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Selected *Saccharomyces cerevisiae* yeast strains and accurate separation of distillate fractions reduce the ethyl carbamate levels in alembic cachaças

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ABSTRACT

Ethyl carbamate (EC) is a potentially toxic compound that may be present at concentrations above maximum limits established in alcoholic beverages, such as cachaça. Most traditional alembic cachaça is produced on a small scale using empirical knowledge. The fermentation step is conducted using yeasts that are endogenous to the sugar cane, and the distillation process is relatively uncontrolled. In this study, gas chromatography coupled with mass spectrometry was used to determine the EC levels in distillate musts and fractions produced by spontaneous or selected *Saccharomyces cerevisiae* strains. The aim was to verify the influence of selected strains as starters for fermentation compared with spontaneous fermentation on EC formation. The distillate fractions from these two production processes were also analysed. Our results demonstrated higher levels of EC (which surpass the limits defined by Brazilian law) in cachaças produced by spontaneous fermentation (50%) compared with the selected strains (30%); and the distillation step showed great contribution for the reduction of the compound. From must to distillate fractions we found an average decrease on EC levels of 62% using selected strains and 44% for the spontaneous fermentation. In addition, careful separation of the distillation fractions was crucial for producing high-quality and safe beverages.

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1. Introduction

Cachaça is the typical sugar cane spirit produced exclusively in Brazil and is appreciated worldwide. However, the quality can be improved primarily through new technologies for selecting appropriate *Saccharomyces cerevisiae* yeast strains for fermentation as well as using suitable vats and distillation equipment (Gomes, Silva, Marini, Oliveira, & Rosa, 2007). In Minas Gerais, which is the most important cachaça-producing region in the country, many distilleries use a traditional production process; thus, it is difficult to generate and maintain a high-quality product (Badotti, Gomes, & Rosa, 2012). Most alembic distilleries produce cachaça through spontaneous fermentation by preparing the inoculum inside the fermentation vat using the natural fermentative microbiota in sugar cane juice, which can last for 20 days. In addition to spontaneous fermentation, selected *S. cerevisiae* strains isolated from fermentation vats can be used (Souza et al., 2012) and are advantageous over the first process due to, for example, rapid and efficient fermentation, improved stress tolerance, low acetic acid production as well as lower flavour variations (Badotti, Belloch, Rosa, Barrio, & Querol, 2010). After fermentation, the must is distilled, which is performed in copper stills that generate three different fractions. Compounds with greater volatility and more solubility in ethanol than water, such as methanol and acetaldehydes, are in the initial boiling samples and are referred to as *head*. The second and purest fraction, which is used in commercial purposes, is the *heart*, which composes 80–85% of the total distillate and is comprised primarily of ethanol as well as higher alcohols. The last fraction or *tail* comprises compounds that are less volatile than ethanol, such as acetic acid and 5-hydroxymethylfurfural (HMF). When the *head* and *tail* fractions are not entirely separated, the sensory quality of cachaça is degraded (Cardeal, Souza, Gomes da Silva, & Marriott, 2008; Reche et al., 2007).

Cachaça is the third most consumed beverage worldwide, and producers are highly interested in increasing the annual exportation

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rates, which are low (15 million litres) compared with the levels produced (1.3 billion litres). Thus, this market has high potential for exploration, considering that this product may be marketable in other countries (ABRABE, 2013). The primary obstacle to enhanced quality is controlling the large variations among different batches and formation of undesirable or toxic substances.

According to Lachenmeier et al. (2010), the Brazilian population has a significant risk of developing cancers through high consumption of alcoholic beverages due to ethyl carbamate (EC). In 2010, the World Health Organization's International Agency for Research on Cancer (IARC, 2010) classified this compound as probably carcinogenic to humans. The Normative Instruction no13 (Brazil, 2005) defines the identity and quality standards for cachaça and considers methanol, ethyl carbamate (EC), 2-butanol, 1-butanol and acrolein as organic contaminants as well as arsenic, copper and lead as inorganic contaminants. The maximum level established for EC is 150 μg L^{-1}, the same value adopted by Canadian laws (Conacher & Page, 1986).

EC, which is also known as urethane ($C_2H_5COONH_2$), is found in several types of fermented foods and beverages (Ough, 1976). In sugar cane juice fermentation, cyanide compounds are the primary contributors to EC formation, and two chemical pathways have been proposed by Guerain and Leblond (1992) for EC formation from cyanides. The first begins with cyanide complexation to Cu^{2+} and culminates with cyanate formation, which may react with alcohol to form ethyl carbamate. The second is based on free radical production, which catalyses cyanide oxidation to cyanate; this may occur during beverage storage. The factors that influence EC formation from cyanide include pH, light, ethanol content, temperature, and Cu^{2+} concentration (Battaglia, Conacher, & Page, 1990). However, EC may be produced during fermentation through a reaction between urea and ethanol, both of which are produced during yeast metabolism (Kodama, Suzuki, Fujinawa, de la Teja, & Yotosuzuka, 1994). The amino acids citrulline, arginine and carbamoyl phosphate are also considered EC precursors (Weber & Sharypov, 2009).

EC analyses for certain cachaça brands showed that concentrations of this compound are typically above the legal limit (Andrade Sobrinho et al., 2009; Baffa Júnior, Mendonça, Pereira, Pereira, & Soares, 2011; Labanca, Glória, & Afonso, 2008; Nóbrega, Pereira, Paiva, & Lachenmeier, 2009; Nóbrega, Pereira, Paiva, & Lachenmeier, 2011). However, the mechanisms and stage of ethyl carbamate formation in cachaças remain unclear. In this study, three *S. cerevisiae* strains that were previously isolated from fermentation environments and selected using specific fermentation parameters were used to evaluate the influence of yeast on EC formation in controlled processes. The distillate fractions from the cachaças produced either by spontaneous or selected strains were also evaluated. As far as we know, this is the first study addressing EC contamination levels in cachaça must samples at distillery scale using selected strains. The primary goal of the current research was to correlate the EC levels with selected strains and contribute such information to producing high-quality cachaças.

2. Material and methods

2.1. Experiment conditions and sample collection

The experiments were performed in two distilleries located in Minas Gerais using the protocol described in Gomes et al. (2007). The fermentation processes were performed using three previously selected *S. cerevisiae* strains Badotti et al. (2010) and spontaneous fermentation. The distillation process followed the protocol traditionally used in each distillery. The entire experiment was such that each vat was at maximum capacity (1000 L) on the same day with the same sugar cane juice lot. The fermentation was performed without any homogenizing instruments. Three must samples (7, 14 and 21 days after fermentation began) were collected. These samples were transported at 4 °C in sterile flasks and processed in 24 h. Next, clean up of the must samples was done using centrifugation at 3000 rpm for 15 min and filtration with a 0.45 μm membrane (Millipore, USA) for further analysis.

The *head*, *heart* and *tail* fractions from the distilled must were separated, filtered and stored at −20 °C for further analysis. This procedure was performed during the cachaça production seasons 2011 and 2012.

2.2. Sample preparation

The ethyl carbamate stock solution was prepared at 100 mg L^{-1} using an aqueous/ethanol solution 40% v/v. Working solutions were prepared from the stock solution through serial dilution at the concentrations 1000 μg L^{-1}, 500 μg L^{-1}, 250 μg L^{-1}, 100 μg L^{-1}, 50 μg L^{-1}, 10 μg L^{-1} and 5 μg L^{-1}. Quantification was performed using external standardisation.

2.3. Instrumental analysis

The samples were analysed using a HP6890 Series Plus gas chromatograph coupled with a mass spectrometer (Agilent Technologies 5975C inert MSD equipped with Triple-Axis Detector) and a multipurpose autosampler (Gerstel, Baltimore. MD). The *m*/*z* 62, 74, and 89 ions were monitored for EC. Aliquots at 1 μL were injected, and the inlet was operated in the splitless mode. The calibration curves and EC analyses were performed at a 40 °C initial temperature and 0.7 min initial time. The temperature was then increased at 3 °C/min to 125 °C. Next, the temperature was increased at 35 °C/min to 220 °C, where it was maintained for 5 min. Chromatographic separation was performed using a fused silica capillary column (Innowax, 30 m × 0.32 mm I.D., film thickness 0.25 μm); helium was the carrier gas.

2.4. Validation studies

The performance parameters evaluated were precision, recovery, as well as analyte limits of detection (LOD) and quantification (LOQ). The limits of detection and quantification were calculated using the standard deviation ratio for the response to the calibration curve slope. The value generated was multiplied by three to generate the LOD and by ten for the LOQ. The solutions used to construct the curve were prepared in triplicate at five concentrations from 5.0 to 1000.0 μg L^{-1}. The calibration curve was constructed by plotting the *m*/*z* 62 area on the *y* axis and the EC concentrations on the *x* axis. The mathematical relationship between peak area and concentration for the species analysed was expressed through the equation for the line and coefficient of determination (R^2). Values at $R > 0.9900$ demonstrated optimum adjustment of the data to the regression line (Ribani, Bottoli, Collins, Jardim, & Melo, 2004).

Accuracy was determined using reproducibility and intermediate precision. Interday intermediate precision was generated in triplicate at 100.0 μg L^{-1} for three different days. The reproducibility or intraday precision was evaluated using six replicates at a single concentration (100.0 μg L^{-1}). The results were expressed as a relative standard deviation. Accuracy was evaluated through recovery experiments using a random sample enriched with EC. Acceptable values for recovery in residue analyses are typically between 70 and 120% with precision values up to 20%. However, depending on the sample analytical complexity, this value can be 50–120% with a 15% precision (Ribani et al., 2004).

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