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Yeast ratio is a critical factor for sequential fermentation of papaya wine by *Williopsis saturnus* and *Saccharomyces cerevisiae*

Pin-Rou Lee,[1] Stephanie Hui Chern Kho,[1] Bin Yu,[2] Philip Curran[2] and Shao-Quan Liu[1]*

[1]*Food Science and Technology Programme, Department of Chemistry, National University of Singapore, Science Drive 3, Singapore 117543.*

[2]*Firmenich Asia Pte Ltd, Tuas, Singapore.*

Summary

The growth kinetics and fermentation performance of *Williopsis saturnus* and *Saccharomyces cerevisiae* at ratios of 10:1, 1:1 and 1:10 (*W.*:*S.*) were studied in papaya juice with initial 7-day fermentation by *W. saturnus*, followed by *S. cerevisiae*. The growth kinetics of *W. saturnus* were similar at all ratios, but its maximum cell count decreased as the proportion of *S. cerevisiae* was increased. Conversely, there was an early death of *S. cerevisiae* at the ratio of 10:1. *Williopsis saturnus* was the dominant yeast at 10:1 ratio that produced papaya wine with elevated concentrations of acetate esters. On the other hand, 1:1 and 1:10 ratios allowed the coexistence of both yeasts which enabled the flavour-enhancing potential of *W. saturnus* as well as the ethyl ester and alcohol-producing abilities of *S. cerevisiae*. In particular, 1:1 and 1:10 ratios resulted in production of more ethyl esters, alcohols and 2-phenylethyl acetate. However, the persistence of both yeasts at 1:1 and 1:10 ratios led to formation of high levels of acetic acid. The findings suggest that yeast ratio is a critical factor for sequential fermentation of papaya wine by *W. saturnus* and *S. cerevisiae* as a strategy to modulate papaya wine flavour.

Introduction

In the recent years, increasing non-*Saccharomyces* yeasts have been recognized for their significant contributions and desirable effects on the sensory characteristics of the wine through the quantitative and qualitative diversity of the products and by-products of fermentation (Ciani and Maccarelli, 1998). However, these non-*Saccharomyces* yeasts are not vigorous or competitive fermenting microorganisms under oenological conditions; thus, they may be only employed as adjunct cultures in conjunction with strongly fermentative *Saccharomyces cerevisiae* strains for the completion of fermentation. Indeed, the use of mixed starters in winemaking enhanced the complexity of wine flavours and had advantages over the spontaneous and pure *S. cerevisiae* fermentations (Ciani *et al*., 2006; Rodríguez *et al*., 2010). Nevertheless, the impacts on wine aroma and quality by the multi-starter cultures are determined by the strains used and the inoculation strategy (Toro and Vazquez, 2002; Ciani *et al*., 2006).

Based on our knowledge, the majority of these studies was focused on the mixed-culture fermentations of selected non-*Saccharomyces* yeasts such as *Candida, Torulaspora, Kloeckera* and *Hanseniaspora* with *S. cerevisiae* (Ciani *et al*., 2006; 2010; Rodríguez *et al*., 2010). There are still numerous other non-*Saccharomyces* yeasts, which are potentially suited for making good quality wine, but remain less unknown because of a lack of research and development. The genus *Williopsis* (formerly *Hansenula*) used in this study was reported as being an important producer of esters, especially *Williopsis saturnus* strains that synthesized significant amounts of volatile branched-chain acetate esters (e.g. isoamyl acetate and isobutyl acetate) (Vandamme, 2003).

To date, only a few studies have evaluated the likelihood of *W. saturnus* in simultaneous fermentation with *S. cerevisiae* and indicated the improvement of aroma and characteristics of papaya and longan wine (Lee *et al*., 2010; Trinh *et al*., 2011). As for sequential fermentations, limited studies have been conducted. Interestingly, Clemente-Jimenez and colleagues (2005) and Rodríguez and colleagues (2010) emphasized that sequential fermentation is the most adequate strategy of strain combination, where the kinetic behaviour resembles a successful spontaneous fermentation and produces wine with differential aromatic quality, relative to simultaneous fermentation. Furthermore, several studies reported the 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 (Ciani *et al*., 2006; Moreira *et al*., 2008; Lee *et al*., 2010), whereas

Received 28 July, 2012; accepted 8 October, 2012. *For correspondence. E-mail chmLsq@nus.edu.sg; Tel. (+65) 6516 2687; Fax (+65) 6775 7895.

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sequential fermentation allowed the persistence of non-*Saccharomyces* yeasts with low fermentative power that would extend or maximize their contact with the juice matrix (Clemente-Jimenez *et al*., 2005; Ciani *et al*., 2006).

For these reasons, sequential fermentations of *W. saturnus* and *S. cerevisiae* were performed in our previous study (Lee *et al*., 2012). However, the papaya wines produced did not acquire fermentation characteristics from both yeasts due to the early growth arrest and low inoculum level of *S. cerevisiae* (Lee *et al*., 2012). Hence, in the present study, we studied sequential fermentation in papaya wine by the utilization of different culture ratios of *W. saturnus* and *S. cerevisiae*, especially ratios with higher cell counts of *S. cerevisiae* than those used in the previous study (Lee *et al*., 2012). We reported on the fermentation behaviour and the metabolic interactions of *W. saturnus* and *S. cerevisiae* in these sequential cultures with respect to the production of ethanol and other volatile compounds that would contribute to the organoleptic characteristics of papaya wine.

Results and discussion

Evolution of biomass and enological properties

The evolution of *W. saturnus* and *S. cerevisiae* is shown in Fig. 1. In all the yeast ratios, *W. saturnus* multiplied incessantly, reaching the late log phase at day 7 and remained stationary as fermentation progressed to completion until day 17 (Fig. 1). Although the growth kinetics of *W. saturnus* was similar at different ratios, its maximum cell count decreased slightly as the inoculated proportion of *S. cerevisiae* was increased. On the other hand, *S. cerevisiae* decreased markedly upon inoculation at day 7 and then remained relatively stable in the 10:1 ratio, while the same yeast stayed almost constant throughout fermentation in the 1:1 and 1:10 ratios. As a consequence, high viable cell densities of both yeasts coexisted and there was no early death of *W. saturnus*.

These results differed from those of our previous study (Lee *et al*., 2012) in which there was no succession of yeasts in the sequential fermentation with the inoculation of *S. cerevisiae* into the papaya juice partially fermented by *W. saturnus*, and the fermentation was dominated by *W. saturnus*. This was likely due to the higher ratio of *W. saturnus* to *S. cerevisiae* (1000:1) used in the previous study. Conversely, Toro and Vazquez (2002) revealed a sharp decrease of *Candida cantarellii* upon the inoculation of *S. cerevisiae* in sequential fermentation. The rapid reduction of *S. cerevisiae* in the 10:1 ratio of *W.* : *S.* could be due to the killer-toxins (also known as mycocins) produced by *W. saturnus*, which are antagonistic against *Saccharomyces* yeasts such as *S. cerevisiae* VL1 and *S. bayanus* CVC-NF74 in yoghurt and cheese systems (Liu and Tsao, 2009; 2010). *Williopsis saturnus* also exhibits retardation and inhibition against other yeasts such as *Candida kefir* and *Kluvyveromyces marxianus* (Liu and Tsao, 2009; 2010). Takasuka and colleagues (1995) and Guyard and colleagues (2002) reported that the *Williopsis* mycocins inhibit the growth of yeasts by interfering with β-1,3 glucan synthesis, which disturbs the synthesis of yeast cell walls and thus, resulting in cell lysis and death. On the other hand, the persistence of both yeasts in the 1:1 and 1:10 ratios could be due to the high initial cell counts of *S. cerevisiae* that were able to overcome the inhibitory effects caused by the mycocins of *W. saturnus*. This hypothesis is supported by the findings

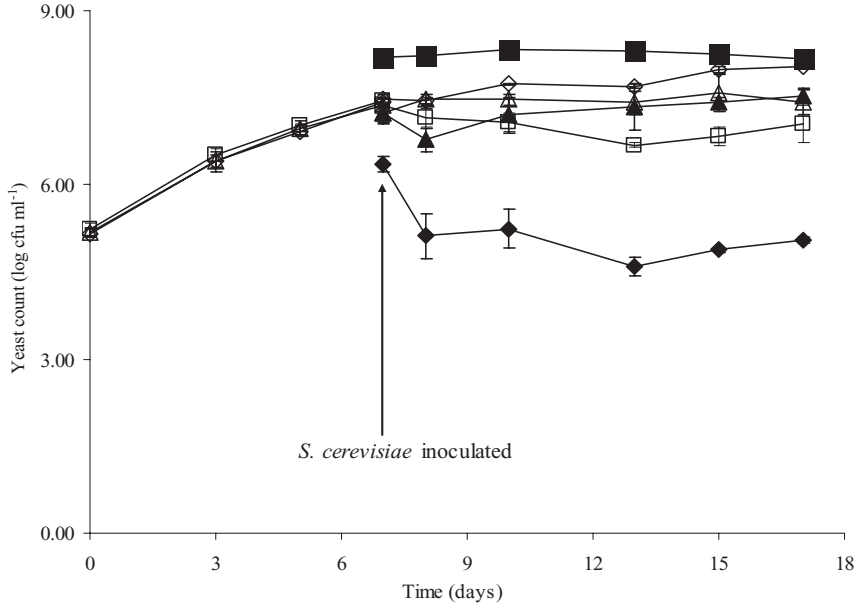

Fig. 1. Evolution of viable yeasts in papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. NCYC2251 (◊):R2 (♦) = 10:1; NCYC2251 (Δ):R2 (▲) = 1:1; NCYC2251 (□):R2 (■) = 1:10. The data are presented as the means ± standard deviation ($n = 3$).

Table 1. Physicochemical parameters of papaya wine (day 17) fermented with sequential cultures of *W. saturnus* and *S. cerevisiae* at different ratios (*W. saturnus*:*S. cerevisiae*).

	Day 0	Ratio 10:1	Ratio 1:1	Ratio 1:10
pH	3.53 ± 0.03[a]	3.54 ± 0.01[a]	3.53 ± 0.03[a]	3.56 ± 0.01[a]
°Brix	11.00 ± 0.07[a]	6.60 ± 1.00[b]	3.71 ± 0.10[c]	3.65 ± 0.17[c]
Ethanol (ml l^{-1})	0.06 ± 0.00[a]	13.84 ± 0.84[b]	38.31 ± 2.02[c]	39.71 ± 1.97[c]
Sugars (g l^{-1})				
Fructose	41.62 ± 1.98[a]	22.63 ± 5.97[b]	ND	ND
Glucose	46.07 ± 2.14[a]	11.91 ± 7.26[b]	ND	ND
Organic acids (g l^{-1})				
Acetic acid	ND	0.45 ± 0.05[a]	0.67 ± 0.02[b]	0.83 ± 0.04[c]
Citric acid	4.51 ± 0.20[a]	2.90 ± 0.13[b]	3.42 ± 0.22[c]	3.39 ± 0.15[c]
Malic acid	5.50 ± 0.34[a]	4.11 ± 0.18[bc]	4.30 ± 0.11[c]	3.71 ± 0.25[b]
Oxalic acid	0.04 ± 0.00[a]	0.07 ± 0.01[b]	0.05 ± 0.00[a]	0.07 ± 0.01[b]
Pyruvic acid	0.86 ± 0.10[a]	0.88 ± 0.01[a]	0.97 ± 0.01[a]	0.89 ± 0.07[a]
Succinic acid	3.17 ± 0.19[a]	4.09 ± 0.05[b]	2.78 ± 0.08[a]	3.68 ± 0.22[c]
Tartaric acid	0.90 ± 0.05[a]	0.77 ± 0.01[b]	0.34 ± 0.01[c]	0.39 ± 0.04[c]

a,b,c,d Statistical analysis at 95% confidence level with same letters in the same row indicating no significant difference.
ND, not detected.

in Liu and Tsao (2010), which showed that the inhibitory effect of *W. saturnus* is regulated by the initial cell count of the target yeast and is effective especially at lower levels of the target yeast.

Total soluble solids (°Brix), sugar consumption, organic acids, ethanol and pH changes are presented in Table 1. Generally, the papaya wine produced by the sequential fermentation of 1:1 ratio had most of the physicochemical properties similar to that produced by the 1:10 ratio, except for acetic, malic, oxalic and succinic acids (Table 1). Among the fermentations, the 1:10 ratio produced papaya wine with the highest ethanol content of 39.71 ml l^{-1} (Table 1). This was in agreement with the highest sugar consumption and high *S. cerevisiae* yeast count in the culture ratio of 1:10 (Fig. 1). The higher ethanol content in the 1:1 and 1:10 ratios was attributed to the higher inoculum levels of *S. cerevisiae*, which is the principal yeast for ethanol production (Nissen *et al*., 2000). As a result, the papaya wines produced by the 1:1 and 1:10 ratios may have better sensory characteristics of ethanol, i.e. fullness, body and mouth-warming effect as compared with the wine produced by the 10:1 ratio. In addition, ethanol affects aroma sensations in wine due to interactions with other compounds, which modify their volatility (Swiegers *et al*., 2008) and is also an important precursor to ethyl esters that are significant contributors of fruity character in wines (Luebke, 1980).

Evolution of volatiles and aroma qualities of papaya wines

Numerous volatiles (e.g. alcohols, aldehydes, esters, fatty acids, monoterpenes, ketones and volatile phenols) contributing to the sensory properties of papaya wine were produced and further transformed by the different ratios of *W. saturnus* and *S. cerevisiae*. Selected volatiles in the final papaya wines were analysed (Table 2). Some of these volatiles increased continuously, while others increased initially and then remained unchanged or declined gradually, being similar to the kinetic trends observed in Lee and colleagues (2010). Volatiles that were initially present, especially fatty acids, sulfur compound and esters (e.g. butyric acid, benzyl isothiocyanate and methyl butyrate) responsible for the typical papaya flavour (Pino *et al*., 2003), were metabolized to trace levels (Table 2).

Among the volatiles, ethanol and higher alcohols were the major compounds produced (Tables 1 and 2). The kinetic changes of these alcohols were similar in all the fermentations, where the alcohols increased gradually during the early stage of fermentation by *W. saturnus* and increased rapidly upon the inoculation of *S. cerevisiae*, then either remained stable or declined slightly (Fig. 2). 2-Ethylhexanol indigenous to the juice was utilized by the yeasts (data not shown). The sequential fermentation of 10:1 ratio consistently produced the lowest amounts of alcohols, whereas the 1:1 and 1:10 ratios produced comparable amounts of ethanol and higher alcohols except for isobutyl and 2-phenylethyl alcohols (Tables 1 and 2). The 1:10 ratio produced significantly higher concentrations of these alcohols than the 1:1 ratio (Table 2). This could be ascribed to the greater inoculum size and viable yeast count of *S. cerevisiae* (Fig. 1), and its higher metabolic ability to produce higher alcohols (Lee *et al*., 2010). Among the higher alcohols, 2-phenylethyl alcohol exceeded its corresponding odour threshold value of 10 mg l^{-1} (Table 2), especially for the 1:10 ratio with 64.47 mg l^{-1} 2-phenylethyl alcohol, which is expected to impart more floral and rose-like notes.

Higher alcohols are important precursors for the formation of fruity esters. The ratio of the contents of higher alcohols to esters is known to influence the sensory properties of fermented beverages. Particularly, wines with

Table 2. Concentrations of major volatile compounds (mg l^{-1}) in papaya wine at day 17 fermented with sequential cultures of *W. saturnus* and *S. cerevisiae* at different ratios (*W. saturnus*:*S. cerevisiae*).

Compounds quantified	RI[e]	Day 0	Ratio 10:1	Ratio 1:1	Ratio 1:10	Odour threshold[f] (mg l^{-1})
Acids						
Acetic acid[2]	1454	47.66 ± 0.09[a]	494.15 ± 17.23[b]	872.17 ± 25.91[c]	991.64 ± 88.89[d]	280
Isobutyric acid[1,2]	1568	0.00 ± 0.00[a]	0.16 ± 0.01[b]	0.10 ± 0.00[b]	0.42 ± 0.05[c]	8.10[g]
Butyric acid[2]	1628	11.27 ± 1.19[a]	2.46 ± 0.23[b]	0.50 ± 0.02[c]	0.56 ± 0.03[c]	2.20
Hexanoic acid[1,2]	1846	0.29 ± 0.02[a]	1.81 ± 0.14[b]	1.56 ± 0.53[b]	2.02 ± 0.23[b]	8.00
Benzoic acid[1]	2455	3.88 ± 0.25[a]	7.72 ± 0.44[b]	5.06 ± 0.05[c]	6.41 ± 0.62[d]	–
Octanoic acid[1,2]	2062	0.08 ± 0.01[a]	0.57 ± 0.05[b]	0.76 ± 0.07[c]	0.58 ± 0.06[b]	8.80
Decanoic acid[1,2]	2275	0.26 ± 0.00[a]	0.46 ± 0.04[b]	0.89 ± 0.09[c]	0.56 ± 0.05[b]	6.00
Dodecanoic acid[1,2]	2487	0.69 ± 0.00[a]	0.87 ± 0.04[b]	0.89 ± 0.04[b]	0.78 ± 0.01[c]	1.00[j]
Alcohols						
Isobutyl alcohol[2]	1084	0.00 ± 0.00[a]	1.31 ± 0.06[b]	1.99 ± 0.14[c]	2.82 ± 0.27[d]	40.00
Active amyl alcohol[2]	1210	0.00 ± 0.00[a]	0.11 ± 0.02[b]	0.79 ± 0.16[c]	0.63 ± 0.05[c]	65.00
Isoamyl alcohol[2]	1222	0.00 ± 0.00[a]	1.19 ± 0.11[b]	2.12 ± 0.15[c]	2.16 ± 0.16[c]	30.00
2-Phenylethyl alcohol[2]	1938	0.00 ± 0.00[a]	14.85 ± 1.72[b]	39.72 ± 2.80[c]	64.47 ± 4.20[d]	10.00
Aldehydes						
Benzaldehyde[2]	1539	0.03 ± 0.00[a]	0.01 ± 0.00[b]	0.00 ± 0.00[c]	0.00 ± 0.00[c]	3.50[h]
O-Tolualdehyde[2]	1668	0.01 ± 0.00[a]	0.07 ± 0.00[b]	0.04 ± 0.00[c]	0.01 ± 0.00[a]	–
Esters						
Ethyl hexanoate[2]	1217	0.00 ± 0.00[a]	0.02 ± 0.00[b]	0.05 ± 0.00[c]	0.06 ± 0.01[d]	0.05
Ethyl octanoate[2]	1430	0.00 ± 0.00[a]	0.15 ± 0.03[b]	1.52 ± 0.07[c]	1.62 ± 0.04[d]	0.02
Ethyl decanoate[2]	1638	0.00 ± 0.00[a]	0.14 ± 0.03[a]	1.64 ± 0.04[b]	1.17 ± 0.18[c]	0.20
Ethyl dodecanoate[2]	1844	0.00 ± 0.00[a]	0.96 ± 0.01[b]	1.47 ± 0.21[c]	1.29 ± 0.03[c]	1.20[j]
Ethyl tetradecanoate[2]	2050	0.00 ± 0.00[a]	0.10 ± 0.00[b]	0.15 ± 0.01[c]	0.07 ± 0.01[d]	0.80[j]
Isobutyl octanoate[2]	1541	0.00 ± 0.00[a]	0.03 ± 0.00[b]	0.04 ± 0.00[b]	0.04 ± 0.00[b]	0.80[j]
Isoamyl octanoate[2]	1652	0.00 ± 0.00[a]	0.03 ± 0.00[b]	0.26 ± 0.01[c]	0.23 ± 0.01[d]	0.125[j]
Ethyl acetate[2]	899	0.00 ± 0.00[a]	267.32 ± 31.90[b]	208.02 ± 29.76[b]	214.14 ± 4.42[b]	7.50
Isoamyl acetate[1,2]	1095	0.00 ± 0.00[a]	1.02 ± 0.02[b]	0.26 ± 0.01[c]	0.52 ± 0.05[d]	0.03
2-Phenylethyl acetate[1,2]	1827	0.00 ± 0.00[a]	1.64 ± 0.12[b]	1.49 ± 0.10[b]	1.96 ± 0.29[c]	0.25

a,b,c,d Statistical analysis at 95% confidence level with same letters in the same row indicating no significant difference.
e Experimentally determined linear retention index on the DB-FFAP column, relative to C5–C40 hydrocarbons.
f,g,h,i,j Odour thresholds collated from literatures [[f]Bartowsky and Pretorius (2009), [g]Salo (1970), [h]Buttery *et al.* (1990), [i]Ferreira *et al.* (2000) and [j]Li *et al.* (2008) respectively].
1,2 Retention index in agreement with literature values [Duarte *et al.* (2010) and Lee *et al.* (2012) respectively].
RI, retention index.

increased contents of esters possess an enhanced fruity flavour that could be improved if the higher alcohol contents were to decrease (Moyano *et al.*, 1994). A new sulfur-containing alcohol, 2-(methylthio)ethanol, was produced in all fermentations especially at 1:1 and 1:10 ratios (Fig. 2), which is reported for the first time in papaya wine and could be derived from L-methionine catabolism by the yeasts. This volatile sulfur compound has been commonly detected in other wines such as white wines, Tinta Negra Mole red wine and Italian sparkling wines (Perestrelo *et al.*, 2006; Fedrizzi *et al.*, 2010). The heavy sulfur compound cannot be eliminated and may impart French bean and cauliflower-like aroma to wine near its flavour threshold of 250 μg l^{-1} (Darriet *et al.*, 1999). However, Perestrelo and colleagues (2006) reported that most of the sulfur compounds identified in wines are usually found at levels below their threshold values. It is not known whether all the yeast ratios used in this study would result in any flavour impact due to 2-(methylthio)ethanol in the papaya wine.

Volatile fatty acids are another important group of volatiles produced by the yeasts (Table 2). The kinetic changes of the volatile fatty acids were similar in all the fermentations with trends comparable to the alcohols in Fig. 2, except for butyric acid that was metabolized (Table 2). The sequential fermentation of 1:1 ratio produced the highest amount of most fatty acids, except for acetic, isobutyric, hexanoic and benzoic acids (Table 2). The 1:10 ratio would have been expected to produce the most C8, C10 and C12 fatty acids, given that *S. cerevisiae* is known to be the main producer of these acids. These results indicate some kind of interaction between *W. saturnus* and *S. cerevisiae* at 1:1 ratio that favoured production of these fatty acids and this interaction merits further research. The sequential fermentation of 1:10 ratio produced the highest amount of acetic acid (991.64 mg l^{-1}), followed by the 1:1 and 10:1 ratios with 872.17 mg l^{-1} and 494.15 mg l^{-1} acetic acid, respectively (Table 2), which were in line with the acetic acid results obtained by HPLC (Table 1). This could be in part due to the hydrolysis by *S. cerevisiae* of some acetate esters such as ethyl acetate produced by *W. saturnus*. The high level of acetic acid produced in all fermentations (Table 2), especially those in the 1:1 and 1:10 ratios

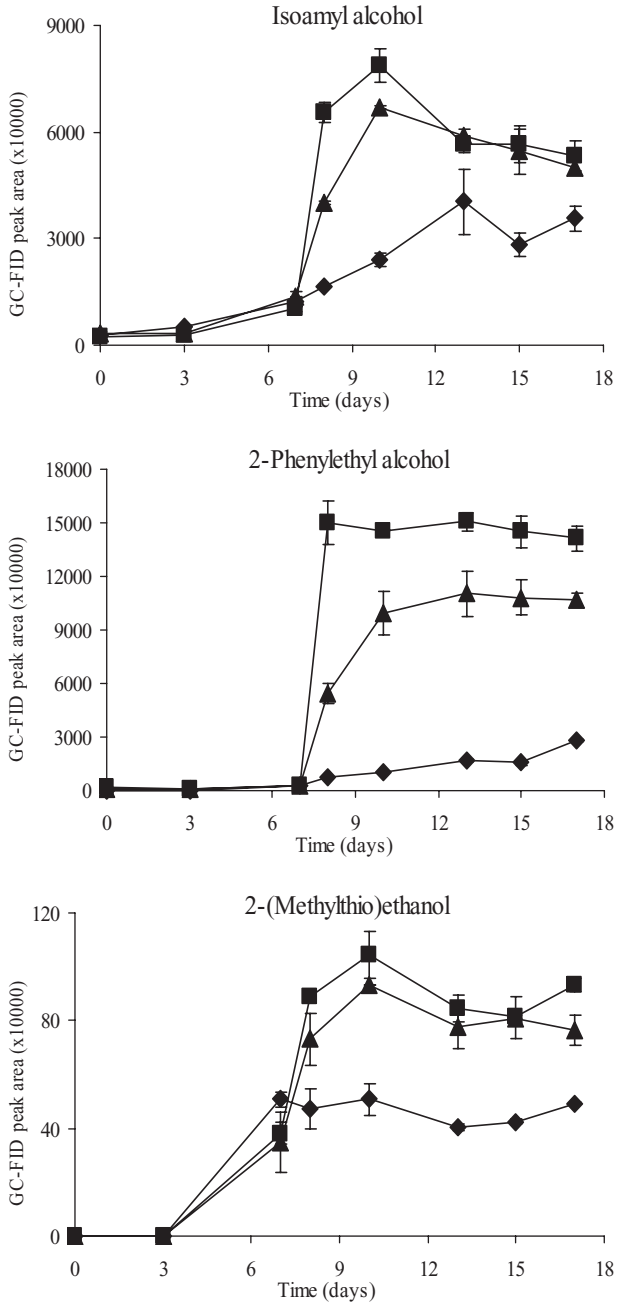

Fig. 2. Changes of higher alcohols and 2-(methylthio)ethanol during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* and *S. cerevisiae*. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■).

may be expected to exert some adverse effects (e.g. acidic, vinegar and pungent flavours) on the aromatic quality of the papaya wine, but this was not confirmed in sensory evaluation presented below. The results of our study differed from those of Kapsopoulou and colleagues (2007), who highlighted that sequential fermentation reduced the acetic acid content of wine. This discrepancy could be attributed to the domination of *S. cerevisiae* in their sequential fermentation and different non-*Saccharomyces* yeast (*Kluyveromyces thermotolerans*) used in the latter study.

Esters constitute the other major fermentation-derived volatiles that include acetate esters, ethyl esters and other medium to long-chain esters (Table 2). The kinetic changes of esters varied with the ester type. Most of the ethyl esters increased slowly at the initial stage of fermentation by *W. saturnus*, followed by substantial increases upon the inoculation of *S. cerevisiae* and then either remained stable or experienced a steady or sharp decline (Fig. 3). Acetate esters, on the other hand, increased substantially during the initial stage of fermentation and decreased sharply upon the inoculation of *S. cerevisiae*, except for ethyl acetate and 2-phenylethyl acetate in the 10:1 and 1:1 ratios (Fig. 3). The evolution or net accumulation of esters in wine is the result of the balance between yeast ester-synthesizing enzymes and esterase enzymes promoting their hydrolysis in the respective yeasts (Lilly *et al.*, 2006). The results of the present study differed from the findings in our previous study (Lee *et al.*, 2012); in the latter study, there was no significant modification of esters with the inoculation of *S. cerevisiae* into the papaya wine partially fermented by *W. saturnus*. It is reported that the volatiles produced by one of the yeasts can be metabolized by the other (Ciani *et al.*, 2010) and redox interactions existed between yeasts (Cheraiti *et al.*, 2005).

The sequential fermentation of 1:1 ratio produced the highest amount of ethyl esters and other miscellaneous esters, except for ethyl hexanoate, ethyl octanoate and acetate esters (Table 2). This correlated with the higher volatile fatty acid production in the 1:1 ratio (Table 2), which are essential precursors for ethyl ester formation (Saerens *et al.*, 2008). The sequential fermentation of 10:1 ratio, on the other hand, produced the highest concentrations of most acetate esters, whereas the 1:10 ratio had the highest amount of 2-phenylethyl acetate, ethyl hexanoate and ethyl octanoate (Table 2). The high viable yeast population of *W. saturnus* against *S. cerevisiae* in the 10:1 ratio accounted for the higher acetate ester production, as *W. saturnus* is a good producer of acetate esters (Park *et al.*, 2009; Trinh *et al.*, 2011). This is in agreement with the lower levels of higher alcohols in the 10:1 ratio (Table 2), which served as precursors, together with acetyl-CoA, for acetate esters (e.g. isoamyl acetate) synthesis by the action of alcohol acetyltransferase (Park *et al.*, 2009). *Saccharomyces cerevisiae*, the principal wine yeast, is a known potent producer of ethyl esters that contribute pleasant fruity and floral odours to wine aroma. Surprisingly, the 1:10 ratio with the highest *S. cerevisiae* did not produce the uppermost amount of most ethyl esters (Table 2). This could be due to the coexistence of both yeasts in the 1:10 ratio (Fig. 1), which may modulate the ester formation capability of *S. cerevisiae*. This sug-

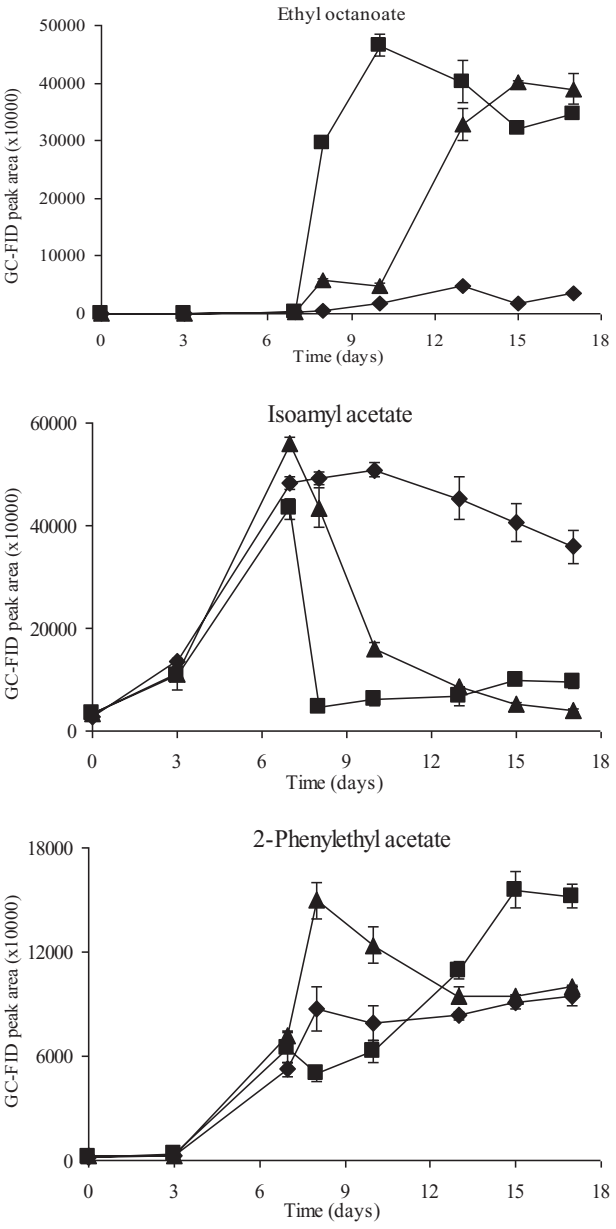

Fig. 3. Changes of ethyl octanoate and acetate esters during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* and *S. cerevisiae*. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■).

gestion is supported by the findings in Cheraiti and colleagues (2005) in that one species or strain in mixed-culture fermentation may impact on the metabolic behaviour of another strain.

Ethyl hexanoate and ethyl octanoate were reported as the odour-active compounds in papaya wine (Pino and Queris, 2011). The concentrations of these ethyl esters in the 1:1 and 1:10 ratios were higher than their threshold values, suggesting that they can contribute pleasant fruity, floral and honey-like flavours to the final wine bouquet (Luebke, 1980). Other ethyl esters (ethyl decanoate and ethyl dodecanoate) produced by both the 1:1 and the 1:10 ratios were also higher than the threshold values. Similarly, these ethyl esters can add pleasant and fruity notes to the papaya wine, but may impart rancid and soapy flavours to the wine bouquet when their concentration was too high (Li *et al.*, 2012). On the other hand, the concentrations of acetate esters in all the fermentations could contribute to the floral (rose) and fruity (banana) notes (Luebke, 1980), especially for the 10:1 and 1:10 ratios with the highest amount of isoamyl acetate and 2-phenylethyl acetate respectively (Table 2). However, the high concentration of ethyl acetate produced by all the ratios was considered detrimental to the wine quality, as ethyl acetate at high levels (200 mg l^{-1}) exerts a solvent-like aroma (Etievant, 1991).

Principal component analysis (PCA) was applied to the ethanol (Table 1) and volatile compounds (Table 2) to discriminate the common characteristics as well as to reveal the diversity in the volatile composition among the papaya wines. The PCA result indicates distinctive volatile compositions and clear separation among the papaya wines (Fig. 4). The papaya wine produced by the sequential fermentation at 10:1 ratio was mainly characterized by ethyl acetate and those volatiles associated with papaya juice (e.g. butyric acid and benzaldehyde). Conversely, the sequential fermentation at 1:1 ratio was associated with more medium-chain fatty acids and ethyl esters such as ethyl decanoate, ethyl dodecanoate and ethyl tetradecanoate. The papaya wine produced by sequential fermentation at 1:10 ratio was distinguished with a high percentage of acetic acid, ethyl hexanoate, ethyl octanoate, isobutyl octanoate, ethanol and higher alcohols.

Sensory analysis

The papaya wine produced by the 10:1 ratio had most of the sensory attributes similar to the other ratios, but there are substantial differences among the ratios that resulted in the differentiation of aroma profiles (Fig. 5). Wine fermented by the 1:10 ratio had more noticeable yeasty, sweet and fusel notes than the 10:1 ratio (Fig. 5), which was probably due to the high levels of 2-phenylethyl acetate, ethyl esters and higher alcohols (Table 2). On the other hand, the wine produced by the 1:1 ratio possessed less buttery and cocoa notes regardless of the significant amount of 3-hydroxy-2-butanone detected (data not shown). There were no significant differences in the aroma profiles in all the papaya wines regardless of the different ratios, which differed from those found for the volatile compounds determined by GC-MS/FID (Tables 1 and 2) and PCA result (Fig. 4). This might be attributed to the complex nature of the papaya wine matrix where the non-volatile compounds such as phenolic compounds,

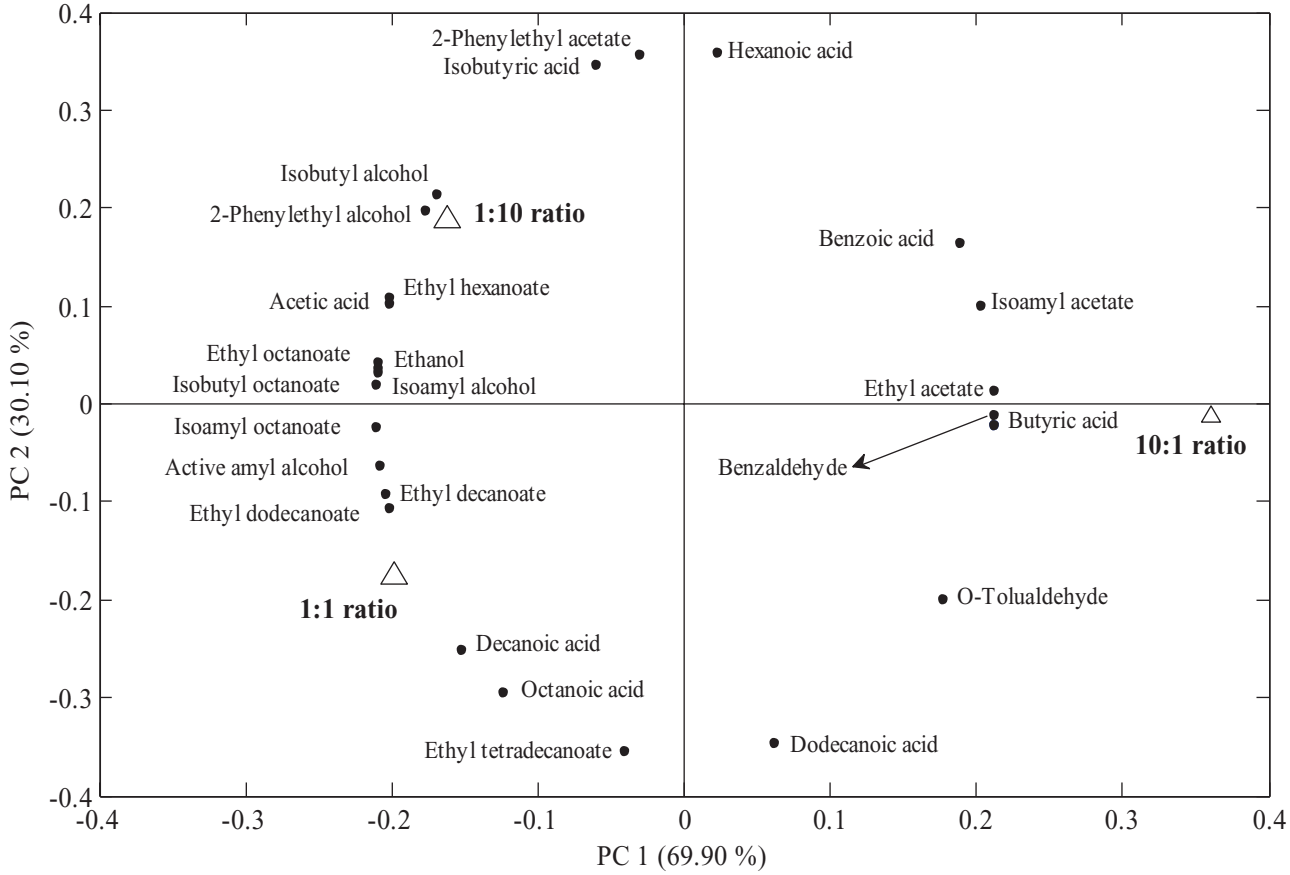

Fig. 4. Bi-plot of principal component analysis of the major volatile compounds in papaya wines fermented by sequential cultures of *W. saturnus* NCYC2251 and *S. cerevisiae* R2 at different ratios (*W. saturnus*:*S. cerevisiae*).

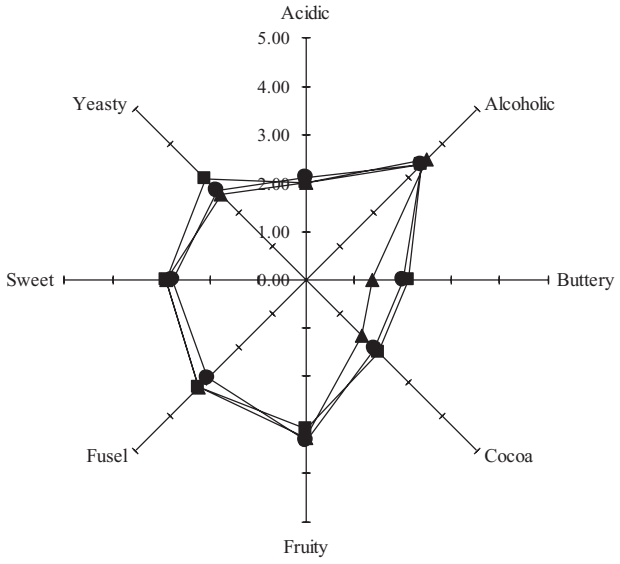

Fig. 5. Aroma profile of papaya wines (day 17) fermented with different ratio of sequential cultures of *W. saturnus* and *S. cerevisiae*. 10:1 ratio (●); 1:1 ratio (▲); 1:10 ratio (■).

organic acids and carbohydrates, or other volatile compounds that significantly impact on aroma volatility and perception (Guth and Fritzler, 2004).

In conclusion, the ratio of *W. saturnus* NCYC2251 to *S. cerevisiae* R2 was crucial for the survival of yeasts which had significant impacts on the production of volatile compounds such as alcohols, fatty acids and esters. Among the yeast ratios, the 1:1 and 1:10 ratios (*W.* : *S.*) enabled the coexistence of both yeasts and enhanced the production of desirable volatile compounds through synergistic effects. The use of sequential fermentation with *W. saturnus* and *S. cerevisiae* at a sufficiently higher ratio of the latter provides a feasible strategy to alter the papaya wine volatile profile.

Experimental procedures

Preparation of yeast cultures and papaya juice

Williopsis saturnus var. *markii* NCYC2251 and *S. cerevisiae* var. *bayanus* Lavin R2 were obtained from National Collection of Yeast Cultures (Norwich, UK) and Lallemand (Brooklyn Park, Australia) respectively. *Williopsis saturnus* was propagated and maintained according to the procedure

described in Lee and colleagues (2010), while *S. cerevisiae* (freeze-dried form) was stored at –80°C before use. Papayas of the Sekaki cultivar were washed, juiced and centrifuged at 32 140 *g* for 15 min at 4°C. The initial sugar concentration of papaya juice was 11.11 °Brix (containing 41.62 g of fructose and 46.07 g of glucose per litre of juice) and the pH was 4.98. The juice was brought to pH 3.5 by the addition of 1 M DL-malic acid and sanitized by adding 100 mg l^{-1} potassium metabisulfite ($K_2S_2O_5$); moreover, sterility check was performed by plate counting.

Fermentation conditions

Triplicate sequential fermentations were carried out with aliquots of 280 ml^{-1} sanitized papaya juices at 20°C by inoculation with ~ 10^5 cfu ml^{-1} *W. saturnus* (pre-culture grown in the same medium at 25°C for 96 h) for 7 days. After 7 days (late log phase of *W. saturnus* with a viable cell count of ~ 10^7 cfu ml^{-1}), fermentations were inoculated with ~ 10^6 cfu ml^{-1}, ~ 10^7 cfu ml^{-1} and ~ 10^8 cfu ml^{-1} of *S. cerevisiae* to obtain ratios of 10:1, 1:1 and 1:10 (*W. saturnus*:*S. cerevisiae*) respectively. Before inoculation, *S. cerevisiae* (freeze-dried form) was reconstituted in sterile nutrient broth (Lee *et al*., 2012) and concentrated by centrifugation to obtain an initial density of 1.17×10^{10} cfu ml^{-1}. All fermentations were maintained up to 17 days under static conditions.

Yeast enumeration and analytical determinations

Enumeration of wine yeasts was performed by plating on potato dextrose agar (PDA) (39 g l^{-1}, Oxoid, Basingstoke, Hampshire, England) that allowed *W. saturnus* to be morphologically distinguished from *S. cerevisiae* colonies. Plates were incubated at 25°C for 2 days before counting. The total soluble solids (°Brix) and pH values were measured using a refractometer (ATAGO, Japan) and pH meter (Metrohm, Switzerland) respectively. Sugars determinations were carried out on a Zorbax carbohydrate column (Agilent, Santa, Clara, CA, USA) using a mixture of acetonitrile and water (80:20 v/v) as the mobile phase at a flow rate of 1.4 ml min^{-1}, and connected to a low temperature evaporative light scattering detector (ELSD-LT). Organic acids were analysed by a Supelcogel C-610 H column (300 × 7.8 mm, Supelco, Bellefonte, PA, USA) using 1 ml l^{-1} sulfuric acid as the mobile phase at a flow rate of 0.4 ml min^{-1} with photodiode array detection (Lee *et al*., 2012).

The volatile compounds were analysed in triplicate, by optimized headspace (HS) solid-phase microextraction (SPME) method (extracted at 60°C for 50 min with 250 r.p.m. agitation), coupled with gas chromatography (GC)-mass spectrometer (MS) and flame ionization detector (FID) (Lee *et al*., 2010). A Carboxen/PDMS fibre (85 μm) (Supelco, Sigma-Aldrich, Barcelona, Spain) was used for extraction of volatiles. A DB-FFAP capillary column (60 m × 0.25 mm I.D. and 0.25 μm film thickness) with stationary phase of polyethylene glycol modified with nitroterephthalic acid (Agilent, Santa Clara, CA, USA) was used for separation. Both injector and detector (FID) temperatures were set at 250°C. The carrier gas was helium at 1.2 ml min^{-1}. The identification of volatile compounds was carried out by matching the mass spectra against NIST 8.0 and Wiley 275 MS libraries, and confirmed with Linear Retention Index (LRI) values and retention times of pure standards (Firmenich, Singapore). The volatile compounds were quantified by comparing concentrations with the corresponding standard solutions using the external standard calibration method. Samples were analysed in triplicate.

Sensory evaluation

The sensorial evaluation of papaya wines was done by a panel of eight well-trained flavourists (three females and five males) from Firmenich Asia (Singapore) who are experienced in wine tasting and in aroma evaluation. There were eight sensory descriptors for the papaya wine aroma: acidic, alcoholic, buttery, cocoa, fruity, fusel, sweet and yeasty. The wine samples were coded and presented randomly to the panel, and the aroma intensity on each sensory descriptor was rated on a hedonic scale from 0 (uncharacteristic) to 5 (very strong).

Statistical analysis

Analysis of variance (ANOVA) using SPSS 17.0 software for Windows (SPSS, Chicago, IL) was applied to the experimental data to determine significant differences between the samples. The statistical level of significance was set at $P < 0.05$. PCA was performed using the software Matlab R2008a (Mathworks, Natick, MA, USA).

Conflict of interest

None declared.

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