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RESEARCH PAPER

Prospecting and engineering yeasts for ethanol production under inhibitory conditions: an experimental design analysis

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Abstract

The recently discovered wild yeast *Wickerhamomyces* sp. UFFS-CE-3.1.2 was analyzed through a high-throughput experimental design to improve ethanol yields in synthetic media with glucose, xylose, and cellobiose as carbon sources and acetic acid, furfural, formic acid, and NaCl as fermentation inhibitors. After Plackett–Burman (PB) and central composite design (CCD), the optimized condition was used in a fermentation kinetic analysis to compare this yeast's performance with an industrial *Saccharomyces cerevisiae* strain (JDY-01) genetically engineered to achieve a higher xylose fermentation capacity and fermentation inhibitors tolerance by overexpressing the genes *XYL1*, *XYL2*, *XKS1*, and *TAL1*. Our results show that furfural and NaCl had no significant effect on sugar consumption by UFFS-CE-3.1.2. Surprisingly, acetic acid negatively affected glucose but not xylose and cellobiose consumption. In contrast, the pH positively affected all the analyzed responses, indicating a cell's preference for alkaline environments. In the CCD, sugar concentration negatively affected the yields of ethanol, xylitol, and cellular biomass. Therefore, fermentation kinetics were carried out with the average concentrations of sugars and fermentation inhibitors and the highest tested pH value (8.0). Although UFFS-CE-3.1.2 fermented glucose efficiently, xylose and cellobiose were mainly used for cellular growth. Interestingly, the genetically engineered strain JDY-01 consumed ~ 30% more xylose and produced ~ 20% more ethanol. Also, while UFFS-CE-3.1.2 only consumed 32% of the acetic acid of the medium, JDY-01 consumed > 60% of it, reducing its toxic effects. Thus, the overexpressed genes played an essential role in the inhibitors' tolerance, and the applied engineering strategy may help improve 2G ethanol production.

Keywords *Wickerhamomyces* sp. · *Saccharomyces cerevisiae* · Fermentation · Glucose · Xylose · Acetic acid

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Introduction

Second-generation (2G) biorefineries use lignocellulosic residues as raw material, and this production process depends on the hydrolysis of these biomasses rich in cellulose and hemicellulose. As a result of this hydrolysis, mainly the hexose glucose, the pentose xylose, and the disaccharide cellobiose (consisting of two glucose molecules joined together by a ß-1,4 glycosidic bond) are obtained—cellobiose, though, is present in a proportion of at least five times smaller in relation to the two mentioned monosaccharides [1–4]. Therefore, the production of 2G ethanol depends on the efficient fermentation of glucose, xylose, and cellobiose. However, the yeast species currently used in the bioethanol production (*Saccharomyces cerevisiae*) is capable of fermenting only the hexose among these three carbohydrates. To overcome this problem, 2G ethanol depends either on the

selection of new species that can metabolize not only glucose but also xylose and cellobiose, or on the construction of genetically modified strains of *S. cerevisiae* to enable it to ferment these two carbohydrates [5, 6].

Biorefineries, however, can produce more than one product from the same lignocellulosic residue. In fact, in addition to ethanol, more recently, xylitol has also been widely discussed in the literature, given its numerous applications, especially in the pharmaceutical, cosmetics, and food industries [7–9]. In this sense, while glucose and cellobiose can be fermented to ethanol by the yeasts used in the process, xylose can be converted either into ethanol or into the so-referred sugar-alcohol.

For xylose fermentation into ethanol, yeast cells are required to be able to express the enzymes xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK). In subsequent reactions, these enzymes will convert xylose to xylitol, xylulose, and, finally, xylulose-5P, which is then destined for the pentose phosphate pathway. Also, it is desirable for XR and XDH to be capable of recycling the same coenzyme in both oxide-reduction reactions (NADH/NAD+), thus avoiding a possible redox imbalance inside the cells, which tends to accumulate xylitol to the detriment of ethanol production [6, 10]. Indeed, this causes some species of yeast to end up only generating xylitol from xylose, which also, as mentioned above, presents itself as an important biotechnological product [11, 12].

However, the fermentative capacity is not the only desirable characteristic for the microorganism to be used in the process. Inhibitor compounds of the fermentative metabolism, such as furfural, formic acid, and predominantly acetic acid, are generated in the pretreatment and hydrolysis stages of the process, which are carried out before fermentation. Due to the presence of these compounds, to guarantee efficiency in the process, it is necessary to select yeasts (prospecting wild species or strains) tolerant to their toxic effects [3, 13, 14] or to genetically engineer *S. cerevisiae* to increase its tolerance to those inhibitor compounds. Thus, in the context of genetic-engineered yeasts, besides the heterologous expression of XR and XDH enzymes recycling the same coenzyme, and the overexpression of XK, some authors have pointed to the modification of the genes that encode the enzymes of the pentose phosphate pathway (PPP) as a strategy to increase tolerance to the inhibitory compounds [15–19]. In their work with laboratory strains of *S. cerevisiae,* Hasunuma et al. [20, 21] demonstrated that increasing tolerance to inhibitors through overexpression of PPP enzymes (such as the transaldolase encoded by the *TAL1* gene) consequently increased ethanol yields in fermentations carried out under different concentrations of weak acids (acetic acid and formic acid). However, as most of these studies used laboratory strains of *S. cerevisiae*, there is still uncertainty regarding the reproducibility of the results when conducted in industrial strains or strains derived from them and isolated from industrial environments, which have genomic adaptations different from those found in laboratory yeasts [22, 23]. Indeed, several reports have started to show that the phenotypic consequences of genomic modifications (e.g., gene deletions) can vary considerably between different strain backgrounds [24], an issue that can have significant implications in metabolic engineering strategies for generating optimized industrial yeast strains [25, 26].

Another desirable characteristic of an industrial yeast is its tolerance to the osmotic stress caused by high sugar concentration. This is, in fact, one of the differentials of *S. cerevisiae*, which is widely recognized for its high fermentative performance even at sugar concentrations as high as 200 g/L [27]. Not that this is a rule for every bioprocess, but normally the higher the sugar concentration, the higher the productivity of the target product, in such a way that the prospection or development of new yeasts includes higher tolerance to a hyperosmotic environment as a targeted trait. Although the industrial fermentation vats are known to select and domesticate xerotolerant yeasts [28], nature also presents niches with reduced water activity where resistant wild strains can be found, and this has recently shown to be quite promising [29].

Last but not least, besides the factors mentioned above, the biorefineries' water footprint has also been identified as a parameter to be optimized [30]. In this sense, the employment of seawater in the fermentation vats is envisaged, partially or totally replacing the use of freshwater. For this, however, fermenting microorganisms must also tolerate high concentrations of salt in addition to the characteristics mentioned above [31–33].

In this context, the present work compared the biotechnological potential of two taxonomically distant yeast strains: the recently isolated wild yeast *Wickerhamomyces* sp. UFFS-CE-3.1.2 [1] and a new genetically modified industrial strain derivative from the widely known *S. cerevisiae* PE-2 [34–36]. After subjecting UFFS-CE-3.1.2 to two subsequent experimental design analyses in synthetic media with the carbohydrates glucose, xylose, and cellobiose, as well as the inhibitors furfural, acetic acid, formic acid, and NaCl, the optimized culture condition was applied to a fermentation kinetics assay to compare the performances of both the wild and genetically engineered strains.

Materials and methods

Yeasts

Two yeasts were used: the wild yeast *Wickerhamomyces* sp. UFFS-CE-3.1.2, previously isolated from decaying wood samples [1], and the genetically modified strain *S. cerevisiae*

JDY-01. This industrial recombinant strain was constructed based on the efficient industrial fuel ethanol yeast strain PE-2 [36]. This diploid *S. cerevisiae* strain was initially transformed with the chromosome-integrative plasmid pAUR-XKXDHXR [37] allowing overexpression of xylose reductase (XR, encoded by *XYL1*) and xylitol dehydrogenase (XDH, encoded by *XYL2*) from *Scheffersomyces stipitis*, as well as xylulokinase (XK, encoded by *XKS1*) from *S. cerevisiae* [38, 39]. Briefly, plasmid pAUR-XKXDHXR was digested with *Bsi*WI, and then chromosomally integrated into the *AUR1* locus of the yeast strain. After 90 min cultivation on rich YP medium (10 g/L yeast extract and 20 g/L peptone, pH5.0) containing 20 g/L glucose, the transformed cells were selected on plates of the same medium containing 20 g/L agar and 0.5 mg/L aureobasidin A (Takara Bio, Kyoto, Japan), producing strain MP-P5 (isogenic to PE-2, but *AUR1-C*::P_{PGK1}-*XKS1*-T_{PGK1}, P_{PGK1}-*XYL2*-T_{PGK1}, P_{PGK1}-*XYL1*-T_{PGK1}). Yeast transformation was performed by the lithium acetate method [40]. Strain MP-P5 was further improved by promoting the overexpression of the *S. cerevisiae TAL1* gene encoding the transaldolase enzyme of the non-oxidative pentose phosphate pathway [41, 42]. The promoter region of this *TAL1* gene was modified according to the polymerase chain reaction (PCR)-based gene replacement procedure [43]. The *kanMX*-P_{ADH1} module from plasmid pFA6a-kanMX6-PADH1 [43] was amplified using primers TAL1-Kan^r-F and TAL1-PADH1-R (Table 1), and the PCR product of 2394 bp (flanked by 40 bp of homology to the promoter and start regions of the *TAL1* gene) containing the *kanMX6* gene and the constitutive promoter of the *ADH1* gene was used to transform competent yeast cells. After 2-h cultivation on YP-20 g/L glucose, the transformed yeast cells were plated on the same medium containing 20 g/L agar and 200 mg/L G-418 (Merck Sigma Aldrich Brasil, Barueri, Brazil) and incubated at 28 °C. G-418-resistant isolates were tested for proper genomic integration of the *kanMX*-P_{ADH1} cassette at the *TAL1* locus by diagnostic PCR using 3 primers (V-TAL1-F, V-TAL1-R, and V-kan^r-F; Table 1). This set of three primers amplified a 501 bp fragment (primers V-TAL1-F and V-TAL1-R) from a normal TAL1 locus, or yielded a 2,700 bp fragment (primers V-TAL1-F and V-TAL1-R) or a 1479 bp fragment (primers V-kan$_r$-F and V-TAL1-R) if the *kanMX*-P_{ADH1} module was correctly integrated at the promoter region of the *TAL1* gene, producing strain JDY-01.

Table 1 Primers used in this study

Primers:	Sequence (5′ → 3′)[a]
TAL1-Kanr-F	**GTGTATGTGTACACCTGTATTTAATTTCCTTACTCGCGGG** *CCAGCTGAAGCTTCGTACGC*
TAL1-PADH1-R	**AGTTGTTAGCAACCTTTTGTTTCTTTTGAGCTGGTTCAGA** *CATTGTATATGAGATAGTTG*
V-TAL1-F	GAGCTACTGGTTGCTGTGAC
V-TAL1-R	GCAATAGAGCCGAAATCACC
V-kan^r-F	CCGGTTGCATTCGATTCC
RT-PCR TAL1-F	GGCCCAAGTTACTTTGATTTCC
RT-PCR TAL1-R	TCGGCTTCACCCTTGTAATC
RT-PCR ACT1-F	TGGATTCCGGTGATGGTGTT
RT-PCR ACT1-R	CGGCCAAATCGATTCTCAA

[a]Bold sequences are homologous to the upstream and downstream region of the target *TAL1* gene and its promoter that was modified, and italicized sequences allow amplification of the transformation module present in plasmid pFA6a-kanMX6-PADH1 [37]

Culture media and growth conditions

Cells were grown in cotton-plugged Erlenmeyer flasks with 1/5 of their volume filled with culture medium in a shaker with controlled temperature and agitation. Before each experimental design, the yeasts were pre-grown for 48 h at 30 °C and 150 rpm in YP rich media containing 20 g/L of glucose until they reached the exponential growth phase (OD_{570nm} ~ 3,5). Then the cells were inoculated in minimal YNB synthetic media (6.7 g/L of yeast nitrogen base) plus different concentrations of glucose, xylose, cellobiose, acetic acid, furfural, formic acid, and NaCl, according to the experimental designs described below. Likewise, the cultures were carried out in different pH ranges, temperature, and agitation according to the matrices presented in Tables 2 and 3.

Plackett–Burman's experimental design

The Plackett–Burman (PB) experimental design matrix was assembled and analyzed using the Protimiza Experimental Design software [44] with the following variables: temperature, pH, agitation, and concentration of inoculum, sugars, and inhibitors (Table 2). The inoculum of each PB fermentation was prepared with yeasts pre-grown as described above. Then the cells were centrifuged (5000*g*, 3 min), washed twice with distilled water, and resuspended in the fermentation culture medium to reach an initial cell concentration of 1, 3, or 5 g/L (Table 2). After 48 h fermentation, cells were centrifuged (5000*g*, 3 min), and supernatants were filtered (0.45 µm) for subsequent quantification of sugar consumption by HPLC as described in the "Analytical methods".

Central composite design

The central composite design (CCD) was also assembled and analyzed with Protimiza software. Pre-grown cells were inoculated at the concentration of 1:100 (v/v—volume of pre-inoculum per volume of medium to be inoculated). Yeasts were cultivated at 30 °C and 150 rpm in

Table 2 Plackett–Burman design and analysis with 11 variables and 3 responses in 48 h batch fermentation by *Wickerhamomyces* sp. UFFS-CE-3.1.2

Assay	Variables											Responses		
	Temp. (°C)	pH	Inoculum (g/L)	Glucose (g/L)	Xylose (g/L)	Cellobiose (g/L)	Acetic acid (g/L)	Formic acid (g/L)	Furfural (g/L)	NaCl (g/L)	Agitation (rpm)	Sugar consumption (%)		
												Glucose	Xylose	Cellobiose
1	40	3	1	10	40	2	0	1	2	0	150	0.1	0.1	0.2
2	40	7	1	10	10	8	0	0	2	35	0	100.0	3.9	7.9
3	40	7	5	10	10	2	5	0	0	35	150	100.0	75.5	100.0
4	40	7	5	40	10	2	0	1	0	0	150	100.0	31.4	68.6
5	20	7	5	40	40	2	0	0	2	0	0	100.0	6.8	8.3
6	40	3	5	40	40	8	0	0	0	35	0	60.0	0.0	0.0
7	20	7	1	40	40	8	5	0	0	0	150	45.0	12.4	3.2
8	40	3	5	10	40	8	5	1	0	0	0	2.4	3.0	11.6
9	40	7	1	40	10	8	5	1	2	0	0	43.3	25.3	6.5
10	20	7	5	10	40	2	5	1	2	35	0	100.0	0.4	5.2
11	20	3	5	40	10	8	0	1	2	35	150	9.3	9.3	8.7
12	40	3	1	40	40	2	5	0	2	35	150	2.7	2.8	2.1
13	20	7	1	10	40	8	0	1	0	35	150	89.2	1.3	0.0
14	20	3	5	10	10	8	5	0	2	0	150	12.8	6.5	8.1
15	20	3	1	40	10	2	5	1	0	35	0	1.9	1.9	0.0
16	20	3	1	10	10	2	0	0	0	0	0	100.0	4.6	52.5
17	30	5	3	25	25	5	2.5	0.5	1	17.5	75	100.0	39.8	100.0
18	30	5	3	25	25	5	2.5	0.5	1	17.5	75	100.0	49.0	100.0
19	30	5	3	25	25	5	2.5	0.5	1	17.5	75	100.0	51.5	100.0

Table 3 Central composite design and analysis with three variables and seven responses in 48 h batch fermentation by *Wickerhamomyces* sp. UFFS-CE-3.1.2

Variables			Responses					
Total sugar (g/L)	pH	Acetic acid (g/L)	Sugar consumption (%)			Yield (mg/g)		
			Glucose	Xylose	Cellobiose	Ethanol[a]	Xylitol[b]	Biomass[a]
22.0	2.0	0.0	0.00	0.00	0.00	0.00	0.00	12.61
88.0	2.0	0.0	0.00	0.00	0.00	0.00	0.00	3.07
22.0	8.0	0.0	100.00	88.07	100.00	0.00	50.50	706.82
88.0	8.0	0.0	100.00	19.91	7.71	101.93	82.15	143.47
22.0	2.0	5.0	0.00	0.00	0.00	0.00	0.00	11.25
88.0	2.0	5.0	0.00	0.00	0.00	0.00	0.00	2.13
22.0	8.0	5.0	100.00	100.00	100.00	0.00	0.00	665.91
88.0	8.0	5.0	100.00	18.20	14.42	110.16	24.55	140.06
55.0	5.0	2.5	100.00	38.97	61.13	61.38	23.00	328.64
55.0	5.0	2.5	100.00	42.21	68.67	65.23	29.92	348.64
55.0	5.0	2.5	100.00	40.20	69.75	83.01	34.66	340.45

[a]Data are expressed as mg of ethanol per g of total sugar available at the beginning of the fermentation

[b]Data are expressed as mg of xylitol per g of xylose available at the beginning of the fermentation

[c]Data are expressed as mg of dry yeast cells per g of total sugar available at the beginning of the fermentation

YNB medium containing 1.0 g/L of furfural, 0.5 g/L of formic acid, 17.5 g/L of NaCl, and different concentrations of carbohydrates and acetic acid (Table 2). The pH was also analyzed as a variable of this CCD. In Table 3, the column "total sugars" represents the combination of the three carbohydrates analyzed: 40 g/L of glucose, 40 g/L of xylose, and 8 g/L of cellobiose (88 g/L total sugar); 25 g/L glucose, 25 g/L xylose, and 5 g/L cellobiose (55 g/L total sugar); and 10 g/L glucose, 10 g/L xylose, and 2 g/L cellobiose (22 g/L total sugar). The remaining sugars and the concentration of xylitol and ethanol produced were determined by HPLC as described in the "Analytical methods".

Fermentation kinetics

For the fermentation kinetics, YNB media containing 25 g/L of glucose, 25 g/L of xylose, and 5 g/L of cellobiose (55 g/L of total sugars) were inoculated at the concentration of 1:100 (as described above). The inhibitors were added to the culture medium at concentrations of 1.0 g/L of furfural, 0.5 g/L of formic acid, 2.5 g/L of acetic acid, and 17.5 g/L of NaCl, and the pH was adjusted to 8.0. The assays were carried out with agitation of 150 rpm and a temperature of 30 °C. A total of ten samples were collected from each culture during 48 h of incubation to determine cellular growth by turbidity measurements at 570 nm (OD_{570nm}) [45], and sugars, acetic acid, ethanol, and xylitol concentration through HPLC analysis (see below).

Analytical methods

During cell cultures, samples were periodically harvested, centrifuged (5000*g*, 3 min), and filtered (0.45 µm) for subsequent quantification of carbohydrates, ethanol, xylitol, and acetic acid by high-performance liquid chromatography (HPLC) as described by Tadioto et al. [8]. The analyses were performed using an LCMS-2020 chromatograph (Shimadzu) with a refractive index detector (RID-10, Shimadzu). For all compounds, a column for organic acids (Aminex HPX-87H, Bio-Rad) was used with a flow rate of 0.6 mL/min, using 5 mM of H_2SO_4 as mobile phase at a temperature of 50 °C. Calibration curves were used with four different concentrations of each analyzed compound.

Quantitative RT-PCR analysis

Quantitative RT-PCR (qRT-PCR) was conducted to verify the overexpression of the *TAL1* gene in strain JDY-01 when compared to the expression of this gene in strain MP-P5. The yeast strains were grown in YP-20 g/L glucose medium to mid-log phase, centrifuged (5000*g*, 4 min at 4 °C), washed with cold distilled water, and according to the manufacturer's protocols, the total RNA of the cell pellets was extracted using the RNeasy® Mini Kit (Qiagen Brazil, São Paulo, Brazil). The total RNA of each sample (1 ug) was reverse transcribed to cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen). The qRT-PCR reactions were performed with the QuantiFast® Sybr Green PCR Kit and the Rotor-Gene® Q equipment (Qiagen) using the primers for the *TAL1* gene (primers RT-PCR TAL1-F and RT-PCR TAL1-R,

Table 1) and for the actin gene (*ACT1*) that was selected as the endogenous reference gene (primers RT-PCR ACT1-F and RT-PCR ACT1-R, Table 1). A dissociation curve was generated for each assay to confirm the amplification of only one product. The $2^{-\Delta\Delta CT}$ method [46] was used to calculate the relative expression levels of the *TAL1* gene relative to the *ACT1* gene, for each yeast strain, in triplicate.

Results and discussion

High-throughput analysis of a recently discovered *Wickerhamomyces* sp. strain

Although it has been only recently isolated from rotten wood [1], the wild yeast *Wickerhamomyces* sp. UFFS-CE-3.1.2 has already displayed high biorefinery employment potential when it was submitted to lignocellulosic [1, 3] and pectin-rich hydrolysates [47] even with seawater-based media [48]. Indeed, we have previously shown that the wild strain UFFS-CE-3.1.2 showed 50% higher ethanol productivity in seawater-based papaya hydrolysates than the well-known industrial *S. cerevisiae* CAT-1 [48], another widely used yeast in the Brazilian fuel ethanol industry [22, 35, 38, 49]. Thus, aiming to reach even higher performances with the yeast *Wickerhamomyces* sp. UFFS-CE-3.1.2, we designed a widely comprehensive Plackett–Burman (PB) experimental plan to evaluate the effects of 11 variables on glucose, xylose, and cellobiose metabolism by the cells. The data in Table 2 show that UFFS-CE-3.1.2 is able to metabolize the three most abundant sugars in lignocellulosic hydrolysates, however, with a marked advantage for glucose, corroborating our previous studies [1, 3, 47, 48]. Indeed, this preference for glucose metabolism is a prevailing feature in yeasts [50–52]. After glucose, cellobiose was the most consumed sugar, although the concentration of this disaccharide was always lower than that of the glucose and xylose, which is usual in lignocellulosic hydrolysates [1–4].

The PB statistical analysis showed that the pH effect (calculated t) was significantly positive for glucose and xylose consumption considering $p \leq 0.05$, and for cellobiose consumption considering $p \leq 0.1$ (Fig. 1), indicating that higher pH values favor sugar metabolization. Sugar concentration also had a significant effect on the percentual of their consumption, although, in this case, the influence was always negative, meaning that higher sugar concentration led to lower consumption (in percentual terms). Glucose uptake was negatively affected by high glucose and cellobiose titer; cellobiose consumption was impaired by itself and xylose; and the percentual of xylose internalized was only decreased in higher concentrations of this pentose (Fig. 1). In fact, the negative effects of one sugar on another have been described elsewhere [52–54], and it was somehow already expected that the higher the sugar concentration, the lower the percentual of sugar consumption by the yeast cells.

Besides the sugar concentration and pH, all the other variables had no consistent influence on the metabolization of the three carbohydrates analyzed; some of them had significant effects on one response but not on the other two (Fig. 1). Surprisingly, this was the case with acetic acid, which is known to impair sugar consumption, especially under low pH values, when it is in its undissociated (liposoluble) form, easily diffusing through the plasma membrane lipid bilayer and reaching the cell cytoplasm. In the cytosol, pH values higher than the external environment provide the dissociation of acetic acid to the ionic form, releasing protons that decrease cytoplasmic pH and consequently may damage the cell and thus produce lower ethanol yield [55–58].

Moreover, our data showed that this carboxylic acid exerted a significantly negative effect ($p \leq 0.05$) only on glucose consumption, although most data in the literature indicate that xylose fermentation is usually more impaired by acetic acid [3, 57, 59]. Nevertheless, considering that acetic acid is probably the most common fermentation inhibitor in second-generation biorefinery processes, we decided to include it as a variable in a next step experimental design (see below). It should also be noted that NaCl had no significant influence on sugar consumption by UFFS-CE-3.1.2.

Since curvature (which represents a favorable trend for the average values of the variables) had a significant positive effect ($p \leq 0.05$) on the three responses analyzed (Fig. 1), the variables other than sugars, pH, and acetic acid were kept in their central values in the central composite design (CCD) that followed the PB analysis. In the CCD, glucose, xylose, and cellobiose concentrations were taken together as one variable: total sugar concentration (Table 3). Once again, acetic acid showed no significant effect on xylose and cellobiose consumption (data not shown), even though it significantly impaired ethanol and xylitol yields (Fig. 2a, b). Higher pH values always favored yeast metabolism, and sugar concentration had significant positive effects on ethanol and xylitol but a negative effect on cellular biomass yields (Fig. 2).

In the next step, UFFS-CE-3.1.2 was subjected to a fermentation kinetic analysis. To this end, except for pH (which has consistently proven to have a beneficial influence at alkaline values), the other analyzed variables were kept in their central values (see "Fermentation kinetics" at "Materials and methods"). The kinetic analysis suggests that glucose was almost entirely fermented into ethanol, while xylose and cellobiose were mostly used as carbon sources for cellular growth (Fig. 3). Although xylose has not entirely been exhausted by the yeast cells, our data show an improvement in the consumption rate of this pentose compared with a previous study [3], indicating that PB and CCD led to an optimization of the UFFS-CE-3.1.2 fermentation performance.

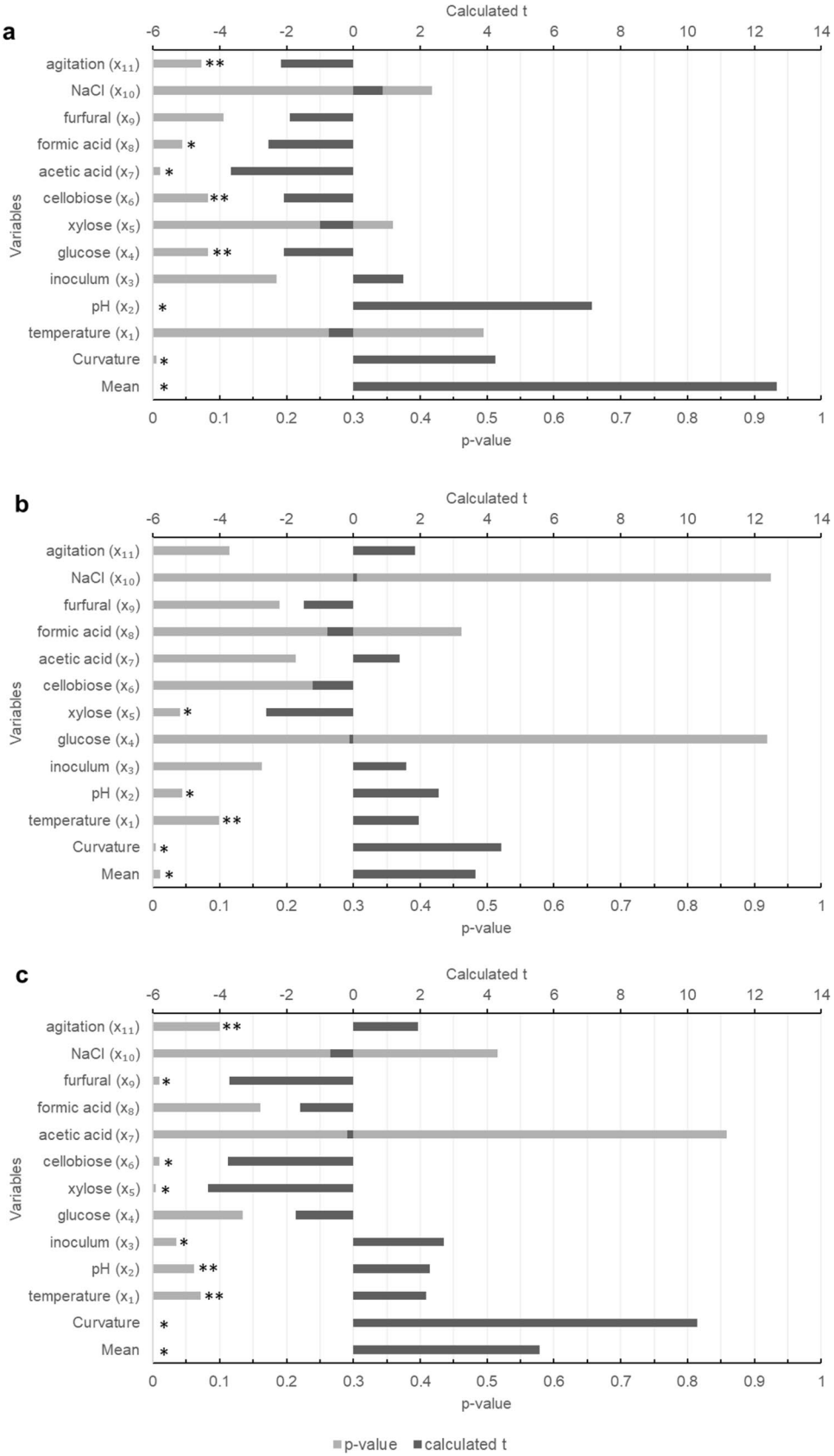

Fig. 1 Calculated *t* and *p* value for *Wickerhamomyces* sp. UFFS-CE-3.1.2 Plackett–Burman's experimental design (PB), considering the percentual of glucose (**a**), xylose (**b**), and cellobiose (**c**) consumption as responses. Significant effects are labeled with * for $p < 0.05$ and with ** for $p < 0.1$

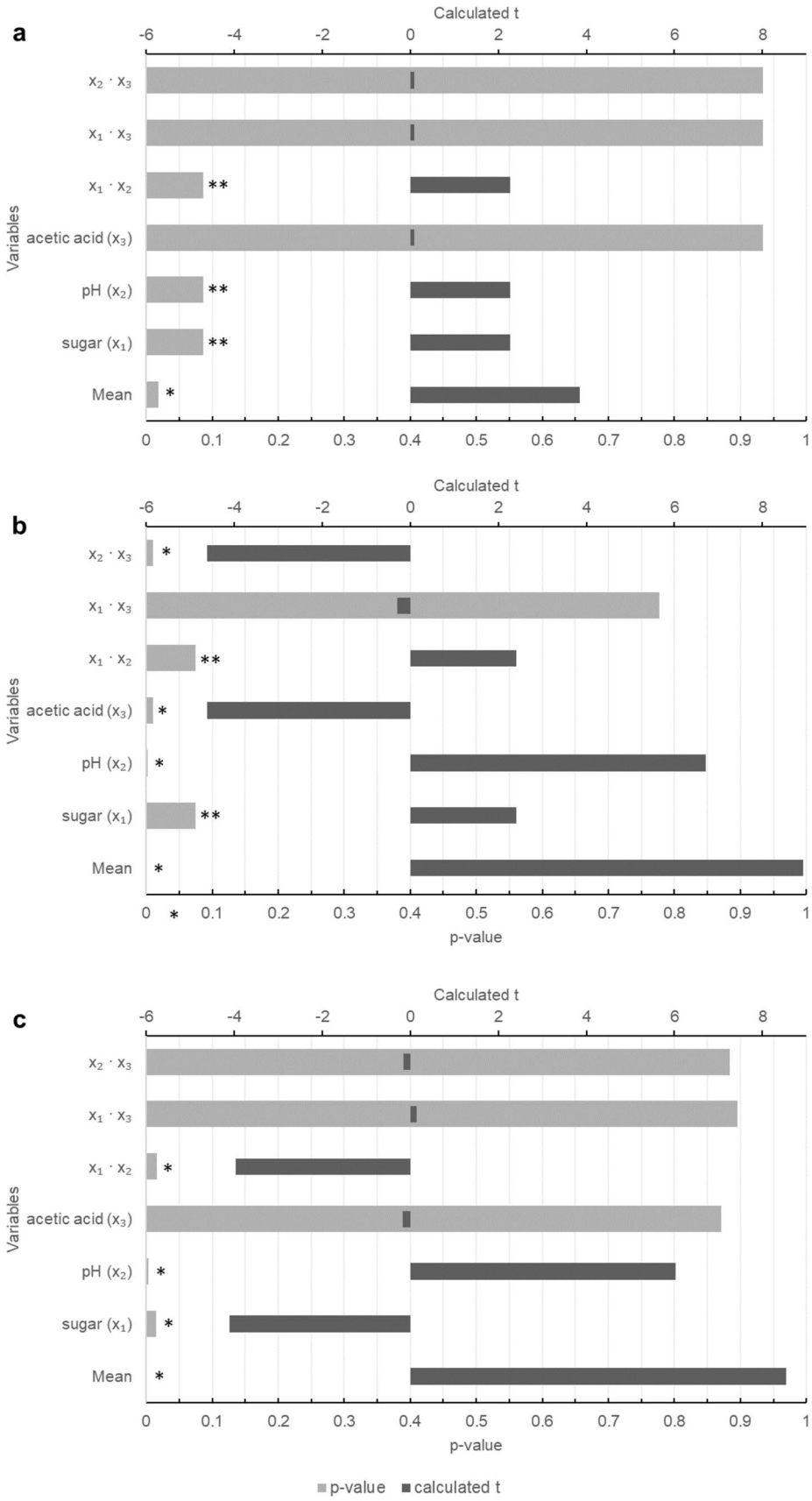

Fig. 2 Calculated *t* and *p* value for *Wickerhamomyces* sp. UFFS-CE-3.1.2 central composite design (CCD), considering the yields of ethanol (**a**), xylitol (**b**), and cellular biomass (**c**) as responses. Significant effects are labeled with * for $p < 0.05$ and with ** for $p < 0.1$. Coefficients of determination: 79.42% (ethanol yield), 96.13% (xylitol yield), 94.52% (biomass yield)

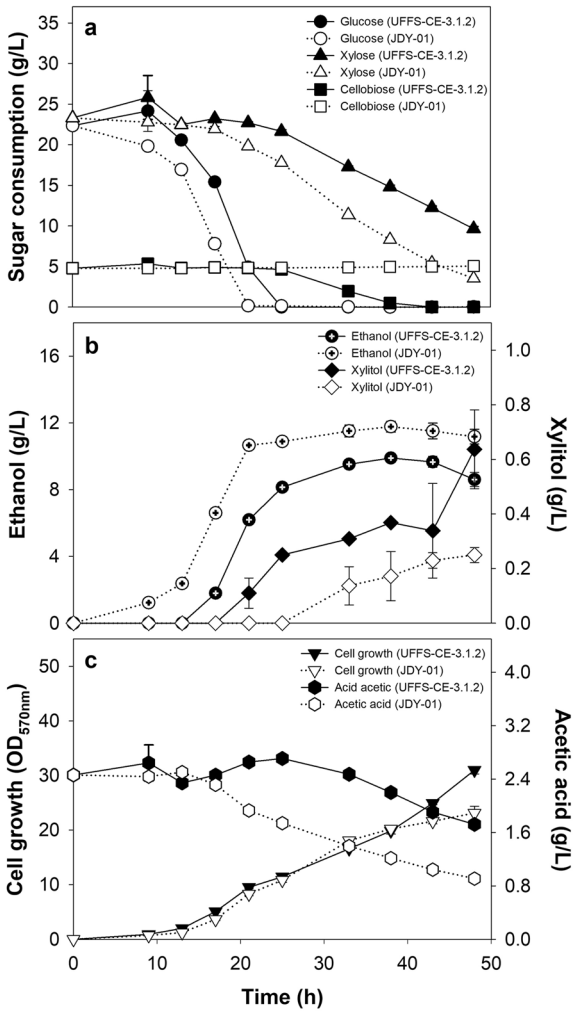

Fig. 3 Fermentation kinetics of *Wickerhamomyces* sp. UFFS-CE-3.1.2 (closed symbols) and *S. cerevisiae* JDY-01 (open symbols) in the presence of fermentation inhibitors. During 48 h incubation period, samples were harvested from the media for the quantification of glucose (circle), xylose (triangle), or cellobiose (square) consumption (**a**), ethanol (cross-hair circle), and xylitol (diamond) production (**b**), and cellular growth (inverted triangle) and acetic acid concentration (hexagon) in the medium (**c**). The data are expressed as averages ± standard errors from three completely independent experiments

Moreover, it is worth noting that this better performance was achieved in the presence of acetic acid (2.5 g/L), formic acid (0.5 g/L), furfural (1.0 g/L), and NaCl (17.5 g/L), which have shown to inhibit sugar metabolism at similar concentration ranges [21, 31, 60, 61].

Only a small amount of the xylose consumed by the cells (~5%) was secreted in the media as xylitol (Fig. 3b), indicating that the common redox imbalance is not impairing the first two reactions in xylose metabolization (catalyzed by XR and XDH) by *Wickerhamomyces* sp. UFFS-CE-3.1.2. In contrast, this also indicates a low potential of this yeast for xylitol production in a multiproduct biorefinery context since previous studies have shown xylitol yields by wild yeasts up to 11 times higher in similar fermentation conditions [8, 62, 63].

Comparing the wild yeast with a genetically engineered industrial strain

Although bioprospection of wild yeasts has shown to be a promising approach for making many biorefinery processes economically viable [64–66], genetic engineering has also proven to be a feasible alternative to improve residual biomasses conversion into bioproducts [5, 67, 68]. In this sense, we decided to compare the wild yeast *Wickerhamomyces* sp. UFFS-CE-3.1.2 fermentation performance with that of a genetically modified industrial *S. cerevisiae* strain. The recombinant fuel ethanol *S. cerevisiae* strain JDY-01 is derived from strain PE-2, an efficient fermenting diploid strain used in first-generation bioethanol production in Brazil [36], but unable to ferment xylose [4, 69]. However, in this study, PE-2 was initially transformed with a chromosome-integrative plasmid to overexpress the three enzymes (XR, XDH, and XK) required for xylose utilization, yielding strain MP-P5. This strain was further improved by promoting the overexpression of the *S. cerevisiae* transaldolase encoded by the *TAL1* gene, a rate-limiting enzyme of the non-oxidative pentose phosphate pathway required for improved xylose consumption [41, 42]. Our PCR analysis revealed that, in strain JDY-01, only one of the *TAL1* genes had its promoter region replaced by the *kanMX*-P_{ADH1} module, and thus the other *TAL1* gene present in this diploid strain retained its normal promoter.

Interestingly, *S. cerevisiae* JDY-01 in the presence of the inhibitors (acetic and formic acids, furfural, and NaCl) consumed glucose faster than the wild yeast *Wickerhamomyces* sp. UFFS-CE-3.1.2, and consumed ~30% more xylose, displaying a ~20% higher ethanol production, even with no cellobiose utilization (Fig. 3). As expected, this higher ethanol production reflects its lower xylitol production and cellular growth, indicating that the engineered *S. cerevisiae* strain deviates more sugar (glucose and xylose) to the alcoholic fermentation route than *Wickerhamomyces* sp. UFFS-CE-3.1.2, thus being more suitable for the 2G ethanol industry. At this point, it is important to remember that the strain JDY-01 lacks a periplasmic ß-glucosidase (or a cellobiose transporter plus an intracellular ß-glucosidase) to allow it to consume and ferment cellobiose, which could ensure an even higher ethanol yield if this recombinant yeast were further modified to ferment this disaccharide.

As it can be claimed that the overexpression of XR, XDH, and XK may be the major reason behind the better fermentative performance of JDY-01, it is worth mentioning that this strain also consumed more xylose and produced more ethanol than its parental strain MP-P5 (data not shown), which

overexpresses the same three enzymes. The only difference between these two strains is the strong promoter P_{ADH1} upstream of the *TAL1* gene in strain JDY-01. When *TAL1* expression was analyzed through qRT-PCR, we observed that this gene was 5.4 times upregulated in JDY-01 compared to MP-P5. Thus, JDY-01 higher transaldolase activity most likely led this strain to increase its xylose consumption capacity even in the presence of fermentation inhibitors. This is corroborated by the acetic acid consumption by JDY-01, since during the 48 h incubation, this strain consumed over 60% of this acid available in the medium (Fig. 3c), probably using it as a carbon source and thus reducing its toxic effects. Significantly, strain UFFS-CE-3.1.2 only consumed 32% of the acetic acid, mostly at the end of the fermentation.

By consuming acetic acid at such a rate, JDY-01 shows to be able to use it as a carbon source. Ending or decreasing the repressive catabolic effect of glucose, acetate may be converted into acetyl-CoA and then stimulate the glyoxylate cycle and gluconeogenesis [60, 70]. By doing this, the strain JDY-01 not only has the toxic effect of acetic acid reduced but also has it used as an energy source [71, 72]. Thus, our results suggest that, as previously hypothesized [15, 20, 21], overexpression of *TAL1* may increase yeast tolerance to the so-referred carboxylic acid.

Moreover, it should be noted that the fermentative performance observed for the strain JDY-01 was achieved in the presence of 17.5 g/L of NaCl, which corresponds to approximately half the concentration of this salt in seawater [73]. In this sense, our data indicate that the engineered strain *S. cerevisiae* JDY-01 could handle 2G ethanol production in a lower water footprint condition, considering, for instance, a situation where freshwater and seawater are used in a ratio of 1:1 (v/v) in the wort.

It is also worth noting that our results not only presented a new engineered *S. cerevisiae* strain with high potential to be employed in the 2G ethanol industry but also a highly feasible approach to improve xylose fermentation by this yeast species. Considering the genotypic and phenotypic variability among several *S. cerevisiae* strains [23, 74], it would be interesting to reproduce the same genetic modifications herein analyzed in other genetic backgrounds. With the aim of increasing water security during 2G ethanol production, the marine *S. cerevisiae* strains that Zaky and coworkers [32, 33, 75, 76] have been isolating and characterizing, for instance, are certainly worth trying.

Conclusion

The newly discovered wild yeast *Wickerhamomyces* sp. UFFS-CE-3.1.2 has previously shown biotechnological potential when analyzed in seawater-based lignocellulosic and pectin hydrolysates. In the present study, a high-throughput experimental design was used to improve its fermentation performance in the presence of the main sugars and inhibitors found in the biorefinery vats. Although acetic acid, furfural, formic acid, and NaCl showed to play a low (or no) effect on its sugar metabolism, this yeast was unable to ferment xylose when subjected to intermediary concentrations of these inhibitory compounds. In this situation, the strain *Wickerhamomyces* sp. UFFS-CE-3.1.2 consumed ~62% of the xylose available through the respiratory route generating cellular biomass. Nevertheless, the experimental designs that were carried out highly improved the xylose consumption capacity of this yeast (compared to our previous studies) and allowed the cells to fully consume cellobiose.

On the other hand, the genetically engineered strain *S. cerevisiae* JDY-01 consumed over 83% of xylose from the medium and produced 20% more ethanol than the wild yeast in the presence of the same fermentation inhibitors. Furthermore, this higher fermentative performance was achieved despite lacking cellobiose consumption, which was completely metabolized by the wild yeast. Thus, if JDY-01 is additionally engineered to ferment this disaccharide, it will very likely be able to further increase ethanol yield in a seawater-based biorefinery context.

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Author contributions VT, CM, AG, LD, and ACL carried out the experimental design assays and the fermentation kinetic analysis. JRD, BRS, and MP engineered the *S. cerevisiae* strains. OF carried out the HPLC analysis. AM, HT, BUS, and SLAJ participated in designing the study and provided financial support. SLAJ also wrote the manuscript, which was revised and approved by all authors.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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