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Comparative study and rapid yeast characterization by molecular analysis for industrial application

Estudo comparativo e caracterização rápida de leveduras por análise molecular para aplicação industrial

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Abstract

Yeasts are one of the most important microorganisms currently used in fermentative processes, and well-adapted *Saccharomyces cerevisiae* strains derived from exhaustive selection are considered an essential, economically viable yeast starters in the industry as bioethanol-producing plants. In order to assure the performance of industrial production, the use of molecular tools is essential for the rapid detection of contamination by wild strains. Morphological identification of yeasts isolated from bioethanol-producing plants was compared with a molecular technique based on restriction fragment length polymorphism of mitochondrial DNA (mtDNA-RFLP). A detailed study of smooth and rough colonies was performed by scanning electron microscopy (SEM). The data showed that the mtDNA-RFLP technique was a highly efficient discriminating tool for yeast strains, allowing correct identification although morphological changes occur in response to stress due to the expression of genes involved in behavior change and/or chromosome rearrangements observed by karyotyping. The majority of the yeasts showed any genetic difference when compared with industrial strains (80-100% confidence). Macroscopically different colonies (smooth or rough) were genetically similar. SEM revealed distinct budding patterns when genotypically similar strains were compared. Therefore the present study suggests that yeasts with similar genotypes can evolve into distinct phenotypes depending on stress conditions.

Keywords: Ethanol fuel, industrial PE-2 strain, PFGE karyotype, colony morphology

Resumo
Leveduras são um dos microrganismos mais importantes utilizados em processos fermentativos, e linhagens bem adaptadas de *Saccharomyces cerevisiae*, oriundas de seleção exaustiva, são consideradas essenciais e economicamente viáveis na indústria como usinas produção de bioetanol. Para garantir o desempenho da produção industrial, o uso de ferramentas moleculares é essencial para a rápida detecção de contaminação por linhagens selvagens. A identificação morfológica de leveduras isoladas de usinas de produção de bioetanol foi comparada com a técnica molecular RFLP (*Restriction Fragment Length Polymorphism*) do DNA mitocondrial (RFLP-mtDNA). Um estudo detalhado sobre colônias lisas e rugosas foi realizado por microscopia eletrônica de varredura (MEV). Os dados mostraram que o método de RFLP-mtDNA foi uma ferramenta discriminante altamente eficiente para linhagens de leveduras, permitindo uma correta identificação embora mudanças morfológicas ocorram em resposta ao estresse devido à expressão de genes envolvidos na mudança de comportamento e/ou rearranjamentos cromossômicos observados por cariotipagem. A maioria das leveduras de cada usina não apresentou diferença genética quando comparada com as linhagens industriais (80-100% de confiança). Colônias macroscopicamente diferentes (lisas ou rugosas) eram geneticamente semelhantes. MEV revelou padrões distintos de brotamento quando linhagens geneticamente similares foram comparadas. Portanto, o presente estudo sugere que leveduras com genótipos semelhantes podem evoluir para fenótipos distintos, dependendo das condições de estresse.
Palavras-chave: Etanol combustível, linhagem industrial PE-2, PFGE-cariótipo, morfologia de colônias

INTRODUCTION

Saccharomyces cerevisiae strains applied in industrial purpose should present desirable characteristic, especially concerning rapid fermentation rate and high yield, biomass production and fast growth. Fermentation conditions such as temperature and pH, high sugar and alcohol levels, as well as competition with wild yeasts are stressful for industrial yeast, and can promote a selective pressure that favors the survival of highly adapted strain. The wild yeast is generally fast-growing, which can affect the productivity performance of industrial strain selected as starter culture [(1)]. Continuous monitoring in the fermentative process is essential to avoid wild yeast contamination.

The identification of yeasts was traditionally carried out using fermentation tests and/or morphological criteria, which are laborious, time-consuming and unable to distinguish interspecies variations. In addition, a stressful process can affect the behavior of same yeast strain and change its phenotype, i.e., the colony morphology. The isolation of yeasts that form smooth or rough colonies from an ethanol production process is common. The formation of smooth or rough colonies is related to the capacity for producing extracellular matrices (EM) and/or budding patterns and pseudohyphal development [(2–5)]. Several studies have used scanning electron microscopy (SEM) in an effort to clarify cell behavior in colony formation [(3,4,6)].

Molecular techniques have been successfully used to discriminate industrial contaminants. One of the most commonly-used methodologies for the routine identification of yeasts is molecular karyotyping by pulsed-field gel electrophoresis (PFGE), a technique that separates chromosomes according to their size [(7)]. Even though this monitoring technique is used in both beverage fermentation and ethanol fuel production at several distilleries, it is very time-consuming, technically demanding and

impractical for industrial purposes. Furthermore, several studies have suggested that yeast chromosomes may become rearranged as a form of adaptation to stressful industrial environments [8–13]. This suspicion has proven relevant in light of the high levels of polymorphism found in industrial strains used for wine and ethanol production as starter culture.

Recent studies have targeted the development of molecular techniques for rapid, efficient and low-cost fermentation process monitoring. Restriction fragment length polymorphism of mitochondrial DNA technique (mtDNA-RFLP) was previously described for differentiating wine yeasts [14–17], and because of its advantages it is currently recommended to distinguish variants from starters and contaminating strains in industrial use [18]. This method consists of isolating the total DNA of the yeast and then using a restriction endonuclease that recognizes G-C sequences. The enzyme recognizes a high number of G-C sites in the nuclear DNA but only a few in mitochondrial DNA, which allows DNA fragments obtained can be visualized in agarose gel [15].

Although molecular methods have been continuously improved and advanced, an accessible rapid tool adapted to industrial-scale production would be crucial to assure bioproduct quality. This comparative study was carried out to apply mtDNA-RFLP as a feasible, reproducible, low-cost and reliable molecular tool that could be used in association with the morphological technique. A detailed study about smooth and rough colonies was later performed by SEM using genotypically similar strains. No comparative assessment was reported for Brazilian industrial strains from the ethanol fermentation process. For this, the electrophoretic profile of isolates with smooth and rough colonies from different bioethanol plants were analyzed.

MATERIAL AND METHODS

Yeasts, media, and general methods

All yeasts used in this study are listed in Table 1. Yeast strains were grown in YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} bacto-peptone, 20 g L^{-1} glucose) for routine maintenance and DNA isolation. The samples provided by Biovale Company (São Pedro do Ivaí, PR, Brazil) were collected between 2006-2008 in several plants of ethanol production from Paraná and São Paulo States. Each sample was diluted and an aliquot was surface-plated on YPD agar and incubated at 30 °C for 48 hours. The yeasts obtained were previously characterized / selected by their morphology before molecular characterization. The industrial *S. cerevisiae* strains CAT-1 and PE-2 (Fermentec, Piracicaba, Brazil), SA-1 (CTC, Piracicaba, Brazil), as well as the probiotic *S. boulardii* strain (Floratil - Merck KGaA, Darmstadt, Germany) were obtained from our house collection (G. M. Andrade-Nóbrega, Microbiology Genetics Laboratory, General Biology Department, State University of Londrina, Brazil) and used as references of DNA profiles. Cultures were grown to stationary phase in YPD liquid, and cells were centrifuged (3,500 *g*, 4 ^{o}C, 5 min), the pellet was washed with sterile water and resuspended in the appropriate buffer prior to DNA purification.

Restriction fragment length polymorphism of mitochondrial DNA (mtDNA-RFLP)

The DNA preparation for mtDNA-RFLP was performed using a rapid 10-min protocol by vortexing with glass beads in the presence of detergents, phenol, chloroform, and isoamyl alcohol as described in [19]. Total DNA released from the yeasts was digested with *Hinf* I enzyme (Invitrogen, Carlsbad, USA) in a reaction

containing 1 - 1.7 µg of DNA and incubated at 37 °C for 5 h, following the manufacturer's instructions. Electrophoresis was performed at 5 V cm^{-1} in 1.3 % (w/v, g) agarose gel stained with ethidium bromide.

Pulsed-Field Gel Electrophoresis (PFGE)

Intact chromosomal yeast DNA was prepared as described in [7] with some modifications. Cells were resuspended in 0.15 mL of pH 7.5 TE buffer (10 mM Tris / 50 mM EDTA) and stored in 1% low melting point agarose plugs with lyticase (20 mg mL^{-1} / pH 7.5 10 mM phosphate sodium). The plugs were incubated in 50 mL conical centrifuge tubes containing pH 7.5 LET buffer (10 mM Tris / 500 mM EDTA) at 37°C for 8 h. The LET buffer was then replaced with pH 9.5 NDS buffer (10 mM Tris / 500 mM EDTA / 1 % *sodium* lauroylsarcosine / 2 mg mL^{-1} fresh proteinase K), and the tubes were incubated overnight at 50 °C under agitation. The plugs were washed 4 x in pH 7.5 TE buffer (1 h between washes) and kept refrigerated at 4 °C (in pH 7.5 TE buffer) until use. PFGE was performed using the contour-clamped homogeneous electric field (CHEF) method in the Gene Navigator® system (Pharmacia Biotech, Uppsala, Sweden), at 6 V cm^{-1} with 70 s pulses for 15 h and 120 s pulses for 11 h. The chromosomes were separated in 1 % (w/v, g) agarose gel using a cooled pH 8 0.5 x TBE buffer (Tris / Borate / EDTA) at 14 °C. The gel was then stained with ethidium bromide.

Cluster analysis

Binary data for presence (1) or absence (0) of DNA fragments from the electrophoretic profiles was used to determinate the matrix. The matrix resulting from analysis was used to calculate the Jaccard index of similarity, and the relationship between isolates and reference strains was estimated by dendrogram construction using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA). Cluster formation at 80 % of similarity was considered in this study. Analysis was performed using the NTSYS-pc v2.1 program [20], and the resampling test (Bootstrap: 2000 replications) was performed using the WinBoot program [21].

Morphological study by scanning electron microscope (SEM)

Selected isolates were submitted to detailed structural analysis of colony by SEM using the protocol described in [22], applying cell lyophilization instead of the dehydration steps with ethanol. The glutaraldehyde cell fixation step was eliminated to assure the intact colonies. The samples were inoculated onto cellulose acetate filters (0.45 mm pore size) that were settled on the surface of YPD agar plates. This procedure assured colony growth without direct contact with agar. The plates were incubated for 48 h at 30 °C and the filters with colonies were then freeze-dried (1 hour at - 47 °C to 0.0063 mBar). The dried colonies were placed with dry carbon tape on aluminum stubs, covered with a layer of 20 nm colloidal gold, and the stubs were analyzed using a Quanta 200 SEM (FEI Company, Oregon, USA) at 20 KV and photographed at different magnifications.

RESULTS

Molecular characterization

The isolates from the ethanol plants were previously characterized by their morphology before molecular characterization. Two rough colonies and one smooth colony were selected from each plant, providing a total of 24 isolates (Table 1).

Table 1. Yeasts used in this study and profile (morphological and molecular) of yeasts isolated from ethanol fermentation plants in the state of Paraná and São Paulo.

Reference strain	**Description**			
PE-2	Industrial *S. cerevisiae* strain			
CAT-1	Industrial *S. cerevisiae* strain			
SA-1	Industrial *S. cerevisiae* strain			
S. boulardii	Probiotic yeast			
Plant Unit Crop / year	**Isolate**	**Colony morphology**	**RFLP-mtDNA Profile**	**PFGE Profile**
Londra	Y1	Smooth	CAT-1	CAT-1
Sept. / 2006	Y2	Rough	CAT-1	Non-specific
	Y3	Rough	Non-specific	Non-specific
Goioerê	Y4	Smooth	PE-2	PE-2
Apr. / 2007	Y5	Rough	PE-2	PE-2
	Y6	Rough	Non-specific	Non-specific
Pioneiros	Y7	Smooth	CAT-1	CAT-1
Oct. / 2006	Y8	Rough	CAT-1	CAT-1
	Y9	Rough	CAT-1	CAT-1
Pioneiros	Y10	Smooth	SA-1	SA-1
Jun. / 2007	Y11	Rough	SA-1	SA-1
	Y12	Rough	SA-1	SA-1
Vale do Ivaí	Y13	Smooth	CAT-1	CAT-1
May / 2007	Y14	Rough	CAT-1	CAT-1
	Y15	Rough	CAT-1	CAT-1
Vale do Ivaí	Y16	Smooth	SA-1	SA-1
Aug. / 2008	Y17	Rough	SA-1	Non-specific
	Y18	Rough	SA-1	SA-1
Da Calda	Y19	Smooth	PE-2	PE-2
Oct. / 2008	Y20	Rough	Non-specific	Non-specific
	Y21	Rough	PE-2	Non-specific
Cooperval	Y22	Smooth	PE-2	PE-2
Nov. / 2006	Y23	Rough	PE-2	PE-2
	Y24	Rough	Non-specific	Non-specific

PCR amplification of ITS1-5.8S-ITS2 ribosomal DNA locus (rDNA) resulted in 850-bp fragments for all isolates and reference strains (data not shown). Yeasts were then submitted to the mtDNA-RFLP technique. Digestion of mitochondrial DNA using *Hinf* I allowed differentiation of the *S. cerevisiae* strains. Figure 1a shows that each reference strain (CAT-1, PE-2, SA-1 and *S. boulardi*) had different restriction profiles. In total, eight different profiles were obtained and allowed the formation of 8

distinct groups with 100 % of similarity based on the Jaccard index (Figure 1b). The reference strains produced 4 different groups. Out of 24 isolates, 20 were clustered into the industrial strains groups (CAT-1, PE-2 and SA-1). Isolates from Londra 2006 plant Y1 and Y2, with smooth and rough colony morphology, respectively, were included in the CAT-1 group, while Y3 with rough colony morphology had no specific profile and was included in a different group (49 % bootstrap value). In addition Y1 and Y2, all isolates from Pioneiros 2006 [Y7 (smooth), Y8 (rough) and Y9 (rough)] and Vale do Ivaí 2007 [Y13 (smooth), Y14 (rough) and Y15 (rough)] plants were also included in the CAT-1 group (99 % bootstrap value). The SA-1 group clustered all isolates from the Pioneiros 2007 [Y10 (smooth), Y11 (rough) and Y12 (rough)] and Vale do Ivaí 2008 [Y22 (smooth), Y23 (rough) and Y24 (rough)] plants (100% bootstrap value). Isolates Y4 (smooth) and Y5 (rough) from the Goioerê 2007, Y10 (smooth) and Y11 (rough) from the Pioneiros 2007, Y19 (smooth) and Y21 (rough) from the Da Calda 2008 and Y22 and Y23 from the Cooperval 2006 plants were included in the PE-2 group (99 % bootstrap value). Like Y3, isolates Y6 (rough) from Goioerê 2007, Y20 (rough) from the Da Calda 2008 and Y24 (rough) from the Cooperval 2006 plants had non-specific profile and were separately included in different groups (34, 61 and 49 % bootstrap values).

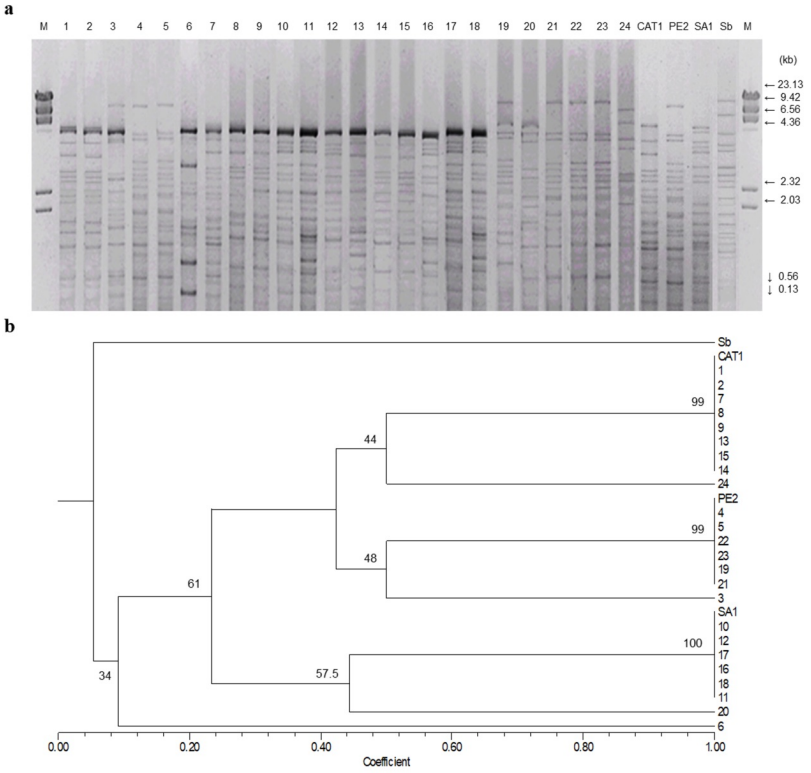

Figure 1. Electrophoretic profiles of yeasts' mtDNA digested with the *Hinf* I enzyme (a) and UPGMA dendrogram of the reference strains and isolates from ethanol plants constructed by RFLP-mtDNA analysis (b); molecular marker λ *Hind* III (M); lines 1-3: isolates from Londra 2006 (Y1, Y2 and Y3); lines 4-6: isolates from Goioerê 2007 (Y4, Y5 and Y6); lines 7-9: isolates from Pioneiros 2006 (Y7, Y8 and Y9); lines 10-12: isolates from Pioneiros 2007 (Y10, Y11 and Y12); lines 13-15: isolates from Vale do Ivaí 2007 (Y13, Y14 and Y15); lines 16-18: isolates from Vale do Ivaí 2008 (Y16, Y17 and Y18); lines 19-21: isolates from Da Calda 2008 (Y19, Y20 and Y21); lines 22-24: isolates from Cooperval 2006 (Y22, Y23 and Y24). The wider bands represent undigested total DNA. Numbers on the dendrogram branches indicate the bootstrap values (2000 replications).

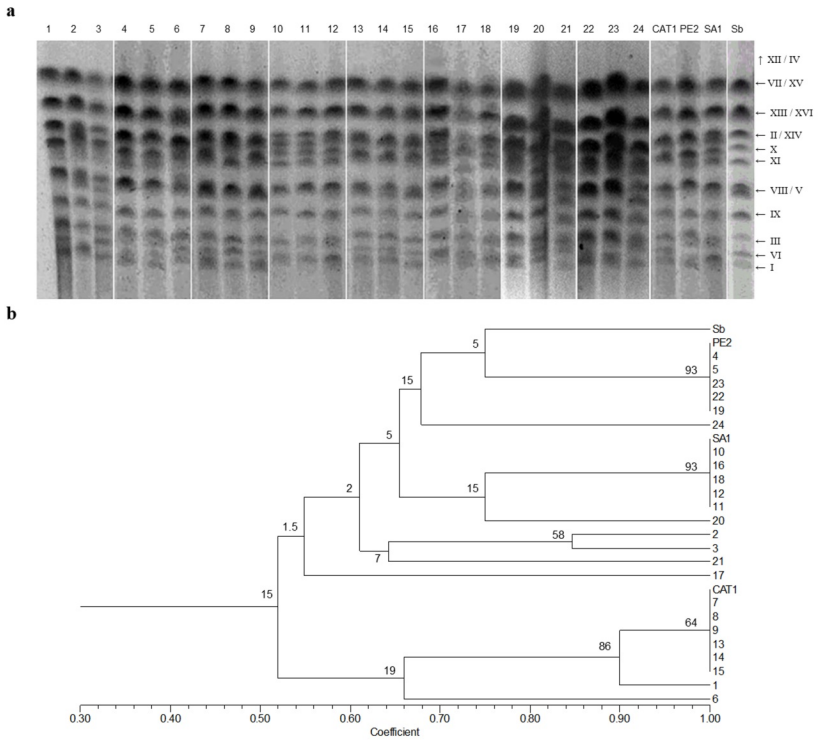

Figure 2. Electrophoretic karyotyping (a) and UPGMA dendrogram of the reference strains and isolates from ethanol plants constructed by PFGE analysis. lines 1-3: isolates from Londra 2006 (Y1, Y2 and Y3); ines 4-6: isolates from Goioerê 2007 (Y4, Y5 and Y6); lines 7-9: isolates from Pioneiros 2006 (Y7, Y8 and Y9); lines 10-12: isolates from Pioneiros 2007 (Y10, Y11 and Y12); lines 13-15: isolates from Vale do Ivaí 2007 (Y13, Y14 and Y15); lines 16-18: isolates from Vale do Ivaí 2008 (Y16, Y17 and Y18); lines 19-21: isolates from Da Calda 2008 (Y19, Y20 and Y21); lines 22-24: isolates from Cooperval 2006 (Y22, Y23 and Y24). Roman numerals represent the yeast chromosomes. Numbers on the dendrogram branches indicate the bootstrap values (2000 replications).

The mtDNA-RFLP analysis showed that strains with smooth and rough colonies had the same restriction profile for each ethanol plant. Karyotyping by PFGE technique confirmed these results for most of the samples, except for Londra 2006 and Da Calda 2008 plants, which isolates with rough colonies had different restriction profiles from their respective smooth colony isolates (Figure 2a). Thus PFGE analysis formed 10 distinct groups with 80 % of similarity based on the Jaccard index (Figure 2b). The reference strains were included into 4 different groups. As well as in mtDNA-RFLP, isolates Y3, Y6, Y20 and Y24 had non-specific profile in PFGE technique and were clustered in groups apart from the reference strains (15 and 5 % bootstrap values). The isolates Y2, Y17, and Y21 were also clustered in groups apart from the reference strains in PFGE analysis (15, 1.5 and 2 % bootstrap values).

Colony morphology study by SEM

Isolates Y4 and Y5 from the Goioerê 2007 plant, with rough and smooth colonies, respectively, were closely related genetically. Therefore, these isolates were analyzed by SEM for detailed observation of colony morphology.

SEM revealed the cell organization in both types of colonies (Figure 3).

Smooth colonies were characterized by individualized cells; some cells showed small buds attached to the mother cell, exhibiting random, axial (the new bud emerges adjacent to the scar), and polar budding modes (bipolar means that new buds emerge from either the distal or the proximate poles; monopolar means that buds emerge from the pole opposite the birth pole). Cells were distributed throughout the colony, resulting in a smooth and compacted appearance (Figure 3, left). In contrast, cells in rough colonies had an irregular appearance, indicating the presence of interconnecting structures on the surface that linked the cells into clusters. The formation of such clusters could be associated with the specific budding pattern in rough colonies. Unlike smooth colonies, rough colonies are twisted by a polarized cell-budding pattern, which results in a chain of connected cells in pseudohyphae (Figure 3, right). The production of extracellular matrix (EM) may also be involved in the formation of rough colonies.

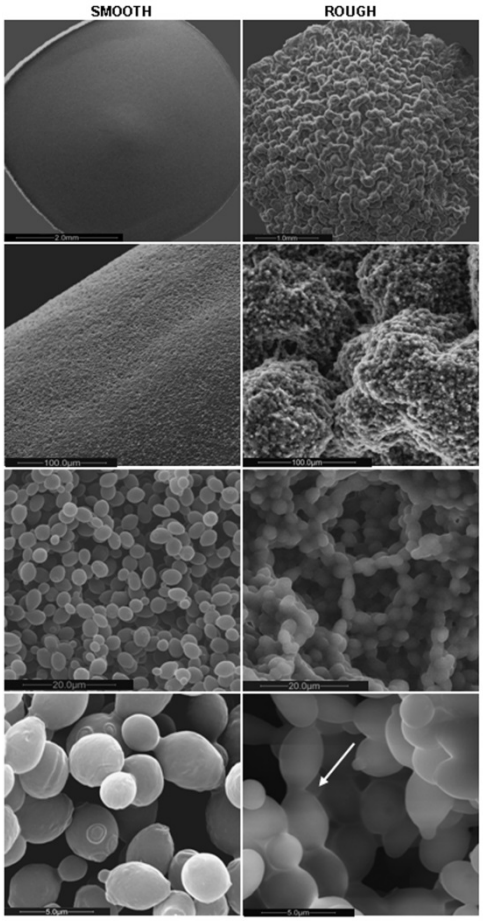

Figure 3. Budding pattern and organization of cells in smooth and rough colonies analyzed with SEM. In polarized budding, cells remained connected (arrows). Smooth colonies (left) had cells with small buds attached to the mother cell, exhibiting random, axial and polar budding modes. These cells were individualized and distributed throughout the entire colony, resulting in a smooth appearance. Rough colonies (right) had a polarized budding pattern, which resulted in a cluster of chains of connected cells organized into a larger aggregated structure. Cells interconnected cells like pseudohyphae (arrow) led the colony surface to have an irregular appearance.

DISCUSSION

The yeasts isolated from ethanol plants were previously characterized as *S. cerevisiae* strains by rDNA-PCR. Most of non-*S. cerevisiae* contaminant yeasts of ethanol fermentation have produced PCR fragments ranging from 400 to 700 bp [23]. The RFLP-mtDNA method using the *Hinf* I restriction enzyme was efficient in differentiating strains and revealed defined patterns for each industrial *S. cerevisiae* strain commonly used in ethanol plants in southern and southeastern Brazil. Other rapid molecular techniques described in the literature as PCR-based were unable to distinguish such minor differences within species. The PFGE method is considered as gold standard for molecular typing and was utilized to corroborate the results of mtDNA-RFLP. Except for some isolates, the results obtained with the mtDNA-RFLP were similar to those from the PFGE technique, which is significant considering the sensitivity of the latter for differentiating strains within the same species. Based on the Jaccard index of similarity, PFGE formed 10 distinct groups versus 8 from mtDNA-RFLP analysis. Nevertheless, the high level of polymorphism found in industrial strains increases the discriminatory power of the karyotyping technique, which can lead to error in strain differentiation.

The PFGE technique is often used to verify chromosome rearrangements. Studies using this technique have suggested that chromosome rearrangement resulted in length polymorphisms in the starter industrial strains of different Brazilian distilleries [1]. Argueso et al. [11] detected rearrangements at sites distal to the core region essential genes (telomeres) in the chromosome VI in a strain with PE-2 genetic background (JAY270) and, in addition to chromosome VI, at least six more chromosomes were polymorphic between their homologs (III, V, IX, XI, XIV and VII / XV). In this present work, polymorphisms in the chromosomes V / VIII, VI / I and X/ XI were fundamental to cluster the isolates into the group of the reference industrial strains (see Figure 2a).

Because mitochondrial DNA does not follow Mendelian segregation patterns, i.e., it does not undergo recombination like other homologous chromosomes of nuclear DNA, its genetic basis is highly preserved and variations between species and strains depend on the number of introns found among the genes [24]. This characteristic could explain the divergence results for isolates Y2, Y17 and Y21 in PFGE technique. We suggest that such isolates correspond to variations of industrial strains that underwent chromosome recombination, since mtDNA-RFLP technique showed that these samples were closely related due to high bootstrap values (99-100 % for mtDNA-RFLP versus 86-93 % for PFGE). Therefore, it can be concluded that although the mtDNA-RFLP technique efficiently discriminates related strains, it cannot reveal genome variability.

In this study, it was found that the smooth and rough samples presented similar profiles among themselves, which suggests that the behavior of these yeasts have been influenced by environmental conditions. The monitoring of yeasts via colony morphology is a routine procedure since wild yeasts tend to form colonies with an appearance distinct from laboratory and industrial strains, i.e., “domesticated” strains. However, the ethanol fermentation process is a stressful environment for yeasts because they are submitted to high temperatures and concentrations of ethanol, acidic pH, osmotic stress, nutrient deprivation, bacterial contamination and other wild yeasts, as well as being recycled throughout the harvest period. Upon being exposed to these different stress factors, yeasts must generate a quick cellular response to protect cell components against damage that may lead to changes in the expression of different genes.

Recently, Reis et al. [25] attempted unsuccessfully to distinguish between the colony phenotypes by the microsatellite analysis. However, they found that, unlike the smooth colonies, the rough colonies of *S. cerevisiae* exhibit an enhanced resistance to stressful conditions as high concentrations of glucose and ethanol. According to results of the microarray analysis, overexpression of genes that codify transposons (Ty elements) as well as several genes in subtelomeric regions may have interfered in the differentiation of yeast colony morphology as a response to environmental signs, such as stress or nutrient starvation [(4)]. It was demonstrated that formation of pseudohyphae and invasive growth in *S. cerevisiae* are regulated by Ty1 transposition, which could be induced by nitrogen starvation [(26)]. High-level expression of Ty elements and several other factors in the cell biological process in response to stress was founded in *S. cerevisiae* under ethanol-stress [(27)], whereas for genes related to DNA transposition and recombination were more expressed under osmotic stress in [(28)].

Budding patterns and pseudohyphal development are related to smooth or rough colony formation. Although polarized cell division is genetically controlled under ploidy influence in *S. cerevisiae* [(29)], it was demonstrated that a colony's cell budding pattern is not always uniform. Vopálenská et al. [(5)] observed colonies formed by haploid strains which should grow exclusively in axial mode in liquid culture were budding in bipolar mode and randomly in solid medium. Unconformity with diploid strains was also observed: many cells that should bud only in polarized mode in liquid medium were also budding randomly. This behavior may actually reflect the nutritional conditions present in the medium, since some areas of the colony would have access to fewer nutrients, which would affect the cell budding pattern. Studies have shown that cultivation in nitrogen-deprived mediums active a pathway which induces the pseudohyphae formation in yeasts [(2,5,26,30,31)]. Studies on "domestication" of wild *S. cerevisiae* strains, cells with altered colony morphology cultivated in rich medium initially underwent no changes, but after some time under favorable laboratory conditions slowly became smooth, eventually becoming indistinguishable from laboratory strains [4]. The same behavior was observed in our laboratory: visibly rough colonies changed to an almost smooth morphology and could be distinguished only by stereoscope. Smooth colonies with rough sections were also observed, which corroborates that cells gradually adapt to the new environment as less energy consumption is necessary. Therefore, the function of polarized growth would be to allow the cells to find nutrients and substrates farther from their initial colonization site.

Besides its importance in cell migration, a polarized budding pattern may help with the secretion of EM, since plasma membrane traffic is also polarized [(2,29)]. In the present study, SEM images revealed the presence of matrix covering the surface of the rough colonies. The EM production may also be involved in colony morphology as demonstrated by loss of EM during transitions in colony morphology in SEM images [4]. Figure 3 of the current work shows cells connected by the poles and distributed widely into ring-like structures. Therefore, the presence of EM does not indicate a lack of nutrients, but a way for the cells to interact at a distance.

The present results show that the mtDNA-RFLP method is simple, rapid, reliable and economical, and can be recommended for the practical molecular identification of yeast strains. In an industrial application, this technique, sometimes coupled to morphological characterization, can assure the early detection of contamination by wild strains. PFGE and SEM analysis suggest that yeasts with similar genotypes can evolve into distinct phenotypes depending on stress conditions, changing from a smooth to a rough colony pattern.

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