MONITORING SACCHAROMYCES CEREVISIAE POPULATIONS BY mtDNA RESTRICTION ANALYSIS AND OTHER MOLECULAR TYPING METHODS DURING SPONTANEOUS FERMENTATION FOR PRODUCTION OF THE ARTISANAL CACHAÇA

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ABSTRACT

An ecological study on Saccharomyces cerevisiae populations in spontaneous fermentation has been conducted in three vats of a cachaca distillery in Minas Gerais, Brazil. Ninety-seven yeast isolates were collected at the beginning, the middle and at the end of the production period, and were identified by standard methods. Differentiation between the indigenous S. cerevisiae strains isolated was performed by mitochondrial DNA (mtDNA) restriction analysis, RAPD-PCR, and PCR fingerprint using an intron splice primer. Analysis of the mtDNA restriction profiles revealed 12 different patterns, 11 corresponding to indigenous yeasts (I to XI) and one (XII) to a commercial strain of the bakery yeast. Pattern II (53.6% of the population) and pattern IV strains were present in all the vats. Pattern IV strain raised from the middle to the end of the period reaching proportions near those of pattern II strain. PCR methods allowed the differentiation of 41 molecular profiles. Both methods showed population fluctuation of S. cerevisiae strains along the period of cachaca production and among different vats of the distillery.

Key words: Saccharomyces cerevisiae, cachaca, fermentation, molecular diversity

INTRODUCTION

Saccharomyces cerevisiae is the prevalent yeast species during the spontaneous fermentation of artisanal Brazilian cachaca (5,9,12,19). The starter ferment is prepared by various methods, including the development of the fermentative microbiota in the sugar-cane juice alone, or mixing of sugar-cane juice with crushed corn, rice or soy meal (9,12). The identification and strain characterization of S. cerevisiae populations are of great importance to understand the cachaca fermentation process, since the quality of the beverage may depend upon the strains prevailing during the process, mainly in terms of their dynamics and occurrence (10,11,12,13). Different techniques based on the detection of molecular polymorphism have been used for the characterization of cachaca yeast strains. Pulsed field gel electrophoresis (PFGE) allowed detecting the occurrence of different S. cerevisiae strains during the annual cycle of cachaca production (May to December), and even in different fermentation vats of the same distillery (5,12). Although PFGE is a valuable method for discriminating strains, there are some factors that limit its applicability, since it is complex and time-consuming. Apart from this, the analysis of a large number of samples is difficult. A method of PCR fingerprinting developed by de Barros Lopes (1,2), using an intron splicer primer, has proved useful to show the molecular diversity of S. cerevisiae strains isolated from different fermentative vats during cachaca production (5,12). This method, associated with RAPD (randomly amplified polymorphic DNA)-PCR analysis, afforded the distinction among indigenous strains of S. cerevisiae during cachaca fermentation. Guerra et al. (5) suggested that the genetic diversity observed in S. cerevisiae populations may be the consequence of a large number of individual genotypes within the species. RAPD and PCR fingerprinting techniques are simple; yet, their reproducibility demands special care and occasionally the interpretation of results can be difficult (4,5,20).
The mitochondrial DNA restriction analysis (mtDNA-RFLP) has been used by various authors to characterize and differentiate *Saccharomyces cerevisiae* industrial strains (6,8,14). mtDNA-RFLP is simple, reliable, fast and cost-effective, and has been reported to afford the differentiation among yeast strains isolated from the same must and to monitor the persistence and prevalence of a specific yeast strain during the whole fermentative process (15-18,21,22).

In the present study, we compare the use of mtDNA restriction analysis, RAPD-PCR, and PCR fingerprints for the molecular characterization of the prevalent *S. cerevisiae* populations in a distillery during the production period of the artisanal cachaça.

**MATERIALS AND METHODS**

**Yeast isolation and identification**

Samples were collected from three circular vats of 1,000 l of capacity (A, B and C) at the beginning (03/07/2000), the middle (12/08/2000), and at the end (24/10/2000) of the annual cycle of cachaça production. The starter ferment was obtained by natural fermentation of the sugar-cane juice mixed crushed corn. The samples were collected during the tumultuous fermentation or at the end of the fermentative cycle. This distillery does not resort to centrifugation to carry out cell recycling, and the yeasts take on average 4 h to decant spontaneously, with the starter strain corresponding to 25% of the vat’s volume. For the isolation of *S. cerevisiae* strains, serial 10-fold dilutions of samples were made and 0.1 ml inoculated in triplicate on SCY agar (sugarcane juice 10%, yeast extract 0.5%, agar 2%, and chloramphenicol 0.01%). Ten yeast colonies were selected representing the prevalent morphotype from each sample of the different vats, at the highest dilution plate. Also, at least one isolate of each distinct colony morphotype was collected for physiological and molecular characterization. The yeasts were identified by standard physiological tests (7).

**Mitochondrial DNA restriction analysis**

The mitochondrial DNA (mtDNA) was purified as described by Querol et al. (14), modified by López et al. (8). The mtDNA was digested with *Hind* restriction endonuclease (Invitrogen), as prescribed by the manufacturer. The restriction fragments were separated by 1.0% agarose gel electrophoresis, with 1X TAE buffer (45 mmol l⁻¹ Tris-borate, 1 mmol l⁻¹ EDTA, pH 8), stained with ethidium bromide, visualized under UV-light and photographed.

**DNA extraction and PCR analysis**

Yeast DNA was extracted as described by Pataro et al. (12). For PCR fingerprinting, the primer EI1 (5’-CTGGCTTGGGTG TGATATCT-3’), complementary to the intron consensus splicing sites, was used (1). For RAPD analysis, the primers OPA1 (5’-CAGGCCCTCTC-3’), and M13 (5’-GAGGTTGGCCTTCT-3’) (5) were used. The RAPD-PCR assay was performed in a 10-ml volume and the reaction mixture contained 1 ng DNA template, 10 pmol the primer, 1.5 mM MgCl₂, 50 mM KCl, 10 mM of Tris/HCl pH 8.5, 125 Mm dNTP’s and 1.5 U Taq DNA polymerase. Reactions were carried out in a thermal cycler (model PTC 100, MJ Research, Inc). RAPD-PCR conditions were: 5 min at 95°C followed by two annealing cycles of 2 min at 30°C, extension for 30 s at 72°C and denaturation for 30 s at 95°C. Thirty-two additional annealing cycles of 2 min at 40°C, extension of 30 s at 72°C and denaturation of 30 s at 95°C were also performed. After the last cycle, a final annealing of 2 min at 40°C and final extension of 5 min at 72°C were performed. PCR fingerprinting assays with primer EI1 were performed as described by Guerra et al. (5). PCR products were separated by agarose gel electrophoresis (1.0%, TAE 1X buffer), stained with ethidium bromide, visualized under UV-light and photographed. The phenetic analysis of RAPD and PCR fingerprinting results were made by comparing the number and sizes of DNA bands amplified from the various *S. cerevisiae* strains. The dendrogram was obtained by the Unweight Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm, based on the data matrix constructed with the results of both the PCR methods combined, and built using the TFPGA (Tools for Population Genetic Analyses) computer package program.

**RESULTS AND DISCUSSION**

*Saccharomyces cerevisiae* was the only species isolated from the fermentation vats during the cachaça production period, with populations ranging between 5.0 x 10⁶ and 3.39 x 10⁹ cfu.ml⁻¹. The variations in *S. cerevisiae* may be explained by the circumstance as of sample collection, with higher counts observed during tumultuous fermentation and lower counts seen at the end of the fermentative cycle. Ninety-seven *S. cerevisiae* isolates were analyzed based on their mtDNA restriction patterns, RAPD and PCR fingerprinting profiles. Table 1 presents the *S. cerevisiae* morphotypes sampled, their mtDNA patterns, and estimate counts for the three vats at the beginning, the middle and at the end of the annual period of cachaça production. *S. cerevisiae* isolates with rough colonial texture and irregular margin were obtained from the three vats, with populations lower than 1.0 x 10⁸ cfu.ml⁻¹, at the middle and the end of the production period. The mtDNA restriction analysis provided 12 unique patterns for the 97 isolates that would therefore be considered as individual strains (Fig. 1). The strains presenting the mtDNA-RFLP patterns identified as II and IV were present in all the three vats during the fermentation season. Isolates showing the mtDNA pattern II (52 isolates) presented the highest yeast counts during the production period (Table 1, Fig. 2). It is worth noticing that *S. cerevisiae* strain, showing pattern IV, appeared at the middle of the period in all the three vats, and increased in vats A and B at the end of the fermentative period. Isolates that...
shared the rough Colonial texture and irregular margin presented
the same mtDNA restriction pattern (pattern IV) (Table 1). The
S. cerevisiae strain with pattern III was also present but in
lower counts, in all the vats. The presence of pattern XII in vat
C at the end of the season was a consequence of the addition of
bakery yeast by the producer. This vat had problems in the
must fermentation, specially concerning delays in the
fermentative cycles, and the producer added bakery yeasts with
a view to improving its fermentative performance.

The mtDNA-RFLP that, as far as we know, was applied here
for the first time for studying the dynamic of S. cerevisiae
populations involved in the cachaca fermentation, revealed
high strain diversity, and indicated the predominance of two
strains (patterns II and IV), in all vats (Fig. 2). This molecular
technique allowed to correlate the morphotypes with rough
colonial texture and irregular margins to a specific mtDNA
restriction pattern (pattern IV) (Table 1, Fig. 1), although not all
the isolates with this pattern exhibited rough colonies. Besides,
mtDNA-RFLP was able to indicate the appearance of specific
mtDNA patterns, suggesting that a succession of yeast strains
would occur, and even that certain strains would prevail at the
final stages of cachaca production.

RAPD analysis with primer M13 was not efficient to
discriminate the S. cerevisiae strains (data not shown), and only
the results obtained with primer OPA1 were used to differentiate
the yeast strains. Figure 3 shows the results of RAPD with primer
OPA1 and PCR fingerprinting analysis with primer EI1 for the S.
cerevisiae isolates from each vat. The isolates were grouped in
distinct clusters, and the similarity was clearly associated with
the production periods sampled. In vat A, four main clusters
(‘a’, ‘b’, ‘c’, and ‘d’) were observed, corresponding to 17 different
composite molecular patterns. Cluster ‘a’ comprised two
molecular profiles with one isolate from the middle and the other
from the end of the fermentative period, but both showing the
same mtDNA restriction pattern (IV). Cluster ‘b’ was composed
by nine isolates, all from the beginning of the season, with 70%
similarity, corresponding to mtDNA patterns I and II. The mtDNA
pattern I occurred exclusively in vat A, and was also signaled by
an exclusive PCR profile (profile 6), which also occurred only in
and the end of the period; by clusters ‘e’ and ‘g’ (vat B, isolates from the beginning and the end of the period); and by clusters ‘i’ and ‘j’ (vat C, isolates from the beginning and the middle of the period). The isolates with mtDNA pattern XII, occurring only in vat C, corresponding to commercial bakery yeast, grouped in cluster ‘k’ (Fig. 3, vat C). Pattern XII isolates represented the PCR profiles 40 and 41, with approximately 70% similarity to each other.

Some of the advantages reported for the use of the RAPD and PCR fingerprinting over other molecular techniques for the analysis of populations are the small quantities of template DNA, simplicity and speed of execution, and the relatively low costs involved, all these aspects that allow to work with large number of polymorphic loci randomly sampled across the whole genome (3). Due to the multilocus nature of the RAPD-PCR marker, this method can be both useful and appropriate to estimate intraspecific diversity. The aspects in the method that are considered difficult to deal with are the relative lack of reproducibility of the banding patterns, the need for several reactions with different primers (the RAPD-PCR discrimination power depends highly on the primers used), and the need for statistical analysis in order to achieve data meaningfulness (3).

In previous works on the genetic diversity of *Saccharomyces cerevisiae* prevalent in the fermentation for the production of *cachaça*, Pataro et al. (12), employing PFGE and PCR fingerprint analysis, found high degrees of genetic polymorphism among isolates from three different distilleries and also among strains isolated from the same vat at different fermentation periods. Guerra et al. (5) also observed a high molecular diversity among the *S. cerevisiae* isolates, using PFGE, during the daily cycles of *cachaça* production. However, the authors showed that the RAPD-PCR profiles had few differences among the isolates, suggesting that they were closely related.

In the present work, we isolated 10 colonies of the prevalent morphotype of *S. cerevisiae* from each of three individual vats from the same distillery, sampled at different moments during the fermentation season (at the beginning, the middle and at the end). Our results of RAPD and PCR fingerprinting analysis showed that the technique allowed the profiling of related yeast isolates, with a good power to genetically discriminate closely related indigenous yeast strains (as indicated by the existence of 41 profiles among 97 isolates), and otherwise, revealing strain families (clusters, ~ 70% similarity) according to their origin during the production period (Fig. 3).

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**Table 1.** Frequency of occurrence of *Saccharomyces cerevisiae* populations and their respective mtDNA restriction profiles from three vats of fermentation, at the beginning, middle and end of the period for *cachaça* production.

<table>
<thead>
<tr>
<th>Period</th>
<th>Vat A</th>
<th>Vat B</th>
<th>Vat C</th>
<th>Vat A</th>
<th>Vat B</th>
<th>Vat C</th>
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<tbody>
<tr>
<td></td>
<td>Plain texture and regular margin</td>
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<td></td>
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<tr>
<td></td>
<td>Rough texture and irregular margin</td>
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<td><strong>Beginning</strong></td>
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<tr>
<td></td>
<td>221 (II)*</td>
<td>33.9 (I)</td>
<td>267 (II)</td>
<td>233.7 (II)</td>
<td>33 (IX)</td>
<td>67.8 (V)</td>
</tr>
<tr>
<td><strong>Middle</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>5.58 (II)</td>
<td>3.05 (II)</td>
<td>0.15 (II)</td>
<td>≤ 0.1 (IV)</td>
<td>≤ 0.1 (IV)</td>
<td>≤ 0.1 (IV)</td>
</tr>
<tr>
<td></td>
<td>0.62 (III)</td>
<td>0.38 (VI)</td>
<td>0.38 (VII)</td>
<td>0.38 (X)</td>
<td>0.20 (IV)</td>
<td>0.05 (VI)</td>
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<td></td>
<td>0.12 (VIII)</td>
<td>0.1 (XI)</td>
<td>0.12 (VIII)</td>
<td>0.12 (VI)</td>
<td>0.24 (IX)</td>
<td>0.12 (X)</td>
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<tr>
<td><strong>End</strong></td>
<td></td>
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<tr>
<td></td>
<td>0.58 (IV)</td>
<td>0.47 (II)</td>
<td>300 (XII)</td>
<td>≤ 0.1 (IV)</td>
<td>≤ 0.1 (IV)</td>
<td>___</td>
</tr>
</tbody>
</table>

*No. of isolates (mtDNA restriction profiles); values expressed as 10⁷ cfu ml⁻¹.
Figure 3. Dendrogram of the Saccharomyces cerevisiae strains based on the RAPD and PCR fingerprinting profiles obtained by primers OPA1 and EI1, respectively. Roman numerals indicate mtDNA restriction patterns type shared by several strains.
The genotypic diversity during the *cachaça* fermentation season strongly indicates the necessity to employ effective methods to differentiate *S. cerevisiae* strains. The use of the mtDNA-RFLP, RAPD and PCR fingerprinting analysis to monitor *S. cerevisiae* revealed a high genetic polymorphism among the isolates from the same vat, from different vats of the same distillery, and among isolates sampled at different moments of the annual period of production of the artisanal *cachaça*. Our results are in accordance with those observed in different previous population studies on ecology and genetic structure of indigenous yeast populations of wineries, indicating a high level of polymorphism in the structure of the nuclear and mitochondrial genomes (3,5,6,12,14,16,18,21,22).

The methods employed here have applicability, being informative on distinct aspects, peculiar to the genetic structure of the population and subpopulations under study. mtDNA-RFLP was accurate in discriminating strains, fast to execute, easy to perform, reliable and economic. Apart from this, the data generated were easy to interpret (14). On the other hand, the RAPD-PCR technique, although demanding careful and laborious procedures for clustering analysis and for estimation of similarity, was informative on the genetic relatedness among the isolates.

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