



Research article

Improving *Saccharomyces cerevisiae* acid and oxidative stress resistance using a prokaryotic gene identified by functional metagenomics

Luana de Fátima Alves^{a,b,c}, Jonatã Bortolucci^a, Valeria Reginatto^a,
María-Eugenia Guazzaroni^a, Solange I. Mussatto^{d,*}

^a Department of Biology, Faculdade de Filosofia, University of São Paulo, Ciências e Letras de Ribeirão Preto, Ribeirão Preto, 14040-901, São Paulo, Brazil

^b Department of Biochemistry, Faculdade de Medicina de Ribeirão Preto, University of São Paulo, Ribeirão Preto, 14040-900, São Paulo, Brazil

^c The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet, Building 220, 2800, Kongens Lyngby, Denmark

^d Department of Biotechnology and Biomedicine, Technical University of Denmark, Søtofts Plads, Building 223, 2800, Kongens Lyngby, Denmark

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ABSTRACT

Innovations in obtaining products from lignocellulosic biomass have been largely based on the improvement of microorganisms and enzymes capable of degrading these materials. To complete the whole process, microorganisms must be able to ferment the resulting sugars and tolerate high concentrations of product, osmotic pressure, ion toxicity, temperature, toxic compounds from lignocellulose pretreatment, low pH, and oxidative stress. In this work, we engineered laboratory and industrial *Saccharomyces cerevisiae* strains by combining a gene (*hu*) recovered from a metagenomic approach with different native and synthetic promoters to obtain improved acid and oxidative stress resistance. Laboratorial strains harboring *hu* gene under the control of the synthetic stress responsive PCCW14v5 showed increased survival rates after 2 h exposure to pH 1.5. The *hu* gene was also able to significantly enhance the tolerance of the industrial strain to high concentrations of H₂O₂ when combined with PTEF1, PYGP1 or PYGP1v7 after 3 h exposure.

1. Introduction

Most of the chemicals and fuels currently used are derived from fossil sources, which are limited and non-sustainable on a long-term period [1,2]. In this sense, the change towards bio-based chemicals produced from renewable feed stocks, i.e. lignocellulose, is a necessary emergent field since this source is much more abundant and, most important, it does not compete with food for supplies [3,4]. Ethanol produced from lignocelluloses is the main example of a cost-effective and reproducible alternative to fossil fuels. For example, microbial fermentation of sugars from sugarcane and corn starch to ethanol is the source of around 100 billion liters of fuel ethanol annually produced in the world using *Saccharomyces cerevisiae* [5], which is the primary ethanol-producing microorganism used in the industry. Additionally to fuel ethanol, since the production of the first non-native molecules in *S. cerevisiae* – lactic acid and xylitol – other different compounds have been produced in engineered yeast, varying from fuels to bulk chemicals to pharmaceutical

* Corresponding author.

E-mail addresses: smussatto@dtu.dk, solangemussatto@hotmail.com (S.I. Mussatto).

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and nutraceutical ingredients [6].

Despite the promising use of *S. cerevisiae* to produce a range of fine chemicals, the development of a robust cell factory able to produce the desired chemical at high titer, rate, yield and, survive to the harsh conditions associated to the specific production processes is still one of the main factors limiting its wide application. In industrial fermentations, *S. cerevisiae* faces many stresses, high concentrations of toxic chemicals and harmful conditions that can compromise product yields and titers. Physical and chemical extracellular stresses include high temperatures, osmotic pressure, low pH, the presence of lignocellulosic biomass derived inhibitors, and oxidative stress resulting from thermo-chemical pretreatment of lignocellulose [7] for which extra operations for process conditioning to yeast tolerance increase the costs and extend the production time.

One of the most critical problems faced during *S. cerevisiae*-mediated bio production is low pH stress [8,9]. Acid stress can be imposed to the culture from the start, or gradually occur by the accumulation of acidic products during fermentation, for example during the production of acidic molecules, such as lactic acid [10]. Additionally, the use of low pH fermentation on the production of some acidic compounds would make the product recovery more straightforward in an industrial setting, especially when recovering organic acids [11]. In the case of lactic acid production at low pH, the product would exist in its undissociated form, simplifying the chemical recovery of the free acid and minimizing the formation of salt by-products normally formed in this process [12]. Usually, several organic acids are produced in prokaryotic organisms [13]; however, many of those prokaryotes are generally unable to grow and produce these compounds at low pH values where the compounds occur predominantly in their undissociated form. In this case, production of these compounds at lower pH values using a more acid-tolerant microorganism as *S. cerevisiae* could increase product yield and titers, reduce the need for pH control systems, and simplify the downstream steps, reducing the cost of the whole process. Native *S. cerevisiae* generally can tolerate low pH values [14–16]. However, one of the most harmful stresses faced by it during the alcoholic fermentation for production of fuel ethanol is the step of cell recycling, which consists in recovering the cells by centrifugation and treating the “yeast cream” using sulphuric acid (pH 1.8 to 2.5 during 1–2 h), to reduce bacterial contamination (Basso et al., 2010). This process causes physiological disturbances in yeast cells, resulting in reduction of intracellular pH, with cell viability drop and lower ethanol yields [17,18].

Another severe stress that affects *S. cerevisiae* during fermentation is oxidative stress, which is a result from an imbalance between generation and elimination of reactive oxygen species (ROS) that can occur naturally during respiration or oxidation of nutrients to obtain energy. Accumulation of ROS during fermentation process is also linked to some toxic by-products resulting from lignocellulose pretreatment, like furfural [19–21]. These active oxygen species pose significant damage to almost all cell components, including DNA, lipids, and proteins, and to cellular redox balance [22]. In this way, improving the tolerance of *S. cerevisiae* chassis cells to low pH stress and tolerance to ROS could benefit a more efficient bio production, offer elimination of conditioning steps operations for yeast tolerance and therefore reduce the costs of the process in industrial-scale ethanol production.

To develop robust *S. cerevisiae* strains for bio-based production, synthetic biology and metabolic engineering have emerged as promising tools for design, assembly, and optimization of natural and non-native biochemical pathways [23,24]. In this context, metagenomics findings appear as potential candidates to expand the synthetic biology toolbox and further, these newly biological parts can be used to improve specific characteristics in microorganisms used in different applications and expand the horizon in microbial engineering. Despite promising, the use of metagenomics findings as a tool for metabolic engineering is still poorly explored [25,26].

In the present study, we selected a metagenomic gene (*hu*), that was recovered from a functional metagenomic approach and conveyed acidic tolerance to a range of bacteria [27] to engineer laboratory and industrial *S. cerevisiae* strains to survive in extremely low pH and presence of oxidative stress. In brief, the *hu* gene mentioned above has a putative function of a histone-like protein and was recovered from a functional screening of metagenomic libraries from planktonic and rhizosphere microbial communities of the Tinto River, an extremely acidic environment. In the approach, the gene was identified by using the gram-negative *Escherichia coli* as host after exposing the cells to a pH 1.8. Then, *hu* gene was transferred to the gram-negative *Pseudomonas putida* and to the gram-positive *Bacillus subtilis* bacteria and the cells were similarly exposed to a low pH stress (3.8 and 4.0, respectively). In all experiments, the hosts harboring *hu* gene showed increased ability to survive in the low pH tested when compared with the wild types, indicating a broad-host range of functionality [27]. Additionally, histone-like proteins are known by playing important roles in the regulation of genes involved in stress resistance mechanisms in bacteria, including genes responding to acid and oxidative stress [28–30]. Considering the above, the *hu* gene has a significant potential for engineering other microorganisms and increasing its robustness. Here, we combined the metagenomic *hu* gene with different yeast promoters - varying from native constitutive (PTEF1), native (PYGP1) and synthetic stress-responsive (PYGP1v7 and PCCW14v5) promoters [11], to generate engineered strains with improved ability to survive in very low pH and high concentrations of hydrogen peroxide. We further checked ethanol production and resistance of the engineered strains after cell recycling.

2. Material and methods

2.1. Strains, chemicals, and media

The *S. cerevisiae* strains CEN.PK113-32D and Ethanol Red used in this study were kindly provided by Professor Irina Borodina (DTU Biosustain). Yeast cells were grown at 30 °C in standard synthetic complete (SC) media (6.7 g/L yeast nitrogen base without amino acids, 20 g/L D-glucose, 1.7 g/L yeast synthetic drop-out media supplement – without leucine). The media was supplemented with 200 mg/L G418 sulfate when indicated. For the survival experiments, YNB/glucose media (7 g/L yeast nitrogen base without amino acids, 20 g/L glucose) was supplemented with hydrogen peroxide to the indicated concentrations used in the assays (2.5, 5.8 or 11.5 mM), or HCl was added to reach the pH indicated (pH 1.3, 1.5, 2.5 or 3.0, depending on the experiment). All cloning procedures were made

using *Escherichia coli* DH10B, which was grown in standard Luria–Bertani (LB) media containing 100 mg/mL ampicillin. Codon-optimized synthetic gene was ordered as a gBlock from Integrated DNA Technologies. Sequencing results were obtained through Eurofins Genomics (Ebersberg, Germany) using Mix2Seq kit.

2.2. Cloning and strain constructions

The *hu* gene identified by a functional metagenomic screening [27] was selected to engineer the *S. cerevisiae* strains CEN.PK113-32D (laboratory) and Ethanol Red (industrial). The codon-optimized gene sequence was combined with the native TEF1 promoter and the three stress-responsive promoters – the native YGP1 and the synthetic YGPv7 and PCCW14v5 - by USER methodology. The bio-bricks (gene and promoters) were amplified by PCR using Phusion U polymerase (Thermo Fischer) and template DNA as indicated. A complete list of the primers and DNA templates used in this study is summarized in Table 1. The fragments were assembled by USER cloning following the manufacturer's recommendations. The integration cassettes used to transform both strains were constructed in two different EasyClone vectors – pCfB2192 and pCfB2225 – for integration of the cassettes into the genome of the laboratory strain CEN.PK113-32D and the Ethanol Red industrial strain, respectively (Fig. 1). Both plasmids were designed to integrate the construct into chromosome XII (on site 2) [31]. After transformation with plasmids, *E. coli* DH10B was grown on LB plates with 100 mg/mL ampicillin. The transformants were checked by colony PCR and sequenced. The selected plasmids were extracted, linearized with *NotI* (Thermo Fischer Scientific) and transformed in *S. cerevisiae* strains using the LiAc/SS carrier DNA/PEG method [32]. For the selection of yeast strains, SC plates supplemented with 200 mg/L G418 or lacking leucine were used. Strains were checked for correct genome integration of the cassettes by colony PCR. Eight different engineered *S. cerevisiae* strains were generated and tested for resistance to low pH and oxidative stress.

2.3. Low pH and hydrogen peroxide resistance assays

Different colonies were selected for overnight cultivations in YNB/glucose media. From overnight cultures, 2.5×10^5 cells were inoculated in 5 mL of fresh YNB/glucose media (when CEN.PK113-32D was used) or supplemented with G418 antibiotic (when Ethanol Red was used). The cultures were incubated at 30 °C, 220 rpm, for 4–5 h until reaching the exponential phase. Then, 2.5×10^5 cells were inoculated in 1 mL of low pH YNB/glucose media (pH 1.3 or 1.5) or in media supplemented with hydrogen peroxide (2.5, 5.8 or 11.5 mM) and incubated at 30 °C. Every 15–30 min, 20 µL of the cultures were collected, diluted in PBS 1x pH 7.4 and the dilutions (10^{-1} , 10^{-2} and 10^{-3}) were plated on YNB/glucose-agar media (for CEN.PK113-32D) or on media supplemented with G418 antibiotic (for Ethanol Red). The plates were incubated at 30 °C for 24 h. Then the cells were counted to define the survival percentage of the engineered strains in comparison with the controls. All survival rates were determined comparing the time points with the time-zero point (just after inoculation in the low pH media or in the media supplemented with hydrogen peroxide).

2.4. Growth curves in low pH culture media

Three colonies were selected and cultivated overnight in YNB/glucose media (for CEN.PK113-32D cultivations) or in media supplemented with G418 antibiotic (for Ethanol Red cultivations). The cultures were diluted in 5 mL of fresh YNB/glucose media (pH 3

Table 1
List of primers used in this study.

Primer ID	Sequence 5' - 3'	Description	Template DNA for amplification
pTEF1_F (PV1F)	CACGCGAUGC ACACACCATAGCTTCAAAATGTT	amplification of TEF1 promoter	<i>S. cerevisiae</i> genomic DNA
pTEF1_R (PG1R)	ACCTGCACUTT GTAATTAACCTAGATTAGATT	amplification of TEF1 promoter	<i>S. cerevisiae</i> genomic DNA
pYGP1pr_F (PV1F)	CACGCGAUTCATTGATTC CAATGTTCTCAC	amplification of YGP1 and YGP1v7 promoters	<i>S. cerevisiae</i> genomic DNA/pASR0067.YGP1v7
pYGP1pr_R (PG1R)	ACCTGCACUTTTCTATTACTG TATTACTTA	amplification of YGP1 promoter	<i>S. cerevisiae</i> genomic DNA
pYGP1v7_R (PG1R)	ACCTGCACUTTTGTTGTTT TATGTGTGTTT	amplification of YGP1v7 and CCW14v5 promoters	<i>S. cerevisiae</i> pASR0067.YGP1v7 and pASR0067.CCW14v5
CCW14v5_F (PV1F)	CACGCGAUCACGAGATTTTTCG CACCCAT	amplification of CCW14v5 promoter	<i>S. cerevisiae</i> pASR0067.CCW14v5
HU_F_optimized (GPIF)	AGTGCAGGUA AAACA ATG TCTAAAGAAAAGAAGTT	amplification of <i>hu</i> -codon optimized gene	gBlock DNA
HU_R_optimized (GV1R)	CGTGCGAU TTAAGCAGCAGTTTTAGAAGC	amplification of <i>hu</i> -codon optimized gene	gBlock DNA
ADH1_test_fw	GAAATTCGCTTATTTAGAAGTGTC	<i>E. coli</i> colony PCR and DNA sequencing	different templates
CYC1_test_rv	CTCCTTCCTTTTCGGTTAGAG	<i>E. coli</i> colony PCR and DNA sequencing	different templates
2220	CCTGCAGACTAGTGCTGAG	<i>S. cerevisiae</i> colony PCR	different templates
894	GGCCCTGATAAGGTTGTTG	<i>S. cerevisiae</i> colony PCR	different templates
2221	GTTGACACTTCTAAATAAGCGAATTTTC	<i>S. cerevisiae</i> colony PCR	different templates
893	CGAAGAAGCGCTGCAATTC	<i>S. cerevisiae</i> colony PCR	different templates

USER overhang in bold, AAAACA – Kozak sequence, **ATG** – start codon, **TAA** – stop codon.

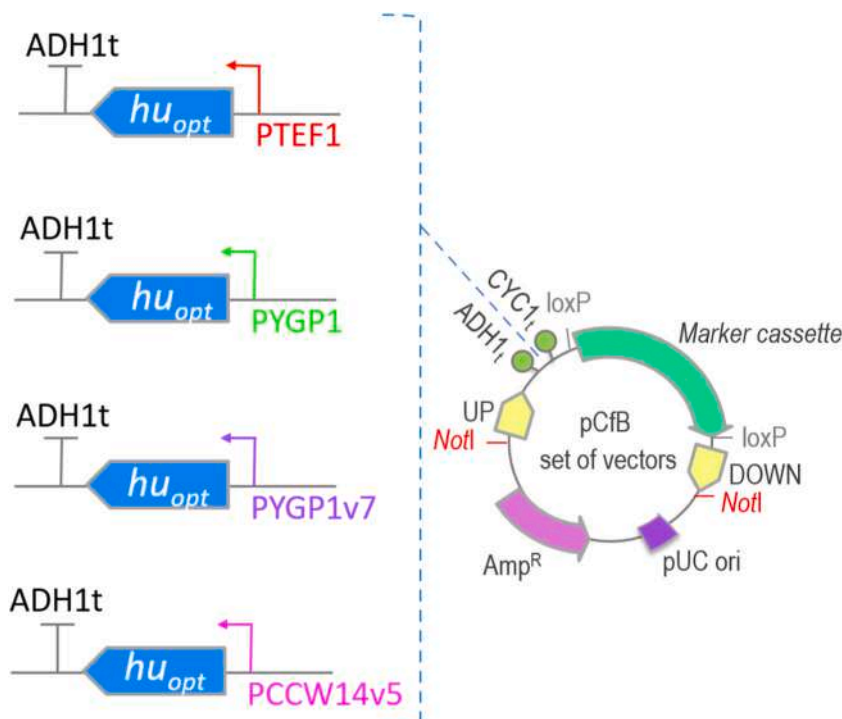


Fig. 1. Schematic representation of the pCfB general vector backbone showing the cassettes cloned in the USER site. Functional elements of the plasmid backbone are shown: Amp^R, ampicillin resistance gene; pUC ori, origin of replication for *E.coli*; ADH1_t and CYC1_t, transcriptional terminators; UP and DOWN, integration targeting sequences; loxP, sites that allow marker rescue mediated by CreA site-specific recombinase; NotI, restriction sites recognized by the endonuclease NotI; Marker cassette, Kleeu2 (auxotrophic marker in pCfB2192 – used in CEN.PK113-32D strain) and kanMX (resistance to G418 in pCfB2225 – used in Ethanol red strain). Cloned in the USER site is the *hu* gene under control of constitutive promoter PTEF1 or the stress-responsive promoters PYGP1, PYGP1v7, or PCCW14v5. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and pH 2.5) to reach an OD_{600nm} of 0.1 ± 0.008 at the starting point. The growth was monitored by measuring the OD_{600nm} during 48 h. The experiments were carried out in 50-mL tubes at 30 °C and 180 rpm.

2.5. Yeast cell recycling experiments

These experiments intended to reproduce the recovery of yeast cells that is performed in industrial ethanol production process, by cells washing with H₂SO₄ [67]. One fermentation cycle comprised the recovery of yeast cells from fermentation followed by acid treatment.

The three *S. cerevisiae* strains (*S. cerevisiae* CEN.PK113-32D harboring the empty cassette - as negative control -, harboring the *hu* gene under the control of PTEF1, and PCCW14v5) were employed in fermentative assays followed by acid cell wash. Fermentative assays were performed in 10-mL serum bottles filled with 8 mL of YNB medium, with 20 g/L of initial cell concentration, 50 g/L of glucose and pH 6.0 ± 0.5 . The bottles were flushed with nitrogen gas after the addition of medium and inoculum and sealed with a butyl rubber stopper and an aluminum cap. The fermentations were carried out for 6 h at 30 °C and 100 rpm. Samples were collected at the beginning and at the end of fermentation to monitor the optical density at 600 nm. Glucose and ethanol concentrations were measured by high performance liquid chromatography (HPLC), according to [33].

After fermentation, the cells were recovered by centrifugation (4000 rpm, 15 min), resuspended in 20 mL saline solution (NaCl 0.85%, peptone 1 g/L), and maintained at 4 °C overnight. The acid treatment was completed by recovering the yeasts from the saline solution by centrifugation (4000 rpm, 15 min) and resuspending them in 8 mL pure water at pH 2.0 adjusted with 2 M H₂SO₄. The cells were kept in this condition for 30 min. After that, cells followed centrifugation and resuspension in 8 mL fresh YNB medium for a new fermentation cycle. Ten sequential fermentation cycles were performed. In addition, a final fermentation was assayed (cycle 11), after keeping the yeast cells at pH 2.0 for one week at 4 °C.

3. Results

3.1. *hu* gene improves the survival of engineered laboratory and industrial strains after exposure to pH 1.5

To set a baseline for subsequent experiments, both *S. cerevisiae* strains, CEN.PK113-32D and Ethanol Red, transformed with the marker cassette (without *hu* gene and promoters) were grown in different low pH YNB media supplemented with glucose 20 g/L and defined as negative controls (for further details see Fig. S1A). Acid resistance assays showed that both *S. cerevisiae* strains showed survival percentages under 10% after 30 min of exposure to pH 1.3 (survival = $5.77 \pm 1.45\%$ for Ethanol Red and $0 \pm 0.0\%$ for CEN-PK113-32D). On the other hand, at pH 1.5, laboratory and industrial strains showed survival percentages under 10% after 75 and 90 min, respectively (survival = $5.96 \pm 2.34\%$ and $9.18 \pm 6.1\%$, respectively). The pH and times necessary to inhibit the survival of the negative control strains to at least 10% were selected as the conditions for the experiments with the engineered strains and we set up all the acid experiments for 2 h exposure to pH 1.5.

S. cerevisiae CEN.PK113-32D strains engineered with the *hu* gene under control of the constitutive TEF1 promoter and the synthetic stress responsive CCW14v5 promoter showed increased survival in comparison with the negative control after 1 h ($p = 0.008$ and $p = 0.003$, respectively) (Fig. 2A) and after 2 h ($p = 0.02$ and $p = 0.002$, respectively) (Fig. 2B) of exposure to pH 1.5. Additionally, the strain engineered with *hu* gene under the control of YGP1 promoter showed a significant increase on the survival percentage ($p = 0.044$) after 1 h at pH 1.5 (Fig. 2A). On the other hand, *S. cerevisiae* Ethanol Red strains engineered with the *hu* gene under control of

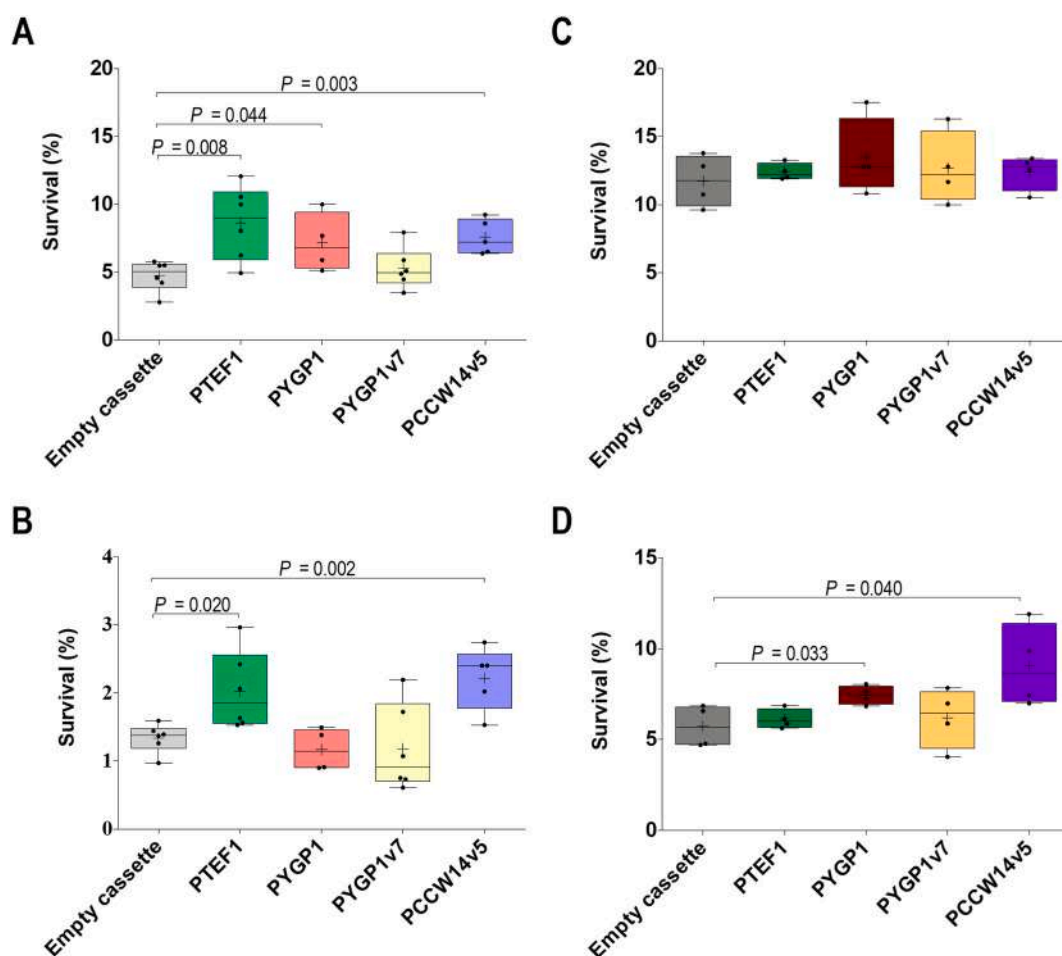


Fig. 2. Survival rates of *S. cerevisiae* CEN.PK113-32D and Ethanol red strains after exposure to extremely low pH. Survival percentages of the engineered *S. cerevisiae* CEN.PK113-32D harboring *hu* gene under control of four different promoters after A. 1 h and B. 2 h exposure to pH 1.5. Survival percentages of the engineered *S. cerevisiae* Ethanol red harboring *hu* gene under control of four different promoters after C. 1 h and D. 2 h exposure to pH 1.5. The boundaries of the boxes indicate the 25th and 75th percentiles; the lines within the boxes indicate the median, and the whiskers mark the minimum and maximum values obtained. “+” indicate the means. *P*-values comparing promoters with empty cassette values (*t*-test) are shown in the graphics when $P < 0.05$. The experiments were carried out in YNB media pH 1.5 supplemented with 2% glucose and without selective pressure. Independent experiments were carried out at least three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

YGP1 and CCW14v5 promoters showed increased survival after 2 h exposure to pH 1.5 ($p = 0.033$ and $p = 0.04$, respectively) (Fig. 2D) but not after 1 h exposure (Fig. 2C). The *hu* gene was also tested under control of the native constitutive PPGK1, but it did not increase the survival of the engineered strains after exposure to pH 1.5 (data not shown).

Table 2 summarizes the increase in the fold change of the engineered strains after exposure to pH 1.5 for 1 h and 2 h. As can be seen, the engineered laboratory *S. cerevisiae* CEN.PK113-32D strain harboring the *hu* gene under the control of the native constitutive TEF1 promoter showed survival capability increased by 1.8-fold after 1 h exposure to pH 1.5 and the improvement was maintained even after 2 h (1.5-fold) (Fig. 2A and B). Similarly, CEN.PK113-32D strain harboring the *hu* gene under the control of the synthetic stress-responsive CCW14v5 also showed increased survival capability after 1 and 2 h under pH 1.5 (1.6-fold) (Fig. 2A and B). Additionally, the strain harboring *hu* gene under the control of the native stress-responsive YGP1 promoter also showed increased survival capability after 1 h at pH 1.5 (1.5-fold) (Fig. 2A). It was also observed an increase in the survival of the Ethanol Red strains harboring the *hu* gene under the control of YGP1 and CCW14v5 promoters after 2 h exposure to pH 1.5 (1.3 fold and 1.5-fold, respectively). The fold changes were calculated according to the survival percentage means of the engineered strains and the negative control.

The growth of the engineered *S. cerevisiae* strains was not affected in low pH media cultivations. Growth curves at pH 3.0 (Fig. S3 A and B) and 2.5 (Fig. S3 C and D) had the same behavior for the engineered strains and the controls in both laboratory and industrial strains tested. *S. cerevisiae* strains (control and engineered strains) were also tested in pH 2.0 and were not able to grow.

3.2. *hu* gene improves the survival of engineered industrial strains after exposure to high concentration of hydrogen peroxide

Negative control *S. cerevisiae* CEN.PK113-32D and Ethanol Red strains were grown in YNB media supplemented with glucose 20 g/L and in different concentrations of H₂O₂. Oxidative stress resistance assays showed that laboratory *S. cerevisiae* CEN.PK113-32D strain showed survival percentages under 10% after 120 min of exposure to 2.5 mM H₂O₂ (survival = $0.3 \pm 0.01\%$) (for further details see Fig. S1B). On the other hand, the industrial Ethanol Red strains showed increased natural resistance to oxidative stress and was able to keep its survival percentages above 20% even after 3 h exposure to 8.3 mM H₂O₂ (survival = $20.16 \pm 4.55\%$). The survival percentage of industrial strain was reduced to less than 10% after 2 h of exposure to a concentration of 11.5 mM H₂O₂ (survival = $7.18 \pm 2.07\%$). (Fig. S1B). The H₂O₂ concentrations and times necessary to inhibit the survival of the negative control strains to at least 10% were selected as the conditions for the experiments with the engineered strains and we set up all the oxidative stress experiments for 3 h exposure to 2.5 mM and 11.5 mM for laboratory and industrial strains, respectively.

Oxidative stress assays showed that the engineered *S. cerevisiae* Ethanol Red strains harboring *hu* gene under the control of TEF1, YGP1 and YGP1v7 promoters were significantly more resistant ($p = 0.049$, $p = 0.019$ and $p = 0.045$, respectively) than the negative control after 3 h of exposure to 11.5 mM H₂O₂ (Fig. 3B). Increase in the survival percentages were not observed after 1 h or 2 h of exposure (Figs. S2C and D). Similarly, engineered laboratory *S. cerevisiae* CEN.PK113-32D strains did not show increase in the survival percentages after exposure to 2.5 mM H₂O₂ (Fig. 3A, S2A and B).

The average of survival (%) were increased in 2.18-, 2.59-, 2.13- and 2.2-fold in the engineered Ethanol Red strains harboring *hu* gene under the control of TEF1, YGP1, YGP1v7 and CCW14v5 promoters, respectively - after 3 h of exposure to H₂O₂. Considering the engineered laboratory strains, the average was increased in 1.15- and 1.7-fold in the strains harboring *hu* under the control of TEF1 and YGP1v7 promoters, respectively (Table 3). However, the increase was not significant in the engineered laboratory strains neither in the engineered industrial strain harboring *hu* under the control of PCCW14v5.

3.3. *hu* gene did not improve ethanol production after 11 cycles of cell recycling

We selected the engineered strains that performed the best in the acid resistance experiments to check their ability to survive and produce ethanol in fermentation conditions using cell recycling steps - similarly to the industrial process used by ethanol plants. During 10 fermentations cycles, the OD at 600 nm of CEN-PK113-32D (harboring the empty cassette - negative control), CEN-PK113-32D harboring *hu* gene under the control of TEF1 promoter, and CEN-PK113-32D harboring *hu* gene under the control of CCW14v5 promoter increased about 0.050 and 0.100, after 6 h of fermentation (Fig. S4), indicating their growth capacity. However, there were

Table 2

Fold change of engineered strains survival after exposure to pH 1.5

Parental strain	Promoter	Survival average (%)		Fold change	
		1 h	2 h	1 h	2 h
<i>S. cerevisiae</i> CEN.PK113-32D	–	4.74 ± 1.12	1.34 ± 0.21	n.a.	n.a.
	TEF1	8.65 ± 2.72	2.03 ± 0.57	1.82	1.51
	YGP1	7.19 ± 2.16	1.17 ± 0.31	1.51	–
	YGP1v7	5.31 ± 1.51	1.18 ± 0.64	–	–
	CCW14v5	7.60 ± 1.27	2.22 ± 0.46	1.6	1.65
<i>S. cerevisiae</i> Ethanol Red	–	11.74 ± 1.90	5.73 ± 1.14	n.a.	n.a.
	TEF1	12.41 ± 0.62	6.13 ± 0.54	–	–
	YGP1	13.48 ± 2.84	7.46 ± 0.52	–	1.3
	YGP1v7	12.69 ± 2.66	6.19 ± 1.64	–	–
	CCW14v5	12.40 ± 1.29	9.05 ± 2.29	–	1.57

n.a – not applicable.

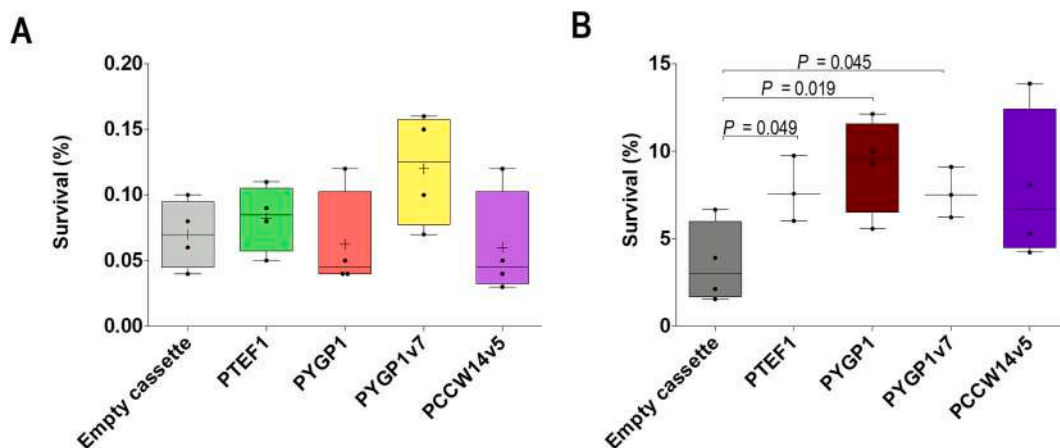


Fig. 3. Survival rates of *S. cerevisiae* CEN.PK113-32D and Ethanol red strains after exposure to H_2O_2 . A. Survival percentages of the engineered *S. cerevisiae* CEN.PK113-32D harboring *hu* gene under control of four different promoters after 3 h exposure to H_2O_2 2.5 mM. B. Survival percentages of the engineered *S. cerevisiae* Ethanol red harboring *hu* gene under control of four different promoters after 3 h exposure to H_2O_2 11.5 mM. The boundaries of the boxes indicate the 25th and 75th percentiles; the lines within the boxes indicate the median, and the whiskers mark the minimum and maximum values obtained. “+” indicate the means. *P*-values comparing promoters with empty cassette values (*t*-test) are shown in the graphics when $P < 0.05$. The experiments were carried out in YNB media supplemented with 2% glucose and without selective pressure. Independent experiments were carried out at least three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3

Fold change of engineered strains survival after exposure to hydrogen peroxide.

Parental strain	Promoter	Survival average (%)	Fold change
		3 h	3 h
<i>S. cerevisiae</i> CEN.PK113-32D	–	0.07 ± 0.02	n.a.
	TEF1	0.08 ± 0.02	1.15
	YGP1	0.06 ± 0.04	–
	YGP1v7	0.12 ± 0.04	1.7
	CCW14v5	0.06 ± 0.04	–
<i>S. cerevisiae</i> Ethanol Red	–	3.57 ± 2.3	n.a.
	TEF1	7.79 ± 1.88	2.18
	YGP1	9.24 ± 2.73	2.59
	YGP1v7	7.63 ± 1.45	2.13
	CCW14v5	7.87 ± 4.31	2.2

n.a – not applicable.

no significant differences in the glucose consumption and final ethanol concentration among the three strains (Fig. 4A and B).

After the first acid treatment, i.e., during the first fermentation cycle, glucose consumption and ethanol concentration were similar for the three strains (ca. 20 and 9 g/L, respectively). In cycles 6 and 10, glucose consumption enhanced to 40 g/L, giving 14 g/L of ethanol. In the additional fermentation cycle (cycle 11), with a prolonged exposure of the strains to acid pH, in 24 h the negative control strain, strain harboring *hu* gene and PTEF1, and strain harboring *hu* gene and PCCW14v5 consumed 36, 4.0, and 27 g/L of glucose, resulting in 14, 1.0 and 9 g/L of ethanol, respectively. However, after 48 h, consumption and ethanol production levels were very similar for the three strains (Fig. 4C and D).

4. Discussion

Fuel ethanol produced from renewable sources is a global commodity product largely produced in Brazil. Together with the US, Brazil provides 85% of the worldwide ethanol production [18]. Currently, Brazilian sugarcane ethanol industries use a singular step in the ethanol fermentation process: the recycling of yeast cells [34]; a process which includes a centrifugation step and the separation of the yeast cells from the fermented medium after the fermentation step. Then the cells are treated with water-diluted sulphuric acid (pH 2.0–2.5 for 1–2 h) and used in a new fermentation cycle. The cell recycling decreases the fermentation time and increases the fermentation yields and ethanol productivity [35]. Although the cell recycling process presents evident advantages in industrial ethanol production, the low pH can affect the cell viability and productivity [8,35,36].

This study reports the combination of a histone-like protein gene (*hu*) recovered from a functional metagenomic approach with different native and synthetic promoters to engineer the laboratory *S. cerevisiae* CEN.PK113-32D and the industrial *S. cerevisiae* Ethanol

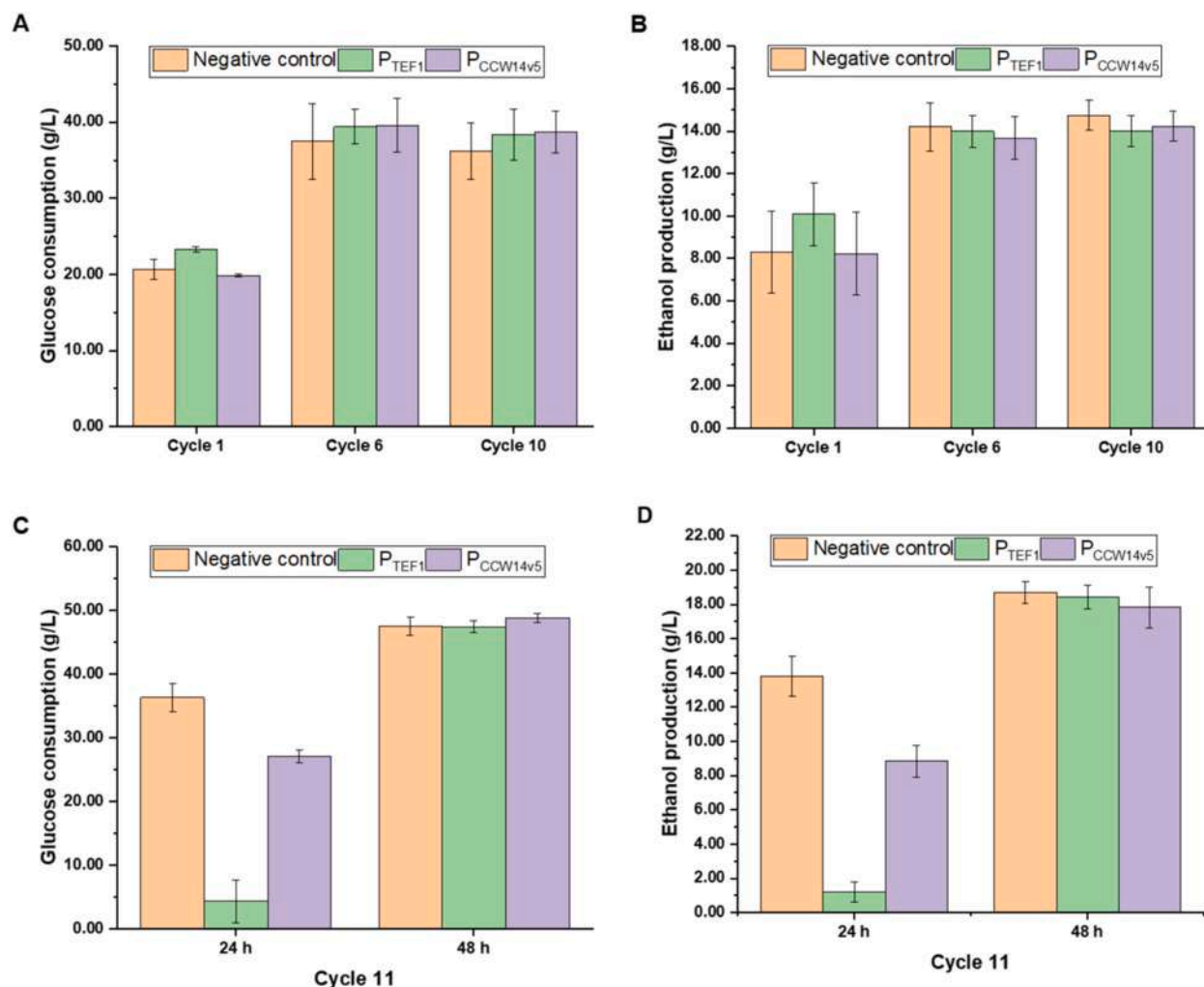


Fig. 4. Glucose consumption and ethanol production during fermentation/recycling cycles. *S. cerevisiae* CEN.PK113-32D control (harboring empty cassette – in absence of gene and promoters) and engineered with *hu* gene under the control of TEF1 and CCW14v5 promoters showing A. glucose consumption and B. ethanol production on fermentation/recycling cycles 1, 6 and 10 after 6 h of fermentation. C. Glucose consumption and D. ethanol production in cycle 11 (after 1 week of acid treatment) after 24 and 48 h fermentation. Independent experiments were carried out in biological triplicates.

Red strains that survived in extremely low pH. The *hu* gene, that remarkably was able to expand the capability of Gram-negative and Gram-positive bacteria to survive under acid stress [27] was also cloned into *Arabidopsis thaliana* and the transgenic plants showed increased growth and longer roots when the F2 generation was cultivated in pH 3.15 and 4.5, respectively (Guazzaroni et al., data not published). Moreover, together with two other genes identified through a functional metagenomics approach - *clpP* and *rbp* [27] -, *hu* gene was combined in synthetic operons that resulted in an increase of around 40% on the survival percentage of *E. coli* [37].

In prokaryotes, histone-like HU proteins are critical structural proteins involved in the maintenance of the nucleoid structure and that play important roles in gene regulation. In *E. coli*, HU protein regulates the expression of as many as 8% of the genome, either by acting together with transcription regulators or by binding directly to DNA [28,30]. *E. coli* HU mutants display multiple growth defects and show increased sensitivity to various stress conditions such as heat shock, cold shock, osmotic and oxidative stress [38]. The deficiency of histone-like proteins has been studied in a number of prokaryotes and has shown that it can result in alterations in the outer membrane composition, perturbation of cell division, decreased cellular growth and increased sensitivity to UV, oxidative stress and low pH [29,39,40]. For example, in *Mycobacterium tuberculosis*, the histone-like protein Lsr2 showed ability to protect DNA against reactive oxygen intermediates [41]. In *Deinococcus radiodurans* the histone-like protein DrHU protects the DNA against hydroxyl radical attack [42].

In *S. cerevisiae*, the mitochondrial histone HM is the major DNA binding protein and is necessary for maintenance of the mitochondrial genome during growth on fermentable sugars. Megraw and collaborators demonstrated that *E. coli* HU protein can functionally complement the function of *S. cerevisiae* HM histone in yeast strains lacking HM and, at the same time, HM can substitute HU in *E. coli*. Both proteins are functional homologous, but they did not share sequence similarity [43]. Our evolutionary relationship

analysis showed the relation among the metagenomic histone-like *hu* used in this study and other 9 histone-like proteins from the HU family that were characterized under stressful conditions (Fig. S5). For example, in *Thermoplasma acidophilum*, which is a thermophilic acidophilic archaea, the HTa protein is an abundant protein that is able to wrap the DNA and protect it from nuclease digestion [44]. Also, in *Helicobacter pylori*, the mutation in the histone-like protein *hup* leads to sensitivity to both oxidative and acid stress [45]. *H. pylori* colonizes stomachs of approximately 50% of humans and during the process, induces a strong inflammatory response that leads to the generation of a number of reactive oxygen species (ROS) [46]. In the present study we showed that the metagenomic *hu* gene can expand the survival capacity of both laboratory and industrial *S. cerevisiae* strains in extremely low pH and high concentrations of H₂O₂, depending on the promoter used. In microbial engineering, the use of strong and constitutive promoters (i.e., PTEF1, PPGK1, PTDH3, PHXT7) for simplicity are often preferred for gene expression due to their stable level of gene expression under varying conditions [47,48] and they are often used for overexpression of key enzymes in metabolic pathways [49,50].

Besides the definition of constitutive, it is already known that the strength of some promoters can vary in some conditions. Many of the constitutive promoters lose their characteristics when subjected to non-optimal conditions for *S. cerevisiae* cultivation (such as lacking glucose, presence of other carbon sources, biomass pretreatment inhibitors, low pH), making the choice of “constitutive” promoters inappropriate in some cases [51,52]. Thus, it is important to test new biological parts, such as promoters that can have better performance under e.g., stress conditions, exploring conditions that are experienced during the fermentation process. Here, it was showed that, in general, engineered strains harboring *hu* gene under the control of the strong native and constitutive PTEF1 and the synthetic stress responsive PCCW14v5 displayed better performance in the harsh conditions tested (low pH and oxidative stress). However, engineered *S. cerevisiae* strains harboring *hu* gene under the control of PPGK1 did not show improved ability to survive in pH 1.5.

Although usually considered having similar promoter strengths (strong constitutive promoters), the strengths of PTEF1 and PPGK1 can vary depending on the cultivation conditions. Sun and collaborators characterized 14 constitutive promoters from *S. cerevisiae* (including PTEF1 and PPGK1) and showed that the strength of the promoters tested varied up to six-fold depending on the condition, being PTEF1 the strongest promoter in all conditions tested [52]. Partow and collaborators characterized a set of native *S. cerevisiae* promoters and found that TEF1 and HXT7 promoters showed the strongest activity under different glucose concentrations and, although PTEF1 and PPGK1 showed comparable strengths in the first time-point measured (8 h), in glucose-limited conditions (after 24 h) PPGK1 had its strength dropped by half, while PTEF1 showed an increase of around 50% [53]. In other study, Xiong and collaborators investigated the activity of 8 native *S. cerevisiae* promoters (including PTEF1 and PPGK1) and one synthetic promoter under different stress conditions (temperature, presence of acetic acid or furfural) and carbon sources (glucose and/or xylose). They observed that the strengths of PADH1 and PPGK1, which are generally, regarded as ‘constitutive’ promoters, decreased significantly under certain conditions, suggesting that these promoters have a condition-specific activity, under stressful conditions [51].

Low pH is one of the most important stress conditions in fermentation for ethanol production. Acidic environments are common in fermentation processes because the low pH is usually maintained in the reactors to prevent bacterial contamination [9], since bacterial contaminants in ethanol production are strongly inhibited at pH below 4.0 [54]. Moreover, *S. cerevisiae* is frequently challenged by bacterial contamination, since the incoming substrate might contain microorganisms and the fermentation is carried out in non-aseptic conditions [8]. Additionally, in Brazilian ethanol production processes, *S. cerevisiae* is recycled several times (usually up to 6 months) for use in subsequent fermentations, which reduces the fermentation time (6–10 h) and cost of yeast propagation, as the carbon source is not deviated for cell multiplication but rather to ethanol production. It is well known that fermentation yield is higher in processes with recycling of yeast cells; however, it increases the chances of contamination [17,55].

Bacterial contamination can cause loss of fermentation sugars and other nutrients, increase in the formation of by-products (lactic acid and acetic acid) and premature yeast flocculation, which can lead to reduction of ethanol yields and productivity and ‘hung’ or ‘stuck’ fermentations [56,57]. To prevent bacterial contamination, the yeast usually receives a treatment with diluted sulphuric acid at pH 1.8–2.5 for 1–2 h between the fermentative cycles, which can result in reduction of yeast viability and low ethanol yield. Yamakawa and collaborators showed that the treatment of *S. cerevisiae* flocculated cultures (induced by contamination with *Lactobacillus fermentum*) with diluted sulphuric acid under pH 2.65 was highly effective for cell flocculation dispersion after 1 h exposure. However, at the lowest pH tested (1.8), the lowest cell viability was observed (85.9%), suggesting yeast cell damage [35]. In another study, Reis and collaborators showed that the treatment of rough *S. cerevisiae* strains with diluted sulphuric acid at pH 1.0 and 1.5 during 2 h severely affected its growth, while the smooth strain PE-02 did not show significant decrease in the growth after the same acid treatments [58].

Presence of rough strains are undesirable during the alcoholic fermentation, due to its association with disturbances in the process [59]. Here, *S. cerevisiae* harboring *hu* gene under the control of PCCW14v5 has shown higher survival capacity compared with the parental Ethanol Red and CEN.PK113-32D strains under low pH stress (Table 2). However, its superiority in terms of ethanol production could not be proved during the 11 fermentation cycles performed. In industrial scale, yeast recovery is carried out about six months twice a day. Thus, a long-term fermentation cycles experiment could better evaluate the acid resilience of the engineered strain. Additionally, the growth of the engineered *S. cerevisiae* strains was not affected in low pH media cultivations (Fig. S3 A). These results can be explained by the methodology used to identify *hu* gene in the functional metagenomics screening [27]. The gene showed activity in an extremely low pH that was not suitable for bacterial growth, responding to an extreme stress condition to increase the survival of the bacteria tested, but not increase or allow bacterial growth. The same occurred in our previous experiments with the engineered *S. cerevisiae* strains challenged by low pH.

S. cerevisiae is also exposed to oxidative stress, which is, in alcoholic fermentation processes, generated mainly by the presence of furfural or use of H₂O₂ in biomass pretreatments. Furfural is linked to an increase in ROS due to its large dipole moment from the aldehyde group [60]. Allen and collaborators showed that after 8 h of exposure to 25 and 50 mM of furfural, *S. cerevisiae* cells showed ROS accumulation equivalent to cells exposed to 5 mM of H₂O₂ [19]. In another study, Zhao and collaborators showed that a

S. cerevisiae mutant strain (Taf25) exhibiting significant increase in oxidative stress tolerance had a shorter lag-phase in a simulated fermentation environment in presence of 4 mM H₂O₂ and furthermore, it showed a faster increase in cell mass after 24 h, which was 12 h earlier than that of the control strain [22]. In the case of using alkaline hydrogen peroxide (AHP) for biomass pretreatment, the concentration of H₂O₂ can vary from 0.2 to 10% (58.8 mM–2.94 M) depending on the recalcitrance of the material. It has been shown that pretreatments at high H₂O₂ concentrations during shorter times are more efficient [61,62]. The main problem faced when using H₂O₂ for biomass pretreatment is to remove it before fermentation. Removal can be done by separating solid-liquid fractions, with the washing of the solid fraction or by the addition of catalase; however, both options increase the cost of ethanol production [63,64]. Hence, considering all the above mentioned, the development of strains that display a phenotype of higher tolerance to ROS and to extremely low pH is an achievement highly desirable for the economic feasibility of industrial-scale ethanol production.

One of the major bottlenecks in the production of fine-chemicals from lignocellulose is related to the production processes, which might be economically competitive when compared to the traditional method currently used, a condition that is dependent of several factors, including the yeast performance during the process, the adoption of recycling techniques – especially of the high-cost additives such as the enzymes, microorganisms, and chemicals. The present study successfully showed the use of a metagenomic gene in combination with different promoters to engineer *S. cerevisiae* strains that were able to survive in very low pH and high concentrations of hydrogen peroxide. Improving yeasts performance at low pH can potentially help minimizing contamination, increasing product yield, reducing the need for pH control systems, and making the product recovery more straightforward, especially in the case of organic acids. Additionally, engineering a good ethanol-producer yeast as *S. cerevisiae* with a gene that also convey resistance to oxidative stress, aiming to guarantee the survival of the populations under the stress conditions generated by furfural, which is one of the most important inhibitors present in sugarcane bagasse hydrolysates, is an innovative solution that can accelerate the development of more efficient fermentation processes for the production of ethanol and fine chemicals from lignocellulosic biomass.

5. Limitations of the study

The present work showed the use of a metagenomic gene in combination with different promoters for the generation of engineered *S. cerevisiae* strains that were able to survive in very low pH and high concentrations of hydrogen peroxide. However, we were unable to prove the superiority of the engineered strains in ethanol production or growth after 11 rounds of fermentation/cell recycling experiments. A long-term fermentation/cell recycling cycles would better evaluate the acid resilience of these engineered strains.

Apart from the cell recycling process, *S. cerevisiae* faces many other stressful conditions, as the presence of inhibitory molecules generated during the pretreatment methods. For example, phenolic and aromatic compounds (4-hydroxybenzoic acid, vanillin, syringic acid) generated from the breakdown of lignin [65]; and acetic acid and furan aldehydes (furfural and 5-hydroxymethyl furfural – that can be further degraded to form weak acids) derived from the degradation of the hemicellulose [66]. The strains generated in this study have a broad scenario for possible applications, given the variety of inhibitors that can be generated during the pretreatment methods and further experiments would evaluate their resistance in presence of these inhibitors.

Author contribution statement

Luana F. Alves: conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; wrote the manuscript.

Jonatã Bortolucci: performed the experiments; analyzed and interpreted the data.

Valeria Reginato: conceived and designed the experiments; contributed with reagents, materials, analysis tools or data.

María-Eugenia Guazzaroni: conceived and designed the experiments; contributed with reagents, materials, analysis tools or data.

Solange I. Mussatto: conceived and designed the experiments; contributed with reagents, materials, analysis tools or data.

All authors reviewed and approved the final version of the manuscript submitted.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2023.e14838>.

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