of a library of the transcription factor encoding gene *SPT15* in which genetic diversity was obtained by error-prone PCR, resulted in a *S. cerevisiae* strain with improved ethanol tolerance (Alper, H. et al. 2006). The use of knockout and overexpression libraries in an integrative manner is another promising alternative for the construction of improved phenotypes (Jin, Y.-S. et al. 2007). However, given the tremendous genetic diversity created by these approaches, the design of proper methods for evaluation of library quality and for isolation of the relevant phenotypes is a critical step (Klein-Marcuschamer, D. et al. 2010).

Evolutionary engineering

Evolutionary engineering, also referred to as adaptive laboratory evolution (ALE) or long-term adaptation, is a method for strain development based on the continuous evolution of a population by applying a long-term selective pressure that will give an advantage to the desired phenotype (Sauer, U. 2001). During the process, variants that are fitter for that particular environment will eventually replace the initial population (reviewed recently by Çakar et al. (Çakar, Z. P. et al. 2012) and Dragotsits et al. (Dragosits, M. et al. 2013)).

The initial population, originating from a wild-type or mutagenized strain, can be cultivated under batch (by serial transfers) or continuous operation modes (e.g. chemostats). In batch experiments, the cells have to handle significant changes in the environmental conditions because the nutrients are being consumed while toxic

metabolic products are produced; and generally the transfer to the new batch is done before the cultures reach stationary phase in order to avoid stationary phase adaptation (Dragosits, M. et al. 2013). In evolutionary engineering experiments using chemostat cultivations, the physiological state remains stable; there, mutations that improve the growth rate or increase the residence time in the fermenter (such as adherence or biofilm formation phenotype) will be advantageous (Sauer, U. 2001). Table 3 is a summary of some evolutionary engineering experiments with yeast that were aimed at improving robustness to environmental stressors.

During the evolutionary process, there are two major sources of genetic variation that may account for an improved phenotype (given that a proper selection method is applied): standing genetic variation or new mutations. The first type refers to allelic variations that are already present in the initial population as neutral (or slightly deleterious) but whose fixation becomes important during the selection process (Burke, M. K. 2012). Since for most of the evolutionary experiments with *S. cerevisiae* the starting population is isogenic, these allelic variations will be present only in those populations that have been previously mutagenized. On the other hand, the frequency of new mutations, i.e. the mutation rate, depends on the presence (or not) of *mutator alleles*. Strong mutator genes studied in bacteria, for example, can increase the mutation rate by 1,000 fold (Taddei, F. et al. 1997). Mutation rate will also vary *within the genome*, with some regions being more prone to mutations than others. For instance, in *S. cerevisiae*, intragenic tandem repeats in the gene encoding the cell surface protein Flo1 can change the length of the protein and as a result the adhesion properties and the capacity for biofilm formation of Flo1 are modified (Verstrepen, K. J. et al. 2005). Finally, the mutation rate will also vary *with the environment*; there have been studies showing that the mutation rate is higher under stress conditions (Sauer, U. 2001; Szafraniec, K. et al. 2001).

It is actually difficult to determine for how long the evolutionary experiment should continue, i.e. at what point the prolongation of the experiment will be paid back with improvement in fitness. This benefit ratio will depend, among other factors, on the mutation rate and the selection pressure; also the increase in fitness is not a linear function of the number of generations (Sauer, U. 2001; Dragosits, M. et al. 2013). In the examples given below, the numbers of generations necessary to achieve the improved phenotype varied between 20 and 500 (Table 3).

In order to determine when it is appropriate to stop a long-term adaptation experiment, and also as a general way of monitoring the evolutionary progress, it is important that evaluation at the single-clone level should run in parallel with the ALE experiment (Sauer, U. 2001). Such analysis allows the early identification of relevant trade-off or cross-benefit events that may appear in alternative environments (Dragosits, M. et al. 2013).

Table 3. Evolutionary engineering experiments with *S. cerevisiae*

Strain	Set-up, environmental stressor/ result	No. of generations	Reference
TMB3400 (industrial)	Batch selection, **furfural/** reduced lag phase and higher viability in furfural-containing media and hydrolysate	300	(Heer, D. et al. 2008)
FY2 derivative S288C (laboratory)	Batch selection, YNB + **lignocellulosic hydrolysate /** evolved strains with 57%, 12%, 22%, and 24% increase in growth rate in the presence of hydrolysate, acetic acid, HMF, and furfural, respectively	Up to 463	(Almario, M. P. et al. 2013)
CEN.PK (laboratory) EMS mutagenized	Chamostat and batch selection, **multiple abiotic stresses**/102-, 89-, 62- and 1,429-fold increase in resistance to freezing-thawing, temperature, ethanol, and oxidative stress	Up to 68	(Çakar, Z. P. et al. 2005)
TMB3400 (industrial)	Batch and chemostat selection, **cocktail of inhibitors and spruce hydrolysate/**83% increase in growth rate, reduction of lag phase for batch; ~3.5-fold increase in furaldehyde conversion rate for chemostat	429 and 97	(Koppram, R. et al. 2012)
TMB3001 (laboratory)	Chemostat selection, **bagasse hydrolysate/** 2 times higher ethanol yield and productivity	353 **hours**	(Martin, C. et al. 2007)
Ethanol Red^{R} (industrial)	Batch cultivations, 50% (v/v) **spruce hydrolysate** and **39°C/**evolved strain capable of growing and fermenting in the presence of inhibitors at 39°C without pre-adaptation	280 approx.	(Paper III)

It is also important to mention that there are also limitations in the development of certain phenotypes through evolutionary engineering, as shown by recent studies aimed at improving acetic acid tolerance in *S. cerevisiae*. For these studies, the tolerant phenotype was not recovered after storage of the evolved populations (Wright, J. et al. 2011)(Sánchez i Nogue, personal communication). Instead, both groups reported the inducible character of the trait (Wright, J. et al. 2011; Sànchez i Nogué, V. et al. 2013).

3.2.3 Combined approaches

Considering the variation in fermentation procedures (SSF, SHF, fed-batch, etc.) and especially in view of the diversity of the properties of lignocellulosic hydrolysates, it is evident that the requirements as to what would be considered "the best strain" will vary from process to process.

On the one hand, non-targeted approaches appear to be more suitable for improvement of several traits simultaneously, especially if the stresses have synergistic effects. On the other hand, the need for a wider range of substrates in *S. cerevisiae* for economically feasible fermentations from lignocellulosic hydrolysates has been largely facilitated by rational metabolic engineering strategies. These two facts therefore support the idea that development of suitable strains would require a combination of approaches. This is exemplified by the recent development of the pentose-consuming and inhibitor-tolerant strain GSE16 (Demeke, M. M. et al. 2013a; Demeke, M. M. et al. 2013b). The original strain Ethanol Red® (an industrial strain used in first-generation production of ethanol) was genetically modified with xylose (and arabinose) pathways and subjected to chemical mutagenesis, genome shuffling, and evolutionary engineering for xylose fermentation and hydrolysate tolerance, resulting in strain GS1.11-26 (Demeke, M. M. et al. 2013a). A further step to recover a loss in aerobic growth capacity was performed by meiotic recombination of GS1.11-26 with a segregant of the parental strain Ethanol Red with high inhibitor tolerance, resulting in GSE16 (Demeke, M. M. et al. 2013b).

4. Inverse metabolic engineering

The concept of reverse engineering—widely applied in diverse disciplines—refers to the process of identifying the technological principles behind an object or system by analyzing its structure and function. The process generally involves the identification and analysis of the different components that make up the device, and the subsequent reconstruction should allow the elucidation of the principles behind its functioning (Eilam, E. 2005). In this chapter, application of this concept in the field of biotechnology is described, and the main genome-wide approaches used for the analysis of biological systems at the molecular level are introduced. I also include some successful examples and lessons learned when applying these approaches, paying special attention to the *S. cerevisiae* stress response during ethanol production.

4.1 The inverse metabolic engineering cycle

The application of the concept of reverse engineering to study biological systems is known either as "**inverse metabolic engineering**" or "**reverse metabolic engineering**" (Bailey, J. E. et al. 1996; Oud, B. et al. 2012). It can be defined as a cycle of steps aimed at identifying the molecular mechanisms behind a phenotype of interest. The phenotypes under study might include, for example, superior strains selected by screening (such as microorganisms from collections or targeted environments), or strains improved by non-targeted approaches (such as the ones discussed in Chapter 4).

A simplified scheme for the reverse metabolic engineering cycle and its supportive elements is shown in Figure 7. Once the superior phenotype has been identified, the next step—and also the key step—in the cycle is the elucidation at the molecular level of the basis of the superior performance. The identification at the genetic level of the factor(s) responsible for the improved phenotype makes the next step of the cycle possible; that is, the partial (or total) reconstruction of the phenotype by introducing such factors into a different strain. This newly constructed strain can be modified again through non-targeted approaches and re-enter the cycle, facilitating the generation and evaluation of optimal pathway configurations (Oud, B. et al. 2012). As pointed out by Oud et al., the information generated through the iteration of the steps during the reverse engineering process cannot only be appraised in the context

of strain improvement for industrial applications (Oud, B. et al. 2012). The significance of this information is also of great value for the construction of knowledge that will lead us to a better understanding of biological systems.

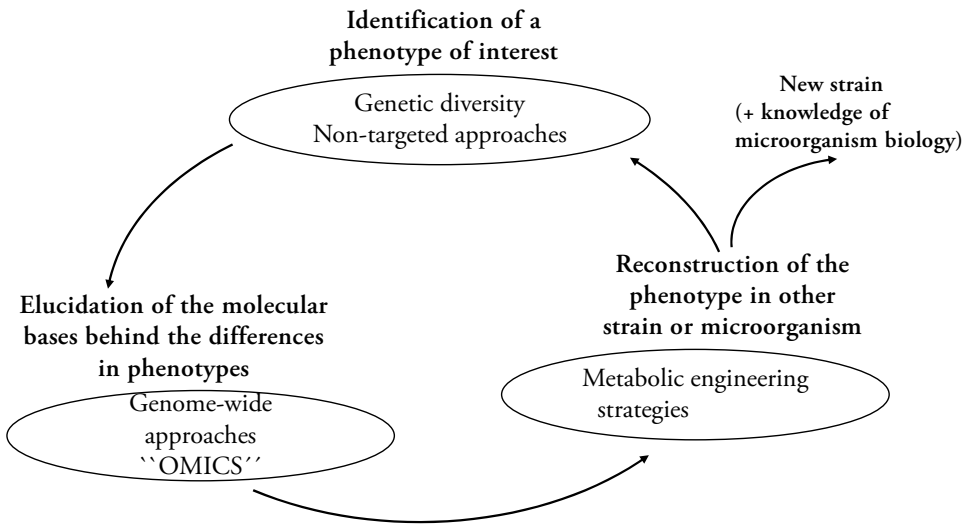

Figure 7. Simplified scheme for the reverse metabolic engineering cycle and (encircled) approaches used at each step of the cycle.

4. 2 Identification of the molecular bases of phenotypic variance

Based on the different levels of biological information represented by DNA, transcripts, proteins, and metabolites, amongst other molecules, various genome-wide analytical approaches (and their related technologies) have been developed. In contrast to more traditional approaches, these so-called "omics" technologies share the following features (Zhang, W. et al. 2010):

> They are built on high-throughput data and the processing of the information is done using a top-down strategy; that is, by decomposing a system into its smaller elements;

The analysis attempts to understand cell functioning as a whole, by integrating the information obtained from the measurement of the different molecular species and how they correlate with each other as a system;

The usefulness of the large amount of data generated is largely dependent on the development of bioinformatics tools (including the statistics associated with them) that permit us to store, organize, visualize, and overall to interpret the enormous amount of complex biological data.

Based on the above characteristics, it is clear that the significance of these technologies goes beyond their use in the setting of reverse metabolic engineering studies. Their application in other type of research questions has revealed, in a relatively short time, a significant amount of relevant information regarding function, regulation, and interaction of and between cell components (Yoon, H.-J. et al. 2002; Francesconi, M. et al. 2014).

The following subsections describe some examples where the main "omics" technologies have been used for identification of relevant genes and mechanisms involved in single or combined stress tolerance in *S. cerevisiae*, especially in the context of inverse metabolic engineering.

4.2.1 Transcriptomics

The first publications reporting genome-wide transcriptional analysis of *S. cerevisiae* were available in 1997 (DeRisi, J. L. et al. 1997; Lashkari, D. A. et al. 1997). Since then, transcriptomics has become the most common and well-developed technique for genome-wide studies, facilitated in part by its relatively low price and the availability of diverse bioinformatics packages for data interpretation (Daran-Lapujade, P. et al. 2008). Hybridization-based approaches are the technology most frequently used for transcriptome analysis, and involve incubation of cDNA that has been previously labelled (e.g. with fluorophores) with commercial high-density oligo microarrays or custom-made microarrays.

The use of DNA microarrays has enabled the generation of large amounts of information about the type and level of transcripts that are altered in *S. cerevisiae* as a response to several environmental conditions (Lelandais, G. et al. 2010). In a pioneering study of stress responses using transcriptomics, changes in transcript levels were recorded over time while cells were facing different environmental conditions; this led to the identification of a set of genes whose expression was altered under nearly every condition tested, and that was considered to take part in a general stress response named the environmental stress response (Gasch, A. P. et al. 2000) (See Chapter 2).

The transcriptional response of *S. cerevisiae* to hydrolysate-derived inhibitors (in their single or combined form) has primarily led to the identification of key biological functions and possible mechanisms related to the survival and adaptation response of the cell. For instance, transcriptome profiling showed that alterations in pathways that supply energy and regeneration of cofactors as well as processes involved in the degradation of damaged proteins and protein modification were mechanisms of adaptation in *S. cerevisiae* while grown in the presence of HMF (Ma, M. et al. 2010). Reverse metabolic engineering with the use of transcriptome technology was recently used for screening for acetic acid tolerance in around 500 *S. cerevisiae* strains, and it led to the identification of a superior phenotype with high capacity for adaptation to the weak acid acetate (Haitani, Y. et al. 2012). Global gene expression analysis also identified the transcription factor *HAA1* as another potential candidate, and later studies showed that overexpression of *HAA1* did indeed result in improved acetic acid tolerance in *S. cerevisiae* (Tanaka, K. et al. 2012). Another successful story on the use of transcriptomics in reverse metabolic engineering was the identification of *ADH6* as a potential candidate for the construction of yeast strains with increased tolerance to HMF (Petersson, A. et al. 2006). In this particular case, the interpretation of the microarray data was facilitated by focusing the analysis on reductase and dehydrogenase genes, since previous results had highlighted differences in reductase capacity between the strains being studied (Nilsson, A. et al. 2005).

Despite these successes, a general outcome is the complexity of the cellular responses to these kinds of environmental stress. Also, the need to identify the biological function of many as yet uncharacterized ORFs has been highlighted, as they appeared to take part in these responses (Ask, M. et al. 2013a).

The extensive research performed using transcriptome profiling has also highlighted critical aspects of experimental design and interpretation of data for this approach. For example, chemostat is usually preferred over batch for comparison of strains or conditions, since this mode of microbial growth eliminates the growth rate as a variable (Daran-Lapujade, P. et al. 2008). A second factor to consider is the speed at which the transcriptional response takes place since, as previously commented, upregulation (or downregulation) of many genes can take place only within a narrow timespan (Gasch, A. P. et al. 2000; Yamamoto Noritaka et al. 2008). Regarding interpretation of data, it has been observed that generally only a small fraction of genes with significant changes in transcription are directly correlated with fitness under the conditions used for comparison (Sundström, L. et al. 2010), so the identification of positive leads becomes more complicated. The consideration of these and other factors during the design of transcriptome experiments surely affect the ease of interpretation of the data, and therefore the chances of identifying positive leads, especially when the nature of the experiment *per se* is complex.

In this context, **RNA-seq technology**, a recently developed method for mapping and quantifying the transcriptome, is expected to facilitate and improve its analysis. Briefly, the RNA population is converted to cDNA fragments which are sequenced

using high-throughput sequencing technology. After sequencing, the reads are aligned to a reference genome or assembled *de novo*, resulting in a genome-scale transcription map describing the quantity of individual reads and their genomic location (Wang, Z. et al. 2009). This approach offers several benefits over hybridization-based approaches for the study of transcriptomes, including low background noise, much larger dynamic range to quantify gene expression level, and lower costs for mapping transcriptomes of large genomes (Wang, Z. et al. 2009).

4.2.2 Proteomics

The low correlation found in many studies between the mRNA level and the protein level indicates that transcriptome analysis cannot always be used to predict protein expression adequately (Gygi, S. P. et al. 1999; Olivares-Hernández, R. et al. 2010). Proteome analysis, i.e. the study of the set of proteins present in a cell (organism or organelle) at given time, is an approach with high potential for describing many biological properties—such as quantification of protein expression, localization and identification of posttranscriptional modifications, mapping of the interactions of proteins with ligands, and identification of the rate at which these properties change under particular conditions (Patterson, S. D. et al. 2003).

At present, two-dimensional electrophoresis (2DE) is the technique commonly used for protein separation, usually followed by mass spectrometry for the identification of protein(s) of interest. Applications include quantitative expression profiling, and it also provides information about the molecular weight and isoelectric point (pI) of the proteins. However, the method has limitations regarding its reproducibility and capacity to separate acidic, basic, hydrophobic, and low-abundance proteins (Chandramouli, K. et al. 2009).

In general, the complexity of proteomic studies is higher than that of transcriptome profiling; and the technology faces important challenges, such as improvement of methods for separation of membrane-bound proteins, and the development of analytical tools for processing and analyzing the large amounts of data that are generated (Chandramouli, K. et al. 2009).

Regarding stress tolerance in *S. cerevisiae*, the contribution of proteomic approaches has been qualitative so far, i.e. the outcomes of the studies have helped to better describe the mechanisms and processes used by the cell to adapt and respond to a particular stress (Kim I et al. 2007; Lin, F.-M. et al. 2009; Martínez-Pastor, M. et al. 2010; Westman, J. O. et al. 2012). However, the findings have not yet been translated into metabolic engineering strategies to create more robust phenotypes. Nevertheless, the development of the various proteomics technologies is booming, and improvements in sensitivity, throughput, and proteome coverage can be expected in the near future (Chandramouli, K. et al. 2009; Mallick, P. et al. 2010).

4.2.3 Metabolomics

Metabolomics refers to the unbiased identification and quantification of all metabolites (i.e. low-molecular-weight molecules) in a biological system (Dunn, W. B. et al. 2005). The metabolites (both intracellular and the ones that are secreted) represent the final product of genetic expression and regulation; and their relevance to cellular metabolism can be appreciated for instance by their influence on enzyme kinetics (e.g. by product inhibition),and their role as signaling and protective molecules (e.g. sphingolipids and glutathione, respectively), among other biological functions (Bergdahl, B. et al. 2012).

A typical workflow of a metabolomic study includes: (a) sample preparation (quenching of the sample is generally necessary due to the speed at which the metabolic processes take place); (b) extraction (for example, polar/non-polar, or hot alcoholic extractions); (c) separation (e.g. by gas chromatography or high performance liquid chromatography (HPLC)); and (d) detection (mainly done by mass spectrometry-based techniques) (Kell, D. B. 2004; Dunn, W. B. et al. 2005; Villas-Bôas, S. G. et al. 2005).

Despite the relatively low number of metabolites estimated in *S. cerevisiae* (around 600 - 1,100) (Dunn, W. B. et al. 2005; Mo, M. et al. 2009), many technical challenges, such as the metabolic compartmentation of the cells, remain to be solved to obtain a complete and accurate determination of its metabolome (Oud, B. et al. 2012). Nevertheless, the determination of a set of metabolites present under a certain set of physiological conditions and at a particular time has proven to be a relevant source of functional information (Goodacre, R. et al. 2004).

Metabolic studies can offer complementary evidence—to transcriptomics and proteomics analyses—about how different phenotypes respond to a particular form of environmental stress. And from the integration of such information, relevant metabolic pathways and signaling mechanisms can be identified. For instance, by analyzing time course data of transcripts and metabolites obtained from yeast responding to high and low temperatures, it was observed that the changes in metabolite levels happened before the changes in transcript levels of the enzymes associated with the corresponding metabolite (either via substrate interactions or product interactions); such types of correlations proved to be useful for determining the direction of metabolic reactions (Walther, D. et al. 2010).

Hasunuma et al. recently showed the potential of metabolomics in the context of reverse metabolic engineering and stress tolerance in *S. cerevisiae* (Hasunuma, T. et al. 2011). In that study, the negative effect of acetic acid during xylose fermentation was studied by a metabolomic approach. In the presence of the acid, a slower rate through the non-oxidative part of the pentose phosphate pathway was revealed from an increase in the level of the associated metabolites. And further overexpression of the

enzymes transaldolase or transketolase increased the production of ethanol in the presence of either acetic acid or formic acid (Hasunuma, T. et al. 2011).

Lipidomics

Given the relevance of lipids in biological functions and structural diversity, they are given special consideration in metabolomics studies. The term **lipidomics** refers to the characterization of the molecular species of lipids that are present in a biological sample (i.e. the lipidome), and it is being developed as a special approach within the metabolomics field (Wolf, C. et al. 2008; Dennis, E. A. 2009).

An increasing number of studies have indicated that lipids are more than structural components of membranes or sources of energy (Roberts, L. D. et al. 2008). For example, sphingolipids have a significant role in *S. cerevisiae*, especially in response to heat stress. As mentioned in Chapter 2, long-chain bases (LCBs) have been suggested to have a role as intracellular signaling molecules during heat stress, which results, among other things, in the ubiquitination of proteins (Dickson, R. 2010). Meier and co-workers also showed that sphingoid bases were required for initiation of translation of heat shock proteins (Meier, K. D. et al. 2006). And although the initial studies regarding lipids structure, localization, and function in *S. cerevisiae* have mainly been done with mutant strains (Daum, G. et al. 1998), the technical advances in lipid identification in combination with other approaches have been of particular value for elucidation of many other additional functions of lipids, such as carbon source utilization, sporulation, and cell wall integrity (Cowart, L. A. et al. 2005).

To the best of my knowledge, lipidomics has not been used in the context of inverse metabolic engineering in *S. cerevisiae* for improvement of robustness. However, lipidomic profiling methods have gained importance in the recent years for the study of responses of *S. cerevisiae* to different forms of stress. For example, an extensive data set regarding the changes in the lipidome of *S. cerevisiae* when subjected to different growth conditions revealed the large variability of the yeast lipidome (Klose, C. et al. 2012). Also, the association between membrane composition and ethanol tolerance has been further investigated by connecting the diversity of lipidomes in several strains of *S. cerevisiae* to their fermentation capacity and final ethanol concentrations (Henderson, C. M. et al. 2013a). Lipidomic studies have also indicated changes in membrane lipid composition as a response of *S. cerevisiae* to hydrolysate-derived inhibitors (Xia, J.-M. et al. 2008; Lindberg, L. et al. 2013). Likewise, comparison between the cellular lipid composition of the evolved strain ISO12 and the parental strain ER (paper III) suggested changes in membrane composition as a mechanism for surviving long-term exposure to the combination of stresses (i.e. high temperature and hydrolysate inhibitors) (paper IV). It is therefore reasonable to expect that the contributions of the aforementioned studies will help in identifying relevant targets to be used in metabolic engineering approaches for improvement of strain robustness.

4.2.4 Genomics

The first complete genome sequence of a free-living organism to be published was that of the bacterium *Haemophilus influenzae* Rd in 1995 (Fleischmann RD et al. 1995). About one year later, the *S.cerevisiae* genome became the first completely sequenced eukaryotic genome, as a result of the work performed by an international group spanning over 19 countries and 94 laboratories (Goffeau A et al. 1996; Engel, S. R. et al. 2013).

Since then, DNA sequencing has been characterized by a fast technological development associated with significant cost reduction, and the number of organisms whose genomes have been completely sequenced is now more than 180. (See Genome news network: http://www.genomenewsnetwork.org).

Studies on *S. cerevisiae* have particularly benefited from these technical advances, since sequencing of a particular strain with full and deep coverage can now be performed within days; also the assembly is very much facilitated by the availability of a reference genome (re-sequencing).

The research questions approached by sequencing are of different kinds. For example, comparative genomics has helped us to understand the origin of yeasts, and the discovery of significant evolutionary events such as whole-genome duplication (Kellis, M. et al. 2004). It has also contributed to the study of phylogenetic relationships and speciation (Piškur, J. et al. 2004) and to our understanding of adaptation mechanisms at the genome level (Burke, M. K. 2012; Kvitek, D. J. et al. 2013). Significant genetic diversity between *S. cerevisiae* strains of different origins (e.g. wine, brewing, bioethanol) and in particular between laboratory and industrial strains has also been confirmed by whole-genome comparisons. These differences include, for instance, the greater number of Ty transposons in S288c relative to industrial strains and a higher level of heterozygosity in industrial strains (Borneman, A. R. et al. 2011).

In the context of *inverse metabolic engineering*, whole-genome sequencing has been described as the most suitable genome-wide approach for the identification of relevant changes at the molecular level (Oud, B. et al. 2012). Advantages include the fact that sequencing results are not affected by experimental variation, and individual mutations generally have much less impact in other parts of the genome (in comparison to the effect of single transcript or protein level variation in the transcriptome and proteome, respectively) (Oud, B. et al. 2012). However, the probability of achieving a successful identification of the mutation(s) responsible for a phenotype of interest rely on factors associated with both technology constraints and the complexity of the genotype and phenotype under study. Regarding technology constraints, the use of the laboratory strain S288c as reference genome may not be optimal for the analysis of industrial strains because of the genetic differences already discussed. The characteristics of the genotype will also determine the probability of successfully identifying relevant mutations; here again, the polyploidy of industrial

strains will add complexity to the sequencing data, especially if it is not possible to obtain haploid cells due to sporulation deficiencies. Moreover, the number of genetic alterations will depend on the complexity of the phenotype under selection. For instance, around 20 non-conservative single nucleotide variations (SNVs) and insertions/deletions (INDELS) were obtained after selection for growth on galactose as a sole carbon source (400 generations) (Hong, K.-K. et al. 2011). In contrast, the whole-genome sequence comparison of the evolved tolerant strain ISO12 and the parental strain revealed a large number of SNVs and INDELs (~9,000), and around 190 larger rearrangements (unpublished data); this complicates the analysis of relationship between genotype and phenotype.

As expressed by Chu and Corey in relation to sequencing projects (Chu, Y. et al. 2012), "it is easy to initiate a project, but it is difficult to obtain and interpret data to adequately answer experimental questions". This assertion, however, could be extended for all types of omics projects, especially regarding interpretation of the biological meaning of the data obtained. For analysis of the highly complex and large volume of data sets generated in these types of studies, the development of special bioinformatics software packages is required. A common approach used in the interpretation of omics data is enrichment analysis. For this, a large number of interesting genes are systematically mapped to the associated biological annotation, such as gene ontology terms, followed by a statistical analysis to highlight the most overrepresented biological annotations (Huang, D. W. et al. 2008). There are different software packages (publicly available tools) for functional analysis, including DAVID (the database for annotation, visualization, and integrated discovery) (Dennis Jr, G. et al. 2003), GOstat (Beissbarth, T. et al. 2004) and GOToolBox (Martin, D. et al. 2004). The different types of functional annotations can be used for identification of the biological functions that are more relevant to the research question.

"Live as if you were to die tomorrow. Learn as if you were to live forever."

Mahatma Gandhi

Conclusions and future perspectives

The aim of this study was to improve the tolerance of, and to understand the response mechanisms of *S. cerevisiae* to hydrolysate-derived inhibitors and high temperature, in order to improve fermentation efficiency during second-generation ethanol production. Several conclusions can be drawn from the results and several suggestions can be made for future studies.

Tolerance to lignocellulosic inhibitors

Several proteins that could be involved in the response and tolerance to lignocellulosic inhibitors were investigated in more detail. Information was obtained at the molecular level on the unusual NADH-dependent reduction of HMF by a mutated Adh1p. Notably, the substitution of the amino acid tyrosine by cysteine at position 295 was found to be the key alteration for reduction of HMF by the mutated Adh1p variant with NADH as cofactor. Activity measurements of the *in vitro* and *in vivo* capacity of different Adh1p variants to reduce HMF suggested that there was a maximum level up to which the detoxification capacity of a strain could be improved with higher reduction activity; above that level, other biological processes probably controlled the detoxification process. A further step in this study would be to analyze the effects of amino acid changes on the tertiary structure of the protein, and to use this information to gain insights into the mechanisms of interaction of the enzyme with the substrate and cofactor.

In addition, another aim of the work was to investigate whether tolerance mechanisms identified in laboratory strains could be relevant in the industrial context. Overexpression of the transcription factor *YAP1* and the mitochondrial NADH-cytochrome b5 reductase encoding gene *MCR1* did indeed result in industrial strains with enhanced capacity to ferment hexose in the presence of undetoxified hydrolysate. The results confirmed the significance of the background strain for the actual outcome of the genetic modification, and the data revealed that xylose consumption was not improved in the modified strains. The new strains could therefore be used for the fermentation of hydrolysates in which the concentration of xylose is negligible (e.g. spruce). On the other hand, a negative interaction seen between *YAP1* overexpression and xylose utilization highlighted not only the differences in the metabolic capacity of *S. cerevisiae* to ferment both types of sugars but also emphasized the need for further improvements in xylose utilization. Further studies using *YAP1*-overexpressing *S. cerevisiae* strains carrying both types of xylose fermentation

pathways would confirm whether this negative interaction is specific for the "highly" altered background of GSE16, or whether it is a general effect. If the second option is verified, analysis at the molecular level in both types of xylose-consuming strains may provide hints about other key reactions that limit xylose metabolism in *S. cerevisiae* and new insights into improving pentose fermentation in the presence of inhibitors.

Tolerance to multiple stressors

An industrial *S. cerevisiae* strain with improved tolerance to the synergistic effect of hydrolysate-derived inhibitors and high temperatures (ISO12) was obtained by an evolutionary engineering strategy. Preliminary evaluation of ISO12 during SSF at 39°C showed a higher glucose consumption rate and higher ethanol production rate for the evolved strain than for the parental strain during the first 24 hours, which was later limited by the rate of sugar hydrolysis. Further characterization of the strain indicated significant alterations in the lipid profile of ISO12, suggesting that membranes may be an important target during the adaptation process. The evolved strain ISO12 and populations from different moments of the evolutionary engineering experiment are potential candidates for more detailed studies on the mechanisms of adaptation of *S. cerevisiae* to long-term exposure to high temperature and hydrolysate. For instance, it would be interesting to ascertain whether the reduced growth rate and furaldehyde reduction capacity observed in ISO12 are the result of random mutations, or whether they are instead necessary regulations to allow other more important reactions to take place under combined forms of stress. A following step in the development of more robust strains to be used in second-generation ethanol processes would be to evaluate the new strains during SHF or SSF processes. The high osmolarity, the low water activity, the presence of lignin, and the limitations in mixing rates during these types of fermentations are major stressors that are normally underestimated during evaluations with the liquid fraction of hydrolysates.

Finally, there is no doubt that the rapid development of the technologies related to omic approaches will continue to expand and become increasingly used in the studies of microbial cell factories. These technologies may represent a challenge for microbiologists, since the development of bioinformatics skills within the discipline is becoming fundamental in order to manage and interpret the large amounts of data generated. Still, such technologies are accelerating our understanding of the cellular functions in *S. cerevisiae* and other organisms, and hopefully many challenges in medicine, agriculture, and environmental issues will soon be overcome.

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