JULIANA CUNHA AMORIM

AVALIAÇÃO DA EFICIÊNCIA DO USO DE INÓCULO MISTO DE LEVEDURAS PARA FERMENTAÇÃO DO CALDO DE CANA E PRODUÇÃO DE CACHAÇA

LAVRAS – MG
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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Microbiologia Agrícola, para a obtenção do título de Mestre.

Orientador
Prof. Dr. Whasley Ferreira Duarte

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2015

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RESUMO

Cachaça é a bebida destilada, obtida a partir da fermentação do caldo cana-de-açúcar, produzida exclusivamente no Brasil, com teor alcoólico de entre 38% e 48% v/v, a 20 °C. A microbiota de processos tradicionais de fermentação é complexa e consiste de leveduras e bactérias. Este trabalho foi realizado com os objetivos de avaliar a eficiência de um inóculo misto de leveduras, composto por *Saccharomyces cerevisiae* CA11 e *Pichia caribbica* CCMA 0198, na fermentação do caldo de cana-de-açúcar, para a produção de *cachaça*; utilizar a técnica de MALDI-TOF MS para a caracterização das leveduras inoculadas pelo perfil das proteínas e realizar a caracterização do perfil sensorial e de metabólitos produzidos por GC-FID, GC-MS e HPLC. Com base nos resultados deste estudo, conclui-se que a coinoculação de *S. cerevisiae* e *P. caribbica* influenciou positivamente a qualidade final da *cachaça*. Na análise sensorial, a *cachaça* produzida pela fermentação com inóculo misto apresentou melhor aceitação para os atributos aparência, aroma, sabor e aspectos gerais. Além disso, a técnica de MALDI-TOF MS demonstrou-se útil para o acompanhamento do inóculo misto de leveduras no processo de fermentação do caldo de cana para a produção de *cachaça*.

Palavras-chave: *cachaça*, fermentação, não *Saccharomyces*, metabólitos, MALDI-TOF MS.

ABSTRACT

Cachaça is a distilled beverage obtained from the fermentation of sugar cane produced exclusively in Brazil, with alcohol content between 38 and 48% v/v 20°C. The microbial of traditional fermentation processes is complex and consists of yeast and bacteria. The objective of this study was to evaluate the efficiency of a mixed yeast inoculum, composed by *Saccharomyces cerevisiae* CA11 and *Pichia caribbica* CCMA 0198, in the fermentation of sugar cane juice for the *cachaça* production, use MALDI-TOF MS technique to characterize the inoculated yeast by the proteins profile and performing the characterization of the sensory profile and metabolites produced by GC-FID, GC-MS and HPLC. Based on the results of this study, it was concluded that co-inoculation of *S. cerevisiae* and *P. caribbica* influenced positively the final quality of *cachaça*. In the sensorial analysis, *cachaça* produced by fermentation with mixed inoculum showed better acceptance for the attributes appearance, aroma, flavor and general aspects. In addition, MALDI-TOF MS technique proved to be useful tool for monitoring the mixed yeast inoculum during the fermentation of sugar cane juice to produce *cachaça*.

Keywords: *Cachaça*. Fermentation. Non-*Saccharomyces*. Metabolites. MALDI-TOF MS.

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CAPÍTULO 1

1 INTRODUÇÃO

Segundo a legislação brasileira, *cachaça* é a denominação típica e exclusiva da aguardente de cana produzida no Brasil, com graduação alcoólica de 38% v/v a 48% v/v, a 20 ºC, obtida pela destilação do mosto fermentado do caldo de cana-de-açúcar, com características sensoriais peculiares, podendo ser adicionada de açúcares até 6 g/L, expressos em sacarose (BRASIL, 2005).

A *cachaça* é a segunda bebida alcoólica mais consumida no Brasil, superada apenas pela cerveja. A produção nacional é de 1,8 a 2 bilhões de litros anuais (90% industrial e 10% artesanal), gerando receita da ordem de US$ 500 milhões e empregando cerca de 600 mil pessoas.

Atualmente, no Brasil, existem aproximadamente 40 mil produtores, sendo o estado de São Paulo o maior produtor de *cachaça* industrial e Minas Gerais o maior produtor de *cachaça* de alambique, com mais de 5.000 alambiques (apenas 976 registrados) e 200 milhões de litros por ano.

O consumo per capita de *cachaça* no Brasil é de 11,5 litros/ano, superando tradicionais consumidores de destilados, como os alemães, os húngaros e os poloneses (entre 9 e 10 litros/ano). Da produção total, 14,8 milhões de litros são exportados, sendo os dois maiores importadores a Alemanha (6 milhões de litros) e a França (500 mil litros), consumindo a *cachaça* principalmente na forma de caipirinha (SERVIÇO BRASILEIRO DE APOIO ÀS MICRO E PEQUENAS EMPRESAS - SEBRAE, 2014).

No Brasil, existem dois processos de produção da *cachaça*, os quais diferem pela quantidade de bebida produzida e pelo método de destilação empregado. A *cachaça* de alambique é aquela destilada em alambique de cobre, sem a adição de açúcar, corante ou outros ingredientes. O destilado é separado em três frações, “cabeça”, “coração” e “cauda”, e os pontos de corte entre as

frações podem variar de acordo com a geometria do alambique e o grau alcoólico inicial do mosto fermentado. Já na *cachaça* industrial, o mosto fermentado é destilado em coluna de destilação contínua e hidratado para a obtenção do teor alcoólico estabelecido por lei.

Na fermentação espontânea (fermento caipira), prática comum no Brasil, os microrganismos presentes no caldo de cana, nos equipamentos (moendas e dornas) ou trazidos por insetos que visitam as áreas de produção são os responsáveis pelo processo, convertendo a sacarose do caldo de cana em etanol, gás carbônico e compostos secundários. Tais compostos secundários (ésteres, aldeídos, álcoois superiores e outros) proporcionam o sabor e o aroma característicos da *cachaça*.

O principal microrganismo presente no fermento caipira (obtido por meio da fermentação natural de uma mistura com receita variável - farelo de arroz, fubá de milho, suco de laranja ou limão, entre outros) é a levedura *S. cerevisiae.* Essa levedura é a mesma utilizada na preparação do fermento de panificação, na produção de álcool combustível e nos processos fermentativos que dão origem à maioria das bebidas alcoólicas conhecidas. Diferentes linhagens da mesma são empregadas para a produção de vinhos e de cervejas, e também para praticamente todas as bebidas destiladas. *Saccharomyces cerevisiae* predomina na indústria de bebidas por sua capacidade de tolerar as condições de fermentação, como alta concentração inicial de açúcares, acidez elevada, variações na temperatura, disponibilidade de nutrientes e, principalmente, teores crescentes de etanol. Esses aspectos peculiares selecionam linhagens de leveduras que apresentam mecanismos de resistência aos estresses que sofrem durante a fermentação.

Microrganismos, como bactérias e leveduras não *Saccharomyces*, também estão presentes durante o processo fermentativo e podem contribuir para a formação do *flavor* da *cachaça*. Existem vários estudos que reportam o uso de

culturas mistas de *S. cerevisiae* e outros microrganismos na produção de bebidas alcoólicas fermentadas (como vinhos de frutas) ou destiladas (tequilas), mas há poucos registros do uso de inóculos mistos de leveduras na produção de *cachaça*. Dessa forma, este trabalho foi realizado com o objetivo de avaliar a eficiência de um inóculo misto de leveduras na fermentação do caldo de cana-de-açúcar para a produção de *cachaça*, além de testar metodologias para a identificação e a caracterização das leveduras, a partir do perfil proteico e metabólico das mesmas.

2 REFERENCIAL TEÓRICO

2.1 Cachaça

Cachaça é uma designação típica e exclusiva da bebida destilada de cana-de-açúcar produzida no Brasil, com grau alcoólico de 38% a 48% v/v, a 20 °C, que é obtida pela destilação do caldo de cana-de-açúcar fermentado (BRASIL, 2005). É a terceira bebida destilada mais consumida no mundo, atrás apenas da vodca e do soju, e a segunda bebida mais consumida no Brasil. A produção anual de *cachaça* é em torno de 2 bilhões de litros, com média de consumo de 11,5 litros por indivíduo por ano (SEBRAE, 2008).

2.2. Processo de produção

A *cachaça* é produzida a partir da moagem da cana-de-açúcar fresca, liberando cerca de 20% p/p de caldo que será fermentado em uma dorna simples e aberta após se ajustar a concentração de açúcares (SCHWAN et al., 2001).

O inóculo microbiano natural (pé de cuba) é, geralmente, preparado por um método chamado fermento caipira e, normalmente, constituído por um mosto com caldo de cana, arroz, farinha de milho, biscoitos salgados com a adição de suco de limão ou laranja para reduzir o pH. A adição de caldo de cana-de-açúcar fresco ocorre a cada dia, de cinco a sete dias (SCHWAN et al., 2001; VICENTE et al., 2006). A cultura iniciadora é utilizada para inocular a dorna principal (dorna de fermentação), tipicamente com menos de 1.000 litros, e uma única batelada de fermentação é, muitas vezes, completada em 24 horas. O caldo fermentado é, então, enviado para um alambique de cobre. Na dorna de fermentação é deixado um volume entre 10% e 20% de caldo fermentado que contém as leveduras, constituindo o pé-de-cuba. O processo é repetido pela

reposição do caldo de cana fresco. Altos níveis de contaminação bacteriana, atrasos na fermentação, presença de leveduras não floculantes e sabores inaceitáveis no destilado normalmente significam que um inóculo deve ser preparado mais do que apenas uma vez por temporada (SCHWAN et al., 2001).

2.3. Leveduras selecionadas

No processo tradicional de fermentação, a microbiota é complexa e consiste de leveduras como *Kluyveromyces marxianus*, *Pichia heimii*, *Hanseniaspora uvarum*, *P. subpelliculosa*, *Debaryomyces hansenii*, *P. methanolica*, *S. cerevisiae* e algumas bactérias ácido láticas, ácido acéticas e bactérias pertencentes à família Enterobacteriaceae (SCHWAN et al., 2001). Devido a esta complexa microbiota, a *cachaça* artesanal, normalmente, tem qualidades sensoriais adicionais devido aos metabólitos e aos voláteis únicos a cada espécie de levedura presente durante o processo de fermentação.

Atualmente, na fermentação para a produção de bebidas, tem-se utilizado leveduras selecionadas, de modo a se obter fermentações mais rápidas, confiáveis, com redução dos riscos de ocorrência de contaminações bacterianas e fermentações lentas (VALERO et al., 2005). Como principais vantagens, o uso de leveduras selecionadas permite rápido início da fermentação, baixo risco de contaminação, melhor uniformidade nas taxas de fermentação, baixa competição por nutrientes, maior rendimento da bebida, baixas concentrações de açúcares residuais, capacidade de floculação, não produção de sulfeto de hidrogênio (H_2S), baixa produção de ácido acético, elevada produção de compostos desejáveis do sabor, alto rendimento alcoólico e produção eficiente de etanol, o que proporciona manutenção das qualidades sensoriais da bebida (BERNADI et al., 2008; CAMPOS et al., 2010; GOMES et al., 2007; OLIVEIRA et al., 2004,

2005; SCHWAN et al., 2001; SILVA; ROSA; OLIVEIRA, 2006; VIANNA et al., 2008).

Desde o início dos anos 1980 até os dias atuais, *S. cerevisiae*, ou "levedura do vinho", vem sendo extensivamente utilizada como iniciadora no processo de fermentação (VALERO et al., 2005). Além das características já citadas, para muitas aplicações industriais nas quais *S. cerevisiae* é utilizada, um comportamento floculante adequado é, certamente, uma das mais importantes características de uma levedura iniciadora (VERSTREPEN et al., 2003).

2.3.1 Leveduras não Saccharomyces

Os efeitos das interações entre *S. cerevisiae* e não *Saccharomyces*, como culturas iniciadoras na qualidade de algumas bebidas, têm sido estudados nos últimos anos. Lee et al. (2010) relataram que a fermentação da cultura mista de *Williopsis saturnus* e *S. cerevisiae* apresentou a capacidade de produção de um vinho de papaia com uma vasta gama de compostos voláteis em concentrações diferentes daquelas obtidas com as culturas puras. Viana et al. (2009) mostraram que uma seleção cuidadosa de leveduras não *Saccharomyces* pode facilitar a obtenção de culturas iniciadoras com uma produção de ésteres otimizada sem comprometer a qualidade do vinho.

Muitos precursores dos componentes aromáticos, como os monoterpenos (limoneno, óxido de linalol, geraniol, nerol, citronelol, α-terpineol, etc.), encontram-se, inicialmente, em sua forma inodora ou glicosilada formando, por exemplo, β-D-glicopiranosídeos, quando ligados à glicose (BOIDO et al., 2002). Além dos β-D-glicopiranosídeo, compostos aromáticos podem estar conjugados com outros açúcares, formando estruturas mais complexas, como α-L-ramnopiranosídeo e α-L-arabinofuranose (SWANGKEAW et al., 2011). Atualmente, a liberação do aroma é otimizada utilizando-se preparações enzimáticas comerciais de origem fúngica,

principalmente de *Aspergillus* spp. As composições dessas preparações variam e elas são, na realidade, uma mistura de glucanases não específicas (VILLENA; IRANZO; PÉREZ, 2007).

A hidrólise enzimática via β-glicosidase produzida por leveduras é um método alternativo que pode ou não modificar o padrão de distribuição natural do aroma, dependendo da especificidade do substrato e da atividade de β-glicosidase (SWANGKEAW et al., 2011). Duarte, Amorim e Schwan (2013) conseguiram relacionar a atividade de β-glicosidase ao aumento da quantidade de compostos terpênicos em caldo de cana fermentado por diferentes leveduras, o que foi útil na seleção das mesmas para uso na produção de *cachaça*.

2.4 Metabolitos microbianos

Os metabólitos são os produtos finais a jusante da expressão de genes e processos de regulação celular, e as alterações em seus níveis podem ser consideradas como a resposta final dos sistemas biológicos a variações ambientais (FIEHN, 2002). A análise do perfil metabólico constitui uma boa ferramenta para investigar a resposta de estirpes de leveduras industriais ao ambiente da fermentação.

2.4.1 Metabólitos microbianos – compostos voláteis formadores de aroma

Etanol e gás carbônico constituem os dois principais compostos formados no processo de fermentação. Em menores quantidades, vários outros compostos são também produzidos na fermentação (LAMBRECHTS; PRETORIUS, 2000).

As leveduras promovem a conversão dos açúcares em produtos como etanol, glicerol, aldeídos, cetonas, ésteres e ácidos e estes compostos contribuem para formar as características de *flavour* da *cachaça* (DUARTE et al., 2011).

Dentre os compostos produzidos em maiores quantidades pelas leveduras na fermentação estão ácido acético, glicerol, ácido succínico e ácido lático (ANTONELLI et al., 1999).

2.4.1.1 Etanol

Bebidas alcoólicas são consumidas primariamente devido ao fato de que o etanol constitui um componente significativo das mesmas. O etanol é o principal constituinte da *cachaça* e um dos principais responsáveis pela caracterização sensorial da bebida. As leveduras são capazes de produzir etanol a partir do metabolismo de açúcares de baixo peso molecular, que podem ser transportados para o citoplasma celular (PATERSON; SWANTSON; PIGGOTT, 2003). Este composto é formado na via glicolítica, em um mecanismo de duas reações. Na primeira reação, o piruvato é descarboxilado, produzindo acetaldeído e liberando CO_2. Em uma segunda reação, o acetaldeído é reduzido para produzir o etanol (LEHNINGER; NELSON; COX, 2006).

2.4.1.2 Glicerol

Na fermentação, a produção do glicerol pelas leveduras se dá no início do processo e considera-se que sua produção ocorre com o consumo dos primeiros 50 g de açúcares (RIBÉREAU-GAYON et al., 2006). Este composto desempenha importante papel na viabilidade celular de leveduras, com o fornecimento de precursores para a síntese de fosfolipídios, que são componentes das membranas celulares durante o período de crescimento da levedura, além da proteção osmótica das leveduras em condições de alta concentração de açúcar; contribuição para a manutenção do equilíbrio redox da célula e a geração da energia (ATP) necessária para o crescimento celular

(SWIEGERS et al., 2005). O glicerol é um constituinte importante para a qualidade da *cachaça* (DUARTE et al., 2011), visto que, em baixas concentrações, é o responsável por dar corpo à bebida.

2.4.1.3 Álcoois superiores

Vários álcoois com mais que dois átomos de carbono são produzidos durante a fermentação e são chamados de álcoois superiores (RIBÉREAU-GAYON et al., 2006). Os álcoois superiores constituem um grupo de compostos encontrados em grande número nas bebidas alcoólicas, nas quais exercem papel importante no aroma. Devido ao seu mecanismo de formação, são também chamados de álcoois de fusel e os principais encontrados nas bebidas são álcool isoamílico (3-metil-butanol-1), propílico (propanol-1), isobutílico (2-metil-propanol-1), sec-butílico (butanol-2), amílico (2-metil-butanol-1), butílico (butanol-1), hexílico (hexanol-1) e 2-feniletanol (PENTEADO; MASINI, 2009; VILELA et al., 2007). A formação dos álcoois superiores pela ação das leveduras ocorre tanto diretamente, a partir da utilização de açúcares, quanto a partir de aminoácidos pela reação de Ehrlich (RIBÉREAU-GAYON et al., 2006).

Na *cachaça*, 2-feniletanol é um dos principais álcoois superiores encontrados (DUARTE et al., 2010, 2011). Este composto é um carreador de aroma e sua presença pode contribuir para as nuances florais na bebida (WONDRA; BEROVIC, 2001). A característica aromática desse composto muda, com a sua oxidação, de “rosas” a “jacinto”. Os álcoois com até cinco átomos de carbono apresentam odores característicos tradicionalmente associados com bebidas destiladas, sendo responsáveis diretos pelo odor da bebida, destacando-se os álcoois amílico e propílico (CARDOSO et al., 2013).

2.4.1.4 Ésteres

Ésteres são compostos de grande importância para o aroma da *cachaça*. Muitos são compostos secundários (originados na fermentação) e apresentam descritores aromáticos, como “banana”, “abacaxi”, “maçã”, “pêra”, etc. (CLARKE; BAKKER, 2004). Os ésteres são formados pelas leveduras durante a fermentação pela ação da acil-CoA, a qual tem grande importância na formação de ácidos orgânicos (BERRY; SLAUGHTER, 2003). A síntese dos ésteres envolve um ácido graxo, um álcool e uma CoA. A acetil-CoA presente na formação do acetato de etila é obtida pela descarboxilação oxidativa do piruvato, mas outras acil-CoA são formadas por reação de acilação da CoASH catalisada pela acil-CoA sintetase (SWIERGERS; PRETORIUS, 2005).

Os ésteres de maior importância aromática são acetato de etila, acetato de isoamila, acetato de isobutila, acetato de 2-feniletila e decanoato de etila (SWIERGERS et al., 2005). O acetato de etila é o éster predominante na *cachaça* (DUARTE et al., 2011; NONATO et al., 2001), produzido em pequenas quantidades por leveduras durante a fermentação. Esse é responsável, quando presente em pequenas porções, pela incorporação de um aroma agradável de frutas na *cachaça* (SOUSA et al., 2012). Por outro lado, em grandes quantidades (acima de 150 mg/L), confere à *cachaça* um sabor enjoativo e indesejado (MALLOUCHOS et al., 2003).

A quantidade e a proporção dos diferentes ésteres influenciam a percepção de aroma nas bebidas, sendo essas características dependentes de fatores como o tipo e a quantidade de fermento, a temperatura, a aeração e a agitação na fermentação, além da qualidade do caldo (CARDOSO et al., 2013).

2.4.1.5 Ácidos orgânicos

Os ácidos orgânicos são compostos de grande importância, pois têm influência sobre diversas propriedades organolépticas, como aroma, sabor e cor das bebidas alcoólicas. Estes compostos são divididos em ácidos voláteis e não voláteis. Os ácidos voláteis apresentam cadeia de carbono curta e, na *cachaça*, o principal representante deste grupo é o ácido acético, cuja quantidade geralmente encontrada pode corresponder a 60% a 95% do conteúdo de ácidos voláteis (OLIVEIRA et al., 2005). Os ácidos graxos de cadeias longas, tais como hexanoico, octanoico, decanoico e dodecanoico, têm efeitos menores sobre o sabor e o aroma das bebidas destiladas (SOUFLEROS et al., 2001). Ácidos com cadeia de carbono variando entre C3 e C16 são sintetizados pelas leveduras durante a fermentação alcoólica e têm influência sobre o aroma. O aroma das bebidas alcoólicas recebe interferência, principalmente, dos ácidos graxos de cadeia curta, como ácido isobutírico, ácido butírico, ácido propiônico, ácido isovalérico, ácido hexanoico, ácido octanoico e ácido decanoico (ABBAS, 2006). Os ácidos butírico, isobutírico e isovalérico são considerados compostos minoritários, mas os seus odores podem ser tão fortes quanto o de ácido acético. Dessa forma, estes ácidos contribuem significativamente no aroma de vinhos e de bebidas destiladas.

2.5 MALDI-TOF MS

A técnica de MALDI-TOF MS (do inglês *matrix assisted laser desorption/ionization - time of flight mass spectrometry*, ou ionização/dessorção a laser, assistida por matriz com analisador de tempo de vôo) tem demonstrado ser um método versátil para analisar macromoléculas de origem biológica (FENSELAU; DEMIREV, 2001), geralmente utilizado no diagnóstico clínico (CASSAGME et al., 2013; DHIMAN et al., 2011; GOYER et al., 2012;

MARKLEIN et al., 2009; MARVIN; ROBERTS; FAY, 2003; STEVENSON et al, 2010; VEEN; CLAAS; KUIJPER, 2010) e representa uma ferramenta promissora para a identificação rápida e fiável de leveduras.

Desde sua invenção, na década de 1980, a MALDI-TOF MS tem sido adotada para aplicações em muitas áreas das ciências da vida (HILLENKAMP; KARAS, 2000). Para uma análise de MALDI-TOF MS, as células são preparadas e cocristalizadas com a matriz, de maneira que produzam uma quantidade suficiente de íons de tamanho médio (2 a 20 kDa) no espectro de massas. A identificação de amostras microbiológicas por este método baseia-se na aquisição de impressões digitais de massas (*fingerprints*) e na subsequente comparação dos dados obtidos com uma base de dados.

A adaptação mais imediata de MALDI-TOF MS tem sido na área da proteômica, quando a análise de proteínas digeridas permite a identificação de pequenas quantidades de proteínas eluídas a partir de géis de eletroforese, em combinação com a existência de um número crescente de sequências genômicas (WELKER; MOORE, 2011).

Embora a técnica de MALDI-TOF MS tenha sido exaustivamente empregada na identificação de bactérias e fungos filamentosos, principalmente na área clínica (WELKER; MOORE, 2011), não há um protocolo realmente efetivo para a identificação de leveduras envolvidas em processos fermentativos.

3 CONSIDERAÇÕES FINAIS

Com base nos resultados deste estudo, conclui-se que a coinoculação de *S. cerevisiae* CA11 e *P. caribbica* influencia positivamente a qualidade final da *cachaça*, principalmente devido aos compostos identificados por GC-FID, CG-MS e HPLC. Como uma consequência das diferenças na composição química, a qualidade sensorial também foi influenciada positivamente.

O processo de destilação não afetou a qualidade final das bebidas, permitindo que as diferenças entre a *cachaça* produzida usando *S. cerevisiae* CA11 e inóculo misto de *P. caribbica* e *S. cerevisiae* possam ser atribuídas ao processo de fermentação. A *cachaça* produzida com o inóculo misto também apresentou grande aceitação na avaliação sensorial, especialmente por parte dos provadores mais jovens, mostrando o potencial dessa bebida como um produto novo, que pode ser apropriado para um determinado nicho de mercado.

A produção de *cachaça* com sabor e aroma acima do padrão existente no mercado e com maior rendimento do produto final irá impactar direta e positivamente a produção de bebidas, reduzindo custos e permitindo que seja agregado valor ao produto e sua inserção em um mercado ávido por uma bebida diferenciada. Além disso, a técnica de MALDI-TOF MS foi útil para o acompanhamento dos inóculos mistos de leveduras no processo de fermentação, em comparação com plaqueamento tradicional, resultando em economia de tempo e recursos.

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CAPÍTULO 2

Evaluation of *Saccharomyces cerevisiae* and *Pichia caribbica* in a mixed inoculum for *cachaça* production and the use of MALDI-TOF for monitoring selected inocula in vat fermentation

Evaluation of *Saccharomyces cerevisiae* and *Pichia caribbica* in a mixed inoculum for *cachaça* production and the use of MALDI-TOF for monitoring selected inocula in vat fermentation

(Preparado de acordo com as normas da revista *International Journal of Food Microbiology* – artigo enviado para publicação)

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Abstract

Cachaça is a unique sugar cane spirit, produced exclusively in Brazil, with an alcohol content of 38–48% v/v at 20°C. The microbiota of traditional fermentation processes is complex and consists of both yeasts and bacteria. The aims of this study were, first, to evaluate the use of mixed culture of *S. cerevisia e* CA11 and *Pichia caribbica* CCMA 0198 for the fermentation of sugar cane juice to produce *cachaça*, and secondly, to evaluate the use of MALDI-TOF as a tool to characterize and monitor selected inocula based on their protein profile. The conditions of fermentation in vats were determined by analyzing different combinations of sugar cane juice Brix degree (14, 16, and 18) and the population size of *P. caribbica* in mixed inoculum (10^5, 10^6, and 10^7 cells/mL). After that, the vat fermentations were conducted in three consecutive batches. The *cachaça* produced by the mixed culture of *P. caribbica* and *S. cerevisiae* showed the highest concentration of volatile compounds associated with good sensory descriptors; these compounds included 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). The *cachaça* produced by the mixed inoculum found greater acceptance in the sensorial analysis for taste and aroma, especially with younger panelists. The extraction of proteins from samples collected directly from sugar cane juice under fermentation (without the traditional plating step) and MALDI-TOF/MS analysis allowed the monitoring and distinction

between mixed inoculum (*S. cerevisiae* and *Pichia caribbica*) and pure inoculum of *S. cerevisiae* during the fermentative process using different Brix degrees (14, 16, and 18) and population sizes of *P. caribbica* in mixed inoculum (10^5, 10^6, and 10^7 cells/mL). In addition, the MALDI-TOF technique was efficient for monitoring the studied inocula for three consecutive fermentation batches in stainless steel vats. In conclusion, we found that improvements could be achieved in the fermentation process with mixed inoculum to improve the quality of *cachaça*, rather than pure of *S. cerevisiae* culture. The MALDI-TOF was useful for monitoring the mixed and pure inocula of yeast in the fermentation process for *cachaça* production.

Keywords: co-inoculation, non-*Saccharomyces*, sugar cane spirit

1. Introduction

Cachaça is the second most widely consumed alcoholic beverage in Brazil, with a production capacity of 2 billion liters/year and about 4000 trademarks, surpassed only by beer. It is the third most widely distilled beverage in the world, although exports reach just over 1% of production; its ethanol content is between 38% (v/v) and 48% (v/v) at 20°C (Brazil, 2005). In 2013, *cachaça* was exported to 59 countries by more than 60 exporting companies, generating revenues of US$ 16.59 million, an increase of 10.71% compared to 2012. There was also a 13.23% increase in volume, with a total of 9.21 million liters.

Cachaça can be described as a complex mixture of flavor compounds in an ethanol/water matrix (Nonato et al., 2001; Souza et al., 2006) that contains higher alcohols, ethyl esters, aldehydes, ketones, and organic acids, which are responsible for the distinct aroma of the beverage (Duarte et al., 2011a; Nonato et al., 2001). Among flavor compounds, 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 Czupryński, 2006). The physicochemical and organoleptic characteristics of *cachaça* depend on several factors involved in distillation, aging (Vicente et al., 2006), and especially, fermentation conditions.

Different yeasts, such as *Kluyveromyces marxianus*, *Pichia heimii*, *Hanseniaspora uvarum*, *Pichia subpelliculosa*, *Debaryomyces hansenii*, *Pichia methanolica*, *Saccharomyces cerevisiae*, and bacteria, such as certain lactic and acetic acid bacteria and several bacteria belonging to the Enterobacteriaceae family, comprise the complex microbiota of traditional fermentation processes (Schwan et al., 2001). As a result, artisanal *cachaça* spirits typically have a unique smell and taste. Bacteria and non-*Saccharomyces* yeast present during the fermentation process can contribute to the formation of the flavor of *cachaça*. Several studies have reported on the use of mixed cultures of *S. cerevisiae* and other microorganisms in the production of wines (Viana et al., 2008; Zott et al., 2008) and spirits (Duarte et al., 2011a; López-Alvarez et al., 2012); however, there is only one record regarding the use of mixed inocula of yeasts in the production of *cachaça* (Duarte et al., 2013). In this previous work, it was demonstrated that non-*Saccharomyces* with high β-

glucosidase activity can contribute to increased levels of volatile compounds, including some esters and monoterpenic (Duarte et al., 2013).

Matrix assisted laser desorption ionization—time of flight/mass spectrometry (MALDI-TOF/MS) has been demonstrated to be a versatile method of analyzing macromolecules from their biological origin (Demirev and Fenselau, 2008), it is already commonly used in clinical diagnosis (Goyer et al., 2012; Marvin et al., 2003; van Veen et al., 2010), and it represents a promising tool for the rapid and reliable identification of yeasts. To conduct a MALDI-TOF/MS analysis, cells are prepared and co-crystallized with a matrix in a way that yields a sufficient number of medium-sized ions (2–20 kDa) in the mass spectra. The identification of microbiological samples by this method relies on the acquisition of mass fingerprints and the subsequent comparison of the obtained data with a database. Only a few studies have used MALDI-TOF/MS to identify yeasts used in fermentation processes (Moothoo-Padayachie et al., 2014; Usbeck et al., 2014), and to the best of our knowledge, there are no MALDI-TOF/MS studies aiming at monitoring yeast selected inocula (mixed and pure inocula) during the fermentation of sugar cane juice.

Thus, the aims of this study were to evaluate the efficiency of a mixed yeast inoculum for the production of *cachaça* and to assess the use of MALDI-TOF/MS to characterize and monitor the inoculated yeasts as pure and mixed inocula.

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 *Pichia caribbica* CCMA 0198 (former *P. caribbica* UFLA CAF733) belonging to the collection of the Laboratory of Microbiology/DBI Federal University of Lavras and *Saccharomyces cerevisiae* CA11 (LNF Latino Americana, Bento Gonçalves - Brazil).

2.2 Determination of the best conditions for the use of the mixed inoculum

To evaluate the effect of sugar cane Brix degree (ºBrix) and the population of *P. caribbica* in the mixed inoculum over the fermentative process, three different ºBrix (14, 16, and 18) and inoculum sizes (10^5, 10^6, and 10^7 cells/mL) of the non-*Saccharomyces* yeast were evaluated.

The fermentation processes were conducted in test tubes containing 10 mL of must, as shown in Table 1. Samples were taken during the fermentation to determine the profile of the yeast population. The volatile and non-volatile compounds in the fermented sugar cane were analyzed by gas chromatography flame ionization detection (GC-FID) and high-performance liquid chromatography (HPLC) to determine the best combination of inoculum size and ºBrix based on the fermentative performance (described below) and the production of desirable volatile compounds. The fermentation was conducted in triplicate. Upon analyzing the results, the best condition obtained was validated by fermentation in stainless vats, with subsequent distillation.

Table 1 Treatments tested with three Brix and three non-*Saccharomyces* population sizes.

Treatments	
14°Brix – 10^5 cells/mL	1
14°Brix – 10^6cells/mL	2
14°Brix – 10^7cells/mL	3
16°Brix – 10^5cells/mL	4
16°Brix – 10^6cells/mL	5
16°Brix – 10^7cells/mL	6
18°Brix – 10^5cells/mL	7
18°Brix – 10^6cells/mL	8
18°Brix – 10^7cells/mL	9

2.3 Vats 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) as described by Duarte et al. (2010). Using a platinum loop, the yeasts were inoculated into tubes containing 1 mL YPD, after which they were incubated at 28°C for 24 hours. After 24 hours, the contents of the tubes were transferred to tubes containing 9 mL YPD, and they were incubated for 24 hours at 28°C. In the next step, the yeast culture (10 mL) was transferred to an Erlenmeyer flask containing 90 mL YPD, which was incubated for 24 hours at 28°C. Finally, the

contents of the tubes (100 mL) were transferred to an Erlenmeyer flask containing 900 mL YPD, and they were incubated for 24 hours at 28°C. From that moment on, every 24 hours, 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 until enough cells were obtained for the inocula; the sizes were predefined as described above.

The fermentation experiments were conducted in stainless steel vats containing 15L sterile sugar cane juice 16 ºBrix at room temperature (±28°C). The yeasts were inoculated together with populations of 10^7 cells/mL (*P. caribbica*) and 10^8 cells/mL (*S. cerevisiae*); the volume of the inoculum corresponding to 10% of the volume of sugar cane juice to be fermented was used to start the fermentation. In addition to the mixed inoculum, *S. cerevisiae* in pure culture was used as a fermentation control. Three batches were processed, and the end of each was set when the °Brix stabilized (around 24 hours). To avoid stressing the yeast cells, the vats were fed by adding certain amounts of 16 °Brix sugar cane juice until the Brix inside the vats reached 7 °Brix, and fed back when the Brix was around 3. This procedure was repeated until the volume was

completed to 15L. During the fermentation, samples were taken at the beginning (T0) and end of each batch (T24) to determine the profile of the yeast population. The fermented sugar cane was analyzed by GC-FID and HPLC. The fermentations were conducted in duplicate.

2.4 Analysis of survival and yeast growth

Yeast growth was measured by plate count. Samples were taken during fermentation and diluted appropriately with 0.1% peptone water. In fermentation with mixed cultures, cell enumerations of selected non-*Saccharomyces* were performed using lysine agar (LA) containing 66 g/L medium lysine (HiMedia®, Mumbai), 10 mL/L, 50% potassium lactate, pH 4.8; the enumeration of *S. cerevisiae* cells was determined as the difference between the count of the YPD plates and the count of the LA plates (Nissen et al., 2003). The plates containing YPD and LA were incubated at 28°C and counted after 48 hours of incubation.

2.5 Evaluation of fermentation performance

The fermentation performance for different conditions of °Brix and inoculum size was based on the conversion factors of the substrates (sucrose, glucose, and fructose) into ethanol ($Y_{p/s}$), glycerol ($Y_{g/s}$), and acetic acid ($Y_{ac/s}$); volumetric productivity of ethanol (Q_p); conversion efficiency of sugars into ethanol (E_f); and total conversion of sugars (*Conv.*) (Duarte et al., 2011b; Oliveira et al., 2004).

2.6 Distillation

The distillation was carried out in a copper alembic with a working capacity of 40 L. Thirty liters of fermented sugar cane juice from each batch were added to the alembic; the temperature was maintained between 91 and 97°C to allow a distillation rate of approximately 1 L/h. Approximately 10% of the distillate (the first fraction) was collected separately ("head"). The "heart" (*cachaça*) was collected until an ethanol concentration reached approximately 42% (v/v). A volume of approximately 10% of the distillate, corresponding to the "tail," was collected at the end of the distillation (Campos et al., 2010). The produced *cachaça* was stored in 700mL glass bottles for subsequent HPLC, GC-FID, GC-MS, and sensory analyses.

2.7 HPLC analysis

Ethanol, glycerol, acetic acid, and carbohydrates (glucose, sucrose and fructose) were analyzed in fermented sugar cane juice during the evaluation of the best fermentation conditions; and ethanol and acetic acid were analyzed in *cachaça*s. These compounds were identified and quantified using HPLC using a Shimadzu chromatograph (Shimadzu Corp., Japan) equipped with a UV-Vis detector (SPD-10Ai) and a refractive index detector (RID-10A). An ion exclusion column (Shim-pack SCR-101H, 7.9 mm x 30 cm) was operated at a temperature of 30°C using 100 mmol/L perchloric acid at a flow rate of 0.6 mL/min. The UV absorbance (210 nm) was used for acids detection, while RID was used to detect sugars, glycerol and ethanol. The compounds were identified by comparing their retention times to the retention times of certified known standards. The quantification was performed using an external calibration methodology. All samples were examined in duplicate (Duarte et al., 2011b).

2.8 GC-FID analysis

Major volatile compounds were analyzed directly after the filtration of samples (0.22 μm pores) without any other prior treatments in fermented sugar cane (experiment for determination of the best fermentation conditions and vat fermentation) and in the *cachaça* from three different batches. The minor volatile compounds in fermented sugar cane juice were determined after extraction and concentration of the headspace compounds with SPME as previously described by de Souza et al. (2009).

The analysis were performed using a gas chromatography (GC) Shimadzu model 17A, equipped with an flame ionization detector (FID) and using an DB Wax column (30 m x 0.25 mm i.d. x 0.25 μm) (J&W Scientific) operated under conditions described by Duarte et al. (2011b). The major volatile compounds were analyzed by the injection of 1 μL of each sample in the split mode (1:10), while for minor volatile compounds the fiber was introduced in the GC injector for thermal desorption at 240°C for 5 min. The subsequent compound identification was accomplished by comparing the retention times of the sample peaks with those of known standards injected in the same conditions. The resulting

measurements were expressed in 4-nonanol (internal standard) equivalents. For the major volatile compounds, 4-nonanol was used at a concentration of 123.76 mg/L, and 312 μg/L for the minor volatile compounds (Duarte et al., 2010, 2011a).

2.9 GC–MS analysis

The minor volatile compounds of *cachaça* were determined after extraction and concentration of the headspace compounds with 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. To sample and concentrate the headspace compounds a 50/30 μm DVB/Carboxen/PDMS StableFlex SPME fiber with manual holder (Supelco, Bellefonte, PA, USA) was used. The SPME parameters used were 60°C and 25 min for extraction (de Souza et al. 2009). The fiber was introduced in the GC injector for thermal desorption at 240°C for 5 min.

GCMS-QP2010 SE system (Shimadzu) was used for sample analysis. Samples were injected into the FFAP column (30 m x 0.25 mm i.d. x 0.25 μm) using splitless injection mode (30 s at 25 psi). The

splitless was opened after 2 minutes. After waiting for 5 min at 50°C, the temperature of the column oven was increased by 3°C/min up to 190°C and was maintained at 190°C for 10 min. High-purity helium was used as carrier gas with a constant flow of 1.2 mL/min. After 6 min the mass spectra, *m/z*, were acquired continuously from 45 to 1000. The temperature of the ion source of electrons was 230°C and the quadrupole was 150°C. Analyses of mass spectra were made by comparing them to those of pure standards (when available) and using the NIST library (2011). The resulting measurements were expressed in 4-nonanol (internal standard) equivalents used at concentration of 125 μg/L.

2.10 Monitoring inoculated yeasts using MALDI–TOF

2.10.1 Media and cultivation conditions

To evaluate the efficiency of the MALDI-TOF/MS technique for monitoring inocula during the fermentation of sugar cane juice, proteins were extracted from different samples. First, from pure cultures of yeast cultivated in YPD agar, samples from mixed inocula (10^7 cells/mL *P. caribbica* and 10^8 cells/mL of *S. cerevisiae*) cultivated in YPD broth, and

samples from mixed inocula (10^7 cells/mL *P. caribbica* and10^8 cells/mL of *S. cerevisiae*) fermenting 16°Brix sugar cane juice were extracted. In the second step, MALDI-TOF/MS was used to monitor the mixed inoculum used under different populations of *P. caribbica* (10^5, 10^6, and 10^7 cells/ml) and sugar cane juice with different °Brix (14, 16, and 18 °Brix) (Table 1). Finally, MALDI-TOF/MS was used to monitor the mixed inoculum and pure inoculum of 16 °Brix *S. cerevisiae* fermenting sugar cane juice for three successive batches; the samples were withdrawn at the beginning and at the end of each batch.

All extractions from colonies were made according to the method of Bruker (2007), and all extractions from liquid cultures were made according to the method of Usbeck et al. (2013), as described below.

2.10.2 Extraction from colonies

Pure cultures of *S. cerevisiae* and *P. caribbica* were cultivated on YPD agar medium at 30°C for 24 hours. Yeast cells were collected from the plate and suspended in 300 µL of ultrapure water by vigorous vortexing, and 900 µL of absolute ethanol were added to the cell suspension. The cell suspension was homogenized and centrifuged at

15,000 rpm for two minutes, and the supernatant was discarded. Centrifugation was repeated to completely remove all residual liquid. The ethanol-exposed cell pellet was air-dried in a laminar-flow hood at room temperature for five minutes. For yeast protein extraction, 50 µL of 70% formic acid (v/v) was added to the pellet, and the contents were thoroughly mixed by vortexing. Thereafter, 50 µL of 100% acetonitrile were added to the solution and mixed thoroughly by vortexing. The suspension was centrifuged at 15,000 rpm for two minutes, and 1 µL of supernatant containing extracted yeast proteins was applied to a specified spot on a steel MALDI target plate and air-dried in a laminar-flow hood at room temperature for five minutes. All samples were prepared in triplicate and each replicate was spotted three times on the MALDI target.

2.10.3 Liquid extraction

The liquid extractions for MALDI-TOF/MS analysis were made according to the method of Usbeck et al. (2013), with minor modifications. First, 900 µL of cell suspension were transferred to a 1.5mL tube and centrifuged at 15,000 rpm at 4°C for two minutes; the supernatant was discarded and the pellet was mixed thoroughly with 300

µL ultrapure water. Then, 900 µL absolute ethanol were added and mixed. The tube was centrifuged at 15,000 rpm at 4°C for two minutes. Ethanol was decanted for five minutes, after which the tube was centrifuged again at 15,000 rpm at 4°C for two minutes. The supernatant was discarded and the pellet was air-dried for a minimum of 30 minutes, until the solvent evaporated completely. Next, 50 µL of 70% formic acid were added to the dried pellet and mixed thoroughly until the extract was resuspended. An equivalent volume of acetonitrile (ACN) was added and the sample was mixed again. The mixture was centrifuged at 15,000 rpm at 4°C for two minutes, after which 1 µL of the supernatant was spotted onto a MALDI target and dried at room temperature. All extractions were performed in triplicate and each replicate was spotted three times on the MALDI target.

After each of the described extraction methods, the target spots were covered with 1 µL matrix solution of α-cyano-4-hydroxy-cinnamic acid (CHCA) (Bruker Daltonik), which was freshly prepared in 50% ACN and 2.5% trifluoroacetic acid, with a final concentration of 10 mg CHCA/mL.

The MALDI-TOF/MS analyses were performed using a Microflex LT spectrometer (Bruker Daltonik) and FlexControl software (Version 3.0) operated according to parameters described by Usbeck et al. (2013). For each spectrum, 240 single spectra, acquired in 40-shot steps from different positions of the target spot, were summarized. An external calibration was performed at regular intervals using a bacterial test standard (Bruker Daltonik).

The raw spectra were converted to a text file containing *m/z* and peak intensities using FlexAnalysis software (Version 3.4). The text files were then imported into mMass software version 5.5.0 (Niedermeyer and Strohalm, 2012), where they were smoothed, the baseline was subtracted, and the signal intensities were normalized. Peak picking was based on total average spectrum, using a signal-to-noise ratio threshold of 5. The peaks were aligned by generating a consensus peak list using SPECLUST (Alm et al., 2006), available at http://bioinfo.thep.lu.se/speclust. This consensus peak list was then used for the cluster analysis.

2.11 Sensory analysis

Sensory analysis of *cachaça* was performed with 61 untrained panelists that used to consume distilled beverages. The panelists, aged between 18 and 60 years, consisted of students and workers at the Federal University of Lavras. Each panelist received 2 samples of *cachaça*. Randomized, 10 mL samples were served in clear 25 mL glasses. Distilled water was provided for rinsing of the palate during testing. Evaluations were performed in the mornings between 9:00 and 10:00 a.m. and were conducted at room temperature (22 to 25°C) under white light. *Cachaça* samples were evaluated for taste, aroma, appearance and overall acceptability according to the hedonic scale of 9 categories: Dislike Extremely = 1, Dislike Much = 2; Dislike Moderately = 3; Dislike Slightly = 4, Neither Dislike nor Like = 5, Like Slightly = 6; Like Moderately = 7; Like Much = 8, Like Extremely = 9 (Duarte et al., 2011a).

2.12 Statistical analysis

Principal Component Analyses, normality test, non-parametric Wilcoxon test and cluster analyses were performed using Xlstat 2014.5 software (Addinsoft's, New York, NY). Cluster analyses were made

using Unweighted Pair-Group Average (PGA) and Pearson dissimilarity. The normality of the data from sensorial analysis was checked using Shapiro-Wilk test at 0.5 of significance and the differences between means was verified by Wilcoxon test ($p < 0.1$).

3 Results and Discussion

3.1 Determination of the conditions of use of the selected mixed inoculum

Nine different assays were performed (Table 1), combining different populations of *P. caribbica* (10^5, 10^6, and 10^7cells/mL) in a mixed inoculum fermenting three different °Brix (14, 16, and 18 °Brix). The results of the sugars, ethanol, glycerol, acetic acid, and kinetic parameters were generally independent on the initial population of *P. caribbica* in the mixed inoculum (Table 2). The residual amounts of fructose in the fermentation broth gradually increased with increasing initial °Brix values (Table 2). As observed with residual fructose, it was also possible to observe a steady increase in ethanol and glycerol concentrations associated with the use of sugar cane juice with greater °Brix, with the highest concentrations of glycerol and ethanol, 8.58 g/L

and 97.18 g/L, respectively, found when sugar cane juice was fermented at 18 °Brix with a mixed inoculum containing the 10^7 cells/mL population of *P. caribbica* (Table 2).

In addition to the higher ethanol concentrations obtained with the use of 18 °Brix sugar cane juice, it was observed that the fermentation with a higher initial content of soluble solids displayed higher ethanol productivity (Q_p) 2.49–2.56 g/L/h) and ethanol yield ($Y_{p/s}$) (0.41–0.42 g/g) and higher conversion efficiencies of sugar into ethanol (E_f), ranging from 81.18% (18 °Brix and 10^7cells/mL) to 83.01% (18 °Brix and 10^8cells/mL).

The acetic acid concentrations did not vary depending on the °Brix and *P. caribbica* population. In our previous work (Duarte et al., 2013), we observed that mixed inoculum in 16 °Brix sugar cane juice displayed increased fructose consumption compared to a pure *S. cerevisiae* culture. Based on this result, we decided to test the fermentation with 18 °Brix; however, even having observed the highest ethanol, glycerol, and kinetic parameters ($Y_{p/s,}$ $Q_{p,}$ and E_f) for sugar cane juice with 18 °Brix, we also observed the least total sugar consumption (*Conv*) in this situation, which is consistent with the high residual levels of sugars, especially fructose

(31.12–35.87 g/L) (Table 2). This reduced consumption of sugars might be related to a lower rate of cell proliferation, which can increase the risks of contamination and reduce efficiency of the selected inoculum, mainly due to the fact that the fermentative process is conducted with recycled cells.

Table 2 Concentrations (g/L) of sugars, alcohols and acetic acid in fermented sugar cane juice and kinetic parameters for mixed inoculum in different Brix and *P. caribbica* population.

Treatments	Compounds (g/L)						Parameters			
	Sucrose	Glucose	Fructose	Glycerol	Ethanol	Acetic acid	$Y_{p/s}$ (g/g)	Ef (%)	Conv. (%)	Q_p (g/L h)
14°Brix-10^5	1.80±0.01	0.25±0.30	0.86±0.42	6.48±0.54	74.06±2.36	0.14±0.01	0.35	67.87	99.21	1.95
14°Brix-10^6	1.85±0.01	0.08±0.06	3.99±1.49	6.29±0.73	73.70±2.82	0.12±0.01	0.35	69.41	97.11	1.94
14°Brix-10^7	1.89±0.02	0.29±0.36	2.59±1.41	6.65±0.71	72.01±4.53	0.08±0.01	0.34	66.54	97.97	1.89
16°Brix-10^5	2.19±0.01	0.22±0.19	20.19±1.87	7.24±0.78	85.50±0.77	0.14±0.02	0.39	75.80	87.65	2.25
16°Brix-10^6	2.32±0.10	0.05±0.01	10.98±6.62	6.96±0.14	86.39±1.83	0.11±0.02	0.37	71.94	93.33	2.27
16°Brix-10^7	2.28±0.03	0.25±0.23	11.06±1.47	7.60±0.89	85.86±0.11	0.12±0.01	0.36	70.97	93.22	2.26
18°Brix-10^5	2.76±0.00	0.53±0.50	35.58±2.10	8.38±0.90	96.68±1.18	0.13±0.02	0.42	83.01	80.56	2.54
18°Brix-10^6	2.67±0.10	0.53±0.51	31.12±1.68	8.58±1.22	97.18±0.37	0.32±0.02	0.42	82.71	82.67	2.56
18°Brix-10^7	2.69±0.03	0.54±0.30	35.87±2.27	8.15±0.93	94.59±0.27	0.12±0.01	0.41	81.18	80.43	2.49

Volatile compounds with relevance to the taste and aroma of *cachaça* were analyzed by GC-FID. The *P. caribbica* population and Brix did not show a clear influence (in general) on the production of volatile compounds. However, for 18 °Brix and an initial *P. caribbica* population of 10^7cells/mL, some significant changes in volatile composition were observed; furfural, volatile fatty acids, and higher alcohols were found in higher concentrations (8.39 mg/L, 74.84 mg/L, and 890.53 mg/L, respectively) (Table 3). According to García-Llobodanin et al. (2008), furfural (with acetaldehyde and methanol) is one of the most negative compounds in distillate. Volatile acids are desirable, since they are present in low concentrations in the beverage; aromatic descriptors for these compounds include terms as “bitter,” “wax” (Meilgaard, 1975), “rancid,” and “sweaty” (Siebert et al., 2005). Some higher alcohols, such as amyl alcohols, can negatively affect the aroma of the beverage when present in high concentrations, with descriptors such as “alcoholic,” “sweet,” and “choking” (Falqué et al., 2001).

Table 3 Concentrations (mg/L) of major volatile compounds in the different Brix and *P. caribbica* population.

N°	Compounds	Treatments									Descriptors
		14°Brix-10^5	14°Brix-10^6	14°Brix-10^7	16°Brix-10^5	16°Brix-10^6	16°Brix-10^7	18°Brix-10^5	18°Brix-10^6	18°Brix-10^7	
	Alcohols (5)										
1	1-Propanol	23.04±15.10	21,23±8,34	30.42±1.70	40.44±1.95	23.05±6.08	11.25±0.45	9.71±0.43	42.1±1.46	114.35±0.52	Alcohol (C)
2	2-Methyl-1-propanol	56.96±0.81	44,95±4,29	53.31±2.64	60.94±4.70	28.84±5.06	17.77±2.24	11.57±0.64	47.35±1.48	45.30±6.29	Malty (A)
3	2-Methylbutanol-1 3-Methylbutanol-1	194.71±2.58	165,80±19,17	180.84±6.02	233.52±23.05	163.59±36.82	66.78±5.63	51.90±5.91	192.05±4.79	589.27±0.26	Alcohol. banana. medicinal. solvent (C)
4	Furfurylalcohol	4.18±0.60	6,05±1,56	5.61±0.57	22.20±2.57	18.48±4.97	5.46±0.77	5.66±0.81	23.95±3.10	68.41±1.28	Sugar cane. Woody (C)
5	2-Phenylethanol	25.25±1.11	22,42±3,71	28.24±4.25	30.44±2.77	20.99±4.45	9.19±0.94	7.71±1.17	26.76±4.09	73.20±6.03	Roses.sweetish. perfumed (C)
	Total alcohols (5)	**304.13**	**260,45**	**298.42**	**387.54**	**254.95**	**110.45**	**86.55**	**332.21**	**890.53**	
	Acetates (3)										
6	Ethyl acetate	12.76±0.98	8,70±0,08	2.24±2.66	14.54±0.62	8.40±1.88	3.97±0.39	2.97±0.33	11.11±0.40	9.45±0.16	Solvent. fruity. sweetish (C)
7	Propyl butyrate	4.67±1.31	6,43±0,92	5.45±1.72	6.70±0.33	7.20±1.62	1.91±0.16	1.27±0.20	6.20±0.41	23.04±6.33	
8	Phenylethyl acetate	0.76±0.11	1,78±0,21	3.17±0.82	3.79±0.29	3.62±0.87	1.33±0.11	1.17±0.13	4.93±0.08	19.51±3.83	Roses. honey. Apple. sweetish (C); flowery (B)
	Total acetates (3)	**18.19**	**16,90**	**17.86**	**25.02**	**19.22**	**7.21**	**5.42**	**22.25**	**52.00**	
	Monoterpenic alcohols (2)										
9	Verbenone	4.47±1.39	2,99±1,10	4.21±1.65	5.81±1.05	19.90±4.51	2.52±1.57	0.84±0.03	5.24±1.12	32.22±0.98	-
10	β-Citroneloll	3.77±0.67	4,56±0,61	3.98±0.78	6.89±0.26	8.53±2.04	1.65±0.07	1.70±0.20	8.05±0.95	21.24±2.59	Citronella (D)
	Total monoterpenic alcohols (2)	**8.24**	**7,55**	**8.19**	**12.70**	**28.43**	**4.18**	**2.54**	**13.29**	**53.46**	
	Aldehydes (2)										
11	Acetaldehyde	16.91±1.43	21,36±0,49	23.27±5.62	26.12±7.30	11.22±2.37	8.45±1.62	3.63±1.70	23.83±10.55	10.44±1.31	Green leaves. fruity (C)
12	Furfural	0.47±0.20	0,41±0,04	0.78±0.16	2.14±0.03	0.62±0.21	0.40±0.01	0.28±0.06	1.65±0.72	8.39±1.96	Paper. husk (C)

nd: not detected. (A) CZERNY et al. (2008); (B) SIEBERT et al. (2005); (C) MEILGAARD (1975)

Table 3 (continued)

N°	Compounds	Treatments									Descriptors
		14°Brix-10^5	14°Brix-10^6	14°Brix-10^7	16°Brix-10^5	16°Brix-10^6	16°Brix-10^7	18°Brix-10^5	18°Brix-10^6	18°Brix-10^7	
	Total aldehydes (2)	**17.38**	**21,77**	**24.05**	**28.26**	**11.84**	**8.85**	**3.91**	**25.48**	**18.83**	
	Volatile acids (8)										
13	Propionic acid	2.93±0.04	2,28±0,04	2.41±0.70	3.02±0.23	3.29±0.79	1.11±0.16	0.77±0.06	3.65±1.13	9.82±1.42	Acetic acid. (C); vinegar (B)
14	Isobutyric acid	2.66±0.47	3,25±1,07	2.82±0.79	2.25±0.41	2.41±0.46	0.97±0.05	0.48±0.00	2.28±0.14	5.56±0.81	Sweaty. bitter (C); rancid (B)
15	Hexanoic acid	1.66±0.41	1,88±0,37	1.43±0.20	2.06±0.27	1.66±0.54	0.76±0.01	0.45±0.02	1.19±0.01	5.51±1.35	Goaty. fatty acid. sweaty (C)
16	2-Ethyl caproic acid	0.49±0.07	0,98±0,25	0.47±0.08	nd	0.91±0.54	0.27±0.09	0.27±0.00	0.37±0.03	1.73±0.56	-
17	Octanoic acid	2.89±0.13	2,94±0,13	2.80±1.05	3.12±0.01	3.14±1.15	1.06±0.09	0.44±0.10	3.17±0.70	6.91±1.10	Fatty acid. vegetable oil (C);
18	Nonanoic acid	2.67±0.61	1,60±0,59	1.72±0.17	6.28±0.23	6.43±3.07	1.32±0.10	2.15±0.64	8.22±0.16	37.14±2.02	-
19	Decanoic acid	1.24±0.32	11,05±0,83	1.06±0.03	1.00±0.01	1.65±0.54	0.35±0.02	0.28±0.03	0.85±0.20	3.59±1.58	Waxy. rancid. soapy (C);
20	Benzoic acid	2.44±0.24	2,38±0,48	2.46±1.01	2.64±0.05	1.80±0.62	0.25±0.01	0.32±0.05	2.38±0.88	4.58±1.10	-
	Total volatile acids	**16.98**	**26,35**	**15.17**	**20.37**	**21.30**	**6.09**	**5.15**	**22.11**	**74.84**	
	Others (1)										
21	1.1-Diethoxiethane	0.69±0.01	0,45±0,11	0.29±0.02	0.71±0.14	0.58±0.13	0.23±0.01	0.06±0.01	0.20±0.04	2.03±0.52	-

nd: not detected. (A) CZERNY et al. (2008); (B) SIEBERT et al. (2005); (C) MEILGAARD (1975)

After analyzing the data of residual sugars, ethanol, fermentative performance, and volatile compounds, the choice of 18 °Brix was disregarded due to the high levels of fructose, low value of *Conv*, and the highest concentrations of furfural and volatile fatty acids.

Taking into account the intermediate values of ethanol (85.50–86.39 g/L), Q_p (2.25–2.27 g/L/h), *Conv* (87.65–93.33%), and higher alcohols (110.45–387.54 mg/L), 16 °Brix was chosen for subsequent steps of the work. In addition, the 10^7 cells/mL population was chosen because in the fermentation process of *cachaça* production, yeasts are subjected to ethanol levels of up to 8% (Campos et al., 2010), and it is usual to recycle the cells, thereby justifying the use of higher initial populations of non-*Saccharomyces* yeast for use in the mixed inoculum.

3.2 Fermentation in stainless steel vats

Vat fermentations were carried out in duplicate using 15L of 16 °Brix sugar cane juice with mixed inocula of *P. caribbica* (10^7 cells/mL) and *S. cerevisiae* CA11 (10^8 cells/mL) and with pure *S. cerevisiae* (10^8 cells/mL) as a control. The *P. caribbica* population in the mixed

inoculum was stable during the entire process, ranging from 7.51 to 7.84 CFU/mL at T0, and from 7.51 to 7.87 CFU/mL at T24. The populations were similar to the populations in our previous study (Duarte et al., 2013). Although the *S. cerevisiae* population in the mixed inoculum was stable (8.20–8.25 CFU/mL) during the first batch, there was a small decrease in the population during the second batch, ranging from 8.29 (T0) to 7.54 (T24) CFU/mL, and an increase during the third batch (7.77–8.49 CFU/mL). When *S. cerevisiae* was used in pure culture to ferment sugar cane juice, small differences in the cell densities were observed between the first and third batches. At T0, the yeast populations ranged from 8.06 to 7.23 log CFU/mL, while at T24 of the first and third batches, the yeast populations were 8.01 and 8.24 log CFU/mL, respectively. The differences in cell population found among the batches was probably due to the removal of yeast cells with the fermented sugar cane juice at the end of each batch fermentation; these changes in yeast population due to the removal of cells after fermentation was reported by Duarte et al., (2011a).

3.3 Chemical analysis of distilled beverage

The HPLC, GC-FID, and GC-MS results are presented as averages of three batches. As shown in Table 4, it was observed that the main purpose of fractionation was achieved, allowing the adjustment of the alcohol content of the heart fraction (*cachaça*). However, the act of fractionating the distillate is important, not only to ensure that the heart fraction has the correct content of ethanol, but also to ensure the presence of compounds favorable to the flavor and aroma of *cachaça*, and to reduce the amounts of undesirable compounds, such as acetic acid (Campos et al., 2010; Duarte et al., 2011a, 2011b; Reche et al., 2007).

The ethanol concentration in *cachaça* produced by *S. cerevisiae* in three consecutive batches ranged from 302.37 g/L to 353.30 g/L, with an average (three batches) of 322.56 g/L (40.88% v/v). The ethanol concentration of *cachaça* obtained from fermentation with the co-inoculation ranged from 312.71 g/L to 328.60 g/L, with an average of 317.48 g/L (40.24% v/v) for the three batches. These values are in agreement with the limit values proposed by Brazil (2005). Methanol, a particularly undesirable alcohol in brandy that originates from pectin degradation, was not found in the *cachaça* produced by co-inoculation or by pure *S. cerevisiae* inoculum.

Table 4 Concentrations of compounds identified in head, heart, and tail fractions (g/L).

Compounds	*S. cerevisiae*			Mixed inoculum		
	B1	B2	B3	B1	B2	B3
	Head			Head		
Ethanol	411.54	453.09	409.59	340.76	409.31	447.68
Acetic acid	0.080	0.019	0.007	0.020	0.021	0.017
	Heart			Heart		
Ethanol	312.02	353.30	302.37	328.60	312.71	311.12
Acetic acid	0.093	0.036	0.022	0.014	0.017	0.023
	Tail			Tail		
Ethanol	253.69	219.73	194.57	150.84	168.21	137.77
Acetic acid	0.064	0.044	0.027	0.002	0.024	0.038

nd not detected.

The acetic acid concentration found in the *cachaça* produced by co-inoculation was lower than the concentration found in samples from fermentation with *S. cerevisiae* (Table 4), although concentrations found in this work are still within the Brazilian law (Brazil 2005). The highest amounts of acetic acid in the *cachaça* produced by *S. cerevisiae* were 0.093 and 0.36 g/L in the first and second batches, respectively; the average concentrations for the three consecutives batches were 0.018 g/L for mixed inoculum and 0.05 g/L for pure *S. cerevisiae* culture. The values found for *S. cerevisiae* are similar to those reported by Duarte et al. (2011a), endorsing the fact that the production of acetic acid by mixed inoculum is lower than that produced by pure *S. cerevisiae* culture. Acetic

acid is one of the most important compounds in *cachaça*, due to its effect on the acidity of the beverage. According to Silva et al. (2006), acidity can significantly and negatively influence the sensory quality of *cachaça*.

A total of eight major volatile compounds were identified in the *cachaça* produced in this study (Table 5). The *cachaça* produced by co-inoculation contained the highest concentration of acetaldehyde (38.73 mg/L). While this compound can exert a negative influence on the beverage, at a concentration of 38.73 mg/L, it probably did not negatively affect the quality, with contributing descriptors associated with green leaves, paper, and husk (Meilgaard, 1975).

Ethyl acetate is the main acetate found in *cachaça* (Duarte et al., 2011a), and according to Souza et al. (2012), at low concentrations (50–80 mg/L), ethyl acetate has a positive impact on flavor. The mixed inoculum produced the highest amount of ethyl acetate (53.72 mg/L) measured in the *cachaça* evaluated in this work (Table 5). In our previous work (Duarte et al., 2013), we demonstrated that the yeast with the highest *β*-glucosidase activity produced a fermented sugar cane juice with higher concentrations of some volatile compounds, including acetates. The higher concentration of ethyl acetate found in the *cachaça* produced

by co-inoculation might be provided by *P. caribbica*, probably because of its *β*-glucosidase activity.

Isoamyl alcohols (2-methylbutanol-1 + 3-methylbutanol-1) were the most abundant compounds measured in both distilled beverages, which are also the major higher alcohols of *cachaça*, according to Duarte et al. (2011a). These compounds are associated with "banana" and "sweetish" descriptors (Czerny et al., 2008), which makes them favorable to the taste and aroma of the *cachaça*. The *cachaça* produced by co-inoculation presented a higher concentration of isoamyl alcohols than the *cachaça* produced by only *S. cerevisiae* (874.37 and 622.28 mg/L, respectively). Another important higher alcohol, 2-phenylethanol, was found in the *cachaça* produced by the mixed inoculum of *P. caribbica* and *S. cerevisiae* at a concentration of 15.89 mg/L, while a pure inoculum of *S. cerevisiae* produced 13.21 mg/L of 2-phenylethanol (Table 5). This compound is an aroma carrier, and its presence may contribute to the floral nuances of the beverage (Wondra, 2001). Based on the profiles of major compounds, it was possible to observe an increase in the concentration of compounds whose aroma descriptors are associated with the improvement of the quality of the beverage.

Table 5 Concentrations (mg/L) of major volatile compounds by GC-FID in the heart fraction (*cachaça*).

Compounds	Inoculum								Descriptors
	Mixed inoculum				*S. cerevisiae*				
	B1	B2	B3	**Mean**	B1	B2	B3	**Mean**	
Acetaldehyde	27.60±0.57	59.97±4.38	28.62±1.56	**38.73**	33.36±0.79	27.78±0.77	20.04±0.14	**27.06**	Green leaves, fruity (B)
Ethyl acetate	44.85±4.56	79.82±6.24	36.49±2.47	**53.72**	63.08±3.14	37.64±1.27	25.22±0.25	**41.98**	Solvent, fruity, sweetish (B)
1-propanol	134.35±1.82	148.61±12.40	130.54±11.33	**137.83**	151.96±6.18	142.01±8.80	145.20±1.48	**146.39**	Alcohol (C)
2-methyl-1-propanol	361.88±12.02	252.65±19.42	344.87±31.45	**319.80**	169.16±10.50	199.83±14.44	97.08±2.64	**155.36**	Malty (A)
2-methylbutanol-1	130.45±13.28	149.19±10.23	111.96±8.38	**130.53**	105.70±5.59	71.23±3.19	56.02±3.58	**77.65**	Alcohol, banana, medicinal, solvent (B)
3-methylbutanol-1	779.25±24.35	634.58±54.55	813.69±65.86	**743.84**	609.54±25.40	620.36±21.40	404.00±2.15	**544.63**	Alcohol, banana, sweetish, aromatic (B)
Furfuryl alcohol	nd	0.83±0.09	1.30±0.56	**1.07**	0.65±0.13	1.34±0.44	0.54±0.10	**0.84**	Sugar cane, Woody (B)
2-phenylethanol	20.37±0.90	8.40±0.11	18.90±1.78	**15.89**	7.89±0.42	21.64±1.48	10.09±1.31	**13.21**	Roses,sweetish, perfumed (B)

nd not detected. (A) CZERNY et al. (2008), (B) MEILGAARD (1975).

A total of 42 minor volatile compounds were identified by GC-MS in the *cachaça* (Table 6). Some esters, such as ethyl hexanoate, ethyl octanoate, and 2-phenylethyl acetate, were found at higher concentrations (114.11 µg/L, 0.69 µg/L, and 2.77 µg/L, respectively) in *cachaça* produced by co-inoculation (Table 6). The results are consistent with those of our previous study (Duarte et al., 2013), in which we demonstrated the efficiency of the mixed inoculum of *P. caribbica* and *S. cerevisiae* in producing esters and the correlation between high activity of β-glycosidase and production of high concentration of some esters that positively affected the aromatic profile of fermented sugar cane juice. Because these compounds—ethyl hexanoate and ethyl octanoate—are associated with "fruity" "green apple" (Siebert et al., 2005; Meilgaard 1975) and "apple," "fruity" (Meilgaard 1975), "sweet" (Siebert et al., 2005), respectively, they are important to the flavor of the beverage. Nascimento et al. (2008) found similar results for ethyl hexanoate and ethyl octanoate in *cachaça*s produced using different alembics compared with our work. Phenylethyl acetate and ethyl dodecanoate were measured at slightly higher concentrations in *cachaça* produced by a pure culture of *S. cerevisiae*; these results disagree with the results of Duarte et al.

(2013), in which phenylethyl acetate was produced in a higher concentration by mixed inocula. The change in the concentration of some compounds compared to the results of Duarte et al. (2013) might have been caused by the distillation process (at 90–97°C), in which some compounds cannot be fully volatilized due to their boiling points or result from the separation of the three fractions—head, heart, and tail—of the distillate (Duarte et al., 2011a).

The monoterpenic alcohols a-terpineol, b-citronellol, and geraniol were found in concentrations of 0.45 µg/L, 2.47 µg/L, and 0.24 µg/L, respectively, in the *cachaça* produced by the mixed inoculum (Table 6). Higher concentrations of monoterpenic compounds were expected in the *cachaça* produced by the mixed inoculum, based on our previous work (Duarte et al., 2013) and due to the fact that non-*Saccharomyces* yeasts are known producers of β-glucosidase, which transforms inactive forms of compounds into their active aromatic forms, improving the sensory qualities of the beverage (Maturano et al., 2012). Although the mixed inoculum resulted in the production of higher amounts of some monoterpenes, the pure *S. cerevisiae* inoculum resulted in a *cachaça* with approximately twice as much linalool (0.88 µg/L).

Table 6 Minor volatile compounds (µg/L) in *cachaça* produced with mixed inoculum and pure *S. cerevisiae* by GC-MS.

N°	Compounds	LRI	Mixed inoculum				*S. cerevisiae*				*Descriptors*
			B1	B2	B3	**Mean**	B1	B2	B3	**Mean**	
	Alcohols										
1	1-Butanol*	1202	12.98	12.50	13.47	**12.98±0.49**	4.13	4.89	5.64	**4.89±1.07**	Malty, solvent-like (A); fusel, spirituous (C)
2	3-Methyl-1-Pentanol*	1325	0.61	0.64	0.42	**0.56±0.11**	1.18	0.83	0.48	**0.83±0.50**	-
3	1-Hexanol*	1356	0.55	0.36	0.28	**0.39±0.14**	0.08	0.19	0.29	**0.19±0.15**	Coconut, green leaves, unpleasant (D)
4	Trans-3-Hexen-1-ol*	-	0.23	0.12	0.08	**0.14±0.07**	0.70	0.42	0.14	**0.42±0.40**	-
5	1-Heptanol**	1455	0.61	0.47	0.31	**0.46±0.15**	0.64	0.52	0.40	**0.52±0.17**	Coconut, ketonic solvent, unpleasant (D)
6	4-Octanol**	-	2.78	2.48	2.24	**2.50±0.27**	0.42	2.08	3.73	**2.08±2.35**	-
7	1-Octanol**	1556	1.27	0.86	0.90	**1.01±0.23**	1.63	1.04	0.45	**1.04±0.83**	Coconut, walnut, oily (D)
8	1,2-propanediol*	-	9.86	9.52	10.20	**9.86±0.34**	0.08	1.75	3.42	**1.75±2.36**	-
9	1,3-butanediol*	1742	0.57	0.62	0.52	**0.57±0.05**	0.04	0.16	0.28	**0.16±0.17**	-
10	1-Dodecanol**	1758	1.03	1.22	0.85	**1.03±0.19**	0.45	0.61	0.76	**0.61±0.22**	-
	Acetates										
11	Ethyl hexanoate*	1231	106.66	121.55	114.11	**114.11±7.44**	23.60	35.37	29.49	**29.49±8.32**	Fruity, green apple (C, D)
12	Ethyl Octanoate*	1431	0.89	0.69	0.48	**0.69±0.21**	nd	nd	nd	**-**	Apple, fruity (E); sweet (C)
13	Furfuryl acetate*	-	1.05	0.95	0.84	**0.95±0.10**	0.74	0.51	0.29	**0.51±0.32**	-
14	Phenyl acetate*	1613	0.09	0.14	0.11	**0.11±0.03**	0.04	nd	nd	**0.01±0.03**	-
15	Diethyl succinate*	1679	5.88	6.90	6.39	**6.39±0.51**	3.91	3.50	3.08	**3.50±0.59**	Wax, tallow, rancid, soap (E); fatty (C)
16	2-Phenylethyl acetate	1780	3.32	2.77	2.22	**2.77±0.55**	2.44	1.55	0.67	**1.55±1.25**	Apple, honey, roses, sweet (D)
17	Phenylethyl acetate*	1805	0.19	0.22	0.16	**0.19±0.03**	0.29	0.25	0.21	**0.25±0.06**	Roses, honey, Apple, sweetish (C); flowery (B)
18	Ethyl dodecanoate**	1832	15.37	15.49	15.62	**15.49±0.13**	16.60	17.24	15.97	**16.60±0.45**	-
19	Ethyl tetradecanoate**	2041	3.81	5.22	7.06	**5.37±1.63**	1.20	0.81	0.42	**0.81±0.56**	-
20	Ethyl 9-hexadecenoate	2237	1.75	1.41	1.08	**1.41±0.34**	1.32	0.88	0.44	**0.88±0.62**	-
	Monoterpenic alcohols										
21	Linalool*	1546	0.35	0.36	0.38	**0.36±0.01**	1.38	0.88	0.37	**0.88±0.72**	Citrus-like, bergamot (A)
22	Menthol*	1628	106.65	118.28	95.03	**106.65±11.62**	103.36	106.17	108.99	**106.17±3.98**	-
23	a-Terpeniol*	1685	0.43	0.45	0.46	**0.45±0.01**	0.18	0.24	0.29	**0.24±0.08**	Pine, terpenoids (D)
24	b-citronellol*	1764	2.47	2.92	2.01	**2.47±0.46**	1.13	1.62	2.10	**1.62±0.69**	Citronella (E)
25	Geraniol*	1846	0.15	0.24	0.32	**0.24±0.09**	0.17	0.18	0.19	**0.18±0.01**	Rose-like, citrus-like (A)

ND not detected; B# corresponds to the consecutive batches. *pure standards **NIST library. (A) CZERNY et al. (2008). (B) GUTH (1997) (C) SIEBERT et al. (2005). (D) Meilgaard (1975). (E) Ribéreau-Gayon et al. (2000).

Table 6 (continued)

N°	Compounds		Mixed inoculum				*S. cerevisiae*				*Descriptors*
			B1	B2	B3	**Mean**	B1	B2	B3	**Mean**	
	Aldehydes										
27	Benzaldehyde**	1509	1.17	1.66	1.42	**1.42±0.25**	4.07	2.39	0.72	**2.39±2.37**	-
	Volatile acids										
28	Propanoic acid**	1543	3.96	3.12	3.54	**3.54±0.42**	0.31	0.21	0.10	**0.21±0.15**	Vinegar (C)
29	Butanoic acid*	1614	0.96	1.15	1.33	**1.15±0.18**	1.89	1.44	1.00	**1.44±0.50**	Banana (C)
30	Hexanoic acid*	1847	1.60	1.94	1.77	**1.77±0.17**	4.71	4.13	3.54	**4.13±0.83**	Fatty acids, vegetable oil (E); cheese, sweaty (C)
31	Heptanoic acid**	1950	0.93	0.74	0.55	**0.74±0.19**	0.11	0.27	0.42	**0.27±0.22**	-
32	Octanoic acid*	2059	3.25	3.65	2.86	**3.25±0.40**	4.60	2.81	1.01	**2.81±2.53**	Fatty acids, vegetable oil (E); rancid, harsh (C)
33	Nonanoic acid*	2085	1.37	0.96	0.55	**0.96±0.41**	1.75	1.31	0.87	**1.31±0.62**	-
34	n-Decanoic acid**	2273	0.40	0.40	0.39	**0.40±0.00**	nd	nd	nd	**-**	Wax, tallow, rancid, soap (E); fatty (C)
35	Dodecanoic acid**	2486	2.04	1.76	1.49	**1.76±0.27**	5.86	3.09	0.32	**3.09±3.91**	-
36	Hexadecanoic acid**	-	5.74	5.68	5.62	**5.68±0.06**	6.05	4.78	3.50	**4.78±1.81**	Fatty acids, vegetable oil (E); cheese, sweaty (C)
	Others										
37	2-Buten-1-one**	-	1.99	2.56	3.12	**2.56±0.56**	2.45	2.58	2.72	**2.58±0.19**	-
38	2-Nonanone*	1381	0.21	0.25	0.28	**0.25±0.04**	nd	nd	nd	**-**	
39	p-Ethylphenol**	2173	1.09	1.23	0.96	**1.09±0.13**	nd	nd	nd	**-**	-

ND not detected; B# corresponds to the consecutive batches. *pure standards **NIST library. (A) CZERNY et al. (2008). (B) GUTH (1997) (C) SIEBERT et al. (2005). (D) Meilgaard (1975). (E) Ribéreau-Gayon et al. (2000).

The concentrations of most volatile acids were increased when *P. caribbica* was used in a mixed inoculum with *S. cerevisiae*, with the exception of hexanoic acid, which was found at 4.13 mg/L when sugar cane juice was fermented by pure *S. cerevisiae* (Table 6).

Taking into account the HPLC, GC-FID, and GC-MS results, it was noted that, in general, higher concentrations of more impactful aromatic compounds (ethyl esters, acetates, higher alcohols, and monoterpenics alcohols) were found in the *cachaça* produced by mixed inoculum. The positive impact of these compounds on the aroma and flavor can be correlated with the sensory quality confirmed by the better acceptance of *cachaça* produced by the mixed inoculum, described below.

3.4 Sensory evaluation

The tasters were provided with a mixed sample consisting of equal parts of the three batches. To analyze the correlation between tasters and attributes evaluated in the sensory analysis, the data (tasters and grades for each attribute) were submitted for principal component analysis

(PCA) as internal preference mapping. Figure 1 shows the results of the PCA. The second and third principal components (PC1, and PC2) accounted for 73.26% of the total variance and allowed for differentiation between panelists who preferred the *cachaça* made with the mixed inoculum and those who preferred the *cachaça* made only with *S. cerevisiae*. On the negative side of PC2, the group of panelists was correlated with the attributes of taste, aroma, appearance, and overall impression of the *cachaça* made with only *S. cerevisiae*; on the positive side of PC2, the other group was characterized by the attributes of *cachaça* made with the mixed inoculum. Twenty-five tasters preferred the *cachaça* produced with only *S. cerevisiae*, while 31 tasters preferred the *cachaça* produced by the mixed inoculum.

The average scores for aroma, taste and overall impression were, respectively, 7.33, 6.98 and 7.21 for *cachaça* produced by the mixed inoculum, and 6.84, 6.47 and 6.60 for *cachaça* produced by *S. cerevisiae*. The average scores for these attributes were significantly (Wilcoxon test $p < 0.1$) higher for *cachaça* produced by mixed inoculum. These results indicate that the *cachaça* made with *S. cerevisiae* and *P. caribbica* was

preferred by the panelists because there was a correlation between the attributes of this beverage and a greater number of tasters.

The results obtained in the sensory analyses can be correlated with those obtained from the chemical characterization (Tables 4, 5, and 6). The use of a mixed inoculum made up of *S. cerevisiae* and *P. caribbica* enabled the production of more esters (which provide the sweet taste and smell of fruit and flowers) and higher alcohols (which provide the taste and smell of coconut and honey and smell of roses). In addition, the *cachaça* produced with non-*Saccharomyces* in a mixed inoculum enabled a reduction in the amount of aldehydes (related to hangover) and in the content of acids that increase the acidity (burning sensation) and gives the beverage smells and unpleasant tastes, such as rancid, wax, and soap. The *cachaça* produced with non-*Saccharomyces* yeast had an average acceptance higher than that obtained with commercial yeast *S. cerevisiae* only, which was characterized as “softer” and “less strong,” although it has the same alcohol content. In addition to the superior flavor and aroma, the non-*Saccharomyces* yeast also results in higher productivity, due to synergistic action with *S. cerevisiae* in the consumption of sugar cane juice.

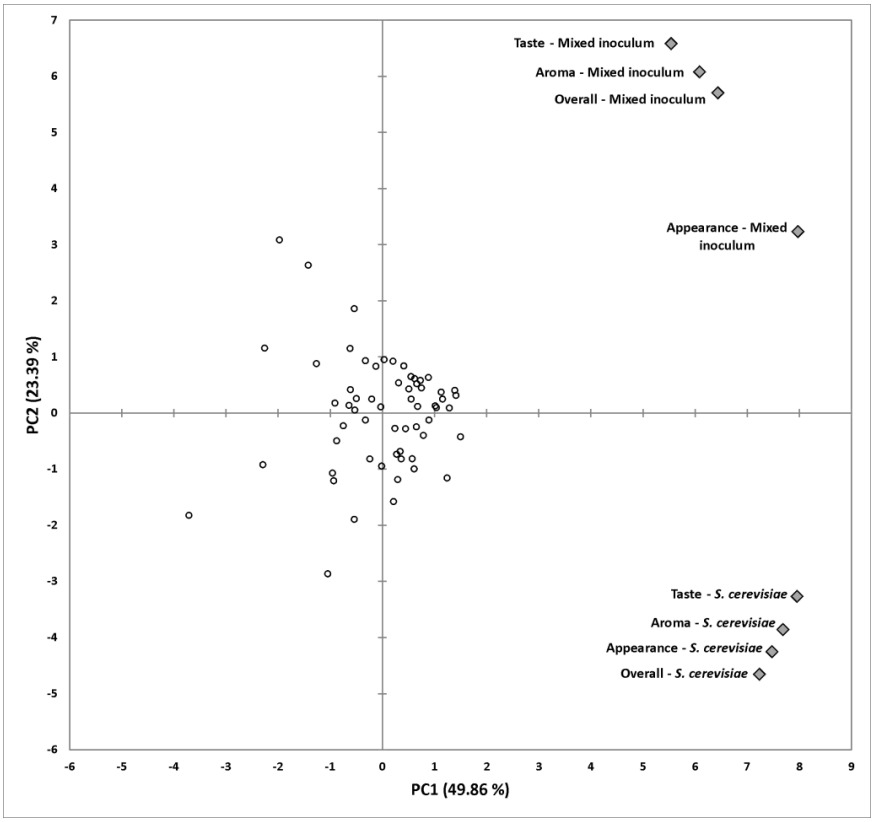

Fig. 1 Principal component analyses of sensorial attributes of the *cachaça*s produced with pure culture of *S. cerevisiae* and co-inoculation of *P. caribbica* and *S. cerevisiae*. Open circles: tasters

3.5 MALDI-TOF

In this work, for the first time, a study aimed to use the MALDI-TOF/MS technique for monitoring a mixed inoculum of yeasts during fermentation of sugar cane juice to produce *cachaça*. For this purpose, first, mass spectra to be used as reference spectra were obtained from pure yeast colonies of *P. caribbica* and *S. cerevisiae* grown on YPD agar, using the extraction method recommended by Bruker (2007). Figure 2 shows typical spectra of protein for pure and mixed cultures, which were considered the reference mass spectra, as most of the works use protein extraction from colonies using Bruker's (2007) extraction protocol. A few works (Usbeck et al. 2013; Moothoo-Padayachie et al., 2013) have reported the use of protein extraction from broth.

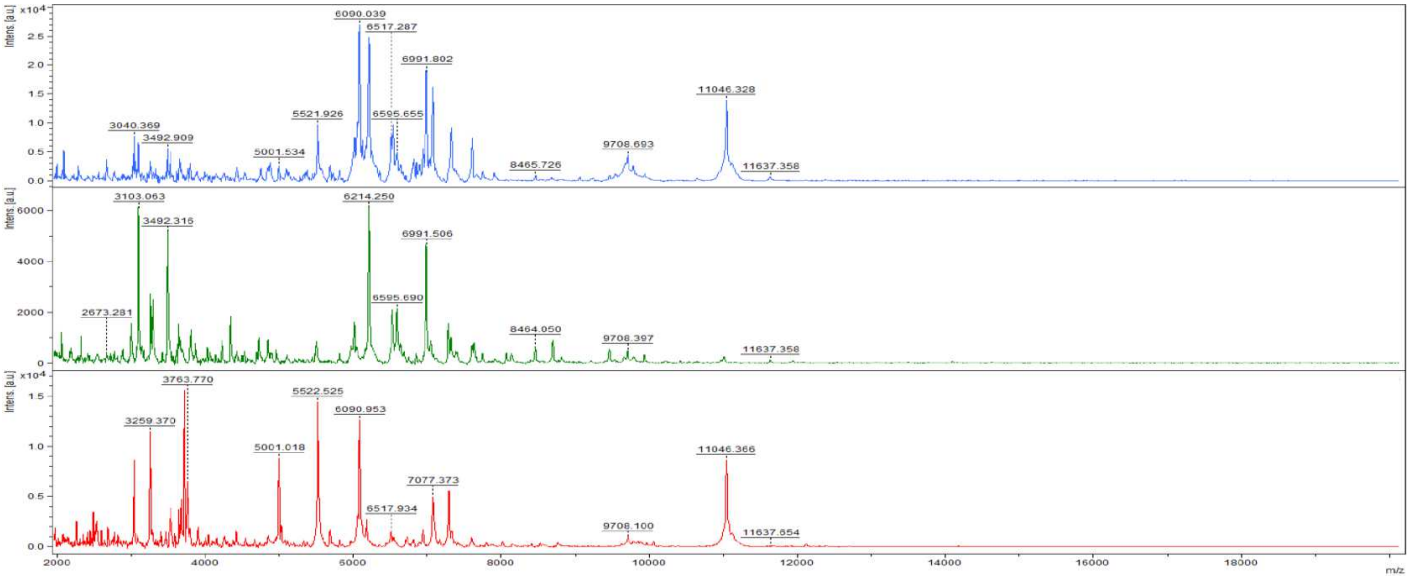

Fig. 2 Typical MALDI-TOF mass spectral profiles of mixed inoculum in sugar cane juice (blue), *S. cerevisiae* in YPD agar (green) and *P. caribbica* in YPD agar (black).

The monitoring of the inoculum during the fermentation process is facilitated if the protein extraction can be made directly from the fermenting must, as there is no need to cultivate the cells in plates, which is a step that involves the expenditure of time and resources. Therefore, we tested the methodology proposed by Usbeck et al. (2013). First, mass spectra were obtained from pure and mixed cultures of *S. cerevisiae* and *P. caribbica* cultivated in YPD broth in order to confirm the non-interference of sugarcane juice in the quality of spectra. Second, mass spectra from the mixed inoculum in sugar cane juice were acquired. Cluster analysis of the mass spectra obtained from the *S. cerevisiae*, *P. caribbica*, and mixed inoculum (YPD and sugar cane juice) showed that the mass spectra of pure cultures were grouped separately (Fig. 3). After verifying that the technique worked for pure cultures, protein extractions were made from the mixed inoculum of *S. cerevisiae* and *P. caribbica* cultivated in YPD medium and sugar cane juice. As shown in Fig. 3, the obtained spectra for both cultures in YPD broth as sugar cane were similar to each other and were different from pure cultures, confirming the feasibility of using MALDI-TOF for monitoring yeasts during sugar cane juice fermentation.

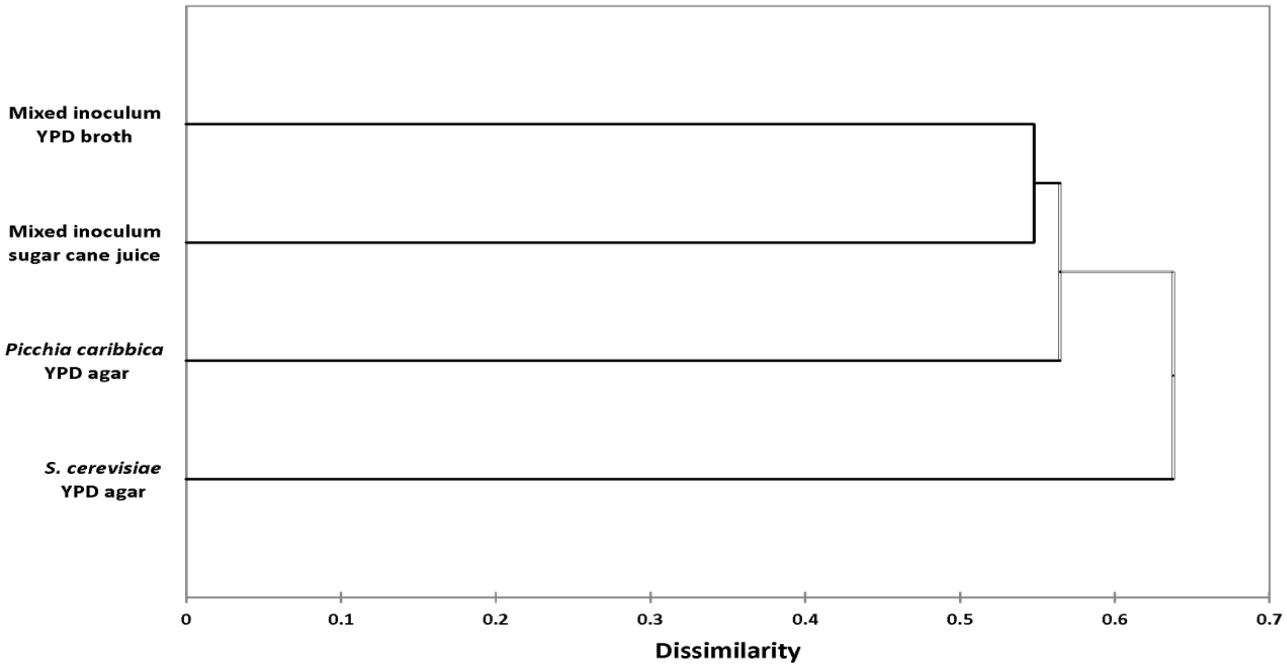

Fig. 3 Dendrogram of protein profile of pure and mixed inoculum of *S. cerevisiae* and *P. caribbica* in different grown media (YPD and 16° Brix sugar cane juice).

From there, to confirm the robustness of the MALDI-TOF technique, protein extraction and mass analyses were conducted for the fermentation experiments with different populations of *P. caribbica* (10^5, 10^6, and 10^7 cells/mL) and Brix (14, 16, and 18 °Brix) (Table 1). The obtained mass spectra were similar to each other (Fig. 4), showing that the MALDI-TOF/MS technique is amenable to use for monitoring yeasts as mixed inoculum even under different fermentation conditions and *P. caribbica* population. Usbeck et al. (2013) demonstrated that the yeast's parameters, such as nutrients, growth path, cell density and presence or absence of oxygen, exert a small influence on the mass profile obtained by MALDI-TOF. In the case of cell density, these authors reported that there was no clear qualitative or quantitative influence on the obtained mass spectra.

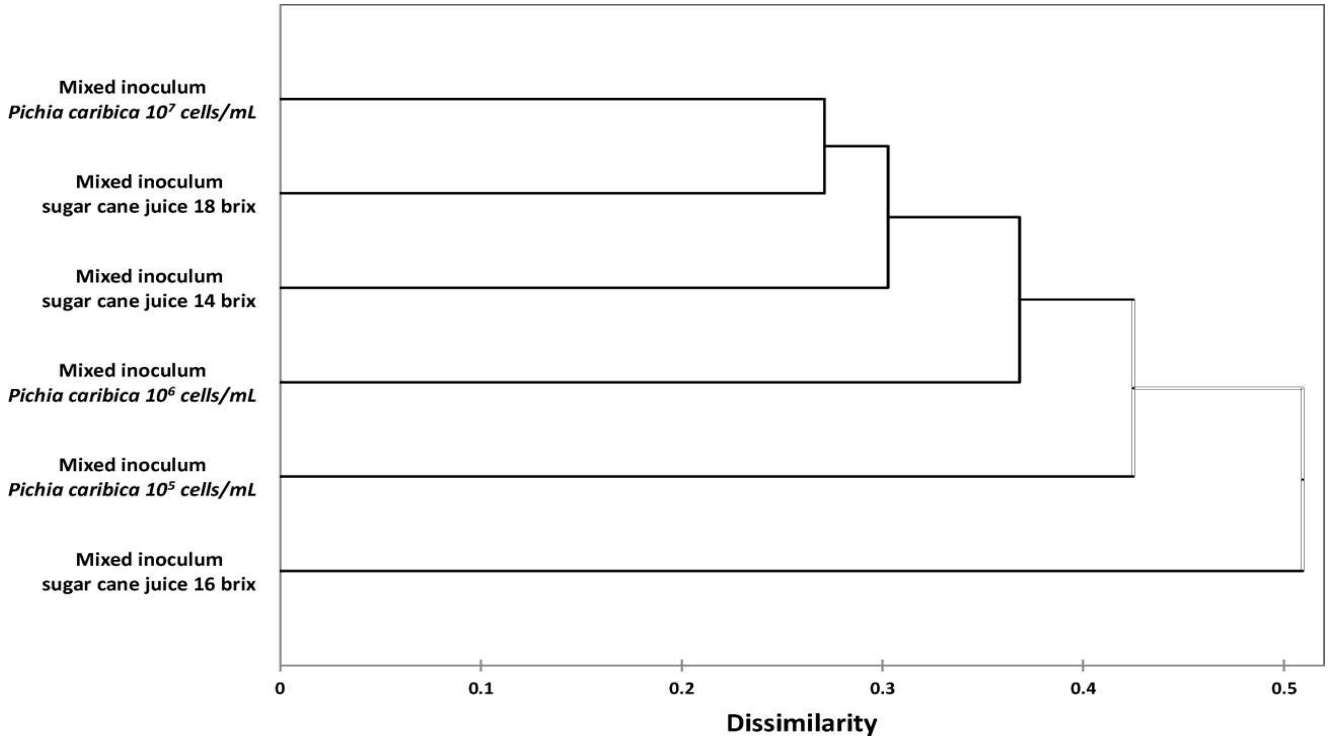

Fig. 4 Dendrogram protein profile of mixed inoculum (*S. cerevisiae* and *P. caribbica*) in different conditions of non-Saccharomyces population size (10^5, 10^6 and 10^7 cells/mL) and sugar cane juice brix (14, 16 and 18).

The *cachaça* production process usually involves several cycles of fermentation using the same inoculum. Therefore, the MALDI-TOF/MS technique was also tested for monitoring a mixed inoculum (*P. caribbica* and *S. cerevisiae*) and a pure culture of *S. cerevisiae* during three consecutive batches with samples collected from the vats at T0 and T24 of each batch. The cluster analysis shown in Fig. 5 indicates that the mass spectra of the mixed inoculum in all three cycles were similar to each other, clustered in one arm of the dendrogram (G1). At the same time, these spectra differed from the mass spectra obtained from pure *S. cerevisiae* culture, which were similar to each other (in all three batches) and grouped together in another arm of the dendrogram (G2). The T24 and T0 of each batch, although grouped in one large group, formed slightly distinct subgroups, which could be a result of interference regarding whether or not the medium was fermented. However, it was possible to differentiate between the mixed inoculum and the pure inoculum, both at the beginning and at the end of each batch, for three batches. These data seem to suggest that the optimized ethanol/formic acid procedure is capable of generating significantly different spectral signals for different selected inocula that may be used for monitoring the

cachaça fermentative process, using pure *S. cerevisiae* or mixed inoculum of *S. cerevisiae* and *P. caribbica*, even under variable conditions such as yeast population, sugar content, and cell recycle. The protein extraction directly from fermented must makes the MALDI-TOF/MS analysis more attractive for use in fermentation processes, as it is faster than traditional plating methods.

The potential of MALDI-TOF/MS as a rapid biotyping tool for identification of a mixed inoculum during the sugar cane fermentation process was established for the first time. Since its introduction in the late 1980s, MALDI-TOF/MS has proven to be a rapid tool for the identification of bacteria (Ilina et al., 2010; Mellmannn et al., 2008), but it has been used less than yeast (Qian et al., 2008). Studies on yeast are still few in number, and most have been limited to clinical yeast, such as *Candida* strains, up to the species level (Qian et al., 2008; van Veen et al., 2010) and to some fermentative yeast such as *Saccharomyces*, *Wickerhamomyces,* and *Debaryomyces* (Moothoo-Padayachee et al., 2013; Usbeck et al., 2013b, 2014). It has been found that the direct procedure that involves depositing a thin layer of yeast cells overlaid with a matrix on a MALDI target plate does not yield sufficient diagnostic

signals (Usbeck et al., 2013a; van Veen et al., 2010). This result might be attributed to the cell wall structure of yeast cells, which makes effective extraction of proteins difficult (Qian et al., 2008). In this study, parameters like yeast population in a mixed inoculum and sugar content in cultivation medium were considered during the optimization of an ethanol/ formic acid extraction protocol proposed by Usbeck et al. (2013) that was previously developed by Bruker Daltonik (Bruker, 2007) for the broad-based MALDI-TOF/MS identification of yeast.

The MALDI-TOF/MS technique provides a meaningful prediction of the probable application field very quickly, which is helpful for monitoring the mixed inoculums of yeast compared with traditional plating and sequencing analyses, which are time - and money - consuming. These traditional analyses cannot be completely replaced by MALDI-TOF MS analysis, but it is a tool for the preliminary decision.

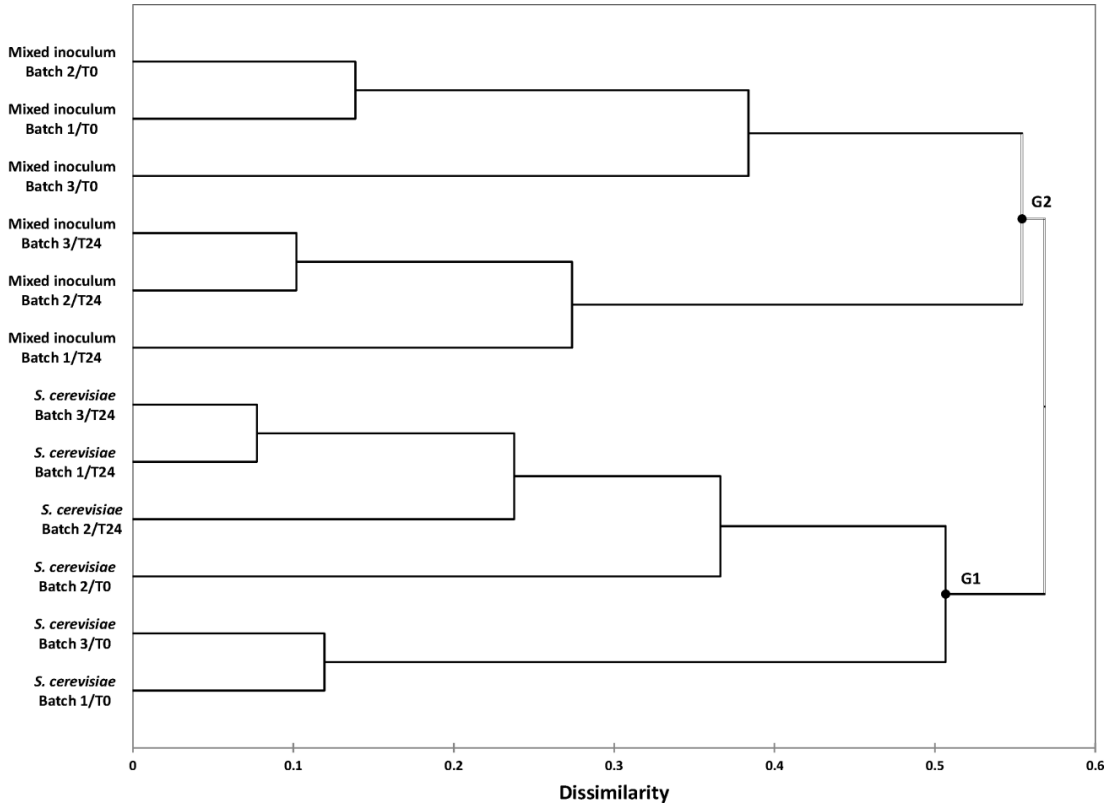

Fig. 5 Dendrogram protein profile of mixed inoculum (*S. cerevisiae* and *P. caribbica*) and pure *S. cerevisiae* during three fermentation batches.

4 CONCLUSIONS

Based on the results of this study, we can conclude that co-inoculation of *S. cerevisiae* and *P. caribbica* positively influenced the final quality of the *cachaça*, mainly due to the compounds identified by GC-FID, GC-MS, and HPLC. As a consequence of the differences in chemical composition, the sensory quality was also positively influenced. The distillation process did not affect differently the final quality of the beverages, allowing the differences between *cachaça* produced using *S. cerevisiae* and using mixed inoculum (*P. caribbica* + *S. cerevisiae*) to be assigned to the fermentation process. Acceptance in the sensory evaluation, especially for younger panelists, showed the potential of the *cachaça* from the mixed inoculum as a new product that might be appropriate for a particular niche market. The production of *cachaça* with taste and aroma above the existing standard in the market will impact *cachaça* production directly and positively, as well as adding value to the spirit in a market with a high demand for a differentiated product. The protein extraction directly from the medium and the MALDI-TOF/MS technique proved to be helpful for monitoring the mixed yeast inoculum

in the fermentation process, compared with traditional plating and sequencing analysis, which are time- and money-consuming.

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