ISSN 1330-9862
(FTB-2876)

original scientific paper

Effect of Precursors on Volatile Compounds in Papaya Wine Fermented by Mixed Yeasts

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Received: July 3, 2011
Accepted: November 25, 2011

Summary

The impact of the addition of fusel oil or amino acids on the volatile compounds in papaya wine fermented with a mixed culture of *Saccharomyces cerevisiae* var. *bayanus* R2 and *Williopsis saturnus* var. *mrakii* NCYC 2251 at a ratio of 1:1000 was studied. Fusel oil addition increased the fraction of alcohols and promoted the production of isoamyl octanoate, isoamyl decanoate and isobutyl decanoate, while decreased the fraction of ethyl acetate and 2-phenylethyl acetate. The addition of amino acids enhanced the formation of total volatile fatty acids, 2-phenylethanol and some ethyl esters. The papaya wine with added amino acids possessed more acidic and buttery notes than the control, while that with added fusel oil had an overall aroma profile comparable to that of the control. This study suggests that papaya juice fermentation with mixed yeasts in conjunction with the added fusel oil or selected amino acids may be another method of modulating the flavour of papaya wine.

Key words: amino acids, volatile compounds, fermentation, fusel oil, mixed culture, papaya wine

Introduction

Saccharomyces cerevisiae, the yeast used in alcoholic fermentation, is primarily responsible for the formation of main metabolic products and also several other flavour compounds (*1*). Many studies suggest that apart from *Saccharomyces* species, non-*Saccharomyces* yeasts are also ecologically and metabolically significant in the wine fermentation, which has laid the platform for more creative and controlled exploitation of various yeasts in wine production (*2*). Nevertheless, *Saccharomyces* yeast is still essential to complete the wine fermentation due to the low stress tolerance of non-*Saccharomyces* yeasts (*3*). For this reason, several authors have studied fermentations with mixtures of yeasts and produced wine and fruit wine with enhanced quality and differential characteristics (*4,5*).

Other than yeasts, the nitrogen composition of the must is another important factor that affects both the aroma and the rate of fermentation through the increase of biomass production, the stimulation of sugar utilization and the prevention of stuck and sluggish fermentations (*6*). Dickinson *et al.* (*7*) and Etschmann *et al.* (*8*) determined the importance of amino acids, especially the branched-chain and aromatic amino acids, in the formation of higher alcohols by yeasts.

Papaya (*Carica papaya*) was chosen for this study due to its high nutrient content (*9*) and abundant supplies

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*Corresponding author; Phone: ++65 6516 2687; Fax: ++65 6775 7895; E-mail: chmLsq@nus.edu.sg in the tropical region. As compared to grapes, papaya has relatively low amounts of amino acids (*10*) and hence nitrogen supplementation in form of amino acids or ammonium would be beneficial for obtaining desirable papaya wine quality. Besides these two supplementation methods, Lee *et al.* (*11*) have proven the feasibility of adding fusel oil into papaya juice as aroma precursor to enhance desirable volatile production (*e.g.* ethanol and esters). Until now, the addition of amino acids or fusel oil to wine during fermentation has mainly been conducted using either single *S. cerevisiae* or non-*Saccharomyce*s yeasts (*11–13*). Regarding mixed yeast cultures, limited studies have been conducted with longan wine fermentation (*14*) and hence, there is a need for further exploration into the use of both mixed cultures and flavour precursors to enhance the volatile compounds in nitrogen-deficient fruit musts.

Considering the increasing applications of mixed cultures in wine fermentation and consumer demand for new types of wine, it will be of value to assess the impact of fusel oil or amino acid addition on the volatile compounds in mixed culture fermentation. The aim of this work is to investigate the effects of fusel oil or amino acid addition on the fermentation by a mixed culture of *Saccharomyces cerevisiae* var. *bayanus* R2 and *Williopsis saturnus* var. *mrakii* NCYC 2251 and the formation of volatile compounds in papaya wine. The ratio of mixed culture used in this study was 1:1000, which corresponded to that in Lee *et al.* (*5*). This ratio enabled the survival of *W. saturnus* in the early stage of fermentation and encouraged metabolic interactions between the yeast species, which improved the organoleptic attributes of papaya wine. *W. saturnus* was chosen due to its ability to produce high levels of esters (*5,14*). Given the capability of *W. saturnus* to produce desirable volatiles, it can potentially enhance the fruity flavour and impart unique oenological characteristics to wines.

Materials and Methods

Microorganisms and media

Saccharomyces cerevisiae var. *bayanus* (Lalvin R2^{TM}) was acquired from Lallemand Inc. (Brooklyn Park, Australia) and *Williopsis saturnus* var. *mrakii* NCYC 2251 was obtained from the National Collection of Yeast Cultures (Norwich, UK). All the yeasts were propagated and maintained according to Lee *et al.* (*5*). The papaya used in the fermentation (Sekaki cultivar, sugar content=11.11 °Bx, pH=4.98) was prepared based on the procedure described in Lee *et al.* (*5*). Potato dextrose agar (PDA; 39 g/L, Oxoid Basingstoke, Hampshire, UK) was used to assess the growth of both *Saccharomyces* and non-*Saccharomyces* wine yeasts.

Experimental fermentations

Mixed culture fermentations with *S. cerevisiae* and *W. saturnus* were carried out in triplicate for 21 days at 20 °C in 300-mL Erlenmeyer flasks containing 250 mL of sterile papaya juice. Each flask was simultaneously inoculated with approx. 10^2 CFU/mL of *S. cerevisiae* R2 and approx. 10^5 CFU/mL of *W. saturnus* var. *mrakii* NCYC 2251. Before inoculation, each yeast species was cultured for 48 h following the procedure described by Lee *et al.* (*5*). Subsequently, either 0.05 % by volume (418.5 mg/L) of fusel oil (Firmenich Asia Pte Ltd, Singapore), comprised (in %): isoamyl alcohol 47, active amyl alcohol 13.26, isobutyl alcohol 16.62, ethanol 9.06, other minor volatiles and water 14.06 (*11*), or 0.05 % (500 mg/L) of an amino acid mixture (Sigma-Aldrich, Oakville, ON, Canada), containing 0.0125 % (125 mg/L) L-leucine, L-isoleucine, L-valine and L-phenylalanine each, except for the control were added to the flasks. The yeast growth was assessed by plating on PDA agar and incubating at 25 °C for 48 h, which allowed non-*Saccharomyces* (wrinkled, rough and dull) to be distinguished morphologically from *Saccharomyces* yeast colonies (shiny, defined round shape and smooth).

Oenological parameters and volatile compound analysis

The sugar content (°Bx) and pH were determined using a refractometer (ATAGO Co. Ltd, Tokyo, Japan) and pH meter (Metrohm Ltd, Herisau, Switzerland), respectively. The sugar and organic acids were analyzed following the protocol described by Lee *et al.* (*11*) with modifications of the sugar analysis method. The determination of sugars was done using a ZORBAX® carbohydrate column (Agilent, Santa Clara, CA, USA) and assessed by evaporative light scattering detector (ELSD). The column was eluted at 40 °C with a degassed mobile phase containing acetonitrile and deionized water (80:20 by volume), at a flow rate of 1.4 mL/min. Volatile compounds were determined and quantified by optimized HS-SPME-GC-MS/FID, as described by Lee *et al.* (*5*). The volatile compounds were identified by comparison with commercial reference compounds provided by Firmenich Asia Pte Ltd, by comparison of retention indices with those described in the literature and the mass spectra with those contained in the Wiley's NIST/EPA/NIH mass spectral library (Wiley/NIST, Hoboken, NJ, USA). Quantification of the selected volatile compounds was similar to that reported by Lee *et al.* (*11*). All samples were analyzed in triplicate.

Sensory analysis of wines

The papaya wines were evaluated by a 5-member expert tasting panel. Descriptors were recorded and classified into eight categories (acidic, alcoholic, buttery, cocoa, fruity, fusel, sweet and yeasty). The papaya wine samples were only sniffed and the aroma intensity of each sensory descriptor was rated on a 5-point hedonic scale. The data were processed to obtain the modified frequency (MF) as described by Torrens *et al.* (*15*). The MF was calculated with the following formula:

$$\mathrm{MF}=(\mathrm{F}\cdot\mathrm{I})^{1/2}/\% \qquad /1/$$

where F is the detection frequency of an aromatic attribute expressed as percentage, and I is the average value of the maximum intensity expressed as percentage.

Statistical analysis

Test of significance for the experimental data was accomplished by employing one-way analysis of variance (ANOVA), using Microsoft Office Excel 2003 (Microsoft Corp., Redmond, WA, USA). Principal component analysis (PCA) was performed using the software MATLAB® R2008a (MathWorks, Natick, MA, USA).

Results and Discussion

Evolution of oenological parameters during papaya wine fermentation

All the fermentations showed similar characteristics in terms of pH changes, total sugar content (°Bx), sugar consumption and organic acid changes regardless of the added amino acids or fusel oil (Table 1). The addition of fusel oil or amino acids did not affect ethanol formation, which was found at volume fractions of 2.70–3.49 % (Table 1). The results of our study are in accordance with those of Trinh *et al*. (*14*), where the control samples and those with added amino acids produced similar ethanol fractions. In contrast, Lee *et al.* (*11*) observed an increase in ethanol production with the addition of fusel oil.

Acetic acid is an undesirable organic acid in alcoholic beverages that imparts vinegary off-odour at concentrations near its flavour threshold of 0.7–1.1 g/L (*16*). The papaya wine fermentation with 0.1 % (by volume) fusel oil reduced acetic acid production in a previous study (*11*). The results of the current study differ from the previous one; here the concentrations of acetic acid produced were higher in the samples with added fusel oil than in the control (Table 1). This may be due to the fact that different volume fractions of fusel oil (0.1 and 0.5 %) and only a *Williopsis saturnus* monoculture were used in the study of Lee *et al.* (*11*).

The *W. saturnus* yeast grew initially and then its growth either remained stationary or declined, especially during the fermentation with added fusel oil, while the *S. cerevisiae* multiplied continuously and overtook the *W. saturnus* population by day 6 (Fig. 1). In particular, the samples with added fusel oil had the lowest *W. saturnus* cell population of $1.69\cdot10^5$ CFU/mL at day 21 (Fig. 1). This could be attributed to the higher amount of acetaldehyde produced as compared to the control and the samples with added amino acids (Table 2; *17*), where acetaldehyde could regulate the growth and survival of yeasts in mixed culture fermentation (*18*). The reduced growth and early death of non-*Saccharomyces* yeasts in mixed culture fermentation has also been observed in other studies (*4*,*5*,*19*), and this has generally been correlated with their inability to survive the increasing fractions of ethanol produced in the fermentation (*20*). However, Pina *et al.* (*21*) discovered that some non-*Saccharomyces* yeasts such as *Hanseniaspora guilliermondii* had similar ethanol tolerance to that of *S. cerevisiae.* Hence, the progressive disappearance of non-*Saccharomyces* yeasts in the mixed culture fermentation has been attributed to other factors such as presence of toxic compounds, cell-cell contact mechanism and quorum sensing (*3*).

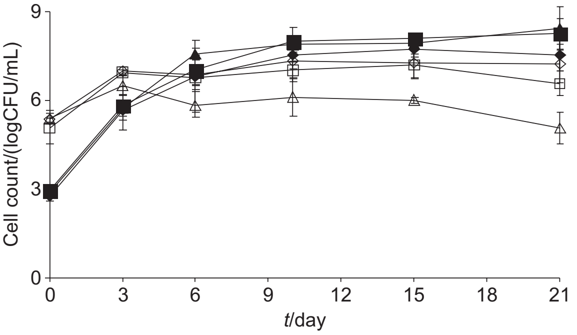

Fig. 1. Evolution of yeasts in papaya wine during the fermentation of the mixed culture of *Saccharomyces cerevisiae* R2/*Williopsis saturnus* NCYC2251 (1:1000) with flavour precursors added; (◆) *S. cerevisiae* var. *bayanus* R2 (control), (◇) *W. saturnus* var. *mrakii* NCYC2251 (control), (▲) *S. cerevisiae* var. *bayanus* R2 with fusel oil added, (△) *W. saturnus* var. *mrakii* NCYC2251 with fusel oil added, (■) *S. cerevisiae* var. *bayanus* R2 with amino acids added, and (□) *W. saturnus* var. *mrakii* NCYC2251 with amino acids added

Table 1. Oenological parameters of papaya wine (day 21) fermented by a mixed culture of *Saccharomyces cerevisiae* and *Williopsis saturnus* in the presence of the added flavour precursors

	Day 0	Control	φ(fusel oil)=0.05 %	$\frac{m(\text{amino acids})}{V(\text{mixture})}$=0.05 %
pH	(3.54±0.01)^{a}	(3.74±0.02)^{b}	(3.75±0.0)1^{b}	(3.73±0.01)^{b}
Sugar/°Bx	(11.11±0.01)^{a}	(3.41±0.18)^{b}	(3.40±0.07)^{b}	(3.49±0.07)^{b}
φ(ethanol)/%	(0.004±0.00)^{a}	(3.49±0.29)^{b}	(2.70±0.36)^{c}	(2.86±0.13)^{c}
γ(sugar)/(g per 100 mL)				
fructose	(4.91±0.44)^{a}	(0.03±0.00)^{b}	(0.03±0.00)^{b}	(0.03±0.00)^{b}
glucose	(5.32±0.50)^{a}	(0.04±0.00)^{b}	(0.04±0.00)^{b}	(0.04±0.00)^{b}
γ(organic acid)/(g per 100 mL)				
acetic acid	(0.027±0.007)^{a}	(0.072±0.001)^{b}	(0.076±0.001)^{c}	(0.081±0.002)^{d}
citric acid	(0.226±0.002)^{a}	(0.205±0.005)^{b}	(0.210±0.001)^{c}	(0.202±0.005)^{b}
malic acid	(0.954±0.007)^{a}	(0.593±0.014)^{b}	(0.576±0.017)^{c}	(0.575±0.019)^{c}
tartaric acid	(0.021±0.002)^{a}	(0.010±0.000)^{b}	(0.010±0.000)^{b}	(0.010±0.000)^{b}

^{a,b,c,d}Statistical analysis at 95 % confidence level with the same letters indicating no significant difference

Kinetic changes of volatile compounds during papaya wine fermentation in the presence of flavour precursors

During papaya wine fermentation, a diversity of volatile compounds were produced by the mixed culture of *S. cerevisiae* and *W. saturnus* that modulated the fermentation bouquet of the resultant papaya wine. These volatile compounds include alcohols, aldehydes, esters, volatile fatty acids, ketones, volatile phenol and monoterpene (Table 2). Volatiles that were originally present in the papaya juice, namely benzyl isothiocyanate, benzaldehyde, β-damascenone and some fatty acids (butyric and hexanoic acids) were metabolized to trace levels (Table 2).

The kinetic changes of volatile fatty acids were similar in all fermentations regardless of the fusel oil or amino acid additions and the trends were similar to those in the work of Lee *et al.* (*22*). The addition of fusel oil or amino acids increased the acetic acid formation, which was similar to the trend in the results for organic acids (Table 1). Octanoic and decanoic acids were the major volatile fatty acids in papaya wine with relative peak areas (RPA) of 0.57–0.84 and 1.43–1.89 %, respectively (Table 2). The addition of fusel oil reduced the formation of octanoic and decanoic acids as compared to the control (Tables 2 and 3; *23,24*). In the samples with added amino acids, the highest amount of total volatile fatty acids with RPA of 3.96 % was produced (Table 2), which was consistent with those obtained by Garde-Cerdán and

Table 2. Major volatile compounds (GC-FID peak area·10^6) and their relative peak areas (RPA) identified in papaya wine at day 21 fermented by a mixed culture of *Saccharomyces cerevisiae* and *Williopsis saturnus* in the presence of the added flavour precursors

Compound	Method	RI	Control		φ(fusel oil)=0.05 %		$\frac{m(\text{amino acids})}{V\,(\text{mixture})}$=0.05 %		Odour*
			Peak area	RPA/%	Peak area	RPA/%	Peak area	RPA/%	
Acids									
acetic acid	TI	1458	(9.09±0.71)^a	0.42	(9.79±0.57)^a	0.52	(9.37±0.58)^a	0.46	acidic, pungent, vinegar-like
propanoic acid	TI	1544	(0.65±0.07)^a	0.03	(0.47±0.01)^b	0.03	(0.71±0.05)^a	0.03	acidic, cheesy, pungent
butyric acid	TI	1633	(1.59±0.10)^a	0.07	(1.72±0.14)^a	0.09	(1.52±0.01)^a	0.07	acidic, buttery, cheesy
hexanoic acid	TI	1849	(1.54±0.05)^a	0.07	(1.50±0.06)^a	0.08	(1.34±0.08)^b	0.07	acidic, cheesy, fruity
octanoic acid	RF, TI	2064	(17.70±0.15)^a	0.82	(10.70±0.24)^b	0.57	(17.10±1.54)^a	0.84	acidic, cheesy, fatty, sweaty
9-decenoic acid	TI	2341	(0.81±0.05)^a	0.04	(1.44±0.04)^b	0.08	(1.65±0.12)^c	0.08	creamy, fatty, milky
decanoic acid	TI	2278	(30.90±0.67)^a	1.43	(28.80±1.31)^a	1.54	(38.50±1.18)^b	1.89	buttery, condensed, milky
dodecanoic acid	TI	2490	(6.91±0.70)^a	0.32	(5.21±0.35)^b	0.28	(8.79±0.03)^c	0.43	fatty, soapy, waxy
tetradecanoic acid	TI	2702	(0.71±0.00)^a	0.03	(0.54±0.04)^b	0.03	(0.77±0.07)^a	0.04	creamy, oily, waxy
hexadecanoic acid	TI	2912	(0.86±0.00)^a	0.04	(0.73±0.06)^a	0.04	(0.82±0.07)^a	0.04	creamy, fatty, waxy
Subtotal			**70.76**	**3.27**	**60.90**	**3.26**	**80.57**	**3.96**	
Alcohols									
ethanol	RF, TI	951	(1520±45.40)^a	70.22	(1290±94.20)^b	68.99	(1340±37.2)^b	65.91	alcoholic, solventy
1-propanol	TI	1040	(0.96±0.03)^a	0.04	(1.02±0.04)^a	0.05	(0.60±0.06)^b	0.03	alcoholic, fermented, solventy
isobutyl alcohol	RF, TI	1092	(5.40±0.58)^a	0.25	(8.49±0.45)^b	0.45	(4.92±0.29)^a	0.24	breathtaking, fermented, whiskey
active amyl alcohol	RF, TI	1213	(4.37±0.11)^a	0.20	(16.50±0.31)^b	0.88	(4.60±0.48)^a	0.23	alcoholic, fermented, fusel
isoamyl alcohol	RF, TI	1215	(12.20±0.07)^a	0.56	(44.60±2.05)^b	2.39	(12.20±0.25)^a	0.60	alcoholic, fermented, whiskey
2-phenylethanol	RF, TI	1929	(26.10±0.65)^a	1.21	(32.50±1.15)^b	1.74	(32.60±0.46)^b	1.60	floral, honey, rosy
Subtotal			**1569.03**	**72.48**	**1393.11**	**74.51**	**1394.92**	**68.61**	
Aldehydes									
acetaldehyde	TI	732	(4.30±0.08)^a	0.20	(5.10±0.20)^b	0.27	(4.14±0.29)^a	0.20	aldehydic, ethereal, fruity
benzaldehyde	TI	1540	(0.65±0.04)^a	0.03	(0.53±0.04)^b	0.03	(0.82±0.05)^c	0.04	bitter almond, cherry, sweet
o-tolualdehyde	TI	1669	(2.46±0.03)^a	0.11	(3.20±0.02)^b	0.17	(2.21±0.12)^c	0.11	bitter almond, cherry pit, sweet
Subtotal			**7.41**	**0.34**	**8.83**	**0.47**	**7.17**	**0.35**	
Esters									
methyl octanoate	TI	1385	(0.56±0.04)^a	0.03	(0.54±0.06)^a	0.03	(0.64±0.03)^a	0.03	citrus, green, fruity

Table 2. – continued

Compound	Method	RI	Control		φ(fusel oil)=0.05 %		$\frac{m(\text{amino acids})}{V\,(\text{mixture})}$=0.05 %		Odour*
			Peak area	RPA/%	Peak area	RPA/%	Peak area	RPA/%	
methyl decanoate	TI	1595	$(4.04\pm0.13)^{a}$	0.19	$(3.67\pm0.18)^{a}$	0.20	$(3.98\pm0.20)^{a}$	0.20	fatty, cognac, oily
methyl dodecanoate	TI	1804	$(1.27\pm0.01)^{a}$	0.06	$(0.86\pm0.06)^{b}$	0.05	$(1.33\pm0.11)^{a}$	0.07	creamy, coconut, waxy
ethyl butyrate	TI	1039	$(1.56\pm0.04)^{a}$	0.07	$(2.01\pm0.08)^{b}$	0.11	$(1.78\pm0.02)^{c}$	0.09	fruity, ripe, sweet
ethyl hexanoate	TI	1223	$(2.88\pm0.10)^{a}$	0.13	$(3.55\pm0.28)^{b}$	0.19	$(3.51\pm0.24)^{b}$	0.17	fruity, pineapple-like, winey
ethyl octanoate	RF, TI	1432	$(35.20\pm2.76)^{a}$	1.63	$(36.50\pm0.15)^{a}$	1.95	$(42.30\pm1.79)^{b}$	2.08	fruity, cognac, yeasty
ethyl nonanoate	TI	1534	$(0.09\pm0.00)^{a}$	0.00	$(0.58\pm0.05)^{b}$	0.03	$(0.10\pm0.00)^{a}$	0.00	cognac, fatty, oily
ethyl 9-decenoate	TI	1694	$(10.40\pm0.24)^{a}$	0.48	$(21.30\pm0.43)^{b}$	1.14	$(17.30\pm0.05)^{c}$	0.85	fatty, fruity
ethyl decanoate	RF, TI	1643	$(211\pm3.71)^{a}$	9.75	$(221\pm10.30)^{a}$	11.82	$(252\pm5.58)^{b}$	12.39	fatty, fruity, winey
ethyl dodecanoate	RF, TI	1848	$(50.70\pm0.29)^{a}$	2.34	$(33.10\pm2.04)^{b}$	1.77	$(57.30\pm5.15)^{c}$	2.82	fruity, oily, waxy
ethyl tetradecanoate	TI	2053	$(1.58\pm0.07)^{a}$	0.07	$(0.88\pm0.06)^{b}$	0.05	$(1.80\pm0.17)^{a}$	0.09	creamy, oily, waxy
ethyl 9-hexadecenoate	TI	2288	$(3.25\pm0.24)^{a}$	0.15	$(3.85\pm0.20)^{b}$	0.21	$(4.78\pm0.01)^{c}$	0.24	creamy, waxy
ethyl hexadecanoate	TI	2259	$(2.38\pm0.23)^{a}$	0.11	$(1.72\pm0.13)^{b}$	0.09	$(2.56\pm0.09)^{a}$	0.13	creamy, fruity, milky
benzyl isothiocyanate	TI	2130	$(0.59\pm0.04)^{a}$	0.03	$(0.46\pm0.01)^{b}$	0.02	$(0.77\pm0.03)^{c}$	0.04	horseradish-like, hot, pungent
isobutyl octanoate	TI	1551	$(0.45\pm0.03)^{a}$	0.02	$(0.65\pm0.03)^{b}$	0.03	$(0.54\pm0.01)^{c}$	0.03	fatty, fruity, winey
isoamyl octanoate	TI	1660	$(1.58\pm0.01)^{a}$	0.07	$(4.03\pm0.05)^{b}$	0.22	$(1.93\pm0.13)^{c}$	0.09	cognac, fatty, oily
isobutyl decanoate	TI	1756	$(0.90\pm0.05)^{a}$	0.04	$(1.61\pm0.06)^{b}$	0.09	$(1.04\pm0.03)^{c}$	0.05	brandy, cognac, oily
isoamyl decanoate	TI	1866	$(1.89\pm0.23)^{a}$	0.09	$(6.80\pm0.12)^{b}$	0.36	$(2.74\pm0.09)^{c}$	0.13	cognac, green, waxy
isoamyl dodecanoate	TI	2071	$(0.36\pm0.02)^{a}$	0.02	$(0.74\pm0.06)^{b}$	0.04	$(0.44\pm0.02)^{a}$	0.02	alcoholic, fatty, yeasty
methyl acetate	TI	845	$(0.47\pm0.05)^{a}$	0.02	$(0.31\pm0.03)^{b}$	0.02	$(0.61\pm0.02)^{c}$	0.03	ethereal, estery, fruity
ethyl acetate	RF, TI	907	$(111\pm8.55)^{a}$	5.13	$(27.80\pm2.93)^{b}$	1.49	$(86.70\pm7.48)^{c}$	4.26	ethereal, fruity, solventy
propyl acetate	TI	992	$(0.56\pm0.03)^{a}$	0.03	$(0.17\pm0.02)^{b}$	0.01	$(0.40\pm0.06)^{c}$	0.02	ethereal, fruity, pear-like
isobutyl acetate	RF, TI	1019	$(0.41\pm0.01)^{a}$	0.02	$(0.43\pm0.00)^{b}$	0.02	$(0.39\pm0.01)^{a}$	0.02	floral, fruity, mixed fruit-like
active amyl acetate	RF, TI	1108	$(0.55\pm0.05)^{a}$	0.03	$(0.84\pm0.07)^{b}$	0.04	$(0.43\pm0.03)^{a}$	0.02	banana-like, fruity, ripe
isoamyl acetate	RF, TI	1110	$(19.80\pm1.55)^{a}$	0.91	$(20.30\pm1.43)^{a}$	1.09	$(15.60\pm0.16)^{b}$	0.77	banana-like, fruity, sweet
2-phenylethyl acetate	RF, TI	1833	$(50.80\pm3.95)^{a}$	2.35	$(9.57\pm0.39)^{b}$	0.51	$(46.70\pm3.82)^{a}$	2.30	floral, rosy, honey
ethyl phenyl acetate	TI	1799	$(0.33\pm0.03)^{a}$	0.02	$(0.68\pm0.06)^{b}$	0.04	$(0.32\pm0.02)^{a}$	0.02	cocoa-like, fruity, honey, rosy
citronellyl acetate	TI	1664	$(0.81\pm0.04)^{a}$	0.04	$(0.38\pm0.01)^{b}$	0.02	$(0.60\pm0.06)^{c}$	0.03	floral, fruity, rose
Subtotal			**515.41**	**23.81**	**404.33**	**21.63**	**548.59**	**26.98**	
Ketones									
3-hydroxy-2-butanone	TI	1302	$(0.78\pm0.07)^{a}$	0.04	$(0.94\pm0.04)^{b}$	0.05	$(0.63\pm0.04)^{c}$	0.03	buttery, creamy, sweet
β-damascenone	TI	1835	$(0.31\pm0.02)^{a}$	0.01	$(0.41\pm0.02)^{b}$	0.02	$(0.33\pm0.01)^{a}$	0.02	fruity, floral, woody
Subtotal			**1.09**	**0.05**	**1.35**	**0.07**	**0.96**	**0.05**	
Phenol									
2,4-di-*tert*-butylphenol	TI	2318	$(0.77\pm0.03)^{a}$	0.04	$(0.69\pm0.03)^{b}$	0.04	$(0.66\pm0.03)^{b}$	0.03	herbal, phenolic
Monoterpene									
β-citronellol	TI	1767	$(0.29\pm0.01)^{a}$	0.01	$(0.50\pm0.03)^{b}$	0.03	$(0.28\pm0.00)^{a}$	0.01	citronella, oily, rose
Total			**2164.76**		**1869.71**		**2033.15**		

[a,b,c]Statistical analysis at 95 % confidence level with the same letters indicating no significant difference

TI=tentative identification by mass spectrum, RF=identification by mass spectrum and retention time identical with a reference compound

RI=retention index experimentally determined on the DB-FFAP column, relative to C_5–C_{40} hydrocarbons

RPA=percentage of the GC-FID peak area of corresponding volatiles in the total peak area of all the identified volatiles

*odour descriptions obtained from Luebke (*17*)

Ancín-Azpilicueta (*12*) where amino acids were added during spontaneous must fermentation. Fatty acids are produced by yeasts or through acid catabolism, which contributes to fresh flavour but imparts an unpleasant flavour at concentrations above 20 mg/L (*25*).

Among the volatile compounds, alcohols (ethanol and higher alcohols) formed the largest group of volatiles accounting for more than 68.61 % RPA in all the papaya wines (Table 2). Consistent with the findings by Lee *et al.* (*22*), most of the alcohols were continuously produced in all fermentations, except for active amyl alcohol and isoamyl alcohol in the fermentations with added fusel oil, which remained essentially unchanged. This may be due to the relative rate of utilization and production of higher alcohols by the yeasts. Those with added fusel oil had the highest concentrations of most of the higher alcohols (except for 2-phenylethanol): isoamyl alcohol (246.61 mg/L), active amyl alcohol (40.96 mg/L) and isobutyl alcohol (12.97 mg/L) (Table 3), which was in line with the trends observed in a *W. saturnus* monoculture with fusel oil (*11*). This was mainly attributed to the addition of fusel oil, in which case higher alcohols (isoamyl, active amyl and isobutyl alcohols) were the major volatile compounds (*26*) and contributed to 3.72 % RPA in papaya wine on day 21 (Table 2). Higher alcohols could also be formed from sugars and/or through the Ehrlich pathway by yeasts during fermentation (*16*). The addition of amino acids resulted in the highest amount of 2-phenylethanol of 17.46 mg/L, followed by the samples with added fusel oil and the control of 14.49 and 10.12 mg/L, respectively (Table 3). The enhanced production of 2-phenylethanol in the samples with added amino acids was in agreement with other studies, where L-phenylalanine was responsible for 2-phenylethanol formation *via* the Ehrlich pathway (*8*,*14*), and a direct relationship is generally observed between the final amount and the initial amino acid fraction in spontaneous must fermentation (*12*).

Esters (21.63–26.98 % RPA), including acetate esters, ethyl esters, methyl esters and other medium- to long-chain esters (Table 2), were the next major constituents in papaya wine after ethanol and fusel alcohols. The kinetic changes of these esters were similar in all the fermentations and corresponded to those found in the study of Lee *et al.* (*22*), where the esters increased initially and either remained relatively stable or declined, with the exception of 2-phenylethyl acetate when fusel oil was added, which decreased throughout fermentation (Fig. 2).

The impact of fusel oil and amino acids on ester production varied with esters. The addition of fusel oil increased the production of isoamyl octanoate, isoamyl decanoate and isobutyl decanoate, while reduced the formation of ethyl acetate, 2-phenylethyl acetate and citronellyl acetate (Tables 2 and 3). The reduced production of these esters might be attributed to the precursors (acetyl-CoA/acetic acid and alcohols) (*27*) being diverted to the biosynthesis of other acetate esters. Alternatively, the enhanced production of isoamyl acetate with the addition of fusel oil was also reported by Yilmaztekin *et al.* (*26*) in a *W. saturnus* monoculture. This phenomenon could be associated with the addition of fusel oil, which provided a supplementary source of higher alcohols (*26*).

The addition of amino acids produced most of the esters comparable to the control except for some ethyl esters, isoamyl acetate, ethyl acetate and citronellyl acetate (Tables 2 and 3). Similarly to fusel oil addition, samples with added amino acids significantly reduced the isoamyl acetate, ethyl acetate and citronellyl acetate production (Tables 2 and 3), which may be attributed to

Table 3. Concentrations of major volatile compounds (in mg/L) in papaya wine (day 21) fermented by a mixed culture of *Saccharomyces cerevisiae* and *Williopsis saturnus* in the presence of the added flavour precursors

Compound	Control		φ(fusel oil)=0.05 %		$\frac{m(\text{amino acids})}{V(\text{mixture})}$=0.05 %		γ(odour threshold)*
	Mean	OAV	Mean	OAV	Mean	OAV	mg/L
isoamyl alcohol	$(76.03\pm3.67)^{a}$	2.53	$(246.61\pm11.06)^{b}$	8.22	$(85.73\pm1.16)^{a}$	2.86	30.00
active amyl alcohol	$(7.42\pm0.32)^{a}$	0.11	$(40.96\pm3.57)^{b}$	0.63	$(11.88\pm0.76)^{c}$	0.18	65.00
isobutyl alcohol	$(1.71\pm0.03)^{a}$	0.04	$(12.97\pm1.43)^{b}$	0.32	$(1.66\pm0.02)^{a}$	0.04	40.00
2-phenylethanol	$(10.12\pm0.05)^{a}$	1.01	$(14.49\pm1.35)^{b}$	1.45	$(17.46\pm0.73)^{c}$	1.75	10.00
octanoic acid	$(2.24\pm0.12)^{a}$	0.25	$(1.53\pm0.03)^{b}$	0.17	$(2.21\pm0.17)^{a}$	0.25	8.80
ethyl octanoate	$(0.48\pm0.04)^{a}$	24.00	$(0.49\pm0.01)^{a}$	24.50	$(0.54\pm0.01)^{b}$	27.00	0.02
ethyl decanoate	$(5.59\pm0.21)^{a}$	27.95	$(5.71\pm0.19)^{a}$	28.55	$(6.08\pm0.03)^{b}$	30.40	0.20
ethyl dodecanoate	$(2.55\pm0.25)^{a}$	2.13	$(2.23\pm0.06)^{b}$	1.86	$(2.90\pm0.28)^{c}$	2.42	1.20^{+}
ethyl acetate	$(50.32\pm0.94)^{a}$	6.71	$(6.65\pm0.62)^{b}$	0.89	$(45.06\pm0.99)^{c}$	6.01	7.50
isoamyl acetate	$(4.52\pm0.15)^{a}$	150.67	$(4.65\pm0.21)^{a}$	155.00	$(2.22\pm0.01)^{b}$	74.00	0.03
active amyl acetate	$(0.02\pm0.00)^{a}$	0.13	$(0.05\pm0.00)^{b}$	0.31	$(0.02\pm0.00)^{a}$	0.13	0.16
isobutyl acetate	$(0.0020\pm0.00)^{a}$	0.00	$(0.0022\pm0.00)^{b}$	0.00	$(0.0019\pm0.00)^{a}$	0.00	1.60
2-phenylethyl acetate	$(2.01\pm0.07)^{a}$	8.04	$(0.42\pm0.01)^{b}$	1.68	$(1.69\pm0.05)^{c}$	6.76	0.25

^{a,b,c}Statistical analysis at 95 % confidence level with the same letters indicating no significant difference

OAV=odour activity values calculated by dividing the concentration of volatile compounds by its odour threshold value

*from Bartowsky and Pretorius (*23*)

$^{+}$from Ferreira *et al.* (*24*)

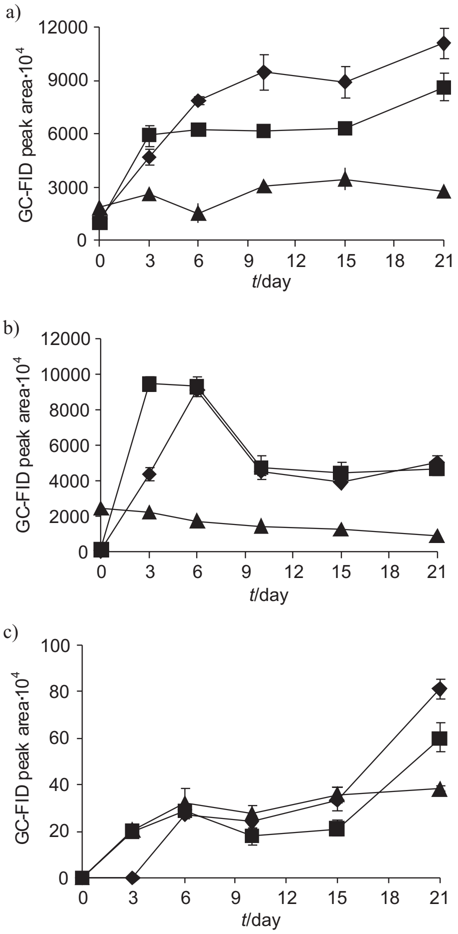

Fig. 2. Changes of acetate esters in papaya wine during the fermentation of the mixed culture of *Saccharomyces cerevisiae* R2/*Williopsis saturnus* NCYC2251 (1:1000) with flavour precursors added; (◆) control, (▲) φ(fusel oil)=0.05 %, (■) m(amino acids)/V(mixture)=0.05 %: a) ethyl acetate, b) 2-phenylethyl acetate, c) citronellyl acetate

fewer precursors (acetyl-CoA/acetic acid) available for the biosynthesis of these esters. The reduced formation of isoamyl acetate in our study contradicted with the findings of Hernández-Orte *et al.* (*13*), where the addition of amino acids in a *S. cerevisiae* monoculture produced a comparable amount to the control (without the amino acids added). Moreover, the addition of amino acids produced the highest concentrations of ethyl esters such as ethyl octanoate (0.54 mg/L), ethyl decanoate (6.08 mg/L) and ethyl dodecanoate (2.90 mg/L) (Table 3). The enhanced production of ethyl esters may be associated with the increased amount of total fatty acids produced with amino acid addition (Table 2), as fatty acids (or their intermediates such as fatty acyl CoAs) are precursors for ethyl ester production (*28*). On the contrary, Garde-Cerdán and Ancín-Azpilicueta (*12*) reported no correlation of ethyl ester formation with the quantity of amino acids added. The results of our study differ from those of Trinh *et al.* (*14*), who reported the enhancement of acetate ester production by the addition of corresponding single amino acids to a mixed culture used for longan juice fermentation. This could be attributed to the higher fractions of single amino acids added and the domination of *Williopsis saturnus* (with higher alcohol acetyltransferase activity) over *S. cerevisiae* (*14*). Similarly, Garde-Cerdán and Ancín-Azpilicueta (*12*) found a direct correlation between the formation of isoamyl acetate and the quantity of amino acids added; nevertheless, the results are different for 2-phenylethyl acetate (*29*). Additionally, mixed culture fermentation was employed in the present study, where *S. cerevisiae*, with low alcohol acetyltransferase activity (*30*), overtook the *W. saturnus* populations and dominated the fermentation (Fig. 1), which further accounted for the insignificant improvement of acetate ester production with the addition of amino acids.

Among the different esters, citronellyl acetate was detected for the first time in papaya wine, to the best of our knowledge. This compound had previously not been discovered in other studies (*5,11,22*) and was also not an indigenous compound in the papaya juice. This could probably be due to the yeast metabolism of β-citronellol and acetyl-CoA/acetic acid during fermentation. In spite of its low concentration, citronellyl acetate can contribute to the overall fruity and floral notes in the papaya wine near its flavour threshold of 0.25 mg/L (*31*).

The miscellaneous volatile compounds including ketones, aldehydes and volatile phenols were metabolized to trace levels during fermentation except for acetaldehyde, 3-hydroxy-2-butanone (acetoin) and β-citronellol, which were formed (Table 2). The addition of fusel oil produced the highest amount of these volatile compounds (Table 2). Acetaldehyde is quantitatively the most important carbonyl compound produced during alcoholic fermentation that further enhances the fruity characteristic of wine with a sensory threshold of around 0.5 mg/L (*32*). The presence of acetaldehyde is responsible for the accumulation of acetoin, which would be subsequently reduced to 2,3-butanediol at the later stage of fermentation and develop off-flavour in alcoholic beverages (*33*). Hence, the addition of amino acids with the lowest fraction of acetoin (RPA of 0.03 %) would be more preferable. β-Citronellol is another important contributor to wine aroma that imparts citronella and rose notes at concentrations near its low flavour threshold of 0.08 mg/L (*23*). It can be released from glycosides through enzymatic hydrolysis or transformed from geraniol by *S. cerevisiae* to enhance the flavour of wine (*34*).

PCA was performed on the volatile compounds from Tables 2 and 3 in order to illustrate graphically the correlations between the volatile compounds and the addition of different precursors, and also to reveal the diversity among the papaya wines. The PCA results of the selected volatile compounds in Table 3 are presented, as these volatile compounds contributed to the model in a meaningful way (≥ 40 % explained variance) (Fig. 3) and it is a representation of the PCA results from Table 2. The PCA biplot indicates distinctive volatile compositions among the papaya wines and differentiation between the additions of different precursors by the first two principal components (Fig. 3). The addition of fusel oil (with positive scores) presented a high percentage of volatile compounds such as isoamyl, active amyl, isobutyl acetates and alcohols as compared to the control. Conversely, the addition of amino acids (positioned in the upper left quadrant) resulted in papaya wines with the largest fractions of volatile ethyl esters, ethyl octano-

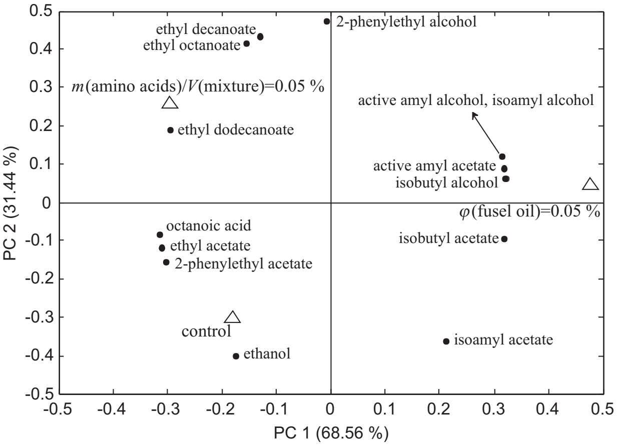

Fig. 3. Principal component analysis biplot for the selected volatile compounds in papaya wine during the fermentation of the mixed culture of *Saccharomyces cerevisiae* R2/*Williopsis saturnus* NCYC2251 (1:1000) with flavour precursors added

ate, ethyl decanoate and ethyl dodecanoate. The papaya wine without the addition of precursors (with negative scores) was distinguished by the following components: ethyl acetate, 2-phenylethyl acetate, octanoic acid and ethanol.

Sensory evaluation of wine samples

All papaya wines were assessed by expert panelists, using selected sensory descriptors for describing the sensorial characteristics and capable of distinguishing one from another. The aroma profiles of all the papaya wines are shown in Fig. 4. As shown in Fig. 4, cocoa, fruity, fusel, sweet and yeasty notes were similar in all the papaya wines regardless of the addition of fusel oil or amino acids. Those fermented with the added amino acids possessed noticeably more acidic and buttery but less alcoholic notes than the control (Fig. 4), which corresponded to the high total volatile fatty acids and lower ethanol content (Tables 2 and 3). Despite the lower ethanol level with the addition of fusel oil, the papaya wine had alcohol note and slightly more fusel notes comparable to the control (Fig. 4). This may be due to the synergistic effects of the higher levels of fusel alcohols present in the wines with added fusel oil (Table 3). Overall, there were no significant differences in the aroma profiles in all the papaya wines regardless of the different flavour precursors added, which differs from those found for the volatile compounds determined by GC-MS/FID (Tables 2 and 3) and PCA result (Fig. 3). This may be attributed to the complexity of the papaya wine matrix and the presence of different volatile compounds having a synergistic effect, hence impacting on the individual flavours well below their individual threshold concentrations (*27*).

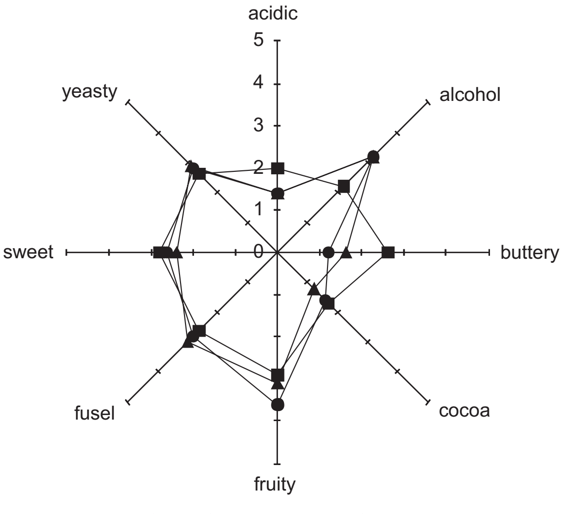

Fig. 4. Aroma profiles of papaya wines fermented by the mixed culture of *Saccharomyces cerevisiae* R2/*Williopsis saturnus* NCYC 2251 (1:1000) with flavour precursors added; (●) control, (▲) φ(fusel oil)=0.05 %, (■) *m*(amino acids)/*V*(mixture)=0.05 %

Conclusion

In this study, the impact of the addition of fusel oil or amino acids on the fermentation by a mixed culture of *S. cerevisiae* and *W. saturnus* on the formation of volatile compounds and aroma profiles was assessed during papaya juice fermentation. The papaya wine obtained with the addition of amino acids contained more volatile acids and ethyl esters. Moreover, with the addition of fusel oil, papaya wine with higher fraction of fusel alcohols and other esters such as isoamyl octanoate, isoamyl decanoate and isobutyl decanoate was obtained. However, no significant differences were observed in the overall flavour profile of all the wines. Further research is required to ascertain the effect of these additives at higher fraction or different ratio on the formation of volatile compounds and flavour complexity.

Acknowledgement

The authors acknowledge and express sincere gratitude to the flavourists from Firmenich Asia Pte Ltd (Singapore) for their participation and professional comments during sensory evaluation. This work was supported, in part, by an ARF grant from Ministry of Education of Singapore (WBS No. R-143-000-507-112).

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