CHAPTER V

FERMENTATION OF PINEAPPLE JUICE WITH SINGLE AND MIXED STARTER CULTURES OF YEASTS

5.1 Introduction

The use of mixtures of different species and strains of yeasts as starter cultures (multi-starter cultures) to induce desirable fermentation of alcoholic beverages has been considered in previous research but application of this concept is still relatively new (Fleet, 2008). There were a few techniques in the application of multi-starter cultures to fermentation processes, and include simultaneous inoculation of the mixture of species, inoculation of the different species in some defined sequence, and use of the organisms as immobilized cells as described in Chapter 2. The advantages of these strategies in wine fermentation processes are to improve the quality of wine in several ways, such as enhancement of desirable volatile compounds (Rojas et al., 2003), biological deacidification (Magyar and Panyik, 1989; Herraiz et al., 1990; Di Maro, Ercolini and Coppola, 1990; Ciani, 1995), and enhancement of glycerol in wine (Ciani and Ferraro, 1996; Ciani and Maccarelli, 1998; Ferraro, Fatichenti and Ciani, 2000).

In this Chapter, species of yeasts isolated from pineapple fermentations in previous Chapters, namely, *S'codes ludwigii* (Sl) and *H. uvarum*1 (Hu1) including commercial *S. cerevisiae*, are used as mixed cultures for conducting pineapple juice fermentations. *S'codes ludwigii* was selected since it could produce the highest

amount of ethanol content relative to the other yeasts isolates. In addition, it showed the other fermentation characteristics, which were similar to the commercial *S. cerevisiae*. *H. uvarum* was also selected since it could generate 2-phenylethyl acetate, which reported as a volatile compound giving rosy and flowery odors as mentioned in Chapter 4. Therefore, these two yeast isolates were selected to apply as multi-starter cultures for pineapple wine fermentation. Fermentation characteristics, such as yeast population profile, pH, ethanol production, sugar utilization, organic acid utilization and production and production of major volatile compounds, were investigated. A main goal was to determine if such use of multi-starter cultures could increase the sensorial characters of pineapple wine product.

5.2 Materials and methods

Two yeast isolates from Chapter 4, *S'codes ludwigii* and *H. uvarum*, in the form of multi-starter cultures were investigated for their fermentation patterns and volatile compounds produced during pineapple wine fermentation. *S. cerevisiae*, the main yeast for general wine fermentation, was also used as the starter culture in this study. The experimental designs of multi-starter culture fermentations are showed in Table 5.1. Each experiment was conducted in duplicate.

Table 5.1 The experimental designs of multi-starter cultures used in pineapple juice fermentation

Experiment	Yeast culture used in fermentation
1	*S. cerevisiae*
2	*H. uvarum*
3	*S'codes ludwigii*
4	*S. cerevisiae* + *H. uvarum*
5	*S. cerevisiae* + *S'codes ludwigii*
6	*S'codes ludwigii* + *H. uvarum*
7	*S. cerevisiae* + *S'codes ludwigii* + *H. uvarum*

5.2.1 Determination of fermentation profile

Sterile pineapple juice (Tipco®, Thailand) was used as the base. Its sugar concentration was increased to 22°brix by adding sucrose (CSR, Australia), This juice (500 ml) was then transferred into 500 ml Erlenmeyer flask. Potassium metabisulphite (KMS) was added to a final concentration of 50 mg l^{-1} and left overnight to conduct decontamination. The starter cultures were prepared in sterile pineapple juice (Tipco®, Thailand) with rotation at 200 rpm, at 25°C for 20-24 hours for by orbital incubator shaker (Gallenkamp, UK.) and used to inoculate pineapple juice fermentations. Each starter culture was inoculated to the prepared pineapple juice at initial population of 10^6 cell ml^{-1}. The pineapple juice fermentations were conducted at 25°C for 1 week in duplicate. The fermented juices were routinely analyzed for yeast population. Concentrations of ethanol, sugars and organic acids and pH (Activon Model 210, Australia) of the fermented juices were analyzed every 2 days and volatile compounds were analyzed in only the last day of fermentation. The ethanol production rates during day 0 to day 2 were calculated using Excel software version 2003 (Microsoft, USA.). Samples for yeast populations were analyzed immediately. Samples for chemical analysis were stored at -30°C until analysis.

5.2.1.1 Determination of yeast population by cultural method

The fermented pineapple juice was serially diluted in 0.1% peptone water. The yeasts in each dilution were isolated by spread inoculation of 0.1 ml onto plates of MEA agar (Oxoid, England) and also WL nutrient agar (Oxoid,

England) to distinguish yeast colonies and incubated at 25°C for 2-4 days. Yeast colonies were counted. The analysis was done in duplicate.

5.2.1.2 Preparation of pineapple wine for HPLC analysis

The pineapple wine samples were prepared using the procedures described in Sections 4.2.3 in Chapter 4.

5.2.1.3 Analysis of ethanol, sugars and organic acids

Ethanol, sugars and organic acids were analyzed by HPLC using the procedures described in Sections 4.2.3 in Chapter 4.

5.2.2 Analysis of volatile compounds

Volatiles were analyzed by HS-SPME coupled with GC-MS as described in Section 4.2.4 Chapter 4.

5.3 Results and discussion

5.3.1 Yeast, sugar and ethanol profiles during pineapple wine fermentation by single and mixed starter cultures of yeasts

The fermentation profiles of pineapple juice inoculated with mixed starter cultures listed in the Table 5.1 were evaluated. Figures 5.1-5.3 illustrate the

fermentation patterns with single cultures of *S. cerevisiae*, *S'codes ludwigii* and *H. uvarum* respectively as explained in Chapter 4.

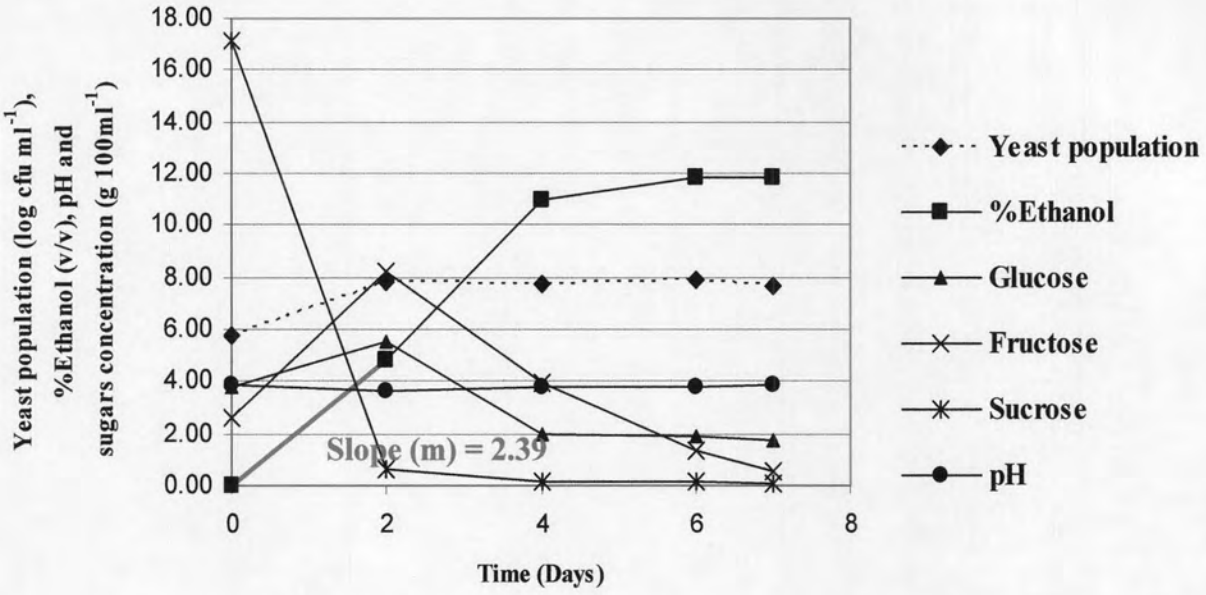

Figure 5.1 The changes of yeast population, ethanol, sugar concentrations and pH of pineapple juice during fermentation with *S. cerevisiae* (from Figure 4.1)

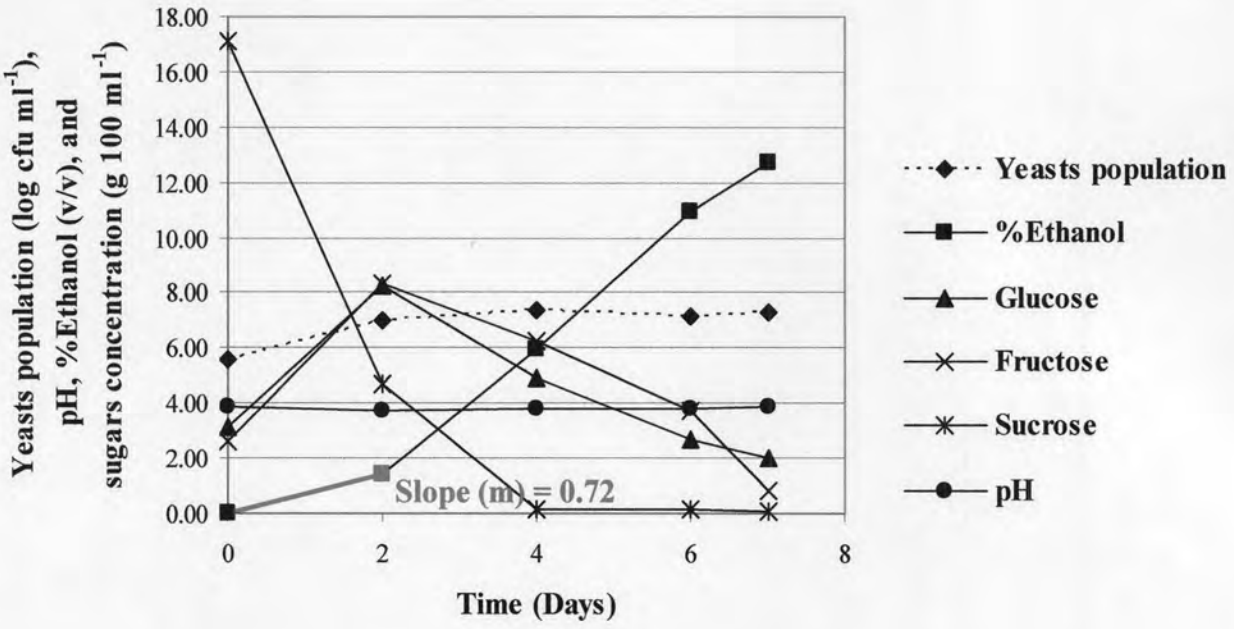

Figure 5.2 The changes of yeast population, ethanol, sugar concentrations and pH of pineapple juice during fermentation with *S'codes ludwigii* (from Figure 4.2)

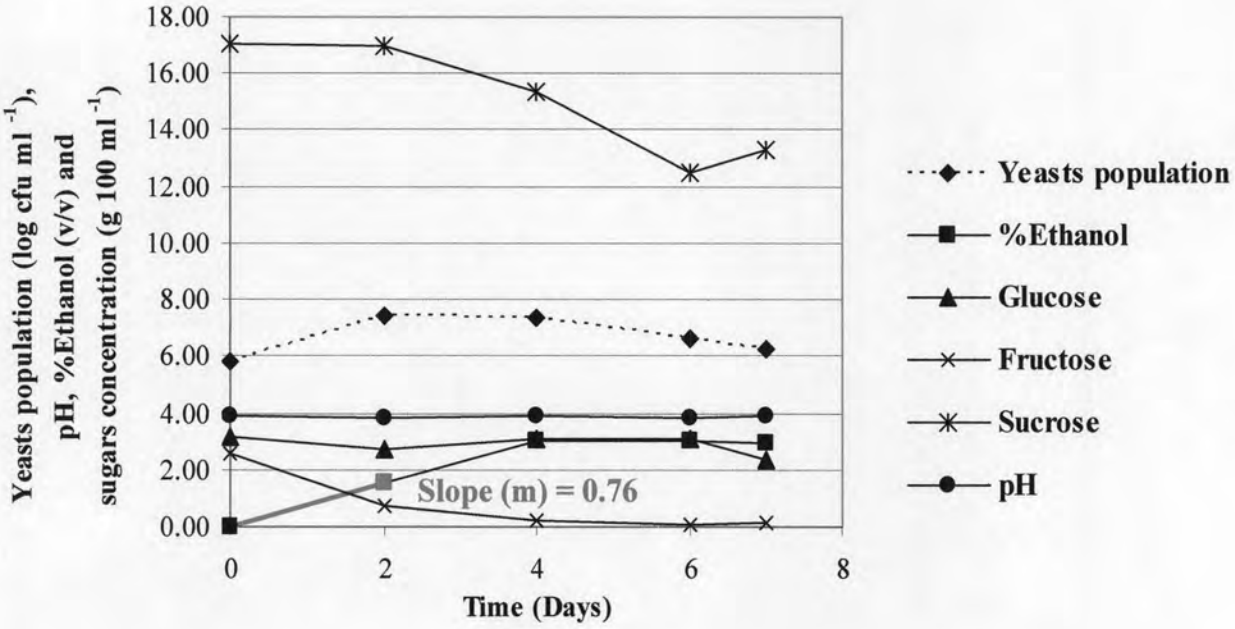

Figure 5.3 The changes of yeast population, ethanol, sugar concentrations and pH of pineapple juice during fermentation with *H. uvarum* (from Figure 4.3)

5.3.1.1 Fermentation with mixed cultures of *S. cerevisiae* and *H. uvarum*

Figure 5.4 shows the pattern of fermentation with mixed cultures of *S. cerevisiae* and *H. uvarum*. During day 1 and day 2, the populations of both yeasts increased approximately 2 log cycles. After day 2, the *S. cerevisiae* population remained stable through to the last day, whereas viable populations of *H. uvarum* were not observed after day 4 of fermentation. Ethanol production was observed at the early stage of fermentation, and rapidly increased to an amount of 11% (v/v) during day 1 to day 4, then shifted to slowly increase to a maximum amount of 12% (v/v) through day 6. For sugar determination, sucrose decreased through day 4, then was not observed throughout the fermentation as it had been utilized. In contrast with sucrose, glucose and fructose increased during day 0 to day 4, decreased in day 6, and then increased again in the last day of fermentation. The changes of yeast population at the initial stage of fermentation could be that yeasts used O_2 content existing in the substrate for their cell division. Thus, their population increased. After that, the fermentation shifted to anaerobic condition, while *S. cerevisiae* produced invertase enzyme which rapidly degraded sucrose to fructose and glucose. Consequently, the increase of glucose and fructose level in substrate was observed, and then they were utilized for ethanol production. *H. uvarum* also produced ethanol. During day 1 to day 2, the ethanol production rate of mixed cultures (m=2.34) was higher than that of single *H. uvarum* (m=0.76), but similar to that of single *S. cerevisiae* (m=2.39). When the ethanol content increased to an amount of 5.7% (v/v) in day 2, *H. uvarum* started to die off because of the toxicity of alcohol content. *S. cerevisiae* had stress from alcohol toxicity as well, thus the

decreasing of ethanol production rate of *S. cerevisiae* was observed after day 5. Glucose and fructose were utilized until day 6, then increased at the last day of fermentation. This could be from the release of glucose and fructose from dead yeast cells during the late of fermentation.

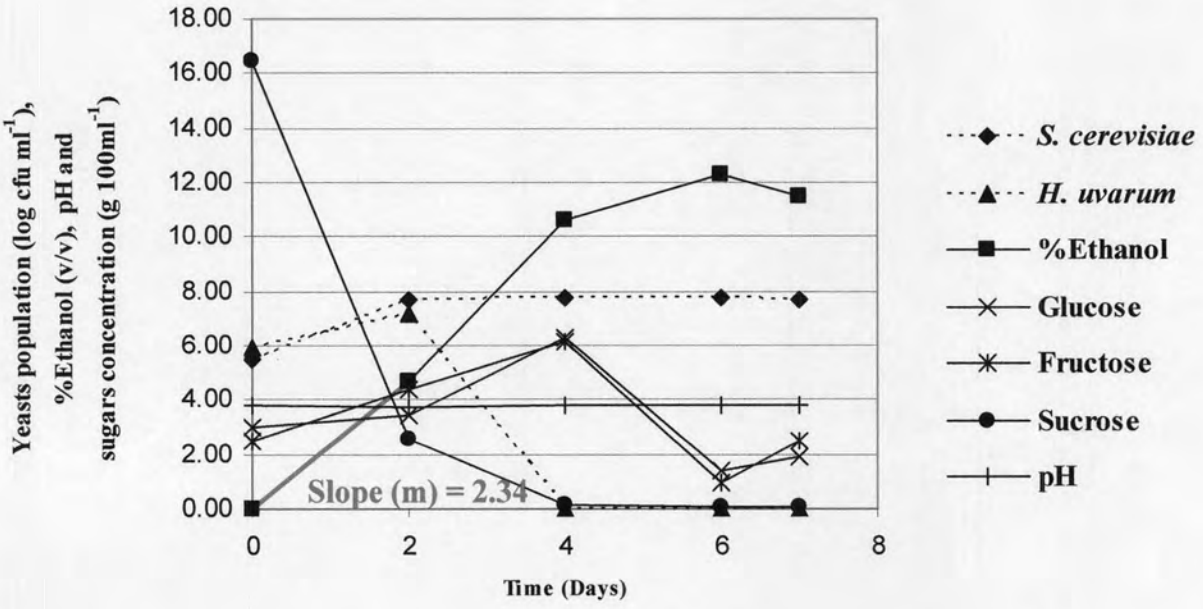

Figure 5.4 The changes of yeast population, ethanol, sugar concentrations and pH of pineapple juice during fermentation with *S. cerevisiae* and *H. uvarum*

5.3.1.2 Fermentation with mixed cultures of *S'codes ludwigii* and *H. uvarum*

The profile of fermentation with mixed cultures of *S'codes ludwigii* and *H. uvarum* is shown in Figure 5.5. The populations of both yeasts increased at the initial stage of fermentation and were stable through day 4, then viable populations of *H. uvarum* decreased after day 4 and was not observed at the last day of fermentation whereas *S'codes ludwigii* still remained stable throughout the fermentation. During day 1 and day 2, the ethanol slightly increased (m=0.87), then shifted to rapidly increased throughout the fermentation, allowing the final ethanol increased to a maximum amount of 12% (v/v) at the last day of fermentation. The patterns of sucrose, glucose and fructose utilization were similar to that of mixed cultures of *S. cerevisiae* and *H. uvarum*. The results showed that *S'codes ludwigii* slowly produced ethanol at the early stage of fermentation. Thus, the ethanol content was only 1.7% (v/v) in day 2. Consequently, *H. uvarum* was still present in the fermentation system to day 4. The longer existence of *H. uvarum* in the fermentation system could provide secondary metabolites produced from *H. uvarum*, such as the floral flavour compounds in wine. During day 1 to day 2, the ethanol production rate of mixed cultures was higher than that of single *S'codes ludwigii* (m=0.72) and that of single *H. uvarum* (m=0.76). Therefore, the fermentation profile of this experiment demonstrates the positive interaction of *S'codes ludwigii* and *H. uvarum* when used as mixed culture fermentation. When the ethanol increased to an amount of 5.7% (v/v) in day 4, *H. uvarum* decreased. However, the ethanol production rate of *S'codes ludwigii* increased throughout the fermentation. Glucose and fructose were utilized until day 4,

then the increase in both sugars was observed in day 6. However, they decreased again in the last day of fermentation. This may be because of the release of glucose and fructose from dead yeast cells, with both sugars then utilized for ethanol production by *S'codes ludwigii* during late of fermentation.

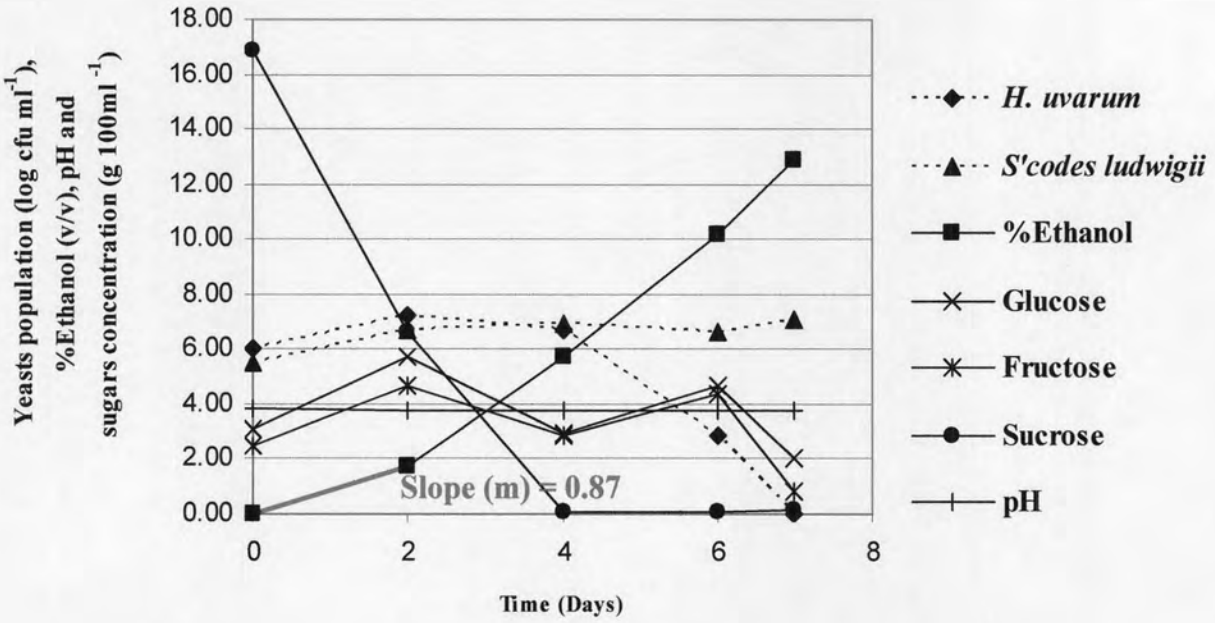

Figure 5.5 The changes of yeast population, ethanol, sugar concentrations and pH of pineapple juice during fermentation with *S'codes ludwigii* and *H. uvarum*

5.3.1.3 Fermentation with mixed cultures of *S. cerevisiae* and *S'codes ludwigii*

The profile of fermentation with mixed cultures of *S. cerevisiae* and *S'codes ludwigii* is illustrated in Figure 5.6. The population of both yeasts increased at the initial stage of fermentation, then remained stable throughout the fermentation. The increasing of ethanol content of this batch was observed throughout the fermentation. During day 1 to day 2, the ethanol production rate of mixed cultures (m=1.60) was higher than that of fermentation by single *S'codes ludwigii* (m=0.72), but lower than that with single *S. cerevisiae* (m=2.39). The ethanol content increased to a maximum amount of 12.7% (v/v) on the last day of fermentation., The patterns of sucrose, glucose and fructose utilization were similar to that of the fermentation with the mixed culture of *S. cerevisiae* and *H. uvarum*. Sucrose was completely degraded by day 4 of the fermentation. Glucose and fructose increased during day 0 to day 2, then were used until day 6, but an increase in glucose levels was observed again at the last day of fermentation, which was due to sucrose degradation.

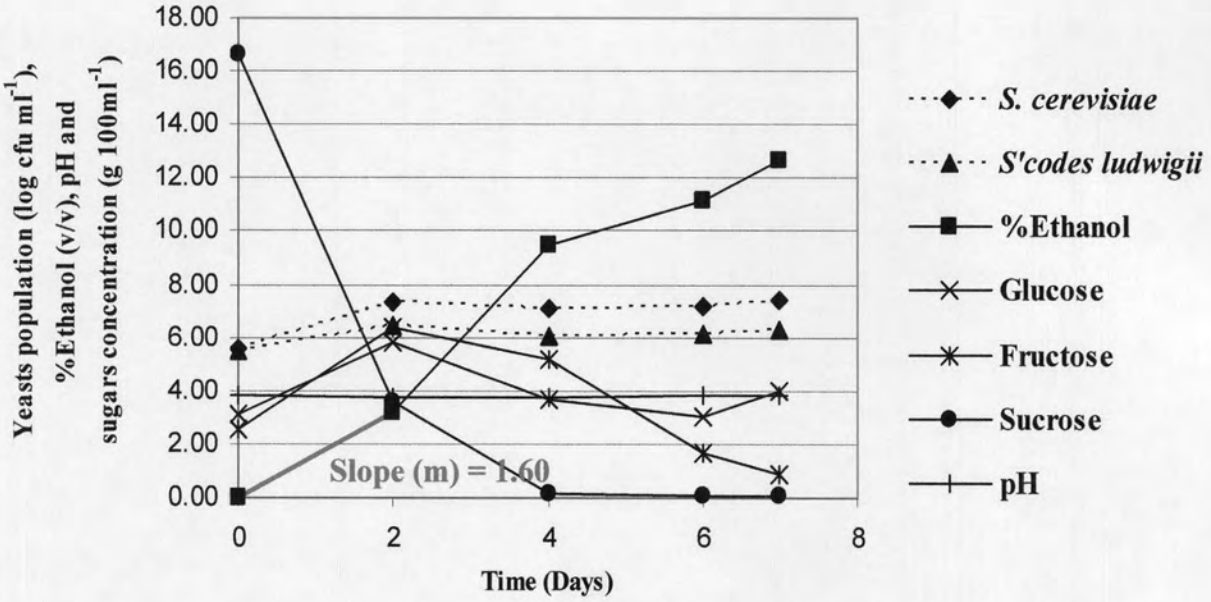

Figure 5.6 The changes of yeast population, ethanol, sugar concentrations and pH of pineapple juice during fermentation with *S. cerevisiae* and *S'codes ludwigii*

5.3.1.4 Fermentation with mixed cultures *S. cerevisiae*, *S'codes ludwigii* and *H. uvarum*

Figure 5.7 shows fermentation profile of pineapple juice inoculated with a mixed starter culture of three yeast species. The population of all yeasts increased during the first 2 days of fermentation. After that, the population of *H. uvarum* dropped and was not observed in the fermentation after day 6. *S. cerevisiae* and *S'codes ludwigii* were detected throughout the fermentation. The content of ethanol increased throughout the fermentation, and was similar to the fermentations with mixed culture of *S'codes ludwigii* and *H. uvarum*, and mixed culture of *S. cerevisiae* and *S'codes ludwigii*. During day 1 to day 2, the ethanol production rate of this mix of cultures (m=1.88) was higher than that of single *S'codes ludwigii* (m=0.72) and *H. uvarum* (m=0.76), but lower than that of single *S. cerevisiae* (m=2.39).The ethanol content was increased to a amount of 13.3% (v/v) at the end day of fermentation. The patterns of sucrose, glucose and fructose utilization were similar to that of mixed cultures of *S. cerevisiae* and *S'codes ludwigii*. Sucrose was completely used by day 4. Glucose and fructose increased during day 0 to day 2, then fructose was utilized throughout the fermentation, while glucose was utilized to day 4, then increased until the end of fermentation.

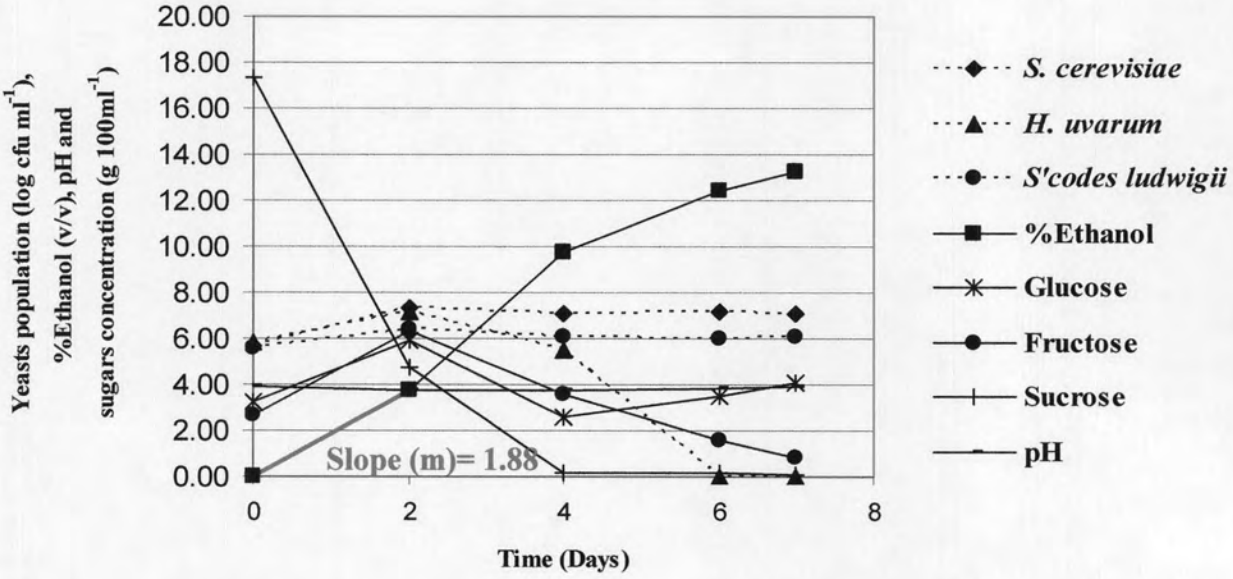

Figure 5.7 The changes of yeast population, ethanol, sugar concentrations and pH of pineapple juice during fermentation with *S. cerevisiae*, *S'codes ludwigii* and *H. uvarum*

In this study, the mixed starter cultures of *S. cerevisiae*, *S'codes ludwigii* and *H. uvarum* were applied to pineapple wine fermentation to investigate their fermentation properties during fermentation. For the mixed starter cultures of *S. cerevisiae* and *H. uvarum*, they could grow in the early stage of fermentation. After that, the population of *H. uvarum* declined, then could not be detected until the end of fermentation. The disappearance of *H. uvarum* might have occurred because of its weaker ethanol tolerance, or from the production of other toxic compounds besides ethanol (Goto, 1980; Fleet, Lafon-Lafourcade and Ribéreau-Gayon, 1984; Martinez, Millan and Ortega, 1989; Schütz and Gafner, 1993; Lema et al., 1996; Constantí et al., 1997; Egli et al., 1998; Cocolin, Bisson and Mills, 2000; Fleet, 2003a). However, some recent studies reported that the early death of non-*Saccharomyces* yeasts during mixed cultures fermentations is associated with the cell–cell contact-mediated mechanism which results in high cell concentrations of *S. cerevisiae* yeasts (Nissen and Arneborg, 2003; Nissen, Nielsen and Arneborg, 2003 and Arneborg et al, 2005). The similar fermentation characteristics of *S. cerevisiae* and *S'codes ludwigii* have been found in the last chapter. It is well known that *S. cerevisiae* is major yeast generally used for wine and alcoholic beverages fermentation. *S'codes ludwigii* has been reported as being able to tolerate high levels of SO_2 and ethanol content (Romano et al., 1999). Hence, it was frequently found as spoilage yeast in wine and alcoholic beverage products (Beuchat and Deák, 1996; Romano et al., 1999).

The fermentation profiles of pineapple wine fermentation by mixed starter cultures revealed that yeast isolates play an important role in ethanol production and utilization of sucrose, glucose and fructose for their ethanol production. The mixed cultures of *S. cerevisiae* and *S'codes ludwigii* produced more

ethanol during the fermentation. The rapid ethanol production of *S. cerevisiae* in the initial stage of fermentation caused the disappearance of *H. uvarum* from the fermentation system in day 2 when mixed cultures of *S. cerevisiae* and *H. uvarum* was used as starter cultures. Whereas, the slow ethanol production of *S'codes ludwigii* in the initial stage of fermentation caused *H. uvarum* to exist longer in the fermentation system when mixed cultures of *S'codes ludwigii* and *H. uvarum* was used as starter cultures.

The chemical substances produced during fermentation that influenced the organoleptic properties of wine were organic acids and volatile compounds, which were secondary metabolites produced by yeast during alcoholic fermentation. Therefore, the production of these compounds generated by multi-starter cultures during natural fermentation was further studied.

5.3.2 Organics acids of pineapple wine fermented by mixed starter cultures

Organic acids are the substances that influence on the sensory characteristics of wine. Several organic acids are produced and degraded during alcoholic fermentation. The appearance of organic acids content in wine depends on the substrate and starter cultures used for wine fermentation. To study the organic acids production of mixed starter cultures, the concentration of organic acids of fermented pineapple juice by mixed starter cultures was investigated throughout fermentation. The organic acid profiles of pineapple wine fermentation were investigated by HPLC during fermentation as mentioned in Materials and Methods. The organic acids determined were citric acid, malic acid, tartaric acid, succinic acid, lactic acid, fumaric acid, formic acid and acetic acid which their results were illustrated in Figure 5.8.

5.3.2.1 Citric acid

The initial citric acid of pineapple musts were in range of 5.9 to 7.6 g l^{-1}. Citric acid content of every sample did not significantly change throughout the fermentation and remained in range of 6.9 to 7.7 g l^{-1}. Citric acid in pineapple juice could not be utilized by three yeast isolates used in this study as mentioned in Chapter 4. Clarke and Bakker (2004) reported that the taste description of citric acid is fresh acid and that the pleasant level of this acid in wine is 0.02-0.08%. Radler (1993) reported that the citric acid content of grape must and wine was in the range of 0-0.5 g l^{-1}, while Ribéreau-Gayon et al. (2000) stated that the concentration of citric acid found in grape and wine prior to the malolactic fermentation process was between 0.5-1.0 g l^{-1}. This

high level of citric acid content in pineapple wine samples could produce an exceedingly sour taste in wine.

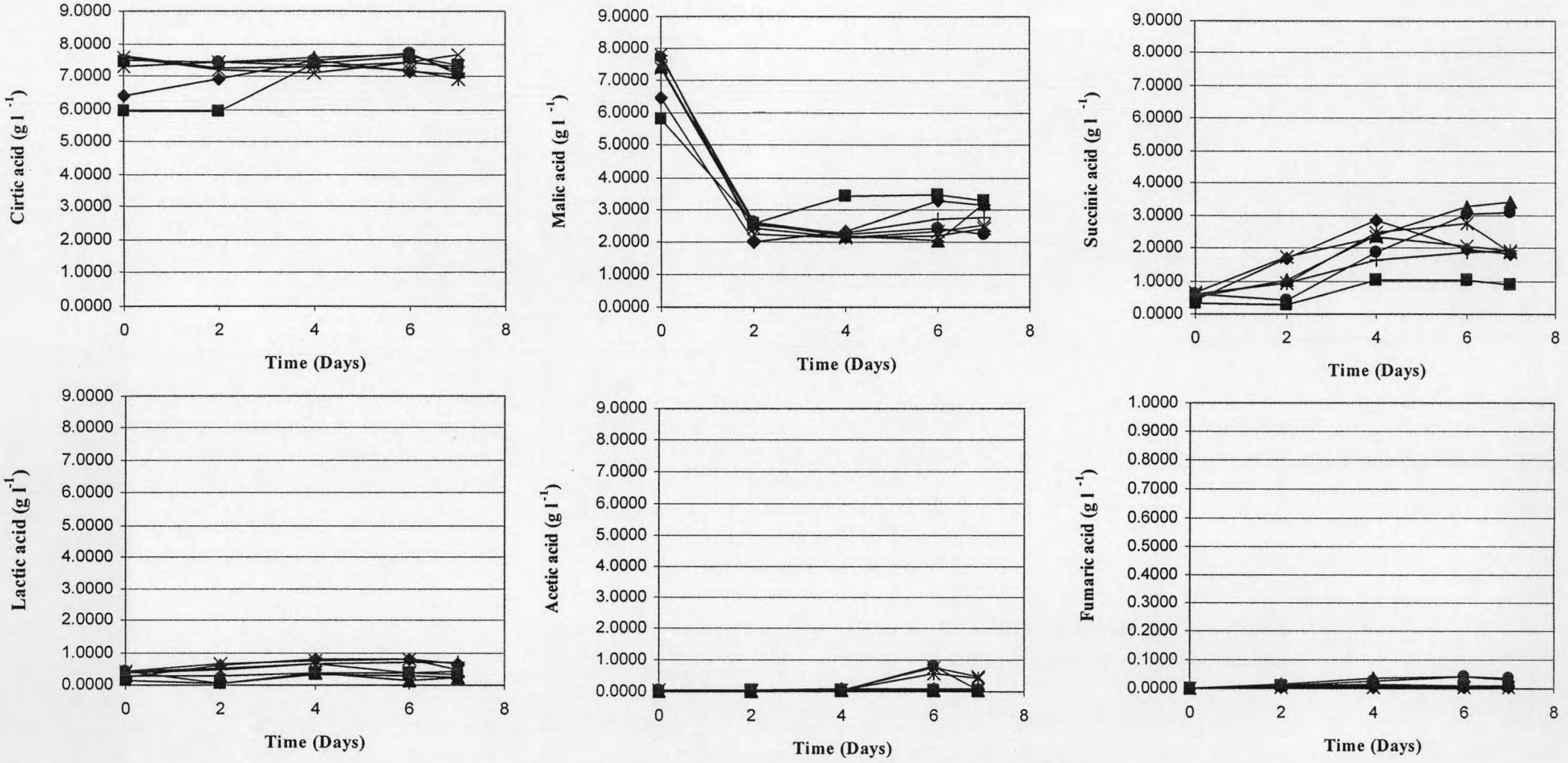

Figure 5.8 Changes in the concentrations of organic acids during fermentation of pineapple juice by multi-starter cultures; ◆ = *S. cerevisiae*, ▲ = *S'codes ludwigii*, ■ = *H. uvarum*, × = mixed cultures of *S. cerevisiae* and *H. uvarum*, ● = mixed cultures of *S'codes ludwigii* and *H. uvarum*, ∗ = mixed cultures of *S. cerevisiae* and *S'codes ludwigii*, + = mixed cultures of *S. cerevisiae*, *S'codes ludwigii* and *H. uvarum*

5.3.2.2 Malic acid

Malic acid of all samples was significantly reduced in the first two days of fermentation, then steady throughout the fermentation. The final concentrations of malic acid detected in pineapple wines remained in range of 2.2 to 3.3 g l^{-1}. Malic acid was the second major organic acid found in pineapple juice. It was found that malic acid was decomposed by all mixed yeast cultures in the same aspect. The decomposing of malic acid at the initial stage of fermentation could be that yeast isolates enzymatic decarboxylated malic acid to pyruvate and CO_2 by malate enzyme, which depends upon the yeast species used in fermentation (Ribéreau-Gayon et al., 2006). The metabolic pathways of malic acid were mentioned in Chapter 2. Radler (1993) reported that L-malic acid content of grape must and wine was in the range of 0.1-6 g l^{-1}. The decreasing of malic acid by yeast cultures could be advantageous to reduce the harsh acid taste of malic acid to the smooth acid taste of lactic acid in pineapple wine (Jackson, 2000).

5.3.2.3 Succinic acid

The concentration of succinic acid of every sample increased during day 0 to day 4. After that, the succinic acid of single *S. cerevisiae*, single *H. uvarum*, mixed cultures of *S. cerevisiae* and *H. uvarum*, and mixed cultures of *S. cerevisiae* and *S'codes ludwigii* decreased until the end of fermentation. Whereas the succinic acid content of single *S'codes ludwigii*, mixed cultures of *S'codes ludwigii* and *H. uvarum*, and mixed cultures of *S. cerevisiae*, *S'codes ludwigii* and *H. uvarum* were increased throughout the fermentation. However, the succinic acid of mixed

cultures of *S. cerevisiae*, *S'codes ludwigii* and *H. uvarum* was lower than that of single *S. cerevisiae* and mixed cultures of *S'codes ludwigii* and *H. uvarum*. The succinic acid content of single *S'codes ludwigii* was higher than that of other samples. Succinic acid is commonly a by-product of alcoholic fermentation. Therefore, an increase in succinic acid level was found during day 0 to day 4. The succinic acid could be formed a few different ways (the oxidation of glutamic acid, degradation of sugars or Wood-Werkman reaction), as stated in Chapter 2.

5.3.2.4 Lactic acid

The concentration of lactic acid of every sample slightly increased throughout the fermentation. D(-)-lactic acid could be formed from the degradation of L(+)-malic acid by yeast isolates used as starter culture during the alcoholic fermentation (Schreier, 1979). However, this result agreed with some previous reports that the production of lactic acid by wine yeasts during fermentation was an insignificantly small amount (Radler, 1993; Fleet, 2001).

5.3.2.5 Fumaric acid

The changes of fumaric acid content during fermentation, the results show that the increase in fumaric acid content detected in all samples was insignificant, which is consistent with the previous report which stated that fumaric acid is rarely found during wine fermentation (Whiting, 1976). Fumaric acid could control growth of lactic acid bacteria and be used as an acidulant in wine Panchal (1990).

5.3.2.6 Acetic acid

With changes of acetic acid content during fermentation, it was found that the acetic acid content of all samples were steady throughout the fermentation. After that, the acetic acid of mixed cultures of *S. cerevisiae* and *H. uvarum*, mixed cultures of *S'codes ludwigii* and *H. uvarum*, and mixed cultures of *S. cerevisiae* and *S'codes ludwigii*, rapidly increased in day 6, then decreased until the end of fermentation.

The investigation of changes of acetic acids during pineapple wine fermentation by mixed culture starters revealed that there was an insignificant difference between the concentrations of acetic acid of pineapple wine fermented by single and mixed starter cultures. However, the influence of mixed starter of two cultures on acetic acid content was observed during the fermentation. The increasing of acetic acid content of mixed starter of two cultures was found in day 6, then acetic acid decreased until the end of fermentation. This decreasing of acetic acid could be associated with the esterification part of acetic acid and ethanol to form ethyl acetate which gives a fruity characteristic in wine products. However, the last concentrations of acetic acid of these three samples decreased in day 7 and did not exceeded 1.5 g l^{-1}, which was acetic acid content that could not be harmful to wine quality as reported by Fleet (2001). This incident should not be ignored and needs further determination, since acetic acid could negatively affect wine flavor quality.

5.3.3 Volatile compounds of pineapple wine fermented by mixed starter cultures

To understand the interaction of mixed starter cultures to the organic acids production. In this study, the volatile compounds of pineapple juice fermented by mixed starter cultures were determined. Their results were displayed in Table 5.2. The chromatograms of volatile compounds analysis of all samples are illustrated in Appendix E.

5.3.3.1 Fermentation with mixed cultures of *S. cerevisiae* and *H. uvarum*

Apart from ethanol, the main volatile compounds of mixed culture of *S. cerevisiae* and *H. uvarum* were ethyl decanoate and ethyl dodecanoate. In addition, isoamyl alcohol, ethyl octanoate and ethyl hexadecanoate was also found in this sample. Notably, these main volatile compounds were similar to those of single *S. cerevisiae*.

The volatile compounds of pineapple wines were analysis to identify the main volatile compounds produced by mixed starter cultures. The interaction of mixed starter cultures on volatile compounds of pineapple wine was investigated. In Chapter 4, *S. cerevisiae* and *S'codes ludwigii* produced ethyl decanoate and ethyl dodecanoate as

Table 5.2 Main volatile compounds of fermented pineapple juice by mixed starter cultures

Order	Main volatile compounds of pineapple wines*						
	S. cerevisiae (Sc)	*S'codes ludwigii* (Sl)	*H. uvarum* (Hu)	Sc + Hu	Sl + Hu	Sc + Sl	Sc + Sl + Hu
1	Ethanol (22.2)	Ethanol (25.9)	Ethanol (12.8)	Ethanol (26.4)	Ethanol (25.4)	Ethanol (21.4)	Ethanol (29.3)
2	Ethyl decanoate (8.8)	Ethyl decanoate (13.3)	2-phenylethyl acetate (6.0)	Ethyl decanoate (5.8)	Ethyl decanoate (4.7)	Ethyl decanoate (9.7)	Ethyl dodecanoate (5.1)
3	Ethyl dodecanoate (8.2)	Ethyl dodecanoate (8.3)	Ethyl acetate (2.6)	Ethyl dodecanoate (5.8)	Ethyl dodecanoate (3.8)	Ethyl dodecanoate (8.1)	Ethyl decanoate (4.7)
4	Isoamylalcohol (4.6)	Isoamylalcohol (5.9)	Ethyl dodecanoate (2.4)	Isoamyl alcohol (3.3)	Isoamyl alcohol (3.7)	Isoamylalcohol (5.1)	Isoamyl alcohol (3.1)
5	Ethyl octanoate (3.2)	Ethyl tetradecanoate (2.3)	Ethyl decanoate (1.9)	Ethyl octanoate (2.6)	Ethyl acetate (1.2)	Ethyl octanoate (3.4)	Acetaldehyde (1.7)
6	Ethyl hexadecanoate (1.8)	Ethyl octanoate (2.1)	Ethyl hexanoate (0.8)	Ethyl hexadecanoate (2.0)	2-Phenylethyl acetate (0.9)	Ethyl acetate (2.5)	Ethyl octanoate (1.7)
7	Acetaldehyde (1.5)	Ethyl acetate (1.9)	Butanoic acid, 2-methyl-, ethyl ester (0.6)	Ethyl acetate (1.1)	Ethyl oleate (0.9)	Acetaldehyde (1.8)	Ethyl acetate (1.7)
8	Ethyl hexanoate (1.3)	Acetaldehyde (1.1)	Ethyl octanoate (0.5)	Ethyl hexanoate (1.1)	Decanoic acid (0.8)	Ethyl hexanoate (1.8)	Ethyl hexanoate (1.0)
9	Isoamyl acetate (1.3)	Decanoic acid (1.0)	Dodecanoic acid (0.5)	1-Butanol, 3-methyl-, acetate (0.9)	Ethyl tetradecanoate (0.8)	1-Butanol, 3-methyl-, acetate (1.7)	Decanoic acid (0.7)
10	Ethyl acetate (1.3)	Dodecanoic acid (1.0)	1H-Indole-3-ethanol, acetate (ester) (0.5)	Decanoic acid (0.9)	Ethyl octanoate (0.7)	Decanoic acid (1.5)	Isoamyl acetate (0.7)

* The number in the parenthesis is volatile compound content (% composition)

main volatile compounds when pineapple juice used as a substrate. These ethyl esters give fruitiness characteristic in pineapple wine (Clarke and Bakker, 2004). *H. uvarum* produced a higher amount of 2-phenylethyl acetate, which can produce flowery and fruity characteristics in pineapple wine (Rychlik, Schieberle and Grosch., 1998). According to the fermentation profile of mixed culture of *S. cerevisiae* and *H. uvarum* (Figure 5.4), the amount of *H. uvarum* population gradually declined and could not be detected since day 4 of fermentation because of its weaker tolerance to ethanol content. The main yeast, *S. cerevisiae*, could govern and complete the fermentation. Consequently, the main volatile compounds of pineapple wine fermented by mixed cultures of *S. cerevisiae* and *H. uvarum* were similar to those of single *S. cerevisiae*.

5.3.3.2 Fermentation with mixed cultures of *S'codes ludwigii* and *H. uvarum*

Ethyl decanoate and ethyl dodecanoate were the main volatile compounds found of mixed cultures of *S'codes ludwigii* and *H. uvarum*. In addition, there were isoamylalcohol, ethyl acetate and 2-phenylethyl acetate found in this fermented juice. Noticeably, the combination of main volatile compounds generated by *S'codes ludwigii* and *H. uvarum* was observed in this sample. 2-Phenylethyl acetate was found as the main volatile compounds of single *H. uvarum* and mixed cultures of *H. uvarum* and *S'codes ludwigii* in pineapple wine fermentation.

Interestingly, there was a combination of their volatile compounds in this pineapple wine. Concerning the fermentation profile of this mixed starter culture (Figure 5.5), the slow increasing of ethanol content of *S'codes ludwigii*

could extend the existence of *H. uvarum* in fermentation until day 4. Hence, *H. uvarum* could stay alive and produce both volatile and non-volatile compounds during the fermentation. It then started to decline until could not be observed at the last day of fermentation. Ethyl acetate was a main volatile compound produced by this mix of starter cultures. This finding agrees with previous studies that apiculate yeast can produce the large amount of ethyl acetate in grape must (Herraiz et al., 1990; Ciani and Picciotti, 1995; Ciani and Maccarelli, 1998; Romano, 2002; Romano et al., 2003; Ciani, Beco and Comitini, 2006). Furthermore, 2-phenylethyl acetate was also detected to be a main volatile compound produced by this mix of starter cultures. It is well known that 2-phenylethyl acetate is a volatile compound producing flowery and fruity characteristics in wine (Rychlik, Schieberle and Grosch., 1998; Clarke and Bakker, 2004). It can enhance the complexity of wine flavours in winemaking. This substance was suggested to be a main volatile compound produced by *H. uvarum* (Moreira et al., 2008) and *H. guilliermondii* (Rojas et al., 2003; Moreira et al., 2005; Moreira et al., 2008).

5.3.3.3 Fermentation with mixed cultures of *S. cerevisiae* and *S'codes ludwigii*

Ethyl decanoate and ethyl dodecanoate were found as the main volatile compounds of mixed culture of *S. cerevisiae* and *S'codes ludwigii*. In addition, isoamylalcohol, ethyl octanoate and ethyl acetate was also found in this sample. The results showed that these main volatile compounds were similar to those of single *S. cerevisiae* and single *S'codes ludwigii*. However, ethyl tetradecanoate, a main volatile compound of *S'codes ludwigii*, was not found in this sample.

The volatile compounds produced from mixed cultures of *S. cerevisiae* and *S'codes ludwigii* were a combination of volatile compounds of both starter cultures. However, the appearance of a lower amount of ethyl tetradecanoate, a main ester produced by *S'codes ludwigii*, in this pineapple wine could be because there was a greater population of *S. cerevisiae* than those of *S'codes ludwigii* throughout the fermentations (Figure 5.6).

5.3.3.4 Fermentation with mixed cultures of *S. cerevisiae*, *S'codes ludwigii* and *H. uvarum*

Ethyl dodecanoate and ethyl decanoate were the major volatile compounds generated by mixed cultures of *S. cerevisiae*, *S'codes ludwigii* and *H. uvarum*. Isoamyl alcohol, acetaldehyde and ethyl octanoate was also found in this fermented juice. This result showed that these main volatile compounds were similar to single *S. cerevisiae* and single *S'codes ludwigii*.

The results revealed that the presence of *S. cerevisiae* could have an affect on the existing of *H. uvarum* during fermentation because of the ethanol production of *S. cerevisiae*. There was no ethyl acetate and 2-phenylethyl acetate produced by *H. uvarum* found to be the main volatile compounds of this pineapple wine. This result corresponded with their fermentation profile as performed in 5.3.1. The fermentation profile of this mix of starter cultures indicated that the decreasing of *H. uvarum* have been found since day 2 until it could not be observed in day 6 of fermentation (Figure 5.6).

These results concluded that the volatile compounds of pineapple wine produced by mixed starter cultures were depended on the fermentative behavior of the main starter culture. *H. uvarum* could not show its main volatile compounds in wine product when *S. cerevisiae* was used in combination with main starter cultures. Conversely, *S'codes ludwigii* could extend the survival of *H. uvarum* during the fermentation. *H. uvarum* could carry out the fermentation and produce the desirable volatile compounds in wine before it started its decline phase. These volatile compounds could increase the complexity of pineapple wine flavors.