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# Comparison between Two Selected *Saccharomyces cerevisiae* Strains as Fermentation Starters in the Production of Traditional Cachaça

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## ABSTRACT

*Two* Saccharomyces cerevisiae *strains were tested as the starter yeasts in a traditional cachaça distillery. The strains used were* S. cerevisiae *UFMG-A829, isolated from a cachaça fermentation process, and* S. cerevisiae *K1-V1116, obtained from the wine industry. The permanence of each strain in the fermentation must was determined by RAPD (Random Amplified Polymorphic DNA)-PCR, with primer M13. Both yeast strains were prevalent in the vats for approximately 30 days. Indigenous non-*Saccharomyces *and indigenous* S. cerevisiae *strains were isolated in lower counts during the fermentation period. Indigenous* S. cerevisiae *strains were molecularly distinct when compared to the starter yeasts. The two yeasts appeared promising starter yeasts in the fermentation process to produce traditional cachaça.*

**Key words**: cachaça, *Saccharomyces cerevisiae*, starter strains, fermentation

## INTRODUCTION

Indigenous or traditional fermented foods are those popular products that can be prepared in the household or in cottage industries using relatively simple techniques and equipment (Aidoo et al. 2006). Some of these products have undergone industrial development and are also now manufactured on a large scale (Wood, 1998; Hui et al., 2004). Cachaça produced in the state of Minas Gerais can be considered a traditional beverage, since it is prepared according to the definition as above. The state of Minas Gerais is the largest producer of traditional cachaça in Brazil. The number of cachaça producing plants is estimated to be around 8,000, accounting for approximately 230 million liters per year. Traditional cachaça is a product of the distillation of sugarcane must in copper alembics. The fermentation process is spontaneous, and involves exclusively the

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indigenous microbiota present in the must and equipments (Morais et al., 1997). Fermentation cycles last an average of 24 h and natural yeast preparations (named "fermento caipira") are used throughout the production season (May to December). During the fermentation cycle, it is possible to isolate different yeast species in the vats. Nevertheless, *Saccharomyces cerevisiae* is the prevailing species (Morais et al., 1997; Pataro et al., 1998, 2000; Guerra et al., 2001). Pataro et al. (2000) and Guerra et al. (2001) have detected different *S. cerevisiae* strains in the fermentation vats during the season, and this strain diversity may cause the sensory properties of cachaça to vary during the production.

*S. cerevisiae* strains have been used as fermentation starters for the majority of fermented and distilled beverages (Kunkee and Bisson, 1993; Cedeño, 1995; Gutiérrez et al., 1997; Fahrasmane and Parfait, 1998; Romano et al., 2003; Victoria Lopez et al., 2003; Valero et al., 2005; Melo et al., 2007). Among the advantages of using selected strains are faster fermentation start-up, lower adverse contamination risks and preservation of the sensory characteristics of the beverage from season to season. Dorneles et al. (2005) studied the influence of using selected *S. cerevisiae* in the elaboration of Terci red wine from Colombo, Parana, Brazil. The authors showed that the use of selected yeasts contributed to the improvement of the physical-chemical parameters and wine quality obtained, reducing undesirable components in the finished product, like the volatile acidity and methanol, when compared to the artisanal wine.

Oliveira et al. (2004) defined some fermentation characteristics in the selection of starter yeasts to use in the production of cachaça. The fermentation yield, ethanol content in the wine and the maximum specific rate for cell growth were identified as important parameters in the selection of strains. The physiological characteristics of the selected *S. cerevisiae* strains, such as the ability to grow at high temperatures and at high alcohol concentrations, osmotolerance, high levels of invertase activity and trehalose accumulation, and production of low levels of volatile acidity are also desirable characteristics that allow these strains to dominate the cachaça fermentation process (Pataro et al. 2002). This study aimed to compare the use of one indigenous and one commercial *S. cerevisiae* strain as starters to the fermentation process of traditional cachaça production, with observation of the permanence times of the starter yeasts.

## MATERIALS AND METHODS

### Strain origin and physiologic tests

Two *S. cerevisiae* strains were used in the experiments. Strain UFMG-A829 was obtained from the yeast collection of the Laboratory of Yeast Ecology and Biotechnology, Department of Microbiology, Federal University of Minas Gerais. The strain was isolated from spontaneous fermentation during the cachaça production process (Pataro et al. 2000). This strain was compared to a commercial strain of *S. cerevisiae* K1-V1116 (Lallemand – Canada) that is one of the most widely active dry wine yeasts in the world. This strain is a rapid starter with constant and complete fermentation at 10 and 35 $^{o}$C, and it is capable of surviving difficult conditions such as low nutrient musts and high levels of sulfur dioxide ($SO_2$) and sugar (http://www.lalvinyeast.com/strains.asp). Methods for the physiological tests of osmotolerance, maximum growth and fermentation temperature, resistance to ethanol, invertase activity, and accumulation of trehalose have been presented in detail by Pataro et al. (1998) and Gomes et al. (2002).

### Large-scale fermentations

Fermentations were carried out in a distillery in the city of Esmeraldas, state of Minas Gerais. For the preparation of the starter strains, strain UFMG-A829 was grown in modified Sabouraud agar (glucose 2%, peptone 1%, yeast extract 0.5%, and agar 2%) at room temperature for 24 h. One milliliter of a suspension containing 1 x $10^7$ cells was inoculated in flasks containing 100 ml SCY broth (sugarcane juice 50%, glucose 0.5%, yeast extract 0.5%, and distilled water 50%). The flasks were incubated in a rotatory shaker (New Brunswick Scientific) at 150 rpm, 25 ± 1°C for 24 h. After this period, the pre-inoculum was transferred to flasks containing 5 L SCY broth, and incubated at room temperature (25 ± 3°C) for 24 h. Ten liters of this second pre-inoculum was added to the vats. The fermentations were conducted in 1,500-L stainless steel vats. The pre-

inoculum was mixed with 90 L sugarcane juice at 8° Brix. After 24 h, a 200-L volume of sugarcane juice (10° Brix) was added to the fermentation vat. On the third day, a 300-L volume of sugarcane juice (12° Brix) was added. On the fourth day, one 400-L volume sugarcane juice (20° Brix) was then added to the vat containing the selected yeast, thus completing a 1,000-L final fermentation volume. After 24 h, the wine produced was distilled, and a new fermentation cycle started. For strain K1-V1116, 500 g of the dry yeast were inoculated directly into the fermentation vat containing 500 L sugarcane juice diluted to 8° Brix. On the second day, the volume inside the vat was completed to 1,000L, and on the third day the wine was distilled. Fermentation was terminated when the Brix scale reading for the wine was zero. The wine was subsequently distilled in a copper still (alembic). This distillery did not resort to centrifugation to carry out cell recycling, and the yeasts, on average, took 4 h to decant spontaneously, with the starter strain corresponding to 25% of the vat's volume. Samples of the sugarcane must were collected on days 3, 5, 11, 18, 25 and 34 after the first distillation to produce cachaça during the two experiments which were carried out. The duration of the experiments was determined with the daily observation of cachaça volatile acidity levels and fermentation times. During the experiments, vat contents were discarded when the volatile acidity of the cachaça, expressed as acetic acid, reached values over 150 mg $ml^{-1}$ anhydrous alcohol, the maximum limits established by Brazilian law (Brazil, 2005), or when the fermentation cycle, normally between 18 and 24 h, lasted over 30 h.

**Yeast counts and identification**

For the determination of yeast populations, 0.1 mL aliquots of appropriate decimal dilutions were spread on SCY (sugarcane juice 50%, glucose 0.5%, yeast extract 0.5%, distilled water 50%, and agar 2%), and lysine agar [Yeast carbon base (Difco) 1.17%, lysine 0.056%, agar 2%, and chloramphenicol 0.01%] for non-*Saccharomyces* species. Plates were incubated at 25° C for 5 days. After growth, ten yeast colonies from each fermentation vat were selected, at the highest dilution plate of SCY agar, all of which represented the same morphotype. Also, at least one sample of each distinct colony morphotype was collected for physiological and molecular characterization. Each different yeast morphotype from lysine agar was counted and purified for identification. The yeast isolates were maintained in YM (yeast extract 0.5%, malt extract 0.5%, glucose 1%, peptone 0.5%, and agar 2%) slants or liquid nitrogen. The yeasts were identified by methods proposed by Yarrow (1998) using the taxonomic keys presented by Kurtzman and Fell (1998).

**DNA extraction and RAPD assay**

Yeast DNA was extracted as described by Pataro et al. (2000). For the PCR assays, the primer M13 (5'-GAGGGTGGCGGTTCT-3') was used (Torriani et al., 1999; Guerra et al., 2001). The PCR assay was conducted as described by Guerra et al. (2001). PCR products were analyzed on 1% agarose gel electrophoresis (1X Tris-acetate – TAE buffer), stained with ethidium bromide and visualized under UV light and photographed.

## RESULTS AND DISCUSSION

The two starter strains tested were able to grow at temperatures of up to 41°C, to ferment glucose at 46° C, and to grow in the 10% ethanol medium. Apart from this, the strains were also able to grow in a medium containing 50% glucose and 8% ethanol. The results showed that the strains studied were adapted to the conditions observed in the fermentation vats. The average temperature of the cachaça fermentations, as practiced in the state of Minas Gerais, is around 30° C and may go up to 40° C in the hotter regions of the state (Pataro et al. 2002). The data obtained for tolerance to high glucose and ethanol concentrations, as well as the ability to grow and to ferment at high temperatures, were similar to the results obtained by Pataro et al. (1998, 2002) for yeast isolated during the cachaça fermentation processes.

Strain UFMG-A829 exhibited 110 µmol of reducing sugars per mg/cells.min of invertase activity, and strain K1-V1116 produced 40 µmol of reducing sugars per mg/cells.min. Studies carried out by Pataro et al. (1998, 2002) have shown an elevated invertase activity in *S. cerevisiae* strains isolated from the traditional fermentation processes to produce cachaça. The values observed for these strains reached 100 µmol reducing sugars/ mg cells.min. The daily addition, to the fermentation vats, of sugar cane juice with approximately 16% sugar made this environment selective for yeast strains that exhibited a high

invertase activity.

The intracellular trehalose level did not vary considerably between the two strains. Strain UFMG-A829 accumulated 28.4 μmol glucose wet weight, and strain K1-V1116 built up more than 40 μmol glucose/g wet weight. The strains that produce high trehalose levels may prevail for longer within the fermentation process, as they pose a better resistance against the stress conditions inherent to the process (Pataro et al., 2002). The trehalose accumulation capacity may be involved in the survival of *S. cerevisiae* strains in *cachaça* fermentation environments.

*S. cerevisiae* UFMG-A829 prevailed throughout the fermentation process for 34 days during the two experiments carried out (Table 1). After this time, the volatile acidity values of the produced cachaça were above the maximum limits established by Brazilian law (Brazil, 2005), and the vat contents were discarded. The non-*Saccharomyces* isolates in vats inoculated with strain UFMG-A829 were identified as belonging to the genera *Candida*, *Schizosaccharomyces* and *Pichia*. *S. cerevisiae* K1-V1116 prevailed in the process during the 28 days in the first experiment and 34 days in the second. In the vats inoculated with this strain, the non-*Saccharomyces* yeasts isolated belonged to species of the genera *Candida*, *Kloeckera*, *Rhodotorula* and *Torulaspora*. The variations in the numbers of *S. cerevisiae* populations may be explained by the circumstance of sample collection, with higher counts observed during tumultuous fermentation and lower counts seen at the end of the fermentative cycle. Both starter strains presented similar yields of cachaça (about 170 l for each 1,000 l of distilled wine) during the time that these strains were prevalent in the fermentation vats in both experiments (data not shown).

**Table 1 –** Numbers (cfu.ml$^{-1}$) of yeast species recovered at different times from vats inoculated with two *Saccharomyces cerevisiae* starter strains in a cachaça distillery.

| | **Yeasts** | **Times[1]** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **I** | **II** | **III** | **IV** | **V** | **VI** |
| | ***S. cerevisiae* UFMG-A 829** | | | | | | |
| A[2] | *Candida apis* | - | - | 1 | - | - | - |
| | *C. famata* | - | - | 2 | - | - | - |
| | *C. gropengiesseri*-like [4] | 1 | - | - | - | - | - |
| | *S. cerevisiae* indigenous | 3 | - | - | - | - | - |
| | *Pichia membranifaciens* | 2 | - | - | - | - | - |
| | *S. cerevisiae* UFMG-A 829 | >300 | 86 | 19.1 | 26 | 9.43 | 0.36 |
| B[3] | *C. parapsilosis* | - | 1 | - | - | - | - |
| | *S. cerevisiae* indigenous | - | 1 | 1 | - | - | 1 |
| | *Schizosaccharomyces pombe* | - | 1 | - | - | - | - |
| | *S. cerevisiae* UFMG-A 829 | >300 | 8 | 3 | 24.3 | 146 | <1x10$^4$ |
| | | | | | | | |
| | ***S. cerevisiae* K1-V1116** | | | | | | |
| A[2] | *C. famata* | - | 2 | 8 | - | - | |
| | *C. gropengiesseri*-like [4] | 1 | - | - | - | - | |
| | *Kloeckera apis* | 40 | - | - | - | - | |
| | *Rhodotorula mucilaginosa* | - | 1 | - | - | - | |
| | *S. cerevisiae* indigenous | 5 | - | - | - | - | |
| | *Torulaspora delbrueckii* | - | - | 2 | - | - | |
| | *S. cerevisiae* K1-V1116 | >300 | >300 | 1400 | 8.76 | 1.03 | |
| B[3] | *C. famata* | - | 1 | - | - | - | - |
| | *S. cerevisiae* indigenous | - | - | 3 | - | - | 1 |
| | *S. cerevisiae* K1-V1116 | 4.3 | 10.7 | 56 | 14 | 0.43 | 0.04 |

Values expressed as 10$^6$ cfu.ml$^{-1}$.

[1]Times I, II, III, IV, V and VI after the start of fermentation were at 3, 5, 11, 18, 28 and 34 days, respectively.

[2]A = first experiment (July of 2000).

[3]B = second experiment (August of 2001); [4] Similar to *C. gropengiesseri.*

The molecular profiles of the 10 *S. cerevisiae* isolates obtained by RAPD-PCR analysis at each collection were identical to the respective starter strains used (Fig. 1). Indigenous *S. cerevisiae* isolates with rough colonial texture and irregular margins were obtained from the vats with population counts higher than the selected start strains at the end of the second experiment. The RAPD-PCR profiles of five of these isolates from vats inoculated with strain UFMG-A829 are shown in Figure 2. Three of these isolates presented molecular profiles identical to this starter strain, and two of them presented molecular profiles which were identical to each other, while being different from the selected starter strain. These latter isolates were considered to be indigenous *S. cerevisiae* of the fermentation process in this distillery. Torriani et al. (1999) successfully used the M13 primer to identify *senso stricto Saccharomyces* strains and also for intra-species differentiation. The authors were able to differentiate *S. cerevisiae* strains with different phenotypes using the primer M13. Lieckfeldt et al. (1993), using the M13 primer, showed the differences among 23 *S. cerevisiae* strains associated with different fermentation products (wine, beer, spirits, as well as bakery products).

Guerra et al. (2001) were able to differentiate the *S. cerevisiae* strains from the spontaneous fermentation for cachaça production using primer M13. In that study, the authors detected a high degree of genetic polymorphism among the strains within a 24-h fermentation cycle for the production of cachaça. In the present study, this technique was also accurate when used to discriminate the isolates of the two strains used as starters during the fermentation for cachaça production.

The characterization of strains that could potentially be used as starters and the observation of such strains using molecular techniques are important for the determination of the time these strains remain in the fermentation process. The results of the present study revealed that two strains were able to prevail in the process for approximately 30 days. Therefore, with periodic yeast replacement, it should be possible to maintain a higher degree of cachaça homogeneity throughout the production season and between the seasons, thus affording a higher quality standard for the beverage.

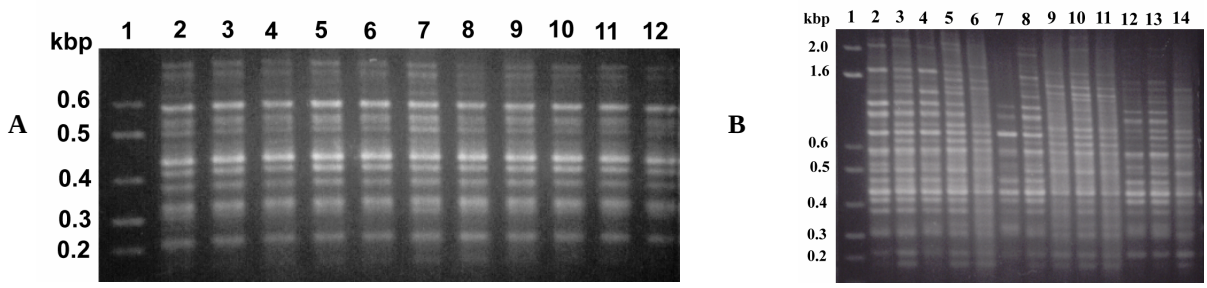


**Figure 1** - RAPD-PCR comparison between the respective starter strains inoculated in fermentation vats with two isolates from the prevailing morphotype of each collection during the experiment carried out in a cachaça distillery. Fig. 1A: lanes: 1, 1 kbp ladder; 2, *Saccharomyces cerevisiae* K1-V116; 3 and 4, *S. cerevisiae* K1-V116 re-isolated after 3-day fermentation; 5 and 6, *S. cerevisiae* K1-V116 re-isolated after 5-days; 7 and 8, *S. cerevisiae* K1-V116 re-isolated after 11-days; 9 and 10, *S. cerevisiae* K1-V116 re-isolated after 18-days; 11 and 12, *S. cerevisiae* K1-V116 re-isolated after 28-days. Fig. 1B: lanes: 1, 1 kbp ladder; 2, *S. cerevisiae* UFMG-A829; 3 and 4, *S. cerevisiae* UFMG-A829 re-isolated after 3-days of fermentation; 5 and 6, *S. cerevisiae* UFMG-A829 re-isolated after 5-days; 7 and 8, *S. cerevisiae* UFMG-A829 re-isolated after 11-days; 9 and 10, *S. cerevisiae* UFMG-A829 re-isolated after 18-days; 11 and 12, *S. cerevisiae* UFMG-A829 re-isolated after 28-days, 13 and 14, *S. cerevisiae* UFMG-A829 re-isolated after 34-days.

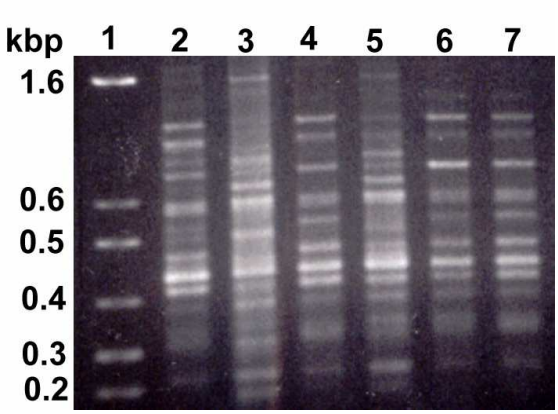


**Figure 2 -** RAPD-PCR comparision between starter strain UFMG-A 829 and *S. cerevisiae* isolates with rough colonial texture and irregular margins. Lanes: 1- 1 kbp ladder; 2- *S. cerevisiae* UFMG-A 829; 3 and 5 indigenous *S. cerevisiae*; 4, 6 and 7 identical to *S. cerevisiae* UFMG-A 829.

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## RESUMO

Duas linhagens de *Saccharomyces cerevisiae* foram testadas como iniciadoras em uma destilaria de cachaça. Foram utilizadas as linhagens de *S. cerevisiae* UFMG-A829, isolada de fermentação de cachaça, e *S. cerevisiae* K1-V1116, de origem vinícola. A permanência de cada linhagem durante a fermentação foi determinada por RAPD (Random Amplified Polymorphic DNA)-PCR, utilizando o iniciador M13. As duas linhagens predominaram nas dornas de fermentação por aproximadamente 30 dias. Leveduras não-*Saccharomyces* e *S. cerevisiae* indígenas foram isoladas em menor proporção durante o experimento. As linhagens de *S. cerevisiae* indígenas apresentaram perfis moleculares distintos em relação às linhagens iniciadoras. As duas linhagens foram promissoras para serem utilizadas como iniciadoras do processo fermentativo para a produção da cachaça.

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