



# Fermentation of D-xylose to Ethanol by *Saccharomyces cerevisiae* CAT-1 Recombinant Strains

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## Abstract

Ethanol production by the D-xylose fermentation of lignocellulosic biomass would augment environmental sustainability by increasing the yield of biofuel obtained per cultivated area. A set of recombinant strains derived from the industrial strain *Saccharomyces cerevisiae* CAT-1 was developed for this purpose. First, two recombinant strains were obtained by the chromosomal insertion of genes involved in the assimilation and transport of D-xylose (*Gal2-N376F*). Strain CAT-1-XRT was developed with heterologous genes for D-xylose metabolism from the oxo-reductive pathway of *Scheffersomyces stipitis* (*XYL1-K270R*, *XYL2*); and strain CAT-1-XIT, with D-xylose isomerase (*xylA* gene, XI) from *Streptomyces coelicolor*. Moreover, both recombinant strains contained extra copies of homologous genes for xylulose kinase (XK) and transaldolase (TAL1). Furthermore, plasmid (pRS42K::XI) was constructed with *xylA* from *Piromyces* sp. transferred to CAT-1, CAT-1-XRT, and CAT-1-XIT, followed by an evolution protocol. After 10 subcultures, CAT-1-XIT (pRS42K::XI) consumed 74% of D-xylose, producing 12.6 g/L ethanol (0.31 g ethanol/g D-xylose). The results of this study show that CAT-1-XIT (pRS42K::XI) is a promising recombinant strain for the efficient utilization of D-xylose to produce ethanol from lignocellulosic materials.

**Keywords** D-xylose isomerase · Bioethanol · *Saccharomyces cerevisiae* · Lignocellulosic biomass

Lucía Coimbra and Karen Malan contributed equally to the study.

## Highlights

- CAT-1-XRT strain, with *TAL1/Gal2-N376F* and *XYL1-K270R/XYL2* from *Scheffersomyces stipitis* and *XKS1* in both *GRE3* alleles, was not able to grow on D-xylose.
- CAT-1-XIT strain, with *TAL1/Gal2-N376F* genes, and *xylA* from *Streptomyces coelicolor* and *XKS1* in both *GRE3* alleles, was not able to grow on D-xylose.
- Evolved CAT-1-XRT and CAT-1-XIT with plasmid pRS42K::XI with *xylA* gene from *Piromyces* sp. had dissimilar growth on D-xylose.
- Evolved CAT-1-XIT (pRS42K::XI) showed the best performance to ferment D-xylose to ethanol.

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## Introduction

Biofuel production has been developed with increased intensity in recent decades. This is associated with the uncertainty of the future of petroleum reserves and oil prices and the need to reduce greenhouse gas emissions [1]. Biofuels are generally obtained from biomass, that is, from living organisms or their metabolic wastes. The use of lignocellulosic biomass for biofuel production emerged from the need to increase yield per area dedicated to cultivation, thereby reducing potential competition with food crops for food purposes [2] and displacement of natural habitats. According to the International Energy Agency (IEA) report, the world production of biofuel was estimated at a record of 162 billion liters in 2019 (7% annual increase) [3]. The COVID-19 pandemic altered all scenarios, including biofuel production, which was reduced by 13% worldwide, reaching 2017 levels. However, the biofuel demand was recovered in 2021, returning to near 2019 level, with an expected growth of 5% in 2022 and 3% in 2023 [4]. The IEA report proposes the need to take political decisions aimed at promoting renewable and

clean energy and its efficient use once post-crisis recovery processes are addressed [3].

Lignocellulosic biomass consists of three main structural components: cellulose, hemicellulose, and lignin. Cellulose, composed exclusively of glucose monomers, and heteropolymer hemicellulose are the two fractions that contain potentially fermentable sugars. The conversion of lignocellulosic biomass into alcohol requires breaking the recalcitrant matrix of biomass and recovering the sugars through a pretreatment, which generally involves physico-chemical procedures. During the pretreatment process, hemicellulose is usually hydrolyzed and solubilized in the liquid fraction, where D-xylose is a component. Cellulose and part of the remaining lignin are recovered in the solid fraction. During pretreatment, inhibitory compounds can be generated, such as furfural, phenols, 5-hydroxymethylfurfural, and acetic acid, which hinder the stages of enzymatic cellulose hydrolysis and microbial fermentation [5, 6].

At the industrial level, bioethanol is generally produced by the fermentation of 6-carbon sugars, using selected strains of *Saccharomyces cerevisiae* [7]. Industrial strains have several advantages, including good ethanol production, tolerance to high fermentation product concentrations, and resistance to osmotic stress from intracellular glycerol accumulation [8]. However, pentoses are not metabolized by the native strains of *S. cerevisiae* [9].

Multiple strategies have been developed to take advantage of all fermentable sugars contained in lignocellulosic biomass, including bio-prospecting new organisms able to consume hexoses and pentoses and the use of genetic engineering techniques with some strains of *S. cerevisiae* [9–11]. Most recombinant strains of *S. cerevisiae* that can ferment D-xylose involve the incorporation of homologous and heterologous genes. Some studies include the expressions of genes that code for permeases with high affinity for pentoses, and have good transport activity even in the presence of glucose [12, 13].

Regarding the expressions of metabolic pathways to assimilate D-xylose in yeasts, studies have focused on two main pathways that involve a common intermediate, xylulose. In yeasts, filamentous fungi, and other eukaryotes [14] that can grow using pentoses as carbon source, D-xylose is first reduced by D-xylose reductase (XR) to xylitol [15], which in turn is oxidized to xylulose by xylitol dehydrogenase (XDH). This route is known as the oxo-reductive pathway. In bacteria and some anaerobic fungi, D-xylose isomerase (XI) is responsible for the direct conversion of D-xylose to xylulose. Later, in both pathways, xylulose is then phosphorylated to xylulose-5 phosphate by xylulokinase (XK) [15]. A large proportion of these studies have focused on the heterologous expressions of genes encoding the oxo-reductive pathway (*XYL1/XYL2*) from *Scheffersomyces stipitis* [16] and the XI pathway (*xylA* gene) from *Piromyces* sp. [17].

In addition, overexpressions of xylulose kinase (XK) and some enzymes of the pentose phosphate pathway (PPP), such as transaldolase (TAL1), transketolase (TKL1), L-ribulose-5-phosphate 4-epimerase (RPE1), and ribose-5-phosphate isomerase (RKI1) of *S. cerevisiae*, have improved ethanol production [18].

The aim of this study was to obtain a recombinant strain from the industrial strain of *S. cerevisiae* CAT-1 [19, 20], commercialized by Fermentec and widely used in South America for ethanol production, that can ferment D-xylose and glucose. The strategy takes advantage of some already known characteristics of CAT-1, an industrial strain well adapted to withstand the adverse conditions generated during the fermentation process. Several industrial strains, including CAT-1, have been the subject of study during the recent years. These strains have been evaluated in terms of both the expressions of the different pathways involved in the metabolism of D-xylose [21–23] and the behavior of these recombinant strains cultured in the presence of either a mixture of D-xylose and glucose or hemicellulose hydrolysate [21, 24]. In other works, the expressions of enzymes involved in the hydrolysis of hemicellulose fractions together with the pentose catabolism genes were also analyzed [22]. In general, the expressions of D-xylose metabolism genes have been cloned in 2 $\mu$  plasmids to evaluate the separate or simultaneous expressions of the *xylA* (XI) gene of the anaerobic bacterium *Clostridium phytofermentans* and the *XYL1-N272D/XYL2* genes of *S. stipitis*.

The approach of this work involved the use of classical strategies for yeast genetic engineering and heterologous gene expression, some of which were successfully used in previous studies. The strain was initially modified by the chromosomal insertion of genes to separately express the pathways of the D-xylose catabolism previously mentioned: the oxo-reductive pathway from *S. stipitis* and D-xylose isomerase from *Streptomyces coelicolor*. In addition, a high copy number plasmid (pRS42K::XI) with the *xylA* (XI) gene from *Piromyces* sp. was also transferred to CAT-1, CAT-1-XRT, and CAT-1-XIT and then D-xylose consumption and ethanol production were evaluated in all the strains.

## Materials and Methods

### Strains, Plasmids, and Growth Conditions

The yeast and bacterial strains used in this work are summarized in Table 1. The recombinant DNA procedures performed, including the construction of plasmids, involved the use of *Escherichia coli* TOP10 (Invitrogen, USA) as host. TOP10 was cultured at 37 °C in Luria–Bertani medium (diluted 1/2) [25]. When needed, 50  $\mu$ g/mL kanamycin (Km), 50  $\mu$ g/mL ampicillin (Amp), and 20  $\mu$ g/mL X-gal were added to the medium.

**Table 1** Bacterial strains, yeast clones and plasmids used

Strains	Characteristics	Reference
<i>Escherichia coli</i> TOP10	<i>mcrAΔ</i> ( <i>mrr-hdRMS-mcrBC</i> ) $\Phi$ 80 <i>lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL</i> (Str <sup>R</sup> ) <i>endA1 nupG</i>	Invitrogen, USA
<i>Saccharomyces cerevisiae</i>		
CAT-1	Diploid strain used for industrial production of bioethanol	[20]
CAT-1-XRT( <i>kanMX hphMX</i> )	Recombinant strain derived from CAT-1 with <i>XYL1-K270R/XYL2/XKS1/loxP-kanMX-loxP</i> and <i>Gal2-N276F/TAL1/loxP-hphMX-loxP</i> in both <i>GRE3</i> alleles	This study
CAT-1-XIT( <i>kanMX hphMX</i> )	Recombinant strain derived from CAT-1 with <i>xylA/XKS1/loxP-kanMX-loxP</i> and <i>Gal2-N276F/TAL1/loxP-hphMX-loxP</i> in both <i>GRE3</i> alleles	This study
CAT-1-XRT	Recombinant strain derived from CAT-1-XRT ( <i>kanMX hphMX</i> ) without antibiotic resistance genes, obtained after a evolution protocol (10 subcultures) (evolution A)	This study
CAT-1-XIT	Recombinant strain derived from CAT-1-XIT ( <i>kanMX hphMX</i> ) without antibiotic resistance genes, following a evolution protocol (10 subcultures) (evolution A)	This study
CAT-1-XRT(pRS42K::XI)	Recombinant strain derived from CAT-1-XRT with pRS42K::XI G418 <sup>R</sup>	This study
CAT-1-XIT(pRS42K::XI)	Recombinant strain derived from CAT-1-XIT with pRS42K::XI G418 <sup>R</sup>	This study
CAT-1 (pRS42K::XI)	Recombinant strain derived from CAT-1 with pRS42K::XI G418 <sup>R</sup>	This study
Plasmid Characteristics Reference		
pCR2.1	Lineal vector with 3'-T overhangs linked to a topoisomerase, for TOPO® TA cloning of PCR products. Amp <sup>R</sup> y Km <sup>R</sup>	TOPO-TA cloning kit. Invitrogen, USA
pBluescript II SK (±) (pSKII)	Cloning vector (phagemid)	[26]
pSKII_MCS1	Cloning vector derived from pBluescript II SK, with MCS including <i>KpnI NdeI PacI AscI MfeI NotI</i> sites. Amp <sup>R</sup>	This study
pSKII_MCS2	Cloning vector derived from pBluescript II SK, with MCS including <i>KpnI NdeI, PacI, AscI, MfeI</i> sites. Amp <sup>R</sup>	This study
pUC57	Cloning vector. Amp <sup>R</sup>	GenScript Corporation
pUG6	Vector with <i>loxP-pAgTEF1-kanMX-tAgTEF1-loxP</i> . Amp <sup>R</sup> , G418 <sup>R</sup>	[27]
pUG75	Vector with <i>loxP-pAgTEF1-hphMX-tAgTEF1-loxP</i> . Amp <sup>R</sup> , G418 <sup>R</sup>	[28]
pSH66	Shuttle vector, with <i>pGAL1-cre</i> Amp <sup>R</sup> , CloNAT <sup>R</sup>	[28]
pRS42K	Shuttle vector (2μ ori, high copy number in yeasts) Amp <sup>R</sup> , G418 <sup>R</sup>	[29]
pRS42K::XI	Vector pRS42K with <i>xylA</i> gene from <i>Piromyces</i> sp. with TPI1 promoter cloned in <i>EcoRV</i> site	This study

*S. cerevisiae* CAT-1 and recombinant yeast strains were cultured at 30 °C in yeast peptone (YP) medium (10 g/L yeast extract and 20 g/L bacteriological peptone), YP (1/5) medium (2 g/L yeast extract and 4 g/L bacteriological peptone), or MM medium (minimal medium; 1 g/L yeast extract, 1 g/L malt extract, 0.4 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 5 g/L KH<sub>2</sub>PO<sub>4</sub>). Carbon source concentrations (glucose or D-xylose) are indicated in each case. When necessary, 200 μg/mL geneticin (G418), 300 μg/mL hygromycin, or 100 μg/mL clonNAT (nourseothricin) was added to the medium. Solid media were prepared including 18 g/L agar.

### Genetic and Recombinant DNA Techniques

Primers are shown in Table 2 and were purchased from Macrogen (Seoul, South Korea). PCR reactions were performed

using Mango Mix (Bioline, UK), in accordance with standard procedures. The genomic DNA of *S. cerevisiae* CAT-1 was used as template for *XKS1*, *TAL1*, or *GRE3* amplification. The *kanMX* gene flanked by *loxP* sequences was amplified using pUG6 as template (Table 1). PCR products designed to be cloned in blocks 1, 2, or 3 were obtained by incorporating restriction sites at both ends. These amplicons were cloned in pCR®2.1-TOPO using the protocol included in the TOPO-TA Cloning Kit (Invitrogen, USA). In the case of *loxP-hphMX-loxP*, the fragment was obtained by the digestion of pUG75 with *NotI* (Table 1). Selected inserts were then subcloned in the corresponding sites, as indicated in each case. Reactions with restriction enzymes and T4 ligase were performed in accordance with the manufacturer's instructions (Thermo Scientific, USA). Ligation reaction products were used to transform chemically competent *E. coli* TOP10 cells [25]. The plasmid purification protocol

**Table 2** Oligonucleotides used for the constructions and verification of the constructions

Name	Sequence	Temp. hybrid (°C)	Size (bp)
Xylulose quinase <i>XKS1</i> ( <i>S. cerevisiae</i> CAT-1 as template) used for construction of block 1			
XKPvuIFor	5'CGATCGACTTTAATGTTGTGTTTCAGTAA3'	60	1.837
XKBamHIRRev	5'GGATCCAGGGCATGATAAATTATTCAAACA3'	60	
loxP-pAgTEF1- <i>kanMX</i> -tAgTEF1-loxP (pUG6 as template) used for construction of block 1			
loxkmloxAflIIFor	CTTAAGAGCGGGTGTGGCGGGTGTGTC	65	1.925
loxkmloxAflIIRev	CTTAAGCAGCGAGTCAGTGAGCGAGGAAGC	65	
Xylulose kinase <i>XKS1</i> ( <i>S. cerevisiae</i> CAT-1 as template) used for construction of block 3			
XKEcoRIFor	5'GAATTCACCTTTAATGTTGTGTTTCAGTAA3'	56	1.837
XKBamHIRRev	5'GGATCCAGGGCATGATAAATTATTCAAACA3'	56	
loxP-pAgTEF1- <i>kanMX</i> -tAgTEF1-loxP (pUG6 template) used for construction of block 3			
loxkmloxAflIIFor	5'CTTAAGAGCGGGTGTGGCGGGTGTGTC3'	65	1.925
loxkmloxAflIIRev	5'CTTAAGCAGCGAGTCAGTGAGCGAGGAAGC3'	65	
<i>TAL1</i> ( <i>S. cerevisiae</i> CAT-1 as template) used for construction of block 2			
TAL1SacIFor	5'GAGCTCATTACTTCTCGAACTCGTCACATA3'	60	1.065
TAL1SalIRev	5'GTCGACTAATATTTCCGAGATACTTCCTAA3'	60	
loxP-pAgTEF1- <i>hphMX</i> -tAgTEF1-loxP (pUG75 as template) used for construction of block 2			
LoxhygroforKpnI	5'GGTACCACGCGGCTACAATTAAT3'	66	1.989
LoxhygrorevKpnI	5'GGTACCAGGGAGACCGGCAGATC3'	66	
Oligonucleotides used to verify the insertion of block 3 in the chromosome of CAT-1			
VerifXIcromFor	5'CGTATGTGAATGCTGGT3'	45	1.350
VerifXIcromRev	5'ACTGAAGGGGGAAGGTG3'	45	
Oligonucleotides used to verify the presence of <i>GRE3</i> in the chromosome of CAT-1			
GRE3 For	5'TTACTACTTCTAGGGGG3'	45	1.370
GRE3 Rev	5'TTAATTTCAATTACCG3'	45	

according to Ausubel and coworkers [25] was performed. Yeast DNA was purified using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA). DNA fragments were purified from agarose gels using the PureLink Rapid Gel Extraction Kit (Invitrogen, USA), in accordance with the manufacturer's instructions. DNA was sequenced at MacroGen (Seoul, South Korea) and DNA sequence analysis was performed using blastn and blastp programs and the GenBank databank (<http://www.ncbi.nlm.nih.gov>).

### Construction of Vectors Carrying Specific Multicloning Site

Two vectors with a specific multicloning site (MCS) were constructed from pBluescript II SK ( $\pm$ ) (Table 1), which

contains infrequent restriction sites. These modifications appeared not to alter the *lacZ* gene expression, as it was possible to visualize  $\beta$ -galactosidase activity when X-gal was incorporated as substrate in the medium.

Different oligonucleotides with partially complementary sequences were synthesized (Table 3). The oligonucleotides were suspended in a 10 mM Tris buffer at pH 8.0, 50 mM NaCl, and 1 mM EDTA and mixed at equimolar concentrations. The mixture was incubated in a thermoblock for 5 min at 95 °C. Then, the tubes were allowed to cool slowly until reaching room temperature to promote the hybridization of complementary strands. Table 3 shows the sequences of the oligonucleotides used and the restriction sites incorporated.

Independently, pBluescript II SK was digested with *KpnI* and *NotI*. The DNA fragment corresponding to the vector

**Table 3** Construction of vectors from pBluescript II SK

Name of MCS	Sequence	Restriction sites of MCS
MCS1	5'CCATATGTTAATTAAGGCGCGCCCAATTGGC3' 3'CATGGGTATACAATTAATTCGCGCGGGTTAACC GCCG5'	<i>KpnI</i> , <i>NdeI</i> , <i>PacI</i> , <i>AscI</i> , <i>MfeI</i> , <i>NotI</i>
MCS2	5'CCATATGTTAATTAAGGCGCGCCCAATTGTAGCT3' 5'ACAATTGGGCGCGCCTTAATTAACATATGGGTAC3'	<i>KpnI</i> , <i>NdeI</i> , <i>PacI</i> , <i>AscI</i> , <i>MfeI</i>

digested with both enzymes was purified and ligated to the DNA fragment formed by the oligonucleotides designed to create MCS1 (Table 3). The new vector was named pSKII\_MCS1.

On the other hand, pBluescript II SK was digested with *KpnI* and *SacI*. Then, the digested vector was purified and ligated with the DNA fragment formed with the oligonucleotides designed for MCS2. The new vector was named pSKII\_MCS2.

### Construction of DNA Blocks for Chromosomal Integration

Three gene blocks were independently synthesized using GeneCust (Custom Services for Research, Luxembourg). Heterologous genes were optimized for codon usage in *S. cerevisiae* and in some cases, sequences included modifications to substitute an specific amino acid in the protein, such as *Gal2-N276F* [13] or *XYL1-K270R* [30]. Each block, cloned in pUC57 (Table 1), was designed including at both ends an infrequent restriction site (*AscI*) and 100 nucleotides identical to specific regions of the *GRE3* gene. These blocks were then subcloned in pSK\_MCS1 or pSK\_MCS2 (Table 1). This allowed the subcloning of additional PCR fragments such as the *XKS1*, *TAL1*, *kanMX*, and *hphMX* genes in selected restriction sites (Fig. 1).

As shown in Fig. 1A, block 1 contains *XYL1-K270R* [30] and *XYL2* ORFs from *S. stipitis*, flanked by *cycC* and *ADH1* promoters and *ADH1* and *TEF1* terminators, respectively. Following this region, the *GAP* promoter and *CYC1* terminator are flanking the *PvuI* and *BamHI* sites, which were included for cloning *XKS1* ORF. Then, the block also has a *Cfr9I* (*XmaI*) site, designed to clone *loxP-kanMX-loxP* amplicon. *GRE3* sequences, incorporated for fragment integration, correspond to sites at both ends of the gene, promoting the deletion of most of this allele. This procedure assures the integration of the following block, which carries internal sequences of *GRE3*, and should recognize the remaining allele. In summary, block 1 isolated from pUC57 was first subcloned in the *AscI* site of pSKII\_MCS2, and then *XKS1* and *loxP-kanMX-loxP* amplicons were inserted in their corresponding sites. Block 1 was then obtained, by digesting pSKII\_MCS2::block 1.2 with *AscI*.

As shown in Fig. 1B, block 2 contains *Gal2-N376F* flanked by the *CYC1* promoter and *ADH1* terminator. This region is followed by the *ADH1* promoter and terminator, flanking *SacI* and *SalI* sites, designed to clone a copy of the *TAL1* gene. Then, the block contains a *NotI* site, which was included to clone the fragment *loxP-hphMX-loxP* from pUG75 (Table 1). Then, block 2 was subcloned in the *AscI* site of pSKII\_MCS2, followed by the insertion of *TAL1* and *loxP-hphMX-loxP* amplicons. Block 2 was obtained by the digestion with *AscI*.

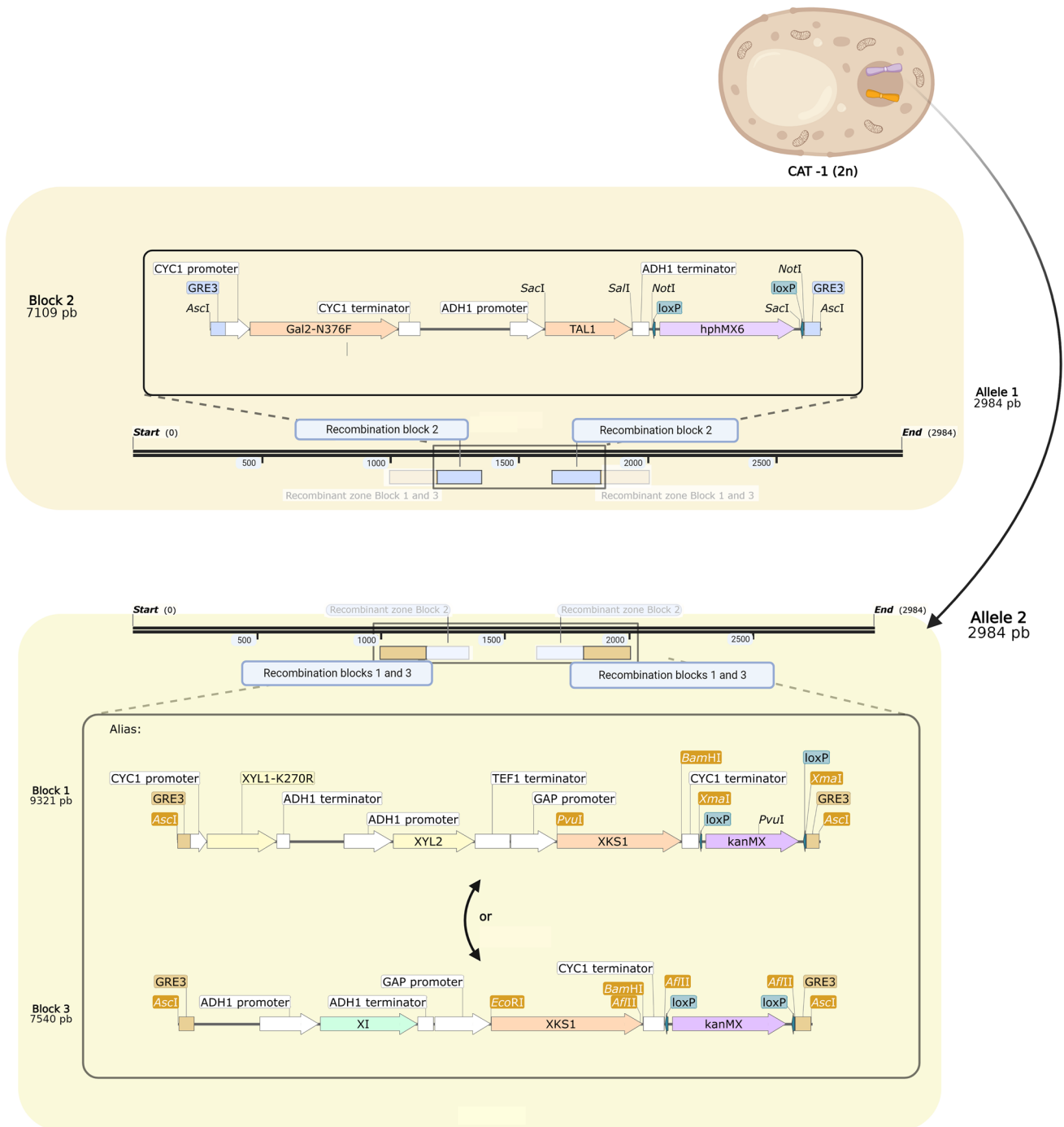
As shown in Fig. 1C, block 3 contains the *xylA* gene from *S. coelicolor* flanked by the *ADH1* promoter and terminator. This region is followed by the *GAP* promoter and *CYC1* terminator, flanking *EcoRI* and *BamHI* sites, designed to clone *XKS1* amplicon. This region is followed by a *AflIII* site, designed to clone the *loxP-kanMX-loxP* amplicon. As mentioned for block 1, this fragment includes nucleotidic sequences of *GRE3* at both ends, assuring the deletion of the remaining *GRE3* allele. Resuming, block 3 was subcloned in the *AscI* site of pSKII\_MCS1, followed by the insertion of both amplicons (*XKS1* and *loxP-kanMX-loxP*). Block 3 was then obtained after the digestion with *AscI*. The DNA of the selected clones derived from CAT-1 were used to verify the correct insertion of each block in the chromosome.

### Construction of Plasmid pRS42K::XI

A fragment containing the *Piromyces* sp. *xylA* gene preceded by the pTPI1 promoter and *CYC1* terminator was cloned in 2 $\mu$  plasmid pRS42K (Table 1) previously digested with *EcoRV*.

### Strain Construction

Blocks were transferred by chemical transformation [31], followed by antibiotic resistance selection. The DNAs of the selected clones were used to verify the correct insertion of each block in the chromosome. Blocks 1 and 3 were first transformed, and recombinant strains were selected from G418 resistant clones and verified by PCR. Then, block 2 was transformed into the selected clones containing block 1 and separately in selected clones with block 3. Recombinant strains were selected on YP medium with 20 g/L dextrose (YPD) agar plates containing G418 and hygromycin as previously described. The selected clones, named as CAT-1-XRT(*kanMX hphMX*) and CAT-1-XIT(*kanMX hphMX*), contained block 1-block 2 and block 3-block 2, respectively. An evolution protocol was applied, as described below (evolution A). First, colonies able to grow in YP medium with 20 g/L D-xylose (YPX) agar with G418 and hygromycin were transformed with the plasmid pSH66 (Table 1; *natMX*), followed by a protocol previously described [28]. A number of G418<sup>r</sup> Hph<sup>s</sup> clones were obtained. Then, a protocol of subcultures in YPD without antibiotic allowed for the selection of clones CloNAT<sup>s</sup>. The absence of antibiotic resistance genes was also verified by PCR. These constructs were named CAT-1-XIT and CAT-1-XRT. Finally, transformation with pRS42K::XI was performed, obtaining strains CAT-1-XRT (pRS42K::XI), CAT-1-XIT (pRS42K::XI), and CAT-1 (pRS42K::XI), which were included as controls. These strains were subjected to evolution protocol B (see below).



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**Fig. 1** Blocks with genes in tandem inserted in *GRE3* alleles

## Evolution Protocols

An adaptive evolution protocol (evolution A) was applied to strains *CAT-1*, *CAT-1-XIT(kanMX hphMX)* and *CAT-1-XRT(kanMX hphMX)*. This involved successive passage of suspended cells from 30 mL of culture to

fresh medium. The primary cultures of each strain were obtained in 50 mL of YPX grown in 200-mL flasks and incubated for approximately 48 h in an orbital shaker (200 rpm). Cells were collected by centrifugation in 50-mL Falcon tubes, suspended in 1 mL YPX and transferred to 30 mL YPX in 40-mL vials with septum. These

vials were then incubated for 24 h with agitation and the procedure was repeated 10 times.

In the case of CAT-1 (pRS42K::XI), CAT-1-XRT (pRS42K::XI), and CAT-1-XIT (pRS42K::XI), primary cultures were incubated for 24 h in 30 mL YPD and G418. Then, the cells were washed twice and suspended in 1 mL MM (evolution B). This suspension was transferred to 30 mL MM with G418 and 20 g/L D-xylose in 40-mL vials with septum. The cultures were incubated for 48 h with agitation. This procedure was also repeated 10 times.

### Selection of Recombinant Strains From Cultures Submitted to the Evolution Protocol

The culture resulting from CAT-1-XIT (pRS42K::XI) in the 10th step of the evolution protocol was used to select single clones with the best performance for D-xylose consumption and ethanol production. Then, serial dilutions of the culture were plated on YPX plus G418. Following the incubation at 30 °C, 10 clones were selected randomly. These clones were cultured in triplicate in 20 mL YPX G418 to determine the ethanol production after 72 h of incubation.

### Evaluation of the Ethanol Production

A primary culture of each cell pool was obtained in YPD, including G418 when necessary. The cells washed and suspended in ultrapure water were used to inoculate 40-mL vials with 20 mL YP (1/5) medium or MM, both with 20 g/L D-xylose, at an initial  $OD_{600nm}$  of 0.5 (Shimadzu UV1800). Samples were collected aseptically at different times for biomass determination and D-xylose and ethanol concentrations in the supernatant. YP (1/5) plus D-xylose was used in these assays instead of YPX to minimize the presence of residual sugars and to ensure the selective conditions for D-xylose consumption.

### Analytical Determinations

The biomass concentration was determined by transferring 1.5 mL of cell suspensions to dried microfuge tubes (at 60 °C for 48 h), previously weighed in an analytical balance. The samples were centrifuged for 6 min at 5000 rpm, and supernatants were stored at –20 °C for the determination of D-xylose and ethanol concentrations. Cell pellets were washed two times and dried in an oven at 60 °C for 48 h or until a constant weight was attained. D-xylose and ethanol concentrations were determined using a high-performance liquid chromatography with infrared spectroscopy system (HPLC-IR) (Shimadzu, Japan), equipped with an Aminex 87H ion exchange column (Biorad, USA)

and using 0.05 N sulfuric acid as mobile phase. The flow rate was 0.6 mL/min at 35 °C.

Biomass and ethanol and D-xylose concentrations were statistically analyzed using the Infostat statistical package with analysis of variance (ANOVA). Data that met assumptions for the ANOVA model were compared using the Tukey test. If the ANOVA assumptions were not met, a non-parametric Kruskal Wallis test was used. In all cases, a  $p$ -value < 0.05 was used.

## Results and Discussion

### Recombinant Strains Construction

Despite expressing all enzymes of the oxo-reductive pathway, the wild-type strains of *S. cerevisiae* cannot assimilate D-xylose and produce ethanol; instead, they excrete xylitol to the medium [32]. This inability is due to a redox imbalance in the NADH levels during the conversion of D-xylose to ethanol [33]. The *GRE3* gene encodes a reductase that reduces D-xylose to xylitol mainly using NADPH as cofactor. Then, xylitol dehydrogenase preferentially uses  $NAD^+$  [14]. Under these growing conditions, there would be no renewable source of  $NAD^+$  in *S. cerevisiae*.

In an initial strategy, genes required to express a selected pathway for D-xylose metabolism were incorporated into the chromosome through homologous recombination. DNA fragments were designed so that the genes were transcribed under the control of strong and medium promoters, organized in tandem. In general, strong promoters were used in all cases, except for *XYL1-K270R*, in which case a constitutive medium promoter was included to avoid the potential accumulation of xylitol. According to previous studies, both alleles of the *GRE3* gene were chosen for integration [24, 34]. Thus, this strategy was associated with the deletion of *GRE3*, thereby reducing the potential accumulation of xylitol. Previous studies have shown that recombinant strains derived from CAT-1 carrying both *GRE3* alleles did not produce high amounts of xylitol compared with other recombinant industrial strains such as PE-2, although an appreciable amount of the metabolite was detected in those constructions [23].

The oxo-reductive pathway included the expression of two heterologous genes from *S. stipitis*, *XYL1* and *XYL2*, optimized for *S. cerevisiae* codon use. According to other studies, these two genes should be actively expressed in this yeast [16, 35]. In addition, as mentioned previously in the section related to redox imbalance, *XYL1* includes a point mutation (K270R), which ensures an increased affinity for cofactor NADH [30]. On the other hand, an attempt was also made to express the D-xylose isomerase pathway by the insertion of *S.*

*coelicolor xylA* gene, which was optimized for codon use in *S. cerevisiae*. Unlike the previous case, no published studies regarding the expression of this gene were found and poor results were obtained with the expression of this enzyme from non-extremophilic bacteria. Most genes that code for D-xylose isomerase of bacterial origin are not expressed efficiently in yeasts [36]. In fact, attempts were made to express *xylA* genes from *E. coli* [37] and *Clostridium thermosulfurogenes* [38] in *S. cerevisiae*. The negative results were attributed to the inability of this yeast to express *xylA* or to synthesize an active enzyme [39]. The first successful attempt was made with the *xylA* gene of the thermophilic bacterium *Thermus thermophilus* in a *S. cerevisiae* strain [40]. Furthermore, codon optimization was shown to improve the performance of heterologous D-xylose isomerase pathways in *S. cerevisiae*, achieving significant increases in the yield and productivity of ethanol. On the other hand, directed evolution was also an effective strategy to improve the kinetic properties of XI in *S. cerevisiae* [36].

Both constructions were complemented with the insertion of a mutated *Gal2-N376F* gene, which codes for a permease with high affinity for D-xylose. Farwick et al. [13] analyzed a collection of mutants in *Gal2* and *Hxt7* transport genes [13]. Substitution of asparagine codon at position 376 for phenylalanine in the *Gal2* gene (*Gal2-N376F*) was the most effective modification, as it

eliminated glucose transport activity, obtaining a high affinity for D-xylose.

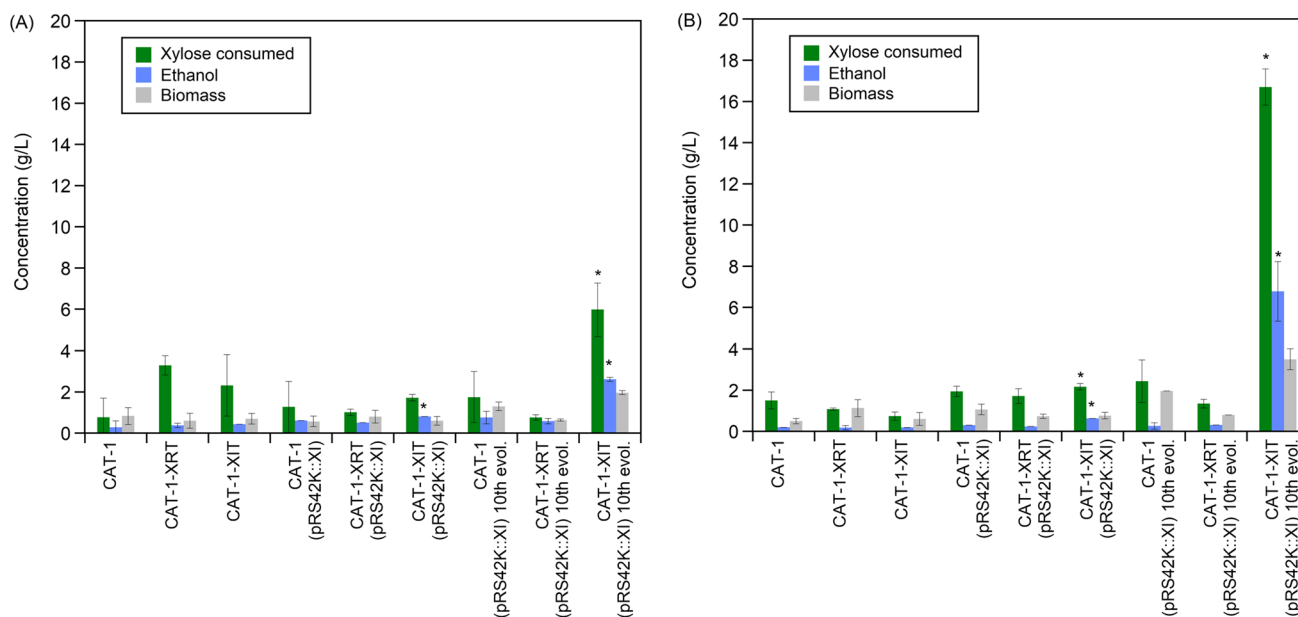
Extra copies of the *XKS1* and *TALI* genes were also included in the cassettes. Lineal constructions were transferred by chemical transformation to CAT-1 cells, G418<sup>r</sup> Hph<sup>r</sup> clones were used for the evolution protocol, and antibiotic resistance genes were then eliminated by the expression of Cre from pSH66 [41], as described in the Materials and Methods section.

## Evaluation of D-xylose Fermentation

Selected constructs, whether or not subjected to evolution (10 subcultures, evolution A), consumed very little D-xylose after long incubation periods in YP (1/5) medium with 20 g/L D-xylose media and did not produce ethanol (Fig. 2B). These results suggest that in the case of recombinant strains carrying genes of the oxo-reductive pathway, the expression levels of heterologous and, perhaps, homologous genes were not sufficient to show a desired phenotype after the evolution protocol.

In the case of recombinant strains carrying the *xylA* gene, the problem could be most likely related to the absence of an active *xylA* gene expression. Beyond the bacterial origin of the *S. coelicolor xylA* gene, this specific gene had not been previously analyzed.

Phenotypes obtained with these constructions could be generated by different causes. Considering previous studies



**Fig. 2** Concentration of D-xylose, production of ethanol and biomass of the different constructions evaluated: CAT-1-XRT, CAT-1-XIT, CAT-1 (pRS42K::XI), CAT-1-XRT (pRS42K::XI), CAT-1-XIT (pRS42K::XI), CAT-1 (pRS42K::XI) (10th evolution aliquot), CAT-1-XRT (pRS42K::XI) (10th evolution aliquot), CAT-1-XIT

(pRS42K::XI) (10th evolution aliquot) and control strain CAT-1. The assay was done in screw-capped vials with septum, to maintain limited O<sub>2</sub> access. Culture media were MM (A) and YP (1/5) (B) with 20 g/L D-xylose. Asterisks indicate significant differences compared to the control strain CAT-1



that used the haploid and diploid strains of *S. cerevisiae*, a possible explanation could be associated with the low expression levels of the *XYL1/XYL2* genes in one case (CAT-1-XRT) and the absence of XI activity in another (CAT-1-XIT). Thus, the expression level of the heterologous *xylA* gene from *Piromyces* sp. was increased, which was known to be actively expressed in *S. cerevisiae* [42]. Furthermore, the expression of the *xylA* gene also eliminates the need to ensure the balance of cofactors. This gene was cloned in a multicopy plasmid to assure high expression levels (pRS42K) [29].

Biomass, D-xylose consumption, and ethanol production were determined in the cultures of different strains, incubated for 72 h at 30 °C (Fig. 2A and B). The strains analyzed were CAT-1-XRT, CAT-1-XIT, CAT-1 (pRS42K::XI), CAT-1-XRT (pRS42K::XI), CAT-1-XIT (pRS42K::XI), CAT-1 (pRS42K::XI) (10th evolution aliquot), CAT-1-XRT (pRS42K::XI) (10th evolution aliquot), CAT-1-XIT (pRS42K::XI) (10th evolution aliquot), and CAT-1 as control. The assay was developed in bottles with a screw cap and septum to maintain limited O<sub>2</sub> access. In general, the phenotypes of the strains analyzed presented similar behaviors in both MM and YP (1/5) media when compared with CAT-1, although the D-xylose consumption and ethanol production were higher in some clones grown in YP (1/5) medium with 20 g/L D-xylose (Fig. 2A and B). Strains with the *S. coelicolor xylA* gene in their genome (block 3-block 2) and multicopy plasmid (pRS42K::XI) showed significant differences from CAT-1 both in ethanol production and D-xylose consumption. Furthermore, clones contained in the cell pool CAT-1-XIT (pRS42K::XI) (10th evolution) far exceeded the ethanol production and D-xylose consumption of others. However, the ethanol production and D-xylose consumption were higher in YP (1/5) medium with 20 g/L D-xylose (Fig. 2B) than in MM with 20 g/L D-xylose (Fig. 2A). CAT-1-XIT (pRS42K::XI) contained in the pool of the 10th evolution produced 6.8 g/L ethanol when cultured in YP (1/5) medium with 20 g/L D-xylose, consuming approximately 83.5% of the total D-xylose. Under these conditions, the ethanol yield by this strain was 0.41 g ethanol/g D-xylose consumed. On the other hand, the clones cultured in MM with 20 g/L D-xylose produced a lower concentration of ethanol (2.6 g/L), consuming only 30% of the total D-xylose, but reaching a higher ethanol yield (0.43 g ethanol/g D-xylose consumed). In any case, both the MM and YP (1/5) medium contain other components that can be used as fermentable carbon sources; therefore, although these are in low quantities, these yields cannot be assigned specifically to D-xylose. Regarding biomass, in both media, no significant differences were found between the cultures. Xylitol was not detected in the supernatant of any cultures analyzed.

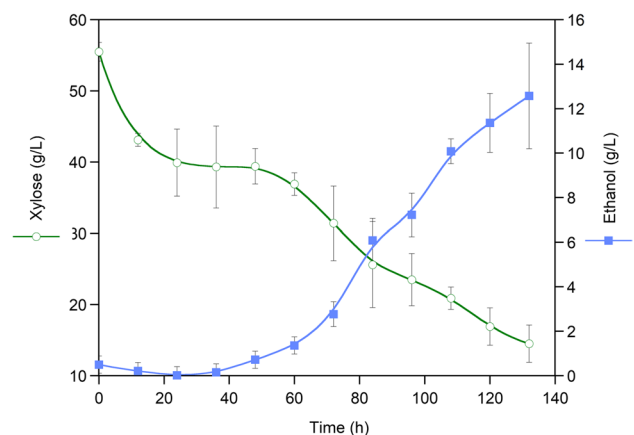
The results showed that CAT-1-XIT (pRS42K::XI) (10th evolution aliquot) was cultured in YP (1/5) medium in the

presence of different D-xylose concentrations of 32, 38, 41, 61, 68, and 80 g/L for 72 h. The D-xylose conversion ranged from 46.6 to 96.3%, the ethanol production ranged from 9.3 to 13.4 g/L, and the biomass concentration ranged from 2.5 to 3.6 g/L. Under these growth conditions, the strain consumed no more than 45 g/L D-xylose, even at higher initial D-xylose concentrations. This behavior could be promoted by different factors. The commercial strain CAT-1 tolerates high concentrations of ethanol and would not be expected to limit the process. This could be due to the relatively low concentration of some other components in the medium that could be growth limiting. It could also be due to the expression levels of the introduced metabolic pathway or the transport activity of D-xylose, which could be limiting to cell growth.

Figure 3 shows the D-xylose and ethanol concentrations for CAT-1-XIT (pRS42K::XI) (10th evolution aliquot) cultured in YP (1/5) medium with an initial D-xylose concentration of 55 g/L. After 132 h, 12.6 g/L of ethanol was achieved with a D-xylose consumption of 74%, which corresponds to a fermentation efficiency of 61% of the theoretical value and a productivity of 0.1 g/(Lh). A long lag phase (48 h) was observed, during which low biomass growth and sugar consumption were determined. After 72 h of incubation, the presence of glycerol was detected in the medium (data not shown).

A control of CAT-1-XIT (pRS42K::XI) (10th evolution aliquot) cultured in YP (1/5) medium in the absence of D-xylose was also used to evaluate the production of ethanol from other components of the medium (e.g., residual sugars in peptone and yeast extract). This culture produced almost undetectable concentrations of ethanol after 11 days of incubation (132 h).

Then, 10 clones were isolated from the 10<sup>th</sup> evolution culture of CAT-1-XIT (pRS42K::XI). These clones were



**Fig. 3** Profile of concentrations of D-xylose (blue) and ethanol (green) in the medium from a culture of CAT-1-XIT (pRS42K::XI) (10th evolution aliquot) over time. Error bars represent standard deviation

used to determine the D-xylose consumption and ethanol production when grown in YPX medium. Non-parametric statistical tests for both compounds (ethanol production and D-xylose consumption) indicated the absence of significant differences between the 10 clones with 22 g/L D-xylose consumed and between 8 and 9 g/L of ethanol produced. These results suggest that the cell populations were homogeneous in terms of ethanol production yield, obtaining a value of  $0.4 \text{ g ethanol/g D-xylose consumed}$ .

The results obtained from different studies focused on enabling yeast strains to ferment D-xylose to ethanol are difficult to compare because different strategies were used and the post-construction steps involved protocols for directed evolution, random mutagenesis, and genome shuffling, which may introduce unpredictable genome modifications [34, 43]. In these communities of organisms subjected to random modifications, the “best” clones that can present a “desired” phenotype are selected, such as the fermentation of D-xylose to ethanol in this case. A complementary activity should include a genomic analysis of “best” clones to identify genes that could potentially be altered in a targeted way. In general, studies have involved the expressions of heterologous genes, both of the oxo-reductive pathway or D-xylose isomerase cloned in plasmids. The absence of growth of the recombinant strains CAT-1-XRT and CAT-1-XIT, even after applying a directed evolution protocol, could be explained by the low expression levels of heterologous enzymes [44], and in the case of XI, the enzyme may even be inactive. However, in the case of recombinant CAT-1-XRT, we estimate that the expressions of *XYL1* and *XYL2* somehow affected the metabolic network of the cell. This is based on the fact that when transferring the plasmid with the *Piromyces* sp. XI gene, only the CAT-1-XIT recombinant strain grew and fermented D-xylose. However, it cannot be ruled out that XI from *S. coelicolor* may play a role that favors growth in the D-xylose of the recombinant strain containing XI cloned in the multicopy plasmid.

Some studies conducted by other authors with industrial strains, including CAT-1, have been oriented to the use of high copy number plasmids to express the heterologous genes *XYL1-XYL2* of *S. stipitis* and *xylA* of *C. phytofermentans*, and extra copies of their own genes, such as *XKS1* and *TAL1* [21–24]. In general, genes encoding relatively high-affinity transporters were not included. In turn, both *GRE3* genes were deleted only in those constructions in which the xylitol production was appreciable and could affect the yield of bioethanol production (PE-2Δ*GRE3*). In those studies, a recombinant strain derived from CAT-1 did not exhibit the best production values compared with the other strains derived from PE-2. By comparing cultures designed under partially similar conditions, it was found that strain CAT-1-X [24] [CAT-1 (pMEC1049), with *XYL1-272D/XYL2/XKS1/TAL1*

and *HphMX4* genes] grown in YPX medium with 41.8 g/L as initial D-xylose concentration reached a maximum ethanol concentration of 8.7 g/L and a yield of  $0.23 \text{ g ethanol/g D-xylose consumed}$  after 48 h of culture (ratio between the ethanol produced to the D-xylose consumed when the maximum ethanol concentration was achieved). The final xylitol concentration in the medium, apparently at 72 h of culture, was 3.6 g/L. These values are relatively similar to those obtained with our construction, although culture conditions were different, especially considering the initial inoculum, which might have affected the times necessary to complete the fermentation of D-xylose in the medium. In our case, cultures in YP (1/5) medium with 55 g/L D-xylose reached 12.6 g/L of ethanol, with a yield of  $0.31 \text{ g ethanol/g D-xylose consumed}$  at 132 h. The different yield values could be due to different causes, including the absence of xylitol due to the directed mutagenesis of both *GRE3* alleles.

CAT-1-XRT (pRS42K::XI) exhibited a limited ability to consume D-xylose and produce ethanol. This result would be in agreement with the behavior previously shown by other recombinant strains carrying genes for both pathways [21]. According to that study, XI activity was strongly reduced in diploid strain PE-2Δ*GRE3* carrying *XYL1-XYL2* genes from *S. stipitis*, although a different behavior was observed in CA11. Cunha et al. [21] analyzed the phenotype of two diploid strains, PE-2Δ*GRE3* and CA11, and their derivative clones containing plasmids with genes for the oxo-reductive and/or D-xylose isomerase (*Clostridium phytofermentans*) pathways. These strains were analyzed in a synthetic D-xylose-containing medium and non-detoxified corn cob lignocellulosic hydrolysate. On the basis of the previous study, the authors propose that the heterologous genes for the oxo-reductive pathway could be involved in the oxidation of potential inhibitors, improving the performance of the corresponding recombinant strains. However, when these clones were grown in YPX medium, NADH and NADPH-dependent XR activities and XDH activities were greatly reduced in PE2Δ*GRE3* carrying *XYL1-XYL2* and *xylA* genes compared with those containing just *XYL1-XYL2* genes. The same happened with the XI activity level, which was higher in the clones containing only the *xylA* gene. In the case of CA11, however, the opposite situation was observed. The main difference between the two strains is related to the different xylitol levels detected in the supernatant of PE2Δ*GRE3* (*XYL1-XYL2-XI*) compared with CA11 (*XYL1-XYL2-XI*). However, it was estimated that these differences in enzymatic activities should be attributed to the reductive balance in both strains. Even considering that in this case, the *XYL1-XYL2* genes appeared to be expressed at very low levels, these enzymes could affect the global reductive balance and result in very poor ability to ferment D-xylose by CAT-1-XRT(pRS42K::XI) compared with CAT-1-XIT(pRS42K::XI). The recombinant strains with multicopy plasmid that produced ethanol by consuming

D-xylose also harbored extra copies of *XKS1*, *TAL1*, and the mutated gene for D-xylose transport, *Gal2-N376F*.

## Conclusions

Two recombinant strains derived from *S. cerevisiae* CAT-1 that contained in their genomes a set of genes involved in D-xylose metabolism (CAT-1-XRT carrying specifically the *XYL1-K270R/XYL2* genes of *S. stipitis* and CAT-1-XIT with the *xylA* gene of *S. coelicolor*) were obtained and compared in terms of their ability to ferment D-xylose to produce ethanol. Following a directed evolution protocol, only CAT-1-XIT expressing the *xylA* gene of *Piromyces* sp. cloned in a high copy number plasmid fermented most D-xylose and produced 12.6 g/L ethanol. The low production levels detected with the CAT-1-XRT strain harboring the same plasmid suggest some type of metabolic imbalance. These phenotypes indicate the need to deepen the study of global metabolism in yeasts when trying to express heterologous pathways, with particular attention to the potential imbalance of reductive power.

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**Author contribution** All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Lucía Coimbra, Karen Malan, Alejandra Fagúndez, Belén Fernández, and Martín Pratto. The first draft of the manuscript was written by Silvia Batista, Claudia Lareo, and Mairan Guigou and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data Availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author.

## Declarations

**Conflict of Interest** The authors declare no competing interests.

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