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ORIGINAL RESEARCH PAPER

Cell recycling during repeated very high gravity bio-ethanol fermentations using the industrial *Saccharomyces cerevisiae* strain PE-2

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Abstract A very high gravity (VHG) repeated-batch fermentation system using an industrial strain of *Saccharomyces cerevisiae* PE-2 (isolated from sugarcane-to-ethanol distillery in Brazil) and mimicking industrially relevant conditions (high inoculation rates and low O_2 availability) was successfully operated during fifteen consecutive fermentation cycles, attaining ethanol at 17.1 ± 0.2% (v/v) with a batch productivity of 3.5 ± 0.04 g l^{-1} h^{-1}. Moreover, this innovative operational strategy (biomass refreshing step) prevented critical decreases on yeast viability levels and promoted high accumulation of intracellular glycerol and trehalose, which can provide an adaptive advantage to yeast cells under harsh industrial environments. This study contributes to the improvement of VHG fermentation processes by exploring an innovative operational strategy that allows attaining very high ethanol titres without a critical decrease of the viability level thus minimizing the production costs due to energy savings during the distillation process.

Keywords Bio-ethanol production · Industrial strain PE-2 · *Saccharomyces cerevisiae* · Repeated-batch system · Very high gravity fermentation

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Introduction

The application of process engineering strategies for the development of high productivity fermentation systems is now considered a key issue in the bio-ethanol industry. An economically relevant factor associated with industrial bio-ethanol production is reaching high ethanol titers (thus obtaining significant savings in energy consumption during the distillation process) over a succession of short fermentation cycles, in which yeast cells at the end of a batch cycle are reused as inoculum for the next cycle (hence increasing cell density in the fermenters while reducing the costs associated with fresh yeast biomass propagation). The so-called repeated-batch fermentation systems with reuse of the yeast cells has already been investigated and reported in the literature, including in beer fermentations (Verbelen et al. 2009a). The maintenance of a high fraction of viable

yeast cells throughout the fermentation cycles is a prerequisite to carry out a long-lasting succession of fermentation cycles (Bai et al. 2008; Laluce et al. 2009). For the production of high levels of ethanol from fermentable sugars, a promising system consists in combining very high gravity (VHG) technology with repeated-batch operation using biomass recycling. Such system requires a robust strain able to efficiently convert the sugars to ethanol at high speed (high ethanol yield and productivity) with high tolerance to osmotic stress, ethanol and other inhibitors (either formed during raw-materials pre-treatments or produced during fermentation).

The sugarcane-to-ethanol fermentation processes implemented in Brazil use very high cell densities to ferment broths (cane juice and/or diluted molasses) containing up to 150–200 g total sugar l^{-1} (mainly sucrose), producing ethanol at 8–11% (v/v) with high productivities (each fermentation cycle lasts only 6–11 h) (Wheals et al. 1999). A few highly tolerant and productive yeast strains were isolated from sugarcane-to-ethanol distilleries showing high tolerance to ethanol and combining high fermentation efficiency with prolonged persistence in the fermentation system. In recent years, such strains have been widely adopted by the industry (Stambuk et al. 2009). One of the most successful examples is the PE-2 wild isolate, which is currently used by about 30% of Brazilian distilleries, generating ca. 10% of the world's bio-ethanol supply (Argueso et al. 2009).

In a previous study, we evaluated the fermentation performance of 8 strains isolated from distillery environments in Brazil under VHG batch conditions. PE-2 and CA1185 isolates were selected based on their higher ethanol titre and productivity (Pereira et al. 2010b). To gather detailed yeast physiological information of PE-2 and CA1185 isolates in VHG conditions, relevant physiological parameters were measured throughout the different batch fermentation stages and the results reveal their robust physiological background under these intensified fermentation conditions (improved accumulation of trehalose, glycogen and sterols relatively to CEN.PK 113-7D laboratory strain) (Pereira et al. 2011). Moreover, a critical ethanol concentration (140 g l^{-1}) above which viability and trehalose concentration decrease significantly was identified (Pereira et al. 2011). This physiological parameter guides the implementation of VHG repeated-batch system and sustains its optimization.

In a well-established bio-ethanol production process, several strategies can be used for further process improvement including to find the optimal balance between final ethanol concentration, initial glucose content, fermentation time, biomass recycling and yeast cell viability. Those parameters strongly influence the economical benefits of industrial bio-ethanol production plants. Hence, the aim of this work was to evaluate and optimize the performance of the *Saccharomyces cerevisiae* PE-2 industrial isolate in a repeated-batch fermentation system with cell recycling under VHG conditions. Moreover, we aimed at devising an operational strategy that permitted attaining very high ethanol titres without resulting in a critical decrease of yeast viability levels.

Materials and methods

Yeast

The yeast used was *S. cerevisiae* strain PE-2, which was isolated from a sugarcane-to-ethanol distillery in Brazil (Basso et al. 2008). This strain was previously selected for its high fermentation performance under VHG conditions (Pereira et al. 2010b). 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 previously optimized VHG medium (Pereira et al. 2010a) consisting of 313–400 g glucose l^{-1}, 44.3 g corn steep liquor (CSL) l^{-1}, 2.3 g urea l^{-1}, 3.8 g $MgSO_4 \cdot 7H_2O$ l^{-1} and 0.03 g $CuSO_4 \cdot 5H_2O$ l^{-1}. The CSL was kindly provided by a starch manufacturer (COPAM, Portugal) and its handling in the laboratory as well as its main composition have been previously reported (Pereira et al. 2010a). The pH of the medium was adjusted to 5.5 with 1 M NaOH. The medium was aerated by stirring with a magnetic bar (length of 3 cm) at >850 rpm during 20 min before transferring to 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 glucose l^{-1}, 20 g

peptone l^{-1} and 10 g yeast extract l^{-1}. After incubation at 30°C and 150 rpm for 22–24 h (OD_{600} of 7–7.5), the cell suspension was aseptically harvested by centrifugation (10 min at 4,800×*g*, 4°C) and resuspended in ice-cold 0.9% (w/v) NaCl to 200 mg fresh yeast (FY) ml^{-1}. This concentrated cell suspension was then used to inoculate 40 ml of fermentation medium with $\sim 10^8$ cells ml^{-1} (unless otherwise stated) to start the fermentation.

Fermentations were conducted 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 (or 27°C where stated) with 150 rpm orbital agitation.

The progress of fermentation was followed by mass loss (resulting from CO_2 production), and samples for analyses were taken just at the ending point. At each time-point, the standard deviation between the independent biological replicates ($n = 2$) was less than 2% of the average value for the CO_2 production.

Repeated-batch operation

For repeated-batch operation, the first fermentation cycle was initiated with the inoculation of 11 mg FY ml^{-1} (approximately 1×10^8 cells ml^{-1}) into 40 ml fermentation medium. At the end of the first cycle, the accumulated yeast biomass was totally recycled to inoculate the second cycle. Briefly, cells were separated from the fermentation broth by centrifugation (10 min at 4,800×*g*, 4°C) and the cell pellet was resuspended in 40 ml fresh and aerated medium. This cell suspension was transferred to a fermentation flask to initiate the second cycle. This procedure was repeated for biomass recycling in the following cycles, as long as final yeast viability remained >50%. When the viability at the end of a fermentation cycle dropped below 50%, only a fraction of the biomass (ca. 11 mg FY ml^{-1}) was recycled to the following cycle.

Analytical methods

Determination of yeast concentration and viability

For fresh and dry yeast (DY) mass determinations, a sample of the fermentation (20 ml) was centrifuged for 10 min at 4,800×*g* (4°C) in a pre-weighed dried tube. The supernatant was completely removed and the tube was dried inside and outside and weighed again to give the FY mass. Then, the yeast pellet was washed with 20 ml distilled water, centrifuged, dried overnight at 105°C and the tube was finally weighed again to give the DY mass.

Total and viable cell counts were determined with an optical microscopic using a Neubauer improved counting chamber. The yeast cell viability was determined by the methylene blue staining method (Mills 1941). The percentage of viable cells was calculated by the ratio between viable (non-stained) and total cell counts.

Extracellular metabolites: glucose, glycerol and ethanol

Fermentation samples were centrifuged (10 min at 4,800×*g*, 4°C) and the supernatants were used for the quantification of extracellular metabolites. Glucose, glycerol and ethanol were analyzed by HPLC using a Varian MetaCarb 87H column eluted at 60°C with 5 mM H_2SO_4 at 0.7 ml min^{-1} linked to a refractive index detector.

Intracellular metabolites: trehalose and glycerol

Yeast cells were collected from fermentation medium by centrifugation (5 min at 4,800×*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}. Samples (100 mg FY) from this yeast suspension were boiled in a water bath for 5 min and 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 (Ferreira et al. 1997) using the same conditions described above for the extracellular metabolites. The final concentrations of intracellular trehalose and glycerol were normalized to yeast dry mass. Deviations (ranges) between technical duplicates (duplicate samples from the same yeast suspension) were determined to be lower than 7% of the average value for the analyses of trehalose and glycerol.

Determination of fermentation parameters

Ethanol conversion yield was calculated by the ratio between the final ethanol concentration 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 g^{-1} 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). Biomass yield was calculated as the ratio between final biomass (DY) concentration and the glucose consumed.

Results and discussion

Impact of initial glucose levels and temperature on the fermentation performance

Ethanol tolerance of yeast is negatively affected by increasing the temperature of fermentation (Casey and Ingledew 1986). Thomas et al. (1993) tested the fermentation of VHG wheat mashes at 20, 25, 30 and 35°C and found that fermentations were complete at 20 and 25°C whilst becoming sluggish and incomplete at 30 and 35°C. At 20°C, the maximum ethanol titre produced was 23.8% (v/v) with yeast extract supplementation, dropping to 18% (v/v) at 30°C (Thomas et al. 1993). Hence, lowering the fermentation temperature can be exploited as a process engineering strategy to alleviate the ethanol inhibition and improve the fermentation performance under VHG conditions.

In order to optimize the process conditions for repeated-batch experiment, the impact of temperature and initial glucose levels on PE-2 fermentation performance was studied. Therefore, fermentations with very-high glucose levels (>360 g l^{-1}) at 27 and 30°C (Table 1) were performed to test whether the *S. cerevisiae* PE-2 strain could attain higher ethanol titres than previously reported at 30°C (Pereira et al. 2011). In fermentations with 364 g glucose l^{-1} at 27 and 30°C the yeast cells fermented most of the glucose (residual of 6 and 35 g l^{-1}, respectively), reaching ethanol titres of 21.6 and 19.3% (v/v), respectively (Table 1). Thus, as expected, decreasing the fermentation temperature to 27°C resulted in increased glucose consumption and final ethanol titre, although batch ethanol productivity decreased by 22% (Table 1). Moreover, strain PE-2 showed quite high osmotic stress tolerance, growing in and fermenting VHG medium with 400 g glucose l^{-1}. However, the VHG fermentations were incomplete, leaving 60 and 80 g glucose l^{-1} and attaining 20.1 and 18.2% (v/v) ethanol at 27 and 30°C, respectively (Table 1).

Our results (see Table 1) showed that increasing the initial glucose from 343 to 364 g l^{-1} and decreasing the temperature from 30 to 27°C represents a considerable improvement in the final ethanol concentration (from 19.1 to 21.6%, v/v) and ethanol yields (86–93%) whereas the batch ethanol productivity was significantly lowered (2.51–1.31 g l^{-1} h^{-1}). Hence, fermenting at 30°C with an initial concentration around 340 g glucose l^{-1} arises as the most efficient and economic option for attaining VHG

Table 1 The effect of temperature and initial glucose concentration on fermentation performance and final ethanol titre attained by PE-2 industrial strain

	S_o	P_t (%, v/v)	S_f (g l^{-1})	X_f (g l^{-1})	$Y_{x/s}$ ($g_{biomass}$ $g_{glucose-1}$)	Y (%)	q (g l^{-1} h^{-1})
30°C	343[a]	19.1 ± 0.4	0.0 ± 0.0	11.0 ± 0.5	0.032 ± 0.001	86 ± 2	02.51 ± 0.05
	364	19.3 ± 0.2	35.5 ± 0.2	10.8 ± 0.1	0.033 ± 0.000	91 ± 1	1.60 ± 0.02
	400	18.2 ± 0.2	80.4 ± 0.5	8.4 ± 0.1	0.026 ± 0.000	88 ± 1	1.51 ± 0.02
27°C	364	21.6 ± 0.3	6.2 ± 0.1	10.2 ± 0.0	0.028 ± 0.000	93 ± 1	1.31 ± 0.02
	400	20.1 ± 0.2	59.9 ± 1.7	9.1 ± 0.1	0.027 ± 0.000	91 ± 1	1.27 ± 0.01

Values are average ± range of independent biological duplicates

S_o initial glucose concentration P_t final residual glucose concentration, X_f final biomass (dry weight) concentration, $Y_{x/s}$ biomass yeild, Y ethanol yeild, % of the theortical, q ethanol producivity.

[a] Kinetics parameters of PE-2 strain in VHG using the same process conditions (Pereira et al. 2011)

fermentation completion (all glucose consumed), high batch productivities and very high ethanol levels at the end of the process. Such very high ethanol titres allow significantly reduced costs in the distillation process, which remains as one of the main constraints in the industrial production of bio-ethanol. Moreover, the use of higher fermentation temperature (30°C instead of 27°C) also allows energy savings through a reduction in cooling costs associated to fermentation vessels over-heating, principally in regions/seasons of high ambient temperature.

Repeated-batch system for VHG bio-ethanol fermentations using PE-2 strain

Influence of cell density on CO_2 productivity in consecutive VHG batch fermentations

The influence of cell density (inoculum concentration) on CO_2 productivity was studied in VHG batch fermentations (Fig. 1). The batch CO_2 productivity increased with increasing inoculum concentration (Fig. 1). The use of very high cell densities (85 and 167 mg FY ml^{-1}) gave high CO_2 productivities (ca. 6 g l^{-1} h^{-1}) through three consecutive batch fermentations. However, severe decreases in fermentation productivity were observed along the consecutive batches. Specifically, in fermentations inoculated with 85 and 167 mg FY ml^{-1} the batch CO_2 productivity in the third batch was 21% and 66% lower relatively to the first batch, respectively (Fig. 1). These impaired productivities correlated with sharp drops in yeast cell viability. Particularly, in the fermentations inoculated with 167 mg FY ml^{-1} the yeast viability declined from 80% (beginning of the first batch) to 20% (final of the third batch). Possibly, this drastic decrease on viability levels was a result of the absence of yeast growth during the sequential batch fermentations and of the high ethanol concentration (15–16%, v/v) attained in these VHG processes.

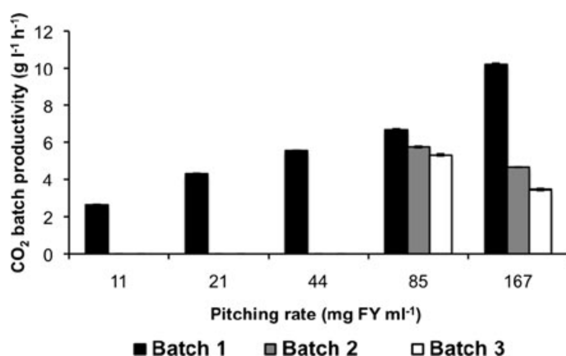

Fig. 1 Correlation between pitching rate (inoculum concentration) and CO_2 batch productivity during VHG (313 ± 11 g glucose l^{-1}) fermentations by industrial PE-2 strain. The batch CO_2 productivity was calculated at the point in which a CO_2 concentration of 121 ± 4 g l^{-1} was reached. Consecutive batch fermentations were only conducted for 85 and 167 mg FY ml^{-1} pitching rate. *Error bars* represent the range between independent biological duplicates

An innovative consecutive batch fermentation process for VHG fermentations

In industry it is common practice to recycle the yeast biomass for several consecutive batches in order to reduce the time and cost for inoculum preparation. For instance, in brewing the yeast is reused 5–20 times depending on the particular brewery (Huuskonen et al. 2010). In Brazilian bio-ethanol distilleries, the yeast is also recycled by centrifugation and in many cases the recycling spans the entire 8 months sugarcane harvesting season (Basso et al. 2008). Moreover, the use of consecutive batch fermentation processes with cell recycling permits the accumulation of high cell densities that can enhance the ethanol productivity of the systems (Van Hoek et al. 2000). In this perspective, the performance of the robust PE-2 strain was evaluated in a repeated-batch VHG fermentation process operated for 15 cycles with cell recycling. Therefore, we developed a strategy consisting in inoculating the initial batch cycle only with 11 mg FY ml^{-1} and recycling the totality of the yeast biomass produced (50–90 mg FY ml^{-1}) in the following cycles. However, when the cell viability at the end of fermentation was lower than 50%, only a fraction (ca. 11 mg FY ml^{-1}) of the yeast recovered was used to inoculate the following cycle in order to allow yeast growth and viability restoration.

The kinetic fermentation data and some evaluated yeast's physiologic parameters during the operation of the repeated-batch system for 15 cycles are shown in Table 2. All fermentations were completed within 29–50 h (exceptions were cycles 6, 7 and 13, which were inoculated with 11 mg FY ml^{-1} with viability levels <50%), depending on the concentration of biomass recycled (11–89 mg FY ml^{-1}), cell viability

Table 2 Repeated-batch VHG fermentations with the industrial strain PE-2

n	t_f (h)	$Biomass_{initial}$ (mg FY ml^{-1})	S_o (g l^{-1})	S_f (g l^{-1})	Co_2 (g l^{-1})	P_f (g l^{-1})	P_t (%, v/v)	G_E (g l^{-1})	q (g l^{-1} h^{-1})	Y (%)	X (cells ml^{-1}) $\times 10^8$	V (%)	T (mg g DY^{-1}	G_I (mg g DY^{-1}
1	39.1	11 ± 0	324	2.2 ± 0.0	136 ± 4	135 ± 2	17.0 ± 0.2	9.5 ± 0.2	3.45 ± 0.04	82 ± 1	5.4 ± 1.0	89 ± 1	n/d	n/d
2	39.2	57 ± 2	323	16.7 ± 1.8	133 ± 2	131 ± 1	16.6 ± 0.2	13.7 ± 0.7	4.10 ± 0.03	79 ± 1	5.3 ± 0.2	82 ± 6	183.5 ± 15.3	n/d
3	29.4	70 ± 1	343	3.3 ± 0.2	140 ± 1	135 ± 0	17.1 ± 0.0	14.8 ± 0.1	4.59 ± 0.01	78 ± 0	5.9 ± 0.0	67 ± 5	n/d	n/d
4	30.5	80 ± 0	343	3.3 ± 0.2	140 ± 1	135 ± 0	17.7 ± 0.0	15.6 ± 0.1	4.43 ± 0.00	78 ± 0	4.6 ± 0.2	63 ± 4	160.7 ± 16.0	n/d
5	38.6	89 ± 1	343	11.0 ± 0.1	132 ± 1	136 ± 6	17.2 ± 0.8	17.9 ± 0.3	3.51 ± 0.16	80 ± 4	7.0 ± 0.7	27 ± 1	93.4 ± 3.2	n/d
6	65.8	11 ± 0[a]	324	23.5 ± 1.9	128 ± 1	125 ± 0	15.9 ± 0.0	n/d	1.91 ± 0.01	82 ± 1	8.2 ± 0.3	49 ± 0	91.0 ± 0.0	n/d
7	64.3	11 ± 0[a]	324	2.2 ± 0.1	137 ± 2	137 ± 1	17.3 ± 0.1	n/d	2.13 ± 0.02	83 ± 1	3.7 ± 0.1	n/d	n/d	n/d
8	49.8	42 ± 4	343	2.2 ± 0.2	131 ± 2	136 ± 2	17.3 ± 0.3	12.9 ± 0.2	2.74 ± 0.05	78 ± 1	4.0 ± 0.3	84 ± 1	69.9 ± 7.5	21.5 ± 1.9
9	29.3	59 ± 01	343	5.2 ± 3.0	137 ± 3	138 ± 3	17.5 ± 0.4	15.7 ± 0.2	4.72 ± 0.12	80 ± 1	5.8 ± 1.5	72 ± 0	60.9 ± 4.9	28.2 ± 0.3
10	33.8	74 ± 1	343	n/d	136 ± 0	n/d	n/d	n/d	n/d	n/d	n/d	72 ± 3	47.0 ± 2.4	31.9 ± 0.0
11	30.7	76 ± 0	343	3.9 ± 1.6	135 ± 0	138 ± 0	17.4 ± 0.0	18.0 ± 0.0	4.49 ± 0.00	79 ± 0	4.8 ± 0.1	66 ± 3	43.5 ± 6.3	30.4 ± 0.0
12	30.0	88 ± 1	343	9.9 ± 0.2	132 ± 1	134 ± 1	16.9 ± 0.1	17.9 ± 0.1	4.46 ± 0.03	79 ± 0	6.4 ± 0.1	42 ± 1	33.0 ± 0.1	28.5 ± 0.1
13	63.1	11 ± 0	324	1.3 ± 0.0	129 ± 0	136 ± 3	17.2 ± 0.3	13.8 ± 0.0	2.15 ± 0.04	82 ± 2	5.9 ± 0.4	77 ± 5	59.7 ± 3.4	25.3 ± 0.0
14	32.6	46 ± 2	343	10.8 ± 0.9	132 ± 1	135 ± 1	17.1 ± 0.2	14.9 ± 0.1	4.14 ± 0.04	80 ± 1	3.0 ± 0.2	69 ± 3	56.9 ± 2.6	25.8 ± 0.3
15	59.7	72 ± 2	343	0.0 ± 0.0	137 ± 2	142 ± 0	18.0 ± 0.1	17.0 ± 0.1	2.38 ± 0.01	81 ± 1	3.9 ± 0.2	0 ± 0	0.0 ± 0.0	31.0 ± 1.1

Values are average ± standard deviation of 4 biological replicates for the first and second cycle, and average ± range of independent biological duplicates for the remaining cycles

n batch number in repeated-batch fermentation, t_f fermentation time of each batch, S_o initial glucose concentration, S_t final residual glucose concentration, P_t final ethanol concentration, P_f final ethanol titre, G_E final glycerol concentration, *q* ethanol productivity, *Y* ethanol yeild, % of the theortical, *X* final cell number of each batch, *V* final viability of each batch, *T* final intercellular trehalose concentration, G_t final intercellular glycerol concentration, *n/d* not determine

[a] In batch cycles 6, 7, and 13 only sa fraction of the biomass was recycled to the following cycle (about 11 mg FY l^{-1})

(27–89%) and initial sugar concentration (324–343 g glucose l^{-1}). In the first five fermentation cycles almost all glucose was consumed (residual <17 g l^{-1}), allowing the production of high ethanol levels of 17% (v/v) with ethanol yields of 78–82%. Moreover, the batch ethanol productivity ranged from 3.45 to 4.59 g l^{-1} h^{-1}.

High viability of yeast biomass is vital for the process to work efficiently. At the end of cycle 5, viability dropped to 27% and therefore the next two cycles were inoculated with only a fraction of yeast biomass (ca. 11 mg FY ml^{-1}), which allowed biomass refreshing (yeast growth) and viability restoring to 84% at the end of cycle 8. Then, until the twelfth cycle, glucose consumption, ethanol titres (ca. 17%) and productivities (2.74–4.72 g l^{-1} h^{-1}) remained quite high. At the end of the twelfth fermentation cycle the cell viability dropped to 42%, recovering to 77% after a refreshing cycle (cycle 13). Taken together, the results showed that this innovative VHG fermentation system could be operated at least during fifteen consecutive batch fermentations with average final ethanol concentrations, ethanol yields and batch productivities of 17.1 ± 0.2% (v/v), 80 ± 1% and 3.51 ± 0.04 g l^{-1} h^{-1}, respectively.

In biotechnological processes a promising strategy to improve the volumetric productivity is the enhancement of suspended yeast cells in a batch fermentor by increasing the pitching rate (Verbelen et al. 2009a, b). The process strategy devised (cycle for refreshing biomass when the yeast viability levels decayed <50%) allowed the successful operation of this system during 15 consecutive cycles maintaining yeast viability at reasonable levels. Also, in beer repeated-batch fermentations in order to keep the yeast cells viable after recycling and, consequently, obtain an optimal fermentation performance another approaches were also investigated. The common practice is aeration of the wort before pitching (strategy that we also performed in this work) thereby providing O_2 for lipid synthesis during the first stages of the fermentation (Ohno and Takahashi 1986). Another process engineering strategies to maximize the performance of yeast cells during consecutive batch fermentations include the oxygenation (aeration) of cropped yeast slurries (Verbelen et al. 2009b), the addition of the required lipids to wort (Casey et al. 1983) and the supplementation of cropped cells with unsaturated fatty acids (sometimes suggested as an alternative to aeration) (Moonjai et al. 2002).

Under VHG conditions, high substrate concentrations may exert inhibition on yeast cells (Cahill et al. 2000). Glycerol is synthesized as a metabolic by-product during alcoholic fermentation essentially to maintain the redox balance in yeast cells. Moreover, the production and intracellular accumulation of glycerol is known to play an essential role as a compatible solute to counteract the hyperosmotic stress (Michnick et al. 1997). In the first fermentation cycle, the PE-2 industrial strain produced 9.5 g glycerol l^{-1} increasing to 18 g glycerol l^{-1} at the end of the fifth cycle; increases on extracellular glycerol also occurred between cycles 8 and 11 (Table 2). The levels of glycerol accumulated intracellularly were also measured at the end of cycles 8–15, maintaining around 21–32 mg glycerol g DY^{-1}. These values are markedly higher than those observed at the end (48 h) of the single batch fermentation using the same process conditions (343 g glucose l^{-1}; 30°C, PE-2 strain) (Pereira et al. 2011). In the single VHG fermentation, the intracellular glycerol levels were maximal (34–42 mg g DY^{-1}) at 12 h and after this point sharply dropped attaining a concentration of 6.0 mg g DY^{-1} at the end of the process (48 h) (Pereira et al. 2011). Possibly, during the repeated recycling procedure the yeast adapted to retain higher intracellular glycerol levels to cope with the elevated and periodically varying osmotic stress.

In the initial batch cycles, yeast cells accumulated high content of intracellular trehalose (>150 mg trehalose g DY^{-1}) (Table 2). During the fifth fermentation cycle a drastic loss of cell viability (63–27%) was accompanied by a massive decrease of intracellular trehalose from 161 to 93 mg g DY^{-1}. In the next cycles the intracellular trehalose content decreased progressively to 33 mg g DY^{-1} (cycle 12). During the thirteenth cycle, the cell viability restoration (42–77%) was accompanied by an increase from 33 to 60 mg trehalose g DY^{-1}. The fifteenth fermentation cycle was extended until 60 h attaining a maximum ethanol production titre of 18% (v/v). However, a critical decrease on intracellular trehalose concentration (0 mg g DY^{-1}) and cell viability (0%) was observed. Thus, the loss of cell viability along the fermentation cycles seems to be associated with the drop of intracellular trehalose content of yeast cells. This decrease in the trehalose levels could be explained by the fact this metabolite (a small soluble

molecule) may leak from cells with fragile/compromised membranes or could be used 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 a result, intracellular trehalose content can be an important parameter of the yeast's physiological state in VHG repeated-batch bio-ethanol fermentation system, which is in agreement with previous reports (Basso et al. 2008; Cot et al. 2007; Pereira et al. 2011). Kaino and Takagi (2008) reported that during YPD fermentations yeast cells rapidly increased the glycerol and trehalose levels in response to the addition of sorbitol (1 M) or ethanol (9%, v/v), respectively. Thus, the increase of glycerol and trehalose levels can act as stress protection mechanisms counteracting the hyperosmotic pressure developed during yeast recycling for a fresh sugarcane juice and the high ethanol levels at the end of batch cycles. Additionally, a global analysis of yeast genes expression has shown that genes involved in glycerol and trehalose metabolism are up regulated by osmotic and ethanol stresses, respectively (Alexandre et al. 2001). Also, our results suggest that the increased biosynthesis of intracellular trehalose and glycerol were a direct response to high ethanol levels and osmotic stress developed during yeast recycling to a fresh high-sugar medium, respectively. Moreover, high accumulation of these key intracellular metabolites by PE-2 cells during consecutive VHG fermentations indicates that this strain is prepared to survive under this stressful environment (high sugar and ethanol levels) and to attain high fermentation performance during the next fermentation cycles.

As reported by Pereira et al. (2011), the viability of yeasts collapsed abruptly when the fermentation process decelerated and thus, harvesting the yeast for recycling to subsequent fermentations as soon as fermentation stops is critical to maintain high viability levels during the next consecutive batch fermentations. In this previous study, it was concluded that the critical ethanol concentration above which viability and trehalose concentration decrease drastically was 17.7% (v/v), meaning that PE-2 cells should be re-pitched before reaching this value. This physiological information was of great importance to implement this efficient VHG repeated-batch system with biomass recycling during 15 consecutive cycles.

With the increased competition and cost pressure in the bio-ethanol industry, process engineering strategies will be a step forward for a further improvement in the concepts of industrial ethanol yield and energy savings. The repeated-batch strategy followed in this work, mimicking industrially relevant conditions (high inoculation rates and low oxygen availability) and using very-high levels of initial sugar (324–343 g l^{-1}), allowed the production of an average ethanol concentration of $17.1 \pm 0.2\%$ (v/v) with a batch ethanol productivity of 3.51 ± 0.04 g l^{-1} h^{-1} during 15 consecutive fermentation cycles. Hence, this stands as an effective repeated-batch process for industrial implementation.

Conclusions

An innovative VHG repeated-batch fermentation system using the industrial strain PE-2 was successfully operated during fifteen consecutive fermentation cycles, attaining a final ethanol concentration of $17.1 \pm 0.2\%$ (v/v) with a batch ethanol productivity of 3.51 ± 0.04 g l^{-1} h^{-1}. Moreover, the yeast biomass refreshing strategy permitted yeast growth to maintaining high viability levels, which is crucial for industrial implementation, especially considering the fact that the yeast is re-pitched for several fermentation cycles. The increased biosynthesis of intracellular glycerol and trehalose by this industrial PE-2 isolate may contribute for its improved tolerance to the elevated and periodically varying osmotic stress during yeast recycling and the high ethanol levels accumulated at the end of each fermentation cycle, respectively.

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