



Physiological comparisons among *Spathaspora passalidarum*, *Spathaspora arborariae*, and *Scheffersomyces stipitis* reveal the bottlenecks for their use in the production of second-generation ethanol

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Abstract

The microbial conversion of pentoses to ethanol is one of the major drawbacks that limits the complete use of lignocellulosic sugars. In this study, we compared the yeast species *Spathaspora arborariae*, *Spathaspora passalidarum*, and *Scheffersomyces stipitis* regarding their potential use for xylose fermentation. Herein, we evaluated the effects of xylose concentration, presence of glucose, and temperature on ethanol production. The inhibitory effects of furfural, hydroxymethylfurfural (HMF), acetic acid, and ethanol were also determined. The highest ethanol yield (0.44 g/g) and productivity (1.02 g/L.h) were obtained using *Sp. passalidarum* grown in 100 g/L xylose at 32 °C. The rate of xylose consumption was reduced in the presence of glucose for the species tested. Hydroxymethylfurfural did not inhibit the growth of yeasts, whereas furfural extended their lag phase. Acetic acid inhibited the growth and fermentation of all yeasts. Furthermore, we showed that these xylose-fermenting yeasts do not produce ethanol concentrations greater than 4% (v/v), probably due to the inhibitory effects of ethanol on yeast physiology. Our data confirm that among the studied yeasts, *Sp. passalidarum* is the most promising for xylose fermentation, and the low tolerance to ethanol is an important aspect to be improved to increase its performance for second-generation (2G) ethanol production. Our molecular data showed that this yeast failed to induce the expression of some classical genes involved in ethanol tolerance. These findings suggest that *Sp. passalidarum* may have not activated a proper response to the stress, impacting its ability to overcome the negative effects of ethanol on the cells.

Keywords Xylose-fermenting yeast · Ethanol production · Co-fermentation · Ethanol tolerance

Introduction

Second-generation (2G) ethanol is produced from lignocellulosic biomass, a renewable energy source alternative to fossil fuels. This biomass is mainly composed of cellulose and hemicellulose, which can be hydrolyzed into hexoses and pentoses and then converted into ethanol by fermenting microorganisms [1–4]. One of the largest challenges for the efficient production of 2G ethanol is the microbial conversion of pentose into ethanol. These sugars comprise 25–40% of the lignocellulosic biomass, with xylose representing the main pentose present in lignocellulosic hydrolysates [5]. Ethanol production from xylose would largely contribute to the economic viability of 2G ethanol.

Strains of *Saccharomyces cerevisiae*, which are widely used in the industrial production of first-generation (1G)

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ethanol, do not naturally ferment pentoses due to the absence of specific transporters and the low expression of genes encoding the enzymes for xylose metabolism [6–9]. Additionally, the strains of *S. cerevisiae* genetically engineered for this purpose still present limitations, which results in xylose-fermenting rates not as high as those obtained with glucose [10]. Thus, to improve xylose use, it is important to enhance our knowledge about the complex regulation regarding the metabolism of this sugar. In this regard, the study and domestication of natural pentose-fermenting microorganisms have been considered as an interesting alternative in the field of 2G ethanol.

The yeasts *Spathaspora arborariae*, *Scheffersomyces stipitis*, and *Spathaspora passalidarum* can convert xylose into ethanol, making them potential candidates for lignocellulosic biomass fermentation [5, 11–16]. For example, *Sp. arborariae* was isolated from rotting wood samples collected at the National Park of Serra do Cipó and at the Rio Doce State Park [13]. The strain *Sc. stipitis* NRRL 7124 presents an endosymbiotic relationship with the beetle *Odontotaenius disjunctus*, which inhabits decaying wood [16, 17]. *Spathaspora passalidarum* NRRLY 27,907 has also been isolated from the intestine of wood-eating beetles [18]. The species *Sc. stipitis* and *Sp. passalidarum* are considered promising for industrial fermentation because they present good fermentative yields under anaerobic conditions [14, 19].

The species studied here were chosen according to the following criteria: *Sc. stipitis* for being the xylose-fermenting species with more scientific information accumulated so far, but with much yet to be investigated about its metabolism; the yeast *Sp. passalidarum* because it recently emerged as the most promising species for ethanol production from xylose; and *Sp. arborariae*, a yeast that belongs to the same genus as *Sp. passalidarum* but has few studies available in the literature. Thus, we suppose that a comparison with *Sp. arborariae* could provide important information about the physiological evolution of this genus of xylose-fermenting yeasts.

Besides the ability to generate high ethanol yields from xylose, other factors that influence ethanol production from hydrolysates should be considered when evaluating fermenting microorganisms. During fermentation, microorganisms are exposed to stressful industrial conditions that can compromise their growth and cellular metabolism, such as osmotic stress due to the high initial sugar concentrations, elevated temperatures, especially during the summer and in tropic countries, the generation of inhibitors from biomass pretreatment, including furfural, 5-hydroxymethylfurfural (HMF), and acetic acid, as well as ethanol accumulation in the culture medium [20].

In this context, our main research question was as follows: What are the physiological limitations of xylose-fermenting yeasts that impede their use for ethanol

production? The hypothesis proposed and tested is that these yeasts can ferment xylose with high yields, but the low tolerance to fermentation conditions is the limiting factor in the process.

To date, respective studies have focused on the fermentative capacity of *Sp. arborariae*, *Sp. passalidarum*, and *Sc. stipitis*, while there are few comparative studies that show how those factors affect xylose fermentation in these yeasts. Furthermore, the relevant aspects of yeast physiology that will orientate strain selection or breeding programs are not widely addressed. Thus, the present work aimed to characterize the fermentative capacity of xylose-fermenting yeast strains (*Sp. arborariae* HM 19.1A, *Sp. passalidarum* NRRLY 27,907, and *Sc. stipitis* NRRL 7124) under different cultivation conditions. The results presented here contribute to the comprehension of the physiology of xylose fermentation and highlight the bottlenecks for future breeding programs.

Materials and methods

Strains and media

We used the yeasts *Sp. arborariae* HM 19.1A, *Sp. passalidarum* NRRLY 27,907, and *Sc. stipitis* NRRL 7124 in this study. For cell maintenance and activation, YPD medium [2% yeast extract, 1% peptone, and 2% glucose (w/v)] was used. To obtain solid medium, 2% agar (w/v) was added to YPD. All yeasts were stored and maintained in 20% glycerol (v/v) at -80 °C.

Fermentation assays

Yeasts were grown overnight in YPX (20 g/L yeast extract, 10 g/L peptone, and 20 g/L xylose) under agitation of 120 rpm at 28 °C. The cells were centrifuged and inoculated into the fermentation medium (5.0 g/L yeast extract, 5.0 g/L peptone, 2.0 g/L NH₄Cl, 1.0 g/L KH₂PO₄, 0.3 g/L MgSO₄·7H₂O) [16] containing xylose (40, 80, or 100 g/L). The initial OD_{600 nm} was adjusted to 2 [21], corresponding to 3.8 g/L of cell dry weight (CDW) for *Sp. arborariae*, 1.5 g/L for *Sp. passalidarum*, and 3.9 g/L for *Sc. stipitis*. The flasks were incubated at 28°C for 70 h at 120 rpm. The best concentration of xylose for ethanol production was determined and used to evaluate the effects of different temperatures (28, 32, and 35 °C) on the fermentation efficiency of the yeasts. Co-fermentation was carried out by adding glucose (20 or 40 g/L) to the medium containing a predetermined xylose concentration and at a certain temperature.

Tolerance assays

Tolerance tests to furfural, HMF, and acetic acid were carried out at the temperature at which the optimum fermentative parameters were determined (see above). The assays were performed in 125-mL Erlenmeyer flasks containing fermentation medium supplemented with xylose (20 g/L) and one of the inhibitors (2.5 g/L furfural, 0.5 g/L HMF, or 3 g/L acetic acid) at 120 rpm for 60 h. The inhibitor concentrations were chosen based on the literature [5, 22]. Ethanol tolerance assays were performed under the same conditions, except for the addition of 2, 4, or 6% (v/v) ethanol instead of the pretreatment compounds.

Fermentation parameters

Ethanol yield ($Y_{P/S}$, g/g) was estimated using Eq. 1, in which Pf is ethanol final mass (g), Pi is ethanol initial mass (g), Si is sugar initial mass (g), and Sf is sugar final mass (g).

$$Y_{P/S} = \frac{(Pf - Pi)}{(Si - Sf)} \quad (1)$$

Ethanol volumetric productivity (Q_p , g/L.h) was estimated using Eq. 2, in which Pf is ethanol final concentration (g/L), Pi is ethanol initial concentration (g/L), and t is fermentation period (h).

$$Q_p = \frac{(Pf - Pi)}{t} \quad (2)$$

Fermentation efficiency (Y%) was estimated using Eq. 3, in which the experimental $Y_{P/S}$ (g/g) was divided by the theoretical $Y_{P/S}$ (0.51 g/g), and the result multiplied by 100.

$$Y = \frac{Y_P(\text{experimental})}{Y_P(\text{theoretical})} \times 100 \quad (3)$$

Total sugar consumption rate (g/L.h) was estimated using Eq. 4, in which Sf is sugar final concentration (g/L), Si is sugar initial concentration (g/L), and t is consumption period (h).

$$\text{Total sugar consumption rate} = \frac{(Sf - Si)}{t} \quad (4)$$

A calibration curve correlating OD600 nm with CDW (g/L) was constructed for each carbon source and for each yeast to evaluate their growth. The specific growth rate (h^{-1}) was determined by the angular coefficient from a linear regression of the plot \ln OD (600 nm) versus time (h) during the yeasts' exponential (log) growth phase. The consumption yield (g/g) was determined by the angular coefficient from a linear regression of the plot CDW (g/L) versus the sugars'

concentrations (glucose or xylose) (g/L). The specific sugar consumption rate ($g_{\text{sugar}}/g_{\text{CDW}} \cdot h$) was determined by multiplying the consumption yields (g/g) for each sugar (xylose and glucose) by the specific growth rate (h^{-1}) of each yeast. Unpaired two-tailed t test (GraphPad Prism 5) was used for the statistical analysis.

Analytical methods

Quantitative determination of substrate consumption and metabolite production was carried out by high-performance liquid chromatography (HPLC) using the Bio-Rad Aminex HPX-87 column at 45 °C. Sulfuric acid (5 mM), applied at a flow rate of 0.7 mL/min, was used as the eluent for separation. The column was coupled to an RID-10A refractive index detector.

Total RNA extraction and quantitative real-time PCR (qPCR) analysis

Sp. passalidarum cells pre-cultured in YPX medium overnight were transferred to 125-mL Erlenmeyer flasks containing 20 mL of fresh medium (initial OD600 nm ~ 0.20). The flasks were incubated at 32 °C at 150 rpm until the cells reached the mid-exponential growth phase (OD600 nm ~ 1.0). The cultures' volumes were divided into two new flasks, with one containing the cells growing without ethanol and the other having the cells treated with ethanol to a final concentration of 4% (v/v). The flasks were incubated for 2 h in the conditions described above. Three independent biological replicates were performed.

Total RNA extraction was performed with RNeasy Mini kit (Qiagen). The samples were quantified by Qubit 3.0 Fluorometer (Invitrogen) and treated with RNase-free DNase I (Promega). cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the following cycle: 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C. The qPCR analysis was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) with StepOne™ Real-Time PCR System (Applied Biosystems). The assays were carried out in technical duplicate using the conditions as follows: 10 min at 95°C and 40 cycles of 15 s at 95°C, 1 min at 60°C. Eight *Sp. passalidarum* genes orthologs of *S. cerevisiae* genes that are involved in ethanol response (*TDH1*, *ADH1*, *ADH7*, *TPS1*, *HSP30*, *HSP104*, *SOD1*, and *MSN2/4*) were selected for qPCR analysis. Primers were designed with Primer3Plus [23], and nucleotide specificity was evaluated by Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>) [22].

Relative mRNA quantification was performed by the standard curve method. A standard curve was obtained for each gene by plotting the average Ct versus the \log_{10} of

different cDNA concentrations from control sample (0.39 – 50.0 ng/ μ L). The results were normalized using *ACT1* and *RDN18* [24] as internal controls. Unpaired two-tailed t test (GraphPad Prism 5) was used for the statistical analysis. Primer sequences and genes accession numbers are available in Supplementary File S1.

Results

Effects of xylose concentration and temperature on ethanol production

Xylose fermentation by *Sp. arborariae*, *Sp. passalidarum*, and *Sc. stipitis* was evaluated at different concentrations of xylose at 28°C (Fig. 1). The species *Sp. arborariae* produced the largest amount of biomass and the lowest ethanol concentration and was the only yeast that produced xylitol under the tested conditions (Fig. 1a-c). The effects of different xylose concentrations on the fermentation parameters were

analyzed (Table 1). Ethanol volumetric productivity (Q_p) was higher in *Sp. passalidarum*, which is consistent with the lowest biomass achieved. Fermentation efficiencies (Y%) did not vary as a function of xylose concentration.

The highest ethanol concentration (g/L) was achieved when 100 g/L of xylose was used. The highest ethanol yields were obtained at 40 h of fermentation for *Sp. passalidarum* ($Y_{P/S}$ 0.40 g/g), 70 h for *Sp. arborariae* ($Y_{P/S}$ 0.32 g/g), and 50 h for *Sc. stipitis* ($Y_{P/S}$ 0.35 g/g). Overall, xylose was completely consumed in less than 70 h, except for *Sp. arborariae* grown in 100 g/L, which consumed about 92% of xylose (Fig. 1). As 100 g/L sugar favored the production of high ethanol concentrations, it was chosen to evaluate the effects of different temperatures in further fermentation assays.

For *Sp. arborariae*, biomass and ethanol production were lower at 35 °C (Fig. 2a-c, Table 2). At 32 and 35 °C, the yeast presented higher yields of xylitol (0.28 and 0.34 g/g, respectively) compared to ethanol (0.19 and 0.18 g/g, respectively). The highest ethanol yield (0.32 g/g) was achieved at 28 °C after 70 h of fermentation (Table 2). Biomass

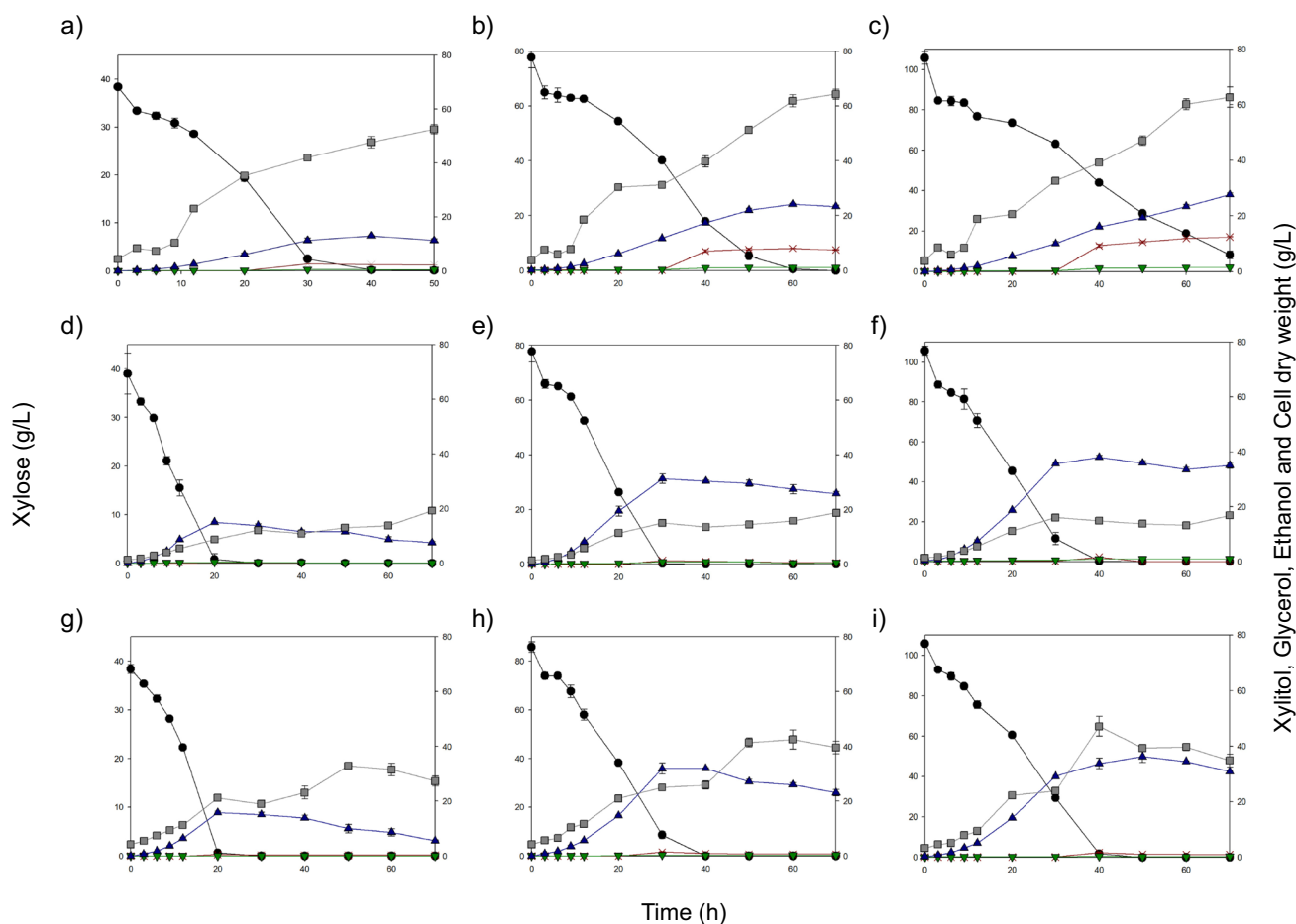


Fig. 1 Fermentation kinetics in 40, 80, or 100 g/L of xylose. *Spathaspora arborariae* (a, b, and c), *Spathaspora passalidarum* (d, e, and f), and *Scheffersomyces stipitis* (g, h, and i). Fermentation lasted 50 h in

(a) and 70 h in the other experiments (b-i). Xylose (●); xylitol (X); glycerol (▼); ethanol (▲); cell dry weight (■)

Table 1 Effect of different xylose concentrations on the fermentation parameters of the yeasts analyzed in this study

Yeast strains	Xylose (g/L)	CDW ¹ (g/L)	Ethanol (g/L)	Y _(P/S) ² (g/g)	Q _P ³ (g/L.h)	Y% ⁴	Time (h)
<i>Spathaspora arborariae</i>	40	47.7 ± 2.1	12.35 ± 1.0	0.32 ± 0.03	0.31 ± 0.02 ^b	62.78 ± 6.2 ns	40
	80	61.9 ± 2.3	23.80 ± 0.6	0.31 ± 0.02	0.39 ± 0.01 ^a	60.18 ± 3.5 ns	60
	100	62.7 ± 3.6	27.58 ± 0.7	0.32 ± 0.01	0.39 ± 0.01 ^a	62.84 ± 1.5 ns	70
<i>Spathaspora passalidarum</i>	40	8.7 ± 0.4	14.40 ± 1.2	0.37 ± 0.01	0.70 ± 0.07 ^b	71.98 ± 0.6 ns	20
	80	15.1 ± 0.7	30.00 ± 2.5	0.38 ± 0.04	0.99 ± 0.08 ^a	75.08 ± 9.1 ns	30
	100	14.9 ± 0.3	36.90 ± 1.9	0.40 ± 0.03	0.91 ± 0.04 ^a	78.83 ± 5.9 ns	40
<i>Scheffersomyces stipitis</i>	40	21.2 ± 0.3	15.30 ± 1.0	0.40 ± 0.03	0.75 ± 0.05 ns	78.70 ± 7.1 ns	20
	80	25.9 ± 1.5	31.10 ± 1.6	0.36 ± 0.01	0.77 ± 0.04 ns	70.05 ± 2.2 ns	40
	100	39.7 ± 1.3	33.70 ± 4.7	0.35 ± 0.05	0.67 ± 0.09 ns	68.36 ± 9.8 ns	50

1—Cell dry weight; 2—yield of ethanol [Y_(P/S)]; 3—ethanol productivity [Q_P]; 4—fermentation efficiency (Y%). Statistical analyses compared the effects of different treatments on each yeast species. ± Standard deviation; ns not significant. The values highlighted with different letters are statistically different in the t test (p < 0.01)

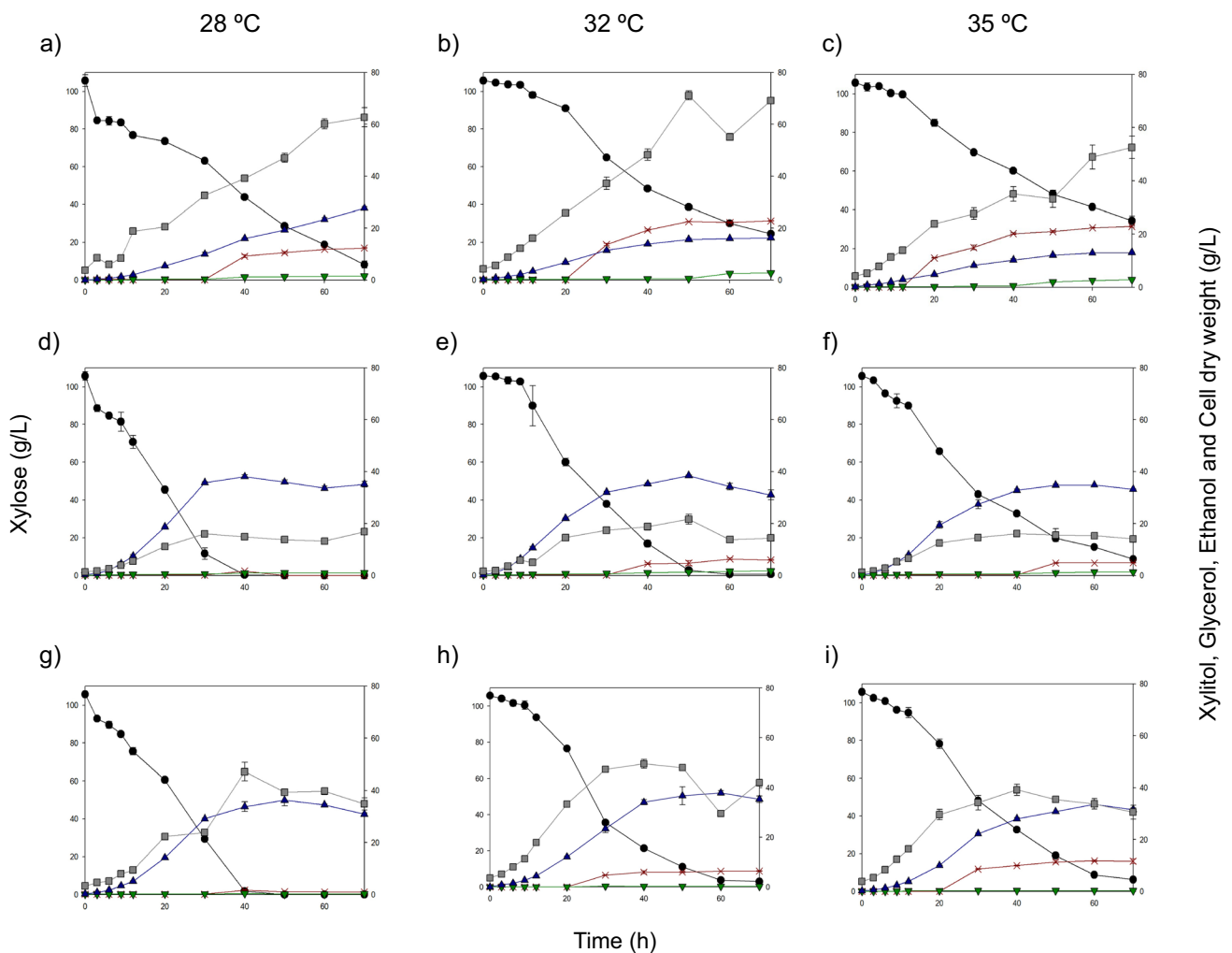


Fig. 2 Fermentation kinetics in fermentation medium containing 100 g/L of xylose. Fermentation was carried out at different temperatures by *Spathaspora arborariae* (a, b, and c), *Spathaspora passali-*

darum (d, e, and f), and *Scheffersomyces stipitis* (g, h, and i). Xylose (●); xylitol (×); glycerol (▼); ethanol (▲); cell dry weight (■)

Table 2 Effect of different temperatures on the fermentation parameters of yeasts grown in fermentation medium containing 100 g/L of xylose

Yeast strains	Temperature (°C)	CDW ¹ (g/L)	Ethanol (g/L)	Y(P/S) ² (g/g)	QP ³ (g/L.h)	Y% ⁴	Time (h)
<i>Spathaspora arborariae</i>	28	62.7 ± 3.6	27.58 ± 0.7	0.32 ± 0.01	0.39 ± 0.01 ^a	62.84 ± 1.5 ^a	70
	32	69.2 ± 0.2	16.91 ± 1.3	0.19 ± 0.04	0.24 ± 0.02 ^b	37.52 ± 7.6 ^b	70
	35	52.5 ± 4.2	12.71 ± 0.6	0.18 ± 0.01	0.18 ± 0.01 ^c	35.55 ± 3.2 ^b	70
<i>Spathaspora passalidarum</i>	28	14.9 ± 0.3	36.92 ± 1.9	0.40 ± 0.03	0.92 ± 0.05 ^a	78.83 ± 5.9 ^{ab}	40
	32	21.7 ± 1.9	30.68 ± 2.3	0.44 ± 0.03	1.02 ± 0.08 ^a	87.01 ± 5.3 ^a	30
	35	15.3 ± 0.2	33.88 ± 1.6	0.37 ± 0.01	0.56 ± 0.02 ^b	73.17 ± 2.9 ^b	60
<i>Scheffersomyces stipitis</i>	28	39.75 ± 1.3	33.74 ± 4.6	0.35 ± 0.05	0.67 ± 0.09 ns	68.36 ± 9.8 ns	50
	32	29.5 ± 0.7	36.85 ± 1.8	0.36 ± 0.02	0.61 ± 0.03 ns	70.53 ± 3.4 ns	60
	35	33.75 ± 2.0	32.60 ± 1.8	0.34 ± 0.03	0.54 ± 0.03 ns	66.97 ± 5.5 ns	60

1—Cell dry weight; 2—yield of ethanol [$Y_{(P/S)}$]; 3—ethanol productivity [Q_p]; 4—fermentation efficiency (Y%). Statistical analyses compared the effects of different treatments on each yeast species. ± Standard deviation; ns not significant. The values highlighted with different letters are statistically different in the t test ($p < 0.01$)

formation and ethanol production by *Sp. passalidarum* were similar regardless of the temperature (Fig. 2d–f). In contrast to *Sp. arborariae*, *Sp. passalidarum* produced more ethanol than xylitol (Fig. 2a–f). Importantly, ethanol volumetric productivity (Q_p) and fermentation efficiency (Y%) were higher at 32°C when compared to the other two species (Table 2). The species *Sc. stipitis* also produced similar levels of ethanol and biomass at the evaluated temperatures (Fig. 2g–i, Table 2). The highest ethanol concentration (36.8 g/L) was obtained after 60 h of fermentation at 32°C, whereas xylitol production was temperature-dependent, as observed in *Sp. arborariae*. However, xylitol concentration did not exceed ethanol concentration (Fig. 2g–i).

The yeasts showed faster xylose consumption at 28 °C compared to the other temperatures (Fig. 2a, d and g), and the sugar was completely consumed by *Sp. passalidarum* and *Sc. stipitis* within 40 h of fermentation (Fig. 2d and g). On the other hand, *Sp. arborariae* did not completely assimilate xylose at any temperature (Fig. 2a–c). The species *Sp. passalidarum* consumed 100% of xylose within 60 h at 32 °C and 92% within 70 h at 35 °C (Fig. 2e and f). Similarly, *Sc. stipitis* assimilated 97 and 94% of xylose at 32 and 35 °C, respectively (Fig. 2h and i). Based on the presented results, further experiments were carried out at 28°C for *Sp. arborariae* and at 32°C for *Sp. passalidarum* and *Sc. stipitis*.

Glucose and xylose co-fermentation

In the co-fermentation assays, the yeasts consumed all glucose between 20 and 30 h of fermentation (Fig. 3). The rates of total sugar consumption were higher in *Sp. passalidarum* and *Sc. stipitis* than in *Sp. arborariae* (Table 3).

In media containing 20 g/L glucose, ethanol production by *Sp. passalidarum* and *Sc. stipitis* was 29.28 and 29.27 g/L, respectively, whereas 25.25 and 32.62 g/L were achieved when 40 g/L glucose was used. The amount of

ethanol produced by *Sp. arborariae* was similar under both conditions. Glucose decreased the fermentation efficiency (Y%) of *Sp. arborariae* and *Sp. passalidarum* in the co-fermentation assays, although this parameter was unaffected in *Sc. stipitis*. Ethanol productivity by yeasts was statistically higher in the absence of glucose (Table 4).

Xylitol production in 20 or 40 g/L glucose was higher in *Sp. arborariae* (13.3 and 15.8 g/L), followed by *Sp. passalidarum* (6.5 and 8.1 g/L) and *Sc. stipitis*, which produced xylitol concentrations below 6 g/L (Fig. 3). Again, *Sp. arborariae* produced more xylitol when compared to the other yeasts.

Effects of compounds generated from pre-treatment on the fermentative capacity of yeasts

The effects of furfural, HMF, and acetic acid on the fermentative capacity of *Sp. arborariae*, *Sp. passalidarum*, and *Sc. stipitis* were analyzed (Fig. 4). In the absence of inhibitors, *Sp. arborariae* consumed all xylose and reached the maximum ethanol production (7.7 g/L) within 30 h (Fig. 4a). In the presence of furfural, the yeast had a long lag phase, and biomass was effectively produced only after 30 h of cultivation (Fig. 4d). Growth coincided with xylose consumption and depletion of furfural. In the presence of HMF, biomass production was similar to that of the control, xylose consumption occurred in the initial hours of cell cultivation, and ethanol production was not impaired (Fig. 4g).

In the control condition, almost all xylose was consumed by *Sp. passalidarum* within 9 h of fermentation, and ethanol production reached 10.2 g/L (Fig. 4b). Furfural also extended the yeast's lag phase and slowed xylose consumption. Effective biomass production occurred only after the inhibitor was completely depleted (20 h). The ethanol concentration reached its maximum (9.1 g/L) at 30 h (Fig. 4e). Hydroxymethylfurfural concentration was reduced by 42.4%

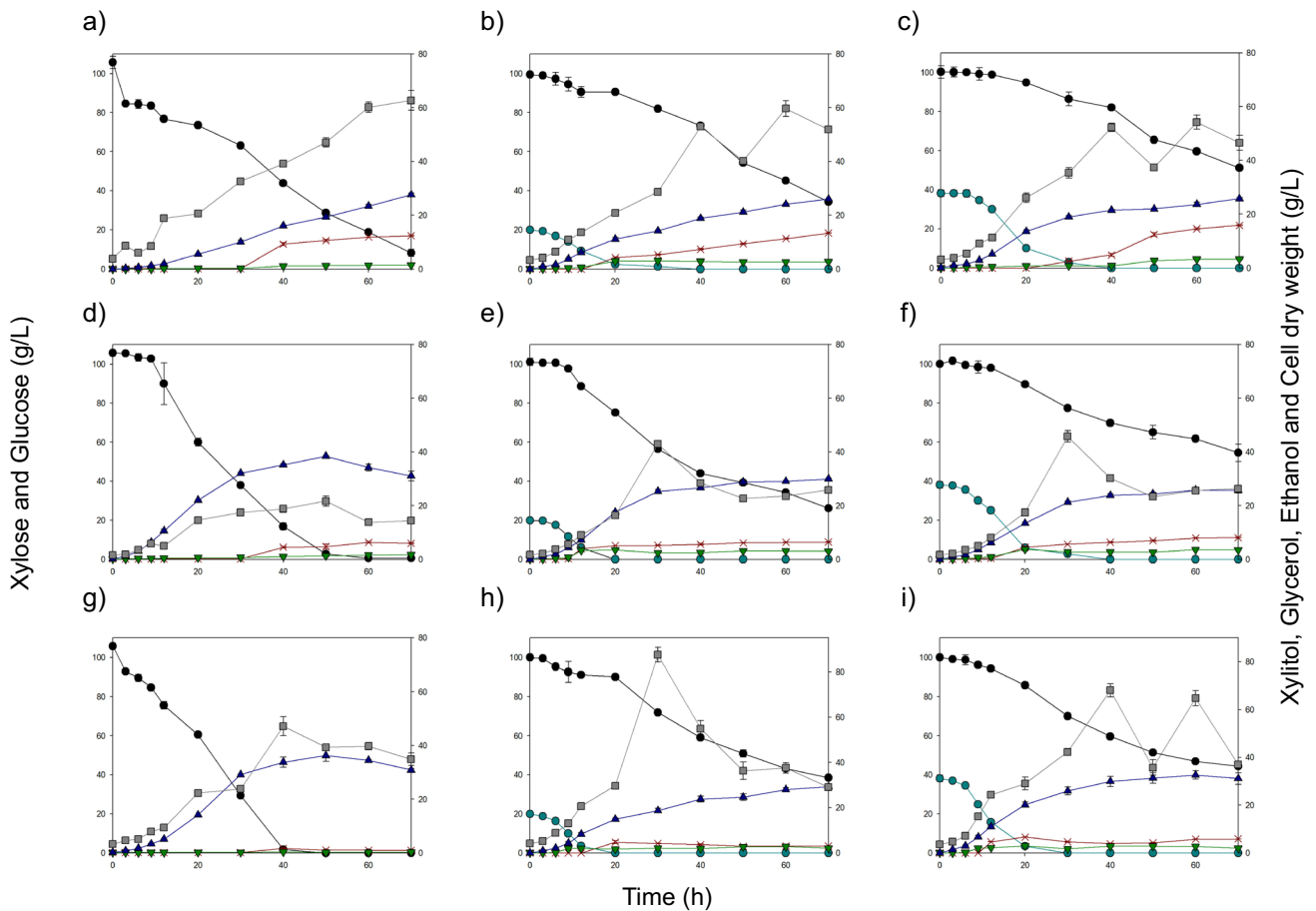


Fig. 3 Co-fermentation kinetics in 100 g/L of xylose and 0, 20, or 40 g/L of glucose, respectively. *Spathaspora arborariae* (a, b, and c), *Spathaspora passalidarum* (d, e, and f), and *Scheffersomyces stipitis* (g, h, and i). Glucose (●); xylose (●); xylitol (X); glycerol (▼); ethanol (▲); cell dry weight (■)

Table 3 Evaluation of sugar yeast consumption in co-fermentation assays

Yeast strains	Carbon source concentration (g/L)	Total sugar consumption rate (g/L. h)	Specific sugar consumption rate (g _{sugar} . g ⁻¹ _{CDW} . h ⁻¹)
<i>Spathaspora arborariae</i>	Xylose 100	1.22 ± 0.02	0.085
<i>Spathaspora passalidarum</i>		1.51 ± 0.01	0.40
<i>Scheffersomyces stipitis</i>		1.46 ± 0.06	0.11
<i>Spathaspora arborariae</i>	Xylose 100 /	0.68 ± 0.05/ 0.50 ± 0.01	0.12/ 0.14
<i>Spathaspora passalidarum</i>	Glucose 20	1.31 ± 0.11/ 0.99 ± 0.02	0.26/ 0.32
<i>Scheffersomyces stipitis</i>		0.73 ± 0.45/ 0.99 ± 0.02	0.08/ 0.15
<i>Spathaspora arborariae</i>	Xylose 100 /	0.42 ± 0.02/ 0.95 ± 0.04	0.02/ 0.14
<i>Spathaspora passalidarum</i>	Glucose 40	0.75 ± 0.05/ 0.95 ± 0.05	0.11/ 0.31
<i>Scheffersomyces stipitis</i>		1.08 ± 0.1/ 1.27 ± 0.06	0.05/ 0.21

± Standard deviation

within 3 h. After 12 h, a complete depletion of xylose was seen and 11.8 g/L of ethanol had been produced (Fig. 4h). No significant difference in ethanol yield ($Y_{P/G}$) was found when comparing the control and HMF conditions.

The species *Sc. stipitis* produced 8.1 g/L of ethanol after 30 h when grown in the presence of only xylose (Fig. 4c). Unlike the other species, *Sc. stipitis* reduced 60% of furfural within 3 h and 99% within 6 h; ethanol production was

Table 4 Evaluation of yeast fermentation parameters in co-fermentation assays

Yeast strains	Glucose (g/L)	CDW ¹ (g/L)	Ethanol (g/L)	Y _(P/S) ² (g/g)	Q _p ³ (g/L.h)	Y% ⁴	Time (h)
<i>Spathaspora arborariae</i>	0	62.7 ± 3.6	27.58 ± 0.7	0.32 ± 0.01	0.39 ± 0.01 ^a	62.84 ± 1.5 ^a	70
	20	51.9 ± 1.0	26.03 ± 0.02	0.29 ± 0.01	0.37 ± 0.01 ^{ab}	57.71 ± 0.6 ^{ab}	70
	40	46.5 ± 2.8	25.21 ± 0.9	0.28 ± 0.02	0.36 ± 0.01 ^b	55.42 ± 3.8 ^b	70
<i>Spathaspora passalidarum</i>	0	17.4 ± 1.0	30.68 ± 2.4	0.44 ± 0.03	1.02 ± 0.08 ^a	87.01 ± 5.3 ^a	30
	20	25.8 ± 0.4	29.28 ± 1.2	0.32 ± 0.01	0.42 ± 0.02 ^b	62.27 ± 2.4 ^b	70
	40	25.6 ± 0.6	25.25 ± 1.0	0.32 ± 0.03	0.42 ± 0.02 ^b	62.68 ± 6.1 ^b	60
<i>Scheffersomyces stipitis</i>	0	29.2 ± 0.8	36.85 ± 1.8	0.36 ± 0.02	0.61 ± 0.03 ^a	70.53 ± 3.4 ns	60
	20	27.2 ± 3.3	29.27 ± 1.4	0.36 ± 0.02	0.42 ± 0.02 ^c	70.13 ± 3.9 ns	70
	40	64.9 ± 3.2	32.62 ± 1.7	0.35 ± 0.04	0.54 ± 0.03 ^b	68.38 ± 7.2 ns	60

1—Cell dry weight; 2—yield of ethanol [Y_(P/S)]; 3—ethanol productivity [Q_p]; 4—fermentation efficiency (Y%). Statistical analyses compared the effects of different treatments on each yeast species. ± Standard deviation; ns not significant. The values highlighted with different letters are statistically different in the t test (p < 0.01)

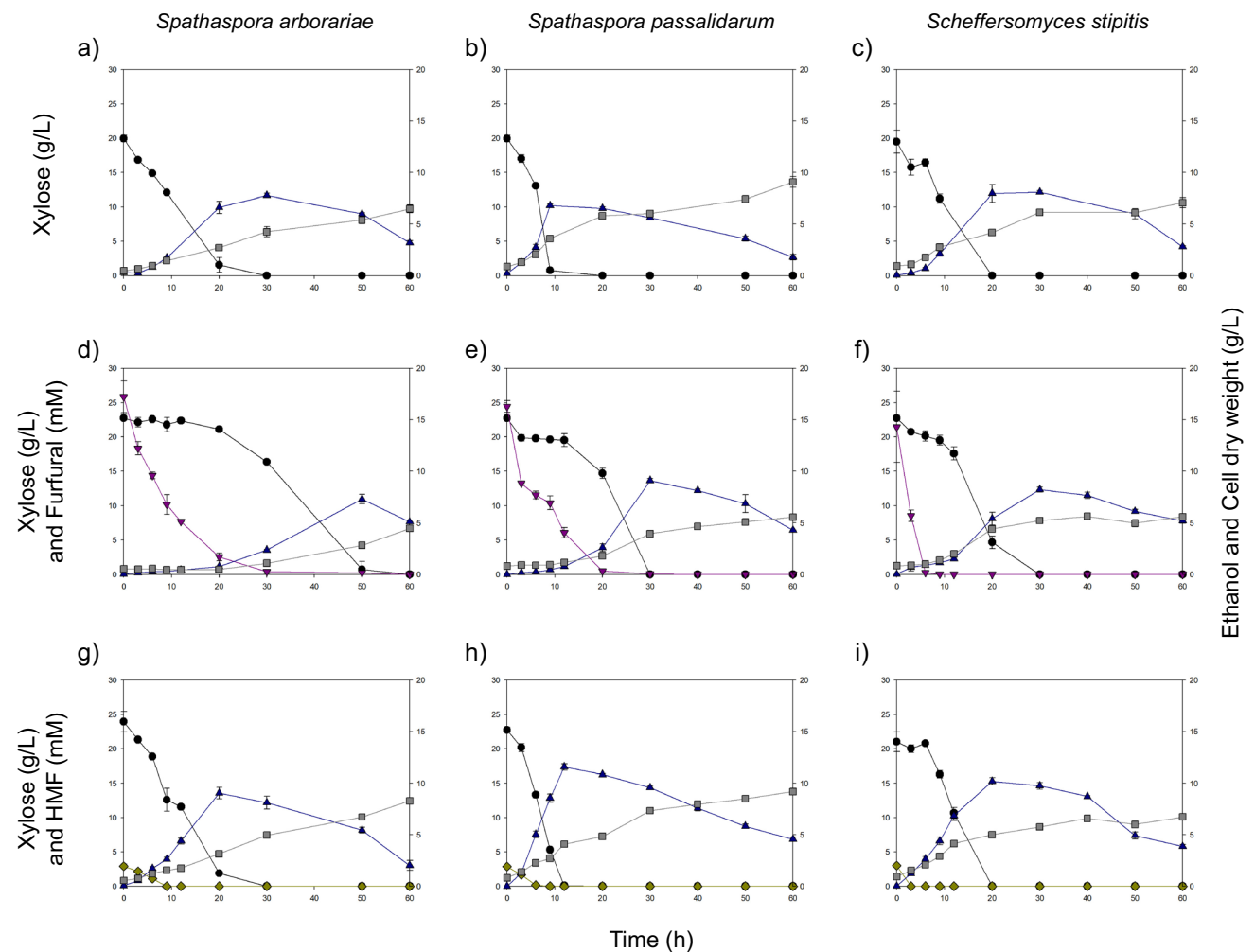


Fig. 4 Yeast fermentation profiles in the absence and presence of inhibitors. Furfural or hydroxymethylfurfural (HMF) was added into the YPX media at different concentrations. *Spathaspora arborariae*

(a, d, and g), *Spathaspora passalidarum* (b, e, and h), and *Scheffersomyces stipitis* (c, f, and i). Xylose (●); ethanol (▲); furfural (▼); hydroxymethylfurfural (◆); cell dry weight (■)

8.23 g/L at 30 h (Fig. 4f). In the presence of HMF, xylose was completely consumed within 20 h, when the maximum ethanol production of 10.2 g/L was achieved. The HMF was depleted after 3 h (Fig. 4i).

The yeasts were not able to grow and produce ethanol in the presence of (3 g/L) acetic acid, and therefore, its concentration did not change over time (data not shown). During the experiment, only small amounts of xylose were consumed, especially in the early hours. For these reasons, the fermentative parameters were not determined.

Effect of ethanol on fermentation

The species *Sp. arborariae*, *Sp. passalidarum*, and *Sc. stipitis* consumed all xylose within 30, 12, and 20 h, respectively, when grown in media containing 2% (v/v) ethanol (Fig. 5a-c). The presence of 4% (v/v) of ethanol reduced the yeasts growth (Fig. 5d-f). At this condition, *Sp. arborariae* did not consume all sugar within the 60 h of fermentation (Fig. 5a). Xylose consumption was also delayed in *Sp. passalidarum* and *Sc. stipitis* in this ethanol concentration. However, *Sp. passalidarum* consumed all sugar within 20 h, whereas *Sc. stipitis* consumed about 98% of xylose within 60 h (Fig. 5b and c). The species *Sp.*

arborariae, *Sp. passalidarum*, and *Sc. stipitis* did not grow at 6% (v/v) of ethanol (Fig. 5d-f), and under this condition, xylose concentration was reduced by 26, 36.7, and 22.5%, respectively (Fig. 5a-c).

Effect of ethanol on the expression of classical stress response genes in *Sp. passalidarum*

The yeast *Sp. passalidarum* was selected for further molecular studies in the presence of ethanol stress. The expression of eight canonical genes with well-documented roles in different mechanisms of stress response in *S. cerevisiae* was analyzed in *Sp. passalidarum* under ethanol stress [25, 26]. These genes are involved in several processes such as carbon metabolism and fermentation (*ADH1*, *ADH7*, *TDH1*, and *TPS1*), protein disruption (*HSP104*), ATPase regulation (*HSP30*), superoxide detoxification (*SOD1*), and regulation of transcription in response to different stresses (*MSN2/4*). Under stress, the expression of the genes *TDH1*, *ADH1*, *TPS1*, *HSP30*, and *MSN2/4* was down-regulated, while the expression of *SOD1* was up-regulated. No significant difference was detected in the expression of *ADH7* and *HSP104* (Fig. 6).

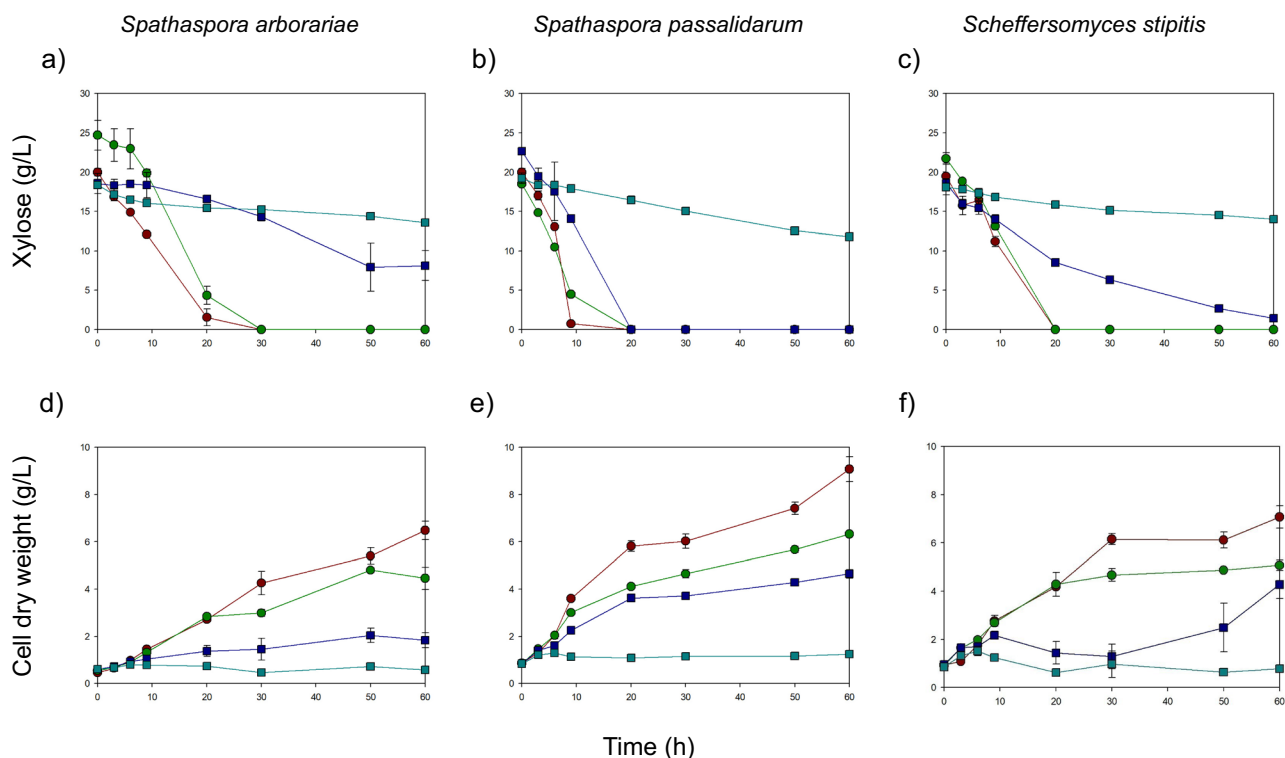
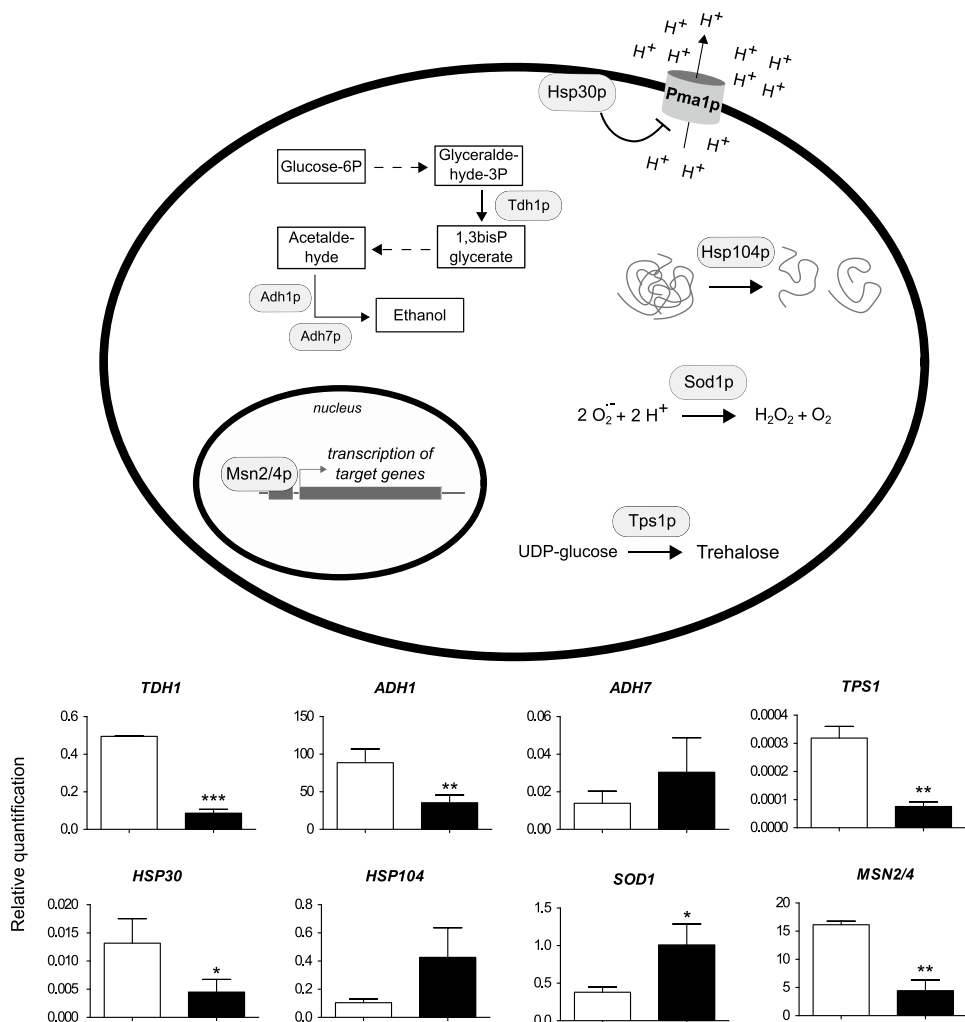


Fig. 5 Xylose consumption and biomass production in the presence of different concentrations of ethanol by *Spathaspora arborariae* (a and d), *Spathaspora passalidarum* (b and e), and *Scheffersomyces stipitis* (c and f). Control (●); ethanol 2% (●); ethanol 4% (■); ethanol 6% (■)

Fig. 6 Expression of genes related to ethanol response in *Spathaspora passalidarum*. The expression profile of genes involved in *S. cerevisiae* response to ethanol (*TDH1*, *ADH1*, *ADH7*, *TPS1*, *HSP30*, *HSP104*, *SOD1*, and *MSN2/4*) was analyzed in *Sp. passalidarum*. Total RNA was extracted from cells grown in the absence (white column) or presence (black column) of 4% (v/v) ethanol after 2 h of stress exposure. The graphs represent the estimated mean and standard deviation values. Asterisks denote statistical significance by the T test (*, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001)



Discussion

Ethanol production of *Sp. arborariae* HM19.1A, *Sp. passalidarum* NRRLY 27,907, and *Sc. stipitis* NRRL 7124 was evaluated at three different xylose concentrations. In agreement with other reports [14, 27], we observed that the yeasts consumed all substrate present in the medium and ethanol was the main product formed. Overall, *Sp. passalidarum* presented the best yields and ethanol production values, followed by *Sc. stipitis*. Veras and collaborators [14] also reported good performances of *Sp. passalidarum* and *Sc. stipitis* upon the comparison of the fermentative capacity of different xylose-consuming yeasts. Similarly, Cadete and collaborators [24] showed that *Sp. passalidarum* stands out as the best xylose fermenter when compared to other yeasts of the same genus.

Spathaspora arborariae had the lowest ethanol yield and presented the highest xylitol production. Xylitol is a by-product of the xylose assimilation pathway. First, xylose is reduced to xylitol by the enzyme xylose reductase (XR),

which is then oxidized to xylulose by the enzyme xylitol dehydrogenase (XDH). Xylulose is phosphorylated by the enzyme xylulokinase, forming xylulose-5-phosphate, which is metabolized by the pentose-phosphate pathway and then directed to glycolysis, where ethanol is produced [28]. Other studies have also demonstrated xylitol production in synthetic media and lignocellulosic hydrolysates by *Sp. arborariae* [5, 29]. In the present study, higher xylitol concentrations were obtained with increasing temperatures. Overall, other yeast species that produce xylitol have also shown better yields at cultivation temperatures ranging from 30 to 37 °C [30], which can be due to changes in the enzymatic activities of xylose reductase and xylitol dehydrogenase. Several optimal temperature values for the XR enzyme are reported in the literature, varying according to the yeast species [31]. Future studies with *Sp. arborariae*, the yeast that produced the highest xylitol amounts, should investigate this interesting feature aiming at xylitol production.

The simultaneous consumption of glucose and xylose is an important feature for microorganisms that ferment

lignocellulosic biomass as these two sugars are the most abundant carbohydrates in this substrate [32]. Our results demonstrate that glucose reduced the rate of xylose consumption in the co-fermentation assays. Increasing concentrations of glucose resulted in a greater reduction in the xylose consumption rate of all yeasts tested. This might be due to sugar competition by transporters [16] and inhibition of the synthesis and/or activity of xylose metabolism enzymes [16, 32]. However, even after glucose depletion, the estimated xylose consumption rates were lower when compared to the fermentation assay containing only xylose. Thus, the presence of glucose possibly affects the cellular metabolism in a global way, as suggested by Conrad and collaborators [33]. Collectively, our results show that the presence of glucose decreased ethanol productivity of the yeasts and reduced ethanol yields. In fact, it was recently shown that glucose inhibits xylose metabolization in *Sp. passalidarum* by decreasing the expression of genes and the activity of key enzymes of xylose metabolism [34].

The final ethanol concentration produced in the presence of furfural and HMF was similar to the amount observed in the control condition. The presence of 2.5 g/L of furfural delayed yeast cell growth and ethanol production until the inhibitor could no longer be detected in the medium. Some *S. cerevisiae* strains can reduce furfural to furfuryl alcohol, which is less toxic to the cell, a process known as in situ detoxification [35]. Probably, the xylose-fermenting yeasts evaluated in the present study have a similar reducing mechanism. Some methods of lignocellulosic biomass pretreatment can also minimize furfural concentration in the raw material. Tanaka and collaborators [36] demonstrated that a pretreatment with two-step acid hydrolysis at a moderate temperature reduced furfural content in the hydrolysate. Another alternative to minimize inhibitor effect is to increase the initial cell concentration for fermentation with non-detoxified hydrolysates. A higher initial cell concentration of *Blastobotry adenivorans* degraded furfural in non-detoxified acid hydrolysate more quickly, leading to complete consumption of sugars and increased ethanol production [37].

Fermentation was not inhibited by 0.5 g/L HMF. Our study shows that the yeasts were able to metabolize HMF and consume xylose simultaneously with no influence on the lag phase, indicating that a detoxifying mechanism is present in the yeasts. According to the literature, some strains of *S. cerevisiae* can convert hydroxymethylfurfural to 2,5-bis-hydroxymethylfuran, a compound that is less toxic to the cells [38]. Despite our results showing that HMF did not affect the yeasts fermentation in synthetic medium, the synergistic effect of this compound along with the other pretreatment inhibitors in lignocellulosic hydrolysates can interfere with yeasts growth and fermentation abilities. Thus, the generation of microorganism

with evolved higher tolerance to pretreatment inhibitors is an important strategy to overcome this issue [5, 39, 40].

Acetic acid can be highly toxic to yeasts due to diffusion through the plasma membrane and dissociation in the cytoplasm, which reduces the intracellular pH and affects cell metabolism [41]. Our results show that acetic acid affected cell growth, xylose consumption, and ethanol production of the xylose-fermenting yeasts. Other authors have also reported a high toxicity of acetic acid for yeasts such as *S. cerevisiae* and *Sc. (Pichia) stipitis* [41–43]. The detoxification of hydrolysates can occur before fermentation to remove or decrease the concentration of the inhibitory compounds, reducing their harmful effects on the process [44].

One of the most common stresses that yeasts face during fermentation is the accumulation of ethanol. At high concentrations, ethanol promotes a complex stress that affects different cellular processes and structures, which can end up limiting yeast cell growth, viability, and fermentative activity [45]. The yeasts studied in this work have a low ethanol tolerance profile as they were unable to grow at 6% (v/v) ethanol. The species *Sp. passalidarum* was less affected by 4% (v/v) ethanol and presented a higher ethanol tolerance compared to the other yeasts. Still, the improvement of *Sp. passalidarum* ethanol tolerance would largely contribute to accelerating its biotechnological use for 2G bioethanol production since the ethanol titer obtained in these fermentations varies between 5 and 7% [20]. Such ethanol concentrations are higher than the ones this yeast can support and remain fermenting.

Based on the screening we have performed in this work with the three xylose-fermenting yeasts, we noticed that their low ethanol tolerance was an interesting subject to further our studies. Ethanol is the final product of fermentative metabolism. Thus, the production and tolerance to high ethanol concentrations are desirable traits for fermentative processes. Much effort has been employed so far to understand the stress response of *S. cerevisiae* [45–56]. The phenotype of low ethanol tolerance presented by non-conventional yeasts has not received much attention. Our results pointed out that *Sp. passalidarum* was the best candidate for the context of xylose fermentation among the yeasts studies. Thus, we performed gene expression analysis with *Sp. passalidarum* growing in a medium containing 4% (v/v) ethanol for 2 h to investigate the expression of classical genes involved in yeast ethanol response. The qPCR results showed that the genes encoding the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase 1 (*TDHI*) and the fermentation enzyme alcohol dehydrogenase 1 (*ADHI*) were down-regulated by ethanol, while the expression of the gene encoding *ADH7*, another alcohol dehydrogenase, was not significantly altered. This profile suggests that even in mild ethanol concentrations, *Sp. passalidarum* reduces glycolytic flux and

ethanol production to minimize the stress effect on the cells that would enhance with ethanol accumulation.

TPS1 and *HSP30* genes were also down-regulated in *Sp. passalidarum* under stress, which is the opposite to the demonstrated in the literature for *S. cerevisiae* [46]. Trehalose accumulation in *S. cerevisiae* increases resistance to several adverse environmental conditions, and the overexpression of *TPS1* (trehalose-6-phosphate synthase) improves ethanol tolerance [47]. Hsp30p is a membrane heat-shock protein that regulates H⁺-ATPase Pma1p activity, having roles in preventing ATP depletion during cytosolic deacidification in the presence of ethanol [48, 49]. The expression of *HSP104*—a chaperone with disaggregase function that is responsive to a wide range of environmental stimuli [50]—was not significantly altered by ethanol stress in our assay. In *S. cerevisiae*, the expression of *HSP104* increases with increasing ethanol concentrations [51], and its expression is regulated by several transcription factors, such as Msn2/4p and Hsf1p [52]. In *Sp. passalidarum*, the transcription of this gene was less effective and the expected up-regulation was not observed.

Interestingly, *SOD1* (Cu/Zn superoxide dismutase), which is involved in the detoxification of superoxides, was the only up-regulated gene among the ones analyzed in our study. The expression of this gene is induced by oxidative stress, which is also caused by ethanol [53]. The formation of superoxides can promote cellular apoptosis and affect the structures of lipids, proteins, and nucleic acids [54]. The overexpression of *SOD1* improves ethanol tolerance in *S. cerevisiae* [55]. Thus, *Sp. passalidarum* probably reduced part of the damage caused by reactive oxygen species (ROS) and maintained cell viability by a similar mechanism.

The *MSN*-like gene of *Sp. passalidarum* was down-regulated under ethanol stress. Msn2p and Msn4p are partially redundant stress-responsive transcription factors of *S. cerevisiae* that regulate the expression of more than 200 genes in response to various environmental stresses, including ethanol [46, 56]. *Sp. passalidarum* presents only one copy of these transcription factors. The down-regulation of this gene in *Sp. passalidarum* may have affected the gene expression profile of this yeast and its final response to ethanol stress.

Conclusion

Among the three xylose-fermenting yeasts evaluated in the present study, *Sp. passalidarum* stood out as the best candidate to in-depth the studies regarding ethanol production from xylose based on its fermentative parameters in the conditions assessed. When analyzing the physiological responses of *Sp. passalidarum* in different sugar concentrations and temperatures, presence of glucose, furfural, HMF, acetic acid, and ethanol, our data showed that the negative effect of glucose on xylose metabolism, and the

low tolerance to acetic acid and ethanol appear as the most important aspects to be improved to increase its performance for the production of 2G ethanol.

Regarding the ethanol stress response of *Sp. passalidarum*, our expression analysis showed that some genes important for cells facing the effects of ethanol challenge were down-regulated in this yeast subjected to 4% (v/v) ethanol. It suggests that *Sp. passalidarum* may not have activated a proper response to cope with ethanol stress, which could have contributed to the low tolerance profile exhibited by the yeast. Future studies investigating how *Sp. passalidarum* regulates gene expression in response to ethanol stress can contribute to obtaining more robust strains of this yeast with increased ethanol tolerance.

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Author Contribution LF, CA, and WS conceived and designed the research. VC, LR, FA, and PF conducted the experiments. AG performed the statistical analysis. LF, VC, LR, FA, and AG analyzed the data. All authors contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Availability of data and material Not applicable.

Code availability Not applicable.

Declarations

Conflicts of interest The authors declare that they have no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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