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ORIGINAL ARTICLE

Innately robust yeast strains isolated from grape marc have a great potential for lignocellulosic ethanol production

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Abstract Bioethanol from lignocellulose is an attractive alternative to fossil fuels, and *Saccharomyces cerevisiae* is the most important ethanol producer. However, yeast cells are challenged by various environmental stresses during ethanol production on an industrial scale, and robust strains with a high tolerance to inhibitors, temperature and osmolality are needed for the effective feasibility of lignocellulosic ethanol. To search for such innately more resistant yeast, we selected grape marc as an extreme environment due to limited nutrients, exposure to solar radiation, temperature fluctuations, weak acids and ethanol. Using a temperature of 40 °C as the key selection criterion, we isolated 120 novel *S. cerevisiae* strains from grape marc and found high ethanol yields (up to 92 % of the theoretical maximum) when inoculated at 40 °C in minimal media with a high sugar concentration. For the first time, this work assessed yeast tolerance to inhibitors at 40 °C, and the newly isolated yeast strains displayed interesting abilities to withstand increasing levels of single inhibitors or cocktails containing a mixture of inhibitory compounds. The newly isolated strains showed significantly higher fermentative abilities and tolerance to inhibitors than the industrial and commercial benchmark *S. cerevisiae* strains. The strong physiological robustness and fitness of a few of these *S. cerevisiae* yeast strains support their potential industrial application and encourage further studies in genetic engineering to enhance their ethanol performance in terms of rate and yield through the co-fermentation of all available carbon sources.

Keywords Lignocellulosic bioethanol · Yeast robustness · Thermo-tolerance · Inhibitor-tolerance · Grape marc · Strain selection

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Introduction

Bioethanol production from renewable resources, such as lignocellulose, is considered a promising alternative to fossil fuels (Galbe and Zacchi 2002; Hamelinck et al. 2005). Ideally, the raw substrate for bioethanol production should be non-edible biomass, such as energy crops, spruce or birch, as well as agricultural by-products, including grain residues and sugarcane bagasse (Kim and Dale 2004; Demirbas 2009; Favaro et al. 2012a, 2013a).

Because native lignocellulosic biomass is highly refractory to degradation, pre-treatments are needed to make the cellulose more accessible to subsequent enzymatic saccharification. However, these methods also result in the co-production of a number of degradation compounds from the hemicellulose (mainly furfural and acetic acid), lignin (phenolic compounds) and cellulose [5-hydroxmethyl-2-furaldehyde (HMF)] fractions. These by-products, present in both the hydrolysate liquor and water-insoluble solid (WIS) fractions of the pre-treatment slurry, impair cellular metabolism and growth, thereby reducing the productivity of the process (Larsson et al. 1999; Almeida et al. 2007; den Haan et al. 2013); consequently, the development of robust yeast strains with improved production rates and resistance is of crucial importance.

A variety of detoxification strategies have been developed to remove or decrease the level of inhibitors from lignocellulosic hydrolysates. However, concerns regarding the techno-

economic feasibility of these methods have been raised, namely, the additional expense of the process steps and the loss of fermentable sugars (Jönsson et al. 2013). Therefore, several countermeasures alternative to detoxification have been proposed to alleviate the challenges associated with inhibitors. Since the concentrations of toxic compounds and sugars in hydrolysates depend on the starting materials, as well as on the conditions during pre-treatment and hydrolysis (Galbe and Zacchi 2007), less recalcitrant feedstock can be selected and mild pre-treatment conditions can be applied (Galbe and Zacchi 2007; Favaro et al. 2013b). Alternatively, a number of avenues have been explored to make conditions more favourable for the fermenting microorganism (Wingren et al. 2003).

Advanced improvements in the optimisation of yeast robustness may require novel metabolic engineering tools, such as protein engineering, metabolic engineering and rational metabolic engineering, as elegantly described by Patnaik (2008) and Sagt (2013). Moreover, several breeding methods, including mutagenesis (Zhu et al. 2008), long-term adaptation (Balakumar et al. 2001; Liu et al. 2005), protoplast fusion, evolution engineering (Cakar 2005) and genome shuffling (Shi et al. 2009; Zhang and Geng 2012), have been previously employed to increase the ethanol production and thermotolerance of yeast. However, yeast strains exhibiting multitolerance to high temperature, inhibitors and ethanol levels have not yet been developed. Furthermore, laboratory strains were mainly utilised in most of these previous studies, and these may be difficult to use in industrial processes because of their generally low industrial fitness (Martin and Jönsson 2003; Garay-Arroyo et al. 2004).

One promising approach is to select for yeast strains with native resistance to inhibitors that could serve as a platform for engineering the ability to utilise xylose or arabinose as a carbon source for ethanol production. The use of naturally robust strains prevents interference with cloned genetic material, as could be the case when recombinant strains are subjected to hardening techniques.

In addition, there is a need to develop thermo-tolerant yeast capable of growth and fermentation at elevated temperatures compatible with optimal cellulase and hemicellulase activities to reduce the cost of cooling during fermentation. Thus, improving the robustness of *Saccharomyces cerevisiae* under the stress of heat and inhibitors is fundamental for the effective conversion of lignocellulose to ethanol.

Whereas many quality reports dealt with the pre-treatment of lignocellulosic materials tailored to maximise sugar release from the feedstock (reviewed in Galbe and Zacchi 2002, 2007), very few have considered yeast strains based on their innate resistance, fermentative traits and industrial scale adaptability (Lindén et al. 1992; Favaro et al. 2012a, 2013a). Moreover, the targets of previous screening or selection surveys for tolerant *S. cerevisiae* yeast were mainly individual stresses, such as high temperature (Babiker et al. 2010; Chen et al. 2013), weak acids or furans and aldehyde resistance (Martin and Jönsson 2003; Garay-Arroyo et al. 2004), while isolating and identifying yeast with a tolerance to multiple stresses apparently received little attention. However, employing naturally tolerant *S. cerevisiae* would, in fact, be a more realistic approach towards developing the second generation bioethanol industry, since it is the combined effect of the stressors that causes the greatest challenge to the success of industrial cellulosic ethanol production (van Maris et al. 2006).

To search for such robust, thermo-tolerant and strongly fermenting yeast, we assessed grape marc as an extreme environment because of the limited availability of nutrients, such as nitrogen and carbon, exposure to solar radiation, temperature fluctuations (between 20 and 45 °C), low pH and concentrations of ethanol and weak acids (Favaro et al. 2013c). Using a temperature of 40 °C as a key selection criterion, we isolated 120 *S. cerevisiae* strains from grape marc and evaluated these for their fermentative ability, as evidenced from glucose consumption and ethanol production in a minimal medium supplemented with high concentrations of glucose and xylose. The new yeast collection was subsequently screened for tolerance to inhibitors at 40 °C in yeast extract, peptone and dextrose (YPD) broth supplemented with increasing concentrations of single inhibitors or cocktails of inhibitory compounds. The effect of the culture pH on the tolerance of the yeast to inhibitor was also addressed.

Materials and methods

Yeast strains, isolation and genetic identification

The following four benchmark *S. cerevisiae* yeast strains were used in this study: the laboratory strain *S. cerevisiae* Y294, (ATCC 201160); the top fermenting beer strain *S. cerevisiae* DSM 70449; the commercial wine strain *S. cerevisiae* EC1118 obtained from Lallemand Fermented Beverages; the industrial strain *S. cerevisiae* 27P (Favaro et al. 2012b).

New yeast strains were isolated from grape marc collected from the 2011 vintage immediately following grape crushing, from a winery located in Italy. After storage for 30 days at the winery, 50 g of marc was dispersed in 500 mL of sterile physiological water (0.85 % NaCl) and the solution subjected to serial dilution before being plated on WL (Wallerstein Laboratory, Oxoid, UK) medium containing 200 μg/mL chloramphenicol (Sigma-Aldrich, USA) to contain bacterial growth and incubated at different temperatures (38, 40, 42 and 44 °C) for 72 h. Yeast colonies were isolated and then purified by growing on yeast and mould agar medium (YM; Oxoid) at 40 °C for 48 h. Isolates were stored at −80 °C in YM medium containing 20 % (v/v) glycerol.

Genetic identification of the strains was achieved using a 750-bp fragment of the ITS1–5.8S–ITS2 sequence following Montrocher et al. (1998). A 5-μL aliquot of cell suspension was heated at 94 °C for 5 min and then subjected to 30 cycles of PCR amplification (initial denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s). Amplification products were checked by agarose gel electrophoresis and then subjected to Sanger sequencing using an ABI protocol for Taq-Dye Terminator Sequencing on an automated ABI377 sequencer (Applied Biosciences, USA). The obtained sequences were edited with Chromas Lite 2.1.1 (Technelysium Pty Ltd, Australia), and species identification was performed following BLASTn alignment (http://blast.ncbi.nlm.nih.gov/Blast) with those present in the GenBank public database. For species identification, a sequence similarity level of 98 % was considered with a >90 % coverage.

Fermentative abilities of *S. cerevisiae* strains in minimal broth supplemented with high concentrations of sugars

In total, we evaluated 120 *S. cerevisiae* strains for their fermentative ability in Must Nutritive Synthetic (MNS) minimal medium supplemented with different concentrations of glucose and/or xylose (20 % glucose, 10 % glucose and 5 % xylose) following the method described by Delfini (1995). MNS broth was specifically chosen because of its composition which can be considered to be quite similar to that of several poor industrial media (Dahod 1999) and to resemble the pre-industrial scale composition of bioethanol broth, where primarily $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$ and small amounts of yeast extract or corn steep liquor are usually added during the fermentation step (Graves et al. 2006; Sassner et al. 2008).

In brief, each glass serum bottle was filled with 100 mL of MNS medium and then sealed using rubber stoppers with a needle for the removal of CO_2 produced during fermentation. Pre-cultures of *S. cerevisiae* strains, grown overnight in YPD broth, were collected, centrifuged and washed twice with sterile physiological water (0.85 % NaCl). Yeast cells were then inoculated, at an average cell concentration of 7.5×10^4 cells per millilitre, into serum bottles each containing 100 mL MNS broth. The incubation was performed in the static condition at 25 and 40 °C. The pH of medium was set at 3.5 using KOH (5 M). Fermentative vigour was monitored daily by measuring bottle weight loss in relation to CO_2 production. Results were reported, using a conversion factor of 2.118 (Delfini 1995), as grams of glucose utilised per liter of MNS. The experiments were carried out in triplicate. Samples were withdrawn after 7 and 21 days, filtered through a sieve (pore size 0.22 μm) and analyzed for their glucose and ethanol contents by high performance liquid chromatography (HPLC) as indicated in the section Analytical methods and calculations.

Screening for tolerance to inhibitors

The newly isolated yeast strains together with the reference strains were evaluated for their tolerance to inhibitors YPD medium formulated with 20 g/L glucose and increasing concentrations of weak acids (acetic, formic, lactic acid) and furans (furfural and HMF), supplemented as single compound or formulated as inhibitor cocktails.

The inhibitor levels were 1.80, 3.60, 5.40 and 7.20 g/L for acetic acid (Merk, Germany); 0.61, 1.22, 1.83 and 2.44 g/L for formic acid (Sigma-Aldrich); 1.72, 3.45, 5.17 and 6.89 g/L for lactic acid (Sigma-Aldrich); 0.69, 1.38, 2.08 and 2.77 g/L for furfural (Sigma-Aldrich); 0.94, 1.86, 2.81 and 3.75 g/L for HMF (Sigma-Aldrich). Inhibitors were also formulated into four cocktails (A, B, C and D) by mixing each increasing dose of each toxic compound, as reported in Table 1. Higher concentrations of a single inhibitor were also tested as follows: concentrations of 9.0, 10.8, 12.6 and 14.4 g/L for acetic acid; 3.05, 3.66; 4.27 and 4.88 g/L for formic acid; 3.46 and 4.15 g/L for furfural; 4.69, 5.63 and 6.56 g/L for HMF.

Yeast cells, grown overnight at 40 °C in YPD broth at 100 rpm, were incubated under aerobic conditions in 0.9 mL of medium at 40 °C. A low cell density (7.5×10^5 cells/mL) was specifically used in this experiment to facilitate our search for robust yeast cells capable of withstanding both high temperature and high inhibitor(s) levels. The experiments were performed in triplicate.

After 40 h, optical density at 600 nm (OD_{600}) was measured. For each strain, the tolerance was evaluated as relative growth (OD_{600} value, %) by comparing the growth in the medium with and without the inhibitors.

The effects of pH on the tolerance of yeast to inhibitors were also considered. To this aim, the pH of YPD broth was left unchanged or, alternatively, adjusted to 4.5, 5.0 or 5.5, after the addition of the inhibitor(s), using NaOH (5 M) or HCl (5 M). This pH range was assessed because it is characteristic of many bioethanol production processes.

Table 1 Composition of synthetic inhibitor cocktails added to YPD broth

Inhibitor (g/L)	Cocktail A	Cocktail B	Cocktail C	Cocktail D
Acetic acid	1.80	3.60	5.40	7.20
Formic acid	0.61	1.22	1.83	2.44
Lactic acid	1.72	3.45	5.17	6.89
Furfural	0.69	1.38	2.08	2.77
HMF	0.94	1.86	2.81	3.75

Analytical methods and calculations

Samples were taken before and during MNS fermentation, filtered through a sieve (pore size 0.22 μm) and analysed by HPLC for glucose, xylose, xylitol, glycerol, acetic acid and ethanol. Monosaccharide analysis was performed with high-performance anion-exchange chromatography with pulsed amperometric detection. The system was equipped with a PA1 column and auto-sampler (Dionex Corporation, Sunnyvale, CA, USA) and the mobile phase used was 1 mM NaOH at a flow rate of 1 mL/min at room temperature (Favaro et al. 2013b).

Organic acids and ethanol were separated on an Aminex HPX-87H column (Bio-Rad, USA) at 65 °C with 5 mM H_2SO_4 as the mobile phase, at a flow rate of 0.5 mL/min. The system (Shimadzu, Japan) was equipped with refractive index detector (Shimadzu) and a cation-H refill cartridge (Bio-Rad).

All experiments were conducted in triplicate, and data were analyzed on Microsoft Excel using one-way analysis of variance. A probability value (P) <0.05 was considered to be statistically significant.

Results and discussion

Isolation and screening for efficient fermenting, thermo- and osmo-tolerant *S. cerevisiae* strains

Although ability to produce ethanol by fermentation at high temperature is considered to be an essential phenotypic trait for a lignocellulosic bioethanol yeast (Banat et al. 1998; Chen et al. 2013), there is a limited number of reports on the screening and selection of yeast able to grow and ferment at or above 40 °C (reviewed in Babiker et al. 2010). We therefore carried out yeast isolations on WL plates incubated at 38, 40, 42 and 44 °C to select for thermo-tolerant and robust yeast from grape marc, which is an unexplored source of microbial biodiversity. We found that a large number of colonies grew at 38 °C and that there was limited or no growth at all at 42 and 44 °C, respectively; therefore, we selected colonies from plates incubated at 40 °C for the isolation of thermo-tolerant strains for further study and genotyping. As described in the Materials and methods, 120 of these isolates showed similarities of the ITS1–5.8S–ITS2 sequence of more than 98 % with the ITS1–5.8S–ITS2 sequence of *S. cerevisiae* type strains (Kurtzman and Robnett 2003) and were first screened for their ability to consume glucose at 40 °C in the MNS minimal broth supplemented with either 200 g/L glucose or a combination of glucose (100 g/L) and xylose (50 g/L). These carbon sources were considered to be representative of the hexose and pentose content in most lignocellulosic hydrolysates (Galbe and Zacchi 2002; Hamelinck et al. 2005). In this study, the ability of the yeast to consume glucose was defined as fermenting vigour and expressed in terms of grams of glucose consumed per liter of MNS broth, as described in the Materials and methods.

Four benchmark strains of *S. cerevisiae* were included in this study. Two *S. cerevisiae* strains, namely DSM 70449 and 27P, had been previously adopted for ethanol production from different lignocellulosic substrates (Almeida et al. 2007; Favaro et al. 2012b), whereas the oenologically relevant *S. cerevisiae* strain EC1118 (Egli et al. 1998) and the laboratory strain Y294 (Martin and Jönsson 2003) were evaluated as additional benchmarks.

Our collection of newly isolated yeast strains produced interesting fermentative performances; Fig. 1 shows the fermenting ability of the 40 best performing yeast strains. At 25 °C in MNS broth with 200 g/L glucose, the majority of the selected isolates readily metabolised glucose (Fig. 1a). Of these, seven *S. cerevisiae* strains (F19, F47, F199, Fm84, Fm87, Fm88 and Fm92) exhibited outstanding fermentative performance, consuming all of the glucose. Surprisingly, the majority of the yeast strains isolated from grape marc showed better fermenting abilities than those of benchmark strains. In comparison, *S. cerevisiae* DSM 70449, the top fermenting beer yeast, showed a significantly lower sugar uptake, while the reference *S. cerevisiae* strains 27P and EC1118, although having the most prominent fermenting phenotype among the tested reference strains, metabolised glucose much slower. As expected, the laboratory *S. cerevisiae* Y294 exhibited only limited fermenting vigour, utilising no more than 110 g/L glucose (Fig. 1a).

In the presence of xylose (Fig. 1b), only four *S. cerevisiae* strains (F66, F100, F199 and Fm92) were able to completely deplete glucose, while the other yeast strains did not use all of the glucose available in the broth, as confirmed also by HPLC analysis (data not shown). In general, the newly isolated strains, with the exception of *S. cerevisiae* Fm4, had better fermentative vigour than that the benchmark yeast.

As shown in Fig. 2, at higher temperature (40 °C) all of the strains showed a rapid consumption of glucose within the first days of culture, followed by a slowing down of fermentation,, as detected by HPLC analysis; consequently, considerable amounts of glucose were still in the broth (data not shown). In MNS medium supplemented with 200 g/L glucose, the control *S. cerevisiae* strains consumed up to 80 g/L of glucose, while the majority of the new *S. cerevisiae* yeast strains had relatively higher fermenting abilities (Fig. 2a), with *S. cerevisiae* F55 and F156, capable of utilising >120 g/L glucose. The latter strain showed a fermenting vigour that was1.5-fold higher than those of the benchmark yeast 27P and EC1118.

Similar behaviour was observed following the incubation of the yeast strains in MNS broth formulated with 100 g/L glucose and 50 g/L xylose, where only the reference

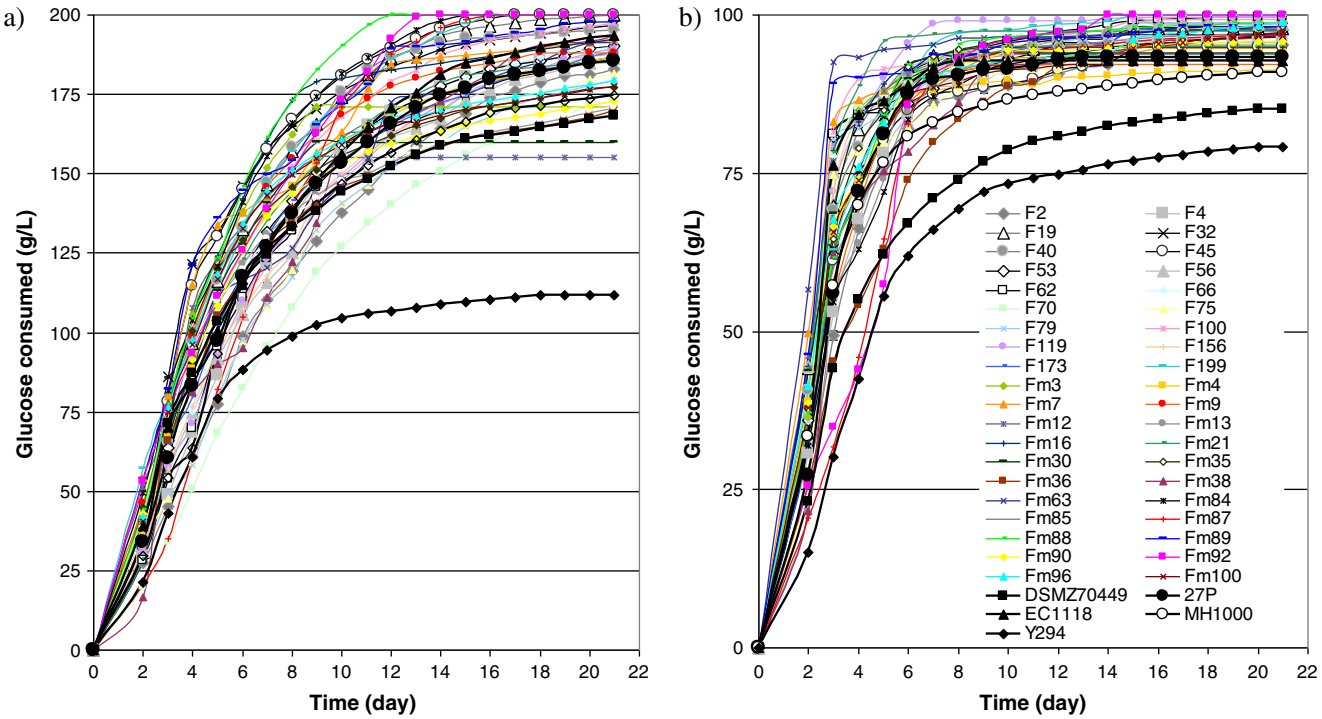

Fig. 1 Cumulative sugar utilisation (grams of glucose consumed per litre of MNS medium) of *Saccharomyces cerevisiae* strains following incubation at 25 °C in MNS medium with 200 g/L glucose (**a**) and with 100 g/L glucose + 50 g/L xylose (**b**). All experiments were conducted in triplicate, and the relative standard error was always <5 % (not reported)

S. cerevisiae strain 27P showed fermentative vigour comparable to that measured for a cluster of the newly isolated *S. cerevisiae* yeast strains (Fig. 2b). In contrast, the other reference yeast strains generally exhibited a low capability

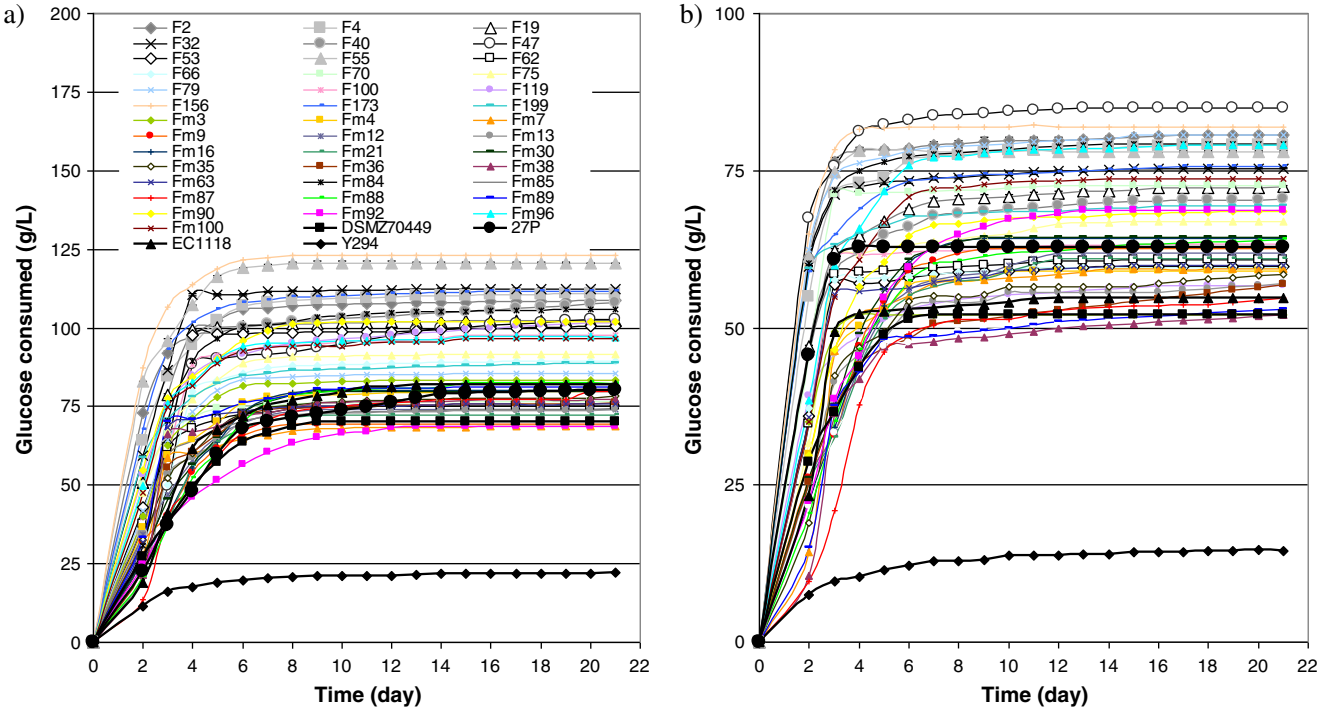

Fig. 2 Cumulative sugar utilisation (grams of glucose consumed per litre of MNS medium) of *S. cerevisiae* strains following incubation at 40 °C in MNS medium with 200 g/L glucose (**a**) and with 100 g/L glucose + 50 g/L xylose (**b**). All experiments were conducted in triplicate, and the relative standard error was always <5 % (not reported)

of withstanding higher temperature and osmotic stress, since they consumed only up to 50 g/L glucose while the results confirmed that laboratory strain Y294 also had the poorest fermenting ability at 40 °C. Among the 40 selected new *S. cerevisiae* yeast strains assessed, the most proficient were *S. cerevisiae* F2, F47, F55, F79, F156 and Fm96, all of which showed a degree of glucose consumption that was much higher than those achieved by the reference yeast. For example, the glucose consumed by *S. cerevisiae* F47 was almost sixfold greater than that of the weakest control yeast strain, Y294, and 1.4-fold higher than that of the best reference yeast strain, 27P.

Ethanol performances at 25 and 40 °C

Due to the large volume of data generated from the screening of the 120 newly isolated yeast strains, we performed a deeper analysis of the consumption of the carbon source and ethanol production only for the top eight strains in terms of fermentative vigour and for the three benchmark *S. cerevisiae* 27P, EC1118 and DSM 70449. The laboratory strain Y294, which exhibited poor fermentative vigour, was not included.

As reported in Table 2, when incubated at 25 °C in MNS medium containing 200 g/L glucose, *S. cerevisiae* strains F47, Fm84 and Fm90 fermented glucose into ethanol with a peak yield of about 97 % of the theoretical (0.51 g of ethanol per gram of glucose). This fermentative efficiency is higher than that recently reported for other *S. cerevisiae* strains cultured at high glucose concentrations (Ortiz-Muniz et al. 2010; He et al. 2012; Favaro et al. 2013b). Moreover, this value was significantly greater than that of the reference yeast DSM 70449, which yielded the lowest ethanol concentration (about 78 g/L), while the excellent suitability of both *S. cerevisiae* 27P and EC1118, producing >90 g/L ethanol, to be used in bioethanol and wine processes, respectively, was confirmed (Favaro et al. 2013d; Aceituno et al. 2012).

In the medium supplemented with 100 g/L glucose and 50 g/L xylose, the newly isolated strains achieved ethanol levels higher than those obtained by the reference strains. Interestingly, *S. cerevisiae* strains Fm84 and Fm90 produced about 48 g/L ethanol, which was 1.09-fold higher than the alcohol concentration detected for the best control yeast, *S. cerevisiae* 27P. Comparing the ethanol yields obtained by the strains in both media (MNS with 200 g/L glucose and MNS with 100 g/L glucose + 50 g/L xylose), it would appear that the presence of xylose in the MNS broth affected glucose uptake and ethanol production; indeed fermentation was slowed down in all of the yeast strains, but particularly in the reference strains, and all of the glucose available in the broth was not consumed (Table 2). For example, *S. cerevisiae* DSM 70449 had a much lower ethanol yield (80 % of the theoretical value) in MSN medium supplemented with both glucose and xylose than in MNS broth supplemented with only 200 g/L glucose, thereby leaving a considerable amount

Table 2 Sugar consumption and product formation by selected *Saccharomyces cerevisiae* isolates and benchmark strains after 21 days of fermentation at 25 °C in MNS broth supplemented with glucose alone (200 g/L) or with glucose (100 g/L) + xylose (50 g/L)

Medium	*Saccharomyces cerevisiae* isolates and benchmark strains[a]										
	27P	EC1118	DSM 70449	F2	F4	F32	F47	F156	F173	Fm84	Fm90
MNS with 20 % glucose											
Glucose (g/L)	5.8	6.6	31.0	11.7	2.3	10.4	–	9.7	11.5	–	24.1
Glycerol (g/L)	5.8	6.8	5.9	4.3	4.4	5.1	3.6	5.8	4.5	6.0	4.5
Ethanol (g/L)	93.7	94.2	78.1	90.9	97.2	90.6	99.0	92.5	89.3	99.3	85.6
Ethanol yield[b]	0.48	0.49	0.46	0.48	0.49	0.48	0.50	0.49	0.47	0.50	0.49
Ethanol yield (%)[c]	94	96	91	95	96	94	97	96	93	97	97
MNS with 10 % glucose and 5 % xylose											
Glucose (g/L)	5.1	7	14.8	4.5	4.3	1.9	3.7	2.2	2.2	–	1.5
Xylose (g/L)	48.2	45	46.7	48.7	47.1	45.2	44	47.1	48.3	48.1	46.5
Xylitol (g/L)	1.7	4.8	2.9	1.3	2.6	4.6	5.8	2.4	1.6	1.4	3.6
Glycerol (g/L)	3.6	3.9	3.6	3.2	3.3	3.7	3.2	3.2	2.9	2.8	3.4
Ethanol (g/L)	43.8	43.4	34.9	44.2	45.4	46.5	44.5	47	45	48.2	47.5
Ethanol yield[b]	0.46	0.47	0.41	0.46	0.47	0.47	0.46	0.48	0.46	0.48	0.47
Ethanol yield (%)[c]	90	92	80	91	93	93	91	94	90	95	93

All experiments were conducted in triplicate, and the relative standard error was always <5 % (not reported)

[a] Underlined strains are the control strains; all other strains (no underlining) are *S. cerevisiae* strains isolated from grape marc

[b] Ethanol yield as gram ethanol/gram consumed glucose

[c] Ethanol yield as % of theoretical maximum (0.51 g/g from glucose)

of glucose in the exhausted broth. However, further studies are in progress to investigate this interesting finding in more depth in order to elucidate the possible negative effect of high xylose concentrations on the glucose-to-ethanol conversion by *S. cerevisiae*. Nevertheless, as reported in Table 2, *S. cerevisiae* strains F156, Fm84 and Fm90 achieved remarkable ethanol yields (about 94 % of the theoretical maximum) and their fermenting efficiencies are a valuable asset considering those displayed by the reference yeast and other *S. cerevisiae* strains (Matsushika et al. 2009; Zhang and Geng 2012).

When incubated at 40 °C in MNS with 200 g/L glucose, the selected strains produced ethanol concentrations ranging between 47.1 and 56.2 g/L, with the latter corresponding to an ethanol yield of 92 % of the theoretical maximum. In comparison, the reference strains showed significantly lower fermenting efficiencies, with *S. cerevisiae* strain 27P being the most efficient at 86 % of the theoretical maximum (Table 3).

In general, the benchmark yeast strains had consumed only half of the supplied glucose by the end of the fermentation period, pointing to ethanol inhibition, which is known to increase with temperature (Banat et al. 1998; Babiker et al. 2010). Similar results were observed in the MNS broth supplemented with 100 g/L glucose and 50 g/L xylose (Table 3). Although all of the yeast strains showed reduced ethanol efficiency in the presence of xylose, the production of ethanol by the newly isolated *S. cerevisiae* strains ranged from 32.4 to 38.1 g/L, while the reference strains were characterised by a much lower fermenting performance. *S. cerevisiae* strains Fm84 and Fm90 exhibited the highest ethanol yields (about 92 % of the theoretical value), which was 1.14-fold higher than the yield achieved by the best control strain, 27P.

Overall, the fermentative parameters exhibited at 40 °C by the new collection of yeast isolated from grape marc are of great interest when compared to those reported in the literature (Babiker et al. 2010). Hacking et al. (1994) compared 55 yeast strains for glucose fermentation at elevated temperatures, achieving yields of 50 % of the theoretical maximum with 12 strains at 40 °C. Thermo-tolerant strains have also been isolated from hot climates or regions. Pellegrini and colleagues screened 457*S. cerevisiae* cultures, ultimately determining that the yeast DBVPG 1849, isolated from Ethiopian wine, was the most efficient fermenting strain at 40 °C, with an ethanol yield of nearly 85 % of the theoretical maximum (Pellegrini et al. 1999). Considering that the yeast DBVPG 1849 has the highest glucose-to-ethanol conversion yield at 40 °C described to date for a *S. cerevisiae* species, this is the first account of a *S. cerevisiae* yeast capable of fermenting glucose at 40 °C with ethanol yields close to 92 % of the theoretical maximum in the presence of high concentrations of sugars. Such fermenting efficiency is even higher than that detected at 40 °C for UV mutants (*S. cerevisiae* UV1 and UV2) and few genome shuffled *S. cerevisiae* yeast evolved from the UV1 and UV2 strains (Shi et al. 2009).

Table 3 Sugar consumption and product formation by selected *S. cerevisiae* isolates and benchmark strains after 21 days of fermentation at 40 °C in MNS broth supplemented with glucose (200 g/L) or with glucose (100 g/L) + xylose (50 g/L)

Medium	*Saccharomyces cerevisiae* isolates and benchmark strains[a]										
	27P	EC1118	DSM 70449	F2	F4	F32	F47	F156	F173	Fm84	Fm90
MNS with 20 % glucose											
Glucose (g/L)	110.8	105.6	125.1	90.6	89.4	87.5	96	80.1	89.5	94	99.3
Glycerol (g/L)	4.6	2.6	3.5	5	4.5	5.2	4	4.5	4.3	3.8	4
Ethanol (g/L)	39	38.6	30	50.5	51.4	52.1	49.1	56.2	49.9	49.9	47.1
Ethanol yield[b]	0.44	0.41	0.4	0.46	0.47	0.46	0.47	0.47	0.45	0.47	0.47
Ethanol yield (%)[c]	86	80	78.00 %	91	91	91	92	92	89	92	92
MNS with 10 % glucose and 5 % xylose											
Glucose (g/L)	38.1	43.4	47.8	24.4	21	26.6	16.6	18.5	25.2	21	26
Xylose (g/L)	47.4	46.7	46.3	48.5	46.8	46	46.9	48.3	49.3	49.1	48.5
Xylitol (g/L)	2.2	2.1	2.8	3.3	3	3.8	3	3.8	3.6	2.5	2.5
Glycerol (g/L)	3	2.5	2.8	3.2	2.7	3.2	3.3	2.7	3	2.5	2.9
Ethanol (g/L)	25.5	22.7	19.5	33.9	34.3	32.4	38.1	38	34.1	37.3	34.6
Ethanol yield[b]	0.41	0.4	0.37	0.45	0.45	0.44	0.46	0.47	0.45	0.47	0.47
Ethanol yield (%)[c]	81	79	73	88	88	87	89	91	89	92	92

All experiments were conducted in triplicate, and the relative standard error was always <5 % (not reported)

[a] Underlined strains are the control strains; all other strains (no underlining) are *S. cerevisiae* strains isolated from grape marc

[b] Ethanol yield as gram ethanol/gram consumed glucose

[c] Ethanol yield as % of theoretical maximum (0.51 g/g from glucose)

In previous studies, the thermo-tolerance of *S. cerevisiae* strains has been far screened by incubating the strains in complex media, such as yeast extract, peptone, glucose (YPD) and/or similarly composed broths (Hacking et al. 1994; Pellegrini et al. 1999; Shi et al. 2009; Babiker et al. 2010). Consequently, the fermenting abilities of the strains selected in our study are even more significant considering that (1) they were achieved in MNS minimal broth, whose composition is quite similar to that of several industrial broths (Dahod 1999) and 2) the fermentations were based on low initial inoculum size (about 10^5 cells/mL).

Tolerance to inhibitors in YPD broth

The 120 newly isolated *S. cerevisiae* strains together with the four benchmark yeast strains were studied for their growth at 40 °C in the presence of increasing concentrations of inhibitory compounds (weak acids and furans), either formulated as a single toxic component or combined in inhibitor cocktails. Aerobic growth in the presence of inhibitors was chosen as the parameter to evaluate the yeast collection in terms of inhibitor tolerance.

The first set of experiments were conducted without adjusting the pH value after the addition of the inhibitor(s) and, consequently, the pH dropped mainly due to the addition of weak acids. This allowed us to screen the strains for the ability to withstand mostly the undissociated acidic forms. This experimental rationale turned out to be useful in selecting the most tolerant yeast as *S. cerevisiae* strains generally displayed strong but variable tolerance phenotypes to each inhibitory compound and, above all, to the inhibitor cocktails (data not shown). Among the aliphatic acids tested, lactic acid did not affect cell growth to any extent since in the presence of the highest amount of lactic acid considered in our study (6.89 g/L), all of the strains produced relative growth values statistically similar to those obtained in the absence of the acid. Similar results have been recently reported by Albers and Larsson (2009) who found that lactic acid added to YPD in the range of 0 to 8 g/L did not hinder the growth and metabolism of other laboratory and industrial *S. cerevisiae* strains. Therefore, additional experiments using this weak acid as the sole inhibitory compound are not reported here, although we continued to use lactic acid as a component of the inhibitor cocktails as it could act as additional acidic stress on yeast metabolism.

The addition of acetic and formic acid reduced yeast growth although the toxic effects of these acids were lower than those powered by furans. Interestingly, the hindering effects of the cocktails were powerful as the majority of the 124 strains screened were able to withstand only cocktail formulation A, which contained the combination of the lowest amount of each inhibitor (data not shown).

To further investigate the tolerance to yeast strains to inhibitors, we evaluated ten *S. cerevisiae* strains, selected on the basis of their tolerance to each toxic compound, for their

Table 4 Influence of weak acids (acetic and formic acid) and furans (furfural and HMF) on growth at 40 ° C in YPD medium of selected newly isolated *S. cerevisiae* strains and the benchmark *S. cerevisae* 27P, EC1118, DMS70449 and Y294. After inhibitor(s) addition pH medium was adjusted to 4.5. Values, reported as relative growth (%) of the optical density measured for each strain after 40 h growth in YPD without inhibitor, are the means of three replicates. Standard error was always less than 4 % (not reported). Bold and grey fonts are used for values equal to or higher than 90 and equal to or lower than 50, respectively

Inhibitor	g/L	mM	27P	EC1118	DSM 70449	Y294	F156	Fm3	Fm12	Fm30	Fm38	Fm64	Fm85	Fm89	Fm90	Fm96
Acetic acid	1.80	30	**95**	**95**	**90**	**92**	**93**	**95**	**98**	**91**	**98**	**98**	**96**	**99**	**97**	**97**
	3.60	60	**93**	**93**	87	88	**92**	**93**	**96**	88	**95**	**98**	**96**	**98**	**97**	**94**
	5.40	90	**90**	85	83	83	89	**91**	**94**	87	**92**	**94**	**93**	**96**	**94**	**92**
	7.20	120	82	78	74	72	85	89	**93**	86	**90**	**91**	**90**	**93**	**91**	**90**
Formic acid	0.61	13	**99**	**99**	**96**	**96**	**99**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
	1.22	27	**99**	**99**	**94**	**96**	**98**	**99**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**99**
	1.83	40	**98**	**99**	**94**	**96**	**98**	**99**	**100**	**100**	**99**	**99**	**100**	**100**	**100**	**98**
	2.44	53	**98**	**99**	**93**	**95**	**97**	**98**	**100**	**98**	**98**	**98**	**100**	**100**	**100**	**98**
Furfural	0.69	7	**95**	85	84	89	88	85	**90**	85	**94**	**97**	86	**93**	**90**	**90**
	1.38	14	83	76	64	83	80	82	88	83	**92**	**95**	82	**90**	87	88
	2.08	22	70	22	44	28	70	77	87	56	**91**	**91**	81	89	86	68
	2.77	29	47	8	18	0	51	63	25	20	43	57	78	85	85	52
HMF	0.94	7	**95**	89	86	85	**90**	**90**	**93**	**91**	**95**	85	**91**	87	89	**92**
	1.86	15	88	81	79	78	81	87	**91**	83	**92**	71	81	75	78	87
	2.81	22	75	65	66	60	75	82	87	67	87	69	69	73	75	74
	3.75	30	51	45	44	35	55	70	76	45	82	40	61	70	72	56
Cocktail	A		86	75	75	81	78	76	87	83	88	84	77	**90**	88	83
	B		79	66	41	43	65	68	77	61	76	77	70	78	78	70
	C		50	20	16	17	51	66	69	28	62	72	64	70	65	60
	D		9	0	0	0	0	8	7	4	8	18	11	16	14	5

Underlined strains are the control strains; all other strains (no underlining) are S. cerevisiae strains isolated from grape marc

ability to grow at 40 °C in YPD broth with the pH adjusted to 4.5 after addition of the inhibitors. Reference yeast were included in the experiment for comparison (Table 4).

On a molar basis, formic acid and acetic acid had a similar inhibiting effect on cell growth. Accordingly, when exposed to the highest dose of formic acid (53 mM), the yeast strains showed relative growth values ranging from 93 to 100 %, similar to those detected in the broth supplemented with 60 mM acetic acid. However, additional doses of acetic acid impaired cell growth and, when exposed to the highest concentration (120 mM), the selected yeast strains showed relative growth values ranging from 85 to 93 % of the growth achieved in the medium without acetic acid, with strains Fm12 and Fm89 showing the most promise. The performance of the control strains exhibited a similar trend, although their values were in most cases lower than those exhibited by the newly isolated yeast strains. Among the reference yeast, the laboratory strain *S. cerevisiae* Y294 had a tolerance to the inhibitors that was comparable with that of the wildtype yeasts *S. cerevisiae* EC1118 and DSM 70449. This result is in accordance with those reported on laboratory strains exhibiting few phenotypic traits comparable to those of robust industrial yeast (Martin and Jönsson 2003).

Of the furans tested, furfural was the most toxic, as evident from the 30 % decrease in relative growth of the yeast strains that was observed following the addition of 2.08 g/L furfural. Strains Fm89 and Fm90 exhibited the greatest degree of tolerance at 2.77 g/L furfural. Similarly, supplementation with HMF also resulted in severe decreases in growth, even though these responses were not as striking as those with furfural. As a result, in the presence of 2.81 g/L HMF, the yeast strains showed relative growth values ranging from 67 to 87 % of the culture growth achieved in the medium without this inhibitor, with the strains Fm12 and Fm38 showing the highest level of tolerance also at 3.75 g/L.

Inhibitor cocktails, formulated as described in Table 1, severely hindered cell growth, with the benchmark yeast strains being the most sensitive (Table 4). Although cocktails A and B generally resulted in strong growth inhibition, cocktails C and D had the highest negative effects on yeast growth. Nevertheless, *S. cerevisiae* strains Fm12, Fm64 and Fm89, when exposed to cocktail C, exhibited the highest degree of

Table 5 Influence of weak acids (acetic and formic acid) and furans (furfural and HMF) on growth at 40 °C in YPD medium of selected *S. cerevisiae* strains and of the most tolerant control yeast 27P. After inhibitor(s) addition pH medium was adjusted to 4.5 or to 5.0 and 5.5 in the case of the cocktails A, B, C, D. Values, reported as relative growth (%) of the optical density measured for each strain after 40 h growth in YPD without inhibitor, are the means of three replicates. Standard error was always less than 4 % (not reported). Bold and grey fonts are used for values equal to or higher than 90 and equal to or lower than 50, respectively.

Inhibitor	g/L	mM	27P	Fm12	Fm38	Fm64	Fm89	Fm90
Acetic acid	9.00	150	69	80	78	76	78	77
	10.80	180	21	68	58	68	63	63
	12.60	210	5	56	23	55	50	53
	14.40	240	0	10	0	6	5	3
Formic acid	3.05	66	**92**	**95**	85	**96**	**96**	**95**
	3.66	79	**91**	**93**	80	**94**	**97**	**93**
	4.27	92	82	86	76	**93**	**93**	**90**
	4.88	105	70	88	72	86	88	87
Furfural	3.46	36	40	0	0	76	0	0
	4.15	43	0	0	0	0	0	0
HMF	4.69	38	15	58	58	30	55	56
	5.63	46	0	21	25	14	18	29
	6.56	54	0	5	8	0	0	0
Cocktail pH 5.0	A		89	**91**	**92**	**92**	**93**	**92**
	B		84	84	82	83	83	81
	C		71	74	70	76	75	78
	D		36	59	62	60	63	64
Cocktail pH 5.5	A		**94**	**96**	**96**	**95**	**94**	**96**
	B		87	88	88	87	86	88
	C		75	80	78	81	78	79
	D		51	73	70	70	72	74

Underlined strain is the control strain; all other strains (no underlining) are S. cerevisiae strains isolated from grape marc

tolerance, with a relative growth of about 70 %. In contrast, cocktail D, formulated with 7.20 g/L acetic acid, 2.44 g/L formic acid, 6.89 g/L lactic acid, 2.77 g/L furfural and 3.75 g/L HMF, supported only a slight growth by a few of the tested strains, suggesting that each inhibitory compound may have synergistically challenged the yeast to grow under this multiple environmental stress.

To investigate the tolerance to inhibitors of the five most promising S. cerevisiae strains, we supplemented YPD broth with higher concentrations of each toxic compound. We also evaluated the tolerance of the strains to inhibitors in cocktails A, B, C and D, adjusting the pH to 5.0 and 5.5. The reference yeast strain 27P, which had a tolerance to inhibitors that was generally comparable to that of the newly isolated strains, was included in the experiment (Table 5).

The yeast strains had high relative growth values at the tested acetic acid levels (9, 10.8, 12.6 and 14.4 g/L). This finding can be explained in the context of the specific environment they originated from. Grape marc is quite rich in acetic acid (Ribereau-Gayon et al. 2007; Favaro et al. 2013c). In contrast, as reported in Table 5, the reference *S. cerevisiae* strain 27P was revealed to be the most sensitive of the tested yeast strains.

Strikingly, the newly isolated strains were able to grow in the presence of higher formic concentrations (Table 5) with relative growth values ranging from 72 to 88 % with 4.88 g/L formic acid. The most resistant strains, Fm64, Fm89 and Fm90, also tolerated further increases in the concentration of this aliphatic acid of up to 8.0 g/L (data not shown).

Only strains *S. cerevisiae* 27P and Fm64 grew in YPD supplemented with 3.46 g/L furfural, with these newly isolated strains showing significantly higher resistance than the reference yeast. HMF was less toxic than furfural as, with the exception of the industrial strain 27P, the yeast had the potential to grow in the presence of up to 5.63 g/L HMF (Table 5).

The results obtained with four inhibitor cocktails at pH 5.0 and 5.5 show that for each strain the higher pH, the more pronounced the tolerance, with *S. cerevisiae* Fm89 and Fm90 exhibiting relative growth values of more than 63 and 72 % at pH 5.0 and 5.5, respectively, in cocktail D. The reference yeast strain 27P was inhibited to a higher extent under identical conditions, showing, values of only 36 and 51 %, respectively.

Taking the results obtained with the cocktails at pH 4.5 into consideration (Table 4), it clearly appears that pH plays a central role in the tolerance of yeast strains to inhibitors. As a result, the pH value(s) usually set in many biomass-to-ethanol processes on the basis of the optimal values for the commercial hydrolytic enzymes (amylases, cellulases, xylanases) should be carefully defined to also take into account the amount and type of inhibitors present in the starting or pre-treated materials in order to boost the tolerance of the yeast strain to the toxicity of the inhibitors.

Overall, the promising inhibitor tolerance phenotypes detected in YPD at different pH values were notable, as many published studies on *S. cerevisiae* inhibitor endurance used predominantly YPD or YPD-based media, but with the pH adjusted to higher values (up to 6.5), which would have decreased the inhibiting power of the aliphatic acids to which the cultures were exposed (Albers and Larsson 2009; Koppram et al. 2012). In addition, the reported surveys were conducted at 30 °C while in our study the tolerance of yeast strains to inhibitors was evaluated for the first time at 40 °C. Moreover, to facilitate our search for robust yeast strains, we specifically designed our study to start with a cell inoculum size (about 10^6 cells/mL) tenfold lower than that normally used in similar experimental activities (Larsson et al. 2001; Martin and Jönsson 2003; Garay-Arroyo et al. 2004).

To our knowledge, this is the first account describing the isolation, characterisation and selection of *S. cerevisiae* yeast strains based on high fermenting abilities and inhibitor tolerance at 40 °C. The new thermo-tolerant yeast collection screened for tolerance to inhibitors at 40 °C show a great potential to tolerate inhibitor(s) concentrations much higher than those exhibited by commercial and industrial yeast generally used in the biomass-to-ethanol route. For example, *S. cerevisiae* strain Fm90, selected as one of the best fermenting isolates (Tables 2 and 3), showed a promising inhibitor tolerance phenotype (Tables 4, 5). As a result, we have demonstrated that the choice of grape marc as a source of microbial diversity was effective in terms of isolating new strains capable of coping with the most significant stresses prevalent in large-scale bioethanol production. Moreover, the phenotypic differences observed between the screened yeast isolates indicated that the selection of strain is decisive when contemplating the design of a process involving fermentation in the presence of lignocellulosic hydrolysates.

The strong physiological robustness and fitness of a few of our newly isolated *S. cerevisiae* strains support their potential industrial application and encourage further genetic engineering to enhance their ethanol performance through the co-fermentation of all available carbon sources.

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