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Isolation of *Saccharomyces cerevisiae* strains producing higher levels of flavoring compounds for production of "cachaça" the Brazilian sugarcane spirit

Maristela de Araújo Vicente [a], Luciano Gomes Fietto [a], Ieso de Miranda Castro [a], Ana Nery Gonçalves dos Santos [b], Maurício Xavier Coutrim [b], Rogelio Lopes Brandão [a,*]

[a] *Laboratório de Biologia Celular e Molecular, Núcleo de Pesquisas em Ciências Biológicas, ICEB II, Departamento de Farmácia, Escola de Farmácia, Universidade Federal de Ouro Preto, Campus do Morro do Cruzeiro. 35.400-000 – Ouro Preto – Minas Gerais – Brazil*

[b] *Laboratório de Análises Físico-Químicas, Departamento de Química, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto – Minas Gerais – Brazil*

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Abstract

In Brazil, spontaneous fermentation and open vessels are still used to produce cachaça (the Brazilian sugarcane spirit) and this fermentation is characterized by mixed cultures with continuous succession of yeast species. This work shows the development of a methodology for isolation of yeasts, particularly *Saccharomyces cerevisiae*, used in the production of cachaça. According to the proposed strategy, the strains were selected for their ability to adapt to stress conditions encountered during fermentation of the sugarcane juice such as high sucrose concentration; high temperatures and high alcohol concentration; for their capacity to flocculate; and for their higher fermentative ability. For strains with such characteristics, specific procedures were employed to select for 5,5,5-trifluoro-DL-leucine (TFL) and cerulenin-resistant strains, since these characteristics are related to a higher capacity of production of the flavoring compounds isoamyl alcohol and caproic acid, respectively. The effectiveness of such a selection strategy was documented. Taken together, the results obtained present the development of a new strategy to isolate yeast strains with appropriated characteristics to be used in the cachaça industry. Moreover, the results obtained offer an explanation for the great variability in terms of chemical composition found in products obtained even in a single distillery.

Keywords: Sugar-cane spirit; *Saccharomyces cerevisiae*; Flavoring compounds

1. Introduction

Cachaça (pronounced kha-sha-ssa) is the most popular distilled beverage produced in Brazil. The annual production reaches 2 billion liters, representing an economic activity estimated to be $ 2.5 billion in U.S. dollars. Due to the internationalization of cachaça, some confusion over rum and cachaça identities has arisen. Cachaça is the denomination of the typical Brazilian spirit produced from the distillation of fermented sugarcane juice, whereas rum, traditionally produced in Caribbean countries, is a sugarcane spirit obtained by the distillation of cooked fermented sugar cane juice and molasses. Furthermore, analytical data of different compounds have been obtained providing a very good discrimination between the two classes of beverages (Cardoso et al., 2004).

The starter for cachaça production is prepared as a mixture of crushed corn, lemons, rice, and starch as sources of supplementary substrates forming a paste and left for spontaneous fermentation at room temperature for 12–24 h. Then sugarcane juice is added to the paste until it is submerged and left in rest for more than 24 h. A new amount of sugarcane juice is added when release of CO_2 indicating fermentative activity by yeast strains naturally present in sugarcane juice is observed; the mixture is left at rest by 24 h once more. This procedure is repeated until a volume corresponding to 20% of the substrate volume to be fermented in the vats is obtained (5–20 days). Thus, the number of yeast increases enough for the

* Corresponding author. Fax: +55 31 3559 1680.
E-mail address: rlbrand@nupeb.ufop.br (R.L. Brandão).

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initiation of a semi-continuous fermentation cycle, taking an additional 24 h.

The cachaça has an ethanol content between 38% (v/v) and 48% (v/v) at 20 °C and also exhibits higher alcohols, ethyl esters, aldehydes, ketones and organic acids that are responsible for the distinct aroma of the beverage (Nonato et al., 2001). Esters formed from the organic acids and alcohols during fermentation and secondary fermentation play an important role in the formation of the sensory features interfering in the formation of a typical bouquet in spirits (Kłosowski and Czuprynski, 2006).

Among flavoring compounds found in other beverages like sake, isoamyl acetate is the main contributor to the fruity and sweet aroma (Yoshikawa, 1999); however, its content is particularly low in the cachaça. This ester is synthesized from isoamyl alcohol and acetyl coenzyme A by the action of the alcohol acetyltransferase and hydrolyzed by esterases (Yoshikawa, 1999; Mason and Dufour, 2000; Kłosowski and Czuprynski, 2006). The isoamyl alcohol can be produced from α-ketoisocaproate, an intermediary in the leucine biosynthetic pathway. In this pathway, the enzyme α-isopropyl malate synthase (α-IPM) promotes the conversion of α-ketoisovalerate in α-isopropyl malate being regulated by feedback inhibition when the levels of L-leucine are high (Casalone et al., 1997). In this case, there is a decrease in the synthesis of isoamyl alcohol (Ashida et al., 1987; Cavalieri et al., 1999). Yeast strains that overproduce L-leucine can be obtained if the feedback inhibition of α-IPM is lost; in such strains, there is also an increase in the production of higher alcohols and their esters, particularly isoamyl alcohol and isoamyl acetate. A successful method to isolate such strains is to expose cells to 5,5,5-trifluor-DL-leucine (TFL), an L-leucine analog, and to select the TFL-resistant strains (Satyanarayana et al., 1968; Bussey and Umbarger, 1970). Very recently, a new mutation in *LEU4* gene (that encodes for α-IPM) that eliminates leucine feedback inhibition and causes hyperproduction of isoamyl alcohol was identified (Oba et al., 2005).

Another important flavoring compound produced by yeast is the ester ethyl caproate (Yoshikawa, 1999). Its synthesis and accumulation in yeast cells is dependent on the presence of the precursors (ethanol and caproic acid) as well as the control of alcohol acyl transferase and esterase enzymes (Verstrepen et al., 2003). The biosynthesis of fatty acids is catalyzed by fatty acid synthetase that is inhibited by cerulenin (Ichikawa et al., 1991). Therefore, a strategy used to isolate strains with a higher capacity to produce fatty acids is to obtain cerulenin-resistant strains.

In the present study, *Saccharomyces cerevisiae* were isolated from fermentation vats of a cachaça distillery and the strains characterized according to their abilities to ferment, flocculate, and produce flavor compounds. The utilization of selected strains with such abilities can benefit the Brazilian cachaça industry by contributing for the reduction of the costs of production by lower time of fermentation and easier separation of the yeast cells from fermented must at the end of the fermentation process and by increasing the levels of the desired flavoring compounds in the beverage.

2. Materials and methods

2.1. Sample collection and yeast identification

The yeast strains described in this work were isolated from a cachaça distillery in Ouro Preto, State of Minas Gerais, Brazil. Samples of must were collected from fermentation vats in sterile 250-ml flasks. Following collection, flasks were placed on ice, transported to the laboratory and processed within 1 h.

Must samples were diluted in sterile distilled water (1:10; 1:100; 1:1000) and 0.1 ml of each dilution were spread on plates containing agar (Difco, Code 1545-01, Difco Laboratories, Detroit, USA) (1.5%, w/v), peptone (Difco, Code 211840) (2%, w/v) and yeast extract (Difco, Code 1515-17) (1%, w/v) supplemented with glucose (20%, w/v) (Sigma, Code G8270, Sigma Chemical Co, St. Louis, USA), chloramphenicol (Sigma Code C0378) (0.1%, w/v) and ethanol (8%, w/v) (Quimex, Code QX 160.1000, F. Maia Indústria e Comércio Ltda, São Paulo, Brazil). Plates were incubated at 30 °C or at 37 °C for 3 days. These procedures permitted to isolate yeast strains, primarily identified by basic morphological characteristics (Pataro et al., 2000).

The isolated strains were submitted to biochemical and molecular methods for species identification. The biochemical methods were based on the utilization of carbon and nitrogen sources, as previously described (Vaughan-Martini and Martini, 1993; Sanni and Lönner, 1993). The molecular method for yeast identification was based on the amplification and sequence analysis of the ribosomal DNA internal transcribed spacer region (ITS) (White et al., 1990). The primers used to amplify the rDNA internal transcribed spacer region were ITS1 (CGG GAT CCG TAG GTG AAC CTG CGG) and ITS4 (CGG GAT CCT CCG CTT ATT GAT ATG C) (Invitrogen, Invitrogen Life Technologies, Carlsbad, USA). The amplification reaction was done in 30 μl (final volume) containing 20 pmol of each primer, 300 ng of genomic DNA template, 0.25 mM each dNTP (Amershan Biosciences, Code 27-2035-02, Amershan Biosciences, Piscataway, USA), 1.5 mM $MgCl_2$ (Sigma, Code M2670) and 0.5 U Taq polymerase (Invitrogen, Code 10966-018). The reactions were run for 40 cycles with denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min. The PCR products were cloned into the pBluescript II SK (+/−) vector (Stratagene, Code 212205, Strategene, La Jolla, USA) and sequenced using the Big Dye™ Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Code 439032, Applied Biosystems, Foster City, USA). Sequences of the PCR products from different strains were compared with the ITS region deposited in the *S. cerevisiae* genome database (http://genome.www.standford.edu:5555/cgi-bin/blastsgd) and the percentage of similarity among the fragments was calculated using the Blast program.

2.2. Mutagenesis of yeast by UV radiation

Mutagenesis of yeast strains was done by UV radiation. The yeast cells were grown until the middle of the exponential

phase ($OD_{600\ nm}$ approximately 1); then, they were collected by centrifugation, washed and resuspended in sterile and distilled water. Appropriate dilutions of this suspension in sterile and distilled water (1:10; 1:100; 1:1000) were spread directly in Petri dishes containing yeast nitrogen base without amino acids 0.67%, w/v (Difco, Code 0919-15); glucose 2%, w/v and 1 mM 5,5,5-trifluor-DL-leucine (TFL, Lancaster, Code 12890, Lancaster Synthesis, Morecambe, England). Each plate was exposed to different UV doses (2–15 mJ). Resistant colonies appearing after 3–5 days were isolated as TFL-resistant strains.

2.3. Physiological characteristics of the Saccharomyces strains

To examine the capacity to produce H_2S, *S. cerevisiae* strains isolated from the fermentation vats and identified by biochemical methods were inoculated on Bismuth Sulfite Agar Medium (Merck, Code 1.05418.0500, E. Merck, Darmstadt, Germany) and incubated at 30 °C for 3 days. Colonies that exhibit brown or black color are H_2S producers (Jiranek et al., 1995). Positive strains were discarded.

The strains that do not produce H_2S were evaluated according to their ability to flocculate during the cellular growth in YP liquid medium (peptone 2% w/v and yeast extract 1% w/v). Cells were inoculated into tubes containing 4 ml YP medium with glucose (2%, w/v) and grown in a rotatory incubator (New Brunswick, Model G25, New Brunswick, Edison, USA) at 200 rpm and 30 °C until absorbance at 600 nm reached 1.0. Flocculent strains were selected by their capacity to sediment at the bottom of the tube in the end of fermentation as described by Verstrepen et al. (2001).

Flocculent yeast strains were submitted to tests of ethanol tolerance and higher temperatures. Colonies were transferred to YP medium supplemented with glucose (2%, w/v) and different concentrations of ethanol (10%, w/v; 15%, w/v; or 20%, w/v) and incubated at 30 °C for 3 or 4 days. Ethanol-tolerant cells (up to 15% w/v ethanol) were transferred to plates containing YP medium plus glucose (2%, w/v) and ethanol (15%, w/v) and incubated at different temperatures (30 °C; 37 °C; 42 °C) also for 3 or 4 days. The ethanol- and temperature (up to 37 °C)-tolerant cells were selected to continue the process.

2.4. Determination of invertase and α-IPM activities

Measurement of specific activity of invertase was measured as described by Goldstein and Lampen (1975) with the modification introduced by Celenza and Carlson (1989). The assay was carried out at pH 5.1 and 37 °C and it is based on the hydrolysis of sucrose by measuring the amount of glucose liberated (nmol glucose^{-1} min mg protein^{-1}). The invertase activity was measured in growth cells under repressed (glucose) and derepressed (raffinose, Sigma, Code R0250) conditions. The α-isopropyl malate syntase (α-IPM) activity was determined by the measurement of the amount of coenzyme A (Sigma, Code A2056), generated during the incubation of the enzyme with 0.2 μmol acetyl coenzyme A and 1.0 mol μ-ketoisovalerate (Aldrich, Code 06416DX, Aldrich Chemical Co, Milwaukee, USA) at 30 °C. After 10 min, by adding 0.75 ml of ethanol, the reaction was interrupted. Then, 0.5 ml of a 1 mM of 5,5-dithiobis-(2-nitrobenzoate) (DNTB) (Lancaster, Code 9868, Lancaster Synthesis, Morecambe, England) solution in 20 mM Tris–HCl buffer pH 8.0 was added. DNTB reacts with coenzyme A forming a yellow anion measured at 412 nm in a spectrophotometer. The molar absorptivity for this complex is 13,600. The activity was expressed in μmol of coenzyme A min^{-1} mg protein^{-1} (Ulm et al., 1972). Protein was determined according to Lowry et al. (1951).

2.5. Killer toxin production

The killer toxin production was followed using *Candida glabrata* NCYC 388 and *S. cerevisiae* NCYC 1006 as sensitive references strains (Pataro et al., 1998). Briefly, YM agar (yeast extract 0.3%, malt extract 0.3% w/v [Biobras, Code 180-3, Biobras SA, Montes Claros, Brazil], peptone 0.5% w/v and glucose 1%) supplemented with 0.003% methylene blue (Sigma, Code M9140) and buffered to pH 4.2 with sodium acetate (Sigma, Code S5636) was previously seeded with *C. glabrata* and *S. cerevisiae*. Then, isolated strains were inoculated and identified as killer if they produced a halo of dark blue dead cells.

2.6. Isolation of TFL- and cerulenin-resistant strains

The selected *S. cerevisiae* strains presenting higher invertase activity (greater than 10,000 U mg protein^{-1} when grown on raffinose) were inoculated in YP medium with glucose (2%) and incubated at 30 °C for 3 days. Then, the strains were transferred to other plates containing minimal medium (YNB) supplemented with glucose (2% w/v) and 1 mM of TFL or with 25 μM of cerulenin (Sigma, Code C2389, Sigma Chemical CO, Steinhein, Germany) (Ashida et al., 1987; Ichikawa et al., 1991). These plates were incubated at 30 °C for 3 days. The colonies that developed on the plates containing the inhibitors were isolated as resistant strains.

2.7. Fermentation tests in culture media

The cellular growth in liquid medium and the fermentative capacity were tested in three different strains. Two of them were selected through the strategy here described (named M70 and M24); the other one was obtained from strain M24 by UV mutagenesis (named M24mt). All were pre-grown in tubes with 4 ml of YP medium plus glucose (2%, w/v) at 30 °C for 24 h. Part of this culture, sufficient to give an initial absorbance of 0.15 at 600 nm, was transferred to sterile Erlenmeyer flasks containing 150 ml of minimal medium containing 16% (w/v) sucrose (Sigma, Code S5016). Then, these flasks were placed in a rotatory incubator (200 rpm) at 30 °C. To evaluate the cellular growth at determined times, as indicated in the figures, 50 μl of samples were diluted with 950 μl of fresh medium and absorbance at 600 nm was measured. Fifteen-milliliter samples also were taken from the growth medium, centrifuged at 1000×*g* for 5 min at 4 °C and the resultant supernatant divided

in two parts. Five-milliliter samples were used in the measurements of sucrose consumption, as well as ethanol and glycerol production. Ten-milliliter samples were distilled in a microdistillator to determine the concentration of isoamyl alcohol, isoamyl acetate, caproic acid and ethyl caproate. We developed this extractive methodology because in the cachaça production, the beverage is obtained by distillation of the fermented sugarcane must.

Ethanol production was determined by using a potassium dichromate method as described by Salik and Povoh (1993). Glycerol was determined enzymatically using a commercial kit (Boehringer, Code 148270, Boehringer, Mannheim, Germany). Sucrose consumption was measured in 500-μl samples transferred to 1.5 ml Eppendorf tubes in which 10 μl HCl 1 M was added. Then, these tubes were heated in a Thermomixer Compact (Eppendorf, Code 5350 000.013, Eppendorf AG, Hamburg, Germany) by 15 min at 95 °C. The samples were neutralized with 10 μl NaOH 1 M and glucose originated from the acid hydrolysis of sucrose was measured using the classical glucose/peroxidase method (Goldstein and Lampen, 1975).

The analysis of the content of isoamyl alcohol, isoamyl acetate, caproic acid and ethyl caproate were performed in a gas chromatograph (Varian Model 3380, Varian Incorporation, Palo Alto, USA) equipped with a split–split less inlet injector and flame ionization detector (FID) presenting a capillary polyethylene glycol column (30 m × 0.25 mm with 0.25-μm-thick stationary phase) (Perkin Elmer, Type COL-ELITE WAX-3, Perkin Elmer Instruments, Shelton, USA). One microliter of sample was injected with a split ratio of 1:80. The injector and detector temperatures were 225 °C and 265 °C, respectively. The make-up gas was nitrogen at 30 ml/min. The temperature profile was 30 °C for 5 min, raised to 150 °C at 25 °C min^{-1} and held for 10 min, raised to 200 °C at 50 °C min^{-1} and held for 5 min. The identification of isoamyl alcohol, isoamyl acetate, caproic acid and ethyl caproate was performed through chemical standards addition, while quantitative determination of the flavoring compounds was accomplished by internal standard method. 1-Hexanol (Merck, Code 804393, E. Merck, Darmstadt, Germany) was used as internal standard (Nonato et al., 2001; Boscolo et al., 2000).

2.8. Reproducibility of results

All experiments were performed at least three times with consistent results. Standard deviations are indicated in the figures. The data also were evaluated statistically by ANOVA analysis of covariance (orthogonal designs) followed by a one-way analysis of the means from different curves (Snedecor and Cochran, 1993).

3. Results and discussion

3.1. Isolation of strains with desired characteristics for cachaça production

The most remarkable characteristics of the production of cachaça in Brazil are related to the preparation of the starter and to the conditions of the fermentation. The fermentation of sugarcane can be considered spontaneous, since traditionally there is no utilization of selected yeast strains. Indeed, it is characterized by a high diversity of microbial species present in the starter, predominantly yeasts. On the other hand, the fermentative process is also performed in successive cycles with daily additions of sugarcane juice also containing new yeast strains (Morais et al., 1997; Pataro et al., 1998, 2000; Silva-Filho et al., 2005). As a consequence, the chemical composition of the beverage is quite variable during the annual period of production: May to November.

Using the methodology described, 1344 yeast strains were isolated directly from samples of diluted fermented must, previously collected in distilleries that were preliminarily identified based on morphological characteristics. All strains demonstrated the ability to grow in similar conditions as those encountered during fermentation of the sugarcane must: high sucrose concentration (20%, w/v) and at temperatures like 37 °C. Furthermore, they are all ethanol resistant (up to 15% v/v). Following standard methods for identification of the yeast genus *Saccharomyces* (Vaughan-Martini and Martini, 1993; Sanni and Lönner, 1993), the initial number of isolated strains was reduced to 1304 yeast strains. From this group, we chose randomly 99 strains (approximately 7.5% of the total) to perform further tests. Among those, 27 strains did not follow the expected behavior typical for *S. cerevisiae*, according to Vaughan-Martini and Martini (1993). The 72 remaining strains were tested for the capacity to produce H_2S, an undesirable compound that must be absent in fermented beverages (Ribeiro and Horii, 1999) with only one strain being excluded (results not shown). The ability to flocculate the 71 remaining strains was evaluated resulting in the selection of 46 flocculent strains. All flocculent strains also demonstrated high ethanol tolerance (15%, v/v) even when incubated at 37 °C (data not shown).

We further examined the level of invertase activity of the flocculent strains, since a high invertase activity is essential for yeast growth in media containing sucrose as the principal carbon source (Ekunsanmi and Odunfa, 1990) in two different conditions: repressive (in the presence of glucose, there is repression of the expression of the encoding invertase gene (SUC2), Gancedo, 1998), and non-repressive with raffinose as carbon source. Fig. 1 shows that almost all strains present the typical behavior, i.e., low activity when glucose is the carbon source, and high activity when cells were grown on raffinose. By this way, we selected eight strains presenting the invertase activity greater than 10,000 U mg $protein^{-1}$ when grown on raffinose.

Since many other wild dominant strains found in the fermentation vats from the distilleries present the ability to produce killer toxins, we tested the “killer” behavior of the these eight strains. All of them inhibited the growth of the toxin-sensitive *C. glabrata* and *S. cerevisiae* strains (data not shown).

3.2. Selection of S. cerevisiae strains with high production of flavoring compounds

Considering that higher alcohols and esters are important to the flavoring characteristics of cachaça, we tested the resistance

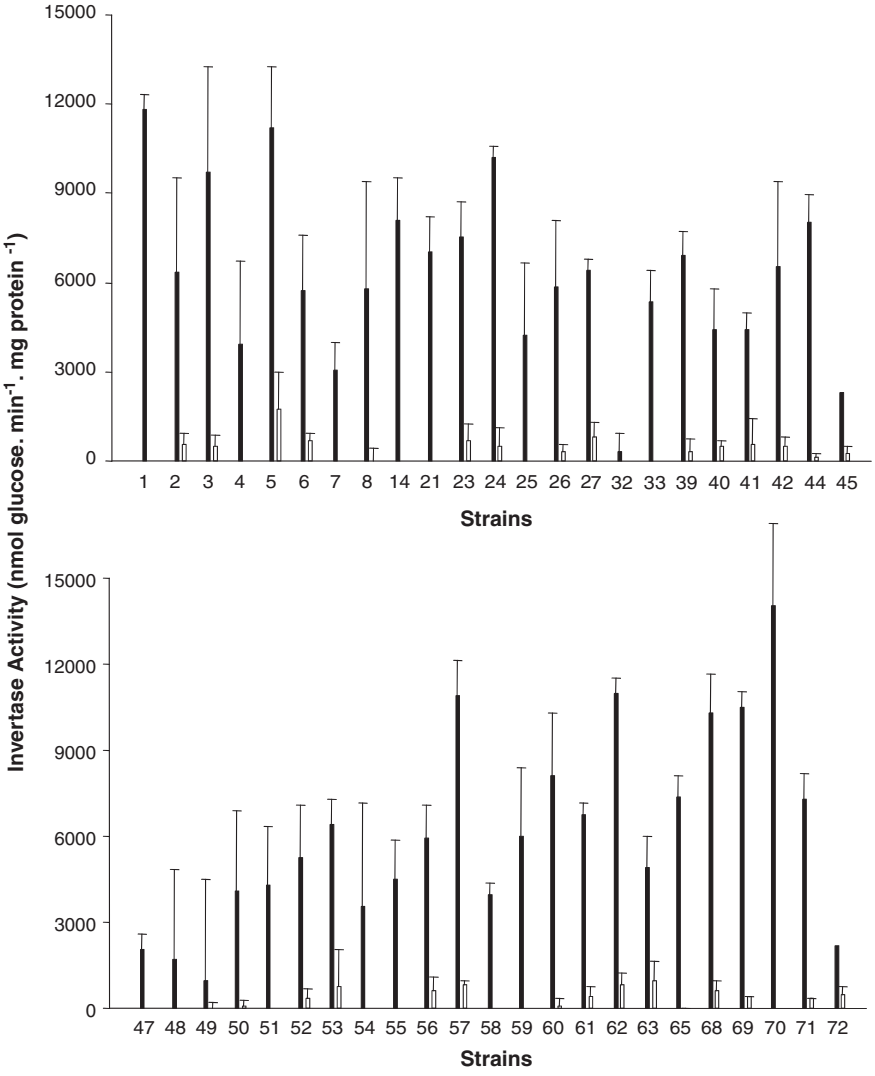

Fig. 1. Invertase activity under repressive (white bars) and non-repressive (black bars) growth conditions from different flocculent yeast strains. The cells were grown on 4% glucose (white bars) or on 2% raffinose (black bars) up to OD_{600nm} 1.0–1.5. The enzyme activity was measured in cellular extracts from cells obtained from both growth conditions.

of the yeast strains to TFL and cerulenin, since these drugs have been used to select strains with improved capacity to produce isoamyl alcohol and caproic acid, precursors of isoamyl acetate and ethyl caproate, respectively (Ashida et al., 1987; Ichikawa et al., 1991). Contrary to the strategy proposed by Arikawa et al. (2000) that generated mutants through chemical treatment, *S. cerevisiae* strains presenting good adaptation to the fermentative conditions for the production of cachaça and also for natural resistance to TFL and cerulenin were selected. Table 1 shows that among eight selected strains, only M70 strain is resistant to both compounds, while M24 strain is sensitive to both.

We confirmed the taxonomic identity of these two strains by cloning and sequencing the rDNA internal transcribed spacer region (ITS). The differences in the rDNA ITS have been used to identify yeast species (Valente et al., 1996; Lopes et al., 1998). Sequences analysis of the rDNA ITS from M70 and M24 strains showed complete identity with the sequence deposited in the *S. cerevisiae* database.

In agreement to the resistance to TFL, the α-IPM activity of M70 strain was not affected by the presence of increasing leucine concentrations; contrarily, it is even activated in low leucine concentrations (Fig. 2). In spite of its sensitivity to TFL, the α-IPM activity found in M24 strain seems to be not

Table 1
5,5,5-trifluoro-DL-leucine (TFL) and cerulenin resistance of different strains of *S. cerevisiae*

Strain	Cellular growth in presence of drugs[a]	
	TFL	Cerulenin
M 5	+	–
M 62	+	–
M 69	+	–
M 70	+	+
M 1	–	+
M 24	–	–
M 57	–	+
M 68	–	+

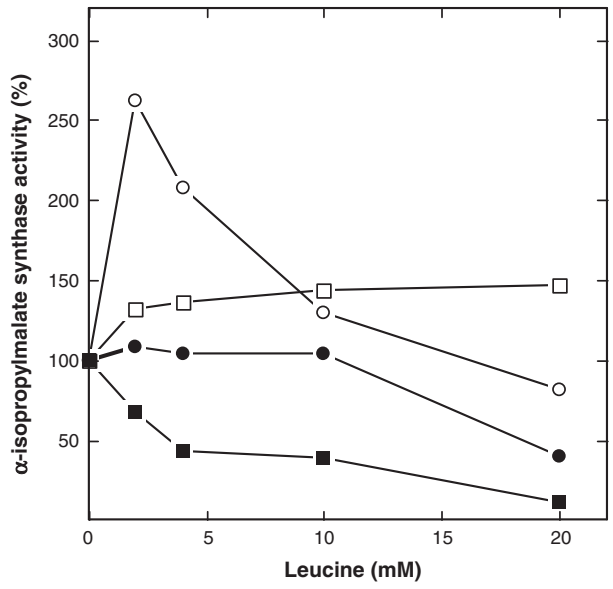

Fig. 2. α-Isopropyl malate synthase (α-IPM) activity from two yeast strains M70 (○) and M24 (●), one UV mutant obtained from strain M24 (□, named M24mt) and haploid wild-type strain W303 (■), used as control of leucine-sensitive α-IPM activity). The activity was measured in the absence or in the presence of increasing concentrations of leucine. The enzymatic activity was originally expressed as the amount of enzyme, which catalyzes the formation of 1 μmol of coenzyme A min^{-1} mg $protein^{-1}$. Results are expressed as percentage of the activity observed without leucine.

inhibited at least until 10 mM of leucine. In agreement with Cavalieri et al. (1999), these results suggest the presence of different alleles regarding the gene encoding for α-IPM p.

In order to further characterize these strains, we decided to evaluate several parameters such as cellular growth, sucrose consumption and ethanol production (Fig. 3). Our results showed no differences in these parameters indicating that both strains present comparable fermentative properties. Nevertheless, the M70 strain showed higher production of isoamyl alcohol and isoamyl acetate ($p<0.05$) (Fig. 4 panels A and B, respectively). These results indicate that the isolation strategy used in this work was able to discriminate yeast strains that, although presenting similar fermentative profiles, produced different levels of flavoring compounds important for the beverage sensorial properties.

On the other hand, we observed that only the cerulenin-resistant strain M70 produced detectable levels of caproic acid (Fig. 5). The maximal production is achieved around 10 h with a strong decrease towards the end of fermentation. However, we did not detect any trace of ethyl caproate in the distilled obtained from the fermented medium produced by both strains.

It is already known that ethyl caproate can be synthesized from caproyl-Coa and ethanol, a reaction catalyzed by the enzyme alcohol acyltransferase, or from caproic acid and ethanol by action of specific esterases (Asano et al., 2000; Kłosowski and Czuprynski, 2006). Thus, since cerulenin is a

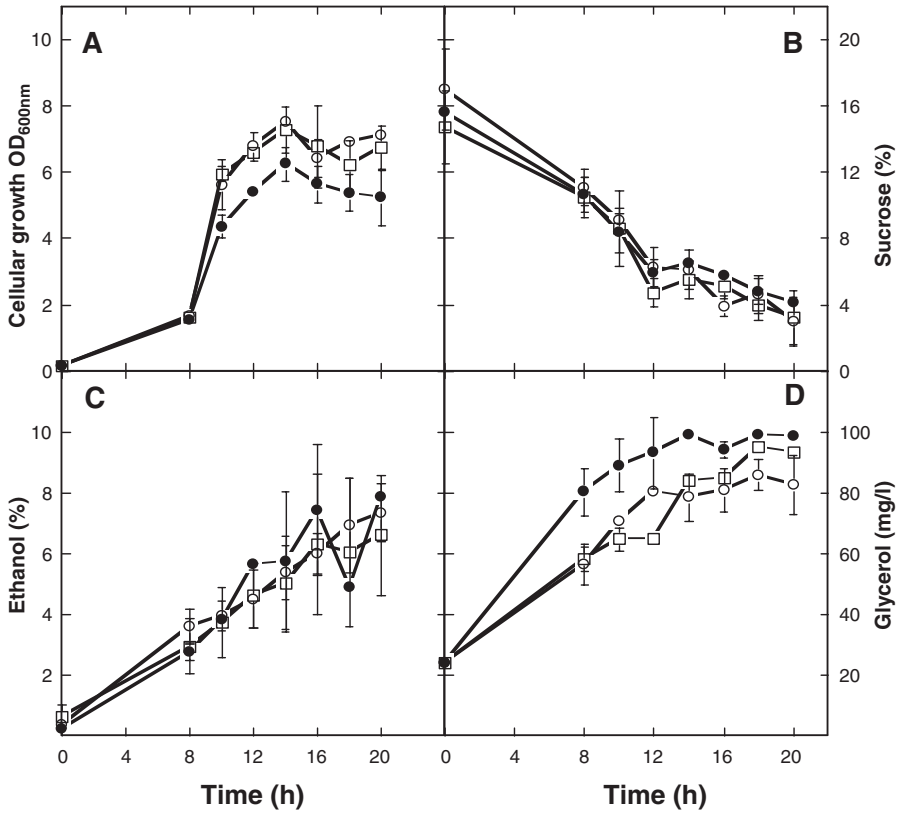

Fig. 3. Cellular growth (Panel A); sucrose consumption (Panel B); and production of ethanol (extracellular concentration) (Panel C) or glycerol (extracellular concentration) (Panel D) from yeast strains M70 (○), M24 (●) and one UV mutant obtained from strain M24 (□, named M24mt) grown in minimal medium with sucrose as carbon source.

specific inhibitor of fatty acid synthase and that cerulenin-resistant mutants normally produce higher amounts of ethyl caproate (Ichikawa et al., 1991), a reasonable explanation for the absence of ethyl caproate production can be related to control the activities of both enzymes (different levels of expression and/or activity of the enzyme(s) involved in this specific esterification reaction). It was proposed that an alcohol acyltransferase, encoded by the *EHT1* gene, is apparently involved in the generation of ethyl caproate and other esters even so, it has not been studied in detail (Mason and Dufour, 2000). In this sense, it is already known that the expression of *ATF1* and *ATF2* genes that encode for the most prominent alcohol acetyltransferase activities in yeast cells are regulated negatively by the availability of oxygen and unsaturated fatty acids. Although the Atf1 p and Atf2 p activities are not required for the synthesis of such ester, a similar regulation process could also affect the activity of the specific alcohol acyltransferase. Thus, more experiments will be necessary to clarify this specific point. Recently (Verstrepen et al., 2005), it was also demonstrated that the *S. cerevisiae* alcohol acetyl transferase Atf1 p is localized in lipid particles.

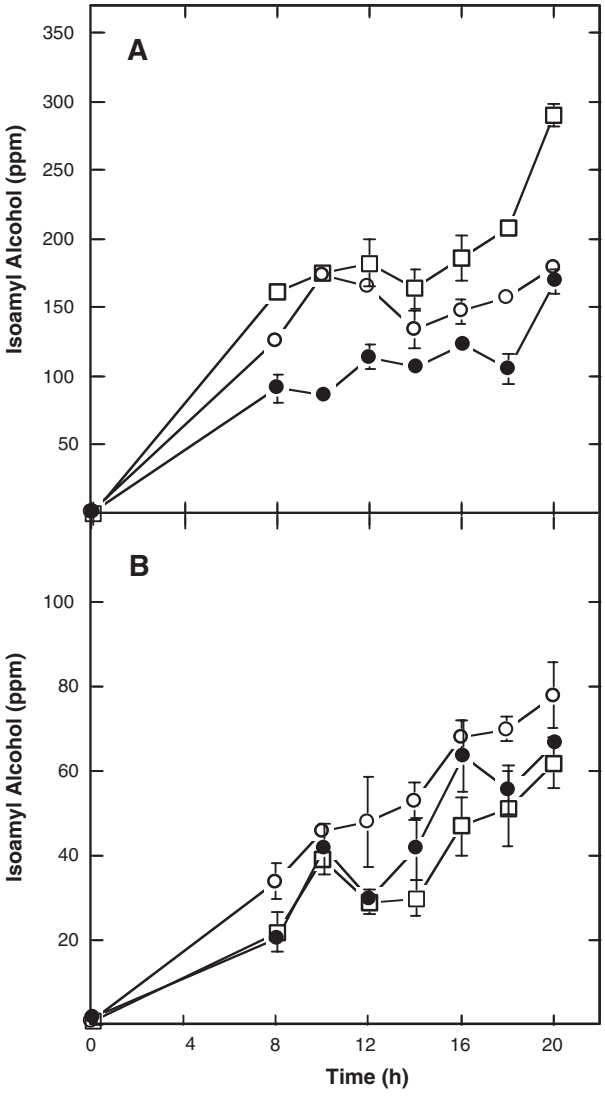

Fig. 4. Isoamyl alcohol (Panel A) and isoamyl acetate (Panel B) production during the cellular growth of two isolated yeast strains M70 (○), M24 (●) and one UV mutant obtained from strain M24 (□, named M24mt) strains in minimal medium presenting sucrose as carbon source.

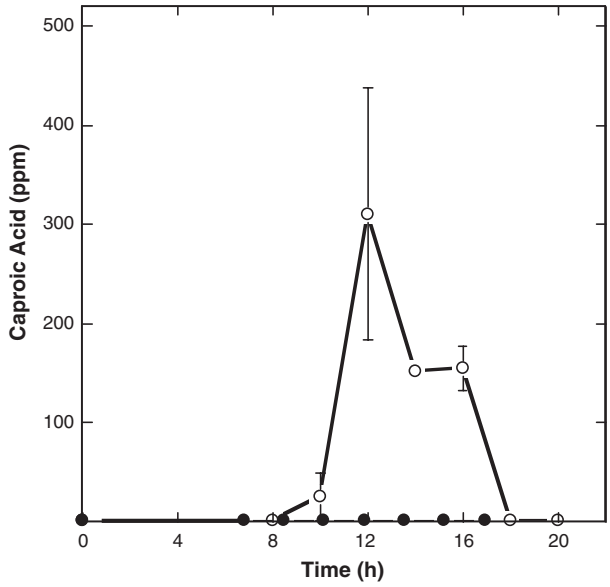

Fig. 5. Caproic acid produced during the cellular growth of two isolated yeast strains M70 (○) and M24 (●) strains in minimal medium presenting sucrose as carbon source.

3.3. Development of TFL-resistance by using sensitive strain

By using UV radiation, a TFL-resistance mutant was obtained from the TFL-sensitive M24 strain. Fig. 2 shows an activating effect on the α-IPM activity observed in higher concentrations of leucine. Interestingly, Casalone et al. (1997) performed a genetic and biochemical characterization of many *S. cerevisiae* mutants resistant to TFL that permitted the identification and classification of these mutants in clusters. Genetically, two groups were found: one comprising strains with dominant mutations probably located on the *LEU4* gene which is coding for the α-IPM enzyme, and other with recessive mutations. Even among these strains with dominant mutations, there were clear differences (as high as 100%) in the production of isoamyl alcohol (Casalone et al., 1997).

However, in spite of the fact that the production of isoamyl alcohol by M24mt strain was higher ($p > 0.05$) than the parental strain (M24) (Fig. 4 panel A), the levels of isoamyl acetate are comparable in both strains (Fig. 4 panel B), indicating that these strains probably present lower alcohol acetyltransferase activity than strain M70. In this regard, it has been previously shown that the alcohol acetyltransferase is extremely unstable at room temperature even “in vivo” (Lyness et al., 1997). Therefore, a possible explanation for these observations would be a decreased stability of this enzyme in both M24 strains as compared to M70 strain.

4. Conclusion

According to official records, around 9000 distilleries exist in the Minas Gerais State, which is responsible for near 20% of

the total production every year in Brazil. Therefore, we believe that the methodology shown here will have a positive impact in the industry, offering to the producers the possibility of choice between the utilization of the traditional starter or an innoculum prepared with selected strains already adapted to their specific conditions. Up to now, fermentation characteristics have been used as the sole criteria for selection of yeast strains isolated from distilleries. Moreover, the results presented here can be used to explain why the final quality of the Brazilian cachaça is so variable when compared to different beverages also obtained by fermentation. It is already known that during the period of production (May to November), the yeast populations are in continuous succession with *S. cerevisiae* as the prevalent species (Pataro et al., 1998, 2000). Thus, by combining classical methods of yeast identification with experiments to test some physiological parameters of the yeast strains, we were able to develop a new strategy to isolate strains that can be used by the Brazilian producers (Vicente et al., 2003) contributing to improve the quality of the cachaça produced by different distilleries.

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