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Combining Xylose Reductase from *Spathaspora arborariae* with Xylitol Dehydrogenase from *Spathaspora passalidarum* to Promote Xylose Consumption and Fermentation into Xylitol by *Saccharomyces cerevisiae*

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Abstract: In recent years, many novel xylose-fermenting yeasts belonging to the new genus *Spathaspora* have been isolated from the gut of wood-feeding insects and/or wood-decaying substrates. We have cloned and expressed, in *Saccharomyces cerevisiae*, a *Spathaspora arborariae* xylose reductase gene (*SaXYL1*) that accepts both NADH and NADPH as co-substrates, as well as a *Spathaspora passalidarum* NADPH-dependent xylose reductase (*SpXYL1.1* gene) and the *SpXYL2.2* gene encoding for a NAD^+-dependent xylitol dehydrogenase. These enzymes were co-expressed in a *S. cerevisiae* strain over-expressing the native *XKS1* gene encoding xylulokinase, as well as being deleted in the alkaline phosphatase encoded by the *PHO13* gene. The *S. cerevisiae* strains expressing the *Spathaspora* enzymes consumed xylose, and xylitol was the major fermentation product. Higher specific growth rates, xylose consumption and xylitol volumetric productivities were obtained by the co-expression of the *SaXYL1* and *SpXYL2.2* genes, when compared with the co-expression of the NADPH-dependent *SpXYL1.1* xylose reductase. During glucose-xylose co-fermentation by the strain with co-expression of the *SaXYL1* and *SpXYL2.2* genes, both ethanol and xylitol were produced efficiently. Our results open up the possibility of using the advantageous *Saccharomyces* yeasts for xylitol production, a commodity with wide commercial applications in pharmaceuticals, nutraceuticals, food and beverage industries.

Keywords: xylose reductase; xylitol dehydrogenase; *Spathaspora*; *Saccharomyces*; xylitol; xylose

1. Introduction

Industrial biotechnology will play an increasing role in creating a more sustainable global economy. Lignocellulosic biomass, an abundant and renewable feedstock, is an attractive raw material for fuels and chemicals production, since it does not compete with food and feed production [1,2]. The major

sugars from the hydrolysis of these feedstocks (e.g., sugarcane bagasse, corn stover, grasses, etc.) are the hexose D-glucose and the pentose D-xylose. To develop an economically feasible industrial process, it is necessary to efficiently consume and metabolize both sugars [3,4], in a scenario where xylose is not as readily consumed as glucose by the *Saccharomyces* yeasts [5]. The efficient consumption of pentose sugars is, therefore, important in the overall bioconversion of plant biomass for the production of liquid fuels and chemicals. While several yeast species have been shown to be able to consume this sugar (including recombinant *S. cerevisiae* strains), the efficiency and rates of xylose utilization are slow and/or inefficient, challenging the industrial production of lignocellulosic valuable chemical compounds [6]. Therefore, there is still a need for the development of new yeasts capable of efficient xylose consumption for fuels and chemicals production.

The discovery of xylose-fermenting yeasts in the early 1980s [7,8] was considered a milestone, with numerous xylose-fermenting yeasts been reported, and some even improved, over the following years [9]. While most bacteria and some fungi convert, directly, xylose into xylulose through a xylose isomerase, in the case of the yeasts known as being capable of xylose utilization, its metabolism occurs via a xylose reductase and xylitol dehydrogenase [10], followed by a xylulokinase that channels xylulose into the pentose-phosphate pathway [5]. While the xylose reductase/xylitol dehydrogenase pathway may present unbalanced co-substrate requirements for these two enzymes, and thus xylitol accumulation and secretion, it is more thermodynamically advantageous than the xylose isomerase pathway, allowing faster xylose assimilation by engineered yeast strains [11,12]. Therefore, xylose-consuming yeast species could be used directly for fuels and chemicals production, or may be a source of genes, enzymes and/or sugar transporters to engineer industrial *S. cerevisiae* strains for improved chemicals production from renewable biomass [13,14].

Recently, a new genus of yeasts (*Spathaspora*) that consume and ferment xylose to various degrees have been isolated from the gut of wood-feeding insects and/or wood-decaying substrates, with a characteristic single elongate ascospore with curved ends [15–17]. An analysis of the activities of the two key enzymes involved in the initial steps of xylose metabolism (xylose reductase and xylitol dehydrogenase), revealed that the majority of *Spathaspora* species analyzed (a total of nine) had high xylose reductase activity, mostly NADPH-dependent, with the exception of three species: *Sp. arborariae*, *Sp. gorwiae*, and *Sp. passalidarum* [16,18,19] that clearly accepted both co-substrates (NADPH and NADH) for xylose reduction, a key factor for efficient consumption and fermentation of this sugar by yeasts [20].

The known genome of *Sp. passalidarum* [21] has two genes encoding xylose reductases, *SpXYL1.1* and *SpXYL1.2*, and recent data show that the *SpXYL1.2* gene encodes for a xylose reductase with higher activity with NADH, allowing efficient anaerobic xylose consumption and fermentation by recombinant *S. cerevisiae* strains [19,22]. Regarding xylitol dehydrogenase activity, all the *Spathaspora* yeasts analyzed showed a NAD^+-dependent activity, and, again, the *Sp. passalidarum* strains stand out with the highest xylitol dehydrogenase activity [16,19]. The genome of *Sp. passalidarum* also contains two genes encoding xylitol dehydrogenases (*SpXYL2.1* and *SpXYL2.2*), and one of them (*SpXYL1.1*) has already been cloned and expressed in *S. cerevisiae* [23]. Considering that *Spathaspora* species may constitute a suitable platform for new genes encoding enzymes involved in xylose consumption to engineer industrial *S. cerevisiae* yeasts [24], in the present work, we extended our knowledge regarding the *Spathaspora* xylose-utilizing enzymes by cloning and expressing in *S. cerevisiae* the *Sp. arborariae SaXYL1* xylose reductase that accepts both NADH and NADPH as cofactors, as well as the *Sp. passalidarum SpXYL1.1* gene encoding for a NADPH-dependent xylose reductase, and the other *SpXYL2.2* gene encoding for a highly-active xylitol dehydrogenase. Regardless of which gene for xylose reductase and xylitol dehydrogenase were combined, the main product of xylose consumption by the recombinant *S. cerevisiae* yeasts was xylitol, a polyol of significant interest in odontological, pharmaceutical and food industries [25,26].

2. Materials and Methods

2.1. Strains, Media and Growth Conditions

The yeast strains used in this study are listed in Table 1. *Escherichia coli* strain DH5α was used for cloning, and was grown in Luria broth (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) supplemented with ampicillin (100 mg/L). Yeasts were grown on rich YP medium (1% yeast extract, 2% Bacto peptone) or synthetic complete (YNB) medium (0.67% yeast nitrogen base without amino acids, supplemented with adequate auxotrophic requirements), containing 2% glucose or xylose. The pH of the medium was adjusted to pH 5.0 with HCl. When required, 2% Bacto agar, 200 mg/L geneticin sulfate (G-418, Sigma-Aldrich Brasil Ltda., São Paulo, SP, Brazil) or 0.5 g/L zeocin (Invivogen, San Diego, CA, USA) were added to the medium. Cells were grown aerobically at 28 °C with shaking (160 rpm) in cotton plugged Erlenmeyer flasks filled to 1/5 of the volume with medium. Cellular growth was followed by turbidity measurements at 600 nm ($OD_{600\ nm}$), and culture samples were harvested regularly, centrifuged (5000× *g*, 1 min), and their supernatants used for the determination of substrates and fermentation products, as described below. The maximum specific growth rate (μ_{max}, h^{-1}) was determined by the slope of a straight line between *ln* OD_{600nm} and time (h) during the initial (~48 h) exponential phase of growth on xylose.

Table 1. Yeast strains, plasmids and primers used in this study.

Strains, Plasmids and Primers	Relevant Features, Genotype or Sequence	Source
Yeast strains:		
Sp. arborariae UFMG-HM.19.1A^T	Isolated from rotting wood in Minas Gerais, Brazil	[16]
Sp. passalidarum UFMG-CM-Y474	Isolated from rotting wood in Roraima, Brazil	[27]
S. cerevisiae CEN.PK2-1C	*MAT*a *leu2–3112 ura3–52 trp1–289 his3*-Δ1 *MAL2–8*^{c} *SUC2*	[28]
S. cerevisiae ASY-1	Isogenic to CEN.PK2-1C, but *KanMX*-P_{ADH1}::*XKS1*	This work
S. cerevisiae ASY-2	Isogenic to ASY-1, but *pho13*Δ::*LoxP-Ble*^{R}*-LoxP*	″
Plasmids:		
pFA6a-kanMX6-PADH1	*KanMX6*-P_{ADH1}	[29]
pUG66	*LoxP-Ble*^{R}*-LoxP*	″
pPGK	2μ *URA3* P_{PGK1}-T_{PGK1}	[30]
pTEF-423	2μ *HIS3* P_{TEF}-T_{CYC1}	[31]
pGPD-423	2μ *HIS3* P_{GPD}-T_{CYC1}	″
pPGK-*SpXYL1.1*	2μ *URA3* P_{PGK1}-*SpXYL1.1*-T_{PGK1}	This work
pPGK-*SaXYL1*	2μ *URA3* P_{PGK1}-*SaXYL1*-T_{PGK1}	″
pGPD-*SaXYL1*	2μ *HIS3* P_{GPD}-*SaXYL1*-T_{CYC1}	″
pPGK-*SpXYL2.2*	2μ *URA3* P_{PGK1}-*SpXYL2.2*-T_{PGK1}	″
pTEF-*SpXYL2.2*	2μ *HIS3* P_{TEF}-*SpXYL2.2*-T_{CYC1}	″
Primers: [1]		
XR-F	ATGAATTCATGGCTACTATTAAATTATCCTCAGGT	This work
XR-R	TTGGATCCTTAAACAAAGATTGGAATGTTGTCC	″
XDH-*Sp*-F	ATGAATTCATGGTTGCTAATCCCTCTTTAGTG	″
XDH-*Sp*-R	CTGGATCCCTACTCTGGTCCATCAATTAAACACTT	″
Prom_PGK_54_F	ACAGATCATCAAGGAAGTAATTATC	″

Table 1. *Cont.*

Ter_PGK_65_R	TTAGCGTAAAGGATGGGGAAAGAG	″
TEF-XDH-*Sp*-F	ATAGATCTATGGTTGCTAATCCCTCTTTAGTG	″
TEF-XDH-*Sp*-R	CTAGATCTCTACTCTGGTCCATCAATTAAACACTT	″
XKS1-Kanr-F	**ATTCGGCCAATGCAATCTCAGGCGGACGAATAAG GGGGCC***CCAGCTGAAGCTTCGTACGC*	″
XKS1-PADH1-R	**AAACCTCTCTTGTCTGTCTCTGAATTACTGAACACAA** *CATTGTATATGAGATAGTTG*	″
V-XKS1-F	CAAGCGACGCAGGGAATAGCC	″
V-XKS1-R	CTTCGTTCAGTCTCTGTTGTGAGC	″
V-kan^r-F	CCGGTTGCATTCGATTCC	″
DE-PHO13-F	**CTTATAGCTTGCCCTGACAAAGAATATACAACTCG GGAAA***CCAGCTGAAGCTTCGTACGC*	″
DE-PHO13-R	**TTCAAAAAGTAATTCTACCCCTAGATTTTGCATTGC TCCT***GCATAGGCCACTAGTGGATC*	″
V-PHO13-F	GGAAGTAGATTGTTCGACGC	″
V-PHO13-R	GATACGCCGTTCGATGCAG	″
V-Ble^r-F	CCTTCTATGAAAGGTTGGGC	″

[1] Underlined sequences indicate restriction enzyme sites (*Bgl*II, *Bam*HI or *Eco*RI) used for cloning, bold sequences are homologous to the upstream and downstream region of the target genes that were modified, and italicized sequences allow amplification of the transformation modules present in plasmids pFA6a-kanMX6-PADH1 and pUG66.

For batch fermentations, cells were pre-grown in synthetic complete YNB medium containing 2% glucose for 20 h at 28 °C, the cells were collected by centrifugation at 6000× *g* for 5 min at 4 °C and washed twice with sterile water, and inoculated at a high cell density (10.0 ± 0.5 g of dry yeast/L), into 25 mL of synthetic YNB medium containing 2% xylose. Batch fermentations were performed at 30 °C in closed 50 mL bottles with a magnetic stir bar, to allow mild agitation (100 rpm). Samples were collected regularly and processed, as described above.

2.2. Molecular Biology Techniques

Standard methods for bacterial transformation, DNA manipulation and analysis were employed [32]. Yeast transformation was performed by the lithium acetate method [33]. To over-express the *XKS1* gene encoding xylulokinase in *S. cerevisiae* CEN.PK2-1C, the promoter region of this gene was modified according to the polymerase chain reaction (PCR)-based gene replacement procedure, as described previously [29]. Briefly, the *kanMX*-P_{ADH1} module from plasmid pFA6a-kanMX6-PADH1 (Table 1) was amplified with primers XKS1-Kanr-F and XKS1-PADH1-R (Table 1), and the resulting PCR product of 2394 bp (flanked by ~40 bp of homology to the promoter and start regions of the *XKS1* gene) containing the truncated and constitutive promoter of the *ADH1* gene was used to transform competent yeast cells. After 2 h cultivation on YP-2% glucose, the transformed cells were plated on the same medium containing G-418 and incubated at 28 °C. G-418-resistant isolates were tested for proper genomic integration of the *kanMX*-P_{ADH1} cassette at the *XKS1* locus by diagnostic colony PCR using 3 primers (V-XKS1-F, V-XKS1-R and V-kan^r-F; Table 1). This set of 3 primers amplified a 1557-bp fragment (primers V-XKS1-F and V-XKS1-R) from a normal *XKS1* locus, or yielded a 3871-bp fragment (primers V-XKS1-F and V-XKS1-R) and a 2889–bp fragment (primers V-kan^r-F and V-XKS1-R) if the *kanMX*-P_{ADH1} module was correctly integrated at the promoter region of the *XKS1* gene, producing strain ASY-1 (*KanMX*-P_{ADH1}::*XKS1*, Table 1).

We further improved the capacity of xylose utilization in this strain by deleting the *PHO13* gene of *S. cerevisiae*, a gene encoding for an alkaline phosphatase known to suppress xylose utilization by recombinant yeast strains [34–36]. Briefly, the *LoxP-Ble*^{R}*-LoxP* knockout cassette from plasmid

pUG66 (Table 1) was amplified with primers DE-PHO13-F and DE-PHO13-R (Table 1), and the resulting PCR product of 1265-bp (flanked by ~40 bp of homology to the upstream and downstream regions of the *PHO13* locus) containing the *Ble*^{R} gene was used to transform ASY-1 competent yeast cells. After 2-h cultivation on YP-2% glucose, the transformed cells were plated on the same medium containing zeocin and incubated at 28 °C. Zeocin-resistant isolates were tested for proper genomic integration of the *LoxP-Ble*^{R}*-LoxP* cassette at the *PHO13* locus by diagnostic colony PCR using 3 primers (V-PHO13-F, V-PHO13-R and V-Ble^{r}-F; Table 1). This set of 3 primers amplified a 1654-bp fragment (primers V-PHO13-F and V-PHO13-R) from a normal *PHO13* locus, or yielded a 1900-bp fragment (primers V-PHO13-F and V-PHO13-R) and a 965–bp fragment (primers V-Ble^{r}-F and V-PHO13-R) if the *LoxP-Ble*^{R}*-LoxP* module replaced and deleted the *PHO13* gene, producing strain ASY-2 (*KanMX*-P$_{ADH1}$::*XKS1* and *pho13*Δ::*LoxP-Ble*^{R}*-LoxP*, Table 1).

Based on the genome sequence of *Sp. arborariae* [37] and *Sp. passalidarum* [21], primers were designed (Table 1) to amplify the xylose reductase encoded by the *SaXYL1* or *SpXYL1.1* genes, and the xylitol dehydrogenase encoded by the *SpXYL2.2* gene, introducing restriction sites for cloning into multicopy shuttle vectors containing strong and constitutive promoters and terminators (pPGK, p423-TEF and p423-GPD, Table 1), as well as the *URA3* or *HIS3* genes used as selective markers. The genomic DNA from the *Sp. passalidarum* and *Sp. arborariae* strains was purified using a YeaStar Genomic DNA Kit^{TM} (Zymo Research, Irvine, CA, USA). The amplified DNA fragments, originally cloned into the pPGK plasmid, had their 5′ and 3′ ends sequenced (ACTGene Analíses Moleculares Ltda., Alvorada, RS, Brazil) using primers Prom_PGK_54_F and Ter_PGK_65_R (Table 1) to confirm the identity of the cloned genes.

2.3. Enzyme Assays

Cell-free extracts for assays of xylose metabolizing enzymes were prepared with the yeast protein extraction reagent Y-PER (Pierce, Rockford, IL, USA) after cultivation yeast cells on YP-2% xylose, or 2% glucose (for *S. cerevisiae* strains over-expressing the cloned genes). Protein concentrations in the cell-free extracts were determined with the Micro-BCA kit (Pierce, Thermo Fisher Scientific Inc., Sinapse Biotecnologia, São Paulo, SP, Brazil), using a Biowave II spectrophotometer (Biochrome WPA, Cambridge, UK). Xylose reductase activity was measured by monitoring the oxidation of NADH or NADPH at 340 nm [10,38] at 30 °C in 45.5 mM potassium phosphate buffer (pH 6.0), using 0.15 mM NADH or NADPH and 200 mM xylose as substrate. The kinetic parameters of xylose reductase for each substrate was determined using 1–400 μM NADH or NADPH, or 0.5–600 mM xylose, under the conditions described above. Xylitol dehydrogenase activity was measured by monitoring the reduction of NAD^+ or $NADP^+$ at 340 nm [10,38] at 35 °C in 50 mM Tris–HCl buffer (pH 9.0) containing 50 mM $MgCl_2$, 300 mM xylitol, and 1 mM NAD^+ or $NADP^+$. The kinetic parameters of xylitol dehydrogenase for each substrate was determined using 1–4000 μM NAD^+, or 0.1–500 mM xylitol, under the same conditions. The xylulokinase activity was determined by a coupled assay to measure ADP production as previously described [39] in 0.2 M Tris–HCl buffer (pH 7.0) containing 2.3 mM $MgCl_2$, 10 mM NaF, 2.5 mM ATP, 0.25 mM phosphoenolpyruvate, 3.5 mM reduced glutathione, 10 U of pyruvate kinase, 15 U of lactate dehydrogenase, 0.2 mM NADH, and 4.25 mM xylulose. One unit of enzyme activity was defined as the amount of enzyme that reduced or oxidized 1 μmol of $NAD(P)^+$ or NAD(P)H per minute. These enzymatic activities were determined using a Cary 60 UV-VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The kinetic parameters (K_m and V_{max}) of the cloned xylose reductases and xylitol dehydrogenase enzymes expressed in *S. cerevisiae* were determined by fitting the experimental data to the Michaelis-Menten equation, using SigmaPlot v. 11.0 (Systat Software Inc., San Jose, CA, USA).

2.4. Analytical Methods

Xylose, ethanol, xylitol, glycerol, and acetate were determined by high performance liquid chromatography (HPLC), equipped with a refractive index detector (RI-2031Plus; JASCO, Tokyo,

Japan) using an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). The HPLC apparatus was operated at 40 °C using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.1 mL/min and 0.01 mL injection volume. The following calculations were considered: products yield ($Y_{p/s}$, g/g) were determined by correlating ΔP produced (xylitol, ethanol, glycerol or acetate) with ΔS consumed (xylose or glucose) at time of maximal substrate consumption. The xylitol volumetric productivity (Q_p, g/L/h) was determined by the slope of a straight line between xylitol concentration (g/L) and time (h) during maximum xylitol production.

3. Results and Discussion

Our analysis for xylose reductase in *Sp. arborariae* UFMG-HM19.1A^T showed activity with both co-substrates (NADH and NADPH); nevertheless, the NADH-dependent activity was ~25% of that observed with NADPH. The NAD^+-dependent xylitol dehydrogenase, however, was not active in the presence of $NADP^+$ (Table 2). Similarly, the *Sp. passalidarum* strain UFMG-CM-Y474 showed xylose reductase activity with both NADH and NADPH, and the highest xylitol dehydrogenase (NAD^+-dependent) activity (~1.3 U/[mg protein], Table 2) among all the *Spathaspora* yeasts analyzed (data not shown). Based on the *Sp. passalidarum* and *Sp. arborariae* genome sequences [21,37], we designed primers to amplify the *SaXYL1* and *SpXYL1.1* genes (both open reading frames -ORFs- with 957 nucleotides, encoding for proteins of 318 amino acids), and the *SpXYL2.2* gene (an ORF with 1089 nucleotides, encoding for a protein of 362 amino acids), and cloned these three genes into the pPGK multicopy plasmid for expression in the *S. cerevisiae* CEN.PK2-1C yeast strain.

Table 2. Xylose reductase and xylitol dehydrogenase activities of selected *Spathaspora* yeasts.

	Specific Activity (U/[mg Protein])			
	Xylose Reductase		**Xylitol Dehydrogenase**	
Co-Substrate:	**NADPH**	**NADH**	**$NADP^+$**	**NAD^+**
Yeast:				
Sp. arborariae UFMG-HM.19.1A^T	0.455 ± 0.006	0.118 ± 0.002	0.001 ± 0.0	0.25 ± 0.01
Sp. passalidarum UFMG-CM-Y474	0.580 ± 0.020	0.380 ± 0.010	0.003 ± 0.0	1.29 ± 0.06

As shown in Table 3, these genes were functional in *S. cerevisiae*, with a xylose reductase activity encoded by the *SaXYL1* gene that accepts both co-substrates, with a NADH-dependent activity that is ~30% the activity measured with NADPH, while the xylose reductase encoded by the *SpXYL1.1* gene accepted only NADPH as co-substrate. The determination of the kinetic parameters of the *SaXYL1* enzyme cloned in *S. cerevisiae* (Table 4) revealed an enzyme with high-affinity for both NADH and NADPH, as described also for *Sp. passalidarum* [18], but with a maximal capacity (V_{max}) with NADH, which is ~35–40% the V_{max} determined with NADPH. Indeed, this gene is closely related to other known yeast xylose reductases that accept both co-substrates (NADH and NADPH) from *Candida tropicalis* [40], *C. parapsilosis* [41], *C. intermedia* [42], and *Scheffersomyces stipitis* [43]. It is worth noting that the predicted (but not functionally characterized) xylose reductase from *Sp. roraimanenses* (a species with strictly NADPH-dependent xylose reductase activity, see [19]) is 98% identical to the enzyme (encoded by *SaXYL1*) cloned from *Sp. arborariae*, differing in only two amino acids: an aspartate (D^{29}) in *Sp. roraimanenses* xylose reductase is substituted by a glutamate (E^{29}) in *SaXYL1*, and the amino acid arginine (R^{60}) from the *Sp. roraimanenses* enzyme is replaced by a lysine (K^{60}) in *SaXYL1*. Further work would be required to verify if these two amino acid substitutions are indeed responsible for the NADH-dependent xylose reductase activity of *SaXYL1*. The cloned *SpXYL1.1* xylose reductase, a gene with 93% identity with *SaXYL1*, is a NADPH-dependent enzyme with affinities for NADPH and xylose similar to the enzyme cloned from *Sp. arborariae* (Table 4), but with much higher maximal

capacity (V_{max} = ~4.5 U/[mg protein]) than the cloned *SaXYL1* enzyme. Thus, this enzyme is similar to other NADPH-dependent xylose reductases from *Pachysolen tannophilus* [44] and *Debaryomyces nepalensis* [45].

Table 3. Xylose reductase and xylitol dehydrogenase activities of cloned genes expressed in *S. cerevisiae* CEN.PK2-1C.

	Specific Activity (U/ [mg Protein])			
	Xylose Reductase		Xylitol Dehydrogenase	
Co-Substrate:	**NADPH**	**NADH**	**$NADP^+$**	**NAD^+**
Plasmid:				
pPGK	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
pPGK-*SaXYL1*	2.66 ± 0.02	0.81 ± 0.30	0.01 ± 0.01	0.01 ± 0.01
pPGK-*SpXYL1.1*	3.00 ± 0.17	0.05 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
pPGK-*SpXYL2.2*	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	2.20 ± 0.21

Table 4. Kinetic parameters of the xylose reductase and xylitol dehydrogenase encoded by genes *SaXYL1*, *SpXYL1.1*, and *SpXYL2.2* expressed in *S. cerevisiae* CEN.PK2-1C.

Plasmid:	**Substrate:**	**K_m**	**V_{max} (U/[mg Protein])**
pPGK-*SaXYL1*	NADH	12.8 ± 3.0 μM	1.0 ± 0.04
	NADPH	26.1 ± 11.0 μM	2.6 ± 0.4
	Xylose (NADH)	29.5 ± 13.0 mM	1.0 ± 0.1
	Xylose (NADPH)	57.5 ± 10.0 mM	2.8 ± 0.2
pPGK-*SpXYL1.1*	NADPH	65.9 ± 28.2 μM	4.7 ± 0.6
	Xylose (NADPH)	53.3 ± 6.1 mM	4.2 ± 0.1
pPGK-*SpXYL2.2*	NAD^+	0.52 ± 0.16 mM	2.4 ± 0.2
	Xylitol	86.0 ± 13.0 mM	4.3 ± 0.2

Regarding the *SpXYL2.2* gene (Table 3), it also encodes for a functional and highly active (2.2 U/[mg protein]) xylitol dehydrogenase when expressed in *S. cerevisiae*, but showing relatively low affinity (Table 4) for both substrates (NAD^+ and xylitol), when compared with *Sp. passalidarum* cells [18]. Unfortunately, we do not have any kinetic parameters of the *SpXYL2.1* enzyme cloned in *S. cerevisiae* by Mamoori and co-workers [23]. Nevertheless, the enzyme encoded by *SpXYL2.2* is similar to xylitol dehydrogenases cloned and characterized from other yeasts [46,47].

We next tested the functionality of these enzymes by their co-expression in *S. cerevisiae*. For the first approach, we expressed the *SaXYL1* or *SpXYL1.1* genes from plasmid pPGK (with *URA3* as the selectable marker) and the *SpXYL2*.2 gene in plasmid p423-TEF (with *HIS3* as the selectable marker) in the strain CEN.PK2-1C. Although the activities were expressed as expected in the yeast strain (see Tables 3–5), these cells were unable to grow or consume xylose from the medium (data not shown). To allow growth on xylose, the xylulokinase gene (*XKS1*) gene from *S. cerevisiae* was over-expressed to increase the flux of carbon into the pentose-phosphate pathway. While the CEN.PK2-1C strain showed a xylulokinase activity of 0.04 ± 0.01 U/[mg protein], the engineered P_{ADH1}::*XKS1* strain ASY-1 (Table 1) showed a ~4-fold increase in xylulokinase activity (0.15 ± 0.04 U/[mg protein]). As such, it was observed growth and xylose consumption when the *S. cerevisiae* ASY-1 strain was transformed with either the pPGK-*SpXYL1.1* and pTEF-*SpXYL2.2*, or the pPGK-*SaXYL1* and pTEF-*SpXYL2.2* plasmids (Figure 1).

Table 5. Xylose reductase and xylitol dehydrogenase activities co-expressed in *S. cerevisiae*.

		Specific Activity (U/[mg Protein])			
		Xylose Reductase		Xylitol Dehydrogenase	
	Co-Substrate:	**NADPH**	**NADH**	**$NADP^+$**	**NAD^+**
Strain:	Plasmids:				
ASY-1	pPGK-*SpXYL1.1* pTEF-*SpXYL2.2*	3.05 ± 0.17	0,05 ± 0.01	0.01 ± 0.01	0.40 ± 0.10
ASY-1	pPGK-*SaXYL1* pTEF-*SpXYL2.2*	2.41 ± 0.25	0.89 ± 0.23	0.01 ± 0.01	0.48 ± 0.03
ASY-2	pPGK-*SaXYL1* pTEF-*SpXYL2.2*	2.37 ± 0.22	0.81 ± 0.22	0.01 ± 0.01	0.48 ± 0.07
ASY-2	pGPD-*SaXYL1* pPGK-*SpXYL2.2*	5.12 ± 1.34	2.11 ± 0.66	0.01 ± 0.01	2.95 ± 0.34

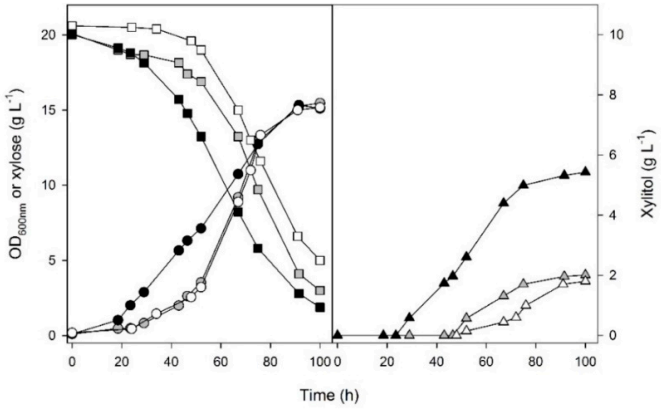

Figure 1. Time course of aerobic cell growth (OD_{600nm}, circles), xylose consumption (squares), and xylitol production (triangles) in synthetic complete (YNB) medium containing 2% xylose by yeast cells of strain ASY-1 transformed with plasmids pPGK-*SpXYL1.1* and pTEF-*SpXYL2.2* (white symbols), or pPGK-*SaXYL1* and pTEF-*SpXYL2.2* (gray symbols), or strain ASY-2 (black symbols) transformed with the same two plasmids (pPGK-*SaXYL1* and pTEF-*SpXYL2.2*).

Figure 1 and Table 6 shows that the ASY-1 strain over expressing the *SaXYL1* xylose reductase that accepts both co-substrates (NADH and NADPH) consumes more xylose that the same strain over-expressing the NADPH-dependent *SpXYL1.1* xylose reductase, although the differences are not statistically significant. For both strains the only product of xylose consumption (besides biomass) was ~2 g/L xylitol, and, while the final yields were similar for both strains, the volumetric xylitol productivity by the strain over-expressing the *SaXYL1* xylose reductase was higher than the strain over-expressing the *SpXYL1.1* enzyme (Table 6).

In order to improve xylose consumption, we deleted in the ASY-1 strain the *PHO13* gene of *S. cerevisiae*, a gene encoding for an alkaline phosphatase known to suppress xylose utilization by recombinant *S. cerevisiae* strains [34]. Indeed, it has been recently reported that *PHO13* deletion leads to up-regulation of the pentose phosphate genes, including *TAL1* (encoding transaldolase) and *TKL1* (encoding transketolase), an up-regulation mediated by the transcription factor *STB5*, enhancing xylose consumption by recombinant *S. cerevisiae* cells [35,36]. As can be seen in Figure 1, the P_{ADH1}:*XKS1* plus *pho13*Δ strain ASY-2 transformed with the same pPGK-*SaXYL1* and pTEF-*SpXYL2.2* plasmids, which showed improved xylose consumption and significantly higher growth rates on this carbon source, increased the production of xylitol to ~5.5 g/L, and, consequently, significantly higher volumetric productivities (Table 6).

Table 6. Xylose consumption and product formation by recombinant *S. cerevisiae* cells.

Strains and Plasmids:	Xylose Consumption (%) [a]	μ_{max} (h^{-1})	$Y_{p/s}^{ethanol}$ (g/g)	$Y_{p/s}^{glycerol}$ (g/g)	$Y_{p/s}^{acetate}$ (g/g)	$Y_{p/s}^{xylitol}$ (g/g)	$Q_p^{xylitol}$ (g/L/h)
Aerobic growth:							
ASY-1 pPGK-*SpXYL1.1* pTEF-*SpXYL2.2*	75.7 ± 4.1	0.070 ± 0.005	0	0	0	0.135 ± 0.025	0.040 ± 0.008
ASY-1 pPGK-*SaXYL1* pTEF-*SpXYL2.2*	79.9 ± 4.9	0.072 ± 0.004	0	0	0	0.152 ± 0.035	0.058 ± 0.001
ASY-2 pPGK-*SaXYL1* pTEF-*SpXYL2.2*	85.5 ± 4.6	0.139 ± 0.015 *	0	0	0	0.230 ± 0.068	0.087 ± 0.017 #
Batch fermentation:							
ASY-2 pPGK-*SaXYL1* pTEF-*SpXYL2.2*	87.5 ± 1.6	NA [b]	0.130 ± 0.018	0.068 ± 0.036	0.013 ± 0.009	0.614 ± 0.022	0.513 ± 0.033
ASY-2 pGPD-*SaXYL1* pPGK-*SpXYL2.2*	85.4 ± 4.3	NA	0.123 ± 0.003	0.089 ± 0.031	0	0.572 ± 0.041	0.370 ± 0.095

Values are the average of two biological duplicates of aerobic growth on 2% xylose or batch fermentation of 2% xylose by the indicated strains. The errors indicated are the standard error of the mean. [a] Percentage of xylose consumed after 100 h of aerobic growth, or after 50 h of batch fermentation. [b] Not applicable. * Significantly different ($p < 0.05$) when compared to the two other specific growth rates. # Significantly different ($p < 0.05$) when compared with the results obtained with the *SpXYL1.1* + *SpXYL2.2* genes (one-way ANOVA).

A recent publication [48] also overexpressed the *Sp. passalidarum* xylose metabolizing enzymes, as well as a *Piromyces* sp. xylose isomerase, in *Aureobasidium pullulans*, in order to improve xylose utilization by this yeast-like fungus. The best xylose consumption, and product production (pullulan and melanin), were obtained when the *SpXYL1.2* and *SpXYL2.2* genes were introduced into *A. pullulans*, a direct consequence of the higher activities achieved with these xylose reductase and xylitol dehydrogenases enzymes from *Sp. passalidarum* [48].

Figure 2 shows the xylose fermentation kinetics during batch fermentations with high cell densities by the ASY-2 strain transformed with the pPGK-*SaXYL1* and pTEF-*SpXYL2.2* plasmids. Xylitol (~10 g/L) was the main product of xylose fermentation, but some ethanol (<2 g/L), glycerol (<0.5 g/L), and acetate (<0.3 g/L) were also produced (Figure 2 and Table 6). Due to this acetate production, the pH of the medium dropped from an initial pH of 5.0 into pH ~3.5. Considering that xylose was not completely consumed during the batch fermentation, in a further attempt to increase the consumption of xylose, we changed the plasmids/promoters of the two cloned genes to improve their activities. When the ASY-2 strain was transformed with the pGPD-*SaXYL1* and pPGK-*SpXYL2.2* plasmids, a 2-fold higher xylose reductase activity and a 6-fold higher xylitol dehydrogenase activity were obtained (Table 5). However, xylose consumption was not improved in this new recombinant strain. Although the recombinant strain produced no acetate (and consequently the pH of the medium dropped just to pH 4.5) and less xylitol and ethanol, the production of glycerol was only slightly increased (Figure 2, Table 6), indicating that other factors may limit xylose utilization by our engineered yeast strains. For example, the bottleneck may be a consequence of the relatively low affinity of the cloned *SpXYL2.2* xylitol dehydrogenase for both NAD^+ and xylitol (Table 4), or the intracellular pools of reduced or oxidized and NADH/NADPH ratio of co-substrates [49,50], or even the low affinity of yeast sugar permeases for xylose transport [5,14,51]. Nevertheless, the ASY-2 strain transformed with the pPGK-*SaXYL1* and pTEF-*SpXYL2.2* plasmids (Table 6) showed xylitol yields ($Y_{p/s}$ = 0.614 g xylitol/g xylose) and volumetric productivities (Q_p = 0.513 g/L/h) as good as or superior to those reported by other naturally xylose fermenting yeasts [52–55] or even engineered *S. cerevisiae* strains [38,49,50]. It is important to note that the maximal expected theoretical yield for the biotransformation of xylose into xylitol is 0.905–0.917 g xylitol/g xylose consumed, depending on how the cells will regenerate the

NADH/NADPH consumed in the reduction of xylose, and that no carbon is used for cell growth or production of other metabolites [56].

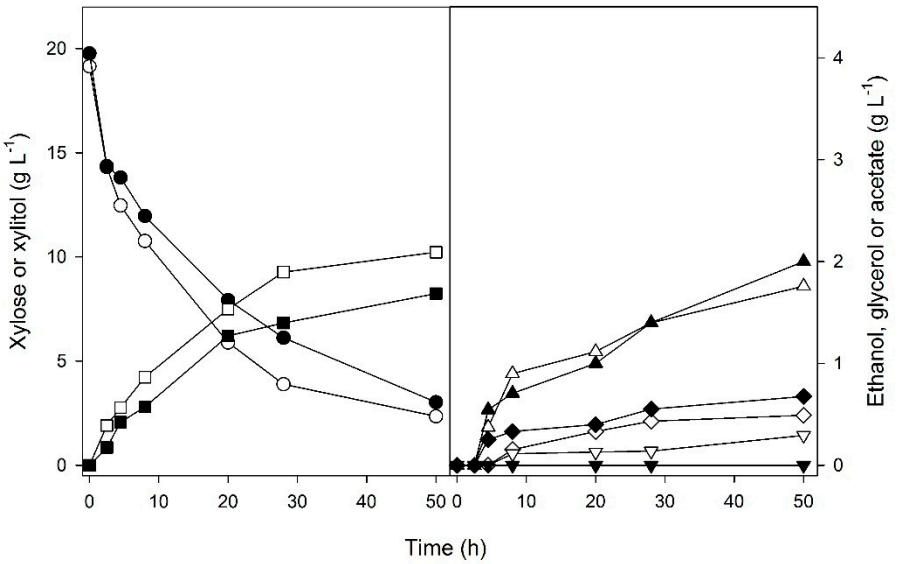

Figure 2. Time course of xylose consumption (circles), and xylitol (squares), ethanol (triangles), glycerol (diamonds), or acetate (inverted triangles) production during 2% xylose batch fermentations in YNB medium by yeast cells of strain ASY-2 transformed with plasmids pPGK-*SaXYL1* and pTEF-*SpXYL2.2* (white symbols), or plasmids pGPD-*SaXYL1* and pPGK-*SpXYL2.2* (black symbols).

Finally, Figure 3 shows the fermentation kinetics during batch co-fermentations of 2% glucose plus 2% xylose with high cell densities by the ASY-2 strain transformed with the pPGK-*SaXYL1* and pTEF-*SpXYL2.2* plasmids. As expected for *S. cerevisiae*, glucose is rapidly consumed in the first 5 h of incubation, while xylose consumption was significantly slower and only 66.45 ± 3.19% of the pentose was consumed after 50 h of incubation. Ethanol (~9.7 g/L, $Y_{p/s}^{ethanol}$ = 0.460 ± 0.001 g ethanol/g glucose), glycerol (~2 g/L, $Y_{p/s}^{glycerol}$ = 0.094 ± 0.001 g glycerol/g glucose), and a small amount of acetate (<0.5 g/L, $Y_{p/s}^{acetate}$ = 0.021 ± 0.001 g acetate/g glucose) were produced during glucose consumption, although we cannot rule out the possibility that some of these products could come from the ~3.6 g/L of xylose assimilated during this period of glucose consumption. From xylose consumption, ~8.5 g/L of xylitol ($Y_{p/s}^{xylitol}$ = 0.614 ± 0.030 g xylitol/g xylose) and ~0.8 g/L of acetate ($Y_{p/s}^{acetate}$ = 0.061 ± 0.012 g acetate/g xylose) were produced (Figure 3). While the xylitol yield was similar to the one obtained during batch fermentation of 2% xylose (see Table 6), the acetate produced during the co-fermentation of glucose and xylose also promoted a drop of the pH of the medium to pH ~3.5. Furthermore, we also observed a drop in the volumetric xylitol productivity (Q_p = 0.238 ± 0.025 g/L/h) during glucose-xylose co-fermentation, when compared with fermentation of just xylose by these cells (see Table 6). From a biorefinery perspective, higher xylose concentrations (>100 g/L) should be tested, in order to obtain higher xylitol titers (>60 g/L), as these fermented broths containing xylitol need to be clarify, concentrated (>750 g/L), and cooled in order to favor its crystallization, all this downstream processing contributing significantly to the overall costs of the product [57,58]. Thus, further research should improve the fermentative production of this interesting sugar alcohol with wide commercial applications in pharmaceuticals, nutraceuticals, food and beverage industries.

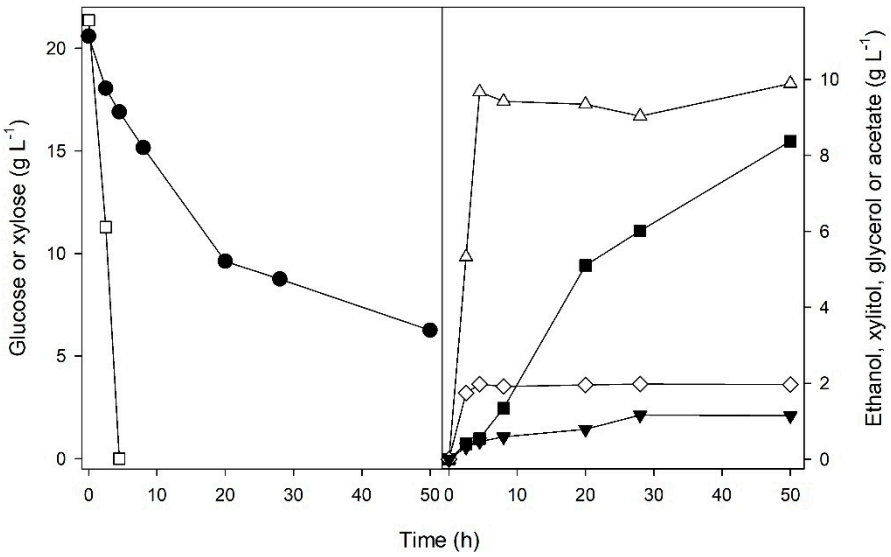

Figure 3. Time course of xylose (black circles) and glucose (white squares) consumption, and xylitol (black squares), ethanol (white triangles), glycerol (white diamonds), or acetate (inverted black triangles) production during 2% xylose plus 2% glucose batch co-fermentations in YNB medium by yeast cells of strain ASY-2 transformed with plasmids pPGK-*SaXYL1* and pTEF-*SpXYL2.2*.

4. Conclusions

In the present work, we cloned and expressed, in *S. cerevisiae*, the *Sp. arborariae* xylose reductase (*SaXYL1*) gene that accepts both NADH and NADPH as cofactors, as well as a *Sp. passalidarum* NADPH-dependent xylose reductase (*SpXYL1.1* gene), and the *SpXYL2.2* gene encoding a NAD^+-dependent xylitol dehydrogenase. The co-expression of both the *SaXYL1* and *SpXYL2.2* enzymes in a P_{ADH1}:*XKS1*/*pho13*Δ *S. cerevisiae* strain allowed efficient growth and xylose consumption by the cells, producing xylitol as the major fermentation product.

Author Contributions: Conceptualization, C.F. and B.U.S.; investigation, methodology, and formal analysis, A.M., A.A.d.S., D.D.A., G.F.G.; resources, C.A.R., C.F. and B.U.S.; writing—review and editing, C.F. and B.U.S.; project administration and funding acquisition, E.P.S.B., C.F. and B.U.S. All authors have read and agreed to the published version of the manuscript.

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