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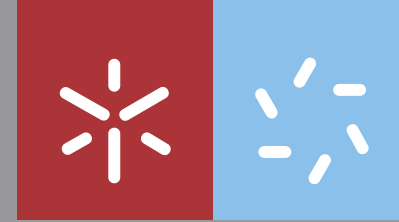


Lorena Donzella

Characterization and engineering of sugar transporters in the yeast *Kluyveromyces marxianus* for biotechnological applications



June 2022



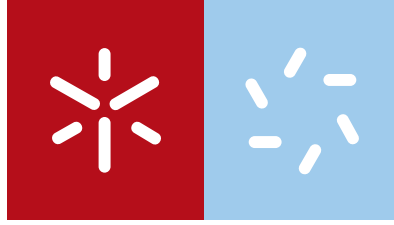
Universidade do Minho
Escola de Ciências



University College Cork
School of Microbiology

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**Characterization and engineering of
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Kluyveromyces marxianus
for biotechnological applications**

Doctoral Thesis
Joint Doctorate in Biology and Microbial Biotechnology

Work supervised by
Professor Maria João Sousa
and
Professor John P. Morrissey

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To my little siblings, I love you and I hope to be of inspiration for you:

Gabri grazie per avermi fatto capire che ti mancavo quando stavo all'"Inglese" e mi chiedevi quando sarei tornata.

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Spero siate orgogliosi di me, vi amo.

STATEMENT OF INTEGRITY

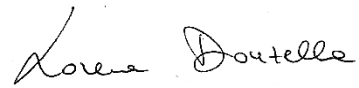
I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

The work presented in this thesis must be considered confidential and under embargo for 1 year.

Date: June 2022

Signature

A handwritten signature in black ink, appearing to read 'Lorena Donzella', written in a cursive style.

Lorena Donzella

RESUMO

A utilização sustentável e eficiente de hidrolisados lignocelulósicos por leveduras em biorefinarias, requer o consumo simultâneo de todos os açúcares presentes nesta matéria-prima, nomeadamente, hexoses e pentoses. Este aspeto está, no entanto, limitado pelo baixo número de transportadores específicos para pentoses conhecidos e pela reduzida taxa de consumo de pentoses pela levedura quando a glucose está presente. *Kluyveromyces marxianus* é uma levedura não convencional que tem atraído atenção considerável devido às suas características com grande interesse industrial, como a capacidade de assimilação natural de pentoses, elevadas termotolerância e taxa de crescimento.

Neste contexto, o principal objetivo da presente tese foi estudar o transporte de pentoses em *K. marxianus*, de forma a selecionar e modificar um transportador que permitisse o co-consumo de glucose e xilose para a aplicação em biorefinarias.

A fim de identificar novos transportadores de pentoses, foi criada uma estirpe de *K. marxianus*, na qual foram eliminados 12 genes que codificavam transportadores da superfamília MFS (major facilitator superfamily) e que não apresentava crescimento em pentoses (xilose ou arabinose). Usando esta plataforma Δ PT, identificamos vários transportadores de *K. marxianus* que recuperaram a capacidade da estirpe utilizar xilose e/ou arabinose. Estes transportadores foram caracterizados quanto à cinética de transporte de pentose e glucose utilizando açúcares marcados com ^{14}C . Um destes transportadores mostrou uma alta afinidade tanto para a xilose como para a glucose, embora a afinidade para a glucose ainda fosse 150 vezes maior do que para a xilose. Usando em análise *in silico* foram selecionamos aminoácidos-alvo deste transportador para mutagénese dirigida, numa tentativa de aumentar a afinidade para a xilose e diminuir o transporte de glucose. Vários mutantes foram construídos e caracterizados por ensaios radioativos e por avaliação do seu crescimento em misturas de açúcares. Um destes mutantes apresentou uma afinidade mais reduzida para glucose aumentando, simultaneamente a sua afinidade para a xilose, mostrando-se muito promissor. A reprogramação bem sucedida deste transportador foi usada para facilitar o co-consumo eficiente de hexoses e pentoses numa nova estirpe de *K. marxianus*, encurtando significativamente o tempo de fermentação em bioreatores com misturas destes açúcares.

Palavras chave: levedura, transportadores de açúcares, xilose, biorefinarias, *Kluyveromyces spp.*

ABSTRACT

Sustainable and efficient use of lignocellulosic hydrolysates in yeast-based biorefineries requires the simultaneous consumption of all the sugars present in this raw material, hexoses and pentoses. This represents a key bottleneck because of the low number of specific pentose transporters identified and the slow rate of pentose uptake into the yeast cell when glucose is present. *Kluyveromyces marxianus* is a non-conventional yeast that has attracted considerable attention due to its traits of industrial interest, like the ability of naturally assimilating pentoses, high thermotolerance and fast growth rate.

In this thesis, the main goal was to investigate and engineer pentose uptake in *K. marxianus* to obtain a transporter that would allow co-consumption of glucose and xylose for the application in biorefineries.

In order to identify novel pentose transporters, a *K. marxianus* screening platform (Δ PT) was constructed by deleting 12 genes that encoded transporters of the major facilitator superfamily (MFS). Using this Δ PT platform, which was unable to grow on pentoses, we identified several native *K. marxianus* transporters that recovered the native strain's ability to grow on either xylose and/or arabinose. Kinetic studies on the most promising candidates using ^{14}C -labelled sugars were carried on. One member of these transporters showed high affinity for both xylose and glucose, though the affinity for glucose was still 150-fold higher than for xylose. Using *in silico* analysis we selected target amino acids of this transporter for site-directed mutagenesis in an attempt to convert it into a specific xylose transporter. Several mutants were constructed and characterised by radioactive uptake and growth assays in bioreactors on mixture of sugars. One of these mutants decreased its ability to transport glucose but still transported xylose with even higher affinity kinetics. The successful reprogramming of this transporter to increase its preference for xylose over glucose was used to facilitate efficient co-consumption of hexose and pentose sugars in a novel *K. marxianus* strain, significantly shortening the time of the whole process.

Key words: yeast, sugar transporters, xylose, biorefineries, *Kluyveromyces spp.*

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LIST OF ABBREVIATIONS

MFS	Major Facilitator Superfamily
TMD	Transmembrane domain
TMS	Transmembrane spanning
WGD	Whole Genome Duplication
SSF	Simultaneous Saccharification and Fermentation
LCB	Lignocellulosic biomass
Hxt	Hexose transporters
Hgt	High-affinity Glucose Transporters
Fsy1	Fructose proton symporter
Frt	Fructose transporter
Lac12	Lactose permease
Lac4	β -galactosidase
Cel2	Cellobiase
BMS	Mannosidase
BGL	β -galactosidase
EGC	Endo- β -1,4-glucanase
KMAR_	<i>Kluyveromyces marxianus</i> NBRC1777 gene annotation
Sc	<i>Saccharomyces cerevisiae</i>
Cg	<i>Candida glabrata</i>
Td	<i>Torulaspora delbruekii</i>
Kl	<i>Kluyveromyces lactis</i>
Km	<i>Kluyveromyces marxianus</i>
Ag	<i>Eremothecium gossypii</i>
Dh	<i>Debaryomyces hansenii</i>
Ps	<i>Scheffersomyces stipitis</i>
Yl	<i>Yarrowia lipolytica</i>
Sp	<i>Schizosaccharomyces pombe</i>
ΔPT	Null pentose transporters platform strain
GRAS	Generally Recognized As <i>Safe</i>
QPS	Qualified presumption of <i>safety</i>
PCR	Polymerase chain reaction
HPLC	High Performance Liquid Chromatography

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Table 4.4. List of KMAR_10531 mutants constructed in this study.

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PUBLICATIONS

The work performed during this PhD resulted in the following peer-reviewed scientific publications:

- Identification of novel pentose transporters in *Kluyveromyces marxianus* using a new screening platform Lorena Donzella, Javier A. Varela, Maria Joao Sousa and John P. Morrissey. FEMS Yeast Research, 21, 2021 - foab026 <https://doi.org/10.1093/femsyr/foab026>
- Engineering a native *Kluyveromyces marxianus* transporter to reprogramme the balance between hexose and pentose uptake. Lorena Donzella et al. (Manuscript in preparation- under review for a patent application)
- Diversification of sugar transporters through niche specialization offers opportunity for biotechnological applications. Lorena Donzella et al. (Manuscript in preparation)

Peer-reviewed scientific publications not included in this thesis:

- Hexose transport in *Torulaspora delbrueckii*: identification of Igt1, a new dual-affinity transporter. Andreia Pacheco, Lorena Donzella, Maria Jose Hernandez-Lopez, Maria Judite Almeida, Jose Antonio Prieto, Francisca Rande-Gil, John P. Morrissey, Maria Joao Sousa. FEMS Yeast Research, 20, 2020 - foaa004. <https://doi.org/10.1093/femsyr/foaa004>

Contributions to the work reported in this thesis

Chapter 1. Lorena Donzella (LD) and John Morrissey (JM) contributed to the idea of the work. LD performed the bioinformatic analysis. LD and JM wrote the manuscript. JM and Maria João Sousa (MJS) reviewed and corrected the manuscript. The work was performed in UCC.

Chapter 2. JM, MJS, LD conceived the project. All the authors designed and interpreted the experiments. LD performed the experiments and the bioinformatic analysis. LD, MJS and JPM wrote the manuscript. The bioinformatic work was performed in UMinho and the experimental part in UCC.

Chapter 3. LD, MJS, JM contributed to the experimental design of the experiments. LD performed the experiments, the bioinformatic analysis, analysed the data and wrote the chapter. The experiments were performed in UCC.

Chapter 4. LD and MJS designed the experiments. LD performed the experiments and the bioinformatics analysis. LD and MJS analysed the data. LD wrote the chapter, MJS and JM reviewed and corrected it. The experiments were performed in UMinho.

Chapter 5. LD, MJS, JM conceived the experimental design. LD performed the experiments, Carlos Belloch Molina (CBM) assisted with the bioreactor experiments. LD wrote the chapter and MJS and JM reviewed and corrected it. The work was performed in UCC.

CONFERENCES CONTRIBUTIONS

Oral presentations

- Yeast4Bio COST Action CA18229 WG Meeting – Bari, Italy – April 2022 – Oral Presentation
“Novel *Kluyveromyces marxianus* platform strain enables the identification of promising pentose transporters for glucose/xylose co-consumption”. [Lorena Donzella](#), Carlos Belloch Molina, John Morrissey, Maria João Sousa
- MicroBiotec 21 – Online Conference, Portugal – November 2021 - Oral Presentation
“Engineering native *Kluyveromyces marxianus* transporter to reprogramme the balance between hexose and pentose uptake”. [Lorena Donzella](#), John Morrissey, Maria João Sousa
- PYFF7 Physiology of Yeasts and Filamentous Fungi - Milan, Italy, June 2019 – Oral Presentation
"Analysis of candidate pentose transporters in *Kluyveromyces marxianus*". [Lorena Donzella](#), Javier Varela, Maria João Sousa, John Morrissey

Poster presentations

- Microbiology Society Annual Conference – Belfast, UK – April 2022 – Poster Presentation
“Engineering a native *Kluyveromyces marxianus* transporter to reprogramme the balance between hexose and pentose uptake”. [Lorena Donzella](#), Carlos Belloch Molina, John Morrissey, Maria João Sousa
- ICY15 meets 30 ICYGMB – International Conference on Yeast Genetics and Molecular Biology – Online Conference - August 2021 - Poster Presentation. “Studying native *Kluyveromyces marxianus* transporters to reprogramme the balance between hexose and pentose transport”. [Lorena Donzella](#), John Morrissey, Maria João Sousa
- EFB 2021 – European Federation of Biotechnology – Online Conference – May 2021
– Flash Poster Presentation “Identification of novel pentose transporters in *Kluyveromyces marxianus* using a new screening platform”. [Lorena Donzella](#), Maria João Sousa, John Morrissey
- 5th Applied Synthetic Biology Meeting in Europe – Online Conference – November 2020 - Poster Presentation
“Multiple *Kluyveromyces marxianus* xylose and arabinose transporters identified using a novel pentose-deficient host strain”. [Lorena Donzella](#), Javier Varela, Maria João Sousa, John Morrissey
- MicroBiotec19 – Coimbra, Portugal, December 2019 – Poster presentation
"Hexose transport in *Torulaspora delbrueckii*: identification of Igt1, a new dual-affinity transporter". Andreia Pacheco, [Lorena Donzella](#) ... Maria João Sousa
- 36th SMYTE Small Meeting on Yeast Transport and Energetics. - Martina Franca, Italy, September 2018 – Poster presentation. “Enhancing yeast sugar transporters capacity under stress conditions”. [Lorena Donzella](#), John Morrissey, Maria João Sousa

THESIS OUTLINE

Background and Aim of the Thesis

Kluyveromyces marxianus is a yeast belonging to the fungal sub-phylum *Saccharomycotina*, known also as the “budding yeast subphylum”. Phylogenetically, *Kluyveromyces* is in the same family (Saccharomycetaceae) as *Saccharomyces*, but *Kluyveromyces* did not undergo the whole genome duplication (WGD). Instead, the *Kluyveromyces*, *Lachancea* and *Eremothecium* genera cluster together forming what is sometimes referred to as the “KLE clade” (Kurtzman, 2003; Marcet-Houben et al., 2015). *K. marxianus* is defined as non-conventional yeast and can be isolated for a great variety of habitats, which results in high metabolic diversity and polymorphism. This yeast is found in fermented milk products (which permits its GRAS and QPS qualification) as well as non-dairy environments such as decaying fruit (Fonseca et al., 2008; Lane et al., 2010). *K. marxianus* is characterized by attractive traits from the industrial point of view, such as its thermotolerance, being able to grow at temperature between 44°C and 52°C, and a rapid growth rate. As consequence, this yeast has attracted considerable attention for numerous biotechnological applications, as an alternative to the commonly used *S. cerevisiae*. Traditional biotechnological applications of *K. marxianus* involve enzyme production, among which β -galactosidase – responsible of the cleavage of lactose into glucose and galactose – and inulinase – needed for the degradation of plant fructans and the consequent sustainable release of simple sugars from complexed raw materials. Moreover, the great degree of genome variation confers *K. marxianus* strains with the capability of assimilating a wide range of substrates, including 6-carbon sugars (glucose, mannose and galactose), disaccharides (lactose and cellobiose) and pentoses (xylose and arabinose) (Lane et al., 2011; Signori et al., 2014). This makes this yeast suitable for bioconversion of raw materials, such as lignocellulosic biomass (LCB), which comprises various hexose and pentose sugars. LCB is considered a credible alternative to fossil resources for the production of biofuels and biochemicals due to its low cost, substantial availability and potential to reduce CO₂ emissions (Liu et al., 2019). LCB has a heterogeneous composition, as it consists of cellulose (40-50%), hemicellulose (20-50%) and lignin (10-40%). Cellulose is a polymer mainly made of glucose, while hemicellulose is a polymer of both hexose (glucose) and pentose (e.g. xylose) sugars. The pretreatment of lignocellulosic biomass releases a mix of six and five-carbon sugars that need to be completely utilized in order to make the biorefinery process economically competitive (Sánchez Nogué et al., 2015; Wei et al., 2015; Bertacchi et al., 2022). Not all microorganisms are able to naturally assimilate pentose sugars and even if they do, they show bottlenecks that make the process inefficient and long. One of the main impediments is that native yeast sugar transporters have significant higher preference for glucose than pentoses as xylose and arabinose, with the consequence that pentoses enter in the cell and are assimilated only when all glucose is depleted

(Li et al., 2016; Wang et al., 2016). As a result, the process becomes time-consuming and biphasic and not suitable for application in industry.

In this thesis, the main goal was to investigate pentose uptake in *K. marxianus* with the final aim of obtaining a transporter that would allow co-consumption of glucose and xylose in order to apply this yeast cell factory to industry.

Outline of the Thesis

Firstly, in **Chapter 1**, a study of the evolution of sugar transporters' families was conducted retrieving 139 transmembrane sugar transporters belonging to the Major Facilitator Superfamily (MFS) from 10 complete genome sequences of species spanning the Ascomycete phylum, and connections between the possess of specific transporters and the habitat of isolation of the yeasts were made. It is known that hexose uptake in yeast mainly occurs via Hxt and Hgt families of transporters. Hxt-like transporters, annotated as Kht-like in *K. marxianus*, were found in all the yeasts considered. However, there were some differences in their copy number, with an expansion in some of the analyzed species. Their phylogeny and synteny were investigated and one hypothesis of their evolution was made. On the other hand, Hgt-like transporters and a specific fructose transporter (Fsy1), which are present in *K. marxianus*, are absent in *S. cerevisiae*. *S. cerevisiae* can easily take up glucose and fructose by Hxt transporters, which work with a passive diffusion mechanism, therefore it might have lost Hgt and Fsy1 proteins as they work by coupling proton symport, this requiring energy (indirectly consuming ATP) and perhaps causing a fitness disadvantage (Coelho et al. 2013). We also showed that *K. marxianus*, *K. lactis*, *P. stipitis* and *D. hansenii* have clusters of sugar transporters associated with hydrolases for the utilization of disaccharides like lactose and cellobiose. Other yeasts like *Y. lipolytica* possesses a family of glycerol transporters consistent with the capacity of this yeast to use triglycerides as a carbon source. More families of transporters were studied and they are described in Chapter 1. Some transporters were found to be promiscuous, being able to uptake different sugars with various affinities. The main outcome of the work reported in this chapter was increased appreciation of the high plasticity of transporters. Some evolved to have a broad spectrum of sugars to allow the yeast to grow in various environments whereas others became more specific for sugars present in higher concentrations in the yeast's environment. The diversity in sugar transporters and consequent ability of yeasts to use various sugars becomes useful when planning the application of yeast cell factories in industry, therefore choosing the right yeast or

studying transporters that could be heterologously expressed in a host strain is important for biotechnological purposes.

Although transport systems for glucose, galactose, lactose and cellobiose were previously described (Varela et al., 2019; Varela et al., 2019b), there was limited knowledge on pentose transport in *K. marxianus*. To fill up this gap, in **Chapter 2**, an extensive bioinformatic analysis was performed and 27 putative proteins were predicted to belong to MFS and involved in sugar uptake in this yeast. Among these proteins, two clusters of transporters called Hgt-like and Kht-like were known to be responsible for the uptake of hexose sugars. Kht-like transporters are homologous to the well-known *S. cerevisiae* Hxt-like that are characterized by high and low affinity for glucose. It was known that pentose uptake can occur through hexose transporters, the most famous example is *S. cerevisiae* Gal2 that is a galactose transporter, which carry xylose import as well (Subtil et al., 2012) but various Hxt-like transporters showed also low affinity for pentoses (Nijland et al., 2020; Shin et al., 2017). For this reason and after a phylogenetic analysis with known xylose transporters, 12 candidate transporters genes were predicted to be involved in pentose import and a *K. marxianus* platform strain, called Δ PT, unable to uptake and grow on xylose and arabinose was constructed with a CRISPR-Cas9 method. This Δ PT strain lacked the 12 predicted transporters which comprised 5 members of the Hgt-family, 6 members of the Kht-family and an additional transporter called KMAR_60179. The Δ PT strain was a suitable tool to test and characterize novel pentose transporters. Therefore, in order to study these transporters, each of the 12 deleted ones was individually expressed in the Δ PT strain. Among these, we identified several putative xylose or arabinose transporter proteins that recovered the null strain's ability to grow on these pentoses. Two proteins of the Hgt-family (KMAR_10527 and KMAR_10531) conferred growth in media with both high and low concentrations of either xylose and arabinose, while six members of the Kht-family and other two Hgt-like proteins displayed growth only at high xylose concentrations, indicating solely low affinity transport activity. KMAR_60179 is an orthologue of the *S. cerevisiae* glycerol transporter Stl1, but, unlike Stl1, it was also found to be a low affinity xylose transporter.

It is known that *K. marxianus*, as other microorganisms, suffers an intrinsic system of catabolite repression that prevents the utilization of other carbon sources in presence of glucose, which is the favorite carbon source. This phenomenon works at two levels: genetic repression of genes involved in the use of alternative carbon sources and competitive inhibition of sugar membrane transporters. As said before, the main goal of this thesis was to enable co-consumption of glucose and xylose in an efficient bio-process. Therefore, there was an interest in understanding how glucose repression works in this yeast

in order to avoid the inhibition on xylose metabolism and transport. Therefore, in **Chapter 3** we reviewed what it is known about *K. marxianus* glucose repression system, comparing it with that in *S. cerevisiae* one and we highlighted the inhibitory effect of glucose on pentose transporters. In order to study the latter, we tested each Hgt or Kht transporter and KMAR_60179 expressed in the Δ PT strain on media containing xylose or arabinose supplemented with the toxic glucose analogue 2-DOG. All Hgt and Kht transporters showed a large impairment in the growth when 2-DOG was in the media, showing that all of them can transport it inside the cell. Only KMAR_60179 was able to support the growth on xylose even in presence of 2-DOG, showing to be insensitive to 2-DOG and being able to uptake xylose. Thus, KMAR_60179 was not being inhibited by glucose, making it a promising candidate for future studies. At this point there was an interest in characterizing deeper the identified pentose transporters, so kinetic studies of two of the most interesting candidates (KMAR_10531 and KMAR_60179) were performed using ^{14}C -glucose and ^{14}C -xylose and they are shown in **Chapter 4**. Although the Hgt-like transporter, KMAR_10531 was found to have 150-fold higher affinity for glucose than for xylose, it was still one of the best transporters for xylose import reported in literature. We decided then to use *in silico* analysis to select target amino acids of this transporter that could be subjected to site-directed mutagenesis in an attempt to get a shift from glucose preference to xylose. It was known that a conserved sequence motif in the first transmembrane domain (TMD1) was involved in sugar specificity and that some residues in TMD8 are essential for the inhibition of xylose transport by glucose in other yeasts like *S. cerevisiae* (Farwick et al., 2014; Nijland et al., 2020; Young et al., 2014). Based on the 3D structure of KMAR_10531, we chose to mutate some residues in both TMD1 and TMD8. One variant KMAR_10531 N³²⁵V, in which a conserved asparagine in TMD8 was substituted by a valine, showed reduced ability to transport glucose but increased xylose affinity. In **Chapter 5**, we tested this engineered transporter expressed in Δ PT platform strain and in the wild type strain in flasks and bioreactors and compared the growth with the one conferred by the native transporter. The conditions to scale up the bioprocess from flasks to bioreactors were optimized and pH was followed along the fermentation in the bioreactors. Interesting a drastic pH increased was observed during xylose consumption and this resulted in the inability of the native transporter KMAR_10531 to complete the consumption of xylose in bioreactor. When the pH was controlled and set at acidic values, KMAR_10531 was able to overcome this limitation carrying xylose transport and enabling its total consumption. From this study, it emerged that this transporter might work as proton-symporter needing a proton gradient to work. The variant KMAR_10531 N³²⁵V, on the other hand, expressed in Δ PT platform strain enabled efficient co-consumption of glucose and xylose both in flasks and bioreactor, depleting all the xylose in less than 60h. The improved variant

KMAR_10531 N³²⁵V expressed in *K. marxianus* ΔPT platform strain represents a promising solution for complete valorization of all sugars present in lignocellulosic biomass for biorefineries.

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Chapter 1

*Diversification of sugar transporters through niche specialization offers
opportunity for biotechnological applications*

1.1 Introduction

Sugar transport is often the parameter that limits yeast growth and fermentation (Kuanyshev et al., 2021). Considerable research effort has been made in the past decades to study various aspects of sugar metabolism in yeast, showing that the uptake of sugar in the yeast cell plays a crucial role in the control of the metabolic flux, having influence on catabolic repression and the equilibrium between respiratory and fermentative metabolism.

Biochemical characterization of sugar uptake in yeast cells started around six decades ago (Cirillo, 1968; Cirillo, 1962), followed by the first isolation 30 years ago of a hexose transporter gene (Tschopp et al., 1986), encoding the *Saccharomyces cerevisiae* galactose permease Gal2. After that, a plethora of monosaccharide transporters (called Hxt) of this yeast was identified and characterized (Nijland et al., 2020). Nowadays, due to development of bioinformatic tools and sequencing methods, a massive amount of information has been produced and a multitude of putative sugar transporters and sugar sensors have been identified in yeast genomes, allowing a deeper study of their evolution and relationship (Donzella et al., 2021; Pacheco et al., 2020; Varela et al., 2019, Díaz-Fernández et al., 2020).

Sugar transporters mainly belong to the major facilitator superfamily (MFS), which includes proteins from bacteria, archaea and eukarya. These proteins are integral plasma-membrane polypeptides that exhibit high structural conservation, although they may share little sequence similarity. MFS sugar transporters consist of two sets of six hydrophobic transmembrane-spanning (TMS) α -helices connected by hydrophilic loop (Leandro et al., 2009) (Figure 1.1).

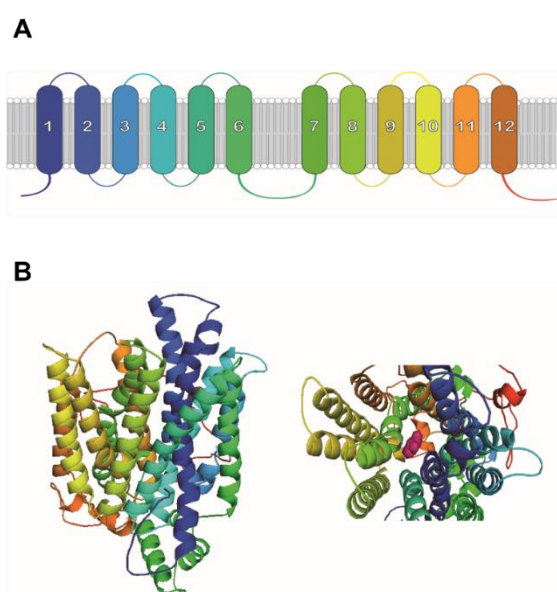


Figure 1.1 Topology of yeast sugar transporters belonging to the MFS. The 12 transmembrane domains are represented as cylinders (A). 3D-structure of a sugar transporter bound to the ligand in pink (B).

The mechanism of action of most of these sugar transporters is facilitated diffusion, a mechanism that permits the movement of a solute molecule favored by its concentration gradient (with no need of external energy) across the membrane (Leandro et al., 2009). Some monosaccharide transporters are able to transport the sugar against its gradient of concentration by coupling the transport with the simultaneous movement of protons in the same direction, hence they are known as proton-symporters and they are energy-consuming systems. Finally, even though they share a similar structure with other members of MSF, some members of the family (i.e. Snf3 or Rgt2 in *S. cerevisiae*) are unable to transport sugars but they act as sensors, signaling the presence of sugars at the membrane, influencing gene expression (Rolland et al., 2002).

Although the evolution of MFS has been discussed previously, there has not been a lot of consideration of links between an organisms' adaptation to specific natural environments and the evolution of transporters. Multiple studies showed that sugar transporters and sensors are present in different copy numbers amongst yeasts (Donzella et al., 2021; Lin et al., 2011; Pacheco et al., 2020; Rodrussamee et al., 2011; Varela et al., 2019). The gain, loss and diversification of transporters over the course of evolution is a strong indication of niche adaptation as it represents specialization to use specific sugars therefore it is valuable to study this process. Here, we took advantage of the availability of high quality genomes to examine this question in budding yeasts. For this analysis, we selected nine species spanning the Saccharomycotina [*Saccharomyces cerevisiae* (*Sc*), *Candida glabrata* (*Cg*), *Torulaspora delbrueckii* (*Td*), *Kluyveromyces lactis* (*Kl*), *Kluyveromyces marxianus* (*Km*), *Eremothecium gossypii* (syn. *Ashbya gossypii*) (*Ag*), *Debaryomyces hansenii* (*Dh*), *Scheffersomyces stipitis* (syn. *Pichia stipitis*) (*Ps*) and *Yarrowia lipolytica* (*Yl*)] and also *Schizosaccharomyces pombe* (*Sp*), which represents the Taphrinomycotina, the basal sub-phylum in the Ascomycetes (**Figure 1.2**).

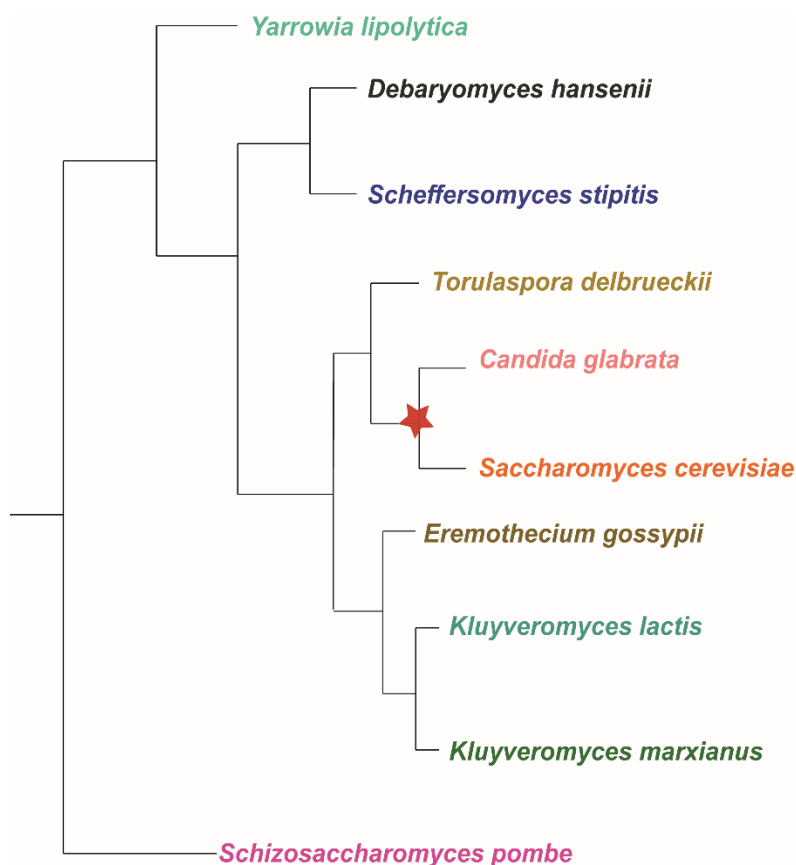


Figure 1.2. Phylogenetic relationship of the yeasts included in this study. The star indicates the Whole Genome Duplication (WGD) event, which as discussed in the text is now believed to be a hybridisation between parents that were basal in the *Torulaspora* and the *Eremothecium*/*Kluyveromyces* lineages.

Complete genome sequences were retrieved and explored leading to the identification of 139 candidate MFS-type putative sugar transporters (Table 1.1). Multiple sequence alignments and phylogenetic analysis was also performed, enabling us to establish the detailed relationships between the MFS proteins within and between species. (Figure 1.3). The extent to which these proteins have been previously studied varies substantially. In some cases, especially for *S. cerevisiae* hexose transporters (HXT family), very detailed knowledge including kinetics of sugar transport is known, whereas for others, function has been inferred based on sequence similarity. In some cases, this was based on limited information and thus multiple transporters now have gene names that do not reflect their substrate range. Other transporters are identified here for the first time and do not have gene names, though now based on our more complete dataset, it is possible to infer potential substrates for some of them. For the purposes of our analysis, we labeled clades and sub-clades based on the known or presumed functions, notwithstanding the limitations already mentioned. We discuss these clades below, offering insights where possible on the implications arising from integrated analysis that considers known functions and evolutionary history.

Figure 1.3. Phylogenetic tree of 139 putative sugar transporters belonging to different yeast species *Saccharomyces cerevisiae* (Sc - orange), *Candida glabrata* (Cg - pink), *Schizosaccharomyces pombe* (Sp - fuxia), *Torulaspora delbrueckii* (Td - ocher), *Kluyveromyces lactis* (Kl - teal), *Kluyveromyces marxianus* (Km - green), *Ashbya gossypii* (Ag - brown), *Debaryomyces hansenii* (Dh - black), *Pichia stipitis* (Ps - blue), *Yarrowia lipolytica* (Yl - light blue). Transporters with putative similar function are put in colored squares. The *S. cerevisiae* strain EC1118 fructose-symporter Fsy1 is included in the tree (marked with *) as reference for the specific fructose transporter cluster. Proteomes of *S. cerevisiae*, *C. glabrata*, *S. pombe*, *T. delbrueckii*, *K. lactis*, *K. marxianus*, *A. gossypii*, *D. hansenii*, *P. stipitis* and *Y. lipolytica* yeast species were retrieved from Uniprot and filtered out for the transporters belonging to the Major Facilitator Superfamily (MFS). Transporters belonging to the MFS, with 11 or more transmembrane domains were considered as potential sugar transporters. Synteny and evolutionary conservation were inspected using the Yeast Gene Browser (YGOB, www.ygob.ucd.ie) (Byrne and Wolfe 2005). Sequence alignment was performed using MUSCLE algorithm 3.8 (Edgar et al. 2004). Neighbor joining was used for the complete phylogenetic tree comprising 139 sugar transporters. The phylogenetic analysis was performed using MEGA6/MUSCLE (Tamura et al. 2013) and the trees were viewed with FigTree software (<http://tree.bio.ed.ac.uk/software/figtree/>).

Table 1.1. Number of sugar transporters and related features of the yeast strains studied in this work.

	C- source	Environment of isolation	Crabtree	WGD	HXT-like	HGT-like	Sensors	Fructose	Lactose	Cellobiose	Maltose	Glycerol
<i>S. cerevisiae</i> S288C	Glucose, Fructose, Maltose	Vineyards, fruits	+	+	 							
<i>C. glabrata</i> ATC 2001	Glucose, Trehalose	Microbial flora of the mouth gastrointestinal tract, skin, vagina	+	+	 							
<i>S. pombe</i> 972 / ATCC 24843	Glucose, Glycerol, Sucrose, Raffinose, Maltose	Fermented beverages, grapes, beer	+	-	 							
<i>T. delbrueckii</i> ATCC 10662	Glucose, Fructose, Maltose	Vineyards, musts, oak, soil	+	-								
<i>K. marxianus</i> NBRC1777	Glucose, Fructose, Lactose, Xylose, Arabinose	Soil	v	-								
<i>K. lactis</i> CBS2359	Glucose, Fructose, Maltose, Lactose	Dairy	-	-								
<i>A. gossypii</i> ATCC 10895	Glucose, Fructose, Sucrose	Pathogen of cotton or citrus fruits	-	-								
<i>D. hansenii</i> CBS 767	Glucose, Glycerol	Various	-	-								
<i>P. stipitis</i> CBS 6054	Glucose, Cellobiose, Xylose, Arabinose	Guts of wood-inhabiting passalid beetles	-	-								
<i>Y. lipolytica</i> CLIB 122	Glucose, Glycerol	Various	-	-								

Expansion and evolution of the *HXT* family

Hexoses uptake occurs via two classes of transporters belonging to the MFS, called Hxt (hexose transporters) and Hgt (high-affinity-glucose transporters), which have various affinity and specificity for diverse sugars (Figure 1.3). Hxts are undoubtedly the best-studied family and many of these proteins have been extensively characterised in *S. cerevisiae*. It is a large family that has been subject to expansion in most species. This is particularly evident in *S. cerevisiae*, which has 17 genes encoding for Hxts. Reconstructing the evolutionary trajectory of HXTs is very instructive for understanding this class of transporters more generally. There are multiple examples of orthologous genes that arose via speciation as well as paralogous genes that arose in particular lineages post-speciation. This is further complicated by the whole genome duplication (WGD) that gave rise to the lineage that includes *S. cerevisiae* and *C. glabrata*, as this was, in fact, a hybridisation between parents at the base of the ZT (*Torulaspora*) and KLE (*Kluyveromyces*) lineages (Marcet-Houben & Gabaldón, 2015; Wolfe, 2015). Finally, as well as duplications and acquisitions, there are also instances of gene loss in the phylogenetic history.

The tree presented in Figure 1.4 focuses on the Hxts and shows additional relevant features. It is clear that the *HXT* family originated from a single transporter gene and since the furthest related species in the Saccharomycotina, *Y. lipolytica* (YAL), has just one copy, this may represent the ancestral state. There are multiple Hxts in *S. pombe* (SCHPO), but these cluster together indicating expansion within that species. Moving from *Y. lipolytica* in the Saccharomycotina, the next two diverging species, *S. stipitis* (genes annotated as PICS) and *D. hansenii* (DH), show independent intraspecies expansion of the ancestral HXT (four and three copies, respectively). The situation becomes more complex when we move to within the Saccharomycetacea, which includes *E. gossypii* (annotated as AG), *S. cerevisiae* (Sc), *Kluyveromyces* (Kl and KMAR), *T. delbrueckii* (TDEL) and *C. glabrata* (CAGL). *E. gossypii* shows expansion of a single HXT, resembling earlier diverging yeasts, but all other species carry expansions of genes that arose at various stages during speciation. Thus, there are multiple clades carrying orthologous sets of genes. From examination of the phylogenetic tree, and also taking gene synteny into account, we propose a particular pattern and sequence by which the HXT family evolved in the Saccharomycetacea (Figure 1.5). The major underlying event was a duplication of the ancestral HXT gene to create a locus with two paralogues. In the ZT lineage there was a further duplication of one of these genes such that there were now three of these genes. Although there have been various expansions and losses, and duplication of some of the genes to other regions of the genome, the locus has largely remained intact and syntenic throughout the Saccharomycetacea and thus is the key to understanding the protein family.

K. lactis and *K. marxianus* are the most straightforward species to analyse first (green labelling in Figure 1.4). *K. lactis* retains the two original genes, which are now named *KHT1* and *KHT2*, and that have been studied as glucose transporters in that species. In some strains studied, there was actually a recombination between *KHT1* and *KHT2*, giving rise to a chimeric gene, *RAG1* (Weirich et al., 2004). In *K. marxianus*, both *KHT1* and *KHT2* have been subject to gene duplications such that there are now two copies of *KHT1* and four copies of *KHT2*. As with *K. lactis*, is also reported that recombination can occur within the locus with some *K. marxianus* strains showing variable numbers (Varela et al, 2019). It is not yet clear whether the intra-KHT recombination that has been described represents true natural diversity or is a laboratory “artefact” but in any case, it illustrates how variation can arise at the locus. *T. delbrueckii*, *S. cerevisiae* and *C. glabrata* also have multiple copies of both the *KHT1*-like and the *KHT2*-like gene but the pattern of expansion is different. Considering first, the *KHT1*-like gene, there are two clades, represented by *TDELOE2280* and *TDELOE2290*. Considering that one of the WGD parents is related to *Torulaspora*, it is inferred that ancestral *KHT1* duplicated early in the ZT lineage and thus the ancestors of *T. delbrueckii*, *S. cerevisiae* and *C. glabrata* all had two *KHT1*-like genes. This is still the case in *T. delbrueckii* (*TDELOE2280* and *TDELOE2290*) but in *S. cerevisiae* one of the genes subsequently duplicated giving rise ultimately to *HXT1* and *HXT3*. This is also seen in *C. glabrata* indicating that it may be preceded that speciation event but low bootstraps prevent a definitive conclusion. Regarding the ancestral *KHT2*-like gene, there was also a duplication in *T. delbrueckii* to give rise to ancestors of *LGT1* and *IGT1*, but the close relationship of these two sequences shows that this was recent duplication within the species. *S. cerevisiae* carries eight genes that are most likely to derive from the *KHT2*-like ancestral gene. We focus first on *HXT4*, *HXT6* and *HXT7* as these are the genes that are in the same genomic location (locus) as all the other *KHT*-like genes. It is clear that *HXT6* and *HXT7* arose for a very recent duplication within *S. cerevisiae* and we will refer to *HXT6/7* as the ancestor and prior to this duplication, two genes were present *HXT6/7* and *HXT4*. Again, considering that the WGD combined parents form the ZT and KLE lineages, we propose that one parental sub-genome donated *HXT6/7* and the other *HXT4*. Although Figure 1.5 has a suggestion, it is not possible to say which sub-genome provided which copy. It is implicit in this phylogenetic reconstruction that ancestrally in the *S. cerevisiae* lineage there were four copies of a *KHT1*-like gene, though now, *S. cerevisiae* possesses three HXTs in this family. In addition, the extant *HXT1* and *HXT3* arise from a tandem duplication, though now they are on different chromosomes. Both of these can be explained by recombination between homologous regions of the parental chromosomes, giving rise to loss of one allele and switching of *HXT5* and *HXT3*. The consequence of all these events are the two extant *S. cerevisiae* loci: *HXT7* – *HXT6* –

HXT3 and *HXT4* – *HXT1* – *HXT5*. As will be discussed later, in all species studied, all the genes at this locus encode glucose transporters and it is therefore reasonably to conclude that glucose transport is the original function of these proteins.

Analysis of *S. cerevisiae* HXTs has always been complicated because of additional expansions. Some are also seen in other species and thus can be dated to certain points on the evolutionary tree. Some are specific to *S. cerevisiae* but others may be indicators of ancient duplications where the orthologous gene was lost from both the ZT and KLE lineages. An example of this would be the deep-branching the clade with *HXT13*, *HXT15*, *HXT16* and *HXT17*. The clade with *HXT2* and *HXT10* (arising from a recent duplication within *S. cerevisiae*) is also interesting as it also has a *T. delbrueckii* orthologue. The branching position, supported by good bootstraps, indicates that the ancestor of this gene even precedes the duplication of the “original” *HXT* that gave rise the *KHT1*-like and *KHT2*-like genes. It is therefore concluded that the orthologous gene was retained in the ZT lineage but lost in the KLE lineage. The additional *S. cerevisiae* genes within the *KHT2*-like clade have mixed ancestry. The galactose transporter, Gal2, arose in the ZT lineage as a duplication of one of the *KHT2*-like ancestral genes and was provided to *S. cerevisiae* by that parent at the WGD event with *HXT8* being a paralogue. *HXT9*, *HXT11* and *HXT12* are paralogues within *S. cerevisiae* and cluster strongly with *Torulaspora* *IGT1* and *LGT1*. There was a single ancestral gene in the *S. cerevisiae* parent that, as well as giving rise to *HXT6/7* also duplicated to a distal locus giving the *HXT9/11/12* ancestor. *C. glabrata* was not extensively discussed above but it follows a similar pattern of expansion via tandem duplication. *HXTs* are ancestral in the ascomycetes and the paradigm of expansion of a single ancestral copy via sequential tandem duplications is also seen in *S. pombe*. In this case, it is known that certain members of the Hxts work not only as hexose transporters, but they are involved in the transport of other substrates or in different cellular function. For instance, in *S. pombe*, among eight proteins (Ght1-8) homolog to Hxts, Ght4 is responsible for the uptake of gluconate instead of glucose (Heiland et al., 2000).

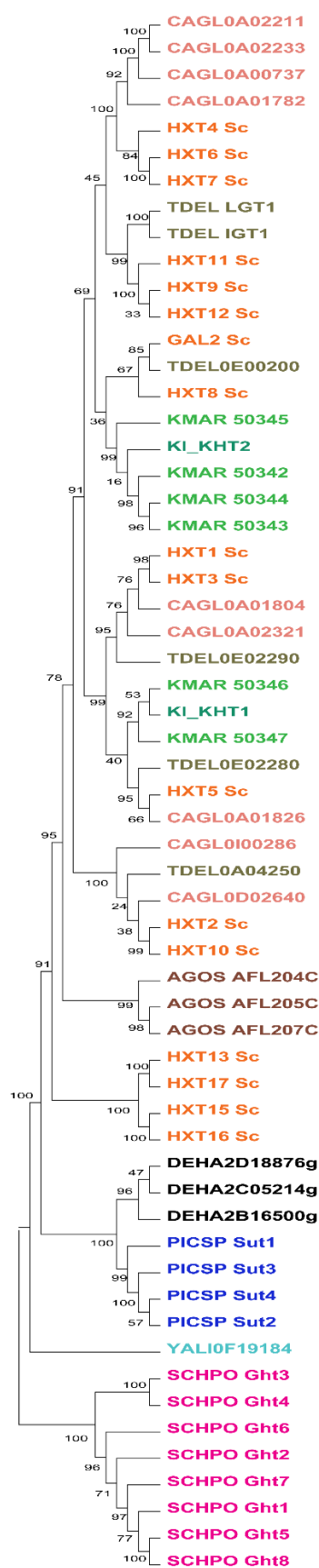
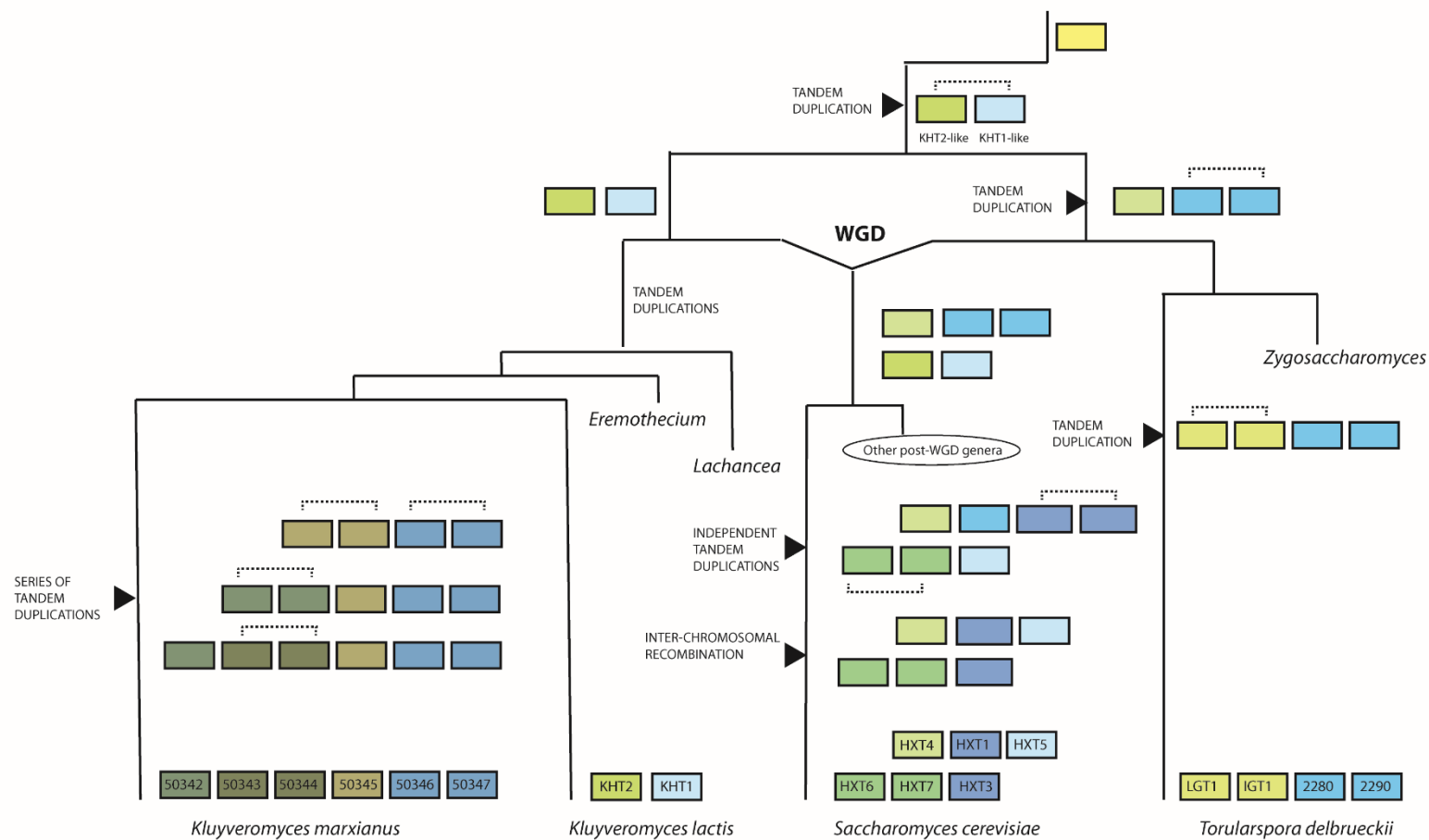


Figure 1.4 Maximum-likelihood tree with 500 times bootstrap **of Hxt proteins among various yeast species.** The colour-code is the same as the one used in Figure 2. The phylogenetic analysis was performed using MEGA6/MUSCLE (Tamura et al. 2013).

Figure 1.5 Hypothesis of evolution of Hxt ancestral locus. Dashed lines represent isolated duplication events which originated multiple copies of transporters. The figure was created considering YOGB synteny and phylogeny between the sugar transporters genes.



Evolution in Transporter Genes is related to the yeast adaptation to their habitats

Before going in detail on each single class of sugars and the transporters that can mediate their uptake, it is important to understand that sugar transporters are characterized by high plasticity and promiscuity. One sugar transporter can have a broad uptake spectrum of sugars, while some transporters evolved to be very specific for sugars present in higher concentrations in the yeast's environment. For example, some glucose transporters belonging to the Hxt and the Kgt families can also mediate the import of xylose, arabinose and/or galactose with various affinities (Knoshaug et al., 2015b; Subtil & Boles, 2012) or lactose and cellobiose can share a sugar transporter (Varela et al., 2019b; Varela et al., 2019) or a glycerol transporter was found to also be a xylose transporter (Donzella et al., 2021).

Glucose uptake – Hxt- and Hgt-like transporters

As shown in the previous paragraph, the hexose transporter genes (*HXTs*) have increased their copy number during the evolution of *S. cerevisiae* lineage. Some authors previously speculated glucose limitation could trigger duplication of hexose transporters Hxt6 and Hxt7 in *S. cerevisiae* (Brown et al., 1998; Dunham et al., 2002). In its natural environment, *S. cerevisiae* can experience a broad range of sugar concentrations for example in rotting fruit or in decomposing leaves. It is likely that, for this reason, this yeast developed a complex and highly regulated system of hexose transporters with specific features which allows the cell to optimize the uptake and metabolism of the sugar at various concentrations (Horák, 2013). A total number of 20 putative members of the MFS have been identified in *S. cerevisiae*, transporters belonging to the Hxt class (Hxt 1-17), Gal2 and Snf3 and Rgt2. Glucose uptake occurs mainly by 7 of these transporters with various affinities: *HXT1* and *HXT3* encode for low affinity, *HXT2*, *HXT4* and *HXT5* for middle affinity and *HXT6* and *HXT7* for high affinity transporters. Their expression is strictly regulated by the concentration of glucose in the media, except for the expression of *HXT5*, which is regulated by growth rate rather than extracellular glucose (Horák, 2013). The function and expression of Hxt8 to Hxt17 transporters is not well defined, but it is known that Hxt9 and Hxt11 might be related to the subfamily of ATP-binding cassette transporters involved in the resistance of antifungals and xenobiotics and Hxt 13-15-16-17 are polyol transporters (Jordan et al., 2016). *GAL2* encodes for a transporter that mediate D-galactose uptake and the other two genes, *SNF3* and *RGT2*, encode for glucose sensors.

As shown in Figure 1.4 and 1.5, homologous of *S. cerevisiae* *HXT* genes are present in all the yeasts investigated, but some yeasts have an additional family of transporters called Hgt, which at first it was thought would allow glucose import with high affinity. In the reality, the specificity of these transporters is broader than thought and includes, beside glucose, galactose, xylose, arabinose. In the dairy yeast *K. lactis* and in the wine yeast *T. delbrueckii* there are respectively one (Billard et al., 1996) and two Hgt-like transporters, but in the non-conventional yeast *Kluyveromyces marxianus*, we observe an expansion in all the hexose transporters (both Hxts, as shown in the previous paragraph, and Hgts). This yeast possesses 5 tandem copies of *HGT1* on chromosome 1 and a further single homologue on chromosome 5 (Figure 1.3). Khts and Hgts transporters of *K. marxianus* are reported to have several functions and a broad specificity for both hexose and pentose sugars deriving from plant sources, which represent the primary environment of isolation of this yeast (Donzella et al., 2021; Varela et al., 2019a).

All the characterized Hgt transporters are proton symporters (Knoshaug et al., 2015, Donzella et al. in submission). In the halotolerant yeast *D. hansenii*, we also detected a high number of sugar transporters, among which 4 copies belonging to the *HGT* clusters and 3 copies to the *KHTs* that have various affinity both for hexoses and pentoses. It is reported that some of its hexose transporters display sugar-associated proton uptake exclusively in the presence of salts as NaCl or KCl (Nobre et al., 1999). We believe that the ones that show the proton symport behaviour belong to the Hgt family, as all the known Hxt mediate facilitated diffusion with no energy expense, although this needs to be better investigated.

It is possible that active transport based on coupling the sugar uptake with proton translocation in the same direction across the membrane (known as sugar/proton symport) was the mechanism of import present in the ancestral yeast. This would explain why most of the transporters belonging to various families - Hgt-like, Lac12-like, Glycerol and Fructose transporters (discussed further in the following paragraphs) - are found to be dependent on proton gradient. Moreover, this is also consistent with the fact that all the known maltose transporters are also proton symporters (Henderson et al., 2017), even though they share an ancestor with Hxt-like transporters (Figure 1.3). The proton motive force that drives this kind of transporters is maintained by export of protons by the H^+ -ATPases therefore this transport system requires energy for its activity. We can speculate that Hxts might have lost the proton symport activity or evolved to not be dependent on proton gradient and therefore were favoured in the course of evolution in some yeasts rather than Hgts. In fact, Hxts are not proton-gradient consuming transporters and for yeasts that prefer aerobic fermentation rather than respiration, like *S. cerevisiae*, saving energy becomes important as fermentation is less energetically efficient than respiration. The same conclusion

can be made regarding the loss of the specific fructose-proton symporter Fsy1 in *S. cerevisiae* (next paragraph).

Fructose uptake – Fsy1 proton symporter

It is known that seven *S. cerevisiae* Hxt transporters (Hxt1-7) also mediate fructose uptake and a specific active fructose/H⁺ symporter is absent in this yeast (Rodrigues De Sousa et al., 1995). As specific fructose transporters (Fsy1) work coupling proton symport, it is hypothesized that in presence of high fructose concentrations, the presence of Fsy1 could cause fitness disadvantage as it requires energy (ATP), having a bad impact on cell energy metabolism (Coelho et al., 2013). For this reason, *S. cerevisiae* might have lost this transporter, as fructose can be easily taken up by Hxt-like transporters, without energy expenditure.

However, a specific fructose-proton symporter (Fsy1 marked with an asterisk in Figure 1.3) is present in *S. cerevisiae* strain EC1118 and other *Saccharomyces* species involved in fermented beverages production. For this reason we decided to look into specific fructose symporters of various yeast species (Figure 1.6). Fructose symporters (Fsy1-like) were found in various species of the Saccharomycotina, including *P. stipitis* and *D. hansenii*, and within the Saccharomycetacea, spanning between ZT clade and KLE clade, with the exception of *E. gossypii*, which lacks a specific fructose transporter, but we can assume that its uptake occurs through other hexose transporters, since it is able to use fructose as carbon source.

There are quite a few works (Anjos et al., 2013; Coelho et al., 2013; Galeote et al., 2010) that state that *FSY1* was acquired in *S. cerevisiae* EC1118 by horizontal gene transfer from an unidentified yeast lineage, conferring the yeast significant advantages during the wine fermentation process. From our data, it is clear that *FSY1* was acquired from one of the yeasts belonging to the ZT clade (often present during wine fermentation). This Sc_EC1118 fructose/H⁺ symporter has higher identity and form a cluster with the *T. delbrueckii* and *Z. rouxii* ones (pink square in Figure 1.6) rather than with the *Kluyveromyces* ones (green square in Figure 1.6).

Orthologous genes of this fructose/H⁺ symporter are found in other members of the *Saccharomyces* genus like *S. uvarum*, *S. pastorianus* and *S. eubayanus*, clustering together with the ones of the ZT clade and *S. cerevisiae* EC1118. However, the acquisition of *FSY1* in these *Saccharomyces* species seems to be due to a different horizontal gene transfer event, specifically from a *Torulaspora* specie. This event

might have happened right after the divergence of the *S. cerevisiae* ancestor and the rest of *Saccharomyces* species, as the *FSY1* share high similarity within these other *Saccharomyces* species.

Given that we also found this specific symporter in the *Kluyveromyces* species, we can assume that the Fsy1 transporter was already present at the ancestral level before the WGD. Another theory is that when the WGD occurred, the ZT parental sub-genome donated *FSY1* to the *S. cerevisiae* lineages and in the course of evolution some strains might have lost it. *FSY1* is in a conserved genomic region called “region C” where various genes display synteny with the genes of species closely related to the *Saccharomyces* clade (Galeote et al., 2010; Novo et al., 2009). Therefore, this region is quite conserved among Saccharomycetacea species, which is consistent with the theory that this region and Fsy1 might have already been in the ancestor. To resolve these options, more studies are needed, taking in consideration more yeast species and exploring in more detail region C.

In other yeasts, fructose uptake occurs via Hxt transporters, but it is worthy to report that in *Yarrowia lipolytica*, a transporter responsible for fructose uptake with high affinity (*YHT1*) clusters with sensors and not with fructose symporters or Hxt-like transporters.

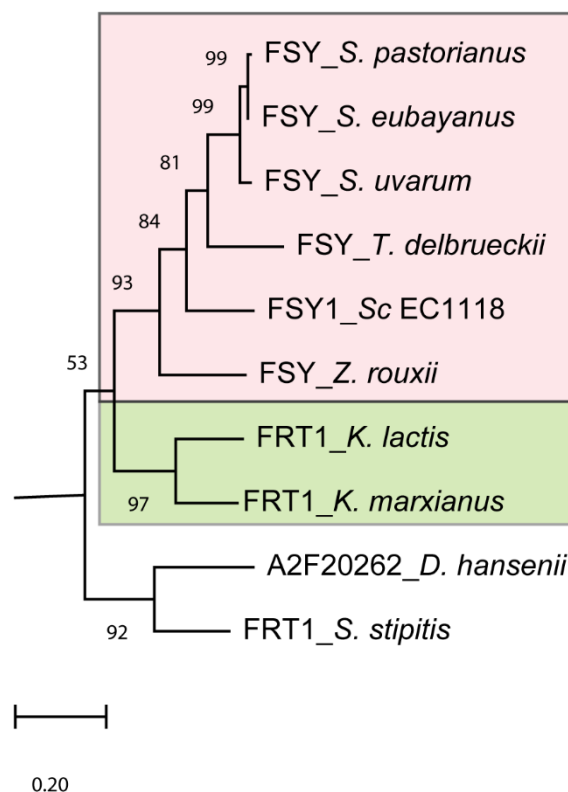


Figure 1.6 Maximum-likelihood phylogenetic analysis of specific fructose/H⁺ symporter of various yeast species. Transporters more related to the ZT clade and the KLE clade are shown in a pink and a green box, respectively.

Pentose uptake – various transporters

S. cerevisiae cannot naturally assimilate pentoses and it lacks specific D-xylose or L-arabinose transporters. Even so, it can uptake these pentoses via endogenous Hxt family of D-glucose transporters as well as the galactose permease Gal2 (Rojas et al., 2021; Vasylyshyn et al., 2020). The latter can transport xylose with low-affinity, but it is competitively inhibited by D-glucose, meaning that xylose starts to be transported and metabolized only when D-glucose is depleted from the media. For this reason, various approaches have been tested to provide *S. cerevisiae* with a specific pentose transporter (Farwick et al., 2014; Nijland et al., 2020). The non-conventional yeast *K. marxianus* is known to be a native-pentose utilizer and various transporters are reported to be involved in xylose and arabinose uptake with different affinities. Arabinose is transported inside the cell via some members of the Hgt cluster of transporters, as for the mutant Δ/hgt the growth in arabinose is suppressed (Chapter 2 - Donzella et al., 2021). Xylose is taken up with high affinity by two members of Hgts (KmAXT1 and KMAR_10531), which are also responsible for glucose and galactose uptake respectively, and with low affinity by other hexose transporters belonging either to the Hgts or Khts. Surprisingly it is reported that another transporter (KMAR_60179) not in cluster with the hexose transporters can also import xylose with low-affinity (Knoshaug et al., 2015; Varela et al., 2019, Donzella et al., 2021).

P. stipitis is an excellent xylose-fermenting organism and it is provided with xylose transporters often used heterologously in other yeasts (Katahira et al., 2008; Runquist et al., 2010). It is characterized by a plethora of transporters able to import both xylose or arabinose. These transporters are found either among Hxts (annotated like Sut) or they are assembled in a cluster between glycerol and cellobiose transporters (in light green in Figure 1.3). Among the latter, Xut1, exhibits moderate sugar transport rates but with preferences for D-xylose over glucose. *D. hansenii* also shows the ability to transport xylose through a transporter found in the same cluster with Ps_Xut1 and Ps_Xut3, called XylHP (DEHA2C11374p) which can uptake xylose and glucose (Ferreira et al., 2013).

Glycerol uptake – Stl1-like transporters

Glycerol uptake occurs in *S. cerevisiae* via a glycerol/H⁺ symporter Stl1 involved in response to high osmolarity (Ferreira et al., 2005). In *T. delbruekii*, *A. gossypii*, *P. stipitis*, *K. lactis* and *K. marxianus* only one potential glycerol transporter is predicted. Curiously, Km_60179 which is equivalent to Sc_Stl1 is reported to be also involved in xylose uptake with low-affinity (Donzella et al., 2021), but no other glycerol

transporter is reported to play the same role in pentose uptakes. As mentioned in the previous paragraph, some specific xylose transporters of *P. stipitis* and *D. hansenii* form a cluster between glycerol and cellobiose transporters, supporting the idea that these transporters are very plastic and evolved new functionality to satisfy the need of the yeast which can live in environments rich in plant disaccharides (cellobiose) and plant pentoses and hexoses.

In *D. hansenii* it is remarkable to underline the expansion of glycerol:H⁺ symporters (8 members). Breuer et al., 2006 reported that the ability of *D. hansenii* to maintain osmotic balance is strictly related to the activity of several transporters under salt stress conditions. Some of these key transporters are symporters of glycerol and sodium which work in a tight equilibrium with sodium-proton antiporters and sodium-potassium pumps.

We also observed the amplification of the glycerol cluster of transporters in *Y. lipolytica* and this is most likely connected to the capacity of this yeast to use lipids as triglycerides as carbon source. Based on the sequence similarity with Sc_Stl1, six proteins from *Y. lipolytica* grouped together with known glycerol transporters and they are responsible for the uptake of glycerol at low concentrations (Erian et al., 2022).

Disaccharides uptake: Lactose, cellobiose, maltose – cluster permease and hydrolase

In the complete phylogenetic tree in Figure 1.3, we can observe a disaccharide cluster, which comprises three sub-clades, one of which is defined as cellobiose (in magenta), one that includes various proteins annotated as lactose transporters – Lac12-like (in blue) and another one with proteins with unknown functions (left in black). The cellobiose cluster appears to be a sister clade of the xylose and glycerol transporters clade. Most of the yeasts rich in xylose and cellobiose transporters (*P. stipitis* and *D. hansenii*) are also characterized by various cellobiose transporters. The use of cellobiose relies on a transporter (annotated as Cel1 in some yeasts) that imports the disaccharide and a β -glucosidase (Cel2), which cleaves the cellobiose in two molecules of glucose inside the cell. The transporter and the enzyme are found in cluster on the same chromosome often in diverging position sharing the promoter. This cluster is found in multiple copies on various chromosomes in *K. lactis*, *P. stipitis* and *D. hansenii* (Figure 1.7). *K. marxianus* is also provided with a β -glucosidase, but the uptake of cellobiose occurs via a transporter annotated as Lac12, as it can transport lactose as well (Varela et al., 2019). Lactose utilization also requires the activity of the cluster system which consists of *LAC12*, encoding a lactose permease for the uptake of this sugar, and *LAC4*, encoding a β -galactosidase for the hydrolysis of lactose into

glucose and galactose. The lactose assimilation capability is one of the main traits that distinguishes *K. marxianus* and *K. lactis* from the model yeast *S. cerevisiae*, which lacks this trait (Lane et al., 2010; Gethins et al., 2016; Morrissey et al., 2015), and both these yeasts show this cluster (Lac12-Lac4). Lactose utilization in *K. marxianus* is strain related and depends on the polymorphisms of the gene encoding for the transporter Lac12: strains carrying the functional allele (LAC12-B), in which the transporter can efficiently transport lactose - named dairy strains - show strong growth on lactose, while those carrying the non-functional allele (LAC12-A) display a poor lactose consumption. A complex evolutionary history, characterized by human domestication, sees a dairy lineage of *K. marxianus* donating the LAC genes to *K. lactis* (Ortiz-Merino et al., 2018; Varela et al., 2019). Both *D. hansenii* and *P. stipitis* also show some transporters which cluster together with the known Lac12 of *K. marxianus* and they have, next to the gene encoding for the transporter, a gene encoding for a β -galactosidase, therefore the cluster Lac12-Lac4 is also present in these yeasts. Unlike *Kluyveromyces* species though, these yeasts are not well-known for lactose utilization and very little information related to this is found in literature (Breuer et al., 2006). It is known that the ability of *P. stipitis* of using lactose is strain specific and variable (Kurtzman et al., 2011). This could be due to a similar phenomenon as in *K. marxianus*, in which a functional allele carrying a high affinity lactose transporter is needed for the efficient utilization of lactose. Lactose uptake and assimilation is a novel evolved function and the original function of all these proteins might have been originally different.

The presented clusters represent a set of genes coding for proteins with physiologically related functions that confer a survival advantage when coinherited. In many cases, the clusters appear to have formed and then the pairs of genes diverged together. Knowing the ecological niche of these yeasts which is mostly woody-plant environment and that these yeasts all can transport and utilize cellobiose, our theory is that the original ancestral cluster comprised a transporter and a hydrolase for a plant disaccharide (a dimer with β 1,4 bond-type, for example a breakdown product of pectin). Then, because of the structural similarity with lactose and the selective pressure imposed by human domestication in dairy environments, it is possible that the transporter and the hydrolase evolved to become more specific for lactose in the case of *K. marxianus*. While for other yeasts, like *P. stipitis* and *D. hansenii*, it is possible that these clusters are unspecific for lactose and they have a broader specificity.

Similar gene clusters patterns are found for the utilization of maltose in *S. cerevisiae*, *T. delbrueckii*, *P. stipitis*, *D. hansenii* and *K. lactis* (Alves-Araújo et al., 2004; Silva et al., 2022; Viigand et al., 2018). In this case, the cluster consists in a maltose permease in association with α -glucosidase, which cleaves

maltose in two molecules of glucose. Usually there are three copies of this pair (transporter and enzyme) in various chromosomes of these yeasts, but *P. stipitis* shows an expansion in these clusters with four normal loci (one permease and one glucosidase sharing the promoter) and one locus on chromosome 6 in which two maltose permeases and two highly divergent glucosidases (one of which resembles more a xylosidase) are found next to each other (Jeffries et al., 2009). On the other hand, the inability of *K. marxianus* to use maltose is consistent with the absence of a specific maltose transporter. This cluster might have been lost after the divergence between *K. marxianus* and *K. lactis* as this yeast shows two maltose permeases.

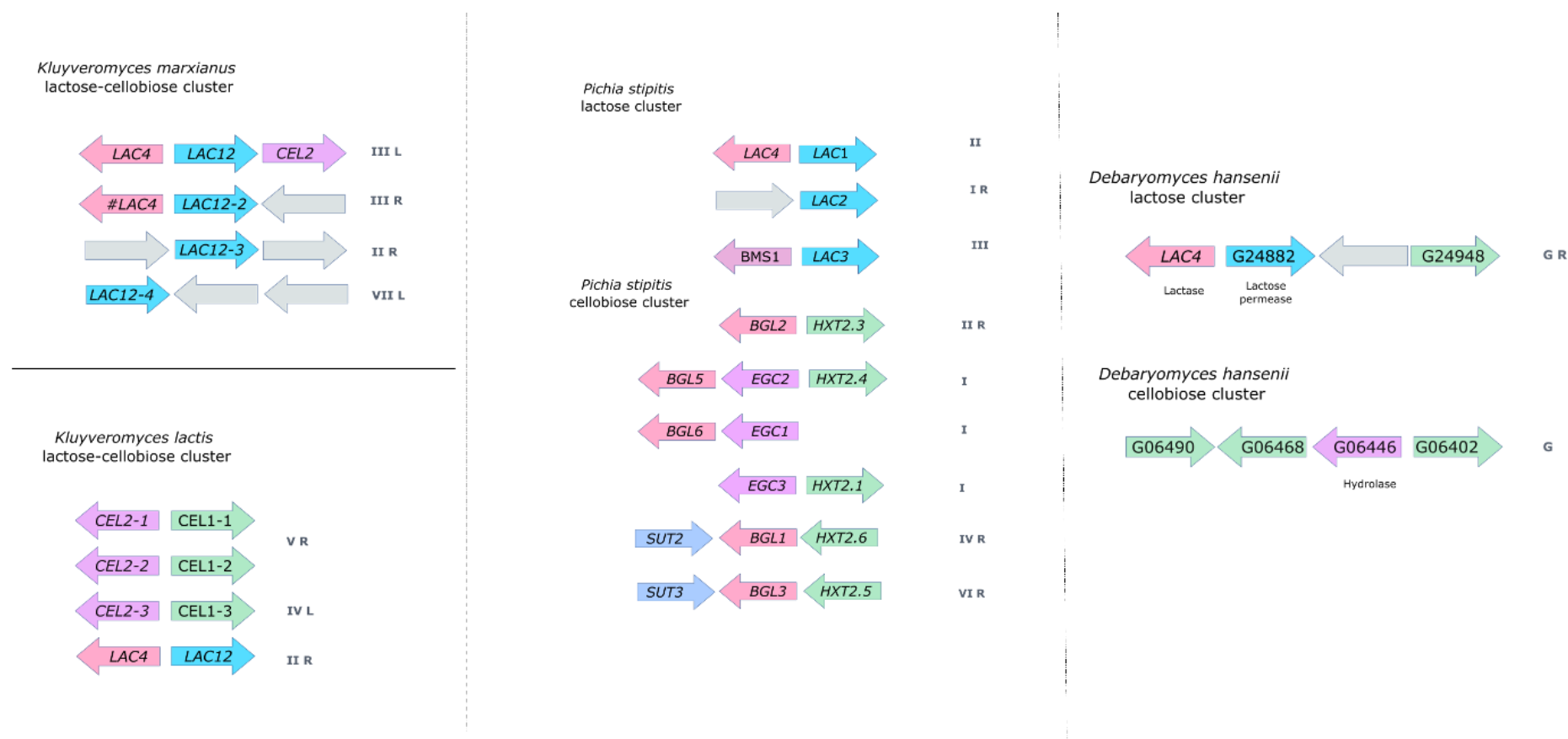


Figure 1.7 LAC and CEL gene clusters in *K. marxianus*, *K. lactis*, *P. stipitis* and *D. hansenii*. In pink and purple colours are reported the enzymes that cleave the sugars (LAC4 : β -galactosidase; CEL2 : cellobiase; BMS : β -galactosidase/mannosidase; BGL : β -galactosidase; EGC : endo- β -1,4-glucanase), in light blue, green and blue are respectively indicated the lactose transporters, cellobiose transporters and hexose transporters.

Sensors – Snf3- Rgt2 like proteins

Except for *S. pombe*, all species appear to maintain a constant number (between 1-3) of sensor genes during their evolution. The post-whole-genome-duplication (WGD) yeasts have two sensors (Snf3 and Rgt2). Some pre-WGD yeasts are also characterized by two sensors genes like *P. stipitis*. Lin et al. (2011) reported that the *S. cerevisiae* sensors Snf3 and Rgt2 resulted from the WGD after the divergence of *S. cerevisiae* lineage from *C. albicans* lineage, and they derive from a common ancestor originated from *C. albicans* Snf3. In *S. cerevisiae*, Snf3 and Rgt2 belong to the family of the Hxt proteins, but they lost the ability to transport glucose and became specialized in respectively sensing extracellular low or high glucose levels. In *C. glabrata*, Cg_Snf3 and Cg_Rgt2 shared 59 and 60% of identity with the two orthologs of *S. cerevisiae* and the glucose signalling pathway is conserved between the two yeasts. Cg_Snf3 can sense glucose in very low concentrations (0.01-1%) consistent with the habitat in which this yeast is found (e.i. niches with low glucose levels as human tissues) (Van Ende, Wijnants, & Van Dijck, 2019).

On the other hand, *K. lactis* possesses only one gene *RAG4* that encodes for the orthologue of Snf3/Rgt2 of *S. cerevisiae*. This sensor protein appears to have the dual function of signalling both high and low concentrations of glucose and oxygen sensing in this yeast (Betina et al., 2001; Micolonghi et al., 2011). No sensors are yet characterized in *K. marxianus*, but we predicted one transporter-like protein KMAR_60499 which clusters with Sc_Snf3 and Sc_Rgt2 and Kl_Rag4 (see Chapter 3).

In *Y. lipolytica*, Yht1 together with another transporter Yht2 resulted closely related to the branch of sensors known in other yeasts, but surprisingly these transporters are shown to be true transporter with actual activity (Lazar et al., 2017) and common sugar sensors (with no transport activity) are not yet identified in this yeast. This could possibly be an indication that sensors evolved from a transporter that lost its function.

Relation between number of Hxt and preference in metabolism

As shown in Table 1.1, the expansion in number of these transporters is reported to be related to the preference of the yeast to perform aerobic fermentation. For instance, *Y. lipolytica*, which is exclusively respiratory (Crabtree negative) has one single *HXT* copy; *Kluyveromyces* species which prefer aerobic respiration (variable Crabtree effect) show 2-6 copies while *Saccharomyces* species which prefer aerobic fermentation (Crabtree positive) have up to 17 copies (Lin et al., 2011).

The whole genome duplication (WGD) event, which took place approx. 100 million years ago, could have strengthened the Crabtree effect in the *Saccharomycetaceae* lineage. Despite the fact that there could be differences in other yeasts depending on the coupling of the oxidation of mitochondrial NADH to the reduction of ubiquinone (Juergens et al., 2020), in general, fermentation produces far less energy in the form of ATP than respiration metabolism e.g. in *S. cerevisiae* respectively only 2 ATP instead of 18 ATP (Pfeiffer et al., 2014). For this reason, to support an efficient metabolism, more transporters are needed to import glucose faster. Consequently, more ethanol is produced, accumulated and also catabolized by some yeasts in a “make-accumulate-consume (ethanol)” strategy (Rozpedowska et al., 2011). Not all the Crabtree positive yeasts follow this strategy, for example *S. pombe* also prefers aerobic fermentation, but it diverged from the hemiascomycetes lineage around 330-420 million years ago (Wood et al., 2002). Hence, it is hypothesized that the Crabtree effect originated independently in this yeast from *Saccharomyces* lineage, so the fermentative lifestyle results different and the yeast can also poorly assimilate ethanol, not following the “make-accumulate-consume” strategy (Hagman, Säll, Compagno, & Piskur, 2013; Lin & Li, 2011). Moreover, in this yeast we have a consistency between the expansion in number of Hxts (8), absence of Hgts and the preference for aerobic fermentation, confirming the relation between these phenomena. On the other hand, *P. stipitis*, Crabtree negative yeast, has a greater respiration capacity than *S. cerevisiae* and it is well known for fermenting pentoses to ethanol, only when oxygen becomes limiting (Papini et al., 2012). Just 4 transporters belonging to the Hxts cluster have been identified in this yeast (Sut 1-4) and they are responsible for the uptake of xylose as well as glucose. However, this yeast shows many other transporters that do not cluster neither with Hgts nor with Hxts, which are involved in the uptake of sugars present in its isolation environment, like pentoses or cellobiose.

We observe a large expansion of hexose transporters (Hxts) in *S. cerevisiae* (17) and, to a lower extent, in *C. glabrata* (9). Nevertheless, both these species lack Hgt-like porters, in contrast to other yeasts investigated in this work (*K. lactis*, *K. marxianus*, *T. delbruekii*, *D. hansenii*, *P. stipitis*, *Y. lipolytica*) and a fructose transporter Fsy1 (Figure 1.3 and Table 1.1). *HGT* genes are found in several fungi, so Hgts and Hxts transporters must have separated before the divergence of fungi. *HGT* genes are also not found in yeasts that strongly prefer aerobic fermentation, like *S. cerevisiae*. They resulted lost during evolution of aerobic fermentation. To overcome this loss, Hxts porters have experienced a rapid expansion in these yeasts.

Potential applications of yeasts provided with various sugar transporters

In this work we aimed to show how the study of transporters is useful for novel biotechnological applications, from biorefinery to food industry. The growing concern over consumption of fossil fuels for chemical synthesis has increased the interest in developing microbial bioprocesses for the production of these compounds in a sustainable fashion (Li et al., 2020; Z. Liu, Moradi, Shi, & Darvishi, 2021). Choosing the right yeast to develop efficient microbial cell factories that can produce a wide variety of valuable compounds at the highest yield and from various feedstocks is crucial (Guirimand et al., 2020). The transport of the sugars deriving from various feedstocks and raw materials is the first step of the bioprocess and conversion into valuable products and most of the time represents one of the major bottlenecks. As underlined, the yeast species here studied show a plethora of importers with diverse affinity for a broad range of sugars. It is important to underline the plasticity of sugar transporters, which evolved over the centuries to adapt to specific environments, gaining new functions, being able to import a broad range of substrates or, on the other hand, becoming more specific for some kind of sugars. Some of these transporters are presented in various copies and the major driving force for difference in numbers among various yeasts is shown to be the abundance of the sugar in the habitat in which the microorganism has evolved (Table 1.1).

The model yeast *S. cerevisiae* has been fully studied in its metabolism and sugar transport system. Nevertheless, it has been industrially used for centuries in the fermented beverage production and more recently for bioethanol from dedicated crops, there has been an increase interest in exploiting different yeasts, called non-conventional, that show peculiar metabolic traits (i.e. natural ability to transport and assimilate pentoses and/or lactose) that could be interesting for industrial bioprocesses (Navarrete et al., 2020).

Based on the kind of transporters here identified and their metabolism, we could say yeasts like *P. stipitis*, *D. hansenii*, *K. marxianus* and *A. gossypii* are good candidates for the utilization of pentoses even under stress conditions since they are provided with various transporters that can uptake xylose and arabinose. This is important for the bio-conversion of 2nd generation sugars deriving from lignocellulosic biomass (LCB), of which up to 40% is represented by xylose. *P. stipitis* and *K. marxianus* have also been used for simultaneous saccharification and fermentation – SSF and conversion of LCB due to their ability to produce lignocellulolytic enzymes to release sugars monomers (*P. stipitis*) and thermotolerance (*K. marxianus*) (Fonseca et al., 2008; Jeffries et al., 2009). The filamentous hemiascomycete *A. gossypii* is known for the industrial production of riboflavin, but recently its potential as microbial cell factory for the

conversion of xylose into biolipids was investigated (Aguar et al., 2015). Moreover, it is provided with 3 Hxt transporters that enable the uptake of various sugars that have been mutated to allow the co-consumption of glucose and xylose deriving from lignocellulosic biomass (Díaz-Fernández et al., 2020). Apart from being provided with various hexose and pentose transporters and being able to assimilate them, the most interesting trait of *D. hansenii* is its resistant to osmotic and halo-stress conditions, making this yeast suitable for using wood hydrolysates also rich in salts (for example coming from seaweed) for bioremediation, food applications or production of xylitol or other sugar alcohol (Breuer et al., 2006; Cho et al., 2013; Nobre et al., 1999). It is also remarkable the superior transport capacity of glycerol (9 members) which make this yeast together with *Y. lipolytica* (6 members) a strong cell factory for the conversion of raw glycerol, which is a by-product of the biodiesel manufacturing process (Erian et al., 2022). Both *D. hansenii* and *Y. lipolytica* are oleaginous yeasts, meaning that they are able to produce and accumulate high amounts of lipids, that can then be used as building blocks for various bioproducts, for example bioplastics or biodiesel. *Y. lipolytica* is also able to use lipids as carbon source, so it is frequently used for the revalorization of waste cooking oils (Domínguez et al., 2010; Liu et al., 2015). For this reason, it is not surprising that *Yarrowia* is not provided with a lot of hexose transporters as it prefers other carbon sources like glycerol or triglycerides over glucose. Among *Kluyveromyces* species, *K. marxianus* and *K. lactis* are provided with the machinery needed to assimilate lactose (a lactose transporter and a β -galactosidase), which is the main component of cheese whey, a by-product of the dairy industry. There has been an increase interest in revalorizing this substrate in the past decade and *Kluyveromyces* ssp. has been often used in this regard, in particular for ethanol or β -galactosidase production from whey permeate (Alves et al., 2019; Diniz et al., 2014; Sampaio et al., 2019). As we said in previous paragraphs, in *K. marxianus*, lactose and cellobiose share a transporter to enter in the cell. Knowing the main environment from which this yeast can be isolated (soil) it is possible to speculate that the lactose transporters arisen from the evolution of plant disaccharide (cellobiose) transporters, giving the yeast the ability to grow in various substrates.

Here, we also show that in the evolution and divergence of the yeast species, duplication events occurred and there is a strong correlation between the expansion of families of transporters (e.g. the hexose transporters Hxts) and the change in metabolism (switch from aerobic respiration to aerobic fermentation). This allowed some yeast to acquire new functions and us to take advantage of these new features. The yeast *S. cerevisiae* has always been used for wine, beer and bread making, but there is an interest in exploiting new yeasts that could have promising traits and improve the final product. Yeast like *S. pombe* and *T. delbrueckii* for example are rich in hexose transporters, which import sugars like glucose

or fructose into the cell and they are Crabtree positive, allowing fermentation and production of ethanol even in aerobic conditions. Other characteristics like the malic acid deacidification ability to reduce the harsh acidity of some wines (Benito et al., 2018) and flavor- and aroma- enhancing properties (Fernandes et al., 2021; Manzanares et al., 1998). This makes these yeasts interesting for modern winemaking industry.

With this study, we aimed to give an overview of the various yeasts that can be used as microbial cell factories in industrial bioprocess due to their evolved beneficial natural phenotypes (i.e. ability to transport and assimilate a broad spectrum of sugars) that translate to several advantages compared to the traditional *S. cerevisiae*.

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CHAPTER 2

Identification of novel pentose transporters in
Kluyveromyces marxianus using a new screening platform

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Abstract

The capacity of yeasts to assimilate xylose or arabinose is strongly dependent on plasma membrane transport proteins. Because pentoses comprise a substantial proportion of available sugars in lignocellulosic hydrolysates, their utilisation is centrally important for the development of second generation biorefineries. Relatively few native pentose transporters have been studied and there is intense interest in expanding the repertoire. To aid the identification of novel transporters, we developed a screening platform in the native pentose-utilising yeast *Kluyveromyces marxianus*. This involved the targeted deletion of twelve transporters of the major facilitator superfamily (MFS) and application of a synthetic biology pipeline for rapid testing of candidate pentose transporters. Using this *K. marxianus* Δ PT platform, we identified several *K. marxianus* putative xylose or arabinose transporter proteins that recovered a null strain's ability to grow on these pentoses. Four proteins of the Hgt-family were able to support growth in media with high or low concentrations of either xylose or arabinose, while six Hxt-like proteins displayed growth only at high xylose concentrations, indicating solely low affinity transport activity. The study offers new insights into the evolution of sugar transporters in yeast and expands the set of native pentose transporters for future functional and biotechnological studies.

2.1 Introduction

Kluyveromyces marxianus is now considered a credible alternative to *Saccharomyces cerevisiae* for specific biotechnological applications (Karim et al., 2020; Morrissey et al., 2015; Nurcholis et al., 2020; Rajkumar et al. 2021). Intrinsic traits like rapid growth, thermotolerance, recognised safe status, and capacity to grow on diverse sugars are important contributory factors. Its natural ability to assimilate pentoses is of particular interest because xylose and arabinose comprise up to 40% of available sugars in lignocellulosic biomass (LCB) (Takkellapati et al. 2018). The future development of the circular bioeconomy requires the use of residual or dedicated biomass such as LCB, and viable biorefineries require the use of microbial cell factories that can efficiently use all available sugars in a feedstock. Although transport systems for glucose, lactose and galactose are well-described, there is limited knowledge on pentose transporters in *K. marxianus*.

It is known that monosaccharide uptake in yeasts mainly occurs through the Hxt class of transporters, which are a subset of the major facilitator superfamily (MFS) (Leandro et al., 2009). Certain Hxt proteins enable efficient import of hexoses, but their lower affinity for xylose and arabinose hinders pentose consumption in the presence of glucose and contributes to the diauxic growth observed on mixtures of these sugars (Wang et al., 2016; Young, et al., 2014). The expansion of the *HXT* genes is well-studied in *S. cerevisiae* (Özcan & Johnston, 1999), and previously we showed that these genes are also duplicated in *K. marxianus*, where they encode a mixture of high and low affinity glucose transporters (Varela et al. 2019a). Unlike *S. cerevisiae*, *Kluyveromyces* species also encode proteins in the Hgt family of sugars transporters. As is the case with the *HXT* genes, the *HGT* genes are expanded in *K. marxianus*. The product of one of these genes, Hgt1, has been extensively characterised in *Kluyveromyces lactis* as a high affinity glucose transporter, as well as a galactose transporter (Baruffini et al., 2006; Billard et al., 1996). Our prior work confirmed orthologous function for the first gene in the *K. marxianus* *HGT* cluster (Varela et al., 2019a), and other researchers demonstrated that the protein encoded by this gene was able to transport arabinose and xylose and thus named the gene *KmAXT1* (Knoshaug et al., 2015). Only Hgt1 / KmAxt1 has a known activity and previous work showed that other Hgts are either capable of low affinity galactose transport or have no known substrates (Varela et al., 2019a).

Because of the importance of pentose transport for biotechnology applications, we were interested in establishing whether any other of the Hgt proteins, or indeed other putative transporters, were able to transport arabinose and/or xylose in *K. marxianus*. Platform strains unable to transport sugars are valuable tools to identify novel transporters and to study transport kinetics (Alves-Araújo et al., 2005;

Wieczorke et al., 1999; Wijsman et al., 2019). Although it is possible to engineer *S. cerevisiae* to use pentoses quite efficiently, we reasoned that a platform in a yeast strain that could natively use pentose sugars would be a powerful resource that could also facilitate studies linking transport with metabolism and physiology. Accordingly, we constructed a *K. marxianus* strain deleted in 12 genes that was completely unable to grow on either xylose or arabinose. We designated this strain *K. marxianus* Δ PT and, following a screen of candidate transporters, we identified several novel xylose transporters, two of which are also involved in arabinose uptake. This work contributes to a better knowledge of sugar membrane transporters in *K. marxianus* and offers resources and tools to researchers interested in identifying, characterising and exploiting pentose transport proteins.

2.2 Materials and Methods

Strains and Growth Conditions

K. marxianus strains used and constructed in this study are listed in Table 2.1. Strains were routinely cultured at 30 °C in YP medium (10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone) or minimal medium (MM) (5 g L⁻¹ Urea, 3 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ MgSO₄·7H₂O, 100 µg mL⁻¹ vitamin solution, 100 µg mL⁻¹ trace elements solution), supplemented with 20 g L⁻¹ of the appropriate sugar (D, Glucose; LAC, lactose). Composition of the vitamin and trace element solutions is reported in the Supplementary File 1. Where required for selection, 200 µg mL⁻¹ of Hygromycin B (Sigma-Aldrich, St. Louis, MI, United States) was added to agar plates.

Table 2.1: Strains used in this study.

Strain	Genotype	Parental strain	Mutation coordinates	Origins
<i>K. marxianus</i> NBRC1777	Wild-type strain			NITE Biological Research Centre, Japan
<i>K. marxianus</i> Δhgt	Δkmar_10531, Δkmar_10530, Δkmar_10529, Δkmar_10528, Δkmar_10527	<i>K. marxianus</i> NBRC1777	HGT: CHR I Δ1,131,464-1,145,439	Varela et al., 2019a
<i>K. marxianus</i> Δkht	Δkmar_50347, Δkmar_50346, Δkmar_50345, Δkmar_50344, Δkmar_50343, Δkmar_50342	<i>K. marxianus</i> NBRC1777	KHT: CHR V Δ 1251-4853, Δ 4926-16261, Δ 16417-20938	This study
<i>K. marxianus</i> ΔhgtΔkht	Δhgt Δkht	<i>K. marxianus</i> Δkht	HGT: CHR I Δ1,131,464-1,145,439 KHT: CHR V Δ 1251-4853, Δ 4926-16261, Δ 16417-20938	This study
<i>K. marxianus</i> ΔPT	Δhgt Δkht Δkmar_60179	<i>K. marxianus</i> Δhgt Δkht	HGT: CHR I Δ1,131,464-1,145,439 KHT: CHR V Δ 1251-4853, Δ 4926-16261, Δ 16417-20938 60179 : CHR VI Δ 134-1710	This study
<i>S. cerevisiae</i> CEN.PK 113-7D	Wild-type strain			Nijkamp et al., 2012

Serial dilutions on 2% agar plates were used to determine the capacity of wild-type and engineered strains to use specific sugars. For these experiments, a single colony was inoculated into 5 mL YPLAC in a 15 mL Sterilin tube and this was incubated overnight at 30°C. Cells were then harvested by centrifugation,

washed with water, and a serial dilution was prepared in water. 5 μL of each dilution were spotted onto minimal media (MM) plates containing D-Glucose, α -Lactose, D-Xylose, L-Arabinose or glycerol to a final concentration of 20 or 1 g L^{-1} . Plates were incubated for up to 120 h at 30 $^{\circ}\text{C}$ and the growth was assessed. To validate the results of the plate assays by testing growth in liquid, a single colony of each engineered strain was inoculated into 10 mL MM media (2% xylose or 2% arabinose) in a 100 mL flask and incubated for 96h at 30 $^{\circ}\text{C}$. Cells were harvested by centrifugation, the supernatant was used for sugar consumption analysis with high-performance liquid chromatography (HPLC) and the pellet was used to determine dry cell weight DCW, expressed as g L^{-1} . Growth curves on xylose were performed in 500 mL shake flasks as follows. Overnight inoculum in YPLAC was prepared as for the serial dilution experiments and 100 ml of 20 g L^{-1} xylose MM medium was inoculated to a starting $A_{600\text{nm}}$ of 0.1. The flasks were incubated at 30 $^{\circ}\text{C}$ on an orbital shaker at 200 rpm and $A_{600\text{nm}}$ was measured every 6-12 hours for 160 h. Consumption of xylose was determined by measuring the sugar remaining in the medium after various periods of growth. 1.5 mL of culture were harvested and spun down to prepare supernatant for HPLC. For the analysis, 800 μL of supernatant were injected into a Hewlett-Packard (HP) 1090 instrument using a Bio-Rad HPX-87H hydrogen ion resin column and an HP 1047A external refractive index detector. The mobile phase, 0.001 N H_2SO_4 , was run at 55 $^{\circ}\text{C}$ at a flow rate of 0.6 ml min^{-1} . Standards were used quantify sugars. *E. coli*/DH5 α was used for cloning purposes. The strain was grown in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} bactopectone, 10 g L^{-1} NaCl) or LB agar plates supplemented with 100 $\mu\text{g mL}^{-1}$ ampicillin.

***In silico* identification of Potential Pentose Sugar Transporters**

Potential sugar porters were identified by the combination of different bioinformatic approaches. The predicted proteome of *K. marxianus* NBRC 1777 was retrieved from the NCBI Assembly platform (<https://www.ncbi.nlm.nih.gov/>) and then compared against the TransportDB 2.0 database to identify possible sugar transporters (Elbourne et al., 2017). After filtering out hits with low identity (<35%) and coverage (<80%), the resulting sequences were submitted to the TMHMM server for the prediction of transmembrane domains. All proteins predicted to contain 11 or more transmembrane domains were considered as potential sugar transporters. Sequences belonging to the MFS superfamily were extracted from this list and the 27 predicted proteins remaining further analysed to try to predict function. Synteny and evolutionary conservation were inspected using the Yeast Gene Browser (YGOB, www.ygob.ucd.ie) (Byrne & Wolfe, 2005). Homologous sequences from *S. cerevisiae* and *K. lactis* were recovered and sequence alignment was performed using the MUSCLE algorithm 3.8 (Edgar et al., 2004) and visualized using Jalview v.2 (Waterhouse et al., 2009). Maximum likelihood phylogenetic analysis with 600 times bootstrap and tree building of the putative sugar transporters compared with known xylose transporters were performed using MEGA6/MUSCLE (Tamura et al., 2013). Preliminary 3D-studies were performed to compare the binding site of KMAR_60179 transporter from *K. marxianus* to the known glycerol transporter Sc_Stl1 from *S. cerevisiae* in order to evaluate the capability of transport of xylose. Protein structure prediction was performed using the ITASSER platform (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) (Yang & Zhang, 2015) and the structures were visualized and investigated with PyMOL v.2.3 (Delano, 2002). Three different naming conventions have been used for *K. marxianus* genes, depending on the strain annotated. Since our study uses strain NBRC 1777, we retained the designations from this annotation. To enable comparison to previous studies, however, we include a table showing the equivalence of the different transporters with which we worked (Table 2.2)

Table 2.2: Equivalence of KHT and HGT genes in different *K. marxianus* genomes. Genes marked with an asterisk have arisen by recombination between two genes. / indicates that the gene is absent.

Homology	NBRC1777	CBS 6556	DMKU3-1042
KHT-like	KMAR_50347	KMXK_OE03690	KLMA_50364
KHT-like	KMAR_50346	KMXK_OE03680	KLMA_50363

KHT-like	KMAR_50345	KMXK_OE03670	KLMA_50362
KHT-like	KMAR_50344		
		KMXK_OE03660*	KLMA_50361*
KHT-like	KMAR_50343		
KHT-like	KMAR_50342	KMXK_OE03650	KLMA_50360
HGT-like	KMAR_10527	KMXK_OA02960	KLMA_10545
HGT-like	KMAR_10528	KMXK_OA02950	KLMA_10546*
HGT-like	KMAR_10529	KMXK_OA02940	/
HGT-like	KMAR_10530	KMXK_OA02930	KLMA_10546*
HGT-like	KMAR_10531	KMXK_OA02920	KLMA_10547
LAC12-3p	KMAR_20788	KMXK_OB08450	KLMA_20830
LAC12-1p	KMAR_30003	KMXK_OH00120	KLMA_30010
LAC12-2p	KMAR_30701	KMXK_OC07340	KLMA_30728
LAC12-4p	KMAR_80005		KLMA_80005
YDR387C	KMAR_20551		KLMA_20580
YBR241C	KMAR_20383		KLMA_20402
Polyol transporter 2	KMAR_20602		KLMA_20638
STL1	KMAR_30579		KLMA_30607
HGT-like	KMAR_50027	KMXK_E00380	KLMA_50032
STL1	KMAR_60179	KMXK_OF03890	KLMA_60180
Glucose sensor	KMAR_60499		KLMA_60507
YFL040W	KMAR_70042		KLMA_70050
	KMAR_70126		KLMA_70145
FTR1	KMAR_80099		KLMA_60440
ITR1/2	KMAR_80266	KMXK_OG02840	KLMA_80273
Hxt14	KMAR_80370	KMXK_OG03930	KLMA_80389
	KMAR_80407		KLMA_70003

Construction of *K. marxianus* mutants

For general screens of gene function, we used the CRISPR-Cas9 system previously developed in our group (Juergens et al. 2018, Varela et al. 2019a) to generate inactivating mutations by non-homologous end-joining (NHEJ) in *K. marxianus* NBRC 1777. This uses pUCC001 (all plasmids listed in Table 2.3), which contains the Cas9 gene and the elements required to express the guide RNA (gRNA) molecule for targeted double stranded breaks. The design of the gRNA sequences specific to target genes was performed using the software sgRNAcas9 (Xie, Shen, Zhang, Huang, & Zhang, 2014) and CRISPRdirect (Naito, Hino, Bono, & Ui-Tei, 2015). For cloning into pUCC001, 20nt target and its reverse complement primers were ordered with suitable overhangs for cloning via Golden Gate Assembly (New England Biolabs, Inc., Ipswich, MA, United States). The presence of the gRNA target sequences in the pUCC001 plasmid was confirmed by colony PCR using the forward target primer and BSA-R. All primers used in this study were ordered from Sigma-Aldrich® and are listed in Table 2.4.

Table 2.3: CRISPR-Cas9 and complementation plasmids used in this study. Restriction enzyme sites are reported in superscript and sgRNA target are indicated in bold.

CRISPR-Cas9 Plasmids	Relevant characteristics	Source
pUDP002	ori amp ^R panARS(OPT) AgTEF1p-hph-AgTEF1t ScTDH3pBsaI BsaI ScCYC1t AaTEF1p- Spcas9D147Y P411T-ScPHO5t	Juergens et al., 2018
pUCC001	[pUDP002] HH-BsaI BsaI-gRNA scaffold-HDV	Rajkumar et al., 2019
pUCC001- HGT	[pUCC001] sgRNA-HGT	Varela et al. 2019a
pUCC001- KHT	[pUCC001] sgRNA-KHT	This study
pUCC001- 20551	[pUCC001] sgRNA-KMAR_20551	This study
pUCC001- 30579	[pUCC001] sgRNA-KMAR_30579	This study
pUCC001- 50027	[pUCC001] sgRNA-KMAR_50027	This study
pUCC001- 60179	[pUCC001] sgRNA-KMAR_60179	This study
Complementation plasmids	Relevant characteristics	Source
pYTK001	Storage vector for cloning gene parts	Lee et al., 2015
pYTK_CYC1	[pYTK001] CYC1 TT	
pYTK013	[pYTK001] pTEF1	Rajkumar et al., 2019
p426	ori amp ^R URA3	Varela et al. 2019a

	pTEF1- HGT/KHT – CYC1 TT	
p426-E03650	[p426]-TEF1-E03650	Varela et al. 2019a
p426-E03670	[p426]-TEF1-E03670	Varela et al. 2019a
p426-50345	[p426]-TEF1-50345	Varela et al. 2019a
p426-50344	[p426]-TEF1-50344	Varela et al. 2019a
p426-50343	[p426]-TEF1-50343	Varela et al. 2019a
p426-A02920	[p426]-TEF1-A02920	Varela et al. 2019a
p426-A02930	[p426]-TEF1-A02930	Varela et al. 2019a
p426-A02950	[p426]-TEF1-A02950	Varela et al. 2019a
p426-A02960	[p426]-TEF1-A02960	Varela et al. 2019a
pTU-DO-G418	ampR KmARS/CEN7 KanMX Bsal sfGFP Bsal	Rajkumar and Morrissey, 2020
pTU-DO-G418- 50342	pTEF1-KMAR_50342-CYC1 TT	This study
pTU-DO-G418- 50343	pTEF1-KMAR_50343-CYC1 TT	This study
pTU-DO-G418- 50344	pTEF1-KMAR_50344-CYC1 TT	This study
pTU-DO-G418- 50345	pTEF1-KMAR_50345-CYC1 TT	This study
pTU-DO-G418- 50346	pTEF1-KMAR_50346-CYC1 TT	This study
pTU-DO-G418- 10527	pTEF1-KMAR_10527-CYC1 TT	This study
pTU-DO-G418- 10528	pTEF1-KMAR_10528-CYC1 TT	This study
pTU-DO-G418- 10529	pTEF1-KMAR_10529-CYC1 TT	This study
pTU-DO-G418- 10530	pTEF1-KMAR_10530-CYC1 TT	This study
pTU-DO-G418- 10531	pTEF1-KMAR_10531-CYC1 TT	This study
pTU-DO-G418- 50027	pTEF1-KMAR_50027-CYC1 TT	This study
pTU-DO-G418- 60179	pTEF1-KMAR_60179-CYC1 TT	This study
pTU-DO-G418- 30579	pTEF1-KMAR_30579-CYC1 TT	This study
pTU-DO-G418- Sc_STL1	pTEF1-Sc_STL1-CYC1 TT	This study
pTU-DO-G418- Sc_GAL2	pTEF1-Sc_GAL2-CYC1 TT	This study

Table 2.4: Primers used in this study for CRISPR gRNA target design, CRISPR-CAS9 genomic indel mutations diagnosis and *kmr_60179* repair fragment construction. The gRNA primers are designed to be 20nt in length and not include the PAM sequence at the 3' end (NGG). For cloning into the CRISPR-plasmid pUCC001, the forward gRNA target primers present overhangs 5'-cgtc-3' and the reverse target primers 5'-aaac-3' (indicated in lower case). For clean disruption of *kmr_60179*, the repair fragment was constructed using primers RF_60179 F and R of 95 bp which overlap for 15 bp (indicated with capital letters).

gRNA Targets CRISPR

<i>kmar_20551_Target_1F</i>	cgtcCGGTAGGAAGTATACTTTGA
<i>kmar_20551_Target_1R</i>	aaacTCAAAGTATACTTCCTACCG
<i>kmar_50027_Target_1F</i>	cgtcGTGATTGCAGGTATGGGTGT
<i>kmar_50027_Target_1R</i>	aaacACACCCATACCTGCAATCAC
<i>kmar_30579_Target_1F</i>	cgtcTCAAAGTGTCTCGTGGAGAA
<i>kmar_30579_Target_1R</i>	aaacTTCTCCACGAGACAGTTTGA
<i>kmar_60179_Target_1F</i>	cgtcACACTTCTACGATCCCAGTT
<i>kmar_60179_Target_1R</i>	aaacAACTGGGATCGTAGAAGTGT
<i>kmar_all_KHT_Target_1F</i>	cgtcTAGTTGGTACAGTAACCCAA
<i>kmar_all_KHT_Target_1R</i>	aaacTTGGGTTACTGTACCAACTA

Diagnostic primers

<i>kmar_50347_diag-F</i>	ATGTCTAATCAATTGACGGC
<i>kmar_50342_diag-R</i>	TTATTTTCATCATAGCCTTGACCATG
<i>kmar_20551_diag-F</i>	GTAATCCACTGTTTCACAGCT
<i>kmar_20551_diag-R</i>	CTCCATGCTGCCGGA
<i>kmar_50027_diag-F</i>	TACTTCAATCACCCGAATGCA
<i>kmar_50027_diag-R</i>	CTGCGCATCAATTATCACTTG
<i>kmar_30579_diag-F</i>	CCTTTTGACCAACAAAGAATCC
<i>kmar_30579_diag-R</i>	CCCCATAACACATTGAATAAACAATG
<i>kmar_60179_diag-F</i>	AGCAATGACAGACACGC
<i>kmar_60179_diag-R</i>	ACGGTTGACAGCGTC

KMAR_60179 Repair fragment

RF_60179_F	ggtcgggacggctcttgagaatcgccatcacgggtggcggttaccgggttttcgctattcgggtacgacc aaggtttgaTGTCGGTATTATTAAACCCACAGGCTGAA
RF_60179_R	tttcagcggtacccgagacgtcttcttcttcttggggaagttgggagtctacaggagcagcagcagc agcatcagcTTCAGCCTGTGGGTTTAATAATACCGGACA

Following cloning, the disruption plasmids were transformed into *K. marxianus* NBRC1777 using a variation of the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl, 2007). Briefly, an overnight yeast culture in YPLAC were used to inoculate a shake flask containing YPLAC medium at an initial A_{600nm} of 0.5. Cultures were then incubated at 30 °C until an A_{600nm} of 2.0 was reached, then harvested and transformed with 300 ng of plasmid DNA. After heat shock, cells were recovered in 1 mL YPLAC medium for 3 h and plated on selective plates containing hygromycin B 100 $\mu\text{g mL}^{-1}$. Colonies were recovered and restreaked on selective medium (hygromycin B) to confirm presence of the plasmid. Colony PCR was carried out using diagnostic primers (diag. F and R) for each gene, and PCR products were then sequenced to determine whether an inactivating mutation had been introduced during repair of the dsDNA break. To allow loss of the pUCC001 plasmid from mutants, strains were grown on YPLAC broth overnight without selection and diluted 1/10 into fresh YPLAC next morning. This was repeated twice, and then single colonies were tested to confirm that they were Hygromycin B sensitive and hence had lost the plasmid. This allowed sequential construction of mutants where required. All sequencing was carried out by GATC Services – LightRun Tube Eurofins Genomics.

A variation of the aforementioned CRISPR-Cas9 system based on homology dependent repair (HDR) was used for construction of stable mutants that did not have the potential to revert. Previously, we showed that it was possible to inactivate the entire *HGT* gene cluster using a single gRNA that targeted a common sequence present in all *HGT* genes generating strain Δhgt (Varela et al. 2019a). This deletion arises due to recombination between the cut first and last genes in the cluster and the same strategy was used to inactivate the entire *KHT/HXT* locus generating strains Δkht and $\Delta hgt\Delta kht$ (Figure 2.1).

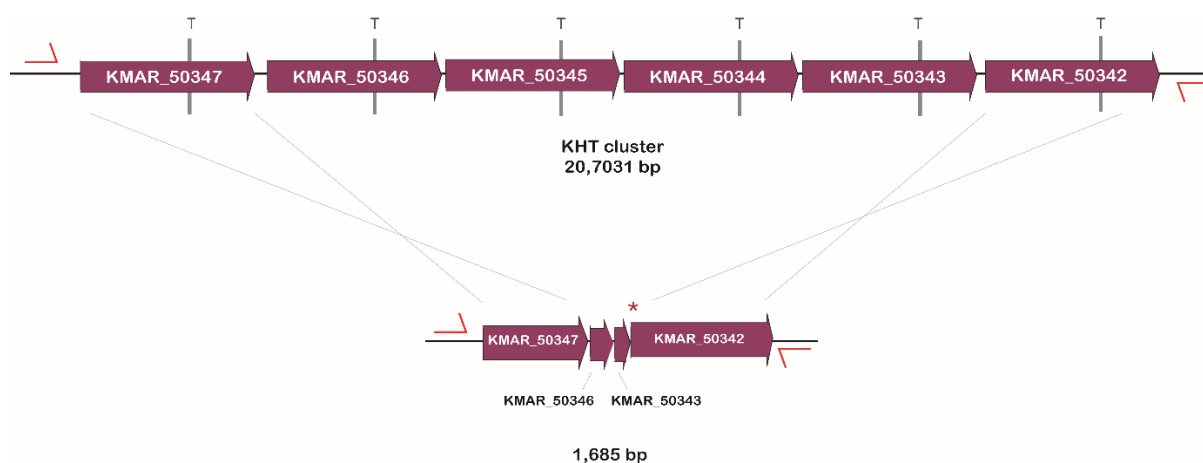


Figure 2.1: Construction of KHT deletion mutants on *K. marxianus* NBRC1777 wild type and Δhgt background. Scheme showing the repair event occurred upon targeting a common sequence (in grey) of the KHT genes with the CRISPR-Cas9 system. Recombination between the two ends of the KHT loci is shown by

grey shading. Recombination event between *KMAR_50347* and *KMAR_50342* led to the loss of functionality of the KHT cluster. The recombined sequence of 1685 bp contains: 490 bp of *KMAR_50347*, 80 bp of *KMAR_50346*, 72 bp of *KMAR_50343* and 1043 bp of *KMAR_50342*. The stop codon (marked with a red asterisk in position 721-723), obtained as consequence of the deletion, results in two truncated proteins of 240 AA and 321 AA, respectively, both lacking 7 fundamental transmembrane domains to be functional. Primers *KMAR_50347_diag_F* and *KMAR_50342_diag_R* used to screen the mutant colonies are shown as red arrows.

For disruption of *KMAR_60179*, a repair fragment was constructed by designing primers RF_60179 F and RF_60179 R, which shared a 30bp overlap and had 80b homology to the 5' and 3' regions flanking *KMAR_60179*, respectively. The 5' and 3' homology arms were annealed and extended by overlap extension PCR using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). Successful construction of the final repair fragment of 190 bp was confirmed by gel electrophoresis and 1 µg repair fragment was then co-transformed with 200 ng pUCC001-60179 into *K. marxianus* $\Delta hgt\Delta kht$ to generate *K. marxianus* $\Delta hgt\Delta kht\Delta kmr_60179$ (later named *K. marxianus* ΔPT). All deletion mutants were confirmed by diagnostic colony PCR with sequencing across the deletion (Figure 2.2). The precise co-ordinates of deletions are listed in Table 2.1, using the genomic co-ordinates from the NBRC1777 genome sequence (Inokuma, et al. 2015).

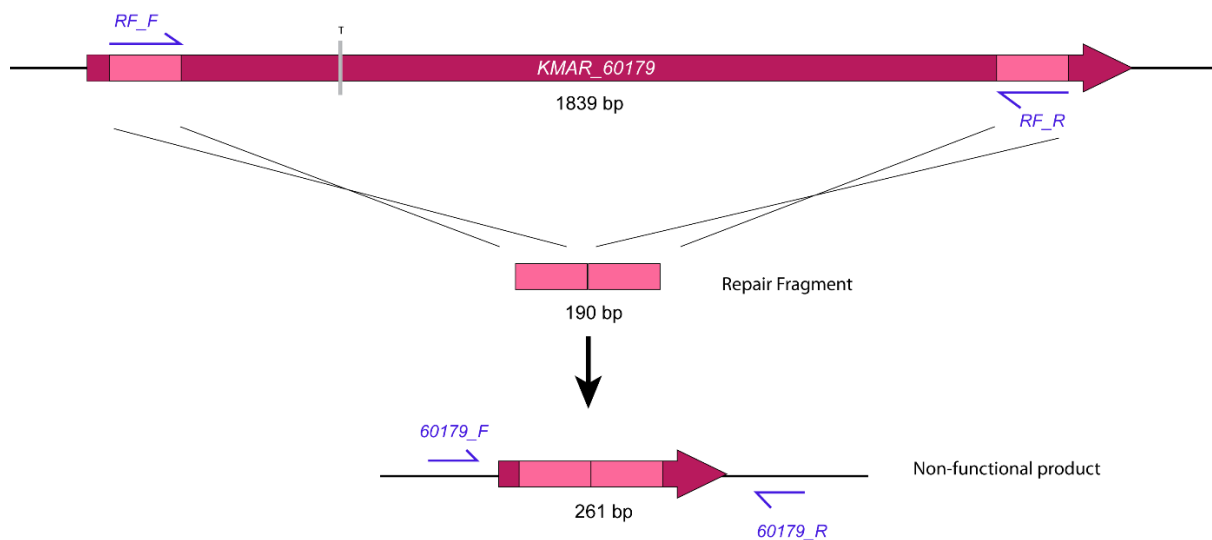


Figure 2.2: Schematic representation of *KMAR_60179* clean deletion. For disruption of *KMAR_60179*, a repair fragment was constructed using *K. marxianus* NBRC 1777 genomic DNA as the template, designing primers RF_60179 F and R for the homology arms at the extremities of *KMAR_60179* of 95 bp (in pink). The 5' and 3' homology arms were then joined by overlap extension PCR. The final repair fragment of 190bp was then co-transformed with the CRISPR-Cas9 plasmid pUCC001-60179 (carrying a specific target sequence recognized by the Cas9 complex, showed in grey). into *K. marxianus* $\Delta hgt\Delta kht$ to generate *K. marxianus*

$\Delta hgt\Delta kht\Delta kmar_60179$ (later named *K. marxianus* ΔPT). The deletion mutant was confirmed by diagnostic colony PCR and sequencing across the deletion with primers 60179_F and 60179_R.

Construction of plasmids expressing candidate pentose transporters and complementation assays

For expression studies, the *KHT* and *HGT* genes and *KMAR_60179* were cloned into the base plasmid pTU-DO-G418 using a Golden Gate assembly as shown in Figure 2.3. pTU-DO-G418 is similar to pMTU-DO-G418 but carries an ampicillin rather than a kanamycin resistance gene (Rajkumar et al. 2020). We use the YTK standard in *K. marxianus* as previously reported by Lee et al., 2015 and Rajkumar et al., 2019. The strategy entailed *in vitro* assembly of the *S. cerevisiae* *TEF1* promoter (p*TEF1*), the open reading frame of the transporter, the *S. cerevisiae* *CYC1* transcriptional terminator (*CYC1* TT) and the plasmid backbone. The p*TEF1* part was already available in the plasmid, YTK013, and a similar storage plasmid with *CYC1* TT was made by amplifying the part from *S. cerevisiae* CENPK 113-7D with primers ScCYC1 F-R and cloning into the storage plasmid YTK001 to create plasmid YTK_CYC1. For construction of the expression vectors for *KMAR_60179*, *KMAR_10529*, *KMAR_50027*, *KMAR_50346*, *KMAR_30579*, *ScSTL1* and *ScGAL2*, the open reading frames were amplified by PCR with primers containing overlapping regions for p*TEF1* and the *CYC1* TT and the vector assembled as previously described (Rajkumar et al. 2019). *KMAR_10527*, *KMAR_10528*, *KMAR_10530*, *KMAR_10531*, *KMAR_50342*, *KMAR_50343*, *KMAR_50344*, *KMAR_50345* had already been cloned between p*TEF1* and *CYC* TT in the p426 plasmid series (Varela et al., 2019a), and, in this case, the entire cassette promoter-gene-terminator was amplified from the p426 plasmid with primers ScTEF1-F and ScCYC1_R and cloned into pTU-DO-G418 by Golden Gate assembly, as described above. All the PCR amplifications were performed using Q5 High-Fidelity Master Mix (New England Biolabs). The resulting plasmids were introduced in competent *E. coli* DH5 α cells (Invitrogen 11319-019) by heat shock and the cells were plated on LB plates with ampicillin 100 $\mu\text{g mL}^{-1}$. After 24 h, white colonies were picked and screened by colony PCR with K1F and Bsmbl_TUDOG418_R primers and plasmids were extracted from the positive colonies and sequenced to check for correct assembling. *K. marxianus* strains were transformed as described earlier but with selection for G418 resistance. The plasmids used for the expression of candidate transporters are reported in Table 2.3.

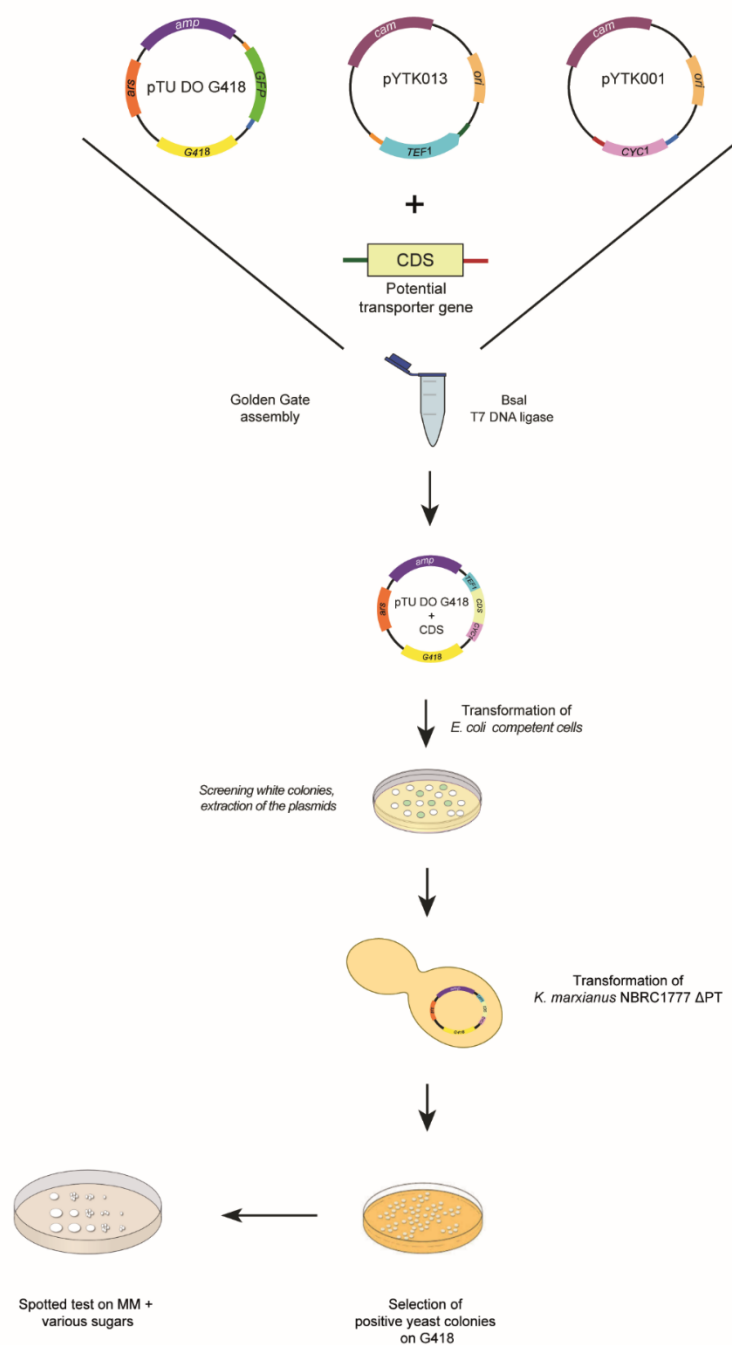


Figure 2.3: A scheme summarizing the complementation assay performed in order to overexpress interesting transporters in *K. marxianus* ΔPT. ScTEF1 promoter, ScCYC1 terminator from *S. cerevisiae* and the relevant genes from *K. marxianus* were amplified by PCR with primers containing specified 4bp overlaps. PCR products were then purified and assembled in pTUDOG418 using a Bsal Golden Gate assembly. The resulting plasmids were then introduced in competent *E. coli* cells by heat shocked and plated on LB+ Amp. After 24 h, white colonies, in which the GFP was substitute with the expression cassette, were screened by colony PCR, plasmids were extracted and the platform strain *K. marxianus* NBRC1777 ΔPT was transformed with selection for G418. Spotted test with serial dilutions on MM plates with lactose, xylose, arabinose and glycerol to test the capacity of wt and engineered strains to use specific sugars.

2.3 Results

Identification of putative sugar transporters in *Kluyveromyces marxianus*

A bioinformatic approach was taken to identify all potential sugar transporters in *K. marxianus*. To do this, the predicted proteome of *K. marxianus* NBRC1777 was compared to the TransportDB 2.0 database to identify candidate MFS transporters. These were then screened on the TMHMM server to select proteins with at least 11 transmembrane domains. This analysis yielded 27 predicted proteins of the Major Facilitator Superfamily (MFS). These 27 proteins were then compared to homologous proteins from *K. lactis* and *S. cerevisiae* to establish gene orthology (Figure 2.4). The majority of the proteins were distributed in clusters that include at least one characterised protein from *S. cerevisiae* and therefore function can be inferred. There are, however, a number of clusters containing proteins present in one or more *Kluyveromyces* species that are absent from *S. cerevisiae*. First, both *Kluyveromyces* species encode the lactose transporter Lac12, though this gene has been amplified to 4 copies in *K. marxianus* (*KMAR_20788*, *KMAR_80005*, *KMAR_30701*, *KMAR_30003*). Second, *K. lactis* encodes three copies of a dedicated cellobiose transporter (*KLLA0E14653*, *KLLA0D00374*, *KLLA0E25015*) and, as reported, this gene is absent in *K. marxianus* (Varela et al., 2019b). Third, both species encode Hgt1 for high affinity glucose transport, though, again as reported, this gene family is expanded in *K. marxianus* where 5 tandem copies are present on chromosome 1 and a further single homologue on chromosome 5. Fourth, both *Kluyveromyces* species encode the fructose transporter Frt1 that is absent in *S. cerevisiae*, as well as two uncharacterised proteins of related sequence (marked “A” in Figure 1). *FSY1* is known to have been acquired by horizontal gene transfer in wine strains and is not a native *S. cerevisiae* gene (Galeote et al., 2010). The maltose transporter cluster is present in both *S. cerevisiae* and *K. lactis* but is absent in *K. marxianus*, consistent with the species’ maltose negative phenotype. Interestingly, *Kluyveromyces* yeasts encode a deeper branching transporter of unknown function that is related to maltose transporters (marked D in Figure 1). In a similar fashion, there are deeper branching *Kluyveromyces*-specific proteins that are related to the inositol transporter Itr1/2 and the glycerol transporter Stl1 (marked B and C, respectively, in Figure 1). Substantial differences between the two *Kluyveromyces* species and *S. cerevisiae*, and between *K. lactis* and *K. marxianus* are evident when examining candidate transporters in the Hxt-like family of proteins. Orthologues of the *S. cerevisiae* high and low affinity Hxt glucose transporters are present in *Kluyveromyces* and the expansion of these in *K. marxianus* relative to *K. lactis* was previously described (Varela et al., 2019a). The *K. lactis* *HXT* homologues were originally named *KHT1* (*RAG1*) and *KHT2*, thus we retain the *KHT* nomenclature in

this study, though the ancestral relationship is clear. Thus, *K. marxianus* NBRC1777 has two proteins related to the low affinity glucose transporter Kht1 (Rag1) and four proteins related to the high affinity glucose transporter Kht2. These are in tandem repeats present in syntenic location with the ancestral *S. cerevisiae* *HXT* locus (*HXT4* – *HXT1* – *HXT5* / *HXT7* – *HXT6* – *HXT3*). *Kluyveromyces* also have a Hxt14 orthologue but lack orthologues of other Hxt-like proteins such as the galactose transporter, Gal2, or the polyol transporters Hxt15/16 and Hxt13/17 (Jordan et al., 2016).

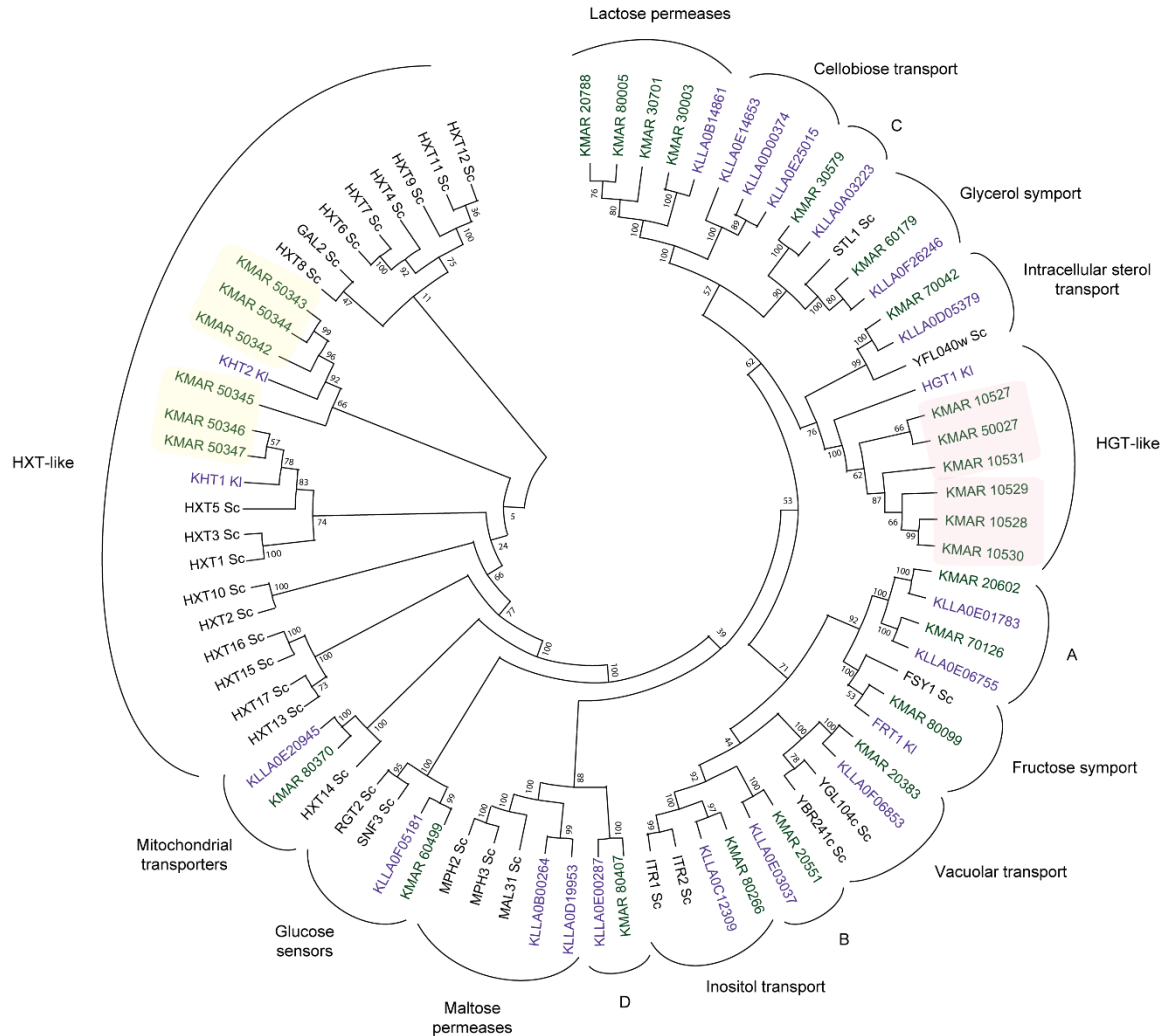


Figure 2.4: Phylogenetic comparison of potential MFS transporters between *K. marxianus*, *K. lactis* and *S. cerevisiae*. Bioinformatic analysis identified 27 predicted proteins in *K. marxianus* NBRC 1777 that satisfied the criteria of being potential sugar transporters (MFS family with 11 or 12 transmembrane domains) (in green). Homologous sequences in *S. cerevisiae* and *K. lactis* (in black and purple, respectively) were retrieved and phylogenetic analysis carried out using MEGA6/MUSCLE. The tree is derived from maximum-likelihood analysis with 600 times bootstrapping. The expanded Kht/Hxt and Hgt families in *K. marxianus* are highlighted with yellow and pink squares, respectively. Labelling is used to indicate the known or predicted function of proteins where data are available. The labels A – D mark *Kluyveromyces* proteins of unknown function that lack clear orthologues in *S. cerevisiae*. ScFsy1 is a fructose transporter that was acquired by horizontal gene transfer in wine strains and is not a native *S. cerevisiae* protein.

S. cerevisiae is unable to efficiently transport pentose sugars, and since we were especially interested in identifying transporters of xylose and/or arabinose, we also performed a phylogenetic analysis comparing the 27 *K. marxianus* MFS proteins to known pentose transporters of *Scheffersomyces stipitis*, *Debaryomyces hansenii*, *Meyerozyma guilliermondii* (syn. *Pichia guilliermondii*) and *Penicillium chrysogenum* (Figure 2.5). The *M. guilliermondii* and *P. chrysogenum* proteins cluster with the *Kluyveromyces* Hgt-like family whereas the *Scheffersomyces* and *Debaryomyces* proteins form a separate unrelated cluster. In this regard, it is notable that the only *K. marxianus* protein that was known at this time to transport pentoses, kmAxt1, is encoded by a *HGT*-like gene, *KMAR_10527* (Knoshaug et al., 2015) whereas the most efficient *S. cerevisiae* protein that can transport xylose is an engineered derivative of the Hxt family protein, Gal2 N376F (Farwick et al. 2014). Since both of these gene families are expanded in *K. marxianus*, we considered it possible that additional pentose transporters might be found in either of the gene clusters.

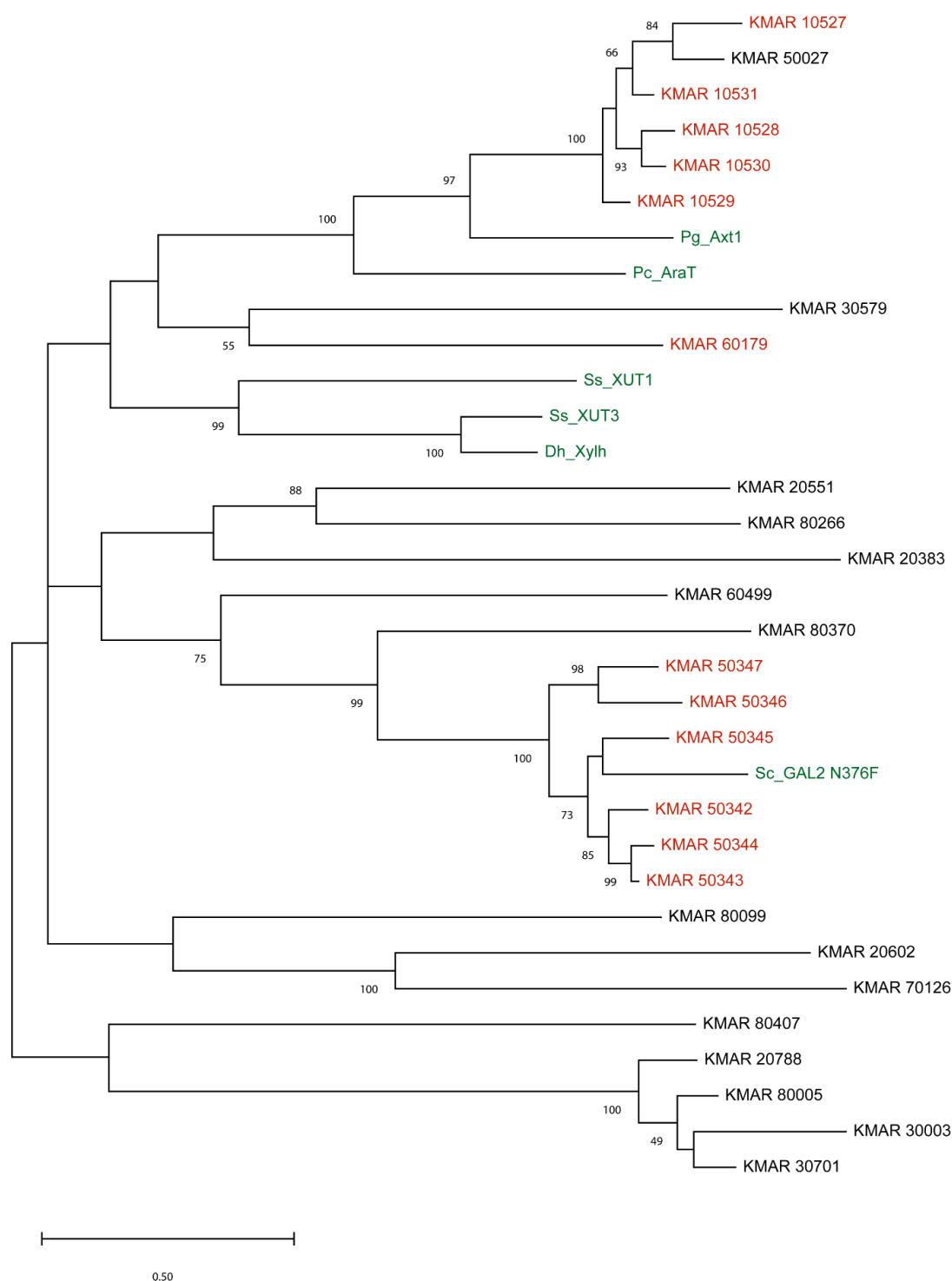


Figure 2.5: Maximum-likelihood phylogenetic analysis of sugar transporters in *K. marxianus* compared with known xylose-arabinose transporters Xut1 (XP_001385583) and Xut3 (XP_001387138) of *S. stipitis*, Xylh (XP_458169) of *D. hansenii*, Gal2 N376F (NP_013182) of *S. cerevisiae*, Axt1 (XP_002562738) of *P. guiliermondii* and AraT (XP_001482096) of *P. chrysogenum* (in green). The candidate transporters knocked out in *K. marxianus* NBRC1777 to create the pentose transporters null strain Δ PT are reported in red.

Construction of a platform *K. marxianus* strain unable to grown on pentose sugars

We decided to generate a *K. marxianus* pentose-negative strain to enable us to express individual transporters to establish if additional pentose transporters could be found. In a previous study, we used CRISPRCas9 to delete the five tandem *HGT* genes and here we employed the same method to delete the entire cluster of *KHT*-like genes in a single step. For this, we designed a CRISPR sgRNA to cut within a conserved region of the *KHT* genes. Following transformation with a plasmid expressing this sgRNA and Cas9, we were able to recover transformants in which all six genes were deleted due to a recombination between the first and the last gene, which generated a non-functional gene (Figure 2.1). The same method was used in both the wild-type and the Δhgt strain, thus we had three strains Δhgt , Δkht , and $\Delta hgt \Delta kht$. We tested the capacity of these mutants to grow on lactose (positive control condition), glucose, xylose and arabinose (Figure 2.7A). The Δhgt strain failed to grow on arabinose but showed no growth defects on glucose or xylose. The Δkht mutant did not show any phenotype but $\Delta hgt \Delta kht$ was mildly impaired for growth on glucose, failed to grow on arabinose, and grew more slowly on xylose. The slower growth of $\Delta hgt \Delta kht$ was more apparent at earlier timepoints (seen in Figure 2.5) but the timepoint of 96h (on xylose and arabinose) was chosen here to detect the influence of transporters that might have delayed or low expression. These data indicate that all functional arabinose transporters are encoded within the *HGT* cluster, that proteins capable of transporting xylose are found in both the *HGT* and *KHT* clusters, and that at least one other xylose transporter is encoded elsewhere in the genome. In an effort to identify this additional xylose transporter, we used CRISPR-mediated NHEJ to systematically inactivate other genes that had been identified in the bioinformatic analysis in the $\Delta hgt \Delta kht$ strain (Figure 2.5) as well as in the parental wild-type (Figure 2.6).

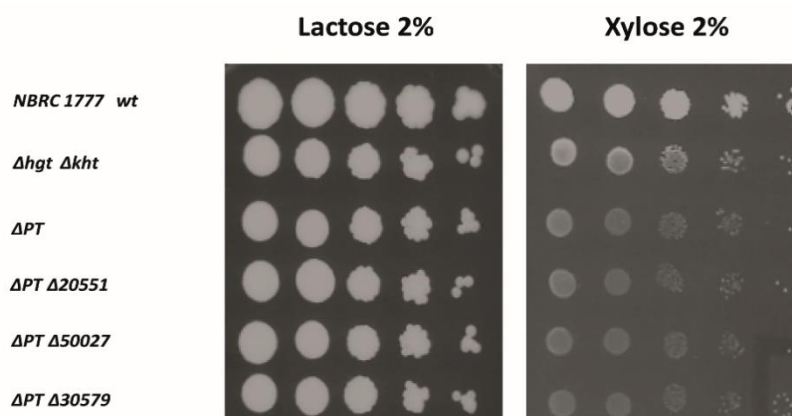


Figure 2.6: Growth phenotype of *K. marxianus* putative pentose transporters null strains. Yeast strains were grown on YPLAC to an OD_{600} of 2 and then washed, serial diluted and spotted onto MM plates supplemented with lactose or xylose 2%. No major differences in growth between ΔPT and the other mutants were observed on YPLAC plates.

Mutation of *KMAR_20551*, *KMAR_50027* or *KMAR_30579* did not show a phenotype but inactivation of *KMAR_60179* in the $\Delta hgt \Delta kht$ background yielded a strain unable to grow on xylose. For subsequent analysis, we wanted to be sure that the mutant could not revert, so we generated a precise deletion of *KMAR_60179* in the $\Delta hgt \Delta kht$ background using a CRISPR homology dependent repair (HDR) method and named this pentose transporter-deficient strain *K. marxianus* ΔPT as it failed to grow on pentose sugars (Figure 2.7A). As agar plate assays often show slight background growth, we also carried out a liquid growth assay on 2% xylose (Figure 2.7B) or 2% arabinose (Figure 2.7C), which showed a complete absence of growth for the ΔPT strain, confirming its suitability as a screening host. Interestingly, $\Delta hgt \Delta kht$ also failed to grow, which is consistent with an idea that the slower growth seen on plates for this strain (Figure 2A) is due to a transporter that may have either low activity or a low level of expression. Alternatively, it may be that, under the growth conditions of this experiment, the gene is expressed on solid but not liquid medium.

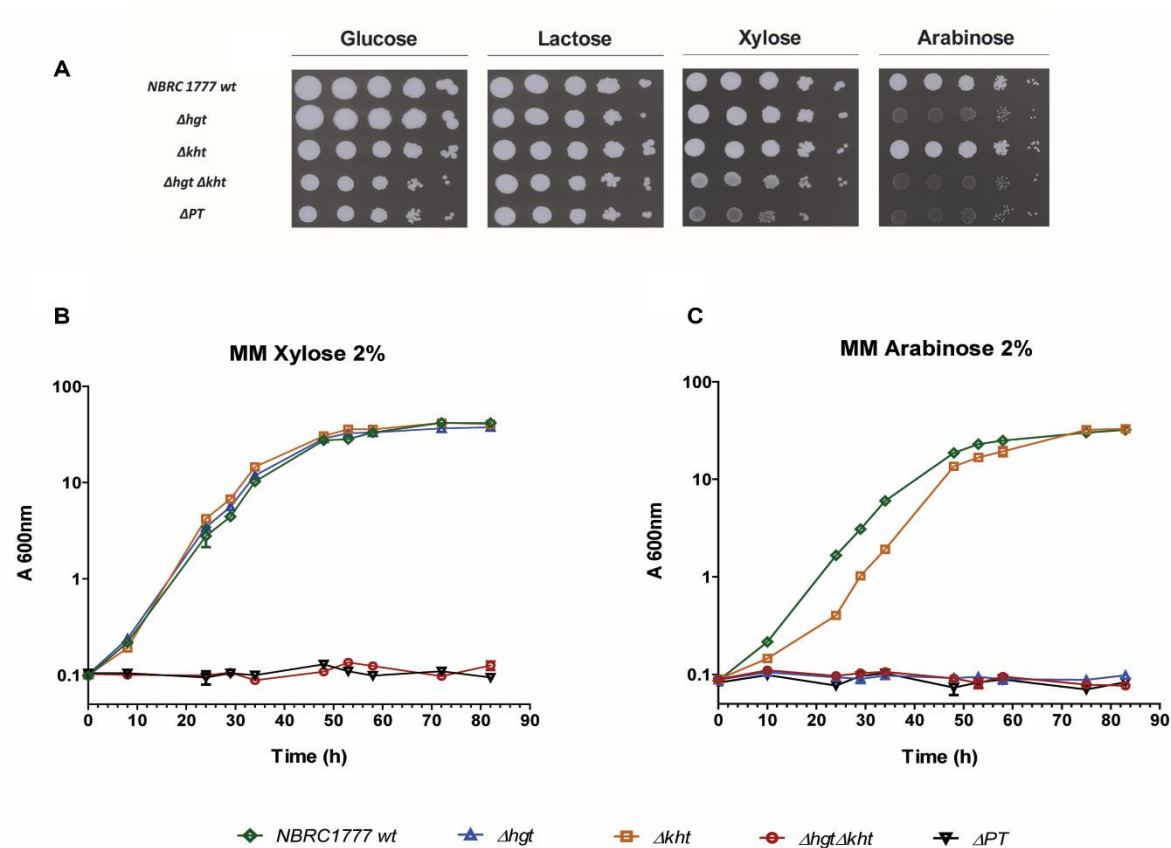


Figure 2.7: The *K. marxianus* platform strain ΔPT is unable to grow on the pentose sugars, xylose and arabinose. A. Growth of mutants on MM containing various sugars (2%). The Δhgt mutant failed to grow on arabinose, indicating that all the functional arabinose transporters are encoded within the *HGT* cluster. The double mutant $\Delta hgt \Delta kht$ grew slowly on xylose indicating both the presence of at least one xylose transporter in the KHT/HXT cluster and at least one other xylose transporter elsewhere in the genome. The ΔPT strain, which also deleted *KMAR_60179* in the $\Delta hgt \Delta kht$ background, failed to grow on either xylose or arabinose indicating the inactivation of all functional pentose transporters. **B-C** *K. marxianus* NBRC 1777 wild-type (green diamonds), Δhgt (blue triangles), Δkht (orange squares), $\Delta hgt \Delta kht$ (red circles) and ΔPT (black triangles) were pre-grown on lactose medium and inoculated into 2% xylose or arabinose and cultivated in flasks at 30°C for 96h.

Characterization of new putative pentose transporters

The pentose-negative strain Δ PT was then used to establish which of the *HGT* and *KHT* genes encoded functional pentose transporters. For this, we individually cloned candidate genes into a plasmid where expression was driven from the constitutive *ScTEF1* promoter. These plasmids were transformed into *K. marxianus* Δ PT, and strains expressing each of the thirteen genes were plated on medium containing xylose or arabinose at 2% or 0.1% concentration (Figure 2.8). The two sugar concentrations were used to distinguish potential high and low affinity transporters, as low affinity transporters would only support growth at the higher sugar concentration. Subsequently, all the strains were tested in a liquid assay where biomass (cell dry weight) and % sugar consumed after 96 h of growth was determined (Table 2.5). Two of the Hgt-like proteins, KMAR_10527 (KmAxt1) and KMAR_10531 supported growth at 0.1 and 2% concentrations of both sugars on plates and in liquid they enabled consumption of >90% xylose and 45-66% arabinose. Two other Hgt-like proteins, KMAR_10529 and KMAR_50027, only allowed growth on these sugars at 2% on plates and led to consumption of 66-59% xylose and 42-59% arabinose. The remaining two Hgt-like proteins, KMAR_10528 and KMAR_10530 transport neither sugar on plates nor in liquid growth. These data are consistent with a conclusion that KMAR_10527 (KmAxt1) and KMAR_10531 are high affinity and KMAR_10529 and KMAR_50027 low affinity pentose transporters. The Δ PT strains expressing the *KHT* genes reintroduced individually displayed only growth at 2% xylose on plates and showed xylose consumption ranging from 55-85% in liquid. A similar phenotype was seen for a strain expressing *KMAR_60179*, indicating the five Kht proteins and KMAR_60179 have characteristics of a low-affinity xylose transporter. For reasons unexplained, we were unsuccessful in our efforts to clone the sixth Kht-encoding gene *KMAR_50347*. Based on the sequence similarity to KMAR_50346 (Figure 2.4), however, it is likely that KMAR_50347 is functionally similar to the other Kht proteins with regard to pentose transport.

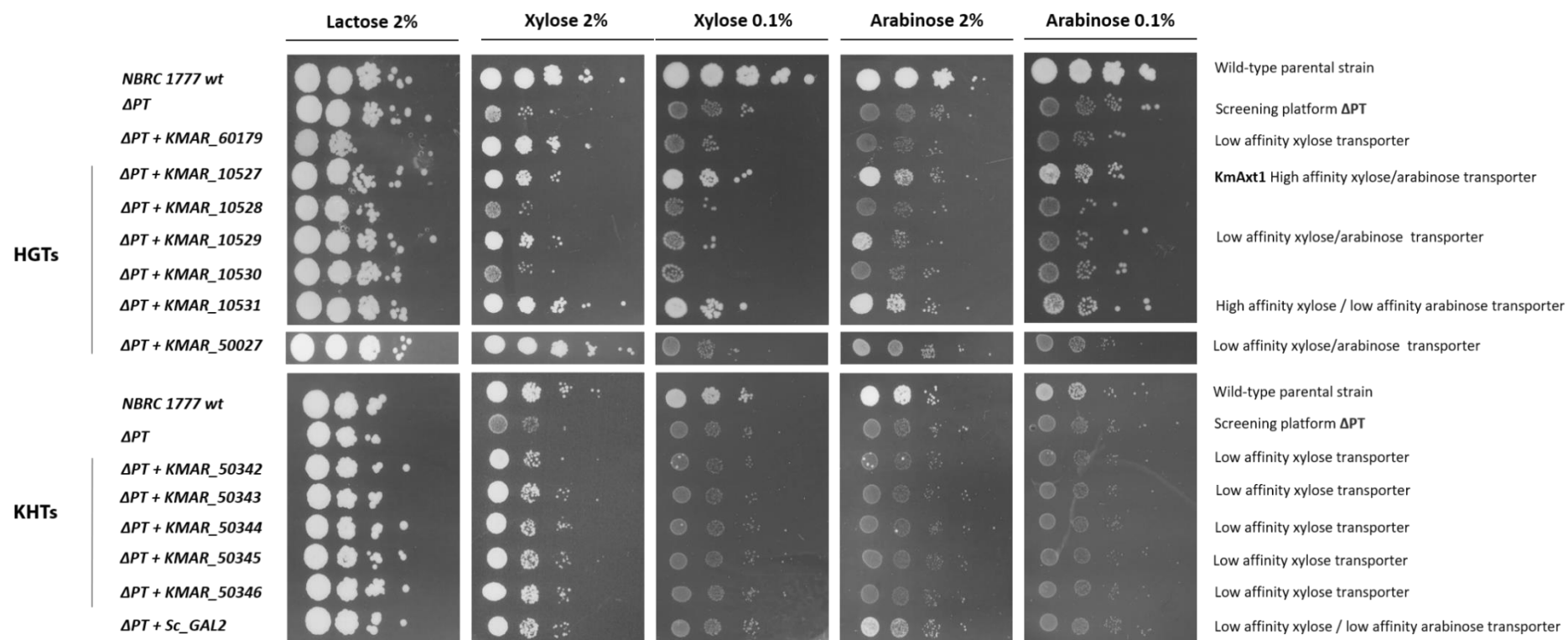


Figure 2.8: Identification of pentose transporters in *K. marxianus* ΔPT . Candidate native *K. marxianus* pentose transporters were individually expressed on a plasmid in the screening platform *K. marxianus* ΔPT . Strains were pre-grown on 2% lactose and then plated in serial dilution on agar plates containing either 2% or 0.1% xylose or arabinose as indicated. Plates were photographed after 96h of growth at 30°C.

Table 2.5. Growth and sugar consumption in 2% liquid medium. Each strain expressing the indicated gene was grown for 96h with either 2% xylose or 2% arabinose as the sole carbon source. Biomass was determined by harvesting the cells and measuring dry cell weight (DCW) and sugar consumption was assessed by measuring residual sugar using HPLC. Data were compiled from triplicate assays.

	Xylose 2%		Arabinose 2%	
	DCW (g L ⁻¹)	Sugar Consumption (%)	DCW (g L ⁻¹)	Sugar Consumption (%)
Δ PT	0.1	2.5 ± 2	0.1	0.98 ± 0.9
Δ PT + <i>KMAR_60179</i>	3.56 ± 0.04	52.64 ± 0.52	0.1 ± 0.2	0.4 ± 0.92
Δ PT + <i>KMAR_10527</i>	6.83 ± 0.33	90.42 ± 0.2	4.05 ± 0.63	45.36 ± 4.74
Δ PT + <i>KMAR_10528</i>	0.16 ± 0.05	3.23 ± 2.64	0.11 ± 0.015	2.46 ± 2.35
Δ PT + <i>KMAR_10529</i>	5.12 ± 0.40	66.28 ± 2.82	3.98 ± 0.48	42.16 ± 1.56
Δ PT + <i>KMAR_10530</i>	0.13 ± 0.005	4.73 ± 1.48	0.11 ± 0.005	7.47 ± 2.95
Δ PT + <i>KMAR_10531</i>	7.52 ± 0.34	94.63 ± 0.14	6.18 ± 0.5	65.85 ± 1.4
Δ PT + <i>KMAR_50027</i>	4.23 ± 0.05	59.34 ± 2.02	3.15 ± 1.41	59.5 ± 2.52
Δ PT + <i>KMAR_50342</i>	6.96 ± 0.36	79.93 ± 3.1	-	-
Δ PT + <i>KMAR_50343</i>	7.94 ± 0.82	85.1 ± 1.01	-	-
Δ PT + <i>KMAR_50344</i>	5.97 ± 0.61	55.58 ± 10.9	-	-
Δ PT + <i>KMAR_50345</i>	4.32 ± 0.40	58.29 ± 6.12	-	-
Δ PT + <i>KMAR_50346</i>	6.39 ± 2.15	68.61 ± 15.4	-	-
Δ PT + <i>Sc_GAL2</i>	2.73 ± 0.57	31.27 ± 1.8	2.03 ± 1.02	36.75 ± 1.25

The identification of *KMAR_60179* as a xylose transporter was unexpected since this is a homologue of *S. cerevisiae* Stl1, which has been extensively studied as a glycerol uptake protein. To determine whether *KMAR_60179* also transported glycerol, the Δ PT strain overexpressing *KMAR_60179*, was grown on medium with glycerol as the sole carbon source (Figure 2.9). The growth impairment of *K. marxianus* Δ PT was rescued by *KMAR_60179*, confirming it as a glycerol, as well as a xylose, transporter (Figure 2.8). *Sc_STL1* and *KMAR_30579* (present in the same branch with *Sc_Stl1* and *KMAR_60179* in Figure 2.4) were overexpressed in the platform strain as well, to check their ability to rescue growth on both glycerol and xylose (Figure 2.9). Any growth on glycerol conferred by these two transporters was minimal and they do not allow growth on xylose at either of the tested concentrations (2% and 0.1%). *In silico* modelling and comparison of the binding pockets of *KMAR_60179* and *Sc_Stl1* offers a possible explanation for the difference in the capacity to transport xylose, since the binding pocket of *Sc_Stl1* appears tighter and may be unable to accommodate xylose (Figure 2.10). We cannot discount the possibility that *Sc_STL1* was not correctly expressed or localised but as the focus of this study was on *K. marxianus* genes, we did not investigate this further.

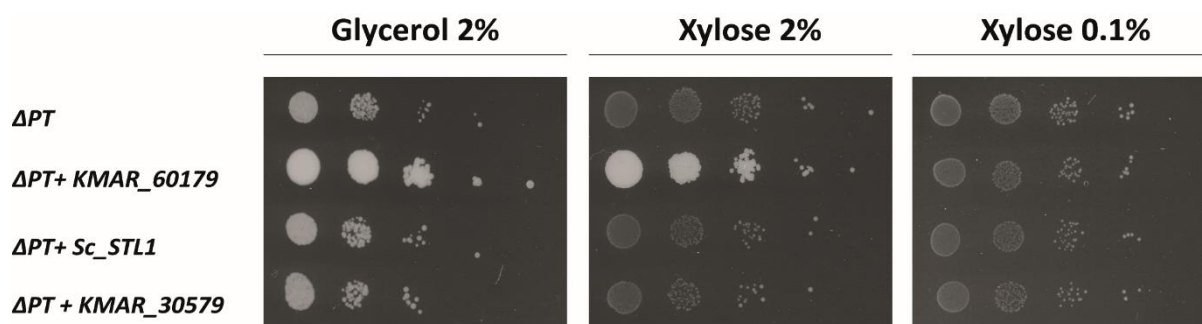


Figure 2.9: The *K. marxianus* Stl1 protein, KMAR_60179, can mediate both glycerol and xylose transport. The two *K. marxianus* genes with homology to *S. cerevisiae* *STL1*, and *Sc_STL1*, were individually expressed on a plasmid in *K. marxianus* ΔPT . Following pre-growth on 2% lactose, strains were plated in serial dilution on agar plates containing 2% glycerol or 2% or 0.1% xylose as indicated. Plates were photographed after 96h of growth at 30°C.

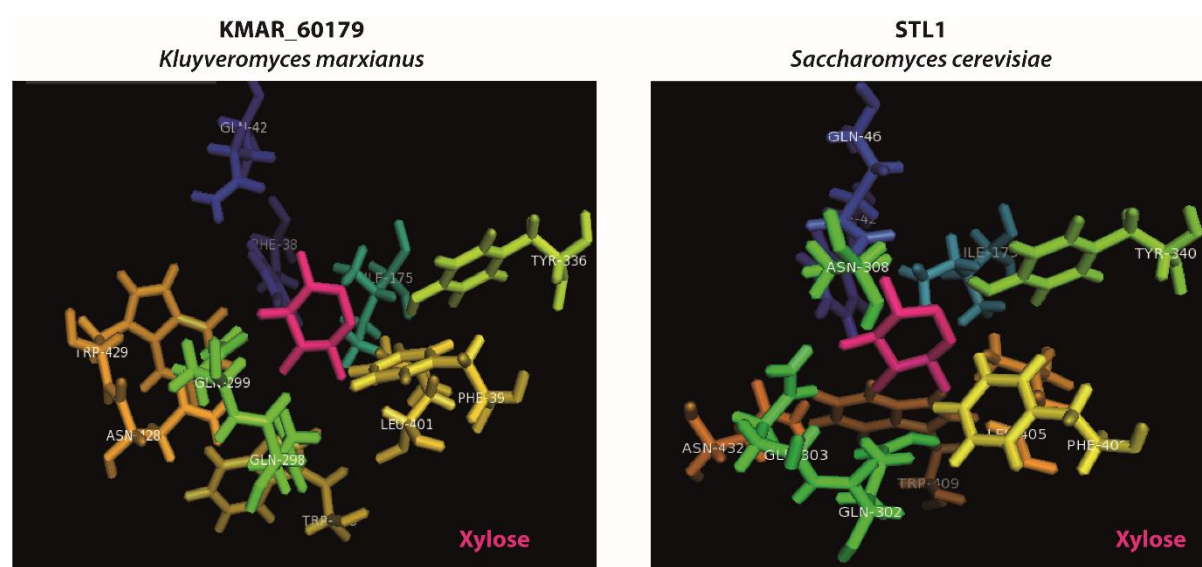


Figure 2.10: KMAR_60179 (on the left) and Sc_STL1 (on the right) binding sites. Amino acids within 4 Å from the ligand (xylose in pink) are reported. The amino acids in the binding pockets of the two transporters result to be highly conserved and the main difference is in the size of the pore, being the one of Sc-STL1 tighter due to the presence of the ASN308 really close to the ligand.

To demonstrate the potential of the platform strain for the identification of heterologous transporters, *S. cerevisiae* *GAL2* was expressed in the ΔPT strain and assessed in the same manner as was done with the *K. marxianus* genes (Figure 2.8; Table 2.5). As expected, Gal2 rescued the growth of the platform strain only on 2% xylose and arabinose 2% (plates) and allowed consumption of 31-37% sugar in the liquid assay, confirming that, in its native form, it is a low affinity pentose transporter (Farwick et al., 2014).

Xylose uptake is the main limiting factor for the complete consumption of this sugar in Δ PT strain

To assess the potential of *K. marxianus* Δ PT as a platform for functional characterization as well as identification of pentose transporters, *K. marxianus* Δ PT overexpressing either the high affinity transporter, KMAR_10531, or the low affinity transporter, KMAR_60179, was grown in liquid culture with sugar consumption monitored (Figure 2.11). Following pre-growth on YPLAC, strains were inoculated in minimal medium with 2% xylose or 2% arabinose as the sole carbon source. Strains expressing either transporter displayed growth on xylose (Figure 5A) but only KMAR_10531 supported growth on arabinose. Although the growth rate on xylose was not dramatically different, with slightly faster growth permitted by KMAR_10531 ($\mu_{\max}$ of 0.06 h⁻¹ vs 0.07 h⁻¹), there was an approximately two-fold difference in biomass generated: $A_{600} \sim 25$ (KMAR_10531) *versus* $A_{600} \sim 13$ (KMAR_60179). The strain expressing *KMAR_10531* also completely depleted xylose after 85h of growth whereas 50% of the xylose remained unused by the strain expressing *KMAR_60179* (Figure 5A). On arabinose (Figure 5B), the growth rate ($\mu_{\max}$ 0.065 h⁻¹) of the strain expressing *KMAR_10531* and the final cell density reached ($A_{600} \sim 23$) were comparable to those on xylose. These findings are consistent with the data shown in Figure 2.8 that indicated that KMAR_10531 is a high affinity transporter for both xylose and arabinose whereas KMAR_60179 is a low affinity xylose transporter and does not transport arabinose.

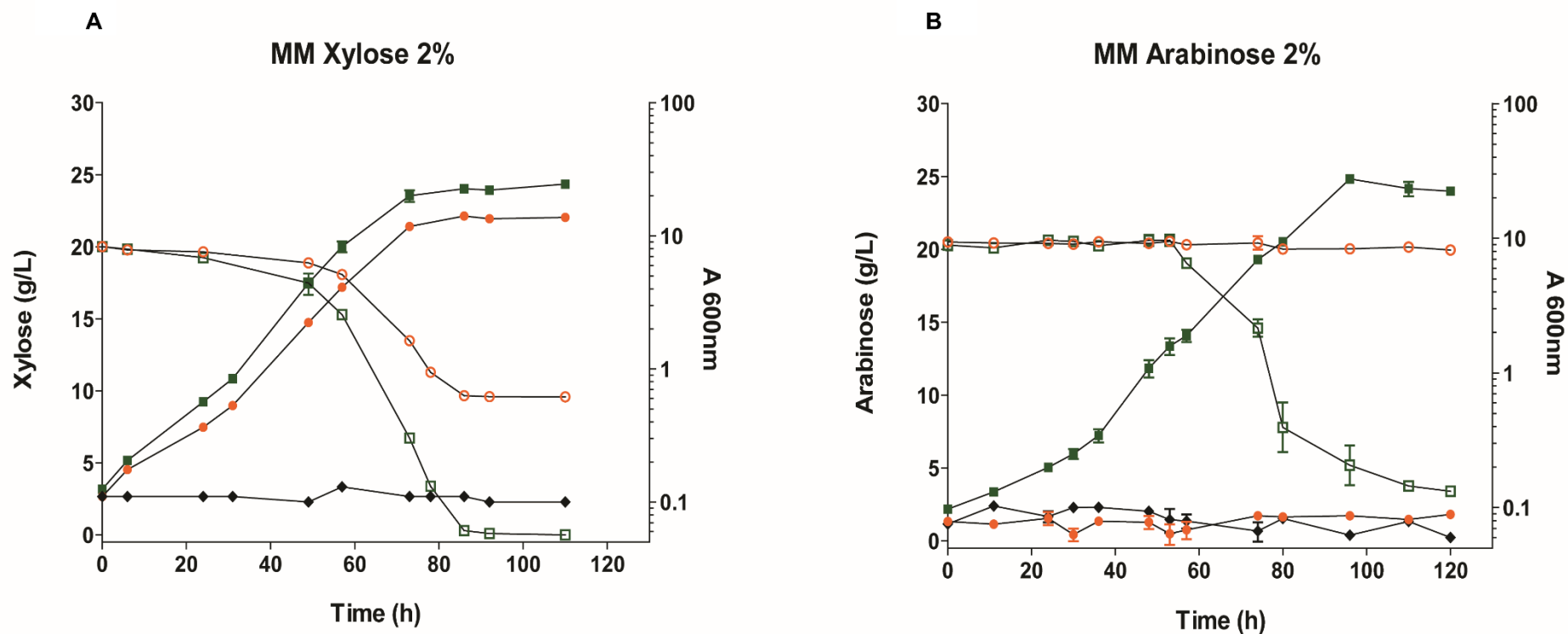


Figure 2.11: Application of *K. marxianus* Δ PT to compare the performance of putative low and high affinity pentose transporters. Genes encoding the candidate high affinity (KMAR_10531) and low affinity (KMAR_60179) pentose transporters were expressed from the *TEF1* promoter on the TU-DO-G418 plasmid in *K. marxianus* Δ PT. Following pre-growth on 2% lactose, the strains carrying *KMAR_10531* (green squares), *KMAR_60179* (orange circles), or empty plasmid (black diamonds) were inoculated in MM with 2% xylose (A) or arabinose (B) and grown at 30°C. Growth (solid symbols) and xylose or arabinose concentration (open symbols) were measured for 120h.

2.4 Discussion

The aims of this study were to expand our knowledge of sugar transporters in *K. marxianus* and to create a platform resource for study of pentose transporters in a native pentose-utilising yeast. This type of platform has been extensively used in *S. cerevisiae* for the functional study of native and heterologous sugar transporters, notably with strain EBY.VW4000, which lacks all functional hexose transporters (Alves-Araújo et al, 2005, Pacheco et al., 2020; Wiczorke et al., 1999). More recently CRISPR-SpCas9 was used to delete the 21 hexose transporters in a *S. cerevisiae* CEN.PK background, to create a more stable strain for future studies (Wijsman et al. 2019). We successfully constructed an analogous platform in *K. marxianus* for the study of pentose transporters. *K. marxianus* Δ PT only contains 3 genomic deletions but lacks twelve genes. It is stable, grows well on lactose and fructose, and only requires the addition of a transport protein to grow on xylose or arabinose. This is desirable if metabolic or physiological studies are to be performed as manipulation of metabolic enzymes is not required. We illustrated the potential by confirming that two of the newly native *K. marxianus* identified transporters and *S. cerevisiae* Gal2 indeed displayed characteristics of pentose transport with high or low affinity. This platform can now be used for studies of transport kinetics, for characterisation of engineered transporters, and for identification of additional pentose transporters from other species.

One of the key questions that we wanted to address was how many of the *K. marxianus* HGTs encoded pentose transporters. We confirmed previous findings that KMAR_10527 (KmAxt1) is able to perform high affinity xylose/arabinose transport and determined that KMAR_10531 has similar capability. Two further Hgts, KMAR_10529 and KMAR_50027 are low affinity xylose/arabinose transporters. These four proteins appear to comprise the full capacity of *K. marxianus* to transport arabinose as deletion of the HGT cluster creates an arabinose-deficient strain. None of the Hxt (Kht) proteins transported arabinose, though four were capable of low affinity xylose transport. This may well reflect a similar situation in *S. cerevisiae*, where only low affinity xylose transport occurs as that species lacks the HGT orthologues. Although the predicted low affinities might suggest that the *K. marxianus* Hxt proteins are less significant than the high affinity Hgt transporters for growth on xylose, it should be noted that it was necessary to delete the genes to create a xylose-negative platform strain (*K. marxianus* Δ PT), indicating that one or more of the Hxts is physiologically relevant. There is a conserved motif in the first transmembrane domain of MFS sugar transporters that is reported to be involved in substrate selectivity and kinetics so we compared this motif between the Hgt and the Hxt proteins (Knoshaug et al., 2016; Pacheco et al., 2020). All the Hxt proteins have the glucose-specific motif sequence G-G-F-V-F-G-W-D, whereas the Hgt

proteins all have the motif X-G-X-X-F-G-F-D, as well as other similarities to known arabinose transporters (Figure 2.12).

Another interesting feature is that three of the four Hgt proteins that can transport arabinose were previously shown to be also able to transport galactose (Varela et al, 2019a). These findings are consistent with a report that galactose transporters are often able to facilitate L-arabinose, due to the similar stereochemistry between those two sugars (Young et al., 2014). The correlation between galactose and arabinose transport was not absolute, however.

1. KMAR_50347	G	G	F	V	-	F	G	W	D	T	G	T	I	S	G	F	V
2. KMAR_50346	G	G	F	V	-	F	G	W	D	T	G	T	I	S	G	F	V
3. KMAR_50345	G	G	F	I	-	F	G	W	D	T	G	T	I	S	G	F	V
4. KMAR_50344	G	G	F	V	-	F	G	W	D	T	G	T	I	S	G	F	V
5. KMAR_50343	G	G	F	V	-	F	G	W	D	T	G	T	I	S	G	F	V
6. KMAR_50342	G	G	F	V	-	F	G	W	D	T	G	T	I	S	G	F	V
7. Sc_GAL2 N376F	G	G	F	M	-	F	G	W	D	T	G	T	I	S	G	F	V
8. KMAR_10527	S	G	L	M	-	F	G	F	D	I	S	S	M	S	S	M	I
9. KMAR_10528	S	G	L	M	-	F	G	F	D	I	S	S	M	S	S	M	I
10. KMAR_10529	S	G	L	M	-	F	G	F	D	I	S	S	M	S	S	M	I
11. KMAR_10530	S	G	L	M	-	F	G	F	D	I	S	S	M	S	S	M	I
12. KMAR_10531	S	G	L	M	-	F	G	F	D	I	S	S	M	S	S	M	I
13. KMAR_50027	S	G	L	M	-	F	G	F	D	I	S	S	M	S	S	M	I
14. Pc_AraT	G	G	A	L	-	F	G	F	D	V	S	S	M	S	A	W	I
15. Pg_Axt1	A	G	M	M	-	F	G	F	D	I	S	S	M	S	A	F	V
16. KMAR_60179	T	G	F	S	L	F	G	Y	D	Q	G	L	M	S	G	I	I
17. KLLA0F26246	T	G	F	S	L	F	G	Y	D	Q	G	L	M	S	G	I	I
18. Sc_STL1	T	G	F	S	L	F	G	Y	D	Q	G	L	M	A	S	L	I
19. Ss_XUT1	G	G	L	V	-	Y	G	Y	N	Q	G	M	F	G	Q	I	S
20. Ss_XUT3	G	G	L	L	-	F	G	Y	D	Q	G	V	I	S	G	I	V
21. Dh_Xylh	G	G	L	L	-	F	G	Y	D	Q	G	V	I	S	G	I	V

Figure 2.12: Comparison of a conserved motif in the first transmembrane span among promising sugar transporters of *K. marxianus*, specific xylose transporters of *S. stipitis* (XUT1 and XUT3) and *D. hansenii* (Xylh) and arabinose transporters of *P. chrysogenum* (AraT) and *P. guilliermondii* (Axt1).

One of the more unexpected findings was that KMAR_60179, the *K. marxianus* equivalent of *S. cerevisiae* Stl1, is a low affinity xylose transporter as well as a glycerol transporter. Sc_Stl1 has been extensively characterised as a glycerol/H⁺ symporter involved in the response to high osmolarity and there are no previous reports of its involvement in xylose transport (Ferreira & Lucas, 2007; Ferreira et al., 2005; Zhao et al., 1994). Differences in the size of the binding pocket, which appears tighter than the one of KMAR_60179, could underlie this phenotype. Our preliminary work indicated that Sc_Stl1 may not transport xylose but more detailed analysis to check expression, localisation, and other aspects, would be required to fully investigate this. Further work would also be needed to investigate whether Stl1 plays the same role with respect to the osmotic response in both *K. marxianus* and *S. cerevisiae*.

The findings of this study are relevant both for fundamental understanding of sugar transport in yeasts and for biotechnological applications. For the latter, the new pentose transporters that we identified can serve as the basis for protein engineering for transporters with new kinetic functionality. In this respect, the fact that the best candidate transporters from *K. marxianus* are only distantly related to the xylose-transporting Hxt derivative (Gal2) is positive as it indicates that the solution space for pentose transporters is quite large. There are also implications for the study of sugar transport and the evolution of transport proteins, though the study also raises as yet unanswered questions in this area. In general, it is difficult to conclude that just because a protein can transport a sugar when expressed heterologously, it does this under normal conditions. Nonetheless, the results with the deletions suggest that most of the proteins with which we worked make some contribution to transport of the relevant sugars. It also seems likely, however, that at least some of the Hgts, and indeed the other MFS transporters identified, transport as yet unknown substrates. It is not known why *K. marxianus* displays this relatively recent expansion of *HGT* genes but it is clear that the proteins are evolving to be functionally distinct. Further study of these proteins may offer new insights into some of the forces and processes that underlie yeast evolution.

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CHAPTER 3

Effect of glucose on pentose assimilation and transport in

Kluyveromyces marxianus

Abstract

Kluyveromyces marxianus, as other microorganisms, possesses an intrinsic system of glucose repression that prevents the utilization of other carbon sources in presence of this sugar. In order to enable *K. marxianus* to simultaneously use both hexose and pentose sugars deriving from lignocellulosic biomass, there is an interest in understanding how glucose repression phenomenon works in this yeast to avoid the inhibition on other sugars, like xylose. Here, glucose repression effects on xylose metabolism and pentose transporters were preliminary investigated. Mutants lacking either Mig1 or Rag5, two fundamental transcriptional regulators believed to be involved in glucose repression, and engineered strains expressing the xylose reductase gene, *XYL1*, under constitutive promoters were constructed and tested to evaluate glucose repression release. Moreover, strains carrying single sugar transporters were as well tested on MM xylose supplemented with 2-deoxyglucose (2-DOG) in order to identify a transporter not inhibited/repressed by glucose. Kmar_60179 emerged as the only transporter not inhibited by glucose.

3.1 Introduction

Kluyveromyces marxianus is a non-conventional yeast that has attracted considerable attention for various biotechnological applications, such as production of bioethanol, hydrolytic enzymes, food biomass and food additives (Lane et al., 2010; Varela et al., 2017). Apart from its considerable thermotolerance and ability to encode enzymes such as inulase or β -galactosidase, one of the most promising traits is the fact that *K. marxianus* can assimilate a wide range of substrates, including pentoses which represent more than 40% of lignocellulosic hydrolysates. Consequentially, there is interest in exploiting the beneficial traits of *K. marxianus* for production of bioproducts such as bioethanol, from raw materials like 2nd generation sugars coming from lignocellulosic biomass. Although, *K. marxianus* is naturally able to use pentoses, it is subjected to an intrinsic system of glucose repression, like other yeasts, that prevents the utilization of other carbon sources in presence of glucose (Carlson et al., 1999; Gancedo et al., 1998; Rolland et al., 2002). To make the entire process economically feasible, there is a need for simultaneous consumption of all hexose (glucose) and pentose (xylose and arabinose) sugars deriving from the raw material to shorten the time of the process and increase the production yield. Therefore, there is a need to analyse glucose repression mechanism in order to then construct a xylose-glucose co-utilization strain. In this study, we review what it is known about *K. marxianus* glucose repression system, comparing its mechanism with *Saccharomyces cerevisiae* and we highlight the inhibitory effect of glucose on pentose transporters.

3.1.2. Glucose repression in yeast. What is it known in *Kluyveromyces marxianus*?

In the well-studied model yeast *S. cerevisiae*, the glucose repression mechanism is regulated by a crosstalk between several glucose signalling pathways in which Snf3/Rgt2 are involved in glucose sensing and uptake in the cells, the Snf1/Mig1 pathway plays a fundamental role negatively regulating the expression of the genes involved in the utilization of alternative carbon sources and the cAMP-dependent protein kinase A pathway senses cell's energy status and affects expression of the Hexose Transporters (*HXT*) genes (Hardie et al., 1998; Kayikci et al., 2015; Özcan et al., 1999; Papamichos-Chronakis et al., 2004; Persson et al., 2022). Glucose signalling and repression mechanism (Figure 3.1) are not well clarified in *K. marxianus*.

In *S. cerevisiae*, Snf3 and Rgt2 belong to the family of the Hxt proteins, but they lost the ability to transport glucose and became specialized in sensing extracellular glucose levels. Snf3 senses low levels of extracellular glucose, while Rgt2 detects high levels of the sugar. The cells can sense extracellular glucose levels, consequently, regulate glucose uptake, and hereby activate glucose repression. In contrast,

Kluyveromyces lactis possesses only one gene *RAG4* that encodes for the orthologue of Snf3/Rgt2 of *S. cerevisiae*. This sensor protein appears to have the dual function of signalling both high and low concentrations of glucose and oxygen sensing in *K. lactis* (Betina et al., 2001; Micolonghi et al., 2011). The orthologous sensor in *K. marxianus* has not been described.

In *S. cerevisiae*, in presence of high glucose concentrations, Snf3/Rgt2 mediate a signal that induces the expression of Hxt transporters in order to increase the import of glucose which inside the cell triggers a cascade of repression in the utilization of other carbon sources. On the other hand, in glucose starvation conditions, factors like Mth1/Std1 are activated and they bind Rgt1, that together with Cyc8-Tup1 repression complex binds the promoter of *HXT*, preventing expression (Ahuatzi et al., 2007; Alipourfard et al., 2019; Nurcholis et al., 2020). In general, low-affinity transporters are expressed and activated when glucose is abundant, while high-affinity transporters are repressed (Kayikci et al., 2015).

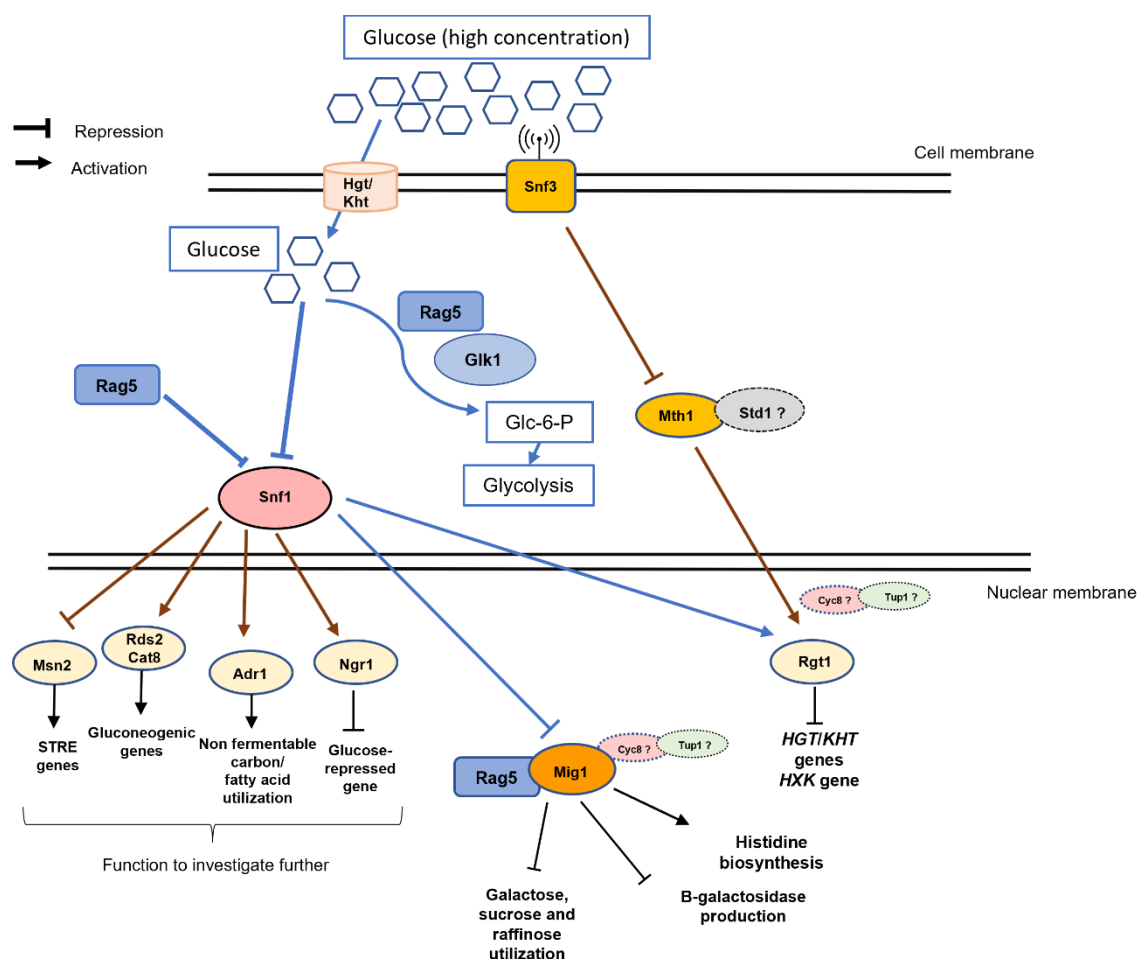


Figure 3.1. Putative glucose repression mechanism in *Kluyveromyces marxianus*. In presence of high glucose concentration, glucose enters in the cell via Hgt/Kht transporters, is phosphorylated by the hexokinase Rag5 or the glucokinase Glk1 and is metabolized via glycolysis. External glucose is sensed by Snf3 (encoded by *KMAR_60499*) which activates various responses including enhancing the expression of hexose transporters. At

the same time, glucose inside the cell triggers a series of repression events which involve suppression of Snf1 with consequent repression of the metabolism of alternative carbon sources. The glucose phosphorylation signal generated by Rag5 appears to play an important role being involved directly or indirectly in the repression of Snf1. Genes indicated with dashed line and interrogation point have been not found yet in *K. marxianus* or their role is uncertain.

Mig1 and Hxk2 play fundamental roles as regulators of glucose repression together with Snf1 in *S. cerevisiae*. The Snf1 kinase complex is involved in the regulation of *HXTs*, glucose repressed genes, but also in several processes related to cell proliferation, aging, stress response and amino acid biosynthesis acting on various target proteins (Mig1, Adr1, Cat8, Rds2, Nrg1) (Usaite et al., 2009). In high glucose concentrations, Snf1 is inactive and excluded from the nucleus, allowing one of its main targets Mig1 to be dephosphorylated and active in the nucleus. Here, Mig1 together with the hexokinase Hxk2 and the co-repression complex Cyc8-Tup1 binds the upstream regions of promoters of genes involved in the assimilation of other carbon sources, respiration and gluconeogenesis causing their repression. On the other hand, when glucose is limited, Snf1 is active and phosphorylates Mig1 allowing release of glucose repression and the expression of glucose-repressed genes. In *K. marxianus*, the disruption of *Snf1* was proved to be critical for xylose utilization, as knocking out this gene prevented the strain from growing on xylose even in absence of glucose. Therefore, it was established that Snf1 pathway is the main signalling pathway for glucose repression of xylose utilization in *K. marxianus* (Hua et al. 2019).

In *S. cerevisiae* there are two hexokinases (*HXK1* and *HXK2*) and one glucokinase (*GLK1*) and as said before Hxk2 is reported to be crucial in glucose repression. On the other hand, in *Kluyveromyces* there is only one hexokinase, called *HXK1/RAG5*, main responsible for glucose and fructose phosphorylation and involved in glucose repression (Bär et al., 2003; Mates et al., 2014) and one glucokinase (*GLK1*) that is not essential for hexose phosphorylation in this yeast (Zhang et al., 2017).

Some studies were conducted to elucidate the role of the *S. cerevisiae* orthologues of the main regulators involved in the Snf1/Mig1 pathway in *K. marxianus* (Hua et al., 2019; Nurcholis et al., 2019a). It was shown that Km_Mig1 and Km_Rag5 share some functions with the orthologues Sc_Mig1 and Sc_Hxk2, in particular regarding the negative regulation of *INU1* (called also *SUC2*) involved in sucrose utilization and repression of galactose and raffinose utilization in presence of glucose (Lertwattanasakul et al., 2011, Zhou et al., 2013), but in contrast with *S. cerevisiae*, Km_Mig1 does not seem to be involved in glucose repression of xylose utilization. Xylose assimilation pathway consists of three steps: xylose is first reduced to xylitol by *XYL1*, then oxidised to xylulose by *XYL2* and finally phosphorylated to xylulose 5-P by *XYL3*. Xylulose 5-P enters in the pentose phosphate pathway (PPP). Several putative Mig1 binding sites have

been found on the promoters of *XYL1* and *XYL2*, but the deletion of *MIG1* does not release glucose repression on xylose. The mechanism of glucose repression of genes related to xylose metabolism in this yeast remains still unclear but both Km_*XYL1* and Km_*XYL2* were shown to be repressed by glucose (Rodrussamee et al., 2011, Hua et al. 2019).

Other functions for Km_Mig1 have been proposed as its involvement in histidine biosynthesis (Nurcholis et al., 2019b) and its deletion was found beneficial to increase β -galactosidase production and activity (Zhou et al. 2013). The latter has been fully studied in *K. lactis*. Here, Kl_Mig1 does not directly mediate the glucose repression of *LAC4* (β -galactosidase), but it represses the expression of Kl_*GAL1* (encoding for a galactokinase) in presence of glucose and the lower level of Gal1 then prevents Lac9-mediated transcription activation of *LAC4* (Dong & Dickson, 1997; Liu et al., 2022).

In *S. cerevisiae* and *K. lactis*, the hexokinase Km_*RAG5* is involved in transcriptional regulation of low-affinity glucose transporter/s (Rag1 in *K. lactis*) (Betina et al., 2001; Prior et al., 1993). Nurcholis et al. 2019 showed that the deletion of *RAG5* in *K. marxianus* (*km_rag5* mutant) reduced the transcriptional level of *RAG1*. This decrease in *RAG1* expression results in reduction in glucose uptake with consequent slowdown in glucose consumption. Lately it was discovered that in *K. marxianus* there is an expansion in the sugar transporter family, Rag1 is not the only low-affinity glucose transporter in this yeast, in which we actually find 2 copies – Kmar_50346 and Kmar_50347 (Donzella et al., 2021; Varela et al., 2019).

The deletion of *RAG5* in *K. marxianus* also caused a retardation in growth in a media where xylose is used as only carbon source, suggesting that hexose transporters including Rag1 are responsible for xylose import in *K. marxianus*. This hypothesis was then confirmed by our studies on pentose transporters in this yeast (Donzella et al. 2021). Rodrussamee et al. (2011) pointed out how the presence of glucose in the media delayed the utilization of galactose, xylose and arabinose in *K. marxianus*, opening up the possibility that transporters for these sugars are sensitive to glucose. Different studies already confirmed this in *S. cerevisiae*, showing glucose have a negative effect on utilization of other carbon sources by competitively inhibiting their transporters (Farwick et al., 2014; Rojas et al., 2021; Subtil et al., 2012), but the latter has not been confirmed yet in *K. marxianus*.

Various questions remain still unsolved on how glucose repression system works in *K. marxianus* and if glucose acts at the transport level, inhibiting the transport of other carbon sources in this yeast.

In this work, we tried to elucidate the differences between the main regulators involved in glucose repression in *S. cerevisiae* and *K. marxianus*, first focusing on the signalling pathway with an *in-silico*

analysis of the orthologue of Sc_Snf3/Rgt2. Then we investigated the Snf1/Mig1 pathway, creating disrupted mutants of Km_*MIG1* and Km_*RAG5* to better study their role in glucose repression on pentoses and we also constructed a mutant in which the promoter of the xylose reductase gene *XYL1* - which reduces xylose to xylitol - was replaced by constitutive promoters in an attempt to reduce the repression induced by glucose. To study these mutants, we compared their growth on pentoses in presence of 2-deoxyglucose (2-DOG). 2-DOG is a glucose analogue that lacks the hydroxyl group on carbon 2 in the glucose backbone. It can be transported into the cells by hexose transporters, be phosphorylated by hexokinase to 2-DOG-6P and trigger glucose repression, but it cannot be metabolized for growth (O'Donnell et al., 2015). Accumulation of 2-DOG-6P leads to inhibition of the hexokinase itself, hampering the metabolism of glucose and creating starvation-like conditions in the cells (Schmidt et al., 2021). Moreover, 2-DOG appears to lead to more fragile and damaged yeast cell walls, trapping cellular uridine and guanosine-nucleotide pools or competitive, inhibiting endogenous precursors needed for cell wall synthesis (Laussel & Léon, 2020).

In previous works (Donzella et al., 2021; Varela et al., 2019), we constructed a platform strain unable to transport xylose into the cell. Here, we use this platform to individually express transporters to establish if any are insensitive to glucose competition. We identified one promising transporter not subjected to glucose inhibition, opening up the possibility to use it for simultaneous consumption of glucose and xylose.

3.2 Materials and Methods

Strains, media and culture conditions

K. marxianus strains (Table 3.1) were pre-cultured in rich growth media YP (yeast extract 10 g/L and peptone 20 g/L) supplemented with 2% lactose. For growth assays, yeast strains were inoculated at A_{600nm} 0.1 in 250 mL shake flasks with 50 mL minimal medium MM (5 g L⁻¹ Urea, 3 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ MgSO₄·7H₂O, 100 µg mL⁻¹ vitamin solution, 100 µg mL⁻¹ trace elements solution), supplemented with various concentrations of the appropriate sugar (D, Glucose; X, Xylose) and grown at 30 °C with 200 rpm agitation. For glucose repression assessment, 2-DOG was added to the flask with a concentration of 0.1%. To observe growth characteristics of various *K. marxianus* mutants, spot tests were carried by pre-growing the yeast strains on YPLAC overnight up to OD 2.0, washing twice and spotting on plates of MM

supplemented with 2% glucose, xylose and arabinose added with increasing concentrations of 2-DOG (0, 0.01, 0.1%).

Table 3.1: Strains used in this study.

Strain	Genotype	Parental strain	Characteristics	Origins
<i>K. marxianus</i> NBRC1777	Wild-type strain			NITE, Japan
$\Delta mig1$	NBRC1777 $\Delta mig1$	<i>K. marxianus</i> NBRC1777	CHR2: SNPs between 118,189-118,208	This study
$\Delta rag5$	NBRC1777 $\Delta rag5$	<i>K. marxianus</i> NBRC1777	CHR6: SNPs between 869,713 – 869,732	This study
Xyl1p-PDCp swap	NBRC1777 Xyl1p-PDC1p swap	<i>K. marxianus</i> NBRC1777	CHR1: Δ 1,411,730-1,412,583 :: pPDC1	This study
Xyl1p-TEFp swap	NBRC1777 Xyl1p-TEF1p swap	<i>K. marxianus</i> NBRC1777	CHR1: Δ 1,411,730-1,412,583 :: pTEF1	This study
<i>K. marxianus</i> ΔPT	$\Delta hgt \Delta kht$ $\Delta kmar_60179$	<i>K. marxianus</i> $\Delta hgt \Delta kht$	HGT: CHR I Δ 1,131,464- 1,145,439 KHT: CHR V Δ 1251-4853, Δ 4926-16261, Δ 16417-20938 60179 : CHR VI Δ 134-1710	Donzella et al. 2021

Bioinformatics

The FASTA file containing the amino-acidic sequences of Sc_Snf3/Rgt2 and Km_Snf3 (code: Kmar_60499) were obtained from the GenBank database (available at <http://www.ncbi.nlm.nih.gov/GenBank/index.html>). Prediction of the transmembrane domains was performed submitting the protein sequences to TMHMM 2.0 server (Krogh et al., 2001). Alignment of Sc_Snf3, Sc_Rgt2 and KMAR_60499 was performed by Muscle (Edgar, 2004) and visualized by Jalview v.2 (Waterhouse et al., 2009). Synteny conservation was inspected using the Yeast Genome Order Browser (YGOB, B, www.ygob.ucd.ie) (Byrne & Wolfe, 2005). The sequences of the regulators Mig1 and Rag5 and Xyl1 genes were retrieved from NCBI. Xyl1 promoter characterization was carried out using YeTFaSCo database to identify potential transcription factor binding site (De Boer & Hughes, 2012).

Construction of mutants to release glucose repression on xylose metabolism

The construction of all the *K. marxianus* mutants was made using CRISPR-Cas9 system previously developed in our group (Donzella et al., 2021; Rajkumar & Morrissey, 2022; Varela et al., 2019). *K. marxianus* $\Delta mig1$ (*kmar_20054*) and $\Delta rag5$ (*kmar_60404*) strains were constructed by indel mutations in the genes *MIG1* and *RAG5*, caused by double stranded break guided by a gRNA (designed to

specifically target the genes to knock out) expressed in a pUCC001 plasmid containing Cas9. The design of the gRNA sequences specific to target genes was performed using the software sgRNAcas9 (Xie et al., 2014) and CRISPRdirect (Naito et al., 2015). For cloning into pUCC001, 20nt target and its reverse complement primers were ordered with suitable overhangs for cloning via Golden Gate Assembly (New England Biolabs, Inc., Ipswich, MA, United States). The presence of the gRNA target sequences in the pUCC001 plasmid was confirmed by colony PCR using the forward target primer and BSA-R. Following cloning, the disruption plasmids were transformed into *K. marxianus* NBRC1777 using a variation of the LiAc/SS carrier DNA/PEG method (Gietz & Schiestl, 2007).

The creation of *K. marxianus* NBRC1777 mutants with *XYL1* promoter replaced were made following the protocol described by Rajkumar et al. 2022. Briefly, a 20nt gRNA sequence was designed to specifically target the 1000bp upstream sequence of *XYL1*. The gRNA sequence was then cloned by Golden Gate to the plasmid pUCC001. In order to allow the replacement of *XYL1* promoter with a constitutive promoter (p*PDC1* or p*TEF1*), *K. marxianus* NBRC1777 was co-transformed with pUCC001 plasmid carrying the gRNA target and a repair fragment constructed as following. The repair fragment for promoter replacement was constructed by amplify the constitutive strong promoter of interest (p*PDC1* or p*TEF1*) with 85bp primer F_swap - designed to be 65 bp complementary to the beginning of the promoter to be substituted (p*XYL1*) and 20bp of the new promoter - and with 85bp primer R-swap – designed with 20bp of the downstream of the new promoter and 65bp complementary to the upstream of the CDS of the *XYL1* gene (Figure 3.2). Replacement of the promoter of *Xyl2* gene were unsuccessful, nevertheless different attempts were made. Possibly *Xyl2* is an essential gene and it's difficult to engineer.

All the plasmids and primers used in this study are listed respectively in Table 3.2 and 3.3.

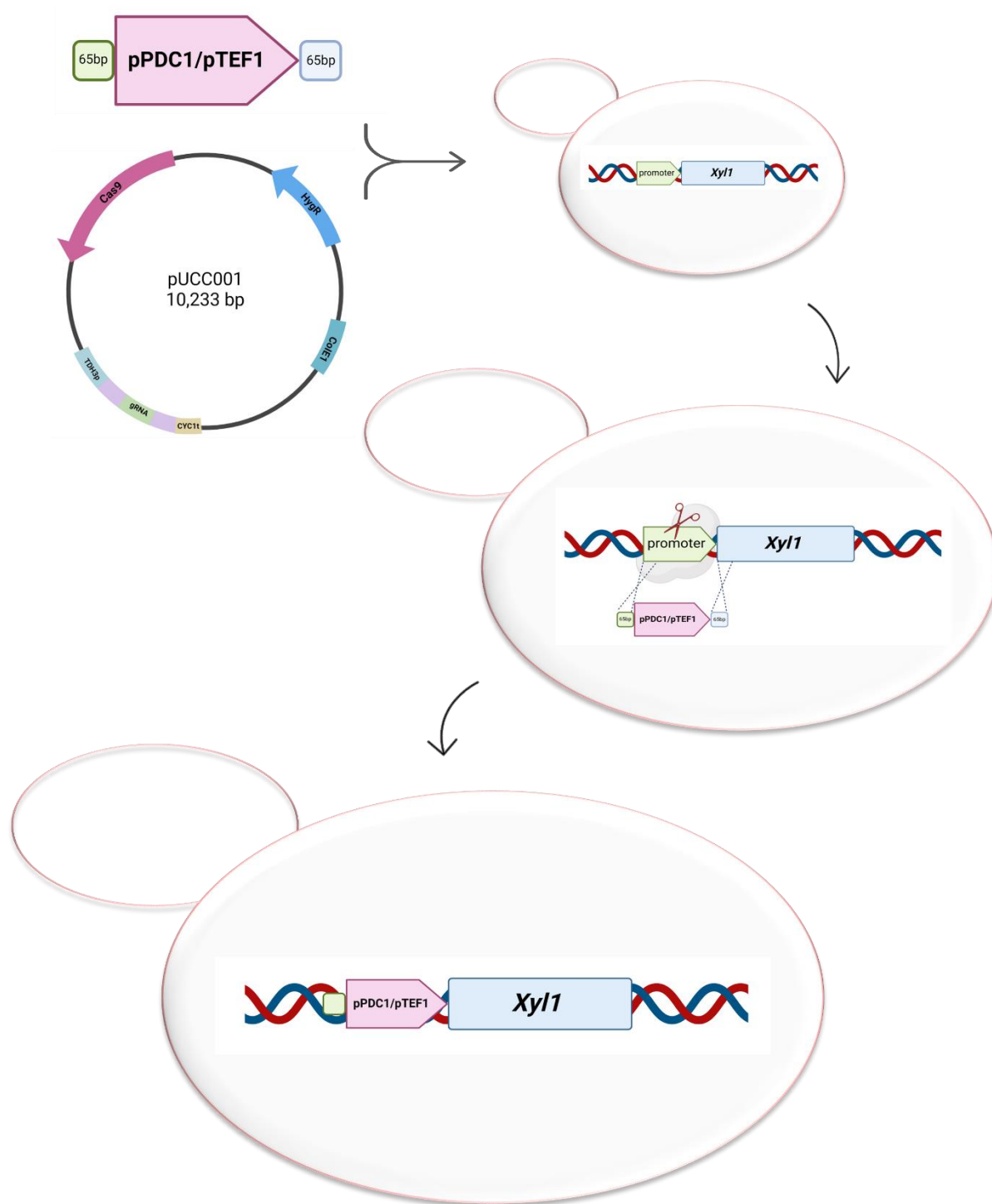


Figure 3.2: Schematic representation of promoter replacement strategy used in this study. *K. marxianus* NBRC1777 was co-transformed with pUCC001 plasmid carrying the gRNA target for *XYL1* promoter and a repair fragment carrying the substitute constitutive promoter (pPDC1 or pTEF1).

Table 3.3: CRISPR-Cas9 and complementation plasmids used in this study.

CRISPR-Cas9 Plasmids	Relevant characteristics	Source
pUCC001	[pUDP002] HH-BsaI BsaI-gRNAscaffold-HDV	Rajkumar et al., 2019
pUCC001- mig1	[pUCC001] sgRNA-KMAR_mig1	This study
pUCC001- rag5	[pUCC001] sgRNA-KMAR_rag5	This study
pUCC001- Xyl1pr	[pUCC001] sgRNA-KMAR_Xyl1pr	This study

Complementation plasmids	Relevant characteristics	Source
pTU-DO-G418	ampR KmARS/CEN7 KanMX BsaI sfGFP BsaI	Rajkumar and Morrissey, 2020
pTU-DO-G418- 50342	pTEF1-KMAR_50342-CYC1 TT	Donzella et al. 2021
pTU-DO-G418- 50343	pTEF1-KMAR_50343-CYC1 TT	Donzella et al. 2021
pTU-DO-G418- 50344	pTEF1-KMAR_50344-CYC1 TT	Donzella et al. 2021
pTU-DO-G418- 50345	pTEF1-KMAR_50345-CYC1 TT	Donzella et al. 2021
pTU-DO-G418- 50346	pTEF1-KMAR_50346-CYC1 TT	Donzella et al. 2021
pTU-DO-G418- 10527	pTEF1-KMAR_10527-CYC1 TT	Donzella et al. 2021
pTU-DO-G418- 10528	pTEF1-KMAR_10528-CYC1 TT	Donzella et al. 2021
pTU-DO-G418- 10529	pTEF1-KMAR_10529-CYC1 TT	Donzella et al. 2021
pTU-DO-G418- 10530	pTEF1-KMAR_10530-CYC1 TT	Donzella et al. 2021
pTU-DO-G418- 10531	pTEF1-KMAR_10531-CYC1 TT	Donzella et al. 2021
pTU-DO-G418- 50027	pTEF1-KMAR_50027-CYC1 TT	Donzella et al. 2021
pTU-DO-G418- 60179	pTEF1-KMAR_60179-CYC1 TT	Donzella et al. 2021

Table 3.4: Primers used in this study for CRISPR gRNA target design, CRISPR-Cas9 genomic indel mutations diagnosis and *XYL1* promoter replacement (swap) repair fragment construction. The gRNA primers are designed to be 20nt in length and not include the PAM sequence at the 3' end (NGG). For cloning into the CRISPR-plasmid pUCC001, the forward gRNA target primers present overhangs 5'-cgtc-3' and the reverse target primers 5'-aaac-3' (indicated in lower case). For replacement of p*XYL1* with a constitutive promoter (p*PDC1* or p*TEF1*), the repair fragments were constructed using primers swap_ F and swap_ R of 85 bp (the 20nt overlapping on the new constitutive promoter – either p*PDC1* or p*TEF1*– are indicated with capital letters).

gRNA Targets CRISPR	
<i>kmar_20054_Target_1F</i>	cgtcAATCAAGATTTCTGCGCAA
<i>kmar_20054_Target_1R</i>	aaacTTGGCGCAGAAATCTTGATT
<i>kmar_60404_Target_1F</i>	cgtcAGTTTTCGAATGGATCTTCT
<i>kmar_60404_Target_1R</i>	aaacAGAAGATCCATTCGAAAAC
<i>pXYL1_Target_1F</i>	cgtcGTGTTTACCTGTATTACCTG
<i>pXYL1_Target_1R</i>	aaacCAGGTAATACAGGTAAACAC

Diagnostic primers	
<i>kmar_20054_diag-F</i>	GATGAGTTGACGAGACACAG
<i>kmar_20054_diag-R</i>	GTCTATCAATGAACCTAAAGGATGGG
<i>kmar_60404_diag-F</i>	TACAGATAGCAGAAACACCACAA
<i>kmar_60404_diag-R</i>	CTTGATCAACGATACCACCG
<i>pXYL1- pPDC1_diag-F</i>	GAATAATGGAATGGCCTTGATTTC
<i>pXYL1-pTEF1_diag_F</i>	AGAAGTAACAGCAGGAAAGAAAAAC
<i>pXYL1-swap_diag_R</i>	CAACTTGATGGCTTCGTACA

Promoter Swap	
<i>pXYL1- pPDC1_swap_F</i>	ccaagaggtagagagcacaagtaacgcaacccaattacgtaatgcgacagattaattgattgaATCCAGC GAATATACAGCGT
<i>pXYL1-pPDC1_swap_R</i>	ccaagccgactagcggcatcttgatccattgttcaaggtaactgttggtgaggtatgtcatGCAATTATTT GGTTTGGGTGTG
<i>pXYL1-pTEF1_swap_F</i>	ccaagaggtagagagcacaagtaacgcaacccaattacgtaatgcgacagattaattgattgaCCAACGC ATATGCTGCAATCA
<i>pXYL1-pTEF1_swap_R</i>	ccaagccgactagcggcatcttgatccattgttcaaggtaactgttggtgaggtatgtcatTTAATGTTA CTTCTCTGGAGTTAGAACT

Complementation of putative pentose transporter in Δ PT strain to check for glucose repression

In order to study glucose inhibition at the level of transporters, individual *HGT* and *KHT* genes were previously cloned into pTU-DO-G418 plasmid under the expression of a constitutive promoter as per method described in Donzella et al. 2021. The plasmids were then transformed in *Kluyveromyces marxianus* Δ PT platform strain. Finally, the transformants were tested on solid media containing 2% glucose, xylose or arabinose added with 2-DOG at different concentrations (0, 0.01% and 0.1%).

3.3 Results and Discussion

Putative differences between Snf3/Rgt2 sensors of *S. cerevisiae* and orthologues in *K. marxianus*

In *S. cerevisiae*, Snf3/Rgt2 belong to the family of the Hxt (hexose transporters) proteins, but they lost the ability to transport glucose and became specialized in sensing extracellular glucose levels. It is known that in *K. lactis* there is just one sensor called Rag4 involved in glucose and hypoxia sensing (Micologhi et al., 2011), but there is a lack of information about this in *K. marxianus*. For this reason, we identified the orthologue of Snf3/Rgt2 system in this yeast. A single orthologue of Sc_Snf3/Rgt2 was identified on chr6 (KMAR_60499) of *K. marxianus* based on sequence homology and confirmed by examining synteny. The sequences were then further investigated by inspecting the membrane topology (TMHMM 2.0 server) and alignment (MUSCLE) (Figure 3.3). *K. marxianus* Snf3 (encoded by KMAR_60499) is a protein of 813 aa, shorter than Sc_Snf3 (884 aa) but longer than Sc_Rgt2 (763 aa) and Kl_Rag4 (716 aa) and consists of 12 transmembrane domains. The comparison of the sequences of *S. cerevisiae*, *K. lactis* and *K. marxianus* sensors mainly reveals differences at the N-term and C-term. In the C-term of the four compared proteins, only 25 amino acids are conserved and this sequence has been shown to be essential for the signalling function in *S. cerevisiae* (Vagnoli et al., 1998). In this yeast, the disruption of the downstream genes of Snf3 pathway results in the release of glucose repression on the assimilation of other carbon sources, as glucose is not sensed and Hxt transporters are not induced to transport this hexose sugar inside the cells causing subsequent repression events. In *K. lactis*, the disruption of *RAG4* causes a severe reduction in the expression of the low-affinity glucose permease (Rag1). Moreover, Rag4 is involved in glucose repression on lactose and galactose utilization, so in the disrupted mutant kl_rag4 the level of enzyme necessary for the utilization of these carbon sources (β -galactosidase) was

derepressed in presence of glucose (Betina et al., 2001). In contrast with this, Hua et al. 2019 reported that the disruption of the downstream genes of Snf3 or PKA signal pathway in *K. marxianus* does not release glucose repression on other carbon sources. The latter could be explained considering the difference we found in C-terminal of Snf3 between the four proteins, that could result in a different mechanism of sensing and pathway in the two yeasts. Indeed, since KMAR_60499 is the only Snf3/Rgt2 type of sensor in *K. marxianus*, it may have a dual function of signalling high and low concentrations of glucose. More studies would be needed to better investigate this first step of glucose sensing mechanisms in this non-conventional yeast.

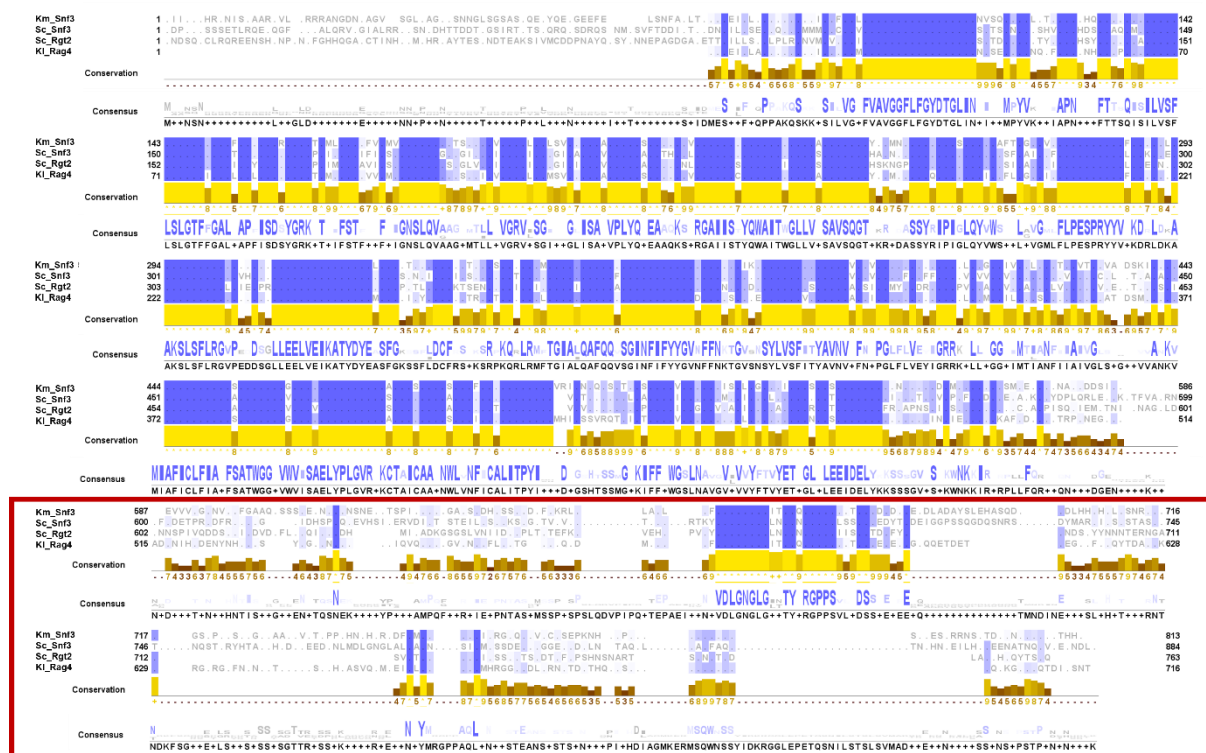


Figure 3.3: Amino acid alignments between Km_Snf3 (Kmar_60499), Sc_Snf3/Rgt2 and Kl_Rag4. Amino acid residues are coloured according to their percentage identity – in dark blue >80%, light blue >60%, white no identity. The consensus displays the most frequent residue in each column of the alignment. Highlighted in the red box, differences in the C-term of Km_Snf3, Sc_Snf3, Sc_Rgt2, Kl_Rag4, that could result in a different mechanism of action of the proteins.

Investigation of glucose repression on xylose utilization in *K. marxianus*

To examine the effect of glucose repression on pentose utilization, Km_*MIG1* and Km_*RAG5* disrupted mutants ($\Delta mig1$ and $\Delta rag5$) were constructed and then tested on solid media containing 2% glucose, xylose or arabinose supplemented with various concentrations of 2-DOG (0, 0.01, 0.1%) (Figure 3.4).

Mig1 is the target of Snf1 and its knock out is proven to shift the sugar preference from glucose to other carbon sources and metabolic patterns in *S. cerevisiae*, consequentially the disruption of *MIG1* in *K. marxianus* is expected to release glucose repression on xylose. Consistent with what was reported by Nurcholis et al. 2019 and Hua et al. 2019, the deletion of *MIG1* does not alleviate glucose repression on xylose or on arabinose in *K. marxianus*, indicating that there might be other signalling pathways that regulate glucose repression on xylose. In contrast, however, our results with $\Delta rag5$ dislike from those obtained by Hua et al. 2019. Surprisingly, the knockout of *Rag5* does not cause any phenotype change, showing a inhibition of the growth on xylose in presence of 2-DOG. RAG5 is described as the only hexokinase that can generate glucose repression-related events (Hua et al. 2019), but the fact that its deletion does not release glucose repression on xylose could be explained if glucose is phosphorylated by GLK1 causing the same repression signals. The latter should be further investigated.

As reported by Rajkumar et al., 2019a, among three xylose-inducible promoters (p*XYL1*, p*XYL2*, p*ALD4*), *XYL1* promoter (p*XYL1*) is the one less induced by the presence of xylose in the media and *XYL1* is the only gene involved in xylose metabolism that was previously shown to be repressed in presence of glucose (Rodrussamee et al. 2011). This reason together with the identification in *XYL1*pr of putative Mig1 binding sites, led us to replace the promoter of *XYL1* with a constitutive promoter. Strains in which the *XYL1* promoter was replaced by the constitutive promoters pPDC1 and pTEF1 (p*XYL1*-pPDC1 swap and p*XYL1*-pTEF1 swap) were constructed in *K. marxianus* NBRC1777 wt background and the mutants were tested on plates made as described above.

The swap of p*XYL1* with p*PDC1* causes an impairment of the growth on xylose and arabinose even in absence of 2-DOG, most probably due to the slight inhibition of *PDC1*pr in presence of xylose (Lertwattanasakul et al., 2015), while the *XYL1*pr swap with *TEF1*pr does not relieve glucose repression on xylose or arabinose when 2-DOG is added.

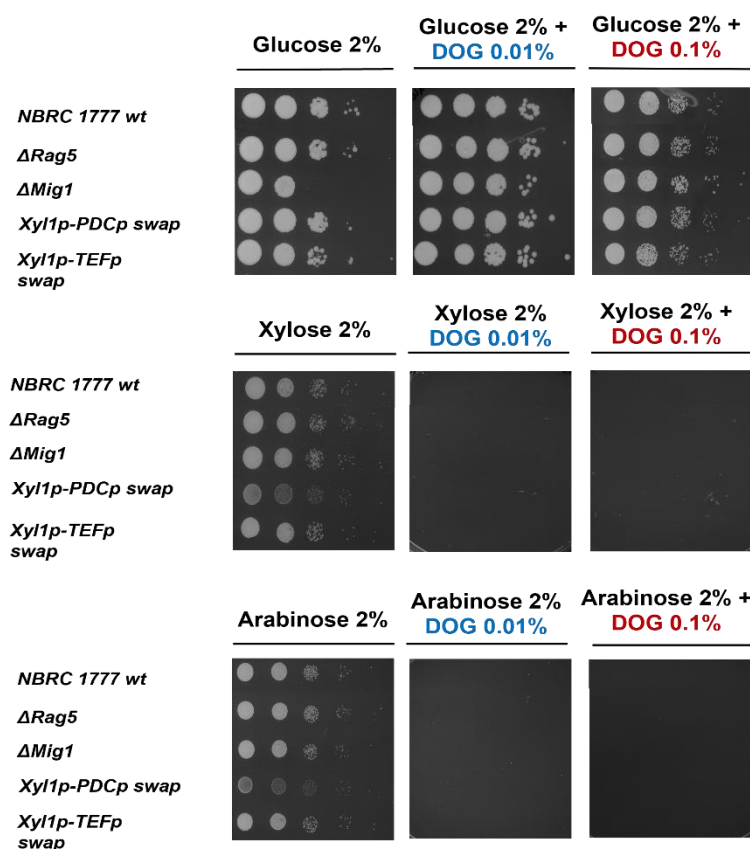


Figure 3.4: Glucose repression effect on different mutants. $\Delta mig1$ and $\Delta rag5$ lack Mig1 and Rag5 transcriptional factors, respectively. *XYL1p-PDC1p swap* and *XYL1p-TEF1p swap* are mutants in which the promoter of *Xyl1* gene was swapped with constitutive promoters *PDC1* and *TEF1*, respectively. *K. marxianus* NBRC1777 wt was used as control. The glucose repression effect was assessed checking the growth of mutants on MM glucose/xylose/arabinose 2% plates added with different concentrations of 2-DOG (none, 0.01%, 0.1%)

Glucose repression influence on xylose uptake in *K. marxianus*

One of the possible targets of glucose repression on other carbon sources is the sugar uptake. It is in fact known that pentose uptake mainly occurs by hexose transporters (Hxt and Hgt) and it is one of the main bottlenecks which prevents the simultaneous consumption of glucose and xylose, as glucose is the preferred substrate of the transporters and it enters first in the cell and starts the cascade of repression on alternative sugars. At the same time, the reduced intracellular accumulation of xylose, due to the transport inhibition, slows down metabolism, not activating xylose inducible pathways (Escalante-Chong et al., 2015; Farwick et al., 2014; Lane et al., 2018). The effect of glucose repression at the level of sugar import was investigated to identify potential pentose transporters not inhibited by glucose. Plasmids carrying individual hexose transporters (*HGTs* or *KHTs*) and the gene encoding the low-affinity xylose transporter *KMAR_60179* were previously constructed (Donzella et al., 2021), then transformed in *Kluyveromyces marxianus* Δ Pt platform strain and tested on solid media containing glucose, xylose or arabinose supplemented with 2-DOG at different concentrations (0, 0.01, 0.1%) (Figure 3.5).

The results showed large impairment in growth of the wild type strain (NBRC1777 wt) on xylose or arabinose added with 2-DOG because, in this strain, 2-DOG can enter inside the cell via several hexose transporters present (Hgt or Kht) resulting then toxic. The growth of this strain resulted reduced even on glucose when 2-DOG is added in higher concentration (0.1%) (Figure 3.6). The platform strain Δ Pt - which lacks all the main hexose transporters and is unable to grow on pentose (Donzella et al. 2021) - looks to be less sensitive to glucose repression because 2-DOG cannot enter in the cell. A slight background growth was observed for the empty Δ Pt strain in plates of xylose and arabinose + 2-DOG, but Δ Pt strain was shown unable to sustain growth in liquid culture either in presence or absence of 2-DOG (Donzella et al., 2021). When any of the hexose transporters, Hgt or Kht, were expressed in Δ Pt strain, a complete inhibition of the growth of these strains was observed on pentoses added with 2-DOG even in a minimal concentration (0.01%). The fact that Δ Pt expressing individual Hgt/Kht is inhibited in presence of 2-DOG indicates the ability of any Hgt and Kht of transporting 2-DOG. As glucose analogue, 2-DOG is taken up by Hgt and Kht hexose transporters but, having the 2-hydroxyl group replaced by hydrogen, it cannot undergo further glycolysis after its phosphorylation. In NBRC1777 wt and Δ Pt expressing individual Hgt/Kht strains, 2-DOG is transported inside the cells by one or more of this hexose transporters and it is accumulated as it cannot be metabolized, resulting toxic (O'Donnell et al., 2015; Soncini et al., 2020).

On the other hand, when the transporter Kmar_60179 is expressed in Δ PT, this strain resulted resistant to 2-DOG showing better growth both in plate and in liquid culture (Figure 3.7) on xylose with or without 2-DOG added in the media compared with the platform strain Δ PT and the wild-type strain NBRC1777. Consistent with the spotted test, the wild type strain NBRC 1777 is subjected to glucose repression, as no growth is observed on xylose in presence of 2-DOG, while Δ PT + KMAR_60179 shows a similar growth and xylose consumption profile on MM xylose with or without 2-DOG, with a slightly longer lag phase in presence of 2-DOG. Δ PT + KMAR_60179 lacks the majority of hexose/pentose transporters (Hgts, Khsts), so it doesn't allow the import of 2-DOG in the yeast cell which occurs through hexose transporters (consequently 2-DOG cannot exert glucose repression on the metabolism) and Kmar_60179 allows the uptake of xylose but not of 2-DOG, making it an interesting xylose transporter insensitive to carbon catabolite inactivation and not inhibited by glucose.

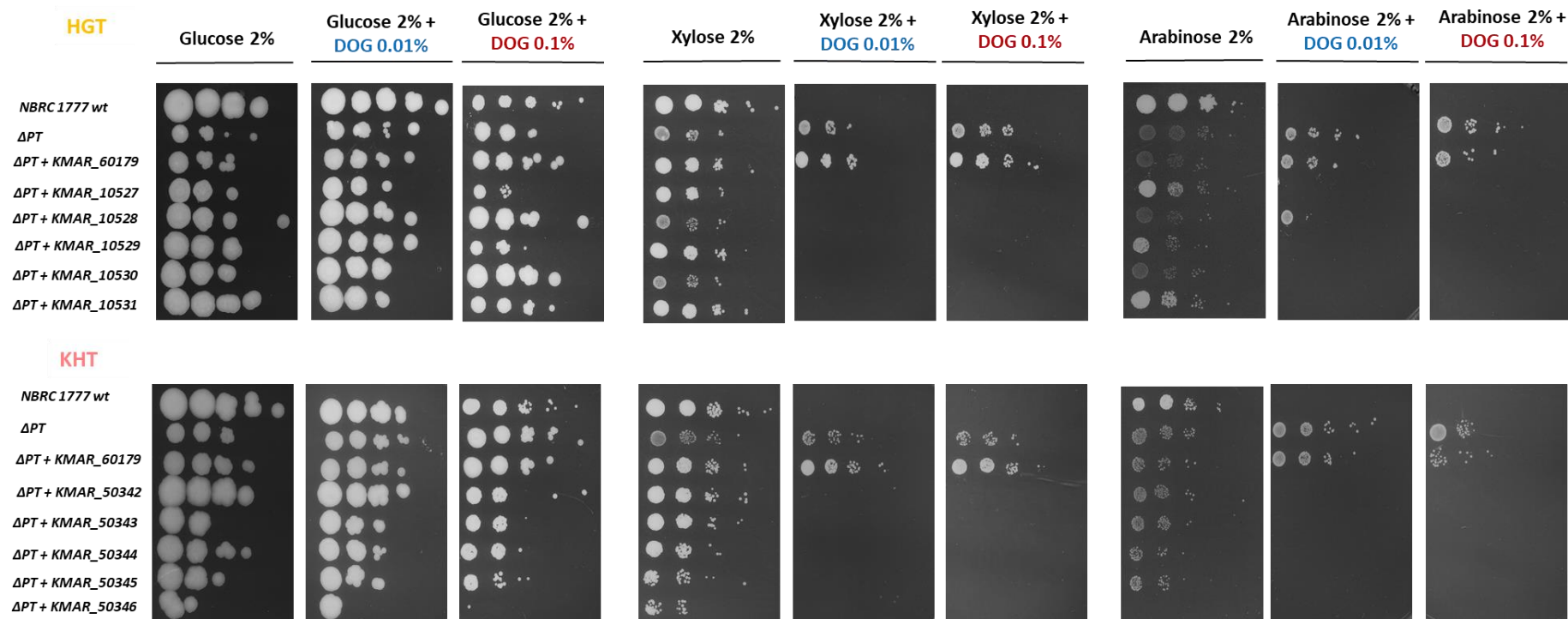


Figure 3.5: Glucose inhibition effect on pentose transporters. Spotted assay of Δ PPT expressing individual transporters (Hgt, Kht and Kmar_60179) on 2% glucose, xylose or arabinose added with various concentrations of 2-DOG (0, 0.01, 0.1%).

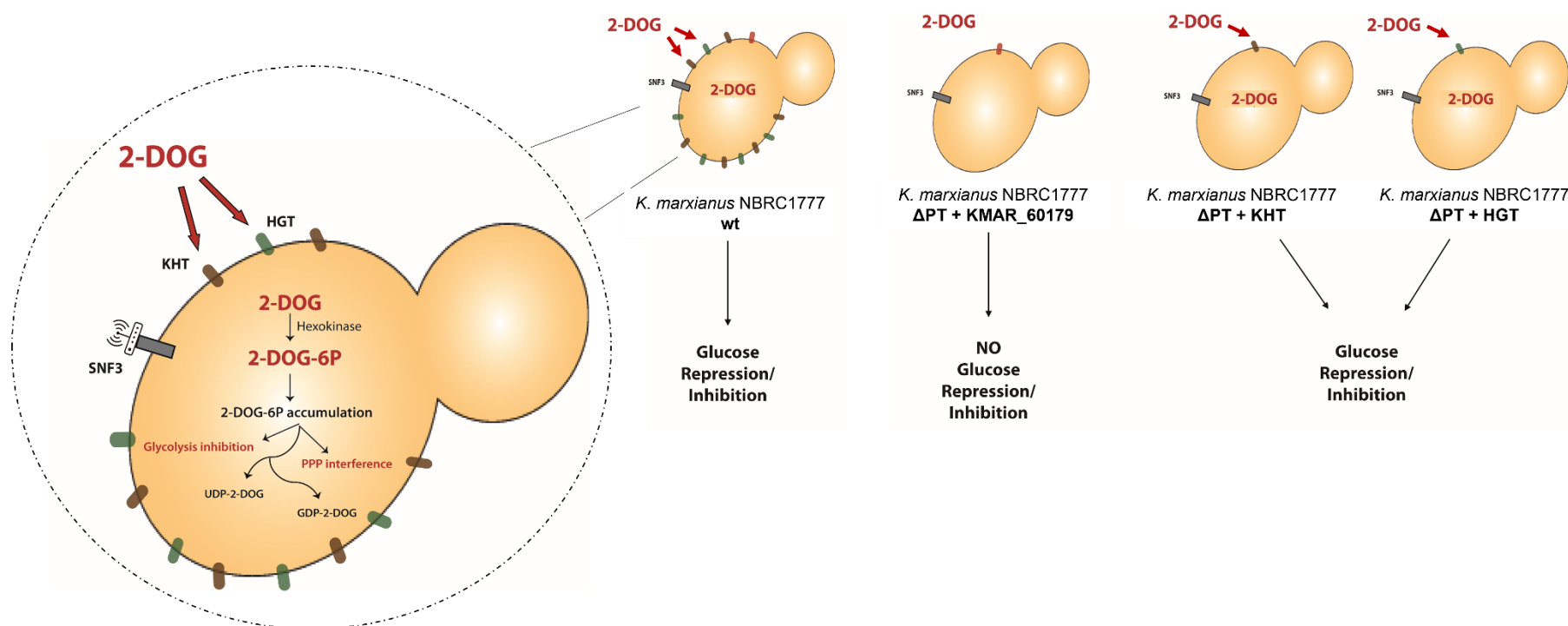


Figure 3.6: Schematic representation of glucose repression/inhibition on various strains grown on MM xylose added with 0.1% 2-DOG. Detail on mechanism of action of 2-DOG on *K. marxianus* NBRC1777 wt and Δ PT expressing individual HGTs and KHTs is reported in a dashed circle on the left. When 2-DOG enters in the cell via HGTs or KHTs, is phosphorylated in 2-DOG-6P and accumulates. It causes block of glycolysis, interferes with pentose phosphate pathway (PPP) and traps cellular uridine- or guanosine-nucleotide (UDP-2-DOG and GDP-2-DOG) required for correct cell wall synthesis leading to more fragile cell wall with consequent damage and inhibition of the growth. 2-DOG is not transported in the Δ PT platform strain or in Δ PT expressing only KMAR_60179.

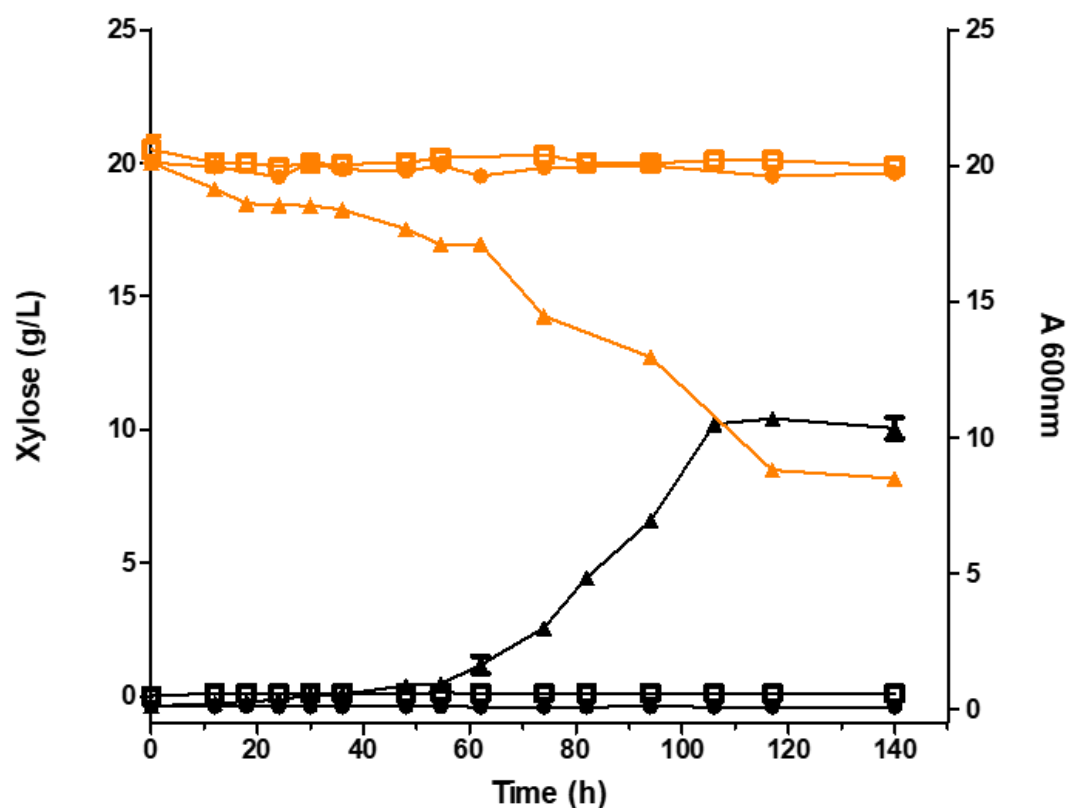


Figure 3.7: Liquid growth assay of NBRC 1777 wt (circles), ΔPT (open squares) and $\Delta PT+KMAR_60179$ (triangles) on MM xylose 2% + 2-DOG 0.1%. Growth and xylose consumption are respectively reported in black and orange lines.

Conclusions

Functional analysis of regulators involved in glucose repression has been extensively covered in *S. cerevisiae*, while in *K. marxianus* just a few studies have been conducted in this regard. In this work, we investigated glucose repression in *K. marxianus* with a perspective on its involvement in xylose metabolism and also studying glucose inhibition at pentose transporters level.

It was already shown that, in contrast with what observed in *S. cerevisiae*, the disruption of the downstream genes of SNF3 or PKA signal pathway couldn't alleviate glucose repression on other carbon sources in *K. marxianus* (Hua et al. 2019). Here we showed that the main difference between SNF3 in *S. cerevisiae* and its orthologous in *K. marxianus* (KMAR_60499) is in the C-term tail of the protein. It is known that the long C-term in Sc_SNF3 is involved in the glucose signalling pathway, explaining the putative different function of this protein in *K. marxianus*. Moreover, it was confirmed that Mig1 is not involved in the process of glucose repression on xylose utilization, but surprisingly from our result neither

the deletion of *Rag5* improves any growth on xylose when the glucose analogue 2-DOG is present, opening up the possibility that another glucose's phosphorylation mechanism can lead to the same repression signal.

It is known that one of the major bottlenecks for simultaneous consumption of glucose and xylose deriving from lignocellulosic biomass is the import of the sugars inside the cell. Most of the membrane sugar transporters have higher preference for glucose rather than for xylose and once glucose is inside the cell can exert glucose repression mechanisms often involving the endocytosis of transporters, leaving in this way xylose behind. In order to identify pentose transporters insensible to glucose inhibition, individual transporters were overexpressed in a *K. marxianus* platform strain previously constructed (Donzella et al. 2021) and the growth of the transformants was assessed on glucose, xylose and arabinose in presence of 2-DOG. All HGT and KHT transporters were shown to be sensible to glucose and could transport 2-DOG inside the cell causing the inhibition of the growth. On the other hand, one transporter, KMAR_60179, was not inhibited by glucose and conferred growth on xylose even in presence of 2-DOG, proving the fact that this transporter is specific for xylose and cannot carry glucose import. This data results relevant in our search for a specific xylose transporter not inhibited by glucose, which could enable xylose uptake simultaneous with glucose uptake and the co-consumption of both sugars at the same time, reducing the time of all the fermentation process. A further characterization based on bioinformatic analysis and radioactive glucose and xylose uptakes of all the identified *K. marxianus* pentose transporters is described in the next chapter.

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CHAPTER 4

Engineering a native *Kluyveromyces marxianus* transporter to reprogramme
the balance between hexose and pentose uptake

This chapter is in preparation for submission.

Abstract

Sustainable and efficient use of lignocellulosic hydrolysates in yeast-based biorefineries requires the simultaneous consumption of both the hexose and pentose sugars present in this feedstock. This represents a key bottleneck because of slow uptake of pentoses and the preferential use of glucose by most yeasts. We previously developed a pentose transporter screening platform in the pentose utilising yeast *K. marxianus* and used this to screen for native *K. marxianus* pentose transporters. We identified various high or low affinity transporters based on growth assays and now, in this study, we performed kinetic studies on two of the most interesting candidates using ^{14}C -labelled sugars. One of these transporters, KMAR_10531, showed high affinity for glucose and middle affinity for xylose, with the affinity for glucose 150-fold higher than for xylose. Using *in silico* analysis we selected target amino acids of this transporter for site-directed mutagenesis in an attempt to get a shift from glucose preference to xylose. Several mutants were constructed and again characterised by ^{14}C -labelled glucose and xylose uptake. Here, we describe a variant KMAR_10531 N³²⁵V which notably reduced the ability to transport glucose but still transported xylose with high affinity kinetics. This successful reprogramming of the transporter to increase its preference for xylose over glucose enabled efficient co-consumption of hexose and pentose sugars in an engineered *K. marxianus* strain.

4.1 Introduction

In the context of the circular economy, the utilisation of lignocellulosic biomass has attracted considerable attention as a sustainable alternative to fossil fuel in order to produce sustainable bio-based molecules by means of yeast cell factories. Lignocellulosic biomass (LCB) is mainly composed of the polysaccharides cellulose and hemicellulose and woody compound lignin, that go through a pre-treatment step and hydrolysis for the release of sugars. The resulting hydrolysate is a mixture of sugars - hexoses (mainly D-glucose – 60-70%) and pentoses (D-xylose 30-40% and L-arabinose ~5%) - that need to be completely utilized to develop profitable second generation biorefineries (Zabed et al. 2016). Most of the conversion processes of LCB into value-added products, like bio-ethanol, are carried on using the common *Saccharomyces cerevisiae*, however this yeast lacks the metabolic pathway for the assimilation of xylose and/or arabinose and specific pentose transporters to transport these sugars into the cells (Gopinarayanan et al. 2019, Patiño et al. 2019, Ochoa-Chacón et al., 2021). It is possible to engineer *S. cerevisiae* to utilize pentoses, but this still comes with substantial limitations as discussed below. Non-conventional yeasts offer desirable characteristics due to their unique biochemical properties and potential applications in biotechnological processes. Among them, the yeast *Kluyveromyces marxianus* is characterized by desired traits from the industrial point of view, like high thermotolerance, fast growth and natural ability to assimilate a broad range of substrates including xylose and arabinose. Although *K. marxianus* is provided with the key enzymes required for xylose and arabinose metabolism, it does not carry out simultaneous consumption (co-consumption) of glucose and pentoses, which is required by biorefineries to obtain a complete and economically feasible process. The major bottleneck of 2nd generation biomass sugars co-consumption is related to the competition for transporters. Indeed, the majority of MFS transporters display higher affinity for glucose and so, import of pentose sugars is observed only once glucose is depleted from the media, giving a typical diauxic growth and making the process time-consuming (Kim et al. 2012). In several studies, enhanced xylose uptake was obtained by engineering native *S. cerevisiae* hexose transporters to make them specific for pentoses (Hamacher et al. 2002, Sedlank et al., 2004, Young et al. 2014, Rojas et al. 2021), but heterologous expression of these transporters in new platform strains shows limited success due to poor expression and stability and/or low turnover (Young et al. 2011, Nijland et al. 2020, Sánchez et al. 2021). In a previous work, we identified a number of *K. marxianus* pentose transporters using a pentose-negative *K. marxianus* platform strain (Δ PT), obtained inactivating a collection of 12 different genes predicted to encode sugar transporters. These candidate transporters were then individually overexpressed in Δ PT strain and tested on different concentrations of xylose or arabinose to determine whether any could transport pentoses

(Donzella et al. 2021). Among all the identified transporters, two stood out either because of higher affinity for pentose sugars (KMAR_10531), or for its specificity for xylose (KMAR_60179). KMAR_10531 was able to recover growth of the Δ PT strain on both xylose and arabinose when present in the media in low (0.1%) and high (2%) concentration showing a high affinity for both sugars. On the other hand, KMAR_60179 was specific just for xylose but with a lower affinity – growth of Δ PT was recovered only with 2% xylose (Donzella et al. 2021). In the present work, we kinetically characterized these two native *K. marxianus* pentose transporters by radioactive uptake assays. After *in silico* studies of the binding site, we rationally engineered KMAR_10531 to increase its affinity for xylose and reduce its ability to uptake glucose, creating various mutants and targeting a positive mutation in a critical conserved asparagine residue in the transmembrane domain 8 (TMD8). Overexpressing this transporter in *K. marxianus* platform strain Δ PT we obtained a strain which allows co-consumption of xylose and glucose (see Chapter 5).

4.2 Materials and Methods

Strains, media and culture conditions

K. marxianus strains (Table 4.1) were routinely pre-cultured in YPLAC (yeast extract 10 g/L; peptone 20 g/L; 2% lactose) to avoid inducing glucose repression. For growth assays, yeast transformants were inoculated at A_{600nm} 0.1 in 250 mL shake flasks with 50 mL minimal medium MM (5 g L⁻¹ Urea, 3 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ MgSO₄·7H₂O, 100 µg mL⁻¹ vitamin solution, 100 µg mL⁻¹ trace elements Verduyn solution prepared as per Donzella et al. 2021), supplemented with various concentrations of the appropriate sugar (D, Glucose; X, Xylose) and grown at 30 °C with 200 rpm agitation. To screen for mutant transporters not inhibited by glucose, spot tests were carried on pre-growing the yeast strains on YPLAC overnight up to OD 2.0, washing twice and spotting in plates of MM supplemented with 2% glucose, xylose and arabinose adding increasing concentrations of 2-DOG (0, 0.1, 0.5%).

Precultures for growth assays were grown for 15 h on YPLAC media with G418 in 100 mL shake flasks at 30 °C and 200 rpm. Promising mutants were tested in flasks of 250 mL containing 50 mL of culture media of MM glucose or xylose 2%. The cultures were started at A_{600nm} of 0.1 and cultivated at 30 °C and 200 rpm, and the A_{600nm} was measured every 3-6 h for 75 h. Consumption of sugars remaining in the medium at each time point was measured by HPLC. 1.5 mL of culture were harvested by centrifugation and 800 µL of supernatant were injected into a Hewlett-Packard (HP) 1090 instrument using a Bio-Rad HPX-87H hydrogen ion resin column and an HP 1047A external refractive index detector. The mobile phase, 0.001 N H₂SO₄, was run at 55 °C at a flow rate of 0.6 ml min⁻¹.

Table 4.1: Strains used in this study. Mutation coordinates for *K. marxianus* ΔPT are reported in Chapter 2.

Strain	Genotype	Parental strain	Origins
<i>K. marxianus</i> NBRC1777	Wild-type strain		NITE Biological Research Centre, Japan
ΔPT	$\Delta hgt \Delta kht \Delta kmar_{60179}$	<i>K. marxianus</i> $\Delta hgt \Delta kht$	Donzella et al. 2021
$\Delta PT + KMAR_{10531}$	$\Delta hgt \Delta kht \Delta kmar_{60179+}$ pTU- DO-G418- 10531	<i>K. marxianus</i> ΔPT	This study
$\Delta PT + KMAR_{60179}$	$\Delta hgt \Delta kht \Delta kmar_{60179+}$ pTU- DO-G418- 60179	<i>K. marxianus</i> ΔPT	This study
$\Delta PT + Sc_GAL2$	$\Delta hgt \Delta kht \Delta kmar_{60179+}$ pTU- DO-G418- <i>Sc_GAL2</i>	<i>K. marxianus</i> ΔPT	This study
$\Delta PT + KMAR_{10531}$ N³²⁵F	$\Delta hgt \Delta kht \Delta kmar_{60179+}$ pTU- DO-G418- 10531 N ³²⁵ F	<i>K. marxianus</i> ΔPT	This study
$\Delta PT + KMAR_{10531}$ N³²⁵V	$\Delta hgt \Delta kht \Delta kmar_{60179+}$ pTU- DO-G418- 10531 N ³²⁵ V	<i>K. marxianus</i> ΔPT	This study
$\Delta PT + KMAR_{10531}$ N³²⁵T	$\Delta hgt \Delta kht \Delta kmar_{60179+}$ pTU- DO-G418- 10531 N ³²⁵ T	<i>K. marxianus</i> ΔPT	This study
$\Delta PT + KMAR_{10531}$ G³⁹F	$\Delta hgt \Delta kht \Delta kmar_{60179+}$ pTU- DO-G418- 10531 G ³⁹ F	<i>K. marxianus</i> ΔPT	This study
$\Delta PT + KMAR_{10531}$ L⁴⁰F	$\Delta hgt \Delta kht \Delta kmar_{60179+}$ pTU- DO-G418- 10531 L ⁴⁰ F	<i>K. marxianus</i> ΔPT	This study
$\Delta PT + KMAR_{10531}$ L⁴⁰F-F⁴²M	$\Delta hgt \Delta kht \Delta kmar_{60179+}$ pTU- DO-G418- 10531 L ⁴⁰ F-F ⁴² M	<i>K. marxianus</i> ΔPT	This study
$\Delta PT + KMAR_{10531}$ L⁴⁰F-M⁴¹L	$\Delta hgt \Delta kht \Delta kmar_{60179+}$ pTU- DO-G418- 10531 L ⁴⁰ F-M ⁴¹ L	<i>K. marxianus</i> ΔPT	This study

Protein structure prediction

Secondary structure of KMAR_10531 was predicted and visualized using the Protter program (Omasits et al. 2014). 3-D protein structure prediction of KMAR_10531 was obtained using the I-TASSER online platform from the Yang Zhang's Research Group and it was studied comparing it to the structural template XylE (PDB ID: 4GBZ). The XylE structure was obtained from the crystal structure of the transporter complexed with xylose or glucose, giving important information about substrate binding. XylE belongs to the major facilitator superfamily (MFS) and exhibits high structural similarity to known hexose transporters. The 3D coordinates of XylE structures bound to glucose or xylose (4GBZ and 4GBY respectively) are retrieved from the protein databank PDB. Figures were prepared with PyMol v2.3.

Site direct mutagenesis of KMAR_10531

Two different site-direct mutagenesis methods using overlap PCR were used to generate specific mutations of KMAR_10531. The first approach consisted in the amplification of the cassette pTEF1-KMAR_10531-CYC1TT from the plasmid pTU-DO-G418-10531 with two sets of primers carrying the desired mutation (Figure 4.1A). The two PCR fragments that were obtained were first assembled to a level I plasmid YTK001 via Golden Gate cloning and then the entire cassette carrying the mutation was assembled again into the backbone pTU-DO-G418.

Several of the mutants were constructed using a more rapid approach as described by Rendulić et al. 2021. In this case, the entire pTEF1-KMAR_10531-CYC1TT plasmid was amplified with ACCUZYME MIX (Bioline, London, UK) using overlapping forward and reverse primers carrying the desired mutation (Soares-Silva et al. 2009, Rendulić et al. 2021) (Figure 4.1B). The resulting PCR product was then treated with DpnI, a restriction enzyme that cleaves only when its recognition site (5'-GATC-3') is methylated, which occurs in bacteria, and therefore destroys the template plasmid but not the PCR-amplified version. The treated mix was transformed into competent *E. coli* cells, which were then plated on LB agar plates + 100 µg/ mL Ampicillin for 24-48h. Colonies were grown overnight, plasmids extracted with Sigma GenElute™ Miniprep Kit and checked for the mutation by sequencing, carried out by YouTubeIT service - Stab Vida. Finally, the *K. marxianus* platform strain ΔPT was transformed with the positive plasmids carrying the native and mutated *K. marxianus* transporters and Sc_Gal2 and empty plasmid as controls, and plated on YPLAC supplemented with G418 for resistance following a variant of LiAc/SS carrier DNA/PEG method (Gietz and Schiestl, 2007). The primers, plasmids used in this study and the created mutants are reported in Table 4.2, 4.3 and Table 4.4 respectively.

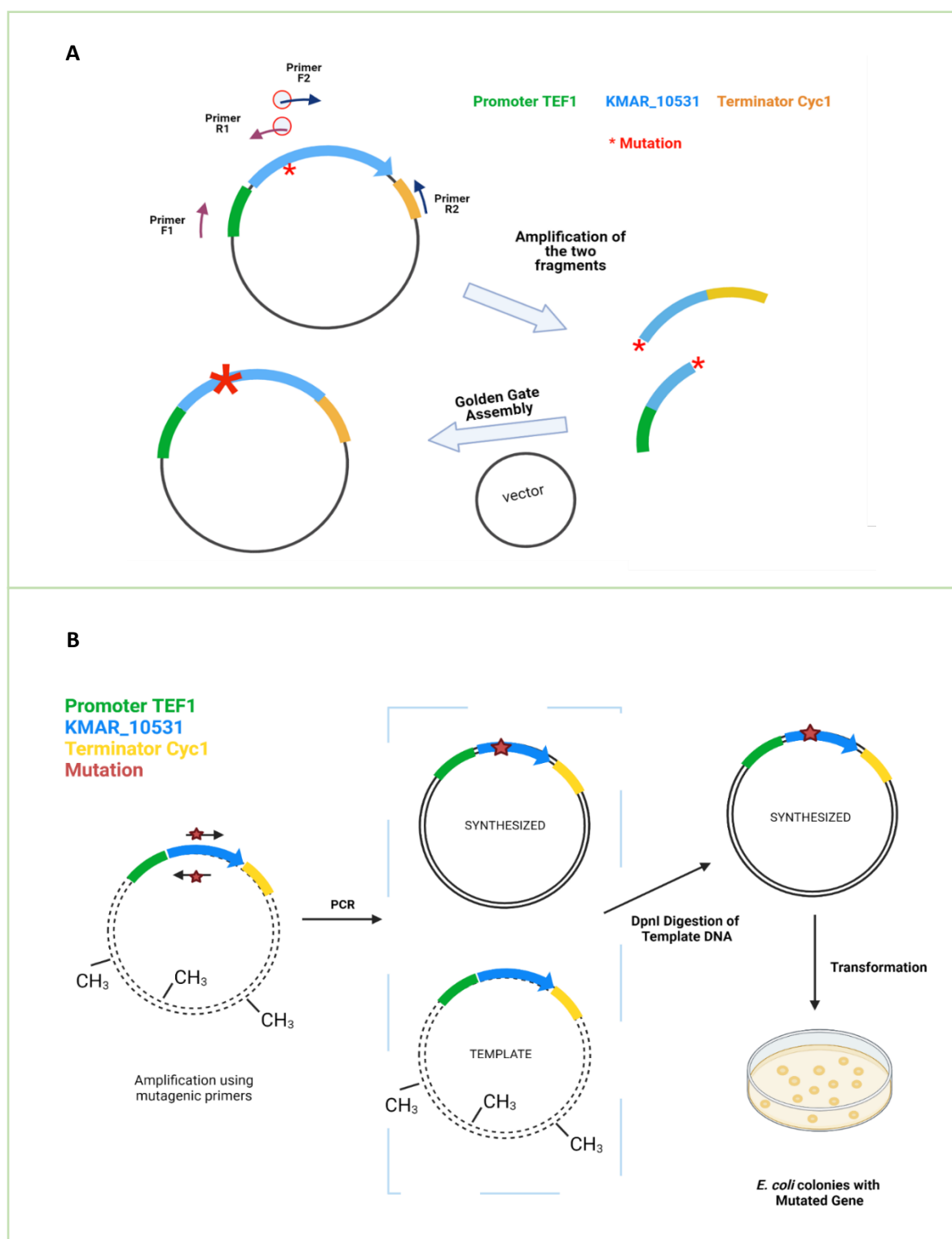


Figure 4.1: A. Rational mutagenesis approach n.1 The amplification of the cassette pTEF1-KMAR_10531-CYC1TT from the plasmid pTU-DO-G418-10531 was performed with two sets of primers carrying the desired mutation in the gene. The two obtained fragments were then assembled via Golden Gate to the backbone pTU-DO-G418. **B. Rational mutagenesis approach n.2** The entire pTEF1-KMAR_10531-CYC1TT plasmid was amplified using overlapping forward and reverse primers carrying the desired mutation. The resulting PCR product was then treated with DpnI, which digests the methylated template plasmid. The new synthesized plasmid carrying the mutation is finally transformed into competent *E. coli* cells.

Table 4.2: Primers used for rational mutagenesis. *Primers have been used in the first approach of rational mutagenesis. **Primers used in the second type approach of rational mutagenesis. In red are reported the nucleotides changed to obtain the desire mutation. Primers 10531_diagF_Id and 10531_diagR_Id have been used to check the mutations.

Primer	Sequence
*YTK_10531_F	GCATCGTCTCATCGGTCTCATATGTCATTGAAAGACAAGATTTTG
*YTK_10531_R	ATGCCGTCTCAGGTCTCAGGATTAGTTAGAGTTTGAGTTTGAGTT
*10531_G39-F_R1	CACGTCTCA GA AGGAAATACATGAGATCAAAC
*10531_G39-F_F2	TTCGTCTCAC TT CCTTATGTTCCGTTTTGATA
**10531_N325-F_Forw	ATTCAATACGTCTTG TC GTGCTAATGACTATTCC
**10531_N325-F_Rev	GGAATAGTCATTAGCAC GAA CAAGACGTATTGAAT
**10531_N325-V_Forw	ATTCAATACGTCTTG GT GTGCTAATGACTATTCC
**10531_N325-V_Rev	GGAATAGTCATTAGCAC CAC CAAGACGTATTGAAT
**10531_N325-T_Forw	ATTCAATACGTCTTG ACT GTGCTAATGACTATTCC
**10531_N325-T_Rev	GGAATAGTCATTAGCAC AGT CAAGACGTATTGAAT
**10531_L40-F_Forw	ATCTCATGTATTTCCGGT TT CATGTTCCGTTTTGA
**10531_L40-F_Rev	TCAAAACCGAACAT GAA ACCGGAAATACATGAGAT
**10531_L40F+F42M_Forw	TCATGTATTTCCGGT TT CATG ATG GGTTTTGATATC
**10531_L40F+F42M_Rev	GATATCAAAAC CAT CAT GAA ACCGGAAATACATGA
**10531_L40-F+M41-L_Forw	CTCATGTATTTCCGGT TT C CTT TTCCGTTTTGATA
**10531_L40-F+M41-L_Rev	TATCAAAACCGAA AAGGAA ACCGGAAATACATGAG
10531_diagF_Id	TTTGCTTCGTAATATTAATTACCAGGG
10531_diagR_Id	GATTGACTGTTAGAGTCAAGGTTAGA

Table 4.3: Complementation plasmids used in this study.

Complementation plasmid	Relevant characteristics	Source
pTU-D0-G418	ampR KmARS/CEN7 KanMX Bsal sfGFP Bsal	Rajkumar and Morrissey, 2020
pTU-D0-G418- 10531	pTEF1-KMAR_10531-CYC1 TT	Donzella et al. 2021
pTU-D0-G418- 60179	pTEF1-KMAR_60179-CYC1 TT	Donzella et al. 2021
pTU-D0-G418- Sc_GAL2	pTEF1-Sc_GAL2-CYC1 TT	Donzella et al. 2021
pTU-D0-G418- 10531 N³²⁵F	pTEF1-KMAR_10531 N ³²⁵ F-CYC1 TT	This study
pTU-D0-G418- 10531 N³²⁵V	pTEF1-KMAR_10531 N ³²⁵ V-CYC1 TT	This study
pTU-D0-G418- 10531 N³²⁵T	pTEF1-KMAR_10531 N ³²⁵ T-CYC1 TT	This study
pTU-D0-G418- 10531 G³⁹F	pTEF1-KMAR_10531 G ³⁹ F-CYC1 TT	This study
pTU-D0-G418- 10531 L⁴⁰F	pTEF1-KMAR_10531 L ⁴⁰ F -CYC1 TT	This study
pTU-D0-G418- 10531 L⁴⁰F-F⁴²M	pTEF1-KMAR_10531 L ⁴⁰ F-F ⁴² M -CYC1 TT	This study
pTU-D0-G418- 10531 L⁴⁰F-M⁴¹L	pTEF1-KMAR_10531 L ⁴⁰ F-M ⁴¹ L -CYC1 TT	This study

Table 4.4: List of KMAR_10531 mutants constructed in this study. The position of the targeted residues and the replacing amino acids (last column) are reported in single letter code (N asparagine, G glycine, L leucine, F phenylalanine, M methionine, V valine, T threonine).

Transporter	Position of targeted residues	Site-direct mutagenesis
KMAR_10531	N ³²⁵	F/V/T
	G ³⁹	F
	L ⁴⁰	F
	L ⁴⁰ F ⁴²	F ⁴⁰ M ⁴²
	L ⁴⁰ M ⁴¹	F ⁴⁰ L ⁴¹

Radiolabelled sugars uptake assays

Cells were grown in YPLAC medium G418 (200 mg/L) for plasmid maintenance. Cells were collected after 24 h in mid-growth phase and washed twice before suspension in water at 30-40 mg dry cell weight/mL (DCW/mL). The assays were performed as following: 20 μ L of yeast suspension (30-40 mg DCW/mL) was transferred to microtubes containing 20 μ L of potassium phosphate buffer 100 μ M, pH 5.0, and incubated for 2 min at 30 °C. The reaction was started by adding 10 μ L of an aqueous solution of the labelled substrate (glucose or xylose), incubated at 30 °C for 20s and stopped by adding 0.5 mL of cold glucose/xylose 500 mM. The microtubes with stopped reaction mixtures were centrifuged for 5 min at 13000 rpm, 4 °C, the supernatant was discarded, and the cell pellet was resuspended in ice-cold deionized water (1 mL) and centrifuged again. The supernatant was discarded, and the cell pellet was resuspended in 1 mL of scintillation liquid (Opti-Phase HiSafe II; LKB FSA Laboratory Supplies, Loughborough, UK). Radioactivity was measured in a Packard Tri- Carb 2200 CA liquid scintillation spectrophotometer with disintegrations per minute correction. The following radioactive labelled substrates were utilized: D-[U-¹⁴C] Glucose (Amersham Biosciences, 287 mCi/mmol) and D-[U-¹⁴C] Xylose (Moravek, Inc., 51.3 mCi/mmol). All the measurements were performed in triplicate. Assays were performed in 20s to maintain initial uptake rates after appropriate experiments ensured that uptake was linear for at least 1 min. Transport activity is described as nmole labelled sugar transported/mg dry cell weight/ min. The transport kinetics best fitting the experimental initial uptake rates and the kinetic parameters, were determined by a computer-assisted non-linear regression analysis (GraphPAD Software version 4.00, San Diego, CA, USA).

Competition assays

The inhibiting effect of xylose and arabinose on glucose transport was determined carrying out competition experiments by measuring the uptake of glucose at each concentration (0.1, 0.5, 1, 2.5, 4, 8, 16 or 32 mM) in the presence of an excess of the other unlabelled sugar (150 mM). Calculation of the apparent K_m (Michaelis constant) and V_{max} (maximal initial uptake velocity) was done by nonlinear regression analysis and global curve fitting in GraphPad Prism v. 8.0.0 (GraphPad Software, San Diego, California USA) with values of three independent measurements.

3.3 Results

Glucose and xylose consumption by two promising native transporters in mixture-sugars medium

We have previously constructed a *K. marxianus* strain (platform strain Δ PT) incapable of growing on xylose (Donzella et al. 2021). We found that reintroduction of two *K. marxianus* transporters, KMAR_10531 and KMAR_60179 into the Δ PT strain could recover growth on xylose. Now, we were interested in assessing the behaviour of these strains in mixed glucose and xylose medium and understanding how xylose consumption is affected by the presence of the glucose. Therefore, the two mutants were tested in a liquid MM medium with mixture of sugars – xylose 2% + glucose 0.1% (Figure 4.2).

Δ PT + KMAR_10531 consumed glucose within 12 h and then it started depleting xylose, using it completely in 72 h. In contrast, Δ PT + KMAR_60179 took almost 30 h to deplete glucose and then xylose started to be used until 72 h when its consumption stopped and 10 g/L sugar remained in the medium. These data suggested that KMAR_10531 could carry out xylose transport with high affinity and that KMAR_60179 seemed to be a low affinity xylose transporter and showed that both strains preferred glucose over xylose.

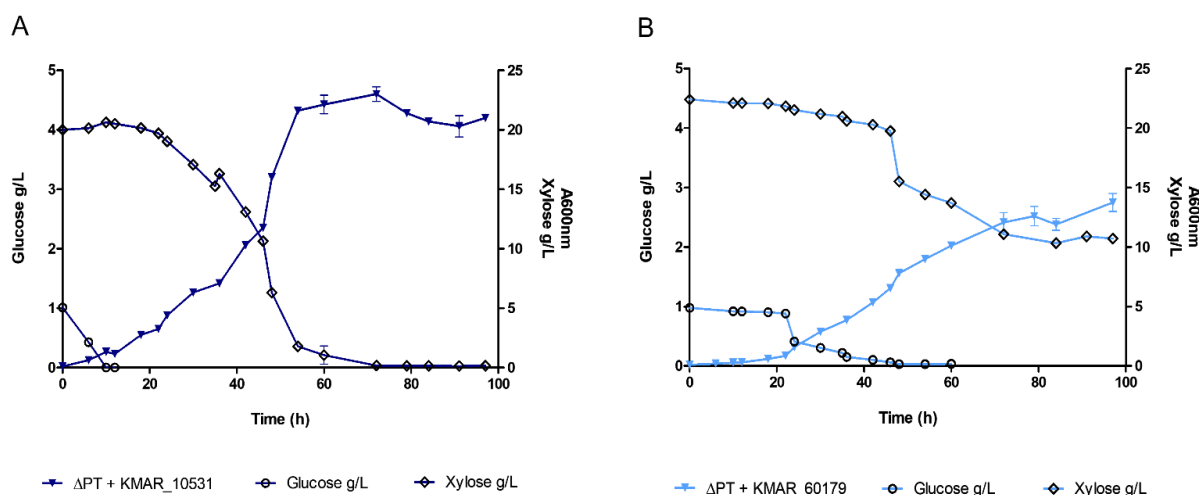


Figure 4.2: Growth assays and sugars consumption of Δ PT + KMAR_10531 (A) and Δ PT + KMAR_60179 (B) on MM xylose 2% + glucose 0.1%. Yeast strains are reported in triangles, xylose in open diamonds and glucose in open circles.

Since it was known from the previous study that the platform Δ PT strain retained some residual activity to transport glucose, to assess if the consumption of glucose observed in the mixed sugar medium was due to the activity of the KMAR_10531 and KMAR_60179 transporters or to another transporter still present in our platform strain, the Δ PT + KMAR_10531 and Δ PT + KMAR_60179 strains were grown in MM 2% glucose along with the empty platform strain Δ PT and the NBRC1777 wt strain (Figure 4.3). In these assays, the Δ PT and the Δ PT + KMAR_60179 strains grew identically, showing that KMAR_60179 did not give any additional capacity to transport glucose transport. In contrast, the Δ PT + KMAR_10531 strain grew almost as well as the wild type, demonstrating that KMAR_10531 is an efficient glucose transporter and the rapid glucose consumption that was seen in Figure 4.2 was due to the activity of KMAR_10531.

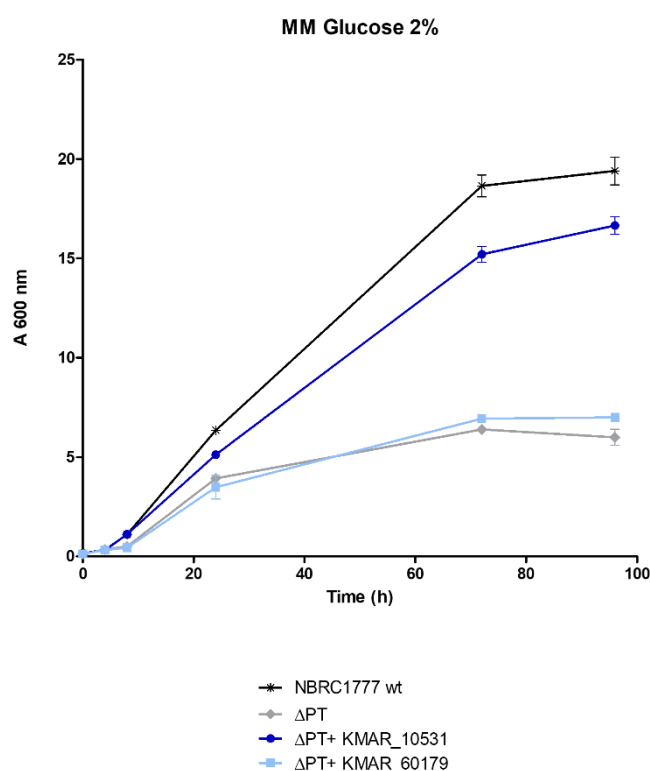


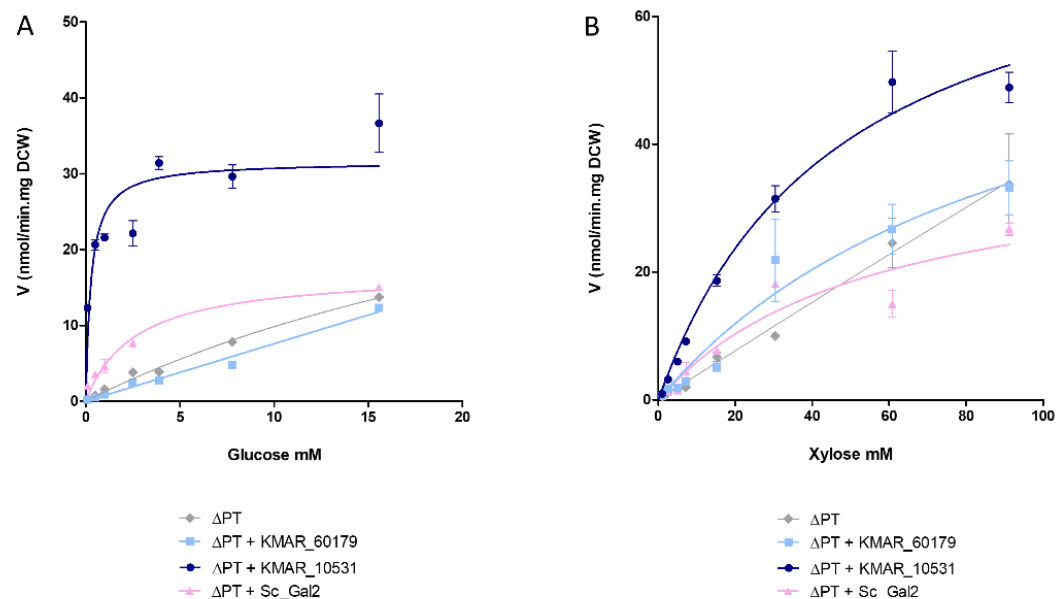
Figure 4.3: Growth assays of *K. marxianus* NBRC1777 wt and Δ PT expressing promising transporters on MM glucose 2%. Wt strain is reported in black line, Δ PT in grey line, Δ PT + KMAR_10531 in blue line and Δ PT + KMAR_60179 in light blue line.

Glucose and xylose uptake of KMAR_10531 and KMAR_60179 native transporters

To characterize the glucose and xylose import activity of KMAR_10531 and KMAR_60179 transporters, next we performed sugar uptake assays with radiolabelled substrates. *K. marxianus* Δ PT host strains individually overexpressing each of these transporters were grown on YPLAC media, yeast cells were harvested after 24 h, and then used for D-(U-14C)-Glucose or D-[U-14C]-Xylose uptakes assays. In these assays, we used glucose concentrations between 0.1 and 15 mM and xylose concentrations between 0.1 and 96 mM. *K. marxianus* Δ PT was also transformed with Sc_Gal2 and an empty vector to use as controls (Figure 4.4).

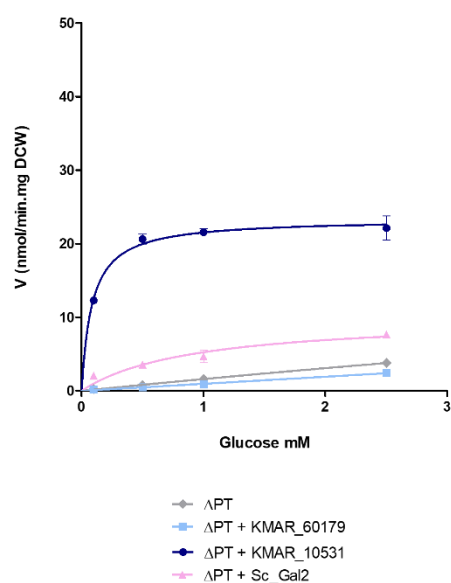
The kinetics of the platform strain Δ PT and the Δ PT overexpressing KMAR_60179 showed a slight background D-glucose uptake, consistent with the poor growth observed on this carbon source, typical of diffusion or very low affinity transport (Figure 4.4 A). KMAR_60179 overexpressed in *K. marxianus* Δ PT showed very low affinity xylose component with a K_m of 98 ± 58 mM and a V_{max} of 70.1 ± 25.5 nmol/mg/min (Figure 4.4 B). The kinetics of glucose transport of *K. marxianus* Δ PT overexpressing KMAR_10531 showed that this transporter mediates high affinity glucose transport, with best saturation kinetics in the range 0.1 – 2.5 mM, and showed a K_m of 0.28 ± 0.1 mM and a V_{max} of 31.6 ± 1.9 nmol/mg/min (Figure 4.4 A and 4.5). On the other hand, xylose uptake kinetics of Δ PT +KMAR_10531 showed a K_m 46.9 ± 9.5 mM and a V_{max} comparable to KMAR_60179 (79.4 ± 7.6 nmol/mg/min), confirming that this transporter is able to uptake xylose with low affinity (Figure 4.4 B).

In order to verify the platform strain Δ PT, we overexpressed Sc_Gal2 and analysed glucose and xylose uptakes. As expected, Gal2 showed to be a good glucose transporter with K_m and V_{max} parameters comparable with the ones reported in literature (2.6 ± 0.6 mM and 17.1 ± 1.6 nmol/mg/min respectively) and a middle-low affinity xylose transporter (K_m 56 ± 25 mM and V_{max} 39.3 ± 8.8 nmol/mg/min) (Fig. 4.4). The differences in transport parameters of Gal2 for xylose obtained in this study when compared the ones reported in literature (Kuanyshev et al., 2021, Farwick et al 2014), could be attributed either to slightly different behaviour of Gal2 when expressed in *K. marxianus*, or to the differences in the range of xylose concentrations tested. In fact, the technical difficulty to reach higher concentrations than 96 mM of the D-[U-14C]-Xylose due to the low specific activity of the stock, results in being in a range below the saturation concentration for KMAR_60179 and Sc_Gal2, making a precise estimation of the K_m for xylose complicated for these transporters, resulting also in the higher standard deviations shown in the table (Fig. 4.4B).



		ΔPT	ΔPT + KMAR_10531	ΔPT + KMAR_60179	ΔPT + Sc_Gal2
A. Glucose uptake (0-15 mM)	Vmax	Ambiguous	31.6 ± 1.9	Ambiguous	17.1 ± 1.6
	Km	Ambiguous	0.28 ± 0.1	Ambiguous	2.6 ± 0.6
B. Xylose uptake (0.1-96mM)	Vmax	Ambiguous	79.4 ± 7.6	70.1 ± 25.5	39.3 ± 8.8
	Km	Ambiguous	46.9 ± 9.5	97.6 ± 58.2	56.3 ± 25.2

Figure 4.4: Radiolabeled substrate uptake of promising native transporters. Michaelis-Menten plot of ^{14}C -Glucose (**A**) and ^{14}C -Xylose (**B**) initial uptake rates in cells of *K. marxianus* ΔPT transformed with KMAR_10531 (in dark blue), KMAR_60179 (in light blue), Sc_Gal2 (in pink) and empty plasmid used as control (in grey). The velocity is reported as nmol sugar taken up per minute per mg cell dry weight (nmol min⁻¹ mgCDW⁻¹). Mean values and standard deviation of triplicate measurements are shown. The kinetic values (Km and Vmax) are reported in the table below. “Ambiguous” is a term coined by GraphPad to describe input data that did not fit in the Michaelis-Menten model.



		Δ PT	Δ PT + KMAR_10531	Δ PT + KMAR_60179	Δ PT + Sc_Gal2
A. Glucose uptake (0-2.5 mM)	Vmax	Ambiguous	23.4 $\pm$ 0.7	Ambiguous	10.1 $\pm$ 1.9
	Km	Ambiguous	0.09 $\pm$ 0.01	Ambiguous	0.9 $\pm$ 0.4

Figure 4.5: Detail on the ^{14}C -Glucose uptake in a range between 0.1 and 2.5 mM glucose. Michaelis-Menten plot of ^{14}C -Glucose (A) and ^{14}C -Xylose (B) initial uptake rates in cells of *K. marxianus* Δ PT transformed with KMAR_10531 (in dark purple), KMAR_60179 (in light blue), Sc_Gal2 (in pink) and empty plasmid used as control (in grey). The velocity is reported as nmol sugar taken up per minute per mg cell dry weight ($\text{nmol min}^{-1}\text{mgCDW}^{-1}$). Mean values and standard deviation of triplicate measurements are shown. “Ambiguous” is a term coined by GraphPad to describe input data that did not fit in the Michaelis-Menten model.

Construction of KMAR_10531 mutants with higher affinity for xylose

Though *K. marxianus* KMAR_10531 was the best transporter in terms of xylose uptake, it still prefers glucose having a much higher affinity for this sugar. In order to get a shift from glucose preference to xylose, we studied the 3D-protein structure of KMAR_10531 obtained by modelling on the structural template of XylE (PDB ID: 4GBZ) looking for potential amino acid residues involved in the binding site and/or preference of sugars (Figure 4.6). Then, we used site direct mutagenesis approaches to try to decrease the affinity of KMAR_10531 for glucose and favour *K. marxianus* xylose transport.

KMAR_10531 comprises 562 amino acids predicted to be arranged in 12 transmembrane domains by TMHMM 2.0 with the typical structure of Major Facilitator Superfamily (MFS) members (Figure 4.6A). It

shows in the first transmembrane domain TMD1 a conserved sequence motif involved in sugar specificity. It is in fact known that this TMD1 specific conserved protein motif could be modified to rewire yeast sugar transporter preference (Young et al., 2014). We conducted a site direct mutagenesis in some residues of this conserved motif $S^{38}G^{39}L^{40}M^{41}F^{42}$ in KMAR_10531 in an effort to change sugar selectivity. We mutated the small non-polar residue Glycine (G)³⁹ and the positive charged residue Leucine (L)⁴⁰ independently to a bulky aromatic residue Phenylalanine (F), that we predicted from the homology model that could cause steric effect on hexoses, inhibiting their transport. We also changed Phenylalanine (F)⁴², to a Methionine (M) as this specific change had been reported to attenuate growth on glucose (Young et al., 2014). Two combinations of mutations in this conserved motif were tested as well: $L^{40}F-M^{41}L$ and $L^{40}F-F^{42}M$. The seven created mutants are reported in Table 4.4.

Separate to the domain linked to sugar specificity in TMD1, some residues in TMD8 are reported to be essential for the inhibition of xylose transport by glucose in *S. cerevisiae* hexose transporters (Farwick et al., 2014; Nijland et al., 2020; Shin et al., 2017; Vasylyshyn et al., 2020; Zhang et al., 2016). In *S. cerevisiae* Gal2, mutating Asparagine ³⁷⁶ (N) to Phenylalanine (F) results in the complete abolition of glucose transport and increased affinity for xylose. Therefore, we chose to replace the residue $N^{376/325}$ in KMAR_10531 with a (F) and we also evaluated two other possible substituents at this position: Valine (V) and Threonine (T).

We individually cloned each KMAR_10531 mutant into ΔPT and grew the strains on MM xylose for 140 h to evaluate differences in the growth on this sugar (Figure 4.7). The mutation $G^{39}F$ results in an impairment of the growth on xylose while the mutations $L^{40}F$, $L^{40}F-F^{42}M$ and $N^{325}T$ showed the same growth tendency on xylose characterized by a longer lag phase compared with the wild type transporter. On the other hand, the mutants $L^{40}F-M^{41}L$, $N^{325}F$, $N^{325}V$ grew at approximately the same rate as the wild type strain, but the $L^{40}F-M^{41}L$ mutant reached a lower final biomass.

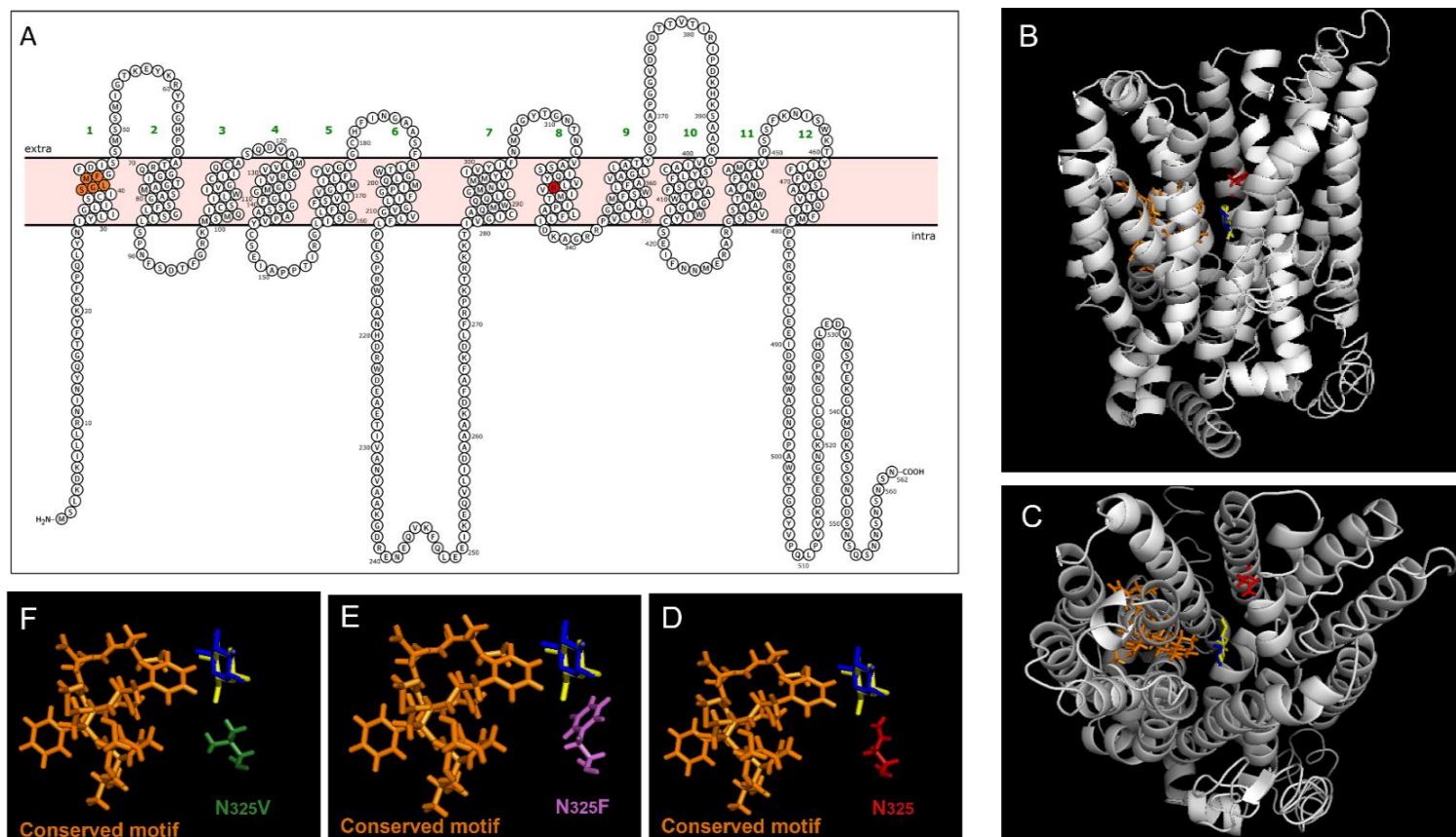


Figure 4.6: Predicted secondary and 3D structures of KMAR_10531. The protein consists in 562 aminoacids distributed in 12 transmembrane domains. The residues considered for mutagenesis are showed in red and orange (A). Cartoon representation of KMAR_10531 structure modelled to the XylE (I-TASSER), view parallel to the membrane (B) and perpendicular (C). Ligands, glucose and xylose, are respectively reported in yellow and blue. The conserved motif 38-SGLMF-42 of the first transmembrane span TM1 is reported in orange and a promising residue N325 present in TM8 in red. Figures were prepared with program Pymol v.2.3 (DeLano 2002). (D) Promising mutations in Asparagine residue (in red) in position 325 – substitution with Phenylalanine is reported in purple (E) and with Valine is reported in green (F).

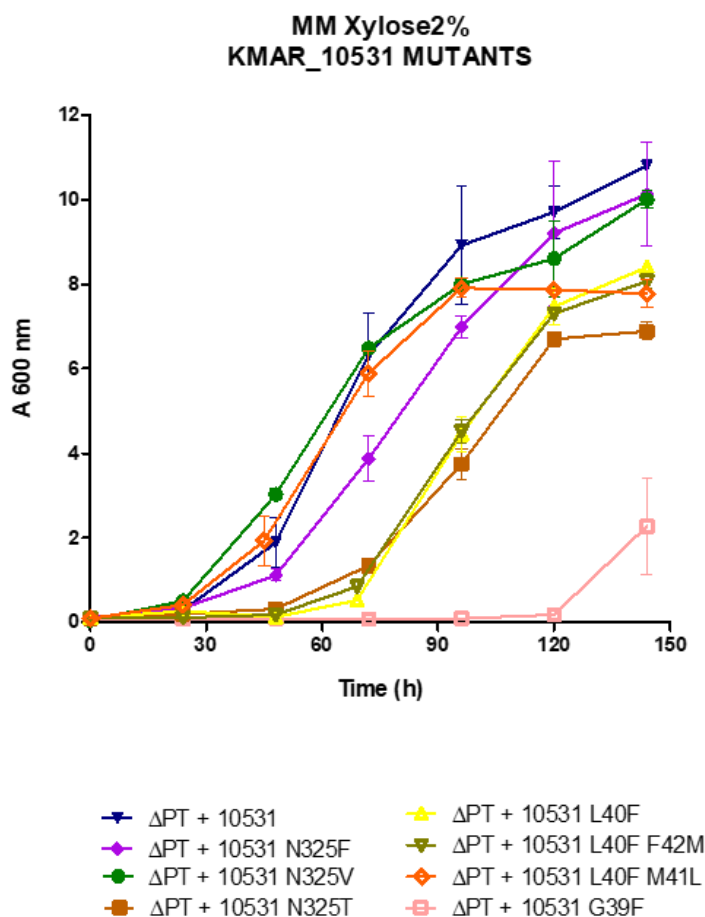


Figure 4.7: Growth assays on xylose 2% of *K. marxianus* ΔPT strain transformed with KMAR_10531 and its mutants. Cells were pre-grown overnight on YPLAC media, washed, inoculated in MM xylose 2% at A_{600nm} 0.1 and grown at 30°C 200rpm. Growth was assessed for 140h. Experiments were performed in triplicates with error bars showing SD.

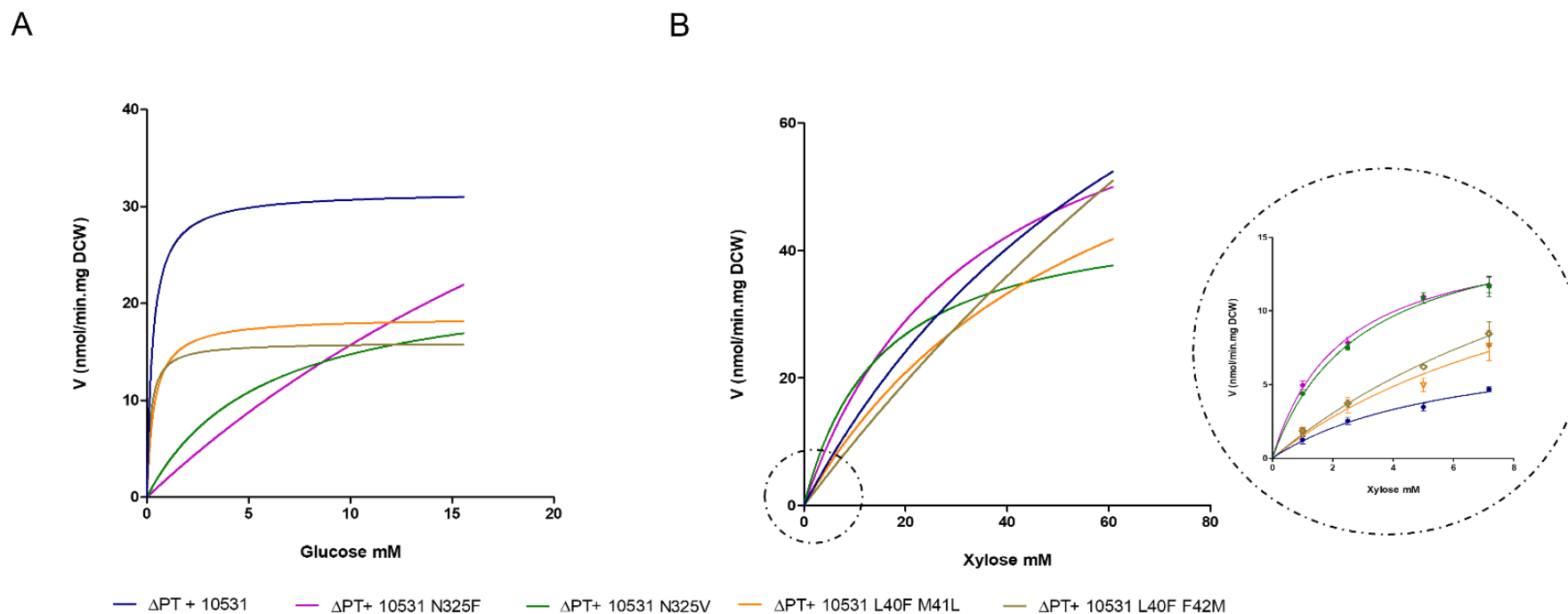
KMAR_10531 mutants' transport characterization

Based on the demonstrated capacity to still confer growth on xylose (Figure 4.7), it was decided to further characterise the following four mutants using radiolabelled sugar uptake assays: L⁴⁰F-M⁴¹L; L⁴⁰F-F⁴²M; N³²⁵F and N³²⁵V (Figure 4.8). The two mutants in TMD1 (L⁴⁰F-M⁴¹L and L⁴⁰F-F⁴²M) showed affinity (Km) for glucose in the same range but a reduced maximum velocity compared to the wt transporter. On the other hand, the two TMD8 mutants N³²⁵F and N³²⁵V had decreased affinity for glucose: the change of N³²⁵ to phenylalanine (N³²⁵F) led to a complete loss of the high affinity component for glucose while the change to valine (N³²⁵V) causes a decrease in Km of 95% for this sugar (Figure 4.8 A).

As for the xylose uptake, the mutation L⁴⁰F-M⁴¹L only slightly decreased the affinity of the transporter, while the mutation L⁴⁰F-F⁴²M had a more negative effect on K_m (Figure 4B). The N³²⁵F mutation improves the affinity for xylose (K_m 33.4±6.6 mM) relative to the native transporter (K_m 46.9±9.5 mM) without affecting the V_{max} constant. The best mutation was N³²⁵V where the K_m (14.9±3.6 mM) was decreased by over 3-fold relative to the native transporter (K_m 46.9±9.5 mM), though the V_{max} did show some reduction. The increase in affinity is best visualised at the low range of xylose concentrations, where the wild type transporter was almost unable to transport xylose, while the two mutants show higher transport activity (Figure 4.8 B detail in dashed circle).

To determine how the best mutations influenced the preference of the transporter for pentoses over hexoses, competition experiments were carried out to assess the effect of xylose and arabinose on glucose transport, by measuring the uptake of glucose at each concentration in the presence of an excess of the unlabelled pentose sugars (Figure 4.9). Glucose transport in the mutants N³²⁵F and N³²⁵V was considerably more inhibited by the presence of xylose or arabinose when compared with the wild type transporter (Figure 4.9 A). In the presence of 150 mM of xylose or arabinose, the apparent K_m of KMAR_10531 N³²⁵V (Figure 4.9 B) for glucose increases 5.8 and 4-fold (33.1 mM and 22.8 mM) respectively, compared to the K_m of the transporter in presence of only glucose (5.7 mM) keeping a similar V_{max} , indicating the competitive inhibition of the uptake of the two sugars. For the mutant KMAR_10531 N³²⁵F (Figure 4.9 C) the competitive inhibition in presence of either xylose or arabinose on glucose transport is also characterized by an increase in K_m , respectively, ~2 and 4.5-fold (45 mM and 117.9 mM) compared to K_m on only glucose (26.5 mM). Interesting, for the wild type transporter KMAR_10531, the presence of arabinose also shows a slightly higher inhibition than xylose. However, this difference might not be significant due to the use of Lineweaver-Burk representation, in which the reciprocal of the reaction's rate is plotted causing that small errors in measurement are more noticeable.

To check how the different competitive inhibition effects could impact the utilization of xylose or arabinose in the presence of glucose, KMAR_10531 mutants were plated on solid MM containing 2% xylose or arabinose added with 0, 0.1 or 0.5% 2-DOG – a synthetic glucose analog that can be imported into cells and interfere with glycolysis and ATP generation hampering growth (Figure 4.10). In accordance with the previous data, among all the mutants, KMAR_10531 N³²⁵F and KMAR_10531 N³²⁵V look the most promising as they confer resistance to 2-DOG, allowing the growth on MM xylose 2% even when we use a high concentration of 2-DOG (0.5%). This assay together with the competitive inhibition assay showed that N³²⁵V mutation favors more xylose transport than the other mutations.



		$\Delta PT + 10531$	$\Delta PT + 10531 N^{325}F$	$\Delta PT + 10531 N^{325}V$	$\Delta PT + 10531 L^{40}F M^{41}L$	$\Delta PT + 10531 L^{40}F F^{42}M$
A. Glucose uptake (0.1-15 mM)	Vmax	31.6 ± 1.9	76.1 ± 43.5	23.07 ± 3.5	18.58 ± 1.3	16.0 ± 1.10
	Km	0.28 ± 0.1	38.44 ± 28.7	5.6 ± 2.0	0.36 ± 0.14	0.18 ± 0.08
B. Xylose uptake (0.1-60 mM)	Vmax	79.4 ± 7.6	77.4 ± 8.3	47.0 ± 4.5	82.4 ± 10.6	148.8 ± 45.8
	Km	46.9 ± 9.5	33.4 ± 6.57	14.9 ± 3.6	59.0 ± 12.1	130.8 ± 53.0

Figure 4.8 Radiolabelled substrate uptake of KMAR_10531 and mutants expressed in the platform ΔPT . Michaelis-Menten plot of ^{14}C -Glucose (**A**) – range 0.1-15 mM - and ^{14}C -Xylose (**B**) – range 0.1-60 mM - initial uptake rates in transformants with detail on the range 0.1-7 mM in dashed circle. The velocity is reported as nmol sugar taken up per minute per mg cell dry weight ($\text{nmol min}^{-1} \text{mgCDW}^{-1}$). Mean values and standard deviation of triplicate measurements are shown.

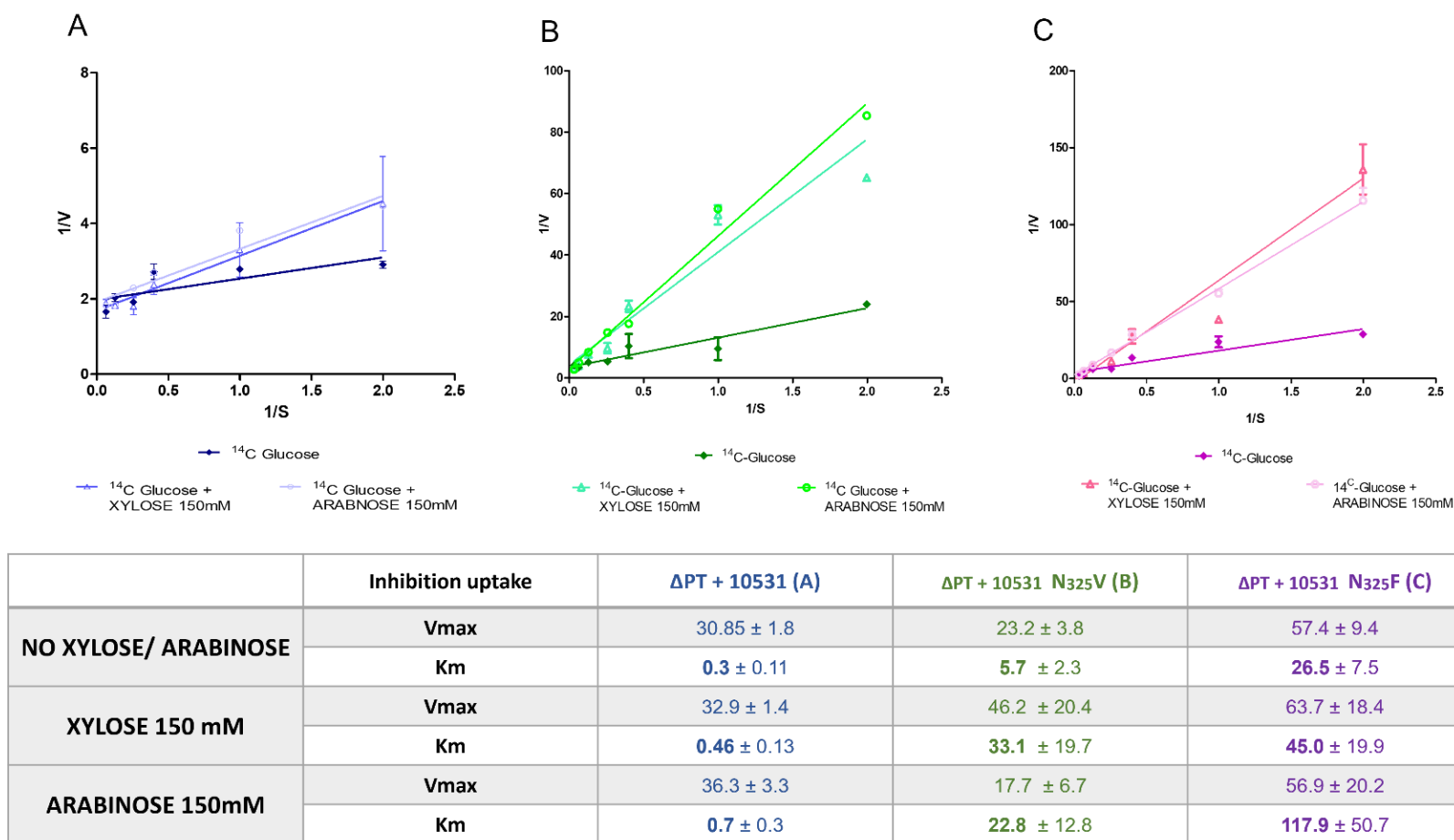


Figure 4.9 Competitive inhibition assays. Lineweaver-Burk plot of the initial uptake rates of glucose transport in the absence or presence of xylose or arabinose. The assay was carried out by measuring the uptake of glucose at each concentration (0.1, 0.5, 1, 2.5, 4, 8, 16 or 32 mM) in the presence of an excess of the other unlabelled sugar (150 mM) in *K. marxianus* ΔPT strain transformed with KMAR_10531 (in blue), KMAR_10531 N³²⁵V (in green) and KMAR_10531 N³²⁵F (in purple). The velocity is reported in $\text{nmol min}^{-1} \text{mgCDW}^{-1}$.

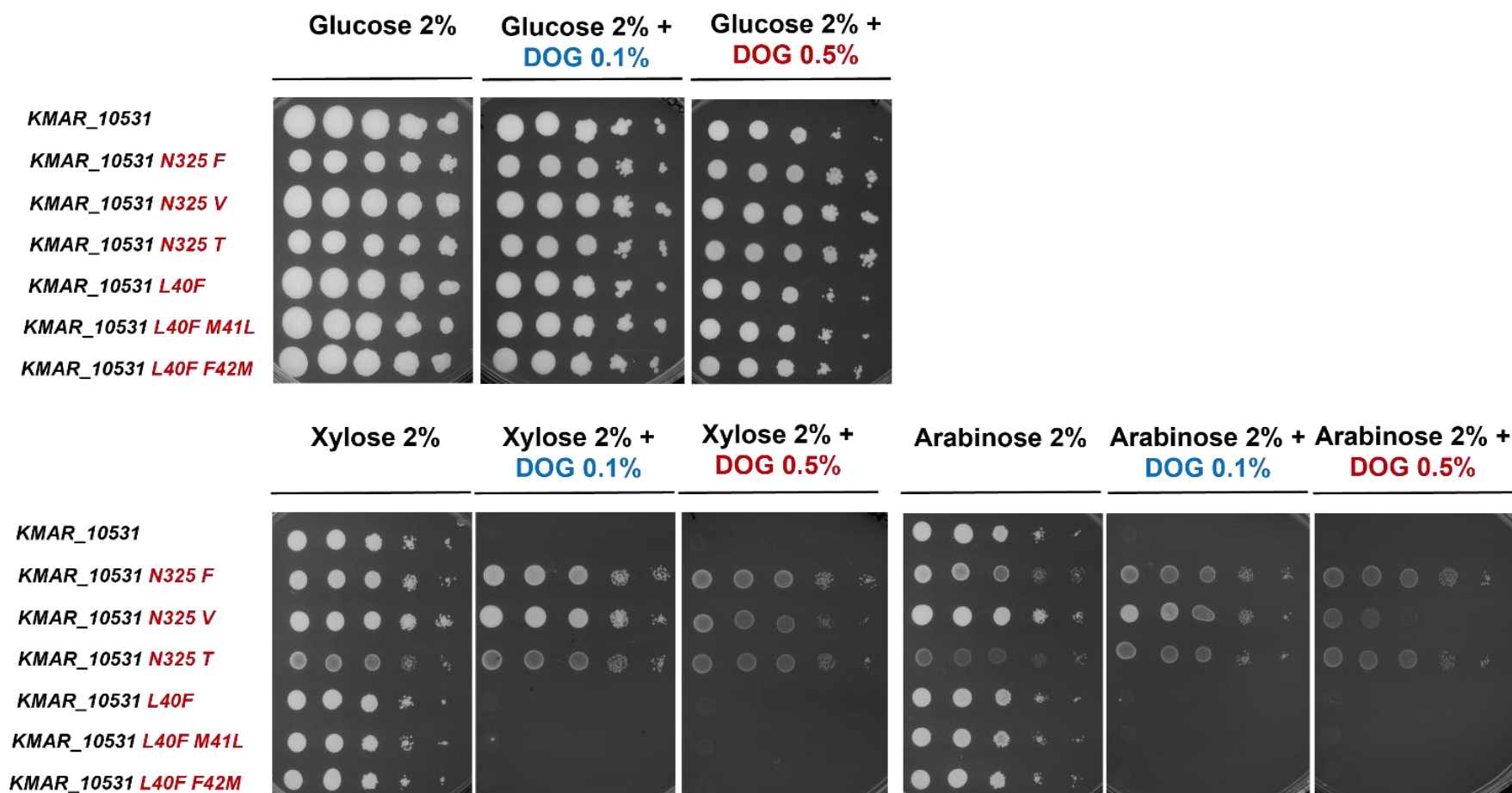


Figure 4.10: Growth comparison of *K. marxianus* Δ PT expressing *KMAR_10531* wild type and promising transporter's mutants. To examine the effect of 2-DOG as glucose analogue on xylose and arabinose transport, cells were pre-grown on YPLAC and then plated in serial dilution on agar plates with 2% glucose, xylose or arabinose added with 0.1 and 0.5 % (w/v) 2-DOG. Plates were photographed after 96h of growth at 30°C.

Discussion

In this study, we characterized two native *K. marxianus* transporters (KMAR_10531 and KMAR_60179) for glucose and xylose transport, when expressed in a *K. marxianus* platform strain Δ PT lacking all pentose transporters previously described (Chapter 2) (Donzella et al. 2021). Uptake kinetics (Figure 4.4-4.5) of these transporters showed that *K. marxianus* is provided with a middle-low (KMAR_10531) and low (KMAR_60179) affinity transporters for xylose. However, as for other transporters, KMAR_10531, the best xylose transporter, also mediates glucose transport with high affinity, and assimilation of this sugar when present in the medium will be favoured over xylose. In fact, one of the major challenges in co-consumption of glucose and xylose deriving from lignocellulosic biomass is the competitive inhibition between both sugars at the level of sugar transporters and the lack of a specific xylose transporter. In order to make possible a faster uptake of xylose and a simultaneous utilization of both pentose and hexose sugars in this yeast, a rational mutagenesis approach was used to construct a set of mutants of the transporter KMAR_10531, which showed already good xylose import compared to other native transporters reported in literature (Nijland et al., 2020). The most promising mutants were then characterized with radiolabelled substrate uptake (Figure 4.8) and a variant of *K. marxianus* KMAR_10531 (N³²⁵V) which improved xylose transport (Figure 4.8B) was identified. Different combinations of mutations of KMAR_10531 in a conserved motif (³⁸SGLMF⁴²) of the first transmembrane span TMD1 reported to be in charge of controlling the selectivity and efficiency of monosaccharide transport in MFS transporters and to be responsible for rewiring xylose transport in *S. stipitis* Rgt2, *C. intermedia* Gsx1 and *S. cerevisiae* Hxt7 (Young et al., 2014) were found to reduce velocity in glucose import, but have no significant effect in improving xylose uptake. Young et al. 2014 also reported that residue 39 controls the efficiency of the transport and a substitution of G³⁹ with a phenylalanine (F) would cause a reduction in growth on hexoses without altering xylose growth rate in *C. intermedia*. Performing this mutation in KMAR_10531 caused a loss in xylose uptake (Figure 4.7), but curiously we find this substitution naturally present in KMAR_60179, which, indeed doesn't allow glucose import and still imports xylose. Prior to this work, multiple mutations and variants in *S. cerevisiae* Gal2 - a galactose permease able to slightly transport xylose and arabinose, but strongly inhibited by glucose - have been proposed to increase xylose uptake. Among these, an asparagine in the transmembrane domain 8 (TMD8) has been found crucial to alleviate glucose competition on this and other transporters (Farwick et al., 2014; Nijland et al., 2020; Shin et al., 2017; Rojas et al. 2021, Nijland et al. 2014). In *K. marxianus*, altering the same residue in position 325 in KMAR_10531 was found to play a fundamental

role to reprogramme the balance between hexose and pentose uptake. Substitutions of N³²⁵ with a valine (V) or a phenylalanine (F) were made and characterized. The most promising mutation, N³²⁵V increased affinity for xylose while decreasing it for glucose, losing the ability to import glucose with high affinity in favour of a high affinity xylose uptake component (Figure 4.8). This caused a shift in specificity from glucose to xylose, compared to the wild type transporter. The substitution of N³²⁵ with the large hydrophobic residue F causes a stronger reduction of glucose uptake compared to the wild type or N³²⁵V, probably due to the steric constrain. The substitution N³²⁵F proved to be more beneficial for xylose uptake in other transporters, i.e. *S. cerevisiae* Gal2, where it not only abolished hexose uptake but improved xylose import as well. In our transporter KMAR_10531, N³²⁵F shows a lower affinity for this sugar compared with N³²⁵V thus there are differences between the KMAR_10531 and Gal2 proteins. As for other transporters, in KMAR_10531, the residue N³²⁵ is located close to the binding pocket, at 4 Å from the ligand, hence its substitution results critical in the interactions of the transporter with glucose and/or xylose. Substitution of the asparagine to valine N³²⁵V results better, increasing affinity of the transporter for xylose. This substitution with a smaller-sized amino acid (V) would not result in narrowing the translocation pore of the transporter, but more likely changes of van der Waals interactions altering the structure of the substrate binding pocket, leading to reduction of glucose affinity and increase in xylose's (Kasahara et al., 2011).

We also studied the competitive inhibition of xylose and arabinose on glucose transport in mutants of KMAR_10531 compared with the wild type transporter and confirmed that the mutations introduced increased the inhibition of glucose transport by the pentoses (Figure 4.9). Moreover, we assessed glucose inhibition on growth on xylose and arabinose for all the constructed mutants using 2-DOG, a glucose analogue that can be imported into cells and interfere with glycolysis and ATP generation (Nurcholis et al., 2018). We observed that the mutations in residue N³²⁵ are sufficient to overcome the inhibition of glucose allowing growth even when 2-DOG was present at high concentration (0.5%) (Figure 4.10).

Summing up, in this study we identified and characterized a variant of a *K. marxianus* transporter KMAR_10531 N³²⁵V with very promising results for simultaneous consumption of glucose and xylose. In the next chapter, the behavior of platform ΔPT and *K. marxianus* wild type strains expressing this transporter in flasks and bioreactors with mixed sugars media, will be characterized.

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CHAPTER 5

Mixed sugars fermentation by engineered *Kluyveromyces marxianus* strain:
assessment of growth and xylose utilization

Abstract

In the previous chapter, we showed the creation of a Hgt-like mutant transporter, KMAR_10531 N^{325V} improved in the high affinity uptake of xylose at the expense of glucose import. Here, we assessed the growth of *K. marxianus* strains expressing this engineered transporter on mixtures of sugars (glucose and xylose) and we optimized the conditions to scale up the bioprocess from flasks to bioreactors. The bioreactor assays allowed to follow the pH of the medium along fermentation, and interestingly identified a drastic increment of pH during the consumption of xylose. This was reflected in the inability of the strain with the native transporter KMAR_10531 to complete the xylose consumption in bioreactor, problem that was solved when the pH was kept at acidic values. In fact, it emerged that this transporter needs a proton gradient to function, raising the question of whether KMAR_10531 works as a proton-symporter.

This work is the first step for the implementation and scaling up of this bioprocess, even so this improved transporter already represents a promising solution for the complete valorization and bioconversion of all the sugars present in lignocellulosic biomass in biorefineries.

5.1 Introduction

Not all the microorganisms are able to naturally assimilate pentose sugars, which are one of the main components of lignocellulosic biomass (Liu et al., 2019), and even if they do, they show bottlenecks that make the conversion process inefficient and long. One of the main impediments is that native yeast sugar transporters have significant higher preference for glucose than xylose, causing that xylose enters in the cell and is assimilated only when all glucose is depleted (diauxic shift) (Li et al., 2016; Wang et al., 2016). Moreover, catabolite repression (see Chapter 3) plays an important role in the inefficiency of the conversion of hexose and pentose sugars, as glucose inside the cell it triggers a cascade of events which mostly ends up with the repression of the utilization of other carbon sources (Kim et al., 2010). For all these reasons, it is desirable to have a transporter that does not efficiently transport glucose and instead can transport xylose with high affinity to address the aforementioned limitations. In Chapter 4, we showed the construction of such transporter in *Kluyveromyces marxianus*, a very valuable non-conventional yeast that, besides other biotechnological relevant characteristics, it is also able to naturally assimilate pentose sugars. Various mutations in the high affinity glucose transporter, KMAR_10531, were made and the change in an arginine residue (N)³²⁵ was found to be enough to enable a better uptake and consumption of xylose in the presence of glucose. Here, we assessed the growth on mixture of sugars of the *K. marxianus* platform strain Δ PT expressing some of the improved transporter mutants.

Traditional shake flasks are widely used in the early stage of developing bioprocesses, because of their simplicity and low cost. The flask step represents the first asset for the development and optimization of a process in a fast way, providing indicative data. Even so, for the implementation of a bioprocess and characterization for potential commercialization, scale up to larger volumes is needed. Scaling up to lab-scale bioreactor systems is the second step, crucial in order to guarantee the reproducibility or even improve the entire process when increasing volumes. Parameters such as aeration, agitation, pH are not controlled or monitored in the simple flask system, but it is important to examine them in larger scale since they influence the transport of solute through the membrane and the physiological behavior of yeasts.

In this work, flasks and bioreactors experiments were conducted optimizing the growth of the yeast strains and then a comparison with the best transporter mutant expressed in the platform and in the wild type strain was conducted. Optimization of the aeration and pH was performed to enable the growth of the microorganisms and the consumption of the sugars in bioreactors. The Δ PT strain expressing

KMAR_10531 N^{325V} stood out as a strain with promising application for co-consumption of glucose and xylose deriving from lignocellulosic biomass.

5.2 Materials and Methods

Strains and culture conditions

K. marxianus strains used in this study are reported in Table 5.1. Strains were pre-cultured in YPLAC media (yeast extract 10 g L⁻¹, peptone 20 g L⁻¹, lactose 20 g L⁻¹) for 15-20h. *E. coli* DH5 α cells carrying the plasmids of interest were grown in LB media (10 g L⁻¹ peptone, 5 g L⁻¹ yeast extract and 5 g L⁻¹ sodium chloride) added with 100 μ g mL⁻¹ ampicillin for 20 h. Plasmids were extracted with Sigma GenEluteTM Miniprep Kit. *K. marxianus* yeast cells were then transformed with the plasmids of interest using a variation of the LiAc/SS carrier DNA/PEG method (Gietz & Schiestl, 2007) and plated on YPLAC agar plates supplemented with G418.

Table 5.1 Yeast strains used in this study.

Strain	Genotype	Parental strain	Characteristics	Origins
<i>K. marxianus</i> NBRC1777	Wild-type strain			NITE, Japan
<i>K. marxianus</i> NBRC1777 + KMAR_10531	NBRC1777 [pTU-DO-G418-KMAR_10531]	<i>K. marxianus</i> NBRC1777	Expresses native KMAR_10531	This study
<i>K. marxianus</i> NBRC1777 + KMAR_10531 N^{325V}	NBRC1777 [pTU-DO-G418-KMAR_10531 N ^{325V}]	<i>K. marxianus</i> NBRC1777	Expresses mutant KMAR_10531 N ^{325V}	This study
<i>K. marxianus</i> ΔPT	$\Delta hgt \Delta kht \Delta kmar_{60179}$	<i>K. marxianus</i> $\Delta hgt \Delta kht$	see Chapter 2	Donzella et al. 2021
<i>K. marxianus</i> ΔPT + KMAR_10531	$\Delta hgt \Delta kht \Delta kmar_{60179}$ [pTU-DO-G418-KMAR_10531]	<i>K. marxianus</i> $\Delta hgt \Delta kht$	Expresses native KMAR_10531	This study
<i>K. marxianus</i> ΔPT + KMAR_N^{325F}	$\Delta hgt \Delta kht \Delta kmar_{60179}$ [pTU-DO-G418-KMAR_10531 N ^{325F}]	<i>K. marxianus</i> $\Delta hgt \Delta kht$	Expresses mutant KMAR_10531 N ^{325F}	This study
<i>K. marxianus</i> ΔPT + KMAR_N^{325V}	$\Delta hgt \Delta kht \Delta kmar_{60179}$ [pTU-DO-G418-KMAR_10531 N ^{325V}]	<i>K. marxianus</i> $\Delta hgt \Delta kht$	Expresses mutant KMAR_10531 N ^{325V}	This study
<i>K. marxianus</i> ΔPT + KMAR_N^{325T}	$\Delta hgt \Delta kht \Delta kmar_{60179}$ [pTU-DO-G418-KMAR_10531 N ^{325T}]	<i>K. marxianus</i> $\Delta hgt \Delta kht$	Expresses mutant KMAR_10531 N ^{325T}	This study

Flasks and Bioreactors fermentations and HPLC analysis

Precultures for flasks and bioreactors experiments were grown for 15 h on YPLAC media with G418 in 100 mL shake flasks at 30 °C and 200 rpm. Promising mutants were tested in flasks of 250 mL containing 50 mL of culture media of mix of sugars - MM added with xylose and glucose in different ratio (respectively xylose 2% + glucose 0.5%; xylose 2% + glucose 1%; xylose 2% + glucose 2%). The cultures were started at A_{600nm} of 0.1 and cultivated at 30 °C and 200 rpm, and the A_{600nm} was measured every 3-6 h for 75 h. *K. marxianus* mutants $\Delta PT + KMAR_10531$ and $\Delta PT + KMAR_10531 N325V$ were also tested in batch cultivation conducted in 4 L Ez-Control (Applikon Biotechnology) Bioreactors with 2 L working volume. The media used was MM Glucose 2% + Xylose 2% supplemented with 1 mL/L of Antifoam C (Sigma Aldrich). Each bioreactor was inoculated to A_{600nm} 0.02 and the experiment was conducted in triplicate at 30 °C. Aeration was optimized based on the culture requirements. It was first controlled by stirrer until the yeast reached A_{600nm} 1.0 (at ~ 15 h) and then was set at 0.5 L min⁻¹ (0.25 vvm – volume of air sparged per unit volume of growth medium per minute) until 24 h, finally it was increased to 1 L min⁻¹ (0.5 vvm) until the end of the experiment. A_{600nm} and dry cell weight (DCW) was measured every 4 h for 60 h. Dry cell weight was determined by collecting 10 mL of cells in weighed tubes, centrifuging for 5 min at 5000 rpm, discarding the supernatant, letting the pellet dry at 50 °C for 72 h, weighing the tubes and calculating the tare. Concentrations of glucose and xylose remaining in the medium at each time point either for flasks or bioreactors was measured by HPLC. For this, 1.5 mL of culture were harvested by centrifugation and 800 μ L of supernatant were injected into a Hewlett-Packard (HP) 1090 instrument using a Bio-Rad HPX-87H hydrogen ion resin column and an HP 1047A external refractive index detector. The mobile phase, 0.001 N H₂SO₄, was run at 55 °C at a flow rate of 0.6 mL min⁻¹.

5.4 Results

Fermentation performance of transporter's mutants on mixture of sugars

To assess the potential of new transporter variants for the co-consumption of glucose and xylose, platform *K. marxianus* ΔPT strain was individually transformed with plasmids carrying three different variations of KMAR_10531 in asparagine (N)³²⁵ (Table 5.1). Strains were tested in flasks with MM with 2% xylose and increasing glucose concentration (0.5%, 1%, 2%) to check for glucose-inhibition of xylose utilisation. On MM xylose 2% + glucose 0.5%, the strain expressing the native protein $\Delta PT + KMAR_10531$ consumes

glucose first, fully depleting it after 22 h, and then consumes xylose (Figure 5.1 A). Maximum biomass is reached after about 50 h but even after 72 h xylose is not fully consumed with ~5 g/L remaining in the medium. Δ PT + KMAR_10531 N³²⁵T consumes glucose with the same rate as the native transporter but it is not able to consume xylose. Compared with the wt transporter, mutation N³²⁵F (Figure 5.1 B) slows down glucose consumption– glucose is completely consumed after 36 h - but does not improve xylose transport – ~ 10 g/L of xylose are still present in the media after 70 h. On the other hand, mutation N³²⁵V (Figure 5.1 C) mirrors the consumption tendency for glucose of the wt transporter, but this mutation improves xylose consumption, as all xylose is consumed within 48 h and overall biomass production is also increased by ~ 20%.

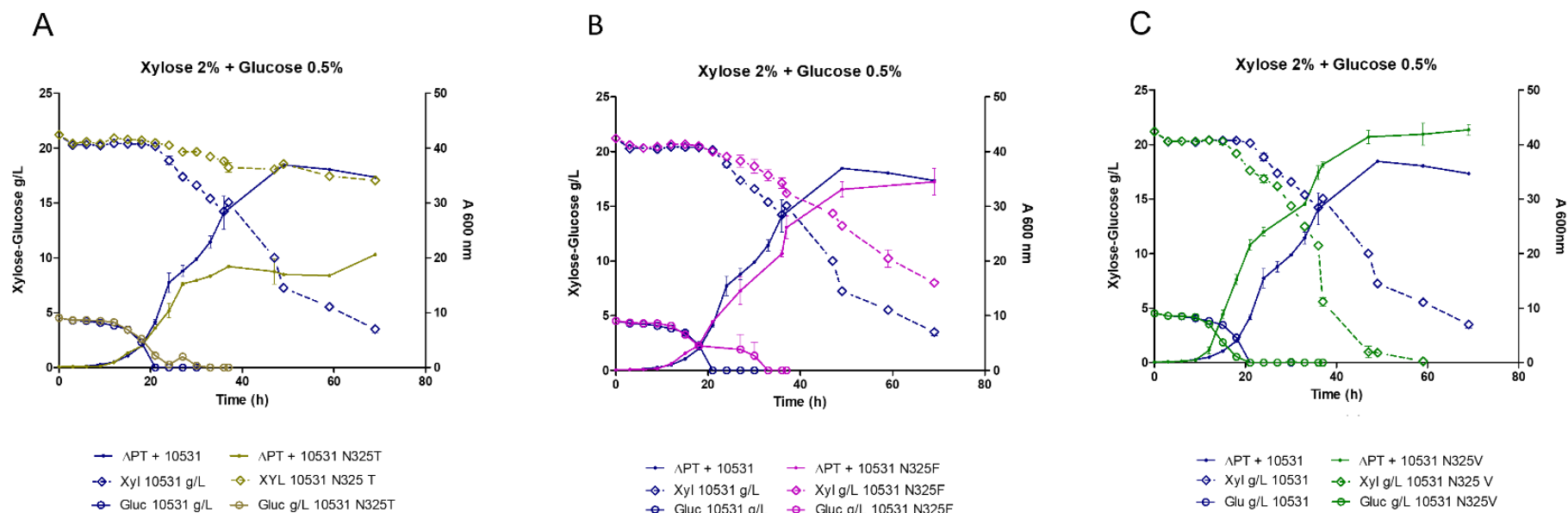


Figure 5.1 Fermentation profiles of *K. marxianus* Δ PT strain expressing KMAR_10531 transporter variants in N³²⁵ on MM xylose 2% + glucose 0.5% in flasks. KMAR_10531 wild type (dark blue) and the mutants N³²⁵T (A, brown), N³²⁵F (B, purple), N³²⁵V (C, green) were overexpressed in the platform strain Δ PT and tested in flasks at 30 °C for 72 h. The consumption of xylose (open diamonds, dashed lines) and glucose (open circles, full lines) and growth curves (closed symbol, full lines) are shown. The error bars and mean values of three independent cultures are reported.

After this first screening, we decided to further characterize the positive mutation N³²⁵V increasing the concentration of glucose to 1% and 2%. When glucose in the medium is 1% (Figure 5.2 A), the native transporter Δ P_T + K_{MAR_10531} consumed it completely within 22 h, but it is not able to exhaust the xylose and after 48 h there are still 5 g/L of this sugar in the medium. Increasing the quantities of glucose, it is clearer how mutation N³²⁵V improved the co-consumption of glucose and xylose, as both sugars simultaneously start to be consumed after 6 h. Glucose is completely depleted by this mutant after 30 h and xylose is exhausted after 48 h.

When glucose in the medium is increased to 2% (Figure 5.2 B), we could observe a clear double step of uptake and consumption of glucose and xylose: the native transporter K_{MAR_10531} is more efficient in transporting glucose in the media, causing its complete depletion in 24 h, while xylose consumption starts when glucose is totally used and it takes around 70 h to be completely consumed. The mutated transporter K_{MAR_10531} N³²⁵V confers Δ P_T the capability to simultaneously consume both sugars, with all the glucose being consumed in 48 h and xylose in 60 h (Figure 5.2 B).

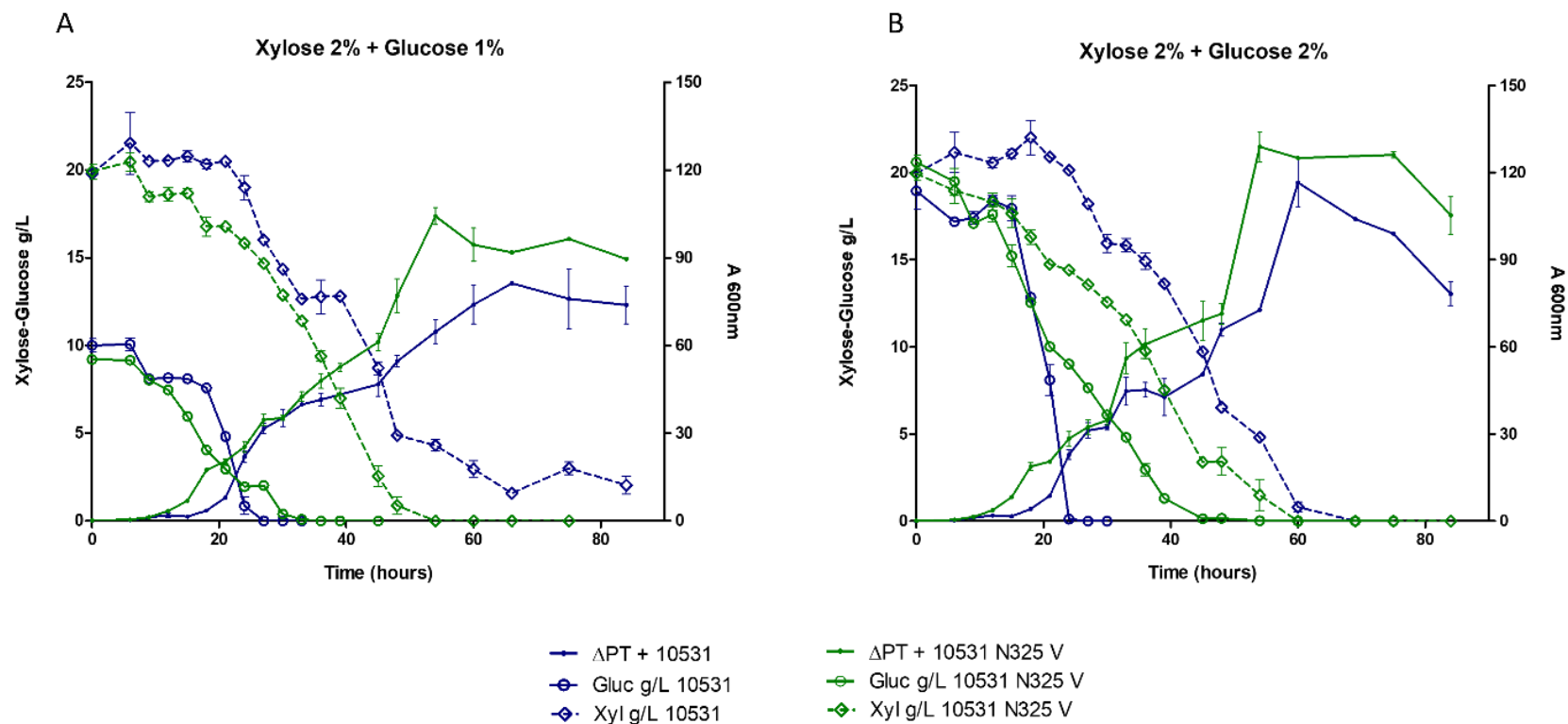


Figure 5.2 Fermentation profiles of *K. marxianus* KMAR_10531 wild type (dark blue) and the mutant KMAR_10531 N³²⁵V (green) on MM xylose 2% + glucose 1% (A) and on MM xylose 2% + glucose 2% (B). The transporters were overexpressed in the platform strain Δ PT and tested in flasks at 30 °C for 84 h. The consumption of xylose (open diamonds, dashed lines) and glucose (open circles, full lines) and growth curves (closed symbol, full lines) are shown. The error bars and mean values of three independent cultures are reported.

Next a set of growth experiments were performed in bioreactors where it was possible to measure and control process parameters. In flask experiments, as the ones shown above, oxygen is present in the gaseous phase above the liquid surface and transferred by diffusion into solution, therefore in this system agitation and aeration are a relative low shear mixing system and it is important to limit the liquid /flask volume to 20% to have useful gas transfer rates. On the other hand, aeration conditions in the bioreactor have been optimized to maximise yeast growth. Thus, during initial stages, oxygenation is maintained simply by stirring but during later growth air is supplied by sparging, which also introduced shear pressure. It was important to ensure that the tank was not either over-aerated causing oxidative stress, nor under aerated as xylose cannot be consumed. In bioreactors, using MM xylose 2% + glucose 2%, $\Delta PT + KMAR_10531 N^{325}V$ reached a biomass of 16 g/L consuming both sugars in the medium in 60 h, confirming its improved behaviour when compared with the ΔPT transformed with the native transporter that did not consume all the xylose in 60 h of assay, producing ~ 11 g/L biomass at the end of the process (Figure 5.3 A). Surprisingly, ΔPT transformed with the native transporter is less efficient in the bioreactor than in flasks, still having ~ 11 g/L of xylose in the medium after 60 h (Figure 5.3 B). An explanation for this was achieved by monitoring and controlling pH in the bioreactor. The initial pH was 5.5 and in $\Delta PT + 10531$, a decrease in pH to ~ 3.9 is observed around 28 h, due to the accumulation of acidic by-products and proton efflux during glucose metabolism. This is followed at 32 h by a rapid increase to pH ~ 8.5 during the assimilation of xylose that started after glucose depletion. On the other hand, for $\Delta PT + 10531 N^{325}V$, the shift from glucose to xylose metabolism is not clearly reflected in the pH, as the strong decrease of pH due to glucose metabolism is not observed, and the increase in pH up to 8.5 is observed around 27 h. Since the increase in pH commenced simultaneously with xylose consumption, it is likely that this is due to depletion of protons from the medium during xylose transport, suggesting that the transporter is a proton-symporter, co-transporting protons with xylose. This hypothesis is also supported by the fact that keeping the pH at 5.5 during all the process, solves the xylose consumption limitation of the native transporter $KMAR_10531$. In fact, this allows a proton gradient at membrane level which may help the transporter to import xylose into the cell and complete its depletion in 65 h (Figure 5.4).

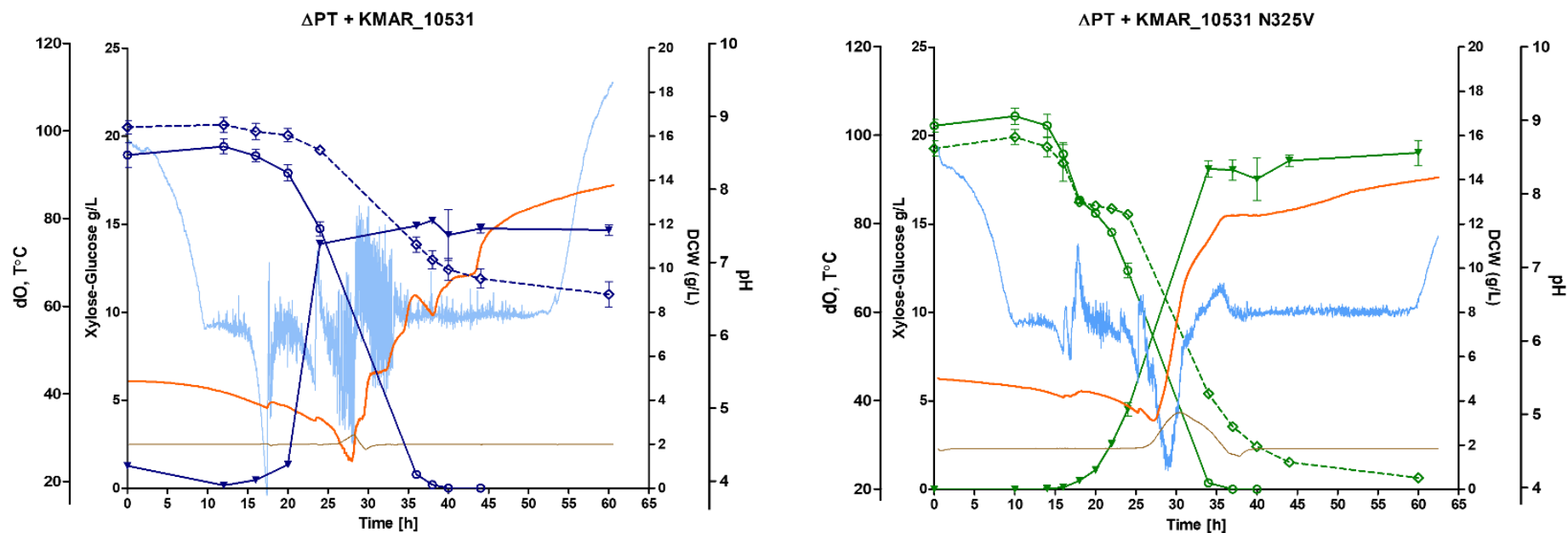


Figure 5.3: Bioreactor growth and sugar consumption of *K. marxianus* ΔPT expressing KMAR_10531 wild type and the mutant KMAR_10531 N^{325V} on MM xylose 2% + glucose 2%. KMAR_10531 wild type (A, dark blue) and the mutant N^{325V} (B, green) were overexpressed in the platform strain ΔPT and tested in Applikon bioreactors at 30 °C for 65 h. The consumption of xylose (open diamonds, dashed lines) and glucose (open circles, full lines) and growth curves (closed symbol, full lines) are shown. pH (orange, full line), T (brown, full line) and dO₂ (light blue, full line) were monitored. The error bars and mean values of three independent cultures are reported.

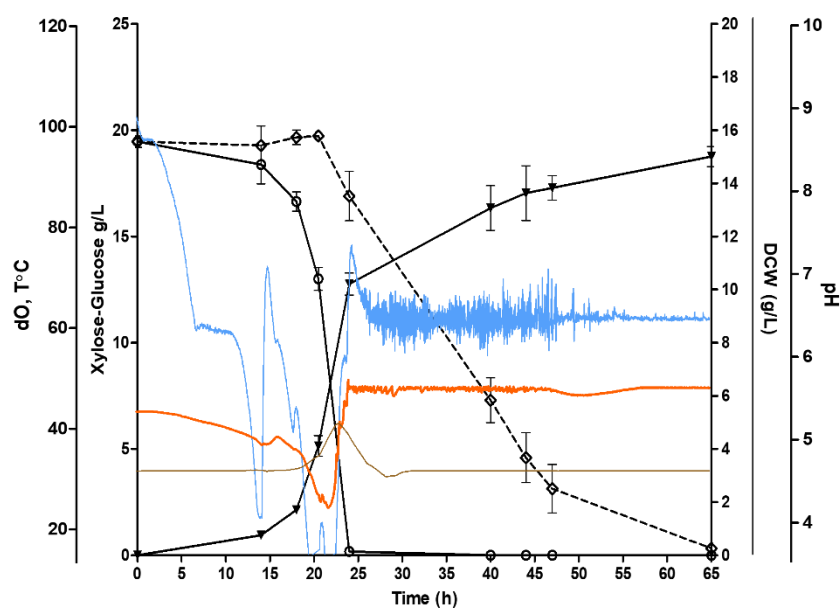


Figure 5.4: Bioreactor growth and sugar consumption of *K. marxianus* Δ PT expressing KMAR_10531 wild type on MM xylose 2% + glucose 2% controlling the pH. KMAR_10531 wild type was overexpressed in the platform strain Δ PT and tested in Applikon bioreactors at 30 °C for 65 h. The consumption of xylose (open diamonds, dashed black line) and glucose (open circles, full black line) and growth curves (closed symbol, full black line) are shown. pH (orange, full line), T (brown, full line) and dO₂ (light blue, full line) were monitored. pH was controlled supplementing HCl 1M to keep it at 5.5. The error bars and mean values of three independent cultures are reported.

Potential of engineered Δ PT strain as chassis for biorefineries

We wanted to investigate whether expression of the improved transporter would also lead to efficient co-consumption of xylose with glucose in a wild-type strain. For this, KMAR_10531 and KMAR_10531 N^{325V} were expressed in *K. marxianus* NBRC1777 (Figure 5.5 A-C) and in the platform strain Δ PT (Figure 5.5 B-D) and their growth on MM Xylose 2% + Glucose 2% was evaluated and compared. When KMAR_10531 is expressed in the wild type strain (Figure 5.5 A in blue), glucose import occurs faster than in the untransformed strain, confirming its expression and activity as an efficient glucose transporter. However, there is no effect on xylose uptake and, similarly to what is observed for the untransformed strain, xylose utilisation only starts when glucose is depleted and all xylose is consumed after 78 h reaching a final A_{600nm} of ~ 45 (Figure 5.5 C). When the wt strain expresses the mutant KMAR_10531 N^{325V} (Figure 5.5 A in green), glucose is consumed in the first 18 h and xylose in 78 h, with the growth slightly faster than the untransformed strain and similar to the strain expressing the native transporter in the first 24h of the process. These results show that expression of either the KMAR_10531 wt or the improved variant in the wild type strain does not confer an advantage for the simultaneous consumption of hexoses and pentoses.

In contrast, the expression of KMAR_10531 N^{325V} in the platform strain Δ PT (Figure 5.5 B-D) allows the co-consumption of both sugars, as shown above, slowing down glucose uptake and so possibly avoiding the cascade events caused by glucose repression on xylose and accelerating the pentose uptake. The process is speeded up from 78 h to less than 60 h and the biomass reached is 16 g/L corresponding to A_{600nm} of ~ 120 .

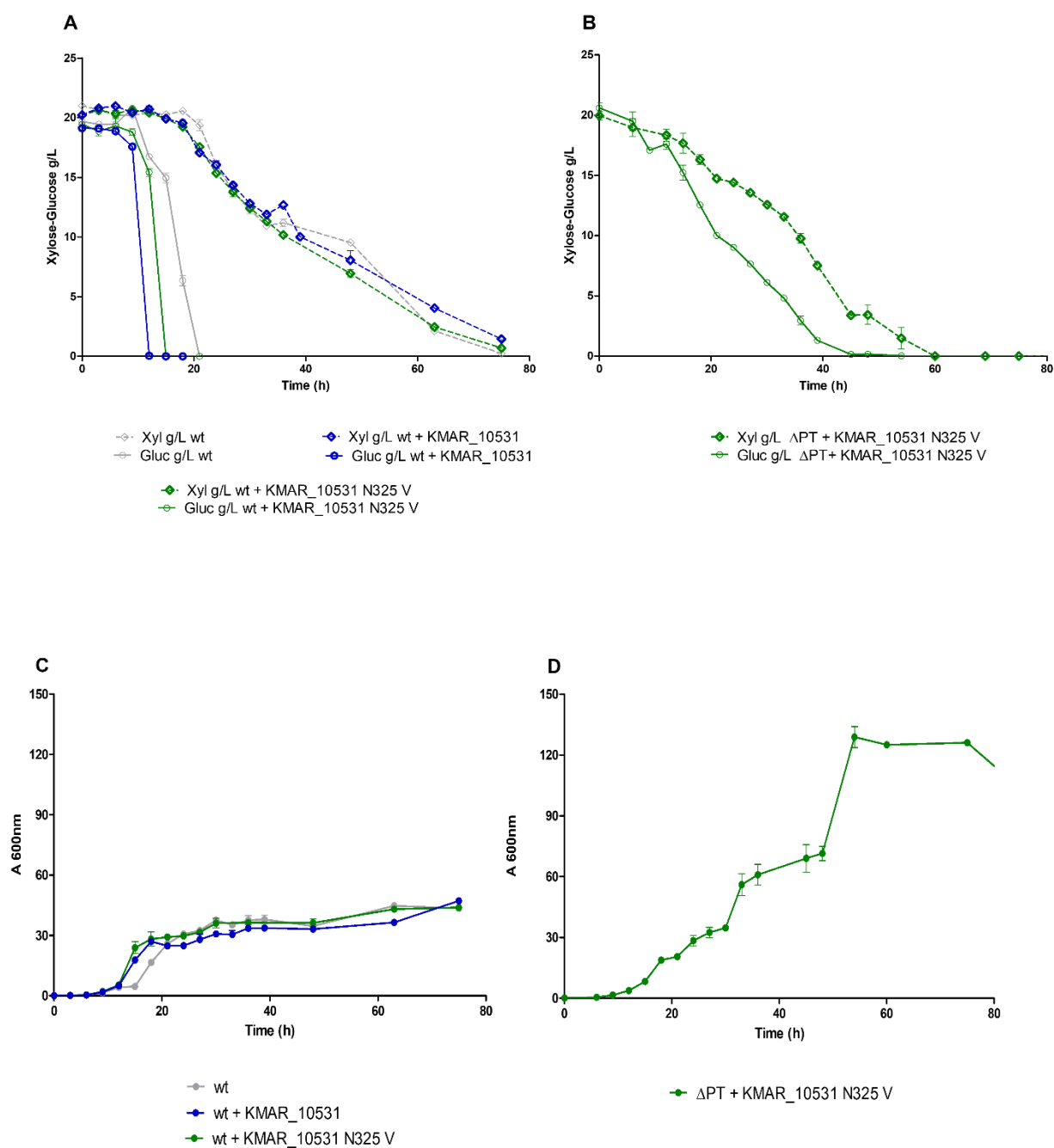


Figure 5.5 Sugar consumption (A and B) and biomass accumulation (C and D) of *K. marxianus* NBRC1777 wt strain (A and C) vs Δ PT platform strain (B and D) expressing an empty vector as control (in grey), KMAR_10531 (in blue) and the mutant KMAR_10531 N³²⁵V (in green). The consumption of xylose (open diamonds, dashed lines) and glucose (open circles, full lines) and A_{600nm} (closed symbol, full lines) are shown.

Discussion

In this study, we showed the potential of the Δ PT strain as chassis for biorefineries. The characterized KMAR_10531 and its mutants in a conserved asparagine residue N³²⁵ were transformed in the platform strain Δ PT and their growth in flasks and bioreactors on mixture of sugars was compared. It was clear that scaling up from flasks to bioreactors led to changes in variables as aeration, agitation and pH that had an impact on strain performance. For example, *K. marxianus* Δ PT expressing KMAR_10531 native transporter, in contrast with what is observed in flasks, was unable to consume all xylose in presence of glucose in the initial bioreactor experiments. In this strain, we observed a diauxic growth in which glucose is first used and then xylose starts to be imported and metabolized but, at 60 h, 11 g/L of xylose was still in the media and the sugar was not fully consumed. We could hypothesize that aeration (1 L min⁻¹), which does not occur in flasks, plays a fundamental role in this regard inducing oxidative stress during the slower xylose consumption phase. However, the incomplete xylose consumption could be solved if the pH is kept at 5.5 (Figure 5.4), showing that maintaining the proton gradient overcomes the limitation of xylose consumption and providing a strong indication that KMAR_10531 is a proton symporter. Comparison with data from other well-characterised sugar transporters supports this idea. It is reported that unpaired charged residues and formation of salt bridges in the hydrophobic core of the transporter have a crucial role in proton translocation. In the transport proteins Mal11 of *S. cerevisiae* and XylE of *E. coli*, three amino acid residues have been pointed out to mediate proton coupling (Henderson et al., 2017; Wisedchaisri et al., 2014). In KMAR_10531 we found that two of these residues are present, the negative charged Asp⁴⁵, conserved in both Mal11 (Asp¹²³) and XylE (Asp²⁷) and the positive charged Arg¹²⁸, conserved just in *E. coli* (Arg¹³³). This is not surprising as, despite sharing important structural features with *E. coli* XylE, it is reported that this key salt bridge network is not conserved in Sc_Mal11. Hence, we can conclude that also KMAR_10531 might have a different mechanism in making and breaking salt bridges and consequently proton-coupling. More studies to elucidate this aspect are needed. The mutant Δ PT + KMAR_10531 N³²⁵V is able to co-consume glucose and xylose and achieve total depletion of xylose within 60 h in reactors even without controlling the pH. In the mutant Δ PT + KMAR_10531 N³²⁵V, due to the higher affinity for xylose, the uptake of this pentose occurs at a comparable speed as glucose, overcoming the diauxic bottleneck. Thus, both sugars are metabolized at the same pace, causing a constant and high metabolic requirement of oxygen, that in the reactors is supplied at 1 L min⁻¹ during the last stage of exponential phase.

These results reveal that in *K. marxianus* Δ PT strain the expression of a transporter with similar affinity for both xylose and glucose is sufficient to enable simultaneous consumption of both sugars, in contrast to what is reported for *S. cerevisiae* (Rojas et al., 2021). Clearly, this might be related to the glucose repression mechanism in Δ PT strain and more generally in *K. marxianus* that is different from the one of *S. cerevisiae* (Nurcholis et al., 2019). Unfortunately, details related to the interferences of glucose on xylose metabolism are not sufficiently investigated in *K. marxianus* but, given the pool of transporters *K. marxianus* is provided with and the enzymes for the assimilation of pentoses, we know that *K. marxianus* has evolved differently from *S. cerevisiae*, therefore the catabolite cascade is also expected to be different (see Chapter 3).

With regard to potential for the development of cell factories and bioprocesses, it was hoped that expression of the KMAR_10531 N³²⁵V variant in the wild-type strain would allow simultaneous co-consumption of glucose and xylose. This turned out not to be the case, however, most probably due to the activity of some of the multiple other glucose transporters in a wild-type strain. The higher concentration of glucose inside the yeast cell due to the efficient uptake by these endogenous transporters (not present in the Δ PT strain) possible triggers glucose repression of xylose metabolic enzymes and degradation of transporters that could uptake xylose. It would be interesting to combine studies on the alleviation of glucose repression with expression of the variant transporter. Even in the absence of this, however, a potential chassis is available as the Δ PT strain expressing KMAR_10531 N³²⁵V allows simultaneous co-consumption of glucose and xylose, reducing significantly the fermentation time in mixed sugars media. The significant reduction in duration of the process and increase in biomass produced, is related to the nature of the platform strain, which lacks 12 genes involved in sugar transport. It is important to precise that no ethanol formation was observe when either the wild type strain or the platform strain were grown on mixture of sugars, therefore in Δ PT strain, energy most probably is saved not expressing those 12 genes and it is directed for the production of biomass. At the same time, glucose cannot efficiently enter in the yeast, and this prevents the start of a cascade of events related to repression on other carbon sources involving both transporters and metabolism.

In conclusion, the engineered Δ PT strain expressing a transporter with similar affinities for xylose and glucose represents a promising chassis strain for the complete utilization of sugars from lignocellulosic biomass in biorefineries for the development of a circular sustainable economy.

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CONCLUSIONS AND FUTURE PERSPECTIVES

Outlook on the findings of this thesis

Sugar transport across the cell membrane is the first step in sugar assimilation in living organisms. It often represents one of the major bottlenecks for the entire conversion process of carbon sources into bio-products, which is a reason why it should be considered when designing and constructing microbial cell factories. The research described in this thesis contributes to the study of evolution of sugar transporters in different yeast species (**Chapter 1**) and more in detail to the characterization of novel transporters in the non-conventional yeast *Kluyveromyces marxianus*. The overall research goal of this thesis was to identify novel pentose transporters to apply for glucose and xylose co-consumption in a lignocellulosic biomass revalorization scenario. There is a limited knowledge on this subject in *K. marxianus*, but this yeast offers traits, like its natural ability of assimilating pentose sugars, that makes it interesting from the industrial and biotechnological point of view. A deep bioinformatic analysis was performed on *K. marxianus* and 27 genes were identified as candidates to encode for sugar transporters in this yeast. Subsequently, a novel *K. marxianus* platform strain Δ PT, in which 12 transporters responsible for pentose uptake were knocked out using a CRISPR-Cas9 system, was constructed (**Chapter 2**). This platform strain was unable to uptake and grow on xylose and arabinose. The 12 deleted transporters were then individually expressed in the Δ PT strain and characterized for growth both on solid and liquid media. Activity of the most promising ones were also studied using radioactive uptake assays (**Chapter 4**). Various novel transporters belonging to the Hgt and the Kht families and an additional transporter KMAR_60179 were found to be involved in the uptake of xylose and arabinose. One Hgt-like member (KMAR_10531), which is a high affinity xylose and glucose transporter but still preferred glucose, was chosen as subject for rational mutagenesis approach in order to improve its affinity for xylose while decreasing affinity for glucose. Finally, one mutant KMAR_10531 N³²⁵V enabled the co-consumption of xylose and glucose in the platform strain Δ PT, both in flask and bioreactors experiments (**Chapter 5**), overcoming the glucose repression that affects all yeasts, including *K. marxianus*, which prevents the utilization of other carbon sources in presence of glucose (discussed in **Chapter 3**).

K. marxianus Δ PT strain as powerful tool to study sugar transporters and their evolution

The constructed platform strain represents a powerful tool that enables the identification and functional characterization of native and/or heterologous pentose transporters. Based on the results discussed in **Chapter 2**, the platform strain still grows on glucose as it does not lack all the hexose transporters, therefore it could be possible in the future to develop it further by adding the deletion of the remaining glucose and fructose transporters and/or the known four copies of genes encoding lactose transporters

(Lac12-1, Lac12-2, Lac12-3, Lac12-4). That would offer opportunities for further investigations of other transporters with various sugar specificities (for example Hgts or Lac12-like transporters from various yeast species) and could contribute to our understanding of the evolution of transporters, which was discussed in **Chapter 1**. An improved platform strain lacking lactose transporters could, for example, be used for the study of uncharacterized disaccharide transporters of other yeast species like *Pichia stipitis* or *Debaryomyces hansenii*. It is most likely that the *K. marxianus* lactose cluster, comprising the transporter (Lac12) to uptake the sugar and the enzyme for its degradation (β -galactosidase) (Naumov, 2008; Varela et al., 2017), evolved from a cluster involved in the utilization of another disaccharide, most probably plant-derived. The original transporter might have been a disaccharide transporter (maybe cellobiose) that happened to be able to also import lactose (because of the structural resemblance to the original substrate) with low rate. Disaccharide assimilation relies on hydrolase activity (glucosidase or galactosidase). Knowing that yeasts like *P. stipitis* and *D. hansenii* are provided with a pool of disaccharide transporters and hydrolases (Jeffries et al., 2009), it is possible that the disaccharide cluster was already present at the ancestral level and it subsequently evolved in *K. marxianus* to become efficient for lactose utilization in a human domestication scenario. We can hypothesize that the weak transport of lactose into the cell by the original disaccharide transporter has resulted in an evolutionary selective pressure for the β -galactosidase *LAC4* evolution and an efficient lactose transporter Lac12 that we found in dairy strains of *K. marxianus*. Further experiments are needed to unfold the evolutionary path of lactose utilization and maybe identify novel disaccharides transporters.

Studying plasticity of sugar transporters for enhanced biotechnological solutions

As underlined in **Chapter 1**, the evolutionary plasticity of sugar transporters lets them acquire new functions to adapt to diverse environments and increase the spectrum of substrates they can uptake. Most of the knowledge available about the function of all sugar transporters is with reference to what is known in *S. cerevisiae* (Leandro et al., 2009). However, we showed that there is a huge diversity and various transporters are still unknown and it is important to explore and unlock their potential. For example, most of the studies available on sugar transporters are based on Hxt-like transporters and the interesting features of Hgt-like transporters were not predicted or well-investigated before this study. Based on this, it is important to further investigate the plasticity of sugar transporters from various yeasts to take advantage of their peculiar characteristics that could be used in microbial cell factories for biotechnological applications. As example, in the previous paragraph we mentioned how we could study the evolution of lactose utilization and characterize the unknown lactose transporters of other yeasts

species like *P. stipitis* and *D. hansenii*. This could lead to the identification of novel sugar transporters that could be of help in the use of lactose, which is one of the main components of cheese whey, the main side-stream of the dairy industry. Moreover, it could be interesting to understand the relationship between disaccharide transporters, like cellobiose and lactose, with glycerol transporters that seems to be phylogenetically close and to have broad sugar specificity as some of these transporters are annotated as being capable of also transporting pentose sugars.

In fact, as shown in **Chapter 2**, among various transporters, one transporter not belonging either to Hgts or Khts, KMAR_60179, was identified and characterized as low affinity xylose transporter (**Chapter 4**). This was surprising as this transporter is homologous to *S. cerevisiae* Stl1 that is a known glycerol transporter. It was then confirmed that KMAR_60179 can also uptake glycerol. This transporter could be engineered to increase its affinity for xylose and then an ambitious application could be using it for co-transport of xylose and glycerol, which are both derived from cheap raw materials, in a yeast provided with the metabolic machinery for the assimilation of both these substrates, like *K. marxianus*.

Tearing down catabolite repression impairing glucose transport

In **Chapter 4** and **5**, to improve xylose uptake, transporter mutants were constructed with rational protein engineering approaches and characterized with radioactive uptake assays. One mutated transporter KMAR_10531 N^{325V} stood out as it was able to uptake xylose with increased affinity while the affinity for glucose decreased about 20 times, making xylose a much more efficient competitive inhibitor of glucose transport. This mutated transporter when re-introduced in the platform strain Δ PT enabled the co-consumption of glucose and xylose present in the media in the same concentrations. It is important to emphasize that the native transporter KMAR_10531 is a Hgt-like protein and this is the first study in which an Hgt-like protein is the subject of rational mutagenesis and kinetics characterization for redirection of its specificity towards xylose. Moreover, having such a transporter expressed in the *K. marxianus* platform strain Δ PT avoids the need of further engineering the yeast backbone for pentose utilization, as *K. marxianus* is naturally able to use pentoses, making the construction of the microbial cell factory effortless. In **Chapter 5**, we showed how having a transporter with increased affinity for xylose and decreased affinity for glucose expressed in a platform strain lacking most of the hexose transporters could help solving the glucose repression phenomenon that we discussed in **Chapter 3**. In fact, in Δ PT + KMAR_10531 N^{325V}, glucose is transported into the yeast cell at a slow rate (since it lacks the 12 most relevant glucose transporters and KMAR_10531 N^{325V} has been engineered to decrease glucose transport) and simultaneously with xylose, reducing the cascade of events related to catabolite

repression triggered by glucose when enters first and in high concentrations in the cell. On the other hand, we did not observe the same behavior in the wild type strain expressing this engineered transporter and a sequential consumption of glucose and xylose was observed as in the untransformed wild type strain. This strain is provided with the pool of 12 hexose transporters that quickly transport glucose inside the cell triggering glucose repression. High glucose concentration inside the cell can start a series of repression events on the assimilation of xylose and also unleash the degradation by endocytosis of some transporters not needed by the cell (like Hxt or Hgt that can also import xylose) (Barata-Antunes et al., 2021). In this regard, it could be interesting studying the ubiquitination and degradation of these transporters and strategies to avoid it, for example changing the ubiquitination signal target domain that is located in the N- or C- cytosolic tail (Tamayo Rojas, 2021).

Applications of the mutated transporter for lignocellulosic biomass revalorization

Future studies will see the application of the engineered strain $\Delta\text{PT} + \text{KMAR}_{10531} \text{N}^{325\text{V}}$ on real substrates, named lignocellulosic biomass hydrolysates. Some strains of *K. marxianus* are known to be quite tolerant to stress conditions occurring during the utilization of lignocellulosic biomass, for example the use of high temperature for processes of simultaneous saccharification and fermentation (SSF) of the biomass or inhibitors produced during the pretreatment of the biomass, like acetic acid (Leonel et al., 2021, Du et al., 2022). Therefore, assessment of the growth of *K. marxianus* NBRC1777 strain on real substrates will be the first starting point. After that, further engineering will be needed for applications of this engineered strain for the productions of various products. Some possible approaches could be to delete the xylulose dehydrogenase gene (*XYL2*) to lead to the production and accumulation of xylitol (Zhang et al., 2021) or substituting the *K. marxianus* xylose reductase and xylitol dehydrogenase genes with a xylose isomerase to avoid any redox imbalance under oxygen-limited conditions allowing xylose fermentation to ethanol (Wang et al., 2013). Some studies are reported in this regard (Bedř et al., 2021) and a key aspect to consider is the optimization of the oxygenation in the system as this could affect xylitol yield.

The results obtained in this PhD work elucidate the role of sugar transporters in the development of microbial cell factories and contribute to obtain a better understanding of how the diversity and plasticity of sugar transporters of various yeast species can be used for biotechnological and sustainable applications. We set a foundation stone for the construction of a non-conventional yeast platform strain that could be used in biorefineries, but there is still room for improvement.

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