Used but not Sensed - The Paradox of D-xylose Metabolism in Saccharomyces cerevisiae

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Abstract

The realization that the extraction and combustion of fossil fuels is having serious effects on the environment and the climate, together with the ever-growing need for fuels, has led to the development of the concept of the biorefinery. Biorefineries are refineries in which fossil resources, such as oil, are replaced by renewable biomaterials to produce biofuels and biochemicals. Non-edible biomass is used in a lignocellulose-based refinery, which avoids the conflict between fuel and food production, but a number of inherent technical challenges must be overcome. The robust and genetic engineering-friendly yeast *Saccharomyces cerevisiae* (baker's yeast) is a promising platform organism for biomass fermentation, but it lacks functional assimilatory pathways to utilise D-xylose, the second most abundant sugar in a wide range of lignocellulosic materials. During the past two decades, recombinant forms of *S. cerevisiae* have been developed able to efficiently convert D-xylose to ethanol. However, the rate of conversion is slow, and D-xylose appears not to be recognised by *S. cerevisiae* as a fermentable sugar.

This thesis is focused on investigating the role of the sugar sensing and signalling routes in the unusual behaviour of *S. cerevisiae* on D-xylose. A panel of in vivo biosensors coupled to D-glucose signalling routes was used under different physiological conditions and in the presence of different genetic modifications. The green fluorescent protein gene (yEGFP3) was coupled to different endogenous yeast promoters known to be regulated by at least one of the three main sugar pathways: Snf3p/Rgt2p, cAMP/PKA and SNF1/Mig1p.

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Further validation of the role of sugar signalling was obtained by using targeted deletants known to improve the D-xylose consumption rate without being directly associated with D-xylose catabolic routes. Notably, it was found that the signalling response on D-xylose changed from a low D-glucose signal in the background strain, to simultaneous signalling of high and low D-glucose in the best strain (*ira2*Δ*isu1*Δ). Since *IRA2* is a repressor of PKA activity, this finding supported the hypothesis of the malfunction of PKA activity on D-xylose due to poor sensing through this route.

This study also focused on understanding whether the sensing signal observed in the presence of high concentrations of D-xylose could be linked to a metabolite acting as a pathway regulator. Using strains in which *PGI1*, which encodes an isomerase enabling the reversible conversion of glucose-6-phosphate and fructose-6-phosphate, had been deleted, it was possible to link changes in signalling to disturbances in the levels of glycolytic intermediates. The findings presented in this thesis support the hypothesis of a dysfunctional sugar signalling mechanism on D-xylose, and show that the phenotype is a result of the lack of membrane sensing in connection with alterations in intracellular signalling.

Key words: *Saccharomyces cerevisiae*; sugar sensing/signalling; D-xylose; XR/XDH; GFP biosensor; cAMP/PKA; Snf3p/Rgt2p; SNF1/Mig1p; flow cytometry

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MADE IN SWEDEN

À minha querida Batcham, Lourdes Ofugi

Abstract

The realisation that the extraction and combustion of fossil fuels is having serious effects on the environment and the climate, together with the ever-growing need for fuels, has led to the development of the concept of the biorefinery. Biorefineries are refineries in which fossil resources, such as oil, are replaced by renewable biomaterials to produce biofuels and biochemicals. Non-edible biomass is used in a lignocellulose-based refinery, which avoids the conflict between fuel and food production, but a number of inherent technical challenges must be overcome. The robust and genetic engineering-friendly yeast *Saccharomyces cerevisiae* (baker's yeast) is a promising platform organism for biomass fermentation, but it lacks functional assimilatory pathways to utilise D-xylose, the second most abundant sugar in a wide range of lignocellulosic materials. During the past two decades, recombinant forms of *S. cerevisiae* have been developed able to efficiently convert D-xylose to ethanol. However, the rate of conversion is slow, and D-xylose appears not to be recognised by *S. cerevisiae* as a fermentable sugar.

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Popular Scientific Summary

The worldwide demand for energy has increased dramatically during the past decades. Combustion of these fuels has resulted in increasing levels of greenhouse gases, leading to global warming and climate change. However, fossil fuels such as coal, oil and gas still play a dominant role in the world's economy, technology and geopolitics. Sustainable alternatives are therefore needed, based on the use of renewable feedstock. Recent years have seen the development of so-called biorefineries, i.e. refineries in which petroleum-based resources are replaced by biomass for the production of biofuels, value-added bio-based products and chemicals, and power. Renewable feedstocks such as sugarcane (in Brazil) and corn (in the USA) are already being used to produce what is often called first-generation (1G) biofuels. However, due to concerns associated with using food crops to produce biofuels, agricultural waste, such as sugarcane straw, bagasse and corn stover, are now being considered for the production of second generation (2G) biofuels. The sustainability of a 2G biorefinery can be further increased by implementing a circular bioeconomy approach: starting with a biological waste resource and closing the loop with recyclable or biodegradable by-products.

The implementation of any new technology is usually associated with challenges, and the 2G biorefinery is no exception. The first challenge is associated with the raw material. Lignocellulosic biomass is a much more complex substrate than food crops, and physical, chemical and/or biological pre-treatment is required before the resulting mixture of sugars can be fermented by a microorganism to produce the target compound. *Saccharomyces cerevisiae*, commonly known as baker's yeast, brewer's yeast or simply yeast, is one of the most common microorganisms utilised in industry due to its good performance in fermentation, its robustness under harsh process conditions, and the fact that it is safe (non-pathogenic). However, *S. cerevisiae* can only ferment C6 sugars, i.e. those with six carbons (e.g. D-glucose), while lignocellulosic materials contain high amounts of C5 sugars, i.e., sugars with five carbons (e.g. D-xylose). This results in a low product yield. Over the past two decades, the metabolism of *S. cerevisiae* has been genetically modified to enable it to utilise D-xylose. However, D-xylose fermentation is still much slower than D-glucose fermentation, the sugar preferred by this yeast.

As humans, we are sensitive to the environment around us through our five senses of sight, hearing, smell, taste and touch. Yeast perceives its environment in a similar way, through sensors on the cellular membrane. For instance, if the yeast is stressed, for example, by a lack of nutrients, its metabolism will slow down. It has been suggested that, despite genetic modifications to create a

pathway for D-xylose assimilation, *S. cerevisiae* does not "sense" the presence of D-xylose in its environment. At the beginning of this work, it was not known whether *S. cerevisiae* could sense D-xylose or not, since this sugar is not naturally fermented by this microorganism. Neither was known what type of signal this yeast would give in the presence of D-xylose.

To study this, a green fluorescent protein (GFP) was introduced into the yeast, making it possible to measure the activation or repression of three sugar signalling routes by detecting the fluorescence. Using this so-called biosensor system, it was shown that the poor consumption of D-xylose by *S. cerevisiae* was due to the inability of yeast to sense D-xylose in its environment. It was also found that when the yeast was genetically modified so that it could metabolise D-xylose (by adding a D-xylose pathway), a high concentration of D-xylose led to the same signal as for a low D-glucose concentration, i.e. similar to the response to a low level of nutrient.

In a yeast strain genetically modified to consume D-xylose even more efficiently, it was found that the genetic modifications changed the D-xylose recognition signal from that corresponding to a low D-glucose signal to that corresponding to a high D-glucose signal, indicating that the yeast sensed a good supply of nutrients.

Finally, attention was directed to identifying whether specific metabolites produced by glycolysis, the main catabolic route for D-glucose, played a role in the D-xylose signal. The results indicated that glucose-6-phosphate was involved in triggering the sugar signalling cascade.

Resumo de Divulgação Científica

Nas últimas décadas, a demanda mundial por energia aumentou dramaticamente e, por esta razão, o uso de combustíveis fósseis resultou em níveis crescentes de gases de efeito estufa, levando ao aquecimento global e às mudanças climáticas. Ainda assim, esses combustíveis não-renováveis, como carvão, petróleo e gás, ainda desempenham um papel dominante na economia, tecnologia e geopolítica do mundo. Por outro lado, torna-se necessário o uso de matérias-primas renováveis como forma de produção de energia mais sustentável e menos poluente.

Nos últimos anos, foram desenvolvidas as chamadas biorrefinarias, ou seja, refinarias nas quais os recursos baseados em petróleo são substituídos por biomassa para a produção de biocombustíveis, bio-produtos de valor agregado, produtos químicos, e energia. Matérias-primas renováveis, como a cana-de-açúcar (no Brasil) e o milho (nos EUA) já estão sendo usadas para produzir o que é comumente chamado de biocombustível de primeira geração (1G). No entanto, devido às preocupações associadas ao uso de plantio de alimentos para a produção de biocombustíveis, os resíduos agrícolas, como palha de cana, bagaço e palha de milho, são considerados como alternativas na produção de biocombustíveis de segunda geração (2G). A sustentabilidade de uma biorrefinaria 2G pode ser ainda otimizada com uma bioeconomia circular: onde o processo se inicia a partir do uso de bio-resíduos e fechando o ciclo com produtos recicláveis ou biodegradáveis.

Em geral, a implementação de qualquer nova tecnologia é acompanhada por novos desafios e a biorrefinaria 2G não é uma exceção à regra. O primeiro desafio está associado à matéria-prima. A biomassa lignocelulósica é um substrato mais complexo do que o obtido diretamente de fontes destinadas à alimentação, visto que são necessários pré-tratamentos físicos, químicos e/ou biológicos antes que a mistura resultante de açúcares possa ser fermentada por microorganismos para produzir um determinado composto. *Saccharomyces cerevisiae*, popularmente conhecida como levedura de pão, levedura de cerveja ou simplesmente levedura, é um dos microrganismos mais comuns utilizados na indústria, devido ao seu bom desempenho na fermentação, sua robustez sob diversas etapas do processo industrial e pela sua segurança (não patogênica). Contudo, *S. cerevisiae* pode fermentar apenas tipo de açúcares C6, isto é, aqueles com seis carbonos (D-glucose), enquanto os materiais lignocelulósicos contêm quantidades elevadas de açúcares C5, em outras palavras, os açúcares com cinco carbonos (D-xilose); o que resulta em um baixo rendimento do produto final. Nas duas últimas décadas, o metabolismo da *S. cerevisiae* tem sido geneticamente modificado a fim de permitir a utilização da D-xilose. No

entanto, a fermentação de D-xilose ainda é muito mais lenta que a fermentação de D-glicose, o açúcar preferido por esta levedura.

A baixa eficiência na fermentação da D-xilose pela *S. cerevisiae* está relacionada com a dificuldade de percepção da presença desse açúcar. Quando se trata de percepção da esfera em que vivemos, nós, seres humanos, somos sensíveis ao ambiente através dos nossos cinco sentidos: a visão, a audição, o olfato, o paladar e o tato. A levedura também percebe seu ambiente de maneira similar, através de sensores na membrana celular. Por exemplo, se a levedura estiver sob condições de estresse devido a falta de nutrientes, seu metabolismo poderá diminuir. Desta forma, apesar das modificações genéticas para criar uma via de assimilação de D-xilose, foi observado que a *S. cerevisiae* não "sente" a presença de D-xilose ao seu redor. No início deste trabalho, não havia conhecimento se *S. cerevisiae* era capaz de detectar a D-xilose, uma vez que este açúcar não é naturalmente fermentado por este microrganismo, tampouco se sabia que tipo de sinal esta levedura desencadearia na presença de D-xilose.

No intuito de estudar tais questões, uma proteína verde fluorescente (GFP) foi introduzida na levedura, tornando-se possível medir a ativação ou inibição de três vias de sinalização de açúcar a partir da fluorescência desta proteína. Usando este sistema chamado biossensor, evidenciou-se que o baixo consumo de D-xilose pela *S. cerevisiae* está relacionado à incapacidade da levedura para detectar, em seu meio, a D-xilose. Verificou-se também, que quando a levedura foi geneticamente modificada para que pudesse metabolizar a D-xilose (adicionando uma via de metabolismo desse açúcar), uma alta concentração de D-xilose desencadeou o mesmo sinal que o de uma baixa concentração de D-glicose, ou seja, semelhante à resposta de um baixo nível de nutrientes.

Diante desses resultados, foram realizadas modificações genéticas ainda mais eficientes para consumir D-xilose em uma cepa de levedura. Verificou-se, assim, que as modificações genéticas alteraram o sinal de reconhecimento da D-xilose (onde a alta concentração de D-xilose é reconhecida como uma baixa concentração de D-glicose) para um sinal similar ao de uma alta concentração de D-glicose. Portanto, isso indica que a partir dessas modificações genéticas, a levedura passou a reconhecer a D-xylose como um bom suprimento de nutrientes.

Por fim, visando compreender esse processo de reconhecimento, foram analisados quais metabólitos específicos produzidos pela via glicolítica, a principal via para metabolizar a D-glicose, possam ser os responsáveis por tal sinalização celular que a *S. cerevisiae* emite na presença da D-xilose. Assim, os resultados indicaram que pelo menos um dos compostos da via glicolítica, glicose-6-fosfato, está envolvido no desencadeamento da cascata de sinalização celular a partir da presença de D-xilose.

List of Publications

This thesis is based on the following papers, which will be referred to by Roman numerals.

I. Assessing the effect of D-xylose on the sugar signaling pathways of *Saccharomyces cerevisiae* in strains engineered for D-xylose transport and assimilation

Osiro, K. O., Brink, D. P., Borgström, C., Wasserstrom, L., Carlquist, M., Gorwa-Grauslund, M. F. (2018). FEMS Yeast Research 18 (1).

II. Exploring the D-xylose paradox in *Saccharomyces cerevisiae* through in vivo sugar signalomics of targeted deletants

Osiro, K. O., Borgström, C., Brink, D. P., Fjölnisdóttir, B. L., Gorwa-Grauslund, M. F. Submitted for publication.

III. Using phosphoglucose isomerase-deficient *Saccharomyces cerevisiae* to further understand the role of glycolytic intermediates in strains engineered for D-xylose utilisation

Borgström, C., Osiro, K. O., Lundberg, E., Gorwa-Grauslund, M. F. Manuscript.

My contributions to the papers

Paper I I participated in the design of the study and was in charge of the strain construction. I planned, designed and performed the enzymatic assays and the flow cytometry assays. I contributed actively to discussions on the data analysis. I wrote the initial draft and finalised the manuscript.

Paper II I participated in the design of the study. I planned and designed the gene deletions and supervised Birta Líf Fjölnisdóttir in the construction of the strains. I performed most of the flow cytometry experiments, planned and performed the anaerobic cultivations, and contributed to the analysis of flow cytometry, anaerobic, aerobic and HPLC data. I helped draft the manuscript.

Paper III I participated in the design of the study, supervised Dominika Miroszewska in the first design of the *PGI1* deletion, and co-supervised Ester Lundberg in the strain construction, enzymatic assay and growth experiments. I performed the flow cytometry analysis. I helped draft the manuscript.

Contents

1. Background

1.1. Circular bioeconomy and biorefineries

The need for sustainable production of energy and chemicals, based on renewable resources, is driven by environmental, economic, industrial and geopolitical factors. The energy sector today is still mainly based on fossil fuels, the combustion of which is the major source of anthropogenic greenhouse gas (GHG) emissions, comprising roughly 70% of the total emissions (Höök & Tang, 2013). It is now widely accepted that GHGs contribute significantly to the climate change that has been observed in recent decades. It has been estimated that human activities have led to an average increase in temperature of 1°C compared to pre-industrial levels (Hoegh-Guldberg et al., 2018). Unless countries respect the Paris Agreement to reduce GHG emissions over the next twelve years, the increase will exceed 1.5°C, leading to severe consequences for the planet (Hoegh-Guldberg et al., 2018; Law et al., 2018).

Apart from the environmental perspectives, economic and geopolitical crises have also resulted from the extraction and use of oil and coal. Supply factors have a powerful influence in determining oil prices (Gallo et al., 2010), which directly affect international economy and geopolitics. For example, the USA still produces almost 18 thousand barrels of petroleum per day; being the largest producer in the world, according to the U.S. Energy Information Administration (EIA) (Figure 1A). However, oil reserves are mainly located in other geographical areas (Figure 1B), which could explain some of the USA's foreign political decisions over the past two decades (Bromley, 2006; Stokes, 2007).

Effects on the environment and climate, geopolitical conflicts resulting from the depletion of fossil resources, and the ever-growing demand for fuels and chemicals have led to the emergence of the biorefinery concept. A biorefinery is a refinery in which renewable plant-based feedstock is used instead of oil to produce fuels and chemicals (Fernando et al., 2006; Kamm & Kamm, 2004).

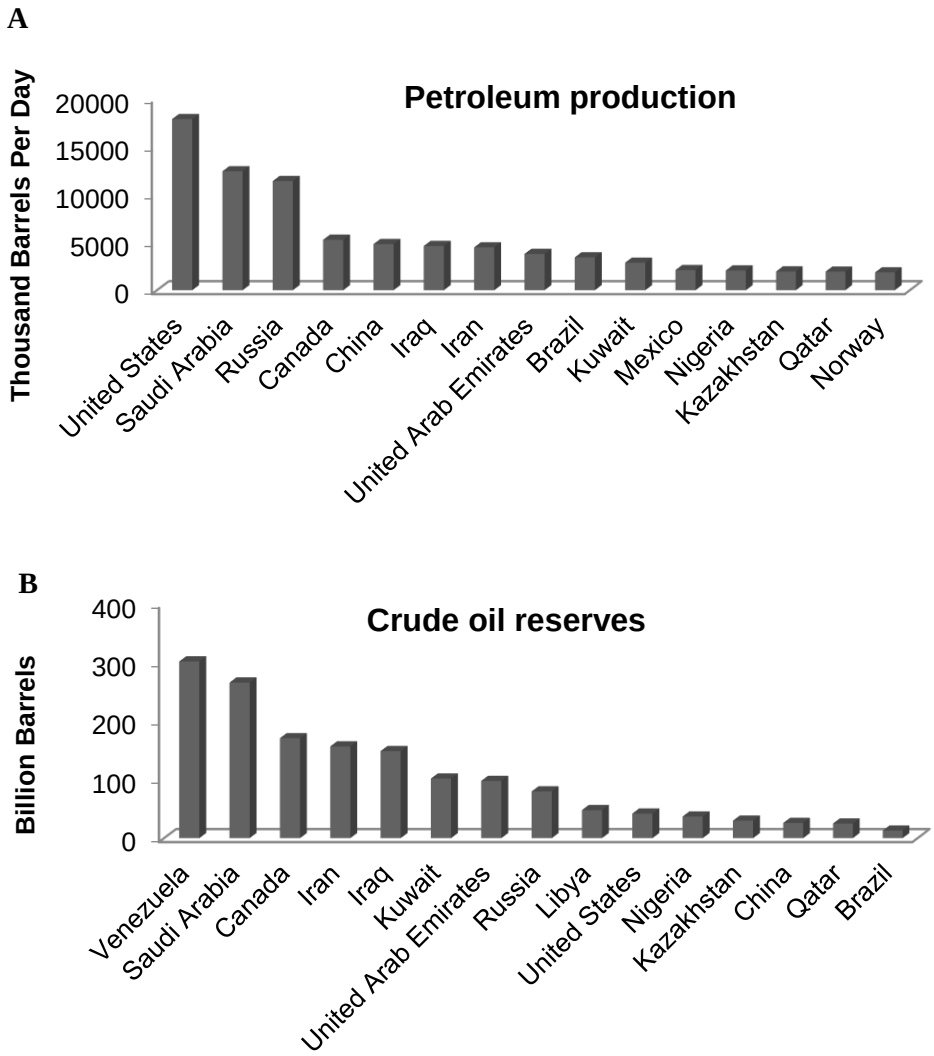

Figure 1. The top 15 petroleum producing countries (A) and the location of crude oil reserves (B) Source: https://www.eia.gov/beta/international/

The industrial production of biofuels from plant mass, i.e. bioethanol, has mainly been based on sugarcane and maize, followed by wheat, sugar beet and sorghum; often referred to as first generation (1G) biofuels. However, the use of food crops for fuel production has led to ethical problems (Mohr & Raman, 2013; Rulli et al., 2016). For instance, the agricultural resources used to meet the demand of the top 14 bioethanol consumers per year (≈ 85% of global

consumption) could feed 200 million people (Rulli et al., 2016). Apart from the use of food crops for energy production, another concern is the increase in agricultural land area required to meet the demand for biofuel production. Agricultural expansion affects the soil, water, local population and biodiversity of the ecosystem (Lynd, 1996; Mohr & Raman, 2013). Brazil is the world leader in ethanol production based on sugarcane (Lopes et al., 2016), and negative effects of agribusiness growth, such as the dispossession of the indigenous population, have been reported (Fernandes et al., 2010; Sauer & Pereira, 2012; Sullivan, 2013). Deforestation to grow sugarcane for biofuel production is another problem that affects the Amazon rainforest and its biodiversity (Gao et al., 2011).

Second generation (2G) biofuels, utilising biomass from agro-industrial waste such as sugarcane straw and bagasse, corn stover and wheat straw, provide an alternative to 1G production (Kamm & Kamm, 2004; Mohr & Raman, 2013; Moncada & Aristizábal, 2016). The concept of 2G biorefineries has gained increasing attention as the production of biofuels can be combined with the production of bio-based chemicals, power, heat and electricity (Demirbas, 2009; Farzad et al., 2017).

The terms bioeconomy and circular economy are frequently associated with the development of biofuels and biorefineries, where the aim is to avoid the use of fossil resources, and achieve sustainable processes with a low carbon footprint (Carus & Dammer, 2018). However, the strategy employed to reduce fossil carbon utilisation in the concept of circular economy is to introduce the recycling of materials at the end of the consumption chain (Carus & Dammer, 2018). This concept differs from the traditional linear economy – which starts with production and ends with waste accumulation – as it moves towards a closed circle, where the recycled product returns to the starting point of production (Zabaniotou, 2017). The concept of bioeconomy, on the other hand, focuses on the replacement of non-renewable fossil carbon with renewable lignocellulosic biomass from forestry, agriculture and the marine environment, including waste material (Kircher, 2014).

The circular bioeconomy is thus the overlap between the circular economy and the bioeconomy, which integrates the recycling of organic materials and nutrients, the utilisation of waste materials, the production of bio-based products, re-utilisation, sharing, recycling and remanufacturing of the final product, aiming at minimum input and waste generation throughout the process (Carus & Dammer, 2018; D'Amato et al., 2017). The utilisation of lignocellulosic biomass in a biorefinery to produce bio-based products from the whole biomass is in line with the concept of the circular bioeconomy, provided the bioproduct generated is either biodegradable or recycled, and agricultural management policies promote sustainable practice (Figure 2).

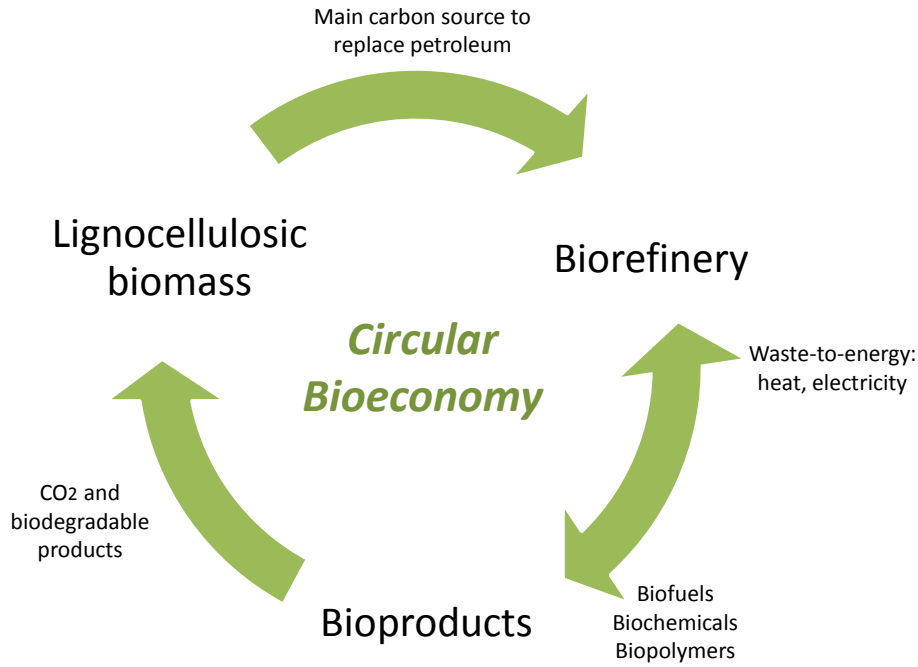

Figure 2. A biorefinery in the circular bioeconomy perspective

1.2. Challenges associated with lignocellulosic materials

Lignocellulosic biomass, such as grass and woody biomass, consists of three major components: cellulose (20–50%), hemicellulose (15–35%) and lignin (10–30%) (Figure 3); their proportions depend on the biomass source (Chundawat et al., 2011; Pauly & Keegstra, 2008). For instance, rice straw, corn stover and sugarcane bagasse are xylan-rich lignocellulosic materials, containing 24.5%, 21.5% and 21.1% xylan, respectively (Zhao et al., 2012a).

Cellulose, a D-glucose polysaccharide, is the main component in the plant cell wall, which provides strength and stability to the plant with the aid of hemicellulose links (Kumar et al., 2008). Hemicellulose is one of the most common polymers found in nature after cellulose (Lachke, 2002). This heterogeneous polysaccharide is composed of pentose sugars (D-xylose, D-arabinose) and hexose sugars (i.e., D-mannose, D-glucose and D-galactose). Finally, lignin is a complex polyaromatic polymer made up of three basic subunits: p-hydroxyphenyls, guaicyls and syringyls (Boerjan et al., 2003).

Lignin is responsible for the stiff plant cell wall in conjunction with the hemicellulose structure, affording resistance against insects and pathogens (Rubin, 2008).

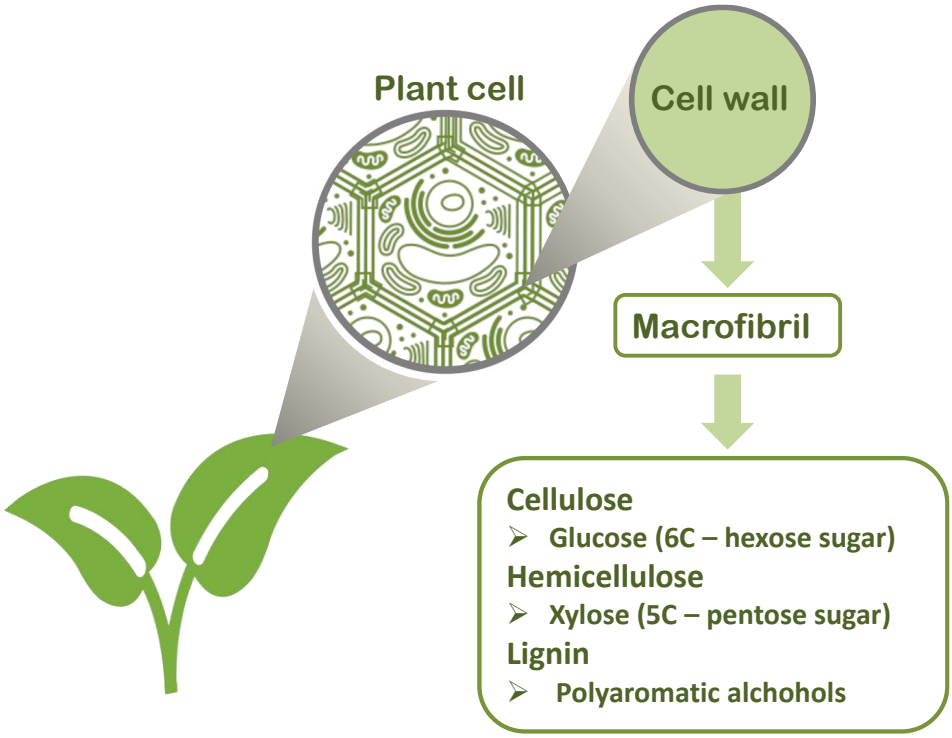

Figure 3. The major components of lignocellulosic biomass: cellulose, hemicellulose and lignin. (Adapted from Rubin (2008))

The chemical composition, arrangement and interactions between these three polymer structures contribute to the recalcitrance of lignocellulosic materials to degradation (Himmel et al., 2007; Zhao et al., 2012a). Therefore, physico-chemical pre-treatment and enzymatic hydrolysis are necessary to convert the complex cellulose and hemicellulose polysaccharides into fermentable sugars (Figure 4) (Zhao et al., 2012b). However, inhibitors (such as furan derivatives, weak acids and phenolic compounds) are also generated together with monomeric sugars during this process. This leads to additional challenges in the enzymatic catalysis and fermentation steps (Almeida et al., 2007; Jönsson & Martín, 2016).

The sugar composition of lignocellulosic biomass also presents a challenge compared to 1G biomass, which contains only hexose sugars that are easily fermented (Naik et al., 2010). D-xylose, the second most abundant sugar in nature, found in lignocellulose, can constitute up to one-third of the available sugars (Lachke, 2002). It is therefore of the utmost importance to use or design a microorganism that can efficiently convert the D-xylose fraction of lignocellulose, to achieve high yield and productivity in the fermentation stage.

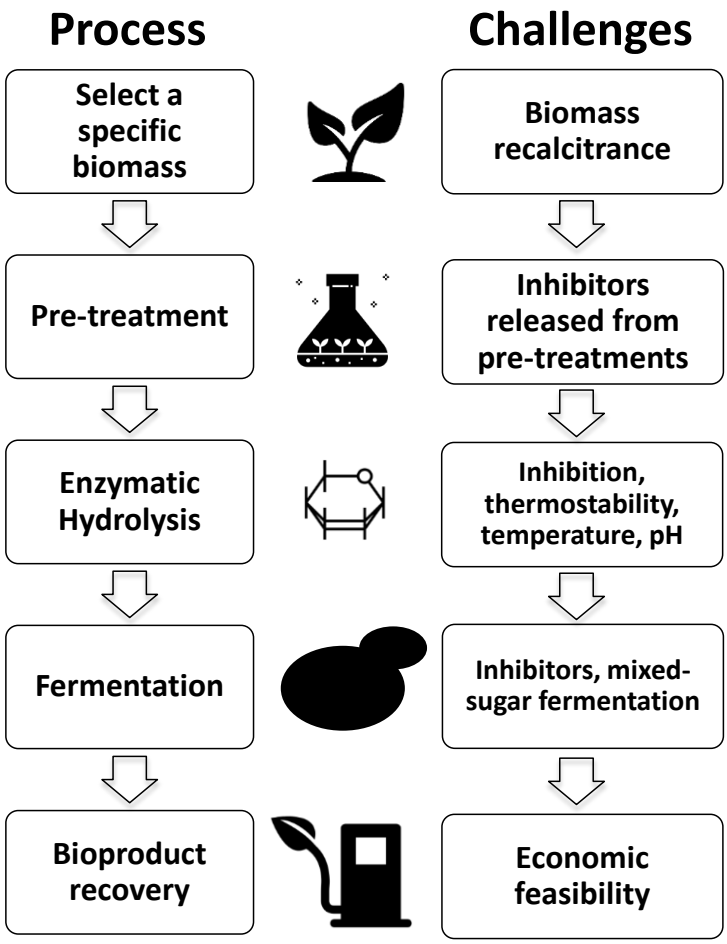

Figure 4. General overview of the biorefinery process and related challenges in each of the process steps

1.3. *Saccharomyces cerevisiae* in biorefineries: limitations and advantages

The fermentation step in lignocellulose-based biorefineries requires the use of a microorganism that can endure harsh process conditions, such as the presence of inhibitory compounds, low pH and high osmotic pressure (Hong & Nielsen, 2012). The eukaryote *Saccharomyces cerevisiae*, also known as baker's yeast, is a promising candidate because it is natively able to efficiently ferment D-glucose, and has the robustness required for biotechnological processes (Almeida et al., 2007). It has therefore been used for the production of a broad range of products, from food (beer, bread, wine), to the more recent production of various chemicals, and it is classified as "generally regarded as safe" (Ostergaard et al., 2000).

S. cerevisiae is also commonly used as a model organism for the study of biological processes in eukaryotes (Karathia et al., 2011). The numerous studies performed with baker's yeast have contributed to the rapid progress made in molecular biology techniques (Hong & Nielsen, 2012). For example, the expansion in bioinformatics and the development of powerful molecular tools for *S. cerevisiae* engineering (e.g. CRISPR-Cas9) was made possible by early genome sequencing and extensive physiological studies of this host (Goffeau, 2000; Goffeau et al., 1996; Stovicek et al., 2015). This has paved the way for the optimization of *S. cerevisiae* as a microbial factory, including its use in lignocellulose-based biorefineries. One major drawback, however, is that *S. cerevisiae* is unable to ferment the pentose sugar D-xylose.

Other eukaryotes found in nature are capable of assimilating D-xylose, e.g. the yeasts *Pichia stipitis*, *Pachysolen tannophilus* and *Candida shehatae*; however they can seldom be used under industrial conditions as they do not have the same ability as *S. cerevisiae* to adapt to harsh conditions (Rudolf et al., 2008; Skoog & Hahn-Hägerdal, 1988), and they require oxygen for D-xylose metabolisation. Metabolic engineering has therefore been applied to *S. cerevisiae* to add pathways for D-xylose utilisation. Two well-studied D-xylose pathways have been successfully introduced and optimized: the oxido-reductive pathway (Kötter et al., 1990; Kötter & Ciriacy, 1993) and the isomerization pathway (Brat et al., 2009; Kuyper et al., 2003). However, engineered *S. cerevisiae* strains still do not recognise D-xylose as a fermentable sugar (Bergdahl et al., 2013; Feng & Zhao, 2013; Klimacek et al., 2010; Runquist et al., 2009; Salusjärvi et al., 2006), leading to low fermentation rates on D-xylose.

1.4. Scope and outline of this work

In the *S. cerevisiae* strains engineered for D-xylose utilisation to date, the yield of ethanol produced from D-xylose approaches the maximum theoretical yield (0.51 g ethanol/g D-xylose) (Chu & Lee, 2007; Jin et al., 2004), but the growth and fermentation rates on D-xylose are still much lower than in D-glucose fermentation. Also, D-xylose fermentation does not always activate the same metabolic pathways as D-glucose fermentation (Runquist et al., 2009; Salusjärvi et al., 2006), which raises the question of whether D-xylose is recognised as a fermentable sugar by *S. cerevisiae*. It has been hypothesized that the lack of D-xylose recognition is related to the way in which D-xylose is sensed, and how the sugar signalling pathways are affected, or not, by this pentose sugar. Therefore, the aim of the work presented in this thesis was to unravel the D-xylose paradox – it is used but not sensed – by studying the complex regulatory sugar signalling pathways in *S. cerevisiae*.

First, a panel of *in vivo* fluorescent biosensors developed by Brink et al. (2016) was used to monitor the three main sugar signalling pathways (**Paper I**). In order to study the signalling routes involved in the utilisation of D-xylose, two modifications were made to the biosensor strains: (i) the addition of a specific D-xylose transporter, to investigate intracellular and non-metabolised D-xylose (GAL2-N376F), and (ii) the addition of this transporter and an oxidoreductase D-xylose pathway, to study the signalling response when D-xylose was metabolised in these D-xylose-utilising strains. Deletion targets reported to improve D-xylose consumption by strains carrying the D-xylose isomerase pathway (Sato et al., 2016) were also deleted in biosensor strains carrying XR/XDH pathway (C5 biosensors) (**Paper II)** to determine whether deleting these genes improved D-xylose consumption through modifications of the sugar signalling. In the final study (**Paper III**), one of the first glycolytic genes was deleted in C5 biosensors to investigate whether there was any major intermediate metabolite (e.g. glucose-6-phosphate (G6P), fructose-1,6-bisphosphate (F1,6bP) or trehalose-6-phosphate (T6P)) whose level or ratio was a key regulator for activating/inactivating one or several of the sugar signalling pathways.

Chapter 2 provides an overview of the state of the art in D-xylose fermentation by *S. cerevisiae*, while Chapter 3 describes the sugar sensing and signalling routes in *S. cerevisiae*, and their known interactions with the central carbon metabolism. The use of biosensors for physiological studies is described in Chapter 4. Chapter 5 summarises the findings of this work, and Chapter 6 gives a future outlook.

2. D-Xylose Fermentation in *Saccharomyces cerevisiae*

> "*S. cerevisiae* and other fermenting yeasts are able to produce (…) xylitol from D-xylose and D-xylulose, but to a lesser extent. This indicates that fermenting yeasts also possess enzymes responsible for oxido-reduction of pentoses." (Gong et al., 1981)

S. cerevisiae is unable to ferment D-xylose to ethanol. However, as stated above, this yeast indeed does carry endogenous genes for the oxido-reduction of D-xylose to xylulose, an intermediate that can be further channelled into the non-oxidative pentose phosphate pathway (PPP) via xylulose kinase. The poor, or complete lack of, growth on D-xylose has been attributed to the low expression level of the corresponding enzymes: D-xylose reductase (XR), for the reduction of D-xylose to xylitol, and xylitol dehydrogenase (XDH), for the oxidation of xylitol to xylulose (Batt et al., 1986; Toivari et al., 2004; Träff et al., 2002). Several endogenous genes have been reported to carry these activities: *YJR096W, GCY1, GRE3* and *YPR1* encoding XR activity, while *XYL2, SOR1, SOR2* and *XDH1* encode proteins with activity for xylitol oxidation. In the last step of D-xylose conversion, *XKS1* represents the only known gene encoding xylulose kinase (XK) activity (Chang et al., 2007; Toivari et al., 2004; Träff et al., 2001; Wenger et al., 2010).

In addition to low expression, endogenous genes also exhibit poor enzymatic activities. For example, a strain overexpressing *GRE3* and *ScXYL2* has been reported to grow more slowly and accumulate more xylitol than a strain engineered with XR and XDH genes from the natural D-xylose-utilising yeast *Pichia stipitis* (Toivari et al., 2004). Therefore, metabolic engineering of *S. cerevisiae* for D-xylose utilisation has been the subject of much study during the past few decades, in exploring different approaches to improving the utilisation of this alternative carbon source (Hahn-Hägerdal et al., 2001; Jeffries, 2006). The present chapter reviews not only the modifications that are necessary to obtain efficient conversion of D-xylose via the central metabolism, but also other, less obvious changes that have led to improvements in D-xylose fermentation.

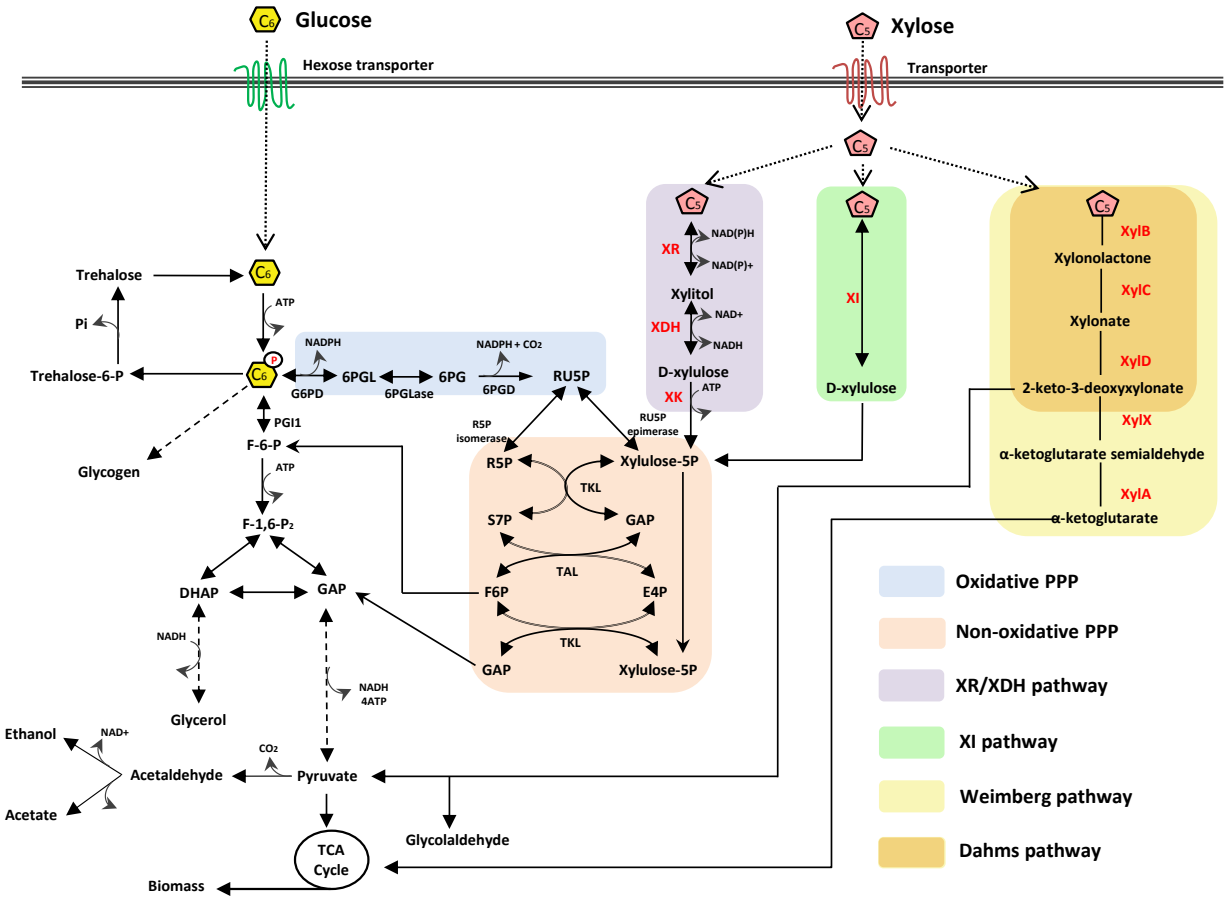
Glucose
Hexose transporter
Xylose
Transporter
Trehalose
Pi
Trehalose-6-P
ATP
NADPH
NADPH + CO2
6PGL
6PG
RU5P
G6PD
6PGLase
6PGD
PGI1
F-6-P
Glycogen
ATP
F-1,6-P2
DHAP
GAP
NADH
Glycerol
NADH
4ATP
Ethanol
NAD+
Acetaldehyde
CO2
Pyruvate
Acetate
TCA
Cycle
Biomass
Glycolaldehyde
R5P
isomerase
RU5P
epimerase
Xylulose-5P
R5P
TKL
S7P
GAP
TAL
F6P
E4P
TKL
GAP
Xylulose-5P
XR
NAD(P)H
NAD(P)+
Xylitol
XDH
NAD+
NADH
D-xylulose
XK
ATP
XI
D-xylulose
XylB
Xylonolactone
XylC
Xylonate
XylD
2-keto-3-deoxyxylonate
XylX
α-ketoglutarate semialdehyde
XylA
α-ketoglutarate
Oxidative PPP
Non-oxidative PPP
XR/XDH pathway
XI pathway
Weimberg pathway
Dahms pathway

Figure 5. Metabolic map showing connections between glycolysis and the D-xylose pathways introduced in *S. cerevisiae*. Glycolysis: C6 hexagon icon: D-glucose, G6P hexagon icon with a P: glucose-6-phosphate, F-6-P: fructose-6-phosphate, F-1,6-P2: fructose-1,6-bisphosphate, DHAP: dihydroxyacetone phosphate, GAP: glyceraldehyde-3-phosphate, PGI1: glucose-6-phosphate isomerase; Oxidative PPP (blue background): 6PGL: 6-phosphogluconolactone, 6PG: 6-phosphogluconate, RU5P: ribulose-5-phosphate, G6PD: glucose-6-phosphate dehydrogenase, 6PGLase: 6-phosphogluconolactonase, 6PGD: 6-phosphogluconate dehydrogenase, R5P isomerase: ribose-5-phosphate isomerase, RU5P epimerase: ribulose-5-phosphate 3-epimerase; Non-oxidative PPP (pink background): R5P: ribose-5-phosphate, Xylulose-5P: xylulose-5-phosphate, S7P: sedoheptulose-7-phosphate, GAP: glyceraldehyde-3-phosphate, F6P: fructose-6-phosphate, E4P: erythrose-4-phosphate, TKL: transketolase, TAL: transaldolase; D-xylose routes consisting of D-xylose reductase (XR) and xylitol dehydrogenase (XDH) pathway (purple background), D-xylose isomerase (XI) pathway (green background) or Weimberg pathway (yellow background): XylB: D-xylose dehydrogenase, XylC: D-xylono-γ-lactone lactonase, XylD: D-xylonate dehydratase; XylX: 2-keto-3-deoxy-D-xylonate dehydratase, XylA: α-ketoglutarate semialdehyde dehydrogenase. The Dahms pathway (brown background) overlaps with the Weimberg pathway

2.1. Essential modifications for D-xylose utilisation

2.1.1. Introduction of D-xylose pathways

There several pathways to metabolise D-xylose, which are illustrated in Figure 5. These pathways and their introduction into *S. cerevisiae* are described below. The combination of XR (EC 1.1.1.10), XDH (EC 1.1.1.9) and XK (EC 2.7.1.17) activities constitutes the so-called oxido-reductive pathway, commonly found in most yeast and fungi species that possess the ability to grow on D-xylose in nature (Jeffries, 1983). In order to confer D-xylose catabolism on *S. cerevisiae*, *XYL1* (D-xylose reductase) and *XYL2* (D-xylitol dehydrogenase) genes from *Scheffersomyces stipitis* were first introduced (Amore et al., 1991; Kötter & Ciriacy, 1993; Tantirungkij et al., 1993). This was because *S. stipitis* D-xylose reductase is one the few described XRs that can use both NADH and NADPH as cofactor, whereas most XRs (including those in *S. cerevisiae*) use only NADPH. Therefore, XR from *S. stipitis* is more compatible with XDH, which uses NAD^+ only. Nonetheless, a high level of xylitol was secreted, which was associated with a remaining cofactor imbalance due to the higher affinity of *S. stipitis* for NADPH than NADH (Kötter & Ciriacy, 1993; Verduyn et al., 1985).

Different protein engineering strategies have been used in attempts to either increase the affinity of XR for NADH, or to change the cofactor preference of XDH for $NADP^+$ (Bengtsson et al., 2009; Jeppsson et al., 2006; Kostrzynska et al., 1998; Watanabe et al., 2007a; Watanabe et al., 2007b). However, the most efficient strategy so far has proven to be the use of a *XR* gene from a *Spathaspora passalidarum* isolate from the Brazilian ecosystem. This isolate harbours two D-xylose reductases: the NADPH-dependent *XYL1.1* and the

NAD(P)H-dependent *XYL.1.2*; the latter having higher affinity for NADH than NADPH (Cadete et al., 2016). The expression of *XYL1.2* in *S. cerevisiae* (strain TMB3504) led to significant increases in ethanol yield and productivity concomitant with low xylitol yield under anaerobic fermentation of D-xylose to ethanol (Cadete et al., 2016).

An alternative route for D-xylose assimilation is the D-xylose isomerase (XI) (EC 5.3.1.5) pathway, consisting of the one-step isomerization of D-xylose to D-xylulose (Jeffries, 1983). A major advantage of this route is that it does not require redox cofactors in the conversion step. However, XI is predominantly found in bacteria, and rarely in eukaryotes (Jeffries, 1983). It therefore took many years to find XI genes that could be actively expressed in a functional protein in *S. cerevisiae* (Amore et al., 1989; Gárdonyi & Hahn-Hägerdal, 2003; Sarthy et al., 1987). *xylA* from *Thermus thermophilus* was the first functional D-xylose isomerase expressed in *S. cerevisiae*, although it afforded very low activity (Walfridsson et al., 1996). Much later, the expression of the XI gene from the fungus *Piromyces* in *S. cerevisiae* proved to be a cornerstone in the use of XI for D-xylose fermentation, providing a considerable improvement in XI activity, probably due to its gene eukaryotic background. After *Piromyces* XI, several XI genes with strong homology to this gene were efficiently expressed in *S. cerevisiae*, such as the codon-optimized gene from *Clostridium phytofermentans* (Brat et al., 2009). However, it was necessary in all cases to increase the number of copies of the gene, either by evolution or by genetic engineering, in order to obtain sufficient flux through the XI pathway (Bracher et al., 2018; Lee et al., 2012b; Sato et al., 2016).

The third, and less explored, catabolic route for D-xylose metabolism is the Weimberg pathway, a pentose oxidation pathway that was first reported in *Pseudomonas fragi* bacterium by Weimberg (1961), but which has also been found and characterized in other prokaryotes, such as the bacterium *Caulobacter crescentus* and the archea *Haloferax volcanii* (Johnsen et al., 2009; Stephens et al., 2007; Weimberg, 1961). The Weimberg pathway consists of D-xylose oxidation in five reaction steps to α-ketoglutarate, which enters the tricarboxylic acid (TCA) cycle, bypassing the non-oxidative pentose phosphate pathway. The enzymes that compose these five oxidation steps are: D-xylose dehydrogenase (XylB), D-xylono-γ-lactone lactonase (XylC), D-xylonate dehydratase (XylD), 2-keto-3-deoxy-D-xylonate dehydratase (XylX) and α-ketoglutarate semialdehyde dehydrogenase (XylA) (Kim & Woo, 2018). Another pathway that can be derived from the Weimberg pathway enzymes is the Dahms pathway, which cleaves the intermediate 2-keto-3-deoxy-D-xylonate with an aldolase and generates pyruvate and glycolaldehyde (Dahms, 1974). The Weimberg pathway from *Caulobacter crescentus* was recently introduced into *S. cerevisiae* to test the possibility of accumulating high-value products from TCA cycle intermediates; however accumulation of the xylonate

intermediate indicated that one or several of the downstream steps in the pathway were not functional (Wasserstrom et al., 2018).

2.1.2. The role of the pentose phosphate pathway

The PPP can be divided in two phases: one oxidative and the other non-oxidative (Figure 5). The oxidative PPP phase is one of the main sources of NADPH inside the cell, through the multi-step conversion of glucose-6-phosphate to ribulose 5-phosphate (Stincone et al., 2015). However, D-xylose assimilation via the isomerase or oxido-reductive pathways occurs via non-oxidative PPP, and xylulose-5-phosphate is a metabolic intermediate (Barnett, 1976). The flux of the non-oxidative phase of the PPP passes through the generation of diverse phosphorylated sugars, consisting of three, four, five, six and seven carbons, finally leading to the formation of fructose-6-phosphate and glyceraldehyde-3-phosphate, which are intermediates of glycolysis (See the review by Stincone, A. et al. 2015 for more detailed information.)

It was shown early on that sedoheptulose-7-phosphate accumulated in D-xylose- and xylulose-metabolising strains, indicating low activity of the non-oxidative PPP in *S. cerevisiae* (Kötter & Ciriacy, 1993; Senac & Hahn-Hägerdal, 1991). Therefore, transaldolase (*TAL1*) and transketolase (*TKL1*) genes were overexpressed together, or not, with the other two genes encoding ribulose-5-phosphate 3-epimerase (*RPE1*) and ribulokinase (*RKI1*), resulting in increased D-xylose consumption in strains optimized for both the XR-XDH and the XI routes (Karhumaa et al., 2007; Karhumaa et al., 2005; Kuyper et al., 2005; Lee et al., 2012a; Matsushika et al., 2012; Zhou et al., 2012). The non-oxidative PPP was also recently engineered to achieve simultaneous utilisation of D-glucose and D-xylose, by deleting the *RPE1* gene (encoding RPE), which converts ribulose-5-phosphate into xylulose-5-phosphate. Under these conditions, D-xylose became a crucial precursor for xylulose-5-phosphate synthesis, thus forcing D-xylose uptake in the presence of D-glucose (Shen et al., 2015).

Using an inverse metabolic engineering approach, improved aerobic growth rate on D-xylose was observed in strains overexpressing *SOL3* (oxidative PPP) and *TAL1* (non-oxidative PPP); showing 19% and 24% faster growth, respectively, compared to the reference strain (Bengtsson et al., 2008). *SOL3*, a gene that encodes 6-phosphogluconolactonase, might assist D-xylose consumption by favouring NADPH regeneration in the oxidative PPP, thus, increasing cofactor availability for a recombinant strain with an NAD(P)H-dependent XR.

2.1.3. D-Xylose transport

Although there is no D-xylose-specific transport system in *S. cerevisiae*, D-xylose can enter the cell by facilitated diffusion using some of the hexose transporters (Kotyk, 1965; Kotyk & Kleinzeller, 1963). *S. cerevisiae* has been reported to possess eighteen hexose transporters (Hxt1p-17p and Gal2p), but only seven of them (Hxt1p-7p) are well characterized, and the main responsible for D-glucose transport (Moysés et al., 2016; Reifenberger et al., 1997). However, growth on hexose could be restored by *SNF3* deletion in a strain where at least 20 transporters (including the maltose transporter family) were inactivated, indicating the existence of more unidentified hexose transporters (Wieczorke et al., 1999).

Hexose transporters are classified according to their affinity (K_m) for D-glucose. Transporters with a K_m value in the range of 1-2 mM are high-affinity hexose transporters (Hxt6p, Hxt7p, and Gal2p), a value around 10 mM corresponds to intermediate-affinity hexose transporters (Hxt2p, Hxt4p and Hxt5p), and a value between 50-100 mM to low-affinity hexose transporters (Hxt1p and Hxt3p) (Diderich et al., 1999; Reifenberger et al., 1997). Specific low- to high-affinity transporters (Hxt1p, Hxt2p, Hxt4p, Hxt5p, Hxt7p and Gal2p) have been described to be D-xylose-permissive during co-fermentation of D-glucose and D-xylose by *S. cerevisiae* (Hamacher et al., 2002; Sedlak & Ho, 2004). Hxt7p and Hxt5p showed higher D-xylose uptake than other transporters (Sedlak & Ho, 2004). However, D-xylose uptake was found to be inhibited by the presence of D-glucose in all cases (Saloheimo et al., 2007; Sedlak & Ho, 2004).

Limitation of D-xylose catabolism by the transport step has recently become more apparent in strains that already have efficient D-xylose metabolism, or under conditions of low D-xylose concentration (Cai et al., 2012). So far, the most promising strategies to improve D-xylose transport have relied on the mutation of specific amino acids from endogenous hexose transporters. For instance, it was recently shown that an asparagine-to-phenylalanine mutation in the Gal2p transporter (Gal2p-N376F) led to co-fermentation of mixed sugars by *S. cerevisiae*, revealing high D-xylose specificity and affinity (K_m = 91.4 mM), as well as the loss of the ability to transport hexose sugars (Farwick et al., 2014). In addition, an asparagine-to-isoleucine mutation at the same amino acid (Gal2p-N376I) allowed *S. cerevisiae* to grow on L-arabinose in the presence of D-glucose (Verhoeven et al., 2018). The conserved asparagine residue among D-glucose transporters appears to be a determinant for sugar specificity, since Hxt7p-N370F also conferred the same phenotype of transporting D-xylose only (Farwick et al., 2014). Another laboratory evolution approach generated the mutant Hxt7p-F79S with a low affinity for D-xylose (K_m = 228.8 ± 45.9 mM),

but a two-fold increase in velocity (Vmax = 186.4 ±20.1 $nmol \cdot min^{-1} \cdot mg^{-1}$) compared to the native transporter (Apel et al., 2016).

2.2. Additional modifications known to improve D-xylose utilisation

Although the systematic engineering approaches described above were needed to generate D-xylose-fermenting yeasts, it is important to consider D-xylose utilisation in the overall context of the cell. Some genes that are not directly involved in the conversion of D-xylose to glycolytic intermediates but are instead involved in different cell process, such as redox balancing, signalling, transcriptional regulation and mitochondrial steps, have also been shown to increase the efficiency of D-xylose utilisation (Table 1). These are discussed below.

2.2.1. Redox balance

As an alternative to XR-XDH cofactor engineering, some metabolic engineering strategies have focused on modifying the level of NAD(P)H/$NAD(P)^+$ cofactors inside the cell, by modifying other redox enzymes in the network. The overall objective was to increase NADPH availability to XR, and NAD^+ availability to XDH. For instance, *ZWF1* was deleted (to limit NADPH production) together with the overexpression of an $NADP^+$-dependent Gdp1 in glycolysis to force the production of NADPH instead of NADH during glycolysis (Verho et al., 2003). Similarly, the *GDH1* gene was deleted to prevent the use of NADPH for ammonium assimilation, and was complemented with the overexpression of NADH-dependent counterparts (encoded by *GDH2* or *GLT1*-*GLN*) to increase NAD^+ availability (Roca et al., 2003).

2.2.2. *PHO13*

Deletion of *PHO13*, an alkaline phosphatase gene, was shown to improve D-xylose consumption by *S. cerevisiae* strains engineered with XR/XDH (Ni et al., 2007; Vleet et al., 2008) or XI pathways (Lee et al., 2014), whereas the deletion of this gene did not affect the D-glucose metabolism (Kim et al., 2015). Further analysis of *pho13*Δ strains showed up-regulation of PPP genes (Kim et al., 2015). In addition, *PHO13* disruption promoted the high expression of 12 genes in the TCA cycle/respiratory chain and 125 cell cycle genes showed differences

in transcription levels during D-xylose fermentation under oxygen-limited conditions (Bamba et al., 2016).

Table 1. Additional target genes known to improve D-xylose consumption in *S. cerevisiae*

Gene	Modification	Function	Pathway used	Reference
ZWF1	Deletion	glucose-6-phosphate dehydrogenase	XR/XDH	Verho et al. (2003)
GDP1	Over-expression	$NADP^+$-dependent D-glyceraldehyde-3-phosphate dehydrogenase	XR/XDH	Verho et al. (2003)
GDH1	Deletion	NADPH-dependent glutamate dehydrogenase	XR/XDH	Roca et al. (2003)
GDH2	Over-expression	NADH-dependent glutamate dehydrogenase	XR/XDH	Roca et al. (2003)
GLT1	Over-expression	Glutamate synthetase	XR/XDH	Roca et al. (2003)
GLN1	Over-expression	Glutamine synthetase	XR/XDH	Roca et al. (2003)
PHO13	Deletion	Haloacid dehalogenase type IIA phosphatase	XI and XR/XDH	Ni, H. (2007), Lee et al. (2014)
PGM2	Over-expression	Phosphoglucomutase that catalyse D-glucose-1-phosphate to glucose-6-phosphate	XR/XDH	Sanchez et al. (2010)
HXK2	Mutation	Hexokinase isoenzyme 2 (D-glucose phosphorylation)	XR/XDH	Bergdahl et al. (2013)
MIG1	Deletion	Transcription factor involved in glucose repression	XR/XDH	Roca et al. (2004)
MIG2	Deletion	Transcription repressor that cooperates with *MIG1*	XR/XDH	Roca et al. (2004)
YLR042C	Deletion	Non-essential gene with unknown function	XR/XDH	Bengtsson et al. (2008), Parachin, N. S. (2010)
MNI1	Deletion	Putative S-adenosylmethionine-dependent methyltransferase	XR/XDH	Bengtsson et al. (2008)
RPA49	Deletion	α-subunit of RNA polymerase A	XR/XDH	Bengtsson et al. (2008)
VPS13	Over-expression	Protein involved in prospore membrane morphogenesis	XR/XDH	HamediRad et al. (2018)
MDH1	Over-expression	Mitochondrial malate dehydrogenase	XR/XDH	HamediRad et al. (2018)
COX5α	Over-expression	Cytochrome c oxidase	XR/XDH	HamediRad et al. (2018)
CDC11	Down-regulation	Protein related to cell cycle division	XR/XDH	HamediRad et al. (2018)
HOG1	Deletion	Mitogen-activated protein kinase	XI	Sato, T. K. (2016), **Paper II**
IRA2	Deletion	GTPase-activating protein	XI and XR/XDH	Sato, T. K. (2016) **Paper II**
ISU1	Deletion	Scaffolding protein for mitochondrial Fe-S cluster biogenesis	XI and XR/XDH	dos Santos, L. (2016), Sato, T. K. (2016), **Paper II**
GRE3	Deletion	Unspecific aldose reductase	XI and XR/XDH	Kuyper, et al (2005), Karhumaa, K. et al. (2007), Sato, T. K. (2016)
SSK2	Deletion	Suppressor of sensor kinase	XI	dos Santos, L. (2016)
CYC8	Deletion	General transcriptional co-repressor	XI and XR/XDH	Zha, J. et al. (2013), Nijland et al. (2017)

2.2.3. *PGM2*

Overexpression of *PGM2*, which encodes an enzyme involved in the inter-conversion of glucose-6-phosphate and glucose-1-phosphate, and is known to benefit galactose fermentation, was also found to improve anaerobic D-xylose fermentation, increasing the D-xylose consumption rate and ethanol yield (Sanchez et al., 2010). It was hypothesized that *PGM2* could help convert ribose-5-phosphate to ribose-1-phosphate, thereby contributing to increased biomass formation and helping to pull the carbon flux from D-xylose (Sanchez et al., 2010).

2.2.4. *HXK2*

Hexokinase 2 (Hxk2p) both catalyses the phosphorylation of D-glucose to glucose-6-phosphate and regulates the expression of sucrose-assimilating gene *SUC2*, and the enzyme is known to be inhibited by D-xylose (Fernández et al., 1986). While Hxk2p has a closed conformation at high D-glucose conditions, Hxk2p changes to an open conformation under low D-glucose and under xylose conditions; Hxk2p is then released from the *SUC2* promoter, which allows the expression of this gene (Vega et al., 2016). By mutating a single amino acid (Phe159Tyr) on the active site of Hxk2p, it was possible to significantly reduce the inhibitory effect of D-xylose *in vitro* (Bergdahl et al., 2013). This may provide a route for more efficient sugar mixture (D-glucose and D-xylose) fermentation rates by manipulation of this enzyme, which is also involved in D-glucose signalling mechanisms (Bergdahl et al., 2013; Bisson & Kunathigan, 2003; Gancedo, 1998; Moreno & Herrero, 2002).

2.2.5. *MIG1/2*

The deletions of *MIG1* and *MIG2*, genes related to the regulation of sugar uptake (cf. Chapter 3), have been shown to improve xylose uptake in mixed sugar chemostat cultivations with glucose and xylose in a XR/XDH strain (Roca et al., 2004). The improvement was attributed to the increased expression of hexose transporter genes. *MIG1* deletant presented an increase of 25% in xylose consumption rate during chemostat cultivations (Roca et al., 2004).

2.2.6. Other modifications

Deletion of *YLR042C, MNI1* (also known as *HPM1*) or *RPA49* has been shown to improve aerobic growth rates on D-xylose by 173%, 62% and 90%,

respectively, compared to a reference D-xylose-consuming strain (Bengtsson et al., 2008). *YLR042C*, a putative non-essential gene, has also been found to improve the D-xylose consumption rate, as well as ethanol and xylitol yields, under anaerobic conditions (Parachin et al., 2010). Overexpression of *VPS13*, *MDH1* and *COX5A*, and down-regulation of *CDC11* were also found to enhance D-xylose utilisation and increase ethanol productivity (HamediRad et al., 2018). However, it has not yet been possible to connect any of these changes to D-xylose metabolism.

Recently, the improvement in D-xylose consumption of a XI-engineered and evolved strain was associated with unexpected epistatic interactions between a kinase involved in osmotic regulation (Mitogen-activated protein kinase - MAPK) (encoded by *HOG1*), a regulator of protein kinase A (PKA) (encoded by *IRA2*), a scaffold protein for mitochondrial iron-sulphur (Fe-S) cluster biogenesis (encoded by *ISU1*) and an endogenous D-xylose reductase (encoded by *GRE3*) (Sato et al., 2016). Among these, *GRE3* is the only gene directly involved in D-xylose catabolism as it encodes an aldose reductase able to reduce D-xylose to xylitol (Kuhn et al., 1995). *GRE3* deletion has already been found to improve D-xylose utilisation in XI-carrying strains by reducing the formation of xylitol (Träff et al., 2001), which is an inhibitor of XI (Yamanaka, 1969). However, the biological functions of the other three genes have not previously been connected to D-xylose metabolism. Two of the targets are known to be connected to signalling routes (*HOG1* and *IRA2*), which suggests an interesting link between signalling and D-xylose uptake. *ISU1*, *HOG1* and *IRA2* are the objects of the study described in **Paper II**, and they will be further discussed in Section 2.3.

The inactivation of other genes associated with the HOG/osmoregulation pathway has been found to have a positive impact on D-xylose utilisation. For instance, the deletion of *SSK2*, which encodes one of the kinases that activate the HOG1 MAPKKK signalling pathway via Pbs2 phosphorylation (see Figure 8 in Section 2.3.2) was found to enhance xylose metabolism (dos Santos et al., 2016). The same was described for the deletion of *CYC8* (Nijland et al., 2017; Zha et al., 2014), which is part of the Sko1–Cyc8–Tup1 co-repressor complex that controls the expression of osmotically regulated promoters, and is activated upon Hog1p phosphorylation (Proft & Struhl, 2002). Thus, three independent studies found mutations in regulatory proteins in the same pathway (dos Santos et al., 2016; Nijland et al., 2017; Sato et al., 2016), which underlines the role of the HOG1 pathway as a key player in a regulatory network to improve C5 consumption. This also indicates that many of the new target genes associated with an improvement in D-xylose utilisation are somehow interconnected (Figure 6).

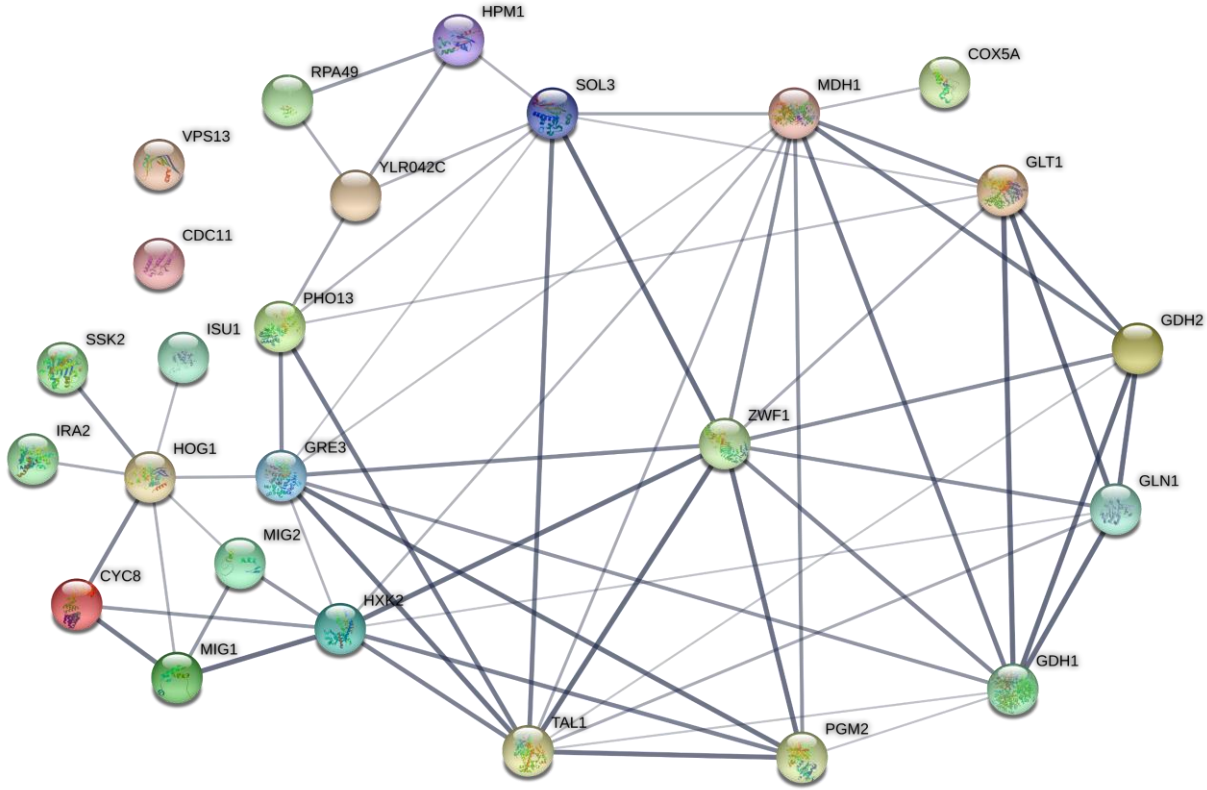

Figure 6. Prediction of protein–protein interactions between the additional target genes known to improve D-xylose consumption in *S. cerevisiae*. Line thickness indicates the strength of data support. (Source: STRING database (https://string-db.org))

2.3. A closer look at *ISU1*, *HOG1* and *IRA2*

As discussed above, combining the deletion of *ISU1, HOG1* and *IRA2* genes in a XI strain had a positive effect on D-xylose utilisation, although no direct connection could be established with D-xylose catabolism (Sato et al., 2016). This section reviews the current knowledge on these three genes, including some deductions made from the present work.

2.3.1. *ISU1*

ISU1 together with its redundant protein Isu2p (sharing more than 80% identity in amino acid sequence) is regulated by the Fe-regulatory factor Aft1p (Garland et al., 1999). *ISU1* and *ISU2* are essential to the cells, and cell death is observed when both genes are deleted (Garland et al., 1999; Schilke et al., 1999).

The Fe-S cluster is a prosthetic group that is integrated into the apoproteins with the aid of a complex set of proteins. There are two main systems of Fe-S-protein biogenesis in eukaryotes: the iron-sulphur cluster (ISC), machinery in mitochondria, and the cytosolic Fe-S-protein assembly (CIA) (Lill & Mühlenhoff, 2006). The overall mechanism for biogenesis in mitochondria (Figure 7) starts with a sulphur donor from a cysteine desulphurase (such as the Nfs1–Isd11 complex) and an iron donor (provided by Yfht1). The cysteine desulphurase liberates the sulphur (S^0) from cysteine to sulphide (S^{2-}) forming an IscS-bound persulphide on a conserved cysteine residue. The reduction of sulphur (S^0) to sulphide (S^{2-}) is performed by ferredoxin reductase and ferredoxin as an electron transfer. Isu1p acts as a platform for Fe-S cluster biosynthesis to later transfer this cluster to apoproteins, which are converted into holoproteins (Lill et al., 2012; Lill & Mühlenhoff, 2006; Nature, 2009).

Fe-S proteins play an essential role in the gene expression process (i.e. DNA replication, DNA repair and translation), the biosynthesis of cofactors and amino-acids, the citric-acid cycle and cell respiration (Nature, 2009). Therefore, deletion of *ISU1*, the scaffold protein involved in Fe-S cluster assembly, can affect some essential cellular activities.

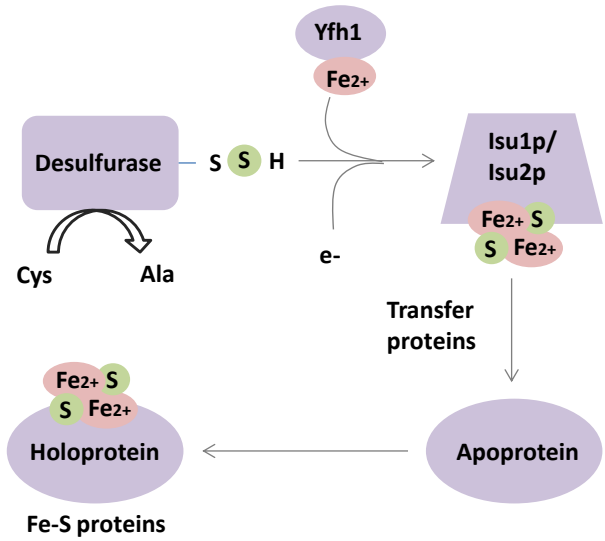

Figure 7. Schematic view of iron-sulphur cluster (Fe-S) biogenesis in mitochondria. (Adapted from Nature, 2009.)

Indeed, it has been reported that the deletion of *ISU1* leads to increased accumulation of iron in the mitochondria, decreased activity of a Fe-S protein (aconitase) and suppression of the oxidative damage in the absence of cytosolic copper/zinc superoxide dismutase (*sod1*Δ) (Garland et al., 1999). Excess iron increases the reactive oxygen species (ROS) via Haber–Weiss chemistry, where $OH^{\bullet}$ (the hydroxyl radical) is generated by the reaction between Fe^{2+} (ferrous) and H_2O_2 (hydrogen peroxide), leading to oxidative stress in the cell (Gomez et al., 2014; Pérez-Gallardo et al., 2013). Hydroxyl radicals have high reactivity with a wide range of biomolecules, including proteins, DNA, RNA, lipids and carbohydrates (Turrens, 2003), severely damaging the cell. Cells in which *ISU1* had been deleted were found to exhibit lower ethanol tolerance, survival and growth in the presence of ethanol than wild-type cells (Pérez-Gallardo et al., 2013). Ethanol toxicity is related to disturbances in iron homeostasis, which is connected to ROS overproduction, as mentioned above.

Despite these negative effects, it has been found in two independent adaptive laboratory evolution studies on engineered *S. cerevisiae* that the inactivation of *ISU1* led to faster D-xylose conversion in the evolved strains (dos Santos et al., 2016; Sato et al., 2016). Improvement in D-xylose consumption rate was observed under both aerobic and anaerobic conditions by Sato et al. (2016), whereas, dos Santos et al. (2016) reported the effect only under semi-anaerobic conditions. In both cases, *S. cerevisiae* was engineered with a XI pathway prior to the adaptive evolution experiment. It was therefore hypothesized that the

increase in cellular iron concentration caused by *ISU1* deletion favoured XI activity, since XI is a metalloenzyme (Sato et al., 2016). Metal cations with ionic radii ≤ 0.8 Å, such as Mg^{2+}, Mn^{2+}, Co^{2+} and Fe^{2+} act as cofactors, boosting XI activity (Kovalevsky et al., 2010). Indeed, the addition of iron enhanced D-xylose fermentation and increased XI activity (dos Santos et al., 2016). However, in the present study on XR-XDH strains (**Paper II**), the combination of *isu1*Δ and *ira2*Δ also conferred the highest specific rates of D-xylose consumption and ethanol production compared to other deletion strains under anaerobic conditions, which indicates that the positive effect of *ISU1* deletion goes beyond this first explanation.

Although deletion of *ISU1* is connected with ROS generation, it has been shown that at low levels of ROS, yeast cells can adapt and become more resistant by activation of the oxidative PPP to generate NADPH (Ayer et al., 2014). Therefore, the increased ROS level resulting from the deletion of *ISU1* could also increase the PPP flux and consequently aid D-xylose metabolism.

2.3.2. *HOG1*

Hog1p (high osmolarity glycerol 1) is a protein kinase, and a member of the mitogen-activated protein (MAP) family (Brewster & Gustin, 2014). Besides its function as a regulator during osmotic stress, this kinase also regulates a wide range of responses under conditions of environmental stress, from cell behaviour to survival, controlling cell cycle progression in the G1 phase, transport, transcription and translation (Brewster & Gustin, 2014; Dinér et al., 2011; Escoté et al., 2004). The activation of the HOG pathway in yeast (Figure 8) starts with two osmosensors at the membrane level: Sln1p and Sho1p (Posas et al., 1996). The two main branches connect through Pbs2p, a key scaffold protein, to activate Hog1p. The Sln1p branch is controlled by turgor pressure between the cell membrane and the cell wall; under normal osmolarity, Sln1p launches the phosphorelay system by autophosphorylation at His576 (Posas et al., 1996). Sln1p activation leads to the phosphorylation of Ssk1p, preventing Ssk1 from triggering Ssk2p and Pbs2p, thus inhibiting the Hog1 MAPK cascade (Maeda et al., 1995). Therefore, the HOG pathway is activated when the turgor pressure of the cell is reduced by increasing the extracellular osmolarity, preventing Ssk1p inhibition and activating Pbs2p and the MAPK cascade via Ssk2p (Brewster & Gustin, 2014). Sho1p is the other branch, which recruits a variety of proteins (not shown in the simplified version of Figure 8) that phosphorylate and activate Ste11p, and consequently, the Hog1p cascade (Brewster & Gustin, 2014).

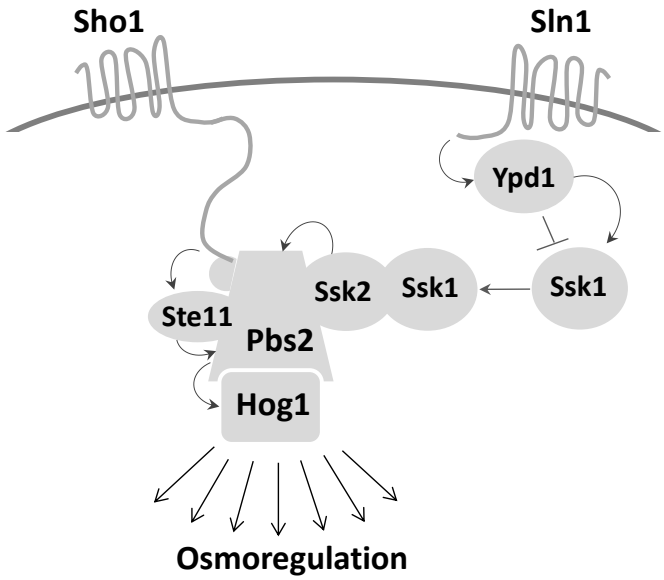

Figure 8. Simplified model of the HOG pathway in yeast. Arrows indicate phosphorylation reactions. (Adapted from Brewster, J. L. et al., 2014)

The deletion of *HOG1* results in a hyper-osmosensitive phenotype in *S. cerevisiae* strains (Albertyn et al., 1994), although glycerol accumulation can suppress this phenotype (Babazadeh et al., 2014). *HOG1* gene deletion also leads to unusual cell morphology, with larger cell size and abnormal bud-like extensions (**Paper II**) (Brewster & Gustin, 2014). Sato et al. (2016) also found that loss of function of *HOG1* and *ISU1* promoted an epistatic interaction which enabled rapid aerobic fermentation of D-xylose due to elevated levels of mitochondrial respiratory proteins in a strain of *S. cerevisiae* engineered with XI. These results therefore indicate a connection between D-xylose utilisation with the Fe-S cluster machinery and the Hog1/MAPK signalling system by some, as yet unknown, mechanism. It is known that Hog1p activation leads to up-regulation of the *GRE3* gene (Garay-Arroyo & Covarrubias, 1999); thus, *HOG1* could also indirectly influence D-xylose metabolism in XI strains. Indeed, *HOG1* deletion did not lead to the same benefits in the *S. cerevisiae* W303 strain engineered with XR/XDH (which is less susceptible to the presence of xylitol than XI strains), even with the addition of *isu1*Δ (**Paper II**).

2.3.3. *IRA2*

Ira2p is involved in the cAMP-PKA sugar signalling route (described in more detail in Chapter 3), where it regulates the intracellular cAMP level by negative regulation of the Ras proteins, thus, being an antagonist to the function of Cdc25p (Figure 9) (Tanaka et al., 1989; Tanaka et al., 1990b). Ira2p activity is regulated by ubiquitination (the addition of ubiquitin, which works as a degradation imprint), which leads to its inactivation and the enhancement of Ras1/2/p activation (Phan et al., 2010). The ubiquitination of Ira2p is modulated by Ubp3 (Li & Wang, 2013), and also by Gpb1/Krh2, which induces proteolysis by binding to Ira2p (Phan et al., 2010).

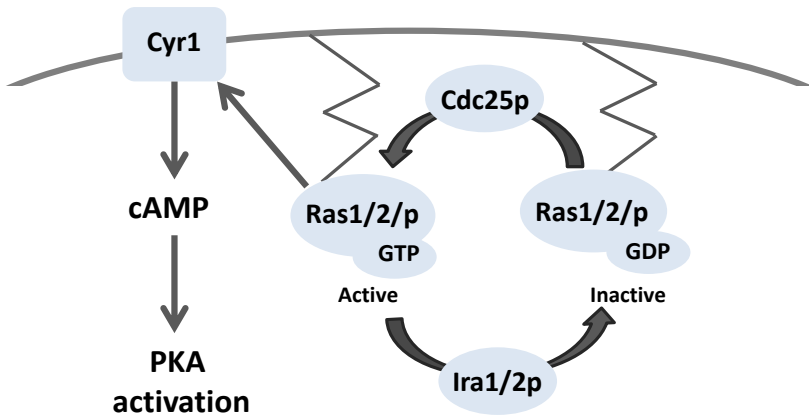

Figure 9. Schematic view of the role of Ira2p activity in the cAMP/PKA pathway. (Adapted from Brink et al. (2016))

Deletion of *IRA2* is expected to abolish the negative regulation of Ras1p/2p and increase the active mode of Ras proteins (Ras-GTP) (Tanaka et al., 1990b; Thevelein & de Windey, 1999), leading to a high PKA activity (Colombo et al., 2004; Peeters et al., 2006; Robinson et al., 1968). The cascade effect of PKA activation triggered by D-glucose favours rapid growth, represses stress response, induces glycolytic flux and leads to the transcription of many genes that are required for respiratory metabolism (Fabrizio et al., 2003; Li et al., 2018b; Park et al., 2005; Yi & Huh, 2015). In the case of PKA hyperactivation, cells are prevented from being arrested at the G1 phase of the cell cycle, even under conditions of carbon starvation, leading to restrained accumulation of glycogen and trehalose (Yi & Huh, 2015).

However, *IRA2* deletion has also been reported to lead to several adverse effects: failure to sporulate and sensitivity to heat shock and nitrogen starvation

(Tanaka et al., 1990b), lower growth rate (Li et al., 2018b and **Paper II**), a decrease in stationary phase survival (Yi & Huh, 2015), and the inability to reach the oxidative growth phase (Russell et al., 1993). These effects are in accordance with the facts that the activation of Ras1p/2p results in early cell death, and that the constitutive activation of PKA also promotes an abrupt decrease in yeast chronological life span (Fabrizio et al., 2003). *IRA2* deletion also leads to the defective disassembly of V-ATPase (Bond & Forgac, 2008) that is involved in proton transport across the plasma or intracellular membrane, sensitivity to oxidative stress inducers and to freeze-thawing (Lee & Ueom, 2001; Park et al., 2005). These effects could be a consequence of the inhibiting stress response by high PKA activity. Flocculation is another phenotype observed with *IRA2* deletion, which is due to activation of *FLO10* gene, provoking cell-to-cell adherence and leading to cell sedimentation (Halme et al., (2004) and **Paper II**).

IRA2 has been connected with the improvement in anaerobic D-xylose utilisation by XI strains, together with an epistatic interaction with *ISU1* (Sato et al., 2016). An increase in specific D-xylose consumption rate and specific ethanol formation rate has also been observed in this present work when deleting *IRA2* in a different strain background containing another D-xylose route (XR-XDH) (**Paper II**). These results indicate that there is a connection between the PKA route and D-xylose fermentation and underlines the importance of understanding how D-xylose is sensed by the cells.

3. D-Glucose Sensing and Signalling

Three pathways are involved in the sensing of D-glucose through different mechanisms: (i) the regulation of D-glucose transport (Snf3p/Rgt2p), (ii) the regulation of genes related to alternative carbon sources than glucose (SNF1/Mig1p), and (iii) the regulation of growth, cell cycle, metabolism, and stress response (cAMP/PKA). Although each plays an important regulatory role, they have different cross-talk, in that a gene can be regulated by more than one specific pathway. For instance, *SUC2* gene is mainly regulated by the SNF1/Mig1p pathway, but it is also influenced by the cAMP/PKA and Snf2p/Rgt2p pathways (Figure 10). These three pathways are described in detail below.

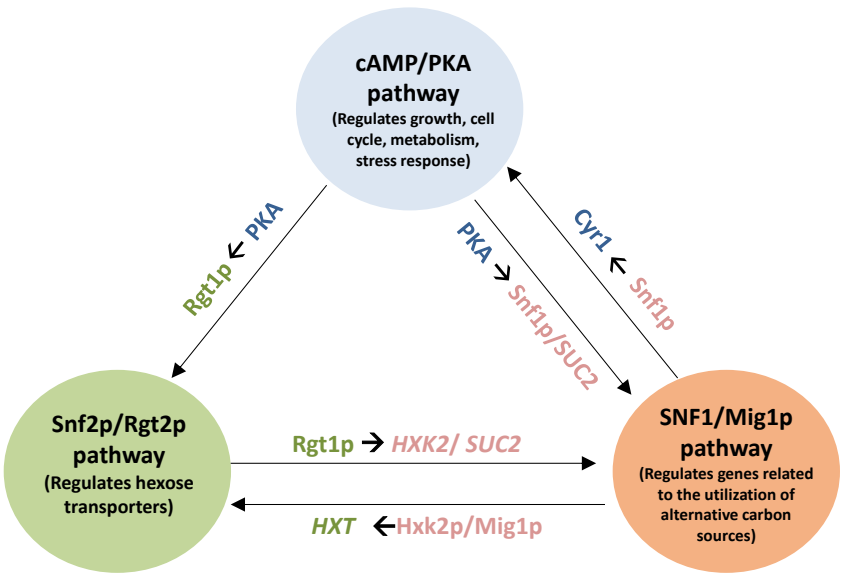

Figure 10. Cross-talk between the three main sugar signalling pathways.

3.1. Signalling pathways

3.1.1. The cAMP/PKA pathway

In *S. cerevisiae*, the change from a D-glucose-free to a D-glucose-rich environment triggers a transient increase in the level of cAMP, which leads to a phosphorylation cascade mediated by PKA (Gancedo, 2008; Kraakman et al., 1999). cAMP synthesis from ATP (Cook et al., 1957) is catalysed by a peripheral membrane protein, adenylate cyclase (Cyr1p) (Kim et al., 2013). When D-glucose is available, the activation of Cyr1p is triggered by G-proteins: Ras-GTP and the Gpr1p-Gpa2p system in their active form (Figure 8) (Kraakman et al., 1999; Rolland et al., 2002).

Ras1p and Ras2p are homologous GTP-binding proteins with a farnesyl and palmitoyl group to be hold on the cytoplasmic side of the plasma membrane and to facilitate the protein–protein interaction for biological activity (Bhattacharya et al., 1995). The GTP-bound form is the active mode of Ras1p/2p, and it is achieved with the help of Cdc25p, which catalyses GTP/GDP exchange. In contrast, Ira1p/2p induces Ras proteins inactivation (Ras-GDP) by the hydrolysis of GTP (Broek et al., 1987; Jones et al., 1991; Tanaka et al., 1989; Tanaka et al., 1990a).

Gpa2p is a heterotrimeric G-α-protein that interacts with the membrane-bound sensor Gpr1p, establishing a G-protein-coupled receptor system that induces the activation of cAMP synthesis with the aid of Cyr1p (Kraakman et al., 1999). The Gpr1p sensor has seven transmembrane segments located at the cell surface that detect extracellular D-glucose and direct the configuration of the active form of GTP-Gpa2p (Kraakman et al., 1999; Rolland et al., 2000; Xue et al., 1998). Gpa2p can also be inactivated by Rgs2p, a protein responsible for accelerating the hydrolysis of GTP attached to Gpa2p (Versele et al., 1999; Versele et al., 2001; Virgilio, 2012).

The addition of D-glucose and intracellular acidification leads to the accumulation of the active forms of Ras-GTP and Gpa1-GTP, respectively, which induces Cyr1p, cAMP synthesis and further activation of PKA (Colombo et al., 2004; Peeters et al., 2006; Robinson et al., 1968). PKA is a heterotetrameric holoenzyme composed of two Tpkp catalytic subunits, which can be redundantly encoded by *TPK1*, *TPK2* and *TPK3*, and two regulatory subunits of *BCY1* (Santangelo, 2006; Toda et al., 1987a; Toda et al., 1987b). Despite the high degree of similarity between the sequences of *TPK1*, *TPK2* and *TPK3*, the subunits are not functionally redundant, which leads to unique

signatures and a multiple function in *S. cerevisiae* signalling (Robertson et al., 2000).

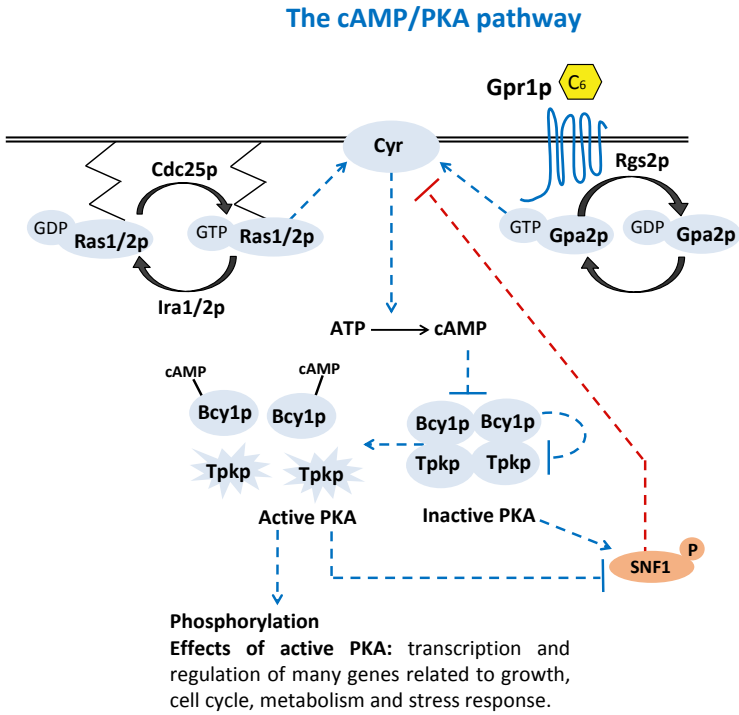

Figure 11. Schematic illustration of the cAMP/PKA pathway.

PKA is in its inactive form when the four subunits are attached to each other, and is activated when cAMP binds to the regulatory subunit Bcy1p, dissociating it from the two catalytic subunits (e.g. Tpk1p and Tpk2p) (Figure 11) (Gancedo, 2008; Santangelo, 2006). PKA becomes active in response to D-glucose, and signals to the cell that the environment is favourable for growth, by inducing genes related to the cell cycle and metabolism, and reducing the stress response (Table 2) (Rubio-Texeira et al., 2010). Under low D-glucose conditions, PKA has the opposite response; arresting in the G1 phase of the cell cycle, inducing stress response, and acquiring reserve carbohydrates, such as trehalose and glycogen (Rubio-Texeira et al., 2010). The cross-talk between the signalling pathways can be exemplified by the case when SNF1, a protein that regulates pathways related to the alternative carbon source (Section 3.1.2), also phosphorylates Cyr1p, decreasing cAMP and reinforcing the inactive state of PKA, which in turn is unable to negatively regulate SNF1 (Figure 10) (Nicastro et al., 2015).

Table 2. Metabolic and physiologic effects of PKA activation

	Effects of PKA activation	References
Induce / Positive regulation	Ribosome biogenesis (Ribi) and ribosomal protein (RP) genes	Klein and Struhl (1994)
	BAT1 gene (Tpk1p regulation): gene involved in exit from stationary phase, iron homeostasis and mitochondrial DNA stability	Robertson et al. (2000)
	Pseudohyphal growth (Tpk2p regulation)	Robertson et al. (2000)
	Genes involved in trehalose degradation and water homeostasis (Tpk2p regulation)	Robertson et al. (2000)
	Growth and increase cell biomass	Klein and Struhl (1994); Zurita-Martinez and Cardenas (2005)
	Low-affinity hexose transporters via Rgt1p phosphorylation (i.e. *HXT1*)	Kim and Johnston (2006)
	Glycolytic genes	Santangelo (2006)
	Protein phosphatases (PP2A and PP1), specifically dephosphorylate serine/threonine amino acids	Castermans et al. (2012)
Repress / Negative regulation	Fructose 1,6-bisphosphatase (gluconeogenesis)	Gancedo et al. (1982); Pohlig and Holzer (1985)
	Stress-responsive genes (i.e. *MSN2* and *MSN4*)	Smith et al. (1998)
	Glycogen and trehalose accumulation	Smith et al. (1998)
	Rim15p (a protein kinase involved in adaptation process to enter in the stationary phase)	Reinders et al. (1998)
	Genes involved in iron uptake (Tpk2p regulation)	Robertson et al. (2000)
	Heat-shock genes (i.e. *HSP12*, *HSP26*) by inhibiting the transcription factor Hsf1	Ferguson et al. (2005)
	Trehalose-6-phosphate synthase (TPS) complex by the phosphorylation of one of the regulatory subunits, Tps3p	Trevisol et al. (2014)
	Invertase (*SUC2*)	Gancedo et al. (2015)
	Sak1p and SNF1 proteins	Nicastro et al. (2015)

3.1.2. The SNF1/Mig1p pathway

The SNF1/Mig1p pathway is involved in the regulation of carbon catabolite repression (CCR). This pathway is activated in the absence of D-glucose, and alleviates CCR in order to express essential genes for growth on alternative carbon sources (such as sucrose, maltose and galactose), and non-fermentable carbon sources (i.e. glycerol and ethanol) (Zaman et al., 2008). This pathway is also involved in other cellular process related to inositol, glycogen accumulation, meiosis, morphology and aging (Ashrafi et al., 2000; Honigberg & Lee, 1998; Kuchin et al., 2002; Shirra & Arndt, 1999).

SNF1 is a protein kinase complex, consisting of three subunits: the α-catalytic subunit, the γ-Snf4 regulatory subunit, and the β-subunit, which is composed of Sip1p, Sip2p or Gal83p (Hedbacker & Carlson, 2008; Kim et al., 2013). SNF1 activation is triggered when Sak1p, Tos3p or Elm1p kinases phosphorylate SNF1 at Thr20 when D-glucose is limited (Hong et al., 2003). However, SNF1 localization (cytoplasm or nucleus) is determined by the Gal83 β-subunit depending on the carbon source, for example, the presence of D-glucose induces SNF1 nuclear exclusion (Vincent et al., 2001).

It was first hypothesized that SNF1 activation was regulated by the AMP:ATP ratio, however, no proof could be found to support this hypothesis (Hardie et al., 1998). When D-glucose is available, the phosphatase Reg1p-Glc7p modulates SNF1 and Hxk2p kinases to repress the genes responsible for the utilisation of alternative carbon sources (e.g. *SUC2*). The dephosphorylation of SNF1 and Hxk2p by Reg1p-Glc7p leads to their inactivation and activation, respectively (see Figure 12) (Fernández-García et al., 2012; Sanz et al., 2000). However, active SNF1 inhibits Reg1p-Glc7p via phosphorylation in a positive feedback loop (Zaman et al., 2008). Thus, SNF1 activation is regulated by Reg1p-Glc7p and Hxk2p, whereas SNF1 localization is regulated in a different manner.

D-glucose is not the only sugar that triggers the nuclear exclusion of SNF1 (with Gal83p subunit). Galactose and 2-deoxy-glucose also promote the same effect as D-glucose on SNF1-Gal83p (nuclear exclusion) (Vincent et al., 2001). This indicates that glucose-6-phosphate may be a candidate for the signalling role of SNF1 localization (Vincent et al., 2001). Moreover, low SNF1 activity has been detected in the presence of 2-deoxy-glucose (a D-glucose derivative that can be phosphorylated but not metabolised), whereas 6-deoxy-glucose (a D-glucose derivative that cannot be phosphorylated due to the lack of a hydroxyl group at carbon 6) had no effect on SNF1 activity (Hedbacker & Carlson, 2006; Kim et al., 2013). Despite these findings, the signalling mechanism behind SNF1-Gal83p localization remains unclear.

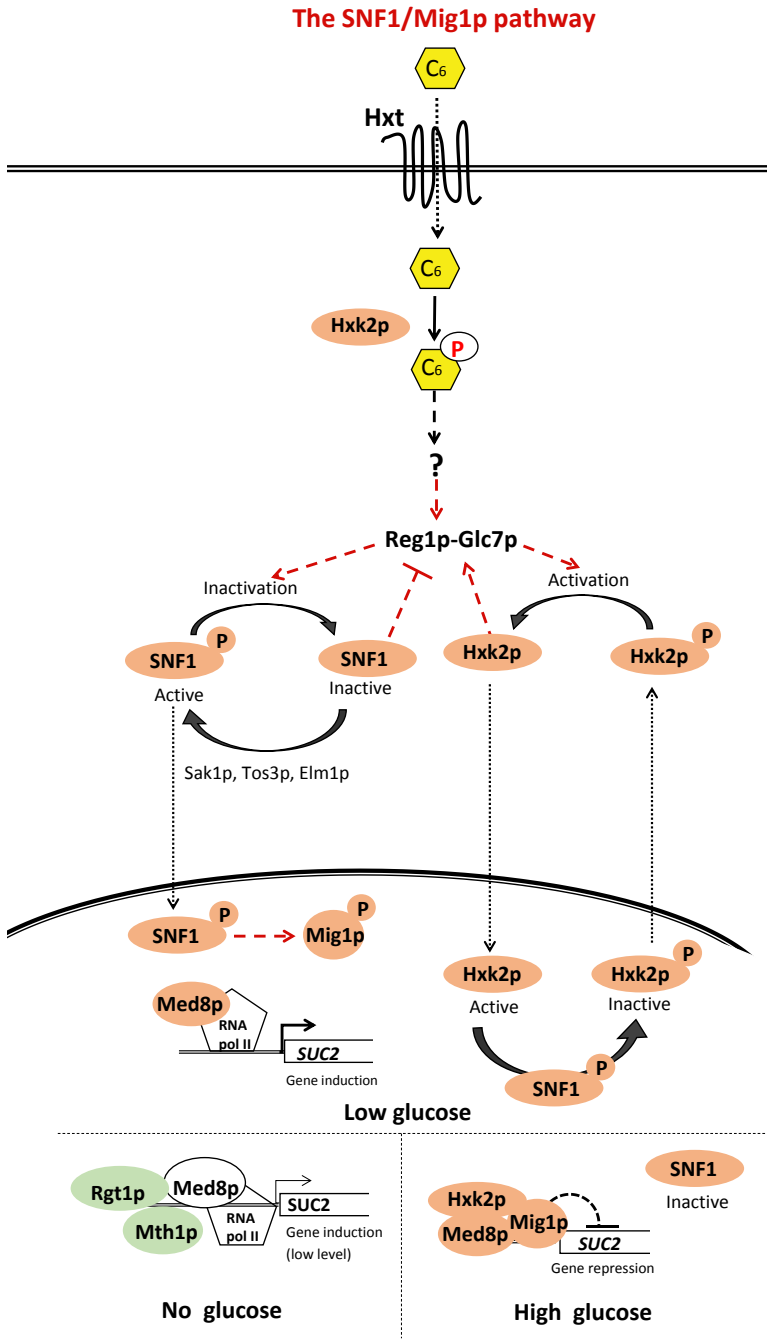

Figure 12. Schematic illustration of the SNF1/Mig1p pathway.

In parallel, the Reg1p–Glc7p complex positively regulates Hxk2p by dephosphorylation at Ser14, allowing the nuclear import of the active form of Hxk2p when D-glucose is available (Figure 12) (Fernández-García et al., 2012). Therefore, the activation of Hxk2p forms a DNA repressor complex with Mig1p in order to repress the genes that are responsible for the use of alternative carbon sources, such as *SUC2* (Ahuatzi et al., 2007). Active Hxk2p also interacts with the mediator factor Med8p, which facilitates gene repression by hindering RNA polymerase activation (de la Cera et al., 2002; Gancedo & Flores, 2008). Apart from Hxk2p, Mig1p, together with the Ssn6p–Tup1p complex, helps to reinforce CCR in a D-glucose-rich environment (Gancedo, 2008; Kayikci & Nielsen, 2015).

In the absence of D-glucose, active SNF1 enters into the nucleus and inactivates Hxk2p by phosphorylation at Ser14, which leads to Hxk2p nuclear export (Figure 12) (Fernández-García et al., 2012). Therefore, in the absence of Hxk2p, Mig1p is phosphorylated by SNF1 and loses its repressive competence, allowing the expression of genes involved in the utilisation of alternative carbon sources (Ahuatzi et al., 2007). Besides the regulation of CCR, Hxk2p and Mig1p also play an important role in the regulation of sugar transporters, assisting in the induction of *HXT1* and the repression of *HXT2/4* at high D-glucose levels, and switching the regulation at low D-glucose concentrations (Özcan & Johnston, 1995; Westholm et al., 2008). If Hxk2p and Mig1p are regarded as transcription factors of hexose transporters this provides a cross-link between the SNF1/Mig1p and Snf3p/Rgt2p pathways (Figure 10).

3.1.3. The Snf3p/Rgt2p pathway

Hexose transporter genes are primarily regulated by the Snf3p/Rgt2p pathway. Snf3p and Rgt2p are membrane sensors characterized by 12 transmembrane domains and a long cytoplasmic domain at the C-terminus that provides the intracellular signal through interaction with other proteins in the pathway (Özcan et al., 1996). Both sensors appear to be evolutionarily derived from hexose transporters that have lost their transporter function (Kim et al., 2013). Despite the above similarities, these two sensors have different affinities for D-glucose, which leads to a different *HXT* expression. The difference in affinity may be correlated to the dissimilarity in their C-terminal tails, having only a short conserved sequence of 17 nearly identical amino acids (Özcan et al., 1996). Snf3p has two of these sequences, while it occurs only once in Rgt2p. Thus, it appears that hexose is sensed by the 12 membrane-spanning domains, whereas the transduction of D-glucose signal might be amplified by the tails through the anchoring of Mth1p and Std1p (Horák, 2013; Moriya & Johnston, 2004).

High levels of D-glucose activate the Rgt2p sensor, which triggers a signalling cascade, inducing the expression of low-affinity hexose transporter genes (for example *HXT1*). In contrast, Snf3p is regulated by low D-glucose concentration, and stimulates the transcription of high-affinity hexose transporter genes, such as *HXT2/4* (Kim et al., 2013; Özcan et al., 1996). It has also been suggested that Snf3p and Rgt2p may regulate D-glucose uptake by sensing the internal-to-external ratio of D-glucose levels (Karhumaa et al., 2010).

In the presence of D-glucose, Snf3p/Rgt2p sensors activate the yeast casein kinase I (Yck1p and Yck2p) that is attached to the plasma membrane via a palmitoyl moiety. This prompts the phosphorylation of Mth1p and Std1p, which are co-repressors of hexose transporter genes (Babu et al., 2004; Kim et al., 2013; Moriya & Johnston, 2004). The phosphorylated Mth1p and Std1p are then ubiquitinated by the SCF–Grr1p ubiquitin ligase complex, and then degraded with the aid of 26S proteasome (Flick et al., 2003). The degradation of Mth1p and Std1p repressors is part of the response needed to express hexose transporter genes (Figure 13).

However, the regulation of the expression of hexose transporter genes also depends on regulators that are associated with other signalling routes. For instance, Mth1p and Std1p act together with Rgt1p, whose phosphorylation depends on the cAMP/PKA pathway (see Figure 10; section 3.1.1; Table 2), to modulate the expression of transporter genes as a function of D-glucose availability (Kayikci & Nielsen, 2015; Kim et al., 2013). At high D-glucose levels, Rgt1p phosphorylation relieves the repression of *HXT* genes (Kim & Johnston, 2006; Kim et al., 2003). However, the mechanism of Rgt1p regarding transcription regulation is more complex, as it is able to repress or induce gene expression in yeast, depending on its degree of phosphorylation (Jouandot et al., 2011).

There are four serine sites on Rgt1p that can be phosphorylated by PKA. Phosphorylation on at least one of these three serines (Ser-202, Ser-283 or Ser-284) is sufficient for Rgt1p to become an activator. However, upon the phosphorylation of four serine residues (Ser-146, Ser-202, Ser-283 and Ser-284) Rgt1p is dissociated from the general repressor complex, Ssn6p–Tup1p, and then released from the *HXT* promoters (Jouandot et al., 2011) (Figure 13). Rgt1p also plays a role in the repression of the *HXK2* gene. A disruption in the *RGT1* gene in the absence of D-glucose leads to an 18-fold increase in the level of *HXK2* expression (Palomino et al., 2005). Another kind of cross-talk that can be seen between the Snf3p/Rgt2p and SNF1/Mig1p pathways, is with *SUC2* repression by Rgt1p in the absence of D-glucose (Figure 10) (Gancedo et al., 2015).

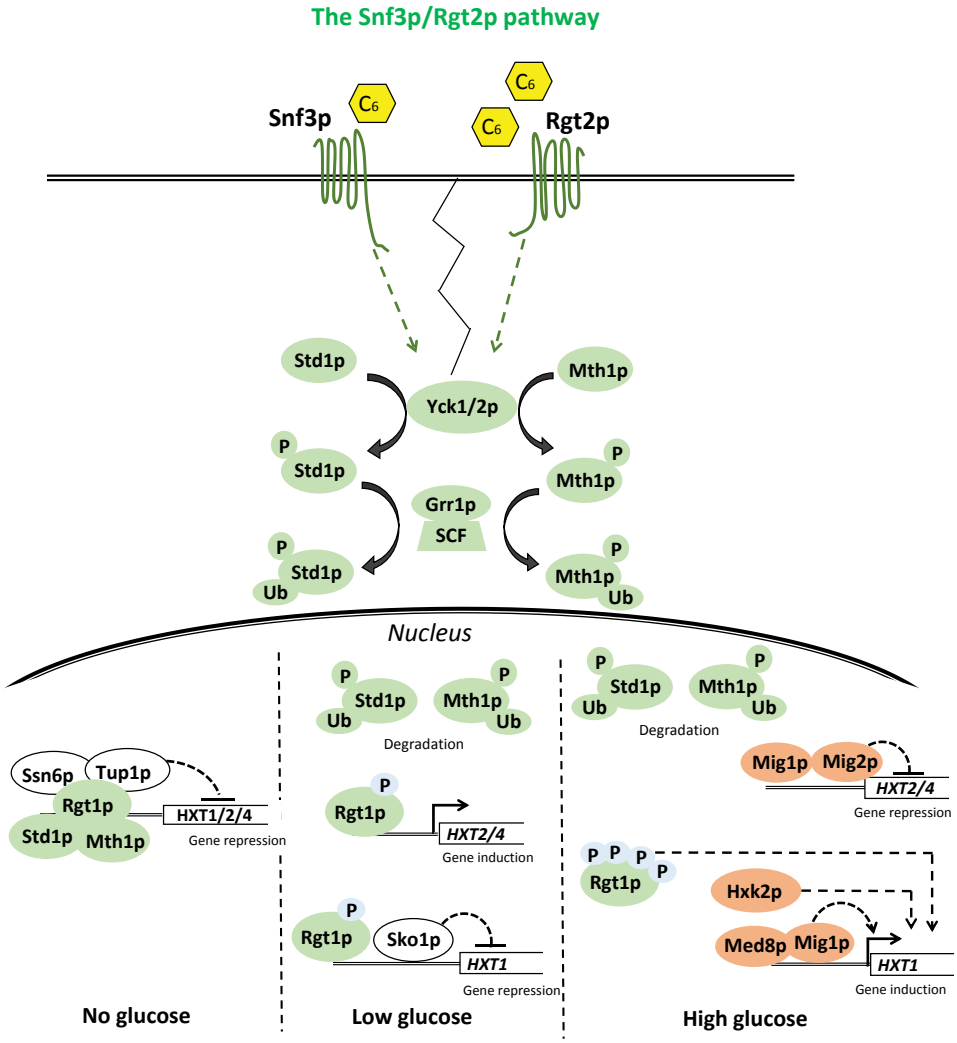

Figure 13. Schematic illustration of the Snf3p/Rgt2p pathway.

Low D-glucose conditions repress *HXT1* despite Rgt1p being active when one serine is phosphorylated (Jouandot et al., 2011). This regulation comes from the HOG pathway (see Figure 8, Section 2.3.2) through the Sko1p–Tup1p–Ssn6p complex, which binds directly to the *HXT1* promoter to act as an inhibitor (Tomás-Cobos et al., 2004). When the level of D-glucose is high, *HXT1* (low-affinity hexose transporter) is induced and activated by Rgt1p (Polish et al., 2005), Mig1p/2p (Lutfiyya et al., 1998; Westholm et al., 2008) and Hxk2p (Özcan & Johnston, 1995). In addition, the Sko1p repressor is phosphorylated

by Hog1p when the extracellular osmolarity is increased, due to the high level of D-glucose, and is released from the *HXT1* promoter (Tomás-Cobos et al., 2004).

Although the Mth1p and Std1p repressors are degraded under high D-glucose conditions, the high-affinity hexose transporters (i.e. *HXT2/4*) are repressed by other regulators: Mig1p and Mig2p from the SNF1/Mig1p pathway (Section 3.1.2) (Westholm et al., 2008). In contrast, low levels of D-glucose induce the transcription of *HXT2/4*, since the Mig1p/2p repressors are phosphorylated and inactivated by SNF1 (see Section 3.1.2) (Chandrashekarappa et al., 2016; Westholm et al., 2008). Finally, the absence of D-glucose leads to the repression of low- and high-affinity hexose transporter genes (Gancedo, 2008; Kayikci & Nielsen, 2015; Kim et al., 2013; Zaman et al., 2008).

3.2. The role of intracellular metabolites in sugar signalling

Apart from the membrane sensors, it has been suggested that specific intracellular metabolites could act as a flux sensor, regulating the metabolic flux and promoting sugar homeostasis (Huberts et al., 2012; Rolland et al., 2002). Proposed flux regulators include glycolytic intermediates, and the ratios between them; glucose-6-phosphate (G6P) and fructose-1,6-bisphosphate (F1,6bP) being interesting candidates (Figure 14) (Huberts et al., 2012).

As mentioned above (Section 3.1.2), glucose-6-phosphate may be involved in the regulation of SNF1 (Hedbacker & Carlson, 2006; Kim et al., 2013; Vincent et al., 2001). Mutations on Hxk1p, Hxk2p and Glk1p (enzymes able to phosphorylate D-glucose) found in an evolved *S. cerevisiae* strain revealed that reducing the D-glucose phosphorylation rate contributed to the alleviation of D-glucose repression (Lane et al., 2018). Furthermore, G6P also has the ability to induce the activity of glycolytic isoenzyme enolase 2 which catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate (Müller et al., 1995).

Another sugar phosphate that may affect sugar signalling pathways is F1,6bP, a putative Ras1p/2p activator, affecting cAMP synthesis and PKA activation (Peeters et al., 2017). F1,6bP has also been proposed as a good target to sense and regulate metabolic flux as its concentration has a direct correlation with sugar uptake (Huberts et al., 2012). F1,6bP has also been reported to activate pyruvate kinase (Susan-Resiga & Nowak, 2003) and to repress the activation of 6-phosphofructokinase (Przybylski et al., 1985).

Trehalose-6-phosphate (T6P) has also been considered as a flux sensing molecule due to its capacity to strictly control gluconeogenic gene repression

(i.e. *FBP1* and *PKA1*) (Vicente et al., 2018). Since T6P is closer to the upstream glycolysis, it has been suggested that it would be a better internal sensor for monitoring the flux than F1,6bP. This assumption is also based on a Michaelis-Menten correlation between T6P concentration and fermentation capacity, showing a positive influence of this metabolite on fermentation (Vicente et al., 2018). T6P is known to have a signalling function in plants. There is also some evidence of SNF1 inhibition in yeast through T6P, but no clear mechanism has been identified (Deroover et al., 2016; Yuhua et al., 2009).

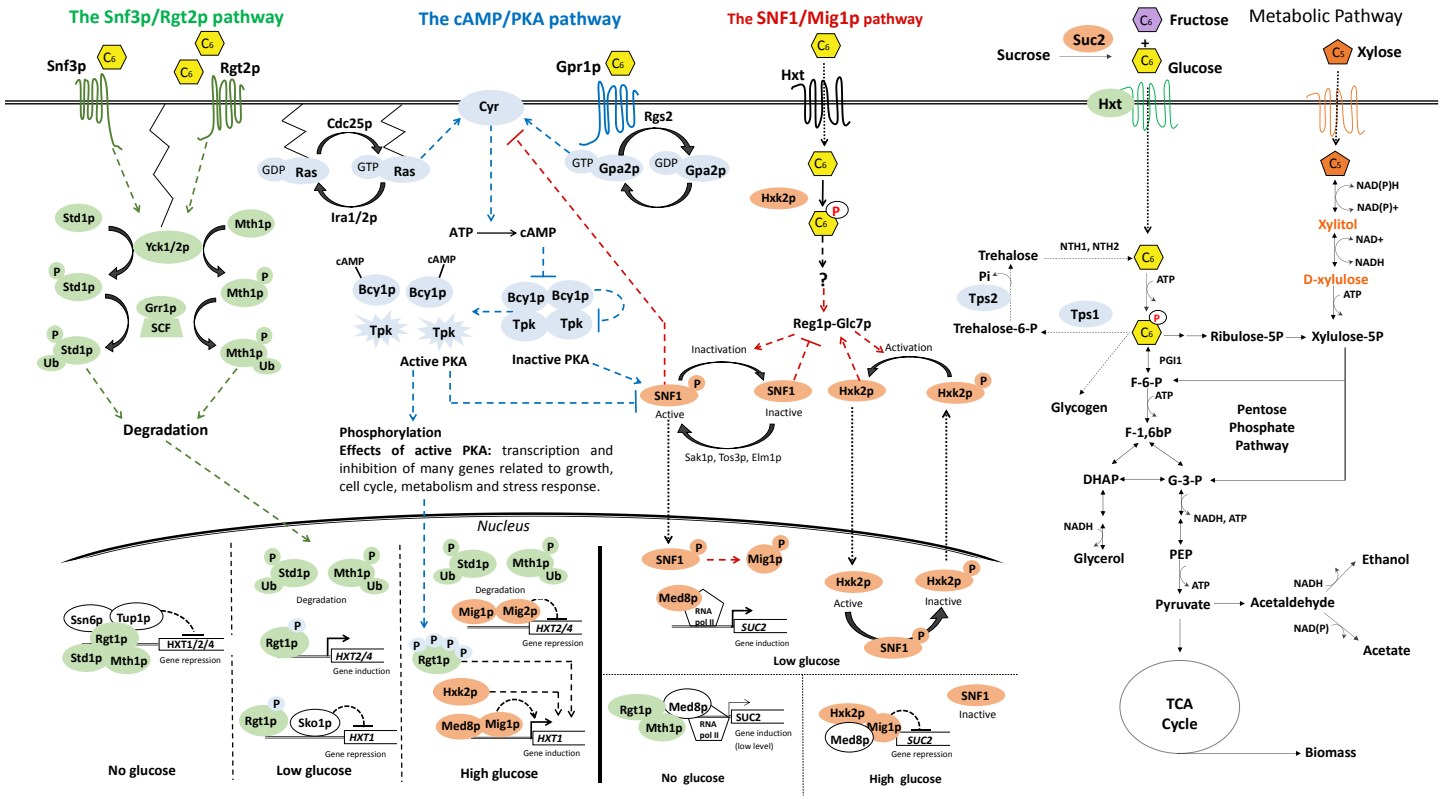

Figure 14. Illustration of the three main signalling pathways (Snf3p/Rgt2p, cAMP/PKA, and SNF1/Mig1p) and their connection to sugar metabolism.

4. Biosensor as a Tool for Physiology Studies

4.1. What is a biosensor?

A biosensor is defined as an analytical element that records and monitors a biological event, analyte or process, and transduces it into a recordable signal (Su et al., 2011; Zhou et al., 2015). The signal measured from biosensors can be detected in different ways: optically, electrochemically, mechanically, calorimetrically or acoustically, to provide information on the functionality of living cells (Su et al., 2011). Optical and electrochemical techniques are most common in microbial biosensors. Electrochemical detection includes several methods, such as amperometry, conductometry, potentiometry and voltammetry, while optical methods are mainly based on colorimetry, fluorescence and luminescence (Park et al., 2013; Su et al., 2011).

4.2. Application of biosensors in *S. cerevisiae*

Optical biosensors can provide useful information on cell physiology. For example, fluorescence- and luminescence-based sensors have been designed for *S. cerevisiae* to provide information on gene regulation and expression (Li et al., 2000), protein localization (Valadi et al., 2004), growth and membrane robustness (Carlquist et al., 2012), redox changes (Dardalhon et al., 2012; Knudsen et al., 2014), quantitative and/or qualitative analyte concentrations (Ameen et al., 2016; Li et al., 2018a; Li et al., 2015; McNabb et al., 2005; Mohsin & Ahmad, 2014; Umeyama et al., 2013; Wang et al., 2016), intracellular pH (Aabo et al., 2011; Imai & Ohno, 1995; Orij et al., 2009; Ullah et al., 2012; Valkonen et al., 2013; Valli et al., 2006; Valli et al., 2005; Weigert et al., 2009) and mating (Rodrigues et al., 2001)(Table 3). In the present work, fluorescence-based biosensors were used to study sugar signalling pathways (**Papers I-III**).

Fluorescence is the most common technique used in *S. cerevisiae*, where different mechanisms and strategies are used to monitor yeast cell response, or to determine the intracellular level of specific compounds. Examples of these are: fluorescence resonance energy transfer (FRET), proteins or promoters tagged with other fluorescent proteins, a pH-sensitive mutant of a green fluorescent protein (GFP), and fluorescent dyes, by converting the protein activity or the ligand factor into an optical signal (VanEngelenburg & Palmer, 2008) (Table 3).

4.3. Green fluorescent protein in reporter systems

Reporter genes, i.e. a fluorescent, luminescent or colorimetric detections whose gene open reading frame is coupled to a chosen promoter or fused to another protein, are widely used to monitor gene regulation and expression (Naylor, 1999). Among the systems available, GFP is very common in reporter systems of different types of organisms (Naylor, 1999). Shirmomura et al. discovered GFP in the jellyfish *Aequorea victoria* in 1962, and it was first called aequorin. GFP emits green light *in vivo* (508-509 nm); however, when extracted from the living organism and incubated with Ca^{2+}, it emits blue light (470 nm) (Morise et al., 1974; Shimomura et al., 1962).

Wild-type GFP has two excitation peaks. The main excitation peak is at 395 nm, and gives an emission peak at 508 nm, while the minor excitation peak at 475 nm generates a maximum emission peak at 503 nm (Tsien, 1998). It has been reported that the fluorescence at 395 nm decreased while that at 475 nm increased in wild-type GFP when the temperature was increased from 15°C to 65°C, and that at 78°C, the fluorescence was reduced by 50% (Ward et al., 1982). GFP fluorescence is also oxygen-dependent, only emitting fluorescence when oxygen is available (Heim et al., 1994). GFP can therefore be used as an O_2 (Takahashi et al., 2005) and temperature sensor (Donner et al., 2012).

GFP has been engineered to allow its use in a wide range of applications (Chalfie, 2009). In particular, a number of GFP variants have been developed through amino acid substitution, where each class has a particular set of excitation and emission wavelengths, providing a broad range of colour (Tsien, 1998). However, most of these GFP derivatives do not have the same stability over a broad pH range as the wild-type GFP (Patterson et al., 1997).

Table 3. Biosensors applied in *S. cerevisiae*

Monitoring of	Biosensor type	Biosensor mechanism	Application	Reference
Gene Regulation and expression	Fluorescence: GFP	Reporter gene: *GAL1*p promoter coupled to the GFP gene	Monitoring the regulation and expression of the *GAL1* gene	Li et al. (2000)
	Luminescence: *Renilla* luciferase	Reporter gene: *PGK1*p or *SPT15*p promoter coupled to *Renilla* luciferase	Quantifying gene expression	McNabb et al. (2005)
Analyte (metabolite or compound)	Fluorescence: GFP	Reporter gene: *JEN1*p promoter coupled to the GFP gene	Monitoring sugar concentration	Chambers et al. (2004)
	Fluorescence: Venus	Reporter gene: *metO*p promoter coupled to the Venus gene	Synthetic gene circuits to determine the intracellular level of S-adenosylmethionine	Umeyama et al. (2013)
	Fluorescence: CFP and YFP	FRET sensor, CFP: donor (light emitter), YFP: acceptor, MetN: reporter element	Monitoring the intracellular level of L-methionine	Mohsin and Ahmad (2014)
	Fluorescence: tdTomato	Reporter gene: *GPM1*p promoter followed by *fapO* operator (*FapR*-Regulated) coupled to tdTomato	Monitoring the intracellular level of malonyl-CoA	Li et al. (2015)
	Fluorescence: CFP and YFP	FRET sensor, CFP: donor (light emitter), YFP: acceptor, L-lysine binding bacteria periplasmic protein: reporter element	Monitoring the intracellular level of L-lysine	Ameen et al. (2016)
	Fluorescence: GFP	Reporter gene: *Tsynth8_pCYC1noTATA* synthetic promoter coupled to the GFP gene	Synthetic gene circuits to determine the intracellular level of galactose- and β-oestradiol	Li et al. (2018a)
Redox changes	Fluorescence: YFP	Reporter gene: rxYFP nucleus and cytosol targeting	Monitoring glutathione redox changes	Dardalhon et al. (2012)
	Fluorescence: GFP	Reporter gene: *GPD2*p promoter coupled to the GFP gene	Monitoring perturbations in the NADH/NAD+ co-factor balance	Knudsen et al. (2014)
Intracellular pH	Fluorescence: CFDA-SE dye	5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester, a dye with pH-dependent emission spectra	Monitoring intracellular pH	Aabo et al. (2011); Imai and Ohno (1995); Weigert et al. (2009)
	Fluorescence: carboxy SNARF-4F dye	Carboxy seminaphthorhodafluor, a dye with pH-dependent emission spectra	Monitoring intracellular pH	Valli et al. (2006); Valli et al. (2005)
	Fluorescence: pHluorin	Reporter gene: pH-sensitive mutant of GFP	Monitoring intracellular pH	Orij et al. (2009); Ullah et al. (2012); Valkonen et al. (2013)
Protein localization and stability	Fluorescence: GFP	Gpd1p and Gpd2p tagged on GFP	Monitoring protein localization	Valadi et al. (2004)
	Calorimetric: β-galactosidase	Reporter gene: *RPN4*p promoter coupled to *LacZ*	Monitoring protein stability	Osipov et al. (2011)
Population heterogeneity and cell membrane robustness	Fluorescence: GFP	Reporter gene: *RPL22A*p promoter coupled to the GFP gene	Monitoring population heterogeneity and cell membrane robustness	Carlquist et al. (2012)
Mating	Fluorescence: DsRed	Reporter gene: DsRed using the pUG vector	Monitoring mating *in vivo*	Rodrigues et al. (2001)
Sugar regulon, sensing and signalling	Fluorescence: GFP	Reporter gene: synthetic hybrid promoter coupled to the GFP gene and the *xylR* gene (D-xylose specific repressor)	Development of D-xylose biosensor that is able to control gene expression in the presence of D-xylose	Teo and Chang (2015),Wang et al. (2016)
	Fluorescence: GFP	Reporter genes: *HXT1*p/*2*p/*4*p, *SUC2*p, *TPS1*p/*2*p, *TEF4*p promoters coupled with the GFP gene	Monitoring the three main sugar signalling pathways	(Brink et al., 2016) Papers I-III

Codon optimization of the GFP-encoding gene seems to be necessary for the development of a specific biosensor for each host. Different GFP types have been codon-optimized for *S. cerevisiae*, showing an increase of up to 101-fold in expression levels and fluorescence intensity (Kaishima et al., 2016). The yeast-enhanced GFP gene (yEGFP3) is an example of a codon-optimized, versatile reporter gene suitable for expression in *S. cerevisiae*, *C. albicans* and other fungal species (Cormack et al., 1997). However, there is a clear difference in the fluorescence signal depending on whether yEGFP3 is expressed by utilising a multicopy plasmid, or the gene is integrated on the chromosome. A multicopy plasmid gives a significantly higher fluorescence intensity, while chromosomal integration gives a more well-defined fluorescence peak, better stability, and lower population heterogeneity caused by variation in gene copy number, and is thus more appropriate for single-cell analysis (Knudsen et al., 2014; Lee et al., 2015).

4.4. Population heterogeneity

The average phenotype of a whole cell population is usually taken into consideration in bioprocess measurements. However, it is known that microbial populations have a certain degree of heterogeneity (Fernandes et al., 2011). In some cases, undesired phenotypes may lead to lower yield and productivity (Hewitt et al., 2000). Nonetheless, heterogeneity has also been reported to provide potential benefits during adaptation processes, or when cell robustness is needed (Avery, 2006; Carlquist et al., 2012). Heterogeneity can arise from cell ageing, the stage of the cell cycle, epigenetic regulation and even stochastic gene expression (Avery, 2006). Heterogeneity can be measured using fluorescence-based reporter systems in combination with analysis at the single-cell level, for example, using flow cytometry.

Flow cytometry is used to measure light scattering (forward scatter and side scatter) (Figure 15A), and fluorescence (Figure 15B) at the single-cell level and can provide useful information on the degree of population heterogeneity under different conditions. Forward-scattered light can be used to determine cell size, as it is detected on the same axis as the laser beam. Side-scattered light is collected at an angle of roughly 90° to the laser beam, and can be used to study internal cellular complexity and granularity (Adan et al., 2017).

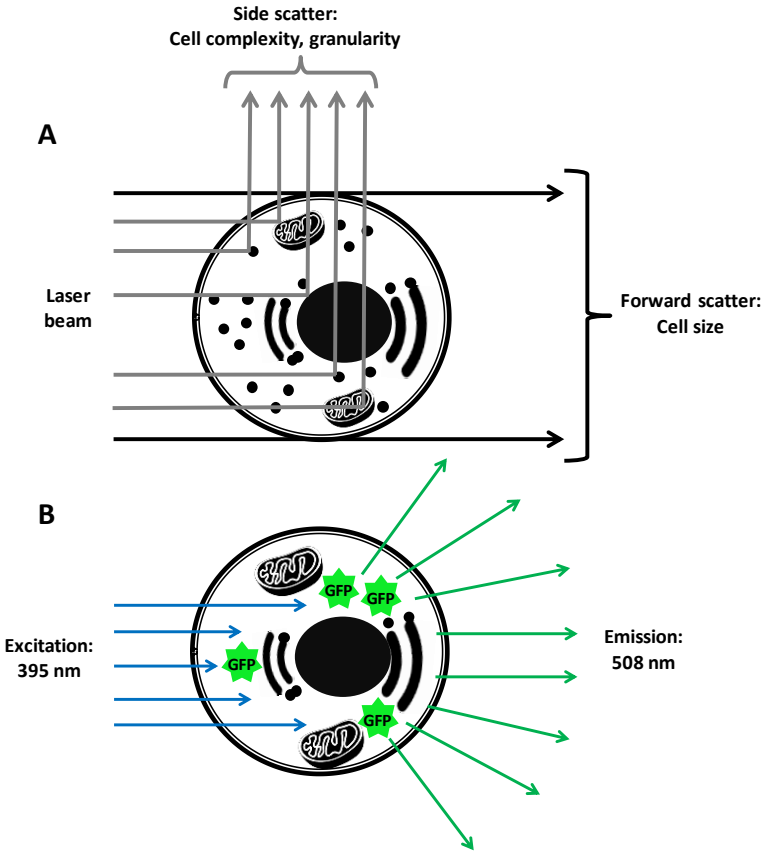

Figure 15. Illustration of forward scatter and side scatter (A), and fluorescence intensity (B), using flow cytometry. The green icons inside the cell in (B) represent GFP fluorescence. Adapted from Adan, A. et al. (2017).

A common problem associated with flow cytometry is autofluorescence. In general, cells have an inherent autofluorescence (Figure 16A), usually arising from NADH, riboflavins and flavin coenzymes (Aubin, 1979), and it is important to distinguish this from the biosensor fluorescence (Mosiman et al., 1997). Flow cytometry measurements are usually presented as shown in Figure 16, where the number of counts is plotted as a function of fluorescence intensity (Macey, 2007). An increase in the fluorescence intensity will cause a shift of the peak to the right, indicating induction of the fluorescent biosensor, while a shift to the left indicates repression (Figure 16B). When the biosensor fluorescence overlaps or is close to the autofluorescence intensity, this indicates repression (Figure 16B). Moreover, overlapping peaks or two or more distinct peaks indicate population heterogeneity (Figure 16C) (Macey, 2007).

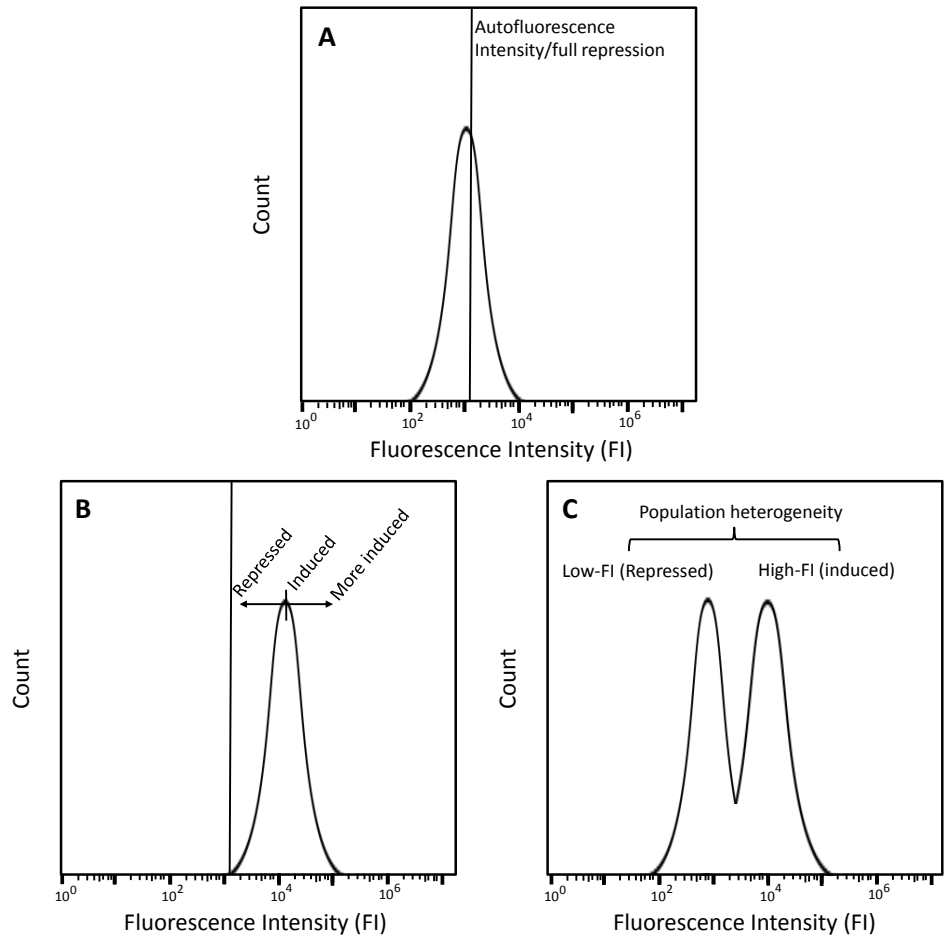

Figure 16. Hypothetical case of a fluorescence biosensor in yeast analysed with flow cytometry to illustrate (A) autofluorescence intensity or repression represented by the vertical line at about 10^3 FI; (B) shows repressed and induced states with the vertical line representing the autofluorescence intensity; and (C) shows population heterogeneity.

4.5. Biosensors for sugar signalling

Fluorescent protein-based biosensors, used as reporter genes, are extremely useful in elucidating the dynamics of signalling networks and transduction processes in living cells in real time (Depry et al., 2013; Mehta & Zhang, 2011). GFP is being used to expand and deepen our knowledge on signalling pathways

in research fields ranging from diseases to biotechnology and biofuels (Depry et al., 2013; Newman et al., 2011).

GFP-based biosensors have been developed to help understand and improve ethanol fermentation from mixed sugars by *S. cerevisiae*, with particular focus on the challenges associated with D-xylose utilisation. One of the strategies employed was the design of a D-xylose biosensor able to both sense D-xylose and regulate protein expression in its presence (Teo & Chang, 2015; Wang et al., 2016). The circuit consists of transcription factors (XylRs) that bind to D-xylose, a XylR-binding operator sequence from bacteria and a synthetic hybrid promoter coupled to the gene of interest. Its applicability was demonstrated with GFP: in the presence of D-xylose, the sugar bound XylR, which released the gene operator and allowed the transcription of GFP (Figure 17). This D-xylose-monitoring/regulation gene circuit is an advanced tool that can regulate D-xylose consumption and fine-tune the expression of pathway as a function of D-xylose availability (Teo & Chang, 2015; Wang et al., 2016).

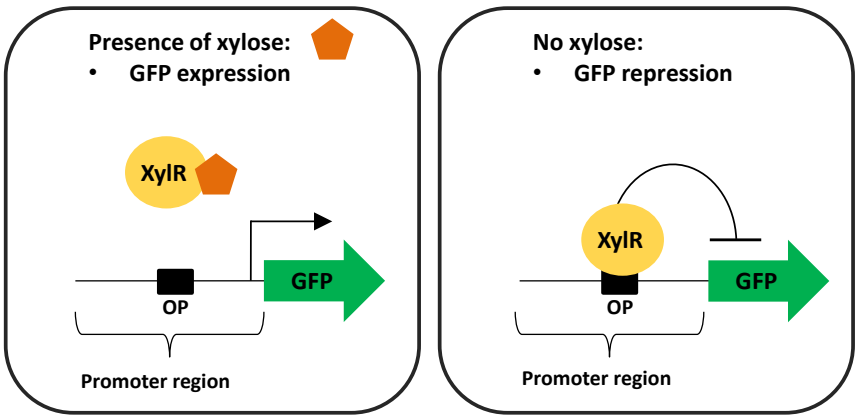

Figure 17. Schematic view of the biosensor circuit designed to control gene expression in the presence of D-xylose. Adapted from Wang et al. (2016).

Our research group has recently developed and validated a non-invasive *in vivo* reporter system in *S cerevisiae* to monitor the three main sugar signalling pathways in response to changes in carbon source (Brink et al., 2016). For this purpose, the yEGFP3 gene was coupled to eight different promoters and chromosomally integrated, generating eight strains in total (named TMB371X), with one biosensor per strain (Brink et al., 2016). The promoters chosen were *HXT1*p/*2*p/*4*p, *TPS1*p/*2*p, *TEF4*p, *SUC2*p, and *CAT8*p, as they represent regulation targets of the three sugar sensing pathways (see Section 3.1 above, Figure 18 and Table 4 below):

- *HXT1*p/*2*p/*4*p are principally regulated by the Snf3p/Rgt2p pathway. *HXT1*p is positively regulated by high D-glucose levels, while *HXT2*p/*4*p are expressed under low D-glucose conditions.
- *TPS1*p/*2*p (trehalose-related genes) and *TEF4*p (translation-related gene) promoters coupled to GFP are biosensors associated with the cAMP/PKA pathway. A high level of trehalose has been observed upon D-glucose depletion and stress, while the decrease in trehalose is triggered by D-glucose abundance (Lillie & Pringle, 1980; Panek & Mattoon, 1977). *TEF4*p, in contrary to *TPS1*p/2p, is regulated by high D-glucose levels and cAMP/PKA activation.
- The *SUC2*p (whose promoter is from the invertase encoding gene) and *CAT8*p (a promoter from an alternative carbon source response activator) biosensors were developed to monitor the regulation of alternative carbon sources than D-glucose via the SNF1/Mig1p pathway, presenting an induction under low D-glucose conditions.

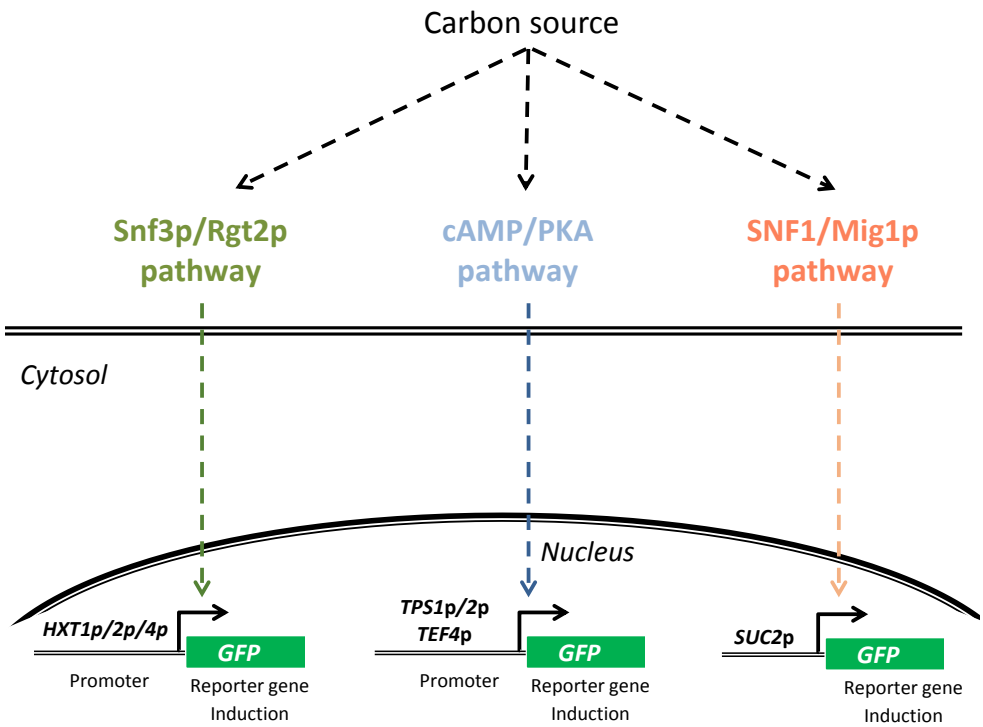

Figure 18. Reporter gene designs previously developed and used to monitor the three main sugar signalling pathways in *S. cerevisiae*.

Table 4. Summary of induction and repression conditions for each biosensor (Extracted and adapted from Brink, D. el al., 2016).

Signalling pathway	Promoter	Induction condition	Repression condition	Reference
Snf3p/Rgt2p	*HXT1* (Low-affinity hexose transporter)	High D-glucose (4% w/v)	Low D-glucose (0.1% w/v)	Özcan and Johnston (1995)
	HXT2 (High-affinity hexose transporter)	Low D-glucose (0.1% w/v)	High D-glucose (4% w/v)	Özcan and Johnston (1995)
	HXT4 (High-affinity hexose transporter)	Low D-glucose (0.1% w/v)	High D-glucose (4% w/v)	Özcan et al. (1996); Özcan and Johnston (1995)
SNF1/Mig1p	*SUC2* (Invertase)	Low D-glucose (0.1% w/v)	High D-glucose (2% w/v) and depleted glucose (0% w/v)	Özcan et al. (1997)
	CAT8 (Alternative carbon source response activator)	Low D-glucose (0.2% w/v)	High glucose (4% w/v)	(Hedges et al., 1995)
cAMP/PKA	*TPS1* (Trehalose-6-phosphate synthase (56 kDa subunit))	Glucose limitation, stress conditions (e.g. heat, nutrient starvation, oxidative stress)	High D-glucose; however, a basal expression level has been observed when growing on rapidly fermentable sugars	Parrou et al. (1999); Winderickx et al. (1996)
	TPS2 (Trehalose-6-phosphate synthase (102.8 kDa subunit))			
	TEF4 (Translation elongation factor)	-	Stress conditions	Grousl et al. (2013); Olarewaju et al. (2004)

Interconnection and cross-talk between pathways could not be avoided when using these promoters (see Chapter 3, Figure 10). For example, the SNF1/Mig1p and cAMP/PKA pathways also influence *HXT1*p/*2*p/*4*p via Mig1p/2p and PKA, respectively. Although this increased the complexity of the analysis and interpretation of the data, it also increased the robustness of the study, as different biosensors converged into a similar signalling pattern (Brink et al., 2016). These biosensors, and derivatives of them, have also been utilised to investigate the response of *S. cerevisiae* to the presence of D-xylose (**Papers I-III**), as described in the next chapter.

5. D-Xylose Sensing by *S. cerevisiae*

5.1. Fermented but not a fermentable sugar

D-glucose is the preferred sugar by *S. cerevisiae*, and will be metabolised first, while other carbon sources will be consumed only when the D-glucose is depleted (Lewis et al., 1993). This phenomenon is called carbon catabolite repression, and is the result of the regulation of the sugar signalling and sensing pathways by the D-glucose concentration (Gancedo, 1998). For instance, high D-glucose concentration will repress galactose and sucrose consumption through the SNF1/Mig1p signalling pathway (Belinchón & Gancedo, 2007; Johnston, 1987).

The implementation of the capacity of *S. cerevisiae* to ferment D-xylose has mostly focused on careful optimization of all the steps that enable D-xylose to be channelled into glycolytic intermediates (see Chapter 2). In other words, there are now efficient routes for D-xylose assimilation. However, D-xylose can still not be assimilated simultaneously with D-glucose, despite the development of specific D-xylose transporters (Section 2.1.3). Moreover, the maximum specific growth rate on D-xylose is still significantly lower than on D-glucose: 0.22 h^{-1} for the best strains (Kuyper et al., 2005) *vs*. 0.3-0.4 h^{-1} on D-glucose under anaerobic conditions (Nissen et al., 2000; Papapetridis et al., 2018). However, only a few studies so far have described a possible link between D-xylose and CCR and, more generally, sugar signalling.

The identification of genes that are typically expressed on non-fermentable carbon sources (i.e. *SUC2, HXK1, HXT5, HXT13, ACS1, MAL11, MAL31,* and *MAL32*) along with the activation of gluconeogenesis, respiration, TCA and glyoxylate cycle in D-xylose-grown cells indicate that *S. cerevisiae* does not recognise D-xylose as a fermentable sugar (Table 5) (Matsushika et al., 2014; Runquist et al., 2009; Salusjärvi et al., 2008; Zeng et al., 2016). The high activity of TCA cycle induced by D-xylose could be one of the reasons behind

of low ethanol production, since 2 carbons are lost as CO_2 (Zeng et al., 2016). These transcriptome, proteome and metabolome studies have shown that the response of *S. cerevisiae* to D-xylose is very similar to its response to carbon starvation (Bergdahl et al., 2012; Klimacek et al., 2010; Matsushika et al., 2013; Salusjärvi et al., 2006). Furthermore, proteome results had shown different phosphorylation forms of glycolytic enzymes, such as Hxk2p, Pgk1p, Eno1 and Eno2p, in the presence of D-xylose (Salusjärvi et al., 2008).

Table 5. Genes upregulated or downregulated in the presence of xylose (**MTH1* and *HXT2* were found to be upregulated (Jin, Y. S. et al., 2004 and Salusjärvi. et al., 2008) and downregulated (Zeng, W.Y. et al., 2016)).

	Genes related to:	**References**
Upregulation	Gluconeogenesis	Salusjärvi, L. et al (2008) Runquist, D. et al (2009) Matsushika, A. et al (2014) Zeng, W.Y. et al (2016)
	Genes related to the oxidative PPP	Runquist, D. et al (2009) Zeng, W.Y. et al (2016)
	TCA and glyoxylate cycles	Jin, Y. S. et al (2004) Salusjärvi, L. et al (2008) Matsushika, A. et al (2014) Zeng, W.Y. et al (2016)
	Respiration	Jin, Y. S. et al (2004) Salusjärvi, L. et al (2008) Matsushika, A. et al (2014) Zeng, W.Y. et al (2016)
	Acetaldehyde and acetyl-CoA metabolism	Salusjärvi, L. et al (2008) Zeng, W.Y. et al (2016)
	Genes typically expressed on non-fermentable carbon sources: *SUC2, HXK1*, *HXT5, HXT13,* maltose metabolism genes	Jin, Y. S. et al (2004) Salusjärvi, L. et al (2008) Zeng, W.Y. et al (2016)
	Sugar signalling: *MTH1**, *ADR1, CAT8, RGT1*	Jin, Y. S. et al (2004) Salusjärvi, L. et al (2008) Zeng, W.Y. et al (2016)
	High-affinity transport (e.g. *HXT2*, *HXT6 and HXT7*)	Jin, Y. S. et al (2004) Salusjärvi, L. et al (2008)
Downregulation	Glycolysis	Salusjärvi, L. et al (2008)
	Low-affinity glucose transporter (e.g. *HXT1*, *HXT2 HXT3*)	Jin, Y. S. et al (2004), Zeng, W.Y. et al (2016)
	Sulphur metabolism	Zeng, W.Y. et al (2016)
	Heme biosynthesis from uroporphyrinogen	Zeng, W.Y. et al (2016)
	Tryptophan degradation	Zeng, W.Y. et al (2016)
	Sugar signalling: *MTH1**, *STD1, MIG1, HXK2*	Salusjärvi, L. et al (2008) Zeng, W.Y. et al (2016)

It has also been shown that D-xylose decreases the glucose-phosphorylating activity of Hxk2p that is one of the main regulators of carbon catabolite repression, through an autophosphorylation mechanism (Fernández et al., 1986; Heidrich et al., 1997). An Hxk2p variant, with Phe159Tyr mutation in the active-site could partly overcome the inactivation, leading to higher catalytic activity in the presence of D-xylose; however no positive effect was observed

on D-xylose fermentation, indicating that other elements than Hxk2p were needed to boost D-xylose utilisation (Bergdahl et al., 2013).

Some connection has been suggested between changes in the sugar sensing pathways and the presence of xylose. For instance, increased levels of *SUC2, HXK1,* and *HXT16,* and moderate expression of genes related to the cAMP/PKA pathway were obtained in xylose grown-cells (Salusjärvi et al., 2008). This activation of genes related to the use of alternative carbon source pointed towards an activation of SNF1/Mig1p by xylose. In parallel, a study with Mig1p, that is part of the SNF1/Mig1p pathway (cf. Section 3.1.2) indicated a possible repressing effect of xylose on *SUC2*: deletion of *MIG1* resulted in some derepression of *SUC2* on glucose; however a lower invertase activity (encoded by *SUC2* gene) was obtained when the strain was cultivated in a mixture of xylose and low glucose, as compared to low glucose only (Roca et al., 2004).

Since xylose is not recognised as a non-fermentable carbon by *S. cerevisiae*, the high respiratory metabolism and the induction of stress response (e.g. Msn2p/4p) (Matsushika et al., 2014) may also increase the production of ROS and affect cell fitness. This may partly explain why mutations on *IRA2, ISU1,* and *HOG1* genes were found to be beneficial (Sato et al., 2016). For instance, high PKA activity driven by *IRA2* deletion leads to the expression of glycolytic enzymes and transporter but also to the inhibition of Msn2p/4p (c.f. 3.1.1, Table 2). It is however difficult to get an overall picture from individual mutants and understanding of how xylose influences the sugar signalling pathways is still at its infancy.

5.2. Design of biosensors for D-xylose sensing

The biosensor panel strains described in the previous chapter are useful in assessing the sugar signalling response of *S. cerevisiae* to different carbon sources, including D-xylose. However, as this panel (based on TMB371X) lacked the capacity to metabolise D-xylose, derivative strains were first constructed to map the sensing pattern of D-xylose when this pentose sugar was only internalised (strains TMB372X) or fully metabolised (strains TMB375X) (Table 6) (**Paper I**).

The next set of strains was made by deleting *IRA2, ISU1* and/or *HOG1* genes in the C5-biosensors TMB375X because these deletions, that are not directly related to xylose catabolism, led to enhanced xylose rate aerobically and anaerobically in a XI strain background (dos Santos et al., 2016; Sato et al.,

2016). Thus, it became possible to check whether the improvement in xylose utilisation was related to sensing and/or to other factors such as the strain background or the type of xylose pathway that was used (**Paper II**). Different deletion combinations were performed, generating single, double and triple deletions and it was found that the strain with the highest specific rates for xylose consumption and ethanol production was combining deletions of *IRA2* and *ISU1* (TMB379X in Table 6) (**Paper II**).

In the last construction step, *PGI1* gene encoding the enzyme involved in the conversion of glucose-6-phosphate to fructose-6-phosphate (Figure 1) was deleted in the C5-biosensors TMB375X, resulting in strains TMB390X (Table 6). This was performed to investigate whether glycolytic intermediates generated from xylose could trigger an intracellular sensing response (**Paper III**). For instance, if either G6P or T6P were major or exclusive metabolite responsible for regulating some of the sugar signalling routes, *PGI1* deletion could give a different biosensor response than the background strain.

Table 6. Different engineering approaches applied to biosensor strains to obtain a broad view of the response of sugar signalling pathways to the presence of D-xylose

Strain	Genetic modification	D-xylose analysis	References
TMB371X	• Biosensor: promoter + GFP	Mostly external D-xylose	Brink et al. (2016)
TMB372X	• Biosensor: promoter + GFP • GAL2mut (D-xylose transporter)	External and internal D-xylose	Paper I
TMB375X	• Biosensor: promoter + GFP • GAL2mut (D-xylose transporter) • Overexpression of non-oxidative PPP • 3 copies of D-xylose pathway (XR/XDH)	External, internal, and metabolised D-xylose	Paper I
TMB379X	• Biosensor: promoter + GFP • GAL2mut (D-xylose transporter) • Overexpression of non-oxidative PPP • 3 copies of D-xylose pathway (XR/XDH) • *ira2*Δ *isu1*Δ	External, internal, and efficiently metabolised D-xylose	Paper II
TMB390X	• Biosensor: promoter + GFP • GAL2mut (D-xylose transporter) • Overexpression of non-oxidative PPP • 3 copies of D-xylose pathway (XR/XDH) • *pgi1*Δ	External, internal, and metabolised D-xylose blocking the synthesis of glucose-6-phosphate through D-xylose metabolism	Paper III

Since the biosensors from series TMB371X had been studied and validated previously (Brink et al. 2016), only a few were chosen for further experiments. The first important requirement was to use one biosensor for each signalling

pathway. Secondly, biosensors should have different regulation patterns (i.e. one should be induced by high D-glucose and another by low D-glucose). These requirements led to the selection of biosensor strains with the following promoters: *HXT1*p (induced by high D-glucose), *SUC2*p (induced by low D-glucose) and *TPS1*p (induced by low D-glucose), for further genetic modifications (**Paper II**). In order to further simplify the study described in **Paper III**, only the *HXT1*p and *SUC2*p biosensors were used, since *HXT1*p is also regulated by cAMP/PKA, and could replace *TPS1*p.

5.3. How our biosensor strains enlighten part of the D-xylose paradox?

Brink et al. (2016) have previously shown that non-recombinant strains of *S. cerevisiae* did not give any signalling response to the presence of extracellular xylose, which indicated that xylose was not recognised at the cell membrane as a sugar to be fermented. However, using the set of C5-biosensor strains described in Section 4.5, it was now possible to demonstrate that xylose does trigger a sugar signalling response, although different from its glucose counterpart (**Papers I-III**). A summary of the main findings is discussed below and summarised in Table 7.

Assimilated but non-metabolised xylose results in population heterogeneity (Paper I)

Population heterogeneity was mostly observed in the biosensor strains developed to monitor intracellular xylose by the sole addition of a xylose transporter (Gal2mut). This population heterogeneity was also found in few biosensor strains lacking the transporter (*HXT2*p/*4*p) (Brink et al., 2016) but not in strains having, the xylose pathway (XR/XDH and non-oxidative PPP genes) in addition to the transporter (**Paper I**). The use of single-cell analysis based on flow cytometry was fundamental in visualizing this phenomenon that might result from difference in the background expression of the yeast endogenous xylose metabolism (Toivari et al., 2004) and/or difference in intracellular xylose levels between cells.

High levels of xylose trigger a similar signalling response as low levels of glucose (Paper I)

Using biosensor strains capable of assimilating D-xylose, it was possible to show that a high D-xylose level triggers a similar response in sugar signalling as a low D-glucose level. This was observed for the three signalling pathways

(**Paper I**): the high D-xylose condition only induced the expression of high-affinity hexose transporters (*HXT2*p/*4*p biosensors), which are regulated by Snf3p/Rgt2p pathway and known to be induced on low level of D-glucose. In addition, *HXT1* (encoding low affinity transporter) was repressed whereas it is known to be induced on high D-glucose levels (cf. section 4.5; Table 4) (Brink et al., 2016, **Paper I**) . This was in agreement with previous transcriptomics studies on C5 strains where *HXT1* was not induced by the presence of D-xylose (Zeng et al., 2016), while *HXT2* was both upregulated (Salusjärvi et al., 2008) and down-regulated (Zeng et al., 2016) under D-xylose conditions. The induction of the *SUC2*p biosensor in the presence of a high D-xylose level also emphasized the low D-glucose signal obtained from the C5 biosensor strains. D-xylose also led to the induction of *TPS1*p/*2*p in the same manner as it was observed for low D-glucose. Expression of trehalose-related genes was indeed observed in the analysis of a transcriptome data from an industrial and laboratory *S. cerevisiae* strains when cultivated on D-xylose (Matsushika et al., 2014).

The response to low D-glucose, also observed for high D-xylose, led to the hypothesis of a low PKA activity in xylose-metabolising strains; active PKA is known to induce *HXT1*p (Jouandot et al., 2011; Kim & Johnston, 2006), and repress *SUC2*p (Gancedo et al., 2015) and *TPS1*p/*2*p (Boy-Marcotte et al., 1998) which is the completely opposite signal from the one observed on high D-xylose and low D-glucose (Figure 19).

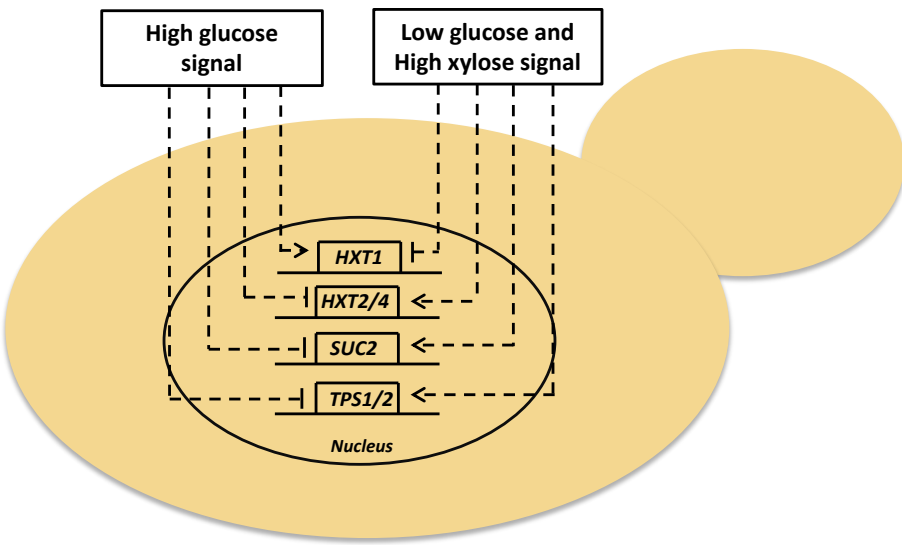

Figure 19. Biosensor regulation during high and low D-glucose and high D-xylose signals in *S. cerevisiae*. Arrows indicate gene induction whereas a vertical line indicates gene repression.

Table 7. Response to the presence of high D-xylose (50 g-L^{-1}) for the biosensors strains (*HXT1*p/*2*p/*4*p, *TPS1*p/*2*p, *TEF4*p and *SUC2*p) with and without additional genetic modifications: D-xylose transporter (Gal2mut), PPP genes, Oxido-reductive xylose pathway (XXX), deletion of *IRA2* and *ISU1* genes (*ira2*Δ*isu1*Δ), and deletion of phosphoglucose isomerase gene (*pgi1*Δ). Blue columns: biosensor strains constructed to analyse the response to extracellular, transporter and metabolised D-xylose. Red column: biosensor strains constructed to study the influence of disturbance in the level of glycolytic intermediates. Green line: summary from each column. *SP: subpopulation

				High D-xylose				
				TMB371X Brink et al. 2016	**TMB372X** Paper I	**TMB375X** Paper I	**TMB379X** Paper II	**TMB390X** Paper III
				Analysing what is the signalling from a xylose condition				Mechanism behind of xylose signalling
				Biosensor	**Biosensor** **Gal2mut**	**Biosensor** **Gal2mut** **PPP** **XXX**	**Biosensor** **Gal2mut** **PPP** **XXX** ***ira2*Δ*isu1*Δ**	**Biosensor** **Gal2mut** **PPP** **XXX** ***pgi1*Δ**
	Induction condition	**Repression condition**	**Biosensor strain**	**Extracellular xylose**	**Intracellular xylose**	**Metabolised xylose**	**Efficiently metabolised xylose**	**Cannot produce G6P**
Snf3p/Rgt2p pathway	**Glucose 40 g/L (6 h)**	**Glucose 5 g/L (14 h)**	***TMB37X2* (*HXT1*p)**	Repression	Repression	Repression	Induction	Repression
	Glucose 1 g/L (6 h)	**Glucose 40 g/L (14 h)**	***TMB37X3* (*HXT2*p)**	Repression	SP* High-FI peak partially induced	Induction	No data	No data
	Glucose 1 g/L (6 h)	**Glucose 40 g/L (14 h)**	***TMB37X4* (*HXT4*p)**	Repression	SP High-FI peak partially induced	Induction	No data	No data
cAMP/PKA pathway	**Glucose 1 g/L (6 h)**	**Glucose 40 g/L (14 h)**	***TMB37X7* (*TPS1*p)**	Repression	SP with high-FI peak repressed	Induction	Repression	No data
	Glucose 1 g/L (6 h)	**Glucose 40 g/L (14 h)**	***TMB37X8* (*TPS2*p)**	Repression	SP with high-FI peak repressed	Induction	No data	No data
	Glucose 40 g/L (6 h)	**Ethanol 3%**	***TMB37X9* (*TEF4*p)**	Repression	Repression	Induction	No data	No data
SNF1/Mig1p Pathway	**Glucose 1 g/L (6 h)**	**Glucose 40 g/L (14 h)**	***TMB37X5* (*SUC2*p)**	Repression	Repression	Induction	SP with high-FI peak induced and predominant	Induction
Summary of all the biosensors signalling from each set of engineered strain (e.g. TMB371X, TMB372X, TMB375X, etc.)				**No signal**	**Limited and heterogeneous signal**	**Low D-glucose signal**	**High and low D-glucose signal**	**Role of metabolites upstream and downstream of Pgi1p in xylose signalling**

Constitutive activation of PKA by *IRA2* deletion could explain the increased D-xylose specific uptake rate (Paper II)

Deletion of the *IRA2* gene in the C5 biosensor strains led to improved D-xylose consumption rate; in parallel, the xylose signal changed from a *low D-glucose*-like to a *high D-glucose*-like signal (**Paper II**). The change was observed in *ira2*Δ and *ira2*Δ*isu1*Δ strains with induction of *HXT1*p and repression of *TPS1*p, a typical feature of a *high D-glucose* signal. As *IRA2* deletion/inactivation leads to constitutive activation of PKA (Colombo et al., 2004; Peeters et al., 2006; Robinson et al., 1968), these results confirmed the hypothesis that D-xylose conditions led to a low PKA activity in the C5 biosensor strains. However, a *glucose*-like signal was also observed with *SUC2*p biosensor. Therefore, both *high* and *low D-glucose* like signals co-existed in the *ira2*Δ*isu1*Δ strain on D-xylose. It also highlighted that the D-xylose paradox is indeed related to sensing and signalling issues (Table 7).

Sato et al. (2016) reported that the triple deletion (*ira2*Δ*isu1*Δ*hog1*Δ) in a BY4741 strain carrying the XI pathway was the most efficient for anaerobic and aerobic D-xylose consumption, whereas in the present study a W303 strain with the double deletion of *ira2*Δ*isu1*Δ with the XR/XDH pathway showed the best rates of D-xylose consumption and ethanol production. Therefore, the improvement in the specific rates of D-xylose consumption and ethanol production was also dependent on the strain background and/or the type of D-xylose pathway used for engineering, and not only to sugar signalling.

The generation of the artificial *high D-glucose* signal by *IRA2* deletion results in the overexpression of transporters that can take up D-xylose; it also fakes a status of glucose abundance that probably leads to an increase in glycolytic flux and a reduction in the stress response in the presence of D-xylose. However, deletions of *IRA2* and *ISU1* also affect the cell growth, probably as a result of ATP depletion, leading to low volumetric ethanol productivity, which is not suitable for industrial applications.

Glycolytic intermediates are suspected to trigger the xylose signalling response (Paper III)

Since extracellular D-xylose could not interfere with signalling through the membrane sensors (Brink et al., 2016, **Paper I**), one or several intracellular metabolites should be responsible for inducing the observed sugar signalling on high D-xylose. Also, low D-xylose levels (5 $g.L^{-1}$ xylose) (**Paper I**) presented a signalling in between the low D-glucose and the starvation signals, indicating an essential role of the flux or level of this/these intracellular metabolite(s). G6P, F1,6bP and T6P are interesting candidates because they are already known to

affect glucose signalling (cf. Section 3.2). All of them can be generated from D-xylose that is first converted to D-xylulose that enters the PPP to join glycolysis at the level of fructose-6-phosphate and glyceraldehyde-3-phosphate (Figure 1). G6P and T6P can be generated from fructose-6-phosphate via the *PGI1*-encoded phosphoglucose isomerase, because Pgi1p is a bidirectional enzyme that is functional under both glycolysis and gluconeogenesis. Indeed, G6P was detected in D-xylose-grown cells, although in low concentrations (Belinchón & Gancedo, 2003). Glycogen and trehalose accumulation in D-xylose-fermenting *S. cerevisiae* strains have also confirmed the presence of a gluconeogenic flux on D-xylose (Jin et al., 2004; Portugal-Nunes et al., 2017; Runquist et al., 2009).

When *PGI1* gene was deleted in the C5 biosensor strains, significant changes were observed in the sugar signalling response to a range of carbon source entering glycolysis upstream and downstream of Pgip (**Paper III**):

- The *HXT1*p biosensor was only induced under conditions where G6P level was expected to be sufficiently high, i.e. with 20 $g.L^{-1}$ glucose in the background strain or with 1 $g.L^{-1}$ glucose with the deletion of *PGI1*. However, too high G6P levels that were foreseen when combining high D-glucose and *PGI1* deletion, led to a reduction of the signal. This would indicate that the level of G6P exerts a key role in the regulation of *HXT1* induction via the Ras complex.
- The mechanism behind derepression of the *SUC2*p biosensor was more complex to comprehend. If G6P was the major or exclusive metabolite responsible for triggering the high D-xylose signal, deletion of *PGI1* would prevent signalling from the *SUC2*p biosensor, since xylose would not be able to undergo gluconeogenesis and produce G6P. However, high D-xylose conditions continued to give a low D-glucose signal when the *SUC2*p biosensor was evaluated in the *PGI1* deletant, redirecting attention to other factors, e.g. F1,6bP. From the overall results on different sugars (**Paper III**), it was hypothesised that a combination of factors must be involved: level of G6P but also the activation/inactivation of Hxk2p and/or the level of glycolytic intermediates downstream of G6P.

Education does not change the world.
Education changes people.
People change the world.

Paulo Freire

6. Outlook

Recombinant C5 strains of *S. cerevisiae* can internalise, metabolise and ferment D-xylose to ethanol, but the rate of D-xylose consumption is still considerably lower than that of D-glucose. Using non-invasive biosensors, the present thesis demonstrated a strong connection between the low rate of D-xylose consumption and the abnormal sugar sensing and signalling observed on D-xylose. It was found that, despite the fact that the yeast is not able to recognise D-xylose as a fermentable sugar, D-xylose does trigger a sugar sensing response. The response was only observed when D-xylose was internalised, indicating that extracellular sugar sensors were not activated. In addition, the response, which was only triggered by high levels of D-xylose, was similar to the cell response to low levels of glucose, explaining the respiratory phenotype previously observed for C5 strains.

The *low D-glucose*-like signal could be changed to a *high D-glucose*-like signal on D-xylose by artificially boosting the PKA activity through *IRA2* deletion. However, a complete deregulation of PKA may not be considered for industrial applications, because it results in lower biomass formation and reduces the resistance to stress (Li et al., 2018b; Park et al., 2005). Moreover, the change in signal was only observed in biosensors connected to the cAMP/PKA pathway.

In the search for intracellular factors that could explain the signal observed for high D-xylose, it was found that the level of G6P could play a major role on the induction/repression of low-affinity hexose transporter genes via the Ras complex. However, experiments with the *SUC2*p indicated that it was not the only factor and some glycolytic intermediates downstream of G6P could be involved. Therefore, quantification of intracellular glycolytic intermediate should be performed on different carbon sources and in strains having or not *PGI1* deletion. This would increase our understanding of the intracellular regulatory factors that are connected to sugar sensing/signalling and help manipulating the cell metabolism to boost D-xylose uptake.

In the present work, the constructed non-invasive biosensors were essential in drawing the above conclusions about sugar signalling. However, a large number of strains had to be constructed and evaluated. In order to simplify future work,

biosensors for the three sensing routes could be implemented into one single strain, by using fluorescent proteins with different excitation/emission range for each sensor. This three-colour biosensor system would be useful to monitor the three sensing pathways simultaneously and to screen for populations with a specific signalling pattern. For example, it could be used as a tool to identify and sort out populations with the aid of a fluorescence-activated cell sorting equipment to find a signal close to "*high D-glucose* signal" in the presence of D-xylose.

A major, and yet not exploited, advantage of the biosensor strains is that it opens the possibility for online monitoring of sugar signalling and sensing, for instance, using a flow cytometer coupled to a bioreactor. In combination with a three-colour biosensor, this represents a very powerful tool to follow the sensing response of the cells to various pulses and conditions in real time.

To conclude, this thesis provided insights about the cell response to D-xylose and gave possible tracks to increase the ability of *S. cerevisiae* to take up D-xylose. It also highlighted the potential of biosensors to follow and decipher complex physiological responses in microbial systems.

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References

Aabo, T., Glückstad, J., Siegumfeldt, H., Arneborg, N. 2011. Intracellular pH distribution as a cell health indicator in *Saccharomyces cerevisiae*. *Journal of the Royal Society Interface*, **8**(64), 1635-1643.

Adan, A., Alizada, G., Kiraz, Y., Baran, Y., Nalbant, A. 2017. Flow cytometry: basic principles and applications. *Critical Reviews in Biotechnology*, **37**(1), 163-176.

Ahuatzi, D., Riera, A., Peláez, R., Herrero, P., Moreno, F. 2007. Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. *Journal of Biological Chemistry*, **282**(7), 4485-4493.

Albertyn, J., Hohmann, S., Thevelein, J.M., Prior, B.A. 1994. *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Molecular and Cellular Biology*, **14**(6), 4135-4144.

Almeida, J.R.M., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G., Gorwa-Grauslund, M.F. 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *Journal of Chemical Technology and Biotechnology*, **82**(4), 340-349.

Ameen, S., Ahmad, M., Mohsin, M., Qureshi, M.I., Ibrahim, M.M., Abdin, M.Z., Ahmad, A. 2016. Designing, construction and characterization of genetically encoded FRET-based nanosensor for real time monitoring of lysine flux in living cells. *Journal of Nanobiotechnology*, **14**(1), 49.

Amore, R., Kötter, P., Küster, C., Ciriacy, M., Hollenberg, C.P. 1991. Cloning and expression in *Saccharomyces cerevisiae* of the NAD(P)H-dependent xylose reductase-encoding gene (*XYL1*) from the xylose-assimilating yeast *Pichia stipitis*. *Gene*, **109**(1), 89-97.

Amore, R., Wilhelm, M., Hollenberg, C.P. 1989. The fermentation of xylose - an analysis of the expression of *Bacillus* and *Actinoplanes* xylose isomerase genes in yeast. *Applied Microbiology and Biotechnology*, **30**, 351-357.

Apel, A.R., Ouellet, M., Szmidt-Middleton, H., Keasling, J.D., Mukhopadhyay, A. 2016. Evolved hexose transporter enhances xylose uptake and glucose/xylose co-utilization in *Saccharomyces cerevisiae*. *Scientific Reports*, **6**(1), 19512.

Ashrafi, K., Lin, S.S., Mancheste, J.K., Gordon, J.I. 2000. Sip2p and its partner Snf1p kinase affect aging in *S. cerevisiae*. *Genes & Development*, **14**, 1872–1885.

Aubin, J.E. 1979. Autofluorescence of viable cultured mammalian cells. *Journal of Histochemistry & Cytochemistry*, **27**(1), 36-43.

Avery, S.V. 2006. Microbial cell individuality and the underlying sources of heterogeneity. *Nature Reviews Microbiology*, **4**(8), 577-587.

Ayer, A., Gourlay, C.W., Dawes, I.W. 2014. Cellular redox homeostasis, reactive oxygen species and replicative ageing in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, **13**, 60–72.

Babazadeh, R., Furukawa, T., Hohmann, S., Furukawa, K. 2014. Rewiring yeast osmostress signalling through the MAPK network reveals essential and non-essential roles of Hog1 in osmoadaptation. *Scientific Reports*, **4**, 4697.

Babu, P., Deschenes, R.J., Robinson, L.C. 2004. Akr1p-dependent palmitoylation of Yck2p yeast casein kinase 1 is necessary and sufficient for plasma membrane targeting. *Journal of Biological Chemistry*, **279**, 27138–27147.

Bamba, T., Hasunuma, T., Kondo, A. 2016. Disruption of *PHO13* improves ethanol production via the xylose isomerase pathway. *AMB Express*, **6**(4).

Barnett, J.A. 1976. The utilization of sugars by yeasts. *Advances in Carbohydrate Chemistry and Biochemistry*, **32**, 125-234.

Batt, C.A., Caryallo, S., Easson, D.D., Akedo, M., Sinskey, A.J. 1986. Direct evidence for a xylose metabolic pathway in *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering*, **28**(4), 549-53.

Belinchón, M.M., Gancedo, J.M. 2007. Glucose controls multiple processes in *Saccharomyces cerevisiae* through diverse combinations of signaling pathways. *FEMS Yeast Research*, **7**(6), 808-818.

Belinchón, M.M., Gancedo, J.M. 2003. Xylose and some non-sugar carbon sources cause catabolite repression in *Saccharomyces cerevisiae*. *Archives of Microbiology*, **180**(4), 293-297.

Bengtsson, O., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F. 2009. Xylose reductase from *Pichia stipitis* with altered coenzyme preference improves ethanolic xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Biotechnology for Biofuels*, **2**(9).

Bengtsson, O., Jeppsson, M., M., S., Parachin, N.S., Uwe Sauer, U., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F. 2008. Identification of common traits in improved xylose-growing *Saccharomyces cerevisiae* for inverse metabolic engineering. *Yeast*, **25**, 835–847.

Bergdahl, B., Heer, D., Sauer, U. 2012. Dynamic metabolomics differentiates between carbon and energy starvation in recombinant *Saccharomyces cerevisiae* fermenting xylose. *Biotechnology for Biofuels*, **5**(34).

Bergdahl, B., Sandström, A.G., Borgström, C., Boonyawan, T., van Niel, E.W.J., Gorwa-Grauslund, M.F. 2013. Engineering yeast hexokinase 2 for improved tolerance toward xylose-induced inactivation. *PLOS One*, **8**(9).

Bhattacharya, S., Chen, L., Broach, J.R., Powers, S. 1995. Ras membrane targeting is essential for glucose signaling but not for viability in yeast. *Cell Biology*, **92**(7), 2984-2988.

Bisson, L.F., Kunathigan, V. 2003. On the trail of an elusive flux sensor. *Research in Microbiology*, **154**, 603–610.

Boerjan, W., Ralph, J., Baucher, M. 2003. Lignin biosynthesis. *Annual Review of Plant Biology*, **54**, 519-546.

Bond, S., Forgac, M. 2008. The Ras/cAMP/protein kinase A pathway regulates glucose-dependent assembly of the vacuolar (H+)-ATPase in yeast. *Journal of Biological Chemistry*, **283**(52), 36513-36521.

Boy-Marcotte, E., Perrot, M., F., B., Boucherie, H., Jacquet, M. 1998. Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in *Saccharomyces cerevisiae*. *Journal of Bacteriology*, **180**, 1044–1052.

Bracher, J.M., Martinez-Rodriguez, O.A., Dekker, W.J.C., Verhoeven, M.D., van Maris, A.J.A., Pronk, J.T. 2018. Reassessment of requirements for anaerobic xylose fermentation by engineered, non-evolved *Saccharomyces cerevisiae* strains. *FEMS Yeast Research*, **19**(1).

Brat, D., Boles, E., Wiedemann, B. 2009. Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, **75**, 2304-2311.

Brewster, J.L., Gustin, M.C. 2014. Hog1: 20 years of discovery and impact. *Cell Biology*, **7**(343).

Brink, D.P., Borgström, C., Tueros, F.G., Gorwa-Grauslund, M.F. 2016. Real-time monitoring of the sugar sensing in *Saccharomyces cerevisiae* indicates endogenous mechanisms for xylose signaling. *Microbial Cell Factories*, **15**(183).

Broek, D., Toda, T., Michaeli, T., Levin, L., Birchmeier, C. 1987. The *S. cerevisiae* CDC25 gene product regulates the RAS/adenylate cyclase pathway. *Cell*, **48**(5), 789-99.

Bromley, S. 2006. Blood for oil? *New Political Economy*, **11**, 419-434.

Cadete, R.M., de las Heras, A.M., Sandström, A.G., Ferreira, C., Gírio, F., Gorwa-Grauslund, M.F., Rosa, C.A., Fonseca, C. 2016. Exploring xylose metabolism in *Spathaspora* species: *XYL1.2* from *Spathaspora passalidarum* as the key for efficient anaerobic xylose fermentation in metabolic engineered *Saccharomyces cerevisiae*. *Biotechnology for Biofuels*, **9**(1), 167.

Cai, Z., Zhang, B., Li, Y. 2012. Engineering *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: reflections and perspectives. *Biotechnology Journal*, **7**(1), 34-46.

Carlquist, M., Fernandes, R.L., Helmark, S., Heins, A.L., Lundin, L., Sørensen, S.J., Gernaey, K.V., Lantz, A.E. 2012. Physiological heterogeneities in microbial populations and implications for physical stress tolerance. *Microbial Cell Factories*, **11**(94).

Carus, M., Dammer, L. 2018. The Circular Bioeconomy—Concepts, Opportunities, and Limitations. *Industrial Biotechnology* **14**(2).

Castermans, D., Somers, I., Kriel, J., Louwet, W., Wera, S., Versele, M., Janssens, V., Thevelein, J.M. 2012. Glucose-induced posttranslational activation of protein phosphatases PP2A and PP1 in yeast. *Cell Research*, **22**(6), 1058-1077.

Chalfie, M. 2009. GFP: lighting up life (Nobel Lecture). *Angewandte Chemie International Edition*, **48**, 5603 – 5611.

Chambers, P., Issaka, A., Palecek, S.P. 2004. *Saccharomyces cerevisiae JEN1* promoter activity is inversely related to concentration of repressing sugar. *Applied and Environmental Microbiology*, **70**, 8–17.

Chandrashekarappa, D.G., McCartney, R.R., O'Donnell, A.F., Schmidt, M.C. 2016. The β subunit of yeast AMP-activated protein kinase directs substrate specificity in response to alkaline stress. *Cellular Signalling*, **28**(12), 1881-1893.

Chang, Q., Griest, T.A., Harter, T.M., Petrash, J.M. 2007. Functional studies of aldo-keto reductases in *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta*, **1773**(3), 321-329.

Chu, B., Lee, H. 2007. Genetic improvement of *Saccharomyces cerevisiae* for xylose fermentation. *Biotechnology Advances*, **25**(5), 425-441.

Chundawat, S.P.S., Beckham, G.T., Himmel, M.E., Dale, B.E. 2011. Deconstruction of lignocellulosic biomass to fuels and chemicals. *Annual Review of Chemical and Biomolecular Engineering*, **2**(1), 121-145.

Colombo, S., Ronchetti, D., Thevelein, J.M., Winderickx, J., Martegani, E. 2004. Activation state of the Ras2 protein and glucose-induced signaling in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, **279**(45), 46715-46722.

Cook, W.H., Lipkin, D., Markham, R. 1957. The formation of a cyclic dianhydrodiadenylic acid (I) by the alkaline degradation of adenosine-5'-triphosphoric acid (II) *Journal of the American Chemical Society*, **79**(13), 3607–3608.

Cormack, B.P., Bertram, G., Egerton, M., Gow, N.A.R., Falkow, S., Brown, A.J.P. 1997. Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in *Candida albicans*. *Microbiology*, **143**(2), 303-311.

D'Amato, D., Droste, N., Allen, B., Kettunen, M., Lähtinen, K., Korhonen, J., Leskinen, P., Matthies, B.D., Toppinen, A. 2017. Green, circular, bioeconomy: A comparative analysis of sustainability avenues. *Journal of Cleaner Production*, **168**, 716-734.

Dahms, S.A. 1974. 3-Deoxy-D-pentulosonic acid aldolase and its role in a new pathway of D-xylose degradation. *Biochemical and Biophysical Research Communications*, **60**(4), 1433-1439.

Dardalhon, M., Kumar, C., Iraqui, I., Vernis, L., Kienda, G., Banach-Latapy, A., He, T., Chanet, R., Faye, G., Outten, C.E., Huang, M.E. 2012. Redox-sensitive YFP sensors monitor dynamic nuclear and cytosolic glutathione redox changes. *Free Radical Biology and Medicine*, **52**, 2254–2265.

de la Cera, T., Herrero, P., Moreno-Herrero, F., Chaves, R.S., Moreno, F. 2002. Mediator factor Med8p interacts with the hexokinase 2: implication in the glucose signalling pathway of *Saccharomyces cerevisiae*. *Journal of Molecular Biology*, **319**(3), 703-714.

Demirbas, M.F. 2009. Biorefineries for biofuel upgrading: a critical review. *Applied Energy*, **86**, S151–S161.

Depry, C., Mehta, S., Zhang, J. 2013. Multiplexed visualization of dynamic signaling networks using genetically encoded fluorescent protein-based biosensors. *European Journal of Physiology*, **465**, 373–381.

Deroover, S., Ghillebert, R., Broeckx, T., Winderickx, J., Rolland, F. 2016. Trehalose-6-phosphate synthesis controls yeast gluconeogenesis downstream and independent of SNF1. *FEMS Yeast Research*, **16**(4).

Diderich, J.A., Schepper, M., van Hoek, P., Luttik, M.A., van Dijken, J.P., Pronk, J.T., Klaassen, P., Boelens, H.F., de Mattos, M.J., van Dam, K., Kruckeberg, A.L. 1999. Glucose uptake kinetics and transcription of *HXT* genes in chemostat cultures of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, **274**(22), 15350-15359.

Dinér, P., Veide, V.J., Kjellén, J., Migdal, I., Andersson, T., Gebbia, M., Giaever, G., Nislow, C., Hohmann, S., Wysocki, R., Tamás, M.J., Grøtli, M. 2011. Design, synthesis, and characterization of a highly effective Hog1 inhibitor: a powerful tool for analyzing MAP kinase signaling in yeast. *PLOS One*, **6**(5).

Donner, J.S., Thompson, S.A., Kreuzer, M.P., Baffou, G. 2012. Mapping intracellular temperature using green fluorescent protein. *Nano Letters*, **12**, 2107−2111.

dos Santos, L., Carazzolle, M., Nagamatsu, S., Sampaio, N., Almeida, L., Pirolla, R., Borelli, G., Corrêa, T., Argueso, J., Pereira, G. 2016. Unraveling the genetic basis of xylose consumption in engineered *Saccharomyces cerevisiae* strains. *Scientific Reports*, **6**(38676).

Escoté, X., Zapater, M., Clotet, J., Posas, F. 2004. Hog1 mediates cell-cycle arrest in G1 phase by the dual targeting of Sic1. *Nature Cell Biology*, **6**(10).

Fabrizio, P., Liou, L.L., Moy, V.N., Diaspro, A., Valentine, J.S., Gralla, E.B., Longo, V.D. 2003. *SOD2* functions downstream of Sch9 to extend longevity in yeast. *Genetics* **163**, 35–46.

Farwick, A., Bruder, S., Schadeweg, V., Oreb, M., Boles, E. 2014. Engineering of yeast hexose transporters to transport D-xylose without inhibition by D-glucose. *PNAS*, **111**(14), 5159-5164.

Farzad, S., Mandegari, M.A., Guo, M., Haigh, K.F., Shah, N., Görgens, J.F. 2017. Multi-product biorefineries from lignocelluloses: a pathway to revitalisation of the sugar industry? *Biotechnology for Biofuels*, **10**(87).

Feng, X., Zhao, H. 2013. Investigating glucose and xylose metabolism in *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* via 13C metabolic flux analysis. *AIChE Journal*, **59**(9), 3195-3202.

Ferguson, S.B., Anderson, E.S., Harshaw, R.B., Thate, T., Craig, N.L., Nelson, H.C. 2005. Protein kinase A regulates constitutive expression of small heat-shock genes in an Msn2/4p-independent and Hsf1p-dependent manner in *Saccharomyces cerevisiae*. *Genetics*, **169**(3), 1203-1214.

Fernandes, B.M., Welch, C.A., Gonçalves, E.C. 2010. Agrofuel policies in Brazil: paradigmatic and territorial disputes. *The Journal of Peasant Studies*, **37**, 793–819.

Fernandes, R.L., Nierychlo, M., Lundin, L., Pedersen, A.E., Puentes Tellez, P.E., Dutta, A., Carlquist, M., Bolic, A., Schäpper, D., Brunetti, A.C., Helmark, S., Heins,

A.L.L., Jensen, A.D., Nopens, I., Rottwitt, K., Szita, N., van Elsas, J.D., Nielsen, P.H., Martinussen, J., Sørensen, S.J., Lantz, A.E., Gernaey, K.V. 2011. Experimental methods and modeling techniques for description of cell population heterogeneity. *Biotechnology Advances*, **29**(6), 575-599.

Fernández-García, P., Peláez, R., Herrero, P., Moreno, F. 2012. Phosphorylation of yeast hexokinase 2 regulates its nucleocytoplasmic shuttling. *Journal of Biological Chemistry*, **287**(50), 42151-42164.

Fernández, R., Herrero, P., Fernández, M.T., Moreno, F. 1986. Mechanism of inactivation of hexokinase PII of *Saccharomyces cerevisiae* by D-xylose. *Journal of General Microbiology*, **132**(12), 3467-3472.

Fernando, S., Adhikari, S., Chandrapal, C., Murali, N. 2006. Biorefineries: current status, challenges, and future direction. *Energy & Fuels*, **20**, 1727-1737.

Flick, K.M., Spielewoy, N., Kalashnikova, T.I., Guaderrama, M., Zhu, Q., Chang, H., C., Wittenberg, C. 2003. Grr1-dependent inactivation of Mth1 mediates glucose-induced dissociation of Rgt1 from *HXT* gene promoters. *Molecular Biology of the Cell*, **14**(8), 3230-3241.

Gallo, A., Mason, P., Shapiro, S., Fabritius, M. 2010. What is behind the increase in oil prices? Analyzing oil consumption and supply relationship with oil price. *Energy*, **33**(10), 4126-4141.

Gancedo, C., Flores, C.L. 2008. Moonlighting proteins in yeasts. *Microbiology and Molecular Biology Reviews*, **72**, 197–210.

Gancedo, J.M. 2008. The early steps of glucose signalling in yeast. *FEMS Microbiology Reviews*, **32**(4), 673-704.

Gancedo, J.M. 1998. Yeast carbon catabolite repression. *Microbiology and Molecular Biology Reviews*, **62**(2), 334–361.

Gancedo, J.M., Flores, C.L., Gancedo, C. 2015. The repressor Rgt1 and the cAMP-dependent protein kinases control the expression of the *SUC2* gene in *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta*, **1850**(7), 1362-1367.

Gancedo, J.M., Mazón, M.J., Gancedo, C. 1982. Inactivation and phosphorylation of yeast fructose1,6-bisphosphatase. *Biochemical Society Transactions*, **10**(5), 326-7.

Gao, Y., Skutsch, M., Drigo, R., Pacheco, P., Masera, O. 2011. Assessing deforestation from biofuels: Methodological challenges. *Applied Geography*, **31**(2), 508-518.

Garay-Arroyo, A., Covarrubias, A.A. 1999. Three genes whose expression is induced by stress in *Saccharomyces cerevisiae*. *Yeast*, **15**, 879–892.

Gárdonyi, M., Hahn-Hägerdal, B. 2003. The *Streptomyces rubiginosus* xylose isomerase is misfolded when expressed in *Saccharomyces cerevisiae*. *Enzyme and Microbial Technology*, **32**(2), 252-259.

Garland, S.A., Hoff, K., Vickery, L.E., Culotta, V.C. 1999. *Saccharomyces cerevisiae ISU1* and *ISU2*: members of a well-conserved gene family for iron-sulfur cluster assembly. *JMB*, **294**(4), 897-907.

Goffeau, A. 2000. Four years of post-genomic life with 6,000 yeast genes. *FEBS Letters*, **480**(1), 37-41.

Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M., Louis, E.J., Mewes, H.W., Murakami, Y., Philippsen, P., Tettelin, H., Oliver, S.G. 1996. Life with 6000 Genes. *Science*, **274**(5287), 546-567.

Gomez, M., Pérez-Gallardo, R.V., Sánchez, L.A. 2014. Malfunctioning of the iron–sulfur cluster assembly machinery in *Saccharomyces cerevisiae* produces oxidative stress via an iron-dependent mechanism, causing dysfunction in respiratory Complexes. *PLOS One*, **9**(10).

Gong, C.S., Chen, L.F., C., F.M., Chiang, L.C., Tsao, G.T. 1981. Production of ethanol from D-xylose by using D-xylose isomerase and yeasts. *Applied and Environmental Microbiology*, **41**(2), 430-436.

Grousl, T., Ivanov, P., Malcova, I., Pompach, P., Frydlova, I., Slaba, R., Senohrabkova, L., Novakova, L., Hasek, J. 2013. Heat shock-induced accumulation of translation elongation and termination factors precedes assembly of stress granules in *S. cerevisiae*. *PLOS One*, **8**(2).

Hahn-Hägerdal, B., Wahlbom, C.F., Gárdonyi, M., van Zyl, W.H., Otero, R.R.C., Jönsson, L.J. 2001. Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. *Advances in Biochemical Engineering/Biotechnology*, **73**, 53-84.

Halme, A., Bumgarner, S., Styles, C., Fink, G.R. 2004. Genetic and epigenetic regulation of the *FLO* gene family generates cell-surface variation in yeast. *Cell*, **116**(3), 405-415.

Hamacher, T., Becker, J., Gárdonyi, M., Hahn-Hägerdal, B., Boles, E. 2002. Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. *Microbiology*, **148**(9), 2783-2788.

HamediRad, M., Lian, J., H., L., Zhao, H. 2018. RNAi assisted genome evolution unveils yeast mutants with improved xylose utilization. *Biotechnology and Bioengineering*, **115**, 1552–1560.

Hardie, D.G., Carling, D., Carlson, M. 1998. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annual Review of Biochemistry*, **67**, 821-855.

Hedbacker, K., Carlson, M. 2006. Regulation of the nucleocytoplasmic distribution of Snf1-Gal83 protein kinase. *Eukaryotic Cell*, **5**(12), 1950–1956.

Hedbacker, K., Carlson, M. 2008. SNF1/AMPK pathways in yeast. *Frontiers in Bioscience*, **13**, 2408–2420.

Hedges, D., Proft, M., Entian, K.D. 1995. *CAT8*, a new zinc cluster-encoding gene necessary for derepression of gluconeogenic enzymes in the yeast *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, **15**(4), 1915-22.

Heidrich, K., Otto, A., Behlke, J., Rush, J., Wenzel, K.W., Kriegel, T. 1997. Autophosphorylation-inactivation site of hexokinase 2 in Saccharomyces cerevisiae. *Biochemistry*, **36**(8), 1960-1964.

Heim, R., Prasher, D.C., Tsien, R.Y. 1994. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *PNAS*, **91**, 12501-12504.

Hewitt, C.J., Caron, G.N.V., Axelsson, B., McFarlane, C.M., Nienow, A.W. 2000. Studies related to the scale-up of high-cell-density *E. coli* fed-batch fermentations

using multiparameter flow cytometry: effect of a changing microenvironment with respect to glucose and dissolved oxygen concentration. *Biotechnology and Bioengineering*, **70**(4), 381-390.

Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D. 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science*, **315**(5813), 804-807.

Hoegh-Guldberg, O., Jacob, D., Taylor, M., Bindi, M., Brown, S., Camilloni, I., Diedhiou, A., Djalante, R., Ebi, K.L., Engelbrecht, F., Guiot, J., Hijioka, Y., Mehrotra, S., Payne, A., Seneviratne, S.I., Thomas, A., Warren, R., Zhou, G. 2018. Impacts of 1.5ºC global warming on natural and human systems.

Hong, K.K., Nielsen, J. 2012. Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries. *Cellular and Molecular Life Sciences*, **69**, 2671–2690.

Hong, S.P., Leiper, F.C., Woods, A., Carling, D., Carlson, M. 2003. Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *PNAS*, **100**, 8839–8843.

Honigberg, S.M., Lee, R.H. 1998. Snf1 kinase connects nutritional pathways controlling meiosis in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, **18**, 4548–4555.

Höök, M., Tang, X. 2013. Depletion of fossil fuels and anthropogenic climate change - A review. *Energy Policy*, **52**, 797–809.

Horák, J. 2013. Regulations of sugar transporters: insights from yeast. *Current Genetics*, **59**, 1-31.

Huberts, D.H., Niebel, B., Heinemann, M. 2012. A flux-sensing mechanism could regulate the switch between respiration and fermentation. *FEMS Yeast Research*, **12**(2), 118-128.

Imai, T., Ohno, T. 1995. The relationship between viability and intracellular pH in the yeast *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, **61**(10), 3604-3608.

Jeffries, T.W. 2006. Engineering yeasts for xylose metabolism. *Current Opinion in Biotechnology*, **17**(3), 320–326.

Jeffries, T.W. 1983. Utilization of xylose by bacteria, yeasts, and fungi. *Pentoses and Lignin. Advances in Biochemical Engineering/Biotechnology*, **27**.

Jeppsson, M., Bengtsson, O., Franke, K., Lee, H., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F. 2006. The expression of a *Pichia stipitis* xylose reductase mutant with higher KM for NADPH increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* **93**.

Jin, Y.S., Laplaza, J.M., Jeffries, T.W. 2004. Stoichiometric network constraints on xylose metabolism by recombinant *Saccharomyces cerevisiae*. *Metabolic Engineering*, **6**(3), 229-238.

Johnsen, U., Dambeck, M., Zaiss, H., Fuhrer, T., Soppa, J., Sauer, U., Schönheit, P. 2009. D-xylose degradation pathway in the halophilic archaeon *Haloferax volcanii*. *Journal of Biological Chemistry*, **284**(40), 27290-27303.

Johnston, M. 1987. A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. *Microbiological Reviews*, **51**(4), 458-476.

Jones, S., Vignais, M.L., Broach, J.R. 1991. The CDC25 protein of *Saccharomyces cerevisiae* promotes exchange of guanine nucleotides bound to ras. *Molecular and Cellular Biology*, **11**(5), 2641-2646.

Jönsson, L.J., Martín, C. 2016. Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource Technology*, **199**, 103–112.

Jouandot, D., Roy, A., Kim, J.H. 2011. Functional dissection of the glucose signaling pathways that regulate the yeast glucose transporter gene (*HXT*) repressor Rgt1. *Journal of Cellular Biochemistry*, **112**(11), 3268-3275.

Kaishima, M., Ishii, J., Matsuno, T., Fukuda, N., Kondo, A. 2016. Expression of varied GFPs in *Saccharomyces cerevisiae*: codon optimization yields stronger than expected expression and fluorescence intensity. *Scientific Reports*, **6**(35932).

Kamm, B., Kamm, M. 2004. Principles of biorefineries. *Applied Microbiology and Biotechnology*, **64**, 137-145.

Karathia, H., Vilaprinyo, E., Sorribas, A., Alves, R. 2011. *Saccharomyces cerevisiae* as a Model Organism: A Comparative Study. *PLOS One*, **6**(2).

Karhumaa, K., Fromanger, R., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F. 2007. High activity of xylose reductase and xylitol dehydrogenase improves xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, **73**, 1039–1046.

Karhumaa, K., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F. 2005. Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. *Yeast* **22**(5), 359-368.

Karhumaa, K., Wu, B., Kielland-Brandt, M.C. 2010. Conditions with high intracellular glucose inhibit sensing through glucose sensor Snf3 in *Saccharomyces cerevisiae*. *Journal of Cellular Biochemistry*, **110**(4), 920-925.

Kayikci, Ö., Nielsen, J. 2015. Glucose repression in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, **15**(6).

Kim, D., Woo, H.M. 2018. Deciphering bacterial xylose metabolism and metabolic engineering of industrial microorganisms for use as efficient microbial cell factories. *Applied Microbiology and Biotechnology*, **102**(22), 9471-9480.

Kim, J.H., Johnston, M. 2006. Two glucose-sensing pathways converge on Rgt1 to regulate expression of glucose transporter genes in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, **281**(36), 26144-26149.

Kim, J.H., Polish, J., Johnston, M. 2003. Specificity and regulation of DNA binding by the yeast glucose transporter gene repressor Rgt1. *Molecular and Cellular Biology*, **23**, 5208–5216.

Kim, J.H., Roy, A., Jouandot II, D., Cho, K.H. 2013. The glucose signaling network in yeast. *Biochimica et Biophysica Acta*, **1830**(11), 5204-5210.

Kim, S.R., Xu, H., Lesmana, A., Kuzmanovic, U., Au, M., Florencia, C., Oh, E.J., Zhang, G., Kim, K.H., Jin, Y.S. 2015. Deletion of *PHO13*, encoding haloacid

dehalogenase type II A phosphatase, results in upregulation of the pentose phosphate pathway in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, **81**(5), 1601-1609.

Kircher, M. 2014. The emerging bioeconomy: industrial drivers, global impact, and international strategies. *Industrial Biotechnology*, **10**(1).

Klein, C., Struhl, K. 1994. Protein kinase A mediates growth-regulated expression of yeast ribosomal protein genes by modulating *RAP1* transcriptional activity. *Molecular and Cellular Biology*, **14**(3), 1920-1928.

Klimacek, M., Krahulec, S., U., S., Nidetzky, B. 2010. Limitations in xylose-fermenting *Saccharomyces cerevisiae*, made evident through comprehensive metabolite profiling and thermodynamic analysis. *Applied and Environmental Microbiology*, **76**, 7566–7574.

Knudsen, J.D., Carlquist, M., Gorwa-Grauslund, M. 2014. NADH-dependent biosensor in *Saccharomyces cerevisiae*: principle and validation at the single cell level. *AMB Express*, **4**, 81.

Kostrzynska, M., Sopher, C.R., Lee, H. 1998. Mutational analysis of the role of the conserved lysine-270 in the *Pichia stipitis* xylose reductase. *FEMS Microbiology Letters*, **159**(1).

Kötter, P., Amore, R., Hollenberg, C.P., Ciriacy, M. 1990. Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene, *XYL2*, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant. *Current Genetics*, **18**(6), 493-500.

Kötter, P., Ciriacy, M. 1993. Xylose fermentation by *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, **38**, 776-783.

Kotyk, A. 1965. Properties of the sugar carrier in baker's yeast. *Folia Microbiologica*, **13**(1), 12–19.

Kotyk, A., Kleinzeller, A. 1963. Transport of D-xylose and sugar space in baker's yeast. *Folia Microbiologica*, **8**(3), 156–164.

Kovalevsky, A.Y., Hanson, L., Fisher, S.Z., Mustyakimov, M., Mason, S.A., Forsyth, V.T., Blakeley, M.P., Keen, D.A., Wagner, T., Carrell, H.L., Katz, A.K., Glusker, J.P., Langan, P. 2010. Metal ion roles and the movement of hydrogen during reaction catalyzed by D-xylose isomerase: a joint x-ray and neutron diffraction study. *Cell Press*, **18**(6), 688-699.

Kraakman, L., Lemaire, K., Ma, P., Teunissen, A.W., Donaton, M.C., Van Dijck, P., Winderickx, J., de Winde, J.H., Thevelein, J.M. 1999. A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Molecular Microbiology*, **32**(5), 1002-1012.

Kuchin, S., Vyas, V.K., Carlson, M. 2002. Snf1 protein kinase and the repressors Nrg1 and Nrg2 regulate *FLO11*, haploid invasive growth, and diploid pseudohyphal differentiation. *Molecular and Cellular Biology*, **22**(12), 3994-4000.

Kuhn, A., van Zyl, C., van Tonder, A., Prior, B.A. 1995. Purification and partial characterization of an aldo-keto reductase from *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, **61**(4), 1580–1585.

Kumar, R., Singh, S., Singh, O.V. 2008. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *Journal of Industrial Microbiology & Biotechnology*, **35**(5), 377-391.

Kuyper, M., Harhangi, H.R., Stave, A.K., Winkler, A.A., Jetten, M.S., de Laat, W.T., den Ridder, J.J.J., Op den Camp, H.J., van Dijken, J.P., Pronk, J.T. 2003. High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Research*, **4**(1), 69-78.

Kuyper, M., Hartog, M.M.P., Toirkens, M.J., Almering, M.J.H., Winkler, A.A., van Dijken, J.P., Pronk, J.T. 2005. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisia*e strain for rapid anaerobic xylose fermentation. *FEMS Yeast Research* **5**, 399-409.

Lachke, A. 2002. Biofuel from D-xylose — The second most abundant sugar. *Resonance*, **7**(5), 50-58.

Lane, S., Xu, H., Oh, E.J., Kim, H., Lesmana, A., Jeong, D., Zhang, G., Tsai, C., S., Jin, Y., S., Kim, S.R. 2018. Glucose repression can be alleviated by reducing glucose phosphorylation rate in *Saccharomyces cerevisiae*. *Scientific Reports*, **8**(1), 2613.

Law, A., Saunders, P., Middleton, J., McCoy, D. 2018. Global warming must stay below 1.5°C. *BMJ* **363**.

Lee, G.H., Ueom, J.H. 2001. Protection of metal stress in *Saccharomyces cerevisiae*: cadmium tolerance requies the presence if two ATP-binding domains of Hsp104 protein. *Bulletin of the Korean Chemical Society*, **22**(5), 514-518.

Lee, M.E., DeLoache, W.C., Cervantes, B., Dueber, J.E. 2015. A highly characterized yeast toolkit for modular, multipart sssembly. *ACS Synthetic Biology*, **4**(9), 975-986.

Lee, S.H., Kodaki, T., Park, Y.C., Seo, J.H. 2012a. Effects of NADH-preferring xylose reductase expression on ethanol production from xylose in xylose-metabolizing recombinant *Saccharomyces cerevisiae*. *Journal of Biotechnology*, **158**, 184–191.

Lee, S.M., Jellison, T., Alper, H.S. 2012b. Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, **78**, 5708 –5716.

Lee, S.M., Jellison, T., Alper, H.S. 2014. Systematic and evolutionary engineering of a xylose isomerase-based pathway in *Saccharomyces cerevisiae* for efficient conversion yields. *Biotechnology for Biofuels*, **7**(122).

Lewis, J.G., Northcott, C.J., Learmonth, R.P., Attfield, P.V., Watson, K. 1993. The need for consistent nomenclature and assessment of growth phases in diauxic cultures of *Saccharomyces cerevisiae*. *Journal of General Microbiology*, **139**, 835-839.

Li, J., Wang, S., VanDusen, W.J., Schultz, L.D., George, H.A., Herber, W.K., Jeong Chae, H.J. 2000. Green fluorescent protein in *Saccharomyces cerevisiae*: Real-time studies of the *GAL1* promoter. *Biotechnology and Bioengineering*, **70**(2), 187-196.

Li, J., Xu, Z., Chupalov, A., Marchisio, M.A. 2018a. Anti-CRISPR-based biosensors in the yeast *S. cerevisiae*. *Journal of Biological Engineering*, **12**(11).

Li, S., Giardina, D.M., Siegal, M.L. 2018b. Control of nongenetic heterogeneity in growth rate and stress tolerance of *Saccharomyces cerevisiae* by cyclic AMP-regulated transcription factors. *PLOS Genetics*, **14**(11).

Li, S., Si, T., Wang, M., Zhao, H. 2015. Development of a synthetic malonyl-CoA sensor in *Saccharomyces cerevisiae* for intracellular metabolite monitoring and genetic screening. *ACS Synthetic Biology*, **4**(12), 1308-1315.

Li, Y., Wang, Y. 2013. Ras protein/cAMP-dependent protein kinase signaling is negatively regulated by a deubiquitinating enzyme, *Ubp3*, in yeast. *Journal of Biological Chemistry*, **288**(16), 11358-11365.

Lill, R., Hoffmann, B., Molik, S. 2012. The role of mitochondria in cellular iron–sulfur protein biogenesis and iron metabolism. *Biochimica et Biophysica Acta* **1823**, 1491–1508.

Lill, R., Mühlenhoff, U. 2006. Iron-sulfur protein biogenesis in eukaryotes: components and mechanisms. *Annual Review of Cell and Developmental Biology*, **22**, 457-486.

Lillie, S.H., Pringle, J.R. 1980. Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. *Journal of Bacteriology*, **143**, 1384-1394.

Lopes, M.L., Paulillo, S.C.L., Godoy, A., Cherubin, R.A., Lorenzi, M.S., Giometti, F.H.C., Bernardino, C.D., Neto, H.B.A., Amorim, H.V. 2016. Ethanol production in Brazil: a bridge between science and industry. *Brazilian Journal of Microbiology*, **47**, 64-76.

Lutfiyya, L.L., Iyer, V.R., DeRisi, J., DeVit, M.J., Brown, P.O., Johnston, M. 1998. Characterization of three related glucose repressors and genes they regulate in *Saccharomyces cerevisiae*. *Genetics*, **150**(4), 1377-1391.

Lynd, L.R. 1996. Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment, and policy. *Annual Review of Energy and the Environment*, **21**(1), 403-465.

Macey, M.G. 2007. Principles of flow cytometry. in: *Flow Cytometry: Principles and Applications*, (Ed.) M.G. Macey, Humana Press Inc.

Maeda, T., Takekawa, M., Saito, H. 1995. Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. *Science*, **269**(5223), 554-558.

Matsushika, A., Goshima, T., Fujii, T., Inoue, H., Sawayama, S., Yano, S. 2012. Characterization of non-oxidative transaldolase and transketolase enzymes in the pentose phosphate pathway with regard to xylose utilization by recombinant *S. cerevisiae*. *Enzyme and Microbial Technology*, **51**, 16-25.

Matsushika, A., Goshima, T., Hoshino, T. 2014. Transcription analysis of recombinant industrial and laboratory *Saccharomyces cerevisiae* strains reveals the molecular basis for fermentation of glucose and xylose. *Microbial Cell Factories*, **13**, 16.

Matsushika, A., Nagashima, A., Goshima, T., T., H. 2013. Fermentation of xylose causes inefficient metabolic state due to carbon/energy starvation and reduced glycolytic flux in recombinant industrial *Saccharomyces cerevisiae*. *PLOS One*, **8**(7).

McNabb, D.S., Reed, R., Marciniak, R.A. 2005. Dual luciferase assay system for rapid assessment of gene expression in *Saccharomyces cerevisiae*. *Eukaryotic Cell*, **4**, 1539–1549.

Mehta, S., Zhang, J. 2011. Reporting from the field: genetically encoded fluorescent reporters uncover signaling dynamics in living biological systems. *Annual Review of Biochemistry*, **80**(1), 375-401.

Mohr, A., Raman, S. 2013. Lessons from first generation biofuels and implications for the sustainability appraisal of second generation biofuels. *Energy Policy*, **63**(100), 114-122.

Mohsin, M., Ahmad, A. 2014. Genetically-encoded nanosensor for quantitative monitoring of methionine in bacterial and yeast cells. *Biosensors & Bioelectronics*, **59**, 358-364.

Moncada, J., Aristizábal, V. 2016. Design strategies for sustainable biorefineries. *Biochemical Engineering Journal*, **116**, 122-134.

Moreno, F., Herrero, P. 2002. The hexokinase 2-dependent glucose signal transduction pathway of *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews*, **26**(1), 83-90.

Morise, H., Shimomura, O., Johnson, F.H., Winant, J. 1974. Intermolecular energy transfer in the bioluminescent system of *Aequorea*. *Biochemistry*, **13**.

Moriya, H., Johnston, M. 2004. Glucose sensing and signaling in *Saccharomyces cerevisiae* through the Rgt2 glucose sensor and casein kinase I. *PNAS*, **101**(6), 1572-1577.

Mosiman, V.L., Patterson, B.K., Canterero, L., Goolsby, C.L. 1997. Reducing cellular autofluorescence in flow cytometry: an in situ method. *Cytometry (Communications in clinical cytometry)*, **30**, 151–156

Moysés, D.N., Reis, V.C., de Almeida, J.R., de Moraes, L.M., Torres, F.A. 2016. Xylose fermentation by *Saccharomyces cerevisiae*: challenges and prospects. *International Journal of Molecular Sciences*, **17**(3), 207.

Müller, S., Boles, E., May, M. 1995. Different internal metabolites trigger the induction of glycolytic gene expression in *Saccharomyces cerevisiae*. *Journal of Bacteriology*, **177**, 4517–4519.

Naik, S.N., Goud, V.V., Rout, P.K., Dalai, A.K. 2010. Production of first and second generation biofuels: A comprehensive review. *Renewable and Sustainable Energy Reviews*, **14**(2), 578-597.

Nature, L.R. 2009. Function and biogenesis of iron–sulphur proteins. *Nature Review Insight*, **460**, 831–838.

Naylor, L.H. 1999. Reporter gene technology: the future looks bright. *Biochemical Pharmacology*, **58**, 749–757.

Newman, R.H., Fosbrink, M.D., Zhang, J. 2011. Genetically encodable fluorescent biosensors for tracking signaling dynamics in living cells. *Chemical Reviews*, **111**, 3614–3666.

Ni, H., Laplaza, J.M., Jeffries, T.W. 2007. Transposon mutagenesis to improve the growth of recombinant *Saccharomyces cerevisiae* on D-xylose. *Applied and Environmental Microbiology*, **73**(7), 2061–2066.

Nicastro, R., Tripodi, F., Gaggini, M., Castoldi, A., Reghellin, V., Nonnis, S., Tedeschi, G., Coccetti, P. 2015. Snf1 Phosphorylates Adenylate Cyclase and Negatively Regulates Protein Kinase A-dependent Transcription in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, **290**(41), 24715-24726.

Nijland, J.G., Shin, H.Y., Boender, L.G.M., de Waal, P.P., Klaassen, P., Driessen, A.J.M. 2017. Improved xylose metabolism by a *CYC8* mutant of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, **83**(11), 1-12.

Nissen, T.L., Hamann, C.W., Kielland-Brandt, M.C., Nielsen, J., Villadsen, J. 2000. Anaerobic and aerobic batch cultivations of *Saccharomyces cerevisiae* mutants impaired in glycerol synthesis. *Yeast*, **16**, 463-474.

Olarewaju, O., Ortiz, P.A., Chowdhury, W.Q., Chatterjee, I., Kinzy, T.G. 2004. The translation elongation factor eEF1B plays a role in the oxidative stress response pathway. *RNA Biology*, **1**(2), 89-94.

Orij, R., Postmus, J., Beek, A., Brul, S., Smits, G.J. 2009. In vivo measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in *Saccharomyces cerevisiae* reveals a relation between intracellular pH and growth. *Microbiology*, **155**(1), 268-278.

Osipov, S., Tutyaeva, V., Preobrazhenskaya, O., Karpov, V. 2011. A rapid method for liquid β-galactosidase reporter assay in *Saccharomyces cerevisiae*. *World Journal of Microbiology and Biotechnology* **27**, 1255–1259.

Ostergaard, S., Olsson, L., Nielsen, J. 2000. Metabolic Engineering of *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews*, **64**(1), 34-50.

Özcan, S., Dover, J., Rosenwald, A.G., Wölfl, S., Johnston, M. 1996. Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. *PNAS*, **93**(22), 12428-12432.

Özcan, S., Johnston, M. 1995. Three different regulatory mechanisms enable yeast hexose transporter (*HXT*) genes to be induced by different levels of glucose. *Molecular and Cellular Biology*, **15**(3), 1564-1572.

Özcan, S., Vallier, L.G., Flick, J.S., Carlson, M., Johnston, M. 1997. Expression of the *SUC2* gene of *Saccharomyces cerevisiae* is induced by low levels of glucose. *Yeast*, **13**, 127–137.

Palomino, A., Herrero, P., Moreno, F. 2005. Rgt1, a glucose sensing transcription factor, is required for transcriptional repression of the *HXK2* gene in *Saccharomyces cerevisiae*. *Biochemical Journal*, **388**(2), 697-703.

Panek, A.D., Mattoon, J.R. 1977. Regulation of energy metabolism in *Saccharomyces cerevisiae:* Relationships between catabolite repression, trehalose synthesis, and mitochondrial development. *Archives of Biochemistry and Biophysics*, **183**, 306-316

Papapetridis, I., Goudriaan, M., Vitali, M., Keijzer, N.A., Broek, M., van Maris, A.J.A., Pronk, J.T. 2018. Optimizing anaerobic growth rate and fermentation kinetics in

Saccharomyces cerevisiae strains expressing Calvin-cycle enzymes for improved ethanol yield. *Biotechnology for Biofuels*, **11**(1), 17.

Parachin, N.S., Bengtsson, O., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F. 2010. The deletion of *YLR042c* improves ethanolic xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Yeast*, **27**, 741–751.

Park, J.I., Grant, C.M., W., D.I. 2005. The high-affinity cAMP phosphodiesterase of *Saccharomyces cerevisiae* is the major determinant of cAMP levels in stationary phase: involvement of different branches of the Ras–cyclic AMP pathway in stress responses. *Biochemical and Biophysical Research Communications*, **327**, 311–319.

Park, M., Tsai, S.L., Chen, W. 2013. Microbial biosensors: engineered microorganisms as the sensing machinery. *Sensors*, **13**(5), 5777-5795.

Parrou, J.L., Enjalbert, B., Plourde, L., Bauche, A., Gonzales, B., François, J. 1999. Dynamic responses of reserve carbohydrate metabolism under carbon and nitrogen limitations in *Saccharomyces cerevisiae*. *Yeast*, **15**, 191–203.

Patterson, G.H., Knobel, S.M., Sharif, W.D., Kain, S.R., Piston, D.W. 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophysical Journal*, **73**(5), 2782-2790.

Pauly, M., Keegstra, K. 2008. Cell-wall carbohydrates and their modification as a resource for biofuels. *The Plant Journal* **54**(4), 559-568.

Peeters, K., Van Leemputte, F., Fischer, B., Bonini, B.M., Quezada, H., Tsytlonok, M., Haesen, D., Vanthienen, W., Bernardes, N., Gonzalez-Blas, C.B., Janssens, V., Tompa, P., Versées, W., Thevelein, J.M. 2017. Fructose-1,6-bisphosphate couples glycolytic flux to activation of Ras. *Nature Communications*, **8**(1), 922.

Peeters, T., Louwet, W., Geladé, R., Nauwelaers, D., Thevelein, J.M., Versele, M. 2006. Kelch-repeat proteins interacting with the Galpha protein Gpa2 bypass adenylate cyclase for direct regulation of protein kinase A in yeast. *PNAS*, **103**(35), 13034-13039.

Pérez-Gallardo, R.V., Briones, L.S., Díaz-Pérez, A.L., Gutiérrez, S., Rodríguez-Zavala, J.S., Campos-García, J. 2013. Reactive oxygen species production induced by ethanol in *Saccharomyces cerevisiae* increases because of a dysfunctional mitochondrial iron–sulfur cluster assembly system. *FEMS Yeast Research*, **13**(8), 804-819.

Phan, V.T., Ding, V.W., Li, F., Chalkley, R.J., Burlingame, A., McCormick, F. 2010. The RasGAP proteins Ira2 and neurofibromin are negatively regulated by Gpb1 in yeast and ETEA in humans. *Molecular and Cellular Biology*, **30**(9), 2264-2279.

Pohlig, G., Holzer, H. 1985. Phosphorylation and inactivation of yeast fructose-1,6-bisphosphatase by cyclic AMP-dependent protein kinase from yeast. *Journal of Biological Chemistry*, **260**, 13818-1382.

Polish, J.A., Kim, J., H., Johnston, M. 2005. How the Rgt1 Transcription Factor of *Saccharomyces cerevisiae* Is Regulated by Glucose. *Genetics*, **169**(2), 583-594.

Portugal-Nunes, D.J., Pawar, S.S., Lidén, G., Gorwa-Grauslund, M.F. 2017. Effect of nitrogen availability on the poly-3-d-hydroxybutyrate accumulation by engineered *Saccharomyces cerevisiae*. *AMB Express*, **7**(1), 35.

Posas, F., Wurgler, M., Maeda, T., A., W.E., Thai, T.C., Saito, H. 1996. Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1–YPD1–SSK1 "two-component" osmosensor. *Cell*, **86**, 865–875.

Proft, M., Struhl, K. 2002. Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. *Molecular Cell*, **9**, 1307–1317.

Przybylski, F., Otto, A., Nissler, K., Schellenberger, W., Hofmann, E. 1985. Effects of fructose 1,6-bisphosphate on the activation of yeast phosphofructokinase by fructose 2,6-bisphosphate and AMP. *Biochimica et Biophysica Acta*, **831**(3), 350-352.

Reifenberger, E., Boles, E., Ciriacy, M. 1997. Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. *European Journal of Biochemistry* **245**, 324-333.

Reinders, A., Bürckert, N., Boller, T., Wiemken, A., de Virgilio, C. 1998. *Saccharomyces cerevisiae* cAMP-dependent protein kinase controls entry into stationary phase through the Rim15p protein kinase. *Genes & Development*, **12**, 2943–2955.

Robertson, L.S., Causton, H.C., Young, R.A., Fink, G.R. 2000. The yeast A kinases differentially regulate iron uptake and respiratory function. *PNAS*, **97**(11), 5984-5988.

Robinson, G.A., Butcher, R.W., Sutherland, E.W. 1968. cyclic AMP. *Annual Review of Biochemistry*, **37**, 149-174.

Roca, C., Haack, M.B., Olsson, L. 2004. Engineering of carbon catabolite repression in recombinant xylose fermenting *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, **63**(5), 578-583.

Roca, C., Nielsen, J., Olsson, L. 2003. Metabolic engineering of ammonium assimilation in xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production. *Applied and Environmental Microbiology*, **69**(8), 4732–4736.

Rodrigues, F., van Hemert, M., Steensma, H.Y., Côrte-Real, M., Leão, C. 2001. Red fluorescent protein (DsRed) as a reporter in *Saccharomyces cerevisiae*. *Journal of Bacteriology*, **183**, 3791–3794.

Rolland, F., De Winde, J.H., Lemaire, K., Boles, E., Thevelein, J.M., Winderickx, J. 2000. Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. *Molecular Microbiology*, **38**(2), 348-358.

Rolland, F., Winderickx, J., Thevelein, J.M. 2002. Glucose-sensing and -signalling mechanisms in yeast. *FEMS Yeast Research*, **2**(2), 183-201.

Rubin, E.M. 2008. Genomics of cellulosic biofuels. *Nature*, **454**(7206), 841.

Rubio-Texeira, M., Zeebroeck, G.V., Voordeckers, K., Thevelein, J.M. 2010. *Saccharomyces cerevisiae* plasma membrane nutrient sensors and their role in PKA signaling. *FEMS Yeast Research*, **10**, 134–149.

Rudolf, A., Baudel, H., Zacchi, G., Hahn-Hägerdal, B., Lidén, G. 2008. Simultaneous saccharification and fermentation of steam-pretreated bagasse using

Saccharomyces cerevisiae TMB3400 and *Pichia stipitis* CBS6054. *Biotechnology and Bioengineering*, **99**(4), 783-790.

Rulli, M., Bellomi, D., Cazzoli, A., Carolis, G., D'Odorico, P. 2016. The water-land-food nexus of first-generation biofuels. *Scientific Reports*, **6**(1), 22521.

Runquist, D., Hahn-Hägerdal, B., Bettiga, M. 2009. Increased expression of the oxidative pentose phosphate pathway and gluconeogenesis in anaerobically growing xylose-utilizing *Saccharomyces cerevisiae*. *Microbial Cell Factories*, **8**(1), 49.

Russell, M., Bradshaw-Rouse, J., Markwardt, D., Heideman, W. 1993. Changes in gene expression in the Ras/adenylate cyclase system of *Saccharomyces cerevisiae*: correlation with cAMP levels and growth arrest. *Molecular Biology of the Cell*, **4**, 757-765.

Saloheimo, A., Rauta, J., Stasyk, O.V., Sibirny, A.A., Penttilä, M., Ruohonen, L. 2007. Xylose transport studies with xylose-utilizing *Saccharomyces cerevisiae* strains expressing heterologous and homologous permeases. *Applied Microbiology and Biotechnology*, **74**(5), 1041-1052.

Salusjärvi, L., Kankainen, M., Soliymani, R., Pitkänen, J.P., Penttilä, M., Ruohonen, L. 2008. Regulation of xylose metabolism in recombinant *Saccharomyces cerevisiae*. *Microbial Cell Factories*, **7**(1), 18.

Salusjärvi, L., Pitkänen, J.P., Aristidou, A., Ruohonen, L., Penttilä, M. 2006. Transcription analysis of recombinant *Saccharomyces cerevisiae* reveals novel responses to xylose. *Applied Biochemistry and Biotechnology*, **128**(3), 237-274.

Sanchez, R., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F. 2010. Cross-reactions between engineered xylose and galactose pathways in recombinant *Saccharomyces cerevisiae*. *Biotechnology for Biofuels*, **3**(1), 1-9.

Santangelo, G.M. 2006. Glucose signaling in *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews* **70**(1), 253-282.

Sanz, P., Alms, G.R., Haystead, T.A., Carlson, M. 2000. Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase. *Molecular and Cellular Biology*, **20**(4), 1321-1328.

Sarthy, A.V., McConaughy, B.L., Lobo, Z., Sundstrom, J.A., Furlong, C.E., Hall, B.D. 1987. Expression of the *Escherichia coli* xylose isomerase gene in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **43**, 1996-2000.

Sato, T.K., Tremaine, M., Parreiras, L.S., Hebert, A.S., Myers, K.S., Higbee, A.J., Sardi, M., McIlwain, S.J., Ong, I.M., Breuer, R.J., Narasimhan, R., McGee, M.A., Dickinson, Q., Reau, A., Xie, D., Tian, M., Reed, J.L., Zhang, Y., Coon, J.J., Hittinger, C.T., Gasch, A.P., Landick, R. 2016. Directed evolution reveals unexpected epistatic interactions that alter metabolic regulation and enable anaerobic xylose use by *Saccharomyces cerevisiae*. *PLOS Genetics*, **12**(10), 1-31.

Sauer, S., Pereira, S. 2012. Agrarian structure, foreign investment in land, and land prices in Brazil. *The Journal of Peasant Studies*, **39**, 873–898.

Schilke, B., Voisine, C., Beinert, H., Craig, E. 1999. Evidence for a conserved system for iron metabolism in the mitochondria of *Saccharomyces cerevisiae*. *PNAS*, **96**(18), 10206-10211.

Sedlak, M., Ho, N.W. 2004. Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant *Saccharomyces* yeast. *Yeast* **21**(8), 671-684.

Senac, T., Hahn-Hägerdal, B. 1991. Effects of increased transaldolase activity on D-xylulose and D-glucose metabolism in *Saccharomyces cerevisiae* cell extracts. *Applied and Environmental Microbiology*, **57**(6), 1701-1706.

Shen, M.H., Song, H., Li, B.Z., Yuan, Y.J. 2015. Deletion of D-ribulose-5-phosphate 3-epimerase (*RPE1*) induces simultaneous utilization of xylose and glucose in xylose-utilizing *Saccharomyces cerevisiae*. *Biotechnology Letters*, **37**(5), 1031-1036.

Shimomura, O., Johnson, F.H., Saiga, Y. 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *Journal of Cellular and Comparative Physiology*, **59(**3), 223-239.

Shirra, M.K., Arndt, K.M. 1999. Evidence for the involvement of the Glc7-Reg1 phosphatase and the Snf1-Snf4 kinase in the regulation of *INO1* transcription in *Saccharomyces cerevisiae*. *Genetics*, **152**, 73–87.

Skoog, K., Hahn-Hägerdal, B. 1988. Xylose fermentation. *Enzyme and Microbial Technology*, **10**(2), 66-80.

Smith, A., Ward, M.P., Garrett, S. 1998. Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation. *The EMBO Journal*, **17**, 3556–3564.

Stephens, C., Christen, B., Fuchs, T., Sundaram, V., Watanabe, K., Jenal, U. 2007. Genetic analysis of a novel pathway for D-Xylose metabolism in *Caulobacter crescentus*. *Journal of Bacteriology*, **189**(5), 2181-2185.

Stincone, A., Prigione, A., Cramer, T., Wamelink, M.M.C., Campbell, K., Cheung, E., Olin-Sandoval, V., Grüning, N.M., Krüger, A., Tauqeer, A.M.T., Keller, M.A., Breitenbach, M., Brindle, K.M., Rabinowitz, J.D., Ralser, M. 2015. The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. *Biological Reviews*, **90**, 927-963.

Stokes, D. 2007. Blood for oil? Global capital, counter-insurgency and the dual logic of American energy security. *Review of International Studies*, **33**, 245–264

Stovicek, V., Borodina, I., Forster, J. 2015. CRISPR–Cas system enables fast and simple genome editing of industrial *Saccharomyces cerevisiae* strains. *Metabolic Engineering Communications*, **2**, 13-22.

Su, L., Jia, W., Hou, C., Lei, Y. 2011. Microbial biosensors: a review. *Biosensors and Bioelectronics*, **26**, 1788–1799.

Sullivan, L.S. 2013. Identity, territory and land conflict in Brazil. *Development and Change*, **44**, 451–471.

Susan-Resiga, D., Nowak, T. 2003. The proton transfer step catalyzed by yeast pyruvate kinase. *Journal of Biological Chemistry*, **278**(15), 12660-12671.

Takahashi, E., Takano, T., Numata, A., Hayashi, N., Okano, S., Nakajima, O., Nomura, Y., Sato, M. 2005. Advances in experimental medicine and biology. in: *Oxygen Transport to Tissue XXVI*, (Eds.) N. Back, I.R. Cohen, D. Kritchevsky, A. Lajtha, R. Peoletti, Vol. 566.

Tanaka, K., Matsumoto, K., Toh-E, A. 1989. *IRA1*, an inhibitory regulator of the RAS-cyclic AMP pathway in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **9**(2), 757-768.

Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M.S., Gibbs, J.B., Matsumoto, K., Kaziro, Y., Toh-e, A. 1990a. *S. cerevisiae* genes *IRA1* and *IRA2* encode proteins that may be functionally equivalent to mammalian ras GTPase activating protein. *Cell*, **60**(5), 803-807.

Tanaka, K., Nakafuku, M., Tamanoi, F. 1990b. *IRA2*, a second gene of *Saccharomyces cerevisiae* that encodes a protein with a domain homologous to mammalian ras GTPase-activating protein. *Molecular and Cellular Biology*, **10**(8), 4303–4313.

Tantirungkij, M., Nakashima, N., T., S., Yoshida, T. 1993. Construction of xylose-assimilating *Saccharomyces cerevisiae*. *Journal of Fermentation and Bioengineering* **75**(2), 83-88.

Teo, W.S., Chang, M.W. 2015. Bacterial XylRs and synthetic promoters function as genetically encoded xylose biosensors in *Saccharomyces cerevisiae*. *Biotechnology Journal*, **10**, 315–322.

Thevelein, J.M., de Windey, J.H. 1999. Novel sensing mechanisms and targets for the cAMP–protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. *Molecular Microbiology*, **33**, 904-918.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J.D., McMullen, B., Hurwitz, M., Krebs, E.G., Wigler, M. 1987a. Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, **7**(4), 1371-1377.

Toda, T., Cameron, S., Sass, P., Zoller, M., Wigler, M. 1987b. Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. *Cell*, **50**, 277-287.

Toivari, M.H., Salusjärvi, L., Ruohonen, L., Penttilä, M. 2004. Endogenous xylose pathway in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, **70**(6), 3681-3686.

Tomás-Cobos, L., Casadomé, L., Mas, G., Sanz, P., Posas, F. 2004. Expression of the *HXT1* low affinity glucose transporter requires the coordinated activities of the HOG and glucose signalling pathways. *Journal of Biological Chemistry*, **279**(21), 22010-22019.

Träff, K.L., Cordero, O.R.R., van Zyl, W.H., Hahn-Hägerdal, B. 2001. Deletion of the *GRE3* aldose reductase gene and its influence on xylose metabolism in recombinant strains of *Saccharomyces cerevisiae* expressing the *xylA* and *XKS1* genes. *Applied and Environmental Microbiology*, **67**(12), 5668-5674.

Träff, K.L., Jönsson, L.J., Hahn-Hägerdal, B. 2002. Putative xylose and arabinose reductases in *Saccharomyces cerevisiae*. *Yeast*, **19**(14), 1233-1241.

Trevisol, E., Panek, A.D., Mesquita, J.F., Eleutherio, E. 2014. Regulation of the yeast trehalose–synthase complex by cyclic AMP-dependent phosphorylation. *Biochimica et Biophysica Acta*, **1840**(6), 1646-1650.

Tsien, R.Y. 1998. The green fluorescence protein. *Biochemistry*, **67**(1), 509-544.

Turrens, J.F. 2003. Mitochondrial formation of reactive oxygen species. *The Journal of Physiology*, **552**, 335-344.

U.S. Energy Information Administration, E. 2018 Total petroleum and other liquids production. in: *International Energy Statistics*, https://www.eia.gov/beta/international/.

Ullah, A., Orij, R., Brul, S., Smits, G.J. 2012. Quantitative analysis of the modes of growth inhibition by weak organic acids in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, **78**, 8377– 8387.

Umeyama, T., Okada, S., Ito, T. 2013. Synthetic gene circuit-mediated monitoring of endogenous metabolites: identification of *GAL11* as a novel multicopy enhancer of s-adenosylmethionine level in yeast. *ACS Synthetic Biology*, **2**(8), 425-430.

Valadi, Å., Granath, K., Gustafsson, L., Adler, L. 2004. Distinct intracellular localization of Gpd1p and Gpd2p, the two yeast isoforms of NAD+-dependent glycerol-3-phosphate dehydrogenase, explains their different contributions to redox-driven glycerol production. *Journal of Biological Chemistry*, **279**(38), 39677-39685.

Valkonen, M., Mojzita, D., Penttilä, M., Bencina, M. 2013. Noninvasive high-throughput single-cell analysis of the intracellular pH of *Saccharomyces cerevisiae* by ratiometric flow cytometry. *Applied and Environmental Microbiology*, **79**, 7179 –7187.

Valli, M., Sauer, M., Branduardi, P., Borth, N., Porro, D., Mattanovich, D. 2006. Improvement of lactic acid production in *Saccharomyces cerevisiae* by cell sorting for high intracellular pH. *Applied and Environmental Microbiology*, **72**, 5492–5499.

Valli, M., Sauer, M., Branduardi, P., Borth, N., Porro, D., Mattanovich, D. 2005. Intracellular pH distribution in *Saccharomyces cerevisiae* cell populations, analyzed by flow cytometry. *Applied and Environmental Microbiology*, **71**, 1515–1521.

VanEngelenburg, S.B., Palmer, A.E. 2008. Fluorescent biosensors of protein function. *Current Opinion in Chemical Biology*, **12**, 60–65.

Vega, M., Riera, A., Fernández-Cid, A., Herrero, P., Moreno, F. 2016. Hexokinase 2 is an intracellular glucose sensor of yeast cells that maintains the structure and activity of Mig1 protein repressor complex. *Journal of Biological Chemistry*, **291**(14), 7267-7285.

Verduyn, C., van Kleef, R., Frank, J., Schreuder, H., van Dijken, J.P., Scheffers, W.A. 1985. Properties of the NAD(P)H-dependent xylose reductase from the xylose-fermenting yeast *Pichia stipitis*. *The Biochemical Journal*, **226**(3), 669-677.

Verho, R., Londesborough, J., Penttilä, M., Richard, P. 2003. Engineering redox cofactor regeneration for improved pentose fermentation in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, **69**, 5892–5897.

Verhoeven, M.D., Bracher, J.M., Nijland, J.G., Jonna Bouwknegt, J., Daran, J.M.G., Driessen, A.J.M., van Maris, A.J.A., Pronk, J.T. 2018. Laboratory evolution of a glucose-phosphorylation-deficient, arabinose-fermenting *S. cerevisiae* strain

reveals mutations in *GAL2* that enable glucose-insensitive L-arabinose uptake. *FEMS Yeast Research*, **18**(6).

Versele, M., de Winde, J.H., Thevelein, J.M. 1999. A novel regulator of G protein signalling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2. *The EMBO Journal*, **18**(20), 5577-5591.

Versele, M., Lemaire, K., Thevelein, J.M. 2001. Sex and sugar in yeast: two distinct GPCR systems. *EMBO Reports*, **2**(7), 574-579.

Vicente, R.L., Spina, L., Gómez, J.P.L., Dejean, S., Parrou, J.L., François, J.M. 2018. Trehalose-6-phosphate promotes fermentation and glucose repression in *Saccharomyces cerevisiae*. *Microbial Cell* **5**(10), 444-459.

Vincent, O., Townley, R., Kuchin, S., Carlson, M. 2001. Subcellular localization of the Snf1 kinase is regulated by specific subunits and a novel glucose signaling mechanism. *Genes & Development*, **15**(9), 1104-1114.

Virgilio, C. 2012. The essence of yeast quiescence. *FEMS Microbiology Reviews*, **36**(2), 306-339.

Vleet, J.H.V., Jeffries, T.W., Olsson, L. 2008. Deleting the para-nitrophenyl phosphatase (pNPPase), *PHO13*, in recombinant *Saccharomyces cerevisiae* improves growth and ethanol production on D-xylose. *Metabolic Engineering*, **10**, 360–369.

Walfridsson, M., Bao, X., Anderlund, M., Lilius, G., Bülow, L., Hahn-Hägerdal, B. 1996. Ethanolic fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus xylA* gene, which expresses an active xylose (glucose) isomerase. *Applied and Environmental Microbiology* **62**, 4648-4651.

Wang, M., Li, S., Zhao, H. 2016. Design and engineering of intracellular-metabolite-sensing/regulation gene circuits in *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering*, **113**(1), 206-215.

Ward, W.W., Prentice, H.J., Roth, A.F. 1982. Spectral perturbations of the *Aequorea* green-fluorescent protein. *Photochemistry and Photobiology*, **35**, 803-808.

Wasserstrom, L., Portugal-Nunes, D., Almqvist, H., Sandström, A.G., Lidén, G., Gorwa-Grauslund, M.F. 2018. Exploring D-xylose oxidation in *Saccharomyces cerevisiae* through the Weimberg pathway. *AMB Express*, **8**(1), 33.

Watanabe, S., Saleh, A.A., Pack, S.P., Annaluru, N., Kodaki, T., Makino, K. 2007a. Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein-engineered NADH-preferring xylose reductase from *Pichia stipitis*. *Microbiology*, **153**, 3044-3054.

Watanabe, S., Saleh, A.A., Pack, S.P., Annaluru, N., Kodaki, T., Makino, K. 2007b. Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein engineered NADP+-dependent xylitol dehydrogenase. *Journal of Biotechnology* **130**(3), 316-319.

Weigert, C., Steffler, F., Kurz, T., Shellhammer, T.H., Methner, F.J. 2009. Application of a short intracellular pH method to flow cytometry for determining *Saccharomyces cerevisiae* vitality. *Applied and Environmental Microbiology*, **75**, 5615–5620.

Weimberg, R.J. 1961. Pentose oxidation by *Pseudomonas fragi*. *Journal of Biological Chemistry*, **236**, 629-635.

Wenger, J.W., Schwartz, K., Sherlock, G. 2010. Bulk segregant analysis by high-throughput sequencing reveals anovel xylose utilization gene from *Saccharomyces cerevisiae*. *PLOS Genetics*, **6**(5).

Westholm, J., Nordberg, N., Murén, E., Ameur, A., Komorowski, J., Ronne, H. 2008. Combinatorial control of gene expression by the three yeast repressors Mig1, Mig2 and Mig3. *BMC Genomics*, **9**(1), 601.

Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C.P., Boles, E. 1999. Concurrent knock out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Letters*, **464**(3), 123-128.

Winderickx, J., de Winde, J.H., Crauwels, M., Hino, A., Hohmann, S., Van Dijck, P., Thevelein, J.M. 1996. Regulation of genes encoding subunits of the trehalose synthase complex in *Saccharomyces cerevisiae*: novel variations of STRE-mediated transcription control? *Molecular and General Genetics*, **252**, 470-482

Xue, Y., Batlle, M., Hirsch, J.P. 1998. *GPR1* encodes a putative G protein-coupled receptor that associates with the Gpa2p Gα subunit and functions in a Ras-independent pathway. *The EMBO Journal*, **17**(7), 1996-2007.

Yamanaka, K. 1969. Inhibition of D-xylose isomerase by pentitols and D-lyxose. *Archives of Biochemistry and Biophysics*, **131**, 502-506.

Yi, D.G., Huh, W.K. 2015. UDP-glucose pyrophosphorylase Ugp1 is involved in oxidative stress response and long-term survival during stationary phase in *Saccharomyces cerevisiae*. *Biochemical and Biophysical Research Communications*, **467**(4), 657-663.

Yuhua, Z., Primavesi, L.F., Jhurreea, D., Andralojc, P.J., Mitchell, R.A.C., Powers, S.J., Schluepmann, H., Delatte, T., Wingler, A., Paul, M.J. 2009. Inhibition of SNF1-related protein kinase 1 activity and regulation of metabolic pathways by trehalose-6-phosphate. *Plant Physiology*, **148**, 1860–1871.

Zabaniotou, A. 2017. Redesigning a bioenergy sector in EU in the transition to circular waste-based bioeconomy - A multidisciplinary review. *Journal of Cleaner Production*, **177**, 197-206.

Zaman, S., Lippman, S., Zhao, X., Broach, J.R. 2008. How *Saccharomyces* responds to nutrients. *Annual Review of Genetics*, **42**(1), 27-81.

Zeng, W.Y., Tang, Y.Q., Gou, M., Xia, Z.Y., Kida, K. 2016. Transcriptomes of a xylose-utilizing industrial flocculating *Saccharomyces cerevisiae* strain cultured in media containing different sugar sources. *AMB Express*, **6**(1), 51.

Zha, J., Shen, M., Hu, M., Song, H., Yuan, Y. 2014. Enhanced expression of genes involved in initial xylose metabolism and the oxidative pentose phosphate pathway in the improved xylose-utilizing *Saccharomyces cerevisiae* through evolutionary engineering. *Journal of Industrial Microbiology & Biotechnology*, **41**(1), 27-39.

Zhao, X., Zhang, L., Liu, D. 2012a. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuels, Bioproducts and Biorefining*, **6**(4), 465-482.

Zhao, X., Zhang, L., Liu, D. 2012b. Biomass recalcitrance. Part II: Fundamentals of different pre-treatments to increase the enzymatic digestibility of lignocellulose. *Biofuels, Bioproducts and Biorefining*, **6**(5), 561-579.

Zhou, H., Cheng, J., Wang, B.L., Fink, G.R., Stephanopoulos, G. 2012. Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. *Metabolic Engineering*, **14**, 611-622.

Zhou, Q., Son, K., Liu, Y., Revzin, A. 2015. Biosensors for cell analysis. *Annual Review of Biomedical Engineering*, **17**(165–190).

Zurita-Martinez, S.A., Cardenas, M.E. 2005. Tor and cyclic AMP-protein kinase A: two parallel pathways regulating expression of genes required for cell growth. *Eukaryotic Cell*, **4**(1), 63-71.