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KATHARINA DE OLIVEIRA BARROS

Diversity, evolution, and adaptation of yeasts able to assimilate and ferment lignocellulose-related sugars

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ATA DA DEFESA DE TESE DE **KATHARINA DE OLIVEIRA BARROS**

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Às 12:00 horas do dia **05 de agosto de 2021**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora composta pelos Drs. Daniel de Assis Santos (Departamento de Microbiologia/ICB /UFMG), Boris U. Stambuk (Universidade Federal de Santa Catarina - UFSC), Dana Opulente, Marc-André Lachance (University of Western Ontario, Ontário - Canadá), o Prof. Dr. Carlos Augusto Rosa – Orientador e Chris Todd Hittinger (Coorientador) para julgar o trabalho final "Diversity, evolution, and adaptation of yeasts able to assimilate and ferment lignocellulose-related sugars", da aluna **Katharina de Oliveira Barros**, requisito final para a obtenção do Grau de **DOUTOR EM CIÊNCIAS BIOLÓGICAS: MICROBIOLOGIA**. Abrindo a sessão, o Presidente da Comissão, Prof. Dr. Carlos Augusto Rosa, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para a apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. A candidata foi considerada **APROVADA**. O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ata, que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 05 de agosto de 2021. O candidato tem 60 (sessenta) dias, a partir desta data, para entregar a versão final da tese ao Programa de Pós-graduação em Microbiologia da UFMG e requerer seu diploma.

Membros da Banca:

Prof. Dr. Daniel de Assis Santos

Dr. Boris U. Stambuk

Dra. Dana Opulente

Dr. Marc-André Lachance

De acordo:

Prof. Dr. Carlos Augusto Rosa

(Orientador)

Chris Todd Hittinger

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Profa. Danielle da Glória de Souza

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I dedicate this work to my father, Jorge Barros, who always encouraged me to read, study, travel, get to know other cultures, and, most importantly, do my best to be kind and have empathy with others. I miss him every single day and my thoughts frequently turn to my boy from Rio and "*Eterno Simpatia*". I also dedicate this dissertation to my mom.

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Abstract

Production of ethanol, other fuels and chemicals from lignocellulosic materials depend on efficient D-xylose conversion. Novel xylose-fermenting yeasts species, including the ones assigned to the genera *Spathaspora* and *Scheffersomyces*, have been described and are potential sources of genes to improve xylose fermentation in *Saccharomyces cerevisiae*. Therefore, the aims of this study were to isolate, identify, and characterize the diversity of yeasts able to assimilate lignocellulosic sugars, study the factors behind the xylose fermentation by *Spathaspora* and *Scheffersomyces* species to understand the different behaviours presented by them, and develop a strain of *Spathaspora passalidarum* able to grow in sugarcane bagasse hydrolysate to investigate the genetic basis of the adaptation. A total of 569 yeast strains were isolated from 60 rotting wood samples collected in three areas of Brazilian Amazonian rainforest. Diversity analyses indicate a dryland forest as the most diverse area and Carú, a floodplain forest, as the least. Of the 89 species identified, 52 were previously known and 37 were new. *Sugiyamaella* was the most prevalent genus identified, followed by *Kazachstania*. *Schwanniomyces polymorphus, Scheffersomyces amazonensis*, and *Wickerhamomyces edaphicus* were the most frequently isolated yeast species. Xylose fermentation assays performed with *Scheffersomyces* and *Spathaspora* species under different aeration conditions exhibited *Scheffersomyces parashehatae, Scheffersomyces xylosifermentans*, and *Sp. passalidarum* as remarkable xylose fermenters under moderate aeration condition. They achieved the highest yields of ethanol among the species tested. However, the determination of enzyme activities and cofactors usage of xylose reductase (XR) and xylitol dehydrogenase (XDH) combined with the fermentation results revealed the great negative impact of the high aeration on the xylose catabolism in these species. The findings also indicate that the degree of the enzyme affinity by the cofactor is extremely important for the generation of ethanol. In the third part of this work, ultraviolet-induced mutagenesis followed by adaptive laboratory evolution (ALE) were undertaken to adapt *Sp. passalidarum* to the inhibitors present in hydrolysates, especially acetic acid. The mutant MT01 showed improved growth in defined medium with xylose and acetic acid. It was subjected to ALE and the evolved strains ME3.5.5 and MEH30.1 were able to grow in sugarcane bagasse hydrolysate (SBHH). Four genes contain disruptive heterozygous mutations, including *CYR1* that encodes adenylate cyclase, and they are candidates for causing increased growth in SBHH.

Keywords: Yeasts, Diversity, Brazilian Amazonia, Rotting-wood, Lignocellulose, Hydrolysates, Xylose, Fermentation, Aeration, *Spathaspora*, *Scheffersomyces*.

Resumo

A produção de etanol a partir de materiais lignocelulósicos depende da conversão eficiente da pentose D-xilose. Novas espécies de leveduras fermentadoras de xilose, incluindo aquelas atribuídas aos gêneros *Spathaspora* e *Scheffersomyces*, foram descritas e são potenciais doadoras de genes para melhorar a fermentação de xilose em *Saccharomyces cerevisiae*. Os objetivos deste estudo foram isolar, identificar e caracterizar a diversidade de leveduras capazes de assimilar açúcares lignocelulósicos, estudar os fatores por trás da fermentação da xilose de espécies pertencentes aos gêneros *Spathaspora* e *Scheffersomyces* para entender os diferentes comportamentos apresentados por elas, e desenvolver uma linhagem de *Spathaspora passalidarum* capaz de crescer em hidrolisado de bagaço de cana de açúcar para investigar a base genética da adaptação. Um total de 569 isolados foram obtidos a partir de 60 amostras de madeira em decomposição coletadas em três áreas da Floresta Amazônica Brasileira. As análises de diversidade mostraram UFAM (terra firme) como a área com maior diversidade e Carú (floresta de várzea) apresentou a menor diversidade. Das 89 espécies identificadas, 52 são espécies descritas e 37 são possíveis espécies novas. *Sugiyamaella* foi o gênero mais prevalente, seguido por *Kazachstania. Schwanniomyces polymorphus*, *Scheffersomyces amazonensis* e *Wickerhamomyces edaphicus* foram as espécies de leveduras mais frequentemente isoladas. Na segunda etapa deste estudo, ensaios de fermentação de xilose realizados com as espécies *Scheffersomyces* e *Spathaspora* sob diferentes condições de aeração mostraram *Scheffersomyces parashehatae, Scheffersomyces xylosifermentans* e *Sp. passalidarum* como potenciais fermentadoras de xilose em condição moderada de aeração. Elas alcançaram os maiores rendimentos de etanol entre as espécies testadas. No entanto, a determinação das atividades enzimáticas da xilose redutase (XR) e xilitol desidrogenase (XDH) combinada aos resultados da fermentação revelou o grande impacto negativo da alta aeração no metabolismo da xilose por essas espécies. Os resultados dos experimentos também indicam que o grau de afinidade da enzima pelo cofator é extremamente importante para a geração do etanol. Na terceira parte deste trabalho, mutagênese induzida por ultravioleta e evolução adaptativa foram realizadas para adaptar *Sp. passalidarum* aos inibidores presentes no hidrolisado de bagaço de cana de açúcar, principalmente ao ácido acético. O mutante MT01 apresentou crescimento em meio definido suplementado com ácido acético e então foi submetido a evolução adaptativa. As linhagens evoluídas ME3.5.5 e MEH30.1 foram capazes de crescer em hidrolisado de bagaço de cana de açúcar. Foram encontradas mutações heterozigotas em quatro genes, incluindo *CYR1*, que codifica a adenilato ciclase. Esses genes podem ser responsáveis pelo desempenho das linhagens evoluídas no hidrolisado de bagaço de cana de açúcar.

Palavras-chave: Leveduras, Diversidade, Amazônia Brasileira, Madeira em decomposição, Lignocelulose, Hidrolisado, Xilose, Fermentação, Aeração, *Spathaspora*, *Scheffersomyces*.

FIGURE LIST

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1. INTRODUCTION

Fossil fuels are still the major source of energy and the demand keeps increasing according to the International Energy Agency (IEA, 2020). Environmental changes in response to the high consumption and the depletion of non-renewable fuels sources in addition to the instability of the oil market have influenced the search for renewable energy sources. Second-generation ethanol (lignocellulosic ethanol) is an eco-friendly fuel and it has potential to reduce the emission of greenhouse gases (Mat Aron et al., 2020). This type of ethanol can be generated from lignocellulosic biomass such as forestry or agricultural residues. For example, sugarcane bagasse is abundant in Brazil and its surplus could potentially be used for this purpose. Thus, efforts have been made to optimize the fermentation process to obtain 2G ethanol on a large scale.

A challenge related to an advantageous production involves the lack of robust microorganisms to utilize the sugar content from the lignocellulosic biomass, especially xylose, the second most sugar in this substrate. Species from the genera *Spathaspora* and *Scheffersomyces* such as *Spathaspora passalidarum* and *Scheffersomyces stipitis* have been described as good xylose-fermenting yeasts when they grow in defined medium, but their fermentative performance in sugarcane bagasse hydrolysates is poor. Another challenge is the steps to processing the raw material. Dilute acid pretreatment is cheaper and the processing time is shorter compared to other methods, but the concentration of microbial inhibitors released is high. Native xylose-fermenting yeasts resistant to toxic compounds are relevant to use this type of pretreatment without additional steps of detoxification. Evolutionary experiments and random mutagenesis were used to develop tolerant xylose native fermenters to acetic acid and furfural (Morales et al., 2017; Senatham et al., 2016; Biazi et al., 2021). Such studies were limited to consideration of the conversion and growth rates and did not consider the genetic

basis of this tolerance. This knowledge would yield insight into the possible genetic modification strategies to improve the performance of xylose fermenters in hydrolysates.

Within the genera *Spathaspora* and *Scheffersomyces* some species successfully conduct the pathway to the xylulose-5-P, which enters into the central carbon metabolism and ethanol is produced as the main product of fermentation. Other species get stuck into the first step, accumulating xylitol. Redox imbalance has been suggested as the main obstacle for the pathway in anaerobic or oxygen-limiting conditions (Moysés et al., 2016). But some species accumulate xylitol independent of the aeration level and others cannot even utilize the sugar. Except for *Spathaspora arborariae, Sp. passalidarum*, and *Sch. stipitis*, little is known about the xylose trait of the other members of the genera and the impact of aeration level on their XR/XDH. Why some species vigorously use the pentose and other species bearing the same necessary machinery for xylose fermentation do not display the same potential is a broad question that the answer remains unclear.

Yeasts naturally able to ferment xylose have been isolated from different biomes of Brazil. Brazilian Amazonia plays crucial roles in biodiversity conservation and carbon storage. Works related to yeast diversity in the region have identified potential new species with ability to assimilate/ferment lignocellulose-related sugars and provided information about their occurrence, for example, in rotting wood (Souza et al., 2017; Cadete et al., 2012). This substrate is a promising environment for the isolation and description of novel yeast species. However, these works used a defined medium as isolation media. There is only one study which used sugarcane bagasse hydrolysate to isolate the yeasts (Morais et al., 2020). Distinct types of hydrolysate in addition to minimal media can be a strategy to increase the number of different species sampling and the number of species up to grow in hydrolysates.

Therefore, the overall aims of the present work were to evaluate the diversity of the community yeasts associated with rotting wood from Brazilian Amazonian rainforest, isolate

new species as well, increase our comprehension about xylose trait and its constraints, and develop a native xylose-fermenting yeast with ability to grow in sugarcane bagasse hydrolysate to evaluate the genetic basis of the adaptation.

2. LITERATURE REVIEW

2.1. Biofuels

High consumption of fossil fuels, its limited supply and consequences for the environment have driven the search for sustainable fuels (Anderson et al., 2020). First generation (1G) biofuels - such as bioethanol produced from sugarcane and corn starch, and biodiesel from oil-seed crops - stand out as renewable energy and as an alternative to displace the fossil fuels (Baig et al., 2019). However, they are generated from cultivable crops that demand high investments in the production of the raw materials and creates a competition for lands that could be used for food production (Bezerra and Ragauskas, 2016; Nosrati-Ghods et al., 2018). Unlike the 1G, 2G biofuels are made from florestal or agricultural residues and do not compete for farmlands (Herrmann et al., 2018). Lignocellulose is an abundant raw material on the planet and a potential source of renewable energy (Andlar et al., 2018). It is low cost, does not directly use food crops and, consequently, does not require investments in areas for planting and agricultural inputs (Maga et al., 2019). This feedstock can be used to produce, for example, 2G ethanol which is one of the most important renewable fuels. Developing an efficient conversion of lignocellulose into biofuels has become a world priority to create an environmentally friendly production of this energy at feasible costs (Kumar et al., 2020; Herrmann et al., 2018). Therefore, sustainable processing of lignocellulosic materials presents a wide field for further development. A broad variety of raw materials can be used, including sugarcane bagasse, rice straws, corn cobs, switchgrass (Tayyab et al., 2018). Since they are abundant substrates and can be converted into ethanol, it is possible to produce this biofuel on large scales and at low cost (Branco, Serafim and Xavier, 2019; Tayyab et al., 2018).

2.2. Lignocellulosic biomass: structure and composition

Lignocellulose is the largest structural part of plants and consists of cellulose (35-55%), hemicellulose (15-30%) and lignin (2-20%), in addition to other constituents in smaller amounts, such as pectin, fatty acids and minerals (Wyman et al., 2019). The concentrations of these components differ according to the type of plant (Isikgor and Becer, 2018). Cellulose is the main component of lignocellulosic biomass, being a polysaccharide that presents as a linear glucose polymer and exhibits a high degree of polymerization (Andlar et al., 2018). Cellulose consists exclusively of β-D-glucopyranose units linked by β-1,4 glycosidic bonds, and intermolecular hydrogen bonds are also present allowing the cellulose to have a rigid structure (Chen et al., 2019). This structure is formed by more organized regions, called crystalline regions, and amorphous portions which are less ordered (Kulasinski et al., 2014; Ravindran and Jaiswal, 2016).

Hemicellulose is a highly branched heteropolymer of hexoses (D-mannose, D-galactose and D-glucose) and pentoses (such as D-xylose and L-arabinose) joined by β-1,4 glycosidic bonds (Prajapati et al., 2018; Kumar and Shukla, 2016). It also contains carboxylic acids, including 4-O-methylglucuronic acid and D-glucuronic acid (Ravindran and Jaiswal, 2016). The composition of hemicelluloses varies according to the type of the plant, and their classification is related to the main heteropolymer present in the structure like xylans, mannans and glucans (Kumar and Shukla, 2016). But in general, the hemicellulose is a backbone of xylan chains (with β -1.4 bonds between D-xylose subunits), with several branches of mannose, arabinose, galactose, glucuronic acid and others (Berglund et al., 2016). Thus, the xylan constitutes the main component of hemicellulose and xylose is the second highest sugar abundant in biomass, behind only the glucose content (Wang et al., 2018). The main role of hemicelluloses is the strengthening of the cell wall due to the interaction with cellulose through hydrogen bonds and lignin through covalent interactions. They have a low degree of

polymerization when compared to cellulose and they are completely amorphous (Wang et al., 2018; Berglund et al., 2016). Due to its heterogeneous constitution, hemicellulose is the most susceptible cell wall component to hydrolysis, which facilitates the obtainment of sugars (Prajapati et al., 2018).

Cellulose and hemicellulose are sources of fermentable sugars for industrial purposes, while the lignin can be used to generate heat and electricity (Kumar and Shukla, 2018). Other possible applications of lignin have been studied, ranging from production of chemicals and polymers to pharmaceutical products (Mármol et al., 2021; Bajwa et al., 2019). Lignin is a heterogeneous macromolecule and comprises aromatic rings linked that form an amorphous arrangement (Ralph et al., 2019). Lignification is associated with the development of vascular systems in plants, which provides resistance to degradation and environmental stresses (Ragauskas et al., 2014).

2.3. Conversion of lignocellulosic biomass into fermentable sugars

The initial step for ethanol production from lignocellulosic biomass consists of a pretreatment of the raw material by physical, chemical, physicochemical, or biological methods (Zhang et al., 2020a; Liu et al., 2019; Hassan et al., 2018). They increase the surface area of the biomass, promote the delignification with the separation of cellulose and hemicellulose fractions, and decrease the cellulose crystallinity (Robak and Balcerek, 2020).

Physical pretreatment reduces the polymerization of cellulose, making it accessible to the hydrolysis process. This procedure can be performed by mechanical reduction (by reducing particle size through grinder), freezing, microwave, and extrusion (Rajendran and Taherzadeh, 2014). Physical-chemical pretreatment methods aim at separating the lignocellulosic matrix, increasing the propensity for bioconversion (Mupondwa et al., 2017). An example of this is the Ammonia Fiber Expansion (AFEX), where the mix of biomass and ammonia (in a determined

ratio) is submitted to high pressure at 120ºC (Jin and Dale, 2018). The advantage of this process is the generation of lower concentrations of microbial inhibitor compounds (Zhang et al., 2020a).

In chemical pretreatment, acid, alkaline, ionic liquid, and organosolv are used (Robak and Balcerek, 2020). Acid pretreatment breaks the lignin structure through the solubilization of hemicellulose, and promotes an increase of cellulose accessibility before the hydrolysis (Woiciechowski et al., 2020). It is an economical and efficient approach (Robak and Balcerek, 2020). Alkaline break the heterocyclic bonds of esters between the monomers of the hemicellulose chains, causing the structural alteration of lignin, increasing the porosity of cellulose and decreasing its crystallinity (Tayyab et al., 2018; Mupondwa et al., 2017). Alkali pretreatment releases toxic compounds, but less than the acid method (Wirawan et al., 2020). Organic solvents, on the other hand, promote delignification by solubilization of lignin and it is considered eco-friendly since the solvent can be recovered by distillation and recycled for pretreatment (Tayyab et al., 2018). This method decreases the generation of inhibitors (Zhao et al., 2009). Ionic liquids are considered to be one of the most promising pretreatment approaches, despite the high price, because of the modifiable ions, great biomass solubility, low volatility, and excellent thermal stability (Robak and Balcerek, 2020).

In biological pretreatments, the lignocellulosic material is subjected to action of certain enzymes, mainly produced by filamentous fungi (Sharma et al., 2019). The primary microorganisms used mainly include white fungi e.g., *Phanerochaete chrysosporium* and *Ceriporia lacerata*); brown fungi (e.g., *Serpula lacrymans* and *Coniophora puteana*), and soft-rot fungi (e.g., *Paecilomyces* sp. and *Cadophora* spp.) (Zhang et al., 2020b). They are capable of modifying the chemical composition and structure of lignocellulosic biomass, making it more accessible to subsequent enzymatic digestion during hydrolysis. The advantages of this technique are the non-use of chemicals, low energy costs, mild operating conditions, and

greener (Abo et al., 2019; Ferdes et al., 2020). However, the efficiency is low, and microorganisms may consume not only lignin, but also polysaccharides that would be used in the fermentation step (Abo et al., 2019).

After the pretreatment stage, the hydrolysis step occurs releasing sugars that will be fermented to produce ethanol (Chen and Fu, 2016). The hydrolysis process can be carried out using diluted or concentrated inorganic acids, or enzymes (Abo et al., 2019; Kumar et al., 2016; Jung et al., 2013). Hydrolysis with dilute acid has the advantage that the reaction time is shorter. However, the temperature and pressure applied to the process are high and result in the degradation of considerable amounts of fermentable sugars (Jung et al., 2013). On the other hand, hydrolysis with concentrated acid allows lower temperature and pressure conditions. The disadvantages of this process include time-consuming and the requirement of corrosion-resistant equipment, which makes this method costly (Loow et al., 2016; Abo et al., 2019).

In biological hydrolysis, the deconstruction of the cellulose polymer chain is catalyzed by the synergistic action of the cellulase group, which comprises three types of enzymes: exo-1,4-β-D-glucanases, endo-1,4-β-D-glucanases, and 1,4-β-D-glucosidases (Sindhu et al., 2016; Malherbe and Cloete, 2002). Endoglucanases randomly cleave glycosidic bonds from the amorphous regions of cellulose, releasing the oligosaccharides. Exoglucanases (cellobiohydrolases and glucanohydrolases) act on either the reducing or the nonreducing ends of a cellulose chain to liberate cellobiose as the major product (Barruetabeña et al., 2019; Sindhu et al., 2016). Glucosidases hydrolyzed cellobiose, aryl-glucopyranosides, and cellodextrins generating glucose monomers (Kumar et al., 2016; Bezerra and Ragauskas, 2016).

Due to the heterogeneous nature of hemicellulose, it requires the participation of different types of enzymes as endoxylanases, β-xylosidases, α-arabinofuranosidases, α-glucuronidases, xylanases, phenolic acid esterases, and p-coumaric esterases (Gonçalves et al., 2015; Ali et al., 2017). The enzymes endo-1,4-β-xylanase and β-xylosidase are the main types

of xylanases responsible for degrading this xylan. In this depolymerization process, xylanases cleave the ß-1,4 bonds of the xylan into smaller oligomers - xylobiose - which are converted to xylose through the β-xylosidases enzymes (Moreira et al., 2016). These enzymes have been extensively studied and can be produced by filamentous fungi, bacteria, and yeasts. Xylanolytic yeasts include species from the *Sugiyamaella*, *Lodderomyces/Candida albicans*, *Clavispora/Candida* clades, and from the genera *Scheffersomyces, Naganishia, Aurobasidium, Kwoniella,* and *Papiliotrema* (Morais et al., 2013; Lara et al., 2014; Sena et al., 2017).

Enzymatic process requires a previous pretreatment and is carried out at milder temperatures than those applied using acids, usually ranging from 45 to 50ºC (Palacios-Bereche et al., 2018). However, the high concentration of glucose and cellobiose formed during the hydrolysis inhibits the enzymatic activity (Miao et al., 2012). To get around this problem, the saccharification process can occur simultaneously with the fermentation, a method called Simultaneous Saccharification and Fermentation (SSF) (Hazeena et al., 2019). The combination of the two reactions allows low amounts of sugars to remain in the medium due to their concomitant consumption during fermentation, which decreases the inhibition of enzymatic activity (Nguyen, Yang and Bae, 2017).

A positive feature of acid hydrolysis is that acids can penetrate in the lignin structure and promote the hydrolysis of cellulose and hemicellulose into fermentable sugars even though no pretreatment has been carried out before. This is not possible in enzymatic hydrolysis (Abo et al., 2019). Furthermore, a major limitation in the application of industrial enzymatic hydrolysis is the high cost associated with the enzymes used in the process, whether due to their purchase or production. Another negative point is the reaction time, which is still quite long (about seven days) (Sindhu et al., 2016).

2.4. Microbial inhibitors present in lignocellulosic hydrolysates

Due to the harsh conditions used in the pretreatment and hydrolysis step, the lignin and sugars present in the polymeric fractions can be degraded, leading to the formation of inhibitory substances toxic to the microorganisms (Franden et al., 2013). These inhibitors are divided into aliphatic acids, furan derivatives, and phenolic compounds (Palmqvist and Hahn-Hägerdal, 2000a). As shown in figure 1, furfural is formed by the dehydration of pentoses present in hemicellulose (D-xylose and D-arabinose) and the formation of hydroxymethylfurfural (5-HMF) occurs through the dehydration of hexoses Sjulander and Kikas et al., 2020). Phenolic compounds are produced by the cleavage of lignin; levulinic and formic acids are generated by the degradation of furan; and acetic acid by hydrolysis of the acetyl radical of the hemicellulose structure (Taherzadeh and Karimi, 2011; Palmqvist and Hahn-Hägerdal, 2000b). The concentrations and types of inhibitors present in the hydrolysate vary according to the lignocellulosic raw material used and the conditions employed in the pretreatment (Zhang et al., 2019; Yang et al., 2018; Bazoti et al., 2017).

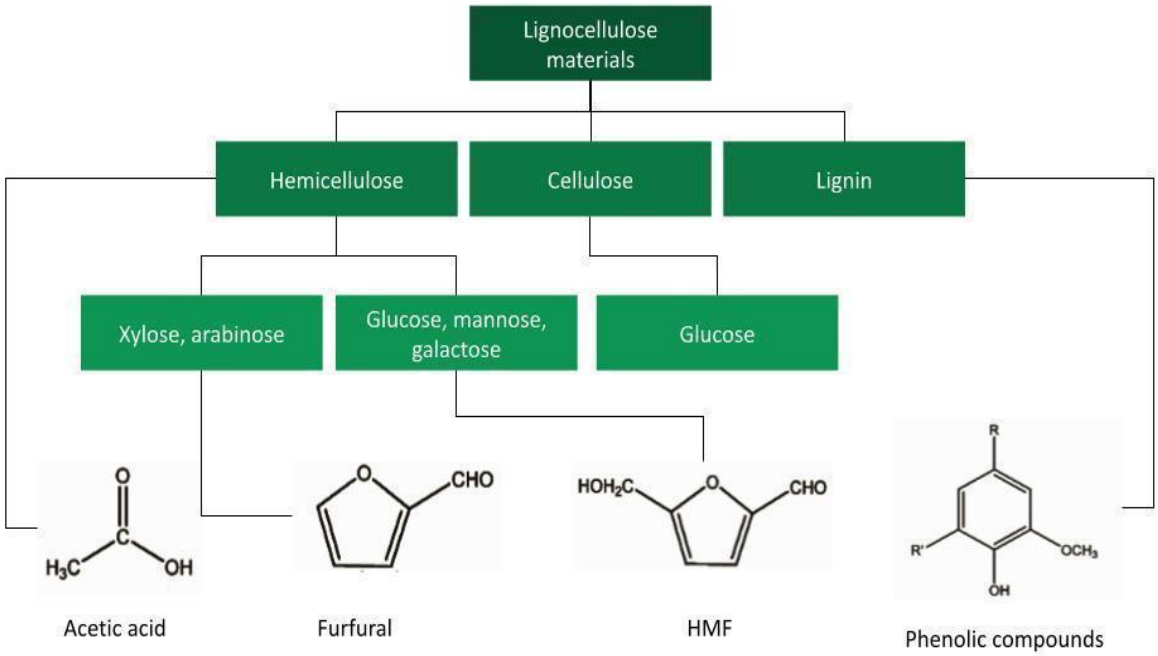

Figure 1. Inhibitory compounds commonly found in lignocellulosic hydrolysates, formed from the degradation of lignin and sugars. Adapted from Palmqvist e Hahn-Hägerdal (2000b).

As furfural and 5-HMF have an aldehyde function in the molecule, they are very reactive and can interfere with microbial growth and impair fermentation by reacting with biological molecules, causing damage to the microorganism's cell membrane (Luo et al.,2018). According to transcriptome analysis of *Zymomonas mobilis*, furfural up- or down-regulated genes involved with cell wall/membrane biogenesis, metabolism, and transcription (He et al., 2012). The mechanisms of action proposed for these compounds are the direct inhibition of enzymes (alcohol dehydrogenase, pyruvate dehydrogenase, and aldehyde dehydrogenase), and interference in the balance of cofactors due to the high requirement of NADH for the reduction of furans to their alcoholic forms, which are less inhibitory (Palmqvist and Hahn-Hägerdal, 2000b; Pietrowski et al., 2014). Phenolic compounds are molecules that have a hydroxyl group (–OH) directly attached to an aromatic ring carbon (Khoddami et al., 2013). They have low water solubility and one of their main properties is their antimicrobial effect. They act by

interfering with cell membrane integrity by increasing its permeability, in addition to inactivating enzymes essential for metabolism (Jönsson and Martín, 2016).

Acetic acid is the inhibitor present in the highest concentration in lignocellulosic hydrolysates (Morales et al., 2017; Pal et al., 2016; Ding et al., 2011). Its undissociated form is hydrophobic and therefore it is able to penetrate the cytoplasmic membrane of the microorganism (Kumar, Cheon and Kim, 2014). It dissociates and leads to the accumulation of acetate and protons with consequent acidification of the cytosol, lowering the local pH (Guan and Liu, 2020). The availability of ATP decreases due to its use by ATPases to remove excess protons formed by the dissociation of acids within the cell. Consequently, the active transport of nutrients is impaired and, therefore, so are the kinetics of microbial growth (Kumar, Cheon and Kim, 2014; Morales et al., 2017).

The presence of inhibitors interferes in the fermentation process, decreasing the ethanol yield and productivity by the microorganism (Morales et al., 2017; Pal et al., 2016). The inhibition provided by the mixture of these compounds in the hydrolysate may be the result of a synergistic effect between them, which plays a greater inhibitory role than the presence of a single toxic compound (Jönsson and Martín, 2016). Although detoxification can be performed to decrease the concentration of inhibitors, this is a step that involves an additional cost (Pan et al., 2019; Morales et al., 2017; Pal et al., 2016; Harner et al. 2014). Importantly, approaches such as random mutagenesis and adaptive laboratory evolution (ALE) have been employed in natural xylose-fermenting yeasts to increase tolerance to hydrolysate inhibitors (Biazi et al., 2021; Morales et al., 2017; Hou and Yao, 2012).

2.5. Transport and metabolism of D-xylose

Uptake of xylose into the yeast cell is essential for ethanol production (Zhao et al., 2020). It can occur by facilitated diffusion (passive transport), with the concentration gradient and without energy consumption (Wang et al., 2016). Xylose uptake may also happen by active transport

with energy consumption (active transport), against the concentration gradient and mediated by specific transporter proteins which generally carry protons with sugar in the same direction (symport) or in the opposite direction (antiport) (Hahn-Hägerdal et al., 2007; Li, Schmitz and Alper, 2016). Transport of pentoses in *S. cerevisiae* occurs by passive transport mediated by native HXT hexose transporters (Patiño et al., 2019; Verhoeven et al., 2018). More than twenty genes (*HXT1* to *HXT17*, *GAL2*, and the glucose sensors *SNF3* and *RGT2*) are involved in the transport of hexoses in this species (Nijland et al., 2020; Roy et al., 2016). Although *S. cerevisiae* is not able to ferment pentoses, the hexose permeases Hxt4, Hxt5, Hxt7 and Gal2 are capable of transporting xylose and/or arabinose (Wang et al., 2016). However, these transporters have different affinities for different substrates and the affinity for glucose over pentoses is always greater (Apel et al., 2016).

Scheffersomyces stipitis and *Scheffersomyces shehatae*, two native xylose-fermenting yeasts, have two xylose transport systems: facilitated diffusion transporters (low-affinity) and proton symporters (high-affinity) (Kilian and Van Uden, 1988). *Sch. stipitis* harbors specific genes for high-affinity xylose transporter, *XUT1* to *XUT7*, as well as genes expressing hexose transporters with lower transport capacity, *RGT2*, *SUT1*, *SUT2*, *SUT3*, and *SUT4* (Moon et al., 2013; Young et al., 2011; Weierstall et al., 1999). However, glucose competes with xylose for the low-affinity system and inhibits xylose transportation by the high-affinity system non-competitively (Sharma et al., 2018). Leandro et al. (2006) discovered a high affinity proton-xylose/glucose symport system (gene *GXS1*) and a low affinity xylose/glucose system (gene *GXF1*) in *Candida intermedia*. Cloning and expression of the Gxf1 transporter in *S. cerevisiae* increased the xylose fermentation performance of this yeast in xylose fermentation. Nevertheless, the expression of the Gxs1 symport exhibited low glucose/xylose transport activity (Leandro et al., 2009). The search for pentose transporters in native xylose-fermenting yeasts has been used as a strategy in metabolic engineering, but the xylose consumption rates

are still relatively low when expressed in *S. cerevisiae* (Wu et al., 2020; Hou et al., 2017). This is another challenge to cost-effective lignocellulosic ethanol, since the pentose is often consumed after the glucose depletion (Tran et al., 2020).

In yeasts, xylose metabolism occurs through redox reactions followed by the addition of phosphate groups (Fig. 2). Xylose is reduced and converted into xylitol by the activity of the enzyme xylose reductase (XR - EC 1.1.1.21), which is dependent on NAD(P)H (Veras et al., 2019). The xylitol can remain in the cell and be excreted into the medium, or it can be oxidized into xylulose by the enzyme xylitol dehydrogenase (XDH - EC 1.1.1.9), which has affinity for NAD^+. Xylulose is phosphorylated to xylulose-5-phosphate by the catalysis of xylulose kinase (XK - EC 2.7.1.17). The enzymes XR, XDH, and XK are encoded by the genes *XYL1*, *XYL2*, and *XYL3*, respectively (Wohlbach et al., 2012). The phosphorylation product, xylulose-5-P, is transformed via pentose phosphate pathway (PPP) into glyceraldehyde-3-phosphate and fructose-6-phosphate, which are metabolized in the glycolysis (via Embden Meyerhof) to glyceraldehyde-3-phosphate. This molecule is converted into dihydroxyacetone phosphate (DHAP) or/and pyruvate. Pyruvate can be decarboxylated to acetaldehyde by pyruvate decarboxylase (Pdc, encoded by *PDC1*) and reduced to ethanol by the alcohol dehydrogenase I (Adh, encoded by *ADH1*). It also is converted to acetyl-CoA that participates in the tricarboxylic acid (TCA) cycle (Hahn-Hägerdal et al., 1994; Nanda et al., 2013).

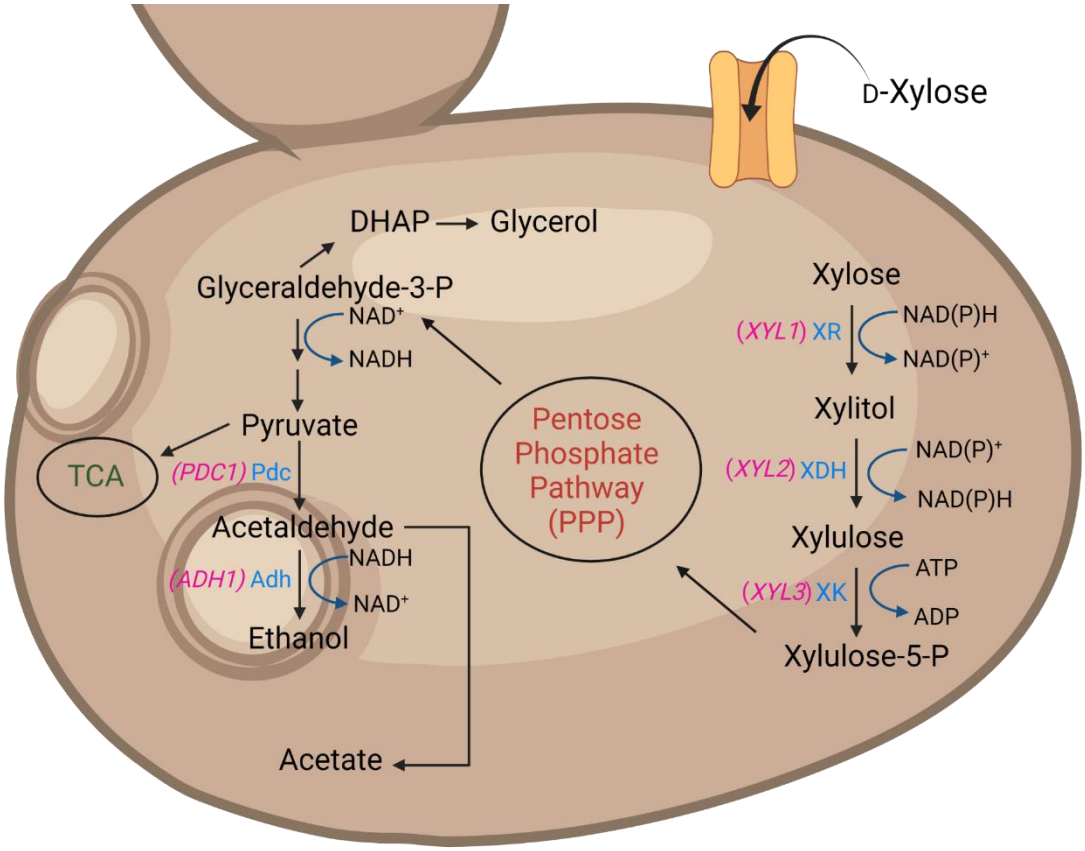

Figure 2. Xylose catabolism in yeasts. Adapted from Wohlbach et al. (2012).

Besides the XR-XDH route in yeasts, there are two other xylose pathways: the xylose isomerase and Weimberg pathways. In the first one, the enzyme xylose isomerase (XI- EC. 5.3.1.5) converts xylose directly into xylulose, which undergoes phosphorylation and participates in the non-oxidative phase of PPP (Cunha et al., 2019). It is present in bacteria and few fungi (Zha et al., 2021). In the Weimberg pathway, xylose is oxidized by xylose dehydrogenase (XylB) to xylono-γ-lactone and converted to xylonate by xylono-γ- lactone lactonase (XylC). Xylonate is dehydrated by xylonate dehydratase (XylD) and 3-keto-2-deoxy-xylonate dehydratase (XylX) to form α-ketoglutarate semialdehyde, which is oxidized to α-ketoglutarate by α-ketoglutarate semialdehyde dehydrogenase (XylA) and enters the TCA cycle (Zha et al., 2021; Borgström et al., 2019). This pathway is present in bacteria such as *Caulobacter crescentus* and *Corynebacterium glutamicum* (Ruchala and Sibirny, 2020). Genes related to Weimburg pathway were expressed in *S. cerevisiae* for production of valuable

chemicals from xylose such as dicarboxylic acids and diols. Some genes were not functional on the yeast which showed poor growth in xylose (Borgstrom et al., 2019; Wasserstrom et al., 2018).

The XR-XDH pathway is the most explored for developing recombinant *S. cerevisiae* strains (Zha et al., 2021). *S. cerevisiae* is the predominant microorganism used for industrial alcoholic fermentation because it displays tolerance to the stress conditions, including ethanol, and even to some inhibitors from the lignocellulosic hydrolysates (Patiño et al., 2019). Due to the great majority of *S. cerevisiae* wild-type strains cannot use xylose, recombinant strains have been developed to confer the necessary enzymatic apparatus for this purpose (Young, Lee and Alper, 2010). Nonetheless, our understanding about the regulation of xylose metabolism is limited compared to the knowledge of the regulatory networks of glucose metabolism in yeasts. This is a challenge for reprogramming *S. cerevisiae* to ferment xylose with the same potential as it ferments glucose (Sato et al., 2016).

Engineered and evolved *S. cerevisiae* strains depend upon epistatic interactions among specific genes to rapidly metabolize xylose aerobically or anaerobically, and genetic mutations in these genes are responsible for synergistically altering metabolic pathways to improve the rate of xylose conversion (dos Santos et al., 2016; Sato et al., 2016). Nonetheless, these mutations cause loss of function in important genes, such as *HOG1* and *IRA2*, both of which are known to result in reduced stress tolerance (Sato et al., 2016). Thus, challenges remain in developing yeast strains capable of fermenting xylose from lignocellulosic hydrolysates (Almeida et al, 2011; Sato et al., 2016). In this framework, the development of xylose-fermenting *S. cerevisiae* strains should not be the only route to be followed, and the domestication of native xylose-fermenting yeasts arises as an alternative for direct use in industrial 2G ethanol production (Cadete et al., 2017).

2.6. Xylose reductase (XR) and xylitol dehydrogenase (XDH)

Xylose reductase and xylitol dehydrogenase are placed in different families of proteins. The first one is an aldose reductase belonging to the aldo-keto reductases (AKRs) superfamily (Lugani et al., 2021). Aldehyde reductases, hydroxysteroid dehydrogenases and dihydrodiol dehydrogenases are also included in this protein superfamily (Jez et al., 1997). They are characterised by their common $(\alpha/\beta)_8$-barrel three-dimensional fold (Fig.3) and reaction mechanism in catalysing the conversion of carbonyl substrates into their respective alcohols (Lugani et al., 2021; Mindnich et al., 2009). All the AKR are NAD(P)H-dependent and they are present across the tree of life (Jez et al., 1997).

Xylitol dehydrogenase is a member of the alcohol dehydrogenases (ADHs) of medium chain dehydrogenase/reductase (MDR) superfamily (Lunzer et al., 1998), which also are ubiquitous enzymes (Asokumar et al., 2018). The superfamily is classified into eight subfamilies based on amino acid sequence alignment and the structural similarity of substrates. Xylitol dehydrogenase belongs to the polyol dehydrogenase (PDH) subfamily that contains sorbitol dehydrogenase (SDH - EC 1.1.1.14) (Watanabe et al., 2005). They use $NAD(P)^+$ as a cofactor to catalyze the oxidation of alcohols to the corresponding aldehydes or ketones. Polyol dehydrogenases have an important role in the bioconversion between rare sugar and alcohol (Lu et al., 2019; Baker et al., 2009). As mentioned above, XDH is responsible for the conversion of xylitol into xylulose. Together, XR and XDH are involved in the first steps of xylose catabolism, and a balance between them may be essential for the success of the xylose pathway in yeasts (Zhu et al., 2021).

Although alcoholic fermentation is traditionally anaerobic, many yeasts have the ability to ferment in the presence of oxygen (Correia et al., 2018). The oxidoreductase enzymes, XR and XDH, have different affinities for the cofactors involved in the metabolism of xylose. *Sch. stipitis* and many of xylose-fermenting yeasts have XR with high affinity for NADPH over

NADH, but the XDH depends on NAD^+ with low affinity for $NADP^+$ (Son et al., 2018). Therefore, the regeneration of NADPH and NAD^+ is necessary to maintain the redox balance. NADPH is regenerated by directing part of the fructose-6-phosphate produced in the oxidative step to the PPP (Christodoulou et al., 2019). Under aerobic conditions, NADH can be reoxidized by the respiratory chain with molecular oxygen as electron acceptor. However, in anaerobic or low aeration condition, another electron acceptor is required to reoxidize NADH otherwise the cell is unable to maintain the redox balance resulting in xylitol accumulation in the cell (Granström et al., 2007).

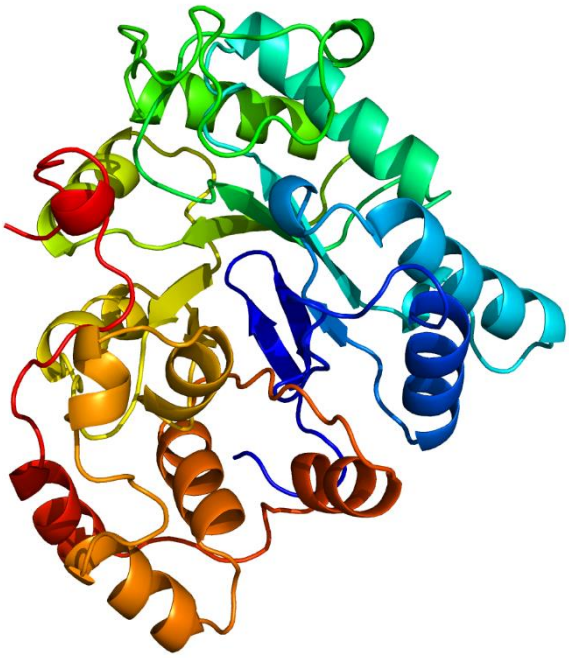

Figure 3 - Three dimensional structure prediction of xylose reductase (XR) from *Scheffersomyces parashehatae*. The structure was generated by Phyre[2] (http://www.sbg.bio.ic.ac.uk).

Aldo-keto reductases contains a Ile-Pro-Lys-Ser motif and conserved Arg situated five residues downstream, as well as the GlyXXXGlyXGly region for the binding of coenzyme in which the last glycine residue is substituted by Glu or Asp in yeast XRs (Geijer et al., 2020; Hossain et al., 2018). To overcome the imbalance of cofactors, studies were directed to

understand the nature of cofactors binding site of the enzyme and change the XRs specificity. Xylose reductase from *Sch. stipitis* and *Candida tenuis* were mutated through introduction of single or double mutations at the conserved motif in the primary sequence of the enzyme (Runquist et al. 2010; Bengtsson et al. 2009; Liang et al. 2007; Kostrzynska et al. 1998). Such studies reported the involvement of some residues (e.g. Lys270, Asn272 and Arg276) by the increase or reduction affinity of the enzyme variants for NADPH/NADH. However, the attempts to change XRs specificity for the cofactor resulted in decreased affinity towards NADPH without significant increase in affinity for NADH (Hossain et al., 2018).

2.7. Native xylose-fermenting yeasts

Decomposing plant materials, gut and larvae of insects, and tree barks are suitable habitats for yeasts due to the presence of sugars that can be assimilated by them. Native xylose-fermenting yeasts are usually isolated from these substrates (Cadete et al., 2017). *Pachysolen tannophilus* was found in a wood extract sample by Bodin and Adzet in 1957. This finding had a great significance because this yeast was reported to convert xylose into ethanol when yeast was not recognized to ferment aldopentoses (Kurtzman, 1983). This yeast reached 52% of ethanol yield in aerobic conditions, but no production was observed under anaerobic conditions (Slininger et al., 1982; Schneider et al., 1981). Later, other yeast species of the subphylum Saccharomycotina were isolated and recognized as xylose-fermenting yeasts.

Scheffersomyces stipitis (*Pichia stipitis*) is the most extensively investigated among the species of the genus *Scheffersomyces* due to producing significant amounts of ethanol from xylose as the sole carbon source (Damiani et al., 2015; Ferreira et al., 2011; Jeffries et al., 2007; Agbogbo and Coward-Kelly, 2008; Du Preez et al., 1989). Members of the genus were isolated from wood-boring insect larvae and their frass, from the guts of beetles and from rotting wood (Kurtzman, 2010). The genus was proposed by Kurtzman and Suzuki (2010) based on

combined analysis of the small subunit (SSU) and partial large subunit (LSU) rRNA genes. They reclassified species previously harbored by the genus *Pichia* (e.g. *P. stipitis*, *P. segobiensis*, and *P. spartinae*) and species assigned to *Candida* (e.g. *C. shehatae*, *C. coipomoensis*, and *C. ergatensis*). Although the ITS region and D1D2 domain are suitable to identify yeast species, multilocus analysis has been made for *Scheffersomyces* members to generate a more reliable phylogeny (Lopes et al., 2018; Liu et al., 2016; Suh et al., 2013; Urbina et al., 2013; Urbina and Blackwell 2012). The genus accommodates twenty-one species (Jia et al., 2020), but results from such multilocus analysis with extended taxon sampling indicate that a phylogenetic re-evaluation of *Scheffersomyces* species may be necessary (Kurtzman and Robnett 2013; Suh et al., 2013).

More recently, species of the genus *Spathaspora*, such as *Sp. passalidarum* and *Sp. arborariae* have been isolated and identified (Nguyen et al., 2006; Cadete et al., 2009), with promising results for the production of ethanol from xylose, especially *Sp. passalidarum* (Cadete et al., 2016; Hou, 2012). *Sp. passalidarum* was the first described species of the genus, isolated from the intestine of the beetle *Odontotaenius disjunctus* belonging to the family Passalidae, in Louisiana, USA (Nguyen et al., 2006). In 2009, *Sp. arborariae* was isolated from decaying wood collected in the Atlantic Forest and Cerrado ecosystems in Brazil (Cadete et al., 2009). These yeasts are capable of efficiently fermenting xylose and producing ethanol, being potential species for the production of this biofuel from lignocellulosic biomass (Cunha-Pereira et al., 2011; Cadete et al., 2016). Currently, *Sp. passalidarum* is considered the best yeast among naturally xylose fermenters and a promising genetic treasure for improving bioethanol production (Selim et al. 2020; Cadete and Rosa, 2018). It is capable of producing ethanol close to the maximum theoretical yield (0.51 g g^{-1}) and consuming xylose rapidly in defined media under oxygen-limiting conditions (Hou, 2012; Long et al., 2012, Cadete et al., 2016). The genus comprises more than fifteen species, most of them produce mainly ethanol and only five species

were reported to produce ethanol from xylose: *Sp. passalidarum*, *Sp. arborariae*, *Spathaspora piracicabensis*, *Spathaspora gorwiae*, and *Spathaspora hagerdaliae* (Varize et al., 2018; Cadete et al., 2016; Lopes et al., 2016). The last one produced xylitol in a similar amount as ethanol from xylose as the sole carbon source (Lopes et al., 2016).

Species belonging to other genera also have the ability to achieve ethanol production from xylose. *Kluyveromyces marxianus* was one of the first yeast species described to ferment xylose as the sole carbon source to ethanol (Goshima et al. 2013; Margaritis and Bajpai, 1982). Species belonging to the genus *Sugiyamaella* are associated with rotting plant materials, soil, and gut of insects (Cadete et al. 2017) and they are recognized by their ability to convert xylose to ethanol and produce xylanolytic enzymes (Moraes et al. 2020; Sena et al. 2017). A single strain of *Wickerhamomyces* sp. (UFF-CE-3.1.2), isolated from rotting wood, reached ethanol production in 72 h of fermentation in non-detoxified and diluted 1:3 (v/v) sugarcane bagasse hydrolysate, containing 0.25% of acetic acid (Bazoti et al., 2017). Species such as *Candida tropicalis*, *Candida boidinii*, *Meyerozyma guilliermondii*, *Cyberlindera galapagoensis*, and *Cyberlindnera xylosilytica* also ferment xylose, but they produce xylitol and do not achieve significant ethanol yields (López-Linares et al., 2020; Cadete et al., 2015; Guamán-Burneo et al. 2015; Wang et al., 2015; de Arruda et al., 2016).

Although several species that are able to ferment xylose accumulate xylitol, it is relevant studying those yeasts, in addition to the yeasts considered ethanol producers, to increase our understanding of the metabolism of pentoses and its limitations. An extended knowledge of this metabolic trait can contribute to improving the strategies of genetic manipulation to generate artificial diversity and select superior strains for industrial production of ethanol.

3. AIMS

Aim 1 - Analyze the diversity of yeasts able to assimilate lignocellulose-related sugars from rotting wood samples collected from dryland and floodplain ecosystems in the Brazilian Amazonian rainforest. In addition, isolate new species and yeasts preadapted to lignocellulosic hydrolysates.

1a. Isolate yeast species associated with rotting wood using minimal medium with xylose or xylan, corn cob hemicellulosic hydrolysate, and sugarcane bagasse hydrolysate as isolation media.

1b. Identify the species and analyze alpha and beta diversity of the three sites of collection.

Aim 2 - Understand the distinct xylose fermentation performance of species belonging to the main xylose-fermenting yeasts clades - *Spathaspora* and *Scheffersomyces*.

2a. Conduct xylose fermentation assays using twenty-four species of the genera *Spathaspora* and *Scheffersomyces* and outgroups to verify the xylose consumption and formation of end-products in high, moderate, and low aeration.

2b. Determine the enzyme activities and the cofactor preference of xylose reductase and xylitol dehydrogenase from those species in high and moderate aeration.

2c. Express *XYL1* gene from *Scheffersomyces* species in *Saccharomyces cerevisiae* and submit the transformants to enzyme assays to determine the enzyme activities and the cofactor specificity.

Aim 3 - Develop a Brazilian *Spathaspora passalidarum* strain able to grow in sugarcane bagasse hemicellulosic hydrolysate (SBHH) and investigate the origin of the tolerance.

3a. Submit the strain *Sp. passalidarum* UFMG-CM-Y469 to random mutagenesis using ultraviolet light.

3b. Select the mutants based on growth rate and ethanol production in defined medium containing xylose and acetic acid and then SBHH.

3c. Perform adaptive laboratory evolution (ALE) in defined medium with acetic acid and then SBHH.

3d. Select the evolved strains based on the results of fermentation in SBHH.

3e. Perform a whole genome sequencing on the ancestral UFMG-CM-Y469 and evolved strains to identify *de novo* mutations related to the tolerance.

CHAPTER I

Diversity of yeasts associated with rotting wood from three areas of Brazilian Amazonian rainforest

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Introduction

Yeast species are present in live and decaying plant parts and they have a beneficial interaction with insects (Cadete et al. 2017). While the yeasts provide nutrients to the insects by contributing to food digestion, the insects carry the yeasts to different habitats (Stefanini et al., 2018). Several yeast species found in the gut and frass of wood-boring insects were also found in their habitats, like species of the *Spathaspora*, *Scheffersomyces,* and *Sugiyamaella* clades (Shi et al., 2021; Lopes et al., 2018a; Nguyen et al., 2006). Yeasts found in wood-boring insects and their habitats have been shown the ability to assimilate and/or ferment the lignocellulose-related sugars, specially pentoses from the hemicellulosic fraction (Barros et al., 2020). As lignocellulose is an abundant raw material found in nature and is also massively produced as an agricultural waste product (Faostat, 2017), those yeasts have been studied about their

capacity to generate value-added products (e.g. ethanol, xylitol, and xylanases) from lignocellulosic materials (Kumar et al., 2021).

Until 2020, the Brazilian Amazonia portion corresponded to 58.9% of Brazilian territory, extending along the North region of the country (IBGE, 2020). Amazonia is not only the world's most diverse rainforest but is also the region in tropical America that has contributed most to its total biodiversity (Antonelli et al., 2018). This biome is classified as a hotspot biodiversity region, areas characterized by outstanding concentrations of endemic species and anthropogenic pressure (Myers et al., 2000). Although the term is related mostly to plants, it can be extended, for example, to invertebrates and yeasts, since there is an insect-yeast-plant association (Péter et al., 2017; Myers et al., 2000). The first study to investigate the Brazilian Amazonian Forest aiming to isolate and identify new D-xylose-fermenting yeasts associated with rotting wood was conducted by Cadete et al. (2012). A total of 224 yeast strains were isolated from 40 rotting wood samples collected in different areas of the forest. Other studies were carried out in this forest with the goal to isolate xylose fermenters and/or xylanase-producing yeasts (Moraes et al., 2020; Souza et al., 2017).

Despite the success of those previous collections in the Brazilian Amazonia, Cadete et al. (2012) and Souza et al. (2017) used minimal media or yeast extract malt to isolate the species. A single work collected in the region using sugarcane bagasse hydrolysate as isolation medium (Moraes et al. 2020). To explore the diversity of the Amazonian rainforest by targeting the group of yeasts with the ability to assimilate lignocellulosic sugars, we collected rotting wood samples in three sites of the forest (drylands and floodplain areas). To isolate the maximum number of species, undiluted and diluted hemicellulosic hydrolysates from different raw materials as well as minimal media with xylose or xylan were used as isolation media. The hydrolysates were also used as a form to isolate species preadapted to the inhibitors.

Material and methods

Yeast isolation and identification

Yeasts were isolated from rotting wood samples of unidentified trees collected in three areas of the Brazilian Amazonian rainforest, in the Amazon state. The areas were Campus II of Universidade Federal do Amazonas (UFAM) (03º 05.654' S, 58º 27.464' W), Piquiá Sol Nascente Community (03º 01.045' S, 58º 28.830' W), and Carú (03º 35.923' S, 58º 44.905' W). They are located in the municipality of Itacoatiara, which is 270 Km east of Manaus, the capital of Amazon state. It presents a tropical climate, where the average annual temperature is between 25.5 and 27.5ºC, and the annual precipitation ranges between 1,500 and 2,100 mm, with rains concentrated in the summer months and a humid season throughout the year (Alves et al., 2019). Twenty samples of rotting wood were collected from each area in February 2019. The samples were stored in sterile plastic bags and transported under refrigeration to the laboratory immediately after the collecting. For each sample, 0.5 g was placed, separately, in flasks with 10 mL sterile sugarcane bagasse and corncob hemicellulosic hydrolysate at three different concentrations (undiluted, diluted 1:2 and diluted 1:5), without nutritional supplementation (the protocol of preparation of these hydrolysates is described below). The samples were also inoculated in the culture medium YNB-xylose (0.67% yeast nitrogen base, 2% xylose), and YNB-xylan (0.67% yeast nitrogen base, 2% xylan). The sugarcane bagasse hemicellulose hydrolysate was diluted with sterile distilled water. The tubes were incubated at 30ºC on an orbital shaker (New Brunswick, USA) at 200 rpm until the growth was detected. Then 0.5 mL of each culture was transferred to tubes containing 5 mL of sugarcane bagasse hydrolysate in the same concentrations listed previously, and the tubes were incubated as described above. After yeast growth, one loopful of culture from each tube was streaked on YM agar (glucose 1 %, yeast extract 0.3 %, malt extract 0.3 %, peptone 0.5 %, agar 2 %, and chloramphenicol 0.02 %) (Sena et al., 2017). The plates were incubated at 25ºC until the yeast colonies developed. The different yeast morphotypes were purified by repeated streaking on

YM agar plates and preserved at -80ºC for later identification. The yeasts were characterized physiologically and morphologically using standard methods (Kurtzman et al., 2011).

Isolates with identical morphological and physiological characteristics were grouped together and subjected to microsatellite-primed $(GTG)_5$ PCR fingerprinting (Libkind et al., 2003). Yeast strains with identical PCR fingerprinting patterns were grouped and putatively considered to belong to the same species (Sampaio et al., 2001; Gomes et al., 2015; Lopes et al., 2018b). At least half of the yeast isolates of each molecular group were identified by sequencing of the ITS-5.8S region and the D1/D2 variable domains of the large subunit rRNA gene. Species identifications were performed by analysis of the sequences of the ITS-5.8S region and the D1/D2 variable domains (White et al., 1990; O'Donnell, 1993; Kurtzman and Robnett, 1998; Lachance et al., 1999). The amplified DNA was cleaned and sequenced in an ABI 3130 Genetic Analyzer automated sequencing system (Life Technologies, California, USA) using BigDye v3.1 and POP7 polymer. The sequences were assembled, edited, and aligned with the program MEGA7 (Kumar et al., 2016). They were compared with annotated yeast sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST at http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990).

Hemicellulosic hydrolysates

Both sugarcane bagasse hemicellulosic hydrolysate (SBHH) and corncob hemicellulosic hydrolysate (CHH) were obtained by acid hydrolysis in bioreactor under the following conditions: 121°C during 20 min; sulfuric acid 98% with ratio of 1:10 (100mg H_2SO_4 per gram of sugarcane bagasse or corncob). The liquid fraction was recovered by vacuum filtration, the pH was adjusted to 5.5 with calcium oxide (CaO), and treated with 2.4% w/v activated vegetable charcoal in the form of refined powder. Then they were autoclaved at 111ºC for 15 min. No detoxification step was performed. Concentrations of sugars and acetic acid were determined by high-performance liquid chromatography - HPLC (Shimadzu, Kyoto, Japan),

using the following conditions: Supelco Analytical C-610 H column (Sigma-Aldrich, USA), maintained at 45°C; volume injection of 20µL; refractive index detector RID 10-A (Shimadzu, Kyoto, Japan); 5 mM H_2SO_4 mobile phase as eluent at a flow rate of 0.6 mL min^{-1}. Furfural and hydroxymethylfurfural were measured by HPLC using a dual k absorbance detector in a 276 nm wavelength (Waters 2487, Milford-MA-USA) and Waters Resolve 5 µL Spherical C18 column (3.9 x 300 mm) at 25ºC; solution of acetonitrile–water 1:8 (v/v) with 1% of acetic acid as eluent, at a flow of 0.8 mL/min and injection volume of 20 µL (Rodrigues et al., 2010).

The compositions of the hydrolysates were: 0.004% 5-HMF, 0.03% furfural, 0.5% acetic acid, 0.6 glucose, and 9% xylose for CHH; 0.013% 5-HMF, 0.02% furfural, 0.5% acetic acid, 0.2% glucose, and 2% xylose for SBHH.

Diversity analyses

To assess the relative abundance and visualize species richness and species evenness of each collection site, we constructed rank abundance curves (RACs) and an accumulation species-richness curve. First, we calculated the rank abundance distribution for each area, which presents an abundance of each species in the sample from the most common (rank one) to the less abundant (highest rank). We fitted a model for this distribution, which is chosen from specific models by its AIC (Akaike Information Criterion). Only a curve of the best model was presented. If the curve from an area becomes steep, it means that there are few species in that location and the species are common. If the curve has a shape in L, it means there are some species in common (left side from the L) and rare species (right side of the L). As the curve is higher, there are more species in the area. Then we constructed a cumulated richness curve for each location and medium representing how many species were found in each sample size. The line shows the average number of species, while the colored bands have a margin of error for this value. If the curve of an area is above the curve of another one, the richness in this area is greater than the other. A rarefaction curve was constructed by randomly re-sampling the pool

of N samples several times and then plotting the average number of species found on each sample. The curve initially grows rapidly as the most common species are found and then slightly flattens as the rarest species remain to be sampled. Alpha diversity analysis was made in R version v3.6.3 using the RStudio v1.3.1073 platform and the package *vegan* (Oksanen et al., 2020). In addition, beta-diversity analysis was conducted to examine similarities and dissimilarities among the different collection sites based on the yeast species using the R packages *vegan* and *ape* (Paradis and Schliep, 2019).

Results and discussion

Yeast identification

A total of 569 yeast isolates were obtained from 60 rotting wood samples of three areas of Amazon Rainforest in Brazil. Two hundred thirty-five were obtained from samples collected in the Piquiá area, 219 from UFAM, and 115 from Carú. Considering the culture media, 128 strains were isolated in YNB-xylan, 118 in YNB-xylose, 45 in undiluted SBHH, 68 in SBHH 1:2, 78 in SBHH 1:5, 18 in undiluted CCHH, 46 in CCHH 1:2, and 68 in CCHH 1:5 (Fig. 1). Three hundred thirty-six isolates were submitted to sequencing of D1D2 domain and ITS region, and 89 species were identified, of which 52 were previously known and 37 represented possible novel species. Thirty-two species were represented by a single isolate, and 57 species were isolated more than once. These species are shown in Table S1, which shows the occurrence and number of each species by isolation site and each culture medium. Ascomycetous yeasts were prevalent. The considerably number of identified species, including undescribed species, is likely due to the greater number of media used during the isolation and possibly due to the geographically distinct regions used as sampling sites, which is expected to increase the probability of obtaining a higher diversity of species.

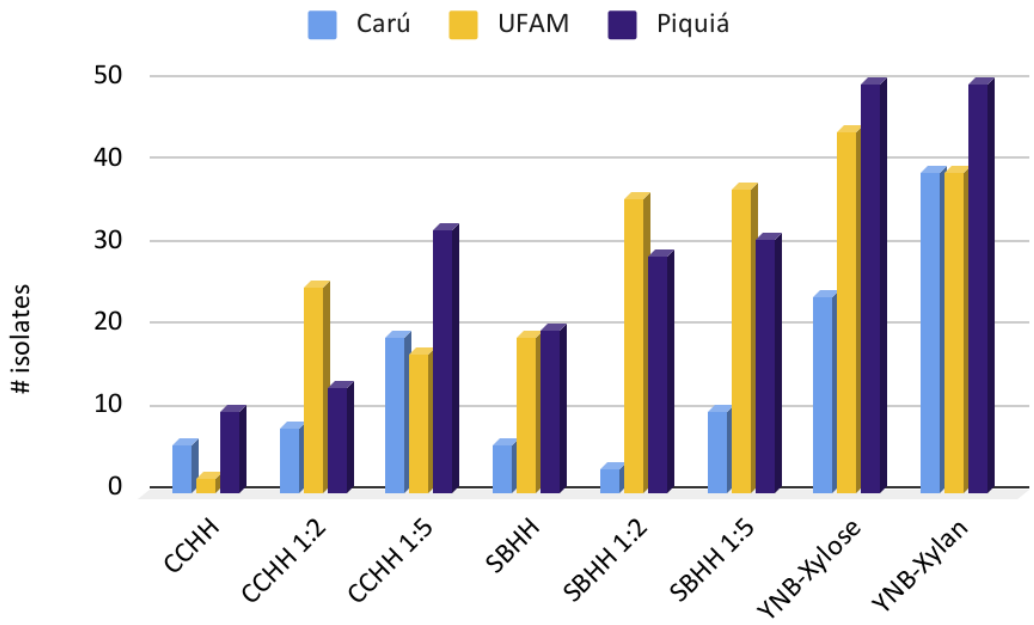

Figure 1. Distribution of isolates per medium and area.

Sugiyamaella was the most prevalent genus identified in this work, represented by 10 species (*Su. bahiana*, *Su. smithiae*, *Su. valenteae*, *Su. xylolytica* and six novel species), followed by *Kazachstania* with nine species (*K. kunashirensis, K. martiniae, K. serrabonitensis, K. wufongensis*, and five new species). Table 1 shows the novel species, their most closely related species, and the percent sequence identity. *Sugiyamaella* species were isolated from all the collection areas in the different culture media utilized, including the hydrolysates. The *Kazachstania* members were recovered mainly in un- and diluted hydrolysates from samples of UFAM and Piquiá. Only two species (*Kazachstania* sp.1 and *K. kunashirensis*), an isolate of each one, did grow in YNB-xylose. The species of this genus are ubiquitous (Jacques et al., 2016), and already have been found, for example, in sourdoughs (Urien et al., 2019), soil (Lee et al., 2009), gut of passalid beetles (Suh and Zhou, 2011), rotting wood (Jacques et al., 2016), gastrointestinal tract infections (Alvarez-Perez et al., 2012), and water reservoirs of bromeliads (Araújo et al., 2012). *Kazachstania* sp.1, with a single isolate, and *Kazachstania* sp.2 are

phylogenetically closest to *K. yasuniensis* and *K. unispora*, respectively. *Kazachstania* sp.3, sp.4, and sp.5 are closest to *K. martiniae*, *K. africana*, and *K. servazzii*, respectively. Due to *Sugiyamaella* species being able to convert D-xylose into ethanol and xylitol (Morais et al., 2013) and produce xylanases (Morais et al., 2020; Lara et al., 2014), they are interesting for biotechnological purposes. Members of this clade are associated with wood-boring insects, frass and habitat of insects and they are frequently found in decaying plant materials (Cadete et al., 2017). Among the three known *Sugiyamaella* species isolated in this work, three (*Su. bahiana*, *Su. valenteae*, and *Su. xylolytica*) were described by Sena et al. (2017). Only in that study, they found seven new species of the genus in rotting wood samples collected in different regions of Brazil. The new species *Sugyamaella* sp.1 to sp.6 are related to *Sugiyamaella* sp., *Su. carassensis*, *Su. lignohabitans*, *Su. bonitensis, Su. paludigena*, and *Su. trypani,* respectively. At the moment, the genus accommodates 29 species, including 13 new accommodations, which were transferred from *Candida* genus (Shi et al., 2021; Urbina et al., 2013).

Table 1. Identification of novel yeast species isolated in Amazonian Rainforest sites based on the sequences of the ITS region and D1/D2 domains of the large subunit of the rRNA gene.

Species	# strains	Identity % (D1/D2)	Top BLAST results
Candida sp.1	1	98	*Candida* sp. NRRL Y-7615
Candida sp.2	1	99	*Candida* sp. YPN 49
Candida sp.3	3	100	*Candida* sp. NRRL Y-2544
Candida sp.4	1	99	*Candida* sp. BG02-7-21-004Q-1-2
Candida sp.5	2	100	*Candida* sp. UFMG-XMD-6.1
Candida sp.6	1	99	*Candida* sp. CLIB 1735
Candida sp.7	1	100	*Candida* sp. P31
Candida sp.8	5	100	*Candida* sp. UFMG-CM-Y6396
Candida sp.9	3	94	*Candida thailandica*
Candida sp.10	1	95	*Candida* sp. UWO(PS)99-305.2
Cyberlindnera sp.1	4	100	*Cyberlindnera* sp. UFMG-CM-Y519
Cyberlindnera sp.2	2	95	*Cyberlindnera fabianii*
Debaryomyces sp.	3	100	*Debaryomyces* sp. UFMG-CM-Y343
Galactomyces sp.	3	99	*Galactomyces* sp. UFMG-CM-Y2982
Kazachstania sp.1	6	100	*Kazachstania* sp. CLQCA 24SC-045
Kazachstania sp.2	1	100	*Kazachstania* sp. LN650649
Kazachstania sp.3	3	99	*Kazachstania martiniae*
Kazachstania sp.4	2	93	*Kazachstania africana*
Kazachstania sp.5	1	98	*Kazachstania servazzii*
Nakaseomyces sp.	11	99	*Nakaseomyces* sp. UFMG-CM-Y6196
Scheffersomyces sp.	3	99	*Scheffersomyces* sp. UFMG-CM-Y365
Spathaspora sp.1	2	97	*Spathaspora boniae*
Spathaspora sp.2	2	98	*Spathaspora arborariae*
Spencermartinsiella sp.1	2	100	*Spencermartinsiella* sp. UFMG-CM-Y3197
Spencermartinsiella sp.2	5	91	*Spencermartinsiella. ligniputridi*
Sugiyamaella sp.1	14	91	*Sugiyamaella carassensis*
Sugiyamaella sp.2	2	97	*Sugiyamaella* sp. UFMG-CM-Y6964

Species	# strains	Identity % (D1/D2)	Top BLAST results
Sugiyamaella sp.3	2	95	*Sugiyamaella. lignohabitans*
Sugiyamaella sp.4	5	94	*Sugiyamaella bonitensis*
Sugiyamaella sp.5	1	97	*Sugiyamaella trypani*
Sugiyamaella sp.6	1	99	*Sugiyamaella paludigena*
Torulaspora sp.1	4	94	*Torulaspora globosa*
Torulaspora sp.2	1	98	*Torulaspora delbrueckii*
Vanderwaltozyma sp.	2	95	*Vanderwaltozyma verrucispora*
Wickerhamiella sp.	2	99	*Wickerhamiella* sp. UFMG-CM-Y2274
Yarrowia sp.	1	95	*Yarrowia* sp. Y7
Yamadazyma sp.	2	93	*Yamadazyma mexicana*

When phenotype characteristics were used as the main criterion to classify yeasts, many morphologically indistinguishable species were grouped as *Candida*. Because of that, several taxonomic changes have been made in the taxa based on DNA analysis (Daniel et al., 2014). Due to the reclassification of dozens of species being classified as belonging to the genus *Candida*, we cannot say that species of this genus were most prevalent in our study. We found 26 species assigned as *Candida*, which is a mix of different clades (*C. albicans, C. blattae, C. maltosa*, *C. orthopsilosis*, *C. tropicalis* - *Lodderomyces/Candida albicans* clade; *C. boidinii* - *Ogataea* clade*; C. boleticola*, *C. natalensis*, - *Kurtzmaniella* clade*; C. ghanaensis, C. gorgasii*, *C. jaroonii*, *C. michaelii* - *Yamadazyma* clade*; C. intermedia*, *C. pseudointermedia* - *Clavispora* clade*; C. palmioleophila* - *Candida glaebosa* clade; *C. melibiosica*, *C. saopaulonensis* - *Metschnikowia* clade; and 10 novel species). *C. melibiosica* (11 strains) and *C. maltosa* (9) were the most frequent species among them. *C. melibiosica* is related with xylitol and arabitol production (Gong et al., 1983) and it is associated with soil and rotting wood (Han et al., 2019, Morais et al., 2013). *C. maltosa* was isolated from soil and water waste (Komagata et al., 1964) and it is known for its ability to grow on hexadecane and fatty acids (Zvonarev et al., 2017;

Mauersberger et al., 1996). The sequences of the D1/D2 domains and ITS region of the novel species *Candida* sp.1 to *Candida* sp.8, and *Candida* sp.10. were identical or similar to species which had their sequences deposited as *Candida* sp. in GenBank (Table 1). Their most closely related described species are *C. maritima* (*Cyberlindnera*), *C. vrieseae* (*Yamadazyma*), *Kuraishia piskuri, Diutina ranongensis*, *C. gorgasii* (*Yamadazyma*), *Wickerhamiella musiphila*, *C. asparagi* (*Clavispora*), *C. thaimueangensis* (*Pichia*), and *Metschnikowia gelsemii,* respectively. *C. thailandica* (*Clavispora*) is the most closely related species of the novel species *Candida* sp.9.

Schwanniomyces polymorphus (20 isolates), *Scheffersomyces amazonensis* (16 isolates), and *Wickerhamomyces edaphicus* (16 isolates), *Sugyamaella* sp.1 (14 isolates), and *K. serrabonitensis* (13 isolates) were the species most frequently isolated. While *Schw. polymorphus* was found in all the areas and isolated mainly in YNB-xylan and YNB-xylose. Fifteen isolates of *Sch. amazonensis* were obtained only from UFAM in all the media, except for undiluted hydrolysates, and an isolate from Caru in SBHH diluted to 1:5. Soil was suggested as the natural habitat of *Schw. polymorphus* (Suzuki and Kurtzman, 2010). Later, this was the most frequently isolated species from rotting wood samples collected at Atlantic rainforest in Brazil in YNB-xylan and YNB-xylose (Morais et al., 2013). This species is also associated with the infrabuccal pocket in the carpenter ant *Camponotus vicinus* (Hymenoptera: Formicidae), but its role in the ant development is not clear (Mankowski et al., 2021). Species belonging to the *Scheffersomyces* clade are commonly found in rotting wood samples, which indicates they are adapted to this substrate (Kurtzman, 2011). Previous studies isolated *Sch. amazonensis*, first classified as *C. amazonensis*, from rotting wood sampled at Brazilian Amazonian rainforest (Cadete et al., 2012) and Atlantic rainforest (Lopes et al., 2018b). It indicates that rotting wood could be its ecological niche. *Sch. amazonensis* is described to be a good xylose and cellobiose-fermenting yeast, producing mainly xylitol and ethanol as end-products (Lopes et al., 2018b,

Cadete et al., 2016). *W. edaphicus* strains were isolated from UFAM and Carú samples in both hydrolysate types with and without dilution. This was the only species isolated in undiluted CCHH from UFAM and Carú samples. *W. edaphicus* was isolated from soil samples collected in Thailand and it was reported that this species is able to grow in a medium containing 50% and 60% glucose, and 10% NaCl (Limtong et al., 2009). Considering the high concentration of sugar and the mix of inhibitors in CCHH hydrolysate, it is possible that this species is adapted to grow in high osmolarity and adverse environments.

Likewise, *Rhodotorula mucilaginosa* (11 isolates), *Nakaseomyces* sp.1 (11 isolates), and *Apiotrichum mycotoxinivorans* (9 isolates) had a significant number of individuals. The sequences of the D1/D2 domain from *Nakaseomyces* sp.1. showed the undescribed *Nakaseomyces* sp. UFMG-CM-Y6196 as the closer related species, which was picked up from wood samples in Atlantic rainforest (Ribeiro, unpublished data). *Nakaseomyces* sp.1. and *Rh. mucilaginosa* were isolated from samples of all the collection sites, and *A. mycotoxinivorans* from UFAM and Carú. Although *Rh. mucilaginosa* can be associated with human diseases (Ioannou et al., 2019), this ubiquitous basidiomycetous is found in a wide range of aquatic and terrestrial habitats, such as freshwater lakes, ocean, living and decaying plant parts (Sampaio, 2011). Biotechnological applications for this yeast have been proposed that include carotenoid (Cheng and Yang, 2016; Libkind et al., 2004), and biopolymers production (Hamidi et al., 2020). *A. mycotoxinivorans* is a basidiomycetous yeast used as a microbial feed additive against mycotoxins (Sun et al., 2020). It was found in rotting wood and water samples and presented xylanase and amylase activities (Carvalho et al., 2021; Carla et al., 2014).

Thirty-two species were represented by single isolates. Twenty-one were isolated in the area of UFAM, and among them, 12 have been earlier described (*C. (Yamadazyma) gorgasii*, *C. (Yamadazyma) michaelii*, *C.* (*Metschnikowiaceae*) *saopaulonensis*, *Cyberlindnera xylosilytica*, *Debaryomyces hansenii*, *Debaryomyces nepalensis*, *Galactomyces candidus*,

Meyerozyma guilliermondii, *Pichia fermentans*, *Saccharomyces cerevisiae*, *Su. bahiana*, and *Schwanniomyces vanrijiae*) and 9 are new (*Candida* sp.1, *Candida* sp.2, *Candida* sp.4, *Kazachstania* sp.2, *Kazachstania* sp.5, *Sugyamaella* sp.5., *Sugyamaella* sp.6, *Torulaspora* sp.2, and *Yarrowia* sp.). Nine species were found in the Piquiá area, four already identified (*C. (Yamadazyma) jaroonii*, *Diutina rugosa*, *Meyerozyma caribbica*, and *Scheffersomyces shehatae*) and 3 are new (*Candida* sp.6, *Candida* sp.7, and *Candida* sp.10). The remaining 4 species were from Carú (*C. albicans*, *Cryptococcus flavus*, *Rhodotorula alborubescens*, and *Su. valenteae*). Thirteen of these species were isolated in YNB-xylan, three in YNB-xylose, two in SBHH, 2 in SBHH 1:2, four in SBHH 1:5, one in CCHH, 3 CCHH 1:2, and four in CCHH 1:5.

Some single isolates might represent transient species within the sampling sites. Indeed, studies in which *S. cerevisiae* were isolated from rotting wood showed that they are present in extremely low numbers compared to the dominating yeast species (Morais et al., 2020; Hui et al., 2013). It is associated with oak bark, exudates, leaves, and soil (Hittinger, 2013). In the case of *S. cerevisiae* and sister species, the isolation from those environments was done using enrichment media (Sampaio and Gonçalves, 2008). *M. caribbica* and *M. guilliermondii* are widely distributed in nature and they also have been found in decaying plant tissues (Ali et al., 2017; Lopes et al., 2018b; Morais et al., 2020), as well as *C. albicans*, which is known as human commensal and opportunistic pathogen, but it is sporadically found in plant materials (Lachance et al., 2011). *Cy. xylosilytica* was first isolated from lignocellulosic materials (Cadete et al., 2015) and species of this clade are frequently recovered from this type of substrate (Boontham et al., 2017; Zheng et al., 2017). In this work, we found two new species belonging to the clade *Cyberlindnera*. *Cyberlindnera* sp.1 is related to *Cy. dasilvae* (Barros et al., 2021) and *Cyberlindnera* sp.2, which is closer to *Cy. fabianii*.

Two new species belonging to *Spathaspora* and one of *Scheffersomyces* were also isolated. *Sp. boniae*, *Sp. arborariae*, and *Scheffersomyces* sp. UFMG-CM-Y365 are the closest

related species. They are the remarkable genera with several species able to ferment xylose and produce great amounts of ethanol (Nakanishi et al., 2017). Those species have been found in the gut, frass, and larva of wood-boring insects and in their habitats (Lopes et al., 2016; Urbina and Blackwell, 2012; Nguyen et al., 2006). Although we isolated described species belonging to *Scheffersomyces*, none of known *Spathaspora* species was found in this work.

Yeast diversity in UFAM, Piquiá and Carú areas

Relative species abundance is a component of biodiversity and refers to how common or rare a species is relative to other species in a defined location or community (Avolio et al., 2019). The results of the rank abundance curve showed that yeasts were most abundant in the UFAM area, followed by Piquiá and Carú (Fig. 2). The cumulative number of species (collector's curve) also presented UFAM as the area with the more even community since its curve climbed more rapidly than Piquiá and Carú (Fig. 3). The collector's curve of a population gives the expected number of observed species and the order in which samples are added to the total affects the shape of the curve (McCabe, 2011). Variation in curve shape due to accumulation order arises from sampling error, as well as from real heterogeneity among the units sampled. To eliminate this arbitrariness, the sample order may be randomized (Colwell and Coddington, 1994). Having this in mind, we also constructed a rarefaction curve representing a number of observed species, in which the pooled samples were randomized 1000 times. The rarefaction curve is a plot of the number of species against the number of samples. The species richness was highest in UFAM (Fig. 4). Evaluating the beta diversity between the areas, the difference between Piquiá-UFAM is slightly lower compared to Piquiá-Carú (Fig. 5). The lowest similarity was found between UFAM and Carú.

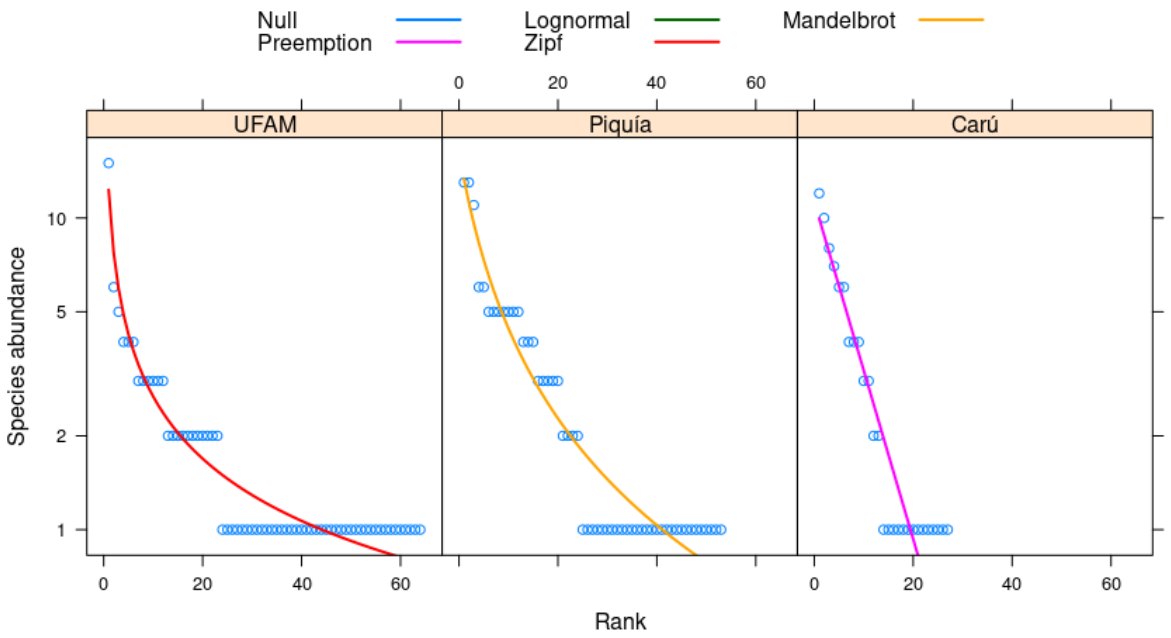

Figure 2. Rank abundance distribution per collecting site (UFAM, Piquiá, and Carú). A steep gradient indicates low evenness, and a shallow curve means high evenness. As higher the curve, more yeast species were found in the area. Carú site presented the area with less species and low evenness and UFAM showed high evenness and more species. The legend shows the types of curves.

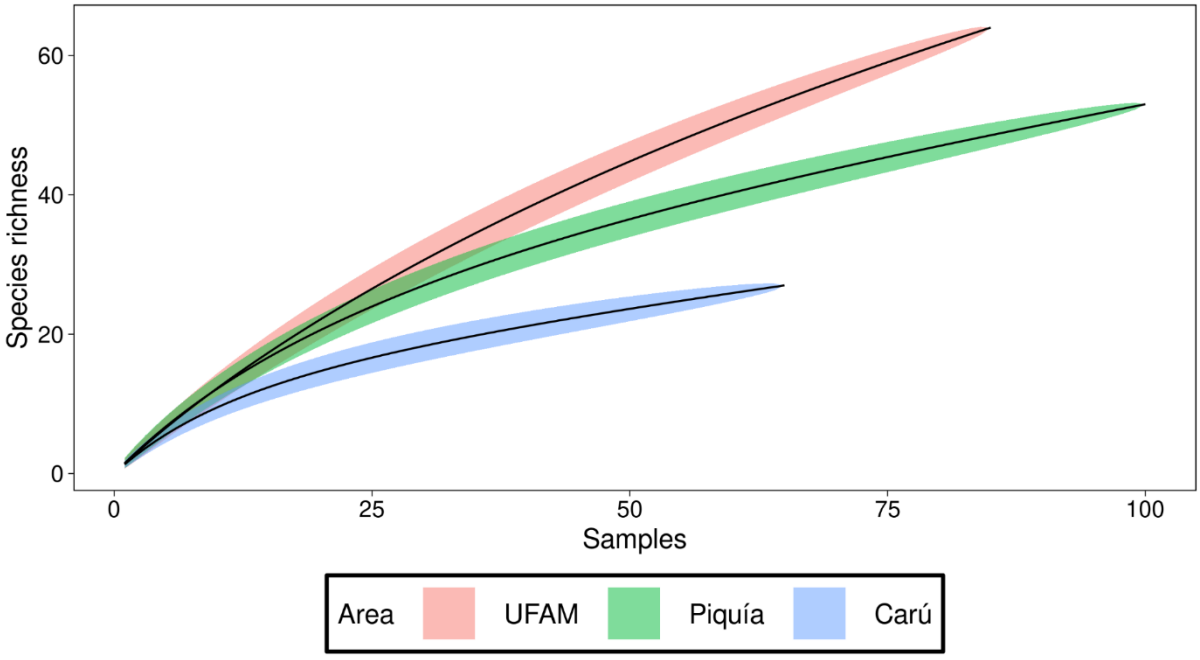

Figure 3. Cumulative number of yeast species discovered in the collecting sites.

While the Carú is a floodplain forest, known as *várzea*, UFAM and Piquiá are dryland forest (*terra firme*) characterized by a dense ombrophilous forest. *Várzea* forests are annually flooded by water white rivers with consequent enrichment of the soil (Parolin et al., 2004), but

they present less diverse and tree species richness than the adjacent drylands (Balslev et al., 1987). This was also reported by Ritter et al. (2020) for the fungal community, in which the richness was lower in floodplain areas than in drylands of Brazilian Amazonian forest. It was suggested that floodplain areas are poorer in species than dryland forests because of the impact of the annual flood. Regarding the distinct and important ecosystems of Amazonian, studies put them in the follow order of decreasing plant and animal diversity (Myster, 2016; Ritter et al., 2019): dryland tropical forests (terra-firme); *várzeas*; forests seasonally flooded by unfertile black water rivers (*igapós*); and naturally open areas associated with white-sand soils (*campinas*) (Ritter et al., 2019; Myster, 2016). Based on this work, the area characterized as *varzea* showed a lower diversity than the dryland forests concerning a group of yeasts able to assimilate lignocellulosic sugars.

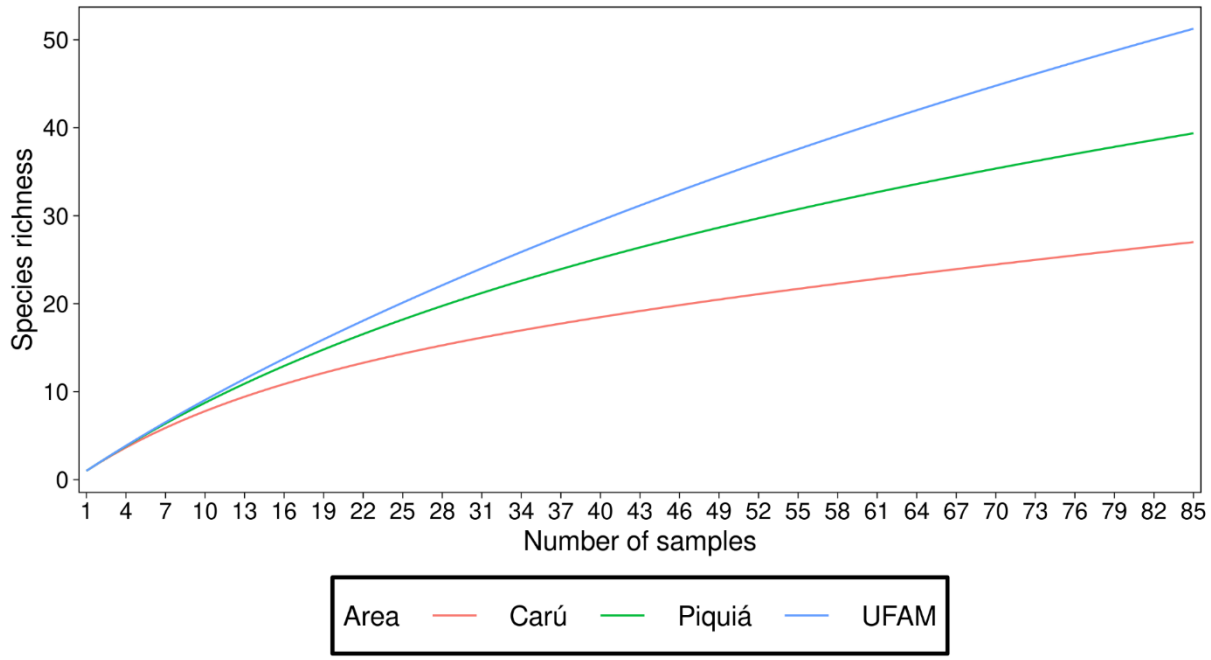

Figure 4. Rarefaction curve of the observed number of yeast species per location.

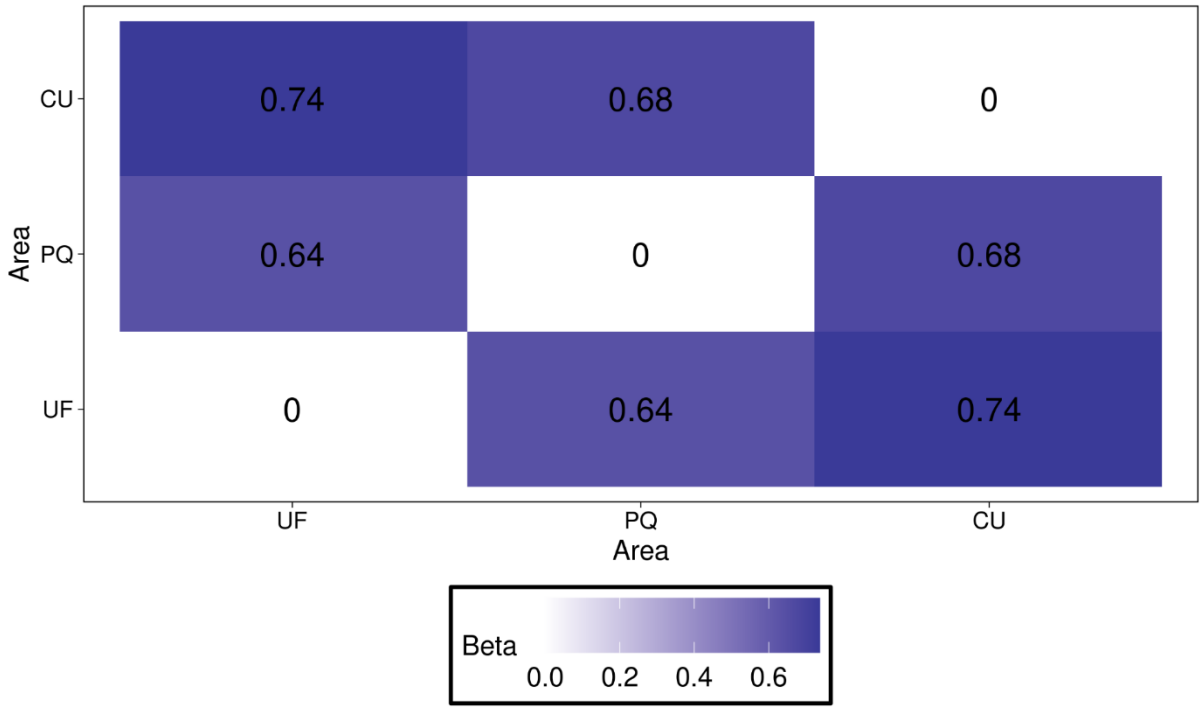

Figure 5. Beta diversity of the locations using a normalized scale from 0 to 1. The higher the value, more distinct the yeast diversity between the areas. Abbreviations: UF, UFAM; PQ, Piquiá; CU, Carú.

We used eight types of isolation medium to select species with ability to assimilate the lignocellulose-related sugars. Those media include YNB-xylan and YNB-xylose, which have already been used to isolate yeast species from rotting wood samples targeting ethanol and xylanase production (Morais et al., 2013). To increase the number of different species, two hemicellulosic hydrolysates from sugarcane bagasse and corncob were used. Both raw materials went through the same sort of pretreatment, but they had a different composition, especially the sugars concentration. Indeed, some species that were isolated from the hydrolysates, un- and diluted, were not found in YNB-xylan and xylose in this work and earlier studies (Guamán-Burneo et al., 2015; Lara et al., 2014; Morais et al., 2013). Among those species are *S. cerevisiae*, *W. edaphicus*, *Vanderwaltozyma yarrowii*, *Rh. alborubescens*, *M. caribbica*, *Pichia fermentans*, *Pichia kluyveri*, *Su. valenteae*, *K. martiniae*, *K. wufongensis*, *C. ghanaensis*, *C. saopaulonensis*, and 13 new species of the clades *Spathaspora*, *Cyberlindnera*, *Kazachstania*, *Vanderwaltozyma*, *Yarrowia*, *Torulaspora*, and *Candida*/*Lodderomyces albicans*. However,

fewer species were isolated in the hydrolysates and more species in YNB-xylan. This result was expected given the hydrolysates are much more selective than the other media due to the inhibitors and different sugars in their composition. The use of dilute hemicellulosic hydrolysate favors the isolation of yeast species capable of growing in the presence of inhibitors. These yeasts could have potential to be used or be a source of genes in experiments of genetic improvement to obtain good strains able to grow and ferment the sugars of lignocellulosic hydrolysates for generating products with biotechnological interests, for example, ethanol and xylitol (Morais et al., 2020).

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CHAPTER II

Factors influencing the xylose catabolism in the species assigned to the genera *Scheffersomyces* and *Spathaspora*

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Introduction

Xylose is the most abundant pentose of the hemicelluloses and the availability of robust microorganisms up to ferment this sugar is required for a profitable ethanol production from lignocellulosic materials (Liu et al., 2021; Zhao et al., 2020). The budding yeast *Saccharomyces cerevisiae* is a key in many biotechnological applications and one of the best understood microorganisms at the mechanistic level (Parapouli et al., 2020; Hittinger, 2013). However, it has distinct intrinsic traits compared to several non-conventional yeast species, which were not completely investigated (Riley et al., 2016). A peculiar frame of this fact is that *S. cerevisiae* does not have the ability to metabolize xylose (Sato et al., 2016), while some species do it remarkably well (Selim et al., 2020).

Species belonging to the genera *Spathaspora* and *Scheffersomyces* are known by the association with insects as well as their habitats and by the natural ability to assimilate and/or ferment xylose (Cadete and Rosa, 2017; Urbina and Blackwell, 2012). *Spathaspora passalidarum*, *Scheffersomyces stipitis*, *Scheffersomyces segobiensis*, *Scheffersomyces shehatae*, *Scheffersomyces coipomoensis*, and *Scheffersomyces ergatensis* were the first

members assigned to their respective clades (Kurtzman and Suzuki, 2010; Nguyen et al., 2006). The first two species have been source of genes for metabolic engineering to confer *S. cerevisiae* the ability to ferment xylose (Cadete et al., 2016; Jeffries and Jin, 2004).

They harbor the genes *XYL1*, *XYL2*, and *XYL3*, which encode the enzymes xylose reductase (XR) (reduces xylose into xylitol), xylitol dehydrogenase (XDH) (converts xylitol into xylulose), and xylulokinase (XK) (catalyzes the phosphorylation of xylulose), respectively (Wohlbach et al., 2011). While most xylose-fermenting species share a XR requiring NADPH as cofactor with low affinity for NADH, the XDH is NAD^+-dependent (Geijer et al., 2020). This different enzyme requirement gives rise to imbalance of cofactors with consequent xylitol accumulation in environments with absent or low oxygen level, since the NAD^+ is not or hardly regenerated (Granström et al., 2007). Unlike *Sch. stipitis*, *Sp. passalidarum* bears two copies of *XYL1*, named *XYL1.1* and *XYL1.2*. The first one encodes XR with NADPH affinity, and the enzyme encoded by the second copy prefers NADH over NADPH (Cadete et al., 2016). Interestingly, the xylose metabolism in this species is driven towards ethanol production instead of xylitol accumulation even in low aeration (Bonan et al., 2020; Hou, 2012).

The overall aim of our study was to understand why some species belonging to the genera *Spathaspora* and *Scheffersomyces* can efficiently conduct the xylose pathway, while others cannot. We hypothesized that the cofactor affinity of XR has an essential role in the great xylose metabolism capability of some yeasts as well as the XDH may be a limiting factor for some species. Although several members of those genera were described in the last years, little information is available for most of them, and the earlier studies are almost confined to *Sp. passalidarum* and *Sch. stipitis*. To assess the xylose fermentation phenotypes of species assigned to the genera *Scheffersomyces* and *Spathaspora*, we performed a series of experiments designed to test utilization, fermentation of xylose in different aeration levels, and to determine the enzyme activities of XR and XDH.

Material and methods

Yeast strains and fermentation conditions

Twenty-four strains of *Scheffersomyces* and *Spathaspora* species (Table S1) were obtained from the USDA Agricultural Research Service (ARS) NRRL Culture Collection in Peoria, Illinois, USA; Collection of Microorganisms and Cells of Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; and CBS Yeast Collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands.

Strains from all yeast species were initially plated from freezer stock on yeast extract peptone dextrose (YPD) plates and grown for single colonies. A colony of each strain was pre-cultured in 3.0 mL of YPD medium (1% yeast extract, 2% peptone, 2% glucose) overnight at 30ºC under 200 rpm. Cells were recovered by centrifugation at 2600 *g* for 10 min, washed with sterile water, and suspended in the fermentation medium at 0.5 $g_{CDW}\ L^{-1}$ (~ 0.3 OD_{600}) of cell concentration (Cadete et al., 2016). To evaluate the performance of the yeasts in different aeration conditions, we used 125-mL shake-flasks (SF) - moderate aeration, and 250-mL baffled-flasks (BF) - high aeration, both containing 50 mL of YPX (1% yeast extract, 2% peptone, 5% xylose). Fermentation assays were also carried out in 125-mL SF with 125 mL of YPX - low aeration - to test the behaviour of some yeasts in low oxygenation availability. The oxygen available in each flask was measured by a dissolved oxygen meter (Mettler Toledo F4-Field FiveGo, USA). The flasks were incubated at 30ºC under 200 rpm for 72 h and samples were taken every 24 h. Cell growth was determined by collecting 1.9 mL of the culture. The cells were recovered by centrifugation and dried in a speed vacuum concentrator. The ethanol, xylitol, and biomass yields ($Y_{p/s}^{et,\ xyl}$, $Y_{x/s}$, g g^{-1}), volumetric productivity of ethanol and xylitol ($Qp^{et,\ xyl}$, g L^{-1} h^{-1}), and consumption of D-xylose were determined as described previously (Cadete et al., 2016).

HPLC analysis

One milliliter of samples for end-product analysis were collected throughout the fermentation, centrifuged at 13500 x *g* for 5 minutes, and supernatants were stored at -20 ºC for analysis. For each sample, glucose, xylose, glycerol, xylitol, succinic acid, lactic acid, formic acid, acetic acid, pyruvate, cellobiose, and ethanol were separated by high-performance liquid chromatography (HPLC) and subsequently quantified using refractive index detection (RID). HPLC-RID was conducted with an Agilent 1260 Infinity system (Agilent Technologies, Palo Alto, CA) equipped with an Aminex HPX-87H anion-exchange column, 300 by 7.8 mm (Bio-Rad, Hercules, CA). Samples were diluted with 9 volumes of H_2O, injected into the HPLC-RID system (50-l injection volume), and eluted isocratically with 0.02 N H_2SO_4 at a flow rate of 0.5 ml/min (RID flow cell, 45°C; column, 50°C). Reference compounds (Thermo Fisher) were diluted in H_2O and used to generate a standard curve. Analyte concentrations were calculated using Chem Station software version B.04.03 (Agilent Technologies) (Schwalbach et al., 2012). Plots were constructed in R v3.6.3 using the RStudio v1.3.1073 platform.

Plasmid construction and Saccharomyces cerevisiae *transformation*

pRSCENSynHyg-TDH3p-GFP-CYC1t is a CEN plasmid containing the Hygromycin B resistance marker. It also has the *TDH3* promoter, which is from the *TDH3* gene that encodes glyceraldehyde-3-phosphate dehydrogenase enzyme involved in metabolism of fermentable sugars and non-fermentable carbon sources (Duveau et al., 2017). It was constructed by digesting DNA fragments with the enzymes *Nhe*I-HI and *Eco*RI-HI, followed by recovery and amplification in *Escherichia coli* by selection on LB (1% tryptone, 1% NaCl, 0.5% yeast extract) + carbenicillin. Modifications of parts were introduced during PCR using oligonucleotides.

Plasmid pRSCENSynHyg-TDH3p -GFP-CYC1t was used for metabolic engineering of xylose fermentation in *S. cerevisiae* S288C. *Spathaspora passalidarum* UFMG-CM-Y469

XYL1.1 and *XYL1.2* genes, *Scheffersomyces xylosifermentans* CBS 12540^T *XYL1* gene, and *Sheffersomyces parashehatae* CBS 12535^T *XYL1* gene were, respectively, amplified with the designed primers oHKB29/oHKB30, oHKB37/oHKB38, oHKB31/oHKB32, and oHKB33/oHKB34 (Table S2). Phusion Hot Start High-Fidelity DNA Polymerase was used for PCR amplification (Thermo Scientific, Waltham, USA). The PCR product was purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The vector inserted in *E. coli* was purified with ZR Plasmid Miniprep – Classic kit (Zymo Research, Irvine, USA) and digested with two endonucleases, *Nhe*I-HI and *Eco*RI-HI (New England BioLabs, Ipswich, USA), to excise the GFP. *S. cerevisiae* was grown in glass tubes with 3 mL of YPD medium and incubated at 30ºC overnight, then inoculated in 5 mL of YPD at 0.2 OD_{600}, and incubated until 0.1 OD_{600}. Transformation of yeast strains was done using the lithium acetate/PEG-4000/carrier DNA method according to Gietz and Woods (2001), using a specific temperature for *S. cerevisiae* (30 and 42ºC). After the transformation, the colonies were selected by replica plating using YPD agar with 50mg/mL of Hygromycin B. Colonies were submitted to Sanger sequencing to confirm the gene insertion.

Enzyme activities

Yeasts were grown in YPX medium as described previously (Cadete et al., 2016), using both SF and BF. In the case of *Saccharomyces cerevisiae* transformants, the yeasts were inoculated in YPD medium. Cells were recovered in the mid-log phase, washed with sterile water, and submitted to crude cell-free extracts with Y-PER® Yeast Protein Extraction Reagent (Thermo Fisher). Protein concentrations in the cell-free extract were determined by BCA Protein Assay Kit (Thermo Fisher). Enzyme activities were determined by oxidation or reduction of the coenzymes at 340 nm using Tecan® (Infinite M-1000, Switzerland) at 25ºC and a total measuring time of 1h30min. Kinetic parameters of XR for xylose reduction were obtained in a reaction mixture containing 200 mM triethanolamine buffer pH 7.0, 10 mM NAD(P)H, 2 M D-

xylose, cell-free extract and deionized water, while kinetic parameters of XDH for xylitol oxidation were obtained in a reaction mixture of glycine buffer 200 mM pH 9.0, $MgCl_2$ 500 mM, $NAD(P)^+$ 60 mM, xylitol 2 M, cell-free extract, and deionized water (Cadete et al., 2016).

We used 96-well plates (Corning® 96 Well Clear Flat Bottom UV-Transparent, Darmstadt, Germany) with 250 uL as final volume. Extract proteins from the yeasts *Spathaspora passalidarum* UFMG-CM-Y469 were used as positive control and *Saccharomyces cerevisiae* 288SC as a negative control. In addition, negative controls containing the reaction mixture without substrate (xylose or xylitol) were done for each sample. Enzyme activities were determined in quadruplicate. A value of 6220 mM^{-1} cm^{-1} was used for the absorption coefficient of NAD(P)H. One unit was defined as the generation of 1 μmol NAD(P)H per min. The specific enzyme activities were given in units (U) per mg protein. Data analyses and plots were performed in R v3.6.3 using the RStudio v1.3.1073 platform.

Results and discussion

Scheffersomyces *and* Spathaspora *species switch their end-products in moderate and high aeration*

Xylitol is an intermediate of the xylose catabolism that is accumulated by several species due to a bottleneck in oxidizing it into xylulose (Palladino et al., 2021; López-Linares et al., 2018). If the xylitol is converted into xylulose, this molecule is phosphorylated to xylulose-5-phosphate, which goes to the pentose phosphate pathway (PPP) (Atzmüller et al., 2020). After reactions resulting in the formation of pyruvate, this molecule is converted into ethanol by pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (Adh) in alcoholic fermentations (Bruder and Boles, 2017). Both ethanol and xylitol were the major bioproducts from xylose fermentation among *Spathaspora* and *Scheffersomyces* species. Not frequently, glycerol was produced. We referred to ethanol and xylitol producers in which the metabolism was driven

mainly to one of these metabolites in SF, since it was the tested condition that most favored the metabolite production. The ethanol producers were *Scheffersomyces xylosifermentans*, *Sch. parashehatae*, *Sch. virginianus*, *Sch. shehatae*, *Sch. stipitis*, *Sch. illinoinensis*, *Sch. cryptocercus*, *Spathaspora arborariae*, *Sp. passalidarum*, and *Sp. gorwiae*. The xylitol producers were represented by *Scheffersomyces coipomoensis*, *Sch. insectosa*, *Sch. amazonensis*, *Sch. quercinus*, *Sp. brasiliensis*, *Sp. suhii*, *Sp. roraimanensis*, *Sp. girioi*, *Sp. hagerdaliae*, *Sp. xylofermentans*, and *Candida (Spathaspora) materiae*.

Of the twenty-four yeast strains of *Spathaspora* and *Scheffersomyces*, three were not capable of fermenting xylose. *Sch. spartinae* and *Sch. gosingicus* showed low growth and modest consumption of sugar with no production of any metabolite, and *Sch. ergatensis* did not grow in YPX at all. The fermentative parameters related to the consumption of D-xylose and the production of biomass, ethanol, and xylitol under moderate and high aeration are summarized in Table S2.

Among the ethanol producers, *Sp. passalidarum, Sch. xylosifermentans,* and *Sch. parashehatae* are remarkable species. They consumed 99% of xylose and reached the highest levels of ethanol in 24 h of fermentation (Fig. 1). At this point, they started to respire the ethanol to keep proliferating. The behavior observed in moderate aeration was not similar under high aeration. The dissolved oxygen level in BF (4.74 mg L^{-1}) is double the oxygen present in SF (2.63 mg L^{-1}), which had a great impact on the performance of the yeasts. For example, the maximum ethanol yield of *Sch. xylosifermentans* was 0.42 g g^{-1} (21 g L^{-1}), with 0.87 g $h^{-1}L^{-1}$ of productivity, and the consumption of xylose content was 99% in 24 h under moderate oxygen limiting-condition, this species used 70% of the sugar in 72 h and no ethanol yield was detected during the batch in BF (Fig. 2). Surprisingly, the best xylose fermenters produced xylitol and/or glycerol, instead of ethanol, as the main fermentation product under high aeration. *Sch. xylosifermentans* and *Sch. parashehatae* accumulated 0.12 g g^{-1} (4.10 g L^{-1}) and 0.16 g g^{-1} (8.10

g L^{-1}) of xylitol, respectively, and *Sp. passalidarum* produced 8.32 g L^{-1} of glycerol. The maximum quantity of these end-products took place between 48 and 72 h of fermentation. *Sp. passalidarum* and *Sch. parashehatae* consumed 99% of the xylose in 72 h. The increased oxygen level favored the production of biomass. *Sp. passalidarum*, *Sch. xylosifermentans*, and *Sch. parashehatae* achieved, in this order, biomass yields of 0.36 g g^{-1}, 0.43 g g^{-1}, and 0.31 g $g^{-1}$of biomass in BF, and 0.14, 0.19, and 0.22 g g^{-1} in SF (Table S2).

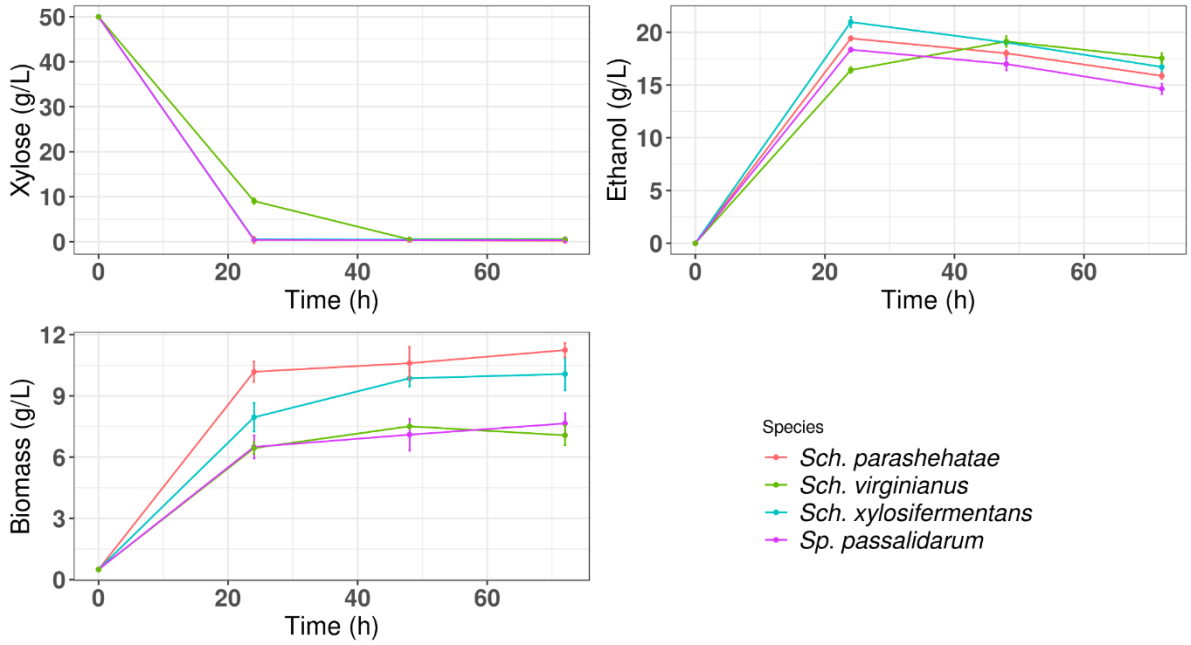


Figure1. Fermentation performance by *Scheffersomyces parashehatae*, *Sch. virginianus*, *Sch. xylosifermentans*, and *Spathaspora passalidarum* under moderate aeration. Error bars indicate the standard deviation from the two independent replicates.

Scheffersomyces virginianus also carried on the xylose pathway favorably under moderate aeration as well, reaching 0.39 g g^{-1} (19 g L^{-1}) of ethanol yield in 48 h of fermentation and depleting 99% of the xylose (Fig. 1). Along with this yeast are *Sch. shehatae*, *Sch. illinoinensis*, *Sch. cryptocercus*, *Sch. stipitis*, and *Sp. arborariae*, with the ethanol yields ranging from 0.29 to 0.36 g g^{-1}(Fig. S1A). The metabolite production by them was also switched

from ethanol to xylitol in BF, except for *Sch. illinoinensis* (Fig. S1B). It was the only species in the ethanol producer group able to produce ethanol in high aeration. In this condition, *Sp. arborariae* did not accumulate xylitol, but generated negligible glycerol yield. *Sch. cryptocercus* and *Sch. stipitis* were the species that reached the highest and the lowest amount of xylitol in BF, respectively. *Sch. gorwiae* produced low ethanol yield in both aeration conditions, but also presented a little xylitol production under high oxygen level. This species took up 46% of the sugar in moderate aeration, and it consumed 93% of the xylose in high aeration.

For all the bioethanol producers, cell biomass increased substantially when the yeasts did grow in BF (Fig. 2; Table S2; Fig. S1B). It was expected that the metabolism was directed mainly towards cellular respiration in high aeration, producing high yield of ATP and using it for cell multiplication. In general, under moderate aeration the yeasts produced more total weight of end-products (ethanol/xylitol) while with plenty of oxygen this did not occur. Despite the xylitol production by the ethanol producers, this result suggests that most of the carbon was converted into carbon dioxide (CO_2). A possible interpretation is that these yeasts may have produced ethanol which was simultaneously consumed generating CO_2.

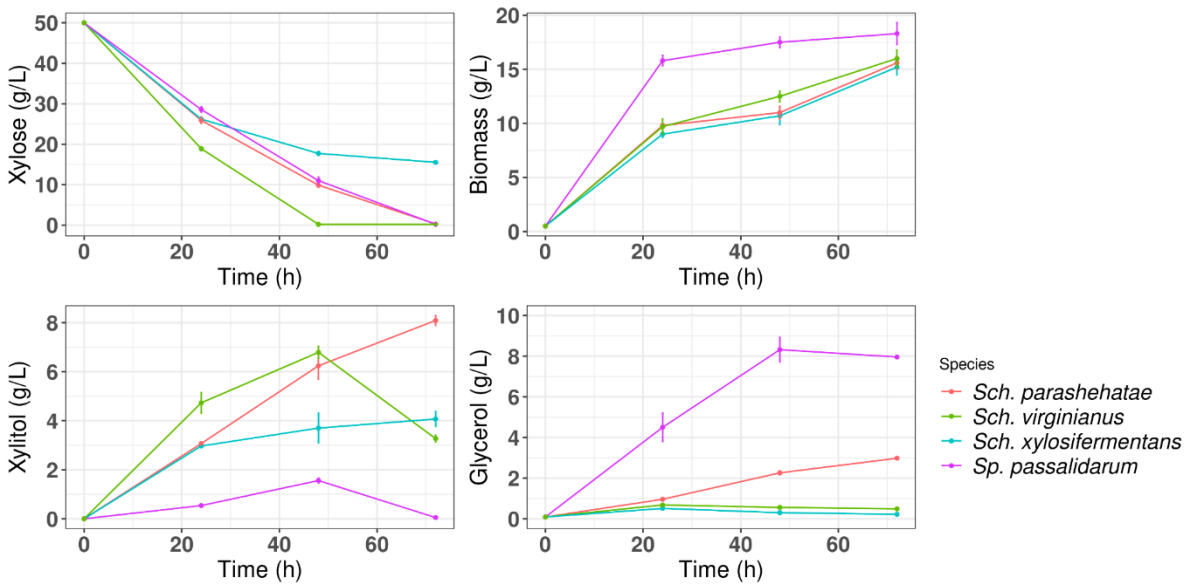


Figure 2. Growth, xylose consumption, and production of glycerol and xylitol under high aeration by *Scheffersomyces parashehatae*, *Sch. virginianus*, *Sch. xylosifermentans*, and *Spathaspora passalidarum*. Error bars indicate the standard deviation from two independent replicates.

Regarding the xylitol producers, the yeasts accumulated considerable amounts of this polyol in moderate condition with yields and productivity ranging 0.32-0.68 g g^{-1} and 0.27-0.75 g h^{-1} L^{-1}, respectively (Fig. S2A). *Scheffersomyces insectosa*, *Sch. amazonensis*, *Sch. quercinus*, *Sp. hagerdaliae*, and *C. materiae* kept producing xylitol in high aeration, but with low yields and productivity (Fig. S2B). However, *Sch. coipomoensis* produced 0.17 g g^{-1} (6.51 g L^{-1}) of ethanol. And only species of the genus *Spathaspora* produced glycerol as the main product of fermentation, including *Sp. xylofermentans*, *Sp. roraimanensis*, *Sp. brasiliensis*, and *Sp. girioi* in BF.

Considering that the NADH is reoxidized by the respiratory chain with oxygen as the final electron acceptor (Dekker et al., 2021), anaerobic conditions may result in accumulation of NADH with repression of the metabolic activity (Vandeska et al., 1995). The stock of NADH varies with the degree of the oxygen-limiting condition. For example, when the yeasts *Sch. xylosifermentans*, *Sch. parashehatae*, *Sp. passalidarum*, and *Sch. virgianus* were submitted to an environment supplied with low aeration (1.54 mg L^{-1} dissolved oxygen), they still reached high levels of ethanol, but the maximum production was delayed and the ethanol productivity decreased (Table 1) compared to the moderate aeration. Under high aeration, the oxygen available could handle the reoxidation of NADH and enough NAD^+ would be expected to be available for XDH to oxidize xylitol into xylulose and achieve ethanol as final product. However, the fermentation results showed an accumulation of xylitol by most ethanol producers. A recent study showed that the aeration can negatively affect the optimal activity of enzymes, such as invertase and catalase (Ge et al., 2020). We hypothesized that the excess of oxygen is exerting a sort of repression on XDH.

Table 1: Sugar consumption and product formation from 50 g L^{-1} of xylose under low aeration.

Species	Time (h)	Xylose (%)*	Ethanol (g L^{-1})	$Y_{p/s}^{et}$ (g g^{-1})†	Qp^{et} (g/h L^{-1})‡	η^{et} (%)	Xylitol (g L^{-1})	$Y_{p/s}^{xyl}$ (g g^{-1})†	Biomass (g_{CDW} L^{-1})
Sp. passalidarum	18	20	3.09 ± 0.1	0.29	0.17	57	1.05 ± 0.1	0.10	2.09 ± 0.1
	24	38	6.10 ± 0.50	0.32	0.25	62	2.08 ± 0.25	0.11	2.88 ± 0.06
	48	87	15.1 ± 0.34	0.35	0.31	68	3.96 ± 0.3	0.09	3.60 ± 0.1
	72	99	18.0 ± 0.47	0.36	0.25	71	4.23 ± 0.3	0.09	3.90 ± 0.8
Sch. xylosifermentans	18	35	7.69 ± 0.33	0.43	0.43	84	0.13 ± 0.01	0.01	1.33 ± 0.1
	24	51	11.2 ±0.14	0.44	0.47	86	0.29 ± 0.3	0.01	2.07 ± 0.48
	48	96	21.0 ±0.1	0.43	0.43	84	0.69 ± 0.2	0.01	2.82 ± 0.1
	72	99	21.4 ±0.2	0.43	0.30	85	0.69 ± 0.1	0.01	3.24 ± 0.2
Sch. parashehatae	18	32	6.64 ±0.21	0.41	0.37	79	0.98 ± 0.3	0.06	2.50 ±0.23
	24	55	10.6 ±0.4	0.39	0.44	75	2.10 ± 0.1	0.08	3.45 ±0.1
	48	98	19.1 ±0.02	0.39	0.40	76	3.49 ± 0.1	0.07	5.47 ±0.11
	72	99	18.9 ±0.2	0.39	0.26	75	3.41 ± 0.14	0.07	5.25 ±0.49
Sch. virginianus	18	20	4.22 ±0.38	0.41	0.23	81	0.41 ± 0.24	0.04	1.90 ± 0.3
	24	35	7.05 ±0.16	0.40	0.29	78	0.79 ± 0.2	0.04	2.52 ±0.3
	48	77	15.4 ±0.2	0.40	0.32	78	2.28 ± 0.48	0.06	3.50 ± 0.4
	72	97	19.5 ±0.06	0.40	0.27	79	2.65 ± 0.2	0.05	3.77 ± 1.3

* xylose consumption (%) – initial D-xylose consumed.
† $Y_{p/s}^{et}$ (g g^{-1}) and $Y_{p/s}^{xyl}$ (g g^{-1}) – ethanol, and xylitol yield, respectively: correlation between ethanol or xylitol (ΔP) produced with sugar (ΔS) consumed.
‡ Qp^{et}(g L^{-1} h^{-1}) – ethanol productivity: ratio between ethanol concentration (g L^{-1}) and time (h).
‣ η^{et} (%) – conversion efficiency: percentage of the maximum theoretical ethanol per g D-xylose).

Mean values ± SD from three independent experiments.

According to our understanding of *S. cerevisiae* metabolism, the production of glycerol by *Spathaspora passalidarum* in high aeration is difficult to explain, especially since *Sp. passalidarum* produced a considerable amount in BF. Glycerol is a link to maintain the redox balance in the absence of oxygen when ethanol flux is overloaded (Medina et al., 2010).

Species with multiple XYL1 copies accumulate xylitol under moderate and high aeration

Recently, a study reported the presence of multiple copies of *XYL1* in *Candida intermedia*, which harbors three copies of the gene, one of which (called by *XYL1.2*) encodes a XR with more affinity for NADH (Geijer et al., 2020). It displays dual cofactor specificity like the *XYL1.1* from *Meyerozyma guilliermondii* and *XYL1.2* from *Sp. passalidarum* (Geijer et al., 2020; Cadete et al., 2016). During our search for homologs across the genera *Spathaspora* and *Scheffersomyces* and some closely related species, we found that *Candida blattae*, *Candida carpophila*, and *Meyerozyma caribbica* also have the gene duplication. We tested those yeasts, including *C. intermedia* and *M. guilliermondii*, in YPX under moderate and high aeration to check their xylose fermentation performance. Even though these species have multiple copies of *XYL1*, the duplication gene does not mean that the organism will have a fitness advantage (Diss et al., 2017). Indeed, all the outgroups accumulate xylitol in both conditions, but less yield was achieved in BF compared to SF (Fig 5). *C. intermedia* and *C. blattae* also produced a low amount of ethanol. The maximum xylose consumption in SF was achieved by *C. blattae*. It consumed 75% of the sugar in 72 h, while the others consumed between 40 to 60%. On the other hand, the xylose depletion was almost complete in high aeration by all the outgroups in 48 h (Fig. 6).

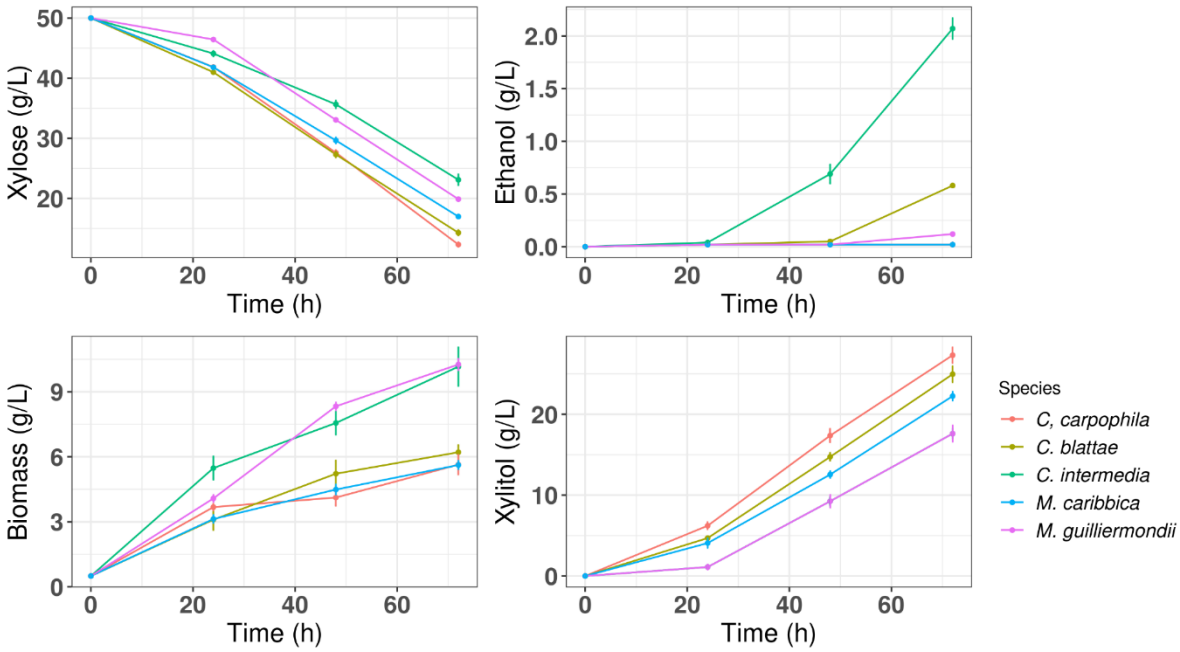


Figure 3. Time course of substrate consumption and product formation under moderate aeration fermentation by outgroups that bear multiple copies of *XYL1* gene. Error bars indicate the standard deviation from the three independent replicates.

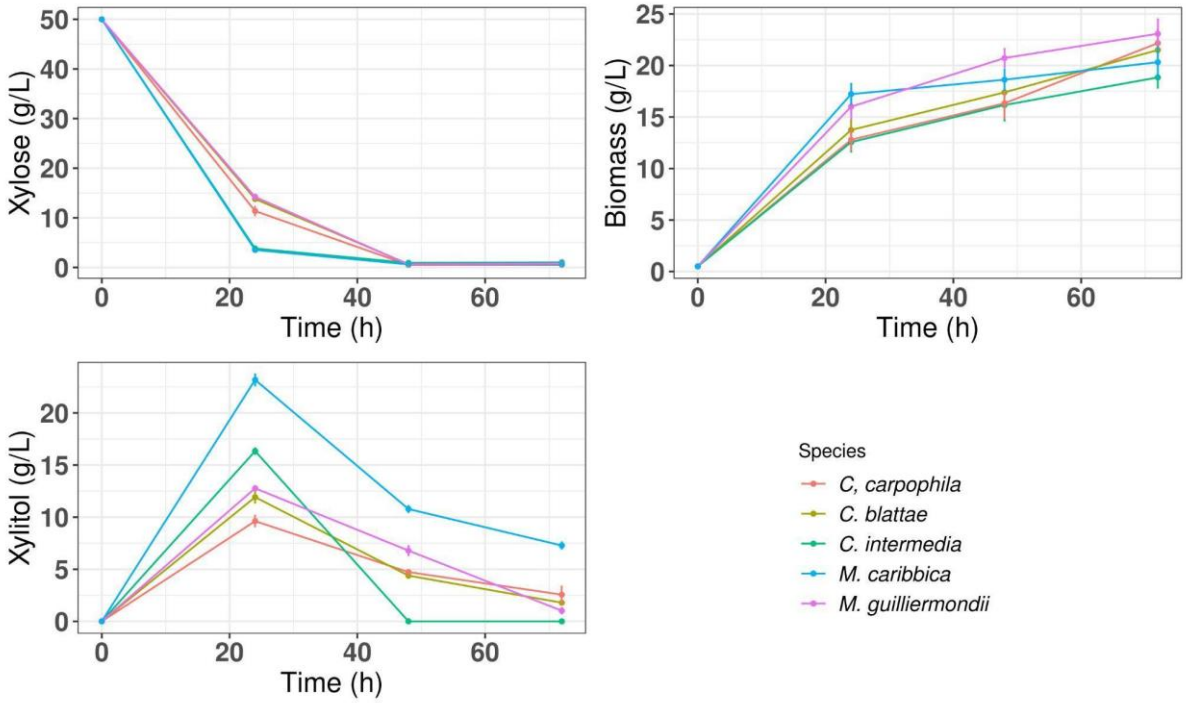


Figure 4. Fermentation performance by the outgroups under high aeration. Error bars indicate the standard deviation from the three independent replicates.

Xylose reductases from Scheffersomyces species have dual-cofactor affinity

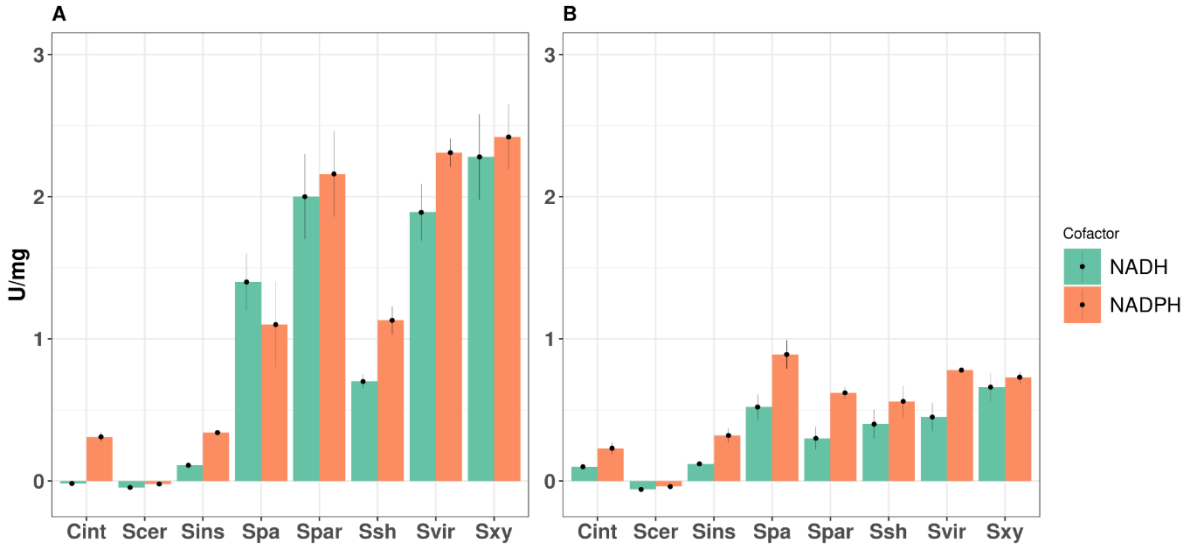
To determine the cofactors preference of the enzymes associated with the first steps of xylose metabolism and to evaluate the influence of oxygen on their affinity, we measured the enzyme activities of XR and XDH from *Sp. passalidarum*, *Sch. parashehatae*, *Sch. virginianus*, *Sch. xylosifermentans*, *Sch. shehatae*, *Sch. insectosa*, and *C. intermedia* (Fig 5). For that, NAD(P)H and xylose were the substrates for XR, $NAD(P)^+$ and xylitol for XDH. We used the crude cell extract after growing the yeasts in YPX under moderate and high aeration.

Xylose reductase from *Sp. passalidarum* used NADH and NADPH under moderate aeration, but the activity was higher for NADH (Fig. 5A). The opposite occurred in BF and the enzyme used more NADPH (Fig. 5B). A similar result was obtained previously, in which high aeration led XR from *Sp. passalidarum* NRRL Y-27907 to switch the cofactor preference (Bonan et al., 2020). We associated this behavior with the presence of two copies of *XYL1*. The paralogous genes may have different levels of expression in both conditions. Cadete et al. (2016) showed that *XYL1.2* is 15-16-fold more expressed in UFMG-CM-Y469 (the same strain used in this work) and 9-10-fold in NRRL Y-27907 than *XYL1.1* in moderate and severe oxygen-limiting conditions. It does make sense since the second copy encodes NADH-preferring XR, and the yeast may use it as a mechanism to maintain the redox balance when the oxygen level is low. The XDH from *Sp. passalidarum* is strictly NAD^+-dependent (Fig. 5C, D). In high oxygen availability, the activity of the enzyme was decreased, but among the other species tested in this experiment, it was the yeast with the highest XDH activity. This result is interesting because *Sp. passalidarum* accumulated negligible amounts of xylitol in BF, and the main fermentation product was glycerol, unlike the other species.

Sch. xylosifermentans, *Sch. parashehatae*, *Sch. shehatae*, and *Sch. virginianus* harbor only a copy of the gene and their xylose reductases presented dual cofactor affinity. Although they had a preference for NADPH, they also showed a high activity with NADH, especially the

XRs from *Sch. xylosifermentans*, *Sch. parashehatae*, and *Sch. virginianus* (Fig. 5A). Correlating the enzyme activity with the xylose fermentation results, the considerable activity with NADH and NADPH as substrates may facilitate the fast xylose consumption and the high ethanol yields produced by these species under moderate and low aeration conditions. In addition, XDH also expressed notable activity in RF (Fig. 5C). Interestingly, this activity decreased significantly in BF, where they produced xylitol (Fig. 5D).

If we compare those species to *Sch. insectosa* and *C. intermedia*, which are xylitol producers, the XDHs of both yeasts presented low activity under moderate and high aeration. The same occurred with XRs, which also showed dual-cofactor specificity but with low enzyme activity. Xylose reductase from *C. intermedia* did not use NADH in moderate aeration condition. *C. intermedia* accumulated more xylitol in BF than in SF, and the xylose was completely consumed with high aeration. In the case of *Sch. insectosa*, the total xylose content was consumed in 72 h of fermentation and xylitol in both conditions.



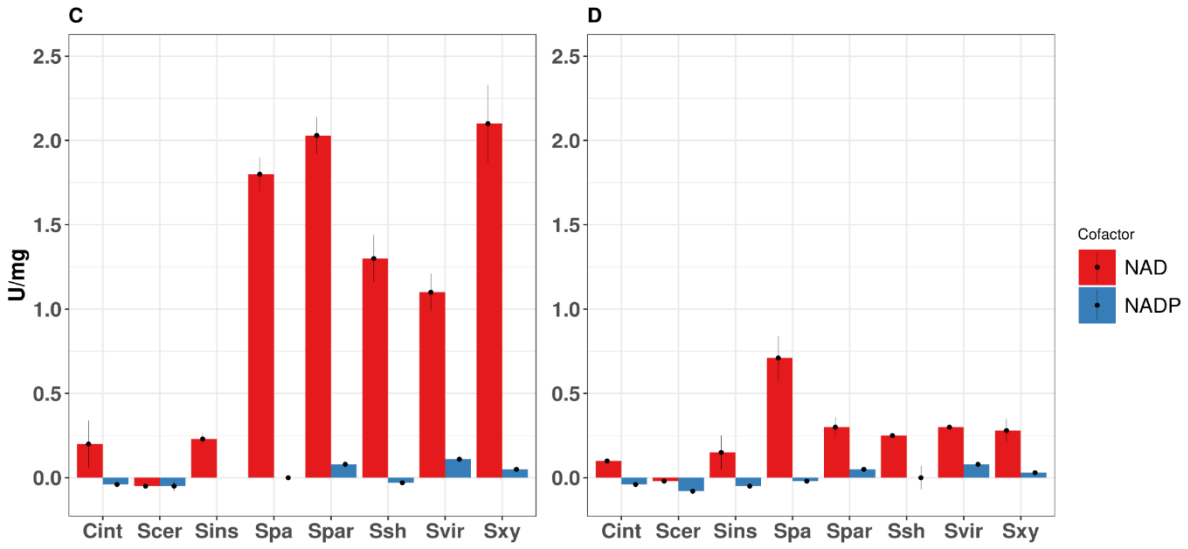


Figure 5. Xylose reductase (XR) and xylitol dehydrogenase (XDH) activities A, C) under moderate aeration, and B, D) high aeration expressed in units (U) per mg protein [U (mg protein)$^{-1}$]. Abbreviations: Cint - *Candida intermedia*, Scer - *Saccharomyces cerevisiae* (negative control), Sins - *Scheffersomyces insectosa*, Spa - *Spathaspora passalidarum* (positive control), Spar - *Scheffersomyces parashehatae*, Svir - *Scheffersomyces virginianus*, Sxy - *Scheffersomyces xylosifermentans*. Error bars indicate the standard deviation from the three independent replicates.

To characterize the XR from *Sch. xylosifermentans* (ScxyXR) and *Sch. parashehatae* (ScpaXR), their genes were individually expressed in *S. cerevisiae* S288C. We used *XYL1.1* and *XYL1.2* from *Sp. passalidarum* (SpaXR1 and SpaXR2, respectively) as positive controls. As the plasmid had a constitutive promoter - *TDH3* - the yeasts were cultured in YPD, and then we performed the enzyme activity assay. All the XRs showed dual-cofactor affinity, except for SpaXR1, which had strict preference for NADPH as reported earlier (Cadete et al., 2016). The NADH/NADPH ratio was higher than one for SpaXR2 and ScxyXR, which indicates that the enzymes have more affinity for NADH than NADPH (Table 3). Considering the standard deviation of the ScxyXR, the enzyme has a similar affinity for both cofactors.

Table 3: Enzyme activities from XR in *S. cerevisiae* S288C transformants.

Species	NADH	NADPH	Ratio NADH/NADPH
S. cerevisiae S288C + *XYL1.1 Sp. passalidarum*	0.02 ± 0.01	0.70 ± 0.05	0.03
S. cerevisiae S288C + *XYL1.2 Sp. passalidarum*	0.60 ± 0.12	0.34 ± 0.1	1.80
S. cerevisiae S288C + *XYL1 Sch. xylosifermentans*	1.36 ± 0.20	1.30 ± 0.07	1.05
S. cerevisiae S288C + *XYL1 Sch. parashehatae*	0.54 ± 0.10	1.1 ± 0.09	0.49

Mean values ± SD from three independent experiments.

The best xylose-fermenting yeasts harbor genes that encode XR with affinity for NADH and NADPH in addition to their high activity. The XDH from them also catalyzes the reactions efficiently in moderate aeration. Based on these results and correlating them with the ones from the fermentation assays, when the oxygenation was increased both enzymes had their activities reduced. Indeed, fermentation performance is negatively influenced by oxygen availability as shown here and in earlier works (Veras et al., 2017; Hagman and Piškur, 2015). Combining the enzyme activities shown in Figure 5 and the fact that xylose was still consumed and xylitol was produced by *Sch. xylosifermentans*, *Sch. parashehatae*, and *Sch. virginianus* in BF, these results imply that the excess of oxygen has a greater impact on the XDH than XR.

Xylose reductase is the first enzyme in the xylose pathway and it controls the rate of xylose utilization. Also, high affinity for cofactors binding ensures that it will be tightly bound to the enzyme and the reaction will proceed even with small concentration of the cofactor in the cell (Hossain et al. 2018). *Sch. insectosa* achieved 0.56 g g^{-1} (26.1 g L^{-1}) of maximum xylitol yield and 90% of xylose was consumed in 48 h. The values in BF were 0.36 g g^{-1} (18.2 g g^{-1}) of maximum xylitol yield and 99% of xylose taken up in 48 h. The xylitol yield was closer to the maximum theoretical yield of xylitol (0.917 g g^{-1}) in SF than the one in BF, and the consumption of xylose was high in both conditions. Associating this result with the enzyme activities, the XR from this species showed low activity for NAD(P)H and XDH for NAD^+ in SF and BF. Under moderate aeration, which still allows oxygenation for NAD^+ regeneration,

Sch. insectosa accumulated a considerable amount of xylitol as well as several species and xylose was completely consumed. These results suggest that the level of the affinity between enzyme and cofactor is essential to the success of the xylose pathway and XDH may be the main limiting factor of some yeasts, especially xylitol producers.

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Supplementary material

Table1: Species strains used in this study and their respectives isolation sources.

Species	Strain	Source
Sch. amazonensis	UFMG-HMD-26.3	rotting wood (Cadete et al., 2012)
Sch. coipomoensis	NRRL Y-17651^T	rotten log (Urbina and Blackwell, 2013)
Sch. cryptocercus	NRRL Y-48824^T	gut of wood cockroach (Urbina and Blackwell, 2013)
Sch. ergatensis	NRRL Y-17652^T	larva of bark beetles (Urbina and Blackwell, 2013)
Sch. gosingicus	CBS 11433^T	soil (Chang et al., 2011)
Sch. illinoinensis	NRRL Y-48827^T	rotten wood (Urbina and Blackwell, 2012)
Sch. insectosa	NRRL Y-12854^T	*Leptura maculicornis* (Kurtzman and Suzuki, 2010)
Sch. parashehatae	CBS 12535^T	larva of *O. disjunctus* (Suh et al., 2013)
Sch. quercinus	NRRL Y-48825^T	rotten wood (Urbina and Blackwell, 2012)
Sch. shehatae	NRRL Y-12858^T	soil (Urbina and Blackwell, 2012)
Sch. spartinae	NRRL Y-7322^T	oyster grass (Kurtzman and Suzuki, 2010)
Sch. stipitis	NRRL Y-7124^T	beetle (Kurtzman and Suzuki 2010)
Sch. virginianus	NRRL Y-48822^T	rotten wood (Urbina and Blackwell, 2012)
Sch. xylosifermentans	CBS 12540^T	insect tunnel (Suh et al., 2013)
Candida (Sp.) materiae	UFMG-07C151B^T	rotting wood (Barbosa et al., 2009)
Sp. arborariae	UFMG-HMD-32.1	rotting wood (Cadete et al., 2009)
Sp. brasiliensis	UFMG-HMD-19.3	rotting wood (Cadete et al., 2013)

Species	Strain	Source
Sp. girioi	UFMG-CM-Y302^T	rotting wood (Lopes et al., 2016)
Sp. gorwiae	UFMG-CM-Y312^T	rotting wood (Lopes et al., 2016)
Sp. hagerdaliae	UFMG-CM-Y303^T	rotting wood (Lopes et al., 2016)
Sp. passalidarum	UFMG-CM-Y469	rotting wood (Cadete et al., 2013)
Sp. roraimanensis	UFMG-HMD-23.2^T	rotting wood (Cadete et al., 2013)
Sp. suhii	UFMG-CM-Y475^T	rotting wood (Cadete et al., 2013)
Sp. xylofermentans	UFMG-CM-Y478^T	rotting wood (Cadete et al., 2013)

Table 2: Primer sequences used in this study.

Codes / Species gene / Direction	Sequences
oHKB29 (*Sp. passalidarum XYL1.1*)_F	ATGGCTACTATTAAATTATCCTC
oHKB30 (*Sp. passalidarum XYL1.1*)_R	TTAAACGAAGATTGGAATGT
oHKB37 (*Sp. passalidarum XYL1.2*)_F	ATGTCTTTTAAATTATCTTCAGGTTAT
oHKB38 (*Sp. passalidarum XYL1.2*)_R	TTAAACAAAGATTGGAATATGGTC
oHKB31 (*Sch, xylosifermentans XYL1*)_F	ATGGTTGCCCCAATCCCACA
oHKB32 (*Sch, xylosifermentans XYL1*)_R	TTAAACGAAGATTGGGATATGGTCCCAGTC
oHKB33 (*Sch. parashehatae XYL1*)_F	ATGAGCCCAAGCCCAATTCC
oHKB34 (*Sch. parashehatae XYL1*)_R	TTAAACGAAGATTGGAATGTGGTCCCA

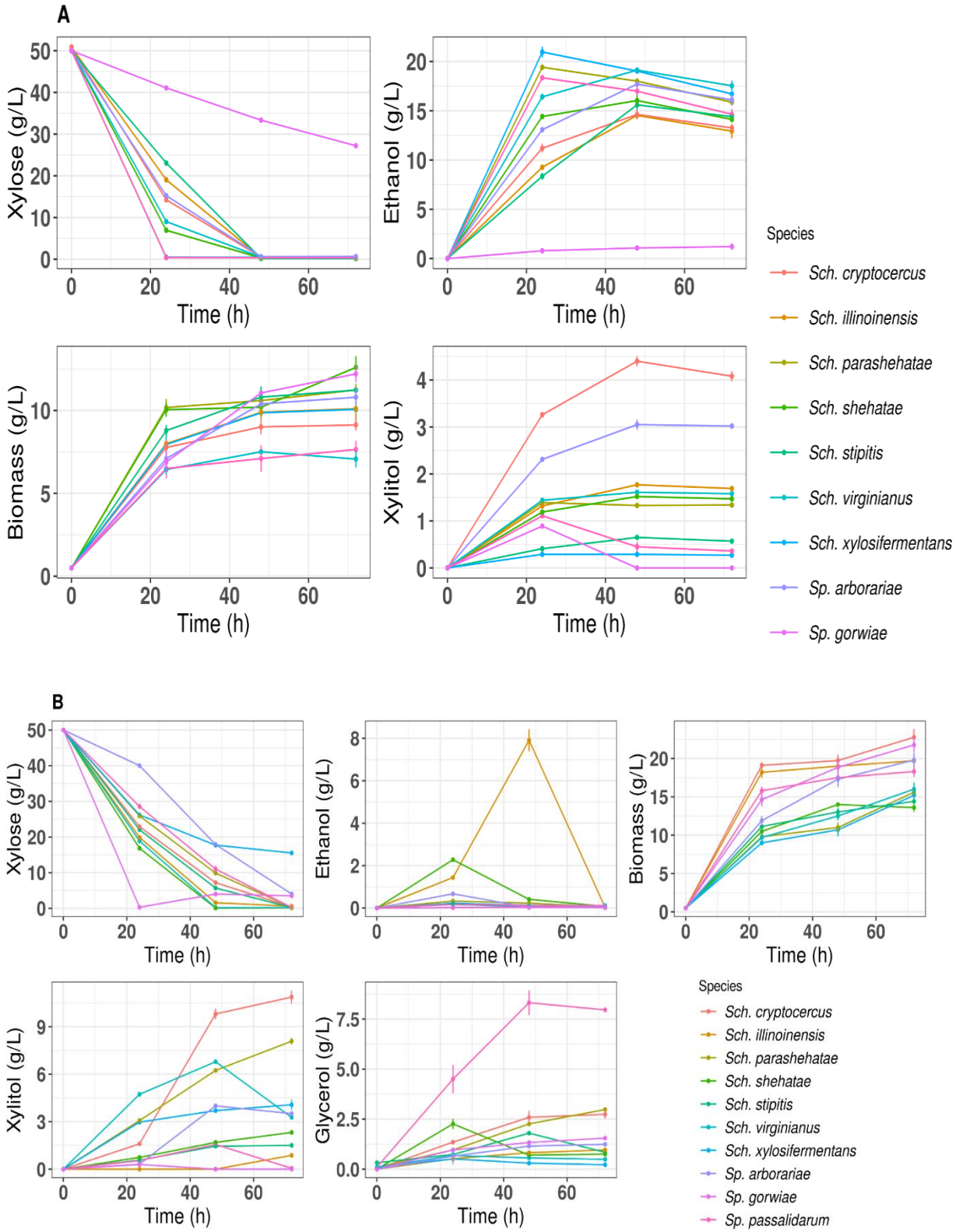
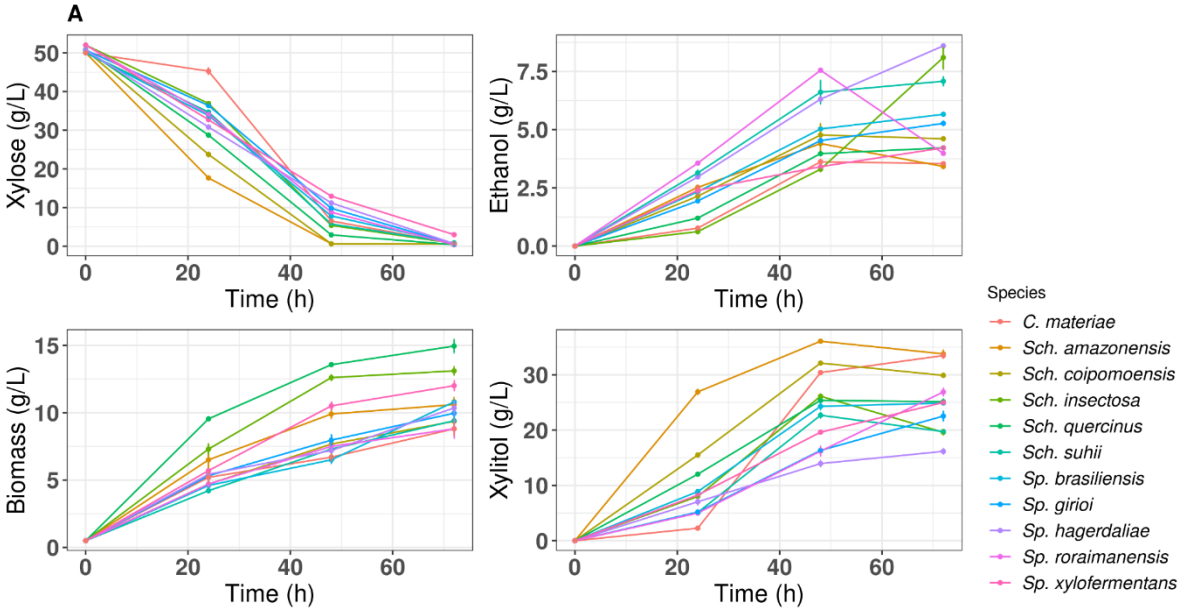


Figure S1. Consumption of xylose and production of biomass, ethanol, xylitol, and glycerol by the ethanol producers under A) moderate aeration and B) high aeration conditions.


A
Xylose (g/L)
Ethanol (g/L)
Biomass (g/L)
Xylitol (g/L)
Time (h)
Species
C. materiae
Sch. amazonensis
Sch. coipomoensis
Sch. insectosa
Sch. quercinus
Sch. suhii
Sp. brasiliensis
Sp. girioi
Sp. hagerdaliae
Sp. roraimanensis
Sp. xylofermentans

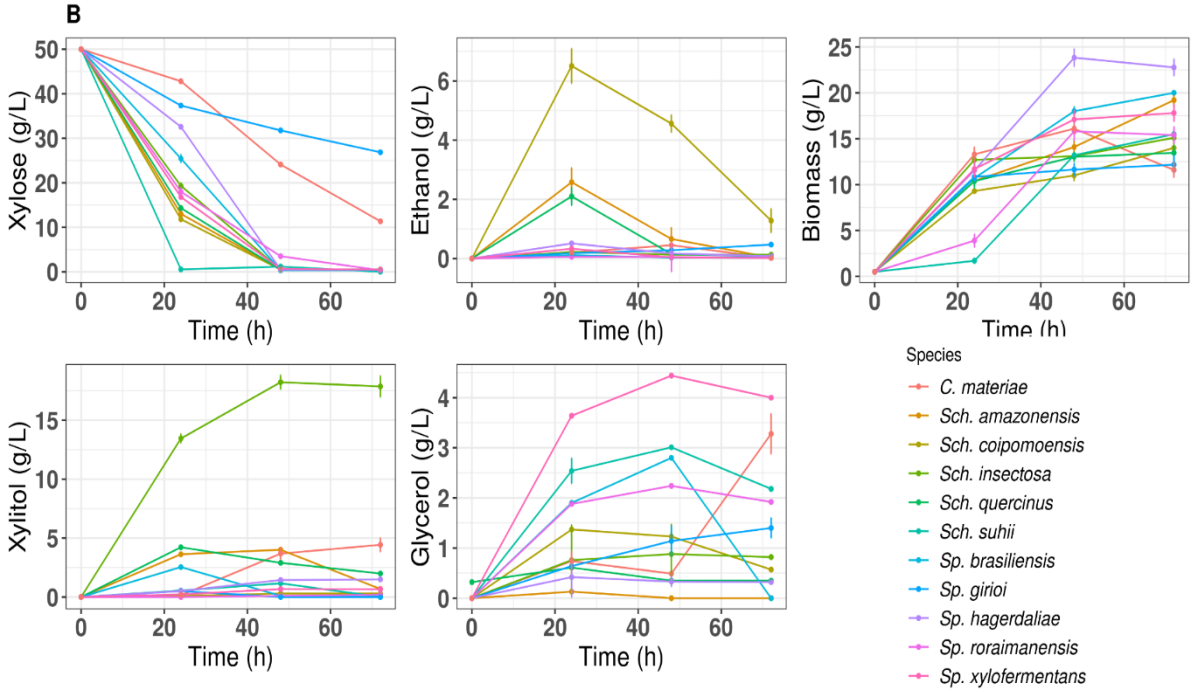


Figure S2. Growth, consumption of xylose and formation of bioproducts by the xylitol producers under A) moderate aeration and B) high aeration conditions.

Table S2: Production of ethanol and xylitol from D-xylose cultures by the new *Spathaspora* and *Scheffersomyces* species.

Aeration condition	Yeast species	Xylose consumption (%)*	Biomass (g L^{-1})	$Y_{x/s}$ (g g^{-1})†	Ethanol (g L^{-1})	$Y_{p/s}^{et}$ (g g^{-1})†	Qp^{et} (g/h L^{-1})‡	η^{et} (%)•	Xylitol (g L^{-1})	$Y_{p/s}^{xyl}$ (g g^{-1})†	Qp^{xyl} (g/h L^{-1})‡	η^{xyl} (%)•	Time (h)▼
Moderate	*Sch. amazonensis*	99	9.90 ± 0.16	0.20	4.40 ± 0.17	0.09	0.09	18	36.1 ± 0.37	0.73	0.75	80	48
	Sch. coipomoensis	99	16.2 ± 0.51	0.18	4.77 ± 0.03	0.10	0.10	19	32.1 ± 1.20	0.64	0.67	70	48
	Sch. cryptocercus	99	9.01 ± 0.10	0.17	14.6 ± 0.42	0.29	0.30	57	4.40 ± 0.04	0.09	0.09	10	48
	Sch. illinoinensis	99	9.89 ± 0.43	0.19	14.5 ± 0.34	0.29	0.30	56	1.77 ± 0.12	0.04	0.04	4	48
	Sch. insectosa	99	13.1 ± 0.25	0.25	8.10 ± 0.13	0.16	0.11	31	19.6 ± 0.89	0.38	0.27	61	72
	Sch. parashehatae	99	10.1 ± 0.19	0.22	19.4 ± 0.20	0.39	0.81	78	1.39 ± 0.12	0.03	0.06	3	24
	Sch. quercinus	94	13.5 ± 0.05	0.29	4.00 ± 0.02	0.08	0.08	16	25.4 ± 0.35	0.53	0.53	58	48
	Sch. shehatae	99	10.2 ± 0.16	0.24	16.0 ± 0.15	0.32	0.33	63	1.52 ± 0.10	0.03	0.03	3	48
	Sch. stipitis	99	10.8 ± 0.28	0.22	15.6 ± 0.31	0.31	0.33	61	0.65 ± 0.02	0.01	0.01	1	48
	Sch. virginianus	99	7.51 ± 0.11	0.13	19.1 ± 0.28	0.39	0.40	76	1.61 ± 0.06	0.03	0.03	4	48
	Sch. xylosifermentans	99	7.95 ± 0.31	0.19	21.0 ± 0.50	0.42	0.87	82	0.29 ± 0.00	0.01	0.01	1	24

Aeration condition	Yeast species	Xylose consumption (%)*	Biomass (g L^{-1})	$Y_{x/s}$ (g g^{-1})†	Ethanol (g L^{-1})	$Y_{p/s}^{et}$ (g g^{-1})†	Qp^{et} (g/h L^{-1})‡	η^{et} (%)·	Xylitol (g L^{-1})	$Y_{p/s}^{xyl}$ (g g^{-1})†	Qp^{xyl} (g/h L^{-1})‡	η^{xyl} (%)·	Time (h)‖
Moderate	*Candida materiae*	99	8.80 ± 0.20	0.17	3.54 ± 0.11	0.07	0.05	14	33.5 ± 0.25	0.68	0.47	74	72
	Sp. arborariae	99	10.4 ± 0.08	0.21	17.7 ± 0.20	0.36	0.37	70	3.05 ± 0.29	0.06	0.06	7	48
	Sp. brasiliensis	98	10.8 ± 0.30	0.21	5.66 ± 0.06	0.12	0.08	23	24.9 ± 0.12	0.51	0.35	55	72
	Sp. girioi	99	9.96 ± 0.10	0.19	5.27 ± 0.12	0.11	0.07	21	22.5 ± 0.31	0.45	0.31	49	72
	Sp. gorwiae	47	12.2 ± 0.22	0.50	1.21 ± 0.10	0.05	0.02	10	0.00 ± 0.00	0.00	0.00	0	72
	Sp. hagerdaliae	99	10.3 ± 0.50	0.20	8.60 ± 0.04	0.17	0.18	34	16.2 ± 0.41	0.32	0.22	35	48
	Sp. passalidarum	99	6.50 ± 0.23	0.14	18.4 ± 0.13	0.37	0.76	73	1.11 ± 0.01	0.02	0.05	2	24
	Sp. roraimanensis	100	7.50 ± 0.05	0.16	7.55 ± 0.01	0.17	0.16	34	16.2 ± 0.22	0.38	0.34	41	48
	Sp. suhii	98	9.40 ± 0.31	0.18	7.08 ± 0.20	0.14	0.10	28	19.7 ± 0.17	0.40	0.27	44	72
	Sp. xylofermentans	100	12.0 ± 0.20	0.23	4.22 ± 0.03	0.09	0.06	17	25.0 ± 0.23	0.51	0.35	56	72
High	*Sch. amazonensis*	99	19.2 ± 1.32	0.37	0.03 ± 0.00	0.00	0.00	0.1	0.68 ± 0.00	0.01	0.01	2	72
	Sch. coipomoensis	99	14.0 ± 0.72	0.27	1.28 ± 0.03	0.03	0.02	5	0.30 ± 0.00	0.01	0.00	1	72

Aeration condition	Yeast species	Xylose consumption (%)*	Biomass ($g\ L^{-1}$)	$Y_{x/s}$ ($g\ g^{-1}$)†	Ethanol ($g\ L^{-1}$)	$Y_{p/s}^{et}$ ($g\ g^{-1}$)†	Qp^{et} ($g/h\ L^{-1}$)‡	η^{et} (%)•	Xylitol ($g\ L^{-1}$)	$Y_{p/s}^{xyl}$ ($g\ g^{-1}$)†	Qp^{xyl} ($g/h\ L^{-1}$)‡	η^{xyl} (%)•	Time (h)‖
High	*Sch. cryptocercus*	99	22.7 ± 0.72	0.45	0.02 ± 0.00	0.00	0.00	0.1	10.9 ± 0.08	0.22	0.15	24	72
	Sch. illinoinensis	99	19.7 ± 1.45	0.38	0.03 ± 0.01	0.00	0.00	0.1	0.87 ± 0.00	0.02	0.01	2	72
	Sch. insectosa	99	15.1 ± 0.19	0.29	0.13 ± 0.08	0.00	0.00	0.5	17.9 ± 0.14	0.36	0.25	39	72
	Sch. parashehatae	99	15.6 ± 0.58	0.31	0.06 ± 0.01	0.00	0.00	0.2	8.10 ± 0.20	0.16	0.11	18	72
	Sch. quercinus	100	13.5 ± 0.12	0.27	0.08 ± 0.04	0.00	0.00	0.3	1.99 ± 0.01	0.04	0.03	5	72
	Sch. shehatae	100	13.6 ± 0.20	0.25	0.08 ± 0.01	0.00	0.00	0.3	2.32 ± 0.02	0.05	0.03	5	72
	Sch. stipitis	100	14.4 ± 0.42	0.29	0.04 ± 0.00	0.00	0.00	0.2	1.50 ± 0.05	0.03	0.02	3	72
	Sch. virginianus	100	16.0 ± 0.70	0.32	0.12 ± 0.00	0.00	0.00	0.5	3.27 ± 0.10	0.07	0.05	7	72
	Sch. xylosifermentans	70	15.2 ± 0.91	0.43	0.12 ± 0.03	0.00	0.00	0.1	4.10 ± 0.29	0.12	0.06	13	72
	Candida materiae	77	11.6 ± 0.25	0.29	0.02 ± 0.05	0.00	0.00	0.1	4.43 ± 0.13	0.11	0.06	13	72
	Sp. arborariae	92	19.8 ± 0.72	0.05	0.09 ± 0.01	0.00	0.00	0.4	0.00 ± 0.00	0.00	0.00	0	72
	Sp. brasiliensis	99	20.0 ± 0.89	0.38	0.05 ± 0.00	0.00	0.00	0.2	0.00 ± 0.00	0.00	0.00	0	72

Aeration condition	Yeast species	Xylose consumption (%)*	Biomass (g L^{-1})	$Y_{x/s}$ (g g^{-1})†	Ethanol (g L^{-1})	$Y_{p/s}^{et}$ (g g^{-1})†	Qp^{et} (g/h L^{-1})‡	η^{et} (%)‣	Xylitol (g L^{-1})	$Y_{p/s}^{xyl}$ (g g^{-1})†	Qp^{xyl} (g/h L^{-1})‡	η^{xyl} (%)‣	Time (h)⁋
High	*Sp. girioi*	47	12.2 ± 0.13	0.49	0.47 ± 0.02	0.02	0.01	4	0.00 ± 0.00	0.00	0.00	0	72
	Sp. gorwiae	93	21.7 ± 0.35	0.46	0.02 ± 0.00	0.00	0.00	0.1	3.51 ± 0.03	0.08	0.05	8	72
	Sp. hagerdaliae	99	22.7 ± 1.12	0.44	0.08 ± 0.03	0.00	0.00	0.3	1.49 ± 0.29	0.03	0.02	3	72
	Sp. passalidarum	100	18.3 ± 1.08	0.36	0.12 ± 0.01	0.00	0.00	0.5	0.05 ± 0.01	0.00	0.00	0.1	72
	Sp. roraimanensis	99	15.4 ± 0.12	0.29	0.02 ± 0.00	0.00	0.00	0.1	0.19 ± 0.08	0.00	0.00	0.4	72
	Sp. suhii	100	15.5 ± 0.42	0.30	0.02 ± 0.01	0.00	0.00	0.1	0.00 ± 0.00	0.00	0.00	0	72
	Sp. xylofermentans	99	17.8 ± 0.15	0.34	0.02 ± 0.00	0.00	0.00	0.1	0.66 ± 0.02	0.01	0.01	1	72

* xylose consumption (%) – initial D-xylose consumed.
† $Y_{x/s}$ (g g^{-1}), $Y_{p/s}^{et}$ (g g^{-1}) and $Y_{p/s}^{xyl}$ (g g^{-1}) – biomass, ethanol, and xylitol yield, respectively: correlation between biomass, ethanol or xylitol (ΔP) produced with sugar (ΔS) consumed.
‡ Qp^{et} and Qp^{xyl} (g L^{-1} h^{-1}) – ethanol productivity: ratio between ethanol concentration (g L^{-1}) and time (h).
‣ η^{et} and η^{xyl} (%) – conversion efficiency: percentage of the maximum theoretical ethanol or xylitol yield (0.511 g ethanol per g D-xylose and 0.917 g xylitol per g D-xylose).
⁋ Time of maximum ethanol production (g L^{-1}) reached by each species in shake flasks; and the time of the end of fermentation for baffled flasks, when the most species reached the high cell concentration.

CHAPTER III

Evolved strain of *Spathaspora passalidarum* producing ethanol from sugarcane bagasse hemicellulosic hydrolysate

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INTRODUCTION

Production of ethanol from lignocellulosic materials is based on the transformation of carbohydrates, including the ones from cellulosic and hemicellulosic fractions (mainly glucose and xylose) (Harner, 2015). Therefore, microorganisms are necessary to enable the fermentation of all types of sugars present in lignocellulosic hydrolysates. Although *Saccharomyces cerevisiae* is the most widely used microorganism for industrial alcoholic fermentation, it cannot ferment xylose because it lacks the xylose assimilation pathway (Ruchala and Sibirny, 2020). The genus *Spathaspora* described by Nguyen et al. (2006) was from a single species, *Sp. passalidarum*, initially isolated from the gut of the *Odontotaenius disjunctus* beetle belonging to the Passalidae family, in Louisiana — USA (Nguyen et al., 2006). Phylogenetic analysis of the complete genome of *Sp. passalidarum* showed that it belongs to the CUG clade of yeasts that decode serine instead of leucine, in which *Scheffersomyces stipitis* is included (Wohlbach et al., 2011). Currently, *Sp. passalidarum* is one

of the most efficient species for the conversion of xylose to ethanol (Cadete and Rosa, 2018) because it contains genes that encode essential proteins that drive the metabolism of that pentose to ethanol, including a NADH-preferring xylose reductase (Wohlbach et al., 2011).

Sp. passalidarum ferments xylose efficiently and achieves high yields of ethanol in rich medium containing xylose (Hou, 2012; et al., 2006, Cadete et al., 2016). However, toxic compounds formed during the pretreatment of the lignocellulosic biomass affect the growth and fermentation performance of the species in hydrolysates (Morales et al., 2017). Hydrolysate-derived inhibitors include acetic acid, phenolic and furaldehyde-derived compounds obtained from the hydrolysis of the acetyl groups of the hemicellulose fraction, degradation of lignin, and dehydration of sugars, respectively (Palmqvist and Hahn-Hägerdal, 2000a). Acetic acid is the major inhibitor present in lignocellulosic hydrolysates (Pattra et al., 2008; Pal et al., 2016; Morales et al., 2017). Its undissociated form goes through the plasma membrane by diffusion and once within the cell it is dissociated, which leads to accumulation of protons and acidification of cytosol (Palmqvist and Hahn-Hägerdal, 2000b). To recover the intracellular pH the ATP is used to pump out the protons from the cell and the active transport of nutrients is jeopardized, affecting cell growth and fermentation capacity (Guan and Liu, 2020). The mix of acetic acid and other inhibitors creates a synergy, in which the harmful effect is greater than the effect caused by only a compound (Ding et al., 2011).

Use of lignocellulosic sugars by the xylose-fermenting yeasts for the so-called “second generation (2G)” ethanol production also depends on strategies that exceed the toxic effects of the inhibitors. Approaches such as random mutagenesis, adaptive laboratory evolution (ALE), and genome shuffling (Su et al., 2018; Morales et al., 2017; Harner, 2015) are used to enhance the tolerance of yeasts to inhibitors present in plant hydrolysates due to the detoxification stage is an additional cost for 2G ethanol production and treatments using enzymes might be expensive (Kumar et al., 2018). Considering that acetic acid is the strongest inhibitor present in

sugarcane bagasse hydrolysate (SBHH) and the synergy by mixing several inhibitors, we hypothesized that Ultraviolet (UV)-induced mutagenesis and ALE in medium containing acetic acid as sole inhibitor would facilitate the adaptation of the yeast in SBHH, which has several toxic compounds, including furfural and 5-hydroxymethylfurfural (5-HMF). We aimed to improve the inhibitor tolerance of the Brazilian strain of *Sp. passalidarum*, UFMG-CM-Y469, through UV mutagenesis followed by ALE in rich medium plus acetic acid and then SBHH, and identify *de novo* mutations related to the improved phenotype in hydrolysate through analysis of whole genome sequencing (WGS) of the ancestral and potential evolved strains.

MATERIAL AND METHODS

Yeast strain and UV mutagenesis

Spathaspora passalidarum strain UFMG-CM-Y469 (=UFMG-HMD-1.1) was subjected to UV mutagenesis. It was isolated by Cadete et al. (2012) from rotting wood samples collected from Brazilian Amazonian Forest area. The strain was obtained from the Coleção de Microrganismos e Células da Universidade Federal de Minas Gerais - UFMG (Collection of Microorganisms and Cells of Universidade Federal de Minas Gerais), Belo Horizonte, Brazil.

The yeast strain was streaked on yeast malt agar (1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, and 2% agar). After 48 h, a single colony was propagated into 15 mL tubes containing 5 mL of YPX (2% xylose, 2% peptone, and 1% yeast extract). The tubes were incubated in an orbital shaker under 200 rpm, at 30ºC for 24 h. The cells were recovered, washed with sterile deionized water, and the cell concentration was adjusted to 1 x 10^7 cell/mL for each strain. To determine the survival curve, the cultures were subjected to a series of 10-fold dilutions. One hundred microliters of each dilution were inoculated in YPX agar plates. The plates were placed in a Crosslinker CL-1000 (UVP, England), which wavelength is 365 nm, and exposed to several intensities of UV radiation (1,000, 1,500, 2,000, 3,000, 4,000, and

5,000 μJ/cm^2). The plates were incubated at 30ºC for 48 h, and the survival rate was calculated as previously described (Hou and Yao, 2012).

After determining the intensity of UV by survival curve, mutagenesis was carried out according to Hou and Yao (2012) (Fig. 1a). Cells were pre-cultured overnight in YPX, and the cell concentration adjusted to 1 x 10^7 cell/mL. One hundred microliters were spotted onto YPXAC (2% xylose, 2% peptone, and 1% yeast extract, 0.25%acetic acid) agar plates. Plates were subjected to 1,500 μJ/cm^2, covered with aluminum foil immediately after the exposition of UV to avoid photoreactivation repair and incubated at 30ºC. After 96 h, the colonies were transferred to YPXAC plates by replica plate and incubated at 30ºC for 48 h. Then the colonies were transferred to YPX agar plates and after 48 h plated back to YPXAC. Single colonies were grown in GYMP broth (2% glucose, 0.5% yeast extract, 0.5% malt extract, 0.2% Na_2PO_4), and stored with 20% glycerol at -80°C. Three independent UV mutagenesis assays were made.

Microtiter-plates growth curves and shake flasks fermentation assays in YPXAC and SBHH

To compare the growth in acetic acid of the ancestral and mutants, the strains were cultured overnight into 5 mL of YPX. After pre-culture, cells were recovered, washed with sterile water, and an aliquot of each strain was transferred to a 96-well plate, containing YPX medium with and without 2 g L^{-1} of acetic acid, with the initial OD_{600} of 0.1. The final volume was 250 μL. Medium without inoculation was used as negative control. The plate was placed in Tecan (Infinite, Switzerland) at 30ºC. Absorbance at 600 nm was monitored every 30 min for 144 h. Kinetic parameters were calculated with R package growthrates in R v3.6.3 using the RStudio v1.3.1073 platform.

Mutants selected from the growth curves and ancestral were submitted to fermentation assays with different concentrations of acetic acid (2 and 3 g L^{-1}). The strains were pre-cultured in YPX during 24 h and 0.5 g L^{-1} of cells were inoculated into 125 mL shake flasks with 50 mL

YPXAC (3% and 5% xylose) and SBHH. Samples were taken at regular intervals of 2 h during 12 h for medium with 3% of xylose, and every 24 hours for 72 h, centrifuged at 13500 x *g* for 10 minutes (Cadete et al., 2016). Supernatants were stored at -20ºC. Cell growth was determined by correlating optical density - OD_{600} (Biospectro SP-22) with the cell dry weight (CDW). Concentrations of xylose, ethanol, xylitol were determined by high-performance liquid chromatography - HPLC (Shimadzu, Kyoto, Japan), using the following conditions: Supelco Analytical C-610 H column (Sigma-Aldrich, USA), maintained at 45°C; volume injection of 20µL; refractive index detector RID 10-A (Shimadzu, Kyoto, Japan); 5 mM H_2SO_4 mobile phase as eluent at a flow rate of 0.6 mL min^{-1}. Graphs were constructed using the ggplot2 package in R v3.6.3 using the RStudio v1.3.1073 platform.

Lignocellulosic hydrolysates

Sugarcane bagasse hemicellulosic hydrolysate was obtained by acid hydrolysis in 250L bioreactor under the following conditions: 120°C during 20 min; sulfuric acid 98% with ratio of 1:10 (100mg H_2SO_4 per gram of sugarcane bagasse). The liquid fraction was recovered by filtration, and the pH was adjusted to 5.5 with calcium oxide (CaO) and autoclaved at 111ºC for 15 min. The final concentrations in SBHH of sugars and inhibitors were determined by HPLC (Cadete et al., 2012). No detoxification and supplementation were performed.

Switchgrass hydrolysate (ASGH) was produced by Ammonia Fiber Expansion (AFEX) pretreatment and enzyme-loading method (Serate et al, 2015). Glucan was loaded in the biomass to reach the final content of 7% in order to obtain a hydrolysate with ~60 g L^{-1} glucose. Water was added to the 7% glucan loading AFEX-pretreated switchgrass biomass and it was autoclaved for 2 h at 121ºC. Then the pH was adjusted with HCl (~37–38% HCl, w/v) and a mixing of cellulase (NS 22257) and xylanase (NS 22244) from Novozymes (Franklinton, NC, USA) was added. The total enzyme loadings were 80 mg protein g^{-1} glucan of biomass for cellulase and 13 mg protein g^{-1} glucan of biomass for xylanase. After 2-4 days of mixing, the

hydrolysis was carried out at 50 °C with stirring at 700 rpm for 7 days. Solids were removed by centrifugation at 8200 × *g* at 4 °C for 10–12 h, the supernatant was filtered through 0.5 μm GVS Maine Glass Prefilters (Thermo Fisher Scientific Inc. Waltham, MA, USA) and 0.2 μm Filter Units (VWR International, Radnor, PA, USA), and stored at 4 °C (Zhang et al., 2020).

Evolution experiments

The selected mutant MT01 and UFMG-CM-Y469 were submitted to adaptive evolution (Fig. 1b). They were evolved aerobically in 50mL-shake flasks containing 20 mL of YPXAC (2% xylose) at 30ºC under 200 rpm. An aliquot of 100 μL was transferred to a fresh medium every 24 h. After five passages, for example, in the same acetic acid concentration, it was increased by 0.5 g L^{-1} until 3.5 g L^{-1}. Cells from the last concentration of acetic acid were cultured in YPXAC agar and subjected to a new concentration of inhibitor for 300 generations in the case of MT01. During the evolution experiment, cultures from each passage were diluted to check the viability in YPXAC agar plates, then single colonies were isolated and cryopreserved. Evolution experiment in YPXAC was followed by ALE using SBHH. This adaptation started with a culture of ME3.5.5 from the last passage in YPXAC. The evolved strain was inoculated in 50mL-shake flasks containing 20 mL of medium (70% sterile water and 30% SBHH) and the dilution was decreased by 10% in each round. This process was repeated until 60 and 70% (v/v) of the hydrolysate, with direct transfer to a new concentration of hydrolysate.

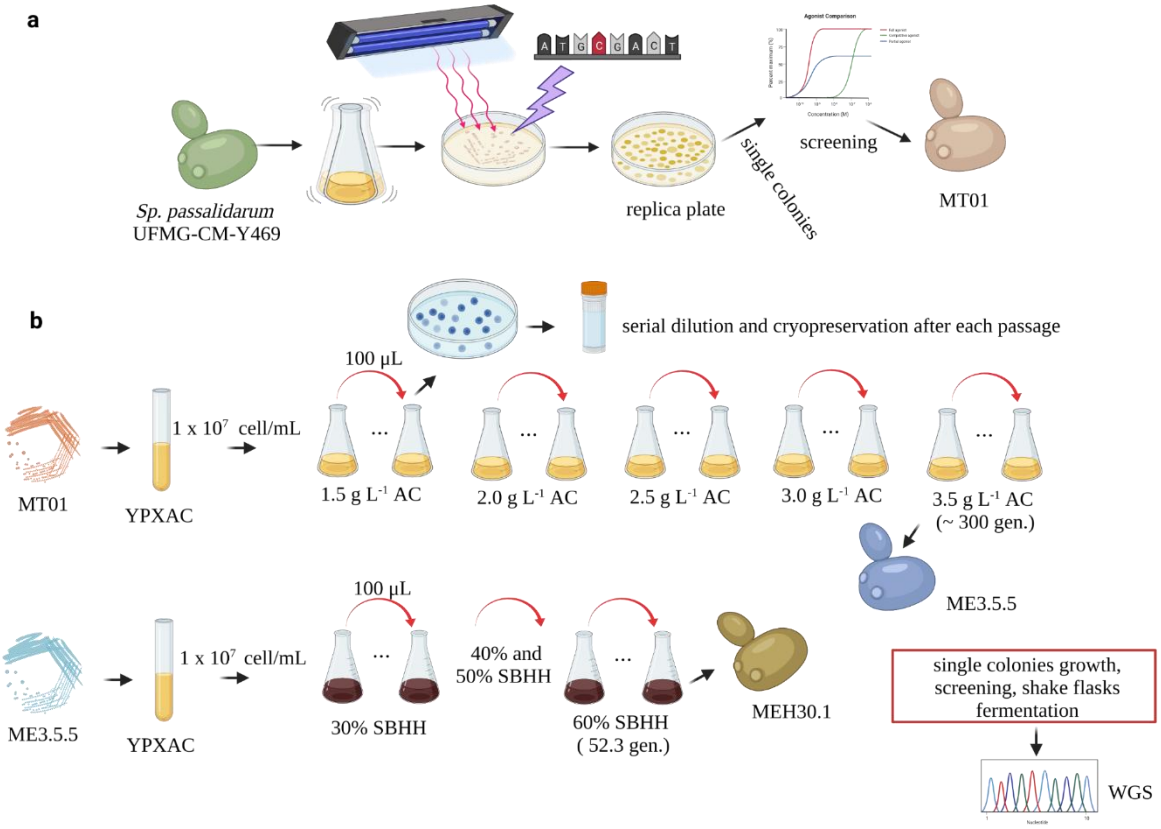

Figure 1. Diagram of a) UV mutagenesis experiment and b) adaptive evolution of MT01 and ME3.5.5. Abbreviations: WGS - Whole Genome Sequencing, SBHH - sugarcane bagasse hydrolysate, gen - generations, AC - acetic acid. Figure was generated in Biorender (https://app.biorender.com/).

Fermentation assays in YPX and SBHH

Wild-type and evolved strains were inoculated in YPX medium, and the flasks were incubated at 30°C in an orbital shaker at 200 rpm for 24 h. The cells were recovered as described above and suspended in YPX (5% xylose, 2% peptone, and 1% yeast extract), SBHH, and ASGH. The fermentation was performed with 125 mL shake flasks with 50 mL of medium, and incubated at 30°C and 200 rpm. The fermentation was monitored by collecting samples every 24 h of fermentation in YPX and SBHH, and every 6 and 18 h for ASGH. The samples were stored at -20°C until analysis. The cell concentrations were determined by counting the OD_{600}. After determining the cell concentration, the samples were centrifuged at 2600 × *g* for 10 min, and the supernatant was stored at -20°C for analysis. The experiment was performed in triplicate.

Xylose, glucose, xylitol, ethanol and acetic acid levels were determined using high-performance liquid chromatography (Shimadzu, Kyoto, Japan) with a Supelcogel C610H ion exclusion column (Sigma-Aldrich, USA) at 45°C and a refractive index detector RID-10A (Shimadzu, Kyoto, Japan). The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1}.

Genome sequencing

To confirm that all the mutant and evolved strains were still *Sp. passalidarum*, D1/D2 sequences of large subunit (26S) of ribosomal DNA of all selected strains were performed (Kurtzman and Robnett 1998). Later, we extracted genomic DNA from the strains UFMG-CM-Y469, ME355, and MH30.1 using phenol:chloroform method and Illumina library prep (Shen et al. 2018). Cells were grown to saturation in YPD (1% yeast extract, 2% peptone, 2% glucose) broth, collected by centrifugation with approximately 500 mL 0.5 mm acid-washed beads (Sigma #G8772), and resuspended in DNA lysis buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, 1% SDS, 2% Triton X-100 in water). Samples were extracted twice with 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma #P2069), precipitated overnight at -80ºC in 100% ethanol, collected by centrifugation, washed twice with 70% ethanol, dissolved in 10 mM Tris-Cl (pH 8), and treated with RNase A (VWR #97064-064) for 30 min at 37ºC. Libraries were prepared with NEBNext Ultra DNA Library Prep kit Illumina (NEB #E7370L), which was performed according to the manufacturer's protocol. Libraries were submitted for 2x150 bp sequencing on an Illumina NovaSeq 6000 instrument.

Analysis of Variants

Adapters were first trimmed from de-multiplexed paired end reads using Trimmomatic (Bolger et al., 2014). Trimmed reads were then aligned to a *Sp. passalidarum* reference assembly (accession #AEIK00000000, Wohlbach et al 2011) using BWA mem (https://arxiv.org/abs/1303.3997). Variants were called using freebayes v1.3.1 using standard filters. Resulting vcf files were filtered for shared and unique variants using vcf tools. Candidate

mutations were manually confirmed in IGV. Genes containing mutations were identified first by blasting the mutated region to the annotated reference genome (AEIK00000000). Nucleotide annotations matching the mutated region were then blasted against annotated *S. cerevisiae* proteins using Blastx. *S. cerevisiae* proteins with significant scores were considered homologous.

Genomes were independently examined for structural variants using Samtools depth (Li et al., 2009). Euploidy of all genomes was confirmed by dividing median chromosome coverage by median genome-wide coverage for each chromosome. Structural variants unique to evolved strains were screened through visual inspection of chromosome coverage plots.

RESULTS

Sp. passalidarum mutants

To induce mutations, it is recommended a UV dosage that leads to a survival of 5 to 10% of the microorganism to select mutants with a high mutation rate (Lawrence, 2002). The UV intensity selected for the mutagenesis assay was 1500 μJ/cm^2, which resulted in the survival of 9% of cells of *Sp. passalidarum* UFMG-CM-Y469 strain. After UV radiation, cells were transferred to new plates with YPX and then transferred to YPXAC by replica plate. This procedure was repeated three times. At the end, 55 colonies were recovered from YPX plus 0.25% of acetic acid plates. The total colonies were obtained from the three independent UV mutagenesis experiments. The size of the colonies varied from large to petite. After the last transfer, petite colonies were reduced significantly.

Six mutants (MT01, MT02, MT37, MT53, MT87, and MT101) showed enhanced cell growth compared to the parental strain. A triplicate shake flask experiment was performed to compare those mutants with the parental strains in YPX and YPXAC to verify whether the mutants were able to produce ethanol and still ferment xylose in both conditions. MT01 was

the only mutant to exhibit growth, xylose consumption, and ethanol production superior to the wild-type strain in YPXAC (Fig. 2). From 30 g L^{-1} of xylose, MT01 consumed 41% of the sugar in 12 h, produced 5.31 g L^{-1} (0.43 g g^{-1}) of ethanol, and 2.30 g L^{-1} of biomass. This mutant produced 43% more ethanol than UFMG-CM-Y469, which reached 3 g L^{-1} of ethanol and 2.13 g_{CDW} L^{-1} of biomass, and consumed 34% of xylose in YPX plus 2 g L^{-1} of acetic acid. In YPX, trade-offs were not observed. MT01 showed a maximum ethanol yield of 0.46 g g^{-1} (13 g L^{-1}) and consumed 95% of sugar between 10 and 12 h.

In agar plates plus acetic acid, the growth of UFMG-CM-Y469 is limited to 1.5 g L^{-1} of acetic acid. But this growth is extended in the liquid medium plus inhibitor and it still ferments xylose and produces ethanol in 2 g L^{-1} of acetic acid. Increasing this concentration to 3 g L^{-1}, the parental strain consumed 12 g L^{-1} of xylose, but it did not reach ethanol yield and accumulated a little amount of xylitol (Fig. 2). The mutant MT01 produced 2.76 g L^{-1} of ethanol in 72 h, but it also accumulated xylitol. Both strains consumed acetic acid.

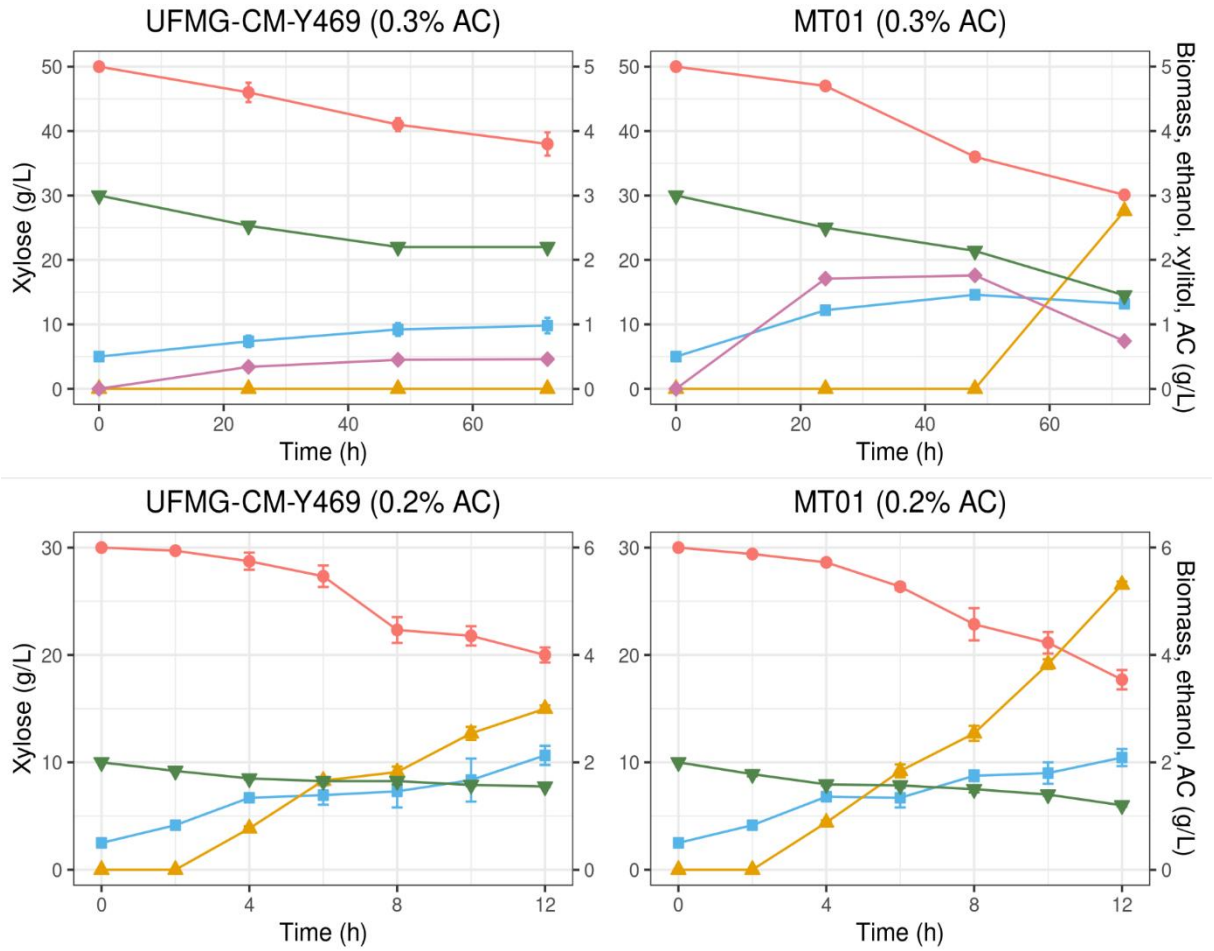

Figure 2. Shake flasks fermentation of YPXAC (2 g L^{-1} acetic acid) in 12 h and (b) YPXAC (3 g L^{-1} acetic acid) in 72 h by *Sp. passalidarum* MT01 and parental strain UFMG-CM-Y469. Symbols: pink filled circles, xylose; yellow filled triangles, ethanol; purple filled diamonds, xylitol; blue filled squares, biomass; green filled triangle down, acetic acid (AC). Error bars indicate the standard deviation from triplicate shake flasks.

Mutants MT02, MT37, MT53, MT87, and MT101 did not show desirable phenotype, in this case enhanced ethanol production, in shake flasks experiments. MT37 and MT101 lost the ability to ferment xylose, while MT02, MT53, and MT87 had a similar or inferior performance compared to the parental strain UFMG-CM-Y469. As the wild-type strain is not able to grow in SBHH and produce any metabolite, we tested MT01 in the hydrolysate to check whether the mutant would grow in this medium, but no growth was observed. MT01 was the mutant selected to be adapted in SBHH due to the results of shake flasks experiments using YPX.

Evolved strains were able to grow and produce ethanol in SBHH

Because the mutant MT01 showed enhanced ethanol production in the presence of acetic acid compared to the ancestral strain it was selected to be submitted to ALE. While the adaptation of UFMG-CM-Y469 was carried out until 2 g L^{-1} acetic acid, because the cells stopped growing, we adapted MT01 for many generations until the concentration of 3.5 g L^{-1} acetic acid. After ~300 generations, the evolved strain ME3.5.5 from the last round was subjected to ALE in SBHH. We obtained the strain MEH30.1 from the last cycle of 60% of SBHH, which showed ethanol production and cell growth in the hydrolysate.

The hydrolysate used in this work was prepared with the hemicellulosic fraction from the sugarcane bagasse. Its composition was 2% xylose, 0.2% glucose, 0.45% acetic acid, 0.013% HMF, 0.02% furfural. The experimental evolution was carried out with a maximum of 70% of the hydrolysate, but we tested the evolved strains without diluting the medium. The evolved strains were able to grow and produce metabolites in SBHH. In the triplicate shake flasks experiment, ME3.5.5 obtained from adaptation in YPXAC reached the maximum ethanol yield, 0.42 g g^{-1} (9 g L^{-1}), in 72 h of fermentation (Fig. 3a). Similar behaviour was observed for the strain MEH30.1, which is from ALE in SBHH (70%). It also showed the maximum ethanol yield in 72 h, about 0.43 g g^{-1} (9.6 g L^{-1}). The complete utilization of xylose and acetic acid by the evolved strains occurred in 72 h and 96 h of fermentation, respectively. As the glucose was present in a little amount in this hydrolysate, it was depleted fast and the yeasts were able to start to consume xylose in the first 24 h. ME3.5.5 and MEH30.1 presented a maximum specific growth rate (μ_{max}) of 0.04 and 0.05, respectively. They were also tested in YPX to check the occurrence of trade-offs. From 50 g L^{-1} of xylose, high amounts of ethanol were produced by all the yeasts of fermentation and xylose was almost completely consumed during 24 h. At this time they produced 0.42 g g^{-1} (~20 g L^{-1}) ethanol and presented a μ_{max} of 0.12 h^{-1}.

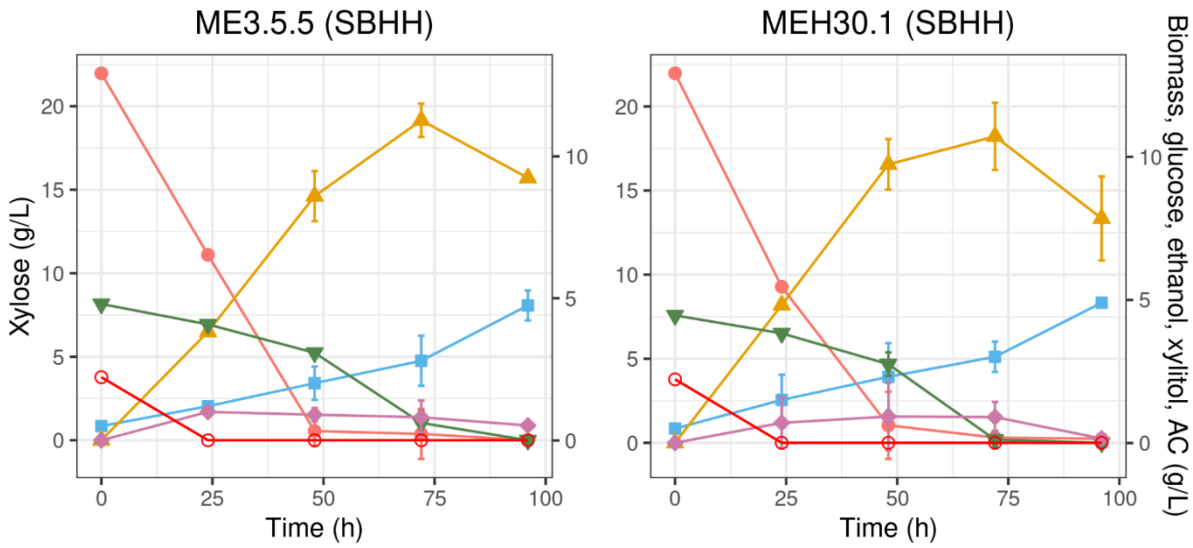

Figure 3. Shake flasks fermentation of SBHH by evolved strain ME3.5.5 from ALE with YPXAC and MEH30.1 strain from adaptation in SBHH. Symbols: pink filled circles, xylose; red circles, glucose; yellow filled triangles, ethanol; purple filled diamonds, xylitol; blue filled squares, biomass; green filled triangle down, acetic acid (AC). Error bars indicate the standard deviation from triplicate shake flasks.

Considering we used hemicellulosic hydrolysate, other sugars (e.g. arabinose, mannose, cellobiose, and galactose) in addition to xylose and glucose were present. Unfortunately, it was not possible to measure the other sugars present in the SBHH. This is a possible explanation for the continued production of ethanol after the xylose and glucose depletion.

Performance of evolved strains in AFEX

As the SBHH is produced from hemicellulosic fractions, we decided to test the native and evolved strains in a hydrolysate prepared with both hemicellulosic and cellulosic fractions of a lignocellulosic material. AFEX hydrolysate has higher content of glucose and xylose, about 60 g L^{-1} and 40 g L^{-1}, respectively, than SBHH. On the other hand, inhibitors are present in lower amounts: 2.8 g L^{-1} acetic acid and 0.04 g L^{-1} furfural. The evolved strains had similar performance of the parental UFMG-CM-Y469 in this hydrolysate (Fig. 4), showing pretty much the same values of production of metabolites, biomass, and consumption of sugars and acetic acid. Negligible amounts of xylose were used during the first 72 h of fermentation. As expected, the pentose was fully consumed after 72 h, when the glucose was already depleted as well acetic

acid. The maximum ethanol yield was 33.6 g L^{-1} in 144 h. But the maximum productivity, 0.329 g L^{-1} h^{-1}, took place in 90 h of fermentation with a ethanol yield of 0.498 g g^{-1} (29.6 g L^{-1}). At this time, the yeasts started to accumulate xylitol. The strains reached about 8 g L^{-1} of biomass with μ_{max} of 0.14 h^{-1}.

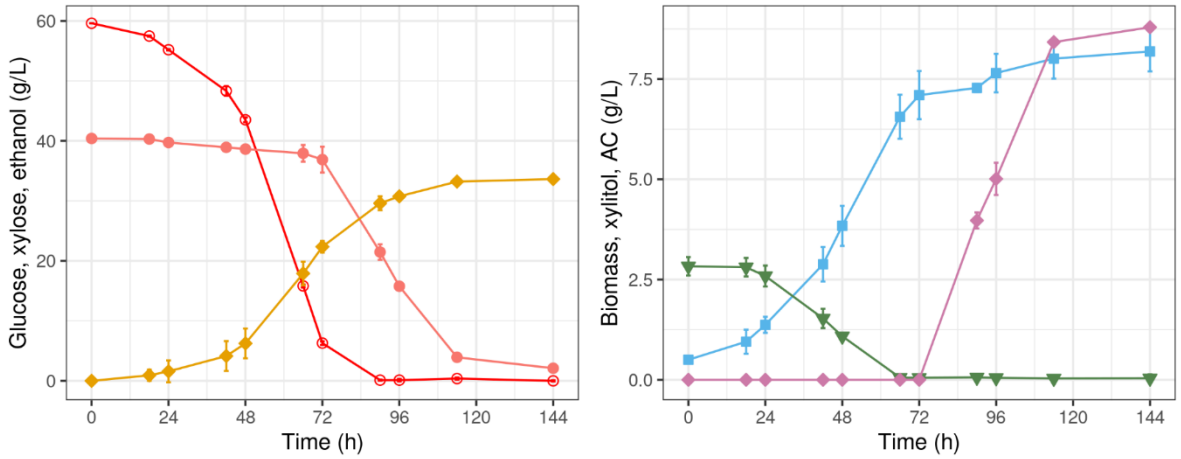

Figure 4. Shake flasks fermentation of AFEX hydrolysate by the native strain UFMG-CM-Y469. Symbols: pink filled circles, xylose; red circles, glucose; yellow filled triangles, ethanol; purple filled diamonds, xylitol; blue filled squares, biomass; green filled triangle down, acetic acid (AC). Error bars indicate the standard deviation from triplicate shake flasks.

De novo mutations

To evaluate the genetic basis of the adaptation, we performed whole genome sequencing on UFMG-CM-Y469 and two evolved strains ME3.5.5 and MEH30.1. Depth plots revealed no aneuploidies or large CNVs in the ancestor or any of the evolved strains. Using variant calling, we identified two types of variations: single-nucleotide polymorphism (SNPs) and small insertions and deletions (indels). First, we looked for mutations shared by the three evolved strains, which were absent in the ancestor UFMG-CM-Y469. Five heterozygous mutations were identified among the strains, one intergenic and four coding sequences mutations (Table 1). A C583T mutation resulting in a nonsynonymous L195F change was found in a gene encoding a poorly conserved hypothetical protein (XM_007377149.1). The remaining three coding-sequence mutations are all disruptive mutations that occurred in conserved genes with

Saccharomyces cerevisiae homologs. A C1208T mutation resulting in a non-synonymous P403L substitution was found in the *Sp. passalidarum* homolog of *ERT1* (XM_007373880.1), a C492A mutation causing a nonsense Y143* mutation was found in the homolog of *NPL4* (XM_007372648.1), and an out-of-frame deletion of TGT3009:3011 in the *CYR1* homolog causes a D1003 frameshift.

We also looked for unique variants in each evolved strain. We were unable to find any mutations unique to ME30.1. Therefore, we have identified mutations putatively responsible for the phenotypes of MT01 and ME3.5.5 but were unable to identify the basis of adaptation in MEH30.1. Because we did not sequence MT01, we cannot differentiate between UV-derived mutations underlying improved performance in MT01 and adaptive mutations acquired by ME3.5.5.

Table 1: Five heterozygous mutations shared by the evolved strains and absent in the ancestor.

Annotation[†]	***S. cerevisiae* homolog**	**Mutation Type**
conserved hypothetical protein	*ERT1*	nonsynonymous
intergenic	-	-
nuclear protein localization protein 4	*NPL4*	nonsense
adenylate cyclase	*CYR1*	frameshift
hypothetical protein	-	nonsynonymous

[†]Based on annotation of genome # AEIK00000000 (Wohlbach et al., 2011)

DISCUSSION

A challenge of ethanol production from lignocellulosic hydrolysates is the toxic compounds generated during the pretreatment/hydrolysis, which might suppress the microorganism metabolism. Acetic acid is the major inhibitor present in lignocellulosic hydrolysates (Pattra et al., 2008; Pal et al., 2016; Morales et al., 2017). The association of this inhibitor with furfural

and phenolic compounds plays a role in the inhibition of amino acids and carbon metabolism intermediates with remarkable decreases of pyruvate and succinate. A comparison among those inhibitors individually placed the acetic acid as the compound with the most harmful effects in yeast growth and metabolism (Ding et al., 2011). Developing microorganisms with enhanced acetic acid and other toxic compounds tolerance may help for the better understanding of traits of biotechnological desired phenotypes that can be driven to the genetic manipulation in order to reach those traits.

In this work, a strain of *Sp. passalidarum* was subjected to UV mutagenesis followed by ALE. It was subjected to only a round of UV mutagenesis due to the rate of induced mutation being directly proportional to the radiation dose to which the microorganism was exposed (Gou, Bloom and Kruglyak, 2019). The colony's size was the first phenotypic trait noticed after the UV radiation. The occurrence of large colonies suggests a primary mechanism of resistance to decrease the amount of UV and reduce its effect, since the increased size can maintain larger reserves of water and other resources (Goldman and Travisano, 2011). Also, large colonies can be related to an increased efficiency of lipid metabolism, like a compensatory effect on the lipid synthesis (Wang et al., 2014). Petite colonies are associated with respiratory deficiency (RD) mutation (Stewart, 2017) and their presence in YPXAC plates was significant. After the transfers by replica plate, the number of petites decreased significantly, indicating that they were not true petite mutants (Mo et al, 2001).

Although several colonies were recovered after the replica plate, only three mutants showed growth with significant difference compared to the native strain and one exhibited better fermentative parameters. Also, some mutants lost the ability to ferment xylose. Two possible explanations for those results are mutations that lead to a loss of a specific phenotype and DNA repair mechanisms. The photoreaction repair was avoided by wrapping the plates with aluminum foil immediately after the UV exposure. However, the damaged DNA could be

repaired by other mechanism such as nucleotide excision repair (NER), which recognize the damage and perform a excision on it and then this part is resynthesized by polymerases and the ends joined by ligases (Feltrin et al., 2020). The cells also can accumulate mutations in genes, such as the ones related to regulatory pathways, that result in a loss in fitness (Gibson et al., 2020).

A mutant MT01 was selected to be submitted to experimental evolution based on the results of shake flasks fermentation. MT01 showed enhanced tolerance to acetic acid compared to the ancestor since it was able to ferment xylose in the highest concentration of acetic acid tested in shake flasks containing YPXAC. However, this performance was not observed in SBHH given that MT01 did not grow in hydrolysate. It is not surprising since the hydrolysate presented a high amount of acetic acid as well the mixture of other inhibitors, which led to a synergistic effect.

After the adaptive evolution, the evolved strains could ferment xylose to ethanol in SBHH. This suggested that the stress caused by the selective pressure on the yeasts resulted in more adapted cells. Interestingly, no trade-offs were observed in the evolved strains ME3.5.5 and MEH30.1. Trade-offs are common in evolution experiments and strains that do not show this feature are interesting for biotechnology purposes (Van den Bergh et al., 2018; Dragosits and Mattanovich, 2013). The wild-type strain was able to grow and reach end-products in AFEX hydrolysate with similar behaviour of the evolved strains. We attributed this result to the lower concentrations of inhibitors, especially acetic acid present in this hydrolysate.

A major goal of this study was to map the genetic basis of improved growth and fermentation in hydrolysate conditions. Mutagenesis of UFMG-CM-Y469 generated strain MT01, which showed improved acetic acid tolerance. Therefore, the improved phenotype of evolved strain ME3.5.5 in YPXAC is presumably due to additive contributions of mutations acquired in the original mutagenesis of UFMG-CM-Y469 and those acquired during the

adaptive evolution of MT01. Five mutations were identified in ME3.5.5, four of which disrupt in gene-coding sequences. Because we did not sequence MT01, we cannot hypothesize which of these mutations underlies the initial improved acetic acid tolerance of MT01 and which mutations drove the adaptive increase in acetic acid tolerance in ME3.5.5. However, the source of mutation is irrelevant to the goal of identifying candidate loci for improving acetic acid tolerance in *Sp. passalidarum.*

Four genes contain disruptive heterozygous mutations in ME3.5.5 and thus are candidates for genetic modification of increased growth in YPXAC and SBHH. These genes include one hypothetical protein and three conserved genes with *S. cerevisiae* homologs. Homologs of the *S. cerevisiae* genes *ERT1* (36% amino acid identity)*, NPL4* (49% amino acid identity)*,* and *CYR1* (43% amino acid identity) acquired a nonsynonymous, nonsense, and frameshift mutation, respectively. Of these, *CYR1* has a previously established connection to xylose fermentation (Wu et al. 2020). *CYR1* encodes adenylate cyclase which converts AMP to cAMP in response to glucose stimulation of Gpr1p. Elevated levels of cAMP then activate PKA activity, upregulating glycolysis and downregulating gluconeogenesis. Elevated cAMP and associated elevation in PKA signaling have been implicated in increased ability to ferment xylose in *S. cerevisiae* (Wu et al. 2020). However, the frameshift we observe truncates the protein and eliminates the C-terminal catalytic domain. Mutations to this domain have been previously shown to inactivate Cyr1p and correspondingly decrease cAMP levels (Vanhalewyn et al. 1999). The truncation we observe is heterozygous, however, which may not cause a straight-forward decrease in adenylate cyclase activity.

We were unable to identify mutations responsible for the improved growth of MEH30.1 relative to ME3.5.5. No mutations were unique to either strain and all heterozygous mutations in ME3.5.5 remained heterozygous in MEH30.1. The ancestral strain used (UFMG-CM-Y469) is a unique isolate and not derived from the reference genome isolate (NRRL Y-27907). We

considered that additional mutations may be obscured in regions unique to UFMG-CM-Y469, but we did not find any additional variants when using a mapping-free approach (Standage et al. 2019). The basis of adaptation in MEH30.1 thus remains unknown. However, the similar phenotype between MEH30.1 and ME3.5.5 and the results of variant analysis suggest that the evolution experiment in SBHH was not effective.

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7. SUMMARY AND FUTURE DIRECTIONS

Chapter 1

A total of 569 yeasts were isolated, of which 89 were identified and 37 new species, from 60 samples of rotting wood collected in dryland (UFAM and Piquiá) and floodplain (Carú) areas from Brazilian Amazonia. The total of isolates obtained in this work is greater compared to the number of yeast strains obtained by Cadete et al. (2012) (224 yeasts isolated from from 40 samples of rotting wood; 33 species identified, 7 novel species) and Morais et al. (2020) (330 isolates from 200 samples of rotting wood; 80 species identified, 14 novel species). This result can be associated with the isolation media. While in this work it was used YNB-xylan, YNB-D-xylose, and two types of hydrolysates undiluted and dilute, the earlier works used only minimal media with xylose or xylan (Cadete et al., 2012), or SBHH undiluted and dilute (Morais et al., 2020).

Dryland sites were more diverse than the floodplain area. An interpretation for this result is the impact of the seasonal flood affecting the soil structure and fertility, which reduces the nutrient availability (González Macé et al., 2016) and alters the yeast community. More species were isolated in minimal media with YNB-xylan and YNB-xylose. Sugarcane bagasse and corncob acid hydrolysate contain several compounds such as acetic acid, furfural and hydroxymethylfurfural, which are toxic for microorganisms. Despite being restrictive, it was possible to isolate strains capable of growing in SBHH and CCHH as isolation media. It also increased the number of species sampling since there were species that only did grow in hydrolysates.

Some species obtained in this work were previously isolated in Brazilian Amazonia and reported as dominant in rotting wood samples, such as *Schwanniomyces polymorphus, Scheffersomyces amazonensis*, and species belonging to the genus *Sugiyamaella* (Morais et al., 2013; Cadete et al., 2012; Kurtzman, 2011). Regarding the main xylose-fermenting clades,

Scheffersomyces stipitis, Sch. shehatae, *Scheffersomyces* sp. and two new *Spathaspora* species were found in this work, but they were less frequent. Described *Spathaspora* species were not isolated. Some species were represented by single isolates such as *Saccharomyces cerevisiae*, a workhorse yeast strain of biotechnology and food industries. Despite its importance, the natural occurrence of *S. cerevisiae* is not well known. In this work, it was isolated in corncob hydrolysate dilute 1:2 in the UFAM area. Morais et al. (2020) isolated six strains of this species in three areas of Atlantic rainforest, in Brazil, in sugarcane bagasse hydrolysate. This species was not isolated in SBHH in this work. Interestingly, the CCHH hydrolysate has high sugar content as well as the SBHH used by the earlier work (4.8% of xylose). It seems that the sugar content is a differential to isolate this species.

As reported here, several described and novel species were isolated. The next experiments suggested for this work is to estimate the growth kinetic parameters of the yeasts in hemicellulosic acid hydrolysates and then select the species to test the fermentation in the same medium.

Chapter 2

Twenty-four species of *Scheffersomyces* and *Spathaspora* clades were submitted to fermentation assays in YPX (5% xylose, 2% peptone, and 1% yeast extract) under moderate (125mL-shake flasks, 50 mL YPX) and high (250mL- baffled flasks, 50 mL YPX) aeration conditions. Ethanol and xylitol were the major products formed in moderate oxygen-limiting conditions. Based on the main product of fermentation in shake flasks the species were classified as ethanol or xylitol producers. *Scheffersomyces xylosifermentans*, *Sch. parashehatae*, and *Sp. passalidarum* showed the highest ethanol yields among the species tested. When they were cultured in baffled flasks, the xylose consumption was delayed, xylitol was accumulated, and the cell biomass increased significantly. This behaviour was observed

for all the ethanol producers but *Scheffersomyces illinoinensis* which still achieved ethanol yield in high aeration. In case of *Sp. passalidarum*, glycerol was the major end-product. Xylose fermentation by *Scheffersomyces virginianus* in addition to the three best ethanol producers was tested under low aeration (125-SF, 125 mL YPX) and they still achieved good yields of ethanol, however, the ethanol productivity decreased in relation to the moderate aeration.

In parallel, xylose fermentation by outgroups possessing multiple copies of the *XYL1* gene was also undertaken. *Candida intermedia, Candida blattae, Candida carpophila*, *Meyerozyma guilliermondii*, and *Meyerozyma caribbica* accumulate xylitol in both aeration conditions used. A copy of the *XYL1* from *C. intermedia* and *M. guilliermondii* was reported to encode NADH-preferring XR (Geijer et al. 2020) as *XYL1.2* from *Sp. passalidarum*, but they did not show similar fitness.

Less than half of the species tested in this work reached ethanol production in satisfactory levels and most of the yeasts accumulated xylitol in moderate and high aeration. Therefore, this chapter was directed to the hypotheses that the level of affinity between cofactors and the enzymes xylose reductase (XR) and xylitol dehydrogenase (XDH) has a great role in the distinct utilization of xylose among *Scheffersomyces* and *Spathaspora* species and the XDH may be a limitation for some species. For that, enzyme assays were conducted to determine the enzyme activities of XR and XDH from the best xylose-fermenting species as well *Scheffersomyces insectosa*, and *C. intermedia*, which are xylitol producers.

The enzyme assays showed that XR from the *Scheffersomyces* species display dual-cofactor specificity. They have preference for NADPH, but the activity with NADH was expressive. When *XYL1* from *Sch. xylosifermentans* was expressed in *Saccharomyces cerevisiae*, the enzyme assay was performed and the the ratio NADH/NADPH was one, which means that the XR has similar affinity for both cofactors. The dual-cofactor specificity of XR may be one of the reasons for the xylose fermentation ability of *Sch. xylosifermentans*, *Sch.*

parashehatae and *Sch. virginianus* in low aeration. *Scheffersomyces insectosa* also presented XR with affinity for NADPH and NADH, but the activity was low.

The activity of XR and XDH was affected by the high level of oxygen. However, the impact was greater on XDH than XR according to the results in Figure 5 of Chapter II. This result suggests that the impact on the XR with high aeration may have delayed xylose uptake by *Sch. xylosifermentans*, *Sch. parashehatae*, and *Sp. passalidarum* under that condition. In the case of the two first species, the effect of high oxygenation on XDH could have caused the production of xylitol. The findings of the fermentation assays and enzyme activity experiments suggest that the degree of the affinity between enzyme and cofactor is essential to the xylose pathway and XDH may be a limitation of some yeasts, especially xylitol producers. Xylose reductase and XDH from *Sch. insectosa* were the only enzymes tested among the yeasts that produce xylitol. Although the results support the hypothesis that XDH from this species is a constraint for xylose fermentation due to the low activity, it would be interesting to determine the enzyme activity from other xylitol producers and perform enzyme kinetic assays.

Currently, RNA sequencing (RNA-seq) data from *Sp. passalidarum* and *Sch. parashehatae* are available for analysis. The yeasts were grown in moderate and high aeration to identify differentially expressed genes related to the utilization of xylose. In addition, a phylogenomic approach is being undertaken to determine the phylogenetic placement of the species assigned to the genera *Spathaspora* and *Scheffersomyces* species and related species. As well the phylogenetic reconstruction using the amino acid sequences of the *XYL1* and *XYL2* genes.

Chapter III

Spathaspora passalidarum UFMG-CM-Y469 was submitted to Ultraviolet (UV)-induced mutagenesis and the mutants were selected by the growth and production of ethanol in a defined

medium containing xylose and acetic acid (YPXAC). The mutant MT01 presented improved fitness compared to the ancestral strain. Then adaptive laboratory evolution (ALE) was conducted with the mutant and the wild-type strain increasing the acetic acid concentrations in the defined medium. While the first one evolved until 0.35% of acetic acid, the native strain did grow until 0.2%. After ~300 generations, ME3.5.5 from the last round of acetic acid concentration was subjected to ALE with hemicellulosic sugarcane bagasse hydrolysate (SBHH) from 30 to 60%. The yeast evolved over ~52 generations and MEH30.1 was obtained.

A major goal of this study was to map the genetic basis of improved growth and fermentation in hydrolysate conditions. For that, fermentation of SBHH by evolved and native strains was undertaken. While the wild-type strain did not grow, ME3.5.5 and MEH30.1 were able to grow and produce ethanol. To evaluate the genetic basis of the adaptation, we performed a whole genome sequencing on UFMG-CM-Y469 and two evolved strains ME3.5.5 and MEH30.1. Five mutations were identified in ME3.5.5, four heterozygous mutations of which were disrupted in gene-coding sequences. No unique mutations were found in MEH30.1. Since both strains presented similar fitness, these results indicate that the ALE in SBHH was not productive.

The genes where the mutations occurred are homologs of the *S. cerevisiae* genes *ERT1*, *NPL4*, and *CYR1*, which acquired a nonsynonymous, nonsense, and frameshift mutation, respectively. *CYR1* encodes adenylate cyclase which converts AMP to cAMP in response to glucose stimulation of Gpr1p. Elevated cAMP and associated elevation in PKA signaling have been implicated in increased ability to ferment xylose in *S. cerevisiae*. Future work to measure cAMP levels in mutant backgrounds may help resolve the relationship between cAMP signaling and YPXAC growth in *S. passalidarum.*

8. CONCLUSIONS

-Isolation of new species and strains apt to grow in lignocellulosic-related sugars is an important taxonomic, evolutionary and biotechnological contribution to studies aimed at understanding and applying this metabolic trait. This study showed that the use of lignocellulosic hydrolysates as an isolation media provided yeast species capable of growing in the presence of inhibitors. Bioprospecting studies can be applied to these yeasts to select species with desirable phenotypes and be source of genes or target of genetic manipulation for second-generation ethanol production. In addition, the isolation of 37 possible new yeast species from rotting wood collected in ecosystems of the Amazonian rainforest shows the importance of scientific studies involving research work in this biome.

-By evaluating the xylose metabolism in *Scheffersomyces* and *Spathaspora* species, ethanol and xylitol are the major products formed from xylose fermentation and this production is directly influenced by oxygen availability. High aeration leads to an increase of cell biomass and carbon dioxide while moderate oxygen-limiting conditions provide more weight of xylitol and ethanol. Xylitol dehydrogenase and xylose reductase are affected by high aeration and their activities decrease considerably, but the impact is higher in XDH than XR which may be an answer for the production of xylitol by ethanol producers. For xylitol producers, it seems that XDH is the major limitation since this enzyme from *Scheffersomyces insectosa* shows low activity with NAD^+ and the species consume xylose, but accumulate xylitol in both aeration conditions tested.

Scheffersomyces species harbor a single copy of *XYL1* which encodes xylose reductase with dual-cofactor specificity. The enzymes from *Scheffersomyces xylosifermentans*, *Scheffersomyces parashehatae*, and *Scheffersomyces virginianus* are NADPH-preferring XRs, but they also present high activity with NADH. Along with the high activity with the cofactor, the dual specificity plays a role when the aeration is reduced because it can avoid the redox

imbalance. The findings of this work in addition to the RNA-seq data can bring fundamental insights about the xylose metabolism and, as consequence, contribute to generate strains for the conversion of xylose into biofuels.

-Random mutagenesis and evolution experiments are great tools to adapt species to stress conditions. Mutants and evolved strains grow in defined medium supplemented with acetic acid and sugarcane bagasse hydrolysate. A heterozygous mutation in the gene encoding adenylate cyclase was found in the evolved strains and it can be involved in the improved fitness in hydrolysate.

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