Food Research International 85 (2016) 76–83

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Sugar cane spirit (cachaça): Effects of mixed inoculum of yeasts on the sensory and chemical characteristics

Juliana Cunha Amorim, Rosane Freitas Schwan, Whasley Ferreira Duarte *

Department of Biology, University of Lavras (UFLA) — Campus Universitário, CEP 37200-000 Lavras, MG, Brazil

ARTICLE INFO

Article history:
Received 19 February 2016
Received in revised form 31 March 2016
Accepted 9 April 2016
Available online 14 April 2016

Keywords:
Co-inoculation
Non-*Saccharomyces*
MALDI TOF
GC–MS
GC–FID
HPLC

ABSTRACT

The main goal of this study was to produce cachaça using a mixed inoculum of *Saccharomyces cerevisiae* and *Meyerozyma caribbica* and characterize the produced beverage using HPLC, GC–FID, GC–MS and sensorial analysis. Additionally, the use of MALDI-TOF as a tool to characterize and monitor pure and mixed inocula fermenting sugar cane juice was also evaluated. Vat fermentations were carried out for three consecutive batches using autoclaved 16 °Brix sugar cane juice fermented by a mixed inoculum of *M. caribbica* 10^7 cells/mL and *S. cerevisiae* 10^8 cells/mL. The cachaça produced by the mixed culture of *M. caribbica* and *S. cerevisiae* showed the highest concentration of volatile compounds associated with good sensory descriptors such as ethyl hexanoate (114.11 μg/L), 2-phenylethyl acetate (2.77 μg/L), a-terpineol (0.45 μg/L), b-citronellol (2.47 μg/L), and geraniol (0.24 μg/L). This beverage consequently showed greater acceptance in the sensorial analysis for taste and aroma, especially by younger panelists. The feasibility of MALDI-TOF use under studied conditions was demonstrated by the comparison of the results obtained from yeast cultivation in YPD broth, YPD agar and sugar cane juice, showing that there was no interference of sugar cane juice in protein profile. The results obtained from MALDI-TOF analysis showed that the protein extraction directly from sugar cane juice under fermentation, without the traditional plating step, allowed the distinction between mixed and pure inocula even under different *M. caribbica* populations and Brix degrees.

1. Introduction

Cachaça is the Brazilian sugar cane spirit produced from fermented sugar cane juice with an ethanol content between 38% (*v*/*v*) and 48% (*v*/*v*) at 20 °C (Brazil, 2005). In 2013, cachaça was exported to 59 countries generating revenues of US$ 16.59 million. The beverage is a complex mixture of flavor compounds in an ethanol/water matrix (de Souza, Vásquez, Del Mastro, Acree, & Lavin, 2006; Nonato, a, Carazza, Silva, Carvalho, & de L Cardeal, 2001) that contains higher alcohols, ethyl esters, aldehydes, ketones, and organic acids, which are responsible for its distinct aroma (Duarte, de Sousa, Dias, & Schwan, 2011a; Nonato et al., 2001). The physicochemical and organoleptic characteristics of cachaça depend on several factors involved in quality control (Granato, de Oliveira, Caruso, Nagato, & Alaburda, 2014), distillation, aging (Vicente et al., 2006), and especially, the fermentation process due to the metabolites produced by yeasts. The importance of the metabolites, mainly high alcohols and esters, produced by yeasts during fermentation is reported by Souza et al. (2012) and Vidal et al. (2013). Along with these works, most of the published papers have focused on the use of selected *Saccharomyces cerevisiae* to produce cachaça (Campos et al., 2010; Silva et al., 2009; Vicente et al., 2006). However, the use of non-*Saccharomyces* yeasts was reported by Duarte, Amorim, and Schwan (2013) showing that three different mixed inocula of non-*Saccharomyces* and *S. cerevisiae* with high β-glucosidase activity could enhance the profile of desirable volatile compounds in fermented sugar cane juice, specially the mixed inoculum of *Meyerozyma caribbica* and *S. cerevisiae*. From this first work, further research with this mixed inoculum is needed to study its behavior when fermenting larger volumes of sugar cane juice in consecutive batches with inoculum recycling to produce the distillated beverage.

The monitoring of yeast population during controlled fermentation using mixed inocula is often made with plate count followed by the use of a molecular technique, such as restriction fragment length polymorphism — RFLP (Viana, Belloch, Vallés, & Manzanares, 2011). Another technique frequently used is quantitative polymerase chain reaction — qPCR (Andorrà, Berradre, Mas, Esteve-zarzoso, & Guillamón, 2012; Wang, Esteve-Zarzoso, Cocolin, Mas, & Rantsiou, 2015). The matrix assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF MS) is frequently used in clinical diagnosis of yeasts and bacteria (Goyer et al., 2012; van Veen, Claas, & Kuijper, 2010), however, in recent years, some studies have used MALDI-TOF MS to identify yeasts used in different fermentation processes such as in the production of ethanol, baking, wine and beer (Moothoo-Padayachie, Kandappa, Krishna, Maier, & Govender, 2013; Spitaels et al., 2014, 2015; Usbeck, Wilde, Bertrand, Behr, & Vogel, 2014). Although this

* Corresponding author.
E-mail address: whasleyduarte@dbi.ufla.br (W.F. Duarte).

http://dx.doi.org/10.1016/j.foodres.2016.04.014
0963-9969/

technique is not fully consolidated for monitoring yeasts in fermentative processes, its cost, time consumption and reliability make it an interesting tool in experiments with selected yeasts. To the best of our knowledge, there are no published works on the production of sugar cane spirit using mixed yeast inoculum and its physical–chemical and sensorial characterization. Also, there are no MALDI-TOF MS studies related to monitoring selected yeast inocula in cachaça fermentation. Therefore, the aims of this study were to evaluate the efficiency of mixed yeast inoculum for the production of cachaça in three consecutive batches and to characterize the produced beverage using HPLC, GC–FID, GC–MS and sensorial analysis. Additionally, the MALDI-TOF MS was assessed as a tool to monitor the inoculated yeast fermenting sugar cane juice under different conditions.

2. Materials and methods

2.1. Yeast

The fermentations were performed using the mixed inoculum selected in a previous work (Duarte et al., 2013). This inoculum was composed of the yeast *M. caribbica* CCMA 0198 (former *Pichia caribbica* UFLA CAF733) belonging to the collection of the Microbiology Laboratory/University of Lavras and *S. cerevisiae* CA11 (LNF Latino Americana, Bento Gonçalves — Brazil).

2.2. Vat fermentations

Yeast strains previously maintained in YPD + 20% glycerol at −80 °C were reactivated and grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). The inoculum was obtained according to Duarte et al. (2010). Briefly, after reactivation in 1 mL of YPD, the inocula were transferred to increasing volumes up to 1 L. From this point, every 24 h, the cells obtained were withdrawn from the vial in sterile water and stored in a refrigerator at 4 °C; a small amount (10%) was left in the flask and refed with sterile YPD broth. This process was repeated for 3 days until enough cells were obtained for the inocula. Yeast viability and population were checked by methylene blue staining and microscopic examination.

The mixed inoculum composed by 10^7 cells/mL of *M. caribbica* and 10^8 cells/mL of *S. cerevisiae* was used to ferment 15 L of autoclaved (121 °C, 20 min) 16 °Brix sugar cane juice. The reasons for choosing 10^7 cells/mL of *M. caribbica* population and 16 °Brix will be presented below. Brix degree was adjusted using distilled water and the initial pH of the must was 5.7. To avoid yeast cell stress, the fermentation was conducted as fed batch. The first batch was started with yeast cells inoculated in 5 L of sterilized sugarcane juice. After that, the Brix degree was monitored every 1 h and, when Brix inside the vats reached 3, a new amount of sugar cane juice was added until the Brix inside the vats was 7. This procedure was repeated four times until the volume of the vats was completed to 15 L. Once the vats were filled, this point was considered as the initial time (T0) of the fermentation. Three batches were processed at room temperature, and the end (T24) of each was set when the °Brix stabilized (around 0–1 °Brix after approximately 24 h of fermentation). Samples were taken at T0 and T24 to determine the yeast population, for protein extraction (MALDI-TOF analysis) and HPLC analysis. The cell enumerations of selected *M. caribbica* were performed using lysine agar (LA) (HiMedia®, Mumbai). The enumeration of *S. cerevisiae* cells was determined as the difference between the cell count in the YPD plates and the cell count in the LA plates (Nissen, Nielsen, & Arneborg, 2003). The fermentations were conducted in duplicate.

2.3. Distillation

The fermented sugar cane juice from each batch (2 replicates of 15 L) was distilled in copper alembic with a distillation rate of approximately 1 L/h. Approximately 10% of the distillate ("head") was collected separately. The "heart" fraction (cachaça) was collected until an ethanol concentration reached approximately 42% (*v*/*v*) (Campos et al., 2010). The produced cachaça was stored in glass bottles for subsequent HPLC, GC–FID, GC–MS, and sensory analysis.

2.4. Chemical analysis

Carbohydrates and ethanol were analyzed in sugar cane juice, while acetic acid and ethanol were determined in cachaça by HPLC according to the methodology proposed by Duarte, Amorim, Lago, de A. Dias, and Schwan (2011b). All samples were examined in duplicate.

Before HPLC and GC–FID analysis, samples were filtered manually using syringe and 0.22 μm pore filter. The major volatile compounds analysis by CG-FID were performed according to Duarte et al. (2010, 2011b).

The minor volatile compounds of cachaça were determined after extraction and concentration of the headspace compounds with solid phase micro extraction (SPME). Aliquots of 500 μL of the cachaça samples were diluted with 4.5 mL of deionized water containing 0.25 g of NaCl. This solution was hermetically closed with a teflon septum and screw cap in a 15 mL vial. The SPME parameters used were 60 °C and 25 min for extraction (Souza, Cardeal, Augusti, Morrison, & Marriott, 2009). After the extraction with a 50/30 μm DVB/Carboxen/PDMS StableFlex SPME fiber and manual holder (Supelco, Bellefonte, PA, USA) the thermal desorption in the GC injector was performed at 240 °C for 5 min. The analyzes were performed using a GC–MS-QP2010 SE system (Shimadzu) equipped with a free fatty acid phase (FFAP) column (30 m × 0.25 mm i.d. × 0.25 μm) operated at 50 °C for 5 min, increased by 3 °C/min up to 190 °C and maintained at 190 °C for 10 min. High-purity helium was used as carrier gas with a constant flow of 1.2 mL/min. The injections were performed using splitless mode (30 s at 25 psi). The splitless was opened after 2 min. After 6 min the mass spectra were acquired continuously from 45 to 1000 *m*/*z*. The temperature of the ion source of electrons was 230 °C and the quadrupole was 150 °C. The analyses of mass spectra were made by comparing them to those of pure standards (when available) and using the Nist library version 2011. The resulting measurements were expressed in 4-nonanol (internal standard) equivalents used at a concentration of 125 μg/L.

2.5. Monitoring inoculated yeasts using MALDI–TOF

The efficiency for monitoring the studied inocula by MALDI-TOF MS and protein extraction from colonies or directly from cell suspension in culture media (YPD broth and sugar cane juice), was evaluated in three different experiments.

A first assay was performed to evaluate the MALDI TOF capacity for distinction between mixed yeast inoculum and pure yeast inocula, the efficiency of protein extraction from yeast colonies (Bruker, 2007) or from cell suspension (Usbeck, Kern, Vogel, & Behr, 2013) and, to check possible interferences of sugar cane juice on the yeast mass spectra. The conditions compared in this assays were: (i) mass spectra from pure cultures of yeasts cultivated in YPD agar, (ii) mass spectra from mixed inoculum (10^7 cells/mL of *M. caribbica* and 10^8 cells/mL of *S. cerevisiae*) cultivated in YPD broth and, (iii) mass spectra from mixed inoculum (10^7 cells/mL *M. caribbica* and 10^8 cells/mL of *S. cerevisiae*) fermenting 16 °Brix sugar cane juice.

In the second assay, to check the effects of non-*Saccharomyces* population and sugar content (°Brix) on the protein profiles, the MALDI-TOF MS was used to monitor the mixed inoculum composed by different populations of *M. caribbica* (10^5, 10^6, and 10^7 cells/mL) fermenting sugar cane juice with different °Brix (14, 16, and 18 °Brix). The assays with YPD broth and sugar cane juice were carried out in 250 mL Erlenmeyer flasks containing 100 mL of sterilized media incubated without agitation for 24 h at 28 °C. The first and second assays were