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Plasmid-mediate transfer of *FLO1* into industrial *Saccharomyces cerevisiae* PE-2 strain creates a strain useful for repeat-batch fermentations involving flocculation–sedimentation

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ABSTRACT

The flocculation gene *FLO1* was transferred into the robust industrial strain *Saccharomyces cerevisiae* PE-2 by the lithium acetate method. The recombinant strain showed a fermentation performance similar to that of the parental strain. In 10 repeat-batch cultivations in VHG medium with 345 g glucose/L and cell recycling by flocculation–sedimentation, an average final ethanol concentration of 142 g/L and an ethanol productivity of 2.86 g/L/h were achieved. Due to the flocculent nature of the recombinant strain it is possible to reduce the ethanol production cost because of lower centrifugation and distillation costs.

1. Introduction

Bio-ethanol fermentation can be improved by media and process optimization and selection of robust microorganisms (Pereira et al., 2010a,b). One of the process strategies currently applied in the fermentation industry is very high gravity (VHG) technology. Compared to conventional fermentations, this approach decreases process water requirements and increases overall plant productivity and final ethanol concentrations (usually above 15%, v/v), and thus allows considerable savings in energy for distillation (Li et al., 2009). However, high osmotic stress and ethanol inhibition affect the microorganism and result in incomplete fermentations. *Saccharomyces cerevisiae* strains are the most frequently used microorganisms for large-scale industrial bio-ethanol fermentations due to their ability to ferment a wide range of sugars and to deal with the harmful VHG stresses (Bai et al., 2008). *S. cerevisiae* CA1185 and PE-2 are industrial strains exhibiting high fermentation performance and stress tolerance under VHG conditions (Pereira et al., 2011). PE-2, isolated from a Brazilian sugar cane-to-ethanol distillery and now intensively used for bio-ethanol production in Brazil, consumed 330 g glucose/L achieving 19.2% (v/v) ethanol, using a VHG optimized medium (Pereira et al., 2010a).

The application of a repeat-batch system with yeast recycling achieves high ethanol productivities (Choi et al., 2009; Li et al., 2009; Ma et al., 2009); however, the need for separating the fermented medium from cells at the end of each batch by filtration or centrifugation (Bai et al., 2008; Sakurai et al., 2000) makes the process expensive. A yeast flocculation–sedimentation process would appear to be able to reduce the cost of the repeat-batch process (Li et al., 2009).

Flocculation is already utilized in a variety of applications such as winemaking (Govender et al., 2010), brewing (Van Mulders et al., 2010) or wastewater treatment (Liu et al., 2009). Wang et al. (2008) transferred a vector harboring the *FLO1* gene into *S. cerevisiae* and Verstrepen et al. (2001) and Zhao et al. (in press) integrated the gene into the yeast's genome to introduce a flocculation phenotype. *FLO1* is the most studied gene related to flocculation (Russell et al., 1980). It encodes a large flocculation protein rich in Ser/Thr amino acids (Watari et al., 1994) and when the protein is incorporated into the cell wall, its N-terminal part has the capacity to selectively bind mannose-sugars present on the wall of other cells (Bidard et al., 1995). The binding efficiency depends on several factors such as pH, temperature or the amount of specific components (e.g. cations, sugars, oxygen, ethanol) (Soares, 2011).

In the present work, *FLO1*-mediate flocculation capacity was introduced into the robust industrial strain, *S. cerevisiae* PE-2, and the recombinant strain was exploited in a VHG repeat-batch system.

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2. Methods

2.1. Yeasts

S. cerevisiae PE-2 (Basso et al., 2008) was maintained at 4 °C on YPD [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose and 2% (w/v) agar].

2.2. Construction of flocculent strains

2.2.1. Plasmid construction

Plasmid pET13.1, an 11.6-kb shuttle vector that contains sequences from plasmid pBR322 as well as the 2μ origin of replication for *S. cerevisiae*, the yeast gene *LEU2* and two copies of the yeast copper resistance gene *CUP1* (Henderson et al., 1985; Penttilä et al., 1987) was used as cloning vector. The restriction site *Hind*III in pET13.1 was used to introduce a cassette consisting of the *PGK* promoter and terminator. The pET13.1 with the *Hind*III*PGK* cassette was named pLD6. The *FLO1* gene with the *ADH1* promoter and terminator was obtained from the plasmid pBR-ADH1-*FLO1* (Watari et al., 1994) by isolation of the corresponding 6.5-kb fragment after digestion with restriction enzyme *Bam*HI. Plasmid pLD7 was obtained by insertion of the 6.5-kb *FLO1* fragment into the *Bam*HI restriction site of pLD6.

2.2.2. Yeast transformation and selection of flocculent transformants

Standard recombinant DNA methods were used. Enzymes were used as recommended by manufacturers. *Escherichia coli* was transformed by electroporation according to protocols from Bio-Rad. DNA plasmid isolation from the recombinant yeast strain was done according to Hoffman and Winston (1987). Vector pLD7 was replicated and isolated from *E. coli* cultures and introduced into PE-2 using the lithium acetate method (Gietz et al., 1992). The selection of transformants was made on NEP agar plates (g/L) (2, $MgSO_4{\cdot}7H_2O$; 2, $(NH_4)_2SO_4$; 3, KH_2PO_4; 0.25, $CaCl_2{\cdot}2H_2O$; 2, yeast extract; 3, peptone; 40, glucose; 20, agar) (Henderson et al., 1985) supplemented with 1.5 mM $CuSO_4$ as selective marker. After 3 d of incubation at 30 °C, cells from 28 colonies with a diameter larger than 1.5 mm were transferred into test tubes containing 5 mL of YPD medium (1% yeast extract, 2% peptone, 2% glucose) supplemented with 1.5 mM $CuSO_4$. The tubes were incubated at 30 °C with 180 rpm for 20 h. Transformants that showed a flocculation phenotype were spread on NEP agar plates supplemented with 1.5 mM $CuSO_4$, allowed to grow at 30 °C for 3 d and then stored at 4 °C.

2.3. Media and fermentations

2.3.1. Inoculum culture

The yeast for inoculation was grown in Erlenmeyer flasks filled to 40% of their volume with medium containing (g/L) 50, glucose; 20, peptone; and 10, yeast extract. The medium for recombinant strains also included 1.5 mM $CuSO_4$. After incubation at 30 °C with 150 rpm for 24 h (OD_{600} of 4–5), the cell suspension was aseptically harvested by centrifugation (10 min at 7500×*g*, 4 °C) and cells were resuspended in ice-cold NaCl 0.9% (w/v) solution to 200 mg fresh yeast (FY)/mL. This concentrated cell suspension was mixed with an appropriate culture medium volume to achieve a cell density of about 1.3×10^8 cells/mL.

2.3.2. Fermentation media

Fermentations were performed in a previously optimized VHG medium (Pereira et al., 2010a), consisting of (g/L) 300–380, glucose; 44.3, corn steep liquor (CSL); 2.3, urea; 3.8, $MgSO_4{\cdot}7H_2O$; and 0.03, $CuSO_4{\cdot}5H_2O$ (corresponding to 0.12 mM Cu^{2+}). The CSL was kindly provided by COPAM (Portugal) and its handling and main composition has been previously reported (Pereira et al., 2010a). The pH of the medium was adjusted to 4.5 with 1 M NaOH. The medium was aerated by stirring with a magnetic bar (length of 3 cm) at >850 rpm for 20 min before inoculation to achieve an oxygen concentration of >95% saturation.

2.3.3. Shake-flask fermentations

Shake-flask fermentations were performed in 100 mL Erlenmeyer flasks fitted with perforated rubber stoppers with glycerol-filled air-locks to permit CO_2 exhaustion while avoiding the entrance of air. The cultures were incubated at 30 °C with 150 rpm orbital agitation.

2.3.4. Repeat-batch system in shake-flasks

Repeat-batch operation was initiated with the inoculation of 11 mg FY/mL (approximately 1.3×10^8 cells/mL) into 40 mL of fermentation medium. At the end of the first fermentation cycle, the yeast cells were allowed to sediment (in a 50 mL tube) for 10 min and then a volume of upper liquid, corresponding to 90% of the working volume, was collected. The remaining liquid containing the majority of the yeast cells was diluted in fresh aerated medium to a final volume of 40 mL. This procedure for biomass recycling was repeated in the following cycles. The progress of fermentation was followed by mass loss (resulting from CO_2 production) and samples for analyses were taken at the beginning and end of fermentation. At each time-point, the standard deviation between replicates was less than 6% of the average value for the CO_2 production.

2.3.5. Repeat-batch system in an air-lift reactor

Bioreactor fermentations were performed in an air-lift reactor (internal recirculation type through a concentric draft tube with an enlarged degassing zone; made in house) with a working volume of 6.1 L (Klein et al., 2005). The reactor was equipped with a cooling jacket that allowed the temperature control at 30 ± 1 °C and a condenser in the top to avoid ethanol evaporation. Agitation was provided by sterile air entering at the base of the reactor at a flow rate of 0.1 vvm. For repeat-batch operation, the yeast biomass was recycled by a flocculation–sedimentation process. At the end of each batch, the aeration was stopped for 15 min, allowing the flocculated cells to sediment at the bottom of the bioreactor. The clarified medium was then removed through the feed/outlet port, leaving a remaining volume of approximately 600 mL (corresponding to a recycling rate of 10%). The aeration was restarted and fresh medium was fed to the bioreactor to start the subsequent fermentation run. This procedure was repeated during ten consecutive batches. Initial batch was inoculated with a 200 mg FY/mL suspension to obtain 11 mg FY/mL (about 1.3×10^8 cells/mL). Fermentation monitoring was performed using the DNS method (Miller, 1959) for reducing sugars quantification.

2.4. Analytical procedures

2.4.1. Determination of yeast concentration and viability

For fresh and dry yeast mass determinations, a sample of the fermentation broth (20 mL) was centrifuged for 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 again to give the fresh yeast (FY) mass. Then, the yeast pellet was washed with 20 mL of distilled water, centrifuged, dried over-night at 105 °C and the tube was finally weighed to give the dry yeast (DY) mass. The methylene-blue staining method (Mills, 1941) was used to count viable and non-viable cells using a microscope and improved Neubauer counting chamber. Yeast viability was calculated as the ratio of viable (non-stained) and total cells counts.

2.4.2. Extracellular compounds – glucose, glycerol and ethanol

A sample of fermentation medium was centrifuged (10 min, 4800×*g*, 4 °C) and the supernatant was stored at −20 °C. Specified compounds were quantified by HPLC (High-Performance Liquid Chromatography), using a Varian MetaCarb 87H column eluted at 60 °C with 0.005 M H_2SO_4 at a flow rate of 0.7 mL/min, and a refractive-index detector.

2.4.3. Intracellular compounds – trehalose, glycerol and sterols

After centrifugation (10 min at 4800×*g*, 4 °C) the yeast pellet was washed with ice-cold NaCl 0.9% (w/v) and resuspended in the same solution to a concentration of 200 mg FY/mL. From this suspension, duplicate samples were taken for the determination of trehalose, glycerol and sterols. For trehalose and glycerol determination, 0.5 mL samples were boiled in a water bath for 5 min and stored frozen (−20 °C). After thawing, trehalose and glycerol were quantified in the extracts by HPLC using the same conditions as described in Section 2.4.2. Sterols were determined after centrifugation of 0.5 mL samples (10 min at 4800×*g*, 0 °C). The supernatant was completely removed and the tubes were subjected to a clean nitrogen flow to expel air from the headspace and stored at −80 °C. Samples were incubated at 80 °C for 90 min with 5 mL alkaline aqueous ethanol (3.6 M KOH in 80% v/v ethanol) in screw-cap tubes. After an extraction process with pentane, the sterol quantification was performed by a spectrophotometric assay using a filipin complex (Sigma F9765) (Rowe et al., 1991). 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).

2.4.4. Flocculation assay

Yeast's flocculation capacity was evaluated by a flocculation assay, previously described by Soares et al. (1992), with slight modifications. Briefly, yeast cells were washed twice with NaCl 15 g/L (pH 3) deflocculating solution and a 25 mL volume of cell suspension was placed in a 25 mL graduated cylinder. Then, 1 mL of 100 mM $CaCl_2$ was added to induce flocculation. Samples (0.2 mL) were taken every 2 min from the same position in the graduated cylinder and the absorbance was measured at 600 nm. An assay without $CaCl_2$ served as control. Sedimentation profiles were obtained by plotting the percentage of yeast cells in suspension for each sample point.

2.5. Determination of fermentation parameters

Ethanol conversion yield was calculated as the ratio of the final ethanol concentration and the initial glucose concentration (1) and was expressed as a percentage (%) of the theoretical conversion yield, i.e. the yield considering a production of 0.511 g of ethanol/g of glucose:

$$Y_n = \frac{\frac{P_{f,n}}{S_{i,n}}}{0.511} \times 100 \quad \text{for the cycle } n \tag{1}$$

Ethanol productivity was defined as the ratio between final ethanol concentration and fermentation time (2):

$$q_n = \frac{P_{f,n}}{t_{f,n}} \tag{2}$$

3. Results and discussion

The overall strategy presented on this study consists in two distinct phases: incorporation of the flocculation capacity into industrial *S. cerevisiae* PE-2 strain; application of one of the transformants in a repeat-batch system where yeast could be recycled by flocculation.

3.1. Yeast transformation and selection of flocculent transformants

Of the 28 colonies originally picked from the selection medium, only two (FL20 and FL 21) showed a flocculation phenotype (Supplementary Data, Fig. S1). The two strains harbored plasmid pLD7 (Fig. 1). When grown in YPD without added copper, these transformants lost the flocculation ability after 5 consecutive cultivations. This loss was accompanied by a loss of the plasmid.

3.2. Fermentation performance of FL20 and FL21

FL20 and FL21 produced similar amounts of ethanol as the parental strain PE-2 (Table 1). For instance, with an initial glucose concentration of 293 g/L, PE-2 strain was able to produce 141 ± 5 g ethanol/L. With a very similar amount of glucose (292 g/L), FL20 and FL21 strains produced 139 ± 3 and 140 ± 2 g ethanol/L, respectively. This fact indicates that transformation procedure did not affect their capacity to attain high ethanol concentrations. In terms of ethanol productivity, PE-2 and flocculent strains presented values ranging from 2.34 to 2.63 g/L/h without considerable differences between PE-2 and recombinant strains. Moreover, the CO_2 production profiles of the recombinant strains were very similar comparing to those of PE-2 (data not shown). Only slightest differences in the biomass concentrations were detected between strains with values ranging from 8 to 10 g (DCW)/L, and no significant differences in the ethanol theoretical yields (90–94%) were found.

3.3. Repeat-batch fermentation with flocculent strains in shake-flasks

The performance of recombinant strain FL20 over 5 cycles is summarized in Table 2 and CO_2 profiles are presented in Fig. 2. The CO_2 production profiles suggest similar fermentation kinetics and efficiencies during the first 3 cycles. In the 4th cycle, a reduction in glucose consumption rate was observed (Table 2). A possible explanation may be the reduced viability of the cells in that cycle, meaning less cells fermenting. The reduced viability at the end of the 5th cycle can be explained by the fact that this cycle was prolonged after total glucose exhaustion. In all cycles, except cycle 4, 129–136 g ethanol/L was produced within approximately 47 h (Table 2) and the final ethanol productivity ranged from 2.71 to 3.24 g/L/h. The average ethanol productivity and final ethanol concentrations are identical to those obtained with PE-2 over

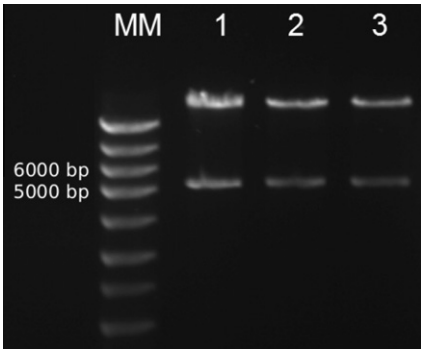

Fig. 1. Agarose gel electrophoresis of digested pLD7 and the plasmid isolated from the recombinant yeasts FL20 and FL21. Lanes: 1 pLD7 used for yeast transformation digested with *Bam*HI; 2, 3 plasmid isolated from *S. cerevisiae* FL20 and FL21 strains, respectively, and recloned in *Escherichia coli* TOP10 (Invitrogen) digested with *Bam*HI; MM size marker (NZYDNA Ladder III, NZYTech).

Table 1
Fermentation kinetic parameters of *S. cerevisiae* PE-2 (parental strain) and recombinant flocculent strains in single batch VHG fermentations. Values are average ± range of at least 2 independent biological replicates.

Strain	Initial glucose (g/L)	Final ethanol (g/L)	Residual glucose (g/L)	Ethanol productivity (g/L/h)	Final biomass (g_{DCW}/L)	Ethanol theoretical yield (%)
PE-2	293.4 ± 0.0	141.2 ± 4.7	0.0 ± 0.0	2.34 ± 0.08	8.4 ± 0.2	94 ± 3
	323.0 ± 0.9	149.0 ± 1.0	0.0 ± 0.0	2.37 ± 0.13	9.7 ± 0.8	90 ± 2
FL20	291.7 ± 0.0	138.7 ± 2.7	0.9 ± 0.0	2.62 ± 0.05	10.1 ± 0.2	93 ± 2
	321.6 ± 0.0	152.2 ± 1.5	0.0 ± 0.0	2.38 ± 0.02	8.3 ± 0.3	93 ± 1
FL21	291.7 ± 0.0	139.5 ± 1.5	1.0 ± 0.0	2.63 ± 0.03	9.9 ± 0.3	94 ± 1
	321.6 ± 0.0	153.4 ± 2.5	1.6 ± 0.1	2.40 ± 0.03	9.9 ± 0.3	93 ± 2

Table 2
Fermentation kinetic parameters of the flocculent strain FL20 in a repeat-batch system performed in shake-flask during 5 cycles. Values are average ± range of two independent biological replicates.

Cycle	Fermentation time (h)	Final ethanol (g/L)	Residual glucose (g/L)	Ethanol productivity (g/L/h)	Final viability (%)	Ethanol theoretical yield (%)
1	42.0	135.9 ± 1.4	0.0 ± 0.0	3.24 ± 0.03	61 ± 5	80 ± 1
2	47.3	130.4 ± 0.2	14.2 ± 2.4	2.76 ± 0.00	62 ± 7	81 ± 0
3	47.6	128.8 ± 3.0	0.6 ± 0.3	2.71 ± 0.06	54 ± 3	77 ± 2
4	48.0	107.8 ± 2.2	48.0 ± 0.9	2.25 ± 0.05	48 ± 6	67 ± 1
5	47.6	135.3 ± 1.6	0.0 ± 0.0	2.84 ± 0.03	20 ± 7	73 ± 1

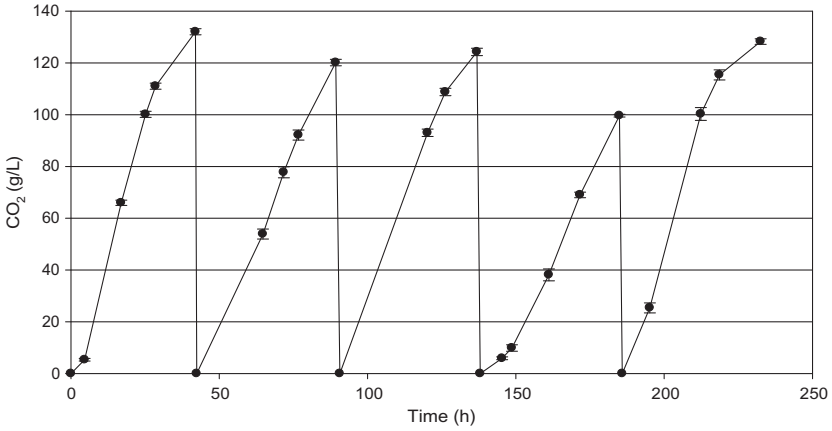

Fig. 2. CO_2 production profiles of *S. cerevisiae* FL20 recombinant strain in a repeat-batch system (5 cycles) using a VHG optimized medium (320 g glucose/L).

15 batch cycles with the yeast recovered by centrifugation (Pereira et al., 2012). The final ethanol concentration is however slightly lower than those obtained with a single batch system (73–81% vs. 90–94% for the ethanol theoretical yield).

In repeat-batch fermentation with the recombinant FL20 strain, the bio-ethanol production system was simplified due to cells flocculation ability (centrifugation was eliminated with the consequent reduction in capital and maintenance costs and energy consumption) and productivity was increased when comparing to single batch with the same yeast strain. Productivity increase comes from the reduction on the time required for cell growth, therefore increasing initial fermentation rate that is considerably lower for the single-batch systems. In the present work, an average ethanol productivity of 2.38 g/L/h was obtained when FL20 was used in a single batch fermentation (322 g glucose/L), this value being smaller than the 2.76 g/L/h obtained for the repeat-batch operation in shake-flasks. Also, yeast populations can become more robust in such type of operation when comparing to single batch system as the inoculum comes from cells that have already been physiologically adapted, in the previous cycle, to the environmental stress of the fermentation culture. In a natural way, cells with higher tolerance to environmental stress will be those that will originate new cells, from one cycle to another (Silva-Filho et al., 2005).

3.4. Repeat-batch fermentations with FL20 in an air-lift reactor

Profiles of glucose consumption and ethanol production are illustrated in Fig. 3 and parameters related to yeast performance are presented in Table 3. The fermentation medium was saturated with oxygen before yeast inoculation, but after a few hours, the dissolved oxygen concentration was near zero and remained close to zero throughout the process. A constant fermentation rate during most of the cycles (except 3rd and 10th cycle) was observed with fermentation times between 44 and 48 h. The final ethanol concentration ranged from 135 to 150 g/L, with an average value of 142 ± 5 g/L. During 3 cycles, all the initial glucose was consumed, and residual glucose concentrations for the remaining cycles were 19 to 58 g/L (average of 40.5 ± 14.6 g/L). The theoretical ethanol yield was always higher than 78%, with an average of 86%.

Values for ethanol productivity ranged from 2.00 to 3.31 g/L/h with an average value of 2.86 g/L/h, the highest values being obtained for the 1st, 4th and 8th cycles. The final biomass concentration showed a considerable variation between cycles ranging from 12.35 g/L at the 1st cycle, to a maximum of 27.66 g/L on the 4th cycle. Differences in cell viability were observed. At the end of the 1st batch viability was around 80%, but dropped to 54% at the end of the 2nd batch. Thereafter, viability increased again and reached 80%, close to the initial levels. These variations were not correlated

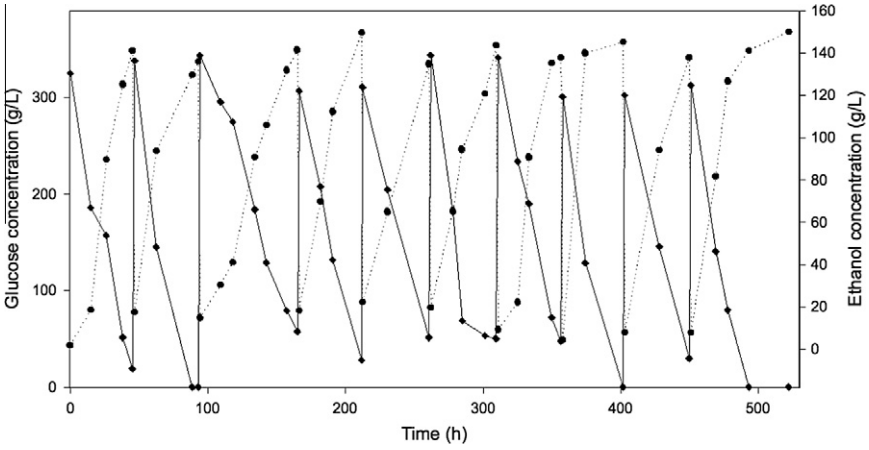

Fig. 3. Profiles of ethanol (●) and glucose (♦) concentration during a succession of 10 repeated batches, performed in an air-lift bioreactor.

Table 3
Fermentation kinetic parameters of the flocculent strain FL20 in a repeat-batch system performed in an air-lift bioreactor during 10 consecutive runs (n.d. – not determined).

n	t_f (h)	S_i (g/L)	S_f (g/L)	P_f (g/L)	q (g/L/h)	V (%)	Y (%)	X_f (g_{DCW}/L)	pH	G_i (mg/g_{DY})	T (mg/g_{DY})	TS (mg/g_{DY})
1	45.3	324.9	19.2	141.2	3.11	80.9 ± 3.47	85	12.35	4.5	5.72	91.94	12.02
2	46.3	337.8	0.0	136.1	2.94	54.6 ± 8.86	79	n.d.	5.0	n.d.	n.d.	9.91
3	70.7	343.2	57.4	141.3	2.00	60.2 ± 4.26	81	20.78	4.9	4.88	43.61	15.68
4	45.5	306.6	27.9	149.6	3.29	67.5 ± 7.21	95	27.66	4.9	10.43	41.74	8.29
5	48.3	310.7	51.3	134.6	2.79	62.8 ± 1.83	85	18.68	4.8	18.15	67.34	20.89
6	47.5	343.7	50.2	143.7	3.03	70.9 ± 5.66	82	18.39	5.1	16.18	30.67	9.62
7	45.7	341.3	47.6	137.9	3.02	74.9 ± 2.80	79	12.36	4.8	n.d.	42.97	9.03
8	43.9	300.6	0.0	145.2	3.31	77.3 ± 2.54	95	21.74	6.0	15.46	33.20	12.76
9	46.8	302.2	29.9	137.9	2.95	79.1 ± 1.60	89	18.00	5.1	14.08	41.88	9.03
10	71.1	312.5	0.0	150.0	2.11	73.6 ± 3.44	94	14.23	5.8	5.36	35.04	10.21

n – cycle number, t_f – fermentation time, S_i – initial glucose concentration, S_f – residual glucose concentration, P_f – final ethanol concentration, q – ethanol productivity, V – final viability, Y – percentage of ethanol theoretical yield, X_f – final biomass concentration, G_i – final intracellular glycerol concentration, T – final trehalose concentration, TS – final total sterols concentration.

with the levels of intracellular glycerol, trehalose and total sterols, despite a close association between these compounds and cell viability (Pereira et al., 2011). During the first 3 runs, the final glycerol concentrations were lower than 6 mg/g_{DY} and from the 4th cycle on, they ranged from 10 to 18 mg/g_{DY}. Final trehalose concentrations varied between 30 and 67 mg/g_{DY} (average of 42.06 ± 11.34 mg/g_{DY}), from the 3rd to the 10th cycle, which is considerably lower than the amount obtained for the 1st cycle (91.94 mg/g_{DY}). The total sterol content in the cells showed a low variability, ranging from 8 to 13 mg/g_{DY}. Exceptionally, at the end of 3rd and 5th cycles, total sterols content was 15.68 and 20.89 mg/g_{DY}, respectively.

Industrial strains present a higher robustness relatively to the main stresses that arises from VHG practice (Bai et al., 2008; Pereira et al., 2011). In this study, the yeast viability along the consecutive fermentations was higher than 60%, even with a constant exposition of cells to high ethanol concentrations. Additionally, the levels of intracellular glycerol, trehalose and total sterols were very close to those obtained in a previous work with PE-2 strain in a single batch system (Pereira et al., 2011). The maximum glycerol concentration achieved in the first cycle was 64 mg/g_{DY} at 15 h of fermentation, and at the end of the cycle (ca. 45 h) this parameter dropped to 5.72 mg/g_{DY} (data not shown). This is consistent with data obtained with the PE-2 strain in a single batch system where a maximum of approximately 42 mg/g_{DY} at 12 h was obtained, decreasing afterwards to approximately 6 mg/g_{DY} at 48 h. The final intracellular glycerol concentration for the remaining cycles (with the exception of 3rd and 10th cycles) was considerably higher with an average of 14.86 mg/g_{DY}. It is possible that the prolonged cell's exposition to a high-solute environment in the 3rd cycle could act as an effective selection process for the more resistant cells in terms of osmotic stress therefore suggesting a possible enhancement of cells robustness over the entire process (Kaino and Takagi, 2008). Relatively to the trehalose levels, they also presented a similar behavior compared with the previously reported data for the PE-2 strain in a single batch system. It was observed that a maximum level of 91.94 mg/g_{DY} was achieved around 45–48 h of fermentation, corresponding to the end of the first cycle (data not shown). The final concentration on the remaining cycles was considerably lower comparatively to the first batch, which could be caused by a higher cell growth that occurs in the first cycle, a relation already reported by Devantier et al. (2005). Other explanation can be the fact that cells from the 1st cycle came from a pre-growth procedure, being collected at the beginning of stationary growth-phase, the point where storage compounds are maximal. After the 2nd cycle, it was observed a drastically decrease on trehalose accumulation which seems to be associated to a severe drop on cells viability. Finally, concerning the levels of total sterols, no increase was verified along the several cycles despite the reasonable increase registered on yeast cell viability. The association between sterol content of cell membrane and cell viability was widely studied by several authors (e.g. Daum et al., 1998), nevertheless no influence was detected for this parameter on these process conditions.

This work describes and evaluates innovative techniques for bio-ethanol production at different scales, with a particular relevance to the use of flocculation associated to a highly productive repeat-batch fermentation system. The relevance of this comes

from the fact that, despite the large number of studies that refer the repeat-batch system, the yeast strain used does not correspond to a high-ethanol production strain (Choi et al., 2009; Horiuchi et al., 2000; Kida et al., 1992; Li et al., 2009; Ma et al., 2009; Silva et al., 2010; Wang et al., 2008). In our work, the industrial and high-ethanol tolerant *S. cerevisiae* PE-2 strain was used (Pereira et al., 2010b).

After a succession of ten cycles, FL20 strain produced an average of 142 ± 5 g ethanol/L. Choi et al. (2009) using the flocculent *S. cerevisiae* CHFY0321 achieved an average of only 84.5 g ethanol/L, using a medium with approximately 177 g glucose/L and Ma et al. (2009), using the *S. cerevisiae* ATCC24860, obtained an average ethanol concentration of 77 g ethanol/L. The closest values to those obtained in the present work were achieved by Li et al. (2009) that, using a medium with 264 g glucose/L, produced an average of 119.5 g ethanol/L, which still is 15.6% lower than our ethanol concentration values. The achievement of these outstanding ethanol concentrations in successive batch fermentations does not follow the most common approach, which consists on enhancing ethanol productivity by using lower initial sugar concentrations and consequently attaining shorter fermentation processes. The present work is focused on the production of very high ethanol concentrations using high-sugar concentration media, which leads to prolonged fermentations, resulting consequently in a decrease on ethanol productivity. Nevertheless, 2.86 g/L/h ethanol productivity was achieved in the described conditions and could still be improved through the reduction of the residual glucose concentration (average of 28.4 g/L; Table 3). By doing this we would not only get an improvement of productivity but also a reduction of the pollution load, associated to sugar content in the fermented broth, and thus an increase of the ethanol conversion yield, which means less amounts of unfermented glucose as well as a reduction in distillation costs.

Bayrock and Ingledew (2001) as well as Wang et al. (2007) also achieved an average ethanol concentration of 132 and 136 g/L, respectively. Although these values are comparable with the ones obtained in the present work, both works present some serious constraints. In the first one, ethanol productivity was only 1.16 g/L/h (continuous system) and in the second one the fermentation medium had an expensive nutritional supplementation (peptone).

3.5. Stability of yeast flocculation

Considering that flocculation was granted through the introduction of a plasmid into the cell, it is possible that this phenotype could suffer specific variations caused by a partial loss of the plasmid. Since selective pressure by copper is necessary for the retention of the plasmid in the culture, but can also have a negative effect on the yeasts' fermentative capability, different $CuSO_4$ concentrations were explored. For the lower copper concentrations (0.12 and 0.62 mM) no effect on the fermentation ability was detected while for the higher copper concentration (1.12 mM) an ethanol production decrease of ca. 3% was observed. Taking these results into account, we chose to use copper in a lower concentration (0.12 mM) because we wanted to use the previous optimized fermentation medium and we would also select for the flocculent cells by sedimentation. In the presence of this amount of copper, the yeast cultures flocculated consistently during the 10 cycles in the air-lift reactor. Results of flocculation assays performed at 3 different points are presented in Fig. 4. The sedimentation profiles show that cells lost some of their flocculation capacity over time. Yeast cells collected at the end of inoculum culture presented a flocculation rate of 93.7%, what is expectable since the number of cell divisions until then was relatively small and the inoculum culture medium contained the selective marker (1.5 mM $CuSO_4$). For the two other points (5th and 10th cycle), where cells were collected from the fermentation medium, the situation was considerably different because cells have been subjected to more cell divisions and the fermentation medium does not have copper in the required concentration to act as selective pressure. Also, we must consider the similarities of these 2 points (5th and 10th cycle) despite the time difference between them. The reason for that could be the low rate of growth after the 4th cycle, as could be seen through the variation of final dry weight (Table 3). Increasing the copper content in the medium could be a good option to reduce plasmid loss, and consequently the variations in flocculation. The use of a 0.62 mM $CuSO_4$ concentration would allow for an improvement on flocculation with no effects on fermentation performance. Another solution can be the integration of the gene into the genome of the strain PE-2. Nevertheless, for ten consecutive cycles the yeast flocculation ability was adequate for recovering yeast cells by sedimentation.

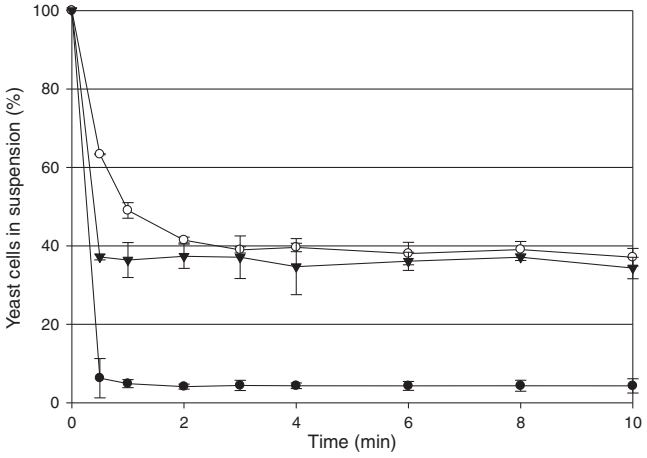

Fig. 4. Sedimentation profiles of FL20 flocculent strain obtained by the method of Soares et al. (1992), at 3 different points of the overall process performed on the airlift: end of inoculum culture (●); middle of 5th cycle (○); end of 10th cycle (▼).

4. Conclusions

The introduction of a plasmid-borne flocculation gene into industrial strain *S. cerevisiae* PE-2 resulted in a flocculating strain that achieved average ethanol concentrations and final productivities similar to those of the parental strain. The flocculating strain FL20 was studied in a repeated-batch system and applied with high success for 10 consecutive fermentations, with yeast cells recycled over the entire process. An average ethanol concentration of 142 g/L was obtained, with a final productivity of 2.86 g/L/h. The amounts of specific intracellular metabolites confirmed an efficient response to VHG stress by the flocculent strain, similar to previous reports for the parental strain in single batch fermentation. Since loss of the plasmid and concomitant loss of flocculation capability under non-selective conditions were observed, it might be necessary to stably integrate the flocculation gene into one of the yeast's chromosomes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.12.089.

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