RESEARCH ARTICLE

Yeast selection for fuel ethanol production in Brazil

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Abstract

Brazil is one of the largest ethanol biofuel producers and exporters in the world and its production has increased steadily during the last three decades. The increasing efficiency of Brazilian ethanol plants has been evident due to the many technological contributions. As far as yeast is concerned, few publications are available regarding the industrial fermentation processes in Brazil. The present paper reports on a yeast selection program performed during the last 12 years aimed at selecting *Saccharomyces cerevisiae* strains suitable for fermentation of sugar cane substrates (cane juice and molasses) with cell recycle, as it is conducted in Brazilian bioethanol plants. As a result, some evidence is presented showing the positive impact of selected yeast strains in increasing ethanol yield and reducing production costs, due to their higher fermentation performance (high ethanol yield, reduced glycerol and foam formation, maintenance of high viability during recycling and very high implantation capability into industrial fermenters). Results also suggest that the great yeast biodiversity found in distillery environments could be an important source of strains. This is because during yeast cell recycling, selective pressure (an adaptive evolution) is imposed on cells, leading to strains with higher tolerance to the stressful conditions of the industrial fermentation.

Introduction

Energy crises and environmental concerns are making bioethanol an attractive renewable fuel source. Fuel bioethanol for transportation has received considerable attention in Brazil, mainly after the first oil crisis of 1973, leading the country to launch a national program to replace part of gasoline by ethanol (Amorim & Leão, 2005). Since then, ethanol production has greatly increased, making Brazil the largest producer and exporter for many decades (Richard, 2006). Recently, however, Brazil has been surpassed in terms of volumetric bioethanol production by the United States (Valdes, 2007), reaching 21 billion liters per year during the 2007/2008 crop season (Oliveira *et al.*, 2007). In the United States, most of the ethanol is produced from corn, amounting to 24 billion liters in 2007 (RFA, 2008), with an estimated energy balance of 1.3 (units of energy from the produced ethanol per 1 unit of energy used for its production). On the other hand, Brazil produces ethanol from sugar cane with an estimated energy balance of 8.0 for this particular crop (Leite, 2005). In Brazil, nearly all the ethanol produced is used as biofuel, today representing $>40\%$ of total gasoline consumed in the country. Scientific and technological advances, for example, regarding sugar cane varieties, agricultural and fermentation process management and engineering have led to increased efficiency in Brazilian distilleries. The benefits of suitable *Saccharomyces cerevisiae* strains for bioethanol fermentations have been less visible.

The Brazilian fermentation process is quite unusual in that yeast cells are intensively recycled ($>90\%$ of the yeast is reused from one fermentation to the next), resulting in very high cell densities inside the fermenter (10–17% w/v, wet basis), which contributes to a very short fermentation time.

Sugar cane juice and molasses (in varying proportion) are used as substrates, and ethanol concentrations of 8–11% (v/v) are achieved within a period of 6–11 h at 32–35 °C. After fermentation, yeast cells are collected by centrifugation, acid washed (the yeast slurry is treated with diluted sulfuric acid at pH 2.0–2.5 for 1–2 h, in order to reduce bacterial contamination) and repitched, comprising at least two fermentation cycles per day during a production season of 200–250 days (Wheals *et al.*, 1999). High cell densities, cell recycling and high ethanol concentration, all contribute to reduced yeast growth, which in turn leads to high ethanol yields (90–92% of the theoretical sugar conversion into ethanol).

Ethanol plants in Brazil traditionally use baker's yeast as starter cultures, because of its low cost and availability at the required amount. For example, some distilleries start with 1–12 tons of pressed baker's yeast. However, we have shown previously (Basso *et al.*, 1993) by karyotyping analyses of yeast samples from five distilleries that baker's yeast and two other *S. cerevisiae* strains used (TA and NF) were unable to compete with indigenous (wild) yeasts that contaminated the industrial processes. It was also observed that only a wild strain (JA-1) previously isolated from one distillery could survive the recycling process, and in most of the distilleries a succession of different indigenous *S. cerevisiae* strains performed the industrial fermentation (Basso *et al.*, 1993). More recently, Silva-Filho *et al.* (2005) demonstrated, by PCR-fingerprinting of yeast samples from six distilleries, that indigenous strains could be more adapted to the industrial process than commercial ones, also identifying dominant strains.

The reason why such starter yeast strains were unable to survive could be due to the stressful conditions imposed by industrial fermentation. High ethanol concentration, high temperature, osmotic stress due to sugar and salts, acidity, sulfite and bacterial contamination are recognized stress conditions faced by yeast during the industrial processes (Alves, 1994), some of them acting synergistically (Dorta *et al.*, 2006) and particularly with cell recycling.

Additional factors can act upon yeast, affecting fermentation performance, such as the presence of toxic levels of aluminum and potassium in the medium. The acidic condition of fermentation renders aluminum (absorbed by sugarcane in acid soils) in its toxic form (Al^{3+}), reducing yeast viability, cellular trehalose levels and fermentation rate, with negative impacts on ethanol yields (Basso *et al.*, 2004). The toxic effects of aluminum can be partially alleviated by magnesium ions and completely abolished in a molasses-rich medium, suggesting the presence of chelating compounds in this substrate (Basso *et al.*, 2004). High potassium levels, especially in molasses, also exert a detrimental effect on yeast performance (Alves, 1994).

In this paper, a strategy for suitable yeast strain selection for Brazilian bioethanol fermentations is presented. This strategy uses the industrial process itself with cell recycling in order to impose selective pressure on the indigenous yeast population. *Saccharomyces cerevisiae* strains with prevalence and persistence in industrial fermentations were selected for improved fermentation performance.

Materials and methods

Yeast strains

Nearly 350 different *S. cerevisiae* strains were analyzed in this work. From these, 24 strains were introduced in industrial fermentations, and their designation and origin are presented in Table 1.

Table 1. Yeast strains introduced in industrial fermentations, their designation and origin

Strains and designation	Origin
Baker's yeast	Three commercial brands (Fleischmann, Itaiquara and Mauri)
IZ-1904	Biological Science Department, Esalq/Usp, Piracicaba, Brazil
TA	Genetics Department, Esalq/Usp, Piracicaba, Brazil
BG-1, CR-1, SA-1	Copersucar, Brazil (isolated from ethanol plants in 1993)
M-26	Unesp, Assis, Brazil (isolated from ethanol plant)
Y-904	Mauri, Brazil (distilling yeast)
PE-2, PE-5, VR-1, BR-1	Isolated in this work from ethanol plants in 1994
BR-2, ME-2, VR-2	Isolated in this work from ethanol plants in 1995
MA-3	Isolated in this work from ethanol plants in 1996
MA-4	Isolated in this work from ethanol plants in 1997
CAT-1, CB-1, NR-1	Isolated in this work from ethanol plants in 1998
BT-1	Isolated in this work from ethanol plants in 2000
AL-1	Isolated in this work from ethanol plants in 2002

Karyotyping of yeast isolates from distillery samples

Depending on the season, 20–78 distilleries were engaged in this study and most of them, except 2 (in North–Northeast region), were located in Brazilian Centre-Southern region, where $>90\%$ of the Brazilian ethanol is produced (comprising the States of São Paulo, Minas Gerais, Paraná and Mato Grosso do Sul). Yeast samples (amounting to 1160 during the 1993–2005 period) from distilleries were aseptically collected after the centrifugation step and plated on YPD agar medium (yeast extract 10 g L^{-1}; peptone 20 g L^{-1}; glucose 20 g L^{-1}; agar 20 g L^{-1}) supplemented with chloramphenicol and tetracycline (100 μg mL^{-1} each) to suppress bacterial contaminants. After growth at 28–30 °C for 48 h (or 4–5 days in case of reduced growth yeasts), a small portion (1–4 mg of wet weight) of 11 isolated colonies from each sample (picked up randomly and representing all the biotypes observed) were individually treated according to the protocol of Blondin & Vézinhet (1988). Pulsed-field gel electrophoresis (PFGE) was performed as a transverse alternating-field electrophoresis (TAFE) version using a Gene-Line (Beckman) instrument set at 170 mA for 4 h and 4-s pulses, followed by 150 mA for 18 h and 60-s pulses. The CHEF-DRIII (BioRad) was also used with three running blocks at 6 V cm^{-1}: 1 h with 5 s pulses, 8 h with 60-s pulses and 12 h with 100-s pulses (Lopes, 2000).

Laboratory fermentations

Three hundred and fifty strains maintained in lyophilized form (reactivated at least every 2 years) were reactivated and propagated at 30 °C in molasses medium diluted to 10% (w/v) total sugar (sucrose, glucose and fructose expressed as hexose content). The medium volume was doubled every 24 h without shaking or supplying air, and yeast biomass was collected by centrifugation (800 ***g*** for 20 min). Fermentation trials were carried out at 33 °C in 150-mL centrifuge vials, simulating the industrial fermentation process as far as possible. A fermentation substrate containing *c.* 20% (w/v) total sugar (composed of cane juice and molasses – 50% of the sugar obtained from each source) was added in three equal portions spaced by 1.5 h upon addition of yeast suspension of a given strain. The yeast suspension (with 33% yeast biomass, w/v wet weight) represented *c.* 30% of the total fermentation volume, similar to industrial conditions. After fermentation, yeast cells were collected by centrifugation (800 ***g*** for 20 min), weighed, treated with sulfuric acid (pH 2.5 for 1 h) and reused in a subsequent fermentation, comprising at least five fermentation cycles. Samples run in triplicate for each strain and for each fermentation cycle, were analyzed for ethanol (steam distillation, followed by densimetry using an AP Paar electronic densimeter DMA48 model), yeast viability (methylene blue staining; Zago *et al*., 1989), glycerol, sucrose, glucose and fructose [high performance chromatography (HPAEC), Dionex DX-300, on a CarboPac PA1 4 × 250 mm column, using 100 mM NaOH as the mobile phase at a flow rate of 0.9 mL min^{-1} and pulsed-amperometric detection]. Yeast cell glycogen and trehalose were also estimated at the beginning of the fermentation assay and at the end of the last cycle. Trehalose was selectively extracted by trichloroacetic acid at 0 °C for 20 min (Trevelyan & Harrison, 1956) and measured by the anthrone reaction (Brin, 1966) or by HPAEC, giving similar results. Glycogen was determined by the Becker method modified according to Rocha-Leão *et al.* (1984).

Aerobic strain propagation, reintroduction into industrial fermentations and yeast population dynamics

The selected strains from the above-mentioned laboratory trials (14 presenting desirable fermentation features) as well as strains from other sources (listed in Table 1, except baker's yeasts) were individually and aerobically cultured (30–32 °C) in molasses medium in a 250-L high oxygen transfer fermenter (Heinrich FRINGS, GmbH). The inoculum originated from 20 L yeast suspension (1.5–2.0% w/v, wet weight) grown in molasses medium with 10% (w/v) sugar, as described previously, but with mechanical stirring (150 r.p.m.). After addition of 20 L sterilized mineral solution (containing 17.4 g K_2HPO_4, 13.2 g $(NH_4)_2SO_4$, 6 g urea, 9.8 g $MgSO_4 \cdot 7H_2O$, 0.6 g $ZnSO_4 \cdot 7H_2O$ and 0.2 g $MnSO_4 \cdot H_2O$) to the fermenter, 20 L sterilized molasses medium (20% w/v total sugar) was pumped at a flow rate of 1 L h^{-1}. The fermenter was supplied with filtered air at a flow rate of 10 m^3 h^{-1}. After feeding, another 40 L mineral solution was added and the fermenter was fed once again with 20 L molasses medium (20% w/v total sugar), resulting in a final 120 L yeast suspension (10–12% w/v, wet weight) with 95–100% cell viability. These selected strains were introduced into industrial fermentations of up to 78 Brazilian distilleries (as is shown in Table 2, depending on the crop season) during 1993–2005, and the strains' population dynamics were monitored by karyotyping of approximately monthly collected yeast samples from distilleries. They were introduced as a mixture of selected strains (0.5 kg wet weight of each strain) with or without baker's yeast (1–12 tons of commercially available pressed yeast). After the 1996 campaign, some selected yeast strains were available in an active dry form and used in larger amounts (10–100 kg per distillery).

Results and discussion

Prevalence of indigenous strains in industrial fermentation

Karyotyping by means of PFGE has proved to be a powerful technique for differentiation between *S. cerevisiae* strains in Brazilian fuel ethanol plants. Nevertheless, the TAFE system was superior when compared with the CHEF system, presenting sharp bands and allowing a better differentiation between strains. All strains used were easily identified by their unique profile (Fig. 1).

When this study was initiated in 1993, baker's yeasts (Fleishmann and Itaiquara) JA-1, TA and IZ-1904 were the available strains, frequently used in distilleries. For the next crop season (1994/95), BG-1, CR-1 and SA-1 strains (isolated by Copersucar, Brazil) also became available. Until August 1993, Fleischmann and Itaiquara baker's yeast karyotype profiles were different from each other, but subsequently these two commercial brands of baker's yeast presented the same karyotype profile, also corresponding to Mauri baker's yeast (data not shown).

Following up some of these strains in industrial fermentations during the first two crop seasons, it became apparent that, in most of the distilleries, baker's yeast was rapidly replaced by wild strains in a period as short as 20–30 days of recycling, while in a few others, this substitution occurred later (50–60 days), in spite of 250 days of recycling during the fermentation season. The same trend occurred with another traditionally used strain, IZ-1904, and with many others that were evaluated as an attempt to improve industrial fermentation (TA and all other brands of baker's

Table 2. Number of distilleries and samples involved in this study in each crop season, as well as the number of karyotyping analysis performed

Crop season	Number of distilleries and samples*	Number of karyotyping analysis	Introduced strains†
1993/1994	20 (43)	473	Baker's(8), TA(10), JA-1(8), IZ-1904(4)
1994/1995	25 (109)	1199	Baker's(5), CR-1(20), JA(20),SA-1 (20), IZ-1904(4)
1995/1996	25 (98)	1078	Baker's(5), PE-2(13), PE-5(11), SA-1(25), VR-1(25), BR-1(2)
1996/1997	30 (130)	1430	Baker's(8), PE-2(28), ME-2(14), SA-1(1), VR-1(28), VR-2(28), BR-2(2)
1997/1998	39 (117)	1287	Baker's(17), CR-1(1), BG-1(7), PE-2(30), SA-1(4), VR-1(30), MA-3(4)
1998/1999	39 (111)	1221	Baker's(16), BG-1(10), CR-1(2), PE-2(25), SA-1(5), Y-904(1), MA-4(4)
1999/2000	37 (130)	1430	Baker's(15), BG-1(11), CAT-1(2), SA-1(5), PE-2(24), VR-1(10), Y-904(1), CB-1(1), NR-1(2)
2000/2001	71 (205)	2255	Baker's(17), BG-1(19), CAT-1(4), CR-1(2), PE-2(41), SA-1(9), VR-1(6)
2001/2002	71 (217)	2387	Baker's(15), BG-1(25), CAT-1(13), CR-1(2), M-26(1), PE-2(53), VR-1(10), Y-904(1), BT-1(3)
2002/2003	70 (205)	2255	Baker's(11), BG-1(18), CAT-1(19), CR-1(1), M-26(1), PE-2(49), Y-904(1)
2003/2004	78 (265)	2915	Baker's(17), BG-1(20), CAT-1(27), PE-2(54), SA-1(6), AL-1(3)
2004/2005	70 (237)	2607	Baker's(12), BG-1(23), CAT-1(24), PE-2(52), SA-1(9)

Also presented here are the strains introduced in industrial fermentations for each crop season, and the number of distilleries in which these strains were introduced. All the distilleries, except two of them, are located in Brazilian Centre-Southern region.

*Number of samples from the distilleries (in parentheses).

†Number of distilleries in which the strains were introduced (in parentheses).

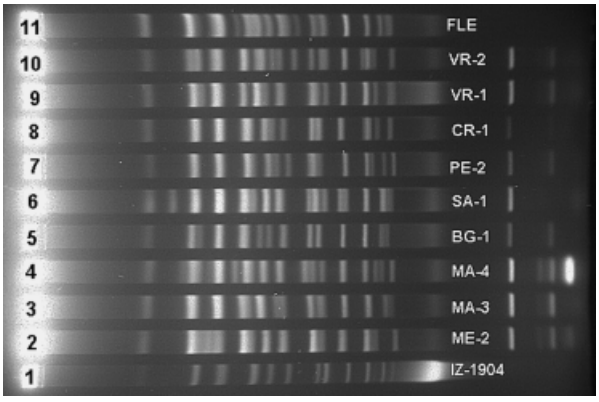

Fig. 1. Karyotyping profiles of some strains used in this work obtained by PFGE as the TAFE system. All strains presented a unique profile. BG-1, CR-1, ME-2, MA-3, MA-4, PE-2, SA-1, VR-1, VR-2 and IZ-1904 strains were isolated from ethanol distilleries; FLE is a baker's yeast strain.

yeast commercially available in Brazil). All these starter cultures were overcome by indigenous yeast strains, mostly *S. cerevisiae*, as predicted by the numbers of bands in the karyotype profile (Vaughan-Martini *et al.*, 1993; Zolan, 1995). During these first two crop seasons, it was observed that only the strains that were previously isolated from the industrial environment (such as JA-1, CR-1 and SA-1) showed longer persistence, being able to establish themselves in industrial fermentations for up to 180–190 days of recycling.

A great biodiversity is found in distilleries

A great biodiversity was observed in industrial fermentations (Fig. 2a), each distillery with its own population, showing a succession of different strains. Prevalent strains were found infrequently (Fig. 2b), and even more rarely – strains with persistence. Prevalent and persistent strains received special attention because this suggested competitiveness and stress tolerance during industrial fermentation, respectively.

Therefore, in the following seasons, the karyotyping analysis was performed not only to follow up introduced strains but also to identify prevalent and persistent indigenous strains.

Unfortunately, most of the indigenous yeast strains, irrespective of their dominance and persistence, showed undesirable fermentation features, such as excessive foam formation, high sedimentation rate (even during fermentation), longer fermentation time and high residual sugar after fermentation. This was demonstrated by a previous screening of 340 different indigenous strains isolated from up to 50 different distilleries during the 1993–2005 crop seasons. These strains were subjected to fermentation trials using industrial substrates (cane juice and molasses) and evaluated for foam formation, flocculation and residual sugar (incomplete fermentation). It was noticed that 67% of the strains produced excessive amount of foam, 33% were flocculent and 53% were not able to metabolize all sugar from the medium, even after fermentation time of 21 h. Unfortunately, some strains presented two or three of these undesirable features. Only 20% of the evaluated indigenous strains did not present such constraints.

High foam-producing strains do not allow the use of fermenters' total capacity and also consume more antifoam products, thus increasing ethanol production costs. When agar-plated, 40% of the indigenous strains showed rough morphology colonies, and within such strains, a great

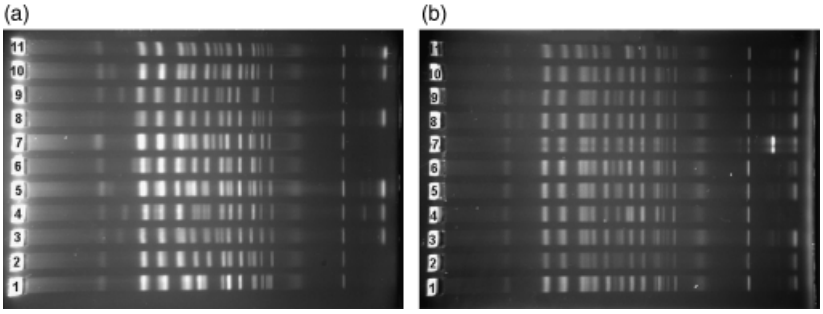

Fig. 2. Karyotyping profiles of yeast population from samples collected in the middle of the crop season, demonstrating the replacement of the starter baker's yeast by a great biodiversity of indigenous strains in one distillery (a). Karyotyping profiles of a yeast population from another distillery presenting a prevalent strain (lines 1–10); minor differences between these lines can be considered as chromosomal rearrangements (b). Numbers refer to 11 isolated colonies from each sample.

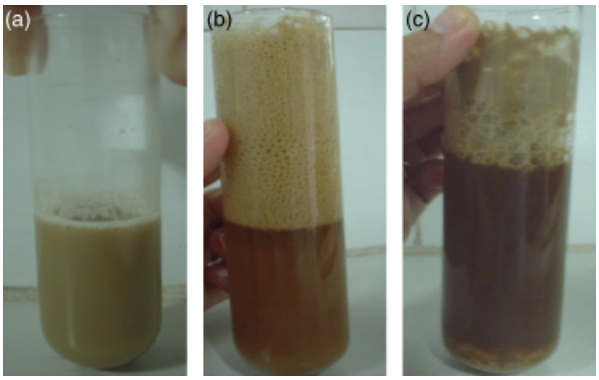

Fig. 3. Industrial substrate fermentation performed by: a selected yeast strain, PE-2 (a); a foam-producing strain (b); and a flocculent foam-producing strain (c). Strain C also presented cell flotation, resulting in low cell density during fermentation.

majority showed undesirable fermentation features. It was noticed that a rough morphology was frequently associated with pseudohyphal growth and a high sedimentation rate during fermentation. Additionally, flocculent or high sedimentation strains impair the centrifugation step of the industrial process. Most of the foam-producing strains also lead to yeast flotation, and both flotation and flocculation (Fig. 3) reduce the yeast contact with the substrate, increasing the fermentation time and resulting in high residual sugar concentration after fermentation. The industrial process as devised in most Brazilian plants operates better with a homogeneous yeast suspension during fermentation. Few ethanol plants in Brazil operate without centrifugation, and in these particular cases, higher sedimentation rate strains are required. This is also used in small-sized 'cachaça' (a Brazilian potable spirit) fermentation facilities (Silva *et al.*, 2006).

These findings point to a frequent practice used today by some distilleries that wrongly select yeast just by collecting the population at the end of the fermentation season. Indeed, such strains could be stress-tolerant and may present some dominance in the fermenter, but a great majority of them do not present desirable fermentation features.

Non-*Saccharomyces* yeasts were also found

In only a few distilleries could non-*Saccharomyces* (mainly *Schizosaccharomyces pombe, Dekkera bruxellensis* and *Candida krusei*) be identified, and even so with a low prevalence (these non-*Saccharomyces* yeasts represent $< 5\%$ of the total strains observed). In an isolated episode, *S. pombe* was found in a higher proportion than an *S. cerevisiae* introduced strain (CAT-1) in a distillery operating with a low ethanol concentration (*c.* 6% v/v). When compared with CAT-1 in a fermentation trial with a final ethanol concentration of 7.8% v/v, this isolated *S. pombe* showed, respectively, a longer fermentation time (20 and 7 h), lower cell viability (45% and 90%) and reduced growth. These effects were more pronounced at higher ethanol concentrations (9.0%), and this observation suggested that such yeasts will hardly compete with *S. cerevisiae* in a regular operating ethanol plant. There are also some reports on the high frequency of *D. bruxellensis* strains in ethanol plants in the northeast region of Brazil (Guerra, 1998; Liberal *et al.*, 2007), but the prevalence of such non-*Saccharomyces* strain could be due to an oxygenation step in these fermentation processes, since Delia *et al.* (1994) and Guerra (1998) observed a stimulating effect of oxygen on growth of this yeast.

The search for suitable indigenous strains

After discarding high-foaming, flocculating and high residual sugar strains, only a small fraction (68 out of 340 evaluated strains) of the indigenous population were found to be promising strains. They were further screened in fermentation trials simulating the industrial fermentation for high ethanol yield, low glycerol formation, maintenance of high viability during recycling and high trehalose and glycogen at intracellular levels. From this screening, it was possible to select 14 strains that presented suitable fermentation profiles regarding the desirable parameters.

These selected strains presented remarkable physiological and technological parameters. Table 3 shows the fermentation characteristics of a selected strain (PE-2) as compared with baker's yeast. Ethanol yield, as the fraction of

Table 3. Physiological and technological parameters of selected PE-2 strain and baker's yeast strain during fermentation cycles using sugar cane juice and molasses as substrates at 33 °C and attaining up to 9.1% (v/v) ethanol

	Strains	
Fermentation parameters	Baker's yeast	PE-2
Ethanol yield (%)*	88.1 (± 1.01)	92.0 (± 1.12)
Glycerol (%)*	5.40 (± 0.25)	3.38 (± 0.33)
Biomass gain (%)†	5.8 (± 0.61)	8.2 (± 0.84)
Viability (%)‡	48 (± 1.1)	94 (± 1.9)
Trehalose (% dry basis)§	4.0 (± 0.22)	9.5 (± 0.29)
Glycogen (% dry basis)§	9.0 (± 0.43)	16.0 (± 0.51)

Data are the average (± SD) of five fermentation cycles run in triplicate.
*Sugar fraction converted into either ethanol or glycerol (g per 100 g of sugar).
†Average biomass increase per fermentation cycle.
‡Cell viability at the end of the last fermentation cycle (% viable cells).
§Cell storage carbohydrate at the end of the last fermentation cycle.

metabolized sugar converted to ethanol, was higher for the selected strain. Despite the apparently low difference in ethanol yield between PE-2 and baker's strain, this difference (3.1) amounts to an increase of 2.1 million liter of ethanol per crop season in a medium-capacity distillery. Table 3 also indicates that glycerol formation is inversely related to ethanol yield and that a low-glycerol-producing strain would have a huge impact on industrial yields. It is well known that glycerol formation is coupled to yeast growth (Nordstrom, 1966; Oura, 1973); hence, it was expected that the selected strain, presenting a more vigorous growth, would produce more glycerol. Because glycerol is also formed in response to stress factors (notably osmostress) (Walker, 1998), it can be considered that fermentations with cell recycling, as performed in this work, impose stress conditions on baker's yeast. Reduced cell viability, as well as low glycogen and trehalose intracellular levels, observed in baker's yeast, indicate that this strain does not survive under these fermentation conditions, and this might explain the short-lived nature of this yeast in industrial fermentations. It is worth noting that the PE-2 fermentation profile was very similar to BG-1, CAT-1, CR-1, SA-1 and VR-1 strains (data not shown).

There is some evidence that trehalose acts as a stress protectant and, together with glycogen, can help yeast to cope with several adverse physiological conditions (Walker, 1998). In our experience with fuel ethanol yeast strains, cell viability declines dramatically when trehalose levels are reduced beyond 0.2% (dry cell weight basis; data not shown), and perhaps the capacity of the selected strains to sustain high trehalose levels during recycling (Table 3) may account for their improved stress tolerance.

The yeast population dynamics in industrial fermentations

The last and decisive step of this yeast selection program was the reintroduction of the 14 selected strains not only in distilleries from where they were isolated, but in as many ethanol plants as possible in order to assess their implantation capability in industrial fermentations. The first-selected strains introduced were BR-1, PE-2, PE-5, SA-1 and VR-1 in the 1995/1996 crop season (Table 2), because they were isolated during the former season (1994/1995) (Table 1). As soon as a promising strain arose, it was introduced in the next crop season. This was performed during the 1993–2005 period involving up to 78 distilleries per crop season, in which a mixture of different strains were introduced and monitored by karyotyping (of approximately monthly collected yeast samples). As many as 12 760 isolated colonies were karyotyped during this period (Table 2). It was also very important to cover several crop seasons (campaigns), because changes in the weather, sugar cane varieties and industrial processing conditions could affect yeast performance. Indeed, some strains that presented good implantation capabilities during the 1994–1998 seasons (as JA-1, CR-1, VR-1 and VR-2) were not able to establish themselves in industrial fermentations during the following seasons. It can be suggested that the increasing molasses proportion in substrates during the 1998–2005 period, due to higher international sugar prices (resulting in a greater production and utilization of molasses, a by-product of sugar industry), also contributed to additional stresses upon yeast. High-concentration molasses substrates are well known to exert a suppressive effect in industrial ethanol fermentations (Amorim & Lopes, 2005).

Figure 4a shows the dynamics of a yeast population in a distillery that started the fermentation process with 0.5 kg (wet weight) of each selected strain (PE-2, SA-1 and VR-1) mixed with 1 ton of pressed baker's yeast. After 29 days of recycling, baker's yeast was not found and all selected strains were present in different proportions. Only the PE-2 strain was able to dominate over the introduced strains, as well as over the contaminant indigenous yeasts, representing the total biomass in the fermenter at 193 days of recycling. Figure 4b also presents the yeast population dynamics in another distillery where PE-2, BG-1 and CAT-1 strains were introduced (10 kg of each in active dry form). Similarly, one can see a better adaptability of the PE-2 strain in this distillery. The CAT-1 strain quickly dominated the fermentation but disappeared after 80 days of recycling, whereas the BG-1 strain could not be implanted, in this particular distillery. These data suggest that variations (not yet studied) might be affecting strains' adaptability in some distilleries.

The results from Fig. 4a clearly show that the selected yeast strains successfully compete with baker's yeast,

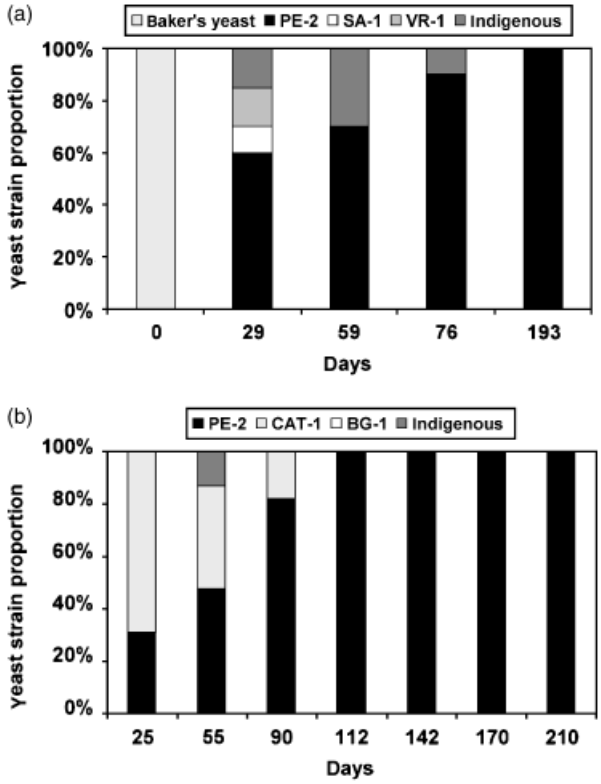

Fig. 4. Yeast population dynamics in two distilleries evaluated by karyotyping of isolated colonies during a crop season. (a) Distillery A started the industrial fermentation with 1 ton of baker's yeast and 0.5 kg of each of the selected strains (PE-2, SA-1 and VR-1). 0 refers to the starting day (only baker's yeasts were detected). (b) Distillery B started the fermentation with a mixture (10 kg of each strain in active dry form) of PE-2, CAT-1 and BG-1 (this strain was not able to implant in this distillery).

commencing from such a disproportional biomass ratio at the start of fermentation (0.5 kg of selected yeast strains vs. 1 ton of baker's yeast; in some distilleries, even 12 tons). When starting with several selected strains, it was apparent that, in most cases, one of the strains showed better adaptability to a particular distillery, as shown in Fig. 4b. However, no correlation between strain adaptability and some particular industrial process feature could be found (fed batch or continuous fermentation, high or low molasses proportion, final ethanol concentration and operating temperature). Nevertheless, Silva-Filho *et al.* (2005) found that a yeast cell PCR-fingerprint profile (P18) was dominant in cane juice-fermenting distilleries, while P6 and P1A profiles were dominant in molasses medium.

In a parallel study, it was also observed that such selected indigenous yeast strains, contrary to baker's yeast, sporulated abundantly, producing asci with three or four spores in a few hours (< 24 h) and generated some rough colonies (Lopes, 2000; Lopes *et al.*, 2002). Rough colonies (associated with pseudohyphal growth) were also observed in selected strains during their implantation in industrial fermentations (0–10% of the colonies for each strain).

Table 4. Implantation performance of different strains, evaluated by their persistence and prevalence in industrial fermentations of 20–78 distilleries during 12 crop seasons (1993–2005)

Introduced strains	Permanence (%)*	Prevalence (%)†
PE-2	58 (±4.1)	54 (±5.2)
CAT-1	51 (±4.3)	45 (±5.8)
BG-1	42 (±5.1)	65 (±4.8)
SA-1	32 (±5.0)	44 (±6.6)
VR-1	25 (±4.3)	15 (±3.0)
CR-1	7 (±6.2)	6 (±6.3)
Others‡	0	0

Data are presented as the average (± SD) of the values at the end of each crop season (collected between 160 and 250 days of recycling).
*Proportion of distilleries (%) where the introduced strain was able to be implanted.
†Strain proportion (%) in distilleries where it was implanted.
‡All other introduced strains.

Lopes (2000) and Lopes *et al.* (2002) isolated 273 PE-2 variants with chromosomal rearrangements from 12 distilleries that used this yeast as the starter culture. Considering these changes in the electrophoretic profile (due to chromosomal rearrangements), the discriminatory power of the karyotyping technique was greatly increased (not published). We could observe a range of 0–40% of chromosomal rearrangements during the implantation period for PE-2, CAT-1 and BG-1 strains (depending on the distillery), but this was more frequent during the late period of the season. Future work will be performed to identify any technological advantage of these variants, mainly regarding better adaptability or higher stress tolerance during fuel ethanol fermentations.

Few selected yeast strains appear to be useful for industrial fermentations

Unfortunately, most of the 14 laboratory-selected strains showed poor implantation capability, when considering the whole period of this study (12 years), even though some strains were able to dominate in a few distilleries during particular crop seasons in Brazil. Few strains were consistently able to contribute good fermentation performance for many distilleries and for many seasons. Table 4 presents the best-performing strains with their implantation capabilities, comprising three strains isolated in this study (PE-2, CAT-1 and VR-1) and others (BG-1, CR-1 and SA-1) isolated by Copersucar (Brazil), showing their dominance and prevalence in industrial fermentations. It can be seen in Table 4 that, during 12 crop seasons, the PE-2 strain, for instance, was implanted in 58% of the distilleries where it was introduced, representing 54% of the yeast population at the end of the crop season (160–200 days of recycling).

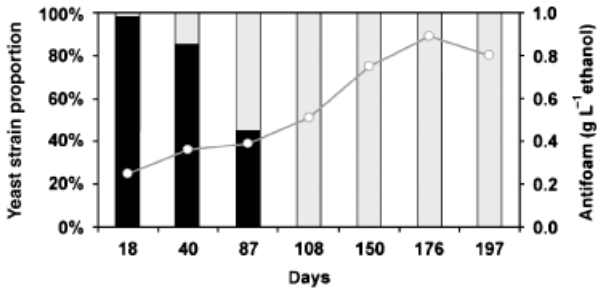

Fig. 5. The antifoam consumption (open circles) expressed as $g L^{-1}$ (produced ethanol) and the yeast population dynamics in a distillery starting the industrial fermentation with the PE-2 strain (black bars). This selected strain was later replaced by several indigenous foam-producing strains (gray bars) during the fermentation season.

To date, there are no similar data covering so many distilleries for such a long time period during which many process variations can be expected in the Brazilian bioethanol industrial sector. PE-2, CAT-1 and BG-1 showed a remarkable capacity of competing with indigenous yeast, surviving and dominating during industrial fermentations, and they are currently the most widely used strains in ethanol plants in Brazil. During 2007/2008, PE-2 and CAT-1 strains were used in about 150 distilleries, representing *c.* 60% of the fuel ethanol produced in Brazil.

Once implanted into a distillery, selected strains may reduce ethanol production costs not only by increasing ethanol yield or simplifying fermentation handling/operations but also through reducing antifoam consumption. Foam production is an important economic issue, and Fig. 5 shows the relationship between antifoam consumption and yeast population dynamics in a distillery during 197 days of operation. In this plant, the PE-2 strain was used as the starter culture and quickly became the dominant strain, but was subsequently replaced by foam-producing indigenous strains. There was a direct relationship between the presence of the selected strain and the low consumption of antifoam products, indicating potential economical benefits, albeit for limited periods.

Conclusions

In *c.* 40% of the distilleries involved in this study, no selected yeast strains could be implanted successfully and compete with indigenous yeasts. This represents economic losses due to the combined effects of foaming, flocculation, high glycerol formation and high residual sugar. Newly selected yeasts, likely isolated from the industrial plants in question, may prove suitable for specific distillery fermentations and will augment the efficiency of Brazilian fuel ethanol plants in the future.

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