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Robust industrial *Saccharomyces cerevisiae* strains for very high gravity bio-ethanol fermentations

Francisco B. Pereira, Pedro M.R. Guimarães, José A. Teixeira, and Lucília Domingues*

IBB–Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

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The application and physiological background of two industrial *Saccharomyces cerevisiae* strains, isolated from harsh industrial environments, were studied in Very High Gravity (VHG) bio-ethanol fermentations. VHG laboratory fermentations, mimicking industrially relevant conditions, were performed with PE-2 and CA1185 industrial strains and the CEN.PK113-7D laboratory strain. The industrial isolates produced remarkable high ethanol titres (>19%, v/v) and accumulated an increased content of sterols (2 to 5-fold), glycogen (2 to 4-fold) and trehalose (1.1-fold), relatively to laboratory strain. For laboratory and industrial strains, a sharp decrease in the viability and trehalose concentration was observed above 90 g l^{-1} and 140 g l^{-1} ethanol, respectively. PE-2 and CA1185 industrial strains presented important physiological differences relatively to CEN.PK113-7D strain and showed to be more prepared to cope with VHG stresses. The identification of a critical ethanol concentration above which viability and trehalose concentration decrease significantly is of great importance to guide VHG process engineering strategies. This study contributes to the improvement of VHG processes by identifying yeast isolates and gathering yeast physiological information during the intensified fermentation process, which, besides elucidating important differences between these industrial and laboratory strains, can drive further process optimization.

[**Key words:** Bio-ethanol production; Very high gravity fermentation; *Saccharomyces cerevisiae*; Industrial strains; Stress tolerance]

VHG processes are very attractive and promising for bio-ethanol production allowing significant improvements in the overall productivity thus minimizing the production costs due to energy savings (1). The use of VHG technology imposes increased stressful conditions to the yeast cells, which have been associated with the loss of yeast viability during VHG fermentation, reduced fermentation rates and incomplete fermentations (2). Thus, the successful implementation of VHG technology in bio-ethanol production requires the development of yeast strains that efficiently ferment high sugar concentrations (>250 g l^{-1}) (3). Such strains must be resistant to the multiple stresses found in the process, including the osmotic stress that results from the high sugar concentrations, the ethanol stress at the end of fermentation, the anaerobic conditions established in the large-scale bioreactors and the cell recycling procedures for utilization of the yeast biomass for several consecutive fermentation cycles (4).

The microflora of traditional and industrial fermentation processes constitutes a potential source of microbial natural isolates that exhibit at least some of the desired physiological characteristics for VHG processes. Specifically, stress-tolerant yeasts can be found in alcoholic fermentation processes, such as the “cachaça” (typical Brazilian distilled beverage obtained from sugarcane) fermentation and bio-ethanol production plants in Brazil (5,6). In these harsh environments, the yeast is subjected to several stresses. At the start-up of fermentation when the diluted sugar cane juice is added to the natural ferment, yeast cells undergo osmotic stress due to the high concentration of sugars in the sugar cane juice (around 16 ºBrix). Various other stresses such as heat, ethanol shock and/or starvation are common conditions in “cachaça” (and bio-ethanol) fermentation vats and often these stress conditions overlap (7).

In contrast to the large amount of knowledge about the stress response, genetic and physiological background of standard laboratory strains (8,9), very few studies provide data about stress resistance and protective mechanisms of indigenous strains isolated from traditional or industrial fermentations. Although, these isolates are potential candidates to overcome the stressful conditions imposed to yeast cells by VHG stress conditions and thus to drive this technology further, the use and characterization of these isolates in VHG fermentations has not been reported. Therefore, it is extremely relevant to understand how these industrial yeast cells adapt to environmental stress conditions and elucidate the physiological bases for its application in VHG fermentation processes.

In a previous study, we evaluated the fermentation performance of 8 strains isolated from distillery environments in Brazil under VHG conditions (6). The strains that exhibited the higher ethanol titre and productivity were PE-2 and CA1185. The strain PE-2 is used by about 30% of sugarcane-to-ethanol distilleries in Brazil, generating about 10%

* Corresponding author. Tel.: +351 253 604400; fax: +351 253 678986.
E-mail address: luciliad@deb.uminho.pt (L. Domingues).

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of the world's bio-ethanol supply (10), and characteristics of its genome have been reported in recent studies (10,11). In this study, we investigated in detail the kinetics of glucose fermentation by industrial strains PE-2 and CA1185 in VHG laboratory fermentations mimicking industrially relevant conditions, i.e., high sugar and ethanol concentrations, high inoculation rates and low oxygen availability. Moreover, relevant physiological parameters (viability; intracellular concentrations of trehalose, glycogen, sterols and glycerol) were measured throughout the different batch fermentation stages with the aim of identifying traits that contribute to the high robustness and fermentation performance of these industrial strains in VHG stress conditions. The haploid laboratory strain CEN.PK113-7D, which showed better fermentation performance than its diploid counterpart CEN.PK122 and than haploid S288C (7), was used as reference.

MATERIALS AND METHODS

Yeasts The two industrial *S. cerevisiae* strains used were strain PE-2, which was isolated from a sugarcane-to-ethanol distillery in Brazil (5), and strain CA1185, which was isolated from a "cachaça" distillery also in Brazil. These strains were selected based on their high fermentation performance under VHG conditions (6). The haploid strain CEN.PK113-7D (9) was included as a laboratory reference strain. Stock cultures were maintained on YPD [1% (w/v) yeast extract, 2% (w/v) bacto peptone and 2% (w/v) glucose] agar plates at 4°C.

Media and fermentations Fermentations were performed in a VHG medium previously optimized (12) consisting of 335–343 g l^{-1} glucose, 44.3 g l^{-1} corn steep liquor (CSL), 2.3 g l^{-1} urea, 3.8 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 0.03 g l^{-1} $CuSO_4 \cdot 5H_2O$. The CSL was kindly provided by a starch manufacturer (COPAM, Portugal) and its handling in the laboratory as well as its main composition has been previously reported (12). The pH of the medium was adjusted to 5.5 with NaOH 1 mol l^{-1}. The medium was aerated by stirring with a magnetic bar (length of 3 cm) at >850 rpm for 20 min before inoculating the fermentation flasks, with the oxygen concentration reaching >95% of air saturation.

The yeast for inoculation was grown in Erlenmeyer flasks filled to 40% of the total volume with medium containing 50 g l^{-1} glucose, 20 g l^{-1} peptone and 10 g l^{-1} yeast extract. After incubation at 30°C and 150 rpm for 18–22 h (OD_{600} of 7–7.5), the cell suspension was aseptically harvested by centrifugation (10 min at 4800×*g*, 4°C) and resuspended in ice-cold 0.9% (w/v) NaCl to a concentration of 200 mg fresh yeast (FY) ml^{-1}. This concentrated cell suspension was then used to inoculate 40 ml of fermentation medium with about 1×10^8 cells ml^{-1}. Fermentations were done in 100 ml Erlenmeyer flasks fitted with perforated rubber stoppers enclosing glycerol-filled air-locks (to permit CO_2 exhaustion while avoiding the entrance of air) and incubated at 30°C with 150 rpm orbital agitation.

For each strain, fourteen replicate fermentations were initiated and two of those flasks were stopped at each time-point (0.5, 12, 24, 48, 72, 84 and 96 h) for analytical characterization. The progress of fermentation was followed by mass loss (resulting from CO_2 production). At each time-point, the standard deviation between replicates was less than 2% of the average value for the CO_2 production.

Analytical procedures Fresh and dry yeast mass were determined using a sample from the fermentation broth (20 ml) collected by centrifugation (10 min at 4800×*g*, 4°C) in a pre-weighed dried tube. The supernatant was completely removed and the tube was dried inside and outside and weighed to give the fresh yeast (FY) mass. Then, the yeast pellet was washed with 20 ml of distilled water, centrifuged, dried overnight at 105°C and the tube was weighed again to give the dry yeast (DY) mass. The yeast cell viability was determined by the methylene blue staining method (13). The percentage of viable cells was calculated by the ratio between viable (non-stained) and total cell counts. Glucose, glycerol and ethanol were analyzed by HPLC using a Varian MetaCarb 87 H column eluted at 60°C with 0.005 mol l^{-1} H_2SO_4 at a flow rate of 0.7 ml min^{-1}, and a refractive-index detector.

For microscopic observation, the yeast strains were cultivated in YPD medium at 30°C and 150 rpm for 18–22 h (OD_{600} of 7–7.5). The culture (1.5 ml) was transferred to a micro-centrifuge tube, allowed to sediment, resuspended in 200 µl of 3% (v/v) glutaraldehyde and after washed, first in water and subsequently in increasing concentrations of ethanol (30%, 70% and 100%, v/v). A small amount of the resulting pellet was dried for 3 days in a desiccator and finally covered with gold for visualization in a Leica Cambridge S360 scanning electron microscope (SEM).

For intracellular analyses, yeast cells were harvested from fermentation by centrifugation (5 min at 4800×*g*, 4°C), washed with ice-cold 0.9% (w/v) NaCl and resuspended in ice-cold 0.9% NaCl to a concentration of 200 mg FY ml^{-1}. From this yeast suspension, duplicate samples were taken for the determination of trehalose and glycerol, glycogen and sterols, as follows. For trehalose and glycerol, 0.5 ml samples were boiled in a water bath for 5 min and stored frozen (−20°C). After thawing, the samples were centrifuged (5 min at 13,400×*g*, 4°C) and the supernatant was collected to a weighed tube. The precipitate was suspended in 0.5 ml of distilled water, centrifuged again and the supernatant was added to the first one. The tube was weighed again to give the extract mass. Trehalose and glycerol were quantified in this extract by HPLC, using the same conditions as described above for the extracellular metabolites. For glycogen, 0.2 ml samples were centrifuged (5 min at 13,400×*g*, 4°C), the supernatant was carefully removed and the pellet was stored frozen (−20°C). The following procedures were modified from the method reported by Schulze et al. (14). After thawing the pellets, 0.5 ml of 40 mmol l^{-1} $C_2H_3NaO_2$ pH 4.8 were added and the samples were incubated for 10 min in a boiling water bath. Then, cells were disintegrated using glass beads (0.5 mm diameter) in a FastPrep Instrument (*Q-BIO gene*). The liquid above and between the beads was collected to a weighed tube with addition of 0.5 ml of 40 mmol l^{-1} $C_2H_3NaO_2$ pH 4.8; this procedure was repeated three times and the supernatants were combined in the same tube. The tube was weighed again to give the extract mass. Glycogen content in the extract was determined by enzymatic breakage to glucose using a commercial *Aspergillus niger* amyloglucosidase (10 mg ml^{-1}; Roche 102857). Briefly, 50 µg of enzyme were added to 25 µl of extract and the mixture was incubated at 37°C for 90 min. Controls were run without the addition of amyloglucosidase. A standard Type III glycogen from rabbit liver (10 mg ml^{-1}; Sigma G8876) was used to check that the enzymatic reaction was working properly. After the enzyme treatment, 970 µl of 50 mmol l^{-1} HEPES buffer pH 8.0 were added, the mixtures were centrifuged at 13,400×*g* for 5 min and the glucose in the supernatants was determined by HPLC. For sterols, 0.5 ml samples were centrifuged (10 min at 4800×*g*, 0°C) and the supernatant was completely removed. The tubes were then subjected to a clean nitrogen flow to expel air from the headspace and stored at −80°C. Sterols were extracted from yeast as follows. Samples (100 mg FY) were incubated at 80°C for 90 min with 5 ml alkaline aqueous ethanol (3.6 mol l^{-1} KOH in 80% v/v ethanol) in screw-cap tubes. After cooling to room temperature, 5 ml of MilliQ water and 5 ml of pentane were added and the tubes were shaken for 10 min. Then, the upper pentane layer was collected and evaporated to dryness under a flow of nitrogen (10 min). The resulting sterol extracts were stored at −20°C under nitrogen. Prior to analysis, N-butanol (1 ml) was added to dissolve the solid. Sterols quantification was performed by the spectrophotometric assay using filipin complex (Sigma F9765), as described by Rowe et al. (15). The final concentrations of intracellular metabolites were normalized to yeast dry mass (considering that the dry yeast corresponds to 30% of the fresh yeast mass). Deviations (ranges) between technical duplicates (duplicate samples from the same yeast suspension) were determined to be lower than 10% of the average value for the analyses of all intracellular metabolites.

Determination of fermentation parameters Ethanol conversion yield was calculated by the ratio between the maximum ethanol concentration produced and the glucose consumed (difference between the initial and residual glucose concentrations). It was expressed as a percentage (%) of the theoretical conversion yield, i.e., the yield considering a production of 0.511 g of ethanol per g of glucose. Ethanol productivity was defined as the ratio between final ethanol concentration and total fermentation time (fermentation was considered to be complete when the weight of the flasks stabilized). Specific rates of ethanol production (g ethanol g FY^{-1} h^{-1}) were calculated from the change in ethanol concentration and the average FY concentration for each sampling interval. Biomass conversion yield was calculated as the ratio between final biomass (DY) concentration and the glucose consumed.

RESULTS

Fermentation kinetics and physiological parameters The fermentation kinetics and relevant physiological parameters of industrial strains PE-2 and CA1185 and laboratory strain CEN.PK 113-7D were determined in VHG batch fermentations (335–343 g l^{-1} glucose).

Fig. 1 shows the time-course of the concentrations of the main extracellular metabolites (glucose, ethanol, CO_2 and glycerol), yeast biomass (fresh yeast mass and cell number), yeast viability and pH of the fermenting broth for the three strains. The industrial strains converted the glucose to ethanol much faster than the laboratory strain (Fig. 1A). Moreover, the industrial strains fermented nearly all of the glucose (residual <3 g l^{-1}) producing over 19% (v/v) ethanol, while the laboratory strain left a residual of ca. 90 g l^{-1} of glucose unfermented after 96 h, and consequently produced only 14% (v/v) ethanol (Fig. 1A; Table 1). Nevertheless, the glucose to ethanol conversion yield was identical for the three strains (Table 1). Expectedly, under the oxygen-limiting conditions used in this study, which resemble the typical anaerobic conditions established in alcoholic fermentation processes in large vessels, the ethanol and CO_2 production profiles were quite similar (Fig. 1A and B).

Yeast growth was monitored along the fermentation by determining the concentration of fresh yeast (wet weight) and by cell counting (Fig. 1C and D, respectively). Yeast dry weight was also determined at the end of fermentations (Table 1). The profiles show that yeast growth occurred mainly during the initial 24 h, after which

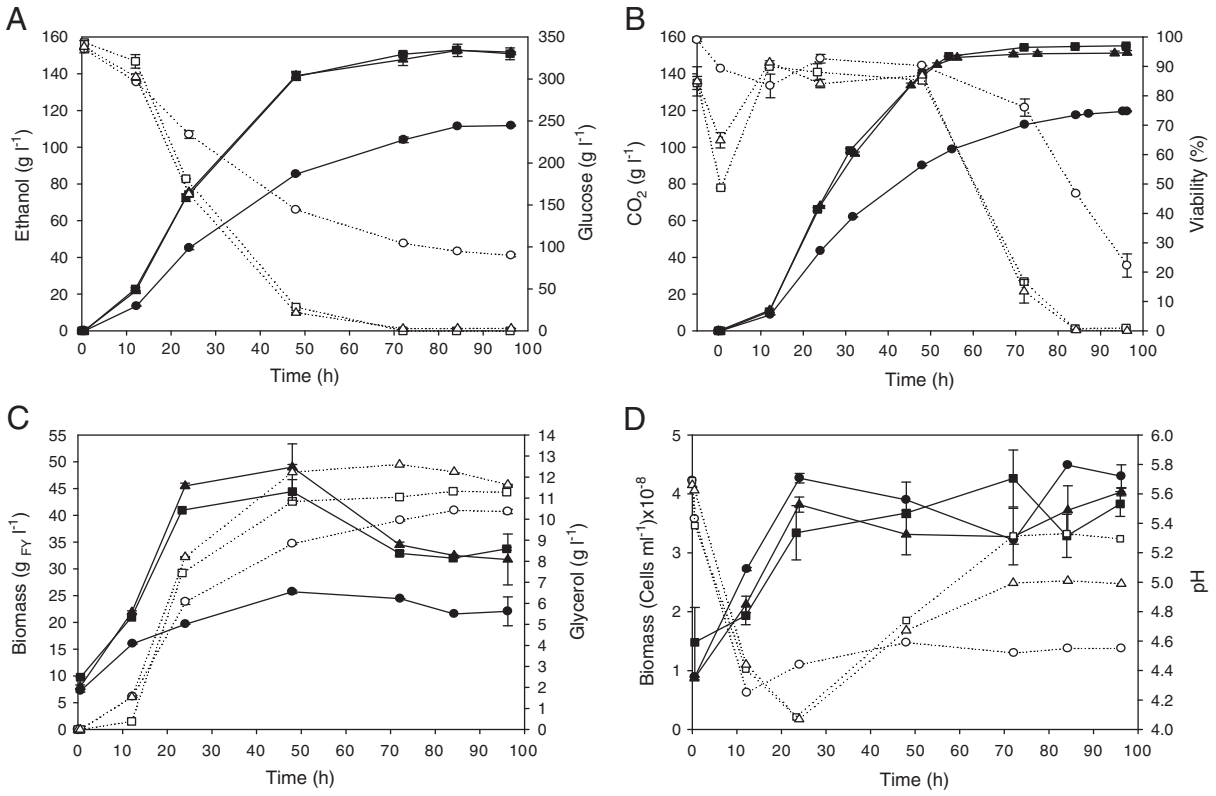

FIG. 1. Evolution of the main parameters during VHG fermentations with CEN.PK113-7D (circles), PE-2 (squares) and CA1185 (triangles) *S. cerevisiae* strains: (A) ethanol and glucose concentrations; (B) CO_2 concentration and cell viability; (C) fresh yeast mass and glycerol concentrations; (D) cell counts and pH. Solid symbols and full lines correspond to the parameters represented in the primary axis (ethanol, CO_2, fresh yeast and cell counts) while open symbols and dotted lines relate to the secondary axis (glucose, viability, glycerol and pH). Error bars indicate the ranges between independent biological duplicates.

growth stopped or increased only slightly until 48 h (Fig. 1C and D). The number of cells was similar for the three strains, increasing from ca. 1×10^8 cells ml^{-1} to ca. 4×10^8 cells ml^{-1} on the stationary phase (after 24 h) (Fig. 1D), which corresponds to an average of two divisions per cell. In contrast, the fresh yeast concentration was much higher (up to 2-fold higher at 24 h) for the two industrial strains than for CEN.PK113-7D (Fig. 1C), which was substantiated by the dry yeast concentration measurements at the end of fermentations (Table 1). These observations indicate that the cells of the industrial strains grew to higher sizes compared to the laboratory strain CEN.PK113-7D, which was supported by microscope measurements (Fig. 2). The reduction observed in the fresh yeast concentration after 48 h (Fig. 1C) was probably due to viability loss by a large fraction of the cells (see below) and associated cell lysis.

The two industrial strains suffered a marked viability drop upon inoculation in VHG medium from over 85% (before inoculation, represented at −5 h in Fig. 1B) to 50–65% (0.5 h in Fig. 1B), most likely due to the strong osmotic shock. Nevertheless, the viability recovered to about 90% at 12 h and kept above 85% until 48 h. Then, viability sharply dropped to 13–17% at 72 h and to less than 1% at 96 h (Fig. 1B). The strain CEN.PK113-7D resisted better to the osmotic shock and upon inoculation there was only a slight and progressive viability loss (from 99% before inoculation to 89% at 0.5 h and to 83% at 12 h). Then, viability increased to over 90% at 24–48 h, progressively dropping afterwards (Fig. 1B).

In all cases, the pH profile declined from around 5.5 at the start of the fermentation to a minimum of 4.1 (PE-2 and CA1185) and 4.3 (CEN.PK113-7D) at 12–24 h of fermentation. Until the end of fermentation, pH increased to 4.5, 5.0 and 5.3 in CEN.PK113-7D, CA1185 and PE-2 fermentations, respectively (Fig. 1D).

The specific rates of ethanol production were maximal during yeast growth, markedly decreasing during the uncoupling phase (Fig. 3). Moreover, the specific rates of ethanol production were similar for the industrial and the laboratory strains (Fig. 3), which indicated that faster fermentation by the industrial strains resulted from higher accumulation of yeast biomass. Comparing to laboratory strain CEN.PK113-7D, this higher biomass accumulation was the result of cell mass increase rather than yeast division, since the cell counts were identical for the three strains although fresh yeast mass was higher (up to 2-fold) for the industrial strains relatively to CEN.PK113-7D (Fig. 1). The specific rates of glucose consumption followed essentially the same trends as ethanol production (data not shown).

TABLE 1. Kinetic parameters of laboratory (CEN.PK113-7D) and industrial (PE-2 and CA1185) *S. cerevisiae* strains in VHG fermentations. Values are average ± range of independent biological duplicates.

	S_0 (gl^{-1})	P_f (gl^{-1})	P_t (%, vol/vol)	S_f (gl^{-1})	G_f (gl^{-1})	X_f (gl^{-1})	$Y_{x/s}$	Y(%)	q ($gl^{-1}\,h^{-1}$)
CEN.PK 113-7D	335	112 ± 0	14.2 ± 0.0	89.8 ± 1.5	10.4 ± 0.1	6.5 ± 0.5	0.027 ± 0.002	89 ± 1	1.31 ± 0.00
PE-2	343	151 ± 3	19.1 ± 0.4	0.0 ± 0.0	11.3 ± 0.3	11.0 ± 0.5	0.032 ± 0.001	86 ± 2	2.51 ± 0.05
CA1185	338	152 ± 0	19.2 ± 0.0	2.9 ± 0.0	11.6 ± 0.0	9.1 ± 0.0	0.027 ± 0.000	89 ± 0	2.53 ± 0.00

S_0, initial glucose concentration. P_f, final ethanol concentration. P_t, final ethanol titre. S_f, final residual glucose concentration. G_f, final glycerol concentration. X_f, final biomass (dry weight) concentration. Yx/s, biomass yield, g biomass g $glucose^{-1}$. Y, ethanol yield, % of the theoretical. q, ethanol productivity.

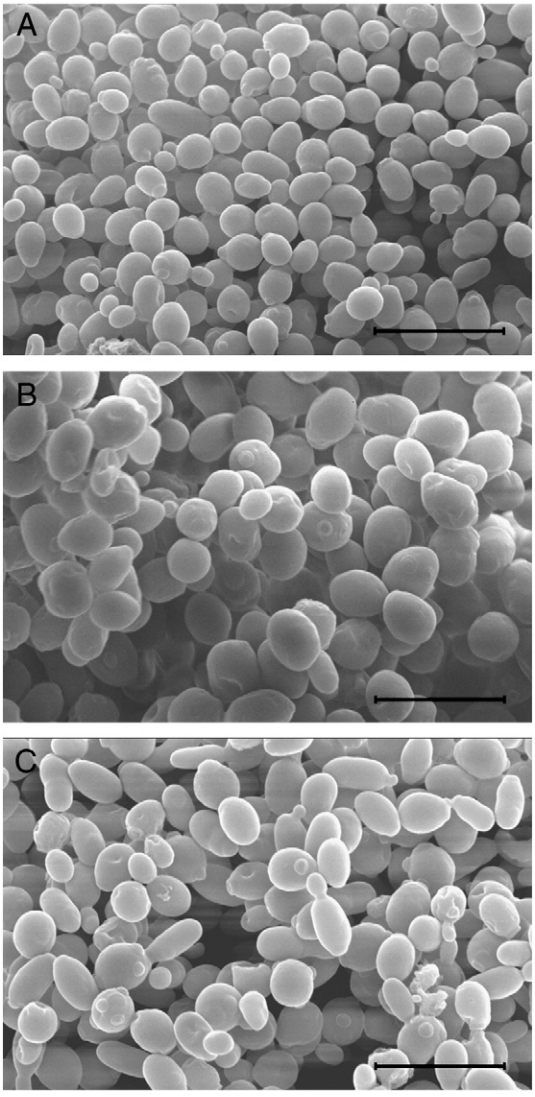

FIG. 2. Microscopic observation of cell size and morphology of: (A) laboratory CEN. PK113-7D strain. Bar = 10 μm; (B) industrial PE-2 strain. Bar = 10 μm; (C) industrial CA1185 strain. Bar = 10 μm.

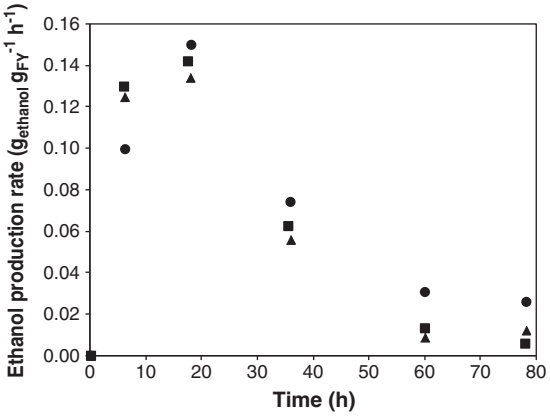

FIG. 3. Evolution of the specific rates of ethanol production throughout VHG fermentations with CEN.PK113-7D (circles), PE-2 (squares) and CA1185 (triangles) *S. cerevisiae* strains.

In Fig. 4 the viability values are plotted against the ethanol concentrations (Fig. 4A) or the specific ethanol production rates (Fig. 4B). Viability drop was associated with ethanol concentrations (Fig. 4A). For the two industrial strains, viability remained >85% up to 140 g l^{-1} ethanol, sharply declining at higher ethanol concentration. In the case of laboratory strain CEN.PK113-7D, the viability decline occurred at much lower critical ethanol concentration (85–100 g ethanol l^{-1}), highlighting the robustness of the industrial isolates for intensified fermentation processes. Moreover, independently of the yeast strain the sharp drop in viability coincided with the strong deceleration in fermentation (specific ethanol production rates <0.04 g ethanol g FY^{-1} h^{-1}) that occurred after 48 h of fermentation (Fig. 4B).

Intracellular metabolites dynamics Many studies have reported the role of relevant metabolites as indicators of the yeast's physiological state in response to osmotic and ethanol stresses, which are the main stress barriers to yeast fermentation performance in VHG media (16,20,23). Through the fermentation process, the changes in compounds such as, trehalose, glycogen, sterols and intracellular glycerol may constitute different adaptation mechanisms, which allow the yeast cells to cope with fermentation-related stresses.

Trehalose and glycogen constitute carbon and energy reserves of the yeast cell and their levels suffer large variations in response to different environmental changes (14,22). In exponentially growing cells, trehalose and/or glycogen reserves increase upon exposure to a variety of stress conditions (e.g., nutrient deprivation, high temperature, high ethanol or weak organic acids concentrations). Hence, these carbohydrates are considered typical hallmarks of rapid adaptations of yeast cells to environmental changing conditions (22). Sterols are essential lipid components of eukaryotic membranes and have been shown to be responsible for a number of important physical

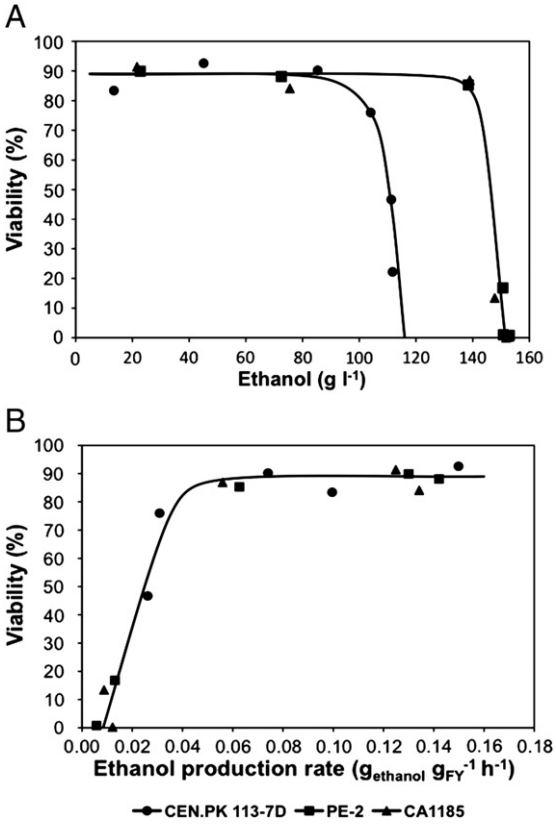

FIG. 4. Yeast viability as a function of (A) ethanol concentration or (B) specific rates of ethanol production during VHG fermentations with CEN.PK113-7D (circles), PE-2 (squares) and CA1185 (triangles) *S. cerevisiae* strains.

characteristics of membranes, particularly as important regulators of membrane permeability and fluidity. Although other membrane lipids also play a role in defining these properties, eukaryotic cells are unable to maintain viability without sterol (25). Moreover, the intracellular accumulation of glycerol is known to play an essential role as a compatible solute to counteract the hyperosmotic stress (20).

So, to characterize the effect of stressful VHG fermentation conditions on the different yeast strains, the levels of relevant intracellular metabolites (trehalose, glycogen, sterols and glycerol) were studied throughout the fermentation process (Fig. 5). Remarkable increases (>16-fold) were seen in the intracellular trehalose concentrations of the three strains from 12 to 24 h (Fig. 5A). The highest intracellular concentrations of trehalose were observed at the point in which fermentations were nearly complete (48 h for the industrial strains and 72 h for CEN.PK113-7D). These maximum intracellular trehalose levels were slightly higher (1.1-fold) in the industrial strains relatively to the laboratory strain. After this point, which coincided with the highest levels of ethanol stress and the following sharp drop in viability, the concentrations of trehalose measured decreased drastically, possibly because of cell lysis and because trehalose (a small soluble molecule) may leak from cells with fragile/compromised membranes (Fig. 5A). This decrease in the trehalose levels could also be at least partly explained by the use of trehalose as reserve energy source to resist and minimize the damaging stress conditions at the end of VHG fermentation (high ethanol levels and depletion of essential nutrients for yeast maintenance).

As shown in Fig. 5B, glycogen began to accumulate from 12 h of fermentation, with a sharp increase after the onset of the stationary phase (24–48 h). Unlike trehalose, glycogen continuously accumulated even when the yeast cell viability significantly dropped. This is probably due to the fact that glycogen is a hardly soluble polymer that cannot easily escape from the yeast cells. PE-2 and CA1185 industrial strains accumulated more glycogen than CEN.PK 113-7D laboratory strain. The highest difference was observed at 48 h when glycogen levels were 2.6-fold higher in PE-2 and 4.2-fold higher in CA1185 as compared to CEN.PK113-7D (Fig. 5B). In the laboratory strain, a mobilization of glycogen occurred during the initial 12 h of fermentation (levels decreased over 4-fold, from about 40 to 9 mg glycogen g DY^{-1}) (Fig. 5B). Conversely, in the industrial strains the initial levels of glycogen were much lower (2–5 mg g DY^{-1}) and no mobilization of glycogen was observed during the initial phase of fermentation (Fig. 5B).

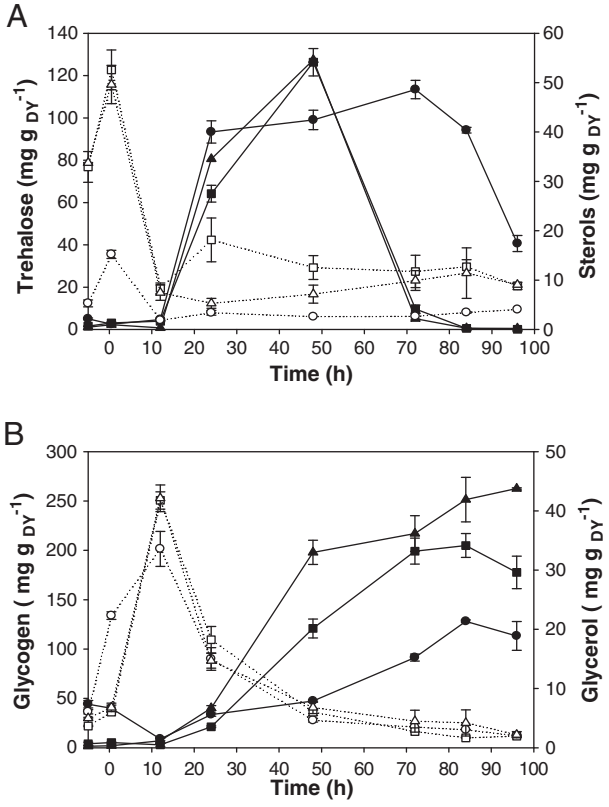

FIG. 5. Levels of intracellular metabolites in the course of VHG fermentations with CEN.PK113-7D (circles), PE-2 (squares) and CA1185 (triangles) *S. cerevisiae* strains: (A) trehalose (solid symbols) and sterols (open symbols); (B) glycogen (solid symbols) and glycerol (open symbols). Error bars indicate the ranges between independent biological duplicates. The levels of intracellular metabolites measured in the yeast suspensions used to inoculate the fermentations are represented at −5 h.

The accumulation of intracellular glycerol along the fermentations was also represented in Fig. 5B. In the yeast suspensions used for inoculation (represented at −5 h), the intracellular glycerol levels were similar for laboratory strain CEN.PK113-7D (6 mg g DY^{-1}) and for the industrial yeasts (4–5 mg g DY^{-1}). Interestingly, after 0.5 h of fermentation the intracellular glycerol concentration strongly increased (3.6-fold) in CEN.PK113-D, while the corresponding increases in the industrial strains were much lower (1.4 to 1.7-fold). After 12 h, the intracellular glycerol levels were maximal (34–42 mg g DY^{-1}) for the three strains. After this point, intracellular glycerol concentrations dropped, coincidently with sugar consumption and consequent osmotic stress release.

In the yeast suspensions used to inoculate the fermentations, the total sterols content of the industrial yeasts was about 6-fold higher than that of the laboratory strain (Fig. 5A). Under this study's conditions, the yeast strains initiated the production of sterols immediately following inoculation. During the initial 0.5 h, the sterols contents increased 2.9-fold in the laboratory strain and 1.5 to 1.6-fold in the industrial strains. At 0.5 h of fermentation, PE-2 and CA1185 industrial strains reached a maximum accumulation of 53 and 50 mg sterols g DY^{-1} respectively, which was around 3-fold higher than produced by the laboratory CEN.PK113-7D strain (15 mg sterols g DY^{-1}) (Fig. 5A). From that point on, the sterols content strongly decreased to about 8–12 mg g DY^{-1} for PE-2 and CA1185 strains and to ca. 3 mg g DY^{-1} for CEN.PK 113-7D strain (Fig. 5A) maintaining around these values until the end of fermentations.

It is noteworthy that in VHG fermentations the intracellular levels of trehalose, glycogen, glycerol and sterols in the diploid strain CEN.PK122 (data not shown) were identical to those in the haploid strain CEN.PK113-7D. This indicates that the higher accumulation of these metabolites in industrial strains PE-2 (which is diploid, (10)) and CA1185 (whose ploidy is unknown) was not a result of their ploidy.

DISCUSSION

Two industrial strains isolated from distilleries in Brazil were for the first time applied and physiologically characterized under VHG batch fermentations, being able to produce very high levels of ethanol (above 19%, v/v) with quite high productivity (>2.5 g l^{-1} h^{-1}). Interestingly, the fermentations were characterized by a long production phase uncoupled to yeast growth (after 24 h, when yeast growth essentially stopped) during which more than half of the ethanol was produced (Fig. 1). Similar uncoupling phase was seen in fermentations with laboratory strain CEN.PK113-7D, although the onset of this phase occurred at much lower ethanol concentration (45 g l^{-1} for CEN.PK113-7D against 70–75 g l^{-1} for the industrial yeasts) (Fig. 1). Such observations are in agreement with previously reported results for a fed-batch aerated fermentation system with the diploid laboratory strain CBS8066 (16,17). In such process, metabolic changes known to take place in glucose-grown stationary phase yeast cells were observed, although these changes were not triggered by

nutrient or oxygen depletion. Therefore, it was hypothesized that the onset of this "stationary-like" phase was a result of high ethanol level simulating a condition of nutrient limitation due to inhibition of uptake of carbon, nitrogen and phosphate (17). Interestingly, our results emphasize that this uncoupling phase is critical for the production of very high concentrations of ethanol under typical industrial conditions, and it is not a specific feature of the fed-batch aerated fermentation process. In brewing fermentations growth arrest is generally accepted to be the result of nutrient limitations, in particular because oxygen is no longer available to synthesize the sterols and unsaturated fatty acids needed to make new membranes (18). Similarly, under our conditions, oxygen-limitation could explain the onset of the uncoupling phase. Upon inoculation into air-saturated VHG medium, yeasts started synthesizing sterols. Then, at a first stage of fermentation process, anaerobic conditions were probably established in the fermentation flasks, with the exhaustion of oxygen preventing the biosynthesis of new sterols. Consequently, from that point on, the pool of sterols had to be divided between mother and daughter cells, which eventually led to growth arrest. The same rational could be applied to unsaturated fatty acids (whose levels were not measured in this work).

The three strains responded to the osmotic shock upon inoculation into VHG medium by increasing the intracellular levels of glycerol. However, the increase was higher in CEN.PK113-7D cells contributing to their survival (viability of 89%), while a large fraction of the industrial strains cells lost viability within 0.5 h. This indicated a higher capacity of the laboratory strain to cope with hyperosmotic shock, which probably resulted from this rapid accumulation of intracellular glycerol (Fig. 5B) that acts as a chemically inert osmolyte counteracting the hyperosmotic pressure (19,20). Nevertheless, it is conceivable that the drop in viability upon exposure to osmotic shock may represent an adaptive advantage of the industrial yeasts studied. These yeasts are cropped at the end of each fermentation batch and reused to inoculate a subsequent batch with very high cell density; in many Brazilian distilleries this biomass recycling procedure spans the entire 8 months sugarcane harvesting season (5). Therefore, these yeasts are periodically subjected to high osmotic shock following inoculation in fresh sugarcane juice. The death of a hyperosmotic sensitive sub-population upon re-inoculation in high-sugar medium could release micro-nutrients to sustain fast growth of the hyperosmotic resistant cells, which would rapidly take over the culture. This is consistent with our observation that viability of the industrial strains was restored at 12 h (Fig. 1B), which was accompanied by notable accumulation of intracellular glycerol (Fig. 5B).

Our results indicate a clear link between the viability drop and the ethanol concentration (Fig. 4A) and it was possible to identify a different critical ethanol concentration above which viability decreases for the laboratory and industrial strains (Fig. 4A). For the two industrial strains, viability remained >85% up to 140 g l^{-1} ethanol, sharply declining at higher ethanol concentration. In the case of strain CEN.PK113-7D, the viability decline occurred at much lower critical ethanol concentration (85–100 g l^{-1} ethanol), confirming lower ethanol resistance for the laboratory strain. Moreover, as pointed by Guimarães and Londesborough (21), these results emphasize the importance of harvesting the yeast for recycling to subsequent fermentations as soon as fermentation stops in order to avoid severe drop in viability. So, to implement a VHG repeated-batch system using these industrial isolates, the critical ethanol concentration above which viability and trehalose concentration decrease drastically is 140 g l^{-1}, meaning that yeasts should be re-pitched before reaching this value.

There was a drastic drop in viability towards the end of fermentations for the three strains, which coincided with the strong deceleration in fermentation that occurred after 48 h (specific ethanol production rates < 0.04 g ethanol g FY^{-1} h^{-1}) (Fig. 4B), which is in agreement with the results obtained by Guimarães and Londesborough (21). These authors observed during high gravity and VHG brewing fermentations that viability drop was related with the fermentation deceleration phase, probably as a result of critical ATP decline. Therefore, below certain critical ethanol production rates (under present work's conditions ca. 0.04 g ethanol g FY^{-1} h^{-1}) the production of ATP become too low to support the demand of maintenance reactions those are necessary to keep the yeast cells viable. This maintenance ATP demand probably augments during the late stages of fermentation as the ethanol stress upsurges. So, interestingly, there is an association between viability decrease with low ethanol production rates (deceleration of fermentation process) and this phenomenon, as can be observed in Fig. 4B, is strain independent.

Comparatively to laboratory CEN.PK113-7D strain, the industrial strains showed increased accumulation of the reserve carbohydrates trehalose and glycogen (Fig. 5). Devantier et al. (23) found that trehalose levels were 15 to 25-fold higher in VHG fermentations comparatively to standard medium with 20 g l^{-1} glucose. This is in line with the very high levels of trehalose accumulation observed during this work's fermentations (Fig. 5A). Nevertheless, the peak levels of intracellular trehalose in the industrial strains were only slightly higher (1.1-fold) relatively to the laboratory strain. Conversely, glycogen accumulation was markedly higher (2 to 4-fold higher peak levels) in the industrial strains than in the laboratory strain (Fig. 5B). Dake et al. (24) reported that increasing ethanol concentrations (from 2 to 8% v/v) in the culture medium resulted in increasing levels of the two pools of glycogen in yeast cells (i.e., cytoplasmic water-soluble glycogen and cell-wall-bound insoluble glycogen), suggesting that there is a correlation between accumulation of glycogen and yeast's adaptation to ethanol. Our results indicate a similar correlation in the course of VHG fermentations, with the glycogen levels increasing in a similar manner for the three strains up to about 100 g l^{-1} (Fig. 6). In contrast to the laboratory strain, the industrial strains were able to continue producing ethanol over 140 g l^{-1} with concurrent increases in glycogen (Fig. 6). It is nonetheless noteworthy that strain CA1185 accumulated considerably more glycogen than strain PE-2 (Figs. 5B and 6), although their ethanol production kinetics were very similar. Taken together, our results suggest that increased accumulation of trehalose and especially glycogen possibly provide an adaptive advantage to the industrial strains under the harsh conditions typical of the fermentation systems from which these were isolated (in which some of the main stresses are common to VHG fermentations).

The industrial strains PE-2 and CA1185 exhibited increased sterol levels (2 to 5-fold during fermentation) relatively to laboratory CEN.PK113-7D strain (Fig. 5A). This ability to accumulate higher sterol levels

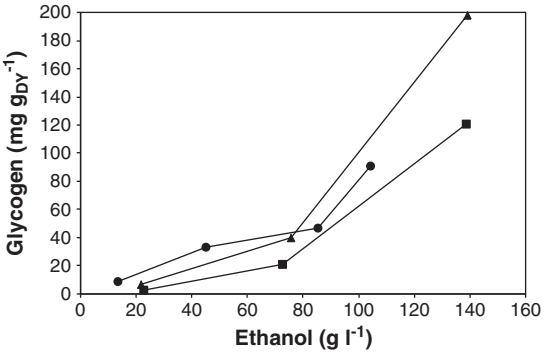

FIG. 6. Intracellular glycogen levels as a function of ethanol concentration during VHG fermentations with CEN.PK113-7D (circles) (data from 12 to 72 h of fermentation), PE-2 (squares) and CA1185 (triangles) (data from 12 to 48 h of fermentation) *S. cerevisiae* strains.

is likely to be another important adaptation of the industrial strains to cope with VHG fermentation stresses. It is particularly important under oxygen-limiting conditions since oxygen is needed for sterol biosynthesis. Possibly, the industrial yeasts were able to channel a higher fraction of the limited amount of oxygen available at the beginning of fermentation to synthesize essential lipids, including sterols.

In conclusion, our results show that these industrial isolates PE-2 and CA1185 have an outstanding performance in VHG fermentation, producing remarkable high ethanol titres (>19%, v/v) with quite high productivity ($>2.5\ g\ l^{-1}\ h^{-1}$), and reveal their robust physiological background under these intensified fermentation conditions. Under VHG conditions, these isolates were characterized by improved accumulation of trehalose, glycogen and sterols, relatively to laboratory strain, which can be related with enhanced stress tolerance and fermentative performance. Interestingly, we have observed that the sharp drop in viability and trehalose concentration coincided with the strong deceleration in fermentation for both laboratory and industrial strains. Nevertheless, the critical ethanol concentration, above which this happens, was significantly higher for industrial isolates ($140\ g\ l^{-1}$).

The robustness of these industrial isolates as well as the detailed physiological information gathered in this study will potentiate VHG technology applications.

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