Universidade do Minho

Escola de Engenharia

Joana Filipa Torres Pinheiro da Cunha

ENGINEERING YEAST TOLERANCE TO INHIBITORY LIGNOCELLULOSIC BIOMASS

Dissertação de Mestrado

Mestrado em Bioengenharia

Trabalho efectuado sob a orientação de:

Professora Doutora Lucília Domingues

e co-orientação de:

Doutora Carla Oliveira

Outubro de 2013

Agradecimentos

Em primeiro lugar gostaria de agradecer às minhas orientadoras, Professora Doutora Lucília Domingues e Doutora Carla Oliveira, pela orientação, apoio, partilha de saber e pela disponibilidade.

À Tatiana e ao Francisco por toda a ajuda, disponibilidade, pelo acompanhamento incansável ao longo de todo o trabalho e por todas as palavras de incentivo.

Aos restantes colegas do grupo LD, Sofia M., Sofia C., Fred, Claúdia, Diana, Maikon, Alóia, Eduardo e Daniel, por toda a ajuda e companheirismo.

Aos colegas da PBMS, principalmente à Carla, à Débora, à Ana e ao Franklin, por estarem sempre disponíveis a ajudar, por proporcionarem um excelente ambiente de trabalho e por todos os momentos de descontracção.

A todas as moradoras do 50, principalmente Jé, Pobeira, Mika, Su e Analuce, por toda a amizade e alegre companhia, e por durante 5 anos fazerem com que viver em Braga se tornasse um verdadeiro "sentir em casa".

A todos os amigos da "Happy Family" que durante estes anos encheram de alegria e boa disposição a vida académica, e a menos académica também. À Cat, ao Pontinho e ao Nuno, porque a vossa companhia torna cada dia melhor que o anterior, e porque esta universidade sem vós estaria praticamente vazia. À Joaninha, porque mesmo à distância o "contacto visual" mantém-se. À Ana, pela ajuda e por ser a prova de que "amigos de intervalo" se podem tornar em amigos para a vida. À Mika, pela grande amizade, alegria contagiante, por toda a ajuda e partilha, e basicamente por teres tornado estes 6 anos "altaméiintes".

À Ju, ao Eduardo, à Marta, ao Hugo, à Rita, ao Paulo e ao Tiago por toda a vossa amizade e por fazerem com que voltar à terra seja sempre um prazer.

À minha família, principalmente à minha mãe e ao meu irmão, sem vós nada disto seria possível.

Financial support:

This work was funded by Fundação para a Ciência e a Tecnologia (FCT), Portugal: project GlycoCBMs FCT PTDC/AGR-FOR/3090/2012 – FCOMP-01-0124-FEDER-027948.

Abstract

The progressive depletion of fossil fuels reserves in the last years led to the necessity for biotechnological manufacturing based on lignocellulosic feedstocks. Lignocellulosic biomass, such as straw, is an abundant low-cost source for production of biofuels, such as bioethanol, that does not compete for food needs. However, lignocellulose-to-ethanol process involves pre-treatment of biomass to obtain readily fermentable sugars, which leads to the accumulation of inhibitory by-products (e.g. furan derivatives, phenolic compounds, organic acids). Significant progress has been made in the understanding of the determinants of yeast tolerance to lignocellulose biomass-derived inhibitors, as well as to high ethanol concentrations. Nevertheless, further knowledge at the genetic level is of essential importance for the improvement of second generation bioethanol conversion technology.

In a previous work, 5 genes, *ERG2*, *PRS3*, *RAV1*, *RPB4* and *VMA8*, were found to contribute to the maintenance of cell viability and/or for maximal fermentation rate in wheat straw hydrolysate. Taking into account the negative effects reported from single overexpression of *ERG2*, *RAV1* and *VMA8* under non-stressful conditions, these genes were not considered as good targets for genetic engineering in the present work. Furthermore, *ZWF1*, a gene essential for yeast response to the presence of acetic acid, was added to the set of genes considered in the present study. To attempt to overcome the fermentation hurdles resultant from the inhibitory load mentioned above, molecular biology tools were used to: (1) unravel *HAA1*, *PRS3* and *RPB4* role in adaptation to toxic biomass hydrolysates, evaluating their expression levels, by qRT-PCR, in the outstanding-fermenting *Saccharomyces cerevisiae* PE-2 when exposed to acetic acid, HMF and furfural, and (2) improve yeast tolerance and adaptation by overexpressing these genes in the auxotrophic *S. cerevisiae* BY4741, using multi-copy vectors, and assessing the effects in *Eucalyptus globulus* wood hydrolysate.

Increased *HAA1*, *PRS3* and *RPB4* expression levels were observed at the late lag and/or initial stationary phases of the fermentation in the presence of inhibitors. However, the overexpression of these genes under the control of the strong constitutive *ScPGK1* promoter has not resulted in improved growth and fermentation profiles. On the other hand, the overexpression of *HAA1* and *PRS3* genes under the regulation of their native promoters resulted in fermentations profiles with a reduced lag-phase. These results indicate that *PRS3* and principally *HAA1* overexpression play an important role in the adaptation to lignocellulosic-based stress, and are good candidates for yeast engineering to improve bioethanol production.

Resumo

A diminuição progressiva das reservas de combustíveis fósseis nestes últimos anos levou à necessidade de uma indústria biotecnológica baseada em matérias-primas lenhocelulósicas. A biomassa lenhocelulósica, tal como a palha, é uma fonte abundante de baixo preço para a produção de biocombustíveis, como o bioetanol, que não compete com as necessidades alimentares. Contudo, o processo de conversão de biomassa lenhocelulósica a etanol envolve um pré-tratamento da biomassa para obtenção imediata de açúcares fermentescíveis, levando à acumulação de produtos inibitórios (ex. derivados de furano, compostos fenólicos, ácidos orgânicos). Avanços significativos têm sido efectuados no que concerne à compreensão de determinantes da tolerância de leveduras a inibidores derivados da biomassa lenhocelulósica, tal como a concentrações elevadas de etanol. No entanto, um maior conhecimento a nível genético é essencial para o melhoramento de tecnologias para a conversão de bioetanol de segunda geração.

Num trabalho anterior, 5 genes, *ERG2*, *PRS3*, *RAV1*, *RPB4* e *VMA8* foram identificados como importantes para a manutenção da viabilidade celular e/ou para maximizar a taxa de fermentação em hidrolisados de palha de trigo. Considerando os efeitos negativos reportados da sobre-expressão singular dos genes *ERG2*, *RAV1* e *VMA8* na ausência de stress, estes genes foram considerados, neste trabalho, como não sendo bons alvos para engenharia genética. Adicionalmente, o gene *HAA1*, essencial na resposta à presença de ácido acético em leveduras, foi acrescentado ao conjunto de genes considerado neste estudo. Na tentativa de ultrapassar os problemas fermentativos acima referidos, ferramentas de biologia molecular foram usadas para: (1) desvendar o papel dos genes *HAA1*, *PRS3* e *RPB4*, na adaptação a hidrolisados de biomassa tóxicos, avaliando os seus níveis de expressão por qRT-PCR, no excepcional organismo fermentativo *Saccharomyces cerevisiae* PE-2 quando exposto a ácido acético, HMF e furfural, e (2) melhorar a tolerância e adaptação da levedura através da sobre-expressão destes genes na estirpe auxotrófica *S. cerevisiae* BY4741, usando vectores multi-cópia, e avaliar os efeitos em hidrolisado de madeira de *Eucalyptus globulus*.

Níveis de expressão aumentados dos genes *HAA1*, *PRS3* e *RPB4* foram observados no final da fase de adaptação e/ou no inicio da fase estacionária da fermentação na presença de inibidores. Todavia, a sobre-expressão destes genes sob o controlo do promotor constitutivo e forte *ScPGK1* não demonstrou um melhoramento dos perfis de crescimento e fermentativos. Em contrapartida a sobre-expressão dos genes *HAA1* e *PRS3* sob a regulação dos seus promotores nativos resultaram em perfis de fermentação com reduzida fase de adaptação. Estes resultados indicam que a sobre-expressão do *PRS3* e principalmente do *HAA1* tem um papel importante na adaptação ao *stress* derivado de biomassa lenhocelulósica, sendo bons candidatos para a engenharia genética de leveduras, de modo a obter melhorias na produção de bioetanol.

Table of contents

List of figures

List of tables

List of abbreviations

°C	degree Celsius
µg	microgram
µl	microliter
µM	micromolar
ADH1	alcohol dehydrogenase I
Ag	*Ashbya gossypii*
amp	ampicillin
bp	base pairs
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DTT	Dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EWH	*Eucalyptus globulus* wood hydrolysate
G	Gram
G	relative centrifugal force
G418	Geneticin
h	Hour
HMF	5-hydroxymethyl furfural
IPTG	β-D-1-thiogalactopyranoside
K	kilo (103)
kV	Kilovolt
L	Liter
LB	Luria-Bertani medium
LiOAc	Lithium Acetate
M	molar concentration
min	Minute
Ml	Mililiter
mM	Milimolar
mRNA	messenger ribonucleic acid
ms	Millisecond
Ng	nanogram
Nm	nanometer
OD	optical density
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PGK1	3-phosphoglycerate kinase
RNA	ribonucleic acid
RNase	Ribonuclease

Rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S	second
SAP	Shrimp Alkaline Phosphatase
Sc	*Saccharomyces cerevisiae*
SD	Synthetic Defined
SHF	Separate Hydrolysis and Fermentation
SOC	Super Optimal broth with Catabolite repression
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TEF	Translation elongation factor 1 α
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
WSH	Wheat Straw Hydrolysate
WT	Wild Type
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside
YPD	Yeast extract Peptone Dextrose medium

AIMS

In recent years the necessity for biotechnological manufacturing based on lignocellulosic feedstocks has become evident. However, the required pre-treatment in the production of lignocellulosic bioethanol leads to the accumulation of by-products inhibitory to yeast growth. A set of genes required for tolerance to stress induced by sole inhibitors was already identified, however, little is known about tolerance to multi-stress induced by multiple inhibitors. Based on genome-wide results, 5 genes, *ERG2*, *PRS3*, *RAV1*, *RPB4* and *VMA8*, were previously found to contribute to the maintenance of cell viability in wheat straw hydrolysate and/or for maximal fermentation rate in this substrate. However, there are no studies describing the outcome in ethanol productivity of lignocellulosic-based fermentations using recombinant *S. cerevisiae* overexpressing these genes.

Taking into account that single overexpression of *ERG2*, *RAV1* and *VMA8* under non-stressful conditions was found to result in a negative effect on ergosterol biosynthesis, toxicity for the yeast cell and in a diminished growth rate, respectively, these genes were not considered as good targets for genetic engineering. Furthermore, *HAA1*, a gene essential for yeast response to the presence of acetic acid [1, 2] and which overexpression was found to enhance tolerance to this inhibitor, was equally studied. Therefore, to expand our understanding of the underlying molecular mechanisms involved in yeast response to the multiple stresses occurring during lignocellulose fermentations under industrially relevant conditions, we specifically aimed to:

1. study *HAA1*, *PRS3* and *RPB4* expression in *S. cerevisiae* PE-2 by qRT-PCR during fermentation in the presence of lignocellulose-related inhibitors;
2. create *S. cerevisiae* strains overexpressing *HAA1*, *PRS3* and *RPB4* genes under the control of their native promoters or of the strong constitutive *ScPGK1* promoter;
3. evaluate the effect of these genes' overexpression in terms of bioethanol production, in fermentations of *Eucalyptus globulus* wood hydrolysate.

1. INTRODUCTION

1.1. Biofuels

The world faces the progressive depletion of its fossil fuels reserves, the most important of the available energetic resources, resulting in a constant rise of the oil prices. Also, the excessive consumption of these fuels during the last decades has greatly contributed to generating high levels of pollution, mostly in large urban areas, associated with elevated greenhouse gas emissions. This allied with the growing demand for energy for transportation, heating, industrial processes, among others, have denoted the need for a substitute for the fossil fuels [3].

The renewable energy industry is currently well developed, however it is mostly focused in the production of electrical energy (wind, solar, and tidal energy, hydro- and geo-energy). Liquid fuels are the basis of more than half of the energy consumed at the present, instigating the necessity to exploit other energy resources, such as biofuels [4].

Biofuel is any fuel that is produced from biomass, which consists of biological matter from dead or even living organisms (being, in this context, usually plant-based). The principal fuels produced from biomass are ethanol, methanol, biodiesel and hydrogen [5]. This work will focus on biomass-based ethanol (or bioethanol) production.

1.2. Bioethanol

Bioethanol is considered as a good alternative to substitute gasoline. Although its energy equivalent is 68% lower than petroleum-based fuel, it has a cleaner combustion [6], which results in a lower emission of toxic substances [7]. The replacement of gasoline by ethanol results in a reduction of more than 80% of carbon emissions, and completely eliminate the release of acid-rain-causing sulfur dioxide [8].

Currently, the leader country on the production of bioethanol is the United States of America (USA), followed by Brazil [9]. In 2012, the worldwide bioethanol production for the fuel market was over 82 thousand millions liters, of which 62% were produced in USA and 25% in Brazil [10]. The principal biomasses sources used in the process of ethanol production are corn (in USA) and sugar cane (in Brazil), among others. The ethanol produced from the easily obtained sugars from feedstocks is denominated first generation bioethanol. However, the utilization of these sources directly compete with the food and animal feed industry, and may not be sufficient to meet the increasing demand for fuel ethanol [11]. Moreover, the utilization of this bioethanol results in a greenhouse gases production not as low as desirable [11].

1.2.1. Second generation bioethanol

The concerns regarding the feedstocks depletion, led to the necessity of a new generation of bioethanol produced from non-food biomass. This is called second generation bioethanol and is based in lignocellulosic biomass, the most abundant and sustainable raw material worldwide, which occurs as a byproduct, thus eliminating the competition with food and feed industry [12]. Such lignocellulosic biomass consists of agricultural (e.g. cereal straw, sugar cane bagasse), industrial (e.g., glycerol from biodiesel production by transesterification), municipal (organic components of solid wastes) and forestry (e.g. wood residues) wastes, and similar sources [13, 14].

As already denoted, the establishment of the second generation bioethanol eliminates the potential conflict between the use of land for food and for bioethanol production[15]. Moreover, the lignocellulosic material is geographically more evenly distributed than the fossil fuels, providing security of supply, and that fact might also provide employment in less-developed regions, especially in rural areas[15]. Another advantage of this lignocellulose-based bioethanol is the low greenhouse gas emission, and consequently low environmental impact [15].

Whereas the first generation bioethanol industry is well settled, the technology for bioethanol production based on lignocellulosic biomass is still developing. Furthermore, the process of conversion of lignocellulosic biomass is more complex than that of sugar- and starch-based sources, as a result of the rigid and complex molecular polymeric structure of cellulosic biomass (lignocellulose is highly resistant to chemical attack, solubilisation and bioconversion) [12]. The fact that cellulosic materials contain five different sugar monomers (glucose, galactose, mannose, xylose, arabinose), while starch is only composed of glucose [16], is another factor of complexity. Several biotechnological companies and government-funded laboratories have already engineered enzymes and microorganisms to optimize the technology to produce bioethanol from lignocellulose sources, and many are working to increase the efficiency of this process.

1.2.2. Bioethanol production from lignocellulosic biomass

Lignocellulosic biomass is mostly comprised of lignin, hemicellulose and cellulose. Pre-treatment and hydrolysis procedures results in the formation and liberation of a large range of compounds (Figure 1.1). Hemicellulose degradation liberates xylose, mannose, acetic acid, galactose, and glucose. Cellulose is hydrolysed to glucose. Furfural and 5-hydroxymethyl furfural

(HMF) are formed from dehydration of pentoses and hexoses, respectively. Phenolic compounds are generated from the partial breakdown of lignin [17, 18].

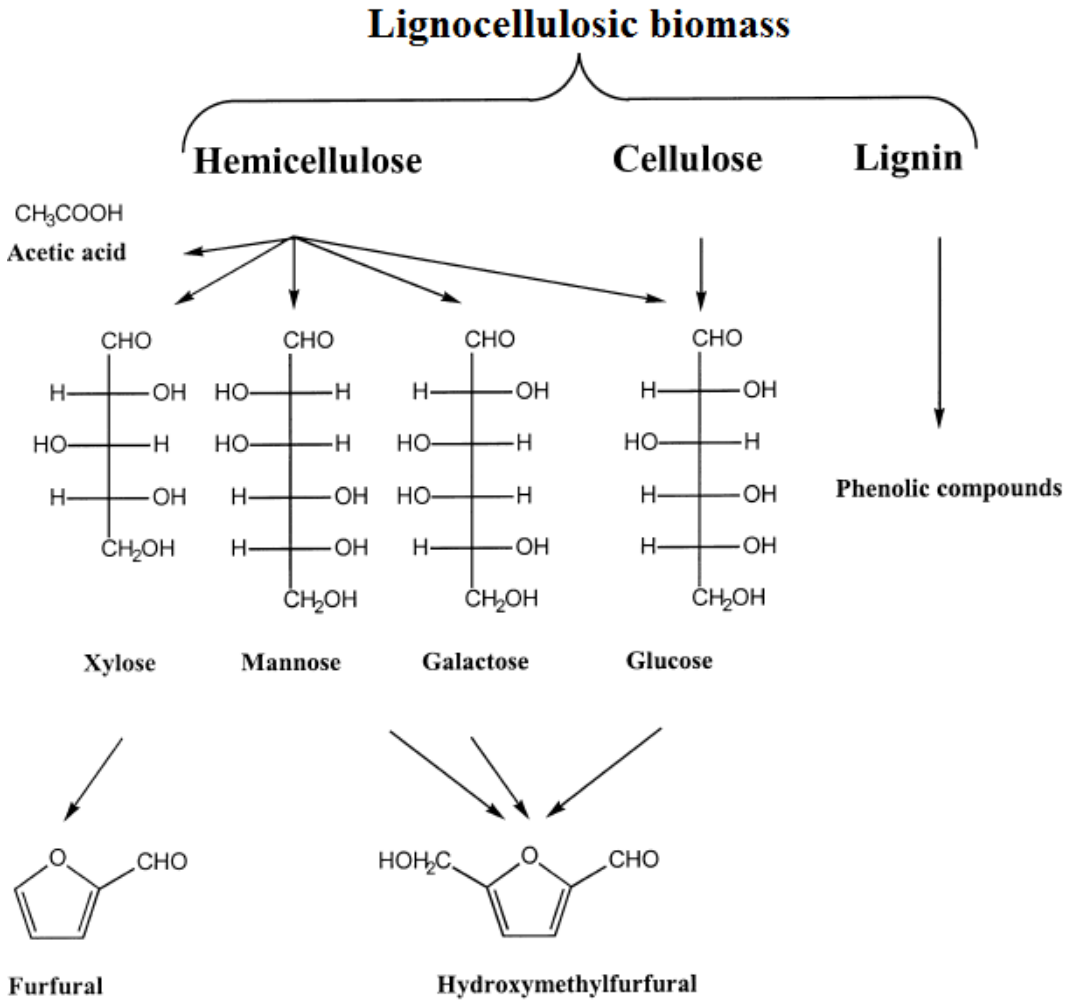

Figure 0.1. Reactions occurring during pre-treatment and hydrolysis of lignocellulosic materials. Adapted from Palmqvist and Hahn-Hagerdal [17].

The method for ethanol production generally consists of three steps: (1) formation of a solution of fermentable sugars, (2) fermentation of these sugars into ethanol, and (3) separation and purification of the produced ethanol, usually by distillation (Figure 1.2) [19]. Production of ethanol from lignocellulosic material mainly differs from sugar- and starch-based production in the step of obtainment of fermentable sugars, being of a greater complexity. The extraction of sugars from sugar crops is a relatively simple procedure, consisting only in a milling step. Starch cannot be used directly for ethanol production, so, in processes that use starch-based materials (e.g. corn, wheat), a step of saccharification is necessary after milling. This saccharification step basically consists of an enzymatic hydrolysis, with α-amylase and amyloglucosidase, resulting in a total breakdown of starch into glucose[6].

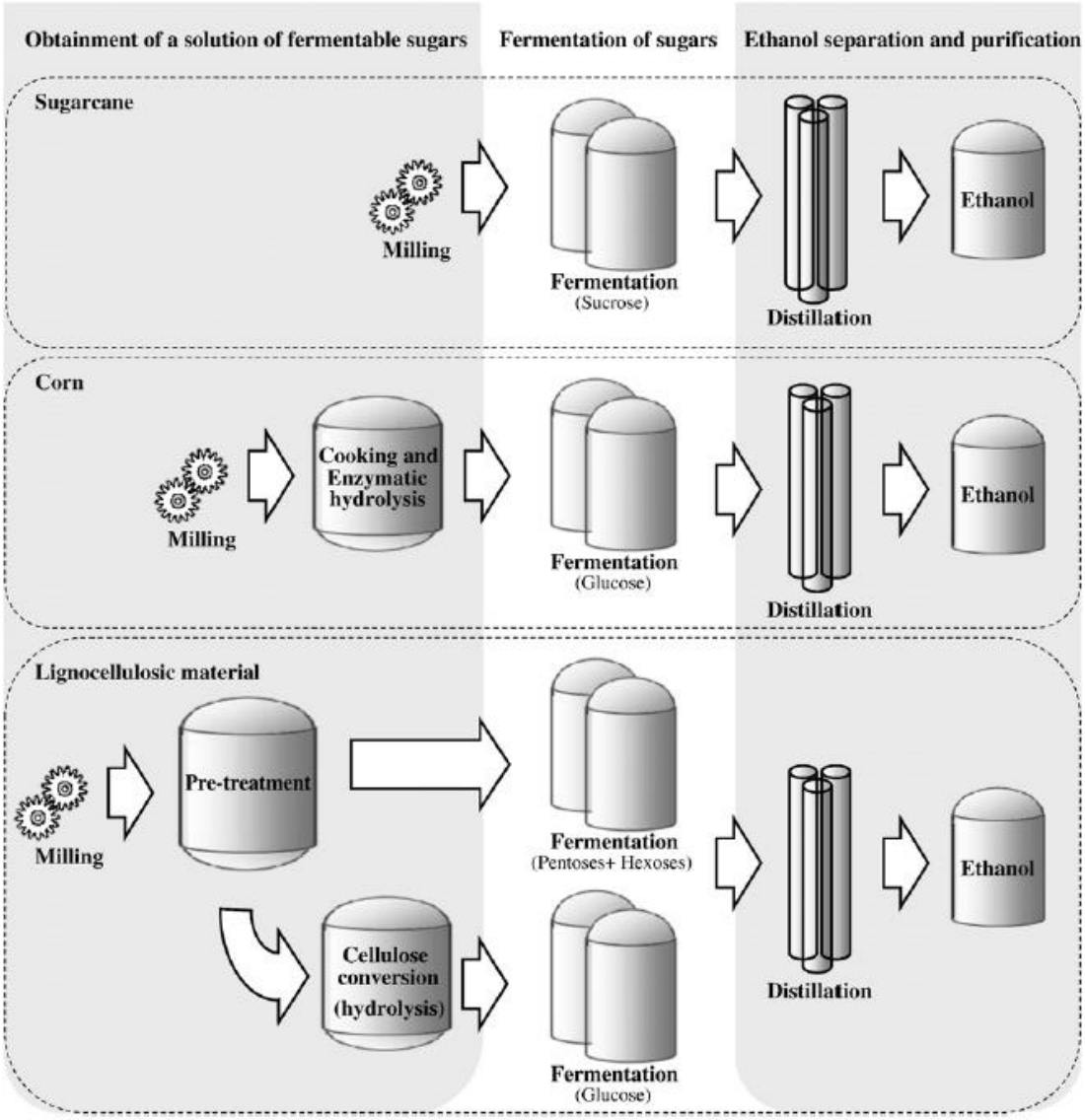

Figure 0.2. Main raw materials and processes used for ethanol production. Reproduced from Mussatto *et al.* [6].

When using lignocellulosic biomass an additional step is necessary before hydrolysis (Figure 1.2)[6]. This step is denominated pre-treatment and generally involves a milling process and a chemical pre-treatment (e.g. diluted acid, alkaline, solvent extraction, steam explosion) to make cellulose and hemicellulose more accessible to the subsequent steps [20]. It should be performed with a minimum formation of compounds capable of inhibiting fermenting microorganisms [18].

In 1976, Gauss *et al.* [21] presented the idea of simultaneously performing the enzymatic hydrolysis and fermentation. The main advantage of this process, later on denominated Simultaneous Saccharification and Fermentation (SSF), is the avoidance of the end-product inhibition of the hydrolysis step. Furthermore, with this process glucose does not need to be separated from the lignin fraction after the hydrolysis, avoiding the potential loss of sugar. This

combination of steps also decreases the number of vessels needed and consequently the investment costs (estimated to be more than 20% reduced). However some drawbacks are present when comparing to the Separate Hydrolysis and Fermentation (SHF) process, where fermentation takes place subsequently, and independently from, the saccharification step [22]. The SSF process operates at non-optimal conditions for hydrolysis, as the optimum temperature for enzymatic hydrolyses is generally higher than that of fermentation (at least when yeast is the fermenting organism in use). Therefore a compromise between fermentation and hydrolysis must be found in order to optimize the temperature parameter and consequently, higher dosages of hydrolytic enzymes are required. In addition, the enzyme reutilization is difficult, as they strongly bind to the substrate. Considering that these cellulases account for an important part of production costs, it is necessary to reduce the enzyme doses to be utilized, e.g. by the addition of surfactants [23, 24].

S. cerevisiae is the most used organism in industrial processes involving alcoholic fermentation. This preference is result of: its GRAS (generally regarded as safe) status; its good fermentable capacity and ethanol tolerance, which allows high ethanol production (up to 20% (v/v)) [25, 26]; its rapid growth under anaerobic condition, important for the oxygenation problem in large-volume industrial fermentations [27]; being one of the best studied organisms, in terms of scientific and industrial knowledge. Furthermore, its easily genetic manipulation and fully sequenced genome, makes *S. cerevisiae* the ideal organism for improvement by genetic engineering.

Even though *S. cerevisiae* is one of the most effective ethanol-producing microorganism, it is unable to utilize pentose sugars for growth or fermentation [22]. The necessity to create a recombinant *S. cerevisiae* strain with the ability to ferment both hexose and pentose sugars emerged, and was already obtained in some studies [28-30] allowing the possibility of a Simultaneous Saccharification and Co-Fermentation (SSCF).

One of the major challenges faced in the production of lignocellulosic bioethanol is the generation of inhibitory compounds during the biomass pre-treatment and hydrolysis steps. These inhibitors of microbial growth comprise furan derivatives (furfural and HMF), several phenolic compounds (e.g. vanillin) and organic acids (e.g. acetic acid) (Figure 1.1). The success of lignocellulosic biomass utilization is necessarily dependent on the development of recombinant *S. cerevisiae* strains capable of withstand, survive, and function in the different stresses imposed

during the bioethanol production processes, including inhibition by the above mentioned compounds, as well as by increasing ethanol concentration, wide pH and osmotic shifts.

1.3. Genes associated with *S. cerevisiae* tolerance to ethanol, acetic acid, vanillin, furfural and HMF

Genome-wide screenings for deletion mutants of *S. cerevisiae* with differential susceptibility to stress induced by ethanol[31], acetic acid[32], vanillin[33] and furfural and/or HMF[34] are described in the literature. These disruptome analyses allowed the registration of phenotypes showing susceptibility (growth inhibition) to the induced-stresses, leading to the identification of genes required for yeast tolerance to the referred stressors. A set of mutants were identified as having increased sensitivity to ethanol (254 genes), to acetic acid (648 genes), to vanillin (76 genes) and to furfural/HMF (62 genes) [31-34].

Gene expression analysis techniques, such as microarray or quantitative real-time PCR (qRT-PCR), are of great importance for understanding the molecular mechanisms of acquired tolerance to inhibitors stress. Using these tools, several studies have reported the identification of key genes related to the genomic adaptation to: acetic acid [35, 36], HMF [35, 37], furfural [35, 36], both furfural and HMF [38, 39], and lignocellulosic-based hydrolysates [35].

Furfural and HMF are considered the most potent inhibitors of yeast growth and fermentation [40-42]. These furans inhibit central enzymes, such as pyruvate dehydrogenase, acetaldehyde dehydrogenase, and alcohol dehydrogenase [43]. Moreover, exposure to high levels of furfural may cause cellular membrane, chromatin, and actin damage [44]. The yeast capacity to reduce these compounds appears to play a major role in tolerance to hydrolysates inhibition [42, 45]. A broad set of genes possibly playing a role in tolerance to/reduction of furfural/HMF have been identified (e.g. alcohol dehydrogenase (*ADH*), aldehyde dehydrogenase (*ALD*) and pyruvate dehydrogenase genes (*PDH*) [43, 46]). Furthermore, overexpression of some genes have been proved to be associated with improved growth, fermentation rate and/or ethanol production in the presence of inhibitory concentrations of furfural and/or HMF (Table 1.1) [34, 45, 47-52]. However, the overexpression of some other genes (*GND1, RPE1, TKL1, GRE3, ALD4* - that had been described as potential determinants of resistance to these inhibitors), resulted in no benefit in terms of growth, ethanol production or fermentation rate in the presence of furfural/HMF inhibitory concentrations [34, 48, 49].

Table 0.1. List of genes, that, when overexpressed, are reported to play a protective role against inhibitory concentrations of furfural and/or HMF.

Gene	Cellular function	Inhibitor	Effect	Author
ZWF1	Involved in pentose phosphate pathway and in adaptation to oxidative stress	Furfural and HMF	Improved growth and ethanol production	Gorsich *et al.* [44]; Park *et al.* [48]
MSN2	Involved in stress response (including oxidative stress)	Furfural	Improved fermentation rate and ethanol production	Sasano *et al.* [47]
ALD6	Aldehyde dehydrogenase required for conversion of acetaldehyde to acetate	Furfural and HMF	Improved growth and ethanol production	Park *et al.* [48]
ADH6	NADPH-dependent alcohol dehydrogenase; may be involved in fusel alcohol synthesis or in aldehyde tolerance	HMF	Improved growth and ethanol production; reduction of HMF	Park *et al.* [48]; Peterson *et al.* [45]; Liu *et al.* [49]; Almeida *et al.* [50]
ADH1	Alcohol dehydrogenase required for the reduction of acetaldehyde to ethanol	HMF	Improved ethanol production; reduction of HMF	Almeida *et al.* [50]
ARI1	NADPH-dependent aldehyde reductase, utilizes aromatic and alophatic aldehyde substrates	Furfural and HMF	Improved growth	Liu and Moon [51]
GRE2	Involved in stress response (including oxidative stress)	HMF	Improved growth; reduction of HMF	Moon and Liu [52]

Acetic acid is one of the principal yeast inhibitors in lignocellulosic hydrolysates [53]. It inhibits specific growth and significantly increases the lag-phase of yeast and reduces ethanol production [54]. Stress related with the presence of this weak acid affects many pathways, such as fatty acid biosynthesis, alternative electron transport chain, and lactate, formate, and ethanol fermentation pathways [55]. Additionally acetic acid is also capable of inducing apoptosis through TOR pathway in yeast [56]. The *HAA1* gene has been described to be essential for the *S. cerevisiae* response to the presence of acetic acid [1, 2], and its overexpression has been proved to enhance acetic acid tolerance [57].

1.3.1. Genes associated with *S. cerevisiae* tolerance to the simultaneous presence of ethanol, acetic acid, vanillin, furfural and HMF in Wheat Straw Hydrolysate

Even though a large range of genes have already been described to play a role in the yeast tolerance to stress induced by one inhibitor, there are no information in the literature about how the expression of these genes can affect the yeast tolerance to the stresses present during lignocellulosic fermentation (environment with multi-stress induced by different inhibitors).

Among the genes identified as required for tolerance to ethanol, acetic acid, vanillin and furfural, none have been found to provide simultaneous protection to the 4 inhibitors[58]. However, 11 genes have been found that confers resistance to ethanol, acetic acid and vanillin (6 genes: *ERG2*, *ERG24*, *END3*, *GCS1*, *RAV1* and *TPS1*) or furfural (5 genes: *NAT3*, *PPA1*, *PRS3*, *RPB4* and *VMA8*) (Figure 3, Table 1.2).

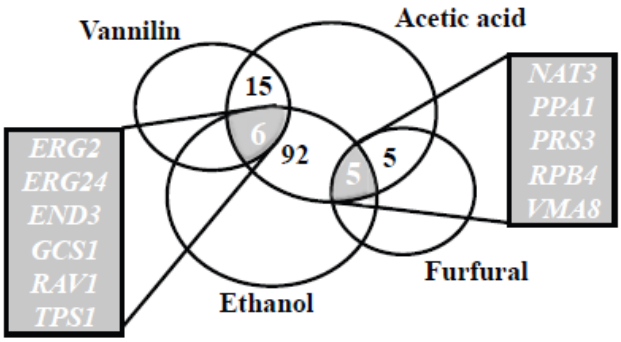

Figure 0.3. Comparison of the yeast genes described as determinants of resistance to inhibitory concentrations of ethanol, acetic acid and furfural or vanillin. Adapted from Pereira et al, 2011 [58].

To understand if these 11 genes are able to confer tolerance in the presence of multiple stressors, and not only in the presence of one, fermentations with single deletion mutants were performed in wheat straw hydrolysate (WSH; containing ethanol, acetic acid, vanillin and furfural) [58]. The results showed impaired growth of the Δ*erg2*, Δ*prs3*, Δ*rpb4* and Δ*vma8* mutants [58], i.e., from the set of 11 genes mentioned, only *ERG2*, *PRS3*, *RPB4* and *VMA8* were required for yeast growth in WSH. The fermentation profiles of all of the 11 mutants were analysed and, consistently, the Δ*erg2*, Δ*prs3*, Δ*rpb4* and Δ*vma8* mutants were the ones with the lowest ethanol production, exhibiting also the lowest fermentation rates [58]. The Δ*rav1* mutant, which showed no impairment in growth in the hydrolysate and generated the same final concentration of ethanol as the wild type strain, exhibited a significant lower fermentation rate [58]. From the 11 genes identified as required for tolerance to ethanol, acetic acid, vanillin or furfural, only five genes, *ERG2*, *PRS3*, *RAV1*, *RPB4* and *VMA8*, were found to contribute to the fermentation rate and/or to the maintenance of cell viability in WSH [58].

Table 0.2. Cellular function of the 11 genes required to provide tolerance to ethanol, acetic acid and vanillin or furfural. The cellular function was obtained from *Saccharomyces* Genome Database (http://www.yeastgenome.org).

Gene	Cellular function
Stressors: Ethanol, acetic acid and vanillin	
END3	Protein involved in endocytosis, actin cytoskeletal organization and cell wall morphogenesis
ERG2	Sterol isomerase involved in ergosterol biosynthesis
ERG24	Sterol reductase involved in ergosterol biosynthesis
GCS1	ADP-ribosylation factor GTPase activating protein, involved in ER-Golgi transport
RAV1	Subunit of the RAVE complex which promotes assembly of the V-ATPase holoenzyme
TPS1	Synthase subunit of trehalose-6-phosphate synthase/phosphatase complex necessary for trehalose biosynthesis
Stressors: Ethanol, acetic acid and furfural	
NAT3	Catalytic subunit of the NATB N-terminal acetyltransferase involved in protein acetylation
PPA1	Proteolipid subunit of the membrane domain of the vacuolar H^+-ATPase (V-ATPase)
RPB4	RNA polymerase II subunit
PRS3	5-phospho-ribosyl-1(alpha)-pyrophosphate synthetase required for nucleotide, histidine and tryptophan biosynthesis
VMA8	Subunit of peripheral membrane domain of the vacuolar H^+-ATPase (V-ATPase)

ERG2 encodes C-8 sterol isomerase, one of the key enzymes involved in ergosterol biosynthesis. The ability to synthesize ergosterol (the major sterol in the plasma membrane of *S. cerevisiae*) has been reported as an important factor in the ethanol tolerance of yeast cells [59-61] indicating a prominent role of this sterol in stabilizing membrane lipids and proteins against the negative effects of ethanol. Consistently with this fact, a *S. cerevisiae* strain overexpressing ergosterol biosynthesis genes was found to have higher growth ability under high ethanol concentration than a laboratory yeast strain [62]. Under non-stressful conditions *ERG2* overexpression or deletion have no effect on growth rate [63]. Also, during bioethanol production processes, a reduction in the transcript levels of ergosterol biosynthetic genes was reported [64],

possibly as a response to the lack of oxygen (this decrease also occurs in winemaking processes [65]). However, studies have shown that overexpression of *ERG2* alone has a negative effect on ergosterol biosynthesis [66, 67].

PRS3 encodes 5-phosphoribosyl-1-pyrophosphate synthetase, responsible for the synthesis of 5-phosphoribosyl-1-pyrophosphate (PRPP), which is required for nucleotide, histidine, and tryptophan biosynthesis. Studies have reported that altering the capacity of the yeast cell for synthesizing PRPP (i.e. using Δ*prs3* strains) causes the cell to display cell wall damage-related phenotypes and interferes with signaling in the cell integrity pathway, suggesting that *PRS3* may have a supporting role in the maintenance of cell integrity [68-70].

RAV1 and *VMA8* are both involved in the assembly and function of the vacuolar membrane H^+-ATPase (V-ATPase): *RAV1* encodes a subunit of the RAVE complex (Rav1p, Rav2p, Skp1p), which promotes assembly of the V-ATPase holoenzyme [71] and *VMA8* encodes a subunit of peripheral membrane domain of the vacuolar H^+-ATPase (V-ATPase) [72]. This last enzyme plays a crucial role in the maintenance of the internal pH within physiological values, especially under stress conditions that result in intracellular acidification, as is the case of stress caused by acetic acid and ethanol [31, 73]. Therefore V-ATPase was identified as a crucial determinant of resistance to these two stressors [31, 32]. Consistently, Δ*rav1* mutants exhibited a substantial reduction in growth on media with low pH value [74], and also a decreased resistance to ethanol [75]. However, overexpression of *RAV1* has been described as toxic for the yeast cell [76]. Regarding the *VMA8* gene, it has been shown that, under non-stressful conditions, overexpression of this gene results in a diminished growth rate, and its deletion has no effect on growth [63].

RPB4 encodes a RNA polymerase II subunit, which interacts with the subunit encoded by *RPB7* (a smaller essential subunit) forming a subcomplex [77] that plays important roles in stress and non-stress related phenotypes (Figure 2) [78]. Deletion of the *RPB4* gene has been found to result in a RNA polymerase II with reduced activity, in impaired growth rate (principally under extreme temperatures) [79-81], and in defective cell wall integrity [82]. It was discovered that this gene plays an important role in the activation of many genes (some of them involved in specific pathways of stress response) [83, 84], and that in its absence, RNA polymerase II cannot transcribe some genes [85]. Farago and collaborators [86] established that *RPB4* is required during temperature, starvation or ethanol stresses. However, this gene has no important role in

osmotic or oxidative stresses [87]. Furthermore, under stress conditions, *RPB4* has been shown to be required for efficient mRNA export to the cytoplasm [86].

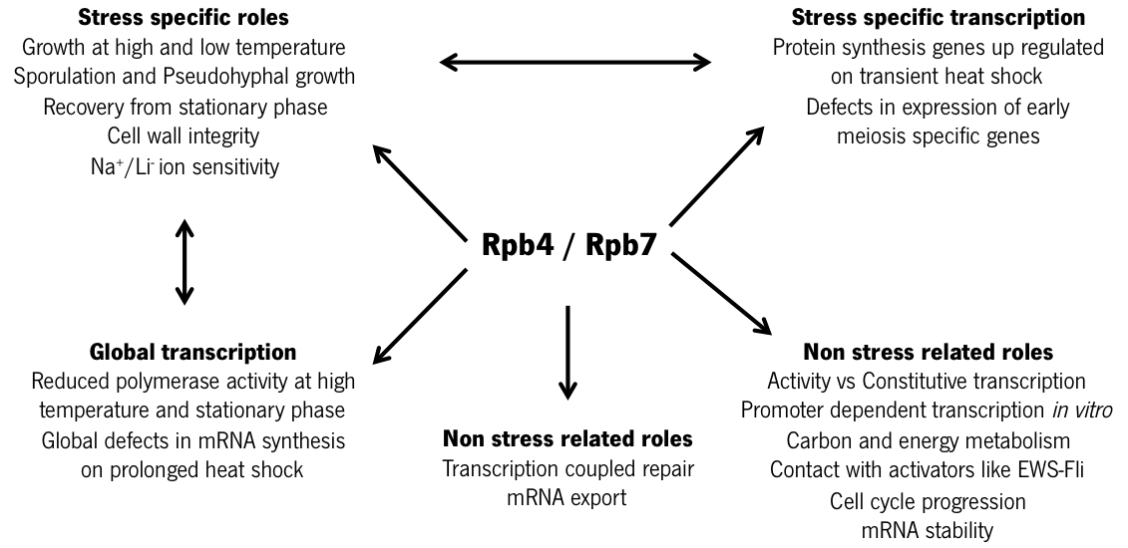

Figure 0.4. Roles played by the *RPB4*/7 sub-complex in stress and non-stress related phenotypes. Reproduced from Sampath and Sadhale, 2005 [78].

Taking into account that single overexpression of *ERG2*, *RAV1* and *VMA8* under non-stressful conditions was found to result in a negative effect on ergosterol biosynthesis, toxicity for the yeast cell and in a diminished growth rate, respectively, these genes were not considered as good targets for genetic engineering. Therefore *PRS3* and *RPB4* genes were selected to be studied, by overexpression and evaluation of their expression levels under inhibitory conditions.

2. MATERIALS AND METHODS

2.1. Sterilization of material, solutions and culture media

All the glass material and culture media for bacteria and yeast were sterilized in autoclave at 121 °C during 20 min. Thermolabile solutions were sterilized by filtration with 0.2 µm filters.

2.2. Strains

Microbial strains used during this work are listed in Table 2.1.

Table 0.1. Microbial strains used during this work.

Strain	Genotype	Source
Escherichia coli TOP10	F– *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15,Δ*lac*X74, *rec*A1, *ara*D139,Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L(StrR), *end*A1, *nup*G	Invitrogen
E. coli NZ5α	*fhu*A2Δ(*arg*F-*lac*Z)U169, *pho*A, *glnV*44,Φ80*lac*ZΔM15, *gyr*A96, *rec*A1, *rel*A1, *end*A1, *thi-*1, *hsd*R17	Nzytech
S. cerevisiae CEN.PK113-7D	*MATα, MAL2-8c,SUC2*	INSA, Toulouse, France
S. cerevisiae BY4741	*MATa, his3Δ1*, *leu2Δ0*, *met15Δ0, ura3Δ0*	EUROSCARF
S. cerevisiae PE-2		Rosane Schwan (Federal University of Lavras, Brazil)

2.3. Bacteria and yeast cells storage

Bacteria and yeast cultures were maintained for up to 2 weeks at 4 °C, in the appropriate selective medium, on inverted agar plates sealed with parafilm. For long time storage, permanent stocks were prepared. A culture grown overnight in appropriate selective liquid medium was 10 fold diluted in fresh medium and grown for more 5-6 h. Afterwards, 0.3 ml of sterile glycerol were added to 1 ml of the culture, mixed by vortexing and incubated on ice for 10 min. The tubes were stored at -80 °C. For culture recovery, the frozen cells were scrapped and spread on appropriate agar medium plate. The permanent stock was stored and re-used.

2.4. Media

E. coli transformant strains were grown in liquid Luria-Bertani (LB) medium supplemented with ampicillin to a final concentration of 100 μg/ml (LB-amp). *S. cerevisiae* strains were grown in liquid Yeast extract Peptone Dextrose (YPD) medium or Synthetic Defined (SD) medium. The transformants selection was made in YPD supplemented with G418 (YPD-G418; to a final concentration of 100 μg/ml in liquid media or 200 μg/ml in solid media) or in Synthetic Defined with Uracil Dropout (SD-Ura) medium. All strains were also grown in the corresponding solid media, obtained by the addition of 2% (w/v) Agar. YPD media was used for aerobic growth (2.18) and, when mentioned, was supplemented with inhibitors: 3.00 g/L of acetic acid, 0.30 g/L of HMF and 1.60 g/L of furfural; or 1.89 g/L of HMF and 2.88 g/L of furfural. Shake-flask fermentation (2.19) was generally performed in EWH, supplemented with 100 g/L glucose (pH 4.5). When using *S. cerevisiae* BY4741, 3% BYauxo Mix was added to the medium to account for the auxotrophies of this strain. Fermentations were also performed in YPD medium and YPD supplemented with 3.11 g/L of acetic acid, 0.33 g/L of HMF and 1.66 g/L of furfural (2.21). The media and solutions mentioned are described in Appendix 1, Table A1.

2.5. Vectors

Vectors used during this work are listed in Table 2.2. All of these vectors contain CoLE1 origin of replication and f1 origin of replication from f1filamentous phage and the gene that confers resistance to ampicillin.

Table 0.2. Vectors used during this work.

Vector	Description	Use	Source
pGEM-T Easy	T-overhangs to improve ligation of PCR products generated by Taq DNA polymerases; LacZ gene for blue-white screening	Ligation of PCR products	Promega
pMI516MCS	Kan selection marker under thecontrol on *AgTEF*promoter and *ScADH1*terminator; URA3 marker; 2-micron origin of replication; *ScPGK1* promoter and terminator	Expression of *S. cerevisiae* genes	Aguiar [88]
YEplac195	URA3 marker; 2-micron origin of replication; LacZ gene for blue-white screening	Expression of *S. cerevisiae* genes with native promoters and terminators	Gietz and Sugino [89]
BHUM1737	YEplac195 containing the *HAA1* gene under the control of its native promoter and terminator in YEplac195	Expression of *S. cerevisiae HAA1* gene	Malcher *et al.* [90]

2.6. Primers

Primers used during this work are listed in Table 2.3.

Table 0.3. Primers used during this work. Underlined are the recognition sites of the restriction enzymes used in the cloning procedures.

Primer name	Sequence (5'->3')	Tm (°C)	Use
PRS3_qPCR_fw	GGCTAGGTCTACAGTTAACAAG	60	*PRS3* expression analysis by qPCR
PRS3_qPCR_rv	GTCCCTAACAGATTCTCCAATAG	61	
RPB4_qPCR_fw	ACGGGAGGAAATAATAAAGATTTG	57	*RPB4* expression analysis by qPCR
RPB4_qPCR_rv	GACGGTTTCTTGGTCTCTAAAT	57	
HAA1_qPCR_fw	CGGAGCACTATCAGATACCTC	61	*HAA1* expression analysis by qPCR
HAA1_qPCR_rv	GGATTGTAAGGATGAAATGGAGG	61	
ACT1_qPCR_fw	GCCGAAAGAATGCAAAAGGA	57	*ACT1* expression analysis by qPCR
ACT1_qPCR_rv	TAGAACCACCAATCCAGACG	59	
ScZWF1_fw	GAATTCATGAGTGAAGGCCCCGTC	66	Amplification of *ZWF1* from *S. cerevisiae* and verification of insertion in the different plasmids
ScZWF1_rv	CTCGAGCTAATTATCCTTCGTATCTTCTGGC	62	
ScZWF1_v1	TGGCATCACCCGTGTAATCGTAGA	57	Verification of *ZWF1* insertion in the different plasmids
ScPRS3_fw	GAATTCATGCCAACAAATTCCATC	62	Amplification of *PRS3* from *S. cerevisiae* and verification of insertion in the different plasmids
ScPRS3_rv	CTCGAGTTATAAGGGATAATTCTTAAATAAATAAG	60	
ScRPB4_fw	GGATCCATGAATGTTTCTACATCAACC	59	Amplification of *RPB4* from *S. cerevisiae* and verification of insertion in the different plasmids
ScRPB4_rv	CTCGAGTTAATAGAGTGTTTCTAGGTTTGAC	60	
ScHAA1_fw	CCGGAATTCATGGTCTTGATAAATGGC	64	Amplification of *HAA1* from *S. cerevisiae* and verification of insertion in the different plasmids
ScHAA1_rv	CCGCTCGAGTCATAACGAAGACATGAAATTATC	68	
PGK_fw	GTTTAGTAGAACCTCGTGAAAC	58	Verification of insertion in the pMI516MCS
PGK_rv	GGCATTAAAAGAGGAGCG	54	
ScZWF1_nat_fw	GTAAGGTGTAGTTTTGCACCC	59	Amplification of *ZWF1* from *S. cerevisiae* with native promotor and terminator regions
ScZWF1_nat_rv	AAATTTTTGCAGACATTTTTGATATATAT	58	
ScPRS3_nat_fw	TTATCTTCATCACCGCCATAC	57	Amplification of *PRS3* from *S. cerevisiae* with native promotor and terminator regions
ScPRS3_nat_rv	ACAAGAGAAACTTTTGGGTAAAATG	59	
ScRPB4_nat_fw	GATTGCTCAAATTAGCATGTGAA	58	Amplification of *RPB4* from *S. cerevisiae* with native promotor and terminator regions and colony PCR
ScRPB4_nat_rv	AATCCTGTCCTTTTTCCTGTTAAATAG	62	

2.7. Plasmid DNA preparation from *E. coli* strains

Plasmid DNA was extracted from *E. coli* strains using a rapid plasmid DNA extraction method or alternately, to obtain higher quantities and purity, a commercial kit.

2.7.1. Rapid plasmid DNA extraction

Cells were collected from a fresh LB-amp agar plate, resuspended in 200 µl of Ultra-Pure (UP) H_2O and mixed by vortexing. Cellular lysis was obtained by the addition of 200 µl of Solution I followed by 4 times inversion to mix. To neutralize and precipitate cell extracts and other contaminants, 200 µl of the Solution II were added, the tube inverted 4 times to mix and incubated for 5 min on ice. The suspension was centrifuged for 2 min at 13200 rpm. The supernatant was mixed with 500 µl of 100% isopropanol and centrifuged for 2 min. The supernatant was carefully removed and the pellet was air dried and resuspended in 30 µl of UP H_2O.

Solution I	1% (w/v) SDS 0.2 M NaOH
Solution II	3 M Potassium acetate 11.5% (v/v) Acetic acid

2.7.2. Commercial kit

The GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) was used according to the manufacturer's protocol. Briefly, cells were collected from a fresh LB-amp agar plate, resuspended in 200 µl of Resuspension Solution and mixed by vortex. Cellular lysis was performed with the addition of 200 µl of Lysis Solution. The sample was gently inverted to mix and allowed to clear for 5 min. Afterwards, 350 µl of Neutralization Solution were added and inverted 4-6 times to mix. The debris were pelleted for 10 min at 13200 rpm. In the meantime, 500 µl of Column Preparation Solution were added to the binding column in a collection tube, spun at 13200 rpm for 1 min and the flow-through discarded. The cleared lysate was transferred into binding column, centrifuged for 1 min and the flow-through discarded. The column was washed with 750 µl of Wash Solution and centrifuged for 1 min. The flow-through was discarded and the column dried by an additional 1 min centrifugation. The column was transferred to a new collection tube and the purified plasmid DNA eluted by addition of 30 µl of Elution Solution followed by 1 min centrifugation.

2.8. Genomic DNA extraction

Genomic DNA was extracted from *S. cerevisiae* CEN.PK113-7D using the Gentra Genomic DNA Purification Kit (Puregene), according to the manufacturer's protocol. Briefly, a cell suspension (grown overnight and containing approximately 1-2 x 10^9 cells) was placed on ice and centrifuged at 2000 g for 3 min. The cell pellet was resuspended with 3 ml of Cell Suspension Solution and 15 µl of Lytic Enzyme Solution was added, followed by 25 times inversion to mix. The cell walls were digested by incubation at 37 ^{0}C for 30 min followed by centrifugation at 2000 g for 3 min. The cell pellet was resuspended in Cell Lysis Solution and the cells lysed by pipetting. The proteins were precipitated by vigorously vortexing after the addition of 1 ml of Protein Precipitation Solution, followed by centrifugation at 2000 g for 10 min. The supernatant containing the DNA was transferred to a clean tube containing 3 ml of 100% isopropanol. The sample was gently inverted 50 times and centrifuged at 2000 g for 3 min. The DNA, visible as a small white pellet, was washed with 3 ml of 70% ethanol. The ethanol was carefully removed after centrifugation at 2000 g for 1 min and the tube air dried for 15 min. The DNA was rehydrated with 500 µl of DNA Hydration Solution and 15 µl of RNase A solution were added. After mixed, the sample was incubated at 37 °C for 30 min and the hydration was completed by incubation for 1 h at 65 °C, and then overnight at room temperature.

2.9. DNA quantification

Nucleic acid concentration and purity was determined in a NanoDrop 1000 Spectrophotometer (Thermo Scientific) by loading 1.5 µl of sample. The absorbance at 260 nm is used to calculate the concentration, in ng/µl. The sample purity is attained by the ratio of absorbance at 260 nm and 280 nm. A value of ~1.8, for DNA, and 2.0, for RNA, is generally accepted as indicative of pure nucleic acid solution. Lower values may indicate the presence of protein, phenol or other contaminants. A secondary measure of nucleic acid purity is the ratio of absorbance at 260 and 230 nm, which should be in the range of 1.8-2.2 for pure nucleic acid solutions. An appreciably lower ratio may indicate the presence of co-purified contaminants.

2.10. DNA Storage

DNA solutions were stored at -20 or 4 °C in TE or EB buffer, or alternately in UP H_2O.

TE buffer	10 mM Tris/HCl pH 8.0 1 mM EDTA
EB buffer	10 mM Tris/HCl pH 8.5

2.11. Amplification of DNA fragments by Polymerase Chain Reaction (PCR)

DNA amplification by PCR was performed using two different enzymes. Amplification of fragments for subcloning procedures was performed with Phusion High-Fidelity DNA polymerase (Finnzymes), while Taq DNA polymerase (NZYTech) was used for colony PCR.

2.11.1. Amplification with Phusion High-Fidelity DNA polymerase

Genes of interest were amplified by PCR technique with Phusion High Fidelity DNA polymerase (Finnzymes) using the primers listed in Table 2.3. The reaction mixture consisted of 20 µl of 5x Phusion HF buffer, 2.5 µl of 20 µM of each Primer (Table 2.3), 2 µl of 10 mM dNTPs, 2 µl of *S. cerevisiae* CEN.PK113-7D genomic DNA (2.8), 1 µl of Phusion HF DNA Polymerase and UP H_2O to the final volume of 100 µL. The amplification of the genes *ZWF1*, *PRS3* and *RPB4* was performed simultaneously, with an initial denaturation step at 98 °C during 1 min, followed by 35 cycles of 10 s denaturation at 98 °C, 30 s annealing at 50 °C and 1 min extension at 72 °C, and with a final extension step of 10 min at 72 °C. The *HAA1* amplification was performed with small modifications: the initial denaturation lasted 30 s, the annealing temperature was 59 °C, the extension took 45 s and with the final extension step 5 min. An additional step of 10 min at 72 °C with the addition of 1 µl of NZYTaq DNA Polymerase (NZYTech) was performed, to add A-overhangs on the PCR products, to enable the ligation reaction to the pGEM-T Easy Vector.

2.11.2. Colony PCR

The colony PCR technique was used for the verification of insertion and correct direction of the different genes/fragments of interest into the different plasmids. Using the tip of a sterile toothpick, a small amount of each colony was added to the bottom of a PCR tube. The cells were microwaved for 2 cycles of 45 s at 900 W and immediately placed on ice. A master mix was prepared, per colony, with 2 µl of 10x Reaction buffer, 0.3 µl of 10 mM dNTPs Mix, 0.6 µl of 50 mM $MgCl_2$, 0.3 µl of 20 µM of each Primer (Table 2.3), 0.2 µl of Taq DNA polymerase and UP H_2O to the final volume of 20 µL. The master mix was distributed by each tube. The PCR

procedure was performed with an initial denaturation at 95 °C during 5 min, followed by 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 50 °C and 1 min, per fragment kb, extension at 72 °C, and with a final extension step of 5 min at 72 °C.

2.12. DNA Electrophoresis

2.12.1. Agarose gel

The analysis of DNA fragments was performed by electrophoresis in agarose gels (usually 1%) in horizontal cells. Green Safe Premium (NZYtech) was added to gels for nucleic acid staining. Loading Dye (1x) was mixed with each sample, which allowed the visualization of the running velocity and increased the samples density (making them denser than the running buffer and allowing them to sink into the well). Electrophoretic runs were performed at 70-100 V, in 1x TAE buffer, until the dye migrated as far as 2/3 of the gel length. Gels were visualized and photographed in a Molecular Imager ChemiDoc™ XRS + Imaging System (Bio-Rad) and analyzed using the Image Lab 4.0 software.

Agarose gel	1% (w/v) Agarose 0.006% (v/v) Green Safe Premium
50x TAE buffer	2 M Tris-base 50 mM EDTA pH 8.0 (with acetic acid)
6x Loading Dye	25% (w/v) glycerol 20 mM EDTA 0.25% (w/v) Bromophenol blue

2.12.2. DNA molecular weight marker

The DNA molecular weight marker used in all gels was NZYDNA Ladder III (NZYTech) which produces a pattern of 14 regularly spaced bands, ranging from 200 to 10000 bp (Table 2.4).

Table 0.4. NZYDNA Ladder III bands molecular weight.

Band	Size (bp)
1	10000
2	7500
3	6000
4	5000
5	4000
6	3000
7	2500
8	2000
9	1400
10	1000
11	800
12	600
13	400
14	200

2.13. DNA purification of PCR products

PCR products were purified using the QIAquick PCR purification Kit (Qiagen) according to the manufacturer's protocol. Briefly, 5 volumes of buffer PB were added to 1 volume of the PCR reaction. The mixed sample was applied to a QIAquick column, placed on a 2 ml collection tube and centrifuged for 1 min. The flow-through was discarded, the column placed back in the same tube and washed with 750 µl of buffer PE followed by 1 min centrifugation. The flow-through was discarded and the QIAquick column centrifuged once more for 1 min to remove residual wash buffer. The column was placed in a clean 1.5 ml tube and the DNA eluted with 30 µl of buffer EB, which was left to stand in the column for 1 min, before centrifugation for 1 min.

2.14. DNA purification from agarose gel

DNA was recovered from agarose gels using the QIAquick Extraction Gel Kit (Qiagen), according to the manufacturer's instructions. Briefly, the DNA fragment to be purified was excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a colorless tube and 3 volumes of buffer QG were added to 1 volume of gel (100 mg~100 µl). The tube was incubated at 50 °C for 10 min, and the expected yellow color, after the gel slice complete dissolution, was confirmed. The sample was mixed with 1 gel volume of isopropanol, transferred to a QIAquick column (placed on a 2 ml collection tube) and centrifuged for 1 min. The flow-through was discarded, the QIAquick column placed back in the same tube and washed with 750 µl of buffer PE, followed by 1 min centrifugation. The flow-through was discarded and the

QIAquick column centrifuged once more for 1 min to remove residual wash buffer. The column was placed into a clean 1.5 ml tube and the DNA eluted with 30 µl of buffer EB, which was left to stand in the column for 1 min, before centrifugation for 1 min.

2.15. Enzymatic modification of DNA

2.15.1. Digestion with restriction endonucleases

Digestion reactions with restriction endonucleases were performed overnight at 37 °C in a final volume of 10-20 µl, using the New England Biolabs enzymes in the appropriate provided10x buffer.

2.15.2. Plasmid DNA dephosphorylation

Digested vectors were dephosphorylated with Fermentas™ Shrimp Alkaline Phosphatase (SAP) to prevent its recircularization during the ligation reaction. The appropriate amount of SAP (1 unit per pmol of plasmid *termini*) was added to the restriction reaction tube (after digestion took place) and incubated for 1 h at 37 °C. The enzyme was inactivated by heating for 15 min at 65 °C.

2.15.3. Ligation reactions

Ligation of DNA fragments to linearized dephosphorylated vectors was performed with T4 DNA Ligase (Promega) at 4 ◦C overnight. The quantity of insert to use was calculated through the formula:

$$\text{ng of insert} = \frac{\text{ng of vector} \times \text{Kb of insert}}{\text{Kb of vector}} \times \text{insert:vector molar ratio} \qquad (1)$$

An insert:vector molar ratio of 3:1 was normally used. The vector quantity generally used was 100 ng. The DNA mix was complemented with 1 µL of 10x Ligase Buffer, 1 U of T4 DNA Ligase and UP H2O to a final volume of 10 µL.

Ligation reactions involving the pGEM-T Easy Vector (Fermentas) were performed with small modifications: 50 ng of vector were used and performed with 2x Rapid Ligation Buffer provided with the vector.

2.16. Transformation of *E. coli* cells

Competent *E. coli* cells were transformed with the constructs resulting from the ligation reactions by heat-shock or electroporation.

2.16.1. Heat-shock method

Transformation using the heat-shock method was performed using NZY5α Competent Cells (NZYtech) according to the manufacturer's protocol. Competent cells were thawed on ice and gently mixed. The ligation reaction (100 to 300 ng of DNA in a maximum volume of 10 µl) was added to 100 µl of competent cells and the tube gently tapped to mix. The cells were incubated on ice for 30 min and heat-shocked for 40 s in a 42 °C water bath. The tubes were placed on ice for 2 min and 900 µl of room temperature SOC medium was added, followed by incubation at 225 rpm and 37 °C for 1 h. The cell suspension was spread on LB-amp agar plates, in appropriate dilutions, and incubated overnight at 37 °C.

SOC medium	2% (w/v) Tryptone
	0.5% (w/v) Yeast Extract
	10 mM NaCl
	2.5 mM KCl
	10 mM $MgSO_4.7H_2O$
	10 mM $MgCl_2.6H2O$
	20 mM Glucose

2.16.2. Electroporation

Transformation by electroporation was performed in an *E. coli* pulser (Bio-Rad) using TOP10 electrocompetent cells stored at -80°C (prepared by our group). A mixture containing 2 µl of the ligation reaction and 40 µl of cells was incubated on ice for 1 min. The mixture was transferred to an ice-cold 0.1 cm cuvette, avoiding the formation of air bubbles and ensuring that the cell suspension was deposited at the bottom of the cuvette. The cuvette was carefully dried before insertion into the electroporation chamber. The electric pulse was performed at 1.8 kV. Immediately after the pulse the cuvette was removed from the chamber, 500 µl of SOC medium were immediately added and the cells resuspended. The cell suspension was transferred to a new tube and the cuvette washed with 500 µl of SOC medium, which were also transferred to the tube. The time constant, with optimal values between 4 and 5 ms, was verified. The cell suspension was incubated at 37 °C and 225 rpm for 1 h and subsequently spread on LB-amp agar plates, in appropriate dilutions, and incubated overnight at 37 °C.

2.17. Transformation of *S. cerevisiae*

S. cerevisiae BY4741 was transformed with the different constructs by the lithium acetate method. Cells were inoculated into 25 ml of liquid YPD medium and grown overnight to approximately $2x10^7$ cells/ml. The cell suspension was 10-fold diluted with fresh and warm (~30 °C) YPD, in a final volume of 25 ml, and grown again to $2x10^7$ cells/ml. The cells were harvested at room temperature for 5 min at 5000 rpm and washed with 25 ml of sterile UP H_2O. The cell pellet was resuspended in 1 ml of sterile UP H_2O, transferred to an 1.5 ml centrifuge tube and the cells pelleted. Cells were washed with 1 ml of TE/LiOAc solution (made fresh from sterile 10x stock), and resuspended in 200 µl of the same solution. The carrier DNA, Sonicated Salmon Sperm (Stratagene), was incubated for 10-15 min at 100 °C and immediately placed on ice. A mixture of 50 µl of the yeast cell suspension, 1 µg (maximum volume of 5 µl) of plasmid DNA and 50 µg of single stranded carrier DNA was prepared in a microcentrifuge tube. Afterwards, 300 µl of sterile 40% Polyethylene Glycol (PEG) 4000 in TE/LiOAc were added and mixed thoroughly. The mixture was incubated for 30 min at 225 rpm and 30 °C, followed by a heat shock at 42 °C for exactly 15 min. The suspension was spun down in the microcentrifuge for 5 s at room temperature and the cell pellet resuspended in 1 ml of 1x TE. The cell suspension was spread on YPD-G418 agar plates, in appropriate dilutions, incubated at 30°C and transformants were visible after 2-5 days.

10x TE buffer	100 mMTris-HCl 10 mM EDTA pH 7.5 (with 10 M NaOH)
10x LiOAc solution	1 M LiOAc pH 7.5 (with diluted acetic acid)
TE/LiOAc	1x TE buffer 1x LiOAc solution
40% PEG-4000 in TE/LiOAc	40% PEG-4000 1x TE buffer 1x LiOAc solution

2.18. Aerobic growth in microplates

The pre-inoculum was carried out in 100 ml Erlenmeyer flasks with 40 ml of growth medium, YPD for the *S. cerevisiae* BY4741 and YPD supplemented with G418 for transformants. One isolated colony was transferred with a loop for each flask and sealed with a cotton plug. The flasks were incubated at 30 °C and 200 rpm overnight (until the mid-exponential phase).

Aerobic growth was performed in YPD medium or YPD supplemented with inhibitors, furfural, HMF and/or acetic acid (and G418), in 24-well microplates (1 ml of final volume per well). The Optical Density at 600 nm (OD_{600nm}) of each pre-inoculum was measured and each microplate well was inoculated to an OD_{600nm} of 0.1. Some wells were not inoculated to serve as control for possible cross-well contaminations. The microplates were incubated at 30 °C and 200 rpm and the growth monitored by OD_{600nm} measurements, until stationary phase was reached, in a Synergy HT Multi-Mode Microplate Reader (BioTek).

2.19. Shake-flask fermentations

Fermentations were performed in EWH (prepared following the method described by Ruiz *et al.* [91]) or YPD with inhibitors, in 100 ml Erlenmeyer flasks fitted with perforated rubber stoppers enclosing glycerol-filled air-locks, which CO_2 exhaustion while avoiding the entrance of air. This system permits a reliable simulation of the low oxygen conditions on industrial fermentations.

The pre-inoculum was carried out in 100 ml Erlenmeyer flasks and 40 ml of growth medium, SD medium for *S. cerevisiae* parent strain and SD-Ura medium for transformants, were distributed for each flask (alternately, YPD and YPD+G418 were used). One isolated colony of each transformant was transferred with a loop for each flask and a cotton plug used to seal the flask. The flasks were incubated at 30 °C and 200 rpm for 22-24 h (until the end of the exponential phase was reached). The following steps were performed on ice. The cell suspension was transferred to 50 ml centrifuge tubes previously weighted and centrifuged at 4 °C and 12000 rpm for 15 min. The supernatant was rejected and the tube walls carefully cleaned. The yeast cell pellet was weighted and resuspended in Saline Solution (0.9% (w/v) NaCl) to a concentration of 200 mg of Fresh Yeast per milliliter (mgFY/ml). The suspension was homogenized by manual agitation. The fermentation media were stirred for 20 min to allow its aeration. When using the URA3 selection marker, uracil was not added to the fermenting media of the transformants (alternately, G418 was used for selection). Precisely 30 ml of the

fermentation media was distributed for each fermentation flask. The flasks were inoculated with the correct amount of the concentrated cell suspension to obtain a final concentration of 5 mgFY/ml (mimicking the high inoculation rates practiced at the industrial level). The suspension was homogenized, the flasks sealed and each lock filled with approximately 1 ml of glycerol. The flasks were incubated at 30 °C and 150 rpm and the fermentations were monitored by the reduction of mass resulting from CO_2 production. Initial and final media samples were collected for glucose, acetic acid, furfural, HMF and ethanol quantification (2.20).

2.20. HPLC quantification

Glucose, acetic acid, furfural, HMF and ethanol were quantified by high performance liquid chromatography (HPLC), upon separation of the different samples in a Varian MetaCarb 87H column, eluted at 60°C with 0.005 M sulfuric acid and at a flow rate of 0.7 mL/min. The peaks corresponding to glucose, acetic acid and ethanol were detected using a refractive index detector, whereas furfural and HMF were detected using an UV detector set at 210 nm.

2.21. Quantitative Reverse Transcriptase PCR (qRT-PCR)

To analyze the expression of the different genes of interest, shake-flask fermentation (2.19) was performed with the industrial strain *S. cerevisiae* PE-2 in YPD medium and YPD medium supplemented with acetic acid, HMF and furfural in concentrations similar to the ones present in EWH. Samples were collected at different phases of the fermentations: late lag-phase (~1 g/L of CO_2 produced), initial exponential phase (~5 g/L of CO_2 produced) and initial stationary phase (~40 g/L of CO_2 produced). Another sample was taken at the early lag-phase (~2 hours of fermentation) from the fermentation in the presence of inhibitors. Each samples contained approximately $2x10^7$ cells, calculated through an OD_{600nm} *vs* Biomass calibration curve, by OD_{600nm} measurements. The cell pellet of each sample was rapidly stored at -70 °C, after washing with Saline Solution.

2.21.1. RNA purification

Total RNA was purified using the RNeasy Plant Mini Kit (Qiagen), according to manufacturer's protocol. Briefly, the stored cell pellets were thawed on ice and resuspended in 600 µl of Buffer RLT (β-Mercaptoethanol (β-ME) was added to Buffer RLT before used) by vortexing. The samples were added to tubes containing 0.5 g of acid-washed glass beads and mixed by vortexing. Cells were disrupted at top speed (6.5 m/s) in the FastPrep®-24 Instrument

(MP Biomedicals) during 4 cycles of 30 s agitation and 5 min of cooling interval. The tubes were removed from the homogenizator and the beads allowed to settle. The lysate was transferred to a new microcentrifuge tube, centrifuged for 2 min at 13200 rpm and the supernatant transferred to another tube. The cell-free homogenized lysate was mixed by pipetting with 1 volume of 70% ethanol. The sample was transferred to an RNeasy spin column placed in a 2 ml collection tube. The lid was gently close and the tube centrifuged for 15 s at 10000 rpm. The flow-through was discarded and 700 µl of Buffer RW1 was added to the column. The tube was centrifuged for 15 s at 10000 rpm to wash the spin column membrane. The flow-through was discarded and 500 µl of Buffer RPE were added to the column, followed by centrifugation for 15 s at 10000 rpm. The flow-through was discarded, another 500 µl of buffer RPE were added and the column was centrifuged for 15 s at 10000 rpm. The column was placed in a new collection tube and centrifuged at 13200 rpm for 1 min. The column was placed into a clean 1.5 ml collection tube and 30 µl of RNase-free water was added directly to the column membrane. The RNA was eluted by centrifugation for 1 min at 10000 rpm. The eluate was placed again on the column membrane, followed by another centrifugation for 1 min at 10000 rpm, to increase the RNA concentration. The RNA quality was verified by 1% agarose gel electrophoresis (2.12.1) and by NanoDrop quantification (2.9).

2.21.2. cDNA synthesis

Single-stranded cDNA was synthesized from the previously purified total RNA with Super ScriptII Reverse Transcriptase (RT) (Invitrogen). Total RNA (1 µg) was combined with 1 µl of oligodT Primer (0.5 µg/µl) and 1 µl of 10 mM dNTP Mix in a total volume of 13 µl completed with DEPC-treated Water. The sample was heated at 65 °C for 5 min in a thermocycler. The tubes were briefly spun and placed on ice for 2 min. Afterwards, 4 µl of 5x First Strand Buffer and 2 µl of 0.1 M dithiothreitol (DTT) were added to each sample tube. The samples were gently mixed, spun in the microcentrifuge and heated at 42 °C for 2 min. The samples were then incubated with 1 µl of SuperScript II RT at 42 °C for 50 min. The reaction was terminated with incubation at 70 °C for 15 min and the tubes were briefly spun, placed on ice and stored at -20 °C.

2.21.3. Quantitative PCR

Oligonucleotides for real-time PCR (Table 2.3) were designed using the Primer Quest tool from OligoAnalizer (IDT. Biotools) followed by a BLAST analysis against the *S. cerevisiae* genome sequence for specificity confidence. Quantitative real-time assays were performed in a CFX96 real-time PCR system (Bio-Rad). Each sample was tested in duplicate in a 96-well plate (Bio-Rad, CA).

The reaction mix (10 µl final volume) consisted of 5 µl of SsoFastEvagreen supermix (Bio-Rad), 0.6 µl of each primer (600 nM final concentration), 2.8 µl of H_2O, and 1 µl of a 1:10 dilution of the cDNA preparation (determined as the appropriate dilution to use from standard curves obtained from 10-fold serial dilutions of cDNA). The absence of genomic DNA in RNA samples was checked by real-time PCR before cDNA synthesis (minus RT control). A blank (No Template Control) was also incorporated in each assay. The thermocycling program consisted of an initial enzyme activation step at 95 °C during 30 s, followed by 40 cycles of 5 s denaturation at 95 °C and 5 s annealing/extension at 54.2 or 55.7 °C. After completion of these cycles, data from the melting-curve were then collected to verify PCR specificity, contamination and the absence of primer dimers.

The PCR efficiency of each primer pair (Eff) was evaluated by the dilution series method using a mix of sample cDNA as the template. Briefly, it was determined from standard curves using the formula $10^{(-1/slope)}$. For the calculations, the base of the exponential amplification function was used (*e.g.* 1.94 means 94% amplification efficiency). Relative expression levels were determined with efficiency correction, which considers differences in primer pair amplification efficiencies between target and reference genes, and results in a more reliable estimation of the "real expression ratio" than the $2\Delta\Delta Ct$ method. For standardization, the results were expressed as target/reference ratio, the reference gene being the genome-encoded actin gene (*ACT1*).

3. RESULTS AND DISCUSSION

3.1. Study of *PRS3*, *RPB4* and *HAA1* expression in a robust *S. cerevisiae* industrial strain during fermentations in control and inhibitory media

To further understand the role that *PRS3*, *RPB4* and *HAA1* may play in yeast's tolerance and adaptation in the presence of inhibitory stress, the expression of these genes was studied during the fermentations of the robust ethanologenic *S. cerevisiae* PE-2 in YPD (control) and in YPD supplemented with acetic acid, HMF and furfural in concentrations similar to those present in EWH (2.4) (Figure 3.1).

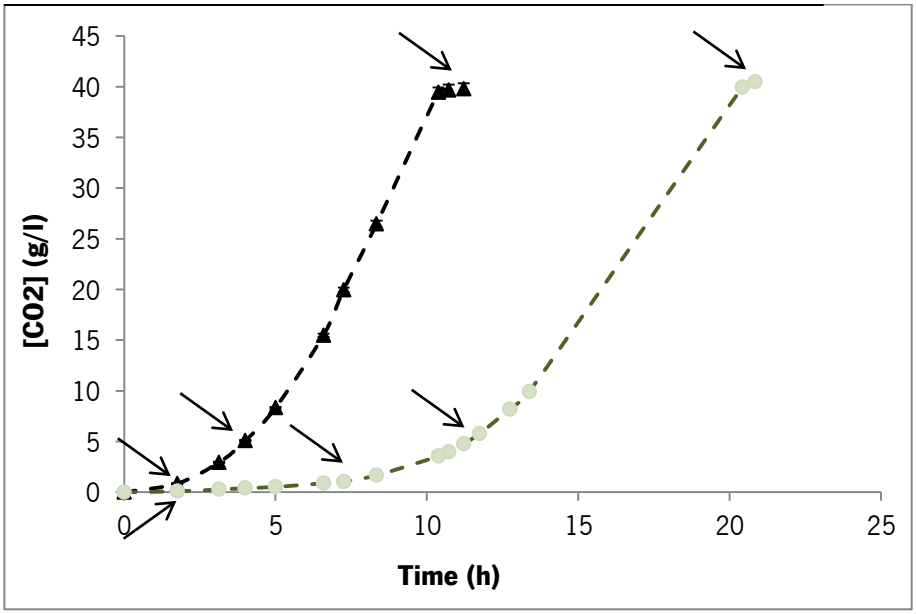

Figure 0.1. Profile of CO_2 production of *S. cerevisiae* PE-2 in YPD (▲) and in YPD supplemented with acetic acid (3 g/L), HMF (0.3 g/L) and furfural (1.66 g/L) (●). Data represents average ± standard deviation obtained from two biological replicates. Samples were collected at the time-points indicated with black arrows.

As previously reported [92], in the inhibitory medium, a much longer lag-phase was also observed in this study. For gene expression analysis, samples were collected at different phases of the fermentations (indicated with arrows in Figure 3.1): late lag-phase, initial exponential phase and initial stationary phase. Another sample was taken at the early lag-phase, from the fermentation in the presence of inhibitors.

3.1.1. RNA quality

After total RNA purification (2.21.1) from the biomass collected at the different time-points, its quality was accessed by agarose gel electrophoresis and NanoDrop quantification. Approximately 700 ng of RNA from each sample were run on an agarose gel (Figure 3.2), and 2 clear and sharp 28S and 18S rRNA bands were visible. A fainter band with a lower molecular weight, corresponding to tRNA, was also observable. mRNA was not detectable on the gel, which

is in agreement with the composition of total RNA from *S. cerevisiae*: approximately 80% of rRNA, 15% of tRNA and 5% of mRNA [93]. This electrophoresis was performed on a native agarose gel (and not in denaturing conditions as recommended), so, the presence of bands above the 28S band may represent different structures of a single RNA species. The RNA integrity could be attained by the ratio of intensity between the 18S and 28S bands. For completely intact RNA a ratio of 0.5 (18S:28S) was expected, and the majority of the samples had approximate values (Figure 3.2). The discrepancy of some of the ratios may be due to non-optimal staining and visualization of the agarose gel.

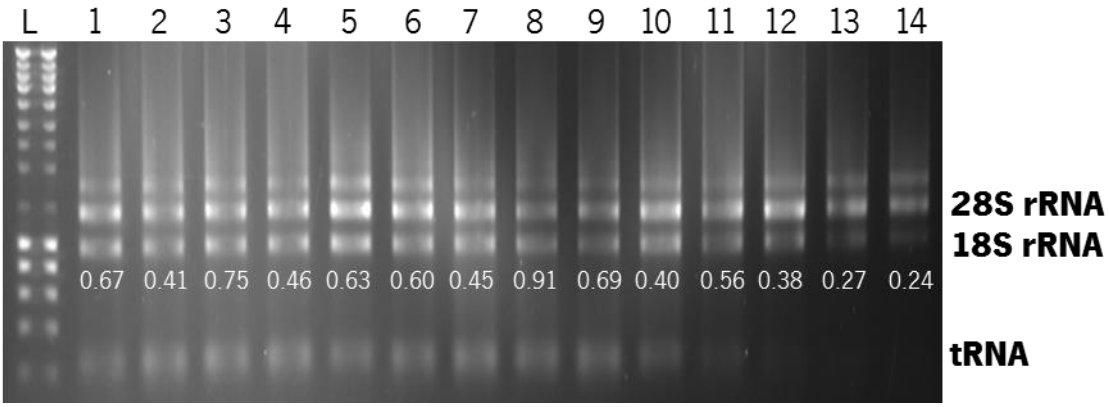

Figure 0.2. Verification of RNA integrity. Samples from the duplicate control fermentations: lag-phase, initial exponential phase, initial stationary phase (lanes 1-6). Samples from the duplicate inhibitory medium fermentations: early lag-phase, late lag-phase, initial exponential phase, initial stationary phase (lanes 7-14). The 18S:28S intensity ratio (calculated using the Image Lab 4.0 software) is indicated in each lane.

The NanoDrop quantification showed concentrations in a range of 138 to 520 ng/µL of total RNA. All the samples presented a ratio of absorbance at 260 nm and 280 nm of ~2.0, which is generally accepted for pure RNA.

3.1.2. Quantitative Reverse Transcriptase PCR

After the cDNA synthesis (2.21.2) some parameters, such as annealing temperature and primer concentration, were optimized in order to obtain better performances of the quantitative PCR (2.21.3). Standard curves were generated by 10-fold serial dilutions of cDNA from the lag-phase of the control fermentation and amplification efficiencies of the primer pairs closer to 100% were obtained (92.6-101.4%), as well as coefficient of determination (R^2) closer to 1 (0.997-1), indicating that optimized qPCR assay conditions were achieved. The expression levels of *PRS3*, *HAA1* and *RPB4* in the cells collected from the fermentations in inhibitory medium were compared with those from the fermentations in control (absence of inhibition) medium at

different phases: late lag-phase (Figure 3.3a), initial exponential phase (Figure 3.3b) and initial stationary phase (Figure 3.3c).

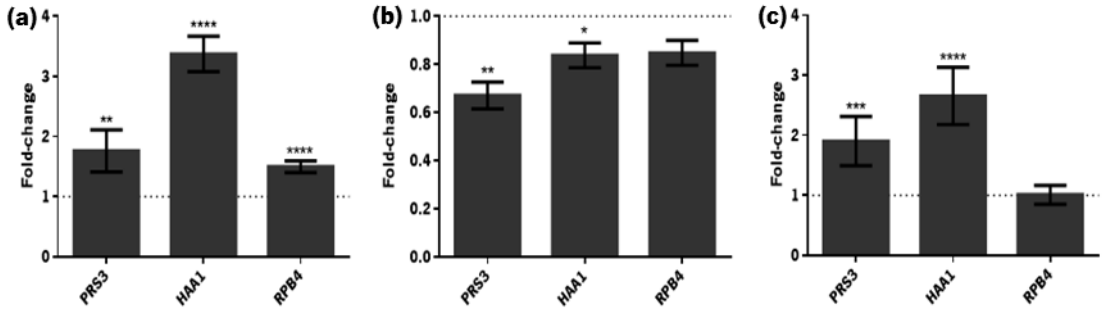

Figure 0.3. Differential expression of *PRS3*, *HAA1* and *RPB4* during different phases of *S. cerevisiae* PE-2 fermentation in inhibitory medium: (a) late lag-phase, (b) initial exponential phase and (c) initial stationary phase. Results are shown as the fold-change in expression relative to that on the same phase of *S. cerevisiae* PE-2 fermentation in the absence of stress (represented by the dotted line). Data represents average ± SEM obtained from two biological replicates and at least two experimental replicates. The statistical significance of the results was quantified using multiple t-tests. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****$ $P \leq 0.0001$.

The inhibitors concentration, as well as glucose and ethanol concentration, in the culture supernatants collected at the different fermentations phases was determined by HPLC (2.20). While acetic acid levels remained approximately constant, HMF and furfural were almost completely depleted at the end of the lag-phase (Figure 3.4).

As mentioned in the introduction, the transcription factor Haa1 is the main player in reprogramming yeast genomic expression in response to acetic acid stress [1, 2], and its overexpression was proved to increase yeast tolerance to the presence of this inhibitor [57]. The observed up-regulation of *HAA1* expression at the late lag-phase in the presence of inhibitors (Figure 3.3a; Figure 3.4) supports its importance in yeast adaptation to stress induced by acetic acid.

The expression of *HAA1* was also found to be up-regulated in inhibitory conditions at the initial stationary phase (Figure 3.3c), where an apparent higher concentration of ethanol was present when compared with the same phase of the control fermentations (Figure 3.1). Increasing amounts of ethanol may accumulate to toxic concentrations during ethanolic fermentation, which is capable of affecting the yeast at the plasma membrane organization and function level, and also by intracellular acidification [94]. Several genes involved in membrane and cell wall composition were identified in genome-wide studies as required for yeast resistance to ethanol-induced stress [30}. Therefore, although *HAA1* was not among the genes identified as determinant for resistance to high ethanol concentrations, it is possible that the observed up-

regulation in its expression under inhibitory conditions at the final stage of fermentation (Figure 3.3c) may be related to the increased ethanol concentration observed in these conditions. *PRS3*, on the other hand, was identified as determinant for resistance to inhibitory concentrations of ethanol [32], and our results demonstrate that its expression was up-regulated at the initial stationary phase in inhibitory fermentation conditions (Figure 3.3c), when ethanol concentration was increased.

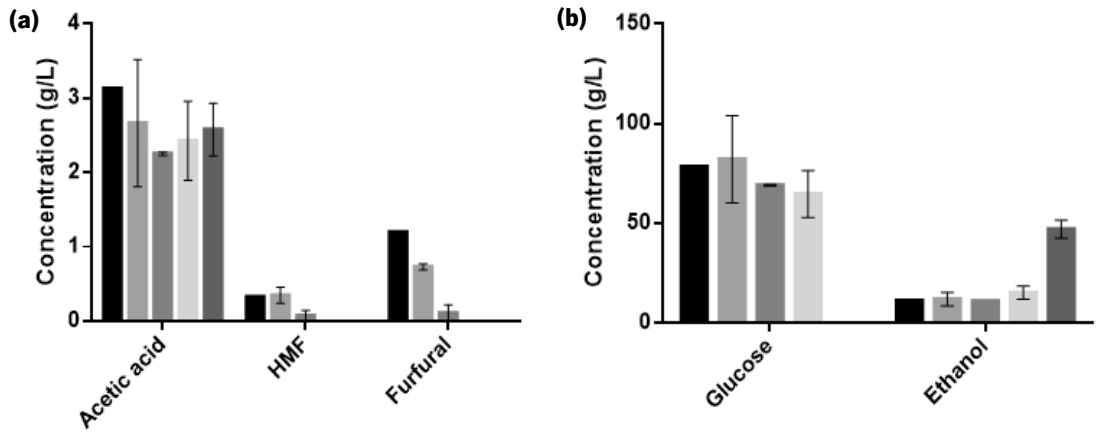

Figure 0.4. Variation of the concentration of the inhibitors, acetic acid, HMF and furfural (a), as well as glucose and ethanol (b), during the fermentation of *S. cerevisiae* PE-2 in inhibitory media: fermentation start (■), early lag-phase (■), late lag-phase (■), initial exponential phase ( ) and initial stationary phase (■). Data represents average ± SEM obtained from two biological replicates.

PRS3 expression was also up-regulated under inhibitory conditions at the late lag-phase (Figure 3.3a). Taking into account that during the lag-phase of fermentation, in the presence of only HMF and furfural, the expression of this gene was previously described to be repressed [38], it is possible that the up-regulation here observed was related to the presence of acetic acid. This gene contributes to the cell integrity, supplying the cell with the key metabolic intermediate PRPP, and it was suggested that it plays a significant role in the remodelling of the cell wall and may have a direct involvement in cell integrity signalling [68]. In fact, the cell envelope permeability to weak acids is dependent of cell wall remodelling [94], supporting the theory that *PRS3* plays a role in yeast adaptation to the presence of acetic acid. The down-regulation of this gene expression at the initial exponential phase (Figure 3.3b), at a time where the yeast have been exposed to acetic acid by prolonged period, may be the result of an already achieved adaptation, i.e. cell wall remodelling.

RPB4 has been found to play a role in the regulation of stress response (e.g. heat shock, starvation) in yeast [80]. Taking into account that this gene was found to be necessary for tolerance to stress caused by the presence of acetic acid, HMF and furfural [58], its up-regulation at the lag-phase in the presence of these inhibitors (Figure 3.3a) may be explained by the yeast need to respond and adapt to the inhibitory stress. This is sustained by the fact that at the following fermentation phases *RPB4* expression was considerably lower (Figure 3.3b and c), as the inhibitory load diminished (Figure 3.4a).

The expression levels of *HAA1*, *PRS3* and *RPB4* along the fermentation (late lag-phase, initial exponential and initial stationary phases) in the presence of inhibitor stress were compared to their expression at the early lag-phase (Figure 3.5). It can be observed that it was in the initial stage of the fermentation (early lag-phase) that these genes were more highly expressed (represented by the dotted line in Figure 3.5), followed by the late lag-phase where the expression levels are still higher than the ones from the subsequent phases (initial exponential and initial stationary phases). These results support the importance of these genes roles in the adaptation phase of *S. cerevisiae* to the presence of inhibitory stress induced by acetic acid, HMF and furfural.

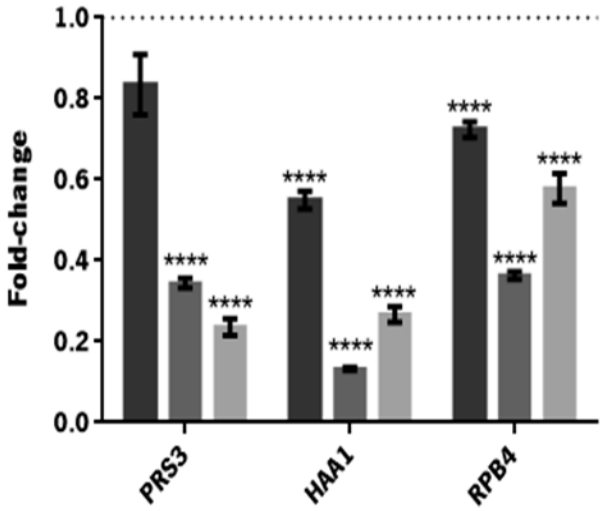

Figure 0.5. Differential expression of *PRS3*, *HAA1* and *RPB4* along a fermentation in inhibitory medium: late lag-phase (■), initial exponential phase (■) and initial stationary phase (■). Results are shown as the fold-change in expression relative to that on the early lag-phase of the same fermentation (represented by the dotted line). Data represents average ± SEM obtained from two biological replicates and at least two experimental replicates, and the statistical significance of the results was quantified using multiple t-tests. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001; **** *P*≤ 0.0001.

Taking into account these results and the information previously available in the literature, this study proceeded with the analysis of the effect of these genes overexpression in fermentations mimicking industrially relevant conditions.

3.2. Construction of recombinant *S. cerevisiae* BY4741 overexpressing *ZWF1*, *PRS3*, *RPB4* and *HAA1* genes under the control of the *ScPGK1* promoter

The *PRS3* and *RPB4* genes were found to contribute to the maintenance of cell viability in wheat straw hydrolysate and for maximal fermentation rate of this substrate [58]. However, the influence of these genes' overexpression in lignocellulosic-based fermentations has not yet been described. To test the effect of overexpressing these genes in *S. cerevisiae* BY4741 fermentations, the multi-copy vector pMI516MCS was used, which contains the strong *S. cerevisiae* phosphoglycerate kinase (*ScPGK1*) constitutive promoter. *ZWF1*, which overexpression has already been found to confer a growth advantage in the presence of lethal concentration of furfural [44], was used as a positive control. Overexpression of the *HAA1* gene has been shown to enhance the tolerance of *S. cerevisiae* to acetic acid [57], one of the major inhibitors present lignocellulosic hydrolysates [53]. Therefore, the effect of the overexpression of this gene was also evaluated in *S. cerevisiae* BY4741 EWH fermentations.

The *ZWF1*, *PRS3* and *RPB4* coding regions were amplified (2.11.1) from genomic DNA of *S. cerevisiae* CEN.PK113-7D with the primers pairs ScZWF1, ScPRS3 and ScRPB4 (Table 2.3), respectively (Figure 3.6), and the corresponding PCR products ligated to the pGEM-T Easy vector (Figure 3.7 and 8). The vectors pJCZ, pJCP and pJCR (containing the *ZWF1*, *PRS3* and *RPB4* genes under the control of the *ScPGK1* promoter, respectively) were constructed by digestion (2.15.1) of the corresponding pGEM-T Easy constructs with EcoRI, SalI/XhoI and BamHI/XhoI (Figure 3.9), respectively, and insertion into pMI516MCS using the EcoRI, SalI/XhoI and BamHI/XhoI sites, respectively (Figure 3.10).

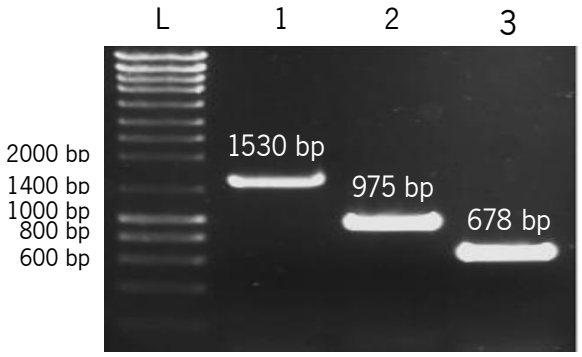

Figure 0.6. PCR amplifications of the coding sequence of the genes of interest from *S. cerevisiae* CEN.PK113-7D. Lane 1: *ZWF1* PCR product; lane 2: *PRS3* PCR product; lane 3: *RPB4* PCR product.

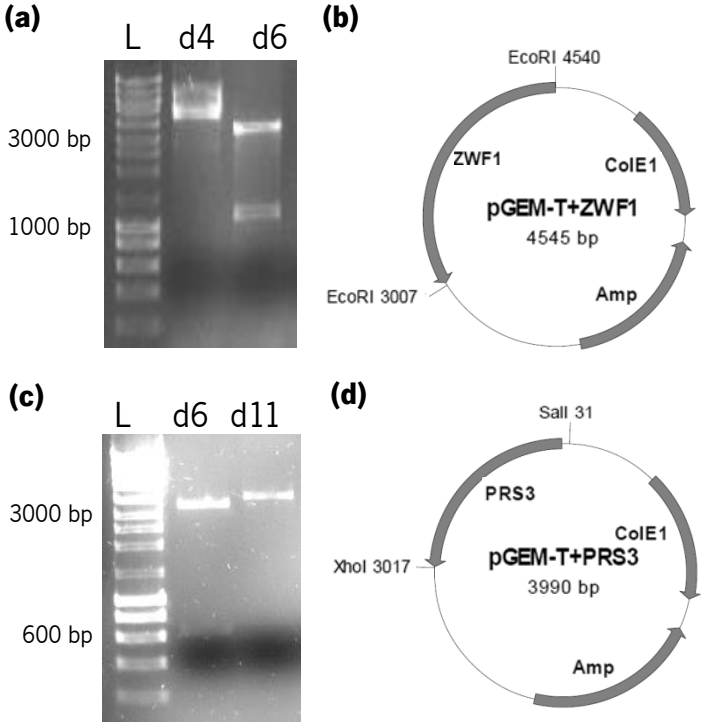

Figure 0.7. Confirmation of insertion of *ZWF1* (a and b) and *PRS3* (c and d) genes into pGEM-T Easy Vector. (a) Resulting pattern of digestion of pGEM-T+ZWF1 with PstI (expected patterns were 4057 and 488 bp or 3451 and 1094 bp). (b) Representation of the pGEM-T Easy vector with *ZWF1* (c) Resulting pattern of digestion of pGEM-T+PRS3 with SacI (expected patterns were 3569 and 421 bp or 3342 and 648 bp). (d) Representation of the pGEM-T Easy vector with *PRS3*.

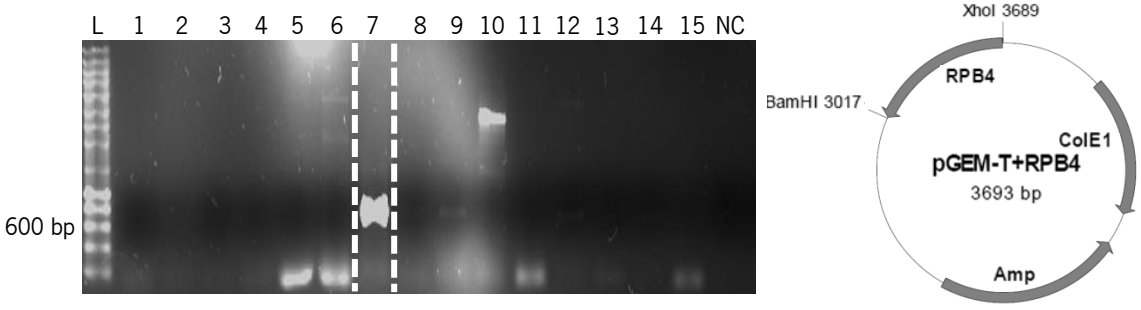

Figure 0.8. Confirmation of insertion of *RPB4* into pGEM-T Easy Vector. (a) Colony PCR of 18 white colonies using ScRPB4 primers (Table 2.3). An amplicon of 678 bp was expected. (b) Representation of the pGEM-T Easy vector with *RPB4*.

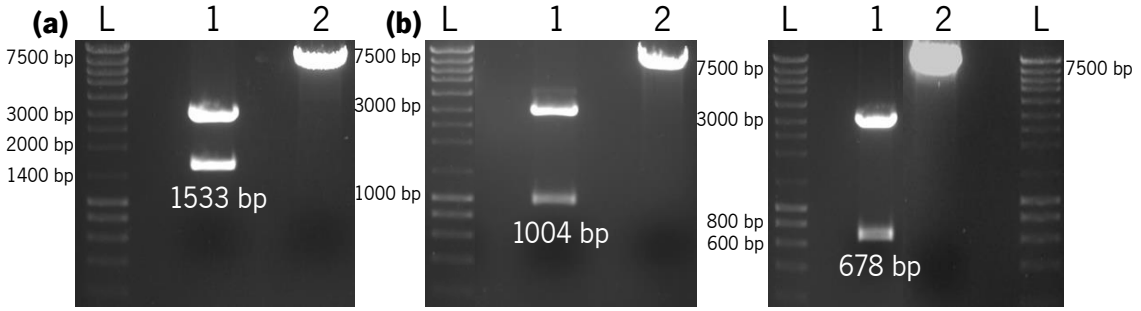

Figure 0.9. Restriction enzyme digestion of the different constructs in pGEM-T Easy Vector and of pMI516MCS Vector. (a) pGEM-T+ZWF1 (1) and pMI516_ MCS (2) were digested with EcoRI. (b) pGEM-T+PRS3 (1) and pMI516MCS (2) were digested with SalI and XhoI. (c) pGEM-T+RPB4 (1) and pMI516_ MCS (2) were digested with BamHI and XhoI.

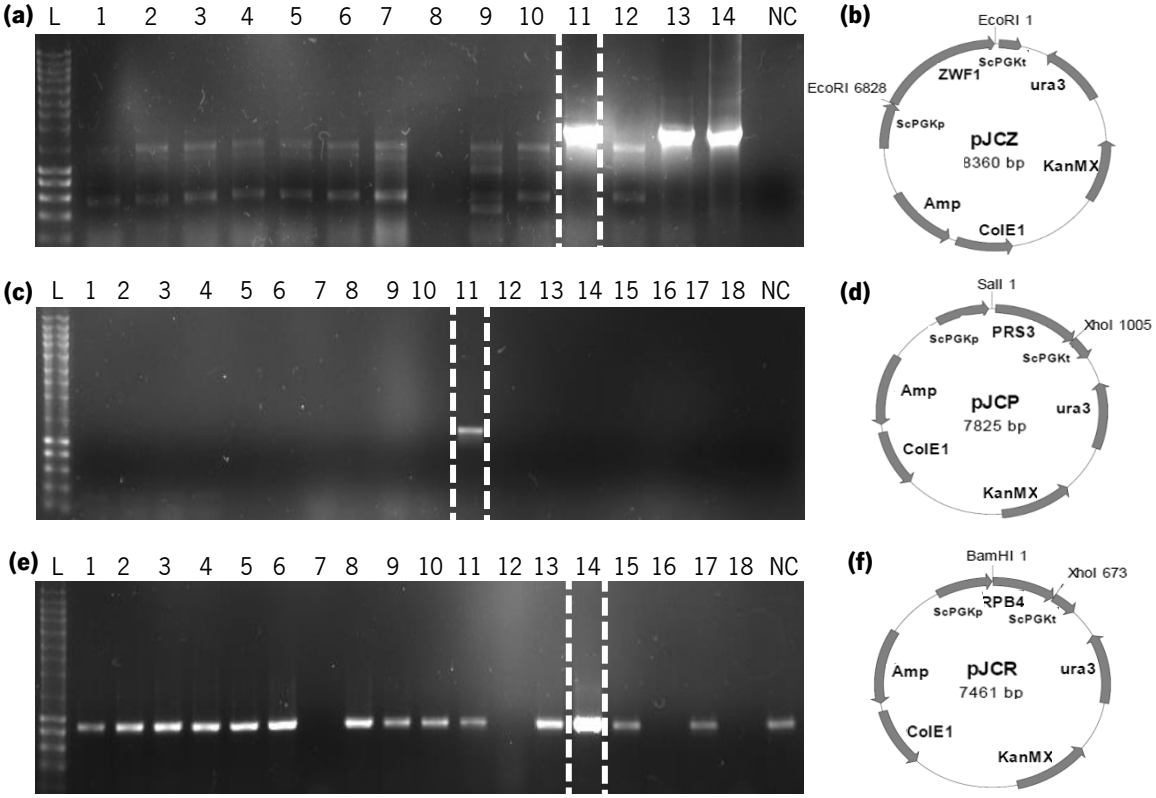

Figure 0.10. Confirmation of correct insertion of *ZWF1*, *PRS3* and *RPB4* genes into pMI516MCS vector. Representation of the pJCZ (b), pJCP (d) and pJCR (f) vectors. (a) Colony PCR of 14 colonies using ScZWF1_fw and PGK_rv primers (Table 2.3), with an expected amplicon of 1758 bp. (c) Colony PCR of 18 colonies using ScPRS3_fw and PGK_rv primers (Table 2.3), with an expected amplicon of 1150 bp. (d) Colony PCR of 18 colonies using ScRPB4_rv and PGK_fw primers (Table 2.3), with an expected amplicon of 878 bp.

For the *ZWF1* gene cloning, the initially selected enzymes were EcoRI and XhoI, however, a mutation in the recognition site for the enzyme XhoI in pGEM-T+ZWF1 was detected by sequencing (Figure 3.11). Taking advantage of the fact that the pGEM-T Easy vector has recognition sites for EcoRI flanking the ligation site for PCR products, both the pGEM-T+ZWF1 construct and the pMI516MCS were digested with this enzyme. The restriction enzymes selected

for digestion of the *PRS3* gene were also EcoRI and XhoI. However, after some unsuccessful transformations, it was presumed that the fact that, in the pGEM-T+PRS3, the XhoI recognition site is located only 3 bp away from a recognition site for EcoRI was affecting the correct function of the XhoI enzyme in the double digestion with the two mentioned enzymes. To overcome this, sequential digestions were performed, with the pGEM-T+PRS3 being digested first with XhoI and after with EcoRI, but this strategy was proved unsuccessful as well. So, taking into account the enzymes present in the Multiple Cloning Sites of pGEM-T Easy and pMI516MCS and the insertion direction of *PRS3* into these vectors, the EcoRI enzyme was substituted by SalI. Therefore, pGEM-T+PRS3 and pMI516MCS were double digested with SalI and XhoI.

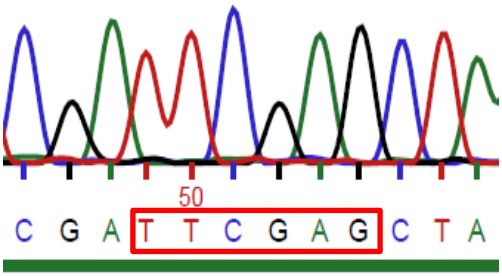

Figure 0.11. Section of the sequencing chromatogram of pGEM-T+ZWF1 showing the mutation on the expected restriction site for XhoI (CTCGAG).

The strategy used for the insertion of the *HAA1* coding sequence into pMI516MCS was the same as that described for the genes *ZWF1*, *PRS3* and *RPB4*. Briefly, the *HAA1* coding region was first amplified from genomic DNA from *S. cerevisiae* CEN.PK113-7D with the primers pair ScHAA1 (Table 2.3) (Figure 3.12) and the resulting PCR product ligated to pGEM-T Easy vector (Figure 3.13). The pJCH vector (containing the *HAA1* gene under the control of the *ScPGK1* promoter) was constructed by digestion (2.15.1) of pGEM-T+HAA1 with EcoRI and XhoI (Figure 3.14) and cloning between the EcoRI and XhoI sites in pMI516MCS (Figure 3.15).

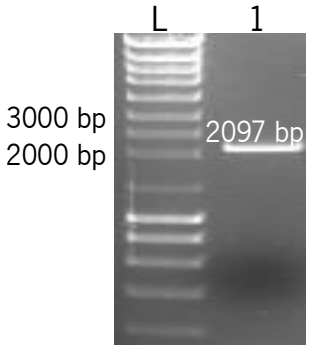

Figure 0.12. PCR amplification of the coding sequence of *HAA1* from *S. cerevisiae* CEN.PK113-7D.

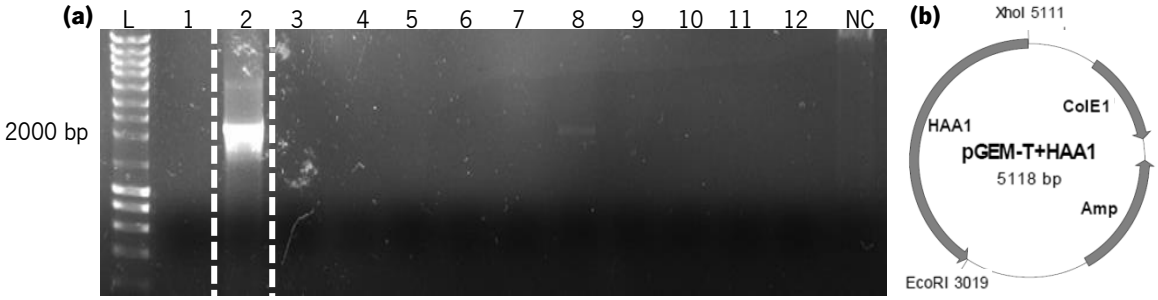

Figure 0.13. Confirmation of insertion of *HAA1* genes into pGEM-T Easy Vector. (a) Colony PCR of 12 white colonies using ScHAA1 primers (Table 2.3) with an expected amplicon of 2103 bp. (b) Representation of the pGEM-T Easy vector with *HAA1*.

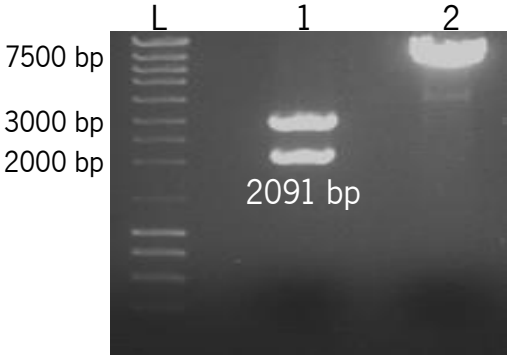

Figure 0.14. Restriction enzyme digestion of pGEM-T+HAA1 (1) and pMI516MCS (2) with EcoRI e XhoI.

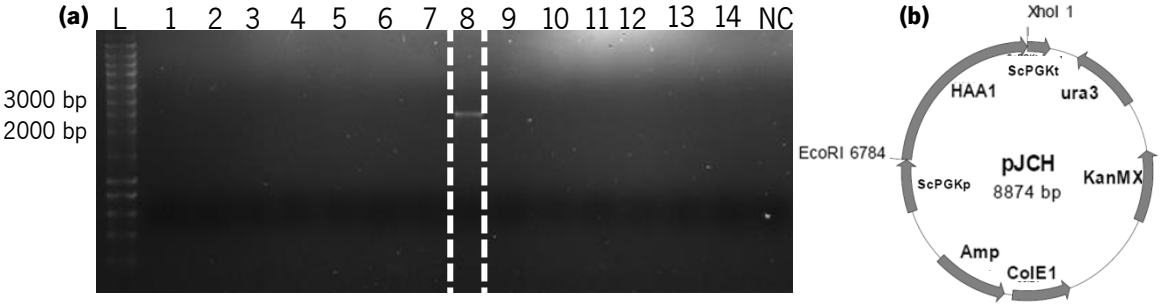

Figure 0.15. Confirmation of correct insertion of *HAA1* into pMI516MCS vector. (a) Colony PCR of 14 colonies using ScHAA1_rv and PGK_fw primers (Table 2.3) with an expected amplicon of 2294 bp. (b) Representation of the pJCH vector.

pJCZ, pJCP, pJCR and pJCH were used to transform *S. cerevisiae* BY4741 (2.17) (Figure 3.16 and 17). As negative control, *S. cerevisiae* BY4741 was also transformed (2.17) with the empty vector pMI516MCS (Figure 3.18). The transformations efficiencies varied from 25 to 100 colonies per µg of DNA. The selection of transformants was made on YPD agar with 200 µg/ml of geneticin (G418). In a previous test, where concentrations between 100 and 300 µg/ml of G418 were used, 200 µg/ml was the lowest concentration of antibiotic capable of hampering the *S. cerevisiae* BY4741 growth on YPD agar.

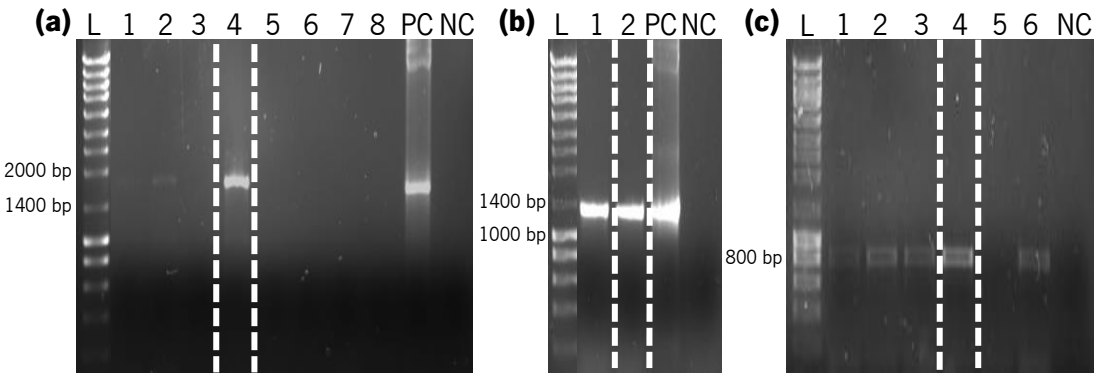

Figure 0.16. Confirmation of transformation of *S. cerevisiae* with pJCZ, pJCP and pJCR. (a) Colony PCR of 8 colonies using ScZWF1_fw and PGK_rv primers (Table 2.3), with an expected amplicon of 1758 bp. (b) Colony PCR of 2 colonies using ScPRS3_fw and PGK_rv primers (Table 2.3), with an expected amplicon of 1150 bp. (c) Colony PCR of 6 colonies using ScRPB4_rv and PGK_fw primers (Table 2.3), with an expected amplicon of 878 bp.

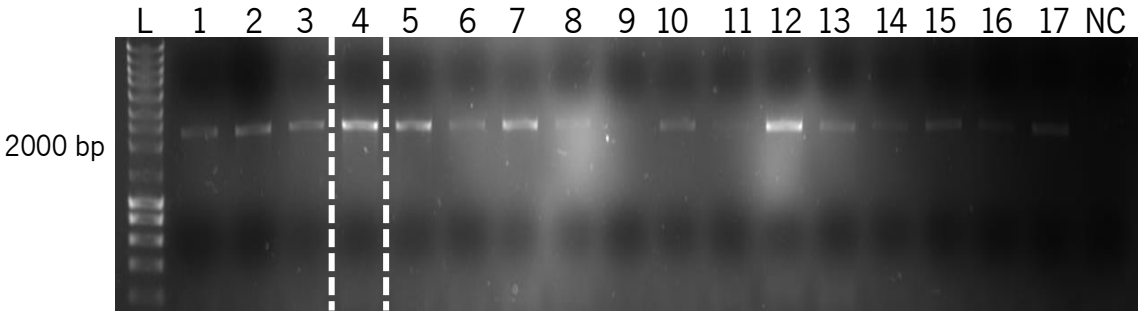

Figure 0.17. Confirmation of transformation of *S. cerevisiae* with pJCH. Colony PCR of 17 colonies using ScHAA1_rv and PGK_fw primers (Table 2.3) with an expected amplicon of 2294 bp.

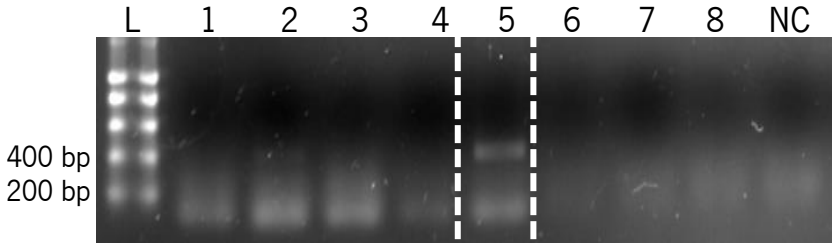

Figure 0.18. Confirmation of transformation of *S. cerevisiae* with pMI516MCS. Colony PCR of 8 colonies using PGK_fw and PGK_rv primers (Table 2.3) with an expected amplicon of 419 bp.

3.2.1. Growth characterization of the overexpressing *S. cerevisiae* strains

S. cerevisiae overexpressing transformants were characterized on their ability to grow aerobically in YPD medium and on YPD supplemented with inhibitors: furfural, HMF and acetic acid (2.18). The similar growth profile of all the transformants in standard YPD (Figure 3.19) showed that the overexpression of *ZWF1*, *PRS3* and *RPB4* had no effect on the yeast growth in the absence of stress.

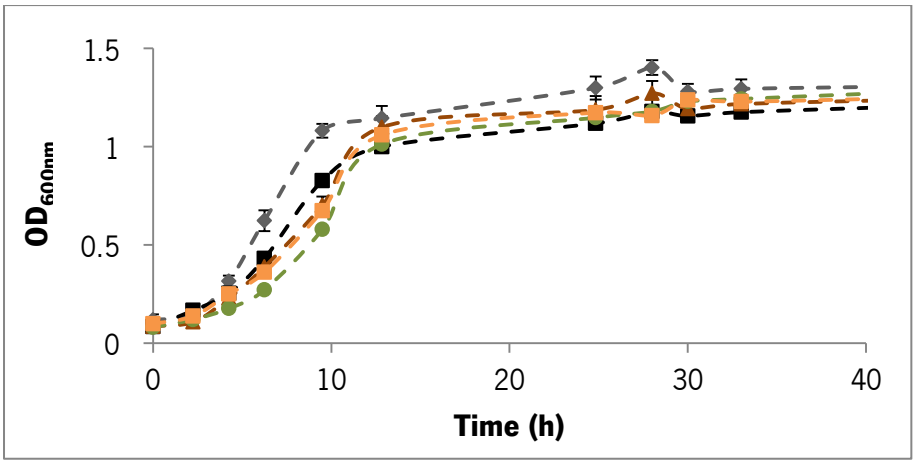

Figure 0.19. Aerobic growth of *S. cerevisiae* BY4741 (♦) (in YPD medium) and of the transformants pMI516MCS (■), pJCZ (▲), pJCP (●) and pJCR (■) in YPD with G418. Data represents average ± standard deviation from three biological replicates.

When grown in the presence of concentrations of inhibitors similar to those present in EWH (Figure 3.20), all the overexpressing transformants showed a slower growth and a substantially longer lag phase than the control *S. cerevisiae* pMI516MCS, suggesting that the overexpression of the genes *ZWF1*, *PRS3* and *RPB4* have a negative effect on the yeast aerobic growth under stress.

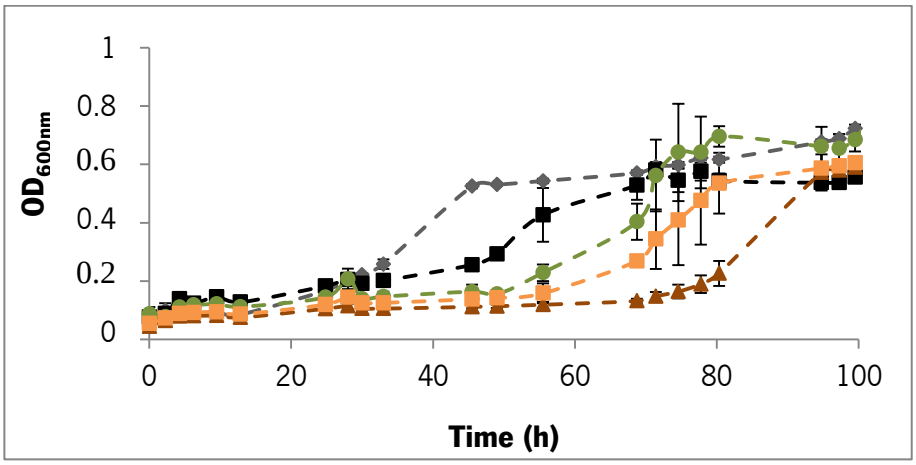

Figure 0.20. Aerobic growth of *S. cerevisiae* BY4741 (♦) (in YPD with 3 g/L of acetic acid, 0.3 g/L of HMF and 1.6 g/L of furfural) and of the transformants: pMI516MCS (■), pJCZ (▲), pJCP (●) and pJCR (■) in YPD medium with G418 and supplemented with the same quantities of the referred inhibitors. Data represents average ± standard deviation from three biological replicates.

The growth was also tested in YPD medium supplemented with higher concentrations of HMF and furfural (1.89 and 2.88 g/L, respectively). In this condition none of the overexpressing transformants were able to exit the lag-phase into exponential growth, despite the fact that both *S. cerevisiae* BY4741 WT and the control *S. cerevisiae* pMI516MCS have successfully reached stationary phase (Figure 3.21). These results are discordant with the already reported effect of *ZWF1* overexpression (under the control of the inducible *MET25* promoter using the pRS425),

which has been described to result in a growth advantage in the presence of concentrations of furfural in a range of 2.88 to 4.80 g/l [44]. This may indicate that the overexpression strategy chosen for this work could be inadequate.

Furthermore, it was observed that the *S. cerevisiae* BY4741 WT has a smaller lag-phase than the control *S. cerevisiae* pMI516MCS. This may be explained by the presence of the G418 antibiotic, which even in the presence of the resistance cassette KanMX, may result in a slower growth. Another possible explanation is the presence of the pMI516MCS vector, as it has been found that the filamentous fungus *Ashbya gossypii TEF-1α* promoter, used in a range of popular marker cassettes [95, 96] (including the KanMX present in this vector), is toxic to *S. cerevisiae* [97]. This indicates that the pMI516MCS vector may be inadequate to this overexpression study.

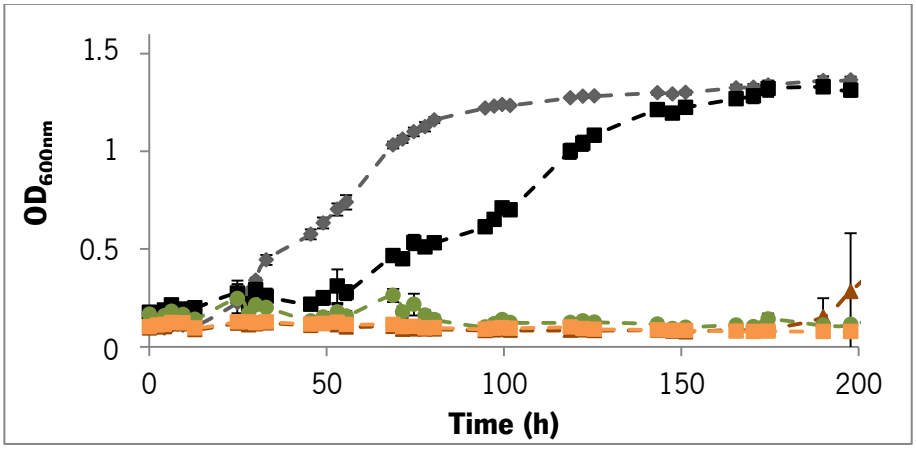

Figure 0.21. Aerobic growth of *S. cerevisiae* BY4741 (♦) (in YPD with 1.89 g/L of HMF and 2.88 g/L of furfural) and of the transformants: pMI516MCS (■), pJCZ (▲), pJCP (●) and pJCR (■) in YPD medium with G418 and supplemented with the same quantities of the referred inhibitors. Data represents average ± standard deviation from three biological replicates.

3.2.2. Effect of *ZWF1*, *PRS3*, *RPB4* and *HAA1* overexpression in lignocellulosic-based fermentations

The role of *ZWF1*, *PRS3*, *RPB4* and *HAA1* overexpression was examined in EWH (70%) shake-flask fermentations (2.19). Under the oxygen-limiting conditions used in these fermentations the profile of CO_2 production obtained provides a suitable assessment of how the fermentation proceeded. Furthermore, the productivity values and ethanol conversion yield were also calculated. The similarity of the fermentation profiles of the transformant strains to that of the control, *S. cerevisiae* pMI516MCS (Figure 3.22) indicates that the overexpression of these genes has no effect in the fermentation process of inhibitory hydrolysates. This fact is

corroborated by the absence of significant differences in the productivity and ethanol yield values obtained (Table 3.1).

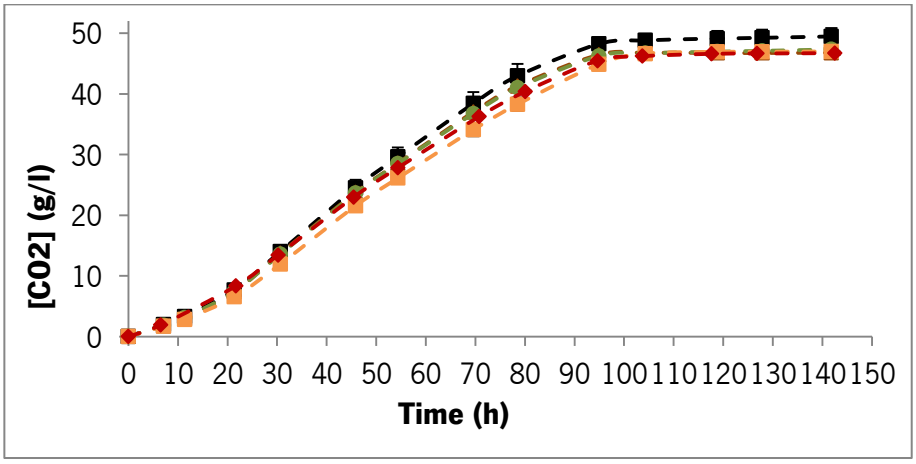

Figure 0.22. Profile of CO_2 production of *S. cerevisiae* BY4741 transformants: pMI516MCS (■), pJCZ (▲), pJCP (●), pJCR (■) and pJCH (♦) in EWH (70%) fermentations. Data represents average ± standard deviation obtained from two biological replicates.

Table 0.1. Effect of the overexpression of *ZWF1*, *PRS3* and *RPB4* genes in EWH fermentations. Data represents average ± standard deviation of two independent experiments and the absence of statistical significance of the results was determined by one-way ANOVA.

***S. cerevisiae* strain**	pMI516MCS	pJCZ	pJCP	pJCR	pJCH
Productivity (g/(l.h))	0.295 ±0.007	0.304 ±0.010	0.292 ±0.005	0.294 ±0.027	0.294 ±0.009
Ethanol yield (%)	41.2 ±1.02	42.5 ±1.41	40.8 ±0.71	41.1 ±3.71	41.1 ±1.20

These results, principally the absence of a positive effect when overexpressing the *HAA1* gene, show that the *ScPGK1* promoter may be inadequate to test overexpression effects on this yeast and indicates that the results obtained may not represent the real role of *ZWF1*, *PRS3* and *RPB4* overexpression in the adaptation to inhibitory lignocellulosic fermentations.

3.3. Study of the effect of *HAA1* overexpression in lignocellulosic-based fermentations

Taking the previous results (3.2.2) into account, the effect of the *HAA1* overexpression using a different vector and native promoters was evaluated in lignocellulosic-based fermentation. For that, *S. cerevisiae* BHUM3731 (YEplac195 vector containing *HAA1* gene under the control of its native promotor) and the *S. cerevisiae* YEplac195 (empty vector control) [90] were tested in

EWH (70%) shake-flask fermentations (2.19). Moreover, the deletion mutant *Δhaa1* (strain lacking the *HAA1* gene) was also tested and its slow fermentation profile (Figure 3.23) and low ethanol productivity (Table 3.2) is consistent with the described essential role of *HAA1* in *S. cerevisiae* response to the presence of acetic acid [1, 2].

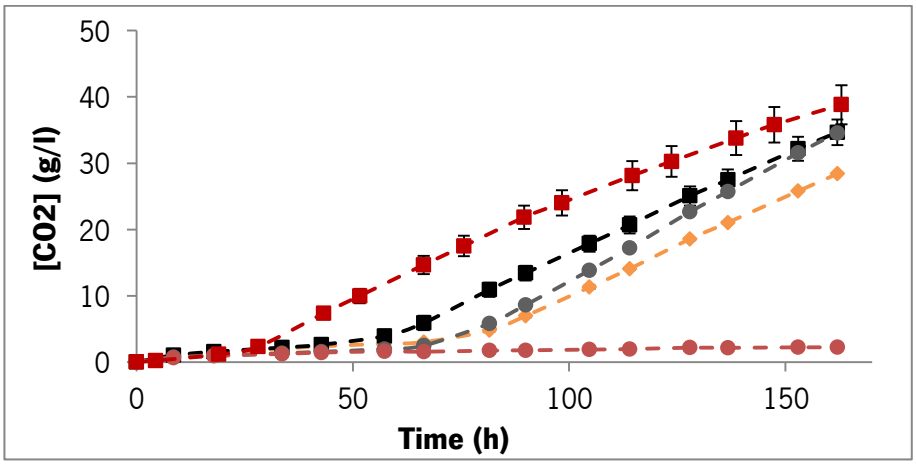

Figure 0.23. Profile of CO_2 production of the deletion mutant *S. cerevisiae* BY4741 *Δhaa1* (♦) and of *S. cerevisiae* BY4741 transformants: YEplac195 (■), BHUM1737 (■), pMI516MCS (●) and pJCH (●) in EWH (70%) fermentations. Data represents average ± standard deviation obtained from two biological replicates.

Table 0.2. Effect of the *HAA1* gene in EWH fermentation. Data represents average ± standard deviation of two biological replicates and the absence of statistical significance of the results was determined by one-way ANOVA.

***S. cerevisiae* strain**	*Δhaa1*	YEplac195	BHUM1737	pMI516MCS	pJCH
Productivity (g/(l.h))	0.177 ±0.000	0.204 ±0.022	0.227 ±0.013	0.163	0.016 ±0.002
Ethanol yield (%)	42.6 ±0.30	44.7 ±3.73	46.3 ±3.71	35.2	32.9 ±17.17

3.3.1. Effect of *HAA1* overexpression under the regulation of its native promotor

The profiles of CO_2 production showed a reduction of almost 50% in the lag phase of the BHUM3731 strain when compared to the control (Figure 3.23), indicating an improved adaptation of the strain overexpressing *HAA1* to the inhibitory compounds present in EWH. Furthermore, the overexpression of this gene seems to result in a slightly higher ethanol productivity and yield (Table 3.2). This result is concordant with the up-regulated expression of *HAA1* at the lag-phase in the presence of inhibitors (Figure 3.3a). Furthermore, at the exponential phase, there seems to be no increased rate of ethanol production by the overexpressing strain

(Figure 3.23). This may be consistent with the down-regulated expression of the *HAA1* gene at this phase (Figure 3.3b), indicative of this gene principal role in the adaptation phase.

3.3.2. Effect of *HAA1* overexpression under the regulation of the *ScPGK1* promoter

The effect of the overexpression of the *HAA1* gene under the control of the *ScPGK1* promoter was evaluated in EWH (70%) fermentations. Taking advantage of the two selection markers of the pMI516MCS vector, this effect was tested with the addition of the antibiotic G418 (Figure 3.22) or in the absence of uracil (Figure 3.23). On both fermentations, the overexpression of the *HAA1* gene under the control of the *ScPGK1* promoter had no positive effect on the fermentation profile when compared to the control strain. Moreover on the fermentation using the URA3 selection marker the pJCH strain was unable to exit the lag phase. A difference in the duration of the lag phase was visible when using different selection markers, probably due to the different pre-inoculum media used: when using the URA3 selection marker, SD-Ura was used in the pre-inoculum, while when using G418 antibiotic the medium used was YPD (supplemented with G418), a rich media, possibly resulting in an inoculum with cells capable of a faster adaptation. The ethanol productivity and yield values support the CO_2 profile results, mainly in the fermentation where the *URA3* marker was used, as a considerably lower productivity was obtained with the overexpressing strain in this condition when compared to the control strain (Table 3.2).

Several studies show that biological systems have evolved to be robust against fluctuations in intracellular biochemical parameters, such as gene expression and protein activities levels [98-101]. However, when this fluctuations surpasses the robustness of the biological systems, e.g. the overexpression of a gene beyond a permissible limit, it results in defects in cellular functions [102]. Recently, it has been revealed that the *S. cerevisiae* cellular system was robust against overexpression of most genes, but sensible to small variations in a specific set of genes [103]. *HAA1* is included in this small group, which may explain the absence of an improved tolerance in *S. cerevisiae* pJCH, where this gene is under control of the strong *ScPGK1* promoter. These results also sustain the possibility that the effects of *ZWF1*, *PRS3* and *RPB4* overexpression observed using the pMI516MCS vector (3.2.2) may not be representative of the real effect that these genes overexpression may have in lignocellulosic fermentations.

3.4. Study of the effect of *ZWF1*, *PRS3* and *RPB4* overexpression under the regulation of the native promoters

3.4.1. Construction of recombinant *S. cerevisiae* BY4741 overexpressing *ZWF1*, *PRS3* and *RPB4* genes under the regulation of the native promoters

Taking into account the previous results, it has been hypothesized that the overexpression of *ZWF1*, *PRS3* and *RPB4* under the control of their native promoters could have a different outcome regarding the tolerance to the presence of inhibitory compounds. Therefore, the genes of interest carrying their native regulatory regions were cloned into the multi-copy vector YEplac195, as this was the vector previously used to overexpress *HAA1* [90] (3.3.1). By using this vector, the possible negative impact of the presence of the *KanMX* cassette was also excluded, as it only carries the *URA3* selection marker.

The *ZWF1*, *PRS3* and *RPB4* genes with corresponding native promoter and terminator sequences were amplified (2.11.1) from genomic DNA of *S. cerevisiae* CEN.PK113-7D (Figure 3.24) with the primers pairs ScZWF1_nat, ScPRS3_nat and ScRPB4_nat (Table 3.3) and the PCR products ligated to pGEM-T Easy vector (Figure 3.25). To construct YEpJCZ, YEpJCP and YEpJCR (containing the *ZWF1*, *PRS3* and *RPB4* genes under the control of their own promoters, respectively) the corresponding pGEM-T Easy constructs were digested with SacI/SphI, for *ZWF1* and *RPB4*, or EcoRI, for *PRS3* (Figure 3.26), and the resulting fragments cloned into the corresponding digested YEplac195 vector (Figure 3.27). YEpJCZ, YEpJCP and YEpJCR were used to transform *S. cerevisiae* BY4741 (2.17)(Figure 3.28), with efficiencies of over 1000 colonies per µg of DNA. The selection of transformants was made in SD-Ura medium plates.

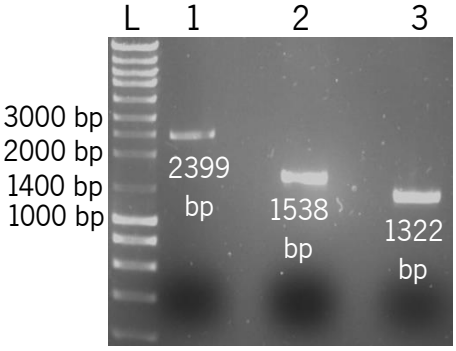

Figure 0.24. PCR amplifications of the genes of interest from *S. cerevisiae* CEN.PK113-7D with native promotor and terminator sequences. Lane 1: *ZWF1* PCR product; lane 2: *PRS3* PCR product; lane 3: *RPB4* PCR product.

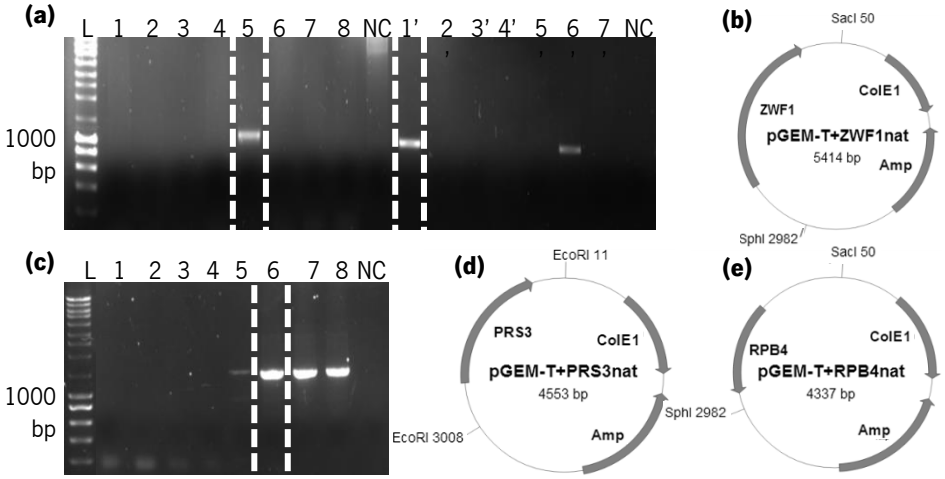

Figure 0.25. Confirmation of insertion of *ZWF1*, *PRS3* and *RPB4* with native regulatory regions into pGEM-T Easy Vector. Representation of pGEM-T Easy with *ZWF1* (b), *PRS3* (d) and *RPB4* (e). (a) Colony PCR of 8 colonies using ScZWF1_V1 and ScZWF1_rv primers (Table 2.3) and an amplicon of 1081 bp was expected; and of 7 colonies using ScPRS3 primers (Table 2.3) and an amplicon of 975 bp was expected. (c) Colony PCR of 8 colonies using ScRPB4_nat primers (Table 2.3) and an amplicon of 1322 bp was expected.

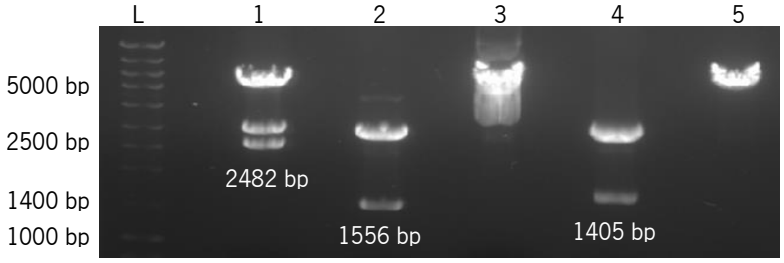

Figure 0.26. Restriction enzyme digestion of the different constructs in pGEM-T Easy Vector and of YEplac195 vector. pGEM-T+ZWF1nat (1), pGEM-T+RPB4nat (2) and YEplac195 (3) were digested with SacI and SphI. pGEM-T+PRS3nat (4) and YEplac195 (5) were digested with EcoRI.

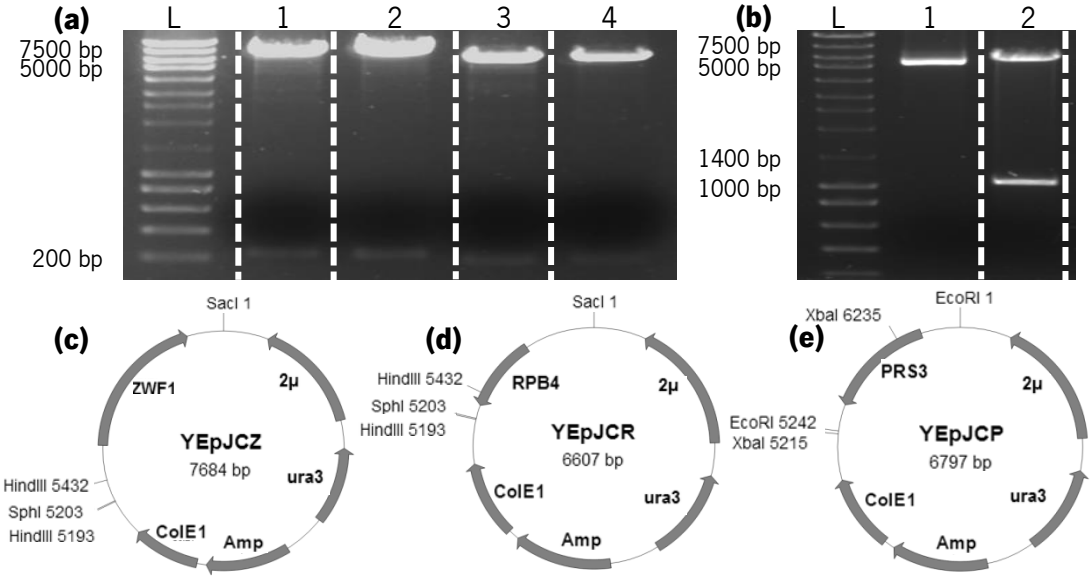

Figure 0.27. Confirmation of insertion of *ZWF1*, *PRS3* and *RPB4* with native regulatory regions into YEplac195 vector. (a) Resulting pattern of digestion of YEpJCZ (1 and 2) and YEpJCR (3 and 4) with HindIII. (b) Resulting pattern of digestion of YEpJCP with XbaI.

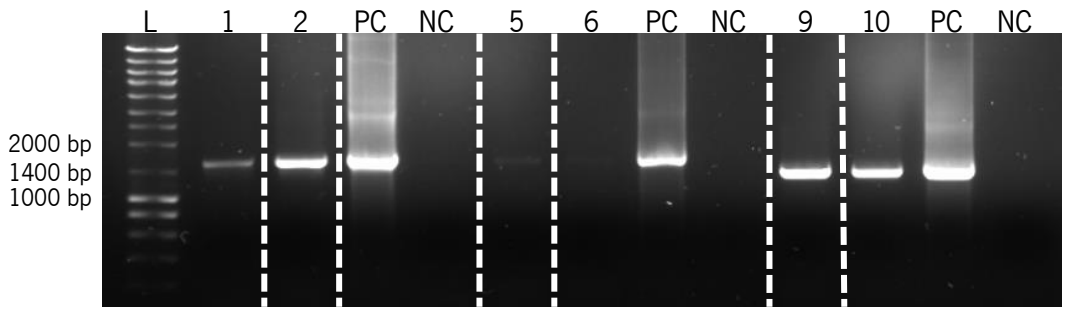

Figure 0.28. Confirmation of transformation of *S. cerevisiae* with YEpJCZ, YEpJCP and YEpJCR. Colony PCR of 2 colonies using ScZWF1 primers (Table 2.3; lanes 1-4) and an amplicon of 1530 bp was expected.. Colony PCR of 2 colonies using ScPRS3_nat primers (Table 2.3; lanes 5-8) and an amplicon of 1538 bp was expected.. Colony PCR of 2 colonies using ScRPB4_nat primers (Table 2.3; lanes 9-12) and an amplicon of 1322 bp was expected.

3.4.2. Effect of *ZWF1*, *PRS3* and *RPB4* overexpression under the regulation of their native promoters in lignocellulosic-based fermentations

The role of *ZWF1*, *PRS3* and *RPB4* overexpression, under the regulation of their native promoters was examined in EWH (60%) shake-flask fermentations (2.19). BHUM1737 strain was used as control for this test, as its positive effect in the reduction of the lag-phase in fermentations in this hydrolysate was previously observed (3.3.1). YEpJCZ, YEpJCP and YEpJCR strains showed a slower production of CO_2 than that of the control YEplac195 strain (Figure 3.29). However, in the initial 40 hours of fermentation, YEpJCP strain showed a slightly higher CO_2 production than the control strain.

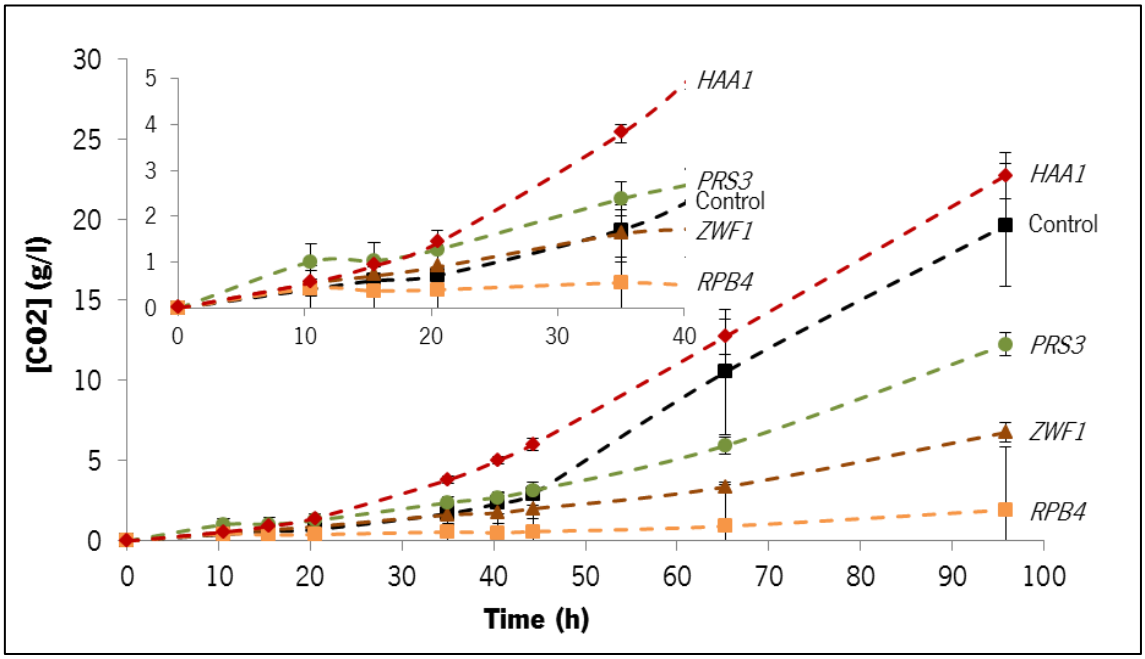

Figure 0.29. Profile of CO_2 production of *S. cerevisiae* BY4741 transformants: YEplac195 (■), YEpJCZ (▲), pJCP (●), pJCR (■) and BHUM1737 (♦) in EWH (60%) fermentations. The smaller graphic focus on the initial 40 hours of the fermentation. Data represents average ± standard deviation obtained from two biological replicates.

Taking into account the results from the expression analysis of *PRS3* under inhibitory conditions (3.1.2), the effect of its overexpression would be expected to be more evident precisely in this phase of the fermentation. Moreover, the effect of the *HAA1* overexpression in the BHUM1737 strain using 60% of EWH (Figure 3.29) was considerably smaller than that observed on 70% of EWH (Figure 3.23), which is closer to the concentration used in the gene expression analysis. This indicates that, in a fermentation with a higher inhibitor stress (e.g. 70% EWH), the positive effect of *PRS3* overexpression in the initial 40 hours of fermentation may be potentiated.

These results indicate *PRS3* and *HAA1*, as genes whose overexpression, under the control of their native promoter using the YEplac195 vector, is capable of improving *S. cerevisiae* adaptation to lignocellulosic hydrolysates. However, a drawback of the use of YEplac195 vector is the absence of an antibiotic resistance gene, which hampers the possibility of testing these overexpressing constructs (using this vector) in industrial robust yeasts, as they are not auxotrophic strains. Therefore, it is necessary to design a new strategy for construction of overexpression strains using a more suitable vector or by genome integration.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

The number of genes identified through genome-wide screenings, whose genetic manipulation is promising in the context of bioethanol process optimization was narrowed down, and its practical importance for maximal performance in lignocellulosic biomass industrial fermentations was confirmed [58]. *PRS3* and *RPB4* genes were selected from this set of genes as the most promising for genetic engineering approaches for yeast improvements in terms of tolerance and response to the multiple stresses occurring during bioethanol fermentations under industrially relevant conditions. Furthermore, the *HAA1* gene was also studied as it had already been described to be essential for the *S. cerevisiae* response to the presence of weak acids, especially acetic acid (one of the major inhibitors in lignocellulosic hydrolysates) [1, 2, 57].

In this study *HAA1*, *PRS3* and *RPB4* expression in *S. cerevisiae* PE-2 was found to be increased at the late lag phase of fermentations in inhibitory medium containing 3 g/l acetic acid, 0.3 g/l HMF and 1.66 g/l furfural. This result clearly relates *HAA1*, *PRS3* and *RPB4* expression to adaptation to inhibitors (acetic acid, HMF and furfural). Moreover *HAA1* and *PRS3* expression was up-regulated at the initial stationary phase, indicating these genes to play a role in yeast tolerance to increasing concentrations of ethanol. As far as we know, it was the first time that genes' expression was compared in different phases of fermentation in inhibitory medium.

In a first approach these genes were overexpressed under the control of the strong *ScPGK1* promoter, using the pMI516MCS vector, which resulted in no positive effect in the adaptation to the inhibitors stress present in lignocellulosic hydrolysate, EWH. When overexpressed using the YEplac195 vector and under the control of their native promoters, the *PRS3* seemed to have a slight positive effect in the adaptation to the lignocellulosic liquor. Furthermore, the *HAA1* overexpression clearly reduced the lag-phase in EWH fermentations. To the extent of our knowledge, for the first time, the *HAA1* overexpression was described to result in a faster adaptation to the inhibitors stress present in lignocellulosic hydrolysates.

In conclusion, we have shown that *HAA1*, *PRS3* and *RPB4* genes play a role in adaptation to inhibitory biomass hydrolysates and that overexpression of *HAA1* and *PRS3* improved *S. cerevisiae* adaptation to the lignocellulosic EWH, accomplishing the aims of this thesis. Furthermore, these results contribute to the progress of development more robust industrial yeast strains, capable of coping with the most significant fermentation stresses and, consequently, to increase ethanol productivity from lignocellulosic biomass.

As mentioned above, when overexpressing *PRS3* (under the control of its native promoter), a slight positive effect was observed in fermentations in medium containing only 60%

of EWH, being necessary further tests (in medium containing 70% of EWH) to evaluate if this small effect can be potentiated by more harsh conditions. Considering that the overexpression effect may vary according with the inhibitory load present in the medium it will be interesting to test the overexpression transformants constructed in fermentation in other lignocellulosic hydrolysates, with different composition and inhibitory ratios. Taking into account the results obtained, *HAA1* and *PRS3* are good candidate genes for further yeast engineering, and the possibility of their simultaneous overexpression should be explored. The use of genetic engineering approaches to increase the expression of the selected genes in industrial strains is the next logical step, to find out whether these manipulations may lead to the generation of more robust industrial yeast strains, able to cope with the most significant fermentation stresses and, thus, to increase ethanol production rate and final ethanol titers. Therefore, we now envision the design of a strategy, based on the Cre-loxP system [104] or homologous recombination at δ-sequences [105], to increase these genes expression by integration in the genome of a robust industrial *S. cerevisiae* strain, such as the PE-2 strain, resulting in more stable overexpressing transformants free of exogenous selection markers. Furthermore, to prevent a toxic effect from excess of overexpression [103] the number of copy genes to integrate should be optimized. After achievement of a stable successively-transformed industrial *S. cerevisiae* a scale-up process will be necessary, to test the strain fermentation performance in a pilot-scale fermenter, in conditions more similar to the ones present in industrial manufacturing plants.

5. REFERENCES

1. Fernandes, A.R., et al., *Saccharomyces cerevisiae adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes.* Biochem Biophys Res Commun, 2005. **337**(1): p. 95-103.
2. Mira, N.P., et al., *Identification of a DNA-binding site for the transcription factor Haa1, required for Saccharomyces cerevisiae response to acetic acid stress.* Nucleic Acids Res, 2011. **39**(16): p. 6896-907.
3. Graziani, M. and Fornasiero, P., *Renewable resources and renewable energy : a global challenge*2007, Boca Raton: CRC Press. 368 p.
4. Zinoviev, S., et al., *Next-generation biofuels: Survey of emerging technologies and sustainability issues.* ChemSusChem, 2010. **3**(10): p. 1106-33.
5. Faaij, A., *Modern Biomass Conversion Technologies.* Mitigation and Adaptation Strategies for Global Change, 2006. **11**(2): p. 335-367.
6. Mussatto, S.I., et al., *Technological trends, global market, and challenges of bio-ethanol production.* Biotechnol Adv, 2010. **28**(6): p. 817-30.
7. Krylova, A., Kozyukov, E., and Lapidus, A., *Ethanol and diesel fuel from plant raw materials: A review.* Solid Fuel Chemistry, 2008. **42**(6): p. 358-364.
8. Lashinsky, A.S., N. D., *How to Beat the High Cost of Gasoline. Forever!*, in *Fortune*2006.
9. *Accelerating Industry: 2012 Ethanol Industry Outlook*, 2012, Renewable Fuels Association.
10. *World Fuel Ethanol Production*. 20/10/2013]; Available from: http://ethanolrfa.org/pages/World-Fuel-Ethanol-Production.
11. Farrell, A.E., et al., *Ethanol can contribute to energy and environmental goals.* Science, 2006. **311**(5760): p. 506-508.
12. Weber, C., et al., *Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels.* Appl Microbiol Biotechnol, 2010. **87**(4): p. 1303-15.
13. Hamelinck, C.N. and Faaij, A.P.C., *Outlook for advanced biofuels.* Energy Policy, 2006. **34**(17): p. 3268-3283.
14. Larson, E.D., *Biofuel production technologies: Status, Prospects and Implications for Trade and Development*, 2008, UNCTAD: Geneva.
15. Hahn-Hagerdal, B., et al., *Bio-ethanol–the fuel of tomorrow from the residues of today.* Trends Biotechnol, 2006. **24**(12): p. 549-56.
16. Wyman, C.E., *Potential synergies and challenges in refining cellulosic biomass to fuels, chemicals, and power.* Biotechnol Prog, 2003. **19**(2): p. 254-62.
17. Palmqvist, E. and Hahn-Hagerdal, B., *Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition.* Bioresour Technol, 2000. **74**(1): p. 25-33.
18. Almeida, J.R.M., et al., *Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae.* Journal of Chemical Technology & Biotechnology, 2007. **82**(4): p. 340-349.
19. Demirbas, A., *Bioethanol from Cellulosic Materials: A Renewable Motor Fuel from Biomass.* Energy Sources, 2005. **27**(4): p. 327-337.
20. Sanchez, O.J. and Cardona, C.A., *Trends in biotechnological production of fuel ethanol from different feedstocks.* Bioresour Technol, 2008. **99**(13): p. 5270-95.
21. Gauss W.F., S.S., Takagi M. , *Manufacture of alcohol from cellulosic materials using plural ferments*, B.R.C.C. Limited, Editor 1976: USA.
22. Olofsson, K., Bertilsson, M., and Liden, G., *A short review on SSF - an interesting process option for ethanol production from lignocellulosic feedstocks.* Biotechnol Biofuels, 2008. **1**(1): p. 7.

23. Alkasrawi, M., et al., *The effect of Tween-20 on simultaneous saccharification and fermentation of softwood to ethanol.* Enzyme and Microbial Technology, 2003. **33**(1): p. 71-78.
24. Xu, F., et al., *Partition of enzymes between the solvent and insoluble substrate during the hydrolysis of lignocellulose by cellulases.* Journal of Molecular Catalysis B: Enzymatic, 2008. **51**(1–2): p. 42-48.
25. Antoni, D., Zverlov, V.V., and Schwarz, W.H., *Biofuels from microbes.* Appl Microbiol Biotechnol, 2007. **77**(1): p. 23-35.
26. Pereira, F.B., et al., *Robust industrial Saccharomyces cerevisiae strains for very high gravity bio-ethanol fermentations.* Journal of Bioscience and Bioengineering, 2011. **112**(2): p. 130-136.
27. Ishtar Snoek, I.S. and Yde Steensma, H., *Factors involved in anaerobic growth of Saccharomyces cerevisiae.* Yeast, 2007. **24**(1): p. 1-10.
28. Bettiga, M., et al., *Arabinose and xylose fermentation by recombinant Saccharomyces cerevisiae expressing a fungal pentose utilization pathway.* Microb Cell Fact, 2009. **8**: p. 40.
29. Karhumaa, K., et al., *Co-utilization of L-arabinose and D-xylose by laboratory and industrial Saccharomyces cerevisiae strains.* Microb Cell Fact, 2006. **5**: p. 18.
30. Sanchez, R.G., et al., *Improved xylose and arabinose utilization by an industrial recombinant Saccharomyces cerevisiae strain using evolutionary engineering.* Biotechnol Biofuels, 2010. **3**.
31. Teixeira, M.C., et al., *Genome-wide identification of Saccharomyces cerevisiae genes required for maximal tolerance to ethanol.* Appl Environ Microbiol, 2009. **75**(18): p. 5761-72.
32. Mira, N.P., et al., *Genome-wide identification of Saccharomyces cerevisiae genes required for tolerance to acetic acid.* Microb Cell Fact, 2010. **9**: p. 79.
33. Endo, A., et al., *Genome-wide screening of the genes required for tolerance to vanillin, which is a potential inhibitor of bioethanol fermentation, in Saccharomyces cerevisiae.* Biotechnol Biofuels, 2008. **1**(1): p. 3.
34. Gorsich, S.W., et al., *Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes ZWF1, GND1, RPE1, and TKL1 in Saccharomyces cerevisiae.* Appl Microbiol Biotechnol, 2006. **71**(3): p. 339-49.
35. Bajwa, P., et al., *Transcriptional profiling of Saccharomyces cerevisiae T2 cells upon exposure to hardwood spent sulphite liquor: comparison to acetic acid, furfural and hydroxymethylfurfural.* Antonie van Leeuwenhoek, 2013. **103**(6): p. 1281-1295.
36. Li, B.Z. and Yuan, Y.J., *Transcriptome shifts in response to furfural and acetic acid in Saccharomyces cerevisiae.* Appl Microbiol Biotechnol, 2010. **86**(6): p. 1915-24.
37. Ma, M. and Liu, Z.L., *Comparative transcriptome profiling analyses during the lag phase uncover YAP1, PDR1, PDR3, RPN4, and HSF1 as key regulatory genes in genomic adaptation to the lignocellulose derived inhibitor HMF for Saccharomyces cerevisiae.* BMC Genomics, 2010. **11**: p. 660.
38. Liu, Z.L., Ma, M., and Song, M., *Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways.* Mol Genet Genomics, 2009. **282**(3): p. 233-44.
39. Ask, M., et al., *The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing Saccharomyces cerevisiae.* Biotechnol Biofuels, 2013. **6**(1): p. 22.
40. Chung, I.S. and Lee, Y.Y., *Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inocula.* Biotechnol Bioeng, 1985. **27**(3): p. 308-15.

41. Olsson, L. and HahnHagerdal, B., *Fermentation of lignocellulosic hydrolysates for ethanol production.* Enzyme and Microbial Technology, 1996. **18**(5): p. 312-331.
42. Taherzadeh, M.J., et al., *Physiological effects of 5-hydroxymethylfurfural on Saccharomyces cerevisiae.* Appl Microbiol Biotechnol, 2000. **53**(6): p. 701-708.
43. Modig, T., Liden, G., and Taherzadeh, M.J., *Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase.* Biochem J, 2002. **363**(Pt 3): p. 769-76.
44. Gorsich, S.W., et al., *Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes ZWF1, GND1, RPE1, and TKL1 in Saccharomyces cerevisiae.* Applied Microbiology and Biotechnology, 2006. **71**(3): p. 339-349.
45. Petersson, A., et al., *A 5-hydroxymethyl furfural reducing enzyme encoded by the Saccharomyces cerevisiae ADH6 gene conveys HMF tolerance.* Yeast, 2006. **23**(6): p. 455-64.
46. Palmqvist, E., Almeida, J.S., and Hahn-Hagerdal, B., *Influence of furfural on anaerobic glycolytic kinetics of Saccharomyces cerevisiae in batch culture.* Biotechnol Bioeng, 1999. **62**(4): p. 447-54.
47. Sasano, Y., et al., *Overexpression of the yeast transcription activator Msn2 confers furfural resistance and increases the initial fermentation rate in ethanol production.* J Biosci Bioeng, 2012. **113**(4): p. 451-5.
48. Park, S.E., et al., *Expression of aldehyde dehydrogenase 6 reduces inhibitory effect of furan derivatives on cell growth and ethanol production in Saccharomyces cerevisiae.* Bioresour Technol, 2011. **102**(10): p. 6033-8.
49. Liu, Z.L., et al., *Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by Saccharomyces cerevisiae.* Appl Microbiol Biotechnol, 2008. **81**(4): p. 743-753.
50. Almeida, J.R.M., et al., *NADH- vs NADPH-coupled reduction of 5-hydroxymethyl furfural (HMF) and its implications on product distribution in Saccharomyces cerevisiae.* Appl Microbiol Biotechnol, 2008. **78**(6): p. 939-945.
51. Liu, Z.L. and Moon, J., *A novel NADPH-dependent aldehyde reductase gene from Saccharomyces cerevisiae NRRL Y-12632 involved in the detoxification of aldehyde inhibitors derived from lignocellulosic biomass conversion.* Gene, 2009. **446**(1): p. 1-10.
52. Moon, J. and Liu, Z.L., *Engineered NADH-dependent GRE2 from Saccharomyces cerevisiae by directed enzyme evolution enhances HMF reduction using additional cofactor NADPH.* Enzyme and Microbial Technology, 2012. **50**(2): p. 115-120.
53. Huang, H., et al., *Identification of crucial yeast inhibitors in bio-ethanol and improvement of fermentation at high pH and high total solids.* Bioresource Technology, 2011. **102**(16): p. 7486-7493.
54. Narendranath, N.V., Thomas, K.C., and Ingledew, W.M., *Effects of acetic acid and lactic acid on the growth of Saccharomyces cerevisiae in a minimal medium.* J Ind Microbiol Biotechnol, 2001. **26**(3): p. 171-7.
55. Mols, M., et al., *Comparative analysis of transcriptional and physiological responses of Bacillus cereus to organic and inorganic acid shocks.* Int J Food Microbiol, 2010. **137**(1): p. 13-21.
56. Almeida, B., et al., *Yeast protein expression profile during acetic acid-induced apoptosis indicates causal involvement of the TOR pathway.* Proteomics, 2009. **9**(3): p. 720-32.
57. Tanaka, K., et al., *Enhancement of acetic acid tolerance in Saccharomyces cerevisiae by overexpression of the HAA1 gene, encoding a transcriptional activator.* Appl Environ Microbiol, 2012. **78**(22): p. 8161-3.

58. Pereira, F.B., et al., *Identification of candidate genes for yeast engineering to improve bioethanol production in very high gravity and lignocellulosic biomass industrial fermentations.* Biotechnol Biofuels, 2011. **4**(1): p. 57.
59. Inoue, T., et al., *Cloning and characterization of a gene complementing the mutation of an ethanol-sensitive mutant of sake yeast.* Biosci Biotechnol Biochem, 2000. **64**(2): p. 229-36.
60. Alexandre, H., Rousseaux, I., and Charpentier, C., *Relationship between Ethanol Tolerance, Lipid-Composition and Plasma-Membrane Fluidity in Saccharomyces-Cerevisiae and Kloeckera-Apiculata.* FEMS Microbiol Lett, 1994. **124**(1): p. 17-22.
61. Swan, T.M. and Watson, K., *Stress tolerance in a yeast sterol auxotroph: role of ergosterol, heat shock proteins and trehalose.* FEMS Microbiol Lett, 1998. **169**(1): p. 191-7.
62. Shobayashi, M., et al., *Effects of culture conditions on ergosterol biosynthesis by Saccharomyces cerevisiae.* Biosci Biotechnol Biochem, 2005. **69**(12): p. 2381-8.
63. Yoshikawa, K., et al., *Comprehensive phenotypic analysis of single-gene deletion and overexpression strains of Saccharomyces cerevisiae.* Yeast, 2011. **28**(5): p. 349-61.
64. Li, B.Z., et al., *Genome-wide transcriptional analysis of Saccharomyces cerevisiae during industrial bioethanol fermentation.* J Ind Microbiol Biotechnol, 2010. **37**(1): p. 43-55.
65. Rossignol, T., et al., *Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation.* Yeast, 2003. **20**(16): p. 1369-85.
66. Zhang, Z., et al., *[Regulation role of sterol C-24 methyltransferase and sterol C-8 isomerase in the ergosterol biosynthesis of Saccharomyces cerevisiae].* Wei Sheng Wu Xue Bao, 2009. **49**(8): p. 1063-8.
67. Veen, M., Stahl, U., and Lang, C., *Combined overexpression of genes of the ergosterol biosynthetic pathway leads to accumulation of sterols in Saccharomyces cerevisiae.* FEMS Yeast Res, 2003. **4**(1): p. 87-95.
68. Wang, K., et al., *Impaired PRPP-synthesizing capacity compromises cell integrity signalling in Saccharomyces cerevisiae.* Microbiology, 2004. **150**(Pt 10): p. 3327-39.
69. Binley, K.M., et al., *The yeast PRS3 gene is required for cell integrity, cell cycle arrest upon nutrient deprivation, ion homeostasis and the proper organization of the actin cytoskeleton.* Yeast, 1999. **15**(14): p. 1459-1469.
70. Hove-Jensen, B., *Heterooligomeric phosphoribosyl diphosphate synthase of Saccharomyces cerevisiae - Combinatorial expression of the five PRS genes in Escherichia coli.* Journal of Biological Chemistry, 2004. **279**(39): p. 40345-40350.
71. Smardon, A.M., Tarsio, M., and Kane, P.M., *The RAVE complex is essential for stable assembly of the yeast V-ATPase.* Journal of Biological Chemistry, 2002. **277**(16): p. 13831-13839.
72. Graham, L.A., Hill, K.J., and Stevens, T.H., *VMA8 encodes a 32-kDa V1 subunit of the Saccharomyces cerevisiae vacuolar H(+)-ATPase required for function and assembly of the enzyme complex.* Journal of Biological Chemistry, 1995. **270**(25): p. 15037-44.
73. Carmelo, V., Santos, H., and SaCorreia, I., *Effect of extracellular acidification on the activity of plasma membrane ATPase and on the cytosolic and vacuolar pH of Saccharomyces cerevisiae.* Biochimica Et Biophysica Acta-Biomembranes, 1997. **1325**(1): p. 63-70.
74. Seol, J.H., Shevchenko, A., and Deshaies, R.J., *Skp1 forms multiple protein complexes, including RAVE, a regulator of V-ATPase assembly.* Nat Cell Biol, 2001. **3**(4): p. 384-91.

75. Yoshikawa, K., et al., *Comprehensive phenotypic analysis for identification of genes affecting growth under ethanol stress in Saccharomyces cerevisiae.* FEMS Yeast Res, 2009. **9**(1): p. 32-44.
76. Brace, E.J., Parkinson, L.P., and Fuller, R.S., *Skp1p regulates Soi3p/Rav1p association with endosomal membranes but is not required for vacuolar ATPase assembly.* Eukaryot Cell, 2006. **5**(12): p. 2104-13.
77. Edwards, A.M., et al., *2 Dissociable Subunits of Yeast Rna Polymerase-Ii Stimulate the Initiation of Transcription at a Promoter Invitro.* Journal of Biological Chemistry, 1991. **266**(1): p. 71-75.
78. Sampath, V. and Sadhale, P., *Rpb4 and Rpb7: a sub-complex integral to multi-subunit RNA polymerases performs a multitude of functions.* IUBMB Life, 2005. **57**(2): p. 93-102.
79. Woychik, N.A. and Young, R.A., *RNA polymerase II subunit RPB4 is essential for high- and low-temperature yeast cell growth.* Mol Cell Biol, 1989. **9**(7): p. 2854-9.
80. Pillai, B., et al., *Whole genome expression profiles of yeast RNA polymerase II core subunit, Rpb4, in stress and nonstress conditions.* J Biol Chem, 2003. **278**(5): p. 3339-46.
81. Choder, M. and Young, R.A., *A portion of RNA polymerase II molecules has a component essential for stress responses and stress survival.* Mol Cell Biol, 1993. **13**(11): p. 6984-91.
82. Bourbonnais, Y., et al., *Multiple cellular processes affected by the absence of the Rpb4 subunit of RNA polymerase II contribute to the deficiency in the stress response of the yeast rpb4 Delta mutant.* Molecular and General Genetics, 2001. **264**(6): p. 763-772.
83. Tan, Q., et al., *Multiple mechanisms of suppression circumvent transcription defects in an RNA polymerase mutant.* Mol Cell Biol, 2000. **20**(21): p. 8124-33.
84. Pillai, B., et al., *Rpb4, a non-essential subunit of core RNA polymerase II of Saccharomyces cerevisiae is important for activated transcription of a subset of genes.* Journal of Biological Chemistry, 2001. **276**(33): p. 30641-7.
85. Rosenheck, S. and Choder, M., *Rpb4, a subunit of RNA polymerase II, enables the enzyme to transcribe at temperature extremes in vitro.* J Bacteriol, 1998. **180**(23): p. 6187-92.
86. Farago, M., et al., *Rpb4p, a subunit of RNA polymerase II, mediates mRNA export during stress.* Mol Biol Cell, 2003. **14**(7): p. 2744-55.
87. Sheffer, A., Varon, M., and Choder, M., *Rpb7 can interact with RNA polymerase II and support transcription during some stresses independently of Rpb4.* Mol Cell Biol, 1999. **19**(4): p. 2672-2680.
88. Aguiar, T., *Understanding the biotechnological potential of Ashbya gossypii*, 2013, University of Minho: Portugal.
89. Gietz, R.D. and Sugino, A., *New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites.* Gene, 1988. **74**(2): p. 527-34.
90. Malcher, M., Schladebeck, S., and Mosch, H.U., *The Yak1 protein kinase lies at the center of a regulatory cascade affecting adhesive growth and stress resistance in Saccharomyces cerevisiae.* Genetics, 2011. **187**(3): p. 717-30.
91. Ruiz, H.A., et al., *Development and characterization of an environmentally friendly process sequence (autohydrolysis and organosolv) for wheat straw delignification.* Appl Biochem Biotechnol, 2011. **164**(5): p. 629-41.

92. Heer, D. and Sauer, U., *Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain.* Microbial Biotechnology, 2008. **1**(6): p. 497-506.
93. Warner, J.R., *The economics of ribosome biosynthesis in yeast.* Trends Biochem Sci, 1999. **24**(11): p. 437-40.
94. Simoes, T., et al., *The SPI1 gene, encoding a glycosylphosphatidylinositol-anchored cell wall protein, plays a prominent role in the development of yeast resistance to lipophilic weak-acid food preservatives.* Appl Environ Microbiol, 2006. **72**(11): p. 7168-75.
95. Wach, A., et al., *New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae.* Yeast, 1994. **10**(13): p. 1793-808.
96. Goldstein, A.L. and McCusker, J.H., *Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae.* Yeast, 1999. **15**(14): p. 1541-53.
97. Babazadeh, R., et al., *The Ashbya gossypii EF-1 α promoter of the ubiquitously used MX cassettes is toxic to Saccharomyces cerevisiae.* FEBS Letters, 2011. **585**(24): p. 3907-3913.
98. Dekel, E. and Alon, U., *Optimality and evolutionary tuning of the expression level of a protein.* Nature, 2005. **436**(7050): p. 588-92.
99. Wagner, A., *Energy constraints on the evolution of gene expression.* Mol Biol Evol, 2005. **22**(6): p. 1365-74.
100. Alon, U., et al., *Robustness in bacterial chemotaxis.* Nature, 1999. **397**(6715): p. 168-71.
101. Little, J.W., Shepley, D.P., and Wert, D.W., *Robustness of a gene regulatory circuit.* EMBO J, 1999. **18**(15): p. 4299-307.
102. Moriya, H., Shimizu-Yoshida, Y., and Kitano, H., *In vivo robustness analysis of cell division cycle genes in Saccharomyces cerevisiae.* PLoS Genet, 2006. **2**(7): p. e111.
103. Makanae, K., et al., *Identification of dosage-sensitive genes in Saccharomyces cerevisiae using the genetic tug-of-war method.* Genome Res, 2013. **23**(2): p. 300-11.
104. Sauer, B., *Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae.* Mol Cell Biol, 1987. **7**(6): p. 2087-96.
105. Oliveira, C., et al., *Development of stable flocculent Saccharomyces cerevisiae strain for continuous Aspergillus niger beta-galactosidase production.* J Biosci Bioeng, 2007. **103**(4): p. 318-24.

APPENDIX 1

Table A1. Composition of solutions and media used for different strains growth.

Media/Solutions	**Composition**
LB medium	1% (w/v) tryptone 0.5% (w/v) yeast Extract 1% (w/v) sodium chloride (NaCl) pH 7.5 (with 10 M NaOH)
YPD medium	2 % (w/v) glucose 2% (w/v) peptone 1 % (w/v) yeast extract
SD medium	2 % (w/v) glucose 0.67% (w/v) nitrogen base w/o aminoacids 3% (v/v) Amino Acid drop out Mix 13.5 mg/l adenine hemisulfate 60.0 mg/l uracil 262 mg/l L-Leucine 119 mg/l L-Threonine 60.0 mg/l L-Histidine pH 5.0 (with 10 MNaOH)
Amino Acid drop out Mix	8.87 g/l L-Aspartic acid 17.5 g/l L-Isoleucine 3.03 g/l L-Lysine 2.77 g/l L-Phenylalanine 3.50 g/l L-Serine 1.00 g/l L-Tyrosine 3.90 g/l L-Valine 11.6 g/l L-Arginine 4.9 g/l L-Methionine 2.73 g/l L-Tryptophan
EWH	0.64 g/l glucose 8.85 g/l xylose 0.18 g/l arabinose 3.11 g/l acetic acid 0.33 g/l HMF 1.66 g/l furfural 1.15 g/l glucooligosaccharides 8.97 g/l xylooligosaccharides 2.55 g/l acetyl groups 2.01 g/l phenolic compounds
BYauxo Mix	2.67 g/l uracil 2.67 g/l histidine 2.67 g/l methionine 8.00 g/l leucine