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Effects of sequentially inoculated *Williopsis saturnus* and *Saccharomyces cerevisiae* on volatile profiles of papaya wine

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ABSTRACT

The effects of sequential inoculation of yeasts *Williopsis saturnus* var. *mrakii* NCYC2251 and *Saccharomyces cerevisiae* var. *bayanus* R2 on the volatile profiles of papaya wine were investigated at an inoculum ratio of 1000 (*W. saturnus*) to 1 (*S. cerevisiae*). Inoculation of *S. cerevisiae* after seven days' fermentation with *W. saturnus* produced papaya wine with more acetate esters and fruitiness than the control (simultaneous inoculation). However, inoculation of *W. saturnus* after two days' fermentation with *S. cerevisiae* resulted in most of the volatile composition being comparable to the control, except for the enhanced amount of ethyl esters. The first inoculated yeast dominated the fermentation. The study suggests that sequential inoculation of non-*Saccharomyces* and *Saccharomyces* yeasts at a certain inoculum ratio may be a valuable tool to manipulate yeast succession and to modulate the volatile profiles and organoleptic properties of papaya wine.

1. Introduction

Recently, researchers have directed attention at other fruits for wine production, rather than grapes. Tropical fruits such as banana, pineapple and mango have been used as substrates for the production of fruit wines (Duarte et al., 2010). There are still a variety of fruits which are potentially suited for making good quality fruit wines, but remain unknown because of a lack of research and development. Among these fruits, papaya (*Carica papaya*) was chosen for this study owing to its abundant supplies and pleasant flavor (Oliveira & Vitória, 2011), rich nutritional content and antioxidants like vitamin C (Sancho, Yahia, & González-Aguilar, 2011).

Wine fermentation is a complex process and is influenced by several factors including yeast strains, fruit cultivar and properties, vinification process, amount of sulfur dioxide and malolactic fermentation (Lilly, Lambrechts, & Pretorius, 2000). Among all these factors, yeast plays the most critical role in affecting the wine flavor by influencing the ecology of the winemaking process, the metabolism and enzymatic activities and the organoleptic impact of individual species or combinations of species on wine flavor. *Saccharomyces cerevisiae* has been commonly used in wine fermentation due its ability to induce reliable and rapid fermentation, ease of control and consistency of fermentations. Nevertheless, increasing studies point to the presence and persistence of non-*Saccharomyces* yeasts in inoculated and spontaneous fermentations (Heard & Fleet, 1985), as well as their contributions to the analytical composition and sensorial characteristics of wine (Garde-Cerdán & Ancín-Azpilicueta, 2006; Lema, Garcia-Jares, Orriols, & Angulo, 1996). This led to the current trend to employ non-*Saccharomyces* yeasts as mixed or sequential cultures with *S. cerevisiae* (Ciani, Comitini, Mannazzu, & Domizio, 2010; Clemente-Jimenez, Mingorance-Cazorla, Martínez-Rodríguez, Las Heras-Vázquez, & Rodríguez-Vico, 2005).

Evidences have highlighted the capability of mixed yeasts in improving the complexity and characteristics of grape and papaya wine (Ciani et al., 2010; Garde-Cerdán & Ancín-Azpilicueta, 2006; Lee, Ong, Yu, Curran, & Liu, 2010a), while results are non-conclusive for sequential fermentations. Ciani, Beco, and Comitini (2006) pointed out limitations of sequential fermentations such as excessive increases in ethyl acetate and the prolonged persistence of non-*Saccharomyces* yeasts at high levels which eventually led to stuck or sluggish fermentations. In contrast, Bely, Stoeckle, Masnuef-Pomarede, and Dubourdieu (2008) and Clemente-Jimenez et al. (2005) reported the improvement of wine quality with enhanced production of desirable flavor compounds and elimination of negative sensorial characteristics in sequential fermentation.

In multistarter fermentations, yeast succession is an essential parameter that affects the chemical composition and the contribution of these yeasts to the overall wine character. Sequential fermentation allowed the persistence of non-*Saccharomyces* (Ciani et al., 2006), while mixed culture fermentation resulted in an early growth arrest of non-*Saccharomyces* due to inhibition by *Saccharomyces* (Lee et al., 2010a). The duration of non-*Saccharomyces* in contact with fruit

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musts is crucial for modifying the flavor composition (Clemente-Jimenez et al., 2005).

With the extended survival of non-*Saccharomyces*, the aim of this study was to investigate the fermentation behavior of *S. cerevisiae* var *bayanus* R2 and *Williopsis saturnus* var. *mrakii* NCYC2251 in sequential cultures as compared to mixed culture. *W. saturnus* was chosen due to its ability to produce high levels of volatile esters especially acetate esters (Trinh, Woon, Yu, Curran, & Liu, 2011). The yeast ratio used corresponded to those in Lee et al. (2010a), where a mixed yeast ratio of 1:1000 (*S. cerevisiae*:*W. saturnus*) improved the analytical and aromatic profiles of papaya wine.

2. Methods and materials

2.1. Microorganisms and yeast culture preparation

S. cerevisiae var. *bayanus* Lavin R2 from Lallemand Inc (Brooklyn Park, Australia) and *W. saturnus* var. *markii* NCYC2251 from National Collection of Yeast Cultures (Norwich, UK) were used in all the fermentations. The yeast cultures were propagated in a sterile nutrient broth (2% w/v glucose, 0.25% w/v yeast extract, 0.25% w/v bacteriological peptone and 0.25% w/v malt extract) for up to 48 h at 25 °C and stored at −80 °C until used (Lee, Ong, Yu, Curran, & Liu, 2010b).

2.2. Papaya juice preparation and fermentations

The papayas (Sekaki cultivar, Malaysia) were processed into juice by mechanical extraction with a Sona juice extractor (Cahaya Electronics, Singapore) and centrifugation at 32,140×g (Beckman Centrifuge, USA) for 15 min to separate the pulp residue and juice. The supernatant was acidified with 1 M DL-malic acid to pH 3.5 and sanitized by 100 ppm potassium metabisulphite ($K_2S_2O_5$) according to the procedure described in Lee et al. (2010b). The efficiency of the sanitation was verified by plate counting. Triplicate fermentations were carried out with 280 mL of sanitized papaya juice in 300-mL conical flasks at 20 °C by inoculation with pre-cultures [grown in the same medium at 25 °C for 72 h (*S. cerevisiae*) and 96 h (*W. saturnus*) until the yeasts achieved 10^7 CFU/mL]. Mixed culture fermentations (MCF, as control) were simultaneously inoculated with final concentrations of ~10^2 CFU/mL of *S. cerevisiae* and ~10^5 CFU/mL of *W. saturnus*. Two types of sequential fermentation were carried out: initial inoculation of ~10^5 CFU/mL of *W. saturnus*, followed by ~10^4 CFU/mL *S. cerevisiae* after seven days (late log phase) (positive sequential fermentation, PSF); initial inoculation of ~10^2 CFU/mL of *S. cerevisiae*, followed by ~10^5 CFU/mL *W. saturnus* after two days (late log phase) (negative sequential fermentation, NSF). PSF was carried out to prolong survival of *W. saturnus* and to maximize its flavor impact. NSF was carried out to examine the behavior of *W. saturnus* and its potential flavor impact. The batch fermentations were carried out for 21 days under static conditions. All samples were taken at days 0, 2, 7, 10, 14 and 21 for the determinations of viable cell counts, pH, °Brix, organic acids, sugars and volatile compounds.

2.3. Analytical determinations

Potato dextrose agar (PDA) and 0.1% (w/v) peptone water (Oxoid, England) were used to assess growth of *S. cerevisiae* and *W. saturnus*. The colonies of *S. cerevisiae* (smooth and shiny) can be morphologically distinguished from those of *W. saturnus* (wrinkled and dull) (Trinh et al., 2011). The °Brix and pH values were measured using a refractometer (ATAGO, Japan) and pH meter (Metrohm, Switzerland), respectively. The sugars and organic acids of papaya juice and wines were evaluated in the manner described by Lee et al. (2010b). Sugar concentrations were determined with a Zorbax carbohydrate column (Agilent, Santa Clara, CA, USA) connected to an ELSD-LT detector. The column was eluted at 40 °C with a mixture of acetonitrile and water (80:20 v/v) mobile phase, at a flow rate of 1.4 mL/min. Organic acids were separated by a Supelcogel C-610H column (300×7.8 mm, Supelco) using 0.1% (v/v) sulphuric acid mobile phase at a flow rate of 0.4 mL/min and the detection was assessed by photodiode array (PDA) at 210 nm.

The determination and quantification of volatile compounds in papaya juice and wines were carried out using headspace (HS) solid-phase microextraction (SPME) sampling combined with gas chromatography (GC)-mass spectrometer (MS) and flame ionization detector (FID) (HS-SPME-GC-MS/FID) according to the procedures described in Lee et al. (2010b). Samples were extracted with HS-SPME at 60 °C for 50 min using a SPME autosampler (CTC, Combi Pal, Switzerland) and thermally desorbed into the injector port at 250 °C for 3 min. Separation was performed with a DB-FFAP capillary column (Agilent, Santa Clara, CA, USA) of 60 m×0.25 mm I.D. and the oven temperature was programmed to run from 50 °C (hold time 5 min) to 230 °C (final hold for 30 min) at 5 °C/min. Identification of the eluted volatile compounds was achieved by matching the mass spectrum against NIST 8.0 and Wiley 275 MS libraries, and confirmed with Linear Retention Index (LRI) value.

Quantification of the selected volatile compounds was similar to that reported in Lee, Yu, Curran, and Liu (2011) and additional volatiles were quantified. Volatiles were quantified using individual external standard solutions dissolved in 10% (v/v) papaya juice-based aqueous solutions, except for ethanol dissolved in 100% (v/v) papaya juice (Lee et al., 2010a; Trinh et al., 2011). All the standards were subjected to similar extraction protocols used for the samples and had R^2 values of at least 0.95. Concentrations of volatile compounds were determined by using the linear regression equation of the corresponding standards. All samples were analyzed in triplicate.

2.4. Sensory analysis

The papaya wines were evaluated by a panel of five well-trained flavorists (three females and two males) from Firmenich Asia. Samples were presented in wine-testing glasses, and then sniffed only. Eight sensory descriptors were selected by consensus to describe the papaya wine aroma: acidic, alcoholic, buttery, cocoa, fruity, fusel, sweet and yeasty notes. The panelists used a 5-point hedonic scale to rate the intensity of each attribute.

2.5. Statistical analysis

Test of significance for the experimental data was accomplished by employing one-way analysis of variance (ANOVA), using Microsoft Office Excel, version 2003. Principal components analysis (PCA) (Matlab R2008a, Mathworks, USA) was applied to discriminate among the means of chemical measurements of volatile compounds from the different fermentations.

3. Results and discussion

3.1. Biomass evolution and metabolic characteristics of yeasts

The evolution of *S. cerevisiae* and *W. saturnus* is shown in Fig. 1. NSF and MCF had similar yeast growth and succession, which was different from PSF. *S. cerevisiae* in both NSF and MCF increased rapidly and then remained stationary, while the same yeast in PSF grew slightly upon inoculation at day 7 and then declined rapidly (Fig. 1). As expected, *W. saturnus* in both NSF and MCF declined rapidly, but the same yeast in PSF multiplied incessantly and achieved a maximum of ~10^8 CFU/mL at day 21 (Fig. 1). The domination of *W. saturnus* in PSF was probably due to the killer toxins produced by *W. saturnus* (Liu & Tsao, 2010), to which *S. cerevisiae* was sensitive (Yap, de Barros Lopes, Langridge, & Henschke, 2000). Mendoza, Manca de Nadra, and Farías (2007) also revealed that the presence of both *Saccharomyces* and