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# Effects of different inoculation strategies of *Saccharomyces cerevisiae* and *Williopsis saturnus* on chemical components of mango wine

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ABSTRACT

This study investigated the effects of different inoculation strategies of *Saccharomyces cerevisiae* MERIT.ferm and *Williopsis saturnus* var. *mrakii* NCYC 500 on volatile composition of mango wine. The inoculation strategies involved positive sequential fermentation (PSF, *W. saturnus* NCYC 500 was first inoculated for 14 days before inactivation, then *S. cerevisisae* MERIT.ferm was inoculated and fermentation continued for another 7 days), negative sequential fermentation (NSF, *S. cerevisiae* MERIT.ferm was first inoculated for 7 days before inactivation; after that, *W. saturnus* NCYC 500 was inoculated at day 14 followed by another 7 days fermentation) and simultaneous mixed culture fermentation (MCF). The growth of *S. cerevisiae* MERIT.ferm in PSF declined slightly immediately after inoculation, which differed from that in NSF and MCF. On the other hand, the growth of *W. saturnus* NCYC 500 was inhibited by prior growth of *S. cerevisiae* MERIT.ferm in NSF and MCF. The volatile profiles of the mango wines varied significantly among different inoculation strategies. PSF produced higher amounts of desirable volatile compounds especially ethyl esters (ethyl esters of hexanoate, decanoate, octanoate), acetate esters (ethyl acetate, isobutyl acetate, isoamyl acetate and 2-phenylethyl acetate) and isoamyl alcohol, which could contribute more fruity and creamy notes to the wines. Therefore, PSF would be a useful strategy for fruit wine making with more complex aroma.



## 1. Introduction

The mango fruit (*Mangifera indica* L.) is one of the most popular and economically important tropical fruits due to its exotic and appealing flavour. The chemical composition of mango fruit varies with location of cultivation, variety, and stage of maturity (Chauhan, Raju, & Bawa, 2010). The *Chok Anan* mango is one of the most popular cultivars consumed for its mild and pleasant flavour in Southeast Asian region due to its higher sugar contents, various organic acids, amino acids, proteins, lipids, vitamins and $\beta$-carotene (Tharanathan, Yashode, & Prabha, 2006). Mango fruit is deemed suitable for making wine, given its high sugar level of around 17%. In addition, mango wine is a potential value added product.

Alcoholic fermentation (AF) is normally conducted by *Saccharomyces* yeasts with the conversion of sugars into ethanol and carbon dioxide, but resulting in less flavour complexity (Lu, Chua, Huang, Lee, & Liu, 2016a, 2017). Recently, non-*Saccharomyces* yeasts have been proved to be beneficial to wine flavour (Lu, Huang, Lee, & Liu, 2015, 2016b). Some species such as *Williopsis saturnus* and *Kloeckera apiculate* produce significantly higher amounts of desirable acetate esters such as isoamyl acetate and 2-phenylethyl acetate, which may impart floral and fruity notes to the wine bouquet (Li, Chan, Yu, Curran, & Liu, 2012; Liu & Tsao, 2010); while other species such as *Torulaspora delbrueckii* produce low amounts of acetic acid (Lu et al., 2016b), *Hanseniaspora* family and *Pichia anomala* produce relatively higher levels of fruity esters (Jolly, Augustyn, & Pretorius, 2006).

AF with non-*Saccharomyces* monocultures is usually incomplete mainly due to their weak fermentative capability and low ethanol tolerance, which resulted in their limited use in commercial wine fermentation despite advantages. Recently, mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts have been explored and utilised to harness the advantages of non-*Saccharomyces* in wine fermentation (Ciani, Comitini, Mannazzu, & Domizio, 2010; Jolly et al., 2006; Li et al., 2012; Lu et al., 2015).

Some studies have demonstrated the enhanced complexities of the wine produced with the use of mixed starters of *Saccharomyces*

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and non-*Saccharomyces* yeasts (Li & Liu, 2016; Lu et al., 2015), while other studies have reported that there is limited contribution of non-*Saccharomyces* yeasts belonging to the genera *Hanseniaspora*, *Kluyveromyces*, *Torulaspora* and *Williopsis* in simultaneous mixed culture fermentations due to their early growth arrest (Bely, Stoeckle, Masneuf-Pomarède, & Dubourdieu, 2008; Clemente-Jimenez, Mingorance-Cazorla, Martínez-Rodríguez, Las Heras-Vázquez, & Rodríguez-Vico, 2005; Varakumar, Kondapalli, & Reddy, 2012). The early demise of the non-*Saccharomyces* yeasts could be attributed to their lower resistance to stresses under oenological conditions.

Some researchers suggested that sequential fermentation is the most adequate strategy for strain combinations, where the kinetic behaviour resembles a successful spontaneous fermentation and produces wine with differential aromatic qualities (Clemente-Jimenez et al., 2005; Lu et al., 2015). This could be due to the prolonged non-*Saccharomyces* yeasts period in controlled sequential fermentation and lack of inhibition from *Saccharomyces* species.

A sequential fermentation of *Pichia fermentans* and *S. cerevisiae* conferred greater complexity to wine through the enhancement of desirable flavour compounds production and glycerol content (Clemente-Jimenez et al., 2005). In addition, sequential fermentation can also be used to favour weak fermentative strains by delaying inoculation of *S. cerevisiae* so that the desirable traits conferred by non-*Saccharomyces* strains can be developed first including good acidity, low volatile acidity, intense fruity ester production (Bely et al., 2008; Lu et al., 2015).

Previously, mango wines were always produced using monocultures of *S. cerevisiae* or a simultaneous inoculation of *S. cerevisiae* and *W. saturnus* (Li et al., 2012; Li & Liu, 2016). To date, there has been no published study about the effects of a controlled sequential fermentation on the volatile composition and quality of mango wine. The objective of this study was to assess the chemical profile of the mango wine after fermentation with *S. cerevisisae* MERIT.ferm and *W. saturnus* NCYC 500 via different inoculation strategies.

## 2. Materials and methods

### 2.1. Mango fruits and mango juice preparation

Mango fruits (*Chok Anan* variety) imported from Malaysia were purchased from a local wholesale market in Singapore. Whole and healthy looking fruits were selected and stored at room temperature until fully ripe. After washing with water to remove dirt and being allowed to air dry naturally at room temperature, the skin and flesh were separated manually. The obtained flesh was then juiced in a commercial juicer (Sona juice extractor, Cahaya Electronics, Singapore) with the resulting puree being centrifuged at 4 °C, 41 415 × *g* (Beckman Centrifuge, Brea, CA, USA) for 15 min and the supernatant was collected and stored at −20 °C before use.

The mango juice (pH 4.55, °Brix 17) was then adjusted to a pH of 3.5 with 50% (w/v) DL-malic acid before the addition of 100 ppm potassium metabisulfite ($K_2S_2O_5$, Goodlife Homebrew center, Norfolk, England). The mixture was left to stand for 24 h at 25 °C for sterilisation. The effectiveness of the sterilisation was verified by streak plating on potato dextrose agar (PDA) plate (39 g/L, Oxoid, Basingstoke, Hampshire, England) and MRS agar (supplemented with 20% apple juice) for yeast and bacteria checking, respectively.

### 2.2. Yeast culture and pre-culture preparation

*Williopsis saturnus* var. *mrakii* NCYC 500 from the National Collection of Yeast Culture (Norwich, UK) and *Saccharomyces cerevisiae* MERIT.ferm from Chr. Han. (Denmark) were purchased in the active freeze dried form. Active dried yeast was propagated in a sterilised nutrient broth (autoclaved for 15 min at 121 °C) consisting of (w/v) 2% glucose, 0.25% bacteriological peptone, 0.25% yeast extract and 0.25% malt extract in deionised water, pH 5.0. Yeast strains were maintained in the nutrient broth and incubated at 25 °C for 48–72 h without aeration and 20% glycerol was added to the culture before being stored at −80 °C. Each pre-culture was prepared with sterilised mango juice inoculated with 10% (v/v) of *S. cerevisiae* MERIT.ferm or *W. saturnus* NCYC 500. The pre-cultures were then incubated at 25 °C for 48–72 h for the yeasts to reach a colony forming units (CFU) over $10^7$ per mL.

### 2.3. Fermentation of mango juice with different inoculation strategies

Three different inoculation strategies were studied. Triplicates of 200 mL of sanitised mango juice in sterilised conical flasks were fermented for 21 days at 20 °C. In simultaneous mixed culture fermentation (MCF), *W. saturnus* NCYC 500 ($10^5$ CFU/mL) and *S. cerevisiae* MERIT.ferm ($10^2$ CFU/mL), in a ratio of 1000:1 were inoculated simultaneously to mango juice to ensure good growth of *W. saturnus*. This is because a high inoculation level of *S. cerevisiae* MERIT.ferm ($10^5$ CFU/mL) resulted in a sharply decline of *W. saturnus* NCYC 500 to undetectable levels within the first few days fermentation (data not shown), which would seriously affect the contribution of *W. saturnus* to wine complexity. For positive sequential fermentation (PSF), *W. saturnus* NCYC 500 ($10^5$ CFU/mL) was inoculated and fermentation was carried out for 14 days before the yeasts were deactivated via ultrasonication (Hielscher-Ultrasound Technology, UIP 1000, 1000 W) for 15 min at 20 kHz. After that, *S. cerevisiae* MERIT.ferm ($10^5$ CFU/mL) was inoculated and the fermentation continued for another 7 days. For negative sequential fermentation (NSF), *S. cerevisiae* MERIT.ferm ($10^5$ CFU/mL) was inoculated and fermentation was carried out for 7 days before the yeasts were deactivated by ultrasonication as described above. *W. saturnus* NCYC 500 ($10^5$ CFU/mL) and glucose were then added and fermentation continued for further 14 days. In NSF, *S. cerevisiae* MERIT.ferm fermentation was halted at day 7 based on a pre-experiment that *S. cerevisiae* was able to complete AF within 7 days. Glucose was replenished to compensate for loss of sugars due to prior fermentation and to ensure the growth of *W. saturnus*.

### 2.4. pH, °Brix and yeast enumeration

Sampling was done at regular intervals throughout the fermentation. The pH and total soluble solids (°Brix) were measured using a refractometer (Atago, Tokyo, Japan) and pH meter (Metrohm, Herisau, Switzerland), respectively. Yeasts cell counts were carried out via the spread plating method on PDA. The plates were incubated at 25 °C for 48 h. Lysine agar is unable to support the growth of *Saccharomyces* yeast (Erten & Tanguler, 2010) and therefore was used to differentiate the growth of *Saccharomyces* and non-*Saccharomyces* yeasts. In addition, the appearance of the colonies of *S. cerevisiae* MERIT.ferm yeast (small, off-white, glossy surface) and *W. saturnus* NCYC 500 (bigger size, a dull, white appearance) was also used to verify.

### 2.5. Analysis of non-volatile and volatile compounds

Sugars (sucrose, glucose and fructose) were analysed by using a Shimadzu HPLC connected to a Zorbax carbohydrate column (150 × 4.6 mm, Agilent, Santa Clara, CA, USA) coupled with an ELSD detector (gain: 5, 40 °C, 350 kPa), eluted by a mixture of acetonitrile and water (80:20, v/v). The flow rate and detection temperature were 1.4 mL/min and 40 °C, respectively. Analysis of organic acids