MODULATION OF PAPAYA WINE FLAVOUR COMPOUND FORMATION BY YEASTS

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NATIONAL UNIVERSITY OF SINGAPORE

2012

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(B. Appl. Sc.(Hons.), National University of Singapore)

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

NATIONAL UNIVERSITY OF SINGAPORE

2012

THESIS DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety, under the supervision of Dr Liu Shao Quan, (in the Food Science and Technology research laboratory, S13-05), Chemistry Department, National University of Singapore, between January 2009 and November 2012.

I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

The content of the thesis has been partly published in:

1. **Lee, P. R.**, Chong, I. S. M., Yu, B., Curran, P., & Liu, S. Q. (**2012**). Effects of sequentially inoculated *Williopsis saturnus* and *Saccharomyces cerevisiae* on volatile profiles of papaya wine. *Food Research International, 45*, 177–183.

2. **Lee, P. R.**, Kho, S. H. C., Yu, B., Curran, P., & Liu, S. Q. (**2012**). Yeast ratio is a critical factor for sequential fermentation of papaya wine by *Williopsis saturnus* and *Saccharomyces cerevisiae*. *Microbial Biotechnology*. (In press). DOI:10.1111/1751-7915.12008.

3. **Lee, P. R.**, Yu, B., Curran, P., & Liu, S. Q. (**2011**). Impact of amino acid addition on aroma compounds in papaya wine fermented with *Williopsis mrakii*. *South African Journal of Enology and Viticulture, 32*, 220–228.

4. **Lee, P. R.**, Yu, B., Curran, P., & Liu, S. Q. (**2011**). Effect of fusel oil addition on volatile compounds in papaya wine fermented with *Williopsis saturnus* var. *mrakii* NCYC 2251. *Food Research International, 44*, 1292–1298.

5. **Lee, P. R.**, Li, X., Yu, B., Curran, P., & Liu, S. Q. (**2011**). Non-*Saccharomyces* yeasts and wine. In: A. S. Peeters (Ed.), *Wine: Types, Production and Health* (Chapter 12). Hauppauge, New York, USA: Nova Science Publishers. ISBN: 978-1-61470-804-9.

6. **Lee, P. R.**, Ong, Y. L., Yu, B., Curran, P., & Liu, S. Q. (**2010**). Evolution of volatile compounds in papaya wine fermented with three *Williopsis saturnus* yeasts. *International Journal of Food Science and Technology, 45*, 2032–2041.

7. **Lee, P. R.**, Ong, Y. L., Yu, B., Curran, P., & Liu, S. Q. (**2010**). Profile of volatile compounds during papaya juice fermentation by a mixed culture of *Saccharomyces cerevisiae* and *Williopsis saturnus*. *Food Microbiology, 27*, 853–861.

8. **Lee, P. R.**, Yu, B., Curran, P., & Liu S. Q. (**2010**). Kinetics of volatile organic compounds during papaya juice fermentation by three commercial wine yeasts. *Nutrition & Food Science, 40*, 566–580.

Lee Pin Rou		1st November 2012
Name	Signature	Date

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest and sincere gratitude to my supervisor Dr Liu Shao Quan for his valuable advice, supervision, guidance and encouragements throughout the entire project. I feel honoured to have him as my supervisor and I have learned a lot from his valuable ideas, profound knowledge, and rich research experience. I am also deeply grateful for his kindness and patience throughout the four years and his countless efforts in guiding me to complete my research project and thesis.

Next, I would like to extend my gratitude to Dr Yu Bin, who has provided me with meticulous guidance, and timely advice on the method development for the solid phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS). I would also like to sincerely thank the flavourists from Firmenich for their precious time and efforts during the wine sensory evaluations.

I would like to express my warmest appreciation to my previous honours year and UROPS students, Miss Ong Yuen-Ling Irene, Miss Irene Saksono, Miss Chong Siew-May Irene, Miss Kho Hui-Chern Stephanie, Mr Saputra Anthony and Mr Toh Mingzhan, for their help and commitment in conducting some fermentation experiments in my research project. I would also like to take this opportunity to thank my fellow postgraduates, Mr Li Xiao, Ms Sun Jingcan, Ms Cheong Mun Wai, Ms Chan Li Jie and Ms Chen Dai, for their advice, technical assistance and moral support rendered throughout the project. I am also grateful for all the technical assistance provided by the staff and laboratory technicians of the Food Science and Technology Programme. Last but not least, I would like to thank my family members and my husband for their unwavering support and encouragement in my life.

PUBLICATIONS LIST

List of publications derived from this thesis

1. **Lee, P. R**., Chong, I. S. M., Yu, B., Curran, P., & Liu, S. Q. (**2013**). Effect of precursors on volatile compounds in papaya wine fermented by mixed yeasts. *Food Technology and Biotechnology, 51*, 92–100.

2. **Lee, P. R.**, Chong, I. S. M., Yu, B., Curran, P., & Liu, S. Q. (**2012**). Effects of sequentially inoculated *Williopsis saturnus* and *Saccharomyces cerevisiae* on volatile profiles of papaya wine. *Food Research International, 45*, 177–183.

3. **Lee, P. R.**, Kho, S. H. C., Yu, B., Curran, P., & Liu, S. Q. (**2012**). Yeast ratio is a critical factor for sequential fermentation of papaya wine by *Williopsis saturnus* and *Saccharomyces cerevisiae. Microbial Biotechnology*. (In press). DOI:10.1111/1751-7915.12008.

4. Chan, L. J., **Lee, P. R.**, Li, X., Chen, D., Liu, S. Q., & Trinh, T. T. T. (**2012**). Tropical fruit wine: an untapped opportunity. In D. Cabel (Ed.), *Food & Beverage Asia. Dec/Jan 2011/2012* (pp. 48–51). Singapore: Pablo Publishing Pte Ltd. ISSN: 2010-2364.

5. **Lee, P. R.**, Yu, B., Curran, P., & Liu, S. Q. (**2011**). Impact of amino acid addition on aroma compounds in papaya wine fermented with *Williopsis mrakii*. *South African Journal of Enology and Viticulture, 32*, 220–228.

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List of related publications

1. **Lee, P. R.**, Toh, M., Yu, B., Curran, P., & Liu, S. Q. (**2013**). Manipulation of volatile compound transformation in durian wine by nitrogen supplementation. *International Journal of Food Science and Technology*, *48*, 650–662.

2. **Lee, P. R.**, Boo, C. X., & Liu, S. Q. (**2013**). Fermentation of coconut water by probiotic *Lactobacillus acidophilus* and *Lactobacillus casei*. *Annals of Microbiology*. (In press). DOI: 10.1007/s13213-013-0607-z.

3. **Lee, P. R.**, Tan, R. M., Yu, B., Curran, P., & Liu, S. Q. (**2012**). Sugars, organic acids, and phenolic acids of exotic seasonable tropical fruits. *Nutrition & Food Science*. (In press).

4. **Lee, P. R.**, Saputra, A., Yu, B., Curran, P., & Liu, S. Q. (**2012**). Biotransformation of durian pulp by mono- and mixed-cultures of *Saccharomyces cerevisiae* and *Williopsis saturnus*. *LWT-Food Science and Technology, 46*, 84–90.

5. Tan, A. W. J., **Lee, P. R.**, Seow, Y. X., Ong, P. K. C., & Liu, S. Q. (**2012**). Volatile sulphur compounds and pathways of L-methionine catabolism in *Williopsis* yeasts. *Applied Microbiology and Biotechnology, 95*, 1011–1020.

6. **Lee, P. R.**, Saputra, A., Yu, B., Curran, P., & Liu, S. Q. (**2012**). Effects of *Saccharomyces cerevisiae* and *Williopsis saturnus* on the volatile and aromatic profiles of grape wine. *Food Biotechnology, 26,* 307–325.

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SUMMARY

This project assessed the biotransformation of volatile and non-volatile papaya constituents with a focus on volatile compounds during fermentation with monocultures and multistarters of *Saccharomyces cerevisiae* and *Williopsis saturnus*. This is in view of developing "papaya wine" as a new tropical fruit wine. Three commercial *S. cerevisiae* wine yeasts, namely strains EC-1118, R2 and Merit.ferm and three *W. saturnus* yeasts: *W. saturnus* var. *mrakii* NCYC2251, *W. saturnus* var. *saturnus* NCYC22 and *W. saturnus* var. *sargentensis* NCYC2727 were screened for their fermentation performances and volatile compound transformation.

S. cerevisiae was the main producer for medium to long-chain fatty acids, alcohols, ethyl esters and terpenoids, while *W. saturnus* produced high levels of acetate esters. Volatiles that were initially present in the papaya juice, especially benzyl isothiocyanate, butyric acid, benzaldehyde and β-damascenone were metabolised to trace levels during fermentation. *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 were selected for subsequent multistarter fermentations due to their relatively optimal formation of ethanol, esters and/or precursors (e.g. higher alcohols), and better growth rate.

The effects of flavour precursors on fermentation performance of *W. saturnus* NCYC2251 using selected amino acids (L-leucine, L-isoleucine, L-valine and L-phenylalanine) and fusel oil were also investigated. The addition of individual amino acids increased the production of corresponding higher alcohols and esters such as isoamyl alcohol, isoamyl acetate, isoamyl butyrate and isoamyl propionate with the addition of L-leucine, whereas the addition of 0.1% (v/v) fusel oil reduced the production of undesirable volatiles such as acetic acid, while increasing the formation of ethanol and acetate esters.

The multistarter fermentations (simultaneous and sequential inoculation) of the two selected *S. cerevisiae* and *W. saturnus* yeasts benefited to some extent from the presence and synergy of both yeasts, depending the yeast ratio. The mixed-culture fermentation (co-inoculation) of *S. cerevisiae* and *W. saturnus* at a ratio of 1:1000 showed the capability of producing papaya wine with a wider range of volatile compounds compared to the pure cultures.

Sequential fermentations of these two yeasts varied with the order of inoculation and the yeast ratio. The yeast that was first inoculated dominated the sequential fermentation. Inoculation of *S. cerevisiae* after seven days' fermentation with *W. saturnus* (positive sequential fermentation) produced papaya wine with more acetate esters and fruitiness than the simultaneous mixed-culture fermentation. However, inoculation of *W. saturnus* after two days' fermentation with *S. cerevisiae* resulted in most of the volatile composition being comparable to the simultaneous mixed-culture fermentation, except for the enhanced amount of ethyl esters. With respect to different yeast ratios, the positive sequential fermentation at the ratio of 10:1 (*W. saturnus*: *S. cerevisiae*) was dominated by *W. saturnus* and produced papaya wine with elevated concentrations of acetate esters. In contrast, the ratios of 1:1 and 1:10 (*W. saturnus*: S. *cerevisiae*) allowed the co-existence of both yeasts which enabled synergistic effects and resulted in the production of more ethyl esters, alcohols, 2-phenylethyl acetate and acetic acid.

These findings suggest that papaya juice fermentation by pure and multistarters of yeasts can be effective in manipulating yeast succession and modulating the volatile composition and organoleptic properties of papaya wine. This may be useful for winemakers in creating novel fruit wines with flavour complexity and distinct style.

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LIST OF ABBREVIATIONS

1,3-BPG	1,3-biphosphoglycerate
β-gal	β-galactosidase
AAT	Alcohol acetyltransferase
ADH II	Alcohol dehydrogenase II
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine-5'-triphosphate
CAS	Chemical Abstracts Service
CFU	Colony-forming units
DPPH	1,1-diphenyl-2-picrylhydrazyl radical scavenging activity
EC	Enzyme Commission
EI	Electron impact ionization
ELSD-LT	Low temperature evaporative light scattering detector
FRAP	Ferric reducing antioxidant power
FW	Fresh weight
G3P	Glyceraldehyde-3-phosphate
GC-FID	Gas chromatography-Flame ionisation detector
GC-MS	Gas chromatography-Mass spectrometry
H_2O	Water
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HS-SPME	Headspace-solid phase microextraction
I.D.	Internal diameter
$K_2S_2O_5$	Potassium metabisulphite

L-PAC	L-phenylacetyl carbinol
LRI	Linear retention index
MCF	Mixed-culture fermentation (co-inoculation)
MF	Modified frequency
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NCYC	National Collection of Yeast Cultures
N.D.	Not detected
NIST	National Institute of Standards and Technology
NSF	Negative sequential fermentation
OAV	Odour activity values
OD	Optical density
ORAC	Oxygen radical absorbance capacity
PC1	First principal component
PC2	Second principal component
PCA	Principal component analysis
PDA	Potato dextrose agar
PDMS	Polydimethylsiloxane
PG	Polygalacturonase
PME	Pectin methylesterase
ppm	Parts per million
ppt	Parts per trillion
PSF	Positive sequential fermentation
PTFE	Polytetrafluoroethylene
QDA	Quantitative descriptive analysis

R^2	Coefficient of determination
RPA	Relative peak area
SO_2	Sulphur dioxide
SPSS	Statistical Program for Social Sciences
TA	Total acidity
TCA	Tri-carboxylic acid
TE	Trolox equivalents
TPP	Thiamine pyrophosphate
TSS	Total soluble solids
UV	Ultraviolet
YAN	Yeast assimilable nitrogen
YPD	Yeast extract peptone dextrose

CHAPTER 1

INTRODUCTION

1.1 Basic knowledge of wine and winemaking

Winemaking, one of mankind's most ancient biotechnologies, took place on sites in Iran from as early as 5400 B.C. (Berkowitz, 1996; Shackford, 2009) and is now one of the most commercially prosperous biotechnological processes. It is usually applied to the production of alcoholic beverages "wine" from grape must or juice involving yeasts and biochemical reactions. Until now, numerous countries practise winemaking and commercialise wine worldwide. Among them, France, Italy, Spain and United States are examples of the top winemaking countries (Wine Institute, 2010).

The winemaking process typically begins with the crushing of fruits to release the juice, followed by maceration (applicable for red wine only) that releases flavour ingredients from the seeds, skins, and pulp as well as promotes the synthesis of additional flavour compounds during fermentation. The enzymes present hydrolyse juice macromolecules into forms readily usable by yeast and bacterial cells. For instance, the action of pectic enzymes enables the release of cellular constituents in juices into the must (Jackson, 2000). Subsequently, alcoholic fermentation may start spontaneously due to the indigenous yeasts derived from the grapes or picked up from the crushing equipment, or by the inoculation of yeast strains of known characteristics. During alcoholic fermentation, sugars are anaerobically converted into ethanol and carbon dioxide by *Saccharomyces* and non-*Saccharomyces* yeasts. Numerous volatile compounds such as esters, carbonyls, higher alcohols, volatile phenols, sulphur

compounds and fatty acids are also produced which contribute to the aroma and flavour, thereby affecting the overall quality of the wine (Swiegers & Pretorius, 2005).

Upon completion of alcoholic fermentation, the wine may be treated to foster a secondary fermentation: malolactic fermentation, by the malolactic bacteria. These bacteria are capable of direct decarboxylation of malic acid to lactic acid and carbon dioxide with the aid of the malolactic enzyme (EC 1.1.1.38) that is present in various lactic acid bacteria, particularly *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Leuconostoc oenos* (now *Oenococcus oeni*) and *Pediococcus damnosus* (Liu, 2002). *Oenococcus oeni* is the preferred species used for malolactic fermentation due to its acid tolerance and flavour profile produced (Liu, 2002). During malolactic fermentation, several aroma compounds are accumulated, such as diacetyl, acetoin, 2,3-butanediol, acetic acid, 2-butanol, diethyl succinate, ethyl acetate, ethyl lactate, ethyl hexanoate and ethyl octanoate, which are capable of further affecting the final wine flavour (Delaquies, Cliff, King, Girad, Hall, & Reynolds, 2000; Henick-Kling, 1995; Jackson, 2000; Lee, Hong, & Lee, 2009; Liu, 2002; Revel, Martin, Pripis-Nicolau, Lonvaud-Funel, & Bertrand, 1999).

The newly fermented wine is protected from or given limited exposure to air in order to restrict oxidation and microbial spoilage. Low doses of sulphur dioxide (SO_2) are also added to protect wine from spoilage organisms. Next, the wine is subjected to maturation that lasts for several weeks or years. The maturation process aids the loss of yeasty odors, the dissipation of excess carbon dioxide, the precipitation of suspended materials, the changes in aroma and the development of an aged bouquet (Jacobson, 2006). After maturation, the wine is racked, where the racking separates the wine from sediments formed during spontaneous or induced clarification. These sediments consist mainly of yeast and bacterial cells, precipitated

tannins, proteins and grape cell remains, which would cause off-flavour production and microbial spoilage if they remained in the wine (Jackson, 2000). Prior to bottling, the wine undergoes a fining process to remove traces of dissolved proteins and other materials such as tannins, to prevent the generation of haziness and soften the wine taste. The wine is also subjected to cold stabilisation and filtration to remove undesirable elements (e.g. potassium acid tartrate crystals, yeasts and microbes) and to enhance stability (Jacobson, 2006). These newly bottled wines are normally aged at the winery for several months to a few years before distribution to the consumers. The aging process harmonises the wine and allows acetaldehyde produced from the oxidation of ethanol by alcohol dehydrogenase II (ADH II) or from bottling (as a consequence of accidental oxygen uptake) to be converted to other non-volatile and volatile compounds such as procyanidins, sotolon and 1,1-diethoxyethane which further improve the wine quality (Peinado & Mauricio, 2009).

1.2 History and trends of tropical fruit wine fermentation

Fruit wine refers to alcoholic beverages made from fruits other than grapes. Tropical fruit wine fermentation such as pineapple and tamarind wine begun as early as 1951 (Czyhrinciw, 1969). Since then, numerous fruit wine-related studies have been conducted or on-going, especially those in the Southeast Asia region due to the limited supply of fresh grapes or unfavorable climatic condition for viticulture. Moreover, this is fueled by the increasing consumers' demand for newer styles of wine. Fruit wines made from apples, banana, pineapple, pupunha, mango, acerola, lychee, longan and raspberry have been produced and some are already commercialised (Duarte, Dias, de Melo Pereira, Gervasio, & Schwan, 2009; Duarte et al., 2010; Pino & Queris, 2010; Trinh, Woon, Yu, Curran, & Liu, 2011). These

proven successes have provided possibilities for producing wines in the tropics made from the local abundant supplies of tropical and subtropical fruits. Most of these fruits are suitable for making a good quality wine due to their appealing and characteristic aromas, as well as nutrient-rich contents.

However, making wine from non-grape must differs significantly from that of grapes due to the dissimilar physical and chemical properties of these tropical fruits. Thus, a simple transfer or adoption of traditional wine-making technology will not result in tropical fruit wine with satisfactory quality. For example, making wine from fruits other than grapes usually requires peeling and mechanical disintegration of the fruit. Also, juices from these fruits are subjected to several conditioning steps, namely the addition of water to dilute the pulp, the amelioration of the juices with sucrose in the case of low initial sugar contents as well as the addition of citric or tannic acids to control the acidity or astringency of the final product. Wine quality is also affected by many factors such as the constituents and quality of the starting materials.

Despite the studies on fruit wine, there are other fruits which have not been fully explored, especially tropical fruits such as mangosteen, durian, papaya, chiku and jackfruit. Hence, this provides opportunities for further research into the utilisation of these fruits for fruit wine innovation, which includes papaya wine fermentation in this project. Furthermore, the selection and utilisation of papaya for winemaking offers an alternative means of reducing post-harvest losses, as large quantities are often wasted during peak harvest periods due to rapid post-harvest deterioration resulting from high heat and humidity, poor handling, poor storage procedures and microbial infestations.

1.3 Objectives of project

Overall Aim

The overall aim of this project was to investigate the fermentation performance, the transformation of papaya constituents and the production of volatile compounds by monocultures and multistarters (simultaneous and sequential inoculation) of *Saccharomyces cerevisiae* and *Williopsis saturnus* with the intention to modulate the papaya wine flavour and to develop a new tropical fruit wine "papaya wine".

Hypothesis

Papaya with its nutrient rich content can be used for wine fermentation and the characteristics volatile production capabilities of *Saccharomyces cerevisiae* and *Williopsis saturnus* via monoculture and multistarters fermentation can modulate and improve the aroma profile of papaya wine.

Specific Objectives

1. To study the impact of wine yeasts on the formation of volatile compounds from papaya fermentation and select one *Saccharomyces* yeast from the three commercial *S. cerevisiae* wine yeast strains, namely EC-1118, R2 and Merit.ferm for subsequent papaya juice fermentations. **(Chapter 4)**

2. To study the impact of *Williopsis* yeasts on the formation of volatile compounds from papaya fermentation and select one strain from the three *Williopsis* yeasts - *W. saturnus* var. *mrakii* NCYC2251, *W. saturnus* var. *saturnus* NCYC22 and *W. saturnus* var. *sargentensis* NCYC2727 for subsequent papaya juice fermentations. **(Chapter 5)**

   [Completed in conjunction with Miss Irene Yuen-Ling Ong, FST Honours project year 2009/2010]

3. To study the effects of amino acid addition on the aroma compound profile of papaya wine fermented by the selected *W. saturnus* yeast. **(Chapter 6)**

   [Some sections completed in conjunction with Miss Irene Saksono, FST UROPS project year 2010]

4. To study the effect of fusel oil addition on the major volatile compounds in papaya wine fermented by the selected *W. saturnus* yeast. **(Chapter 7)**

5. To investigate the fermentation performance and the production of major volatile compounds in mixed-culture (co-inoculation) papaya wine fermentation by the selected *S. cerevisiae* and *W. saturnus* yeasts at a ratio of 1:1000. **(Chapter 8)**

   [Completed in conjunction with Miss Irene Yuen-Ling Ong, FST Honours project year 2009/2010]

6. To investigate the fermentation profile and the evolution of major volatile compounds in papaya wine fermentation by different orders of sequential inoculation of selected *W. saturnus* and *S. cerevisiae* yeasts as compared to simultaneous inoculation (mixed-culture fermentation) at a ratio of 1000:1 (*W. saturnus*: *S. cerevisiae*). **(Chapter 9)**

   [Completed in conjunction with Miss Irene Siew-May Chong, FST Honours project year 2010/2011]

7. To evaluate the fermentation performance and the evolution of major volatile compounds in papaya wine fermented by sequential inoculation of selected *W. saturnus* and *S. cerevisiae* yeasts at different ratios. **(Chapter 10)**

   [Completed in conjunction with Miss Stephanie Hui-Chern Kho, FST Honours project year 2011/2012]

CHAPTER 2

LITERATURE REVIEW

2.1 Nutritional information of papaya fruit

2.1.1 General information of papaya

Papaya (or paw paw, *Carica papaya*) is a melon-like tropical fruit belonging to the family *Caricaceae*. It is believed to be native to tropical America but has been widely grown throughout other tropical and subtropical regions such as Australia, Hawaii, Florida, various parts of Central and South Africa, and South East Asia including Malaysia and Indonesia. There are several cultivars of papaya available worldwide such as Solo and Taiwan from Brazil; Maradol from Cuba, Colombia and Mexico; Sekaki (also known as 'Hong Kong') and Eksotika from Malaysia; and Khack Dum from Thailand (De Oliverira & Vitória, 2011). Papaya is a climacteric fruit and exhibits a characteristic rise in ethylene production during ripening which is accompanied by softening, change in colour and the development of a strong distinct aroma. Papaya is also considered as a delicate and perishable fruit, susceptible to mechanical injury, physiological deterioration, water loss and decay. Papaya has high enzymatic activities of polygalacturonase (PG), pectin methylesterase (PME), β-galactosidase (β-gal), xylanase and cellulase which are responsible for increasing pectin solubility and depolymerisation during ripening (Lazan, Selamat, & Ali, 1995; Manenoi & Paull, 2007; Paull & Chen, 1983).

Papayas are commonly consumed fresh or used as an ingredient for other foods such as jellies, jams and juices. Sometimes, it is used as a therapeutic remedy due to several of its medicinal properties. For instance, the pulp is used in African

hospitals for treating paediatric burns (Starley, Mohammed, Schneider, & Bickler, 1999). Papaya has also been used as a potential renewable energy resource for industrial alcohol production because of its low cost and high availability (Sharma & Ogbeide, 1982). With the continuous research and development, a handful of fermented papaya products with health benefits were developed. These include Atchara, a fermented green papaya in raw coconut water vinegar; fermented papaya preparation, a natural health food made by yeast fermentation of *Carica papaya* Linn (Hiramoto, Imao, Sato, Inoue, & Mori, 2008); and cocktail EM-X, a cocktail with antioxidant properties that is fermented from unpolished rice, papaya and seaweeds (Deiana et al., 2002).

2.1.2 Non-volatile composition of papaya

2.1.2.1 Nutritional composition of papaya

Papayas have high nutritional content, comprising of a wide range of nutrients including protein, fat, carbohydrate, dietary fiber, dietary mineral and vitamin (Table 2.1). Several studies highlighted that papaya is an excellent source of copper, calcium, iron, magnesium, potassium and antioxidants such as vitamin C (ascorbic acid), polyphenols and carotenoids (β-carotene, β-cryptoxanthin) (Gayosso-García Sancho, Yahia, & González-Aguilar, 2011; Peterson, 1991; Richardson & Hyslop, 1992; Rivera-Pastrana, Yahia, & González-Aguilar, 2010; Wall, 2006). In fact, the papaya fruit is ranked first among several exotic fruits, lemon and orange for its vitamin C content (Vinci, Botre, Mele, & Ruggieri, 1995). Lycopene is also present in several papaya cultivars and its content in these papayas can be compared favorably to those in red, ripe tomatoes (2573 and 3025 μg/100 g), a high-lycopene fruit (Wall, 2006). Nevertheless, high amounts of lycopene are only found in red-fleshed papaya

cultivars (from Hawaii) such as Sunrise and SunUp, while the yellow-fleshed cultivars such as Kapoho, Laie Gold and Rainbow do not contain lycopene (Wall, 2006).

Table 2.1. Nutritional composition of papaya (Adapted from Moy, 2003)

Nutrients	**Concentration** (per 100 g of edible pulp)
Moisture (%)	86–89
Carbohydrate	9.5–12.2
Protein (N x 6.25; g)	0.36–0.5
Fat (g)	0.06–0.1
Fiber (g)	0.5–0.6
Ash (g)	0.5–0.6
Ascorbic acid (mg)	40–84
Vitamin A (mg)	11–32*
Thiamin (mg)	0.027–0.04
Riboflavin (mg)	0.043–0.25
Niacin (mg)	0.20–0.33
Calcium (mg)	10–30
Phosphorus (mg)	10–12
Iron (mg)	0.2–4.0
Energy (cal)	40–48

* Vitamin A data assuming 12 mg of *all-trans* β-carotone = 1 μg *all-trans* retinol.

2.1.2.2 pH and organic acid composition of papaya

Papaya is a low-acid fruit with a slight acidic pH ranging from 5.5 to 5.9, accounting for the low tartness of the papaya fruit. This pH value is much higher than the pH values of other tropical fruits which usually range from 3.2 to 4.5 (Moy, 2003). The different types of organic acids found in papaya include α-ketoglutaric, oxalic, citric, galacturonic, ascorbic, L-malic, D-malic, quinic, succinic, tartaric and fumaric acids (Cano, Torija, Marín, & Cámara, 1994; Chan, Chang, Stafford, & Brekke, 1971; Hernández, Lobo, & González, 2009). The organic acid profile is mainly constituted by citric and L-malic acids at 332 mg/100g fresh weight (FW) and 202 mg/100g FW respectively (Hernández et al., 2009), and followed by α-ketoglutaric and ascorbic

acids in lesser concentrations (Chan et al., 1971). These organic acids account for 85% of the total titratable acidity in papaya (Chan et al., 1971).

2.1.2.3 Sugar composition of papaya

Sugars are the main components of total soluble solids (TSS) content measured in ^{o}Brix, which is normally used to indicate the sweetness level or percentage of sugars in fruits. The TSS of papayas varies widely from 5.6 to 13.5% across the different cultivars that origniated from Florida, India and Hawaii (Moy, 2003). Sugars, especially fructose and glucose are the main contributors to the carbohydrate content in papaya. The sugar composition of papaya depends on the continuous sucrose import rather than starch degradation, as papaya mesocarp does not contain measurable starch or other carbohydrate storage compounds (Paull, 1993). With the inactivation of enzyme invertase, the total carbohydrate content for around 10 g per 100 g of edible portion in ripe papaya consists of 48.3% sucrose, 30% glucose and 22% fructose (Moy, 2003). However, when the papaya tissues are macerated, invertase (β-fructofuranosidase, EC 3.2.1.26) would catalyse the hydrolysis of sucrose to fructose and glucose (Zhou & Paull, 2001).

2.1.2.4 Amino acid and phenolic acid composition of papaya

The individual amino acid proportion in papaya fruit is specified in Table 2.2. Aspartic acid is the most dominant amino acid in ripe papaya, followed by glutamic acid, lysine and glycine. Generally, papayas have relatively low amino acid content as compared to grape and other tropical fruits, and the amino acid profile varies significantly across the different fruits (Table 2.2). The amino acid composition of

fruit may be influenced by a variety of factors, including cultural and climatic differences, cultivar, stage of growth, time of harvest, as well as storage and ripening conditions (Clark, Smith, & Boldingh, 1992).

Table 2.2. Comparison of amino acid contents of papaya (mg/100 g pulp) against grape and some other tropical fruits[a]

Amino acid	Papaya (*Carica Papaya)*		Grape (*Vitis vinifera*)	Pineapple (*Ananas comosus*)	Longan (*Dimocarpus longan*)	Mango (*Mangifera Indica*)
	USDA (2011)	Blakesley, Loots, Plessis, and Bruyn (1979)				
Alanine	14	15	22	33	157	82
Arginine	10	11	130	19	35	31
Aspartic acid	49	55	38	121	126	68
Cystine	-	0	10	14	-	-
Glutamic acid	33	37	81	79	209	96
Glycine	18	20	16	24	42	34
Histidine	5	6	22	10	12	19
Isoleucine	8	9	11	19	26	29
Leucine	16	18	22	24	54	50
Lysine	25	22	27	26	46	66
Methionine	2	2	9	12	13	8
Phenylalanine	9	10	19	21	30	27
Proline	10	11	80	17	42	29
Serine	15	17	22	35	48	35
Threonine	11	12	22	19	34	31
Tryptophan	8	-	11	5	-	13
Tryosine	5	6	10	19	25	16
Valine	10	11	22	24	58	42

[a]Data are collated from USDA National Nutrient Database for Standard Reference, Release 24 (2011), unless otherwise stated.

Papayas have good antioxidant properties with 3.0 μmol trolox equivalents (TE)/g FW of oxygen radical absorbance capacity (ORAC), 3.9 μmol trolox equivalents (TE)/g FW of ferric reducing antioxidant power (FRAP) and 65.1 μg gallic acid equivalents/g puree of 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity (DPPH) (Mahattanatawee, Manthey, Luzio, Talcott, Goodner, & Baldwin, 2006; Patthamakanokporn, Puwastien, Nitithamyong, & Sirichakwal, 2008). These

properties are partially attributed to its phenolic constituents (Rivera-Pastrana et al., 2010). Ferulic acid, caffeic acid and rutin are the most abundant phenolics in papaya fruit exocarp, while traces of caffeic, gallic and protocatechuic acids conjugates are present in the papaya mesocarp (Rivera-Pastrana et al., 2010). Most of these phenolic constituents occur naturally in the bound or esterified forms. For example, ferulic acid is covalently conjugated to plant-cell-wall polysaccharides, glycoproteins, polyamines, lignin and insoluble carbohydrate biopolymers (Liu, 2004). The phenolic contents of papayas are influenced by several intrinsic and extrinsic factors such as species, cultivar, environment, handling, degree of maturity and storage conditions (Thomás-Barberán & Espín, 2001).

2.1.3 Volatile composition of papaya

The volatile composition of papaya has been studied by several researchers using various extraction methods (e.g. headspace solid phase microextraction, simultaneous distillation-extraction), which led to the identification of more than 166 volatiles (Almora, Pino, Hernández, Duarte, González, & Roncal, 2004; Flath & Forrey, 1977; Pereira, Pereira, & Câmara, 2011; Pino, Almora, & Marbot, 2003). In addition, Pino et al. (2003) reported that distinctive volatile composition variations existed among the different varieties of papaya, whereby the volatile components of Sri Lankan, Maradol and Colombian papayas were dominated by esters, while benzyl isothiocyanate and terpenoids were the major aroma compounds in the Hawaiian papaya.

Generally, the typical aroma profile of a fully-ripened papaya comprises of a fairly wide range of volatile compounds such as fatty acids, esters, alcohols, aldehydes, ketones and terpenoids (Table 2.3). Among these constituents, methyl

butyrate, ethyl butyrate, 3-methyl-1-butanol, 1-butanol, benzyl alcohol, linalool, α-terpineol, nerol, geraniol, furfural, linalool oxide, hydroxypropanone, (*Z*)-ocimene, limonene, sabinene, (*Z*)-neoalloocimene and benzyl isothiocyanate are the major compounds (Almora et al., 2004; Flath & Forrey, 1977; MacLeod & Pieris, 1983; McGrath & Karahadian, 1994; Pereira et al., 2011; Pino et al., 2003). Esters such as short-chain methyl and ethyl esters (e.g. methyl and ethyl butyrate) are the primary esters that contribute to the fruity and typical papaya flavour; in particular, methyl butyrate, ethyl butyrate, ethyl acetate, ethyl hexanoate and ethyl 2-methylbutyrate have been reported to be the most potent odour compounds in papaya (Balbontín, Gaete-Eastman, Verara, Herrera, & Moya-León, 2007; MacLeod & Pieris, 1983). Many of these esters are formed through the enzymatic degradation of fatty acids during the ripening process (Buttery, 1981). On the other hand, linalool and benzyl isothiocyanate are the major compounds that contribute to the fresh and the pungent off-odour in papaya, respectively (Moy, 2003). Volatile fatty acids belong to another group of compounds with the major representatives being tetradecanoic, hexadecanoic and (*Z*)-9-hexadecenoic acids (Pino et al., 2003). Nevertheless, many of these major fatty acids are not of aromatic importance.

Most of these volatiles occur in free forms but some volatiles are present in bound forms as glycosides. These bound volatile compounds are released or formed by enzymatic hydrolysis during the disruption of cell structure, e.g. during fruit pulp processing. For instance, benzaldehyde, benzyl alcohol, 2-phenylethyl alcohol, benzyl isothiocyanate and (*E*)-3,7-dimethylocta-2,6-dienoic acid are liberated by glycosidases, while linalool and 2,6-dimethyloct-7-ene-2,3,6-triol are released by phosphatase activity (Heidlas, Lehr, Idstein, & Schreier, 1984; Schwab, Mahr, &

Schreier, 1989). These volatiles released by the enzymatic hydrolysis reactions thus impact significantly on the aroma profile of papaya.

Table 2.3. List of volatile compounds present in fully-ripened papaya fruits

Groups	**Volatile compounds**[a]
Acids	Acetic, octanoic, decanoic, dodecanoic, tetradecanoic, pentadecanoic, hexadecanoic, (*Z*)-9-hexadecenoic, (*E*)-9-hexadecenoic, linolenic
Alcohols	Butanol, 2-propanol, isobutyl alcohol, 1-penten-3-ol, 3-pentanol, 1-hexanol, 2-hexanol, 3-hexanol, isoamyl alcohol, 1-octanol, benzyl alcohol, 2-phenylethyl alcohol, 2,6-dimethyl-3,6-epoxy-7-octen-2-ol (*cis* and *trans*), 2,6-dimethyl-2,6-epoxy-7-octen-3-ol (*cis* and *trans*)
Aldehydes	2-Methylbutanal, hexanal, heptanal, benzaldehyde, octanal, nonanal, decanal, phenylacetaldehyde, furfural
Esters	Ethyl butyrate, ethyl 2-butenoate, ethyl 3-hydroxybutyrate, ethyl 2-methylbutyrate, ethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate, ethyl tetradecanoate, ethyl benzoate, methyl butyrate, methyl 2-hydroxybutyrate, methyl 2-butenoate, methyl propanoate, methyl hexanoate, methyl octanoate, methyl decanoate, methyl gernanate, methyl dodecanoate, methyl tetradecanoate, methyl palmitoleate, propyl butyrate, propyl propanoate, isoamyl benzoate
Heteroatom (*N, S*) compounds	Benzyl isothiocyanate, methyl thiocyanate, phenylacetonitrile
Ketones	6-Methyl-5-hepten-2-one, heptan-2-one, 4-hydroxy-4-methylpentan-2-one, hydroxypropanone
Lactones	γ-Hexalactone, γ-octalatone
Terpenoids	Myrcene, α-phellandrene, α-terpinene, β-phellandrene, limonene, germacrene D, (*Z*)- β-ocimene, (*E*)- β-ocimene, γ-terpinene, caryphyllene, o-xylene, sabinene, (*Z*)-neoalloocimene, linalool, terpinen-4-ol, α-terpineol, geraniol, nerol, linalool oxide

[a]Volatile compounds collated from: Almora et al. (2004); Balbontín et al. (2007); Flath and Forrey (1977); MacLeod and Pieris (1983); McGrath and Karahadian (1994); Pereira et al. (2011); Pino et al. (2003).

2.2 Wine fermentation

2.2.1 Biochemistry of alcoholic fermentation

In alcoholic fermentation, simple sugars such as glucose and fructose are metabolised in the yeast cytoplasm via a series of enzymatic reactions, which collectively known as glycolysis, to ethanol and carbon dioxide under anaerobic conditions (Fig. 2.1). Generally, glycolysis occurs entirely in the cytosol of the cell and involves several major steps including the transformation of glucose into fructose 1,6-biphosphate, the cleavage of fructose 1,6-biphosphate to glyceraldehyde-3-phosphate (G3P), the conversion of G3P to 1,3-biphosphoglycerate (1,3-BPG), the transfer of phosphoryl group of the acylphosphate from 1,3-BPG to ADP that yields 3-phosphoglycerate and ATP, and finally the transformation of 3-phosphoglycerate into pyruvate (Fig. 2.1). Each monosaccharide molecule generates two molecules of pyruvate, two molecules of carbon dioxide, two NADH and a net gain of two ATP.

Under anaerobic conditions, respiration is inhibited and pyruvate does not proceed into the Krebs cycle and oxidative phosphorylation pathway. Instead, pyruvate is transformed to acetaldehyde and carbon dioxide by pyruvate decarboxylase. A cofactor, thiamine pyrophosphate (TPP), is required to form a carbanion that readily combines with the pyruvate carbonyl group. Acetaldehyde is then reduced to ethanol by alcohol dehydrogenase and in the process regenerates NAD^+ which is consumed during glycolysis (Fig. 2.1).

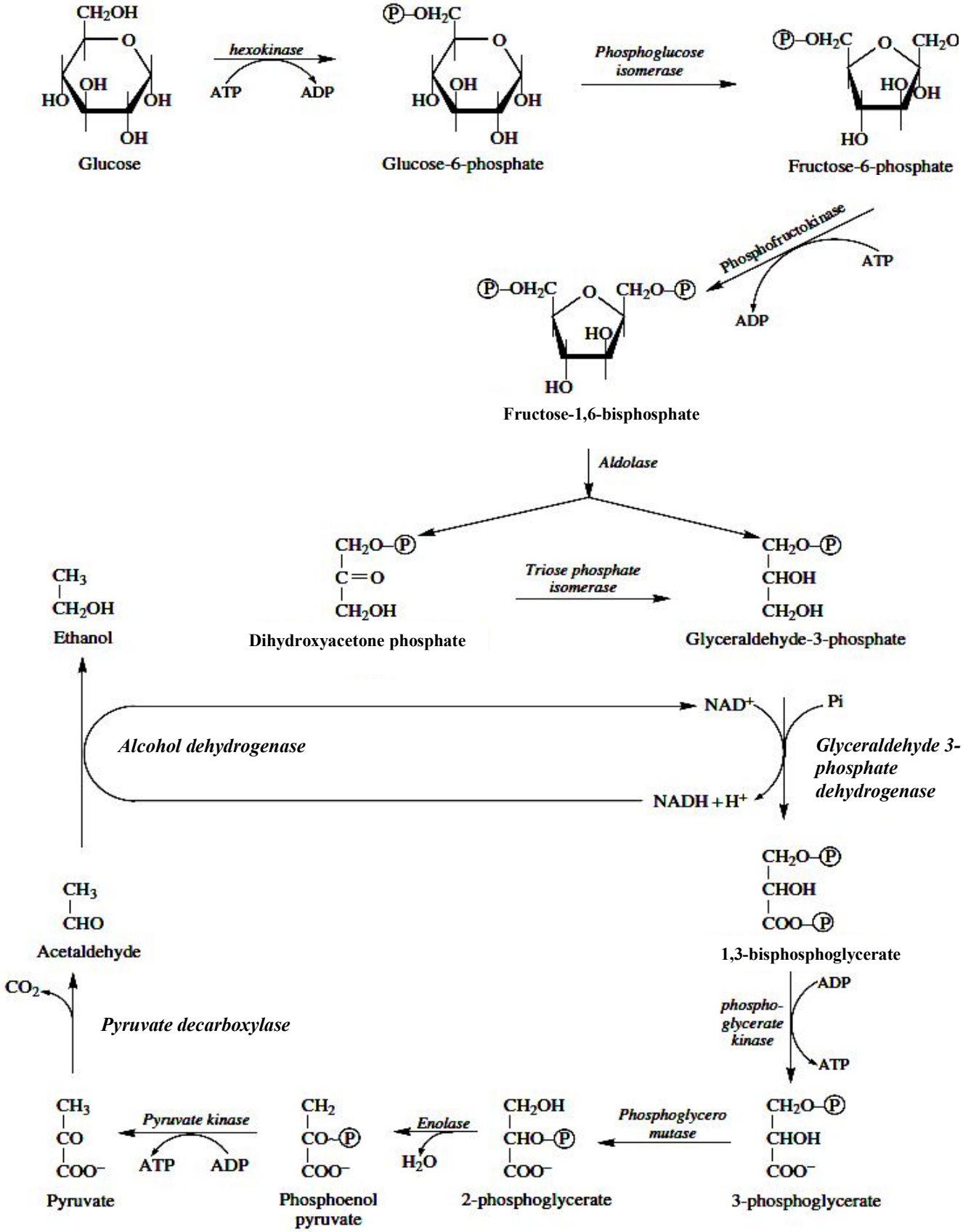

Fig. 2.1. Glycolysis and alcoholic fermentation pathway
(Adopted from Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006)

2.2.2 Volatile compounds produced during wine fermentation

In addition to ethanol production during alcoholic fermentation, non-volatile and odourless compounds undergo transformation into numerous volatile and aromatic compounds such as fatty acids, alcohols, esters, aldehydes, ketones, volatile phenols and terpenoids that make up the "fermentation bouquet" (Janssens, de Pooter, Schamp, & Vandamme, 1992; Swiegers, Bartowsky, Henschke, & Pretorius, 2005). A variety of yeast biochemical mechanisms are involved, including hydrolysis and transformation reactions such as reduction, esterification, decarboxylation, oxidation and metabolite-induced condensation reactions. The accumulation of these compounds in wine depends on the yeast strain, must composition (chemical, physical and nutrient composition) and fermentation conditions. Among these compounds, higher alcohols, esters, volatile fatty acids, carbonyl compounds and volatile sulphur compounds are of particular importance as they contribute the greatest impact on wine aroma.

Higher alcohols, or fusel alcohols (fusel oil), are metabolites from sugar and amino acid catabolism (Ehrlich pathway) that can impart fruity and floral notes at optimal levels, whereas an excess would cause an intense pungency in wine (Swiegers et al., 2005). Higher alcohols are also important precursors for the formation of esters, which are associated with pleasant aromas and characteristic fruity flavours of wine. In the Ehrlich pathway, aminotransferases catalyse the transamination of amino acids to form their respective α-keto acids. The α-keto acids are subsequently decarboxylated to form aldehydes with one carbon atom less by decarboxylase enzymes. Finally, the aldehydes are reduced by alcohol dehydrogenases to form the fusel alcohols (Derrick & Large, 1993). Higher alcohols can be categorised into either aliphatic or aromatic alcohols. Examples of aliphatic fusel alcohols are isoamyl

alcohol from leucine, isobutanol from valine and active amyl alcohol from isoleucine. Aromatic fusel alcohols include 2-phenylethyl alcohol from phenylalanine and tyrosol from tyrosine (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008).

Esters are the most significant contributors of fruity character in wines. The two main groups of fermentation-derived esters that have been long associated with wine fruitiness are acetate esters [ethyl acetate, isobutyl acetate, active amyl acetate, isoamyl acetate, hexyl acetate, and 2-phenylethyl acetate], and fatty acid ethyl esters (C3-C10). Acetate esters can be formed by alcohol acetyltransferases from the respective higher alcohols and acetyl-CoA (Swiegers et al., 2005). Conversely, fatty acid ethyl esters can be formed by enzymatic esterification of the activated fatty acids formed during the early stages of lipid biosynthesis (Suomalainen, 1981) or through alcoholysis catalysed by ethanol hexanoyl transferase or acyl-coenzymeA: ethanol *O*-acyltransferase from ethanol and fatty acyl-CoAs derived from metabolism of fatty acids (Saerens et al., 2006). The final concentration of esters in wine is the result of the balance between yeast ester-synthesising enzymes and esterase enzymes promoting their hydrolysis in the respective yeasts (Lilly, Bauer, Lambrechts, Swiegers, & Cozzolino, 2006).

Wine contains a diversity of straight-chain (C2-C18) and branched-chain fatty acids (e.g. 2-methyl propanoic, 2-methyl butanoic and 3-methyl butanoic acids) that contribute to the complexity of wine but impart an unpleasant flavour at high concentrations (Swiegers & Pretorius, 2005). As the fatty acid chain length increases, volatility decreases and the odour changes from sour to rancid and cheesy (Francis & Newton, 2005). Among them, acetic acid is quantitatively and sensorially the most important volatile fatty acid produced during alcoholic fermentation (Eglinton & Henschke, 1999). Acetic acid is formed by the action of aldehyde dehydrogenases

from acetaldehyde, while straight-chain fatty acids (C4–C12) are by-products of saturated fatty acid metabolism (Ugliano & Henschke, 2009). Fatty acids are formed by fatty acid synthase via the repeated condensation of acetyl-CoA derived from sugar metabolism. In particular, acetyl-CoA is first converted to malonyl-CoA by acetyl-CoA carboxylase; the malonyl-CoA formed is then utilized by the fatty acid synthase complex which undergo repetitive condensation with enzyme bound acetyl-CoA for the synthesis of fatty acids (Lambrechts & Pretorius, 2000). However, branched-chain fatty acids (e.g. 2-methylbutyric and 3-methylbutyric acids) are derived from oxidation of the aldehydes formed from α-keto acids during amino acid metabolism (Ugliano & Henschke, 2009).

Yeasts produce various carbonyl compounds including aldehydes, ketones, keto acids and lactones from sugar metabolism. Among them, acetaldehyde is quantitatively the most important carbonyl compound that is mainly produced by *S. cerevisiae* from pyruvate through the glycolytic pathway with concentrations ranging from 50 to 120 mg/L (Suomalainen & Lehtonen, 1979), and together with its low sensory threshold, it would impart an undesirable green flavour to wine. However, in some wines, high concentrations of acetaldehyde are generally associated with oxidation off-flavors (aldehydic) that are responsible for giving the distinctive characteristic of dry wines such as Spanish Sherries, French *vin jaune* and Sardinian Vernaccia (Schreier, 1979). Acetaldehyde also acts as a precursor for acetoin production (Collins, 1972) in the early phase of fermentation but the latter is reduced to 2,3-butanediol at a later stage (Guymon & Crowell, 1965). This helps to reduce the off-flavour development such as creamy or buttery aroma in alcoholic beverages.

Sulphur compounds are usually found at low concentrations in wine but can strongly affect the aroma profile due to their low odour thresholds (Vermeulen, Gijs,

& Collin, 2005). The majority of the sulphur volatiles are formed by the metabolism of sulphate, sulphites and sulphur-containing amino acids present in the must as well as pesticides by yeasts (De Mora, Eschenbruch, Knowles, & Spedding, 1986). These compounds include thiols, disulphides, trisulphides and thioesters with typical odour descriptors such as rotten egg-, onion-, garlic- and cabbage-like (Moreira, Mendes, Pereira, Guedes de Pinho, Hogg, & Vasconcelos, 2002).

2.2.2.1 Analysis of volatile compounds in wine

Traditionally, liquid-liquid extraction, simultaneous distillation/extraction, and dynamic and static headspace sampling methods have been used for analysis of wine flavour (Ferreira, Rapp, Cacho, Hastrich, & Yavas, 1993; Gil, Mateo, Jiménez, Pastor, & Huerta, 1996; Soles, Ough, & Kunkee, 1982; Stashenko, Macku, & Shibamato, 1992). However, these methods are time-consuming; resulting in extensive solvent waste and solvent costs, and can result in the loss of some important volatiles depending on solvent selectivity and volatility. In addition, liquid–liquid extractions frequently require heating of the sample, which can result in degradation and/or artifact formation.

Conversely, solid-phase microextraction (SPME) is now widely used for analysis of aroma volatiles in many food and beverage matrices, especially those in wines (Alves, Nascimento, & Nogueira, 2005; Campillo, Penalver, & Hernandez-Cordoba, 2008; García, Reichenbaher, Denzer, Hurlbeck, Bartzsch, & Feller, 1997; Kafkas et al., 2006). SPME is a solvent-free sampling technique that is not only faster and easier than solvent extractions and distillations; it is also highly reproducible and sensitive. SPME can also closely reflect the true volatile flavor profile of the wine than those generated by distillation and solvent extraction processes (Carasek &

Pawliszyn, 2006) and the detection limits can reach parts per trillion (ppt) levels for certain compounds (Pawliszyn, 1997). Generally, SPME involves the concentration of analytes by adsorption (or absorption) onto a polymeric material that is coated onto the end of a fused silica fiber. Extraction is based on partitioning of the analyte among the three phases present in the sampling vial: the liquid, the headspace of the vial and the SPME fiber (Pawliszyn, 1997). The quantity of analyte extracted by the fibre is proportional to its concentration in the sample as long as equilibrium is reached or, in the case of short time pre-equilibrium, provided with the help of convection or agitation. The three modes of SPME are direct extraction, membrane protected extraction and headspace extraction. For direct extraction, the SPME device is inserted into the sample to which allows the analyte to transfer directly from the sample matrix into the fibre. For membrane protected extraction, the extraction is similar to direct extraction but the SPME devices have a selective membrane which provides protection to the membrane and add a degree of selectivity. In headspace (HS) extraction, the SPME device is placed in a region of air above the sample (HS) to adsorb volatile compounds while excluding interference from high molecular weight and non-volatiles. As the fibre is not in contact with the sample, it is protected from damage which allows modification of the sample such as changing the pH or temperature to improve extraction. As compared to direct and membrane protected extractions, headspace extraction enables more accurate absorption of volatiles as it reduces the non-volatile matrix effect from restricting the volatile absorption.

2.2.3 Yeast strains and evolution of inoculation strategies in wine fermentation

The key microorganisms in wine fermentation are fermentative yeasts that transform fruit juice/must into a distinctive and highly-flavoured alcoholic beverage.

Therefore, the selection of yeasts and the corresponding inoculation strategies are essential extrinsic factors. Interestingly, with the advancement in the understanding of biochemistry, ecology, molecular biology and physiology of yeasts, it has led to the dynamic evolution of inoculation strategies of yeast in wine fermentation in order to cater for the growing demands for different types and style of wines.

Traditionally, *S. cerevisiae* is commonly used for inoculation in large scale wine fermentation due to its high ethanol tolerance, catabolic efficiency, homogeneity of fermentation and ease of control. *S. cerevisiae* also has the ability to hydrolyse conjugated aroma precursors in juice that improve wine aroma (Zoecklein, Marcy, Williams, & Jasinski, 1997) and produce different flavour profiles when fermenting the same grape juice with different strains or species of *Saccharomyces* yeasts. In addition, the volatile thiols responsible for the characteristic nuances of wines made from the Sauvignon Blanc grape variety, are principally formed during alcoholic fermentation by the metabolic action of some *S. cerevisiae* yeast strains from S-cysteine precursors in the must (Murat, Masneuf, Darriet, Lavigne, Tominaga, & Dubourdieu, 2001). However, it has been reported that wines produced with *Saccharomyces* yeast monocultures lack flavour complexity and vintage variability as compared to the wines produced from spontaneous fermentation (Lambrechts & Pretorius, 2000). This is because spontaneous or natural alcoholic fermentation is a complex process carried out by a succession of yeasts from different genera and species (Romano, Fiore, Paraggio, Caruso, & Capece, 2003). However, spontaneous fermentation is usually not favoured by wine-makers as it is an uncontrolled process, where the impact of the different types of yeasts on the wine aroma and flavour may not be consistent (Ciani, Comitini, Mannazzu, & Domizio, 2010).

As compared to *S. cerevisiae*, non-*Saccharomyces* yeasts have been considered as wild or spoilage yeasts and are not favourable for fermentation due to the potential production of larger amounts of ethyl acetate and acetic acid (Ciani et al., 2010). Nevertheless, in the recent years, wine-makers and researchers have recognised the ability of non-*Saccharomyces* to produce esters and other volatile compounds that can improve the fermentation bouquet of wine (Gil et al., 1996; Jolly, Augustyn, & Pretorius, 2006; Romano, Suzzi, Comi, & Zironi, 1993; Romano, Suzzi, Comi, Zironi, & Maifreni, 1997) and thus, there was a re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking. Some authors have also stated that non-*Saccharomyces* yeasts excrete enzymes that may be responsible for giving the wine the unique characteristics. For example, β-glucosidase involved in the flavour-releasing processes has been described in species of *Candida, Kloeckera, Pichia, Hansenula, Hanseniaspora* (Charoenchai, Fleet, Henschke, & Todd, 1997) and *Metschinikowia* (Fernandez, Ubeda, & Briones, 2000). Protease involved in the reduction of protein-induced haze formation in wines has also been reported in species of *Candida*, *Kloeckera*, *Pichia*, *Hanseniaspora* and *Debaryomyces* (Charoenchai et al., 1997; Dizy & Bisson, 2000; Strauss, Jolly, Lambrechts, & van Rensburg, 2001).

Despite the capability of non-*Saccharomyces* yeasts in the production of flavour compounds that can modulate the wine quality, *Saccharomyces* yeast is still essential to complete wine fermentation due to its higher stress-tolerant ability than non-*Saccharomyces* yeasts. Hence, leading to the exploration of the usage of multistarter (simultaneous or sequential inoculation) of non-*Saccharomyces* and *Saccharomyces cerevisiae* yeasts that takes advantage of the flavour-enhancing

potential of the former and the ethanol-producing ability of the latter (Ciani et al., 2010; Lambrechts & Pretorius, 2000; Romano et al., 2003).

Since then, several multistarter fermentations (sequential or simultaneous inoculation) with improved complexity and enhanced wine quality have been reported (Clemente-Jimenez, Mingorance-Cazorla, Martınez-Rodrıguez, Las Heras-Vazquez, & Rodrıguez-Vico, 2005; Moreira, Mendes, Guedes de Pinho, Hogg, & Vasconcelos, 2008; Soden, Francis, Oakey, & Henschke, 2000). For example, simultaneous mixed-culture fermentation of *Debaryomyces vanriji* and *S. cerevisiae* increased the geraniol concentration in Muscat wine (Garcia et al., 2002), while sequential fermentation of *Pichia fermentans* and *S. cerevisiae* conferred greater complexity to wine through the enhancement of desirable flavour compounds production and glycerol content (Clemente-Jimenez et al., 2005). Other studies even highlighted the use of multistarter fermentations to reduce the negative sensorial characteristics and for biological acidification of wines (Bely, Stoeckle, Masneuf-Pomarède, & Dubourdieu, 2008; Kapsopoulou, Mourtzini, Anthoulas, & Nerantzis, 2007; Moreno, Millan, Ortega, & Medina, 1991). Simultaneous inoculation of *Torulaspora delbrueckii* and *S. cerevisiae* at a ratio of 20:1 produced 53% and 60% reductions in the volatile acidity and acetaldehyde, respectively (Bely et al., 2008), while a mixed-culture (co-inoculation) of *Kluyveromyces thermotolerans* and *S. cerevisiae* could achieve up to a 70% increase in titratable acidity and consequently a reduction of 0.3 pH units for biological acidification (Kapsopoulou et al., 2007).

2.2.4 Fermentation conditions affecting yeast growth and evolution

Since yeast is critical in affecting the wine quality, there is a need to identify the various factors affecting the yeast growth. Some important factors that affect the

yeast growth and evolution in alcoholic fermentation are fermentation temperature, sulphur dioxide concentration, nutrients availability and the ratio of yeasts in multistarter fermentation.

Fermentation temperature is one of the important vinification factors that affect the rate of yeast growth and alcoholic fermentation. Cultures such as *S. cerevisiae* exhibited high cell population and kinetics at temperatures between 20 and 30°C (Mendoza & Farias, 2010), while *Kloeckera apiculata* grew and survived better than *S. cerevisiae* in fermentations performed below 20°C and dominated fermentations at 10°C (Heard & Fleet, 1988). Simiarly, Erten (2002) revealed that *K. apiculata* could survive longer and even dominate over *S. cerevisiae* in the early phase of mixed-culture (co-inoculation) fermentation at low temperatures as compared to fermentations conducted above 20°C. Killian and Ough (1979) and Torija et al. (2003) highlighted the increasing number of fermentations conducted at low temperatures (10–15°C) due to the enhancement of volatile production and improvement of wine quality. However, these low temperatures could easily cause sluggish or stuck fermentations due to the restriction in yeast growth (Torija et al., 2003). Molina, Swiegers, Varela, Pretorius, and Agosin (2007) also pointed out that higher concentration of ethyl esters related to fresh and fruity aroma was produced in fermentation at 15°C, but fermentation at higher temperature (28°C) produced more flowery related aroma compounds (e.g. 2-phenylethyl acetate).

Other fermentation conditions such as sulphur dioxide (SO_2) addition would also have an impact on the yeast growth and succession. Addition of SO_2 to must to control oxidation reactions and restrict the growth of the indigenous yeast population is a well-established practice in winemaking. SO_2 is highly toxic to most non-*Saccharomyces* yeasts, while strains of *Saccharomyces* in general are quite resistant to

it (Benda, 1982; Romano & Suzzi, 1993a). The total concentration of SO_2 in grape juice during fermentation consists of the bound and free forms. The undissociated molecular form of free SO_2 is the most important antimicrobial agent. Generally, it is accepted that 0.5 to 2.0 mg/L of molecular SO_2 is necessary to obtain a good biological stability. Thereafter, the amount of SO_2 would affect the yeast growth rate especially to the non-*Saccharomyces* yeast in the fermentation medium and the subsequent volatile compound production.

The growth of yeasts especially those in the multistarter fermentation is subjected to nutrient limitations. The difference in cell concentrations during wine fermentations reflects the fact that the non-*Saccharomyces* yeasts have higher growth requirements for certain nutrients than *S. cerevisiae* (Mauricio, Guijo, & Ortega, 1991). An example is the oxygen availability, where it is required by yeasts for the synthesis of cellular membrane lipid compounds, especially ergosterols and unsaturated fatty acids (Bonciu, 2009). This is especially so during the cell growth where most of the oxygen was used for several functions such as ring cleavage of proline (Ingledew, Magaus, & Sosulski, 1987), mitochondrial development and energy supply. Visser, Scheffers, Batenburg-Van Der Vegte, and Van Dijken (1990) showed that *S. cerevisiae* is capable of rapid growth under limited nutrient conditions such as strictly anaerobic environment, whereas other yeasts, including the wine-related genera *Candida* and *Torulaspora*, grew poorly under the same conditions. The growth and evolution of yeasts during multistarter fermentations may alternatively be due to the fact that non-*Saccharomyces* species are less tolerant than *S. cerevisiae* under low available oxygen conditions. This hypothesis is supported by the findings in Hansen, Nissen, Sommer, Nielsen, and Arneborg (2001) and Panon (1997), who revealed that higher oxygen concentration allowed longer co-existence of non-

Saccharomyces and *Saccharomyces* yeasts, while oxygen limitation led to the death of non-*Saccharomyces* yeasts during simultaneous mixed-culture fermentation with *S. cerevisiae*.

Another example is the nitrogen content (e.g. amino acids, peptides and ammonium salts), where nitrogen is required by yeast to build biomass which directly affects the rate of wine fermentation. Moreover, nitrogen availability also affects the production of volatile compounds and sugar utilisation (Arias-Gil, Garde-Cerdan, & Ancin-Azpilicueta, 2007; Sablayrolles, 2009). Generally, a minimum of 140 mg N/L yeast assimilable nitrogen (YAN) is required to complete fermentation within the normal range of sugars (Butzke, 1998). The significance of nitrogen content and availability are closely related to the nitrogen requirement by the yeasts. Studies revealed that significant variation in the nitrogen requirement and consumption existed amongst different strains of yeasts (Andorrà, Berradre, Rozès, Mas, Guillamón, & Esteve-Zarzoso, 2010; Manginot, Rouston, & Sablayrolles, 1998). Yeast strains with low nitrogen requirements had a high specific fermentation rate and were highly effective in using nitrogen for protein synthesis (Manginot et al., 1998). On the other hand, Torrea, Fraile, Garde, and Ancín (2003) revealed a positive correlation between nitrogen demand and volatile production, where strains with a higher nitrogen demand produced a higher concentration of esters during fermentation. This may possibly be due to nitrogen nutrients being the essential precursors for the formation of esters and alcohols and thus, regulated their production. Furthermore, Andorrà et al. (2010) commented that mixed-culture (co-inoculation) fermentation of *S. cerevisiae*, *Hanseniaspora uvarum* and *Candida zemplinina* have higher and more complex amino acids consumption than their pure cultures, leading to better synthesis of volatile compounds.

The ratio of *Saccharomyces* to non-*Saccharomyces* yeasts is another important parameter that determines the extent of growth based on their interactions and the quality of the resultant wine in multistarter fermentations (Bely et al., 2008; Comitini et al., 2011; Trinh et al., 2011; Viana, Gil, Valles, & Manzanares, 2009). The period of yeast viability, governed by the yeast ratio in multistarter fermentation, is important as it allows maximum contribution by the intended yeast strains. Viana et al. (2009) studied the mixed-culture fermentations (co-inoculation) of *H. osmophila* and *S. cerevisiae* at ratios of 90:10, 75:25, 50:50, 25:75, 10:90 and 5:95 and reported that a ratio of 90:10 was capable of producing wines with enhanced 2-phenyethyl acetate production. Conversely, Trinh et al. (2011) highlighted the early growth arrest of *W. saturnus* in simultaneous mixed-culture fermentation of *S. cerevisiae* and *W. saturnus* at a ratio of 1:100; however it was more effective in modulating and improving the aromatic profiles of the longan wine than the ratio of 1:1000.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials, yeast strains and culture media

Papaya fruits (Sekaki cultivar, Malaysia) were purchased from the local fruit wholesale centre in Singapore. D(-)Fructose, D(+)glucose, L-valine, L-phenylalanine, L-leucine, L-isoleucine, acetic, citric, DL-malic, DL-tartaric, lactic, oxalic, pyruvic and succinic acids were purchased from Sigma-Aldrich (Oakville, ON, Canada). Potato dextrose agar (PDA), lysine agar, bacteriological peptone, yeast extract and malt extract were purchased from Oxoid (Hampshire, England).

The pure reference compounds used in the identification and quantitative analysis of the volatile compounds and the fusel oil were obtained from Firmenich Asia Pte Ltd (Singapore) and Merck (Darmstadt, Germany). Table 3.1 shows the composition of the fusel oil used and determined using the volatile compound analysis method described below. Food-grade DL-malic acid was purchased from Suntop (Singapore). Potassium metabisulphite was obtained from The Goodlife Homebrew Centre (Norfolk, England). Sulphuric acid was purchased from VWR (Pennsylvania, USA). The methanol and acetonitrile obtained from Tedia (Fairfield, USA) were of HPLC grade.

The following yeast strains were used in this study: *Saccharomyces cerevisiae* var. *bayanus* EC 1118 and R2 (Lallemand Inc, Brooklyn Park, Australia) and *S. cerevisiae* Merit.ferm (Chr.-Hansen, Copenhagen, Denmark), *Williopsis saturnus* var. *mrakii* NCYC2251, *W. saturnus* var. *saturnus* NCYC22 and *W. saturnus* var. *sargentensis* NCYC2727 (National Collection of Yeast Cultures, Norwich, UK). All strains were obtained in freeze-dried form.

Yeast strains were propagated in nutrient broth comprised of 2% (w/v) glucose, 0.25% (w/v) yeast extract, 0.25% (w/v) bacteriological peptone and 0.25% (w/v) malt extract. The components in nutrient broth were dissolved in deionised water, adjusted to pH 5.0 by 1.0 M HCl and sterilised by autoclaving at 121^{o}C for 15 min. Yeast cultures were incubated at 25^{o}C for up to 48 h without aeration and dispensed in one mL aliquots and stored at -80^{o}C until use.

Table 3.1. Composition of fusel oil

Components	**Wt.%**
Ethanol	9.06
Active amyl alcohol (2-methyl-1-butanol)	13.26
Isoamyl alcohol (3-methyl-1-butanol)	47.00
Isobutyl alcohol	16.62
n-Propanol	0.49
Active amyl acetate	0.08
Isoamyl acetate	0.55
Isobutyl acetate	0.12
Ethyl decanoate	0.22
Ethyl octanoate	0.13
Ethyl dodecanoate	0.11
Water and other minor volatile compounds	12.36

3.2 Preparation and pretreatment of papaya juice

The papayas with an initial sugar concentration of 11-11.70 °Brix (containing 3.72-5.48 g of fructose and 3.78-5.32 g of glucose per 100 mL of juice) and pH 4.98 were washed, peeled, cut and processed into juice by mechanical extraction with a Sona juice extractor (Cahaya Electronics, Singapore) and centrifuged at 32,140×*g* (Beckman Centrifuge, USA) for 15 min at 4^{o}C to separate the pulp residue and juice. The supernatant was acidified with 1 M DL-malic acid to pH 3.5 and sanitised by 100 ppm potassium metabisulphite ($K_2S_2O_5$) at 20°C for 24 h. The efficiency of the sanitation was verified by plate counting.

3.3 Preparation of starter cultures and fermentation conditions

Yeast starter cultures (pre-cultures) were prepared using sanitised papaya juice, inoculated with 8% and 10% (v/v) of *S. cerevisiae* and *W. saturnus*, respectively. The pre-cultures were then incubated at 25^{o}C for 72 h (*S. cerevisiae*) and 96 h (*W. saturnus*) until the yeasts achieved 10^7 CFU/mL. However, the *S. cerevisiae* pre-culture used in the sequential fermentations with different ratios of *W. saturnus* and *S. cerevisiae* (**Chapter 10**) was prepared from the freeze-dried yeasts rehydrated in sterile nutrient broth according to the producer's instructions, where 15g of freeze-dried *S. cerevisiae* var. *bayanus* R2 was reconstituted in 100 mL of sterile nutrient broth for 25 min at 35°C and concentrated by centrifugation at 4,248×*g*, 4^{o}C for 25 min (Sigma 3-18K centrifuge, Osterode am Harz, Germany) to obtain an initial density of $1.17x10^{10}$ CFU/mL. Plating was carried out on PDA agar to assess yeast growth.

Replicate or triplicate laboratory-scale fermentations were conducted using a simple batch system in sterile conical flasks containing 250-300 mL of sterile papaya juice at 20^{o}C for 14-21 days (plugged with cotton wool, then wrapped with aluminum foil) and subjected to various fermentation designs by inoculation of corresponding yeast pre-cultures described below:

- Fermentations were inoculated with ~10^5 CFU/mL of the three *Saccharomyces* yeasts that included *S. cerevisiae* var. *bayanus* EC 1118, R2 and *S. cerevisiae* Merit.ferm (**Chapter 4**).
- Fermentations were inoculated with ~10^5 CFU/mL of the three *Williopsis* yeasts that included *W. saturnus* var. *mrakii* NCYC2251, *W. saturnus* var. *saturnus* NCYC22, *W. saturnus* var. *sargentensis* NCYC2727 (**Chapter 5**).

- Fermentations were inoculated with ~10^5 CFU/mL of *W. saturnus* var. *mrakii* NCYC2251 and added with either 0.05% (w/v) of L-leucine, L-isoleucine, L-valine or L-phenylalanine, except for the control (**Chapter 6**).
- Fermentations were inoculated with ~10^5 CFU/mL of *W. saturnus* var. *mrakii* NCYC2251 and added with either 0.1% (v/v) (837 mg/L) or 0.5% (v/v) (4185 mg/L) (final concentrations quoted in parentheses) of fusel oil with density of 0.837 g/mL at 20°C, except for the control (**Chapter 7**).
- Mixed-culture fermentations were simultaneously inoculated with ~10^2 CFU/mL of *S. cerevisiae* var. *bayanus* R2 and ~10^5 CFU/mL of *W. saturnus* var. *mrakii* NCYC2251. Pure culture fermentations (monocultures) were also carried out under the same conditions (**Chapter 8**).
- Two types of sequential fermentations were carried out: initial inoculation of ~10^5 CFU/mL of *W. saturnus* var. *mrakii* NCYC2251, followed by ~10^4 CFU/mL of *S. cerevisiae* var. *bayanus* R2 after seven days (late log phase of *W. saturnus* growth) (positive sequential fermentation, PSF); initial inoculation of ~10^2 CFU/mL of *S. cerevisiae* R2, followed by ~10^5 CFU/mL of *W. saturnus* NCYC2251 after two days (late log phase of *S. cerevisiae* growth) (negative sequential fermentation, NSF). Mixed-culture fermentations (MCF, as control) simultaneously inoculated with ~10^2 CFU/mL of *S. cerevisiae* R2 and ~10^5 CFU/mL of *W. saturnus* NCYC2251. PSF was carried out to prolong survival and persistence of *W. saturnus* and to maximise its flavour impact, while NSF was carried out to examine the behavior of *W. saturnus* and its potential flavour impact (**Chapter 9**).
- Three different ratios of sequential fermentations were carried out with initial inoculation of ~10^5 CFU/mL of *W. saturnus* var. *mrakii* NCYC2251 and followed by the inoculation of ~10^6 CFU/mL, ~10^7 CFU/mL and ~10^8 CFU/mL of *S.*

cerevisiae var. *bayanus* R2 after seven days (late log phase of *W. saturnus* growth with ~10^7 CFU/mL) to achieve ratios of 10:1, 1:1 and 1:10 (*W. saturnus*: *S. cerevisiae*), respectively (**Chapter 10**).

3.4 Analytical determinations and yeast enumeration

For all fermentations, samples were taken at the indicated time points and subjected to microbiological and chemical analyses. The total soluble solids (^{o}Brix), pH and optical density (at 600 nm) were measured using a refractometer (ATAGO, Tokyo, Japan), pH meter (Metrohm, Herisau, Switzerland) and spectrometer (UV mini-1240, Shimadzu, Kyoto, Japan), respectively.

Sugars and organic acids (g/100 mL) were determined using Shimadzu modular chromatographic system (Kyoto, Japan) equipped with LC-20AD XR pumps, a SPD-M20A photodiode array detector, a low temperature evaporative light scattering detector (ELSD-LT), a SIL-20AC XR autoinjector and controlled via LC solution software version 1.25. Cell-free samples were obtained by centrifugation of the growth medium at 4,248×*g*, 4^{o}C for 25 min (Sigma 3-18K centrifuge, Osterode am Harz, Germany), filtered through a 0.20 µm RC membrane (Sartorius, Gottingen, Germany) and stored at -50^{o}C before analysis. Samples were analysed in triplicate. The identification and quantification of compounds were carried out by comparing retention time, spectrum and concentration with reference standards.

Organic acids were analysed with a Supelcogel C-610 H column (300 × 7.8 mm, Supelco, Bellefonte, PA, USA) using 0.1% (v/v) sulphuric acid mobile phase at a flow rate of 0.4 mL/min at 40^{o}C and detection was assessed by photodiode array at 210 nm. However, various columns were used for the separation of sugars in the different fermentations but quantification and detection were assessed by ELSD-LT

(gain: 5; 40°C; 350 kPa). The utilisation of different columns in the various fermentations was due to the availability of columns and technical difficulties such as high column back-pressure and shifted in retention time of sugars with extended column usage. Nevertheless, the results obtained for the initial sugar composition of papaya juice and the sugar consumption trend in the different fermentations were consistent (**Chapters 4-10**). For the different *Williopsis* yeasts and mixed-culture fermentations (**Chapters 5 and 8**), the sugar separation was achieved with a Prevail carbohydrate ES column (5 µm particle size, 150 x 4.6 mm) using an isocratic elution mobile phase of acetonitrile and water (78:22 v/v) at a flow rate of 0.5 mL/min at 25°C. Next, for the different strains of *S. cerevisiae* fermentation and *W. saturnus* NCYC2251 fermentations with the addition of selected amino acids or fusel oil (**Chapters 4, 6 and 7**), the determination of sugar was conducted on a Pinnacel II amino column (150 × 4.6 mm, Restek), using a mixture of acetonitrile and water (80:20 v/v) mobile phase at a flow rate of 1 mL/min at 40°C. For the remaining fermentations (**Chapters 9 and 10**), the separation of sugars was performed on a Zorbax carbohydrate column (150 x 4.6 mm, Agilent, Santa Clara, CA, USA) using a mixture of acetonitrile and water (80:20 v/v) as the mobile phase with a flow rate of 1.4 mL/min at 40°C.

Yeast growth during fermentations was assessed by viable cell quantification using the classical plate count method. Wine samples were diluted in 0.1% (w/v) peptone water before plating. Yeasts were enumerated by spread plating on PDA agar and incubated at 25°C for 48 h before colony counting. In mixed-culture and sequential fermentations, the colonies of *W. saturnus* (wrinkled, rough and dull) were morphologically differentiated from those of *S. cerevisiae* (shiny, defined round shape

and smooth). Lysine agar which is unable to support the growth of *S. cerevisiae* (Erten & Tanguler, 2010; Lin, 1975) was used to check the *W. saturnus* populations.

3.5 Volatile compound analysis

The volatile compounds in the papaya wines were determined and quantified by the optimised headspace (HS) solid-phase microextraction (SPME) method, coupled with gas chromatography (GC)-mass spectrometer (MS) and flame ionisation detector (FID) (HS-SPME-GC-MS/ FID). HS-SPME has been mainly used as a qualitative or semi-quantitative method for the analysis of wine aroma compounds (García et al., 1997; Trinh et al., 2011). The use of HS-SPME for quantitative purposes is strongly affected by the nature of the matrix, the amount of sample, desorbing conditions, the fibre coating, the extraction temperature, the extraction time, etc (Burman, Albertsson, & Hoglund, 2005). Nevertheless, Baptista, da P Tavares, and Carvalho (1998) and Campillo et al. (2008) stated that SPME could also be used as a quantitative method for accurate and precise analysis of volatiles, as long as consistent and optimised sampling conditions were utilised.

The fibre used for the absorption of volatiles was an 85 μm-fused silica fibre coated with carboxen/polydimethylsiloxane (PDMS) (Supelco, Sigma-Aldrich, Barcelona, Spain). Papaya wine sample of 5 mL (pH adjusted to 2.5 by using 1 M HCl) was sealed in a 20-mL vial with a septum lined with polytetrafluoroethylene (PTFE). Extraction was performed in the headspace using a SPME autosampler (CTC, Combi Pal, Switzerland) with extraction temperature set at 60°C for 50 min with 250 rpm agitation and the fibre was thermally desorbed into the injector port of Agilent 7890A gas chromatograph (Santa Clara, CA, USA) at 250°C for 3 min. Separation was performed with a capillary column (Agilent DB-FFAP, Santa Clara, CA, USA) of

60 m x 0.25 mm I.D. coated with 0.25 µm film thickness of polyethylene glycol modified with nitroterephthalic acid. The oven temperature was 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. The volatile compounds were identified by matching the mass spectra against those in the NIST 8.0 and Wiley 275 MS libraries, and confirmed with the linear retention index (LRI) values of pure standards or from the literatures. LRI values on the DB-FFAP column were determined using a series of alkanes (C5-C40) run under identical conditions. Samples were analysed in triplicate.

Selected major volatile compounds (**Chapters 5-10**) were quantified using individual external standard solutions that were prepared based on the method of Chen, Begnaud, Chaintreau, and Pawliszyn (2006) with modifications. The individual external standard solutions were prepared and diluted with 10% papaya juice-based aqueous solutions to obtain a range of concentrations, except for ethanol standard that was diluted in 100% papaya juice. All the standards were subjected to similar extraction protocols used for the samples and had R^2 values of at least 0.95 [Appendix A (Table A.1)]. Concentrations of volatile compounds were determined by using the linear regression equations of the corresponding standards. Odour activity values (OAVs) of quantified volatiles were calculated according to their known thresholds from literatures (Bartowsky & Pretorius, 2009; Ferreira, Lopez, & Cacho, 2000).

3.6 Sensory analysis

The papaya wines produced by mixed-culture and sequential fermentations (**Chapters 9 and 10**) were evaluated by a panel of five to eight experienced flavourists (a mixture of females and males) from Firmenich Asia Pte Ltd (Singapore) using quantitative descriptive analysis (QDA) methodology. A constant volume of wine was presented in wine-testing glasses and was arbitrarily coded. Eight sensory descriptors were selected by consensus to describe the papaya wine aroma: acidic, alcoholic, buttery, cocoa, fruity, fusel, sweet and yeasty notes. The papaya wine samples were only sniffed and the aroma intensity of each sensory descriptor was rated on a 5-point hedonic scale, where 0 indicated that the descriptor was not perceivable and 5 indicated that the descriptor had high intensity. The data were processed to obtain the modified frequency (*MF*) for the sensory descriptors as described in Tao, Liu, and Li (2009). The *MF* was calculated with the following formula: $MF(\%) = [F(\%)I(\%)]^{1/2}$, where $F(\%)$ is the detection frequency of an aromatic attribute expressed as percentage and $I(\%)$ is the average intensity expressed as a percentage of the maximum intensity.

3.7 Statistical analysis

An analysis of variance (ANOVA) using SPSS 17.0 software for Windows (SPSS Inc., Chicago, IL) was applied to the experimental data to determine significant differences between the samples. A Scheffé's method was used for pairwise comparisons for results that showed significant ANOVA differences. The confidence limits were based on 95% confidence level (significant difference when $p<0.05$). Principal component analysis (PCA) was performed using the software Matlab

R2008a (Mathworks, Natick, MA, USA) to discriminate among the means of chemical measurements of volatile compounds.

CHAPTER 4

DYNAMICS OF VOLATILE COMPOUNDS DURING PAPAYA JUICE FERMENTATION BY THREE COMMERCIAL WINE YEASTS

4.1 Introduction

The flavour of wine and other alcoholic beverages is a sensory perception that varies with individual, the context of the consumer's experience and the chemical composition of the product, and is an important attribute. The chemical composition of wine is the foundation of the sensory responses and is determined by many factors such as the fruit variety, the soil, the fermentation processes, and winemaking practices (Cole & Noble, 1995). Among these factors, yeasts play a very important role in wine flavour modulation, where a vast number of volatile compounds are formed and modulated by yeasts that significantly impact on the flavour and overall quality of wines. Ethanol, esters, higher alcohols, volatile acids, carbonyl compounds, volatile phenols and sulphur compounds are examples of volatile compounds produced by yeasts during fermentation. Esters, specifically acetate esters and fatty acid ethyl esters are present in all wines and contribute to 'fruity' characters that significantly influence wine aroma (Swiegers & Pretorius, 2005).

Wine is typically produced using a genetically homogeneous subgroup of *Saccharomyces cerevisiae* yeast strains and different strains or species of *Saccharomyces* can produce differential flavour profiles (Carrau, Medina, Farina, Boido, Henschke, & Dellacassa, 2008; Swiegers et al., 2009). In this way, controlling the alcoholic fermentation is an effective method for modulating wine aroma. The aim of this chapter was to evaluate the fermentation performance and the formation of

volatile compounds by three commercial *S. cerevisiae* wine yeasts, namely strains EC-1118, R2 and Merit.ferm, in papaya juice with the intention of selecting one yeast for further studies involving *Saccharomyces* and non-*Saccharomyces* yeasts to enhance papaya wine flavour. This is because multistarter fermentation of *Saccharomyces* and non-*Saccharomyces* yeasts has been intensively studied and applied to grape wine fermentation for flavour modulation and mouthfeel improvement (Fleet, 2008; Viana et al., 2009). Furthermore, commercial non-*Saccharomyces* yeasts comprised of *Kluyveromyces thermotolerans* and/or *Torulapora delbrueckii* (pure strains or blends of non-*Saccharomyces* and *Saccharomyces cerevisiae*) (Chr.-Hansen, Denmark) have emerged in the grape wine industry to enable wine differentiation through the addition of flavour complexity and enhancement of mouthfeel.

4.2 Results and discussion

4.2.1 Fermentation profiles of three commercial wine yeasts

The papaya juice used in this study showed a high potential for papaya wine production with a soluble solids content of about 12°Brix value. The three strains of *S. cerevisiae* yeasts had similar characteristics in terms of growth, pH changes, sugar consumption and organic acids changes [Fig. 4.1, Table 4.1, Appendix B (Fig. B1)]. The viable yeast cell population of all three cultures reached the maximum value on day 14 as shown in Fig. 4.1, where strain Merit.ferm showed the highest growth at 9.11 x 10^7 CFU/mL, followed by strain R2 at 7.96 x 10^7 CFU/mL and strain EC-1118 at 7.09 x 10^7 CFU/mL (Table 4.1). The ^{o}Brix values in all the three cultures displayed rapid reductions from day 0 to day 3 and reached ^{o}Brix values of around 3.80-3.97% at the end of the fermentation. The rate of alcoholic fermentations could be measured

by the decline in the soluble sugars content as these sugars were converted to ethanol, carbon dioxide and other secondary metabolites. The pH value did not fluctuate much over the fermentation period with values maintaining at around pH 3.58-3.67 (Table 4.1).

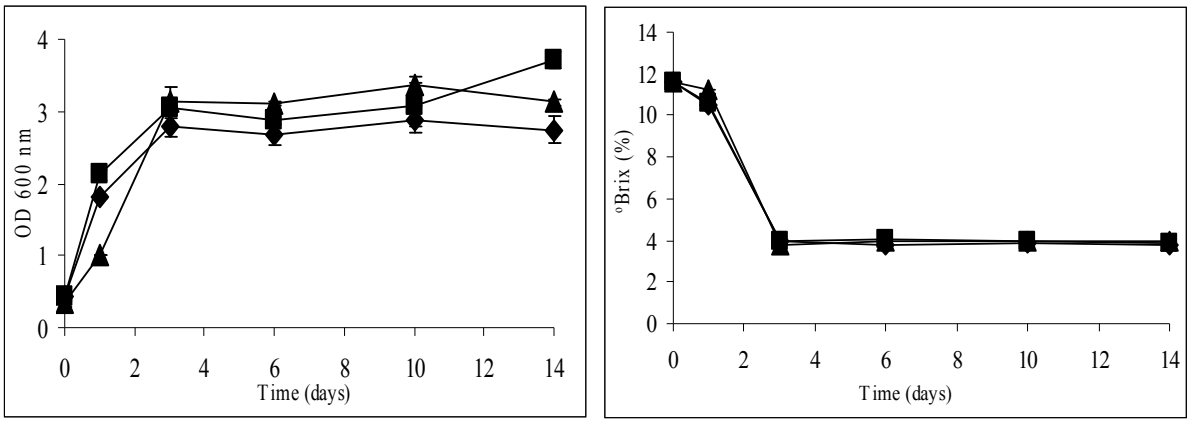

Fig. 4.1. Growth of yeasts (as optical density OD at 600 nm) and °Brix changes during papaya juice fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

The sugar concentrations (fructose and glucose) decreased rapidly as the fermentation progressed, which corresponded to the rapid reductions in the °Brix values [Fig. 4.1, Appendix B (Fig. B1)]. The final fructose and glucose concentrations at day 14 were similar for all the three cultures, with final concentrations at around 0.03 g/100 mL (Table 4.1). Fructose and glucose were utilised by the yeast cells as the source of energy for their growth and multiplication throughout fermentation.

Non-volatile organic acids play important roles in the physical, chemical and microbiological stability of wines besides providing flavour balance in wine by affecting the acidity (Swiegers, Saerens, & Pretorius, 2008). The composition of the organic acids in papaya juice is shown in Table 4.1. The high initial level of malic acid as compared to the other organic acids (Table 4.1) was attributed to the addition of malic acid before the start of fermentation for the acidification of papaya juice. The

original concentration of malic acid in papaya juice before acidification was 0.187 g/100 mL. Generally, the changes in organic acids were similar in all the three cultures [Table 4.1, Appendix B (Fig. B1)]. The malic and tartaric acids decreased, while acetic, citric and succinic acids either increased or remained relatively constant throughout fermentation. Interestingly, succinic acid was not produced by any of the *S. cerevisiae* yeasts, where succinic acid supposes to be the main acid produced by yeast during fermentation (Swiegers et al., 2005). This maybe due to the utilisation of succinic acid for the formation of volatile compounds such as mono- and diethyl succinate which are significant contributors to the body of a wine (Lambrechts & Pretorius, 2000). The significant reduction of malic acid during fermentation was likely due to its uptake and retention by cells of *S. cerevisiae* via passive diffusion (Coloretti, Zambonelli, Castellari, Tini, & Rainieri, 2002; Saayman & Viljoen-Bloom, 2006), rather than degradation, because *S. cerevisiae* neither metabolise D-malic acid (Coloretti et al., 2002) nor degrade L-malic acid efficiently (Redzepovic, Orlic, Majdak, Kozina, Volschenk, & Viljoen-Bloom, 2003). Strain EC-1118 produced the least amount of acetic acid with 0.051 g/100 mL, followed by strain Merit.ferm and strain R2 with 0.088 g/100 mL and 0.089 g/100 mL, respectively (Table 4.1). Generally, acetic acid becomes unpleasant at concentrations near the threshold of 0.07-0.10 g/100 mL and usually values between 0.02 and 0.07 g/100 mL are considered optimal in wine (Lambrechts & Pretorius, 2000; Mendoza & Farias, 2010).

Table 4.1. Oenological parameters of papaya wine (day 14) fermented with three commercial wine yeasts.

	Day 0	Yeast EC-1118	Yeast MERIT.ferm	Yeast R2
pH	3.58 ± 0.01[a]	3.65 ± 0.01[b]	3.67 ± 0.00[c]	3.66 ± 0.02[bc]
[o]Brix (%)	11.60 ± 0.01[a]	3.80 ± 0.09[b]	3.84 ± 0.08[b]	3.97 ± 0.10[b]
Yeast cell count x 10^6(CFU/mL)	0.30 ± 0.06[a]	70.90 ± 14.30[b]	91.10 ± 9.02[b]	79.60 ± 14.70[b]
[*]Estimated ethanol (%, v/v)	-	4.60 ± 0.05[a]	4.58 ± 0.05[a]	4.50 ± 0.06[a]
Sugars (g/100 mL)				
Fructose	3.72 ± 0.08[a]	0.03 ± 0.00[b]	0.03 ± 0.00[b]	0.03 ± 0.00[b]
Glucose	3.78 ± 0.01[a]	0.03 ± 0.00[b]	0.03 ± 0.00[b]	0.03 ± 0.00[b]
Organic acids (g/100 mL)				
Acetic acid	0.038 ±0.002[a]	0.051 ± 0.003[b]	0.088 ± 0.003[c]	0.089 ± 0.003[c]
Citric acid	0.280 ± 0.016[a]	0.256 ± 0.004[a]	0.264 ± 0.011[a]	0.260 ± 0.001[a]
Malic acid	1.002 ± 0.041[a]	0.683 ± 0.004[b]	0.638 ± 0.016[b]	0.679 ± 0.009[b]
Succinic acid	0.190 ± 0.005[a]	0.186 ± 0.014[a]	0.136 ± 0.015[b]	0.164 ± 0.009[ab]
Tartaric acid	0.016 ± 0.001[a]	0.011 ± 0.001[b]	0.010 ± 0.002[b]	0.009 ± 0.001[b]

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

[*]Estimated ethanol concentrations calculated by taking the difference between the initial and final [o]Brix values and multiplied by a factor of 0.59.

4.2.2 Volatile profiles of papaya juice

The major volatiles in papaya juice contained 14 esters, 7 volatile acids, 5 alcohols, 4 ketones, 1 aldehyde and 1 sulphur-containing compound (Table 4.2). The alcohol and volatile fatty acid detected at higher levels were ethanol and butyric acid, respectively. Esters such as methyl hexanoate, ethyl butyrate, methyl butyrate and methyl octanoate identified in papaya juice were the primary esters that contribute to the tropical fruit-like aroma of papaya, being similar to those reported in Pino et al. (2003). The sulphur-containing volatile compound, benzyl isothiocyanate was also found in papaya juice, which was formed by enzymic hydrolysis of glucosinolates during disruption of cell tissues (Tang, 1971) and imparted pungent off-odour in papaya (Moy, 2003). Most of the volatile compounds identified in the papaya juice are similar to those reported elsewhere (Table 4.2). Moreover, several fatty acids and ketones found in the present study, namely butyric acid, hexanoic acid, nonanoic acid, 2-undecanone, β-damascenone and geranylacetone, have not been reported in papaya juice (Table 4.2). The differences in the volatile composition between the present study and other studies (Almora et al., 2004; Flath & Forrey, 1977; Pino et al., 2003; Schwab et al., 1989) might be due to the different cultivars or different extraction/analytical methods used.

Table 4.2. Volatile compounds in papaya juice detected using HS-SPME-GC-MS/FID

Groups	Volatile compounds identified	GC-FID Peak Area	Relative Peak Area (%)	Compound reported in literatures
Acid	Acetic acid	$1.09x10^6$	1.54	Almora et al., 2004; Pino et al., 2003
	Butyric acid	$2.10x10^7$	29.71	
	Hexanoic acid	$1.03x10^6$	1.46	
	Octanoic acid	$1.05x10^6$	1.48	Pino et al., 2003
	Nonanoic acid	$1.24x10^5$	0.18	
	Decanoic acid	$5.97x10^5$	0.84	Pino et al., 2003
	Dodecanoic acid	$8.59x10^5$	1.21	Almora et al., 2004; Pino et al., 2003
Alcohol	Ethanol	$4.54x10^6$	6.40	
	Benzyl alcohol	$4.52x10^5$	0.64	Almora et al., 2004; Flath & Forrey, 1977; Pino et al., 2003; Schwab et al., 1989
	2-Ethylhexanol	$1.02x10^5$	0.14	
	2-Phenylethyl alcohol	$4.31x10^5$	0.61	Pino et al., 2003; Schwab et al., 1989
	Linalool	$2.35x10^4$	0.03	Almora et al., 2004; Flath & Forrey, 1977; Heidlas et al., 1984; Macleod & Pieris, 1983; Pino et al., 2003; Schwab et al., 1989; Winterhalter, Katzenberger, & Schreier, 1986
Aldehyde	Benzaldehyde	$2.22x10^6$	3.13	Pino et al., 2003
Ester	Ethyl butyrate	$1.55x10^5$	0.22	Almora et al., 2004; McGrath & Karahadian, 1994; Pino et al., 2003; Shiota, 1991
	Ethyl heptanoate	$2.61x10^5$	0.37	
	Ethyl dodecanoate	$1.04x10^5$	0.15	Almora et al., 2004; Pino et al., 2003
	Methyl butyrate	$1.36x10^6$	1.93	Almora et al., 2004; McGrath & Karahadian, 1994; Pino et al., 2003; Shiota, 1991
	Methyl hexanoate	$1.37x10^6$	1.94	McGrath & Karahadian, 1994; Shiota, 1991
	Methyl octanoate	$2.62x10^6$	3.77	McGrath & Karahadian, 1994; Pino et al., 2003; Shiota, 1991
	Methyl nonanoate	$9.34x10^4$	0.13	
	Methyl decanoate	$1.91x10^6$	2.70	Pino et al., 2003; Shiota, 1991
	Methyl undecanoate	$5.34x10^4$	0.08	
	Methyl dodecanoate	$2.96x10^6$	4.19	Pino et al., 2003
	Methyl tridecanoate	$4.58x10^4$	0.06	
	Methyl tetradecanoate	$1.65x10^6$	2.33	Pino et al., 2003
	Methyl palmitoleate	$9.91x10^6$	14.00	Pino et al., 2003
	Methyl hexadecanoate	$6.33x10^5$	0.89	
Ketone	6-Methyl-5-hepten-2-one	$8.20x10^6$	11.58	Pino et al., 2003
	2-Undecanone	$7.91x10^4$	0.11	
	β-Damascenone	$2.20x10^6$	3.10	
	Geranylacetone	$3.09x10^6$	4.37	
Heteroatom (*N, S*) compound	Benzyl isothiocyanate	$5.12x10^5$	0.72	Almora et al., 2004; Flath & Forrey, 1977; Heidlas et al., 1984; Macleod & Pieris, 1983; Pino et al., 2003; Schwab et al., 1989; Tang, 1971

4.2.3 Dynamic changes of volatile compounds during papaya juice fermentation

During papaya juice fermentation, a number of volatile compounds such as fatty acids, alcohols, esters, acetaldehyde and acetoin were produced, whereas volatiles indigenous to the juice such as benzaldehyde, benzyl isothiocyanate, β-damascenone and butyric acid were catabolised (Figs. 4.2–4.7).

The profile of production and degradation of fatty acids of C2 to C12 was similar among the three wine yeasts, except for acetic acid [Fig. 4.2, Appendix B (Fig. B2)]. Butyric and hexanoic acids that were present at relatively high concentrations in the juice were utilised during fermentation. The decrease in butyric and hexanoic acids corresponded to the increase in the formation of ethyl butyrate and ethyl hexanoate, respectively (Fig. 4.4). These ethyl esters were probably produced by the esterification of ethanol with the corresponding butyric and hexanoic acids that had undergone a previous activation by combining with coenzyme A. This esterification process is catalysed by the action of alcohol acyltransferase enzyme. On the other hand, octanoic, decanoic and dodecanoic acids increased initially, and then decreased. The initial formation of octanoic, decanoic and dodecanoic acids could be due to the repeated condensation of acetyl-CoA derived from sugar metabolism by fatty acid synthase (Gonzalez-Marco, Jimenez-Moreno, & Ancin-Azpilicueta, 2010). The subsequent decline in these fatty acids coincided with the increase in their corresponding esters. Acetic acid increased consistently throughout fermentation due to the oxidation of acetaldehyde by the enzyme acetaldehyde dehydrogenase. Among the three yeasts, strain EC-1118 produced the least amount of acetic acid, which was in line with the organic acids trend in Table 4.1. Acetic acid is an undesirable volatile acid in alcoholic beverages, imparting a vinegar off-odour and as such, strain EC-1118 was deemed as a desirable yeast for papaya wine production. There were

statistical differences in the amounts of fatty acids at day 14 among or between the yeasts (Table 4.3).

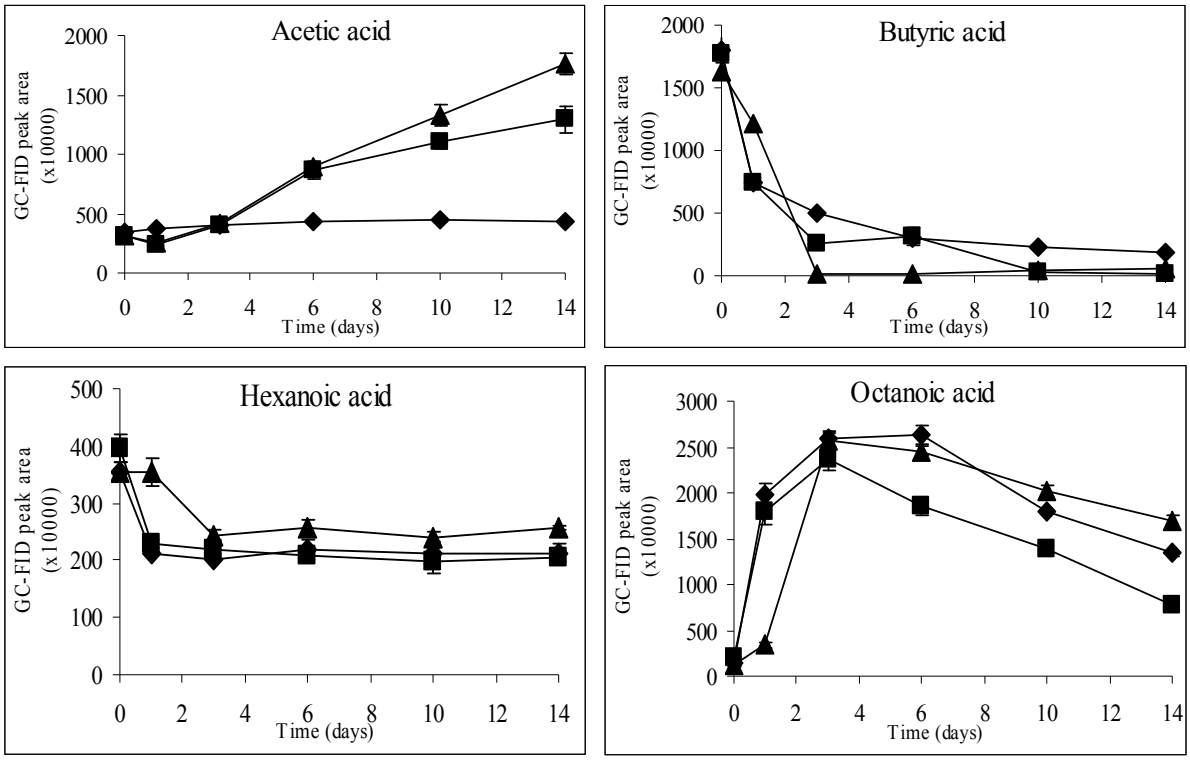

Fig. 4.2. Changes of fatty acids in papaya wine during fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

Ethanol, isobutyl alcohol (2-methyl-1-propanol), isoamyl alcohol (3-methyl-1-butanol) and 2-phenylethyl alcohol were the major alcohols produced by the three yeasts during papaya juice fermentation (Table 4.3). The dynamic changes of alcohols were similar [Fig. 4.3, Appendix B (Fig. B3)], whereas the final amounts of alcohols at day 14 varied significantly with yeasts and with strain EC-1118 consistently producing the least amount of each type of alcohol. Conversely, strain R2 produced the highest amount of ethanol and total alcohols (Table 4.3). Isobutyl alcohol was derived from L-valine and isoamyl alcohol was produced from L-leucine during yeast metabolism (Dickinson et al., 1997; Dickinson, Harrison, & Michael, 1998). 2-Phenylethyl alcohol was formed from L-phenylalanine (Etschmann, Bluemke, Sell, &

Schrader, 2002). Higher alcohols, recognised for their strong and pungent smell and taste, can have a significant influence on the taste and character of wine (Lambrechts & Pretorius, 2000). Higher alcohols could also influence the sensory properties of the wine by serving as precursors for ester formation (Soles et al., 1982) and thus enhancing the fruity flavour of the wine by causing an increase in the content of esters (Valero, Moyano, Millan, Medina, & Ortega, 2002).

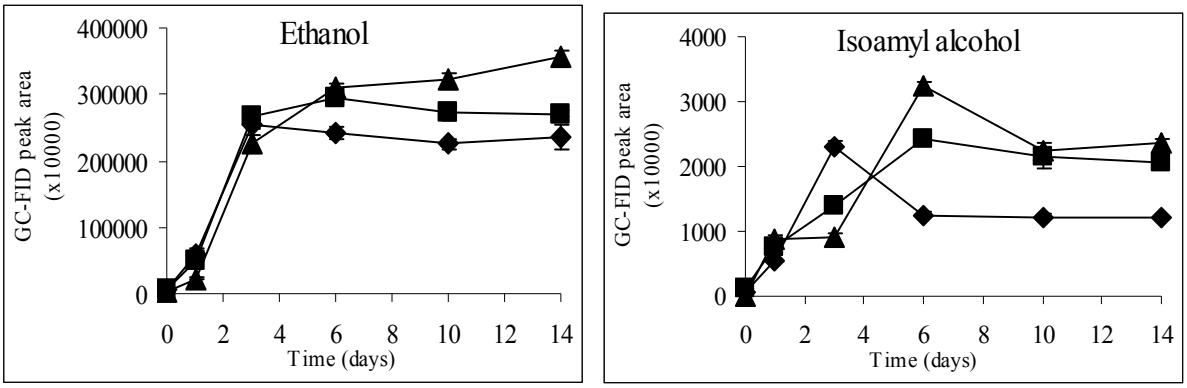

Fig. 4.3. Changes of ethanol and isoamyl alcohol in papaya wine during fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

Esters were the next major volatiles produced by the three yeasts during papaya juice fermentation with relative peak areas (RPA) ranging from 14.41% to 19.15%, which included ethyl esters, methyl esters, acetate esters and other esters (Table 4.3). Some esters increased initially and then remained stable during fermentation, while other esters like isoamyl acetate and 2-phenylethyl acetate increased initially, and was followed by a steady and sharp decline [Figs. 4.4 and 4.5, Appendix B (Figs. B4 and B5)]. The formation of carboxylate esters by yeasts involves the enzymatic reaction between an alcohol group and the CoA-activated acid (Park, Shaffer, & Bennett, 2009), while the degradation of esters could have occurred by hydrolysis due to the wine acidity and the presence of esterase and lipases which

split the ester to its principal alcohol and acid moieties (Sumby, Grbin, & Jiranek, 2010). The formation or degradation of esters can have dramatic effects on the sensorial quality of fermented beverages, depending on the type and quantity of esters. The dynamic changes of esters were similar among the three yeasts with the exception of ethyl butyrate and ethyl hexanoate (Fig. 4.4). The final amounts of some esters at day 14 varied significantly among or between the yeasts at $p<0.05$ (Table 4.3). Among the three yeasts, strain R2 had the highest production of most acetate esters (Table 4.3), which could be linked to the high quantities of alcohols that strain R2 produced (Table 4.3). Conversely, strain EC1118 produced the highest amount of ethyl hexanoate and ethyl dodecanoate (Table 4.3). Acetate esters contribute fruity and floral notes, except for ethyl acetate, which imparts light fruity and solvent-like aroma at excessive levels. Ethyl esters of fatty acids contribute pleasant fruity, floral and honey-like flavours (Luebke, 1980).

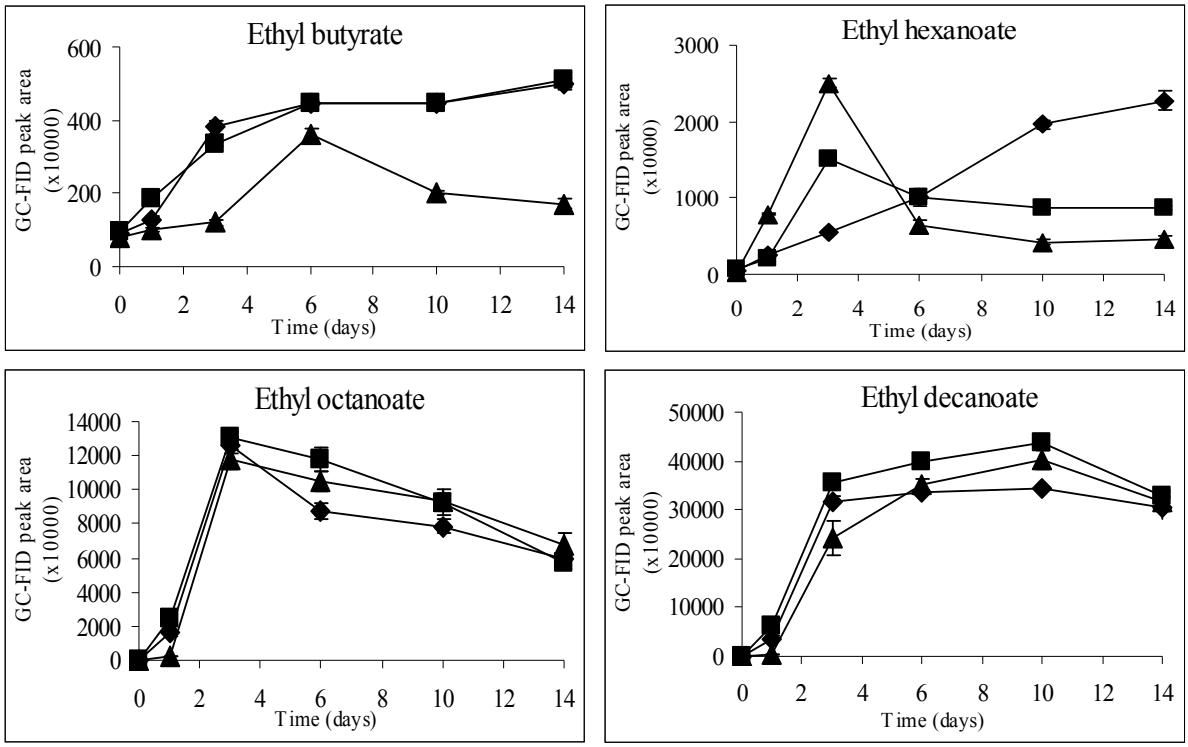

Fig. 4.4. Changes of ethyl esters in papaya wine during fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

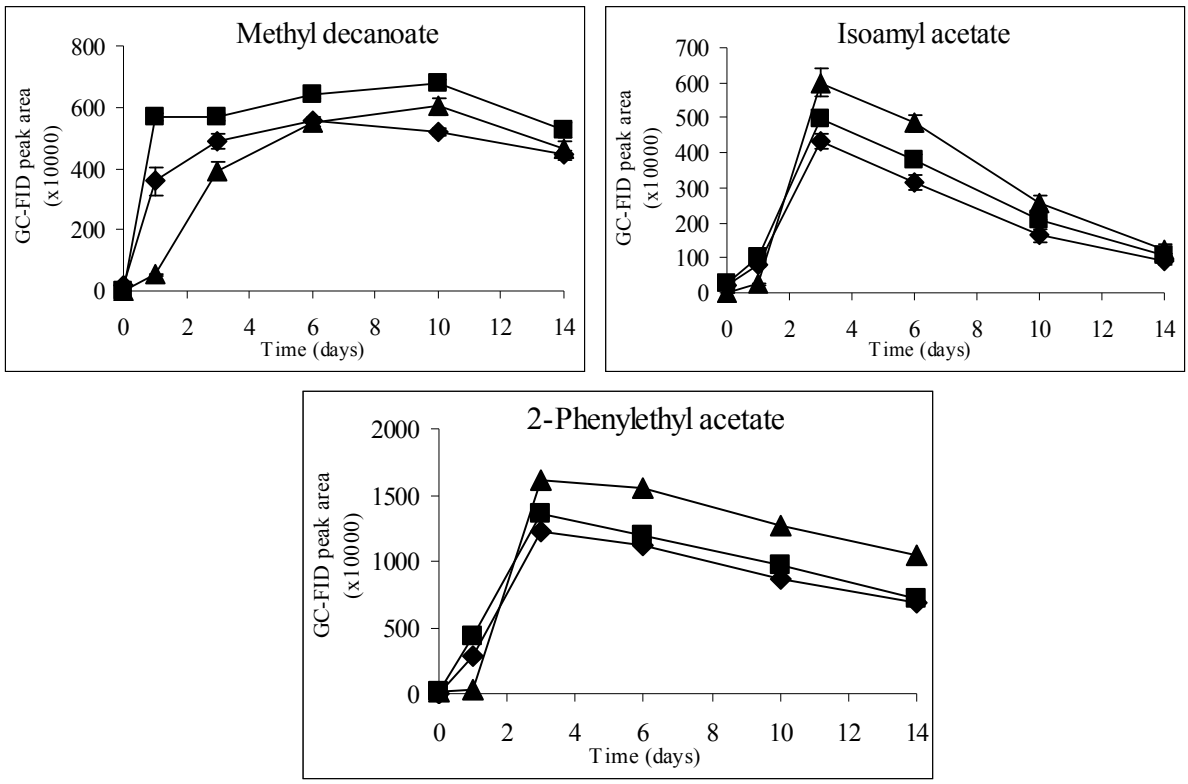

Fig. 4.5. Changes of methyl decanoate and acetate esters in papaya wine during fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

Among the aldehydes, O-tolualdehyde and benzaldehyde (present in the juice) were metabolised to trace levels during fermentation, while acetaldehyde was produced by all three yeasts [Fig. 4.6, Appendix B (Fig. B6)]. The dynamic changes and final amounts of aldehydes were essentially identical among the three yeasts. There were no statistical differences in the final quantities of aldehydes among the yeasts (Table 4.3). Acetaldehyde is a major component of aldehydes constituting more than 90% of the total aldehyde content (Nykanen, 1986) and it plays an important role in the aroma and bouquet of wine. Acetaldehyde originates as an intermediate product of yeast metabolism from pyruvate through the glycolytic pathway and it is also a precursor for acetate and 3-hydroxy-2-butanone (acetoin) production (Collins, 1972) as well as ethanol. The accumulation of acetaldehyde

occurred as sugars are continuously metabolised and due to the need for NAD^+ regeneration under anaerobic conditions (Swiegers et al., 2005). It is well-documented that differences exist in the amounts of acetaldehyde formed by yeasts and that *S. cerevisiae* strains can produce relatively high levels of acetaldehyde from 50 to 120 mg/L (Fleet & Heard, 1993). The reduction of aldehydes observed in this study corresponded to those of van Iersel, Brouwer-Post, Rombouts, and Abee (2000), where higher aldehydes are reduced to their respective alcohols to regenerate cofactors. Moreover, aldehydes could also be oxidised by aldehyde dehydrogenase to form carboxylic acids and eventually esters (Sumby et al., 2010).

Among the ketones, β-damascenone concentration decreased during fermentation, whereas acetoin increased [Fig. 4.6, Appendix B (Fig. B6)]. There were no strain differences in β-damascenone utilisation, whereas strain variations exist in acetoin formation detected at the end of fermentation with strain Merit.ferm producing the highest amount of acetoin with 0.05% (RPA) (Table 4.3). Acetoin (3-hydroxy-2-butanone) was a by-product of *S. cerevisiae* metabolism in the early phase but was reduced to 2,3-butanediol at the later stage (Guymon & Crowell, 1965). The accumulation of acetoin was predominantly due to the presence of increasing amount of acetaldehyde and imparted creamy and butter-like notes to wine (Collins, 1972). Nevertheless, Romano and Suzzi (1993b) proposed three synthetic pathways of 3-hydroxy-2-butanone. Firstly, active acetaldehyde and acetyl-CoA can be condensed to form diacetyl that is reduced to 3-hydroxy-2-butanone. Next, α-acetolactate (derived from the condensation of active acetaldehyde and pyruvate) can be decarboxylated to form 3-hydroxy-2-butanone. Thirdly, active acetaldehyde can be combined with another molecule of acetaldehyde directly to form 3-hydroxy-2-butanone without the need of an intermediate.

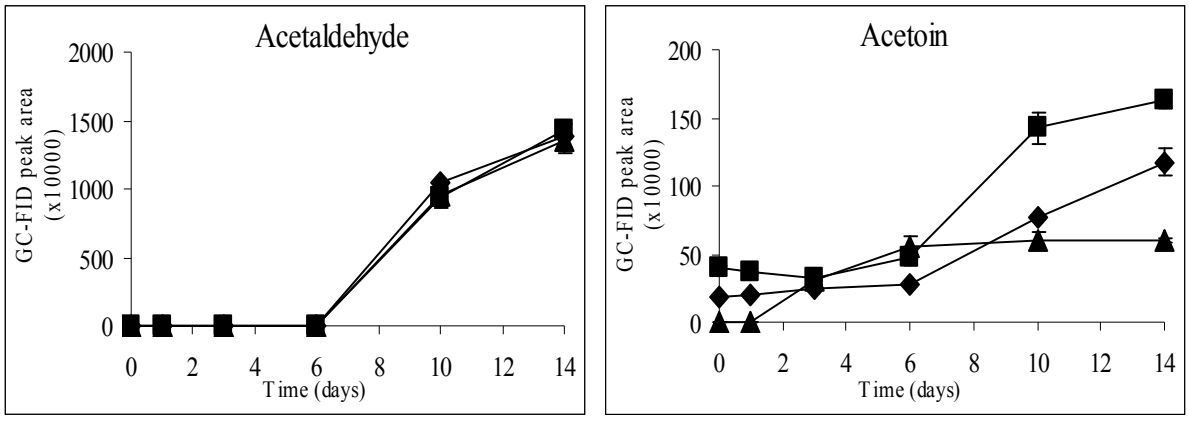

Fig. 4.6. Changes of acetaldehyde and acetoin in papaya wine during fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

Benzyl isothiocyanate (the naturally present sulphur-containing volatile compound in the juice) was almost completely degraded as fermentation progressed. A similar trend was observed for all fermentations (Fig. 4.7). This compound is responsible for the characteristic pungent odour in papaya juice (Moy, 2003) and with its degradation during fermentation, the pungent odour also diminished.

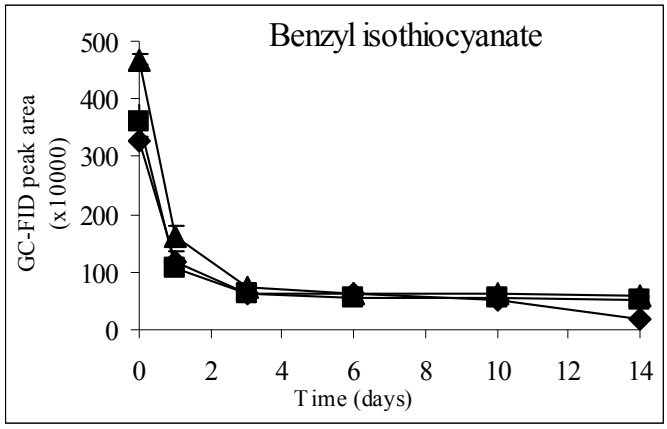

Fig. 4.7. Changes of benzyl isothiocyanate in papaya wine during fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

Table 4.3. Major volatile compounds (GC-FID peak area x 10^6) and their relative peak areas (RPA) identified in papaya wine fermented with three commercial wine yeasts at day 14 and analysed using HS-SPME-GC-MS/FID

No.	Volatile compounds identified	CAS no.[d]	Yeast EC-1118 Peak area	Yeast EC-1118 RPA (%)	Yeast Merit.ferm Peak area	Yeast Merit.ferm RPA (%)	Yeast R2 Peak area	Yeast R2 RPA (%)	Organoleptics[e]
	Acids								
1	Acetic acid	000064-19-7	4.28 ± 0.65[a]	0.14	12.90 ± 1.98[c]	0.37	17.60 ± 1.50[b]	0.40	Acidic, pungent, vinegar-like
2	Butyric acid	000107-92-6	1.89 ± 0.58[a]	0.06	0.15 ± 0.01[b]	0.00	0.15 ± 0.01[b]	0.01	Acidic, buttery, cheesy
3	Hexanoic acid	000142-62-1	2.11 ± 0.19[a]	0.07	2.05 ± 0.11[a]	0.06	2.57 ± 0.05[b]	0.06	Acidic, cheesy, fruity
4	Octanoic acid	000124-07-2	13.40 ± 0.47[a]	0.44	7.79 ± 0.49[c]	0.23	16.80 ± 1.23[b]	0.38	Acidic, cheesy, fatty, sweaty
5	Decanoic acid	000334-48-5	33.90 ± 1.71[a]	1.10	25.20 ± 3.17[b]	0.73	37.20 ± 3.27[a]	0.85	Buttery, condensed, milky
6	Dodecanoic acid	000143-07-7	7.03 ± 0.72[a]	0.23	4.40 ± 0.70[b]	0.13	6.45 ± 0.63[a]	0.15	Fatty, soapy, waxy
	Subtotal		**62.61**	**2.04**	**52.49**	**1.52**	**81.14**	**1.86**	
	Alcohols								
7	Ethanol	000064-17-5	2360 ± 308[a]	76.87	2710 ± 62.00[a]	78.64	3570 ± 308[b]	81.77	Alcoholic, solventy
8	Isobutyl alcohol	000078-83-1	2.91 ± 0.20[a]	0.09	4.88 ± 0.45[c]	0.14	6.73 ± 0.68[b]	0.15	Breathtaking, whisky
9	Isoamyl alcohol	000123-51-3	12.00 ± 1.82[a]	0.39	20.60 ± 2.77[b]	0.60	23.50 ± 2.05[b]	0.54	Alcoholic, fermented, whiskey
10	2-Phenylethyl alcohol	000060-12-8	26.60 ± 3.49[a]	0.87	30.70 ± 1.17[a]	0.89	37.80 ± 2.43[b]	0.87	Floral, honey, rosy
	Subtotal		**2401.51**	**78.22**	**2766.18**	**80.27**	**3638.03**	**83.33**	
	Aldehydes								
11	Acetaldehyde	000075-07-0	13.90 ± 1.46[a]	0.45	14.20 ± 1.02[a]	0.41	13.60 ± 1.57[a]	0.31	Aldehydic, ethereal, fruity
12	Benzaldehyde	000100-52-7	0.46 ± 0.04[a]	0.01	0.39 ± 0.03[a]	0.01	0.35 ± 0.03[a]	0.01	Bitter almond, cherry, sweet
13	O-Tolualdehyde	000529-20-4	2.11 ± 0.31[a]	0.07	2.29 ± 0.25[a]	0.07	2.40 ± 0.26[a]	0.05	Bitter almond, cherry pit, sweet
	Subtotal		**16.47**	**0.54**	**16.88**	**0.49**	**16.35**	**0.37**	
	Esters								
14	Methyl octanoate	000111-11-5	0.91 ± 0.06[a]	0.03	0.83 ± 0.07[a]	0.02	0.94 ± 0.10[a]	0.02	Citrus, green, fruity
15	Methyl decanoate	000110-42-9	4.44 ± 0.26[a]	0.14	5.26 ± 0.43[a]	0.15	4.64 ± 0.48[a]	0.11	Fatty, cognac, oily
16	Methyl dodecanoate	000111-82-0	2.02 ± 0.37[a]	0.07	1.67 ± 0.24[a]	0.05	1.59 ± 0.15[a]	0.04	Creamy coconut, waxy
17	Ethyl butyrate	000105-54-4	5.01 ± 0.27[a]	0.16	5.10 ± 0.33[a]	0.15	1.72 ± 0.23[b]	0.04	Fruity, ripe, sweet
18	Ethyl hexanoate	000123-66-0	22.70 ± 2.25[a]	0.74	8.65 ± 0.41[c]	0.25	4.47 ± 0.48[b]	0.10	Fruity, pineapple-like, winey

Table 4.3. (Continued)

No.	Volatile compounds identified	CAS no.[d]	Yeast EC-1118 Peak area	Yeast EC-1118 RPA (%)	Yeast Merit.ferm Peak area	Yeast Merit.ferm RPA (%)	Yeast R2 Peak area	Yeast R2 RPA (%)	Organoleptics[e]
19	Ethyl octanoate	000106-32-1	60.00 ± 2.19[a]	1.95	57.20 ± 5.36[a]	1.66	67.40 ± 5.19[a]	1.54	Fruity, cognac, yeasty
20	Ethyl 9-decenoate	067233-91-4	27.70 ± 1.26[a]	0.90	46.70 ± 4.61[c]	1.36	64.10 ± 8.05[b]	1.47	Fatty, fruity
21	Ethyl decanoate	000110-38-3	305 ± 3.87[a]	9.93	329 ± 11.10[a]	9.55	317 ± 37.90[a]	7.26	Fatty, fruity, winey
22	Ethyl dodecanoate	000106-33-2	94.40 ± 1.40[a]	3.07	77.50 ± 6.23[b]	2.25	76.70 ± 1.23[b]	1.76	Fruity, oily, waxy
23	Ethyl tetradecanoate	000124-06-1	3.85 ± 0.71[a]	0.13	3.62 ± 0.34[a]	0.11	4.11 ± 0.33[a]	0.09	Creamy, oily, waxy
24	Ethyl 9-hexadecenoate	054546-22-4	20.60 ± 3.31[a]	0.67	28.50 ± 3.86[b]	0.83	32.70 ± 3.68[b]	0.75	Creamy, waxy
25	Ethyl hexadecanoate	000628-97-7	8.64 ± 0.44[a]	0.28	7.72 ± 0.80[a]	0.22	9.99 ± 0.56[b]	0.23	Creamy, fruity, milky
26	Ethyl oleate	000111-62-6	3.43 ± 0.31[a]	0.11	3.10 ± 0.31[a]	0.09	3.61 ± 0.41[a]	0.08	Floral, waxy
27	Isoamyl octanoate	002035-99-6	1.90 ± 0.17[a]	0.06	1.38 ± 0.05[b]	0.04	2.24 ± 0.20[a]	0.05	Cognac, fatty, oily
28	Isobutyl decanoate	030673-38-2	0.60 ± 0.08[a]	0.02	0.80 ± 0.04[c]	0.02	0.96 ± 0.08[b]	0.02	Brandy, cognac, oily
29	Isoamyl decanoate	002306-91-4	3.67 ± 0.48[a]	0.12	3.65 ± 0.11[a]	0.11	3.97 ± 0.34[a]	0.09	Cognac, green, waxy
30	Ethyl acetate	000141-78-6	15.40 ± 1.71[a]	0.50	19.50 ± 1.30[b]	0.57	21.20 ± 2.47[b]	0.49	Ethereal, fruity, solventy
31	Isoamyl acetate	000123-92-2	0.92 ± 0.18[a]	0.03	1.06 ± 0.11[a]	0.03	1.25 ± 0.13[a]	0.03	Banana-like, fruity, sweet
32	2-Phenylethyl acetate	000103-45-7	6.83 ± 0.30[a]	0.22	7.13 ± 0.11[a]	0.21	10.50 ± 0.11[b]	0.24	Floral, rosy, honey
	Subtotal		**588.02**	**19.15**	**608.37**	**17.65**	**629.09**	**14.41**	
	Ketones								
33	3-Hydroxy-2-butanone	000513-86-0	1.18 ± 0.36[a]	0.04	1.63 ± 0.09[c]	0.05	0.60 ± 0.05[b]	0.01	Buttery, creamy, sweet
34	β-Damascenone	023726-93-4	0.12 ± 0.02[a]	0.00	0.15 ± 0.01[a]	0.00	0.15 ± 0.02[a]	0.00	Fruity, floral, woody
	Subtotal		**1.3**	**0.04**	**1.78**	**0.05**	**0.75**	**0.02**	
	Heteroatom (N, S) compound								
35	Benzyl isothiocyanate	000622-78-6	0.19 ± 0.03[a]	0.01	0.52 ± 0.05[b]	0.02	0.58 ± 0.04[b]	0.01	Horseradish-like, hot, pungent
	Total		**3070.1**		**3446.22**		**4365.94**		

[a,b,c]Statistical analysis at 95% confidence level with same letters indicating no significant difference.
[d]CAS number obtained from Wiley MS library.
[e]Odor descriptions obtained from Luebke (1980).

4.2.4 Principal component analysis

To highlight differences between the three commercial wine yeasts, major volatile compounds from Table 4.3 were subjected to principal component analysis (PCA). The PCA discriminated the common characteristics and revealed the diversity in the volatile composition among the different cultures (Fig. 4.8). The first principal component (PC1) accounted for 63.54% of the total variance that characterised the distinction of strain R2 from the other two yeasts, while PC2 explained the remaining 36.46%. Strain R2 (with positive scores) was mainly characterised by alcohols, acetate esters, isoamyl decanoate, ethyl octanoate and hexanoic acid. Conversely, strain EC-1118, positioned on the upper left quadrant, had correlation with methyl dodecanoate, ethyl hexanoate, ethyl dodecanoate, benzaldehyde and butyric acid. Strain MERIT.ferm was more related to acetaldehyde, acetoin and ethyl butyrate.

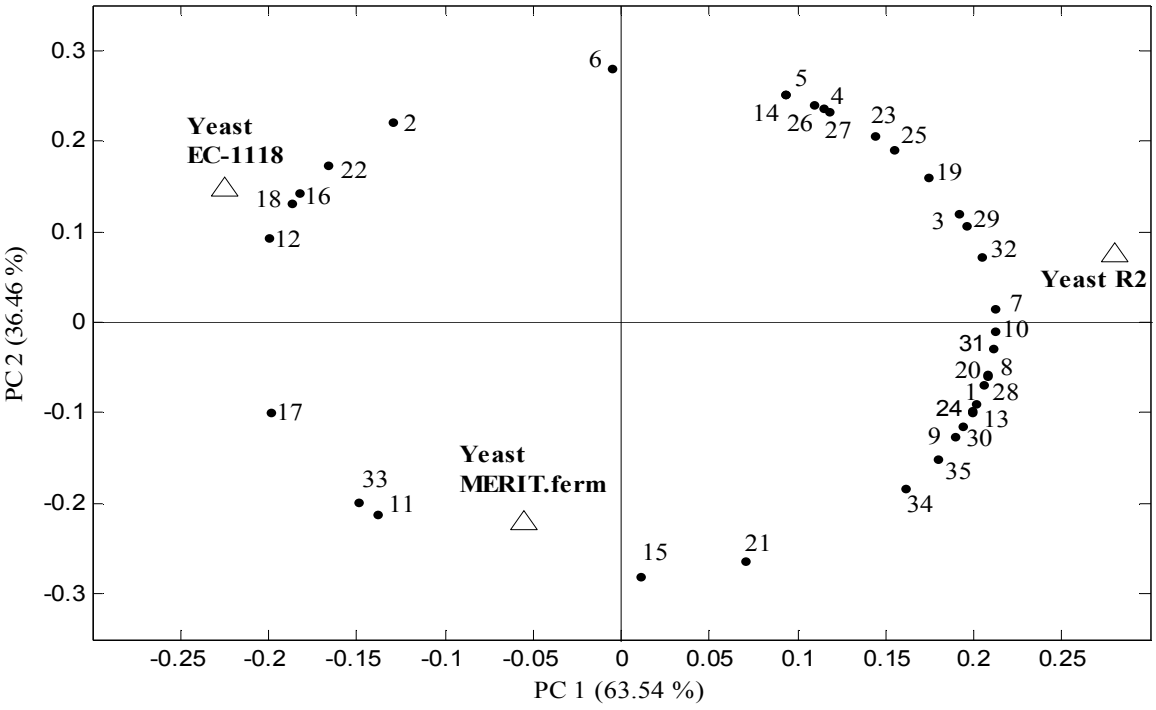

Fig. 4.8. Bi-plot of principal component analysis of the major volatile compounds in papaya wine fermented with three commercial wine yeasts. The major volatile compounds and numbers are given in Table 4.3.

4.3 Conclusions

In this chapter, attempts were made to evaluate the fermentation performance and dynamic changes of volatile compounds during papaya juice fermentation by three commercial wine yeasts (strains EC-1118, R2 and MERIT.ferm). On the one hand, some naturally-occurring volatiles in papaya juice were degraded; on the other hand, a wide range of volatile compounds were produced during papaya juice fermentation including alcohols, volatile fatty acids and especially esters (acetate and ethyl esters). The evolution profiles of volatile compounds during fermentation were similar among the three yeasts, although the volatile composition and final concentrations of some volatile compounds differed significantly at the 95% confidence level. It remains to be ascertained whether these statistical differences translate into sensory differences. Among the three yeast strains, strain R2 seems to be a more suitable candidate for subsequent multistarter fermentations with non-*Saccharomyces* yeast (*W. saturnus*) as compared to the other two yeast strains (**Chapters 8-10**) due to its better profile of ethanol and higher alcohols which are essential precursors for esters formation.

CHAPTER 5

EVOLUTION OF VOLATILE COMPOUNDS IN PAPAPYA WINE FERMENTED WITH THREE *WILLIOPSIS SATURNUS* YEASTS

5.1 Introduction

Traditionally, most wines are produced by *Saccharomyces* yeasts due to homogeneity of fermentation and ease of control. However, these wines lack flavour complexity, stylistic distinction and vintage variability contributed by indigenous yeasts (Lambrechts & Pretorius, 2000). This has led to studies on other yeasts especially non-*Saccharomyces* yeasts. Non-*Saccharomyces* yeasts such as *Hanseniaspora*, *Candida*, *Pichia* and *Metschnikowia* are present in the initial stages of fermentation process. Moreover, these yeasts are reported to influence the final organoleptic properties of the wine, as they are the main producers of some fermentation compounds such as acetic acid, glycerol and esters (Rojas, Gil, Pinaga, & Manzanares, 2001; Romano et al., 1993, 1997). Other studies have also shown their capabilities to contribute positively to wine flavour (Ciani & Maccarelli, 1998; Gil et al., 1996). Strain biodiversity exists in the non-*Saccharomyces* wine yeasts with regard to their levels of enzymatic activities (Manzanares, Rojas, Genoves, & Valles, 2000) and fermentation metabolites (Capece, Fiore, Maraz, & Romano, 2005) that give rise to the unique oenological characteristics of each wine-producing zone.

The genus *Hansenula* (now *Williopsis*) was originally introduced to accommodate the saturn-shaped ascospore-forming, nitrate-assimilating species *W. saturnus* (James, Roberts, & Collins, 1998). It has been reported that *Williopsis* yeasts are potent producers of esters (Inoue, Trevanichi, Fukuda, Izawa, Wakai, & Kimura, 1997) and *W. saturnus*, in particular, can convert higher alcohols into their

corresponding acetate esters such as isoamyl acetate (Janssens et al., 1992; Yilmaztekin, Erten, & Cabaroglu, 2008). *Williopsis* species are able to synthesise high levels of volatile esters in YPD medium, e.g. isoamyl acetate, at concentration range of 12–73 mg/L that has a characteristic banana and pear drops flavour impact (Iwase, Morikawa, Fukuda, Sasaki, & Yoshitake, 1995). It is generally not found from the natural environment like the surfaces of fruits or winery equipments. However, with the production of desirable volatile compounds, *W. saturnus* can potentially enhance the fruity flavour in wines obtained from cultivars with neutral characteristics.

The aim of this chapter was to investigate the fermentation performance and the evolution of volatile compounds by three *Williopsis* yeast strains - *W. saturnus* var. *mrakii* NCYC2251, *W. saturnus* var. *saturnus* NCYC22 and *W. saturnus* var. *sargentensis* NCYC2727 in papaya juice with the aim of selecting one strain for further studies involving *Saccharomyces* and non-*Saccharomyces* yeasts to modulate papaya wine flavour and improve wine quality.

5.2 Results and discussion

5.2.1 Fermentation profiles of three *Williopsis* yeasts

The three strains of *W. saturnus* yeasts showed similar characteristics in terms of pH changes, total soluble solids (^{o}Brix), sugar consumption and yeast growth (Figs. 5.1 and 5.2). The pH value did not fluctuate much over the fermentation period with values maintaining at around pH 3.58 – 3.76 (Table 5.1). The ^{o}Brix value displayed a gradual reduction and reached a final ^{o}Brix value ranging from 3.40 – 5.25% (Table 5.1). Strain NCYC22 had the fastest rate of sugar consumption (Figs. 5.1 and 5.2). The three strains of *W. saturnus* yeasts appeared to be glucophilic, consuming glucose faster than fructose (Fig. 5.2).

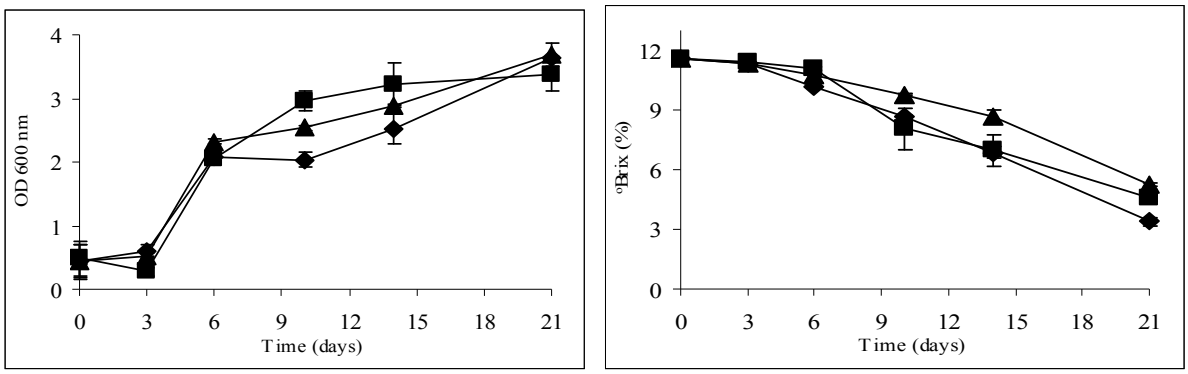

Fig. 5.1. Growth of yeasts (as optical density OD 600 nm) and °Brix changes during papaya juice fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (♦), *W. saturnus* var. *mrakii* NCYC2251 (▲) and *W. saturnus* var. *sargentensis* NCYC2727 (■). (Error bars = standard deviation).

The changes of organic acids were similar in all the three cultures. Malic and tartaric acids decreased, while acetic and succinic acids increased and, citric acid remained essentially unchanged throughout the fermentation with strain NCYC2727 producing the highest amount of acetic acid [Table 5.1, Appendix C (Fig. C1)]. The significant reduction of malic acid corresponded to those observed in **Chapter 4**. Malic acid can be weakly metabolised by wine yeasts to form pyruvate, and subsequently to ethanol during fermentation. However, this pathway was strain-dependent, whereby *Williopsis* yeast was reported to demonstrate weak metabolism of this organic acid (Radler, 1993). Hence, this may be explained by the report of Coloretti et al. (2002) and Saayman and Viljoen-Bloom (2006) that D- and L-malic acid molecules could enter the cells of yeast by means of simple diffusion. Similarly, the decline in tartaric acid could be due to uptake by the yeast or precipitation as potassium hydrogen tartrate, or more commonly known as cream of tartar in wines as yeast do not have the necessary mechanisms required for tartaric acid degradation (Gao & Fleet, 1995). Non-*Saccharomyces* yeasts have been associated with high levels of acetic acid production as compared to *Saccharomyces* yeasts (du Toit &

Pretorius, 2000). However, the amounts of acetic acid produced by strains NCYC2251 and NCYC22 were within the optimal acetic acid concentration range of 0.02-0.07 g/100 mL reported for wine (Lambrechts & Pretourius, 2000). Among the three cultures, strain NCYC2251 manifests the highest production of succinic acid (Table 5.1). These results corresponded to the findings in Ciani and Maccarelli (1998), where the succinic acid production varied significantly amongst non-*Saccharomyces* yeasts. Generally, succinic acid imparts mild and pleasant flavour to the wine, but affects total acidity (TA) of wine with excessive accumulation (Swiegers et al., 2005). Abnormal succinic acid accumulation during fermentation has been associated with several factors such as yeast strains, fermentation conditions and must composition (nutrient content, pH and sulphur dioxide concentration) (Coulter, Godden, & Pretorius, 2004).

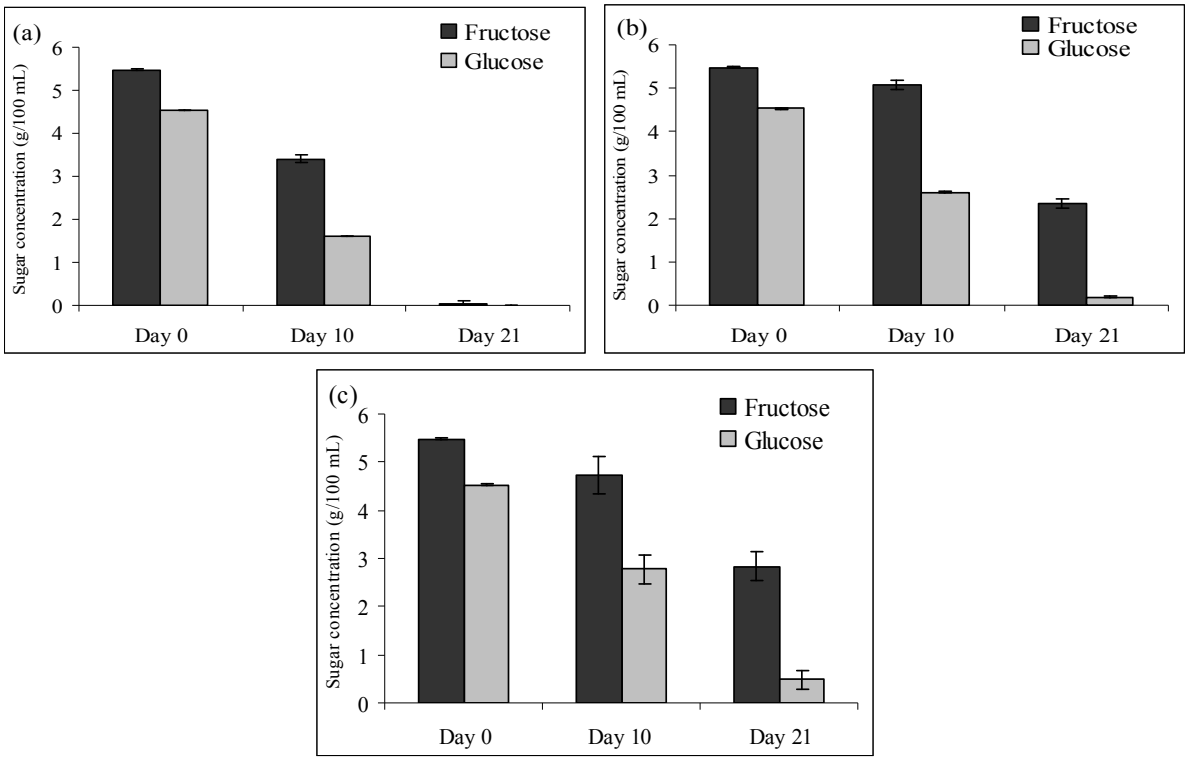

Fig. 5.2. Sugar consumption in papaya wine during fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (a), *W. saturnus* var. *mrakii* NCYC2251 (b) and *W. saturnus* var. *sargentensis* NCYC2727 (c). (Error bars = standard deviation).

The viable yeast cell population of all the three cultures reached the maximum at day 21 with strain NCYC2251 showing the highest growth at 1.67 x 10^8 CFU/mL, followed by strain NCYC22 at 8.00 x 10^7 CFU/mL and strain NCYC2727 grew to a lesser extent, reaching a population of 5.94 x 10^7 CFU/mL from the initial cell population of about 1.5 x 10^5 CFU/mL (Table 5.1). Strain NCYC2251 with a viable yeast cell population twice more than that of strains NCYC22 and NCYC2727 is a better candidate to be used in multistarter fermentations, as during spontaneous fermentation, non-*Saccharomyces* yeasts would normally die off before the *Saccharomyces* wine yeasts due to the former being less ethanol-tolerant, leaving the latter to dominate and eventually complete the fermentation (Cocolin, Bisson, & Mills, 2000; Fleet & Heard, 1993).

Table 5.1. Oenological parameters of papaya wine (day 21) fermented with three *W. saturnus* yeasts.

	Day 0	**Yeast NCYC22**	**Yeast NCYC2251**	**Yeast NCYC2727**
pH	3.58 ± 0.00[a]	3.76 ± 0.01[b]	3.68 ± 0.01[c]	3.67 ± 0.01[c]
[o]Brix (%)	11.60 ± 0.01[a]	3.40 ± 0.21[b]	5.25 ± 0.08[c]	4.58 ± 0.14[d]
Yeast cell count x 10^6(CFU/mL)	0.15 ± 0.01[a]	80.0 ± 1.06[b]	167 ± 43.70[c]	59.40 ± 17.60[b]
Ethanol (%, v/v)	0.03 ± 0.00[a]	2.35 ± 0.10[b]	1.78 ± 0.10[c]	2.16 ± 0.12[b]
Sugars (g/100 mL)				
Fructose	5.48 ± 0.02[a]	0.05 ± 0.06[b]	2.35 ± 0.12[c]	2.83 ± 0.30[d]
Glucose	4.53 ± 0.01[a]	0.01 ± 0.00[b]	0.19 ± 0.02[c]	0.49 ± 0.20[d]
Organic acids (g/100 mL)				
Acetic acid	0.033 ± 0.001[a]	0.058 ± 0.001[b]	0.049 ± 0.004[b]	0.077 ± 0.015[c]
Citric acid	0.244 ± 0.001[a]	0.231 ± 0.001[b]	0.228 ± 0.001[c]	0.260 ± 0.000[d]
Malic acid	1.008 ± 0.006[a]	0.621 ± 0.004[b]	0.748 ± 0.004[c]	0.790 ± 0.002[d]
Succinic acid	0.208 ± 0.005[a]	0.297 ± 0.006[b]	0.412 ± 0.006[c]	0.286 ± 0.010[b]
Tartaric acid	0.044 ± 0.001[a]	0.007 ± 0.001[b]	0.008 ± 0.000[bc]	0.009 ± 0.001[c]

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

5.2.2 Changes in volatile compounds during papaya juice fermentation

During papaya juice fermentation, several classes of volatile compounds including fatty acids, alcohols, esters and aldehydes were produced. However, volatiles that were indigenous to the juice such as benzyl isothiocyanate, benzaldehyde, β-damascenone and some fatty acids (butyric and hexanoic acids) were diminished (Figs. 5.3-5.7).

The evolution of butyric acid was similar among the three yeasts (Fig. 5.3). There were significant differences among the three yeasts in their profile of production and degradation of acetic, hexanoic, octanoic, decanoic and dodecanoic acids [Fig. 5.3, Appendix C (Fig. C2)]. Strain NCYC2727 consistently produced the least amounts of fatty acids such as octanoic acid at 0.28 mg/L, as compared to octanoic acid produced by strains NCYC22 and NCYC2251 at 0.44 mg/L and 3.50 mg/L, respectively (Tables 5.2 and 5.3). Butyric and hexanoic acids present at relatively high concentrations in the juice were utilised by all yeasts during fermentation. Acetic acid and other fatty acids increased initially, and then decreased toward the end of fermentation, except for strain NCYC2727 [Fig. 5.3, Appendix C (Fig. C2)]. The dynamic changes of volatile fatty acids corresponded to those observed in **Chapter 4**. Strain NCYC2251 produced the highest amount of total fatty acids with 2.64% (relative peak area, RPA) (Table 5.2). There were statistical differences in the concentrations of fatty acids at day 21 among or between the yeasts (Table 5.2). Strain NCYC2727 produced the highest amount of acetic acid with 0.63% (RPA), followed by strains NCYC22 and NCYC2251 with 0.30-0.31% (RPA) (Table 5.2), which corresponded to the organic acids results (Table 5.1). Despite the relatively high levels of acetic acid produced, non-*Saccharomyces* yeasts are increasingly being used in wine research due to their capabilities to contribute

positively to wine flavour through the production of esters such as isoamyl acetate and 2-phenylethyl acetate that impart sweet, fruity, flowery-like and banana-like flavours (Erten & Campbell, 2001; Jolly et al., 2006; Romano et al., 1997).

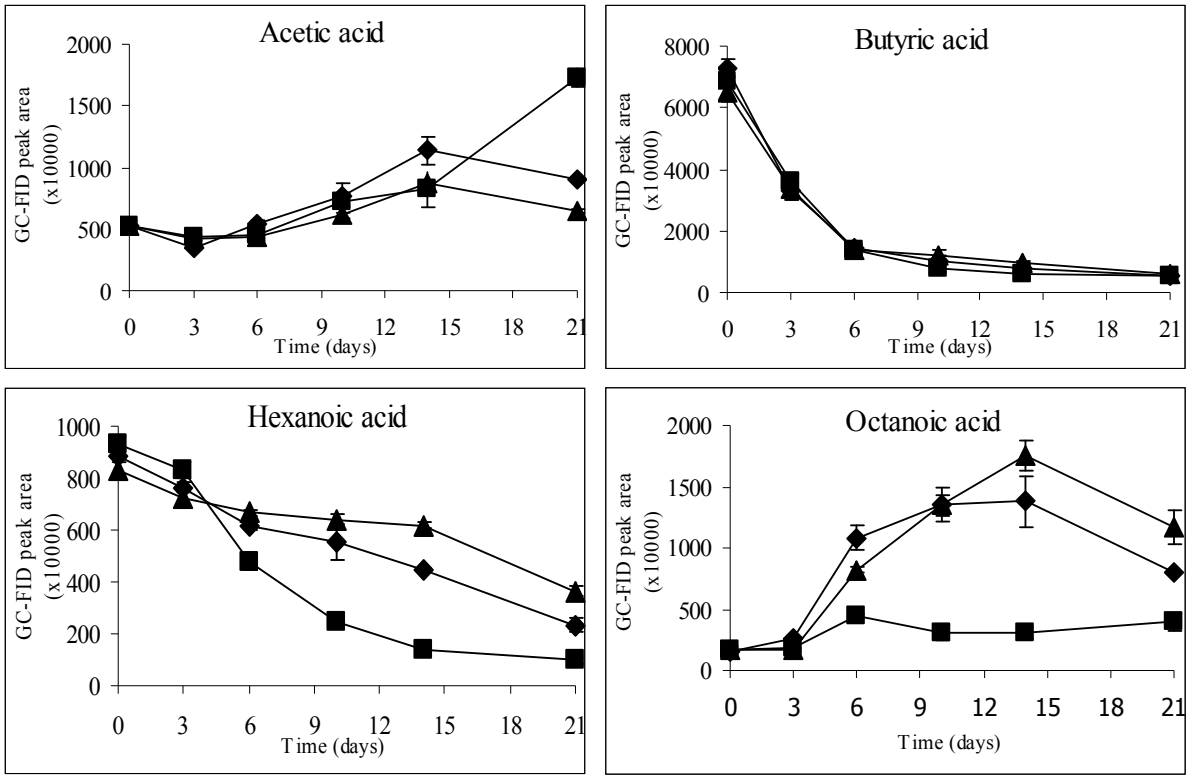

Fig. 5.3. Changes of fatty acids in papaya wine during fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (♦), *W. saturnus* var. *mrakii* NCYC2251 (▲) and *W. saturnus* var. *sargentensis* NCYC2727 (■). (Error bars = standard deviation).

Ethanol, isobutyl alcohol (2-methyl-1-propanol), isoamyl alcohol (3-methyl-1-butanol) and 2-phenylethyl alcohol were the major alcohols produced by the three yeasts during papaya juice fermentation (Table 5.2). 2-Ethylhexanol indigenous to the papaya juice was metabolised to a trace level (Fig. 5.4). The dynamic changes of alcohol formation and catabolism were similar [Fig. 5.4, Appendix C (Fig. C3)], whereas the final amounts of alcohols at day 21 varied significantly between or among the yeasts (Table 5.2) and with strain NCYC2727 producing the highest amount of 2-phenylethyl alcohol at 9.97 mg/L, while strain NCYC2251 produced the

utmost amount of isoamyl alcohol at 12.51 mg/L (Table 5.3). Strain NCYC22 produced the highest amount of ethanol at 1.86 x 10^4 mg/L (2.35% v/v), followed by strains NCYC2727 and NCYC2251 at 1.74 x 10^4 mg/L (2.16% v/v) and 1.41 x 10^4 mg/L (1.78% v/v), respectively (Tables 5.1 and 5.3), which corresponded to the lowest ^{o}Brix value observed in the papaya wine fermented by strain NCYC22 at day 21 (Table 5.1).

W. saturnus yeasts are known to oxidise sugars mainly to carbon dioxide and water, producing only low levels of ethanol and resulting in wine with ethanol levels of 2.8 – 7.8% (v/v) (Erten & Campbell, 2001). This was also observed in this study with low levels of ethanol being produced (Table 5.1). The problem with low-alcohol wines is the loss of sensory characteristics of ethanol, i.e. fullness, body and mouth-warming effect. Ethanol also has a flavour-enhancing effect as a carrier for aroma volatiles, thus the flavour thresholds of acids, esters and higher alcohols in these products would likely be higher in low-alcohol wines compared to normal wines (Vradis & Floros, 1993).

Higher alcohols produced are affected by the type and/or concentration of nitrogenous substances with some amino acids such as branched-chain and aromatic amino acids originally present in the papaya juice being the main precursors. The ratio of higher alcohols to the esters is known to influence the sensory properties of the wine with higher alcohols being the necessary precursors for the formation of some esters. An increase in the content of esters would result in an enhanced fruity flavour (Valero et al., 2002).

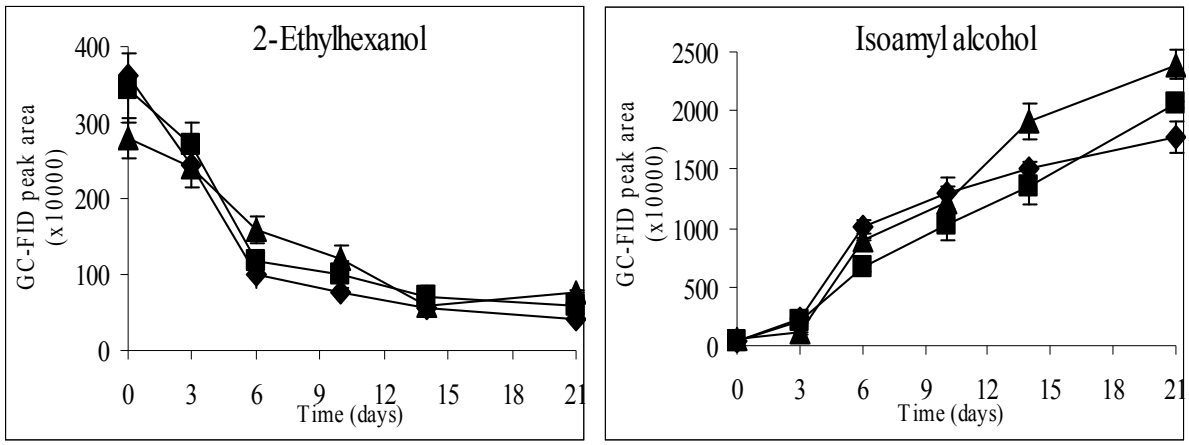

Fig. 5.4. Changes of 2-ethylhexanol and isoamyl alcohol in papaya wine during fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (♦), *W. saturnus* var. *mrakii* NCYC2251 (▲) and *W. saturnus* var. *sargentensis* NCYC2727 (■). (Error bars = standard deviation).

Among the volatile compounds produced by the three yeasts, esters constituted the majority of the volatiles ranging from 32.56 to 42.62% (RPA) (Table 5.2), which included ethyl esters, acetate esters and other esters. The dynamic changes of most esters were similar among the three yeasts, except for some esters such as benzyl acetate, 2-phenylethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl hexadecanoate and methyl esters, leading to strain differentiation (Figs. 5.5 and 5.6). Most of the quantified esters, especially ethyl octanoate, isoamyl acetate and 2-phenylethyl acetate, had concentrations higher than their corresponding odour thresholds and were expected to contribute to the papaya wine aroma (Table 5.3).

Most of the acetate esters tended to increase initially then declined with the exception of ethyl acetate, propyl acetate and 2-phenylethyl acetate, which increased and were stable [Fig. 5.5, Appendix C (Fig. C4)]. Strain NCYC22 consistently produced the highest amount of most acetate esters except for ethyl acetate and methyl acetate (Fig. 5.5, Tables 5.2 and 5.3). The final amounts of acetate esters at day 21 varied significantly between or among the yeasts (Tables 5.2 and 5.3). Among

the acetate esters, ethyl acetate was produced in the largest amount, followed by isoamyl acetate and 2-phenylethyl acetate (Table 5.2). These esters impart desirable fruity and floral notes, except for ethyl acetate at high levels (150-200 mg/L) that imparts light fruity and solvent-like aroma (Jackson, 1994).

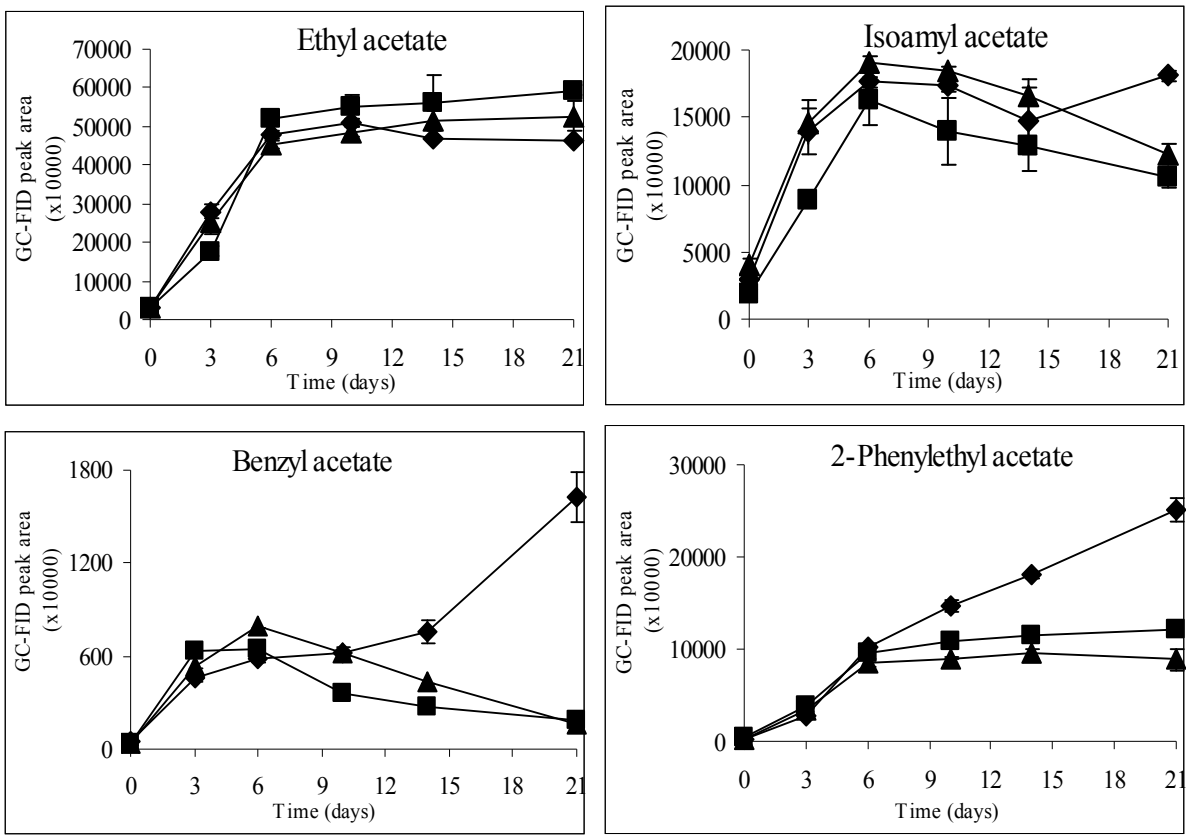

Fig. 5.5. Changes of acetate esters in papaya wine during fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (♦), *W. saturnus* var. *mrakii* NCYC2251 (▲) and *W. saturnus* var. *sargentensis* NCYC2727 (■). (Error bars = standard deviation).

Ethyl esters and other esters generally increased during fermentation except for isoamyl butyrate and methyl octanoate [Fig. 5.6, Appendix C (Fig. C5)]. Strain NCYC2727 producing the lowest amounts of ethyl and methyl esters except for ethyl hexanoate and ethyl hexadecanoate (Tables 5.2 and 5.3). The biosynthesis of ethyl esters was very slow at the beginning of fermentation and increased exponentially after day 6 (Fig. 5.6). This correlated with the amount of ethanol initially present in

the papaya juice and produced during fermentation [Appendix C (Fig. C3)], where ethanol was one of the cosubstrates that regulate the formation of ethyl esters (Saerens, Delvaux, Verstrepen, Van Dijck, Thevelein, & Delvaux, 2008). Moreover, the slow formation of esters at the initial stage of fermentation could also be due to the high metabolic demand for acetyl-CoA for yeast growth. After the active growth phase an equilibrium is established between acetyl-CoA consumption for growth and for ester production (Lilly, Lambrechts, & Pretorius, 2000; Peddie, 1990), which also accounted for the exponential increase in ethyl and methyl esters after Day 6. The final amounts of all esters at day 21 varied significantly among or between the yeasts at $p<0.05$ (Tables 5.2 and 5.3).

Studies have shown that *Williopsis saturnus* yeasts can convert higher alcohols into the corresponding acetate esters by the action of alcohol acetyltransferase in the presence of respective alcohols and acetyl-CoA (Janssens et al., 1992). Esters are responsible for the characteristic fruity odours of wine fermentation bouquet (Rapp & Mandrey, 1986) and as such, strains NCYC22 and NCYC2251 would be more desirable yeasts for papaya wine fermentation due to their relatively high level of ester formation.

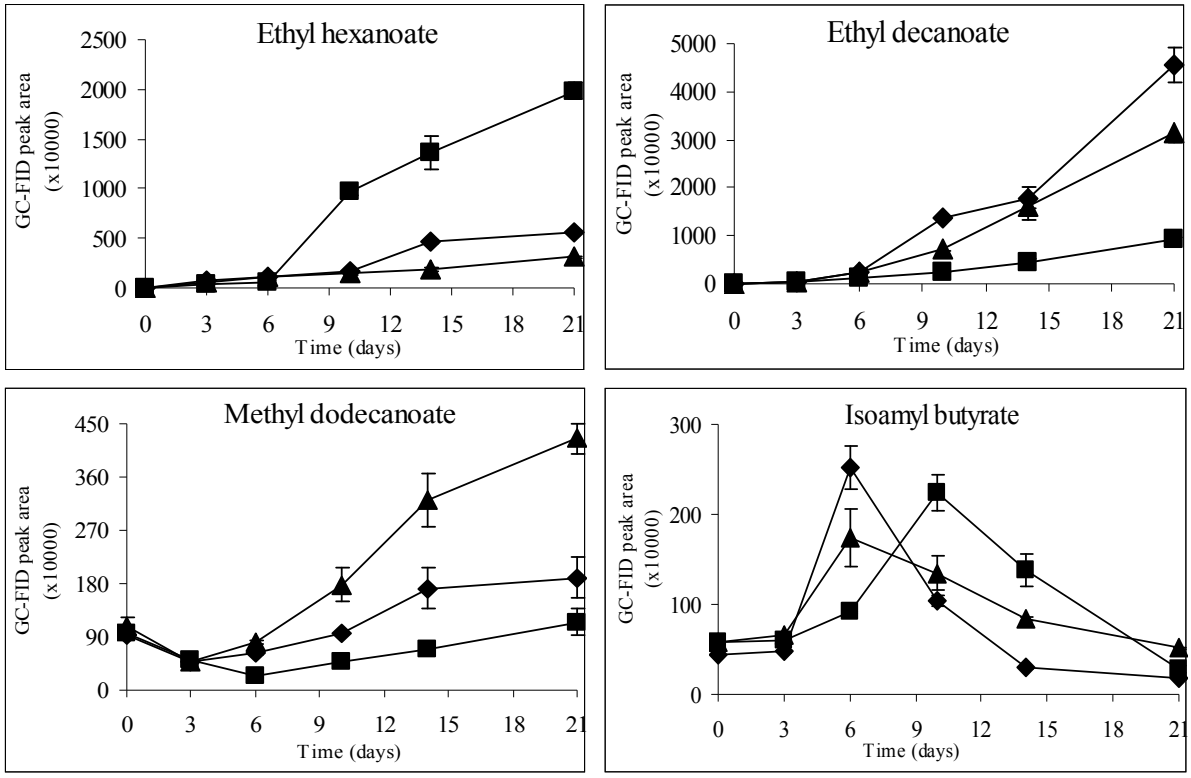

Fig. 5.6. Changes of ethyl esters, methyl dodecanoate and isoamyl butyrate in papaya wine during fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (♦), *W. saturnus* var. *mrakii* NCYC2251 (▲) and *W. saturnus* var. *sargentensis* NCYC2727 (■). (Error bars = standard deviation).

The miscellaneous volatile compounds including aldehydes, ketones and benzyl isothiocyanate, particularly benzaldehyde, 3,4-dimethylbenzaldehyde, β-damascenone and benzyl isothiocyanate (present in the papaya juice), were metabolised to trace levels during fermentation, except that O-tolualdehyde was produced [Fig. 5.7, Appendix C (Fig. C6)]. The reduction of aldehydes during fermentation corresponded to those in **Chapter 4**, except for O-tolualdehyde. Ugliano and Henschke (2009) commented that higher aldehydes, usually produced in trace amounts, can be derived from the biosynthesis of fatty acids from acetyl-CoA, which is derived from acetic acid. The dynamic changes of aldehydes, β-damascenone and benzyl isothiocyanate were similar among the three yeasts, except for benzaldehyde with strain NCYC2727 displaying a more rapid utilisation [Fig. 5.7, Appendix C (Fig.

C6)]. Benzaldehyde is enzymatically converted to L-phenylacetyl carbinol (L-PAC) by yeasts in the presence of pyruvate that is generated from glycolysis (Mahmoud, El-Sayed, & Coughlin, 1990). In a parallel, the undesired reaction part of the benzaldehyde is also reduced by alcohol dehydrogenase to benzyl alcohol (Mahmoud et al., 1990). The results of this study differed from Mahmoud et al. (1990), where there was no production of benzyl alcohol in all the cultures. This may be due to *S. cerevisiae* was used in the study of Mahmoud et al. (1990). The final amounts of the miscellaneous volatile compounds at day 21 varied significantly among the yeasts at $p<0.05$ (Table 5.2).

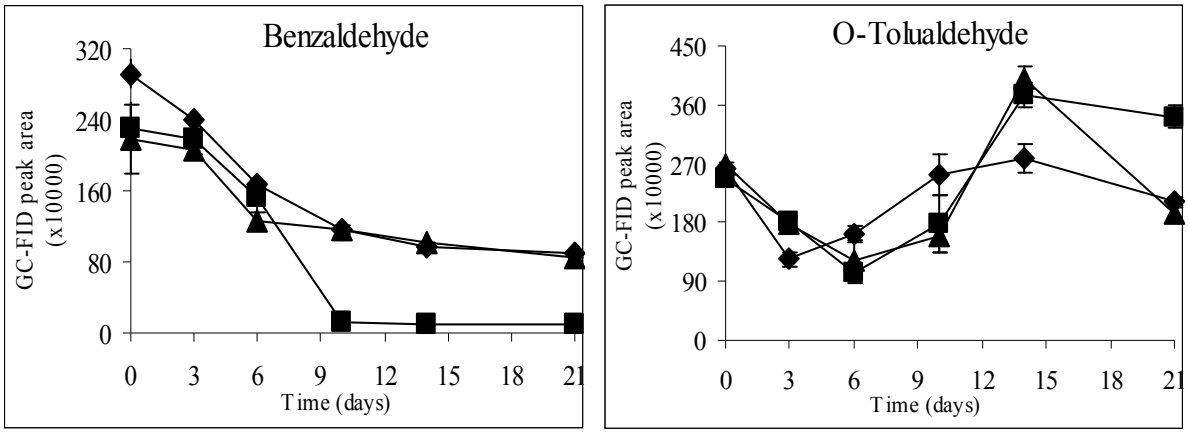

Fig. 5.7. Changes of benzaldehyde and O-tolualdehyde in papaya wine during fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (♦), *W. saturnus* var. *mrakii* NCYC2251 (▲) and *W. saturnus* var. *sargentensis* NCYC2727 (■). (Error bars = standard deviation).

Table 5.2. Major volatile compounds (GC-FID peak area x 10^6) and their relative peak areas (RPA) identified in papaya wine fermented with three *W. saturnus* yeasts at day 21

No.	Compounds identified	CAS no.[d]	LRI[e]	Yeast NCYC22 Peak Area	Yeast NCYC22 RPA (%)	Yeast NCYC2251 Peak Area	Yeast NCYC2251 RPA (%)	Yeast NCYC2727 Peak Area	Yeast NCYC2727 RPA (%)	Organoleptics[f]
	Acids									
1	Acetic acid[3]	000064-19-7	1487	9.02 ± 0.03[a]	0.30	6.53 ± 0.15[b]	0.31	17.30 ± 0.71[c]	0.63	Acidic, vinegar
2	Butyric acid[2]	000107-92-6	1620	5.47 ± 0.19[a]	0.18	6.14 ± 0.09[b]	0.29	5.24 ± 0.56[a]	0.19	Rancid, cheesy
3	Hexanoic acid[1,4]	000142-62-1	1889	2.31 ± 0.27[a]	0.08	3.65 ± 0.17[b]	0.18	0.96 ± 0.12[c]	0.03	Sweet, cheesy
4	Octanoic acid[1,2,4]	000124-07-2	2108	8.00 ± 0.06[a]	0.26	11.70 ± 1.36[b]	0.56	3.98 ± 0.70[c]	0.15	Sweet, cheesy
5	Decanoic acid[1,4]	000334-48-5	2327	4.81 ± 0.76[a]	0.16	12.90 ± 1.34[b]	0.62	4.19 ± 0.69[a]	0.15	Unpleasant, rancid, sour
6	Dodecanoic acid[1]	000143-07-7	2543	5.29 ± 0.89[a]	0.17	14.00 ± 1.41[b]	0.67	6.53 ± 0.57[a]	0.24	Fatty, coconut, bay oil
	Subtotal			**34.90**	**1.15**	**54.92**	**2.64**	**38.20**	**1.39**	
	Alcohols									
7	Ethanol[2]	000064-17-5	943	1900 ± 114[a]	62.76	1090 ± 60[b]	52.32	1750 ± 331[a]	63.80	Strong alcoholic
8	Isobutyl alcohol[2]	000078-83-1	1100	8.56 ± 0.22[a]	0.28	9.45 ± 0.27[b]	0.45	12.20 ± 1.59[c]	0.44	Wine solvent
9	Isoamyl alcohol[4]	000123-51-3	1196	17.80 ± 1.27[a]	0.59	23.90 ± 1.23[b]	1.15	20.50 ± 0.24[c]	0.75	Fruity, nail polish
10	2-Phenylethyl alcohol[4]	000060-12-8	1917	14.80 ± 1.52[a]	0.49	12.10 ± 0.73[b]	0.58	23.00 ± 2.48[c]	0.84	Rose, floral, honey
11	2-Ethylhexanol[4]	000104-76-7	1527	0.42 ± 0.01[a]	0.01	0.78 ± 0.02[b]	0.04	0.58 ± 0.10[c]	0.02	Citrus, fresh, floral, oily, sweet
	Subtotal			**1941.58**	**64.13**	**1136.23**	**54.54**	**1806.28**	**65.85**	
	Aldehydes									
12	Benzaldehyde[2]	000100-52-7	1574	0.90 ± 0.09[a]	0.03	0.86 ± 0.01[a]	0.04	0.09 ± 0.01[b]	0.00	Almond like
13	O-Tolualdehyde	000529-20-4	1705	2.12 ± 0.08[a]	0.07	1.94 ± 0.13[a]	0.09	3.41 ± 0.17[b]	0.12	Fruity, sweet, cherry, chemical
14	3,4-Dimethylbenzaldehyde	005973-71-7	1880	0.67 ± 0.20[a]	0.02	0.44 ± 0.06[a]	0.02	0.11 ± 0.01[b]	0.00	-
	Subtotal			**3.69**	**0.12**	**3.24**	**0.16**	**3.61**	**0.12**	
	Esters									
15	Methyl octanoate[3]	000111-11-5	1411	0.49 ± 0.01[a]	0.02	1.09 ± 0.04[b]	0.05	0.22 ± 0.01[c]	0.01	Powerful, fruity, orange-like

Table 5.2. (Continued)

No.	Compounds identified	CAS no.[d]	LRI[e]	Yeast NCYC22 Peak Area	Yeast NCYC22 RPA (%)	Yeast NCYC2251 Peak Area	Yeast NCYC2251 RPA (%)	Yeast NCYC2727 Peak Area	Yeast NCYC2727 RPA (%)	Organoleptics[f]
16	Methyl decanoate[3]	000110-42-9	1627	1.20 ± 0.16[a]	0.04	1.52 ± 0.14[a]	0.07	0.30 ± 0.06[b]	0.01	Pleasant, fruity, floral
17	Methyl dodecanoate[3]	000111-82-0	1842	1.90 ± 0.34[a]	0.06	4.25 ± 0.25[b]	0.20	1.15 ± 0.22[c]	0.04	Waxy, creamy coconut, mushroom
18	Ethyl butyrate[1]	000105-54-4	1046	4.68 ± 0.37[a]	0.15	5.23 ± 0.23[a]	0.25	2.75 ± 0.22[b]	0.10	Pineapple, banana
19	Ethyl hexanoate[1,2]	000123-66-0	1240	5.68 ± 0.15[a]	0.19	3.17 ± 0.09[b]	0.15	19.20 ± 0.86[c]	0.70	Green banana, estery, fruity, pineapple
20	Ethyl octanoate[1,2,4]	000106-32-1	1434	34.70 ± 3.28[a]	1.15	34.00 ± 4.28[a]	1.63	2.68 ± 0.40[b]	0.10	Pleasant, fruity, floral, apple
21	Ethyl decanoate[1,2]	000110-38-3	1673	45.60 ± 3.59[a]	1.51	31.30 ± 1.98[b]	1.50	9.07 ± 0.70[c]	0.33	Sweet, brandy-like
22	Ethyl dodecanoate[3]	000106-33-2	1885	25.10 ± 1.35[a]	0.83	56.20 ± 2.70[b]	2.70	22.70 ± 1.13[a]	0.83	Sweet, waxy, floral, soapy
23	Ethyl tetradecanoate[3]	000124-06-1	2095	3.03 ± 0.17[a]	0.10	1.43 ± 0.12[b]	0.07	1.49 ± 0.28[b]	0.05	Sweet,waxy
24	Ethyl 9-hexadecanoate[3]	054546-22-4	2337	0.92 ± 0.02[a]	0.03	0.98 ± 0.01[b]	0.05	0.72 ± 0.05[c]	0.03	-
25	Ethyl hexadecanoate[3]	000628-97-7	2307	1.82 ± 0.10[a]	0.06	0.52 ± 0.01[b]	0.02	0.78 ± 0.06[c]	0.03	Waxy, fruity, creamy, milky
26	Isoamyl butyrate[3]	000106-27-4	1272	0.18 ± 0.01[a]	0.01	0.51 ± 0.01[b]	0.02	0.28 ± 0.04[c]	0.01	Fruity
27	Isoamyl propanoate	000105-68-0	1190	0.68 ± 0.02[a]	0.02	1.02 ± 0.07[b]	0.05	0.47 ± 0.04[c]	0.02	Sweet, fruit, banana, pineapple
28	Methyl acetate	000079-20-9	861	1.33 ± 0.05[a]	0.04	1.60 ± 0.26[a]	0.08	2.08 ± 0.10[b]	0.08	Fruity, sweet
29	Ethyl acetate[2]	000141-78-6	901	462 ± 2.79[a]	15.26	527 ± 39.4[b]	25.30	593 ± 13.60[b]	21.62	Pineapple, sweet, fruity
30	Propyl acetate	000109-60-4	1002	8.80 ± 0.31[a]	0.29	5.05 ± 0.04[b]	0.24	7.49 ± 0.27[c]	0.27	Celery, fruity, fusel, raspberry
31	Isoamyl acetate[1]	000123-92-2	1089	181 ± 3.63[a]	5.98	123 ± 7.95[b]	5.90	105 ± 7.32[c]	3.83	Banana, apple, estery
32	Benzyl acetate	000140-11-4	1780	16.20 ± 1.58[a]	0.54	1.60 ± 0.20[b]	0.08	1.88 ± 0.30[b]	0.07	Sweet, floral, fruity, jasmine

Table 5.2. (Continued)

No.	Compounds identified	CAS no.[d]	LRI[e]	Yeast NCYC22 Peak Area	Yeast NCYC22 RPA (%)	Yeast NCYC2251 Peak Area	Yeast NCYC2251 RPA (%)	Yeast NCYC2727 Peak Area	Yeast NCYC2727 RPA (%)	Organoleptics[f]
33	2-Phenylethyl acetate[1]	000103-45-7	1821	251 ± 11.80[a]	8.29	88.40 ± 2.40[b]	4.24	122 ± 7.94[c]	4.45	Rose, honey, floral
	Subtotal			**1046.31**	**34.56**	**887.87**	**42.62**	**893.26**	**32.56**	
	Ketone									
34	β-Damascenone[4]	023726-93-4	1872	0.37 ± 0.02[a]	0.01	0.60 ± 0.03[b]	0.03	0.77 ± 0.06[c]	0.03	Rose, cooked apple
	Heteroatom (N, S) compound									
35	Benzyl isothiocyanate[3]	000622-78-6	2176	0.53 ± 0.02[a]	0.02	0.45 ± 0.02[b]	0.02	0.94 ± 0.06[c]	0.03	Watercress, medicinal horseradish, oily
	Total			**3027.38**		**2083.31**		**2743.06**		

[a,b,c]Statistical analysis at 95% confidence level with same letters indicating no significant difference.
[d]CAS number obtained from Wiley MS library.
[e]Experimentally determined linear retention index on the DB-FFAP column, relative to C5-C40 hydrocarbons.
[f]Odour description obtained from Luebke (1980).
[1,2,3,4]Retention index in agreement with those in the literatures [Duarte et al. (2010), Goodner (2008), Pino et al. (2003) and Segurel, Baumes, Langlois, Riou, and Razungles (2009), respectively].

Table 5.3. Concentration of selected major volatile compounds (mg/L) in papaya wine fermented with three *W. saturnus* yeasts at day 21

Compounds quantified	Yeast NCYC22		Yeast NCYC2251		Yeast NCYC2727		Odor threshold[d]
	Mean	OAV	Mean	OAV	Mean	OAV	
Ethanol	18571± 821[a]	-	14077 ± 2740[b]	-	17403 ± 975[a]	-	-
Isoamyl alcohol	4.57 ± 0.11[a]	0.15	12.51 ± 1.84[b]	0.42	11.06 ± 1.21[b]	0.37	30.00
2-Phenylethyl alcohol	3.49 ± 0.17[a]	0.35	3.10 ± 0.08[b]	0.31	9.97 ± 0.20[c]	1.00	10.00
Octanoic acid	0.44 ± 0.04[a]	0.87	3.50 ± 0.10[b]	7.00	0.28 ± 0.04[c]	0.56	8.80
Ethyl octanoate	0.81 ± 0.01[a]	470.00	0.76 ± 0.00[b]	440.00	0.68 ± 0.00[c]	395.00	0.02
Ethyl decanoate	0.55 ± 0.03[a]	2.78	0.31 ± 0.01[b]	1.57	0.05 ± 0.01[c]	0.26	0.20
Ethyl dodecanoate	0.84 ± 0.02[a]	0.70	1.69 ± 0.03[b]	1.41	0.81 ± 0.02[a]	0.68	1.20[e]
Isoamyl acetate	18.06 ± 1.46[a]	606.47	6.15 ± 0.67[b]	206.47	3.56 ± 0.25[c]	119.41	0.03
2-Phenylethyl acetate	7.21 ± 0.29[a]	29.13	2.34 ± 0.02[b]	9.46	3.58 ± 0.25[c]	14.46	0.25

Abbreviation: OAV = Odour activity values calculated by dividing concentration by the odour threshold value of the compound

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

[d]From Bartowsky and Pretorius (2009).

[e]From 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 unit adjusted to mg/L.

5.2.3 Principal component analysis

Principal component analysis (PCA) was applied to the volatile compounds in papaya wines from Tables 5.2 and 5.3 to obtain an overall relationship between volatile compounds and the three *W. saturnus* yeasts. The PCA result of the quantified major volatile compounds (Table 5.3) is presented as it is a proximate representation of the PCA result from Table 5.2 [Appendix C (Fig. C7)] and indicate distinctive volatile compositions among the papaya wines fermented by the three *W. saturnus* yeasts (Fig. 5.8). The wine produced by strain NCYC22 was more related to acetate esters (e.g. 2-phenylethyl acetate and isoamyl acetate), ethyl octanoate and ethyl decanoate. In the lower right-quadrant, the papaya wine produced by strain NCYC2251 was characterised by isoamyl alcohol, ethyl dodecanoate and octanoic acid. Conversely, strain NCYC2727 resulted in papaya wine with high amount of 2-phenylethyl alcohol.

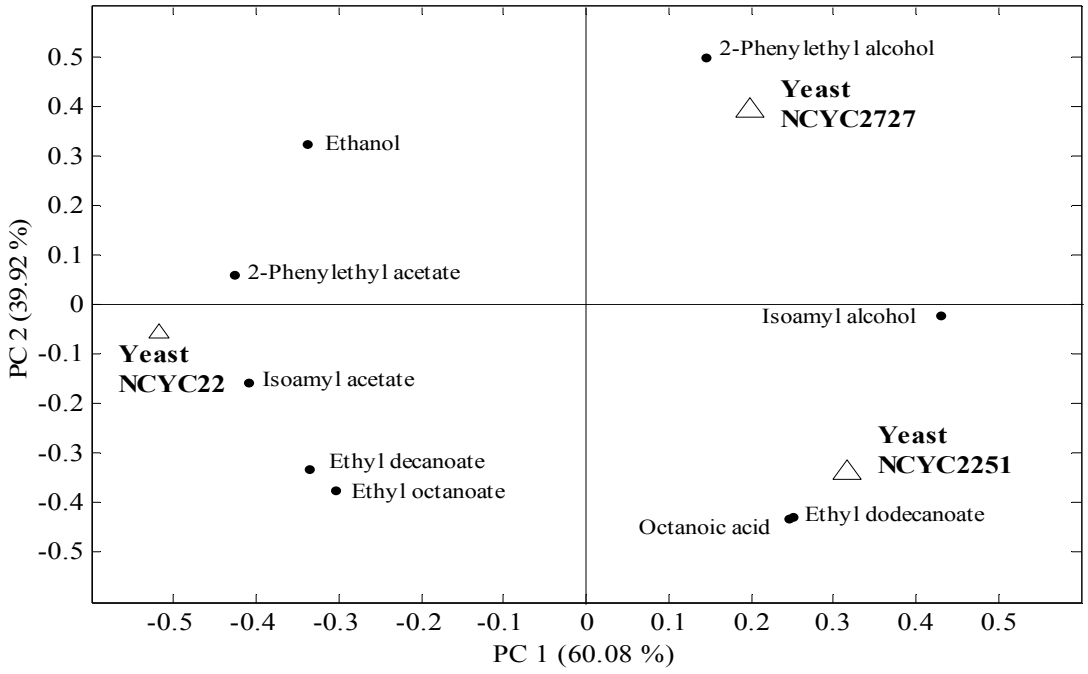

Fig. 5.8. Bi-plot of principal component analysis of the quantified major volatile compounds in papaya wine fermented with three *W. saturnus* yeasts.

5.3 Conclusions

In this chapter, the three *Williopsis* yeasts displayed various capabilities of fermenting papaya juice, leading to the formation and utilisation of numerous volatile compounds during fermentation. The dynamic changes of yeast fermentation and volatile compounds were similar among the three yeasts. However, there were distinctive volatile compounds produced that gave rise to strain differentiation with strain NCYC2251 producing the utmost amount of methyl esters, fatty acids and ethyl dodecanoate, followed by strain NCYC22 with the highest amount of most acetate esters and ethyl esters, and strain NCYC2727 producing the highest amount of ethyl hexanoate, 2-phenylethyl alcohol and acetic acid. Among the *W. saturnus* yeasts, strain NCYC2251 is a more suitable candidate for subsequent multistarter fermentations with *Saccharomyces* yeast due to its favourable growth rate (**Chapters 8-10**). However, its lower acetate ester-forming capability, as compared to strain NCYC22, warrants further research to evaluate the possibility of enhancing ester formation through the addition of selected assimilable nitrogen sources as flavour precursors (e.g. ammonia or amino acids) or fusel oil (as source of higher alcohols) (**Chapters 6 and 7**) in order to produce papaya wine with distinctive characteristics and improved quality.

CHAPTER 6

IMPACT OF AMINO ACID ADDITION ON VOLATILE COMPOUNDS IN PAPAYA WINE FERMENTED WITH *WILLIOPSIS SATURNUS* VAR. *MRAKII* NCYC2251

6.1 Introduction

In wine-making, 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 and metabolism. The degree of nitrogen availability can affect yeast metabolism and thus, volatile compound formation. Several studies have revealed the effects of ammonium addition on the formation of volatile compounds (Barbosa, Falco, Mendes-Faria, & Mendes-Faria, 2009; Hernandez-Orte, Bely, Cacho, & Ferreira, 2006a; Hernandez-Orte, Ibarz, Cacho, & Ferreira, 2005; Moreira, Guedes de Pinho, Santos, & Vasconcelos, 2011). Moreira et al. (2011) found that the addition of ammonium to must with low yeast assimilale nitrogen (YAN) reduced the production of volatile sulphur compounds during fermentation. In other studies, it was also observed that ammonium supplementation also increased ester production which helps to modulate the aroma profile in wine (Barbosa et al., 2009; Hernandez-Orte et al., 2006a). The formation of volatile compounds including higher alcohols, short to medium-chain fatty acids, ethyl esters and acetate esters can be manipulated by the type and/or concentration of nitrogen (Bell & Henschke, 2005; Torrea, Varela, Ugliano, Ancin-Azpilicueta, Francis, & Henschke, 2011). When supplemented with excessive amounts of ammonium, there could be a risk of producing wine with elevated levels of acetic acid, ethyl acetate,

volatile acidity (Bell & Henschke, 2005; Sablayrolles, 2009) or even ethyl carbamate (Ough, Crowell, & Mooney, 1988).

Papayas are relatively low in some amino acids as compared to grapes (Table 2.2). 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; Dickinson, Harrison, Dickinson, & Hewlins, 2000), whereas 2-phenylethyl alcohol 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-CoA. Acetate esters such as isoamyl acetate and 2-phenylethyl acetate are recognised as important flavour compounds in wine that impart characteristic aromas (Rojas et al., 2001, 2003).

Considering the common practice of nitrogen addition in wine-making, high ester-synthesising potential of *Williopsis* yeasts 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 these yeasts. The aim of this chapter was to study the fermentation performance and the 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 influences on aroma compound formation in wine fermentations (Dickinson et al., 1997, 1998, 2000; Hernandez-Orte, Cacho, & Ferreira, 2002).

6.2 Results and discussion

6.2.1 Growth and fermentation behaviour of *W. saturnus* in the presence of different amino acids

All the fermentations showed similar characteristics in terms of yeast growth and total soluble solids (^{o}Brix), regardless of the amino acids added (Fig. 6.1). 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 6.1). The pH did not vary significantly during fermentation with values maintaining at pH 3.57-3.68 (Table 6.1).

Both the sugar consumption and the organic acid changes were not affected by the addition of amino acids. Sugar consumption displayed a gradual reduction during fermentation with preferential utilisation of glucose over fructose [Table 6.1, Appendix D (Fig. D1)], being consistent with the ^{o}Brix trend and sugar consumption pattern of *W. saturnus* observed in **Chapter 5** (Figs. 5.1 and 5.2).

The changes of the organic acids were similar in all fermentations, where citric acid remained constant while malic and tartaric acids decreased slightly, and acetic and succinic acids increased [Table 6.1, Appendix D (Fig. D1)]. The changes of organic acids, especially the reduction of malic and tartaric acids corresponded to those observed in **Chapter 5**. This could be due to the uptake of D- and L-malic acid molecules by yeast via passive diffusion (Coloretti et al., 2002; Saayman & Viljoen-Bloom, 2006) and the precipitation of tartaric acid as potassium hydrogen tartrate (cream of tartar).

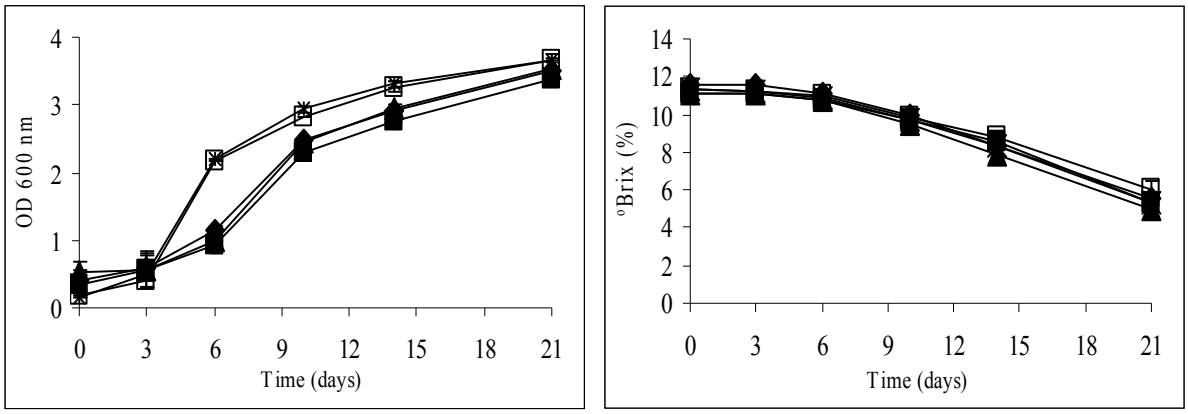

Fig. 6.1. Growth of yeasts (as optical density at OD 600 nm) and °Brix changes in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (∗); 0.05% isoleucine (□). (Error bars = standard deviation).

In wine, acetic acid is of particular importance as it can confer a vinegary odour to the wine. Yeasts are able to produce acetic acid from the oxidation of acetaldehyde by the enzyme acetaldehyde dehydrogenase. The addition of leucine and isoleucine produced slightly higher amounts of acetic acid than the control at 0.051 g/100 mL and 0.054 g/100 mL, respectively (Table 6.1). The acetic acid concentrations obtained in all the fermentations were lower than its odour threshold of 0.07-0.11 g/100 mL (Lambrechts & Pretorius, 2000). The production of succinic acid, on the other hand, was not affected by the addition of amino acids (Table 6.1). These results correlate with the findings in Camarasa, Grivet, and Dequin (2003), where the formation of succinic acid via the fumarate reduction under anaerobic condition operates independently of the nitrogen source, while the additional formation of succinic acid via the oxidative decarboxylation of 2-oxoglutarate (aerobic condition) was affected by glutamate.

Table 6.1. Fermentation parameters of papaya wine (day 21) fermented with *W. saturnus* var. *mrakii* NCYC2251 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}
^{o}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}
Ethanol % (v/v)	0.02 ± 0.00^{a}	2.17 ± 0.07^{bc}	2.37 ± 0.01^{c}	1.61 ± 0.12^{d}	2.12 ± 0.06^{b}	2.06 ± 0.11^{b}
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 ± 0.001^{b}	0.051 ± 0.002^{cd}	0.054 ± 0.001^{d}
Citric acid	0.271 ± 0.001^{a}	0.245 ± 0.003^{b}	0.230 ± 0.003^{c}	0.231 ± 0.001^{c}	0.237 ± 0.002^{d}	0.242 ± 0.003^{b}
Malic acid	0.902 ± 0.024^{a}	0.696 ± 0.013^{b}	0.648 ± 0.013^{c}	0.682 ± 0.014^{d}	0.666 ± 0.022^{e}	0.687 ± 0.004^{bd}
Succinic acid	0.180 ± 0.003^{a}	0.258 ± 0.003^{bc}	0.249 ± 0.003^{b}	0.268 ± 0.026^{c}	0.257 ± 0.001^{bc}	0.259 ± 0.004^{bc}
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.

6.2.2 Dynamic changes of volatile compounds during papaya juice fermentation

During papaya juice fermentation, a number of volatile compounds were produced including fatty acids, alcohols, esters and aldehydes: some were stable, others were metabolised. Volatile 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 (Figs. 6.2-6.6).

The dynamic changes of volatile fatty acids were similar in all the fermentations [Fig. 6.2, Appendix D (Fig. D2)]. Hexanoic, butyric and nonanoic acids present at relatively high concentrations in the juice was utilised, while other fatty acids such as acetic, octanoic, decanoic, dodecanoic and tetradecanoic acids increased during fermentation. The addition of amino acids increased the formation of acetic acid as compared to the control (Table 6.2). The addition of L-phenylalanine increased the utilisation of hexanoic acid but reduced the formation of octanoic acid and other medium to long-chain fatty acids [Fig. 6.2, Tables 6.2 and 6.3, Appendix D (Fig. D2)]. The addition of L-leucine and L-isoleucine produced the highest amount of acetic acid with relative peak areas (RPA) ranging from 0.49 to 0.56% that corresponded to the organic acid results (Table 6.1). 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* yeasts (Romano et al., 2003; Viana, Gil, Genoves, Valles, & Manzanares, 2008). However, the amount of acetic acid produced in this study was within the acceptable range of 0.02-0.07 g/100 mL for wine (Lambrechts & Pretourius, 2000).

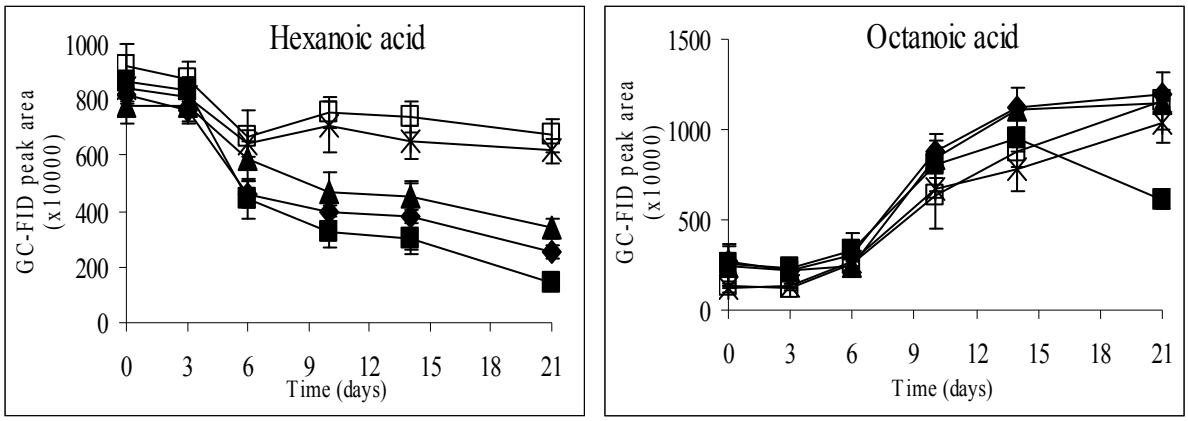

Fig. 6.2. Changes in hexanoic and octanoic acids in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (∗); 0.05% isoleucine (□). (Error bars = standard deviation).

Ethanol, isobutyl alcohol (2-methyl-1-propanol), active amyl alcohol (2-methyl-1-butanol), isoamyl alcohol (3-methyl-1-butanol) and 2-phenylethyl alcohol were the major alcohols produced by strain NCYC2251 during papaya wine fermentation (Fig. 6.3). The effect of the addition of amino acids on ethanol production varied. Amino acid addition significantly increased production of respective higher alcohols (Fig. 6.3).

Studies have shown that with the addition of different amino acids (as additional nitrogen source), *Saccharomyces* yeasts and certain non-*Saccharomyces* yeasts (*K. marxianus*) are capable of producing additional respective higher alcohols from these amino acids via Ehrlich's pathway. In the Ehrlich's pathway, amino acids are primarily transaminated to their respective α-keto acids by aminotransferases. The α-keto acids formed are subsequently decarboxylated to form aldehydes, which was further reduced by alcohol dehydrogenase to form higher alcohols (Dickinson et al., 1997, 1998, 2000; Etschmann et al., 2002; Hazelwood et al., 2008). The results of this study are in accordance with the previous studies, where the fermentations 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-phenylethyl alcohol (17.16 mg/L), respectively (Table 6.3). Those added with either L-leucine or L-isoleucine or L-valine showed markedly increased production of isobutyl alcohol, as compared to the control (Fig. 6.3, Table 6.3).

Based on the concentrations, the fermentation added with L-valine produced a relatively high amount of isobutyl alcohol at 9.17 mg/L (Table 6.3). However, as compared to the semi-quantified results, slight variation was observed, which was probably due to the wine matrix effects on the HS-SPME fiber (Burman et al., 2005), deterioration of the mixed coating on the fiber upon the extraction of wine samples (Bianco, Novario, & Zianni, 2009) and possibly thermal deterioration of the fiber with numerous injections.

The final amounts of alcohols at day 21 varied significantly among the different amino acids added and the control (Tables 6.2 and 6.3). The results of this study differed from those of Garde-Cerdan and Ancin-Azpilicueta (2008) and Hernandez-Orte, Ibarz, Cacho, and Ferreira (2006b), which found that there was no positive correlation between the higher alcohols production and the amino acids added with the exception for 2-phenylethyl alcohol; some such as isoamyl alcohol, even decreased. This may be due to the fact that a mixture of amino acids and different yeasts were used in other studies.

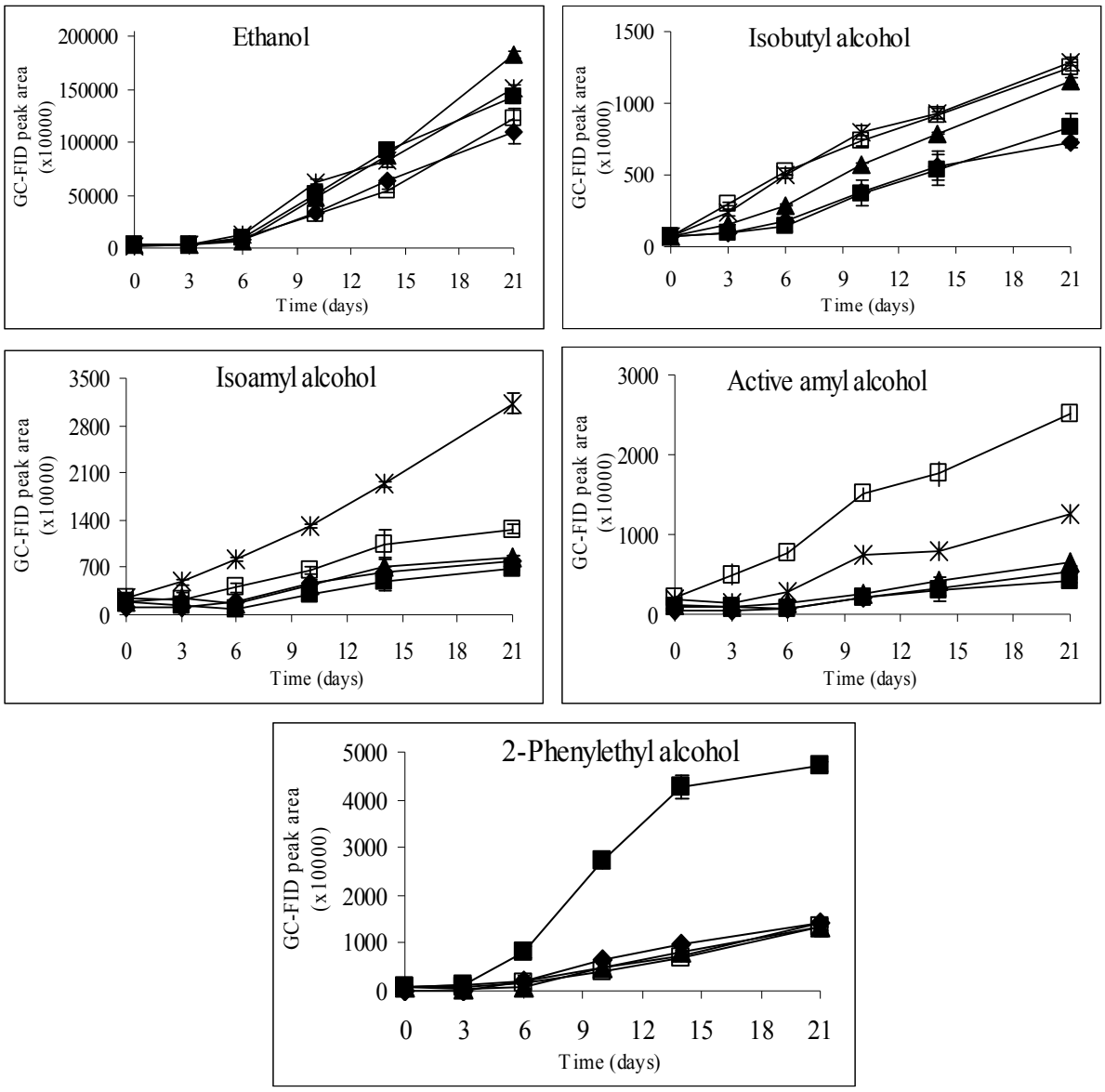

Fig. 6.3. Changes in alcohols in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (∗); 0.05% isoleucine (□). (Error bars = standard deviation).

Esters were the next abundant volatile compounds produced by yeast strain NCYC2251 during papaya juice fermentation ranging from 29.36 to 46.64% (RPA), which included acetate esters, ethyl esters, methyl esters and other esters (Table 6.2). Acetate esters tended to increase initially then declined with the exception of 2-phenylethyl acetate, ethyl acetate and propyl acetate, which increased and remained relatively stable [Fig. 6.4, Appendix D (Fig. D3)]. Ethyl and methyl esters generally

increased during fermentation [Fig. 6.5, Appendix D (Fig. D4)], being consistent with the evolution trends observed in **Chapter 5** (Fig. 5.6). Within the miscellaneous esters, isoamyl propanoate, isoamyl butyrate and 2-phenylethyl butyrate increased initially and followed by a decline (Fig. 6.5).

The impact of amino acid addition on ester production varied with esters. The addition of L-phenylalanine increased production of 2-phenylethyl acetate and 2-phenylethyl butyrate, while reducing formation of isobutyl acetate, isoamyl acetate and benzyl acetate (Figs. 6.4 and 6.5, Tables 6.2 and 6.3). Fermentation with added L-phenylalanine displayed significant production of 2-phenylethyl acetate at 14.30 mg/L (Table 6.3). The increased production of 2-phenylethyl acetate was likely due to the presence of high amounts of 2-phenylethyl alcohol and acetyl-CoA, which provided the necessary precursors for the formation of 2-phenylethyl acetate by the action of alcohol acetyltransferase (AAT) enzymes (Swiegers et al., 2005). The decreased production of other acetate esters upon the addition of L-phenylalanine (Tables 6.2 and 6.3) could be due to competition for and diversion of acetyl-CoA for 2-phenylethyl ester formation or competition for uptake of substrates such as amino acids that may serve as aroma precursors.

L-Leucine addition enhanced the formation of propyl acetate, ethyl butyrate, ethyl hexanoate, ethyl octanoate, methyl octanoate, isoamyl butyrate, isoamyl propanoate and produced the highest amount of isoamyl acetate with 8.29 mg/L, while L-isoleucine addition had the highest amount of active amyl acetate produced with 0.06 mg/L (Figs. 6.4 and 6.5, Tables 6.2 and 6.3). 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 acetyl-CoA produced from 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 enhanced or inhibited uptake of certain amino acids. Further studies are needed to elucidate this.

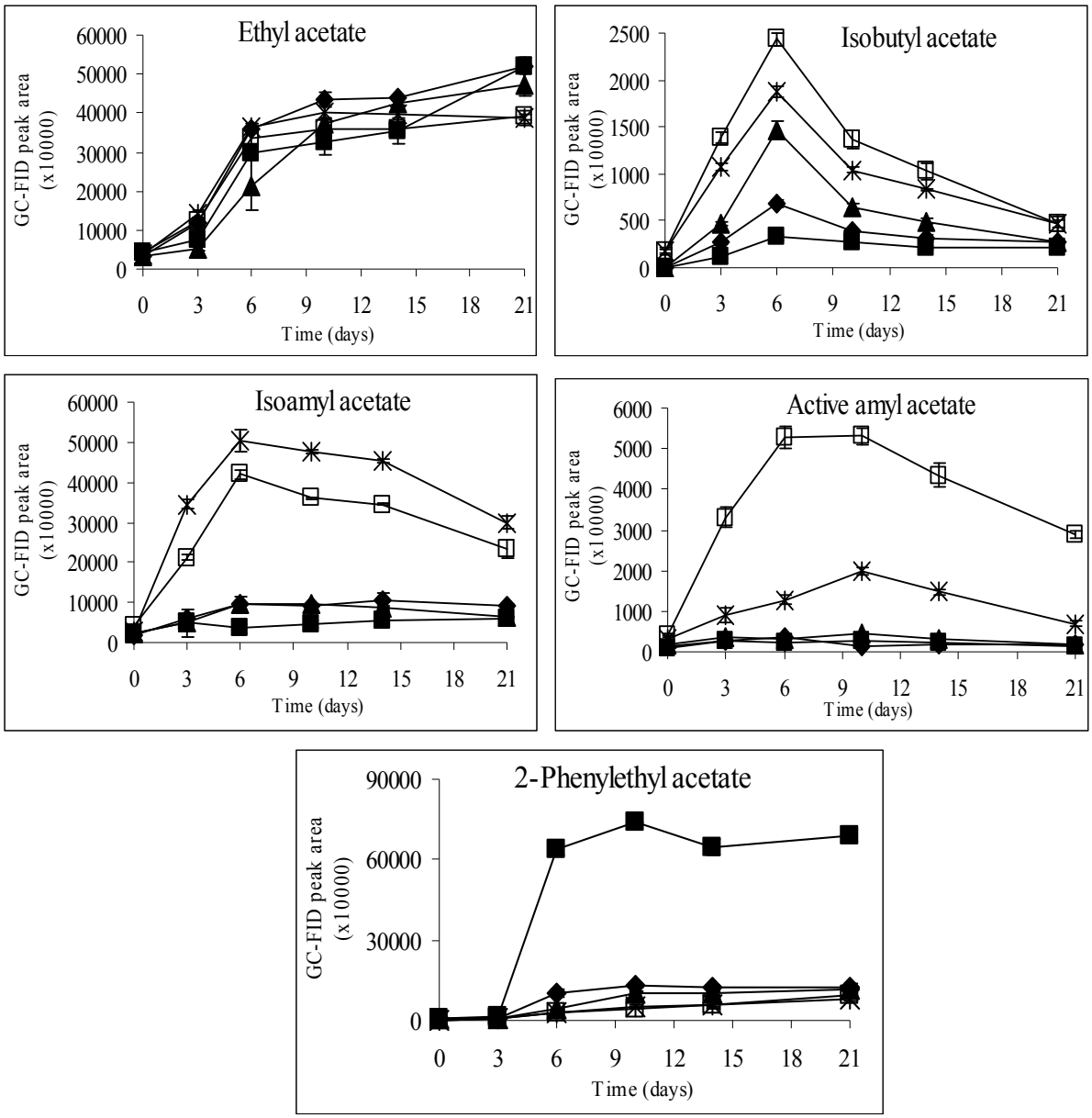

Fig. 6.4. Changes in acetate esters in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (∗); 0.05% isoleucine (□). (Error bars = standard deviation).

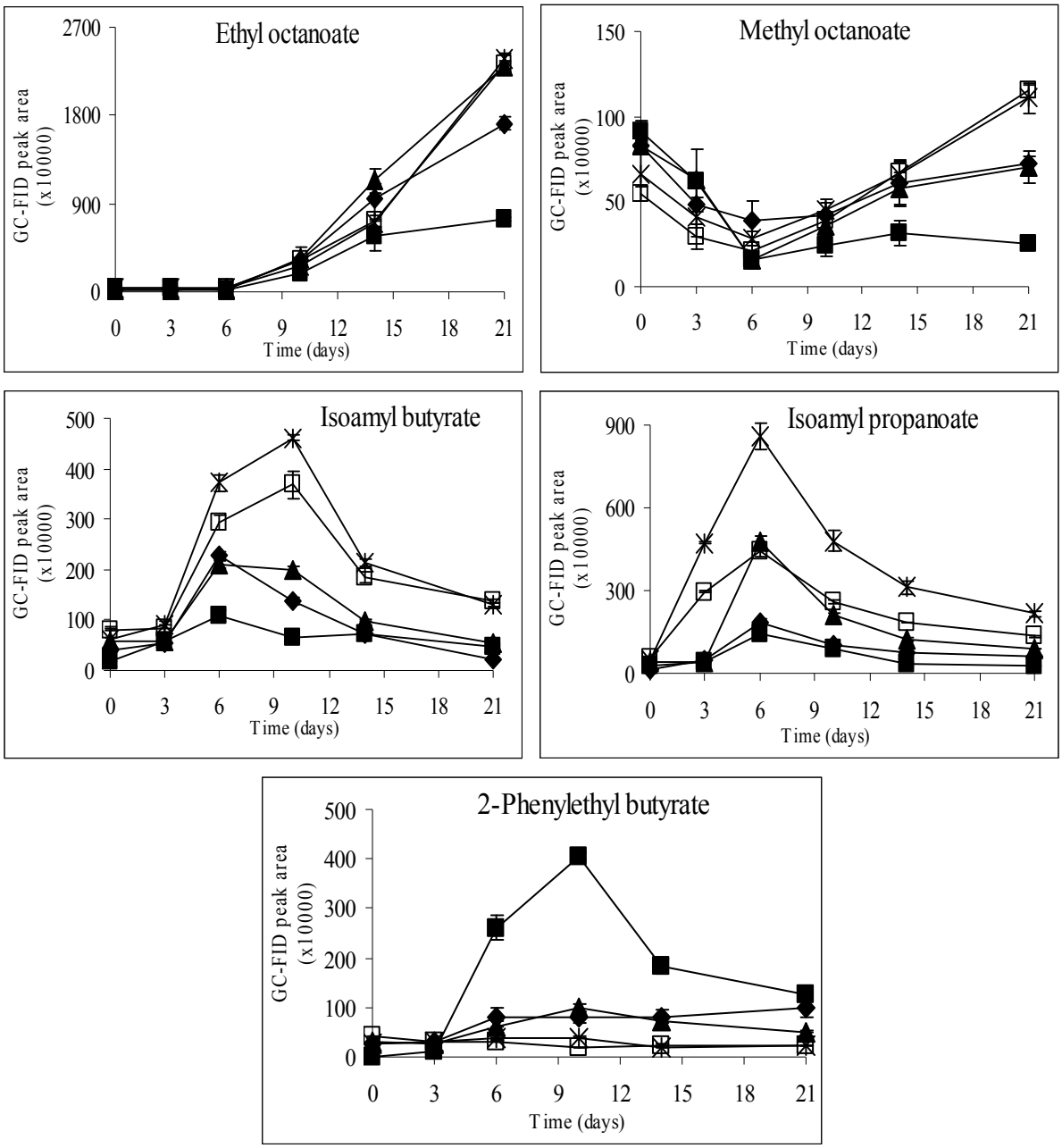

Fig. 6.5. Changes in ethyl decanoate, methyl octanoate and other esters in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (∗); 0.05% isoleucine (□). (Error bars = standard deviation).

The addition of L-valine only slightly increased isobutyl acetate production at 0.009 mg/L (Table 6.3). The addition of amino acids did not affect the formation of ethyl acetate, except for those added with L-leucine and L-isoleucine (Fig. 6.4, Table 6.2). The formation of ethyl octanoate 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 and methyl esters (Tables 6.2 and 6.3). The reduction of ethyl esters with the addition of L-phenylalanine could be related to the reduced *de novo* biosynthesis of fatty acyl-CoA associated with fatty acid and/or sugar metabolism. The effect of L-isoleucine, L-leucine and L-valine additions on other ethyl and methyl esters production varied [Fig. 6.5, Tables 6.2 and 6.3, Appendix D (Fig. D4)]. The final concentrations of esters were dependent on the stability and determined any significant difference at the statistical level, which varied among the different treatments (Tables 6.2 and 6.3).

Among the aldehydes (Fig. 6.6), benzaldehyde (present in the juice) was metabolised to trace levels during fermentation regardless of amino acid added, but the addition of L-phenylalanine increased the benzaldehyde initially, which then declined. O-Tolualdehyde and ethylbenzaldehyde tended to increase during fermentation but their formation was reduced with the addition of amino acids, except for those with L-isoleucine and L-valine added that enhanced the formation of O-tolualdehyde and ethylbenzaldehyde, respectively (Fig. 6.6, Table 6.2). The initial production of benzaldehyde with the addition of L-phenylalanine corresponded to the findings in Okrasa, Guibe-Jampel, Plenkiewicz, and Therisod (2004) who proposed the formation of benzaldehyde from phenylalanine as the oxidative deformylation of phenylacetaldehyde derived from the Ehrlich pathway. The final amounts of aldehydes at day 21 varied significantly among the different amino acids added at $p<0.05$ (Table 6.2). β-Damascenone and benzyl isothiocycanate were metabolised to trace levels and were not affected by amino acid addition [Fig. 6.6, Appendix D (Fig. D5)].

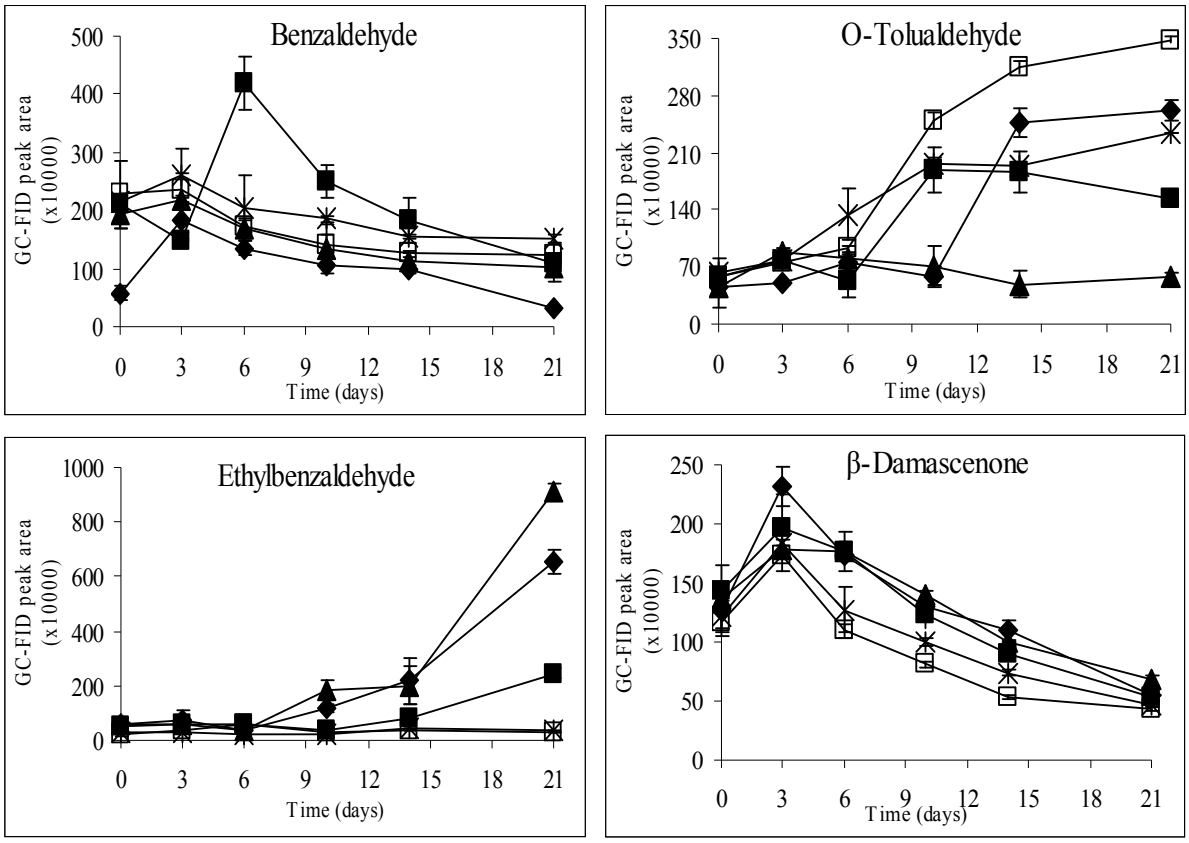

Fig. 6.6. Changes in aldehydes and β-damascenone in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (∗); 0.05% isoleucine (□). (Error bars = standard deviation).

Table 6.2. Major volatile compounds (GC-FID peak area x 10^6) and their relative peak areas (RPA) identified in papaya wine (day 21) fermented with *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added

No.	Compounds identified	LRI[f]	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		Organoleptics[g]
			Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	
	Acids												
1	Acetic acid[3]	1469	6.50 ± 0.44[a]	0.31	8.30 ± 0.91[b]	0.30	9.20 ± 0.13[b]	0.32	12.40 ± 0.32[c]	0.49	12.20 ± 1.14[c]	0.56	Sour, vinegar
2	Butyric acid[2]	1639	5.80 ± 0.94[a]	0.28	9.80 ± 0.23[b]	0.36	4.50 ± 0.13[a]	0.16	10.90 ± 0.72[b]	0.43	11.60 ± 0.81[b]	0.53	Cheesy, rancid, sweat
3	Hexanoic acid[1,4]	1890	2.50 ± 0.81[ab]	0.12	3.40 ± 0.42[a]	0.12	1.40 ± 0.01[b]	0.05	6.20 ± 0.41[c]	0.25	6.70 ± 0.63[c]	0.31	Cheesy, fatty, sour
4	Octanoic acid[1,4]	2110	11.90 ± 0.21[a]	0.58	11.50 ± 0.22[a]	0.42	6.10 ± 0.64[b]	0.21	10.40 ± 1.22[a]	0.41	11.60 ± 1.63[a]	0.53	Cheesy, sweat
5	Nonanoic acid[2]	2219	1.90 ± 0.14[a]	0.09	0.90 ± 0.08[b]	0.03	0.80 ± 0.08[b]	0.03	0.90 ± 0.06[b]	0.04	0.80 ± 0.06[b]	0.04	Fat, green
6	Decanoic acid[4]	2328	8.40 ± 0.21[a]	0.41	6.70 ± 0.32[b]	0.24	4.10 ± 0.69[c]	0.14	5.40 ± 0.22[d]	0.21	5.20 ± 0.14[d]	0.24	Fat, rancid
7	Dodecanoic acid[3]	2544	15.60 ± 1.32[a]	0.75	15.60 ± 1.04[a]	0.57	7.20 ± 0.28[b]	0.25	10.70 ± 0.14[c]	0.43	10.60 ± 0.12[c]	0.49	Bay oil, coconut, fatty
8	Tetradecanoic acid[3]	2757	1.80 ± 0.14[a]	0.09	1.60 ± 0.01[b]	0.06	1.00 ± 0.05[c]	0.03	1.20 ± 0.07[d]	0.05	1.10 ± 0.01[c]	0.05	Fatty, creamy, soapy
	Subtotal		**54.40**	**2.63**	**57.80**	**2.10**	**34.30**	**1.19**	**58.10**	**2.31**	**59.8**	**2.74**	
	Alcohols												
9	Ethanol[2]	948	1100 ± 111.12[a]	53.15	1830 ± 30.84[b]	66.64	1430 ± 58.82[c]	49.65	1510 ± 34.78[c]	60.03	1220 ± 104.43[a]	55.94	Strong alcoholic, sweet
10	Isobutyl alcohol[2]	1099	7.20 ± 0.34[a]	0.35	11.50 ± 0.28[b]	0.42	8.40 ± 0.92[a]	0.29	12.90 ± 0.33[b]	0.51	12.50 ± 0.27[b]	0.57	Ether wine
11	Active Amyl alcohol[4]	1220	5.30 ± 0.13[ab]	0.26	6.50 ± 0.64[a]	0.24	4.20 ± 0.43[b]	0.15	12.50 ± 0.12[c]	0.50	25.10 ± 0.79[d]	1.15	Fusel, onion, pungent, winey
12	Isoamyl alcohol[4]	1221	8.00 ± 0.73[a]	0.39	8.40 ± 0.44[a]	0.31	6.70 ± 0.24[a]	0.23	31.20 ± 1.51[b]	1.24	12.70 ± 0.59[c]	0.58	Burnt, malt, whiskey

Table 6.2. (Continued)

No.	Compounds identified	LRI[f]	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		Organoleptics[g]
			Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	
13	2-Phenylethyl alcohol[4]	1944	14.30 ± 0.58[a]	0.69	13.30 ± 0.72[a]	0.48	47.00 ± 1.14[b]	1.63	14.20 ± 0.74[a]	0.56	13.30 ± 1.78[a]	0.61	Honey, lilac, rose
	Subtotal		**1134.8**	**54.83**	**1869.7**	**68.09**	**1496.3**	**51.95**	**1580.8**	**62.84**	**1283.6**	**58.85**	
	Aldehydes												
14	Benzaldehyde[2]	1553	0.30 ± 0.01[a]	0.01	1.00 ± 0.32[b]	0.04	1.10 ± 0.06[b]	0.04	1.50 ± 0.08[c]	0.06	1.20 ± 0.22[b]	0.06	Almond, burnt sugar
15	O-Tolualdehyde	1684	2.60 ± 0.12[a]	0.13	0.60 ± 0.06[b]	0.02	1.50 ± 0.13[c]	0.05	2.30 ± 0.01[d]	0.09	3.50 ± 0.04[e]	0.16	Almond, cherry pit, coumarin
16	Ethylbenzal-dehyde	1876	6.60 ± 0.41[a]	0.32	9.20 ± 0.32[b]	0.34	2.50 ± 0.24[c]	0.09	0.30 ± 0.02[d]	0.01	0.30 ± 0.01[d]	0.01	Sweet
	Subtotal		**9.50**	**0.46**	**10.80**	**0.39**	**5.10**	**0.18**	**4.10**	**0.16**	**5.00**	**0.23**	
	Esters												
17	Methyl octanoate[3]	1390	0.70 ± 0.05[a]	0.03	0.70 ± 0.12[a]	0.03	0.30 ± 0.02[b]	0.01	1.10 ± 0.09[c]	0.04	1.20 ± 0.03[c]	0.06	Green, orange, sweet, waxy
18	Methyl decanoate[3]	1633	1.30 ± 0.03[a]	0.06	1.30 ± 0.08[a]	0.05	0.90 ± 0.05[b]	0.03	0.90 ± 0.07[b]	0.04	0.70 ± 0.04[b]	0.03	Floral, fruity, oily, wine
19	Methyl dodecanoate[3]	1815	4.10 ± 0.22[a]	0.20	4.10 ± 0.06[a]	0.15	2.00 ± 0.06[b]	0.07	1.70 ± 0.05[c]	0.07	1.70 ± 0.14[c]	0.08	Creamy coconut, soapy, waxy
20	Ethyl butyrate[1]	1034	5.30 ± 0.32[a]	0.26	7.40 ± 0.33[b]	0.27	5.10 ± 0.22[a]	0.18	9.20 ± 0.38[c]	0.37	5.80 ± 0.42[a]	0.27	Fruity, sweet
21	Ethyl hexanoate[1]	1251	2.00 ± 0.14[a]	0.10	1.80 ± 0.12[a]	0.07	1.00 ± 0.13[b]	0.03	4.30 ± 0.02[c]	0.17	4.40 ± 0.14[c]	0.20	Pineapple, sweet
22	Ethyl octanoate[1,4]	1436	17.20 ± 0.64[a]	0.83	23.00 ± 0.73[b]	0.84	7.40 ± 0.22[c]	0.26	23.90 ± 0.59[b]	0.95	23.30 ± 1.04[b]	1.07	Oily, fruity
23	Ethyl decanoate[1]	1649	30.10 ± 1.03[a]	1.45	25.30 ± 1.74[b]	0.92	13.50 ± 0.83[c]	0.47	13.00 ± 0.28[c]	0.52	14.70 ± 0.62[c]	0.67	Fruity, sweet apple, waxy
24	Ethyl dodecanoate[3]	1857	49.00 ± 3.31[a]	2.37	65.70 ± 1.82[b]	2.39	27.00 ± 0.24[c]	0.94	20.50 ± 0.94[d]	0.81	17.40 ± 1.18[d]	0.80	Floral, soapy, sweet, waxy

Table 6.2. (Continued)

No.	Compounds identified	LRI[f]	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		Organoleptics[g]
			Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	
25	Ethyl tetradecanoate[3]	2201	2.50 ± 0.12[a]	0.12	2.80 ± 0.31[a]	0.10	1.30 ± 0.14[b]	0.05	0.20 ± 0.07[c]	0.01	0.20 ± 0.04[c]	0.01	Sweet, waxy
26	Ethyl 9-hexadecenoate[3]	2337	3.40 ± 0.24[a]	0.16	2.60 ± 0.07[b]	0.09	1.70 ± 0.13[c]	0.06	0.80 ± 0.03[d]	0.03	0.80 ± 0.03[d]	0.04	Creamy, waxy
27	Ethyl hexadecanoate[3]	2306	1.40 ± 0.14[a]	0.07	1.10 ± 0.09[b]	0.04	0.90 ± 0.06[c]	0.03	0.50 ± 0.01[d]	0.02	0.50 ± 0.02[d]	0.02	Creamy, fruity, milky
28	Isoamyl butyrate[3]	1275	0.20 ± 0.00[a]	0.01	0.50 ± 0.03[b]	0.02	0.50 ± 0.03[b]	0.02	1.30 ± 0.04[c]	0.05	1.40 ± 0.04[c]	0.06	Green apple, sweet estery, waxy
29	Isoamyl propanoate	1197	0.60 ± 0.08[a]	0.03	0.90 ± 0.02[b]	0.03	0.30 ± 0.04[c]	0.01	2.20 ± 0.13[d]	0.09	1.40 ± 0.04[e]	0.06	Banana, pineapple-like, tropical
30	2-Phenylethyl butyrate	1941	1.00 ± 0.14[a]	0.05	0.50 ± 0.01[b]	0.02	1.30 ± 0.08[c]	0.05	0.20 ± 0.01[d]	0.01	0.20 ± 0.04[d]	0.01	Floral, fruity, musty
31	Methyl acetate	865	2.40 ± 0.14[a]	0.12	2.90 ± 0.33[b]	0.11	1.70 ± 0.23[c]	0.06	1.50 ± 0.01[c]	0.06	1.60 ± 0.12[c]	0.07	Estery, fruity, winey
32	Ethyl acetate[2]	916	521 ± 22.43[a]	25.18	471 ± 29.48[a]	17.15	518 ± 2.13[a]	17.98	386 ± 19.28[b]	15.35	391 ± 6.44[b]	17.93	Ethereal, fruity, pineapple
33	Propyl acetate	1001	5.00 ± 0.07[a]	0.24	6.20 ± 0.53[b]	0.23	5.00 ± 0.44[a]	0.17	8.20 ± 0.14[c]	0.33	4.20 ± 0.12[a]	0.19	Fruity, pear
34	Butyl acetate	1061	1.20 ± 0.09[a]	0.06	1.70 ± 0.13[b]	0.06	1.60 ± 0.06[b]	0.06	2.00 ± 0.04[c]	0.08	1.30 ± 0.14[a]	0.06	Ethereal, fruity, sharp
35	Isobutyl acetate[1]	1029	2.80 ± 0.06[a]	0.14	2.70 ± 0.24[a]	0.10	2.10 ± 0.14[a]	0.07	4.70 ± 0.42[b]	0.19	4.70 ± 0.83[b]	0.22	Banana, ethereal, fruity,
36	Active amyl acetate	1097	1.70 ± 0.52[a]	0.08	1.90 ± 0.07[a]	0.07	1.30 ± 0.14[a]	0.05	6.80 ± 0.74[b]	0.27	28.80 ± 0.92[c]	1.32	Banana, fruity, ripe
37	Isoamyl acetate[1]	1099	91.00 ± 4.02[a]	4.40	63.30 ± 0.73[a]	2.31	60.40 ± 2.49[a]	2.10	300 ± 17.48[b]	11.93	232 ± 22.44[c]	10.64	Banana, fruity with a ripe estery nuance, sweet
38	Benzyl acetate[4]	1753	2.60 ± 0.22[a]	0.13	2.80 ± 0.03[a]	0.10	2.00 ± 0.07[b]	0.07	2.50 ± 0.06[a]	0.10	2.70 ± 0.14[a]	0.12	Floral, fruity, sweet

Table 6.2. (Continued)

No.	Compounds identified	LRI[f]	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		Organoleptics[g]
			Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	
39	2-Phenylethyl acetate[1]	1841	123 ± 11.64[a]	5.94	116 ±13.43[ab]	4.22	688 ± 20.24[d]	23.89	79.80 ± 8.03[bc]	3.17	91.80 ± 5.04[ac]	4.21	Floral rosy, honey, sweet
	Subtotal		**869.50**	**42.01**	**806.20**	**29.36**	**1343.30**	**46.64**	**871.30**	**34.64**	**831.80**	**38.14**	
	Ketone												
40	β-Damascenone[4]	1845	0.60 ± 0.06[a]	0.03	0.70± 0.04[b]	0.03	0.50 ± 0.02[ac]	0.02	0.50 ± 0.02[ac]	0.02	0.40 ± 0.01[c]	0.02	Apple, honey, rose
	Heteroatom (N, S) compound												
41	Benzyl isothiocyanate[3]	2140	0.70 ± 0.02[ab]	0.03	0.80±0.03[a]	0.03	0.80 ± 0.09[a]	0.03	0.60 ± 0.03[bc]	0.02	0.50 ± 0.04[c]	0.02	Medicinal horseradish, oily, watercress
	Total		**2069.50**		**2746.00**		**2880.30**		**2515.40**		**2180.10**		

[a,b,c,d,e]Statistical analysis at 95% confidence level with same letters indicating no significant difference.
[f]Experimentally determined linear retention index on the DB-FFAP column, relative to C5-C40 hydrocarbons.
[g]From Luebke (1980).
[1,2,3,4]Retention index in agreement with those in the literature [Duarte et al. (2010), Goodner (2008), Pino et al. (2003) and Segurel et al. (2009), respectively].

Table 6.3. Concentrations of selected major volatile compounds (mg/L) in papaya wine (day 21) fermented with *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added

Compounds quantified	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[e] (mg/L)
	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	
Ethanol	17122 ± 546[ab]	-	18712 ± 63[a]	-	12673 ± 938[c]	-	16749 ± 440[ab]	-	16242 ± 867[b]	-	-
Isoamyl alcohol	13.53 ± 0.91[ab]	0.45	14.92 ± 1.46[ac]	0.50	11.36 ± 0.93[b]	0.38	19.98 ± 1.35[d]	0.67	17.66 ± 0.92[dc]	0.59	30.00
Active amyl alcohol	0.69 ± 0.03[a]	0.01	0.98 ± 0.06[b]	0.02	0.45 ± 0.05[c]	0.01	1.10 ± 0.01[b]	0.02	1.77 ± 0.15[d]	0.03	65.00
Isobutyl alcohol	2.26 ± 0.22[a]	0.06	9.17 ± 0.77[b]	0.23	1.77 ± 0.18[a]	0.04	6.00 ± 0.21[c]	0.15	6.51 ± 0.32[c]	0.16	40.00
2-Phenylethyl alcohol	2.29 ± 0.13[a]	0.23	2.57 ± 0.37[a]	0.26	17.16 ± 2.48[b]	1.72	2.24 ± 0.10[a]	0.22	1.99 ± 0.26[a]	0.20	10.00
Octanoic acid	0.37 ± 0.04[a]	0.04	0.28 ± 0.03[b]	0.03	0.03 ± 0.00[c]	0.00	0.28 ± 0.01[b]	0.03	0.38 ± 0.02[a]	0.04	8.80
Ethyl octanoate	0.07 ± 0.01[a]	3.50	0.11 ± 0.01[b]	5.50	0.04 ± 0.00[c]	2.00	0.13 ± 0.01[bd]	6.50	0.11 ± 0.01[cd]	5.50	0.02
Ethyl decanoate	0.29 ± 0.04[a]	1.45	0.28 ± 0.00[a]	1.40	0.20 ± 0.02[b]	1.00	0.23 ± 0.01[ab]	1.15	0.25 ± 0.02[ab]	1.25	0.20
Ethyl dodecanoate	3.97 ± 0.40[a]	3.31	4.87 ± 0.15[b]	4.06	3.70 ± 0.10[a]	3.08	3.55 ± 0.19[a]	2.96	3.52 ± 0.30[a]	2.93	1.20[f]
Isoamyl acetate	6.48 ± 0.09[a]	216.00	6.38 ± 0.04[a]	212.67	6.57 ± 0.18[a]	219.00	8.29 ± 0.04[b]	276.33	7.10 ± 0.10[c]	236.67	0.03
Active amyl acetate	0.015 ± 0.002[a]	0.09	0.015 ± 0.001[a]	0.09	0.014 ± 0.002[a]	0.09	0.013 ± 0.002[a]	0.08	0.063 ± 0.004[b]	0.38	0.16
Isobutyl acetate	0.008 ± 0.002[a]	0.01	0.009± 0.001[b]	0.01	0.007 ± 0.001[ac]	0.00	0.005 ± 0.002[c]	0.00	0.007 ± 0.001[a]	0.00	1.60
2-Phenylethyl acetate	1.76 ± 0.16[a]	7.04	1.82 ± 0.08[a]	7.28	14.30 ± 1.64[b]	57.20	1.37 ± 0.11[a]	5.48	1.74 ± 0.10[a]	6.96	0.25

Abbreviation: OAV = Odour activity values calculated by dividing concentration by the odour threshold value of the compound

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

[e]From Bartowsky and Pretorius (2009).

[f]From Ferreira et al. (2000).

6.2.3 Principal component analysis

Principle component analysis (PCA) was applied to all volatile compounds in Tables 6.2 and 6.3 to obtain a more simplified view of the volatile profiles of the papaya wines after the addition of different amino acids. PCA is a projection method that reduces the dimensionality in a data matrix while retaining the most significant information. The PCA of the quantified major volatile compounds (Table 6.3) reveals clear separation among the papaya wines added with the different amino acids (Fig. 6.7), and it is a representation of the PCA result from Table 6.2. The first two principle components (PCs) represented 80.5% of the total variance, thus the remaining PCs made very little contribution to the total variance.

The addition of L-phenylalanine was associated with a high percentage of 2-phenylethyl acetate and 2-phenylethyl alcohol as compared to the control. The wines produced with the addition of L-isoleucine and L-leucine expressed close resemblance that had a correlation with isoamyl alcohol, active amyl alcohol, isoamyl acetate, active amyl acetate and ethyl octanoate. Conversely, the papaya wine produced with the addition of L-valine (upper left quadrant) was more related to ethyl decanoate, ethyl dodecanoate, ethanol, isobutyl alcohol and isobutyl acetate. Interestingly, the control was not associated with any volatile compounds in Fig. 6.7. However, it was characterised by long-chain ethyl esters such as ethyl tetradecanoate, ethyl hexadecanoate and ethyl 9-hexadecanoate in the PCA result from Table 6.2 [Appendix D (Fig. D6)]. This could be due to a lack of external standards to quantify the representative volatile compounds of the control.

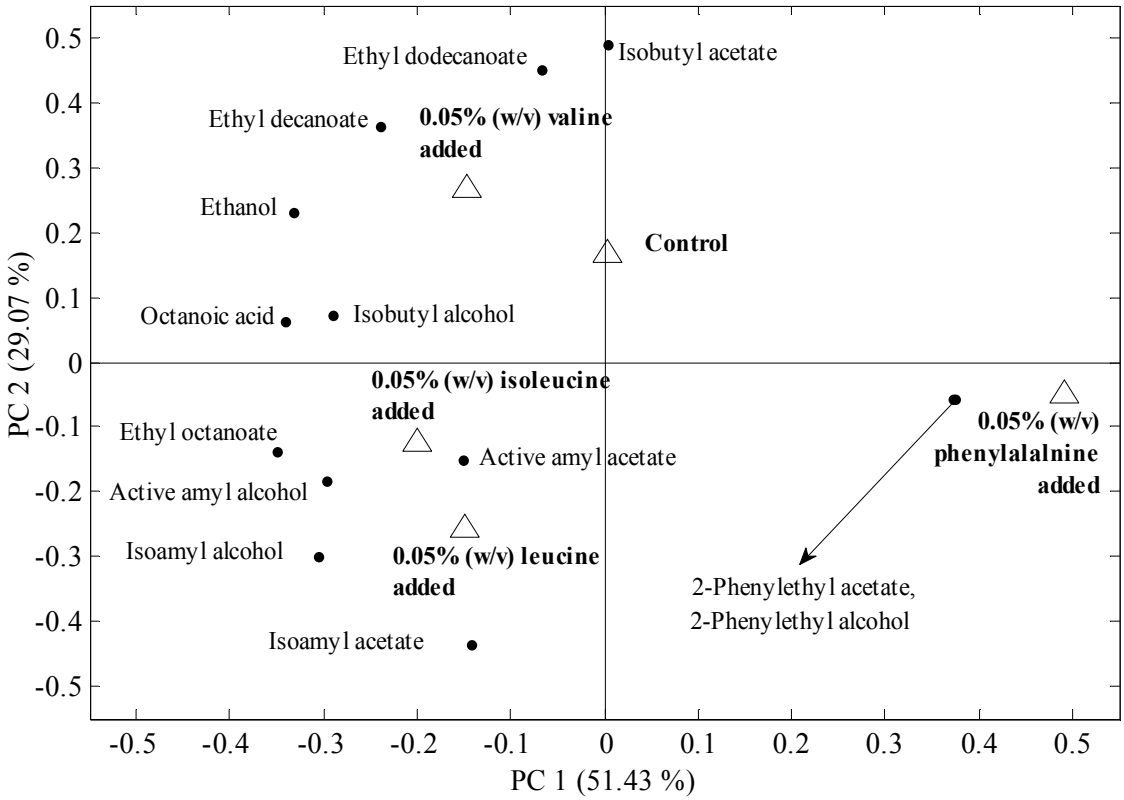

Fig. 6.7. Bi-plot of principal component analysis of the quantified major volatile compounds in papaya wine fermented with *W. saturnus mrakii* NCYC2251 in the presence of the different added amino acids.

6.3 Conclusions

In this chapter, fermentation performance and 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. saturnus* NCYC2251 was capable of producing papaya wine with enhanced amount of targeted aroma-active compounds through the addition of a specific amino acid, and hence can be a valuable tool to modulate the aroma of papaya wine.

CHAPTER 7

EFFECT OF FUSEL OIL ADDITION ON VOLATILE COMPOUNDS IN PAPAYA WINE FERMENTED WITH *WILLIOPSIS SATURNUS* VAR. *MRAKII* NCYC2251

7.1 Introduction

Fusel oil is a by-product of the alcohol distillation industry. Approximately 1 to 11 L of fusel oil is obtained with 1000 L of ethanol from the distillation, depending on the substrate used, nitrogenous substances added and conditions of fermentation and distillation (Patil, Koolwal, & Butala, 2002). The main components of fusel oil are ethanol (13%), butanol (15%), i-amyl alcohols (amyl and isoamyl alcohols, 51%) and small proportions of other secondary alcohols and water (15%) (Yilmaztekin, Erten, & Cabaroglu, 2009). The direct utilisation of fusel oil as a solvent is limited and a large portion of fusel oil is generally discarded due to its relatively undesirable dark-reddish colour and unpleasant odour (Kucuk & Ceylan, 1998). However, studies have suggested that fusel oil has the potential as a valuable raw material for synthesising other chemicals, for example, enzymatic synthesis and/or esterification of fusel oil with butyric acid to yield esters such as ethyl butyrate (Kucuk & Ceylan, 1998; Welsh & Williams, 1989).

The yeast from the genus *Williopsis* (formerly *Hansenula*) is a potent producer of esters (Inoue et al., 1997) and has the capability of converting higher alcohols present in the fusel oil into the corresponding acetate esters (Janssens et al., 1992; Vandamme, 2003; Yilmaztekin et al., 2009) that potentially enhance the fruity flavour in wines. Traditionally, higher alcohols are only formed by yeast via catabolic routes (Ehrlich pathway) in the presence of sufficient amino acids (Sentheshanmuganathan,

1960) or produced *de novo* from sugars (Clemente-Jimenez et al., 2005). Hence, the addition of fusel oil provides an additional source of higher alcohols for ester formation, which is an alternative way to obtain natural acetate esters from cheap agricultural residues.

Given the capability of *W. saturnus* to convert the higher alcohols into respective esters, it would be of value to evaluate the possibility of using fusel oil as an aroma precursor in papaya wine fermentation. This will be more economical as compared to the addition of amino acids for increased ester formation. The aim of this chapter was to investigate the effects of fusel oil addition on the fermentation performance and the volatile compounds formation by *W. saturnus* var. *mrakii* NCYC2251 in papaya juice.

7.2 Results and discussion

7.2.1 Growth and fermentation behaviour of yeast in the presence of different concentrations of fusel oil

Yeast growth, viable cells, total soluble solids (^{o}Brix), sugar consumption, organic acid and pH changes are presented in Fig. 7.1, Table 7.1 and Appendix E (Fig. E1). The addition of 0.1% (v/v) fusel oil had most of the fermentation characteristics similar to the control (no addition), except for the yeast growth that differed slightly. The control has a lag phase of 3 days, while the fermentation added with 0.1% (v/v) fusel oil has a longer lag phase of 6 days (Fig. 7.1). The papaya juice fermentation with 0.1% (v/v) fusel oil added had the highest yeast growth with cell count of 2.32 x 10^8 CFU/mL at day 21, followed by the control at 1.55 x 10^8 CFU/mL from an initial cell population of 2.30 x 10^5 CFU/mL (Table 7.1). This corresponded to the lowest ^{o}Brix value of 3.92% (0.80 g/100 mL fructose and 0.31 g/100 mL glucose) in the

fermentation added with 0.1% (v/v) fusel oil (Table 7.1). The °Brix value trend and sugar consumption were not affected by the addition of 0.1% (v/v) fusel oil [Fig. 7.1, Appendix E (Fig. E1)] and corresponded to the sugar consumption behaviour of this *W. saturnus* strain as observed in **Chapter 5** (Figs. 5.1 and 5.2). The pH changes varied with values maintaining at around pH 3.55-3.74 (Table 7.1). The changes of the organic acids were similar in both the control and that added with 0.1% fusel oil, except that the fermentation added with 0.1% fusel oil had reduced acetic acid production while the control had increased acetic acid production [Table 7.1, Appendix E (Fig. E1)].

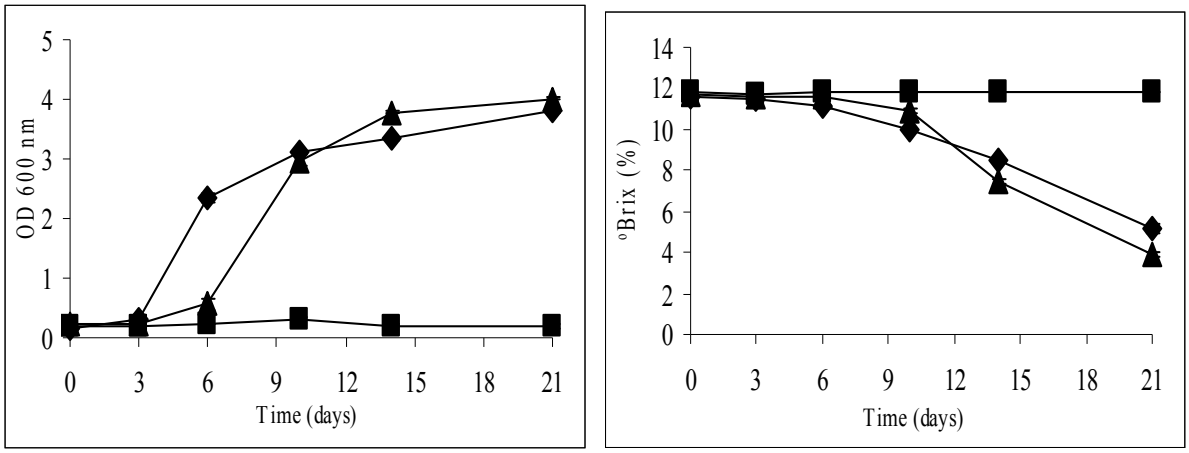

Fig. 7.1. Growth of yeasts (as optical density OD 600 nm) and °Brix changes in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

The fermentation added with 0.5% (v/v) fusel oil showed no growth and no changes in all the fermentation characteristics throughout the 21-day fermentation [Fig. 7.1, Table 7.1, Appendix E (Fig. E1)]. The results of this study differed from those of Yilmaztekin et al. (2009), which found that *W. saturnus* var. *saturnus* can tolerate up to 2% (v/v) of fusel oil and the yeast growth would only decrease significantly when more than 3% (v/v) of fusel oil was added. This may be due to the

fact that fusel oil was added into the fermentation medium at the beginning of the stationary phase or the different subspecies of *Williopsis* yeast used in the study of Yilmaztekin et al. (2009).

Table 7.1. Fermentation parameters of papaya wine (day 21) fermented with *W. saturnus* var. *mrakii* NCYC2251 in the presence of the added fusel oil (v/v)

	Day 0	**Control**	**0.1% fusel oil added**	**0.5% fusel oil added**
pH	3.57 ± 0.01^{a}	3.67 ± 0.01^{b}	3.74 ± 0.01^{c}	3.55 ± 0.00^{a}
^{o}Brix (%)	11.70 ± 0.02^{a}	5.17 ± 0.22^{b}	3.92 ± 0.11^{c}	11.80 ± 0.01^{a}
Yeast cell count x10^{6}(CFU/mL)	0.23 ± 0.01^{a}	155 ± 9.90^{b}	232 ± 15.20^{c}	0.25 ± 0.01^{a}
Sugars (g/100 mL)				
Fructose	3.94 ± 0.01^{a}	1.71 ± 0.05^{b}	0.80 ± 0.05^{c}	4.19 ± 0.27^{a}
Glucose	4.11 ± 0.13^{a}	0.52 ± 0.02^{b}	0.31 ± 0.03^{b}	4.67 ± 0.37^{a}
Organic acids (g/100 mL)				
Acetic acid	0.034 ± 0.003^{a}	0.046 ± 0.002^{b}	0.028 ± 0.002^{a}	0.036 ± 0.003^{a}
Citric acid	0.269 ± 0.003^{a}	0.250 ± 0.003^{b}	0.243 ± 0.001^{b}	0.252 ± 0.011^{ab}
Malic acid	0.929 ± 0.021^{a}	0.707 ± 0.019^{b}	0.604 ± 0.015^{b}	0.932 ± 0.036^{a}
Succinic acid	0.181 ± 0.009^{a}	0.281 ± 0.029^{b}	0.326 ± 0.012^{b}	0.182 ± 0.003^{a}
Tartaric acid	0.017 ± 0.001^{a}	0.008 ± 0.001^{b}	0.008 ± 0.001^{b}	0.013 ± 0.001^{c}

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

7.2.2 Volatile compounds evolution during papaya juice fermentation

During the fermentation, a number of volatile compounds were produced by yeast metabolism including acids, alcohols, esters and aldehydes with alcohols being the most abundant aroma compounds produced. However, those volatile compounds initially present in the juice such as benzyl isothiocyanate, benzaldehyde and butyric acid were catabolised (Figs. 7.2-7.7).

The dynamic changes of fatty acids were similar in all fermentations, except for fatty acids of C8 to C14 fatty acids with 0.1% (v/v) fusel oil addition that

increased initially, and then decreased [Fig. 7.2, Appendix E (Fig. E2)]. Hexanoic and butyric acids present at relatively high concentrations in the juice were utilised by *W. saturnus* during fermentation in the control, while the same fatty acids were either absent or of negligible amounts in the fermentations added with 0.1% (v/v) and 0.5% (v/v) fusel oil (Fig. 7.2). This may due to the addition of fusel oil that altered the initial volatile composition of papaya juice. The fermentation added with 0.5% (v/v) fusel oil had the highest concentrations of octanoic, nonanoic, decanoic, dodecanoic and tetradecanoic acids (Tables 7.3 and 7.4). The formation of these fatty acids in those added with 0.5% (v/v) fusel oil [Fig. 7.2, Appendix E (Fig. E2)] corresponded to the reduction of the corresponding ethyl esters [Fig. 7.5, Appendix E (Fig. E5)]. This could possibly be due to the hydrolysis of ethyl esters with regards to the low pH environment (Bisson, 2008) or the metabolism of non-growing yeast cells. Fatty acids are essential precursors for ethyl esters formation. These ethyl esters are produced enzymatically during the synthesis or degradation of fatty acids (Alves, Lima, Dias, Nunes, & Schwan, 2010), which impart desirable fresh and fruity flavour to the wine (Table 7.3).

Acetic acid is an undesirable volatile in alcoholic beverages and imparts vinegary off-flavour. The result of this study revealed that the addition of fusel oil decreased the formation of acetic acid as compared to the control. The fermentation added with 0.1% (v/v) fusel oil did not produce acetic acid with a final concentration (0.028 g/100 mL) similar to that at day 0 (0.034 g/100 mL) (Table 7.1). The fermentation added with 0.5% (v/v) fusel oil had 0.036 g/100 mL acetic acid, which was also similar to that of day 0 (Table 7.1). These results are somewhat different from the semi-quantified volatile results (Table 7.3), which may be attributed to the

limited absorption capacity of SPME fiber and matrix effects during extraction (Burman et al., 2005).

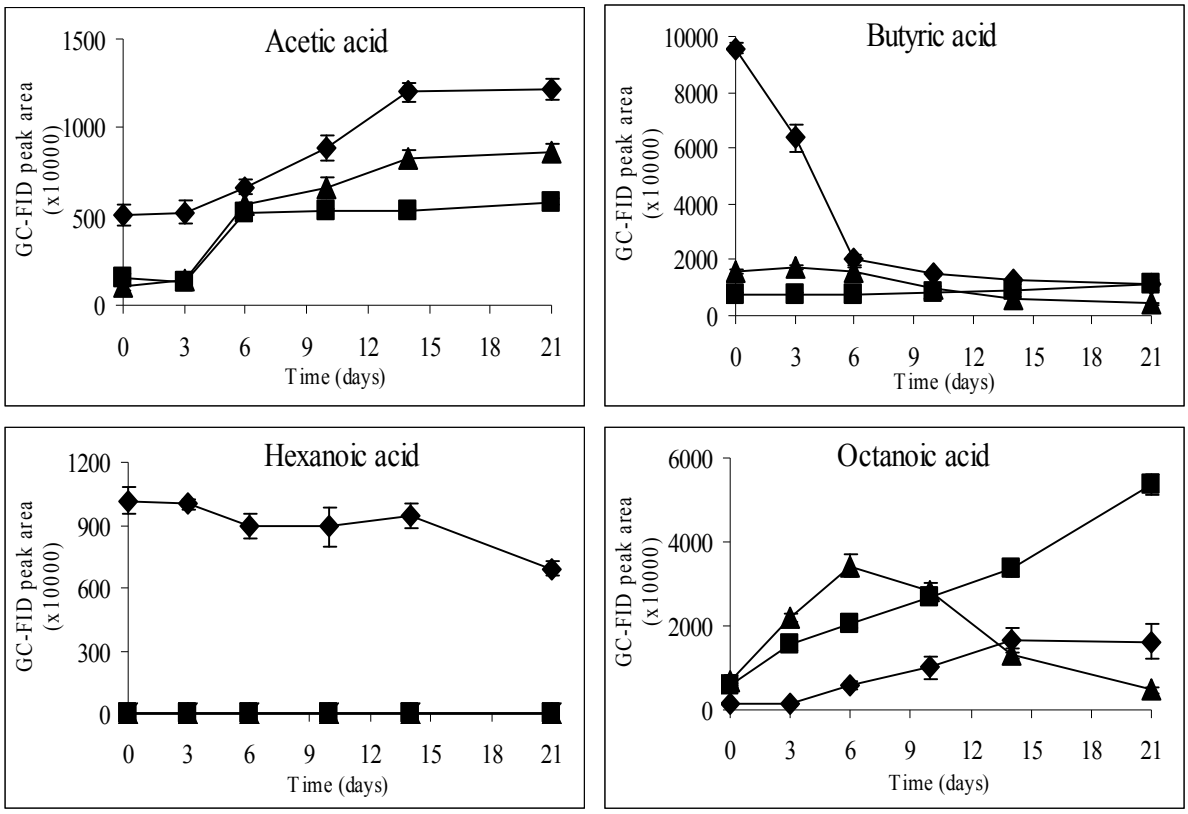

Fig. 7.2. Changes in fatty acids in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

Alcohols (ethanol and higher alcohols) are quantitatively the largest group of volatile compounds with ethanol, isobutyl alcohol (2-methyl-1-propanol), isoamyl alcohol (3-methyl-1-butanol), active amyl alcohol (2-methyl-1-butanol) and 2-phenylethyl alcohol being the major alcohols (Table 7.3). The dynamic changes of the alcohols were similar in all the fermentations, except for 2-ethylhexanol and 1-octanol that were metabolised in the control and in the fermentation added with 0.1% (v/v) fusel oil, respectively [Fig. 7.3, Appendix E (Fig. E3)]. Ethanol was constantly produced throughout the fermentation with the addition of 0.1% (v/v) fusel oil producing the highest amount of ethanol with 3.34% (v/v) (2.65 x 10^4 mg/L) (Table

7.2). The fermentation with the addition of 0.5% (v/v) fusel oil had no ethanol production (Fig. 7.3, Table 7.2), which corresponded to the negative yeast growth in Fig. 7.1 and Table 7.1.

Table 7.2. Ethanol concentrations of papaya wines fermented with *W. saturnus* var. *mrakii* NCYC2251 in the presence of the added fusel oil (v/v) before and after fermentation

	Day 0			Day 21		
	Control	**0.1% fusel oil added**	**0.5% fusel oil added**	**Control**	**0.1% fusel oil added**	**0.5% fusel oil added**
Ethanol % (v/v)	0.02 ± 0.00^{a}	0.02 ± 0.01^{a}	0.08 ± 0.01^{a}	2.64 ± 0.04^{b}	3.34 ± 0.12^{c}	0.06 ± 0.00^{a}

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

2-Phenylethyl alcohol was continuously produced in all the fermentations (Fig. 7.3). Those added with 0.1% (v/v) and 0.5% (v/v) fusel oil had comparable amount of 2-phenylethyl alcohol at 4.49 mg/L and 4.89 mg/L, respectively (Table 7.4). The substantial amount of 2-phenylethyl alcohol detected in those added with 0.5% (v/v) fusel oil even though there was no yeast growth, suggesting that the formation could be due to chemical means or enzymatic activities in the non-growing yeast cells. The other higher alcohols were either increased or decreased, depending on the type and initial level of the higher alcohols [Fig. 7.3, Appendix E (Fig. E3)]. This could be due to the relative rate of utilisation and production of higher alcohols by the yeast. As expected, the fermentation with 0.5% (v/v) of fusel oil added had the highest amount of most of the higher alcohols such as isoamyl alcohol (5053 mg/L); active amyl alcohol (1384 mg/L); isobutyl alcohol (86.24 mg/L) (Tables 7.3 and 7.4). This was mainly attributed to the addition of fusel oil, where these were the major volatile compounds in the fusel oil (Table 3.1). Higher alcohols with concentrations below

300 mg/L contribute to the desirable complexity of wine aroma, while at levels above 400 mg/L, the higher alcohols are regarded as a negative quality factor (Rapp & Mandery, 1986). The fermentation added with 0.5% (v/v) fusel oil had total higher alcohol concentrations higher than 400 mg/L, which is considered negative for wine quality. It should be noted that the final total level of higher alcohols in the fermentation with added 0.1% (v/v) fusel oil was less than 300 mg/L (Table 7.4), which was not expected to exert an adverse impact on wine aroma.

Higher alcohols are normally produced by yeast via Ehrlich's pathway in the presence of sufficient amino acids (Sentheshanmuganathan, 1960). Higher alcohols and acetyl-CoA form the main precursors for acetate ester formation such as branched-chain or aromatic esters that lead to wine flavour complexity, stylistic distinction and vintage variability (Soles et al., 1982). Non-*Saccharomyces* yeasts produce lower levels of higher alcohols as compared to *Saccharomyces* yeasts (Moreira et al., 2008). The addition of fusel oil contributes an additional source of higher alcohols for ester formation.

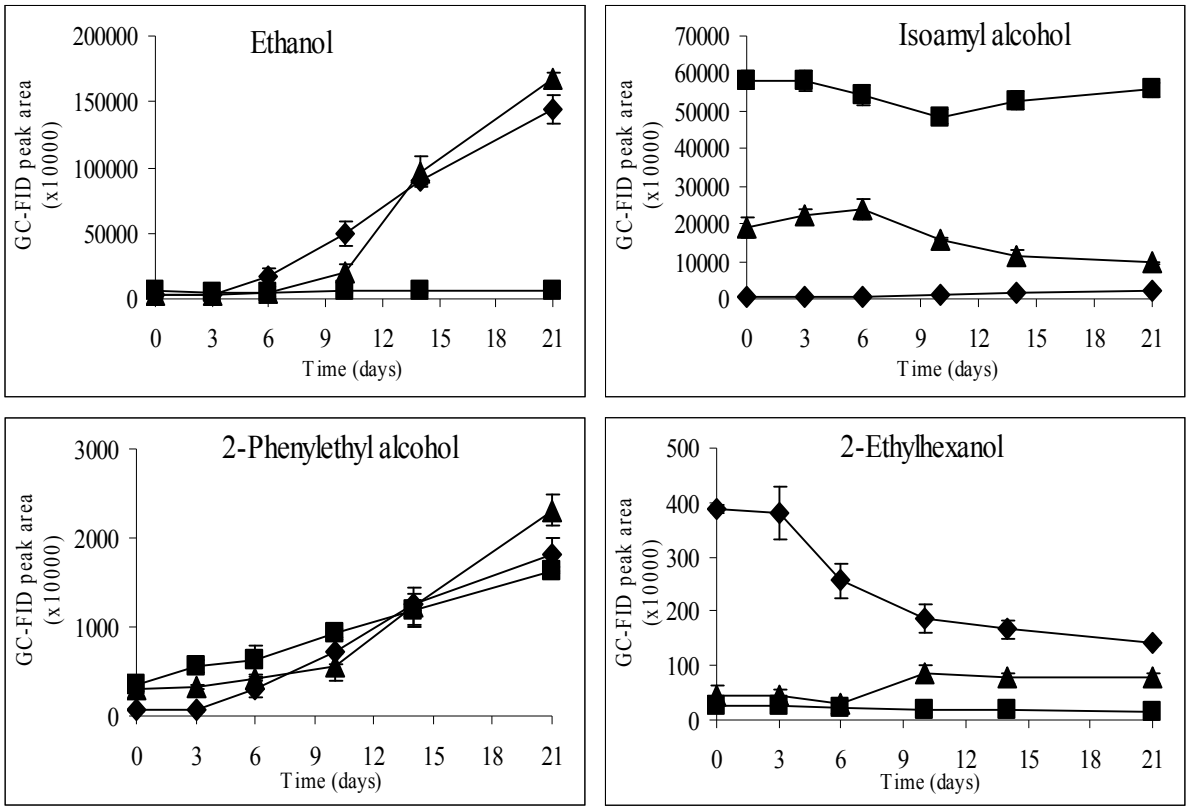

Fig. 7.3. Changes in alcohols in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

Among the other volatile compounds, esters were the next most abundant group of aroma compounds produced ranging from 33.53 to 59.22% (RPA), which included acetate, ethyl, methyl and other esters (Figs. 7.4-7.6, Table 7.3). The fermentations added with fusel oil, especially those with the addition of 0.5% (v/v) fusel oil had high initial level of most of these esters as compared to the control (Figs. 7.4-7.6). This could be due to the presence of these esters in fusel oil, albeit in a small amount (Table 3.1). Acetate esters tended to increase initially then declined with the exception of the fermentation added with 0.5% (v/v) fusel oil, which decreased throughout the fermentation [Fig. 7.4, Appendix E (Fig. E4)]. The addition of 0.1% (v/v) fusel oil consistently produced the highest amount of most acetate esters especially isoamyl acetate (57.65 mg/L), except for ethyl acetate, propyl acetate and

benzyl acetate as compared to the control [Tables 7.3 and 7.4, Appendix E (Fig. E4)]. These acetate esters impart desirable fruity and floral notes, except for ethyl acetate at high levels that imparts light fruity and solvent-like aroma. The rapid increase of isoamyl acetate production was likely due to 3-methyl-1-butanol (isoamyl alcohol) from fusel oil added into the fermentation medium and acetyl-CoA being converted into 3-methyl-1-butyl acetate (isoamyl acetate) via alcoholysis at a much faster rate than other esters being formed (Vandamme & Soetaert, 2002). Alcohol acetyltransferases in the yeasts involved in ester biosynthesis would become saturated when more than 400 mg/L of 3-methyl-1-butanol (Calderbank & Hammond, 1994) and 1000 mg/L of fusel oil (Quilter, Hurley, Lynch, & Murphy, 2003) were added into the fermentation media and hence, there would be no increment in isoamyl acetate levels with further additions of fusel oil beyond these levels. The results of this study are in accordance with these studies, where there was no production of acetate esters in the fermentation added with 0.5% (v/v) fusel oil.

Non-*Saccharomyces* wine yeasts are traditionally associated with high ethyl acetate production that can impart spoilage character to wine at a concentration of 150-200 mg/L (Jackson, 1994). The addition of 0.1% (v/v) fusel oil greatly reduced the ethyl acetate concentration produced by *W. saturnus* with 9.53% (RPA) as compared to the control with 16.70% (RPA) (Fig. 7.4, Table 7.3). The final amounts of acetate esters at day 21 varied significantly among the different concentrations of fusel oil and the control (Tables 7.3 and 7.4).

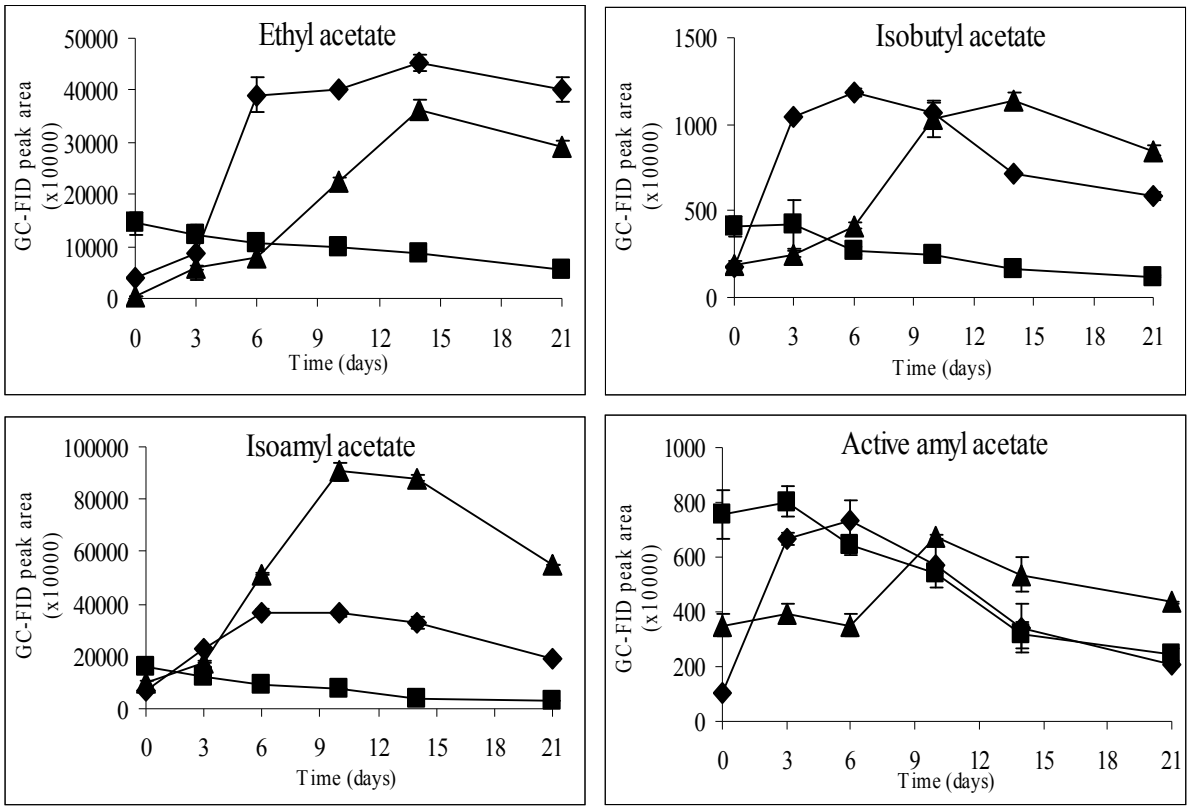

Fig. 7.4. Changes in acetate esters in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

Ethyl and methyl esters generally decreased during fermentation, except for the control where there was an increase. Some methyl esters such as methyl octanoate and methyl decanoate increased initially and then declined in the fermentation added with 0.1% (v/v) fusel oil [Fig. 7.5, Appendix E (Fig. E5)]. For the miscellaneous esters [Fig. 7.6, Appendix E (Fig. E6)], most of them either remained constant or increased gradually and then decreased in the fermentation added with 0.1% (v/v) fusel oil and the control, except for propyl decanoate that decreased continuously with the addition of 0.1% (v/v) fusel oil. The addition of 0.5% (v/v) fusel oil increased the formation of methyl decanoate, isoamyl decanoate, isoamyl dodecanoate, isobutyl decanoate and isoamyl propanoate, while ethyl butyrate, ethyl (*E*)-4-decenoate, isoamyl octanoate and isobutyl octanoate increased and then either remained constant

or decreased [Figs. 7.5 and 7.6, Appendix E (Figs. E5 and E6)]. This was observed even though there was no yeast growth, suggesting that the formation of these esters could be chemical rather than microbiological. Conversely, medium- to long-chain ethyl esters and methyl dodecanoate decreased significantly throughout fermentation in those added with fusel oil [Fig. 7.5, Appendix E (Fig. E5)]. The reduction of these esters in those added with 0.1% (v/v) fusel oil could be attributed to volatilisation and/or the rate of hydrolysis was greater than their formation (Miller, Wolff, Bisson, & Ebeler, 2007), while those added with 0.5% (v/v) fusel oil was likely due to volatilisation and/or hydrolysis due to the acidic condition (Bisson, 2008; Ramey & Ough, 1980).

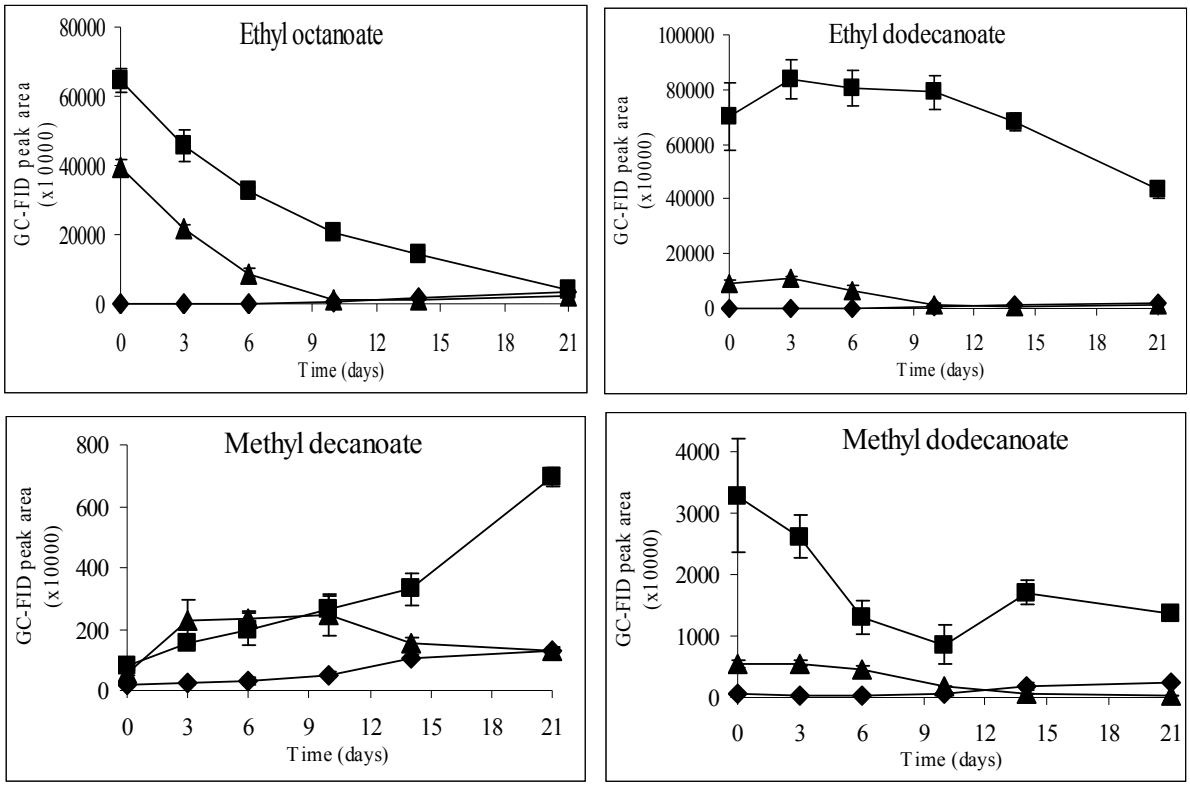

Fig. 7.5. Changes in ethyl and methyl esters in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

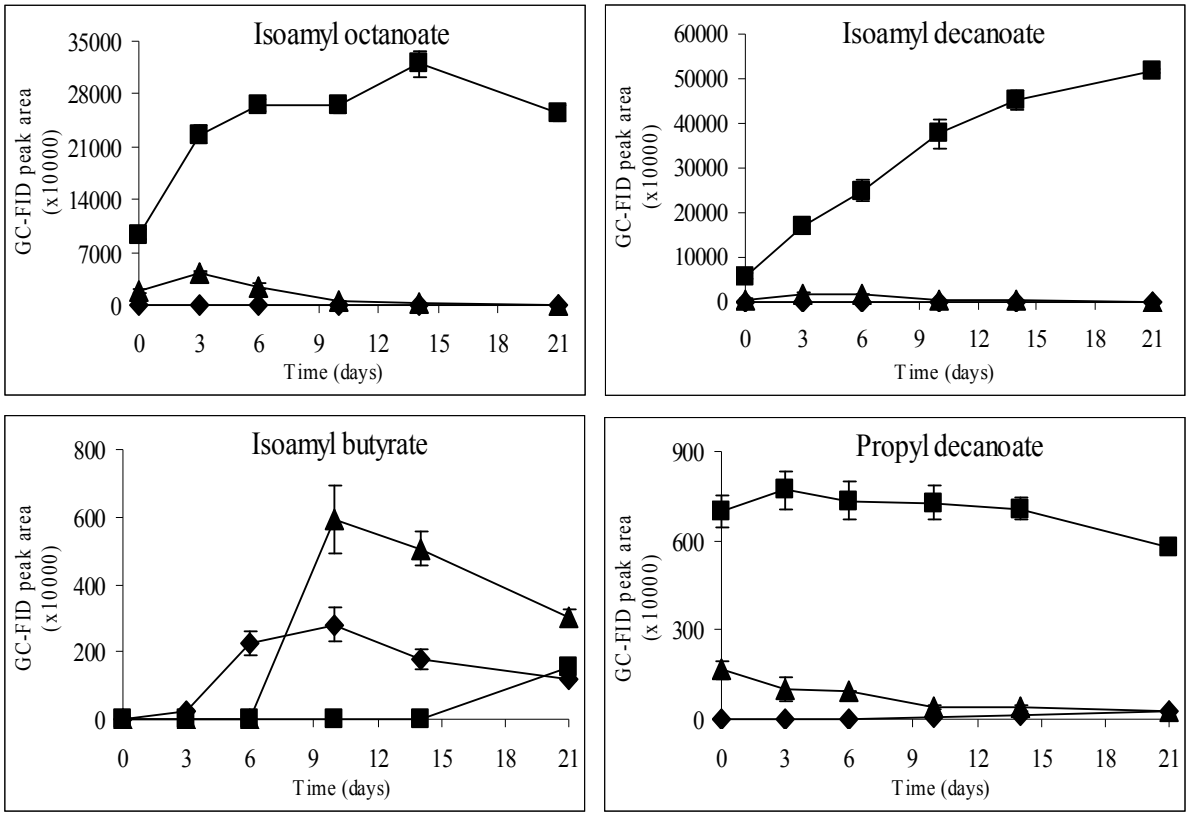

Fig. 7.6. Changes in other esters in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

Most of the miscellaneous volatile compounds, particularly benzaldehyde and benzyl isothiocyanate, either remained constant or metabolised to trace levels, except for O-tolualdehyde, β-damascenone and β-ionone that were formed in the fermentation added with 0.5% (v/v) fusel oil [Fig. 7.7, Appendix E (Fig. E7)], being comparable to the incremental trends in the esters formation. Those with the addition of 0.5% (v/v) fusel oil had the highest amount of aldehydes and ketones at day 21 (Table 7.3), which may contribute to green, fatty, fruity and pungent aromas (Ugliano & Henschke, 2009). β-Damascenone was one of a few compounds which were identified in both fresh papaya juice and wine. There were significant differences in the concentrations of ethyl esters and other major volatile compounds at day 21

among the different concentrations of added fusel oil and the control (Tables 7.3 and 7.4).

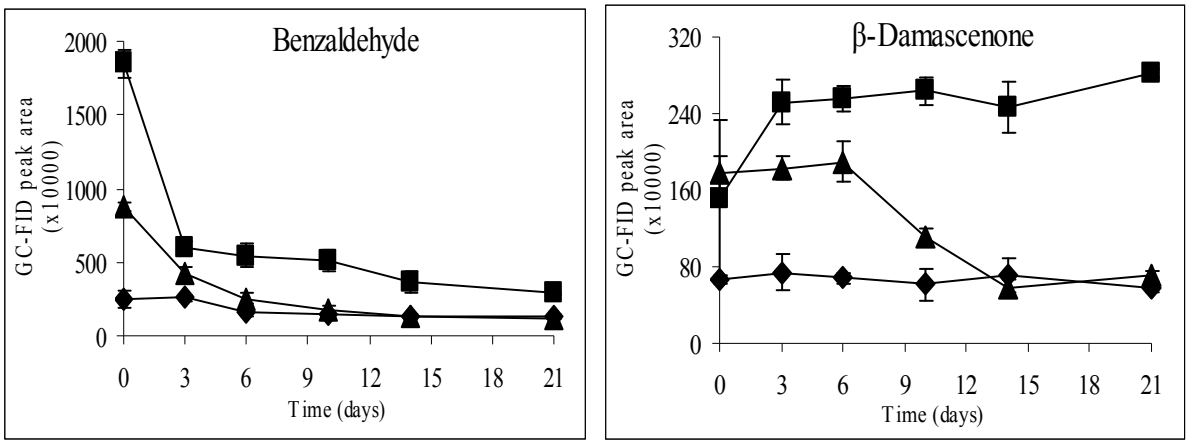

Fig. 7.7. Changes in benzaldehyde and β-damascenone in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

Table 7.3. Major volatile compounds (GC-FID peak area x 10^6) and their relative peak areas (RPA) identified in papaya wine (day 21) fermented with *W. saturnus* var. *mrakii* NCYC2251 in the presence of added fusel oil (v/v)

No.	Compounds identified in this study	LRI[d]	Control		0.1% fusel oil added		0.5% fusel oil added		Organoleptics[e]
			Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	
	Acids								
1	Acetic acid[3]	1470	12.10 ± 0.58[a]	0.50	8.68 ± 0.43[b]	0.29	5.81 ± 0.15[c]	0.17	Acidic, vinegar
2	Butyric acid[2]	1639	10.90 ± 1.71[a]	0.45	4.25 ± 0.21[b]	0.14	11.60 ± 0.24[a]	0.33	Rancid, cheesy
3	Hexanoic acid[1,4]	1860	6.89 ± 0.35[a]	0.29	0.00 ± 0.00[b]	0.00	0.00 ± 0.00[b]	0.00	Sweet, cheesy
4	Octanoic acid[1,4]	2076	16.30± 4.01[a]	0.68	4.69 ± 0.68[b]	0.15	53.80 ± 2.54[c]	1.54	Sweet, cheesy
5	Nonanoic acid[2]	2184	0.66 ± 0.03[a]	0.03	0.58 ± 0.04[a]	0.02	7.48 ± 0.11[b]	0.21	Green, fatty
6	Decanoic acid[4]	2292	9.17 ± 0.37[a]	0.38	4.80 ± 0.28[a]	0.16	307 ± 16.70[b]	8.79	Unpleasant, rancid, sour
7	Dodecanoic acid[3]	2506	15.80 ± 0.75[a]	0.66	2.04 ± 0.39[b]	0.07	31.10 ± 0.43[c]	0.89	Fatty, coconut, bay oil
8	Tetradecanoic acid[3]	2718	1.22 ± 0.11[a]	0.05	0.30 ± 0.03[b]	0.01	3.13 ± 0.17[c]	0.09	Waxy, fatty, soapy, coconut
	Subtotal		**73.40**	**3.04**	**25.34**	**0.83**	**419.92**	**12.03**	
	Alcohols								
9	Ethanol[2]	950	1440 ± 108.56[a]	59.95	1680 ± 40.70[b]	55.23	65.80 ± 1.76[c]	1.89	Strong alcoholic
10	1-Propanol[3]	1038	1.36 ± 0.10[a]	0.06	4.65 ± 0.14[b]	0.15	7.57 ± 0.42[c]	0.22	Sweetish, fusel oil
11	1-Butanol[1,4]	1155	0.13 ± 0.01[a]	0.01	0.74 ± 0.11[b]	0.02	8.11 ± 0.17[c]	0.23	Sweet apricot
12	Isobutyl alcohol[2]	1088	13.10 ± 0.47[a]	0.55	33.20 ± 0.95[b]	1.09	87.50 ± 2.28[c]	2.51	Wine solvent
13	Active amyl alcohol[4]	1222	19.80 ± 1.56[a]	0.82	77.90 ± 2.80[b]	2.56	230 ± 18.20[c]	6.59	Roasted, wine, onion, fruity
14	Isoamyl alcohol[4]	1224	23.70 ± 1.39[a]	0.99	95.60 ± 3.36[b]	3.14	556 ± 12.90[c]	15.93	Whiskey, malt, burnt
15	1-Octanol[2]	1573	0.19 ± 0.00[a]	0.01	0.31 ± 0.03[a]	0.01	3.97 ± 0.15[b]	0.11	Waxy, green, citrus
16	2-Phenylethyl alcohol[4]	1945	18.10 ± 1.82[a]	0.75	23.10 ± 1.74[b]	0.76	16.30 ± 0.32[a]	0.47	Rose, floral, honey
17	2-Ethylhexanol[4]	1501	1.42 ± 0.04[a]	0.06	0.77 ± 0.08[b]	0.03	0.15 ± 0.00[c]	0.00	Citrus, fresh, floral, oily, sweet
	Subtotal		**1517.80**	**63.19**	**1916.27**	**62.99**	**975.40**	**27.94**	
	Aldehydes								
18	Benzaldehyde[2]	1552	1.31 ± 0.09[a]	0.05	1.17 ± 0.03[a]	0.04	2.86 ± 0.08[b]	0.08	Almond like
19	O-Tolualdehyde	1683	2.46 ± 0.24[a]	0.10	3.92 ± 0.28[b]	0.13	4.03 ± 0.51[b]	0.12	Fruity, sweet, cherry, chemical
	Subtotal		**3.77**	**0.16**	**5.09**	**0.17**	**6.89**	**0.20**	

Table 7.3. (Continued)

No.	Compounds identified in this study	LRI[d]	Control		0.1% fusel oil added		0.5% fusel oil added		Organoleptics[e]
			Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	
	Esters								
20	Methyl octanoate[3]	1386	1.47 ± 0.07[a]	0.06	0.81 ± 0.03[b]	0.03	0.57 ± 0.03[c]	0.02	Powerful, fruity, orange-like
21	Methyl decanoate[3]	1600	1.31 ± 0.08[a]	0.05	1.32 ± 0.07[a]	0.04	6.97 ± 0.30[b]	0.20	Pleasant, fruity, floral
22	Methyl dodecanoate[3]	1814	2.31 ± 0.19[a]	0.10	0.31 ± 0.00[b]	0.01	13.60 ± 0.82[c]	0.39	Waxy, creamy coconut, mushroom
23	Ethyl butyrate[1]	1034	8.57 ± 0.04[a]	0.36	4.18 ± 0.31[b]	0.14	8.23 ± 0.14[a]	0.24	Apple
24	Ethyl hexanoate[1]	1218	1.64 ± 0.10[a]	0.07	1.76 ± 0.17[a]	0.06	1.14 ± 0.08[b]	0.03	Apple peel, fruity
25	Ethyl octanoate[1,4]	1433	36.3 ± 1.89[a]	1.51	23.10 ± 2.31[b]	0.76	38.90 ± 2.13[a]	1.11	Pleasant, fruity, floral
26	Ethyl nonanoate	1539	0.51 ± 0.00[a]	0.02	1.04 ± 0.13[b]	0.03	1.83 ± 0.09[c]	0.05	Fruity, apple, tropical, winey
27	Ethyl (E)-4-decenoate	1692	0.43 ± 0.02[a]	0.02	0.95 ± 0.07[a]	0.03	39.20 ± 1.39[b]	1.12	Green, apple waxy nuance
28	Ethyl decanoate[1]	1648	18.90 ± 1.12[a]	0.79	19.20 ± 1.04[a]	0.63	444 ± 13.00[b]	12.72	Sweet, grape
29	Ethyl dodecanoate[3]	1855	17.70 ± 1.64[a]	0.74	11.80 ± 0.70[a]	0.39	433 ± 29.40[b]	12.40	Sweet, waxy, floral, soapy
30	Ethyl tetradecanoate[3]	2065	0.74 ± 0.03[a]	0.03	0.59 ± 0.06[a]	0.02	45.30 ± 3.75[b]	1.30	Sweet,waxy
31	Isoamyl propanoate	1150	0.00 ± 0.00[a]	0.00	8.66 ± 0.46[b]	0.28	13.00 ± 0.74[c]	0.37	Sweet, banana, pineapple
32	Isoamyl butyrate[3]	1251	1.16 ± 0.05[a]	0.05	3.06 ± 0.20[b]	0.10	1.54 ± 0.03[c]	0.04	Fruity
33	Isobutyl octanoate	1556	0.40 ± 0.03[a]	0.02	0.43 ± 0.00[a]	0.01	19.80 ± 0.74[b]	0.57	Fruity, green, oily, floral
34	Isoamyl octanoate	1667	0.28 ± 0.00[a]	0.01	1.08 ± 0.06[a]	0.04	254 ± 10.10[b]	7.28	Sweet, fruity, waxy, green, fatty
35	Propyl decanoate	1739	0.24 ± 0.01[a]	0.01	0.27 ± 0.05[a]	0.01	5.80 ± 0.21[b]	0.17	Waxy, fruity, fatty
36	Isobutyl decanoate	1771	0.21 ± 0.00[a]	0.01	0.38 ± 0.03[a]	0.01	73.20 ± 6.13[b]	2.10	Oily, sweet, brandy, apricot, cognac
37	Isoamyl decanoate	1879	0.00 ± 0.00[a]	0.00	2.02 ± 0.01[a]	0.07	518 ± 5.04[b]	14.84	Waxy, banana, fruity, sweet, cognac

Table 7.3. (Continued)

No.	Compounds identified in this study	LRI[d]	Control		0.1% fusel oil added		0.5% fusel oil added		Organoleptics[e]
			Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	
38	Isoamyl dodecanoate	2085	0.00 ± 0.00[a]	0.00	0.31 ± 0.00[a]	0.01	28.30 ± 1.30[b]	0.81	Winey, fatty, creamy, yeasty, fusel
39	Methyl acetate	851	1.88 ± 0.01[a]	0.08	1.70 ± 0.09[b]	0.06	0.00 ± 0.00[c]	0.00	Fruity, sweet
40	Ethyl acetate[2]	911	401 ± 24.50[a]	16.70	290 ± 13.70[b]	9.53	54.10 ± 3.09[c]	1.55	Pineapple, sweet, fruity
41	Propyl acetate	988	9.43 ± 0.44[a]	0.39	5.86 ± 0.58[b]	0.19	1.05 ± 0.05[c]	0.03	Celery, fruity, fusel, raspberry, pear
42	Butyl acetate	1061	1.56 ± 0.10[a]	0.06	2.02 ± 0.12[b]	0.07	1.21 ± 0.12[c]	0.03	Ethereal, solvent, fruity, banana
43	Isobutyl acetate[1]	1024	5.89 ± 0.22[a]	0.25	8.49 ± 0.27[b]	0.28	1.23 ± 0.03[c]	0.04	Sweet, fruity, ethereal, banana
44	Active amyl acetate	1097	2.09 ± 0.08[a]	0.09	4.33 ± 0.00[b]	0.14	2.45 ± 0.25[a]	0.07	Sweet, banana, fruity, ripe
45	Isoamyl acetate[1]	1099	189 ± 8.30[a]	7.87	551 ± 1.72[b]	18.11	32.30 ± 1.70[c]	0.93	Banana, apple, estery
46	Benzyl acetate[4]	1752	3.06 ± 0.03[a]	0.13	2.46 ± 0.12[b]	0.08	0.62 ± 0.01[c]	0.02	Sweet, floral, fruity, fresh apple
47	2-Phenylethyl acetate[1]	1840	99.10 ± 7.34[a]	4.13	145 ± 10.90[b]	4.77	23.60 ± 0.03[c]	0.68	Rose, honey, floral
48	4-Ethyl phenyl acetate[2]	1808	0.31 ± 0.02[a]	0.01	1.04 ± 0.09[b]	0.03	4.05 ± 0.08[c]	0.12	Strong, sweet, rosy, honey
	Subtotal		**805.49**	**33.53**	**1093.17**	**35.93**	**2066.99**	**59.22**	
	Ketones								
49	2-Undecanone	1609	0.21 ± 0.02[a]	0.01	0.38 ± 0.03[b]	0.01	1.42 ± 0.09[c]	0.04	Fruity with creamy cheese like notes
50	β-Damascenone[4]	1844	0.57 ± 0.03[a]	0.02	0.70 ± 0.06[b]	0.02	2.82 ± 0.05[c]	0.08	Rose, apple, honey
51	β-Ionone[3]	1968	0.13 ± 0.01[a]	0.01	0.19 ± 0.01[a]	0.01	2.75 ± 0.20[b]	0.08	Woody, berry, floral, green, fruity
52	2-Tridecanone[3]	1825	0.19 ± 0.01[a]	0.01	0.33 ± 0.02[a]	0.01	11.90 ± 0.56[b]	0.34	Fatty, waxy, mushroom, coconut
	Subtotal		**1.10**	**0.05**	**1.60**	**0.05**	**18.89**	**0.54**	

Table 7.3. (Continued)

No.	Compounds identified in this study	LRI[d]	Control		0.1% fusel oil added		0.5% fusel oil added		Organoleptics[e]
			Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	
	Heteroatom (N, S) compound								
53	Benzyl isothiocyanate[3]	2139	0.65 ± 0.03[a]	0.03	0.49 ± 0.05[a]	0.02	2.57 ± 0.16[b]	0.07	Watercress, medicinal horseradish
	Total		**2402.21**		**3041.96**		**3490.66**		

[a,b,c]Statistical analysis at 95% confidence level with same letters indicating no significant difference.
[d]Experimentally determined linear retention index on the DB-FFAP column, relative to C5-C40 hydrocarbons.
[e]Odor description obtained from Luebke (1980).
[1,2,3,4]Retention index in agreement with those in the literature [Duarte et al. (2010), Goodner (2008), Pino et al. (2003) and Segurel et al. (2009), respectively].

Table 7.4. Concentrations of selected major volatile compounds (mg/L) in papaya wine (day 21) fermented with *W. saturnus* var. *mrakii* NCYC2251 in the presence of added fusel oil (v/v)

Compounds quantified	Control		0.1% fusel oil added		0.5% fusel oil added		Odor threshold[d]
	Mean	OAV	Mean	OAV	Mean	OAV	
Ethanol	20832 ± 307[a]	-	26532 ± 932[b]	-	445.56 ± 14.70[c]	-	-
Isoamyl alcohol	49.47 ± 2.82[a]	1.65	158.31 ± 11.31[a]	5.28	5053 ± 401.12[b]	168.43	30.00
Active amyl alcohol	17.78 ± 0.73[a]	0.27	101.24 ± 1.68[a]	1.56	1384 ± 56.54[b]	21.29	65.00
Isobutyl alcohol	2.05 ± 0.19[a]	0.05	6.59 ± 0.71[a]	0.16	86.24 ± 4.12[b]	2.16	40.00
2-Phenylethyl alcohol	3.18 ± 0.29[a]	0.32	4.49 ± 0.26[b]	0.45	4.89 ± 0.13[b]	0.49	10.00
Octanoic acid	0.84 ± 0.02[a]	0.10	0.20 ± 0.01[b]	0.02	1.87 ± 0.19[c]	0.21	8.80
Ethyl octanoate	0.13 ± 0.00[a]	6.50	0.07 ± 0.00[b]	3.50	0.10 ± 0.01[c]	5.00	0.02
Ethyl decanoate	0.13 ± 0.00[a]	0.65	0.10 ± 0.01[a]	0.50	1.60 ± 0.28[b]	8.00	0.20
Ethyl dodecanoate	0.55 ± 0.06[a]	0.46	0.14 ± 0.01[a]	0.12	26.19 ± 0.67[b]	21.83	1.20[e]
Isoamyl acetate	9.71 ± 0.47[a]	323.67	57.65 ± 7.79[b]	1921.67	8.60 ± 0.91[a]	286.67	0.03
Active amyl acetate	0.002 ± 0.001[a]	0.01	0.006 ± 0.001[b]	0.04	0.003 ± 0.001[a]	0.02	0.16
Isobutyl acetate	0.17 ± 0.02[a]	0.11	0.53 ± 0.04[b]	0.33	0.0004 ± 0.0001[c]	0.00	1.60
2-Phenylethyl acetate	1.43 ± 0.07[a]	5.72	2.33 ± 0.20[b]	9.32	0.49 ± 0.07[c]	1.96	0.25

Abbreviation: OAV = Odour activity values calculated by dividing concentration by the odour threshold value of the compound

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

[d]From Bartowsky and Pretorius (2009).

[e]From Ferreira et al. (2000).

7.2.3 Principal component analysis

Volatile compounds in the papaya wines from Tables 7.3 and 7.4 were used for principal component analysis (PCA) to obtain a pictorial relationship of the papaya wines based on their volatile composition. The PCA of the quantified major volatile compounds (Table 7.4) is presented in Fig. 7.8 as it is a representation of the PCA result from Table 7.3. The first principal component (PC1) is plotted against the second (PC2), and the separation among different papaya wines from this PC1–PC2 scattered point plot is obvious (Fig. 7.8). Principal component 1 (PC1) accounted for 72.39% of the total variance that distinguished the addition of 0.5% (v/v) fusel oil from both the control and the fermentation added with 0.1% (v/v) fusel oil, while PC2 explained the remaining 27.61% (Fig. 7.8). Those with 0.5% (v/v) fusel oil added was characterised by ethyl decanoate, ethyl dodecanoate and higher alcohols that were mainly contributed by the addition of fusel oil (Table 3.1). Conversely, the papaya wine produced by the addition of 0.1% (v/v) fusel oil was more related with acetate esters (e.g. isoamyl acetate, isobutyl acetate and 2-phenylethyl acetate). The papaya wine without the addition of fusel oil (control), located on the upper-right quadrant, was not associated with any volatile compounds (Fig. 7.8). This was surprising as it produced a variety of volatile compounds during fermentation (Figs. 7.2-7.7) and comprised several volatiles at day 21 (Table 7.3). By comparison, it was correlated with ethyl acetate, propyl acetate, benzyl acetate and 2-ethylhexanol in the PCA result from Table 7.3 [Appendix E (Fig. E8)]. These volatiles were not quantified due to the lack of authentic standards previously and thus, were not reflected in Table 7.4 and the PCA plot (Fig. 7.8).

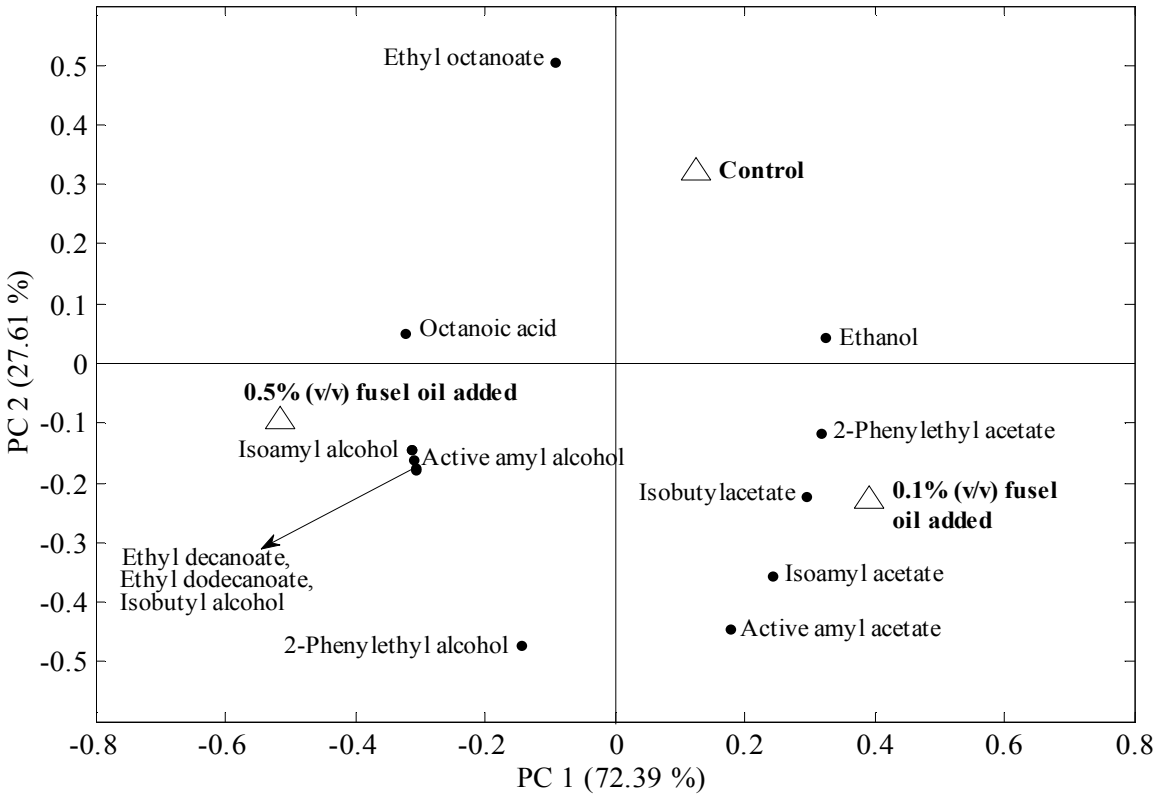

Fig. 7.8. Bi-plot of principal component analysis of the quantified major volatile compounds in papaya wine fermented by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added.

7.3 Conclusions

In this chapter, the impact of fusel oil addition on the fermentation performance and the volatile compounds formation by *W. saturnus* var. *markii* NCYC2251 was assessed during papaya juice fermentation. *W. saturnus* var. *mrakii* NCYC2251 was able to modulate papaya wine fermentation through production of relatively high amounts of esters. This modulation was further impacted by the addition of fusel oil that increased ester production, which might lead to improved aroma differentiation. Overall, *W. saturnus* with 0.1% (v/v) fusel oil added showed a capability of producing papaya wine with higher amounts of ethanol and acetate esters. The addition of 0.5% (v/v) fusel oil had clearly evidenced inhibitory effects on yeast growth. The combination of fusel oil at low concentrations together with non-

Saccharomyces yeast enabled the production of a broader range of flavour-enhancing volatile compounds such as ethanol and acetate esters as compared to the amino acid addition that directed at specific volatile compound enhancement (**Chapter 6**). Hence, this technique can be a way of modulating the papaya wine flavor compound formation and diversifying its flavour, which merits further research including sensory evaluation.

CHAPTER 8

PROFILE OF VOLATILE COMPOUNDS DURING PAPAYA JUICE FERMENTATION BY A MIXED-CULTURE OF *SACCHAROMYCES CEREVISIAE* VAR. *BAYANUS* R2 AND *WILLIOPSIS SATURNUS* VAR. *MRAKII* NCYC2251

8.1 Introduction

Wine fermentation is a complex process characterised by a succession of different yeasts (*Saccharomyces* and non-*Saccharomyces* yeasts). Several authors claim that non-*Saccharomyces* yeasts used in mixed-starter cultures may enhance the organoleptic characteristics of wine due to higher production of important metabolites, such as enhanced glycerol production (Soden et al., 2000) and improved 2-phenylethyl acetate and isoamyl acetate content in wines (Moreira et al., 2008; Viana et al., 2009). In addition, negative attributes of non-*Saccharomyces* yeasts were either suppressed or modified by *Saccharomyces* (Ciani et al., 2010).

In simultaneous mixed-culture fermentations, the ratio of *Saccharomyces* to non-*Saccharomyces* yeasts is an important parameter that determines the quality of the resultant wine (Bely et al., 2008; Comitini et al., 2011). A ratio of 90:10 of *Hanseniaspora osmophila* and *S. cerevisiae* was appropriate to produce wines of desired quality with enhanced 2-phenyethyl acetate production (Viana et al., 2009). However, there is a lack of data on the minimum percentage of non-*Saccharomyces* yeasts in mixed-starters that is required to influence the analytical profile of wines and a reduced percentage of non-*Saccharomyces* yeasts would be more acceptable to the

wine industry to prevent the formation of undesirable flavour compounds and also for more predictable and consistent fermentation.

To date, only a few studies have evaluated the likelihood of *W. saturnus* in simultaneous mixed-culture fermentation with *S. cerevisiae* (Erten & Tanguler, 2010; Trinh et al., 2011). Trinh et al. (2011) demonstrated the potential of improving wine aroma by simultaneous mixed-culture fermentation of *W. saturnus* and *S. cerevisiae*. However, Erten and Tanguler (2010) reported that the use of *W. saturnus* in combination with *S. cerevisiae* produced wines with undesirably high levels of acetic acid. Despite the inconsistency, the use of *W. saturnus* may introduce an element of oenological diversity to the process that goes beyond *Saccharomyces* species, but further research and understanding is required to prevent any unwanted consequences from their use and to exploit their beneficial contributions.

The aim of this chapter was to study the fermentation performance and the production of volatile compounds in mixed-culture (co-inoculation) papaya wine fermentation by *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 at an approximate ratio of 1:1000. This ratio enabled the growth and longer survival of *W. saturnus* in mixed-culture (co-inoculation) fermentation (Trinh et al., 2011), which would encourage metabolic interactions between the yeast species. The *Saccharomyces* and non-*Saccharomyces* yeasts used in this chapter were selected from preliminary screening of different strains of *S. cerevisiae* and *W. saturnus*, based on their fermentation performance and volatile compound formation (**Chapters 4 and 5**).

8.2 Results and discussion

8.2.1 Growth of yeasts in pure and mixed-cultures and changes in non-volatiles

Yeast viable cells, total soluble solids (^{o}Brix), sugar consumption and organic acid profiles of single and mixed-cultures are shown in Fig. 8.1, Table 8.1 and Appendix F (Fig. F1). The fermentation characteristics of the mixed-culture were similar to those of the *S. cerevisiae* monoculture in terms of viable cells, sugar consumption and changes in organic acid amounts. The pH changes were similar in all fermentations, maintaining at around 3.50-3.58 (Table 8.1). The viable yeast cell populations of both pure cultures of *S. cerevisiae* and *W. saturnus* reached the maximum of 1.24 x 10^8 CFU/mL on day 7 and 9.49 x 10^7 CFU/mL on day 14, respectively (Fig. 8.1). In the mixed-culture, *S. cerevisiae* increased rapidly and its cell count was comparable to the *W. saturnus* population by day 3. The cell population of *S. cerevisiae* reached a maximum of 7.26 x 10^7 CFU/mL on day 7, while the *W. saturnus* population peaked at day 3 (6.9 x 10^5 CFU/mL) and declined gradually till the end of fermentation. The results showed that even at a significant higher ratio of *W. saturnus* to *S. cerevisiae*, the population of the *Saccharomyces* yeast still overtook the non-*Saccharomyces* yeast after two days of fermentation.

The maximum viable cell population of *W. saturnus* and *S. cerevisiae* attained in the mixed-culture was lower than that of the corresponding monoculture, being consistent with Mendoza, Manca de Nadra, & Farias (2007) on mixed-cultures (co-inoculation) of *K. apiculata* and *S. cerevisiae*. The early growth arrest of non-*Saccharomyces* species during grape juice fermentation has traditionally been associated with their lower tolerance to ethanol or to other toxic compounds. However, there could be other factors such as oxygen availability, cell–cell contact, quorum sensing and space limitation that may cause the early growth arrest of non-

Saccharomyces yeasts (Arneborg et al., 2005; Hansen et al., 2001; Nissen & Arneborg, 2003; Nissen, Nielsen, & Arneborg, 2003; Panon, 1997). The degree of non-*Saccharomyces* yeast succession during fermentation would in turn affect the final organoleptic properties of the wine.

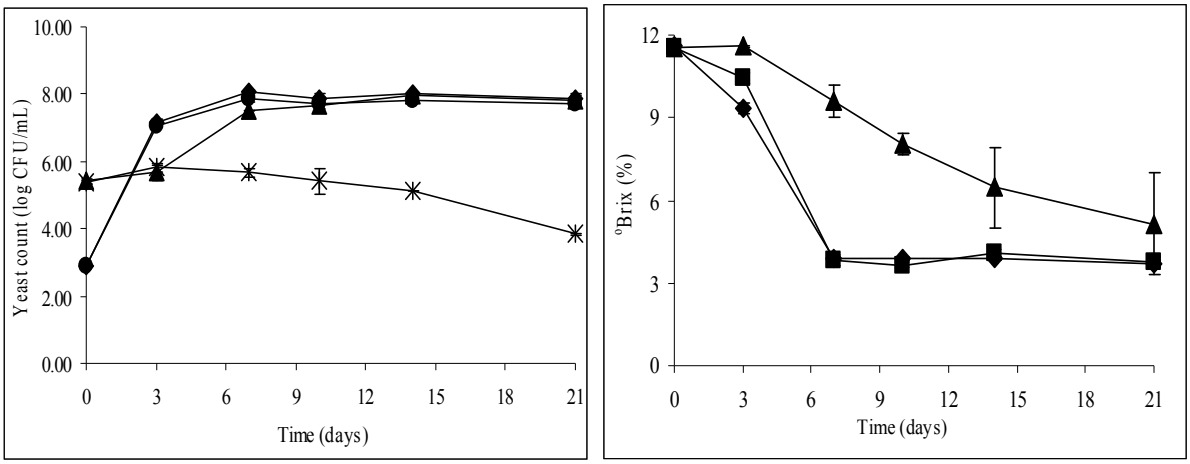

Fig. 8.1. Changes of yeasts (as viable cell counts) and °Brix in papaya wine during mixed-culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■); *S. cerevisiae* var. *bayanus* R2 in mixed-culture (●); *W. saturnus* var. *mrakii* NCYC2251 in mixed-cultures (∗). (Error bars = standard deviation).

The °Brix values in both the mixed-culture and the *S. cerevisiae* monoculture displayed rapid reduction which corresponded to sugar consumption and reached a °Brix value of around 3.7 – 3.8% on day 7, and remained stationary at that level until the end of fermentation (Fig. 8.1). The *W. saturnus* monoculture, on the other hand, had a gradual reduction in the °Brix value over the 21-day fermentation period (Fig. 8.1). The mixed-culture and the *S. cerevisiae* monoculture displayed similar patterns of depletion of glucose and fructose, consuming almost all sugars (Table 8.1). The *W. saturnus* monoculture preferentially utilised glucose over fructose [Table 8.1, Appendix F (Fig. F1)], being consistent with the sugar consumption trend observed in **Chapter 5** (Figs. 5.1 and 5.2).

The changes in organic acids amounts were similar in the monocultures and mixed-culture, where the malic and tartaric acids decreased, while citric, succinic and acetic acids either remained relatively constant or increased (Table 8.1). The organic acid trends corresponded to those observed in **Chapters 4 and 5**. The changes in organic acids in the wines could be due to either cellular uptake or excretion of metabolic products. Transportation of organic acids across the cell membrane could occur by either active transport or simple diffusion depending on the presence of a carrier. Succinic acid was the main carboxylic acid produced during fermentation which was likely to involve the reductive branch of the Krebs cycle (Swiegers et al., 2005). Similarly, other studies highlighted that fermenting and/or anaerobically-grown yeasts contain fumarate reductases, which responsible for the irreversible reduction of fumarate to succinate (Hauber & Singer, 1967; Muratsubaki & Katsume, 1985). Interestingly, succinic acid was only increased in the *W. saturnus* monoculture with 0.263 g/100 mL (Table 8.1), while it did not change significantly in the other fermentations. These results corresponded to the changes of succinic acid observed in **Chapters 4 and 5**, which maybe attributed to its production being highly variable amongst *Saccharomyces* and non-*Saccharomyces* yeasts (Ciani & Maccarelli, 1998; Swiegers et al., 2005) or the utilisation of succinic acid to produce other volatile compounds by the *S. cerevisiae* monoculture and the mixed-culture. The *W. saturnus* monoculture produced the highest amount of acetic acid (Table 8.1). The acetic acid produced was twice more than the optimal acetic acid concentration range of 0.02–0.07 g/100 mL reported for wine (Lambrechts & Pretorius, 2000). Large variations in acetic acid production have been observed in other studies, ranging from about 0.06 g/100 mL to more than 0.34 g/100 mL (Romano et al., 2003; Viana et al., 2008).

Table 8.1. Fermentation parameters of papaya wine (day 21) fermented by a mixed-culture of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251

	Day 0	Control yeast R2	Control yeast NCYC2251	Mixed-culture
pH	3.58 ± 0.02[a]	3.55 ± 0.01[a]	3.50 ± 0.01[b]	3.51 ± 0.01[b]
[o]Brix (%)	11.60 ± 0.02[a]	3.70 ± 0.15[b]	5.14 ± 0.40[c]	3.79 ± 0.16[b]
Ethanol % (v/v)	0.01 ± 0.00[a]	4.76 ± 0.44[b]	2.34 ± 0.04[c]	5.42 ± 0.15[b]
Sugars (g/100 mL)				
Fructose	5.48 ± 0.02[a]	N.D.	3.17 ± 0.30[b]	N.D.
Glucose	5.08 ± 0.03[a]	0.04 ± 0.00[b]	1.25 ± 0.26[c]	0.04 ± 0.00[b]
Organic acids (g/100 mL)				
Acetic acid	0.031 ± 0.004[a]	0.113 ± 0.012[b]	0.131 ± 0.013[b]	0.106 ± 0.010[b]
Citric acid	0.235 ± 0.003[a]	0.236 ± 0.011[a]	0.255 ± 0.021[a]	0.230 ± 0.030[a]
Malic acid	0.949 ± 0.008[a]	0.603 ± 0.021[b]	0.698 ± 0.120[b]	0.595 ± 0.072[b]
Succinic acid	0.203 ± 0.004[ab]	0.207 ± 0.004[b]	0.263 ± 0.022[b]	0.158 ± 0.030[a]
Tartaric acid	0.020 ± 0.001[a]	0.008 ± 0.000[b]	0.009 ± 0.000[b]	0.008 ± 0.000[b]

Abbreviation: N.D. = not detected.
[a,b,c]Statistical analysis at 95% confidence level with same letters indicating no significant difference.

8.2.2 Dynamic changes of volatiles during papaya juice fermentation

During the fermentation, the yeasts involved in the pure and mixed-culture fermentations released secondary products such as higher alcohols, esters, acids and carbonyl compounds with the mixed-culture producing a wider range and higher amounts of volatiles than the pure cultures (Tables 8.2 and 8.3). Volatiles that were originally present in the juice such as benzyl isothiocyanate, benzaldehyde, β-damascenone and certain fatty acids (butyric and hexanoic acids) were diminished (Figs. 8.2-8.7).

The profile of production and degradation of fatty acids of C2 to C12 was similar in all the fermentations, except for hexanoic, octanoic and decanoic acids in the *W. saturnus* monoculture [Fig. 8.2, Appendix F (Fig. F2)]. Most of the fatty acids increased initially, and then decreased towards the end of fermentation, except for acetic, hexanoic and butyric acids (Fig. 8.2). Butyric and hexanoic acids present at

relatively high concentrations in the juice were utilised during fermentation to trace levels by all cultures. The mixed-culture of *S. cerevisiae*/*W. saturnus* had fatty acid formation and utilisation trends similar to those of the *S. cerevisiae* monoculture, but produced slightly higher amounts of total fatty acids with 2.66% (relative peak area, RPA) (Table 8.2). In particular, the mixed-culture produced higher amount of octanoic and decanoic acids than the *S. cerevisiae* monoculture with 1.75 mg/L and 1.26 mg/L, respectively (Table 8.3).

Acetic acid was constantly produced throughout the fermentation with the *W. saturnus* monoculture produced the highest concentration of acetic acid with 0.54% (RPA), followed by the mixed-culture and the *S. cerevisiae* monoculture with 0.42% (RPA) each (Table 8.2), which were consistent with the findings as shown in Table 8.1. Non-*Saccharomyces* yeasts have been associated with high acetic acid production and thus, are traditionally considered as spoilage yeasts (du Toit & Pretorius, 2000). Viana et al. (2009) discovered that acetic acid produced by a mixed-culture (co-inoculation) of *H. osmophila*/*S. cerevisiae* (0.042 g/100 mL) was approximately 3-fold higher than that produced by a *S. cerevisiae* monoculture (0.013 g/100 mL), but they were still within the optimal acetic acid range for wines (Lambrechts & Pretorius, 2000).

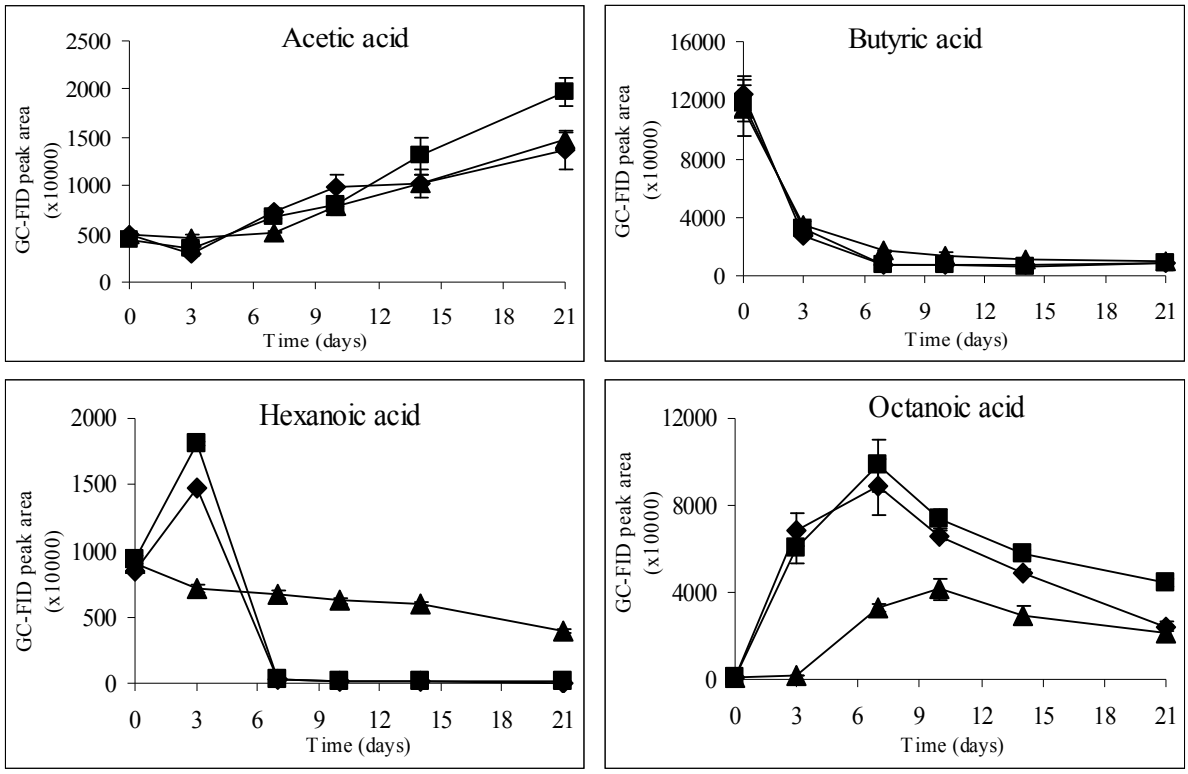

Fig. 8.2. Changes of fatty acids in papaya wine during mixed-culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■). (Error bars = standard deviation).

Ethanol, isobutyl alcohol (2-methyl-1-propanol), isoamyl alcohol (3-methyl-1-butanol) and 2-phenylethyl alcohol were the major alcohols produced by the mixed- and pure cultures during papaya juice fermentation (Table 8.2). The dynamic changes of the alcohols were similar among the different cultures [Fig. 8.3, Appendix F (Fig. F3)], whereas the final amounts of alcohols at day 21 varied significantly (Tables 8.2 and 8.3). The *W. saturnus* monoculture constantly produced the lowest amounts of each type of alcohols, except for isobutyl alcohol (Tables 8.2 and 8.3). 2-Ethylhexanol initially present in the juice was utilised by all yeasts during fermentation (Fig. 8.3). The mixed-culture fermentation produced papaya wine with the highest ethanol concentration of 66.49% (RPA) as compared to the pure cultures (Table 8.2). This corresponded to the ethanol content in the mixed-culture of 4.27 x 10^4 mg/L (5.42%

v/v), followed by the *S. cerevisiae* and *W. saturnus* monocultures of 3.75 x 10^4 mg/L (4.76% v/v) and 1.84 x 10^4 mg/L (2.34% v/v), respectively (Tables 8.1 and 8.3). This could possibly be attributed to the early death and autolysis of non-*Saccharomyces* yeasts (Hernawan & Fleet, 1995), which could provide a source of nutrients for *S. cerevisiae*. In studies by other researchers (Charoenchai et al., 1997; Dizy & Bisson, 2000), some species of non-*Saccharomyces* yeasts such as *K. apiculata* and *Metschnikowia pulcherrima* are significantly proteolytic and could generate amino acids for use by *S. cerevisiae*. Studies have shown that non-*Saccharomyces* yeasts in pure and mixed-cultures (co-inoculation) produced lower amounts of higher alcohols as compared to *S. cerevisiae* (Moreira et al., 2008; Rojas et al., 2003). The results of this study are in accordance with these studies, where the *W. saturnus* monoculture and the mixed-culture produced lower levels of total higher alcohols with 2.1% (RPA) and 3.7% (RPA), respectively, as compared to the *S. cerevisiae* monoculture (Table 8.2). Lower levels of higher alcohols in wine was produced by non-*Saccharomyces* yeast, as higher alcohols were used as precursors for ester formation, leading to wine flavour complexity, stylistic distinction and vintage variability (Soles et al., 1982).

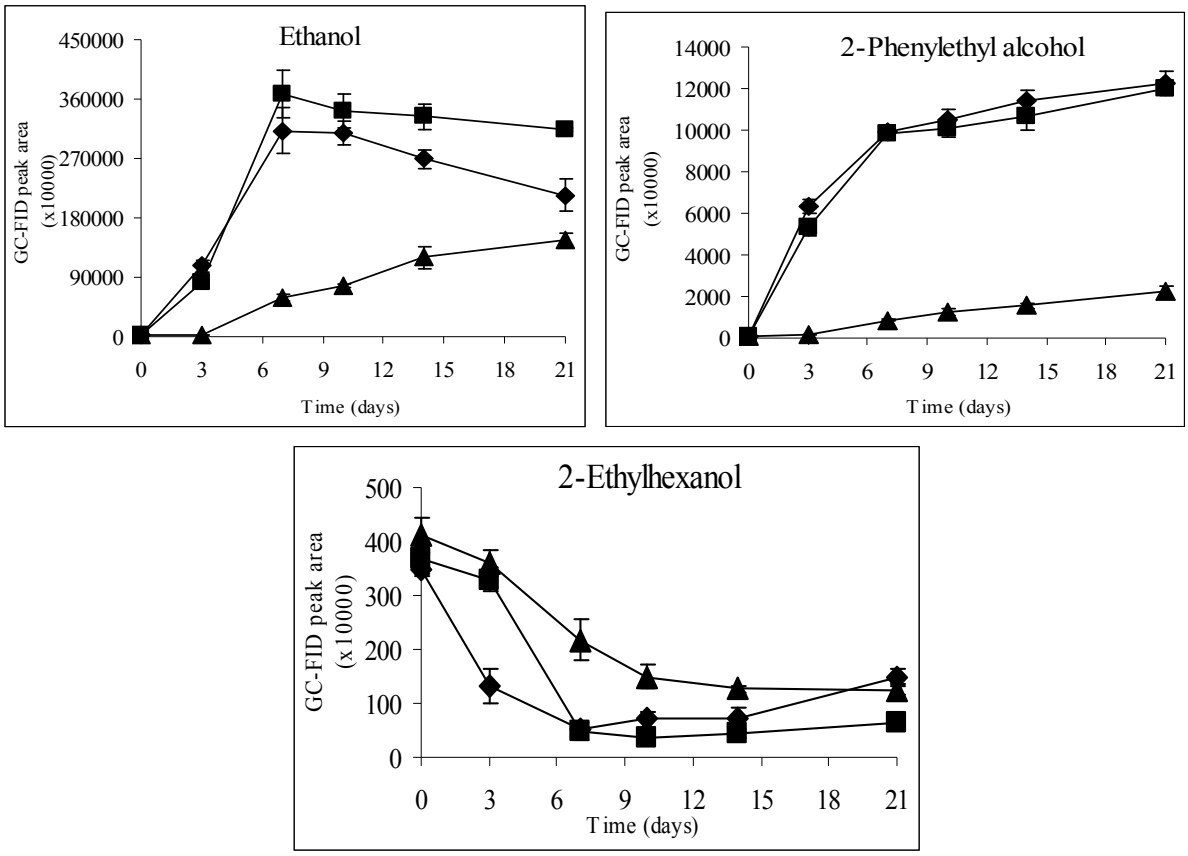

Fig. 8.3. Changes of alcohols in papaya wine during mixed-culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■). (Error bars = standard deviation).

Microorganisms are known to modulate aromatic esters in wine (Sumby et al., 2010). Esters constituted about 25.25 to 41.09% (RPA) of the volatiles produced by all the cultures (Table 8.2), which included methyl, ethyl, acetate and other esters. Acetate and ethyl esters formed the bulk of the esters that contribute, with a lesser extent for ethyl acetate due to its high odour threshold, to fruit and floral notes to the wine aroma. The dynamic changes of most of the esters were similar in the mixed-culture and the *S. cerevisiae* monoculture, but were significantly different from that of the *W. saturnus* monoculture, leading to differential characteristics of wines [Figs. 8.4-8.6, Appendix F (Figs. F4-F6)].

Generally, the *W. saturnus* monoculture produced the highest level of all acetate esters and the maximum amount peaked at day 7, and then declined with the exception of ethyl acetate and 2-phenylethyl acetate, which increased throughout the fermentation [Fig. 8.4, Appendix F (Fig. F4)]. Both the mixed-culture and the *S. cerevisiae* monoculture had much lower levels of acetate ester production, which increased slightly then declined significantly [Fig. 8.4, Appendix F (Fig. F4)]. The mixed-culture fermentation had a slightly higher level of acetate ester production than the *S. cerevisiae* monoculture, in particular, 2-phenylethyl acetate production at 0.46 mg/L at day 21 (Tables 8.2 and 8.3). This was likely due to the higher ester-synthesising activities of the *W. saturnus* present in the mixed-culture. *Williopsis* yeasts are potent producers of esters (Inoue et al., 1997) and *W. saturnus* can convert higher alcohols into the corresponding acetate esters (Janssens et al., 1992). These results corresponded with the lower levels of higher alcohols such as isoamyl alcohol and 2-phenylethyl alcohol (the precursors, together with acetyl-CoA) for isoamyl acetate and 2-phenylethyl acetate synthesis, respectively, by the action of alcohol acetyltransferase (Yoshioka & Hashimoto, 1981). Levels of ethyl acetate of 150–200 mg/L are considered to impart a spoilage character to wine (Jackson, 1994). The level of ethyl acetate produced in the papaya wine fermented with the *W. saturnus* monoculture was 36-fold greater than that produced by the *S. cerevisiae* monoculture (Table 8.3). The high level of ethyl acetate (262 mg/L) produced by the *W. saturnus* monoculture, would expect to exert an adverse effect on the aromatic quality of the papaya wine. The presence of *S. cerevisiae* in the mixed-culture reduced the ethyl acetate concentration produced by the *W.* saturnus yeast significantly, approximately to the same level as that of the *S. cerevisiae* monoculture at the end of fermentation (Fig. 8.4, Table 8.3).

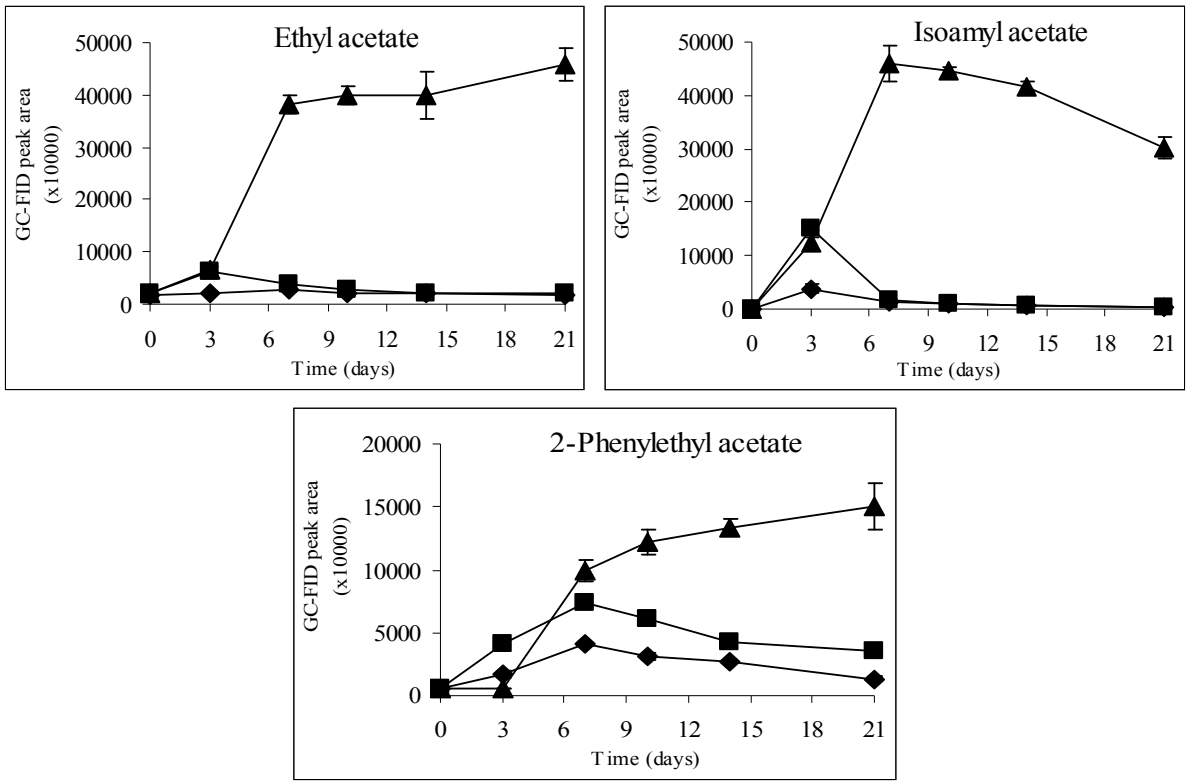

Fig. 8.4. Changes of acetate esters in papaya wine during mixed-culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■). (Error bars = standard deviation).

Ethyl and methyl esters generally increased with some increased continuously, while others increased and either remained constant or declined during fermentation [Fig. 8.5, Appendix F (Fig. F5)], being consistent to the trends observed in **Chapters 4 and 5**. The mixed-culture fermentation produced the highest amounts of these esters followed by the *S. cerevisiae* and *W. saturnus* monocultures, except for ethyl butyrate, methyl octanoate and methyl dodecanoate (Tables 8.2 and 8.3). The final amounts of these esters at day 21 varied significantly among the mixed and pure cultures (Table 8.2). Ethyl esters contribute pleasant fruity, floral and honey-like flavours (Table 8.2). Ethyl esters and other major volatiles have been shown to be significantly higher in wines produced by pure cultures of *S. cerevisiae* and the inoculation of non-*Saccharomyces* yeasts would result in a decreased production of these esters (Herraiz,

Reglero, Herraiz, Martín-Álvarez, & Cabezudo, 1990). In contrast, Moreira et al. (2008) and Rojas et al. (2003) found that *S. cerevisiae* produced wines with levels of ethyl hexanoate that were not affected by the presence of apiculate yeasts in the starter. The results of this study are in accordance with Moreira et al. (2008) and Rojas et al. (2003) and in addition, the mixed-culture fermentation had a slightly higher level of esters than that by the pure cultures, such as ethyl octanoate and ethyl decanoate at 1.2 mg/L and 4.4 mg/L, respectively (Tables 8.2 and 8.3), which indicated the synergistic effects of both yeasts involved. Similarly, Howell, Cozzolino, Bartowsky, Feet, and Henschke (2006) highlighted that mixed-culture (co-inoculation) impacted on the metabolic performance of individual strains within the mixture, implying the potential synergistic effects between the different yeast strains. Further research is needed to confirm this hypothesis.

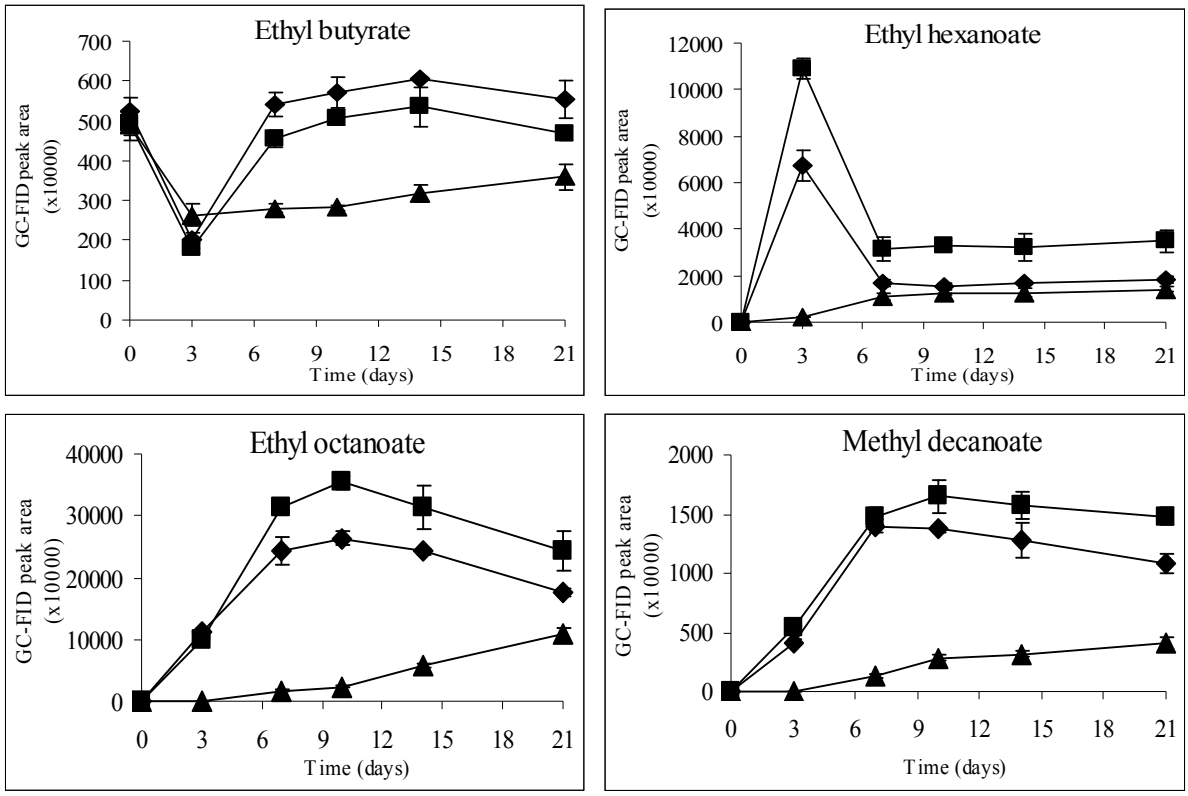

Fig. 8.5. Changes of ethyl esters and methyl decanoate in papaya wine during mixed-culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■). (Error bars = standard deviation).

Among the other esters, isoamyl octanoate, isobutyl decanoate and isoamyl decanoate increased initially and declined slightly towards the end of fermentation, with the mixed-culture displaying similar trends to those of the *S. cerevisiae* monoculture. The *W. saturnus* monoculture had the lowest production of these esters such as isoamyl octanoate of 0.06 mg/L at day 21 [Fig. 8.6, Tables 8.2 and 8.3, Appendix F (Fig. F6)]. The final amounts of these esters at day 21 varied significantly between the mixed and pure cultures at $p<0.05$ (Table 8.2).

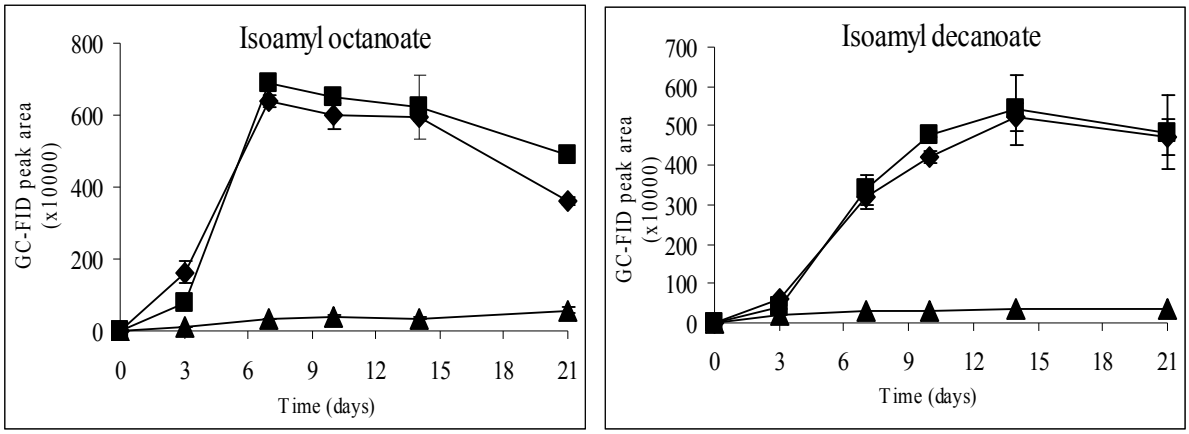

Fig. 8.6. Changes of other esters in papaya wine during mixed-culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■). (Error bars = standard deviation).

The dynamic changes of aldehydes, ketones and benzyl isothiocyanate in both mixed and pure cultures were similar, but their final amounts at day 21 varied significantly (Table 8.2). These volatile compounds especially benzaldehyde, benzyl isothiocyanate and β-damascenone, except for acetaldehyde, were metabolised to trace levels during fermentation [Fig. 8.7, Appendix F (Fig. F7)]. Acetaldehyde was produced by all cultures with the mixed-culture showing the highest production of 0.10% (RPA) (Table 8.2). Acetaldehyde is an important intermediate in ethanol production and can also be oxidised to form acetic acid (Ugliano & Henschke, 2009).

It is also a major component that plays an important role in the aroma and bouquet of wine. Among the various yeasts, *S. cerevisiae* yeasts have the capability to produce relatively high levels of acetaldehyde from 50 to 120 mg/L (Fleet & Heard, 1993). This was observed in this study, with the *S. cerevisiae* monoculture producing higher levels of acetaldehyde than the *W. saturnus* monoculture, which resulted in the mixed-culture acquiring the cumulative effects from both yeasts.

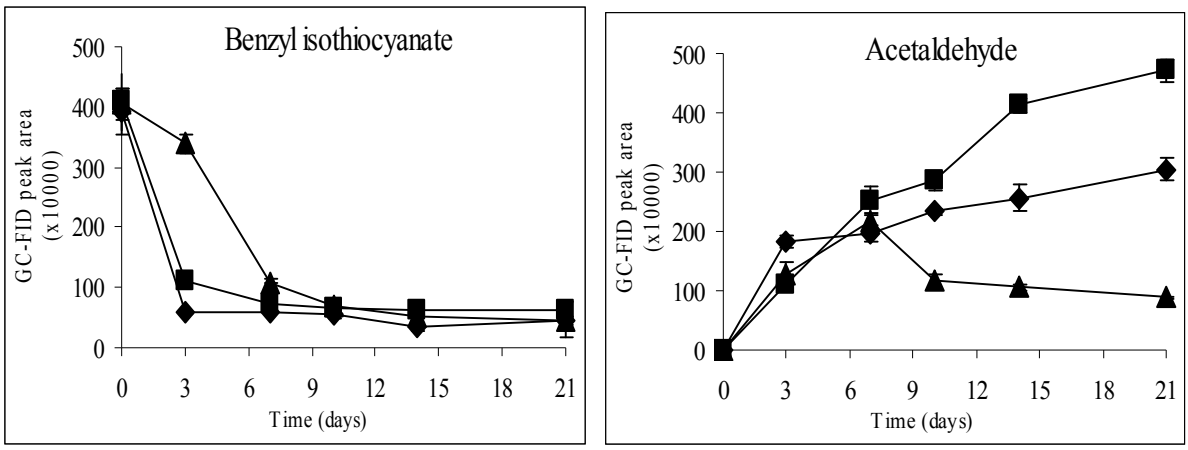

Fig. 8.7. Changes of benzyl isothiocyanate and acetaldehyde in papaya wine during mixed-culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■). (Error bars = standard deviation).

Table 8.2. Major volatile compounds (GC-FID peak area x 10^6) and their relative peak areas (RPA) in papaya wine (day 21) fermented by a mixed-culture of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251

No.	Compounds identified in this study	LRI[d]	Control Yeast R2 Peak Area	Control Yeast R2 RPA (%)	Control Yeast NCYC2251 Peak Area	Control Yeast NCYC2251 RPA (%)	Mixed-culture Peak Area	Mixed-culture RPA (%)	Organoleptics[e]
	Acids								
1	Acetic acid[3]	1460	13.60 ± 1.87[a]	0.42	14.80 ± 0.89[a]	0.54	19.70 ± 1.41[b]	0.42	Acidic, vinegar
2	Butyric acid[2]	1641	8.87 ± 1.13[a]	0.27	9.98 ± 0.83[a]	0.37	8.48 ± 0.49[a]	0.18	Rancid, cheesy
3	Hexanoic acid[1,4]	1890	0.07 ± 0.00[a]	0.00	3.93 ± 0.13[b]	0.14	0.12 ± 0.00[c]	0.00	Sweet, cheesy
4	Octanoic acid[1,4]	2112	24.40 ± 2.51[a]	0.75	21.30 ± 3.54[a]	0.78	44.60 ± 1.68[b]	0.95	Sweet, cheesy
5	Decanoic acid[4]	2328	32.10 ± 1.76[a]	0.99	9.93 ± 0.33[b]	0.36	45.40 ± 4.64[c]	0.96	Unpleasant, rancid, sour
6	Dodecanoic acid[3]	2545	5.87 ± 0.20[a]	0.18	4.62 ± 0.34[b]	0.17	7.13 ± 0.06[c]	0.15	Fatty, coconut, bay oil
	Subtotal		**84.91**	**2.61**	**64.56**	**2.37**	**125.43**	**2.66**	
	Alcohols								
7	Ethanol[2]	943	2150 ± 242[a]	66.06	1480 ± 85[b]	54.22	3130 ± 28[c]	66.49	Strong alcoholic
8	Isobutyl alcohol[2]	1090	8.71 ± 0.11[a]	0.27	9.46 ± 0.22[b]	0.35	8.25 ± 0.39[a]	0.18	Wine solvent
9	Isoamyl alcohol[4]	1196	56.20 ± 3.27[a]	1.73	25.40 ± 2.18[b]	0.93	45.90 ± 2.39[c]	0.97	Fruity, nail polish
10	2-Phenylethyl alcohol[4]	1917	123 ± 5.63[a]	3.78	22.30 ± 3.00[b]	0.82	120 ± 2.00[a]	2.55	Rose, floral, honey
11	2-Ethylhexanol[4]	1500	1.49 ± 0.14[a]	0.05	1.25 ± 0.07[a]	0.05	0.62 ± 0.05[b]	0.01	Citrus, fresh, floral,
	Subtotal		**2339.4**	**71.89**	**1538.41**	**56.36**	**3304.77**	**70.20**	
	Aldehydes								
12	Acetaldehyde[2]	732	3.05 ± 0.20[a]	0.09	0.89 ± 0.02[b]	0.03	4.71 ± 0.19[c]	0.10	Pungent, ethereal, fruity
13	Benzaldehyde[2]	1550	1.94 ± 0.20[a]	0.06	1.25 ± 0.21[b]	0.05	1.71 ± 0.06[a]	0.04	Almond like
14	O-Tolualdehyde	1680	2.22 ± 0.09[a]	0.07	1.86 ± 0.03[b]	0.07	1.73 ± 0.05[c]	0.04	Fruity, sweet, cherry, chemical
	Subtotal		**7.21**	**0.22**	**4.00**	**0.15**	**8.15**	**0.18**	
	Esters								
15	Methyl octanoate[3]	1385	2.37 ± 0.13[a]	0.07	5.18 ± 0.32[b]	0.19	2.63 ± 0.36[a]	0.06	Powerful, fruity, orange-like
16	Methyl decanoate[3]	1640	10.80 ± 0.77[a]	0.33	4.05 ± 0.48[b]	0.15	14.80 ± 0.72[c]	0.31	Pleasant, fruity, floral
17	Methyl dodecanoate[3]	1810	1.53 ± 0.26[a]	0.05	2.99 ± 0.23[b]	0.11	2.34 ± 0.15[c]	0.05	Waxy, soapy, creamy coconut
18	Ethyl butyrate[1]	1024	5.53 ± 0.48[a]	0.17	3.59 ± 0.32[b]	0.13	4.68 ± 0.18[c]	0.10	Pineapple, banana

Table 8.2. (Continued)

No.	Compounds identified in this study	LRI[d]	Control Yeast R2 Peak Area	Control Yeast R2 RPA (%)	Control Yeast NCYC2251 Peak Area	Control Yeast NCYC2251 RPA (%)	Mixed-culture Peak Area	Mixed-culture RPA (%)	Organoleptics[e]
19	Ethyl hexanoate[1]	1250	18.30 ± 1.26[a]	0.56	14.10 ± 1.17[b]	0.52	34.80 ± 4.66[c]	0.74	Banana, estery, fruity
20	Ethyl octanoate[1,4]	1434	176 ± 7.81[a]	5.41	108 ± 11.80[b]	3.96	243 ± 32.20[c]	5.16	Pleasant, fruity, floral, apple
21	Ethyl decanoate[1]	1630	564 ± 20.30[a]	17.33	65.20 ± 9.47[b]	2.39	895 ± 6.68[c]	19.01	Sweet, brandy-like
22	Isoamyl octanoate	1650	3.60 ± 0.12[a]	0.11	0.56 ± 0.08[b]	0.02	4.87 ± 0.14[c]	0.10	Sweet, fruity, Pineapple
23	Isobutyl decanoate	1760	1.45 ± 0.10[a]	0.04	0.92 ± 0.13[b]	0.03	1.81 ± 0.19[c]	0.04	Oily, sweet brandy, apricot
24	Isoamyl decanoate	1860	4.71 ± 0.47[a]	0.14	0.38 ± 0.05[b]	0.01	4.84 ± 0.95[a]	0.10	Waxy, banana, fruity
25	Methyl acetate	850	0.27 ± 0.02[a]	0.01	2.21 ± 0.19[b]	0.08	0.32 ± 0.00[c]	0.01	Fruity, sweet
26	Ethyl acetate[2]	910	17.90 ± 0.58[a]	0.55	459 ± 32.10[b]	16.82	21.30 ± 0.99[c]	0.45	Pineapple, sweet, fruity
27	Isoamyl acetate[1]	1089	2.33 ± 0.34[a]	0.07	302 ± 20.80[b]	11.06	1.73 ± 0.18[a]	0.04	Banana, apple, estery
28	Benzyl acetate[4]	1755	0.00 ± 0.00[a]	0.00	3.48 ± 0.41[b]	0.13	0.28 ± 0.02[c]	0.01	Sweet, floral, fruity, jasmine
29	2-Phenylethyl acetate[1]	1821	13.20 ± 1.73[a]	0.41	150 ± 18.40[b]	5.50	35.90 ± 1.13[c]	0.76	Rose, honey, floral
	Subtotal		**821.99**	**25.25**	**1121.66**	**41.09**	**1268.30**	**26.94**	
	Ketone								
30	β-Damascenone[4]	1840	0.63 ± 0.01[a]	0.02	0.39 ± 0.05[b]	0.01	0.56 ± 0.06[a]	0.01	Rose, cooked apple
	Heteroatom (N, S) compound								
31	Benzyl isothiocyanate[3]	2130	0.46 ± 0.02[a]	0.01	0.45 ± 0.06[a]	0.02	0.61 ± 0.08[b]	0.01	Watercress, oily
	Total		**3254.6**		**2729.47**		**4707.82**		

[a,b,c]Statistical analysis at 95% confidence level with same letters indicating no significant difference.
[d]Experimentally determined linear retention index on the DB-FFAP column, relative to C5-C40 hydrocarbons.
[e]Odor description obtained from Luebke (1980).
[1,2,3,4]Retention index in agreement with those in the literature [Duarte et al. (2010), Goodner (2008), Pino et al. (2003) and Segurel et al. (2009), respectively].

Table 8.3. Concentrations of selected major volatile compounds (mg/L) in papaya wine (day 21) fermented with a mixed-culture of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251

Compounds quantified	Control Yeast R2		Control Yeast NCYC2251		Mixed-culture		Odor threshold[d]
	Mean	OAV	Mean	OAV	Mean	OAV	
Ethanol	37517 ± 3336[a]	-	18441 ± 347[b]	-	42743 ± 3469[a]	-	-
Isobutyl alcohol	1.41 ± 0.02[a]	0.04	1.54 ± 0.14[a]	0.04	1.33 ± 0.18[a]	0.03	40.00
Isoamyl alcohol	108.59 ± 5.46[a]	3.62	21.60 ± 1.11[b]	0.72	94.57 ± 1.33[c]	3.15	30.00
2-Phenylethyl alcohol	23.11 ± 0.83[a]	2.31	2.79 ± 0.07[b]	0.28	22.98 ± 0.05[a]	2.30	10.00
Octanoic acid	0.97 ± 0.10[a]	0.11	0.85 ± 0.14[a]	0.10	1.75 ± 0.07[b]	0.20	8.80
Decanoic acid	0.96 ± 0.04[a]	0.16	0.50 ± 0.08[b]	0.08	1.26 ± 0.10[c]	0.21	6.00
Ethyl octanoate	1.08 ± 0.10[a]	54.19	0.83 ± 0.06[b]	41.50	1.20 ± 0.11[a]	59.82	0.02
Ethyl decanoate	2.99 ± 0.21[a]	14.95	0.26 ± 0.05[b]	1.30	4.40 ± 0.65[c]	22.00	0.20
Ethyl acetate	7.18 ± 0.40[a]	0.96	262 ± 6.90[b]	34.93	11.72 ± 0.43[a]	1.56	7.50
Isoamyl acetate	0.06 ± 0.00[a]	2.04	6.19 ± 0.25[b]	206.33	0.04 ± 0.00[a]	1.33	0.03
2-Phenylethyl acetate	0.24 ± 0.00[a]	0.96	2.50 ± 0.19[b]	10.00	0.46 ± 0.03[c]	1.84	0.25
Isoamyl octanoate	0.18 ± 0.02[a]	1.44	0.06 ± 0.00[b]	0.48	0.14 ± 0.01[c]	1.12	0.125[e]

Abbreviation: OAV = Odour activity values calculated by dividing concentration by the odour threshold value of the compound

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

[d]From Bartowsky and Pretorius (2009).

[e]From Ferreira et al. (2000).

8.2.3 Principal component analysis (PCA)

PCA was applied to the volatile compounds in papaya wines from Tables 8.2 and 8.3. Both the PCA results show similar outcome and thus, PCA of the quantified major volatile compounds from Table 8.3 is presented in Fig. 8.8. The monocultures and mixed-culture were mainly separated along the first principal component (PC1), which explained 90.77% of the total variance, while PC2 explained the remaining 9.23% (Fig. 8.8). The *W. saturnus* monoculture had a high percentage of acetate esters, while the *S. cerevisiae* monoculture was associated with more isoamyl alcohol, 2-phenylethyl alcohol and isoamyl octanoate. The mixed-culture, located on the upper right quadrant, had good correlation with ethanol, decanoic acid and ethyl esters such as ethyl octanoate and ethyl decanoate.

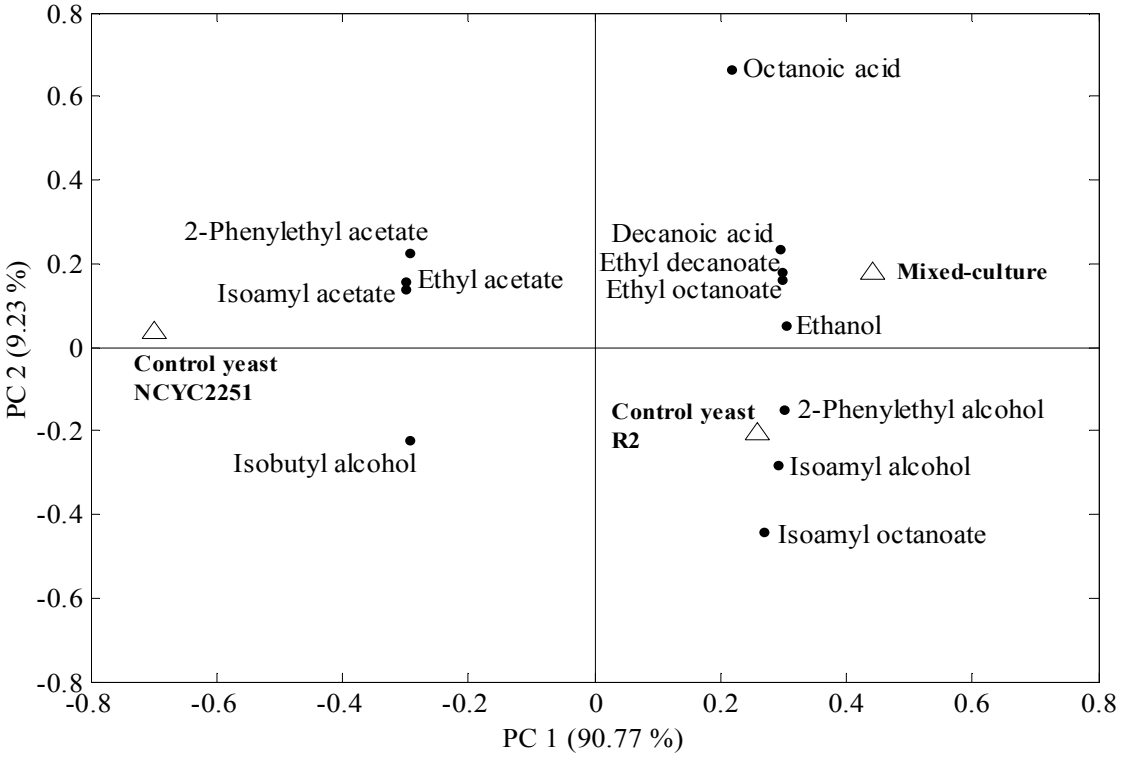

Fig. 8.8. Bi-plot of principal component analysis of the quantified major volatile compounds in papaya wines fermented by mono- and mixed-cultures of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251.

8.3 Conclusions

In this chapter, the fermentation performance and the formation/utilisation of aroma compounds by a mixed-culture (co-inoculation) of *S. cerevisiae* var. *bayanus* R2 /*W. saturnus* var. *mrakii* NCYC2251 were assessed and compared against fermentations using the corresponding single cultures. Overall, the mixed-culture fermentation showed the capability of producing papaya wine with a wider range of volatile compounds and higher amounts of volatile compounds as compared to the pure cultures, with higher levels of acetate esters than the *S. cerevisiae* monoculture and higher alcohols and ethyl esters levels than the *W. saturnus* monoculture. The mixed-culture also produced highest levels of aroma-active esters such as ethyl hexanoate, ethyl octanoate and ethyl decanoate. However, the mixed-culture showed similar trends to those of the *S. cerevisiae* monoculture in the formation and utilisation of most of the volatile compounds.

CHAPTER 9

EFFECT OF SEQUENTIALLY INOCULATED *WILLIOPSIS SATURNUS* VAR. *MRAKII* NCYC2251 AND *SACCHAROMYCES CEREVISIAE* VAR. *BAYANUS* R2 ON VOLATILE PROFILES OF PAPAYA WINE

9.1 Introduction

Recently, researchers have directed attention to the presence and persistence of non-*Saccharomyces* yeasts in inoculated and spontaneous wine fermentations (Heard & Fleet, 1985), as well as their contributions to the analytical composition and sensorial characteristics of wine (Garde-Cerdán & Ancín-Azpilicueta, 2006; Lema, Garcia-Jares, Orriols, & Angulo, 1996). However, these non-*Saccharomyces* yeasts are not vigorous or competitive fermenting microorganisms under oenological conditions; thus, they may be only employed as starter cultures in conjunction with strongly fermentative *S. cerevisiae* strains for the completion of fermentation. This led to the current trend to employ non-*Saccharomyces* yeasts as mixed (co-inoculation) or sequential cultures with *S. cerevisiae* (Ciani et al., 2010; Clemente-Jimenez et al., 2005).

Previous chapter (**Chapter 8**) and other evidences have highlighted the capability of mixed yeasts (co-inoculation) in improving the complexity and characteristics of grape and other fruit wines (Ciani et al., 2010; Garde-Cerdán & Ancín-Azpilicueta, 2006; Trinh et al., 2011), while results are non-conclusive for sequential fermentations. Ciani, Beco, and Comitini (2006) pointed out limitations of sequential fermentations such as excessive production of ethyl acetate and the

prolonged persistence of non-*Saccharomyces* yeasts at high levels, which eventually led to stuck or sluggish fermentations. In contrast, Bely et al. (2008) and Clemente-Jimenez et al. (2005) reported the improvement of wine quality with enhanced production of aromatic compounds and elimination of negative sensorial characteristics to some extent in sequential fermentation.

In multistarter fermentations, yeast succession is an essential parameter that affects the chemical composition and the contribution of these yeasts to the overall wine character. Sequential fermentation allowed the persistence of non-*Saccharomyces* (Ciani et al., 2006), while simultaneous mixed-culture fermentation resulted in an early growth arrest of non-*Saccharomyces* (Viana et al., 2009). The duration of non-*Saccharomyces* in contact with the fruit must is crucial for modifying the flavour composition (Clemente-Jimenez et al., 2005).

With the intention to extend survival and persistence of non-*Saccharomyces*, the aim of this chapter was to investigate the fermentation behaviour of *S. cerevisiae* var. *bayanus* R2 and *Williopsis saturnus* var. *mrakii* NCYC2251 in sequential fermentations [positive sequential fermentation (PSF): inoculation of *S. cerevisiae* into the medium partially fermented by *W. saturnus*; negative sequential fermentation (NSF): inoculation of *W. saturnus* into the medium partially fermented by *S. cerevisiae*], as compared to the mixed-culture fermentation (MCF, co-inoculation). The yeast ratio used corresponded to that described in **Chapter 8**, where a fixed yeast ratio of 1:1000 (S. *cerevisiae* R2: *W. saturnus* NCYC2251) improved the analytical and aromatic profiles of papaya wine.

9.2 Results and discussion

9.2.1 Biomass evolution and metabolic characteristics of yeasts

The evolution of *S. cerevisiae* and *W. saturnus* is shown in Fig. 9.1. Negative sequential fermentation (NSF) and mixed-culture fermentation (MCF) had similar yeast growth and succession patterns, which were different from those of positive sequential fermentation (PSF). *S. cerevisiae* in both NSF and MCF increased rapidly and then remained stationary, while the same yeast in PSF grew slightly upon inoculation at day 7 and then declined rapidly (Fig. 9.1).

As expected, *W. saturnus* in both NSF and MCF declined rapidly, but the same yeast in PSF multiplied incessantly and achieved a maximum of ~10^8 CFU/mL at day 21 (Fig. 9.1). The domination of *W. saturnus* in PSF was probably due to the killer toxins produced by *W. saturnus* (Liu & Tsao, 2010), to which *S. cerevisiae* was sensitive (Yap, de Barros Lopes, Langridge, & Henschke, 2000). These results were contrary to those of Ciani et al. (2006) and Toro and Vazquez (2002), where the non-*Saccharomyces* yeast decreased rapidly upon the sequential inoculation of *S. cerevisiae*. This could be due to the different ratios of yeasts and different species of non-*Saccharomyces* yeasts such as *Candida cantarellii*, *Hanseniaspora uvarum*, *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans* used in Ciani et al. (2006) and Toro and Vazquez (2002).

The domination of *S. cerevisiae* in NSF was generally ascribed to its higher capacity to withstand the harsh changing environmental conditions in winemaking (Pretorius, 2000). Nevertheless, the early growth arrest of *W. saturnus* in both NSF and MCF could be due to several factors such as its lower ethanol tolerance, oxygen availability, toxic compounds, nutrient limitation, quorum sensing and cell-cell

contact mechanism (Arneborg et al., 2005; Fleet & Heard, 1993; Nissen & Arneborg, 2003; Nissen et al., 2003; Panon, 1997).

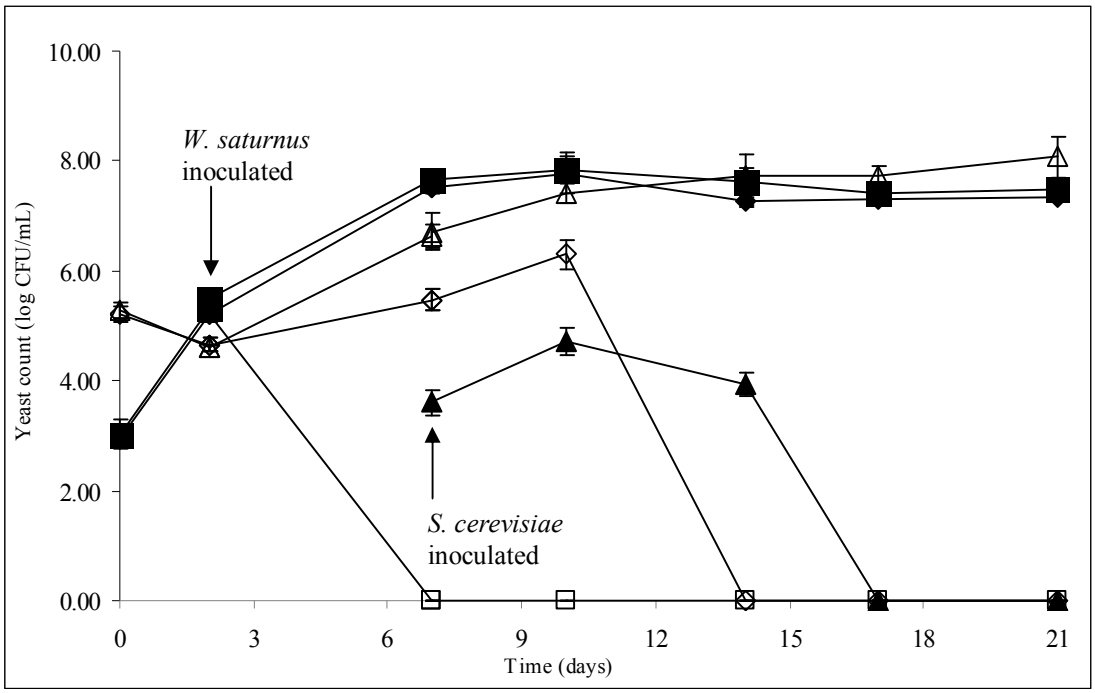

Fig. 9.1. Evolution of yeasts in papaya wine fermentation. *S. cerevisiae* var. *bayanus* R2 in mixed-culture fermentation (♦); *W. saturnus* var. *mrakii* NCYC2251 in mixed-culture fermentation (◊); *S. cerevisiae* R2 in positive sequential fermentation (▲); *W. saturnus* NCYC2251 in positive sequential fermentation (Δ); *S. cerevisiae* R2 in negative sequential fermentation (■); *W. saturnus* NCYC2251 in negative sequential fermentation (□). Positive sequential fermentation: inoculation of *S. cerevisiae* R2 after 7 days' fermentation with *W. saturnus* NCYC2251; negative sequential fermentation: inoculation of *W. saturnus* NCYC2251 after 2 days' fermentation with *S. cerevisiae* R2. Mixed-culture fermentation: co-inoculation of both cultures. (Error bars = standard deviation).

The oenological parameters of NSF, PSF and MCF are shown in Table 9.1 and Appendix G (Fig. G1). Most of the fermentation characteristics of NSF were similar to those of MCF, where the ^{o}Brix, sugars and organic acids decreased significantly except for succinic acid, acetic acid and pH. The pH did not change significantly with values maintaining at around pH 3.50-3.60, while acetic and succinic acids either increased continuously or remained constant.

The oenological parameters of PSF decreased gradually, except for the increased formation of acetic and succinic acids. This is in agreement with the domination of *W. saturnus* in PSF (Fig. 9.1) and the low fermentative ability of the *W. saturnus* yeasts (**Chapter 8**). These results are similar to those in Ciani et al. (2006), where PSF preferentially utilised glucose over fructose and had higher residual sugar levels than MCF. Acetic acid can be produced from the oxidation of acetaldehyde by acetaldehyde dehydrogenase and causes objectionable wine flavour near its threshold of 0.07-0.11 g/100 mL (Lambrechts & Pretourius, 2000). The acetic acid levels produced by all the fermentations were lower than the threshold level and corresponded to the finding of Toro and Vazquez (2002), who found low acetic acid production by both the simultaneous mixed-culture and the sequential fermentations. Succinic acid is a regular by-product in the alcoholic fermentation, which is most likely formed through the reductive branch (via oxaloacetate and malate) of the tri-carboxylic acid (TCA) cycle (Swiegers et al., 2005) and if present at relatively high levels, it could affect wine quality by imparting unusual "salty" or "bitter" taste (Whiting, 1976).

Table 9.1. Oenological parameters of papaya wine (day 21) fermented with mixed and sequential cultures of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251.

	Day 0	Mixed-culture	Positive sequential[e]	Negative sequential[f]
pH	3.52 ± 0.00[a]	3.59 ± 0.00[b]	3.50 ± 0.00[a]	3.60 ± 0.00[b]
°Brix (%)	11.30 ± 0.02[a]	4.09 ± 0.11[b]	8.19 ± 0.38[c]	3.80 ± 0.17[b]
Ethanol (%, v/v)	0.01 ± 0.00[a]	4.74 ± 0.22[b]	1.77 ± 0.08[c]	4.56 ± 0.36[b]
Sugars (g/100 mL)				
Fructose	4.16 ± 0.09[a]	0.02 ± 0.00[b]	3.45 ± 0.14[c]	0.02 ± 0.00[b]
Glucose	4.46 ± 0.11[a]	0.02 ± 0.00[b]	2.09 ± 0.19[c]	0.02 ± 0.00[b]
Organic acids (g/100 mL)				
Acetic acid	0.011 ± 0.001[a]	0.032 ± 0.001[b]	0.043 ± 0.002[c]	0.044 ± 0.003[c]
Citric acid	0.272 ± 0.004[a]	0.236 ± 0.006[b]	0.247 ± 0.002[c]	0.234 ± 0.002[b]
Malic acid	1.054 ± 0.007[a]	0.742 ± 0.011[b]	0.930 ± 0.005[c]	0.720 ± 0.006[b]
Succinic acid	0.176 ± 0.001[a]	0.164 ± 0.007[a]	0.216 ± 0.015[b]	0.178 ± 0.013[a]
Tartaric acid	0.021 ± 0.0008[a]	0.006 ± 0.0001[b]	0.010 ± 0.0009[c]	0.012 ± 0.0009[c]

[a,b,c,d]Statistical analysis at 95% confidence level with same letters indicating no significant difference.
[e]Inoculation of *S. cerevisiae* after 7 days' fermentation with *W. saturnus*.
[f]Inoculation of *W. saturnus* after 2 days' fermentation with *S. cerevisiae*.

9.2.2 Aromatic quality and volatile composition of papaya wine

Controlled mixed (co-inoculation) and sequential cultures of *S. cerevisiae* and non-*Saccharomyces* were able to improve the analytical and aromatic profiles of wines through the metabolic interactions between the different yeast species (Ciani et al., 2010). A wide variety of volatile compounds were produced and modulated by different sequential fermentations. These included volatile fatty acids, alcohols, esters, aldehydes, ketones, volatile phenol and terpenoids (Table 9.2). However, those volatiles that were initially present in the papaya juice were metabolised to trace levels in all the fermentations (Figs. 9.2-9.6).

The dynamic changes of these volatile compounds were similar to those presented in **Chapter 8**, where some volatiles increased incessantly, while others increased initially and then either remained unchanged or declined (Figs. 9.2-9.6).

The different trends of volatile evolution may be due to the diverse rates of enzymatic synthesis and hydrolysis or chemical hydrolysis. These enzymatic activities are affected by factors such as the dominating yeast strains and nutrients, especially nitrogen concentration and must solids (Sumby et al., 2010). The dynamic changes of these volatile compounds influenced the aromatic composition and modulated the fermentation bouquet of papaya wines.

Volatile fatty acids were one of the significant groups of volatile compounds that were produced by yeast during fermentation (Table 9.2). The dynamic changes of volatile fatty acids were similar in both MCF and NSF, where acetic, isobutyric and hexanoic acids increased with the progress of fermentation, while C8 to C12 fatty acids increased initially and then declined [Fig. 9.2, Appendix G (Fig. G2)]. Conversely, these volatile fatty acids in PSF increased continuously throughout fermentation. Butyric acid was metabolised in all the fermentations [Appendix G (Fig. G2)]. NSF and MCF produced comparable amounts of fatty acids (Tables 9.2 and 9.3). In contrast, PSF consistently produced lower levels of fatty acids except for acetic acid (Table 9.2). The domination of *W. saturnus* in PSF might have utilised the majority of the acetyl-CoA for the synthesis of acetate esters leading to insufficient acetyl-CoA available for the synthesis of fatty acids (Lambrechts & Pretorius, 2000) and hence, accounting for the lower levels of fatty acids as compared to those in NSF and MCF.

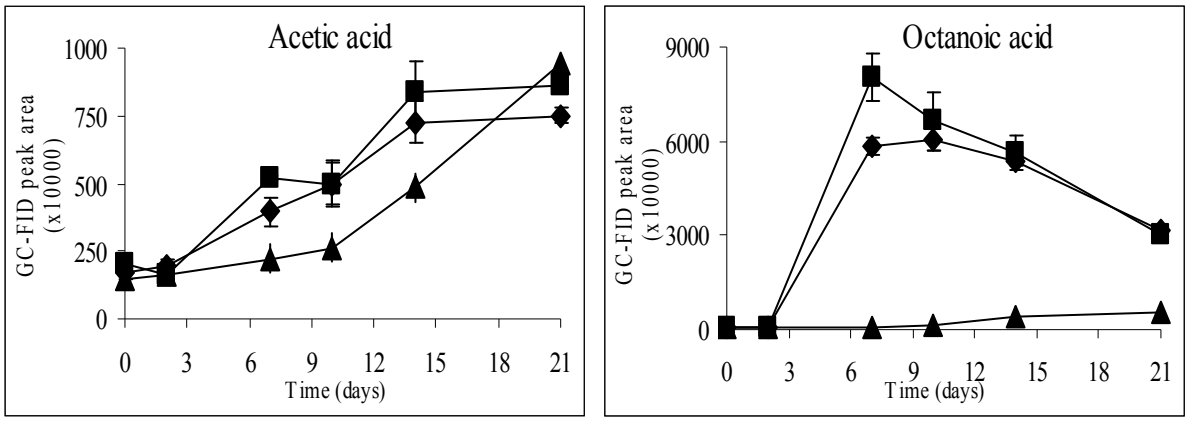

Fig. 9.2. Changes of acetic and octanoic acids during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

Among the volatile compounds, alcohols (ethanol and higher alcohols) constituted the largest group with relative peak areas (RPA) ranging from 53.83% to 78.33% (Table 9.2). The dynamic changes of the alcohols were similar in both NSF and MCF, where the alcohols increased initially and then either became stationary or declined slightly [Fig. 9.3, Appendix G (Fig. G3)]. Hence, the wines produced by NSF and MCF had comparable amounts of ethanol and higher alcohols (Tables 9.1-9.3). In contrast, these alcohols increased gradually throughout fermentation in PSF [Fig. 9.3, Appendix G (Fig. G3)], and PSF had lesser alcohol production and almost 60% lower ethanol than MCF, except for isobutyl alcohol (Tables 9.1-9.3). The lower levels of alcohols in PSF corresponded to the higher levels of acetate esters (Tables 9.2 and 9.3) produced by the dominant *W. saturnus*.

These results differ from those of Ciani et al. (2006) and Toro and Vazquez (2002), where MCF and PSF produced comparable amounts of ethanol and higher alcohols. This discrepancy could be attributed to the domination of *S. cerevisiae* in their PSF and the different species of non-*Saccharomyces* yeasts used (*K.*

thermotolerans, H. uvarum, *T. delbrueckii* and *C. cantarelli*). Higher alcohols are important precursors for the formation of esters, which are formed by transamination of the corresponding amino acids through the Ehrlich pathway or produced *de novo* from sugars (Clemente-Jimenez et al., 2005). However, they have adverse effects on the quality of the final product when present in wines at excessive levels (Thornton, 1991). With the gradual production and rapid utilisation of alcohols in PSF, the probability of the higher alcohols exerting adverse effects would be lower as compared to NSF and MCF.

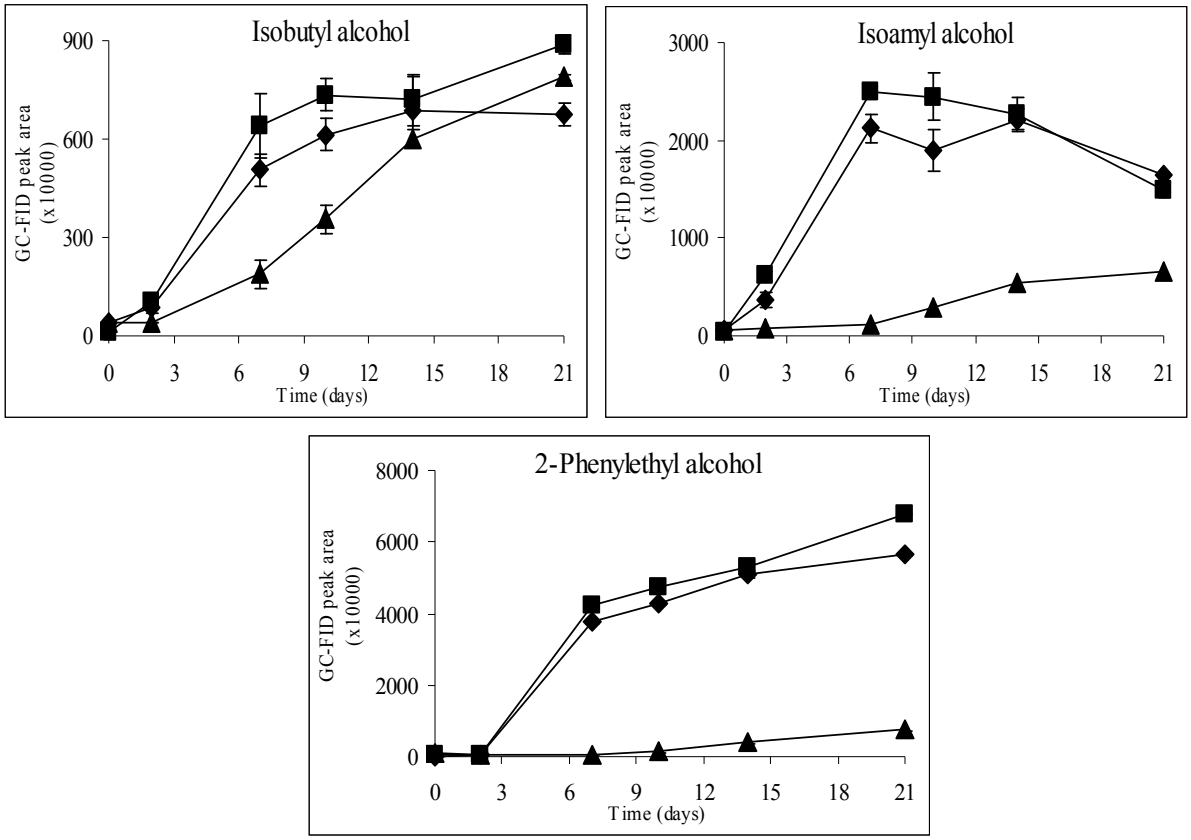

Fig. 9.3. Changes of alcohols during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

Esters are important contributors to the fruity flavours of alcoholic beverages (Russell, 2003). Variable amounts of esters were produced with acetate and ethyl

esters being the majority (Table 9.2). Most of the esters were produced continuously. Some esters increased initially and then remained stable or declined slightly [Figs. 9.4 and 9.5, Appendix G (Figs. G4-G6)]. The dynamic changes of these esters were similar in both NSF and MCF, but were significantly different from those of PSF [Figs. 9.4 and 9.5, Appendix G (Fig. G4-G6)]. The interaction and metabolism of different yeasts would have modulated ester production.

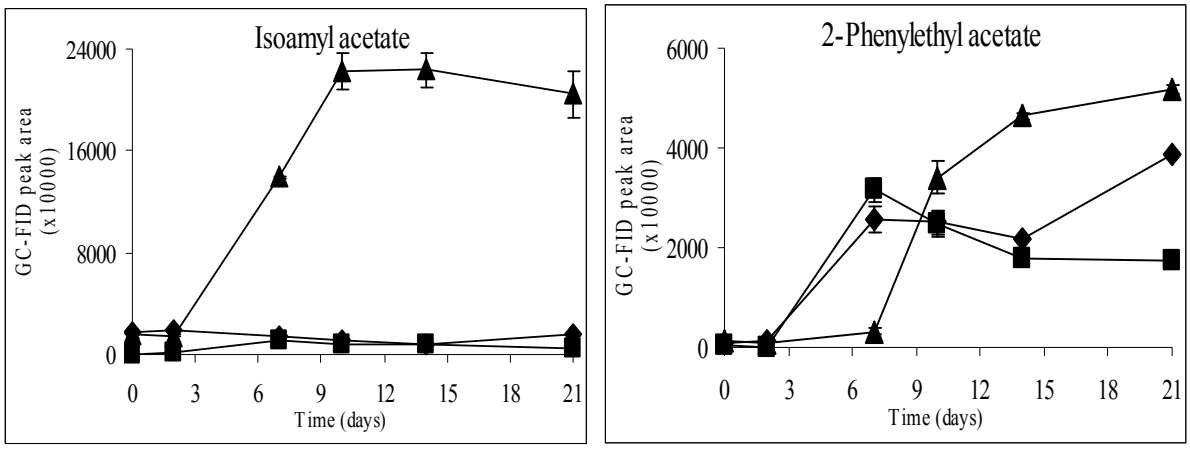

Fig. 9.4. Changes of isoamyl acetate and 2-phenylethyl acetate during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

Generally, NSF and MCF produced comparable amounts of esters, except for isoamyl acetate, isobutyl acetate and some ethyl esters (Tables 9.2 and 9.3). The longer persistence of *W. saturnus* (with high acetate ester-synthesising activities) in MCF accounted for the higher amounts of isoamyl acetate and isobutyl acetate produced. However, the higher *S. cerevisiae* population in NSF gave rise to the higher concentrations of ethyl esters including ethyl octanoate and ethyl dodecanoate as compared to MCF (Tables 9.2 and 9.3), supporting the findings of Rojas et al. (2003). The concentrations of isoamyl acetate and 2-phenylethyl acetate in NSF exceeded their thresholds (Table 9.3) and were expected to exert sensory impacts.

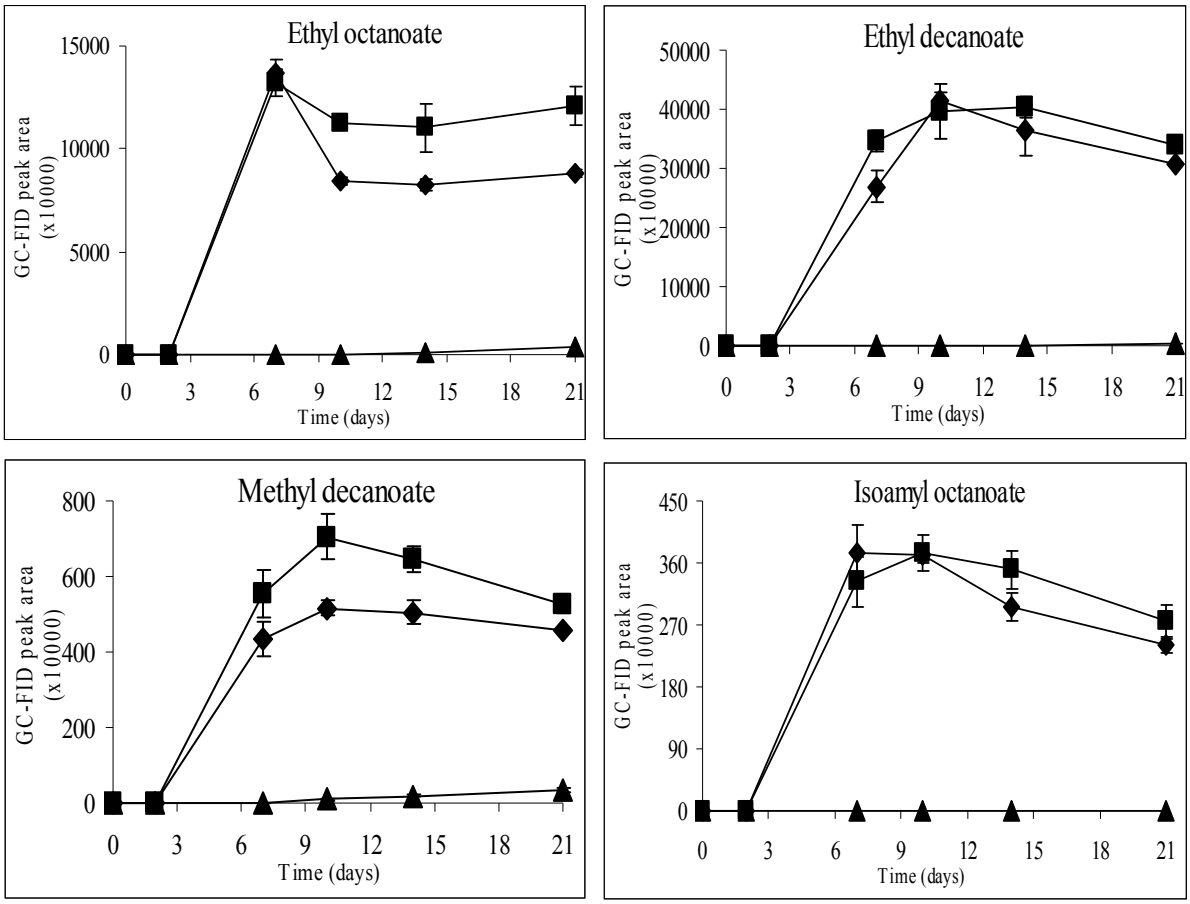

Fig. 9.5. Changes of ethyl esters, methyl decanoate and isoamyl octanoate during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

W. saturnus can potentially enhance the fruity flavour through synthesising important volatile esters especially isoamyl acetate (banana-like) and ethyl acetate (Erten & Tanguler, 2010). Indeed, PSF dominated by *W. saturnus* produced higher amounts of acetate esters such as isoamyl acetate (9.49 mg/L), active amyl acetate (0.31 mg/L), 2-phenylethyl acetate (3.53 mg/L) and ethyl acetate (205.2 mg/L) (Table 9.3). These esters could contribute to the fruity notes and add to the flavour complexity, except for ethyl acetate that could impart solvent-like flavour as its concentration exceeded 200 mg/L (Etievant, 1991).

The results of this study corresponded to the findings in Ciani et al. (2006) where PSF produced higher levels of ethyl acetate than MCF. Acetate esters are formed by alcohol acetyltransferases from the reaction between acetyl-CoA and alcohols that is either ethanol or higher alcohols derived from amino acid metabolism (Saerens et al., 2008). The results of this study agreed with Saerens et al. (2008), where PSF with higher levels of acetate esters resulted in lower levels of corresponding alcohols (Tables 9.2 and 9.3). Moreover, Fukuda et al. (1998) revealed that the resultant amounts of acetate esters were dependent on the balance between the degradation and synthesis of esters governed by esterase and alcohol acetyltransferase, respectively.

Other volatiles including aldehydes, ketones, benzyl isothiocyanate, volatile phenol and terpenoids were also detected. Most of them were metabolised during fermentation except for acetaldehyde, 3-hydroxy-2-butanone (acetoin), β-citronellol and citronellyl acetate that were formed [Fig. 9.6, Appendix G (Fig. G7)]. PSF and NSF produced lower amounts of these volatiles than MCF, except for acetaldehyde and β-citronellol (Table 9.2). NSF had a comparable amount of acetaldehyde to MCF and a higher amount of β-citronellol than MCF (Table 9.2).

Acetaldehyde is an intermediary product of yeast metabolism from pyruvate through the glycolytic pathway and *S. cerevisiae* strains can produce relatively high levels of acetaldehyde from 50 to 120 mg/L (Fleet & Heard, 1993). Hence, the high level of acetaldehyde in both NSF and MCF could be related to the domination of *S. cerevisiae* in these fermentations (Fig. 9.1). The occurrence of β-citronellol in papaya wine was likely due to its production by *S. cerevisiae* yeast. Mateo and Jiménez (2000) revealed that the presence of β-citronellol in wine could be due to hydrolysis of glycosides with bound citronellol or transformation from geraniol and nerol by *S.*

cerevisiae. The results of this research corresponded to those of Mateo and Jiménez (2000), where NSF with a higher *S. cerevisiae* population had a higher amount of β-citronellol than MCF. Conversely, the results of this study did not support other reports that linked non-*Saccharomyces* yeasts with higher β-glucosidase activities than *S. cerevisiae* yeasts and thus, enhancing wine aroma through releasing terpenols (Charoenchai et al., 1997; Fia, Giovani, & Rosi, 2005; Manzanares et al., 2000).

Citronellyl acetate was detected for the first time in papaya wine. This compound was previously not found in papaya wines described in earlier chapters and was also not an indigenous compound in the papaya juice. This could be due to the yeast metabolism of β-citronellol and acetyl-CoA by alcohol acetyltransferase (Oda, Inada, Kobayashi, Kato, Matsudomi, & Ohta, 1996). Nevertheless, Castro, Napoleão, and Oliveria (1998) highlighted the possibility of citronellyl acetate formation through esterification of β-citronellol and acetic acid by lipase. Citronellyl acetate can contribute to the overall fruity and floral notes in the papaya wine near its flavour threshold of 0.25 mg/L (Yamamoto, Shimada, Ohmoto, Matsuda, Ogura, & Kanisawa, 2004). Both NSF and PSF would have little flavour impact from citronellyl acetate as compared to MCF, especially PSF where citronellyl acetate was not detected (Fig. 9.6, Table 9.2).

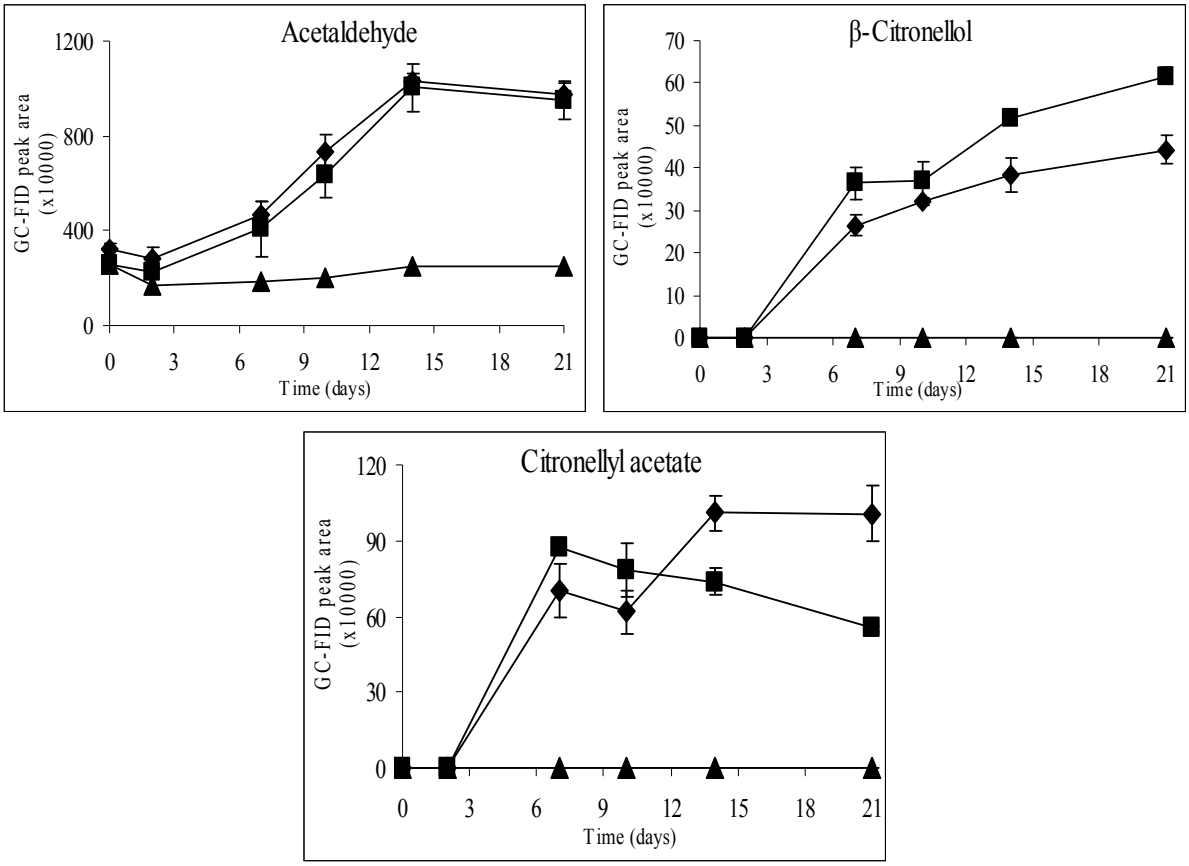

Fig. 9.6. Changes of acetaldehyde and terpenoids during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

Table 9.2. Major volatile compounds (GC-FID peak area x 10^6) and their relative peak areas (RPA) identified in papaya wine (day 21) fermented with mixed and sequential cultures of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251.

			Mixed-culture		Positive sequential[d]		Negative sequential[e]		
No.	**Compounds**	**LRI[f]**	**Peak area**	**RPA (%)**	**Peak area**	**RPA (%)**	**Peak area**	**RPA (%)**	**Organoleptics[g]**
	Acids								
1	Acetic acid[3]	1461	7.52 ± 0.25[a]	0.22	9.46 ± 0.04[b]	0.71	8.61 ± 0.04[c]	0.25	Acidic, pungent, vinegar-like
2	Isobutyric acid	1570	0.75 ± 0.04[a]	0.02	0.61 ± 0.02[b]	0.05	0.64 ± 0.05[b]	0.02	Acidic, cheese, rancid
3	Butyric acid[2]	1630	1.91 ± 0.11[a]	0.05	3.83 ± 0.36[b]	0.29	2.62 ± 0.01[c]	0.07	Acidic, buttery, cheesy
4	Hexanoic acid[1,4]	1847	4.65 ± 0.07[a]	0.13	2.92 ± 0.04[b]	0.22	3.48 ± 0.05[c]	0.10	Acidic, cheesy, fruity
5	Octanoic acid[1,4]	2061	31.70 ± 1.50[a]	0.91	5.39 ± 1.02[b]	0.41	30.00 ± 0.37[a]	0.85	Acidic, cheesy, fatty, sweaty
6	Nonanoic acid[2]	2169	0.40 ± 0.02[a]	0.01	0.28 ± 0.02[b]	0.02	0.95 ± 0.04[c]	0.03	Cheesy, fatty, waxy
7	9-Decenoic acid	2339	4.20 ± 0.16[a]	0.12	0.00 ± 0.00[b]	0.00	4.00 ± 0.06[c]	0.11	Creamy, fatty, milky
8	Decanoic acid[4]	2275	44.20 ± 3.10[a]	1.27	2.70 ± 0.10[b]	0.20	37.40 ± 1.16[c]	1.07	Buttery, condensed, milky
9	Dodecanoic acid[3]	2487	10.00 ± 0.57[a]	0.29	2.93 ± 0.15[b]	0.22	8.17 ± 0.22[c]	0.23	Fatty, soapy, waxy
	Subtotal		**105.33**	**3.02**	**28.12**	**2.11**	**95.87**	**2.73**	
	Alcohols								
10	Ethanol[2]	954	2650 ± 160[a]	75.86	685 ± 15[b]	51.52	2620 ± 63[a]	74.62	Alcoholic, solventy
11	1-Propanol[3]	1040	1.43 ± 0.07[a]	0.04	0.84 ± 0.06[b]	0.06	1.94 ± 0.07[c]	0.06	Alcoholic, fermented, solventy
12	Isobutyl alcohol[2]	1091	6.74 ± 0.35[a]	0.19	7.89 ± 0.09[b]	0.59	8.88 ± 0.29[c]	0.25	Breathtaking, fermented, whisky
13	Active amyl alcohol[4]	1220	5.22 ± 0.08[a]	0.15	8.00 ± 0.42[b]	0.60	6.84 ± 0.54[c]	0.19	Alcoholic, fermented, fusel
14	Isoamyl alcohol[4]	1223	16.40 ± 0.01[a]	0.47	6.65 ± 0.64[b]	0.50	14.80 ± 0.79[c]	0.42	Alcoholic, fermented, whiskey
15	2-Phenylethyl alcohol[4]	1926	56.40 ± 0.61[a]	1.61	7.41 ± 0.33[b]	0.56	67.70 ± 1.67[c]	1.93	Floral, honey, rosy
	Subtotal		**2736.19**	**78.33**	**715.79**	**53.83**	**2720.16**	**77.48**	
	Aldehydes								
16	Acetaldehyde[2]	745	9.75 ± 0.59[a]	0.28	2.46 ± 0.26[b]	0.19	9.47 ± 0.78[a]	0.27	Aldehydic, ethereal, fruity
17	Benzaldehyde[2]	1538	0.70 ± 0.06[a]	0.02	1.00 ± 0.08[b]	0.08	1.22 ± 0.06[c]	0.03	Bitter almond, cherry, sweet
18	O-Tolualdehyde	1666	2.11 ± 0.07[a]	0.06	1.84 ± 0.05[b]	0.14	2.23 ± 0.07[a]	0.06	Bitter almond, cherry pit, sweet
19	2,4-Dimethylbenzaldehyde	1836	0.56 ± 0.05[a]	0.02	0.33 ± 0.05[b]	0.02	0.55 ± 0.06[a]	0.02	Almond, cherry, vanilla
	Subtotal		**13.12**	**0.38**	**5.63**	**0.42**	**13.47**	**0.38**	
	Esters								
20	Methyl octanoate[3]	1378	1.40 ± 0.13[a]	0.04	0.37 ± 0.05[b]	0.03	1.39 ± 0.09[a]	0.04	Citrus, green, fruity

Table 9.2. (Continued)

No.	Compounds	LRI[f]	Mixed-culture Peak area	Mixed-culture RPA (%)	Positive sequential[d] Peak area	Positive sequential[d] RPA (%)	Negative sequential[e] Peak area	Negative sequential[e] RPA (%)	Organoleptics[g]
21	Methyl decanoate[3]	1589	4.57 ± 0.05[a]	0.13	0.32 ± 0.06[b]	0.02	5.24 ± 0.22[c]	0.15	Fatty, cognac, oily
22	Methyl dodecanoate[3]	1800	1.65 ± 0.02[a]	0.05	0.42 ± 0.03[b]	0.03	1.30 ± 0.11[c]	0.04	Creamy coconut, waxy
23	Ethyl butyrate[1]	1037	1.03 ± 0.01[a]	0.03	1.32 ± 0.05[b]	0.10	1.60 ± 0.10[c]	0.05	Fruity, ripe, sweet
24	Ethyl hexanoate[1]	1217	11.90 ± 0.09[a]	0.34	0.00 ± 0.00[b]	0.00	5.82 ± 0.11[c]	0.17	Fruity, pineapple-like, winey
25	Ethyl octanoate[1,4]	1428	88.20 ± 1.76[a]	2.52	3.80 ± 0.15[b]	0.29	121 ± 9.35[c]	3.45	Fruity, cognac, yeasty
26	Ethyl 9-decenoate	1690	56.50 ± 2.57[a]	1.62	0.00 ± 0.00[b]	0.00	76.40 ± 0.53[c]	2.18	Fatty, fruity
27	Ethyl decanoate[1]	1642	307 ± 3.89[a]	8.79	2.85 ± 0.13[b]	0.21	338 ± 5.10[c]	9.63	Fatty, fruity, winey
28	Ethyl dodecanoate[3]	1840	72.00 ± 1.68[a]	2.06	4.87 ± 0.21[b]	0.37	61.20 ± 1.39[c]	1.74	Fruity, oily, waxy
29	Ethyl tetradecanoate[3]	2050	1.92 ± 0.09[a]	0.05	0.24 ± 0.02[b]	0.02	1.51 ± 0.05[c]	0.04	Creamy, oily, waxy
30	Ethyl 9-hexadecenoate[3]	2286	3.62 ± 0.19[a]	0.10	0.00 ± 0.00[b]	0.00	2.33 ± 0.03[c]	0.07	Creamy, waxy
31	Ethyl hexadecanoate[3]	2257	3.82 ± 0.19[a]	0.11	0.18 ± 0.01[b]	0.01	2.45 ± 0.01[c]	0.07	Creamy, fruity, milky
32	2-Methylbutyl hexanoate	1451	0.46 ± 0.00[a]	0.01	0.00 ± 0.00[b]	0.00	0.65 ± 0.04[c]	0.02	Ethereal
33	Propyl octanoate	1513	0.35 ± 0.01[a]	0.01	0.00 ± 0.00[b]	0.00	0.54 ± 0.00[c]	0.02	Coconut, fatty, winey
34	Isobutyl octanoate	1544	1.18 ± 0.01[a]	0.03	0.11 ± 0.00[b]	0.01	0.84 ± 0.03[c]	0.02	Fatty, fruity, winey
35	Isoamyl octanoate	1656	2.40 ± 0.11[a]	0.07	0.00 ± 0.00[b]	0.00	2.75 ± 0.23[c]	0.08	Cognac, fatty, oily
36	Propyl decanoate	1722	0.49 ± 0.05[a]	0.01	0.00 ± 0.00[b]	0.00	0.57 ± 0.01[c]	0.02	Fatty, fruity, waxy
37	Isobutyl decanoate	1754	1.11 ± 0.02[a]	0.03	0.00 ± 0.00[b]	0.00	1.10 ± 0.07[a]	0.03	Brandy, cognac, oily
38	Isoamyl decanoate	1863	3.60 ± 0.24[a]	0.10	0.16 ± 0.02[b]	0.01	3.56 ± 0.26[a]	0.10	Cognac, green, waxy
39	Methyl acetate	845	0.39 ± 0.03[a]	0.01	3.28 ± 0.27[b]	0.25	0.33 ± 0.02[a]	0.01	Ethereal, estery, fruity
40	Ethyl acetate[2]	907	11.90 ± 1.04[a]	0.34	274 ± 21.42[b]	20.61	24.90 ± 0.49[a]	0.71	Ethereal, fruity, solventy
41	Propyl acetate	990	0.00 ± 0.00[a]	0.00	3.23 ± 0.10[b]	0.24	0.00 ± 0.00[a]	0.00	Ethereal, fruity, pear-like
42	Butyl acetate	1066	0.19 ± 0.00[a]	0.01	1.00 ± 0.04[b]	0.08	0.08 ± 0.00[c]	0.00	Banana-like, fruity, sweet
43	Isobutyl acetate[1]	1020	2.73 ± 0.13[a]	0.08	15.20 ± 0.76[b]	1.14	0.00 ± 0.00[c]	0.00	Floral, fruity, mixed fruit-like
44	Active amyl acetate	1105	0.54 ± 0.01[a]	0.02	9.61 ± 0.11[b]	0.72	0.57 ± 0.06[a]	0.02	Banana-like, fruity, ripe
45	Isoamyl acetate[1]	1106	15.20 ± 1.40[a]	0.44	204 ± 18.6[b]	15.34	4.80 ± 0.37[a]	0.14	Banana-like, fruity, sweet
46	Benzyl acetate	1740	0.12 ± 0.00[a]	0.00	1.62 ± 0.03[b]	0.12	0.11 ± 0.00[a]	0.00	Floral, fruity, jasmine-like, sweet

Table 9.2. (Continued)

No.	Compounds	LRI[f]	Mixed-culture Peak area	Mixed-culture RPA (%)	Positive sequential[d] Peak area	Positive sequential[d] RPA (%)	Negative sequential[e] Peak area	Negative sequential[e] RPA (%)	Organoleptics[g]
47	2-Phenylethyl acetate[1]	1827	38.70 ± 0.34[a]	1.11	51.70 ± 1.01[b]	3.89	17.30 ± 0.48[c]	0.49	Floral, rosy, honey
48	Ethyl phenyl acetate[2]	1795	0.43 ± 0.01[a]	0.01	0.08 ± 0.01[b]	0.01	0.45 ± 0.04[a]	0.01	Cocoa-like, fruity, honey, rosy
	Subtotal		**633.40**	**18.13**	**578.36**	**43.49**	**676.79**	**19.27**	
	Ketones								
49	3-Hydroxy-2-butanone[1]	1308	1.73 ± 0.08[a]	0.05	0.22 ± 0.01[b]	0.02	1.43 ± 0.06[c]	0.04	Buttery, creamy, sweet
50	β-Damascenone[4]	1831	0.45 ± 0.03[a]	0.01	0.36 ± 0.00[b]	0.03	0.46 ± 0.04[a]	0.01	Fruity, floral, woody
	Subtotal		**2.18**	**0.06**	**0.58**	**0.04**	**1.89**	**0.05**	
	Phenol								
51	2,4-Di-tert-butylphenol	2314	0.86 ± 0.06[a]	0.02	0.58 ± 0.05[b]	0.04	0.92 ± 0.07[a]	0.03	Herbal, phenolic
	Terpenoids								
52	β-Citronellol[2]	1766	0.44 ± 0.03[a]	0.01	0.00 ± 0.00[b]	0.00	0.62 ± 0.02[c]	0.02	Citronella, oily, rose
53	Citronellyl acetate[2]	1659	1.01 ± 0.11[a]	0.03	0.00 ± 0.00[b]	0.00	0.55 ± 0.02[c]	0.02	Floral, fruity, rose
	Subtotal		**1.45**	**0.04**	**0.00**	**0.00**	**1.17**	**0.04**	
	Heteroatom (N, S) compound								
54	Benzyl isothiocyanate[3]	2124	0.69 ± 0.02[a]	0.02	0.61 ± 0.04[b]	0.05	0.71 ± 0.03[a]	0.02	Horseradish-like, hot, pungent
	Total		**3493.22**		**1329.67**		**3510.98**		

[a,b,c]Statistical analysis at 95% confidence level with same letters indicating no significant difference.
[d]Inoculation of *S. cerevisiae* after 7 days' fermentation with *W. saturnus*.
[e]Inoculation of *W. saturnus* after 2 days' fermentation with *S. cerevisiae*.
[f]Experimentally determined linear retention index on the DB-FFAP column, relative to C5-C40 hydrocarbons.
[g]Odor descriptions obtained from Luebke (1980).
[1,2,3,4]Retention index in agreement with those in the literature [Duarte et al. (2010), Goodner (2008), Pino et al. (2003) and Segurel et al. (2009), respectively].

Table 9.3. Concentrations of selected major volatile compounds (mg/L) in papaya wine (day 21) fermented with mixed and sequential cultures of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251.

	Mixed-culture		Positive sequential[d]		Negative sequential[e]		Odor threshold[f]
Compounds quantified	Mean	OAV	Mean	OAV	Mean	OAV	**(mg/L)**
Ethanol	37395 ± 1770[a]	-	13998 ± 656[b]	-	35991 ± 2816[a]	-	-
Isoamyl alcohol	61.85 ± 1.91[a]	2.06	0.33 ± 0.03[b]	0.01	55.63 ± 3.00[c]	1.85	30.00
Active amyl alcohol	19.05 ± 0.62[a]	0.29	0.63 ± 0.02[b]	0.01	10.06 ± 0.14[c]	0.15	65.00
Isobutyl alcohol	1.91 ± 0.12[a]	0.05	2.49 ± 0.12[b]	0.06	2.00 ± 0.02[a]	0.05	40.00
2-Phenylethyl alcohol	25.23 ± 1.82[a]	2.52	1.24 ± 0.08[b]	0.12	26.94 ± 2.22[a]	2.69	10.00
Octanoic acid	6.36 ± 0.54[a]	0.72	0.70 ± 0.06[b]	0.08	5.33 ± 0.44[a]	0.61	8.80
Ethyl octanoate	0.88 ± 0.01[a]	44.00	0.10 ± 0.00[b]	5.00	0.99 ± 0.07[c]	49.50	0.02
Ethyl decanoate	4.21 ± 0.46[a]	21.05	0.12 ± 0.01[b]	0.60	4.90 ± 0.13[a]	24.50	0.20
Ethyl dodecanoate	5.22 ± 0.12[a]	4.35	0.18 ± 0.02[b]	0.15	6.73 ± 0.39[c]	5.61	1.20[g]
Ethyl acetate	21.49 ± 1.16[a]	2.87	205.2 ± 5.63[b]	27.36	20.91 ± 0.24[a]	2.79	7.50
Isoamyl acetate	1.91 ± 0.01[a]	63.67	9.49 ± 0.39[b]	316.33	0.82 ± 0.06[c]	27.33	0.03
Active amyl acetate	0.03 ± 0.00[a]	0.19	0.31 ± 0.03[b]	1.94	0.03 ± 0.00[a]	0.19	0.16
Isobutyl acetate	0.006 ± 0.00[a]	0.00	0.011 ± 0.00[b]	0.01	0.003 ± 0.00[c]	0.00	1.60
2-Phenylethyl acetate	0.48 ± 0.05[a]	1.92	3.53 ± 0.24[b]	14.12	0.47 ± 0.03[a]	1.88	0.25

Abbreviation: OAV = Odour activity values calculated by dividing concentration by the odour threshold value of the compound

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

[d]Inoculation of *S. cerevisiae* after 7 days' fermentation with *W. saturnus*.

[e]Inoculation of *W. saturnus* after 2 days' fermentation with *S. cerevisiae*.

[f]From Bartowsky and Pretorius (2009).

[g]From Ferreira et al. (2000).

9.2.3 Principal component analysis

Principal component analysis (PCA) was applied to the volatile data (Tables 9.2 and 9.3) to discriminate the typical volatile profile of each papaya wine produced by PSF, NSF and MCF. The PCA bi-plot of the quantified major volatile compounds (Table 9.3) is presented as it shows a similar outcome to the PCA result from Table 9.2, indicating the correlation between the quantified and semi-quantified data. The distributions of the various fermentations in the consensus space (Fig. 9.7) indicate differences between the wines and provide information on the volatile compounds responsible for the differences identified. Principal component 1 (PC1) accounted for 96.20% of the total variance, which separated PSF from MCF and NSF, due to the higher concentrations of isobutyl alcohol and acetate esters. Principal component 2 (PC2) distinguished NSF from MCF due to the larger proportions of ethyl esters and 2-phenylethyl alcohol.

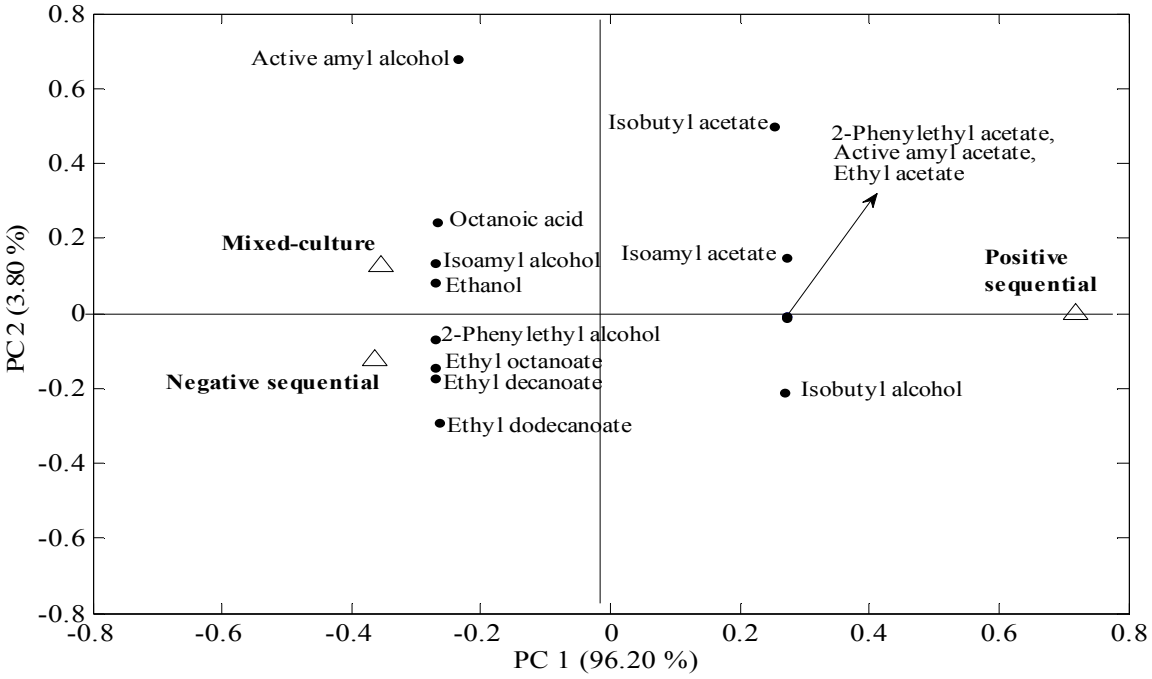

Fig. 9.7. Bi-plot of principal component analysis of the quantified major volatile compounds in papaya wine during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Positive and negative sequential fermentations are defined as in Fig. 9.1.

9.2.4 Sensory characteristics of papaya wine

All the papaya wines were evaluated by experienced panelists and a list of sensory descriptors with more than 40% modified frequency (*MF*) was selected by consensus on the basis of their experience in wine sensory analysis [Appendix G (Table G1)]. The aroma profiles of the papaya wines are represented in a spiderweb diagram as shown in Fig. 9.8. Most of the sensory attributes were similar between NSF and MCF, except for the alcoholic, yeasty and sweet notes that were more noticeable in MCF (Fig. 9.8). The highest level of total alcohols (78.33% RPA) detected in MCF (Table 9.2) may account for the apparent alcoholic note. Wines produced from PSF had more prominent fruity notes than MCF, which was probably due to the high level of esters (43.49% RPA) (Table 9.2). Soden et al. (2000) also revealed that PSF had a different sensory profile from MCF. The results of sensory analysis are in accordance with those found for the volatile compounds identified by GC-MS/FID (Tables 9.2 and 9.3) and PCA (Fig. 9.7). However, there was no statistically significant difference in the overall aroma profiles of all the papaya wines at $p<0.05$, except for alcoholic and fruity notes [Appendix G (Table G2)]. This may be attributed to the large variations in the sensory results and the complex nature of the papaya wine matrix where the non-volatile matrix significantly impacts on the aroma volatility and perception (Guth & Fritzler, 2004).

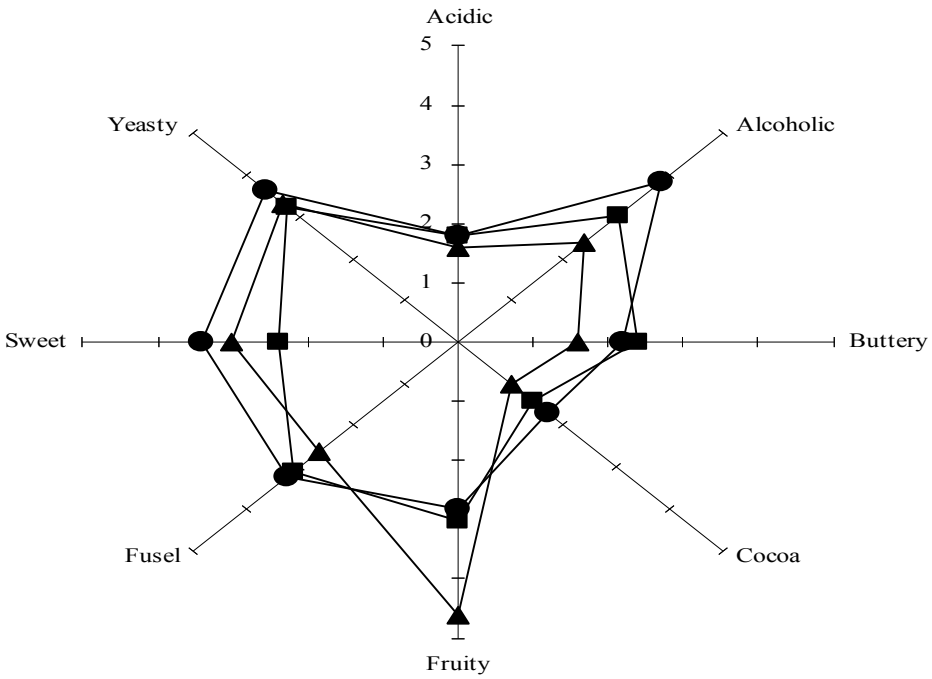

Fig. 9.8. Aroma profile of papaya wines (day 21) fermented with mixed and sequential cultures of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251. Mixed-culture (•); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1.

9.3 Conclusions

In this chapter, fermentation performance, yeast succession and dynamic changes of volatiles were assessed in PSF, NSF and MCF involving *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine fermentation. Most of the fermentation and volatile properties were similar in NSF and MCF, which differed significantly from PSF. Overall, the papaya wine fermented by PSF and NSF had unique flavour and wine quality, where PSF produced larger amounts of acetate esters (ethyl, active amyl, isoamyl, 2-phenylethyl and isobutyl acetates), while NSF produced higher amounts of ethyl esters (ethyl octanoate and dodecanoate) as compared to MCF. The results of this study were promising, but PSF seemed to have not benefited from the *S. cerevisiae* at the inoculum ratio due to early growth arrest of the latter.

CHAPTER 10

YEAST RATIO IS A CRITICAL FACTOR FOR SEQUENTIAL FERMENTATION OF PAPAYA WINE BY *WILLIOPSIS SATURNUS* VAR. *MRAKII* NCYC2251 AND *SACCHAROMYCES CEREVISIAE* VAR. *BAYANUS* R2

10.1 Introduction

Over the years, the use of multistarter cultures in winemaking has gained increasing popularity due to their ability to enhance the complexity of wine flavour through the syngeristic effects from both the *Saccharomyces* and non-*Saccharomyces* yeasts and has advantages over spontaneous and pure *S. cerevisiae* fermentations (Ciani et al., 2006; Rodríguez, Lopes, Barbagelata, Barda, & Caballero, 2010). Generally, the impacts on wine aroma and quality by the multistarter cultures are determined by the strains used and the inoculation strategy (e.g. simultaneous or sequential) (Ciani et al., 2006; Toro & Vazquez, 2002). However, studies reported the limited contribution of non-*Saccharomyces* yeasts belonging to the genera *Candida, Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Torulaspora* and *Williopsis* in simultaneous mixed-culture fermentations due to their early growth arrest (Ciani et al., 2006; Erten & Tanguler, 2010; Jolly, Augustyn, & Pretorius, 2003), while sequential fermentation allowed the persistence of non-*Saccharomyces* yeasts with low fermentative power that would extend or maximise their contact with the juice matrix (Ciani et al., 2006; Clemente-Jimenez et al., 2005).

For these reasons, sequential fermentations of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2 were performed in the previous chapter

(**Chapter 9**). However, the papaya wines produced did not acquire fermentation characteristics from both yeasts due to the early growth arrest and the low inoculum size of *S. cerevisiae* (**Chapter 9**). Hence, in this chapter, the experiment was designed to study sequential fermentation in papaya wine by exploring different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2, relative to the fixed ratio of 1000 to 1 (*W. saturnus*: *S. cerevisiae*) used previously. This chapter reported on the fermentation behaviour and the metabolic interactions of *W. saturnus* and *S. cerevisiae* in sequential fermentation (inoculation of *S. cerevisiae* R2 after 7 days' fermentation with *W. saturnus* NCYC2251) at ratios of 10:1, 1:1 and 1:10 (*W. saturnus*: *S. cerevisiae*) with respect to the production of ethanol and other volatile compounds that would contribute to the organoleptic characteristics of papaya wine.

10.2 Results and discussion

10.2.1 Evolution of biomass and enological properties

The evolution of *W. saturnus* and *S. cerevisiae* is shown in Fig. 10.1. At 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. 10.1). 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 at the 10:1 ratio, while the same yeast stayed almost constant throughout fermentation at the 1:1 and 1:10 ratios. As a consequence, high viable cell densities of both yeasts co-existed at the 1:1 and 1:10 ratios, and there was no early death of *W. saturnus*.

These results differed from those described in the previous chapter (**Chapter 9**) 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 experiment (**Chapter 9**). Conversely, Toro and Vazquez (2002) revealed a sharp decrease of *Candida cantarelli* upon the inoculation of *S. cerevisiae* at 1:1 ratio in sequential fermentation.

Ciani et al. (2010) and Jolly et al. (2006) reported that different interactions could be established between non-*Saccharomyces* and *S. cerevisiae* yeast strains such as mutualism/synergism, amensalism or antagonism and competition. The rapid reduction of *S. cerevisiae* at the 10:1 ratio of *W. saturnus*: *S. cerevisiae* 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 & Tsao, 2009, 2010). *W. saturnus* also exhibits retardation and inhibition against other yeasts such as *Candida kefir* and *Kluvyveromyces marxianus* (Liu & Tsao, 2009, 2010). Guyard et al. (2002) and Takasuka, Komiyama, Furuichi, and Watanabe (1995) reported that the *Williopsis* mycocins inhibit the growth of yeasts by interfering with β-glucan synthesis and thus, disturbing the synthesis of the yeast cell walls. Guyard et al. (2002) also highlighted that the *Williopsis* mycocins have hydrolytic activity against cell wall β-glucan, which disrupts the yeast cell wall integrity and thus, resulting in cell lysis and death. On the other hand, the persistence of both yeasts at 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 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.

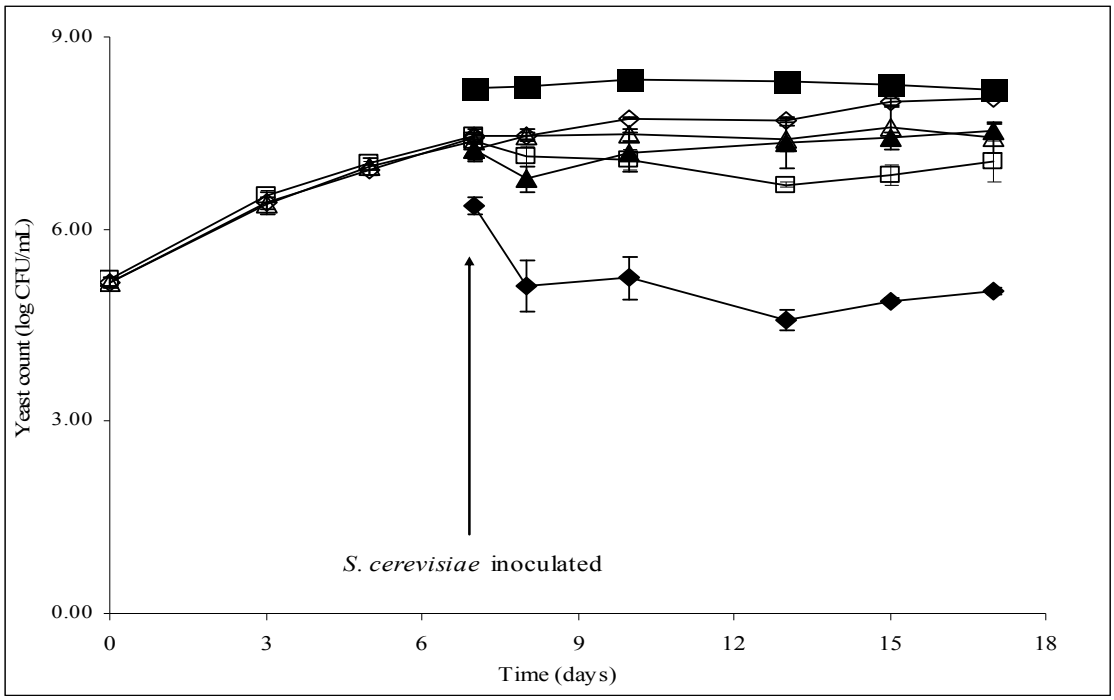

Fig. 10.1. Evolution of viable yeasts in papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. NCYC2251 (◊):R2 (♦) =10:1; NCYC2251 (Δ):R2 (▲) =1:1; NCYC2251 (□):R2 (■) =1:10. (Error bars = standard deviation).

Total soluble solids (°Brix), sugar consumption, organic acids, ethanol and pH changes are presented in Fig. 10.2 and Table 10.1. In all the fermentations, the pH values did not change significantly with values maintaining around 3.53 to 3.56, while the organic acids decreased with the exception for acetic, oxalic, pyruvic and succinic acids that either increased moderately or remained unchanged (Table 10.1). These organic acids may be derived from sugar, amino acid or fatty acid metabolism during yeast metabolism (Boulton, Singleton, & Bisson, 1996) and play important roles in the physical, chemical and microbiological stability of wines besides providing taste-

sensory property to consumers (Swiegers et al., 2008). The ^{o}Brix values at both 1:1 and 1:10 ratios displayed rapid reductions after inoculation of *S. cerevisiae*, which corresponded to the sugar consumption rates and reached the final ^{o}Brix values of around 3.65-3.71% (Fig. 10.2, Table 10.1). The 10:1 ratio, on the other hand, had a gradual reduction in the ^{o}Brix value and sugar consumption rate (Fig. 10.2).

Generally, 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 10.1). Among the fermentations, the 1:10 ratio produced papaya wine with the highest ethanol content of 3.97% (v/v) (Table 10.1). This was in agreement with the highest sugar consumption and the high *S. cerevisiae* yeast count at the ratio of 1:10 (Figs. 10.1 and 10.2). The higher ethanol content at 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, Kielland-Brandt, Nielsen, & Villadsen, 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 to 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).

Table 10.1. Physicochemical parameters of papaya wine (day 17) fermented with sequential cultures of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2 at different ratios (*W. saturnus*: *S. cerevisiae*)

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	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 (%, v/v)	0.01 ± 0.00[a]	1.38 ± 0.08[b]	3.83 ± 0.20[c]	3.97 ± 0.20[c]
Sugars (g/100 mL)				
Fructose	4.16 ± 0.20[a]	2.26 ± 0.60[b]	N.D.	N.D.
Glucose	4.61 ± 0.21[a]	1.19 ± 0.73[b]	N.D.	N.D.
Organic acids (g/100 mL)				
Acetic acid	N.D.	0.045 ± 0.005[a]	0.067 ± 0.002[b]	0.083 ± 0.004[c]
Citric acid	0.451 ± 0.020[a]	0.290 ± 0.013[b]	0.342 ± 0.022[c]	0.339 ± 0.015[c]
Malic acid	0.550 ± 0.034[a]	0.411 ± 0.018[bc]	0.430 ± 0.011[c]	0.371 ± 0.025[b]
Oxalic acid	0.004 ± 0.000[a]	0.007 ± 0.001[b]	0.005 ± 0.000[a]	0.007 ± 0.001[b]
Pyruvic acid	0.086 ± 0.010[a]	0.088 ± 0.001[a]	0.097 ± 0.001[a]	0.089 ± 0.007[a]
Succinic acid	0.317 ± 0.019[a]	0.409 ± 0.005[b]	0.278 ± 0.008[a]	0.368 ± 0.022[c]
Tartaric acid	0.090 ± 0.005[a]	0.077 ± 0.001[b]	0.034 ± 0.001[c]	0.039 ± 0.004[c]

Abbreviation: N.D. = not detected.

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

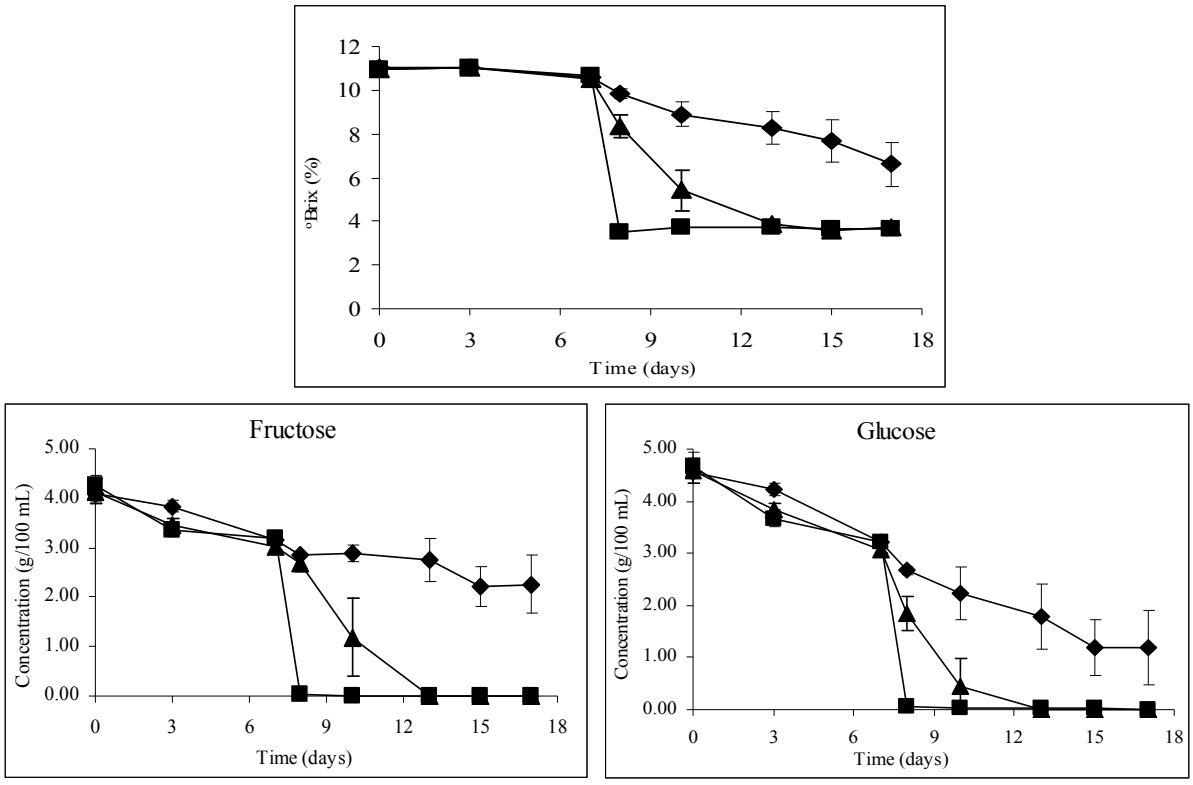

Fig. 10.2. Changes of °Brix and sugars during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

10.2.2 Evolution of volatiles and aroma qualities of papaya wines

Numerous volatiles (e.g. alcohols, aldehydes, esters, fatty acids, terpenoids and ketones) contributing to the sensory properties of papaya wine were produced and further transformed by the different ratios of *W. saturnus* and *S. cerevisiae* (Tables 10.2 and 10.3). Selected major volatiles in the final papaya wines were quantified (Table 10.3). Some of these volatiles increased continuously, while others increased initially and then remained unchanged or declined gradually [Figs. 10.3-10.7, Appendix H (Figs. H1-H7)]. Volatiles that were initially present, especially fatty acids, sulphur-containing compound and esters (e.g. butyric acid, benzyl isothiocyanate and methyl butyrate) responsible for the typical papaya flavour (Pino et al., 2003), were metabolised to trace levels [Figs. 10.3-10.7, Tables 10.2 and 10.3, Appendix H (Figs. H1-H7)].

Volatile fatty acids belong to one of the important groups of volatiles produced by yeasts, which would contribute to the complexity of wine at low levels but impart an unpleasant odour at high concentrations (Swiegers & Pretorius, 2005). The dynamic changes of the volatile fatty acids were similar in all the fermentations, where the fatty acids 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, except for butyric acid that was metabolised [Fig. 10.3, Appendix H (Fig. H1)]. The sequential fermentation at 1:1 ratio produced the highest amounts of most fatty acids including C8, C10, C12 and C14, except for acetic, isobutyric, hexanoic and benzoic acids [Fig. 10.3, Tables 10.2 and 10.3, Appendix H (Fig. H1)]. The 1:10 ratio would have been expected to produce the most C8, C10, C12 and C14 fatty acids, given that *S. cerevisiae* is known to be the main producer of these acids (**Chapters 4 and 8**). 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), followed by the 1:1 and 10:1 ratios with 872.17 mg/L and 494.15 mg/L of acetic acid, respectively (Table 10.3), which were in line with the acetic acid results obtained by HPLC (Table 10.1). This could be in part due to the hydrolysis by *S. cerevisiae* of some acetate esters such as ethyl acetate produced by *W. saturnus*. Conversely, the least amount of acetic acid produced by the 10:1 ratio could be due to the conversion of acetic acid to acetyl-CoA and utilization of acetyl-CoA by the dominant *W. saturnus* yeast to generate higher amounts of acetate esters (Tables 10.2 and 10.3). The high level of acetic acid produced in all fermentations (Table 10.3), especially those at the 1:1 and 1:10 ratios 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 this study differed from those of Kapsopoulou et al. (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 Kapsopoulou et al. (2007). Nevertheless, Bely et al. (2008) reported lower effects on the reduction of acetic acid by a sequential culture of *T. delbrueckii* and *S. cerevisiae* as compared to co-fermentation.

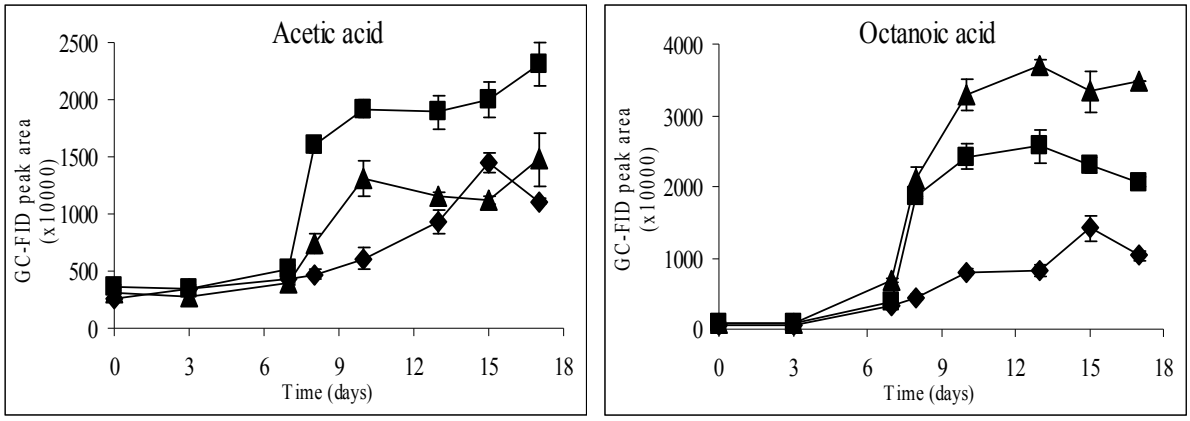

Fig. 10.3. Changes of acetic and octanoic acids during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

Among the volatiles, alcohols were the major compounds produced with relative peak areas (RPA) of 67.75-76.92% (Table 10.2). Ethanol alone made up more than 90% of the volatiles under the alcohol group with the remaining being higher alcohols (Table 10.2). The dynamic 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. 10.4, Appendix H (Fig. H2)]. The substantial decrease in the alcohols concentration after their formation may be due to the rapid utilisation of these alcohols as substrates for ester formation (Park et al., 2009). 2-Ethylhexanol indigenous to the juice was utilised by the yeasts [Appendix H (Fig. H2)].

The sequential fermentation at 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 10.1-10.3). The 1:10 ratio produced significantly higher concentrations of these alcohols than the 1:1 ratio (Tables 10.2 and 10.3). This could be ascribed to the higher

inoculum level and viable yeast count of *S. cerevisiae* (Fig. 10.1), and its higher metabolic ability to produce higher alcohols (**Chapter 8**). Among the higher alcohols, 2-phenylethyl alcohol exceeded its corresponding odour threshold value of 10 mg/L (Bartowsky & Pretorius, 2009), especially for the 1:10 ratio with 64.47 mg/L of 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 increased contents of esters possess an enhanced fruity flavour that could be improved if the higher alcohol contents were to decrease (Moyano, Moreno, Millan, & Medina, 1994). A new sulphur-containing alcohol, 2-(methylthio)ethanol, was produced in all fermentations especially at 1:1 and 1:10 ratios (Fig. 10.4), which is reported for the first time in papaya wine and could be derived from L-methionine catabolism by the yeasts. This volatile sulphur compound has been commonly detected in other wines such as white wines, Tinta Negra Mole red wine and Italian sparkling wines (Fedrizzi, Magno, Finato, & Versini, 2010; Perestrelo, Fernandes, Albuquerque, Marques, & Câmara, 2006). The heavy sulphur compound cannot be eliminated and may impart French bean and cauliflower-like aroma to wine near its flavour threshold of 250 μg/L (Darriet, Lavigne-Cruège, & Tominaga, 1999). However, Perestrelo et al. (2006) reported that most of the sulphur 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.

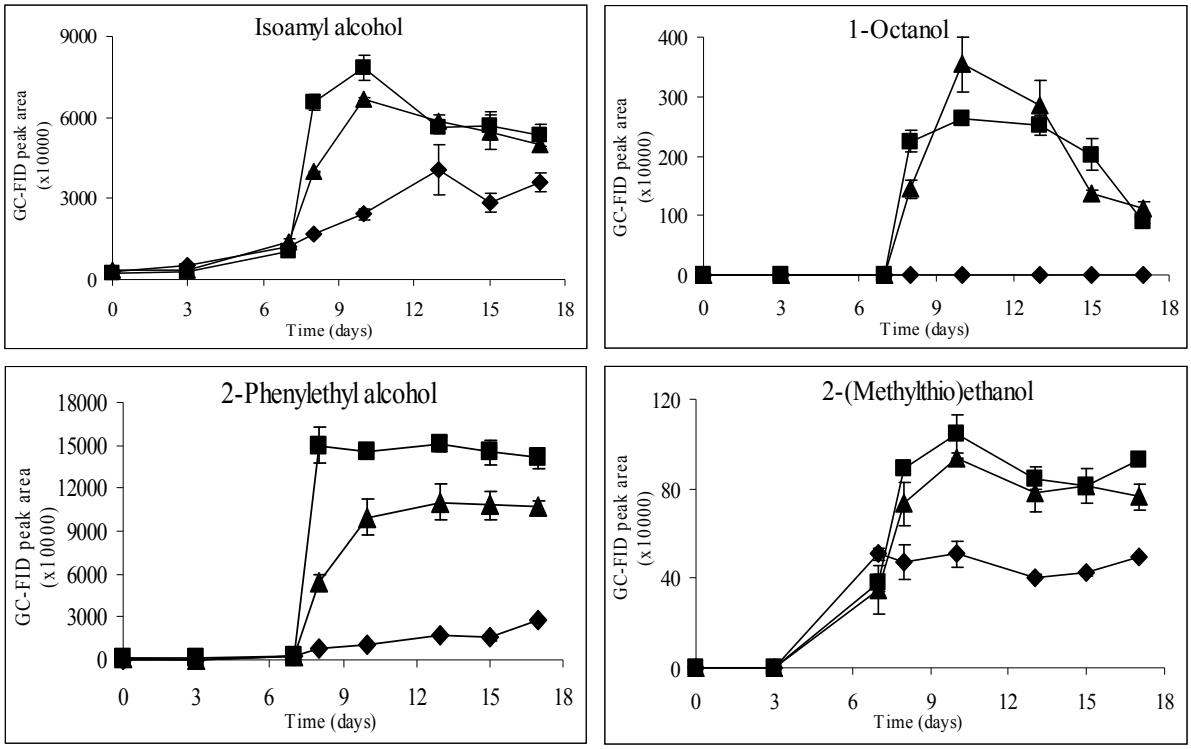

Fig. 10.4. Changes of higher alcohols and 2-(methylthio)ethanol during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

Esters constitute the other major fermentation-derived volatiles (21.56-30.13% RPA) (Table 10.2) that are formed by yeasts via enzymatic condensation of alcohol and CoA-activated acid/acetyl-CoA (Park et al., 2009). These esters included acetate esters, ethyl esters, methyl esters and other medium to long-chain esters (Tables 10.2 and 10.3). The dynamic changes of esters varied with the ester type. Most of the acetate esters increased substantially during the initial stage of fermentation and decreased sharply upon the inoculation of *S. cerevisiae*, except for ethyl acetate, butyl acetate, isoamyl acetate and 2-phenylethyl acetate at the 10:1 and 1:1 ratios [Fig. 10.5, Appendix H (Fig. H3)]. Ethyl, methyl and other esters, on the other hand, increased slowly or remained essentially unchanged 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, except for

isobutyl hexanoate, isobutyl octanoate and 2-phenylethyl octanoate at 10:1 ratio that increased only at day 15 [Fig. 10.6, Appendix H (Figs. H4-H6)]. Methyl butyrate initially present in the papaya juice was metabolised at all the ratios [Appendix H (Fig. H5)]. The evolution and net accumulation of esters in wine is the result of the balance between yeast ester-synthesising enzymes and esterases promoting their hydrolysis in the respective yeasts (Lilly et al., 2006). The results of the current chapter differed from the findings in **Chapter 9**. In the previous chapter (**Chapter 9**), there was no significant modification of esters with the inoculation of *S. cerevisiae* into the papaya wine partially fermented by *W. saturnus*. This was likely due to the low inoculum size of *S. cerevisiae* used in **Chapter 9**. It was reported that the volatiles produced by one of the yeasts can be metabolised by other yeasts (Ciani et al., 2010) and redox interactions existed between yeasts (Cheraiti, Guezenec, & Salmon, 2005).

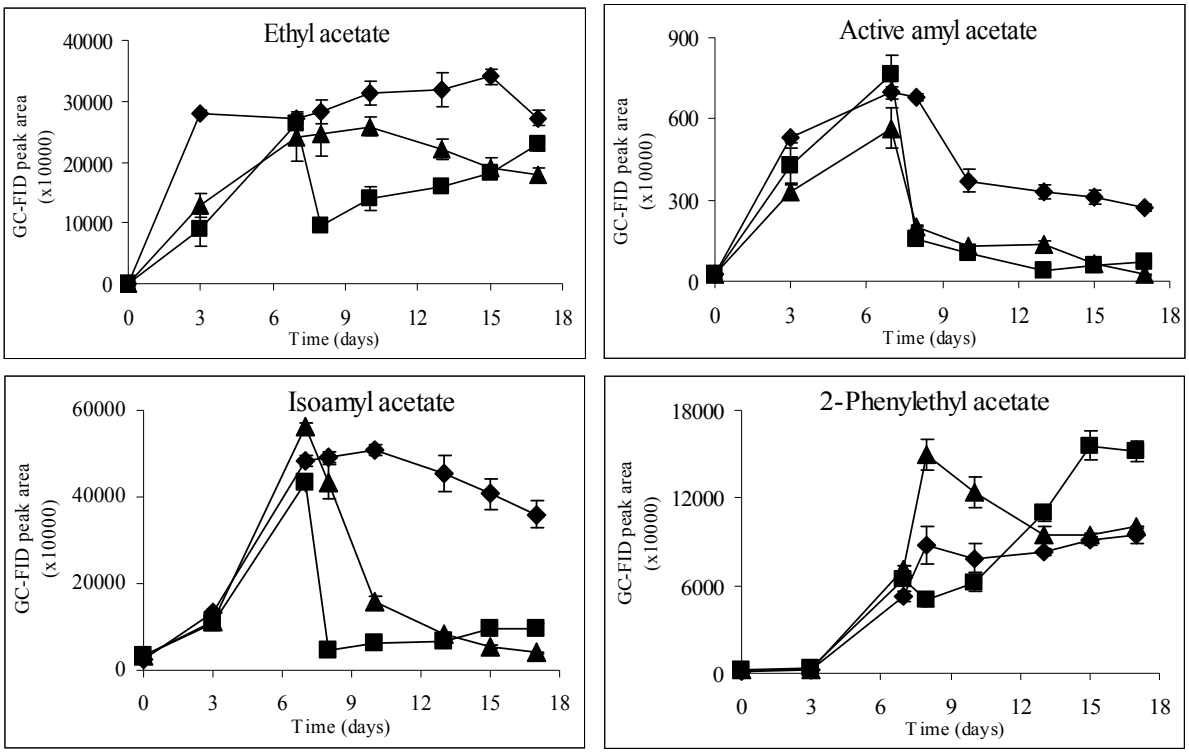

Fig. 10.5. Changes of acetate esters during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

The sequential fermentation at 1:1 ratio produced the highest amounts of ethyl esters, methyl and other miscellaneous esters, except for ethyl butyrate, ethyl hexanoate, ethyl octanoate and acetate esters (Tables 10.2 and 10.3). This correlated with the higher volatile fatty acids production at the 1:1 ratio (Fig. 10.3, Tables 10.2 and 10.3), which are precursors for ethyl ester formation (Saerens et al., 2006, 2008). The sequential fermentation at 10:1 ratio, on the other hand, produced the highest concentrations of most acetate esters, whereas the 1:10 ratio had the highest amounts of 2-phenylethyl acetate, ethyl hexanoate and ethyl octanoate at 1.96 mg/L, 0.06 mg/L and 1.62 mg/L, respectively (Tables 10.2 and 10.3). The high viable yeast population of *W. saturnus* against *S. cerevisiae* at 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 at 10:1 ratio (Tables 10.2 and 10.3), 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).

S. 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* cell count did not produce the uppermost amount of most ethyl esters (Tables 10.2 and 10.3). This could be due to the co-existence of both yeasts at 1:10 ratio (Fig. 10.1), which may modulate the ester formation capability of *S. cerevisiae*. This suggestion is supported by the findings in Cheraiti et al. (2005) in that one species or strain in mixed-culture fermentation may impact on the metabolic behaviour of another strain.

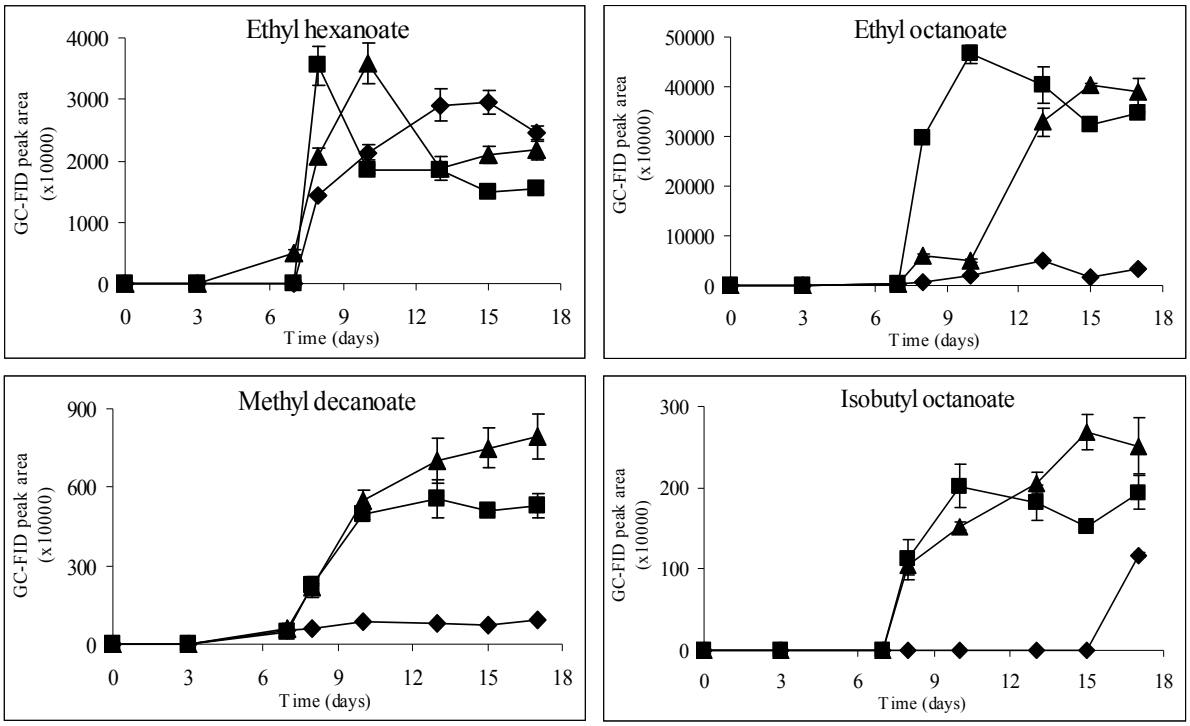

Fig. 10.6. Changes of ethyl esters, methyl decanoate and isobutyl octanoate during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

Ethyl hexanoate and ethyl octanoate were reported as the odour–active compounds in papaya wine (Pino & Queris, 2011). The concentrations of these ethyl esters at 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 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, Yu, Curran, & Liu, 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 10.3).

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) exerts a solvent-like aroma (Etievant, 1991).

Other volatile compounds including aldehydes, ketone, terpenoids and benzyl isothiocyanate were also present in the papaya wines (Tables 10.2 and 10.3). Most of these volatile compounds were metabolised to trace levels, except for acetaldehyde, O-tolualdehyde, terpenoids and 3-hydroxy-2-butanone (acetoin), which were produced [Fig. 10.7, Tables 10.2 and 10.3, Appendix H (Fig. H7)]. These volatiles generally remained stable or decreased slightly 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, except for 3-hydroxy-2-butanone, β-citronellol and citronellyl acetate were not formed at 10:1 ratio [Fig. 10.7, Appendix H (Fig. H7)].

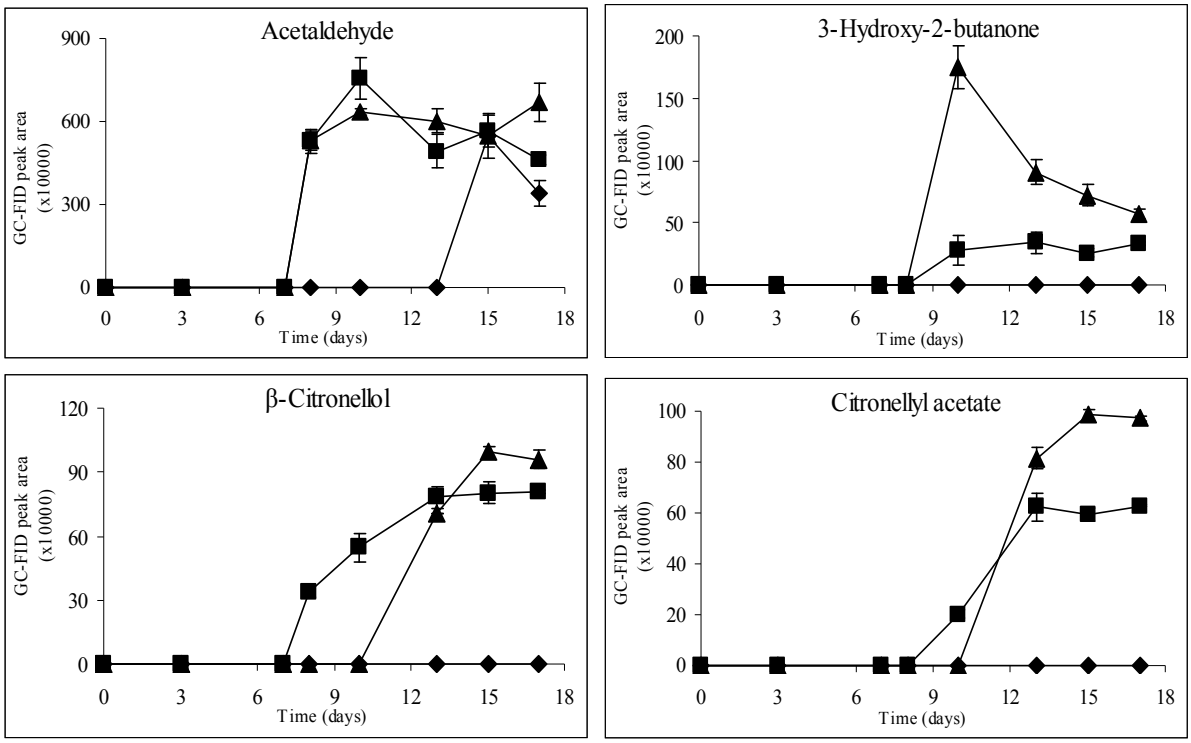

Fig. 10.7. Changes of acetaldehyde, 3-hydroxy-2-butanone and terpenoids during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

The sequential fermentation at 1:1 ratio consistently produced the highest amount of acetaldehyde and 3-hydroxy-2-butanone (Fig. 10.7, Table 10.2). The concentrations of these volatiles in the wines were dependent on the yeast cultures (pure or multistarter) used in the alcoholic fermentation (Bely et al., 2008; Ciani et al., 2006; Toro & Vazquez, 2002) and the accumulation of these by-products can have a negative effect on wine. Ciani et al. (2010) highlighted the reduction of these volatiles in several sequential fermentations, where the actively fermenting *S. cerevisiae* yeast strain can metabolise these volatiles produced by the non-*Saccharomyces* yeasts. The results of this study correlated with Ciani et al. (2010), where there was inverse correlation between the production of these volatiles and the inoculum size of *S. cerevisiae* in the sequential fermentation at 1:1 and 1:10 ratios (Fig. 10.7).

Similarly, the sequential fermentation at 1:1 ratio produced the highest amount of β-citronellol and citronellyl acetate, but there was no production of the terpenoids at 10:1 ratio (Fig. 10.7). The production of β-citronellol and citronellyl acetate at 1:1 and 1:10 ratios could be due to *S. cerevisiae* that released β-citronellol from glycosides through enzymatic hydrolysis or transformed from geraniol and nerol (Mateo & Jiménez, 2000), and followed by transformation of citronellol and acetyl-CoA by the yeasts to yield citronellyl acetate. As a consequence, the papaya wines produced by the 1:1 and 1:10 ratios may be expected to acquire positive flavour attributes from β-citronellol and citronellyl acetate (e.g. citronella, rose and fruity notes) due to their low flavour threshold of 0.08 mg/L and 0.25 mg/L, respectively (Bartowsky & Pretorius, 2009; Yamamoto et al., 2004).

Table 10.2. Major volatile compounds (GC-FID peak area x 10^6) and their relative peak areas (RPA) identified in papaya wine (day 17) fermented with sequential cultures of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2 at different ratios (*W. saturnus*: *S. cerevisiae*)

			Day 0		Ratio 10:1		Ratio 1:1		Ratio 1:10		
No.	**Compounds**	**LRI[e]**	**Peak area**	**RPA (%)**	**Peak area**	**RPA (%)**	**Peak area**	**RPA (%)**	**Peak area**	**RPA (%)**	**Organoleptics[f]**
	Acids										
1	Acetic acid[3]	1454	3.18 ± 0.15[a]	2.02	11.10 ± 0.32[b]	0.39	14.70 ±2.30[c]	0.27	23.10 ± 1.85[d]	0.46	Acidic, pungent, vinegar-like
2	Isobutyric acid	1568	0.00 ± 0.00[a]	0.00	0.87 ± 0.08[b]	0.03	0.55 ± 0.02[c]	0.01	1.69 ± 0.19[d]	0.03	Acidic, cheese, rancid
3	Butyric acid[2]	1628	57.20 ± 1.86[a]	36.42	3.40 ± 0.13[b]	0.12	0.00 ± 0.00[c]	0.00	0.00 ± 0.00[c]	0.00	Acidic, buttery, cheesy
4	Hexanoic acid[1,4]	1846	2.51 ± 0.15[a]	1.60	4.49 ± 0.46[b]	0.16	3.53 ± 0.21[c]	0.06	3.62 ± 0.39[c]	0.07	Acidic, cheesy, fruity
5	Benzoic acid	2455	0.22 ± 0.01[a]	0.14	0.29 ± 0.01[b]	0.01	0.29 ± 0.05[b]	0.01	0.45 ± 0.00[c]	0.01	Balsamic, faint
6	Octanoic acid[1,4]	2062	0.76 ± 0.03[a]	0.48	10.30 ± 0.60[b]	0.36	34.80 ± 0.01[c]	0.64	20.60 ± 0.62[d]	0.41	Acidic, cheesy, fatty, sweaty
7	9-Decenoic acid	2338	0.00 ± 0.00[a]	0.00	0.00 ± 0.00[a]	0.00	1.86 ± 0.05[b]	0.03	0.00 ± 0.00[a]	0.00	Creamy, fatty, milky
8	Decanoic acid[4]	2275	0.90 ± 0.03[a]	0.57	5.86 ± 0.31[b]	0.21	30.00 ± 3.33[c]	0.55	13.20 ± 1.11[d]	0.26	Buttery, condensed, milky
9	Dodecanoic acid[3]	2487	0.43 ± 0.03[a]	0.27	3.37 ± 0.30[b]	0.12	4.03 ± 0.26[c]	0.07	1.33 ± 0.08[d]	0.03	Fatty, soapy, waxy
10	Tetradecanoic acid[3]	2699	0.00 ± 0.00[a]	0.00	0.32 ± 0.04[b]	0.01	0.53 ± 0.02[c]	0.01	0.26 ± 0.00[d]	0.01	Fatty, oily, waxy
	Subtotal		**65.20**	**41.51**	**40.00**	**1.42**	**90.29**	**1.66**	**64.25**	**1.28**	
	Alcohols										
11	Ethanol[2]	944	17.60 ± 0.62[a]	11.21	1820 ± 115[b]	64.41	3780 ± 354[c]	69.53	3630 ± 211[c]	72.26	Alcoholic, solventy
12	1-Propanol[3]	1036	0.00 ± 0.00[a]	0.00	2.34 ± 0.11[b]	0.08	5.32 ± 0.43[c]	0.10	5.10 ± 0.08[c]	0.10	Alcoholic, fermented, solventy
13	Isobutyl alcohol[2]	1084	0.00 ± 0.00[a]	0.00	15.10 ± 0.13[b]	0.53	13.50 ± 0.62[c]	0.25	18.40 ± 1.00[d]	0.37	Breathtaking, fermented, whisky
14	Active amyl alcohol[4]	1210	1.22 ± 0.06[a]	0.78	11.60 ± 0.93[b]	0.41	11.60 ± 1.13[b]	0.21	13.00 ± 0.98[b]	0.26	Alcoholic, fermented, fusel
15	Isoamyl alcohol[4]	1222	2.64 ± 0.12[a]	1.68	35.70 ± 3.49[b]	1.26	49.90 ± 1.75[c]	0.92	53.30 ± 4.19[c]	1.06	Alcoholic, fermented, whiskey
16	Benzyl alcohol[3]	1899	0.63 ± 0.03[a]	0.40	0.66 ± 0.02[a]	0.02	0.96 ± 0.07[b]	0.02	0.81 ± 0.06[c]	0.02	Balsamic, floral, rose
17	2-Ethylhexanol	1500	0.93 ± 0.06[a]	0.59	0.21 ± 0.02[b]	0.01	0.00 ± 0.00[c]	0.00	0.00 ± 0.00[c]	0.00	Citrus, fresh, floral

Table 10.2. (Continued)

No.	Compounds	LRI[e]	Day 0		Ratio 10:1		Ratio 1:1		Ratio 1:10		Organoleptics[f]
			Peak area	RPA (%)	Peak area	RPA (%)	Peak area	RPA (%)	Peak area	RPA (%)	
18	1-Octanol[2]	1559	0.00 ± 0.00[a]	0.00	0.00 ± 0.00[a]	0.00	1.12 ± 0.10[b]	0.02	0.90 ± 0.04[c]	0.02	Aldehydic, green, waxy
19	2-Phenylethyl alcohol[4]	1938	1.30 ± 0.00[a]	0.83	28.00 ± 0.65[b]	0.99	107 ± 4.03[c]	1.97	141 ± 6.94[d]	2.81	Floral, honey, rosy
20	1-Decanol[2]	1775	0.00 ± 0.00[a]	0.00	0.60 ± 0.04[b]	0.02	0.71 ± 0.07[c]	0.01	1.33 ± 0.03[d]	0.03	Fatty, floral, waxy
	Subtotal		**24.32**	**15.48**	**1914.21**	**67.75**	**3970.11**	**73.03**	**3863.84**	**76.92**	
	Aldehydes										
21	Acetaldehyde[2]	727	0.00 ± 0.00[a]	0.00	3.41 ± 0.46[b]	0.12	6.67 ± 0.70[c]	0.12	4.63 ± 0.19[d]	0.09	Aldehydic, ethereal, fruity
22	Benzaldehyde[2]	1539	3.74 ± 0.24[a]	2.38	1.46 ± 0.10[b]	0.05	0.68 ± 0.04[c]	0.01	0.68 ± 0.04[c]	0.01	Bitter almond, cherry, sweet
23	O-Tolualdehyde	1668	5.54 ± 0.33[a]	3.53	11.10 ± 0.70[b]	0.39	14.30 ± 1.32[c]	0.26	2.18 ± 0.05[d]	0.04	Bitter almond, cherry pit, sweet
24	2,3-Dimethylbenzalde-hyde	1840	0.87 ± 0.01[a]	0.55	1.54 ± 0.25[b]	0.05	1.50 ± 0.01[b]	0.03	1.03 ± 0.03[c]	0.02	Almond, cherry, vanilla
	Subtotal		**10.15**	**6.46**	**17.51**	**0.62**	**23.15**	**0.43**	**8.52**	**0.17**	
	Esters										
25	Methyl butyrate[2,3]	991	9.69 ± 0.11[a]	6.17	0.00 ± 0.00[b]	0.00	0.00 ± 0.00[b]	0.00	0.00 ± 0.00[b]	0.00	Etherial, fruity, pineapple
26	Methyl octanoate[3]	1376	0.09 ± 0.00[a]	0.06	1.30 ± 0.14[b]	0.05	4.89 ± 0.46[c]	0.09	4.25 ± 0.41[c]	0.08	Citrus, green, fruity
27	Methyl decanoate[3]	1593	0.00 ± 0.00[a]	0.00	0.94 ± 0.09[a]	0.03	7.94 ± 0.87[b]	0.15	5.28 ± 0.47[c]	0.11	Fatty, cognac, oily
28	Methyl dodecanoate[3]	1798	0.39 ± 0.01[a]	0.25	0.69 ± 0.00[b]	0.02	1.06 ± 0.01[c]	0.02	0.44 ± 0.04[a]	0.01	Creamy coconut, waxy
29	Methyl tetradecanoate[3]	2011	0.45 ± 0.02[a]	0.29	0.45 ± 0.02[a]	0.02	0.38 ± 0.03[ab]	0.01	0.30 ± 0.03[c]	0.01	Fatty, petal, waxy
30	Methyl 9-hexadecenoate[3]	2248	0.33 ± 0.02[a]	0.21	0.21 ± 0.01[b]	0.01	0.44 ± 0.01[c]	0.01	0.35 ± 0.02[a]	0.01	-
31	Ethyl butyrate[1]	1029	1.27 ± 0.04[a]	0.81	4.63 ± 0.60[b]	0.16	2.71 ± 0.26[c]	0.05	2.60 ± 0.18[c]	0.05	Fruity, ripe, sweet
32	Ethyl hexanoate[1]	1217	0.00 ± 0.00[a]	0.00	24.60 ± 1.02[b]	0.87	21.70 ± 1.58[c]	0.40	15.60 ± 1.04[d]	0.31	Fruity, pineapple-like, winey
33	Ethyl octanoate[1,4]	1430	0.00 ± 0.00[a]	0.00	34.80 ± 1.35[b]	1.23	390 ± 27.60[c]	7.17	347 ± 1.82[d]	6.91	Fruity, cognac, yeasty
34	Ethyl nonanoate	1532	0.00 ± 0.00[a]	0.00	0.47 ± 0.01[b]	0.02	0.80 ± 0.07[c]	0.01	0.82 ± 0.05[c]	0.02	Fruity, rum, wine

Table 10.2. (Continued)

No.	Compounds	LRI[e]	Day 0 Peak area	Day 0 RPA (%)	Ratio 10:1 Peak area	Ratio 10:1 RPA (%)	Ratio 1:1 Peak area	Ratio 1:1 RPA (%)	Ratio 1:10 Peak area	Ratio 1:10 RPA (%)	Organoleptics[f]
35	Ethyl 9-decenoate	1690	0.00 ± 0.00^{a}	0.00	0.48 ± 0.04^{a}	0.02	49.50 ± 1.31^{b}	0.91	3.87 ± 0.32^{c}	0.08	Fatty, fruity
36	Ethyl decanoate[1]	1638	0.00 ± 0.00^{a}	0.00	31.50 ± 3.89^{b}	1.11	479 ± 24.40^{c}	8.81	194 ± 9.45^{d}	3.86	Fatty, fruity, winey
37	Ethyl dodecanoate[3]	1844	0.00 ± 0.00^{a}	0.00	8.57 ± 0.97^{b}	0.30	40.20 ± 1.47^{c}	0.74	13.70 ± 1.19^{d}	0.27	Fruity, oily, waxy
38	Ethyl tetradecanoate[3]	2050	0.00 ± 0.00^{a}	0.00	0.76 ± 0.01^{b}	0.03	2.41 ± 0.19^{c}	0.04	1.03 ± 0.08^{d}	0.02	Creamy, oily, waxy
39	Ethyl 9-hexadecenoate[3]	2284	0.00 ± 0.00^{a}	0.00	2.10 ± 0.16^{b}	0.07	6.02 ± 0.12^{c}	0.11	4.98 ± 0.43^{d}	0.10	Creamy, waxy
40	Ethyl hexadecanoate[3]	2256	0.00 ± 0.00^{a}	0.00	0.69 ± 0.02^{b}	0.02	2.67 ± 0.20^{c}	0.05	1.39 ± 0.07^{d}	0.03	Creamy, fruity, milky
41	Propyl octanoate	1508	0.00 ± 0.00^{a}	0.00	0.23 ± 0.01^{b}	0.01	0.87 ± 0.01^{c}	0.02	0.76 ± 0.04^{d}	0.02	Coconut, fatty, winey
42	Propyl decanoate	1718	0.00 ± 0.00^{a}	0.00	0.24 ± 0.02^{b}	0.01	0.89 ± 0.00^{c}	0.02	0.29 ± 0.00^{c}	0.01	Fatty, fruity, waxy
43	Isobutyl hexanoate	1342	0.00 ± 0.00^{a}	0.00	0.14 ± 0.00^{b}	0.00	0.40 ± 0.01^{c}	0.01	0.42 ± 0.01^{c}	0.01	Estery, fruity, green apple
44	Isobutyl octanoate	1541	0.00 ± 0.00^{a}	0.00	1.17 ± 0.02^{b}	0.04	2.51 ± 0.35^{c}	0.05	1.94 ± 0.21^{c}	0.04	Fatty, fruity, winey
45	Isoamyl octanoate	1652	0.00 ± 0.00^{a}	0.00	0.40 ± 0.05^{b}	0.01	4.84 ± 0.21^{c}	0.09	2.14 ± 0.19^{d}	0.04	Cognac, fatty, oily
46	Isobutyl decanoate	1749	0.00 ± 0.00^{a}	0.00	0.14 ± 0.01^{b}	0.00	1.24 ± 0.03^{c}	0.02	0.71 ± 0.00^{d}	0.01	Brandy, cognac, oily
47	Isoamyl decanoate	1861	0.00 ± 0.00^{a}	0.00	0.25 ± 0.02^{b}	0.01	1.98 ± 0.12^{c}	0.04	0.85 ± 0.03^{d}	0.02	Cognac, green, waxy
48	2-Phenylethyl octanoate	2394	0.00 ± 0.00^{a}	0.00	0.30 ± 0.02^{b}	0.01	0.64 ± 0.05^{c}	0.01	0.53 ± 0.05^{c}	0.01	Caramellic, cocoa, waxy
49	Methyl acetate	843	0.00 ± 0.00^{a}	0.00	1.20 ± 0.01^{b}	0.04	1.22 ± 0.08^{b}	0.02	1.14 ± 0.09^{b}	0.02	Ethereal, estery, fruity
50	Ethyl acetate[2]	899	0.00 ± 0.00^{a}	0.00	272 ± 12.70^{b}	9.63	180 ± 10.40^{c}	3.31	229 ± 9.58^{d}	4.56	Ethereal, fruity, solventy
51	Butyl acetate	1056	0.00 ± 0.00^{a}	0.00	1.94 ± 0.19^{b}	0.07	0.00 ± 0.00^{a}	0.00	0.00 ± 0.00^{a}	0.00	Banana-like, fruity, sweet
52	Active amyl acetate	1092	0.24 ± 0.01^{a}	0.15	2.71 ± 0.11^{b}	0.10	0.23 ± 0.02^{a}	0.00	0.73 ± 0.05^{c}	0.01	Banana-like, fruity, ripe
53	Isoamyl acetate[1]	1095	32.70 ± 1.91^{a}	20.82	359 ± 31.10^{b}	12.71	41.00 ± 2.70^{a}	0.75	95.50 ± 11.90^{c}	1.90	Banana-like, fruity, sweet
54	Amyl acetate	1149	0.00 ± 0.00^{a}	0.00	0.90 ± 0.05^{b}	0.03	0.00 ± 0.00^{a}	0.00	0.00 ± 0.00^{a}	0.00	Apple, banana-like, ethereal
55	Benzyl acetate	1740	0.26 ± 0.01^{a}	0.17	2.76 ± 0.39^{b}	0.10	1.49 ± 0.09^{c}	0.03	1.33 ± 0.10^{c}	0.03	Floral, fruity, jasmine-like

Table 10.2. (Continued)

No.	Compounds	LRI[e]	Day 0 Peak area	Day 0 RPA (%)	Ratio 10:1 Peak area	Ratio 10:1 RPA (%)	Ratio 1:1 Peak area	Ratio 1:1 RPA (%)	Ratio 1:10 Peak area	Ratio 1:10 RPA (%)	Organoleptics[f]
56	Octyl acetate	1471	0.00 ± 0.00[a]	0.00	0.97 ± 0.15[b]	0.03	1.06 ± 0.01[b]	0.02	0.00 ± 0.00[a]	0.00	Earthy, green, mushroom
57	2-Phenylethyl acetate[1]	1827	1.96 ± 0.07[a]	1.25	94.80 ± 6.10[b]	3.36	100 ± 0.17[b]	1.84	152 ± 6.76[c]	3.03	Floral, rosy, honey
	Subtotal		**47.38**	**30.16**	**851.34**	**30.13**	**1348.09**	**24.80**	**1082.95**	**21.56**	
	Ketones										
58	3-Hydroxy-2-butanone[1]	1317	0.00 ± 0.00[a]	0.00	0.00 ± 0.00[a]	0.00	0.57 ± 0.04[b]	0.01	0.33 ± 0.01[c]	0.01	Buttery, creamy, sweet
59	4-Methyl-2-heptanone	1189	2.58 ± 0.15[a]	1.64	0.62 ± 0.06[b]	0.02	0.00 ± 0.00[c]	0.00	0.00 ± 0.00[c]	0.00	-
60	6-Methyl-5-hepten-2-one[3]	1333	1.26 ± 0.03[a]	0.80	0.00 ± 0.00[b]	0.00	0.00 ± 0.00[b]	0.00	0.00 ± 0.00[b]	0.00	Citrus, lemongrass-like, musty
61	β-Damascenone[4]	1829	1.81 ± 0.04[a]	1.15	0.00 ± 0.00[b]	0.00	0.00 ± 0.00[b]	0.00	0.00 ± 0.00[b]	0.00	Fruity, floral, woody
	Subtotal		**5.65**	**3.60**	**0.62**	**0.02**	**0.57**	**0.01**	**0.33**	**0.01**	
	Terpenoids										
62	β-Citronellol[2]	1773	0.00 ± 0.00[a]	0.00	0.00 ± 0.00[a]	0.00	0.96 ± 0.04[b]	0.02	0.81 ± 0.01[c]	0.02	Citronella, oily, rose
63	Citronellyl acetate[2]	1659	0.00 ± 0.00[a]	0.00	0.00 ± 0.00[a]	0.00	0.97 ± 0.01[b]	0.02	0.63 ± 0.00[c]	0.01	Floral, fruity, rose
	Subtotal		**0.00**	**0.00**	**0.00**	**0.00**	**1.93**	**0.04**	**1.44**	**0.03**	
	Heteroatom (N, S) compounds										
64	2-(Methylthio)ethanol[3]	1547	0.00 ± 0.00[a]	0.00	0.49 ± 0.01[b]	0.02	0.76 ± 0.06[c]	0.01	0.93 ± 0.03[d]	0.02	Meaty, sulfurous
65	Benzyl isothiocyanate[3]	2123	4.37 ± 0.04[a]	2.78	1.26 ± 0.06[bc]	0.04	1.34 ± 0.13[b]	0.02	1.00 ± 0.02[c]	0.02	Horseradish-like, hot, pungent
	Subtotal		**4.37**	**2.78**	**1.75**	**0.06**	**2.10**	**0.04**	**1.93**	**0.02**	
	Total		**157.07**		**2825.43**		**5435.27**		**5022.63**		

[a,b,c,d]Statistical analysis at 95% confidence level with same letters indicating no significant difference.
[e]Experimentally determined linear retention index on the DB-FFAP column, relative to C5-C40 hydrocarbons.
[f]Odour descriptions obtained from Luebke (1980).
[1,2,3,4]Retention index in agreement with those in the literature [Duarte et al. (2010), Goodner (2008), Pino et al. (2003) and Segurel et al. (2009), respectively].

Table 10.3. Concentrations of selected major volatile compounds (mg/L) in papaya wine (day 17) fermented with sequential cultures of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2 at different ratios (*W. saturnus*: *S. cerevisiae*).

Compounds quantified	Day 0		Ratio 10:1		Ratio 1:1		Ratio 1:10		Odor threshold[e] (mg/L)
	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	
Acids									
Acetic acid	47.66 ± 0.09[a]	0.17	494.15 ± 17.23[b]	1.76	872.17 ± 25.91[c]	3.11	991.64 ± 88.89[d]	3.54	280
Isobutyric acid	0.00 ± 0.00[a]	0.00	0.16 ± 0.01[b]	0.02	0.10 ± 0.00[b]	0.01	0.42 ± 0.05[c]	0.05	8.10[i]
Butyric acid	11.27 ± 1.19[a]	5.12	2.46 ± 0.23[b]	1.12	0.50 ± 0.02[c]	0.23	0.56 ± 0.03[c]	0.25	2.20
Hexanoic acid	0.29 ± 0.02[a]	0.04	1.81 ± 0.14[b]	0.23	1.56 ± 0.53[b]	0.20	2.02 ± 0.23[b]	0.25	8.00
Benzoic acid	3.88 ± 0.25[a]	-	7.72 ± 0.44[b]	-	5.06 ± 0.05[c]	-	6.41 ± 0.62[d]	-	-
Octanoic acid	0.08 ± 0.01[a]	0.01	0.57 ± 0.05[b]	0.06	0.76 ± 0.07[c]	0.09	0.58 ± 0.06[b]	0.07	8.80
Decanoic acid	0.26 ± 0.00[a]	0.04	0.46 ± 0.04[b]	0.08	0.89 ± 0.09[c]	0.15	0.56 ± 0.05[b]	0.09	6.00
Dodecanoic acid	0.69 ± 0.00[a]	0.69	0.87 ± 0.04[b]	0.87	0.89 ± 0.04[b]	0.89	0.78 ± 0.01[c]	0.78	1.00[h]
Alcohols									
Ethanol	50.32 ± 2.76[a]	-	10924 ± 665[b]	-	30230 ± 1595[c]	-	31333 ± 1553[c]	-	-
Isobutyl alcohol	0.00 ± 0.00[a]	0.00	1.31 ± 0.06[b]	0.03	1.99 ± 0.14[c]	0.05	2.82 ± 0.27[d]	0.07	40.00
Active amyl alcohol	0.00 ± 0.00[a]	0.00	0.11 ± 0.02[b]	0.00	0.79 ± 0.16[c]	0.01	0.63 ± 0.05[c]	0.01	65.00
Isoamyl alcohol	0.00 ± 0.00[a]	0.00	1.19 ± 0.11[b]	0.04	2.12 ± 0.15[c]	0.07	2.16 ± 0.16[c]	0.07	30.00
2-Phenylethyl alcohol	0.00 ± 0.00[a]	0.00	14.85 ± 1.72[b]	1.49	39.72 ± 2.80[c]	3.97	64.47 ± 4.20[d]	6.45	10.00
Aldehydes									
Benzaldehyde	0.03 ± 0.00[a]	0.01	0.01 ± 0.00[b]	0.00	0.00 ± 0.00[c]	0.00	0.00 ± 0.00[c]	0.00	3.50[f]
O-Tolualdehyde	0.01 ± 0.00[a]	-	0.07 ± 0.00[b]	-	0.04 ± 0.00[c]	-	0.01 ± 0.00[a]		-
Esters									
Ethyl hexanoate	0.00 ± 0.00[a]	0.00	0.02 ± 0.00[b]	0.32	0.05 ± 0.00[c]	0.96	0.06 ± 0.01[c]	1.18	0.05
Ethyl octanoate	0.00 ± 0.00[a]	0.00	0.15 ± 0.03[b]	7.50	1.52 ± 0.07[c]	76.00	1.62 ± 0.04[c]	81.00	0.02
Ethyl decanoate	0.00 ± 0.00[a]	0.00	0.14 ± 0.03[a]	0.70	1.64 ± 0.04[b]	8.20	1.17 ± 0.18[c]	5.85	0.20
Ethyl dodecanoate	0.00 ± 0.00[a]	0.00	0.96 ± 0.01[b]	0.80	1.47 ± 0.21[c]	1.23	1.29 ± 0.03[c]	1.08	1.20[g]

Table 10.3. (Continued)

Compounds quantified	Day 0		Ratio 10:1		Ratio 1:1		Ratio 1:10		Odor threshold[e] (mg/L)
	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	
Ethyl tetradecanoate	0.00 ± 0.00[a]	0.00	0.10 ± 0.00[b]	0.13	0.15 ± 0.01[c]	0.19	0.07 ± 0.01[d]	0.09	0.80[h]
Isobutyl octanoate	0.00 ± 0.00[a]	0.00	0.03 ± 0.00[b]	0.04	0.04 ± 0.00[b]	0.05	0.04 ± 0.00[b]	0.05	0.80[h]
Isoamyl octanoate	0.00 ± 0.00[a]	0.00	0.03 ± 0.00[b]	0.24	0.26 ± 0.01[c]	2.08	0.23 ± 0.01[d]	1.84	0.125[g]
Ethyl acetate	0.00 ± 0.00[a]	0.00	267.32 ± 31.90[b]	35.64	208.02 ± 29.76[b]	27.74	214.14 ± 4.42[b]	28.55	7.50
Isoamyl acetate	0.00 ± 0.00[a]	0.00	1.02 ± 0.02[b]	34.00	0.26 ± 0.01[c]	8.67	0.52 ± 0.05[d]	17.33	0.03
2-Phenylethyl acetate	0.00 ± 0.00[a]	0.00	1.64 ± 0.12[b]	6.56	1.49 ± 0.10[b]	5.96	1.96 ± 0.29[c]	7.84	0.25

Abbreviation: OAV = Odour activity values calculated by dividing concentration by the odour threshold value of the compound.

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

[e,f,g,h,i]Odour thresholds collated from literatures ([e]Bartowsky and Pretorius (2009), [f]Buttery, Teranishi, Ling, and Turnbaugh (1990), [g]Ferreira et al. (2000), [h]Li, Tao, Wang, and Zhang (2008) and [i]Salo (1970), respectively).

10.2.3 Principal component analysis

Principal component analysis (PCA) was applied to the volatile compounds from Tables 10.2 and 10.3 to discriminate the common characteristics as well as to reveal the diversity in the volatile composition among the papaya wines produced by the different yeast ratios in sequential fermentation. In general, both the PCA results showed similar trends and thus, the PCA result of the quantified major volatile compounds (Table 10.3) is presented in Fig. 10.8.

The PCA result indicates distinctive volatile compositions and clear separation among the papaya wines (Fig. 10.8). The first principal component (PC1) accounted for 69.90% of the total variance that characterised the distinction of the 10:1 ratio from the other ratios, while PC2 explained the remaining 30.10% that separated the 1:1 ratio from the 1:10 ratio.

The papaya wine produced by the sequential fermentation at 10:1 ratio was mainly characterised by ethyl acetate and those volatiles associated with papaya juice (e.g. butyric acid and benzaldehyde). Conversely, the sequential fermentation at 1:1 ratio, positioned on the negative semi-axes, was associated with more fatty acids and ethyl esters such as octanoic acid, decanoic acid, ethyl decanoate, ethyl dodecanoate and ethyl tetradecanoate. The papaya wine produced by sequential fermentation at 1:10 ratio (upper-left quadrant) was distinguished with a high percentage of acetic acid, ethyl hexanoate, ethyl octanoate, isobutyl octanoate, ethanol and higher alcohols.

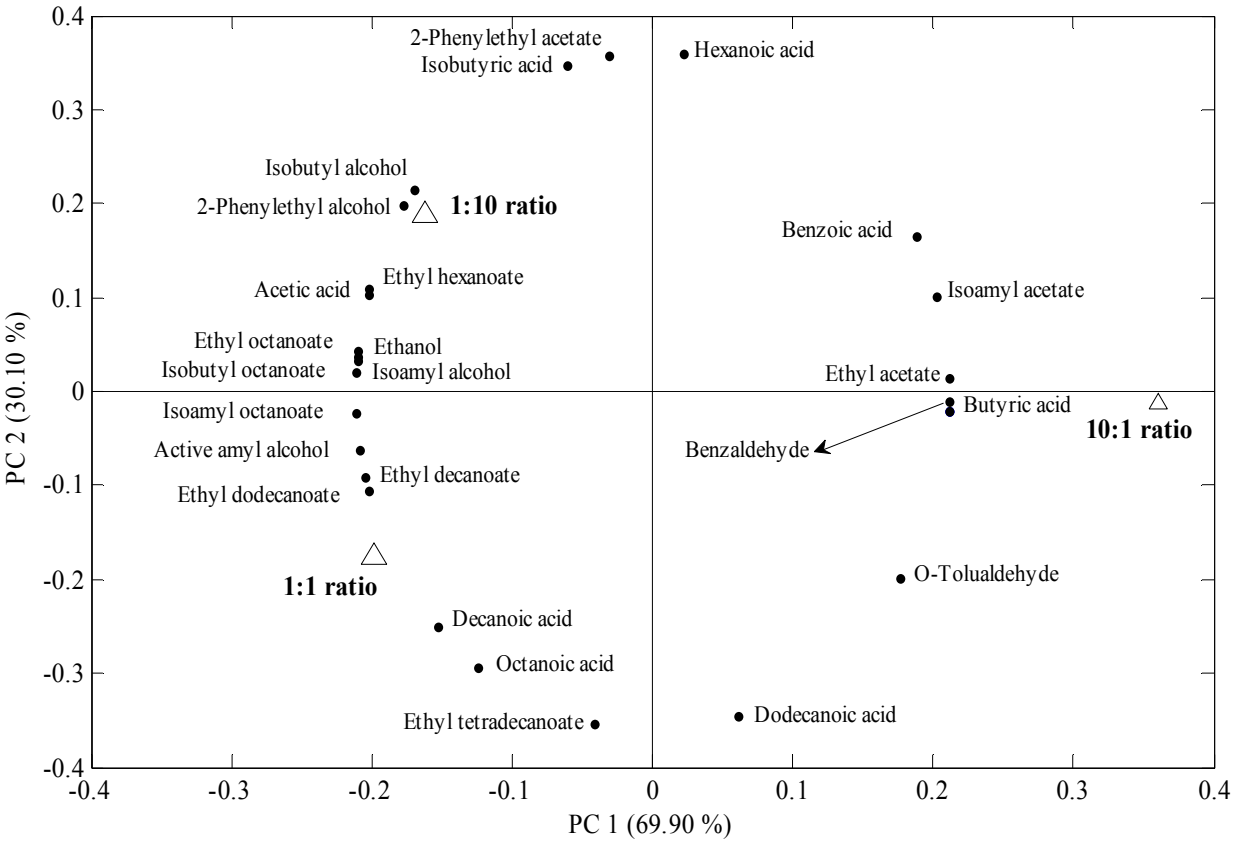

Fig. 10.8. Bi-plot of principal component analysis of the quantified major volatile compounds in papaya wines fermented by sequential cultures of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2 at different ratios (*W. saturnus*: *S. cerevisiae*).

10.2.4 Sensory characteristics of papaya wine

The three papaya wines were evaluated by sensory descriptive analysis using a list of sensory descriptors (>49.05% *MF*) [Appendix H (Table H1)] that was selected by consensus on the basis of the panelists' experience in wine sensory analysis. Generally, the 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. 10.9). The ratio 10:1 was considered to be slightly fruitier than the other ratios, which could be attributed to the higher amount of acetate esters formed (Tables 10.2 and 10.3).

Wine produced by the 1:10 ratio had more noticeable yeasty, sweet and fusel notes than the 10:1 ratio, which was probably due to the high levels of 2-phenylethyl acetate, ethyl esters and higher alcohols (Tables 10.2 and 10.3). On the other hand, the wine produced by the 1:1 ratio possessed less buttery and cocoa notes regardless of the significant amounts of 3-hydroxy-2-butanone and decanoic acid detected (Tables 10.2 and 10.3). Similarly, lower acidity was perceived in the 1:1 and 1:10 ratios as compared to 10:1 ratio, despite the significantly higher amounts of acetic acid present in former two ratios (Tables 10.1 and 10.3). These sensory discrepancies could be due to the complex interaction among the volatile compositions in wine, which led to the masking or suppression by the higher odour-active fruity esters.

Generally, there were no significant differences in the aroma profiles in all the papaya wines regardless of the different ratios [Appendix H (Table H2)], which differed from the volatile compounds (Tables 10.2 and 10.3) and PCA result (Fig. 10.8). This might be attributed to the complex nature of the papaya wine matrix where the non-volatile compounds such as phenolic compounds, organic acids and carbohydrates, or other volatile compounds that significantly impact on the aroma volatility and perception (Guth & Fritzler, 2004). Pineau, Barbe, Van Leeuwen, and Dubourdieu (2009) also pointed out that wine sensory attributes may be the result of interactions between multiple compounds, rendering prediction of aroma proportionally based on compounds present *per se* being inappropriate. Furthermore, not all sensory descriptors can be explained by the studied volatile compounds (Vilanova, Genisheva, Masa, & Oliveira, 2010) and the use of humans as measuring instruments can be subjective due to biasness and variation which exists between individuals (Meilgaard, Civille, & Carr, 1999).

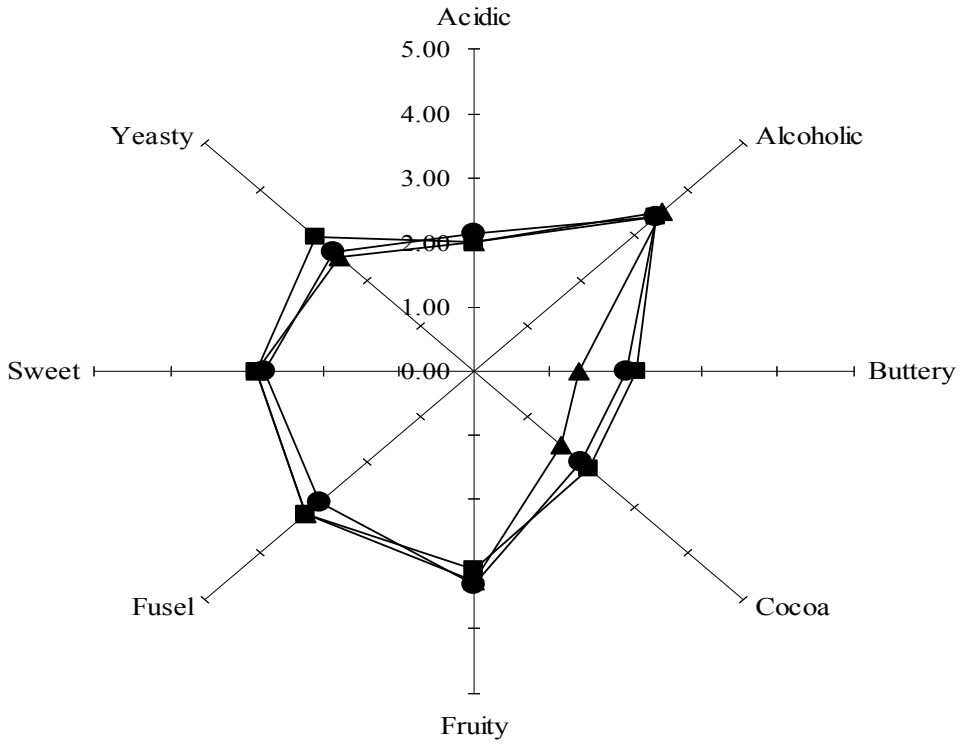

Fig. 10.9. Aroma profile of papaya wines (day 17) fermented with different ratio of sequential cultures of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (●); 1:1 ratio (▲); 1:10 ratio (■).

10.3 Conclusions

In this chapter, the impact of yeast ratio on yeast succession, fermentation performance and volatile formation was assessed during papaya wine fermentation by sequentially inoculating *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. Overall, the ratio of *W. saturnus* NCYC2251 to *S. cerevisiae* R2 was crucial for the survival of yeasts that had significant impacts on the production of a plethora of volatile compounds such as alcohols, fatty acids, esters and terpenoids. Among the yeast ratios, the 1:1 and 1:10 ratios enabled the co-existence of both yeasts and enhanced the production of desirable volatile compounds through synergistic effects. 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 10:1 ratio, on the other hand, was dominated by *W. saturnus* and produced papaya wine with elevated concentrations of acetate esters. 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 and merits further research on the enhancement of their positive attributes while mitigating its shortcomings.

CHAPTER 11

GENERAL CONCLUSIONS AND FUTURE WORK

11.1 General conclusions

S. cerevisiae was the principal yeast responsible for the production of alcohols, ethyl esters, medium to long-chain fatty acids and terpenoids. The three strains of *S. cerevisiae* namely EC-1118, R2 and MERIT.ferm exhibited similar dynamic changes in oenological properties and volatile compounds production. Nevertheless, strain R2 had better production profile of ethanol and higher alcohols as compared to the other two yeast strains, which are essential precursors for esters formation.

W. saturnus was a poor ethanol producer, but modulated the papaya wine fermentation through production of relatively high amounts of fruity or floral acetate esters. Strain differentiation existed amongst strains of the *Williopsis* yeast with regard to the production of volatile compounds. Strain NCYC2251 produced the utmost amount of methyl esters, fatty acids and ethyl dodecanoate, followed by strain NCYC22 with the highest amount of most acetate esters and ethyl esters, and strain NCYC2727 produced the highest amount of ethyl hexanoate, 2-phenylethyl alcohol and acetic acid.

The production of volatile compounds by *W. saturnus* var. *mrakii* NCYC2251 was further modulated through the supplementation of flavour precursors (fusel oil or selected amino acids). *W. saturnus* NCYC2251 was able to significantly enhance the production of targeted aroma-active compounds through the addition of a specific amino acid into papaya juice. L-Leucine addition increased the production of isoamyl alcohol and related 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.

The addition of fusel oil had both volatile modification and growth inhibitory effects on *W. saturnus* NCYC2251, depending on the concentration added. The addition of 0.1% (v/v) enabled the production of a broad range of flavour-enhancing volatile compounds such as ethanol and acetate esters, while reducing the production of undesirable volatiles such as acetic acid. The addition of 0.5% (v/v) fusel oil inhibited yeast growth. Sensory analysis is required to evaluate the relative contribution of each volatile compound to the organoleptic characteristics of papaya wine.

With the adoption of multistarter inoculation, various degrees of yeast succession were experienced during fermentation which in turn affected the final organoleptic properties of papaya wine. Mixed-culture fermentation (co-inoculation) of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 at a ratio of 1:1000 was dominated by *S. cerevisiae*, while *W. saturnus* had an early growth arrest. The mixed-culture had a significant impact on the production of volatile compounds as compared to the monoculture, where it had higher production of acetate esters than the *S. cerevisiae* monoculture and higher concentration of alcohols and ethyl esters than the *W. saturnus* monoculture. The mixed-culture also produced utmost levels of aroma-active esters such as ethyl hexanoate, ethyl octanoate and ethyl decanoate (pleasant fruity, estery and floral aroma) in papaya wine.

Sequential fermentation of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2 was affected by the order of yeast inoculation and yeast ratio. Positive sequential fermentation (PSF) [inoculation of *S. cerevisiae* R2 into the

medium partially fermented by *W. saturnus* NCYC2251] at a ratio of 1000:1 (*W. saturnus*: *S. cerevisiae*) was dominated by *W. saturnus*, while the *S. cerevisiae* had an early growth arrest that limited its flavour contribution. The PSF acquired characteristics from *W. saturnus* and produced papaya wine with more acetate esters and fruitiness than mixed-culture fermentation (MCF) [co-inoculation]. Negative sequential fermentation (NSF) [inoculation of *W. saturnus* NCYC2251 into the medium partially fermented by *S. cerevisiae* R2] was dominated by *S. cerevisiae* and resembled MCF in terms of the changes in oenological parameters and volatiles, except for the enhanced amount of ethyl esters.

The domination of *W. saturnus* in PSF was evidenced even at a ratio of 10:1 (*W. saturnus* NCYC2251: *S. cerevisiae* R2) and produced papaya wine with low ethanol and high acetate esters contents. Increasing the ratio of *S. cerevisiae* to 1:1 and 1:10 (*W. saturnus* NCYC2251: *S. cerevisiae* R2) enabled the co-existence of both yeasts and improved volatile compounds formation through interactions and synergy between the two types of yeasts. The 1:1 and 1:10 ratios resulted in production of more ethyl esters, alcohols and 2-phenylethyl acetate. Nevertheless, the resultant wines fermented with ratios 1:1 and 1:10 were similar due to their comparable yeast population. Moreover, the persistence and interaction of both yeasts at 1:1 and 1:10 ratios led to formation of high levels of acetic acid which might present a challenge for its application.

In conclusion, papaya wines with differential characteristics and aroma profiles have been successfully produced through alcoholic fermentation by monocultures and multistarters (simultaneous and sequential inoculations) of *S. cerevisiae* and *W. saturnus*. The presence of volatile compounds and their concentrations during papaya juice fermentation and in papaya wine were dependent

on the yeast strain and the inoculation strategy. The use of monocultures and multistarters of *S. cerevisiae* and *W. saturnus* in the biotransformation of papaya juice has provided an alternative use for papaya fruit, and may create a new industrial outlet for this fruit.

11.2 Suggestions for future work

11.2.1 Effects of different sequential fermentation techniques on the volatile profile of papaya wine

In this project, the sequential fermentations of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2 were only carried out using different orders of yeast inoculation and yeast ratios. However, there are other methodologies available for conducting sequential fermentation; hence, the effects of different sequential fermentation techniques on the volatile compounds production and sensory quality of papaya wine can be investigated. Earlier inoculation of *S. cerevisiae* into the partially fermented medium can be carried out, where this would still allow early growth of *W. saturnus*, but is followed by partial inactivation of this yeast through inoculation of actively growing *S. cerevisiae*, simulating yeast succession in a spontaneous fermentation. Alternatively, *W. saturnus* can be removed prior to the subsequent inoculation of *S. cerevisiae* through sterile filtration, which could reduce the interaction between the yeasts and the persistence of *W. saturnus* in the fermentation medium that were observed in this project. This is because the interaction or co-existence of both yeasts in sequential fermentation produced high level of acetic acid in this project, which is undesirable for wine aroma and quality.

11.2.2 Evaluation of fermentation conditions on volatile compounds formation in papaya wine fermented by *S. cerevisiae* and *W. saturnus*

In wine fermentation, fermentation conditions are one of the essential factors that determine the type and amount of aroma in the wine (Cole & Noble, 1995; Noble, 1994). The effects of fermentation parameters including temperature, pH, oxygen availability and sulphur dioxide on the oenological parameters and volatile compounds produced by *S. cerevisiae* and *W. saturnus* can be studied. Optimisation with the aid of response surface methodology may be done to find out the optimised conditions to achieve desirable volatile profiles of papaya wine.

11.2.3 Effect of flavour precursors on the volatile compounds production by *S. cerevisiae*

In this study, significant modifications of papaya wine volatile profiles and aroma profile differentiation were achieved through the supplementation of flavour precursors (fusel oil and selected amino acids) in the papaya wine fermented by *W. saturnus* var. *mrakii* NCYC2251 monoculture. Therefore, the possibility and effects of these flavour precursors supplementation in fermentation by *S. cerevisiae* can also be explored. This is because *S. cerevisiae* exhibited significant different fermentation characteristics as compared to *W. saturnus*, which may result in different volatile modulation even with the addition of similar flavour precursors.

11.2.4 Increasing ethanol content in papaya wine

In this study, low concentrations of ethanol were obtained in papaya wines, especially those fermented by the *W. saturnus* monoculture. This may be attributed to

the low sugar concentration of papaya juice and the low fermentative ability of *W. saturnus* in those fermented by the *W. saturnus* monoculture. The possibilities of increasing ethanol content in papaya wine can also be investigated. For examples, papaya juice with enriched sugar concentration can be utilised for fermentation by *S. cerevisiae* or metabolic engineering can be applied to enhance the fermentative rate of *W. saturnus.*

11.2.5 Investigation of the underlying mechanism of the early growth arrest of *W. saturnus* in simultaneous mixed-culture fermentation

In the mixed-culture fermentation (co-inoculation) of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251, the latter had an early growth arrest that limited its contribution to the resultant wine aroma profile. These could be associated with several factors such as lower tolerance to ethanol, presence of toxic compounds, nutrient depletion, oxygen availability, space confinement, quorum sensing and cell–cell contact (Arneborg et al., 2005; Fleet & Heard, 1993; Hansen et al., 2001; Nissen & Arneborg, 2003; Nissen et al., 2003; Panon, 1997). Nevertheless, these factors were investigated using other non-*Saccharomyces* yeasts such as *Hanseniaspora uvarum*, *Kluyveromyces thermotolerans* and *Torulaspora delbrueckii* instead of *W. saturnus* (Arneborg et al., 2005; Hansen et al., 2001; Nissen & Arneborg, 2003). Hence, the underlying mechanism of the early growth arrest of *W. saturnus* in simultaneous mixed-culture fermentation can be determined.

11.2.6 Incorporation of malolactic fermentation into papaya wine

The papaya wine fermentation in this study only comprised of alcoholic fermentation. Future work could incorporate malolactic fermentation after the primary alcoholic fermentation has completed. *O. oeni* is commonly added to wines after alcoholic fermentation to reduce the acidity by metabolising malic acid to lactic acid (Liu, 2002). In addition, other volatile compounds are formed during the malolactic fermentation (Costantini, Garcia-Moruno, & Moreno-Arribas, 2009; Izquierdo Canas, Carcia Romero, Gomez Alonso, & Palop Herreros, 2008), which can further modify the character and aroma of papaya wine.

REFERENCES

Almora, K., Pino, J. A., Hernández, M., Duarte, C., González, J., & Roncal, E. (2004). Evaluation of volatiles from ripening papaya (*Carica papaya* L., var. Maradol roja). *Food Chemistry*, *86,* 127–130.

Alves, J. A., Lima, L. C. O, Dias, D. R., Nunes, C. A., & Schwan, R. F. (2010). Effects of spontaneous and inoculated fermentation on the volatile profile of lychee (*Litchi chinensis* Sonn) fermented beverages. *International Journal of Food Science and Technology*, *45*, 2358–2365.

Alves, R. F., Nascimento, A. M. D., & Nogueira, J. M. F. (2005). Characterization of aroma profile of Madeira wine by sorptive extraction techniques. *Analytica Chimica Acta, 546*, 11–21.

Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J. M., & Esteve-Zarzoso, B. (2010). Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *European Food and Research Technology*, *231*, 215–224.

Arias-Gril, M., Garde-Cerdan, T., & Ancin-Azpilicueta, C. (2007). Influence of addition of ammonium and different amino acid concentrations on nitrogen metabolism in spontaneous must fermentation. *Food Chemistry, 103*, 1312–1318.

Arneborg, N., Siegumfeldt, H., Andersen, G. H., Nissen, P., Daria, V. R., Rodrigo, P.J., et al. (2005). Interactive optical trapping shows that confinement is a determinant of growth in a mixed yeast culture. *FEMS Microbiology Letters, 245*, 155–159.

Balbontín, C., Gaete-Eastman, C., Verara, M., Herrera, R., & Moya-León, M. A. (2007). Treatment with 1-MCP and the role of ethylene in aroma development of mountain papaya fruit. *Postharvest Biology and Technology*, *43*, 67–77.

Baptista, J. A. B., da P Tavares, J. F., & Carvalho, R. C. B. (1998). Comparison of catechins and aromas among different green teas using HPLC/SPME-GC. *Food Research International, 31*, 729–736.

Barbosa, C., Falco, V., Mendes-Faria, A., & Mendes-Faria, A. (2009). Nitrogen addition influences formation of aroma compounds, volatile acidity and ethanol in nitrogen deficient media fermented by *Saccharomyces cerevisiae* wine strains. *Journal of Bioscience and Bioengineering, 108*, 99–104.

Bartowsky, E. J., & Pretorius, I. S. (2009). Microbial formation and modification of flavour and off-flavour. In H. Konig, G. Unden, & J. Frohlich (Eds.), *Biology of Microorganisms on Grapes, in Must and in Wine* (pp. 215–217). New York: Springer.

Bell, S. J., & Henschke, P. A. (2005). Implications of nitrogen nutrition for grapes, fermentation and wine. *Australian Journal of Grape and Wine Research, 11*, 242–295.

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., & Dubourdieu, D. (2008). Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. *International Journal of Food Microbiology, 122*, 312–320.

Benda, I. (1982). Wine and brandy. In G. Reed (Ed.), *Prescott and Dunn's Industrial Microbiology* (pp. 293–402). Westport, USA: AVI Publishing Co.

Berkowitz, M. (1996). World's earliest wine. Archeology (Archaeological Institute of America) 49. Available at: http://www.archaeology.org/9609/newsbriefs/wine.html. (Accessed on 22nd Feb, 2012)

Bianco, G., Novario, G., & Zianni, R. (2009). Comparison of two SPME fibers for the extraction of some off-flavor cork-taint compounds in bottled wines investigated by GC–HRMS. *Analytical and Bioanalytical Chemistry, 393*, 2019–2027.

Bisson, L. F. (2008). Introduction to wine production (Section 3- The alcoholic fermentation). Available at: http://enologyaccess.org/Resources/VEN124/VEN124_10.htm (Accessed on 20th May, 2012)

Blakesley, C. N., Loots, J. G., Plessis, L. D., & Bruyn, G. D. (1979). Gamma irradiation of subtropical fruits. 2. Volatile components, lipids and amino acids of mango, papaya, and strawberry pulp. *Journal of Agricultural and Food Chemistry, 27,* 42–48.

Bonciu, C. (2009). Research concerning the influence of linoleic acid addition in wort comparing with wort aeration. *Innovative Romanian Food Biotechnology, 4*, 24–31.

Boulton, R. B., Singleton, V. L., & Bisson, L. F. (1996). *Principles and Practices of Winemaking*. New York: Chapman & Hall, (Chapter 4).

Burman, L., Albertsson, A. C., & Hoglund, A. (2005). Solid-phase microextraction for qualitative and quantitative determination of migrated degradation products of antioxidants in an organic aqueous solution. *Journal of Chromatography A*, *1080*, 107–116.

Buttery, R. G. (1981). Vegetable and fruit flavors. In R. Teranishi, R. A. Flath, & H. Sugisawa (Eds.), *Flavor Research Recent Advances* (pp. 175–216). New York: Dekker.

Buttery, R. G., Teranishi, R., Ling, L. C., & Turnbaugh, J. G. (1990). Quantitative and sensory studies on tomato paste volatiles. *Journal of Agricultural and Food Chemistry, 38*, 336–340.

Butzke, C. E. (1998). Survey of yeast assimilable nitrogen status in musts from California, Oregon and Washington. *American Journal of Enology and Viticulture, 49*, 220–224.

Calderbank, J., & Hammond, J. R. M. (1994). Influence of higher alcohol availability on ester formation by yeast. *Journal of American Society of Brewing Chemists*, *52*, 84–90.

Camarasa, C., Grivet, J. P., & Dequin, S. (2003). Investigation by 13C-NMR and tricarboxylic acid (TCA) deletion mutant analysis of pathways for succinate formation in *Saccharomyces cerevisiae* during anaerobic fermentation. *Microbiology, 149*, 2669–2678.

Campillo, N., Penalver, R., & Hernandez-Cordoba, M. (2008). Solid-phase microextraction for the determination of haloanisoles in wines and other alcoholic beverages using gas chromatography and atomic emission detection. *Journal of Chromatography A, 1210*, 222–228.

Cano, M. P., Torija, E., Marín, M. A., & Cámara, M. (1994). A simple ion-exchange chromatographic determination of non-volatile organic acids in some Spanish exotic fruits. *Zeitschrift für Lebensmittel-Untersuchung und-Forschung, 199*, 214–218.

Capece, A., Fiore, C., Maraz, A., & Romano, P. (2005). Molecular and technological approaches to evaluate strain biodiversity in *Hanseniaspora uvarum* of wine origin. *Journal of Applied Microbiology*, *98*, 136–144.

Carasek, E., & Pawliszyn, J. (2006). Screening of tropical fruit volatile compounds using solid-phase microextraction (SPME) fibers and internally cooled SPME fiber. *Journal of Agricultural and Food Chemistry, 54*, 8688–8696.

Carrau, F. M., Medina, K., Farina, L., Boido, E., Henschke, P., & Dellacassa, E. (2008). Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: effects of yeast assimilable nitrogen on two model strains. *FEMS Yeast Research, 8*, 1196–1207.

Castro, H. F., Napoleão, D. A. S., & Oliveria, P. C. (1998). Production of citronellyl acetate in a fed-batch system using immobilized lipase. *Applied Biochemistry and Biotechnology, 70–72,* 667–675.

Chan, H. T., Chang, T. S. K., Stafford, A. E., & Brekke, J. E. (1971). Nonvolatile acids of papaya. *Journal of Agricultural and Food Chemistry, 19*, 263–265.

Charoenchai, C., Fleet, G., Henschke, P., & Todd, B. (1997). Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. *Australian Journal of Grape and Wine Research, 3*, 2–8.

Chen, Y., Begnaud, F., Chaintreau, A., & Pawliszyn, J. (2006). Quantification of perfume compounds in shampoo using solid-phase microextraction. *Flavour and Fragrance Journal*, *21*, 822–832.

Cheraiti, N., Guezenec, S., & Salmon, J. M. (2005). Redox interactions between *Saccharomyces cerevisiae* and *Saccharomyces uvarum* in mixed culture under enological conditions. *Applied and Environmental Microbiology, 71,* 255–260.

Ciani, M., Beco, L., & Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *International Journal of Food Microbiology, 108*, 239–245.

Ciani, M., Comitini, F., Mannazzu, I., & Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Research, 10*, 123–133.

Ciani, M., & Maccarelli, F. (1998). Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology, 14*, 199–203.

Clark, C. J., Smith, G. S., & Boldingh, H. L. (1992). Effect of nitrogen fertilisation on the free amino acid composition of kiwifruit during development and postharvest storage. *Scientia Horticulturae, 52*, 85–94.

Clemente-Jimenez, J. M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Las Heras-Vázquez, F. J., & Rodríguez-Vico, F. (2005). Influence of sequential yeast mixtures on wine fermentation. *International Journal of Food Microbiology, 98*, 301–308.

Cocolin, L., Bisson, L. F., & Mills, D. A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters*, *189*, 81–87.

Cole, V. C., & Noble, A. C. (1995). Flavor chemistry and assessment. In A. G. H. Lea, & J. R. Piggott (Eds.), *Fermented beverage production* (pp. 361–385). London, United Kingdom: Blackie Academic and Professional.

Collins, E. B. (1972). Biosynthesis of flavor compounds by microorganisms. *Journal of Dairy Science, 55*, 1022–1028.

Coloretti, F., Zambonelli, C., Castellari, L., Tini, V., & Rainieri, S. (2002). The effect of DL-malic acid on the metabolism of L-malic acid during wine alcoholic fermentation. *Food Technology and Biotechnology, 40*, 317–320.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiology, 28*, 873–882.

Costantini, A., Garcia-Moruno, E., & Moreno-Arribas, M. V. (2009). Biochemical transformations produced by malolactic fermentation. In M. V. Moreno-Arribas, & M. C. Polo (Eds.), *Wine Chemistry and Biochemistry* (pp. 28–41). New York, USA: Springer Science+Business Media.

Coulter, A. D., Godden, P. W., & Pretorius, I. S. (2004). Succinic acid – how it is formed, what is its effect on titratable acidity, and what factors influence its concentration in wine? *Australian and New Zealand Wine Industry Journal, 19*, 16–25.

Czyhrinciw, N. (1969). Tropical fruit technology. In C. O. Chichester (Ed.), *Advances in Food Research* (pp. 196–204). New York, USA: Academic press.

Darriet, P., Lavigne-Cruège, V., & Tominaga, T. (1999). *A paradox: The volatile sulphur compounds responsible for both defects and qualities in wines*. Bordeaux, France: Vigne et Vin Publications International, (pp. 127–133).

De Mora, S. J., Eschnbruch, R., Knowles, S. J., & Speddingm D. J. (1986). The formation of dimethyl disulphide during fermentation using a wine yeast. *Food Microbiology, 3*, 27–32.

De Oliverira, J. G., & Vitória, A. P. (2011). Papaya: Nutritional and pharmacological characterization, and quality loss due to physiological disorders. An overview. *Food Research International*, *44*, 1306–1313.

Deiana, M., Dessi, M. A., Ke, B., Liang, Y. F., Higa, T., Gilmour, P. S., et al. (2002). The antioxidant cocktail effective microorganism X (EM-X) inhibits oxidant-induced interleukin-8 release and the peroxidation of phospholipids in vitro. *Biochemical and Biophysical Research Communications, 296*, 1148–1151.

Delaquies, P., Cliff, M., King, M., Girad, B., Hall, J., & Reynolds, A. J. (2000). Effect of two commercial malolactic cultures on the chemical and sensory properties of Chancellor wines vinified with different yeast and fermentation temperatures. *American Journal of Enology & Viticulture*, *51*, 42–48.

Derrick, S., & Large, P. J. (1993). Activities of the enzymes of the Ehrlich pathway and the formation of branched-chain alcohols in *Saccharomyces cerevisiae* and *Candida utilis* grown in continuous-culture on valine or ammonium as sole nitrogen-source. *Journal of General Microbiology, 139*, 2783–2792.

Dickinson, J. R., Harrison, S. J., Dickinson, J. A., & Hewlins, M. J. E. (2000). An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae. The Journal of Biological Chemistry, 275*, 10937–10942.

Dickinson, J. R., Harrison, S. J., & Michael, J. E. (1998). An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae. The Journal of Biological Chemistry, 273*, 25751–25756.

Dickinson, J. R., Lanterman, M. M., Danner, D. J., Pearson, B. M., Sanz, P., Harrison, S. J., et al. (1997). A ^{13}C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae. The Journal of Biological Chemistry, 272*, 26871–26878.

Dizy, M., & Bisson L. (2000). Proteolytic activity of yeast strains during grape juice fermentation. *American Journal of Enology and Viticulture, 51*, 155–167.

du Toit, M., & Pretorius, I. S. (2000). Microbial spoilage and preservation of wine: using weapons from nature's own arsenal — A review. *South African Journal of Enology and Viticulture*, *21*, 74–96.

Duarte, W. F., Dias, D. R., de Melo Pereira, G. V., Gervasio, I. M., & Schwan, R. F. (2009). Indigenous and inoculated yeast fermentation of gabiroba (*Campomanesia pubescens*) pulp for fruit wine production. *Journal of Industrial Microbiology and Biotechnology*, *36*, 557–569.

Duarte, W. F., Dias, D. R., Oliveira, J. M., Vilanova, M., Teixeira, J. A., Almeida e Silva, J. B., et al. (2010). Raspberry (*Rubus idaeus* L.) wine: Yeast selection, sensory evaluation and instrumental analysis of volatile and other compounds. *Food Research International, 43*, 2303–2314.

Eglinton, J. M., & Henschke, P. A. (1999). The occurrence of volatile acidity in Australian wines. *Australian Grapegrower & Winemaker, 426a*, 7–12.

Erten, H. (2002). Relations between elevated temperatures and fermentation behaviour of *Kloeckera apiculata* and *Saccharomyces cerevisiae* associated with winemaking in mixed cultures. *World Journal of Microbiology and Biotechnology, 18*, 373–378.

Erten, H., & Campbell, I. (2001). The production of low-alcohol wines by aerobic yeasts. *Journal of the Institute of Brewing, 107*, 207–215.

Erten, H., & Tanguler, H. (2010). Influence of *Williopsis saturnus* yeasts in combination with *Saccharomyces cerevisiae* on wine fermentation. *Letters in Applied Microbiology*, *50*, 474–479.

Etievant, P. X. (1991). Wine. In H. Maarse (Ed.), *Volatile Compounds in Food and Beverages* (pp. 483–546). New York: Marcel Dekker.

Etschmann, M. M. W., Bluemke, W., Sell, D., & Schrader, J. (2002). Biotechnological production of 2-phenylethanol. *Applied Microbiology and Biotechnology, 59*, 1–8.

Fedrizzi, B., Magno, F., Finato, F., & Versini, G. (2010). Variation of some fermentative sulfur compounds in Italian “Millesimè” classic sparkling wines during aging and storage on lees. *Journal of Agricultural and Food Chemistry, 58*, 9716–9722.

Fernandez, M., Ubeda, J. F., & Briones, A. I. (2000). Typing of non-*Saccharomyces* yeast with enzymatic activities of interest in winemaking. *International Journal of Food Microbiology, 59*, 29–36.

Ferreira, V., Lopez, R., & Cacho, J. F. (2000). Quantitative determination of the odorants of young red wines from different grape varieties. *Journal of the Science of Food and Agriculture, 80*, 1659–1667.

Ferreira, V., Rapp, A., Cacho, J. F., Hastrich, H., & Yavas, I. (1993). Fast and quantitative-determination of wine flavor compounds using microextraction with Freon 113. *Journal of Agricultural and Food Chemistry*, *41*, 1413–1420.

Fia, G., Giovani, G., & Rosi, I. (2005). Study of β-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. *Journal of Applied Microbiology*, *99*, 509–517.

Flath, R. A., & Forrey, R. R. (1977). Volatile components of papaya *(Carica papaya* L., Solo Variety). *Journal Agricultural and Food Chemistry*, *25*, 103–109.

Fleet, G. H. (2008). Wine yeasts for the future. *FEMS Yeast Research*, *8*, 979–995.

Fleet, G. H., & Heard, G. M. (1993). Yeasts: Growth during fermentation. In G. H. Fleet (Ed.), *Wine Microbiology and Biotechnology* (pp. 27–54). Switzerland: Harwood Academic Publishers.

Francis, I. L., & Newton, J. L. (2005). Determining wine aroma from compositional data. *Australian Journal of Grape and Wine Research, 11*, 114–126.

Fukuda, K., Yamamoto, N., Kiyokawa, Y., Yanagiuchi, T., Wakai, Y., Kitamoto, K., et al. (1998). Balance of activities of alcohol acetyltransferase and esterase in *Saccharomyces cerevisiae* is important for production of isoamyl acetate. *Applied and Environmental Microbiology*, *64*, 4076–4078.

Gao, C., & Fleet, G. H. (1995). Degradation of malic and tartaric acids by high density cell suspensions of wine yeasts. *Food Microbiology, 12*, 65–71.

Garcia, A., Carcel, C., Dulau, L., Samson, A., Aguera, E., Agosin, E., et al. (2002). Influence of a mixed culture with *Debaryomyces vanriji* and *Saccharomyces cerevisiae* on the volatiles of a Muscat Wine. *Journal of Food Science***,** *67*, 1138–1143.

García, D. D. C., Reichenbacher, M., Danzer, K., Hurlbeck, C., Bartzsch, C., & Feller, K. H. (1997). Investigations on wine bouquet components by solid-phase microextraction-capillary gas chromatography (SPME-CGC) using different fibers. *Journal of High Resolution Chromatography*, *20*, 665–668.

Garde-Cerdán, T., & Ancín-Azpilicueta, C. (2006). Contribution of wild yeasts to the formation of volatile compounds in inoculated wine fermentations. *European Food Research and Technology*, *222*, 15–25.

Garde-Cerdán, T., & Ancín-Azpilicueta, C. (2008). Effect of the addition of different quantities of amino acids to nitrogen-deficient must on the formation of esters, alcohols, and acids during wine alcoholic fermentation. *LWT- Food Science and Technology, 41*, 501–510.

Gayosso-García Sancho, L. E., Yahia, E. M., & González-Aguilar, G. A. (2011). Identification and quantification of phenols, carotenoids, and vitamin C from papaya (*Carica papaya* L., cv. Maradol) fruit determined by HPLC-DAD-MS/MS-ESI. *Food Research International, 44*, 1284–1291.

Gil, J. V., Mateo, J. J., Jiménez, M., Pastor, A., & Huerta, T. (1996). Aroma compounds in wine as influenced by apiculate yeasts. *Journal of Food Science, 61*, 1247–1250.

Gonzalez-Marco, A., Jimenez-Moreno, N., & Ancin-Azpilicueta, C. (2010). Influence of nutrients addition to nonlimited-in-nitrogen must on wine composition. *Journal of Food Science, 75*, 206–211.

Goodner, K. L. (2008). Practical retention index models of OV-101, DB-1, DB-5, and DB-Wax for flavor and fragrance compounds. *LWT-Food Science and Technology*, *41*, 951–958.

Guth, H., & Fritzler, R. (2004). Binding studies and computer aided modelling of macromolecule and odorant interactions. *Chemistry and Biodiversity, 1*, 2001–2003.

Guyard, C., Dehecq, E., Tissier, J.P., Polonelli, L., Dei-Cas, E., Cailliez, J.C., et al. (2002). Involvement of β-glucans in the wide-spectrum antimicrobial activity of *Williopsis saturnus* var. *mrakii* MUCL 41968 killer toxin. *Molecular Medicine, 8*, 686–694.

Guymon, J. F., & Crowell, E. A. (1965). The formation of acetoin and diacetyl during fermentation, and the levels found in wines. *American Journal of Enology and Viticulture, 16*, 85–91.

Hansen, E. H., Nissen, P., Sommer, R., Nielsen, J. C., & Arneborg, N. (2001). The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with *Saccharomyces cerevisiae*. *Journal of Applied Microbiology*, *91*, 541–547.

Hauber, J., & Singer, T. P. (1967). Studies on succinate dehydrogenase: Intracellular distribution, catalytic properties and regulation of fumarate reductases in yeast. *European Journal of Biochemistry, 3*, 107–116.

Hazelwood L. A., Daran, J. M., van Maris, A. J. A., Pronk, J. T., & Dickinson, J. R. (2008). The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Applied and Environmental Microbiology, 74*, 2259–2266.

Heard, G. M., & Fleet, G. H. (1985). Growth of natural yeast flora during the fermentation of inoculated wines. *Applied and Environmental Microbiology, 50*, 727–728.

Heard, G. M., & Fleet, G. H. (1988). The effects oftemperature and pH on the growth ofyeast species during the fermentation ofgrape juice. *Journal of Applied Bacteriology, 65*, 23–28.

Heidlas, J., Lehr, M., Idstein, H., & Schreier, P. (1984). Free and bound terpene compounds in papaya *(Carica papaya,* L.) fruit pulp. *Journal of Agricultural and Food Chemistry*, *32*, 1020–1021.

Henick-Kling, T. (1995). Control of malo-lactic fermentation in wine: energetics, flavour modication and methods of starter culture preparation. *Journal of Applied Bacteriology - Symposium Supplement*, *79*, 295–375.

Hernandez-Orte, P., Bely, M., Cacho, J., & Ferreira, V. (2006a). Impact of ammonium additions on volatile acidity, ethanol, and aromatic compound production by different *Saccharomyces cerevisiae* strains during fermentation in controlled synthetic media. *Australian Journal of Grape and Wine Research, 12,* 150–160.

Hernandez-Orte, P., Cacho, J., & Ferreira, V. (2002). Relationship between varietal amino acid profile of grapes and wine aromatic composition. Experiments with model solutions and chemometric study. *Journal of Agricultural and Food Chemistry, 50*, 2891–2899.

Hernandez-Orte, P., Ibarz, M. J., Cacho, J., & Ferreira, V. (2005). Effect of the addition of ammonium and amino acids to musts of Airen variety on aromatic composition and sensory properties of the obtained wine. *Food Chemistry, 89*, 163–174.

Hernandez-Orte, P., Ibarz, M. J., Cacho, J., & Ferreira, V. (2006b). Addition of amino acids to grape juice of the Merlot variety: Effect on amino acid uptake and aroma generation during alcoholic fermentation. *Food Chemistry, 98*, 300–310.

Hernández, Y., Lobo, M. G., & González, M. (2009). Factors affecting sample extraction in the liquid chromatographic determination of organic acids in papaya and pineapple. *Food Chemistry, 114*, 734–741.

Hernawan, T., & Fleet, G. H. (1995). Chemical and cytological changes during the autolysis of yeasts. *Journal of Industrial Microbiology, 14*, 440–450.

Herraiz, T., Reglero, G., Herraiz, M., Martín-Álvarez, P. J., & Cabezudo, M. D. (1990). The influence of the yeast and type of culture on the volatile composition of wines fermented without sulphur dioxide. *American Journal of Enology and Viticulture, 41*, 313–318.

Hiramoto, K., Imao, M., Sato, E. F., Inoue, M., & Mori, A. (2008). Effect of fermented papaya preparation on dermal and intestinal mucosal immunity and allergic inflammations. *Journal of the Science of Food and Agriculture, 88*, 1151–1157.

Howell, K. S., Cozzolino, D., Bartowsky, E., Fleet, G. H., & Henschke, P. A. (2006). Metabolic profiling as a tool for revealing *Saccharomyces* interactions during wine fermentation. *FEMS Yeast Research, 6*, 91–101.

Ingledew, W. M., Magaus, C. A., & Sosulski, F. W. (1987). Influence of oxygen on proline utilization during the wine fermentation. *American Journal of Enology and Viticulture*, *38*, 246–248.

Inoue, Y., Trevanichi, S., Fukuda, K., Izawa, S., Wakai, Y., & Kimura, A. (1997). Roles of esterase and alcohol acetyltransferase on production of isoamyl acetate in *Hansenula mrakii*. *Journal of Agricultural & Food Chemistry, 45*, 644–649.

Iwase, T., Morikawa, T., Fukuda, H., Sasaki, K., & Yoshitake, M. (1995). Production of fruity odor by genus *Williopsis*. *Journal of the Brewing Society of Japan, 90*, 394–396.

Izquierdo Canas, P. M., Carcia Romero, E., Gomez Alonso, S., & Palop Herreros, M. L. L. (2008). Changes in the aromatic composition of Tempranillo wines during spontaneous malolactic fermentation. *Journal of Food Composition and Analysis, 21,* 724–730.

Jackson, R. S. (1994). Chemical constituents of grapes and wine. In S. L. Taylor (Ed.), *Wine Science. Principles and Applications* (pp. 178–219). San Diego: Academic Press.

Jackson, R. S. (2000). *Wine Science. Principles, Practice, Perception*. (2nd ed.). San Diego, California: Academia Press, (Chapter 7).

Jacobson, J. L. (2006). *Introduction to Wine Laboratory Practices and Procedures*. New York: Springer Science+Business Media, (Chapter 7).

James, S. A., Roberts, I. N., & Collins, M. D. (1998). Phylogenetic heterogeneity of the genus *Williopsis* as revealed by 18S rRNA gene sequences. *International Journal of Systematic Bacteriology*, *48*, 591–596.

Janssens, L., de Pooter, H. L., Schamp, N. M. & Vandamme, E. J. (1992). Production of flavours by microorganisms. *Process Biochemistry, 27*, 195–215.

Jolly, N. P., Augustyn, O. P. H., & Pretorius, I. S. (2003). The effects of non-*Saccharomyces* yeasts on fermentation and wine quality. *South African Journal of Enology and Viticulture, 24*, 55–62.

Jolly, N. P., Augustyn, O. P. H., & Pretorius, I. S. (2006). The role and use of non-*Saccharomyces* yeasts in wine production. *South African Journal of Enology and Viticulture, 27*, 15–39.

Kafkas, E., Cabaroglu, T., Selli, S., Bozdogan, A., Kurkcuoglu, M., Paydas, S., et al. (2006). Identification of volatile aroma compounds of strawberry wine using solid-phase microextraction techniques coupled with gas chromatography–mass spectrometry. *Flavour and Fragrance Journal, 21*, 68–71.

Kapsopoulou, K., Mourtzini, A., Anthoulas, M., & Nerantzis, E. (2007). Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. *World Journal of Microbiology and Biotechnology, 23*, 735–739.

Killian, R. E., & Ough, C. S. (1979). Fermentation esters-formation and retention as affected by fermentation temperature. *American Journal of Enology and Viticulture, 30*, 301–305.

Kucuk, Z., & Ceylan, K. (1998). Potential utilization of fusel oil. A kinetic approach for production of fusel oil esters through chemical reaction. *Turkish Journal of Chemistry*, *22*, 289–300.

Lambrechts, M. G., & Pretorius, I. S. (2000). Yeasts and its importance to wine aroma- a review. *South African Journal of Enology and Viticulture, 21*, 97–129.

Lazan, H., Selamat, M. K., & Ali, Z. M. (1995). β-Galactosidase, polygalacturonase and pectinesterase in differential softening and cell wall modification during papaya fruit ripening. *Physiology Plant, 95*, 106–112.

Lee, J. E., Hong, Y. S., & Lee, C. H. (2009). Characterization of fermentative behaviors of lactic acid bacteria in grape wines through ^{1}H NMR- and GC-based metabolic profiling. *Journal of Agricultural and Food Chemistry*, *57*, 4810–4817.

Lema, C., Garcia-Jares, C., Orriols, I., & Angulo, L. (1996). Contribution of *Saccharomyces* and non-*Saccharomyces* populations to the production of some components of Albarino wine aroma. *American Journal of Enology and Viticulture, 47*, 206–216.

Li, H., Tao, Y. S., Wang, H., & Zhang, L. (2008). Impact odorants of Chardonnay dry white wine from Changli county (China). *European Food Research and Technology, 227*, 287–292.

Li, X., Yu, B., Curran, P., & Liu, S. Q. (2012). Impact of two *Williopsis* yeast strains on the volatile composition of mango wine. *International Journal of Food Science and Technology, 47,* 808–815.

Lilly, M., Bauer, F. F., Lambrechts, M. G., Swiegers, J. H., & Cozzolino, D. (2006). The effects of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates. *Yeast, 23*, 641–659.

Lilly, M., Lambrechts, M. G., & Pretorius, I. S. (2000). Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. *Applied and Environmental Microbiology*, *66*, 744–753.

Lin, Y. (1975). Detection of wild yeasts in the brewery. Efficiency of differential media. *Journal of the Institure of Brewing*, *81*, 410–417.

Liu, R. H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *Journal of Nutrition, 134,* 3479–3485.

Liu, S. Q. (2002). Malolactic fermentation in wine - beyond deacidification: A review. *Journal of Applied Microbiology*, *92*, 859–601.

Liu, S. Q., & Tsao, M. (2009). Inhibition of spoilage yeasts in cheese by killer yeast *Williopsis saturnus* var. *saturnus*. *International Journal of Food Microbiology, 131*, 280–282.

Liu, S. Q., & Tsao, M. (2010). Biocontrol of spoilage yeasts and moulds by *Williopsis saturnus* var. *saturnus* in yoghurt. *Nutrition and Food Science*, *40*, 166–175.

Luebke, W. (1980). The Good Scents Company. Available at: http://www.thegoodscentscompany.com/index.html (Accessed on 28th Feb, 2012)

MacLeod, A. J., & Pieris, N. M. (1983). Volatile components of papaya *(Carica papaya* L.) with particular reference to glucosinolate products. *Journal of Agricultural and Food Chemistry*, *31*, 1005–1008.

Mahattanatawee, K., Manthey, J. A., Luzio, G., Talcott, S. T., Goodner, K., & Baldwin, E. A. (2006). Total antioxidant activity and fiber content of select Florida-grown tropical fruits. *Journal of Agricultural and Food Chemistry, 54*, 7355–7363.

Mahmoud, W. M., El-Sayed, A. H. M. M., & Coughlin, R. W. (1990). Production of L-phenylacetyl carbinol by immobilized yeast cells: 1. Batch fermentation. *Biotechnology and Bioengineering, 36*, 47–54.

Manenoi, A., & Paull, R. E. (2007). Papaya fruit softening, endoxylanase gene expression, protein and activity. *Physiology Plant, 131*, 470–480.

Manginot, C., Rouston, J. L., & Sablayrolles, J. M. (1998). Nitrogen demand of different yeast strains during alcoholic fermentation. Importance of the stationary phase. *Enzyme and Microbial Technology*, *23*, 511–517.

Manzanares, P., Rojas, V., Genoves, S., & Valles, S. (2000). A preliminary search for anthocyanin– β-D-glucosidase activity in non-*Saccharomyces* wine yeasts. *International Journal of Food Science & Technology, 35*, 95–103.

Mateo, J. J., & Jiménez, M. (2000). Monoterpenes in grape juice and wines. *Journal of Chromatography A, 881*, 557–567.

Mauricio, J. C., Guijo, S., & Ortega, J. M. (1991). Relationship between phospholipid and sterol contents in *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* and their fermentation activity in grape musts. *American Journal of Enology and Viticulture, 42*, 301–308.

McGrath, M. J., & Karahadian, C. (1994). Evaluation of physical, chemical, and sensory properties of pawpaw fruit (*Asimina triloba*) as indicators of ripeness. *Journal of Agricultural and Food Chemistry, 42*, 968–974.

Meilgaard, M., Civille, G. V., & Carr, B. T. (1999). *Sensory Evaluation Techniques* (3rd ed.). Boca Raton, Florida: CRC Press.

Mendoza, L., & Farias, M. E. (2010). Improvement of wine organoleptic characteristics by non-*Saccharomyces* yeast. In A. Mendez-Vilas (Ed.), *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology* (pp. 908–919). Badajoz, Spain: Formatex Research Center.

Mendoza, L. M., Manca de Nadra, M. C., & Farias, M. E. (2007). Kinetics and metabolic behavior of a composite culture of *Kloeckera apiculata* and *Saccharomyces cerevisiae* wine related strains. *Biotechnology Letters, 29*, 1057–1063.

Miller, A. C., Wolff, S. R., Bisson, L. F., & Ebeler, S. E. (2007). Yeast strain and nitrogen supplementation: Dynamics of volatile ester production in Chardonnay juice fermentations. *American Journal of Enology and Viticulture, 58*, 470–483.

Molina, A. M., Swiegers, J. H., Varela, C., Pretorius, I. S., & Agosin, E. (2007). Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds. *Applied Microbiology and Biotechnology, 77*, 675–687.

Moreira, N., Guedes de Pinho, P., Santos, C., & Vasconcelos, I. (2011). Relationship between nitrogen content in grapes and volatiles, namely heavy sulphur compounds, in wines. *Food Chemistry, 126*, 1599–1607.

Moreira, N., Mendes, F., Guedes de Pinho, P., Hogg, T., & Vasconcelos, I. (2008). Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *International Journal of Food Microbiology***,** *124*, 231–238.

Moreira, N., Mendes, F., Pereira, O., Guedes de Pinho, P., Hogg, T., & Vasconcelos, I. (2002). Volatile sulphur compounds in wines related to yeast metabolism and nitrogen composition of grape musts. *Analytica Chimica Acta, 458*, 157–167.

Moreno, J. J., Millan, C., Ortega, J. M., & Medina, M. (1991). Analytical differentiation of wine fermentations using pure and mixed yeast cultures. *Journal of Industrial Microbiology*, *7*, 191–190.

Moy, J. H. (2003). Papayas. In B. Caballero, P. M. Finglas, & L. Trugo (Eds.), *Encyclopedia of Food Sciences and Nutrition* (pp. 4345–4351). New York, USA: Academic Press.

Moyano, L., Moreno, J., Millan, C., & Medina, M. (1994). Flavour in ‘‘Pedro Ximenez’’ grape must subjected to maceration processes. *Vitis, 33*, 87–91.

Murat, M. L., Masneuf, I., Darriet, P., Lavigne, V., Tominaga, T., & Dubourdieu, D. (2001). Effect of *Saccharomyces cerevisiae* yeast strains on the liberation of volatile thiols in Sauvignon blanc wine. *American Journal of Enology and Viticulture, 52*, 136–139.

Muratsubaki, H., & Katsume, T. (1985). Characterization of fumarate reductase from baker's yeast: Essential sulfhydryl group for binding of FAD. *Journal of Biochemistry, 97*, 1201–1209.

Nissen, P., & Arneborg, N. (2003). Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae. Archives of Microbiology, 180,* 257–263.

Nissen, P., Nielsen, D., & Arneborg, N. (2003). Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell–cell contact-mediated mechanism. *Yeast*, *20*, 331–341.

Nissen, T. L., Kielland-Brandt, M. C., Nielsen, J., & Villadsen, J. (2000). Optimization of ethanol production in *Saccharomyces cerevisiae* by metabolic engineering of the ammonium assimilation. *Metabolic Engineering, 2*, 69–77.

Noble, A. C. (1994). Wine flavour. In J. R. Piggott, & A. Patterson (Eds.), *Understanding natural flavours* (pp. 228–242). Glasgow, United Kingdom: Blackie Academic and Professional.

Nykanen, L. (1986). Formation and occurrence of flavour compounds in wine and distilled alcoholic beverages. *American Journal of Enology and Viticulture, 37*, 84–96.

Oda, S., Inada, Y., Kobayashi, A., Kato, A., Matsudomi, N., & Ohta, H. (1996). Coupling of metabolism and bioconversion: microbial esterification of citronellol with acetyl coenzyme A produced via metabolism of glucose in an interface bioreactor. *Applied and Environmental Microbiology*, *62*, 2216–2220.

Okrasa, K., Guibe-Jampel, E., Plenkiewicz, J., & Therisod, M. (2004). *In vitro* bi-enzymatic synthesis of benzaldehyde from phenylalanine: practical and mechanistic studies. *Journal of Molecular Catalysis B: Enzymatic, 31*, 97–101.

Ough, C. S., Crowell, E. A., & Mooney, L. A. (1988). Formation of ethyl carbamate precursors during grape juice (Chardonnay) fermentation. I. Addition of amino acids, urea and ammonium: effects of fortification of intracellular and extracellular precursors. *American Journal of Enology and Viticulture, 39*, 243–249.

Panon, G. (1997). Influence of oxygen on fermentation pattern in model media containing mixed or sequential cultures of three cider producing yeasts:

Saccharomyces cerevisiae, *Hanseniaspora valbyensis* and *Metschnikowia pulcherrima*. *Sciences Des Aliments, 17*, 193–217.

Park, Y. C., Shaffer, C. E. H., & Bennett, G. H. (2009). Microbial formation of esters. *Applied Microbiology and Biotechnology, 85*, 13–25.

Patil, A. G., Koolwal, S. M., & Butala, H. D. (2002). Fusel oil: composition, removal, and potential utilization. *International Sugar Journal*, *104*, 51–58.

Patthamakanokporn, O., Puwastien, P., Nitithamyong, A., & Sirichakwal, P. P. (2008). Changes of antioxidant activity and total phenolic compounds during storage of selected fruits. *Journal of Food Composition and Analysis, 21*, 241–248.

Paull, R. E. (1993). Pineapple and papaya. In: G. B. Seymour, J. E. Taylor, & G. A. Tucker (Eds.), *Biochemistry of Fruit Ripening* (pp. 302–323). London: Chapman & Hall.

Paull, R. E., & Chen, N. J. (1983). Postharvest variation in cell wall-degrading enzymes of papaya (*Carica papaya* L.) during fruit ripening. *Plant Physiology, 72*, 382–385.

Pawliszyn, J. (1997). *Solid-phase Microextraction: Theory and Practice*. New York: Wiley-VCH.

Peddie, H. A. B. (1990). Ester formation in brewery fermentations. *Journal of the Institute of Brewing*, *66*, 327–331.

Peinado, R. A., & Mauricio, J. C. (2009). Biologically Aged Wines. In M. V. Moreno-Arribas, & M. C. Polo (Eds.), *Wine Chemistry and Biochemistry* (pp. 81–101). New York: Springer Science+Business Media.

Pereira, J., Pereira, J., & Câmara, J. S. (2011). Effectiveness of different solid-phase microextraction fibres for differentiation of selected Madeira island fruits based on their volatile metabolite profile—Identification of novel compounds. *Talanta*, *83*, 899–906.

Perestrelo, R., Fernandes, A., Albuquerque, F. F., Marques, J. C., & Câmara, J. S. (2006). Analytical characterization of the aroma of Tinta Negra Mole red wine: Identification of the main odorants compounds. *Analytica Chimica Acta, 563*, 154–164.

Peterson, R. N. (1991). Pawpaw (Asimina). In N. J. Moore, & J. R. Ballington (Eds.), *Genetic Resources of Temperature Fruit and Nut Crops* (pp. 569–900). Wageningen, The Netherlands: International Society for Horticultural Science.

Pineau, B., Barbe, J. C., Van Leeuwen, C., & Dubourdieu, D. (2009). Examples of perceptive interactions involved in specific "red-" and "black-berry" aromas in red wines. *Journal of Agricultural and Food Chemistry, 57*, 3702–3708.

Pino, J. A., Almora, K., & Marbot, R. (2003). Volatile components of papaya (*Carica papaya* L., *Maradol* variety) fruit. *Flavour and Fragrance Journal*, *18*, 492–496.

Pino, J. A., & Queris, O. (2010). Analysis of volatile compounds of pineapple wine using solid-phase microextraction techniques. *Food Chemistry, 122*, 1241–1246.

Pino, J. A., & Queris, O. (2011). Characterisation of odour-active compounds in papaya (*Carica papaya* L.) wine. *International Journal of Food Science and Technology, 47*, 262–268.

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast*, *16*, 675–729.

Quilter, M. G., Hurley, J. C., Lynch, F. J., & Murphy, M. G. (2003). The production of isoamyl acetate from amyl alcohol by *Saccharomyces cerevisiae*. *Journal of the Institute of Brewing*, *109*, 34–40.

Radler, F. (1993). Yeasts – metabolism of organic acids. In G. H. Fleet (Ed.), *Wine Microbiology and Biotechnology* (pp. 165–182). Chur, Switzerland: Harwood Academic Publishers.

Ramey, D. D., & Ough, C. S. (1980). Volatile ester hydrolysis or formation during storage of model solutions and wines. *Journal of Agricultural and Food Chemistry*, *28*, 928–934.

Rapp, A., & Mandery, H. (1986). Wine aroma. *Experientia*, *42*, 873–884.

Redzepovic, S., Orlic, S., Majdak, A., Kozina, B., Volschenk, H., & Viljoen-Bloom, M. (2003). Differential malic acid degradation by selected strains of *Saccharomyces* during alcoholic fermentation. *International Journal of Food Microbiology, 83*, 49–61.

Revel, G. D., Martin, M., Pripis-Nicolau, L., Lonvaud-Funel, A., & Bertrand, A. (1999). Contribution to the knowledge of malolactic fermentation influence on wine aroma. *Journal of Agricultural and Food Chemistry*, *47*, 4003–4008.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., & Lonvaud, A. (2006). *Handbook of Enology: The Microbiology of Wine and Vinifications.* (Vol. 1, 2nd ed.). Chichester, UK: John Wiley & Sons, Ltd, (Chapter 2).

Richardson, T., & Hyslop, D. B. (1992). Enzymes. In O. R. Fenema (Ed.), *Food Chemistry* (pp. 35–38). New York, USA: Marcel Dekker Incorporated.

Rivera-Pastrana, D. M., Yahia, E. M., & González-Aguilar, G. A. (2010). Phenolic and carotenoid profiles of papaya fruit (*Carica papaya* L.) and their contents under low temperature storage. *Journal of the Science of Food and Agriculture, 90*, 2358–2365.

Rodríguez, M. E., Lopes, C. A., Barbagelata, R. J., Barda, N. B., & Caballero, A.C. (2010). Influence of *Candida pulcherrima* Patagonian strain on alcoholic

fermentation behaviour and wine aroma. *International Journal of Food Microbiology, 138*, 19–25.

Rojas, V., Gil, J. V., Pinaga, F., & Manzanares, P. (2001). Studies on acetate ester production by non-*Saccharomyces* wine yeasts. *International Journal of Food Microbiology, 70,* 283–289.

Rojas, V., Gil, J. V., Pinaga, F., & Manzanares, P. (2003). Acetate ester formation in wine by mixed cultures in laboratory fermentations. *International Journal of Food Microbiology, 86*, 181–188.

Romano, P., Fiore, C., Paraggio, M., Caruso, M., & Capece, A. (2003). Function of yeast species and strains in wine flavour. *International Journal of Food Microbiology, 86*, 169–180.

Romano, P., & Suzzi, G. (1993a). Sulfur dioxide and wine microorganism. In G. H. Fleet (Ed.), *Wine Microbiology and Biotechnology* (pp. 373–393). Chur, Switzerland: Harwood Academic Publishers.

Romano, P., & Suzzi, G. (1993b). Acetoin production in *Saccharomyces cerevisiae* wine yeast. *FEMS Microbiology Letters, 108*, 23–26.

Romano, P., Suzzi, G., Comi, G., & Zironi, R. (1993). Higher alcohol and acetic acid production by apiculate wine yeasts. *Journal of Applied Bacteriology, 73*, 126–130.

Romano, P., Suzzi, G., Comi, G., Zironi, R., & Maifreni, M. (1997). Glycerol and other fermentation products of apiculate wine yeasts. *Journal of Applied Microbiology, 82*, 615–618.

Russell, I. (2003). Understanding yeast fundamentals. In K. A. Jacques, T. P. Lyons, & D. R. Kelsall (Eds.), *The Alcohols Textbook* (pp. 85–119). Nottingham, UK: Nottingham University Press.

Saayman, M., & Viljoen-Bloom, M. (2006). The biochemistry of malic acid metabolism by wine yeast - A review. *South African Journal of Enology and Viticulture, 27*, 113–122.

Sablayrolles, J. M. (2009). Control of alcoholic fermentation in winemaking: Current situation and prospect. *Food Research International, 42*, 418–424.

Saerens, S. M. G., Delvaux, F., Verstrepen, K. J., Van Dijck, P., Thevelein, J. M., & Delvaux, F. R. (2008). Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. *Applied and Environmental Microbiology, 74,* 454–461.

Saerens, S. M. G., Verstrepen, K. J., Van Laere, S. D. M., Voet, A. R. D., Van Dijck, P., Delvaux, F. R., et al. (2006). The *Saccharomyces cerevisiae EHT1* and *EEB1* genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. *Journal of Biological Chemistry, 281*, 4446–4456.

Salo, P. (1970). Variability of odour thresholds for some compounds in alcoholic beverages. *Journal of the Science of Food and Agriculture, 21*, 597–600.

Schreier, P. (1979). Flavour composition of wines. *CRC Critical Reviews in Food Science and Nutrition, 12*, 59–111.

Schwab, W., Mahr, C., & Schreier, P. (1989). Studies on the enzymic hydrolysis of bound aroma components from *Carica papaya* fruit. *Journal of Agricultural and Food Chemistry*, *37*, 1009–1012.

Segurel, M. A., Baumes, R. L., Langlois, D., Riou, C., & Razungles, A. J. (2009). Role of glycosidic aroma precursors on the odorant profiles of Grenache noir and Syrah wines from the Rhone valley. Part 2: Characterization of derived compounds. *Journal International des Sciences de la Vigne et du Vin, 43*, 213–223.

Sentheshanmuganathan, S. (1960). The mechanism of the formation of higher alcohols from amino acids by *Saccharomyces cerevisiae*. *Journal of Biochemistry*, *74*, 568–576.

Shackford, S. (2009). Biomolecular archaeologist uncorks world's oldest known grape wine (Cornell University). Available at: http://ezramagazine.cornell.edu/update/May12/EU.McGovern.uncorks.html (Accessed on 14th Oct, 2012).

Sharma, V. C., & Ogbeide, O. N. (1982). Pawpaw as a renewable energy resource for the production of alcohol fuels. *Energy, 7*, 871–873.

Shiota, H. (1991). Volatiles compounds of pawpaw fruit (*Asimina triloba Dunal*). *Journal of Agricultural and Food Chemistry*, *39*, 1631–1635.

Soden, A., Francis, I. L., Oakey, H., & Henschke, P. A. (2000). Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. *Australian Journal of Grape Wine Research, 6*, 21–30.

Soles, R. M., Ough, C. S., & Kunkee, R. E. (1982). Ester concentration differences in wine fermented by various species and strains of yeasts. *American Journal of Enology and Viticulture*, *33*, 94–98.

Starley, I. F., Mohammed, P., Schneider, G., & Bickler, S. W. (1999). The treatment of paediatric burns using topical papaya. *Burns, 25*, 636–639.

Stashenko, H., Macku, C., & Shibamato, T. (1992). Monitoring volatile chemicals formed from must during yeast fermentation. *Journal of Agricultural and Food Chemistry*, *40*, 2257–2259.

Strauss, M., Jolly, N., Lambrechts, M., & van Rensburg, P. (2001). Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. *Journal of Applied Microbiology, 91*, 182–190.

Sumby, K. M., Grbin, P. R., & Jiranek, V. (2010). Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. *Food Chemistry, 121,* 1–16.

Suomalainen, H. (1981). Yeast esterases and aroma esters in alcoholic beverages. *Journal of the Institute of Brewing, 87*, 296–300.

Suomalainen, H., & Lehtonen, M. (1979). The production of aroma compounds by yeast. *Journal of Institute Brewing, 85*, 149–156.

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., & Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Australian Journal of Grape and Wine Research, 11*, 139–173.

Swiegers, J. H., & Pretorius, I. S. (2005). Yeast modulation of wine flavour. *Advances in Applied Microbiology*, *57*, 131–175.

Swiegers, J. H., Saerens, S. M. G., & Pretorius, I. S. (2008). The development of yeast strains as tools for adjusting the flavor. In D. Havkin-Frenkel, & F. C. Belanger (Eds.), *Biotechnology in Flavor Production* (pp. 1–55). Oxford, UK: Blackwell Publishing Ltd.

Swiegers, J. H., Willmott, R. L., Siebert, T. E., Lattey, K., Bramley, B. R., Francis, I. L., et al. (2009). The influence of yeast on the aroma of Sauvignon Blanc wine. *Food Microbiology, 26*, 204–211.

Tang, C. S. (1971). Benzyl isothiocyanate of papaya fruit. *Phytochemistry, 10*, 117–121.

Takasuka, T., Komiyama, T., Furuichi, Y., & Watanabe, T. (1995). Cell wall synthesis specific cytocidal effect of *Hansenula mrakii* toxin-1 on *Saccharomyces cerevisiae*. *Cellular & Molecular Biology Research, 41*, 575–581.

Tao, Y. S., Liu, Y. Q., & Liu, H. (2009). Sensory characters of Cabernet Sauvignon dry red wine from Changli County (China). *Food Chemistry*, *114*, 565–569.

Thomás-Barberán, F., & Espín, J. C. (2001). Phenolic compounds and related enzymes as determinants of quality of fruits and vegetables. *Journal of the Science of Food and Agriculture*, *81*, 853–876.

Thornton, R. J. (1991). Wine yeast research in New Zealand and Australia. *CRC Critical Reviews in Biotechnology*, *11*, 327–345.

Torija, M. J., Beltran, G., Novo, M., Poblet, M., Guillamon, J. M., Mas, A., et al. (2003). Effects of fermentation temperature and *Saccharomyces* species on the

cell fatty acid composition and presence of volatile compounds in wine. *International Journal of Food Microbiology, 85*, 127–136.

Toro, M. E., & Vazquez, F. (2002). Fermentation behaviour of controlled mixed and sequential cultures of *Candida cantarelli* and *Saccharomyces cerevisiae* wine yeast. *World Journal of Microbiology and Biotechnology*, *18*, 347–354.

Torrea, D., Fraile, P., Garde, T., & Ancín, C. (2003). Production of volatile compounds in the fermentation of chardonnay musts inoculated with two strains of *Saccharomyces cerevisiae* with different nitrogen demands. *Food Control*, *14*, 565–571.

Torrea, D., Varela, C., Ugliano, M., Ancin-Azpilicueta, C., Francis, I. L., & Henschke, P. A. (2011). Comparisons of inorganic and organic nitrogen supplementation of grape juice-Effect on volatile composition and aroma profile of a Chardonnay wine fermented with *Saccharomyces cerevisiae* yeast. *Food Chemistry, 127*, 1072–1083.

Trinh, T. T. T., Woon, W. Y., Yu, B., Curran, P., & Liu, S. Q. (2011). Growth and fermentation kinetics of a mixed culture of *Saccharomyces cerevisiae* var. *bayanus* and *Williopsis saturnus* var. *saturnus* at different ratios in longan juice (*Dimocarpus longan* Lour.). *International Journal of Food Science and Technology, 46*, 130–137.

Ugliano, M., & Henschke, P. A. (2009). Yeasts and wine flavour. In M. V. Moreno-Arribas, & M. C. Polo (Eds.), *Wine Chemistry and Biochemistry* (pp. 314–374). New York, USA: Springer Science+Business Media.

USDA (The United States Department of Agriculture) (2011). USDA National Nutrient Database for Standard Reference, Release 24 [Online]. Agricultural Research Service, Nutrient Data Laboratory Home Page. Available at: http://www.ars.usda.gov/ba/bhnrc/ndl (Accessed on 19th Feb, 2012).

Valero, E., Moyano, L., Millan, M. C., Medina, M., & Ortega, J. M. (2002). Higher alcohols and esters production by *Saccharomyces cerevisiae*. Influence of the initial oxygenation of the grape must. *Food Chemistry*, *78*, 57–61.

van Iersel, M. F., Brouwer-Post, E., Rombouts, F. M., & Abee, T. (2000). Influence of yeast immobilization and aldehyde reduction during the production of alcohol-free beer. *Enzyme and Microbial Technology, 26*, 602–607.

Vandamme, E. J. (2003). Bioflavours and fragrances via fungi and their enzymes. *Fungal Diversity, 13*, 1323–1332.

Vandamme, E. J., & Soetaert, W. (2002). Bioflavours and fragrances via fermentation and biocatalysis. *Journal of Chemical Technology & Biotechnology*, *77*, 1323–1332.

Vermeulen, C., Gijs, L., & Collin, S. (2005). Sensorial contribution and formation pathways of thiols in foods: A review. *Food Reviews International*, *21*, 69–137.

Viana, F., Gil, J. V., Genoves, S., Valles, S., & Manzanares, P. (2008). Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiology*, *25*, 778–785.

Viana, F., Gil, J. V., Valles, S., & Manzanares, P. (2009). Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. *International Journal of Food Microbiology, 135*, 68–74.

Vilanova, M., Genisheva, Z., Masa, A., & Oliveira, J. M. (2010). Correlation between volatile composition and sensory properties in Spanish Albariño wines. *Microchemical Journal, 95*, 240–246.

Vinci, G., Botre, F., Mele, G., & Ruggieri, G. (1995). Ascorbic acid in exotic fruits: A liquid chromatographic investigation. *Food Chemistry, 53*, 211–214.

Visser, W., Scheffers, W. A., Batenburg-Van Der Vegte, W. H., & Van Dijken, J. P. (1990). Oxygen requirements of yeasts. *Applied and Environmental Microbiology, 56*, 3785–3792.

Vradis, I., & Floros, J. D. (1993). Membrane separation processes for wine dealcoholization and quality improvement. *Developments in Food Science, 32*, 501–520.

Wall, M. M. (2006). Ascorbic acid, vitamin A, and mineral composition of banana (*Musa* sp.) and papaya (*Carica papaya*) cultivars grown in Hawaii. *Journal of Food Composition and Analysis*, *19*, 434–445.

Welsh, F. W., & Williams, R .E. (1989). Lipase mediated production of flavor and fragrance esters from fusel oil. *Journal of Food Science*, *54,* 1565–1568.

Whiting, G. C. (1976). Organic acid metabolism of yeasts during fermentation of alcoholic beverages - Review. *Journal of the Institute of Brewing, 82*, 84–92.

Wine Institute (2010). Statistics of world wine production by country. Available at: http://www.wineinstitute.org/files/WorldWineProductionbyCountry.pdf (Accessed on 16th Jan, 2012).

Winterhalter, P., Katzenberger, D., & Schreier, P. (1986). 6,7-Epoxy-linalool and related oxygenated terrpenoids from *Carica papaya* fruit. *Phytochemistry*, *25*, 1347–1350.

Yamamoto, T., Shimada, A., Ohmoto, T., Matsuda, H., Ogura, M., & Kanisawa, T. (2004). Olfactory study on optically active citronellyl derivatives. *Flavour and Fragrance Journal, 19,* 121–133.

Yap, N. A., de Barros Lopes, M., Langridge, P., & Henschke, P. A. (2000). The incidence of killer activity of non-*Saccharomyces* yeasts towards indigenous

yeast species of grape must: potential application in wine fermentation. *Journal of Applied Microbiology*, *89*, 381–389.

Yilmaztekin, M., Erten, H., & Cabaroglu, T. (2008). Production of isoamyl acetate from sugar beet molasses by *Williopsis saturnus* var. *saturnus*. *Journal of the Institute of Brewing, 114*, 34–38.

Yilmaztekin, M., Erten, H., & Cabaroglu, T. (2009). Enhanced production of isoamyl acetate from beet molasses with addition of fusel oil by *Williopsis saturnus* var. *saturnus*. *Food Chemistry*, *112*, 290–294.

Yoshioka, K., & Hashimoto, N. (1981). Ester formation by alcohol acetyltransferase from brewer's yeast. *Agricultural and Biological Chemistry, 45*, 2183–2190.

Zhou, L., & Paull, R. E. (2001). Sucrose metabolism during papaya (*Carica papaya*) fruit growth and ripening. *Journal of the American Society for Horticultural Science, 126*, 351–357.

Zoecklein, B. W., Marcy, J. E., Williams, J. M., & Jasinski, Y. (1997). Effect of native yeasts and selected stains of *Saccharomyces cerevisiae* on glycosyl glucose, potential volatile terpenes, and selected aglycones of white Riesling (*Vitis vinifera* L.) wines. *Journal of Food Composition and Analysis, 10*, 55–65.

Appendix A

Table A.1 Standard curve equations for HS-SPME-GC-MS/FID quantification of selected major volatile compounds

Compounds	Gradient	Y-intercept	Linear range (ppm)	Correlation coefficient (R^2)	LOD (ppm)	LOQ (ppm)	RSD (n = 5) (%)	Recovery (n = 3) (%)	Spiked level for recovery (ppm)
Acetic acid	9.52×10^4	6.79×10^4	0.01-20.00	0.990	0.479	1.597	7.23	120.39	0.983
Isobutyric acid	3.03×10^6	1.07×10^5	0.02-2.00	0.994	0.020	0.065	10.67	109.19	0.113
Butyric acid	3.24×10^6	3.73×10^5	0.015-6.00	0.992	0.108	0.360	4.07	107.94	0.970
Hexanoic acid	1.35×10^7	-7.23×10^5	0.002-20.00	0.998	0.004	0.012	7.83	110.70	0.510
Benzoic acid	1.75×10^5	-3.07×10^4	0.4-13.33	0.998	0.626	2.086	8.99	105.47	2.440
Octanoic acid	2.35×10^7	-6.23×10^5	0.002-13.33	0.999	0.002	0.007	8.83	82.71	0.190
Decanoic acid	4.47×10^7	-1.08×10^7	0.01-13.33	0.996	0.003	0.011	9.17	94.65	0.980
Dodecanoic acid	3.18×10^7	-1.66×10^7	0.04-20.00	0.995	0.005	0.017	10.49	77.01	2.440
Ethanol	1.43×10^5	1.87×10^7	50-2000	0.987	14.74	49.13	2.78	116.77	800
Isobutyl alcohol	4.65×10^6	5.67×10^5	0.05-1.00	0.980	0.036	0.120	7.25	92.79	0.120
Active amyl alcohol	3.08×10^7	1.93×10^6	0.02-1.00	0.950	0.008	0.026	4.41	93.68	0.110
Isoamyl alcohol	3.80×10^7	1.56×10^6	0.0025-1.00	0.982	0.006	0.022	8.58	92.94	0.060
2-Phenylethyl alcohol	6.25×10^6	1.39×10^4	0.002-0.267	0.995	0.001	0.004	9.76	112.74	0.011
Benzaldehyde	2.42×10^8	2.41×10^6	0.005-1.00	0.998	0.003	0.009	11.92	100.92	0.035
O-Tolualdehyde	1.86×10^8	-7.89×10^5	0.001-1.00	0.999	0.0004	0.0013	9.92	93.53	0.056
Ethyl hexanoate	7.17×10^8	4.66×10^6	0.0002-0.4	0.997	0.001	0.003	7.49	86.87	0.023
Ethyl octanoate	3.75×10^8	-2.15×10^6	0.0002-2.0	0.997	0.001	0.005	7.16	77.30	0.080
Ethyl decanoate	1.69×10^8	1.38×10^7	0.01-6.67	0.995	0.008	0.027	4.94	97.44	0.250
Ethyl dodecanoate	1.48×10^8	-6.74×10^6	0.01-6.67	0.997	0.003	0.010	2.61	132.81	0.250
Ethyl tetradecanoate	1.98×10^7	2.70×10^6	0.01-1.0	0.997	0.010	0.032	3.45	80.43	0.120
Isobutyl octanoate	2.33×10^8	-6.42×10^5	0.002-2.00	0.997	0.0004	0.0014	6.20	62.32	0.100
Isoamyl octanoate	5.67×10^7	2.81×10^4	0.0002-0.13	0.998	0.001	0.003	8.82	108.15	0.009
Ethyl acetate	2.42×10^7	4.35×10^6	0.02-1.0	0.953	0.022	0.072	5.55	95.67	0.120
Isobutyl acetate	3.14×10^8	2.64×10^6	0.0001-0.1	0.989	0.0003	0.0011	4.67	89.16	0.005
Active amyl acetate	5.01×10^8	9.00×10^5	0.0001-0.067	0.998	0.0003	0.0010	10.54	102.13	0.004
Isoamyl acetate	7.16×10^8	2.30×10^6	0.0001-0.2	0.996	0.001	0.002	9.27	79.86	0.009
2-Phenylethyl acetate	1.10×10^8	1.09×10^6	0.0001-0.2	0.996	0.001	0.002	3.88	113.63	0.009

Abbreviation: LOD = Limit of detection; LOQ = Limit of quantification.

Appendix B

Supplementary figures for Chapter 4 (Dynamics of volatile compounds during papaya juice fermentation by three commercial wine yeasts)

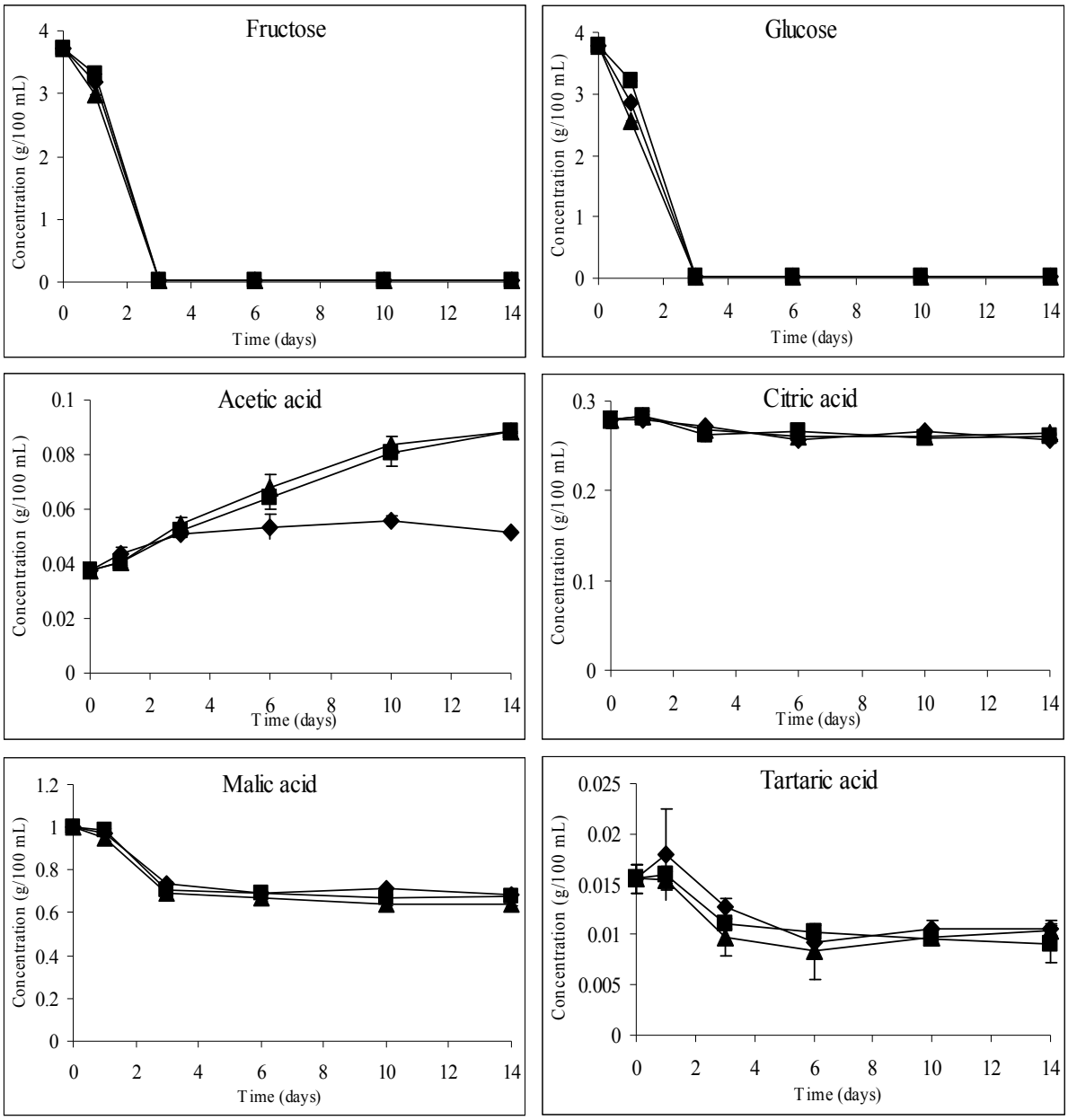

Fig. B1. Sugars and organic acids changes during papaya juice fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

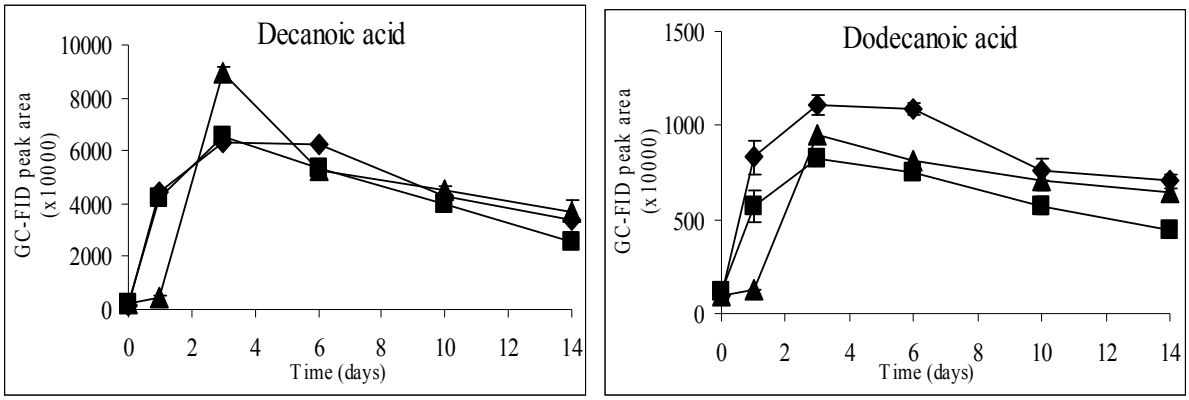

Fig. B2. Changes of dodecanoic acid in papaya wine during fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

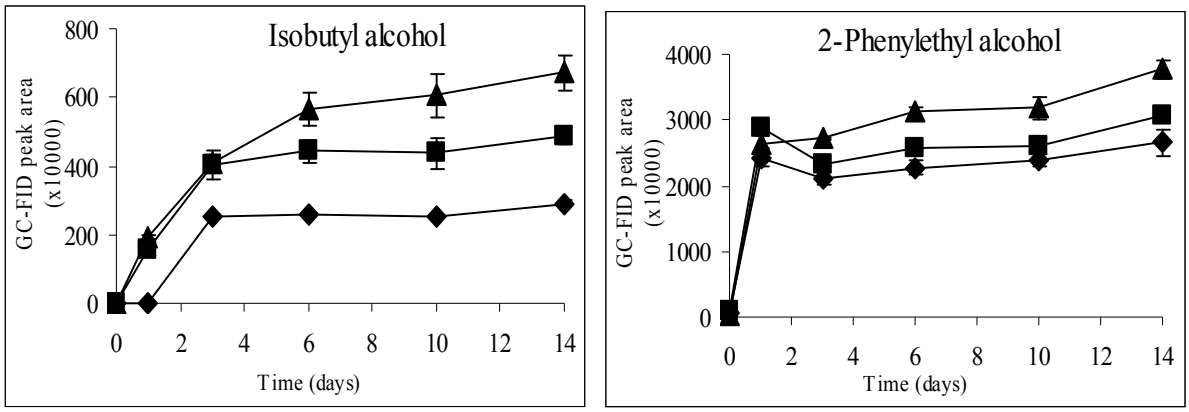

Fig. B3. Changes of higher alcohols in papaya wine during fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

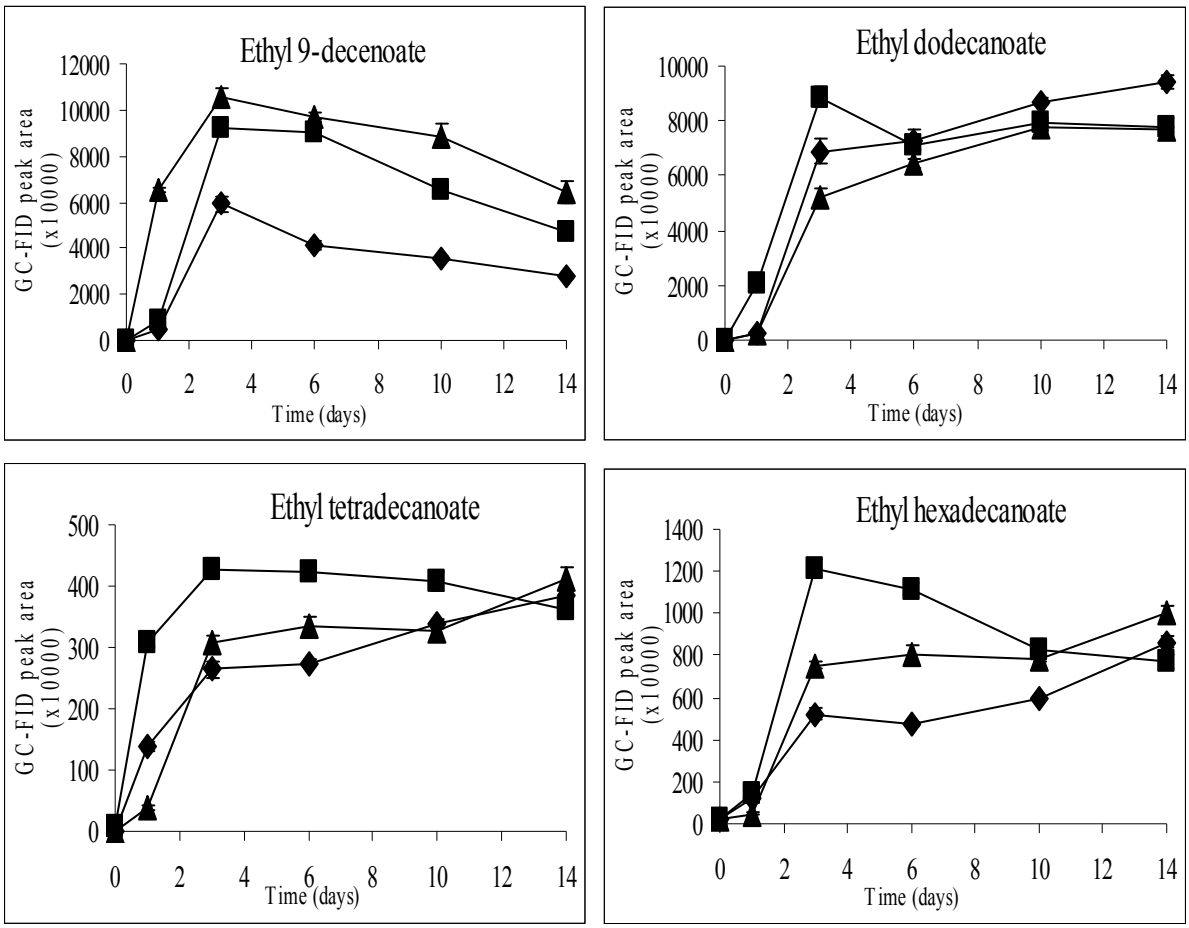

Fig. B4. Changes of ethyl esters in papaya wine during fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

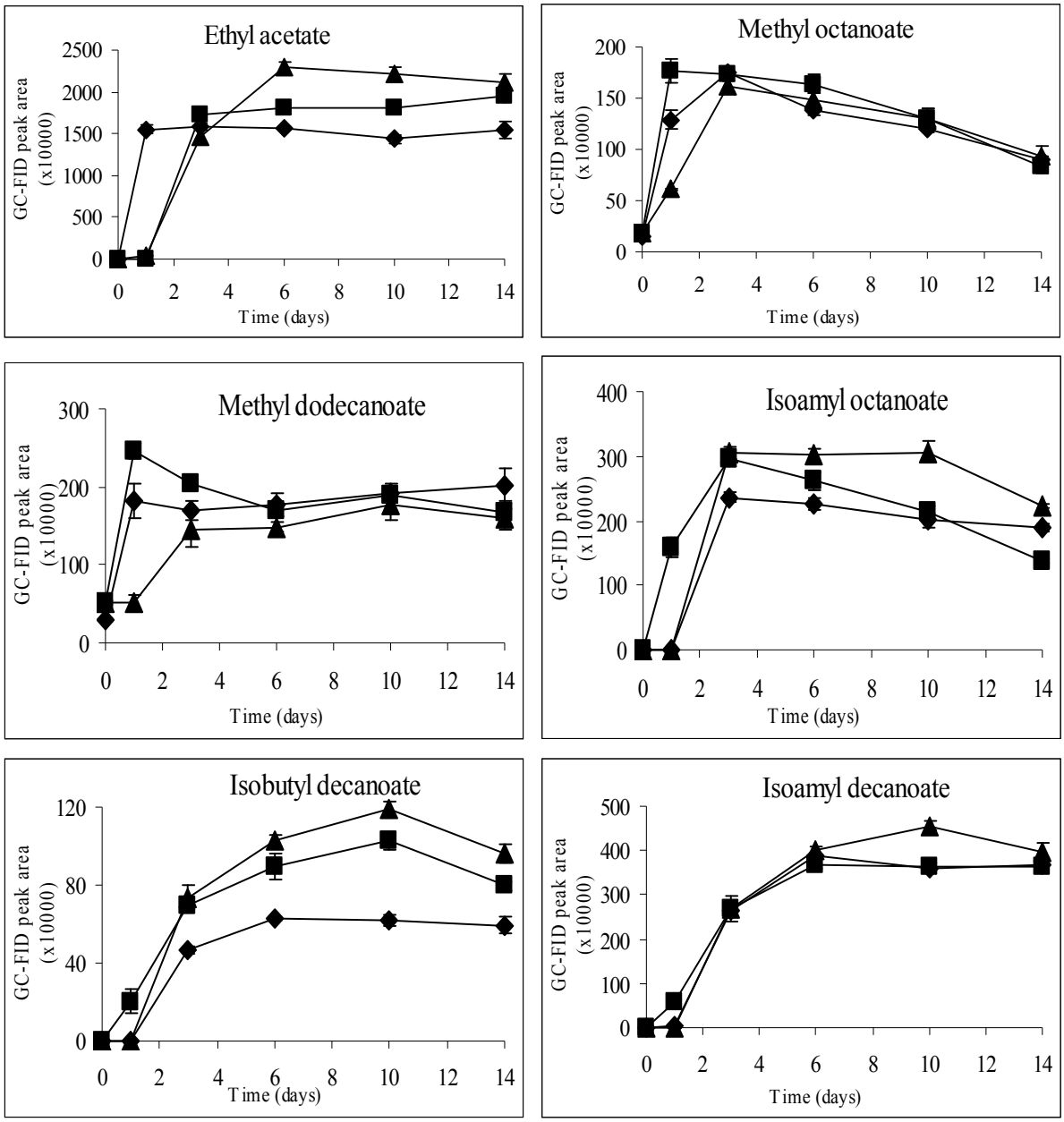

Fig. B5. Changes of ethyl acetate, methyl and other esters in papaya wine during fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

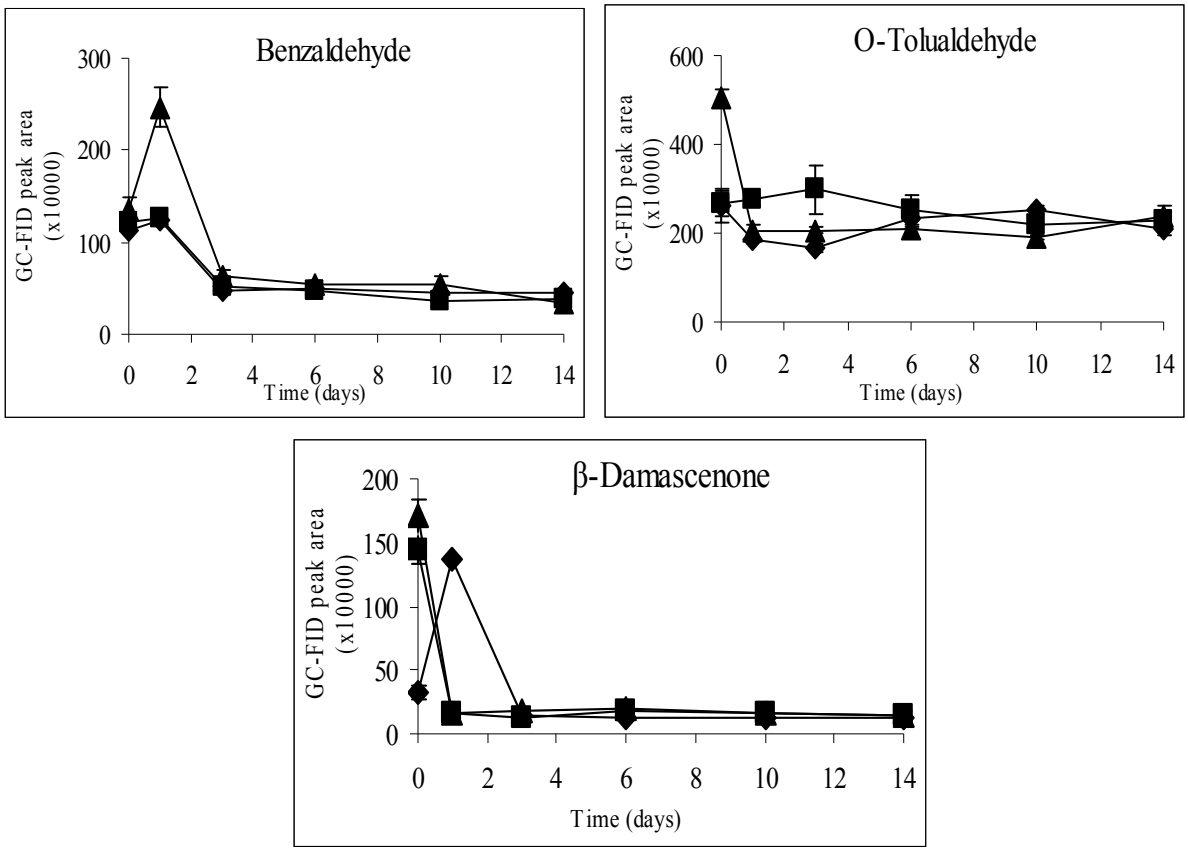

Fig. B6. Changes of aldehydes and β-damascenone in papaya wine during fermentation by three commercial wine yeasts: *S. cerevisiae* var. *bayanus* EC-1118 (♦), *S. cerevisiae* var. *bayanus* R2 (▲) and *S. cerevisiae* MERIT.ferm (■). (Error bars = standard deviation).

Appendix C

Supplementary figures for Chapter 5 (Evolution of volatile compounds in papaya wine fermented with three *Williopsis saturnus* yeasts)

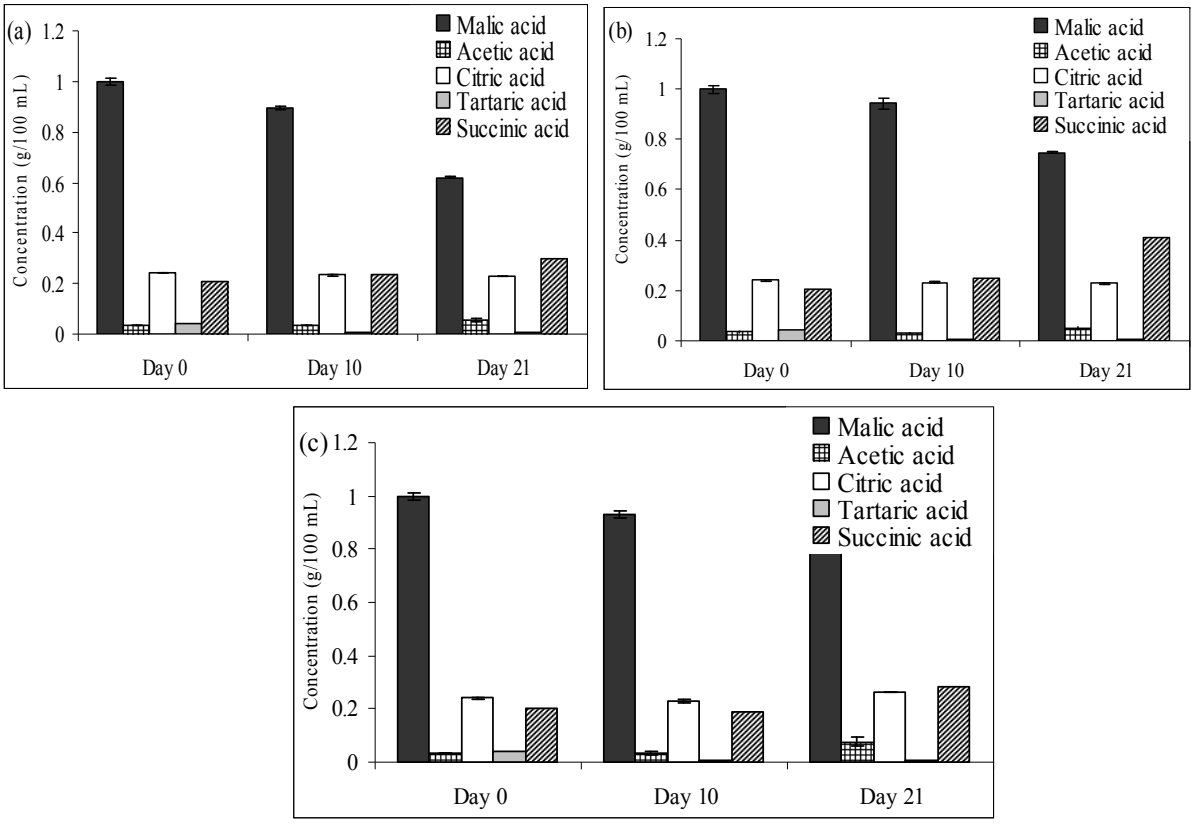

Fig. C1. Organic acids changes during papaya juice fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (a), *W. saturnus* var. *mrakii* NCYC2251 (b) and *W. saturnus* var. *sargentensis* NCYC2727 (c). (Error bars = standard deviation).

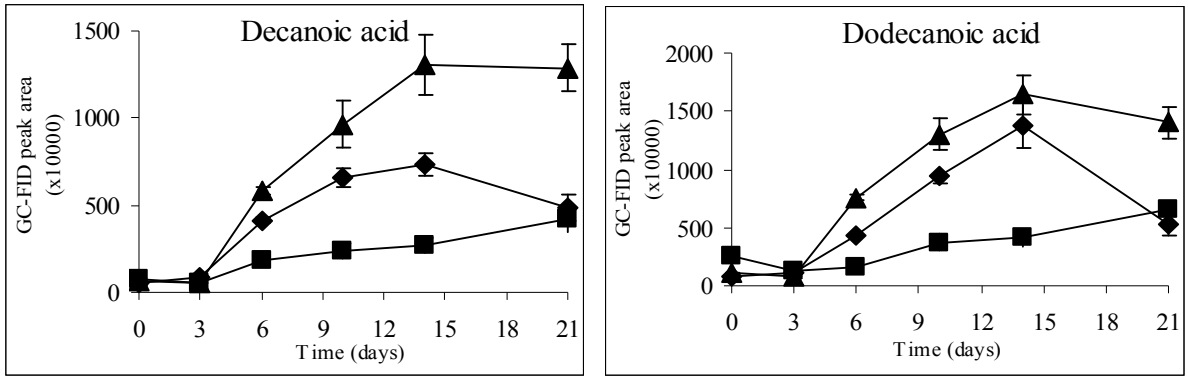

Fig. C2. Changes of fatty acids in papaya wine during fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (♦), *W. saturnus* var. *mrakii* NCYC2251 (▲) and *W. saturnus* var. *sargentensis* NCYC2727 (■). (Error bars = standard deviation).

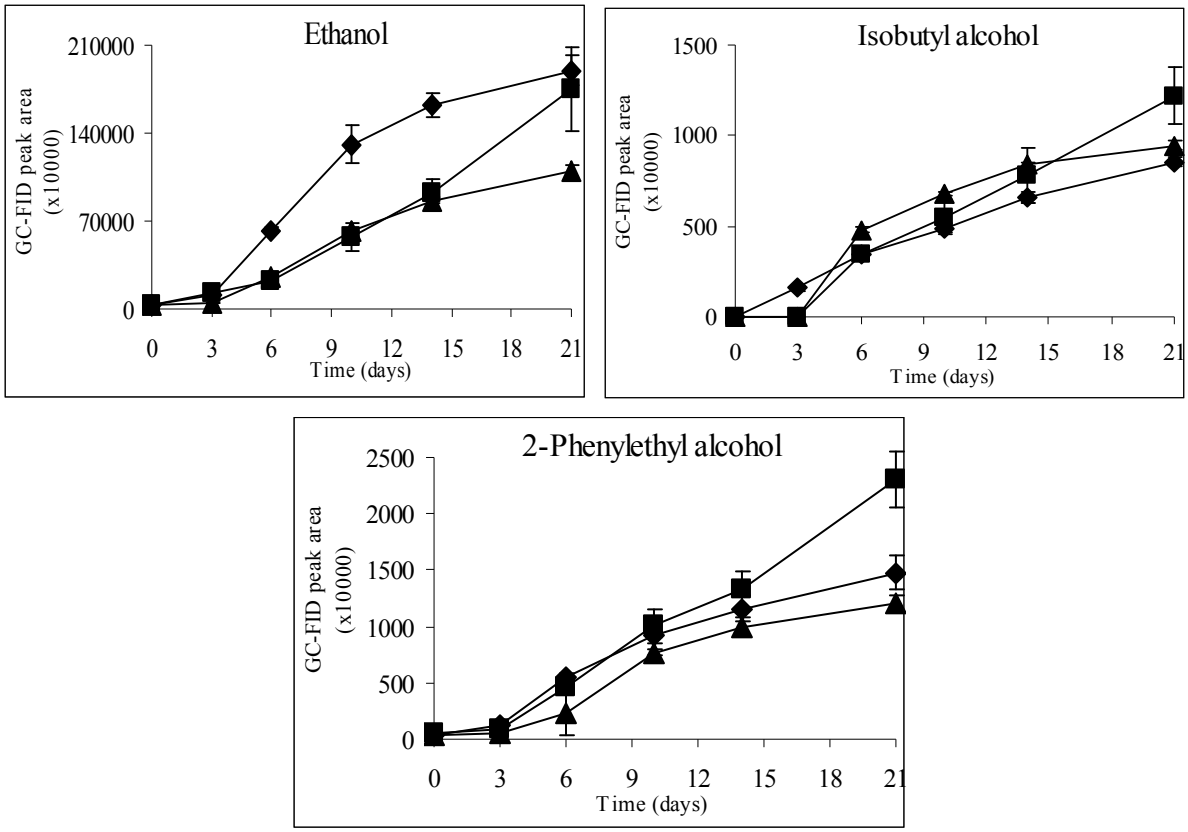

Fig. C3. Changes of alcohols in papaya wine during fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (♦), *W. saturnus* var. *mrakii* NCYC2251 (▲) and *W. saturnus* var. *sargentensis* NCYC2727 (■). (Error bars = standard deviation).

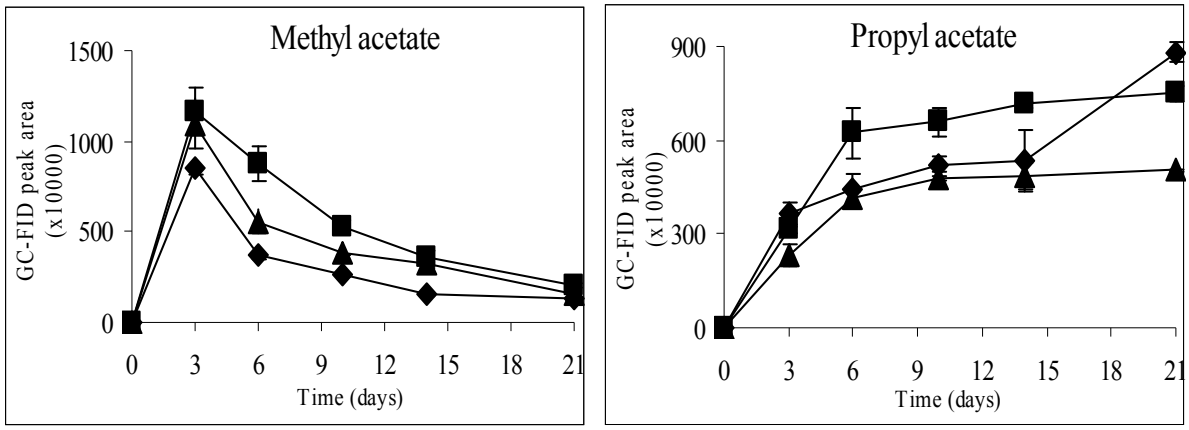

Fig. C4. Changes of acetate esters in papaya wine during fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (♦), *W. saturnus* var. *mrakii* NCYC2251 (▲) and *W. saturnus* var. *sargentensis* NCYC2727 (■). (Error bars = standard deviation).

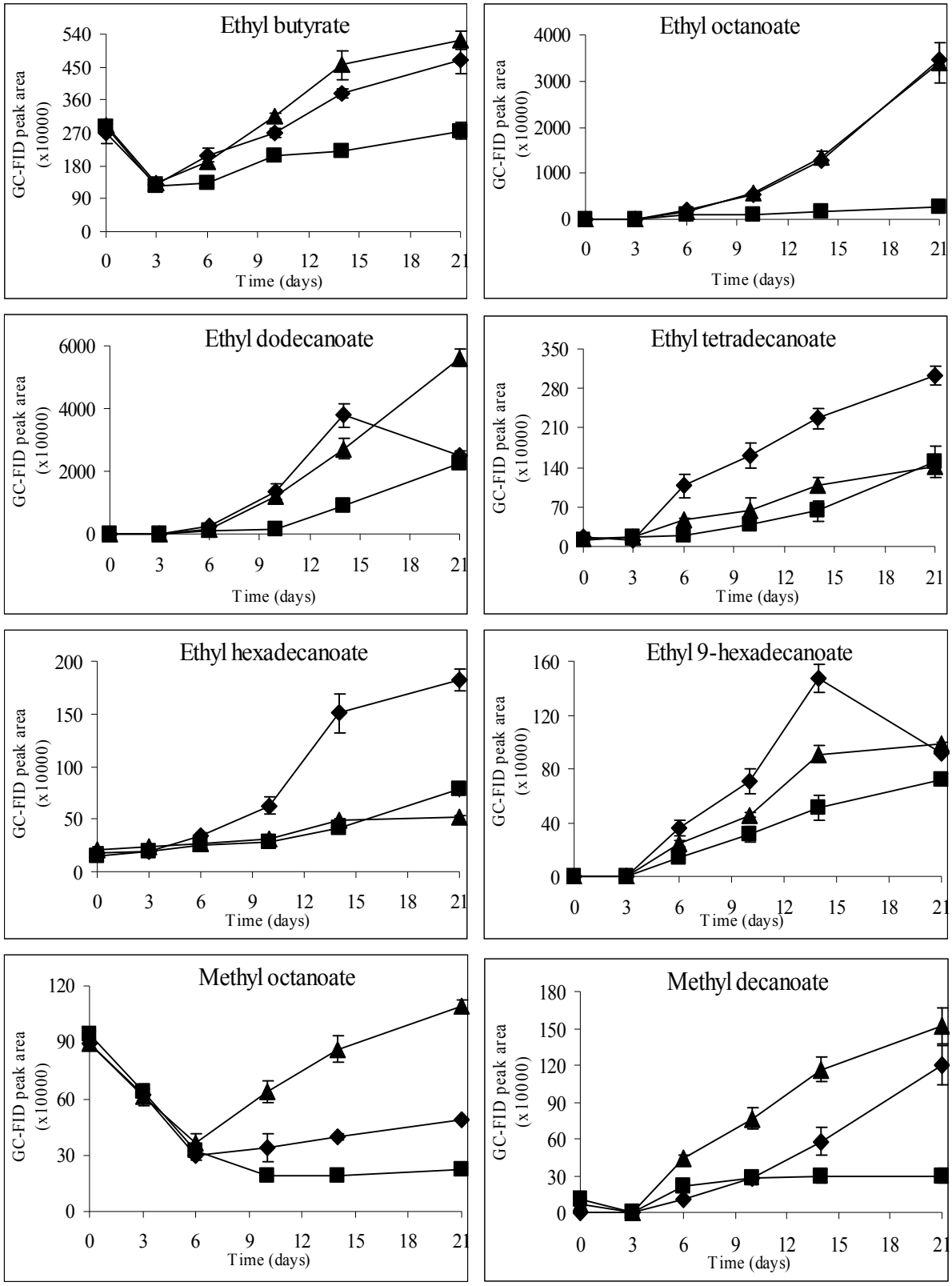

Fig. C5. Changes of ethyl and methyl esters in papaya wine during fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (♦), *W. saturnus* var. *mrakii* NCYC2251 (▲) and *W. saturnus* var. *sargentensis* NCYC2727 (■). (Error bars = standard deviation).

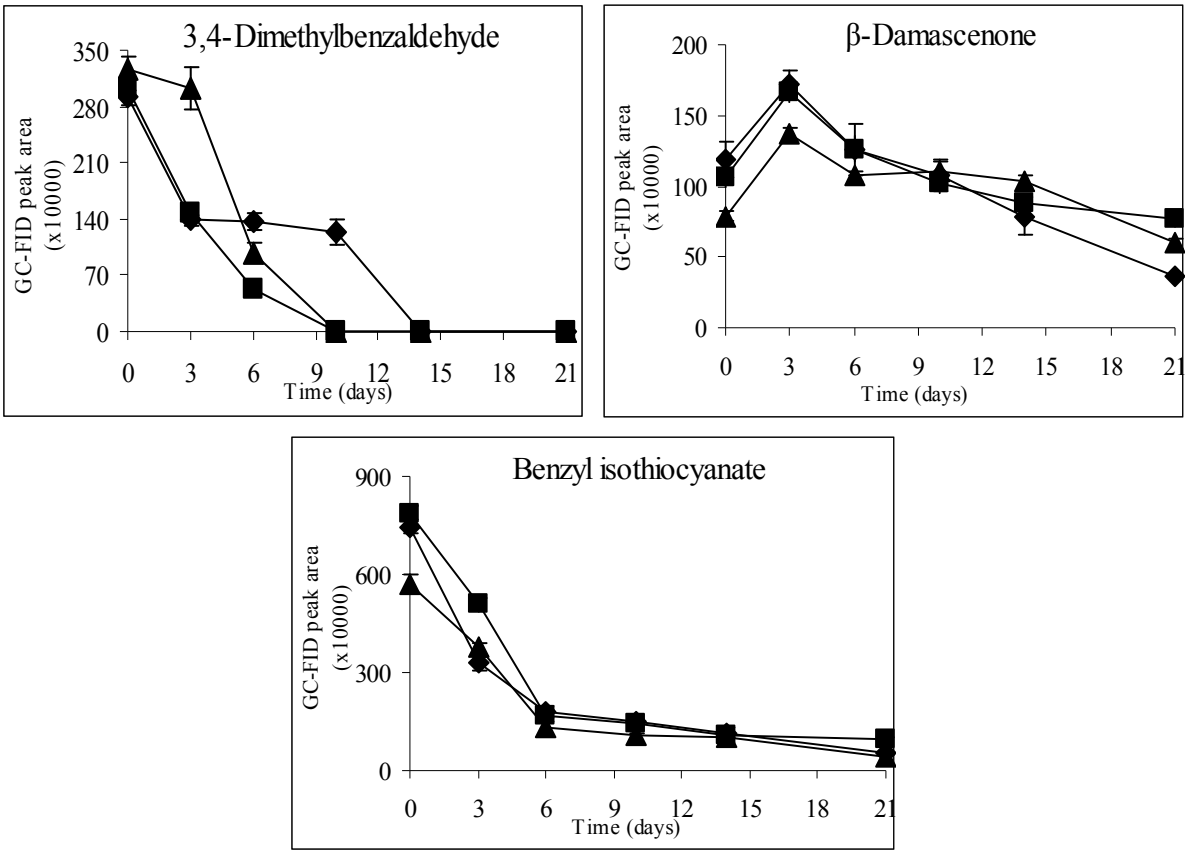

Fig. C6. Changes of 3,4-dimethylbenzaldehyde, β-damascenone and benzyl isothiocyanate in papaya wine during fermentation by three *W. saturnus* yeasts: *W. saturnus* var. *saturnus* NCYC22 (♦), *W. saturnus* var. *mrakii* NCYC2251 (▲) and *W. saturnus* var. *sargentensis* NCYC2727 (■). (Error bars = standard deviation).

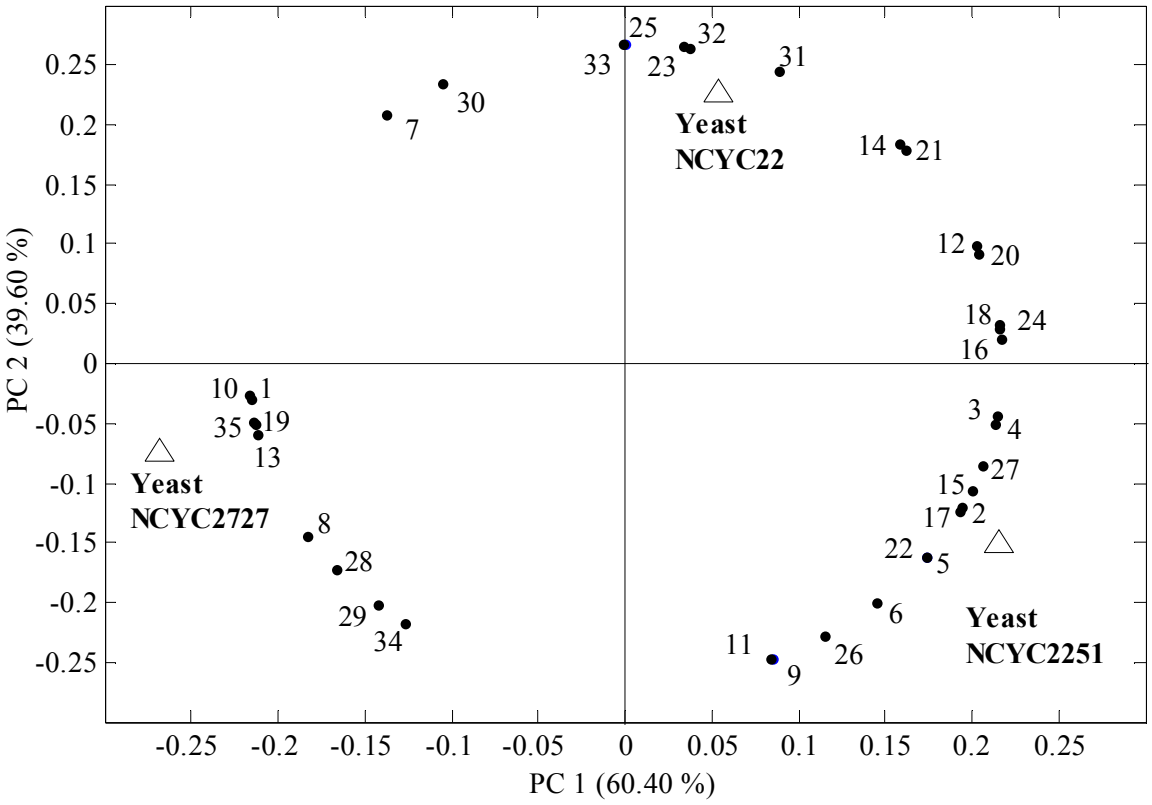

Fig. C7. Bi-plot of principal component analysis of the major volatile compounds in papaya wine fermented with three *W. saturnus* yeasts. The major volatile compounds and numbers are given in Table 5.2.

Appendix D

Supplementary figures for Chapter 6 (Impact of amino acid addition on aroma compounds in papaya wine fermented with *Williopsis saturnus* var. *mrakii* NCYC2251.)

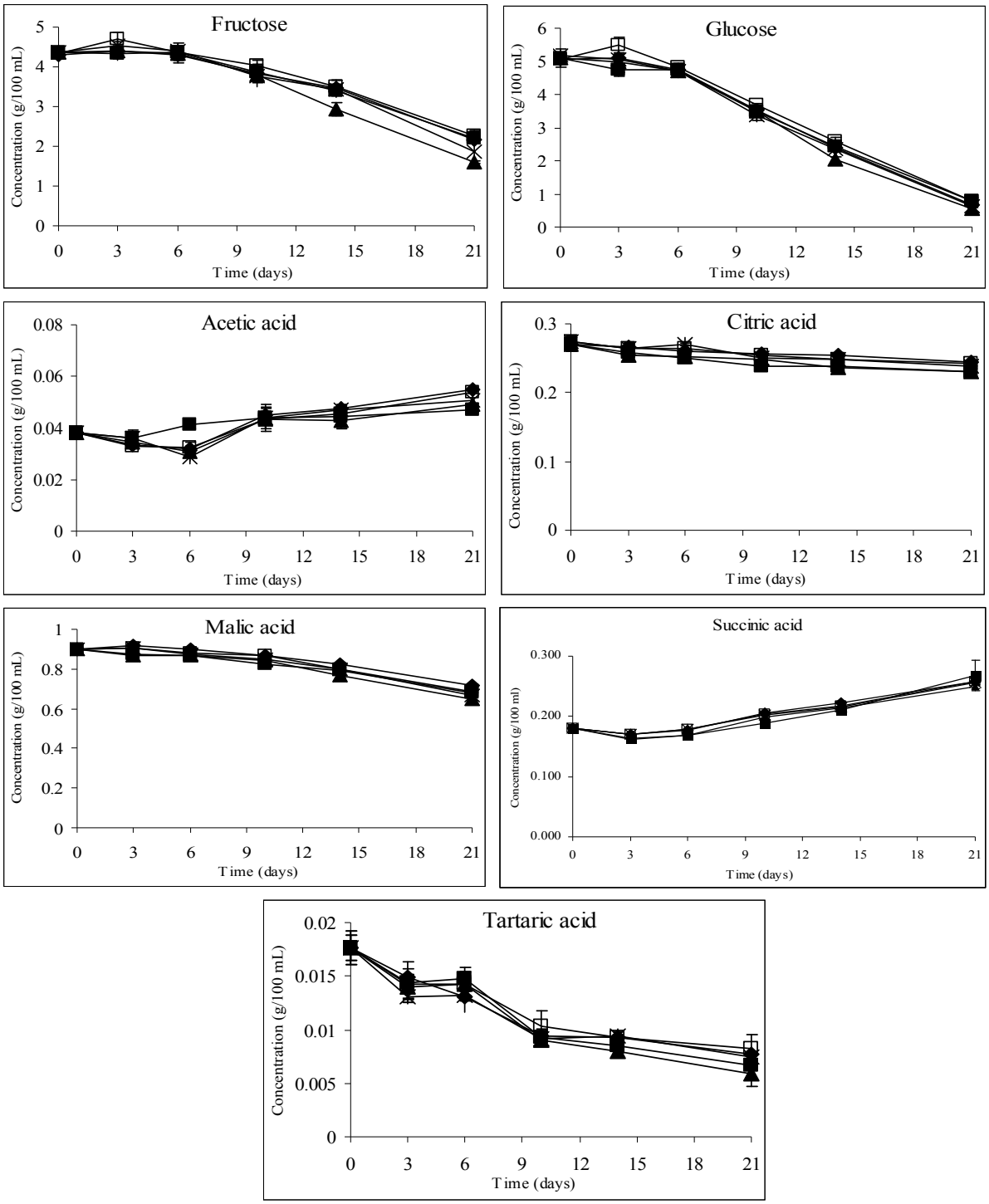

Fig. D1. Sugar consumption and organic acids changes in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (∗); 0.05% isoleucine (□). (Error bars = standard deviation).

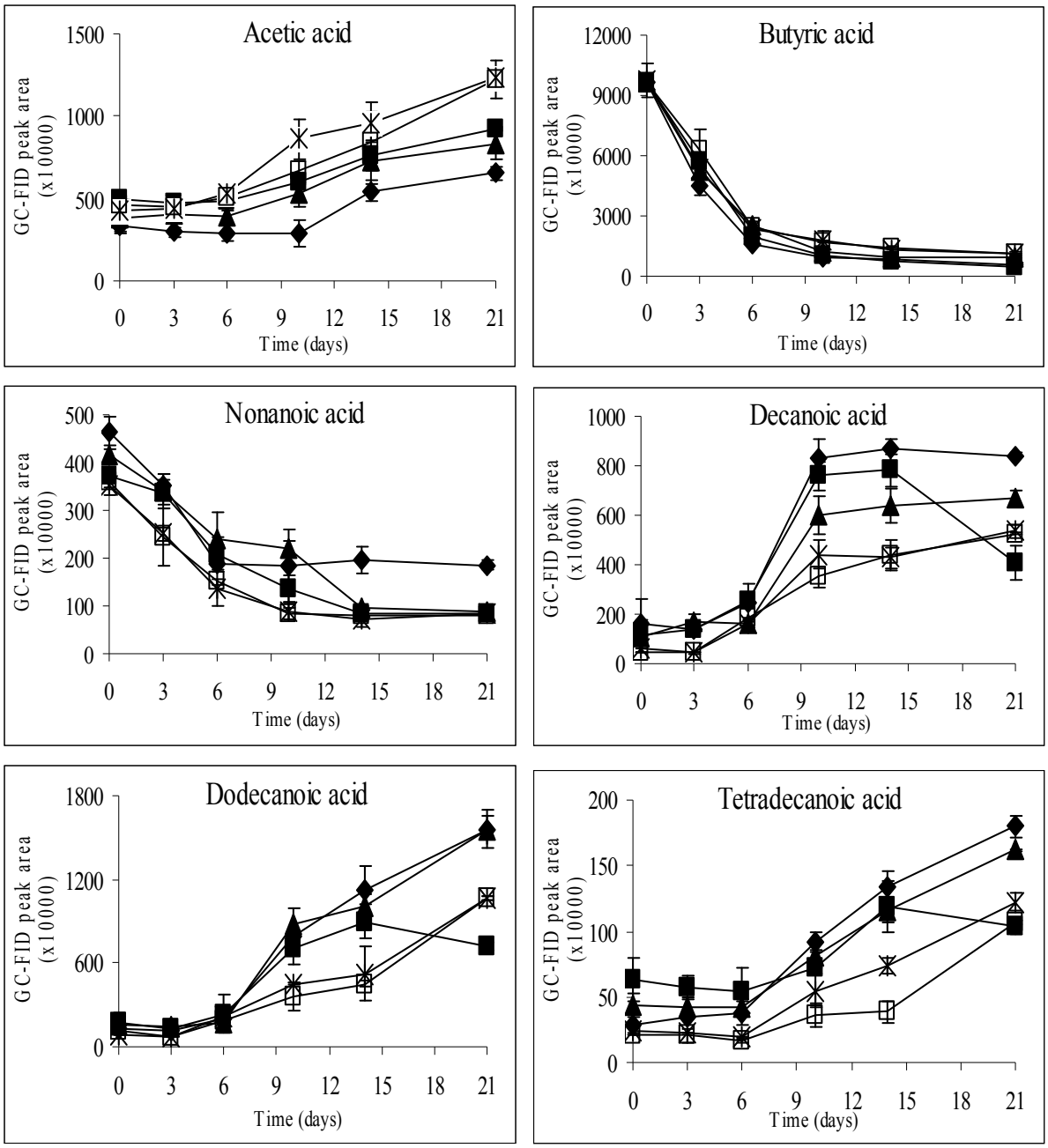

Fig. D2. Changes in fatty acids in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (∗); 0.05% isoleucine (□). (Error bars = standard deviation).

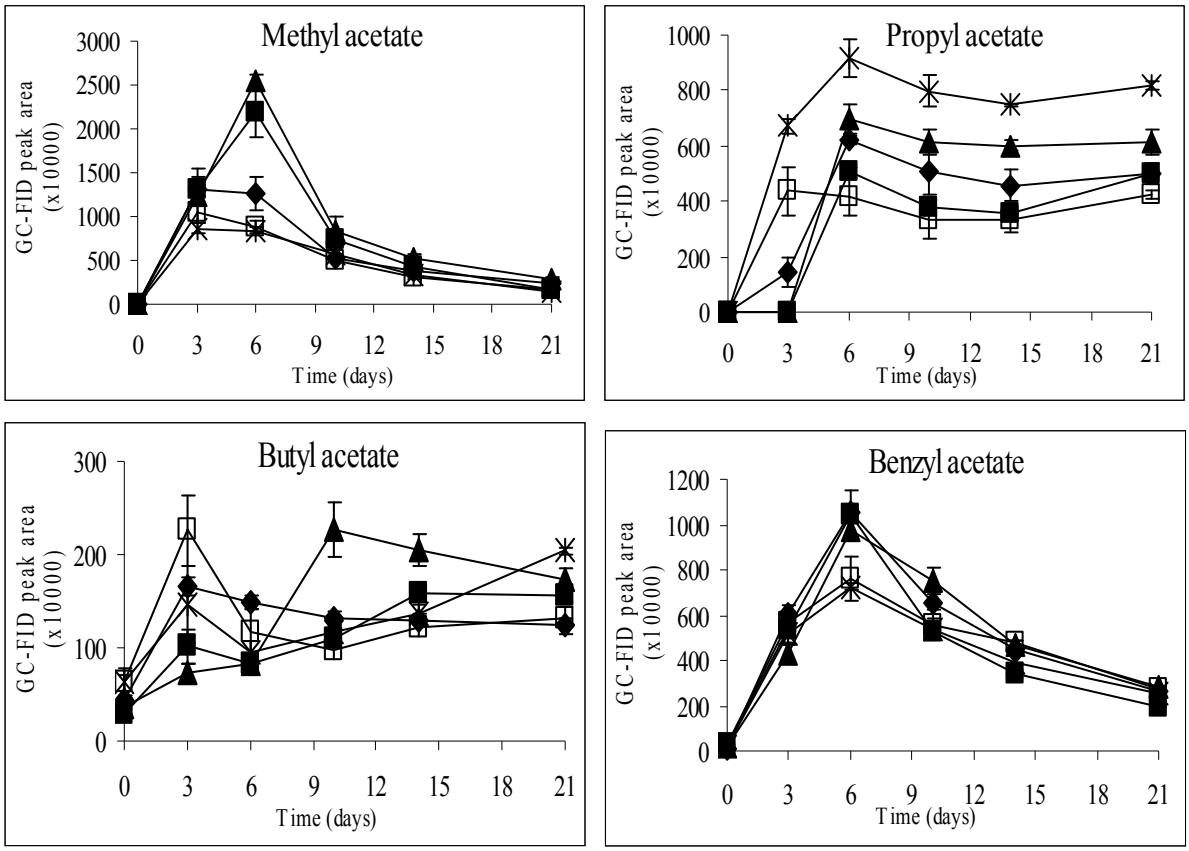

Fig. D3. Changes in acetate esters in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (∗); 0.05% isoleucine (□). (Error bars = standard deviation).

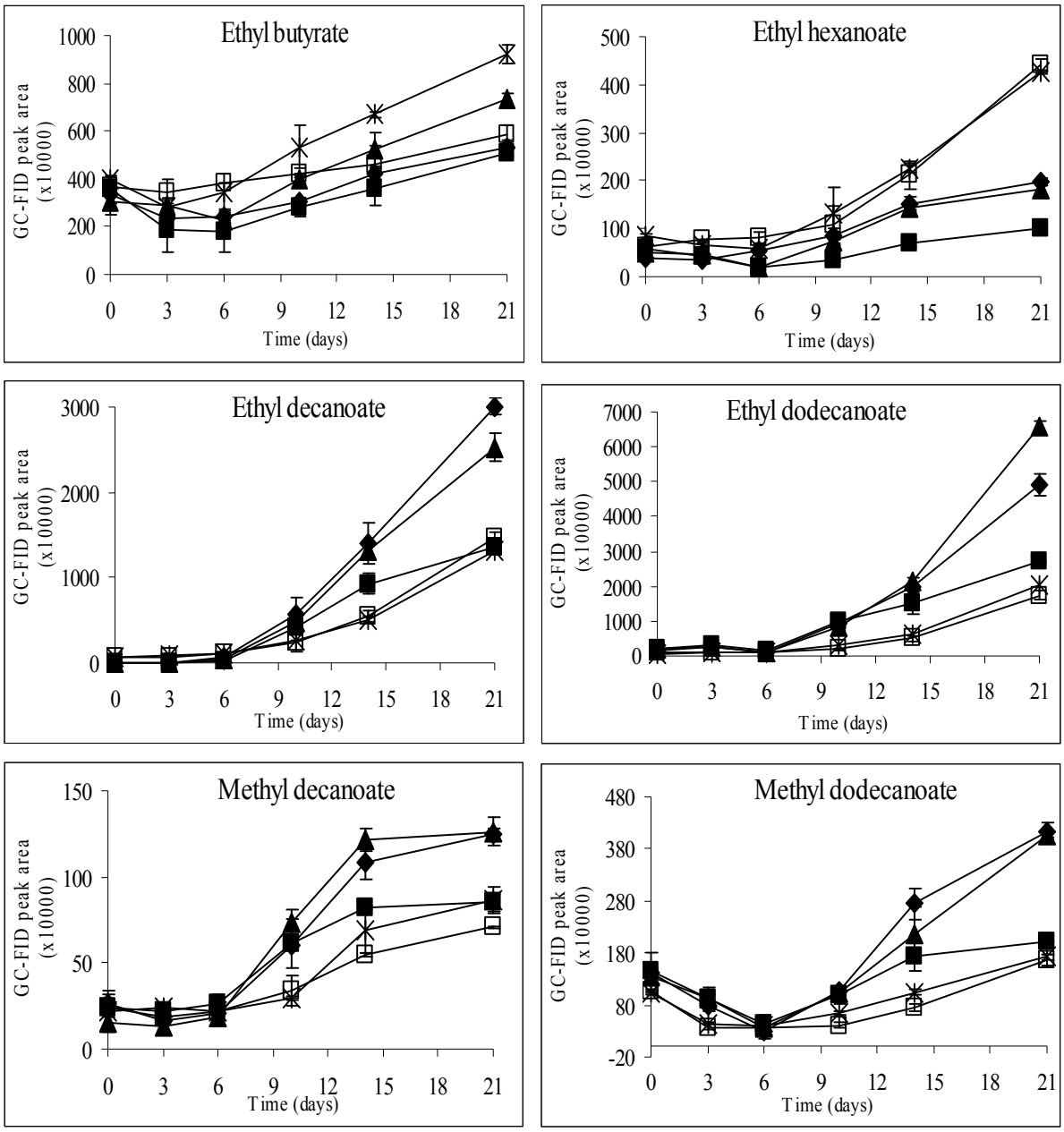

Fig. D4. Changes in ethyl and methyl esters in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (∗); 0.05% isoleucine (□). (Error bars = standard deviation).

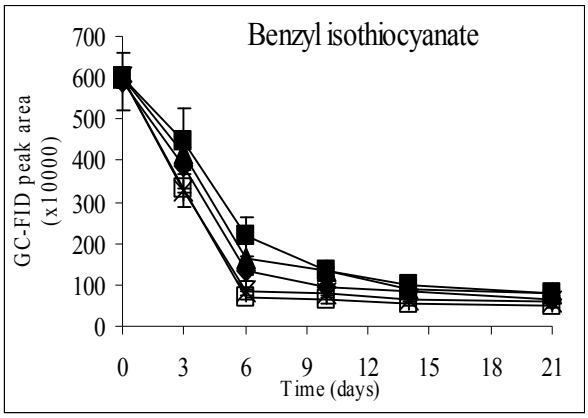

Fig. D5. Changes in benzyl isothiocyanate in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). Control (♦); 0.05% valine (▲); 0.05% phenylalanine (■); 0.05% leucine (∗); 0.05% isoleucine (□). (Error bars = standard deviation).

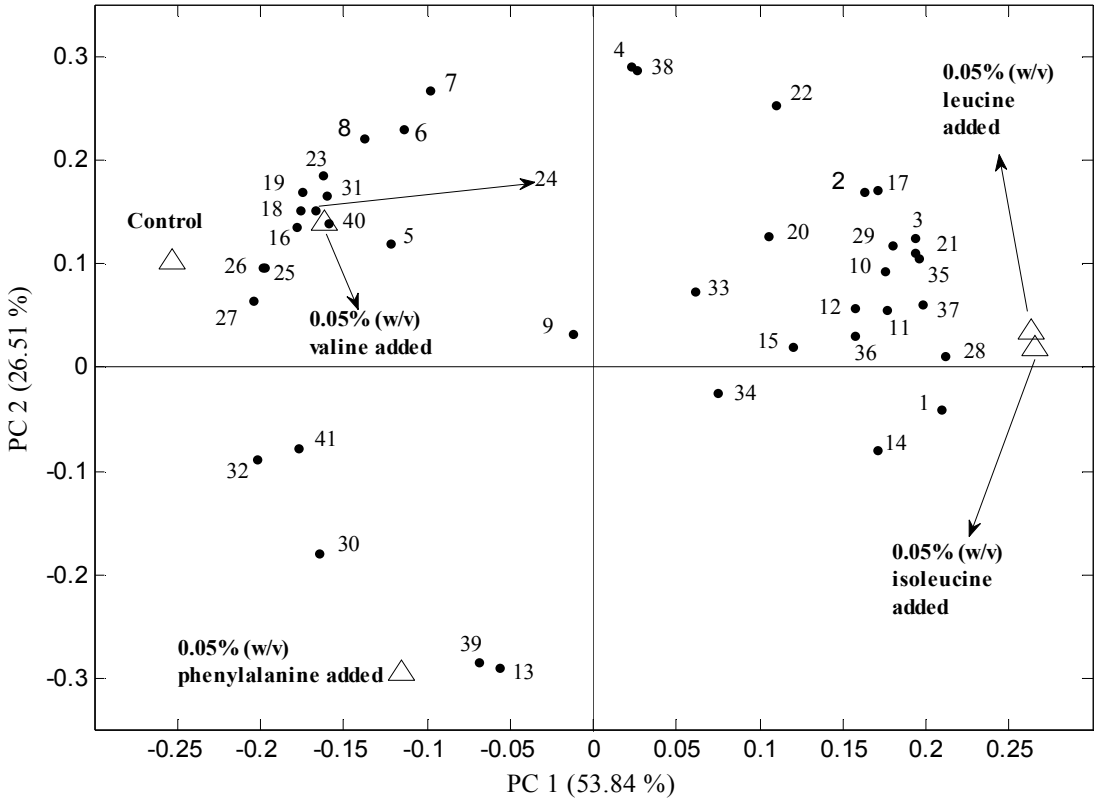

Fig. D6. Bi-plot of principal component analysis of the major volatile compounds in papaya wine fermented by *W. saturnus* var. *mrakii* NCYC2251 with different amino acids added (w/v). The major volatile compounds and numbers are given in Table 6.2.

Appendix E

Supplementary figures for Chapter 7 (Effect of fusel oil addition on volatile compounds in papaya wine fermented with *Williopsis saturnus* var. *mrakii* NCYC2251)

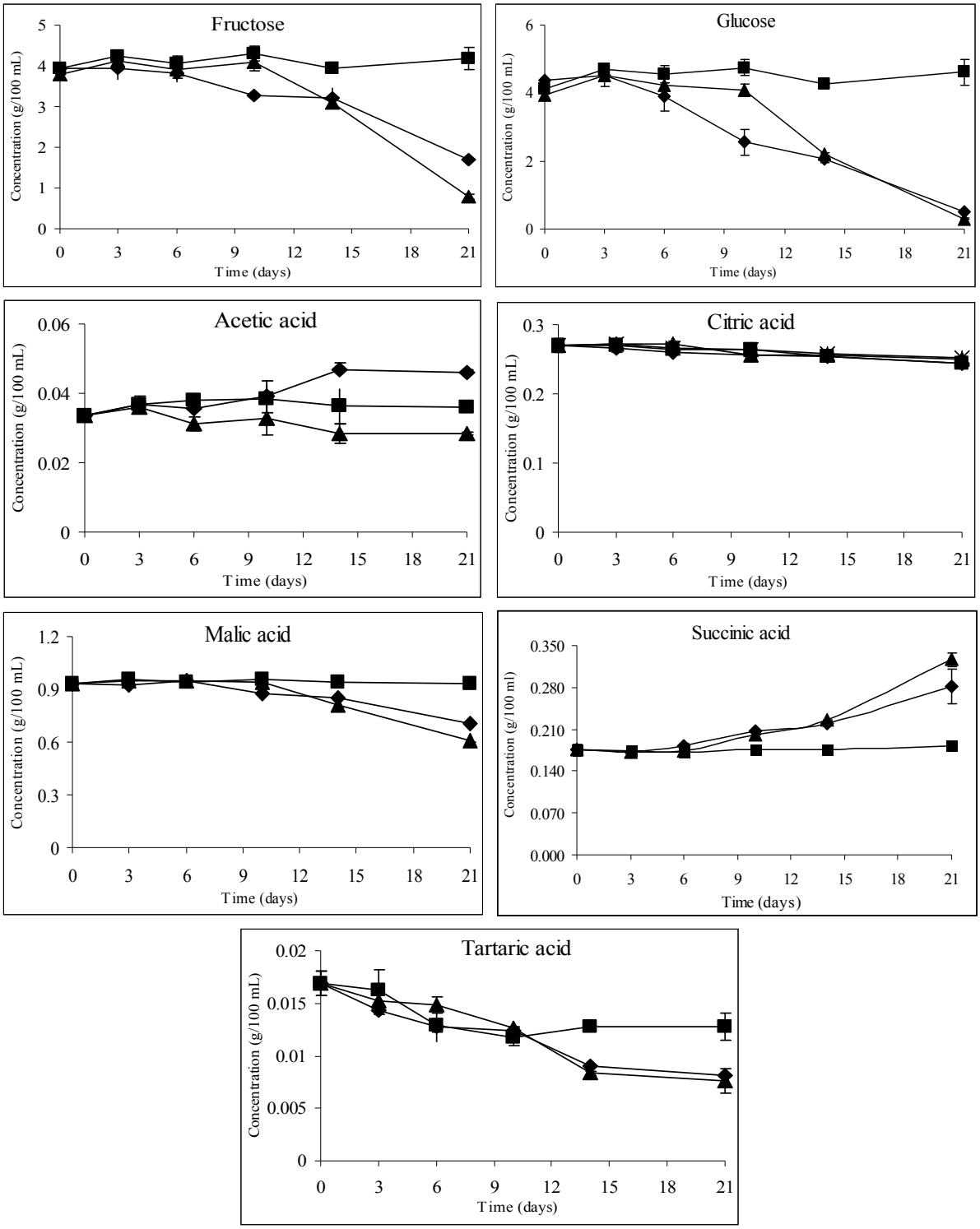

Fig. E1. Sugar consumption and organic acids changes in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

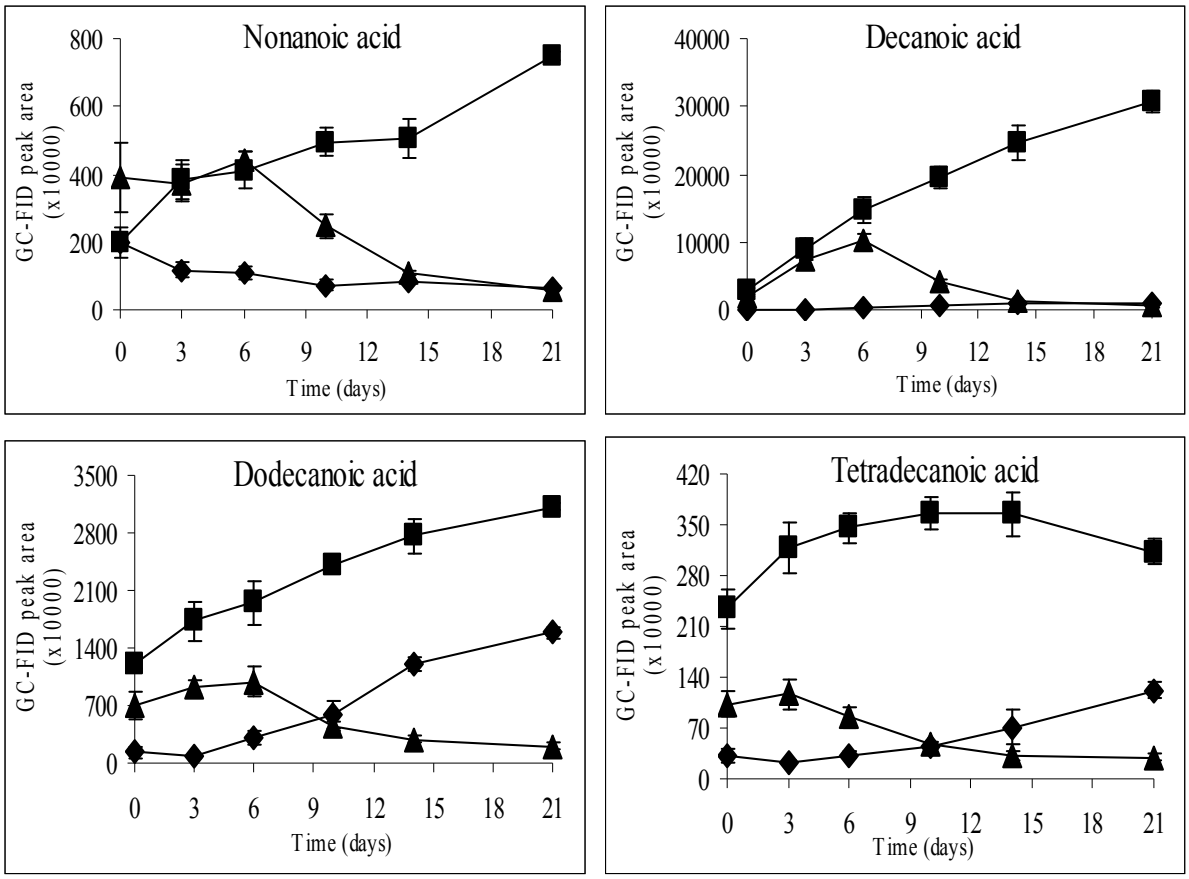

Fig. E2. Changes in fatty acids in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

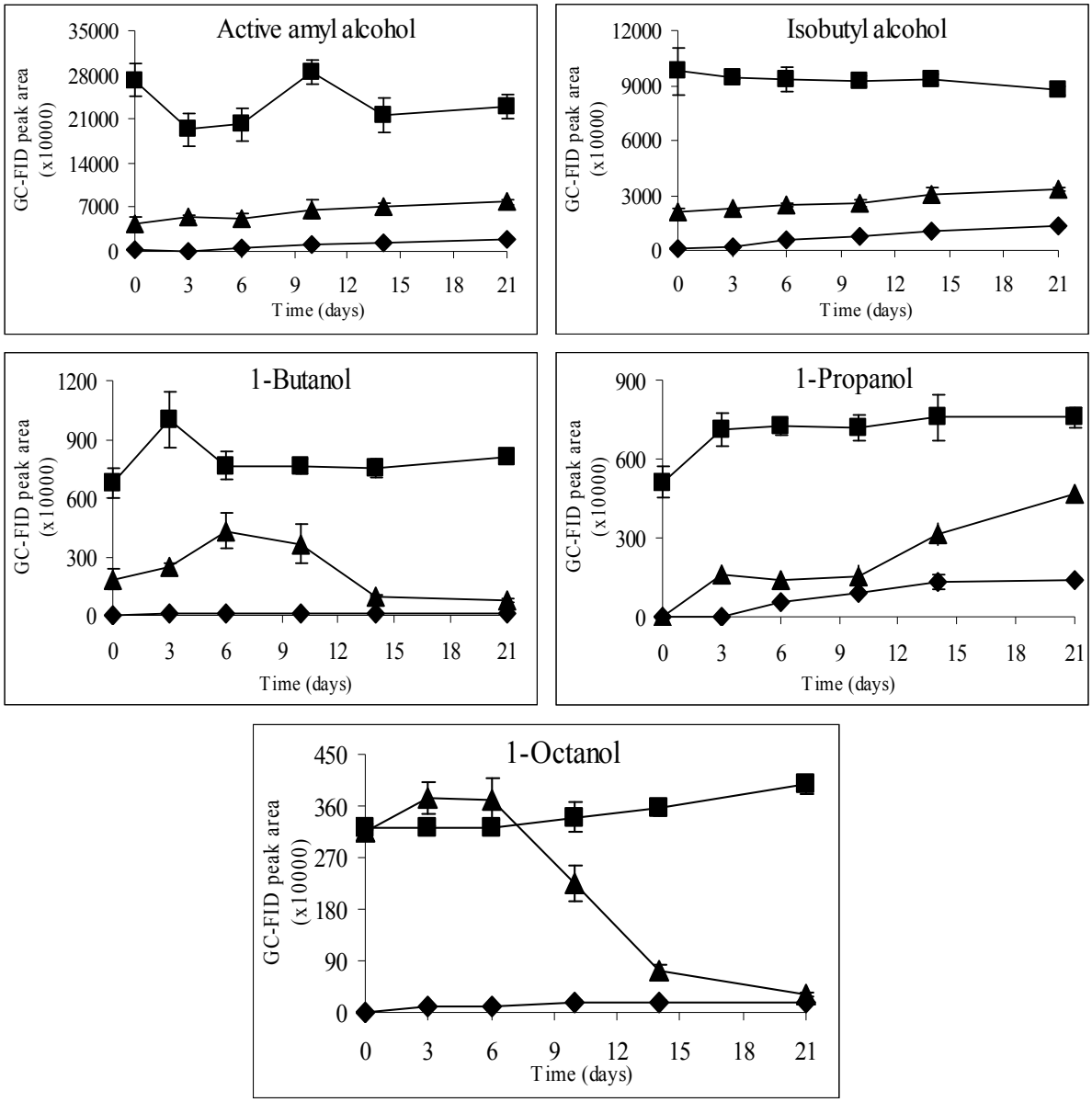

Fig. E3. Changes in alcohols in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

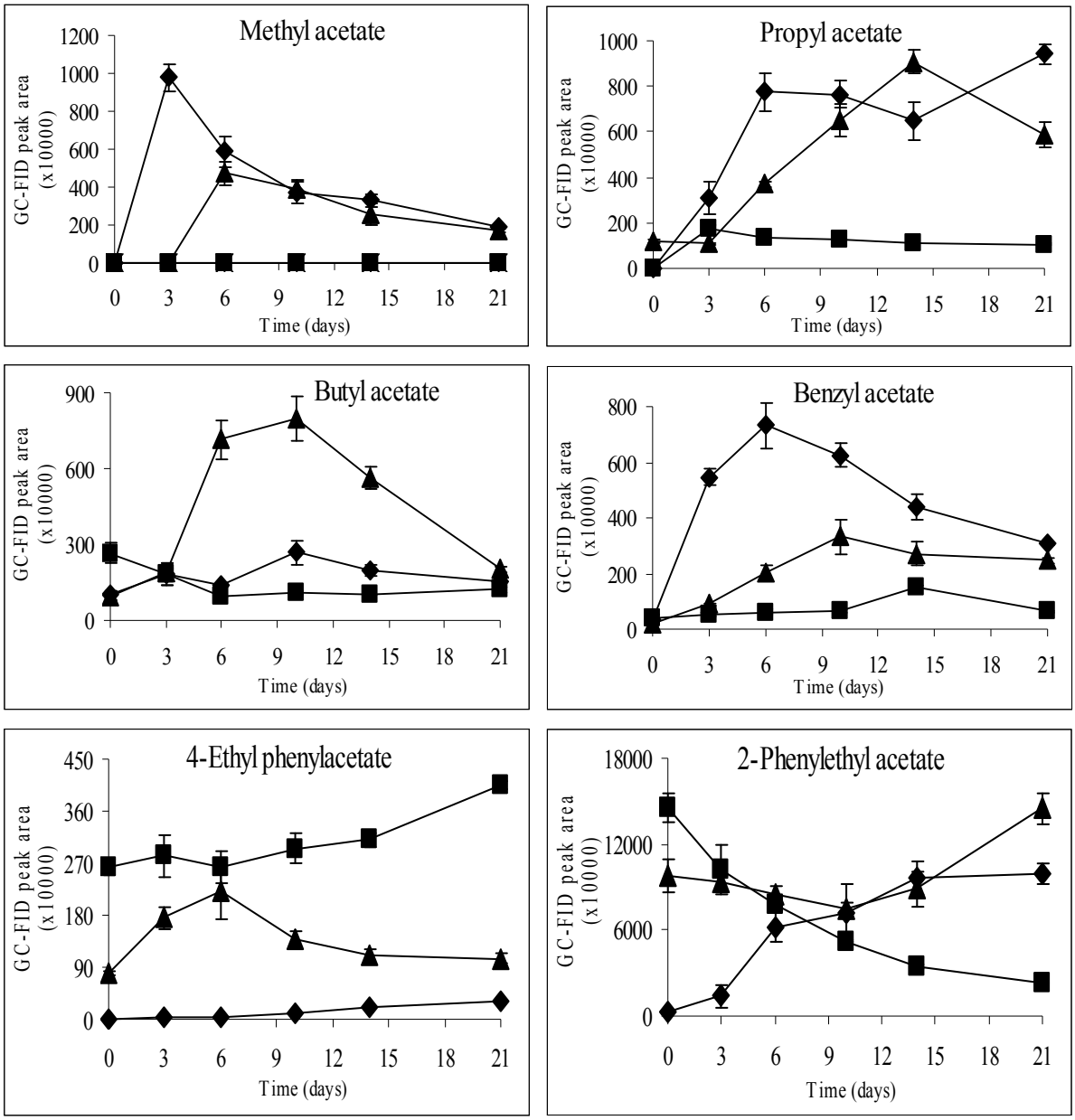

Fig. E4. Changes in acetate esters in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

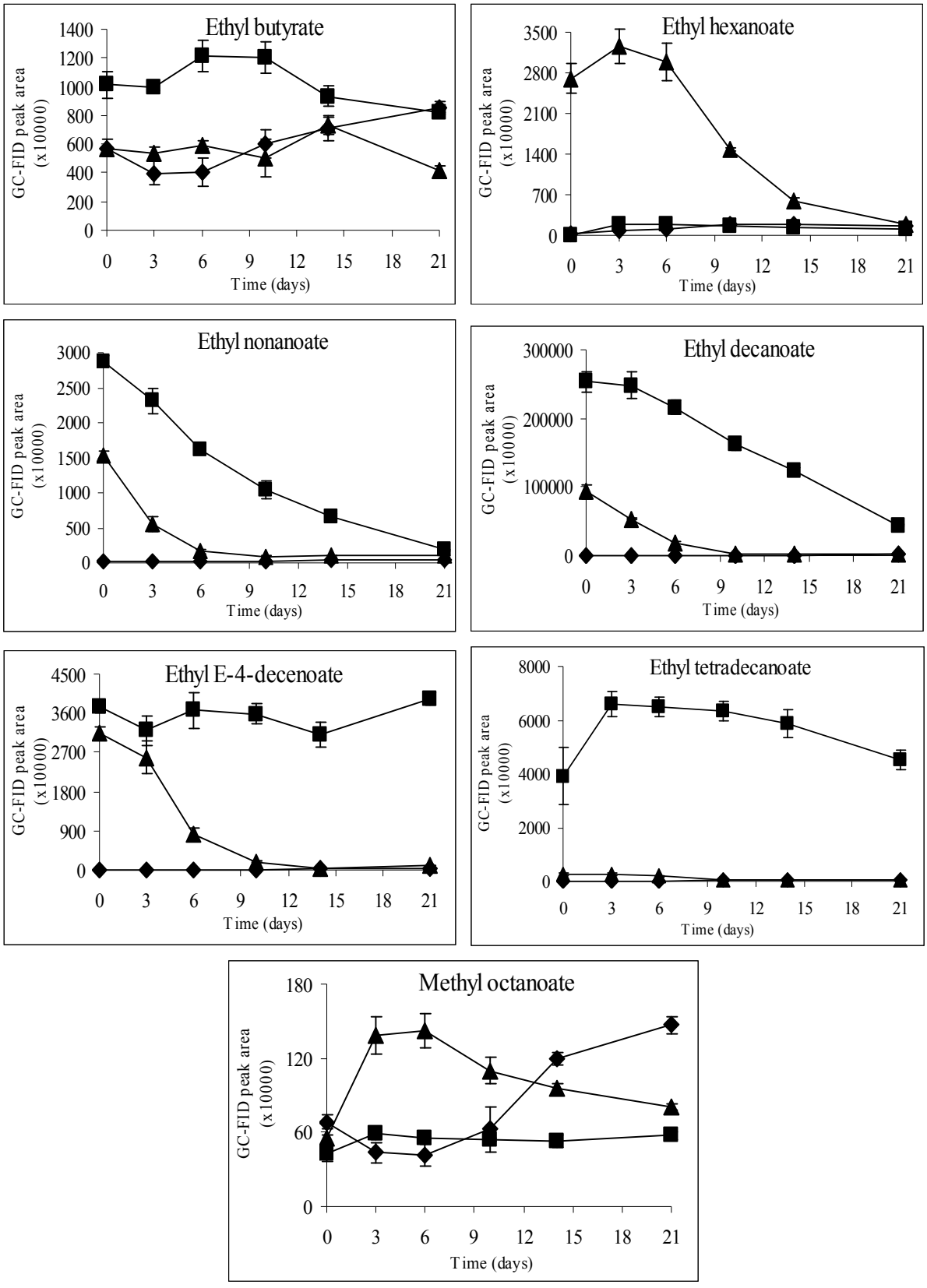

Fig. E5. Changes in ethyl esters and methyl octanoate in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

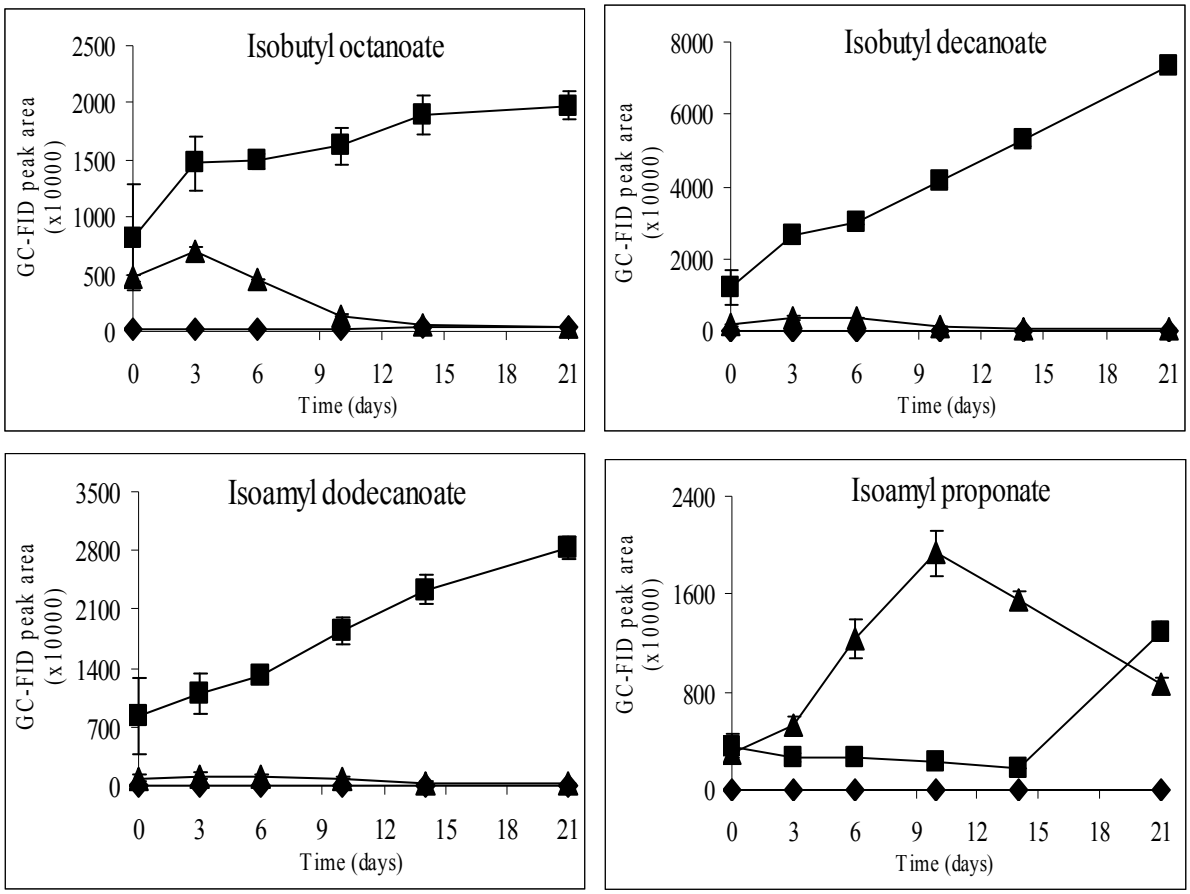

Fig. E6. Changes in other esters in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

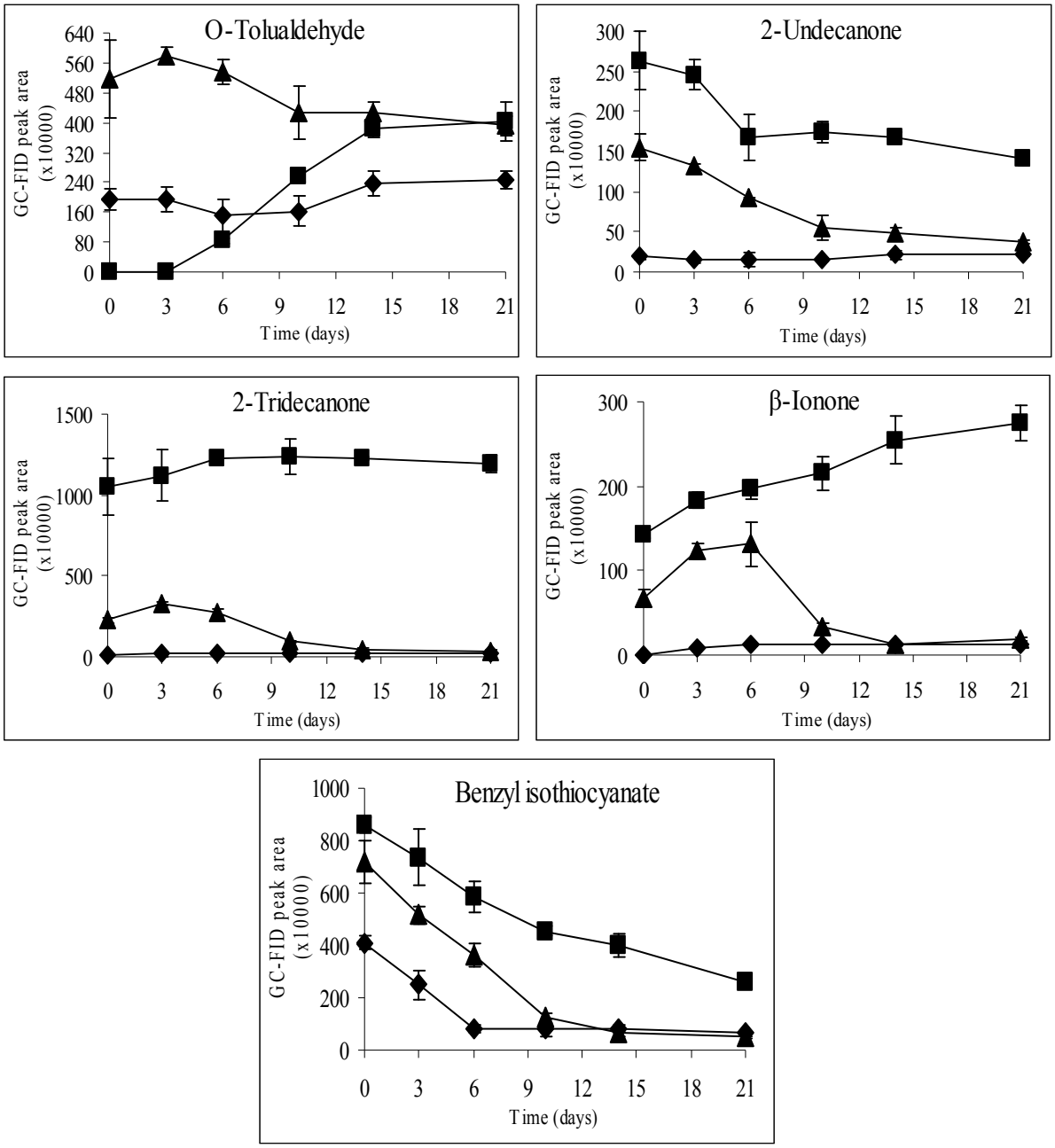

Fig. E7. Changes in O-tolualdehyde, ketones and benzyl isothiocyanate in papaya wine during fermentation by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. Control (♦); 0.1% (v/v) (▲); 0.5% (v/v) (■). (Error bars = standard deviation).

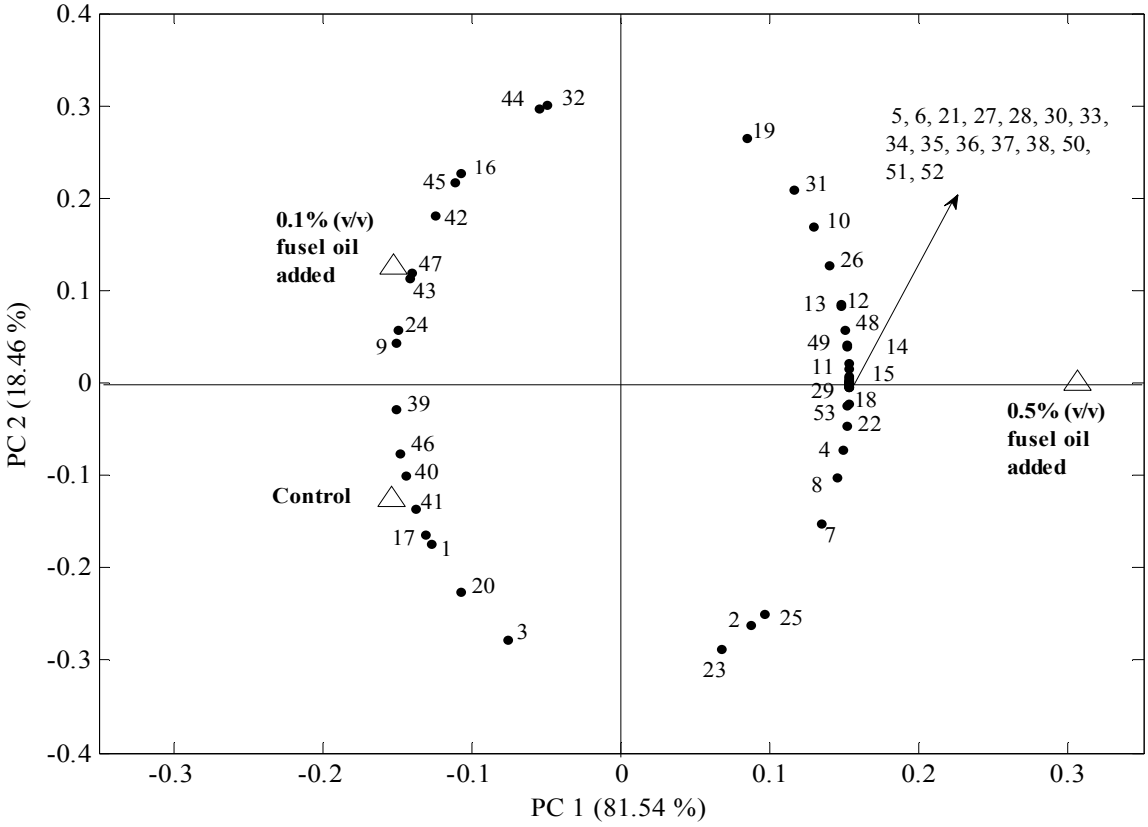

Fig. E8. Bi-plot of principal component analysis of the major volatile compounds in papaya wine fermented by *W. saturnus* var. *mrakii* NCYC2251 with different concentrations of fusel oil added. The major volatile compounds and numbers are given in Table 7.3.

Appendix F

Supplementary figures for Chapter 8 (Profile of volatile compounds during papaya juice fermentation by a mixed-culture of *Saccharomyces cerevisiae* var. *bayanus* R2 and *Williopsis saturnus* var. *mrakii* NCYC2251)

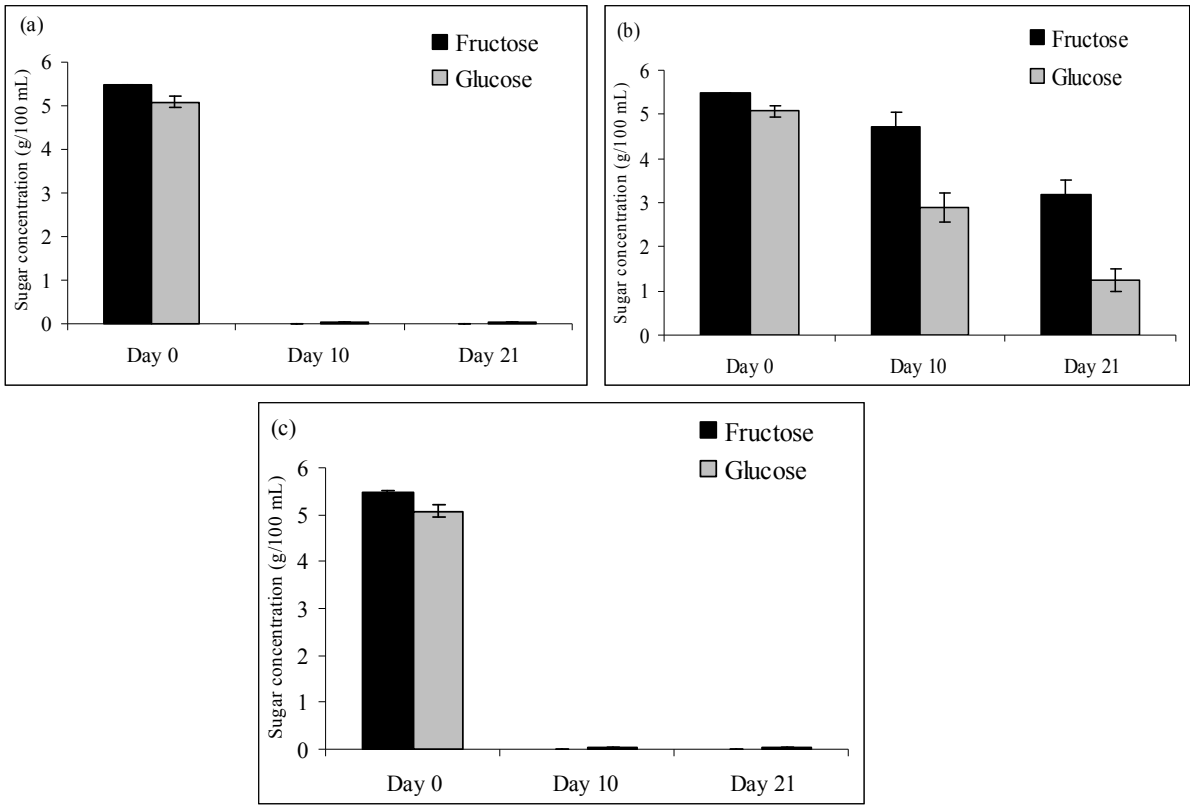

Fig. F1. Sugar consumption by yeasts in papaya wine during mixed culture fermentation. *S. cerevisiae* var. *bayanus* R2 (a); *W. saturnus* var. *mrakii* NCYC2251 (b); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (c). (Error bars = standard deviation).

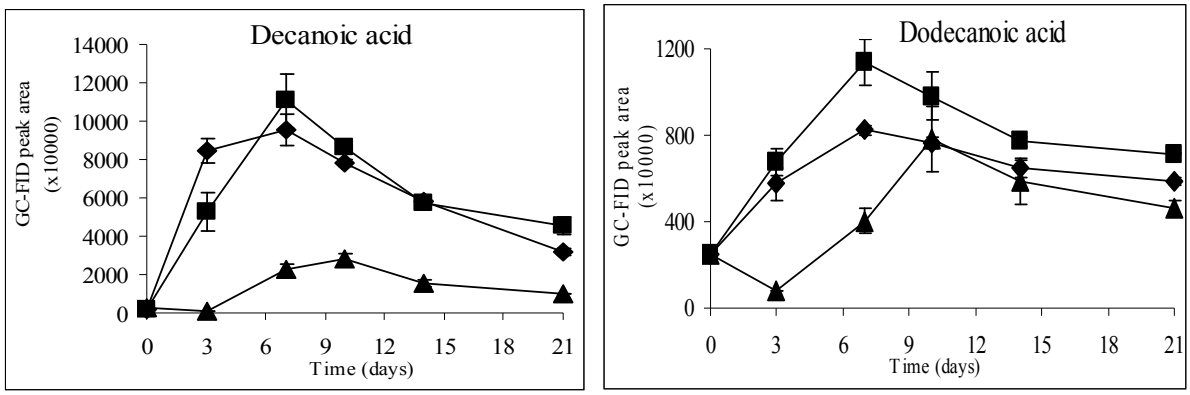

Fig. F2. Changes of decanoic and dodecanoic acids in papaya wine during mixed culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■). (Error bars = standard deviation).

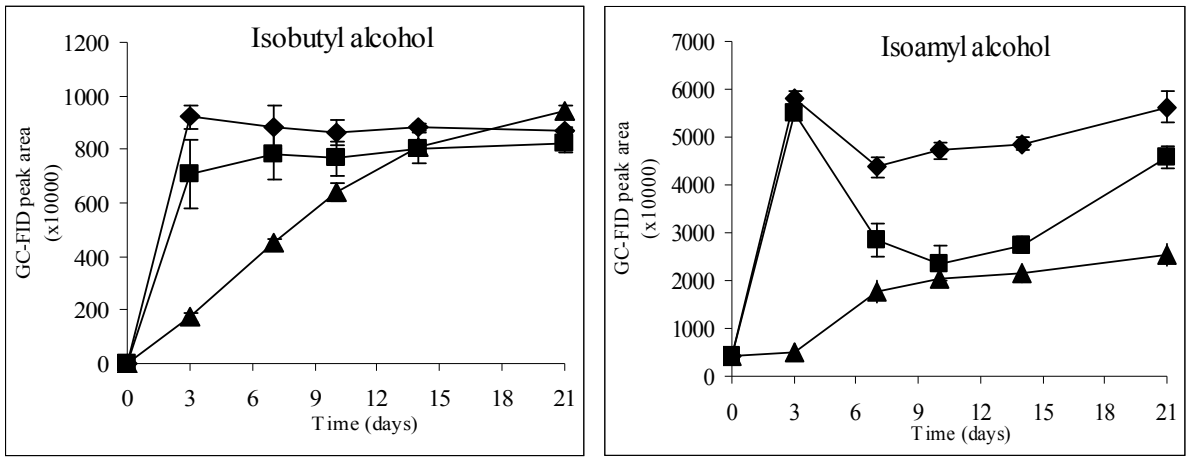

Fig. F3. Changes of isobutyl and isoamyl alcohols in papaya wine during mixed culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■). (Error bars = standard deviation).

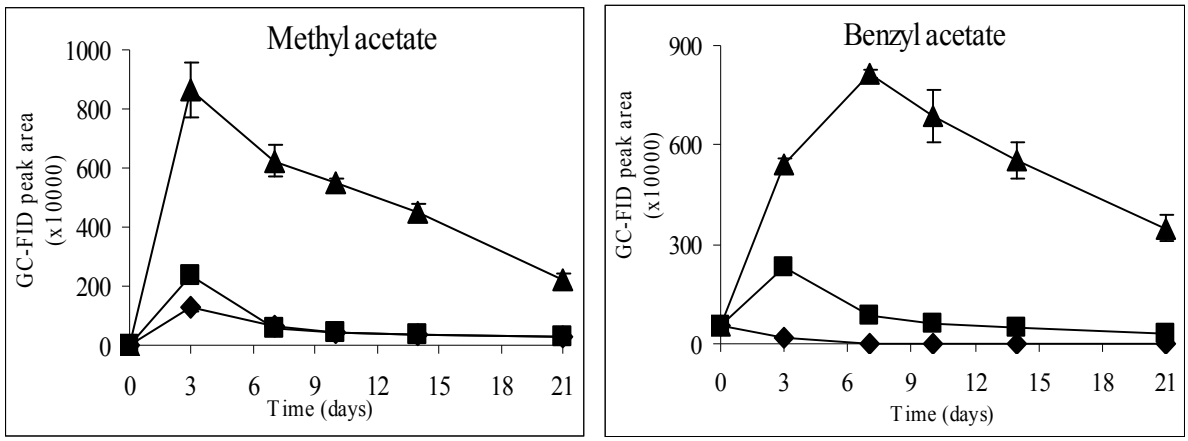

Fig. F4. Changes of methyl acetate and benzyl acetate in papaya wine during mixed culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■). (Error bars = standard deviation).

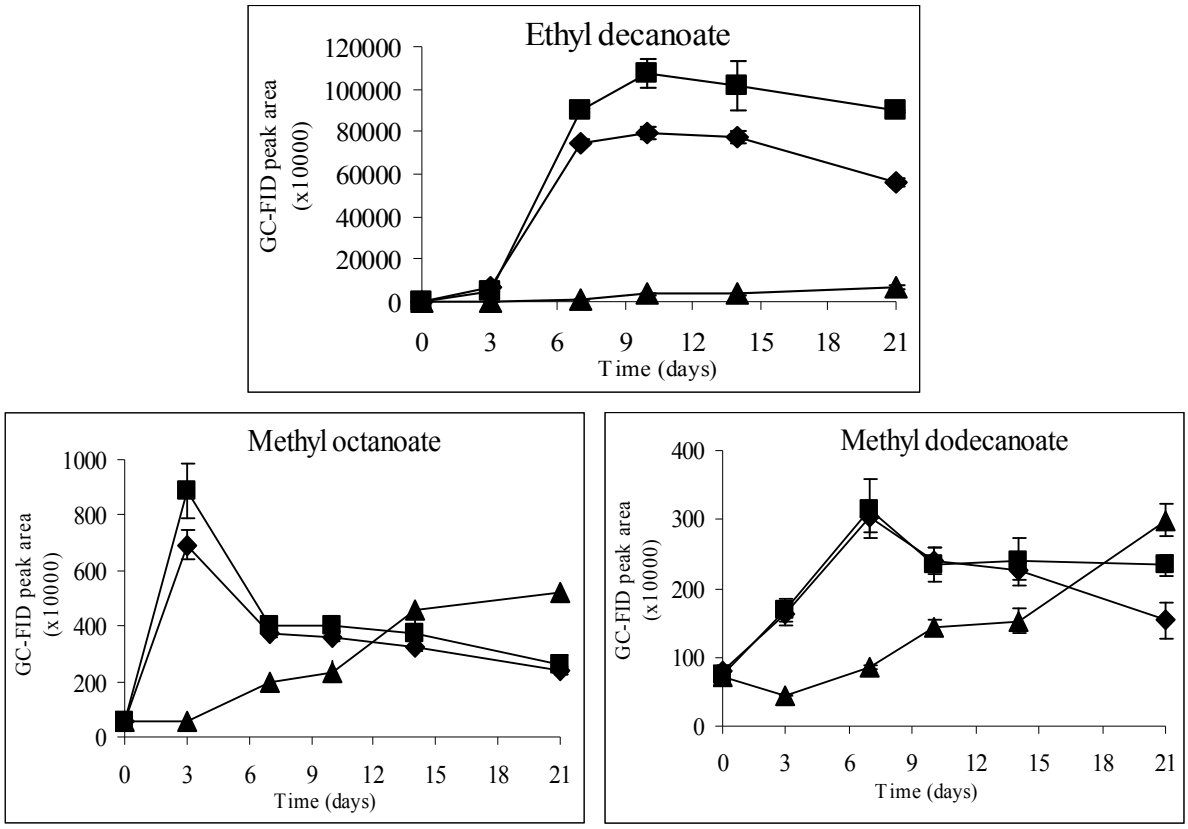

Fig. F5. Changes of ethyl decanoate and methyl esters in papaya wine during mixed culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■). (Error bars = standard deviation).

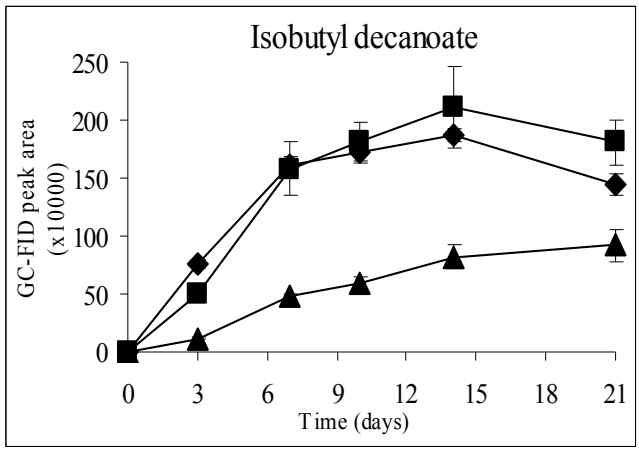

Fig. F6. Changes of isobutyl decanoate in papaya wine during mixed culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251mixed-culture (■). (Error bars = standard deviation).

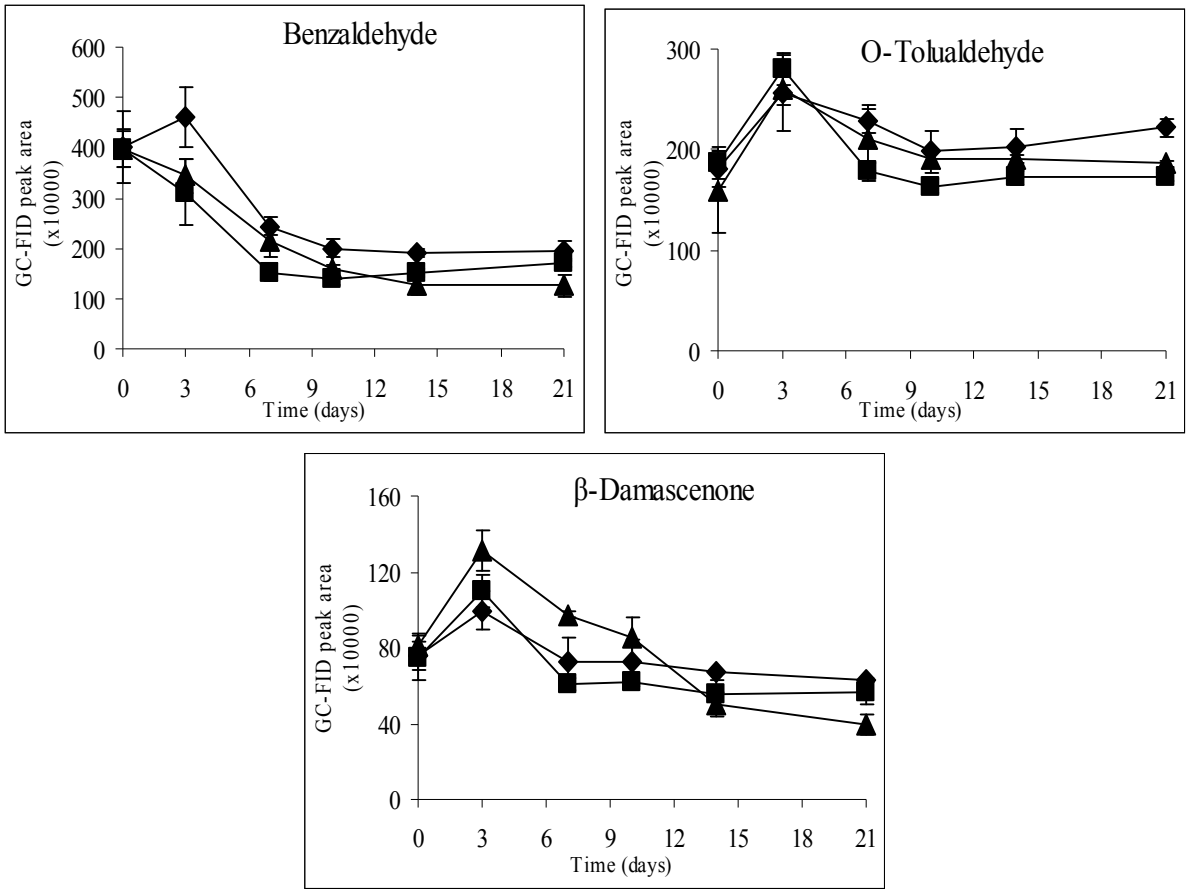

Fig. F7. Changes of aldehydes and β-damascenone in papaya wine during mixed culture fermentation. *S. cerevisiae* var. *bayanus* R2 (♦); *W. saturnus* var. *mrakii* NCYC2251 (▲); *S. cerevisiae* R2–*W. saturnus* NCYC2251 mixed-culture (■). (Error bars = standard deviation).

Appendix G

Supplementary figures and tables for Chapter 9 (Effects of sequentially inoculated *Williopsis saturnus* var. *mrakii* NCYC2251 and *Saccharomyces cerevisiae* var. *bayanus* R2 on volatile profiles of papaya wine)

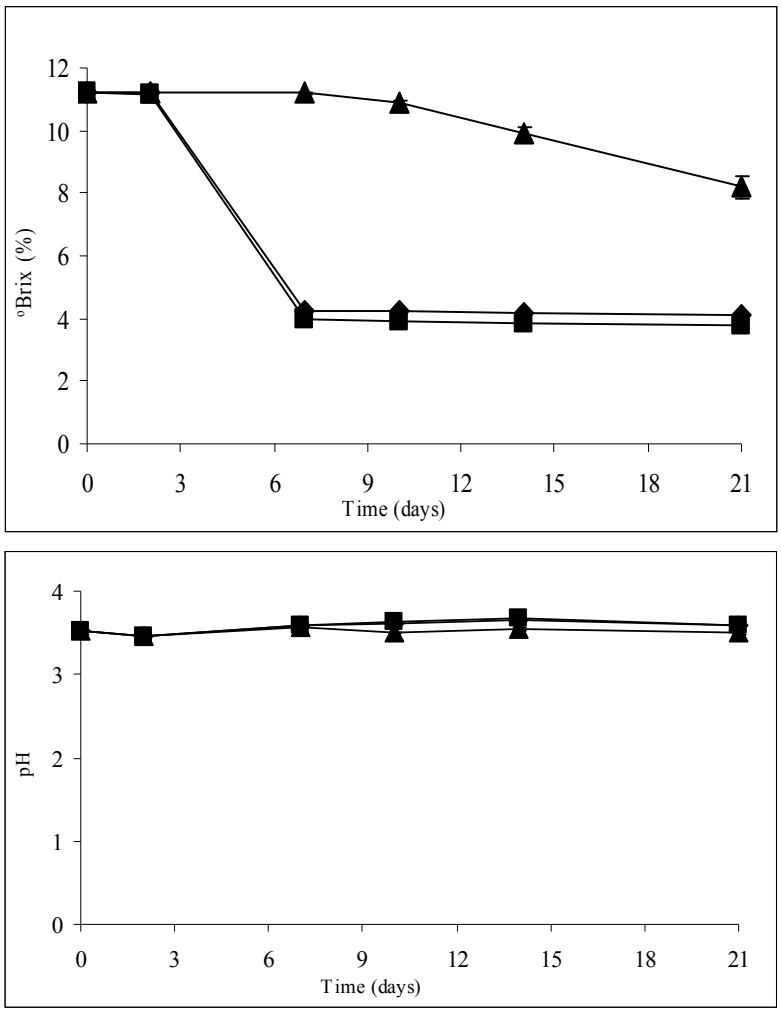

Fig. G1. Brix and pH changes during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

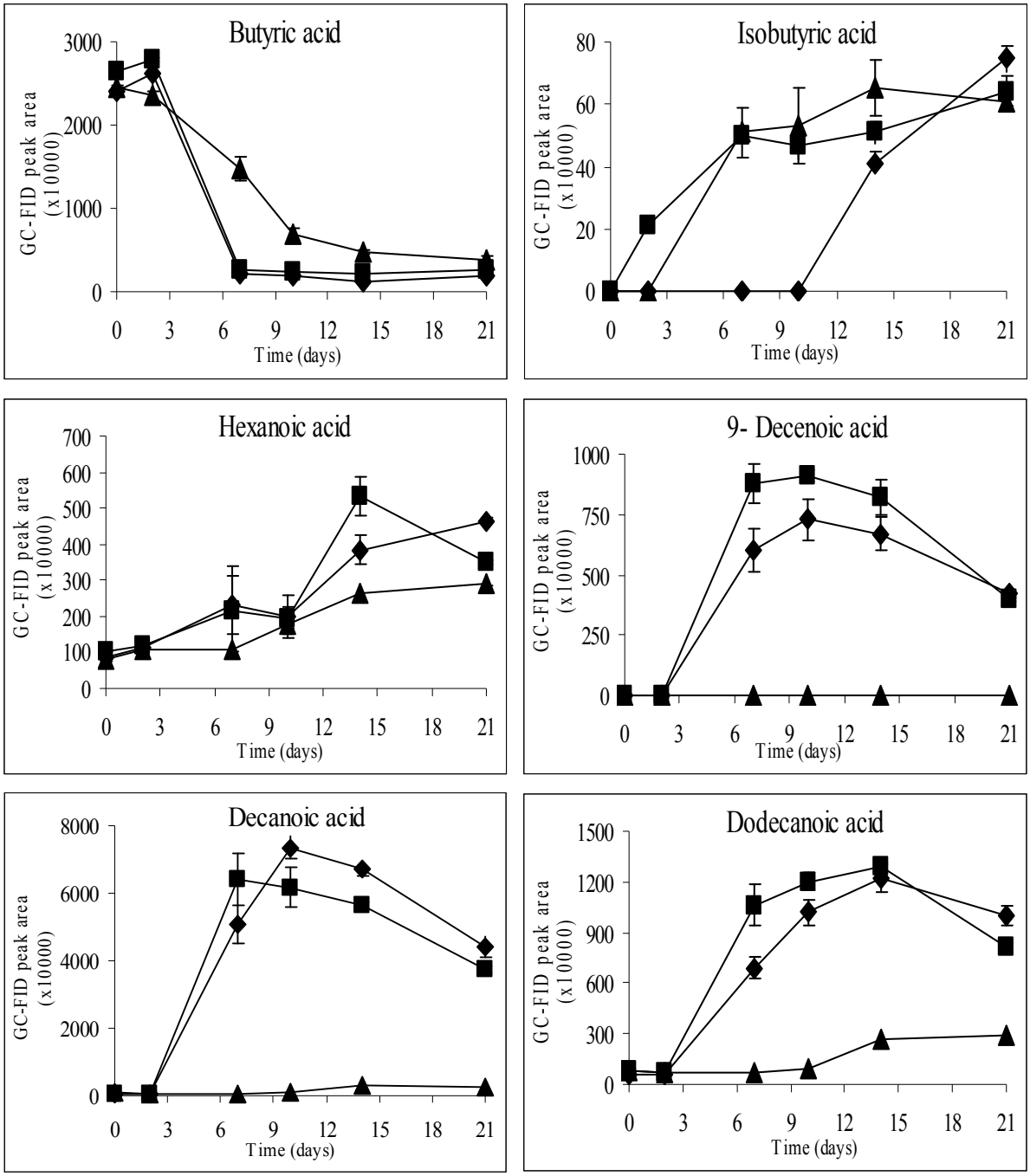

Fig. G2. Changes of fatty acids during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

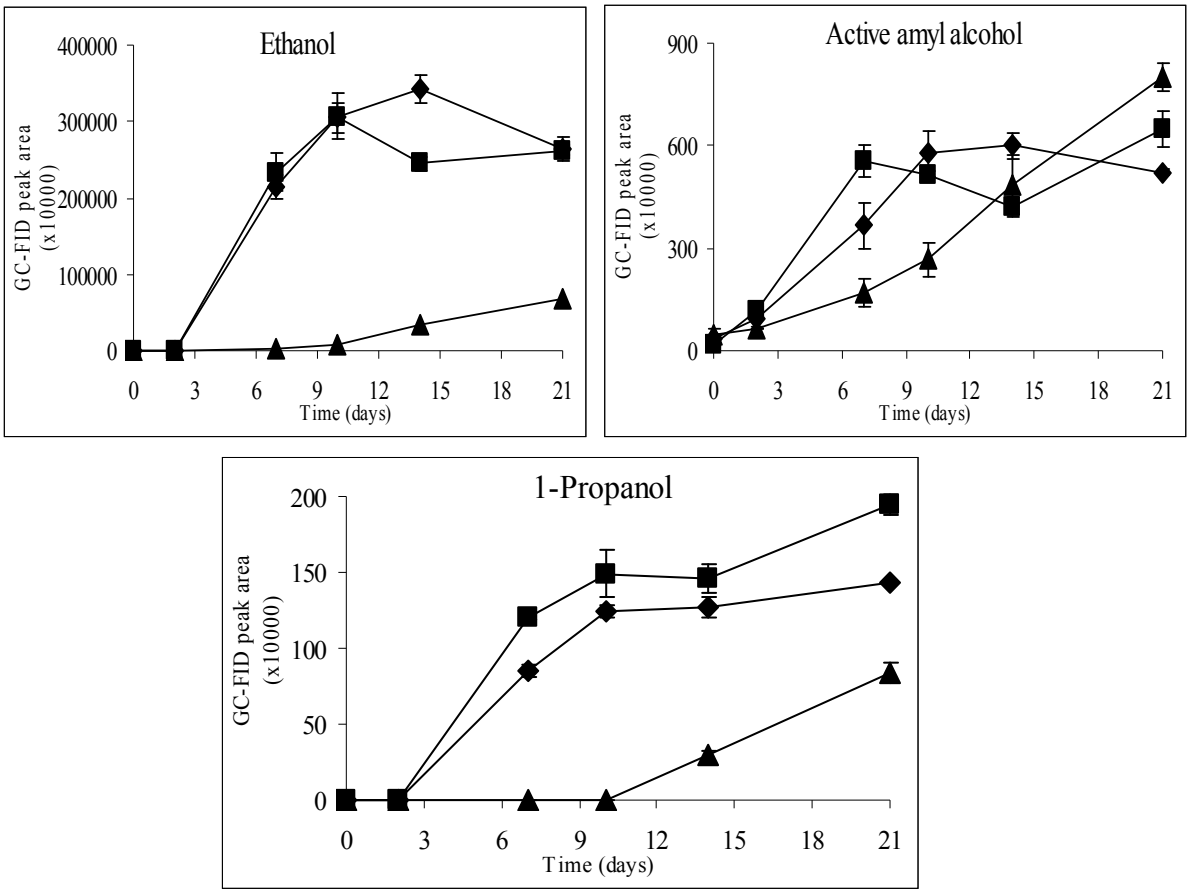

Fig. G3. Changes of alcohols during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

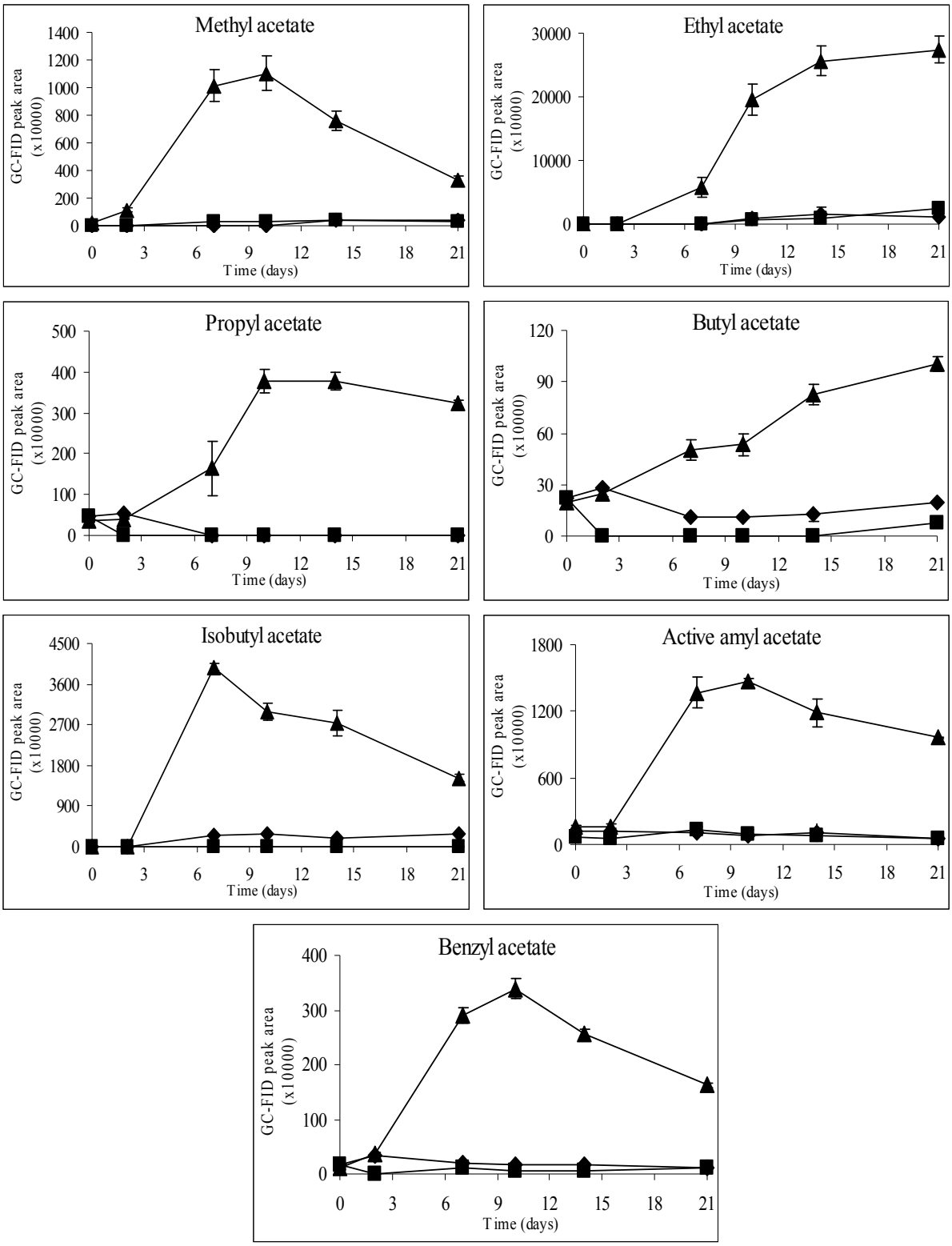

Fig. G4. Changes of acetate esters during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

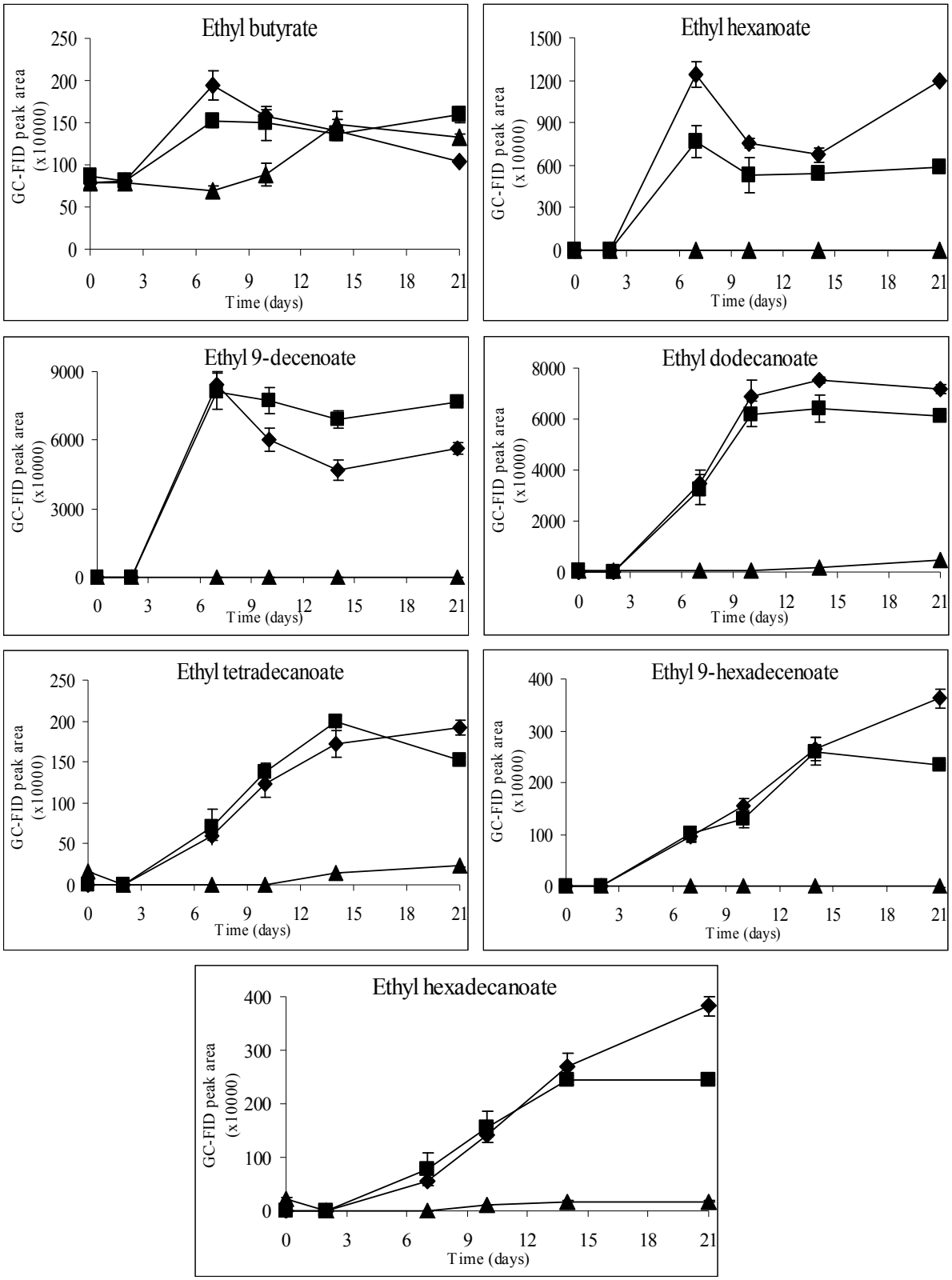

Fig. G5. Changes of ethyl esters during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

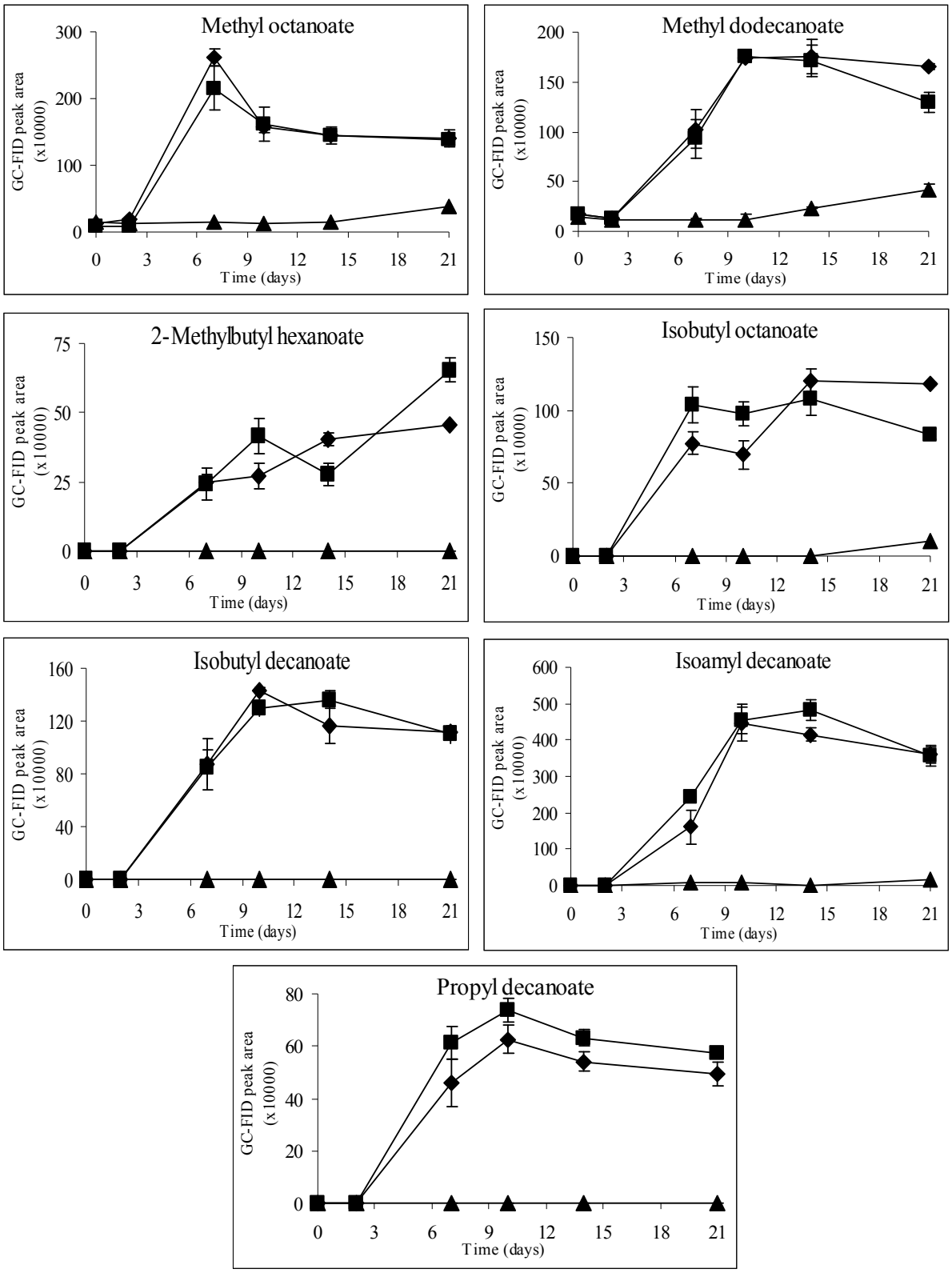

Fig. G6. Changes of methyl and other esters during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

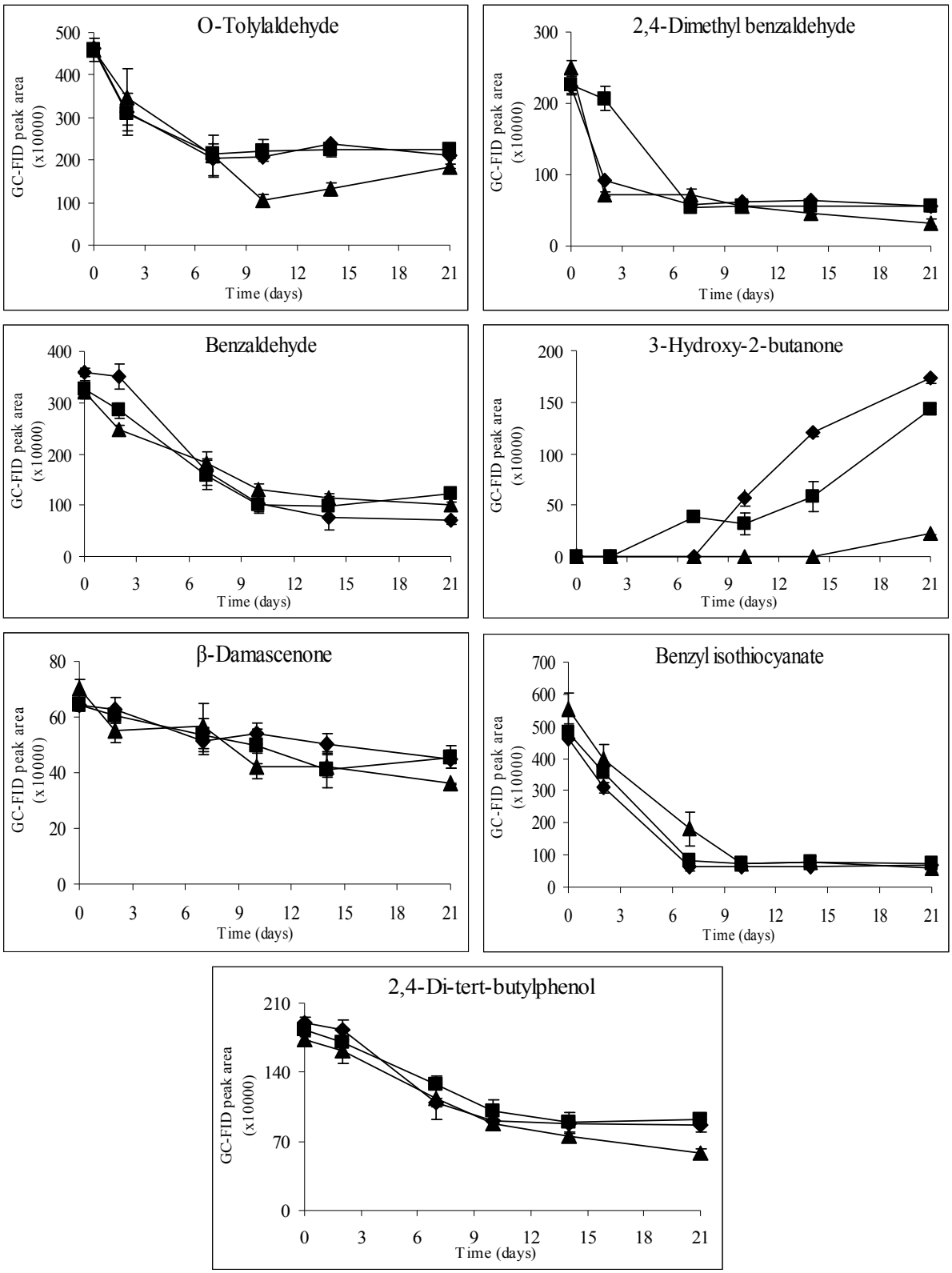

Fig. G7. Changes of miscellaneous volatiles during mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine. Mixed-culture (♦); positive sequential (▲); negative sequential (■). Positive and negative sequential fermentations are defined as in Fig. 9.1. (Error bars = standard deviation).

Table G1. Modified frequency (*MF*%) value of sensory descriptors for the papaya wines (day 21) fermented by mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine.

Sensory descriptors	Mixed culture	Positive sequential[a]	Negative sequential[b]
Acidic	60.00	56.57	60.00
Alcoholic	87.18	69.28	77.46
Buttery	66.33	50.60	69.28
Cocoa	52.15	40.00	52.92
Fruity	74.83	95.92	77.46
Fusel	80.00	72.11	78.74
Sweet	82.46	77.46	69.28
Yeasty	84.85	81.24	80.00

[a]Inoculation of *S. cerevisiae* after 7 days' fermentation with *W. saturnus*.
[b]Inoculation of *W. saturnus* after 2 days' fermentation with *S. cerevisiae*.

Table G2. Sensory parameters of papaya wines (day 21) fermented by mixed and sequential fermentations of *S. cerevisiae* var. *bayanus* R2 and *W. saturnus* var. *mrakii* NCYC2251 in papaya wine.

Sensory descriptors	Mixed culture	Positive sequential[c]	Negative sequential[d]
Acidic	1.80 ± 0.84^{a}	1.60 ± 1.34^{a}	1.80 ± 0.84^{a}
Alcoholic	3.80 ± 0.45^{a}	2.40 ± 0.89^{b}	3.00 ± 0.71^{ab}
Buttery	2.20 ± 1.10^{a}	1.60 ± 0.89^{a}	2.40 ± 0.89^{a}
Cocoa	1.70 ± 1.30^{a}	1.00 ± 0.71^{a}	1.40 ± 0.55^{a}
Fruity	2.80 ± 0.45^{a}	4.60 ± 0.89^{b}	3.00 ± 0.00^{a}
Fusel	3.20 ± 0.84^{a}	2.60 ± 0.89^{a}	3.10 ± 0.74^{a}
Sweet	3.40 ± 0.89^{a}	3.00 ± 0.00^{a}	2.40 ± 0.55^{a}
Yeasty	3.60 ± 0.55^{a}	3.30 ± 0.97^{a}	3.20 ± 0.84^{a}

[a,b]Statistical analysis at 95% confidence level with same letters indicating no significant difference.
[c]Inoculation of *S. cerevisiae* after 7 days' fermentation with *W. saturnus*.
[d]Inoculation of *W. saturnus* after 2 days' fermentation with *S. cerevisiae*.

Appendix H

Supplementary figures for Chapter 10 (Yeast ratio is a critical factor for sequential fermentation of papaya wine by *Williopsis saturnus* var. *mrakii* NCYC2251 and *Saccharomyces cerevisiae* var. *bayanus* R2)

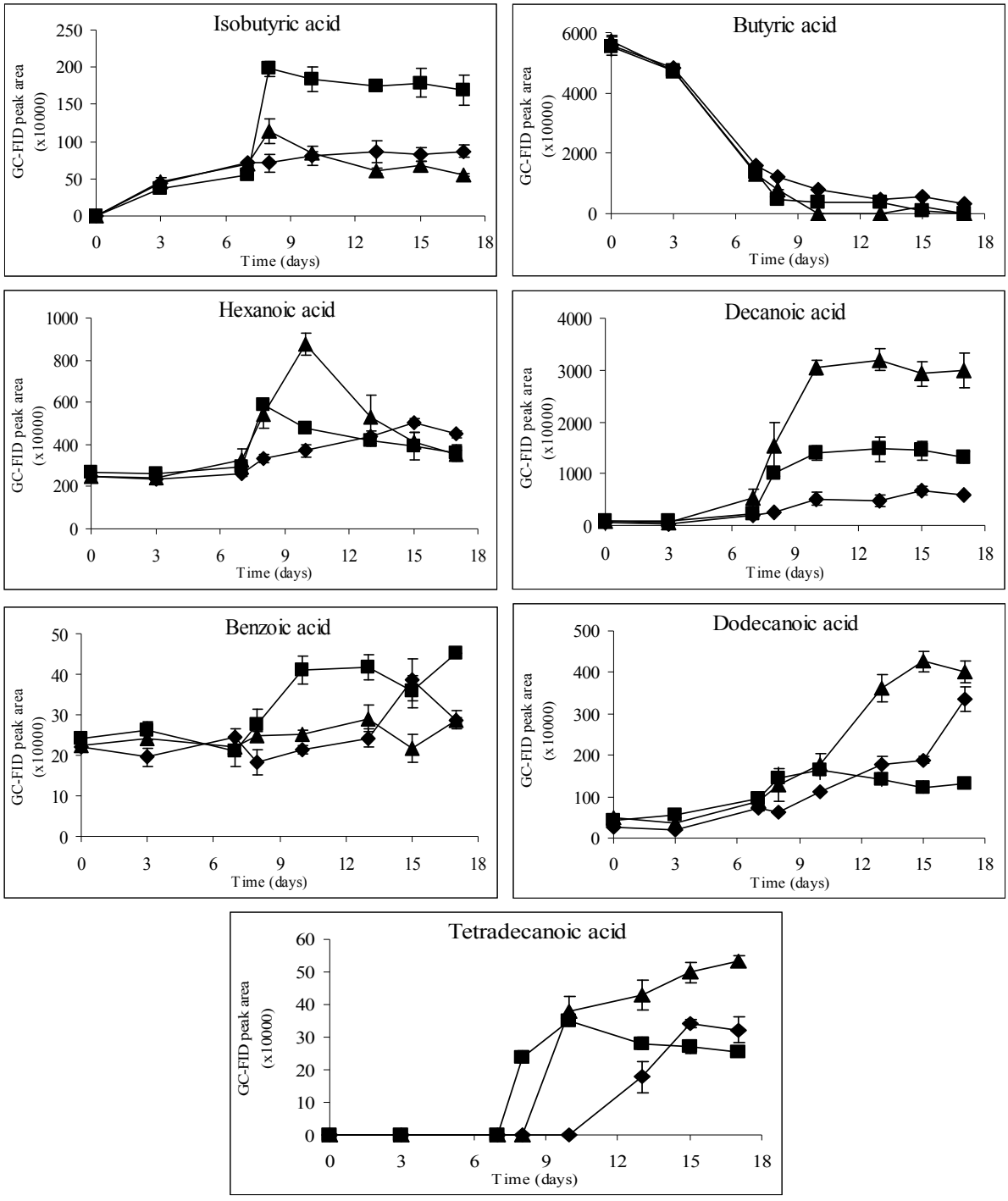

Fig. H1. Changes of fatty acids during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

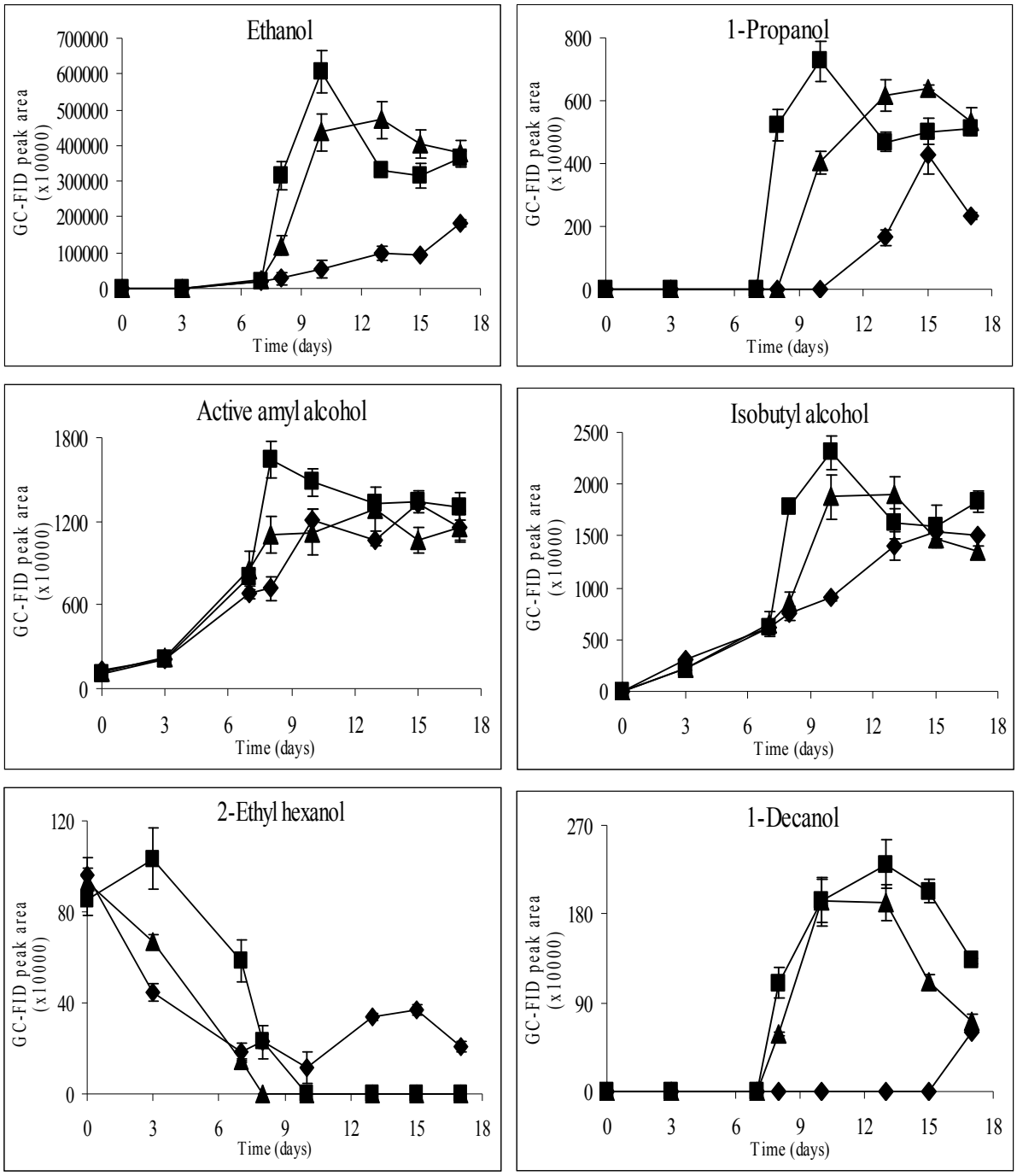

Fig. H2. Changes of alcohols during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

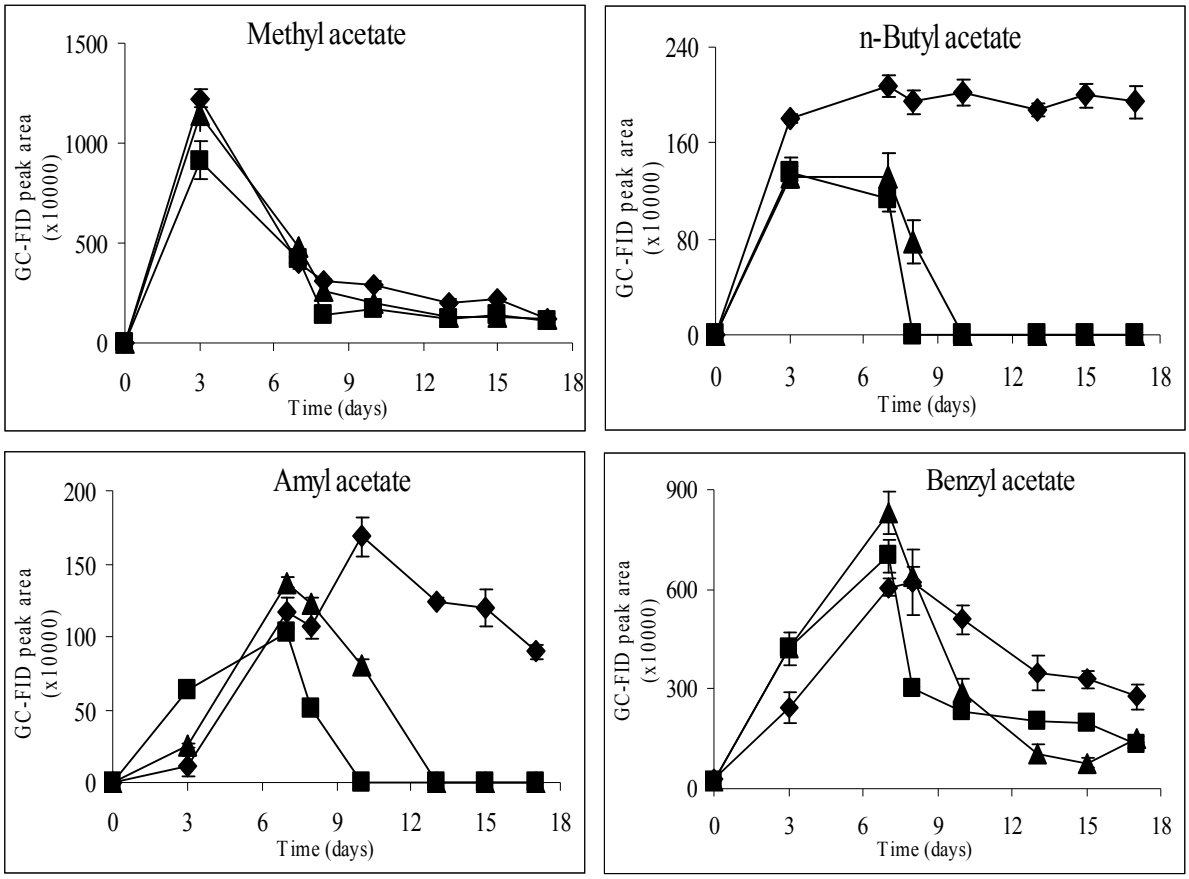

Fig. H3. Changes of acetate esters during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

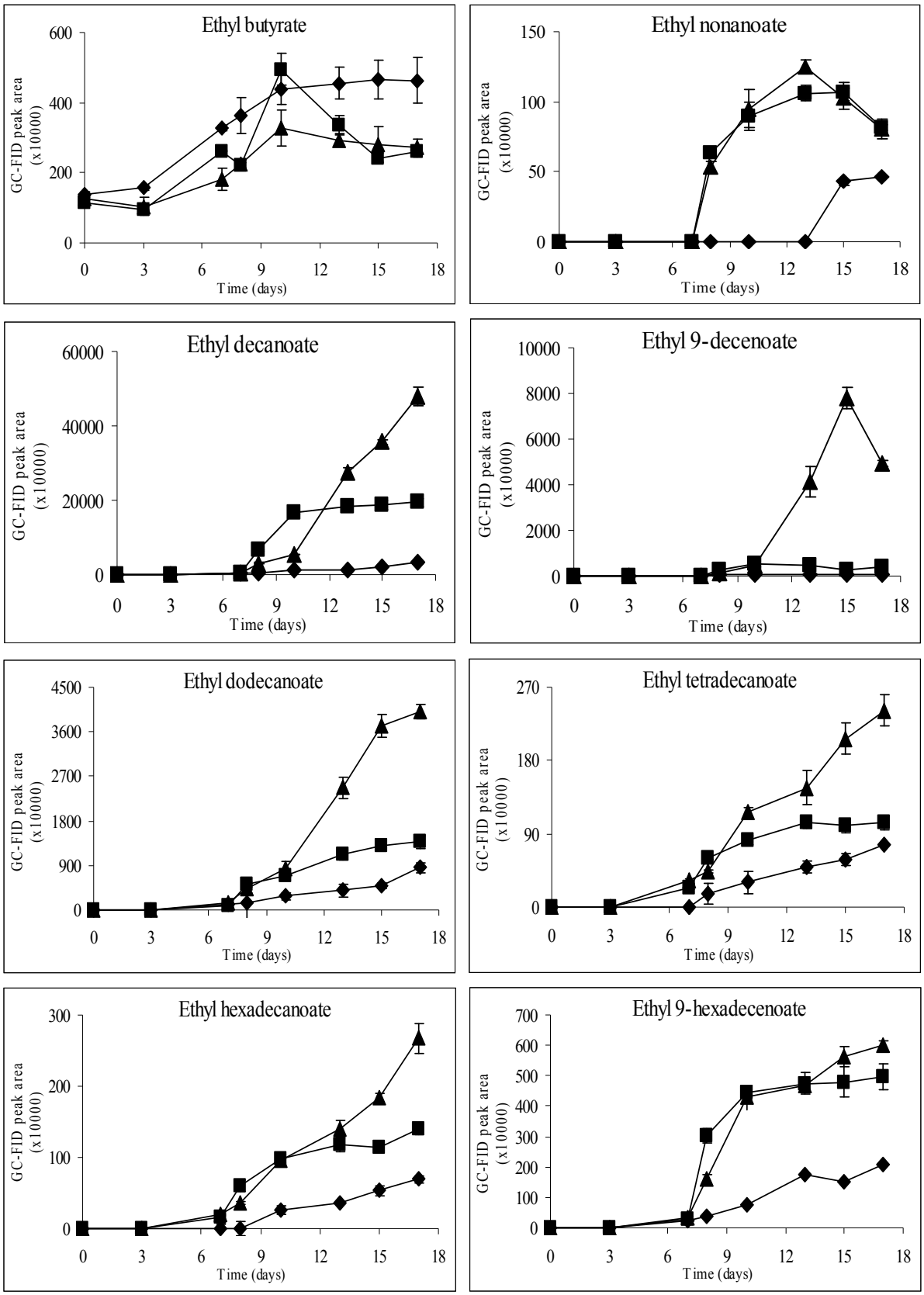

Fig. H4. Changes of ethyl esters during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

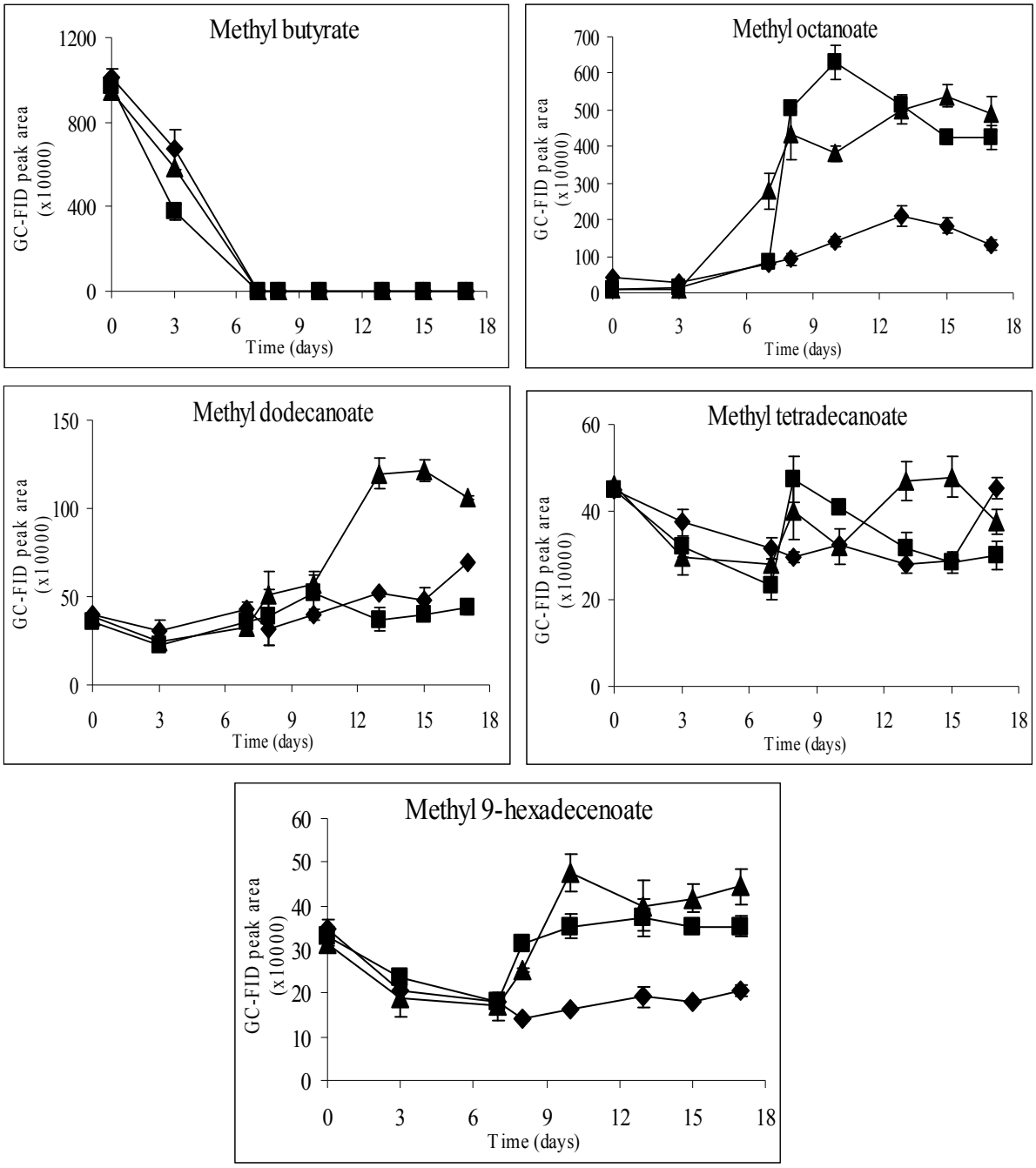

Fig. H5. Changes of methyl esters during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

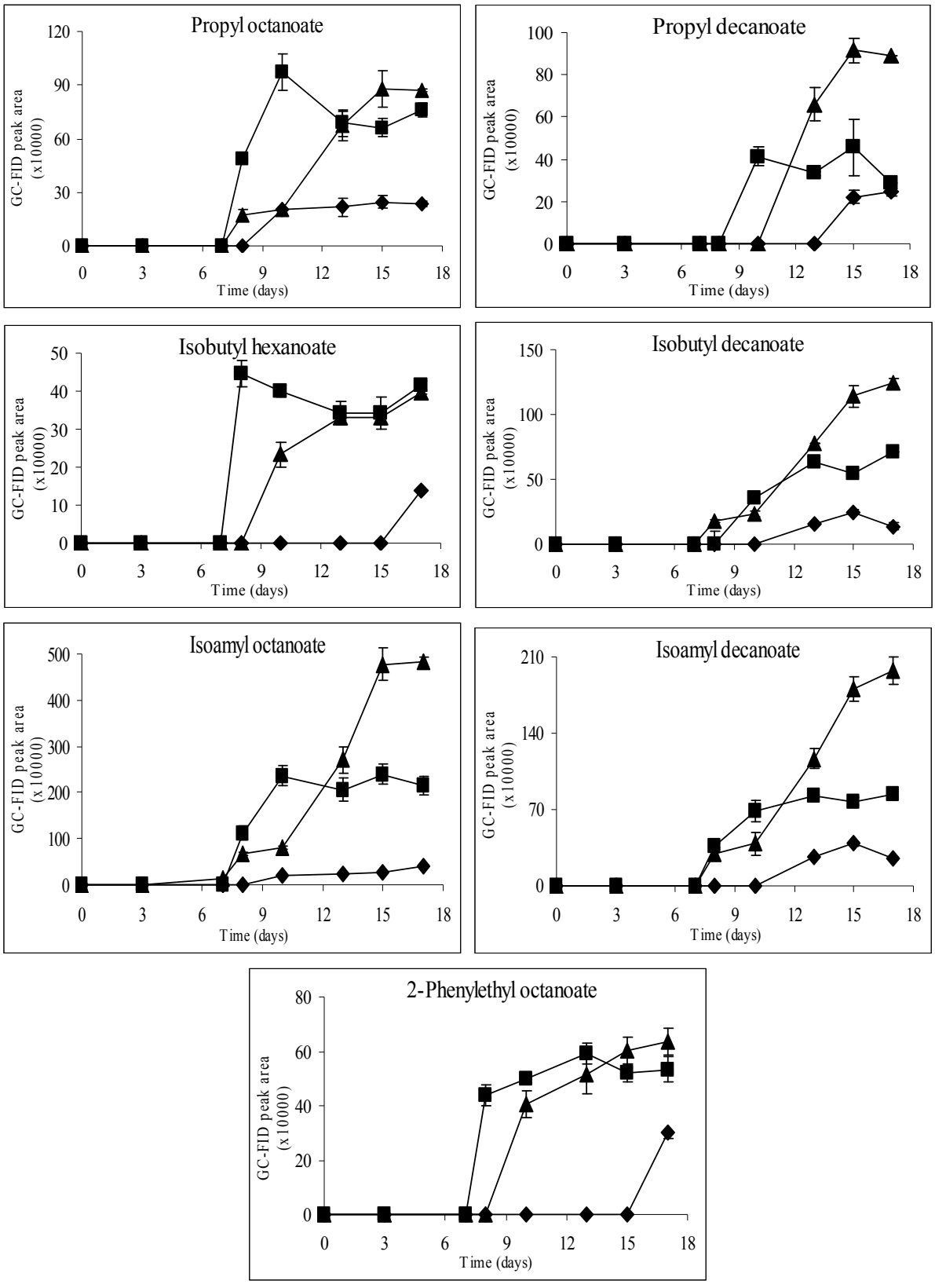

Fig. H6. Changes of miscellaneous esters during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

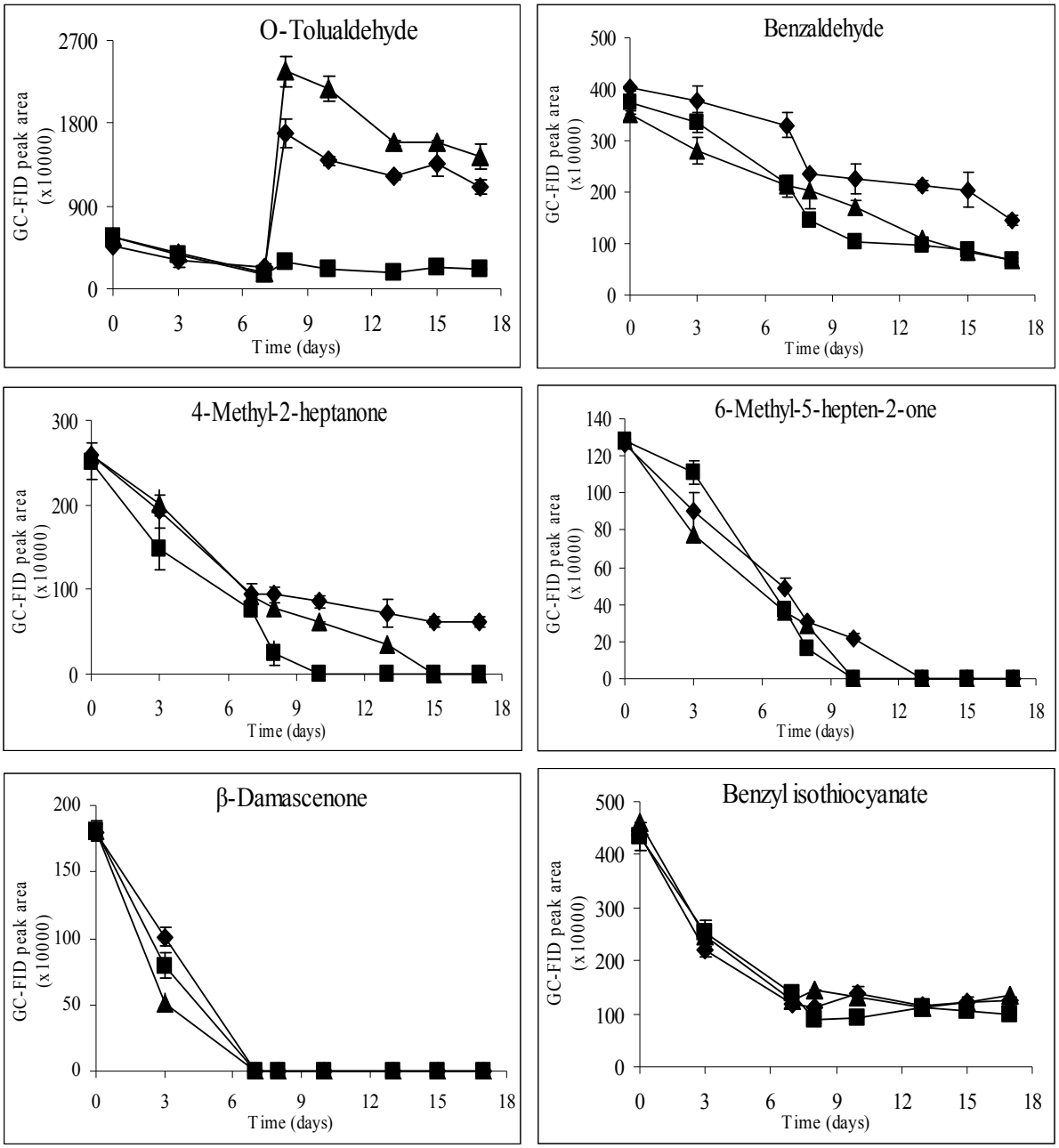

Fig. H7. Changes of miscellaneous volatile compounds during papaya wine sequential fermentation inoculated with different ratios of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2. 10:1 ratio (♦); 1:1 ratio (▲); 1:10 ratio (■). (Error bars = standard deviation).

Table H1. Modified frequency (*MF*%) value of sensory descriptors among the papaya wines (day 17) fermented with sequential cultures of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2 at different ratios (*W. saturnus*: *S. cerevisiae*)

Sensory descriptors	**Ratio 10:1**	**Ratio 1:1**	**Ratio 1:10**
Acidic	60.98	59.16	59.16
Alcoholic	82.16	83.67	82.16
Buttery	63.25	49.05	65.19
Cocoa	59.16	53.33	65.19
Fruity	81.39	80.62	78.26
Fusel	75.83	79.06	79.06
Sweet	74.16	75.83	75.83
Yeasty	72.46	70.71	76.65

Table H2. Sensory parameters of papaya wines (day 17) fermented with sequential cultures of *W. saturnus* var. *mrakii* NCYC2251 and *S. cerevisiae* var. *bayanus* R2 at different ratios (*W. saturnus*: *S. cerevisiae*)

Sensory descriptors	**Ratio 10:1**	**Ratio 1:1**	**Ratio 1:10**
Acidic	2.13 ± 1.36^{a}	2.00 ± 1.41^{a}	2.00 ± 1.41^{a}
Alcoholic	3.38 ± 0.74^{a}	3.50 ± 0.76^{a}	3.38 ± 0.74^{a}
Buttery	2.00 ± 1.07^{a}	1.38 ± 0.92^{a}	2.13 ± 1.13^{a}
Cocoa	2.00 ± 1.07^{a}	1.63 ± 1.06^{a}	2.13 ± 0.99^{a}
Fruity	3.31 ± 0.70^{a}	3.25 ± 0.46^{a}	3.06 ± 1.21^{a}
Fusel	2.88 ± 0.99^{a}	3.13 ± 1.13^{a}	3.13 ± 0.83^{a}
Sweet	2.75 ± 0.89^{a}	2.88 ± 0.83^{a}	2.88 ± 0.99^{a}
Yeasty	2.63 ± 1.30^{a}	2.50 ± 0.93^{a}	2.94 ± 0.68^{a}

[a]Statistical analysis at 95% confidence level with same letters indicating no significant difference.