Impact of Amino Acid Addition on Aroma Compounds in Papaya Wine Fermented with *Williopsis mrakii*

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The impact of amino acid addition on aroma compound formation in papaya wine fermented with yeast *Williopsis saturnus* var. *mrakii* NCYC2251 was studied. Time-course papaya juice fermentations were carried out using *W. saturnus* var. *mrakii* NCYC2251, with and without the addition of selected amino acids (L-leucine, L-isoleucine, L-valine and L-phenylalanine). Yeast growth and changes in sugars, °Brix, organic acids and pH were similar, regardless of amino acid addition. L-Leucine addition increased the production of isoamyl alcohol and some esters such as isoamyl acetate, isoamyl butyrate and isoamyl propionate, while L-isoleucine addition increased the production of active amyl alcohol and active amyl acetate. L-valine addition slightly increased the production of isobutyl alcohol and isobutyl acetate. L-phenylalanine addition increased the formation of 2-phenylethanol, 2-phenylethyl acetate and 2-phenylethyl butyrate, while decreasing the production of most other esters. This study suggests that papaya juice fermentation with *W. saturnus* var. *mrakii* NCYC2251 in conjunction with the addition of selected amino acid(s) can be an effective way to modulate the aroma of papaya wine.

INTRODUCTION

In winemaking, an adequate nitrogen level in the grape must is essential for a successful alcoholic fermentation, as assimilable nitrogen has been identified as a key nutrient that regulates yeast growth. The degree of nitrogen availability can affect yeast metabolism, such as volatile compound formation. Several studies have revealed the effects of ammonium addition on the formation of volatile compounds (Hernandez-Orte *et al*., 2005, 2006). Volatile compounds, including higher alcohols, short to medium-chain fatty acids, ethyl esters and acetate esters, are affected by the type and/or concentration of nitrogen (Bell & Henschke, 2005). If yeast suffers from nitrogen deficiency during wine fermentation, sluggish or stuck fermentation may occur (Bisson & Butzke, 2000; Sablayrolles, 2009). In contrast, when supplemented with excessive amounts of ammonium there could be a risk of producing wine with elevated levels of higher alcohols (Beltran *et al*., 2005), acetic acid (Bely *et al*., 2003), ethyl acetate (Sablayrolles, 2009) or even ethyl carbamate (Ough *et al*., 1988).

The papaya (or paw paw, *Carica papaya*) used in this study is one of the fruits with abundant supply in the tropical region, in contrast to the supply of grapes. However, papayas are relatively low in some amino acids, containing only 9 mg phenylalanine, 16 mg leucine, 8 mg isoleucine and 10 mg valine in every 100 g of edible portion (USDA, 2009), compared to grapes, with 19 mg phenylalanine, 22 mg leucine, 11 mg isoleucine and 22 mg valine per 100g of grape (*Vitis vinifera*) (USDA, 2009). Some amino acids, especially the branched-chain amino acids and aromatic amino acids, are important precursors to aroma compounds. Higher alcohols such as isobutyl alcohol, isoamyl alcohol and active amyl alcohol are derived from L-valine, L-leucine and L-isoleucine respectively (Dickinson *et al*., 1997, 1998, 2000), whereas 2-phenylethanol is formed from L-phenylalanine (Etschmann *et al*., 2002) by *Saccharomyces* yeasts and certain non-*Saccharomyces* yeasts (e.g. *Kluyveromyces marxianus*). These alcohols can be converted into esters, such as branched-chain or aromatic esters, by both *Saccharomyces* and non-*Saccharomyces* yeasts due to the action of alcohol acetyltransferases in the presence of acetyl Co-A. Acetate esters such as isoamyl acetate and 2-phenylethyl acetate are recognised as important flavour compounds in wine, imparting characteristic flavours (Rojas *et al*., 2001, 2003). Non-*Saccharomyces* yeasts excrete various enzymes that are responsible for giving the wine its unique characteristics (Pretorius *et al*., 1999). Glucanase activity has been described in the genus *Candida* (Strauss *et al*., 2001), whereas ß-glycosidase activity has been described

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in species of *Pichia, Hansenula* and *Hanseniaspora* (Charoenchai *et al*., 1997). Among the non-*Saccharomyces* yeasts, *Williopsis* yeasts are potent producers of esters (Inoue *et al*., 1997), and *Williopsis saturnus* in particular has the uppermost ability to convert higher alcohols into the corresponding acetate esters, e.g. isoamyl acetate at a concentration of 12 to 73 mg/L (Iwase *et al*., 1995).

Considering the common practice of ammonia addition, increasing recognition of the roles of non-*Saccharomyces* yeasts in grape and fruit wine fermentation and consumer demand for more unique and stylistic wine, it is of interest to understand the effect of amino acid addition on aroma compound generation by non-*Saccharomyces* yeasts. The aim of this work was to study the fermentation performance and formation of aroma compounds by *W. saturnus* var. *mrakii* NCYC2251 in papaya juice, with and without the addition of L-valine, L-phenylalanine, L-leucine and L-isoleucine. The selection of the four amino acids was based on reports that these amino acids have the most influence on aroma compound formation in wine fermentations (Dickinson *et al*., 1997, 1998, 2000; Hernandez-Orte *et al*., 2002).

MATERIALS AND METHODS

Yeast strain and materials

Freeze-dried *Williopsis saturnus* var. *mrakii* NCYC2251 was obtained from the National Collection of Yeast Cultures (Norwich, UK) and propagated following the procedure described by Lee *et al*. (2010). L-Leucine, L-isoleucine, L-valine, L-phenylalanine, fructose, glucose, acetic acid, citric acid, DL-malic acid and DL-tartaric acid were purchased from Sigma-Aldrich (Oakville, ON, Canada). The pure reference compounds used in the quantitative analysis of the volatile compounds were obtained from Firmenich Asia Ltd (Singapore) and Merck (Darmstadt, Germany). Food-grade DL-malic acid was purchased from Suntop (Singapore). Potato dextrose agar (PDA), bacteriological peptone and malt extract were purchased from Oxoid (Hampshire, England). Potassium metabisulphite was obtained from The Goodlife Homebrew Centre (Norfolk, England).

Fermentation conditions

The preparation and fermentation of the papaya juice were based on the procedure described by Lee *et al*. (2010). Papayas of the Sekaki cultivar were washed, juiced and centrifuged (32,140 x *g* for 15 min at 4°C). The initial Brix was 11.60% and the pH was 4.98. DL-malic acid (1 M) was added to reduce the pH value to 3.55, and the juice was sterilised overnight with 100 ppm of potassium metabisulphite. Laboratory-scale fermentations were carried out in duplicate with conical flasks containing 250 mL of sterile papaya juice at 20°C under static conditions. Each flask was inoculated with ~10^5 cfu/mL of *W. saturnus* var. *mrakii* NCYC 2251 and added with 0.05% (w/v) of L-leucine, L-isoleucine, L-valine or L-phenylalanine, except for the control. Samples were taken during fermentation (Day 0, 3, 6, 10, 14 and 21).

Analytical methods

Total soluble solids (°Brix), pH, optical density, sugars, organic acids and aroma compounds were analysed as described elsewhere (Lee *et al*., 2010), with some modifications. Organic acid separation was carried out on a Supelcogel C-610H column (300 x 7.8 mm, Supelco), using 0.1% sulphuric acid mobile phase at a flow rate of 0.4 mL/min with photodiode array (PDA) detection. The determination of sugar was done on a Pinnacel II amino column (Restek, 150 x 4.6 mm) using a mixture of acetonitrile and water (80:20) mobile phase at a flow rate of 1 mL/min, and assessed by evaporative light scattering detector (ELSD).

Aroma compounds in the papaya wines were determined by the optimised headspace (HS) solid-phase microextraction (SPME) method, coupled with gas chromatography (GC)-mass spectrometer (MS) and flame ionisation detector (FID) (Lee *et al*., 2010). The fibre used for the absorption of volatiles was a 85 µm fused silica fibre coated with Carboxen/PDMS (Supelco, Sigma-Aldrich, Barcelona, Spain). Papaya wine samples of 5 mL were extracted by HS-SPME for 50 min at 60°C and thermally desorbed into the injector port at 250°C for 3 min. Separation was performed with a DB-FFAP column (60 m x 0.25 mm I.D.) and with the oven temperature programmed to run from 50°C (hold time 5 min) to 230°C at a rate of 5°C/min (final hold for 30 min). Helium was the carrier gas at a linear velocity of 1.2 mL/min. The transfer line temperature was 280°C. Mass detector conditions were: electron impact (EI) mode at 70eV; source temperature: 230°C; mass scanning parameters: 3 min → 22 min: m/z 25–280 (5.36 scan/s); 22 min → 71 min: m/z 25–550 (2.78 scan/s) under full-scan acquisition mode. Identification of the eluted compounds was achieved by matching the mass spectrum against NIST 8.0 and Wiley 275 MS libraries, and confirmed by the Linear Retention Index (LRI) value. LRI values on the FFAP column were determined using a series of alkanes (C_5-C_{40}) run under identical conditions. Quantification of the selected volatiles was similar to that described in Lee *et al*. (2010), with additional volatiles included: active amyl alcohol [0.002–0.33 ppm (mg/L)]; isobutyl alcohol (0.02–3.21 ppm); active amyl acetate (0.002–0.07 ppm); isobutyl acetate (0.0001–0.022 ppm); ethyl decanoate (0.001–1 ppm); ethyl dodecanoate (0.001–1 ppm); octanoic acid (0.1–10 ppm) (R^2 values of standard curves were at least 0.98). All samples were analysed in triplicate.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Microsoft Office Excel, version 2003 (Lee *et al*., 2010).

RESULTS AND DISCUSSION

Yeast growth, total soluble solids and pH changes during papaya juice fermentation

All the fermentations showed similar characteristics in terms of growth kinetics and total soluble solids (°Brix) (Table 1). The pH did not vary significantly during fermentation, with values maintained at pH 3.57 to 3.68 (Table 1). Both the sugar consumption and the organic acid changes were not affected by the addition of amino acids. The sugar consumption displayed a gradual reduction during fermentation, with preferential utilisation of glucose over fructose (Table 1). This corresponds to the sugar consumption behaviour of *W. saturnus* reported in Lee *et al*. (2010).

TABLE 1
Fermentation parameters of papaya wine (day 21) fermented with *Williopsis mrakii* in the presence of the added amino acids

	Day 0	Control	0.05% (w/v) valine added	0.05% (w/v) phenylalanine added	0.05% (w/v) leucine added	0.05% (w/v) isoleucine added
pH	3.57 ± 0.01[a]	3.67 ± 0.01[b]	3.68 ± 0.01[b]	3.64 ± 0.01[b]	3.67 ± 0.00[b]	3.65 ± 0.01[b]
°Brix (%)	11.60 ± 0.00[a]	5.50 ± 0.08[bc]	4.95 ± 0.11[c]	5.36 ± 0.07[bc]	5.32 ± 0.12[bc]	5.98 ± 0.50[b]
Yeast cell count x 10^6 (cfu/mL)	0.30 ± 0.01[a]	157 ± 11.70[bd]	136 ± 6.19[c]	139 ± 3.54[c]	156 ± 2.65[b]	174 ± 9.02[d]
Sugars (g/100 mL)						
Fructose	4.32 ± 0.01[a]	2.16 ± 0.10[bc]	1.59 ± 0.04[c]	2.20 ± 0.05[b]	1.87 ± 0.01[bc]	2.25 ± 0.14[b]
Glucose	5.06 ± 0.01[a]	0.69 ± 0.03[bc]	0.55 ± 0.06[c]	0.77 ± 0.01[b]	0.64 ± 0.05[bc]	0.79 ± 0.01[b]
Organic acids (g/100 mL)						
Acetic acid	0.038 ± 0.001[a]	0.046 ± 0.001[b]	0.049 ± 0.001[bc]	0.047 ± 0.00[b]	0.051 ± 0.002[cd]	0.054 ± 0.001[d]
Citric acid	0.271 ± 0.001[a]	0.245 ± 0.00[b]	0.230 ± 0.00[c]	0.231 ± 0.001[c]	0.237 ± 0.002[d]	0.242 ± 0.003[b]
Malic acid	0.902 ± 0.02[a]	0.696 ± 0.01[b]	0.648 ± 0.01[c]	0.682 ± 0.01[d]	0.666 ± 0.02[e]	0.687 ± 0.00[bd]
Tartaric acid	0.018 ± 0.001[a]	0.008 ± 0.00[a]	0.006 ± 0.001[a]	0.007 ± 0.001[a]	0.007 ± 0.00[a]	0.008 ± 0.001[a]

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

The changes in the organic acids were similar in all fermentations, where citric acid remained fairly constant while malic and tartaric acids decreased slightly and acetic acid increased (Table 1). There were statistical differences in the concentrations of organic acids at day 21 among or between the different treatments and the day 0 sample, except for tartaric acid (Table 1). The viable yeast cell populations of all fermentations reached the maximum of approximately 1.36 x 10^8 - 1.74 x 10^8 cfu/mL at the end of fermentation (day 21), from the initial cell population of about 3.0 x 10^5 cfu/mL (Table 1).

Kinetic changes in aroma compounds during papaya juice fermentation

During papaya juice fermentation, a number of aroma compounds were produced, including fatty acids, alcohols, esters and aldehydes: some were stable, others were metabolised. Aroma compounds that were indigenous to the juice, such as benzyl isothiocyanate, β-damascenone and some fatty acids such as butyric and hexanoic acids, were utilised (data not shown).

The kinetics of the acetic and hexanoic acids were similar in all the fermentations (data not shown). Hexanoic acid, which was present at relatively high concentrations in the juice, was utilised, while acetic acid increased during fermentation. The addition of amino acids increased the formation of acetic acid in comparison to the control (Table 2). The addition of L-phenylalanine increased the utilisation of hexanoic acid, but reduced the formation of octanoic acid. The addition of leucine and isoleucine produced the highest amount of acetic acid, with relative peak areas (RPA) ranging from 0.49 to 0.56% that corresponded to the trend in the organic acid results (Table 1). Acetic acid is an undesirable volatile acid in alcoholic beverages and has the capability of imparting a vinegary off-flavour. Great variability in acetic acid production, from about 0.06 g/100 mL to more than 0.34 g/100 mL has been observed for non-*Saccharomyces* yeast (Viana *et al*., 2008). However, the acetic acid produced in this study was within the acceptable range of 0.02 to 0.07 g/100 mL for wine (Lambrechts & Pretorius, 2000). There were statistical differences in the concentrations of fatty acids between the different amino acids added and the control at day 21 (Table 2).

Ethanol, isobutyl alcohol (2-methyl-1-propanol), active amyl alcohol (2-methyl-1-butanol), isoamyl alcohol (3-methyl-1-butanol) and 2-phenylethanol were the major alcohols produced by yeast strain NCYC2251 during papaya juice fermentation (Fig. 1). The effect of the addition of amino acids on ethanol production varied. Amino acid addition significantly increased their respective higher alcohol production (Fig. 1). Studies have shown that, with the addition of different amino acids, *Saccharomyces* yeasts and certain non-*Saccharomyces* yeasts (*Kluyveromyces marxianus*) are capable of producing the respective higher alcohols through a decarboxylation process of the corresponding α-keto acids by Ehrlich's pathway, followed by a reduction to produce the final alcohols (Perez *et al*., 1992; Dickinson *et al*., 1997, 1998, 2000; Etschmann *et al*., 2002). The result of our study are in accordance with these studies, where the fermentation added with L-leucine, L-isoleucine and L-phenylalanine displayed increased production of isoamyl alcohol (19.98 mg/L), active amyl alcohol (1.77 mg/L) and 2-phenylethanol (17.16 mg/L) respectively (Table 3). Those added with either L-leucine, L-isoleucine or L-valine showed markedly increased production of isobutyl alcohol compared to the control (Fig. 1). Based on the concentrations, those added with L-valine produced a relatively high amount of isobutyl alcohol, with 9.17 mg/L (Table 3). However, slight variation was observed in comparison to the semi-quantified results, which was probably due to the matrix effects from the wine on the HS-SPME (Burman *et al*., 2005). The final amounts of alcohols at day 21 varied significantly among the different amino acids added and the control (Tables 2 and 3). The results of our study differ from those of Hernandez-Orte *et al*. (2006) and Garde-Cerdan and Ancin-Azpilicueta (2008), who found that there was no positive correlation between higher alcohol production and the amino acids added, with some, such as isoamyl alcohol, even decreasing. This may

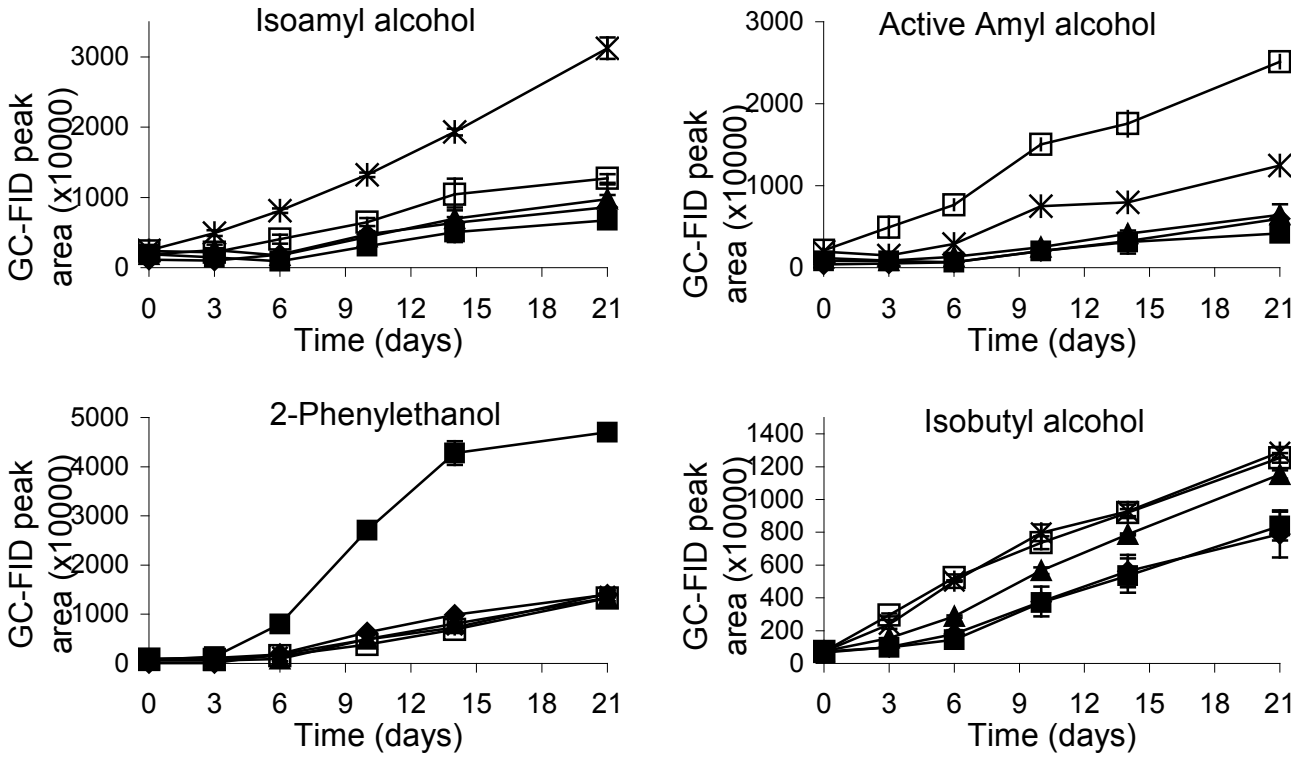

FIGURE 1
Changes in alcohols in papaya wine during fermentation by *Williopsis saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (*); 0.05% isoleucine (□).

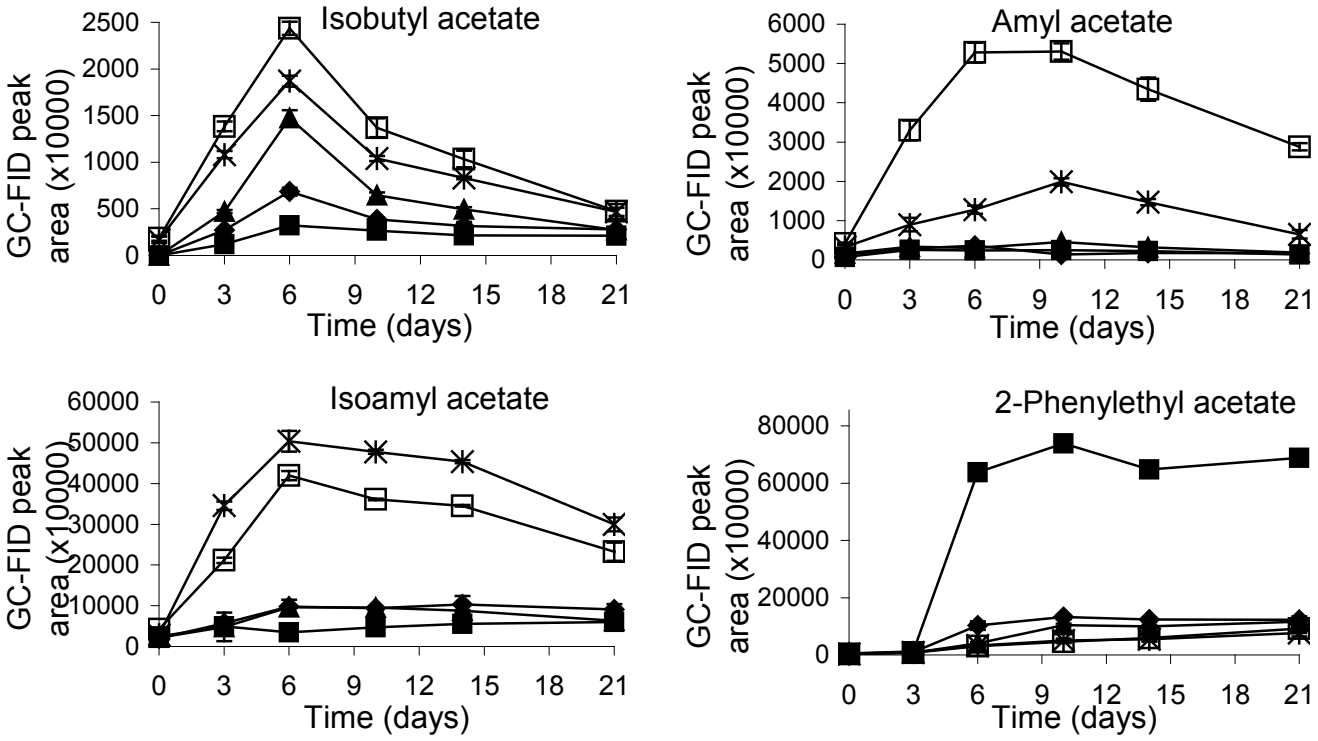

FIGURE 2
Changes in acetate esters in papaya wine during fermentation by *Williopsis saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (*); 0.05% isoleucine (□).

TABLE 2
Major aroma compounds (GC-FID peak area x 10^6) and their relative peak areas (RPA) identified in papaya wine at day 21 with different amino acids added

Compounds identified in this study	RI	Control		0.05% (w/v) valine added		0.05% (w/v) phenylalanine added		0.05% (w/v) leucine added		0.05% (w/v) isoleucine added		Odour descriptor
		Peak area	RPA (%)	Peak area	RPA (%)	Peak area	RPA (%)	Peak area	RPA (%)	Peak area	RPA (%)	
Acids												
Acetic acid[2]	1469	6.5 ± 0.4[a]	0.31	8.3 ± 0.9[b]	0.30	9.2 ± 0.1[b]	0.32	12.4 ± 0.3[c]	0.49	12.2 ± 1.1[c]	0.56	Vinegar[3]; sour[4]
Butyric acid[2]	1639	5.8 ± 0.9[a]	0.28	9.8 ± 0.2[b]	0.36	4.5 ± 0.1[a]	0.16	10.9 ± 0.7[b]	0.43	11.6 ± 0.8[b]	0.53	Rancid, cheesy, sweat[4]
Hexanoic acid[1, 2]	1890	2.5 ± 0.8[ab]	0.12	3.4 ± 0.4[a]	0.12	1.4 ± 0.01[b]	0.05	6.2 ± 0.4[c]	0.25	6.7 ± 0.6[c]	0.31	Sour, fatty, cheesy[3]
Octanoic acid[1,2]	2110	11.9 ± 0.21[a]	0.58	11.5 ± 0.2[a]	0.42	6.1 ± 0.6[b]	0.21	10.4 ± 1.2[a]	0.41	11.6 ± 1.6[a]	0.53	Sweat, cheesy[4]
Nonanoic acid	2219	1.9 ± 0.1[a]	0.09	0.9 ± 0.08[b]	0.03	0.8 ± 0.08[b]	0.03	0.9 ± 0.06[b]	0.04	0.8 ± 0.06[b]	0.04	Green, fat[4]
Decanoic acid[2]	2328	8.4 ± 0.2[a]	0.41	6.7 ± 0.3[b]	0.24	4.1 ± 0.7[c]	0.14	5.4 ± 0.2[d]	0.21	5.2 ± 0.1[d]	0.24	Rancid, fat[4]
Dodecanoic acid[2]	2544	15.6 ± 1.3[a]	0.75	15.6 ± 1.0[a]	0.57	7.2 ± 0.3[b]	0.25	10.7 ± 0.1[c]	0.43	10.6 ± 0.1[c]	0.49	Fatty, coconut, bay oil[3]
Tetradecanoic acid	2757	1.8 ± 0.1[a]	0.09	1.6 ± 0.01[b]	0.06	1.0 ± 0.05[c]	0.03	1.2 ± 0.07[d]	0.05	1.1 ± 0.01[c]	0.05	Waxy, fatty, soapy, creamy[3]; metal[4]
Subtotal		**54.4**	**2.63**	**57.8**	**2.10**	**34.3**	**1.19**	**58.1**	**2.31**	**59.8**	**2.74**	
Alcohols												
Ethanol[2]	948	1100 ± 111[a]	53.2	1830 ±30.8[b]	66.6	1430 ± 58.8[c]	49.7	1510 ± 34.8[c]	60.0	1220 ± 104[a]	56.0	Strong alcoholic[3]; sweet[4]
Isobutyl alcohol[2]	1099	7.2 ± 0.3[a]	0.35	11.5 ± 0.3[b]	0.42	8.4 ± 0.9[a]	0.29	12.9 ± 0.3[b]	0.51	12.5 ± 0.3[b]	0.57	Ether wine[3]
Active Amyl alcohol[2]	1220	5.3 ± 0.1[ab]	0.26	6.5 ± 0.6[a]	0.24	4.2 ± 0.4[b]	0.15	12.5 ± 0.1[c]	0.50	25.1 ± 0.8[d]	1.15	Fusel, winey, pungent[3]; wine, onion[4]
Isoamyl alcohol[2]	1221	8.0 ± 0.7[a]	0.39	8.4 ± 0.4[a]	0.31	6.7 ± 0.2[a]	0.23	31.2 ± 1.5[b]	1.24	12.7 ± 0.6[c]	0.58	Whiskey, malt, burnt[4]
2-Phenylethyl alcohol[2]	1944	14.3 ± 0.6[a]	0.69	13.3 ± 0.7[a]	0.48	47 ± 1.1[b]	1.63	14.2 ± 0.7[a]	0.56	13.3 ± 1.8[a]	0.61	Rose, lilac, honey[4]
Subtotal		**1134.8**	**54.8**	**1869.7**	**68.1**	**1496.3**	**52.0**	**1580.8**	**62.8**	**1283.6**	**58.9**	
Aldehydes												
Benzaldehyde[2]	1553	0.3 ± 0.0[a]	0.01	1.0 ± 0.3[b]	0.04	1.1 ± 0.06[b]	0.04	1.5 ± 0.08[c]	0.06	1.2 ± 0.2[b]	0.06	Almond, burnt sugar[4]
O-Tolualdehyde[2]	1684	2.6 ± 0.1[a]	0.13	0.6 ± 0.06[b]	0.02	1.5 ± 0.1[c]	0.05	2.3 ± 0.01[d]	0.09	2.5 ± 0.04[e]	0.11	Almond, sweet, cherry pit, coumarin[3]
Ethyl-benzaldehyde	1876	6.6 ± 0.4[a]	0.32	9.2 ± 0.3[b]	0.34	2.5 ± 0.2[c]	0.09	0.3 ± 0.02[d]	0.01	0.3 ± 0.01[d]	0.01	Sweet[4]
Subtotal		**9.5**	**0.46**	**10.8**	**0.39**	**5.1**	**0.18**	**4.1**	**0.16**	**4.0**	**0.18**	
Esters												
Methyl octanoate[2]	1390	0.7 ± 0.05[a]	0.03	0.7 ± 0.1[a]	0.03	0.3 ± 0.02[b]	0.01	1.1 ± 0.09[c]	0.04	1.2 ± 0.03[c]	0.06	Waxy, green, sweet, orange[3]
Methyl decanoate[2]	1633	1.3 ± 0.03[a]	0.06	1.3 ± 0.08[a]	0.05	0.9 ± 0.05[b]	0.03	0.9 ± 0.07[b]	0.04	0.7 ± 0.04[b]	0.03	Oily, wine, fruity, floral[3]
Methyl dodecanoate[2]	1815	4.1 ± 0.2[a]	0.20	4.1 ± 0.06[a]	0.15	2.0 ± 0.06[b]	0.07	1.7 ± 0.05[c]	0.07	1.7 ± 0.1[c]	0.08	Waxy, soapy, creamy coconut[3]
Ethyl butyrate[1],[2]	1034	5.3 ± 0.3[a]	0.26	7.4 ± 0.3[b]	0.27	5.1 ± 0.2[a]	0.18	9.2 ± 0.4[c]	0.37	5.8 ± 0.4[a]	0.27	Fruity, sweet[3]; apple[4]
Ethyl hexanoate[1],[2]	1251	2.0 ± 0.1[a]	0.10	1.8 ± 0.1[a]	0.07	1.0 ± 0.1[b]	0.03	4.3 ± 0.02[c]	0.17	4.4 ± 0.1[c]	0.20	Sweet, pineapple[3]; apple peel, fruity[4]
Ethyl octanoate[1],[2]	1436	17.2 ± 0.6[a]	0.83	23 ± 0.7[b]	0.84	7.4 ± 0.2[c]	0.26	23.9 ± 0.6[b]	0.95	23.3 ± 1.0[b]	1.07	Fruity, fat[4]
Ethyl decanoate[1],[2]	1649	30.1 ± 1.0[a]	1.45	25.3 ±1.7[b]	0.92	13.5 ± 0.8[c]	0.47	13 ± 0.3[c]	0.52	14.7 ± 0.6[c]	0.67	Sweet apple, waxy, fruity[3]; grape[4]
Ethyl dodecanoate[2]	1857	49 ± 3.3[a]	2.37	65.7±1.8[b]	2.39	27 ± 0.2[c]	0.94	20.5 ± 0.9[d]	0.81	17.4 ± 1.2[d]	0.80	Sweet, waxy, floral, soapy[3]

TABLE 2 (CONTINUED)

Compounds identified in this study	RI	Control		0.05% (w/v) valine added		0.05% (w/v) phenylalanine added		0.05% (w/v) leucine added		0.05% (w/v) isoleucine added		Odour descriptor
		Peak area	RPA (%)	Peak area	RPA (%)	Peak area	RPA (%)	Peak area	RPA (%)	Peak area	RPA (%)	
Ethyl tetradecanoate[2]	2201	2.5 ± 0.1[a]	0.12	2.8 ± 0.3[a]	0.10	1.3 ± 0.1[b]	0.05	0.2 ± 0.07[c]	0.01	0.2 ± 0.04[c]	0.01	Sweet, waxy[3]
Ethyl 9-hexadecenoate[2]	2337	3.4 ± 0.2[a]	0.16	2.6±0.07[b]	0.09	1.7 ± 0.1[c]	0.06	0.8 ± 0.03[d]	0.03	0.8 ± 0.03[d]	0.04	Creamy, waxy[3]
Ethyl hexadecanoate[2]	2306	1.4 ± 0.1[a]	0.07	1.1±0.09[b]	0.04	0.9 ± 0.06[c]	0.03	0.5 ± 0.01[d]	0.02	0.5 ± 0.02[d]	0.02	Waxy, fruity, creamy, milky[3]
Benzyl isothiocyanate[2]	2140	0.7 ± 0.02[ab]	0.03	0.8±0.03[a]	0.03	0.8 ± 0.09[a]	0.03	0.6 ± 0.03[bc]	0.02	0.5 ± 0.04[c]	0.02	Watercress, medicinal horseradish, oily[3]
Isoamyl butyrate[2]	1275	0.2 ± 0.0[a]	0.01	0.5± 0.03[b]	0.02	0.5 ± 0.03[b]	0.02	1.3 ± 0.04[c]	0.05	1.4 ± 0.04[c]	0.06	Fruity, green apple, sweet estery, waxy[3]
Isoamyl propionate[2]	1197	0.6 ± 0.08[a]	0.03	0.9± 0.02[b]	0.03	0.3 ± 0.0[c]	0.01	2.2 ± 0.1[d]	0.09	1.4 ± 0.04[e]	0.06	Sweet, fruity, banana, pineapple-like, tropical[3]
2-Phenylethyl butyrate	1941	1.0 ± 0.1[a]	0.05	0.5± 0.01[b]	0.02	1.3 ± 0.08[c]	0.05	0.2 ± 0.01[d]	0.01	0.2 ± 0.0[d]	0.01	Sweet, floral, musty, fruity[3]
Methyl acetate[2]	865	2.4 ± 0.1[a]	0.12	2.9± 0.3[b]	0.11	1.7 ± 0.2[c]	0.06	1.5 ± 0.01[c]	0.06	1.6 ± 0.1[c]	0.07	Fruity, estery, winey, etherial[3]
Ethyl acetate[2]	916	521 ± 22.4[a]	25.2	471±29.5[a]	17.2	518 ± 2.1[a]	18.0	386 ± 19.3[b]	15.4	391 ± 6.4[b]	17.9	Sweet, fruity, etherial[3]; pineapple[4]
Propyl acetate[2]	1001	5.0 ± 0.07[a]	0.24	6.2 ± 0.5[b]	0.23	5.0 ± 0.4[a]	0.17	8.2 ± 0.1[c]	0.33	4.2 ± 0.1[a]	0.19	Pear, fruity[3]
Butyl acetate	1061	1.2 ± 0.09[a]	0.06	1.7 ± 0.1[b]	0.06	1.6 ± 0.06[b]	0.06	2.0 ± 0.04[c]	0.08	1.3 ± 0.1[a]	0.06	Etherial, sharp, fruity banana, sweet[3]
Isobutyl acetate[1],[2]	1029	2.8 ± 0.06[a]	0.14	2.7 ± 0.2[a]	0.10	2.1 ± 0.1[a]	0.07	4.7 ± 0.4[b]	0.19	4.7 ± 0.8[b]	0.22	Sweet, fruity, etherial, banana, tropical[3]
Active amyl acetate	1097	1.7 ± 0.5[a]	0.08	1.9± 0.07[a]	0.07	1.3 ± 0.1[a]	0.05	6.8 ± 0.7[b]	0.27	28.8 ± 0.9[c]	1.32	Sweet, banana, fruity, ripe[3]
Isoamyl acetate[1],[2]	1099	91 ± 4.0[a]	4.40	63.3 ± 0.7[a]	2.31	60.4 ± 2.5[a]	2.10	300 ± 17[b]	11.9	232 ± 22.4[c]	10.6	Sweet, fruity with a ripe estery nuance[3]; banana[3], [4]
Benzyl acetate[2]	1753	2.6 ± 0.2[a]	0.13	2.8± 0.03[a]	0.10	2.0 ± 0.07[b]	0.07	2.5 ± 0.06[a]	0.10	2.7 ± 0.1[a]	0.12	Sweet, floral, fruity, jasmine[3]; fresh, boiled vegetable[4]
2-Phenylethyl acetate[1],[2]	1841	123 ± 11.6[a]	5.94	116 ± 13.4[ab]	4.22	688 ± 20.2[d]	24.0	79.8 ± 8[bc]	3.17	91.8 ± 5.0[ac]	4.21	Sweet, honey, floral rosy[3]
Subtotal		**870.2**	**42.1**	**807**	**29.4**	**1344.1**	**46.7**	**871.9**	**34.7**	**832.3**	**38.2**	
						Ketone						
B-Damascenone[2]	1845	0.6 ± 0.06[a]	0.03	0.7± 0.04[b]	0.03	0.5 ± 0.02[ac]		0.5 ± 0.02[ac]	0.02	0.4 ± 0.01[c]	0.02	Rose, apple, honey[4]
Total		**2069.5**		**2746**		**2880.3**		**2515.4**		**2180.1**		

RI: retention index

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

[(1), (2)] MS data and retention index in agreement with those in the literature [Duarte *et al*. (2010) and Lee *et al*. (2010) respectively].

[(3)]From Luebke (1980).

[(4)]From Acree & Arn (2004).

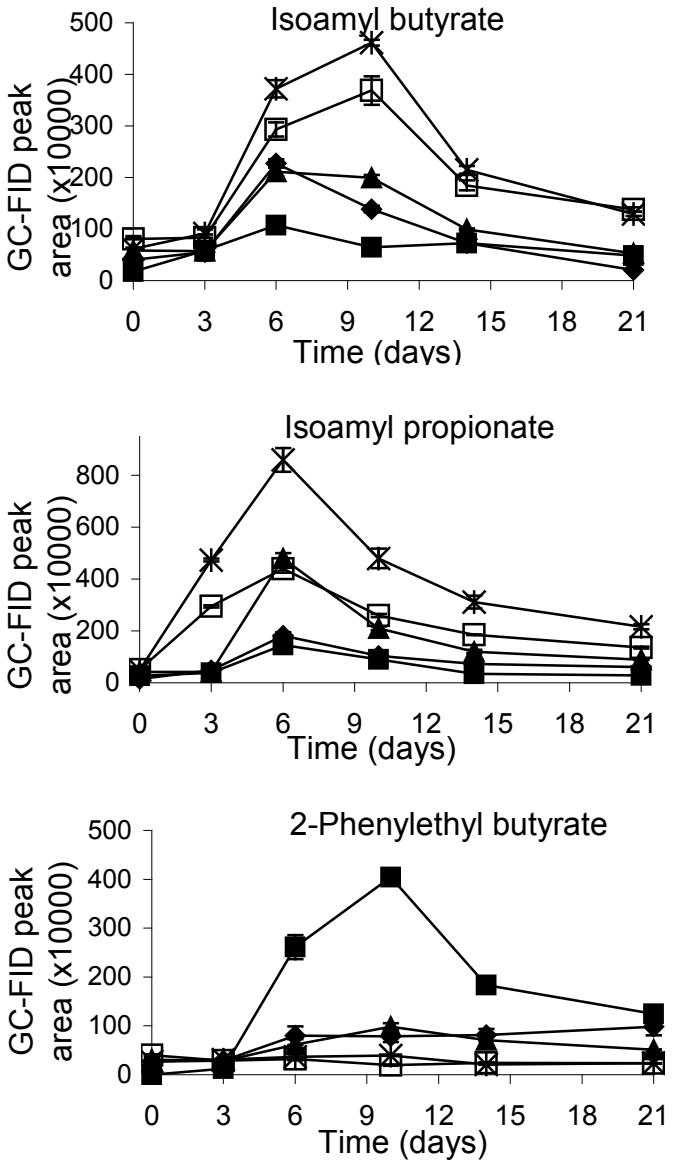

FIGURE 3

Changes in other esters in papaya wine during fermentation by *Williopsis saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (*); 0.05% isoleucine (□).

be due to the fact that a mixture of amino acids and different yeasts were used in these studies.

Esters were the most abundant aroma compounds produced by yeast strain NCYC2251 during papaya juice fermentation, ranging from 29.4 to 46.7% (RPA). They included acetate esters, ethyl esters, methyl esters, and other esters (Figs. 2 and 3, Table 2). Acetate esters tended to increase initially and then decline, with the exception of 2-phenylethyl acetate, which increased and remained stable (Fig. 2). Ethyl esters generally increased during fermentation. Among the miscellaneous esters, isoamyl propionate, isoamyl butyrate and 2-phenylethyl butyrate increased initially, followed by a decline (Fig. 3).

The impact of amino acid addition on ester production varied with esters. The addition of L-phenylalanine increased the production of 2-phenylethyl acetate and 2-phenylethyl butyrate, while it reduced the formation of isobutyl acetate, isoamyl acetate and isoamyl butyrate (Figs. 2 and 3). Fermentation with added L-phenylalanine displayed significant production of 2-phenylethyl acetate, at 14.30 mg/L (Table 3). The increased production of 2-phenylethyl acetate was likely due to the presence of high amounts of 2-phenylethanol and acetyl-CoA, which provided the necessary precursors for the formation of different acetate esters by the action of alcohol acetyltransferase (AAT) enzymes (Viana *et al*., 2008). The decreased production of other acetate esters upon the addition of L-phenylalanine (Fig. 2) could be due to competition for and diversion of acetyl-CoA to 2-phenylethyl ester formation, or competition for the uptake of substrates such as amino acids that may serve as aroma precursors.

L-leucine addition enhanced the formation of propyl acetate, isoamyl butyrate and isoamyl propionate and produced the highest amount of isoamyl acetate, at 8.29

TABLE 3
Concentrations of major aroma compounds (mg/L) in papaya wine fermented with *Williopsis mrakii* with different amino acids added at day 21

Compounds quantified	CAS no.[1]	Control	0.05% (w/v) valine added	0.05% (w/v) phenylalanine added	0.05% (w/v) leucine added	0.05% (w/v) isoleucine added	Odor threshold[2] (mg/L)
Ethanol	000064-17-5	17122 ± 546[ab]	18712 ± 63[a]	12673 ± 938[c]	16749 ± 440[ab]	16242 ± 867[b]	
Isoamyl alcohol	000123-51-3	13.53 ± 0.91[ab]	14.92 ± 1.46[ac]	11.36 ± 0.93[b]	19.98 ± 1.35[d]	17.66 ± 0.92[dc]	30.00
Active amyl alcohol	000137-32-6	0.69 ± 0.03[a]	0.98 ± 0.06[b]	0.45 ± 0.05[c]	1.10 ± 0.01[b]	1.77 ± 0.15[d]	65.00
Isobutyl alcohol	000078-83-1	2.26 ± 0.22[a]	9.17 ± 0.77[b]	1.77 ± 0.18[a]	6.00 ± 0.21[c]	6.51 ± 0.32[c]	40.00
2-Phenylethyl alcohol	000060-12-8	2.29 ± 0.13[a]	2.57 ± 0.37[a]	17.16 ± 2.48[b]	2.24 ± 0.10[a]	1.99 ± 0.26[a]	10.00
Octanoic acid	000124-07-2	0.37 ± 0.04[a]	0.28 ± 0.03[b]	0.03 ± 0.00[c]	0.28 ± 0.01[b]	0.38 ± 0.02[a]	8.80
Ethyl octanoate	000106-32-1	0.07 ± 0.01[a]	0.11 ± 0.01[b]	0.04 ± 0.00[c]	0.13 ± 0.01[bd]	0.11 ± 0.01[cd]	0.02
Ethyl decanoate	000110-38-3	0.29 ± 0.04[a]	0.28 ± 0.00[a]	0.20 ± 0.02[b]	0.23 ± 0.01[ab]	0.25 ± 0.02[ab]	0.20
Ethyl dodecanoate	000106-33-2	3.97 ± 0.40[a]	4.87 ± 0.15[b]	3.70 ± 0.10[a]	3.55 ± 0.19[a]	3.52 ± 0.30[a]	1.2[3]
Isoamyl acetate	000123-92-2	6.48 ± 0.09[a]	6.38 ± 0.04[a]	6.57 ± 0.18[a]	8.29 ± 0.04[b]	7.10 ± 0.10[c]	0.03
Active amyl acetate	000624-41-9	0.015 ± 0.00[a]	0.015 ± 0.00[a]	0.014 ± 0.00[a]	0.013 ± 0.00[a]	0.06 ± 0.00[b]	0.16
Isobutyl acetate	000110-19-0	0.008 ± 0.00[a]	0.009± 0.001[b]	0.007 ± 0.001[ac]	0.005 ± 0.00[c]	0.007 ± 0.001[a]	1.60
2-Phenylethyl acetate	000103-45-7	1.76 ± 0.16[a]	1.82 ± 0.08[a]	14.30 ± 1.64[b]	1.37 ± 0.11[a]	1.74 ± 0.10[a]	0.25

[a,b,c,d] Statistical analysis at 95% confidence level with same letters indicating no significant difference.
[1]CAS number obtained from Wiley MS library.
[2]From Bartowsky and Pretorius (2009).
[3]Ferreira *et al*. (2000). The matrix was an 11% ethanol aqueous solution containing 7 g/L of glycerol and 5 g/L of tartaric acid, with units adjusted to mg/L.

mg/L, while L-isoleucine addition produced the highest amount of active amyl acetate, at 0.06 mg/L (Figs. 2 and 3, Table 3). Similarly to L-phenylalanine addition, the increased production of isoamyl acetate and active amyl acetate was likely due to the increased amounts of respective higher alcohols, together with the acetyl-CoA produced from the sugars and other substrates. The increased production of other esters with the addition of L-leucine and L-isoleucine could be related to the uptake and metabolism of other substrates, such as the enhanced or inhibited uptake of certain amino acids. Further studies are needed to elucidate this.

The addition of L-valine only slightly increased isobutyl acetate production, by 0.009 mg/L (Table 3). The addition of amino acids did not affect the formation and/or degradation of ethyl acetate and benzyl isothiocyanate, except for those added with L-leucine and L-isoleucine (Table 2). The formation of aroma-active ethyl octanoate was increased with the addition of L-leucine, L-isoleucine and L-valine, while the addition of L-phenylalanine reduced the production of most ethyl esters (Tables 2 and 3). The reduction in the ethyl esters with the addition of L-phenylalanine could be related to the reduced *de novo* biosynthesis of fatty acyl Co-A associated with fatty acid and/or sugar metabolism. The effect of L-isoleucine and L-valine additions on the production of other ethyl esters varied (Tables 2 and 3). The final concentrations of esters were dependent on the stability and determined any significant differences at the statistical level, which varied among the different treatments (Table 2).

Comparison of quantified and semi-quantified major volatiles

The quantified and semi-quantified volatiles showed generally similar trends (Tables 2 and 3). However, discrepancies were observed between the semi-quantitative and the quantified results for some volatiles, such as ethanol, isobutyl alcohol and isobutyl acetate. This may be attributed to the deterioration of the mixed coating on the fibre upon the extraction of wine samples (Bianco *et al*., 2009) and possibly thermal deterioration of the fibre with numerous injections. Nevertheless, linear calibration curves obtained in this study had R^2 values of at least 0.98 (data not shown), and the relative standard derivation of the results in Table 3 was less than 14%, indicating moderately good repeatability under the analytical conditions used. In general, HS-SPME is used mainly as a qualitative or semi-quantitative method for the analysis of wine aroma compound evolution (Tao *et al*., 2008). Moreover, Baptista *et al*. (1998) stated that SPME can also be used as a quantitative method for the accurate and precise analysis of volatiles, as long as consistent and optimised sampling conditions are utilised.

CONCLUSION

In this study, fermentation performance and the formation/utilisation of aroma compounds during papaya juice fermentation by *W. saturnus* var. *mrakii* NCYC2251 were assessed, together with the effects of the addition of amino acids, namely L-leucine, L-isoleucine, L-valine and L-phenylalanine. Overall, *W. mrakii* showed a capability to produce papaya wine with a wider range of aroma compounds with the addition of a specific amino acid. Papaya juice fermentation with *W. mrakii*, together with adding a specific

amino acid, can be a valuable tool to modulate the aroma of papaya wine.

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