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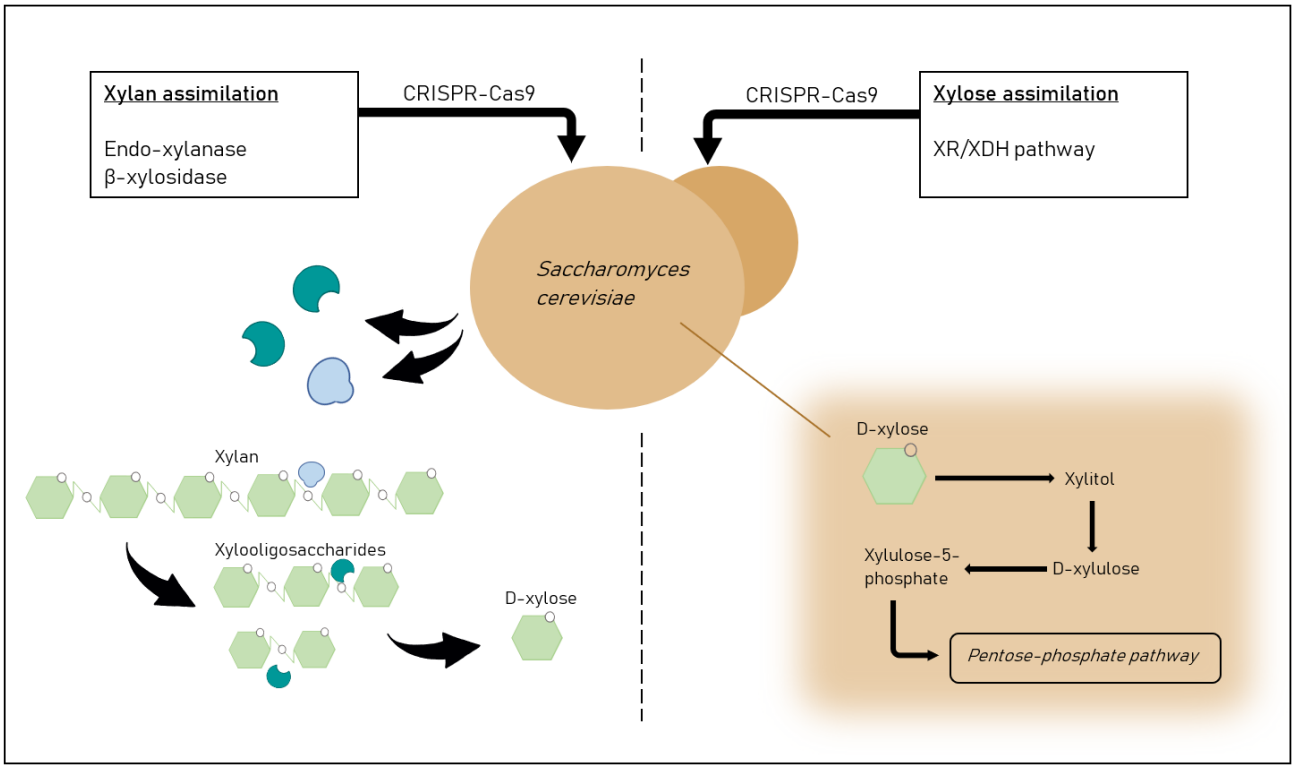

Development of xylan- and xylose assimilating *Saccharomyces cerevisiae* using CRISPR-Cas9 technology

Master's thesis in Biotechnology

ELIN LÖNNQVIST

DEPARTMENT OF BIOLOGY AND BIOLOGICAL ENGINEERING
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2022
www.chalmers.se

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Supervisor: João Heitor Colombelli Manfrão Netto, Department of Biology and Biological Engineering
Examiner: Cecilia Geijer, Department of Biology and Biological Engineering

Master's Thesis 2022
Department of Biology and Biological Engineering
Division of Industrial Biotechnology
Chalmers University of Technology
SE-412 96 Gothenburg
Telephone +46 31 772 1000

Cover: Graphical abstract.

Typeset in LaTeX
Gothenburg, Sweden 2022

Abstract

Biorefineries produce a vast array of products for industrial, pharmaceutical and food applications. To allow for an enviromentally and economically sustainable production, the input biomass should not compete with crops cultivated for other purposes such as food or feed. Instead, lignocellulosic biomass from e.g. fast-growing wood and grasses has emerged as an alternative. However, one of the most important industrial microorganisms, the yeast *Saccharomyces cerevisiae*, is unable to efficiently degrade and utilise the polysaccharides present in the plant cell wall, including hemicellulose, a major constituent of lignocellulosic biomass. This project was aimed at developing a yeast strain able to utilise xylan, one of the most abundant hemicelluloses. A CRISPR-Cas9 system was used to introduce the hemicellulases endo-xylanase and β-xylosidase for degradation of the xylan polymer, and the XR/XDH pathway for assimilation of the xylose monomers by *S. cerevisiae*. The integration of the XR/XDH pathway and the hemicellulases was successful and growth analysis of the resulting strains showed ability to grow on xylose based media as well as on xylooligosaccharides, indicating a functional XR/XDH pathway and β-xylosidase enzyme. β-xylosidase activity was also demonstrated through enzymatic assays. However, capability of degrading the xylan polymer could not be demonstrated via enzymatic assays, indicating insufficient endo-xylanase activity. While further studies are required to optimise the hemicellulase expression in the constructed strain, the xylose-degrading ability of the strain was comparable to that of other genetically modified strains expressing the XR/XDH pathway. Overall, the successful development of an *S. cerevisiae* strain capable of efficiently degrading xylan and utilising xylose would be of great industrial value and further research on this topic should be pursued.

Keywords: xylose assimilating yeast, xylan assimilation, CRISPR-Cas9, metabolic engineering, lignocellulosic biomass, XR/XDH pathway, saccharomyces cerevisiae.

Contents

Abbreviations

Amp	ampicillin
bp	base pair(s)
CDS	coding sequence
CRISPR	clustered regularly interspaced palindromic repeats
DSB	double-stranded break
ER	endoplasmic reticulum
GFP	green fluorescent protein
GRAS	generally regarded as safe
gRNA	guide RNA
HDR	homology-directed repair
Hyg	hygromycin
MQ	Milli-Q (purified water)
Neo	neomycin
NHEJ	non-homologous end joining
ORI	origin of replication
PAM	protospacer adjacent motif
pNP	*para*-nitrophenol
pNPX	*para*-nitrophenol-β-D-xylanopyroside
PPP	pentose-phosphate pathway
RPM	revolutions per minute
sgRNA	single guide RNA
tracrRNA	trans-activating RNA (synonymous to scaffold RNA)
TU	transcriptional unit
UPR	unfolded protein response
XDH	xylitol dehydrogenase
XI	xylose isomerase
XOS	xylooligosaccharides
XR	xylose reductase
YNB	yeast nitrogen base (medium)

1 Background

Plant biomass can be used as raw material for the production of various value-added industrial products, including materials, chemicals and biofuel. Biofuels are considered an important factor in the transition to more environmentally sustainable transports as its production and consumption represents a closed carbon cycle [1], in contrast to the traditional petroleum-based alternatives. However, a persisting issue has been that the starch-based crops, such as sugar cane and maize, used as biomass source within industrial production competes with food resources [1]. Focus has since been shifted towards non-edible crops, consisting mainly of lignocellulose, as a better alternative; the "second-generation biomass" [2]. Such crops include e.g. grasses, agricultural residues and wood [1].

Lignocellulose consists of the three main components cellulose, hemicelluloses and lignin [2]. Hemicellulose constitutes up to 35 % of total biomass and in much of the second generation biomass, the majority of hemicelluloses are xylans [3]. In fact, xylans are the second most abundant polysaccharides on Earth [3]. Structurally, xylans are composed of a xylose backbone to which different substituent groups (xylopyranose, glucoronic acid, acetyl, arabinofuranose, hydrocinnamic, ferulic or *p*-coumaric acids) are bound [2]. Different substituents results in different xylan polysaccharides with different biochemical properties [4].

One of the main organisms used in biorefineries for industrial production of value-added products is the yeast *Saccharomyces cerevisiae*. This yeast is particularly favoured for its ethanol-producing capabilities [5], but, being classified as a GRAS organism, is also suitable for a vast array of other products such as pharmaceuticals, fine chemicals and food products [6]. However, *S. cerevisiae* is unable to degrade xylan, and while an unspecific aldose reductase encoded by the native *GRE3* gene has shown capability of reducing xylose to xylitol [7], xylose utilisation is generally inefficient. This may be resolved through genetic engineering approaches, by introducing genes encoding xylose consumption pathways [8]. Furthermore, by also introducing relevant hemicellulases, one could endow *S. cerevisiae* with the ability to assimilate xylan from lignocellulosic biomass into xylose monomers. Specifically, to digest the xylan polymer into xylose monosaccharide pentoses, a minimum of three types of enzymes are required; endo-1,4-β-xylanases, which hydrolyse xylan into xylooligosaccharides (XOS), β-xylosidase, which hydrolyses the XOS into D-xylose, and finally, enzymes that specifically target the different substituents on the xylan main chain [9]. As xylan cannot enter the cell due to lack of specific transporters for large polysaccharides, these enzymes must either be i) secreted, or ii) displayed on the cell surface. While immobilisation of the enzymes on the cell surface carries benefits such as enzyme recycling, increased synergy and effective enzyme concentration [10], secreted enzymes mimic the "free enzyme system" observed in filamentous fungi, which in turn have shown higher xylanase levels than other xylanolytic yeast and bacteria [11]. Furthermore, secreted enzymes are only limited by the secretion capacity of the organism, and not by the spatial constraints as is the case for cell-surface display [12]. It has previously been noted that xylanases are abundantly expressed several fungal species, such as the *Aspergillus* and *Trichoderma* genera, which are

the most commonly employed in industrial applications [13]. Xylanases from these, as well as from *Bacillus sp.* and the fungus *Scheffersomyces stipitis* have previously been introduced into *S. cerevisiae* via genetic engineering [14],[15],[16]. Finally, after degradation of the xylan polymer, the xylose pentoses must be converted into xylulose-5-phosphate which can enter the central metabolism via the pentose-phosphate pathway to be utilised by *S. cerevisiae*, [17],[18],[19]. In other xylose-utilising yeasts, the conversion from xylose to xylulose, via xylitol, is achieved by two enzymes; xylose reductase (XR) and xylitol dehydrogenase (XDH) [17][18]. Recombinant xylose-utilising *S. cerevisiae* strains have been engineered with genes from the yeast *Scheffersomyces stipitis* (formerly *Pichia stipitis*) *XYL1* and *XYL2*, encoding XR and XDH respectively [20]. It has previously been shown that retaining the XR activity of the native *GRE3* in place of heterologous *XYL1* is undesirable, appointed to aggravated redox factor balance [21],[22]. However, xylose utilisation in *S. cerevisiae* is also limited by its poor xylose uptake, in part due to the low xylose affinity of the transmembrane hexose transporters [23]. It has been found that overexpression of the homologous gene *XKS1*, encoding xylulokinase (XK), can increase the rate of xylose uptake in *S. cerevisiae* [24].

Today, the state-of-the-art for genetic engineering includes the CRISPR-Cas (clustered regularly interspaced palindromic repeats–CRISPR-associated proteins) system, which, since its appearance, has emerged as one of the most important gene editing technologies. In nature, the CRISPR-Cas system has evolved as a part of prokaryotic immune defence against invading bacteriophages [25]. CRISPR structures are characterised by short, recurrent, and typically palindromic sequences of DNA, interspaced with the short sequences derived from foreign DNA – "protospacers" [26]. Protospacer DNA is transcribed into crRNA (CRISPR-RNA), which binds to a second RNA structure, tracrRNA (trans-activating crRNA), and the two-RNA complex combines with a Cas protein [27], e.g. Cas9. Cas proteins are RNA-guided nucleases [28], and will induce double-stranded breaks (DSBs) in DNA sequences complementary to the crRNA, provided they are followed by a di- or tri-nucleotide sequence called a protospacer-adjacent motif (PAM) recognised by the specific Cas protein [26],[27],[29]. For Cas9, the associated PAM is 5'-NGG-3' [29]. Importantly for genetic engineering applications, it has been found that i) the crRNA–tracrRNA complex can be replaced by a single RNA structure, a so-called single guide-RNA (sgRNA), and ii) that the sgRNA element can be used to instruct the Cas protein to induce DSBs in a target sequence of choice [27],[29].

By codon-optimising the Cas9 protein and including a nuclear localisation signal, it can be transformed into and expressed in non-prokaryotic cells [28] along with a well-designed sgRNA to introduce precise DSBs in the host genome to regulate expression of a target gene, or to introduce new genes. For gene knock-ins, the homology-directed repair (HDR) mechanism is utilised, where the donor DNA is flanked by homology arms matching the DNA at either side of the DSB and used as a repair template [30]. With its many possibilities, the CRISPR-Cas9 technology has found applications within research, healthcare, agriculture, diagnostics and therapeutics [27]. In this project, CRISPR-Cas9 was used to

introduce secreted hemicellulases and the XR/XDH pathway into *S. cerevisiae* to develop xylan- and xylose assimilating yeast strains for effective biomass utilisation in industrial applications.

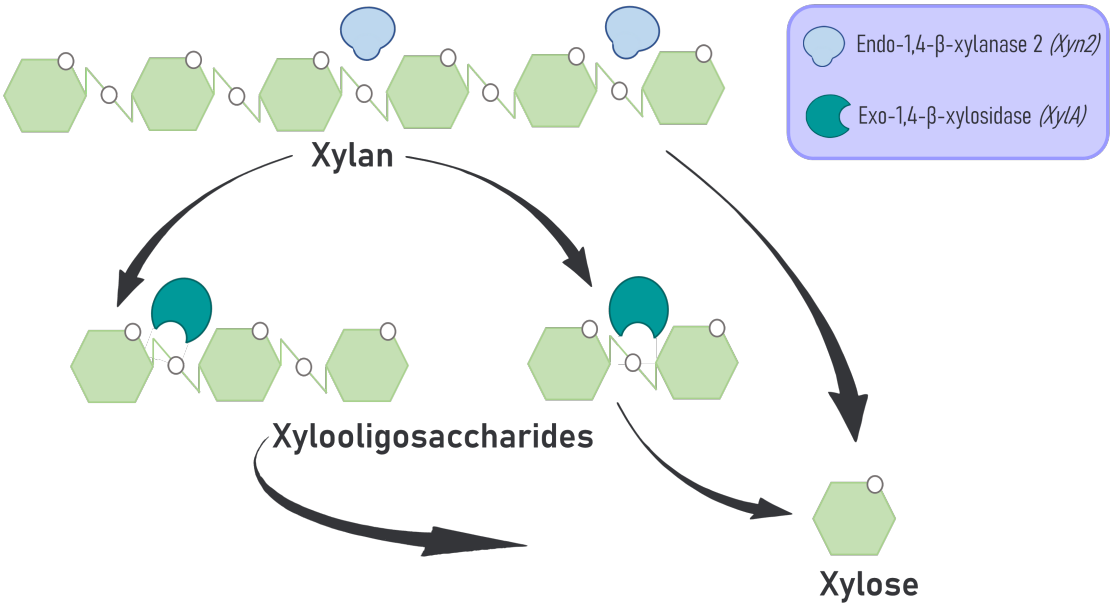


Figure 1: Overview of the breakdown of a xylan polymer to xylose monomers by xylanases *Xyn2* and *XylA*.

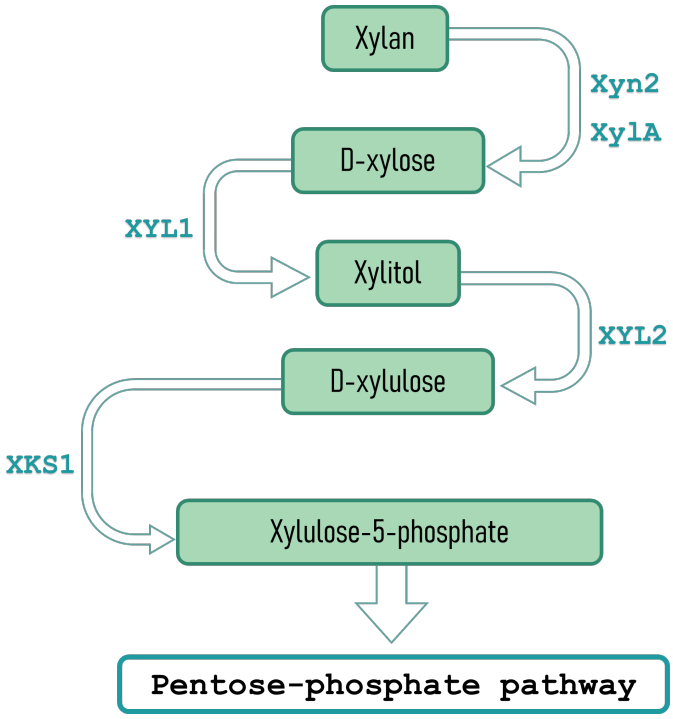


Figure 2: Overview of the intended pathway for xylan and xylose utilisation by yeast.

2 Methodology

A general overview of the methodology is provided in Figure 3. Table 1 details the organisms, genes and vectors relevant to this project.

ID	Type	Description
CEN.PK113-7D	yeast strain	haploid *S. cerevisiae* strain, host strain in this project
CPXGRE3	yeast strain	modified CEN.PK113-7D yeast strain with the XXX cassette integrated at the *GRE3* locus
CEN.PK.XXX	yeast strain	modified CEN.PK113-7D in-house strain with XXX cassette integrated in *HO* locus, host strain for MCXcassette
CX2A	yeast strain	CEN.PK.XXX with MCXcassette integrated at the X2 locus, constructed in this project
diploid XXX	yeast strain	diploid in-house *Saccharomyces cerevisiae* strain with the XXX cassette (allegedly) integrated at the *GRE3* locus in addition to a number of other modifications such as overexpression of genes in the PPP, well-adapted to xylose, used for comparison in this project
pMEL13 [31]	plasmid	sgRNA vector for integration at *GRE3* locus, ampicillin and G418 (geneticin) selective
CSS	plasmid	Cas9 vector, hygromycin selective, Cas9 under control of the galactose-induced *GAL1* promoter
LT58	plasmid	vector for Cas9 and sgRNA targeting the X2 locus, Cas9 under control of the constitutive promoter *TEF1*
XXX [32]	plasmid	in-house plasmid, vector for xylose utilisation genes (XXX cassette = *XYL1*, *XYL2*, *XKS1*) to be inserted into the *GRE3* locus of CEN.PK113-7D
MCX	plasmid	vector for hemicellulases (MCXcassette = *Xyn2*, *XylA*) to be inserted into the *HO* locus, constructed using MoClo assembly
GRE3 (GenBank: DAA06798.1)	gene	gene enconding an unspecific aldose reductase in *S. cerevisiae*, target site for the XXX cassette
HO (GenBank: CAA62447.1)	gene	gene enconding an site-specific endonuclease related to mating in *S. cerevisiae*, target site for *xyn2-xylA* cassette from MCX cassette, OR XXX cassette
X2	intergenic site	target site for MCX cassette
$gRNA_{YST}$	sgRNA	sgRNA targeting the *GRE3* locus, designed using the Yeastriction tool
$gRNA_{CHOP}$	gRNA	gRNA targeting the *GRE3* locus, designed using the CHOPCHOP tool
XYL1 (GenBank: ABN67152.1)	gene	D-xylose reductase (*Scheffersomyces stipits*), housed in XXX plasmid
XYL2 (GenBank: EAZ62959.1)	gene	xylitol dehydrogenase (*Scheffersomyces stipitis*), housed in XXX plasmid
XKS1 (GenBank: DAA08287.1)	gene	xylulose kinase (*Saccharomyces cerevisiae*), housed in XXX plasmid

Xyn2 (GenBank: AAB50278.1)	gene	endo-1,4-β-xylanase 2 (*Trichoderma reesei*), housed in MCX plasmid
XylA (GenBank: OOO12801.1)	gene	exo-1,4-β-xylosidase (*Aspergillus oryzae*), housed in MCX plasmid

Table 1: Overview of strains, plasmids, loci and genes relevant to this project.

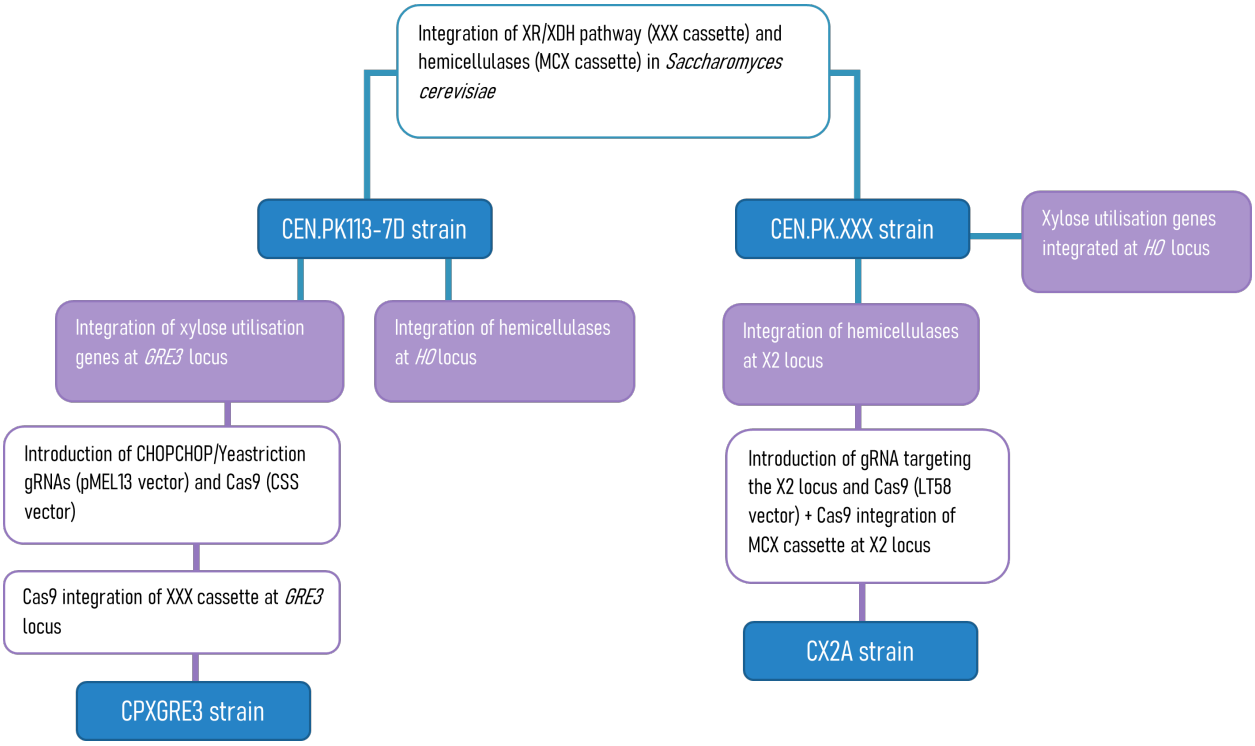


Figure 3: Methodology overview illustrating the two different approaches to the integration of the xylose utilisation genes and hemicellulases. Yeast strains CPXGRE3 and CX2A originate from this project. Colour coding: filled blue = strains, filled purple = genetic modifications (filled white/purple border = specified genetic modification approach).

Media used in this study are summarised in Table 2 and further detailed in Appendix A. For *in silico* design and visualisation of primers and sgRNAs, as well as simulation of cloning and PCR, the SnapGene software, version 6.0.2, (Insightful Science; available at snapgene.com) was utilised. Design and selection of sgRNAs was based on results from the CHOPCHOP [33] and Yeastriction [31] web tools. Primers and sgRNAs were further evaluated using the OligoAnalyzer tool (Integrated DNA Technologies, available at eu.idtdna.com.)

Medium	Type	Carbon source
YPD	complex medium	glucose (2 % w/v)
YP-xylose	complex medium	xylose (2 % w/v)
YP-galactose	complex medium	galactose (2 % w/v)
YNB-xylose	minimal defined medium	xylose (2 % w/v)
Delft-glucose	minimal defined medium	glucose (2 % w/v)
Delft-xylose	minimal defined medium	xylose (2 % w/v)

Table 2: Summary of media used in this study. Yeast nitrogen base (YNB) medium was obtained from Thermo Scientific. The Delft minimal defined medium was first described by Verduyn *et al.* [34]
. Carbon source concentrations are given for the final work solutions.

2.1 Construction of a xylose-utilising strain

The xylose-utilising yeast strain CPXGRE3 was constructed by Cas9-guided integration of the XXX cassette, containing the XR/XDH pathway, at the *GRE3* locus of the CEN.PK113-7D host strain. The following subsections detail this process.

2.1.1 Design and preparation of gRNAs for integration of the XR/XDH pathway at the *GRE3* locus

The CHOPCHOP and Yeastriction tools were used to find optimal gRNA sequences for knockout of the *GRE3* locus. For Yeastriction, results were generated specifically for the yeast strain CEN.PK113-7D, while the reference strain S228c was used for CHOPCHOP. Ultimately, the top result from Yeastriction and CHOPCHOP respectively were chosen. The two gRNAs are detailed in Table 3.

	Yeastriction ($gRNA_{YST}$)	**CHOPCHOP ($gRNA_{CHOP}$)**
Sequence (5'⟶ 3')	GTAATTTACAAAACTCAACTAGG	GCGCCCGTATAGAATCCTGGAGG
DNA strand	(-)	(-)
Reverse complement (5'⟶ 3')	CCTAGTTGAGTTTTGTAAATTAC	CCTCCAGGATTCTATACGGGCGC
Score	1,89	72,23 %
Hairpin: lowest ΔG	0,17 kcal/mol	0,3 kcal/mol
Self-dimer: lowest ΔG	-5,36 kcal/mol	-9,89 kcal/mol

Table 3: Top-ranked sgRNAs from Yeastriction and CHOPCHOP for knockout of the *GRE3* locus in *S. cerevisiae*. Scores are given as for the related design tool (max score Yeastriction = 3, CHOPCHOP = 100 %). The underlined 3 bp sequence indicates the associated PAM sequence located directly upstream of the 20 bp sgRNA sequence in the coding (+) strand of the *GRE3* locus. The PAM sequence is not included in the OligoAnalyzer evaluation or integrated into the vector.

The pMEL13 plasmid, kindly gifted by Jean-Marc Daran (Addgene plasmid #107919), was used as a vector for transformation of the gRNAs into CEN.PK113-7D. The pMEL13 plasmid was extracted from *E. coli* using the GeneJET Plasmid Miniprep Kit (Thermo Scientific). The chosen gRNAs were inserted into the pMEL13 vector via substitution PCR, replacing a pre-existing gRNA sequence in the vector while preserving the tracrRNA also present in the pMEL13 plasmid. The substitution PCR was performed with primers binding directly upstream and downstream of the target substitution site, flanked by the reverse complements to the desired gRNAs, as detailed in Table 4. The substitution PCR was performed using the Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Successful integration of the gRNAs into the pMEL13 vector was verified via sequencing of the PCR products. Positive PCR products were digested with the DpnI restriction endonuclease (Thermo Scientific), which exclusively cuts methylated DNA, to remove any remaining pMEL13 backbones not containing the desired gRNA insert. The DpnI treatment was performed according to the manufacturer's instructions. For re-circularisation of the prepared vectors, competent DH5α *E. coli* were transformed with the prepared sgRNA vectors according to the protocol provided in *Transformation protocol for Escherichia coli* in Appendix B. Transformants were cultured on LB + Amp agar plates. Liquid LB + Amp cultures prepared from the plate colonies were grown overnight at 37 °C and the GeneJET Plasmid Miniprep Kit used to extract the prepared sgRNA vectors.

	Forward primer	**Reverse primer**
Sequence (5'⟶ 3')	GTTTTAGAGCTAGAAATAGCAAGT	GATCATTTATCTTTCACTGCGG
Length	24 bp	22 bp
Melting temp.	51 °C	52 °C
GC content	33 %	41 %
CHOPCHOP F	5'-gcgcccgtatagaatcctggGTTTTAGAGCTAGAAATAGCAAGT-3'	
CHOPCHOP R	5'-ccaggattctatacgggcgcGATCATTTATCTTTCACTGCGG-3'	
Yeastriction F	5'-gtaatttacaaaactcaactGTTTTAGAGCTAGAAATAGCAAGT-3'	
Yeastriction R	5'-agttgagttttgtaaattacGATCATTTATCTTTCACTGCGG-3'	

Table 4: Primers for the integration of the designed gRNAs into the pMEL13 vector. The ordered sequences, the primers flanked by the respective gRNAs, are specified in the two last rows, with the primer sequences in uppercase letters and the gRNA sequences in lowercase letters.

2.1.2 Preparation of the XR/XDH gene cassette

The XXX plasmid (see Table 1), an in-house plasmid containing the xylose utilisation genes *XYL1*, *XYL2* and *XKS1* was linearised by treatment with the PstI and MssI FastDigest restriction endonucleases (Thermo Scientific) to obtain the DNA fragment constituting the XXX cassette. Successful digestion was verified via gel electrophoresis (85 V, 30 min).

2.1.3 Strain construction: integration of xylose utilisation genes

The CEN.PK113-7D host strain was transformed simultaneously with the Cas9 vector and prepared sgRNA vector according to the protocol detailed in *Transformation protocol for Saccharomyces cerevisiae* in Appendix B. For each donor DNA, a total amount of ca. 500 ng was used. The transformants were plated on YPD + G418 + Hyg agar plates and incubated at 30 °C until colonies appeared.

The cells were transformed a second time with the linearised XXX cassette according to the protocol in Appendix B. Approximately 900 ng of the donor DNA was used for the transformation. The transformants were plated on YP-galactose + G418 + Hyg agar plates and incubated at 30 °C. As the Cas9 gene is under control of the *GAL1* promoter in the CSS plasmid, the presence of galactose is required to induce its expression. Insertion of the XXX cassette was investigated via colony PCR. The protocol for the colony PCR is described in Appendix B. Three primers were used for the PCR; a forward primer 5' of the *GRE3* promoter region, a reverse primer in the *GRE3* CDS and a reverse primer in the XXX cassette such that the two possible primer combinations generating fragments of 527 bp (*GRE3* intact) and 1 417 bp (XXX cassette inserted), respectively. The PCR products were analysed via gel electrophoresis (80 V, 45 min). Colonies of positive transformants were re-streaked onto YPD plates. The constructed strain, CEN.PK113-7D with the XXX cassette integrated at the *GRE3* locus, was named CPXGRE3.

2.2 Construction of a xylan-assimilating yeast strain

To endow the strain with the ability to degrade xylan, the hemicellulases *XylA* and *Xyn2* containing the *SED1p* signal peptide for protein secretion were introduced at the X2 intergenic site via the MCX vector, constructed using the MoClo modular cloning system [35].

2.2.1 Construction of the MCX plasmid

The vector with the hemicellulase expression cassette to be integrated at the X2 locus was first assembled *in silico* using the SnapGene software. The required plasmid parts, the promoter–gene–terminator sequence, for each of the two hemicellulases *Xyn2* and *XylA* were first extracted from source DNA using the BsmBI restriction enzyme. A transcriptional unit (TU) for each of the two hemicellulases was constructed by integration of the promoter–gene–terminator sequence in a plasmid backbone via BsaI assembly, where the insert replaced a pre-existing GFP insert. The constructed TUs were subsequently used for the assembly of the final multigene cassette by integrating the promoter–gene–terminator sequences into the final vector backbone via BsmBI assembly, again replacing a pre-existing GFP insert utilised for colony screening.

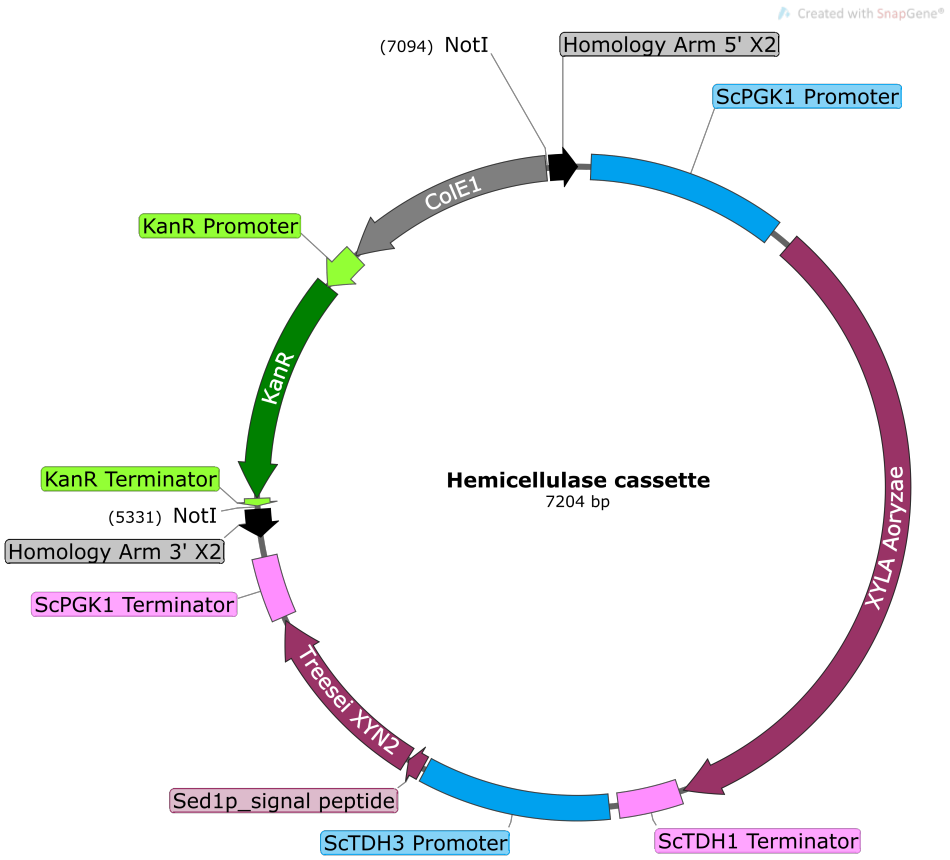


Figure 4: Final MCXplasmid with the two TUs for expression of the gens *Xyn2* and *XylA*. The *Xyn2* and *XylA* genes are shown in purple. In addition to the GFP, the final vector plasmid also contained the *ColE1* ORI for bacteria, MoClo assembly connectors, and the *KanR* selective marker. This figure was generated using the Snapgene software.

The MCX backbone and plasmids with level 1 TUs for *Xyn2* and *XylA* respectively were extracted from *E. coli* cultures using the GeneJET Plasmid Miniprep Kit (Thermo Scientific). The level 2 MCX construct containing the desired hemicellulase genes was constructed via BsmBI assembly according to the protocol described in *BsmBI assembly for level 2 MoClo constructs* in Appendix B. Competent DH5α *E. coli* were transformed with the constructed MCX plasmid according to the transformation protocol detailed in Appendix B. Transformants were plated on LB + Neo agar plates and incubated at 37 °C. Positive transformants were identified by lack of GFP when exposed to UV light and verified via colony PCR. The positive colonies were inoculated in 5 mL LB + Neo medium and incubated at 37 °C overnight. The MCX hemicellulase vector was extracted from the liquid cultures using the GeneJET Plasmid Miniprep Kit (Thermo Scientific). The purified products were quantified using NanoDrop (Thermo Scientific). The MCX hemicellulase cassette was prepared for yeast transformation by digestion of the purified plasmids with the NotI FastDigest (Thermo Scientific) restriction enzyme. To prevent recircularisation of the plasmid, the FastAP al-

kaline phosphatase (Thermo Scientific) was added to the restriction mixture. Successful digestion was verified via gel electrophoresis before inactivating the NotI enzyme.

2.2.2 Strain construction: hemicellulase integration

The CEN.PK.XXX strain was transformed simultaneously with the digested MCX xylanase cassette and the LT58 plasmid containing the Cas9 gene and sgRNA targeting the X2 locus, according to the transformation protocol described in Appendix B. The amount of donor DNA used was approximately 1 000 ng for the MCX cassette and 240 ng for the LT58 plasmid. Transformants were plated on YPD + G418 agar plates and incubated at 30 °C. Successful integration of the MCX hemicellulase cassette at the X2 locus was verified via colony PCR. For the colony PCR, three primers forming two possible primer combinations depending on whether the MCX cassette had been integrated or not were used, producing fragments with distinctive lengths; one forward primer binding at the 3' end of the MCX cassette, another forward primer binding outside of the 5' homology arm at the X2 locus, and one reverse primer binding outside of the 3' homology arm at the X2 locus. Positive transformants were re-streaked to a YPD agar plate and incubated at 30 °C. The constructed strain, the CEN.PK.XXX with the MCX cassette integrated at the X2 locus, was named CX2A.

2.3 Adaptation of constructed strains to xylose

The constructed strains were adapted to xylose by gradual introduction to minimal xylose-based medium. Figure 5 illustrates this procedure. Two colonies each for the CPXGRE3 strain and CX2A were used. The colonies were first inoculated in 5 mL YP-xylose medium in 50 mL centrifuge tubes and grown in at 30 °C shaking incubator until reaching an OD of 1–2. The cells were then washed and transferred to 5 mL YNB-xylose at an initial OD of 0,1 and again grown in the shaking incubator until an OD of 1–2, after which they were washed and transferred to 5 mL Delft-xylose at an initial OD of 0,1. After reaching an OD of 1–2 in Delft-xylose, the cells were plated onto Delft-xylose agar (1,5 %) plates and incubated at 30 °C.

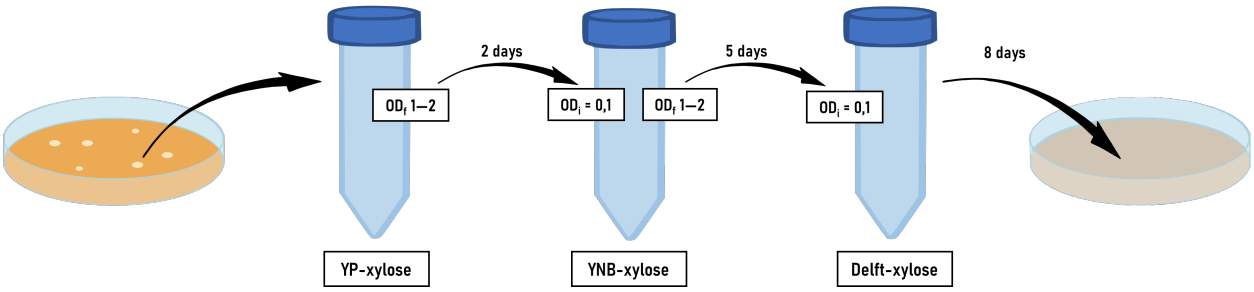


Figure 5: Illustration of the process of adapting the CPXGRE3 and CX2A to xylose. The number of days refers to the time the cells were grown in each medium before being transferred.

2.4 Analysis of the constructed strains

The ability of the constructed strains to utilise xylose and degrade xylan was evaluated and compared to other strains by growth analysis, enzymatic assays and by analysing degradation products via HPLC.

2.4.1 Microplate cultivation

A growth comparison between the constructed strains, negative controls and in-house strains was conducted by microplate cultivation. The growth profiler analysis was initially performed for the non-xylose adapted strains, before the integration of the hemicellulases, to compare the ability to grow in xylose between the constructed CPXGRE3 and the in-house CEN.PK.XXX strain. Two positive CPXGRE3 transformants were inoculated in 5 mL Delft-glucose medium and grown overnight in a 30 °C shaking incubator. Colonies from the CEN.PK.XXX strain, negative control CEN.PK113-7D and positive control diploid XXX were simultaneously inoculated in the same manner. This experiment was performed to select the host yeast strain for later integration of the MCX cassette.

For the xylose adapted cultures, the CEN.PK113-7D negative control, the two CPXGRE3 positive transformants and the CX2A strain were included in the analysis. For each strain or transformant, three colonies were inoculated in Delft-glucose medium and incubated at 30 °C for two days. For the CX2A, only two replicates could be obtained from the liquid cultures and used in the growth profiler experiment.

The cultures were spun down at 4 600 RPM for 7 minutes and the supernatant removed. The cells were washed twice by resuspension in 1 mL sterile MQ water, centrifugation at 8 000 RPM for 5 minutes and the supernatant discarded. The washed cells were resuspended in sterile MQ water and the OD of the suspension measured. An aerobic growth profiler 96-well plate was prepared with 225 µL of Delft-xylose medium and 25 µL of cell suspension with OD 1, for a final OD of 0,1. Additionally, three wells with Delft-xylose medium only were prepared as blanks. For the xylose adapted strains, wells were also prepared with YP-xylose in the same manner, with and without cells, to compare the growth in different media. The plate was incubated in the Growth Profiler 960 (Enzyscreen) at 30 °C, 250 RPM for 6–7 days.

2.4.2 Shake flask cultuivation of the constructed strains in xylose media

The growth of the constructed strains in xylose media was further evaluated via HPLC analysis of growth media, and for the xylose-adapted strains, also with dry biomass and OD measurements.

For the xylose adapted cultures, the CPXGRE3 strain, CX2A strain and CEN.PK113-7D were grown at 30 °C in 5 mL Delft-glucose overnight and subsequently transferred to 500 mL shake flasks with 25 mL Delft-xylose medium with an initial calculated OD of 0,5. Three

biological replicates were used per strain for the xylose adapted cultures, with the exception of the CX2A strain where only two biological replicates could be obtained from the overnight cultures. The cultures were incubated at 30 °C and 1 mL samples for HPLC analysis and dry biomass measurement collected and the OD measured at 0 h, 24 h, 47 h, 70 h, 119 h and 142 h. The culture samples for dry biomass were filtered through filter papers for dry cell mass (Sartorius) using a vacuum filtration unit (Sartorius). The filters were rinsed by filtering MQ water through the filters. All filters were dried before and after use by microwaving at the lowest setting for 10 min and kept in a desiccator until measurement.

All collected HPLC samples were centrifuged at 13 400 RPM for 2–4 min and the supernatant transferred to fresh microcentrifuge tubes. Prior to analysis, the supernatants were diluted with MQ water and the diluted samples filtered through 0,2 micron filters. The samples were kept at -20 °C until analysis. Five HPLC standards were prepared with xylose, xylitol and ethanol. The HPLC analysis was performed using the UltiMate 3000 system (Thermo Scientific) with an analysis time of 20 minutes per sample.

2.4.3 Enzymatic activities

Enzymatic assays were performed to investigate the xylanolytic activity of the constructed CX2A. The β-xylosidase activity was evaluated via the pNP-xylose assay and the xylanase activity was evaluated via the DNS assay.

For the DNS assay, pre-inoculums for three colonies each from the constructed CX2A strain and the CEN.PK.XXX (negative control) were prepared in 5 mL YPD (4 % glucose) and incubated in a 30 °C shaking incubator overnight. The cultures were subsequently transferred to 50 mL YPD medium in 500 mL shake flasks with baffles and incubated at 30 °C. After two days of incubation, the cells were transferred to 50 mL centrifuge tubes and centrifuged at 4 500 RPM for 8 minutes, the supernatant decanted to fresh centrifuge tubes and stored at 8 °, and the cell pellets resuspended in 50 mL fresh YPD medium (2 % glucose) in the same shake flasks, and incubated overnight for further biomass production. As the hemicellulases are secreted by the cells, the DNS assay was performed only using the supernatants (before and after medium change) from the cultures. The shake flask cultures were transferred to 50 mL Falcon tubes, centrifuged at 4 500 RPM for 7 minutes and the supernatant transferred to fresh 50 mL centrifuge tubes. The DNS assay was performed according to the protocol detailed in Appendix B.

For the pNP-xylose assay, supernatant and cell samples were diluted in 0,05 M NaAc buffer. YP and 0,05 M NaAc buffer were used as blanks. To a 96-well plate, 100 µL pNP-xylose (5 mM) was transferred to each well to be used in the assay. To each well, 50 µL diluted sample or blank was added. Technical triplicates were used for both samples and blanks. The prepared plate was incubated at 30 °C in a thermomixer for 30 min, after which 150 µL stop solution (Na_2CO_3, 1 M) was added to each well. The plate was read at 410 nm. The assay was also performed for qualitative results.

2.5 Growth on xylooligosaccharides

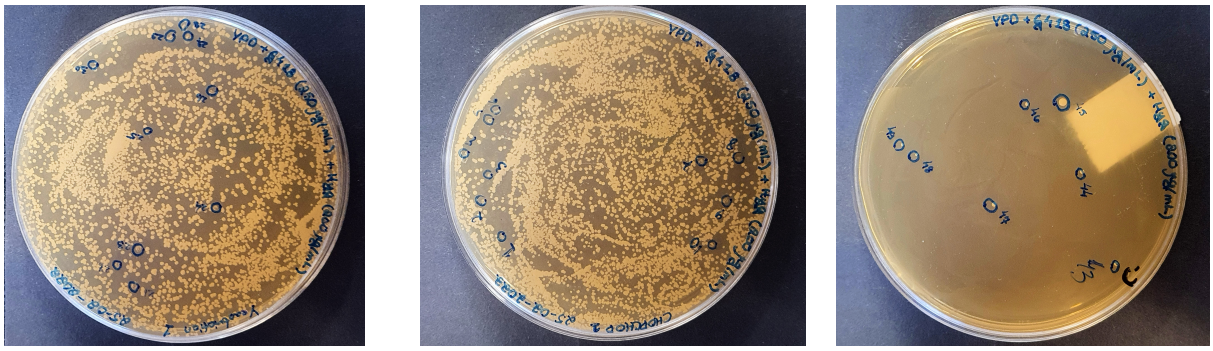
An experiment was performed to evaluate the ability of the CX2A strain to grow on XOS (xylan $\geq$ 95 XOS from corncob, Carlroth). The growth of the CX2A strain on Delft-XOS (2 %) was monitored for seven days by growth profiler analysis using the Growth Profiler 960 (Enzyscreen) and compared to the CEN.PK113-7D negative control.

3 Results

3.1 Strain construction

3.1.1 Transformation with the sgRNA and Cas9 vectors into the CEN.PK113-7D yeast strain

The CEN.PK113-7D strain was transformed simultaneously with the pMEL13 plasmid with the sgRNA and the CSS plasmid containing the Cas9 gene, and subsequently plated on YPD agar plates containing hygromycin and G418 for selection of positive clones (Figure 6).



(a) pMEL13 + $gRNA_{YST}$ transformants. (b) pMEL13 + $gRNA_{CHOP}$ transformants. (c) Negative control pMEL13 transformants, with no plasmid DNA added.

Figure 6: Transformation of the CEN.PK113-7D strain with sgRNA vectors. The transformants were plated on YPD + Hyg + G418 agar plates to select for positive clones, harbouring the HygR gene present in the pMEL13 plasmid. Figures show the plates after three days of incubation.

3.1.2 Integration of the XXX cassette into the CEN.PK113-7D yeast strain

The CEN.PK113-7D strain with the plasmids containing the *GRE3*-targeting sgRNA and the Cas9 gene were transformed with the XXX cassette containing the XR/XDH pathway. The successful integration of the XXX cassette harbouring the xylose utilisation genes was verified via colony PCR (Figure 7), using the CEN.PK113-7D strain as a negative control and the in-house diploid XXX strain, in which the *GRE3* locus had been the target for XXX cassette integration, as a positive control. However, these results revealed that the diploid XXX strain did in fact have an intact *GRE3* locus, i.e. no XXX cassette integrated at the site, and was thus not a true positive control. Two primer pairs were used for the PCR,

amplifying regions of i) 527 bp (*GRE3* intact) and ii) 1 417 bp (XXX cassette integrated). Out of the 20 transformants evaluated, ten each with CHOPCHOP and Yeastriction gRNAs respectively, two transformants were verified as positive, both with $gRNA_{YST}$.

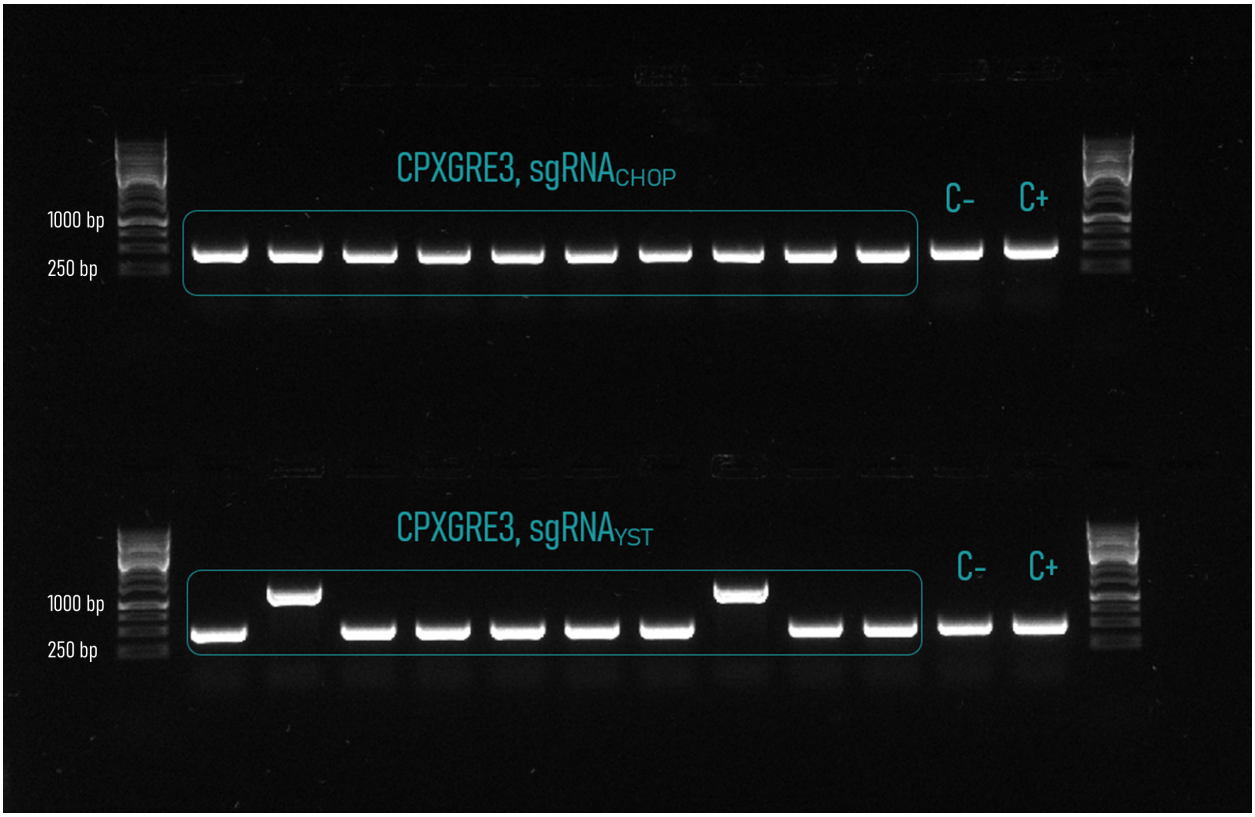


Figure 7: Gel electrophoresis from colony PCR of XXX transformants. Negative control (C-) = CEN.PK113-7D, positive control (C+) = diploid XXX strain. GeneRuler 1 kb DNA Ladder (Thermo Scientific): 250 bp – 500 bp – 750 bp – 1 000 bp – 1 500 bp – (...) – 10 000 bp.

3.1.3 Integration of the MCX cassette at the X2 locus of the CEN.PK.XXX strain

Based on results from growth profiler experiments (see 2.4.1) comparing growth on xylose of the constructed CPXGRE3 strain to the CEN.PK113-7D negative control and two in-house strains, the decision was made to use the in-house strain CEN.PK.XXX to construct a xylan assimilating strain as this strain had displayed more efficient utilisation of xylose than CPXGRE3 in the condition applied in this work. CEN.PK.XXX has the XXX cassette integrated at the *HO* locus.

The CEN.PK.XXX strain was transformed with the MCX plasmid containing the hemicellulase cassette with *XylA* and *Xyn2*. Simultaneously, the strain was transformed with the LT58 plasmid containing a gRNA targeting the X2 locus, and the Cas9 gene under the con-

trol of a constitutive promoter. Colony PCR was performed to verify sucessful integration of the MCX cassette at the X2 locus of the CEN.PK.XXX strain (Figure 8), with primer pairs amplifying regions of i) 765 bp (X2 intact) and ii) 435 bp (MCX cassette insterted).

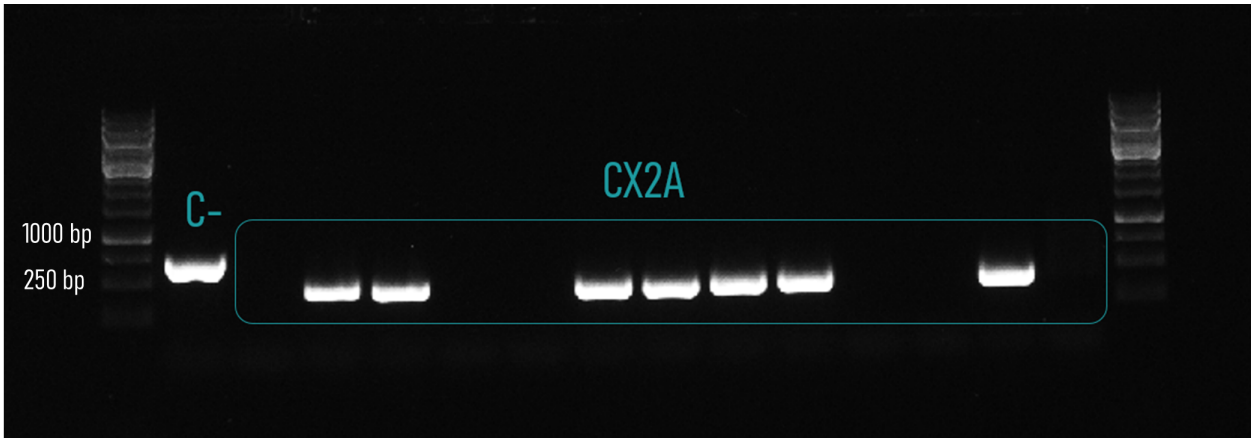
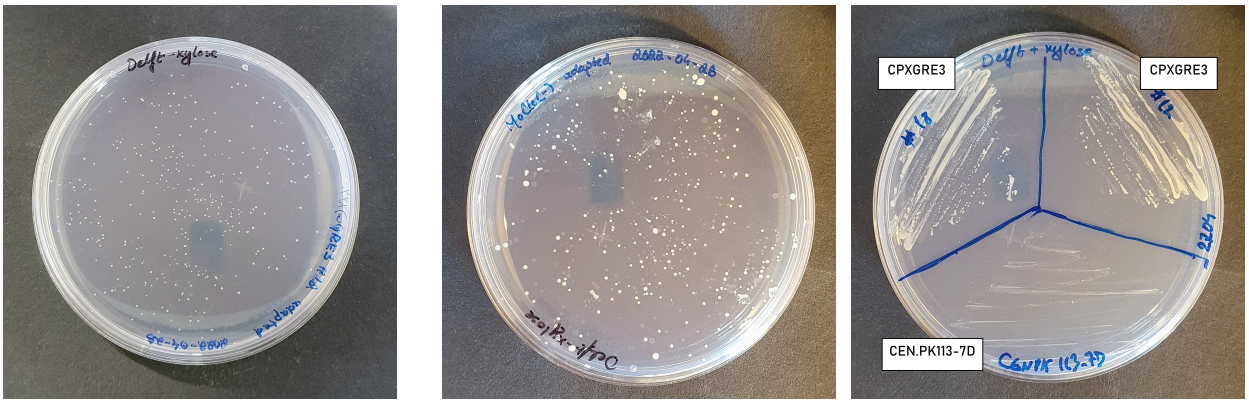


Figure 8: Gel electrophoresis from colony PCR of MCX transformants. Negative control (C-) = CEN.PK113-7D. GeneRuler 1 kb DNA Ladder (Thermo Scientific): 250 bp – 500 bp – 750 bp – 1 000 bp – 1 500 bp – (...) – 10 000 bp.

The gel electrophoresis of the products from the colony PCR with the MCX transformants showed that all seven transformant colonies that produced any band were positive.

3.2 Adaptation of constructed strains to xylose

The construted CPXGRE3 and CX2A strains were adapted xylose by gradual introduction to Delft-xylose, a xylose-based minimal defined medium, as described above (Figure 9). The CEN.PK113-7D strain was included in the adaptation experiments as a negative control.



(a) CPXGRE3

(b) CX2A

(c) Two CPXGRE3 transformants (upper) and the CEN.PK113-7D negative control (lower)

Figure 9: Delft-xylose agar plates with the xylose-adapted strains. Photos of the plates were taken after the same incubation time.

The adapted strains displayed growth on xylose-based minimal defined medium, and more efficient utilisation of xylose than the CEN.PK113-7D negative control, which had been adapted according to the same protocol as the constructed strains.

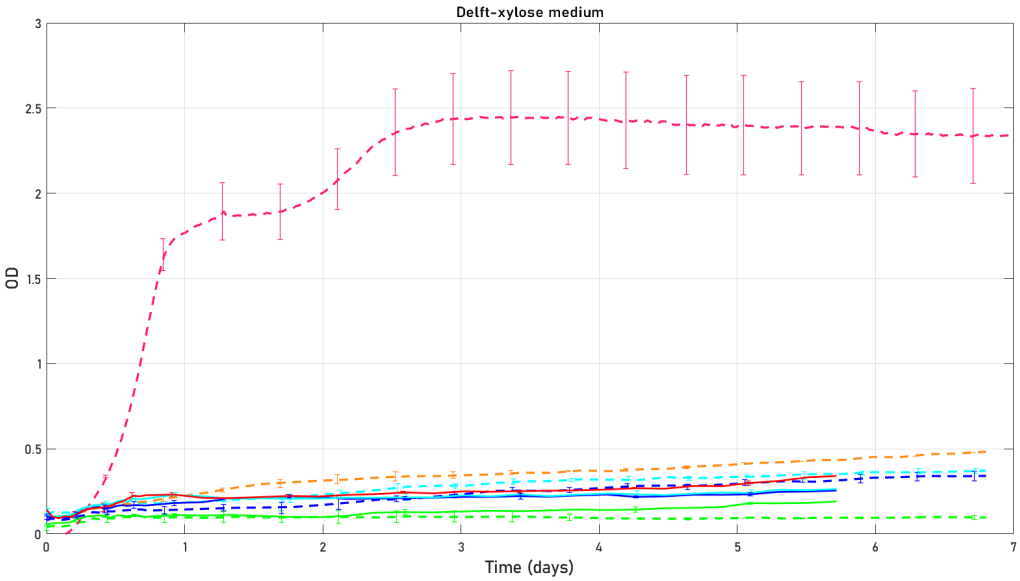
3.3 Xylose utilisation by constructed strains

The ability of the constructed strains to grow on xylose was evaluated by growth profiler analysis, OD and biomass measurements from shake flask cultures and qualitatively by cultivation on agar plates with xylose-based media. Values and measurements are generally the means of three biological replicates, with the exception of the xylose-adapted CX2A strain, where only two biological replicates were available for the experiments.

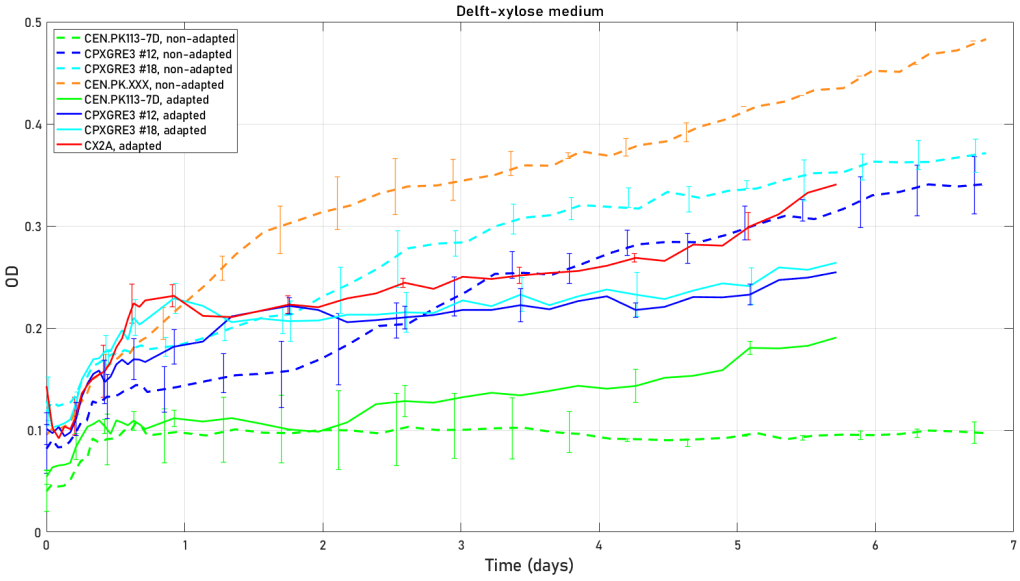
Overall, growth for all xylose adapted strains appeared slower compared to the preceding experiment with the non-xylose adapted strains (Figure 10a, Figure 10b), but the results also suggested that none of the strains had yet reached stationary phase when the experiment was terminated at six days, one day less than the initial comparison between the non-xylose adapted strains.

3.3.1 Growth profiler analysis

Growth profiler experiments were performed for the non-xylose adapted strains and the xylose adapted strains in Delft-xylose (Figures 10a, 10b). The xylose adapted strains were also evaluated in YP-xylose medium (Figure 11, Table 5).



(a) Growth profiler analysis of non-xylose adapted and xylose adapted strains in Delft-xylose medium. The diploid XXX strain is used as a positive control, the CEN.PK113-7D strain as a negative control.



(b) Growth profiler analysis of non-xylose adapted and xylose adapted strains, in Delft-xylose medium, plotted without the diploid XXX strain.

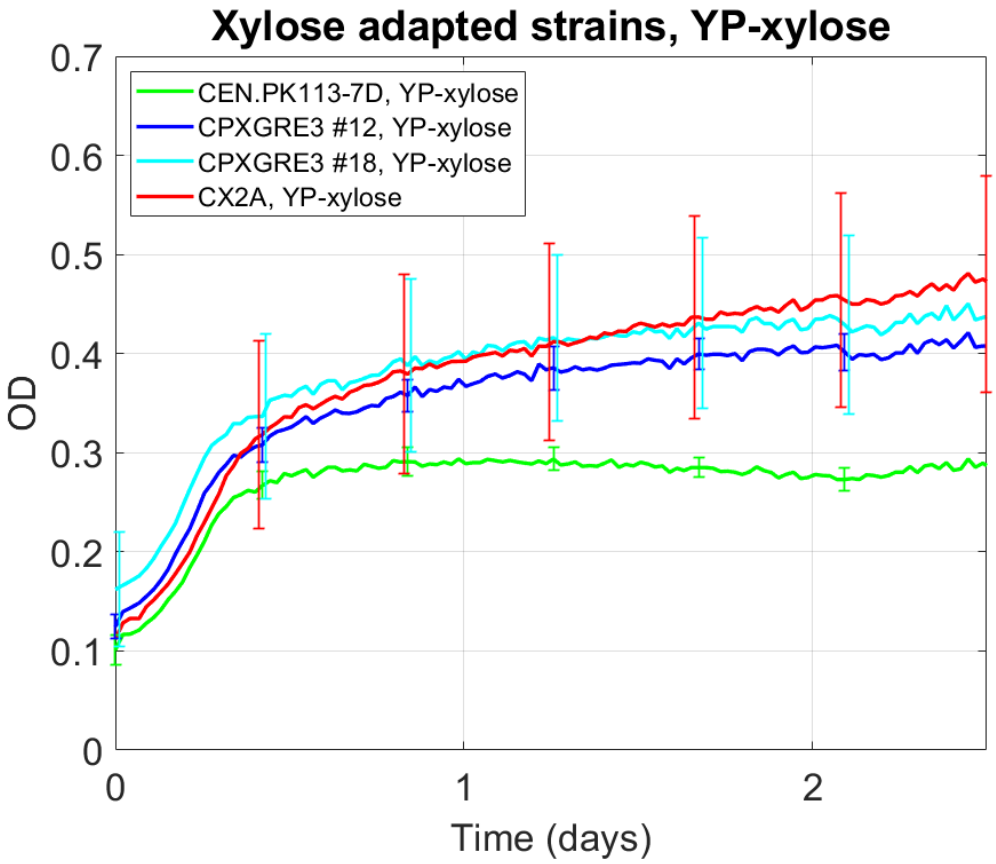


Figure 11: Growth profiler analysis of xylose-adapted strains in YP-xylose medium.

Strain	**Medium**	**ΔOD$_{48\ h}$**	**μ_{max} (h^{-1})**	**Doubling time (h)**
CEN.PK113-7D	YP-xylose	0,1770	0,1337	5,19
CPXGRE3 #12	YP-xylose	0,2820	0,1307	5,30
CPXGRE3 #18	YP-xylose	0,2727	0,1240	5,59
CX2A	YP-xylose	0,2230	0,1423	4,87

Table 5: OD values at 48 h, maximum specific growth rate and doubling time for the xylose adapted strains in YP-xylose from the growth profiler analysis. The maximum specific growth rates μ_{max} and subsequently doubling times are based on exponential curve fitting using 10 data points within the exponential growth interval, with correlation coefficients $R^2 > 0{,}99$.

3.3.2 Shake flask cultivation on xylose of the constructed strains

The graph in Figure 12 shows the OD over time for the shake flask cultures of the xylose-adapted strains on Delft-xylose. It may be noted that the OD is appreciably higher compared to the results from the growth profiler for the same strains in Delft-xylose medium (Figure 10b), particularly for the CPXGRE3 and CX2A strains. Most likely, this result can

primarily be attributed to superior aeration in the shake flasks compared to the microplates used for the growth profiler experiments. In addition, as can be seen in the Figure 10b and Figure 12, the shake flask cultures were started at a slightly higher initial OD.

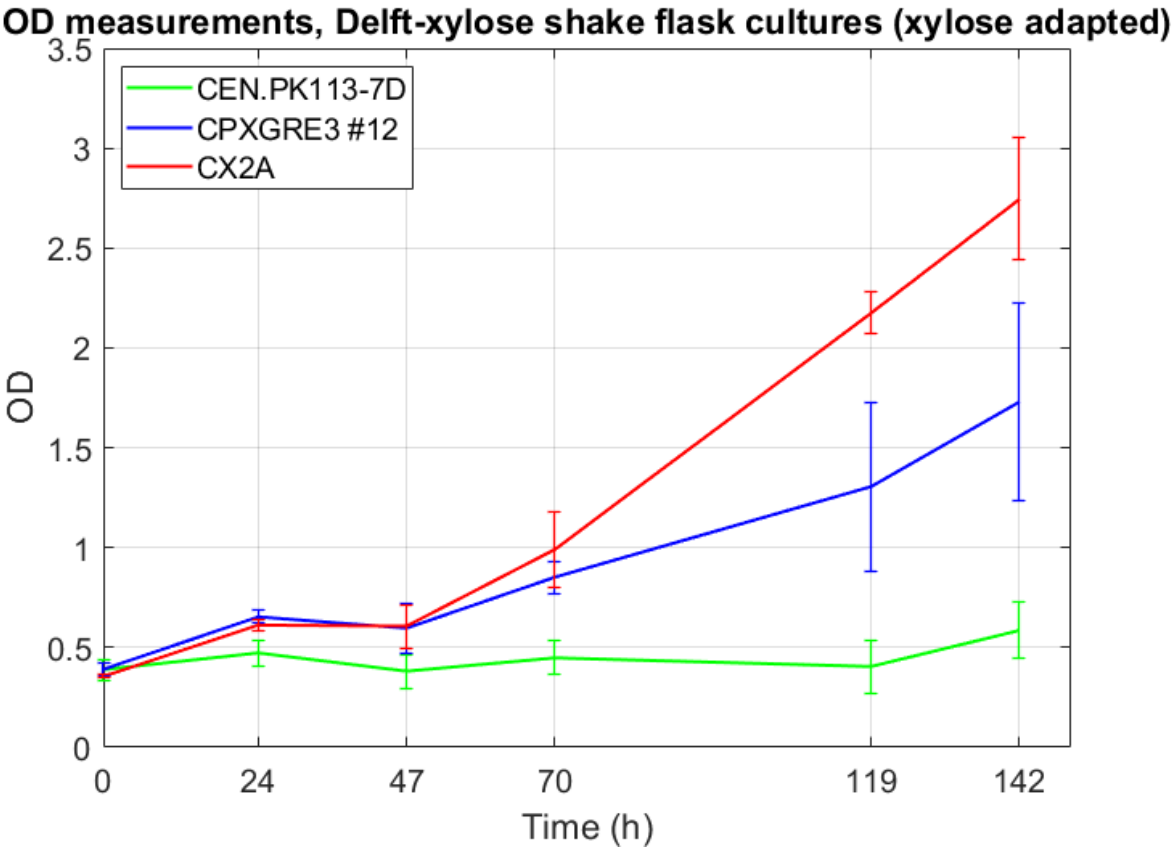


Figure 12: OD measurements at different timepoints post-inoculation for the xylose adapted Delft-xylose shake flask cultures, grown at 30 °C in a shaking incubator. Values are the means of three biological replicates, with the exception of the CX2A strain, where only two biological replicates were available.

Strain	**Medium**	**ΔOD$_{142\ h}$**	**Final biomass content (g/L)**
CEN.PK113-7D	Delft-xylose	0,1960	0,40
CPXGRE3 #12	Delft-xylose	1,3390	0,70
CX2A	Delft-xylose	2,3890	1,20

Table 6: Total increase in OD and final biomass content for the Delft-xylose cultures with the xylose adapted strains.

3.4 HPLC analysis of xylose, xylitol and ethanol content

Table 7 displays the total xylose consumption, xylitol- and ethanol production for the xylose adapted shake flask cultures in Delft-xylose after 142 h of cultivation. Ethanol was only

detected in the supernatants of the constructed strains, indicating the degradation of xylose by these strains. For the CEN.PK113-7D and CPXGRE3 strains, the results showed that the xylose concentration in the supernatant was higher than the initial concentration of 20 g/L. As the strains do not possess any pathway allowing for xylose formation, it is inferred that these results are not reliable, further implying that they cannot be used to calculate yields of xylitol and ethanol on xylose.

Strain	**Xylose in supernatant (g/L)**	**Xylitol produced (g/L)**	**Ethanol produced (g/L)**
CEN.PK113-7D	21,4118	0,1268	0
CPXGRE3#12	21,6363	0,0805	0,2893
CX2A	18,2400	0,1097	0,5385

Table 7: Total xylose in supernatant, xylose- and ethanol production of the xylose adapted strains cultivated for 142 h in shake flasks in Delft-xylose medium.

3.5 Xylanolytic activity

The xylanolytic activity of the constructed strains was evaluated with the DNS assay for endoxylanase activity, and the pNP-xylose asssay for β-xylosidase activity. No activity was detected the DNS assay. However, β-xylosidase activity could be demonstrated with the pNP-xylose assay. For the pNP-xylose assay, both qualitatively and quantitatively for the CX2A strain. The qualitative results are exhibited as the colour change of samples to yellow (Figure 13) as the xylose analogue pNPX is hydrolysed by xylosidase to pNP. The stop solution, The CEN.PK.XXX strain, which lacks the hemicellulase genes and was used as a negative control, did not display this colour change. The qualitative results for the CX2A strain, obtained via absorbance measurements of the assay samples, are shown in Figure 14.

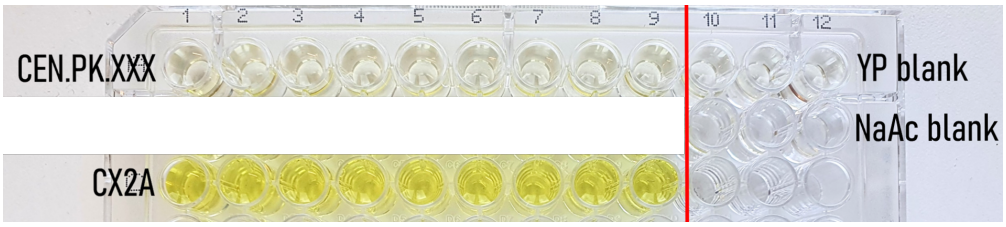


Figure 13: Qualitative results from the pNP-xylose assay with supernatants from the CX2A strain, and CEN.PK.XXX as a negative control.

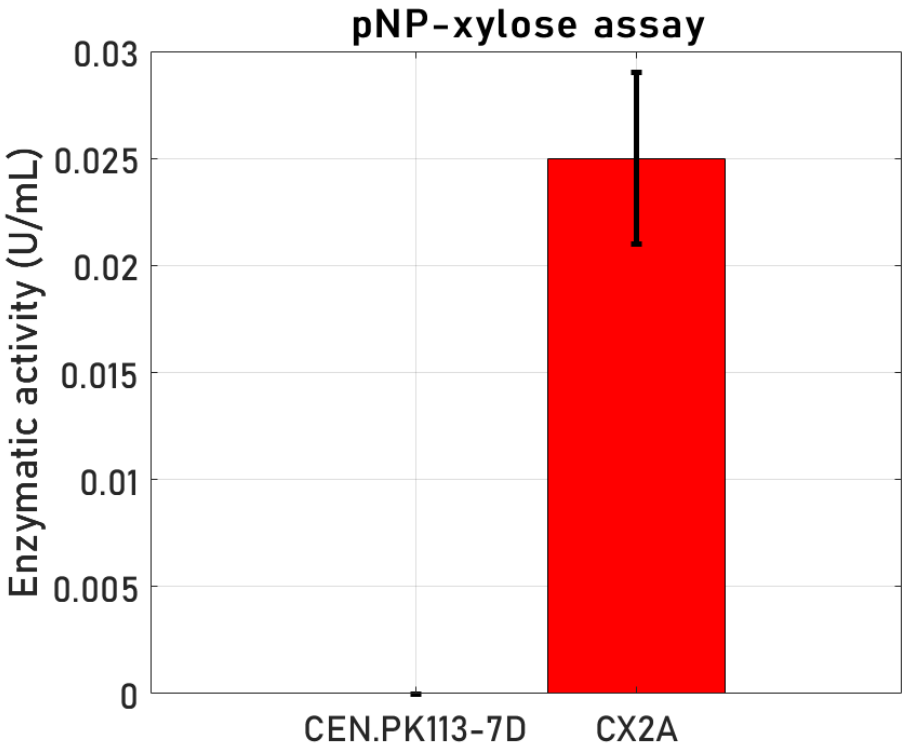


Figure 14: Quantitative results from the pNP-xylose assay with supernatants from the CX2A strain and CEN.PK113-7D as a negative control.

3.6 Growth on xylooligosaccharides

Figure 15 below shows the OD over time for the CX2A and CEN.PK113-7D strains in Delft-XOS (2 %). While the CEN.PK113-7D reached stationary phase at around 12 h of cultivation, probably due to utilisation of remaining sugar monomers in the commercial XOS utilised, the OD of the constructed CX2A strain was still increasing at the final data point after 168 h of cultivation, having then reached an OD of 0,35, compared to 0,18 for the negative control. These results, along with those of the enzymatic assays, indicate that the CX2A lacks sufficient ability to assimilate xylan into XOS, but has functional β-xylosidase to degrade XOS into xylose monomers to use as a carbon source. This further points to the main issue being related to endoxylanase activity, as this enzyme is responsible for the degradation of xylan to XOS.

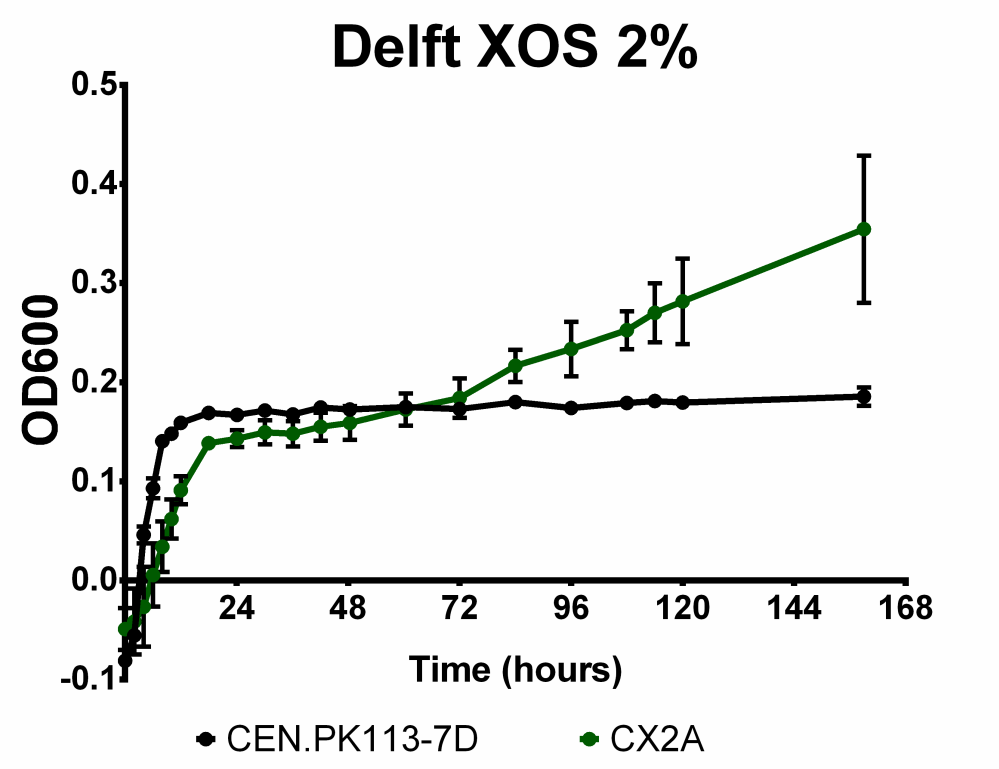


Figure 15: OD measurement of XOS cultures of the CX2A and CEN.PK113-7D strains.

4 Discussion

Efficient utilisation of second-generation biomass, consisting mainly of lignocellulose, for industrial microbial production of value-added products in biorefineries is an important step in the development of a more sustainable production industry. However, *S. cerevisiae*, one of the most favoured industrial microorganisms, is unable to assimilate hemicelluloses such as xylan, which constitute a significant fraction of second-generation biomass.

In this project, a pathway for xylose utilisation consisting of *XYL1* and *XYL2* from *S. stipitis* and *XKS1* from *S. cerevisiae* was first integrated at the *GRE3* locus of the the CEN.PK113-7D yeast strain, using the Cas9 endonuclease. The *GRE3* gene encodes an unspecific aldolase with limited ability to reduce xylose, but its expression has been shown to aggrevate the redox balance [21],[22] for the xylose assimilation pathway introduced. In addition, it may be hypothesised that the aldolase encoded by *GRE3* could compete for substrate with the more efficient xylose reductase encoded by *XYL1*. Interestingly, the diploid XXX strain, which was thought to have the XXX cassette integrated at the *GRE3* locus [32], displayed a negative result in the colony PCR. This was also confirmed in several subsequent PCRs using the same primers (data not shown). This indicates that the diploid XXX in fact does not have the XXX cassette integrated at the *GRE3* locus, but at another location in the genome.

Genome sequencing may be required to determine the unknown location, which may be of interest as an alternative and possibly advantageous integration site for the XXX cassette or other modifications relating to xylose metabolism. Furthermore, these results also indicate that the unspecific aldolase encoded by *GRE3* is intact in the diploid XXX strain, but does not appear to significantly impact xylose utilisation negatively, based on the high growth displayed in the growth profiler experiments (Figure 10a). However, as this strain also has other modifications intended to improve xylose metabolism, further experiments would be required to establish the isolated effect of the intact *GRE3*. While the diploid XXX also has a number of other modifications, in a addition to being a diploid strain, future projects with this strain and its parent strains could focus on evaluating the effects of maintained *GRE3* activity during expression of the XR/XDH pathway.

Growth evaluation of the strains in xylose-based media showed, somewhat surprisingly, lower growth for the xylose adapted strains than for the non-xylose adapted strains. While a part of the explanation could be that the termination of the growth curve one day earlier for the xylose adapted strains obscured potential later developments in growth, it may also be that the expression of additional heterologous genes caused some form of cellular stress, resulting in retarded growth for CX2A. However, the inter-strain differences in growth were more expressed for the Delft-xylose shake flask cultures. Here, the CX2A strain reached a OD of 2,74 when the experiment was ended at 142 h, while the CPXGRE3 strain reached an OD of 1,73 and CEN.PK113-7D an OD of 0,59. The differences in biomass content at the same time point were comparable, with values of 1,20 g/L for the CX2A strain, 0,70 g/L for the CPXGRE3 strain and 0,40 g/L for the CEN.PK113-7D negative control. Better growth conditions in the shake flask, such as better aeration, is likely to have contributed to the improved growth compared to the growth profiler experiments with the same strains. In fact, xylose utilisation in *S. cerevisiae* via the particular XR/XDH pathway from *S. stipitis* has been shown to be significantly impeded in oxygen-limited conditions, likely linked to cofactor imbalances [36],[37],[38].

HPLC analysis of growth media showed that the constructed strains, CPXGRE3 and CX2A, were able to produce ethanol during growth on xylose, with titres of 0,2893 g/L and 0,5385 g/L after 142 h of cultivation. In contrast, the unmodified CEN.PK113-7D strain did not produce any ethanol. Furthermore, the CEN.PK113-7D samples demonstrated a higher xylitol content than the CPXGRE3 and CX2A strains. This may indicate that while the native *GRE3* aldolase reductase is able to reduce xylose to xylitol, but that the strain is unable to further utilise the xylitol due to lack of efficient pathways. While further experiments with more biological replicates would be required to prove any statistical differences, the results from the HPLC analysis overall suggest that the constructed strains have improved ability to utilise xylose as a carbon source. For the xylose-assimilating activity in general, it is probable that additional or alternative approaches to enhancing xylose metabolism would increase the efficiency. For example, further genetic modifications targeting e.g. the PPP or transmembrane transporters could be considered. A longer adaptation period to xylose may

have further enhanced the efficiency to metabolise xylose by the constructed strains, especially if a more targeted approach such as adaptive laboratory evolution (ALE) is employed. This would allow for identification, cultivation and development of high-potential strains from clones with desired phenotypes with no obvious relation to genotype [39], as well as identification of favourable genotypes stemming from natural mutagenesis. However, while some argue that ALE approaches are even a necessary part of developing an efficient xylose utilisation pathway in *S. cerevisiae* [19], this was beyond the scope of this project.

The xylanolytic activity of the CX2A was evaluated via enzymatic assays. Endo-xylanase activity could not be detected by the DNS-assay approach applied in this project. The pNP-xylose assay indicated elevated β-xylosidase activity compared to the negative control. As a final experiment, the ability of the CX2A strain to grow on XOS was evaluated with the CEN.PK113-7D strain as a negative control. Degradation of the short-chain XOS into xylose requires an exolytic enzyme targeting the β-1,4-linkages at the ends of the XOS. As this activity corresponds to that of β-xylosidase (see Figure 1), XOS degradation is indicative of β-xylosidase activity. Results from cultivation of the strains on XOS, together with enzymatic assays, indicated that the CX2A strain has a functional β-xylosidase enzyme to utilise XOS as a carbon source by degradation into xylose monomers, but does not possess the ability to degrade the xylan polymer into XOS, which would require a functional endoxylanase enzyme. A possible explanation is that the substituents of the xylan main chain block access for this enzyme, and that introduction of additional enzymes targeting the substituents would resolve the issue. Due to the heterogenicity of xylans, substituent-targeting enzymes may need to be selected based on the primary biomass source of interest. For example, wood xylans tend to contain and abundance of glucuronic acid substituents [40], which can be targeted by the α-glucoronidase enzyme [11], while α-L-arabonofuranosidase would be required to target the arabinofuranose substituents commonly found in xylan from annual plants [40],[11]. For the purpose of evaluating whether substitutent blockage of endoxylanase is indeed the issue in this project, further enzymatic assays could be performed using xylan from sources known to contain primarily unsubstituted xylan, such as tobacco stalks or esparto grass [40].

The lack of xylanolytic activity may also be related to dysfunctional hemicellulase secretion. Bottlenecks in the secretion of heterologous proteins is a well-known issue for *Saccharomyces cerevisiae*, particularly in that accumulation of unfolded proteins in the endoplasmic reticulum (ER) can trigger the unfolded protein response (UPR) which in turn results in ER stress and in severe cases apoptosis [41]. This form of cellular stress may also be a potential explanation for the reduced growth of CX2A compared to the CEN.PK.XXX parent strain in the growth profiler experiments. While the relation between UPR and the heterologous hemicellulases introduced to *S. cerevisiae* in this project must be investigated separately to correlate the expression of genes related to UPR with that of the genes of interest [41],[42], the hypothesis that accumulation of unfolded proteins may be a reason for the low endoxylanase activity should remain viable. Several studies have shown that engineering of key components in the endoplasmic reticulum (ER) or other elements related to reduced ER

stress may improve the secretion of heterologous proteins [43],[44],[45] which could be seen as an option in future projects.

Another alternative would be to consider cell-surface displayed enzymes rather than secreted. Kruger *et al.* [46] integrated and analysed the activity of xylanase and β-xylosidase in an *S. cerevisiae* strain expressing the XI pathway for xylose utilisation, and found that the highest hemicellulase activities were achieved with cell surface-tethered enzymes during growth on xylose, while secreted enzymes showed higher activities when the strains were grown on glucose. Furthermore, it was concluded that co-expression of the two hemicellulases resulted in the highest growth on xylan and XOS. Overall, there are numerous factors which may interfere with functional hemicellulase activity in recombinant *S. cerevisiae*, demonstrating the substantial challenge in constructing a xylanolytic yeast strain.

5 Conclusion

Saccharomyces cerevisiae is one of the most important industrial microorganisms and developing strains capable of utilising a wide substrate range is crucial for efficient and sustainable production of numerous yeast-derived products. This project was aimed at developing a xylan- and xylose assimilating *S. cerevisiae* yeast strain by Cas9 integration of hemicellulases and the XR/XDH pathway. The strain CPXGRE3 was constructed by integrating the XR/XDH pathway at the *GRE3* locus of the CEN.PK113-7D host strain, and displayed higher growth on xylose-based media than the parent strain in growth profiler experiments and plate cultures, but lower than the in-house strain CEN.PK.XXX, which has the XR/XDH pathway integrated at the *HO* locus. The CEN.PK.XXX strain was selected for Cas9 integration of secreted hemicellulases, endoxylanase (*XylA*) and β-xylosidase (*Xyn2*), producing the CX2A strain. This strain strain displayed ability to grow on xylose, and while significant β-xylosidase activity could be shown both qualitatively and quantitatively, endoxylanase activity could not be detected. However, the strain also demonstrated growth on XOS, which indicates β-xylosidase activity. Thus, the main issue appears to lie in the endoxylanase degratation of the xylan main chain. It is possible that the bottlenecks related to the secretion of heterologous proteins in *S. cerevisiae* hinder efficient hemicellulase activity, and another option for future projects would be to investigate cell wall-tethered hemicellulases. Overall, the objective of constructing a xylose assimilating yeast strain was successfully achieved with the CPXGRE3 strain, while the objective of a xylan assimilating strain was partly achieved with the CX2A. With further optimisation of the expression and display system for the hemicellulases, it is likely that a fully xylanolytic yeast strain can be developed from the constructed strains.

Acknowledgements

I would like my supervisor João Heitor Colombelli Manfrão Netto for the constant support and guidance throughout the project, and my examiner Cecilia Geijer for providing great feedback from which I have been able to improve. I am also grateful to the IndBio division at Chalmers for welcoming me to perform my master's thesis in their facilities. Overall, this project has been a great opportunity for me to learn and gain practical experience in the vast world of molecular biology.

Elin Lönnqvist, Gothenburg, August 2022

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A Growth media

Carbon source stock solutions

Carbon source	Stock concentration
Glucose	200 g/L (20 % w/v)
Xylose	200 g/L (20 % w/v)
Galacose	100 g/L (10 % w/v)

Table 8: Concentrations used for carbon source stock solutions.

YP-based media

Volume	Component
900 mL	MQ water
20 g	peptone
10 g	yeast extract
100 mL	carbon source (10x)*
15 g	agar**
Final volume: 1 L	

Table 9: Recipe for YP-based media. *See Table 8. **For agar media only.

LB-based media

Volume	Component
950 mL	MQ water
10 g	tryptone
10 g	NaCl
5 g	yeast extract
15 g	agar*
Final volume: 1 L	

Table 10: Recipe for LB-based media. *For agar media only.

YNB-based media

Volume	Component
900 mL	MQ water
100 mL	YNB (10x) (Thermo Scientific)
100 mL	carbon source (10x)*
Final volume: 1 L	

Table 11: Recipe for YNB medium. For YNB-agar medium, MQ water was reduced and the volume replaced with 3 % (2x) agar solution. *See Table 8.

Delft medium

Volume	Component	Note
200 mL	Delft salts (5x)	5x solution: 25 g/L $(NH_4)_2SO_4$, 15 g/L KH_2PO_4, 5 g/L $MgSO_4 \cdot {}_7H_2O$
200 mL	potassium phtalate buffer	51 g/L KHP, adjust to pH 5,5
100 mL	carbon source (10x)	see Table 8
1 mL	vitamins (1 000x)	
1 mL	trace elements (1 000x)	
489 mL	MQ water	
Final volume: 1 L		

Table 12: Recipe for Delft medium. See the "Note" column for recipes and directions regarding separate medium stock solutions. For Delft-agar medium, MQ water was reduced and the volume replaced with 3 % (2x) agar solution.

B Protocols

Transformation protocol for *Escherichia coli*

1. Thaw competent *E. coli* were ice.

2. For each transformation mix, add 50 µL competent cell suspension and the donor DNA to a labelled Eppendorf tube.

3. Heat-shock the cells in a 42 °C water bath for 60 s and immediately put on ice for 2 min.

4. To each transformation mix, add 1 000 µL LB medium.

5. Incubate the samples in a 37 °C shaker for 1 h.

6. Centrifuge samples at 5 000 RPM for 5 min.

7. Remove 850 µL of the supernatant.

8. Resuspend the cell pellet in the remaining medium.

9. Spread the cell suspension on (selective) agar plates and incubate the plates at 37 °C.

Transformation protocol for *Saccharomyces cerevisiae*

The transformation protocol described below was used for all transformations of *Saccharomyces cerevisae* in this project. Note: the single-stranded carrier DNA (here denoted ssDNA) used in this protocol is prepared from salmon sperm DNA by sonication for 5 minutes followed by boiling for 5 minutes. The ssDNA may be prepared beforehand and stored frozen at -20 °C.

Volume	**Component**	**Final concentration**
600 µL	lithium acetate (1 M)	0,2 M
2 000 µL	polyethylene glycol 3350 (60 % v/w)	40 % v/w
300 µL	dithiothreitol (1 M)	100 mM
100 µL	MQ water	
Final volume: 3 mL		

Table 13: Recipe for 3 mL one-step buffer. The one-step buffer can be prepared in advanced and kept stored frozen at -20 °C.

1. Grow the cells to be transformed in YPD medium (+ any relevant antibiotics) overnight

(incubate in 30 °C shaker).

2. Measure the OD of the overnight cultures diluted 10x and calculate the volumes corresponding to $5 \cdot 10^7$ cells (OD = 1 $\longleftrightarrow$ $1 \cdot 10^7$ cells/mL).

3. Transfer $5 \cdot 10^7$ cells to an Eppendorf tube and spin down the cells (centrifuge 5 min at 8 000 RPM).

4. Remove the supernatant and wash the cells twice by resuspending the pellet in 1 mL sterile MQ water and centrifuging at 8 000 RPM for 5 min.

5. After the second wash, remove all remaining supernatant by pipetting. Any leftover supernatant may change the concentration of the one-step buffer.

6. Resuspend the washed cell pellet in one-step buffer. The volume of one-step buffer should complete the total volume to either i) 100 µL, if the total volume of DNA (ssDNA + donor DNA) is lower than 10 µL, or ii) 200 µL, if the total volume of DNA (ssDNA + donor DNA) is higher than 10 µL – see next step.

7. Add 50 µg of pre-boiled single-stranded carrier DNA and 50 ng – 1 µg of donor DNA.

8. Incubate in a 45 °C water bath for 45 min, vortexing briefly every 10 min.

9. Add 1 mL of sterile MQ water to the sample and mix by pipetting. Centrifuge the samples at 8 000 RPM for 5 min.

10. Remove the supernatant and resuspend the cell pellet in 1 mL YPD medium. Incubate the sample at 30 °C for 4 h.

11. Centrifuge the samples at 8 000 RPM for 5 min and remove the supernatant. Wash the cells twice by the same procedure as described in step 4.

12. After the second wash, remove all remaining supernatant and resuspend the pellet in 200 µL sterile MQ water.

13. Transfer the cell suspension to selective agar plates and incubate at 30 ° for 3–4 days.

Colony PCR protocol for *Saccharomyces cerevisiae*

The protocol described below details how colony PCRs in this project were performed. The protocol is adapted from Horecka and Chu [47] and the Phire Hot Start II DNA Polymerase (Thermo Scientific) protocol [48]. Colonies to be used in the colony PCR should preferably come from a freshly prepared/re-streaked plate. All work with cell cultures should be carried

out in a LAF bench.

Preparation of donor DNA

1. Select the colonies to be analysed from the agar plates. Preferably, colonies should be well-defined and separated, and fairly large.

2. For each colony + positive and negative control, add 20 µL NaOH solution (0,02 M) to a PCR tube.

3. Transfer a piece of each selected colony to its corresponding tube and suspend in the NaOH. The solution should be cloudy, but not white.

4. Break the cells by incubating them at 98 °C for 15 min.

PCR mix

Prepare the PCR master mix. The recipe in Table 14 is for a single reaction and can be multiplied by the number of desired reactions. To account for pipette inaccuracy, additional reactions may be prepared. PCR master mix components should preferably be added in the order indicated (descending). Pipette to mix.

Volume	**Component**
4,00 µL	5X Phire Green Reaction Buffer
0,40 µL	dNTPs 10 mM
1,00 µL	Primer F 10 µM
1,00 µL	Primer R 10 µM
11,20 µL	MQ water
0,40 µL	Phire Hot Start II DNA Polymerase
Final volume: 18 µL	

Table 14: Recipe for PCR mastermix using Phire Hot Start II DNA Polymerase and 2 µL of template DNA per reaction. Final reaction volume 20 µL. If more than two primers are used, subtract the additional volume from the MQ water. If needed, the MQ water can be decreased to 10,20 µL and the template DNA increased to 3 µL.

For each reaction, add 18 µL of the PCR master mix to fresh PCR tubes. To each tube, add 2 µL of the corresponding donor DNA from the tubes with the lysed cells. The total volume per reaction tube should be 20 µL.

Incubate the samples in a thermocycler according to the programme specified in the protocol for the polymerase.

BsmBI assembly for level 2 MoClo constructs

This protocol specifically describes the BsmBI assembly of the MCXxylanase vector, a level 2 MoClo construct. The level 1 transcriptional units, here for the *Xyn2* and *XylA* genes, should be assembled and extracted prior to executing this protocol.

Combine the the reaction mix specified in Table 15 below.

Volume	**Component**
1 µL	*XylA* TU
1 µL	*Xyn2* TU
0,5 µL	MCXbackbone
0,5 µL	dithiothreitol (20 mM)
1 µL	ligase buffer
0,5 µL	T4 ligase
0,5 µL	BsmBI
5 µL	MQ water
Final volume: 10 µL	

Table 15: Reaction mix for the level 2 BsmBI assembly of the MCXxylanase vector.

Incubate the reaction mix in a thermocycler running the programme specified in Table 16 below.

Initial restriction	37 °C	4:00 min
— 20x —		
Restriction	37 °C	1:00 min
Ligation	16 °C	2:00 min
— 20x —		
Final restriction	37 °C	4:00 min
Inactivation	80 °C	10:00 min
Storage	12 °C	∞

Table 16: Thermocycler programme for the BsmBI assembly of the MCXxylanase vector.

Store the restriction/ligation mixture with the constructs at -20 °C.

DNS assay

The DNS assay was used to investigate the xylanolytic activity of the constructed CX2Astrain. For secreted enzymes, only the supernatants were used. The assay was performed using a 96-well plate and with triplicates of each sample and blank. Two sets of samples were prepared; supernatants added before the incubation at 50 °C incubation and supernatants added after the 50 °C incubation, to serve as references. The cell cultures used for the DNS assay in this project are described in the Methodology section. The protocol used for the DNS assay is detailed below.

1. To each well that will be used for the assay, add 150 µL xylan solution (20 g/L) dissolved in NaAc (0,1 M)

2. To all wells except those reserved for the reference supernatant samples, add 150 µL of the supernatant, or YP/buffer for blanks

3. Incubate the plate at 50 °C for 30 min (thermomixer, no shaking)

4. Place the plate on ice

5. Add the supernatants to the reference wells with only xylan + buffer

6. To a fresh 96-well plate, add 125 µL 2x DNS to the same wells used in the first plate

7. From the first plate, transfer 125 µL from each well to the second plate with the DNS, in the same configuration as the first plate, use the pipette to mix both before and after transferring the samples

8. Incubate the new plate with the DNS and samples at 80 °C for 30 min with shaking at 750 RPM

9. Remove the plate and place in a fume hood for 5–10 min to cool

10. Read the plate at 575 nm using a spectrophotometer plate reader

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