EFFECTIVE BIOETHANOL PRODUCTION FROM PLANTBASED BIOMASS BY GENETICALLY MODIFIED YEAST STRAINS
EFFECTIVE BIOETHANOL PRODUCTION FROM PLANTBASED BIOMASS BY GENETICALLY MODIFIED YEAST STRAINS

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DE ACORDO COM A LEGISLAÇÃO EM VIGOR, NÃO É PERMITIDA A REPRODUÇÃO DE QUALQUER PARTE DESTA Tese.

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ABSTRACT

The exponential use and depletion of fossil fuels reserves have brought to light several environmental and economic concerns. Bioethanol is of major importance in achieving an efficient alternative source of energy, being renewable and environmentally sustainable. The selection of robust yeast strains as chassis for metabolic engineering could contribute to attain a cost-effective lignocellulosic bioethanol processing. In this selection, the background of metabolic engineering strains for xylose fermentation could play an important role, being necessary the evaluation of fermentation performance in several stress environments related with the lignocellulosic bioethanol processing.

In this work, four robust yeast chassis isolated from industrial environments – PE-2, CA11, CAT-1 and CCUG53310 – were engineered with the same xylose metabolic pathway. The recombinant strains were physiologically characterized in synthetic xylose and xylose-glucose medium, on non-detoxified hemicellulosic hydrolysates of two fast growing hardwoods (Eucalyptus globulus and Paulownia tomentosa) and agricultural residues (corn cob and wheat straw) and on E. globulus hydrolysate at different temperatures.

Similar rates of xylose consumption in presence or absence of glucose in synthetic media were obtained by PE-2-X-dGRE and CA11-X, showing co-fermentation of both sugars. On the other hand, CAT-1-X was capable of utilize xylose, but only after glucose depletion, while CCUG53310-X exhibited extreme deficiency in xylose utilization. Low-cost supplementation by agro-industrial residues of hydrolysate fermentation media demonstrated to be a great substitute for commercial supplements which could reduce operational cost of process. All of the strains tested were able to efficiently ferment the different hydrolysates. The highest ethanol yield (0.46 g/g) from Paulownia hydrolysate was obtained by PE-2-X-dGRE3 within 24 h of fermentation at 30 ºC. In fact, this strain showed superior fermentation ability in all the hydrolysates. Nevertheless, an evaluation of thermostolerance of strains showed that, CA11-X exhibited clearly best performance than PE-2-X-dGRE3 at 40 ºC, utilizing all xylose present in the medium. Results here presented demonstrate that the genetic background of the chassis strains plays a major role on their capacity to metabolize xylose, which was here proved to be dependent of the inhibitory composition of the lignocellulosic hydrolysate as well as of the temperature conditions of the fermentation process. These heterogeneous outcomes highlight the importance of carefully addressing the engineering of yeast strains for efficient lignocellulosic ethanol production.
**Resumo**

O uso e a redução exponencial de combustíveis fósseis trouxeram vários problemas a nível econômico e ambiental. O bioetanol é considerado muito importante para alcançar uma fonte de energia eficiente, sendo renovável e ambientalmente sustentável. A seleção de estirpes de levedura robustas como base para engenharia metabólica pode contribuir para alcançar um processo de bioetanol lignocelulósico rentável. Nesta seleção, o background de estirpes geneticamente modificadas para a fermentação de xilose pode desempenhar um papel importante, sendo necessária a avaliação da performance de fermentação em várias condições de stress relacionadas com o processamento de bioetanol lignocelulósico.

Neste trabalho, quatro estirpes de *Saccharomyces cerevisiae* – PE-2, CA11, CAT-1 e CCUG53310 – foram geneticamente modificadas com a mesma via metabólica para o consumo de xilose. As estirpes recombinantes foram caracterizadas fisiologicamente em meio sintético de xilose e glucose-xilose, em hidrolisados hemicelulósicos não-detoxificados de duas madeiras de rápido crescimento (*Eucalyptus globulus* e *Paulownia tomentosa*) e dois resíduos de agricultura (palha de trigo e espiga de milho) e em hidrolisado de *E. globulus* a diferentes temperaturas.

Taxas de consumo de xilose semelhantes na presença ou ausência de glucose em meios sintéticos foram obtidas pelas estirpes PE-2-X-dGRE e CA11-X, mostrando co-fermentação de ambos os açúcares. Por outro lado, CAT-1-X foi capaz de utilizar a xilose, mas apenas após a depleção da glucose, enquanto que CCUG53310-X mostrou grande deficiência na utilização de xilose. Suplementação low-cost com resíduos agroindustriais em fermentações de hidrolisados mostraram ser bons substitutos para suplementos comerciais, o que reduziria os custos do processo. Todas as estirpes testadas foram capazes de fermentar os diferentes hidrolisados. A maior taxa de produção de etanol (0.46 g/g) em *Paulownia* foi obtida pela PE-2-X-dGRE3 em 24h, a 30 ºC, que mostrou superior de capacidade de fermentação em todos os hidrolisados. No entanto, numa avaliação da termotolerância das estirpes, CA11-X mostrou claramente uma melhor performance do que PE-2-X-dGRE3 a 40 ºC, utilizando toda a xilose presente no meio. Os resultados aqui apresentados demonstram que o background genético das estirpes desempenha um papel importante na sua capacidade de metabolizar xilose, tendo sido provado que está dependente da inibição da composição de hidrolisados lignocelulósicos, bem como das condições térmicas e do processo de fermentação. Estas consequências heterogêneas destacam a importância de delinear cuidadosamente a engenharia genética das estirpes de leveduras para a produção eficiente de etanol lignocelulósico.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>AFEX</td>
<td>Ammonia Fibre Explosion</td>
</tr>
<tr>
<td>AR</td>
<td>Aldose Reductase</td>
</tr>
<tr>
<td>CC</td>
<td>Corn cob</td>
</tr>
<tr>
<td>CBP</td>
<td>Consolidated Bioprocessing</td>
</tr>
<tr>
<td>CSL</td>
<td>Corn Steep Liquor</td>
</tr>
<tr>
<td>CW</td>
<td>Cheese whey</td>
</tr>
<tr>
<td>$E_{\text{max}}$</td>
<td>Maximum ethanol in fermentation</td>
</tr>
<tr>
<td>EGW</td>
<td><em>Eucalyptus globulus</em> wood</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FPU</td>
<td>Filter Paper Unit</td>
</tr>
<tr>
<td>$G_i$</td>
<td>Initial glucose in fermentation</td>
</tr>
<tr>
<td>$G_f$</td>
<td>Final glucose in fermentation</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally Recognized As Safe</td>
</tr>
<tr>
<td>HMF</td>
<td>Hydroxymethylfurfural</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HXT</td>
<td>Hexose transporter</td>
</tr>
<tr>
<td>KL</td>
<td>Klason Lignin</td>
</tr>
<tr>
<td>LCM</td>
<td>Lignocellulosic materials</td>
</tr>
<tr>
<td>LSR</td>
<td>Liquid to solid ratio</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose Phosphate Pathway</td>
</tr>
<tr>
<td>PTW</td>
<td><em>Paulownia tomentosa</em> wood</td>
</tr>
<tr>
<td>RFA</td>
<td>Renewable Fuels Association</td>
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<tr>
<td>RYE</td>
<td>Raw yeast extract</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
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<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>S.</td>
<td>Severity factor</td>
</tr>
<tr>
<td>SHCF</td>
<td>Separate Hydrolysis and Co-Fermentation</td>
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<tr>
<td>SHF</td>
<td>Separate Hydrolysis and Fermentation</td>
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<tr>
<td>SSF</td>
<td>Simultaneous Saccharification and Fermentation</td>
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<tr>
<td>SSCF</td>
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</tr>
<tr>
<td>SY</td>
<td>Solid Yield</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
</tr>
<tr>
<td>WS</td>
<td>Wheat straw</td>
</tr>
<tr>
<td>X.</td>
<td>Initial xylose in fermentation</td>
</tr>
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<td>X.</td>
<td>Final xylose in fermentation</td>
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<tr>
<td>XDH</td>
<td>Xylitol Dehydrogenase</td>
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<tr>
<td>XK</td>
<td>Xylulose Kinase</td>
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<tr>
<td>XI</td>
<td>Xylose Isomerase</td>
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<tr>
<td>XR</td>
<td>Xylose Reductase</td>
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<td>Y&lt;sub&gt;E/G+xy&lt;/sub&gt;</td>
<td>Metabolic yield of ethanol from all sugars in the medium</td>
</tr>
<tr>
<td>Y&lt;sub&gt;x/s&lt;/sub&gt;</td>
<td>Biomass yield</td>
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<tr>
<td>YP</td>
<td>Yeast extract and Peptone medium</td>
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<tr>
<td>YPD</td>
<td>Yeast extract, Peptone and Dextrose medium</td>
</tr>
<tr>
<td>YPX</td>
<td>Yeast extract, Peptone and Xylose medium</td>
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1. INTRODUCTION
1.1. Renewable sources for energy: biofuels

The exponential use and depletion of fossil fuels reserves has brought to light several environmental and economic concerns. High levels of pollution associated with great amounts of greenhouse gas emissions (responsible for global warming), geopolitical issues and the constant increase of fossil fuels prices are some of the factors that have triggered the search for new renewable energy sources (Moysés et al., 2016). Also, the emergent demand for energy for heating, industrial processes and transportation, have pointed out the need for a substitute for the fossil fuels (Fornasiero and Graziani, 2011). Since the energetic crisis that the 1970s started to reveal, the research and development focus have to change in order to attain a sustainable production of fuels and chemicals (Hahn-Hägerdal et al., 2007).

Biofuel is, technically, a fuel derived from biomass, biological material obtained from dead or living organisms, such as plants, a concept recently narrowed down to renewable sources of carbon (Lee and Lavoie, 2013). Established technologies developed in the last years for ethanol production are crop-based, using mainly sugarcane juice and corn starch. Since the cost of raw materials are almost half of the bioethanol cost (Zaldivar et al., 2001) and these feedstocks are used in human and animal feed, efforts were made in order to find alternatives. Lignocellulosic biomass plays a major role in this field, being a renewable, widespread and abundant source of organic material in amount enough to satisfy the growing energetic requirements and suitable for the bioconversion into biofuels, without being competitive with food production, providing both economic and strategic benefits (Pereira et al., 2014).

1.1.1. Current scenario for bioethanol production

Ethanol is probably the oldest product of biotechnology. Bioethanol is of major importance in achieving an efficient alternative source of energy, being renewable and environmentally sustainable (Pereira et al., 2010a). Bioethanol is a natural substitute for gasoline, and despite the fact that has approximately less one third of energy than this fossil fuel, due to its oxygen content, has a cleaner combustion, resulting in a reduction of the emission of toxic compounds, and a higher octane rating, offering more knock resistance at a lower cost than any other gasoline additive available (Zaldivar et al., 2001; RFA, 2016). Bioethanol can be utilized as an exclusive fuel for dedicated engines or mixed with gasoline (Hahn-Hägerdal et al., 2006), which is already a reality, being possible to use an
85/15% ethanol-gasoline mixture (Haghighi Mood et al., 2013), and up to 100 % in flex-fuel vehicles. Bioethanol incorporation in gasoline reduces greatly the emission of carbon monoxide and unburned hydrocarbons that form smog (Wyman, 1996). Biomass-derived ethanol use drives to a net reduction of the levels of carbon dioxide (CO₂), the principal greenhouse gas, that can go from 60% to 90%, when compared to gasoline consumption in vehicles (Brown et al., 1998). Besides that, the carbon dioxide released during fuel combustion is recycled through photosynthesis, having no impact on net increase of CO₂ levels in atmosphere. The recent Directive (EU) 2009/28 from the European Union has established that “every Member State is to ensure that the share of energy from renewable sources in all forms of transport by 2020 is at least 10% of the final consumption of energy in transport”. With this, the EU plans to reduce gas emission by at least 6% until the end of 2020 (The European Parliament and the Council of the European Union, 2015).

At the end of the XX century, ethanol production was about 31 thousand million litres per year (Zaldivar et al., 2001). According to the annual report from Renewable Fuels Association, by 2007 this value had already increased to almost 50 thousand million litres. Ethanol production reached its peak in 2015, with an approximate production of 97.2 thousand million litres, which represents an enormous upsurge in both ethanol demand and production (Figure 1.1). United States of America is the major producer of bioethanol, followed by Brazil. These two countries alone are responsible for the production of nearly 85% of total bioethanol produced worldwide (RFA, 2016), being corn the foremost biomass source in the United States of America, while Brazil ethanol production relies predominantly on sugarcane (Chum et al., 2014).

![Figure 1.1. Global Ethanol Production (Adapted from RFA).](image-url)
Bioethanol can be classified in three major categories:

- **First-generation ethanol** is directly related to biomass that is mainly used as feedstock. It is principally produced from fermentation of hexose sugars, such as glucose or sucrose (which is then converted to monomeric components), mainly obtained from sugarcane, and starch, which can also be converted into glucose (Figure 1.2), derived from crops like corn, sugarbeets, sweet shogum (Lee and Lavoie, 2013). By being competitive with food crops and having a relatively high carbon footprint, it is not as attractive as it was expected (Farrell, 2006; Highina et al., 2014). Nevertheless, bioethanol is nowadays the main biofuel produced and commercialized in the world (Van Eylen et al., 2011).

- **Second-generation ethanol** is obtained from lignocellulosic biomass. This raw material is not competitive with food crops, consisting in the use of agricultural and forest residues, municipal solid wastes, among others.

- **Third-generation ethanol** is associated with the use of polysaccharides from macroalgae or accumulated starch in microalgae or direct synthesis by genetic modification (Kumar et al., 2016) The algal biomass still has many geographical and technical challenges to overcome (Lee and Lavoie, 2013; Moncada et al., 2014).

![Figure 1.2. Schematic representation of main biomasses used for first generation bioethanol. Adapted from Zaldivar et al. (2001).)](image)

### 1.2. **Second generation bioethanol**

Although first-generation biofuels are becoming more established in the industry, its competition with feedstock brings some setbacks to its increased demand over the last few years. This led to the necessity of explore an alternative pathway to bioethanol production from non-food biomass –
second-generation bioethanol. It is based in lignocellulosic materials (LCM), the most abundant and sustainable material available in the planet, which are considered by-products and, therefore, non-competitive with food and feed industry (Havlík et al., 2011).

Lignocellulosic biomass is available in many different forms, consisting in organic materials considered agricultural excesses like cereal straw, sugarcane bagasse, forest residues (wood shavings, reclaimed wood, etc.); industrial (residues from pulp and paper industry) and municipal (cardboard, newspapers) wastes; sawdust; lower value timber and other analogous fonts (Domínguez-Bocanegra et al., 2015; Hamelinck and Faaij, 2006; Zaldivar et al., 2001). Another advantage from the utilization of lignocellulosic biomass is its more uniform geographic distribution, ensuring security of supply and possible employment in underdeveloped areas (Hahn-Hägerdal et al., 2006). Also, bioethanol based on lignocellulosic materials results in low greenhouse gas emissions than first-generation bioethanol, reducing the environmental impact (Hahn-Hägerdal et al., 2006; Highina et al., 2014). By 2012 there were already more than ten demonstration scale facilities for second-generation ethanol production, with potential to grow to a commercial scale (AEC, 2012). Since 2013, some commercial-scale facilities have arisen, being expected a great increase in the number of these ethanol plants, with some of them being already under construction (Sánchez Nogué and Karhumaa, 2015). Nevertheless, bioethanol conversion from lignocellulosic materials involves more operational stages than first generation bioethanol (see Figure 1.3). These additional steps are related with chemical composition and structure of LCMs.

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Figure 1.3. Main steps for ethanol production from sugarcane, cereals and lignocellulosic materials.
1.2.1. Lignocellulosic biomass composition

Lignocellulose materials are composed by three different major structural components: cellulose, hemicellulose and lignin (Figure 1.4).

Cellulose ($C_{6}H_{10}O_{5}$) is the most abundant polymer on Earth and the major constituent of lignocellulosic biomass, and in its composition are thousands of $\text{D}-\text{glucose}$ molecules linked in a linear chain by $\beta$(1,4)-glycosidic bonds. Cellulose chains are associated forming cellulose fibrils. Its fibres are linked by a great number of hydrogen bonds (Li et al., 2010) and, for that reason, cellulose is insoluble in water and most organic solvents (Swatloski et al., 2002). Cellulose can be chemically hydrolysed by acid (diluted or concentrated) or by enzymes (Zaldivar et al., 2001).

Hemicellulose ($C_{5}H_{8}O_{4}$) is a vastly branched heteropolymer, constituted partly of xylan (composed by xylose units) and, principally in hardwood and agroindustrial wastes, arabinoxylan, which on its turn is composed by polymers of $\text{D}-\text{xylose}$ and $\text{L}-\text{arabinose}$, pentose sugars not naturally metabolized by yeasts, representing 40% of total sugars in LCM (Tomás-Pejo et al., 2014). Other than pentose sugars, hemicellulose contains other sugar residues: hexoses, like $\text{D}-\text{galactose}$, $\text{L}-\text{galactose}$, $\text{D}-\text{mannose}$, $\text{L}-\text{rhamnose}$ and $\text{L}-\text{fucose}$, and uronic acids ($\text{D}-\text{glucoronic}$). Hemicellulose is more prone to hydrolysis than cellulose (Brigham et al., 1996), and its composition is reliant on the source of the raw material (Wiselogel et al., 1996).

Lignin $[C_{9}H_{10}O_{3}(OCH_{3})_{0.5-1.7}]_{n}$ is an aromatic polymer synthesized from phenylpropanoid precursors (Haghighi Mood et al., 2013). It is a macromolecule, being the most profuse aromatic polymer occurring in nature (Zaldivar et al., 2001). The major phenylpropene monomers in its structure – p-coumaryl, coniferyl and synapyl alcohols – are linked together by a set of linkages, forming a complex matrix that adds compressive strength and rigidity to cell wall (Kang et al., 2014).

Figure 1.4. Schematic representation of lignocellulosic material composition. Arrows represent hydrolysis steps, where the dark arrows represent hydrolysis steps that generate fermentable sugars, while the white arrows represent hydrolysis steps originating not fermentable sugars. Other monomers obtained from hemicellulose are residual compounds like rhamnose, fucose and uronic acids (adapted from Zaldivar et al. (2001)).
1.2.2. Lignocellulosic materials processing

In order to achieve an effective bioethanol production, it is necessary to undergo these materials to treatments that allow the release of monomeric fermentable sugars. The search for an ideal lignocellulose pretreatment has more than two decades. Lynd (1996) had already proposed some prerequisites for this goal: i) production of reactive fibres; ii) high yield of pentoses in non-degraded form; iii) avoid the liberation of inhibitory compounds to the fermentation medium; iv) requisition of small or no size reduction; v) ability to work in reactors with reasonable dimensions and modest cost; vi) no solid residues production; vii) high level of simplicity; viii) effectiveness at low moisture contents. The pretreatment goals the opening of the structure of the fibres and to dissolution of fractions of the biomass (Galbe and Zacchi, 2012; Viikari et al., 2012).

The pretreatment of lignocellulosic materials breaks down its structure and improve the hydrolysis of polysaccharides that constitutes it into fermentable sugars (Figure 1.5). The pretreatment necessary to obtain a more accessible cellulose and hemicellulose can be physical (milling, extrusion, freeze pretreatment, etc.), chemical (acid, alkaline, organosolv, liquid hot water or authydrolysis, etc.), physico-chemical (steam explosion, ammonia fibre explosion (AFEX), among others) or biological (white-rot fungi pretreatment) (Haghighi Mood et al., 2013). Among of steps involved in bioethanol production from lignocellulosic materials, the pretreatment is considered the most expensive individual stage (Yang and Wyman, 2008).

![Figure 1.5. Effect of pretreatment on lignocellulosic structure. Adapted from Haghighi Mood et al. (2013).](image-url)
No single pretreatment exists that fulfils all the necessary requirements for an “ideal” lignocellulosic processing (Romani et al., 2010a). Nevertheless, treatment with water (and without other reaction catalyst) at high temperatures, also known as autohydrolysis, is considered an eco-friendly process that allows the hemicellulose solubilisation into hemicellulose-derived compounds (such as xylo-oligosaccharides and xylose) and the almost total recovery of cellulose and lignin in the solid fraction (Romani et al., 2010). Autohydrolysis treatment is considered as the first step for a biorefinery (Gullón et al., 2012).

Besides pretreatment elevated cost, the major setback of performing pretreatment is the release of toxic compounds. During autohydrolysis treatment of lignocellulosic materials, degradation products considered inhibitors are liberated which can be grouped in three major categories: furans, weak acids and phenolic compounds. Furan compounds such as 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF) are formed by dehydration of pentose and hexose sugars, respectively. Furfural affects the metabolism of the central carbon and, when combined with other aldehydes, acids or alcohols, including ethanol, has a synergistic effect. When in comparison, it is possible to establish that aromatic compounds as phenolic compounds are more toxic than furan, which by its turn is more toxic than furfural, being HMF the less toxic compound. Nonetheless, HMF stays in the medium about four times longer, derived from its low conversion rate (Taherzadeh et al., 2000). Weak acids, from which acetic acid is highlighted, are formed by deacetylation of hemicelluloses (Pereira et al. 2011). On the other hand, phenols compounds are obtained from lignin degradation and include a wide range of compounds, such as 4-hydroxybenzaldehyde, vanillin, and syringaldehyde. In some cases, these compounds have to be removed in order to perform hydrolysis and fermentation in optimal conditions, obtaining maximum conversion (Kang et al., 2014).

After lignocellulose pretreatment, subsequent enzymatic hydrolysis step that releases glucose from cellulose is also required. There is still no microorganism known that is capable to use lignin monomers for ethanol production (Zaldivar et al., 2001). Still, in the production of ethanol from LCM, great amounts of lignin are produced. Towards the attenuation of this downside, current pilot plants of ethanol production from second generation bioethanol production utilize the residual lignin from the process to generate energy or sequester it as ‘biochar’ as a carbon sink, to value this by-product (Doherty et al., 2011).
For the production of bioethanol there are several steps tightly connected. The process starts with feedstock collection and ends with product collection by distillation and waste disposal. In the middle, it is necessary to ensure the transportation of raw material to the manufacturer, which after preparation is hydrolysed and fermented (Olsson and Hahn-Hägerdal, 1996). The optimization of these operations is a crucial condition in an efficient bioethanol plant.

### 1.2.3. Overall view of conversion process: lignocellulosic biomass into bioethanol

As has been addressed above, the conversion of lignocellulosic biomass in order to generate bioethanol is highly sensible and variable-dependent. Starting off, it is fundamental a proper accommodation of feedstock when in the ethanol facility to prevent problems like early fermentation and bacterial contamination. First step of the biomass utilization is pretreatment, performed in order to alter the lignocellulosic structure of the feedstock (Sun and Cheng, 2002), making cellulose more accessible to hydrolysis. Most usual methods are steam explosion and dilute acid pre-hydrolysis, followed by enzymatic hydrolysis (Kang et al., 2014). The main problem of pretreatment and first hydrolysis resides in the formation of inhibitors. To reduce its formation, improving the solubilisation of hemicellulose, sulphuric acid or carbon dioxide are often added in this step in order to reduce temperature and time of process avoiding degradation compounds increase (Hendriks and Zeeman, 2009). Steam explosion has some downsides, such as the release of degradation products that reduce the efficiency of fermentation and partial destruction of xylan fraction, and, for that reason, dilute sulphuric acid (0.5%-1.0%) is normally preferred (Kang et al., 2014). This procedure leads to the release of several inhibitors, like acetic acid, and accumulation of sulphuric acid, needing its removal and neutralisation before fermentation. Pretreatment is highly expensive, representing about one third of total cost of the entire process (Tomás-Pejó et al., 2008).

Second stage hydrolysis aims to the conversion of the cellulose released in first stage hydrolysis into glucose, being newly catalysed by dilute or concentrated acid or cellulose enzymes. The resultant hydrolysate can be detoxified to overcome the inhibitory effect from the compounds released until this phase of the process (Kang et al., 2014). Detoxification step can represent up to 22 % of total cost of bioethanol production (von Sivers et al., 1994).
Fermentation is the process from which hexose (6C) and pentose (5C) sugars are converted into ethanol. Theoretical maximum yield of the process is 0.511 kg of ethanol and 0.489 kg for kg of carbon dioxide (CO\textsubscript{2}) per kg of sugar (Kang et al., 2014). When expressed in function of glucan and xylan, theoretical ethanol yield is 0.719 and 0.736 litres of ethanol per kg, respectively (Kang et al., 2014). Saccharomyces cerevisiae is the most common microorganism used to execute this step, although its intrinsic inability to metabolize pentose sugars like xylose.

When using enzymatic hydrolysis, integration of hydrolysis and fermentation steps of bioethanol production can be achieved by several options that are available.

- In Separate Hydrolysis and Fermentation (SHF), the susceptible cellulose is saccharified by enzymes and the glucose obtained is fermented into ethanol sequentially in separated reactors (Kang et al., 2014). On its side is the fact that each operation can be conducted individually at optimal conditions of both temperature and pH, although the accumulation of glucose (end product of hydrolysis procedure) inhibits the activity of cellulases, as well as high costs for two separate reactors.

- Simultaneous Saccharification and Fermentation (SSF) uses cellulases and hemicellulases from external sources in order to release sugars from solid fraction during the fermentation. The glucose released by enzyme activity is rapidly converted into ethanol by yeast. This configuration presents several advantages when comparing with SHF: it has a great rate of hydrolysis, requiring low enzyme loading, which is traduced in high ethanol yields and reduced risk of contamination. On its downside, temperature of operation has to fit all conditions at once, which does not allow individual optimization for each condition (Zaldivar et al., 2001). On the other hand, Separate Hydrolysis and Co-Fermentation (SHCF) and Simultaneous Saccharification and Co-Fermentation (SSCF) mixing hemicellulosic fraction are alternatives to these two processes that allow the fermentation of both C5 and C6 sugars by a single microorganism strain in the same reactor, which represent a major improvement in terms of the cost of the process, enhancing its commercial viability (Humbird and Aden, 2009; Kazi et al., 2010; Klein-Marcuschamer et al., 2010).

- There are some novel approaches in development, like Consolidated Bioprocessing (CBP), that comprehends the production of all the required enzymes and bioethanol itself using a single type of microorganism in a single reactor. Although it is considered the ultimate evolution of the
conversion technology of biomass into bioethanol, it is expected that several years will be needed in order to determine the microorganisms able to perform in these conditions (Klein-Marcuschamer et al., 2010).

At last, purification of ethanol is required, normally achieved by distillation. Bottom product in the first distillation column (stillage) contains basically lignin and water, alongside unconverted cellulose and hemicellulose. The insoluble fraction can be dehydrated by a pressure filter and then utilized for steam and electricity generation, allowing the ethanol plant a self-supplying energy source, reducing also solid waste disposal and even generating additional income by selling the excess of energy (Humbird et al., 2011; Kazi et al., 2010)

1.3. Challenges of lignocellulosic ethanol fermentation

There are some desirable traits that can bring ethanol production from LCM to another level like the capacity to ferment all sugars simultaneously, the internal ability of a microorganism to degrade cellulose and/or hemicellulose, the growing ability on low pH medium, once that fermentations at a pH lower than 5.0 have less risk of contamination. For that reason, a microorganism that could ferment in very acidic conditions, requires minimal nutrients or has the capability of using low-cost by-products as supplements would represent some advantages. In fact, supplementation facilitates microorganism growth and fermentation, minimizing the toxic effects of the inhibitors (Tomás-Pejó et al., 2012). Utilization of residues from the agro-food industry is attractive since it enables the revalorization of these by-products, improving economic efficiency. There are some agroindustrial residues that have been tested and optimized in the last years to supplement fermentations, providing complex nutrients and nitrogen to fermentation media, from which is possible to highlight corn steep liquor (CSL), raw yeast extract (RYE), cheese whey (CW) and urea (Pereira et al., 2010a). Also, industrial environments are often affected by high temperatures, especially in tropical countries like Brazil, where, due to its expensive costs, no cooling systems are utilized (Ruiz et al., 2012). Significant cost reduction can be achieved by improving yeast thermostolerance, allowing fermentation performance at higher temperatures (Abdel-banat et al., 2010). Additionally, fermentation at high temperatures allow a more effective simultaneous saccharification and fermentation, substantial reduction of contamination and a continuous shift from fermentation to distillation (Ruiz et al., 2012).
1.3.1. Importance of lignocellulose-derived xylose utilization

A cost-effective second generation bioethanol production process obliges an efficient and rapid fermentation of all sugars present in the lignocellulosic biomass derived from cellulose and hemicellulose (Kim et al., 2013). Xylose fermentation could reduce the ethanol production cost up to 25% (Hinman et al., 1989; Tao et al., 2011). In order to allow a sustainable ethanol production as an alternative to fossil fuels, it is necessary to overcome some major challenges.

First, the hydrolysis of lignocellulosic biomass obliges to the use of different procedures and enzyme utilization that it is not necessary in terms of starch-based processes, associated with first-generation ethanol production. Given that, and as stated above, treatment of lignocellulosic biomass itself drives to the release of toxic and acidic compounds that reduces greatly the efficiency of the process. Also, the absence of microorganisms capable of ferment, in an efficient manner, pentose sugars released by lignocellulosic materials hydrolysis is one of the main factors that has been challenging the utilization of lignocellulose (Sánchez Nogué and Karhumaa, 2015). Across sugar-containing crops, the complex structure of lignocellulose is an obstacle to ethanol production, by being resistant to degradation.

Another main challenge of bioethanol production from LCM is the formation of by-products, from which is possible to highlight xylitol formation. Xylitol formation occurs in naturally xylose fermenting yeast strains, such as *S. stipitis*, and in genetic metabolic engineered strains carrying xylose metabolic pathways. It occurs under two major circumstances: a cofactor imbalance between xylose reductase (XR) and xylitol dehydrogenase (XDH) enzymes (Kim et al., 2013), causing a xylitol pool formation in cytosol, in response to a metabolic pathway disruption; and an unspecific aldose reductase (AR), encoded by *GRE3* gene, which is responsible for the reduction of D-xylose to xylitol, utilizing NADPH as a cofactor (Traff et al., 2001). The reduction of xylitol formation is a main objective in order to upsurge bioethanol yield. To achieve that, multiple strategies have been approached over the last years. The overexpression of xylose isomerase (XI) enables the isomerisation of D-xylose to xylulose without xylitol generation, which can be an intermediate of this conversion is a strategy to avoid its formation (Kuyper et al., 2003). Also, XR from *S. stipites* was engineered by random mutagenesis followed by selection in anaerobic batch culture resulting in a mutated XR (N272D) with higher specificity for NADH than NADPH, that was able to decrease the cofactor imbalance caused in the first two steps of xylose metabolism, improving ethanol yield and productivity (Runquist et al.,
Another strategy to avoid xylitol formation is \( \text{GRE3} \) gene deletion, eliminating the main native route of xylitol production (Traff et al., 2001).

### 1.3.2. Genetically modified yeast strains for xylose consumption

As aforementioned, \textit{Saccharomyces cerevisiae} is, almost unanimously, the preferred microorganism for fermentation processes in industrial environments. This results of several characteristics associated with this yeast species: its natural fermenting capability, as it can produce great amounts of ethanol (Çakar et al., 2012; Van Zyl et al., 2007), high tolerance to ethanol and inhibitory compounds (present in lignocellulosic hydrolysates), being more robust than bacteria (Almeida et al., 2007; Matsushika et al., 2009a; Pereira et al., 2011b) and Generally Regarded as Safe (GRAS) status (Eksteen et al., 2003). Nevertheless, it is not capable of fermenting pentose sugars such as xylose and arabinose, which are major constituents of lignocellulosic materials (Çakar et al., 2012), but is able to metabolize xylulose, a keto-isomer of D-xylose (Matsushika et al., 2009a). Some yeast strains like \textit{Pichia stipitis}, now entitled \textit{Scheffersomyces stipitis}, have the ability to utilize xylose, as well as other pentose sugars. Despite the fact that some recent studies over the last decade have been attempting to establish ethanol production on a potentially industrial scale xylose fermentation with \textit{S. stipitis} (Balagurunathan et al., 2012; Ferreira et al., 2011; Nigam, 2001), its ethanol yields are not as high as desirable. Hence, the majority of research studies tend to focus on the use of recombinant \textit{S. cerevisiae} as a preference for effective bioethanol production. In order to do so, a rational metabolic engineering approach involving the heterologous expression of the genes responsible for xylose fermentation from \textit{S. stipitis} are being used. Xylose reductase (XR), encoded by \textit{XYL1} gene, and xylitol dehydrogenase (XDH), encoded by \textit{XYL2} gene, enzymes that, together, allows the conversion of xylose into xylulose, which as aforementioned is utilized by \textit{S. cerevisiae}. The introduction of these genes in this yeast strain generates a recombinant strain able to grow aerobically on xylose (Cai et al., 2012; Çakar et al., 2012).

The necessity to create a recombinant \textit{S. cerevisiae} strain with the ability to ferment both hexose and pentose sugars emerged, and was already obtained in multiple studies (Casey et al., 2010; Ko et al., 2016; Laplace et al., 1993; Romani et al., 2015), allowing the possibility of a Simultaneous Saccharification and Co-Fermentation. One of the major challenges faced in the production of lignocellulosic bioethanol is the generation of inhibitory compounds during the biomass pretreatment...
and hydrolysis steps. These inhibitors of microbial growth comprise furan derivatives (furfural and HMF), several phenolic compounds (e.g. vanillin) and organic acids (e.g. acetic acid). The success of lignocellulosic biomass utilization is necessarily dependent on the development of recombinant \textit{S. cerevisiae} strains capable of withstand, survive, and function in the diverse stresses imposed during the bioethanol production processes, including inhibition by the above-mentioned compounds, as well as by increasing ethanol concentration, wide pH range and osmotic shifts.

- **Xylose metabolic pathways**

  According to the type of microorganism, xylose uptake is performed via different pathways. In bacteria, xylose utilization is driven by a xylose isomerase (XI), which catalyses the isomerization of xylose to xylulose. Instead, yeasts convert xylose to xylitol, by the action of xylose reductase, which is subsequently converted by xylitol dehydrogenase into xylulose that yeasts can naturally utilize. D-xylulose is posteriorly phosphorylated to D-xylulose-5-phosphate by xylulokinase (XK), and then introduced in the pentose phosphate pathway (PPP) to glycolysis (Zhou et al., 2012), which occurs in either one alternative pathways. A third metabolic pathway, possessed by \textit{Archae} organisms, consists in an oxidative xylose catabolic pathway. Here, xylose is oxidized exclusively to the tricarboxylic acid cycle (alternatively, citric acid cycle) intermediate \(\alpha\)-ketoglutarate through the action of multiple enzymes, but since it requires the expression of several genes it has not been used until now to engineer \textit{S. cerevisiae} (Moysés et al., 2016).

  Although the identification of orthologous genes encoding functional xylose reductase and xylitol dehydrogenase in \textit{S. cerevisiae}, it was not capable to grow on xylose alone (Träff et al., 2002). Engineering \textit{S. cerevisiae} with the XI pathway consists in the expression of \textit{xylA} gene, which encodes xylose isomerase. Though many bacterial \textit{xylA} genes, such as \textit{Escherichia Coli} (Sarthy et al., 1987) or \textit{Clostridium thermosulfurogenes} (Moes et al., 1996), the first successful recombinant yeast strain carrying this pathway was only transformed with the eukaryotic \textit{xylA} from the anaerobic fungus \textit{Piromyces sp. E2} (Kuyper et al., 2003). Since then, many other \textit{xylA} genes from different microorganisms have been expressed in \textit{S. cerevisiae} (Moysés et al., 2016). Xylose Isomerase is a meta-ion-dependent enzyme that catalyses the isomerization of xylose to xylulose. Its advantage is the elimination of the cofactor imbalance, producing less amount of xylitol.
As said above, the XR/XDH pathway is characterized by the occurrence of xylitol, an intermediate between xylose and xylulose. Xylose reductase, a NAD(P)H-dependent enzyme, catalyses the conversion of xylose to xylitol, and by its turn, xylitol dehydrogenase – with NAD⁺ as cofactor – converts xylitol into xylulose (Zaldivar et al., 2001). This was actually the first documented gene engineering strategy from Kötter et al. 1990, in which genes \textit{XYL1} and \textit{XYL2} from \textit{S. stipitis} encoding these two enzymes (XR and XDH, respectively) were expressed in a recombinant \textit{S. cerevisiae} strain, enabling yeast cell growth on D-xylose. However, the expression of these genes alone lead to very low levels of ethanol produced, while xylitol production was considerably high (Kim et al., 2013). Numerous metabolic engineering strategies have been tested in order to increase the ability of xylose fermentation (Cai et al., 2012; Matsushika et al., 2009a), but it is still required further improvement of the xylose consumption rate and ethanol yield of the process, which is still mainly performed in laboratory strains, and for that reason, unfitting for bioethanol production in industrial environments (Matsushika et al., 2009a). \textbf{Figure 1.6} shows a representation of the metabolic routes involved in both pathways described above.

\textbf{Figure 1.6.} Xylose metabolic pathways in metabolic engineered yeasts. Xylose can be metabolized by xylose reductase (XR), encoded by \textit{XYL1} gene, and xylitol dehydrogenase (XDH), encoded by \textit{XYL2}. Also, xylose isomerase (XI), encoded by \textit{xylA} gene, can also efficiently yield xylulose. Phosphorylation of xylulose by xylulose kinase (XKS) produces xylulose-5-phosphate, enabling its enter in pentose phosphate pathway (PPP). \textit{HXT} represents the hexose transporters in yeast membranes.
Although it has opened the path to yeast cell growth on D-xylose media solely, results obtained with mere introduction on either one of these metabolic pathways alone is insufficient for an effective fermentation of xylose by recombinant yeast strains (Moysés et al., 2016).

- Strategies for xylose uptake improving:

In order to achieve efficient xylose utilization for bioethanol production it is necessary to perform additional genetic modifications. From above, it is possible to realize that xylulokinase plays a major role in the incorporation of xylulose in the PPP, which represents a key-step in the overall conversion of xylose in ethanol. Also, slow xylulose consumption is tightly connected to low levels of endogenous xylulokinase activity that can limit partially xylose utilization (Deng and Ho, 1990).

Consequently, numerous metabolic engineering strategies have been focusing on the optimization of the XR/XDH/XK ratio (Eliasson et al., 2001; Matsushika and Sawayama, 2008). The overexpression of endogenous \textit{XKS1} gene encoding XK in recombinant xylose-utilizing strains of \textit{S. cerevisiae} harbouring the overexpression of \textit{XYL1} and \textit{XYL2} (XR/XDH pathway) or \textit{xylA} (XI pathway) genes resulted in an improvement in ethanol yields (Matsushika et al., 2009b; Smith et al., 2014). Accomplish a cofactor balance is one main goal for a competent xylose utilization, in order to prevent a xylitol pool formation in cytosol. The strategy aforementioned consisting in engineering XR by random mutagenesis led to an improving in ethanol yield and productivity (Runquist et al., 2010). Khattab et al. (2011) reported a combination of strictly NADPH-dependent XR mutant, derived by site-directed mutagenesis, alongside NADP$^+$-dependent XDH mutant, in order to recycle NADPH between XR and XDH effectively.

Additionally, one of the causes for xylitol formation is an unspecific aldose reductase (AR), which is encoded by the \textit{GRE3} gene on chromosome VIII (Garay-Arroyo and Covarrubias, 1999), that is able to reduce xylose to xylitol (Kuhn et al., 1995). Various studies have accomplished the deletion of the \textit{GRE3} gene, obtaining a recombinant \textit{S. cerevisiae} strain with diminished xylitol formation (Lee et al., 2012; Romani et al., 2015; Traff et al., 2001), which combined with the above-mentioned genetic modifications can improve xylose utilization for efficient bioethanol production. Nevertheless, other approaches have been tested recently, with promising results. The overexpression of the \textit{GRE3} gene, alongside overexpression of \textit{XYL2} and \textit{XKS1}, had been reported to improve ethanol yield and
lower xylitol production when compared to the same strain with the \textit{GRE3} gene deleted, suggesting that a fine-tuning of the \textit{GRE3} expression over its deletion may be a beneficial alternative (Khattab and Kodaki, 2014).

The deletion of other genes like \textit{PHO13}, encoding \textit{p}-nitrophenylphosphatase (Van Vleet et al., 2008), has been shown to upregulate other PPP-gene expression, improving xylose consumption rate, ethanol yield, and it may be that this gene is one of the factors affecting yeast cell growth on xylose (Bamba et al., 2016). Furthermore, the deletion of an aquaglyceroporin \textit{FPSI} that has a role in xylitol transport (Wei et al., 2013) had also shown positive effect in ethanol production.

Overexpression of enzymes from the pentose phosphate pathway is also a recurring strategy. Although many enzymes were tested, the majority of them presented some side effects, such as growth inhibition on glucose (Jeffries, 2006). Nonetheless, overexpression of endogenous \textit{TALI}, encoding a transaldolase, have been proved to increment xylose metabolic rate and ethanol production (alongside other previously described genetic modifications) (Pereira et al., 2016). The overexpression of other enzymes from the non-oxidative part of the pentose phosphate pathway, encoded for genes such as \textit{TKL1}, \textit{RKI1} and \textit{RPE1}, have also been shown improved cell growth on xylose media (Karhumaa et al., 2005).

Lastly, xylose enters the cell via hexose transports that have low affinity for pentose sugars, which has been suggested as a limiting factor in xylose utilization (Eliasson et al., 2000). Given this, mutating hexose transporters in order to improve xylose transport into yeast cell might be an interesting approach. In absence of glucose or in situation of glucose depletion or exhaustion from the medium, some hexose transporter (HXT) proteins with high xylose transport capacity are quickly degraded and detached from the cytoplasmic membrane (Snowdon and van der Merwe, 2012). Substitution of N-terminal lysine residues in the endogenous hexose transporters HXT1 and HXT36 that are exposed to catabolite degradation gave rise to in improved retention at the cytoplasmic membrane in the absence of glucose and triggered improved xylose fermentation upon the depletion of glucose and when cells are cultivated in D-xylose solely, avoiding ubiquitination of hexose transporters in \textit{S. cerevisiae} (Nijland et al., 2016). Also, a single amino acid change in the hexose transporter HXT7 coding sequence led to a mutant HXT7(F79S) that showed improved xylose uptake rates (Reider Apel et al., 2016).
1.3.3. Metabolic engineered robust yeast strains for lignocellulosic bioethanol production

Recent studies have shown that laboratory strains do not exhibit great performance in lignocellulosic fermentations, even with multiples genetic modifications (Romani et al., 2015). In this sense, focus of genetic engineering strategies has been changed to its application on industrial yeast strains. The hardness of pretreatment is irremediably attached to the generation of degradation products as has been mentioned above. Recent work has shown that metabolic engineering of industrial *S. cerevisiae* strains may have different responses depending on the individual genetic background of yeast strains (Cunha et al., 2015). Thus, the use of natural robust yeast strains to overcome this inhibitor challenge is present as interesting alternative strategy. Industrial isolates from harsh industrial environments have a well-established robust background, exhibiting higher fermentation capacity (Mussatto et al., 2010; Pereira et al., 2010b). Also, these strains exhibit improved stress tolerance by the presence of stress factors in the harsh industrial processes, like high sugar and ethanol concentrations, elevated temperatures, drastic pH variations and presence of toxic compounds (Pereira et al. 2011). Microflora from traditional and industrial fermentation processes represents a potential source of microbial natural isolates that present some of the preferred physiological background features for lignocellulosic hydrolysates fermentation, even if they have not been naturally exposed to these particular inhibitors. Some strains of *S. cerevisiae* obtained from Brazilian sugarcane-to-ethanol distilleries ("cachaça" and bio-ethanol plants) have presented high fermentation efficiency with extended tenacity in the fermentation system (Pereira et al., 2012, 2011b, 2010b). Additionally, a flocculating yeast strain isolated from Swedish second-generation plant exhibited high tolerance to ethanol, as well as to osmotic stress and inhibitor presence (Westman et al., 2012). Plenty of work have been done also to evaluate and improve yeast strains thermotolerance (Banat et al., 1996; Mitsumasu et al., 2014; Yu et al., 2008), since it can be a major advantage in the industrial environment, aiding for a cost-efficient process (Pereira et al., 2014; Ruiz et al., 2012). Environmental conditions of stress influence the expression of flocculent character of some laboratory strains, which can be supportive for ethanol production from LCM (Landaeta et al., 2013).

Despite the fact that industrial yeast strains are more tolerant to specific inhibitors present in lignocellulosic hydrolysate, they are challenging to engineer due to their complex background and
limited accessibility of genetic tools (Li et al., 2016). Also, various engineered industrial *S. cerevisiae* strains exhibit variable fermentation performance (Cai et al., 2012). The inhibitory composition of the lignocellulosic hydrolysates has also been shown to be an important determinant of the response of genetically modified yeast (Cunha et al., 2015). Moreover, there are indications that industrial isolates may have different genetic background for pentose metabolism (Li et al., 2015).

Although the many potential candidates to contour the stressful conditions involved in fermentation of lignocellulosic hydrolysates and the increasing evolution in this technology, characterization of these robust industrial yeast strains is still to be reported (Pereira et al., 2014). From this, it is possible to understand that the wide range of variables and barriers that yeast strains face when integrated in second-generation bioethanol processes require also a wide-open screening strategy. Commonly performed screenings for selection of an optimal strain consists in exposing several yeast to the individual/separate effect of stress factors or in testing only one lignocellulosic material (Demeke et al., 2013; Li et al., 2015). Table 1.1 displays various studies performed in recent years in lignocellulosic hydrolysates with metabolic genetic engineered *S. cerevisiae* yeast strains for xylose consumption.

These realities created the necessity for a tailor-made development of lignocellulosic ethanol-producing yeast with a careful selection of the chassis strains. Selection of suitable yeast for xylose metabolic engineering should be addressed from a global perspective taking into account the limitations involved in the process (such as source of hydrolysate containing inhibitors, temperature, and simultaneous hexoses and pentoses consumption). Hence, a more effective approach for evaluation of industrial *S. cerevisiae* isolates should pass by a screening in a broad range of lignocellulosic hydrolysates and in conditions where multiple stresses occurring during lignocellulosic ethanol production are evaluated simultaneously, in a more dose-to-reality approach.
<table>
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<th>Strain</th>
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<tr>
<td>Eucalyptus wood</td>
<td>Hot compressed water (150 °C; 4 h)</td>
<td>SSSF at high solid; Enzyme: 20 FPU/g; 35 % solids</td>
<td>MAR4</td>
<td>XYL1/XYL2/XKS1</td>
<td>XR/XDH</td>
<td>(Fujii et al., 2014)</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>Steam pretreatment (190 °C, 10 min)</td>
<td>SSSF; Enzyme: 20 FPU/g; 7.5 % solids</td>
<td>TMB3400</td>
<td>XYL1/XYL2/XKS1</td>
<td>XR/XDH</td>
<td>(Erdei et al., 2012)</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>Steam pretreatment (190 °C, 10 min)</td>
<td>SSSF; Enzyme: 58 FPU/g; 7.5 % solids</td>
<td>KE6-12</td>
<td>XR/XDH</td>
<td>XYL1/XYL2/XKS1</td>
<td>(Erdei et al., 2013b)</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>Steam pretreatment (190 °C, 10 min)</td>
<td>SSSF; Enzyme: 12% solids; Enzyme: 15 FPU/g</td>
<td>KE6-12</td>
<td>XR/XDH</td>
<td>XYL1/XYL2/XKS1</td>
<td>(Moreno et al., 2013)</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>Steam Explosion (210 °C; 2.5 min)</td>
<td>SSSF; 12% solids; Enzyme: 15 FPU/g</td>
<td>KE6-12</td>
<td>XR/XDH</td>
<td>XYL1/XYL2/XKS1</td>
<td>(Novy et al., 2014)</td>
</tr>
<tr>
<td>Corn cob</td>
<td>Steam pretreatment, SO₂ cat. (185 °C; 5 min)</td>
<td>Fed-batch SSSF; SSSF with hydrolysate; liquid fraction feeding</td>
<td>KE6-12</td>
<td>XR/XDH</td>
<td>XYL1/XYL2/XKS1</td>
<td>(Koppram et al., 2013)</td>
</tr>
<tr>
<td>Corn stover</td>
<td>AFEX pretreatment (100 °C; 30 min)</td>
<td>SSSF with 9 % glucan loading</td>
<td>GLBRCY35</td>
<td>XR/XDH</td>
<td>XYL1/XYL2/XKS1</td>
<td>(Jin et al., 2013)</td>
</tr>
<tr>
<td>Corn stover</td>
<td>Alkaline pretreatment (30 °C; 0.125 g H₂O₂/g biomass, 24 h)</td>
<td>SSSF: 20% solids; Enzyme: 0.2% protein/solid</td>
<td>RN1016</td>
<td>XI</td>
<td>xylA/XKS1</td>
<td>(Sato et al., 2014)</td>
</tr>
<tr>
<td>Corn stover</td>
<td>AFEX pretreatment (140 °C; 15 min)</td>
<td>SHF; Enzyme: 15 mg/g glucan; 6 % glucan</td>
<td>424A (LNHST)</td>
<td>XR/XDH</td>
<td>XYL1/XYL2/XKS1</td>
<td>(Uppugundla et al., 2014)</td>
</tr>
<tr>
<td>Rice straw</td>
<td>Hot water pretreatment</td>
<td>Fermentation of lignocellulosic hydrolysate</td>
<td>MTB-1X/TALADH</td>
<td>XR/XDH</td>
<td>XYL1/XYL2/XKS1/TAL1/ADH</td>
<td>(Hasunuma et al., 2014)</td>
</tr>
<tr>
<td>Sweet sorghum bagasse</td>
<td>Dilute acid thermal pretreatment (190 °C; 105 s; 1.64% H₂SO₄)</td>
<td>SSF: 20% solids; Enzyme: 0.2% protein/solid</td>
<td>RN1016</td>
<td>XI</td>
<td>xylA/XKS1</td>
<td>(Van Eyken et al., 2011)</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>Alkaline pretreatment (0.4 M NaOH; 25 °C; 24 h)</td>
<td>SSSF: 10 mg/g solids; 500 kKat/g DW; 10 % solids</td>
<td>GS1.11-26</td>
<td>XI</td>
<td>xylA/XKS1/TAL1/TKL1/RPE1/RKL1/HKT7/ArA1AraA/AraB/AraO/TAL2/TKL2/evolved</td>
<td>(Demeke et al., 2013)</td>
</tr>
<tr>
<td>Corn stover</td>
<td>AFEX pretreatment (1 g NH₃/g DW; 5 min)</td>
<td>SHF; Enzymes: 15 FPU/g, 64 pNPGU/g glucan; 6%/9% glucan loading</td>
<td>GLBRCY128</td>
<td>XI</td>
<td>xylA/XYL3/TAL1/evolved</td>
<td>(Parreiras et al., 2014)</td>
</tr>
<tr>
<td>Sweet sorghum bagasse</td>
<td>Steam pretreatment (190 °C; 5 min)</td>
<td>Fed-batch SSF</td>
<td>D5A⁺</td>
<td>XI</td>
<td>xylA/XKS1</td>
<td>(Smith et al., 2014)</td>
</tr>
<tr>
<td>Rice straw/oak hard wood</td>
<td>Dilute acid thermal pretreatment (190 °C; 105 s; 1.64% H₂SO₄)</td>
<td>SHF; Enzyme: 16 FPU/g; 15 % solids</td>
<td>SXA-R2P-E</td>
<td>XI</td>
<td>xylA/XKS1/TAL1/XKS1/ΔG/RE3/ΔPHO13/evolved</td>
<td>(Ko et al., 2016)</td>
</tr>
</tbody>
</table>
2. **OBJECTIVES**
As referred before, the multiple stress conditions involved in lignocellulosic hydrolysates fermentation cannot be accessed as individual factors isolated from each other, requiring an integrated approach when screening for chassis strains for genetic metabolic engineering. The main goal of this work was to evaluate the performance of five metabolic engineered yeast strains, carrying the oxidoreductase pathway, alongside overexpression of endogenous xylulokinase and transaldolase, for effective second-generation bioethanol production utilizing different raw materials as feedstock: *Eucalyptus globulus* wood, *Paulownia tomentosa* wood, wheat straw and corn cob.

In order to expand the understanding of how metabolic stresses like inhibitor presence or temperature are correlated with each other and how it affects fermentation profiles, the specific aims of this work were:

1. Evaluation of fermentation and co-fermentation by metabolic engineered yeast strains in synthetic medium containing xylose solely and glucose and xylose, respectively;
2. Evaluation of the substitution of commercial supplementation for agro-industrial by-products on the fermentation of lignocellulosic hydrolysates;
3. Evaluation of fermentation performance of metabolic engineered strains in non-detoxified hemicellulosic hydrolysates from several raw materials;
4. Study the thermotolerance of selected metabolic engineered yeast strains under high temperature in hemicellulosic hydrolysates.
3. MATERIALS AND METHODS
3.1. Sterilization of material, solutions and culture media

Glass material was sterilized in autoclave at 121 °C for 15 min. Culture media for yeast growth were sterilized in autoclave at 112 °C for 20 min, in order to decrease xylose degradation, which is present in all of the media. Thermolabile solutions were sterilized by filtration with 0.2 µm filters.

3.2. Strains

Four industrial *S. cerevisiae* isolates were used as host strains for xylose metabolic engineering in this work: CCUG53310, flocculating strain isolated from a Swedish second-generation bioethanol plant (Purwadi et al., 2007); PE-2 and CAT-1, isolated from Brazilian first-generation bioethanol plants (Basso et al., 2008); and CA11, isolated from Brazilian "cachaça" fermentation processes (Freitas Schwan et al., 2001).

3.2.1. Genetic engineering strategy

Genetic engineering strategy utilized for this work consisted in the introduction of the pMEC1049 vector (Romani et al., 2015), containing the oxidoreductase xylose utilizing pathway, in the above-mentioned industrial strains using the lithium acetate method (Gietz and Akio, 1988). Then, transformants were selected in YPD plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar) containing 300 mg/L of hygromycin. The resultant xylose metabolizing yeast strains were named PE-2-X, CAT-1-X, CCUG53310-X and CA11-X. This vector contains the *XYL1* (N272D; mutated for higher specificity for NADH (Runquist et al., 2010)) and *XYL2* genes from *Scheffersomyces stipitis*, and the endogenous *XKS1* and *TAL1* genes, under different *S. cerevisiae* promoters *TEF1*, *TDH3*, *PGI1* and *FBA1*, respectively. In addition, in the PE-2 strain containing the pMEC1049 vector, both alleles of the *GRE3* gene, which encodes an unspecific aldolase reductase, were deleted, removing the principal native route of xylitol production (Romani et al., 2015), and was also studied in this work, and was named PE-2-X-dGRE3.

3.2.2. Cell storage

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

### 3.3. Hemicellulosic hydrolysates

In order to obtain sugar from lignocellulosic materials, four raw materials were used in this work: *Eucalyptus globulus* wood, *Paulownia tomentosa* wood, corn cob and wheat straw. These lignocellulosic materials were subjected to an autohydrolysis treatment in order to produce liquors enriched in hemicellulose-derived compounds. In a second stage, these liquors were subjected to an acid hydrolysis in order to obtain hemicellulosic hydrolysates (enriched in xylose). **Table 3.1** summaries the condition of autohydrolysis treatment. **Figure 3.1** shows a schematic representation of steps involved in the hemicellulosic hydrolysates production.

**Table 3.1.** Characterization of the raw material concerning treatment and the composition of solid phase and hemicellulosic liquor phase (hydrolysate).

<table>
<thead>
<tr>
<th>Raw material</th>
<th><em>Eucalyptus globulus</em></th>
<th><em>Paulownia tomentosa</em></th>
<th>Wheat straw</th>
<th>Corn cob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Autohydrolysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>conditions</td>
<td>( S_0 = 4.08 );</td>
<td>( S_0 = 4.19 );</td>
<td>( S_0 = 3.92 );</td>
<td>( S_0 = 3.83 );</td>
</tr>
<tr>
<td>Solid Yield (%)</td>
<td>LSR=8 g/g</td>
<td>LSR=8 g/g</td>
<td>LSR=10 g/g</td>
<td>LSR=8 g/g</td>
</tr>
<tr>
<td></td>
<td>71.4</td>
<td>68.9</td>
<td>60.2</td>
<td>64.1</td>
</tr>
</tbody>
</table>

\( S_0 \) is the severity factor of the pretreatment; LSR is the liquid-solid ratio.
3.3.1. Lignocellulosic raw materials

The raw materials utilized in this work were provided by different sources. *Eucalyptus globulus* wood (EGW) samples were obtained from a local pulp factory (ENCE, Galicia, NW Spain), while *Paulownia tomentosa* wood (PTW) was provided by a local wood plantation, located in Foz (Lugo, NW Spain). Wheat straw was supplied by a local farmer (Elvas, Portugal) and corn cob samples were collected locally in the North of Portugal. All materials were milled to pass an 8 mm screen (suitable for autohydrolysis treatments), air-dried and homogenized in a single lot to circumvent differences in composition among aliquots. Aliquots of the different raw materials were subjected to moisture determination (drying at 105 °C to constant weight). All samples were stored in containers with aeration in dark and dry place until use.

3.3.2. Analysis of raw material

In order to determine the structural components of the raw materials, a quantitative acid hydrolysis was performed. On the first stage of the process, polysaccharides were converted into oligosaccharides through the addition of 5 mL of H$_2$SO$_4$ concentrated at 72 % (w/w) to a sample of
0.5 g of raw material, and placed for 1 h in a water bath at 30 °C. After this, distilled water was added to the tube in order to stop the reaction. The tube content was transferred to a 250 mL Schott flask and filled with water until final weight of 148.68 g was achieved. With this, H₂SO₄ concentration dropped to 4 % (w/w), and the solution was introduced in autoclave for 1 h at 121 °C, beginning the second stage of this hydrolysis, where the oligosaccharides were converted into monomers. After cooling the flasks, these were dried and weighted to access the losses during the second stage. Sample was filtered by vacuum in a Büchner flask, using a glass Gooch crucible nr. 3, separating the liquor with the sugars from the lignin fraction retained in the filter. The filter was kept for 24 h in an oven at 105 °C for posterior quantification. The liquor was analysed by HPLC. Polysaccharides content (glucan and xylan) was determined by glucose and xylose concentration in liquor after acid hydrolysis, respectively.

The Klason lignin content (KL) of wood was determined from the solid residue attained in the quantitative acid hydrolysis step and previously dried in oven. After cool down in a desiccator, samples were weighted and lignin content is calculated through the following formula:

$$KL = \frac{m_{gos} - m_R}{m_{nih} \cdot (1 - H)} \cdot (100 - EC)$$ (I)

where $m_{gos}$ is the mass in grams of the Gooch crucible with the dried sample, $m_d$ is the mass in grams of the dried Gooch crucible, $m_{nih}$ is the mass in grams of the initial humid raw material submitted to quantitative acid hydrolysis, $H$ is the humidity of the raw material in g of water/g of humid LCM and EC is content in extracts.

### 3.3.3. Hydrothermal or autohydrolysis pretreatment

Lignocellulosic materials were processed by hydrothermal treatment also named autohydrolysis. The raw materials were mixed with water at different liquid to solid ratios (LSR). LSR for wheat straw pretreatment was 10 kg of water/kg of oven-dried raw material, while the other three raw materials (Eucalyptus globulus wood, Paulownia tomentosa wood and corn cob) had LSR of 8 kg of water/kg of oven-dried raw material. These treatments were performed in a pressurized Parr reactor (Parr Instruments Company) of 3.785 litres internal volume, tailored with four blade turbine impellers, being heated by an external fabric mantle, and cooled by tap water circulating through an internal
loop. In autohydrolysis trials, the reaction media were stirred at 150 rpm and heated, following the
standard temperature profiles up to severity factor \((S_0)\) between 3.83 and 4.19 (Table 3.1), being
cooled afterwards. Operational conditions were selected based on previously reported data (Rivas et
al., 2002; Romaní et al., 2010; Ruiz et al., 2012). After cooling down the system, the media were
immediately filtered by vacuum pressure, separating liquid (liquors) and solid phases.

The effects attained in a certain non-isothermal autohydrolysis trial can be measured in terms
of severity \((S_0)\), which comprises the combined effects of temperature and reaction time along
heating and cooling. \(S_0\) was determined by Lavoie et al. (2010) as:

\[
S_0 = \log R_0 = \log \left[ \frac{R_0}{R_0^\text{HEATING}} + \frac{R_0}{R_0^\text{COOLING}} \right]
\]

\[
S_0 = \log \left[ \frac{t_{\text{HEATING}}}{t_{\text{COOLING}}} \right] = \log \left[ \frac{t_{\text{MAX}}}{t_{\text{MIN}}} \right] = \log \left[ \frac{t_0}{t_1} \right]
\]

where \(R_0\) is the severity factor, \(t_{\text{MAX}}\) (min) is the time needed to achieve the desired
autohydrolysis temperature, \(t_1\) (min) is the time needed for the whole heating–cooling period, and
\(T(t)\) and \(T_0(t)\) stand for the temperature profiles in heating and cooling, respectively. Calculations
were made assuming reported data for \(x\) and \(T_{\text{REF}}\) (14.75 ºC and 100 ºC, respectively).

### 3.3.4. Analysis of the solid phase from autohydrolysis

Solid phase was recovered and weighted for gravimetric determination of the solid yield (SY). After its
separation in the end of the autohydrolysis procedure, this phase was repeatedly washed
with distilled water until a liquid with approximately pH = 7 is obtained. Afterwards, its moisture was
determined, being the yield defined as the grams of final solid phase obtained per 100 grams of
LCM, calculated through the following equation:

\[
\text{SY} = \frac{m_{\text{SPh}} \cdot (1 - H_{\text{SPh}})}{m_{\text{RMn}} \cdot (1 - H_{\text{RMn}})} \cdot 100
\]

where \(m_{\text{SPh}}\) is the humid mass, SP is the solid phase, RM represents the raw material and H is
humidity of the sample. After that, an aliquot is extracted in order to determine its composition
posteriorly through a quantitative acid hydrolysis in section 3.3.2.
3.3.5. Analysis of the solubilized fraction from autohydrolysis

An aliquot of autohydrolysis liquid phase was filtered through 0.20 µm membranes and utilized for HPLC determination of glucose, xylose, arabinose, acetic acid, hydroxymethylfurfural, and furfural, being broadly described further in the section 3.8. A second aliquot was subjected to post-hydrolysis (121 ºC for 20 min and 4 % (w/w) H₂SO₄) for oligosaccharides determination. The solubilized fraction is defined by the grams of LCM solubilized on the process for each 100 grams of initial LCM, and its conversion ratio can be defined by the equation:

\[ SF = 100 - SY \]  

where SF is the solid fraction and SY is the solid yield determined above.

3.3.6. Acid hydrolysis of autohydrolysis liquors: hemicellulosic hydrolysates

Liquors from autohydrolysis treatment of lignocellulosic materials were hydrolysed in order to cleave the xylooligosaccharides into xylose at 121 ºC, 45 min., 1.5 % H₂SO₄. These operational conditions were determined by mathematical adjustment proposed by Garrote et al. (2001). The hydrolysed liquor obtained (hemicellulosic hydrolysate) was neutralised with CaCO₃, achieving a final pH of 5, followed by the separation of the precipitated CaSO₄ from the supernatant by filtration.

3.3.7. Membrane sterilisation

The hemicellulosic hydrolysates obtained previously were filtered through a 0.2 µm membranes (Nalgene), under vacuum pressure into previously sterilised Schott flasks in autoclave, ensuring the sterilisation of the hydrolysate before fermentation.

3.3.8. Supplementation nutritional with low-cost agroindustrial by-products

Lignocellulosic hydrolysates fermentations (media used in results described in section 4.3) were supplemented with low-cost agroindustrial by-products as it was described and optimized by Kelbert et al. (2015). Cheese whey (CW) and raw yeast extract (RYE) were sterilized by pasteurization (60
and added aseptically to the hydrolysate in its solid form. CW was provided by Quinta dos Ingleses (Agro-Livestock Company, Portugal), while RYE was provided by a microbrewery (Fermentum, Portugal), being dried at 60 °C until no weight variation. Corn steep liquor (CSL) in its liquid gross form was sterilized in autoclave at 121 °C for 15 minutes. Stock solutions of urea and potassium metabisulfite \((\text{K}_2\text{O}_5\text{S}_2)\) were prepared and sterilized by filtration and in autoclave \((121 \, ^\circ\text{C}; 15 \text{ min.})\), respectively. Table 3.2 shows the concentrations in which each supplement was added to the hydrolysates (Kelbert et al., 2015).

**Table 3.2.** Low-cost agroindustrial by-products used as nutritional supplements.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheese whey</td>
<td>16.52 g/L</td>
</tr>
<tr>
<td>Raw yeast extract</td>
<td>4.10 g/L</td>
</tr>
<tr>
<td>Corn steep liquor</td>
<td>5.79 g/L</td>
</tr>
<tr>
<td>Urea</td>
<td>0.86 g/L</td>
</tr>
<tr>
<td>Potassium metabisulfite</td>
<td>0.3335 g/L</td>
</tr>
</tbody>
</table>

**3.4. Inoculum preparation**

Yeast strains used for inoculum preparation were preserved in YPX plates (5 g/L of yeast extract, 10 g/L of peptone, 20 g/L of xylose and 20 g/L of technical agar) at 4 °C, being plated regularly. Initially, a pre-inoculum was carried out in 100 ml Erlenmeyer flasks containing 30 mL of growth media, composed by 10 g/L of yeast extract, 20 g/L of peptone and 30 g/L of xylose. One isolated colony of each strain was transferred with a loop for each Erlenmeyer and a cotton plug used to seal the flask. Flasks were then incubated at 30 °C and 200 rpm for 24 h, when the exponential phase of growth was achieved. At this time, the cell suspension was transferred to 50 mL centrifuge tubes previously weighted and centrifuged \((4 \, ^\circ\text{C}; 9000 \text{ rpm}; 15 \text{ min.})\). Supernatant was rejected, the tube walls were cautiously cleaned and yeast cell pellet was weighted and pellets were resuspended in 0.9% \((\text{w/v})\) sodium chloride solution, for a final concentration of 200 mg of Fresh Yeast per millilitre \((\text{mgFY}/\text{mL})\). The suspension was homogenized by manual agitation and the concentrated cell suspensions were used to inoculate the fermentation media.
3.5. **Synthetic fermentation media**

The fermentation experiments were inoculated with 5 mg cells/mL fresh yeast of the above-mentioned suspension and carried out in 100 mL Erlenmeyer flasks, with a working volume of 30 mL at 30 ºC, with orbital shaking (150 rpm) under oxygen-limited condition at pH = 5. Final biomass concentration in the media was measured by dry cell weight in the end of the fermentations. The experiments of this study were performed in duplicate, allowing the standard deviation calculation. The composition of the fermentation media is described below.

- **Xylose medium**

  Xylose medium utilized for fermentation was composed by YP medium (10 g/L of yeast extract and 20 g/L of peptone) containing 45 g/L of xylose, and defined as YPX medium. The flasks were closed with cotton stopper, defining this condition as oxygen-limited, where the culture has contact with air but is under oxygen limitation like in industrial conditions.

- **Xylose and Glucose medium**

  These fermentations were performed in oxygen-limited condition using an YP medium, as described above, containing 20 g/L of glucose and 50 g/L of xylose.

- **Hydrolysate media**

  Hydrolysate media were composed by different lignocellulosic hydrolysates (method of production was described in 3.3) alongside different types of supplementation, carried out in 100 mL Erlenmeyer flasks with a working volume of 30 mL. Commercial supplements referred in section 4.2 consisted in yeast extract and peptone, with concentrations of 10 g/L and 20 g/L, respectively. Alternatively, low-cost supplementation was added to the same hydrolysates in the concentrations described in 3.3.2.
3.6. **Spot dilution growth assay**

Cell growth of the selected yeast strains under different stresses – acetic acid, furfural and temperature –, was assessed through a spot dilution growth assay. Cells were grown overnight in YPX medium (10 g/L of yeast extract, 20 g/L of peptone and 30 g/L of xylose) and collected by centrifugation (2500 g, 15 min, 4 ºC) when in the mid-exponential phase (OD600 aprox. 1.5). Cells were re-suspended in NaCl 0.9% and its density was normalized to an OD600 of 10. A 10-fold serial dilution of this suspension (10^1, 10^0, 10^-1, 10^-2, 10^-3, 10^-4) was prepared and 4 µl of each dilution was spotted onto each plate prepared with the several conditions: YPX plates with 10 g/L of yeast extract, 15 g/L of peptone, 20 g/L of xylose and 20 g/L of technical agar were prepared for control and the inhibitors were added in different concentrations (2.5, 5 and 6 g/L of acetic acid; 1.25, 2.5 and 5 g/L of furfural). Eucalyptus hydrolysate plate was composed by the liquor with the addition of 10 g/L of yeast extract, 15 g/L of peptone and 20 g/L of technical agar. Plates were incubated for 4 days at 30 ºC and 40 ºC and its images were obtained on a transilluminator (ChemiDoc XRS+, BioRad).

3.7. **Determination of fermentation parameters**

Glucose consumption rate (g/L-h) was calculated by the ratio between glucose consumed (G_t0 - G_{tGf}) and time needed for this consumption (t_{Gf} - t_{t0}), where G_{t0} is the concentration of glucose at initial time and G_{tGf} is the final concentration (approximately 0 g/L) for all strains evaluated. t_{t0} is the time initial (0 h) and t_{Gf} is the time needed for consumption of all glucose in the medium.

For xylose consumption rate (g/L-h), two rates were calculated in order to evaluate the differences in the xylose uptake in presence or absence of glucose. Thus, xylose consumption rate in presence of glucose was calculated by ratio between xylose consumed (X_{t0} - X_{tGf}) and time needed for glucose consumption (t_{Gf} - t_{t0}), where X_{t0} is the concentration of xylose at initial time and X_{tGf} is the xylose concentration when the glucose was totally consumed. Xylose consumption rate in absence of glucose was calculated by ratio between xylose consumed (X_{tGf} - X_{tEmax}) and the time needed for this consumption (t_{Emax} - t_{Gf}). Where, X_{Emax} is the concentration of xylose when ethanol achieved the maximum concentration and t_{Emax} is the time needed to achieve the maximal concentration of ethanol. In media with xylose as only carbon source, xylose consumption rate was calculated by ratio between xylose consumed (X_{t0} - X_{tEmax}) and time needed for glucose consumption (t_{Emax} - t_{t0})
Ethanol yield \((Y_{P/S})\) was calculated by the ratio between maximal ethanol produced and sugars consumed in this period of time. Biomass yield \((Y_{X/S})\) was determined by ratio between dry weight of cells and sugars consumed at the end of fermentation. Fermentation parameters from hydrolysates assays were expressed as ethanol obtained in g per 100 g of xylan present in lignocellulosic biomass.

### 3.8. Analytical methods

The samples collected from fermentation runs and from the hydrolysates, as well as the quantifications of components of raw materials during pretreatment autohydrolysis, quantitative acid hydrolysis and post-hydrolysis, were analysed for xylose, glucose, xylitol, acetic acid, ethanol, furfural and HMF concentration by HPLC utilizing an Agilent BioRad 87H column, operating at 60 °C, with a mobile phase 0.005 M \(H_2SO_4\) and flow rate of 0.6 mL/min. The peaks corresponding to xylose, glucose, xylitol, ethanol and acetic acid were detected using a JASCO 830-IR intelligent refractive index detector.

### 3.9. Statistical analysis

Statgraphics Centurion for Windows was used to perform the statistical analysis. Data are presented as means ± standard deviation of duplicate determination. Differences among the different yeast strains were verified using one-way ANOVA test, with subsequent Tukey’s test as a post hoc comparison of means. Statistical significance was established at \(p\) value < 0.05 for the comparisons, and assembled in homogenous groups represented by letters.
4. RESULTS AND DISCUSSION
4.1. Evaluation of metabolic engineered strains for xylose and glucose fermentation in synthetic medium (YPDX and YPX)

4.1.1. Evaluation of yeast strains co-fermentation capacity

Recent studies have shown the importance of the host strain background to construct xylose-fermenting *S. cerevisiae* (Matsushika et al., 2009b; Hector et al., 2011; Li et al., 2015). The co-fermentation of glucose and xylose is central for industrial ethanol processes, since both sugars are present in lignocellulosic biomass (Zaldivar et al., 2001). In this work, glucose and xylose uptake was evaluated in five different robust yeast strains – PE-2-X, PE-2-X-dGRE3, CA11-X, CAT-1-X and CCUG53310-X –, selected due to high tolerance to inhibitor compounds, superior ability for ethanol performance and high ethanol productivities comparing to laboratory strains (Pereira et al., 2014).

*Figure 4.1* and *Table 4.1* show fermentation parameters and main results obtained from fermentation profiles of these strains in YPDX medium.

![Graphs showing fermentation parameters](image-url)
All strains presented a similar glucose consumption rate of about 3 g/L-h, except for CCUG53310-X, which showed a consumption rate of 2 g/L-h (Figure 4.1a). It has been reported that glucose and xylose are sequentially consumed due to the slower xylose consumption in presence of glucose (Kim et al., 2013). The presence of glucose may suppress xylose consumption, since both sugars enter the cell through the same hexose transporters (Kim et al., 2012). Nevertheless, the presence of glucose in very low concentrations (less than 1 g/L) can facilitate xylose consumption (Lee et al., 2002).

In this study, xylose consumption rate was analysed separately, in the presence and absence of glucose (Figure 4.1b). When both sugars were present in the medium, PE-2-X showed the greater xylose consumption rate (more than 2 g/L-h). Nevertheless, after glucose depletion, xylose uptake dropped to about half of the initial rate. In comparison, PE-2-X-dGRE3 presented lower xylose consumption rate in the presence of both sugars. Nonetheless, PE-2-X-dGRE3 kept the same xylose consumption rate when on xylose alone (about 1.4 g/L-h), with no significant differences when compared with PE-2-X without the \textit{GRE3} deletion in the same conditions. These consumption rates can be favourably compared to literature, where rates below 0.5 g/L-h were reported in media containing both glucose and xylose in aerobic conditions (Parreiras et al., 2014). CA11-X showed a reduced rate in the first six hours (until glucose depletion), in comparison with PE-2-X and PE-2-XxdGRE3, and the consumption rate diminished to half of its initial value after that (Figure 4.1b). CAT-1-X seems to present some difficulty in co-fermenting both sugars, being the only strain that had increased consumption rate when xylose was the only carbon source (Figure 4.1b). Regardless of this increase, its values were lower than PE-2-X and PE-2-X-dGRE3, which may also suggest some inhibition from glucose on xylose consumption. CUG53310-X showed great deficiency to utilize xylose in both situations (presence or absence of glucose) as can be observed in Figure 4.1b.

In terms of ethanol yield, PE-2-X-dGRE3, CAT-1-X and CA11-X appear to be the most efficient strains (Figure 4.1c). Regarding xylitol accumulation, CA11-X and CAT-1-X produced low amounts of this by-product (about 5 g/L and 3 g/L, respectively) and CCUG53310 produced less than 2 g/L, due, most probably, to its inefficient xylose consumption (Figure 4.1d). The deletion of the \textit{GRE3} gene in PE-2-X-dGRE3 lead to a reduction in xylitol production of more than 30%, when compared to PE-2-X (Figure 4.1d), which follows the same tendency shown in previous studies (Traff et al., 2001; Träff-Bjerre et al., 2004). Consequently, the ratio between ethanol produced over sugars...
consumed was significantly higher in fermentation with PE-2-X-dGRE3 ($P<0.05$), which states clearly the positive influence of the deletion of GRE3 gene on the reduction of the generation of this by-product and improvement of bioethanol yield (Figure 1d). Nevertheless, xylitol production by PE-2-X-dGRE3 remains more than two-fold higher than the values achieved by CA11-X and CAT-1-X (Figure 1d) showing clearly the importance of the yeast chassis on the outcome of metabolic engineering strategies. Despite the fact that GRE3 deletion removes the major native route of production of this by-product (Träff et al., 2001), xylitol accumulation has been also associated to the activity of an endogenous xylitol dehydrogenase with higher specificity for xylulose (Richard et al., 1999), putative aldose reductases (Garay-Arroyo & Covarrubias, 1999) and a cofactor imbalance in the two-step conversion of xylose into xylulose, resulting in a xylitol pool formation (Eliasson et al., 2001; Almeida et al., 2011).

<table>
<thead>
<tr>
<th>Run</th>
<th>Yeast</th>
<th>Medium</th>
<th>$G_0$ (g/L)</th>
<th>$X_{0s}$ (g/L)$^*$</th>
<th>$G_f$ (g/L)</th>
<th>$X_{fs}$ (g/L)</th>
<th>$E_{max}$ (g/L)</th>
<th>Xylitol (g/L)</th>
<th>$Y_{X/S}$ (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PE-2-X</td>
<td>YPDX</td>
<td>19.8 ± 1.3</td>
<td>52.8 ± 3.5</td>
<td>0.000 ± 0.000</td>
<td>0.375 ± 0.109</td>
<td>16.7 ± 0.0</td>
<td>10.6 ± 0.6</td>
<td>0.171</td>
</tr>
<tr>
<td>2</td>
<td>PE-2-X-dGRE3</td>
<td>YPDX</td>
<td>20.2 ± 1.0</td>
<td>51.0 ± 4.6</td>
<td>0.000 ± 0.000</td>
<td>0.431 ± 0.152</td>
<td>19.4 ± 0.1</td>
<td>7.18 ± 0.39</td>
<td>0.180</td>
</tr>
<tr>
<td>3</td>
<td>CA11-X</td>
<td>YPDX</td>
<td>20.9 ± 2.9</td>
<td>42.9 ± 4.0</td>
<td>0.000 ± 0.000</td>
<td>0.882 ± 0.181</td>
<td>15.3 ± 0.7</td>
<td>5.11 ± 0.24</td>
<td>0.289</td>
</tr>
<tr>
<td>4</td>
<td>CAT-1-X</td>
<td>YPDX</td>
<td>18.7 ± 0.7</td>
<td>42.5 ± 0.1</td>
<td>0.000 ± 0.000</td>
<td>0.650 ± 0.514</td>
<td>15.5 ± 0.4</td>
<td>3.22 ± 0.09</td>
<td>0.262</td>
</tr>
<tr>
<td>5</td>
<td>CCUG53310-X</td>
<td>YPDX</td>
<td>20.6 ± 0.1</td>
<td>43.8 ± 0.2</td>
<td>0.000 ± 0.000</td>
<td>30.2 ± 0.929</td>
<td>8.66 ± 0.22</td>
<td>1.47 ± 0.21</td>
<td>0.401</td>
</tr>
<tr>
<td>6</td>
<td>PE-2-X-dGRE3</td>
<td>YPX</td>
<td>-</td>
<td>48.6 ± 1.3</td>
<td>-</td>
<td>0.000 ± 0.000</td>
<td>10.3 ± 0.2</td>
<td>8.08 ± 0.32</td>
<td>0.278</td>
</tr>
<tr>
<td>7</td>
<td>CA11-X</td>
<td>YPX</td>
<td>-</td>
<td>43.3 ± 1.5</td>
<td>-</td>
<td>0.375 ± 0.025</td>
<td>7.26 ± 0.43</td>
<td>6.73 ± 0.43</td>
<td>0.341</td>
</tr>
<tr>
<td>8</td>
<td>CAT-1-X</td>
<td>YPX</td>
<td>-</td>
<td>41.8 ± 0.8</td>
<td>-</td>
<td>0.252 ± 0.061</td>
<td>8.67 ± 0.10</td>
<td>3.63 ± 0.34</td>
<td>0.374</td>
</tr>
<tr>
<td>9</td>
<td>CCUG53310-X</td>
<td>YPX</td>
<td>-</td>
<td>41.3 ± 0.4</td>
<td>-</td>
<td>34.5 ± 1.0</td>
<td>0.613 ± 0.036</td>
<td>0.53 ± 0.07</td>
<td>0.101</td>
</tr>
</tbody>
</table>

$G_0$ is the glucose concentration at time = 0 h; $X_{0s}$ is the xylose concentration at time = 0 h; $G_f$ is the glucose concentration at final time = 72 h; $X_{fs}$ is the xylose concentration at final time = 72 h; $E_{max}$ is the maximal ethanol concentration; $Y_{X/S}$ is the biomass yield at the end of fermentation (g of dry cell/g of consumed sugar).

4.1.2. Evaluation of yeast strains xylose fermentation capacity

To evaluate yeast behaviour in the presence of xylose as the only carbon source and observe the capacity of modified strains for xylose fermentation, its consumption in synthetic medium was assayed with four of the yeast strains above-mentioned: PE-2-X-dGRE3, CA11-X, CAT-1-X and CCUG53310-X (Figure 4.2 and Table 4.1). It is possible to observe that PE-2-X-dGRE3 showed a higher xylose consumption rate when compared with the other strains tested, consuming about 1
g/L of xylose per hour (Figure 4.2a). In contrast, CCUG53310-X was clearly the less efficient strain, consuming less than 20% of the xylose present in the medium (Table 4.1, Run 9), and at a much slower xylose consumption rate (Figure 4.2a). The high consumption rate of xylose exhibited by PE-2-X-dGRE3 resulted in a consequently higher production of xylitol, achieving 8.08 g/L. CA11-X and CAT-1-X, while presenting similar xylose consumption rates, had statistically significant differences (P<0.05) when comparing xylitol production (6.73 g/L and 3.63 g/L, respectively). Xylitol production by CCUG53310-X was low as a consequence of its low xylose consumption (Figure 4.2b). Ratio between ethanol produced and xylose consumed confirmed the poor efficiency of CCUG53310-X for ethanol production from xylose exclusively, while the other three strains tested in this assay have shown no statistically significant differences between them, achieving ethanol yields of approximately 0.2 g/g (Figure 4.2c). Several works have been carried out regarding xylose consumption in aerobic conditions (Anderlund et al., 2001; Toivari et al., 2001; Pitkänen et al., 2005), with low ethanol yield (less than 0.1 g/g) and relatively high xylitol production (up to 0.62 g/g), with most encouraging results in anaerobic conditions with ethanol yields up to 0.43 g/g and low xylitol production (Karhumaa et al., 2007). Differences on xylitol production, xylose consumption and ethanol concentration among recombinant *S. cerevisiae* strains isolated from different industrial processes (brewery, bakery and food industry) were also reported by Matsushika et al. (2009a) in which a flocculating strain from brewery was the most efficient strain for xylose-to-ethanol production in all conditions evaluated. In the present work, the background of strains isolated from similar fermentation environments, bioethanol and “cachaça” industries, were evaluated and proved to differently influence the fermentation outcome, mainly regarding xylitol production and xylose consumption rate.

Figure 4.2. Fermentation parameters obtained from experiments carried out in YPX medium under conditions listed in Table 4.1: a) Xylose consumption rate; b) maximum xylitol produced; c) ethanol yield, ratio between ethanol produced and xylose consumed.
4.2. Evaluation of yeast strain performances on lignocellulosic hydrolysates

4.2.1. Hydrothermal treatment of lignocellulosic biomass: hemicellulosic hydrolysates

_Eucalyptus globulus_ wood, _Paulownia tomentosa_ wood, corn cob and wheat straw were subjected to hydrothermal treatment (or autohydrolysis treatment) in order to solubilize the hemicellulose fraction into oligosaccharides and monosaccharides and to alter their recalcitrant structures improving the enzymatic saccharification. Table 4.2 showed the chemical composition of raw materials and pretreated lignocellulosic biomasses. After treatment in solid phase, cellulose recovery (measured as glucan) was 93.6, 96.5, 86.5 and 98.03 % for eucalypt, paulownia, wheat straw and corn cob, respectively. On the other hand, the almost complete solubilisation of xylan in liquid phase was achieved for the four lignocellulosic biomasses studied. Second stage of acid hydrolysis of four autohydrolysis liquors were carried out in order to obtain hydrolysates enriched in xylose. Table 1 also showed the chemical composition of four hydrolysates used in this study for the evaluation of xylose consumption by metabolic engineered _S. cerevisiae_ strains. As can be observed, corn cob hydrolysate showed the highest xylose concentration followed by _Eucalyptus_ and wheat straw hydrolysates. Hardwoods achieved a higher acetic acid concentration (6 and 5 g/L for _Eucalyptus_ and _Paulownia_) than agricultural residues. The highest concentration of furfural and HMF was obtained in _Eucalyptus_ hydrolysate.

The hydrolysates obtained from hydrothermal and acid hydrolysis were used as fermentation media in further experiments. For that, the hydrolysates were employed without additional step of detoxification.
Table 4.2. Characterization of lignocellulosic biomasses (composition of solid phase and hydrolysate) and operational conditions of pretreatment.

<table>
<thead>
<tr>
<th>Raw material composition (g of component/100 g of raw material)</th>
<th>Eucalyptus globulus</th>
<th>Paulownia tomentosa</th>
<th>Wheat straw</th>
<th>Corn cob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan</td>
<td>45</td>
<td>40</td>
<td>37</td>
<td>34</td>
</tr>
<tr>
<td>Xylan</td>
<td>16</td>
<td>15</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Arabinan</td>
<td>1.09</td>
<td>-</td>
<td>1.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Acetylgroups</td>
<td>3.0</td>
<td>3.2</td>
<td>2.8</td>
<td>2.4</td>
</tr>
<tr>
<td>Klason Lignin</td>
<td>28</td>
<td>22</td>
<td>27</td>
<td>25</td>
</tr>
</tbody>
</table>

Hydrothermal treatment conditions

<table>
<thead>
<tr>
<th>Solids Yield</th>
<th>S&lt;sub&gt;0&lt;/sub&gt;=4.08; LSR=8 g/g</th>
<th>S&lt;sub&gt;0&lt;/sub&gt;=4.19; LSR=8 g/g</th>
<th>S&lt;sub&gt;0&lt;/sub&gt;=3.92; LSR=10 g/g</th>
<th>S&lt;sub&gt;0&lt;/sub&gt;=3.83; LSR=8 g/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid Yield</td>
<td>71.4</td>
<td>68.9</td>
<td>60.2</td>
<td>64.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>a) Solid phase composition (g of component/100 g of hydrothermally pretreated raw material)</th>
<th>Eucalyptus globulus</th>
<th>Paulownia tomentosa</th>
<th>Wheat straw</th>
<th>Corn cob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan</td>
<td>59</td>
<td>56</td>
<td>63</td>
<td>52</td>
</tr>
<tr>
<td>Xylan</td>
<td>2.1</td>
<td>2.3</td>
<td>7.2</td>
<td>13</td>
</tr>
<tr>
<td>Acetylgroups</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Klason Lignin</td>
<td>34</td>
<td>36</td>
<td>27</td>
<td>25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b) Hydrolysate composition from acid hydrolysis of autohydrolysis liquor (g/L)</th>
<th>Eucalyptus globulus</th>
<th>Paulownia tomentosa</th>
<th>Wheat straw</th>
<th>Corn cob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Xylose</td>
<td>16</td>
<td>14</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>5</td>
<td>6</td>
<td>2.6</td>
<td>4</td>
</tr>
<tr>
<td>HMF</td>
<td>0.3</td>
<td>0.15</td>
<td>0.13</td>
<td>0.2</td>
</tr>
<tr>
<td>Furfural</td>
<td>1.6</td>
<td>0.85</td>
<td>0.35</td>
<td>0.4</td>
</tr>
</tbody>
</table>

4.2.2. Low-cost supplementation in lignocellulosic hydrolysates fermentation using PE-2-X-dGRE3

Once analysed the yeast strains behaviour in synthetic media, it is of major importance to evaluate its performance in real lignocellulosic hydrolysates, where the source of raw material could play an important role. Lignocellulosic hydrolysates present poor nutritional content, creating the necessity of external nutrient supplementation in order to improve ethanol yields (Jørgensen, 2009). In previous work, the supplementation of *E. globulus* hydrolysate with low cost agro-industrial by-products was optimized in order to improve the glucose fermentation and ethanol yield using wild-type PE-2 strain (Kelbert et al., 2015). Here, the PE-2-X-dGRE3 strain was selected to evaluate the effect of substitution of commercial nutrients by these low-cost supplements in xylose fermentation conditions in all of the hydrolysates used in this work. Results in Figure 4.3 show that xylose consumption using both types of supplementation was similar in all hydrolysates.
Figure 4.3. Time course of hemicellulosic hydrolysate fermentation (data expressed as g of xylose and ethanol per 100 g of xylan present in raw material) at oxygen-limited conditions using PE-2×dGRE3: a) Eucalyptus globulus hydrolysate; (b) Paulownia tomentosa hydrolysate; c) Wheat straw hydrolysate; and d) Corn cob hydrolysate. Experiments were performed with different supplementation: low-cost (LC) and commercial (YP) supplements at 30 º C. Glucose concentration was below 1 g/L and acetic acid levels were not inhibitory. Statistical differences were only detected in the ethanol concentrations obtained from corn cob hydrolysate (P<0.05).

Additionally, the different supplementation had no effect on the ethanol production on three of the hydrolysates (Figure 4.3a, b and c), and was slightly superior (P<0.05) with low-cost supplementation in the corn cob hydrolysate (Figure 4.3d). This fact is of relevant importance since the commercial supplementation could be substituted by low-cost supplements in lignocellulosic fermentation processes reducing operational costs. Taking into account these results, subsequent fermentation experiments were carried out with low-cost supplements.
4.2.3. Xylose consumption of metabolic engineered strains on lignocellulosic hydrolysates

Widespread strategy for the evaluation of metabolic or evolutionary engineered strains for xylose consumption is based on screening in a synthetic medium for single inhibitor/stress tolerance (Li et al., 2015) or in only one lignocellulosic hydrolysate and evolutionary engineering to improve tolerance (Demeke et al., 2013; Li et al., 2015). It should be taken into account that the composition of hydrolysates can be heterogeneous since this depends on the pretreatment employed, the conditions of pretreatment and the source of lignocellulosic biomass (Koppram et al., 2014).

In this work, four hydrolysates from fast growing hardwood and agricultural residue biomasses were employed as fermentation media to evaluate the metabolic engineering yeast performances. Due to CCUG53310 poor capacity for xylose fermentation, only three yeast strains were selected (PE-2-X-dGRE3, CA11-X and CAT-1-X) for fermentation in the four hydrolysates with low-cost supplementation (runs listed in Table 4.3). In Figure 4.4, differences among strains using several hydrolysates can be compared, showing the broad range of conditions evaluated in this work.

Table 4.3. Main results of batch fermentation performance of Saccharomyces cerevisiae strains (PE-2 with GRE3 deleted, dGRE3, CA11 and CAT-1 backgrounds with the xylose metabolic pathway, X) in lignocellulose hydrolysates (EGW – Eucalyptus globulus; PTW – Paulownia tomentosa wood; WS – wheat straw; CC – corn cob).

<table>
<thead>
<tr>
<th>Run</th>
<th>Yeast</th>
<th>Hydrolysate</th>
<th>T (°C)</th>
<th>G₀ (g/L)</th>
<th>X₀ (g/L)</th>
<th>Gₖ (g/L)</th>
<th>Xₖ (g/L)</th>
<th>AA (g/L)</th>
<th>E_max (g/100 g Xn)</th>
<th>Yₑ/(G+Xₖ) (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PE-2-X-dGRE3</td>
<td>EGW</td>
<td>30</td>
<td>0.994 ± 0.017</td>
<td>13.2 ± 0.1</td>
<td>0.000 ± 0.000</td>
<td>2.10 ± 0.27</td>
<td>4.30 ± 0.18</td>
<td>16.7 ± 0.2</td>
<td>0.430</td>
</tr>
<tr>
<td>2</td>
<td>CA11-X</td>
<td>EGW</td>
<td>30</td>
<td>1.51 ± 0.79</td>
<td>15.0 ± 0.7</td>
<td>0.000 ± 0.000</td>
<td>0.302 ± 0.111</td>
<td>3.93 ± 0.62</td>
<td>12.8 ± 0.6</td>
<td>0.293</td>
</tr>
<tr>
<td>3</td>
<td>CAT-1-X</td>
<td>EGW</td>
<td>30</td>
<td>1.27 ± 0.03</td>
<td>16.5 ± 0.1</td>
<td>0.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>4.28 ± 0.10</td>
<td>12.0 ± 0.1</td>
<td>0.278</td>
</tr>
<tr>
<td>4</td>
<td>PE-2-X-dGRE3</td>
<td>PTW</td>
<td>30</td>
<td>1.42 ± 0.03</td>
<td>13.2 ± 0.3</td>
<td>0.000 ± 0.000</td>
<td>1.50 ± 0.04</td>
<td>5.56 ± 0.06</td>
<td>15.6 ± 0.2</td>
<td>0.459</td>
</tr>
<tr>
<td>5</td>
<td>CA11-X</td>
<td>PTW</td>
<td>30</td>
<td>1.82 ± 0.42</td>
<td>13.9 ± 2.3</td>
<td>0.000 ± 0.000</td>
<td>0.767 ± 0.417</td>
<td>5.07 ± 0.71</td>
<td>16.1 ± 1.0</td>
<td>0.431</td>
</tr>
<tr>
<td>6</td>
<td>CAT-1-X</td>
<td>PTW</td>
<td>30</td>
<td>2.06 ± 0.10</td>
<td>14.5 ± 0.2</td>
<td>0.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>6.03 ± 0.05</td>
<td>13.9 ± 0.1</td>
<td>0.426</td>
</tr>
<tr>
<td>7</td>
<td>PE-2-X-dGRE3</td>
<td>WS</td>
<td>30</td>
<td>0.958 ± 0.197</td>
<td>13.2 ± 0.5</td>
<td>0.000 ± 0.000</td>
<td>1.14 ± 0.134</td>
<td>2.74 ± 0.26</td>
<td>17.6 ± 1.4</td>
<td>0.400</td>
</tr>
<tr>
<td>8</td>
<td>CA11-X</td>
<td>WS</td>
<td>30</td>
<td>1.38 ± 0.28</td>
<td>14.5 ± 3.2</td>
<td>0.000 ± 0.000</td>
<td>0.878 ± 0.154</td>
<td>2.63 ± 0.33</td>
<td>10.9 ± 0.1</td>
<td>0.249</td>
</tr>
<tr>
<td>9</td>
<td>CAT-1-X</td>
<td>WS</td>
<td>30</td>
<td>1.43 ± 0.09</td>
<td>15.5 ± 0.1</td>
<td>0.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>2.75 ± 0.07</td>
<td>10.2 ± 0.6</td>
<td>0.266</td>
</tr>
<tr>
<td>10</td>
<td>PE-2-X-dGRE3</td>
<td>CC</td>
<td>30</td>
<td>1.12 ± 0.01</td>
<td>21.5 ± 0.4</td>
<td>0.000 ± 0.000</td>
<td>1.44 ± 0.08</td>
<td>3.82 ± 0.14</td>
<td>35.6 ± 0.2</td>
<td>0.424</td>
</tr>
<tr>
<td>11</td>
<td>CA11-X</td>
<td>CC</td>
<td>30</td>
<td>1.73 ± 0.44</td>
<td>24.1 ± 4.7</td>
<td>0.000 ± 0.000</td>
<td>1.06 ± 0.05</td>
<td>3.69 ± 0.53</td>
<td>23.5 ± 0.8</td>
<td>0.279</td>
</tr>
<tr>
<td>12</td>
<td>CAT-1-X</td>
<td>CC</td>
<td>30</td>
<td>2.16 ± 0.19</td>
<td>25.7 ± 1.5</td>
<td>0.000 ± 0.000</td>
<td>0.119 ± 0.051</td>
<td>4.43 ± 0.05</td>
<td>26.4 ± 0.6</td>
<td>0.306</td>
</tr>
<tr>
<td>13</td>
<td>PE-2-X-dGRE3</td>
<td>EGW</td>
<td>40</td>
<td>0.615 ± 0.049</td>
<td>14.3 ± 0.6</td>
<td>0.000 ± 0.000</td>
<td>6.15 ± 0.39</td>
<td>4.37 ± 0.31</td>
<td>14.0 ± 0.2</td>
<td>0.370</td>
</tr>
<tr>
<td>14</td>
<td>CA11-X</td>
<td>EGW</td>
<td>40</td>
<td>0.780 ± 0.028</td>
<td>14.8 ± 0.0</td>
<td>0.000 ± 0.000</td>
<td>4.00 ± 0.000</td>
<td>4.46 ± 0.26</td>
<td>16.1 ± 0.1</td>
<td>0.363</td>
</tr>
</tbody>
</table>

T (°C) is the temperature of incubation during fermentation process; G₀ is the glucose concentration at time = 0 h; X₀ is the xylose concentration at time = 0 h; Gₖ is the glucose concentration at final time = 72 h; Xₖ is the xylose concentration at final time = 72 h; E_max is the maximal ethanol concentration; Yₑ/(G+Xₖ) is ethanol yield from glucose and xylose; AA is the initial concentration of acetic acid in each LCM hydrolysate.
In *E. globulus* wood, xylose consumption rate was very similar for the three yeast strains (more than 0.3 g/L-h), but when considering the ratio between ethanol produced over xylose consumed, PE-2-X-dGRE3 performed significantly better (Figure 4.4a), with a yield of 0.43 g/g in the first 24 hours, close to the theoretical yield, while the two other strains exhibited ratios of about 0.30 g/g (Table 4.3, Run 1-3). In *Paulownia tomentosa* wood hydrolysate, none of the yeast stood out, with all of them showing high ratios of ethanol production over xylose consumption in the first 24 hours (0.43-0.46 g of ethanol/g of sugar) (Figure 4.4b). The lowest xylose consumption rate in this hydrolysate was influenced by more inhibitor loading (Table 4.2). These results show great capability of fermentation by the tested strains even with relatively high concentrations of acetic acid present in this hydrolysate (between 5 and 6 g/L).

In wheat straw hydrolysate, xylose consumption rates were significantly similar for the evaluated strains (Figure 4.4c), showing relevant differences in ethanol yield (PE-2-X-dGRE3 achieved the highest yield of 0.4 g/g). Lastly, in Corn cob hydrolysates, CA11-X presented a xylose consumption rate of 0.47 g/L-h, significantly higher than PE-2-X-dGRE3 (0.41 g/L-h), and close to theoretical yield, with CAT-1-X consuming xylose at a 0.45 g/L-h rate (Table 4.3, Run 10-12). However, despite lower rate consumption, ethanol yield of PE-2-X-dGRE3 was significantly greater than CA11-X and CAT-1, presenting a ratio of 0.43 g of ethanol per g of xylose consumed (Figure 4.4d).

Overall, lower xylose consumption was obtained by fast growing hardwoods (*Paulownia* and *Eucalyptus*) when compared to hydrolysates from agricultural residues, corn cob and wheat straw. This fact could be due to higher acetic acid concentration in hardwood hydrolysates (Table 4.3). Acetic acid is one of the most inhibitory compounds released from hemicellulose during pretreatment, affecting greatly both growth and ethanol fermentation of yeast strains (Demeke et al., 2013). Its effects are known to be more severe for xylose fermentation than for glucose fermentation (Casey et al., 2010) and the presence of acetic acid in concentrations of about 4 to 5 g/L is capable of decreasing xylose consumption (Casey et al., 2010; Demeke et al., 2013). The overall relatively low xylose consumption rates obtained in these hydrolysates comparing with synthetic media (Figure 4.4) may be due to synergistic toxic effects of acetic acid, phenolics, furfural and hydroxymethylfurfural present in the hydrolysates composition (Ko et al., 2016).
**Figure 4.4.** Fermentation parameters (xylose consumption rate and ethanol yield) obtained from experiments carried out under conditions listed in Table 3 using: a) Eucalyptus globulus hydrolysate; b) Paulownia tomentosa hydrolysate; c) Wheat straw hydrolysate; and d) Corn cob hydrolysate.
Direct comparison with results obtained in the literature is not straightforward due to process complexity and heterogeneity that includes: type of pretreatment (hydrothermal or acid), the source of raw material that determines the operational conditions and consequently the inhibitor compounds released, the background of selected strain and the metabolic pathway used. Anyway some good examples can be picked up from literature where interesting ethanol yields were reported for recombinant strains in specific hydrolysates. Koppram et al. (2013) reported efficient ethanol production with a recombinant KE6-12 strain carrying the XR-XDH pathway in simultaneous saccharification and co-fermentation fed-batch assays in corn cob hydrolysate, with ethanol yields up to 0.39 g/g. Ko et al. (2016) reported for the SXA-R2 P-E strain, expressing the XI pathway, in oak hardwood hydrolysate with approximately 6 g/L of acetic acid, an ethanol yield of 0.43 g/g. Studies using wheat straw as feedstock with industrial recombinant yeast strains expressing also the XR-XDH pathway have reported ethanol yields from 0.39 g/g (KE6-12 strain) up to 0.48 g/g, with TMB3400 (Erdei et al., 2012 and 2013b). While these results were obtained in fed-batch fermentation, the ethanol yields obtained in this work stand among those. Besides in this study, the hydrolysates were supplemented with low cost nutrients and not with the commercially available but expensive yeast extract and peptone. Still worth of note are the results obtained in this work that show differences between the engineered strains evaluated depending on the lignocellulosic hydrolysate used, supporting the idea that the selection of yeast for integrated lignocellulosic bioethanol processes should be addressed from a global perspective (considering all hurdles at once). The selection of the yeast chassis cell and advanced engineering strategies should take into account from the very beginning the process conditions and biomass material that will be used in order to attain a high-productivity process.

4.2.4. Thermotolerance evaluation

Thermotolerance is other attractive feature to be evaluated since high temperatures are necessary for the enzymatic saccharification of pretreated lignocellulosic biomass. From the results shown above, it is possible to observe that PE-2-X-dGRE3 appeared to be the yeast strain with more stable behaviour in both synthetic and hydrolysate media, presenting good ethanol yields. In addition, CA11-X has a strong background of thermotolerance in glucose fermentation (Ruiz et al., 2012). For
these reasons, both strains were selected for a further study in *Eucalyptus globulus* hydrolysate at 40 °C.

In order to evaluated several stress conditions simultaneously which generates a large number of fermentations, both yeast strains growth was evaluated in a spot assay in eucalyptus hydrolysate plates, as well as in xylose agar plates with different concentrations of acetic acid and furfural (inhibitors present in EGW hydrolysates), at 30 °C and 40 °C. Figure 4.5 showed the growth of strains at different inoculum load. Results obtained showed efficient growth of both plates at 30 °C in control and Eucalyptus hydrolysate assays, as well as in 2.5 g/L of acetic acid and 2.5 g/L of furfural (Figure 4.5). Despite this, at 40 °C, residual growth was observed in all conditions only in the most concentrated dilution which determines a great influence of inoculum size. From the previous fermentations with these strains, it was observed fermentation ability to perform in media up to almost 6 g/L of acetic acid (*Paulownia tomentosa* hydrolysate). Also, as said before, the strong thermotolerance background should allow cell growth at higher temperatures. These results highpoint the differences between solid and liquid media, inducing once and again that a clearly estimate of the effect of stress factors should not be performed individually.

![Figure 4.5](image)

*Figure 4.5. Growth of *S. cerevisiae* strains on the plate at stress conditions. 4 μL of each suspension from 10-fold serial dilution with initial OD600 of 1.0 was spotted onto the plates. All cell cultivations were incubated at 30 °C and 40 °C for 4 days, for thermotolerance evaluation under the different concentrations of inhibitors.*

In addition, eucalyptus hydrolysate fermentations were carried out in liquid medium in order to evaluate the xylose consumption and ethanol production. Figure 4.6a shows the fermentation profile obtained by these strains. At this temperature, it was possible to observe that CA11-X was
able to consume all xylose present in the medium (Table 4.3, Run 13 and 14) at a higher rate, while PE-2-X-dGRE3 consumed approximately 70 % of xylose, at a rate of 0.2 g/L·h (Figure 4.6b). Probably, a synergetic effect occurs when the temperature is increased from 30 to 40 °C being the hydrolysate more inhibitory for the strains (Kelbert et al., 2016). Ethanol yield was also higher in CA-11-X fermentation, with production of almost 16 g of ethanol per 100 g of xylan in raw material, against less than 14 g of ethanol in the same condition (Table 4.3). Also, when comparing these results with the same fermentations at 30 °C, it is possible to observe that PE-2-X-dGRE3 presents a significant reduction in xylose consumption rate, while CA11-X maintains a constant value. The elevated temperature and presence of inhibitory compounds are known to cause a common stress response to protect yeast cell from damage (Lu et al., 2012). Being CA11-X strain more thermotolerant than the PE-2-X strain, on these specific fermentation conditions, CA11-X was able to cope better with the stressors present on the hydrolysate showing a fermentation performance superior to that of PE-2-X strain.

![Figure 4.6. Thermotolerance evaluation of PE-2-X-dGRE3 (full symbols) and CA11-X (empty symbols) at 40 °C in Eucalyptus globulus hydrolysates: a) Time course profile of fermentation, b) Xylose consumption rate](image)

**4.3. Overall comparison of industrial *S. cerevisiae* backgrounds**

In this work we present a screening approach that allows the identification of potential chassis strains with suitable backgrounds to cope with process-specific challenges that arise during lignocellulosic bioethanol production. An initial screening for evaluation of co-fermentation of glucose and xylose showed differences between the strains inherent capacities for xylose consumption: while PE-2-X, PE-2-X-dGRE3 and CA11-X were capable of simultaneously consume both sugars, CAT-1-X showed difficulties in co-consuming both sugars. Additionally, the CCUG53310-X strain, that has
been described to efficiently produce ethanol from glucose in the presence of lignocellulosic-derived inhibitors (Pereira et al., 2014), has been shown here to be clearly incapable of metabolizing xylose, making it unfeasible as chassis choice for valorization of lignocellulosic hydrolysates with high contents of this sugar. When comparing all strains for their capacity to metabolize xylose in synthetic media, both in the presence and absence of glucose, we observed a superior performance by PE-2-X and PE-2-X-dGRE3. Nonetheless, the PE-2 strain presented an innate propensity for xylitol accumulation, and such strains may benefit from an alternative xylose metabolic pathway, such as the non-cofactor-requiring xylose isomerase pathway. This highlights the importance of adjusting the metabolic engineering strategy to the genetic background of the chassis strains.

In a subsequent evaluation of strains fermentation performance in real lignocellulosic hydrolysates, it was observed that the presence of inhibitory compounds clearly hampered xylose consumption rate comparing with synthetic media, supporting the importance of testing several challenging conditions simultaneously. Moreover, the necessity of adjusting the strain selection to the specific inhibitory load of different hydrolysates was underlined by the heterogeneous results obtained from the four hydrolysates tested: all strains had similar performances in the *Paulownia* hydrolysate, while PE-2-X-dGRE3 achieved superior ethanol yields in the other three hydrolysates.

Despite the distinguished overall results of PE-2-X-dGRE3, the CA11-X strain had a superior performance in fermentation of *E. globulus* hydrolysate at 40 °C, a clear consequence of its thermostolerant background. This result highlights, not only the importance of the strain background, but also the necessity of performing screenings with integration of all the major obstacles for efficient bioethanol production from lignocellulosic material: xylose consumption, inhibitory loads and higher process temperatures.
5. **Conclusions and Future Perspectives**
Conclusions

In this work, a screening approach was addressed in order to identify a suitable chassis *S. cerevisiae* strains capable of coping with the major challenges present in lignocellulosic fermentation process.

The influence of *S. cerevisiae* strains background for xylose consumption has been demonstrated with the differences obtained in the fermentation performances, not being their behaviour same in all conditions evaluates. These heterogeneous outcomes highlight the importance of carefully addressing the engineering of yeast strains for efficient lignocellulosic ethanol production.

- In synthetic media containing glucose and xylose sugars, glucose consumption rates were very similar, not showing significant differences among industrial metabolic engineered strains. Nevertheless, different rates in xylose consumption in presence or absence of glucose were observed among strains. While PE-2 and CA11 recombinant strains (PE-2-X, PE-2-X-dGRE3 and CA11-X) were able to consume glucose and xylose simultaneously, engineered CAT-1 (CAT-1-X) utilizes these sugars sequentially, consuming xylose only after glucose depletion. On the other hand, CCUG-55310 was not efficiently able to ferment xylose in presence of glucose, showing very low xylose consumption. Xylitol production significantly varied among strains, resulting PE-2-X the strain with the highest production. The deletion of GRE3 reduced the xylitol accumulation in comparison with PE-2-X. Nevertheless, the xylitol production obtained by PE-2-X-dGRE3 was even superior to xylitol produced by CAT-1-X and CA11-X.

- In lignocellulosic hydrolysates fermentations, commercial nutrients (peptone and yeast extract) were successfully substituted by low-cost nutritional supplements, obtaining the same profile of fermentation in all hydrolysates studied. The metabolic engineered strains evaluated in this work showed to be capable to ferment non-detoxified hydrolysates from several raw materials (hardwoods and agricultural residues). Despite the overall superior performance of PE-2-X-dGRE3 at 30 °C, when subjected to high temperatures, necessary temperature for the enzymatic hydrolysis, CA11-X was clearly superior, utilizing all xylose present in the medium, which is concomitant with its genetic thermotolerant background. This data supports the idea that the genetic background of the chassis strains plays a major role in fermentation of pentose sugars.
In this work, the screening strategy proposed aims to show that metabolic engineered strains can display different response dependent of stress condition exposed. The selection of one strain in basis on only one condition of stress (temperature, or inhibitors or glucose and xylose) could be not suitable taking account that all these process conditions are present together and not individually.

**Future Perspectives**

Further studies should focus on the refinement of the metabolic genetic engineering here approached. A combination of genetic modification can lead to interesting results that may overtake some of the present challenges. Alternative approaches relative to GRE3 expression levels may be useful to diminish xylitol formation, by focusing on a fine-tuning of its expression, instead of its deletion and simultaneous expression of its homologous xylose reductase, that catalyses the same reaction of aldolase reductase (Khattab and Kodaki, 2014). Results presented here suggest that this approach focusing xylitol reduction may be worth of be applied in CA11 strain, that showed good fermenting ability alongside low by-products formation.

Furthermore, improvements can be achieved in terms of xylose assimilation. The genetic manipulation of hexose transporters, by increasing its affinity for xylose (Reider Apel et al., 2016) or preventing its degradation in the absence on glucose (Nijland et al., 2016), may lead to stimulating outcomes in a near future. Also, additional modifications in the non-oxidative pentose phosphate pathway alongside the expression of the metabolic engineering presented in this work may lead to an improvement in xylose utilization and ethanol yield.

Simultaneous saccharification and fermentation assay can be addressed with the selected strain in order to attain an integrated evaluation of lignocellulose-to-ethanol process. Therefore, whole slurry (liquid and solid phases) from pretreatment could be used as carbon source. Enzymatic saccharification of oligosacharides from autohydrolysis liquors could be evaluated in order to substitute the acid hydrolysis and to obtain a more integrated process. In addition, the high solid loadings should be also approached to achieve higher ethanol titres. For that, different strategies of
SSCF could be carried out attending at stage of process and temperature (presaccharification or hemicellulosic fermentation before cellulose saccharification), assessing the moment of inoculation or enzyme addition with the objective to improve lignocellulosic to ethanol conversion. Scale-up of process should be carried out, being necessary a pre-evaluation of bioreactor design more appropriate for the purpose developed process.
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