Universidade do Minho
Escola de Engenharia

Luís Pedro de Matos Melo Ferraz

Genetically modified yeast strains for effective bioethanol production from lignocellulosic residues

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Mestrado em Bioengenharia

Trabalho efetuado sob a orientação de:

Professora Doutora Lucília Domingues

Professor Doutor Björn Johansson

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DECLARAÇÃO

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À minha família porque sem vocês nada disto seria possível.

ABSTRACT

The growing concern over the shortage of oil reserves, together with the need to preserve the environment, resulted in the search of viable alternative renewable sources for production of sustainable fuels such as 2nd generation bioethanol, produced from lignocellulosic biomass. The extraction of fermentable sugars from these lignocellulosic materials results in an undesirable release of inhibitory compounds, such as acetic acid and furfural, that have a negative impact on yeast growth and ethanol fermentation. *Saccharomyces cerevisiae* is the more suitable microorganism for genetic improvement of ethanol production however, *S. cerevisiae* is not able to metabolize xylose for its own growth or ethanol production which means that about 20 to 30% of lignocellulosic hydrolysate is not used. Furthermore, strains isolated from harsh industrial environments are naturally more robust and, depending on their genetic background, may respond differently to the presence of lignocellulosic-derived inhibitors.

HAA1 and *PRS3* genes have been described to improve yeast tolerance to lignocellulosic inhibitors. *HAA1* overexpression have shown positive effects on yeast tolerance towards acetic acid in industrial strains fermentations carried out in glucose media and in laboratory strains fermentations in xylose media. *PRS3* overexpression have been reported to increase industrial strains tolerance in glucose fermentations performed in real hydrolysate media. However, overexpression of *HAA1* and/or *PRS3* genes in industrial yeast strains capable of xylose fermentation in real lignocellulosic hydrolysates have never been attempted. Taking this into account, and using molecular biology tools, this study aimed to simultaneously overexpress *HAA1* and/or *PRS3* genes and insert a D-xylose metabolic pathway in PE-2Δ*GRE3* and CA11, yeast strains with an industrial background; and evaluate their performance in aerobic growth and fermentation assays in the presence of lignocellulosic-derived inhibitors.

The results obtained with these strains in aerobic growth tests and fermentation assays showed the effect of the overexpression of *HAA1* and *PRS3* increased PE-2Δ*GRE3* capacity towards inhibitors in aerobic growth tests. Furthermore, in fermentations performed in xylose medium containing acetic acid and furfural, the overexpression of *HAA1* and *PRS3* in PE-2Δ*GRE3* strains seem to increase yeast fermenting capacity comparing to the control strain. However, CA11 strains overexpressing both genes did not show increased abilities in the presence of lignocellulosic-derived inhibitors. These different performances showed that the overexpression of the same genes in strains with different background can lead to different outcomes. The overall results of this thesis highlight that the genetic engineering of industrial yeast isolates for improved production of 2nd generation bioethanol must be carefully addressed, and must rely in an integrative approach, considering the strain metabolic background, its capacity to consume xylose, and yeast tolerance towards inhibitors in real lignocellulosic hydrolysates.

RESUMO

A crescente preocupação sobre a diminuição das reservas de petróleo e a necessidade de preservar o ambiente, levou à procura de alternativas viáveis para a produção de combustíveis sustentáveis como é o caso do bio etanol de 2º geração, combustível produzido a partir de biomassa lenhocelulósica. A extração de açucares fermentáveis a partir destes resíduos liberta uma série de compostos inibitórios, como ácido acético e furfural, que causam efeitos negativos no crescimento das leveduras e na produção de etanol. A *Saccharomyces cerevisiae* é o microrganismo mais adequado para produção de etanol e para ser geneticamente modificado. Contudo, a *S. cerevisiae* não é capaz de metabolizar a xilose para o seu crescimento nem para a produção de etanol, o que significa que 20 a 30% dos compostos lenhocelulósicos não são usados. Estirpes isoladas de ambientes industriais são naturalmente mais robustas e dependendo do seu background podem responder de maneiras diferentes à presença de inibidores derivados da lenhocelulose.

Já foi descrito que os genes *HAA1* e *PRS3* melhoram a tolerância das leveduras a estes inibidores. A sobreexpressão do *HAA1* já mostrou efeitos positivos na tolerância das leveduras ao ácido acético em estirpes industriais em fermentações de glucose e em estirpes laboratoriais em meio com xilose. A sobreexpressão do *PRS3* já foi associada ao aumento de tolerância de estirpes industriais em hidrolisados reais. Contudo, a sobreexpressão conjunta do *HAA1* e do *PRS3* em estripes industriais capazes de consumir xilose em fermentações com hidrolisados reais nunca foi realizada. Tendo isto em conta, e usando ferramentas de biologia molecular, o objetivo deste trabalho foi sobreexpressar simultaneamente o *HAA1* e *PRS3* e inserir uma via metabólica de consumo de xilose na PE-2Δ*GRE3* e CA11, que são estirpes com um background industrial; e avaliar o seu desempenho em termos de crescimento aeróbio e em fermentações na presença de inibidores.

Os resultados obtidos em ensaios de crescimento aeróbio mostraram que a sobreexpressão do *HAA1* e do *PRS3* aumentou a capacidade das estirpes de PE-2Δ*GRE3* em termos de resistência aos inibidores. Nos ensaios das fermentações realizadas com esta estirpe em meio de xilose contendo ácido acético e furfural mostraram que a sobreexpressão dos dois genes parece aumentar a capacidade fermentativa das estirpes recombinantes em relação à estirpe controlo. Contudo, nos ensaios realizados com a estirpe CA11, a sobreexpressão dos dois genes não aumentou a capacidade fermentativa da levedura na presença de inibidores. Estes resultados demonstram que a sobreexpressão dos mesmos genes em estirpes diferentes pode levar a resultados diferentes. O resultado global deste trabalho realçou que a modificação genética de leveduras para melhorar a produção de etanol de 2º geração tem de assentar numa abordagem englobando o background de cada estirpe, a sua capacidade de consumir xilose e a sua tolerância a inibidores provenientes de hidrolisados reais.

SCIENTIFIC OUTPUT

Cunha, J, Romaní, A, Ferraz, L, Costa, C, Johansson, B, Domingues, L. Towards a cost-effective bioethanol process: yeast development to overcome challenges derived from lignocellulosic processing. 1st International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability. 2016.

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List of Abbreviations

Amp – Ampicillin

Bp – Base pairs

DNA – deoxyribonucleic acid

dNTP's - Deoxyribonucleotide triphosphates

EDTA - Ethylenediamine tetraacetic acid

EGW – *Eucalyptus globulus* wood

HMF – 5-hydroxymethyl furfural

HPLC – High performance liquid chromatography

LB – Luria-Bertani medium

LiOAc – Lithium acetate

OD – Optical density

PCR – Polymerase chain reaction

PEG - Polyethylene glycol

PRPP - 5-phosphoribosyl-1- pyrophosphate

RNA – ribonucleic acid

Rpm – revolutions per minute

SHF – Separate hydrolysis and fermentation

SOC - Super optimal broth with catabolite repression

SSF – Simultaneous saccharification and fermentation

TAE – Tris-acetate-EDTA

TE – Tris-EDTA

Tris – tris (hydroxymethyl) aminomethane

UP – Ultra pure

XDH – Xylitol dehydrogenase

XKS – Xylulose kinase

XR – Xylose reductase

YPD – Yeast extract peptone dextrose medium

YPX – Yeast extract peptone xylose medium

Aims

Over the last years, climate changes and a possible future shortage of fossil fuels have resulted in a growing attention towards the improvement of lignocellulosic biomass-derived fuel, such as bioethanol. However, the conversion of lignocellulosic materials into fermentable sugars leads to the release of inhibitory compounds, mainly acetic acid and furfural.

S. cerevisiae is the most used microorganism for ethanol production, due to its high fermentation capability and high tolerance to ethanol, acidity and process conditions. Furthermore, isolates from industrial environments are known to have a more robust background and are more capable of withstanding the stress conditions of industrial bioethanol processes. Nonetheless, *S. cerevisiae* is not able to metabolize xylose for its own growth or ethanol production, which means that about 20 to 30% of lignocellulosic hydrolysate is not used. Several studies have reported genetic modifications in *S. cerevisiae* to enable xylose fermentation by the construction of xylose metabolic pathways from fungi or bacteria. Recently, a novel metabolic pathway assembly tool, the Yeast Pathway Kit, has allowed the construction of a plasmid containing the XR/XDH/XK pathway that enabled xylose metabolism comparison between different *S. cerevisiae* strain backgrounds.

HAA1 and *PRS3* genes have been identified as key genes in the tolerance against lignocellulosic-derived inhibitors and their overexpression has been reported to increase yeast tolerance to these inhibitory compounds. Nevertheless, the effect of overexpressing these genes was never studied using an integrated approach combining: (i) metabolic engineering of industrial yeast strains; (ii) consumption of xylose; (iii) tolerance to lignocellulosic-derived inhibitors and (iv) real lignocellulosic hydrolysates.

Taking this into account, and to better understand the importance of *HAA1* and *PRS3* genes, we specifically aimed to:

1. Create *S. cerevisiae* strains capable of D-xylose fermentation and simultaneously overexpressing *HAA1* and/or *PRS3* genes, taking advantage of the robust industrial background of PE-2Δ*GRE3* and CA11 isolates.
2. Evaluate the effect of the overexpression of these genes in terms of xylose consumption and bioethanol production in the presence of lignocellulosic-derived inhibitors.

1. Introduction

1.1. Biofuels

The world is, nowadays, heavily dependent on fossil fuels. The huge consumption of these fuels has led to a diminishing of its sources and even more, to a constant rise of the oil prices. One of the main problems of burning fossil fuels to produce energy is the emission of greenhouse effect gases, especially carbon dioxide, and other contaminants to the atmosphere. These products have been suggested to be contributing to the global warming effects and to changes in the environment and natural element patterns. For those reasons, the increasing demand of energy associated with the dependence on fossil fuels is one of the greatest challenges of the 21st century (Rostagno et al., 2014).

The key challenge for the present world is to discover new renewable energy resources able to replace fossil fuels. However, making economically attractive renewable fuels is a difficult task. Biofuel is any fuel produced from biomass, which consists of biological matter from dead or even living organisms (usually plant-based) (Faaij, 2006). Moreover, biofuels are a promising alternative to fossil fuels because of its ability to reduce greenhouse gases emission, continuous supply of feedstock through the year, ease of cultivation, harvesting and transportation (Adenle et al., 2013; Tye et al., 2011). Biofuels are generated from living organisms and include: biodiesel, bioethanol and biogas (Adam & Shanableh, 2015; Yusuf, 2007). This work will focus on biomass-based ethanol (or bioethanol) production.

1.2. Bioethanol

The world production of bioethanol increased from 50 million m^3 in 2007 to over 100 million m^3 in 2012. Brazil and the United States represent approximately 80% of the world supply (Kang, Appels, Baeyens, Dewil, & Tan, 2014; Kang, Appels, Tan, & Dewil, 2014). Bioethanol feedstocks can be classified into three types: sucrose-containing feedstocks (eg., sugar beet, sweet sorghum and sugarcane), starchy materials (eg., wheat, corn and barley) and lignocellulosic biomass (eg., wood, straw and grasses) (Balat et al., 2008; Bertrand et al., 2016).

1.2.1. First generation Bioethanol

First generation bioethanol is a biofuel produced by the fermentation and distillation of sugar and starch based raw materials (Rostagno et al., 2014). At the beginning of 2016, 25 billion of gallons of bioethanol were being produced worldwide, whereas the first generation bioethanol had a major contribution (Bertrand et al., 2016). The United States is the world's largest producer of bioethanol, producing over 14 billion gallons in 2014 alone. Together, the U.S and Brazil produce 83% of the world's

ethanol, resulting in 21 million m^3 ethanol produced from sugarcane and 60 million m^3 from corn and other grains (Dutta et al., 2014; REN21, 2012). Figure 1.1 shows the world wide ethanol production from 2007 to 2015.

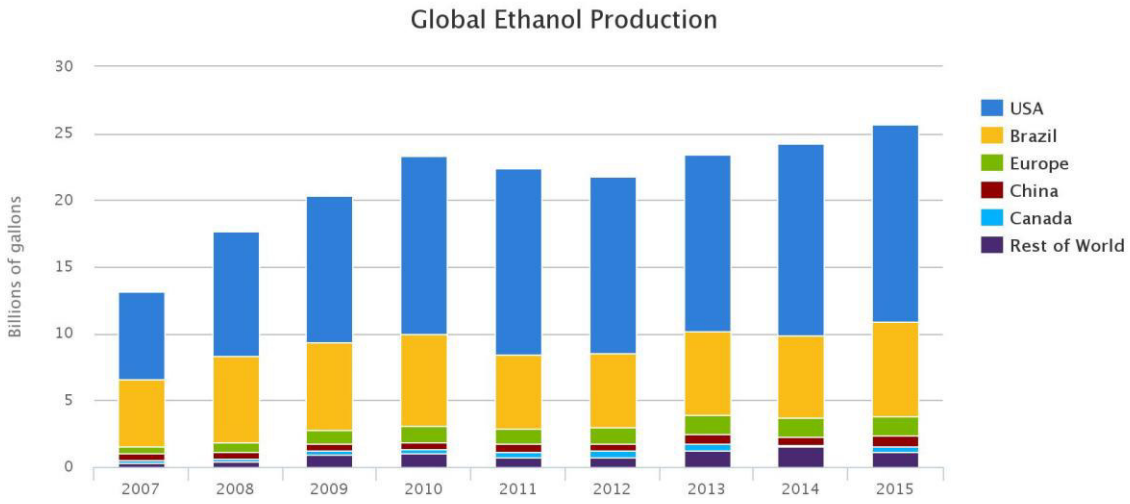

Figure 1.1: Global bioethanol production from 2007 to 2015 (Bertrand et al., 2016).

However, the first generation biofuels create concerns about the environmental impacts which sets limits in the increasing production of biofuels of first generation (Naik et al., 2010). Furthermore, first generation bioethanol requires high feedstocks production, leading to food vs. fuel concerns where one of the reasons for rising food prices is due to the increase in the production of these fuels (Bezerra & Ragauskas, 2016; Laursen, 2006). The concerns regarding the viability of these feedstocks led to the necessity to develop processes that can produce bioethanol from renewable, cheap and abundant sources.

1.2.2. Second generation Bioethanol

Second generation bioethanol could avoid many of these concerns since it relies on nonfood bio resources, such as lignocellulosic biomass. The lignocellulosic biomass is a particularly attractive feedstock because it is the cheapest, most abundant, and fastest growing form of terrestrial biomass (Somma et al., 2010). Indeed, lignocellulosic materials are widely available: forest slashes, crop residues, yard trimmings, food processing waste, and municipal organic refuses can be the feed stock for bioethanol (Guo et al., 2015). Therefore, lignocellulosic materials are a perfect solution to the problem of the competitive use of resources for food and fuel.

Lignocellulosic materials are composed by cellulose, hemicellulose and lignin (Figure 1.2). The cellulose fraction consists mainly of glucose monomers, the hemicellulose fraction is a mixture of hexoses, such as glucose, manose and galactose, and pentose sugars such as xylose and arabinose.

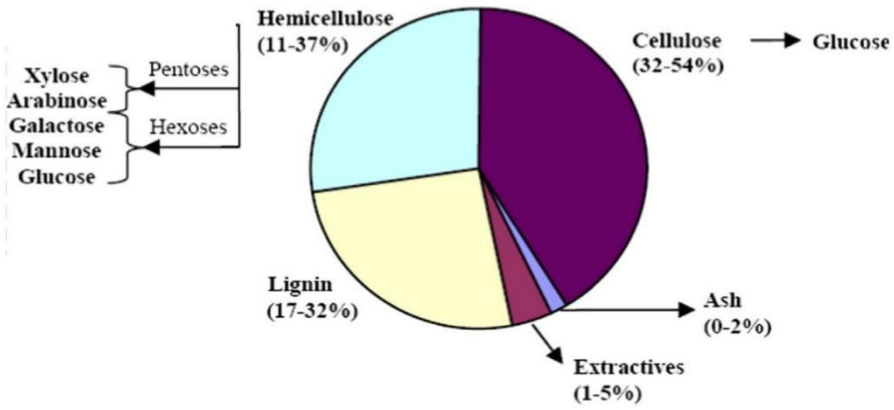

Figure 1.2: Composition of lignocellulosic materials (adapted from (Guo et al., 2015)).

There are various steps necessary for the lignocellulose conversion to ethanol (Figure 1.3). In the first step biomass is pretreated combining physical and chemical reactions and then the sugar monomers are released by enzymatic hydrolysis. The hydrolysis and fermentation steps can be done separately (Separate Hydrolysis and Fermentation, SHF) or through Simultaneous Saccharification and Fermentation (SSF) (Olofsson et al., 2008; Pereira, 2013). In the SHF process, both enzymes and yeast work at their optimal temperature but the efficiency of hydrolysis can be reduced due to end-product inhibition. Regarding to the SSF process, enzymes and yeast work simultaneously at suboptimal temperature conditions, this way, there is no accumulation of end-products avoiding any inhibition (Balat, 2011). Ethanol is then produced using yeast, bacteria or fungi able to ferment different sugar monomers. The final step to obtain pure bioethanol is distillation.

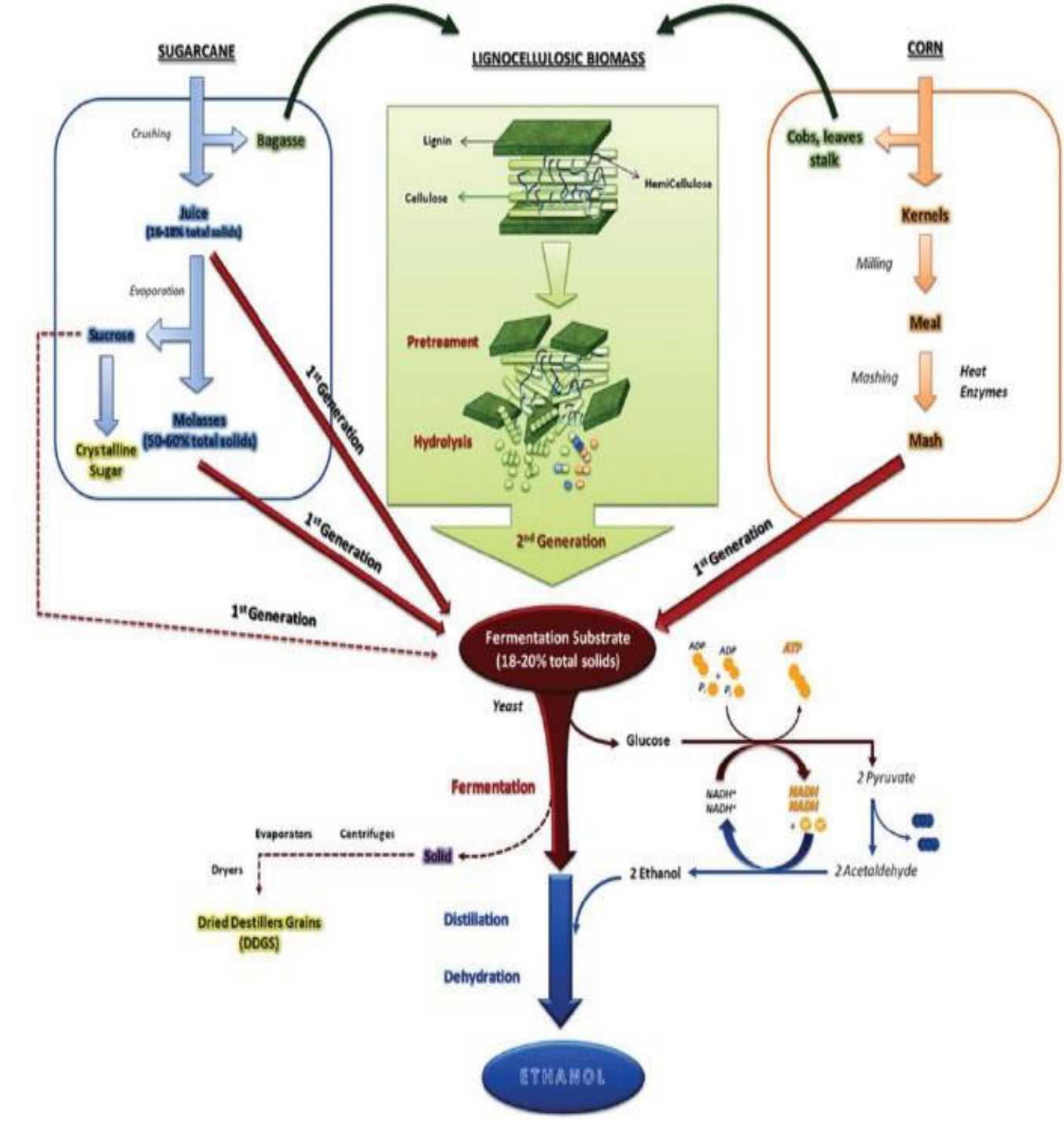

Figure 1.3: Overview of the process for the production of 1st and 2nd generation bioethanol (Rostagno et al., 2014).

The pretreatment is a crucial step to break down the lignin structure and disrupt the crystalline structure of cellulose to increase enzyme accessibility. There are several pretreatments and each one has a specific effect on the cellulose, hemicellulose and lignin fraction. Thus, different pretreatment methods and conditions should be chosen according to the process configuration selected for the subsequent

hydrolysis and fermentation steps. Several pretreatment options have been developed such as steam explosion, auto hydrolysis, ammonia pretreatment, among other (Balat, 2011; Bellesia et al., 2011; Pereira, 2014; Ruiz et al., 2011). Some key properties necessary for a cost effective pretreatment are exposed in Table 1.1 (Tomás-Pejó et al., 2011).

Table 1.1: Key factors in an effective pretreatment (adapted from (Tomás-Pejó et al., 2011)).

Key Factors in an Effective Pretreatment
Solid fraction highly digestible
No sugar degradation
Low amount of toxic compounds
Operation in reasonable size and moderate cost reactors
Nonproduction of solid wastes residues
Obtaining high sugar concentration

Under the extreme conditions observed during the pretreatment step, some toxic compounds are released together with the sugars. These inhibitory compounds can be divided in: phenolic compounds such as aromatic and polyaromatic compounds, furans such as furfural and hydroxymethyl furfural (HMF) and weak acids such as acetic acid and formic acid. Because of the harmful effects of inhibitory compounds to yeast cells, a detoxification step is often added after the pretreatment step to remove these chemical (Taylor et al., 2012). However, this may constitute up to 22% of the total ethanol production cost and should therefore be avoided (Öhgren et al., 2007).

1.2.2.1. *S. cerevisiae* for 2nd generation bioethanol production

The most promising candidate for ethanol production from lignocellulosic materials is the yeast *Saccharomyces cerevisiae*, owing to its high rate of glucose fermentation capability and high tolerance to ethanol, acidity and process conditions, but also to its well-known production, storage and transport systems at commercial scale (Demeke et al., 2013; Olsson & Nielsen, 2000; Wouter Wisselink et al., 2009; Zaldivar et al., 2001).

A drawback of yeast utilization is the incapacity of *S. cerevisiae* to metabolize xylose for its own growth or ethanol production, which means that about 20 to 30% of sugars from lignocellulosic hydrolysate are not used. Furthermore, *S. cerevisiae* shows incapacity to naturally grow in the presence of some inhibitors released from lignocellulosic compounds (Cai et al., 2012; Carroll & Somerville, 2009;

van Maris et al., 2006). To overcome these problems, strategies of genetic engineering of *S. cerevisiae* may be used to improve tolerance to lignocellulosic-based inhibitors and to allow xylose metabolization.

1.2.2.2. Metabolic Engineering for xylose metabolism

As mentioned before, *S. cerevisiae* is unable to metabolize xylose, nevertheless, this microorganism is able to convert xylulose (an isomerized product of xylose) into ethanol, throughout its phosphorylation by xylulokinase (XK), posterior metabolization through the pentose phosphate pathway where it is finally channelled to glycolysis (Cai et al., 2012). Being xylose one of the most abundant sugar present in lignocellulosic hydrolysates, its fermentation is essential for the economics of the process (Kim et al., 2013). In order to overcome this disadvantage, several efforts had been made to engineer *S. cerevisiae* through the construction of xylose metabolic pathways from fungi and bacteria, so it becomes able to ferment xylose (Karhumaa et al., 2007; Matsushika et al., 2009; Wang et al., 2014).

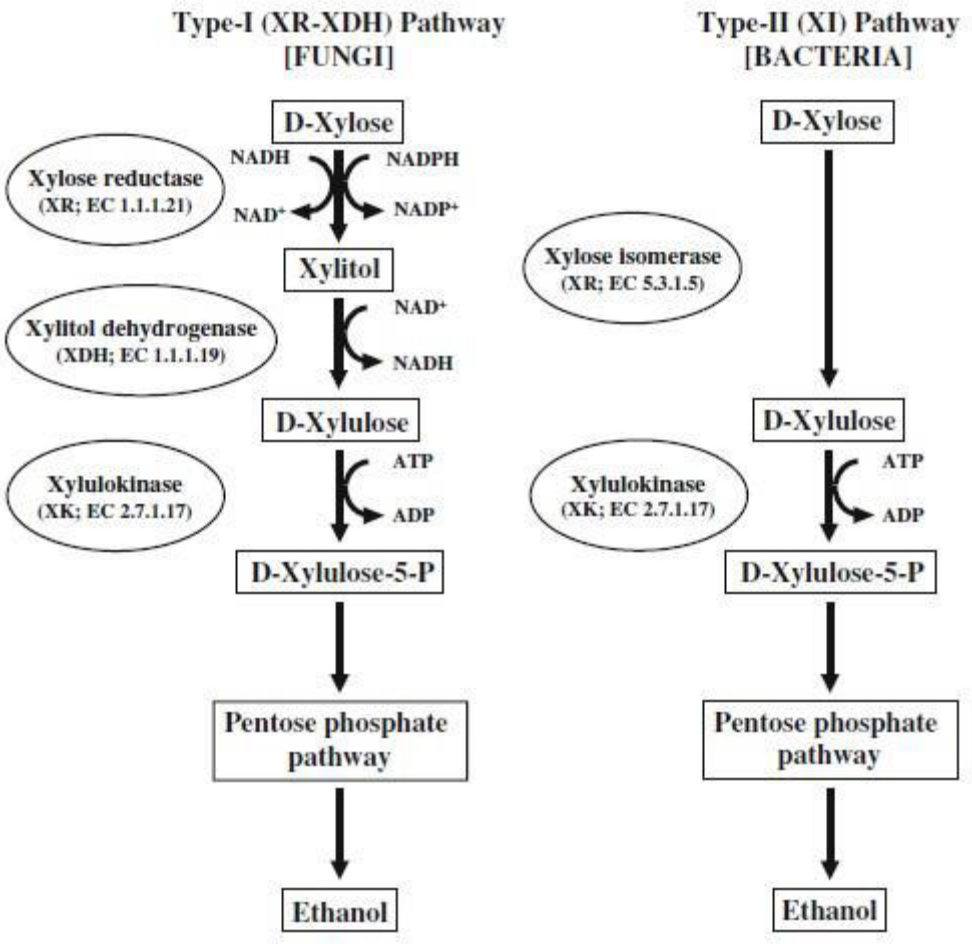

Figure 1.4: Outline of D-xylose metabolic pathways in fungi and bacteria (Matsushika et al., 2009)).

S. cerevisiae was first engineered for the fermentation of D-xylose by Kötter et al (Kötter et al., 1990). The authors report the expression of the genes *SsXYL1* and *SsXYL2* from *Scheffersomyces stipites*, which encode D-xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively (Kötter et al., 1990). While XR is responsible for the reduction of D-xylose to xylitol (using NADPH or NADH as cofactor), XDH catalyze the oxidation of xylitol to D-xylulose, using NAD^+. This strategy, described meanwhile by several authors, has resulted in aerobic growth on D-xylose, but scarce ethanol production and high amounts of xylitol were also produced (reviewed in Kim et al., 2013). The xylitol production is generally affected by two different ways: the cofactor imbalance between XR and XDH and the endogenous aldose reductase *GRE3* that reduces D-xylose to xylitol using NADPH as cofactor (Träff et al., 2001). In order to lower the xylitol formation, a few approaches have been developed: the engineering of XR from *S. stipites* that was able to diminish the cofactor imbalance, improving the ethanol productivity and yield (Runquist et al., 2010); the *GRE3* deletion that removes the native route for xylitol production (Träff et al., 2001) and the use of the xylose isomerase pathway that can carry out the isomerization of xylose without intermediates, reducing the xylitol production (Kuyper et al., 2003);

As mentioned, this second pathway allows direct isomerization of D-xylose to xylulose through heterologous expression of xylose isomerase (XI). The first successful attempt to express this pathway in *S. cerevisiae* was reported by Walfridsson et al. (1996) and consisted in expressing the XI of the thermophilic bacterium *Thermus termophilus*. By using an isomeration instead of a reduction/oxidation conversion of D-xylose to xylulose, the co-factor imbalance problem is avoided. However, reports have shown, that D-xylose utilization in XI expressing strains was found to be inferior to strains expressing the XR/XDH pathway (Bettiga et al., 2008). This problem might be due to the low activity of XI enzyme in *S. cerevisiae* and its inhibition by xylitol (Chang et al., 2007; Toivari et al., 2004). Both pathways are represented in Figure 1.4.

1.2.2.3. Target genes for improvement of *S. cerevisiae* tolerance to inhibitory compounds

One approach to tackle the inhibitor challenge is by using natural robust yeast strains. Industrial isolates are known to be very robust, to show stress tolerance that is developed in the presence of stress factors related with harsh industrial process such as elevated temperatures, pH variations and presence of toxic compounds (Della-Bianca et al., 2013; Pereira, Guimarães, Teixeira, & Domingues, 2011). Moreover, industrial isolates show higher fermentation capacity (Mussatto et al., 2010; Pereira et al., 2010).

During yeast cultivation and fermentation, the inhibitory compounds resultant of lignocellulose pretreatment induce a harsh effect on yeast metabolism reducing the ethanol yield and productivity (Liu, 2006; Mira, Teixeira, & Sá-Correia, 2010; Pereira, Teixeira, Mira et al., 2014). For these reasons, it would be interesting to find key genes able to increase yeast tolerance to multiple inhibitors compounds present in lignocellulosic hydrolysates (Pereira et al., 2014).

These inhibitors can be grouped in two main classes: weak acids and furan compounds. Weak acids have been described to induce a strong intracellular acidification, with negative consequences for the activity of metabolic enzymes. Weak acids also inhibit yeast fermentation by reducing biomass formation and ethanol yield (Almeida et al., 2007; Larsson et al., 1999). On the other hand, yeast cells reduce furan compounds to their less toxic compounds, leading to a lower productivity in the fermentation process and to an increasing of the lag phase. Moreover, furfural and HMF are known to cause DNA, RNA, protein and membrane damage at low concentrations (Ask et al., 2013; Lin et al., 2009).

Several studies have been carried out in order to understand which genes are important to *S. cerevisiae* tolerance to inhibitors (Pereira, Guimarães, Gomes, et al., 2011; Pereira et al., 2014). *PRS3* and *HAA1* have been selected as target genes once their overexpression can lead to a higher resistance of *S. cerevisiae* to hydrolysate-derived inhibitors (Cunha et al., 2015; Inaba et al., 2013; Tanaka et al., 2012).

PRS3 gene 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 identified *PRS3* as a key gene necessary for yeast growth and maximal fermentation rate in the presence of inhibitors (Pereira, Guimarães, Gomes, et al., 2011; Pereira et al., 2014). Moreover, the overexpression of *PRS3* may contribute to increase the carbon flux in favor of metabolic pathways which are important for the regeneration of NADH, a cofactor required for the detoxification of furfural and HMF, and for ethanol production (Cunha et al., 2015).

HAA1 gene was first included into a family of copper regulated transcription factors, based on the identification of a putative copper regulatory domain within its DNA binding domain. However, unlike its homologous proteins, the function of *HAA1* is independent of the copper status of the cell and it was related that *HAA1* gene was found to regulate directly or indirectly the transcription of approximately 80% of the acetic acid-activated genes, suggesting that *HAA1* is the main player in the control of yeast response to this weak acid (Keller et al., 2001; Mira, Becker et al., 2010). Furthermore, the expression of the *HAA1* gene was shown to lead to a reduction of the adaptation period of yeast cells to toxic concentration of weak acids, by decreasing the loss of cell viability during the latency phase (Fernandes et al., 2005; Mira,

Becker, et al., 2010). Tanaka et al. (2012) overexpressed the *HAA1* gene in a laboratory strain and showed that this strain acquired a higher level of acetic acid tolerance in synthetic medium. Inaba et al. (2013) constructed a *HAA1*-overexpressing strain derived from an industrial bioethanol strain, which showed tolerance not only to acetic acid but also to lactate, and this tolerance was dependent on the increased expression of *HAA1* gene. Furthermore, in a molasses medium, this strain showed a higher fermentation ability (ethanol production) in the presence of acetic acid than the wild-type strain (Inaba et al., 2013). Sakihama et al. (2015), overexpressed *HAA1* gene in a recombinant xylose-fermenting *S. cerevisiae* laboratory strain, resulting in an improved culture growth and higher ethanol production in synthetic media containing acetic acid.

Cunha et al. (2015) have reported that the overexpression of the same genes can result in different outcomes depending on the strain and hydrolysate used and for that reason, tolerance engineering has to be customized to the strain background and to the hydrolysate specific inhibitory load used in the process. Therefore, *PRS3* and *HAA1* genes were selected for overexpression in different industrial strains, genetically modified for xylose consumption, in order to take advantage of their more robust background and higher tolerance to lignocellulosic-derived inhibitors.

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 minutes. Xylose-containing media were sterilized at 117 °C.

2.2. Strains and Plasmids

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

Table 2.1: Microbial strains and plasmids used during this work.

Strains	Genotype	Source
E. coli XL1 BLUE	*recA1; hsdR17; re1A; lac* [F << lacIqZM15 Tn10(Tetr)]	Stratagene
E. coli TOP10	F– *mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15,ΔlacX74, recA1, araD139,Δ(ara-leu)7697, galU, galK, rpsL(StrR), endA1, nupG*	Invitrogen
S. cerevisiae CEN.PK 113-5D	*MATa; ura3-52*	(Van Dijken et al., 2000)
S. cerevisiae CEN.PK113-7D	*MATα, MAL2-8c, SUC2*	INSA, Toulouse, France
S. cerevisiae PE-2	Diploid; Isolated from bioethanol plants in 1994	(Basso et al., 2008)
S. cerevisiae PE-2 Δ*GRE3*	PE-2, *GRE3*::natMX4 / *GRE3*::kanMX4	(Romaní et al., 2015)
S. cerevisiae PE-2 Δ*GRE3* pMEC1153	PE-2, *GRE3*::natMX4 / *GRE3*::kanMX4, pMEC1153	(Romaní et al., 2015)
S. cerevisiae PE-2 Δ*GRE3* pMEC9001	PE-2, *GRE3*::natMX4 / *GRE3*::kanMX4, pMEC9001	This work
S. cerevisiae PE-2 Δ*GRE3* pMEC9002	PE-2, *GRE3*::natMX4 / *GRE3*::kanMX4, pMEC9002	This work
S. cerevisiae PE-2 Δ*GRE3* pMEC9003	PE-2, *GRE3*::natMX4 / *GRE3*::kanMX4, pMEC9003	This work

S. cerevisiae CA11	Isolated from Brazilian "cachaça" fermentation process	(Freitas Schwan et al., 2001)
S. cerevisiae CA11 pMEC1153	CA11, pMEC1153	This work
S. cerevisiae CA11 pMEC9001	CA11, pMEC9001	This work
S. cerevisiae CA11 pMEC9002	CA11, pMEC9002	This work
S. cerevisiae CA11 pMEC9003	CA11, pMEC9003	This work

Plasmids	**Property**	**Source**
pMEC1153	PYPK4-TEF1tp-XR(N272D)-TDH3tp-XYL2-PGI1tp-XKS1-FBA1tp-TAL1-PDC1tp, HphMX4	(Romaní et al., 2015)
pMEC9001	PYPK4-TEF1tp-XR(N272D)-TDH3tp-XYL2-PGI1tp-XKS1-FBA1tp-TAL1-PDC1tp, HphMX4, *HAA1* gene under the control of its native promoter and terminator	This work
pMEC9002	PYPK4-TEF1tp-XR(N272D)-TDH3tp-XYL2-PGI1tp-XKS1-FBA1tp-TAL1-PDC1tp, HphMX4, *PRS3* gene under the control of its native promoter and terminator	This work
pMEC9003	PYPK4-TEF1tp-XR(N272D)-TDH3tp-XYL2-PGI1tp-XKS1-FBA1tp-TAL1-PDC1tp, HphMX4, *HAA1* and *PRS3* genes under the control of their respective native promoter and terminator	This work

2.3. Bacteria and yeast cells storage

Bacteria and yeast cells were maintained for up to 2 weeks at 4 °C, in the appropriate medium, on inverted agar plates sealed with parafilm. For long time storage, permanent stocks were prepared. Briefly, a culture grown overnight in appropriated selective liquid medium was 10 times diluted in fresh medium and grown for more 6 hours. Afterwards, 0.3 mL of sterile glycerol were added to 1 mL of the culture, mixed by vortexing and incubated on ice for 10 minutes. The tubes were then stored at -80 °C. For culture recovery, the frozen cells were scraped and spread on appropriate agar medium plate.

2.4. Media and growth conditions

E. coli strains used in this work were grown in Luria-Bertani (LB) medium (1% (w/v) Triptone, 0.5% (w/v) NaCl, 0.5 (w/v) yeast extract) at 37 °C. For transformants selection, LB solid medium was supplemented with 100 mg/L of ampicillin (LB-amp). The yeast strains used in this work were grown at 30 °C. Host strains were grown in Yeast Extract-Peptone-Dextrose (YPD) medium (2% (w/v) Peptone, 1% (w/v) yeast extract and 2% (w/v) glucose). Yeast transformants were selected in YPD solid medium supplemented with 300 mg/L hygromycin and maintained in YPX medium (2% (w/v) Peptone, 1% (w/v) yeast extract and 2% (w/v) xylose). All strains were also grown in the corresponding solid media, obtained by the addition of 2% (w/v) agar. YPX media was used for aerobic growth (2.6) and was supplemented with inhibitors: 1 g/L of furfural, 4 g/L of acetic acid or 1 g/L of furfural and 3 g/L of acetic acid. Shake-flask fermentations (2.7) were performed in YPX supplemented with 1 g/L of furfural or 1 g/L of furfural and 3 g/L of acetic acid or in *Eucalyptus globulus* wood (EGW) hydrolysate supplemented with low-cost nutrients (16.52 g/L cheese whey, 0.86 g/L urea, 5.79 g/L corn steep liquor, 4.10 g/L raw yeast extract, and 0.3335 g/L of potassium metabisulfite ($K_2O_5S_2$), as described by Kelbert et al. (2015)). Stock solutions of urea and $K_2O_5S_2$ were prepared by filtration and sterilized and in autoclave at 121 °C for 15 minutes. Finally, corn steep liquor in its liquid gross from was also sterilized in the same conditions. Cheese whey and raw yeast extract were sterilized by pasteurization at 60 °C for 1 hour and then were added to the hydrolysate in its solid form aseptically. Regarding to cheese way, it was provided by Quinta dos Ingleses (Agro-livestock Company, Portugal), while raw yeast extract was provided by a microbrewery called Fermentum (Portugal), being then dried at 60 °C until there was no weight variation.

Hemicellulosic hydrolysate used in this work was obtained from processing *Eucalyptus globulus* lignocellulosic feedstocks by hydrothermal treatment followed by acid hydrolysis. Conditions of hydrothermal treatment were selected based on previous works (Pereira, Guimarães, Gomes, et al., 2011; Rivas et al., 2002; Romaní et al., 2014). After treatment, solid and liquid phases were separated by filtration. Liquid phases were subjected to a second step of acid hydrolysis with 1.5% (w/w) H_2SO_4 for 45 minutes at 121 °C in an autoclave. Resulting hydrolysates (containing hemicellulose derived compounds) were neutralized with $CaCO_3$ until pH 5 and sterilized by filtration (0.2 μm) to be used as fermentation media. Composition of hydrolysates (sugars, acetic acid and furan compounds) was analysed by HPLC (2.8).

2.5. Molecular Biology

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

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

2.5.1.1. Commercial kit

Plasmid DNA was extracted from *E. coli* strains using a commercial kit from Sigma-Aldrich® named GenElute™ Plasmid Miniprep Kit. The kit 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 minutes. Afterwards, 350 μL of Neutralization Solution were added and inverted 4 - 6 times to mix. The debris were pelleted for 10 minutes at 16100 g. In the meantime, 500 μL of Column Preparation Solution were added to the binding column in a collection tube, spun at 16100 g for 1 minute and the flow-through discarded. The cleared lysate was transferred into binding column, centrifuged for 1 minute and the flow-through discarded. The column was washed with 750 μL of Wash Solution and centrifuged for 1 minute. The flow-through was discarded and the column dried by an additional 1 minute 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 minute centrifugation.

2.5.1.2. 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. It was added 200 μL of Solution I (1% (w/v) SDS; 0.2 M NaOH) in order to obtain cellular lysis and then, the solution was inverted four times to mix. To neutralize and precipitate cell extracts and other contaminants, 200 μL of Solution II (3 M Potassium acetate; 11.5% (v/v) Acetic acid) were added, again, the tube was inverted 4 times to mix and incubated on ice for 5 minutes. Afterwards, suspension was centrifuge for 2 minutes at 16100 g. The supernatant was mixed with 500 μL of 100% isopropanol and centrifuged for 2 minutes. Finally, the supernatant was carefully removed and the pellet was air dried and ressuspended in 30 μL of UP H_2O.

2.5.2. Plasmid DNA preparation from Yeast strains

Two methods for plasmid extraction from yeast were used. In both methods, 1 - 2 μL of the final purified DNA were used for *E. coli* transformation (2.8) and the plasmid was confirmed by enzymatic digestion after being extracted from *E. coli*.

2.5.2.1. Commercial Kit

This method consisted on a protocol adapted to use the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich®), with an additional step to disrupt the cell wall using lyticase (Sigma-Aldrich®). An overnight cell culture was harvested, centrifuged and washed in water. The pellet was mixed with 200 μL of Resuspension solution (Miniprep Sigma-Aldrich® Kit) and 10 μL of lyticase (5 U/μL), and incubated at 37 °C for 2 hours. The solution was further purified as described in section 2.5.1.

2.5.2.2. Phenol/Chloroform/Isoamyl alcohol Method

In this method, yeast cells were grown overnight at 30 °C in a selective medium. Cell suspension was centrifuged for 2 min at 3000 g. The supernatant was discarded and the cells were washed with 0.5 mL of distilled water. Cells were transferred to a 1.5 mL centrifuge tube and pelleted. Afterwards, the cell pellet was ressuspended in 0.2 mL of extraction buffer (2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 100 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.0), and 0.2 mL of solution F/C/I (Phenol stabilized: Chloroform: Isoamyl Alcohol 25:24:1, PanReac AppliChem); and 0.3 g of glass beads were added to the cell suspension. The mixture was homogenized at top speed (6.5 m/s) in a FastPrep®-24 Instrument (MP Biomedicals) during 4 cycles of 30 s agitation with 1 min of cooling interval. After addition of 0.2 mL of TE buffer, the mixture was centrifuged at 16100 g for 5 minutes and the liquid phase was transferred to a new tube and the DNA was precipitated with 1 mL of pure ethanol was added. The tube was inverted 4 - 6 times and then centrifuged for 2 minutes, the supernatant was discarded. The DNA pellet was ressuspended in 50 μL of TE buffer.

2.5.3. DNA Quantification

Nucleic acid concentration was determined in a NanoDrop 1000 Spectrophotometer (Thermo Scientific) by loading 2 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 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.

2.5.4. DNA Storage

DNA solutions were stored at -20 or 4 °C in TE or EB buffer, or alternately in UP H_2O. The TE buffer composition is 10 nm Tris/HCl pH 8.0 and 1 mM EDTA while EB buffer composition is 10 nm Tris/HCl pH 8.5.

2.5.5. 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.

2.5.5.1. 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 2.2. The reaction mixture consisted of 2 μL of 10x Phusion HF buffer, 0.5 μL of 20 μM of each Primer, 0.4 μL of 10 mM dNTPs, 1.9 μL of DNA, 1 μL of Phusion HF DNA Polymerase and UP H_2O to the final volume of 20 μL. The amplification of the genes *PRS3* and *HAA1* was performed simultaneously, with an initial denaturation step at 95 °C during 5 minutes, followed by 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 52°C and 1.5 minutes extension at 72 °C, and with a final extension step of 10 minutes at 72 °C.

2.5.5.2 Primers

Table 2.2: Primers used during this work. Underlined are the homologous recombination sites with pMEC1153 plasmid.

Primer Name	Sequence (5'-3')	TM (ºC)	Use
Haa_f	GGTTTTACCGTGTGCGGAGATCAGGTTCTGATCCCCCAT TTCCCCTTTCT	68	Amplification of *HAA1* gene with native promotor and terminator from BHUM1737 (Malcher et al., 2011)
Haa_r	AGACAAACCGTGGGACGAATTCTTAAGATGCTCGAATAC CTCATCTCTGCG	65	Amplification of *HAA1* gene with native promotor and terminator from BHUM1737 (Malcher et al., 2011)
*PRS3*_f	TAACGATGTAGTACAGCGTTTCCGCTTTTTCACCCTTAT CTTCATCACCGC	64	Amplification of *PRS3* gene with native promotor and terminator from YEpJCP (Cunha et al., 2015)
*PRS3*_r	CATAAGTACCCATCCAAGAGCACGCTTATTCACCAACAA GAGAAACTTTTG	64	Amplification of *PRS3* gene with native promotor and terminator from YEpJCP (Cunha et al., 2015)

2.5.6. DNA Electrophoresis

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) (25% (w/v) glycerol, 20 mM EDTA, 0.25% (w/v) Bromophenol blue) 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 Tris-Acetate-EDTA (TAE, 2 M Tris-base, 50 mM EDTA, pH 8.0 (with acetic acid)) buffer, until the dye migrated as far as 2/3 of the gel length. Gels were visualized and photographed in a Molecular Imager ChemiDocTM XRS + Imaging System (Bio-Rad) and analysed using the Image Lab 4.0 software.

The DNA molecular weight markers used in all agarose gels were NZYDNA Ladder III (NZYTech) or GeneRuler 1 kb DNA Ladder (Thermo Scientific®) which produce a pattern of 14 regularly spaced bands, ranging from 200 to 10000 bp and 250 to 10000 bp, respectively (Table 2.3).

Table 2.3: NZYDNA Ladder III and GeneRuler™ 1kb DNA Ladder bands molecular weight.

	NZYDNA Ladder III	GeneRuler™ 1 kb DNA Ladder
Band	**Size (bp)**	
1	10000	10000
2	7500	8000
3	6000	6000
4	5000	5000
5	4000	4000
6	3000	3500
7	2500	3000
8	2000	2500
9	1400	2000
10	1000	1500
11	800	1000
12	600	750
13	400	500
14	200	250

2.5.7. Enzymatic digestion with restriction endonucleases

Digestion reactions with restriction endonucleases were performed in appropriate provided 10x buffer, in a final volume of 20 µL, during 2 hours at 37 °C. All restriction enzymes (except *AleI* and *PciI*) were purchased from Thermo Fisher Scientific Inc. and *AleI* and *PciI* were obtained from New England Biolabs®.

2.5.8. Bacteria transformation

In order to transform XL1-BLUE bacteria cells, a maximum of 30 µL of DNA was added to 200 µL of cells and put on ice for 30 minutes. Then, the mixture was incubated for 40 seconds at 42 °C in a water bath and placed on ice for 10 minutes. Transformants were incubated with 900 µL of SOC (2% (w/v) Tryptone; 0.5% (w/v) Yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgSO_4.7H_2O$; 10 nM $MgCl_2.6H_2O$ and 20 mM Glucose) at 37 °C for 1 hour with vigorous shaking. Cells were centrifuged for a few seconds at 16100 g. The pellet was ressuspended in 100 µL of water, plated on LB-amp and incubated overnight at 37 °C.

2.5.9. Yeast transformation

The plasmids were constructed by the gap repair technique, using the lithium acetate method. Additionally, yeast cells were also transformed with the different (already constructed) vectors by the lithium acetate method. Cells were inoculated into 20 mL of liquid YPD medium and grown overnight at 30 °C. The cell suspension was diluted with fresh YPD to a OD_{600nm} of 0.1 and grown again to a OD_{600nm} of 0.8. The cells were harvested at room temperature for 5 minutes at 2500 g and washed with 25 mL of sterile H_2O. The cell pellet was resuspended in 1 mL of H_2O, transferred to a 1.5 mL centrifuge tube and the cells pelleted. 300 µL of LiOAc (0.1 M) solution were added to the cells.

A mixture of 50 µL of the yeast cell suspension, 1 µL of digested pMEC1153 plasmid (with *Xho*I for the construction of pMEC9001, with *Ale*I for the construction of pMEC9002, and with *Xho*I and *Ale*I, simultaneously, for the construction of pMEC9003), 4 µL of the PCR product of the genes (which contain homologous recombination sites with the digested plasmid) and 50 µL of Salmon sperm DNA (2 mg/mL) was prepared in a micro centrifuge tube. Alternatively, instead of the PCR products and digested plasmid, an already constructed vector was added to the mixture. Afterwards, 250 µL of a sterile mixture of 900 µL Polyethylene Glycol (PEG) 50% and 100 µL LiOAc (1M) was added and mixed thoroughly. The mixture as incubated for 30 minutes at 200 rpm orbital agitation and 30 °C, followed by a heat shock at 42 °C for 40 minutes and then placed on ice for 1 minute. The suspension was spun down in the micro centrifuge for 5 seconds at room temperature and the cell pellet resuspended in 1 mL of fresh YPD. The cell suspension was transferred to 50 mL centrifuge tubes already containing 1 mL of YPD and incubated for 4 hours at 200 rpm orbital agitation and 30 °C. Afterwards, the suspension was spun down for 10 minutes at 2500 g. Cells were washed with 150 µL of H_2O and then plated in YPD plates supplemented with hygromycin, incubated at 30 °C and transformants were visible after 2-3 days.

2.6. Yeast growth assay under aerobic conditions

The pre-inoculum was carried out in 100 mL Erlenmeyer flasks with 27 mL of fresh YPX and 3 mL of an overnight liquid culture of each strain. Flasks were incubated at 30 °C and 200 rpm orbital agitation orbital agitation for 22-24 hours. The following steps were performed on ice. The cell suspension was transferred for 50 mL tubes and centrifuged for 3 minutes at 2500 g and 4 °C. Pellets were ressupended in 30 mL of a saline solution (0.9% (w/v) NaCl) and mixed cautiously. The Optical Density at 600 nm (OD_{600nm}) was measured and each microplate well was inoculated to an OD_{600nm} of 0.1.

Aerobic growth was performed in YPX medium or YPX supplemented with inhibitors, furfural and/or acetic acid (2.4), in 24-well microplates.

Some wells were not inoculated to serve as control for possible cross-well contaminations. The microplates were incubated at 30 °C and 200 rpm orbital agitation and the growth monitored by OD_{600nm} measurements, in a Synergy HT Multi-Mode Microplate Reader (BioTek).

2.7. Shake-flask fermentations

Fermentations were performed in YPX with inhibitors, 1 g/L of furfural or 1 g/L of furfural and 3 g/L of acetic acid or in *Eucalyptus globulus* wood (EGW) hydrolysate, in oxygen-limited conditions using 100 mL Erlenmeyer flasks sealed with cotton plugs. Under this condition, defined as oxygen-limited, the culture has contact with air but is under oxygen limitation recreating industrial conditions.

The pre-inoculum was carried out in 500 mL Erlenmeyer flasks with 135 mL of fresh YPX and 15 mL of an overnight liquid culture of each strain. The flasks were incubated at 30 °C and 200 rpm orbital agitation for 22-24 hours. 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 2500 g for 15 minutes. The supernatant was rejected and the tube walls carefully cleaned. The yeast cell pellet was weight and resuspended in ice-cold Saline Solution (0.9 % (w/v) NaCl) to a concentration of 250 (or 500) mg of Fresh Yeast per milliliter (mgFY/mL). The suspension was homogenized by manual agitation. Precisely 27.6 mL of the fermentation media was distributed for each fermentation flask. The flasks were inoculated with 2.4 mL of the yeast suspension leading to a final concentration of 20 (or 40) mgFY/mL. The suspension was homogenized and the flasks were sealed. The flasks were incubated at 30 °C and 150 rpm orbital agitation and samples were collected during the fermentation time for xylose, acetic acid, furfural, ethanol and xylitol quantification. Fermentations in synthetic media were inoculated with 20 mgFY/mL, while in the fermentation carried out in EGW hydrolysate, the yeast concentration used was 40 mgFY/mL.

In the end of fermentation, the dry weight was determined according to the following procedure: 15 mL centrifuge tubes were placed at 105 °C for 24 hours, tubes were then left in a desiccator for 15 minutes and weighted. 10 mL of each flask were added to the corresponding centrifuge tube and the tubes were kept on ice. Afterwards, cell suspension was centrifuged for 10 minutes at 2500 g and 4 °C. Yeast pellets were washed with 10 mL of distillate water and centrifuged again. Finally, tubes containing cell pellets were dried at 105 °C for 24 hours and weighted.

2.7.1. Determination of growth and fermentation parameters

Fermentation parameters were calculated at 24 hours of fermentation with PE-2 Δ*GRE3* strains and at 23 hours with CA11 strains. Xylose consumption rate (g/L.h) was calculated as the difference between culture xylose concentrations X_{t0} at the beginning of the culture and X_{txf} at time t_{xf} divided by t_{xf}. Where X_{t0} is the concentration of xylose at initial time (t_0) and t_{xF} is after 23 hours of fermentations in CA11 strains and after 24 hours in PE-2 Δ*GRE3* strains. Ethanol productivity (g/L.h) was calculated as the difference between ethanol concentration E_{t0} at the beginning of the fermentation and E_{tef} at time t_{ef} divided by t_{ef}. Where E_{t0} is the concentration of ethanol at initial time (t_0) and t_{ef} is after 23 hours of fermentations in CA11 strains and after 24 hours in PE-2 Δ*GRE3* strains. Ethanol ($Y_{E/S}$) and xylitol ($Y_{XYL/S}$) yields were calculated as the difference between xylitol or ethanol concentrations at 23 hours of fermentations in CA11 strains and at 24 hours in PE-2 Δ*GRE3* strains and the respective concentration at the beginning of the fermentation divided by the sugars (glucose and xylose for ethanol yield and xylose for xylitol yield) consumed in the same period of time. Biomass yield ($Y_{X/S}$) was determined by the ratio between the dry weight of cells and the sugars consumed at the end of fermentation. In aerobic growth, the percentage of xylose consumed was calculated as the difference between xylose concentration at the beginning of the growth and xylose concentration at 48 hours of the growth divided by initial xylose concentration.

2.8. HPLC quantification

Glucose, xylose, acetic acid, furfural, xylitol and ethanol were quantified by high performance liquid chromatography (HPLC), upon separation of the different samples in a BioRad Aminex HPX-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 xylose, acetic acid, xylitol and ethanol were detected using a Knauer-IR intelligent refractive index detector, whereas furfural was detected using an Knauer-UV detector set at 210 nm.

2.9 Statistical analysis

GraphPad Prism for Windows version 6.01 was used to carry out the statistical analyses. Differences between the fermentation profiles of the over-expressing strain and the control strain were tested by repeated measures one-way ANOVA, followed by Bonferroni post hoc test. Statistical significance was established at $P < 0.05$ for the comparisons.

3. Results

3.1. Construction of pMEC9001, pMEC9002 and pMEC9003 plasmids

In order to simultaneously test the effects of the overexpression of *HAA1* and *PRS3* genes and the ability of industrial yeast strains to consume xylose, *HAA1* and *PRS3* were inserted into the plasmid pMEC1153 (Figure 3.1) (Romaní et al., 2015). This plasmid was previously created using a metabolic pathway assembly tool, the Yeast Pathway Kit (YPK), based on hierarchical homologous recombination of fragments cloned in an *E. coli* positive selection vector (Pereira et al., 2016). The engineered pathway contains four different genes: Ss*XYL1*, Ss*XYL2*, Sc*XKS1* and Sc*TAL1*, under different *S. cerevisiae* promotors *TEF1*, *TDH1*, *PGI1* and *FBA1*, respectively. Ss*XYL1* and Ss*XYL2* genes are from *Scheffersomyces stipitis* and, as previously mentioned, encode for D-xylose reductase (XR) and xylitol dehydrogenase (XDH) respectively (Romaní et al., 2015). XKS encodes for the endogenous xylulose kinase and TAL1 encodes for transaldolase, an enzyme of the pentose phosphate pathway. Furthermore, pMEC1153 has a *hph*MX4 hygromycin resistance gene which allows the transformation of industrial yeast strains (Romaní et al., 2015).

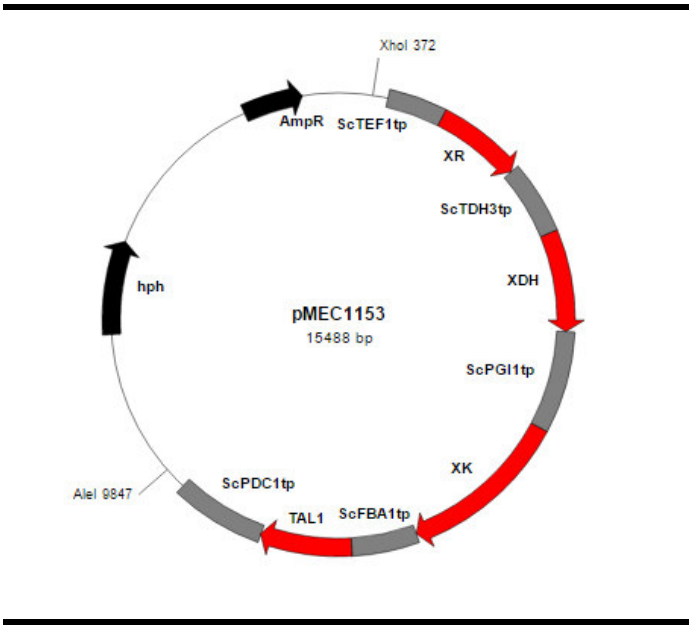

Figure 3.1: Schematic representation of pMEC1153.

The first step for the construction of the new plasmids was the digestion of pMEC1153 with specific restriction enzymes to produce homologous recombination sites with the primers used in *HAA1* and *PRS3* amplification by PCR (Figure 3.2 and Table 2.2).

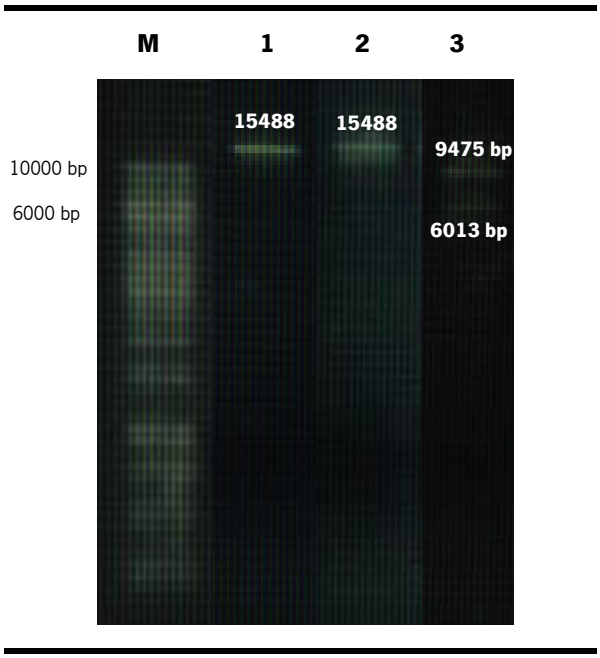

Figure 3.2: Restriction enzyme digestion of pMEC1153. Lane 1: pMEC1153 digestion with *Xho*I (expected band size was 15488 bp); lane 2: pMEC1153 digestion with *Ale*I (expected band size was 15488 bp); lane 3: pMEC1153 digestion with *Xho*I and *Ale*I (expected band sizes were 9475 and 6013 bp). M: 1kb GeneRuler™ DNA ladder.

As mentioned before, the sequence originated by pMEC1153 digestion has homologous recombination sites with the PCR product of genes amplification. The two DNA fragments recombine together directed by the short stretches of homology derived from the vector (Figure 3.3). *HAA1* and *PRS3* genes were amplified from BHUM1737 and YepJCp (Figure 3.4) respectively with primers represented in Table 2.2.

Figure 3.3: Schematic representation of homologous recombination assembly.

The digested pMEC1153 plasmid and the amplified genes were then co-transformed in yeast CEN.PK 113-7D (2.5.9), originating a recombinant yeast clone carrying the plasmid containing the target gene(s). pMEC9001 was produced by inserting the *HAA1* gene in pMEC1153 vector linearized with *Xho*I enzyme (Figure 3.5). On the other hand, pMEC9002 was produced by inserting *PRS3* gene in pMEC1153

vector linearized with *Ale*I enzyme (Figure 3.6). Finally, pMEC9003 contains both *HAA1* and *PRS3* genes in the pMEC1153 vector that was linearized with both restriction enzymes, *Xho*I and *Ale*I (Figure 3.7). After extraction of the constructed plasmids from yeast, and transformation in *E. coli*, the confirmation of the correct construction of all plasmids was achieved by restriction analysis.

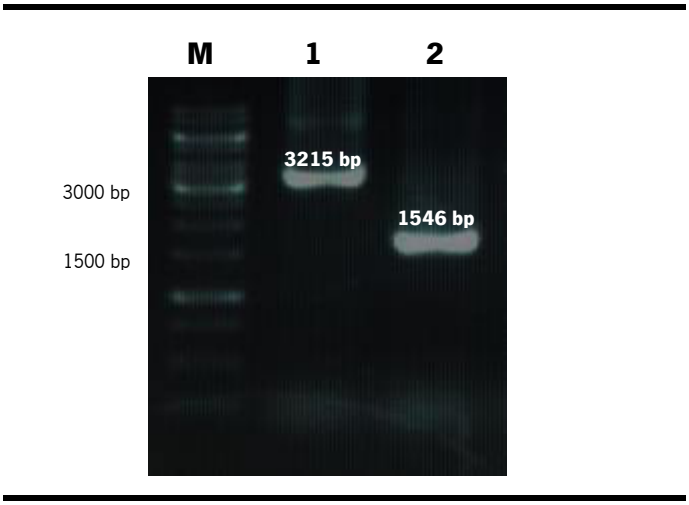

Figure 3.4: PCR amplification of *HAA1* and *PRS3* genes with specific primers. Lane 1: *HAA1* PCR product (expected band sizes 3215 bp); lane 2: *PRS3* PCR product (expected band sizes 1546 bp). M: 1kb GeneRuler™ DNA ladder.

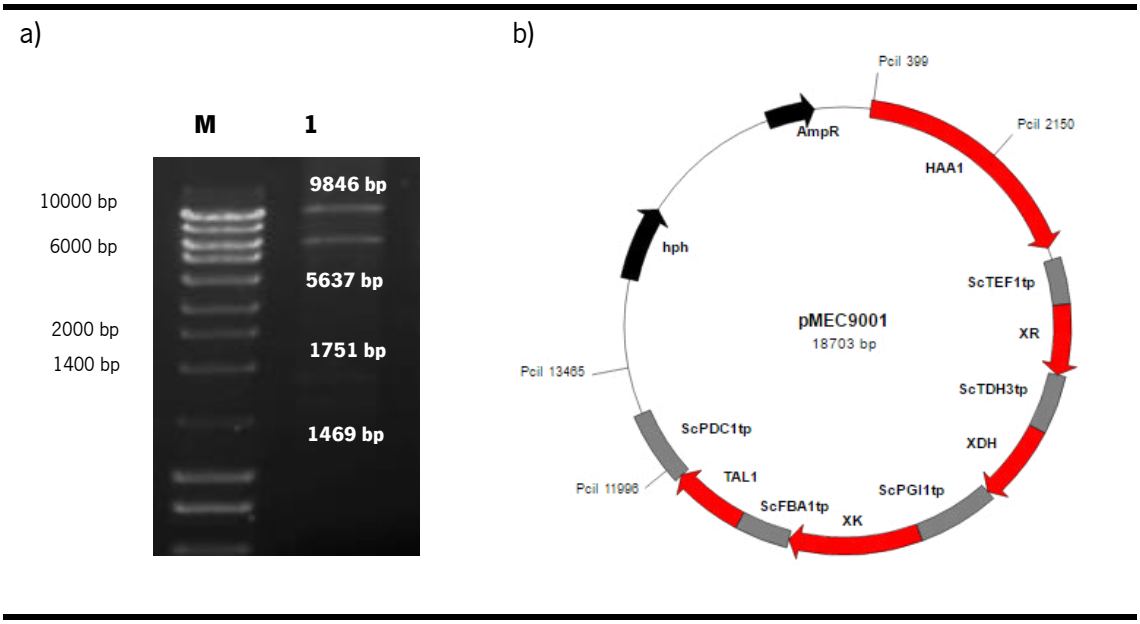

Figure 3.5: a) Confirmation of the construction of pMEC9001 plasmid. Lane 1: Digestion of pMEC9001 with *Pci*I (expected band sizes 9846, 5637, 1751 and 1469 bp). M: NZYDNA Ladder III. b) Schematic representation of pMEC9001 plasmid.

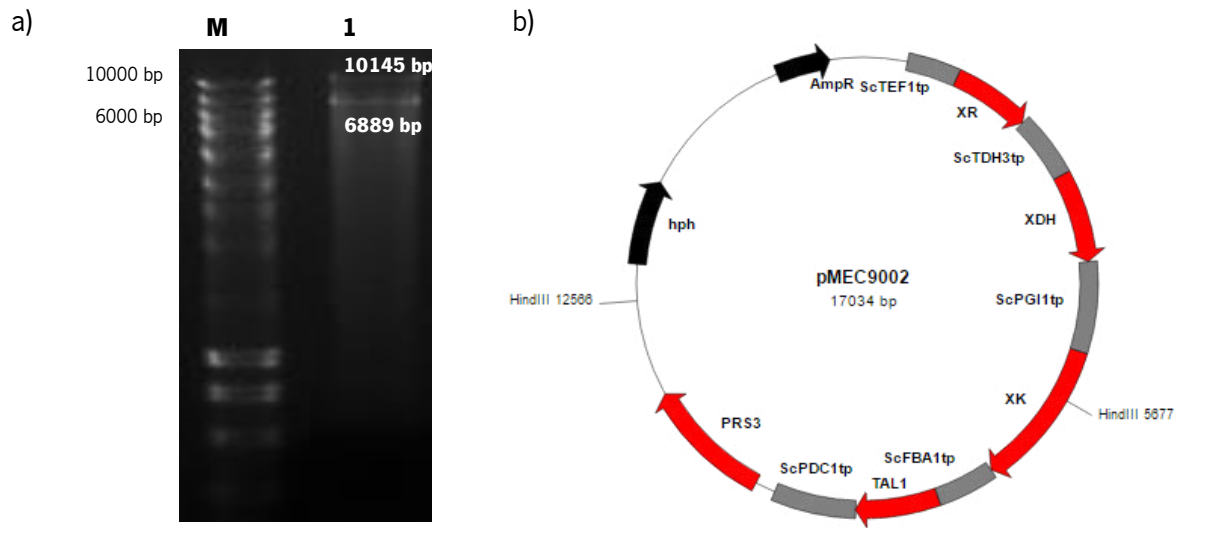

Figure 3.6: a) Confirmation of the construction of pMEC9002 plasmid. Lane 1: Digestion of pMEC9002 with *Hind*III (expected band sizes 10145 bp and 6889 bp). M: NZYDNA Ladder III. b) Schematic representation of pMEC9002 plasmid.

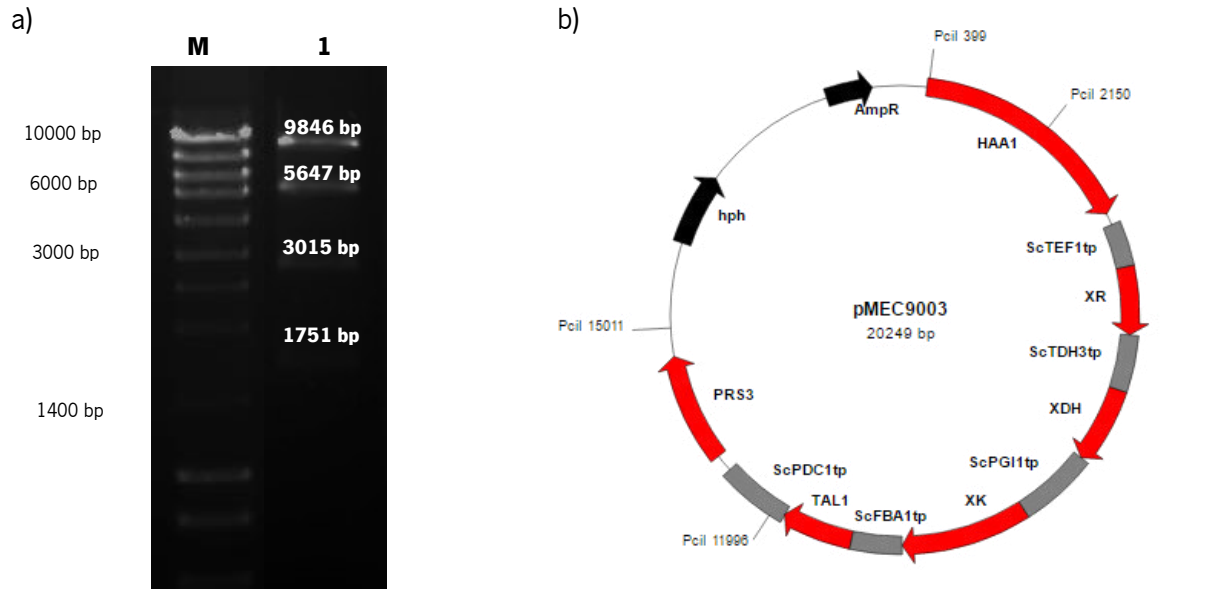

Figure 3.7: a) Confirmation of the construction of pMEC9003 plasmid. Lane 1: Digestion of pMEC9003 with *Pci*I (expected band sizes 9846, 5647, 3015 and 1751 bp). M: NZYDNA Ladder III. b) Schematic representation of pMEC9003 plasmid.

pMEC1153, pMEC9001, pMEC9002 and pMEC9003 were used to transform *S. cerevisiae* strains CA11 and PE-2Δ*GRE3* (2.2). The transformations efficiencies varied from 25 to 500 colonies per µg of DNA. The selection of transformants was made in YPD agar with 300 mg/L of hygromycin.

3.2. Yeast growth assay under aerobic conditions

To further understand the role that *HAA1* and *PRS3* overexpression may play in yeast tolerance in the presence of inhibitors, *S. cerevisiae* CA11 and PE-2Δ*GRE3* strains transformed with the constructed plasmids and with pMEC1153 were characterized on their ability to grow aerobically in xylose medium. Four different media were used: YPX, YPX supplemented with 1 g/L of furfural, YPX supplemented with 4 g/L of acetic acid and YPX supplemented with 1 g/L of furfural and 3 g/L of acetic acid. The inhibitors concentrations were determined by exposing the PE-2Δ*GRE3* pMEC1153 to different concentrations of acetic acid and furfural, and the selected values were the minimal concentrations capable of inducing a significant inhibitory effect in yeast resulting in longer lag-phases (approximately 20 hours).

3.2.1. CA11 Recombinant Strains

In YPX medium and in YPX supplemented with 1 g/L of furfural the control strain (CA11 transformed with the xylose metabolic pathway present in the plasmid pMEC1153) was able to grow faster than other strains (Figure 3.8 A and B). Furthermore, in YPX, the strain harbouring the pMEC1153 vector showed a higher percentage of xylose consumed than the other three recombinant strains overexpressing *HAA1* and/or *PRS3* (Figure 3.9 A), however, in YPX supplemented with furfural, there were no significant differences among all strains (Figure 3.9 B). In YPX supplemented with 4 g/L of acetic acid none of the strains was able to exit the lag-phase into exponential growth (Figure 3.8 C) and there was no xylose consumption in any strain. Regarding to YPX supplemented with 1 g/L of furfural and 3 g/L of acetic acid, all strains showed a slower growth (Figure 3.8 D) and, once more, there was no xylose consumption in any strain.

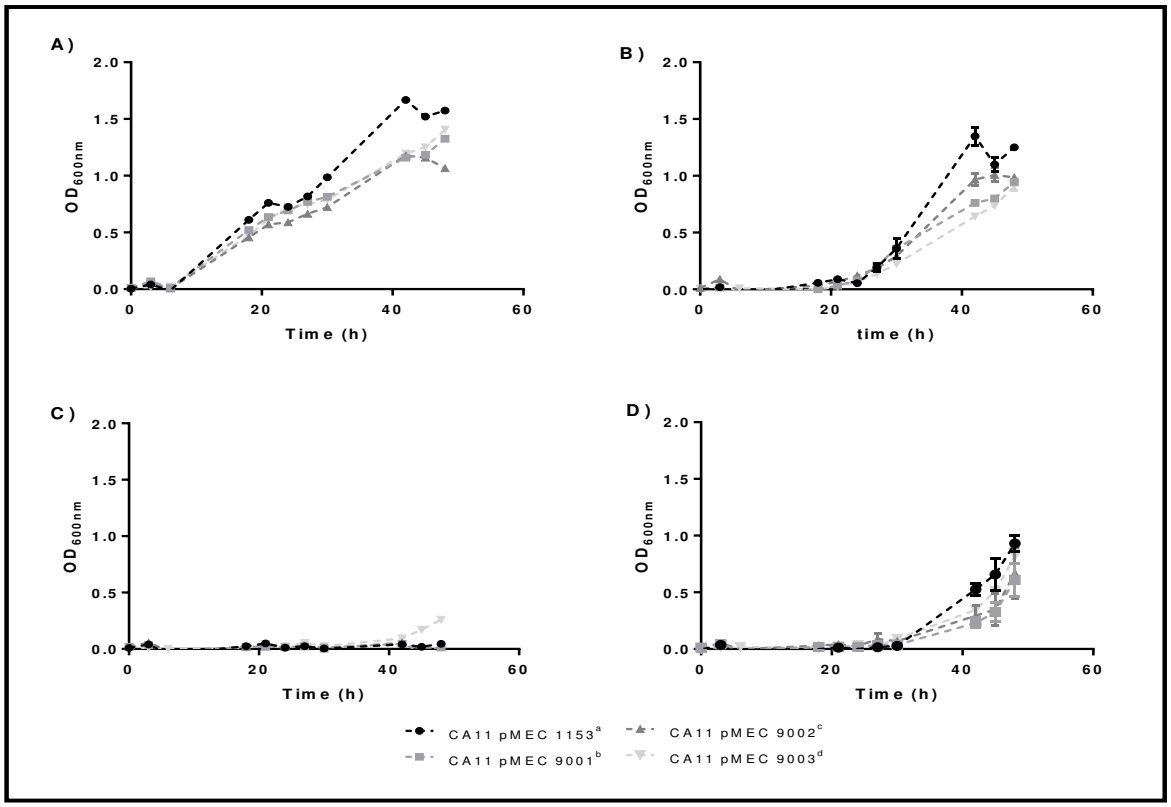

Figure 3.8: Aerobic growth of *S. cerevisiae* CA11 recombinant strains in: A) YPX medium. a,b**; a,c**; a,d**; c,d**. B) YPX with 1 g/L of furfural. a,b*; a,d*; b,d*; c,d*.C) YPX with 4 g/L of acetic acid. a,d**; b,d***; c,d**. D) YPX with 1 g/L of furfural and 3 g/L of acetic acid. pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

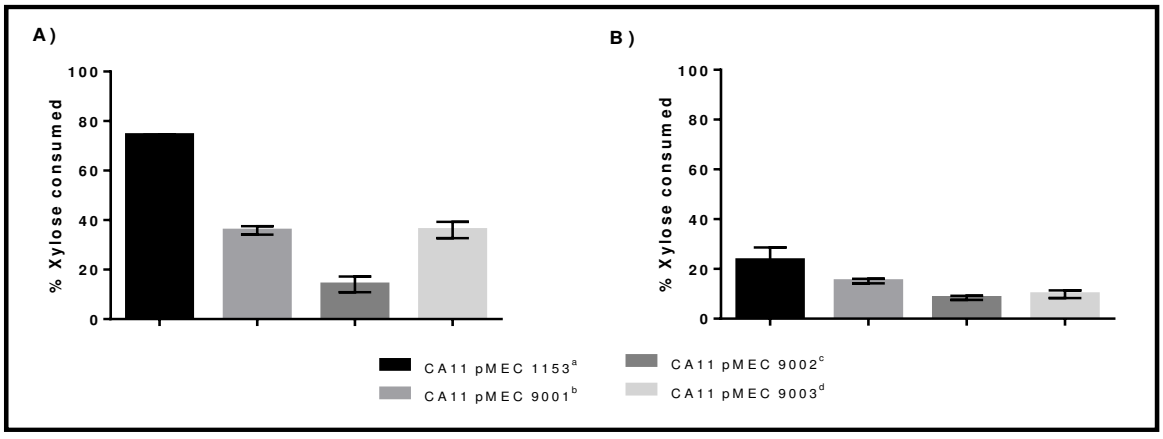

Figure 3.9: Percentage of xylose consumed at 48 hours by *S. cerevisiae* CA11 recombinant strains in: A) YPX medium. a,b**; a,c***; a,d**; b,c *; c,d*. B) YPX with 1 g/L of furfural. pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

3.2.2. PE-2 Δ*GRE3* recombinant Strains

All strains showed a similar growth in YPX and YPX supplemented with 1 g/L of furfural (Figure 3.10 A and B), despite that, in medium supplemented with 1 g/L of furfural all strains showed a longer lag phase. However, in both media, the strain overexpressing *HAA1* and *PRS3* (PE-2 Δ*GRE3* pMEC9003) had a significant lower percentage of xylose consumed (Figure 3.11 A and B) than the other strains. Regarding to YPX medium supplemented with 1 g/L of furfural and 3 g/L of acetic acid, all strains overexpressing *HAA1* and/or *PRS3* genes, showed higher growth rates (Figure 3.10 D) and higher capacity to consume xylose (Figure 3.11 D) than the control strain. Furthermore, in xylose medium supplemented with 4 g/L of acetic acid, the three recombinant strains showed higher performances than the control. Moreover, PE-2 Δ*GRE3* pMEC9003, strain overexpressing *HAA1* and *PRS3* genes simultaneously, showed an improved growth capacity (Figure 3.10 C) and a higher ability to consume xylose (Figure 3.11 C) than strains overexpressing only one of the genes.

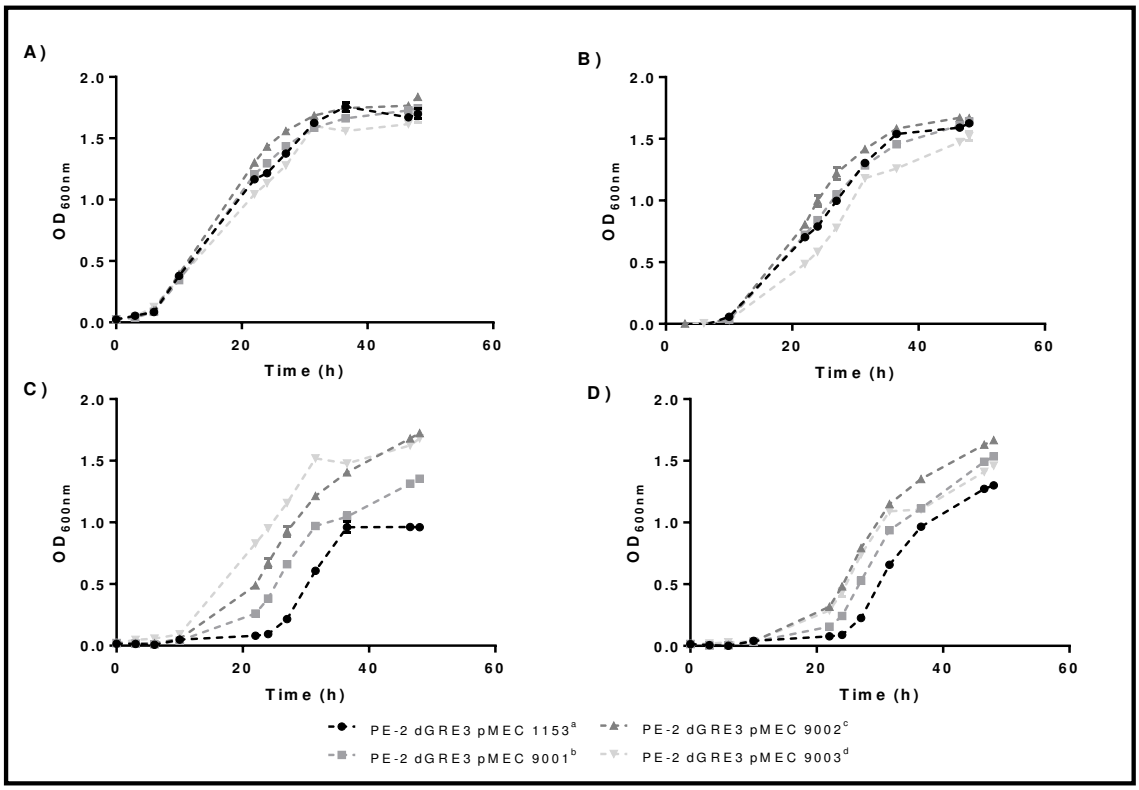

Figure 3.10: Aerobic growth of *S. cerevisiae* PE-2 Δ*GRE3* recombinant strains in: A) YPX medium. a,c*; a,d*; b,d*; c,d**. B) YPX with 1 g/L of furfural. a,d**; b,d**; c,d**.C) YPX with 4 g/L of acetic acid. a,b**; a,c**; a,d***; b,d**; c,d*.D) YPX with 1 g/L of furfural and 3 g/L of acetic acid. a,b**; a,c**; a,d**; c,d*. pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

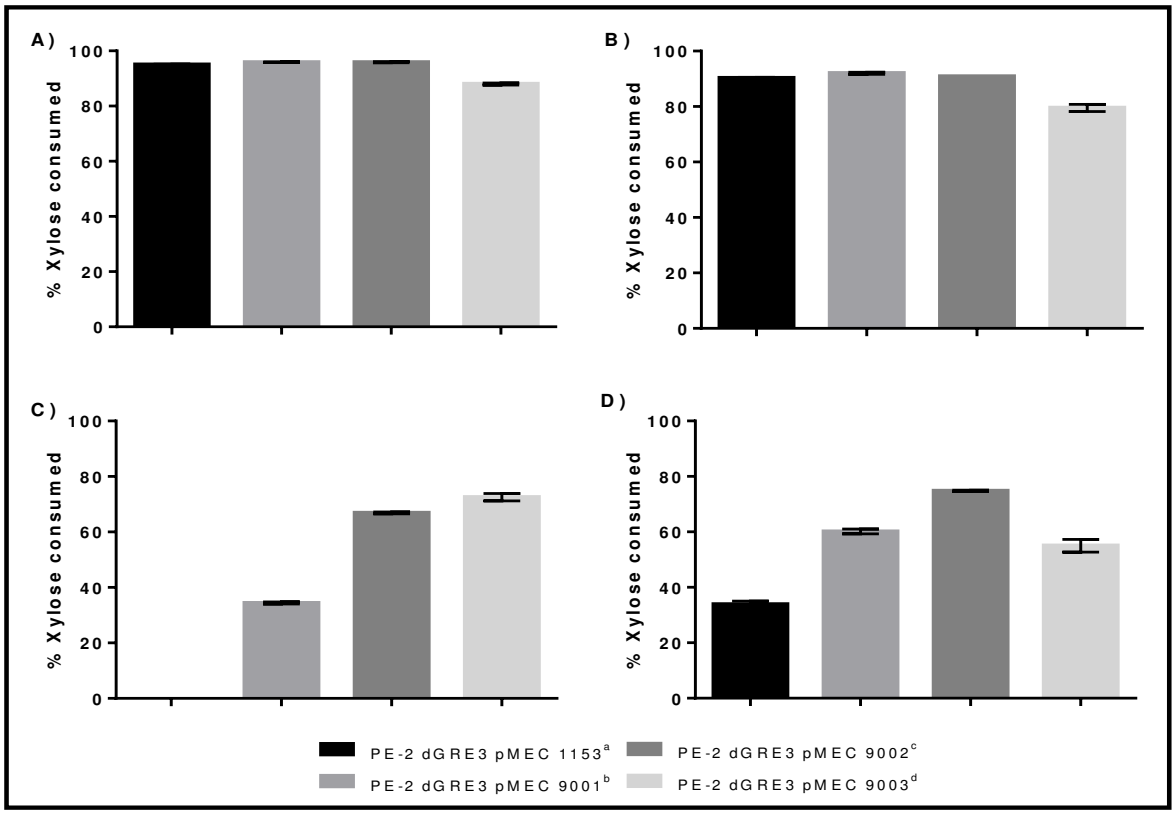

Figure 3.11: Percentage of xylose consumed at 48 hours by S. *cerevisiae* PE-2 Δ*GRE3* recombinant strains in: A) YPX medium. a,d***; b,d ****; c,d ****. B) YPX with 1 g/L of furfural. a,c**; b,d**; c,d**. C) YPX with 4 g/L of acetic acid. a,b****; a,c****; a,d****; b,c****; b,d ****; c,d*. D) YPX with 1 g/L of furfural and 3 g/L of acetic acid. a,b***, a,c***; a,d**; b,c**; c,d**. pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

3.3. Shake-flask fermentations

The effect of the overexpression of *HAA1* and *PRS3* genes was also evaluated in shake-flask fermentations in YPX supplemented with 1 g/L of furfural, in YPX with 1 g/L of furfural and 3 g/L of acetic acid and in an EGW hydrolysate (2.4). As mentioned before, fermentations assays were carried out in oxygen-limited conditions in order to recreate industrial conditions.

3.3.1. CA11 recombinant strains

3.3.1.1. YPX medium supplemented with furfural

Figure 3.12 shows the time-course of the concentration of the main metabolites (xylose, ethanol and xylitol). The strain CA11 pMEC1153 consumed higher amounts of xylose than the recombinant

strains and produced higher ethanol concentrations (Figure 3.12 A and B). However, *HAA1* and *PRS3* overexpressing strains show lower levels of xylitol accumulation than the control strain (Figure 3.12 C). The overexpression of *PRS3* resulted in lower levels of xylose consumption and this result is reflected in a lower production of ethanol. On the other hand, this strain had a significantly higher biomass yield (Table 3.1). Regarding to furfural concentrations, it was verified that all yeast strains were able to detoxify it within the first 19 hours (data not shown).

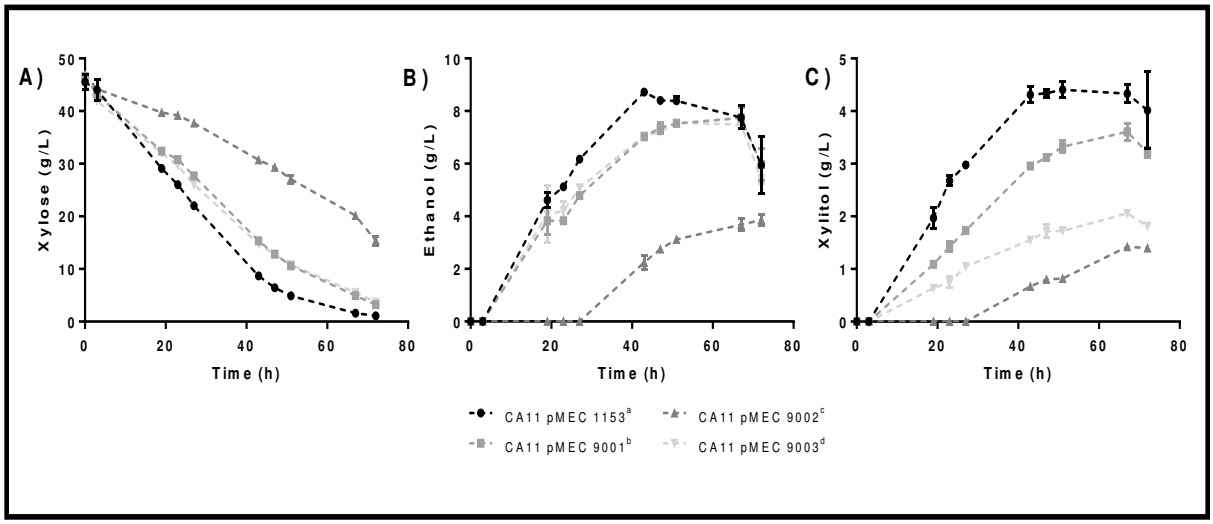

Figure 3.12: Time-course evolution of the main metabolites during CA11 recombinant strains fermentation in the YPX medium supplemented with 1 g/L of furfural. A) Xylose concentration. a,b*; a,c**; a,d*; cd**.B) ethanol concentration. a,b*; a,c**; c,d** C) xylitol concentration. a,b*; a,c**; a,d**; b,d**; c,d** pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

HAA1 and *PRS3* overexpressing strains showed a significantly lower xylose consumption rates than the control strain. The overexpressing of *HAA1* resulted in similar xylose consumption rates comparing to the overexpression of both genes (Figure 3.13 A). Regarding to ethanol productivity, the simultaneous overexpression of *HAA1* and *PRS3* resulted in similar levels comparing to those obtained in the control strain, while the overexpression of *HAA1* or *PRS3* have led to a lower ethanol productivity (Figure 3.13 B). CA11 pMEC1153, CA11pMEC 9001 and CA11pMEC9003 showed similar results in terms of ethanol yield (Figure 3.13 C). Finally, the overexpression of both genes led to a significantly lower xylitol yield comparing to other strains (Figure 3.13 D).

The overexpression of *HAA1* and *PRS3* simultaneously had similar effects that the overexpression of only *HAA1*. CA11 pMEC9001 and CA11 pMEC9003 showed similar results in terms of xylose consumption rate, ethanol productivity and ethanol yield (Figure 3.13 A, B and C). However, the strain

overexpressing *HAA1* and *PRS3* simultaneously showed a significant lower xylitol yield (Figure 3.13 D). The strain overexpressing only *PRS3* had a lower xylose consumption rate and no ethanol production in the first 23 hours of fermentation (Figure 3.13 A and B). Furthermore, this strain showed a much higher final xylose concentration and a much lower concentration of maximum ethanol production (Table 3.1). CA11 pMEC9001 and CA11 pMEC9003 also showed similar results in terms of final xylose concentration and maximum ethanol production (Table 3.1).

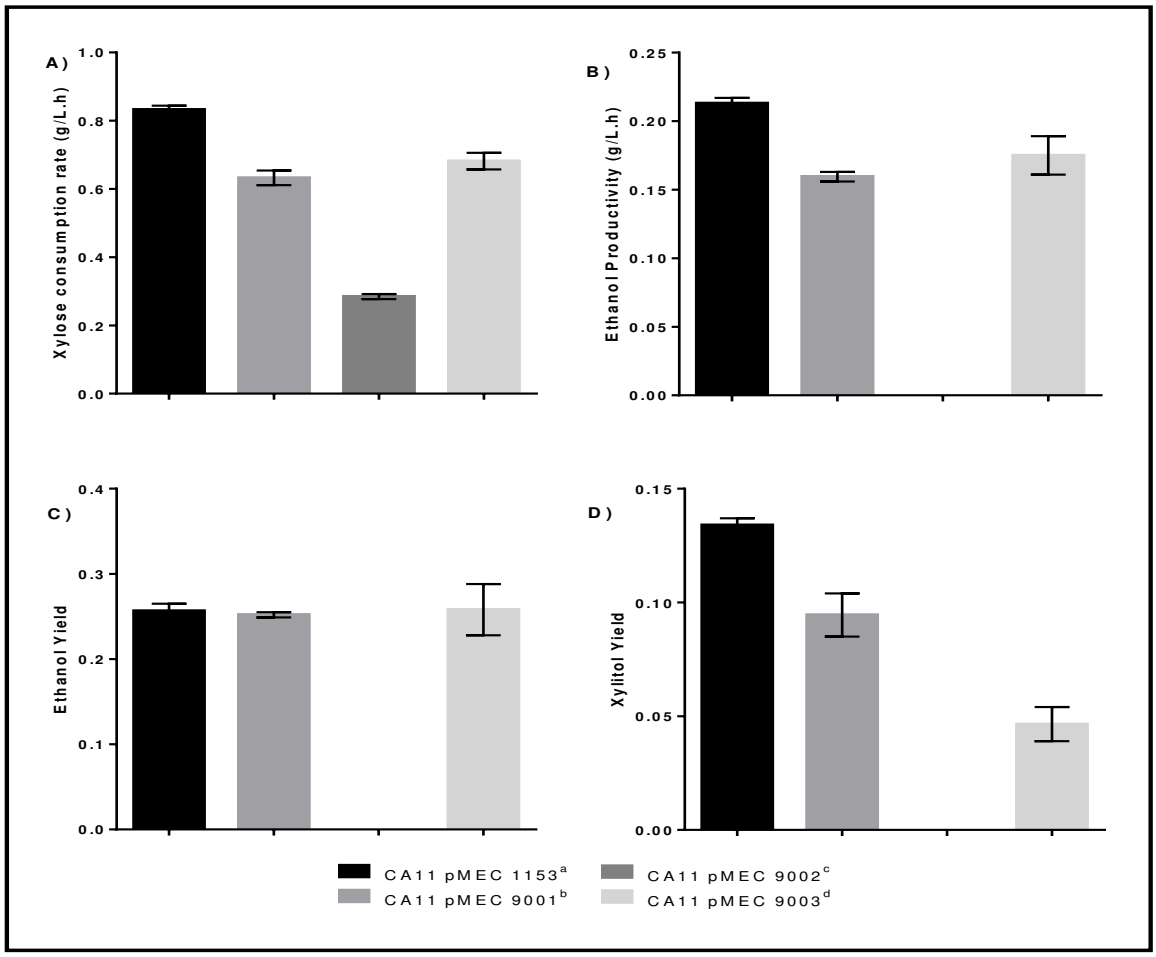

Figure 3.13: Fermentation parameters calculated at 23 hours for CA11 recombinant strains fermentation in the YPX medium supplemented with 1 g/L of furfural. A) Xylose consumption rate (g/L.h). a.b**; a,c***; a,d*; b,c***; c,d***. B) ethanol productivity (g/L.h). a,b*; a,c***; b,c***; c,d***.C) ethanol yield. a,c**; b,c**; c,d**. D) xylitol yield. a,c***; a,d**; b,c**; b,d*; c,d*. pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 3.1: Fermentation parameters of CA11 recombinant strains in YPX with 1 g/L of furfural during fermentation time. Significant differences between the overexpressing strains and the control strain are indicated by (*). Significant differences between the strains overexpressing *HAA1* and *PRS3* simultaneously (d) and the strains overexpressing only *HAA1* (b) or *PRS3* (c) were as follows: final xylose c,d***; maximum ethanol c,d***, biomass yield b,d*, c,d****. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

	CA11 pMEC1153	CA11 pMEC9001	CA11 pMEC9002	CA11 pMEC9003
Final Xylose (g/L)	1.103±0.386	3.211±0.080	15.35±0.88***	3.698±0.062
Maximum Ethanol (g/L)	8.723±0.022	7.736±0.406*	3.862±0.215****	7.557±0.078*
Maximum Xylitol (g/L)	4.408±0.151	3.605±0.163*	1.421±0.046***	2.055±0.031***
Biomass yield	0.340±0.003	0.343±0.001	0.395±0.002****	0.327±0.001*

3.3.1.2. YPX medium supplemented with furfural and acetic acid

Figure 3.14 shows the concentration of the main metabolites during fermentation time. It was verified that all strains except CA11 pMEC9003 showed a similar xylose consumption during fermentation time (Figure 3.14 A). However, CA11 pMEC1153 produced higher levels of ethanol than the recombinant strains (Figure 3.14 B).

In terms of acetic acid (Figure 3.14 C), is possible to see that it is produced by all strains until approximately 70 hours of fermentation. Regarding to xylitol levels (Figure 3.14 D), the strain overexpressing *HAA1* and *PRS3* presented lower production. Total furfural detoxification was achieved before 24 hours of fermentation (data not shown).

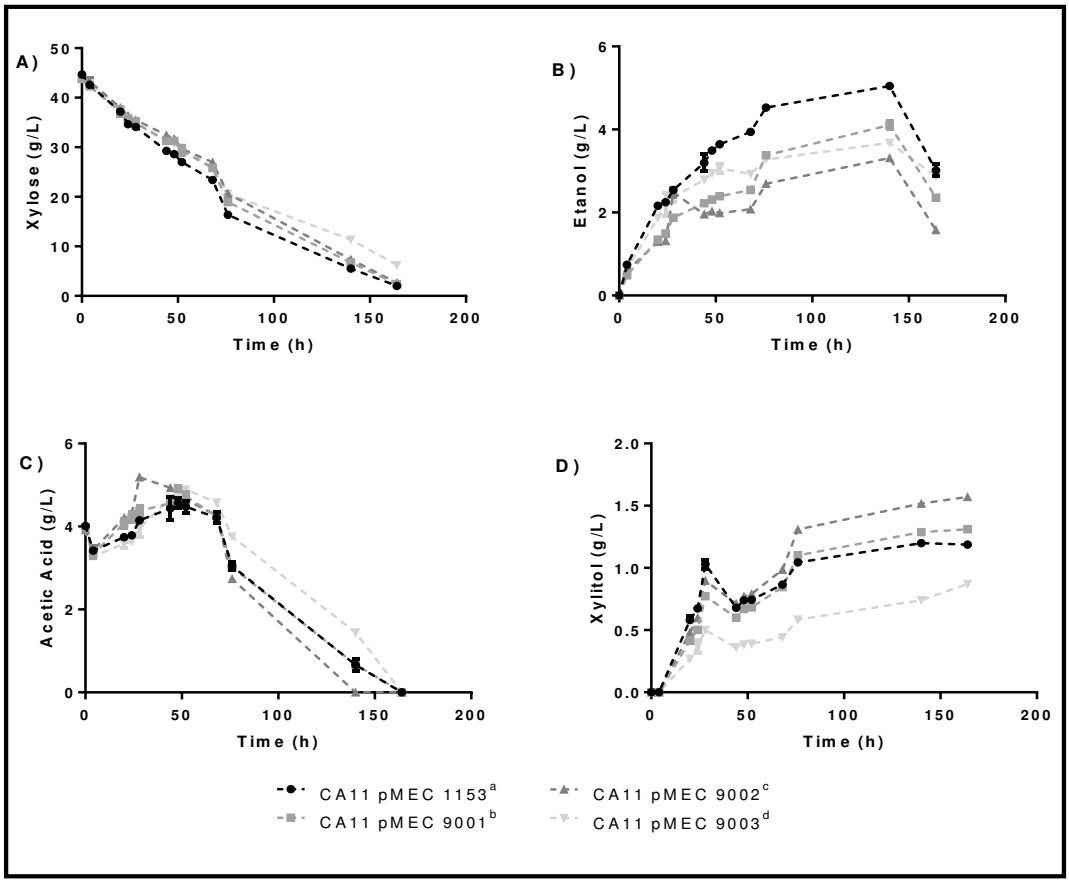

Figure 3.14: Time-course evolution of the main metabolites during CA11 strains recombinant fermentation in the YPX medium supplemented with 1 g/L of furfural and 3 g/L of acetic acid. A) Xylose concentration. a,c*. B) ethanol concentration. a,b**; a,c***; a,d**; d,b***; c,d***. C) acetic acid concentration. D) xylitol concentration. a,b**; a,c**; a,d**; b,d**; c,d***.pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Concerning the main fermentation parameters, shown in Figure 3.15, there were no significant differences between all strains in terms of xylose consumption rate, ethanol productivity or ethanol yield (Figure 3.15 A, B and C). However, CA11 pMEC9003 showed a significantly lower xylitol yield than other strains (Figure 3.15 D).

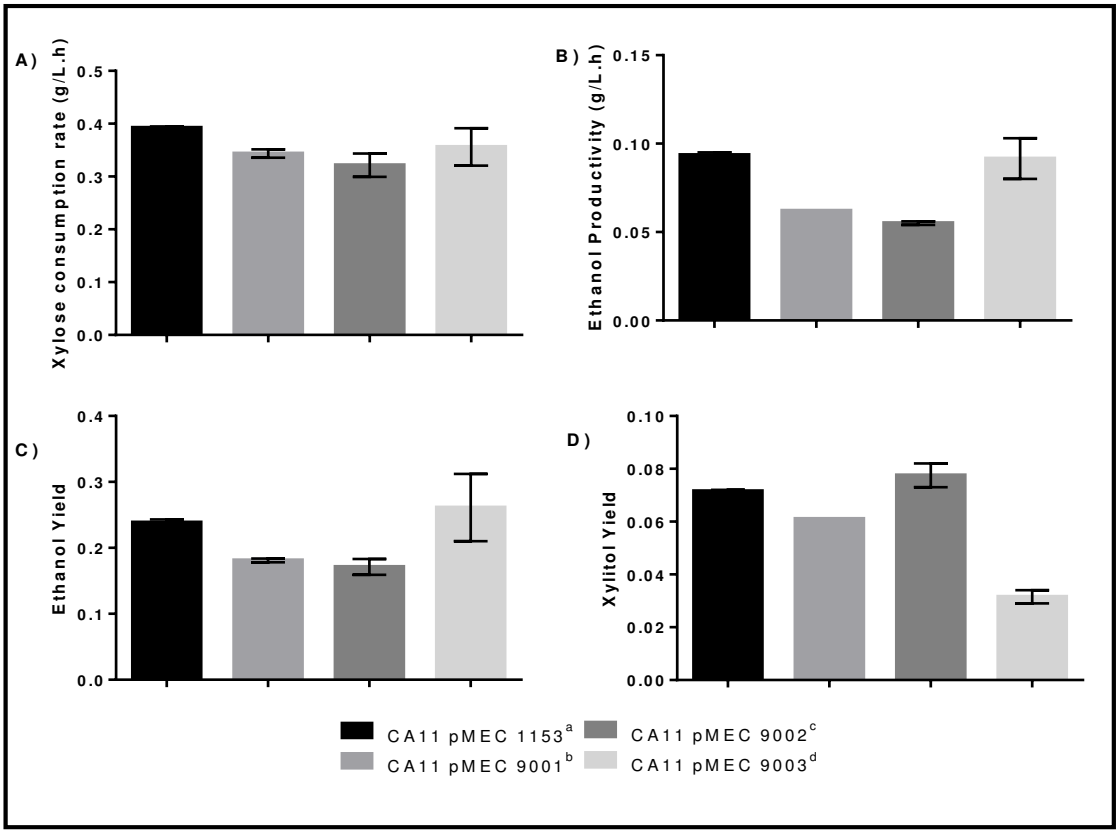

Figure 3.15: Fermentation parameters calculated at 23 hours for CA11 recombinant strains fermentation in the YPX medium supplemented with 1 g/L of furfural and 3 g/L of acetic acid. A) Xylose consumption rate (g/L.h). B) ethanol productivity (g/L.h). C) ethanol yield. D) xylitol yield. a,d**; b,d**; c,d**. pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 3.2: Fermentation parameters of CA11 recombinant strains in YPX with 1 g/L of furfural and 3 g/L of acetic acid during fermentation time. Significant differences between the strains overexpressing *HAA1* and *PRS3* simultaneously (d) and the strains overexpressing only *HAA1* (b) or *PRS3* (c) were as follows: final xylose b,d**, c,d**; maximum xylitol b,d***, c,d****; biomass yield b,d****, c,d****. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

	CA11 pMEC1153	CA11 pMEC9001	CA11 pMEC9002	CA11 pMEC9003
Final Xylose (g/L)	2.002±0.100	2.415±0.061	2.658±0.092	6.231±0.384***
Maximum Ethanol (g/L)	5.128±0.032	4.176±0.090	3.368±0.074	3.740±0.040
Maximum Xylitol (g/L)	1.198±0.011	1.131±0.017	1.572±0.004**	0.8705±0.0195**
Biomass yield	0.272±0.001	0.288±0.000****	0.306±0.001****	0.230±0.000****
Acetic acid initial (g/L)	4.014±0.044	3.977±0.045	3.919±0.025*	3.908±0.054
Maximum Acetic acid (g/L)	4.561±0.118	4.917±0.019	5.186±0.060	4.895±0.018

3.3.2. PE-2 Δ*GRE3* recombinant strains

3.3.2.1. YPX medium supplemented with furfural

Regarding to the results achieved with PE-2 Δ*GRE3* strains in xylose medium supplemented with 1 g/L of furfural, xylose consumption profiles were similar for strains overexpressing *HAA1* or *PRS3* genes and control strain while the strain overexpressing the two genes simultaneously (PE-2 Δ*GRE3* pMEC9003 strain) showed a slower xylose consumption and produced the lowest ethanol concentration (Figure 3.16 A and B). Furthermore, simultaneous overexpression of both genes resulted, once more, in significant lower levels of xylitol accumulation (Figure 3.16 C). In terms of furfural, all yeast strains were able to detoxify it within the first 3 hours of fermentation (data not shown).

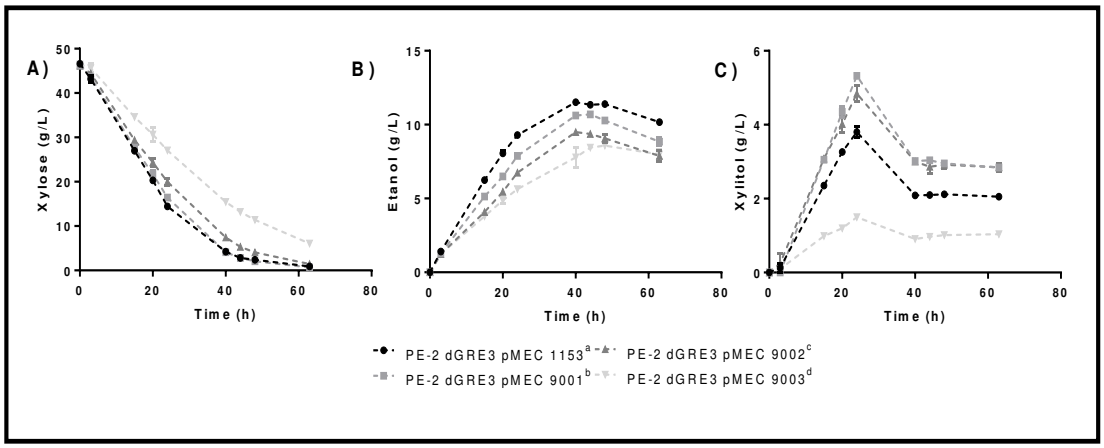

Figure 3.16: Time-course evolution of the main metabolites during PE-2 Δ*GRE3* recombinant strains fermentation in the YPX medium supplemented with 1 g/L of furfural. A) Xylose concentration. a,c*; a,d**; b,d**; c,d**. B) ethanol concentration. a,b*; a,c**; a,d**; b,d**; c,d*. C) xylitol concentration. a,b**; a,c*; a,d**; b,d***; c,d**. pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 3.17 presents the main parameters calculated at 24 hours of fermentation. In terms of xylose consumption rate (Figure 3.17 A), the strain overexpressing *HAA1* gene, PE-2 Δ*GRE3* pMEC9001 showed similar results to the control strain while the other two recombinant strains showed a slower xylose consumption rate. Nevertheless, all strains showed significantly different results regarding to ethanol productivity (Figure 3.17 B) being the control strain the strain with higher ability to produce ethanol in the first 24 hours. Concerning to ethanol yield, PE-2 Δ*GRE3* pMEC1153 and pMEC9003 showed similar results (Figure 3.17 C). In terms of xylitol yield, PE-2 Δ*GRE3* pMEC1153 and pMEC9003 showed also similar results with lower xylitol levels than strains overexpressing *HAA1* and *PRS3* separately (Figure 3.17 D). Regarding the maximum xylitol produced during the fermentation, the simultaneous

overexpression of *HAA1* and *PRS3* resulted in significantly lower levels of xylitol accumulation than the other strains (Table 3.3).

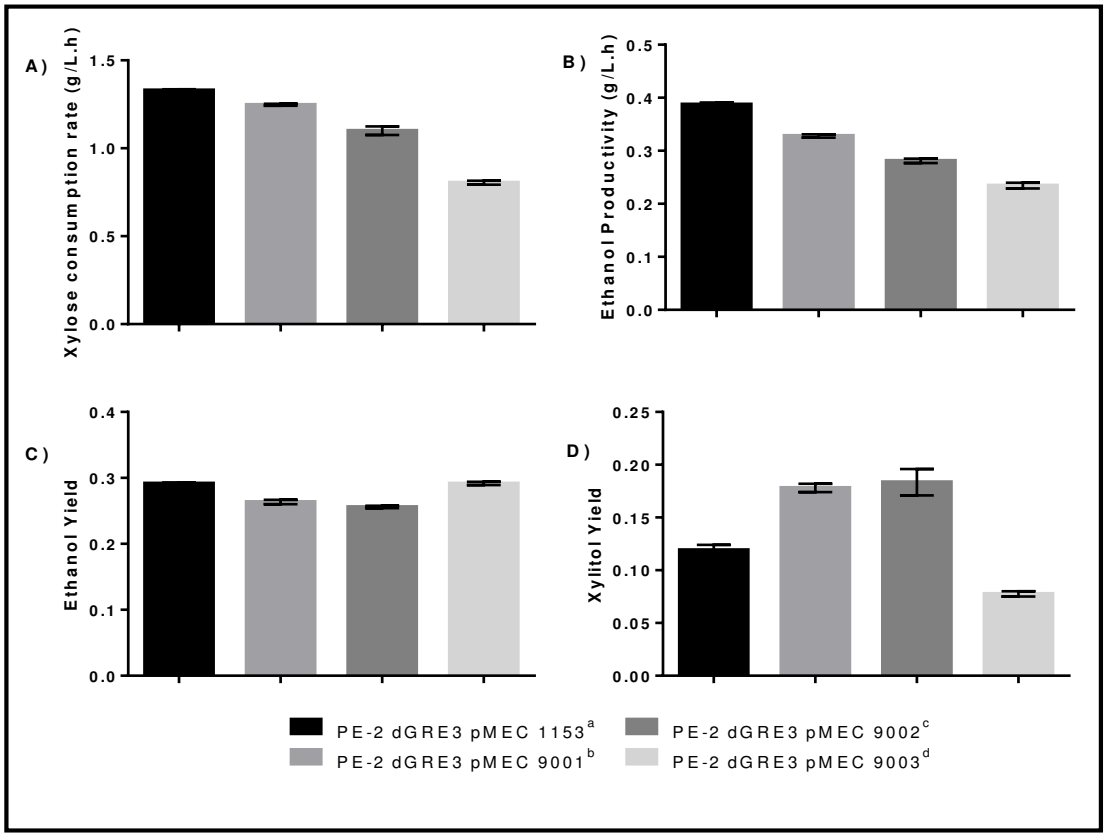

Figure 3.17: Fermentation parameters calculated at 24 hours for PE-2 Δ*GRE3* recombinant strains fermentation in the YPX medium supplemented with 1 g/L of furfural. A) Xylose consumption rate (g/L.h). a,c**; a,d****; b,c*; b,d***; c,d***. B) Ethanol productivity (g/L.h). a,b**; a,c***; a,d****; b,c**; b,d***; c,d **. C) Ethanol yield. a,b**; a,c**; b,c**; c,d**. D) Xylitol yield. a,b*; a,c*; b,d**; c,d**. pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 3.3: Fermentation parameters of PE-2 Δ*GRE3* recombinant strains in YPX with 1 g/L of furfural during fermentation time. Significant differences between the strains overexpressing *HAA1* and *PRS3* simultaneously (d) and the strains overexpressing only *HAA1* (b) or *PRS3* (c) were as follows: final xylose b,d****, c,d****; maximum ethanol b,d***, c,d**; maximum xylitol b,d***, c,d***; biomass yield b,d*. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

	PE-2 Δ*GRE3* pMEC1153	PE-2 Δ*GRE3* pMEC9001	PE-2 Δ*GRE3* pMEC9002	PE-2 Δ*GRE3* pMEC9003
Final Xylose (g/L)	0.891±0.016	0.676±0.006	1.44±0.08*	6.06±0.14****
Maximum Ethanol (g/L)	10.17±0.19	10.69±0.01*	9.497±0.021***	8.585±0.073****
Maximum Xylitol (g/L)	3.803±0.139	5.324±0.085**	4.840±0.220*	1.499±0.072**
Biomass yield	0.242±0.002	0.278±0.002**	0.288±0.003***	0.296±0.000***

3.3.2.2. YPX medium supplemented with furfural and acetic acid

The concentrations of the main metabolites during fermentation in media containing 1 g/L of furfural and 3 g/L of acetic acid are presented on Figures 3.18. Analysing this figure, it is notorious that all strains presented similar xylose consumption and all were unable to completely metabolize the xylose present in the medium (arresting the consumption at 40 hours). Furthermore, all the strains produced acetic acid at an initial stage of fermentation, the overexpressing strains during 40 hours (reaching ca. 6 g/L) and the control strain during 24 hours (reaching ca. 5 g/L) (Figure 3.18 C). Nonetheless, all overexpressing strains reached higher levels of maximum ethanol production comparing to the control strain (Figure 3.18 B) during fermentation time (Table 3.4). Furthermore, after 3 hours of fermentation, furfural was completely detoxified (data not shown).

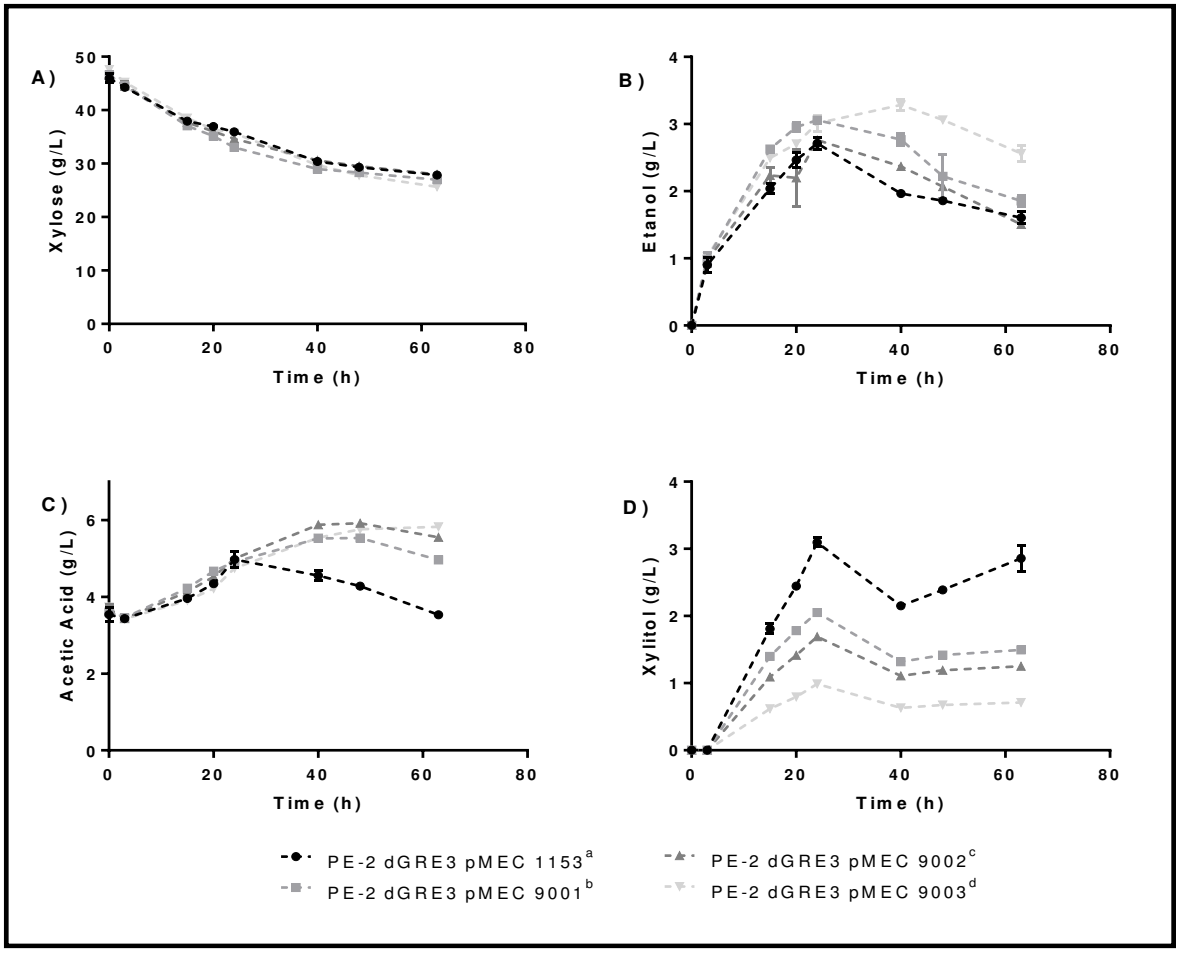

Figure 3.18: Time-course evolution of the main metabolites during PE-2 Δ*GRE3* recombinant strains fermentation in the YPX medium supplemented with 1 g/L of furfural and 3 g/L of acetic acid. A) Xylose concentration. a,b**. B) ethanol concentration. a,b**; a,d**; b,d*; c,d*. C) acetic acid concentration. a,b**; a,c*; a,d**. D) xylitol concentration. a,b***; a,c***; a,d***; b,d***; c,d** pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Despite the incomplete xylose consumption, and considering the first 24 hours of fermentation (Figure 3.18), the strains overexpressing *HAA1* (exclusively or simultaneously with *PRS3*) seem to present higher ethanol productivities (Figure 3.19 B). In terms of xylose consumption rate after the first 24 hours (Figure 3.19 A) there were no significant differences between all strains. Regarding ethanol yield the overexpression of *HAA1* and/or *PRS3* had no effect (Figure 3.19 C). As observed before, the *HAA1* and *PRS3* overexpressing strains showed significant lower levels of xylitol yield in the first 24 hours of fermentation (Figure 3.19 D) and maximum xylitol (Table 3.4).

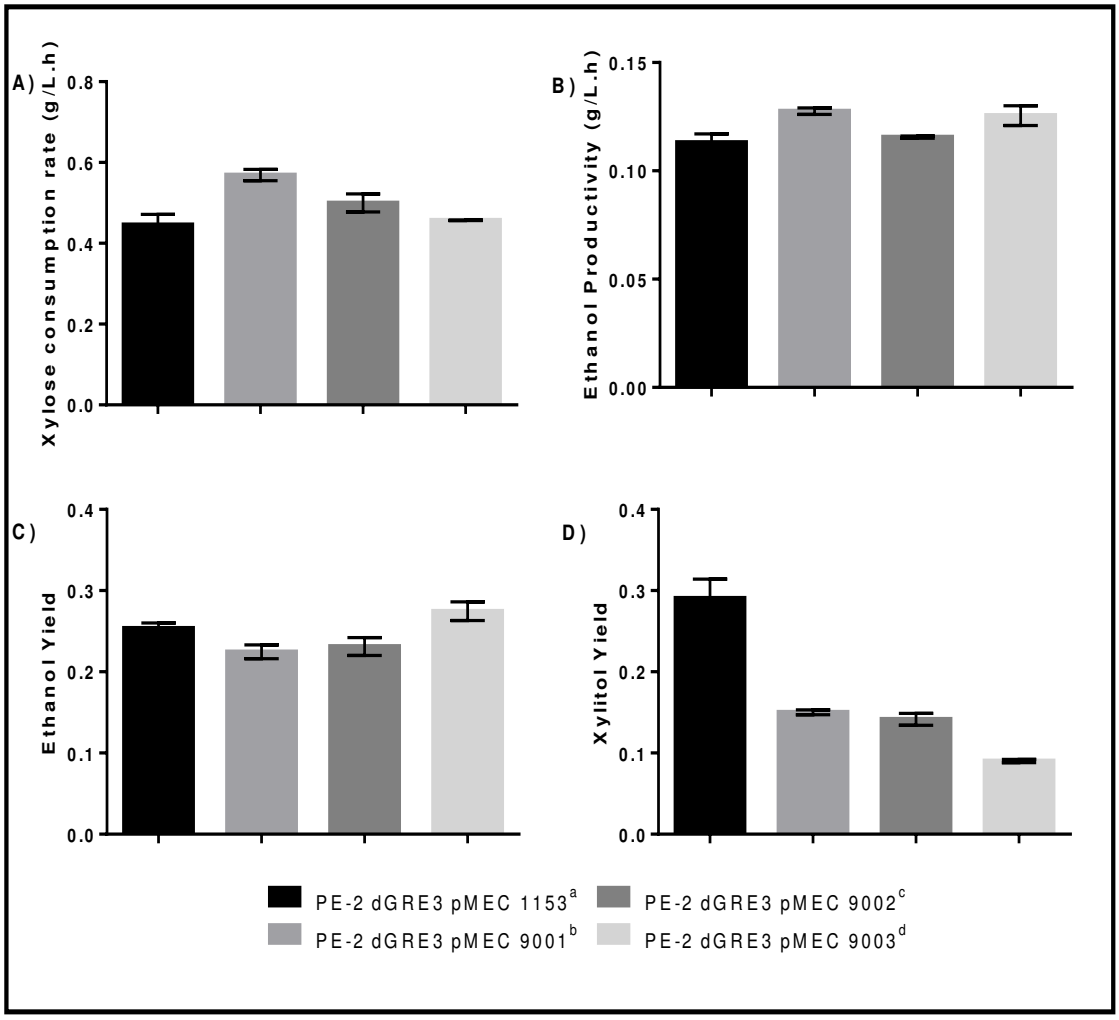

Figure 3.19: Fermentation parameters calculated at 24 hours for PE-2 Δ*GRE3* recombinant strains fermentation in the YPX medium supplemented with 1 g/L of furfural and 3 g/L of acetic acid. A) Xylose consumption rate (g/L.h). B) ethanol productivity (g/L.h). C) ethanol yield. D) xylitol yield. a,b**; a,c**; a,d **. pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 3.4: Fermentation parameters of PE-2 Δ*GRE3* recombinant strains in YPX with 1 g/L of furfural and 3 g/L of acetic acid during fermentation time. Significant differences between the strains overexpressing *HAA1* and *PRS3* simultaneously (d) and the strains overexpressing only *HAA1* (b) or *PRS3* (c) were as follows: final xylose c,d*; maximum ethanol c,d*; maximum xylitol b,d***, c,d**. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

	PE-2 Δ*GRE3* pMEC1153	PE-2 Δ*GRE3* pMEC9001	PE-2 Δ*GRE3* pMEC9002	PE-2 Δ*GRE3* pMEC9003
Final Xylose (g/L)	27.850±0.443	26.964±0.047	27.989±0.044	25.627±0.0416*
Maximum Ethanol (g/L)	2.713±0.087	3.058±0.039	2.762±0.011	3.283±0.088*
Maximum Xylitol (g/L)	3.097±0.070	2.051±0.010***	1.693±0.017****	0.9880±0.0180****
Biomass yield	0.271±0.012	0.262±0.002	0.273±0.006	0.233±0.005
Acetic acid initial (g/L)	3.537±0.181	3.601±0.036	3.685±0.128	3.737±0.008
Maximum Acetic acid (g/L)	4.972±0.212	5.531±0.074	5.920±0.028*	5.826±0.029*

3.3.2.3. EGW Hydrolysate

The role of *HAA1* and/or *PRS3* overexpression was also analysed in *Eucalyptus globulus* wood hydrolysate (Figure 3.20). In this medium, all the strains consumed approximately all the xylose (residual < 2 g/L) (Figure 3.20 A and Table 3.5). Regarding the acetic acid concentration, none of the strains produced this compound (Figure 3.20 C), and all the strains demonstrated the same behaviour, reducing this weak acid to lower concentrations than the initially present in the fermentation medium. Furfural concentrations were reduced, by yeast strains, to zero after 4 hours of fermentation (data not shown).

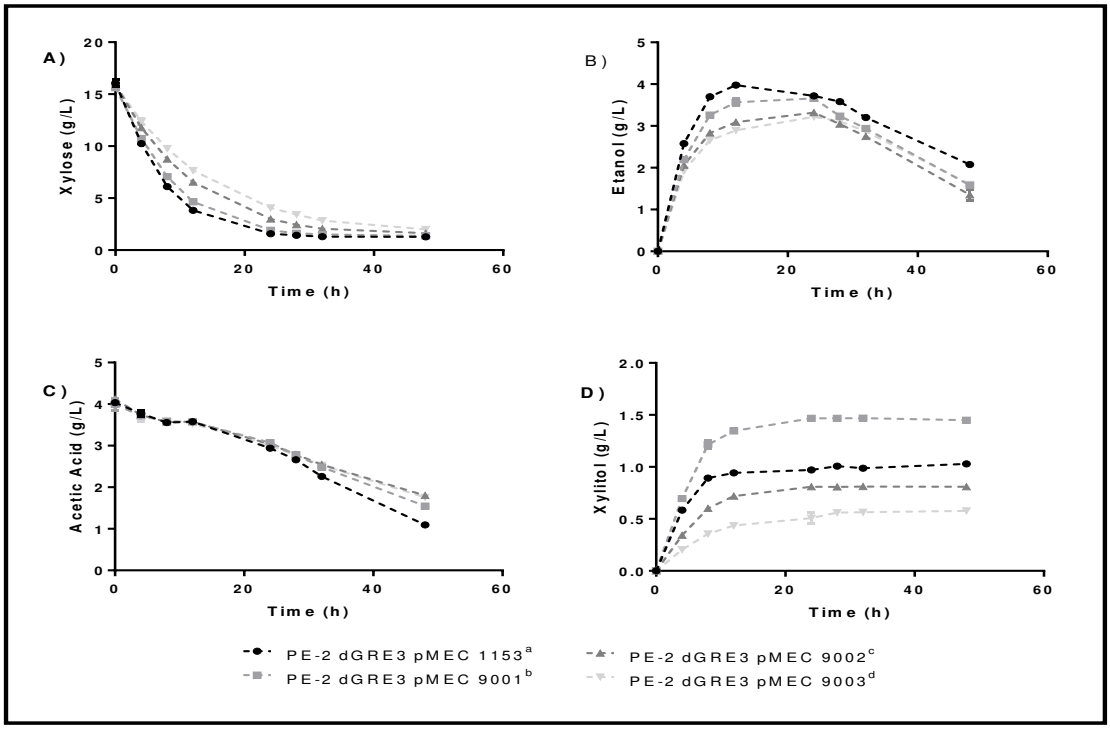

Figure 3.20: Time-course evolution of the main metabolites during PE-2 Δ*GRE3* recombinant strains fermentation in EGW hydrolysates. A) Xylose concentration. a,c**; a,d**; b,d**; c,d**.B) ethanol concentration. a,b*; a,c**; a,d**; b,d*. C) acetic acid concentration. D) xylitol concentration. a,b***; a,c***; a,d***; b,d***; c,d***. pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Observing Figure 3.21 is possible to understand that the overexpression of *HAA1* and/or *PRS3* in PE-2 Δ*GRE3* strains did not lead to improvements on yeast resistance to inhibitors. In fact, PE-2 Δ*GRE3* pMEC1153 showed significantly higher levels of xylose consumption rate (Figure 3.21 A). In terms of ethanol productivity, the PE-2 Δ*GRE3* pMEC1153 and pMEC9001 showed higher productivities than the other two strains (Figure 3.21 B). Regarding to ethanol yield, there were no significant differences between all strains tested (Figure 3.21 C). As observed in the previous fermentations, the simultaneous overexpression of *HAA1* and *PRS3* in the PE-2 Δ*GRE3* strain resulted in lower xylitol yield (Figure 3.21 D) and lower maximal production (Table 3.5).

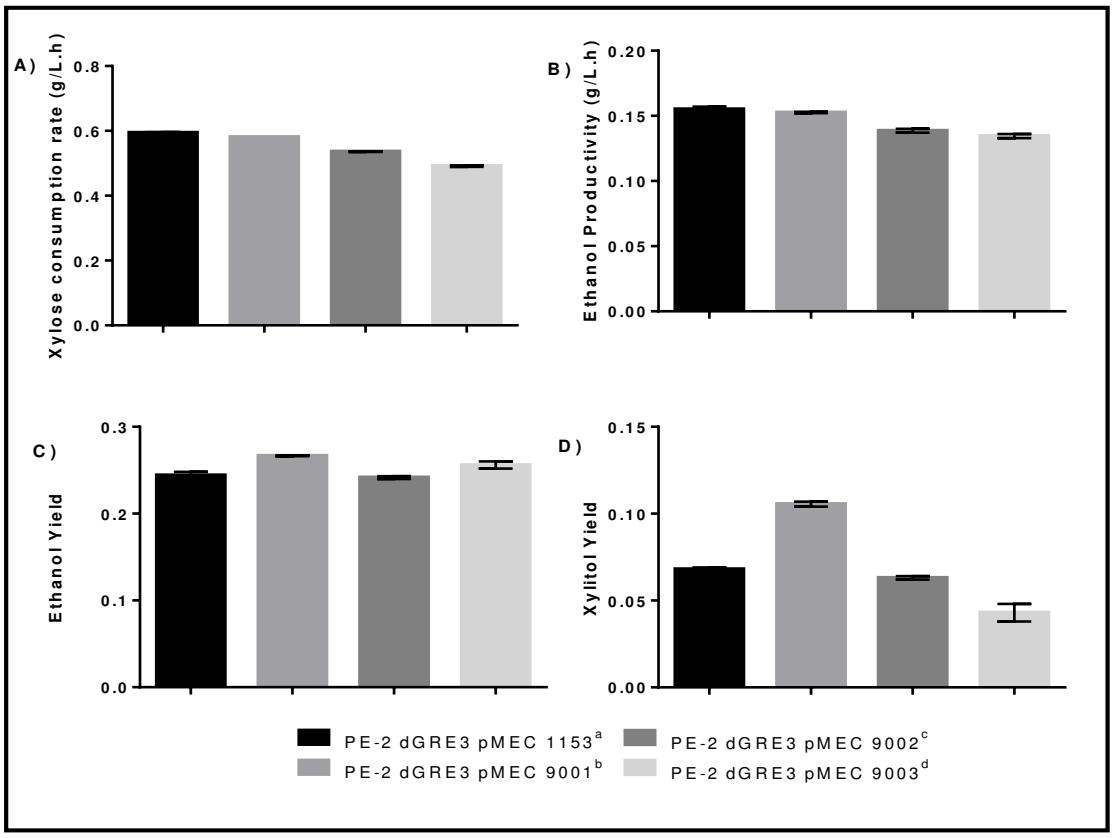

Figure 3.21: Fermentation parameters calculated at 24 hours for PE-2 Δ*GRE3* recombinant strains fermentation in EGW hydrolysates. A) Xylose consumption rate (g/L.h). a,b*; a,c****; a,d****; b,c***; b,d****; c,d***. B) ethanol productivity (g/L.h). a,c**; a,d**; b,c*; b,d**. C) ethanol yield. D) xylitol yield. a,b**; a,d*; b,c**; b,d***; c,d*. pMEC1153: plasmid with xylose metabolic pathway; pMEC9001: pMEC1153 plus *HAA1* gene; pMEC9002: pMEC1153 plus *PRS3* gene; pMEC9003: pMEC1153 plus *HAA1* and *PRS3* genes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 3.5: Fermentation parameters of PE-2 Δ*GRE3* recombinant strains in YPX in EGW hydrolysate. Significant differences between the strains overexpressing *HAA1* and *PRS3* simultaneously (d) and the strains overexpressing only *HAA1* (b) or *PRS3* (c) were as follows: final xylose b,d****, c,d***; maximum ethanol b,d**; maximum xylitol b,d****, c,d****; biomass yield b,d**. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

	PE-2 Δ*GRE3* pMEC1153	PE-2 Δ*GRE3* pMEC9001	PE-2 Δ*GRE3* pMEC9002	PE-2 Δ*GRE3* pMEC9003
Final Xylose (g/L)	1.275±0.006	1.363±0.013	1.624±0.022***	1.999±0.014****
Maximum Ethanol (g/L)	3.979±0.004	3.660±0.003**	3.320±0.030***	3.220±0.037***
Maximum Xylitol (g/L)	1.029±0.003	1.469±0.006****	0.8110±0.0070***	0.5775±0.0055****
Biomass yield	0.9634±0.0030	0.9761±0.0043	1.019±0.005*	1.043±0.010**
Acetic acid initial (g/L)	4.034±0.033	3.999±0.144	4.117±0.028	4.018±0.059
Maximum Acetic acid (g/L)	4.034±0.033	3.999±0.144	4.117±0.028	4.018±0.059

4. Discussion

Previous works performed by our research group described CA11 and PE-2 as two promising yeast strains for bioethanol production from lignocellulosic biomass (Pereira et al., 2010; Pereira, Guimarães, Teixeira, et al., 2011; Pereira, Romaní et al., 2014). These strains were isolated from a Brazilian "cachaça" fermentation process and from a bioethanol plant, respectively, and taking advantage of their robust background, they were genetically modified to efficiently consume xylose and to overexpress *HAA1* and *PRS3* genes. In order to understand the effects of *HAA1* and *PRS3* overexpression in these strains, aerobic growth tests and fermentation assays were carried out in xylose media containing lignocellulosic-derived inhibitors.

Results obtained regarding yeast aerobic growth showed that PE-2 Δ*GRE3* strains overexpressing these genes exhibited an increased capacity to grow and consume xylose in media containing stronger inhibitory conditions (YPX supplemented with 4 g/L of acetic acid and YPX supplemented with 1 g/L of furfural and 3 g/L of acetic acid). In fact, in the condition containing 4 g/L of acetic acid the simultaneous overexpression of *HAA1* and *PRS3* resulted in an increased culture growth and xylose consumption, indicating a putative synergetic effect of overexpressing both genes. On the other hand, CA11 strains overexpressing *HAA1* and *PRS3* did not show improvements in growth in any media containing inhibitors. Moreover, CA11 strains showed much longer lag phases than PE-2 Δ*GRE3* strains in the presence of inhibitors. In previous reports, (Sakihama et al., 2015), *HAA1* gene was also integrated in a xylose-fermenting *S. cerevisiae* harbouring *S. stipits* XR and XDH genes as well as endogenous XK gene. The results obtained with this recombinant *S. cerevisiae* strain in aerobic growth tests in xylose-containing medium also showed an increased resistance of yeast to acetic acid comparing to the control strain.

In fermentation assays performed in YPX medium supplemented with 1 g/L of furfural, the overexpression of *HAA1* and *PRS3* genes did not increased, or affected negatively, yeast fermenting capacity in both strains. It has been reported that the introduction and expression of foreign genes in a host organism may remove resources from host cell metabolism (Glick, 1995) and in these fermentations with a low inhibitory load the overexpression of *HAA1* and *PRS3* may have lead yeast to an unnecessary metabolic burden.

Regarding yeast performances in YPX medium supplemented with 1 g/L of furfural and 3 g/L of acetic acid, all CA11 strains were able to consume almost all of the xylose presented in the medium. However, in PE-2 Δ*GRE3* strains, none of the strains was able to efficiently consume the xylose presented in the medium and consumption stopped at 40 hours. This incomplete fermentation seems to be related with the amounts of acetic acid presented in fermentation medium. Acetic acid is a by-product of *S. cerevisiae* alcoholic fermentation and, in higher concentrations may contribute to the fermentation arrest and reduced ethanol productivity (Casey et al., 2010; Garay-Arroyo et al., 2004; Rasmussen et al., 1995). This result

highlight the importance of using an integrated approach when evaluating genetic engineering of yeast for improved bioethanol production, considering the several drawbacks of the process.

Despite PE-2 Δ*GRE3* pMEC1153 has produced lower amounts of acetic acid than other strains, its fermentation was arrested as well, which may indicate that the overexpression *HAA1* and *PRS3* resulted in a higher tolerance to acetic acid. In terms of ethanol production, results show that the overexpression of the two genes in CA11 strains may not be advantageous in this condition, considering that overexpressing strains had worst performances than the control strain. However, results obtained with PE-2 Δ*GRE3* seem to point to an advantage of overexpressing *HAA1*, principally when combined with *PRS3*, in the presence of furfural and acetic acid. A positive effect of overexpressing *PRS3* in PE-2 fermentations from glucose in lignocellulosic hydrolysates was previously reported by Cunha et al. (2015), which suggested that *PRS3* overexpression may contribute to increase the carbon flux in favour of metabolic pathways important for the regeneration of NADH, a cofactor required for furfural and HMF detoxification and for ethanol production (Figure 4.1). Furthermore, in the xylose-containing media studied in this work, high levels of NADH may be an advantage for the xylose metabolic pathway used in this work, which contains a mutated XR with higher specificity for NADH. Additionally, the positive effects observed with *HAA1* overexpression were expected, as this gene has been reported to be related with yeast resistance to acetic acid (Fernandes et al., 2005). Furthermore, the expression of *HAA1* gene was shown to lead to the reduction of the duration of the adaptation period of yeast cells exposed to toxic concentration of this acid, by decreasing the loss of cell viability occurring during the phase of latency (Fernandes et al., 2005). Tanaka et al. (2012) reported that the overexpression of *HAA1* gene in a laboratory strain led to a higher tolerance to acetic acid. Furthermore, *HAA1*p- regulated genes were shown to have specific activities restricting the influx of the acetic acid and/or promoting a more efficient acetate export. Additionally, the *HAA1* gene is responsible for the activation of SNF1 complex which is involved in carbo hydrate metabolism and necessary for switching from glucose to other carbon sources (Carlson, 1999; Hardie, Carling, & Carlson, 1998).

Regarding PE-2 Δ*GRE3* strains fermentation in EGW hydrolysate the overexpression of *HAA1* and/or *PRS3* did not show an improved performance. Despite presenting similar concentrations of furfural and acetic acid (compared to YPX medium supplemented with 1 g/L of furfural and 3 g/L of acetic acid), this hydrolysate contains other inhibitory compounds, such as HMF or phenolic compounds which may increase the inhibitory severity of this media when comparing to YPX supplemented with 1 g/L of furfural and 3 g/L of acetic acid). Furthermore, it was already reported that genes overexpression can result in different outcomes, in terms of yeast tolerance, depending on the inhibitory composition of the fermentation media (Cunha et al., 2015). Moreover, these differences can also be related with different initial cell concentrations

used. In fermentation performed in YPX medium supplemented with 1 g/L of furfural and 3 g/L of acetic acid, the initial cell concentration was 20 g of wet cells/L while in the fermentation performed in EGW hydrolysate it was 40 g of wet cells/L. Lower initial cell concentrations favour yeast growth while, the use of higher initial cell concentrations favours yeast fermenting capacities instead of yeast growth.

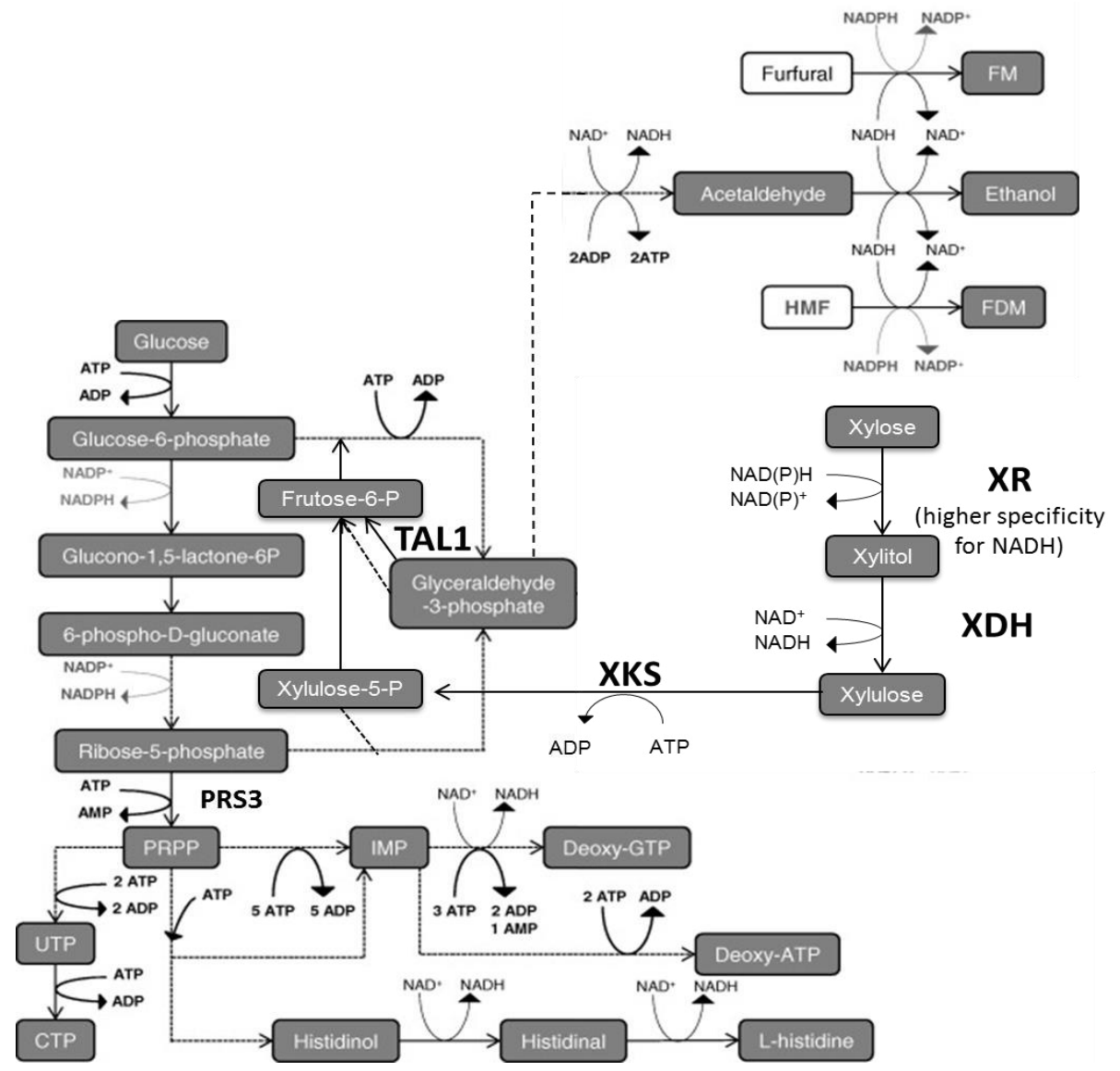

Figure 4.1: Schematic representation of the glucose and xylose metabolic pathways and conversion of furfural and HMF by *S. cerevisiae*. Adapted from Cunha et al. (2015).

Considering that, an initial concentration of 40 g of wet cells/L results in a reduced yeast growth, and the positive effect of genes overexpression in aerobic growth tests, where the initial cell concentration was lower, it might be advantageous, in terms of yeast tolerance and bioethanol production improvement, to use a smaller initial cell concentration.

The accumulation of xylitol, a by-product of fermentation from xylose, has a negative effect in terms of ethanol yields. Hahn-HäGerdal et al. (1991) have already reported that preference for NAD^+ and NADPH cofactors during xylose metabolization through XR/XDH/XK pathway might lead to redox imbalance and higher xylitol production. Despite the fact that a XR with higher specificity for NADH was used in work, xylitol accumulation still occurred. Furthermore, it was observed that the PE-2 Δ*GRE3* strains showed higher levels of xylitol production than CA11 strains, even with the deletion of the principal native route of xylitol formation (Δ*GRE3*). Nevertheless, the simultaneous overexpression of *HAA1* and *PRS3* in both strains showed significant lower levels of xylitol production in all fermentation media tested. These results could possibly be due to a redox balance obtained by the simultaneous overexpression of the genes.

It has been reported that yeast strains isolated from different environmental conditions have different fermentation performances (Francisco Pereira et al., 2014). Accordingly, in this work, differences were observed between CA11 and PE-2 Δ*GRE3* transformant strains in terms of tolerance to lignocellulosic inhibitors, with CA11 presenting lower tolerance capacity of furfural detoxification. Moreover, it has also been previously verified that the overexpression of the same genes in different yeast strains lead to different outcomes (Cunha et al., 2015). The results obtained in this thesis corroborate this effect, as it has been observed that overexpression of *HAA1* and/or *PRS3* lead to different metabolic responses in CA11 and PE-2 Δ*GRE3*.

5. General Conclusions and Future Perspectives

Over the last years, several studies have been made in order to improve *S. cerevisiae* tolerance towards lignocellulosic-derived inhibitors. Genetic manipulation of yeast strains, such as genes overexpression, is one of the approaches used to improve *S. cerevisiae* tolerance to these inhibitors. *HAA1* and *PRS3* overexpression have already been reported to have positive effects in yeast resistance to acetic acid and furfural. However, it is, to the extent of our knowledge, the first time that *HAA1* and/or *PRS3* overexpression was evaluated in xylose fermenting industrial S. *cerevisiae* strains in real lignocellulosic hydrolysates. Taking advantages of the more robust background of CA11 and PE-2 Δ*GRE3* strains, the effect of the overexpression of these genes was evaluated in aerobic growth assays as well as in fermentations performed in inhibitors-containing xylose media.

Tests performed regarding yeast aerobic growth showed that the overexpression of *HAA1* and/or *PRS3* in PE-2 Δ*GRE3* strains resulted in an increased capacity to grow and consume xylose in media containing stronger inhibitory conditions. However, the overexpression of these genes in CA11 strains did not improve yeast growth in any media containing inhibitors. These results clearly indicate that the overexpression of the same genes in different strains result in different outcomes depending on yeast metabolic background.

Concerning the fermentations assays, in xylose media containing 1 g/L of furfural, none of the strains overexpressing *HAA1* and *PRS3* genes showed an increased or a decreased capacity during fermentation time. However, results obtained with PE-2 Δ*GRE3* recombinant strains in fermentations carried out in xylose medium containing 1 g/L of furfural and 3 g/L of acetic acid point to an advantage of overexpressing *HAA1* and *PRS3* simultaneously. However, the overexpression of *HAA1* and *PRS3* genes in CA11 strains did not improved yeast tolerance towards these inhibitors. Moreover, results showed that CA11 and PE-2 Δ*GRE3* strains overexpressing *HAA1* and *PRS3* simultaneously produced always lower amounts of xylitol than the other strains. These results might mean that simultaneously overexpression of the two genes has a crucial role in the production of xylitol. Finally, results showed that simultaneous overexpression of *HAA1* and *PRS3* can lead to different outcomes in different strains and that the overexpression of the two genes showed to increase yeast performance in media containing higher inhibitory levels.

The overall results of this thesis highlight the importance of using an integrated approach for genetic engineering of *S. cerevisiae* for improved production of 2nd generation bioethanol, considering the yeast metabolic background, its capacity to consume xylose, and yeast tolerance towards inhibitors in real lignocellulosic hydrolysates.

Considering that PE-2 Δ*GRE3* produced higher amounts of xylitol than CA11 strains, it may take advantage of a different genetic strategy for xylose consumption, opting for the xylose isomerase (XI) based pathway instead of xylose reductase (XR) and xylitol dehydrogenase (XDH), eliminating a step of xylitol production. Furthermore, it would be interesting to use lower initial cell concentrations in fermentation assays to verified if *HAA1* and/or *PRS3* overexpression, in these conditions, may lead to increase yeast tolerance towards inhibitory compounds. Another interesting approach, considering the thermotolerant background of the CA11 strain, would be to test the CA11 overexpressing strains in xylose fermentation in the presence of inhibitors at higher temperatures increasing the overall stress conditions. The possibility of working at superior temperatures would be an advantage in an SSF process where an equilibrium between the optimal temperature for yeast and enzymes is required.

For a sustainable production of 2nd generation bioethanol, and considering that genes overexpression effect may vary with different media compositions, it would be also interesting to test these transformants strains overexpressing *HAA1* and *PRS3* genes in fermentations with different real lignocellulosic hydrolysates, containing different composition, as well as evaluate their capacity for co-consumption of glucose and xylose.

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