Spontaneous fermentation and selected yeast fermentation for the production of cachaça by cell-recycle batch process

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Abstract

Yeast recycling during alcoholic fermentation for the production of cachaça can stimulate the development of a wide variety of microorganisms resulting from successive fed-batch procedures and the intrinsic characteristics of the process. Thus, whereas yeast recycling is a common practice in cachaça production units, this study aimed to evaluate the microbiological and technological characteristics of fermentation processes using selected and wild yeasts and chemical quality of the distillate. The fermentation was carried out in a batch process using selected wild yeasts for four fermentative cycles. At the beginning of the fermentation, the yeast cell viability and total yeast counts were evaluated. After the fermentation process, the parameters acidity, pH, alcohol content, and total residual reducing sugars of the wines, this being distilled in copper stills were analyzed to determine the physicochemical composition of cachaça. Although the selected yeast fermentation showed higher viable cell counts at the beginning of the first cycle, the wild yeasts adapted to the environmental conditions, with an increase in the viable cells at the beginning of the second cycle. The yeast counts in the recycled yeast increased during the spontaneous fermentation cycles. Lower residual sugar levels were observed in the spontaneous fermentation, leading to a higher alcohol production in wines. The distillates obtained from spontaneous fermentation and selected yeast fermentation presented physicochemical composition within the limits of the Brazilian legislation, suggesting spontaneous fermentation can be carried out efficiently during successive cell recycling, enabling a balanced production of volatile compounds in the distillate.

Keywords: spirit, fermentation process, volatile compounds, cell viability, wine, microorganisms.

Introduction

According to the Normative Instruction n.º 13 of 2005 that regulates the identity and quality standards of sugar cane spirit, *cachaça* is defined a typical and exclusive sugar cana spirit of Brazil, produced by the distillation of fermented sugar cane, presinting 38 – 48% ethanol by volume at 20ºC with peculiar sensory features (BRASIL, 2005a).

Fermentation for the production of cachaça is traditionally performed in a batch system, with yeast recovery by decanting the must of yeast cells after degradation of sugar from wort. Then, the sugarcane must is mixed with the recovered inoculum, thus initiating a new fermentation process (MUTTON et al., 2014; OLIVEIRA FILHO et al., 2016; ALVES et al., 2018).

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Most cachaça distilleries conduct fermentation using wild yeasts, also known as *fermento caipira*. These yeasts are naturally present in sugarcane juice, usually obtained from the spontaneous fermentation by the addition of crushed corn and rice bran to the wort (GABRIEL et al., 2012; MENDONÇA et al., 2016).

Spontaneous fermentation presents great biodiversity of microorganisms (*Saccharomyces cerevisiae, Pichia anomala, Debaryomyces hansenii, Zygosaccharomyces bailii, Rhodotorula mucilaginosa, Kloeckera apis* and others), which are introduced through successive feedings of sugarcane juice during the yeast preparation stage and throughout the fermentation cycles (VICENTE et al., 2006; GOMES et al. 2007; OLIVEIRA et al., 2008; BADOTTI et al., 2010). For contributing to the development of the chemical and sensory profile of the distillates, this population of microorganisms are considered the key to the formation of cachaça *terroir* (GABRIEL et al., 2012; PORTUGAL et al., 2016).

Some authors have shown that the genetic variability can directly affect the operational performance of the process, changing the content and the ratio of the main volatile compounds in the beverage. To reduce the molecular diversity, some authors have suggested the use of selected *Saccharomyces cerevisiae* strains, thus ensuring the high quality and standardization of cachaça (GOMES et al., 2007; NOVA et al., 2009; SILVA et al., 2009; CAMPOS et al., 2010).

We believe that yeast heterogeneity during spontaneous fermentation is important for the formation of chemical compounds responsible for the identity, quality, and characterization of cachaça. Thus, to contribute with more information on the traditional practices of cachaça production, the objective of the present study was to evaluate the effect of cell recycling on the performance of spontaneous fermentation and selected yeast fermentation and to determine the concentration of the main volatile compounds in the distillate.

Material and methods

Sugar cane processing and wort preparation

Sugarcane variety SP 70-1406 grow in the Uberaba-MG region, with a soluble solids content of 22 ºBrix, harvested manually during the 2017/2018 crop was used in the study. The sugarcane juice for the preparation of the must was extracted by conventional milling and diluted with distilled water to 16 ° Brix.

Conduction fermentative process

Fermentation was performed at room temperature in batch system in conical bottom stainless steel vats, with a capacity of 4.5L and a working volume of 2.8L, for four fermentation cycles. For the alcoholic fermentation, the selected yeast of Saccharomyces cerevisia LNF CA-11 (Treatment 1) at a concentration of 10^7 CFU.mL^{-1}, prepared and adapted according to the manufacturer's recommendations, and wild yeasts (Treatment 2) produced by spontaneous fermentation (average of 3,6 x 10^7 CFU.mL^{-1}) prepared with the addition of crushed corn and rice bran to the sugarcane wort were used, as reported by MENDONÇA et al. (2016) with adaptations.

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For each treatment, 2.8L of wort at 16 ºBrix was used, corresponding to two additions of 1.4L, with the second feeding after 60 minutes of processing. At the end of each fermentation cycle (zero Brix degree), the yeast was left to decant, aiming to reuse the inoculum for the subsequent cycle.

After 30 min of the last feeding, an aliquot of wine was removed to evaluate the yeast cell viability using the methylene blue staining and cell counts in the Neubauer Chamber (SILVA et al., 2003).

After fermentation, the wine was collected and analyzed for boiling point (SILVA et al. 2003), pH by direct reading in a digital meter Tekna T-1000, total acidity (g $H_2SO_4.dm^{-3}$) by titration with 0.05N NaOH (COPERSUCAR, 2001), and total residual reducing sugars by the LANE & EYNON method (1934), using a Redutec (Marconi) apparatus.

The recycled yeast was analyzed for total yeast counts in WLN (Wallerstein Laboratories Nutrient Agar) (CECCATO-ANTONINI, 2010) with the addition of ampicillin and nalidixic acid (100 mg / L).

Distillation of wine

To compose the wine samples for the distillation process, the wines from the repetitions of each treatment and fermentation cycles were mixed (*blend*), and distilled in a simple alembic still consisting of a copper boiler, a hat, a cooper pipe, and a condenser. After separation of the head fraction (2% of the volume), the heart fraction was collected and standardized at 42 % $v.v^{-1}$ alcohol, and stored in an amber glass vial for analysis.

Chromatographic analyses

The chemical compounds of the distillates were analyzed according to the official procedures established by the current legislation (BRASIL, 2005b). The acetaldehyde, ethyl acetate, n-propanol, i-butanol, i-amyl alcohol, furfural, acetic acid, methanol, sec-butanol, and 1-butanol contents of the heart fraction were determined by gas chromatography coupled with flame ionization detector (GC-FID). The analyses were performed in a Shimadzu QP-2010 PLUS gas chromatograph with a Stabilwax-DA (Crossbond Carbowax esterified polyethylene glycol, 30 m × 0.18 mm × 0.18 µm) column and flame ionization detector (FID). The detector and injector temperatures were set at 250 ° C, using automatic injection mode, at a split ratio of 1:25 and injection volume of 1.0 µL. The carrier gas (H_2) flow rate was 1.5 $mL.min^{-1}$ with a total flow of 42 $mL.min^{-1}$, and pressure of 252.3 kPa. The column temperature ramp was programmed to start at 40 °C (4 min), increasing up to 120 ° C at a rate of 20 ° $C.min^{-1}$ (1 min) and increasing from 30 °C min^{-1} up to 180 ° C (4 min) (BORTOLETTO; ALCARDE, 2013).

The concentration of ethyl carbamate was determined after 72 hours of distillation, as this compound is formed within 24-48 hours of process (RIFFKIN et al., 1989; AYLOTT et al., 1990). All samples were filtrated on a PVDF membrane filter (13 mm diameter, 0.45 µm pore size) and analyzed in a gas chromatograph (GC) coupled to a Shimadzu GCMSQP2010 Plus mass spectrophotometer (Kyoto, Japan) at ionization of 70 eV using a polar capillary column (esterified with propylene glycol, HPFFAP; 50 m x 0.20 mm x 0.33 µm stationary phase film thickness). The injector and detector interface

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temperatures were 230 and 220 °C, respectively. The temperature ramp was set as starting at 90 °C for 1 min, increasing up to 150 °C at a rate of 10 ° C.min^{-1}, then heating up to 230 °C at a rate of 30 ° C.min^{-1}, and remaining at this temperature for 2 min. A volume of 1.0 µL was injected using a splitless injector model. Helium gas was used at a flow rate of 1.2 mL.min^{-1}. The analysis was monitored by selected ion monitoring of m/z 62 for ethyl carbamate used as the internal standard (RECHE et al., 2007; CLEGG; FRANK, 1988). The quantification was performed by comparing the results with an analytical curve obtained using ethyl carbamate stock solution, with concentration ranging from 50 to 500 µg.L^{-1}.

The analytical parameter of the chromatographic analyses were determined according to the simple linear relationship, described by the equation y = ax + b. The determination of the detection limit (DL), the quantification limit (QL) and the calculation of the regression coefficients of the analytical curves (a, b, r^2), as well as the retention time (RT) obtained for each compound, are shown in Table 1.

Table 1. Retention time (RT), detection limit (DL), and quantification limit (QL) of volatile congener and contaminants, and correlation coefficients (a, b, r^2) of the calibration curves in alcoholic solution (40 % v.v^{-1})

Volatile congener	RT (min)	DL (mg.100 mL anhydrous ethanol^{-1})	QL (mg.100 mL anhydrous ethanol^{-1})	a	b	r²
Acetaldehyde	0.29	0.080	0.266	0.809	-	0.998
Ethyl acetate	1.41	0.044	0.144	0.037	0.0905	0.994
Propanol	4.43	0.054	0.176	0.231	0.0099	0.999
Isobutanol	5.22	0.029	0.098	0.020	0.0037	0.999
Isoamyl alcohol	6.72	0.015	0.044	0.176	0.0145	0.999
Acetic acid	9.15	0.580	1.740	0.623	0.1111	0.994
Contaminants congener	**RT (min)**	**DL (mg.100 mL anhydrous ethanol^{-1})**	**QL (mg.100 mL anhydrous ethanol^{-1})**	**a**	**b**	**r²**
Methanol	1.62	0.159	0.534	0.784	0.0486	0.965
1-butanol	5.99	0.061	0.200	0.203	0.1331	0.997
2-butanol	4.02	0.215	0.710	0.266	0.0024	0.999
Ethyl carbamate	10.15	0.180[a]	0.550[a]	64.714	1241.67	0.9984

Statistical analysis

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Data were analyzed by analysis of variance, the Tukey test at 5% level of probability was applied, using Sisvar statistical software, according to FERREIRA (2011).

Results and discussion

Two different batch fermentations for the production of cachaça were evaluated for the yeast performance during cell recycling. At the beginning of the first cycle, the selected yeast fermentation showed viable cell counts 30% higher than that observed for wild yeast fermentation. However, throughout the cycle, those yeast strains adapted to environmental conditions, thus starting the second cycle with cell viability greater than 80 % (Figure 1).

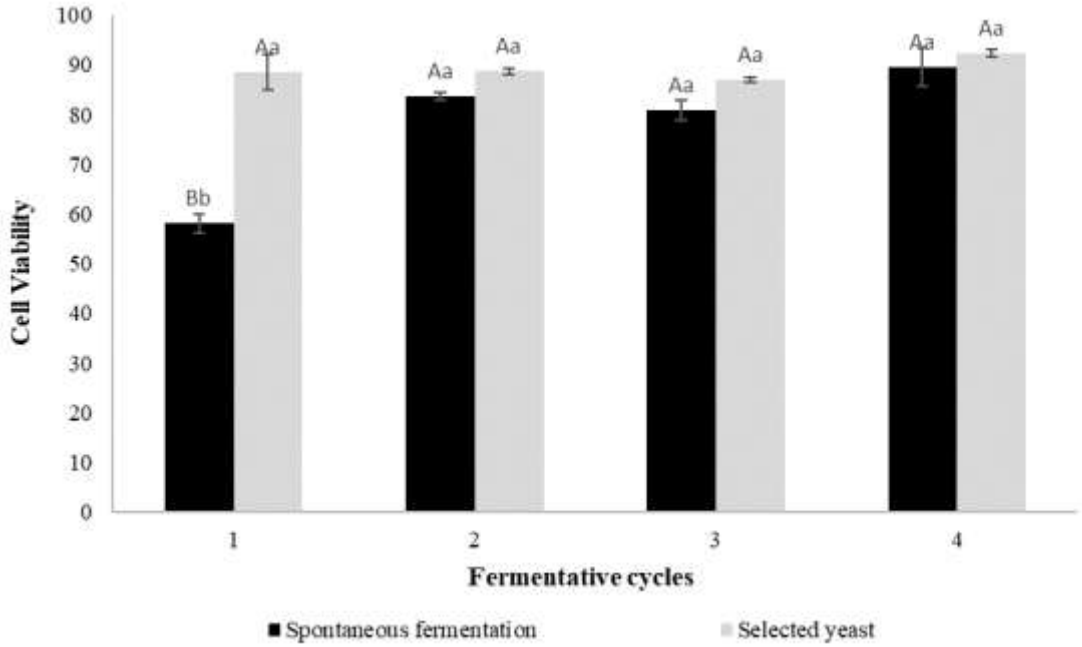

Figure 1. Interaction between treatments and cycles for yeast cell viability during spontaneous fermentation and selected yeast fermentation. Lowercase letters compare averages between formulations at the same cycle. Uppercase letters compare averages of the same formulation at different cycle. Means followed by the same letter do not differ at 5% probability by Tukey test.

The stress conditions during the alcoholic fermentation can activate the adaptive metabolism response, favoring the accumulation of trehalose, increasing cell resistance and viability for the subsequent fermentation cycle (PAREDES et al., 2018). We believe that the first cycle promoted the selection of yeast strains capable of growing under the process conditions established by the spontaneous fermentation (temperature, ethanol concentration, osmotic pressure, and acidity), thus favoring the increase in viable cells.

Spontaneous fermentation led to a highly significant increase ($P < 0.01$) in total recycled yeast counts, while a decrease in yeast counts up to the fourth cycle was observed for the selected yeast fermentation (Figure 2). According to GABRIEL et al. (2012), the previous adaptation and selection of natural yeasts in the culture medium during yeast development may favor its activity during the alcoholic fermentation. PORTUGAL et al. (2016) also found that the population

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of *Saccharomyces cerevisiae* stood out from other classes of microorganisms at the beginning of the tumultuous phase, until the end of the spontaneous fermentation. Probably, the predominance of dominant yeast strains and the characteristics of this step contributed to both the maintenance of the number of viable cells at the beginning of the process and the increase in total yeasts during the successive cell recycling.

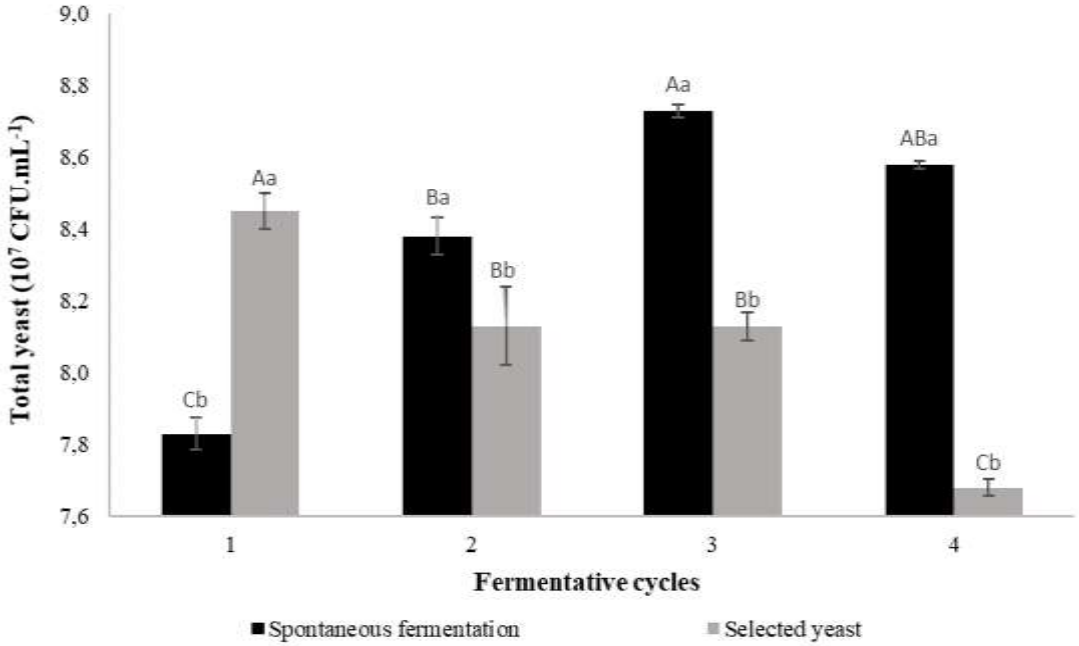

Figure 2. Interaction between treatments and cycles for total yeast counts in the recycled yeast from spontaneous fermentation and selected yeast fermentation. Lowercase letters compare averages between formulations at the same cycle. Uppercase letters compare averages of the same formulation at different cycle. Means followed by the same letter do not differ at 5% probability by Tukey test.

The results of the physicochemical chracterization of the fermented wine presented lower pH values and higher total acidity in wines obtained from the spontaneous fermentation when compared to those obtained using selected yeasts (Figure 3). Although *Saccharomyces cerevis*iae is generally the dominant species in spontaneous fermentation, other microorganisms (lactic acid bacteria, acetic acid bacteria, and non-Saccharomyces yeasts) are also present, contributing to the production of organic acids during the fermentation process (OLIVEIRA et al., 2008; BADOTTI et al., 2010; GABRIEL et al., 2012; PORTUGAL et al., 2016).

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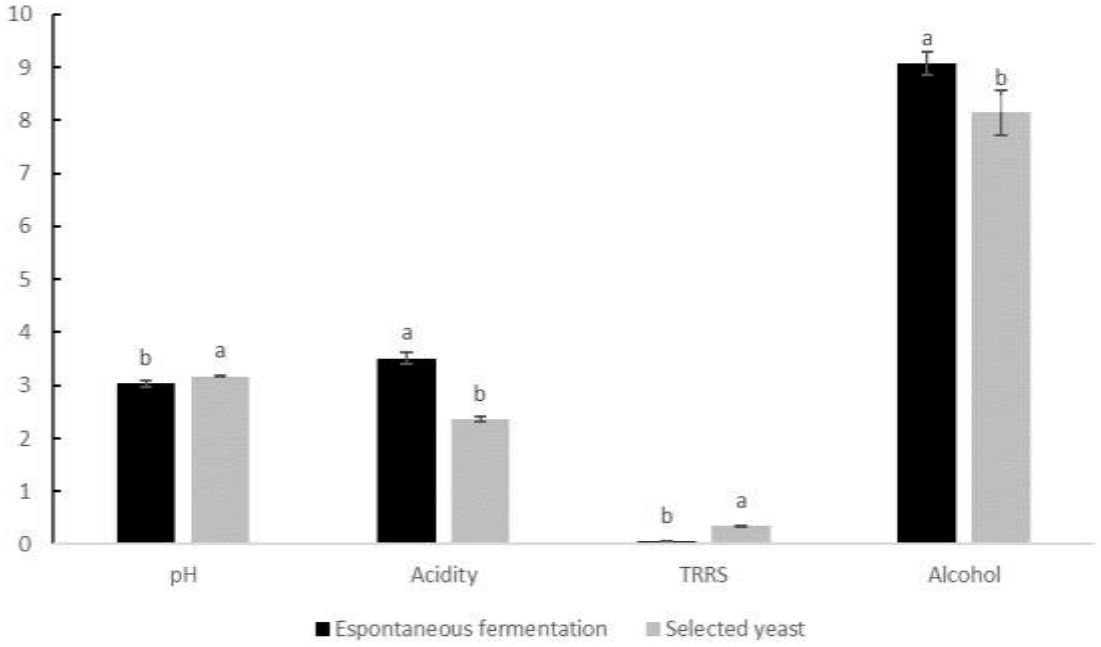

Figure 3. Analysis of variance of the parameters pH, total titratable acidity ($g.H_2SO_4.L^{-1}$), total residual reducing sugars (%), and alcohol content (% $v.v^{-1}$) of wines from spontaneous fermentation and selected yeast fermentation. Means followed by the same letter in the same parameter do not differ at 5% probability by Tukey test.

The fermentative performance of the yeast strains was not affected by the high acidity levels (1.48 fold) in spontaneous fermentation. This result can be evidenced by the increase yeast counts during yeast recycling (Figure 2), which resulted in a lower percentage of residual sugars and a 10% increase in alcohol production in wines (Figure 3).

Studies have shown that the fermentation of sugarcane juice using a blend of *Saccharomyces cerevisiae* and non-Saccharomyces strains presented low concentration of residual sugars, allowing a higher substrate conversion into product, besides contributing to the production of volatile compounds from sugarcane, which are desirable in cachaça (DUARTE et al., 2013; AMORIM et al., 2016; PORTUGAL et al., 2017). In the research presented, there was probably a synergistic interaction between the different yeast strains coexisting in spontaneous fermentation, increasing the consumption of sugars and production of ethanol during the fermentation process.

The cachaça produced by spontaneous fermentation and selected yeast fermentation presented an average ethanol content (1st and 4th cycle) of 38.6% $v.v^{-1}$ and 39.4% $v.v^{-1}$, respectively (Table 2). CAMPOS et al. (2010) studied the production of volatile compounds by different *Saccharomyces cerevisiae* strains, and found an average ethanol concentration in wine and cachaça of 8.1 and 40.4 $mg.100\ mL^{-1}$, respectively. The fermentation performed in this study also led to a balanced production of ethanol in wine (8.6 $g.100mL^{-1}$), allowing the standardization of the alcoholic concentration of the distillate (39 % $v.v^{-1}$) as proposed by the Brazilian legislation (BRASIL, 2005a).

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Table 2. Alcohol content, volatile congeners, and contaminants of cachaça from spontaneous fermentation and selected yeast fermentation in the first and fourth fermentation cycles

Compound	Spontaneous Fermentation		Selected yeasts		Limits (BRASIL, 2005a)
	1º Cycle	4º Cycle	1º Cycle	4º Cycle	
Alcohol content[a]	39.19	38.06	39.54	39.24	38 – 48
Volatile congeners					
Volatile acidity (acetic acid)[b]	42.71	4.60	29.82	18.53	<150
Aldehydes (acetic aldehyde)[b]	9.06	5.20	14.74	10.93	<30
Esters (ethyl acetate)[b]	5.97	3.36	4.65	2.22	<200
Furfural[b]	1.40	2.60	0.68	1.68	<5
n-propanol[b]	19.57	17.18	28.83	33.82	-
i-butanol[b]	16.08	13.40	59.08	18.14	-
i-amylic[b]	205.59	167.26	195.32	119.67	-
Higher alcohols[b]	241.24	197.85	283.23	171.64	<360
Coefficient of congeners[b]	300.38	213.61	333.33	204.99	200 – 650
Contaminants					
Methanol[b]	3.39	3.13	nd	1.48	<20
2-butanol[b]	nd	nd	nd	nd	<10
n-butanol[b]	nd	nd	nd	nd	<3
Copper[c]	9.40	4.60	7.30	4.60	<5
Ethyl carbamate[d]	32.84	42.32	55.71	55.94	<210

[a] % ethanol ($v.v^{-1}$) at 20ºC; [b]$mg.100mL^{-1}$; [c]$mg.L^{-1}$; [d] $\mu g.L^{-1}$.

Table 2 shows a 9.8-fold reduction of the acidity levels of the distillates from the spontaneous fermentation between the 1st and 4th fermentation cycles, while the acidity of the distillate from the selected yeast fermentation decreased by 1.6-fold. Although wines from spontaneous fermentation presented higher acidity levels, the concentration of volatile acids in cachaça was much lower than the limit (<150 $mg.100\ mL^{-1}$) established by the Brazilian legislation (BRASIL, 2005a). PORTUGAL et al. (2016) studied a single process of spontaneous fermentation and found that lactic acid bacteria and acetic acid bacteria actively participated in the fermentation process, contributing to the increase in the total acidity of the wine. The authors also reported that this

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increase did not compromise the yeast performance and the volatile acidity of cachaça (25.3 mg.100 mL^{-1}).

The concentration of acetic aldehyde was 2-fold higher in the distillate produced in the 4th cycle of selected yeast fermentation than wild yeast, and remained below the limit established by legislation for all cycles evaluated. The excessive production of this compound is associated with process failures, including the lack of separation of the head fraction (BORTOLETTO; ALCARDE, 2015). Therefore, it was observed that the fermentations evaluated allowed a balanced production of this aldehyde in the spirit.

Yeast recycling (wild and selected strains) slightly reduced the ethyl acetate levels in the distillate produced in the 4th cycle, remaining with an average value of 2.8 mg.100 mL^{-1}. This result is similar to that found by AMORIM et al. (2016) in cachaça produced using *Saccharomyces cerevisiae* LNF CA11 strain (4.2 mg.100 mL^{-1}). These findings demonstrate that the fermentation process of the present study were effective in the production of ethyl acetate in the recycling conditions studied, which may contribute to the development of the sweet and fruity flavor of cachaça.

High levels of higher alcohols mainly composed by n-propanol, i-butanol, and i-amyl alcohol were observed in the first fermentation cycle for both processes, which reduced in the distillates from the 4th fermentation cycle. Although the compounds n-propanol, i-butanol, and i-amyl alcohols are important for the sensory characterization of the beverage and are involved with the formation of other secondary compounds, at high levels they can cause serious sensory defects (PORTUGAL et al. 2016). The concentration of higher alcohols found in this study (262.23 and 184.74 mg.100 mL^{-1} in the 1st and 4th cycles, respectively) was lower than the maximum established by the Normative Instruction 13 (360 mg.100 mL^{-1}) (BRASIL, 2005a), indicating that cell recycling did not compromise the synthesis of these compounds in both fermentation processes.

The levels of furfural and organic contaminants (ethyl carbamate, methanol, 2-butanol, and n-butyl alcohol) were considerably lower than the limits established by the current legislation (BRASIL, 2005a), demonstrating that the fermentation conditions of this study allowed the production of distillates with low concentrations of these compounds.

The distillates exhibited higher copper levels when compared to the limits allowed by Brazilian legislation (BRASIL, 2005a) for both fermentation conducted in the first cycle. Probably, the copper oxidized in the distillation apparatus increased the concentration of this compound in cachaça from the first cycle, which was lower in the distillates of the 4th fermentation cycle.

Under the experimental conditions studied, the distillates did not exceed the limits of volatile congeners (acetaldehyde, ethyl acetate, higher alcohols, acetic acid, and furfural) established by the Brazilian legislation (BRASIL, 2005a). Therefore, the fermentation by cell recycling can produce adequate volatile compounds levels in the distillate.

Conclusion

The fermentation developed in this study presented similar fermentative performance and the distillates met the standards established by the Brazilian

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legislation. The results suggest that spontaneous fermentation can be carried out efficiently during successive cell recycling, enabling a balanced production of volatile compounds in the distillate. The heterogeneity of microorganisms in spontaneous fermentation can contribute to the formation of chemical compounds responsible for the cachaça *terroir*.

Avaliação da produção de cachaça por processo batelada com recirculação de células utilizando fermentação espontânea e levedura selecionada

Resumo

A recirculação do fermento durante as fermentações destinadas a produção de cachaça pode estimular o desenvolvimento de uma grande variedade de microrganismos, decorrentes das alimentações sucessivas de caldo e das características intrínsecas do processo. Assim, considerando-se que o reaproveitamento das leveduras é uma prática comum nas unidades de produção de cachaça, este trabalho teve o objetivo de estudar o comportamento microbiológico e tecnológico de fermentações conduzidas com leveduras selecionadas e selvagens e a qualidade química do destilado. As fermentações foram avaliadas em processo batelada utilizando-se leveduras selecionadas e selvagens, durante quatro ciclos fermentativos. No início das fermentações foi avaliada a viabilidade celular das leveduras e a concentração de leveduras totais no pé-de-cuba. Após o término do processo fermentativo foram analisados os parâmetros acidez, pH, teor alcoólico e açúcares redutores residuais totais dos vinhos, sendo este destilado em alambique de cobre, para a caracterização da composição química da cachaça. As fermentações conduzidas com leveduras selecionadas apresentaram maiores porcentagens de células viáveis no início do primeiro ciclo, entretanto, posteriormente as leveduras selvagens adaptaram-se as condições do meio, iniciando o segundo ciclo com maiores porcentagens de células viáveis. As contagens de leveduras no fermento reciclado foram aumentadas ao longo dos ciclos para as fermentações espontâneas. Os níveis de açúcares residuais foram menores para as fermentações espontâneas, refletindo em maior produção de álcool nos vinhos. Os destilados obtidos de fermentação espontânea e leveduras selecionadas apresentaram composição química dentro dos limites da legislação brasileira, sugerindo que as fermentações espontâneas podem ser conduzidas eficientemente durante os sucessivos reciclos de células, possibilitando a produção balanceada de compostos secundários no destilado.

Palavras-chave: destilado, processo fermentativo, compostos voláteis, viabilidade celular, vinho, microrganismos.

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