

Biotechnological potential of yeast isolates from *cachaça*: the Brazilian spirit

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Abstract This study identified phenotypic traits appropriate for biotechnological applications of 118 yeasts isolated from *cachaça* distilleries. Different properties were verified: capacity to use alternative carbon sources; ability to tolerate high concentrations of sucrose, ethanol, methanol, aluminum and zinc as well as different pH values and foam production. *Pichia guilliermondii* and *Pichia anomala* strains were identified as the most promising ones for application in the second-generation biofuel industry, showing ability to grow on high glycerol concentrations. Other isolates, identified as *Saccharomyces cerevisiae*, produced bioethanol comparable to the industrial strains, and were therefore ideal for use in the first-generation ethanol industry. Some of these strains also showed high resistance

to aluminum, as observed in sugarcane juice, and to inter-cycle washings with diluted sulphuric acid, as performed in the industrial bioethanol production process. In summary, yeast isolates from *cachaça* distilleries displayed robustness and phenotypic plasticity, which makes them interesting for biotechnological applications.

Keywords *Saccharomyces cerevisiae* · *Pichia anomala* · *Pichia guilliermondii* · *Cachaça* · Biotechnological applications

Introduction

The increasing demand for energy is an irreversible global phenomenon. Intensive use of fossil fuels, the main component of the energy matrix of most societies, is observed for decades as unsustainable in the long term. Thus, search for cleaner, renewable energy sources has increased in the past few decades. Among these, biofuels have been proposed as a short-term solution, fitting a transition stage before better options are available [2, 16]. This is mostly due to their relative chemical similarity to fossil fuels, which reduces the need for large modifications in current combustion engines and fuel distribution logistics. Some countries such as Brazil already have biofuels on their energy matrix. Since the late 1970s, ethanol produced by a few *Saccharomyces cerevisiae* industrial strains has been added to gasoline or directly used in motor vehicles.

Brazilian bioethanol production is commonly performed in very-high-gravity (VHG) fermentations using highly concentrated substrates such as sugarcane juice and molasses and producing high ethanol yields [6], thus reducing the costs of ethanol distillation [13]. In addition, the Brazilian bioethanol production process recycle yeast

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cells. After fermentation, they are collected by centrifugation and incubated in dilute sulphuric acid (pH 2.0–2.5) for 1–3 h. This treatment reduces bacterial contamination but also lowers the vitality of yeast cells, which causes a considerable delay in the start-up of the subsequent fermentation cycle [8]. Moreover, in certain areas in Brazil, the soil is rich in aluminum and/or zinc. When sugarcane is grown on such soils, the aluminum and/or zinc content in the sugarcane juice can be high enough to inhibit yeast fermentation [33]. An additional problem in the alcohol industry is related to the production of foam that impairs the fermentation vats to operate at full capacity. This requires continuous utilization of antifoaming products, thereby increasing ethanol production costs [13]. Altogether, despite the fact that bioethanol processes are industrially well established and operative for 4 decades, these problems configure the need for technological upgrade and performance amelioration.

Another well-developed biofuel is biodiesel, which is produced by the transesterification of triacylglycerols. At present, chemical methods drive most biodiesel production process; however, strong efforts are being made to develop biorefineries for the biological production of esters. On the other hand, the biodiesel production process yields crude glycerol waste [approximately 10 % (v/v) of the whole reaction product]. In view of extremely large amounts of crude glycerol produced worldwide, this is being considered for conversion into valuable goods. Microbial processing of these wastes into valuable goods, namely succinic, citric, or propionic acids, hydrogen, and ethanol, is a promising alternative as some microorganisms are not affected by the chemical contaminants present in crude glycerol [24, 31, 35].

In a totally separate economy sector, the production of *cachaça* (the Brazilian spirit) also starts by fermenting sugarcane juice and molasses. Unlike the case of bioethanol, in this process, yeasts are not washed after each fermentation cycle. In addition, the initial inoculum is not made of selected yeast starters, and fermentations are conducted by a spontaneous community of species and strains. *S. cerevisiae*, the predominant yeast species, has to cope with: (1) competition from many other types of yeast as well as bacteria; (2) high environmental temperature fluctuations owing to the absence of temperature control in industrial premises; (3) low a_w , generated by sugarcane juice at 20 % sucrose (18–20° Brix); and (4) increasing amounts of ethanol during each fermentation cycle. These combined extreme conditions contribute to the natural selection of robust yeast strains with unusual physiological characteristics, which are presumably interesting for diverse biotechnological applications, namely addressing the problems from the above-mentioned bioethanol and biodiesel industrial sectors.

In this study, 118 yeast strains obtained from different *cachaça* distilleries covering a large geographical area of Brazil, including the states of Bahia, Espírito Santo, Minas Gerais and Rio de Janeiro were used. Yeast cells were initially selected according to (1) tolerance to high concentrations of ethanol; sugar and high temperatures; (2) fermentative capacity and (3) ability to flocculate [41]. These *cachaça* yeast isolates were characterized bearing in mind the putative future utilization in the production of bioethanol (first generation) and/or in sustainable management of biofuel wastes, i.e., utilization of residues from bioethanol and biodiesel production to generate yeast-based added value products. Yeast isolates showed unusual high phenotypic plasticity and robustness. Two of them, identified as strains of *Pichia guilliermondii* and *P. anomala*, presented interesting characteristics for biodiesel waste utilization. Another isolate, an *S. cerevisiae* strain, presented remarkably promising characteristics for producing ethanol in the presence of aluminum and was further resistant to continuous acid washing. The present results emphasize that a considerable degree of biotechnological innovation and improvement can be derived from the exploitation of the microbial biodiversity of spontaneous fermentations without the need for cost- and time-consuming laborious genetic manipulation.

Materials and methods

Strains and culture conditions

Yeast strains used in this study originated from sugarcane fermentation vats in *cachaça* distilleries from diverse geographical localizations in Brazil. Yeast colonies were isolated as described previously [41]. The commercial bioethanol strains PE2, CAT1, and Ethanol Red (Fermotec, Brazil) and the laboratory strain *S. cerevisiae* BY4741 were used as controls. For short-term maintenance, yeasts were cultured in YPD [1 % (w/v) yeast extract, 2 % (w/v) peptone, with 2 % (w/v) dextrose] supplemented with 2 % (w/v) agar and kept at 4 °C. Long-term maintenance was guaranteed by the storage of samples from YPD-grown cultures at –80 °C with 30 % (v/v) glycerol. All strains are available in the culture collection of the Federal University of Ouro Preto (propp@ufop.br). Growth assays were performed in 96-microwell plates containing 200 μ L YP [1 % (w/v) yeast extract, 2 % (w/v) peptone] that was differently supplemented. Plates were incubated at 30 °C without shaking. Batch cultures were performed at 30 °C, with 1:5 air-to-liquid ratio and 200 rpm orbital shaking (New Brunswick Model G200). Inoculation was standardized to initial $OD_{600nm} = 0.1$ using an overnight culture. Growth was monitored following OD_{600nm} in a spectrophotometer

BioMate3 (Thermo Scientific). The specific growth rate (μ) was estimated from OD_{600nm} measurements. Drop tests were performed using $10\times$ serial dilutions of YPD-grown $OD_{600nm} \approx 1.0$ batch cultures on sterile deionized water. Plates were incubated at $30\text{ }^{\circ}\text{C}$.

Growth in different carbon sources and under stressful conditions

The ability to use different carbon sources was tested in YP batch cultures with 2 % (w/v) xylose, ferulic acid, *p*-coumaric acid. Additionally, the ability to tolerate the following stress agents was tested in YPD: acetic acid (2.0–6.0 g/L), formic acid (0.5–3.0 g/L), furfural (10–60 mmol/L), hydroxymethyl-2-furaldehyde (HMF) (10–60 mmol/L) [1]. Also, growth on increasing concentrations of glycerol [2, 5, 10, 12, 15, 20, 25 % (w/v)] was tested. Tolerance to increasing concentrations of ethanol or methanol [2, 5, 10, 12, 15, 20 % (v/v)] as well as a wide range of pH values (from 2.5 to 10.5 NaOH or HCl adjusted) was screened in YPD microwell plates. Results from microwell assays of chosen strains were confirmed by drop tests in solid media using tenfold dilutions of an initial inoculum of 10^7 cells/mL. Tolerance to zinc and aluminum was tested by drop tests in solid YPD supplemented with 10 mmol/L AlCl_3 , 5 mmol/L $\text{Al}_2(\text{SO}_4)_3$ or 5 mmol/L ZnSO_4 .

Small-scale fermentations

Small-scale industrial-like batch fermentations were performed on fermentation tubes (5.5×14 cm) (handmade at Katholieke Universiteit Leuven, Belgium) (Fig. 1). These were kept still at $30\text{ }^{\circ}\text{C}$, except for an initial 2-h period with orbital shaking (350 rpm). Fermentations were started at initial $OD_{600nm} = 5.0$ (approximately 5×10^7 yeast cells) and were noninvasively followed by weighing the flasks. Ethanol production was estimated by weight loss. Foaming was monitored by measuring the thickness of the foam layer on top of the fermentation medium. Starter cultures were obtained from pre-cultures of YP with 10 % (w/v) glucose or sucrose (150 mL) and incubated for 48 h at $30\text{ }^{\circ}\text{C}$ and 200 rpm. These were centrifuged (3,000 rpm, 5 min, $4\text{ }^{\circ}\text{C}$), and the pellets were re-suspended in 10 mL YP with 20 % sucrose, 33 % glucose, or 2 % xylose and used to inoculate 90 mL of identical fermentation media, total 100 mL of medium. To test for fermentation in the presence of 10 mmol/L $\text{Al}_2(\text{SO}_4)_3$, cells were pregrown in 3 mL of YPD for 24 h (200 rpm, $30\text{ }^{\circ}\text{C}$). This pre-inoculum was used to inoculate 100 mL of 10 % sucrose, grown for 2 days (200 rpm, $30\text{ }^{\circ}\text{C}$) until the stationary phase. Cells were then harvested by centrifugation and used to inoculate 20 % sucrose supplemented with 10 mmol/L $\text{Al}_2(\text{SO}_4)_3$.



Fig. 1 Fermentation tubes (handmade at Katholieke Universiteit Leuven, Belgium) used to monitor ethanol production through weight loss

Small-scale fermentations with 2 % xylose in the presence of inhibitors acetic acid (2.0 g/L), formic acid (0.5 g/L), furfural (10 mmol/L) and HMF (10 mmol/L) were performed as described.

Acid washing between fermentation cycles

Small-scale fermentation cycles of 24 h in 20 % sucrose were intercalated with an acid washing treatment of yeast cells. At the end of each cycle, approximately 6×10^8 yeast cells were harvested by centrifugation and resuspended for 1 h in sulphuric acid diluted in water to pH 2.0, following which they were harvested again by centrifugation and reused in a subsequent fermentation. This procedure was repeated up to six cycles. The viability of yeasts in vats was

quantified by counting the viable cells in a Neubauer chamber after staining with methylene blue.

Molecular identification of yeast strains

Yeast genomic DNA was extracted [26] and subjected to polymerase chain reaction (PCR) amplification of the 18S rRNA internal transcribed spacer (ITS) regions (*ITS1/5*, *8S/ITS2*) using the primers ITS1 + ITS4 [43]. DNA electrophoresis was performed using standard procedures. MW markers were a 1-kb DNA ladder from Promega and 100-bp DNA ladder from Axygen. The amplicon (880 pb) was analyzed by restriction fragment length polymorphism (RFLP) using *HhaI*, *HaeIII*, and *HinfI* and compared with a control correspondent sequence originating from *S. cerevisiae* BY4741 [15]. Different ITS PCR products were purified using standard procedures and sequenced by capillary electrophoresis (Sanger method) using the ABI3130 platform Life Technologies (Myleus Biotechnology, Belo Horizonte, Minas Gerais, Brazil). Sequences were analyzed using the National Centre for Biotechnology Information (NCBI) database.

Reproducibility

All experiments were conducted in triplicate and repeated twice.

Results

Physiological properties of 118 Brazilian *cachaça* yeast strains

Fermentation vats from *cachaça* distilleries from several states of Brazil were used to isolate 118 yeast strains [41]. These were subjected to a physiological survey, including (1) the ability to grow on biofuel industry wastes; (2) elevated tolerance to high alcohol concentrations and (3) tolerance to other stressful conditions: low pH, the presence of aluminum, zinc or VHG fermentation conditions (Supplementary Material, S1).

Cellular growth in different carbon sources

The chemical composition of crude biodiesel waste varies according to several production constraints, namely the type of fat or oil used in the process [17]. Nonetheless, glycerol is the most relevant compound present in biodiesel wastes [30]. Therefore, the ability of each of the 118 yeast isolates to grow on glycerol (reagent grade) as unique sole carbon and energy source was tested in concentrations

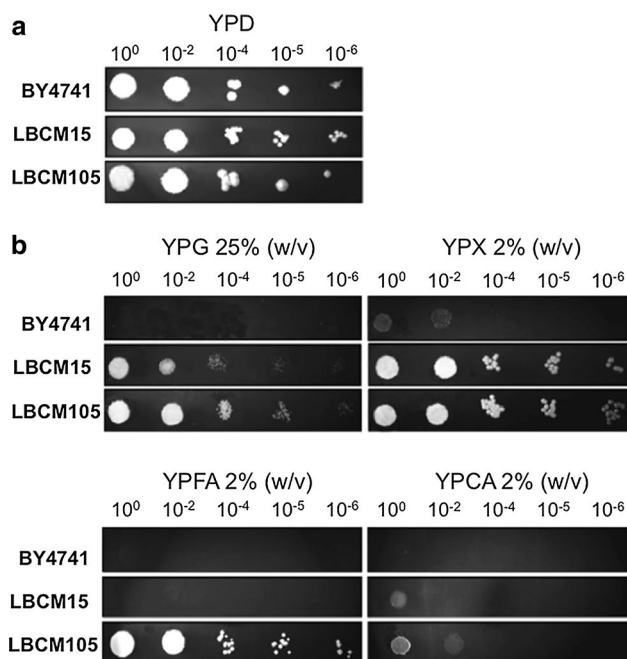


Fig. 2 Viability phenotype of candidate strains (LBCM15 and LBCM105) on **a** glycerol and **b** lignocellulosic component-based media. Drop tests were performed from cellular suspensions containing approximately 10^7 cells/mL in solid medium: YPD, YP + 25 % glycerol, YP + 2 % xylose, YP + 2 % ferulic acid and YP + 2 % coumaric acid (w/v)

spanning from 2 to 25 % (w/v). All strains grew on 2 % (w/v) glycerol, although only a few could grow on glycerol concentrations above 10 % (Supplementary Material, S1). Strains LBCM15 and LBCM105 grew well on 20 and 25 % (w/v) glycerol, respectively, compared with 15 % (w/v) for CAT1, PE2, and Ethanol Red industrial strains used as controls and 5 % (w/v) for the laboratory strain BY4741 (Fig. 2; Table 1).

The bioethanol industry produces very high amounts of a sugarcane fibrous dry waste rich in lignin and hemicellulose, bagasse. This is hardly degraded by microbes, namely yeasts. *Cachaça* yeast isolates were tested for the ability to grow on the most prominent components of sugarcane bagasse, xylose, ferulic acid or coumaric acid, as sole carbon and energy sources. Eight strains (LBCM08, LBCM14, LBCM15, LBCM19, LBCM46, LBCM99, LBCM105 and LBCM106) could grow on xylose, two of which (LBCM15 and LBCM105) could also grow on ferulic acid and one of which (LBCM105) could also grow on coumaric acid (Fig. 2). This last strain was further challenged with compounds that are produced during the pretreatment of lignocellulosic biomass: furfural, acetic acid, formic acid and 5-hydroxymethyl-2-furfuraldehyde (HMF) in the presence of which it could still grow on glucose (Table 2).

Table 1 Different yeast strains with higher potential to use the biodiesel and lignocellulose waste: highest glycerol concentration allowing cellular growth and tolerance to ethanol, methanol

Yeast strains	Growth condition		
	Highest (glycerol) allowing growth as sole carbon source [% (w/v)]	Highest (ethanol) tolerated in YPD [% (v/v)]	Highest (methanol) tolerated in YPD [% (v/v)]
LBCM15	20	12	12
LBCM105	25	15	15
CAT1	15	12	15
PE2	15	12	15
Ethanol Red	15	20	15
BY4741	5	5	5

Table 2 Cellular growth of yeast strains in YPD with different concentrations of acetic acid, formic acid, furfural and 5-hydroxymethyl-2-furaldehyde (HMF)

Yeast strains	Inhibitors											
	Acetic acid (g/L)			Formic acid (g/L)			Furfural (mmol/L)			HMF (mmol/L)		
	2.0	4.0	6.0	0.5	1.5	3.0	10	30	60	10	30	60
LBCM105	+++	+	+	+++	+	+	+	+	+	+++	+	+
PE2	+++	+	+	++	+	+	+	+	+	++	+	+

+++ Growth equal to reference in the absence of inhibitors, ++ intermediate growth compared to reference in the absence of inhibitors, + lower growth than the reference in the absence of inhibitors

Table 3 Ability of *cachaça* yeast strains to endure different stress conditions: growth on YPD supplemented with ethanol or 10 mmol/L of aluminum, or adjusted to low pH with HCl

Yeast strain → growth condition	LBCM03	LBCM05	LBCM15	LBCM36	LBCM37	LBCM47	LBCM54	LBCM69	LBCM78	LBCM79	LBCM97	LBCM105
Highest (ethanol) allowing growth [% (v/v)]	12	12	12	12	10	12	12	12	12	12	10	15
Lowest pH allowing growth	2.5	2.5	2.5	3.4	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Growth w/10 mmol/L AlCl ₃	++	++	+++	+	+++	+++	++	++	++	++	+	+++

+ Empirical growth scale

Tolerance to alcohol

Biodiesel wastes contain high amounts of methanol and less frequently contains ethanol [30]. In addition, regardless of the type of industrial fermentation, yeasts have to cope with very high amounts of alcohol accumulated from each fermentation cycle to the next. Therefore, the tolerance of yeasts to ethanol and methanol up to 20 % (v/v) in glucose-based medium was tested. None of the strains could grow in presence of 20 % (v/v) alcohol. Twelve strains could grow in presence of 15 % (v/v) ethanol (Supplementary Material, S1). Again, strains LBCM15 and LBCM105 stood out for growing in the presence of 12 and 15 % (v/v) methanol and ethanol, respectively (Table 1). In addition, when these strains were grown in YP 20 % of sucrose for 24 h, the industrial strain PE2 produced 106.77 g/L of ethanol, while LBCM15 and LBCM105 produced 61.05 and 64.26 g/L of ethanol, respectively.

Other stress conditions

The bioethanol industry uses cleaning treatments with dilute sulphuric acid (pH 2.0–2.5) to eliminate bacteria contaminations between fermentation cycles [3]. Although that does not apply to the *cachaça* production process, in view of future applications, 118 yeast isolates were characterized with regard to their resistance to low pH in glucose-based medium (Supplementary Material, S1). Half of the strains grew well at pH values as low as 2.4, including the above-mentioned strains LBCM15 and LBCM105 (Table 3). In addition, high pH was tested. No growth was observed at pH above 9.5. The broader pH range allowing growth was 2.4–9.5 observed with strains LBCM15, LBCM37, LBCM47, LBCM97, and LBCM105. Moreover, the soils in which sugarcane is produced often contain elevated amounts of metal ions that pass on to sugarcane juice. This is therefore rich in zinc and aluminum, both of

which are highly toxic to yeasts [3]. Aluminum in the form of $\text{Al}_2(\text{SO}_4)_3$ and AlCl_3 , as well as ZnSO_4 was used to supplement fermentation media (Supplementary Material, S1). From the 118 strains tested, eight could tolerate 10 mmol/L AlCl_3 and 5 mmol/L ZnSO_4 (LBCM03, LBCM05, LBCM47, LBCM54, LBCM69, LBCM78, LBCM79, and LBCM105).

From all the above-mentioned results, two strains, LBCM15 and LBCM105, emerge as being able to tolerate the highest alcohol concentrations, the lowest pH, and the presence of aluminum and zinc (Table 3) as well as being able to grow on 20–25 % glycerol (Fig. 2). This makes them promising candidates for further exploitation in innovative processes of biodiesel waste consumption. These same characteristics also make strain LBCM105, together with strain LBCM47 (able to tolerate low pH and the presence of Al/Zn), good candidates for first-generation ethanol production. Furthermore, the strains LBCM08, LBCM14, LBCM15, LBCM19, LBCM46, and LBCM99, but mainly strain LBCM105, could be considered good candidates for the development of second-generation bioethanol production processes owing to their ability to convert hemicellulose and lignin constituents into biomass (Supplementary Material, S1).

Sugarcane juice-like sucrose fermentations

These results prompted us to assay small-scale ethanol fermentations using sugarcane juice as a substrate for testing the 12 strains that displayed a higher tolerance to ethanol: LBCM07, LBCM10, LBCM13, LBCM17, LBCM23, LBCM25, LBCM28, LBCM44, LBCM47, LBCM65, LBCM67, LBCM105 and LBCM107. Fermentations were performed on YP with 20 % sucrose (average sucrose concentration in sugarcane juice) (Fig. 3a; Supplementary Material, S2A). Strains LBCM10, LBCM13, LBCM25 and LBCM47 could achieve these ethanol yields in 24 h similar to the control industrial strain PE2 (Fig. 3a), while the strains LBCM07, LBCM17, LBCM28, LBCM44, LBCM65, LBCM67, LBCM105, LBCM107 showed slower ethanol production (Supplementary Material, S2A). In addition, the production of foam was monitored (Supplementary Material, S3A, C and D). Strains showed three distinct patterns of foam production throughout fermentation: (1) strains LBCM10, LBCM28 and LBCM44 produced foam during the entire fermentation; (2) strains LBCM13, LBCM17, LBCM23, LBCM65, LBCM67, LBCM105 and LBCM107 produced foam mainly during the first 24 h and gradually decreased production without ceasing; and (3) strains LBCM07, LBCM25 and LBCM47, similarly to the industrial strain PE2, did not produce foam after 72 h.

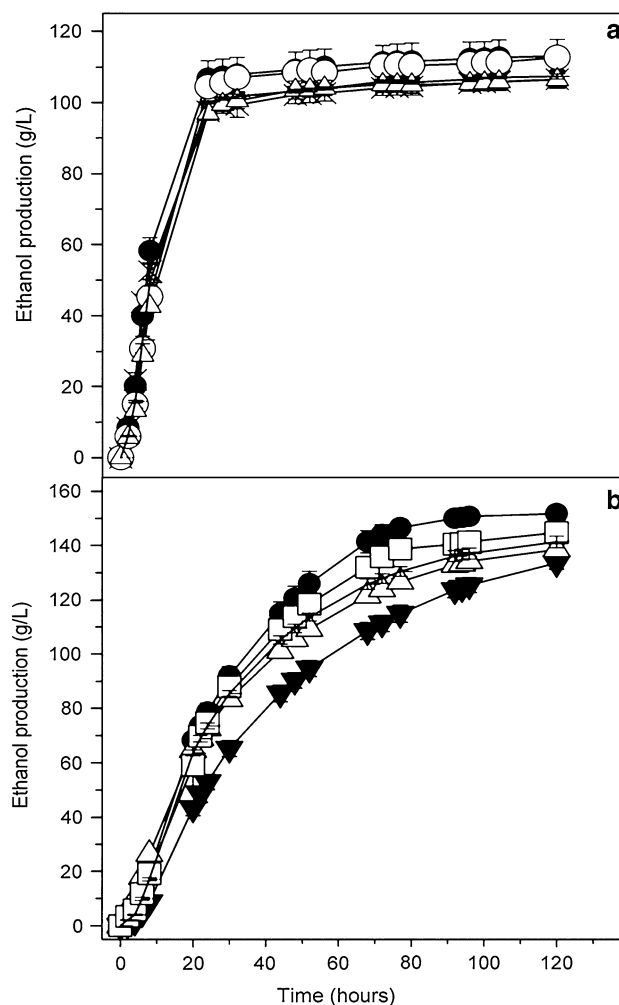


Fig. 3 Profile of ethanol production in small-scale fermentation of different yeast strains in 20 % sucrose (a) and 33 % glucose—VHG fermentation (b). The strains LBCM13 (○), LBCM10 (×), LBCM25 (△), and LBCM47 (–) could achieve ethanol yields close to the maximum in 24 h, as the industrial strain PE2 (●) (a). The strains LBCM17 (▼), LBCM25 (△), LBCM47 (–), and LBCM67 (□) showed higher ethanol production capacity (higher than 90 %) similar the industrial strain PE2 (●) (b)

VHG fermentations

These strains were also subsequently used to mimic VHG fermentations using YP with 33 % glucose. Ethanol production was monitored (Fig. 3b; Supplementary Material, S2B). None of the strains reached the same production levels as the industrial strain PE2. The production of foam was globally much less intense than that in the 20 % sucrose fermentation (Supplementary Material S3B, D and F). Again, three distinct patterns could be distinguished: (1) strain LBCM28 presented a constant although very low foam production; (2) strain LBCM10 gradually decreased production, without ceasing it; and (3) strains LBCM07, LBCM13, LBCM17,

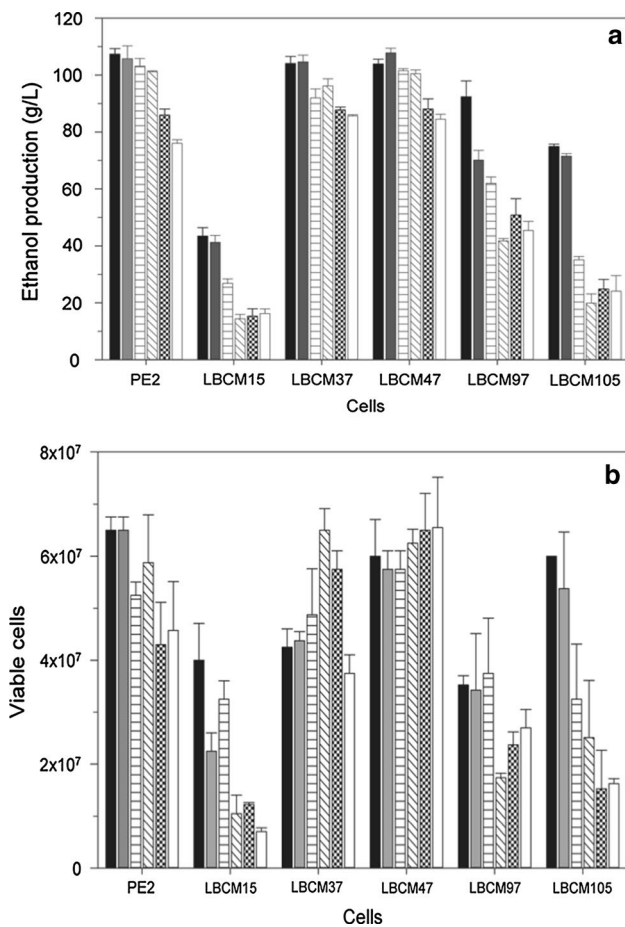


Fig. 4 Effect of low pH treatment on ethanol production during recycling of different yeast strains in small-scale fermentations. The fermentations were performed at 30 °C in 100 mL of YP + 20 % sucrose. Cycles of 24 h were intercalated with washings in diluted sulphuric acid six consecutive times. The different bars represent each cycle of fermentation. Final ethanol production (a). Viable cell number after each fermentation cycles (24 h). The viable cell number was assessed based on cell counting in the end of each fermentation cycle (b). Cycles: first cycle (black bar), second cycle (gray bar), third cycle (white bar with horizontal lines), fourth cycle (white bar with oblique lines), fifth cycle (grid bar) and sixth cycle (white bar)

LBCM23, LBCM25, LBCM44, LBCM47, LBCM65, LBCM67 and LBCM105 as well as the control industrial strain PE2 did not produce foam at all.

Sulphuric acid washings

Bearing in mind the acid cleaning treatments used by the bioethanol industry between fermentation cycles, ethanol production was further measured in the presence of dilute sulphuric acid (pH 2.0). High initial cell densities (approximately 6×10^8 yeast cells) were used to cope with the predicted loss of cell viability. The ethanol produced was quantified (Fig. 4a), and the viability of the culture (Fig. 4b) after the sixth 24-h cycle was monitored. The best

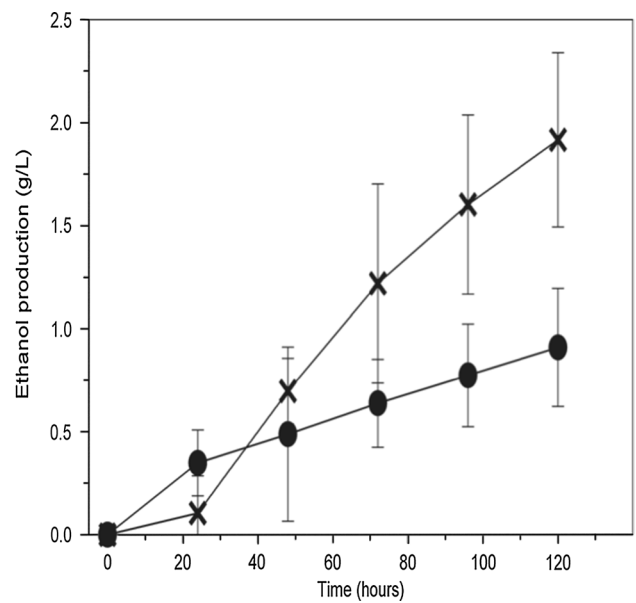


Fig. 5 Production of ethanol from xylose of the strain LBCM105 (X) compared with PE2 (•). The fermentations were performed on YP with 2 % xylose, in static conditions, except for first 2 h when stirring was applied (350 rpm)

results were obtained with strains LBCM37 and LBCM47, for which the total ethanol production was approximately 85 g/L, which was 20 % more than that by the industrial strain PE2 in YP with 20 % sucrose. Cellular viability closely followed the variations in ethanol production after each 24-h cycle. Notably, strain LBCM37 lost significantly more viability than all others, which is quite surprising considering its higher ability to produce ethanol.

Aluminum toxicity

The addition of 10 mmol/L $Al_2(SO_4)_3$ to 20 % sucrose fermentations (not shown) highlighted the isolates able to keep producing ethanol despite the presence of this contaminant. Strains LBCM05 and LBCM47 were the best performers, producing 1.5-fold (69 g/L) more ethanol than the industrial strain PE2 after 133 h of fermentation (not shown), which makes them excellent candidates for first-generation bioethanol production.

Pentose fermentation

Finally, strain LBCM105 was challenged with a small-scale fermentation using YP supplemented with 2 % xylose. This strain fermented xylose and produced 2 g/L ethanol after 120 h of fermentation, while the industrial strain PE2 produced around 1 g/L (Fig. 5). These amounts were 5- and 10-fold smaller, respectively, than the ethanol these strains produce from identical amounts of glucose (approximately

10 g/L). However, this strain (LBCM105) showed a reduction of approximately 30 % in the ethanol production in the presence of the inhibitors furfural, acetic acid, formic acid and HMF (not shown).

Molecular identification of the selected yeast strains

In view of their promising characteristics, yeast strains LBCM15, LBCM25, LBCM47 and LBCM105 were submitted to taxonomic identification by molecular techniques on the basis of 5.8 S-ITS rRNA region amplification and restriction analysis. The restriction profiles of the PCR products of yeast strains LBCM25 and LBCM47 were similar to those of *S. cerevisiae* BY4741 (control), while those of LBCM15 and LBCM105 were different (not shown). The PCR products of strains LBCM15 and LBCM105 were sequenced and revealed 98 and 88 % identity, respectively, to the corresponding regions from the type strains of *P. guilliermondii* (GenBank: FJ527876.1) and *P. anomala* (GenBank: FJ713067.1). The degree of similarity allows classifying LBCM15 and LBCM105 strains as *P. guilliermondii* and *P. anomala*, while strains LBCM25 and LBCM47 are most probably *S. cerevisiae*.

Discussion

The sugarcane fermentation vats from *cachaça* production processes yielded 118 yeast isolates that were used to survey physiological traits bearing in mind specific biotechnological applications. These were as follows: (1) the utilization of bioethanol and biofuel industry wastes and (2) the improvement of ethanol yields from sugarcane juice, namely in VHG fermentation conditions. This demanded the verification of the ability of these yeasts to use carbon sources other than sucrose or glucose and the corresponding ethanol production.

Contamination of fermentations with bacteria, mainly species of *Lactobacillus*, is a well-known significant industrial problem causing production losses up to 22 % [3, 9]. These bacteria can produce lactic and acetic acid, diacetyl, reuterin and proteinaceous compounds affecting yeast growth and ethanol production capacity [9]. Several techniques, such as antibiotics, ammonia and acid treatment are currently being employed in an attempt to inhibit unwanted microbial growth [3, 9]. However, importantly, these yeasts were also tested for their resistance to the high amounts of ethanol accumulated during consecutive cycles, together with the low pH from washes with diluted sulphuric acid performed between fermentation cycles.

Finally, it was also important to test the resistance of these strains and their fermentative ability to the presence

of inhibitory metals present in sugarcane juice, mainly aluminum and zinc.

The reasoning underlying the choice of sugarcane fermentation vats from *cachaça* distilleries for this purpose is related to these fermentations occurring in environments of high osmotic pressure and high temperatures (a physiological trait not explored in this survey) and relying on spontaneous microbial fauna. Strong competition between different microorganisms, together with continuous exposition to considerable levels of alcohol and reutilization of “foot-of-vat” without treatment between fermentative cycles, generates a constant natural selective pressure imposed by environmental conditions [29]. Yeast strains spontaneously occurring in sugarcane fermentation vats must be natural ethanol producers under stressful conditions because the industrial environment favors confined evolution and selection for industrially favorable characteristics. Fermentation vats are thus an excellent source of yeast biodiversity already adapted to harsh industrial environments. Further spontaneous genetic hybridization events between different yeast strains [4] may also contribute to strain amelioration. Results obtained with this physiological survey reflect this industrial natural selection effect and highlight the high plasticity of these yeasts, showing these strains as natural candidates for several possible industrial applications.

Glycerol is the major waste product from the biodiesel industry, presently produced in excessive amounts [30]. Interesting high added value solutions for its use are not available. This happens because crude glycerol from biodiesel contains a significant number of contaminants that preclude its direct utilization for microbial feed [11, 22, 32, 44]. Nevertheless, glycerol and alcohol (more frequently methanol than ethanol) are the most relevant chemicals present in biodiesel wastes [30]. Therefore, yeasts were surveyed for their ability to grow in crescent concentrations of glycerol up to 25 % and in the presence of high concentrations of methanol and ethanol up to 15 %. Two strains, LBCM15 and LBCM105, identified as *P. guilliermondii* and *P. anomala*, respectively, could use glycerol up to 25 % (w/v). Both *P. guilliermondii* and *P. anomala* are good glycerol consumers [21, 34, 38], used in many biotechnological applications [42]. Furthermore, LBCM15 and LBCM105 could tolerate methanol or ethanol up to 15 % (v/v). These strains also presented an impressively resilient ethanol production ability in the presence of aluminum and low pH on either 20 % sucrose or 33 % glucose. In particular, *P. anomala* is recognizably robust because it is able to grow in the presence of high ethanol concentrations [23, 39], at low pH, under high osmotic pressure, and under low oxygen tension [28]. Moreover, in contrast to *S. cerevisiae*, *P. anomala* is tolerant to Zn^{2+} (up to 500 mg/L), Cd^{2+} [40], Hg^{2+} , Ni^{2+} , Cr^{6+} , Pb^{2+} , Cu^{2+} , and Co^{2+} [5, 7, 12]. Isolation of two *Pichia* species from *cachaça* fermentation vats

is not a surprising fact. These species are easily found in water, soil, plants, or crops suitable for ensilage. Furthermore, many authors reported their presence, particularly during early stages of *cachaça* fermentation [36]. Their robustness (to tolerate biotic and abiotic stresses), together with their plasticity (using a diverse set of carbon and energy sources) should explain their almost ubiquitous nature in such harsh conditions as those found in *cachaça* production vats.

Production of bioethanol from sugarcane leads to the accumulation of huge amounts of the sugarcane bagasse, a material composed of hemicellulose and lignin. Its polysaccharide fraction is composed of cellulose and xylose-rich hemicellulose, and its polyphenolic fraction is composed of several aromatic monomers, derived from hydroxycinnamic acids such as ferulic or coumaric acids [10, 27]. *Pichia* species were described as capable of using multiple substrates [19] and are among the few organisms that can metabolize ferulic acid [14, 18, 20, 37]. Accordingly, *P. anomala*, strain LBCM105, could grow on ferulic and coumaric acids as well as ferment xylose. The ethanol produced from xylose by strain LBCM105 was less than in sucrose- or glucose-based fermentations. Still, this strain can be considered for the future improvement of fermentation for second-generation bioethanol production from sugarcane bagasse.

S. cerevisiae is the most robust ethanol-producing microorganism [25, 45]. In this survey, *S. cerevisiae* strain LBCM47 stood out as a good candidate for the improvement of first-generation ethanol production, beyond the already excellent yeasts in the bioethanol industry, CAT1, PE2, and Ethanol Red. Apart from the capacity to produce higher concentrations of ethanol in 20 % sucrose or 33 % glucose (VHG), its capacity to actively survive very low pH (around 2.4) as well as aluminum toxicity suggests that it may ally a better ethanol performance with a high resilience to industrial harsh conditions. In addition, the foam production pattern observed is compatible with its industrial utilization with less expenditure in anti-foam agents.

Well-known processes used for decades (like bioethanol), or for centuries (like *cachaça*), are often not standardized or quality controlled as well as are poorly studied. Studies such as this are mandatory to generate molecular tools for monitoring industrial fermentations. However, more importantly, studies such as this demonstrate the importance of exploring the biodiversity associated with industrial environments, of *cachaça* production processes or others, to find the tools required to solve problems, to improve performance, or to generate novel processes required to ensure sustainability in the long run.

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