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2025

Citation for published version (APA):
Persson, V. (2025). *The Peculiar Xylose Sensing of Saccharomyces cerevisiae*. Department of Applied Microbiology, Lund University.

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The peculiar xylose sensing of *Saccharomyces cerevisiae*

VIKTOR C. PERSSON

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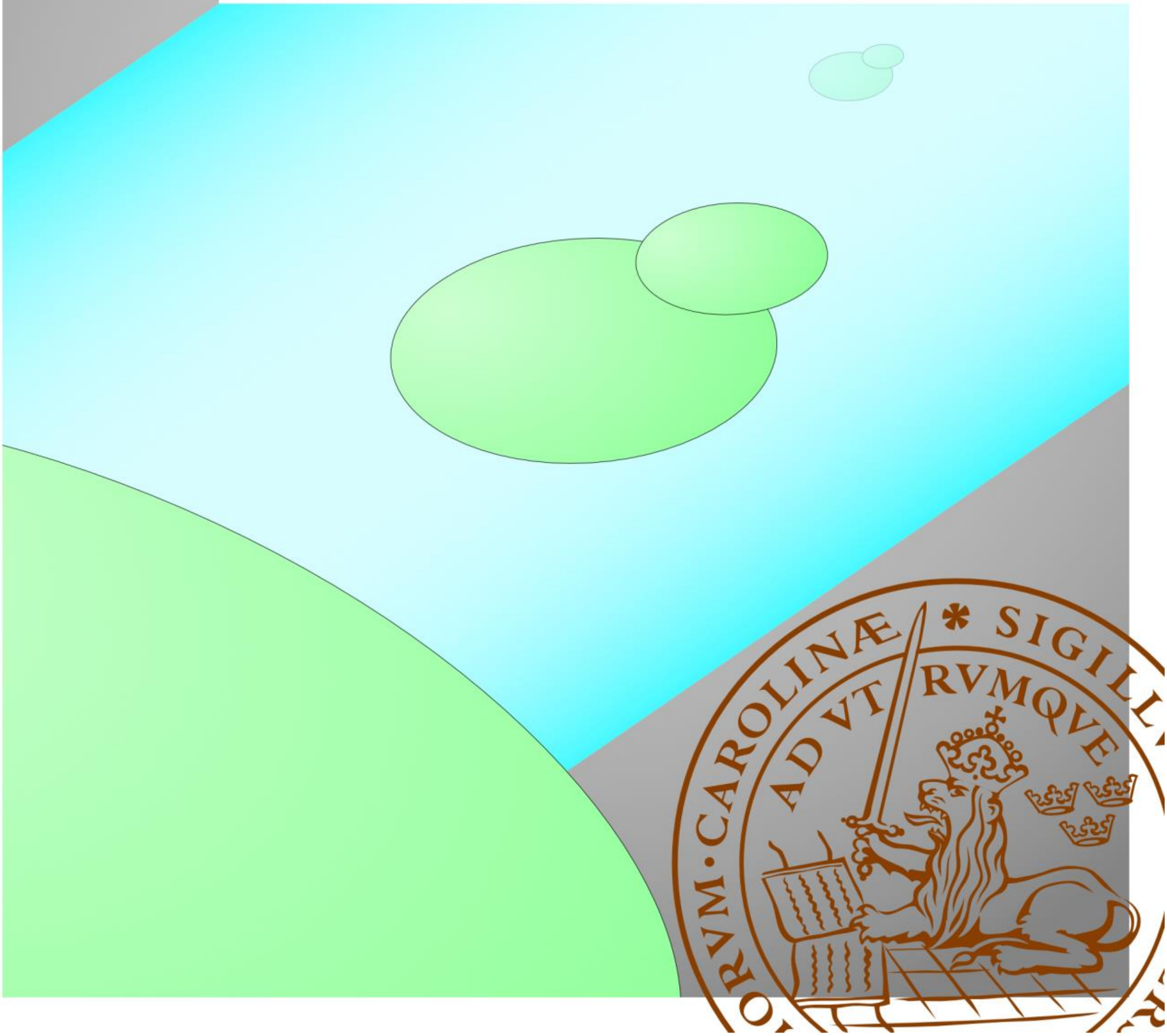

The Peculiar Xylose Sensing of *Saccharomyces cerevisiae*

Viktor C. Persson

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Engineering at Lund University to be publicly defended on 26th of May 2025 at 09.00 in lecture hall KC:B, Kemicentrum, Naturvetarvägen 22, Lund, Sweden

Faculty opponent
Associate Professor Cecilia Geijer, Division of Industrial Biotechnology,
Chalmers Institute of Technology, Gothenburg, Sweden

Organization: Lund University, Division of Biotechnology and Applied Microbiology

Document name: Doctoral Dissertation

Date of issue 2025-05-26

Author(s): Viktor C. Persson

Sponsoring organization: Lund University

Title and subtitle: The Peculiar Xylose Sensing of *Saccharomyces cerevisiae*

Abstract:

Robust and rapid xylose utilization is essential for developing sustainable bioprocesses capable of converting pentose-rich lignocellulosic biomass into renewable chemicals. *Saccharomyces cerevisiae* is a popular choice for industrial bioprocesses due to its high product yields, robust performance, and stress tolerance. Unfortunately, *S. cerevisiae* does not natively utilize xylose. Decades of research have led to the design of engineering strategies that enable and substantially improve the conversion of xylose, but further improvements are still needed to effectively compete with petroleum-based alternatives. Efforts have mostly targeted individual enzymes and proteins related to the central carbon metabolism, leading to a stepwise refinement over time. An alternative approach is to target the cellular regulatory network controlling the overall sugar utilization, enabling simultaneous modulation of dozens of downstream enzymes at once. This concept forms the central theme of my thesis. By investigating the response of *S. cerevisiae* regulatory networks to xylose, and subsequently engineering them to exhibit altered responses, I aimed to enhance xylose utilization for the production of sustainable biochemicals.

Three main pathways detect and respond to glucose and other carbon sources in *S. cerevisiae*: the Snf3p/Rgt2p pathway, the SNF1 pathway, and the PKA pathway. These pathways control sugar transport, alternative carbon utilization, and sugar feasting, respectively. Previous studies of these pathways have shown that xylose is not perceived as a rapidly fermentable sugar, as evidenced by the activation of the SNF1 pathway and lack of PKA activity. In my work, I found evidence that specific metabolic intermediates formed during xylose catabolism (fructose-6-phosphate in particular), and extracellular xylose itself for non-metabolizing cells, are likely behind this response. In addition, I showed that the response was independent from the xylose utilization pathway employed, and that the response occurred in both laboratory and industrial strains. As such, the response is a widespread phenomenon and represents an important target for future strain improvement.

In addition to this, I endeavored to design and implement xylose-specific receptors to co-stimulate all three pathways since this has been demonstrated to improve xylose utilization. Two separate approaches were pursued: 1) chimeric receptors were constructed by combining xylose-binding transporters with signaling domains to trigger the Snf3p/Rgt2p pathway, and 2) mutagenizing the PKA-activating

G-protein coupled receptor Gpr1p. While the chimeric constructs initially showed promise, it was later revealed that the altered sugar signaling response was likely due to residual transport activity rather than signaling. In silico modelling of mutant Gpr1p candidates indicated potential for xylose binding, leading to the construction of a genetic library; however, screening of the library remains to be performed.

Overall, this thesis represents a step forward in the understanding of sugar signaling in *S. cerevisiae* on xylose and ways to alter it for improved pentose utilization.

Key words: *Saccharomyces cerevisiae*, sugar signaling, sensing, cellular regulation, xylose, lignocellulose, SNF1, PKA, Snf3, Rgt2, Gpr1, sustainability, bioeconomy

Language: English

Number of pages: 104

ISBN: 978-91-8096-106-6

Signature

Date 2025-03-31

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LUND
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Coverphoto:
Flow cytometry from the perspective of the cell
by Viktor C. Persson, 2024.

Division of Biotechnology and Applied Microbiology
Faculty of Engineering
Department of Process and Life Science Engineering
Lund University

ISBN, printed version: 978-91-8096-106-6
ISBN, digital version: 978-91-8096-107-3

Printed in Sweden by Media-Tryck, Lund University
Lund 2025

NORDIC SWAN ECOLABEL
Printed matter
3041 0903

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MADE IN SWEDEN

I have found, through painful experience, that the most
important step a person can take
is always the next one.

Table of Contents

1.1. Abstract

Robust and rapid xylose utilization is essential for developing sustainable bioprocesses capable of converting pentose-rich lignocellulosic biomass into renewable chemicals. *Saccharomyces cerevisiae* is a popular choice for industrial bioprocesses due to its high product yields, robust performance, and stress tolerance. Unfortunately, *S. cerevisiae* does not natively utilize xylose. Decades of research have led to the design of engineering strategies that enable and substantially improve the conversion of xylose, but further improvements are still needed to effectively compete with petroleum-based alternatives. Efforts have mostly targeted individual enzymes and proteins related to the central carbon metabolism, leading to a stepwise refinement over time. An alternative approach is to target the cellular regulatory network controlling the overall sugar utilization, enabling simultaneous modulation of dozens of downstream enzymes at once. This concept forms the central theme of my thesis. By investigating the response of *S. cerevisiae* regulatory networks to xylose, and subsequently engineering them to exhibit altered responses, I aimed to enhance xylose utilization for the production of sustainable biochemicals.

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In addition to this, I endeavored to design and implement xylose-specific receptors to co-stimulate all three pathways since this has been demonstrated to improve xylose utilization. Two separate approaches were pursued: 1) chimeric receptors were constructed by combining xylose-binding transporters with signaling domains to trigger the Snf3p/Rgt2p pathway, and 2) mutagenizing the PKA-activating G-protein coupled receptor Gpr1p. While the chimeric constructs initially showed promise, it was later revealed that the altered sugar signaling response was likely due to residual transport activity rather than signaling. *In silico* modelling of mutant Gpr1p candidates indicated potential for xylose binding, leading to the construction of a genetic library; however, screening of the library remains to be performed.

Overall, this thesis represents a step forward in the understanding of sugar signaling in *S. cerevisiae* on xylose and ways to alter it for improved pentose utilization.

1.2. Popular science summary

For thousands of years, mankind has maintained a symbiotic relationship with baker's yeast: we provide fermentable sugars, and it provides us with beer, wine and bread. In recent decades, this partnership has been broadened to also include the production of advanced biochemicals, such as plastics and biofuels.

The use of fermentation for sustainable chemical production has become an important strategy to replace petroleum products and help meet climate goals. However, growing crops for the sole purpose of turning it into biochemicals is not feasible, since this takes up agricultural land that could otherwise be used to feed the growing population. This led scientists to ponder: *"What if, instead of using the corncobs, we use the stalks?"*. Brilliant! Use the crop for food as usual while using the waste for biochemicals, a potential win-win situation. However, this approach comes with a major challenge: the corn stalks contain different types of sugars, some of which are not used by yeast.

We are all familiar with table sugar, scientifically known as sucrose. Depending on your background, you may also have encountered glucose and fructose syrup in the kitchen—the monomeric sugars that compose sucrose. Similar to us, baker's yeast loves these sugars, which are abundant in corn. Most of the sugar in corn kernels is stored as starch, long chains of glucose. However, the stalks contain sugar chains in the form of cellulose and hemicellulose. While the cellulose is entirely made up of glucose, the hemicellulose is rich in other types of sugars, primarily xylose. This poses a problem, as baker's yeast cannot naturally ferment xylose.

Using DNA technologies, it has become possible to design baker's yeast that can utilize xylose. However, the yeast still takes longer to utilize xylose than glucose, as it favors the latter. One potential reason for this is that the yeast does not seem to recognize xylose as a fermentable sugar. Instead, it prepares itself for starvation and slows down its metabolism. Yet, at the same time, the yeast does not appear to be fully starving either.

In this thesis, I aimed to investigate this peculiar response to xylose. By understanding how, and to what extent, xylose is perceived—we might identify ways to trick the yeast into recognizing xylose as more fermentable. Prior research has indicated that improving the use of xylose is possible by tricking baker's yeast into perceiving everything as fermentable. However, this approach had significant drawbacks as the yeast became highly susceptible to stress after consuming all the sugars. Bearing this in mind, I explored ways to trigger the same fermentation response only when xylose was available using modified sugar receptors, with the aim of achieving the best of both worlds.

1.3. Populärvetenskaplig sammanfattning

I tusentals år har människan haft ett avtal med jästsvampen *Saccharomyces cerevisiae*: vi ger den socker i utbyte mot öl, vin, och bröd. Under de senaste decennierna har vi utvidgat denna överenskommelse till att även inkludera produktionen av avancerade biokemikalier som nu används till plast och biobränslen.

Att fermentera fram hållbara biokemikalier har blivit en viktig strategi för att ersätta petroleumprodukter och nå klimatmål. Att odla grödor enbart för att omvandla dem till biokemikalier är dock inte hållbart, eftersom detta tar upp jordbruksmark som annars skulle kunna användas för att föda en växande befolkning. Detta fick forskare att fundera: "Tänk om vi använder stjälkarna istället för själva majsen?" Genialiskt! Använd grödan som vanligt i kosten, och använd restprodukterna för att göra biokemikalier. Men det finns ett stort problem med detta: majsskälkarna innehåller mycket av fel sorts sockerart.

Vi är alla bekanta med strösocker, även känd som sackaros. Beroende på din bakgrund kanske du till och med har stött på glukos- och fruktossirap i köket—de sockerarter som tillsammans bygger upp sackaros. Precis som vi så älskar jäst dessa sockerarter, och majs har massvis av dem. Det mesta av sockret lagras som stärkelse, långa kedjor av glukos. Men istället för stärkelse innehåller majsskälkarna socker i form av cellulosa och hemicellulosa i ett förhållande av 2:1. Medan cellulosa enbart består av glukos, är hemicellulosan fylld med andra typer av sockerarter—främst xylos. Och här uppstår problemet: jästen kan inte använda sig av xylos.

Med hjälp av DNA-teknologi har det blivit möjligt att modifiera bagerijäst så att den kan jäsa xylos. Det tar dock fortfarande längre tid för jästen att använda xylos än det tar att använda glukos. En trolig anledning till detta är att jästen inte verkar se xylos som en jäsbar sockerart. Istället verkar det so matt den förbereder sig inför svält och saktar ner sin metabolism. Men, samtidigt så verkar jästen dock inte vara helt utsvulten heller.

I denna avhandling har jag undersökt denna förunderliga responsen till xylos. Genom att förstå hur, och i vilken utsträckning, jästen uppfattar xylos kan vi kanske hitta sätt att lura jästen att se xylos som mer jäsbart. Tidigare studier har visat att om man får jästen att tro att allting är jäsbart (alltså även luften), så förbättras utnyttjandet av xylos. Detta kommer dock till ett högt pris: jästen blir mycket stresskänslig när sockret tar slut. Med detta i åtanke har jag undersökt sätt att trigga samma jäsrespons, men endast när xylos är tillgängligt, med förhoppningen att uppnå det bästa av båda världar.

1.4. List of publications and scientific contributions

The thesis is based on the following research articles and manuscripts, which can be found indexed by roman numerals at the end of the booklet.

Paper I

Title: *Impact of xylose epimerase on sugar assimilation and sensing in recombinant* Saccharomyces cerevisiae *carrying different xylose-utilization pathways*.

Authors: Viktor C. Persson[‡], Raquel Perruca Foncillas[‡], Tegan R. Anderes, Clément Ginestet, Marie F. Gorwa-Grauslund.

Status: Published in *Biotechnology for Biofuels and Bioproducts*, https://doi.org/10.1186/s13068-023-02422-z, 2023.

Contribution: I conceptualized and designed the project, and then performed: strain construction, flow cytometry, HPLC analysis, data acquisition, data interpretation, statistical analysis, student supervision, original manuscript drafting, and manuscript revision.

Paper II

Title: *Applying the Weimberg pathway in* Saccharomyces cerevisiae *for growth and sensing studies on xylose as the sole carbon source.*

Authors: Viktor C. Persson, Celina Borgström, Nina Egeler, Marie F. Gorwa-Grauslund.

Status: Under final internal revision before submission.

Contribution: I conceptualized major parts of the project, and then performed: strain engineering, plasmid construction, enzyme assays, UHPLC analysis, HPLC analysis, metabolite extraction and derivatization, metabolomics analysis, flask cultivation, bioreactor cultivations, microbial adaptation, flow cytometry, instrument manufacturing, data analysis, data interpretation, statistical analysis, student supervision, project management, original manuscript drafting, and manuscript revision.

Paper III **Title**: *Using phosphoglucose isomerase-deficient (pgi1Δ)* Saccharomyces cerevisiae *to map the impact of sugar phosphate levels on d-glucose and d-xylose sensing*.

Authors: Celina Borgström[‡], Viktor C. Persson[‡], Oksana Rogova, Karen O. Osiro, Ester Lundberg, Peter Spégel,
Marie F. Gorwa-Grauslund.

Status: Published in *Microbial Cell Factories*, https://doi.org/10.1186/s12934-022-01978-z, 2022.

Contribution: I developed screening and culture protocols, and performed: cultivations, flow cytometry, HPLC analysis, metabolite extraction, data analysis, data interpretation, statistical analysis, and extensive manuscript revision.

Paper IV **Title**: *Glucose receptor deletion and engineering: impact on xylose sensing and utilization in* Saccharomyces cerevisiae.

Authors: Bruna C. Bolzico[‡], Viktor C. Persson[‡], Raúl N. Comelli, Marie F. Gorwa-Grauslund.

Status: Submitted to *FEMS Yeast Research*, 2025.

Contribution: I conceptualized and designed major parts of the project, and aided in: plasmid construction, CRISPR integrations, flow cytometry, HPLC analysis, cultivations, data analysis, data interpretation, statistical analysis, project management, and extensive manuscript revision.

Paper V **Title**: *Exploring sugar signaling dynamics in industrial* Saccharomyces cerevisiae *strains with enhanced xylose metabolism*.

Authors: Gisele Cristina de Lima Palermo, Viktor C. Persson, Marie F. Gorwa-Grauslund, Leandro Vieira dos Santos.

Status: Draft manuscript.

Contribution: I conceptualized and designed major parts of the project, and aided in: CRISPR integrations, flow cytometry, HPLC analysis, cultivation, instrument manufacturing, data analysis, data interpretation, statistical analysis, project management, original manuscript drafting, and manuscript revision.

Paper VI

Title: *Rational engineering of Gpr1p for xylose sensing in* Saccharomyces cerevisiae.

Authors: Viktor C. Persson, Marie F. Gorwa-Grauslund.

Status: Draft manuscript.

Contribution: I conceptualized and designed major parts of the project, and performed: strain engineering, plasmid construction, flow cytometry, cultivation, *in silico* modeling and molecular docking, data analysis, data interpretation, statistical analysis, student supervision, project management, original manuscript drafting, and manuscript revision.

Additionally, I have contributed to the following publications, which are not included in the thesis:

Review article

Title: *D-Xylose Sensing in* Saccharomyces cerevisiae*: Insights from D-Glucose Signaling and Native D-Xylose Utilizers*.

Authors: Daniel P. Brink, Celina Borgström, Viktor C. Persson, Karen Ofuji Osiro, Marie F. Gorwa-Grauslund.

Status: Published in International Journal of Molecular Sciences, https://doi.org/10.3390/ijms222212410, 2021.

Contribution: I researched and wrote key chapters, took part in original manuscript drafting, and critically reviewed and revised the manuscript.

Research article

Title: *Evaluation of Pyrophosphate-Driven Proton Pumps in* Saccharomyces cerevisiae *under Stress Conditions*.

Authors: Krishnan Sreenivas, Leon Eisentraut, Daniel P. Brink, Viktor C. Persson, Magnus Carlquist, Marie F. Gorwa-Grauslund, Ed W. J. van Niel.

Status: Published in Microorganisms, https://doi.org/10.3390/microorganisms12030625, 2024.

Contribution: I performed data analysis, data interpretation, and critically reviewed and revised the manuscript.

1.5. Abbreviations

αKG	α-ketoglutarate
ADP	Adenosine diphosphate
AEC	Adenylate energy charge
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate kinase
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CCR	Carbon catabolite repression
CRISPR	Clustered regularly interspaced short palindromic repeat
F16bP	Fructose-1,6-bisphosphate
F6P	Fructose-6-phosphate
FAD	Flavin adenine dinucleotide
FRET	Förster resonance energy transfer
FSC	Forward scatter
G6P	Glucose-6-phosphate
Gal2mut	Mutant of Gal2p with xylose specificity
Gal2RT	Gal2mut fused with Rgt2p signaling domain
Gal2ST	Gal2mut fused with Snf3p signaling domain
GAP	GTPase activating protein
GEC	Guanylate energy charge
GEF	Guanine exchange factor
GFP	Green fluorescent protein
HDR	Homology directed repair
HOG	High osmolarity glycerol
IVHOR	*In vivo* homologous overlap recombination
MFIs	Mean fluorescence intensities
NAD	Nicotinamide adenine dinucleotide

NADP	Nicotinamide adenine dinucleotide phosphate
NHEJ	Non-homologous end joining
PDSE	Post-diauxic shift element
PI	Propidium iodide
PKA	Protein kinase A
PPP	Pentose phosphate pathway
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
SSC	Side scatter
STRE	Stress responsive element
TCA	Tricarboxylic acid
Thr	Threonine
XI	Xylose isomerase
XR/XDH	Xylose reductase/xylitol dehydrogenase

1.6. Key genes and proteins

Bcy1p	Regulatory subunit of PKA
Cyc8p	Transcriptional cofactor (repressor)
Cat8p	Activator of gluconeogenesis genes
Cyr1p	cAMP producing adenylate cyclase
Gpa2p	G-protein alpha subunit linked to extracellular sugar sensing
Gpr1p	G-protein coupled receptor linked to extracellular sugar sensing
Glc7p	General protein phosphatase
HXT	Hexose transporter encoding gene
Ira2p	GTPase-activating protein linked to intracellular sugar sensing
Mig1p	Glucose repression transcription factor
Msn2/4p	Stress response transcription factors
Mth1p	Transcriptional co-repressor (transporters)
Pfk1/2p	Phosphofructokinase 1/2 producing F16bP from F6P
Pgi1p	Phosphoglucose isomerase interconverting G6P and F6P
Ras1/2p	Small GTPases linked to intracellular sugar sensing
Rgt2p	Glucose transporter-inducing receptor
Rds2p	Activator of gluconeogenesis genes
Reg1p	Partner of Glc7p used for SNF1 inactivation
Snf3p	Glucose transporter-inducing receptor
SUC2	Sucrose invertase encoding gene
Sak1p	SNF1 activating kinase
Sip1p	SNF1 complex localization subunit
Snf4p	SNF1 complex regulatory subunit
Std1p	Transcriptional co-repressor (transporters)
Tpk1/2/3p	Catalytic subunits of PKA
TPS1	Trehalose-6-phosphate synthase encoding gene

Chapter 1.

Introduction.

While it is widely acknowledged that global greenhouse gas emissions must be reduced, achieving this requires a comprehensive understanding of their sources and the means to mitigate them. A global emission analysis from 2020 underscored this complexity by examining emissions across sectors and sub-sectors (Ritchie, 2020). The study revealed that while major contributors demand immediate attention, meaningful progress cannot be achieved without also addressing smaller sub-sectors (Ritchie, 2020). This doctoral thesis contributes to these efforts by focusing on the valorization of lignocellulosic biomass, an abundant and renewable resource. Specifically, it explores how the regulatory networks in yeast can be leveraged to reduce emissions from crop burning—the act of burning agricultural waste after harvest—and the forestry industry. By employing yeast to convert waste and residuals into biochemicals, which serve as sustainable alternatives to petroleum-based products, excess emissions could be reduced (Figure 1.1).

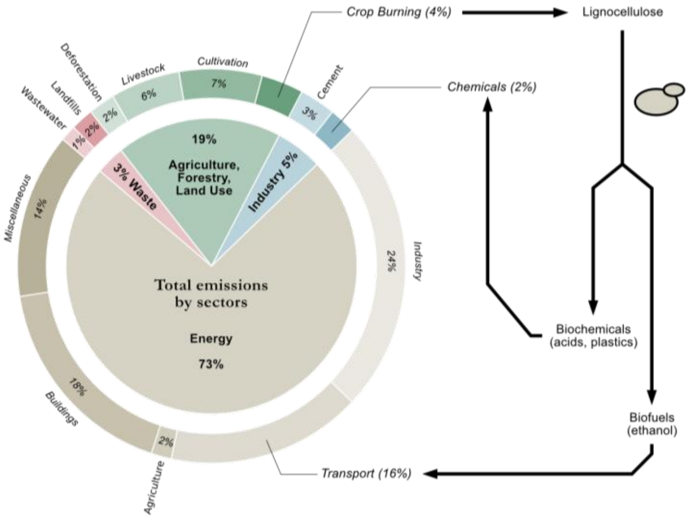


Figure 1.1. Greenhouse gas emissions by sector and sub-sector.

1.1. Lignocellulose

1.1.1. An abundant and renewable feedstock

Post-harvest agricultural residues and forestry byproducts primarily consist of lignocellulose, a heterogeneous material composed of cellulose, hemicellulose, and lignin (Park et al., 2020). While the exact composition varies, these polymers are typically present in a 2:1:1 ratio (Hassan et al., 2018). Cellulose and hemicellulose are both sugar polymers, with cellulose consisting of glucose monomers in a linear chain whereas hemicellulose consists of various pentose and hexose sugars in branched chains (Aditiya et al., 2016). Lignin, in contrast, is a complex polymer of aromatic compounds and is particularly recalcitrant to hydrolysis (Park et al., 2020).

The sugar polymers, especially cellulose, are of particular interest due to their potential microbial conversion into bioethanol and other valuable products (Park et al., 2020). Despite abundant lignocellulosic biomass, its use in industrial bioethanol production remains minimal. In Europe, only 0.5% of bioethanol derives from lignocellulose, while in Brazil and the United States—the world's largest bioethanol producers—it accounts for only 0.2% of total production (IEA, 2023, 2024). Instead, the majority of bioethanol derives from first-generation (1G) agricultural feedstocks such as corn and sugarcane, competing with food production. Notably, approximately half of the total crop weight—in the form of lignocellulose-rich stalks and husks—is used as animal feed or burned rather than being further valorized into valuable chemicals (Jain & Kumar, 2024). Ethanol derived from such lignocellulosic feedstocks is termed second-generation (2G) bioethanol.

1.1.2. Barriers to second-generation bioethanol production

Although 2G bioethanol is gaining traction, it faces technological and economic challenges that hinder large-scale implementation without significant governmental subsidies (IEA, 2023). Barriers include: (i) pretreatment inefficiencies that limit sugar polymer hydrolysis, (ii) low microbial tolerance to process conditions and pretreatment-derived inhibitors, and (iii) inadequate utilization of key sugars by current industrial microbes (Aditiya et al., 2016; Robak & Balcerek, 2018; Roukas & Kotzekidou, 2022). These factors collectively impact the titers, production rates, and yields—critical economic drivers in biomass processing (Wyman et al., 2005).

This thesis focuses on improving pentose sugar utilization by baker's yeast, *Saccharomyces cerevisiae*, which represent a critical issue given that pentose sugars can constitute up to 30% of the total sugars found in lignocellulosic biomass (Gao et al., 2009; Gírio et al., 2010; Mensah et al., 2021). Although emphasis is put on pentose catabolism and ethanol as a product, microbial bioconversion can also be considered for numerous other valuable biochemicals (Sun & Jin, 2021).

1.1.3. Pretreatment, hydrolysis, and inhibitors

For microbes to use the sugars in lignocellulose, the feedstock must first undergo pretreatment and hydrolytic depolymerization (Aditiya et al., 2016). Although pretreatment is among the most expensive steps in the process, it significantly improves the hydrolysis efficiency and thus sugar availability (Wyman et al., 2005).

Various pretreatment methods exist, each with unique advantages and limitations (Table 1.1), but they all share the common goal of increasing surface area and reducing crystallinity (Aditiya et al., 2016; Chundawat et al., 2020; Gírio et al., 2010; Hahn-Hägerdal et al., 2007a; Jain & Kumar, 2024; Park et al., 2020; Robak & Balcerek, 2018; Shrotri et al., 2017; Trček et al., 2015; Tsafrakidou et al., 2023; Wyman et al., 2005; Ziegler-Devin et al., 2021). Pretreatment facilitates depolymerization by shortening polymers, solubilizing oligomers, and fractionating cellulose, hemicellulose, and lignin (Aditiya et al., 2016; Wyman et al., 2005; Ziegler-Devin et al., 2021). Lignin removal and separate valorization is crucial, since aromatic compounds can inhibit enzymatic hydrolysis (Park et al., 2020; Wyman et al., 2005; Ximenes et al., 2011). Effective pretreatment must balance environmental sustainability, cost-efficiency, and minimize the formation of microbial inhibitors (Aditiya et al., 2016; Robak & Balcerek, 2018). Given these considerations (Table 1.1), STEX may be the most viable pretreatment method despite the formation of inhibitors, even though it necessitates additional downstream enzymatic strategies or the selection of inhibitor-tolerant strains.

Table 1.1. Overview of pretreatment options in lignocellulosic bioprocessing.

Method	Description	Advantages	Limitations
Steam Explosion (STEX)	High-pressure steam increasing surface area, shortens fibers, and releases acetic acid.	Scalable, cost-effective process, environmentally friendly.	Generates microbial inhibitors (weak acid, furaldehydes), costly equipment.
Ammonia Fiber Expansion (AFEX)	Anhydrous ammonia under pressure disrupts biomass structure.	Lower inhibitor formation, ammonia can serve as nitrogen source.	High energy costs, hazardous to workers, limited efficacy on lignin-rich biomass.
Ammonia Recycle Percolation (ARP) / Aqueous Ammonia Soaking (AAS)	Ammonia delignifes biomass while minimizing inhibitor formation.	Reduces inhibitors, enables hardwood processing.	Requires precise temperature control, energy-intensive.
Concentrated or dilute acid addition	Acids hydrolyzes fibers and release sugars.	High sugar yields, simple process.	Generates inhibitors, causes equipment corrosion, heat.
Biological Pretreatment	Fungi or enzymes degrade lignin and cellulose.	Environmentally friendly, avoids chemical additives.	Slow processing rates, impractical for large-scale use.

After pretreatment, sugar polymers can be hydrolyzed into monomeric sugars using either acid hydrolysis or enzymatic hydrolysis with commercially available cellulases and hemicellulases (Aditiya et al., 2016). Acid hydrolysis, while effective, suffers from the same drawbacks as acid pretreatment, including lower yields and the formation of inhibitory byproducts (Roukas & Kotzekidou, 2022; Xiao et al., 2004). Acids not only directly inhibit microbial activity by processes such as futile cycling (Trček et al., 2015), they also cause the degradation of hexose sugars into additional inhibitors such as hydroxymethyl-furfural (HMF) and furfural (Robak & Balcerek, 2018). Enzymatic hydrolysis, in contrast, achieves higher yields without introducing additional inhibitors. However, the recalcitrant nature of lignocellulose and the presence of inhibitors necessitate the use of 30 to 50 times more enzyme in 2G bioethanol processes compared to 1G production, significantly increasing operational costs (IEA, 2023). Furthermore, although applying cellulases and hemicellulases together simplifies processing, sugars released from hemicellulose like mannose, xylose, and galactose can inhibit cellulase activity, potentially reducing overall efficiency (Xiao et al., 2004).

Despite these challenges in pretreatment and hydrolysis, several biorefineries are already operational and the 2G bioethanol process has been assessed at a technology readiness level (TRL) of 8 out of 9 (Jain & Kumar, 2024). Achieving full commercialization (TRL 9) requires further improvements to ensure economic viability without reliance on government subsidies (Jain & Kumar, 2024). A key strategy is maximizing sugar conversion efficiency, ensuring that all released sugars, including pentose sugars, contribute to bioproduct formation rather than focusing solely on hexose sugars.

1.2. Xylose and yeast

1.2.1. Pentose utilization

Given that pentose sugars account for nearly one-third of the total sugar content in lignocellulosic feedstocks, with xylose as the predominant one, efficient microbial utilization of xylose is essential for optimizing bioprocessing. Several native xylose-fermenting organisms have been identified in nature, including: *Escherichia coli*, *Zymomonas mobilis*, *Scheffersomyces stipitis*, and *Spathaspora passalidarum* (Bañares et al., 2021; T. W. Jeffries & J. R. H. Van Vleet, 2009; Martinez-Jimenez et al., 2021; Xia et al., 2019). However, many of native xylose utilizers produce undesirable byproducts and exhibit limited tolerance to the harsh industrial conditions encountered in lignocellulosic bioprocessing (Hahn-Hägerdal et al., 2007a; Robak & Balcerek, 2018; Toivola et al., 1984). These conditions include microbial inhibitors from the pretreatment, as well as environmental stressors such as low pH, high osmolarity, and high ethanol concentrations (Hahn-Hägerdal et al., 2007a; Robak & Balcerek, 2018). While these stressors help prevent contamination, they also impose significant demands on production microbes (Robak & Balcerek, 2018). Consequently, native xylose utilizers are often impractical for industrial application without significant efforts towards improving their tolerance. In contrast, *S. cerevisiae* is widely used in industrial bioprocessing due to its robustness, ethanol tolerance, and well-characterized genetic system (Hahn-Hägerdal et al., 2007a). However, it naturally lacks the ability to efficiently metabolize xylose.

This leaves three possible approaches: (i) engineering industrial *S. cerevisiae* strains for efficient xylose utilization, (ii) improving the robustness of native xylose utilizers, or (iii) developing microbial consortia where different organisms specialize in detoxification or sugar utilization. This thesis focuses primarily on the first approach—enabling xylose catabolism in recombinant *S. cerevisiae*.

1.2.2. Genetic tools for *S. cerevisiae*

S. cerevisiae is a widely utilized organism in bioprocesses, owing not only to its tolerance to industrial conditions but also to its genomic stability, high ethanol productivity, and ease of genetic engineering (de Oliveira Vargas et al., 2023). A robust cellular DNA repair system contributes to both maintaining genome stability in diploid strains and enhancing gene editing efficiency in haploid strains, thereby facilitating genetic modifications (Haber, 2018).

Gene editing tools such as the Cas9 endonuclease are capable of inducing double-strand DNA breaks (DSBs) but do not inherently insert or remove genetic material (Rainha et al., 2020). Instead, these modifications rely on the cellular DNA repair mechanisms. In *S. cerevisiae*, two predominant DNA repair pathways govern genome modifications: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Haber, 2018). Notably, HDR is highly active in *S. cerevisiae* compared to most other yeast species, enabling gene replacement through the introduction of a recombinant homologous sequence, either as a linear DNA fragment or an integrative plasmid (Cai et al., 2019; Haber, 2018).

The HDR mechanism in *S. cerevisiae* is sufficiently active to support in vivo homologous overlap recombination (IVHOR), wherein multiple sequentially overlapping DNA sequences can be assembled directly within the yeast cell, as illustrated in Figure 1.2 (Swers et al., 2004). IVHOR played a crucial role in the strain construction strategies employed in the studies linked to this thesis, occasionally enabling the generation of new recombinant yeast strains within a week. However, in certain instances, this approach was unsuccessful, necessitating the traditional construction of plasmids via molecular biology techniques.

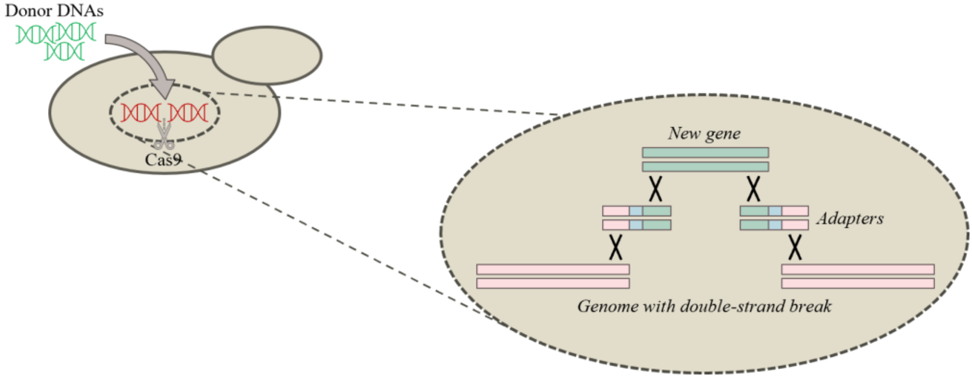


Figure 1.2. *S. cerevisiae*'s powerful recombinatory machinery allows for *in vivo* homologous recombination of multiple DNA fragments at once, minimizing the need to constructing plasmids and speeding up strain construction. Crosses represent homologous recombination events.

1.2.3. Limits in xylose metabolism by recombinant *S. cerevisiae*

Over the past three decades, extensive research has been dedicated to enable efficient xylose metabolism in *S. cerevisiae*. Strategies have included introducing genes from native xylose-utilizing species, upregulating the endogenous pentose phosphate pathway (PPP), and improving xylose transport (Figure 1.3).

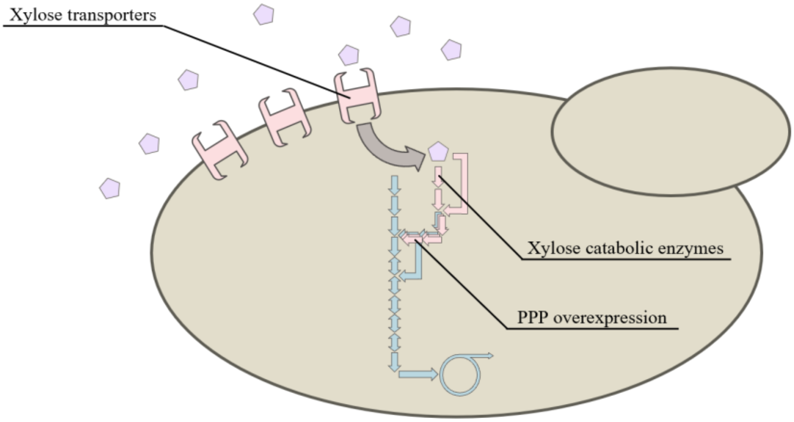


Figure 1.3. Summary of strategies for improved xylose utilization in recombinant *S. cerevisiae*. Modifications are highlighted in pink. The arrows show a simplified version of glycolysis, the PPP, the TCA cycle, and the xylose catabolic pathways.

These modifications have facilitated xylose utilization, leading to the production of ethanol and other valuable biochemicals (Hahn-Hägerdal et al., 2007a; Park et al., 2020; Sun & Jin, 2021). However, this catabolism is yet to be fully optimized as several challenges persist, including inefficient co-consumption of xylose in the presence of high glucose concentrations and relatively slow xylose utilization rates compared to glucose (Brink et al., 2021; Hahn-Hägerdal et al., 2007a; Wasylenko & Stephanopoulos, 2015). The underlying cause of these limitations is only partly understood but is hypothesized to involve issues related to xylose transport and/or dysregulation of central carbon metabolism on xylose (Brink et al., 2021; Hahn-Hägerdal et al., 2007a; Kötter & Ciriacy, 1993; Rojas et al., 2021).

Inefficient transport

In non-engineered *S. cerevisiae*, xylose uptake occurs via promiscuous hexose transporters with relatively low affinity for xylose compared to glucose (Kötter & Ciriacy, 1993; Roca et al., 2004). This results in competitive inhibition when glucose is abundant and inefficient transport when xylose concentrations are low (Kötter & Ciriacy, 1993). Attempts to circumvent this limitation have included overexpressing the endogenous hexose transporters with the highest propensity for xylose transport—such as *HXT1*, *HXT7*, and *GAL2*—although these efforts have not fully overcome the issue (Gonçalves et al., 2014; Nijland & Driessen, 2019). Long term, this is likely not the best approach since the intense overexpression constitutes a loss of energy which could be redirected into product formation.

Efforts to instead engineer mutant hexose transporters with increased xylose affinity and reduced glucose affinity have shown promise—especially those based on the *GAL2*-encoded transporter (Jiang et al., 2020; Nijland & Driessen, 2019; Rojas et al., 2021). Such mutants have enabled more similar uptake rates between glucose and xylose, although it should be noted that this is partly due to limiting the overall glucose uptake which would be counter-productive in an industrial setting. An alternative approach is to search for high-affinity xylose transporters in native xylose-utilizing yeasts; this has led to promising but mixed results (Hector et al., 2008; Jiang et al., 2020; Katahira et al., 2008; Leandro et al., 2006; Nijland & Driessen, 2019; Runquist et al., 2010; Young et al., 2011).

An issue with sensing and regulation

A complex network of regulatory proteins coordinates cellular responses to diverse and changing environmental conditions in *S. cerevisiae* (Brink et al., 2021; Broach, 2012; Conrad et al., 2014). This regulatory system is especially pronounced in response to excess glucose, where yeast cells undergo global transcriptional changes to prioritize glucose utilization, transport, and fermentation (Conrad et al., 2014). Conversely, when glucose levels are low, gene expression shifts to favor ethanol respiration, sucrose hydrolysis, and storage carbohydrate synthesis via gluconeogenesis (Conrad et al., 2014).

Given that *S. cerevisiae* does not natively metabolize xylose, it is unclear how its regulatory networks would respond to the pentose sugar. Perhaps most likely, the cells would exhibit a gene expression patterns characteristic of glucose starvation. Such a response would represent a fundamental challenge, as starvation typically leads to cellular energy preservation strategies such as downregulation of glycolysis and sugar transport, both of which are important for efficient xylose metabolism (Roca et al., 2004; Wasylenko & Stephanopoulos, 2015). However, in recombinant xylose-utilizing *S. cerevisiae*, xylose does elicit a regulatory response (Bergdahl et al., 2012; Bergdahl et al., 2013; Brink et al., 2021; Jin & Jeffries, 2004; Matsushika et al., 2014; Osiro et al., 2018; Salusjärvi et al., 2008; Salusjärvi et al., 2006; Wasylenko & Stephanopoulos, 2015; Wu et al., 2020). This response mirrors that observed under low-glucose conditions, rather than starvation, which could be advantageous in terms of increased expression of xylose-transporting proteins (Osiro et al., 2018; Roca et al., 2004). Unfortunately, this regulatory state is also associated with ethanol respiration and gluconeogenesis—processes that are directly counterproductive to efficient xylose fermentation.

The present thesis aims to investigate the underlying causes of this putative dysregulation and explores the possibility of modifying the associated regulatory mechanisms to enhance xylose utilization for biochemical production. Chapter 2 provides an overview of cellular regulation mechanisms in *S. cerevisiae*, highlights the specific cellular response to xylose, and identifies potential causes of this response. Chapter 3 examines these hypothesized causes through experimental investigation. Chapter 4 explores strategies to modify or circumvent this regulatory state. Finally, Chapter 5 summarizes the key findings, discusses their potential applications, and outlines directions for future research.

Chapter 2.

Sugar signaling in *S. cerevisiae*

In the late 1920s, Herbert G. Crabtree described a phenomenon whereby fermentation would occur despite the presence of oxygen whenever glucose was available (Crabtree, 1929; De Deken, 1966). This seemingly paradoxical phenotype, where substrate-level phosphorylation is employed rather than respiration despite a ten-fold reduction in ATP yield, was studied intensely due to its similarities to aerobic fermentation in cancer cells (Crabtree, 1929; Pfeiffer & Morley, 2014). It turned out that many yeast species, including *S. cerevisiae*, performed aerobic fermentation when high levels of glucose were present in the growth medium—and eventually this became known as the Crabtree effect (De Deken, 1966). The evolutionary purpose is still debated, although most agree that it likely evolved due to competitive advantages of rapid sugar depletion and ethanol accumulation or due to biochemical limitations in respiration and free energy dissipation (Niebel et al., 2019; Pfeiffer & Morley, 2014). Nevertheless, the Crabtree effect represents a good example of the drastic changes in cellular phenotype that can occur when external stimuli is provided. In the following chapter, we will explore the sugar-responsive regulatory system in *S. cerevisiae*, how these mechanisms respond to glucose, and what the present thesis aims to resolve.

2.1. Carbon catabolite repression and sugar signaling

S. cerevisiae has several specific nutrient responses which are directed by signaling mechanisms, ranging from the sensing of nitrogen and phosphate to pH and sugars (Conrad et al., 2014). Many of these signaling pathways act via transcriptional regulators that interact with conserved DNA motifs in target genes, with notable motifs such as Stress Response Elements (STREs), Acetic acid Response Elements (ACREs), and Post-Diauxic Shift Elements (PDSEs) (Folch-Mallol et al., 2004). These motifs allow the signaling pathways to control an array of different genes, rather than just single ones: thereby rewiring the cellular metabolism without the need for hundreds of individual transcription factors.

Whenever a preferred carbon source is available, *S. cerevisiae* and many other yeasts display carbon catabolite repression (CCR)—referring to the repression of gene transcription that occurs in response to specific carbon sources (Gancedo, 1998). This is the case for several sugars such as fructose, galactose, and mannose; but it is particularly relevant in the case of glucose, leading many to use the term glucose repression synonymously with CCR (Gancedo, 1998). Although the Crabtree effect likely occurs primarily due to glucose repression of respiratory genes, CCR and sugar sensing impacts a lot more than just respiration (Malina et al., 2021). For instance, gluconeogenesis and storage carbohydrate synthesis are also repressed; meanwhile, glycolysis and sugar transport are upregulated—which is likely underlying the increase in glycolytic enzyme abundance seen during the Crabtree effect (Brink et al., 2021; Malina et al., 2021).

Transcriptional repression and activation occur via transcription factors, proteins capable of binding DNA and either inhibiting or aiding the transcription process (Willey et al., 2022). Transcription factors accomplish this using DNA-binding protein structures, such as zinc finger domains made of cysteine repeats, and often by recruiting transcription co-factors (Smith & Johnson, 2000). The Cyc8p-Tup1p complex is one such co-factor, acting as a major co-repressor for many genes, and it can be directed to target genes by DNA-binding transcription factors such as Rgt1p or Mig1p (Papamichos-Chronakis et al., 2004). Cyc8p and Tup1p will come up several times in the next segment as they are integral to the signaling pathways responsible for sensing sugar availability. Once recruited to a target gene by DNA-binding repressors, the Cyc8p-Tup1p complex represses transcription either by blocking the transcriptional machinery physically, or by recruiting other global co-repressors such as chromatin remodelers that make the DNA inaccessible to the polymerases (Conrad et al., 2014). Meanwhile, other regulatory proteins can act as transcriptional activators by deactivating these repressors and co-repressors, typically via phosphorylation, either by dissociating them from each other or by impacting their ability to bind DNA (Conrad et al., 2014).

The sugar signaling pathways have historically been divided up into three primary branches: the SNF1 pathway, the cAMP/PKA pathway, and the Snf3p/Rgt2p pathway (Figure 2.1) (Brink et al., 2021; Broach, 2012; Conrad et al., 2014). This categorization most likely stems from generalizations of the purposes of each branch; however, it should be underscored that all of these pathways interact extensively with each other. There are transcription factors which are affected by several pathways at once, and there is cross-talk in which one pathway disables another pathway (Brink et al., 2021; Conrad et al., 2014). These complex interactions allow the cell to respond to different levels of sugar with specific behaviors. Nevertheless, a certain degree of separation between the pathways is applied in the present chapter for the sake of clarity. Focus has been placed on critically reviewing primary evidence to create an up-to-date summary of the pathways and their regulation.

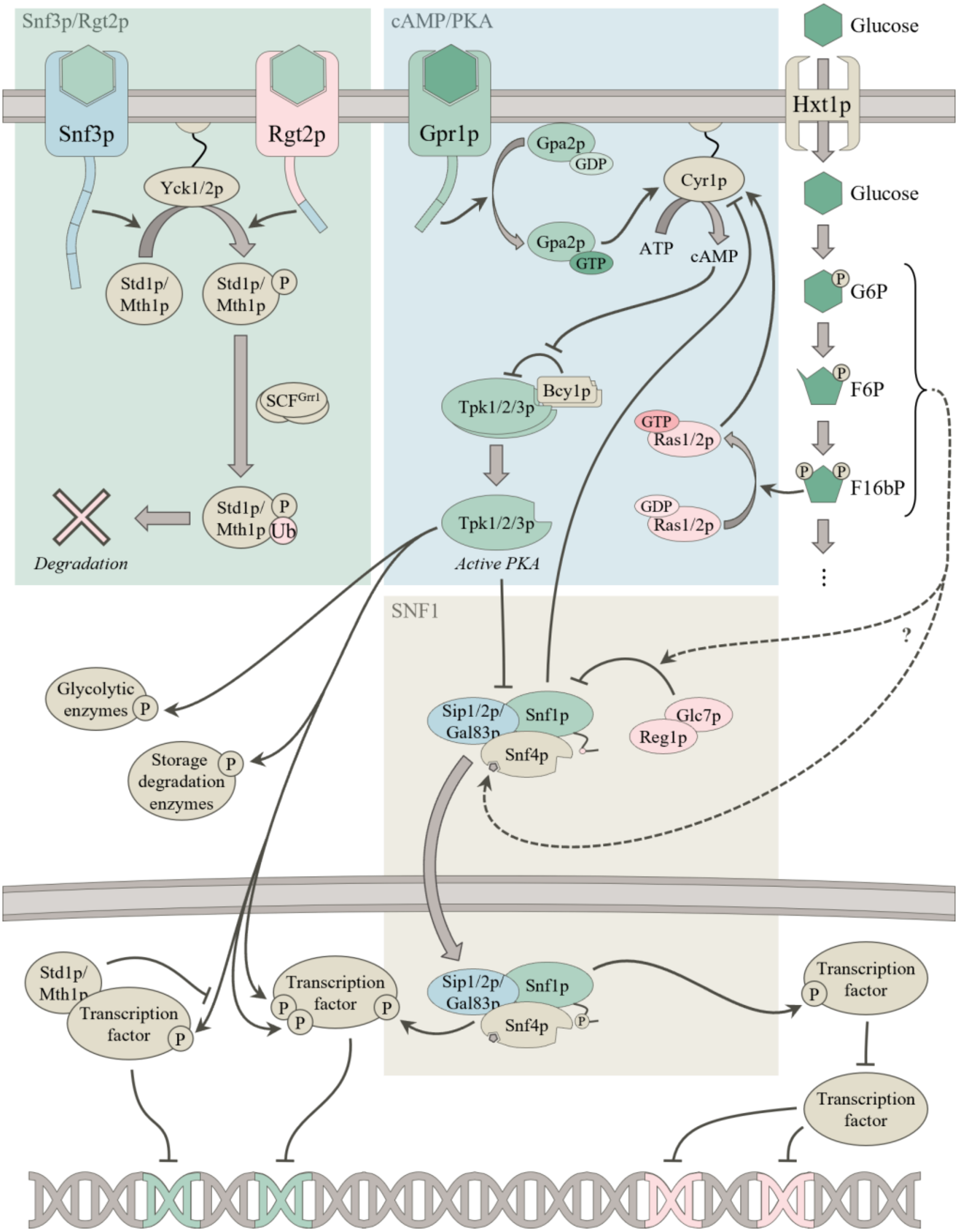


Figure 2.1. The three primary sugar signaling pathways (Snf3p/Rgt2p, SNF1, and PKA) and their interconnections, impacting the activity of the other pathways and sharing downstream targets. The majority of the regulation occurs by phosphorylation of enzymes and transcription factors to alter activity and DNA-binding affinity, respectively. Abbreviations: cAMP, cyclic AMP; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F16bP, fructose-1,6-bisphosphate; SCF, Skp-, Cullin-, F-box-containing.

2.1.1. The SNF1 pathway

The heterotrimeric SNF1 complex is a kinase that controls the expression of a multitude of glucose-responsive genes (Conrad et al., 2014). Commonly described as the main regulator of glucose repression, it has a pronounced role in repressing the expression of genes for alternative sugar utilization as well as respiratory repressors—leading to increased utilization of non-glucose sugars and respiration (Conrad et al., 2014; Frechin et al., 2014). The complex is composed of the Snf1p AMP-activated protein kinase (AMPK) subunit, the Snf4p regulatory subunit, and one of three localization-determining subunits: Gal83p, Sip1p, or Sip2p (Figure 2.2) (Broach, 2012). Like other eukaryotic AMPKs, the SNF1 complex is involved in the cellular energy homeostasis and is responsive to ADP-levels via allosteric sites on the Snf4p regulatory subunit (Mayer et al., 2011). However, the SNF1 complex also appears to respond directly to glucose levels (Broach, 2012; Conrad et al., 2014; Milanesi et al., 2021)—although the underlying mechanism remains ambiguous.

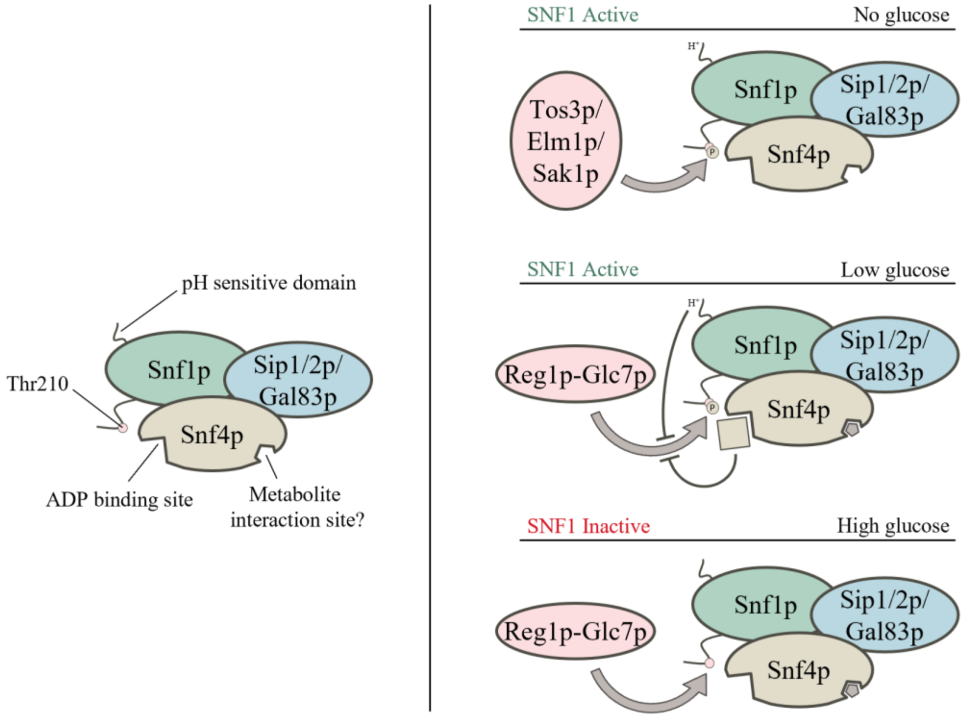


Figure 2.2. The structure of the SNF1 complex (left) and the regulatory changes that trigger SNF1 complex activity under various glucose conditions (right). When glucose is scarce, one of three kinases (Tos3p/Elm1p/Sak1p) phosphorylates Thr210 to activate the complex. When glucose is abundant, the phosphatase complex (Reg1p-Glc7p) dephosphorylates Thr210 to inactivate the complex. ADP binding and a pH-sensitive polyhistidine tract appear to inhibit phosphatase activity. It remains unclear whether the SNF1 complex is capable of responding directly to glucose-derived metabolites or not. While all three localization subunits (Sip1p, Sip2p, Gal83p) are shown in the figure, only one is present per complex.

When glucose is present, the Reg1p-Glc7p complex dephosphorylates threonine 210 (Thr210) of Snf1p leading to its inactivation (Broach, 2012; Conrad et al., 2014). When glucose is depleted, three partially redundant upstream kinases (Tos3p/Elm1p/Sak1p) phosphorylate Thr210 to re-activate the protein kinase (Broach, 2012; Conrad et al., 2014). However, neither the phosphatase complex nor the upstream kinases have altered activity in response to glucose (Conrad et al., 2014). Instead, it seems that the glucose-dependent activity is linked to the SNF1 complex itself. Suggested mechanisms include: i) protein-metabolite interactions between the SNF1 complex and glucose-derived intermediates, ii) intermediate interactions with the Reg1p-Glc7p protein phosphatase complex, or iii) sensing of intracellular pH via a polyhistidine domain in Snf1p—although crosstalk from other signaling pathways makes it difficult to conclusively pinpoint how the regulation occurs (Castermans et al., 2012; Huberts et al., 2012; Kayikci & Nielsen, 2015; Milanesi et al., 2020; Milanesi et al., 2021; Simpson-Lavy & Kupiec, 2023). Regardless of the mechanism behind glucose detection, the outcome is a very dynamic regulatory system that can influence the cellular behavior by altering the activity of enzymes directly (e.g., inactivation of pyruvate kinase and adenylyl cyclase by phosphorylation), the accessibility of DNA for transcription (e.g., by recruiting the SAGA complex to remodel the chromatin), and the localization of downstream transcription factors (e.g., the partitioning of the Mig1p repressor to the nucleus) (Broach, 2012; Conrad et al., 2014; Nicastro et al., 2015).

Mig1p as a target

One of the most important targets of the SNF1 complex is the Mig1p transcriptional repressor, and thus the signaling route is often termed the SNF1/Mig1p pathway. Mig1p binds specific DNA motifs of genes encoding enzymes and transcription factors, such as the extracellular invertase gene (*SUC2*) and gluconeogenesis activators (e.g., *CAT8*), and recruits the general Cyc8p-Tup1p corepressor complex in order to decrease expression levels when glucose is abundant (Broach, 2012; Klein et al., 1998; Papamichos-Chronakis et al., 2004; Roy et al., 2013). When glucose is scarce, the SNF1 complex phosphorylates Mig1p which leads to derepression of these same genes by dissociation of the corepressors and localization of Mig1p to the cytosol—enabling the cell to grow on alternative sugars and non-fermentable carbon sources (Figure 2.3) (Papamichos-Chronakis et al., 2004). The active, dephosphorylated Mig1p transcription factor also participates in the repression of *HXT2* when glucose is abundant (Brink et al., 2021). The SNF1 complex phosphorylates and activates proteins derived from these genes, such as Cat8p and Rds2p (Broach, 2012). The Rds2p transcription factor is of particular note as it shares a lot of targets with Cat8p for induction of gluconeogenesis, but also because it has been suggested to participate in the activation of respiration via Hap4p (Figure 2.3) (Bolotin-Fukuhara, 2017; Broach, 2012). This constitutes only one of the ways by which glucose represses respiration; another alternative way which depends on the PKA pathway will be discussed later.

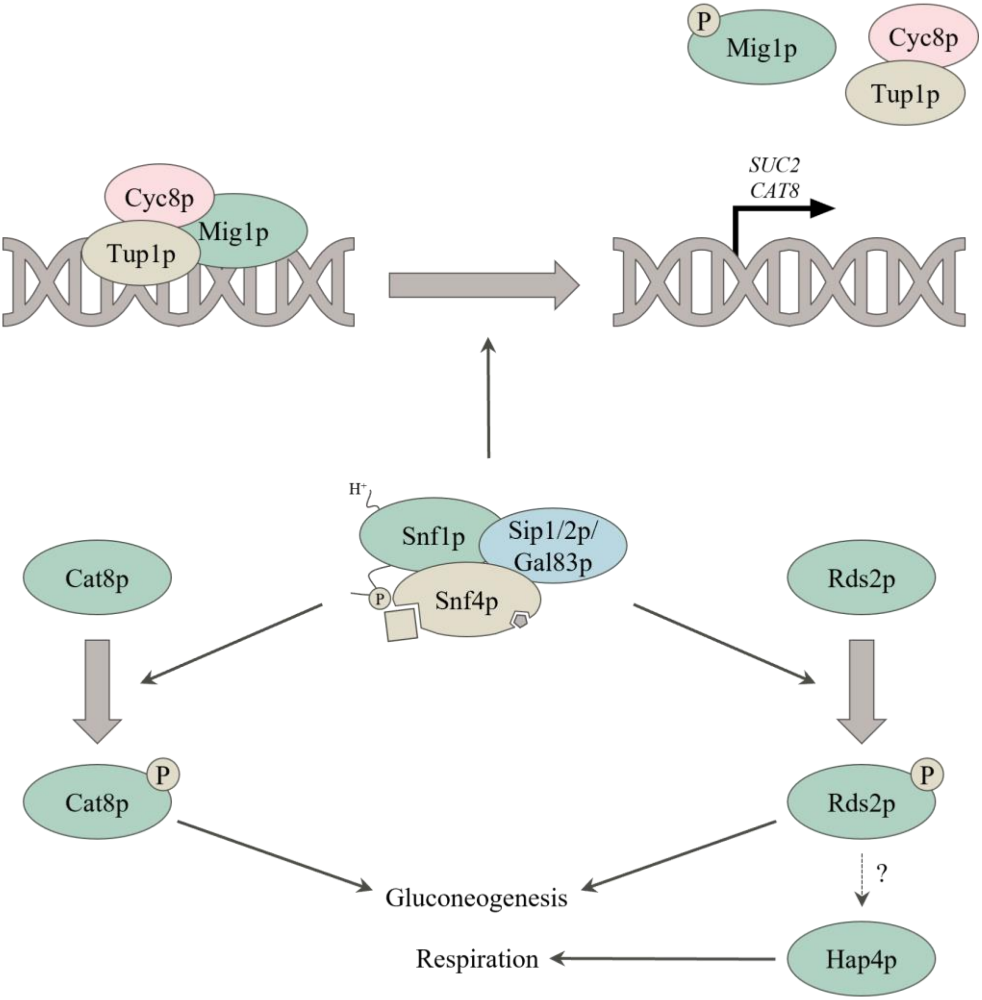


Figure 2.3. Examples of regulatory roles of the active SNF1 complex during glucose scarcity, including the regulation of gene expression via Mig1p and the activation of gluconeogenic regulators such as Cat8p and Rds2p by phosphorylation.

Hxk2p as a target

The role of the predominant hexokinase during growth on glucose, Hxk2p, has long been debated in relation to SNF1-dependent signaling. Hxk2p gained attention for its putative moonlighting role as a co-repressor in cellular regulation by SNF1-dependent nuclear localization (Broach, 2012). It was suggested to occur as a result of acting as a kinase on transcription factors rather than sugars; however, disruption of the sugar phosphorylation activity did not impact the regulatory activity and vice versa (Broach, 2012).

The longstanding model has been that a fraction of Hxk2p proteins localize to the nucleus during glucose-rich conditions and the cytosol during glucose-scarce conditions, in contrast to mammalian homologs which behave in the opposite way (Broach, 2012; Conrad et al., 2014; Lesko et al., 2023). However, a recent study attempting to replicate these findings found contradicting evidence, claiming that previous studies likely lacked sufficient resolution and that the regulatory activity of Hxk2p may be confined to a handful of genes rather than the global gene expression (Lesko et al., 2023). As such, the findings indicate that Hxk2p is likely nuclear-excluded during glucose-abundant conditions (Figure 2.4). While genes such as the previously mentioned *SUC2* still appear to be influenced by Hxk2p, the exact mechanism is unknown (Lesko et al., 2023).

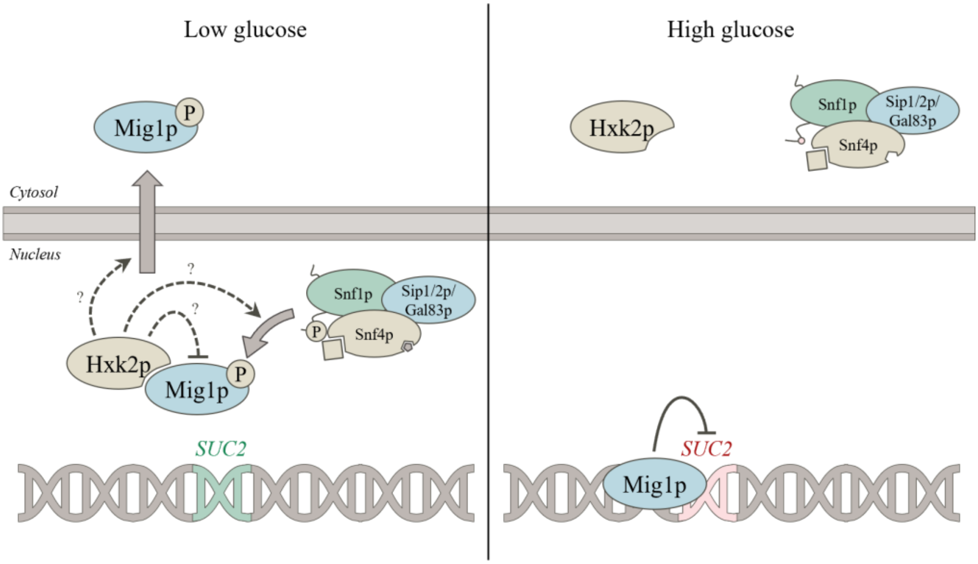


Figure 2.4. Updated model of Hxk2p localization and function under low (left) and high (right) glucose conditions in relation to the SNF1 complex and Mig1p. Hxk2p may influence Mig1p activity: directly via binding, indirectly via altering Mig1p localization, and/or indirectly via improved Mig1p phosphorylation by the SNF1 complex.

The mechanism behind might be based on Hxk2p either inhibiting Mig1p DNA binding, increasing SNF1-dependent phosphorylation of Mig1p, or aiding in the nuclear exclusion of Mig1p (Figure 2.4) based on the observations that: i) Hxk2p has been shown to complex with Mig1p *in vitro*, ii) the disruption of Hxk2p leads to constitutive nuclear Mig1p localization and repression activity, iii) and the Mig1p-binding site is required for nuclear translocation of Hxk2p (Broach, 2012; Lesko et al., 2023).

2.1.2. The cAMP/PKA pathway

Much like the SNF1 complex, protein kinase A (PKA) controls the expression of a vast array of genes (Broach, 2012). PKA is involved in the regulation of activities such as sugar metabolism, stress tolerance, and the cell cycle (Folch-Mallol et al., 2004). The kinase can be activated by extracellular stimulus via the G-protein coupled receptor (GPCR) Gpr1p or by intracellular signals via the Ras1/2p GTPases (Figure 2.5) (Busti et al., 2010; Colombo et al., 1998; Conrad et al., 2014).

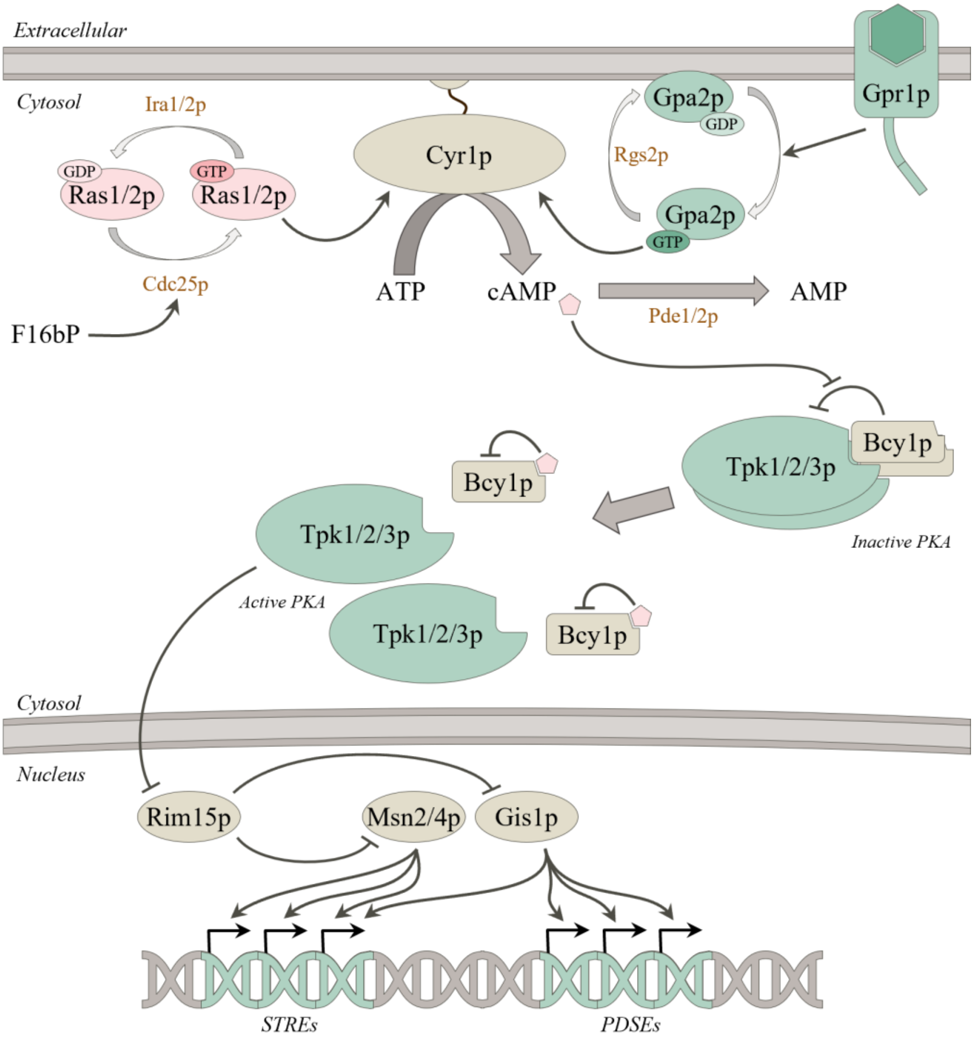


Figure 2.5. Overview of the PKA pathway, including the extracellular signaling branch via Gpr1p/Gpa2p and the intracellular signaling branch via Ras1/2p. Activation of the kinase has wide effects on expression, especially stress response elements (STREs) and post-diauxic shift elements (PDSEs). cAMP, cyclic AMP; F16bP, fructose-1,6-bisphosphate.

The membrane sensor Gpr1p is activated when 4 to 20 g L^{-1} of extracellular glucose is present (Harashima & Heitman, 2002; Lemaire et al., 2004; Rolland et al., 2000). Gpr1p in turn activates Gpa2p, the Gα-subunit of the heterotrimeric G-protein complex, which in its activated GTP-form triggers Cyr1p adenylyl cyclase to produce the secondary messenger cyclic AMP (cAMP) (Harashima & Heitman, 2005; Peeters et al., 2007). Gpa2p is in turn negatively regulated by the Rgs2p GTPase-activating protein (GAP) (Harashima & Heitman, 2005; Peeters et al., 2007). The extracellular activation of the PKA pathway, including attempts at engineering the pathway, will be covered in more detail in Chapter 4.

Ras1/2p is activated when 0.2 to 2 g L^{-1} of extracellular glucose is present in the environment (Brink et al., 2021). Ras1/2p are small GTPases which are activated by the formation of fructose-1,6-bisphosphate from glycolysis via the guanine-exchange factors (GEFs) Cdc25p/Sdc25p (Colombo et al., 1998; Peeters et al., 2017). In its active GTP-form, both Ras1/2p trigger cAMP production by Cyr1p adenylyl cyclase, although Ras2p is thought to play the predominant role in this activity (Conrad et al., 2014; Harashima et al., 2006). Ras1/2p are subsequently deactivated by the Ira1/2p GAPs (Conrad et al., 2014; Harashima et al., 2006). As mentioned above, both Gpr1p and Ras1/2p activation ultimately result in the production of cAMP by the Cyr1p adenylyl cyclase (Broach, 2012).

cAMP acts as a potent secondary messenger which has many cellular effects, and levels are primarily controlled by an enzymatic competition between the Cyr1p adenylyl cyclase and the cAMP-degrading phosphodiesterases Pde1/2p (Broach, 2012). One of the most important roles of cAMP is dissociating the negative regulator Bcy1p from the catalytically active Tpk1/2/3p subunit of PKA, resulting in PKA activation (Conrad et al., 2014). The three homologs of Tpk have partially redundant functionalities but specialize in their targets and localization (Broach, 2012). Once Tpk1/2/3p dissociates from Bcy1p, it goes on to affect the activities of key proteins and transcriptional regulators in the cell. This includes not only proteins involved directly in sugar metabolism, such as trehalase and pyruvate kinase, but also negative feedback regulators such as Pde1/2p (Colombo et al., 2017; Colombo et al., 2022; Conrad et al., 2014). This feedback regulation is very important since constitutive formation of cAMP is detrimental to the cell as it leads to improper activation of PKA and elevated levels of ROS and apoptosis (Conrad et al., 2014).

A particularly notable phenotype pertaining to PKA activation is decreased stress tolerance (Conrad et al., 2014). PKA inhibits Rim15p, which in turn inhibits the Msn2/4p and Gis1p transcription factors responsible for the expression of a wide array of stress tolerance genes and catabolic genes via STREs and PDSEs, respectively (Conrad et al., 2014). One of the targets of Msn2/4p is the superoxide dismutase gene *SOD1*, which has been shown to impact respiratory activity (Reddi & Culotta, 2013). Due to its connection to stress tolerance genes, many industrial and laboratory strains which have been selectively bred in stressful conditions carry mutations in the PKA pathway and especially in *CYR1* (Brink et al., 2021).

2.1.3. The Snf3p/Rgt2p pathway

The expression of suitable transporters at appropriate levels is essential to ensure an efficient uptake of specific sugars without unnecessary energy loss or membrane crowding. This is the primary purpose of the Snf3p/Rgt2p pathway (Figure 2.6). Upon activation of the Snf3p or Rgt2p receptor, the Std1p and Mth1p co-repressors are recruited to the membrane (Schmidt et al., 1999). Phosphorylation of these co-repressors by membrane-anchored Yck1/2p kinases allows the Grr1p-SCF ubiquitinase complex to target them for degradation (Willems et al., 2004). This leaves the Rgt1p repressor open to phosphorylation, which subsequently causes dissociation of the Cyc8p-Tup1p co-repressors and derepression of transporter-encoding genes (Conrad et al., 2014). Crosstalk from the HOG osmolarity pathway (via Sko1p) and the SNF1 pathway (via Mig1p) increases the selectivity of which transporters are expressed during low and high glucose (Brink et al., 2021).

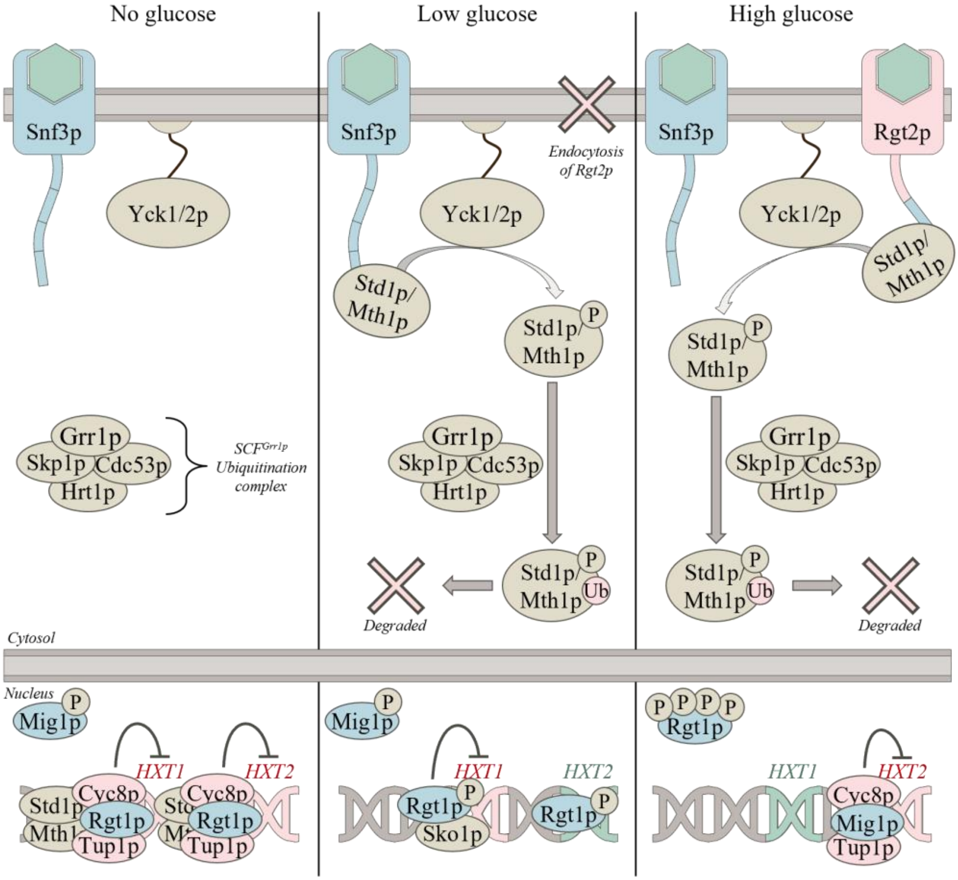


Figure 2.6. Overview of the Snf3p/Rgt2p pathway under various glucose conditions.

The Snf3p receptor primarily mediates responses to glucose scarcity, whereas the Rgt2p receptor is associated with responses to glucose abundance; leading to environmentally tailored expression of high-affinity/low-capacity transporters (Hxt2p) and low-affinity/high-capacity transporter (Hxt1p), respectively (Conrad et al., 2014). Rgt2p responsiveness is heavily influenced by endocytosis and *RGT2* repression at low glucose levels, while Snf3p remains present across conditions (Kim & Rodriguez, 2021; Melton et al., 2021). The Rgt2p endocytosis is thought to be primarily dictated by the intracellular C-terminal domain of the protein, which also harbors the sugar signaling domain, since Snf3p endocytosis is increased if the domains are swapped (Kim et al., 2024). The signaling domains of the receptors both contain conserved 17 amino acid repeats, one in Rgt2p and two in Snf3p, which are important for the activation of the pathway (Brink et al., 2021). The significance of the differences in these domains remains to be elucidated; however, past research has revealed that: i) swapping the sugar signaling domains of the two receptors alters sugar signaling, ii) overexpression of a mutant Rgt2p lacking the signaling domain still shows partial signaling, and iii) expression of only the signaling domain is enough to elicit a partial signaling response (Kim et al., 2024; Moriya & Johnston, 2004; Özcan et al., 1998). All in all, these findings indicate that both the receptors and the signaling domains are functionally distinct and non-redundant.

The co-repressors Mth1p and Std1p also appear to have distinct roles. The expression and degradation of these proteins is closely linked to glucose availability (Figure 2.7) (Roy et al., 2013). Both Mth1p and Std1p are actively degraded via the Snf3p/Rgt2p whenever glucose is available, regardless of whether it is present at low or high concentrations (Broach, 2012; Roy et al., 2014). However, while *MTH1* is repressed by Mig1p and the Cyc8p-Tup1p complex when glucose concentrations are high, *STD1* expression remains constant across conditions (Broach, 2012; Roy et al., 2014). As such, low levels of Std1p are likely present even when glucose is present, perhaps acting as a sensing mechanism to respond rapidly to changes in the environment while Mth1p acts as an enforcer during prolonged glucose starvation.

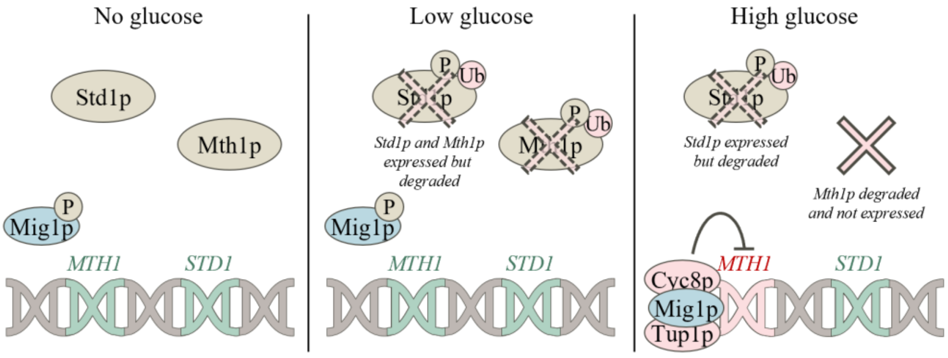


Figure 2.7. The expression and degradation of Std1p and Mth1p in various glucose conditions.

However, there are inconsistencies in this model for the Snf3p/Rgt2p pathway. For instance, overexpression of *YCK1* causes constitutive signaling regardless of receptor activation (Moriya & Johnston, 2004), raising doubts whether the receptors are truly necessary for membrane recruitment. A recent study reported that this can likely be explained by the Yck1/2p kinases having accessory functions in addition to co-repressor phosphorylation, such as phosphorylating the Snf3p and Rgt2p receptors to inhibit endocytosis (Figure 2.7) (Kim et al., 2024; Snowdon & Johnston, 2016). Indeed, the Yck1/2p kinases have multiple important functions in the cell, including the stabilization of maltose permease and the repression of respiration via Sod1p (Broach, 2012; Reddi & Culotta, 2013; Snowdon & Johnston, 2016). Thus, the overexpression of *YCK1* may simply act to increase Snf3p and Rgt2p receptor levels and thus enable increased co-repressor recruitment. This would align with previous reports of *RGT2* overexpression being capable of rescuing the, otherwise fatal, deletion of *YCK1/2* (Snowdon & Johnston, 2016).

Another inconsistency pertains to the localization of Mth1p and its supposed interaction with the membrane-bound receptors and kinases. While Std1p has been shown to localize both to the nucleus and to the membrane (Schmidt et al., 1999), Mth1p demonstrates constitutive nuclear localization (Pasula et al., 2010). Nevertheless, degradation of Mth1p occurs in a glucose-dependent manner and relies on both the receptors and the nuclear localization of ubiquitin ligase complex (Blondel et al., 2000; Landry et al., 2012; Pasula et al., 2010). Adding to the discrepancies, *akr1* palmitoyl transferase deletants which cannot properly palmitoylate and membrane-anchor Yck1/2p still show a glucose-dependent response, indicating that the interaction between the receptors and Yck1/2p may not be required for the degradation of Mth1p (Pasula et al., 2010). Two possible explanations are that: i) there is another unidentified kinase which is triggered upon receptor activation which migrates to the nucleus and phosphorylates Mth1p, and ii) there is an orthogonal mechanism at play which regulates the targeting of the ubiquitin ligase complex towards Mth1p (Figure 2.8). The reason a kinase is suspected to be involved is because the $\mathrm{SCF}^{\mathrm{Grr1}}$ ubiquitin ligase complex typically prefers phosphorylated targets (Willems et al., 2004). An alternative explanation would be that the receptors instead influence $\mathrm{SCF}^{\mathrm{Grr1}}$ complex assembly, which is known to be enhanced by glucose (Li & Johnston, 1997), or its nuclear localization through an unknown mechanism to increase Mth1p degradation.

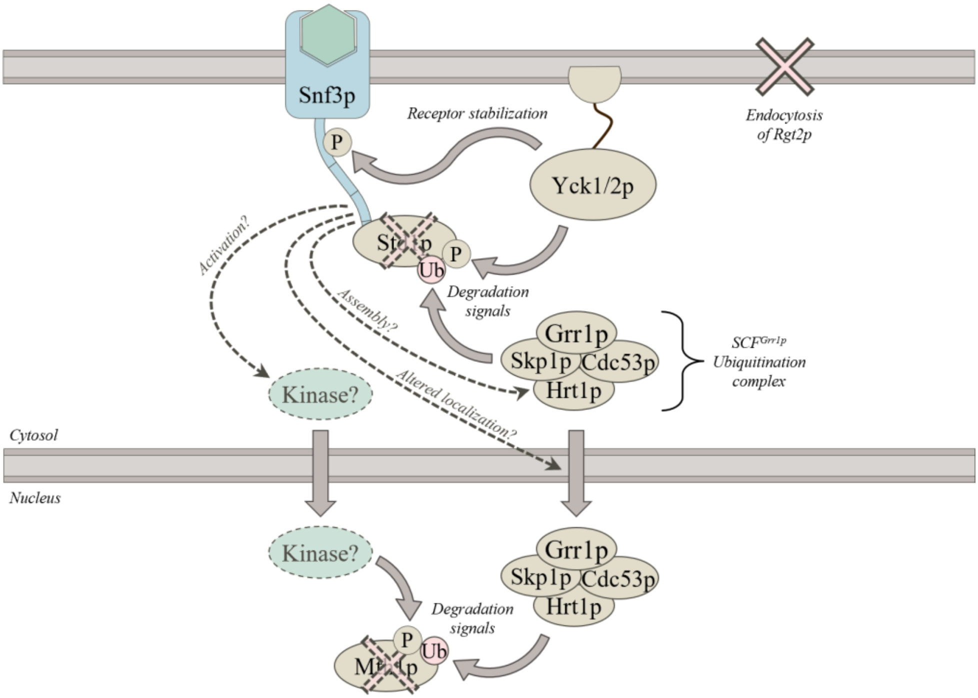
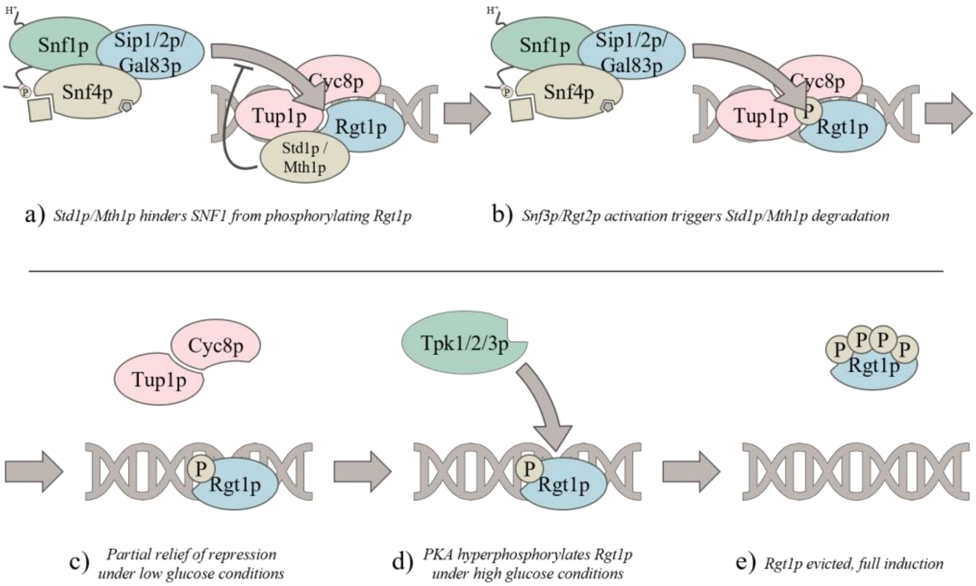


Figure 2.8. Alteration to the model to account for the constitutive nuclear localization of Mth1p.

In any case, degradation of Mth1p/Std1p is essential for exposing the DNA-targeting Rgt1p factor to phosphorylation (Mosley et al., 2003). Although many reviews state that PKA is responsible for partial phosphorylation of Rgt1p upon Mth1p/Std1p degradation, there is no conclusive primary evidence demonstrating this to the best of my knowledge. There is, however, evidence that the partial phosphorylation is SNF1-dependent (Palomino et al., 2006). It remains unclear whether this is a common misconception, or if a key piece of evidence is missing. Based on the activities of the SNF1 and PKA pathways, it is more probable that the SNF1 complex is behind the partial phosphorylation of Rgt1p under low glucose conditions (Figure 2.9a-b). Once Rgt1p becomes partially phosphorylated, it loses the ability to couple with the Cyc8p-Tup1p co-repressor complex which upon release leads to gene de-repression (Figure 2.9c) (Roy et al., 2013). It does not appear to matter if Rgt1p remains bound to the DNA at this stage (Roy et al., 2013).



a) *Std1p/Mth1p hinders SNF1 from phosphorylating Rgt1p*

b) *Snf3p/Rgt2p activation triggers Std1p/Mth1p degradation*

c) *Partial relief of repression under low glucose conditions*

d) *PKA hyperphosphorylates Rgt1p under high glucose conditions*

e) *Rgt1p evicted, full induction*

Figure 2.9. Phosphorylation of Rgt1p likely depends on all three sugar signaling pathways.

Another layer of regulation has been identified, as hyperphosphorylation of all four serine residues in Rgt1p by PKA causes it to instead behave as a transcriptional activator—which is required for full *HXT1* induction (Mosley et al., 2003; Palomino et al., 2006). Perhaps the hyperphosphorylation acts to fully eject the Rgt1p from the gene (Figure 2.9d-e), as supported by *in vitro* assays showing Rgt1p cannot bind the DNA under high glucose conditions, and this simply enables the transcriptional machinery to more easily access the site (Mosley et al., 2003; Roy et al., 2013). This could then be misinterpreted as an activation. Alternative explanations would rely on hyperphosphorylated Rgt1p acting in some way which does not depend on DNA-binding.

2.2. Measuring sugar sensing

2.2.1. Assays and biosensors

As discussed above, sugar sensing can result in a variety of different transcriptional and post-translational changes which impact cellular behavior. Secondary messengers can trigger secondary events with wide-scale consequences—which is beneficial for the cell but makes it difficult for us to fully assay. As such, most studies focus only on a small subset of the regulatory network at a time.

Gene expression can be monitored using, e.g., quantitative polymerase chain reactions (qPCRs), transcription-based reporters, and transcriptomics (Adeniran et al., 2015; Fassbinder-Orth, 2014; Horak & Snyder, 2002; Yu & Nielsen, 2019). Specific protein activities can be assayed using co-factor supplementation or Förster resonance energy transfer (FRET) probes (Adeniran et al., 2015; Skruzny et al., 2019). Phosphorylation of proteins can be measured using western blots or mass spectrometry (Mishra et al., 2017; Ray & Haystead, 2003), whereas protein interactions can be assessed using immunoprecipitation (Tanaka, 2011). Each has their unique benefits and drawbacks, describing different parts of the regulation. However, measuring all at once would be extremely impractical and costly.

In the work related to this thesis, transcription-based fluorescent reporters to monitor gene expression were primarily used (Brink et al., 2016). These were chosen rather than transcriptomics, despite the latter providing far more detailed and global information, because of the dramatically lower costs and time requirements (Adeniran et al., 2015; Yu & Nielsen, 2019). Instead of monitoring nearly all genes at a few time points, the fluorescent reporters allowed for the monitoring of a select number of genes at nearly any time point. In contrast to qPCRs, which could be seen as an intermediate step between the two techniques in allowing you to monitor multiple genes at once, the possibility to easily monitor gene expression on the single cell level with reporters was preferred as this would allow the discovery of subpopulations (Nadal-Ribelles et al., 2024).

Choosing suitable reporter genes, which are controlled by the different sugar signaling pathways, made it possible to indirectly determine whether a pathway was activated or not (Brink et al., 2016). Throughout the present thesis, fluorescent reporters constructed by linking the expression of GFP to the promoters of the genes listed in Table 2.1 were able to provide input on the response of the three sugar signaling pathways to different carbon sources.

Table 2.1. Reporter genes with their respective function and what reporter induction indicates.

Gene	Protein	Interpretation
HXT1	High-capacity transporter	Both Rgt2p and PKA are active. Abundance of carbon in environment.
HXT2	Low-capacity transporter	Snf3p is active and SNF1 is inactive. Scavenging for carbon in environment.
SUC2	Extracellular invertase	SNF1 complex is actively deactivating Mig1p. Alternative sugar utilization genes expressed, likely gluconeogenesis and respiration too.
CAT8/ SIP4/ RDS2	Transcription factors	SNF1 complex is actively deactivating Mig1p. Gluconeogenesis and respiration expressed, likely alternative sugar utilization too.
TPS1	Trehalose synthase	PKA is inactive. Less glycolytic activity and growth, more storage carbohydrate synthesis.

Depending on the research question and the dynamic range of expression—how big the difference is between maximal and minimal expression of the gene—certain promoters may be less optimal than others. For instance, while activation of the *CAT8* promoter may provide a more direct indication of gluconeogenic activity, the gain in dynamic range from using the promoter of *SUC2* may make it a better choice as a reporter of an active gluconeogenesis. Similarly, while the *HXT1* promoter has a larger dynamic range, it depends on PKA activation for full induction (Figure 2.6, right), and studies focusing on the Snf3p/Rgt2p pathway alone under low glucose conditions may benefit more from monitoring the *HXT2* promoter (Figure 2.6, middle). The PKA pathway is perhaps the most difficult one to monitor since many promoters linked to it have low dynamic ranges or may be far downstream of the actual kinase, as is the case with *TPS1* promoter. To the best of my knowledge, there is no mechanistic explanation as to how *TPS1* expression is repressed when PKA is activated, despite the final result being well-documented. Nevertheless, it represents one of the better promoters available.

The promoters linked to the genes outlined above have already previously been evaluated for use as transcription-based reporters—commonly described as fluorescent biosensors (Brink et al., 2016). In my work, I primarily used the *HXT1*p_GFP, *HXT2*p_GFP, *SUC2*p_GFP, and *TPS1*p_GFP biosensors to assess the activation of the three pathways (Table 2.1).

2.2.2. Fluorescence and flow cytometry

Both fluorometry and flow cytometry are capable of measuring the fluorescence of the reporters. Although fluorometry allows faster measurements using more robust equipment, flow cytometry was chosen in the present studies due to its sensitivity and ability to discern single-cell GFP responses.

Flow cytometry analyzes individual events (cells) by passing them through a laser beam in a liquid stream (interrogate), measuring light scatter and fluorescence (Figure 2.10). The forward scatter (FSC) detector estimates cell size, while the side scatter (SSC) detector assesses either granularity in mammalian cells or size in smaller cells like microbes. Additionally, fluorescence detection via wavelength-specific filters to quantify signals from markers such as GFP or propidium iodide (PI) enable the collection of dozens of parameters per cell. The cell size proxy can help filter out debris and normalize fluorescence intensities when morphology varies. Data is stored as a table of fluorescent intensities for each event and can be analyzed using commercial tools like FlowJo or free software such as Python, which supports machine learning techniques like Gaussian mixture models for identifying subpopulations. Results are then summarized as histograms or mean fluorescence intensities (MFIs) for analysis. The statistics of flow cytometry can quickly become complicated, but the simplest way is to simply compare the MFIs of replicates between different experimental conditions.

Additional lasers and filters can be added to increase the versatility of the flow cytometer, and a spatially separated parallel triple-laser system was key for the use of the Förster resonance energy transfer probe (FRET) in one of my projects. As opposed to co-linear laser designs, where multiple lasers are optically aligned to interrogate the event simultaneously, the time delay between the different lasers in parallel designs decrease emission overlap between different fluorophores—potentially allowing for the use of more fluorophores per cell. At the start of my work, the flow cytometers available at the department were all co-linear and emission overlap between fluorophores made it impractical to apply several fluorophores in a single cell. Consequently, only one biosensor was applied to each strain throughout the studies. This drastically increased the workload since every genetic modification spawned multiple new strains that all needed to be verified, tested in control conditions, and assayed separately. Recently, significant improvements have been made which enable the use of two fluorophores in co-linear systems by carefully selecting fluorophores with minimal overlap in spectral emissions (Perruca Foncillas et al., 2023; Torello Pianale & Olsson, 2023).

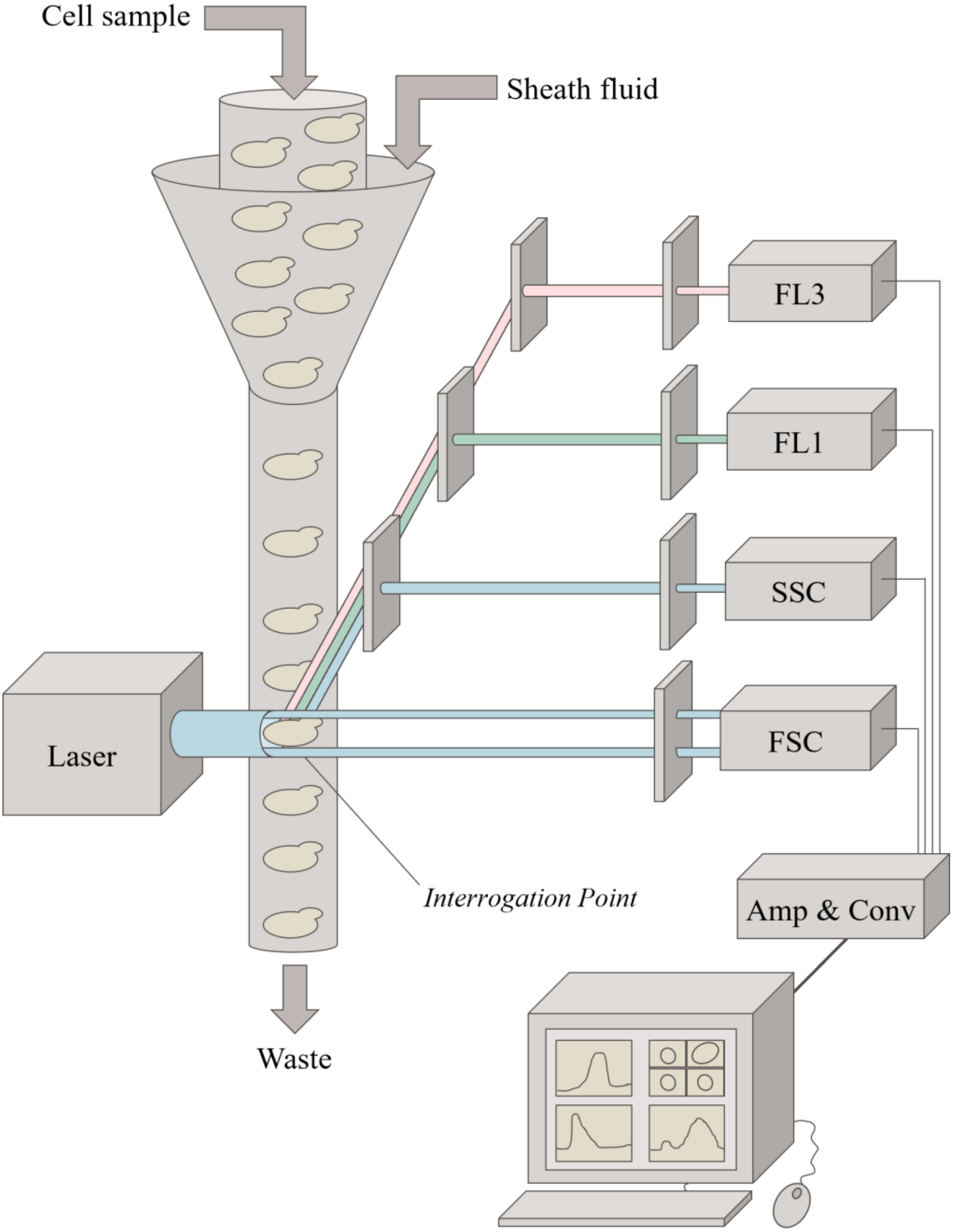


Figure 2.10. Overview of a single laser flow cytometer with one forward scatter (FSC), one side scatter (SSC), and two fluorescence (FL1 & FL3) detectors.

2.3. A peculiar response to xylose

2.3.1. Sensed as non-fermentable

The parts above summarize current knowledge on the sugar signaling pathways and their activation by their intended substrate, glucose. However, far less is known about their responses to xylose. When the effect of xylose on sugar signaling was first explored using fluorescent biosensors, a very peculiar response was observed: *SUC2* and *TPS1* were induced when xylose was actively metabolized (Table 2.2) (Osiro et al., 2018). This implied that the SNF1 complex was active while PKA was not, indicating that respiration and gluconeogenesis were likely being prioritized over fermentation and glycolysis.

Table 2.2. Signaling pathway activation when exposed to low glucose, high glucose, and high xylose.

Condition	**Snf3p/Rgt2p via *HXT1p* biosensor**	**SNF1/Mig1p via *SUC2p* biosensor**	**cAMP/PKA via *TPS1p* biosensor**
High Xylose	2200	5800	8500
Low Glucose	1400	5100	7700
High Glucose	4400	1500	4000

Indeed, the observation of the signaling response were corroborated throughout the literature with studies reporting: i) a lack of repression for respiration and the tricarboxylic acid cycle, ii) induction of genes for growth on non-fermentable sugar sources (*HXK1*, *FBP1*, *PCK1*), iii) increased trehalase activity, and iv) decreased glycolysis activity (Bergdahl et al., 2012; Bergdahl et al., 2013; Brink et al., 2021; Jin & Jeffries, 2004; Matsushika et al., 2014; Salusjärvi et al., 2008; Salusjärvi et al., 2006; Wasylenko & Stephanopoulos, 2015; Wu et al., 2020).

In summary, the evidence pointed towards recombinant *S. cerevisiae* failing to recognize xylose as a non-fermentable carbon source—despite actively fermenting it. Understanding the cause of this sugar signaling state and how to engineer it may bring about dramatic improvements to the utilization of xylose.

2.3.2. Possible underlying causes

Is the response pathway-dependent?

The studies outlined above were all performed in laboratory strains utilizing the xylose reductase/xylitol dehydrogenase (XR/XDH) pathway, prompting several questions: is the response specific to this catabolic pathway, would the same be observed for alternative pathways, and is the response present in industrial strains too? A metabolomic study showed accumulation of glyceraldehyde-3-phosphate on xylose when the xylose isomerase (XI) catabolic pathway was employed instead of the XR/XDH pathway, in line with a lack of proper activation of glycolysis (Wasylenko & Stephanopoulos, 2015). However, this was far from definitive proof that the sugar signaling pathways fell into the same configuration as seen with the XR/XDH pathway. Given that it has also been suggested that the redox imbalance imposed by the cofactor mismatch between XR/XDH may trigger a respiratory response to compensate for NADH accumulation (Jin & Jeffries, 2004), it became important to revisit the sensing response in XI-based strains.

Mechanism of SNF1 activation?

It is not entirely clear how the sugar signaling pathways are able to respond to xylose. Induction of *SUC2* only seems to occur in cells that are actively metabolizing xylose, so it would seem likely that the SNF1 complex is being activated by glycolytic intermediates derived from xylose: so which intermediates are responsible for this response? Knowing this could enable us to engineer the metabolome to avoid the accumulation of certain intermediates.

Extracellular sensing?

Another line of questioning focused on the partial *HXT2* induction observed in non-metabolizing cells exposed to extracellular xylose, with full induction only when xylose metabolism was occurring (Brink et al., 2016; Osiro et al., 2018). Can yeast detect extracellular xylose? Snf3p has been reported to show substrate promiscuity to other hexose sugars, but not towards xylose (Dietvorst et al., 2010). Such activation of Snf3p may be beneficial to the cell by increasing xylose uptake via transporter expression, or it may be detrimental by wasting cellular energy on ineffective transporters.

Engineering strategies to alter sensing?

Attempts at constitutively engineering the sugar signaling pathways for xylose utilization has previously demonstrated detrimental effects on stress tolerance (Colombo et al., 1998; Folch-Mallol et al., 2004; Smith et al., 1998): would it be possible to engineer receptors instead to selectively alter sugar signaling when xylose is present?

2.4. Aim of thesis

In the present thesis, I attempt to shed light on the xylose-induced sugar signaling response of *S. cerevisiae*. By examining the cause and potential solutions, the objective was to improve xylose utilization to enable the production of renewable chemicals for a sustainable future. Chapter 3 will be dedicated to outlining how I investigated the causes, while Chapter 4 will be focusing on attempts at engineering the sugar signaling pathways.

The thesis encompasses six publications, many of which are a result of international and in house collaborations. The first two studies focused on investigating whether the regulatory response to xylose in *S. cerevisiae* was dependent on the introduced xylose catabolism route by studying the response in strains carrying the XI pathway and the oxidative Weimberg pathway (**Papers I and II**). The next study aimed at identifying which metabolic intermediates were linked to the activation of the SNF1 complex and the sugar signaling response (**Paper III**). Then, the putative activation of Snf3p in response to extracellular xylose was explored and chimeric proteins were created with the goal of improving xylose sensing via the Snf3p/Rgt2p pathway (**Paper IV**). To assess the consistency of the sensing response on xylose across strains background, the fluorescent reporters were also used in an industrially derived strain (**Paper V**). Finally, mutagenesis of the G-protein coupled receptor Gpr1p was attempted to enable xylose binding and selective PKA activation for improved utilization (**Paper VI**).

Chapter 3.

Understanding the sensing of xylose

3.1. Recombinant pathways for xylose catabolism

To study and understand the fundamental nature of the xylose response in *S. cerevisiae,* genetic and metabolic engineering were used as tools to perturb the cell. By introducing new pathways, disrupting metabolic reaction steps, and removing extracellular receptors, the underlying cause of the xylose sensing response was gradually identified.

In this thesis, three distinct metabolic routes were employed for xylose utilization: the XR/XDH oxidoreductive pathway, the XI isomerization pathway, and the Weimberg oxidation pathway (Figure 3.1). Each pathway differs in redox neutrality, metabolic intermediates, and level of technical development. The metabolic pathways were implemented and optimized in separate biosensor strains to compare the xylose sensing response as a function of redox imbalance or formation of specific metabolic intermediates.

3.1.1. The oxidoreductive pathway

The XR/XDH pathway is a well-established metabolic pathway that has been used and optimized over the course of the past three decades (Hahn-Hägerdal et al., 2007a; Wasylenko & Stephanopoulos, 2015). It is typically found in microbes inhabiting wood-degradation niches and consists of two enzymes: xylose reductase and xylitol dehydrogenase (Valdehuesa et al., 2018; Wasylenko & Stephanopoulos, 2015). These enzymes catalyze the conversion of xylose into xylulose, which can be endogenously converted into xylulose-5-phosphate by *S. cerevisiae* and metabolized via the pentose phosphate pathway (PPP) followed by glycolysis (Wasylenko & Stephanopoulos, 2015). Three xylose molecules are required to complete one pass through the pathway, optimally resulting in the formation of at least 5 ATP, 5 NADH, and 5 pyruvate after glycolysis—or a maximum of 20.8 ATP equivalents per xylose after respiration.

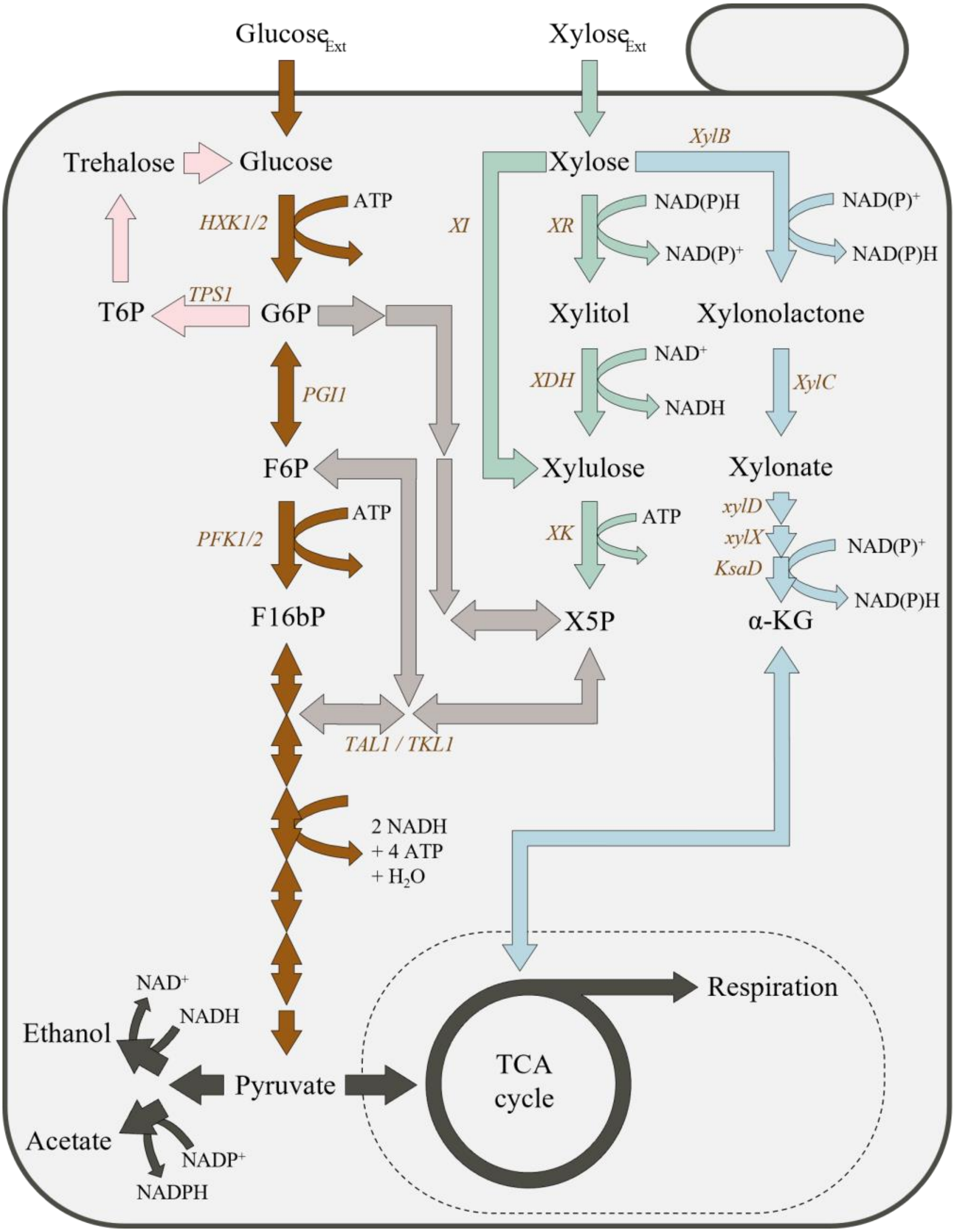


Figure 3.1. Overview of the central carbon metabolism of *Saccharomyces cerevisiae*, along with key genes and recombinant xylose utilization pathways. Metabolites (G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F16bP, fructose-1,6-bisphosphate; X5P, xylulose-5-phosphate; αKG, α-ketoglutarate) and enzymes (XylB, xylose dehydrogenase; XylC, xylonolactonase; XylD, xylonate dehydrogenase; XylX, 2-keto-3-deoxy-xylonate dehydratase; KsaD, α-ketoglutarate semialdehyde dehydrogenase; XR, xylose reductase; XDH, xylitol dehydrogenase; XI, xylose isomerase; XK, xylulokinase) have been abbreviated or omitted to improve clarity.

Initially, limited xylose conversion was observed when the XR/XDH encoding genes were expressed in *S. cerevisiae*. Limited PPP activity and xylose transport represented substantial challenges which were addressed by overexpressing genes encoding xylulokinase, PPP enzymes (*TAL1*, *TKL1*), and transporters. Another issue was the redox imbalance imposed by the NADPH-preference of the *Scheffersomyces stipitis* (formerly *Pichia stipitis*) XR (SsXR) not aligning with the NAD^+-preference of the *Sc. stipitis* XDH (SsXDH). This led to xylitol accumulation and decreased strain performance, especially under anaerobic conditions (Kötter & Ciriacy, 1993).

Since then, a few native xylose utilizing species have been shown to also carry homologs of XR with alternative cofactor preference, allowing the differential expression of NADH-preferring XR under oxygen-limiting conditions presumably to combat this redox imbalance (Barros et al., 2024). Indeed, replacing the SsXR with an XR lacking cofactor preference such as one found in *Spathaspora passalidarum* (SpXR) or *Sc. xylosifermentans* (SxXR) did improve the phenotype of recombinant xylose-fermenting *S. cerevisiae* under anaerobic conditions (Barros et al., 2024; Cadete et al., 2016). Still, it remains unclear whether any residual redox imbalance can be linked to the aforementioned peculiar response on xylose.

3.1.2. The isomerization pathway

The XI pathway is most commonly found in bacteria inhabiting wood-degradation niches such as beetle guts, although a few eukaryotic homologs have also been identified (Kwak & Jin, 2017; Miyamoto et al., 2022). Similar to the XR/XDH pathway, the XI pathway relies on the PPP as a metabolic shunt towards glycolysis (Figure 3.1) (Miyamoto et al., 2022). However, only one enzyme is required for the interconversion between xylose and xylulose which eliminates any potential redox imbalance in the pathway (Miyamoto et al., 2022). Consequently, the XI pathway could be employed to investigate if the aforementioned redox balance in the XR/XDH pathway plays a significant role in sugar signaling on xylose.

While the XI pathway benefits from being redox neutral, achieving efficient xylose utilization with the pathway has proven challenging (Moysés et al., 2016; van Maris et al., 2007). The recombinant XI enzymes require covalent metal ions and, being primarily found in bacteria, often perform better at higher temperatures (Bhosale et al., 1996). Likely due to these factors, efficient xylose utilization typically requires high gene copy numbers to enable sufficient xylose conversion in *S. cerevisiae* (Moysés et al., 2016). Although this can be overcome by using multicopy plasmids, the use of antibiotics-based plasmids is not feasible for industrial use, and integration into dynamic genomic sites such as the delta-region may be preferred (Hahn-Hägerdal et al., 2007a; Sakai et al., 1990). In our study (Paper I), we ultimately had to resort to using multicopy plasmids for xylose utilization to occur via the XI route.

To improve the xylose utilization further, we also introduced a gene encoding xylose epimerase: an enzyme interconverting xylose anomers. Under standard conditions, xylose consists of 33% α-D-xylose, 66% β-D-xylose, and 1% linear xylose (Schmidt et al., 1996). This is of industrial relevance since several xylose-binding enzymes have shown a preference for α-D-xylose over the other anomers (Miyamoto et al., 2022; Schray & Rose, 1971; Vogl & Brecker, 2013). However, xylan polymers in the hemicellulose feedstock consist almost exclusively of β-D-xylose, and as such the conversion to α-D-xylose may pose a bottleneck (Gírio et al., 2010). This may explain why *Lactococcus lactis* carries an epimerase enzyme in its xylan operon (Erlandson et al., 2000). This *L. lactis* enzyme has previously been applied to industrial *S. cerevisiae* strains resulting in improved xylose performance (Sibbesen et al., 2016). In our study, the xylose epimerase gene from *L. lactis* was introduced in strains carrying different homologs of XI (Paper I). Curiously, improvement was only observed for the strain carrying the *Lachnoclostridium phytofermentans*-derived XI (LpXI). Given that the LpXI-based strain had previously shown the highest utilization rates, this may indicate that the anomerization rate only becomes a bottleneck when the rate of isomerization is sufficiently high. Alternatively, it could indicate that different homologs have different anomer preferences.

3.1.3. The Weimberg pathway

In contrast to the XR/XDH and XI pathways, the Weimberg pathway does not rely on substrate-level phosphorylation via glycolysis for xylose utilization (Valdehuesa et al., 2018). Instead, xylose is converted into α-ketoglutarate through a series of oxidations and isomerizations, entering the central carbon metabolism via the tricarboxylic acid (TCA) cycle (Valdehuesa et al., 2018) (Figure 3.1). One xylose molecule is optimally converted into 2 GTP, 7 NADH, and 2 $FADH_2$—or approximately 20.5 ATP per xylose after respiration. Although the energy yield is slightly lower and respiration is a necessity, this conversion occurs without any decarboxylation reaction. This allows all carbon from the xylose molecule to be conserved into the TCA cycle. For comparison, the XR/XDH and XI pathways both result in a net 33% total carbon loss to CO_2 during conversion of pyruvate to acetyl-CoA when attempting to form the same TCA intermediates.

The Weimberg pathway is commonly found in organisms inhabiting aerobic and carbon-limited niches, such as the freshwater bacterium *Caulobacter crescentus* and the soil bacterium *Pseudomonas fragi*, likely due to its carbon conservation and reliance on respiration (Almqvist et al., 2018; Buchert & Viikari, 1988; Weimberg, 1961). Interestingly, parts of the Weimberg pathway are also functionally expressed in bacteria such as *E. coli*, *Corynebacterium glutamicum*, and *P. putida* production; this has led to efforts in reconstituting the missing enzymes into these organisms to enable xylose utilization via the Weimberg pathway, with the end goal of carbon-efficient organic acid production (Bator et al., 2020; Brüsseler et al., 2019; Lu et al., 2021; Radek et al., 2017; Rossoni et al., 2018). Amino acids such as α-ketoglutaric acid, succinic acid, and citric acid not only have intrinsic value, but also serve as excellent starting points for the synthesis of other industrially-relevant chemicals including: itaconate, butanediols, aldonic acid, and γ-polyglutamic acid (Lu et al., 2021; Toivari et al., 2010). Moreover, intermediates within the pathway, such as xylonate, are valuable for applications in concrete dispersal, textile bleaching, and electroplating (Buchert & Viikari, 1988). While bacterial hosts may be more suitable for many of these products, the inherent acid tolerance of *S. cerevisiae* could make it more suitable for the industrial production of carboxylic acids. Unfortunately, implementing the Weimberg pathway in *S. cerevisiae* has proven challenging (Borgström et al., 2019; Tanaka et al., 2025; Wasserstrom et al., 2018).

Efforts to establish the Weimberg pathway in *S. cerevisiae* have involved introducing recombinant genes from *C. crescentus* and *Co. glutamicum* (Borgström et al., 2019; Tanaka et al., 2025; Wasserstrom et al., 2018). This approach has led to strains capable of growth on xylose in bioreactors when primed with glucose (Borgström et al., 2019). Dysregulating the iron metabolism (by deletion of *BOL2*) proved essential for improving the pathway efficiency in *S. cerevisiae*, likely because the bacterial XylD was initially improperly loaded by the eukaryotic iron sulfur cluster biogenesis system (Borgström et al., 2019; Wasserstrom et al., 2018). Similarly, overexpression of the last three enzymes of the pathway was necessary to decrease byproduct accumulation in the form of xylonate (Borgström et al., 2019). Despite these efforts, xylonate formation still occurred, and glucose pre-supplementation and sparging were required for growth on xylose (Borgström et al., 2019). A recent study managed to enable growth on xylose as the sole carbon source by applying phylogenetic analysis and screening to identify new pathway enzymes, although 50% of the xylose was still accumulated as xylonate (Tanaka et al., 2025).

In our study (Paper II), the Weimberg pathway was optimized further and leveraged to study sugar signaling during non-glycolytic xylose utilization. An improved xylose utilization was unexpectedly observed when switching from the CEN.PK laboratory strain background to the W303 signaling background. It remains unclear why this is the case; however, similar dramatic changes in performance of recombinant pathways have previously been reported (Canelas et al., 2010; Strucko et al., 2015). Further adaptation was performed in the W303 background using a bioreactor with xylose supplementation. The new strain required neither air sparging nor glucose pre-supplementation, and no xylonate accumulation could be detected. The maximum specific growth rate of the adapted strain on xylose was rather high, 0.07 h^{-1}, with the isogenic XR/XDH strain showing a maximum rate of 0.10 h^{-1}. However, this is still far from the highest xylose growth rates reported in the literature which are around 0.25 h^{-1} (Govindaswamy & Vane, 2007; Jeppsson et al., 2003; Trivedi et al., 2023). Whole genome sequencing of the adapted Weimberg strain confirmed the integrity of the pathway and revealed a mutations related to mitochondrial function, protein metabolism, and cell cycle modulation (Paper II).

3.2. The cause(s) of the xylose sensing response

3.2.1. Impact of xylose-induced redox imbalances on sensing

While the repression of *HXT1* and induction of *TPS1* seen on xylose is expected due to the lack of glucose, induction of *SUC2* is typically only observed when low levels of glucose are present. Despite this, strains growing on xylose as the sole carbon source via the XR/XDH pathway displayed *SUC2* induction—typically indicative of a respiratory response (Brink et al., 2016; Osiro et al., 2018). The redox imbalance between XR and XDH, resulting in NADH accumulation, has previously been suggested as a potential cause for the respiratory response (Jin et al., 2004). However, the *SUC2* induction was observed in a strain carrying the optimized SpXR/SsXDH enzyme pair which is less redox imbalanced, making this explanation less likely (Cadete et al., 2016; Osiro et al., 2018).

When the redox neutral XI pathway (Paper I) and the NADH-accumulating Weimberg pathway (Paper II) were introduced in the W303 background, the induction of *SUC2* observed on xylose in the XR/XDH strain was still observed—indicating that redox imbalance was not a likely cause for the sensing response (Figure 3.2). Instead, alternative hypotheses were considered, including the impact of: i) xylose-derived intracellular intermediates, ii) intracellular pH, iii) intracellular energy carriers, and iv) extracellular xylose.

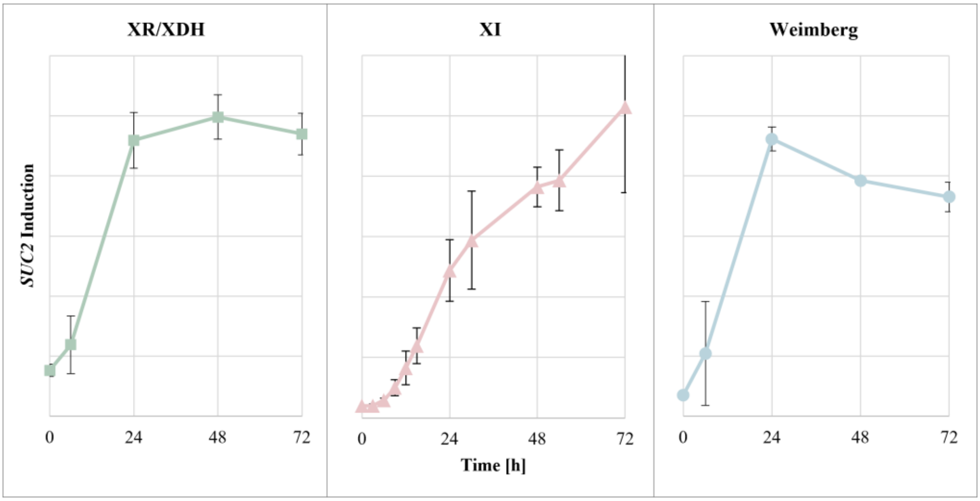


Figure 3.2. The same xylose response was observed regardless of which xylose pathway is introduced. Graphs show mean GFP fluorescence from strains carrying the *SUC2* biosensor and one of the three xylose utilization pathways, adapted from Papers I and II: XR/XDH (left), XI (middle), Weimberg (right).

3.2.2. Impact of xylose-derived intracellular metabolites on sensing

Several intracellular metabolites have been proposed to participate in signaling-related protein-metabolite interactions, including glucose-6-phosphate (G6P), glucose-1-phosphate (G1P), fructose-6-phosphate (F6P), and fructose-1,6-bisphosphate (F16bP) (Deroover et al., 2016; Milanesi et al., 2020; Milanesi et al., 2021; Nunes et al., 2013; Peeters et al., 2017; Vicente et al., 2018). For instance, F16bP has been observed to act as a flux sensor for the PKA pathway in *S. cerevisiae* (Peeters et al., 2017) and for the mammalian SNF1 pathway analog (AMPK) (Zhang et al., 2017). Although the latter appears to rely on the formation of an AMPK complex for which there is no ortholog in yeast; moreover, recent studies have indicated that G6P rather than F16bP may be a flux sensor for the SNF1 pathway (Milanesi et al., 2021). To investigate if intermediates affect the xylose sensing response, we employed a variety of analytical techniques while altering intracellular metabolite levels by: supplying different carbon sources, using different xylose pathways, and deleting enzymes responsible for key glycolytic reactions.

Despite the substantial differences in overall routes, untargeted metabolomics revealed that the XR/XDH and Weimberg strains accumulated similar levels of G6P and F6P (Paper II). Combined with the aforementioned conservation of *SUC2* induction in the Weimberg strains, suggesting SNF1 pathway activation, this is a strong indication that glycolytic intermediates are involved in the xylose response. *PFK1/2* deletants which are unable to form F16bP still showed activation of the SNF1 pathway when supplied with glucose (Milanesi et al., 2021), indicating that either G6P or F6P is specifically involved in the SNF1 complex interaction.

By deleting the *PGI1* gene, encoding the phosphoglucose isomerase responsible for the interconversion between G6P and F6P in glycolysis, we sought to further narrow down the list of causative intermediates. This deletion made XR/XDH-based strains unable to form G6P from xylose and fructose, but able to form it from glucose and galactose. By supplying the deletants with specific sugar mixtures (e.g., xylose, or glucose combined with fructose), we were able to selectively accumulate metabolic intermediates. Using this approach, combined with targeted metabolite profiling, we demonstrated that the xylose sensing response likely stems from the sugar phosphate intermediates downstream of the phosphoglucose isomerase enzyme (Paper III). In combination with the aforementioned results, this indicates that F6P serves as the flux sensing molecule responsible for activating SNF1 complex on xylose. Consistent with this hypothesis, *pgi1* deletants grown on xylose exhibited both increased accumulation of F6P and increased *SUC2* induction compared to the wildtype strain.

3.2.3. Impact of intracellular pH on sensing

Besides intracellular sugar phosphate levels, other key physiological parameters can vary when changing the carbon source. Could alterations to the intracellular pH influence the sugar signaling pathways? Decreased intracellular pH has been linked to decreased PKA activity via Ras1/2p and to increased SNF1 complex activity via a pH-sensitive polyhistidine domain which otherwise inhibits complex assembly (Colombo et al., 1998; Simpson-Lavy & Kupiec, 2022). *S. cerevisiae* exhibits differing intracellular pH depending on glucose availability (Imai & Ohno, 1995; Orij et al., 2011; Simpson-Lavy & Kupiec, 2022). When glucose is abundant, the homeostasis is maintained via ATP-dependent proton efflux pumps which places the intracellular pH at around 7.0–7.4, favoring PKA activation and inhibiting SNF1 activity (Dechant et al., 2010; Isom et al., 2018; Simpson-Lavy & Kupiec, 2022). In contrast, for glucose-deprived cells, intracellular pH often drops to 5.7–6.0 which reinforces high SNF1 activity and low PKA activity (Orij et al., 2009). On xylose, intracellular pH measurements typically remain near 7.0–7.5 in both non-conventional and recombinant xylose-metabolizing yeasts, i.e., substantially higher than the acidic intracellular pH found in truly starved cells and cell incapable of xylose metabolism (5.5) (Lohmeier-Vogel et al., 1995; Lohmeier-Vogel et al., 1998; Lohmeier-Vogel et al., 1996; Sreenivas et al., 2024; Torello Pianale et al., 2021). This high intracellular pH would be expected to activate PKA and inactivate SNF1 if it were the predominant signal; yet the experimental data consistently indicate the opposite, with PKA inactivity and SNF1 complex activity. Consequently, the available evidence does not support intracellular pH fluctuations as the primary cause of the observed xylose-induced SNF1 signaling response.

3.2.4. Impact of intracellular energy carriers on sensing

While ATP levels are intrinsically linked to PKA activity by acting as the substrate for cAMP production, the ADP and AMP levels are known to regulate the activity of the SNF1 complex (Chandrashekarappa et al., 2011; Mayer et al., 2011). ADP and, to a lesser extent, AMP are notably the predominant species involved in protecting SNF1 from phosphorylation-induced autoinhibition (Chandrashekarappa et al., 2011). As such, with increasing levels of ADP, SNF1 activity is expected to increase. ADP levels can be obtained from previous studies where the adenylate energy charge (AEC), the ratio of ATP:ADP:AMP, had been determined during starvation and growth on glucose and xylose (see Table 2.1) (Bergdahl et al., 2012; Klimacek et al., 2010). The data show that ADP levels are elevated on xylose compared to starvation, in line with the reported increased SNF1 activity (Klimacek et al., 2010; Osiro et al., 2018) and the anaerobic induction of the SNF1-controlled *SUC2*p_GFP biosensor (Osiro et al., 2019). Consequently, ADP acting as an allosteric activator of SNF1 during xylose utilization and causing the xylose response should not be ruled out. However, this needs a dedicated investigation.

Table 2.1. Data from Klimacek et al. (2010), showing levels of adenylate and guanylate energy carriers (µmol/g_{cell}) in *S. cerevisiae* when exposed to glucose, xylose, and carbon-less media. The adenylate and guanylate energy charges (AEC and GEC, respectively) represent the energy state of the cell.

	Glucose	Xylose	Starvation
ATP	13	7.2	0.2
ADP	4.7	4.0	0.5
AMP	1.3	2.8	4.7
AEC	0.81	0.66	0.08
GTP	3.0	1.7	0.0
GDP	0.9	0.9	0.2
GMP	0.1	0.8	1.5
GEC	0.86	0.63	0.06

The GTP:GDP ratio has also been suggested to play a role in PKA regulation via the Ras1/2p, which adopt an active form when carrying GTP. This is based on *in vitro* correlations between Ras2p GTP-loading and the GTP:GDP ratio (Rudoni et al., 2001), and may be linked to the low PKA activity seen on xylose since the cellular guanylate energy charge (GEC) changes in a similar way as described for the AEC (Table 2.1) (Klimacek et al., 2010; Myers et al., 2019; Osiro et al., 2018). However, this is largely based on indirect evidence, whereas Ras1/2p have been shown to be directly controlled by F16bP via Cdc25p/Sds25p (Peeters et al., 2017). As such, a lack of F16bP would also explain the decreased PKA activity on xylose. In line with this, strains on xylose showed a preferential accumulation of F6P rather than F16bP (Paper III).

3.2.5. Impact of extracellular xylose on sensing

Besides the intracellular regulation, sugar signaling in *S. cerevisiae* is also connected to the extracellular environment via transmembrane receptors. Conflicting evidence can be found as to whether the glucose receptor Snf3p is able to sense extracellular xylose or not. A study monitoring Mth1p degradation, downstream of Snf3p activation, was unable to demonstrate an effect by xylose (Dietvorst et al., 2010). On the other hand, partial induction of Snf3p-induced *HXT2* reporter was observed when xylose was supplemented (Brink et al., 2016). To shed light on this discrepancy, *SNF3* gene was deleted and the *HXT2* induction was recorded in strains engineered for xylose utilization to various extents (Paper IV).

Deletion of *SNF3* in strains lacking xylose metabolism resulted in decreased induction of the *HXT2* fluorescent reporter, indicating that Snf3p is able to detect extracellular xylose. This aligns with previous findings obtained in *snf3*-null mutants using qPCR (Wu et al., 2020), further cementing the causal link between extracellular xylose sensing and *HXT2* induction. Paper IV differed from previous research in that we conducted assays at the single cell level without requiring additional carbon sources and without being affected by constitutive PKA mutations, offering a complementary perspective on the topic. The previous study also observed a diminished level of *HXT2* induction on xylose compared to glucose in a Snf3p-dependent manner, suggesting lower affinity or incomplete activation by xylose. While our results also showed an overall lower level of *HXT2* induction, we discovered that this reduction was attributable to the Snf3p-dependent formation of subpopulations where only certain cells are induced.

When combining *snf3* deletion with xylose catabolism, the expression of *HXT2* became less dependent upon Snf3p-activation and the non-induced subpopulation was no longer observed (Paper IV). Most likely, the intracellular signals generated by xylose-derived intermediates overshadowed the extracellular signal from Snf3p. As mentioned in Chapter 2, *HXT2* derepression is carefully controlled by the SNF1 complex by phosphorylating Rgt1p to inhibit the recruitment of the Cyc8p-Tup1p co-repressor complex (Palomino et al., 2006; Papamichos-Chronakis et al., 2004; Roy et al., 2013). With increased SNF1 complex abundance and activity due to the presence of F6P and elevated ADP levels, the Snf3p-independence may arise from increased phosphorylation of Rgt1p preventing the initial formation of the Rgt1p-Cyc8p-Tup1p repressor complex (Figure 3.3). Thus, the Std1p/Mth1p co-repressors are unable to fulfill their role in protecting Rgt1p from phosphorylation—making the signal independent of Snf3p activation during xylose utilization (Figure 3.3).

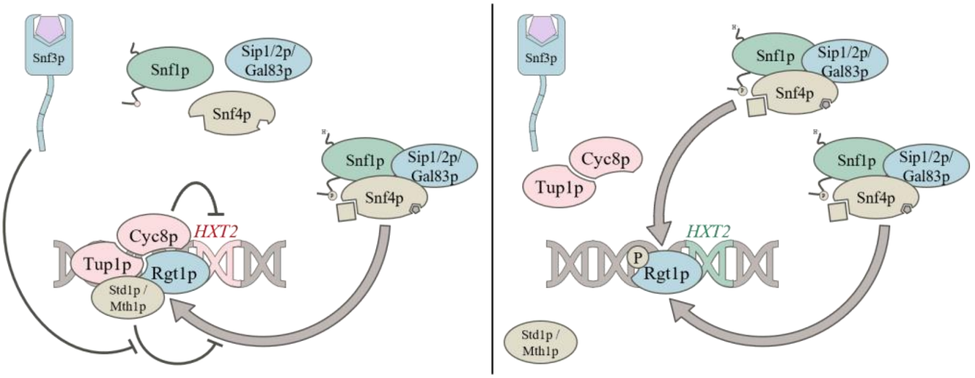


Figure 3.3. Proposed mechanisms for xylose-dependent *HXT2* induction via the Snf3p receptor (left) and the decoupling of Snf3p activation from *HXT2* induction when xylose catabolism is introduced (right).

3.3. Applicability of the results

The results discussed above point towards the xylose-derived sugar signaling response being connected to the formation of metabolic intermediates and to the extracellular sensing of Snf3p. Moreover, the response is not changed by implementing another xylose utilization pathway—highlighting the importance of understanding and engineering said response. However, these conclusions stem from experiments performed in laboratory strains, whereas many of the strains used for industrial bioethanol production are the products of extensive selective breeding with potential mutations in the signaling pathways (Hahn-Hägerdal et al., 2007a).

To test whether our findings were industrially applicable, we also introduced the signaling biosensors in a haploid line derived from the Brazilian cellulose-fermenting PE-2 strain carrying the XR/XDH pathway (Paper V). The function of the biosensors was validated on glucose (Figure 3.4), and the peculiar xylose sensing was shown to also be both present and relevant in industrial strains (Figure 3.5).

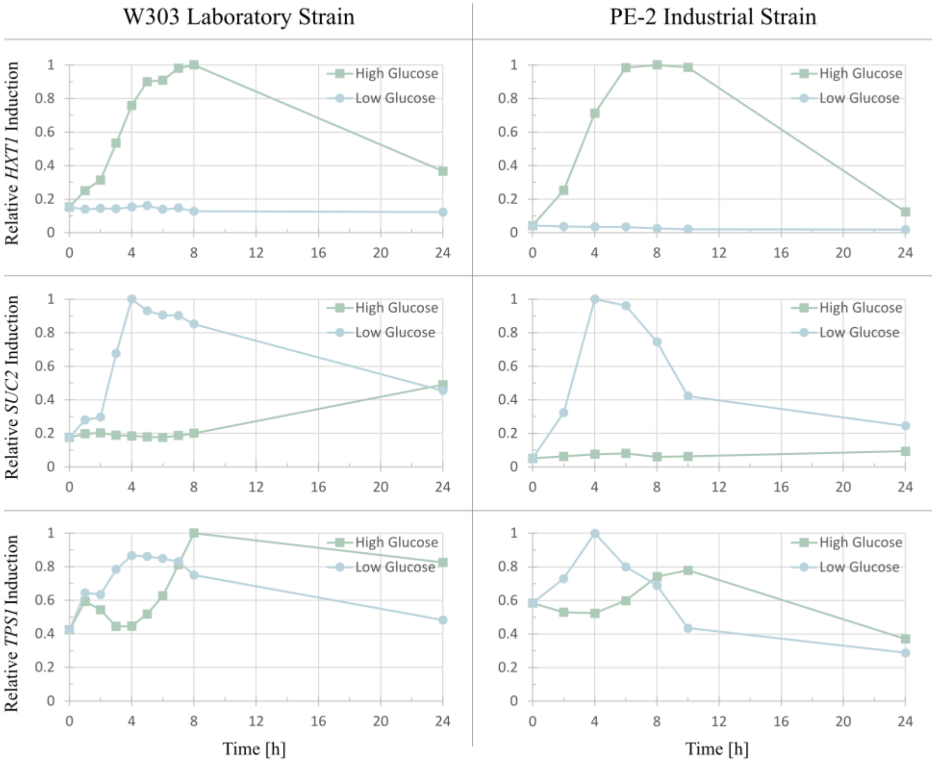


Figure 3.4. Sugar signaling response to high and low levels of glucose show high similarities between laboratory (W303; left) and industrially derived (PE-2; right) strains.

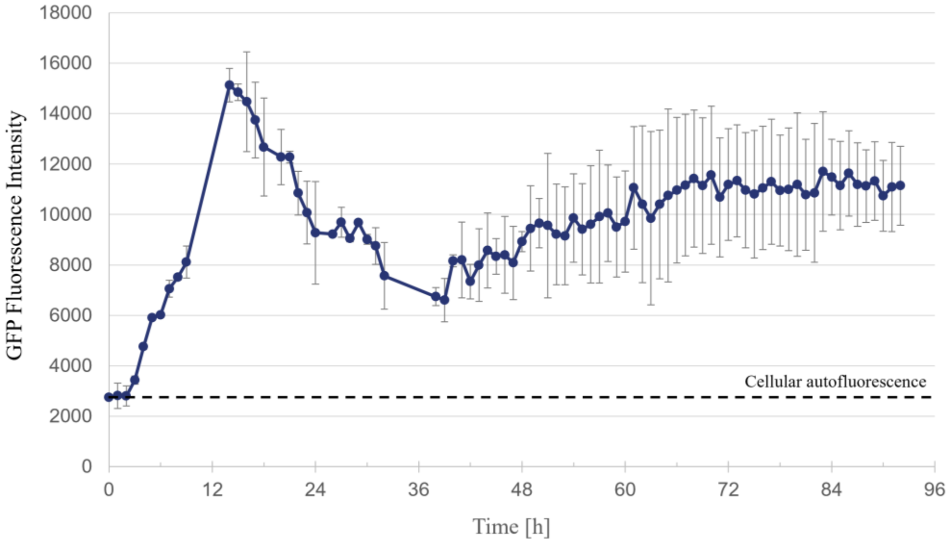


Figure 3.5. Measurements of the *SUC2p*-based biosensor using an automated flow cytometer reveals xylose-derived induction in industrially-derived (PE-2) strains. The induction is indicative of the same SNF1 pathway activation that is reported in laboratory strains during xylose catabolism.

Chapter 4.

Engineering the sensing of xylose

Since the peculiar sugar signaling response in recombinant xylose-utilizing strains is likely driven by the formation of intracellular intermediates, it is difficult to circumvent it entirely. However, it may be possible to rewire the signaling pathways to favor fermentation despite its presence. Engineering sugar signaling for improved xylose utilization has previously been achieved by targeting regulators upstream and effectors downstream in the Snf3p/Rgt2p, SNF1, and PKA pathways (Myers et al., 2019; Osiro et al., 2019; Roca et al., 2004; Wu et al., 2020). While these constitutive mutations improved xylose utilization, they also impacted other cellular phenotypes such as stress tolerance (Peeters et al., 2007). This chapter reviews prior approaches and covers our efforts to mitigate the drawbacks of constitutive mutants by introducing xylose-specific engineered receptors.

4.1. Targeting the PKA pathway

The PKA pathway is mainly active in the presence of high glucose levels, enhancing yeast catabolic activity while suppressing stress responses such as trehalose accumulation and chaperone synthesis (Budhwar et al., 2010; Colombo et al., 1998; Lorenz & Heitman, 1997; Peeters et al., 2007). The activation of the PKA pathway has been shown to enhance xylose utilization in recombinant strains carrying the XR/XDH and XI pathways (Myers et al., 2019; Osiro et al., 2019), although xylose itself does not naturally trigger PKA activation (Brink et al., 2016; Lemaire et al., 2004; Rolland et al., 2000). To elevate PKA activity, constitutive mutants have been developed through: i) deletion or mutation of *BCY1*, ii) deletion of *IRA2*, iii) deletion of *PDE1/2*, and iv) mutations of *GPA2* (Lorenz & Heitman, 1997; Myers et al., 2019; Osiro et al., 2019; Wu et al., 2020). A schematic overview of these targets within the PKA pathway is presented in Figure 4.1.

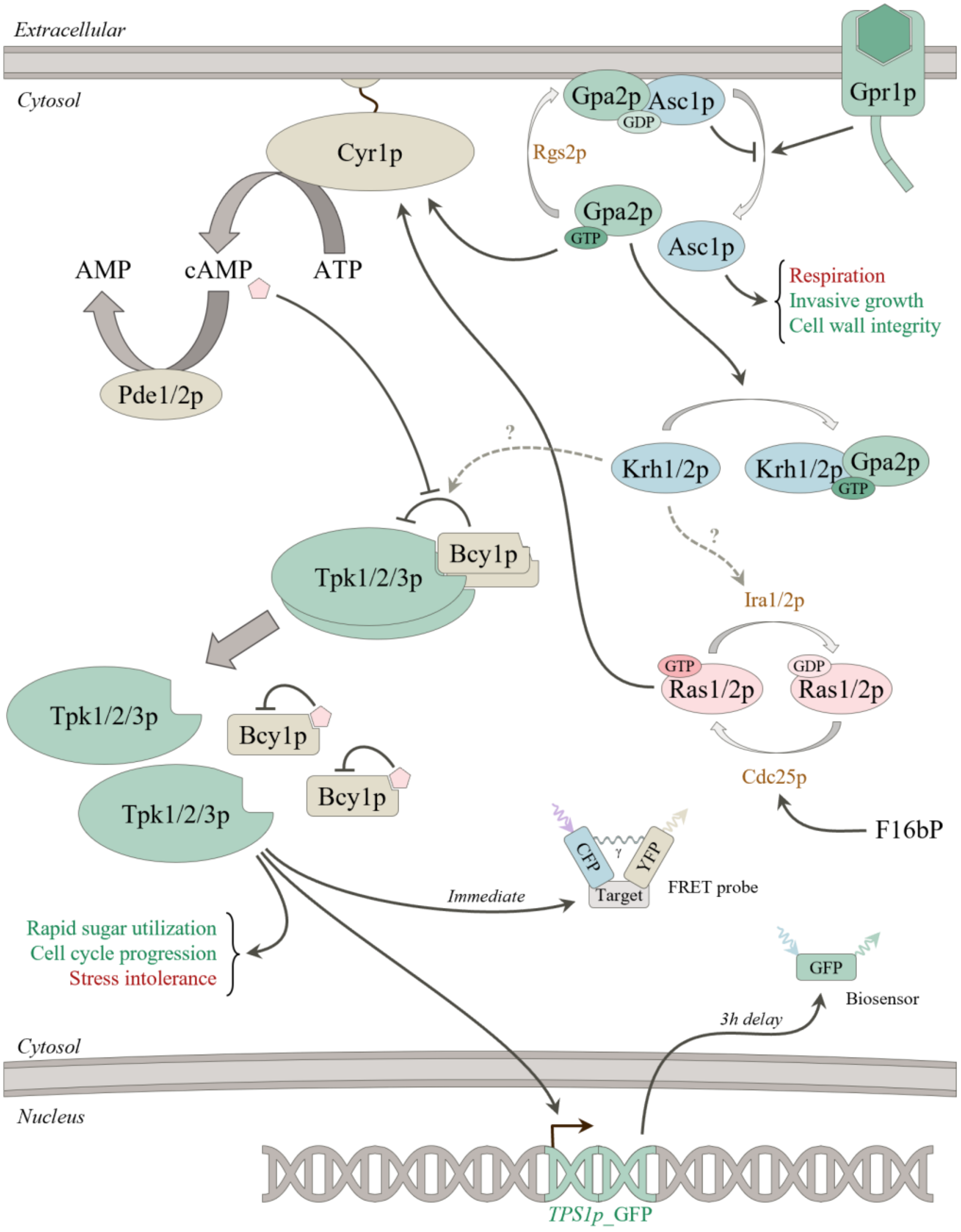


Figure 4.1. Detailed overview of the PKA pathway, including targets for constitutive engineering, physiological outcomes of pathway component activation, and key fluorescent reporters. Green bubbles indicate primary regulatory units, blue indicate secondary regulatory units, pink highlights the intracellular Ras1/2p route, and yellow indicates general proteins. Thick arrows indicate conformations changes or co-factor replacement. Thin arrows indicate positive interactions and hammerheads indicate negative interactions. Question marks with dashed arrows represent positive interactions that are yet to be fully confirmed. Abbreviations: F16bP, fructose-1,6-bisphosphate.

4.1.1. Constitutive PKA mutants for improved xylose utilization

A straightforward target for modifying PKA activity is the negative regulatory subunit Bcy1p. The PKA holoenzyme consists of two catalytically active subunits (Tpk1/2/3p) and two Bcy1p regulatory subunits (Broach, 2012). When cAMP binds to Bcy1p, it triggers its dissociation from the catalytic subunits, thereby activating PKA. Consequently, removing Bcy1p or disrupting its interaction with the catalytic subunits can increase PKA activity (Portela et al., 2003). Deletion of *BCY1* has previously been shown to improve xylose fermentation (Myers et al., 2019). However, this approach had significant drawbacks, including a disruption of the lipid homeostasis resulting in anaerobic growth arrest on xylose and slower aerobic growth on glucose—both of which pose challenges for industrial applications due to impaired strain propagation (Myers et al., 2019; Wagner et al., 2023). Additionally, deletion of *BCY1* is reportedly lethal depending on the strain background (DeWitt et al., 2023). As an alternative, modification of Bcy1p function has shown promise. The addition of a peptide to the C-terminus of Bcy1p improved xylose fermentation with fewer adverse effects on growth (Myers et al., 2019). The mechanism is as yet unknown, although it was hypothesized to result from a partial disruption of Bcy1p function—indicative of it having alternative functions, perhaps aiding in guiding PKA to target substrates (Myers et al., 2019; Smith et al., 2017).

Another approach for increasing PKA activity involved deleting the *IRA2* gene which encodes a negative regulator of Ras2p and consequently PKA (Tanaka et al., 1990). Mutants carrying *ira2*Δ deletion have been identified by several groups using adaptive laboratory evolution to improve xylose fermentation (de Oliveira Vargas et al., 2023; dos Santos et al., 2016; Myers et al., 2019; Osiro et al., 2019; Sato et al., 2016). This improvement occurred in a SNF1-dependent manner, suggesting that the deletion of *IRA2* was involved in SNF1 activation (Myers et al., 2019). The observed improvement was attributed to the co-activation of the PKA and SNF1 pathways, with PKA promoting robust metabolic activity and SNF1 enabling alternative sugar utilization. However, based on the findings presented in this thesis, it is more likely that SNF1 was already activated by the xylose-derived intracellular metabolites and that the deletion of *IRA2* only acted to co-activate the PKA pathway alongside the already activated SNF1 pathway. While a slight increase in SNF1 activity has been reported following *IRA2* deletion (Osiro et al., 2019), this effect was minimal compared to the activation caused by intermediates (Paper III).

Another alternative approach has been to target the low- and high-affinity phosphodiesterases Pde1p and Pde2p, which act as negative feedback regulators of PKA by converting cAMP into AMP (Colombo et al., 1998; Folch-Mallol et al., 2004). Mutant strains carrying the *pde1*Δ*pde2*Δ double deletion have been shown to respond more readily to external cAMP supplementation, lowering the threshold required for PKA activation, and exhibiting marginally improved xylose utilization and ethanol production (Lorenz & Heitman, 1997; Wu et al., 2020).

The final attempted target for achieving constitutive PKA activation was Gpa2p from the extracellular signaling branch. The *GPA2*^{G132V} mutant is known to exhibit constitutive activation, likely due to a reduction in GTPase activity (Lorenz & Heitman, 1997). Although this mutation led to improved xylose utilization, its impact was less pronounced compared to that observed in the *pde1*Δ*pde2*Δ double deletion mutant (Wu et al., 2020). It is important to emphasize that the improvements in xylose utilization and fermentation were relatively minimal in both the *GPA2*^{G132V} and the *pde1*Δ*pde2*Δ strains. Therefore, it remains uncertain whether these mutations can be practically applicable in an industrial setting.

4.1.2. Adverse effects of constitutive PKA activation

Although several of the strategies discussed above have proven successful in activating PKA, leading to improved xylose utilization, issues associated with stress tolerance were encountered. The PKA pathway is tightly linked to cell cycle progression and plays a crucial role in maintaining cells in the correct cycle phase during stationary phase, particularly when nutrients are depleted (Colombo et al., 1998; Folch-Mallol et al., 2004; Werner-Washburne et al., 1993). Consequently, mutants with increased PKA activity failed to properly arrest in the G_1 phase during starvation, which affected cellular fitness (Colombo et al., 1998; Smith et al., 1998). These mutants notably exhibited a lack of stress response gene activation and a lack of storage carbohydrates such as trehalose and glycogen, which normally correlate positively with stress tolerance (Colombo et al., 1998; Folch-Mallol et al., 2004). Overall, this resulted in poor growth on non-fermentable carbon sources, which is likely to be exacerbated under industrial conditions with acid and heat stress (Colombo et al., 1998).

4.1.3. Xylose-specific PKA activation

S. cerevisiae might not be as negatively impacted during nutrient starvation if it was possible to ensure PKA activation only occurred when xylose is present. To explore this, the extracellular branch of the PKA pathway was targeted with the objective of generating mutant Gpr1p receptors capable of xylose-dependent PKA activation.

The Gpr1p receptor and G-protein signaling in S. cerevisiae

As discussed in Chapter 2, Gpr1p interacts with the Gα protein Gpa2p via its C-terminal tail to stimulate cAMP production and activate PKA (Colombo et al., 1998; Conrad et al., 2014; Harashima & Heitman, 2005; Lorenz & Heitman, 1997). The binding specificity of Gpr1p appears restricted to glucose and sucrose, since other hexose sugars (e.g., fructose, galactose, mannose), glucose analogs (e.g., 2-deoxyglucose, 6-deoxyglucose), and xylose fail to activate the receptor (Brink et al., 2016; Lemaire et al., 2004; Rolland et al., 2000).

The G-proteins associated with Gpr1p in *S. cerevisiae* are rather unconventional (Figure 4.1). Rather than the typical heterotrimeric complex, *S. cerevisiae* only has a heterodimeric complex of the Gα-protein Gpa2p and Gβ-protein Asc1p without a conjugal Gγ-protein (Harashima & Heitman, 2005; Peeters et al., 2007; Zeller et al., 2007). The Gγ-protein is usually prenylated and responsible for the localization of the complex to the membrane; instead Gpa2p carries an atypical N-terminal domain that is capable of localizing independently (Harashima & Heitman, 2005; Huang et al., 2019; Zeller et al., 2007). Upon binding of glucose or sucrose to Gpr1p, the intrinsic GEF activity of Gpa2p is activated and GDP is replaced with GTP, which causes the release of Asc1p from Gpa2p (Harashima & Heitman, 2005; Rachfall et al., 2013; Zeller et al., 2007). Next, Gpa2p activates Cyr1p to produce cAMP and trigger PKA activity while Asc1p interacts with ribosomal proteins to inhibit the translation of transcription factors and upregulate respiration, invasive growth, and cell wall integrity (Peeters et al., 2007; Rachfall et al., 2013). Adding to the unconventional nature of this signaling system, Gpa2p has a second Gβ-like partner in addition to Asc1p. The Kelch repeat-containing proteins Krh1/2p (Gpb2/1p) are Gβ-mimics that interact with the GTP-bound form of Gpa2p and function as a bypass to regular Cyr1p-dependent signaling (Harashima & Heitman, 2002, 2005; Peeters et al., 2007). The bypass acts as an orthogonal signaling route, ensuring PKA activity remains low whenever Gpa2p is not active by preventing degradation of the PKA-inhibiting Bcy1p subunit and/or the Ras1/2p-inactivating Ira2p protein (Budhwar et al., 2010; Harashima et al., 2006; Peeters et al., 2006; Peeters et al., 2007). As such, creating a xylose-specific Gpr1p receptor may have additional advantages compared to the constitutive mutants, besides addressing the tolerance issues, as it may disable the Krh1/2p proteins and thus improve PKA activity further.

Gpr1p mutagenesis for xylose sensing

Substitution assays have identified critical amino acids within transmembrane domain VI of Gpr1p, with the sugar binding site likely being located near residues 627 to 648 (Figure 4.2) (Busti et al., 2010). Among these, residues A640, Q644, Y645, and E648 have been shown to be essential for glucose sensing, while residues P627, Y630, I633, W634, P637, and D641 were required for both glucose and sucrose sensing (Busti et al., 2010; Lemaire et al., 2004). It should be noted that the cysteine substitutions affecting sugar responsiveness may also alter receptor activity indirectly by modifying the protein structure—such as occluding the binding site or disrupting signaling domains—rather than directly impacting ligand binding.

With the goal of altering ligand affinity of the receptor, the residues in domain VI represent promising targets for mutagenesis. In addition to the A640C substitution that was previously demonstrated to inactivate Gpr1p glucose sensing (Lemaire et al., 2004), A640V substitution results in constitutively active Gpr1p (Lawenius, 2017). Consequently, mutagenesis of these ten amino acids was prioritized in the present work (Paper VI).

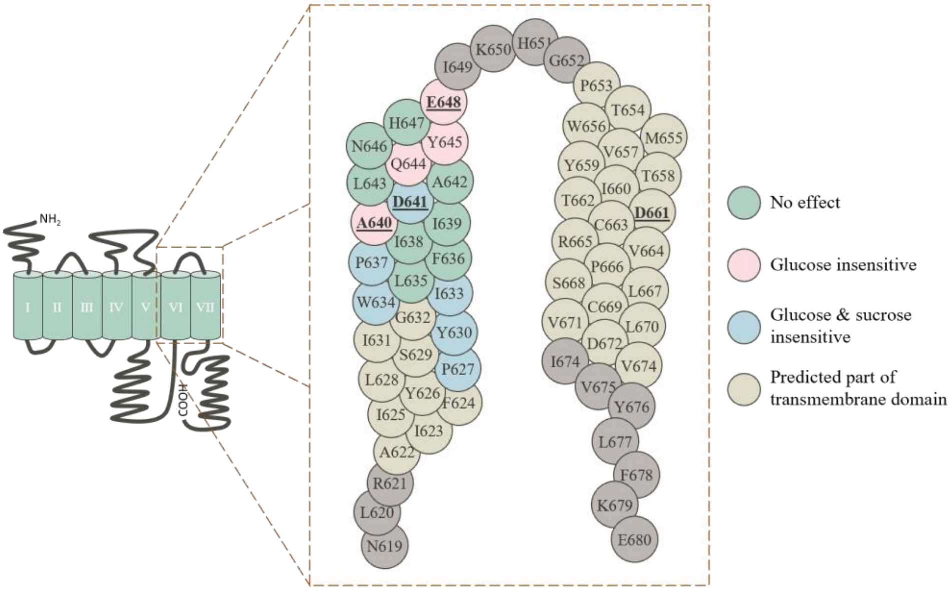


Figure 4.2. Overview of the structure of Gpr1p glucose receptor, including which residues have previously been subjected to cysteine substitution and the impact of substitution on PKA activation. Pink residues disabled glucose activation, blue residues disabled both glucose and sucrose activation, and green residues had no impact. Gray residues have not been investigated. All colored residues are predicted to be part of transmembrane domains VI (left) and VII (right). Bold and underscored residues are predicted by molecular docking simulations to contribute to glucose binding in Paper VI.

Attempting all possible amino acid substitutions in the ten residues associated with glucose and sucrose sensing would result in an astronomical number of variants (20^{10}; roughly 10 trillion combinations) which would be challenging to both generate and screen. To address this, the search space was narrowed down by predicting the sugar-binding residues using *in silico* methods. Based on a TmAlphaFold2 predicted structure (Jumper et al., 2021; Varadi et al., 2021; Varadi et al., 2023), many of the ten critical residues were found facing inwards towards an intramolecular pocket within the protein and molecular docking identified four amino acids as potential glucose binding partners: A640, D641, E648, and D661 (Figure 4.2, see underscored residues in bold) (Lemaire et al., 2004). For xylose, no such interactions could be predicted. By modeling amino acid substitutions, D641E was identified as a possible mutation for enabling xylose binding. D641 was previously identified as critical for both glucose and sucrose activation, while A640 and E648 substitutions only impacted glucose-derived activation (Lemaire et al., 2004). Based on these findings, the D641E substitution was selected for site-directed mutagenesis, together with A640C as a negative control and A640V as a positive control. To increase the likelihood of success and explore potential combinatorial effects, site-saturation mutagenesis was applied to residues A640, D641, and E648 as well.

To ensure that overexpression of native *GPR1* did not inadvertently impact PKA activity, for example by affecting cellular fitness through membrane crowding (Guigas & Weiss, 2016), cells transformed with the unmutated *GPR1* plasmid were screened as controls. PKA activity was then assessed using the *TPS1*p_GFP biosensor constructed by Brink et al. (2016). Overexpression of native *GPR1* did not alter the responsiveness to xylose but conferred a very low level of constitutive PKA activation under all environmental conditions tested (Paper VI). Moreover, the response became more sensitive to low levels of glucose. Nevertheless, these effects were not considered detrimental enough to impact the downstream screening step.

A fluorescence-activated cell sorting (FACS)-based approach was elected for screening. FACS operates similarly to flow cytometry, but allows for the physical separation and sorting of individual events after interrogation. This method can be powerful for screening cell libraries where a suitable fluorescent marker is available, such as labelled antibodies or fluorescent biosensors. However, the *TPS1*p_GFP biosensor might not be the optimal marker for this approach as it requires up to three hours to reach peak expression and degradation is quite slow without active growth. Moreover, the biosensor exhibits an inverse response to PKA activity, complicating both the assays and the interpretation of the results. Therefore, we also evaluated an alternative marker in the form of a Förster resonance energy transfer (FRET) probe based on the PKA-responsive AKAR3 element (Figure 4.3) (Allen & Zhang, 2006; Colombo et al., 2017; Colombo et al., 2022; Shrestha et al., 2015). Although the FRET probe has advantages over the *TPS1*p_GFP biosensor, several technical challenges were encountered when incorporating the FRET probe into our approach. For instance, not all energy is transferred between CFP and YFP, resulting in residual cyan fluorescence alongside yellow fluorescence. The emission profiles of these fluorophores overlapped substantially, and our flow cytometer lacked the necessary filters to distinguish the signals. Due to these constraints and a lack of time, the FRET approach was abandoned in favor of the *TPS1*p_GFP biosensor.

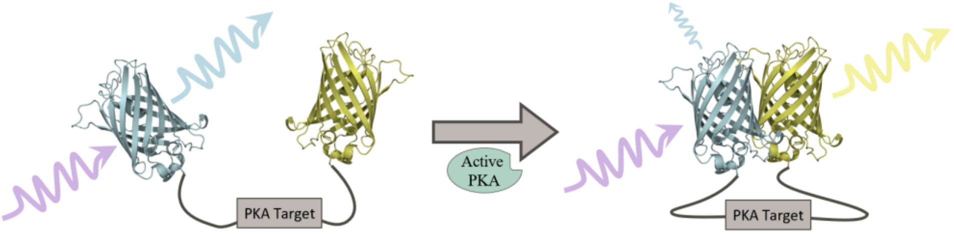


Figure 4.3. The FRET probe contains two fluorescent domains (from cyan and yellow fluorescent protein; CFP and YFP) which are connected by a PKA-substrate domain (AKAR3). Upon phosphorylation, a conformational change brings the fluorophores into close proximity—allowing resonance energy transfer. When CFP absorbs a violet photon, energy can be transferred to YFP which emits a yellow photon. The FRET probe responds directly to PKA activity and can be pre-expressed for faster response rates.

4.2. Engineering of receptors from other sensing routes

The activation of the Snf3p/Rgt2p pathway, alongside the PKA pathway in xylose-utilizing strains, provides a beneficial effect on xylose sensing and utilization (see Chapter 3) (Osiro et al., 2019). However, the reported increase in Snf3p/Rgt2p activity is based on a biosensor targeting the *HXT1* promoter, which is also regulated by the PKA pathway via Rgt1p (Figure 2.1) (Broach, 2012; Conrad et al., 2014). Thus, these findings should be confirmed through independent experiments, such as directly assaying Mth1p degradation. Alternatively, this could be demonstrated by developing receptors capable of xylose-based Snf3p/Rgt2p signaling. In the present thesis, we opted to construct chimeric receptors by fusing the sugar-binding domain of a known xylose transporter with the sugar-sensing domains of the Snf3p/Rgt2p glucose receptors (Paper IV). This approach circumvents the need for detailed knowledge of the residues involved in sugar binding. By enabling xylose sensing, this strategy aimed to enhance xylose utilization while investigating the impact of Snf3p/Rgt2p activation on strain performance.

4.2.1. Chimeric transceptors

Several transceptors—receptors with the ability to transport their substrates—have been identified in nature (Mep1/2p for nitrogen, Pho84p for phosphate, Gap1p for amino acids) (Conrad et al., 2014; Stasyk et al., 2018; Thevelein & Voordeckers, 2009; Van Zeebroeck et al., 2009). Since the Snf3p and Rgt2p receptors share significant homology with glucose transporters (Conrad et al., 2014; Özcan et al., 1996), it might be possible to attach the sugar signaling domains of Snf3p or Rgt2p onto other hexose transporters and retain both functions.

Chimeric fusion transceptors between the Hxt1p/2p glucose transporters and Snf3p or Rgt2p, which retain the ability to transport glucose and induce signaling, have previously been constructed (Kim et al., 2024; Özcan et al., 1998). However, the signaling response was weak—showing only 12 to 24% of the response observed with native Snf3p or Rgt2p; perhaps due to the absence of Yck1/2p-dependent stabilization or signaling properties inherent to the native binding domains (Kim et al., 2024). The latter hypothesis is more likely since overexpression of the chimeric transceptors did not resolve it (Özcan et al., 1998), and may be rooted in the binding domain needing to remain in a specific configuration for a period in order to transmit a signal (Kim et al., 2024; Scharff-Poulsen et al., 2018). Nevertheless, the outcome could be limited to the Hxt1/2p transporter domains as others have not been tested. Since domains from the Gal2p galactose transporter and Hxt2p have previously been shown to be interchangeable (Nishizawa et al., 1995), we fused the Snf3p and Rgt2p signaling domains to a xylose-promiscuous Gal2p transporter (*GAL2*^{N376F}; Gal2mut) (Rojas et al., 2021) in an attempt to create novel xylose receptors capable of activating the Snf3p/Rgt2p pathway (Paper IV).

4.2.2. Xylose-sensing chimeric receptors

At the outset of the project, it was not yet understood that Snf3p sensed extracellular xylose. Instead, the prevailing hypothesis was that intracellular xylose or its partial catabolism were the primary drivers of the observed increase in *HXT2* expression (Brink et al., 2016; Osiro et al., 2018). Accordingly, and to avoid the generation of transceptors, truncated variants of Gal2p were included in the construction of Snf3p- and Rgt2p-based chimeric receptors (Figure 4.4; Gal2STp and Gal2RTp, respectively) as the C-terminal domain has been shown to be essential for functional transport (Nishizawa et al., 1995).

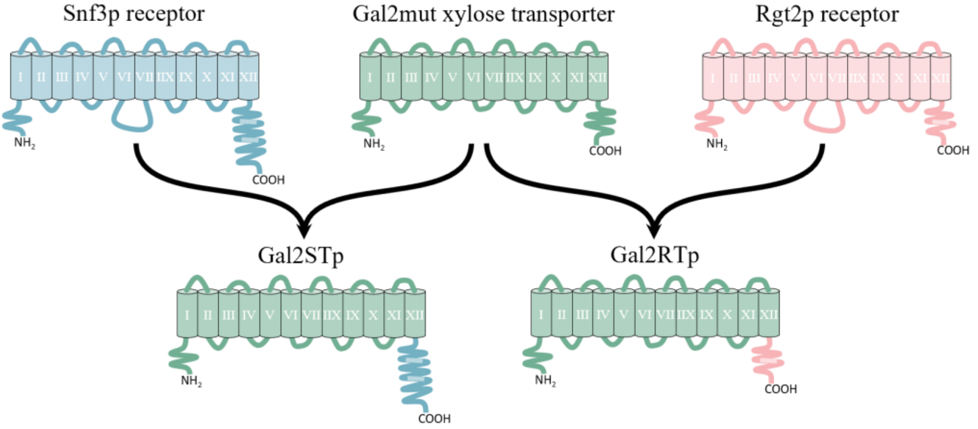


Figure 4.4. Construction of the chimeric receptors Gal2STp and Gal2RTp was achieved by fusing the sugar transporting domain of a xylose-transporting Gal2p mutant (Gal2mut; N376Y) with the signaling domain from the C-terminal domain of the Snf3p and Rgt2p receptors, respectively.

Upon introducing the Gal2STp chimera, the expected increase in *HXT2*p_GFP fluorescence was recorded in the presence of xylose (Paper IV). To verify whether the response was due to the chimera functioning as a receptor rather than as a transporter, several controls were included: fully intact Gal2mut, partially truncated Gal2mut, and fully truncated Gal2mut lacking the C-terminal tail. As fully truncated Gal2p was reported to fail in membrane localization (Rojas et al., 2021), we anticipated a wild-type response from this mutant, which was confirmed experimentally. However, the Gal2STp chimera showed no significant difference in activation compared to a partially truncated or a fully intact Gal2mut (Figure 4.5). This suggested that the Gal2STp chimera caused the response via retained transport functionality rather than by adopting a productive signaling conformation—functioning as transporters or transceptors rather than receptors.

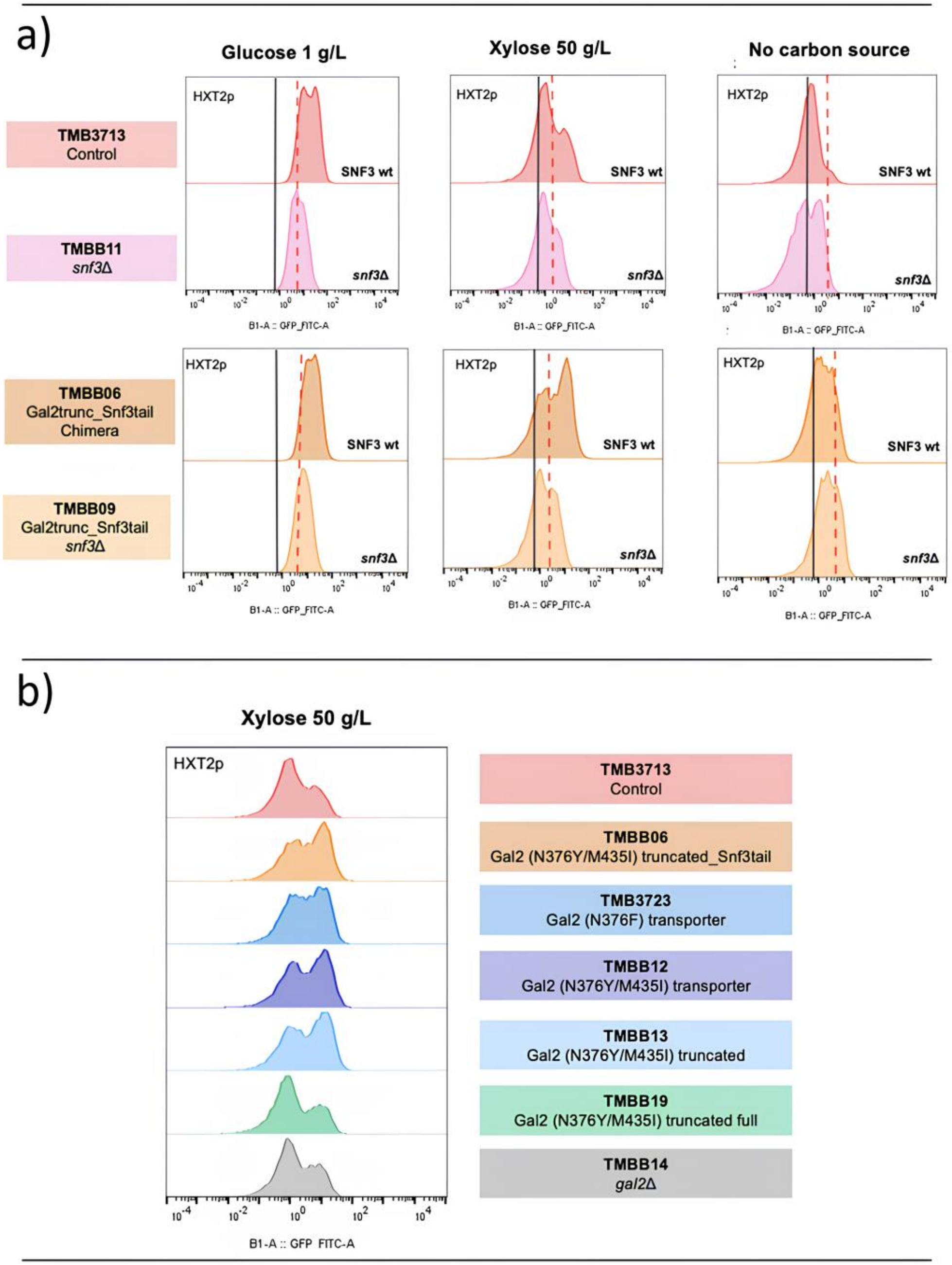


Figure 4.5. Results from the chimeric protein of Gal2mut transport domain with Snf3p signaling domain shows xylose-dependent increase in *HXT2* signal indicative of increased sugar signaling (a); however, truncated Gal2mut transporters without the signaling domain show the same response (b). Graphs are adapted from Paper IV.

In addition to the construction of Gal2STp, a Rgt2p-based chimera called Gal2RTp was also created and assessed for its performance using an *HXT1*p_GFP biosensor strain (Figure 4.4). Initial findings showed promise, as fluorescence levels increased with xylose supplementation (Figure 4.6). This observation was made in the presence of glucose since full *HXT1* expression requires Rgt1p dissociation via PKA hyperphosphorylation (see Chapter 2, Figure 2.9). Unfortunately, a similar increase in fluorescence was observed when an equal amount of salt was added to the medium instead of glucose, suggesting that the response may have been influenced by crosstalk from the HOG osmo-tolerance signaling pathway, which also affects Rgt1p (Brink et al., 2021). The underlying cause of the Gal2RTp chimera's lack of function remains unclear. While the Gal2p domain can likely transport and thus bind xylose, the chimera may suffer from degradation or an inability to adopt a functional signaling conformation. Given the recent revelation that the Rgt2p signaling domain itself serves as an endocytosis signal (Kim et al., 2024), this approach to enable xylose-induced Snf3p/Rgt2p signaling is not likely to work in glucose-free medium. Additional work to disable the degradation would be needed.

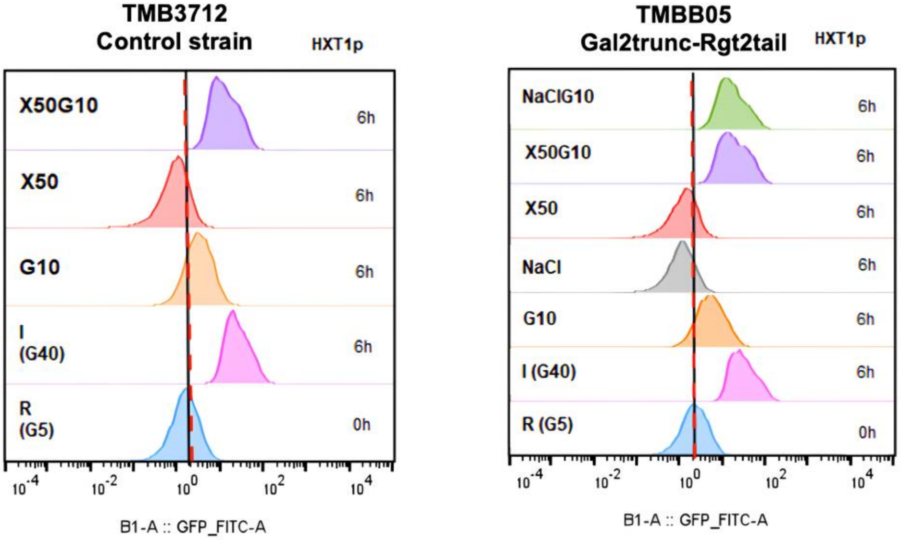


Figure 4.6. Results from chimeric protein of Gal2mut transport domain with Rgt2p signaling domain shows xylose-dependent increase in *HXT1* signal indicative of increased sugar signaling; however, addition of an equimolar amount of NaCl results in similar findings, suggesting that the osmolarity is behind the induction rather than xylose itself.

Chapter 5.

Conclusions and Outlook.

Despite active catabolism, xylose fails to elicit a robust fermentative response in the regulatory network of recombinant *S. cerevisiae*. Instead, a respiratory response is observed which likely leads to inefficiencies in xylose fermentation. At the outset of my thesis, it remained unclear whether this was a widely occurring phenomenon, or specific to laboratory strains. Four different potential causes of the respiratory response were hypothesized: sensing of the redox imbalance of XR/XDH, sensing of extracellular xylose, sensing of intracellular pH or energy carriers, and sensing of intracellular intermediates.

5.1. Thesis conclusions

Overall, the present thesis contributed to a deeper understanding of the peculiar xylose response. First, the same signaling response was observed in industrially-derived strains (Paper V), indicating the wider relevance of our findings. The sensing response also remained similar regardless of the presence of a cofactor imbalance or the specific xylose catabolic pathway used (Paper I-II). In parallel, a new role for the Snf3p receptor in extracellular xylose sensing was unraveled in non-utilizing cells (Paper IV), although it proved not to be a dominant factor during active xylose catabolism. Attempts at introducing xylose-sensitive receptors proved difficult and will require additional efforts to succeed (Papers IV & VI). Finally, evidence for a protein-metabolite interaction-based response was provided, identifying xylose-derived intermediates (likely fructose phosphates) as triggers for SNF1 complex activation (Papers II & III). Based on these results, the intracellular intermediates appear to be the dominant factor behind the xylose sensing response in xylose-utilizing strains (Figure 5.1).

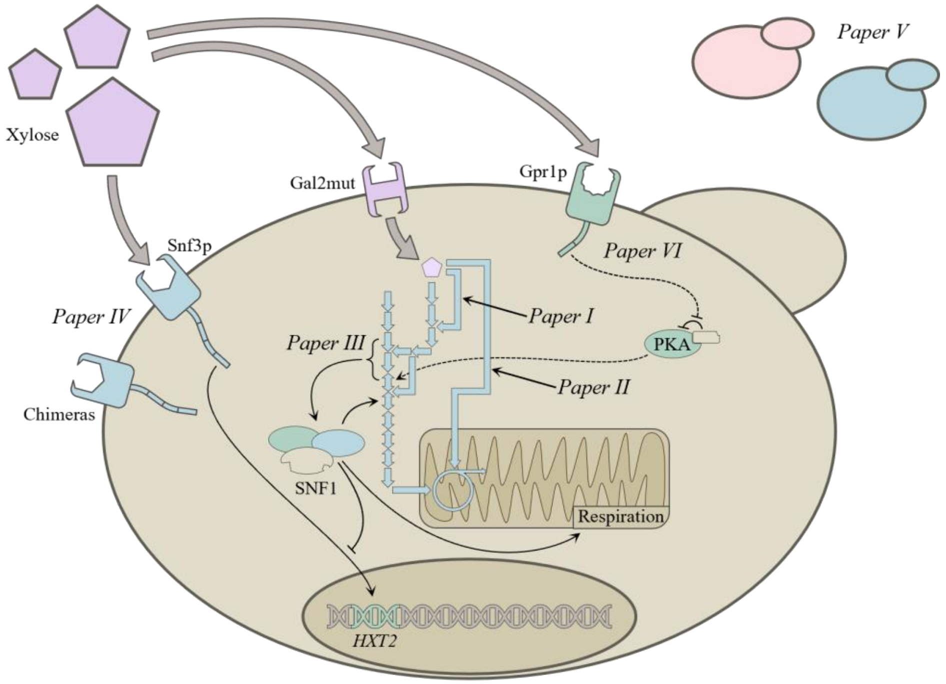


Figure 5.1. Graphical summary of the findings covered in this thesis. The signal transduction has been simplified for clarity.

It has been proposed that G6P acts as a flux signaling molecule for the SNF1 complex (Milanesi et al., 2021). This conclusion came from the observation that deletion of *PFK1/2* (responsible for the formation of F16bP from F6P) did not abolish the glucose-dependent SNF1 complex activation. However, the present thesis´ results showed that the *pgi1* deletion (responsible for the conversion of G6P to F6P) did not abolish SNF1 activation on xylose (Paper III), indicating that G6P may not be responsible for the activation of the complex—at least on xylose. One possible explanation to these contradictory results would be that F6P is the main metabolite acting as the flux signaling molecule for the SNF1 complex in *S. cerevisiae* (similar to F16bP in mammalian cells)—as it is the only metabolite that can form in both *PFK1/2* and *PGI1* deletants under the respective testing conditions. While the aforementioned deletion of *PFK1/2* would indeed lead to G6P accumulation, it would also be possible to accumulate F6P. The targeted metabolite profiling performed in Paper III agrees with this model, showing accumulation of F6P on xylose after deletion of *PGI1*. Further experiments will be necessary to elucidate if and how F6P interacts with the SNF1 complex to affect its activity.

5.2. What comes next?

5.2.1. Further studies on the impact of energy carrier levels

If F6P is not the flux sensing molecule of the SNF1 complex, the observations that SNF1 activation occurs in *pfk1pfk2* double deletants on glucose and in *pgi1* deletants on xylose may be reconciled by ADP-regulation playing the principal role in its activation—i.e., that the energy carrier levels is the true cause of the peculiar xylose sensing response. Rather than focusing on proving F6P influences SNF1 activity directly, it might be possible to determine the significance of the similarities in ADP levels during xylose utilization by developing a SNF1 mutant that is insensitive to ADP and testing for SNF1 activity during xylose fermentation. Alternatively, it may be possible to alter the adenylate energy carrier composition by the addition of chemicals that disrupt the generation of ATP.

5.2.2. Development and optimization of chimeric sensors

I still believe that the creation of a xylose-specific receptor for strongly activating the Snf3p/Rgt2p should be further explored. Testing the effect of introducing a chimera combining the Snf3p binding domain with the Rgt2p signaling domain on xylose would be a suitable first step, as similar constructs have already shown to partially signaling on glucose. For this to work, and for future versions of Gal2RTp to be stable in the absence of glucose, it would also be important to investigate whether the degradation tag in the Rgt2p signaling domain can be disrupted.

To figure out why the Gal2STp chimera did not function properly, it would be interesting to apply it in a transporter-less (hxt-null) strain and investigate whether it retains the capability to transport xylose. Moreover, fusing a GFP to the chimera would provide information on its cellular localization and stability over time. However, it should be kept in mind that both approaches would likely require significantly higher overexpression in order to get measurable results, which might make findings less physiologically relevant.

Another promising avenue would involve boosting the relatively weak xylose response currently observed from native Snf3p, perhaps by improving its binding affinity for xylose or disrupting its ability to bind competing sugars. Alternatively, a chimeric approach involving the systematic replacement of specific segments of the transmembrane domains of Snf3p or Rgt2p with corresponding regions from the mutant Gal2p—rather than substituting the entire binding domain—could provide a promising path forward. This strategy may help pinpoint residues critical for xylose binding, as demonstrated in previous studies (Kasahara et al., 2007; Nishizawa et al., 1995). Monitoring these constructs for xylose sensitivity could provide further insights into developing an effective receptor.

5.2.3. Improving SNF1/PKA co-activation

As mentioned earlier, co-activation of the PKA and SNF1 pathways have shown improved xylose utilization (Myers et al., 2019; Osiro et al., 2019). Both PKA and the SNF1 complex have the ability to regulate the activity of the other pathway. The SNF1 complex phosphorylates Cyr1p to inhibit PKA activation (Nicastro et al., 2015) while PKA can trigger altered localization and degradation of the SNF1 complex (Simpson-Lavy & Kupiec, 2022). It might be possible to increase pathway co-activation by disrupting the SNF1-phosphorylation sites of Cyr1p and the PKA-phosphorylation sites of Snf1p, Sip1p and Sak1p. This might represent an approach which alters sugar signaling without necessarily deregulating the entire system, similar to how mutations of Bcy1p can selectively alter its function and result in different phenotypes compared to full deletion (Myers et al., 2019).

5.3. Inspiration from nature

5.3.1. Native xylose-utilizing yeasts respond to xylose

Many non-conventional yeast (NCY) species are naturally capable of xylose catabolism, a selection of which is displayed in Figure 5.2. These species may have evolved regulatory networks related to xylose utilization which could offer insight into how *S. cerevisiae* could be further engineered.

It has been shown that both *Sc stipitis* and *Candida intermedia* display differential gene expression upon encountering xylose compared to glucose—leading to increased expression of XR- and XDH-encoding genes (Geijer et al., 2020; T. W. Jeffries & J. R. Van Vleet, 2009; Yuan et al., 2011). Similarly, xylose-dependent differential gene expression has been observed in the native utilizers *Kluyveromyces marxianus* (transcriptomics assay; Schabort et al. (2016)), *Pachysolen tannophilus* (targeted mRNA assay; Bolen et al. (1996)), and *Rhodoturola toruloides* (proteomics assay; Tiukova et al. (2019)). To the best of my knowledge, no such comparison has yet been made for *Sp. passalidarum*—perhaps best known for being the source of a widely adopted XR enzyme with increased preference for NADH (Cadete et al., 2016).

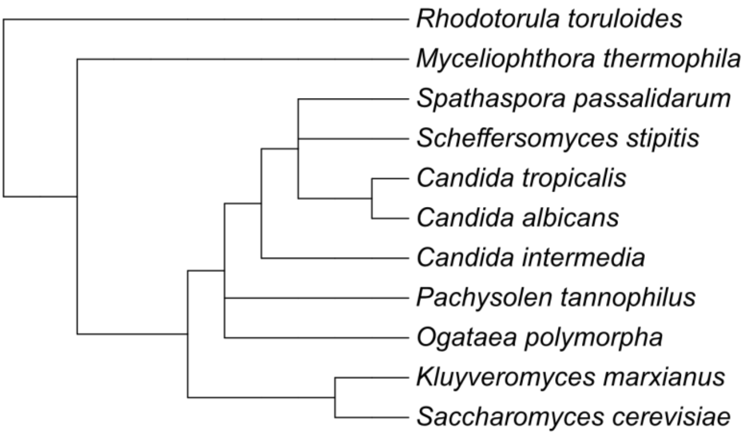


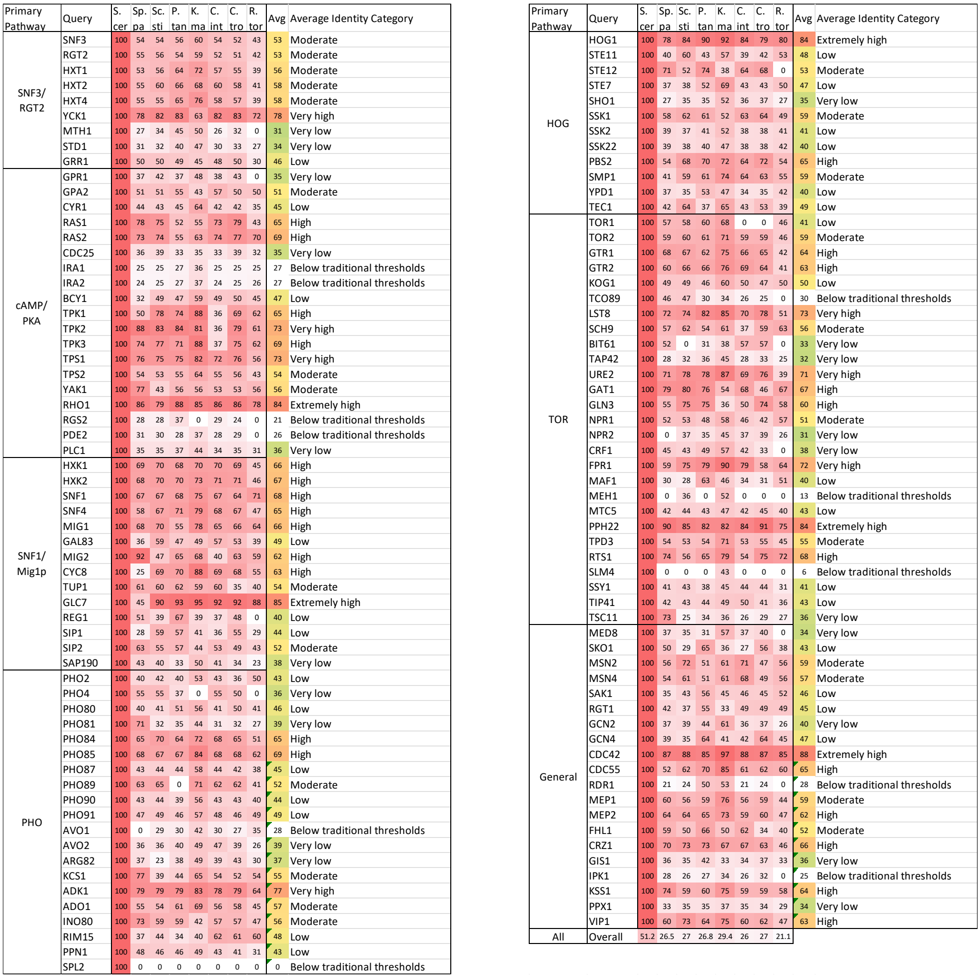
Figure 5.2. Phylogenetic tree based on 18S ribosomal DNA highlighting key xylose-utilizing species and *Saccharomyces cerevisiae*.

Sp. passalidarum is a promising alternative to *S. cerevisiae* for industrial bioethanol processes due to its high ethanol yields, acceptable inhibitor tolerance, and ability to ferment the sugars under full anaerobic conditions (Harner et al., 2015; Hou, 2012; Long et al., 2012; Neitzel et al., 2022). It was initially reported to co-consume glucose and xylose at a very high rate (Hou, 2012; Long et al., 2012); however, the validity of this claim remains uncertain. In my opinion, the first study has insufficient temporal resolution to claim co-consumption (Hou, 2012), whereas the second study does not strictly control aeration (Long et al., 2012). In line with this, later articles have observed glucose repression upon addition of glucose and 2-deoxyglucose (Ribeiro et al., 2021). Interestingly, glucose repression in this species appears to specifically downregulate the expression of XR and XDH (qPCR assays) and subsequently lower the enzyme activity (Ribeiro et al., 2021).

While the transcriptome of *Sp. passalidarum* has been investigated, it has only been used to determine the response to high ethanol concentrations (Albuini et al., 2023) and to determine changes over time when grown under industrial conditions (Neitzel et al., 2022). Therefore an analysis of the *Sp. passalidarum* transcriptome under anaerobic conditions with and without glucose and xylose could be very enlightening and reveal whether this yeast makes other regulatory changes to accomplish improved xylose utilization. For instance, it has previously been shown that *Sp. passalidarum* upregulates the pentose phosphate pathway (PPP) in response to xylose (Wohlbach et al., 2011)—a mechanism that has been recreated in *S. cerevisiae* by increasing the level of PPP enzymes (Hahn-Hägerdal et al., 2007b). Other such findings, applicable to *S. cerevisiae*, might be possible to unveil by studying *Sp. passalidarum* more closely.

5.3.2. Xylose-responsive homologs to *S. cerevisiae* receptors?

An appealing avenue for future research involves identifying xylose-responsive homologs of the *S. cerevisiae* sugar signaling receptors in other yeasts. For instance, *RGT2* expression in *Sc. stipitis* has been shown to increase in response to xylose, hinting on the role of this receptor in xylose sensing (Yuan et al., 2011). As a pre-investigation into this, I performed a local BLAST analysis on 121 genes related to sugar signaling in *S. cerevisiae* (e.g., *RGT2*, *TPK1*, *SNF1*) against the latest genome models of the industrially-relevant non-conventional ascomycetous yeasts *Sp. passalidarum*, *Sc. stipitis*, *Pa. tannophilus*, *K. marxianus*, *C. tropicalis*, *C. intermedia*, and the basidiomycetous yeast *R. toruloides* (Figure 5.3).



Primary Pathway	Query	S. cer	Sp. pa	Sc. sti	P. tan	K. ma	C. int	C. tro	R. tor	Avg	Average Identity Category
SNF3/ RGT2	SNF3	100	54	54	56	60	54	52	43	53	Moderate
	RGT2	100	55	56	54	59	52	51	42	53	Moderate
	HXT1	100	53	56	64	72	57	55	39	56	Moderate
	HXT2	100	55	60	66	68	60	58	41	58	Moderate
	HXT4	100	55	55	65	76	58	57	39	58	Moderate
	YCK1	100	78	82	83	63	82	83	72	78	Very high
	MTH1	100	27	34	45	50	26	32	0	31	Very low
	STD1	100	31	32	40	47	30	33	27	34	Very low
	GRR1	100	50	50	49	45	48	50	30	46	Low
cAMP/ PKA	GPR1	100	37	42	37	48	38	43	0	35	Very low
	GPA2	100	51	51	55	43	57	50	50	51	Moderate
	CYR1	100	44	43	45	64	42	42	35	45	Low
	RAS1	100	78	75	52	55	73	79	43	65	High
	RAS2	100	73	74	55	63	74	77	70	69	High
	CDC25	100	36	39	33	35	33	39	32	35	Very low
	IRA1	100	25	25	27	36	25	25	25	27	Below traditional thresholds
	IRA2	100	24	25	27	37	24	25	26	27	Below traditional thresholds
	BCY1	100	32	49	47	59	49	50	45	47	Low
	TPK1	100	50	78	74	88	36	69	62	65	High
	TPK2	100	88	83	84	81	36	79	61	73	Very high
	TPK3	100	74	77	71	88	37	75	62	69	High
	TPS1	100	76	75	75	82	72	76	56	73	Very high
	TPS2	100	54	53	55	64	55	56	43	54	Moderate
	YAK1	100	77	43	56	56	53	53	56	56	Moderate
	RHO1	100	86	79	88	85	86	86	78	84	Extremely high
	RGS2	100	28	28	37	0	29	24	0	21	Below traditional thresholds
	PDE2	100	31	30	28	37	28	29	0	26	Below traditional thresholds
	PLC1	100	35	35	37	44	34	35	31	36	Very low
SNF1/ Mig1p	HXK1	100	69	70	68	70	70	69	45	66	High
	HXK2	100	68	70	70	73	71	71	46	67	High
	SNF1	100	67	67	68	75	67	64	71	68	High
	SNF4	100	58	67	71	79	68	67	47	65	High
	MIG1	100	68	70	55	78	65	66	64	66	High
	GAL83	100	36	59	47	49	57	53	39	49	Low
	MIG2	100	92	47	65	68	40	63	59	62	High
	CYC8	100	25	69	70	88	69	68	55	63	High
	TUP1	100	61	60	62	59	60	35	40	54	Moderate
	GLC7	100	45	90	93	95	92	92	88	85	Extremely high
	REG1	100	51	39	67	39	37	48	0	40	Low
	SIP1	100	28	59	57	41	36	55	29	44	Low
	SIP2	100	63	55	57	44	53	49	43	52	Moderate
	SAP190	100	43	40	33	50	41	34	23	38	Very low
PHO	PHO2	100	40	42	40	53	43	36	50	43	Low
	PHO4	100	55	55	37	0	55	50	0	36	Very low
	PHO80	100	40	41	51	56	41	50	41	46	Low
	PHO81	100	71	32	35	44	31	32	27	39	Very low
	PHO84	100	65	70	64	72	68	65	51	65	High
	PHO85	100	68	67	67	84	68	68	62	69	High
	PHO87	100	43	44	44	58	44	42	38	45	Low
	PHO89	100	63	65	0	71	62	62	41	52	Moderate
	PHO90	100	43	44	39	56	43	43	40	44	Low
	PHO91	100	47	49	46	57	48	46	49	49	Low
	AVO1	100	0	29	30	42	30	27	35	28	Below traditional thresholds
	AVO2	100	36	36	40	49	47	39	26	39	Very low
	ARG82	100	37	23	38	49	39	43	30	37	Very low
	KCS1	100	77	39	44	65	54	52	54	55	Moderate
	ADK1	100	79	79	79	83	78	79	64	77	Very high
	ADO1	100	55	54	61	69	56	58	45	57	Moderate
	INO80	100	73	59	59	42	57	57	47	56	Moderate
	RIM15	100	37	44	34	40	62	61	60	48	Low
	PPN1	100	48	46	46	49	43	41	31	43	Low
	SPL2	100	0	0	0	0	0	0	0	0	Below traditional thresholds
HOG	HOG1	100	78	84	90	92	84	79	80	84	Extremely high
	STE11	100	40	60	43	57	39	42	53	48	Low
	STE12	100	71	52	74	38	64	68	0	53	Moderate
	STE7	100	37	38	52	69	43	43	50	47	Low
	SHO1	100	27	35	35	52	36	37	27	35	Very low
	SSK1	100	58	62	61	52	63	64	49	59	Moderate
	SSK2	100	39	37	41	52	38	38	41	41	Low
	SSK22	100	39	38	40	47	38	38	42	40	Low
	PBS2	100	54	68	70	72	64	72	54	65	High
	SMP1	100	41	59	61	74	64	63	55	59	Moderate
	YPD1	100	37	35	53	47	34	35	42	40	Low
	TEC1	100	42	64	37	65	43	53	39	49	Low
TOR	TOR1	100	57	58	60	68	0	0	46	41	Low
	TOR2	100	59	60	61	71	59	59	46	59	Moderate
	GTR1	100	68	67	62	75	66	65	42	64	High
	GTR2	100	60	66	66	76	69	64	41	63	High
	KOG1	100	49	49	46	60	50	47	50	50	Low
	TCO89	100	46	47	30	34	26	25	0	30	Below traditional thresholds
	LST8	100	72	74	82	85	70	78	51	73	Very high
	SCH9	100	57	62	54	61	37	59	63	56	Moderate
	BIT61	100	52	0	31	38	57	57	0	33	Very low
	TAP42	100	28	32	36	45	28	33	25	32	Very low
	URE2	100	71	78	78	87	69	76	39	71	Very high
	GAT1	100	79	80	76	54	68	46	67	67	High
	GLN3	100	55	75	75	36	50	74	58	60	High
	NPR1	100	52	53	48	58	46	42	57	51	Moderate
	NPR2	100	0	37	35	45	37	39	26	31	Very low
	CRF1	100	45	43	49	57	42	33	0	38	Very low
	FPR1	100	59	75	79	90	79	58	64	72	Very high
	MAF1	100	30	28	63	46	34	31	51	40	Low
	MEH1	100	0	36	0	52	0	0	0	13	Below traditional thresholds
	MTC5	100	42	44	43	47	42	45	40	43	Low
	PPH22	100	90	85	82	82	84	91	75	84	Extremely high
	TPD3	100	54	53	54	71	53	55	45	55	Moderate
	RTS1	100	74	56	65	79	54	75	72	68	High
	SLM4	100	0	0	0	43	0	0	0	6	Below traditional thresholds
	SSY1	100	41	43	38	45	44	44	31	41	Low
	TIP41	100	42	43	44	49	50	41	36	43	Low
	TSC11	100	73	25	34	36	26	29	27	36	Very low
General	MED8	100	37	35	31	57	37	40	0	34	Very low
	SKO1	100	50	29	65	36	27	56	38	43	Low
	MSN2	100	56	72	51	61	71	47	56	59	Moderate
	MSN4	100	54	61	51	61	68	49	56	57	Moderate
	SAK1	100	35	43	56	45	46	45	52	46	Low
	RGT1	100	42	37	55	33	49	49	49	45	Low
	GCN2	100	37	39	44	61	36	37	26	40	Very low
	GCN4	100	39	35	64	41	42	64	45	47	Low
	CDC42	100	87	88	85	97	88	87	85	88	Extremely high
	CDC55	100	52	62	70	85	61	62	60	65	High
	RDR1	100	21	24	50	53	21	24	0	28	Below traditional thresholds
	MEP1	100	60	56	59	76	56	59	44	59	Moderate
	MEP2	100	64	64	65	73	59	60	47	62	High
	FHL1	100	59	50	66	50	62	34	40	52	Moderate
	CRZ1	100	70	73	73	67	67	63	46	66	High
	GIS1	100	36	35	42	33	34	37	33	36	Very low
	IPK1	100	28	26	27	34	26	32	0	25	Below traditional thresholds
	KSS1	100	74	59	60	75	59	59	58	64	High
	PPX1	100	33	35	35	37	35	34	29	34	Very low
	VIP1	100	60	73	64	75	60	62	47	63	High
All	Overall	51.2	26.5	27	26.8	29.4	26	27	21.1		

Figure 5.3. Identity of the best match (E-value) of proteins homologous to signaling proteins in *S. cerevisiae* (S.cer) found in *Sp. passalidarum* (Sp.pa), *Sc. stipitis* (Sc.sti), *P. tannophilus* (P.tan), *K. marxianus* (K.ma), *C. intermedia* (C.int), *C. tropicalis* (C.tro), and *R. toruloides* (R.tor).

While this approach provides only a basic indication of potential homology and does not guarantee functional equivalence, it serves as a useful starting point to prioritize genes for deeper characterization. The average sequence identity across all 7 species was calculated and each gene classified into the following categories: extremely high (≥80%), very high (70–79%), high (60–69%), moderate (50–59%), low (40–49%), and very low (30–39%) identity. Eleven genes fell below 30% identity, the commonly accepted limit for homology searches, and were excluded from further analysis.

From this analysis, many major components of the SNF1 pathway (e.g., *SNF1*, *SNF4*, *GLC7*) and the PKA pathway (e.g., *TPK1/2/3*, *RAS1/2*), along with key transcriptional regulators (*CYC8*, *MIG1*), exhibited high to extremely high average sequence identity, indicating that these essential regulatory modules are conserved. This would align with the fundamental importance of SNF1 and PKA signaling in metabolism; and indeed, these pathways appear to be conserved in some non-conventional yeasts (Van Ende et al., 2019). At the same time, it has been demonstrated that although the native xylose utilizer *C. albicans* carries functional variants of the SNF1 complex and *MIG1*, the corresponding Mig1p is not subject to control by SNF1 (Harcus et al., 2013; Van Ende et al., 2019). As such, it is important to keep in mind that even if another species contains similar regulatory elements, the interactions between components may be fundamentally different.

The Snf3p/Rgt2p and PKA pathway are of particular interest due to their dependence on extracellular receptors and due to our results indicating that their activation can improve xylose utilization in *S. cerevisiae* (Osiro et al., 2019). The homology search revealed a moderate average identity for potential variants of the *SNF3* and *RGT2* receptors. Although some hits could be caused by misidentification of hexose transporters, there are confirmed cases of functionally conserved orthologs of the Snf3p/Rgt2p receptors, e.g., in *Ogataea polymorpha* (Hxs1p) (Stasyk et al., 2008) and *C. albicans* (Hgt2p) (Van Ende et al., 2019). Nevertheless, if one were to try to confirm the role of these putative receptors, it could be relevant to widen the initial screening to include more hits (in case false positives from transporters) and to look for the presence of a long C-terminal tail.

For the PKA route, only a very low identity was found across species for the Gpr1p receptor whereas moderate identity could be seen for the downstream Gα-encoding *GPA2*. It is also possible that the identity is higher for Gα due to conserved receptor-binding WD-40 domains. Nevertheless, *C. albicans* has confirmed homologs for *GPR1* and *GPA2*— although they appear to sense lactate and/or methionine rather than glucose and sucrose (Van Ende et al., 2019).

5.4. Final thoughts

Our understanding of the metabolism of *S. cerevisiae* has advanced substantially since initial observations of its ability to perform fermentation under aerobic conditions. Various regulatory pathways controlling yeast phenotypic responses have been studied and modelled, leading to developments not only in the understanding of yeast metabolism but to advances in mammalian cell signaling and cancer research. While no model will ever be fully accurate, continued refinement brings us ever closer to practical predictions of cellular phenotypes which can provide meaningful aid to scientific and industrial advancements.

This thesis represents one step forward in our understanding of sugar signaling in *S. cerevisiae*, with emphasis on how the glucose signaling pathways respond to xylose—a sugar that is not natively utilized by the yeast. The thesis also highlights ways in which the sugar signaling pathways can be modified to enable increased xylose utilization in recombinant strains. Ultimately, I hypothesize that the peculiar xylose sensing response—characterized by the activation of the SNF1 pathway in xylose-utilizing strains despite an absence of glucose—is directly linked to the formation of the metabolic intermediate F6P alongside a lack of PKA pathway activation. Although the activation of the SNF1 pathway is linked to increased respiration, which inhibits fermentation processes, it also promotes alternative sugar utilization. Since co-activation of the PKA pathway alongside the SNF1 pathway has been shown to improve xylose utilization, attempting to increase PKA activation rather than decrease SNF1 activation is likely to yield enhanced industrial strains for 2G bioethanol production and other biochemicals. Consequently, engineering strategies that mimic extracellular glucose activation of PKA, such as mutant glucose receptors with xylose affinity, are promising for the development of future industrial xylose fermenting strains.

Acknowledgements

Doing a Ph.D. has been just as fun as I hoped it would be. Research, teaching, learning, collaborating, presenting, succeeding, failing, all of it. The community at Applied Microbiology has been incredible, both during late nights and during game nights. I am deeply grateful to everyone who has been part of this experience: offering guidance, support, and encouragement. To those mentioned below, and to those who are not, thank you for everything. Ultimately, I am excited for what comes next, despite not knowing what comes next: dovie'andi se tovya sagain.

Marie Gorwa-Grauslund, you have played a crucial role in guiding me both in science and in academia as my main supervisor. I am ever grateful for your engaging and inspiring mentorship, and for staying on as my “remote supervisor” despite great personal cost and the loss of many a weekend. Your ability to drive projects while allowing me to develop and make my own mistakes, giving freedom with responsibility, is truly amazing. Throughout my time here, I have felt comfortable speaking my mind and openly discussing issues with projects owing largely to the fact that I always knew I could trust you to do the same. I want to thank you especially for reigning in my twenty thousand ideas, for teaching me the importance of prioritization within research, for giving me the opportunity to try new techniques and take fun courses, and for giving me thorough feedback on grants and writing. It has been an honor to work with and learn from you!

I also want to thank the rest of my supervisors. **Ed van Niel**, you not only acted as my department representative for 3 years but took over a lot of responsibilities when Marie transitioned away from academia. Thank you for taking the time to do this, and for the many interesting lunchroom discussions. **Peter Spégel**, you got me interested in chromatography and mass spectrometry early in during my masters and provided invaluable feedback in the ISP meetings with new ideas and directions. Thank you for your enthusiasm and engagement, for answering my many questions, and for guiding me through the use of untargeted metabolomics and data processing. **Magnus Carlquist**, you are one of the first to introduce me to gene technology and microbial flow cytometry, and have provided a lot of invaluable advice when it comes to academia, projects, and grants. Thank you for always helping put things into perspective and planning ahead, both in practical experiments and careerwise.

Daniel Brink, you have been a mentor and role model since we first worked together during the summer of 2018. Since then, you've also been a colleague, a co-author, a career guide, a project advisor, a programming peer, a writing peer, a dashing rogue, a gamemaster, a meme-lord, but by far most important: a friend. Thank you for always being there, for always providing perspective, for staying late to discuss science, for the many riveting salad diagrams, for the game nights, for everything. You are an amazing person.

I also wish to thank the rest of the seniors at TMB, but especially the following. **Catherine Paul**, you caught my attention early in the masters with your lectures in gene technology, in which I think you were the first to ever introduce me to flow cytometry and CRISPR. Thank you for your engaging and insightful comments on everything from presentations and grants to articles and career choices, and for your practical and emotional support within academia. **Jenny Schelin**, you always seem to find time to help people and I truly look up to your fantastic teaching abilities. Thank you for all your advice and support, both in long-term career matters and in academic matters. **Johannes Hedman**, thank you for offering to be my department representative, for answering my many questions, and for your fantastic music suggestions. **Linda Jansson**, thank your continual support and career advice, and for always brightening my day. **Ghjuvan Micaelu Grimaud**, thank you for taking on the mantle of dissertation chair on a short notice, for your engagement in project meetings in the ME group, and for your advice on grants and resumes. **Celina Borgström**, thank you for introducing me to my research subject, for teaching me the value of quick-and-dirty setups, and for the fun easter egg hunts.

Part of the reason I wanted to do a Ph.D. at TMB was the people, not least the fellow doctoral students and researchers. Thank you to all who partook in this journey, but especially the following. **Krishnan Sreenivas**, thank you for giving me the confidence to try to fix and build equipment on my own, for the office and lab company during perpetual night experiments, for bringing to life some of the funniest characters I've seen, for joining me in endless Sibylla runs, and most importantly for joining me on our highly successful fitness journey. **Matilda Johansson**, thank you for always bringing joy and positivity to any situation, for being a graphical genius and Office wizard, and for being my much needed office chromatography buddy to bounce ideas with. **Nikhil Seshagiri Rao**, thank you for being your guidance and instruction, for being the wacky uncle of the office, and for being the best neighbor ever—we may have left Kämnärs but Kämnärs will never leave us. **Caroline Schleich**, thank you for being an awesome office mate and for the opportunity to get to know you. **Gisele Cristina de Lima Palermo**, thank you for the fantastic collaboration, for the many amazing game nights, for the enthusiasm and excitement you bring to any experiment, for sharing the experience of babysitting a weird Frankensteinian creation we chose to call an 'auto-spectro-photometer' overnight, for showing us amazing hospitality and guiding us around Campinas and your lab, for introducing me to coxinhas, and a special thank you to your husband **Élerson Palermo** for telling us the best strategies. **Bruna Bolzico**, thank you for the incredible collaboration, for your inspiring and motivating enthusiasm and need for knowledge, for being a great writer and scientist, for the fun after-works, for writing possibly the best travel guide ever, for staying in Sweden even though the first apartment was depressing, and for being an inspiration example of never giving up (flying between Argentina, Brazil, Sweden, and Italy, back and forth all the time). **Fredrik Lund**, it feels crazy that I met you on day one of the masters nearly ten years ago, and that we then proceeded to do our M.Sc. theses at the same place, and now doctoral education—what is next? Same postdoc institute? I don't know how to cover it all, but suffice to say I wish to thank you for your continual support throughout the Ph.D., for you input on important projects and strain construction, and for arranging amazing after-work sessions with beer and music competitions together with Tova. On that note, I want to thank **Tova Lindh** as well for always being there, for being incredibly versatile and having the ability to switch between academic subjects like a magician, and for giving me a run for my money when it comes to trying to be the student with the greenest thumbs. **Tage Rosenqvist**, as I mentioned with Fredrik, it is crazy to think that we met nearly 10 years ago, even starting in the same introductory group. I want to thank you for your valuable inputs to my projects during Monday seminars, for being an inspirational academic figure, and for organizing amazing after-work sessions. **Isabela Mendes Bonfim**, thank you for your continual career guidance, for your input on projects using your encyclopedic knowledge of enzymes, and for assuring me that the conference was not predatory. **Isabel Erb**, thank you for your enthusiasm and fun loving attitude, and for your willingness to perform advanced machine learning on bread data. **Lisa Lindahl**, thank you for lending me your expertise as energy drink savant, and for the awesome after-works. **Jacob Miefalk**, thank you for always giving your undivided focus to discussions, for demolishing us in badminton, and for organizing amazing game nights. **Iria Feijóo Rey**, thank you for always bringing great energy to the after-works and for possibly being the best wall climber I have ever seen. **Paulo César Silva**, thank you for always bringing a remaining optimistic in meetings, and for showing me what real volleyball looks like. **Khaled Youssef**, thank you for always bringing enthusiasm to any situation, and for giving me some much needed breaks from my thesis writing with exciting flow cytometry questions. **Raquel Perruca Foncillas**, thank you for being you, for the amazing project we did together, and for the many lively lunch debates over what sandwiches actually are. **Nina Muratovska**, thank you for being a beacon of joy to your surrounding, for showing me what pro-volleyball looks like, for bringing valuable perspectives to projects in the ME group, and for your impressive ability to sustain avocados. **Julia Södergren**, thank you for the amazing after-works and for bringing an amazing sense of humor to the division. And finally the new forensic group, **Sofie Bergstrand**, **Julia Bengtsson**, **Christina Forsberg**, thank you for the opportunity to get to know you and for contributing to the inclusive and friendly environment at TMB.

Throughout my doctoral time, I have taught and worked with some truly amazing students. I want to thank the students participating in the labs of Molecular Cell Biology 2021–2023 and Gene Technology 2024, your enthusiasm and desire for knowledge were a big motivator and source of energy, especially when the pandemic was at its worst and when experiments were going poorly. Along the same lines, I wish to thank **Man Zhang** for joining me in the teaching and bringing a helpful can-do attitude. But perhaps more importantly, I want to thank the master students that I was privileged to supervise in individual research projects and master theses, especially the following. **Nina Egeler**, your enthusiasm for science is contagious, your independence and ability to identify knowledge gaps is incredible, and I felt very lucky to have had you as a student and I look forward to publishing our results. **Tegan Anderes**, your ability to communicate, learn quickly, and implement literature findings were amazing to see in action, and I am very proud of having a publication with you. **Ylva Johansson**, your ability to adapt to changing project plans and embrace new techniques was extremely impressive, and despite the limited time I think we accomplished a lot of important work. I also wish to thank the rest of the master students and project assistants that have worked at TMB over the years, especially **Chiara Siti** and **Sofia Badiali** for their wonderful contributions to the environment at TMB, as well as for repeatedly sampling my forehead and hating my pasta, respectively.

None of this work would've gone nearly as smoothly if it weren't for the amazing work of **Anette Ahlberg** and **Christer Larsson**. Thank you for your substantial and continuous support with all the administrative duties and equipment malfunctions, for being kind, and for being optimistic.

I want to also mention the people who helped me get to the Ph.D. in the first place. I don't know if I would've finished my masters degree on time had it not been for the fun and the partying to displace the stress of the studies. This especially applied to, but is not limited to: **Linnéa Wahlberg**, **Viktor Halldén**, **Ronja Wennerström**, **Charlott Vikenborg**, **Anna-Malin Hallberg**, **Jonatan Jarnås**, **Ebba Fjelkner**, **Gustav Karlsson**, **Cecilia Håkansson**, **Linnéa Petersson**, **Johan Davidsson**, **Fay Stensson**, **Malte Marthin** (and **Tage** and **Fredrik**, as mentioned earlier).

I want to thank my friends for their support and for keeping my mind off work in the downtime. This is especially true for the members of our roleplaying groups, including **Rebecca Grönwall**, **Per Eborn**, **Danne Cheveyo**, **Alex Wyke** (as well as **Gisele**, **Krishnan**, **Élerson**, **Daniel** as mentioned earlier). Your imagination and our collective problem solving (/creation) has given me a lot of energy throughout the years, and I wish to sincerely thank you for this. I will continue to hope to never have to apply any of it in real life, especially when it comes to infrastructure. Along these same lines, I also want to thank by closest friends since high school: **Jenny Martinsson** and **Kasper Malmborn**. Your continual support throughout this Ph.D. during our recurring taco nights (which is apparently our brand now) and insistence on watching strange Swedish TV shows has been instrumental.

Last, but not least, I wish to thank my family and extended family for supporting me throughout my education. **Lynn Persson**, thank you for your optimism and cool headedness, for helping me deal with things going wrong and right, and for the many lyckosparkar. **Ida Hallberg**, thank you for always bringing joy and excitement, for the many incredible baked goods, and for being there when I need you. **Hampus Hallberg**, thank you for being so open and inclusive despite having an excentric scientist as a brother-in-law, for the many amazing barbeques, and for tolerating my 500 questions about car parts. **Ebbe** and **Ella Hallberg**, thank you for being the best nephew and niece one could possibly imagine. **Titti Ekegren**, thank you for motivating me to apply for the Ph.D. position in the first place, for coaching me through the tough times, and for your continual support. **Michael Ekegren**, thank you for your inclusivity, for your advice, and for giving me both a tolerance and an appreciation for the spicy *Capsicum* species. **Alva Ekegren**, thank you for the many game nights, for being an inspiration when it comes to animal husbandry, and for playing the best lordling. Finally, **Amanda Ekegren**, thank you for everything. For your support throughout this education, throughout the late night experiments, and throughout writing this thesis. For your peer review and opinions. For your understanding and patience. For joining me in weird activities like forging. For bringing me to equally strange activities such as camping with swords. For being a masterful chef. For amazing game nights. For being there no matter what.

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The Peculiar Xylose Sensing of *Saccharomyces cerevisiae*

This thesis explores how the yeast regulatory networks respond to the pentose sugar xylose, the second most abundant sugar in plant matter, and how this regulation can be engineered to favor xylose consumption.

Understanding how yeast responds to xylose is critical to enable efficient production of sustainable biofuels and biochemicals from renewable feedstocks, such as lignocellulose. The regulatory networks of *S. cerevisiae* influence hundreds of genes simultaneously, and are responsible for orchestrating an appropriate and efficient response to changes in the cellular environment. When a fermentable sugar such as glucose is detected, the cell makes a multitude of changes to its metabolism to prioritize rapid fermentation to ethanol. However, in the case of xylose, investigations showed that *S. cerevisiae* does not detect xylose as a fermentable carbon source. Thus, the overall metabolism is likely not properly tailored to perform robust and rapid fermentation. Curiously, there was another layer to it though: despite xylose being seen as non-fermentable, the regulatory networks were reporting that a sugar was present at low levels. With xylose being a non-natively utilized sugar in *S. cerevisiae*, this was strange since the networks were expected to report that starvation—a peculiar sensing response, indeed. Moreover, this regulatory state is typically connected to respiration, which may hamper fermentation further.

By applying primarily metabolic engineering, fluorescent biosensor, and metabolomics, we managed to narrow down the cause of this response to metabolic intermediates forming during xylose catabolism, which activate the SNF1 regulatory pathway —the primary suspect being fructose-6-phosphate. Rather than trying to avoid this activation, a more fruitful endeavor might be to attempt to co-activate the PKA regulatory pathway which promotes rapid sugar utilization and fermentation and the Snf3p/Rgt2p pathway which promotes sugar uptake, since this has been shown to improve xylose fermentation in previous publications. We attempt to achieve this by creating novel xylose-specific receptors linked to the regulatory pathways.

Viktor C. Persson has had a passion for science from a young age, deciding to pursue research and academia at the age of 15. Pedagogy has always been a priority, working part-time as an assistant in many courses during his M.Sc. in Biotechnology. His favorite work activities include: teaching, writing, and fika. In his spare time, he likes to pursue a variety of hobbies including board games, music, and forging.