ESCOLA POLITÉCNICA DA UNIVERSIDADE DE SÃO PAULO

Chemical Engineering Department

Metabolic Engineering of *Saccharomyces cerevisiae* for Second Generation Ethanol from Xylo-Oligosaccharides

Dielle Pierotti Procópio

Advisor: Thiago Olitta Basso

São Paulo - SP

2022

Dielle Pierotti Procópio

Metabolic Engineering of *Saccharomyces cerevisiae* for Second Generation Ethanol from Xylo-Oligosaccharides

Versão Corrigida

Tese apresentada à Escola Politécnica da
Universidade de São Paulo para obtenção
do título de Doutora em Ciências.

Área de concentração Engenharia Química

Orientador: Prof. Dr. Thiago Olitta Basso

São Paulo, SP

2022

Este exemplar foi revisado e alterado em relação à versão original, sob responsabilidade única do autor e com a anuência de seu orientador.

São Paulo, 08 de Março de 2022

Assinatura do autor 

Assinatura do orientador 

CATALOGAÇÃO-NA-PUBLICAÇÃO

Procópio, Dielle
Metabolic Engineering of Saccharomyces cerevisiae for Second Generation Ethanol from Xylo-Oligosaccharides / D. Procópio, T. Basso -- São Paulo, 2021.
153 p.

Tese (Doutorado) - Escola Politécnica da Universidade de São Paulo. Poli-Integra.

1.fermentação 3.Saccharomyces cerevisiae 4.xilose 5.etanol I.Universidade de São Paulo. Escola Politécnica. Poli-Integra II.t. IV.Basso, Thiago

Dedico esse trabalho àqueles que fazem esta minha jornada mais feliz: meus pais, Rosemary e Geraldo, meu irmão Diego, e meu marido, Ronaldo. Amo vocês mais que tudo!

AGRADECIMENTOS

Agradeço ao Departamento de Engenharia de Química e à USP por me acolherem e permitirem meu desenvolvimento profissional e pessoal.

Agradeço ao Prof. Dr. Thiago Olitta Basso pela orientação, dedicação, amizade, cervejas, confiança e claro, pelo grande ensinamento científico durante esse tempo. Sua orientação foi um grande diferencial na minha formação.

Agradeço à Profa. Dra. Rosana Goldbeck pela infinita disponibilidade, pela amizade e todo ensinamento, sempre contribuindo para o aprimoramento do meu conhecimento científico. A Profa. Rosana e o Dr. Gleidson Teixeira foram essenciais para determinar os objetivos do meu projeto de doutoramento. Muito obrigada a ambos, por toda dedicação para com este projeto.

Agradeço ao Prof. Dr. Yong-Su Jin e aos membros do JinLab pelo acolhimento, pelos ensinamentos e todo o apoio e dedicação ao meu projeto durante o tempo passado na Universidade de Illinois (UIUC). Apesar das dificuldades pelas quais passamos em função da pandemia do Covid-19, pude concluir com êxito meu propósito, e isso foi graças ao apoio de vocês.

Agradeço à Profa. Dra. Telma Franco, Prof. Dr. Marcelo Brandão, Dra. Lívia Brenelli, e Felipe Ciamponi por toda contribuição durante esses anos.

Agradeço ao Prof. Dr. David Leak e ao Dr. Emanuele Kendrick pelo acolhimento e ensinamentos durante o tempo passado na Universidade de Bath.

Agradeço à CAPES pela concessão da bolsa de doutoramento durante os anos 2017-2018. À FAPESP pelo financiamento do meu projeto doutorado, processo 2018/01759-4, e pela bolsa estágio de pesquisa no exterior BEPE, processo 2019/18075-3.

Agradeço a toda equipe do Laboratório de Engenharia de Bioprocessos (BELa), principalmente ao pessoal do segundo andar, pessoas maravilhosas que se tornaram grandes amigos e que sempre quero manter contato. Vocês tornaram esse percurso mais divertido, apesar de todas as dificuldades ineretens ao desenvolvimento de um projeto de pesquisa. Em especial, quero agradecer a Thais pela constante companhia durante os cultivos em reator e PCRs, e a Thamiris e Gabriel por toda ajuda com o HPLC.

Agradeço a todos amigos e colegas que participaram dessa trajetória, seja aqui no Brasil ou nos Estados Unidos, pelo acolhimento e ensinamentos. Vocês estão presentes em meu coração.

Agradeço imensamente à minha família, pelo apoio, incentivo e amor. Mesmo distantes estiveram sempre presentes na minha mente e em meu coração. À minha mãe Rosemary pelos ensinamentos, inspiração e grande incentivo aos meus estudos. Ao meu pai Geraldo pelos incentivos. Ao meu irmão Diego pelo apoio e amizade. Ao meu marido Ronaldo, por sempre se fazer presente, pelo incentivo, e por todo amparo. Aos meus sogros, Vanilda e Pedro, pelo apoio, carinho e paciência. Sem vocês eu nada seria... Amo muito vocês!!!

Aos Deuses principalmente, que me iluminam e me protegem todos os dias.

Enfim, a todos que de alguma forma contribuíram para a realização deste trabalho.

Muito Obrigada!

Há quem passe por um bosque e só veja lenha para a fogueira

(Liev Tolstoi)

RESUMO GERAL

A expansão da população humana tem levantado importantes questões relacionadas, por exemplo, à necessidade do aumento da demanda por alimentos, água, energia, bem como com os danos ao clima e ao meio ambiente. Nossa maior parte da fonte de energia provém do petróleo e a produção massiva de dióxido de carbono está ligada às mudanças climáticas globais. Os resíduos lignocelulósicos estão atraindo um interesse crescente no mundo inteiro como uma nova matriz energética para substituir o uso de recursos fósseis. Recentemente, com o desenvolvimento de estratégias de engenharia metabólica para a melhoria de linhagens microbianas, a fermentação pela levedura mais amplamente utilizada, a *Saccharomyces cerevisiae*, assumiu uma nova importância, proporcionando um potencial significativo para a produção de biocombustíveis e bioquímicos. Com base nessas afirmações, linhagens de *S. cerevisiae* tem sido modificadas para fermentar açúcares derivados de materiais lignocelulósicos. Entretanto, a fermentação da biomassa lignocelulósica apresenta uma série de desafios, posto que a geração dos açúcares fermentescíveis a partir dos resíduos lignocelulósicos requer pré-tratamentos químicos e físico-químicos severos os quais geram vários compostos tóxicos que inibem o crescimento dessa levedura que, por sua vez, afetará o rendimento dos produtos alvo. É também mencionada a incapacidade da levedura *S. cerevisiae* de fermentar todos os açúcares derivados da biomassa lignocelulósica, os quais devem ser abordados para viabilizar a produção industrial de biocombustíveis e bioquímicos. Portanto, na presente tese, nossos objetivos tiveram como foco alguns destes desafios, incluindo i) desenvolver uma linhagem de levedura capaz de fermentar xilose eficientemente, quando comparada com sua cepa parental. A linhagem evoluída obtida, DPY06, apresentou um aumento de 70% na taxa de consumo de xilose às 72h de cultivo em comparação com a linhagem parental; ii) expandir as capacidades da uma linhagem de *S. cerevisiae* para fermentar xilo-oligossacarídeos. Para atingir tal objetivo, foi construída a linhagem SR8A6S3-CDT_2-$GH43_{2/7}$. Cultivos realizados em xilana hidrolisada resultaram em um rendimento de etanol 84% superior que o cultivo controle; iii) a seleção da uma linhagem de *S. cerevisiae* que é naturalmente mais adaptada ao crescimento na presença dos inibidores tóxicos presentes nos hidrolisados lignocelulósicos. Esse estudo reveleou o excelente desempenho da linhagem industrial SA-1; iv) investigar como uma linhagem industrial de *S. cerevisiae* responde ao ácido *p*-cumárico. Os dados obtidos indicaram importantes mudanças fisiológicas em cultivos de quimióstato na presença de 7 mM de *p*-cumárico e v) investigar a síntese de bio-polímeros por uma linhagem de *S. cerevisiae* capaz de fermentar xilose e acetato.

ABSTRACT

The expansion of the human population has raised important questions concerned, for example, the need for increasing demand for food, water, energy, as well climate, and environmental damage. The majority of energy sources are from petroleum and the massive production of carbon dioxide from high-energy consumption links to global climate changes. Lignocellulosic residues are attracting increasing interest worldwide as a new energy matrix to replace the usage of fossil resources. In more recent years, the development of metabolic engineering strategies for strain improvement, microbial fermentation by the widely used yeast, *Saccharomyces cerevisiae*, has taken on a new dimension, providing significant potential for producing advanced biofuels and biochemicals. In the light of these statements, *S. cerevisiae* strains have been reprogrammed to ferment sugars derived from lignocellulosic materials. However, fermentation of lignocellulosic biomass suggests many challenges, such as the generation of fermentable sugars from lignocellulosic residues requires harsh chemical and physicochemical pre-treatments which would generate various toxic compounds that inhibit the growth of microorganisms which in turn affect the yield of target products, as well as the inability of *S. cerevisiae* cells to ferment all available sugars from lignocellulosic biomass, which must be addressed to make feasible the industrial production of biofuels and biochemicals. Therefore, in the present Thesis, our goals were addressed to face some of these challenges, including i) to develop an evolved yeast strain which is capable of efficiently ferment xylose, when comparing with its parental strain. An evolved strain was developed, DPY06, which exhibited an increase of 70% on xylose consumption rate at 72h of cultivation in comparison to its parental strain; ii) to expand the capabilities of industrial and laboratory *S. cerevisiae* strain to utilize plant-derived xylo-oligosaccharides. To achieve this goal, the engineered strain, SR8A6S3-CDT_2-$GH43_{2/7}$ was constructed. In cultivations using a hydrolyzed xylan, the ethanol yield was 84% higher for the engineered strain in comparison with its parental strain; iii) screening the best fitness of *S. cerevisiae* strain against toxic inhibitors in lignocellulosic hydrolysates. This screening was able to reveal the outstanding performance of one of the industrial strains (SA-1) over the strains evaluated; iv) investigating how *S. cerevisiae* yeast cells respond to the presence of the *p*-coumaric acid. The dataset obtained indicated important physiological changes in glucose-limited chemostat cultivations in the presence of 7 mM; and v) to investigate a polymer synthesis by xylose-acetate utilizing *S. cerevisiae* strain when fermenting glucose, xylose, and acetate.

CONTENTS

INITIAL SUMMARY

Lignocellulosic biomass represents the most abundant material on earth (Chandel et al. 2012) which makes it an important resource in second-generation biofuel and chemicals production. Its composition includes cellulose, hemicellulose, and lignin, in other words, complexes carbohydrates, and phenolic compounds. There are widespread potent organisms able to degrade the polymeric structure that composes cellulose and hemicellulose. These organisms that can be fungi, bacteria, yeast, marine algae, protozoans, snails, crustaceans, and insets (Jeffries 1983; Biely 1985; Kulkarni et al. 1999; Katahira et al. 2004; Schmoll and Schuster 2010) can provide to us the 'tools' to use this abundant carbon source which is lignocellulosic biomass to produce desired products such as biofuels. Alongside the carbohydrates, aromatic compounds are the second most abundant class of organic compounds in nature (Adeboye et al. 2014). They form the building blocks of lignin and they play a crucial role in plants' resistance to diseases and infections (van der Pol et al. 2014; Adeboye et al. 2014; Maurya et al. 2015). During biofuel production, lignocellulosic materials are first subjected to pretreatment processes and hydrolysis which release a wide range of inhibitory compounds that strongly affect the fermentation performance of microorganisms (Palmqvist and Hahn-Hägerdal 2000a; Ask et al. 2013).

The long-term goal of my Ph.D. study was to develop a superior yeast strain host with desirable phenotypes for hemicellulose hydrolysates fermentation, in other words, a robust strain able to convert xylo-oligomers to ethanol. The engineering of xylo-oligosaccharide-consuming *S. cerevisiae* strains is a current goal for more effective utilization of lignocellulosic biomass and the development of economic industrial processes. In **Chapter I**, we present a review of the multiple strategies adopted to developing a new metabolic engineering host to produce hemicellulosic ethanol.

The industrial SA-1 *S. cerevisiae* strain appeared to be more robust in inhibitory compound-containing media than the remaining strains, other industrial and laboratory strains (as shown in **Chapter VI**). According to its differentiated capacity to grow under inhibitory conditions, this was selected for the next goal of my study, to construct a strain able to produce hemicellulosic ethanol. At this step of my Ph.D. study, we intended to expand the sugar catabolism of *S. cerevisiae* strain, though developing a strain able to consume oligomers derived from hemicellulosic hydrolysate materials, instead of monomers only. Previously to achieve a strain able to consume xylo-oligomers, an industrial xylose-utilizing *S. cerevisiae* strain, SA-1 XR/XDH,

derived from SA-1, which expresses xylose oxidoreductase pathway from *Scheffersomyces stipittis* (encoded by *xyl1*, *xyl2*, and *xyl3*), was submitted to evolutionary engineering in xylose-limiting chemostat cultivation for 64 days to improve its xylose-uptake ability, yielding the DPY06 strain, that showed better traits than its parental strain, as presented in **Chapter II**. In **Chapter III**, DPY13 was constructed by genetic manipulations of SA-1 XR/XDH through the transformation of a plasmid that carries an intracellular xylo-oligosaccharide (XOS) consumption pathway from *Neurospora crassa*. Since SA-1 XR/XDH is a polyploid strain, in **Chapter IV**, we isolated a stable haploid of SA-1 XR/XDH, yielding the DPY07 strain, which showed desirable traits as demonstrated. DPY07 was developed to be easier for genetic manipulation and further strain optimization, such as efficient XOS fermentation.

To achieve our goal of achieving a strain able to metabolize XOS, in **Chapter V**, an acetate-utilizing strain was subject to genetic manipulations through the integration of XOS-consumption pathway on it. By simultaneous consumption of xylose, XOS, and acetate, the resulted strain, SR8A6S3-CDT_2-$GH43_{2/7}$, showed ethanol yield higher than its parental strain when cultivated in hemicellulosic hydrolysate and hydrolyzed xylan. Furthermore, the engineered strain was able to produce ethanol through simultaneous co-utilization of XOS, xylose and acetate. When a hemicellulosic hydrolysate was used, SR8A6S3-CDT_2-$GH43_{2/7}$ produced higher ethanol content than the control strain without the XOS consumption pathway. The consumption of XOS, xylose and acetate can expand the capabilities of *S. cerevisiae* to utilize plant-derived and represent the potential to increase the efficiency of second-generation biofuel production.

In **Chapter VI** we investigated numerous inhibitory compounds on physiology parameters of 4 *Saccharomyces cerevisiae* strains to assess their tolerance under the compounds investigated. We selected SA-1 as the best-performance strain considering the duration of lag-phase and growth rate. Therefore, a robust 1G yeast strain showed to be an ideal candidate for lignocellulosic processes. Once a robust industrial strain was selected a thorough investigation was conducted in **Chapter VII**. The influence of *p*-coumaric acid on SA-1 physiology was studied under an anaerobic glucose-limited chemostat and when in the presence of 7 mM *p*-coumaric acid, the ethanol yield was higher.

In the last chapter, **Chapter VIII**, we presented an interesting result found during batch fermentation experiments of acetate-consuming *S. cerevisiae* strain. When it is cultivated under

high amounts of xylose, acetate, and glucose an unknown visible-naked-eye extracellular material was produced which is under investigation.

I would like to describe here the order in which the study was conducted. The choice to present my Ph.D. thesis in that way was to facilitate the understanding regarding the construction of an *S. cerevisiae* strain able to consume XOS, my main goal. However, in the practical, I started studying the influence of lignocellulosic inhibitors on yeast metabolism. After noticing SA-1 presented good performance, this strain was selected as a host for the XOS-consuming pathway. Thereby, I started investigating and improving its xylose uptake through evolutionary engineering in parallel with the introduction of XOS genes into its cell. Finally, SR8A6S3-CDT_2-$GH43_{2/7}$ was constructed while I complete my scholarship at the University of Illinois at Urbana-Champaign, under Dr. Jin's supervision. SR8A6S3 was a strain constructed by his group. During my cultivation with Dr. Jin's strain, I noticed an extracellular material as described in **Chapter VIII**.

References

Adeboye, P. T., Bettiga, M., and Olsson, L. (2014). The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in Saccharomyces cerevisiae in lignocellulose hydrolysates. *AMB Express* 4, 46. doi:10.1186/s13568-014-0046-7.

Ask, M., Bettiga, M., Mapelli, V., and Olsson, L. (2013). The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing Saccharomyces cerevisiae. *Biotechnol. Biofuels* 6, 22. doi:10.1186/1754-6834-6-22.

Chandel, A. K., Chandrasekhar, G., Silva, M. B., and Silvério da Silva, S. (2012). The realm of cellulases in biorefinery development. *Crit. Rev. Biotechnol.* 32, 187–202. doi:10.3109/07388551.2011.595385.

Maurya, D. P., Singla, A., and Negi, S. (2015). An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. *3 Biotech* 5, 597–609. doi:10.1007/s13205-015-0279-4.

Palmqvist, E., and Hahn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. *Bioresour. Technol.* doi:10.1016/S0960-8524(99)00160-1.

van der Pol, E. C., Bakker, R. R., Baets, P., and Eggink, G. (2014). By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels. *Appl. Microbiol. Biotechnol.* 98, 9579–9593. doi:10.1007/s00253-014-6158-9.

CHAPTER I: XYLO-OLIGOSACCHARIDE CONSUMPTION BY ENGINEERED *SACCHAROMYCES CEREVISIAE* TO PRODUCE ETHANOL: A REVIEW

The content of this chapter was published in *Frontiers in Bioengineering and Biotechnology* (doi.org/10.3389/fbioe.2022.825981). Procópio, Dielle Pierotti; Kendrick, Emanuele; Franco, Telma Teixeira; Goldbeck, Rosana; Damasio, André Ricardo de Lima; Leak, David; Jin, Yong-Su; Basso, Thiago Olitta.

Xylo-oligosaccharide consumption by engineered *S. cerevisiae* to produce ethanol: a review

ABSTRACT

The engineering of xylo-oligosaccharide-consuming *Saccharomyces cerevisiae* strains is a promising approach for more effective utilization of lignocellulosic biomass and the development of economic industrial fermentation processes. Extending the sugar consumption range without catabolite repression by including the metabolism of oligomers instead of only monomers would significantly improve second-generation ethanol production This review focuses on different aspects of the action mechanisms of xylan-degrading enzymes from bacteria and fungi, and their insertion in *S. cerevisiae* strains to obtain microbial cell factories able of consume these complex sugars and convert them to ethanol. Emphasis is given in different strategies for ethanol production from both extracellular and intracellular xylo-oligosaccharide utilization by *S. cerevisiae* strains. The suitability of *S. cerevisiae* for ethanol production combined with its genetic tractability indicates that it can play an important role in xylan bioconversion through the heterologous expression of xylanases from other microorganisms.

Keywords: Xylo-oligosaccharides; *Saccharomyces cerevisiae*; lignocellulosic ethanol; xylose; xylanases.

1.1. BACKGROUND

High fermentative capacity and robustness make *Saccharomyces cerevisiae* the organism of choice for ethanol production. *S. cerevisiae* is the most widely used strain in the ethanol industry with a well-characterized genome sequence, besides being an efficient expression system for recombinant enzyme production (Hou et al., 2012; Fang et al., 2017; Oh and Jin, 2020). Industrial *S. cerevisiae* strains are highly tolerant against various stresses in industrial environments, such as low pH, high osmotic pressure, high alcohol concentration, and phage contamination. In addition, this yeast presents high ethanol productivity, resulting from the naturally selective process that occurs during the successive fermentations involving pitching between fermentation tanks (Della-Bianca et al., 2014; Nielsen, 2019).

Economic production of second-generation biofuels should include the efficient and simultaneous co-fermentation of all hydrolysable sugars derived from cellulose and hemicellulose (Li et al., 2015). Lignocellulose-derived ethanol provides environmental and economic benefits, as significant reductions in the disposal of solid wastes, and less air pollution, besides representing a promising industry in the expected transition from fossil to renewable fuels and chemicals. This biofuel is an environmentally friendly liquid fuel because the exhaust carbon dioxide is being taken up by growing plant biomass, contributing to the reduction of CO_2 content in the air, which in turn, contributes to the equilibrium of the earth's atmosphere (Jacobsen and Wyman, 2000; Palmqvist and Hahn-Hägerdal, 2000a; Galbe and Zacchi, 2002).

Hemicellulose and cellulose are the major components of the secondary layers of the cell wall in wood fiber, shaping the well-known natural compost, lignocellulosic biomass, along with lignin and minor components such as extractives and minerals (Gírio et al., 2010). Lignocellulose represents the most abundant source of renewable material on earth. This material can be found in agricultural residues, forestry waste, municipal solid waste, woods, and grasses, making them widely available at low cost, which is advantageous to the industrial context (Palmqvist and Hahn-Hägerdal, 2000b; Dahlman et al., 2003). Hemicellulose is a heterogeneous group of polysaccharides that comprises 15-35% of plant biomass (Gírio et al., 2010). Achieving 31.4% in switchgrass (Sun and Cheng, 2002), 29.3% in willow (Jorgensen et al., 2007), 28.6% in sugarcane bagasse (Fernandes Pereira et al., 2011), 22.1% in corn stover (Jorgensen et al., 2007), 19.7% in birchwood (Jorgensen et al., 2007) and 18% in spruce (Tengborg et al., 1998). Besides different amounts, the distribution of hemicellulose varies significantly between different plants. Depending

on the source of the biomass (softwoods or hardwoods), its structure and composition can also vary. Softwood hemicellulose (pine and spruce, for example) presents a higher proportion of mannose and glucose units than hardwood hemicellulose (such as *Eucalyptus*, willow, and oak), which in turn, has a higher ratio of xylose units typically acetylated (Palmqvist and Hahn-Hägerdal, 2000b; Dahlman et al., 2003). The dominant hemicellulose polymer in hardwood biomass, xylan, is composed of repeating β (1-4)-linked xylose residue backbone, with acetyl and (methyl)glucuronic acid side groups. However, variations exist in its structures between different species (Rennie and Scheller, 2014; Wierzbicki et al., 2019).

Until recently, xylan represented the main component of plant biomass that cannot be efficiently utilized for biofuels production by fermentation using modified *S. cerevisiae* yeast strain. However, in 2004, Katahira and coauthors first demonstrated that a xylose-consuming *S. cerevisiae* strain expressing xylanolytic enzymes was able to produce ethanol from hemicellulose fraction although in lower levels (Katahira et al., 2004). Xylo-oligosaccharides (XOS)-consuming *S. cerevisiae* strains can represent an essential step to reach a more cost-effective second-generation ethanol production, conferring three significant advantages: (i) less intensive pre-treatment conditions would be required – harsh lignocellulosic pretreatment has been applied to release monomers (fermentable sugars), however during this process several yeast growth inhibitors are formed, such as furans, organic acids, phenols, and inorganic salts. Different aspects can interfere with the severity of the pretreatment process, which include holding time, pH, and temperature (Pedersen et al., 2010). The lower severity process can result in high amounts of oligosaccharides, lower monosaccharides, and lower inhibitors compounds, as presented by Brenelli et al. (2020). In their study, the authors evaluated the effect of a mild deacetylation treatment accomplished by hydrothermal pretreatment of raw sugarcane straw and achieve 81.5% of soluble hemicellulose with XOS yields up to 9.8% (w/w of an initial straw). These investigators found that an increase in the pretreatment temperature from 180 °C to 210 °C, achieving a severity factor greater than 4, was accompanied by an increase in xylose production and lower oligosaccharides production. Under a lower severity factor condition (3.95) the crude hydrolysate yielded approximately 13.5 g L^{-1} soluble XOS as well low amounts of arabinose, xylose, formic acid, acetic acid, and furfural were obtained. Increased temperature is related to an increase in the severity of the treatment, resulting also in the formation of inhibitors for both the enzymatic and fermentation processes (Pedersen et al., 2010). It is worth mentioning that the depolymerization of

cellulose and solubilization of hemicellulose and lignin vary according to the proposed pretreatment process and the severity factor applied in the respective process (Lynd et al., 2002)." Moreover, the preparation of hemicellulose hydrolysate includes acid addition, high pressure and temperature which cause environmental pollution and equipment corrosion; therefore, successful ethanol production through XOS fermentation would make the process more environmentally friendly (Woodward and Wiseman, 1982; Gueguen et al., 1997; Nevoigt, 2008; Li et al., 2013); (ii) lower demand for xylanolytic enzymes would be required, achieving lower production costs – the biomass enzymatic hydrolysis is a crucial step in the overall process due to its relatively large contribution to the total cost of lignocellulosic-derived ethanol (Nieves et al., 1997; Galbe and Zacchi, 2002); to maximize xylose yield and minimize the production of inhibitors, higher amounts of xylanolytic enzymes are required for total degradation of xylan and XOS which is far prohibitively expensive on an industrial scale (Galbe and Zacchi, 2002), milder pretreatment methods have been described and (iii) industrial competitive advantages (mainly for recombinant microorganism which are able to uptake and consume XOS internally) - it is expected that XOS-consuming *S. cerevisiae* strains would have a competitive advantage concerning other microorganisms, such as contaminating bacteria and wild *Saccharomyces* and non-*Saccharomyces* species that naturally use xylan as carbon source (Cabrini and Gallo, 1999; Amorim et al., 2011). It is important to point out that, in order to obtain a second-generation ethanol cost-competitive with first-generation ethanol, it is crucial to obtain microorganisms with unique genotype features to hydrolyze hemicellulose internally through recombinant DNA technology, which represents the best option to overcome the barriers to the commercial exploitation of lignocellulosic bioethanol.

Heterologous expression of xylose and XOS-producing enzymes in *S. cerevisiae* has been extensively reported. However, only one study has reported an *S. cerevisiae* strain able to break xylan down in an intracellular environment (Li et al., 2015). For this reason, although there are engineering efforts to improve direct xylan utilization by this microorganism, some limitations still remain, such as the affinity between XOS and cell membrane transporters, and the understanding of metabolic pathways regulation. This overview examines all strategies reported to date adopted for the re-construction of XOS assimilation in *S. cerevisiae* yeast strains, focusing on those that bioethanol could be bio-converted from hemicellulose fraction.

1.2. LATEST TRENDS IN XYLOSE-UTILIZING *S. CEREVISIAE*

Although *S. cerevisiae* strains present all genes required for the xylose fermentation, i.e., xylose reductase (*XR*), xylitol dehydrogenase (*XDH*), and xylulokinase (*XKS1*), only *XKS1* has been functionally expressed. *XKS1* phosphorylates xylulose into xylulose-5-phosphate which is introduced into the central metabolism through the pentose-phosphate pathway. Previous studies have reported that the wild type of *S. cerevisiae* is capable of naturally assimilating xylulose as a sole carbon source, although at a low rate, under aerobic conditions. However, xylulose is a rare pentose not widely available in nature and probably due to this, the challenge of directing xylulose fermentation by *S. cerevisiae* has received little attention (Jeffries, 1983; Eliasson et al., 2000; Mittelman and Barkai, 2017; Patiño et al., 2019). Furthermore, xylose fermentation by *S. cerevisiae* requires additional interventions in endogenous genes expression and/or kinetic properties (Patiño et al., 2019).

With a focus on second-generation bioproducts, it is not surprising that many studies have attempted to develop laboratory and industrial engineered *S. cerevisiae* strains capable of simultaneous glucose and xylose fermentation by the expression of heterologous xylose consumption genes (Eliasson et al., 2000; Kuyper et al., 2005; Kwak and Jin, 2017; Li et al., 2019). Many studies have shown that different mutations can improve xylose fermentation by yeast. In Table 1 we benchmark five xylose-utilizing strains with superior ethanol yields on xylose metabolism.

In nature, pentose assimilation is widespread across many prokaryotes and eukaryotes, such as *Pseudomonas fragi* (Weimberg, 1961), *Klyyveromyces lactis* (Margaritis and Bajpai, 1982), *Scheffersomyces stipitis* (Toivola et al., 1984), *Candida shehatea* (Toivola et al., 1984), *Pachysolen tannophilus* (Smiley and Bolen, 1982; Toivola et al., 1984), *Trichoderma* sp. (Kulkarni et al., 1999), *Aspergillus* sp. (Kulkarni et al., 1999), *Cryptococcus adeliae* (Petrescu et al., 2000), *Pseudoalteromonas haloplanktis* (Van Petegem et al., 2002), *Hansenula polymorpha* (Ryabova et al., 2003), *Bacillus halodurans* (Honda and Kitaoka, 2004), *Bacillus subtilis* (Collins et al., 2006), *Caulobacter crescentus* (Stephens et al., 2007), *Plectosphaerella cucumerina* (Zhang et al., 2007), *Haloferax volcanii* (Johnsen et al., 2009), *Aurebasidium pullulans* (Yegin, 2017). To date, three different pathways for xylose assimilation have been identified in these microorganisms and they are differentiated by the involvement of a phosphorylation step (Figure 1). In the first possibility, xylose is isomerized to xylulose and then phosphorylated to form xylulose-5-P. Two metabolic

pathways have been identified which involve this strategy: the redox pathway, involving the combined activity of pyridine-nucleotide-dependent xylose reductase (XR) and xylitol dehydrogenase (XDH), and the isomerization pathway involving the redox-cofactor-independent xylose isomerase (XI). The main difference between these pathways is the dependence on cofactors (oxido-reduction pathway) or not (isomerization pathway). Both metabolic pathways have been used extensively as targets in engineered *S. cerevisiae* and have been reviewed in detail (Kim et al., 2012, 2013b; Harner et al., 2015; Kwak and Jin, 2017; Bracher et al., 2019). The generation of xylulose-5-phosphate via the oxidoreductase pathway allows a link to glycolysis, the central carbon flux, through the non-oxidative part of the pentose phosphate pathway (Stincone et al., 2015). Optimized *S. cerevisiae* recombinant strains overexpressing XI or XR/XDH have been reported (Table 1). The success of these strategies enables new perspectives on the carbon-source range assimilated by *S. cerevisiae* to be considered. Since xylose assimilation by engineered *S. cerevisiae* strains has become well-established, new approaches have been adopted to enable *S. cerevisiae* to consume XOS instead of xylose and glucose (La Grange et al., 2000, 2001; Fujita et al., 2002; Qian et al., 2003; Katahira et al., 2004; Lee et al., 2009; Sun et al., 2012; Li et al., 2015; dos Reis et al., 2016; Sekar et al., 2016; Zhang et al., 2017a). Scientific interest in this field is increasing steadily, but still much must be done to obtain an efficient XOS-consuming *S. cerevisiae* strain.

An additional xylose assimilation possibility is the so called Weimberg pathway, which is characterized as an oxidative but non-phosphorylating metabolic pathway without xylose to xylulose isomerization. This pathway received much less attention when compared with oxido-reductase and isomerase pathways but recently has gained major attention from research groups (Shen et al., 2020). This pathway provides an alternative entry point for xylose into yeast central metabolism with possibilities to produce new compounds that are intermediates or derivatives from the TCA cycle and provides an interesting route for the production of xylose-derived α-ketoglutarate (Figure 1) (Weimberg, 1961). Recently, the Weimberg pathway enzymes derived from *Caulobacter crescentus* and *Corynebacterium glutamicum* were functionally expressed in *S. cerevisiae*; however, pathway intermediates were detected, indicating that this pathway needs further optimization (Borgström et al., 2019). Some of the drawn hypotheses could explain this incompatibility between prokaryotic and eukaryotic proteins, such as deficiency of enzymatic cofactors, posttranslational modifications of the protein, differences in the internal pH of the

parental and the host cell (Sarthy et al., 1987), and the improper folding of the protein (Gárdonyi and Hahn-Hägerdal, 2003; Xia et al., 2016).

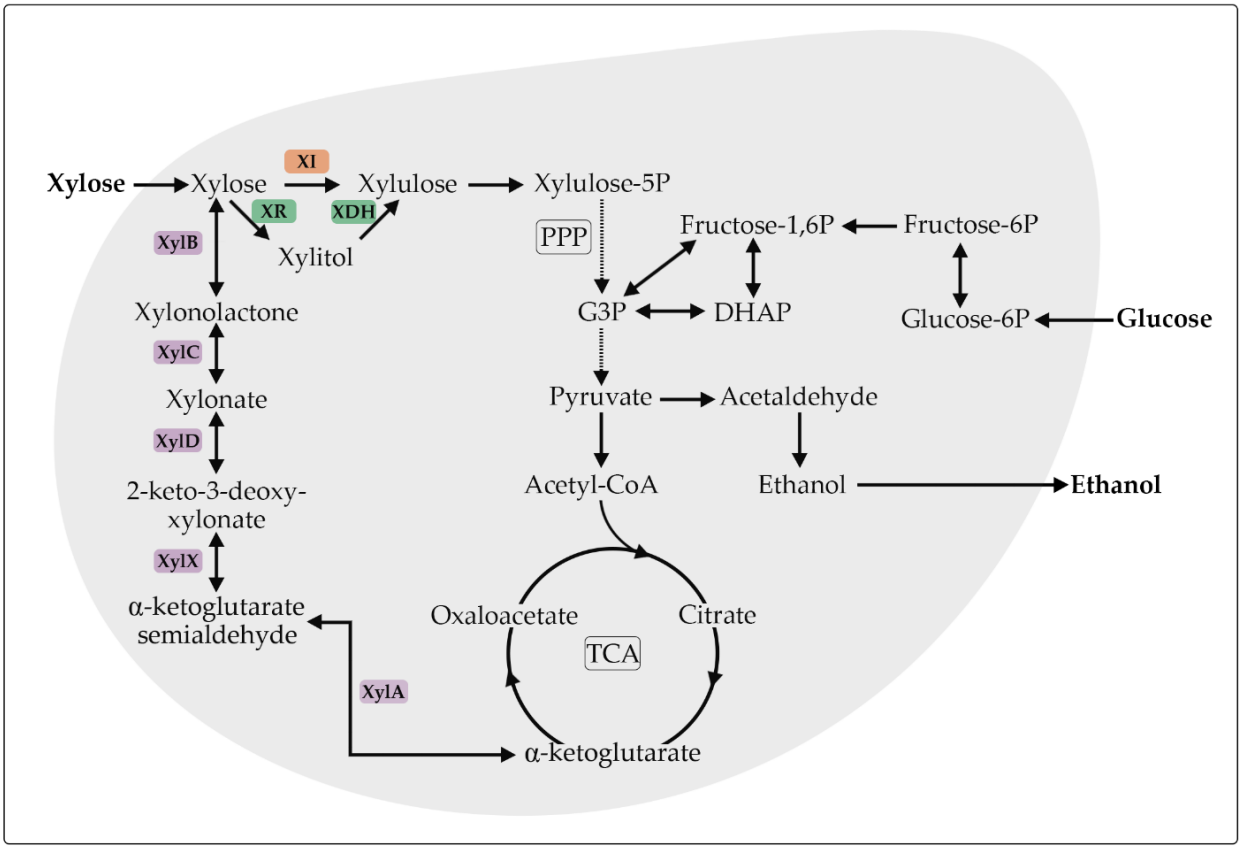

Fig. 1.1. Schematic overview of the xylose degradation pathway associated with the central carbon metabolism in yeast. The orange box indicates the redox-cofactor-independent xylose isomerase (XI), green boxes indicate the pyridine-nucleotide-dependent xylose reductase (XR) and xylitol dehydrogenase (XDH), and purple boxes indicate the five reaction steps of the Weimberg pathway catalyzed by xylose dehydrogenase (XylB), xylonolactonase (XylC), xylonate dehydratase (XylD), 3-keto-2deoxy-xylonate dehydratase (XylX), and α-ketoglutarate semialdehyde dehydrogenase (XylA). Abbreviations: PPP – pentose phosphate pathway, TCA – tricarboxylic acid cycle, G3P – glyceraldehyde 3-phosphate, DHAP – dihydroxyacetone phosphate. Adapted from (Borgström et al. 2019).

Table 1.1. Literature data on engineered, xylo-oxidoreductase and xylose-isomerase -based *S. cerevisiae* strains

Strain	Parental strain	Relevant genotype/ features	Culture conditions	Xylose specific consumption rate (g g^{-1} h^{-1})	Ethanol production rate(g g^{-1} h^{-1})	Ethanol productivity (g L^{-1} h^{-1})	Ethanol yield (g g_{xylose}^{-1})	Reference
LVY34.4	PE-2 (*MATα*)	XI - *Orpinomyces* sp. *XYLA*, *XKS1*, *TAL1*, *RKI1*, *TKL1*, *RPE1*, *Δgre3*, evolved	Microaerobic batch, YPX, 3% xylose, ICW 0.25 g DCW L^{-1}	1.320	0.620	ND	0.460	(Dos Santos et al. 2016)
XUSE	BY4741 (*MATα his3 leu2 met15 ura3*)	XI - *Piromyces* sp. *XYLA*, *XKS1*, *TAL1*, Δ*gre3*, Δ*pho13*, evolved	Microaerobic batch, YSC, 2% xylose, OD_{600} 10	ND	ND	ND	0.400	(Tran Nguyen Hoang et al. 2018)
IMU078	CEN.PK113-5D (*MATa ura3*)	XI – *Piromyces* sp. *XYLA*, *RPE1*, *RKI1*, *TAL1*, *TKL1*, *NQM1*, *TKL2*, *XKS1*, Δ*gre3*	Anaerobic batch, Synthetic medium with L-aspartate instead of ammonium sulphate, 2% xylose, ICW 0.02 g DCW L^{-1}	ND	ND	ND	0.406	(Bracher et al. 2019)
SR8N	D425-2 (*MATa his3 leu2 ura3*)	XR/XDH - *S. stipitis XYL1*, *XYL2* and *XYL3*, *Lactococcus lactis NoxE*, *Δpho13*, *Δald6*	Microaerobic batch, YNB, 4% xylose, OD_{600} 10	ND	ND	1.220	0.391	(Kim et al. 2013c; Zhang et al. 2017b)
YRH1490	PE-2 (*MATα*)	XR/XDH - *S. stipitis XYL1* and *XYL2*, *XKS1*	Microaerobic batch, YPX, 8% xylose, OD_{600} 1	ND	ND	0.310	0.330	(Dias Lopes et al. 2017)

ICW = Initial cell weight
OD_{600} = Initial OD_{600}
ND no data available

1.3. XYLANOLYTIC ENZYMES SYSTEM

The most selective method for the conversion of poly- to monosaccharides is by using enzymes. Most studies on hemicellulases have focused on xylanolytic enzymes, which are responsible for xylan hydrolysis (Gírio et al., 2010). The study of microorganisms able to hydrolyze xylan started more than 130 years ago, probably in 1889 by Hoope-Seyler (Whistler and Masak Jr., 1955). Since then, many organisms with the ability to colonize and grow on plant biomass have been identified. Xylanolytic enzyme producers are widespread, such as fungi, bacteria, yeast, marine algae, protozoans, snails, crustaceans, and insects, of which bacterial and fungal xylanases have the most important role concerning heterologous expression in *S. cerevisiae* (Jeffries, 1983; Biely, 1985; Kulkarni et al., 1999; Katahira et al., 2004; Schmoll and Schuster, 2010). *Trichoderma reesei*, *Trichoderma atroviride*, *Trichoderma virens* (Beg et al., 2001), *Aspergillus niger* (La Grange et al., 2001), *Neurospora crassa* (Li et al., 2015), *Aspergillus foetidus* (Whistler and Masak Jr., 1955), *Bacillus pumilus* (Pan et al., 1991) are some examples of potent xylanolytic enzymes producers. These enzymes have potential for the application of xylanases in several industries, such as in the pulp and paper, food additives, animal feed, textiles, drinks industries, ethanol, and xylitol production (Polizeli et al., 2005). The search for newer microbial xylanases producers is ongoing, together with molecular biology studies on the regulation of xylanases expression and their heterologous expression in non-xylanolytic microorganisms.

Xylans represent a family of complex non-cellulosic branched polysaccharides that consists structurally of linear homopolymeric β-(1,4)-xylopyranosyl units with a diversity of substituted groups, which vary quantitatively and qualitatively according to the plant or the method of isolation. They can be comprised of 4-*O*-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or *p*-coumaroyl groups (Figure 2) (Wong et al., 1988; Collins et al., 2005; Biely et al., 2016). Wood xylan exists as *O*-acetyl-4-*O*-methylglucuronoxylan in hardwoods and as arabino-4-*O*-methylglucuronoxylan in softwoods, which represent the two major forms of xylan in wood, whereas xylans in grasses and annual plants are typically arabinoxylans (Kulkarni et al., 1999). On the other hand, in esparto grass, tobacco stalks, and guar seed husk another type of xylan has been identified, the homoxylans which are composed exclusively of xylosyl residues (Sunna and Antranikian, 1997).

Due to the heterogeneity and complex chemical nature of xylan, its complete breakdown requires the action of a consortium of enzymes with diverse specificities and modes of action (Figure 2). Thus, it is not surprising that xylan-degrading organisms produce a multienzyme system of xylanases that present diverse structures, different mechanisms of action, substrate specificities, hydrolytic activities, and physicochemical characteristics (Beg et al., 2001; Collins et al., 2005; Moreira and Filho, 2016). It is interesting to note that microorganisms can produce multiple forms of the same xylanase family, showing that some factors such as differential processing of mRNA, post-translational modification, proteolytic digestion, and differential expression by distinct alleles of one gene, or even by completely separate genes affect this multiplicity of xylanases secreted (Polizeli et al., 2005; de Vries et al., 2017).

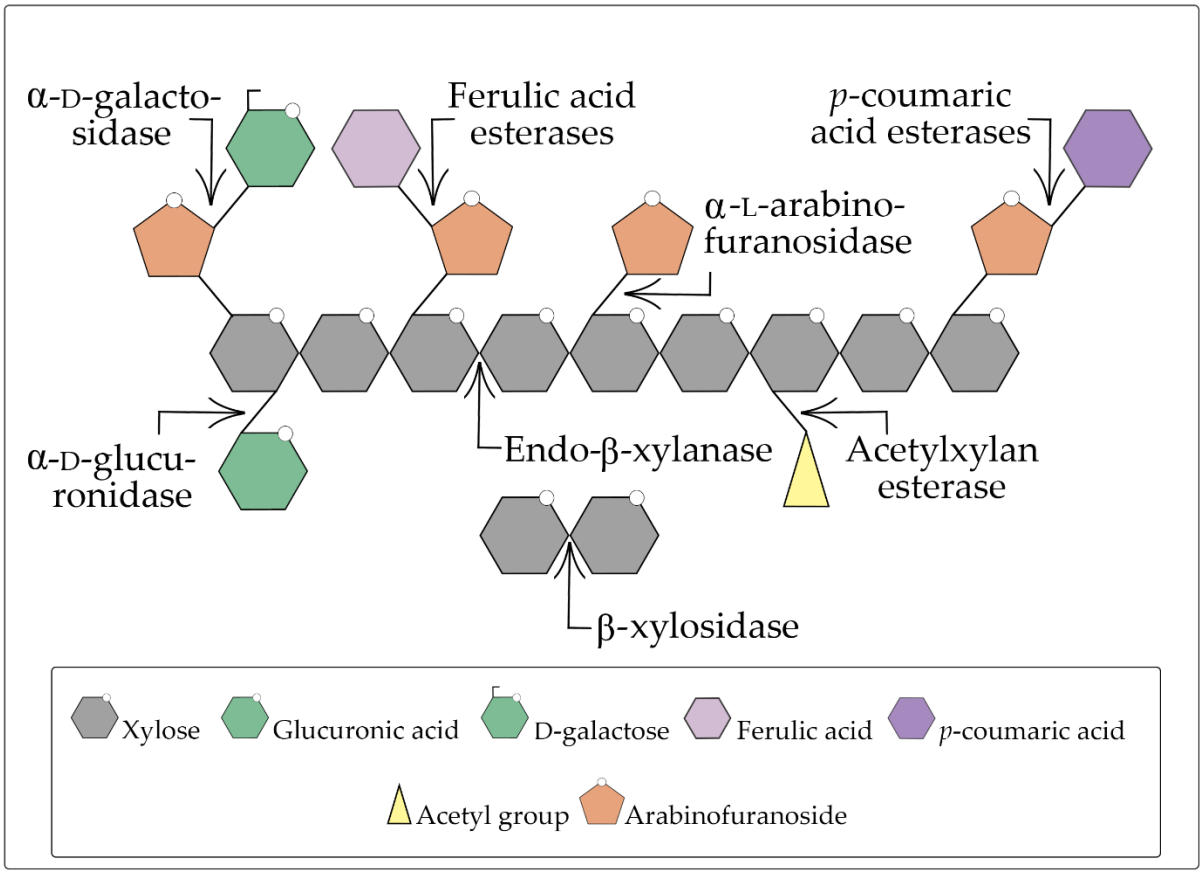

Fig. 1.2. Xylan degradation scheme. The arrows represent each enzyme activity for a determined substrate. Adapted from (Bhardwaj et al. 2019).

The carbohydrate-active enzymes (CAZy – www.cazy.org) database collectively complies and assigns xylanases are glycoside hydrolases (GH) that catalyze the hydrolysis of 1,4-β-D-

xylosidic linkages in xylan. Sequence-based classification has grouped xylanases in two major families GH10 and GH11, but xylanases are also found in other GH families, 3, 5, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, 62, 98 and 141 (Collins et al., 2005; Chakdar et al., 2016; Velasco et al., 2019). GH10 members are highly active on short XOS, thereby indicating small substrate-binding sites. The major enzymes of this family are endo-β-1,4-xylanases (Collins et al., 2005). In contrast, the enzymes that belong to the GH11 family are most active on long-chain XOS. Furthermore, this family is monospecific, which means they are exclusively active on D-xylose-containing substrates (Collins et al., 2005; Chakdar et al., 2016). Some genera of fungi and bacteria produce more than one subfamily of xylanases. However, the majority of the bacterial xylanases belong to the GH10 family, whereas fungal xylanases majorly belong to the GH11 family (Liu et al., 2011). There is a great diversity of xylanases produced among bacterial genera with *Bacillus* presenting a potential source of these enzymes (Subramaniyan and Prema, 2002; Gupta and Verma, 2015; Chakdar et al., 2016). Xylanases obtained from bacterial sources are known to be active and stable in a wide range of pH and temperature, such as temperature from 30 to 60 °C, pH from 5.0 to 9.0 (See some examples in Table 2). These enzymes are produced alone mostly, thereby reducing the downstream process time (Chakdar et al., 2016).

Despite the great diversity of bacterial xylanase producers, filamentous fungi are the major commercial source due to their higher levels of xylanase secretion (Kulkarni et al., 1999; Polizeli et al., 2005). Some works have demonstrated that many fungal species produce xylanase when cultured on cellulose (Mishra et al., 1984; Biely, 1985; Wong et al., 1988), perhaps because the cellulose substrates contain traces of hemicellulose (Polizeli et al., 2005). Nevertheless, in an opposite scenario, several fungal species produce specific xylanases with little or no cellulase background in the presence of xylan, which indicates the xylanolytic and cellulolytic systems are likely to be under separate regulatory control (Biely, 1985; Wong et al., 1988). And interestingly XOS reduced the efficiency of cellulose hydrolysis by cellulase (Zhang et al., 2012; Wang et al., 2018) which cannot be effectively relieved by increasing the loading of the cellulose substrate or cellulase (Wang et al., 2018). However, some fungi species require low pH for the growth and production of xylanases which necessitates additional steps in the subsequent stages which make fungal xylanases less attractive (Chakdar et al., 2016).

Among xylanases, endo-β-xylanases (xylanase or endo-β-1,4-xylanases) and β-D-xylosidases (β-xylosidases or xylosidase) have been most extensively studied. Endo-β-xylanases

(EC 3.2.1.8) randomly cleave the β-1,4 linkages between the xylopyranosyl units from the xylan backbone, producing mixtures of XOS (Biely, 1985; Kulkarni et al., 1999). β-D-xylosidases (EC 3.2.1.37) are known to be the major component of xylanase systems. They are produced by biodegradative microorganisms to hydrolyze XOS releasing D-xylose; however, usually, they do not hydrolyze xylan, with their best substrate being xylobiose and their affinity for XOS being inversely proportional to its degree of polymerization. They act on the non-reducing ends of their substrate, XOS and/or xylobiose (Wong et al., 1988; Collins et al., 2005; Polizeli et al., 2005). A high concentration of xylose in the fermentation broth can inhibit the activity of β-D-xylosidases (Fujii et al., 2011) which leads to the inefficient hydrolysis of hemicellulose and the accumulation of XOS and xylobiose by using microorganisms that do not consume xylose rapidly. Some β-D-xylosidases have been reported to possess α-L-arabinofuranosidase activity, e.g. the enzymes from *A. niger*, *T. reesei*, *T. ethanolicus,* and *Penicillium wortmannin* (Sunna and Antranikian, 1997). Other enzymes, such as α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73), and *p*-coumaric acid esterases (EC 3.1.1.-) catalyze the removal of xylan side groups (Collins et al., 2005). All these enzymes act cooperatively to convert xylans into xylose, XOS, *O*-acetyl, L-arabinose, acetic and glucuronic acids (Beg et al., 2001; Polizeli et al., 2005).

Understanding the enzymes xylanolytic microorganisms produce for hemicellulose breakdown may become an important tool for re-construction of their XOS degradation pathway in non-xylanolytic microorganisms. As mentioned before, complete degradation of xylan is achieved by a variety of modular enzymes. Although many hemicellulolytic enzymes have been studied extensively, little is known about how microorganism cells sense the presence of xylan and uptake hemicellulose-derived products (Polizeli et al., 2005; Delmas et al., 2012; Najjarzadeh et al., 2020). The induction signal for the synthesis of xylanolytic enzymes is supposed to involve transporters of xylose and short XOS released by the action of little amounts of the enzymes produced constitutively, along with lactose, glucose, and even cellulose, that are able to cross the membrane and induce the regulatory machinery (Biely and Petráková, 1984; Royer and Nakas, 1989; Chandra Raj and Chandra, 1995; Christakopoulos et al., 1996a, 1996b; Kulkarni et al., 1999), which suggests a complex induction mechanism of xylanases. In their study, Delmas et al. (Delmas et al., 2012) studied the strategy of the filamentous fungus *A. niger* employ to degrade complex polysaccharides. They showed that wheat straw itself is not initially detected by the *A.*

niger. According to their findings, the overall strategy appears to be an induction of a specific, small scale, sensory response by the onset of carbon starvation, mediated at least partially by alleviation of CreA-dependent catabolite repression, that triggers the release of a small subset of degradative enzymes which initiate degradation on a small scale, in turn releasing sugars that cause the fungus to express its full degradative arsenal (Delmas et al., 2012). More recently, Najjarzadeh and collaborators reported that xylotetraose is more effective than other substrates inducing endoxylanase, while xylohexaose and xylobiose are the best inducers of extracellular β-xylosidase, and cell-bound β-xylosidase, respectively (Najjarzadeh et al., 2020).

The driving force for xylose and xylo-oligomers uptake vary considerably among plant cell wall-degrading microorganisms. Several microbial transport systems show to be regulated by two-component systems, responding to environmental or intracellular signals to alter gene expression (Shulami et al., 2006). The two-component system includes two proteins, a receptor histidine kinase, and a response regulator. Each system uses transient phosphorylation of sensory system and a regulatory response of proteins at a specific histidine or aspartate residue for signal, and thus forms a pathway for phosphoryl transfer (Verhamme et al., 2002). Another example of oligomer transport system was described in bacterial species. Some members of the genus *Bifidobacterium* were found to be able to utilize xylan as a carbon source. A genome sequence analysis of these members have found a variety of genes related to ATP-binding cassette (ABC) sugar transporters (Chen et al., 2019; Liu et al., 2014; Saito et al., 2020).

1.4. HETEROLOGOUS EXPRESSION OF THE XYLANASES IN THE YEAST *S. CEREVISIAE*

Several reports have described the expression of heterologous endo-β-xylanases and β-xylosidase from both prokaryotes and eukaryotes in *S. cerevisiae* to enable the conversion of xylan or XOS into xylose by this species (Table 2). Usually, the complete hydrolysis of xylan requires at least these two enzymes (Biely, 1985): an endo-β-xylanase that cleaves xylan into XOS with diverse degrees of polymerization, followed by the breakdown of XOS to xylose by β-xylosidase (Wang et al., 2018). Although endo-β-xylanases are important for the hydrolysis process, β-xylosidase is considered a key enzyme (Banerjee et al., 2010), since XOS accumulation can reduce the efficiency of cellulases, such as cellobiohydrolase I (CBHI, from *Thermoascus aurantiacus*), cellobiohydrolase II (CBHII, from *Trichoderma reesei*) and endoglucanase II (from *T.*

aurantiacus) (Zhang et al., 2012), which would affect conversion yields in simultaneous saccharification and fermentation (SSF) or consolidated bioprocessing (CBP). Therefore, reducing the concentration of XOS using β-xylosidase represents the best strategy to prevent enzyme inhibition.

The heterogeneous nature of hemicellulose represents a challenge for hemicellulase enzymes. Revising the first-generation ethanol industry, a huge amount of lignocellulose-based materials is formed during ethanol production, especially corn stover, sugarcane straw, and sugarcane bagasse, which are particularly attractive as second-generation ethanol feedstock. As mentioned earlier, their hemicellulose content can achieve up to 28 % in sugarcane bagasse (Fernandes Pereira et al., 2011), 27 to 31 % in sugarcane straw (Almeida and Colombo, 2021), and 22 % in corn stover (Jorgensen et al., 2007). According to the chemical structure of hemicellulose, they present a very similar composition, which includes a high content of acetyl as side groups. Therefore, it is important to highlight that other enzymes would allow more efficient degradation of these hemicellulosic derived materials, such as acetylxylan esterases (EC 3.1.1.72) in combination with xylanase and xylosidase for hydrolysing pre-treated hardwood hemicellulose. The presence of acetyl group degrading enzymes may increase the accessibility of the xylose chain to xylanases.

1.5. *S. CEREVISIAE* AS A PLATFORM TO PRODUCE XYLANOLYTIC ENZYMES

To the best of our knowledge, the first report of xylan-degrading genes expression in *S. cerevisiae* was in 1991, when Pan et al. (1991) described the expression of two enzymes, xylanase (a *xynA* gene product) and β-xylosidase (a *xynB* gene product) from *Bacillus pumilus* in yeast cells. Thereafter, physiological data related to the expression of the intracellular β-xylosidase from *B. pumilus* in *S. cerevisiae* has been published (Crous et al., 1995, 1996; La Grange et al., 1996, 2000). A successful expression of a bacterial β-xylosidase from *B. pumilus* (*xynB*) in yeast cells was achieved by its fusion to a native secretion signal sequence named mating pheromone α-factor (MFα1s) (La Grange et al., 1997). The native open-reading frame of these enzymes starts with the codon TTG which is not recognized by *S. cerevisiae* for the initiation of translation. Even after replacing the TTG codon with an ATG starting codon, no β-xylosidase activity could be detected by the recombinant *S. cerevisiae* Y294 (La Grange et al., 1997).

Although there has been some success in functional expression of bacterial enzymes in *S. cerevisiae* (Table 2), this can be problematic, possibly because of incompatibility with eukaryotic chaperones (Sarthy et al., 1987; Gárdonyi and Hahn-Hägerdal, 2003; Xia et al., 2016). The heterologous expression of eukaryotic xylanases in *S. cerevisiae* naturally shows more compatibility since fungal species share many features, particularly related to transcription, translation, and protein stability (Frommer and Ninnemann, 1995). The first recombinant *S. cerevisiae* strain (namely IAF130) expressing heterologous eukaryotic xylanase was described by Moreau et al. (1992). However, because the cells could not catabolize xylose, the majority of the early reports of recombinant *S. cerevisiae* expressing β-xylosidases only demonstrated the conversion of xylan and XOS into xylose, xylobiose, and xylotriose, but not to ethanol.

Heterologous expression of the *T. reesei* xylanase II (*XYNII*) anchored on the cellular surface was described in the *S. cerevisiae* strain MT8 by using a cell surface engineering system based on α-agglutinin which consist of the fusion of the protein with the C-terminal-half region of an agglutinin. The recombinant strain MT8-1/pCAS1-XYNII was able to hydrolyze birchwood xylan into xylobiose and xylotriose. The proposed work did not aim to present a hydrolytic profile during the growth of MT8-1/pCAS1-XYNII in a medium containing complex sugar. Instead, xylanase activity was measured in both supernatant and pellet fractions from pre-cultured strain. XYNII activity was detected in the cell pellet with no leakage into the supernatant medium (Fujita et al., 2002).

1.6. ETHANOL PRODUCTION FROM XOS BY EXTRACELLULAR EXPRESSION OF XYLANOLYTIC ENZYMES IN *S. CEREVISIAE* STRAINS

The first example of the concept of CBP applied to ethanol production from xylan using recombinant *S. cerevisiae* strain without the addition of exogenously xylan-degrading enzymes was described in 2004 (Katahira et al., 2004). In their work, xylanase II (*XYNII*) from *T. reesei* QM9414 and β-xylosidase (*XylA*) from *Aspergillus oryzae* NiaD300, were co-displayed on the cell surface of xylose-consuming *S. cerevisiae* harboring genes encoding the oxidoreductase pathway from *S. stipitis* and native xylulokinase (XKS) from *S. cerevisiae*. To obtain this strain, the C-terminal region of α-agglutinin was fused to both xylanolytic enzymes. The constitutive expression of *XYNII* and *Xyla* enabled xylan consumption and ethanol production without a lag-phase. The recombinant strain MT8-1/ pUCSXIIXA/ pWX1X2XK produced 7.1 g L^{-1} of ethanol after 62 h of

fermentation in SDC – semi defined medium supplemented with birchwood xylan corresponding to 100 g of total sugar per liter as the sole carbon source. Despite the significant ethanol production, a large amount of xylan remained in the growth medium, suggesting that this strain needs further optimization.

Microbial surface display technology allows the expression of peptides and proteins on the surface of living cells in which proteins are expressed extracellularly, however, the enzymes remain fused at the cell with no leakage into the culture medium (Fujita et al., 2002; Katahira et al., 2004; Tafakori et al., 2012). Other examples of coexpression of xylanolytic enzymes anchored on the *S. cerevisiae* cellular surface considered the expression of bifunctional minihemicellulosomes, with several assembled modules included (Sun et al., 2012). Sun and coauthors constructed a recombinant yeast that directly produced ethanol from birchwood xylan through the expression of bifunctional minihemicellulosomes. This recombinant strain co-displays two complementary xylanases, *XYNII* and an *A. niger* xylosidase (*XDNL*) as well as a mini scaffolding (CipA3), which served as the basis to establish interaction between the enzymes and cell surface. According to their findings, the HZ3345 strain was able to ferment xylan into ethanol. The recombinant *S. cerevisiae* strain also contained an integrated xylose-utilizing pathway (XR, XDH, and XK from *S. stipitis*) to ensure the xylose assimilation. Interestingly, xylose production was immediately observed from XOS, without any lag phase, as previously observed (Katahira et al. 2004). The recombinant HZ3345 strain, produced 0.95 g L^{-1} of ethanol from approximately 3.0 g L^{-1} birchwood xylan after 80 h of cultivation under anaerobic conditions in YPBX (YP supplemented with birchwood xylan) supplemented with Tween and ergosterol.

In a more recent study, a blended bioprospecting approach was applied (Lee et al., 2015) along with rational and evolutionary engineering to improve xylan assimilation in an engineered xylan-catabolizing *S. cerevisiae* strain. The extracellular expression of xylan active enzymes (xylanase 1 – XNA1, xylosidase 2 – XD2, and arabinofuranosidase – AFB) from *Ustilago bevomyces* were cloned into 2-µm plasmids, p423 and p424 under the control of the *GPD* promoter. These plasmids were transformed into the *S. cerevisiae* YSX3 *Δhis3* strain, which has the xylose consumption pathway genes from *S. stipitis* integrated. Before applying the evolutionary approach, the recombinant strains grew slowly on xylan as a sole carbon source, producing 0.26 ± 0.008 g L^{-1} ethanol from YPXN (YP supplemented with 20 g L^{-1} xylan) after five days of cultivation. To improve its ability to assimilate xylan, serial-subcultures in the xylan

medium were used over 3 weeks. After selecting clones with improved traits, the evolved strain was able to produce 23% more ethanol in complex media (YPXN, 2% xylan), 0.32 ± 0.028 g L^{-1}. These results demonstrate the capacity to use whole-cell adaptive evolution to improve xylan metabolism by the cell.

The often-emphasized advantage of the xylose isomerase pathway in comparison with the oxidoreductase pathway was considered by Mert et al. (2016). In the earlier studies (Katahira et al., 2004; Fujii et al., 2011; Sun et al., 2012), engineered *S. cerevisiae* strains with XR/XDH were modified by the introduction of xylanolytic enzymes. Although the authors observed ethanol production from XOS, large amounts of xylose remained in the fermentation broth, probably resulting from a redox imbalance and/or inefficient xylose uptake; this, in turn, can inhibit β-xylosidase activity (Fujii et al., 2011; Peng et al., 2017; Niu et al., 2019). However, early attempts to express xylanases in engineered *S. cerevisiae* strains harboring XI had failed to produce ethanol from beechwood xylan (5%) as the sole carbohydrate source under aerobic growth over 28 days (Mert et al., 2016). The recombinant strain, Y294[YMXI], which carries *T. reesei* endoxylanase (*XYNII*), *A. niger* β-xylosidase (*xlnD*), *S. stipitis* xylulokinase (*xyl3*), and the codon-optimized xylose isomerase encoding gene (*xylA*) from *Bacteroides thetaiotaomicron* was able to break down xylan into trisaccharide, disaccharides, and monosaccharides. However, the growth rate was low probably due to the low consumption of xylose. The small amounts of xylose consumed supported cell biomass synthesis only; ethanol, xylitol, glycerol, and acetic acid production were negligible. It is worth noting that when this recombinant strain Y294[YMXI] was cultivated under similar conditions but using xylose (2%) as the sole carbon source, higher biomass was obtained, and larger amounts of xylose were consumed in a lower cultivation time. Moreover, xylitol production was also observed (Mert et al., 2016). Unfortunately, whether the expressed xylanolytic enzymes were secreted or expressed intracellularly in the Y294[YMXI] strain is not clear. It is important to mention that in previous studies published by this research group, using a *S. cerevisiae* expressing an endoxylanase encoding gene (*xyn2*) and a xylosidase encoding gene (*xlnD*), enzyme activities were detected in the culture supernatant (La Grange et al. 2001), suggesting that these enzymes were secreted by the strain.

Recombinant gene expression can promote a nonspecific metabolic burden which reduces the maximum specific growth rate and production yield of the host, as previously observed (Görgens et al., 2001). In this research, *T. reesei* xylanase II (*XYN2*) was expressed in two

recombinant *S. cerevisiae* strains, Y294 [PGK1-XYN] and Y294 [ADH2-XYN], using two 2-µm yeast plasmids under the control of either the yeast glycolytic phosphoglycerate kinase (*PGK1*) or alcohol dehydrogenase II (*ADH2*) promoters, respectively. *ADH2* is a strong promoter inducible in the absence or at low concentrations of glucose, while *PGK1* is a constitutive promoter. However, no significant difference was observed for *XYN2* expression by Y294 [ADH2-XYN] and Y294 [PGK1-XYN] strains. After 80 h of cultivation in a defined medium (Verduyn et al., 1992) containing 20 g L^{-1} glucose, specific xylanase production levels were 3.2 and 2.6 mg (g biomass)$^{-1}$, respectively. The fermentation parameters of Y294 [PGK1-XYN] and Y294 [ADH2-XYN] were compared with those of the reference strains. In all Y294 [PGK1-XYN] and Y294 [ADH2-XYN] cultivations, a reduction in yeast biomass, ethanol, and glycerol yields were observed as well as specific consumption and production rates of glucose and ethanol, compared with the reference strains. Therefore, the expression of XYN2 from either *PGK1* or *ADH2* promoters resulted in a significant metabolic burden on the host metabolism.

These findings might explain the results obtained by Mert et al. (2016), who found lower biomass production in the engineered strain Y294 [YMXI] during cultivation on xylan than in xylose as sole carbohydrate source. It is likely that the metabolic burden associated with the expression of xylanolytic enzymes impacted xylose isomerase activity. Unfortunately, xylanase activity assays during cultivation on xylan were not reported (Mert et al. 2016). The influence of *ADH2* and *PGK1* on xylanase expression was also examined by (Nuyens et al., 2001). They also used two 2-µm yeast plasmids named pFN3 and pFN4 to insert endoxylanase (*xynA*) of *B. pumilus* PLS into *S. cerevisiae* Y294 strain under the control of these two different promoters. The two engineered yeast strains did not exhibit any xylanase activities until the gene encoding uracil phosphoribosyl transferase (*FUR1*) was disrupted. This step ensured auto-selection of the *URA3*-bearing expression plasmid in a rich growth medium since mutants by *FUR1* disruption allow the growth of the recombinant yeasts in a complex medium without the risk of losing the plasmid (La Grange et al., 1996). However, unlike the work of (Görgens et al., 2001), Y291[pFN3 *fur1::LEU2*], in which the xylanase was under the control of the *ADH2* promoter, exhibited better xylanase activity (and presumably, expression) in the culture supernatant than Y291[pFN4 *fur1::LEU2*], specifically 8.5 nkat mL^{-1} and 4.5 nkat ml^{-1}, respectively.

Recently, Niu et al. (Niu et al., 2019) reported ethanol production from an efficient xylose-utilizing strain, BSPX042, expressing a xylose isomerase gene derived from a bovine rumen

metagenomic study (Ru-*xylA*), cloned in an episomal plasmid (pJXIH-PC, *URA3* as a select marker) carrying the β-xylosidase from *Penicillium oxalicum* (*xyl3A*) and the signal peptide fragment *INU* from *Klyuveromyces*. The recombinant strain, BSGIBX, cultivated in a selective synthetic complete medium supplemented with 20 g L^{-1} XOS, immediately converted XOS into xylobiose and xylotriose after inoculation. The highest ethanol concentration, approximately 4,37 g L^{-1}, was reached at 36 h. When the XOS were pretreated with xylanase, the ethanol concentration reached approximately 9 g L^{-1}. Another important study involving the use of the XI pathway and xylanases is the work of (Tabañag et al., 2018). They expressed five different hemicellulases: endoxylanase (*XYNII*), β-xylosidase (*Bxl1*), acetylxylan esterase (*Axe1*), α-D-glucuronidase (*Glr1*) and α-L-arabinofuranosidase (*Abf1*), all from *T. reesei*, bound to the cell surface of a XI-expressing *S. cerevisiae* strain. Since hemicellulose is a complex structure that requires a consortium of enzymes to break it down completely, the authors explored accessory enzymes to make the main-chain more accessible to main-chain cleaving hemicellulases. The recombinant strain grew on xylan substrates as their sole carbon source and achieved an ethanol titer of 0.96 g L^{-1} after 160h of cultivation.

In the context of a lignocellulosic biorefinery, in order to make full use of cellulose and hemicellulose to produce ethanol, Lee et al. (Lee et al., 2007) investigated constitutive co-expression of endoxylanase (*xynA*) from *Bacillus* spp. and endoglucanase (*egl6*) from *Trichoderma* spp. in *S. cerevisiae* SEY2102 strain. The expression levels of endoxylanase and endoglucanase were investigated during aerobic cultivation on YPD medium. Although fermentative parameters were not investigated, 5.6 U mL^{-1} of endoxylanase was secreted into the extracellular medium, and 1.96 U mL^{-1} was intracellular after 48 h cultivation. However, these findings are still far from achieving the goal of ethanol bioconversion from cellulosic biomass. The most promising strategy for converting cellulosic biomass to ethanol in yeast is certainly the concerted heterologous expression of all main types of hemicellulases and cellulases enzymes to maximize their synergies and improve ethanol production (Nevoigt, 2008).

Similarly to the previous report, Saitoh et al. (Saitoh et al., 2011) have reported the expression of β-xylosidase and β-glucosidase from *T. reesei* on the yeast cell surface based on α-agglutinin engineering system, obtaining the engineered industrial *S. cerevisiae* strain OC2-AXYL2-ABGL2-Xyl2 which also contains the oxidoreductase pathway for xylose consumption. The highest ethanol concentration, 12.5 g L^{-1}, was observed after 48 h in YPKX medium (40 g L^{-}

[1] KC-flock and 40 g L^{-1} xylan from Birchwood) containing 30 g L^{-1} cellulose. The ethanol yield was 0.52 g (g sugar consumed)$^{-1}$. Another example of the coexpression in *S. cerevisiae* of cellulase and hemicellulose enzymes, including an endoxylanase, xylosidase, and glucosidase was reported by (Sakamoto et al., 2012). These authors expressed endoxylanase from *T. reesei*, β-xylosidase from *Aspergillus oryzae*, and β-glucosidase from *A. aculeatus* anchored on the surface cell of the laboratory xylose-assimilating *S. cerevisiae* MN8140/XBX. Therefore, the recombinant strain, MN81/XBXX, expressed XR and XDH from *S. stipitis*, xylulokinase from *S. cerevisiae*, in addition to xylanases, and cellulase enzymes. The strain was reported to ferment cellulose and hemicellulose giving a high ethanol yield, 0.32 g g^{-1}, and concentration of 8.2 g L^{-1} after 72 h, from rice straw under oxygen-limited conditions and initial cell concentration of 100 g of wet cells L^{-1}. When 0.2 g L^{-1} of a commercial hemicellulase was added at the medium, the recombinant strain reached 10.3 g L^{-1} ethanol after 72 h, and the ethanol yield was 0.41 g g^{-1}. The addition of the commercial hemicellulase allows complete hydrolysis of xylobiose after 72h of fermentation, which in turn increased xylose content in the medium. Although the depletion of all xylobiose, after 72h of fermentation 2.2 g L^{-1} xylose remained in the fermentation medium (Sakamoto et al. 2012).

Lastly, (Xiao et al., 2019) also reported the co-expression of both cellulase and xylanase enzymes in *S. cerevisiae* (unfortunately, the authors did not specify what cellulase or xylanase was used in their work). As reported herein, there is a synergistic action between cellulase and xylanase during lignocellulose hydrolysate (Zhang et al., 2012; Wang et al., 2018; Xiao et al., 2019). In the presence of such synergies, pretreated lignocellulosic substrate degradation is more efficient because, since XOS can inhibit cellulase activity, the co-expression strategy would reduce the cellulase activity inhibitors (Zhang et al., 2012; Wang et al., 2018). Inside the concept of synergistic effect, it is worth noting that β-glucosidase did not affect xylanase activity as demonstrated by Chen at al. (2019). The recombinant strains of Xaio et al (Xiao et al., 2019), *INVSc1*-CBH-CA and *INVSc1*-CBH-TS, were cultivated using partly delignified corn stover (PDCS) producing 1.66 g L^{-1} and 1.90 g L^{-1} of ethanol after 120 h cultivation, respectively. This was approximately 4 times higher than the control (a strain that expressed a single cellulase or xylanase). Although the ethanol production did not exceed that of other published works (Katahira et al., 2004; Fujii et al., 2011; Niu et al., 2019) the effective synergistic effect of those enzymes

could improve the saccharification of lignocellulose and increase the ethanol yield during fermentation by *S. cerevisiae*.

It is important to note that most of the investigations using engineered xylan-consuming *S. cerevisiae* cells have been carried out using laboratory strains, except by the work of (Saitoh et al., 2011). However, based on the industrial conditions for ethanol production, i.e., lignocellulosic inhibitors (Almeida et al., 2007), high osmolarity, and low pH, industrial host backgrounds would present more advantages as compared to laboratory strains (Della-Bianca and Gombert, 2013; Cola et al., 2020).

1.7. ETHANOL PRODUCTION FROM INTRACELLULAR XOS UTILIZATION IN *S. CERESISIAE* STRAINS

Economic bioethanol production from lignocellulose requires complete and rapid conversion of both cellulose and hemicellulose on an industrial scale (Li et al., 2015; Mert et al., 2016). This generally includes the pretreatment of lignocellulosic biomass to increase enzyme accessibility, which improves the amount of fermentable sugars from the enzymatic digestion for biomass-to-bioethanol microbial conversion, and subsequent distillation (Palmqvist and Hahn-Hägerdal, 2000a; Katahira et al., 2004; Lynd et al., 2008). Ultimately, engineered *S. cerevisiae* expressing XOS-transporters and producing active xylanolytic enzymes for the intracellular depolymerization of XOS to xylose are important for reducing the xylanases inhibition by its end products and for tackling the issues of microbial contamination in industrial conditions, as well as for taking full advantage of all of the sugars in lignocellulose hydrolysate (Fujii et al., 2011; Niu et al., 2019). There is a large amount of data on the expression of xylanolytic enzymes in *S. cerevisiae* strains, however, few of them report the expression of intracellular XOS hydrolysis system in this yeast (Li et al., 2015). Besides the reduction of xylanases inhibition by its end products, XOS internalization represents an additional advantage over extracellular hydrolysis. The engineered *S. cerevisiae* would grow faster than other contaminant microorganisms in the fermentation tank.

Neither XOS transporters in *S. cerevisiae* nor expression of heterologous XOS-transporters expression have been reported in the works mentioned above. Li et al. (2015) reported the first engineered *S. cerevisiae* strain able to consume XOS intracellularly following uptake by an oligosaccharide-transporter. The recombinant strain expressed two β-xylosidases, GH43-2, and

GH23-7, and one transporter, CDT-2, from *Neurospora crassa* as well as XR/XDH from *S. stipitis* to ensure the internal breakdown of XOS into ethanol (SR8U) (Kim et al., 2013b; Li et al., 2015). The expression of both β-xylosidases was essential for converting XOS into xylose as the XR acted as an XOS reductase, producing xylosyl-xylitol as a potential dead-end product. Although GH43-7 had weak β-xylosidase activity, it rapidly hydrolyzed xylosyl-xylitol into xylose and xylitol. Anaerobic fermentation with this strain, expressing CDT-2, GH43-2, and GH43-7 in an optimized minimum medium (oMM) containing 4% xylose and 3% XOS, produced more than 30 g L^{-1} of ethanol in 72h of cultivation, after supplying an additional 50 g L^{-1} xylose at hour 48 (Li et al. 2015). The authors also performed a co-fermentation of sucrose plus XOS with the strain SR8U carrying the plasmid pXD8.7. According to their report, the recombinant strain could increase 3 g L^{-1} of ethanol concentration comparing cultivations performed in oMM media containing approximately 60 g L^{-1} sucrose (control cultivation) and the media containing approximately 60 g L^{-1} sucrose and 10 g L^{-1} XOS of which 4.2 g L^{-1} represent xylobiose and 2.3 g L^{-1} xylotriose (Li et al. 2015).

Although Fujii et al. (2011) did not have express a specific XOS transporter, their recombinant strain could hydrolyze xylobiose and xylotriose to xylose intracellularly. They have reported an XOS-fermenting yeast strain, D-XSD/XKXDHXR, that was constructed by expression of intracellular *T. reesei* β-xylosidase in a xylose-utilizing *S. cerevisiae* D452-2 strain expressing oxidoreductase pathway from *S. stipitis*. The recombinant strain D-XSD/XKXDHXR produced 4.2 g L^{-1} of ethanol from 10.8 g L^{-1} of xylobiose and 4.1 g L^{-1} of xylotriose after 168 h of fermentation in SCX medium under anaerobic conditions. The group reported xylose accumulation in the fermentative broth, suggesting that xylose uptake was a rate-limiting step, leading to a long XOS fermentation time. The authors claimed that the extracellular xylose accumulation implies that the cell exported the excess of intracellular xylose since no extracellular β-xylosidase activity was detected and the XOS hydrolysis occurred in the intracellular environment. These data suggest xylobiose and xylotriose were transported inside the cell using native transporters of the related *S. cerevisiae* strain, probably by saccharide transporters.

As evident from Table 2, chromosomal integrative approaches have not been widely applied to construct the XOS-utilizing *S. cerevisiae* strain. Although laborious, this approach represents the only feasible strategy for engineering genetically stable yeast strains without a select marker, for industrial applications (Nevoigt, 2008; Fang et al., 2017). Fang et al. (Fang et al. 2017)

reported a promising approach to obtain yeast with a chromosomal multicopy expression of a *Bacillus* sp. xylanase (*xynHB*) in *S. cerevisiae* strain A13. rDNA-mediated integration was used in their work, providing stable expression over 1,011 generations of cultivation, and higher copy numbers of the target gene in the chromosome than from integrating plasmids, i.e., 13.64 copies of *xynHB* gene were found in the A13 genome. Yeast genome contains around 100 rDNA repetitive units which provide ideal homologous recombination sites for the target gene. It is worth noting that gene stability is only observed when the integrated plasmid is smaller than the size of the rDNA unit (9.1 kb) (Lopes et al., 1996). The A13 strain lacked the enzymes required to form xylulose from xylose, therefore ethanol production was not the goal of Fang's research.

Table 1.2**.** Characteristics of xylanase from different microorganisms functionally expressed in *S. cerevisiae*

Organism	Enzyme	Cloning process	Host		Optimum		Activity in *S. cerevisiae*	Reference
			Name	Remarks	Temperature	pH		
Ethanol production has not been reported								
Bacterial species								
B. pumilus PLS	β-xylosidase	pDLG12 – 2µ yeast plasmid	*S. cerevisiae* Y294	*MATα leu2-3 112 ura3-52 his3 trip1-289*	45-50°C	6.6	0.09 nkat mL^{-1}	(La Grange et al. 1996, 1997)
B. pumilus IPO	Xylanase (*xynA*)	pNAX2 - 2µ yeast plasmid	*S. cerevisiae* NA87-11A cir^{+}	*ho MATα leu2-112 pho3 pho5 his3 trip1*	40°C	6.5	0.36 U (mg protein)$^{-1}$	(Panbangred et al. 1983; Pan et al. 1991)
B. pumilus IPO	β-xylosidase (*xynB*)	pYXB – 2µ yeast plasmid	*S. cerevisiae* NA87-11A cir^{+}	*ho MATα leu2-112 pho3 pho5 his3 trip1*	ND	ND	0.28 U (mg protein)$^{-1}$	(Pan et al. 1991)
Caldocellum saccharolyticum	Xylanase (*xynA*)	pFGxyn - 2µ yeast plasmid	*S. cerevisiae* STX329-3A	*MATα ade1 his2 trip1 gal2*	ND	ND	90 U (mg protein)$^{-1}$	(Donald et al. 1994)
Bacillus sp. KK-1	β-xylosidase (*xylB*)	pBX45 – 2µ yeast plasmid	*S. cerevisiae* SEY2102	*MATα ura3-52 leu2-112 his4-519 suc2-Δ9*	ND	ND	2.9 U mL^{-1}	(Kim et al. 2000)
B. pumilus PLS	Xylanase (*xynA*)	pFN3 – 2µ yeast plasmid	*S. cerevisiae* Y294	*MATα leu2-3 112 ura3-52 his3 trip1-289 Δfur*	58°C	6.2	8.5 nkta mL^{-1}	(Nuyens et al. 2001)
		pFN4 - 2µ yeast plasmid			58°C	6.2	4.5 nkta mL^{-1}	

Table 1.2. (Continued)

Penicillium purpurogenum ATCC No. MYA-38	Xylanase (*xynA*)	pYEplac181 – integrating plasmid	*S. cerevisiae* YM335::RY171	*Mata gal4-536 ura3-52 ade2-101 lys2-801 his3-200 met Δgal*	ND	ND	4.52 U mL^{-1}	(Ma and Ptashne 1987; Chávez et al. 2002)
Bacillus spp.	Xylanase (*xynB*)	pAGX3 – 2μ yeast plasmid	*S. cerevisiae* SEY2102	*MATα leu2-112 ura3-52 his4-519 suc2-Δ9*	ND	ND	7.56 U mL^{-1}	(Lee et al. 2007)
Bacillus spp.	Xylanase (*xynB*)	pADEX-1 – 2μ yeast plasmid	*S. cerevisiae* SEY2102	*MATα ura3-52 leu2-112 leu2-3 his4-519 suc2-Δ9*	ND	ND	9.8 U mL^{-1}	(Lee et al. 2009)
Bacillus sp. HY-20	Xylanase (*XylP*)	pGMF-xylP – 2μ yeast plasmid	*S. cerevisiae* SEY2102	*MATα leu2-3 112 ura3-52 his4-519 suc2-Δ9*	ND	ND	70.1 U mL^{-1}	(Kim et al. 2013a)
Bacillus sp. HY-20	Xylanase (*XylP*)	pGMF-xylP – 2μ yeast plasmid	*S. cerevisiae FY833*	*MATa leu- Δ 1 ura3-52 his3-Δ200 lys2-Δ202 trp1- Δ63*	ND	ND	42.4 U mL^{-1}	(Kim et al. 2013a)
Bacillus sp. HBP8	Xylanase (*XynHB*)	pHBM367H - rDNA-mediated integration plasmid	*S. cerevisiea* INV	*MATa his3D1 leu2 trp1-289 ura3-52 his3D1 leu2 trp1-289 ura3-52*	ND	ND	255 U (g DWcell)$^{-1}$	(Fang et al. 2017)

Table 1.2. (Continued)

Fungal species								
Cryptococcus albidus	Xylanase (*XLN*)	pVT100 – 2µ yeast plasmid	*S. cerevisiae* T109-3C	*MATa Cir*$^{+}$ *leu*2-3 *leu*2-112 *his*3-11 *his*3-5 *ra*3 *can*1	ND	ND	1.3 U (mg protein)$^{-1}$	(Moreau et al. 1992)
Aspergillus kawachii IFO4308	β-xylosidase (*xyn*C)	pJC1 - 2µ yeast plasmid	*S. cerevisiae* Y294	*MATα leu2-3 112 ura3-52 his3 trip1-289*	60°C	3	300 nkat mL^{-1}	(Crous et al. 1995)
Aureobasidium pullulans Y-2311-1	Xylanase II (*XynA*)	2µ yeast plasmid	*S. cerevisiae* INVSc1	*MATα his3-Δ1 leu2 trp1-289 ura3-52*	ND	ND	32.9 U mL^{-1}	(Li and Ljungdahl 1996)
A. nidulans G191	Xylanase (*xlnA*)	pYLA1 - 2µ yeast plasmid	*S. cerevisiae* OL1	*MATa leu2-3 112 his3-11 15 ura3-251 337*	ND	ND	65 U mL^{-1}	(Pérez-González et al. 1996)
	Xylanase (*xlnB*)	pYLB1 - 2µ yeast plasmid			ND	ND	25 U mL^{-1}	
T. reesei RutC-30	α-Arabinofuranosidase (a*bfB*)	p17SA – 2µ yeast plasmid	*S. cerevisiae* DBY746	*MATα his3 Δ1 leu2-3 112 ura3-52 trp1-289 cyh*^{R}	ND	ND	171.1 nkat mL^{-1}	(Margolles-Clark et al. 1996)
A. niger ATCC 90196	β-xylosidase	pMLU1 – 2µ yeast plasmid	*S. cerevisiae* Y294	*MATα leu2-3 112 ura3-52 his3 trip1-289 Δfur1*	60°C	4	91 nkat mL^{-1}	(Luttig et al. 1997)
T. reesei	Xylanase II (*XYNII*)	pCAS1 - 2µ yeast plasmid	*S. cerevisiae* MT8-1	*MATα ade leu2 ura3 his3 trip1*	40°C	5	1.78 µmol min^{-1} (g DWcell)$^{-1}$	(Fujita et al. 2002)
Trichoderma spp.	Endoglucanase (GenBank Access No. AY466436)	pAGX1 – 2µ yeast plasmid	*S. cerevisiae* SEY2120	*MATα leu2-112 ura3-52 his4-519 suc2-Δ9*	ND	ND	0.6 U mL^{-1}	(Lee et al. 2007)
C. flavus I-11	Xylanase (*CfXYN1*)	Yep351PGK - 2µ yeast plasmid	*S. cerevisiae* MFL	*leu2*	50°C	3	2.5 U mL^{-1}	(Parachin et al. 2009)

Table 1.2. (Continued)

A. niger IME-216	Xylanase	pUPXR – integrating plasmid	*S. cerevisiae* YS2_2	Industrial ethanol producing strain	ND	ND	74.8 U mL^{-1}	(Tian et al. 2013)
Ethanol production has been reported								
Fungal species								
A. orizae NiaD300	β-xylosidase (XylA)	pUCSXIIXA - cell-surface expressing plasmid	*S. cerevisiae* MT8-1	*MATa ade leu2 his3 ura3 trp1 SsXYL1 SsXYL2 ScXKS1*	ND	ND	234 U (g DWcell)$^{-1}$	(Katahira et al. 2004)
T. reesei QM9414	Xylanase II (XYNII)				ND	ND	16 U (g DWcell)$^{-1}$	
T. reesei QM9414	β-xylosidase	pAUR-XSD – 2μ yeast plasmid	*S. cerevisiae* MA-D4	*MATα leu2 his3 ura3 can1 SsXYL1 SsXYL2 ScXKS1 Δaur*	ND	ND	6 nmol min^{-1} (mg protein)$^{-1}$	(Fujii et al. 2011)
T. reesei	β-xylosidase (*XYL*)	pUCSXylAf - integrating plasmid	*S. cerevisiae* OC-2	*MATa/α SsXYL1 SsXYL2 ScXKS1*	60°C	ND	ND	(Saitoh et al. 2011)
T. reesei	β-xylosidase (*XYNII*)	pδW-GPAGXynII-integrating plasmid	*S. cerevisiae* MT8-1	*Mata ade his leu2 trip1 ura3 SsXYL1 SsXYL2 ScXKS1*	ND	ND	41.2 U (g DWcell)$^{-1}$	(Sakamoto et al. 2012)
A. oryzae	β-xylosidase (*XylA*)	pIHBGXylA - integrating plasmid			ND	ND	16.8 U (g DWcell)$^{-1}$	
T. reesei DSM769	Xylanase II (XynII)	pYD1 – 2μ yeast plasmid	*S. cerevisiae* EBY100 (Invitrogen, Carlsbad, CA)	*SsXYL1 SsXYL2 SsXYL3*	ND	ND	ND	(Sun et al. 2012)
A. niger DSM821	β-xylosidase (*XlnD*)				ND	ND	ND	
A. niger DSM821	α-arabinofuranosidase (*AbfB*)				ND	ND	ND	

Table 1.2. (Continued)

A. terreus	Xylanase β-xylosidase	pRSK2 – 2μ yeast plasmid	*S. cerevisiae* INVSc1	*MATa his3Δ1 leu2 trp1-289 ura3-52 CtXR*	ND	ND	ND	(Li et al. 2013)[a]
N. crassa FGSC 2489	β-xylosidase (GH43-2) β-xylosidase (GH43-7)	pXD8.7 - 2μ yeast plasmid	*S. cerevisiae* SR8U	*MATα ura3 SsXYL1 SsXYL2 SsXYL3 Δpho13 Δald6*	ND	7	ND	(Kim et al. 2013c; Li et al. 2015)
U. bevomyces	Xylanase 1 (*XNA1*)	P423 – 2μ yeast plasmid	*S. cerevisiae* YSX3 *Δhis*	*Mata trp1 can1 cyn1 gal⁺ leu2::LEU2-TDH3P-PsXYL1-TDH3T ura3::URA3-TDHP-PsXYL2-TDH3T Ty3::G418-PsXYL3 YOR202w::hphNT1*	ND	ND	ND	(Lee et al. 2015)
	Xylosidase 2 (*XD2*)	P424 – 2μ yeast plasmid			ND	ND	ND	
	Arabinofuranosidase (*ABF*)	P424 – 2μ yeast plasmid			ND	ND	ND	
T. reesei QM6a	Endoxylanase (*Xyn2*)	pVSDis-TrXyn2 - cell-surface expressing plasmid	*S. cerevisiae* EBY100	*Mata AGA1::GAL1-AGA1::URA3 ura3-52 trp1 leu2-Δ200 his3-Δ200 pep4::HIS3 prb11.6R can1 GAL1 PrXI PrXKS*	ND	ND	1.197 U mg^{-1}	(Tabañag et al. 2018)
	β-xylosidase (*Bxl1*)	pVSDis-TrBxl1 - cell-surface expressing plasmid			ND	ND		
	Acetyl esterase (*Axe1*)	pVSDis-TrAxe1 - cell-surface expressing plasmid			ND	ND		
	α -glucuronidase (*Glr1*)	pVSDis-TrGlr1 - cell-surface expressing plasmid			ND	ND		
	α-arabinofuranosidase (*Abf1*)	pVSDis-TrAbf1 - cell-surface expressing plasmid			ND	ND		

[a] The final goal of this work was xylitol production from xylan.. Li et al. achieved a xylitol yield of 0.71 g xylitol (g xylan)$^{-1}$, and *S. cerevisiae* recombinant strain, Sc-K2, produced 1.94 g L^{-1} xylitol when cultivated in YPD supplemented with 3 g L^{-1} xylan, (Li et al. 2013).

Ss = S. stipitis, Sc = S. cerevisiae, Ct = Candida tropicalis, Pr = Prevotella ruminicola

ND no data available

1.8. CONCLUDING REMARKS AND FUTURE PROSPECTS

Although xylan-degrading enzyme systems have been studied extensively, there are much more missing or points to connect than cellulose-degrading enzyme systems, probably because the structure of xylan is more complex and varies from plant to plant. However, the xylanolytic enzyme system deserves the same attention as the cellulolytic systems because their biotechnological potential is equally important. *S. cerevisiae* wild type strain is not suitable for producing bioethanol, even from a lignocellulosic hydrolysate with minimized production of inhibitors and high concentrations of hemicellulose/cellulose-derived oligosaccharides. A xylo-oligosaccharide-assimilating pathway has been demonstrated to be effective to generate *S. cerevisiae* strains able to convert polysaccharides into monomers. The effective intracellular hydrolysis of XOS has been demonstrated however the development of a strain capable of transport large molecules of XOS is a crucial challenge. Screening for non-glucose specific transporters, such as xylose and xylo-oligomers specific transporters, and intracellular endoxylanases might advance strain improvement for efficient biomass conversion.

Significant improvements towards ethanol production from hemicellulose have been achieved in recent years, as the synergistic effect of overexpressing a combination of β-xylosidases, xylanases, and cellulases have been established (La Grange et al., 2000; Chen et al., 2019; Xiao et al., 2019); however, there are potential limitations for efficient ethanol production from xylan by engineered *S. cerevisiae*, for example, β-Xylosidases with lower inhibition by products are needed for future *S. cerevisiae* engineering in order to achieve the complete conversion of xylobiose or xylotriose into xylose (Niu et al., 2019). In addition, it needs to be recognized that xylan in hemicellulose is typically branched and decorated, requiring accessory enzymes for their removal. Nevertheless, improvements in the technology to engineer and evolve *S. cerevisiae*, together with our current state of knowledge suggest that there is a high potential for the application of xylanolytic enzymes to obtain mono- and oligosaccharides from pretreated lignocellulose followed by fermentation into ethanol, since the cost of such sugars has historically been far too high to attract industrial interest.

Concerning the actual large-scale bioethanol production process from sugar- and starch-containing feedstock, hemicellulosic-derived bioethanol is still in its infancy due to low ethanol yield achieved by the abovementioned engineered *S. cerevisiae* strains. However, it is worth mentioning that lignocellulose hydrolysate contains a mix of carbon sources, such as cello-

oligosaccharides, XOS, and monomers as glucose, and xylose. Naturally, *S. cerevisiae* can consume some mono- and disaccharides, such as D-glucose, D-galactose, D-fructose, D-mannose, maltose, sucrose, and trehalose (Lagunas 1993), which do not represent the totality of the sugar derived from lignocellulose hydrolysate. It is well known in the scientific literature that economically feasible bioethanol production might include the use of all sugars available in the lignocellulose biomass. Thereby, despite the poor fermentation performance of XOS-utilizing *S. cerevisiae* strains as compared to glucose/sucrose fermentations, the simultaneous co-fermentation of lignocellulosic-derived sugars may result in in higher ethanol titer and will maximize the use of the carbon available in lignocellulose feedstock.

1.9. REFERENCES

Almeida, J. R. M., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G., and Gorwa-Grauslund, M. F. (2007). Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae. *J. Chem. Technol. Biotechnol.* 82, 340–349. doi:10.1002/jctb.1676.

Almeida, M. A., and Colombo, R. (2021) Production chain of first-generation sugarcane bioethanol: characterization and value-added application of wastes. *BioEn. Res*. 1-16. doi:10.1007/s12155-021-10301-4.

Amorim, H. V., Lopes, M. L., de Castro Oliveira, J. V., Buckeridge, M. S., and Goldman, G. H. (2011). Scientific challenges of bioethanol production in Brazil. *Appl. Microbiol. Biotechnol.* 91, 1267–1275. doi:10.1007/s00253-011-3437-6.

Banerjee, G., Car, S., Scott-Craig, J. S., Borrusch, M. S., and Walton, J. D. (2010). Rapid optimization of enzyme mixtures for deconstruction of diverse pretreatment/biomass feedstock combinations. *Biotechnol. Biofuels* 3, 1–15. doi:10.1186/1754-6834-3-22.

Beg, Q. K., Kapoor, M., Mahajan, L., and Hoondal, G. S. (2001). Microbial xylanases and their industrial applications: A review. *Appl. Microbiol. Biotechnol.* 56, 326–338. doi:10.1007/s002530100704.

Bhardwaj, N., Kumar, B., and Verma, P. (2019). A detailed overview of xylanases: an emerging biomolecule for current and future prospective. *Bioresour. Bioprocess.* 6, 1–40. doi:10.1186/s40643-019-0276-2.

Biely, P. (1985). Microbial xylanolytic systems. *Trends Biotechnol.* 3, 286–290. doi:10.1016/0167-7799(85)90004-6.

Biely, P., and Petráková, E. (1984). Novel inducers of the xylan-degrading enzyme system of Cryptococcus albidus. *J. Bacteriol.* 160, 408–412. doi:0021-9193/84/100408-05$02.00/0.

Biely, P., Singh, S., and Puchart, V. (2016). Towards enzymatic breakdown of complex plant xylan structures: State of the art. *Biotechnol. Adv.* 34, 1260–1274. doi:10.1016/j.biotechadv.2016.09.001.

Borgström, C., Wasserstrom, L., Almqvist, H., Broberg, K., Klein, B., Noack, S., et al. (2019). Identification of modifications procuring growth on xylose in recombinant Saccharomyces cerevisiae strains carrying the Weimberg pathway. *Metab. Eng.* 55, 1–11. doi:10.1016/j.ymben.2019.05.010.

Bracher, J. M., Martinez-Rodriguez, O. A., Dekker, W. J. C., Verhoeven, M. D., Van Maris, A. J. A., and Pronk, J. T. (2019). Reassessment of requirements for anaerobic xylose fermentation by engineered, non-evolved *Saccharomyces cerevisiae* strains. *FEMS Yeast Res.* 19, 1–15. doi:10.1093/femsyr/foy104.

Brenelli, L. B., Figueiredo, F. L., Damasio, A., Franco, T. T., and Rabelo, S. C. (2020). An integrated approach to obtain xylo-oligosaccharides from sugarcane straw: From lab to pilot scale. *Bioresour. Technol.* 313, 123637. doi:10.1016/J.BIORTECH.2020.123637.

Cabrini, K. T., and Gallo, C. R. (1999). Identificação de leveduras no processo de fermentação alcoólica em usinas do estado de São Paulo, Brasil. *Sci. Agric.* 56, 207–216. doi:10.1590/S0103-90161999000100028.

Chakdar, H., Kumar, M., Pandiyan, K., Singh, A., Nanjappan, K., Kashyap, P. L., et al. (2016). Bacterial xylanases: biology to biotechnology. *3 Biotech* 6, 1–15. doi:10.1007/s13205-016-0457-z.

Chandra Raj, K., and Chandra, T. S. (1995). A cellulase-free xylanase from alkali-tolerant Aspergillus fischeri Fxn1. *Biotechnol. Lett.* 17, 309–314. doi:10.1007/BF01190644.

Chávez, R., Schachter, K., Navarro, C., Peirano, A., Aguirre, C., Bull, P., et al. (2002). Differences in expression of two endoxylanase genes (xynA and xynB) from Penicillium purpurogenum. *Gene* 293, 161–168. doi:10.1016/S0378-1119(02)00720-5.

Chen, P., You, Q., Li, X., Chang, Q., Zhang, Y., Zheng, B., et al. (2019). Polysaccharide fractions from Fortunella margarita affect proliferation of Bifidobacterium adolescentis ATCC 15703 and undergo structural changes following fermentation. *Int. J. Biol. Macromol.* 123, 1070–1078. doi:10.1016/J.IJBIOMAC.2018.11.163.

Chen, C.-H., Yao, J.-Y., Yang-Barbara, Lee, H.-L., Yuan, S.-F., Hsieh, H.-Y., et al. (2019). Engineer multi-functional cellulase/xylanase/β-glucosidase with improved efficacy to degrade rice straw. *Bioresour. Technol. Reports* 5, 170–177. doi:10.1016/j.biteb.2019.01.008.

Christakopoulos, P., Mamma, D., Nerinckx, W., Kekos, D., Macris, B., and Claeyssens, M. (1996a). Production and partial characterization of xylanase from Fusarium oxysporum. *Bioresour. Technol.* 58, 115–119. doi:10.1016/S0960-8524(96)00091-0.

Christakopoulos, P., Nerinckx, W., Kekos, D., Macris, B., and Claeyssens, M. (1996b). Purification and characterization of two low molecular mass alkaline xylanases from Fusarium oxysporum F3. *J. Biotechnol.* 51, 181–189. doi:10.1016/0168-1656(96)01619-7.

Cola, P., Procópio, D. P., Alves, A. T. de C., Carnevalli, L. R., Sampaio, I. V., da Costa, B. L., et al. (2020). Differential effects of major inhibitory compounds from sugarcane-based lignocellulosic hydrolysates on the physiology of yeast strains and lactic acid bacteria. *Biotechnol. Lett.* 42, 571–582. doi:10.1007/s10529-020-02803-6.

Collins, T., Gerday, C., and Feller, G. (2005). Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol. Rev.* 29, 3–23. doi:10.1016/j.femsre.2004.06.005.

Collins, T., Hoyoux, A., Dutron, A., Georis, J., Genot, B., Dauvrin, T., et al. (2006). Use of glycoside hydrolase family 8 xylanases in baking. *J. Cereal Sci.* 43, 79–84. doi:10.1016/j.jcs.2005.08.002.

Crous, J. M., Pretorius, I. S., and Van Zyl, W. H. (1995). Cloning and expression of an Aspergillus kawachfi endo-1,4-β-xylanase gene in Saccharomyces cerevisiae. *Curr. Genet.* 28, 467–473. doi:10.1007/BF00310817.

Crous, J. M., Pretorius, I. S., and Van Zyl, W. H. (1996). Cloning and expression of the α-L-

arabinofuranosidase gene (ABF2) of Aspergillus niger in Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* 46, 256–260. doi:10.1007/s002530050813.

Dahlman, O., Jacobs, A., and Sjoè, J. (2003). Molecular properties of hemicelluloses located in the surface and inner layers of hardwood and softwood pulps. *Cellulose* 10, 325–334. Available at: https://link.springer.com/content/pdf/10.1023/A:1027316926308.pdf [Accessed September 7, 2020].

de Vries, R. P., Riley, R., Wiebenga, A., Aguilar-Osorio, G., Amillis, S., Uchima, C. A., et al. (2017). Comparative genomics reveals high biological diversity and specific adaptations in the industrially and medically important fungal genus Aspergillus. *Genome Biol. 2017 181* 18, 1–45. doi:10.1186/S13059-017-1151-0.

Della-Bianca, B. E., de Hulster, E., Pronk, J. T., van Maris, A. J. A., and Gombert, A. K. (2014). Physiology of the fuel ethanol strain Saccharomyces cerevisiae PE-2 at low pH indicates a context-dependent performance relevant for industrial applications. *FEMS Yeast Res.* 14, 1196–1205. doi:10.1111/1567-1364.12217.

Della-Bianca, B. E., and Gombert, A. K. (2013). Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. 104, 1083–1095. doi:10.1007/s10482-013-0030-2.

Delmas, S., Pullan, S. T., Gaddipati, S., Kokolski, M., Malla, S., Blythe, M. J., et al. (2012). Uncovering the Genome-Wide Transcriptional Responses of the Filamentous Fungus Aspergillus niger to Lignocellulose Using RNA Sequencing. *PLOS Genet.* 8, 1–13. doi:10.1371/JOURNAL.PGEN.1002875.

Dias Lopes, D., Carlos, ·, Rosa, A., Hector, R. E., Bruce, ·, Dien, S., et al. (2017). Influence of genetic background of engineered xylose-fermenting industrial Saccharomyces cerevisiae strains for ethanol production from lignocellulosic hydrolysates. *J Ind Microbiol Biotechnol* 44, 1575–1588. doi:10.1007/s10295-017-1979-z.

Donald, K. A. G., Carle, A., Gibbs, M. D., and Bergquist, P. L. (1994). Production of a bacterial thermophilic xylanase in Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* 42, 309–312. doi:10.1007/BF00902734.

dos Reis, T. F., de Lima, P. B. A., Parachin, N. S., Mingossi, F. B., de Castro Oliveira, J. V., Ries, L. N. A., et al. (2016). Identification and characterization of putative xylose and cellobiose transporters in Aspergillus nidulans. *Biotechnol. Biofuels* 9, 204. doi:10.1186/s13068-016-0611-1.

Dos Santos, L. V., Carazzolle, M. F., Nagamatsu, S. T., Sampaio, N. M. V., Almeida, L. D., Pirolla, R. A. S., et al. (2016). Unraveling the genetic basis of xylose consumption in engineered Saccharomyces cerevisiae strains. *Sci. Rep.* 6, 38676. doi:10.1038/srep38676.

Eliasson, A., Christensson, C., Wahlbom, C. F., and Hahn-Hagerdal, B. (2000). Anaerobic xylose fermentation by recombinant Saccharomyces cerevisiae carrying XYL1, XYL2, and XKS1 in mineral medium chemostat cultures. *Appl. Environ. Microbiol.* 66, 3381–3386. doi:10.1128/AEM.66.8.3381-3386.2000.

Fang, C., Wang, Q., Selvaraj, J. N., Zhou, Y., Ma, L., Zhang, G., et al. (2017). High copy and stable expression of the xylanase XynHB in Saccharomyces cerevisiae by rDNA-mediated integration. *Sci. Rep.* 7, 1–9. doi:10.1038/s41598-017-08647-x.

Fernandes Pereira, P. H., Cornelis, H., Voorwald, J., Odila, M., Cioffi, H., Mulinari, D. R., et al. (2011). Sugarcane bagasse pulping and bleaching: Thermal and chemical characterization. *BioResources* 6, 2471–2482. Available at: https://bioresources.cnr.ncsu.edu/wp-content/uploads/2016/06/BioRes_06_3_2471_FernandesP_VCMDP_SCB_Pulp_Bleach_Th

erm_Chem_Char_987.pdf.
Frommer, W. B., and Ninnemann, O. (1995). Heterologous expression of genes in bacterial, fungal, animal, and plant cells. *Annu. Rev. Plant Physiol. Plant Mol. Bioi* 46, 419–463. doi:0066-4294/95/0601-0419$05.00.
Fujii, T., Yu, G., Matsushika, A., Kurita, A., Yano, S., Murakami, K., et al. (2011). Ethanol production from xylo-oligosaccharides by xylose-fermenting Saccharomyces cerevisiae expressing β-xylosidase. *Biosci. Biotechnol. Biochem.* 75, 1140–1146. doi:10.1271/bbb.110043.
Fujita, Y., Katahira, S., Ueda, M., Tanaka, A., Okada, H., Morikawa, Y., et al. (2002). Construction of whole-cell biocatalyst for xylan degradation through cell-surface xylanase display in Saccharomyces cerevisiae. *J. Mol. Catal. B Enzym.* 17, 189–195. doi:10.1016/S1381-1177(02)00027-9.
Galbe, M., and Zacchi, G. (2002). A review of the production of ethanol from softwood. *Appl. Microbiol. Biotechnol.* 59, 618–628. doi:10.1007/s00253-002-1058-9.
Gárdonyi, M., and Hahn-Hägerdal, B. (2003). The Streptomyces rubiginosus xylose isomerase is misfolded when expressed in Saccharomyces cerevisiae. *Enzyme Microb. Technol.* 32, 252–259. doi:10.1016/S0141-0229(02)00285-5.
Gírio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S., and Bogel-Łukasik, R. (2010). Hemicelluloses for fuel ethanol: A review. *Bioresour. Technol.* 101, 4775–4800. doi:10.1016/j.biortech.2010.01.088.
Görgens, J. F., van Zyl, W. H., Knoetze, J. H., and Hahn-Hägerdal, B. (2001). The metabolic burden of the *PGK1* and *ADH2* promoter systems for heterologous xylanase production by *Saccharomyces cerevisiae* in defined medium. *Biotechnol. Bioeng.* 73, 238–245. doi:10.1002/bit.1056.
Gueguen, Y., Chemardin, P., Labrot, P., Arnaud, A., and Galzy, P. (1997). Purification and characterization of an intracellular β-glucosidase from a new strain of Leuconostoc mesenteroides isolated from cassava. *J. Appl. Microbiol.* 82, 469–476. doi:10.1046/j.1365-2672.1997.00136.x.
Gupta, A., and Verma, J. P. (2015). Sustainable bio-ethanol production from agro-residues: A review. *Renew. Sustain. Energy Rev.* 41, 550–567. doi:10.1016/J.RSER.2014.08.032.
Harner, N. K., Wen, X., Bajwa, P. K., Austin, G. D., Ho, C. Y., Habash, M. B., et al. (2015). Genetic improvement of native xylose-fermenting yeasts for ethanol production. *J. Ind. Microbiol. Biotechnol.* 42, 1–20. doi:10.1007/s10295-014-1535-z.
Heiling, A. (2020). Using CRISPR/Cas9 to regulate enzyme expression levels in the Weimberg pathway for optimal xylose consumption in Saccharomyces cerevisiae. Available at: http://lup.lub.lu.se/student-papers/record/9015858 [Accessed January 22, 2021].
Honda, Y., and Kitaoka, M. (2004). A family 8 glycoside hydrolase from Bacillus halodurans C-125 (BH2105) is a reducing end xylose-releasing exo-oligoxylanase. *J. Biol. Chem.* 279, 55097–55103. doi:10.1074/jbc.M409832200.
Hou, J., Tyo, K. E. J., Liu, Z., Petranovic, D., and Nielsen, J. (2012). Metabolic engineering of recombinant protein secretion by Saccharomyces cerevisiae. *FEMS Yeast Res.* 12, 491–510. doi:10.1111/J.1567-1364.2012.00810.X.
Jacobsen, S. E., and Wyman, C. E. (2000). Cellulose and hemicellulose hydrolysis models for application to current and novel pretreatment processes. in *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology* (Humana Press), 81–96. doi:10.1385/ABAB:84-86:1-9:81.

Jeffries, T. W. (1983). "Utilization of xylose by bacteria, yeasts, and fungi," in *Pentoses and Lignin* (Berlin/Heidelberg: Springer-Verlag), 1–32. doi:10.1007/BFb0009101.

Johnsen, U., Dambeck, M., Zaiss, H., Fuhrer, T., Soppa, J., Sauer, U., et al. (2009). D-xylose degradation pathway in the halophilic archaeon Haloferax volcanii. *J. Biol. Chem.* 284, 27290–27303. doi:10.1074/jbc.M109.003814.

Jorgensen, H., Kristensen, J. B., and Felby, C. (2007). Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels, Bioprod. Biorefining* 1, 119–134. doi:10.1002/bbb.4.

Katahira, S., Fujita, Y., Mizuike, A., Fukuda, H., and Kondo, A. (2004). Construction of a xylan-fermenting yeast strain through codisplay of xylanolytic enzymes on the surface of xylose-utilizing Saccharomyces cerevisiae cells. *Appl. Environ. Microbiol.* 70, 5407–5414. doi:10.1128/AEM.70.9.5407-5414.2004.

Kim, J.-H., Kim, B.-W., Yoon, K.-H., and Nam, S.-W. (2000). Expression of Bacillus sp. β-xylosidase gene (xylB) in Saccharomyces cerevisiae. *Biotechnol. Lett.* 22, 1025–1029. doi:10.1023/a:1005661909095.

Kim, M.-J., Kim, B.-H., Nam, S.-W., Choi, E.-S., Shin, D.-H., Cho, H.-Y., et al. (2013a). Efficient Secretory Expression of Recombinant Endoxylanase from Bacillus sp. HY-20 in Saccharomyces cerevisiae. *J. Life Sci.* 23, 863–868. doi:10.5352/jls.2013.23.7.863.

Kim, S. R., Ha, S. J., Wei, N., Oh, E. J., and Jin, Y. S. (2012). Simultaneous co-fermentation of mixed sugars: A promising strategy for producing cellulosic ethanol. *Trends Biotechnol.* 30, 274–282. doi:10.1016/j.tibtech.2012.01.005.

Kim, S. R., Skerker, J. M., Kang, W., Lesmana, A., Wei, N., Arkin, A. P., et al. (2013b). Rational and Evolutionary Engineering Approaches Uncover a Small Set of Genetic Changes Efficient for Rapid Xylose Fermentation in Saccharomyces cerevisiae. *PLoS One* 8, 1–13. doi:10.1371/journal.pone.0057048.

Kulkarni, N., Shendye, A., and Rao, M. (1999). Molecular and biotechnological aspects of xylanases. *FEMS Microbiol. Rev.* 23, 411–456. doi:10.1111/j.1574-6976.1999.tb00407.x.

Kuyper, M., Hartog, M., Toirkens, M., Almering, M., Winkler, A., Vandijken, J., et al. (2005). Metabolic engineering of a xylose-isomerase-expressing strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res.* 5, 399–409. doi:10.1016/j.femsyr.2004.09.010.

Kwak, S., and Jin, Y. S. (2017). Production of fuels and chemicals from xylose by engineered Saccharomyces cerevisiae: A review and perspective. *Microb. Cell Fact.* 16, 1–15. doi:10.1186/s12934-017-0694-9.

La Grange, D. C., Claeyssens, M., Pretorius, I. S., and Van Zyl, W. H. (2000). Coexpression of the Bacillus pumilus β-xylosidase (xynB) gene with the Trichoderma reesei β-xylanase 2 (xyn2) gene in the yeast Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* 54, 195–200. doi:10.1007/s002530000372.

La Grange, D. C., Pretorius, I. S., Claeyssens, M., and Van Zyl, W. H. (2001). Degradation of Xylan to D-Xylose by Recombinant Saccharomyces cerevisiae Coexpressing the Aspergillus niger β-Xylosidase (xlnD) and the Trichoderma reesei Xylanase II (xyn2) Genes. *Appl. Environ. Microbiol.* 67, 5512–5519. doi:10.1128/AEM.67.12.5512-5519.2001.

La Grange, D. C., Pretorius, I. S., and Van Zyl, W. H. (1996). Expression of a Trichoderma reesei β;-xylanase gene (XYN2) in Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* 62, 1036–1044. doi:10.1128/aem.62.3.1036-1044.1996.

La Grange, D. C., Pretorius, I. S., and Van Zyl, W. H. (1997). Cloning of the Bacillus pumilus β-xylosidase gene (xynB) and its expression in Saccharomyces cerevisiae. *Appl. Microbiol.*

Biotechnol. 47, 262–266. doi:10.1007/s002530050924.
Lagunas R (1993) Sugar transport in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 10:229–242. https://doi.org/10.1111/J.1574-6968.1993.TB05869.X
Lee, J. H., Heo, S. Y., Lee, J. W., Yoon, K. H., Kim, Y. H., and Nam, S. W. (2009). Thermostability and xylan-hydrolyzing property of endoxylanase expressed in yeast Saccharomyces cerevisiae. *Biotechnol. Bioprocess Eng.* 14, 639–644. doi:10.1007/s12257-009-0014-2.
Lee, J. H., Lim, M.-Y., Kim, M.-J., Heo, S.-Y., Seo, J.-H., Kim, Y.-H., et al. (2007). Constitutive coexpression of Bacilus endoxylanase and Trichoderma endoglucanase genes in Saccharomyces cerevisiae. *J. Microbiol. Biotechnol.* 17, 2076–2080. Available at: http://www.jmb.or.kr/submission/Journal/017/JMB017-12-24.pdf.
Lee, S.-M., Jellison, T., and Alper, H. S. (2015). Xylan catabolism is improved by blending bioprospecting and metabolic pathway engineering in *Saccharomyces cerevisiae*. *Biotechnol. J.* 10, 575–575. doi:10.1002/biot.201400622.
Li, X., Chen, Y., and Nielsen, J. (2019). Harnessing xylose pathways for biofuels production. *Curr. Opin. Biotechnol.* 57, 56–65. doi:10.1016/j.copbio.2019.01.006.
Li, X. L., and Ljungdahl, L. G. (1996). Expression of Aureobasidium pullulans xynA in, and secretion of the xylanase from, Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* 62, 209–213. doi:0099-2240/96/$04.00+0.
Li, X., Yu, V. Y., Lin, Y., Chomvong, K., Estrela, R., Park, A., et al. (2015). Expanding xylose metabolism in yeast for plant cell wall conversion to biofuels. *Elife*, 1–16. doi:10.7554/eLife.05896.
Li, Z., Qu, H., Li, C., and Zhou, X. (2013). Direct and efficient xylitol production from xylan by Saccharomyces cerevisiae through transcriptional level and fermentation processing optimizations. *Bioresour. Technol.* 149, 413–419. doi:10.1016/j.biortech.2013.09.101.
Liu, L., Cheng, J., Chen, H., Li, X., Wang, S., Song, A., et al. (2011). Directed evolution of a mesophilic fungal xylanase by fusion of a thermophilic bacterial carbohydrate-binding module. *Process Biochem.* 46, 395–398. doi:10.1016/j.procbio.2010.07.026.
Liu, L., Qin, Y., Wang, Y., Li, H., Shang, N., and Li, P. (2014). Complete genome sequence of Bifidobacterium animalis RH, a probiotic bacterium producing exopolysaccharides. *J. Biotechnol.* 189, 86–87. doi:10.1016/J.JBIOTEC.2014.08.041.
Lopes, T. S., de Wijs, I. J., Steenhauer, S. I., Verbakel, J., and Planta, R. J. (1996). Factors Affecting the Mitotic Stability of High-Copy-Number Integration Into the Ribosomal DNA of Saccharomyces Cerevisiae. *Yeast* 12, 467–477. doi:10.1002/(SICI)1097-0061(199604)12:5<467::AID-YEA933>3.0.CO;2-3.
Luttig, M., Pretorius, I. S., and Van Zyl, W. H. (1997). Cloning of two β-xylanase-encoding genes from Aspergillus niger and their expression in Saccharomyces cerevisiae. *Biotechnol. Lett.* 19, 411–415. doi:10.1023/A:1018327623422.
Lynd, L. R., Weimer, P. J., van Zyl, W. H., and Pretorius, I. S. (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiol. Mol. Biol. Rev.* 66, 506–577. doi:10.1128/MMBR.66.3.506-577.2002/ASSET/38C13E20-0334-4477-8327-F000A9C877DC/ASSETS/GRAPHIC/MR032001411T.JPEG.
Lynd, L. R., Laser, M. S., Bransby, D., Dale, B. E., Davison, B., Hamilton, R., et al. (2008). How biotech can transform biofuels. *Nat. Biotechnol.* 26, 169–172. doi:10.1038/nbt0208-169.
Ma, J., and Ptashne, M. (1987). Deletion analysis of GAL4 defines two transcriptional activating segments. *Cell* 48, 847–853. doi:10.1016/0092-8674(87)90081-X.

Margaritis, A., and Bajpai, P. (1982). Direct fermentation of D-xylose to ethanol by Kluyveromyces marxianus strains. *Appl. Environ. Microbiol.* 44, 1039–1041. doi:10.1128/aem.44.5.1039-1041.1982.

Margolles-Clark, E., Tenkanen, M., Nakari-Seta¨la¨, T., Seta¨la, S., Seta¨la¨, S., Penttila¨*, M., et al. (1996). Cloning of Genes Encoding-L-Arabinofuranosidase and-Xylosidase from Trichoderma reesei by Expression in Saccharomyces cerevisiae Downloaded from. *Appl. Environ. Microbiol.* 62, 3840–3846. doi:10.1128/aem.62.10.3840-3846.1996.

Mert, M. J., la Grange, D. C., Rose, S. H., and van Zyl, W. H. (2016). Engineering of Saccharomyces cerevisiae to utilize xylan as a sole carbohydrate source by co-expression of an endoxylanase, xylosidase and a bacterial xylose isomerase. *J. Ind. Microbiol. Biotechnol.* 43, 431–440. doi:10.1007/s10295-015-1727-1.

Mishra, C., Keskar, S., and Rao, M. (1984). Production and Properties of Extracellular Endoxylanase from Neurospora crassa. *Appl. Environ. Microbiol.* 48, 224–228. doi:0099-2240/84/070224-05$02.00/0.

Mittelman, K., and Barkai, N. (2017). The genetic requirements for pentose fermentation in budding yeast. *G3 Genes, Genomes, Genet.* 7, 1743–1752. doi:10.1534/g3.117.039610.

Moreau, A., Durand, S., and Morosoli, R. (1992). Secretion of a Cryptococcus albidus xylanase in Saccharomyces cerevisiae. *Gene* 116, 109–113. doi:10.1016/0378-1119(92)90637-5.

Moreira, L. R. S., and Filho, E. X. F. (2016). Insights into the mechanism of enzymatic hydrolysis of xylan. *Appl. Microbiol. Biotechnol.* 100, 5205–5214. doi:10.1007/s00253-016-7555-z.

Najjarzadeh, N., Matsakas, L., Rova, U., and Christakopoulos, P. (2020). Effect of Oligosaccharide Degree of Polymerization on the Induction of Xylan-Degrading Enzymes by Fusarium oxysporum f. sp. Lycopersici. *Molecules* 25, 5849. doi:10.3390/molecules25245849.

Nevoigt, E. (2008). Progress in metabolic engineering of Saccharomyces cerevisiae. *Microbiol. Mol. Biol. Rev.* 72, 379–412. doi:10.1128/MMBR.00025-07.

Nielsen, J. (2019). Yeast Systems Biology: Model Organism and Cell Factory. *Biotechnol. J.* 14, 1800421. doi:10.1002/biot.201800421.

Nieves, R. A., Ehrman, C. I., Adney, W. S., Elander, R. T., and Himmel, M. E. (1997). Survey and analysis of commercial cellulase preparations suitable for biomass conversion to ethanol. *World J. Microbiol. Biotechnol.* 14, 301–304. doi:10.1023/A:1008871205580.

Niu, Y., Wu, L., Shen, Y., Zhao, J., Zhang, J., Yi, Y., et al. (2019). Coexpression of β-xylosidase and xylose isomerase in Saccharomyces cerevisiae improves the efficiency of saccharification and fermentation from xylo-oligosaccharides. *Cellulose* 26, 7923–7937. doi:10.1007/s10570-019-02650-3.

Nuyens, F., Van Zyl, W. H., Iserentant, D., Verachtert, H., and Michiels, C. (2001). Heterologous expression of the Bacillus pumilus endo-β-xylanase (xynA) gene in the yeast Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* 56, 431–434. doi:10.1007/s002530100670.

Oh, E. J., and Jin, Y.-S. (2020). Engineering of Saccharomyces cerevisiae for efficient fermentation of cellulose. *FEMS Yeast Res.* 20. doi:10.1093/femsyr/foz089.

Palmqvist, E., and Hahn-Hägerdal, B. (2000a). Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. *Bioresour. Technol.* doi:10.1016/S0960-8524(99)00160-1.

Palmqvist, E., and Hahn-Hägerdal, B. (2000b). Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Elsevier doi:10.1016/S0960-8524(99)00161-3.

Pan, C. X., Xu, W. Z., Akatsuka, H., Negoro, S., Shima, Y., Urabe, I., et al. (1991). Expression of the xylan-degrading genes of Bacillus pumilus IPO in Saccharomyces cerevisiae. *J. Ferment.*

Bioeng. 71, 303–308. doi:10.1016/0922-338X(91)90340-M.

Panbangred, W., Shinmyo, A., Kinoshita, S., and Okada, H. (1983). Purification and properties of endoxylanase produced by bacillus pumilus. *Agric. Biol. Chem.* 47, 957–963. doi:10.1080/00021369.1983.10865760.

Parachin, N. S., Siqueira, S., de Faria, F. P., Torres, F. A. G., and de Moraes, L. M. P. (2009). Xylanases from Cryptococcus flavus isolate I-11: Enzymatic profile, isolation and heterologous expression of CfXYN1 in Saccharomyces cerevisiae. *J. Mol. Catal. B Enzym.* 59, 52–57. doi:10.1016/j.molcatb.2008.12.018.

Patiño, M. A., Ortiz, J. P., Velásquez, M., and Stambuk, B. U. (2019). <scp>d</scp> -Xylose consumption by nonrecombinant <scp> *Saccharomyces cerevisiae* </scp> : A review. *Yeast* 36, yea.3429. doi:10.1002/yea.3429.

Pedersen, M., and Meyer, A. S. (2010). Lignocellulose pretreatment severity – relating pH to biomatrix opening. *N. Biotechnol.* 27, 739–750. doi:10.1016/J.NBT.2010.05.003.

Peng, S., Cao, Q., Qin, Y., Li, X., Liu, G., and Qu, Y. (2017). An aldonolactonase AltA from Penicillium oxalicum mitigates the inhibition of β-glucosidase during lignocellulose biodegradation. *Appl. Microbiol. Biotechnol.* 101, 3627–3636. doi:10.1007/s00253-017-8134-7.

Pérez-González, J. A., De Graaff, L. H., Visser, J., and Ramön, D. (1996). Molecular cloning and expression in Saccharomyces cerevisiae of two Aspergillus nidulans xylanase genes. *Appl. Environ. Microbiol.* 62, 2179–2182. doi:10.1128/aem.62.6.2179-2182.1996.

Petrescu, I., Lamotte-Brasseur, J., Chessa, J.-P., Ntarima, P., Claeyssens, M., Devreese, B., et al. (2000). Xylanase from the psychrophilic yeast Cryptococcus adeliae. *Extremophiles* 4, 137–144. doi:10.1007/s007920070028.

Polizeli, M. L. T. M., Rizzatti, A. C. S., Monti, R., Terenzi, H. F., Jorge, J. A., and Amorim, D. S. (2005). Xylanases from fungi: Properties and industrial applications. *Appl. Microbiol. Biotechnol.* 67, 577–591. doi:10.1007/s00253-005-1904-7.

Qian, Y., Yomano, L. P., Preston, J. F., Aldrich, H. C., and Ingram, L. O. (2003). Cloning, Characterization, and Functional Expression of the Klebsiella oxytoca Xylodextrin Utilization Operon (xynTB) in Escherichia coli. *Appl. Environ. Microbiol.* 69, 5957–5967. doi:10.1128/AEM.69.10.5957-5967.2003.

Rennie, E. A., and Scheller, H. V. (2014). Xylan biosynthesis. *Curr. Opin. Biotechnol.* 26, 100–107. doi:10.1016/j.copbio.2013.11.013.

Royer, J. C., and Nakas, J. P. (1989). Xylanase production by Trichoderma longibrachiatum. *Enzyme Microb. Technol.* 11, 405–410. doi:10.1016/0141-0229(89)90134-8.

Ryabova, O., Chmil, O., and Sibirny, A. (2003). Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast. *FEMS Yeast Res.* 4, 157–164. doi:10.1016/S1567-1356(03)00146-6.

Saitoh, S., Tanaka, T., and Kondo, A. (2011). Co-fermentation of cellulose/xylan using engineered industrial yeast strain OC-2 displaying both β-glucosidase and β-xylosidase. *Appl. Microbiol. Biotechnol.* 91, 1553–1559. doi:10.1007/s00253-011-3357-5.

Saito, Y., Shigehisa, A., Watanabe, Y., Tsukuda, N., Moriyama-Ohara, K., Hara, T., et al. (2020). Multiple Transporters and Glycoside Hydrolases Are Involved in Arabinoxylan-Derived Oligosaccharide Utilization in Bifidobacterium pseudocatenulatum. *Appl. Environ. Microbiol.* 86. doi:10.1128/AEM.01782-20/SUPPL_FILE/AEM.01782-20-S0001.PDF.

Sakamoto, T., Hasunuma, T., Hori, Y., Yamada, R., and Kondo, A. (2012). Direct ethanol

production from hemicellulosic materials of rice straw by use of an engineered yeast strain codisplaying three types of hemicellulolytic enzymes on the surface of xylose-utilizing Saccharomyces cerevisiae cells. *J. Biotechnol.* 158, 203–210. doi:doi:10.1016/j.jbiotec.2011.06.025.

Sarthy, A. V., McConaughy, B. L., Lobo, Z., Sundstrom, J. A., Furlong, C. E., and Hall, B. D. (1987). Expression of the Escherichia coli xylose isomerase gene in Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* 53, 1996–2000. doi:10.1128/aem.53.9.1996-2000.1987.

Schmoll, M., and Schuster, A. (2010). Biology and biotechnology of Trichoderma. *Appl. Microbiol. Biotechnol.* 87, 787–799. doi:10.1007/s00253-010-2632-1.

Sekar, R., Shin, H. D., and DiChristina, T. J. (2016). Direct conversion of cellulose and hemicellulose to fermentable sugars by a microbially-driven Fenton reaction. *Bioresour. Technol.* 218, 1133–1139. doi:10.1016/j.biortech.2016.07.087.

Shen, L., Kohlhaas, M., Enoki, J., Meier, R., Schönenberger, B., Wohlgemuth, R., et al. (2020). A combined experimental and modelling approach for the Weimberg pathway optimisation. *Nat. Commun.* 11, 1–13. doi:10.1038/s41467-020-14830-y.

Shulami, S., Zaide, G., Zolotnitsky, G., Langut, Y., Feld, G., Sonenshein, A. L., et al. (2007). A Two-Component System Regulates the Expression of an ABC Transporter for Xylo-Oligosaccharides in Geobacillus stearothermophilus. *Appl. Environ. Microbiol.* 73, 874–884. doi:10.1128/AEM.02367-06.

Smiley, K. L., and Bolen, P. L. (1982). Demonstration of D-xylose reductase and D-xylitol dehydrogenase in Pachysolen tannophilus. *Biotechnol. Lett.* 4, 607–610. doi:10.1007/BF00127793.

Stephens, C., Christen, B., Fuchs, T., Sundaram, V., Watanabe, K., and Jenal, U. (2007). Genetic analysis of a novel pathway for D-xylose metabolism in Caulobacter crescentus. *J. Bacteriol.* 189, 2181–2185. doi:10.1128/JB.01438-06.

Stincone, A., Prigione, A., Cramer, T., Wamelink, M. M. C., Campbell, K., Cheung, E., et al. (2015). The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. *Biol. Rev.* 90, 927–963. doi:10.1111/brv.12140.

Subramaniyan, S., and Prema, P. (2002). Biotechnology of microbial xylanases: Enzymology, molecular biology, and application. *Crit. Rev. Biotechnol.* 22, 33–64. doi:10.1080/07388550290789450.

Sun, J., Wen, F., Si, T., Xu, J. H., and Zhao, H. (2012). Direct conversion of xylan to ethanol by recombinant Saccharomyces cerevisiae strains displaying an engineered minihemicellulosome. *Appl. Environ. Microbiol.* 78, 3837–3845. doi:10.1128/AEM.07679-11.

Sun, Y., and Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresour. Technol.* 83, 1–11. doi:10.1016/S0960-8524(01)00212-7.

Sunna, A., and Antranikian, G. (1997). Xylanolytic Enzymes from Fungi and Bacteria. *Crit. Rev. Biotechnol.* 17, 39–67. doi:10.3109/07388559709146606.

Tabañag, I. D. F., Chu, I. M., Wei, Y. H., and Tsai, S. L. (2018). Ethanol production from hemicellulose by a consortium of different genetically-modified sacharomyces cerevisiae. *J. Taiwan Inst. Chem. Eng.* 89, 15–25. doi:10.1016/j.jtice.2018.04.029.

Tafakori, V., Torktaz, I., Doostmohamadi, M., and Ahmadain, G. (2012). Microbial cell surface display; its medical and environmental applications. *Iran. J. Biotechnol.* 10. Available at: https://www.researchgate.net/publication/232708479_Microbial_cell_surface_display_its_

medical_and_environmental_applications [Accessed January 21, 2021].
Tengborg, C., Stenberg, K., Galbe, M., Zacchi, G., Larsson, S., Palmqvist, E., et al. (1998). Comparison of SO2 and H2SO4 impregnation of softwood prior to steam pretreatment on ethanol production. in *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology* (Humana Press Inc.), 3–15. doi:10.1007/BF02920119.
Tian, B., Xu, Y., Cai, W., Huang, Q., Gao, Y., Li, X., et al. (2013). Molecular Cloning and Overexpression of an Endo-β-1,4-xylanase Gene from Aspergillus niger in Industrial Saccharomyces cerevisiae YS2 Strain. *Appl Biochem Biotechnol* 170, 320–328. doi:10.1007/s12010-013-0173-7.
Toivola, A., Yarrow, D., and Van Den Bosch, E. (1984). Alcoholic fermentation of D-xylose by yeasts. *Appl. Environ. Microbiol.* 47, 1221–1223. doi:10.1128/aem.47.6.1221-1223.1984.
Tran Nguyen Hoang, P., Ko, J. K., Gong, G., Um, Y., and Lee, S. M. (2018). Genomic and phenotypic characterization of a refactored xylose-utilizing Saccharomyces cerevisiae strain for lignocellulosic biofuel production. *Biotechnol. Biofuels* 11, 268. doi:10.1186/s13068-018-1269-7.
Van Petegem, F., Collins, T., Meuwis, M. A., Gerday, C., Feller, G., and Van Beeumen, J. (2002). Crystallization and preliminary X-ray analysis of a xylanase from the psychrophile Pseudoalteromonas haloplanktis. *Acta Crystallogr. Sect. D Biol. Crystallogr.* 58, 1494–1496. doi:10.1107/S0907444902011666.
Velasco, J., Oliva, B., Mulinari, E. J., Quintero, L. P., da Silva Lima, A., Gonçalves, A. L., et al. (2019). Heterologous expression and functional characterization of a GH10 endoxylanase from Aspergillus fumigatus var. niveus with potential biotechnological application. *Biotechnol. Reports* 24, e00382. doi:10.1016/j.btre.2019.e00382.
Verduyn, C., Postma, E., Scheffers, W. A., and Van Dijken, J. P. (1992). Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* 8, 501–517. doi:10.1002/yea.320080703.
Verhamme, T., Arents, J. C., Postma, P. W., Crielaard, W., and Hellingwerf, K. J. (2002). Investigation of in vivo cross-talk between key two-component systems of Escherichia coli.
Wang, C., Lu, X., Gao, J., Li, X., and Zhao, J. (2018). Xylo-oligosaccharides Inhibit Enzymatic Hydrolysis by Influencing Enzymatic Activity of Cellulase from Penicillium oxalicum. *Energy and Fuels* 32, 9427–9437. doi:10.1021/acs.energyfuels.8b01424.
Weimberg, R. (1961). Pentose Oxidation by Pseudomonas fragi*. *J. Biol. Chem.* 236, 629–635. doi:10.1016/s0021-9258(18)64279-6.
Whistler, R. L., and Masak Jr., E. (1955). Enzymatic Hydrolysis of Xylan. *J. Am. Chem. Soc.* 77, 1241–1243. doi:10.1021/JA01610A042.
Wierzbicki, M. P., Maloney, V., Mizrachi, E., and Myburg, A. A. (2019). Xylan in the middle: Understanding xylan biosynthesis and its metabolic dependencies toward improving wood fiber for industrial processing. *Front. Plant Sci.* 10, 1–29. doi:10.3389/fpls.2019.00176.
Wong, K. K., Tan, L. U., and Saddler, J. N. (1988). Multiplicity of beta-1,4-xylanase in microorganisms: functions and applications. *Microbiol. Rev.* 52, 305–317. doi:10.1128/mmbr.52.3.305-317.1988.
Woodward, J., and Wiseman, A. (1982). Fungal and other β-d-glucosidases—their properties and applications. *Elsevier* 4, 73–79. doi:10.1016/0141-0229(82)90084-9.
Xia, P.-F., Zhang, G.-C., Liu, J.-J., Kwak, S., Tsai, C.-S., Kong, I. I., et al. (2016). GroE chaperonins assisted functional expression of bacterial enzymes in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 113, 2149–2155. doi:10.1002/bit.25980.

Xiao, W., Li, H., Xia, W., Yang, Y., Hu, P., Zhou, S., et al. (2019). Co-expression of cellulase and xylanase genes in *Sacchromyces cerevisiae* toward enhanced bioethanol production from corn stover. *Bioengineered* 10, 513–521. doi:10.1080/21655979.2019.1682213.

Yegin, S. (2017). Single-step purification and characterization of an extreme halophilic, ethanol tolerant and acidophilic xylanase from Aureobasidium pullulans NRRL Y-2311-1 with application potential in the food industry. *Food Chem.* 221, 67–75. doi:10.1016/j.foodchem.2016.10.003.

Zhang, C., Acosta-Sampson, L., Yu, V. Y., and Cate, J. H. D. (2017a). Screening of transporters to improve xylodextrin utilization in the yeast Saccharomyces cerevisiae. *PLoS One* 12, e0184730. doi:10.1371/journal.pone.0184730.

Zhang, G.-C., Turner, T. L., and Jin, Y.-S. (2017b). Enhanced xylose fermentation by engineered yeast expressing NADH oxidase through high cell density inoculums. *J. Ind. Microbiol. Biotechnol.* 44, 387–395. doi:10.1007/s10295-016-1899-3.

Zhang, G. M., Huang, J., Huang, G. R., Ma, L. X., and Zhang, X. E. (2007). Molecular cloning and heterologous expression of a new xylanase gene from Plectosphaerella cucumerina. *Appl. Microbiol. Biotechnol.* 74, 339–346. doi:10.1007/s00253-006-0648-3.

Zhang, J., Tang, M., and Viikari, L. (2012). Xylans inhibit enzymatic hydrolysis of lignocellulosic materials by cellulases. *Bioresour. Technol.* 121, 8–12. doi:10.1016/j.biortech.2012.07.010.

CHAPTER II: ADAPTATIVE LABORATORY EVOLUTION OF AN INDUSTRIAL XYLOSE-UTILIZING *SACCHAROMYCES CEREVISIAE* STRAIN: CONTINUOUS CULTIVATION AS A TOOL FOR SELECTING IMPROVED STRAINS

The content of this chapter is in preparation for submission to *Microorganisms* for peer-review. I will be the first author of the paper and M.Sc. Thais Helena Costa Petrin, M.Sc. Thamiris Guerra Giacon will be the co-authors. I performed the research with the help of the co-authors, and Dr. Thiago Olitta Basso was the director of the research.

Adaptative laboratory evolution of an industrial xylose-utilizing *Saccharomyces cerevisiae* strain: continuous cultivation as a tool for selecting improved strains

ABSTRACT

Production of biofuels from lignocellulosic (LC) residues should be fuelling the energy matrix in the near future. Fermentation of LC hydrolysates poses many scientific and technological challenges as the limitation of *Saccharomyces cerevisiae* in fermenting pentose sugars (derived-hemicellulose). To overcome the inability of *S. cerevisiae* to ferment xylose and present more robustness in the presence of inhibitory compound-containing media, an industrial *S. cerevisiae* strain, SA-1 XR/XDH, presenting oxidoreductase xylose catabolism pathway from *Scheffersomyces stipittis* (encoded by *xyl1*, *xyl2*, and *xyl3*), was cultivated in an aerobic xylose-limited chemostat in a bioreactor operated in the continuous mode to improve its xylose consumption kinetics for 64 days. The evolved DPY06 and its parental strain were evaluated under anaerobic conditions in complex media. DPY06 was able to consume xylose faster, exhibiting an increase of 70% on xylose consumption rate at 72h of cultivation in comparison to its parental strain, which indicating that laboratory evolution improved xylose uptake of SA-1 XR/XDH.

Keywords: evolutionary engineering; xylose; industrial *Saccharomyces cerevisiae*; chemostat cultivation.

2.1. INTRODUCTION

Lignocellulosic biomass is a potential sustainable source of various carbon sources found in several types of raw materials, ranging from urban and industrial waste, wood, and agricultural residues such as corn straw, wheat straw, rice straw, and sugarcane bagasse (Himmel et al. 2007; Cardona and Sánchez 2007). Therefore, this material represents a partially untapped rich source for biotechnology, biofuels, and biomaterials production, besides constituting a renewable alternative to petrochemicals (Guerriero et al. 2016). Cost-effective biofuel production from renewable biomass requires the efficient and complete use of all sugars present in these raw lignocellulosic materials, including all pentose and hexose sugars (Hong and Nielsen 2012; Li et al. 2015).

To overcome the inability of *S. cerevisiae* yeast to metabolize xylose and to make economically profitable second-generation ethanol, for instance, this microorganism has been engineered to express either xylose reductase-xylitol dehydrogenase (XR/XDH) genes (the so-called oxidoreductase pathway), the xylose isomerase (XI) gene, or selected genes from the non-phosphorylating portion of the Weimberg pathway, as presented in Fig. 2.1 (Jeffries 2006; Petrovič 2015; Jo et al. 2017; Borgström et al. 2019; Heiling 2020; Shen et al. 2020; Lee et al. 2021). *S. cerevisiae* yeasts represent a key cell factory already used to produce a wide range of industrial products such as first-generation biofuels, food products, biochemicals, and pharmaceuticals (Hong and Nielsen 2012). Among many *S. cerevisiae* strains, the industrial ones have been intensively engineered for xylose fermentation because of their higher tolerance to the environmental stresses encountered in such production processes, as well as their superior sugar fermentation capabilities as compared to laboratory counterparts (Hong and Nielsen 2012; Della-Bianca and Gombert 2013; Cola et al. 2020; Lee et al. 2021).

Despite the success of genetic engineering allowing laboratory and industrial *S. cerevisiae* strains to use xylose, this yeast faces uptake issues for this pentose, therefore, improvements in efficient xylose utilization have been hampered, in part, by xylose transport capacity (Apel et al.; Nijland et al. 2014; Jiang et al. 2020). *S. cerevisiae* has numerous monosaccharide transporters (*HXT1-17* and *GAL2*) and a few of those (*HXT1*, *HXT2*, *HXT4*, *HXT5*, *HXT7*, and *GAL2)* were identified to be able to transport xylose (Apel et al.; Hamacher et al. 2002; Sedlak and Ho 2004). Moreover, glucose represses xylose transport by the native transporters, limiting the use of them in mixed sugar fermentation (Apel et al.; Sedlak and Ho 2004; Subtil and Boles 2012). Several

studies have attempted to bioprospecting and characterizing heterologous xylose-transporters in *S. cerevisiae*, providing several alternatives for efficient xylose uptake (Saloheimo et al. 2007; Runquist et al. 2009; Young et al. 2011; Apel et al. 2016; Jiang et al. 2020; Podolsky et al. 2021). Other studies have been devoted to improving native transporters using a combination of bioinformatics and mutagenesis. The results of these studies are promising, however, further optimization would certainly improve xylose utilization (Young et al. 2011; Farwick et al. 2014).

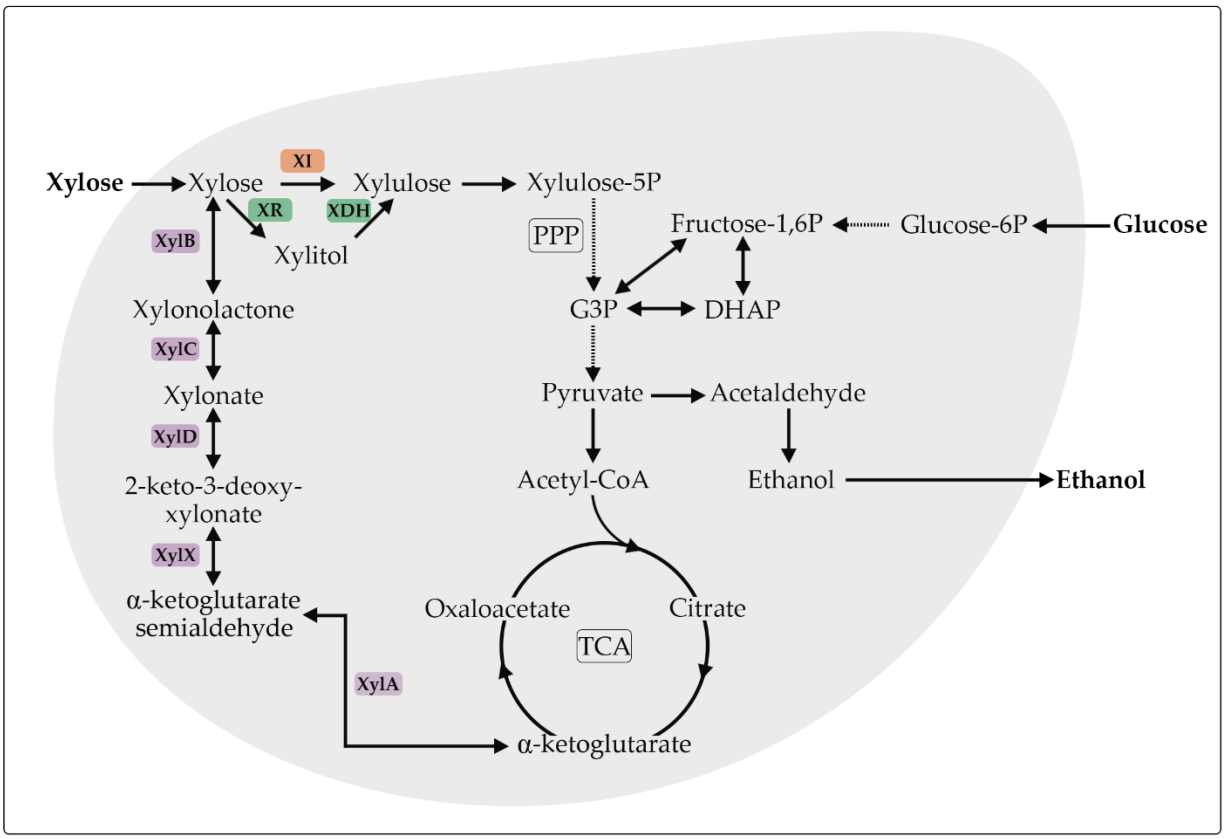

Fig. 2.1. Schematic overview of the xylose degradation pathway associated with the central carbon metabolism in yeast. The orange box indicates the redox-cofactor-independent xylose isomerase (XI), red boxes indicate the pyridine-nucleotide-dependent xylose reductase (XR) and xylitol dehydrogenase (XDH), and purple boxes indicate the five reaction steps of the Weimberg pathway catalyzed by xylose dehydrogenase (XylB), xylonolactonase (XylC), xylonate dehydratase (XylD), 3-keto-2deoxy-xylonate dehydratase (XylX), and α-ketoglutarate semialdehyde dehydrogenase (XylA). Abbreviations: PPP – pentose phosphate pathway, TCA – tricarboxylic acid cycle, G3P – glyceraldehyde 3-phosphate, DHAP – dihydroxyacetone phosphate. Adapted from (Borgström et al. 2019).

In the present work, adaptive laboratory evolution through chemostat cultivations has been used to address the low xylose uptake rate of an industrial xylose-utilizing *S. cerevisiae* strain previously modified with the oxidoreductase pathway from *S. stipittis* (encoded by *XYL1*, *XYL2*,

and *XYL3*) yielding SA-1 XR/XDH (Basso 2015), which was kindly provided by Prof. Luiz Carlos Basso (Escola Superior da Agricultura Luiz de Queiroz –Universidade de São Paulo). This strain was cultivated for 64 days in an aerobic xylose-limited chemostat, resulting in the evolved strain DPY06, that showed significant optimization to produce ethanol from in xylose and glucose under anaerobic conditions.

2.2. MATERIALS AND METHODS

2.2.1. Strains and media

The *S. cerevisiae* SA-1 XR/XDH strain, in which *XYL1*, *XYL2*, and *XYL3* were integrated into the *ura3* locus (Basso 2015), yielding an auxotrophic strain for uracil, was used for the evolutionary engineered experiments. Strains were cultured in the reference defined media previously developed by Verduyn and co-workers (VM) and further adapted by Luttik and co-workers (Verduyn et al. 1992; van Hoek P et al. 2000; Luttik et al. 2000), both for adaptive laboratory evolution cultivations, as well as for shake-flask physiological characterization, respectively. The media was composed with the following components (in $g.L^{-1}$): NH_2CONH_2 (urea), 2.3; KH_2PO_4, 3.0; K_2SO_4, 6.6; $MgSO_4.7H_2O$, 0.5; and trace-elements consisting of (in $mg.L^{-1}$) EDTA, 15, $ZnSO_4.7H_2O$, 4.5, $MnCl_2.2H_2O$, 0.84; $CoCl_2.6H_2O$, 0.3; $CuSO_4.5H_2O$, 0.3; $Na_2MoO_4.2H_2O$, 0.4; $CaCl_2.2H_2O$, 4.5, $FeSO_4.7H_2O$, 3.0, H_3BO_3, 1.0, KI, 0.1. A solution containing vitamins was filter-sterilized and added to the medium to a final concentration of (mg L^{-1}) d-biotin, 0.05; calcium pantothenate, 1.0; nicotinic acid, 1.0; myo-inositol, 25; thiamine.HCl, 1.0; pyridoxine.HCl, 1.0, and p-aminobenzoic acid, 0.20.

Yeast extract-peptone (YP) medium (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}) was also used to grow yeast cells before and after the evolutionary engineering, to analyse its fermentative performance. The two *S. cerevisiae* strains used in this work are displayed in Table 2.1.

Table 2.1. Yeast strains used in this study.

Strain	Description	Reference
SA-1 XR/XDH	Xylose-consuming strain engineered from strain SA-1, *Δura3*	(Basso 2015)
DPY06	Evolved SA-1 XR/XDH strain	This work

2.2.2. Adaptative laboratory evolution in aerobic xylose-limited chemostat cultivations

After the pre-cultivation step of the SA-1 XR/XDH strain at 30 °C and 200 rpm in 500 mL shake flasks containing 100 mL of the VM defined medium with xylose 20 g L^{-1} and uracil 20 mg L^{-1}, yeast cells were inoculated in a 2.0 L water-jacketed model Labors 5 (Infors AG, Switzerland) bioreactor containing 0.8 L of VM defined medium, with 60 g L^{-1} xylose and 80 mg L^{-1} uracil. Bioreactor batch cultivation (BBC) was conducted until exhaustion of xylose (that was monitored by a sharp drop in the CO_2 concentration in the off-gas analysis). After BBC, the mode of cultivation was switched to continuous through the constant addition of fresh VM medium supplemented with 20 g L^{-1} xylose and 80 mg L^{-1} uracil by a peristaltic pump. To maintain the working volume constant a mechanical drain removed fermentative broth from the bioreactor for an appropriated waste vessel. The feeding rate was adjusted during the cultivation to increase the selective pressure on the cells. Compressed air was used to flush the culture vessel (0.5 L min^{-1}). The dissolved oxygen concentration was monitored constantly by a dissolved oxygen electrode (Mettler-Toledo, Columbus, OH, USA). Agitation frequency was set to 800 rpm, the temperature was controlled at 30 °C, and pH was controlled at 5.0 via controlled 2 M KOH solution. Continuous cultivation was performed for 64 days. To isolate potential evolved strains, samples were plated from the continuous cultivation medium in YP medium containing 20 g L^{-1} xylose (YPX) and evaluated under aerobic (oxic) and anaerobic (anoxic) conditions (200 rpm) in shake-flask cultivations, as depicted below.

2.2.3. Shake-flask cultivations for strain characterization

YP medium was used for shake-flasks batch cultivations. After pre-inoculum, yeast cells were harvested by centrifugation at 3,134 × g, at 4°C for 5 min, and washed three times with distilled water. Aerobic and micro-aerobic batch fermentation experiments were performed by inoculating the pelleted yeast cells in 100 mL Erlenmeyer flask with 30 mL containing fermentative medium, composed of YPX (YP medium containing xylose), YPD (YP medium containing glucose), or YPDX (YP medium containing glucose and xylose). All conditions were performed at 30°C. For aerobic conditions, agitation was set at 200 rpm, while under anaerobic and micro-aerobic conditions, agitation was 100 rpm. For anaerobic batch fermentation experiments, pre-cultures were inoculated in a serum bottle containing 30 mL of fermentative

medium, which could be YPX or YPDX. A serum bottle sealed with butyl rubber stoppers was used to ensure strict anaerobic (anoxic) conditions. The serum bottles with fermentation media were then flushed with nitrogen gas, previously passed through a heated, reduced copper column to remove the trace of oxygen. All cultivations were performed in biological triplicates.

2.2.4. Analytical methods

Samples were taken at appropriate intervals to measure cell growth and metabolites. Cell growth was monitored by optical density (OD) at 600 nm using an UV-visible Spectrophotometer (BIomate 5) (Basso et al. 2010). Cell dry mass concentration was determined by gravimetry, according to a protocol described by Olsson and Nielsen (1997). Samples were centrifuged at 15000 rpm for 10 min and supernatants were diluted appropriately and then used for determination of glucose, xylose, xylitol, glycerol, succinate, acetic acid, and ethanol by high-performance liquid chromatography (Agilent Technologies 1200 Series) equipped with a refractive index detector. The Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA) was used and the columns were eluted with 5 mM H_2SO_4 at 60 °C, and the flow rate was set at 0.6 mL/min.

2.3.RESULTS AND DISCUSSION

2.3.1. Physiological characterization of SA-1 XR/XDH in different xylose concentrations

SA-1 XR/XDH strain expresses two copies of a heterologous xylose-assimilating pathway composed of *XYL1, XYL2,* and *XYL3* enzymes which were integrated at the *URA3* locus (Basso 2015). Recombinant *S. cerevisiae* expressing *XYL1* and *XYL2* was found to grow on xylose as the sole carbon source (Kötter et al. 1993). Native *S. cerevisiae* encodes a xylulokinase (*XKS1*) which is expressed at a low level, and which enables *S. cerevisiae* to grow on and ferment xylulose (Rodriguez-Peña et al. 1998). Overexpression of *S. stipitis XYL3* along with high levels of *XYL1* and *XYL2* in *S. cerevisiae* completely inhibited cell growth on xylose, whereas an *S. cerevisiae* transformant expressing *XYL3* at a moderate level was able to grow on xylose (Jin et al. 2003).

A number of studies have been performed to optimize the XR/XDH pathway (Kötter et al. 1993; Walfridsson et al. 1995; Ho et al. 1998; Richard et al. 2000). The first enzyme reaction catalysed by XR (*XYL1*) is a NAD(P)H-dependent, and the second enzyme, XDH (*XYL2*), requires NAD^+ exclusively, Fig 2.2 (Kuyper et al. 2004). The unbalanced cofactor requirement between

XR and XDH, which can result in an accumulation of NADH, has received considerable attention as a major limiting factor of the xylose oxidoreductase pathway in *S. cerevisiae* (Kim et al. 2013b), since engineered *S. cerevisiae* cells expressing XR/XDH pathway presented very slow growth and high amounts of xylitol formation under anaerobic fermentation of xylose (Kuyper et al. 2004), which, the latter compost (xylitol) is crucial to cope with redox balance during oxygen-liming or anoxic conditions.

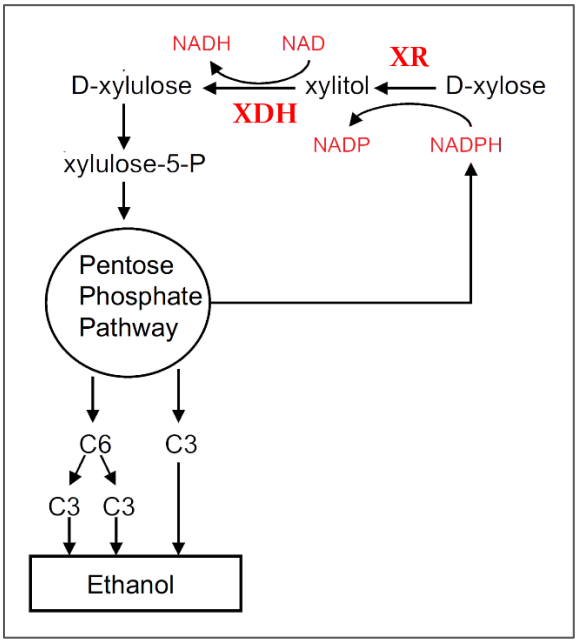

Fig. 2.2. Conversion of xylose into ethanol via XR/XDH. Adapted from Kuyper et al. 2004.

When sufficient oxygen is provided, excessive xylitol formation can be prevented (Winkelhausen and Kuzmanova 1998). Therefore, anaerobic alcoholic fermentation of xylose without xylitol formation is possible when XR and XDH would have matching coenzyme specificities (Kuyper et al. 2004). But, recent studies have demonstrated that further rational and inverse metabolic engineering strategies have improved anaerobic xylose catabolism with lesser xylitol formation of *S. cerevisiae* expressing *XYL1*, *XYL2*, and *XYL3* through concomitant deletion of *PHO13* and *ALD6*, as well as overexpression of *TAL1*, followed by laboratory adaptative evolution (Kim et al. 2013b; Jeong et al. 2020).

Despite SA-1 XR/XDH do not present any additional modification to address the abovementioned problems associated with anaerobic xylose fermentation, this strain was tested under strictly anaerobic (anoxic) conditions on xylose in a batch fermentation to analyze its ability

to metabolize xylose under such conditions. YPX1 (YP containing 10 g L^{-1} xylose), YPX2 (YP containing 20 g L^{-1} xylose), YPX4 (YP containing 40 g L^{-1} xylose), and YPX8 (YP containing 80 g L^{-1} xylose) were used as culture media to evaluate growth and metabolite kinetics of this yeast strain, starting with an initial OD_{600} of 0.3. The results showed that it spent more than 600 h to consumed over 50% xylose when fermenting YPX1 (Fig. 2.3A). In the same time frame, when fermenting 20 g L^{-1} xylose, the SA-1 XR/XDH strain consumed 31% of total available sugar (Fig. 2.3B), 23% of 40 g L^{-1} xylose (Fig. 2.3C), and 11% of 80 g L^{-1} xylose (Fig. 2.3D). Higher availability of xylose amount did not increase xylose consumption by SA-1 XR/XDH. The transport of xylose into the cell is the first metabolic step of xylose conversion. Xylose is taken up by the facilitated diffusion hexose transport system in *S. cerevisiae* but does so at considerably lower rates than glucose (Meinander and Hahn-Hägerdal 1997; Hector et al. 2008). Some studies by Wahlbom et al. (2003b) and Kuyper et al. (2005) suggest that xylose transport may be one of the limiting steps in xylose utilization since the increase in xylose transport in engineered strains results from the increase in hexose transporters. The lower xylose assimilation by SA-1 XR/XDH for higher xylose amounts, however, does not suggest an inefficient xylose uptake by the native transporters of the cell. These results, instead, suggest that under the anaerobic condition the highest xylose concentration (up to 40 g L^{-1}) triggered catabolite repression in SA-1 yeast. The greatest quantity of xylose consumed was observed for YPX4 cultivation. Between the four conditions adopted, YPX1, YPX2, YPX4, and YPX8, the amount of xylose consumed by the industrial transformant yeast increased between the first and third conditions and was reduced by the last one (Table 2.2).

The fact of *S. cerevisiae* does not metabolize xylose naturally, xylose could act as a repressing sugar in "recombinant" metabolism. Xylose metabolism might lead to the formation of an intermediate that acts as a triggering molecule for a carbon catabolite repression cascade, in a manner similar to that of some glucose intermediates. Accordingly, the way the cell recognizes, senses, and signals the presence of xylose must affect the efficiency of its utilization and fermentation. Supporting the abovementioned facts, previous studies of xylose-metabolising *S. cerevisiae* have suggested that xylose has a depressing effect on gene expression (Belinchón and Gancedo 2003; Roca et al. 2004; Salusjärvi et al. 2006, 2008).

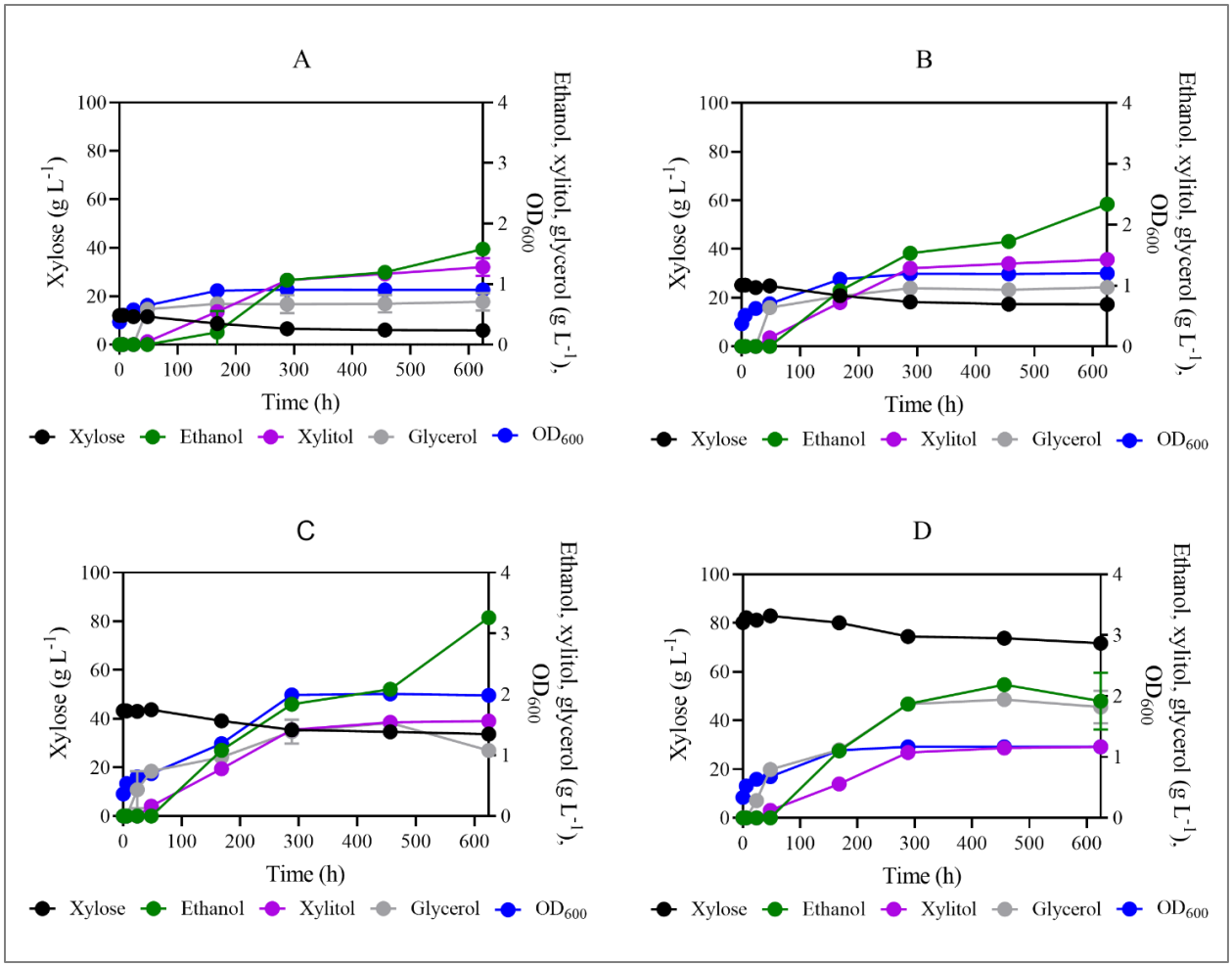

Fig. 2.3. Anaerobic fermentation of SA-1 XR/XDH in YP medium containing 10 g L^{-1} xylose – YPX1 (A), 20 g L^{-1} xylose – YPX2 (B), 40 g L^{-1} xylose – YPX4 (C), or 80 g L^{-1} xylose – YPX8 (D). Fermentations were performed with an initial cell density OD_{600} of 0.3, at 30°C and 100 rpm.

Kim et al. (2013b) observed a catabolite xylose repression for micro-aerobic cultivations of SR7 *S. cerevisiae* strain. When a laboratory strain SR7 (D452-2 wild type strain expressing multiple copies of *XYL1* and two copies of *XYL2* and *XYL3*) was cultured in 40 g L^{-1} xylose as a sole carbon source a long lag time was observed, suggesting that some limitations or inhibitory mechanisms hindering xylose consumption might exist in the SR7 strain. Further, they investigated the relation between initial xylose concentration and specific growth rate. When the initial xylose concentration was increased from 1 to 10 g L^{-1}, the specific growth rate increased as well. However, from the initial 20 g L^{-1} xylose, the growth rate decreased severely. And, for the initial xylose concentration of 40 g L^{-1}, no significant cell growth was observed during 24 h of incubation (Kim et al. 2013b).

Under all analyzed conditions ethanol was detected, with the highest amount in the YPX4 fermentation media. Also, in YPX4 cultivation the engineered strain presented a higher xylose consumption rate as well accumulated higher xylitol amount (Table 2.2). However, the maximum specific xylose consumption rate was observed for cultivations in YPX8 (0.11 ± 0.07 g xylose OD^{-1} h^{-1}) at 24 h of cultivation, even despite under this condition SA-1 XR/XDH presented a lesser quantity of xylose consumption. Therefore, the findings suggested that higher xylose concentration exhibited an inhibitory mechanism hindering xylose consumption in SA-1 XR/XDH strain.

Table 2.2. Fermentation profiles of SA-1 XR/ XDH (SA-1 expressing *XYL1*, *XYL2*, and *XYL3*) when fermenting YPX1 (YP medium containing 10 g L^{-1} xylose), YPX2 (YP medium containing 20 g L^{-1} xylose), YPX4 (YP medium containing 40 g L^{-1} xylose), or YPX8 (YP medium containing 80 g L^{-1} xylose) under anaerobic condition, at 30°C and 100 rpm.

Cultivation condition	**Xylose consumed (g L^{-1})**	**Xylose consumption rate (g L^{-1} h^{-1})**	**Specific xylose consumption rate (g xylose OD^{-1} h^{-1}) [OD][a]**	**Accumulated xylitol production (g L^{-1})**	**Accumulated glycerol production (g L^{-1})**	**Accumulated etanol production (g L^{-1})**	**$Y_{Ethanol}$**
YPX1	6.21 ± 0.13	0.01 ± 0.00	0.04 ± 0.01 [0.57]	1.21 ± 0.15	0.69 ± 0.15	1.6 ± 0.07	0.25 ± 0.02
YPX2	8.23 ± 0.58	0.01 ± 0.00	0.07 ± 0.02 [0.63]	1.43 ± 0.02	0.97 ± 0.03	2.34 ± 0.02	0.28 ± 0.05
YPX4	10.39 ±1.56	0.02 ± 0.00	0.04 ± 0.01 [0.65]	1.56 ± 0.05	1.13 ± 0.09	3.22 ± 0.10	0.32 ± 0.02
YPX8	8.59 ± 1.21	0.01 ± 0.00	0.11 ± 0.07 [0.64]	1.16 ± 0.01	1.97 ± 0.27	2.14 ± 0.47	0.23 ± 0.04

All described condition was performed under anaerobic condition (100 rpm) with initial OD_{600} of 1. All parameters were calculated for up 624 h of fermentation.
[a] OD value at the maximum rate of xylose consumption.
Parameters: $Y_{Ethanol}$, Ethanol yield (g ethanol g consumed $xylose^{-1}$).

Therefore, only the expression of *XYL1*, *XYL2*, and *XYL3* did not allow efficient growth of the SA-1 XR/XDH strain anaerobically on xylose. Although the rate of anaerobic xylose catabolism is relatively low, when comparing anaerobic cultivation of SA-1 XR/XDH in YPX1 with an evolved laboratory strain expressing oxidoreductase pathway from *Pichia stipitis* (Sonderegger and Sauer 2003), the industrial xylose-fermenting strain described here achieved the same ethanol yield (0.25 g ethanol g consumed $xylose^{-1}$) of the 460-generation population from Sonderegger and Sauer's work when cultivated in strictly anaerobic batch culture with 10 g L^{-1} xylose.

Eliasson et al. (2000) included glucose in the anaerobic bioreactor cultivation in the presence of xylose. A modified *S. cerevisiae* strain TMB3001 (CEN.PK 113-7A expressing *XYL1* and *XYL2* genes from *P. stipitis* and the overexpression of endogenous *XKS1* gene) was able to grow aerobically on xylose, but not anaerobically. The xylose uptake rate increased with increasing xylose concentration and decreasing glucose concentration in the feed. However, even at the highest xylose concentration, only 12% of the xylose was consumed (Eliasson et al. 2000).

Heterologous expression of xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*), or of xylose isomerase (*xylA*), either case of which is accompanied by overexpression of xylulokinase (*XKS1* or *XYL3*), are known as the prevalent strategies for metabolic engineering of *S. cerevisiae* to ferment xylose. An interesting study, however, proposed an alternative strategy. Kim et al. (2013a) replaced the expression of *XYL1* by the overexpression of an endogenous aldose reductase (*GRE3*) of D452-2 strain, achieving the engineered *S. cerevisiae* DG23 strain that overexpresses *GRE3*, *XYL2*, and *XYL3*. DG23 strain could ferment xylose (YP medium containing 40 g L^{-1} xylose) under oxygen-limited conditions but, produced lower ethanol yield (0.29 g g^{-1} sugar) than our findings for the engineered industrial yeast (Table 2.2). They also investigated the ability of *S. cerevisiae* D452-2 expressing *XYL1*, *XYL2*, *XYL3* to ferment 40 g L^{-1} xylose in the YP medium, yielding DX123 strain. The yielded strain, DX123, produced 0.23 g ethanol for g sugar under oxygen-limited conditions.

Similar oxygen-limited cultivation was performed with SA-1 XR/XDH. The industrial engineered strain was also cultivated under microaerobic conditions (100 rpm) in the presence of 40 g L^{-1} of xylose, see Fig. 2.3 and Table 2.3. When sufficient oxygen is provided, the engineered strain was able to consume over 90% xylose, producing ethanol within 72h (Fig. 2.4). As previously reported, oxygen (or some other electron-accepting system) is required to resolve the redox imbalance caused by the cofactor difference between XR and XDH (Jin and Jeffries 2004).

Under the same condition (YPX4 and 100 rpm), the YSX3 *S. cerevisiae* strain was cultivated (Jin and Jeffries 2004). Their strain, which contains *XYL1*, *XYL2*, and *XYL3* in the chromosome, was unable to consume completely 40 g L^{-1} xylose in 100 h of cultivation. Indeed, engineered *S. cerevisiae* expressing *XYL1*, *XYL2*, and *XYL3* exhibited inefficient xylose fermentation because of the cofactor requirements of NAD(P)H-specific XR and NAD^+ -specific XDH originated from *S. stipitis* are not matched. The imbalance was hypothesized to cause overall inefficiency of xylose fermentation by engineered *S. cerevisiae* (Kötter et al. 1993; Kim et al.

2013a). Another work used a haploid industrial ethanol-producing *S. cerevisiae* strain (PE-2 derived strain) as the host for the *S. stipitis* XR/XDH expression pathway (Dias Lopes et al. 2017). After batch fermentations under micro-aerobic conditions using YPX8 (YP containing 80 g L^{-1} xylose), the transformant strain YRH1490 achieved a higher ethanol yield (0.310 g ethanol g $xylose^{-1}$) when comparing with the SA-1 strain (Table 2.3).

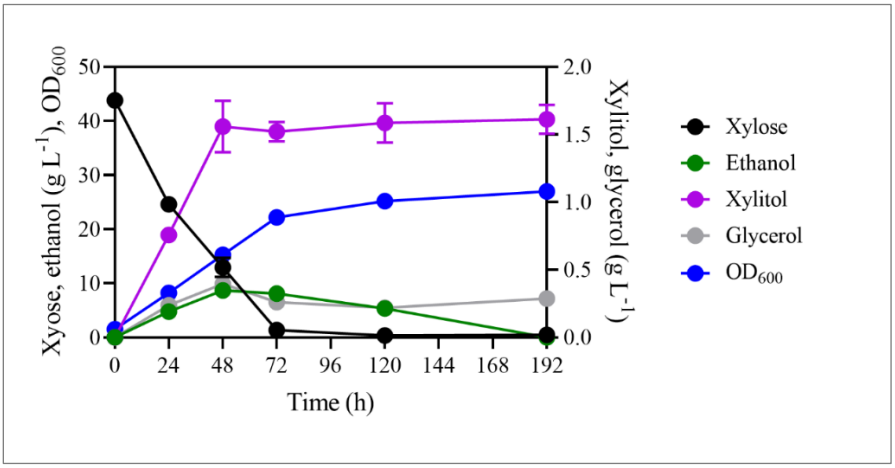

Fig. 2.4. Micro-aerobic fermentation of SA-1 XR/XDH in YP medium containing 40 g L^{-1} xylose (YPX4), with initial cell density OD_{600} of 1, at 30°C and 100 rpm.

Table 2.3. Fermentation profiles of SA-1 XR/XDH (SA-1 expressing *XYL1*, *XYL2*, and *XYL3*) when fermenting YPX4 (YP medium containing 40 g L^{-1} xylose) under micro-anaerobic condition, at 30°C and 100 rpm.

Cultivation condition	**Xylose consumed (g L^{-1})**	**Xylose consumption rate (g L^{-1} h^{-1})**	**Accumulated xylitol production (g L^{-1})**	**Accumulated glycerol production (g L^{-1})**	**Accumulated etanol production (g L^{-1})**	$Y_{Ethanol}$
YPX4	42.49 ± 0.82	0.36 ± 0.00	1.54 ± 0.01	0.26 ± 0.00	8.19 ± 0.34	0.24 ± 0.03

All described condition was performed under micro-anaerobic condition (100 rpm) with initial OD_{600} of 1. All parameters were calculated for up 72 h of fermentation.
Parameters: $Y_{Ethanol}$, Ethanol yield (g ethanol g consumed $xylose^{-1}$).

The abovementioned laboratory strain SR7 (Kim et al. 2013b) could convert 40 g L^{-1} of xylose into ~ 9 g L^{-1} of ethanol in 120 h under micro-aerobic conditions. Kim and co-workers used evolutionary engineering to further improve xylose utilization. Between the second and third transfers, there was a significant increase in growth rate and ethanol yield suggesting accumulation of some advantageous mutation(s), which turned out to be an allele of *PHO13*. While SR7 before evolution took 144 h to metabolize 40 g L^{-1} of xylose into ~ 8 g L^{-1} of ethanol after evolution

(SR7e3) took 24 h to metabolize the same amount of xylose into 13 g L^{-1} of ethanol. Their results suggest that balanced expression of the xylose pathway, coupled with evolutionary engineering could be used to improve the xylose fermentation efficiency of the strains.

Numerous studies attempted to minimize the cofactor imbalance through various metabolic engineering approaches, such as XR/XDH pathway was balanced by random or rational mutagenesis of the proteins (Petschacher et al. 2005; Hahn-Hägerdal et al. 2007; Watanabe et al. 2007; Matsushika et al. 2008; Runquist et al. 2010), or facilitating the regeneration of the cofactors during xylose metabolism through the introduction of heterologous enzymes (Bro and Nielsen 2004; Zhang et al. 2012, 2016). Therefore, without oxygen availability, the engineered strain was not able to ferment xylose efficiently, since the redox imbalance between XR/XDH cofactors requires molecular oxygen to regenerate NAD^+ (Kuyper et al. 2004). As described previously, anaerobic growth on xylose was reported to require additional laboratory evolution, mutagenesis, and/or genetic engineering (Kuyper et al. 2003, 2005; Bracher et al. 2019).

2.3.2. Adaptative laboratory evolution of SA-1 XR/XDH

Evolutionary engineering improved the xylose-fermenting capabilities of a laboratory strain expressing *XYL1*, *XYL2,* and *XYL3*, which also exhibited a shorter lag time on xylose as a sole carbon source than its parental strain (Kim et al. 2013b). As mentioned by Steensels et al. (2014), directed evolution can be used to fine-tune a specific phenotype that is already present in the original population but is not optimal yet. Considering these statements and the abovementioned results, SA-1 XR/XDH can metabolize xylose, however high concentrations of xylose under anaerobic conditions exhibited an inhibitory effect on its xylose catabolism. Therefore, we hypothesized that growth inhibition by high concentrations of xylose would be strong selective pressure for the isolation of suppressor mutants that have improved growth in xylose. To test this hypothesis, we carried out evolutionary engineering of the SA-1 XR/XDH strain by cultivating an aerobic xylose-limited chemostat for 64 days (Fig. 2.5A and Fig. 2.5B), which is characterized by a batch and continuous phase. The initial specific growth rate, calculated during the batch phase, was 0.012 h^{-1}, that corresponding to a doubling time of 58 h, representing 2.41 times lower than the doubling time achieved by Kuyper et al. (2004) using the CEN.PK113-5D strain genetically modified with the *Piromyces* sp. xylose-isomerase (*xylA*) gene when cultivated in VM in shake-flasks.

After the batch phase, denoted by the exhaustion of xylose in the bioreactor, feeding started at a fixed dilution rate. The initial flow rate was based on the maximum growth rate of the batch phase, and the flow was kept within an interval of 8-10 mL h^{-1}. Cells were cultivated at a fixed dilution rate until a decrease in xylose concentration. Therefore, as the residual xylose concentration decreased, the flow rate was augmented. Xylose consumption (Fig. 2.5A), glycerol, and xylitol productions (Fig. 2.5B) were observed during the continuous phase, but ethanol production was not. The intensive aeration would leave to competition for glycolytic NADH between mitochondrial respiration and alcoholic fermentation, thus promote cell growth and reducing the ethanol yield (Kuyper et al. 2004).

After several generations under a selective pressure environment, SA-1 XR/XDH cells end up undergoing mutations and, less adapted cells were eliminated from these cropping, remaining evolved strains with the ability to consume xylose faster (Brown and Oliver 1982; Jansen et al. 2004; Çakar et al. 2012). At the end of the evolutionary experiment, it is possible to observe an increase in cell density, when the dilution rate achieved 0.6 h^{-1}. An increase in the flow rate did not promote the wash-out of the cells from the bioreactor, suggesting the progress of adaptative evolution.

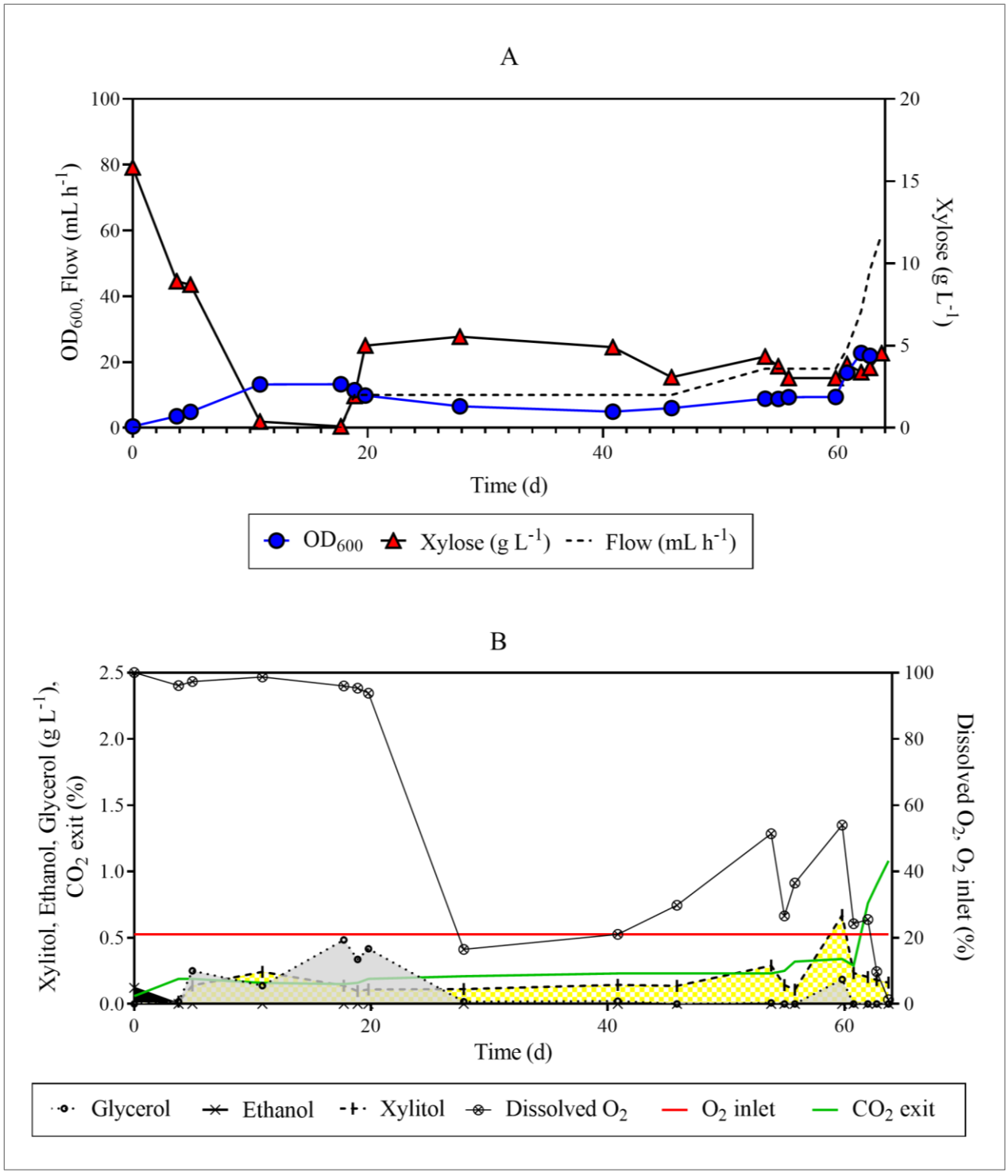

Fig. 2.5. Long-term continuous cultivation of *S. cerevisiae* SA-1 XR/XDH under an aerobic condition with xylose as the carbon source (VM supplemented with xylose). A. OD_{600} values, residual xylose concentration, and pump flow rate. The batch phase lasted for 17 days. B. Glycerol, grey area; Xylitol, yellow area; Ethanol, black area.

Adaptative laboratory evolution represents one of the most practical solutions for

metabolic engineering to induce spontaneous mutations favorable to heterologous metabolism (Shin et al. 2021). Assisted with genome sequencing and omics approaches, spontaneous mutations can be identified. Adaptative evolution in the laboratory relies on genetic diversity and artificial selection, which is analogous to processes that occur in nature during the evolution of new species (Sauer 2001). As mentioned before, Kim et al. (2013b) used evolutionary engineering to further improve xylose utilization, which had yielded a strain able to metabolize xylose faster than its parental strain. Therefore, through extensive efforts in metabolic and evolutionary engineering, recombinant *S. cerevisiae* is now able to convert xylose into ethanol as the sole carbon source (Kim et al. 2013b).

In another study published by Sonderegger and Sauer (2003) the *S. cerevisiae* TMB3001 was maintained in a long-term continuous culture under progressive more restrictive oxygen limitation (150 generations under aerobic condition, almost 100 generations under microaerobic, and 90 generations under anaerobic condition) to select a strain capable of anaerobic growth on xylose alone. Starting from continuous aerobic cultivation on a mixture of xylose and glucose and progressive to anaerobic cultivation on xylose as the sole carbon source, the authors obtained two cell populations. Clones taken from the smaller subpopulation were incapable of anaerobic growth on xylose but produced more ethanol from xylose than the parental strain. Clones taken from the larger subpopulation grew anaerobically on xylose but showed impaired growth on glucose (Sonderegger and Sauer 2003).

Similar continuous cultivation under aerobic conditions was performed by Wahlbom et al. (2003a) with recombinant xylose-utilizing *S. cerevisiae* strain TMB3399. They also performed continuous cultivation under oxygen-limited and anaerobic conditions to obtain mutants that display improved ability to ferment xylose. However, even the best of these mutants, the strain TMB3400, showed only about one-third of the aerobic maximum growth rate obtained with *P. stipitis* CBS 6054 on xylose. Comparing to its parental strain, the TMB3400 strain achieved an ethanol yield of 0.25 while for TMB3399 it was 0.21 g ethanol g xylose^{-1} (Wahlbom et al. 2003a, b).

2.3.3. Fermentative analysis performance of SA-1 XR/XDH and DPY06

To check the improvements derived from the laboratory evolution approach, the evolved strain (hereafter called DPY06) that was isolated at the end of the evolutionary cultivation, and its

parental strain, SA-1 XR/XDH was cultivated under a strict anaerobic (anoxic) condition in a YP medium containing 20 g L^{-1} glucose and 80 g L^{-1} xylose, at 30 °C and 100 rpm in serum bottles sealed with butyl rubber stoppers with an initial OD_{600} of 0.3. In these cultures, DPY06 exhibited different capabilities in consuming xylose in comparison with its parental strain (Fig. 2.6). Laboratory evolution generated a strain able to consume xylose faster (Fig. 2.7A and Table 2.4). Ethanol production was detected in both cultivations, however, DPY06 did show a slight improvement in the volumetric ethanol productivity (Table 2.4). Moreover, despite DPY06 showed higher xylitol accumulation until 168 h of cultivation (Fig. 2.6C), the evolved strain exhibited lower xylitol accumulation at the end of the fermentation than SA-1 XR/XDH, which achieved higher xylitol amounts after 168 h of cultivation.

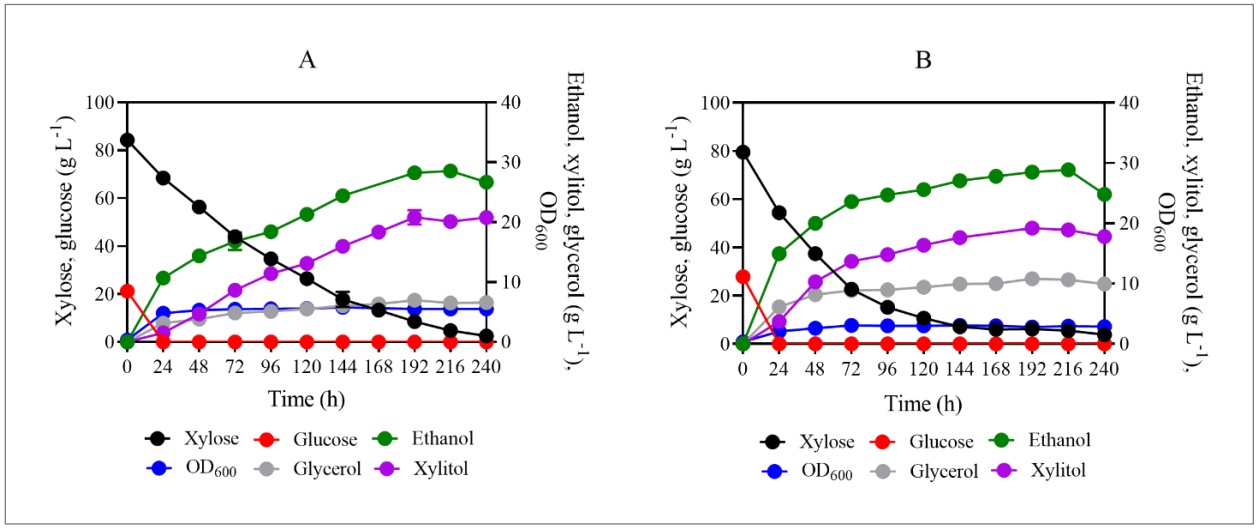

Fig. 2.6. Fermentation profiles of the parental strain SA-1 XR/XDH (A) and DPY06 (B) when fermenting YP supplemented with 20 g L^{-1} glucose and 80 g L^{-1} xylose under anaerobic condition. An initial OD_{600} was adjusted to 0.3. The figure illustrates the means of biological triplicate experiments of each strain SA-1 XR/XDH expressing two copies of XYL1, XYL2, and XYL3; DPY06 an evolved SA-1 XR/XDH.

It is interesting to note that DPY06 used the available carbon mainly to produce ethanol, xylitol, and glycerol; and minor amounts were diverted to yeast biomass in comparison to SA-1 XR/XDH, which achieve double OD_{600} after 24h until the end of the cultivation. Furthermore, practically all biomass was produced during glucose depletion (first 24 h of cultivation – Fig. 2.5), after that, it was no significant increments in it. The xylose consumption and specific xylose consumption rates were determined and DPY06 presented high values for these parameters. Table 2.4 presents these values at 24 and 144 h. Therefore, under the conditions investigated, the isolated

mutant has advantages in xylose fermentation. These results corroborate the hypothesis that the evolutive engineering approach can optimize the sugar uptake by the evolved strain.

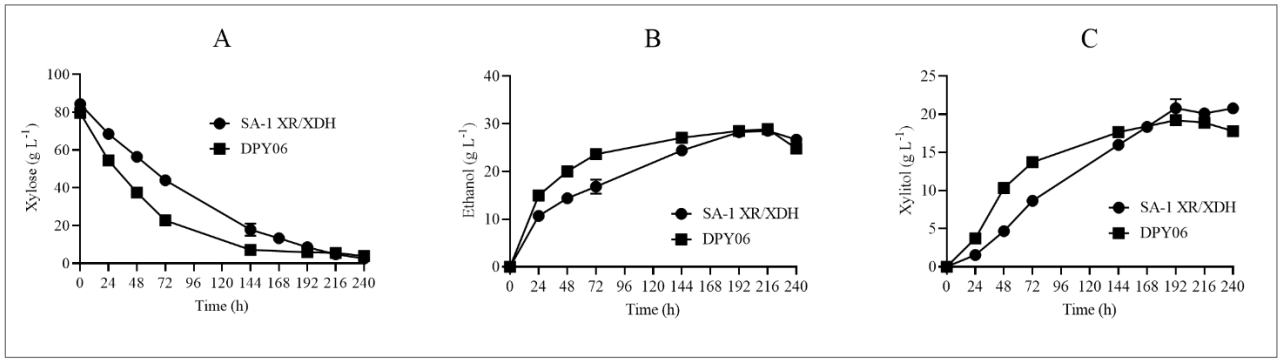

Fig. 2.7. Comparison of xylose consumption (A), ethanol (B), and xylitol (C) production of two xylose-assimilating strains in YP media containing 20 g L^{-1} glucose and 80 g L^{-1} xylose under anaerobic (anoxic) condition. An initial OD_{600} was adjusted to 0.3. The figure illustrates the means of biological triplicate experiments of each strain.

Table 2.4. Fermentation profile of SA-1- XR/XDH and DPY06 under anaerobic conditions

	At 24 h			At 72 h			
	r_{xylose}	r_{xylose}*	$P_{Ethanol}$	r_{xylose}	r_{xylose}*	$P_{Ethanol}$	$Y_{Ethanol}$
SA-1-XR/XDH	0.67 ± 0.07	0.14 ± 0.02	0.45 ± 0.02	0.56 ± 0.00	0.10 ± 0.00	0.24 ± 0.01	0.28 ± 0.00
DPY06	1.04 ± 0.01	0.50 ± 0.01	0.62 ± 0.01	0.79 ± 0.01	0.26 ± 0.00	0.33 ± 0.00	0.27 ± 0.00

Parameters: r_{xylose}, xylose consumption rate (g L^{-1} h^{-1}); r_{xylose}*, specific xylose consumption rate (g L^{-1} OD^{-1} h^{-1}); $P_{Ethanol}$, volumetric ethanol productivity (g L^{-1} h^{-1}); $Y_{Ethanol}$, ethanol yield (g $g_{total\ sugar}^{-1}$).

In a more recent study, Jeong et al (2020) have applied a blended approach, metabolic and evolutionary engineering, to improve xylose assimilation in a laboratory *S. cerevisiae* strain expressing *XYL123*, besides a deletion of *PHO13* (p-nitrophenylphosphatase) and overexpression of *TAL1* (transaldolase). Adaptative evolution approach was applied, and mutants isolated from the evolved cultures showed improved xylose fermentation capabilities, with a growth rate of 0.19 g L^{-1} h^{-1} and ethanol yield of 0.32 g ethanol g $xylose^{-1}$ when fermenting YP medium containing 40 g L^{-1} of xylose, which was higher than DPY06 strain in YPDX under anaerobic condition. Therefore, multiple mutations were probably necessary to endow SA-1 XR/XDH with the ability to grow faster on xylose.

Although many recombinant *S. cerevisiae* strains can convert xylose into ethanol, most of these strains are haploid versions, since they are easy for genetic manipulation but, not for

robustness. On the other hand, industrial diploid or polyploid *S. cerevisiae* strains are harder to manipulate but are preferred for eventual industrial applications because they are typically more tolerant to these toxic compounds and easier to cultivate in large bioreactors (Dias Lopes et al. 2017). These toxic compounds such as furans, organic acids, phenols, and inorganic salts are formed during lignocellulosic pre-treatment. Diploid strain presents a differentiated capacity to grow under inhibitory conditions (Cola et al. 2020). The genetic background of the host strain significantly affects the performance of the recombinant strain (Li et al. 2016). Normally, industrial diploid strains are more robust and better ethanol producers compared to laboratory haploid strains, however, industrial diploid *S. cerevisiae* strains have been less common (Diao et al. 2013; Li et al. 2016).

Furthermore, the SA-1 strain represents a promising platform yeast strain for second-generation ethanol production, since it showed to be more robust in the presence of inhibitory compound-containing media (Cola et al. 2020). In the present study, the laboratory evolutionary engineering approach was a critical step to improve the xylose-fermenting capabilities of the engineered SA-1 strain.

2.4. CONCLUSION

Laboratory adaptive evolution provided an improvement in SA-1 XR/XDH strain, mainly concerning the xylose uptake. This dataset demonstrates the feasibility of this strategy. The evolved strain, DPY06, was able to consume xylose faster, presenting a specific xylose consuming rate 72% higher than the control cultivation, and showed an improvement in the volumetric ethanol productivity, 28% comparing DPY06 and SA-1 XR/XDH at 24 h of cultivation in YP media supplemented with glucose and xylose. The evolutive study was performed in long-term continuous cultivation in synthetic media under controlled conditions. Although we have verified that the evolved strain performs well at elevated sugar concentrations (100 g L^{-1} sugars) under strictly anaerobic conditions, many other parameters relevant for industrial second-generation ethanol production remain to be systematically investigated, as full incorporation of lignocellulosic hydrolysates into the fermentative analysis of studied strain.

2.5. REFERENCES

Apel AR, Ouellet M, Szmidt-Middleton H, et al (2016) Evolved hexose transporter enhances

xylose uptake and glucose/xylose co-utilization in *Saccharomyces cerevisiae*. Sci Rep 6:1–10. https://doi.org/10.1038/srep19512

Basso TO, Dario MG, Tonso A, et al (2010) Insufficient uracil supply in fully aerobic chemostat cultures of *Saccharomyces cerevisiae* leads to respiro-fermentative metabolism and double nutrient-limitation. Biotechnol Lett 32:973–977. https://doi.org/10.1007/s10529-010-0248-2

Basso TP (2015) Improvement of *Saccharomyces cerevisiae* by hybridization for increased tolerance towards inhibitors from second-generation ethanol substrate. Biblioteca Digital de Teses e Dissertações da Universidade de São Paulo

Belinchón MM, Gancedo JM (2003) Xylose and some non-sugar carbon sources cause catabolite repression in *Saccharomyces cerevisiae*. Arch Microbiol 180:293–297. https://doi.org/10.1007/s00203-003-0593-9

Borgström C, Wasserstrom L, Almqvist H, et al (2019) Identification of modifications procuring growth on xylose in recombinant *Saccharomyces cerevisiae* strains carrying the Weimberg pathway. Metab Eng 55:1–11. https://doi.org/10.1016/j.ymben.2019.05.010

Bracher JM, Martinez-Rodriguez OA, Dekker WJC, et al (2019) Reassessment of requirements for anaerobic xylose fermentation by engineered, non-evolved *Saccharomyces cerevisiae* strains. FEMS Yeast Res 19:. https://doi.org/10.1093/femsyr/foy104

Bro C, Nielsen J (2004) Impact of 'ome' analyses on inverse metabolic engineering. Metab Eng 6:204–211. https://doi.org/10.1016/J.YMBEN.2003.11.005

Brown SW, Oliver SG (1982) Isolation of ethanol-tolerant mutants of yeast by continuous selection. Eur J Appl Microbiol Biotechnol 16:119–122. https://doi.org/10.1007/BF00500738

Çakar ZP, Turanlı-Yıldız B, Alkım C, Yılmaz Ü (2012) Evolutionary engineering of *Saccharomyces cerevisiae* for improved industrially important properties. FEMS Yeast Res 12:171–182. https://doi.org/10.1111/j.1567-1364.2011.00775.x

Cardona CA, Sánchez ÓJ (2007) Fuel ethanol production: Process design trends and integration opportunities. Bioresour Technol 98:2415–2457. https://doi.org/10.1016/j.biortech.2007.01.002

Cola P, Procópio DP, Alves AT de C, et al (2020) Differential effects of major inhibitory compounds from sugarcane-based lignocellulosic hydrolysates on the physiology of yeast strains and lactic acid bacteria. Biotechnol Lett. https://doi.org/10.1007/s10529-020-02803-6

Della-Bianca BE, Gombert AK (2013) Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. 104:1083–1095. https://doi.org/10.1007/s10482-013-0030-2

Diao L, Liu Y, Qian F, et al (2013) Construction of fast xylose-fermenting yeast based on industrial ethanol-producing diploid *Saccharomyces cerevisiae* by rational design and adaptive evolution. BMC Biotechnol 13:1–9. https://doi.org/10.1186/1472-6750-13-110

Dias Lopes D, Carlos ·, Rosa A, et al (2017) Influence of genetic background of engineered xylose-fermenting industrial *Saccharomyces cerevisiae* strains for ethanol production from lignocellulosic hydrolysates. J Ind Microbiol Biotechnol 44:1575–1588. https://doi.org/10.1007/s10295-017-1979-z

Eliasson A, Christensson C, Fredrik Wahlbom C, et al (2000) Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying XYL1, XYL2, and XKS1 in mineral medium chemostat cultures. Appl Environ Microbiol 66:3381–6. https://doi.org/10.1128/AEM.66.8.3381-3386.2000

Farwick A, Bruder S, Schadeweg V, et al (2014) Engineering of yeast hexose transporters to

transport D-xylose without inhibition by D-glucose. Natl Acad Sci 111:5159–5164. https://doi.org/10.1073/pnas.1323464111

Guerriero G, Hausman JF, Strauss J, et al (2016) Lignocellulosic biomass: Biosynthesis, degradation, and industrial utilization. Eng. Life Sci 16:1–16. https://doi.org/10.1002/elsc.201400196

Hahn-Hägerdal B, Karhumaa K, Jeppsson M, Gorwa-Grauslund MF (2007) Metabolic Engineering for Pentose Utilization in *Saccharomyces cerevisiae*. Biofuels 108:147–177. https://doi.org/10.1007/10_2007_062

Hamacher T, Boles E, Gárdonyi M, et al (2002) Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. Microbiology 148:2783–2788. https://doi.org/10.1099/00221287-148-9-2783

Hector RE, Qureshi N, Hughes SR, Cotta MA (2008) Expression of a heterologous xylose transporter in a *Saccharomyces cerevisiae* strain engineered to utilize xylose improves aerobic xylose consumption. Appl Microbiol Biotechnol 80:675–684. https://doi.org/10.1007/s00253-008-1583-2

Heiling A (2020) Using CRISPR/Cas9 to regulate enzyme expression levels in the Weimberg pathway for optimal xylose consumption in *Saccharomyces cerevisiae*. Master Thesis of Lund University

Himmel ME, Ding S-YS-Y, Johnson DK, et al (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315:804–807. https://doi.org/10.1126/science.1137016

Ho NWY, Chen Z, Brainard AP (1998) Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. Appl Environ Microbiol 64:1852–1859. https://doi.org/0099-2240/98/$04.00+0

Hong KK, Nielsen J (2012) Metabolic engineering of *Saccharomyces cerevisiae*: A key cell factory platform for future biorefineries. Cell Mol Life Sci 69:2671–2690. https://doi.org/10.1007/s00018-012-0945-1

Jansen MLA, Daran-Lapujade P, de Winde JH, et al (2004) Prolonged maltose-limited cultivation of *Saccharomyces cerevisiae* selects for cells with improved maltose affinity and hypersensitivity. Appl Environ Microbiol 70:1956–1963. https://doi.org/10.1128/AEM.70.4.1956-1963.2004

Jeffries TW (2006) Engineering yeasts for xylose metabolism. Curr Opin Biotechnol 17:320–326. https://doi.org/10.1016/j.copbio.2006.05.008

Jeong D, Oh EJ, Ko JK, et al (2020) Metabolic engineering considerations for the heterologous expression of xylose-catabolic pathways in *Saccharomyces cerevisiae*. PLoS One 15:e0236294. https://doi.org/10.1371/journal.pone.0236294

Jiang Y, Shen Y, Gu L, et al (2020) Identification and characterization of an efficient D-xylose transporter in *Saccharomyces cerevisiae*. J Agric Food Chem 68:2702–2710. https://doi.org/10.1021/acs.jafc.9b07113

Jin YS, Jeffries TW (2004) Stoichiometric network constraints on xylose metabolism by recombinant *Saccharomyces cerevisiae*. Metab Eng 6:229–238. https://doi.org/10.1016/j.ymben.2003.11.006

Jin YS, Ni H, Laplaza JM, Jeffries TW (2003) Optimal growth and ethanol production from xylose by recombinant *Saccharomyces cerevisiae* require moderate D-xylulokinase activity. Appl Environ Microbiol 69:495–503. https://doi.org/10.1128/AEM.69.1.495-503.2003

Jo JH, Park YC, Jin YS, Seo JH (2017) Construction of efficient xylose-fermenting

Saccharomyces cerevisiae through a synthetic isozyme system of xylose reductase from *Scheffersomyces stipitis*. Bioresour Technol 241:88–94. https://doi.org/10.1016/j.biortech.2017.05.091

Kim SR, Kwee NR, Kim H, Jin YS (2013a) Feasibility of xylose fermentation by engineered *Saccharomyces cerevisiae* overexpressing endogenous aldose reductase (GRE3), xylitol dehydrogenase (XYL2), and xylulokinase (XYL3) from *Scheffersomyces stipitis*. FEMS Yeast Res 13:312–321. https://doi.org/10.1111/1567-1364.12036

Kim SR, Skerker JM, Kang W, et al (2013b) Rational and evolutionary engineering approaches uncover a small set of genetic changes efficient for rapid xylose fermentation in *Saccharomyces cerevisiae*. PLoS One 8:e57048. https://doi.org/10.1371/journal.pone.0057048

Kötter P, Ciriacy M, Kotter P, et al (1993) Xylose fermentation by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 38:776–783. https://doi.org/10.1007/BF00167144

Kuyper M, Harhangi HR, Stave AK, et al (2003) High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? FEMS Yeast Res 4:69–78. https://doi.org/10.1016/S1567-1356(03)00141-7

Kuyper M, Toirkens MJ, Diderich JA, et al (2005) Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res 5:925–934. https://doi.org/10.1016/j.femsyr.2005.04.004

Kuyper M, Winkler AA, Van Dijken JP, Pronk JT (2004) Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: A proof of principle. FEMS Yeast Res 4:655–664. https://doi.org/10.1016/j.femsyr.2004.01.003

Lee JW, Yook S, Koh H, et al (2021) Engineering xylose metabolism in yeasts to produce biofuels and chemicals. Curr Opin Biotechnol 67:15–25. https://doi.org/10.1016/j.copbio.2020.10.012

Li H, Shen Y, Wu M, et al (2016) Engineering a wild-type diploid *Saccharomyces cerevisiae* strain for second-generation bioethanol production. Bioresour Bioprocess 3:. https://doi.org/10.1186/s40643-016-0126-4

Li X, Yu VY, Lin Y, et al (2015) Expanding xylose metabolism in yeast for plant cell wall conversion to biofuels. Elife. https://doi.org/10.7554/eLife.05896

Luttik MA, Kötter P, Salomons FA, et al (2000) The *Saccharomyces cerevisiae* ICL2 gene encodes a mitochondrial 2-methylisocitrate lyase involved in propionyl-coenzyme A metabolism. J Bacteriol 182:7007–13. https://doi.org/10.1128/JB.182.24.7007-7013.2000

Matsushika A, Watanabe S, Kodaki T, et al (2008) Expression of protein engineered NADP+-dependent xylitol dehydrogenase increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 81:243–255. https://doi.org/10.1007/s00253-008-1649-1

Meinander NQ, Hahn-Hägerdal B (1997) Influence of cosubstrate concentration on xylose conversion by recombinant, XYL1-expressing *Saccharomyces cerevisiae*: A comparison of different sugars and ethanol as cosubstrates. Appl Environ Microbiol 63:1959–1964. https://doi.org/10.1128/aem.63.5.1959-1964.1997

Nijland JG, Shin HY, De Jong RM, et al (2014) Engineering of an endogenous hexose transporter into a specific D-xylose transporter facilitates glucose-xylose co-consumption in *Saccharomyces cerevisiae*. Biotechnol Biofuels 7:. https://doi.org/10.1186/s13068-014-0168-9

Olsson L, Nielsen J (1997) On-line and in situ monitoring of biomass in submerged cultivations.

Trends Biotechnol 15:517–522. https://doi.org/10.1016/S0167-7799(97)01136-0
Petrovič U (2015) Next-generation biofuels: a new challenge for yeast. Yeast 32:583–593. https://doi.org/10.1002/yea.3082
Petschacher B, Leitgeb S, Kavanagh KL, et al (2005) The coenzyme specificity of *Candida tenuis* xylose reductase (AKR2B5) explored by site-directed mutagenesis and X-ray crystallography. Biochem J 385:75–83. https://doi.org/10.1042/BJ20040363
Richard P, Toivari MH, Penttilão M (2000) The role of xylulokinase in *Saccharomyces cerevisiae* xylulose catabolism. FEMS Microbiol Lett 190:39–43. https://doi.org/10.1111/j.1574-6968.2000.tb09259.x
Roca C, Haack MB, Olsson L (2004) Engineering of carbon catabolite repression in recombinant xylose fermenting *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 63:578–583. https://doi.org/10.1007/s00253-003-1408-2
Rodriguez-Peña JM, Cid VJ, Arroyo J, Nombela C (1998) The YGR194c (XKS1) gene encodes the xylulokinase from the budding yeast *Saccharomyces cerevisiae*. FEMS Microbiol Lett 162:155–160. https://doi.org/10.1111/j.1574-6968.1998.tb12993.x
Runquist D, Fonseca C, Rådström P, et al (2009) Expression of the Gxf1 transporter from *Candida intermedia* improves fermentation performance in recombinant xylose-utilizing *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 82:123–130. https://doi.org/10.1007/s00253-008-1773-y
Runquist D, Hahn-Hägerdal B, Bettiga M (2010) Increased ethanol productivity in xylose-utilizing *Saccharomyces cerevisiae* via a randomly mutagenized xylose reductase. Appl Environ Microbiol 76:7796–7802. https://doi.org/10.1128/AEM.01505-10
Saloheimo A, Rauta J, Stasyk O V., et al (2007) Xylose transport studies with xylose-utilizing *Saccharomyces cerevisiae* strains expressing heterologous and homologous permeases. Appl Microbiol Biotechnol 74:1041–1052. https://doi.org/10.1007/s00253-006-0747-1
Salusjärvi L, Kankainen M, Soliymani R, et al (2008) Regulation of xylose metabolism in recombinant *Saccharomyces cerevisiae*. Microb Cell Fact 7:1–16. https://doi.org/10.1186/1475-2859-7-18
Salusjärvi L, Pitkänen JP, Aristidou A, et al (2006) Transcription analysis of recombinant *Saccharomyces cerevisiae* reveals novel responses to xylose. Appl Biochem Biotechnol 128:237–273. https://doi.org/10.1385/ABAB:128:3:237
Sauer U (2001) Evolutionary Engineering of industrially important microbial phenotypes. Adv Biochem Eng Biotechnol 73:129–169. https://doi.org/10.1007/3-540-45300-8_7
Sedlak M, Ho NWY (2004) Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant *Saccharomyces* yeast. Yeast 21:671–684. https://doi.org/10.1002/yea.1060
Shen L, Kohlhaas M, Enoki J, et al (2020) A combined experimental and modelling approach for the Weimberg pathway optimisation. Nat Commun 11:1–13. https://doi.org/10.1038/s41467-020-14830-y
Shin M, Park H, Kim S, et al (2021) Transcriptomic changes induced by de-activation of lower glycolysis and its advantage on pentose sugar metabolism in *Saccharomyces cerevisiae*. Authorea Prepr. https://doi.org/10.22541/AU.160010788.88280741/V2
Sonderegger M, Sauer U (2003) Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose. Appl Environ Microbiol 69:1990–8
Steensels J, Snoek T, Meersman E, et al (2014) Improving industrial yeast strains: exploiting natural and artificial diversity. FEMS Microbiol Rev 38:947–95.

https://doi.org/10.1111/1574-6976.12073
Subtil T, Boles E (2012) Competition between pentoses and glucose during uptake and catabolism in recombinant *Saccharomyces cerevisiae*. Biotechnol Biofuels 5:. https://doi.org/10.1186/1754-6834-5-14
van Hoek P, van Dijken JP, Pronk (2000) Regulation of fermentative capacity and levels of glycolytic enzymes in chemostat cultures of *Saccharomyces cerevisiae*. Enzyme Microb Technol 26:724–736
Verduyn C, Postma E, Scheffers WA, Van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8:501–517. https://doi.org/10.1002/yea.320080703
Wahlbom CF, Otero RRC, Zyl WH Van, Jo LJ (2003a) Molecular Analysis of a *Saccharomyces cerevisiae* mutant with improved ability to utilize xylose shows enhanced expression of proteins involved in transport, initial xylose metabolism, and the pentose phosphate pathway. Appl Environ Microbiol 69:740–746. https://doi.org/10.1128/AEM.69.2.740
Wahlbom CF, Van Zyl WH, Jönsson LJ, et al (2003b) Generation of the improved recombinant xylose-utilizing *Saccharomyces cerevisiae* TMB 3400 by random mutagenesis and physiological comparison with *Pichia stipitis* CBS 6054. FEMS Yeast Res 3:319–326. https://doi.org/10.1016/S1567-1356(02)00206-4
Walfridsson M, Hallborn J, Penttila M, et al (1995) Xylose-Metabolizing *Saccharomyces cerevisiae* strains overexpressing the Tkl1 and Tali genes encoding the pentose-phosphate pathway enzymes transketolase and transaldolase. Appl Environ Microbiol 61:4184–4190
Watanabe S, Saleh AA, Pack SP, et al (2007) Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein-engineered NADH-preferring xylose reductase from Pichia stipitis. Microbiology 153:3044–3054. https://doi.org/10.1099/mic.0.2007/007856-0
Winkelhausen E, Kuzmanova S (1998) Microbial conversion of d-xylose to xylitol. J Ferment Bioeng 86:1–14. https://doi.org/10.1016/S0922-338X(98)80026-3
Young E, Poucher A, Comer A, et al (2011) Functional survey for heterologous sugar transport proteins, using *Saccharomyces cerevisiae* as a host. Appl Environ Microbiol 77:3311–3319. https://doi.org/10.1128/AEM.02651-10
Zhang GC, Kong II, Wei N, et al (2016) Optimization of an acetate reduction pathway for producing cellulosic ethanol by engineered yeast. Biotechnol Bioeng 113:2587–2596. https://doi.org/10.1002/bit.26021
Zhang J, Tang M, Viikari L (2012) Xylans inhibit enzymatic hydrolysis of lignocellulosic materials by cellulases. Bioresour Technol 121:8–12. https://doi.org/10.1016/j.biortech.2012.07.010

CHAPTER III: ENGINEERING OF INDUSTRIAL XYLOSE-CONSUMING STRAIN TO METABOLIZE XYLO-OLIGOSACCHARIDES

Engineering of industrial xylose-consuming strain to metabolize xylo-oligosaccharides

ABSTRACT

The need to replace fossil fuels due to the political, economic, and especially environmental impact problems has motivated research into renewable energy sources. In this context, the yeast *Saccharomyces cerevisiae*, the common microorganism used to produce bioethanol, has been demonstrated as a highly interesting commercial microorganism. However, this yeast cannot ferment hemicellulose-derived sugar. Sustainable second-generation ethanol should include the efficient and simultaneous co-fermentation of all sugars derived from cellulose and hemicellulose. To face the problem associated with the inability of *S. cerevisiae* to ferment hemicellulose-derived sugar this present research proposal aimed to engineer a xylose-utilizing industrial *S. cerevisiae* strains, SA-1 XR/XDH, to transport and consume xylo-oligosaccharides (XOS). The selected strain was transformed with a plasmid that carries genes for XOS transport and hydrolysis from *Neurospora crassa*. The utilization of XOS transporter and consumption will expand the capabilities of *S. cerevisiae* to utilize plant-derived and represent a potential to increase the efficiency of second-generation biofuel production.

Keywords: lignocellulosic ethanol; industrial *Saccharomyces cerevisiae*; xylo-oligosaccharides; xylose.

3.1. INTRODUCTION

Profitable renewable chemicals and biofuels from plant biomass, lignocellulosic materials, require efficient and complete use of all abundant sugars. This biomass consists essentially of highly compacted materials of cellulose, hemicellulose, and lignin (Pettersen 1984; Murphy and McCarthy 2005; Shen et al. 2013). Despite *S. cerevisiae*, the common microorganism used to produce bioethanol, cannot ferment hemicellulose-derived sugar, like xylose, and XOS (Hahn-Hägerdal et al. 2007) it has been published engineered *S. cerevisiae* strains able to metabolize xylose and XOS (Katahira et al. 2004; Saitoh et al. 2011; Sakamoto et al. 2012; Sun et al. 2012; Li et al. 2015; Lee et al. 2015; Qi et al. 2015; Dos Santos et al. 2016; Tabañag et al. 2018; Bracher et al. 2019). However, with respect to intracellular hydrolysis of XOS – the focus of the present work, just Li et al. (2015) published an engineered *S. cerevisiae* strain able to metabolize intracellularly XOS. Their recombinant strain expressed the same genes used in the present work, two β-xylosidases, GH43-2, and GH43-7, and one transporter, CDT-2, from *N. crassa*, which were inserted in a laboratory strain. In the present study, an industrial *S. cerevisiae* strain was chosen as the host of the abovementioned genes from *N. crassa*.

Fermentation of hemicellulose-derived sugar will demand further improvements for industrial implementation (Jeppsson et al. 2002), for example, providing xylose-fermentation capabilities into *S. cerevisiae* (Jeffries 2006; Jo et al. 2017). Some examples of this xylose catabolism pathway include the oxidoreductase from *Scheffersomyces stipittis* (encoded by *XYL1*, *XYL2*, and *XYL3*) and isomerase pathway from *Piromyces* spp., (encoded by xylA) (Himmel et al. 2007; Lee et al. 2012). As well, providing XOS-fermentation capabilities. The construction of oligo-fermenting *S. cerevisiae* strain will imply reduce operational costs and reduced enzyme loadings during enzymatic hydrolysis of lignocellulosic biomass since less intensive pre-treatment conditions could be used, devising oligosaccharides instead of monomers. It is expected that such yeasts would have a competitive advantage in relation to other microorganisms, such as contaminating bacteria and wild *Saccharomyces* and non-*Saccharomyces* species (Cabrini and Gallo 1999; Amorim et al. 2011), once they would be able to consume oligomers in hydrolysates. Thus, engineering an efficient oligosaccharide-fermenting *S. cerevisiae* strain would be a strategy for improving ethanol production from lignocellulosic materials (Fujii et al. 2011).

It is of utmost importance to mention that the conversion of lignocellulosic biomass to ethanol requires 4 steps: pre-treatment and hydrolysis to transform polymerized sugars, cellulose,

and hemicellulose into fermentable sugars, fermentation, and distillation (Olsson and Hahn-Hägerda 1996). However, while lignocellulosic materials are breaking down during the pre-treatment and hydrolysis step, lignocellulosic-derived inhibitors are formed (van Maris et al. 2006; Kłosowski and Mikulski 2021). The main inhibitors observed in lignocellulosic substrates are sugar degradation products (furans such as furfural and hydroxymethylfurfural - HMF), weak organic acids (such as acetic, glycolic, levulinic, and formic acids), and phenol derivatives. Xylose, mannose, acetic acid, galactose, and glucose are released when hemicellulose is degraded, and cellulose is hydrolyzed to glucose. At high temperatures and pressure, xylose is degraded to furfural (Palmqvist and Hahn-Hägerdal 2000). Similarly, 5-hydroxymethylfurfural (HMF) is formed from hexose degradation. Phenolic compounds are generated from the partial breakdown of lignin (Almeida et al. 2007). Formic acid is formed when furaldehyde compounds are broken down, and levulinic acid is formed by HMF degradation (Palmqvist and Hahn-Hägerdal 2000). The lignocellulosic inhibitors can affect cellular growth and fermentative performance (Almeida et al. 2007; Almario et al. 2013; Adeboye et al. 2014). These lignocellulosic-derived inhibitors, thereby, represent an additional obstacle to second-generation ethanol, since the majority of the compounds abovementioned several affect yeast growth and its fermentative performance (Palmqvist and Hahn-Hägerdal 2000; Kim et al. 2013; Caspeta and Nielsen 2015). But, industrial yeast strains have been showing to be able to withstand with an outstanding performance the presence of inhibitory compounds to detriment of laboratory *S. cerevisiae* strains (Cola et al. 2020), which makes them a good choice for second-generation ethanol.

In this context, this work intended to implement the performance of a XOS pathway by the expression of the pDX8.4, pDX8.6, and pXD8.7 plasmid (kindly provided by Dr. Jamie Cate from the University of California at Berkeley, CA, USA) that carries overexpressed sequence for transport and hydrolysis of XOS, in an industrial xylose-utilizing *S. cerevisiae* strain previously modified with *XYL1*, *XYL2*, and *XYL3*, SA-1 XR/XDH, gently provided by Luis Carlos Basso (Escola Superior da Agricultura Luiz de Queiroz –Universidade de São Paulo), as represented in Fig. 3.1, for more effective utilization of lignocellulosic biomass.

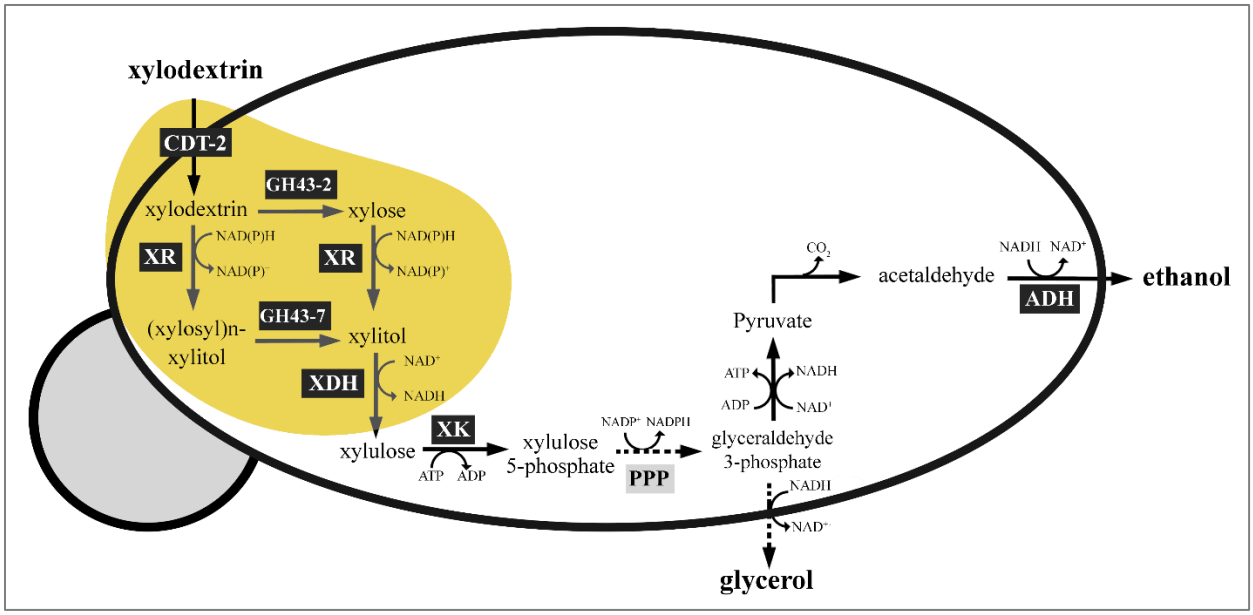

Fig. 3.1. Xylooligosaccharide metabolism by expression of the XOS transport (*CTD-2*) and the expression of the consumption (*GH43-2* and *GH43-7*) pathways from *Neurospora crassa.* Xylose metabolism expression by xylose reductase (*XR*) and xylitol dehydrogenase (*XDH*) from *S. stipitis*.

3.2. GOALS

The present research proposal aims to insert XOS consumption pathway into an industrial xylose-consuming *S. cerevisiae* strain SA-1 XR/XDH. The XOS pathway chosen is the CDT-2 transport and two β-xylosidases, GH43-2 and GH43-7, from *Neurospora crassa*. A metabolic engineering strategy was performed to improve xylose uptake by the host strain.

3.3. MATERIALS AND METHODS

3.3.1. Strains, plasmids, and media

E. coli was cultured in lysogeny broth (LB) medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 10 g/L NaCl) at 37 °C and 100 µ/mL ampicillin (LBA) was added for selection when required. All *S. cerevisiae* strains used and constructed in this work are summarized in Table 3.1. Yeast cells were grown in yeast extract-peptone (YP) medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) containing hemicellulosic hydrolysate (YPHX), and enzymatically-hydrolysed xylan (YPXylan). All cultivations were performed at 30 °C and 200 rpm for aerobic conditions. All plasmids used in this work are summarized in Table 3.2 and Fig. 3.2.

Table 3.1. The yeast strains used in this study.

Strain	Description	Reference
SA-1 XR/XDH	Xylose-consuming strain engineered from strain SA-1	(BASSO et al. 2015)
DPY11	SA-1 XR/XDH expressing CEN/ARS plasmid carrying CDT-2, GH43-2 overexpression cassette	This work
DPY12	SA-1 XR/XDH expressing CEN/ARS plasmid carrying CDT-2, and GH43-7 overexpression cassette	This work
DPY13	SA-1 XR/XDH expressing CEN/ARS plasmid carrying CDT-2, GH43-2, and GH43-7 overexpression cassette	This work

Table 3.2. The plasmid used in this study.

Plasmids	Description	Reference
pXD8.4	pRS316-pCCW12-CDT2-tCYC1-$_{p}$CCW12-GH43-2 tCYC1	(Li et al. 2015a)
pXD8.6	pRS316-pCCW12-CDT2-tCYC1-$_{p}$CCW12-GH43-7 tCYC1	(Li et al. 2015a)
pXD8.7	pRS316-pCCW12-CDT2-tCYC1-$_{p}$CCW12-GH43-2 tCYC1-$_{p}$CCW12-GH43-7 tCYC1	(Li et al. 2015a)

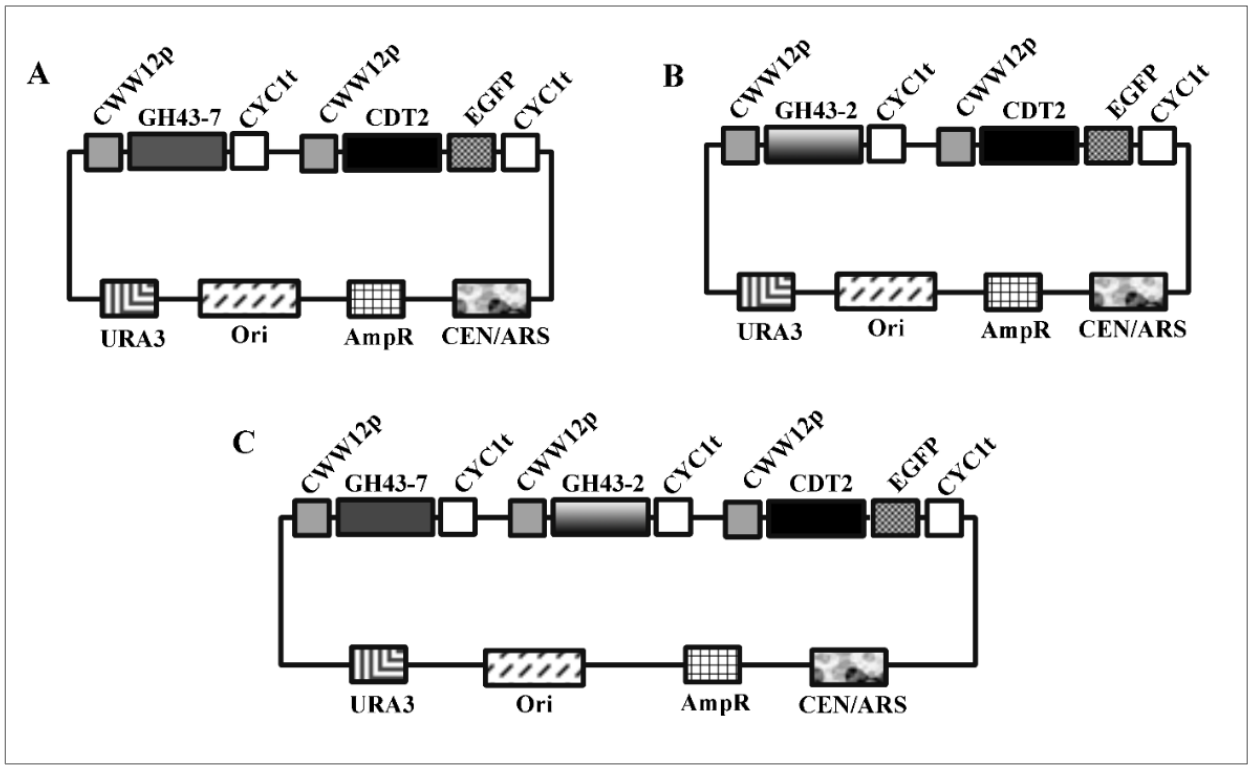

Fig. 3.2. Representative scheme of the XOS consumption plasmids. A, pXD8.6, GH43-7, xylanase from *N. crassa;* CDT-2, XOS transporter from *N. crassa*. B, pXD8.4, GH43-2, xylanase from *N. crassa;* CDT-2, XOS transporter from *N. crassa.* C, pXD8.7, GH43-2, and G43-7, xylanases from *N. crassa;* CDT-2, XOS transporter from *N. crassa*(Li et al. 2015a).

To construct an XOS-utilizing strain expressing separately the episomal plasmids containing the $_{P}$CCW12-CDT-2-$_{T}$CYC1-$_{P}$CCW12-GH43-2-$_{T}$CYC1, $_{P}$CCW12-CDT-2-$_{T}$CYC1-$_{P}$CCW12-GH43-7-$_{T}$CYC1, and $_{P}$CCW12-CDT-2-$_{T}$CYC1-$_{P}$CCW12-GH43-2-$_{T}$CYC1-$_{P}$CCW12-GH43-7-$_{T}$CYC1 overexpressed sequence, were transformed into SA-1 XR/XDH, yielding the DPY11, DPY12, and DPY13. Transformation of yeast cells was carried out with the polyethylene glycol (PEG)-LiAc method (Gietz et al. 1995). SA-1 XR/XDH is an industrial *S. cerevisiae* strain that was previously constructed by the integration of xylose with oxidoreductase pathway from *S. stipittis* (*XYL1*, *XYL2*, and *XYL3*) into *ura3* gene, yielding an auxotrophic xylose-utilizing industrial strain. Before the transformation of the strain, plasmids were digested with the restriction enzyme EcoRI for confirmation of their size.

3.3.2. Batch fermentation

YP medium was used for batch cultivation. After pre-inoculum, yeast cells were harvested by centrifugation at 3,134 ×g, at 4°C for 5 min, and washed three times with distilled water. Washed yeast cells were inoculated in a 100 mL Erlenmeyer flask with 30 mL containing fermentative medium, which could be YPH (YP medium containing hemicellulosic hydrolysate), YPHG (YP medium containing 20 g L^{-1} glucose), or YPHX (YP medium containing 20 g L^{-1} xylose). For aerobic conditions, agitation was set at 200 rpm, while under micro-aerobic conditions, agitation was 100 rpm. Both the condition was performed at 30°C. The hemicellulosic hydrolysate was kindly provided by Dra. Livia Brenelli from Centro Nacional de Pesquisa em Energia e Materiais - CNPEM, Campinas, SP, which was obtained by a hydrothermal pre-treatment (190 °C, 20 min, 10% solids). Table 3.3 shows the composition of the hemicellulosic hydrolysate one time concentrated.

Table 3.3. Hemicellulosic hydrolysate composition after the treatment with commercial endoxylanase GH11.

Compost	AA	FA	FT	AR	AOS	GOS	Xyl	HMF	FL	XOS
Concentration (g L^{-1})	0.77	2.51	6.40	3.93	2.45	6.91	2.74	0.07	0.01	49.67

Description: AA, acetic acid, FA, formic acid, FT, phenolics totals AR, arabinose, AOS, arabino-oligosaccharides, GOS, gluco-oligosaccharides, Xyl, xylose, HMF, hydroxymethyl furfural, FL, furfural, XOS, total xylo-oligosaccharides.

3.3.3. Analytical methods

3.3.3.1. Extracellular metabolites and cell density

Samples were taken at appropriate intervals to measure cell growth and metabolites. Cell growth was monitored optical density (OD) at 600 nm using UV-visible Spectrophotometer (BIomate 5) (Basso et al. 2010). Cell dry mass concentration was determined by gravimetry, according to a protocol described by Olsson and Nielsen (Olsson and Nielsen 1997). Samples were centrifuged at 15000 rpm for 10 min and supernatants were diluted appropriately and then used for determination of glucose, xylose, xylitol, glycerol, succinate, acetic acid, and ethanol by high-performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) equipped with a refractive index detector. The Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA) was used and the columns were eluted with 0.005 N H_2SO_4 at 60 °C, and the flow rate was set at 0.6 mL/min.

3.3.3.2. Xylo-oligosaccharide quantification

The enzymatic products were analyzed by high-performance anion-exchange chromatography with pulsed amperometry detection (HPAEC–PAD) to detect xylose and XOS released by the hemicellulolytic enzyme mixture. Separation was performed using a Dionex ICS-3000 instrument (Thermo Fisher Scientific, Dionex Product, Sunnyvale, CA, USA) with a CarboPac PA100 column (4×250 mm) and CarboPac PA100 guard column (4×50 mm), according to a linear gradient of A (NaOH 500 mM) and B (NaOAc 500 mM, NaOH 80 mM). The gradient program was 15 % of A and 2 % of B at 0–10 min, and 15–50 % of A, and 2–20 % of B at 10–20 min, with a flow rate of 1.0 mL min^{-1}. The integrated peak areas were adjusted based on standards (1 time to 6 times).

3.4. RESULTS AND DISCUSSION

3.4.1. Engineering of industrial XOS-utilizing strains

Effective ethanol and biochemicals production from lignocellulosic materials require efficient use of all carbon sources present in this biomass, which includes cellulose- and hemicellulose-derived (Li et al. 2015). XOS-consuming *S. cerevisiae* strain can represent an essential step concerning second-generation ethanol process, since they can promote the

application of a lesser intensive pre-treatment condition as well a reduction of xylanolytic enzymes requirement, achieving lower costs.

A large amount of data on the heterologous expression of xylanolytic enzymes in *S. cerevisiae* exist in the literature (see Chapter I). Nonetheless, few of them reported ethanol production, and just one of them, intracellular hydrolysis of XOS (Li et a. 2015). To provide to the SA-1 XR/XDH strain the ability to hydrolyze intracellularly XOS, we transformed the referred industrial strain with a plasmid that carries XOS-transport and consumption pathways from *N. crassa.* It was used three plasmids, pDX8.4, pDX8.6, and pDX8.7 which differ in their cassette's conformation (Fig. 3.2). Before the transformation of the strain, plasmids were digested with the restriction enzyme EcoRI for confirmation of their size. Fig. 3.3 present the agarose gel (0.8 %) with the bands of digested and not digested plasmids.

After confirmation, they were transformed into SA-1 XD/XDH strain. The transformed cells were plated in solid medium YNB (Yeast Nitrogen Base) without uracil (URA-) for selecting transformed strains. From the transformations, three strains were obtained, hereafter named DPY11, DPY12, and DPY13.

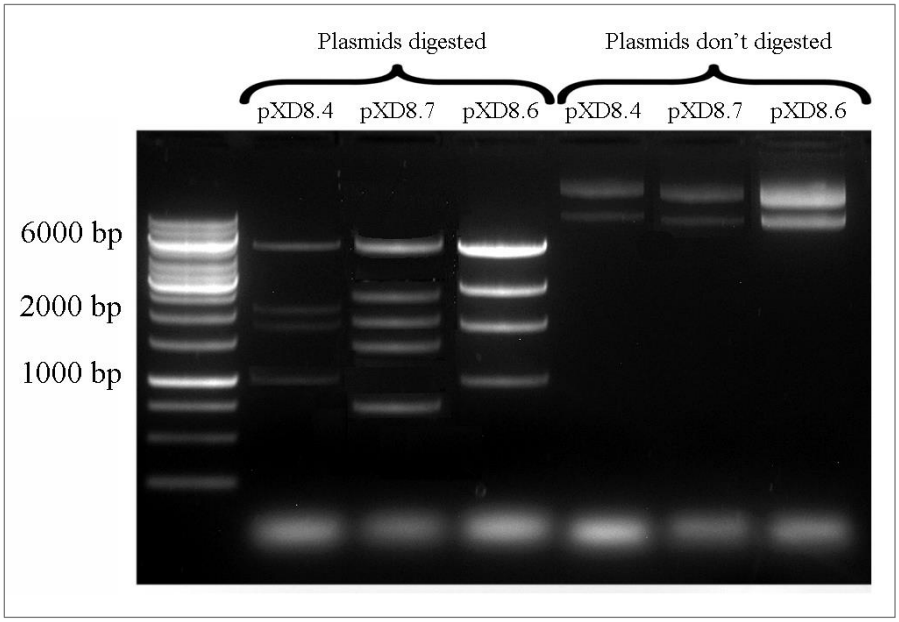

Fig. 3.3. Agarose gel of plasmids digested with EcoRI. Sizes of the bands results of the digested plasmids, pXD8.4: 5598bp, 2252bp, 1878bp, 1071bp; pXD8.7: 5552bp, 2921bp, 1878bp, 1071bp; and pXD8.6: 5552bp, 2921bp, 1878bp, 1071bp.

3.4.2. Evaluation of XOS-consuming yeasts

We evaluated XOS consumption parameters of engineered yeast strains, DPY11, DPY12, and DPY13 in YPH medium under micro-aerobic conditions at 30 °C, with an initial OD_{600} of 10. Figure 3.4 shows the growth profile of the transformant strains. Larsson and co-authors (Larsson et al. 1999) published that acid hemicellulose hydrolysates from spruce strongly inhibited growth and ethanol production of *S. cerevisiae*. Similarly, (Martín and Jönsson 2003) demonstrated a similar yeast response for bagasse acid hemicellulose hydrolysates. Hemicellulose hydrolysates from acid hydrolyzed bagasse inhibited the growth and fermentation of *E. coli* LY01 (Martinez et al. 2001). However, xylose and glucose-fermenting adapted *Zymomonas mobilis* strains were able to produce ethanol in dilute acid hydrolysates from yellow poplar (McMillan et al. 1999).

Despite these data, engineered yeast strains, DPY11, DPY12, and DPY13 were able to consume xylose even in the presence of lignocellulosic inhibitors, as expected for industrial background yeast strain. Xylobiose and xylotriose (X2 and X3) were better consumed by DPY13. It was not observed high variations in X5 concentration for any cultivation. Cell viability was monitored during cultivation, and it remained above 80 % of total viable cells (data not shown).

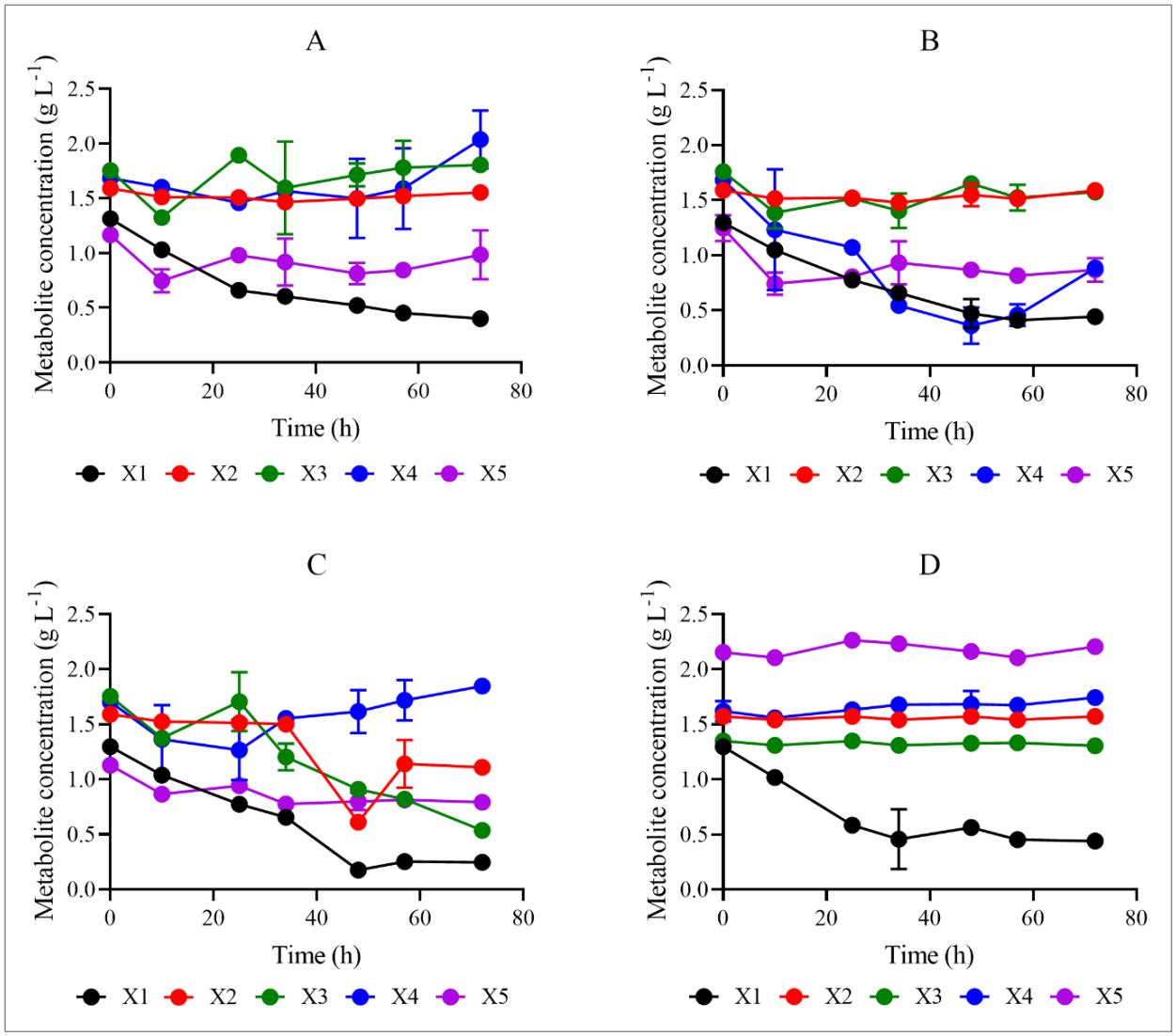

Fig. 3.4. XOS (X2-X5) and xylose concentrations during batch cultivation in YPH (YP medium containing hemicellulosic hydrolysate) of XOS-engineered industrial *S. cerevisiae* strain, DPY11 (A), DPY12 (B), DPY13 (C), and the control SA-1 XR/XDH (D). Cultivations were performed in duplicate at 30 °C and 100 rpm with an initial OD_{600} of 10.

Concerning the previous work where similar construction had been performed (Li et al. 2015), through the expression of the same genes from *N. crassa* in a laboratory xylose-consuming strain. Two β-xylosidases, GH43-2, and GH43-7 were required for the complete conversion of XOS into xylose. According to them, it is essential to the expression of both β-xylosidases for efficient conversion of XOS into xylose when the host strain expresses the oxidoreductase pathway. Therefore, since only DPY13 presents both the enzymes, further cultivations were performed in the hemicellulosic to investigate its fermentative performance (Fig. 3.5).

As shown in Fig. 3.5, DPY13 was able to grow in the presence of the hemicellulosic hydrolysate with ethanol production, for all conditions, YPH, YPGH, and YPXH. Li and co-authors (2015) performed an anaerobic fermentation with their laboratory XOS-consuming strain in an optimized minimum medium (oMM) containing 4% xylose and 3% XOS. Their strain produced more than 30 g L^{-1} of ethanol in 72h of cultivation. In contrast, the highest ethanol production by DPY13 cultivation, 18 g L^{-1} ethanol, was observed for YPHG cultivation containing 2% glucose and 0.6% XOS. It is worth mentioning that in Li's work xylan hydrolysate was used to offer XOS sugars, here, instead, it was used hemicellulosic hydrolysate that, besides the presence of XOS sugars there are inhibitory compounds that negative influence fermentative yeast performance. Thereby, DPY13 presented good performance consuming all xylose during the fermentation.

There are in the literature some works relating ethanol production from XOS by engineered XOS-xylose-fermenting strains displaying xylanolytic enzymes anchored on the cell surface (Fujita et al. 2002; Katahira et al. 2004; Tafakori et al. 2012). This strategy allowed extracellular hydrolysis of XOS to xylose. The recombinant strains were previously modified with a xylose consumption pathway, allowing the conversion of xylose to ethanol. Concerning an industrial host background, Saitoh et al. (2011) have reported the co-expression of β-xylosidase and β-glucosidase from *T. reesei* on the yeast cell surface based on α-agglutinin engineering system, obtaining the engineered industrial *S. cerevisiae* strain OC2-AXYL2-ABGL2-Xyl2 which also contains the oxidoreductase pathway for xylose consumption. The highest ethanol concentration, 12.5 g L^{-1}, was observed after 48 h in YPKX medium (40 g L^{-1} KC-flock and 40 g L^{-1} xylan from Birchwood) containing 30 g L^{-1} cellulose. The ethanol yield was 0.52 g (g sugar consumed)$^{-1}$. Despite Saitoh's work had reported a strain able to consume cellulosic and hemicellulosic-derived, their engineered strain, OC2-AXYL2-ABGL2-Xyl2 was unable to hydrolyse the oligomers intracellularly.

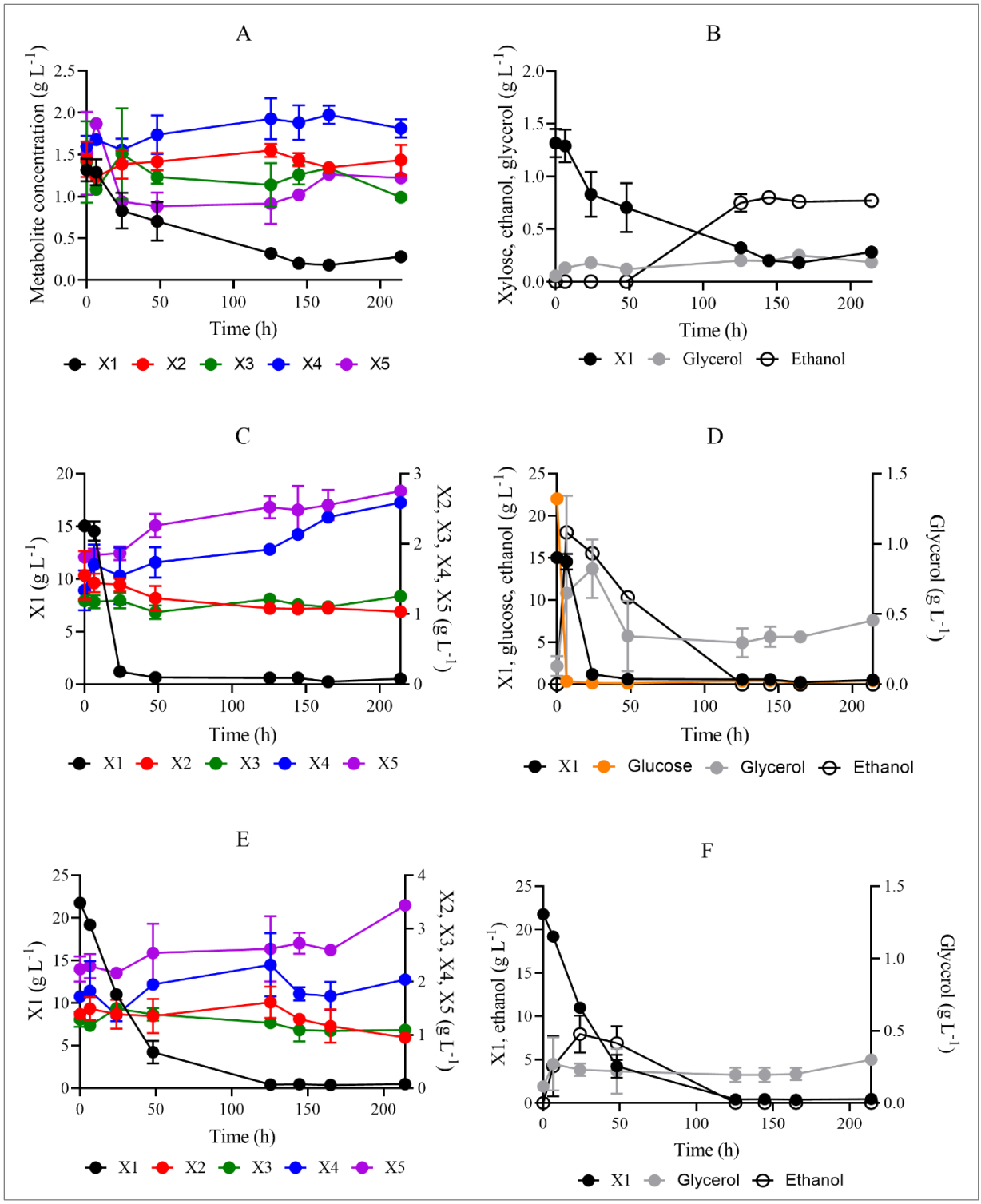

Fig. 3.5. XOS (X2-X5), xylose, ethanol, and glycerol concentrations during batch cultivation in YPH (YP medium containing hemicellulosic hydrolysate), A and B, YPHG (YP medium containing hemicellulosic hydrolysate and 20 g L^{-1} glucose), C and D, and YPHX (YP medium containing hemicellulosic hydrolysate and 20 g L^{-1} xylose), E and F, of XOS-engineered industrial *S. cerevisiae* strain DPY13. Cultivations were performed in duplicate at 30 °C and 100 rpm with an initial OD_{600} of 10.

Hemicellulosic-derived sugar comprises 15-35% of lignocellulosic biomass, representing a large source of renewable material which is available at low cost (Palmqvist and Hahn-Hägerdal 2000; Dahlman et al. 2003; Gírio et al. 2010). To obtain engineered strains able to consume oligomers derived from hemicellulose, mainly concerning intracellular hydrolysis, it represents a potential benefit for bioethanol production since engineered XOS-utilizing *S. cerevisiae* strain would have a competitive advantage concerning other microorganisms, such as contaminating bacteria and wild *Saccharomyces* and non-*Saccharomyces* species that are innately adept at utilizing XOS as carbon source (Cabrini and Gallo 1999; Amorim et al. 2011).

3.5. CONCLUSIONS

DPY13, an engineered SA-1 XR/XDH strain that carries a xylose oxidoreductase pathway (*XYL1*, *XYL2*, and *XYL3*) from *S. stipittis* (XR/XDH pathway), and the XOS transport and consuming genes from *N. crassa* was able to grow in complex media containing hemicellulosic hydrolyzed as the energy source. Furthermore, the strain was also able to consume xylose (X1), xylobiose (X2), and xylotriose (X3). This dataset demonstrates the feasibility of this strategy. However, an episomal expression of XOS-pathway enzymes does not represent an appropriate strategy for the industrial process, further work must be done to integrate this sequence into the genomic DNA of industrial *S. cerevisiae* strain.

3.6. FUTURE STUDIES

Although these studies have constructed a robust host for the hemicellulosic hydrolysates fermentation. Further optimization of the strain could be done to further increase XOS uptake rate and ethanol yield. In addition, the fermentative performance of the control strain, SA-1 XR/XDH, should be done to validate the results exhibited by DPY13. For that, SA-1 XR/XDH should be cultivated in YPH (YP medium containing hemicellulosic hydrolysate), YPHG (YP medium containing 20 g L^{-1} glucose), or YPHX (YP medium containing 20 g L^{-1} xylose) and, besides oligomers quantification, ethanol and glycerol should be measured, as well xylitol from each sample presented in this work.

Additionally, the pathway was not integrated into yeast DNA genomic. It will generate modified strains genetically stable, thereby more feasible for the industrial environment. Below

are some tips to achieve the abovementioned goal – an industrial strain able to produce hemicellulosic ethanol.

- To engineer the previously evolved strain, DPY06 (Chapter IV) through the insertion of the pXD8.7 plasmid that carries both transporter and xylanases.
- To isolate a haploid strain (work in progress, Chapter VI) to make more feasible the integration process.
- To engineer the XOS transport CDT-2 from *N. crassa* through locus-specific integration into *SOR1 locus* of the haploid strain using CRISPR-Cas9;
- To engineer the XOS degrading enzymes *GH43-2* and *GH43-7* from *N. crassa* through locus-specific integration into *GRE3 locus* of the strain previously modified with CDT-2 gene, using CRISPR-Cas9;
- To evaluate major physiological parameters in the presence of xylose, acetate, and XOS under microaerobic and anaerobic conditions.

3.7. REFERENCES

Adeboye PT, Bettiga M, Olsson L (2014) The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates. AMB Express 4:1–10. https://doi.org/10.1186/s13568-014-0046-7

Almario MP, Reyes LH, Kao KC (2013) Evolutionary engineering of *Saccharomyces cerevisiae* for enhanced tolerance to hydrolysates of lignocellulosic biomass. Biotechnol Bioeng 110:2616–2623. https://doi.org/10.1002/bit.24938

Almeida JRM, Modig T, Petersson A, et al (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 82:340–349. https://doi.org/10.1002/jctb.1676

Amorim H V., Lopes ML, de Castro Oliveira JV, et al (2011) Scientific challenges of bioethanol production in Brazil. Appl Microbiol Biotechnol 91:1267–1275. https://doi.org/10.1007/s00253-011-3437-6

Basso TO, Dario MG, Tonso A, et al (2010) Insufficient uracil supply in fully aerobic chemostat cultures of *Saccharomyces cerevisiae* leads to respiro-fermentative metabolism and double nutrient-limitation. Biotechnol Lett 32:973–977. https://doi.org/10.1007/s10529-010-0248-2

Cabrini KT, Gallo CR (1999) Identificação de leveduras no processo de fermentação alcoólica em usinas do estado de São Paulo, Brasil. Sci Agric 56:207–216. https://doi.org/10.1590/S0103-90161999000100028

Caspeta L, Nielsen J (2015) Thermotolerant yeast strains adapted by laboratory evolution show trade-off at ancestral temperatures and preadaptation to other stresses. MBio 6:1–9. https://doi.org/10.1128/mBio.00431-15

Cola P, Procópio DP, Alves AT de C, et al (2020) Differential effects of major inhibitory compounds from sugarcane-based lignocellulosic hydrolysates on the physiology of yeast

strains and lactic acid bacteria. Biotechnol Lett. https://doi.org/10.1007/s10529-020-02803-6
Fujii T, Yu G, Matsushika A, et al (2011) Ethanol production from xylo-oligosaccharides by xylose-fermenting *Saccharomyces cerevisiae* expressing β-xylosidase. Biosci Biotechnol Biochem 75:1140–6. https://doi.org/10.1271/bbb.110043
Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360. https://doi.org/10.1002/yea.320110408
Hahn-Hägerdal B, Karhumaa K, Jeppsson M, Gorwa-Grauslund MF (2007) Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. In: Biofuels. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 147–177. https://doi.org/10.1007/10_2007_062
Himmel ME, Ding S-YS-Y, Johnson DK, et al (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315:804–807. https://doi.org/10.1126/science.1137016
Jeffries TW (2006) Engineering yeasts for xylose metabolism. Curr Opin Biotechnol 17:320–326. https://doi.org/10.1016/j.copbio.2006.05.008
Jeppsson M, Johansson B, Hahn-hägerdal B, Gorwa-grauslund MF (2002) Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing *Saccharomyces cerevisiae* strains improves the ethanol yield from xylose. Appl Environ Microbiol 68:1604–1609. https://doi.org/10.1128/AEM.68.4.1604
Jo JH, Park YC, Jin YS, Seo JH (2017) Construction of efficient xylose-fermenting *Saccharomyces cerevisiae* through a synthetic isozyme system of xylose reductase from *Scheffersomyces stipitis*. Bioresour Technol 241:88–94. https://doi.org/10.1016/j.biortech.2017.05.091
Kim SR, Skerker JM, Kang W, et al (2013) Rational and evolutionary engineering approaches uncover a small set of genetic changes efficient for rapid xylose fermentation in *Saccharomyces cerevisiae*. PLoS One 8:e57048. https://doi.org/10.1371/journal.pone.0057048
Kłosowski G, Mikulski D (2021) Impact of lignocellulose pretreatment by-products on *Sachharomyces cerevisiae* strain ethanol red metabolism during aerobic and anaerobic growth. Mol 2021, Vol 26, Page 806 26:806. https://doi.org/10.3390/MOLECULES26040806
Larsson S, Palmqvist E, Hahn-Hägerdal B, et al (1999) The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb Technol 24:151–159. https://doi.org/10.1016/S0141-0229(98)00101-X
Lee S-M, Jellison T, Alper HS (2012) Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. Appl Environ Microbiol 78:5708–16. https://doi.org/10.1128/AEM.01419-12
Li X, Yu VY, Lin Y, et al (2015) Expanding xylose metabolism in yeast for plant cell wall conversion to biofuels. Elife 1–16. https://doi.org/10.7554/eLife.05896
Martín C, Jönsson LJ (2003) Comparison of the resistance of industrial and laboratory strains of *Saccharomyces* and *Zygosaccharomyces* to lignocellulose-derived fermentation inhibitors. Enzyme Microb Technol 32:386–395. https://doi.org/10.1016/S0141-0229(02)00310-1
Martinez A, Rodriguez ME, Wells ML, et al (2001) Detoxification of dilute acid hydrolysates of lignocellulose with lime. Biotechnol Prog 17:287–293. https://doi.org/10.1021/bp0001720
McMillan JD, Newman MM, Templeton DW, Mohagheghi A (1999) Simultaneous saccharification and cofermentation of dilute-acid pretreated yellow poplar hardwood to ethanol using xylose-fermenting *Zymomonas mobilis*. Applied Biochemistry and Biotechnology 77:649–665. https://doi.org/10.1007/978-1-4612-1604-9_59

Murphy JD, McCarthy K (2005) Ethanol production from energy crops and wastes for use as a transport fuel in Ireland. Appl Energy 82:148–166. https://doi.org/10.1016/j.apenergy.2004.10.004

Olsson L, Hahn-Hägerda B (1996) Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb Technol 18:312–331. https://doi.org/10.1016/0141-0229(95)00157-3

Olsson L, Nielsen J (1997) On-line and in situ monitoring of biomass in submerged cultivations. Trends Biotechnol 15:517–522. https://doi.org/10.1016/S0167-7799(97)01136-0

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bior Techn 74:25–33. https://doi.org/10.1016/S0960-8524(99)00161-3

Pettersen RC (1984) The Chemical Composition of Wood. Adv Chem 207:57–126. https://doi.org/10.1021/ba-1984-0207.ch002

Saitoh S, Tanaka T, Kondo A (2011) Co-fermentation of cellulose/xylan using engineered industrial yeast strain OC-2 displaying both β-glucosidase and β-xylosidase. Appl Microbiol Biotechnol 91:1553–1559. https://doi.org/10.1007/s00253-011-3357-5

Shen D, Xiao R, Gu S, Zhang H (2013) The overview of thermal decomposition of cellulose in lignocellulosic biomass. Cellulose 193–226. https://doi.org/10.5772/51883

van Maris AJA, Abbott DA, Bellissimi E, et al (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Van Leeuwenhoek 90:391–418. https://doi.org/10.1007/s10482-006-9085-7

CHAPTER IV: SCREENING OF AN HAPLOID INDUSTRIAL XYLOSE-UTILIZING *SACCHAROMYCES CEREVISIAE* STRAIN

Screening of a haploid industrial xylose-utilizing *Saccharomyces cerevisiae* strain

ABSTRACT

Industrial polyploid strains harbor numerous beneficial traits for lignocellulosic ethanol production. Sustainable second-generation ethanol should include an efficient and simultaneous co-fermentation of all sugars derived from cellulose and hemicellulose. Here we demonstrated an efficient xylose-consuming haploid strain isolated from an industrial xylose-utilizing *Saccharomyces cerevisiae* strain, SA-1 XR/XDH. We successfully isolate a Matα strain, DPY07, with a similar phenotype to its parental strain. Our main goal in this work was to select a haploid xylose-consuming industrial *S. cerevisiae* for future integration of the efficient transport and consumption xylo-oligosaccharides (XOS) pathway enzymes, CDT-2, GH43-2, and GH43-7, from *Neurospora crassa*, to expand its carbon source, from monomers to oligomers.

Keywords: lignocellulosic ethanol; industrial *Saccharomyces cerevisiae*; haploid strain; xylose.

4.1. INTRODUCTION

A challenge of laboratory *S. cerevisiae* yeast strains for second-generation biofuel productions is their lack of desirable traits to tolerate the environmental stresses in lignocellulosic hydrolysates. Thus, it is necessary to develop a more robust and efficient fermenting strain with the ability to tolerate various toxic compounds found in lignocellulose hydrolysates. Industrial *S. cerevisiae* strains are one of the most potential hosts for superior fermentation of lignocellulosic hydrolysates due to their high tolerance to various stresses in industrial environments, resulted from the natural adaptative evolution during successive fermentations that occur during the ethanol-1G production process (Della-Bianca and Gombert 2013; Nielsen 2019; Cola et al. 2020). However industrial *S. cerevisiae* strains are not well characterized, and it is challenging to introduce designed genetic perturbations due to their complex genetic structures and a high degree of heterozygosity (Zhang et al. 2014).

Rational metabolic engineering strategies with industrial *S. cerevisiae* strain can represent an important role in hemicellulosic hydrolysates bioconversion into ethanol and chemicals (Li et al. 2015). Engineer industrial yeast strain by introducing specific designed genetic perturbations can be difficult (Zhang et al. 2014). Thereby, an option to overcome the barriers of genetic modifications is by the isolation of haploid cells with genetically tractable and desirable traits (Steensels et al. 2014). In this work, a haploid strain was isolated from an industrial xylose-utilizing *S. cerevisiae* strain SA-1-XR/XDH for a future modification through the locus-specific integration of the XOS-consumption pathway, as represented in Fig. 4.1, for more effective utilization of lignocellulosic biomass.

Expand xylose to XOS utilization provides a reduction of the costs associated with the preparation of lignocellulosic biomass to fermentation since less intensive pre-treatment conditions and lower demand for enzymes will be required. Besides, recombinant *S. cerevisiae* strain capable of fermenting oligomers, instead of monomers only, will have competitive advantages in detriment of other microorganisms like bacteria and wild yeast Saccharomyces and non-Saccharomyces species (Amorim et al. 2011) that contaminate the alcoholic fermentation tank and compete with selected yeast strain for carbon sources (Cabrini and Gallo 1999).

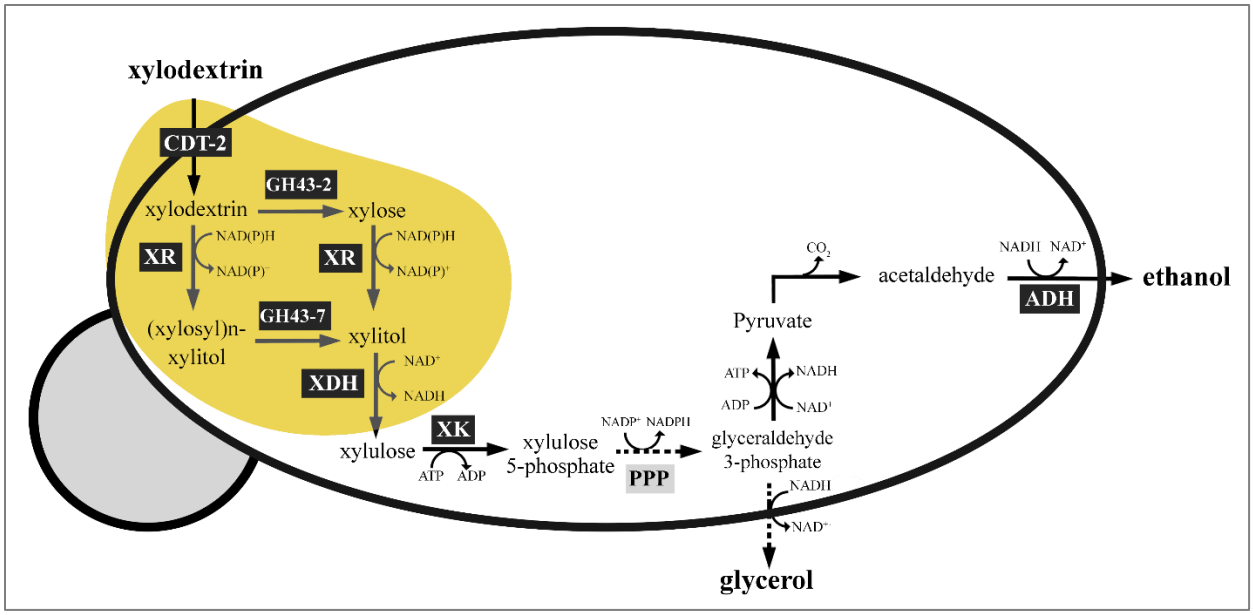

Fig. 4.1. Xylooligosaccharide metabolism by expression of the XOS transport (*CTD-2*) and the expression of the consumption (*GH43-2* and *GH43-7*) pathways from *Neurospora crassa.* Xylose metabolism expression by xylose reductase (*XR*) and xylitol dehydrogenase (*XDH*) from *S. stipitis*.

4.2. GOAL

The present research proposal aimed to isolate a haploid strain from an industrial xylose-utilizing *S. cerevisiae* strain, SA-1-XR/XDH, which carries xylose-oxidoreductase pathway from *Scheffersomyces stipittis*, gently provided by Luiz Carlos Basso (Escola Superior da Agricultura Luiz de Queiroz –Universidade de São Paulo).

4.3. MATERIALS AND METHODS

4.3.1. *S. cerevisiae* strains

All yeast strain used and constructed in this work is summarized in Table 4.1. SA-1 XR/XDH is an industrial *S. cerevisiae* strain that was previously constructed by the integration of xylose with oxidoreductase pathway from *S. stipittis* (*XYL1*, *XYL2*, and *XYL3*) into *ura3* gene, yielding an auxotrophic xylose-utilizing industrial strain (Basso et al. 2015).

Table 4.1. The yeast strains used in this study.

Strain	Description	Reference
SA-1 XR/XDH	Xylose-consuming strain engineered from strain SA-1 (*ura3::XYL1,XYL2,XYL3)* Mat**a**/Matα	(Basso et al. 2015)
DPY07	Haploid (Matα) derived from SA-1 XR/XDH	This work
DPY08	Haploid (Matα) derived from SA-1 XR/XDH	This work
DPY09	Haploid (Matα) derived from SA-1 XR/XDH	This work
DPY10	Haploid (Matα) derived from SA-1 XR/XDH	This work

4.3.2. Sporulation conditions and spore isolation

Sporulation of the diploid SA-1-XR/XDH strain was performed as following. Yeast cells grown overnight on YPD (Sigma Aldrich) were inoculating into a 10 mL sporulation medium (10 g L^{-1} potassium acetate, 1 g L^{-1} yeast extract, and 0.5 g L^{-1} glucose) and incubate at 30 °C, 250 rpm for 7 days until sporulation occurred. Their sporulation efficiency was evaluated by the population of the tetrads. To digest yeast cell wall zymolyase enzyme (ZymoReaserch® 10 units/µL) was used. For that, sporulation medium with spores was spined down, the supernatant removed, and the cell pellet was washed once by centrifugation with 500 µL of water, pelleted, and resuspended in 200 µL of zymolyase enzyme solution (0.5 mg/mL zymolyase) and then incubated for 20 min in a 37 °C water bath. 1 mL autoclaved water was added, and the suspension was placed on ice. to isolated spores, 50 µL of cooled cells were placed onto a YPD (20 g L^{-1} glucose) plate.

4.3.3. Mating-type test

A halo assay based on yeast pheromone response was used to determine strains mating-type. Mating type tester strains (KFY138 for MATα and KFY139 for MAT**a**) were first spread on YPD 20 g/L agar plates. Spores were taken from a YPD plate, suspended in water, and transferred dropwise without dilution to a YPD plate spread with a fresh layer of either the a or the α mating-type tester strains. Plates were incubated at 30 °C until a halo-formation could be observed. The presence of a halo around a colony was used to score its mating type. Query strain on the MATα plate indicates the query is MATα or MAT**a** for colonies plate on MAT**a**.

4.3.4. Fermentation and analytical methods

Batch fermentation experiments were performed by inoculating yeast cells grown on 250 mL Erlenmeyer flask containing 30 mL of fermentation media: yeast extract-peptone (YP)

medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) containing 40 g L^{-1} xylose (YPX), or 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 6 g L^{-1} acetate (YPDXA). Microaerobic batch fermentations were performed at 30 °C and 100 rpm conditions. Aerobic batch fermentation was performed at 30 °C at 250 rpm. Samples were taken at appropriate intervals to measure cell growth and metabolites. Cell growth was monitored optical density (OD) at 600 nm using UV-visible Spectrophotometer (BIomate 5). The samples were centrifuged at 15000 rpm for 10 min and supernatants were diluted appropriately and then used for determination of glucose, xylose, xylitol, glycerol, succinate, acetic acid, and ethanol by high-performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) equipped with a refractive index detector. The Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA) was used and the columns were eluted with 0.005 N H_2SO_4 at 60 °C, and the flow rate was set at 0.6 mL min^{-1}.

4.4. RESULTS AND DISCUSSION

4.4.1. Screening of SA-1 haploid strain

To isolate haploid cells, SA-1-XR/XDH cells were grown in an appropriate medium to induce sporulation. According to Argueso et al. (2009), Brazilian industrial strains are heterothallic which means they have stable mating types, and sporulate easily giving viable spores. As shown in Fig. 4.2, after one-week of cultivation, we could easily find sporulated cells in the culture.

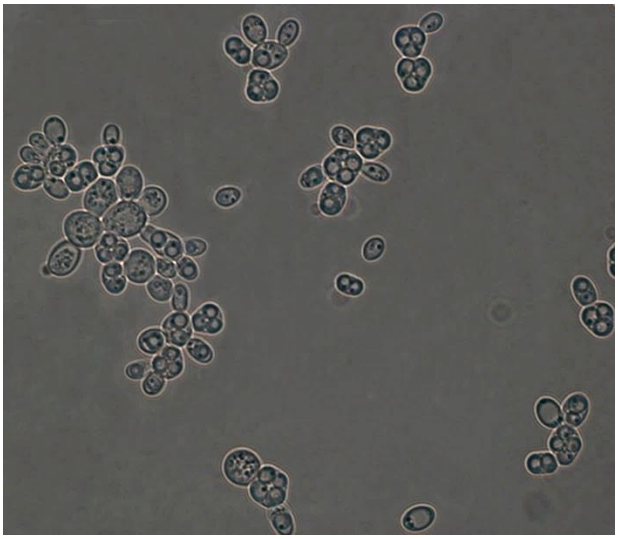

Fig. 4.2. Sporulating SA-1 XR/XDH cells. Cells were visualized using a 100x objective on an inverted microscope. Starvation condition to sporulate strains.

Four Mat*α* haploids colonies, derived from the sporulated SA-1-XR/XDH strain followed by zymolyase treatment, were selected by plating on YPD agar plate spread with a fresh layer of either the **a** or the *α* mating-type tester strains. The colonies 4, 17, 19, and 24 (Fig. 4.3), namely hereafter DPY07, DPY08, DPY09, and DPY10, respectively, were evaluated for xylose uptake and ethanol production in complex media supplemented with 40 g L^{-1} xylose at 30 °C, for 72h under aerobic condition (Fig. 4.4).

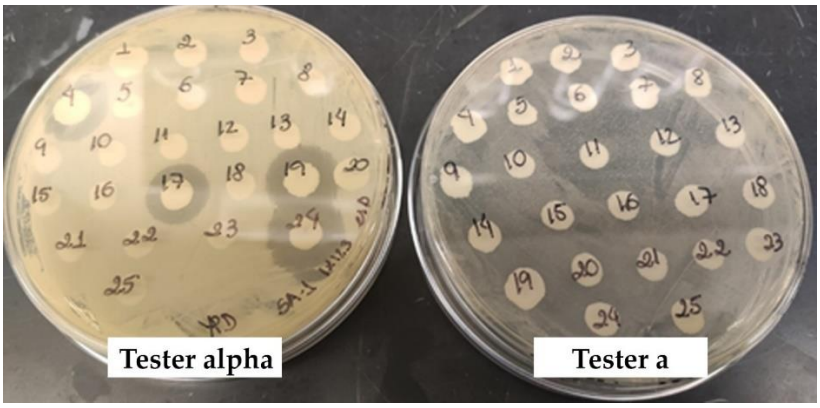

Fig. 4.3. Haploids SA-1 XR/XDH cells. Four haploid colonies were isolated throughout co-plating along with tester *S. cerevisiae* cells. Isolate colonies resulted from the zymolyase treatment of SA-1 XR/XDH sporulated culture.

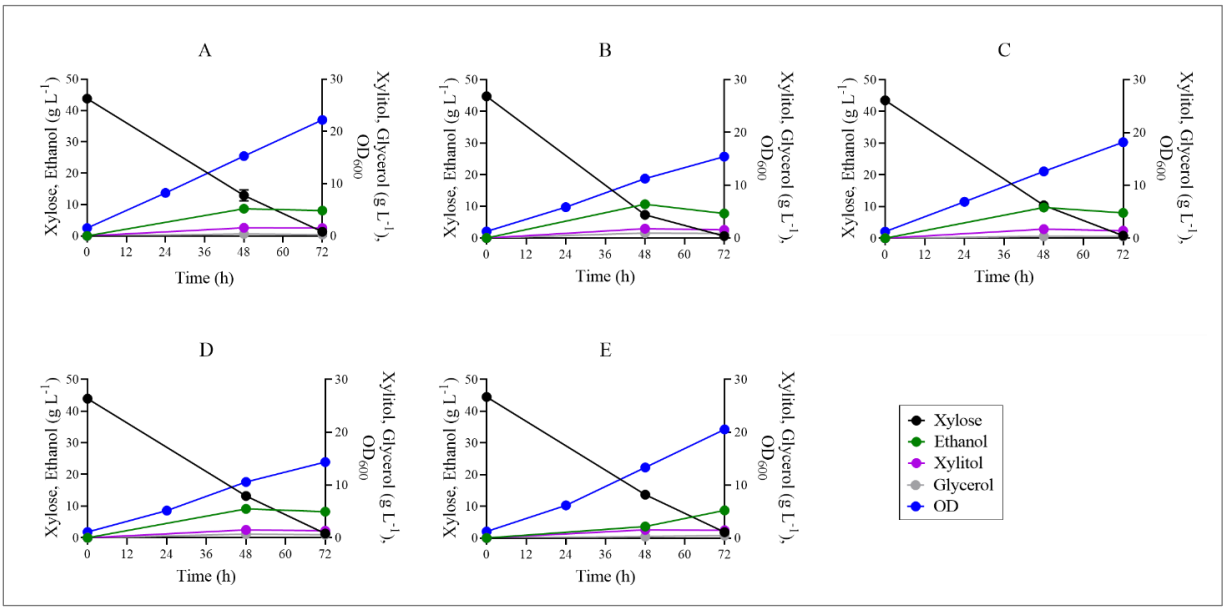

Fig. 4.4. Fermentation profile of the industrial xylose-consuming strain SA-1-XR/XDH (A), and their Mat*α* spores DPY07 (B), DPY08 (C), DPY09 (D), and DPY10 (E) when fermenting YP supplemented with 40 g L^{-1} xylose under aerobic condition. An initial OD_{600} was adjusted to 1. Data are presented as mean values and standard deviation of three independent biological replicates.

Over an initial cell loading of 1 OD at 600 nm, all strains were able to grow and consume all xylose present in the fermentative medium. However, some differences in growth were observed among the four isolated haploid strain and their parental diploid strain (Fig. 4.5). DPY07 displayed higher xylose uptake when compared with the other strains, as well achieved higher ethanol and xylitol concentrations even more than the parental strain. The evaluated strains showed different growth profiles under the condition analyzed, with DPY09 displaying the smallest growth profile. Based on ethanol yield, DPY07 and DPY09 have shown similar value which is higher than SA-1-XR/XDH (Table 4.2). In general, DPY07 was selected for its improved growth under the condition analyzed.

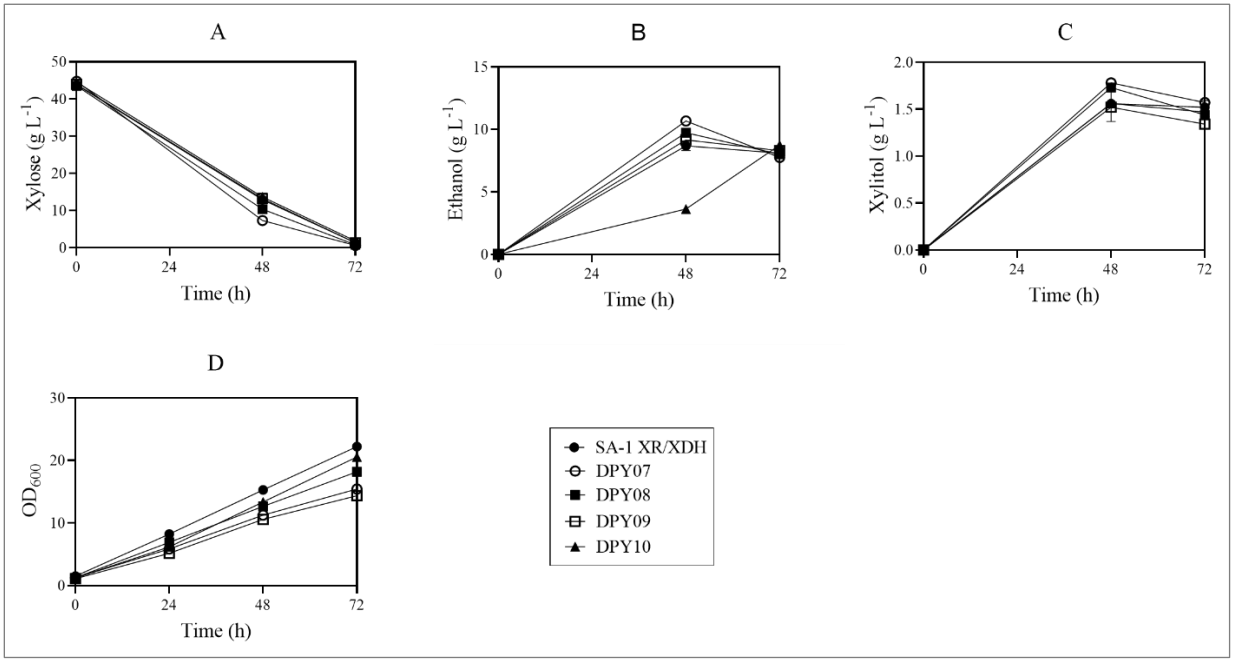

Fig. 4.5. Comparison of xylose consumption (A), ethanol (B), xylitol (C), and biomass (D) production of four Mat*α* haploid strains and their parental strain, SA-1-XR/XDH in YPX media containing 40 g L^{-1} xylose under aerobic condition. An initial OD_{600} was adjusted to 0.3. Data are presented as mean values and standard deviation of three independent biological replicates.

Table 4.2. Fermentation profile of SA-1-XR/XDH and its spores, DPY07, DPY08, DPY09, and DPY10 under aerobic cultivation in YPX (4%) media.

Strain	$Y_{Ethanol}$	$Y_{Xylitol}$	$Y_{Glycerol}$
SA-1 XR/XDH	0.21 ± 0.01	0.04 ± 0.00	0.01 ± 0.00
DPY07	0.29 ± 0.01	0.04 ± 0.00	0.02 ± 0.00
DPY08	0.28 ± 0.01	0.04 ± 0.00	0.01 ± 0.00
DPY09	0.29 ± 0.01	0.03 ± 0.00	0.01 ± 0.00
DPY10	0.12 ± 0.00	0.03± 0.00	0.01 ± 0.00

4.4.2. Comparison of fermentative performance between SA-1XR/XDH and DPY07

In face to obtain further details about the fermentative performance of the parental and the screened strain, SA-1-XR/XDH and DPY07, cultivations were carried out micro-aerobically in YPDXA (20 g L^{-1} glucose, 80 g L^{-1} xylose, and 6 g L^{-1} acetate) in order to mimic the fermentation of lignocellulosic hydrolysates, Fig 4.6A, and Fig. 4.6B, respectively. We include in the present comparison a well-known xylose-acetate-consuming, SR8A6S3 because of its high capacity to ferment xylose and acetate (Zhang et al. 2016).

All strains were able to consume the xylose completely, however, SR8A6S3 consumed all sugar faster than SA-1-XR/XDH or DPY07, while both these strains spent a long time, 240 h. Acetate was almost exhausted for DPY07 cultivations, which did not occur with SA-1-XR/XDH in which residual acetate was 2 g L^{-1} (Fig. 4.6A2). It is worth mentioning that SA-1-XR/XDH and DPY07 were able to produce high amounts of succinate which did not occur with SR8A6S3. With the haploid strain achieving higher values than SA-1-XR/XDH (Fig. 4.7).

Acetate was almost completely consumed by DPY07, indicating this strain presents potential *in-situ* detoxification for this inhibitory lignocellulosic compound. For SA-1-XR/XDH, almost 2 g L^{-1} acetate remained in the supernatant cultivation, from that 6 g L^{-1} that was added at the beginning of the experiment. No significant xylitol was found for DPY07 and its parental strain. As shown in Fig. 4.7, xylose consumption, ethanol, and glycerol production, and OD_{600} profiles are very similar for both strains. Further, DPY06 consumed acetate faster. These findings suggest that the haploid-screened strain presents a similar phenotype as the diploid-parental strain, indicating DPY07 can present desirable traits to tolerate the environmental stresses in lignocellulosic hydrolysates, similar to SA-1 (Cola, et al. 2020).

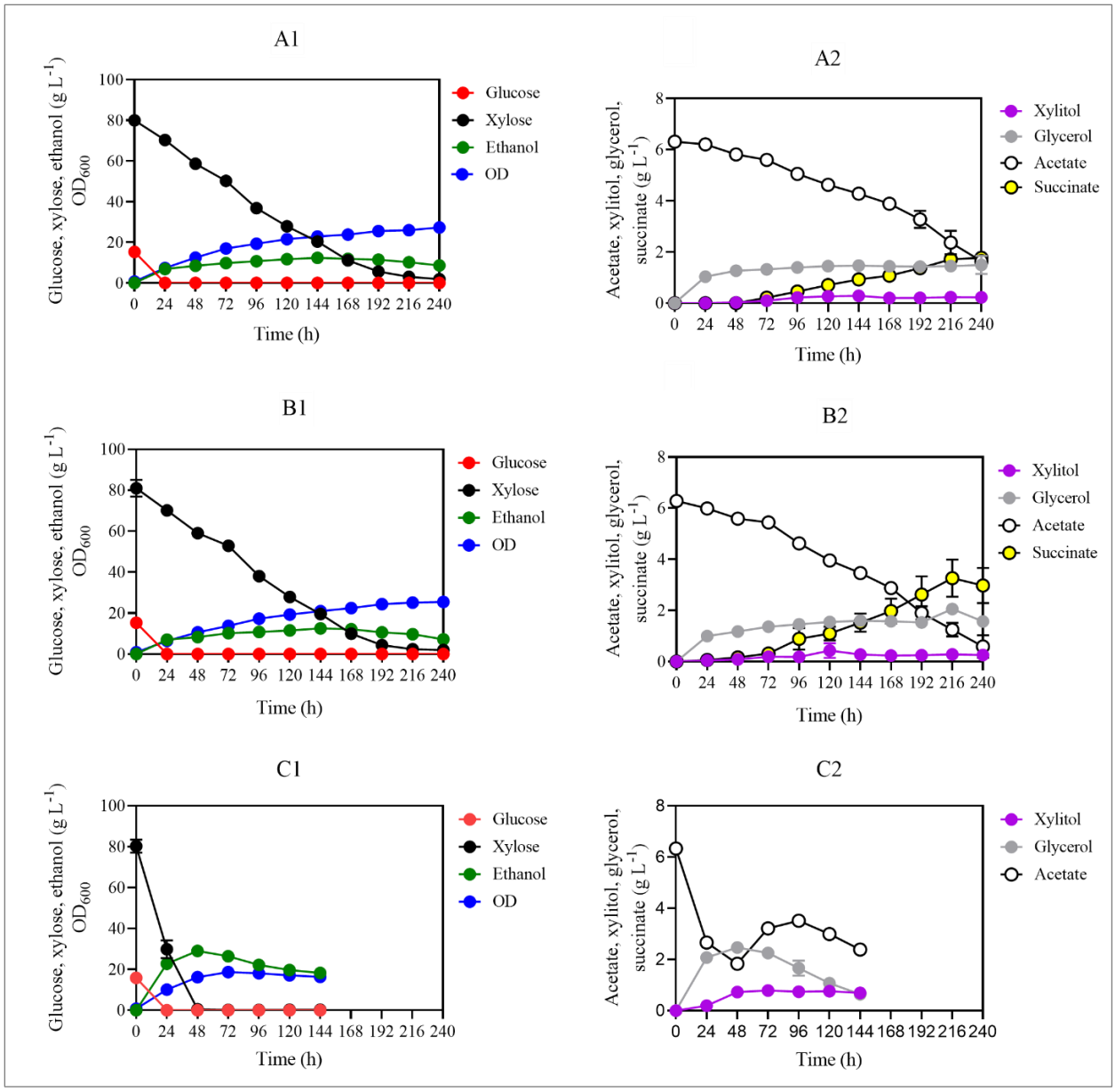

Fig. 4.6. Fermentation profile of a diploid industrial xylose-consuming strain SA-1-XR/XDH (A1 and A2), haploid DPY07 (B1 and B2), and laboratory SR8A6S3 (C1 and C2), when fermenting YP supplemented with 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 6 g L^{-1} acetate under microaerobic condition. An initial OD_{600} was adjusted to 1. Data are presented as mean values and standard deviation of three independent biological replicates.

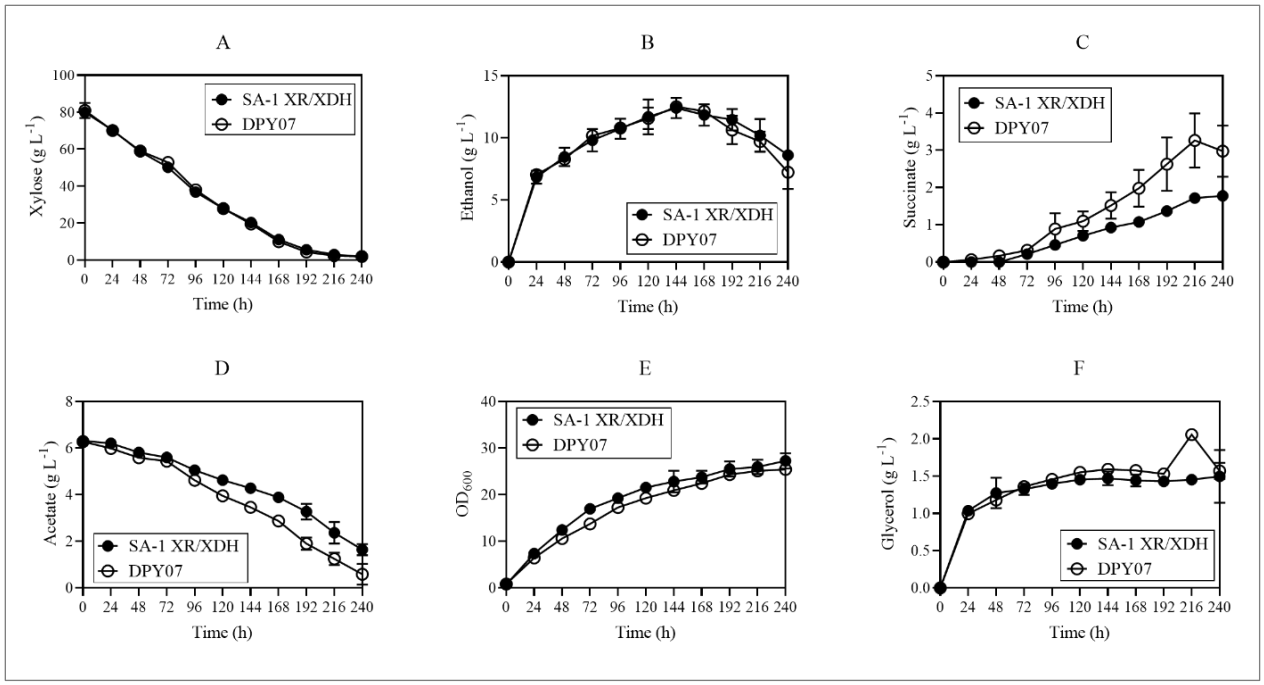

Fig. 4.7. Comparison of xylose (A) and acetate (D) consumption, ethanol (B), succinate (C), OD_{600} (E), and glycerol (F) production of two xylose-assimilating strains, SA-1-XR/XDH and its isolated haploid strain DPY07 in YP media containing 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 6 g L^{-1} acetate under microaerobic condition. An initial OD_{600} was adjusted to 1. Data are presented as mean values and standard deviation of three independent biological replicates.

4.5. PARTIAL CONCLUSIONS

A haploid industrial *S. cerevisiae* strain could be isolated and partially characterized. DPY07 is now amenable to a wide variety of genetic experiments for expanding its carbon source. The selected strain presented higher cell ethanol yield during batch fermentation, also presented higher succinate production when cultivated in complex media supplemented with glucose, xylose and acetate.

4.6. FUTURE STUDIES

To achieve an industrial strain able to produce hemicellulosic ethanol, the following steps could be considered:

- To engineer the XOS transport CDT-2 from *N. crassa* through locus-specific integration into *SOR1 locus* of the DPY07 strain using CRISPR-Cas9;

- To engineer the XOS degrading enzymes *GH43-2* and *GH43-7* from *N. crassa* through locus-specific integration into *GRE3 locus* of the strain previously modified with CDT-2 gene, using CRISPR-Cas9;
- To evaluate major physiological parameters in the presence of xylose, acetate, and XOS under microaerobic and anaerobic conditions.

4.7. REFERENCES

Amorim H V., Lopes ML, de Castro Oliveira JV, et al (2011) Scientific challenges of bioethanol production in Brazil. Appl Microbiol Biotechnol 91:1267–1275. https://doi.org/10.1007/s00253-011-3437-6

Argueso JL, Carazzolle MF, Mieczkowski PA, et al (2009) Genome structure of a *Saccharomyces cerevisiae* strain widely used in bioethanol production. Genome Res 19:2258–70. https://doi.org/10.1101/gr.091777.109

Cabrini KT, Gallo CR (1999) Identificação de leveduras no processo de fermentação alcoólica em usinas do estado de São Paulo, Brasil. Sci Agric 56:207–216. https://doi.org/10.1590/S0103-90161999000100028

Cola P, Procópio DP, Alves AT de C, et al (2020) Differential effects of major inhibitory compounds from sugarcane-based lignocellulosic hydrolysates on the physiology of yeast strains and lactic acid bacteria. Biotechnol Lett. https://doi.org/10.1007/s10529-020-02803-6

Della-Bianca BE, Gombert AK (2013) Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. 104:1083–1095. https://doi.org/10.1007/s10482-013-0030-2

Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360. https://doi.org/10.1002/yea.320110408

Li X, Yu VY, Lin Y, et al (2015) Expanding xylose metabolism in yeast for plant cell wall conversion to biofuels. Elife 2015:1–55. https://doi.org/10.7554/eLife.05896

Nielsen J (2019) Yeast systems biology: model organism and cell factory. Biotechnol J 14:1800421. https://doi.org/10.1002/biot.201800421

Steensels J, Snoek T, Meersman E, et al (2014) Improving industrial yeast strains: exploiting natural and artificial diversity. FEMS Microbiol Rev 38:947–95. https://doi.org/10.1111/1574-6976.12073

Zhang G-C, Kong II, Kim H, et al (2014) Construction of a quadruple auxotrophic mutant of an industrial polyploid *Saccharomyces cerevisiae* strain by using RNA-guided Cas9 nuclease. ASM 80:7694–7701 https://doi.org/10.1128/AEM.02310-14

Zhang GC, Kong II, Wei N, et al (2016) Optimization of an acetate reduction pathway for producing cellulosic ethanol by engineered yeast. Biotechnol Bioeng 113:2587–2596. https://doi.org/10.1002/bit.26021

CHAPTER V: METABOLIC ENGINEERING OF A LABORATORY *SACCHAROMYCES CEREVISIAE* STRAIN FOR SECOND-GENERATION ETHANOL PRODUCTION FROM XYLO-OLIGOSACCHARIDES AND ACETATE

The content of this chapter is in preparation for submission to *Metabolic Engineering*. I will be the first author of the paper, and Dr. Jaewon Lee, Dr. Jonghyeok Shin, Dr. Liang Sun, and Dra. Rosana Goldbeck, will be the co-authors. I performed the research with the help of the co-authors, and Dr. Thiago Olitta Basso and Dr. Yong-Su Jin were the directors of the research.

Metabolic engineering of a laboratory *saccharomyces cerevisiae* strain for second-generation ethanol production from xylo-oligosaccharides and acetate

ABSTRACT

Acetate and xylo-oligosaccharides (XOS)-consuming *Saccharomyces cerevisiae* strain can represent an essential step to reach a more cost-effective second-generation ethanol production. Xylan is one of the most abundant polysaccharide chains present in lignocellulosic residues. Acetic acid is a toxic inevitable component in lignocellulosic hydrolysates released from hemicellulose and lignin breakdown. Engineered *S. cerevisiae* expressing NADPH-linked xylose reductase (XR) and NAD^+-linked xylitol dehydrogenase (XDH) for xylose assimilation, as well NADH-linked acetylating acetaldehyde dehydrogenase (AADH) and acetyl-CoA synthetase (ACS) for a NADH-dependent acetate reduction pathway was used as the host for expressing two β-xylosidases, GH43-2 and GH43-7, and one transporter, CDT-2, from *Neurospora crassa*. Xylitol accumulation from the xylose consumption pathway poses many obstacles for the ethanol production process since its accumulation reduces final ethanol yield. In this work, two endogenous genes that encoding aldose reductase and sorbitol (xylitol) dehydrogenase, *GRE3* and *SOR1*, respectively, which are linked to the innate capacity of xylitol accumulation by the yeast cell, have their activities inactivated through cloning the genes comprising the xylanolytic system. The engineered strain, SR8A6S3-CDT_2-$GH43_{2/7}$ was able to produce ethanol through simultaneous co-utilization of XOS, xylose and acetate. When a hemicellulosic hydrolysate was used, the yielded yeast strain produced 60% more ethanol and 12% less xylitol than the control strain without the XOS consumption pathway. In cultivations using a hydrolyzed xylan, the ethanol yield was 84% higher for the engineered strain in comparison with its parental strain. The consumption of XOS, xylose and acetate can expand the capabilities of *S. cerevisiae* to utilize plant-derived and represent the potential to increase the efficiency of second-generation biofuel production.

Keywords: lignocellulosic ethanol; *Saccharomyces cerevisiae*; xylo-oligosaccharides; xylose; acetate consumption.

5.1. INTRODUCTION

The production of fuel ethanol from sugarcane is a promising industry in the expected transition from fossil to renewable fuels and chemicals (Petrovič 2015). In order to keep this industry fully competitive and profitable, it is of utmost importance to continue investing in the improvement of the production process, as well as in the expansion of alternative feedstock to feed it (Palmqvist and Hahn-Hägerdal 2000a). Considering alternative feedstocks, lignocellulosic materials hold the remarkable potential to increase biofuel production since it represents a sugar-rich residue, as well the cost of their procurement is relatively cheap, besides being an abundant non-food feedstock (Ji et al. 2011; Ko and Lee 2018). The lignocellulosic biofuel production process mainly includes the deconstruction of biomass into fermentable sugars and the conversion of sugars to biofuels (Li et al. 2019). Due to the complex and recalcitrant nature of lignocellulosic biomass, composed of cellulose, hemicellulose, and lignin, harsh pretreatment is required before enzymatic hydrolysis and fermentation, resulting in the production of several by-products that can inhibit the microbial metabolism, such as furans, organic acids, phenols, and inorganics salts (Ask et al. 2013; Kłosowski and Mikulski 2021).

Pretreatment aims to reduce the number of crystalline regions in cellulose, and partial degradation of hemicellulose and lignin to increase the susceptibility of the biomass to enzymatic complexes activity, which in turn are necessary to breakdown polysaccharides into monomers and fermentable sugars (Sarkar et al. 2012; Sharma et al. 2020; Kłosowski and Mikulski 2021). However, during the degradation of hemicellulose and lignin, acetic acid is an unavoidable compost once hemicellulose and lignin are acetylated (Klinke et al. 2004). This compost is toxic to yeast metabolism negatively affecting sugar fermentation and biofuel yield (Klinke et al. 2004; Almeida et al. 2007; Kłosowski and Mikulski 2021). Weak organic acids, such as acetic acid, can diffuse undissociated through the cell membrane and dissociate inside the cell, releasing protons and lowering the internal pH value (Bellissimi et al. 2009; Kłosowski and Mikulski 2021). To overcome the inhibitory effect of acetic acid, Zhang and coauthors (2016) introduced an optimized route for acetate reduction, through the expression of three copies of codon-optimized *adhE* (CO_*adhE*) from *Escherichia coli* three copies of a mutated *ACS* (*ACS*Opt*) from *Salmonellas enterica* into the xylose-fermenting *S. cerevisiae* strain expressing NADPH-linked xylose reductase (XR) and NAD^+-linked xylitol dehydrogenase (XDH), yielding the SR8A6S3 strain. This strategy enables efficient xylose fermentation with 29.7% higher ethanol yield and 70.7%

lower byproducts (xylitol and glycerol) as compared to the control strain without acetate reduction pathway when cultivated in YP medium supplemented with 20 g L^{-1} glucose, 80 g L^{-1} xylose and 8 g L^{-1} acetate under strict anaerobic (anoxic) conditions. Thereby, the possibility of using acetate as an electron acceptor could reduce glycerol and xylitol production during xylose fermentation by the engineered strain. Reduction of acetate to ethanol could serve as an electron sink to alleviate the redox cofactor imbalance resulting from XR and XDH activities (Wei et al. 2013a). In other words, through reductive metabolism of acetate, the surplus NAD^+ from reductive metabolism of acetate could be exploited for xylose metabolism. Thereby, as mentioned by the authors, this strategy can provide multiple benefits for the ethanol industry (Zhang et al. 2016).

Despite SR8A6S3 can deal with acetic acid present in lignocellulosic hydrolysates, there are a large number of other inhibitory compounds that are also released while cellulose, hemicellulose, and lignin are degraded during the pretreatment step (Palmqvist and Hahn-Hägerdal 2000b; Kłosowski and Mikulski 2021). Thereby, less intensive pre-treatment could be considered for achieving less inhibitory compounds production, however, large amounts of cellulase and hemicellulase enzymes cocktails would be required for converting the two major polysaccharides found in the plant cell wall, cellulose and hemicellulose into monomers of glucose and xylose, respectively, posing unsolved economic and logistical challenges for the industry (Lynd et al. 2002; Somerville et al. 2004; Himmel et al. 2007; Chundawat et al. 2011; Li et al. 2015). Thus, to make second-generation ethanol more profitable, it is worth noting that it is crucial to achieving microorganisms with unique genotype features to direct utilization of polysaccharides chains, as cellulose and hemicellulose derived polysaccharides. In previous work, Li and coauthors (Li et al. 2015) expanding xylose utilization by an engineered *S. cerevisiae* strain, for internal breaking down of oligomers to xylose monomers through the expression of two β-xylosidases, GH43-2, and GH23-7, and one XOS-transporter, CDT-2, from *Neurospora crassa* into the xylose-utilizing yeast strain. Their strain could produce more than 30 g L^{-1} of ethanol in 72h of cultivation in optimized minimum medium (oMM) supplemented with 4% xylose and 3% XOS and anaerobic condition.

In this work, we used the SR8A6S3 strain as a platform for the construction of yeast strain able to ferment XOS, xylose, and acetate into ethanol (summarized in Fig. 5.1). Genes derived from *N. crassa* for the XOS consumption pathway were integrated into the SR8A6S3 genome, enabling it synthesizes the transporter CDT-2 and the enzymes GH43-2 and GH43-7. A high expression cassette for CDT-2 was integrated into sorbitol (xylitol) dehydrogenase encoding gene

sor1 through the locus-specific integration tool, CAS-9-based system (Stovicek et al. 2015). Both enzymes GH43-2 and GH43-7 were integrated into aldose reductase encoding gene *gre3* using the same locus-specific integration tool. Our proposal comprises the deletion of *GRE3* and *SOR1* for mitigating xylitol production (Fig. 5.2) and diverted the carbon for ethanol production by the recombinant strain. (Träff et al. 2001; Toivari et al. 2004). The latest engineered strain, SR8A6S3-CDT_2-$GH43_{2/7}$ exhibited ethanol production up to 84% under a micro-aerobic condition in the hemicellulosic hydrolysate.

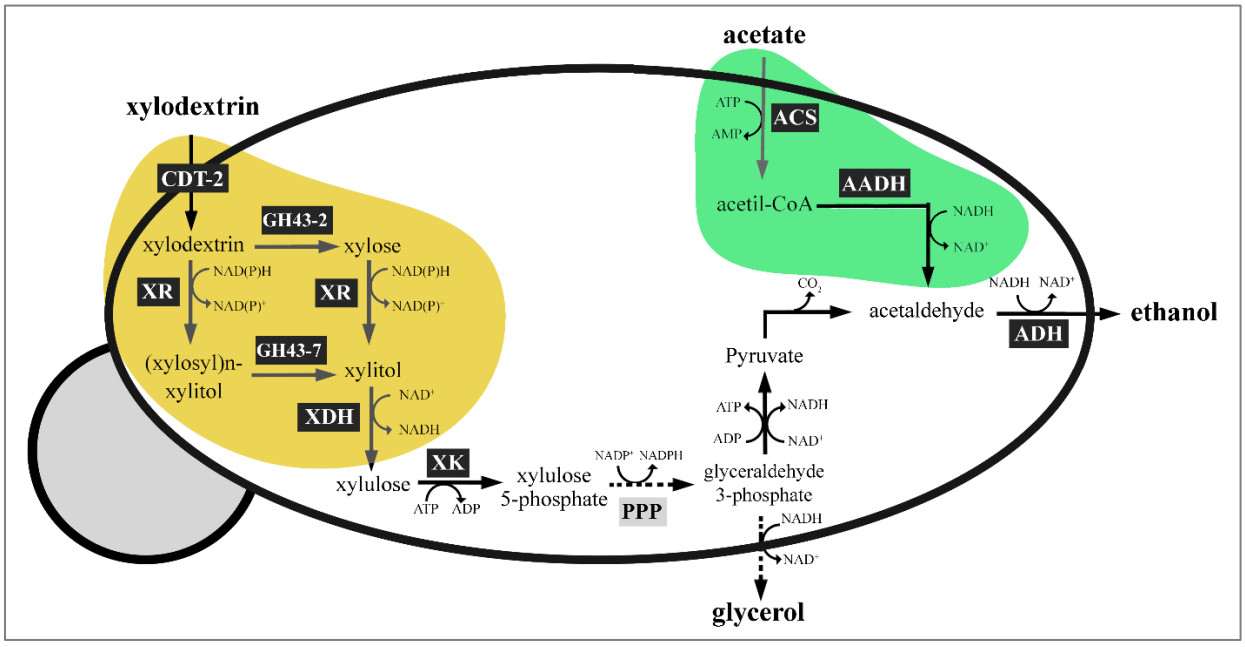

Fig. 5.1. XOS metabolism by expression of the XOS transport (*CTD-2*) and the expression of the consumption (*GH43-2* and *GH43-7*) pathways from *N. crassa*. Xylose metabolism expression by xylose reductase (*XR*) and xylitol dehydrogenase (*XDH*) from *S. stipitis*. The surplus NADH produced during xylose fermentation can be exploited for detoxifying acetate into ethanol through exogenous acetate reduction pathway, which is represented by conversion of acetate into acetyl-CoA by acetyl CoA synthetase enzyme (*ACS*), and by the production of acetaldehyde by acetyl-CoA by heterologous expression of the acetylating acetaldehyde dehydrogenase (*AADH*) and, finally, by the ethanol production from acetaldehyde by the action of the alcohol dehydrogenase (*ADH*).

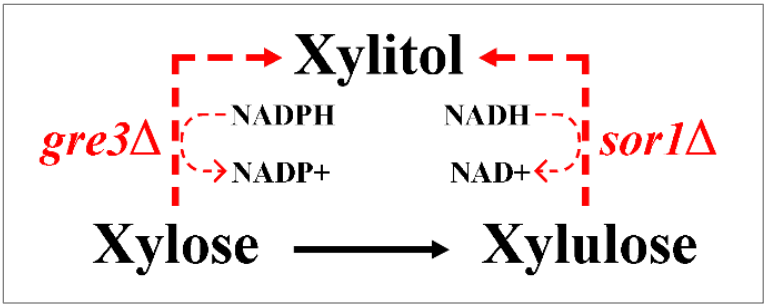

Fig. 5.2. The target genes are to be deleted (*gre3Δ*, *sor1Δ*) by the integration of xylanase genes (GH43-2, GH43-7) and transporter (CDT-2). Adapted from (Jeong et al. 2020).

5.2. GOALS

The present proposal aimed to insert an internal XOS consumption pathway from *N. crassa* (Li et al. 2015) into a previously published laboratory *S. cerevisiae* strain, SR8A6S3 (Zhang et al. 2016), previously engineered with the xylose catabolism pathway including the oxidoreductase enzymes from *S. stipitis* (encoded by *XYL1*, *XYL2*, and *XYL3*) and the acetate reduction pathway from composed by AADH from *E. coli*, and ACS, and *S. enterica*. The specific goals of this project were as follows:

- To evaluate the fermentative performance of two xylose-acetate-utilizing strains, namely SR8A5S3 and SR8A6S3 under different conditions;
- To engineer the XOS transport CDT-2 from *N. crassa* through locus-specific integration into *sor1 locus* of the chosen xylose-acetate-utilizing strain using CRISPR-Cas9;
- To engineer the XOS degrading enzymes *GH43-2* and *GH43-7* from *N. crassa* through locus-specific integration into *gre3 locus* of the chosen xylose-acetate-utilizing strain previously modified with CDT-2 gene, using CRISPR-Cas9;
- To evaluate major physiological parameters in the presence of xylose, acetate, and XOS under microaerobic and anaerobic conditions.

5.3. MATERIALS AND METHODS

5.3.1. Strains and media

E. coli strain DH5α was used for the construction and propagation of plasmids. *E. coli* was cultured in Luria-Bertani (LB) medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 10 g L^{-1} NaCl) at 37 °C and 100 µ/mL ampicillin (LBA) was added for selection when required. All *S. cerevisiae* strains used and constructed in this work are summarized in Table 5.1. Yeast cells were grown in

yeast extract-peptone (YP) medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) containing xylose and acetate (YPXA), or a mixture of glucose, xylose, and acetate (YPDXA), or hemicellulosic hydrolysate (YPH), or hemicellulosic hydrolysate, xylose, acetate (YPXAH), or hydrolysed xylan (YPXy), or hydrolysed xylan, acetate (YPAXy) at 30 °C for fermentation experiments. The hemicellulosic hydrolysate was kindly provided by Dra. Livia Brenelli from Centro Nacional de Pesquisa em Energia e Materiais - CNPEM, Campinas, SP, which was obtained by a hydrothermal pre-treatment (190 °C, 20 min, 10% solids) (Brenelli et al 2020). Table 5.2 shows the composition of the hemicellulosic hydrolysate one time concentrated. Hydrolysed xylan was obtained according to (Bailey et al. 1992).

Table 5.1. The yeast strains used in this study.

Strain	Description	Reference
SR8	Efficient xylose-consuming strain (evolved strain of D452-2 *leu2::LEU*_[RS305_TDH3$_p$_XYL1_TDH3$_T$ *ura3::URA3*_pRS-X123 *his::HIS*_pRS3-X123, and *ald6::AUR1-C* pAUR_d_ALD6)	(Kim et al. 2013)
SR8A5S3	SR8 expressing two copies of COadhE overexpression cassette and three copies of mutant *Salmonella* ACS gene overexpression cassette	(Zhang et al. 2016b)
SR8A6S3	SR8 expressing three copies of COadhE overexpression cassette and three copies of mutant *Salmonella* ACS gene overexpression cassette	(Zhang et al. 2016b)
SR8A6S3-CDT$_2$	SR8A6S3 expressing one copy of CDT2 overexpression cassette	This work
SR8A6S3-CDT$_2$-GH43$_{2/7}$	SR8A6S3 expressing one copy of GH43-2 and GH43-7 overexpression cassette	This work

Table 5.2. Hemicellulosic hydrolysed composition after the treatment with commercial endoxylanase GH11.

Compost	AA	FA	FT	AR	AOS	GOS	Xyl	HMF	FL	XOS
Concentration (g L^{-1})	0.77	2.51	6.40	3.93	2.45	6.91	2.74	0.07	0.01	49.67

Description: AA, acetic acid, FA, formic acid, FT, phenolics totals AR, arabinose, AOS, arabino-oligosaccharides, GOS, gluco-oligosaccharides, Xyl, xylose, HMF, hydroxymethyl furfural, FL, furfural, XOS, total xylo-oligosaccharides.

Yeast strains transformed with plasmids containing antibiotics were propagated on YPD plates supplemented with the corresponding antibiotics, such as clonNAT (100 µg mL^{-1}), geneticin G418 (200 µg mL^{-1}), hygromycin B (200 µg mL^{-1}). SR8A6S3-CDT$_2$ strain was generated by integrating CDT-2 transporter overexpressing gene cassette into the *SOR1* locus of SR8A6S3 genome. To construct an XOS-utilizing strain the $_P$GAP– GH43-7-$_T$CYC-$_P$CCW12-GH43-2-

$_T$CYC1 was integrated at the *GRE3* locus of SR8A6S3-CDT$_2$, yielding the SR8A6S3-CDT$_2$-GH43$_{2/7}$.

5.3.2. Plasmids and strain construction

All plasmids and primers used and constructed in this work are summarized in Table 5.3 and 5.4, respectively. The guide RNA plasmids (Table 5.5) gRNA-sor-K and gRNA-gre-K were amplified from gRNA-cs9-K by using primers pair DPO_089 and DPO-090, DPO_087 and DPO_088 carrying a 20 bp PAM sequence for *SOR1* and *GRE3 locus*, respectively. For genomic integration of CDT-2 through CRISPR-Cas9-based integration in the *SOR1* gene site of SR8A6S3, CDT-2 donor DNA was amplified from pRS425-CDT2 plasmid using a primer pair DPO_081 and DPO_082. Transformants with CDT-2 integration were confirmed by PCR using primers DPO_083 and DPO_084 and the resulted strain was designated as the SR8A6S3-CDT$_2$ (Table 5.1).

Table 5.3. Plasmids used in this study.

Plasmids	Description	Reference
pRS42K	pRS42K	(Liu et al. 2016)
p425-CDT2	pRS425-pPGK1-CDT2-tCYC1	This work
p426GPD	pRS426-pTDH3-tCYC1	Addgene
Cas9-NAT	P414-pTEF1-Cas9-tCYC1-NAT1	Addgene
gRNA-sor-K	pRS42K carrying *SOR1* disruption gRNA cassette	This work
gRNA-gre-K	pRS42K carrying *GRE3* disruption gRNA cassette	This work
p426-GH43$_{2/7}$	pRS426-pTDH3-GH43-7-$_T$CYC-$_P$CCW12-GH43-2 tCYC1	This work

Table 5.4. Primers used in this study.

Name	Sequence (5'- 3')
DPO_059	ATGCCCCTCGTCAAGAACCCCATCCTCCCCGGCTTCAATC
DPO_063	TTACTTCCCAGCCGGCTGCTTTTCCCCACAAATCTTCCCCTCTTCA
DPO_064	CCGGGCTGCAGGAATTCGAT
DPO_065	GGGATCCACTAGTTCTAGAA
DPO_062	CCAGAACTTAGTTTCGACGGATTCTAGAACTAGTGGATCCCATGCCCCTCGTCAAGAACC
DPO_074	GACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGTTACTTCCCAGCCGGCTGC
DPO_057	GTAATATAAATCGTAAAGGAAAATTGGAAATTTTTTAAAGGTAATACGACTCACTATAGG
DPO_058	TTGTTCATATCGTCGTTGAGTATGGATTTTACTGGCTGGAAATTAACCCTCACTAAAGGG
DPO_081	AATCAACAAGAAAAAATACTAAAAAAAAAAAATTGAAAAATGTAAAACGACGGCCAGT
DPO_082	TATATATGGACATGAACCAGTGCCGAAAAGTATTCACTTTACAGGAAACAGCTATGAC
DPO_089	TGTGTCGAACCCTTATCAGTGTTTTAGAGCTAGAAATAGCAAG
DPO_090	ACTGATAAGGGTTCGACACAGATCATTTATCTTTCACTGCGGA
DPO_083	CCGGTCTCGTATCTCCTTT
DPO_084	CTATCAACTGGAAGTAATGCG
DPO_069	GGGGGCCTATCAAGTAAATTACTCCTGGT
DPO_070	GTTCAGATTCACTTCTTGATATTTCC
DPO_087	TCCTCAATCATTCATTGAGAGTTTTAGAGCTAGAAATAGCAAG
DPO_088	TCTCAATGAATGATTGAGGAGATCATTTATCTTTCACTGCGGA

Table 5.5. gRNA used in this study.

gRNA (5'- 3')	Insertion locus	Plasmid	Reference
TGTGTCGAACCCTTATCAGT	*SOR1*	gRNA-sor-K	This study
TCCTCAATCATTCATTGAGA	*GRE3*	gRNA-gre-K	This study

To generate the transformants strains expressing the enzymes GH43-7_GH32-2 gene cassette, this sequence was amplified from genomic DNA of an unpublished evolved XOS-consuming strain, SR8-XD3, from Jin Lab (PI: Dr. Yong-Su Jin). Firstly, its genomic DNA was prepared with the Rapid Yeast Genomic DNA Extraction Kit (Bio Basic Inc., Markham Ontario, CA) and quantified by NanoDrop ND-1000. Primer pairs of DPO_059 and DPO_063 were used to amplify the *GH43-7-$_T$CYC-$_P$CCW12-GH43-2* gene sequence. The PCR product *GH43-7-$_T$CYC-$_P$CCW12-GH43-2* was amplified again using a primer pair of DPO_062 and DPO_074 which has homology with plasmid p426GPD. Similarly, the plasmid p426GPD was amplified using a primer pair of DPO_064 and DPO_065. PCR was performed using forward primer 1.25 µL, reverse primer 1.25 µL, DNA sample 1 µL, Phusion high-fidelity DNA polymerase master mix with HF buffer (New England BioLabs) 12.5 µL, and nuclease-free water 9 µL. Both the linear sequences were transformed in competent *E. coli* Dh5α to form the plasmid p426-GH43$_{2/7}$ (Table 5.3). Digestion was performed to confirm the plasmid assemble. $_P$TDH3-GH43-7-$_T$CYC- $_P$CCW12-GH43-2 $_T$CYC1 donor DNA was amplified from plasmid p426-GH43$_{2/7}$ using a primer pair DPO_057 and DPO_058.

Transformation of yeast cells was carried out with the polyethylene glycol (PEG)-LiAc method (Gietz et al. 1995). One microgram of DNA was used for Cas9 or gRNA plasmid transformation; in the meantime, 1.5 µg of donor DNA was used for homologous recombination. Proper integration was confirmed by PCR using primers DPO_069 and DPO_070. Recombinant strain was designated as the SR8A6S3-CDT$_2$-GH43$_{2/7}$ (Table 5.1).

5.3.3. Fermentation and analytical methods

Anaerobic batch fermentation experiments were performed by inoculating yeast cells grown on YPD into 30 mL fermentation media. Initial cell densities were OD_{600} of ~ 1. A serum bottle sealed with butyl rubber stoppers was used to ensure strict anaerobic conditions. The serum bottles with fermentation media were then flushed with nitrogen gas, which had passed through a heated, reduced copper column to remove the trace of oxygen. Aerobic batch fermentation

experiments were performed in a 125 mL flask with 30 mL of fermentation media (YP medium containing 20 g L^{-1} glucose and/or 80 g L^{-1} xylose and/or 8 g L^{-1} acetate) at 30 °C and 250 rpm. Microaerobic and anaerobic batch fermentations were performed in a 125 mL flask and 100 mL serum bottle flask, respectively, with 30 mL of fermentation media (YP medium containing 20 g L^{-1} glucose and/or 80 g L^{-1} xylose and/or 8 g L^{-1} acetate) at 30 °C and 100 rpm.

After pre-inoculum, yeast cells were harvested by centrifugation at 3,134 ×g, at 4°C for 5 min, and washed three times with distilled water. Washed yeast cells were inoculated in a 100 mL Erlenmeyer flask with 30 mL containing fermentative medium, which could be YPH (YP medium containing hemicellulosic hydrolysate), YPHG (YP medium containing hemicellulosic hydrolysate and glucose 20 g L^{-1}), YPHX (YP medium containing hemicellulosic hydrolysate and xylose 20 g L^{-1}), YPHXA (YP medium containing hemicellulosic hydrolysate, acetate 8 g L^{-1}, and xylose 20 g L^{-1}), YPXyl (YP medium containing hydrolyzed), or YPXylA (YP medium containing hemicellulosic hydrolysate and acetate 8 g L^{-1}). For aerobic conditions, agitation was set at 200 rpm, while under micro-aerobic conditions, agitation was 100 rpm. Both the condition was performed at 30°C.

Samples were taken at appropriate intervals to measure cell growth and metabolites. Cell growth was monitored by optical density (OD) at 600 nm using UV-visible Spectrophotometer (BIomate 5). The samples were centrifuged at 15000 rpm for 10 min and supernatants were diluted appropriately for determination of glucose, xylose, xylitol, glycerol, succinate, acetic acid, and ethanol by high-performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) equipped with a refractive index detector. The Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA) was used and the columns were eluted with 0.005 N H_2SO_4 at 60 °C, and the flow rate was set at 0.6 mL min^{-1}.

5.3.3.1. Xylo-oligosaccharide quantification

The enzymatic products were analyzed by high-performance anion-exchange chromatography with pulsed amperometry detection (HPAEC–PAD) to detect xylose and XOS released by the hemicellulolytic enzyme mixture. Separation was performed using a Dionex ICS-3000 instrument (Thermo Fisher Scientific, Dionex Product, Sunnyvale, CA, USA) with a CarboPac PA100 column (4×250 mm) and CarboPac PA100 guard column (4×50 mm), according to a linear gradient of A (NaOH 500 mM) and B (NaOAc 500 mM, NaOH 80 mM). The gradient

program was 15 % of A and 2 % of B at 0–10 min, and 15–50 % of A, and 2–20 % of B at 10–20 min, with a flow rate of 1.0 mL min^{-1}. The integrated peak areas were adjusted based on standards (×1 to×6).

5.4. RESULTS AND DISCUSSION

5.4.1. Evaluation of the acetate-utilizing strains

Lignocellulosic biomasses represent a great value raw material to produce various chemical products, including bioethanol. They represent the most abundant source of renewable material on earth besides presenting low cost, minimal land usages as well as avoid the competition between food and fuel (Palmqvist and Hahn-Hägerdal 2000a; Dahlman et al. 2003). Employing microorganisms for the bioconversion of plant-derived materials into biofuels provides opportunities for a sustainable future. However, commercial production of lignocellulosic biofuel has been hampered by the high cost of cellulase and hemicellulase enzymes cocktails which are required in large quantities for releasing monosaccharides from plant cell wall polymers (Galbe and Zacchi, 2002; Lynd et al. 2002; Himmel et al. 2007). Though the complex carbohydrates polymers from plant biomass, cello-oligosaccharides, and XOS, are not catabolized by wild-type *S. cerevisiae*, the most used microorganism in the ethanol industry (Matsushika et al. 2009; Galazka et al. 2010; Li et al. 2015). There are spread in nature many cellulolytic and hemicellulolytic organisms which possessing efficient machinery for degrading complex polymer chains, like cellulose and hemicellulose (Biely 1985; Kulkarni et al. 1999; Schmoll and Schuster 2010). In this sense, we promote reconstitution of an XOS-consumption pathway in a well-known xylose fermentative *S. cerevisiae* strain to promote efficient bioconversion of lignocellulose hydrolysate into ethanol. Thereby, an internal hydrolysis hemicellulose machinery composed of two key enzymes for the XOS hydrolysis pathway, GH43-2, and GH43-7, and the transporter CDT-2 that allow XOS transport inside the cell, was integrated into xylose-acetate-consuming *S. cerevisiae* strain (Zhang et al. 2016). The resulting microorganism has a competitive advantage concerning other microorganisms, such as contaminating bacteria and wild *Saccharomyces* and non-*Saccharomyces* species, which are not able to metabolize complex hemicellulose-derived sugars.

Before the integration of the XOS pathway, two acetate-consuming strains, SR8A5S3 and SR8A6S3, were investigated concerning their xylose-acetate fermentative performance under

anaerobic conditions. Over an initial cell loading of 1 Optical Density (OD) at 600 nm, strain SR8A6S3 (Fig. 5.3B) displayed higher rates of ethanol production when compared to strain SR8A5S3 (Fig. 5.3A) and the control strain, SR8 (Fig. 5.3C) (Kim et al. 2013), for fermentations carried out anaerobically in YPDXA media (YP medium containing 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate).

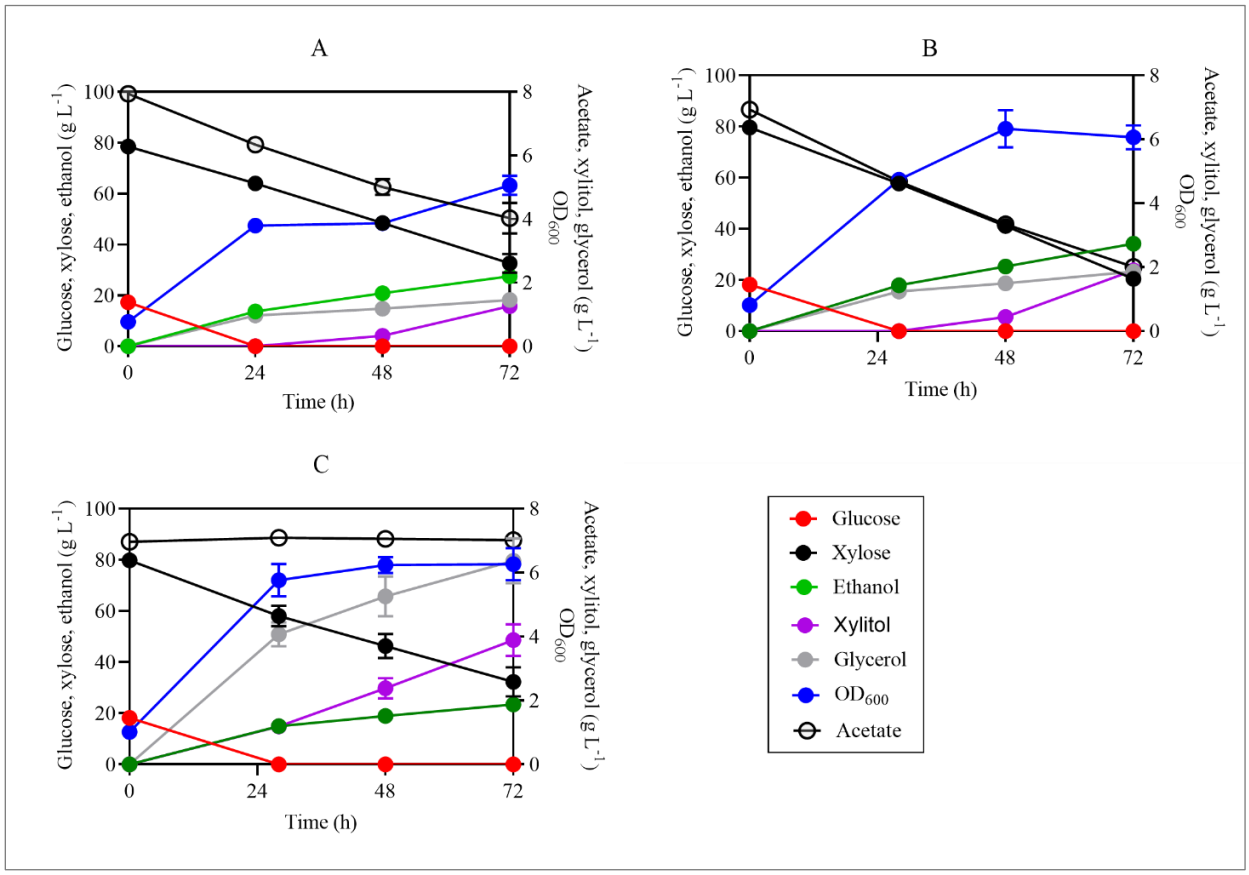

Fig. 5.3. Fermentation profiles of the acetate-consuming strains SR8A5S3 (A), SR8A6S3 (B), and their parental strain SR8 (C) when fermenting YP medium supplemented with 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate under anaerobic condition. Data are presented as mean value and standard deviation of three independent biological replicates.

Both the two published strains SR8A5S3 and SR8A6S3 contain the NADH-dependent acetate reduction pathway through the expression of three copies of the mutant *Salmonella ACS gene* (*ACS*Opt*) and two or three copies of a codon-optimized *adhE* gene (*CO_adhE*), respectively (Zhang et al. 2016). Acetic acid is available at significant amounts in lignocellulosic hydrolysates and, the NADH-dependent acetate reduction pathway with xylose oxidoreductase pathway allows that the surplus NADH generated on xylose metabolism to be oxidized to NAD^+ through reduction

of acetate into ethanol, allowing the anaerobic xylose fermentation and in situ detoxification of acetate in cellulosic hydrolysate through simultaneous co-utilization of xylose and acetate (Wei et al. 2013).

The higher expression of the codon-optimized *adhE* gene optimized the acetate fermentation performance of SR8A6S3 when compared with SR8A5S3 which has one less copy of this gene. Notably, no acetate reduction was observed for SR8 cultivations. The acetate reduction pathway could improve ethanol yield in SR8A5S3 and SR8A6S3 (Table 5.6) compared to strain SR8 by providing cofactors for XR/XDH pathway and to drive co-consumption of xylose and acetate.

Table 5.6. Fermentation profile of SR8A5S3, SR8A6S3, and SR8 when fermenting YP medium supplemented with 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate under anaerobic condition. Data are presented as mean value and standard deviation of three independent biological replicates.

Strain	$Y_{Ethanol}$	$Y_{Xylitol}$	$Y_{Glycerol}$
SR8A5S3	0.41 ± 0.01	0.03 ± 0.00	0.02 ± 0.00
SR8A6S3	0.42 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
SR8	0.36 ± 0.01	0.08 ± 0.00	0.10 ± 0.00

Parameters: $Y_{Ethanol}$, ethanol yield (g $g_{consumed\ sugar}^{-1}$); $Y_{Xylitol}$, xylitol yield (g $g_{consumed\ sugar}^{-1}$); $Y_{Glycerol}$, glycerol yield (g $g_{consumed\ sugar}^{-1}$).

We found that SR8A6S3 was capable of higher xylose and acetate consumptions and ethanol production compared to the SR8A5S3 strain and the control strain (Table 5.7), despite a formation of little more xylitol as a by-product (Fig. 5.3).

Table 5.7. Comparison between initial and residual metabolites of SR8A5S3, SR8A6S3, and SR8 cultivations in YP medium supplemented with 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate under anaerobic condition. Data are presented as mean value and standard deviation of three independent biological replicates.

	SR8A5S3		SR8A6S3		SR8	
	0h	72h	0h	72h	0h	72h
Glucose (g L^{-1})	17.28 ± 0.06	0.00 ± 0.00	18.27 ± 0.14	0.00 ± 0.00	18.24 ± 0.15	0.00 ± 0.00
Xylose (g L^{-1})	78.42 ± 0.28	32.92 ± 3.73	79.86 ± 0.63	20.29 ± 0.60	79.93 ± 0.64	29.26 ± 5.65
Acetate (g L^{-1})	7.94 ± 0.03	4.1 ± 0.48	6.94 ± 0.05	2.03 ± 0.10	6.97 ± 0.04	7.05 ± 0.08
Ethanol (g L^{-1})	0.00 ± 0.00	26.97 ± 1.44	0.00 ± 0.00	34.24 ± 0.30	0.00 ± 0.00	23.94 ± 1.63
OD_{600}	0.78 ± 0.03	5.00 ± 0.31	0.81 ± 0.04	5.9 ± 0.38	0.98 ± 0.00	5.50± 0.26

It is worth noting that the SR8A6S3 strain was able to grow better under the condition analysed. Here we used media conditions chosen previously in the development of these strains, to enable comparisons to previously published results (Zhang et al. 2016). Taking into account the

fermentative parameters and combining them with the previously published work, SR8A6S3 exhibited the most desirable phenotypes for the next steps of this work, which includes the integration of xylanases and XOS-transport genes.

5.4.2. Cas9- based integration of CDT-2 expression cassette into the *SOR1 locus*

Rational metabolic engineering strategies were performed to introduce XOS metabolism in the *S. cerevisiae* SR8A6S3 strain. A previous study reported a heterologous expression of cello-oligosaccharides transporter (CDT-2) and two intracellular xylanases (GH43-2 and GH43-7) from *N. crassa* by *S. cerevisiae* strain. Their engineered strain was able to transport and ferment XOS intracellularly efficiently (Li et al. 2015). In the present study, we first introduced the CDT-2 expression cassette into the xylose-acetate-fermenting SR8A6S3 strain. For the genome editing of XOS-utilizing strains, we used the CRISPR-Cas9 system-based approach, which represents the most promising tool for genome editing (Cong et al. 2013; Dicarlo et al. 2013). Thereby, a CDT-2 cassette under the control of the *PGK* promoter was introduced into the *SOR1* locus (Fig. 5.4). Fig. 5.5 represents the agar plates of transformants SR8A6S3-CDT_2.

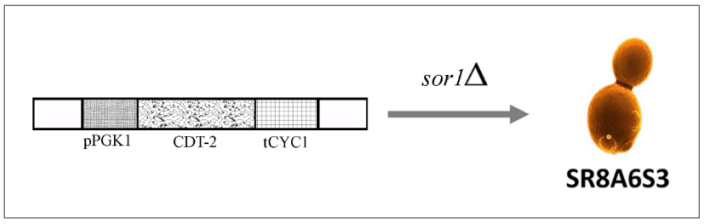

Fig. 5.4. Scheme of CDT-2 cassette under the control of *PGK1* promoter.

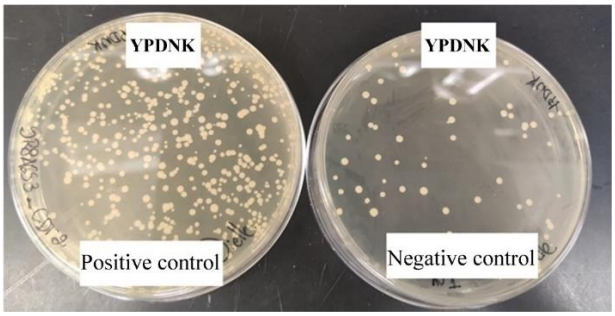

Fig. 5.5. Transformant strains selected by plates containing YPDNK (10 g L^{-1} yeast extract, 20 g L^{-1} peptone; 20 g L^{-1} glucose, 100 µg mL^{-1} clonNAT, 200 µg mL^{-1} Geneticin G418, 20 g L^{-1} agar). Positive control (left) was transformed with Cas9 plasmid, gRNA-sor-K plasmid, and donor DNA (CDT-2 gene cassette). Negative control (right) was transformed with Cas9 plasmid and gRNA-sor-K plasmid, and water.

Despite xylitol has a variety of uses in the food and pharmaceutical industries, this metabolite may face competition from an available carbon source, making less efficient ethanol production. *S. cerevisiae* strains possess genes putatively encoding enzymes capable of xylose reduction as *GRE3, GCY1*, *YPR1*, *YDL124W*, *YJR096W*, and xylitol oxidation as *XYL2*, *SOR1*, *SOR2, XDH1*, which can result in xylitol formation from xylose fermentation (Wenger et al. 2010). To lead a reduction of xylitol production and divert the carbon to ethanol production, CDT-2 gene cassette was introduced into *SOR1* gene site which can oxidize xylose to xylitol (Konishi et al. 2015). Elimination of xylitol from the ethanol fermentation process and possible replacement with increased ethanol amounts could have significant commercial advantages. In this sense, we utilized this endogenous gene of *S. cerevisiae* as a host for chromosomal integration of the XOS transporter CDT-2 for the purpose of increasing ethanol production.

Proper integration of the CDT-2 cassette was confirmed by PCR analysis. Colony PCR was performed directly from 27 colonies of the positive-control plate. Separated PCR products by agarose gel electrophoresis are presented in Fig. 5.6. Once proper integration was confirmed, a positive transformant was then evaluated for growth in xylose and acetate.

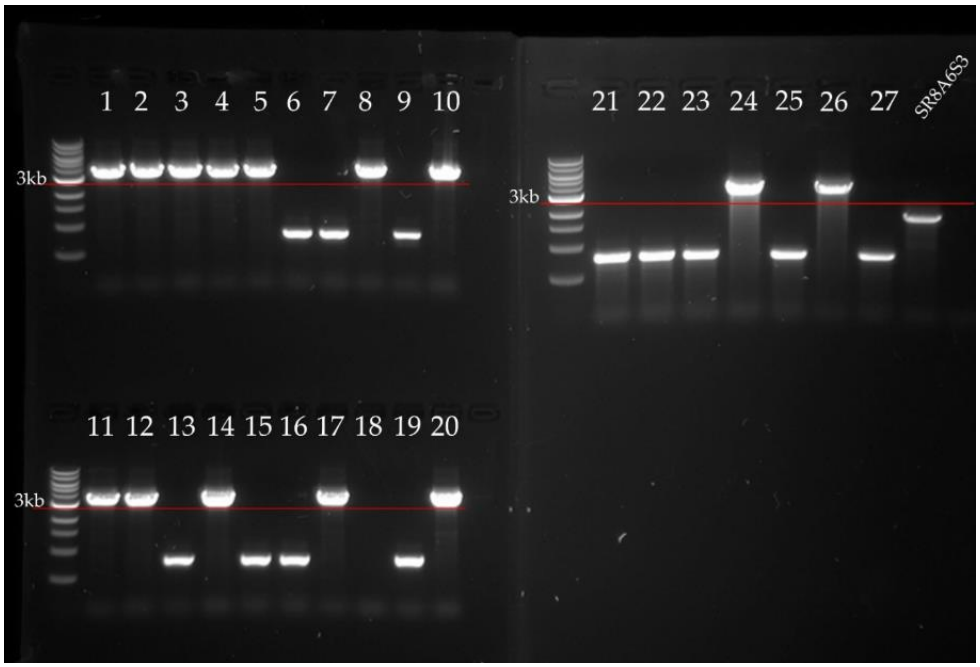

Fig. 5.6. Colony PCR products analyzed using an agarose gel of 0.8% from positive control plate colonies. *sor1*Δ::$_{P}$*PGK1-CDT-2-*$_{T}$*CYC1* mutant cells present a band of 3.4 kb (1, 2, 3, 4, 5, 8, 10, 11, 12, 14, 17, 20, 24, 26), a deletion of the sor1 gene was observed in 6, 7, 9, 13, 15, 16, 19, 21, 22, 23, 25, 27 which band present 0.8 kb. The last band, SR8A6S3, represents the positive PCR control, of which the band has 1.8 kb.

5.4.3. Comparison of xylose and acetate fermentation of SR8A6S3-CDT$_2$ and SR8A6S3 strains

To assess the impact of the genetic modification, a YP-based medium was used to cultivate the recombinant strains. Xylose and acetate fermentation performance of both the strains, SR8A6S3-CDT$_2$ and SR8A6S3, were compared in anaerobic and micro-aerobic batch cultures (Fig. 5.7, 5.8, 5.10, and 5.11). We evaluated for xylose and acetate consumption, and ethanol and xylitol production in high sugar content media (20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate) at 30 °C and an initial cell density of 1 (OD at 600 nm).

Deletion of the *sor1* gene led to decreased xylose consumption, as showed in Fig 5.8A and Table 5.7. However, strain SR8A6S3-CDT$_2$ had slightly higher ethanol yields and produced less xylitol and glycerol as a by-product (Table 5.8). Xylose consumption rate was significantly higher for SR8A6S3 strain at 24h and 72h of cultivation in comparison to the engineered strain. As well in the SR8A6S3-CDT$_2$ cultivation more xylose remained in the fermentative broth after 72h (Fig. 5.8). These results indicate that more substrate carbon could be channeled towards the desired product for the engineered strain. Probably an apparent change of the redox balance influenced the growth and production of metabolites.

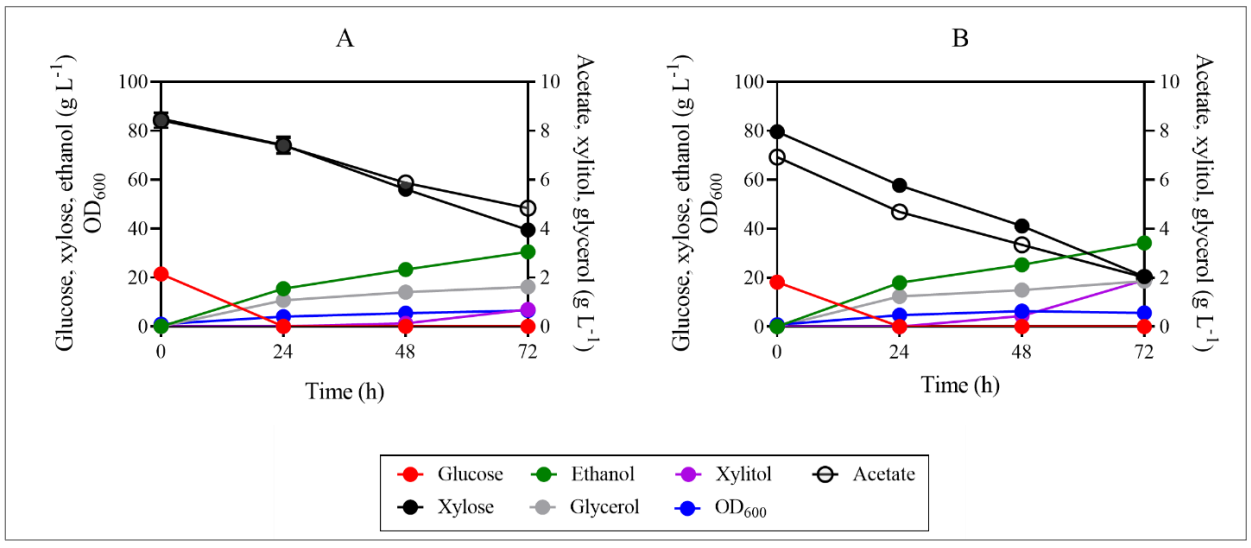

Fig. 5.7. Fermentation profiles of the SR8A6S3-CDT$_2$ (A), SR8A6S3 (B) when fermenting 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate under anaerobic condition. Data are presented as mean values and standard deviation of three independent biological replicates.

Table 5.8. Fermentation profile of SR8A6S3-CDT$_2$ and SR8A6S3 under anaerobic conditions.

	At 24 h				At 72 h				
	r_{xylose}	r_{xylose}*	$P_{xylitol}$	$P_{Ethanol}$	r_{xylose}	r_{xylose}*	$P_{xylitol}$	$P_{Ethanol}$	$Y_{Ethanol}$
SR8A6S3-CDT$_2$	0.37±0.13	0.10±0.03	0.00±0.00	0.62±0.06	0.63±0.03	0.10±0.02	0.01±0.00	0.41±0.01	0.46±0.01
SR8A6S3	0.94±0.06	0.19±0.01	0.00±0.00	0.74±0.00	0.83±0.02	0.14±0.00	0.03±0.00	0.47±0.00	0.44±0.00

Parameters: r_{xylose}, xylose consumption rate (g L^{-1} h^{-1}); r_{xylose}*, specific xylose consumption rate (g L^{-1} OD^{-1} h^{-1}); $P_{xylitol}$, volumetric xylitol productivity (g L^{-1} h^{-1}); $P_{Ethanol}$, volumetric ethanol productivity (g L^{-1} h^{-1}); $Y_{Ethanol}$, ethanol yield (g $g_{consumed\ carbon\ source}^{-1}$).

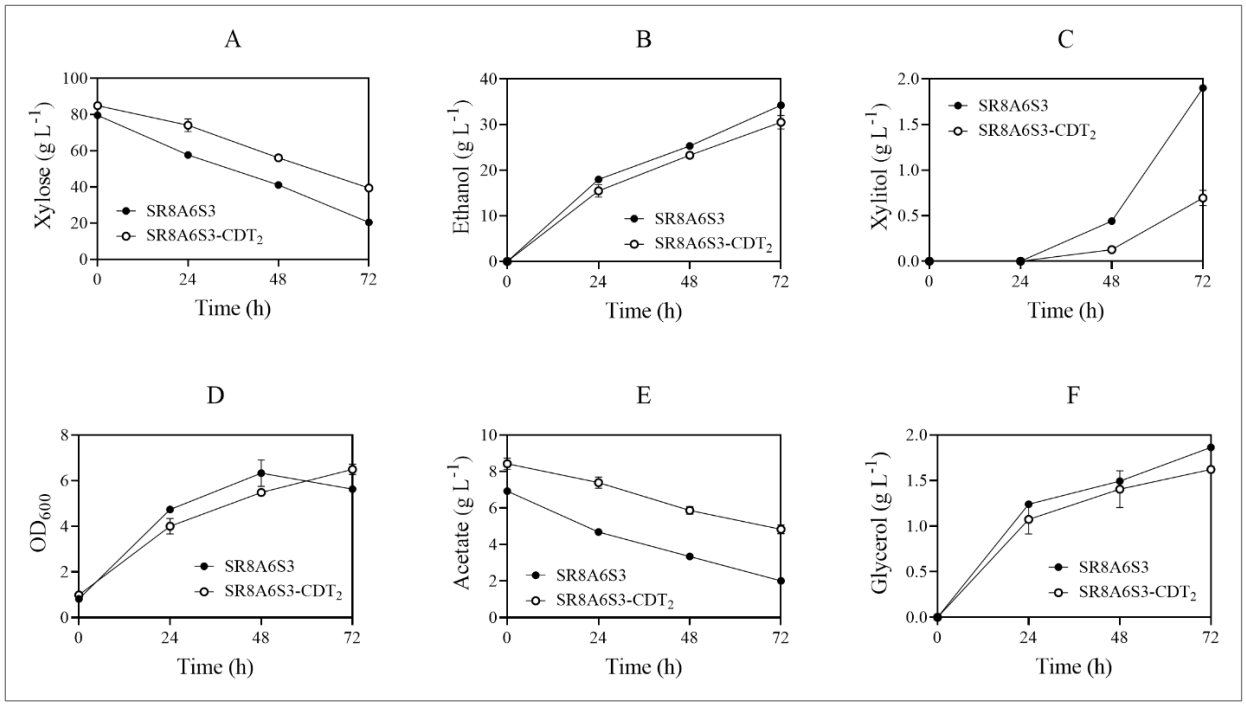

Fig. 5.8. Comparison of xylose (A) and acetate (E) consumption, ethanol (B), xylitol (C), OD_{600} (D), and glycerol (F) production of two xylose-acetate-assimilating strains, SR8A6S3-CDT$_2$ and SR8A6S3, in YP media containing 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate under anaerobic condition. An initial OD_{600} was adjusted to 1. The figure illustrates the means of triplicate experiments of each strain.

Fig. 5.9 shows the main metabolites analysed for comparison of fermentation profiles of SR8A6S3-CDT$_2$ and SR8A6S3. Elimination of xylitol production through *sor1Δ* increases the availability of intracellular NADH, which enabled the recombinant cell to produce more ethanol from grams consumed sugar (ethanol yield). Deletion of the *sor1* gene activity does not eliminate xylitol production since other genes encode enzymes capable of xylose reduction or xylitol oxidation, resulting in xylitol production. However, the xylitol amount reduced from 1.9 g L^{-1} to 0.69 g L^{-1} comparing parental and recombinant strain, respectively (Fig. 5.8C). In other words,

sor1Δ could reduce xylitol production by 63%. In principle, NAD^+ should be available to drive the reaction of xylitol to xylulose.

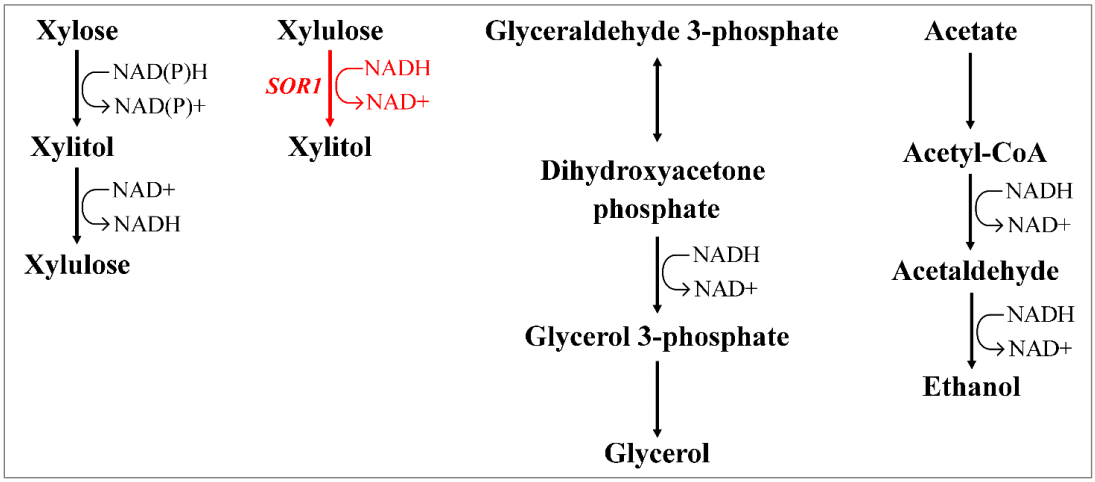

Fig. 5.9. Target metabolites were analysed for comparison of fermentation profiles of the SR8A6S3-CDT_2 and SR8A6S3. The red arrow represents deleted gene/route. Adapted from Jeong et al. (2020).

Under strict anaerobic conditions, ethanol is the most important primary metabolite produced in terms of reoxidation of excess NADH and redox balancing, followed by the production of glycerol (Jain et al. 2011), which are important to support xylulose production from xylitol. When oxygen is available in the flask, redox balancing of NADH / NAD^+ can also occur through the electron transport chain, which can result in less accumulation of xylitol amount in the medium. We could observe this hypothesis during batch cultivations under micro-aerobic, where less xylitol production was observed for both the strains (Fig. 5.11C). Micro-aerobic batch fermentations were performed in complex media YP supplemented with 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate at 30 °C, 100 rpm and an initial cell loading of 1 (OD_{600}). Representative fermentation profiles are shown in Fig. 5.10, whereas Fig. 5.11 shows a comparison between each important metabolite to analyse the influence of *sor1*Δ on xylose and acetate fermentation under micro-aerobic conditions of SR8A6S3 and SR8A6S3-CDT_2.

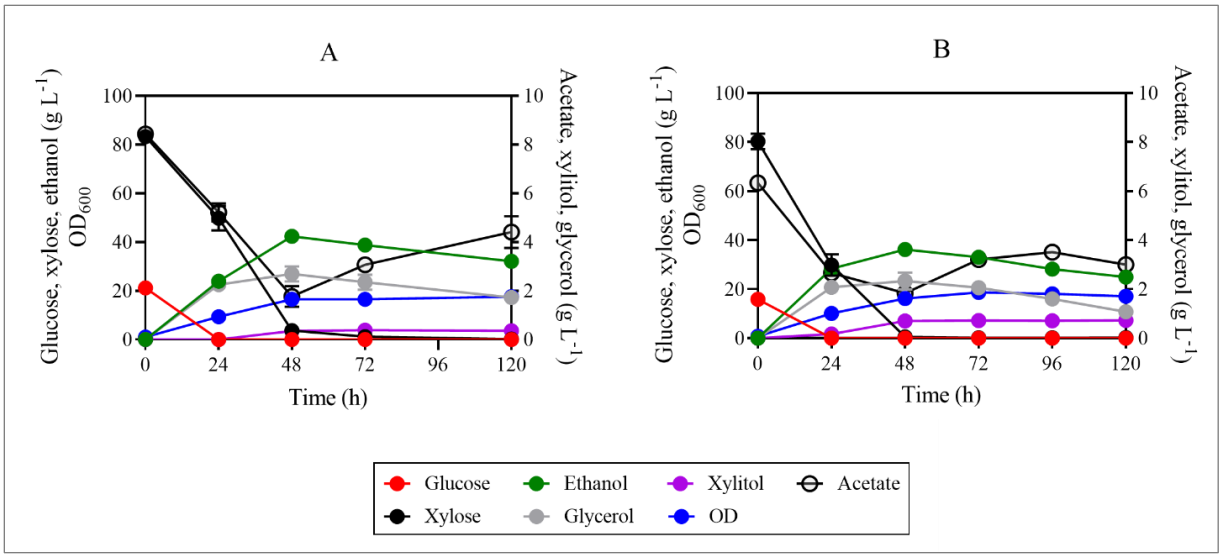

Fig. 5.10. Fermentation profiles of the SR8A6S3-CDT$_2$ (A), SR8A6S3 (B) when fermenting 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate under micro-aerobic condition. Data are presented as mean value and standard deviation of three independent biological replicates.

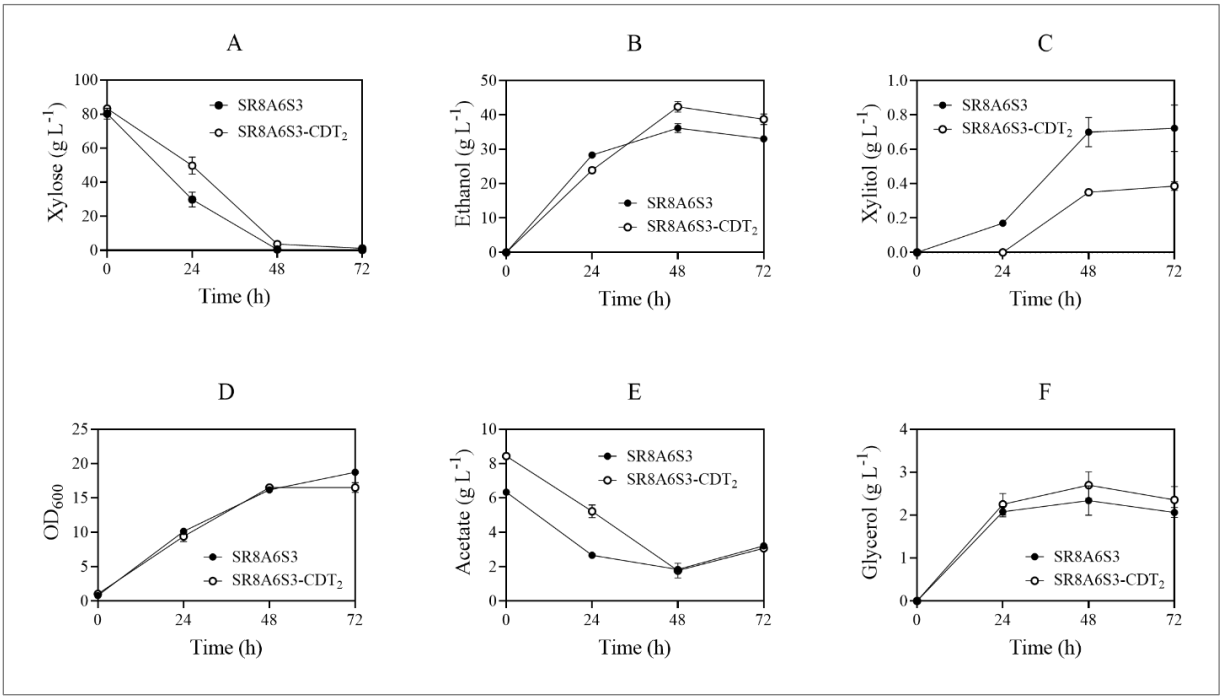

Fig. 5.11. Comparison of xylose (A) and acetate (E) consumption, ethanol (B), xylitol (C), OD_{600} (D), and glycerol (F) production of two xylose-acetate-assimilating strains, SR8A6S3-CDT$_2$ and SR8A6S3, in YP media containing 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate under micro-aerobic condition. An initial OD_{600} was adjusted to 1. The figure illustrates the means of triplicate experiments of each strain.

The recombinant strain SR8A6S3-CDT$_2$ produced a higher total amount of ethanol than SR8A6S3. As expected, xylitol production was lowest in SR8A6S3-CDT$_2$ (Fig. 5.11C). Before 48

h of cultivation, ethanol production was higher for the control strain cultivation, but after that, the modified strain increased the ethanol production, more than SR8A6S3 (Fig. 5.11B). The biomass concentration for both strains remained similar before 48h (Fig. 5.11D). At this condition, SR8A6S3 strains consumed faster acetate than SR8A6S3-CDT_2 (Fig. 5.11E). The resulting engineered yeast strain exhibited a slight increase in glycerol production during micro-aerobic cultivation.

5.4.4. Cas9- based integration of a GH43-2_GH43-7 expression cassette into the *GRE3 locus*

For the newly engineered strains, we hypothesized that XOS genes derived from evolved strains could exhibit a better ability to hydrolysate XOS. Both two enzymes, GH43-2, and GH43-7 were amplified from genomic DNA of an unpublished evolved XOS-consuming *S. cerevisiae* strain SR8-XD3 previously constructed for Jin Lab members, as represented in Fig. 5.12.

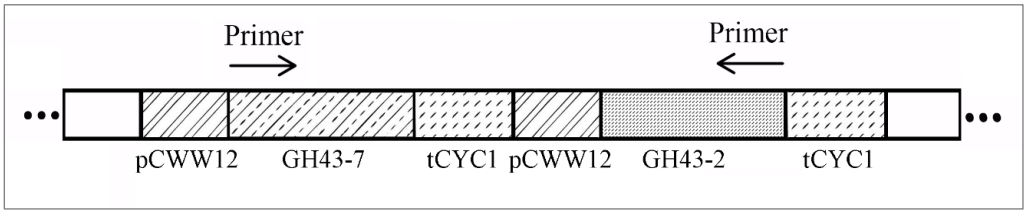

Fig. 5.12. Scheme of xylanases amplification from SR8-XD3.

SR8-XD3 was streaked on YPD plates (20 g L^{-1} glucose) and kept at 30 °C for 48 hours. Genomic DNA of three different colonies was extracted following kit instructions. Using appropriated primer pairs, the GH43-7-$_T$CYC-$_P$CCW12-GH43-2 gene sequence (Fig. 5.12) was amplified. The PCR product size was confirmed using agarose gel (0.8%), Fig. 5.13.

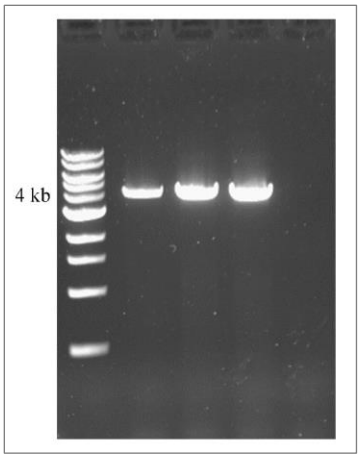

Fig. 5.13. Agarose gel of amplified $GH43_{2/7}$ from the genome DNA of SR8-XD3. Genomic DNA of three different colonies of SR8-XD3 was extracted and used as a template for individual amplification of the GH43-7-$_T$CYC-$_P$CCW12-GH43-2 sequence. Control was performed with the PCR without a DNA sample. Band's size, $GH43_{2/7}$: 3.8 kb.

To assemble the plasmid p426-$GH43_{2/7}$, the previous amplified sequence, GH43-7-$_T$CYC-$_P$CCW12-GH43-2, was amplified again used specific primers to add overhands homology sequence with the p426 plasmid. Fig. 5.14 and 5.15 show the representative scheme of the amplification of both sequences, $GH43_{2/7,}$ and p426. The newest $GH43_{2/7}$ gene cassette was used for the construction of the intracellular XOS hydrolysis pathway in SR8A6S3-CDT_2. A high-efficient Cas9 system for genome editing was used for genome integration of the desired sequence into the *GRE3* gene site.

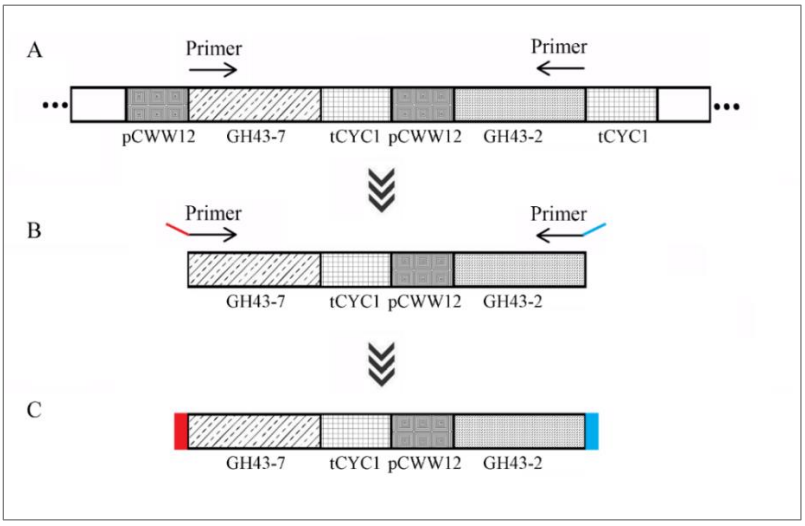

Fig. 5.14. Schematic representation of the amplification of $GH43_{2/7}$ sequence to construct the plasmid p426-$GH43_{2/7}$.

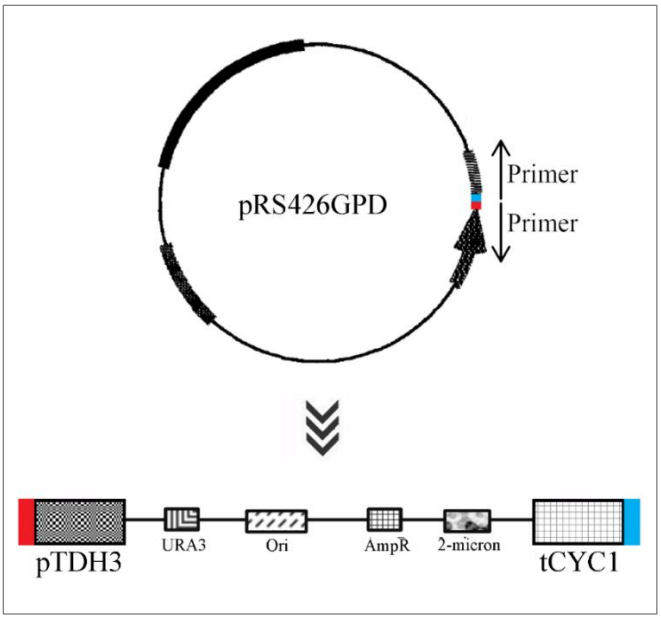

Fig. 5.15. Schematic representation of the amplification of p426 plasmid to construct the plasmid p426-GH43$_{2/7}$.

For overlapping the sequences to form the final plasmid p426-GH43$_{2/7}$ (Fig. 5.16), both linearized sequences were transformed into competent *E. coli* DH5α. From eight different colonies, plasmid DNA was extracted in order to verify proper overlapping of the homologous sequences to yield the p426-GH43$_{2/7}$ plasmid. The extracted plasmids were digested separately with the restriction enzymes BamHI and KpnI for confirmation of their sizes (Fig. 5.17). Three plasmids from the eight extracted presented the expected size for p426-GH43$_{2/7}$, plasmids #3, #4, and #6.

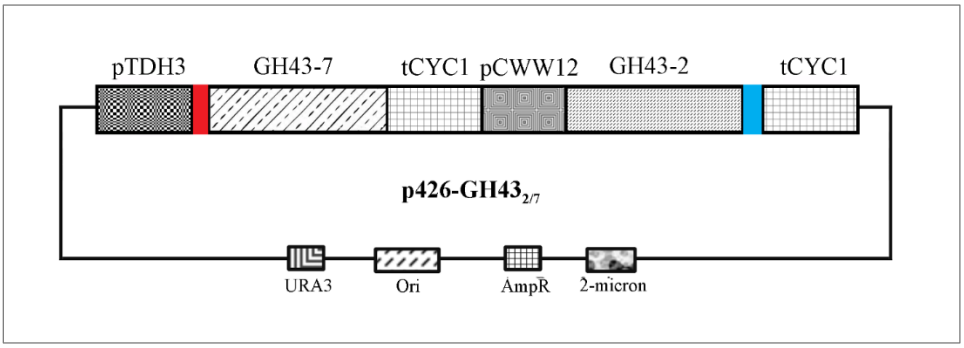

Fig. 5.16. Representative scheme of the p426-GH43$_{2/7}$ vector.

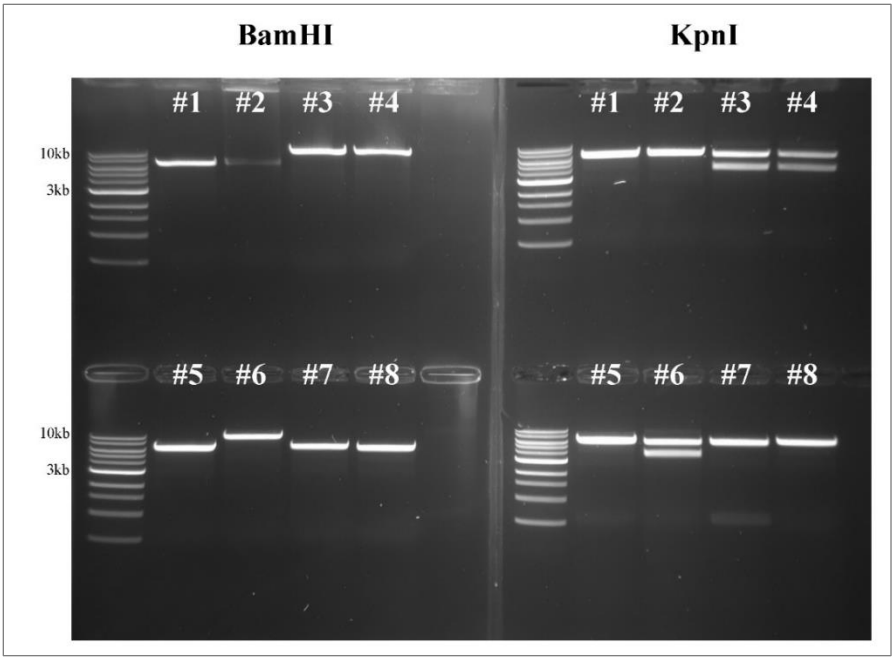

Fig. 5.17. Agarose gel of the digested pRS426-GH43$_{2/7}$ plasmid. Band's size for BamHI digestion, pRS426-GH43$_{2/7}$: 10.5 kb, pRS426: 6.6 kb; KpnI digestion, pRS426-GH43$_{2/7}$: 6.5 kb and 4 kb, pRS426: 6.6 kb.

After confirmation, plasmids #3, #4, and #6 were used as a template for the amplification of the $_{P}$TDH3-GH43-7-$_{T}$CYC- $_{P}$CCW12-GH43-2-$_{T}$CYC1 sequence, which was used to be integrated into the SR8A6S3-CDT$_2$ strain in the *GRE3* gene *locus*. Fig. 5.18 presents the agarose gel (0.8%) with the bands of the amplified GH43$_{2/7}$ gene cassette sequence, which represents the donor DNA for the integration step. It has been published that *GRE3* is an important xylose reducing enzyme expressed by wild *S. cerevisiae* strains in which its deletion was found to decrease xylitol formation by half (Träff et al. 2001). Therefore, our goal with the deletion of *GRE3* is to decrease the amounts of carbon diverted to xylitol formation and increase them for ethanol. For the genome editing for yield SR8A6S3-CDT$_2$-GH43$_{2/7}$, CRISPR-Cas9 system-based approach was used. Fig. 5.19 represents the agar plates of transformants SR8A6S3-CDT$_2$-GH43$_{2/7}$.

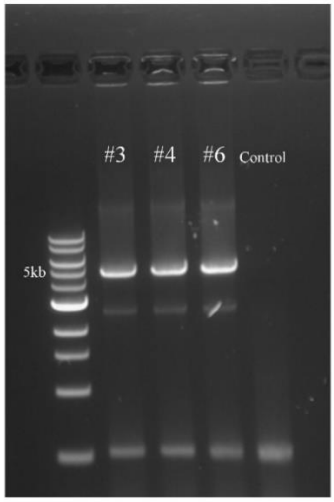

Fig. 5.18. Agarose gel of amplified pTDH3-GH43-7-$_T$CYC-$_P$CCW12-GH43-2 tCYC1gene cassette from three different pRS426-GH43$_{2/7}$ plasmids. Control was performed with the PCR without a DNA sample. Band's size, $_P$TDH3-GH43-7-$_T$CYC-$_P$CCW12-GH43-2 $_T$CYC1: 5 kb.

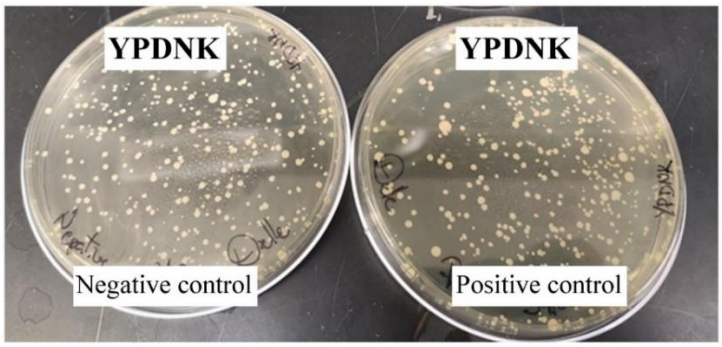

Fig. 5.19. Transformant strains selected by plates containing YPDNK (10 g L^{-1} yeast extract, 20 g L^{-1} peptone; 20 g L^{-1} glucose, 100 µg mL^{-1} clonNAT, 200 µg mL^{-1} Geneticin G418, 20 g L^{-1} agar). Positive control (right) was transformed with Cas9 plasmid, gRNA-gre3 plasmid, and donor DNA (GH43$_{2/7}$ gene cassette). Negative control (left) was transformed with Cas9 plasmid, gRNA-gre3 plasmid, and water.

Proper integration of the GH43$_{2/7}$ sequence cassette into SR8A6S3-CDT$_2$-GH43$_{2/7}$ was confirmed by PCR analysis. Colony PCR was performed directly from 7 colonies of the positive-control plate. Separated PCR products by agarose gel electrophoresis are presented in Fig. 5.20. Once proper integration was confirmed, a positive transformant was then evaluated for growth in xylose and acetate.

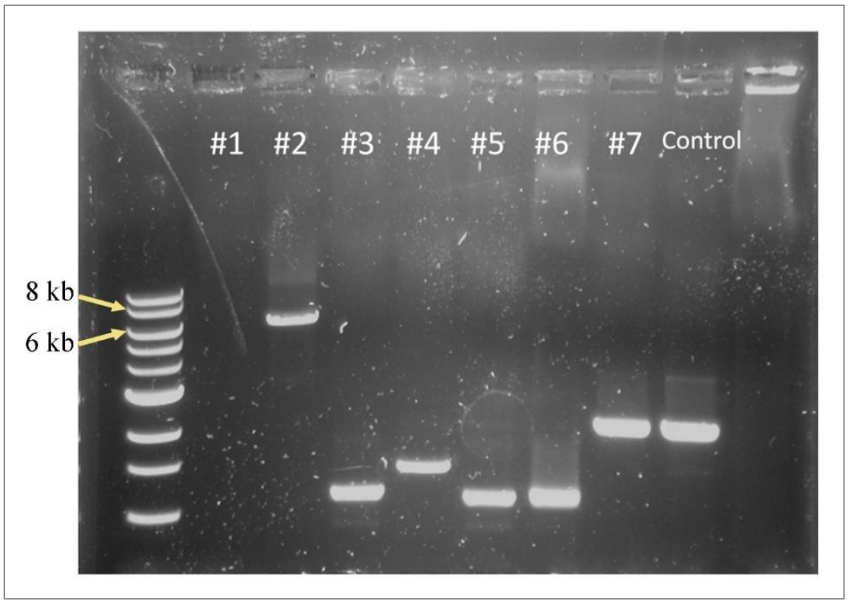

Fig. 5.20. Colony PCR products analyzed using an E-Gel 0.8% agarose from positive control plate colonies. *gre3*Δ:: $_{P}$TDH3-GH43-7-$_{T}$CYC- $_{P}$CCW12-GH43-2-$_{T}$CYC1 mutant cells present a band of 7 kb (colony 2), The last band represents the positive PCR control, of which the band has 1.5 kb.

5.4.5. Comparison of xylose and acetate fermentation of SR8A6S3-CDT$_2$-GH43$_{2/7}$, SR8A6S3-CDT$_2$ and SR8A6S3 strains

To investigate the impact of the genetic modification, a YP-based medium was used to cultivate the strain SR8A6S3-CDT$_2$-GH43$_{2/7}$. Xylose and acetate fermentation performance of the SR8A6S3-CDT$_2$-GH43$_{2/7}$ strain was analysed and compared with SR8A6S3-CDT$_2$ and their parental strain, SR8A6S3. Anaerobic batch cultivation was carried out (Fig. 5.21 and 5.22). We evaluated for xylose and acetate consumption, and ethanol and xylitol production in high sugar content media (20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate) at 30 °C and an initial cell density of 1 (OD at 600 nm).

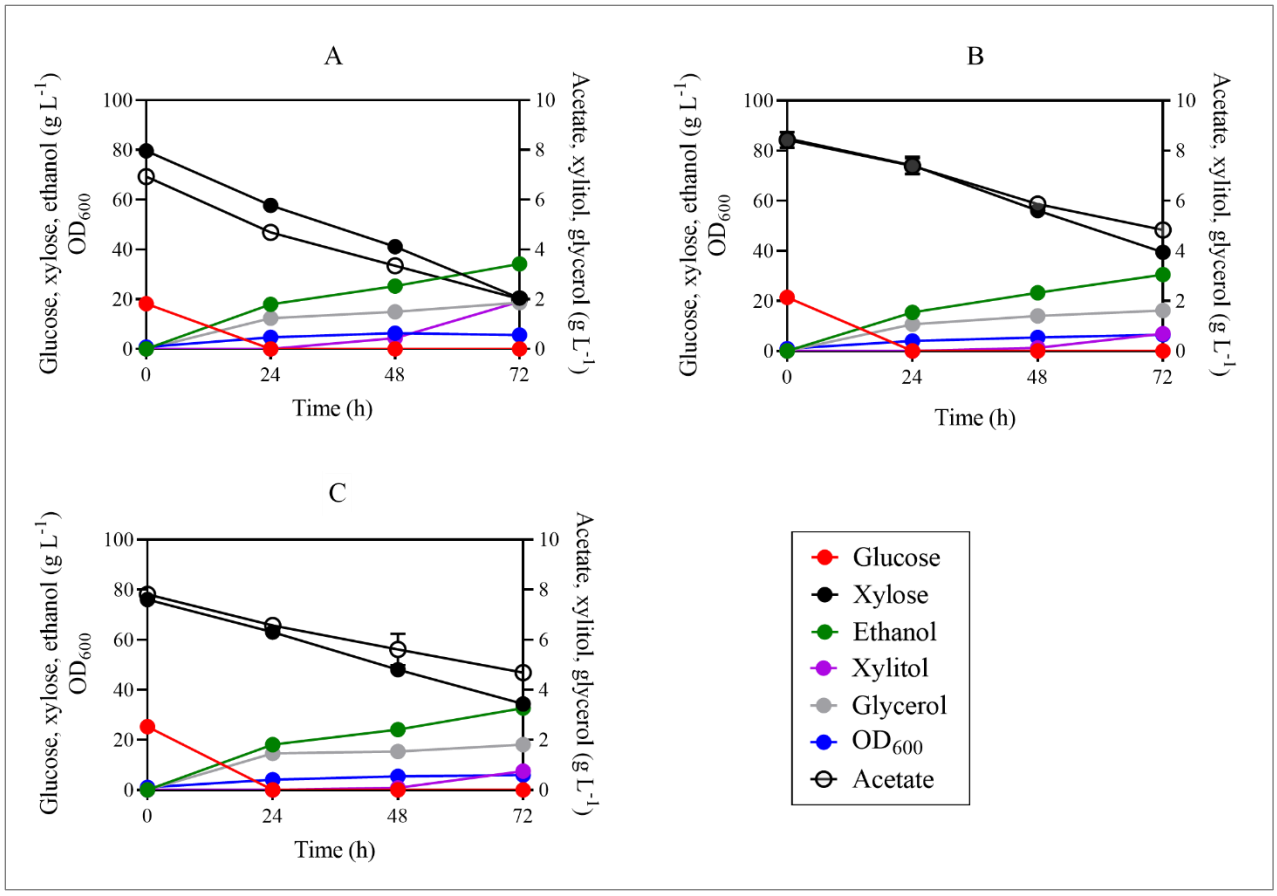

Fig. 5.21. Fermentation profiles of SR8A6S3 (A), SR8A6S3-CDT$_2$ (B), and SR8A6S3-CDT$_2$-GH43$_{2/7}$ (C) when fermenting YP supplemented with 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate) under anaerobic condition. Data are presented as mean value and standard deviation of three independent biological replicates.

Comparing the fermentative profiles showed in Fig. 5.21, SR8A6S3-CDT$_2$-GH43$_{2/7}$ presented improvements on xylose and acetate consumption profiles, as well final ethanol concentration achieved high values. Deletion of the *gre3* gene did not modify xylitol production by *gre3Δ* mutant strain (Fig. 5.22C and Table 5.9). SR8A6S3-CDT$_2$-GH43$_{2/7}$ produced a higher total amount of ethanol than SR8A6S3-CDT$_2$ but smaller than SR8A6S3 (Fig. 5.22B). However, the newest recombinant strain had slightly higher ethanol yields in comparison with SR8A6S3-CDT$_2$ and even more than SR8A6S3 (Table 5.9). Until 48 h SR8A6S3-CDT$_2$-GH43$_{2/7}$ presented the same biomass production profile as SR8A6S3-CDT$_2$, but at 72h its biomass concentration remained lower (Fig. 5.22D). SR8A6S3 consumed faster acetate than SR8A6S3-CDT$_2$ or SR8A6S3-CDT$_2$-GH43$_{2/7}$ (Fig. 5.22E).

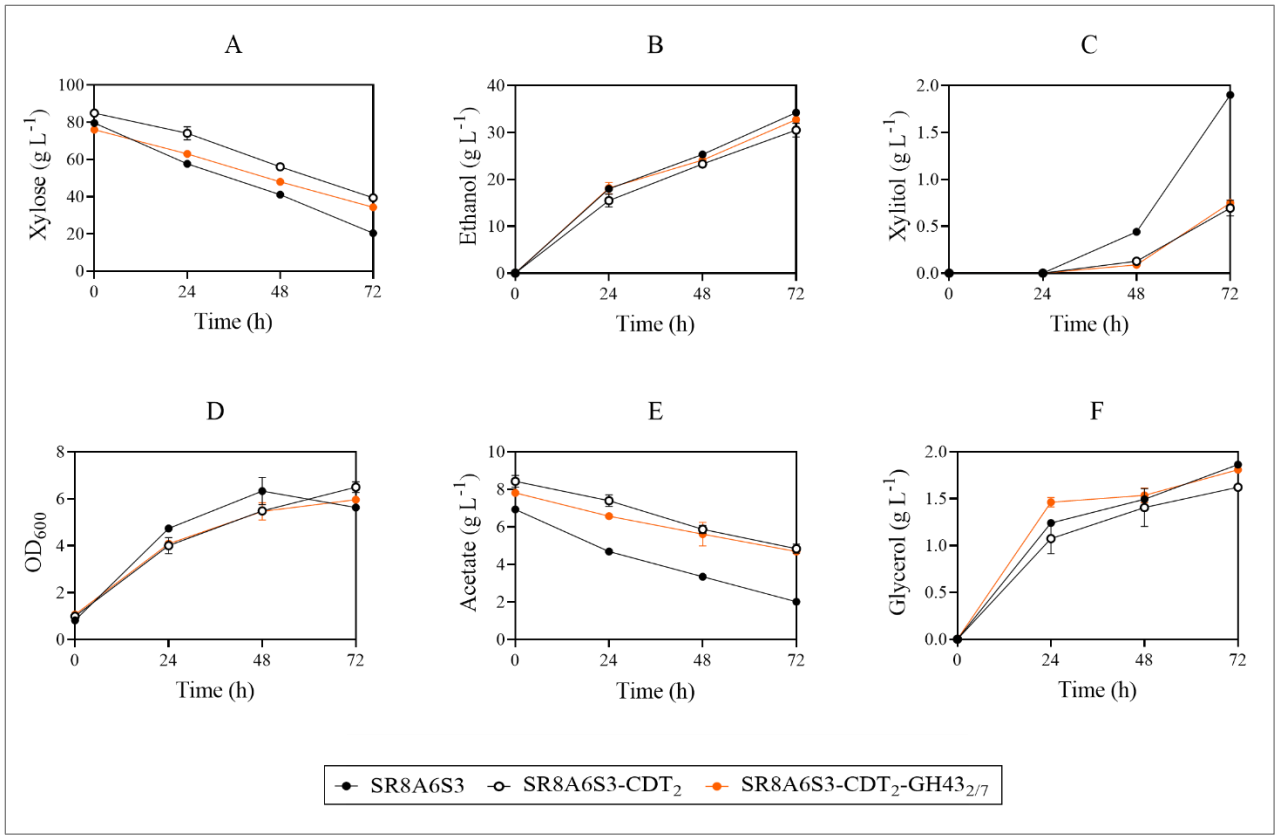

Fig. 5.22. Comparison of xylose (A) and acetate (E) consumption, ethanol (B), xylitol (C), OD_{600} (D), and glycerol (F) production of three xylose-acetate-assimilating strains, SR8A6S3, SR8A6S3-CDT_2, and SR8A6S3-CDT_2-$GH43_{2/7}$ cultivated in YP media containing 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate under anaerobic condition. An initial OD_{600} was adjusted to 1. The figure illustrates the means of triplicate experiments of each strain.

Table 5.9. Fermentation profile of SR8A6S3-CDT_2 and SR8A6S3 under anaerobic conditions.

	At 24 h				At 72 h				
	r_{xylose}	r_{xylose}*	$P_{xylitol}$	$P_{Ethanol}$	r_{xylose}	r_{xylose}*	$P_{xylitol}$	$P_{Ethanol}$	$Y_{Ethanol}$
SR8A6S3-CDT_2-$GH43_{2/7}$	0.54±0.03	0.15±0.01	0.00±0.00	0.77±0.05	0.57±0.01	0.10±0.00	0.01±0.00	0.45±0.00	0.47±0.01
SR8A6S3-CDT_2	0.37±0.13	0.10±0.03	0.00±0.00	0.62±0.06	0.63±0.03	0.10±0.02	0.01±0.00	0.41±0.01	0.46±0.01
SR8A6S3	0.94±0.06	0.19±0.01	0.00±0.00	0.74±0.00	0.83±0.02	0.14±0.00	0.03±0.00	0.47±0.00	0.44±0.00

Parameters: r_{xylose}, xylose consumption rate (g L^{-1} h^{-1}); r_{xylose}*, specific xylose consumption rate (g L^{-1} OD^{-1} h^{-1}); $P_{xylitol}$, volumetric xylitol productivity (g L^{-1} h^{-1}); $P_{Ethanol}$, volumetric ethanol productivity (g L^{-1} h^{-1}); $Y_{Ethanol}$, ethanol yield (g $g_{consumed\ carbon\ source}^{-1}$).

The previous deletion, *sor1Δ* decreased the xylose consumption rate, however, the new deletion *Δgre3* increase this rate at both times analysed, 24 and 72 h, but it continues lower than SR8A6S3 (Table 5.9). Interestingly, the ethanol production rate at 24 h was higher for SR8A6S3-CDT_2-$GH43_{2/7}$ than compared strains.

Elimination of xylitol production through *sor1Δ* and *gre3Δ* increases availability of intracellular NADH and $NADP^+$, which enabled the recombinant cell to produce even more ethanol from grams consumed sugar (ethanol yield) than a single deletion of *sor1Δ*. However, xylitol production suffers few modifications, despite the *GRE3* gene encode a xylose reduction which final product is xylitol (Fig. 5.23).

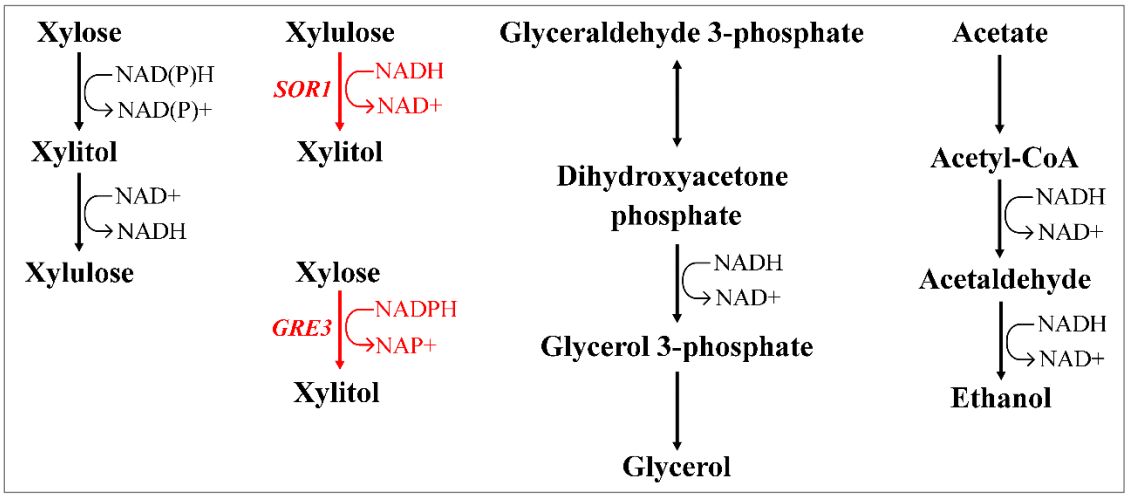

Fig. 5.23. Target metabolites were analysed for comparison of fermentation profiles of the SR8A6S3-CDT_2-$GH43_{2/7}$ and SR8A6S3. The red arrow represents deleted gene/route.

Wenger et al. (2010) screened a large number of *S. cerevisiae* strains, among wild, industrial, and laboratory backgrounds, to determine xylose-positive phenotype. From 647 studied strains, some wine strains appeared as able to grow modestly on xylose. By the application of high-throughput sequencing to bulk segregant analysis, they were able to identify the gene responsible for growth on xylose by the wine *S. cerevisiae* strains, a novel *XDH* gene homologous to *SOR1* (which was called *XDH1*). Next, the authors performed a comprehensive analysis of the involvement of the genes *GCY1, GRE3, YDL124W, YJR096W, YPR1, SOR1, SOR2, XDH1, XYL2, XKS1*, in the *XDH1* background strain (which has a xylose-positive phenotype) by deleting each gene or applying possible combinations of several genes involved. Individual deletion of each putative xylitol dehydrogenase (*SOR1*, *SOR2* and *XYL2*) could increase xylose utilization phenotype relative to the positive control. Further, when all three genes were deleted together (*sor1*Δ *sor2*Δ *xyl2*Δ), the phenotype was further enhanced (Wenger et al. 2010).

In a published study, *GRE3* gene was deleted to improve xylose metabolism of *S. cerevisiae* strain, CEN.PK2-1C, expressing xylose isomerase encoding gene *xylA* from *Thermus*

thermophilus. The recombinant Δ*gre3* strains produced less xylitol than the control cultivation, performed with the CEN.PK2-1C strain (Träff et al. 2001). According to the authors, deletion of the *GRE3* gene in *S. cerevisiae* decreased xylitol formation two- to threefold but not completely. As abovementioned, xylitol may also be formed by the activity of other genes, as endogenous XDH (homologous to *SOR1* gene), through the reduction of xylulose or through putative XR enzymes (Richard et al. 1999; Wenger et al. 2010; Patiño et al. 2019). Similarly, Jeong and co-authors (2020), construct a *S. cerevisiae* strain expressing isomerase pathway (*xylA*) from anaerobic fungus *Orpinomyces* sp. (GenBank No. MK335957). Some additional modifications (Δ*gre3*, Δ*sor1*, *XYL3,* and *TAL1*) were performed in the XI-strain to reduce xylitol accumulation and increase the growth rate (Jeong et al. 2020).

On the other hand, overexpression of the endogenous genes *GRE3* and *XYL2*, coding for nonspecific aldose reductase and a xylitol dehydrogenase, respectively, under endogenous promoters, could promote the growth of *S. cerevisiae* on xylose in the presence of glucose in aerobic shake flask cultivation (Toivari et al. 2004). However, significantly more xylitol was formed by the CEN.PK2 strain overexpressing the *S. cerevisiae* enzymes in comparison to strain which carrying XR and XDH from *P. stipitis*. Also, transcriptional analysis of xylose and glucose cultures show that the expression of *SOR1,* which encodes sorbitol dehydrogenase, was elevated in transformed cultures. Thereby, the presence of xylose resulted in higher XDH activity and induced the expression of the *SOR1* gene which also has XDH activity (Toivari et al. 2004).

According to the abovementioned studies, *GRE3* and *SOR1* genes were considered for improving xylose fermentation from different perspectives. It should be noted that in some of them, Δ*sor1* could increase xylose utilization and, Δ*gre3* and Δ*sor1* promote a decrease in xylitol accumulation. The above results from recent studies are in part consistent with the findings in the present study that the deletion of *GRE3* and *SOR1* from *S. cerevisiae* genome decrease xylitol formation, however, only Δ*sor1* did not increase xylose utilization.

5.4.6. Fermentation of a hemicellulosic hydrolysate by the engineered SR8A6S3-CDT$_2$-GH43$_{2/7}$ strain

In order to evaluate XOS consumption parameters of the latest engineered yeast strain, SR8A6S3-CDT$_2$-GH43$_{2/7}$, we cultivated this strain under micro-aerobic conditions at 30 °C, in YPH (YP medium supplemented with hemicellulosic hydrolysate) with an initial OD_{600} of 10 (Fig.

5.24). Xylobiose (X2) and xylotriose (X3) were the main carbon source available in the medium. As shown in Fig. 5.24, sugar consumption by the genetically manipulated strain was very small, whose levels remained high during the cultivation. Until 48 h of the cultivation, X2 and X3 concentrations varied slightly. Concomitantly, small amounts of ethanol, xylitol, and glycerol were produced. These results suggests that X2 and X3 were not able to interact with inducer-triggered sites within the cell to start the synthesis of xylanolytic enzymes. In their cultivations with the genetically modified strain carrying CDT-2, GH43-2, and GH43-7 from *N. crassa*, Li and authors (2015) observed that XOS fermentation was stimulated by glucose and xylose. According to them the presence of xylose and glucose greatly improved the anaerobic fermentation of XOS. These observations indicate that metabolic sensing in *S. cerevisiae* with the complete XOS pathway may require additional tuning for optimal XOS fermentation (Li et al. 2015).

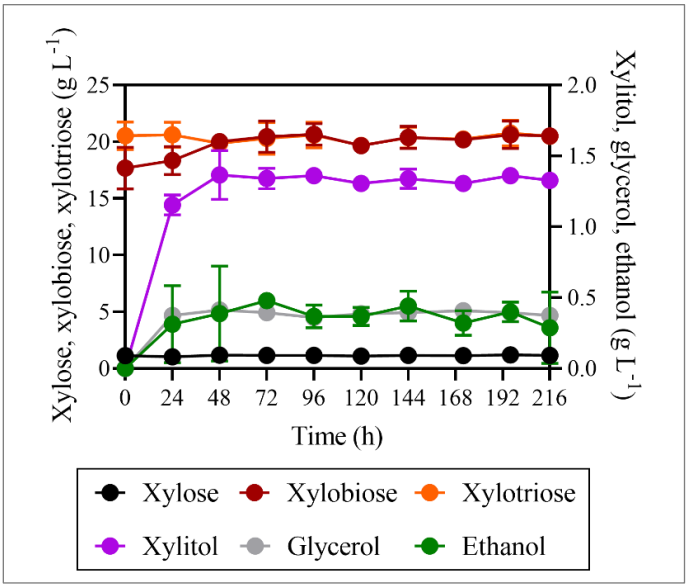

Fig. 5.24. Fermentation profiles of SR8A6S3-CDT$_2$-GH43$_{2/7}$ during batch cultivation in YPH (YP medium containing xylose, acetate, and hydrolyzed hemicellulose). Cultivations were performed at 30 °C and 100 rpm with an initial OD_{600} of 10. Data are presented as mean value and standard deviation of three independent biological replicates.

Notably, we observed that XOS utilization was stimulated by addition of xylose in the fermenting medium (Fig, 5.25). Thus, the presence of xylose improved the consumption of X2 and X3 available in the medium producing 3.78 ± 0.53 g L^{-1} ethanol at 24 h of cultivation. Until 48 h of cultivation, xylose and X2 were completely consumed. Further, we observed simultaneous fermentation of xylose and XOS, with increased ethanol yields, suggesting that the introduction of

the XOS pathway could enhance biofuel production from XOS-containing lignocellulosic hydrolysates.

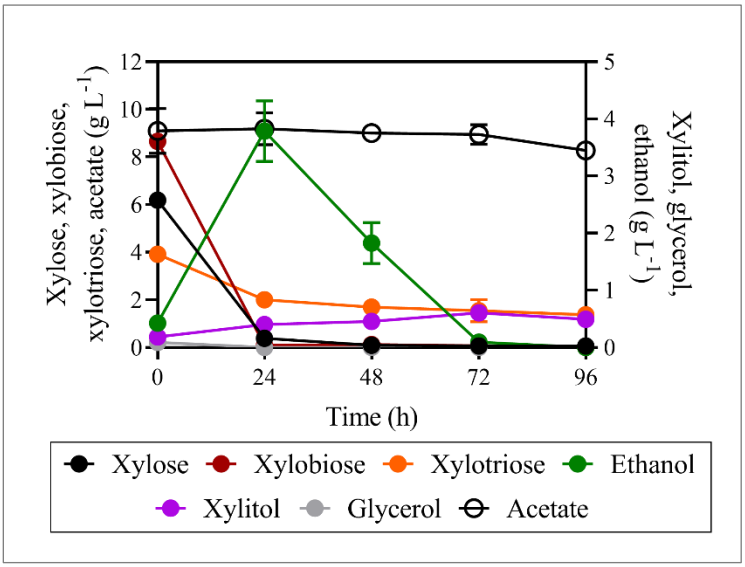

Fig. 5.25. Fermentation profiles of SR8A6S3-CDT$_2$-GH43$_{2/7}$ during batch cultivation in YPXAH (YP medium containing xylose, acetate, and hydrolyzed hemicellulose). Cultivations were performed at 30 °C and 100 rpm with an initial OD_{600} of 20. Data are presented as mean value and standard deviation of two independent biological replicates.

The breakdown of hemicellulose, which is acetylated (Kłosowski and Mikulski 2021) releases highly toxic acetate, degrading the fermentative performance of *S. cerevisiae* (Bellissimi et al. 2009; Li et al. 2015). SR8A6S3 was previously engineered (Zhang et al. 2016) through optimized expression levels of AADH and ACS in the acetate reduction pathway. Thereby, acetate has been consumed into ethanol by the optimized strain (Zhang et al. 2016). We, therefore, tested whether the acetate reduction pathway could be carried out simultaneously with XOS fermentation, as a means to augment ethanol yield from the lignocellulosic hydrolysate. However, to our surprise, acetate was not utilized by the SR8A6S3-CDT$_2$-GH43$_{2/7}$ strain, remaining in the culture supernatant (Fig. 5.25). Unfortunately, it seems that the utilization of X2 and X3 decreases the previous capabilities of the engineered strain to utilize acetate. The observation of an apparent inhibition of the acetate reduction pathway by XOS-consumption pathway suggest the existence of bottlenecks in the engineered strain. Recombinant gene expression can promote a nonspecific metabolic burden which reduces consumption rates and production yield of the host (Görgens et al. 2001).

In a previous study, Zhang et al. (2015) highlighted that three major factors might limit the metabolic fluxes of the acetate reduction pathway, which include the intracellular ATP levels, NADH levels, and the activities of key enzymes (ACS and AADH), being the last the major limiting factor among them. In this study, the expression of key enzymes was not modified through genetic interventions. Therefore, we reasoned the reduction in the acetate consumption by XOS- and xylose-acetate-consuming strains might be due to the perturbations in ATP and NADH levels generated through the addition of a transporter and two enzymes for XOS transport and hydrolysis.

Regarding xylitol production, we observed smaller xylitol accumulation by the SR8A6S3-CDT_2-$GH43_{2/7}$ strain cultivated in YPXAH (YP medium supplemented with xylose, acetate, and hydrolyzed hemicellulose) (Fig. 5.25) under micro-aerobic condition when compared in YPDXA (YP medium supplemented with glucose, xylose, and acetate) under anaerobic conditions (Fig. 5.21C). Moreover, when in the presence of hydrolyzed hemicellulose, SR8A6S3-CDT_2-$GH43_{2/7}$ produced xylitol faster.

To assess the impact of the additional genetic modifications, we cultivated both SR8A6S3-CDT_2-$GH43_{2/7}$ and SR8A6S3 strains in YPXAH (YP medium containing hemicellulosic hydrolysate, xylose, and acetate) with an initial OD_{600} of 1 (Fig. 5.26). High fermentative capacity was observed for SR8A6S3-CDT_2-$GH43_{2/7}$, which produced more ethanol and achieved higher cell density than the control cultivation. Interestingly, despite SR8A6S3 did not express either xylanolytic enzymes or XOS-transporter, X2 concentrations decreased along the cultivation. One of the hypothesis could be the conversion of X2 into the non-metabolizable compound xylosyl-xylitol by XR (xylose reductase), as observed previously by Li and colleagues (2015), or alternatively, that X2 had entered the cell through the natural transport system in *S. cerevisiae*. It is worth pointing out that *S. cerevisiae* can consume mono- and disaccharides, such as maltose, sucrose, and trehalose, which were up taken through the action of membrane transporters (Lagunas 1993). The up taken of sucrose (disaccharides composed of two monosaccharides, glucose, and fructose) can occur via the proton-symport (Mal11p), followed by ATP-driven export of the proton (which require hydrolysis of ATP) (Marques et al. 2018). As abovementioned, besides sucrose, *S. cerevisiae* is able to synthesize trehalose (disaccharide composed of two glucose) as a carbon source for growth (Jules et al. 2004). This disaccharide can be taken up via Agt1p-mediated trehalose transport followed by intracellular hydrolysis catalyzed by trehalase Nth1. Further, *AGT1*/*MAL11* gene is controlled by the *MAL* system. Maltose is transported to the cytosol by an

energy-dependent process coupled to the electrochemical proton gradient (Lagunas 1993). In our cultivations, we observed that X2 concentration dropped for SR8A6S3 cultivation, which did not express CDT-2 transporter, as well as it was not consumed for SR8A6S3-CDT_2-$GH43_{2/7}$ cultivated in YPH without xylose addition. These observations suggest the potential interaction involving *MAL* activator protein for xylose. Further, X2 was consumed faster for the XOS-consuming strain than its parental strain (Fig. 5.27), indicating that the CDT-2 transporter from *N. crassa* was efficiently expressed in *S. cerevisiae*.

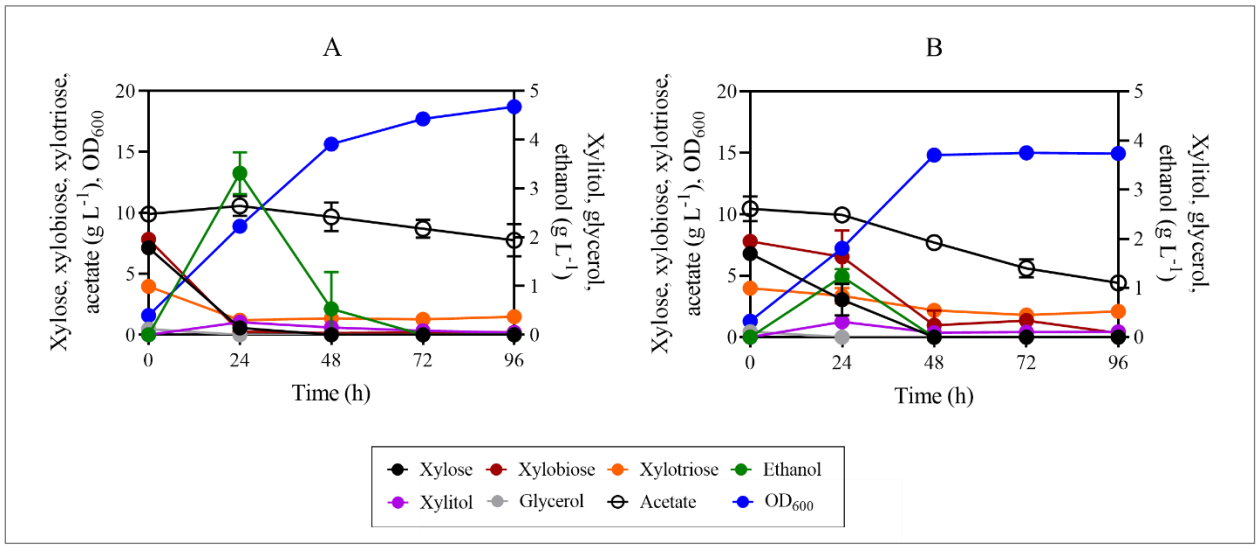

Fig. 5.26. Fermentation profiles of SR8A6S3-CDT_2-$GH43_{2/7}$ (A) and SR8A6S3 (B) during batch cultivation in YPXAH (YP medium containing xylose, acetate, and hydrolyzed hemicellulose). Cultivations were performed at 30 °C and 100 rpm with an initial OD_{600} of 1. Data are presented as mean value and standard deviation of two independent biological replicates.

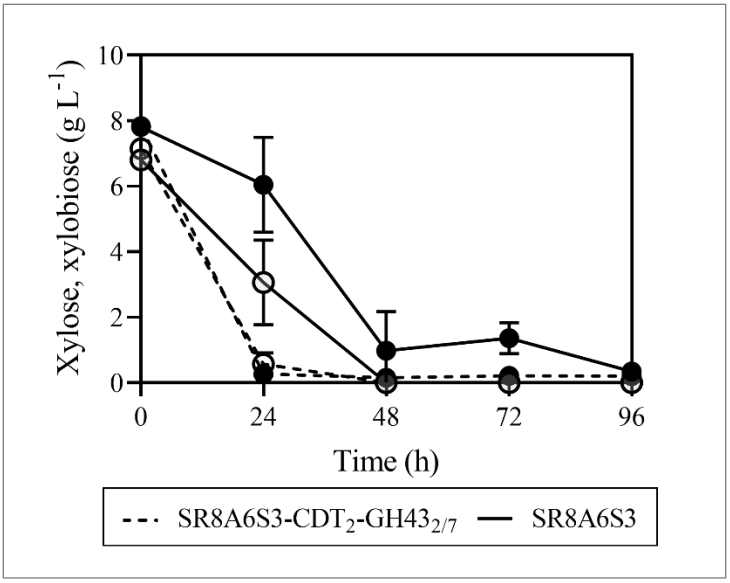

Fig. 5.27. Comparison of xylose (open circle) and xylobiose (filled circle) consumption for SR8A6S3 and SR8A6S3-CDT_2-$GH43_{2/7}$ cultivations in YP media containing xylose, acetate, and hydrolyzed hemicellulose under micro-aerobic condition. The figure illustrates the means of duplicate experiments of each strain.

Although we observed X2 decrease in the cultivations with the SR8A6S3 strain, only in cultivations with the XOS-consuming strain (SR8A6S3-CDT_2-$GH43_{2/7}$) ethanol accumulation was consistent with X2 fermentation, i.e., conversion of X2 into ethanol (Fig. 5.26). Similar to the abovementioned results, the acetate reduction pathway was inhibited for SR8A6S3-CDT_2-$GH43_{2/7}$ cultivated in the hemicellulosic hydrolysate. Despite acetate consumption pathway had also been affected in SR8A6S3 strain cultivation, the parental strain consumed higher acetate amounts than the SR8A6S3-CDT_2-$GH43_{2/7}$ strain

It is worth mentioning that in hemicellulosic hydrolysates, besides the presence of sugars, inhibitory compounds are also present, and they negatively influence fermentative yeast performance (Palmqvist and Hahn-Hägerdal 2000b). In order to mimic the fermentation of hemicellulosic hydrolysate, with the absence of inhibitory compounds, sole hydrolyzed xylan and a mix of hydrolyzed xylan plus acetate, were both supplemented in YP medium, as carbon sources, generating YPXyl and YPAXyl cultivation media, respectively. Thereby, the newly engineered strain and its parental strain were cultivated in YPXy with an initial OD_{600} of 10 (Fig. 5.28B and 5.28D), and in YPAXyl with an initial OD_{600} of 10 (Fig. 5.28A and 5.28C). As expected, the engineered strain, SR8A6S3-CDT_2-$GH43_{2/7}$, produced a higher quantity of ethanol than the parental strain SR8A6S3 in all conditions tested.

Similar to the hydrolysate cultivations, X2 concentrations were decreased by both strains, SR8A6S3-CDT$_2$-GH43$_{2/7}$ and SR8A6S3 (Fig. 5.28). Interestingly, ethanol accumulation was much higher in SR8A6S3-CDT$_2$-GH43$_{2/7}$ cultivations when compared to the parental strain (SR8A6S3) in both media. Furthermore, although in 96 h of cultivation, both the strain could consume the same amount of X2 in YPXyl (Fig. 5.28B and 5.28D), apparently only strain SR8A6S3-CDT$_2$-GH43$_{2/7}$ fermented it to ethanol, achieving 84% more ethanol than SR8A6S3 strain.

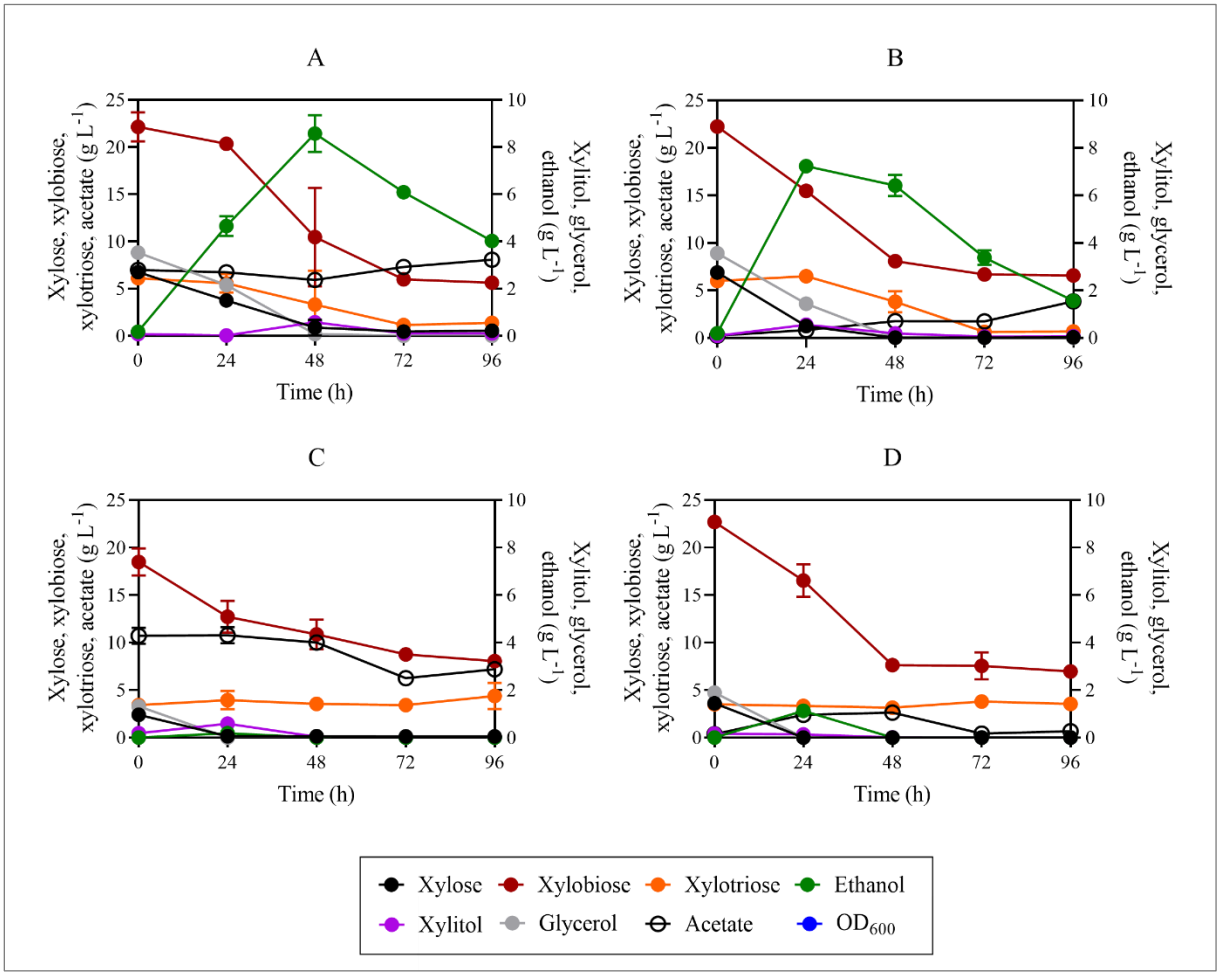

Fig. 5.28. Fermentation profiles of SR8A6S3-CDT$_2$-GH43$_{2/7}$ (A and B) and SR8A6S3 (C and D) during batch cultivation in YPAXyl (YP medium containing hydrolyzed xylan and acetate), A and C, and YPXyl (YP medium containing hydrolyzed xylan), B and D. Cultivations were performed at 30 °C and 100 rpm with an initial OD$_{600}$ of 10. Data are presented as mean value and standard deviation of two independent biological replicates

As shown in Fig. 5.28A and 5.28B, the XOS-consumption strain depleted xylose within 48 h. In YPXyl cultivation SR8A6S3-CDT$_2$-GH43$_{2/7}$ consumed 5.32 ± 0.13 g L^{-1} from 5.99 ± 0.11 g

L^{-1} of X3 available in the medium, and 4.59 ± 0.04 g L^{-1} from 5.95 ± 0.01 g L^{-1} in YPAXyl cultivations. After 72 h of cultivation, no substantial decrease in X3 amount was observed for both cultivations with this strain. SR8A6S3-CDT_2-$GH43_{2/7}$ finished xylose fermentation along with the co-fermentation with X2 and X3, however without consuming acetate at all. SR8A6S3 was able to consume small amounts of acetate, 0.86 ± 0.36 g L^{-1}.

To investigate the impact of the genetic modification, ethanol yield based on grams of consumed xylose was calculated for each condition analyzed in this section (Table 5.10). The ethanol yield of the SR8A6S3-CDT_2-$GH43_{2/7}$ strain increased substantially as compared to the SR8A6S3 strain. This substantial yield increase is due consumed XOS. Through the expression of XOS-consumption pathway, we have made considerable improvements in fermentation utilizing hemicellulosic hydrolysate and hydrolyzed xylan as carbon source.

Table 5.10. Ethanol yield of SR8A6S3-CDT_2 and SR8A6S3.

	Cultivation medium	Initial OD_{600}	$Y_{Ethanol}$
SR8A6S3-CDT_2-$GH43_{2/7}$	YPXAH	1	0.50 ± 0.03
	YPXAH	20	0.58 ± 0.08
	YPXyl	10	1.24 ± 0.04
	YPAXyl	10	1.43 ± 0.05
SR8A6S3	YPXAH	1	0.33 ± 0.08
	YPXyl	10	0.31 ± 0.00
	YPAXyl	10	0.08 ± 0.00

Parameters: $Y_{Ethanol}$, ethanol yield (g $g_{consumed\ xylose}^{-1}$).

XOS combined with xylose could improve bioethanol production from hydrolyzed hemicellulose. Hemicellulosic-derived sugar comprises 15-35% of lignocellulosic biomass, representing a large source of renewable material which is available at low cost (Palmqvist and Hahn-Hägerdal 2000a; Dahlman et al. 2003; Gírio et al. 2010). To obtain engineered strains able to consume oligomers derived from hemicellulose, mainly concerning intracellular hydrolysis, it represents a potential benefit for bioethanol production since engineered XOS-utilizing *S. cerevisiae* strain would have a competitive advantage concerning other microorganisms, such as contaminating bacteria and wild *Saccharomyces* and non-*Saccharomyces* species that are innately adept at utilizing XOS as carbon source (Cabrini and Gallo 1999; Amorim et al. 2011).

5.5. CONCLUSIONS

The dataset presents so far indicates important physiological changes take from the integration of the XOS-consumption pathway into two xylitol-production-related genes. Two yielded strains, SR8A6S3-CDT_2 and SR8A6S3-CDT_2-$GH43_{2/7}$ showed a reduction in xylitol production and improvement in ethanol yield when compared with their parental strain SR8A6S3 in YPDXA cultivations under both aerobic and anaerobic conditions. SR8A6S3-CDT_2-$GH43_{2/7}$ was able to ferment X2 and X3 efficiently for ethanol production and achieving the highest levels of 8.57 ± 0.78 g L^{-1} after cultivation of 48 h in YP supplemented with hydrolyzed xylan and acetate.

5.6. FUTURE STUDIES

Although this study has constructed an efficient strain for expressing the XOS-consumption pathway. Further optimization of the strain could be done to further increase both XOS and acetate uptake rate and ethanol yield. Below are some tips to achieve this goal:

- To perform anaerobic cultivations with SR8A6S3-CDT_2-$GH43_{2/7}$ in hydrolyzed xylan and hemicellulosic hydrolyzed;
- To investigate the effect of CDT-2, GH43-2, and GH43-7 on redox metabolism and energy metabolism in micro-aerobic and anaerobic conditions;
- To improve the XOS fermentation performance through evolutionary engineering.

5.7. REFERENCES

Almeida JRM, Modig T, Petersson A, et al (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 82:340–349. https://doi.org/10.1002/jctb.1676

Amorim H V., Lopes ML, de Castro Oliveira JV, et al (2011) Scientific challenges of bioethanol production in Brazil. Appl Microbiol Biotechnol 91:1267–1275. https://doi.org/10.1007/s00253-011-3437-6

Ask M, Bettiga M, Mapelli V, Olsson L (2013) The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing *Saccharomyces cerevisiae*. Biotechnol Biofuels 6:22. https://doi.org/10.1186/1754-6834-6-22

Bailey, M. J., Biely, P., and Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* 23, 257–270. doi:10.1016/0168-1656(92)90074-J.

Bellissimi E, van Dijken JP, Pronk JT, van Maris AJA (2009) Effects of acetic acid on the kinetics of xylose fermentation by an engineered, xylose-isomerase-based *Saccharomyces cerevisiae* strain. FEMS Yeast Res 9:358–364. https://doi.org/10.1111/j.1567-1364.2009.00487.x

Brenelli, L. B., Figueiredo, F. L., Damasio, A., Franco, T. T., and Rabelo, S. C. (2020). An integrated approach to obtain xylo-oligosaccharides from sugarcane straw: From lab to pilot

scale. *Bioresour. Technol.* 313, 123637. doi:10.1016/J.BIORTECH.2020.123637.
Cabrini KT, Gallo CR (1999) Identificação de leveduras no processo de fermentação alcoólica em usinas do estado de São Paulo, Brasil. Sci Agric 56:207–216. https://doi.org/10.1590/S0103-90161999000100028
Chundawat SPS, Beckham GT, Himmel ME, Dale BE (2011) Deconstruction of Lignocellulosic Biomass to Fuels and Chemicals. Annu Rev Chem Biomol Eng 2:121–145. https://doi.org/10.1146/annurev-chembioeng-061010-114205
Cong L, Ran FA, Cox D, et al (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823. https://doi.org/10.1126/science.1231143
Dahlman O, Jacobs A, Sjoè J (2003) Molecular properties of hemicelluloses located in the surface and inner layers of hardwood and softwood pulps. Cellulose 10:325-334
Dicarlo JE, Norville JE, Mali P, et al (2013) Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. Nucleic Acids Res 41:4336–4343. https://doi.org/10.1093/nar/gkt135
Galazka JM, Tian C, Beeson WT, et al (2010) Cellodextrin transport in yeast for improved biofuel production. Science 330:84–86. https://doi.org/10.1126/science.1192838
Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360. https://doi.org/10.1002/yea.320110408
Gírio FM, Fonseca C, Carvalheiro F, et al (2010) Hemicelluloses for fuel ethanol: A review. Bioresour Technol 101:4775–4800. https://doi.org/10.1016/j.biortech.2010.01.088
Görgens JF, van Zyl WH, Knoetze JH, Hahn-Hägerdal B (2001) The metabolic burden of the *PGK1* and *ADH2* promoter systems for heterologous xylanase production by *Saccharomyces cerevisiae* in defined medium. Biotechnol Bioeng 73:238–245. https://doi.org/10.1002/bit.1056
Himmel ME, Ding S-YS-Y, Johnson DK, et al (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315:804–807. https://doi.org/10.1126/science.1137016
Jain VK, Divol B, Prior BA, Bauer FF (2011) Elimination of glycerol and replacement with alternative products in ethanol fermentation by *Saccharomyces cerevisiae*. 38:1427–1435. https://doi.org/10.1007/s10295-010-0928-x
Jeong D, Oh EJ, Ko JK, et al (2020) Metabolic engineering considerations for the heterologous expression of xylose-catabolic pathways in *Saccharomyces cerevisiae*. PLoS One 15:e0236294. https://doi.org/10.1371/journal.pone.0236294
Jules M, Guillou V, François J, Parrou JL (2004) Two distinct pathways for trehalose assimilation in the yeast *Saccharomyces cerevisiae*. Appl Environ Microbiol 70:2771–2778. https://doi.org/10.1128/AEM.70.5.2771-2778.2004
Ji X-J, Huang H, Nie Z-K, et al (2011) Fuels and chemicals from hemicellulose sugars. Adv Bioch Eng 128:199–224. https://doi.org/10.1007/10_2011_124
Kim SR, Skerker JM, Kang W, et al (2013) Rational and evolutionary engineering approaches uncover a small set of genetic changes efficient for rapid xylose fermentation in *Saccharomyces cerevisiae*. PLoS One 8:e57048. https://doi.org/10.1371/journal.pone.0057048
Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 2004 661 66:10–26. https://doi.org/10.1007/S00253-004-1642-2

Kłosowski G, Mikulski D (2021) Impact of lignocellulose pretreatment by-products on *Saccharomuces cerevisiae* strain ethanol red metabolism during aerobic and anaerobic growth. Mol 2021, Vol 26, Page 806 26:806. https://doi.org/10.3390/MOLECULES26040806

Ko JK, Lee SM (2018) Advances in cellulosic conversion to fuels: engineering yeasts for cellulosic bioethanol and biodiesel production. Curr. Opin. Biotechnol. 50:72–80. https://doi.org/10.1016/j.copbio.2017.11.007

Konishi J, Fukuda A, Mutaguchi K, Uemura T (2015) Xylose fermentation by *Saccharomyces cerevisiae* using endogenous xylose-assimilating genes. Biotechnol Lett 37:1623–1630. https://doi.org/10.1007/s10529-015-1840-2

Lagunas R (1993) Sugar transport in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 10:229–242. https://doi.org/10.1111/J.1574-6968.1993.TB05869.X

Li X, Chen Y, Nielsen J (2019) Harnessing xylose pathways for biofuels production. Curr. Opin. Biotechnol. 57:56–65. https://doi.org/10.1016/j.copbio.2019.01.006 0958-1669/ã

Li X, Yu VY, Lin Y, et al (2015) Expanding xylose metabolism in yeast for plant cell wall conversion to biofuels. Elife 1–16. https://doi.org/10.7554/eLife.05896

Liu JJ, Kong II, Zhang GC, et al (2016) Metabolic engineering of probiotic Saccharomyces boulardii. Appl Environ Microbiol 82:2280–2287. https://doi.org/10.1128/AEM.00057-16

Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506–77. https://doi.org/10.1128/MMBR.66.3.506-577.2002

Marques WL, Mans R, Henderson RK, et al (2018) Combined engineering of disaccharide transport and phosphorolysis for enhanced ATP yield from sucrose fermentation in *Saccharomyces cerevisiae*. Metab Eng 45:121–133. https://doi.org/10.1016/J.YMBEN.2017.11.012

Matsushika A, Inoue H, Kodaki T, Sawayama S (2009) Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. Appl Microbiol Biotechnol 84:37–53. https://doi.org/10.1007/s00253-009-2101-x

Palmqvist E, Hahn-Hägerdal B (2000a) Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. Bioresour. Technol 74:17-24. https://doi.org/10.1016/S0960-8524(99)00161-3

Palmqvist E, Hahn-Hägerdal B (2000b) Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. Bioresour. Technol 74:25-33. https://doi.org/10.1016/S0960-8524(99)00161-3

Patiño MA, Ortiz JP, Velásquez M, Stambuk BU (2019) D-xylose consumption by nonrecombinant *Saccharomyces cerevisiae* : a review. Yeast 36:541–556. https://doi.org/10.1002/yea.3429

Petrovič U (2015) Next-generation biofuels: a new challenge for yeast. Yeast 32:583–593. https://doi.org/10.1002/yea.3082

Richard P, Toivari MH, Penttilä M (1999) Evidence that the gene YLR070c of *Saccharomyces cerevisiae* encodes a xylitol dehydrogenase. FEBS Lett 457:135–138. https://doi.org/10.1016/S0014-5793(99)01016-9

Sarkar N, Ghosh SK, Bannerjee S, Aikat K (2012) Bioethanol production from agricultural wastes: An overview. Renew Energy 37:19–27. https://doi.org/10.1016/J.RENENE.2011.06.045

Sharma B, Larroche C, Dussap CG (2020) Comprehensive assessment of 2G bioethanol production. Bioresour Technol 313:1–9. https://doi.org/10.1016/J.BIORTECH.2020.123630

Somerville C, Bauer S, Brininstool G, et al (2004) Toward a systems approach to understanding plant cell walls. Science 306:2206–2211. https://doi.org/10.1126/science.1102765

Stovicek V, Borodina I, Forster J (2015) CRISPR–Cas system enables fast and simple genome editing of industrial *Saccharomyces cerevisiae* strains. Metab Eng Commun 2:13–22. https://doi.org/10.1016/J.METENO.2015.03.001

Toivari MH, Salusjärvi L, Ruohonen L, Penttilä M (2004) Endogenous xylose pathway in *Saccharomyces cerevisiae*. Appl Environ Microbiol 70:3681–3686. https://doi.org/10.1128/AEM.70.6.3681-3686.2004

Träff KL, Cordero RRO, Van Zyl WH, 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. Appl Environ Microbiol 67:5668–5674. https://doi.org/10.1128/AEM.67.12.5668-5674.2001

Wei N, Quarterman J, Kim SR, et al (2013) Enhanced biofuel production through coupled acetic acid and xylose consumption by engineered yeast. Nat Commun 4:1–8. https://doi.org/10.1038/ncomms3580

Wenger JW, Schwartz K, Sherlock G (2010) Bulk segregant analysis by high-throughput sequencing reveals a novel xylose utilization gene from *Saccharomyces cerevisiae*. PLoS Genet 6:1–17. https://doi.org/10.1371/journal.pgen.1000942

Zhang G-C, Kong II, Wei N, et al (2016) Optimization of an acetate reduction pathway for producing cellulosic ethanol by engineered yeast. Biotechnol Bioeng 113:2587–2596. https://doi.org/10.1002/bit.26021

CHAPTER VI: EVALUATION OF *SACCHAROMYCES CEREVISIAE* STRAINS IN THE PRESENCE OF LIGNOCELLULOSIC INHIBITORS

The content of this chapter was published in *Biotechnology Letters* (doi.org/10.1007/s10529-020-02803-6). Priscila Cola, and I were the co-first authors and performed the experiments together with the help from Adriana Tabosa de Castro Alves, Luciana Rebelo Carnevalli, Ícaro Viana Sampaio, Bruno Labate Vale da Costa, and Dr. Thiago Olitta Basso was the director of the research. All my contribution for this publication was described in this Chapter VI.

Evaluation of *Saccharomyces cerevisiae* strains in the presence of lignocellulosic inhibitors

ABSTRACT

Expanding the global production of lignocellulosic ethanol requires microorganisms with increasing resistance to pretreatment process inhibitors. Understanding how these molecules impact the performance of *Saccharomyces cerevisiae* is of paramount importance to implement strategies to increase its robustness toward lignocellulosic inhibitory compounds, such as evolutionary engineering. In this sense, we hypothesized that the physiological effects of furans (HMF and furfural), organic acids (acetic and levulinic acid), and phenolic compounds (*p*-coumaric and ferulic acid) on *S. cerevisiae* would be unique and would have phenotypic traits demonstrated in the growth pattern of *S. cerevisiae*. For that purpose, four strains, including two laboratories (CEN.PK113-7D and CEN.PK112) and two industrial yeast strains (SA-1 and JAY270, a derivative of PE-2), were evaluated in the presence of these inhibitors. The impact of these compounds on quantitative physiological parameters, such as maximum specific growth rate and conversion yields were assessed in a defined medium. Concentration thresholds were based on previous studies performed by our group as well as on literature data. In cultures containing 20 mM of furfural, CEN.PK113-7D presented the largest extension of the lag phase concerning the other yeasts. In this condition, SA-1 exhibited the lowest lag phase elongation (67% lower than CEN.PK113- 7D and 56% lower than JAY270). A similar trend was observed with 30 mM of HMF, in which SA-1 stood out as compared to the other strains. At 40 mM of furfural and 200 mM of levulinic, no strain could grow. 50 mM acetic acid in a nonbuffered medium exhibited an elevated toxic effect on cell growth, and SA-1 was the only strain able to grow under this condition. Overall, SA-1 is a promising platform yeast strain for second-generation ethanol production and future metabolic and evolutionary engineering strategies, and strain robustness understanding.

Keywords: lignocellulosic ethanol; lignocellulosic inhibitors; *Saccharomyces cerevisiae*; laboratory strains; industrial strains; fermentation.

6.1. INTRODUCTION

Sustainable biofuel production from renewable biomass will require the efficient and complete use of all abundant sugars in the plant cell wall (Li et al. 2015). Lignocellulosic biomass is found in several types of raw materials, ranging from urban and industrials waste, wood and agricultural residues such as corn, wheat, and rice straws, and sugarcane bagasse (Himmel et al. 2007; Cardona and Sánchez 2007). As a plant-cell-wall derived compound, it is a structure characterized by a mesh of polysaccharides, structural proteins, and phenolic compounds that, in addition to protecting the plant cell against external attacks, provide structural and mechanical support to the plant tissue, which makes this matrix a highly compact and treatment-resistant structure (Gírio et al. 2010; Parawira and Tekere 2011). It consists essentially of cellulose microfibrils, representing 30-50 % of plant dry weight, hemicellulose, and lignin, representing between 20-35 % and 5-30 %, respectively (Lynd et al. 2002). The chemical composition variation is due to various factors, as climatic variability. Fig. 6.1 shows a structural scheme of the composition of lignocellulosic biomass (Pettersen 1984; Murphy and McCarthy 2005; Shen et al. 2013).

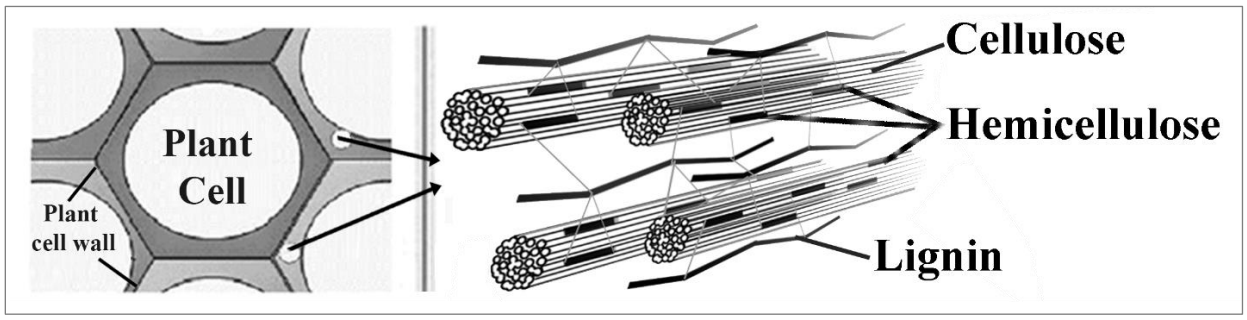

Fig. 6.1. Schematic representation of the proposed cell wall together with the location of the main components of the biomass. Adapted from Murphy and Mccarthy and Shen et al. (Murphy and McCarthy 2005; Shen et al. 2013).

Cellulose is in nature as individual molecules which are composed of D-glucose units, joined by β-1,4-glycosidic bonds (O'Sullivan 1997; Lynd et al. 2002). The representation of its linear chain is presented in Fig. 6.2A. Hemicellulose is a heterogeneous group of polysaccharides that can vary depending on the plant source (softwood or hardwood). Different from cellulose, hemicellulose is structurally amorphous and variable, being composed mainly of D-glucose, D-galactose, and D-mannose, by pentoses, such as D-xylose and L-arabinose, and by uranic acids

such as D-galacturonic, D-glucuronic, and methyl galacturonic acid. It is found in the wall of plant cells, associated with cellulose, and binding non-covalently to the surface of the cellulose fibrils by keeping them in place (Timell 1967). Fig. 6.2B shows a simplified scheme of hemicellulose structure.

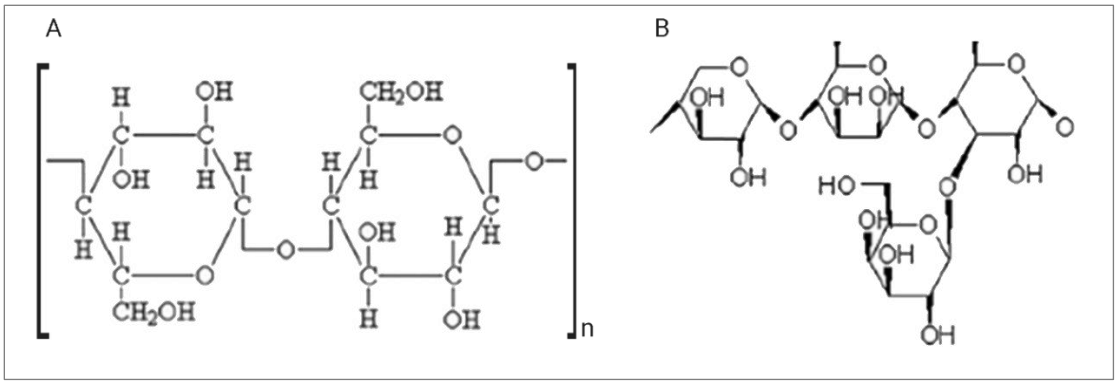

Fig. 6.2. A. Fragment (repeating unit) of a cellulose chain; B. Simplified chemical structure of hemicellulose (Sadeek et al. 2015).

Lignin is a complex compost highly branched and non-crystalline formed by methoxy and phenyl propyl groups deposited during the maturation of the cell wall, aiding the union of the cells. It is composed of nine carbon units derived from the substituted cinnamyl alcohol, which are coumarin, coniferyl, and alcohol syringyl. Its main role is to form a physical seal, promoting the packaging of the plant cell wall. This recalcitrant structure confers impermeability and resistance against microbial attack and oxidative stress. The complex formed by lignin, cellulose, and hemicellulose is called lignocellulose (Buchanan et al. 2003; Hendriks and Zeeman 2009). Fig. 6.3. shows a representative scheme of lignin structure.

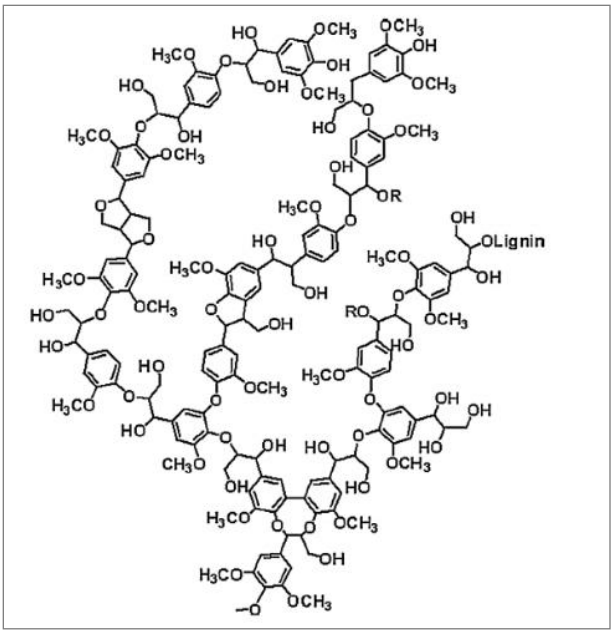

Fig. 6.3. Chemical structure of lignin (Sadeek et al. 2015).

Microbial lignocellulose utilization can represent a promising strategy to overcome the current dependency on petroleum-based fuels. The use of lignocellulosic materials requires several steps before they are converted to biochemicals which makes the process somewhat complex (Parawira and Tekere 2011). Firstly, lignocellulosic biomass must be submitted to harsh conditions (pretreatment) to make this material more susceptible to enzymatic hydrolysis (Sun and Cheng 2002; Parawira and Tekere 2011). The subsequent enzymatic hydrolysis of the cellulose and hemicellulose breaking down polymers into fermentable sugars (Olsson and Hahn-Hägerda 1996). Lignocellulosic pretreatment can also generate compounds that can inhibit microbial metabolism (Ask et al. 2013). 5-hydroxymethylfurfural (HMF) and 2-furaldehyde (furfural) are formed as dehydration products of hexoses and pentoses, respectively, and have been shown to decrease ethanol productivity, induce DNA damage, inhibit cell growth, and inhibit several enzymes in glycolysis, thus posing a serious challenge for the feasibility of chemicals derived from lignocellulosic material (Boyer et al. 1992; Navarro 1994; Larsson et al. 1999; Ask et al. 2013). Weak organic acids such as acetic, glycolic, levulinic, and formic acids are the most common weak acids present in lignocellulosic hydrolysates. Acetic acid is formed by the de-acetylation of hemicelluloses, while formic and levulinic acids are products of HMF breakdown. Formic acid can additionally be formed from furfural under acidic conditions at elevated temperatures (Almeida et al. 2007). They inhibit yeast growth and fermentation by reducing biomass formation

and ethanol yields in which are ascribed to uncoupling and intracellular anion accumulation (Larsson et al. 1999; Almeida et al. 2007). Phenolic compounds are generated from the partial breakdown of lignin (Almeida et al. 2007). It has been suggested that phenolic compounds exert toxic effects on the membrane of the cell in the fermentation of lignocellulosic hydrolysate, which is supported by observations that phenol changes membrane function and influences protein-to lipid rations in the membrane (Keweloh et al. 1990).

During industrial fermentation, yeasts face a variety of stress factors, including a high concentration of ethanol and salts, and high temperatures, for example. In addition to these stress factors, yeast cells are subjected to a continuous recycling process that lasts for the whole harvest season (around 250 days a year). All this dynamic can generate strains more adapted to the stress factors mentioned above as well with high viability maintenance in response to wild yeast and bacterial contaminants (Della-Bianca et al. 2013). In the light of these facts, we investigated the impact of different concentrations of some inhibitors found in lignocellulosic biomass such as furfural, HMF, acetic, levulinic, *p*-coumaric, and ferulic acid on the aerobic physiology of laboratory and industrial *S. cerevisiae* strains by subjecting the cells to a batch fermentation by including separately each inhibitor. We studied the severity of their inhibitory effects on cell growth elongation of the lag phase of lab *S. cerevisiae* strains, CEN.PK113-7D and CEN.PK112, and industrial *S. cerevisiae* strains, SA-1 and JAY270.

6.2. GOAL

The present research proposal aims to evaluate the *S. cerevisiae* performance facing some inhibitory compounds derived from the lignocellulosic hydrolysate.

6.3. MATERIAL AND METHODS

6.3.1. Microorganisms and maintenance

To inhibitors screening, two reference laboratory strains for physiological studies and functional genomics were used, being one haploid CEN.PK 113-7D (van Dijken JP et al. 2000) and one diploid CEN.PK 112 (van den Broek et al. 2015). We also used two industrial strains widely employed in the Brazilian sugarcane ethanol industry, namely SA-1 and JAY270, a derivative of PE-2 (Basso et al. 2008; Della-Bianca et al. 2013). The genotypic characteristics of the strains used in this work are summarized in Table 6.1.

Table 6.1. The **y**east strains used in this study.

***S. cerevisiae* strains**	**Relevant characteristics**	**Reference**
CEN.PK113-7D	MATa, MAL2-8^{c}, SUC2	(van Dijken et al. 2000)
CEN.PK112	MATa, MATα	(van den Broek et al. 2015)
SA-1	MATa, MATα, Amplified SNA/SNZ	(Basso et al. 2008; Della-Bianca et al. 2013)
JAY270	Heterothallic, MAT**a**, MATα, PE-2 derivative	(Argueso et al. 2009)

Stock cultures were grown at 30 °C and 200 rpm in shake flasks on 100 mL YPD medium (yeast extract 10 g L^{-1}, peptone 20 g $L^{-1,}$ and glucose or xylose). After overnight growth, 20 % (final concentration, p/v) glycerol was added and 1 mL aliquots were stored at -80 °C (Della-Bianca and Gombert 2013).

6.3.2. Cultivation conditions

The physiological characterization of yeast in the presence or in the absence (control) of inhibitory compounds, was performed in 50 mL-falcon tubes. Defined medium according to Verduyn et al., (1992) and adapted by Luttik et al., (2000) (Verduyn et al. 1992; Luttik et al. 2000), whose composition is described (in $g.L^{-1}$): NH_2CONH_2 (urea), 2.3; KH_2PO_4, 3.0; K_2SO_4, 6.6; $MgSO_4.7H_2O$, 0.5; and trace elements consisting of ($mg.L^{-1}$) EDTA, 15, $ZnSO_4.7H_2O$, 4.5, $MnCl_2.2H_2O$, 0.84; $CoCl_2.6H_2O$, 0.3; $CuSO_4.5H_2O$, 0.3; $Na_2MoO_4.2H_2O$, 0.4; $CaCl_2.2H_2O$, 4.5, $FeSO_4.7H_2O$, 3.0, H_3BO_3, 1.0, KI, 0.1. A solution containing vitamins was filter-sterilized and added to the medium to a final concentration of (mg L^{-1}) d-biotin, 0.05; calcium pantothenate, 1.0; nicotinic acid, 1.0; myo-inositol, 25; thiamine.HCl, 1.0; pyridoxine.HCl, 1.0, and p-aminobenzoic acid, 0.20. The medium had its pH adjusted to 6.0 with the addition of KOH. Media containing a single inhibitory compound was prepared to add the concentrations reported in Table 6.2. in the defined medium (Verduyn et al. 1992; Luttik et al. 2000). Precultures for all cultivations were grown overnight in an orbital shaker at 30 °C and 150 rpm in 250-mL shake flasks containing 50 mL of defined medium without the addition of any inhibitory compound. Batch fermentations were inoculated to an initial OD_{600} of 0.3 and incubated in an orbital shaker at 30 °C and 150 rpm.

Table 6.2. Lignocellulosic inhibitor compounds screened for their toxicity to *S. cerevisiae* under two different concentrations.

Inhibitory compound	Concentration range tested (mM)	
Acetic acid	50	200
Levulinic acid	25	200
HMF	16	32
Furfural	20	40
p-coumaric	2.5	7
Ferulic acid	0.4	1.3

6.3.3. Analytical methods

6.3.3.1. Extracellular fermentative metabolites and cell dry

Extracellular metabolites and cell dry mass concentrations during cultivations were determined as described by Basso et al. (Basso et al. 2010). Cell dry mass concentration was determined by gravimetry, according to a protocol described by Olsson and Nielsen (Olsson and Nielsen 1997). Biomass concentration was also indirectly monitored by absorbance at 600 nm (Abs 600 nm). The residual carbon source, ethanol, glycerol, and organic acids concentrations were quantified by high-performance liquid chromatography (HPLC) as described by Della-Bianca et al. (Della-Bianca et al. 2014) using an HPLC model Prominence (Shimadzu Corporation, Japan) and an HPX-87H analytical column (Bio-Rad Laboratories, USA) at 60 ºC with 5 mM H_2SO_4 as the mobile phase at 0.6 mL min^{-1}. Ethanol concentrations were corrected for evaporation as described by Medina et al. (Medina et al. 2010).

6.3.3.2. Phenolic compound quantification

The determination and quantification of phenolic compounds present in samples of industrial hydrolysates were based on the modified methodology of Kammerer et al. (Kammerer et al. 2004). We analyzed it by high-performance liquid chromatography (HPLC) model Prominence (Shimadzu Corporation, Japan) and a C18 analytical column (Supelco Inc. model waters Spherisorb ODS- 25 µm, 250 mm x 4,6 mm) at 30 °C with 2 % (v/v) acetic acid in ionized water (eluent A) and acetic acid 0.5 % in ionized water and acetonitrile (50:50, v/v; eluent B) as the mobile phase at 1.0 mL min^{-1} using a gradient program: from 10 to 15 % of B (10 min), 15 % of B isocratic (3 min), 15 to 25% of B (7 min), 25 to 55 % of B (30 min), 55 to 100 % of B (1 min), 100 % of B isocratic (5 min), from 100 to 10 % of B (0,1 min). The total run time was 60 min, with a flow rate of 1.0 mL min^{-1} and an oven temperature of 30 °C. The injection volume for

all samples was 10 μL. Monitoring was performed with a Shimadzu UV detector at wavelengths of 280 nm and 320 nm simultaneously. The concentrations of the compounds were calculated from calibration curves obtained from standard solutions.

6.4. RESULTS AND DISCUSSION

During industrial fermentation, yeasts face a variety of stresses factors, including a high concentration of ethanol and salts, high temperatures, and others. In addition to these stress factors, sugarcane-based fermentation differs significantly from other processes. Yeast cells are subjected to a continuous recycling process that lasts for the whole harvest season (around 250 days a year). This is an important characteristic that highlights the occurrence of heterogeneous yeast population dynamics in which industrial strains become selected by adapting not only to the stress factors mentioned above but also to the rapid growth and high viability maintenance in response to wild yeast and bacterial contaminants (Della-Bianca et al. 2013).

Additional obstacles arise in the second-generation ethanol production process, where lignocellulosic residues are the substrates for fermentation. In addition to the problem of pentose fermentation, physical-chemical pretreatment steps generate various microbial inhibitors that severely affect yeast growth and physiology that compromise the fermentation process (Palmqvist and Hahn-Hägerdal 2000; Skerker et al. 2013; Caspeta et al. 2015). Lignocellulosic-derived inhibitors are formed during the pretreatment of biomass and depend mainly on the type of biomass used and the process conditions (Klinke et al. 2004; van Maris et al. 2006). Many studies have been performed on the formation of by-products during the pretreatment of sugarcane bagasse. Knowledge of the formation of by-products from lignocellulosic material is greatly beneficial when the decomposed lignocellulose is used in a fermentation process. These by-products can result in problems further downstream since they can inhibit the growth and performance of microorganisms during fermentation (van der Pol et al. 2014).

Therefore, we investigated the effects of various inhibitory compounds on cell growth and elongation of the lag phase. Strains evaluated included the lab strain *S. cerevisiae* CEN.PK113-7D and its diploid version CEN.PK112, and two industrial strains from the fuel ethanol industry, namely *S. cerevisiae*, SA-1, and JAY270. Different concentrations of the major lignocellulosic inhibitors, such as furfural, HMF, acetic, levulinic, p-coumaric, and ferulic acids, were investigated.

During this investigation, we have found that the industrial strain *S. cerevisiae* SA-1 was more robust when cultivated in the presence of inhibitory compound-containing media than other strains, since lag phase duration was smaller and growth rate was higher, in most conditions investigated (Fig. 6.4 and Table 6.3).

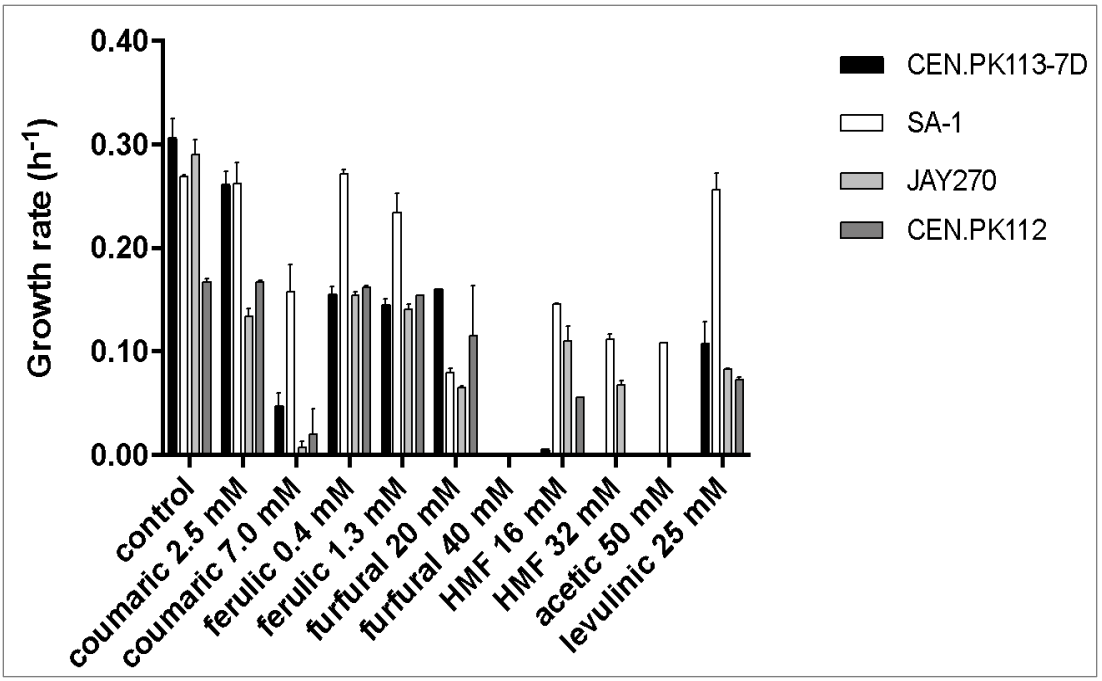

Fig. 6.4. *S. cerevisiae* growth rates in the presence of different concentrations of inhibitor compounds.

Table 6.3. Duration of the lag phase (h) in cultures with different lignocellulosic inhibitory compounds (mM).

Strains	**Control**	**Acetic acid**		**Levulinic acid**		**HMF**		**Furfural**		***p*-coumaric acid**		**Ferulic acid**	
		50	**200**	**25**	**200**	**16**	**32**	**20**	**40**	**2.5**	**7**	**0.4**	**1.3**
CEN.PK113-7D	1	>96	>96	14	>96	>96	>96	30	>96	1	>96	3	6
CEN.PK112	1	>96	>96	3	>96	3	>96	36	>96	3	>96	3	3
SA-1	1	15	>96	3	>96	3	3	3	>96	1	3	1	1
JAY270	1	>96	>96	3	>96	3	3	24	>96	1	>96	1	1

The lab reference strain CEN.PK113-7D did not grow at both concentrations of HMF tested (16 and 32 mM), whereas the industrial strains grew, and their lag phase was only slightly delayed. Remarkably, only the two industrial strains grew at the highest concentration tested (32 mM) (Table 6.3). None of the strains could grow at 200 mM acetic acid or 200 mM levulinic acid

or 40 mM furfural. 50 mM acetic acid in a non-buffered medium exhibited an elevated toxic effect on cell growth, and SA-1 was the only strain able to grow under these conditions. In cultures containing 25 mM of levulinic acid, the inhibitory effect was very intensive for haploid laboratory strain.

6.5. CONCLUSION

The present work investigated the effects of major lignocellulosic inhibitor compounds on important physiological parameters of two laboratories and two industrial *S. cerevisiae* strains, that could serve as candidate microbial platforms in the second-generation ethanol industry. In general, this screening was able to reveal the outstanding performance of one of the industrial strains (SA-1) over the strains evaluated, under the presence of inhibitory compounds. Most of the inhibitors tested had a significantly smaller impact on the growth rate and the lag phase of this strain. This observation corroborates the hypothesis that industrial yeast strains tend to be more resistant to adverse conditions and are therefore the best candidates for further research in second-generation ethanol technology.

6.6.REFERENCES

Almeida JRM, Modig T, Petersson A, et al (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 82:340–349. https://doi.org/10.1002/jctb.1676

Argueso JL, Carazzolle MF, Mieczkowski PA, et al (2009) Genome structure of a Saccharomyces cerevisiae strain widely used in bioethanol production. Genome Res 19:2258–70. https://doi.org/10.1101/gr.091777.109

Ask M, Bettiga M, Mapelli V, Olsson L (2013) The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing *Saccharomyces cerevisiae*. Biotechnol Biofuels 6:22. https://doi.org/10.1186/1754-6834-6-22

Basso LC, de Amorim H V., de Oliveira AJ, Lopes ML (2008) Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res 8:1155–1163. https://doi.org/10.1111/j.1567-1364.2008.00428.x

Basso TO, Dario MG, Tonso A, et al (2010) Insufficient uracil supply in fully aerobic chemostat cultures of *Saccharomyces cerevisiae* leads to respiro-fermentative metabolism and double nutrient-limitation. Biotechnol Lett 32:973–977. https://doi.org/10.1007/s10529-010-0248-2

Boyer LJ, Vega JL, Klasson KT, et al (1992) The effects of furfural on ethanol production by *Saccharomyces cerevisiae* in batch culture. Biomass and Bioenergy 3:41–48. https://doi.org/10.1016/0961-9534(92)90018-L

Buchanan CM, Buchanan NL, Debenham JS, et al (2003) Preparation and characterization of arabinoxylan esters and arabinoxylan ester/cellulose ester polymer blends. Carbohydr Polym 52:345–357. https://doi.org/10.1016/S0144-8617(02)00290-4

Cardona CA, Sánchez ÓJ (2007) Fuel ethanol production: Process design trends and integration

opportunities. Bioresour Technol 98:2415–2457. https://doi.org/10.1016/j.biortech.2007.01.002
Caspeta L, Castillo T, Nielsen J (2015) Modifying Yeast Tolerance to Inhibitory Conditions of Ethanol Production Processes. Front Bioeng Biotechnol 3:184. https://doi.org/10.3389/fbioe.2015.00184
Della-Bianca BE, Basso TO, Stambuk BU, et al (2013) What do we know about the yeast strains from the Brazilian fuel ethanol industry? Appl Microbiol Biotechnol 97:979–991. https://doi.org/10.1007/s00253-012-4631-x
Della-Bianca BE, de Hulster E, Pronk JT, et al (2014) Physiology of the fuel ethanol strain *Saccharomyces cerevisiae* PE-2 at low pH indicates a context-dependent performance relevant for industrial applications. FEMS Yeast Res 14:1196–1205. https://doi.org/10.1111/1567-1364.12217
Della-Bianca BE, Gombert AK (2013) Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. Antonie van Leeuwenhoek, Int J Gen Mol Microbiol. https://doi.org/10.1007/s10482-013-0030-2
Gírio FM, Fonseca C, Carvalheiro F, et al (2010) Hemicelluloses for fuel ethanol: A review. Bioresour Technol 101:4775–4800. https://doi.org/10.1016/j.biortech.2010.01.088
Hendriks ATWM, Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour Technol 100:10–18. https://doi.org/10.1016/j.biortech.2008.05.027
Himmel ME, Ding S-YS-Y, Johnson DK, et al (2007) Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production. Science 315:804–807. https://doi.org/10.1126/science.1137016
Kammerer D, Claus A, Carle R, Schieber A (2004) Polyphenol Screening of Pomace from Red and White Grape Varieties (Vitis vinifera L.) by HPLC-DAD-MS/MS. J Agric foor Chem 52:4360–4367. https://doi.org/10.1021/JF049613B
Keweloh H, Weyrauch G, Rehm HJ (1990) Phenol-induced membrane changes in free and immobilized *Escherichia coli.* Appl Microbiol Biotechnol 33:66–71
Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66:10–26. https://doi.org/10.1007/s00253-004-1642-2
Larsson S, Palmqvist E, Hahn-Hägerdal B, et al (1999) The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb Technol 24:151–159. https://doi.org/10.1016/S0141-0229(98)00101-X
Li X, Yu VY, Lin Y, et al (2015) Expanding xylose metabolism in yeast for plant cell wall conversion to biofuels. Elife. https://doi.org/10.7554/eLife.05896
Luttik MA, Kötter P, Salomons FA, et al (2000) The *Saccharomyces cerevisiae* ICL2 gene encodes a mitochondrial 2-methylisocitrate lyase involved in propionyl-coenzyme A metabolism. J Bacteriol 182:7007–13
Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506–77
Medina VG, Almering MJH, van Maris AJA, Pronk JT (2010) Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. Appl Environ Microbiol 76:190–195. https://doi.org/10.1128/AEM.01772-09
Murphy JD, McCarthy K (2005) Ethanol production from energy crops and wastes for use as a transport fuel in Ireland. Appl Energy 82:148–166

Navarro AR (1994) Effects of furfural on ethanol fermentation by *Saccharomyces cerevisiae*: Mathematical models. Curr Microbiol 29:87–90. https://doi.org/10.1007/BF01575753

O'Sullivan AC (1997) Cellulose: the structure slowly unravels. Cellulose 4:173–207. https://doi.org/10.1023/A:1018431705579

Olsson L, Hahn-Hägerda B (1996) Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb Technol 18:312–331. https://doi.org/10.1016/0141-0229(95)00157-3

Olsson L, Nielsen J (1997) On-line and in situ monitoring of biomass in submerged cultivations. Trends Biotechnol 15:517–522. https://doi.org/10.1016/S0167-7799(97)01136-0

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Elsevier

Parawira W, Tekere M (2011) Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. Crit Rev Biotechnol 31:20–31. https://doi.org/10.3109/07388551003757816

Pettersen RC (1984) The Chemical Composition of Wood. pp 57–126

Sadeek SA, Negm NA, Hefni HHH, Wahab MMA (2015) Metal adsorption by agricultural biosorbents: Adsorption isotherm, kinetic and biosorbents chemical structures. Int J Biol Macromol 81:400–409. https://doi.org/10.1016/j.ijbiomac.2015.08.031

Shen D, Xiao R, Gu S, Zhang H (2013) The Overview of Thermal Decomposition of Cellulose in Lignocellulosic Biomass. In: Cellulose - Biomass Conversion. InTech

Skerker JM, Leon D, Price MN, et al (2013) Dissecting a complex chemical stress: chemogenomic profiling of plant hydrolysates. Mol Syst Biol 9:1–21. https://doi.org/10.1038/msb.2013.30

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresour Technol 83:1–11. https://doi.org/10.1016/S0960-8524(01)00212-7

Timell TE (1967) Recent progress in the chemistry of wood hemicelluloses. Wood Sci Technol 1:45–70. https://doi.org/10.1007/BF00592255

van den Broek M, Bolat I, Nijkamp JF, et al (2015) Chromosomal copy number variation in *Saccharomyces pastorianus* is evidence for extensive genome dynamics in industrial lager brewing strains. Appl Environ Microbiol 81:6253–6267. https://doi.org/10.1128/AEM.01263-15

van der Pol EC, Bakker RR, Baets P, Eggink G (2014) By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels. Appl Microbiol Biotechnol 98:9579–9593. https://doi.org/10.1007/s00253-014-6158-9

van Dijken JP, Bauer J, Brambilla L, et al (2000) An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. Enzyme Microb Technol 26:706–714

van Maris AJA, Abbott DA, Bellissimi E, et al (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Van Leeuwenhoek 90:391–418. https://doi.org/10.1007/s10482-006-9085-7

Verduyn C, Postma E, Scheffers WA, Van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8:501–517. https://doi.org/10.1002/yea.320080703

CHAPTER VII: A THOROUGH UNDERSTANDING OF THE EFFECTS OF *P*-COUMARIC ACID ON *SACCHAROMYCES CEREVISIAE* METABOLISM UNDER GLUCOSE-LIMITED CHEMOSTAT CULTIVATION

The content of this chapter is submitted to *BBA Advances* for peer-review – Annex II. Felipe Eduardo Ciamponi, and I were the co-first authors and performed the experiments with the help from Dr. Natália Farad Murad, Dr. Telma Teixeira Franco, and Dr. Thiago Olitta Basso and Dr. Marcelo Brandão were the director of the research. Briefly, the paper had investigated how *S. cerevisiae* yeast cells responded to the presence of *p*-coumaric acid on global transcriptional and physiological levels. All my contributions to this publication were described in Chapter VII.

A thorough understanding of the effects of *p*-coumaric acid on *Saccharomyces cerevisiae* metabolism under glucose-limited chemostat cultivation

ABSTRACT

During industrial fermentation, yeasts face a variety of stress factors, including a high concentration of ethanol and salts, high temperatures, and pH. Additional obstacles arise in the second-generation ethanol production process, where lignocellulosic residues are the substrates for fermentation. The physical-chemical pretreatment steps generate various microbial inhibitors that severely affect yeast growth and physiology and compromise fermentation efficiency. The understanding of the physiological effects of major inhibitory compounds on the performance of *Saccharomyces cerevisiae* is essential for the effective implementation of strategies that promote the increase of its robustness and improve fermentation performance. In this sense, we investigated the effect of one major phenolic compound, *p*-coumaric acid (pCA), on important quantitative physiological parameters of the industrial strain *S. cerevisiae*, SA-1. The dataset presented indicated important physiological changes in glucose-limited chemostat cultivations in the presence of 7 mM pCA as compared to the control condition (without pCA addition). We observed an increase in consumption and production rates, such as for glucose (26%), CO_2 (12%), and ethanol (53%). On the other hand, we observed a decrease in biomass yield (22%), and the glycerol production rate (19%).

Keywords: lignocellulosic ethanol; *p*-coumaric; *Saccharomyces cerevisiae*; chemostat cultivation.

7.1. INTRODUCTION

Plant-based biofuel and biochemicals require deconstruction and hydrolysis of the lignocellulosic biomass to facilitates the breakdown of cellulose, hemicellulose, and lignin present in the plant cell wall, before conversion to products of interest (Palmqvist and Hahn-Hägerdal 2000; Adeboye et al. 2017). Look into lignin complexes, they are abundant organic compounds that under harsh treatment release several biologically active compounds such as phenolic compounds (Palmqvist and Hahn-Hägerdal 2000; Adeboye et al. 2015, 2017). These compounds are considered an inhibitor of yeast metabolism, expressing different potential inhibitory activity according to their composition (Larsson et al. 2000). Although it is known that phenolic compounds are the most toxic by-products of lignocellulose pretreatment due to their low molecular weight, their mechanism's influence on cellular metabolism and the way yeast cells adapt to them has not been fully understood (Adeboye et al. 2015; Kłosowski and Mikulski 2021).

Phenolic lignin degradation products are a heterogeneous group comprising aromatic compounds that have different functional groups and hence different potential inhibitory activity (Klinke et al. 2004; Almeida et al. 2007), which low molecular-weight phenolic compounds (molecular-weight (MW) < 350 Da) are more inhibitory to *S. cerevisiae* than high MW phenolics (Fiorentino et al. 2003; Almeida et al. 2007). Their effects on microbial growth and product yield are described to be vary variable and can be related to their specific functional groups (Jönsson et al. 2013). One possible inhibition mechanism is that phenolics interfere with the yeast cell membrane by influencing its function and changing its protein-to-lipid ratio (Keweloh et al. 1990; Palmqvist and Hahn-Hägerdal 2000b).

In second-generation biofuel production, furan, acids, and phenolic compounds derivatives of cellulose, hemicellulose, and lignin released into the fermentation broth act as potential inhibitors against fermenting organisms (Larsson et al. 1999; Adeboye et al. 2015). Most of them can be detoxified *in situ* by *S. cerevisiae* strains through their conversion to lesser inhibitory compost (Liu 2011; Kłosowski and Mikulski 2021), including some phenolics compounds, as demonstrated by Adeboye and coauthors (2015). According to the authors, *S. cerevisiae* was able to degrade some phenolic compounds under aerobic conditions, such as *p*-coumaric acid (pCA), coniferyl aldehyde, and ferulic acid (Adeboye et al. 2015), however, in the present study we demonstrated that under anaerobic condition an industrial *S. cerevisiae* strain was not able to metabolize pCA.

The main aim of this study was to investigate the influence of pCA on yeast physiology in a glucose-limited chemostat under the anaerobic condition as well as provide samples for identifying endogenous enzymes that are actively involved in the pCA detoxification process in *S. cerevisiae* SA-1 strain. Alterations in transcription patterns of SA-1, when exposed to 7 mM pCA, were analyzed by Mc.S. Felipe Ciamponi (Universidade Estadual de Campinas – UNICAMP). High-throughput RNA sequencing had been used to achieve the goal. Among the phenol compounds derivatives from lignocellulose hydrolysis, pCA represents the majority of the total composition (van der Pol et al. 2014), therefore understanding its inhibitory effects on *S. cerevisiae* metabolism is important to obtain tolerant strains for second-generation ethanol production.

7.2. GOAL

The present research proposal aims to evaluate the effect of 7 mM pCA on the physiology of *S. cerevisiae* SA-1 using an anaerobic continuous cultivation system.

7.3. MATERIAL AND METHODS

7.3.1. Microorganisms and maintenance

The strain investigated in this study, SA-1, is an industrial *S. cerevisiae* strain (*MATa*/*MATα*). Inoculum cultures were prepared from -80°C glycerol stocks on a defined medium (VM) according to Verduyn et al., (1992) and adapted by Luttik et al., (2000), whose composition is described (in $g.L^{-1}$): NH_2CONH_2 (urea), 2.3; KH_2PO_4, 3.0; K_2SO_4, 6.6; $MgSO_4.7H_2O$, 0.5; 1 mL L^{-1} trace elements solution, 1 mL L^{-1} vitamins solution (Verduyn et al. 1992; Luttik et al. 2000), and 20 g L^{-1} glucose. The pre-cultures were cultivated in Erlenmeyer flasks containing 30 mL of VM supplemented with 20 g L^{-1} glucose. They grew overnight at 30°C in a rotary shaker at 200 rpm.

7.3.2. Chemostat cultivation with pCA

After pre-cultivation of SA-1, yeast cells were inoculated in a 2,0 L water-jacketed model Labors 5 (Infors AG, Switzerland) bioreactor containing 1 L of VM medium containing 20 g L^{-1} glucose and or not (for control cultivation) 7 mM pCA. All chemostat cultivation was carried out in a way to minimize the oxygen diffusion into the system, using Viton “O”-rings and neoprene tubing. Nitrogen gas was used to flush both the culture vessel (0.5 L min^{-1}) and the medium vessel

(MV) (flow not measured) throughout the cultivation. Agitation frequency was set to 800 rpm, the temperature was controlled at 30 °C, and pH was controlled at 5.0 via controlled 2 KOH solution. Bioreactor batch cultivations (BBC) were characterized by the exhaustion of glucose (that were monitored by a sharp drop in the CO_2 concentration in the off-gas). After BBC, the mode of cultivation was switched to continuous mode through the constant addition of fresh VM medium supplemented with 20 g L^{-1} glucose and or not (for control cultivation) 7 mM pCA by a peristaltic pump. To maintain the inside volume constant a mechanical drain constantly removed fermentative broth from the bioreactor for an appropriated vessel. Cultures were assumed to be in steady-state (SS) when, after at least five-volume changes after the latest perturbation, the culture dry weight and the specific carbon dioxide production rate varied less than 2% over volume changes.

7.4. RESULTS AND DISCUSSION

7.4.1. Anaerobic glucose-limited chemostat of SA-1 in the presence of pCA

Phenolic compounds generated and released during hydrolysis can inhibit yeast metabolism. A wide variety of aromatic compounds are formed from lignin, which is partially degraded during the pretreatment step of the lignocellulosic material (Larsson et al. 2000). Because of the diverse nature of phenolic compounds, they present a significant challenge. And, despite that, they are the least studied and understood of all of the inhibitors present in lignocellulosic materials (Adeboye et al. 2015).

In the present study, we focused on the severity of one phenolic compound, pCA, on *S. cerevisiae* SA-1 physiology in a glucose-limited chemostat under anaerobic conditions. When comparing the data obtained with SA-1 strain cultivated in the presence of 7 mM pCA and the absence of pCA (control), we observed an increase in consumption and production rates, such as for glucose (26%), CO_2 (12%), and ethanol (53%). On the other hand, we observed a decrease in biomass yield (22%), and the glycerol production rate (19%) (Table 7.1). These results demonstrated that the carbon source of anaerobic glucose-limited chemostat cultures of the SA-1 strain is mainly diverted to ethanol and CO_2, and minor amounts of glycerol, lactic and acetic acids, with a concomitant formation of yeast biomass (Table 7.1). The presence of 7 mM pCA did not influence cell growth rate.

Table 7.1. Physiology of *S. cerevisiae* SA-1 strain in glucose-limited anaerobic chemostats in the presence and absence of 7 mM pCA at a dilution rate of 0.1 h^{-1}. Specific rates (q) are given in mmol g^{-1} h^{-1}, μ in h^{-1}, X in g DW L^{-1}, $Y_{X/S}$ in g DW g glucose^{-1}, $Y_{Eth/S}$ in g ethanol^{-1} g glucose^{-1}, and C recovery in (%). Data is the average values of duplicate experiments ± deviation of the mean.

Parameters \ Conditions	SA-1 (control)	SA-1 (7 mM p-coumaric acid)
μ	0.38 ± 0.02	0.37 ± 0.03
Residual glucose	0.69 ± 0.05	0.69 ± 0.22
q glucose	-5.80 ± 0.05	-7.34 ± 0.50
q CO2	9.82 ± 1.01	11.04 ± 1.03
q ethanol	8.66 ± 0.34	13.27± 1.17
q glycerol	1.04 ± 0.07	0.84 ± 0.24
q lactate	0.08 ± 0.00	0.06 ± 0.03
q pyruvate	0.02 ± 0.00	0.03 ± 0.02
q acetate	0.00 ± 0.00	0.000 ± 0.000
X	2.645 ± 0.007	2.145 ± 0.02
$Y_{X/S}$	0.11 ± 0.00	0.09 ± 0.00
$Y_{Eth/S}$	0.38 ± 0.02	0.46 ± 0.01
C recovery	98.51 ± 3.35	100.761 ± 0.01

Significant reduction in maximum specific growth rate (reducing from 0.41 ± 0.07 h^{-1} for the control cultivation to 0.29 ± 0.02 h^{-1}) and increase in glycerol production rate (from 0.08 ± 0.006 g g^{-1} to 0.12 ± 0.002 g g^{-1}) of *S. cerevisiae* CEN.PK113-7D was observed by Adeboye et al. (2015) during aerobic growth in batch cultures performed in a bioreactor using yeast minimal mineral medium containing or not (control) 9.7 mM pCA. In a previous study, the authors observed that the growth rate is significantly reduced in a dose-dependent manner (Adeboye et al. 2014). Some studies have shown that biomass yield, growth rate, and ethanol productivity are generally more decreased than ethanol yields of *S. cerevisiae* in the presence of phenols (Klinke et al. 2004; Almeida et al. 2007).

Interestingly, Adeboye and coauthors (2015) observed an *in situ* detoxification of some phenolic compounds. According to them, *S. cerevisiae* CEN.PK113-7D is capable of slow *in situ* catabolic conversion of 9.7 mM pCA under aerobic batch cultivations. They observed the complete conversion of pCA into other phenolic compounds throughout 72 h (Adeboye et al. 2015). We found out that under anaerobic glucose-limited chemostat cultivations pCA is not metabolized by *S. cerevisiae* SA-1, remaining in the fermentative broth. (Fig. 7.1). Larsson et al. (2000) also showed that *S. cerevisiae*, baker's yeast, could convert some inhibitory phenolics to fewer toxic compounds in fermentations performed under oxygen-limited conditions. The authors have revealed that pCA at 1 g L^{-1} decreased growth rate and ethanol productivity to approximately 63%

of the reference fermentation. Biomass and ethanol yield, however, was comparable to the reference (Larsson et al. 2000).

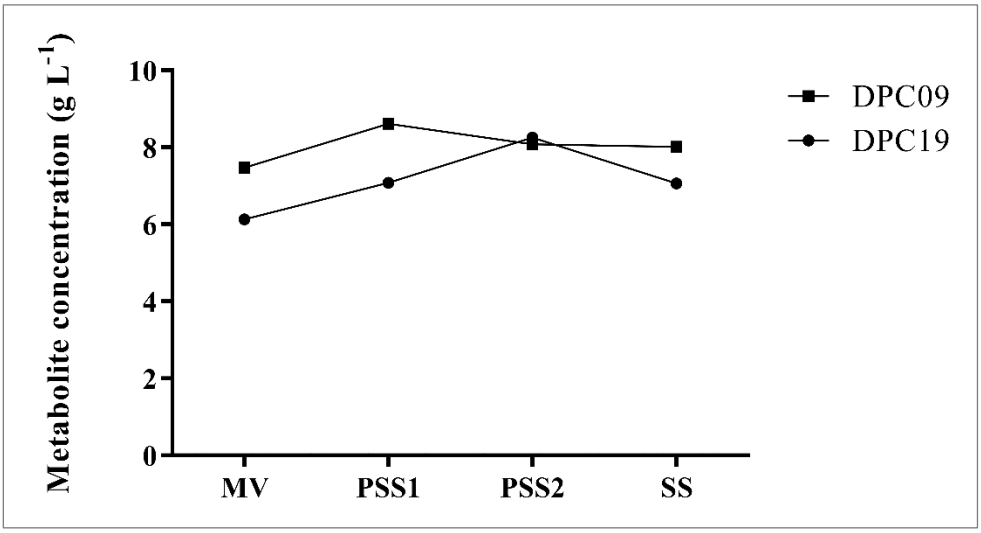

Fig. 7.1. *p*-Coumaric acid concentration during anaerobic glucose-limited chemostat cultivations of *S. cerevisiae* SA-1 strain. MV, medium vessel; PSS1, first pre-steady-state; PSS2, second pre-steady-state; SS, steady-state. DPC09 and DPC19 are the codes for the duplicate runs.

It is possible to notice a variation of the pCA concentration between MV and SS samples for both cultivations, probably resulting from insolubility at higher concentrations. Despite that variation, it did not characterize a consumption by the yeast strain. In other words, under anaerobic conditions, *S. cerevisiae* SA-1 was unable to metabolize pCA compound unlike those results found by Adeboye et al. (2015, 2017) for CEN.PK113-7D cultivated under aerobic batch fermentation, that the endogenous catabolism of pCA led to *in situ* detoxification of it through a process in which this phenolic compound was converted into a less inhibitory compound. They hypothesized that *ALD5*, *PAD1*, *AFT1*, and *AFT2* played significant roles in the catabolism of some phenolic compounds, including pCA (Adeboye et al. 2017). Phenolic compounds have been suggested to affect the integrity of cell membranes, thereby affecting their ability to serve as selective barriers and enzyme matrices (Palmqvist and Hahn-Hägerdal 2000). As related and observed, the phenolic compounds affect the metabolism of fermentative organisms (Adeboye et al. 2015).

The inhibitory effect of phenolic compounds represents a challenge for second-generation biofuel It was reported that the removal of phenolic compounds from the hydrolysate before fermentation with *S. cerevisiae* results in a substantial improvement of the fermentability (Larsson et al. 1999, 2000; Jönsson et al. 2013). Therefore, it is important to understand the cell response to

individual stress factors for reprogramming supporting pathways of in situ detoxification to withstand the stress.

7.5. CONCLUSION

During pretreatment, a diverse array of phenolic compounds is released into the hydrolysates from the depolymerization of lignin. The nature of them varies depending on the pretreatment method and the nature of the biomass (Palmqvist and Hahn-Hägerdal 2000). Among the phenol compounds derivatives from lignocellulose hydrolysis, pCA represents the majority of the total composition (van der Pol et al. 2014). From the results present in this study it could be concluded that the presence of pCA in anaerobic glucose-chemostat cultivations influenced the yeast metabolism, as observed in the increase of glucose consumption ethanol production rate. Interestingly, it was not observed a bioconversion of this phenolic compound into a less inhibitory compound.

7.6. REFERENCES

Adeboye PT, Bettiga M, Aldaeus F, et al (2015) Catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid by *Saccharomyces cerevisiae* yields less toxic products. Microb Cell Fact 14:149. https://doi.org/10.1186/s12934-015-0338-x

Adeboye PT, Bettiga M, Olsson L (2017) ALD5, PAD1, ATF1 and ATF2 facilitate the catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid in *Saccharomyces cerevisiae*. Sci Rep 7:1–13. https://doi.org/10.1038/srep42635

Adeboye PT, Bettiga M, Olsson L (2014) The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates. AMB Express 4:2–10. https://doi.org/10.1186/s13568-014-0046-7

Almeida JRM, Modig T, Petersson A, et al (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae. J Chem Technol Biotechnol 82:340–349. https://doi.org/10.1002/jctb.1676

Fiorentino A, Gentili A, Isidori M, et al (2003) Environmental effects caused by olive mill wastewaters: toxicity comparison of low-molecular-weight phenol components. J Agric Food Chem 51:1005–1009. https://doi.org/10.1021/jf020887d

Jönsson LJ, Alriksson B, Nilvebrant N-O (2013) Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol Biofuels 2013 61 6:1–10. https://doi.org/10.1186/1754-6834-6-16

Keweloh H, Weyrauch G, Rehm H-J (1990) Phenol-induced membrane changes in free and immobilized *Escherichia coli*. Appl Microbiol Biotechnol 1990 331 33:66–71. https://doi.org/10.1007/BF00170572

Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66:10–26. https://doi.org/10.1007/s00253-004-1642-2

Kłosowski G, Mikulski D (2021) Impact of lignocellulose pretreatment by-products on *Sachharomyces cerevisiae* strain ethanol red metabolism during aerobic and anaerobic growth. Mol 2021, Vol 26, Page 806 26:806. https://doi.org/10.3390/MOLECULES26040806

Larsson S, Palmqvist E, Hahn-Hägerdal B, et al (1999) The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb Technol 24:151–159. https://doi.org/10.1016/S0141-0229(98)00101-X

Larsson S, Quintana-Sáinz A, Reimann A, et al (2000) Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by *Saccharomyces cerevisiae*. In: Twenty-First Symposium on Biotechnology for Fuels and Chemicals. Humana Press, Totowa, NJ, pp 617–632

Liu ZL (2011) Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. Appl Microbiol Biotechnol 90:809–825. https://doi.org/10.1007/s00253-011-3167-9

Luttik MA, Kötter P, Salomons FA, et al (2000) The *Saccharomyces cerevisiae* ICL2 gene encodes a mitochondrial 2-methylisocitrate lyase involved in propionyl-coenzyme A metabolism. J Bacteriol 182:7007–13

Palmqvist E, Hahn-Hägerdal B (2000a) Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. Bioresour. Technol.

Palmqvist E, Hahn-Hägerdal B (2000b) Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. Bioresour. Technol.

van der Pol EC, Bakker RR, Baets P, Eggink G (2014) By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels. Appl Microbiol Biotechnol 98:9579–9593. https://doi.org/10.1007/s00253-014-6158-9

Verduyn C, Postma E, Scheffers WA, Van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8:501–517. https://doi.org/10.1002/yea.320080703

CHAPTER VIII: POLYMER SYNTHESIS BY XYLOSE-ACETATE-CONSUMING *SACCHAROMYCES CEREVISIAE* STRAIN

Polymer synthesis by xylose-acetate-consuming *saccharomyces cerevisiae* strain

ABSTRACT

The growing amount of plastic waste coming from daily life has received considerable attention since the high number of synthetic plastics used for an extensive range of activities have impacted our natural environment. In this work, we present a bio-based polymer produced by a xylose-acetate-utilizing *Saccharomyces cerevisiae* strain, SR8A6S3, when fermenting glucose, xylose, and acetate. Further work is needed to investigate specific applications of this extracellular material produced from those cultivations. But the results presented so far indicated that SR8A6S3 represents a potential host strain to produce bio-polymer

Keywords: *Saccharomyces cerevisiae*; polymer; xylose; acetate.

8.1. INTRODUCTION

Industrial polymers are, in the main, petrochemical-based. Thereby, the risk of exhausting the supply of fossil fuels (Kirsch 2020), sustainable biological routes for non-petrochemical renewable polymers should be considered (Byrom 1987). Yeast *Saccharomyces cerevisiae* is an important host for the chemical industry since, through the fermentative process, this microorganism can produce some chemical building blocks at reduced costs chemicals (Borodina and Nielsen 2014). In the expected transition from fossil to renewable resources, several companies produce are working on developing a fermentation process to produce biopolymers (Vink et al. 2004; Borodina and Nielsen 2014). Among microbially produced polymers, polysaccharides, as xanthan gum were probably the first one that was considered for industrial use (Byrom 1987).

S. cerevisiae yeast is an ideal host microorganism for industrial chemical production because it offers advantages including no phage contaminations, easier separation, suitability in large-scale fermentation, and generally higher tolerances. This use has been used extensively for genetic manipulation for producing heterologous compounds (Curran et al. 2013). In designing cell factories, redirection of metabolic fluxes is achieved by manipulating the host genome to increase the activity of the enzymes leading toward the product of interest, through modulating native expression, or by the expression of heterologous genes or synthetic genes and proteins (Borodina and Nielsen 2014). When expressing heterologous genes, it is useful to test multiple gene variants in the face of different environmental characteristics, as medium composition, or concentration of dissolved oxygen available.

The present study was conducted to explore the potential of a published strain SR8A6S3 (Zhang et al. 2016) for polymer production under different conditions. So far, the resulting polymer was not characterized, however, the database presented in this work represents an initial step to understand the process of this polymer production. Future work is necessary for its identification as well as its potential for industrial application.

8.2. GOALS

The present proposal aimed to evaluate polymer production by xylose-acetate-consuming strain, SR8A6S3, previously published (Zhang et al. 2016). Several conditions were considered to investigate the best way by which the target microorganism produces extracellular material.

8.3. MATERIALS AND METHODS

8.3.1. Strains and media

All *S. cerevisiae* strains used in this work are summarized in Table 8.1. Yeast cells were grown in yeast extract-peptone (YP) medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) containing glucose (YPD), xylose (YPX), acetate (YPA), or a mixture of glucose, xylose, and acetate (YPDXA) at 30 °C for fermentation experiments.

Table 8.1. Yeast strains used in this study.

Strain	Description	Mating-type	Reference
SR8	Efficient xylose-consuming strain (evolved strain of D452-2 *leu2::LEU*_[RS305_TDH3$_p$_XYL1_TDH3$_T$ *ura3::URA3*_pRS-X123 *his::HIS*_pRS3-X123, and *ald6::AUR1-C* pAUR_d_ALD6)	Mat**a**	(Kim et al. 2013)
SR8A6S3	SR8 expressing three copies of COadhE overexpression cassette and three copies of mutant Salmonella ACS gene overexpression cassette	Mat**a**	(Zhang et al. 2016)
SR8A6S3-CDT$_2$	SR8A6S3 expressing one copy of CDT2 overexpression cassette	Mat**a**	This work

8.3.2. Fermentation and analytical methods

Anaerobic and aerobic batch fermentation experiments were performed by inoculating yeast cells grown on YPD into 30 mL fermentation media (YP medium containing 20 g L^{-1} glucose and 80 g L^{-1} xylose and 8 g L^{-1} acetates). Initial cell densities were OD_{600} of ~ 1. For anaerobic conditions, a serum bottle sealed with butyl rubber stoppers was used to ensure strict anaerobic conditions. The serum bottles with fermentation media were then flushed with nitrogen gas, which had passed through a heated, reduced copper column to remove the trace of oxygen. Microaerobic batch fermentation experiments were performed using 30 mL of fermentation media (YP medium containing 20 g L^{-1} glucose and/or 80 g L^{-1} xylose and/or 8 g L^{-1} acetate) in a 125 mL flask with initial cell densities OD_{600} of ~ 1. Anaerobic and microaerobic batch fermentations were performed at 30 °C and 100 rpm conditions. Samples were taken at appropriate intervals for microscope analysis (Zeiss Axio Imager A1, Serien-Nr: 3517002128).

8.4. RESULTS

8.4.1. The acetate reduction pathway enables polymer production by *S. cerevisiae*

In a previous report, a xylose-utilizing *S. cerevisiae* strain, SR8, was engineered by the locus-specific integration of the two copies of the acetylating acetaldehyde dehydrogenase (AADH) gene from *E. coli* and two copies of the acetyl CoA synthetase enzyme (*ACS*) gene from *S. enterica* on the *ALD6 locus*, yielding SR8A6S3 (Zhang et al. 2016). From SR8A6S3, another strain was constructed, SR8A6S3-CDT_2 by the locus-specific integration of the transporter CDT-2 gene cassette on the *SOR1* gene, more details of these strains are described in Chapter V.

We carried out aerobic, anaerobic, and micro-aerobic fermentation to evaluate acetate consumption capacities of the above-mentioned strains (SR8, SR8A6S3, and SR8A6S3-CDT_2) in YPDXA medium (YP medium containing 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate) for 72h, in biological triplicates. Under aerobic and anaerobic batch fermentations any strain did not produce any extracellular material (data not shown). It was therefore surprising that extracellular polymer production was observed from micro-aerobic cultivation of SR8A6S3 and SR8A6S3-CDT_2 (Fig. 8.1.). Thus, reduced amounts of dissolved oxygen should be present in the medium to promote this extracellular material formation. Fig. 8.2. shows further electron microscope pictures of the microbial biopolymer produced by SR8A6S3 under micro-aerobic cultivation in YPDXA medium.

Strains SR8A6S3 and SR8A6S3-CDT_2 exhibited significant amounts of the extracellular material after 24 h of cultivation in which it had been possible to note at naked eyes (Fig. 8.3.). It presents as an agglomerated viscous material. Interestingly, SR8 cultivations did not present the production of any extracellular material, which makes us hypothesize that this material has resulted from the integration of the acetate reducing pathway into SR8 (Zhang et al. 2016). As well the integration of CDT-2 gene cassette into the *SOR1* gene site did not modify the way whereby the yeast produced the extracellular material, since SR8A6S3 and SR8A6S3-CDT_2 presented the same polymer production characteristics, at least under the conditions studied so far.

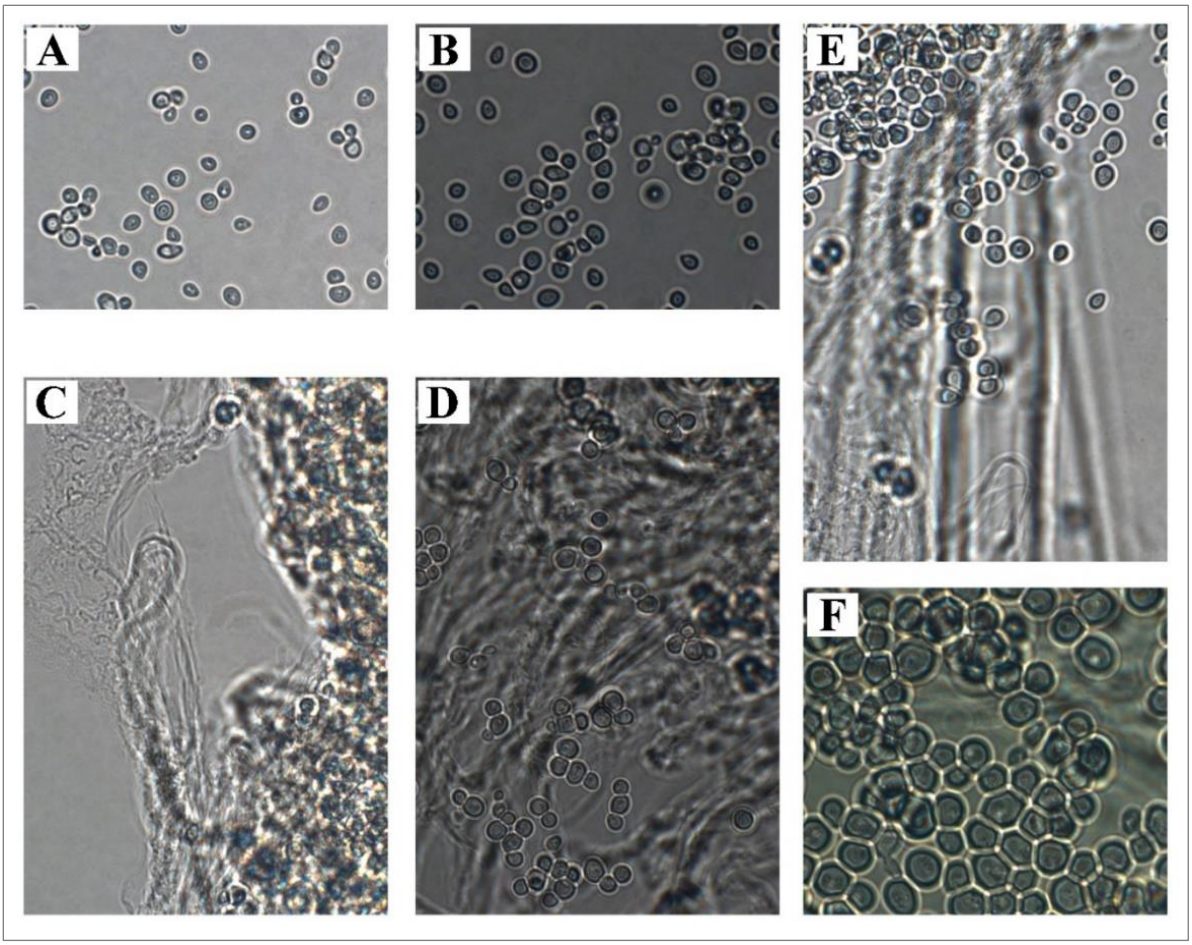

Fig. 8.1. Electron microscope view of the SR8 (A and B), SR8A6S3 (C and D), SR8A6S3-CDT_2 (E and F) cultivations at 24h (A, C and E) and 48h (B, D, and F). Imagens were captured by a 63x oil objective lens (A, B, C, D, and E) or 100x oil objective lens (F).

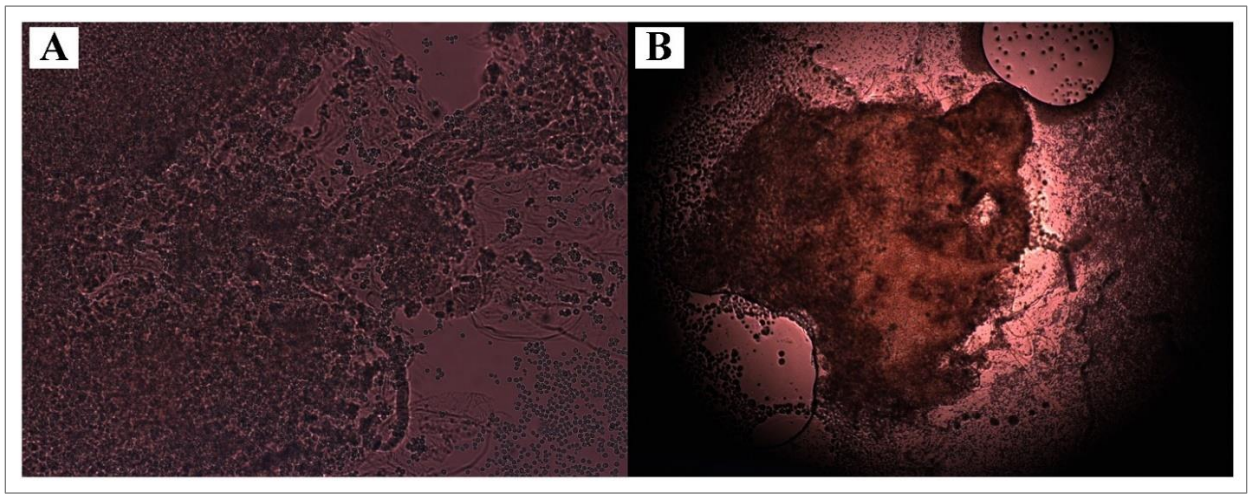

Fig. 8.2. Electron microscope view of the polymeric material produced by SR8A6S3 when cultivated in YPDXA medium under micro-aerobic condition. Imagens were captured by a 10x objective lens (A) or 5x objective lens (B).

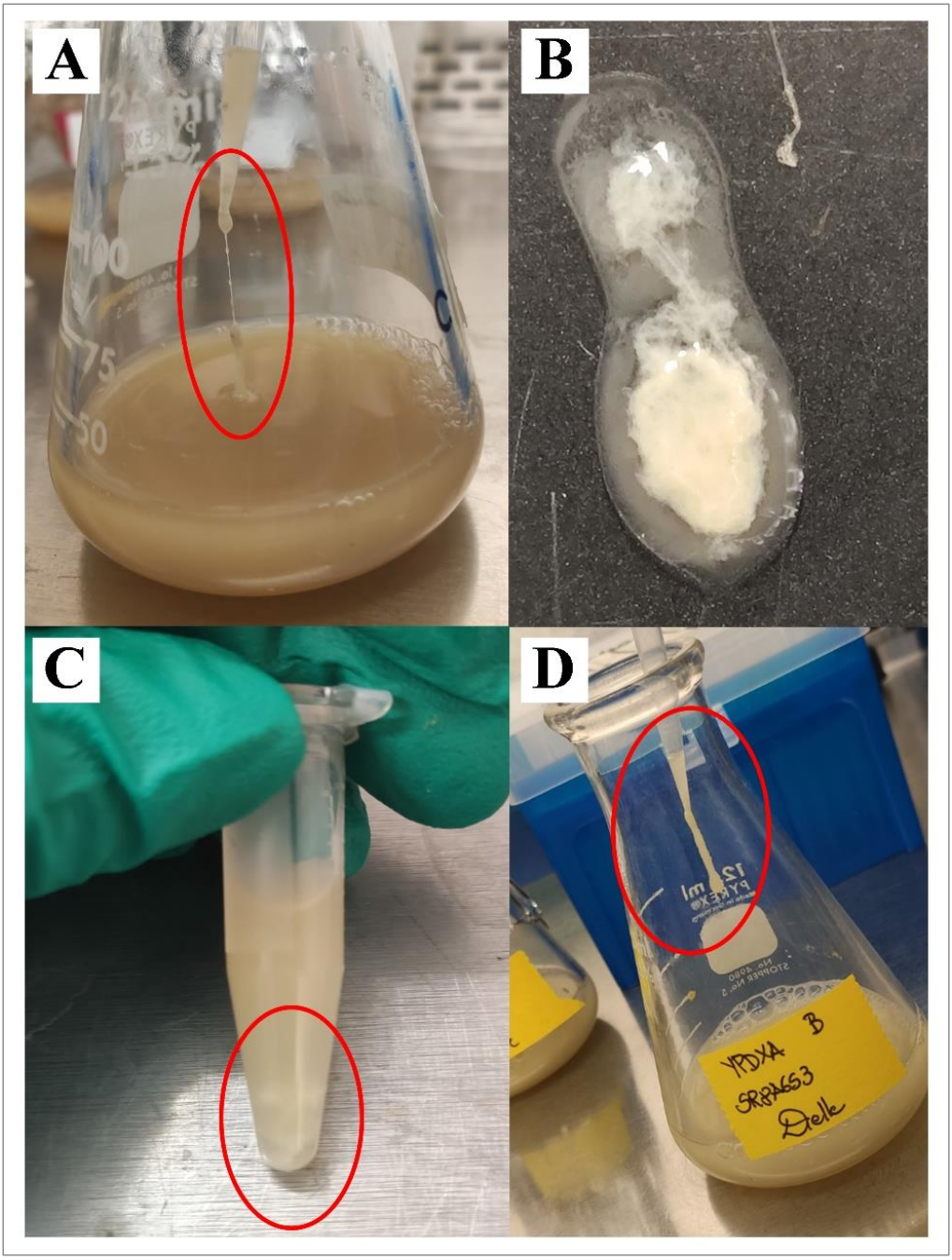

Fig. 8.3. Polymer production by SR8A6S3 when cultivated under a micro-aerobic condition in YPDXA medium (YP medium containing 20 g L^{-1} glucose, 80 g L^{-1} xylose, and 8 g L^{-1} acetate). (A), (B), (C), and (D) were taken at 48h of cultivation. In (A) and (D) is possible to see the material been removed from the fermentative broth. (B) shows a closer picture of the polymer.

From microscope images, only polymer and yeast cells can be observed. Therefore, the extracellular material observed from SR8A6S3 and SR8A6S3-CDT_2 does not come from any

culture contamination. Suspension cells of SR8A6S3 at 72h of cultivation were plated on a YPD agar plate and incubated at 30 °C until colonies grow (Fig. 8.4.)

Fig. 8.4. SR8A6S3 colonies isolated in YPD agar plate. The cell suspension came from the YPDXA cultivation at 72h.

8.4.2. Evaluating the effect of different carbon sources on polymer production by SR8A6S3

To check whether different carbon source influences the production of the polymer by the studied strains, we investigated the growth of the SR8 and SR8A6S3 in the presence of a combination of xylose, glucose, and acetate, in order to each fermentative medium contained only two carbon sources. Therefore, each strain was cultivated in YPDX medium (YP medium containing 20 g L^{-1} glucose and 80 g L^{-1} xylose), YPDA medium (YP medium containing 20 g L^{-1} glucose and 8 g L^{-1} acetate), and YPXA medium (YP medium containing 80 g L^{-1} xylose and 8

g L^{-1} acetate) for 72 h in biological triplicate. Cultivations were performed under micro-aerobic conditions since it was previously observed that only under this condition extracellular material was produced.

As expected, SR8 did not produce any extracellular material during the entire duration of the cultivation, from any fermentative medium analyzed (data not shown). On the other hand, SR8A6S3 produced the polymeric material when cultivated in YPDX and YPXA (Fig. 8.5), which was observed in the supernatant after 48 h of cultivation. It is worth highlighting that this strain produced extracellular material at 24 h of cultivation in the YPDXA medium. Therefore, the absence of acetate or glucose delayed the polymer production in 24 h. Interestingly, in the YPDA medium, we did not observe any external material (data not shown). Thereby, xylose is crucial for its production.

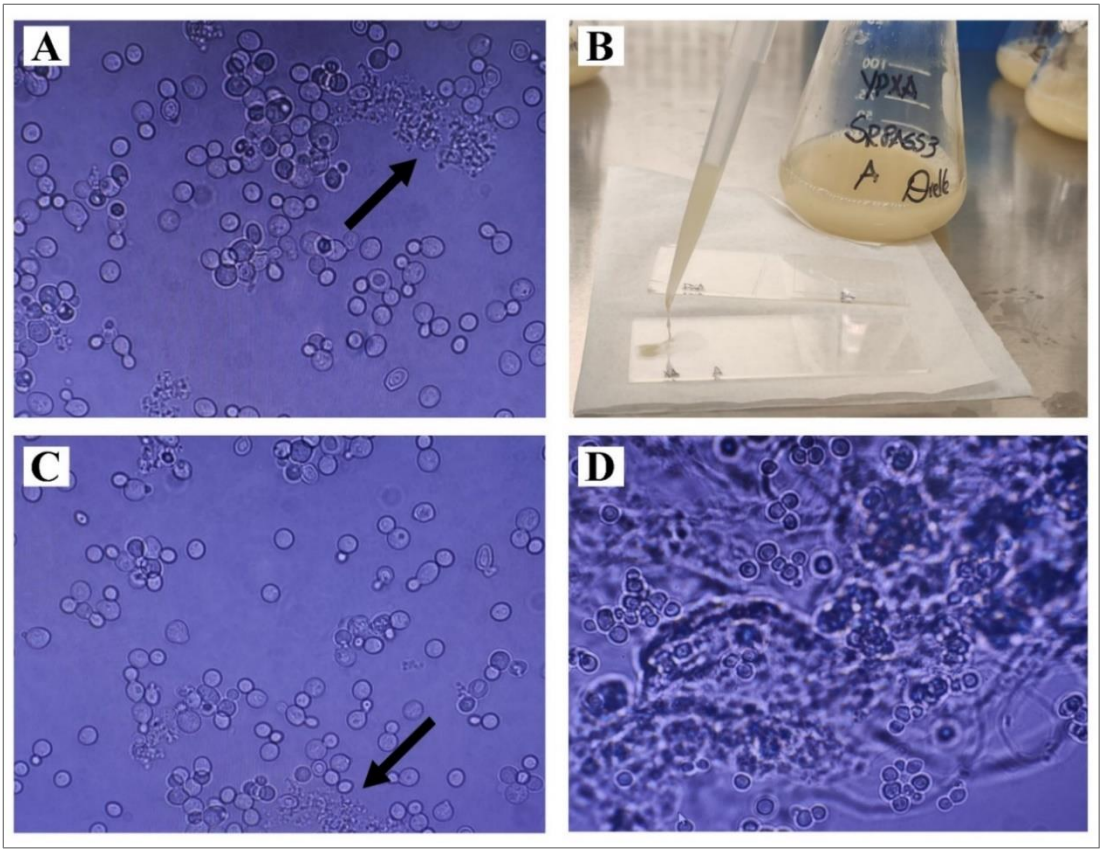

Fig. 8.5. Electron microscope view of the polymeric material produced by SR8A6S3 at 48h of cultivation in YPDX medium (A and C) and in YPXA medium (B and D) under micro-aerobic condition. All microscope images were captured by a 63x oil objective lens. (B) Removal of the extracellular material produced by SR8A6S3 when cultivated under a micro-aerobic condition in a YPXA medium.

When SR8A6S3 was cultivated in YPDX the extracellular material produced presented different properties, as shown in Fig. 8.5A and 8.5B. The viscous characteristic observed in

YPDXA and YPXA cultivations was not observed in YPDX cultivations. Further, when comparing the extracellular material produced in Fig. 8.5A or 8.5C with 8.5D, it is clear to note that the carbon source influenced the bioprocess of polymer production by the cell.

8.5. PARTIAL CONCLUSIONS

The dataset presents so far represents an initial step in the understanding of the conditions which enable the production of polymeric material from SR8A6S3. In all experiments performed in this study, we could observe that the acetate-reducing pathway which was integrated into a xylose-utilizing strain promote intracellular changes that resulted in the formation of this material under specific conditions. Furthermore, xylose must be present in the medium to allow its production.

8.6. FUTURE STUDIES

This study has demonstrated a non-previous mentioned ability of SR8A6S3 to synthesize an extracellular polymer. Further optimization of the strain could be done to further improve the extracellular polymer production. In addition, other conditions could be considered to elucidate the pathways used by the strain to metabolize this material.

8.7. REFERENCES

Borodina I, Nielsen J (2014) Advances in metabolic engineering of yeast *Saccharomyces cerevisiae* for production of chemicals. Biotechnol J 9:609–620. https://doi.org/10.1002/biot.201300445

Byrom D (1987) Polymer synthesis by microorganisms: technology and economics. Trends Biotechnol 5:246–250. https://doi.org/10.1016/0167-7799(87)90100-4

Curran KA, Leavitt JM, Karim AS, Alper HS (2013) Metabolic engineering of muconic acid production in *Saccharomyces cerevisiae.* Metab Eng 15:55–66. https://doi.org/10.1016/j.ymben.2012.10.003

Kim SR, Skerker JM, Kang W, et al (2013) Rational and evolutionary engineering approaches uncover a small set of genetic changes efficient for rapid xylose fermentation in *Saccharomyces cerevisiae*. PLoS One 8:e57048. https://doi.org/10.1371/journal.pone.0057048

Kirsch S (2020) Running out? Rethinking resource depletion. Extr. Ind. Soc. 7:838–840. https://doi.org/10.1016/j.exis.2020.06.002

Vink ETH, Rábago KR, Glassner DA, et al (2004) The sustainability of NatureWorks™ polylactide polymers and Ingeo™ polylactide fibers: an update of the future. Macromol Biosci 4:551–564. https://doi.org/10.1002/mabi.200400023

Zhang GC, Kong II, Wei N, et al (2016) Optimization of an acetate reduction pathway for

producing cellulosic ethanol by engineered yeast. Biotechnol Bioeng 113:2587–2596. https://doi.org/10.1002/bit.26021