CHAPTER IV

DETERMINATION OF FERMENTATION PROFILE OF YEAST ISOLATES IN PINEAPPLE JUICE FERMENTATION

4.1 Introduction

Yeasts are significant in winemaking because they perform the alcoholic fermentation, and produce the desirable secondary metabolites affect the sensory quality of the final wine product. However, they may also cause spoilage of the wine (Fleet, 2002; Romano et al., 2003). Therefore, yeast selected for wine fermentation play the important role in the final quality of produced wine. The basic criteria for selection and development of yeast for wine fermentation are under three categories: (i) properties that affect the performance of the fermentation process, (ii) properties that determine wine quality and character and (iii) properties associated with the commercial production of wine yeasts (Fleet, 2008). To obtain the information about wine making properties of yeast isolates, the fermentation patterns of yeast strains need to be determined. In this Chapter, yeast species isolated in Chapter 3 were primarily screened for their alcoholic fermentation properties. The selected yeast isolates were investigated for their fermentation characteristics, such as development of yeast population, ethanol production, sugar utilization,, production and assimilation of organic acids, pH change to wine, as well as their production of major volatile compounds during pineapple juice fermentation. The fermentation characteristics of the yeast isolates will then be considered in selection of strains for use as starter cultures in the subsequent Chapter.

4.2 Materials and methods

4.2.1 Yeast isolates

Yeasts selected from those described in Chapter 3 were used in the experiments reported in this Chapter. They are listed in Table 4.1, showing their sources of isolation.

4.2.2 Micro-fermentation screening

The yeast isolates from Chapter 3 were initially screened on the basis of their ability of sugars fermentation and production of total alcohol content higher than 5% (v/v) and no characteristic of film formation. Fresh culture was prepared by inoculated one loop of 48 hours yeast colony into 5 ml of sterile pineapple juice (Tipco®, Thailand). Inoculated juice was orbitally shaken 200 rpm at ambient temperature (28-30°C) for approximately 20-24 hours or until cell population number reached 8 log cfu ml^{-1} and was used as starter culture for pineapple juice fermentations. The same pineapple juice was used as the base. Its sugar concentration was adjusted to 22°brix by adding sucrose (Mitrhpol, Thailand). This juice (10 ml) was then transferred into 20 ml sterile test tube with screwed cap. Each starter culture was inoculated into the 22°brix pineapple juice at initial population of 10^6 cell ml^{-1} and mixed thoroughly. The pineapple juice fermentations were conducted at 25°C for 1 week in triplicate. The fermented juices were analyzed for total alcohol concentration by vinometer (Alla, France) at the last day of fermentation.

Table 4.1 Yeasts selected from the enrichment cultures and natural fermentation of pineapple sample from Thailand and their source of isolation

No.	Yeast isolates	Code	Source of isolation
1	*P. guilliermondii*	Pg1	Pinapple fruits at 1 month before harvest stage, enriched in pineapple juice
2	*P. guilliermondii*	Pg2	Pinapple fruits at 2 month before harvest stage, enriched in grape juice with 6% (v/v) ethanol
3	*P. guilliermondii*	Pg3	Pinapple fruits at 3 month before harvest stage, enriched in grape juice
4	*P. guilliermondii*	Pg4	Pinapple fruits at 1 month before harvest stage, enriched in grape juice
5	*P. guilliermondii*	Pg5	Pinapple fruits at 2 month before harvest stage , enriched in grape juice with 6% (v/v) ethanol
6	*Candida* sp.	Cs1	Natural fermentation in day 2
7	*Candida* sp.	Cs2	Pinapple fruits at 2 month before harvest stage, enriched in grape juice with 6% (v/v) ethanol
8	*Candida* sp.	Cs3	Pinapple fruits at 2 month before harvest stage, enriched in pineapple juice
9	*Candida* sp.	Cs4	Natural fermentation in day 5
10	*Candida* sp.	Cs5	Natural fermentation in day 4
11	*H. uvarum*	Hu1	Natural fermentation in day 4
12	*H. uvarum*	Hu2	Natural fermentation in day 3
13	*H. uvarum*	Hu3	Pinapple fruits at 2 month before harvest stage, enriched in grape juice
14	*H. uvarum*	Hu4	Natural fermentation in day 1
15	*C. tropicalis*	Ct1	Pinapple fruits at Harvest stage, enriched in pineapple juice
16	*C. tropicalis*	Ct2	Pinapple fruits at 1 month before harvest stage, enriched in grape juice with 6% ethanol
17	*I. orientalis*	Io	Damaged fruits enriched in pineapple juice
18	*P. fermentans*	Pf	Damaged fruits enriched in grape juice with 6% (v/v) ethanol
19	*S'codes ludwigii*	Sl	Damaged fruits enriched in grape juice with 6% (v/v) ethanol
20	*Z. bailii*	Zb	Damaged fruits enriched in grape juice

4.2.3 Determination of fermentation profile

Yeast isolates selected from micro-fermentation screening were used in more detailed studies of fermentation profiles. Inoculum cultures of these yeasts were prepared in sterile pineapple juice (Tipco®, Thailand) that was incubated with rotation at 200 rpm, at 25°C for 20-24 hours by orbital incubator shaker (Gallenkamp, UK.) and used to inoculate in pineapple juice fermentations.

The same pineapple juice 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 as described in section 4.2.2. 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 the last day of fermentation. Samples for yeast populations were analyzed immediately. Samples for chemical analysis were stored at -30°C until analysis.

4.2.3.1 Determination of yeast population by cultural method

Samples of the fermented pineapple juice were serially diluted in 0.1% peptone water. The yeasts in each dilution were enumerated and isolated by spread inoculation of 0.1 ml onto plates of MEA agar (Oxoid, England) and incubation at 25°C for 2-4 days. This analysis was done in duplicate. Yeast colonies were counted to give populations as log cfu ml^{-1}.

4.2.3.2 Preparation of fermented pineapple juice for chemical analysis by HPLC

Samples were centrifuged in Sorvall® TC-6, tabletop centrifuge (Dupont, USA.) at 3,000 rpm (1,502 xg) to separate yeast cell, pulp and other substances. The juices were then filtered with 0.45 micron syringe filter (Micro Analytix Pty Ltd, Australia) to remove small particles. The filtrates were poured into a vial, which was capped and put in an autosampler tray for injection into the HPLC.

4.2.3.3 Analysis of ethanol

Ethanol concentration in fermented pineapple juices were analysed by HPLC (Waters™ 717 plus Autosampler with Waters™ 600 Controller, Waters Associates Inc., USA.), using the method developed by Davis, Lee and Fleet (1986) and Bell et al. (1991) with some modification. The analytical column (HPX-87H, 300x7.8 mm ion exclusion column, Bio-Rad, USA.) was run at 55°C using orthophosphoric acid in water (0.06%) as mobile phase at a flow rate of 0.5 ml min^{-1}.

Ethanol was detected by a Waters 2414, Refractive Index Detector (Waters Associates Inc., USA.), and data were analyzed by a Millenium software program. The method was calibrated using ethanol at a concentration of 1.0% (v/v) as standard. (Appendix B)

4.2.3.4 Analysis of sugars

Sugars in fermented pineapple juices were analyzed by HPLC (Waters^{TM} 717 plus Autosampler with Waters^{TM} 600 Controller, Waters Associates Inc., USA.), using the method developed by Davis, Lee and Fleet (1986) and Bell et al. (1991) with some modification. The analytical column (HPX-87H, 300x7.8 mm ion exclusion column, Bio-Rad, USA.) was run at 25°C using orthophosphoric acid in water (0.06%) as mobile phase at a flow rate of 0.5 ml min^{-1}. Sugars were detected by a Waters 2414, Refractive Index Detector (Waters Associates Inc., USA.), and data were analysed by a Millenium software program. The method was calibrated using a standard of mixture of glucose, fructose and sucrose, each at a concentration of 5 g l^{-1}. (Appendix C)

4.2.3.5 Analysis of organic acids

Organic acids in fermented pineapple juices were analyzed by HPLC (Waters^{TM} 717 plus Autosampler with Waters^{TM} 600 Controller, Waters Associates Inc., USA.), using the method developed by Davis, Lee and Fleet (1986) and Bell et al. (1991) with some modification. The analytical column (HPX-87H, 300x7.8 mm ion exclusion column, Bio-Rad, USA.) was run at 55°C using

orthophosphoric acid in water (0.06%) as mobile phase at a flow rate of 0.5 ml min^{-1}. Organic acids were detected by a Waters™ 996 Photodiode Array Detector (Waters Associates Inc., USA.), and data were analyzed by a Millenium software program. The method was calibrated using a standard of mixture of citric, tartaric, malic, succinic, lactic and formic acids, each at a concentration of 5 g l^{-1}, fumaric acid at a concentration of 0.1 g l^{-1} and 1% (v/v) acetic acid. (Appendix D)

4.2.4 Analysis of volatile compounds

4.2.4.1 HS-SPME extraction

The volatile compounds were analyzed by headspace-solid phase microextraction (HS-SPME) with GC-MS using the method developed by Bosch-Fusté et al. (2007) with some modification. One milliliter of fermented pineapple juice sample was placed into a 22 ml glass vial (HP, USA.) for each HS-SPME analysis. The vial was tightly capped with PTFE septum (HP, USA.) and placed in the heating box. The sample was maintained for 30 min at 50°C. A 20 mm-fiber with coating of 100μm layer of polydimethylsiloxane (PDMS) was used in this study. The fiber was activated by inserting it into the GC injector at 250°C for 30 min. The SPME fiber was then placed into the headspace and maintained for 30 min at 50°C. After reaching the sampling time, the fiber was removed from the vial and inserted into the GC injection port for desorption for 5 min.

4.2.4.2 GC-MS analysis

Volatile compounds were identified on a mass spectrometer (Agilent Technologies 5973 Network coupled directly online to an Agilent Technologies 6890N Network GC System, USA.). Spectra were obtained on electron impact at 70 eV, scanning from 10-350 amu. The GC system was equipped with HP-Innowax capillary column (0.32mm x 30m x 0.25µm, Agilent Technologies, USA.) and the carrier gas was helium at a flow rate of 1.5 ml min^{-1}. The GC oven temperature was programmed from 50°C and held for 10 min, from 50°C to 250°C at 10°C min^{-1}, and finally 10 min at 250°C. Volatile compounds were injected in splitless mode. These volatile compounds were identified by comparison with reference spectra Willey 6.1 (NY, USA.).

4.3 Results and discussion

4.3.1 Micro-fermentation screening

Yeast isolates obtained from Chapter 3 were screened for alcohol production by performing micro-fermentation tests using sterile pineapple juice as a substrate. Table 4.2 shows alcohol content and film forming characteristics produced by 20 yeast isolates in micro-fermentation.

Issatchenkia orientalis produced the highest alcohol content, followed by *S'codes ludwigii*. Five isolates of *Candida* sp. (Cs1 - Cs5) generated a range of alcohol contents from 3.0 to 6.7% (v/v). Four *H. uvarum* isolates (Hu1 – Hu4)

produced alcohol ranging from 3.7 to 5.2% (v/v), whereas five *P. guilliermondii* isolates (Pg1 – Pg5) produced alcohol ranging from 1.2 to 3.7% (v/v).

Based on film formation ability, *Issatchenkia orientalis*, *C. tropicalis* and *P. fermentans* were observed as film forming yeasts in pineapple juice fermentation. This observation agreed with the previous reports that *I. orientalis*, *C. tropicalis* and *P. fermentans* can form surface filaments during their growth (Kurtzman and Fell, 1998; Barnett et al., 2000). Yeasts were selected for further study based on the following criteria: (i) total alcohol content, (ii) no-film forming ability, and (iii) species previously reported as successful wine fermentative yeasts. Based on these criteria, *S'codes ludwigii*, *H. uvarum*, *Candida* sp. and *Z. bailii* were selected for further experiments. Their cell morphologies were determined, as shown in Appendix A.

Table 4.2 Fermentation characteristics of 20 yeast isolates in pineapple juice micro-fermentation

No.	Yeast isolates	Code	Total alcohol content (%v/v) ± SD.	Film formation
1	*I. orientalis*	Io	10.5 ± 1.0	+
2	*S'codes ludwigii*	Sl	8.0 ± 0.0	-
3	*Candida* sp.	Cs1	6.7 ± 0.8	-
4	*C. tropicalis*	Ct1	6.1 ± 0.9	+
5	*C. tropicalis*	Ct2	6.0 ± 0.9	+
6	*Candida* sp.	Cs2	5.7 ± 1.3	-
7	*Z. bailii*	Zb	5.7 ± 1.2	-
8	*H. uvarum*	Hu1	5.2 ± 0.3	-
9	*H. uvarum*	Hu2	5.0 ± 0.0	-
10	*Candida* sp.	Cs3	4.5 ± 0.9	-
11	*P. fermentans*	Pf	4.0 ± 0.5	+
12	*H. uvarum*	Hu3	4.0 ± 0.5	-
13	*Candida* sp.	Cs4	3.8 ± 0.8	-
14	*H. uvarum*	Hu4	3.7 ± 0.8	-
15	*P. guilliermondii*	Pg1	3.7 ± 1.0	-
16	*Candida* sp.	Cs5	3.0 ± 1.0	-
17	*P. guilliermondii*	Pg2	3.0 ± 0.9	-
18	*P. guilliermondii*	Pg3	2.5 ± 0.5	-
19	*P. guilliermondii*	Pg4	2.3 ± 0.6	-
20	*P. guilliermondii*	Pg5	1.2 ± 0.3	-

+ = positive, - = negative

4.3.2 Fermentation profile of yeast isolates

The changes of total yeast population, pH, and contents of ethanol, sugars and organic acids were investigated during pineapple juice fermentation.

Normally, in wine fermentation, commercial cultures of *S. cerevisiae* are used as starter culture because of advantages such as a rapid and successful process, and the consistent and predictable quality of the wine (Fleet et al., 2002). Therefore, the fermentation profile of *S. cerevisiae* (Angle ®, China), a commercial strain normally used for wine, was also investigated to compare with fermentation profile of yeast isolates in this study.

4.3.2.1 Fermentation with *S. cerevisiae*

Figure 4.1 shows the fermentation pattern of pineapple juice inoculated with *S. cerevisiae*. During day 0 and day 2 of fermentation, its population increased approximately 2 log cycles. After day 2, *S. cerevisiae* numbers were stable through to the last day. Ethanol production was observed at the early stage of fermentation, and rapidly increased to 11% (v/v) during days 1 to 4, then increased slowly to 12% (v/v) through to day 6. For sugar determination, sucrose consistently decreased throughout the fermentation. In contrast with sucrose, glucose and fructose consistently increased during days 0 to 2, then decreased throughout the fermentation. The rapid increase of yeast population at the initial stages of fermentation could be due to utilization of residual O_2 in the juice allowing for faster cell division. After that, the fermentation shifted to anaerobic condition. When the ethanol content reached 11% (v/v) by day 4, *S. cerevisiae* probably suffered stress from alcohol toxicity. Hence, the decreasing of ethanol production rate of *S. cerevisiae* was observed after day 4.

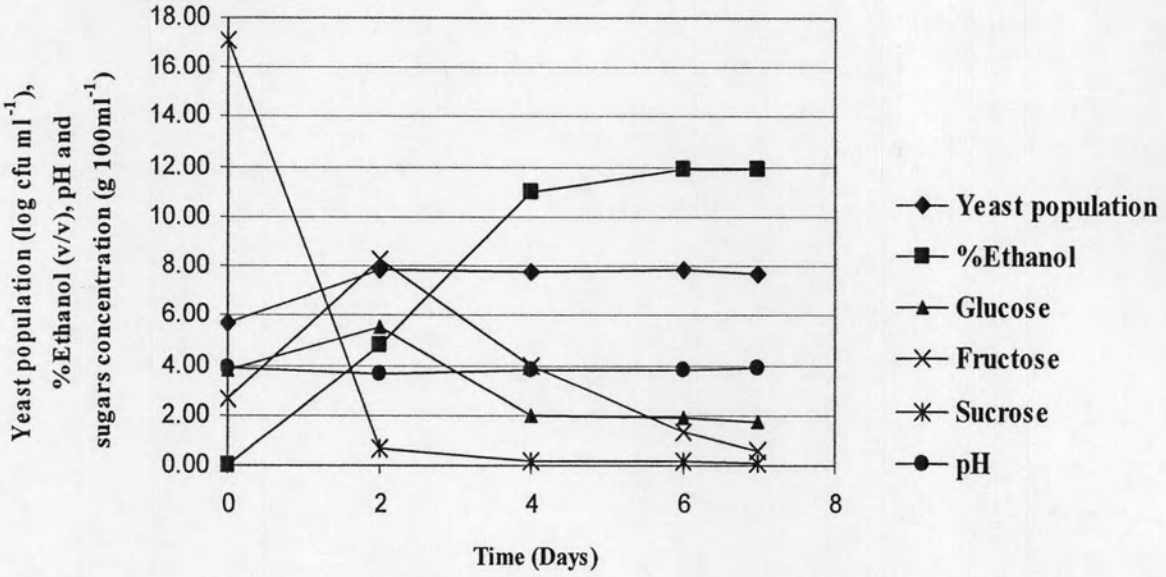

Figure 4.1 Yeast population and changes in the concentrations of ethanol, sugars and pH during fermentation of pineapple juice by *S. cerevisiae*

4.3.2.2 Fermentation with *S'codes ludwigii*

The fermentation pattern of *S'codes ludwigii* (Sl) is shown in Figure 4.2 and was relatively similar to that of *S. cerevisiae*. At the initial stage of fermentation, the population of *S'codes ludwigii* (Sl) increased approximately 2 log cycles. During day 0 and day 2, the ethanol content slightly increased, then shifted to rapidly increase throughout the fermentation, allowing the final ethanol content to reach almost 13% (v/v) in the last day of fermentation. The patterns of sucrose, glucose and fructose utilization were similar to that of *S. cerevisiae*. *S'codes ludwigii* (Sl) showed slow ethanol production at the early stage of fermentation. Thus, the ethanol content was only 1.4% (v/v) in day 2. After that, glucose and fructose were used for ethanol production of *S'codes ludwigii* (Sl) throughout the fermentation.

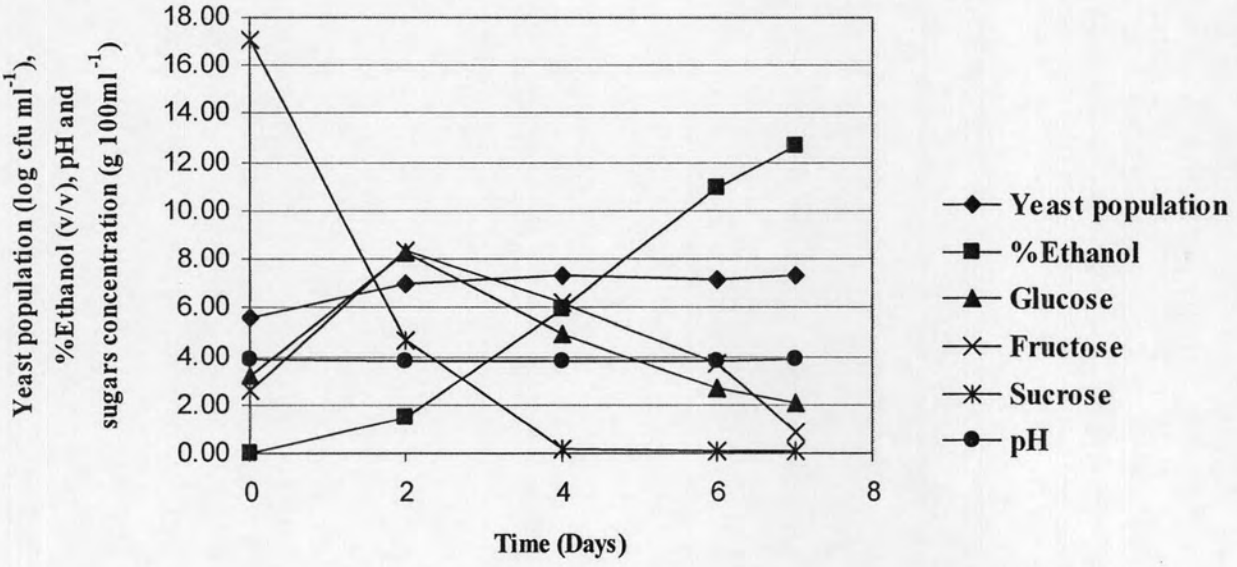

Figure 4.2 Yeast population and changes in the concentrations of ethanol, sugars and pH during fermentation of pineapple juice by *S'codes ludwigii* (Sl)

4.3.2.3 Fermentation with *H. uvarum*

The fermentation profile of *H. uvarum*1 (Hu1) is illustrated in Figure 4.3. The population of *H. uvarum*1 (Hu1) increased at the initial stage of fermentation and remained stable until day 4, then decreased throughout the fermentation. The ethanol content slightly increased to 3.1% (v/v) from day 0 to day 4 of fermentation, and remained constant throughout the fermentation. When sugars were determined, sucrose slightly decreased throughout the fermentation because of the low pH condition of pineapple juice. Fructose was used throughout the fermentation, whereas glucose content was constant during day 0 to day 6, then decreased until the end of fermentation. The fermentation pattern of *H. uvarum*2 (Hu2) was also investigated in this study, as indicated in Figure 4.4. The results show the similarity between the fermentation pattern of *H. uvarum*1 (Hu1) and *H. uvarum*2 (Hu2).

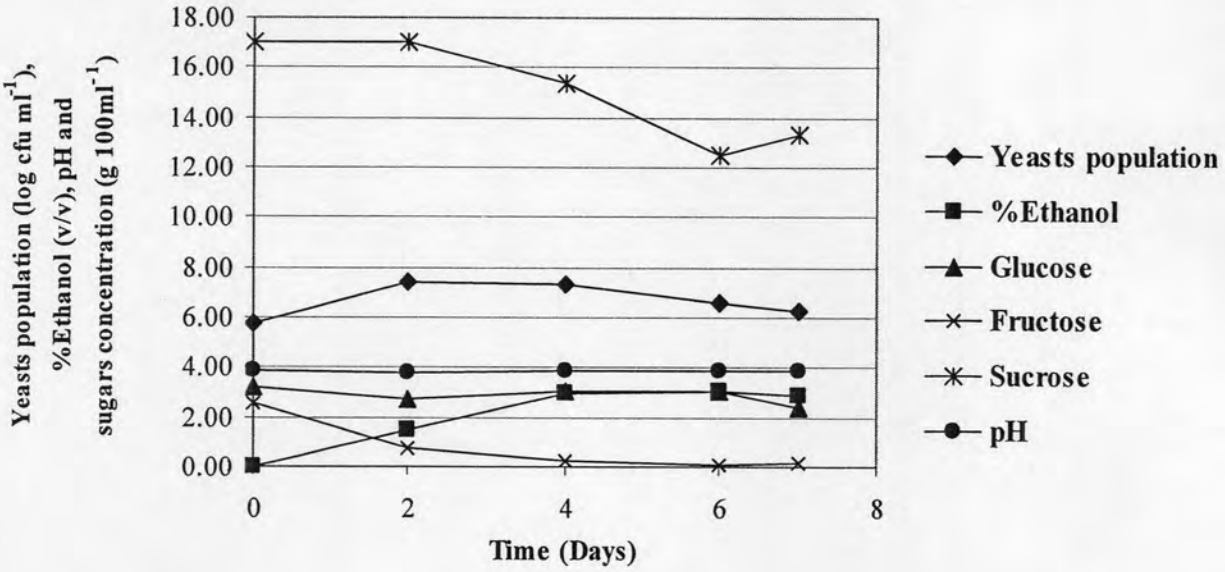

Figure 4.3 Yeast population and changes in the concentrations of ethanol, sugars and pH during fermentation of pineapple juice by *H. uvarum*1 (Hu1)

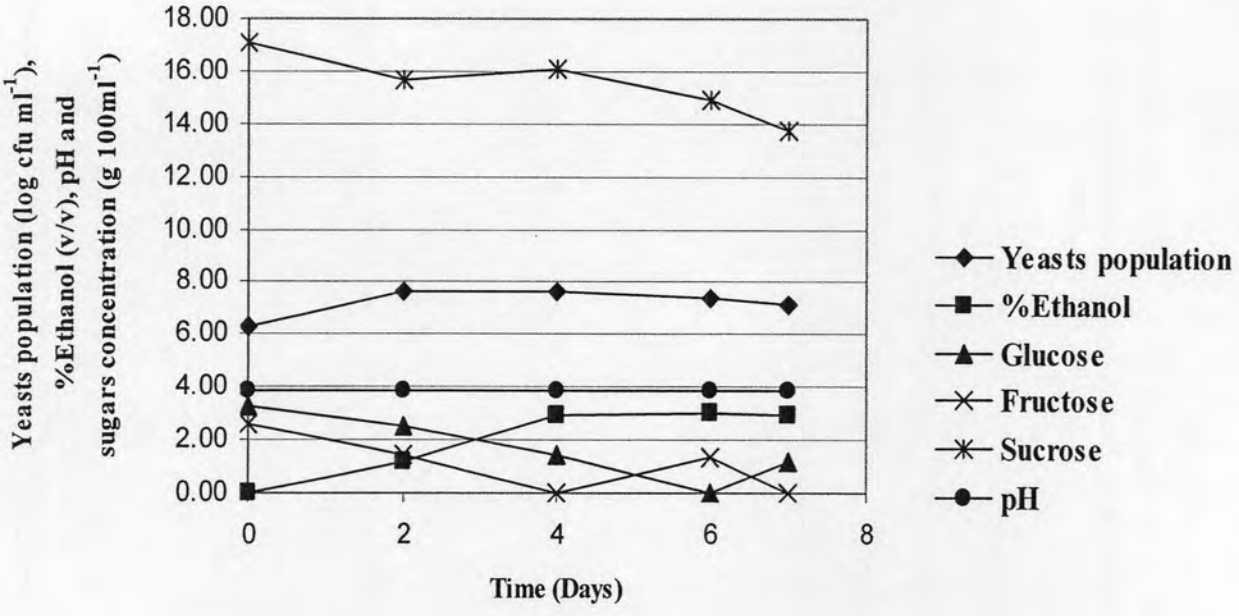

Figure 4.4 Yeast population and changes in the concentrations of ethanol, sugars and pH during fermentation of pineapple juice by *H. uvarum*2 (Hu2)

4.3.2.4 Fermentation with *Z. bailii*

Figure 4.5 shows fermentation pattern of pineapple juice inoculated with *Z. bailii* (Zb). Its population increased approximately 2 log cycles during day 0 and day 6 of fermentation, then decreased until the end of fermentation. Ethanol production was observed at the early stage of fermentation, the ethanol slightly increased from day 0 to day 2, then increased to an amount of 7% (v/v) throughout the fermentation. Sucrose consistently decreased throughout the fermentation. In contrast to sucrose, glucose and fructose consistently increased from day 0 to day 4. Glucose content was stable to day 6, then decreased until the end of fermentation, while fructose consistently decreased throughout the fermentation.

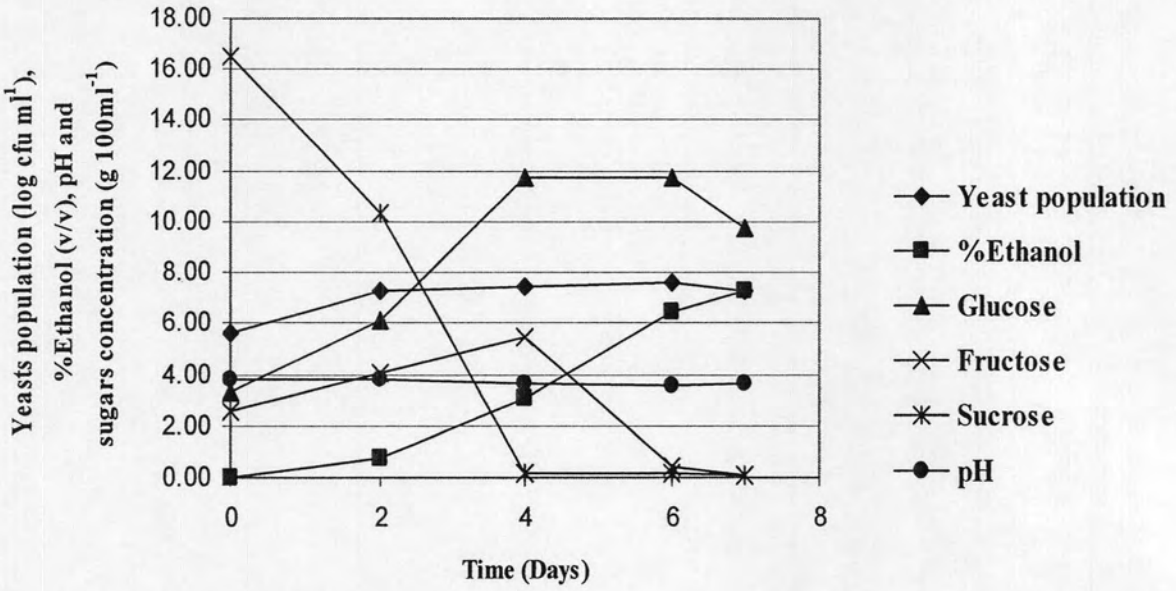

Figure 4.5 Yeast population and changes in the concentrations of ethanol, sugars and pH during fermentation of pineapple juice by *Z. bailii* (Zb)

4.3.2.5 Fermentation with *Candida* sp.

The fermentation profile of *Candida* sp.1 (Cs1) is illustrated in Figure 4.6. The population of *Candida* sp.1 (Cs1) increased at initial stage of fermentation and stable throughout the fermentation. The ethanol content slightly increased to 2.7% (v/v) during days 0 to 4 of fermentation, then was constant throughout the fermentation. For sugar determination, sucrose slightly decreased throughout the fermentation because of low pH condition of pineapple juice. Glucose content decreased until day 4, then slightly increased throughout the fermentation, whereas fructose was used throughout the fermentation. The fermentation pattern of *Candida* sp.2 (Cs2) was also investigated in this study, as shown in Figure 4.7. The similarity between the fermentation pattern of *Candida* sp.1 (Cs1) and *Candida* sp.2 (Cs2) was observed during the fermentation.

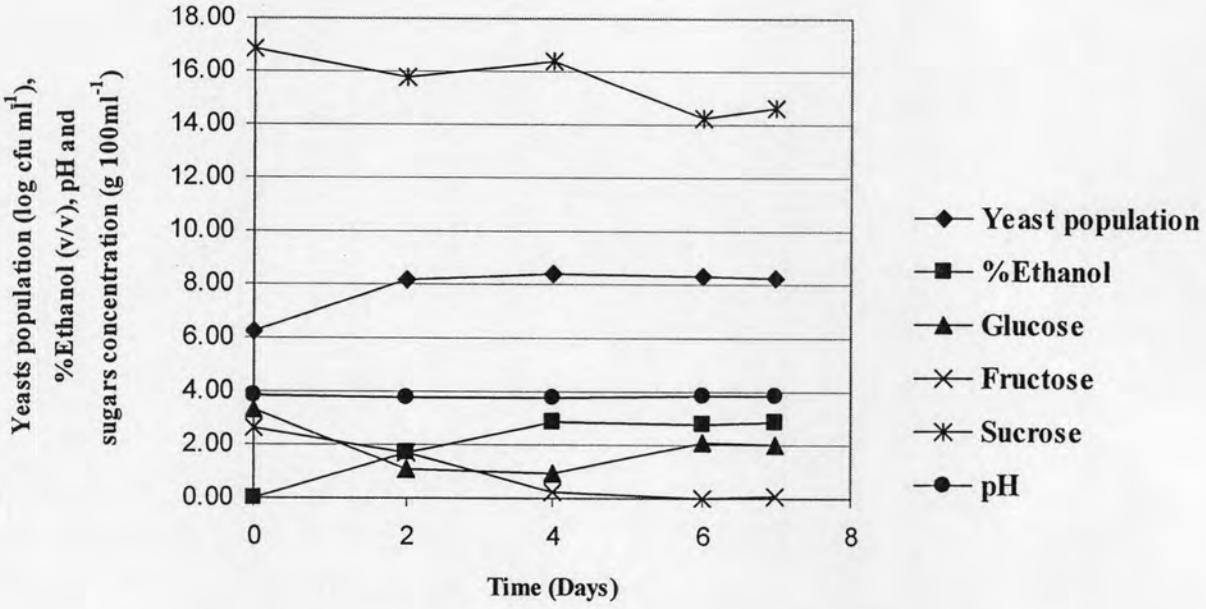

Figure 4.6 Yeast population and changes in the concentrations of ethanol, sugars and pH during fermentation of pineapple juice by *Candida* sp.1 (Cs1)

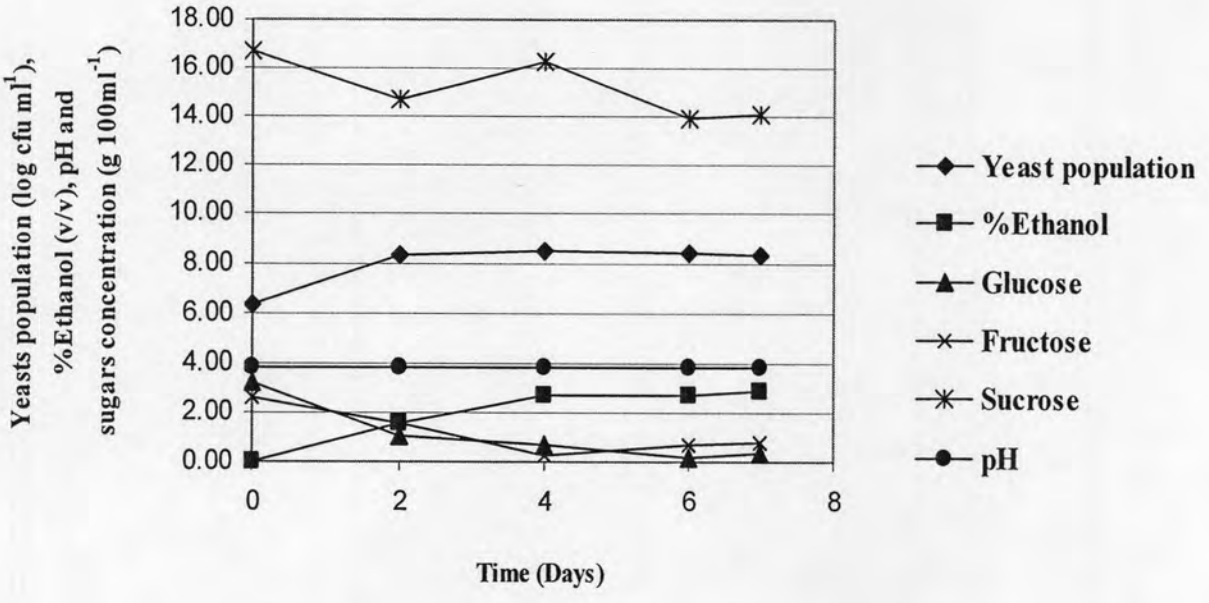

Figure 4.7 Yeast population and changes in the concentrations of, ethanol, and sugars and pH during fermentation of pineapple juice by *Candida* sp.2 (Cs2)

4.3.3 Concentration of organic acids during fermentation of pineapple juice by yeast isolates

Concentrations of organic acids throughout fermentation were monitored by HPLC. The organic acids determined were citric acid, malic acid, succinic acid, lactic acid, fumaric acid and acetic acid, which have been reported to be significant in wine fermentations and to influence the the organoleptic quality of final wine product (Schreier, 1979; Farkaš, 1988; Jackson, 2000; Ribéreau-Gayon et al., 2000; Fleet, 2001). Figure 4.8 illustrates the changes in content of organic acids of pineapple juice during fermentation.

4.3.3.1 Citric acid

Citric acid is the main organic acid in pineapple juice. Therefore, higher citric acid content was found in pineapple juice than other organic acids. For all yeast isolates, the content of citric acid did not significantly change throughout the fermentation and remained in the range of 7.0 to 7.3 g l^{-1}. It had an influence on pH and organoleptic quality of pineapple wine. In this study, all yeast isolates could not degrade citric acid in pineapple juice. This result was consistent with previous reports stating that these yeasts can not use citrate for their growth (Kurtzman and Fell, 1998; Barnett et al., 2000). Consequently, pH value was relatively constant throughout the fermentation (Figure 4.1 to 4.7).

4.3.3.2 Malic acid

On the contrary, these yeasts could reduce malic acid content in pineapple juice during fermentation. Malic acid was the second major organic acid in pineapple juice. It was found that malic acid was decomposed by all yeast isolates. Malic acid contents of all yeast isolates was reduced in the first two days of fermentation, then remained steady throughout the fermentation. The utilization of malic acid at the initial stage of fermentation could be due to enzymatic decarboxylation of 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 malic acid content of final fermented pineapple juices remained in the range of 2.2 to 4.8 g l^{-1}. Apart from decarboxylation of malic acid, the decreasing of malic acid could be due to the transformation of L(+)-malic acid to D(-)-lactic acid during alcoholic fermentation. In addition, malic acid could be directly metabolized to succinic acid. (Schreier, 1979).

4.3.3.3 Succinic acid

Succinic acid contents of every fermentation increased during day 0 to day 4. After that, the succinic acid content for the fermentation with *S. cerevisiae* decreased until the end of fermentation. The succinic acid content with *S'codes ludwigii* (Sl) fermentation increased up to 3.4 g l^{-1} throughout the fermentation. This result agreed with a previous study that *S'codes ludwigii* (Sl) produced higher succinic acid in relation to the other non-*Saccharomyces* yeasts (Ciani and Maccarelli, 1998). The increasing of succinic acid during day 0 to day 4 is

expected since succinic acid is normally a by-product of alcoholic fermentation (Schreier, 1979; Farkaš, 1988; Jackson, 2000; Ribéreau-Gayon et al., 2000; Fleet, 2001). The higher amount of succinic acid could result in a bitter, salty taste in wine. Nevertheless, the production of succinic acid is not associated with any major defects of wine quality (Fleet, 2001). The succinic acid could be formed in a few ways, i.e. the oxidation of glutamic acid, degradation of sugars or Wood-Werkman reaction, as stated in section 2.6.2.1, Chapter 2.

4.3.3.4 Lactic acid

S. cerevisiae showed a slight increase of lactic acid, whereas its content remained relatively stable during fermentation with other species. D(-)-lactic acid could be formed from the degradation of L(+)-malic acid by yeast isolates 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).

4.3.3.5 Fumaric acid

Only *S'codes ludwigii* (Sl) gave a slight increase of fumaric acid content throughout the fermentation, while the increase of fumaric acid content detected in other batches was insignificant. This was consistent with the previous report which stated that fumaric acid was 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).

4.3.3.6 Acetic acid

Fermentations with *Z. bailii* showed a slight increase in acetic acid content, whereas, acetic acid content of other batches insignificantly increased throughout the fermentation. The increasing of acetic acid could be due to the oxidation of acetaldehyde to form acetic acid by yeast isolates during alcoholic fermentation (Farkaš, 1988; Ribéreau-Gayon et al., 2006).

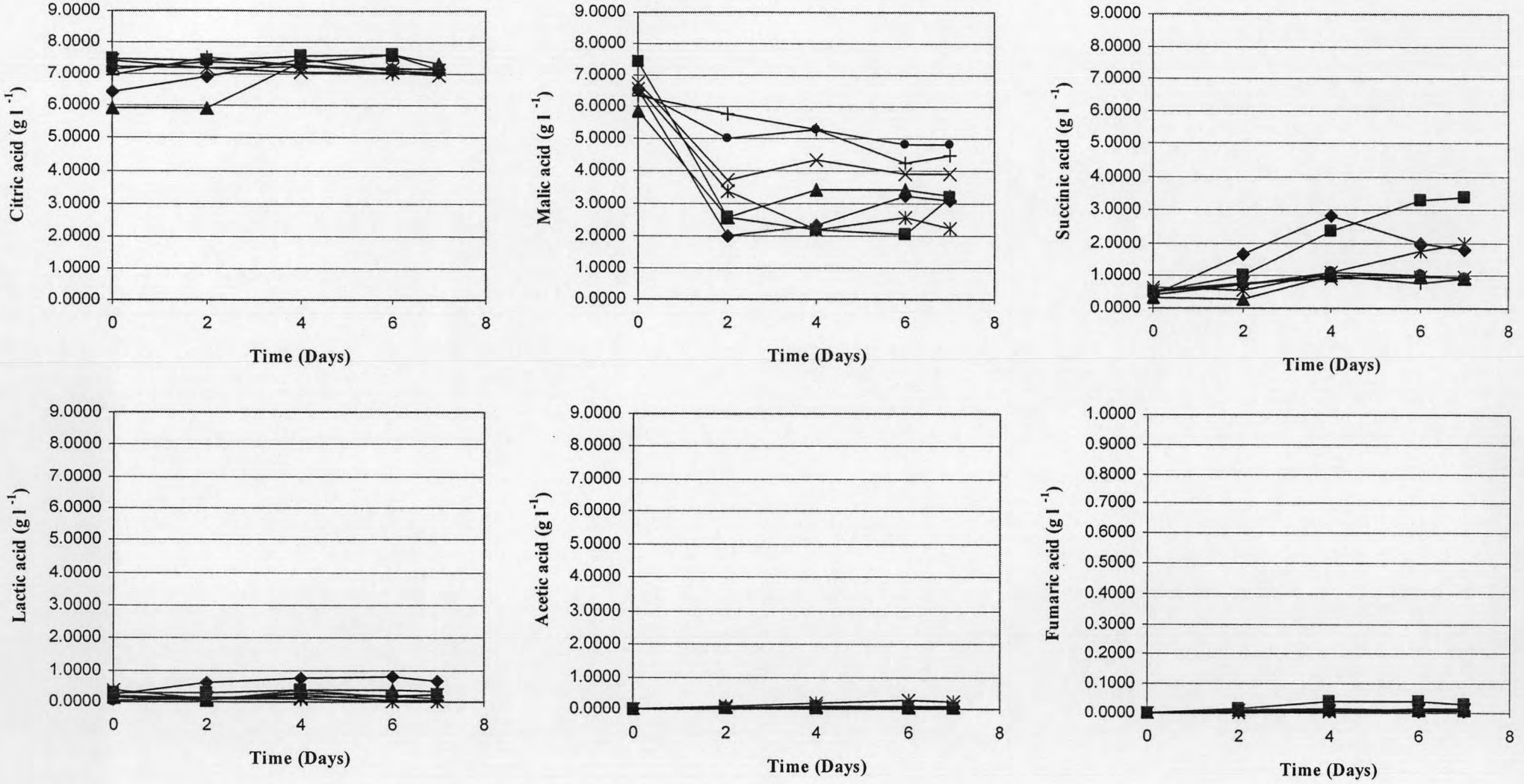

Figure 4.8 Changes in the concentrations of organic acids during fermentation of pineapple juice by different yeasts; ◆ = *S. cerevisiae*, ■ = *S'codes ludwigii*, ▲ = *H. uvarum*1, × = *H. uvarum*2, ∗ = *Z. bailii*, ● = *Candida sp.*1, + = *Candida sp.*2

4.3.4 Analysis of volatile metabolites produced by yeasts during the fermentation of pineapple juice

Apart from nonvolatile compounds, yeasts also produce a number of volatile compounds which are responsible for the aroma of wine and other alcoholic beverages during alcoholic fermentation, such as alcohol, higher alcohols, aldehydes and esters (Schreier, 1979). To study the volatile compounds produced by yeast isolates using pineapple juice as a substrate, the fermented pineapple juices by six yeasts isolates were semi-quantitatively examined using method based on chromatography techniques. Headspace SPME with GC-MS was applied to analyze their main volatile compounds as mentioned in Materials and Methods.

4.3.4.1 Pineapple juice

Ethyl hexanoate was a major ester of the based pineapple juice used in this study. In addition, the other esters were also found as the main volatile compounds of pineapple juice as shown in Table 4.3.

4.3.4.2 Fermentation with *S. cerevisiae*

S. cerevisiae is the main yeast used as a starter culture for wine fermentation. Therefore, its volatile compounds were used as the typical volatile compounds for this study. Apart from ethanol, the main volatile compounds found in this fermented pineapple juice were ethyl decanoate and ethyl dodecanoate.

Table 4.3 Main volatile compounds of fermented pineapple juice by the yeast isolates

Order	Main volatile compounds of fermented pineapple juices*							
	Control	*S. cerevisiae*	*S'codes ludwigii* (Sl)	*H. uvarum* (Hu1)	*H. uvarum* (Hu2)	*Z. bailii* (Zb)	*Candida* sp.1 (Cs1)	*Candida* sp.2 (Cs2)
1	Ethyl hexanoate (15.5)	Ethanol (22.2)	Ethanol (25.9)	Ethanol (12.8)	Ethanol (11.3)	Ethanol (21.0)	Ethanol (9.4)	Ethanol (10.6)
2	1-Butanol, 3-methyl-, acetate (2.4)	Ethyl decanoate (8.8)	Ethyl decanoate (13.3)	2-phenylethyl acetate (6.0)	2-Phenylethyl acetate (4.8)	Isoamyl alcohol (3-Methyl-1-butanol) (7.5)	Isoamyl alcohol (3-Methyl-1-butanol) (2.6)	Isoamylalcohol (7.7)
3	Ethyl 2-methylbutanoate (2.3)	Ethyl dodecanoate (8.2)	Ethyl dodecanoate (8.3)	Ethyl acetate (2.6)	Ethyl acetate (3.1)	Ethyl acetate (2.3)	Ethyl hexanoate (1.4)	Ethyl hexanoate (5.2)
4	Isobutyl hexanoate (1.9)	Isoamylalcohol (4.6)	Isoamylalcohol (5.9)	Ethyl dodecanoate (2.4)	Ethyl dodecanoate (1.9)	Isobutyl alcohol (0.9)	Isoamylalcohol (0.9)	Isoamyl acetate (0.8)
5	Ethyl acetate (0.8)	Ethyl octanoate (3.2)	Ethyl tetradecanoate (2.3)	Ethyl decanoate (1.9)	Ethyl hexanoate (1.1)	2-Phenylethanol (0.8)	2-phenylethyl acetate (0.6)	Ethyl 2-methylbutanoate (0.8)
6	Ethyl butanoate (0.7)	Ethyl hexadecanoate (1.8)	Ethyl octanoate (2.1)	Ethyl hexanoate (0.8)	Ethyl decanoate (0.7)	Ethyl hexadecanoate (0.4)	2-Phenylethanol (0.5)	Ethyl acetate (0.8)
7	2,4-Di-tert-butylphenol (0.3)	Acetaldehyde (1.5)	Ethyl acetate (1.9)	Butanoic acid, 2-methyl-, ethyl ester (0.6)	Ethyl 2-methylbutanoate (0.5)	2-Phenylethyl acetate (0.4)	1-Dodecanol (0.5)	Isobutyl alcohol (0.6)
8	Ethanol (0.3)	Ethyl hexanoate (1.3)	Acetaldehyde (1.1)	Ethyl octanoate (0.5)	Isoamyl acetate (0.3)	Ethyl dodecanoate (0.4)	Isopropyl tetradecanoate (0.5)	2-Phenylethanol (0.6)
9	Ethyl octanoate (0.2)	Isoamyl acetate (1.3)	Decanoic acid (1.0)	Dodecanoic acid (0.5)	1-Propanol, 2-methyl- (0.3)	Ethyl octanoate (0.4)	2,4-Di-tert-butylphenol (0.5)	Ethyl decanoate (0.5)
10	Benzyl benzoate (0.2)	Ethyl acetate (1.3)	Dodecanoic acid (1.0)	1H-Indole-3-ethanol, acetate (ester) (0.5)	Decanoic acid (0.2)	1-Decene (0.4)	Isoamyl acetate (0.4)	5-Phenyl-2-pentanone (0.5)

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

Additionally, Isoamylalcohol was main higher alcohol found in this experiment. The main other volatile compounds of *S. cerevisiae* was showed in Table 4.3.

4.3.4.3 Fermentation with *S'codes ludwigii*

The main volatile compounds produced by *S'codes ludwigii* (Sl) were similar to those of *S. cerevisiae*. Ethyl decanoate and ethyl dodecanoate were found as the main volatile compounds of *S'codes ludwigii* (Sl). Isoamylalcohol was main higher alcohol found in this sample. Additionally, ethyl tetradecanoate was also observed in this fermented pineapple juice.

4.3.4.4 Fermentation with *H. uvarum*

Major volatile compounds of pineapple juice fermented by both isolates of *H. uvarum* (Hu1 and Hu2) was 2-phenylethyl acetate and ethyl acetate. Ethyl dodecanoate and ethyl decanoate were also produced by *H. uvarum*. In addition, the similarity of the volatile compounds of *H. uvarum*1 (Hu1) and *H. uvarum*2 (Hu2) was observed in this study.

4.3.4.5 Fermentation with *Z. bailii*

Isoamyl alcohol (3-Methyl-1-butanol) and ethyl acetate were found as main volatile compounds of *Z. bailii* (Zb), the other higher alcohols, isobutyl alcohol and phenethyl alcohol, was also found in this samples. The type of esters

observed in this fermented juice was similar to those of *S. cerevisiae*, *S'codes ludwigii* (Sl) and *H. uvarum* (Hu1 and Hu2) fermentations, but their quantity was lower.

4.3.4.6 Fermentation with *Candida* sp.

The main volatile compounds of fermented pineapple juice of *Candida* sp.1 (Cs1) were isoamyl alcohol (3-Methyl-1-butanol) and ethyl hexanoate. Some other esters found in this fermented pineapple juice was similar to those of other yeasts. The similarity of the volatile compounds of *Candida* sp.1 (Cs1) and *Candida* sp.2 (Cs2) was observed in this study.

4.3.5 Fermentation properties of yeast isolates

Based on the fermentation profiles of yeast isolates, these results demonstrated their fermentation properties in pineapple wine which could be discussed as following sections.

- Fermentation properties of *S. cerevisiae* in pineapple wine

The fermentation pattern of *S. cerevisiae* was compared with that of the other yeast isolates. The decreasing of sucrose of *S. cerevisiae* fermentation could be that its ability of invertase enzyme production as stated by Dworschack and Wickerham (1958). *S. cerevisiae* rapidly degraded sucrose to fructose and glucose. Consequently, the increasing of glucose and fructose content in substrate was observed, then they were used for ethanol production. Concerning the ethanol

production, the consistent increasing of ethanol content of this batch was observed throughout the fermentation, in particular during day 0 to day 4. Consequently, it could produce the ethanol content up to 11.8% (v/v). In general, wine fermentation, *S. cerevisiae* could generate ethanol content in the range of 6-23% (v/v) (Fleet, 2001).

- Fermentation properties of *S'codes ludwigii* in pineapple wine

S'codes ludwigii (Sl) could produce a higher ethanol concentration than *S. cerevisiae*. In addition, the difference in patterns of ethanol production between *S'codes ludwigii* (Sl) and *S. cerevisiae* were clearly observed. *S'codes ludwigii* (Sl) slowly produced ethanol in the early of fermentation, then shifted to consistently increase after day 2 until the end of fermentation. The slow increase of *S'codes ludwigii* (Sl) could be useful for the adaptation of *S'codes ludwigii* (Sl) in higher ethanol concentration condition during pineapple juice fermentation. This result was consistent with other researcher which reported that this yeast was highly tolerant to ethanol content (Romano and Suzzi, 1990; Ciani and Maccarelli, 1998; Romano et al. 1999).

- Fermentation properties of *H. uvarum* in pineapple wine

Both isolates of *H. uvarum* (Hu1 and Hu2) could not use sucrose for their growth and fermentation, which clearly differed from the sugar assimilation pattern of *S. cerevisiae* and *S'codes ludwigii* (Sl). This finding agreed with the reports that *H. uvarum* can not use sucrose as a carbon source (Kurtzman and Fell, 1998; Barnett et al., 2000). However, the slight decreasing of sucrose content

occurred from the spontaneous inversion of sucrose to be glucose and fructose in low pH conditions (Pennington and Baker, 1990). The low ethanol production was observed from the pineapple juice fermented by *H. uvarum* (Hu1 and Hu2). The results agreed with other research which reported that *H. uvarum* could generate low ethanol content during the micro-fermentation of grape must and orange juice (Mingorance-Cazorla et al., 2003).

- Fermentation properties of *Z. bailii* and *Candida* sp. in pineapple wine

Z. bailii (Zb) showed the ability of degradation of sucrose. However, it produced the ethanol content was only 7% (v/v). Whereas, both isolates of *Candida* sp. (Cs1 and Cs2) could not use sucrose as a carbon source and were low ethanol producers, which was similar to the fermentation pattern of *H. uvarum* (Hu1 and Hu2).

The fermentation profiles of pineapple wine fermentation by yeast isolates revealed that yeast isolates play important roles to the degradation of sucrose and assimilation of glucose and fructose for their ethanol production. In addition, during alcoholic fermentation, several secondary metabolites, organic acids and volatile compounds were also produced by yeasts which these chemical substances influence on the organoleptic properties of wine product. Therefore, the production of these compounds by yeast isolates during the fermentation was investigated in this study.

4.3.5.1 Organic acids production of yeast isolates in pineapple wine fermentations

The organic acids in wine originated from raw material used in wine fermentation and wine fermentation by yeast (Ribéreau-Gayon et al., 2000). Firstly, the organic acids from raw material were different depending on the kind of fruits used in fermentation. The major organic acids in pineapple fruits are citric acid and malic acid (Singleton and Gortner, 1965; Dull, 1971; Camara et al., 1994; Bartolome, Rupbrez and Carmen, 1995; Bartholomew, Paull and Rohrbach, 2003). The main organic acids produced during wine fermentation were lactic acid, succinic acid, fumaric acid and acetic acid (Farkaš, 1988; Ribéreau-Gayon et al., 2000; Fleet, 2001).

The investigation of changes of organic acids during pineapple wine fermentation yeast isolates, the significant difference of malic and succinic acids contents of fermented pineapple juice inoculated with different yeast isolates was observed during fermentation. *S'codes ludwigii* (Sl) could generate succinic acid throughout the fermentation. Consequently, the increasing of succinic acid of *S'codes ludwigii* (Sl) was found throughout the fermentation.

The results of this study suggested that the different yeast isolates exploited for pineapple wine fermentation have an influence on organic acids content of final alcoholic beverages. However, these fermentative yeasts can produce not only the substances which give taste characteristics, but also the volatile compounds which give aroma characteristics to alcoholic beverages. Hence, in the

further studies, the volatile compounds produced by these yeast isolates were primarily screened to know their major volatile compounds of fermented pineapple juices.

4.3.5.2 Volatile compounds production of yeast isolates in pineapple wine fermentations

The main volatile compounds of fermented pineapple juice were a group of esters. Additionally raw material, generally, wine esters originated from enzymatic esterification during the fermentation process. In this study, ethyl decanoate and ethyl dodecanoate were the main esters found in fermented pineapple juices by *S. cerevisiae* and *S'codes ludwigii* which both of volatile compounds was reported as main esters in Kiwi wine (Demyttenaere et al., 2003). However, ethyl hexanoate and ethyl octanoate were reported to be the main ethyl esters of fatty acid produced by yeast during wine fermentation (Ribéreau-Gayon et al., 2000) which differ from the results of this study. It could be that the difference of chemical composition between pineapple and grape juice used as a substrate influences on the volatile compounds of final products (Soles, Ough and Kunkee, 1982). These ethyl esters of fatty acid give pleasant odors, such as anise seed or applelike aroma of ethyl hexanoate, sour apple aroma of ethyl octanoate and floral odor of ethyl decanoate (Saerens et al., 2008), which contribute to the aromatic finesse of wine. Many researchers reported that the organoleptic characteristics of wine are strongly influenced by these esters developed during the yeast fermentation (Schreier, 1979; Zoecklein et al., 1995; Romano, 2002).

2-Phenylethyl acetate was a main esters of fermented pineapple juice by *H. uvarum* (Hu1 and Hu2). Several researches suggested that 2-phenylethyl acetate production was fermentation characteristic commonly observed during wine fermentation by *Hanseniaspora* yeasts, such as *H. uvarum* (Moreira et al., 2008), *H. guilliermondii* (Moreira et al., 2005; Moreira et al., 2008) and *H. osmophila* (Viana et al., 2008). The flavor characteristic of 2-phenylethyl acetate was rosy and flowery odors. Hence, many researchers used this yeast in co-cultures with *S. cerevisiae* to enhance the complexity of wine flavors during fermentation.

For *Z. bailii* (Zb), although some ethyl esters, ethyl dodecanoate and ethyl octadecanoate, which similar to those of *S. cerevisiae* and *S'codes ludwigii* were found in this sample, a low quantity of these volatile compounds were observed. Consequently, it could produce the fermented pineapple juice with low fruitiness aspect.

For both isolates of *Candida* sp. (Cs1 and Cs2), the variety of esters found in this samples was obvious lower than those of other yeast isolates. Their main esters were ethyl hexanoate which was the main volatile compounds of based pineapple juice. This result revealed that both isolates of *Candida* sp. could also produce the fermented pineapple juice with low volatile compounds complexity.

Based on the fermentation properties of each yeasts isolates as described above, *S'codes ludwigii* (Sl) and *H. uvarum*1 (Hu1) were selected to use as starter culture for pineapple wine fermentation in Chapter 5. *S'codes ludwigii* (Sl) could produce the high amount of ethanol content. In addition, it showed other fermentation

characteristics which were similar to *S. cerevisiae* (commercial strain). Thus, it could be developed to be the main specific starter culture for pineapple wine fermentation. For *H. uvarum*1 (Hu1), although its ability in ethanol production and tolerance was lower than other yeasts, *H. uvarum*1 (Hu1) was potential yeast isolates to produce 2-phenylethyl acetate which give rosy and flowery odors in pineapple wine. In addition, It could produce lower acetic acid than *H. uvarum*2 (Hu2). Hence, *H. uvarum*1 (Hu1) could be developed to be the starter culture for initial stage of pineapple wine fermentation to enhance the complexity of volatile compound in pineapple wine. For further study, *S'codes ludwigii* (Sl) and *H. uvarum*1 (Hu1) were used as starter cultures in the form of multi-starter cultures for pineapple wine fermentation.