CHAPTER III

ISOLATION AND IDENTIFICATION OF YEASTS ASSOCIATED WITH PINEAPPLE FRUITS AND NATURAL PINEAPPLE JUICE FERMENTATION

3.1 Introduction

The indigenous yeasts were reported to be wild yeasts producing wine of unique flavors and exceptional quality when used for traditional wine fermentation. (Fleet et al., 2002). To commence the development of pineapple wine making technology, the fundamental knowledge of the indigenous yeast in pineapple wine fermentation requires investigation. Therefore, the indigenous yeasts associated with pineapple fruits and spontaneous pineapple juice fermentation were studied. These yeasts could be selected and developed as species specific starter cultures for pineapple wine fermentation. Further investigation is required to determine their function in fermentation and their roles in pineapple wine flavors.

In this study, the yeasts in spontaneous pineapple juice fermentation were investigated. The experiment was divided into two parts. In the first part, yeasts associated with pineapple fruits during cultivation stages and damaged fruits were determined by performing the isolation of yeast by rinsing and enrichment methods.

In the second part, yeasts in spontaneous fermentation of freshly crushed pineapple juice were investigated. In this part, the yeasts in the natural fermentation of

pineapple samples from Thailand and Australia were studied to determine the influence of climate and region on the yeasts associated with these systems.

The yeast communities in spontaneous fermentations were determined by cultural plating compared to PCR-DGGE analysis. The yeasts isolates obtained throughout the study were identified by three molecular methods, RFLP of the ITS region of yeast rDNA and sequencing analysis of the D1/D2 domain of the 26S rDNA and ITS region of yeast rDNA, and also by the commercial kit, ID 32C system. The competence of the methods in yeasts identification was evaluated prior to use in Section 3.3.1.

3.2 Materials and methods

3.2.1 Pineapple sources

The pineapple samples "Smooth cayanne" (*Ananas comosus* (L.) Merr.) were aseptically collected from the field located in Prachuapkirikhun province, Thailand where they were grown without chemical applications. Each sample represented a particular maturity stage and consisted of a composite of batches (approximately 2-3 fruits for each field) taken randomly from different fields within the field location. Pineapple fruits were collected at different stages of cultivation, namely, stages of 3, 2, 1 month before commercial harvest, harvest and damaged fruits. Duplicate samples were collected. Pineapple fruit samples were directly transported to the laboratory and investigated for the yeasts associated with the pineapple surfaces within 24 hr, using the methodologies as described in the

following sections. Five pineapple fruits of each cultivation stage were used for the study of yeasts associated with pineapple fruit in Section 3.2.1.1

3.2.1.1 Isolation of yeasts associated with pineapple fruit by rinse-plating method

The pineapple fruits from each cultivation stage were aseptically peeled and totally mixed before performing yeast isolations. Fifty grams of pineapple peels were rinsed in 450 ml of 0.1% peptone water with 0.01% Tween 80 by orbital shaking at 150 rpm for 30 minutes. The yeasts in the rinse were isolated by spread inoculation of 0.1 ml of rinse dilutions onto plates of MEA agar with 0.1% Oxytetracycline (1:10) (Oxoid, Basingstoke, England), and incubation at 25°C for 2-4 days. Yeast colonies were counted and representative colonies were isolated and purified by restreaking onto MEA, and then identified by morphological examination, sequencing of the 26S rDNA D1/D2 and ITS region of ribosomal DNA (Kurtzman and Robnett, 1998) and RFLP of the ITS region (Granchi, Bosco and Vicenzini, 1999). The population number of yeast species associated with the pineapples at different cultivation stages was experimental designed by CRD (Completely Randomized Design) and statistically analyzed using SPSS software for window Version 10.0.

3.2.1.2 Isolation of yeasts on pineapple fruit by enrichment method

Ten grams of pineapple peels were added to100 ml of four types of enrichment medium. These were pineapple juice, pineapple juice with 6%

ethanol, grape juice and grape juice with 6% ethanol. The enrichment cultures were incubated at 25°C for 1 week. The predominant yeasts in the cultures were routinely isolated by streaking on to MEA plates everyday. Yeasts colonies were purified and identified as described in Section 3.2.1.1.

3.2.2 Isolation and identification of yeasts from spontaneous fermentations

For study of the yeasts in spontaneous fermentation of pineapple juice, pineapple samples were collected from the farm as mentioned above and from markets located in Bangkok and Sydney, Australia. Whole pineapple fruits at harvesting stage (three fruits per each experiment) were freshly crushed. Crushed juices were filtered and the filtrates for (500 ml) collected in 1,000 ml sterile Erlenmeyer flasks. These steps were handled under aseptic condition. The juices were incubated at 25°C for 6 days. Populations of yeasts, and concentrations of ethanol, sugars and organic acids, and pH (Activon Model 210, Australia) were routinely investigated every 2 days during fermentation. The fermented pineapple juice was serially diluted in 0.1% peptone water. The yeasts in each dilution were isolated and enumerated by spread inoculation of 0.1 ml onto plates of MEA agar (Oxoid, England) and incubation at 25°C for 2-4 days. Yeast colonies were counted. Representative colonies of the different yeasts were purified by restreaking on MEA and then maintained and subcultured until identification. The analysis was done in duplicate Yeast colonies were identified as described in Section 3.2.1.1.

3.2.3 DNA extraction from yeasts

DNA was extracted from pellets of yeast cells according to procedures described by Cocolin, Bisson and Mills (2000). Cells were resuspended in 200 μl of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10mM Tris pH 8.0, 1mM EDTA) and homogenized with 0.3 g of glass beads (0.5 mm in diameter) in a bead beater at 6000 rpm for 1 min in the presence of 200 μl of phenol/ chloroform/isoamyl alcohol (50:48:2). TE buffer (200 μl) (10 mM Tris, 1 mM EDTA, pH 7.6) was mixed with the disrupted cells and the suspension was centrifuged at 16,000 x g for 10 min, 4°C. The supernatant was collected and 2.5 volumes of absolute ethanol were added to precipitate the DNA, which was sedimented by centrifuging at 16,000 x g and 4°C for 10 min, washed with 70% ethanol and then resuspended in 50 μl of TE buffer.

3.2.4 DNA amplification and primers

The D1/D2 domain of the 26S rDNA was amplified by PCR with the forward primer NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and reverse primer LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (Cocolin, Bisson and Mills, 2000). The conditions of this reaction were the same as those just described, except that the concentration of $MgCl_2$ was increased to 2.25 mM. The PCR reaction was run with an initial step at 95°C for 5 min, and 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 2 min, extension at 72°C for 2 min, with a final extension at 72°C for 7 min. Reagents for the PCR were *i-Taq*™ DNA Polymerase (Intron Biotechnology, Korea), and the primers were obtained from Bio Basic Inc. (Canada). The reaction was conducted in a DNA Engine (MJ research

PTC–200, Korea). In Australia, Reagents for the PCR were ABI Amplitaq Gold Polymerase (Applied Biosystems, AUS.), and the primers obtained from Sigma Genosys (Sigma-Aldrich, USA.). The reaction was conducted in GeneAmp® (Applied Biosystems, AUS.).

The ITS region of yeast rDNA was amplified by PCR with the forward primer ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and reverse primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GE-3') described by White et al. (1990). The conditions of this reaction were the same as those just described. The PCR reactions were run with the initial step of 95°C for 5 min, 35 cycles of denaturation at 94°C for 2 min, annealing at 56°C for 2 min, extension at 72°C for 2 min, and a final extension at 72°C for 10 min (Esteve-Zarzoso et al., 1999). Reagents for the PCR were *i-Taq*™ DNA Polymerase (Intron Biotechnology, Korea), and the primers were obtained from Bio Basic Inc. (Canada). The reaction was conducted in a DNA Engine (MJ research PTC–200, Korea). In Australia, Reagents for the PCR were ABI Amplitaq Gold Polymerase (Applied Biosystems, AUS.), and the primers obtained from Sigma Genosys (Sigma-Aldrich, USA.). The reaction was conducted in GeneAmp® (Applied Biosystems, AUS.).

The D1/D2 domain of the 26s rDNA was amplified by a two step nested PCR for DGGE, The first PCR was performed using the forward primer NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and reverse primer NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (O'Donnell, 1993). Amplification was done in a standard reaction mixture containing 10 mM Tris HCl (pH 8.3), 20 mM KCl, 1.5 mM $MgCl_2$, each deoxynucleotide triphosphate at a concentration of 0.2 mM, 1.25 IU

of *Taq* polymerase, each primer at a concentration of 0.2 μM and the DNA template at a final concentration of 10 ng PCR was run for 36 cycles with annealing at 52°C, extension at 72°C for 2 min, and denaturation at 94°C for 1 min (Kurtzman and Robnett, 1998). Reagents for the PCR were ABI Amplitaq Gold Polymerase (Applied Biosystems, AUS.), and the primers obtained from Sigma Genosys (Sigma-Aldrich, USA.). The reaction was conducted in GeneAmp® (Applied Biosystems, AUS.).

The amplicon (approximately 600 bp) from the first PCR was diluted and further amplified with a second PCR using the GC-clamp primer NL1 (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCCATATCAATAAGC-3') and forward primer LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (Cocolin, Bisson and Mills, 2000). The conditions of this reaction were the same as those just described, except that the concentration of $MgCl_2$ was increased to 2.25 mM. The PCR reaction was run with an initial step at 95°C for 5 min, and 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 2 min, extension at 72°C for 2 min, with a final extension at 72°C for 7 min. Reagents for the PCR were ABI Amplitaq Gold Polymerase (Applied Biosystems, AUS.), and the primers obtained from Sigma Genosys (Sigma-Aldrich, USA.). The reaction was conducted in GeneAmp® (Applied Biosystems, AUS.).

3.2.5 RFLP analysis

The PCR products of the ITS region of yeast rDNA (5 μl) were digested for 90 min at 37°C with 5 U of restriction enzyme in 15 μl reaction volumes, using the manufacturer's instructions and conditions. The restriction enzymes used

were *Cfo* I (Promega, USA.), *Hae* III (Toyobo, Japan) and *Hinf* I (Toyabo, Japan). RFLP products were analysed by horizontal agarose gel 2.5% (w/v) electrophoresis, using a 100 bp EZ Load™ Molecular Rulers (Bio-Rad, USA.) as the size standard. Electrophoresis was performed with an Electrophoresis Power Supply EPS 301 (Amersham Pharmacia Biotech, Sweden) at 110 V for 1 h. After electrophoresis, the gels were stained with ethidium bromide and photographed under transluminated UV light. The RFLP profiles were scanned and the size of restriction fragments was measured using a computer program (Carnoy 2.0, Lab of Plant Systematics, Belgium). The restriction fragment profiles were compared with the data of, Guillamón et al. (1998), Esteve-Zarzoso et al. (1999), Granchi, Bosco and Vicenzini (1999) and Sabate et al. (2002) and Prakitchaiwattana (2005).

3.2.6 DGGE analysis

The GC-clamp PCR products were separated according to their sequences using DGGE with a Dcode™ Universal Mutation System (Bio-Rad, USA.). PCR samples were directly applied onto 8% (w/v) polyacrylamide gels in a running buffer (1% TAE) containing 40 mM Tris-acetate, 2 mM Na_2EDTA-H_2O, pH 8.5. The gels were prepared with a denaturing gradient from 30 - 60% of urea and formamide (Cocolin et al., 2002) and a polyacrylamide and bis-acrylamide ratio of 19: 1 (polyacrylamide mixing powder, Bio-Rad). Electrophoresis was performed at a constant voltage of 120 V for 6 hours with a constant temperature of 60°C. After electrophoresis, the gels were stained in 1% TE buffer pH 8, containing ethidium bromide and photographed under UV translumination.

3.2.7 Sequencing analysis of DNA bands and yeast identification

The PCR products from amplification of 26S ribosomal DNA and ITS were sent to a commercial sequencing facility (Macrogen, Souel, Korea). DNA base sequences were analysed by comparison with the GenBank databases of the National Center for Biotechnology Information (NCBI). Searches in GenBank with Blast program were performed to determine the closest known relative of partial 26S rDNA and ITS sequences (Altschul et al., 1997).

In Australia, The PCR products from amplification of 26S ribosomal DNA and ITS were cleaned with QIAquick PCR Purification Kit (Qiagen, USA.), then labeled with dye terminators using the ABI Big Dyeterminator Version 3.1 Kits (Applied Biosystems, AUS.). The labeled PCR products were purified by ethanol precipitation according to the manufacturer's instructions and sent to a commercial sequencing facility (The Ramaciotti Centre, UNSW, AUS.). DNA base sequences were analysed by comparison with the GenBank databases of the National Center for Biotechnology Information (NCBI). Searches in GenBank with Blast program were performed to determine the closest known relative of partial 26S rDNA and ITS sequences (Altschul et al., 1997).

DNA bands from DGGE gel were carefully selected and excised from the gels using sterile razor blades. The pieces of gel were soaked in 20 μl of TE buffer overnight at room temperature to allow diffusion of DNA. Eluted DNA (10 μl) was amplified by PCR using NL1 and LS2 primers. The PCR products were used for sequencing analysis and yeast identification as just described. Yeast species were

identified on the basis of their sequences (greater than 95 % homology with the databases) (Kurtzman and Robnett, 1998; Scorzetti et al., 2002).

3.2.8 Determination of fermentation profile

3.2.8.1 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 and enumerated by spread inoculation of 0.1 ml onto plates of MEA agar (Oxoid, England) and incubation at 25°C for 2-4 days. Yeast colonies were counted. The analysis was done in duplicate.

3.2.8.2 Preparation of fermented pineapple juice for HPLC analysis

The fermented pineapple juice samples were centrifuged by 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 through a 0.45 micron syringe filter (Micro Analytix Pty Ltd., Australia) to separate small particle in samples. The filtrates were poured into a vial, which was capped and put in an autosampler tray for injection.

3.2.8.3 Analysis of ethanol

Ethanol concentrations 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 analysed by a Millenium software program. The method was calibrated using ethanol at a concentration of 1.0 % (v/v) as standard. (Appendix B)

3.2.8.4 Analysis of sugars

Sugars 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 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)

3.2.8.5 Analysis of organic acids

Organic acids 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}. 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, 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)

3.3 Results and discussion

3.3.1 Yeast identification

The traditional cultural approach to yeast identification is very labour-intensive, lengthy and costly. Hence, molecular methods based on DNA analysis are now popular for identification of yeasts because they quickly identify yeasts to genus and species level. The workload is minimal and, usually, reliable data can be obtained within 1-2 days (Kurtzman and Fell, 1998; Barnett et al., 2000; Fernandez-Espinar et al., 2006). Several approaches are being used. Of the various molecular approaches available, RFLP analysis of the ITS region of ribosomal DNA, sequencing of the

D1/D2 domain of the 26S ribosomal DNA, sequencing of the ITS region of ribosomal DNA are finding most application (Kurtzman and Robnett, 1998). RFLP analysis was reported to be most convenient method for yeast identification because of its speed (10 hr), simplicity and cheaper cost (Esteve-Zarzoso et al., 1999; Granchi, Bosco and Vicenzini, 1999; Chen et al., 2000; Fernandez-Espinar et al., 2000). For DNA sequencing analysis, previous literatures reported that the reproducibility of this method has been found to be very high (Lee et al., 1992; Parker et al., 1995; Demeter et al., 1998). However, At least two independent molecular methods were suggested to use for yeast identification (Prakitchaiwattana, 2005).

In this study, yeasts associated with pineapple samples of Thailand and Australia were isolated by performing cultural plating. Total yeast isolates from the fruits were primarily screened and grouped based on their colony and cellular morphological properties. Then, 20% of total yeast isolates of each group was selected as representative and further identified by the following methods. The yeast isolates from pineapples grown in Thailand were then further identified by conventional and three molecular methods (RFLP and sequencing analysis of two DNA regions). The yeast isolates from pineapple grown in Australia were further identified by similar methodologies but ID 32C system (Biomerieux, France) was used instead of RFLP analysis. The results are shown in the following sections.

3.3.1.1 Identification of yeast isolates from Thailand by three molecular methods

Based on morphological identification, 36 yeast isolates were obtained and identified by three molecular analyses. The results are shown in Table 3.1. Colony isolates that were mucoid to butyrous and had pink to red color (Profile 1, Table 3.1) were identified by sequencing of D1/D2 region of 26S rDNA. The sequence gave 100% identity with the database sequence of *R. mucilaginosa*. This sequence also gave 97% identity with *R. mucilaginosa* when identified by sequence of the ITS region. For the RFLP analysis, DNA pattern of this yeast was not identical to any previous RFLP profiles reported. The other yeast isolates that gave the same identification by the two methods (D1/D2 and ITS sequencing analysis) were *I. orientalis*, *H. uvarum*, *H. opuntiae*, *C. tropicalis*, *P. fermentans*, *Z. bailii*, *C. stellata* and *E. hasegawianum* (Profiles 3-4, 5, 6, 10, 11, 12, 13 and 14, respectively, Table 3.1). When identified by RFLP, these isolates did not correspond to both sequencing analyses. Colony isolates that were fluid, mucoid to butyrous and had pink to red color gave a 26S rDNA sequence that did not match with any database sequences. However, their ITS sequence gave 99% identity with *Rh. toluloides* (anamorph *R. glutinis*) (Profile 2, Table 3.1). For RFLP analysis, its RFLP profile matched with the database profile of *R. glutinis*. Colony isolates that were butyrous and had white to cream color gave a sequene of 26S rDNA, with 100% identity with the database sequence of *C. fermentati* (synonyms *C. guilliermondii*; anamorph *P. guilliermondii*) (Profile 7, Table 3.1), which was identical to the ITS sequencing analysis (100% identity with *P. guilliermondii*). The RFLP profile of this isolate also matched with the database profile of *P. guilliermondii*. The yeast isolates that had similar

identification by three methods were *A. pullulans* and *Saccharomycodes ludwigii*. (Profiles 8 and 9, respectively, Table 3.1) Two yeast isolates, with colonies that were butyrous and white to cream color, gave ITS sequences with 98% identity to *Cryptococcus* sp. but their 26S rDNA sequence did not match with 26S rDNA database sequences. Their RFLP profile also did not match any profile reported.

Table 3.1 Identification of yeasts isolated from pineapple fruits and spontaneous fermentation of freshly crushed pineapple juice in Thailand

Profile	Sequencing of D1/D2 region of 26S rDNA	Accession Number	Percent identity	Sequencing of ITS region	Accession Number	Percent identity	Restriction fragments[1]			Closest relative[2]	No. of isolates
							CfoI	*HaeIII*	*HinfI*		
1	*R. mucilaginosa*	DQ132885	100	*R. mucilaginosa*	AF444614	97	320+240	425+215	340+225	No matching	1
2	Unidentified			*Rhodos. toluloides*	AB073266	99	300+230+100	450+230+150	180+150	*R. glutinis*	1
3	*I. orientalis*	DQ466533	100	*I. orientalis*	AY939808	98	200 +180+50	400	250+150	*C. intermedia var intermedia*	1
4	*I. orientalis*	AY601160	98	*I. orientalis*	AY939796	85	230	400+200+ 150+100	270+200+150	*C. montana*	2
5	*H. uvarum*	AY305681	100	*H. uvarum*	AM160628	100	320	850	400+190	No matching	3
6	*H. opuntiae*	DQ872866	100	*H. opuntiae*	DQ872855	100	720	580	450+320	*H. uvarum*	3
7	*C. fermentati*	AY187283	100	*P. guilliermondii*	DQ088676	100	320+290	400	450+380	*P. guilliermondii*	7
8	*A. pullulans*	AB304735	98	*A. pullulans*	EF197817	98	200+100	450+150	300+200	*A. pullulans*	3
9	*S'codes ludwigii*	U73601	98	*S'codes ludwigii*	AB056133	87	350	750	450+280+150	*S'codes ludwigii*	1
10	*C. tropicalis*	AY601158	100	*C. tropicalis*	AY939810	96	270	450	300	*C. albican*	2
11	*P. fermentans*	EF554827	99	*P. fermentans*	AF411062	99	200+100	320+100	250+230+70	*C. sorbata*	1
12	*Z. bailii*	U72161	99	*Z. bailii*	X84640	99	320+300+280	750	400+250+180	*H. guilliermondii*	2
13	*C. stellata*	AJ966340	98	*C. stellata*	AY160766	97	230	500+250	250+250	No matching	1
14	*E. hasegawianum*	AY560616	99	*E. hasegawianum*	AF444522	90	680	650+200	450+350	No matching	1
15	*C. nivariensis*	AY627307	99	*Candida sp.*	AY787833	88	350	600	450+300	*Z. mellis*	5
16	Unidentified			*Cryptococcus* sp.	AF444396	98	250	270+100	250+230	No matching	2

[1] Values refer to the number of base pairs per fragment.

[2] Identification compared with RFLP pattern of Guillamón et al. (1998), Esteve-Zarzoso et al. (1999) and Prakitchaiwattana (2005)

From these results, it was found that 31% of 36 yeast isolates could be identified by all three methods, giving similar identification results. Forty-seven percent of all isolates gave similar identifications by sequencing analysis of two DNA regions. Three percent of yeast isolates identified by ITS sequencing and RFLP analysis gave similar identification results. Fourteen percent of yeast isolates gave totally different identification results by the three methods. Based on the percent of identification results as mentioned above, identification of yeast isolates was best achieved by D1/D2 sequencing analysis in combination with ITS sequencing analysis. This could be potential methodology to be used for yeast isolate identification. RFLP analysis was not efficient enough for identifing these unknown yeasts since these results were frequently different from those obtained by sequencing analysis. The main limitation of this method could be the lack of diversity of information in the ITS-RFLP databases. These limitations have been stated by Esteve-Zarzoso et al. (1999), Heras-Vazquez et al. (2003) and Clemente-Jimenez et al. (2004).

3.3.1.2 Identification of yeast isolates from Australia by two molecular methods and the ID 32 C system

Because of limitations of the RFLP analysis as mentioned above, the ID 32 C system was used instead of RFLP analysis to identify the yeast isolates in Australia. Based on morphological identification, 21 yeast isolates were selected and identified by D1/D2 and ITS analysis and the ID 32 C system. The results are shown in Table 3.2. Colony isolates that were butyrous and had white to cream color, when sequenced by the D1/D2 region of 26S rDNA, gave 99% identity with the database sequence of *P. guilliermondii* (Profiles 1 and 2, Table 3.2). Its ITS

sequence also gave 97% identity with *P. guilliermondii*. By the ID 32 C system, its numerical biocode gave 88.3% identity with *C. famata* (Profile 1, Table 3.2). The yeast isolates identified by two methods (D1/D2 and ITS sequencing analysis) giving identical results were *P. fermentans*, *I. orientalis*, *Candida* sp., *Y. lipolytica*, *Tr. globispora*, *R. mucilaginosa* and *A. pullulans* (Profiles 5-6, 13, 14-15, 16, 17, 18 and 19, respectively, Table 3.2). The isolates with butyrous, white to cream color colonies, when identified by sequencing of 26S rDNA, gave 99% identity with the database sequence of *P. guilliermondii* (Profile 2, Table 3.2). This isolate also gave 97% identity with *P. guilliermondii* when identified by the ITS sequencing. For the ID 32 C system, its numerical biocode gave 89.4% identity with *C. guilliermondii* (anamorph *P. guilliermondii*), which corresponded to the identification result of both sequencing analysis. Yeast isolates which gave similar identities by 2 molecular methods and ID 32 C system were *P. membranifaciens* (anamorph *C. valida*), *H. uvarum* (anamorph *K. apiculata*) and *I. orientalis* (anamorph *C. krusei*) (Profiles 3-4, 8-11 and 12, respectively, Table 3.2). Two yeast isolates with butyrous and white to cream color colonies, gave identification results were totally different by the three methods. By ITS sequencing analysis, they gave 92-98% identity to *Saccharomycetales* sp. whereas 26S rDNA sequencing gave 97-98% identity to *C. edaphicus*. Their numerical biocode gave 94.8% identity to *G. capitatum* and 99.2% identity to *C. grabata* (Profiles 20 and 21, respectively, Table 3.2).

Table 3.2 Identification of yeasts isolated from spontaneous fermentation of freshly crushed pineapple juice in Australia

Profile	Sequencing of D1/D2 region of 26S rDNA	Accession Number	Percent identity	Sequencing of ITS region	Accession Number	Percent identity	API ID 32 C	Percent identity
1	*P. guilliermondii*	EU833236	99	*P. guilliermondii*	EU568993	97	*C. famata*	88.3
2	*P. guilliermondii*	AB260128	99	*P. guilliermondii*	EU568969	97	*C. guilliermondii*	89.4
3	*P. membranifaciens*	AY529509	99	*P. membranifaciens*	AB193175	98	*C. valida*	99.7
4	*P. membranifaciens*	AY529507	98	*P. membranifaciens*	AF411062	96	*C. valida*	99.7
5	*P. fermentans*	EF554827	99	*P. fermentans*	EF061132	92	*C. rugosa*	99.8
6	*P. fermentans*	EF554827	99	*P. fermentans*	FJ231463	90	*C. krusei*	97.8
7	*P. fermentans*	EF554827	98	*Pichia* sp.	AB286072	85	*Cr. humicola*	99.2
8	*H. uvarum*	EU359819	100	*H. uvarum*	DQ872856	94	*K. apis/apiculata*	99.9
9	*H. uvarum*	EF139133	98	*H. opuntiae*	DQ872855	95	*K. apis/apiculata*	99.9
10	*H. uvarum*	EF139133	100	*H. opuntiae*	DQ872855	94	*K. apis/apiculata*	99.9
11	*H. uvarum*	EF139133	98	*H. opuntiae*	DQ872855	98	*K. apis/apiculata*	99.9
12	*I. orientalis*	FJ515259	98	*I. orientalis*	EU315767	95	*C. krusei*	87.9
13	*I. orientalis*	AY305675	98	*I. orientalis*	AB467299	95	*Cr. humicola*	99.2
14	*Candida* sp.	EF460674	100	*Candida* sp.	EF612207	91	*Cr. humicola*	86.6
15	*Candida* sp.	EF460674	99	*Candida* sp.	EF612207	84	*C. rugosa*	99.9
16	*Y. lipolytica*	EU327113	98	*Y. lipolytica*	FJ515197	98	*Cr. humicola*	86.6
17	*Tr. globispora*	EF551317	99	*Tr. globispora*	AF444432	90	*C. guilliermondii*	91.0
18	*R. mucilaginosa*	FJ515267	100	*R. mucilaginosa*	AB193175	98	*Cr. humicola*	98.9
19	*A. pullulans*	FJ515253	98	*A. pullulans*	FJ515198	98	*C. famata*	-
20	*C. edaphicus*	AB247371	98	*Saccharomycetales* sp.	AF411062	92	*G. capitatum*	94.8
21	*C. edaphicus*	AB247371	97	*Saccharomycetales* sp.	EF060939	98	*C. glabrata*	99.2

- = No percent identity

From these results, 38% out of the 21 yeast isolates gave similar identities by all three methods. Forty-eight percent of all isolates identified by sequencing analysis of two DNA regions displayed similar identification results. Fourteen percent of yeast isolates identified by three methods gave totally different identification results. These observations were also consistent with those reported in Section 3.3.1.1. The use of 26S rDNA in combination with ITS sequencing analysis could be a proper methodology for yeast isolates identification. Yeast identification by ID 32 C gave data that were frequently different from the two sequencing methods. This demonstrated that ID 32 C might not be sufficiently efficient for the identification of unknown yeasts, since diversity of information in the ID 32 C Analytical Profile Index databases was limited. In addition, the identity result (in term of % identity) of numerical biocode by ID 32 C system varied depending upon the geographic origin of the yeast isolates (Ramani et al., 1998). However, this method could be used for specific identification of some yeast species, in particular *H. uvarum* (anamorph *K. apiculata*).

According to the results of yeast identification by several methods in this study, approximately 50% of the yeast isolates were identified by using 26S rDNA in combination with ITS sequencing analysis. Therefore, two sequencing analyses were selected as the main methods for the identification of yeast isolates. However, the identification of yeast species was confirmed when results of two identification methods out of three methods were similar. Where there was no matching of those two out of three methods, another identification method was performed using the ID 32 C system or RFLP analysis. However, there were some

yeast isolates that did not give similar identification results by any of the three methods. These yeast isolates would be assigned as unidentified species.

3.3.2 Yeasts associated with pineapple fruits

Both qualitative and quantitative investigations of yeasts associated with pineapple fruits were carried out in this study. Common yeasts associated with pineapple fruits at different cultivation stages were isolated by rinse-plating methods. Enrichment was also performed to isolate fermentative yeasts and/or yeasts which were present at very low population on the fruits.

3.3.2.1 Yeasts isolated by rinse-plating method

Yeasts were rinsed from pineapples with sterile peptone water, and then examined by plating onto MEA. The colonies were counted and identified by the methodologies as mentioned in section 3.3.1. Table 3.3 shows the populations and species of yeasts observed on pineapple skins at different cultivation stages. Yeast populations on the skins of young fruits at 3 months before harvest were low at approximately 3 log cfu g^{-1}, then gradually increased in parallel with maturity development of the fruits. In addition, an increase in yeast species diversity was also observed as the fruit matured. *A. pullulans* was a predominant yeast found on intact fruit skin at the young cultivation stages (65-100%). At harvesting stage, the decrease of *A. pullulans* (33%) was observed, whereas *P. guilliermondii* was more prevalent on the pineapple skin (67%). Additionally, for yeast species found on damaged fruit skins, 100 % of yeast isolates were *Z. bailii* and its population was approximately

6.23 log cfu g^{-1}. Another yeast occasionally found in this study was *Cryptococcus* sp., isolated from the pineapple fruits at 3 months before harvesting.

Table 3.3 Yeasts isolated from pineapple skins at different cultivation stages and damaged fruits by the rinse-plating method

Cultivation stage	Yeast population (log cfu g^{-1})	Yeast isolates	
		Yeast species	Frequency of occurrence (%)
3 months before harvest	3.84^{d} ±0.08	*A. pullulans*	97
		Cryptococcus sp.	3
2 months before harvest	4.01^{c} ±0.09	*A. pullulans*	65
		P. guilliermondii	35
1 month before harvest	4.26^{b} ±0.04	*A. pullulans*	100
Harvesting fruits	4.33^{b} ±0.01	*P. guilliermondii*	67
		A. pullulans	33
Damaged fruits	6.23^{a}±0.04	*Z. bailii*	100

a, b, c and d were statistically significant difference ($P \leq 0.05$)

Aureobasidium pullulans was a predominant species on pineapples throughout cultivation, giving way to the fermentative yeast, *P. guilliermondii*, at full maturity. The population of individual yeasts increased in parallel with maturity development of pineapple fruits. This could be the increasing in concentration of sugars and nutrients in flesh during maturation (Bartholomew, Paull and Rohrbach, 2003). These sugars and nutrients in the inner flesh could osmotically diffuse through tissues to the outer surface of fruit skin when the pineapple surface

becomes wet because of rain or overnight formation of dew. The indigenous yeasts on the fruit skin could assimilate these sugars and nutrients in the fruit exudates for their growth.

In this study, *A. pullulans* was prevalent on intact fruits skin at every cultivation stage. The prevalence of *A. pullulans* might be explained by the fact that this yeast is widely associated with plants and fruits as a phyllospheric organism and has probably evolved to tolerate the temperature, water availability, irradiation, and low nutrients of this environment (Blakeman and Fokkema, 1982; Elad, Kohl and Fokkema, 1994; Fleet, 2003b). Also, it is known to produce extracellular substances that are antagonictic to other organisms, including yeasts (Bhatt and Vaughan, 1962; Leibinger et al., 1997; Castoria et al., 2001). Consequently, its predominance on pineapple surface could eliminate other species and account for the absence of the other yeast as found in this study. As pineapple matures to full ripeness, yeasts became more abundant, increasing to populations of 3 - 4 log cfu g^{-1}. *A. pullulans* present on immature fruits could be isolated from mature fruits, but at this stage, *P. guilliermondii* was more prevalence. *P. guilliermondii* was reported as yeast generally present in natural habitat, such as soil, pond and sea water, and reported as significant yeast found in traditional starter cultures for sake making. In fruit ecosystems, this yeast was found on corrosol fruit (look like custard apple) and damaged grape berries (Barnett et al., 2000; Stringini et al., 2008). As mentioned above, when pineapple fruit develops to maturity, the nutrients and some chemical substances progressively accumulate in pineapple fruit exudates. The antifungal agents could occur from the combination of organic acids such as lactic, acetic or other organic acids and antimicrobial agents in form of enzyme or some peptides (Yang and Clausen, 2004),

and these could impact on *A. pullulans* growth. On the other hand, these substances might be selective for *P. guilliermondii,* since this yeast prefers to grow under acidic condition and can assimilate acid as a carbon source for its growth.

On damaged fruits surfaces, the osmotolerant yeast, *Z. bailii*, became the predominant yeast found in this study. It could be explained that when the fruit skin was damaged, the juice in the inner flesh exuded through the fruit surface. The juice on damaged fruit then evaporated when exposed to sunlight or wind, allowing sugar concentration of the juice to increase. Under this high sugar concentration condition, it is selective for osmotolerant yeasts to grow. *Z. bailii* is an osmotolerant yeast generally found as spoilage yeast in several foods such as mayonnaise, salad dressing, pickles, marzipan, fruit juice and concentrate, soft drinks and wines (Thomas and Davenport, 1985; Kunkee and Bisson, 1993; Fleet, 1999; Martorell et al., 2007). Therefore, it was not surprising to find this yeast in such high sugar concentration area of damaged surface.

The finding of only one species, *Z. bailii,* on pineapple damaged fruit might be explained by the fact that this yeast could also tolerate stress from undesirable conditions (acid and antimicrobial agents in fruit exudates). On the other hand, this yeast might prefer to utilize some vitamins or amino acids available on damaged fruit surfaces. However, many factors involved in the presence of the yeasts on the pineapple surfaces as found in this study require investigation.

3.3.2.2 Yeasts isolated by enrichment method

The enrichment method was used to isolate fermentative yeasts and/or yeast present on pineapple surfaces in low population. The pineapple fruits skins at different cultivation stages were enriched in pineapple juice, pineapple juice with 6% ethanol, grape juice and grape juice with 6% ethanol, then incubated at room temperature for 7 days. Yeasts isolated from pineapple skin of fruits at 3 months before harvest in enrichment cultures are displayed in Table 3.4. In pineapple and grape juice cultures, *H. uvarum* was frequently isolated during day 1 to day 4. Then, *P. guilliermondii* was more frequently isolated from the cultures in day 5 to day 7. In grape juice with 6% ethanol added, *P. guilliermondii* was observed almost everyday, while *H. uvarum* was not found throughout the experiment. In pineapple juice with 6% ethanol added, no yeast species was consistently found. However, *P. guilliermondii* and *H. uvarum* were still occasionally isolated. The other yeasts occasionally isolated from the enriched cultures of pineapple skin were *Candida* sp., *I. orientalis* and *A. pullulans*.

Yeasts isolated from pineapple skin at 2 months before harvest in enrichment cultures were shown in Table 3.5. Overall, yeast species isolated from all cultures were more diverse than species isolated from 3 months fruits. In pineapple juice, *C. tropicalis* was the yeast frequently found throughout 7 days and unidentified yeasts were found almost everyday, while *H. uvarum* and *P. guilliermondii* were observed just in the first three days of enrichment. In grape juice and grape juice added 6% ethanol, *P. guilliermondii* was mainly isolated, whereas in pineapple juice with added 6% ethanol, *H. uvarum* was more prevalently isolated. *Candida* sp.,

Table 3.4 Yeasts isolated from skin of pineapple fruits at 3 months before harvesting stage by enrichment methods

Stage of enrichment culture (Days)	Yeast species isolated			
	Pineapple juice	Pineapple juice with 6% ethanol	Grape juice	Grape juice with 6% ethanol
1	*H. uvarum* Unidentified yeast	*P. guilliermondii*	*H. uvarum*	*P. guilliermondii*
2	*H. uvarum*	*P. guilliermondii*	*H. uvarum* *P. guilliermondii*	*P. guilliermondii* *I. orientalis*
3	*H. uvarum*	*I. orientalis*	*H. uvarum*	*P. guilliermondii*
4	*H. uvarum* *P. gulliermondii* *Candida* sp.	*Candida* sp.	*H. uvarum* *P. gulliermondii*	*A. pullulans* *Candida* sp.
5	*P. guilliermondii* *Z. bailii*	*H. uvarum*	*P. gulliermondii* *A. pullulans*	*P. gulliermondii*
6	*P. gulliermondii* Unidentified yeast	*H. uvarum*	*H. uvarum* *P. guilliermondii*	*I. orientalis* *P. guilliermondii*
7	*P. guilliermondii* Unidentified yeast	*I. orientalis*	*P. guilliermondii*	*P. guilliermondii*

Table 3.5 Yeasts isolated from skin of pineapple fruits at 2 months before harvesting stage by enrichment methods

Stage of enrichment culture (Days)	Yeast species isolated			
	Pineapple juice	Pineapple juice with 6% ethanol	Grape juice	Grape juice with 6% ethanol
1	*Rhodos. toruloides* Unidentified yeast	*P. guilliermondii* Unidentified yeast	*P. guilliermondii*	*E. hasegawianum*
2	*H. uvarum* *P. guilliermondii* *I. orientalis* Unidentified yeast	*P. guilliermondii* *H. uvarum*	*P. guilliermondii* *I. orientalis* *A. pullulans*	*E. hasegawianum* *P. guilliermondii*
3	*H. uvarum* *P. guilliermondii* *C. tropicalis*	*H.uvarum* *I. orientalis*	*I. orientalis* *H. uvarum*	*P. guilliermondii*
4	*C. tropicalis* *Candida* sp. *A. pullulans* Unidentified yeast	*P. guilliermondii* *H. uvarum* *R. mucillaginosa*	*P. guilliermondii* *I. orientalis* *H. uvarum*	*P. guilliermondii* *I. orientalis* *H. uvarum* *Candida* sp.
5	*C. tropicalis* *Candida* sp. *A. pullulans* Unidentified yeast	*P. guilliermondii* *H. opuntiae* *I. orientalis*	*P. guilliermondii*	*P. guilliermondii*
6	*R. mucillaginosa* *I. orientalis*	*H. uvarum*	*H. opuntiae*	*H. opuntiae*
7	*C. tropicalis*	*I. orientalis* *E. hasegawianum* Unidentified yeast	*I. orientalis*	*P. guilliermondii* *I. orientalis* *S'codes ludwigii*

I. orientalis and *A. pullulans* were still occasionally isolated. The other yeasts, such as *R. mucillaginosa*, *H. opuntiae* and *E. hasegawianum* were additionally found.

Table 3.6 displays yeasts isolated from pineapple skin of the fruits in enrichment cultures at 1 month before harvest. In pineapple juice culture, *P. guilliermondii* and *I. orientalis* were mainly isolated almost every day. These yeasts were also mainly observed in pineapple juice with 6% ethanol added. In grape juice culture, *H. uvarum* was prevalence almost everyday, whereas *I. orientalis* and *P. guilliermondii* were occasionally observed just in the last three days. *E. hasegawianum*, which was occasionally observed at the 2 month stage fruits, were still found in this stage. The yeasts additionally found were *C. tropicalis*, *Candida* sp. and *C. stellata* which were isolated from only grape juice culture at day 7.

Yeasts isolated from enriched cultures of pineapple skin of fruits at harvesting stage are shown in Table 3.7. A lesser diversity of yeast species was isolated from the skin of pineapple at this stage. In pineapple juice culture, the most prevalent yeast isolated throughout 7 days was *H. uvarum*. This yeast was also the most prevalent species found in grape juice culture. In pineapple juice with 6% added ethanol and grape juice with 6% ethanol added, only *I. orientalis* was prevalent throughout 7 days. *P. guilliermondii* was found in all cultures in only the first 2 days of enrichment. *C. tropicalis* was also frequently isolated from grape juice enrichment culture. *I. orientalis* was the only yeast found every day in both grape and pineapple juice with 6% ethanol added. On pineapple skin at this cultivation stage, *P. guilliermondii* was occasionally isolated when enriched in pineapple juice and grape juice with added 6% ethanol. The other yeasts occasionally isolated from pineapple

skin at harvesting stage in enriched cultures were *R. mucillaginosa*, *A. pullulans*, *H. opuntiae* and *E. hasegawianum*.

For damaged pineapple fruits, yeasts isolated from the skin are shown in Table 3.8. In pineapple juice with 6% ethanol and grape juice, *Z. bailii* was prevalently isolated, whereas in grape juice with 6% ethanol added, *S'codes ludwigii* was observed to be prevalent. In pineapple juice, *P. fermentans* was the main yeast isolated throughout 7 days. Unidentified yeasts were frequently found in all enrichment cultures during days 1-5. The other yeasts isolated from damaged fruits skins in all enrichment cultures were *Candida* sp., *I. orientalis* and *C. tropicalis*. There was no *H. uvarum* or *P. guilliermondii* observed in enrichment cultures.

Table 3.6 Yeasts isolated from skin of pineapple fruits at 1 month before harvesting stage by enrichment methods

Stage of enrichment culture (Days)	Yeast species isolated			
	Pineapple juice	Pineapple juice with 6% ethanol	Grape juice	Grape juice with 6% ethanol
1	*P. guilliermondii*	*P. guilliermondii*	*H. uvarum*	*P. guilliermondii*
2	*P. guilliermondii*	*P. guilliermondii* *I. orientalis*	*H. uvarum* *Cryptococcus* sp. Unidentified yeast	*P. guilliermondii* *I. orientalis*
3	*I. orientalis*	*P. guilliermondii* *I. orientalis*	*H. uvarum* *Candida* sp.	*I. orientalis*
4	*I. orientalis* *Candida* sp.	*P. guilliermondii* *I. orientalis*	*H. uvarum* *I. orientalis*	*H. uvarum* *C. tropicalis*
5	*P. guilliermondii* *I. orientalis*	*I. orientalis*	*H. uvarum* *I. orientalis* *P. guilliermondii*	*P. guilliermondii* *I. orientalis*
6	*I. orientalis*	*I. orientalis*	*I. orientalis* *P. guilliermondii*	*P. guilliermondii*
7	*P. guilliermondii* *E. hasegawianum*	*E. hasegawianum*	*P. guilliermondii* *C. stellata* *E. hasegawianum*	*P. guilliermondii* *E. hasegawianum*

Table 3.7 Yeasts isolated from skin of pineapple fruits at harvesting stage by enrichment methods

Stage of enrichment culture (Days)	Yeast species isolated			
	Pineapple juice	Pineapple juice with 6% ethanol	Grape juice	Grape juice with 6% ethanol
1	*H. uvarum*	*I. orientalis*	*H. uvarum* Unidentified yeast Unidentified yeast	*P. guilliermondii* *Cryptococcus* sp.
2	*H. uvarum* *P. guilliermondii* *R. mucillaginosa*	*I. orientalis* Unidentified yeast	*H. uvarum* *I. orientalis* *C. tropicalis*	*P. guilliermondii* *I. orientalis*
3	*A. pullulans* Unidentified yeast	*I. orientalis*	*H. uvarum* *C. tropicalis* *Candida* sp.	*I. orientalis*
4	*H. uvarum*	*I. orientalis*	*H. uvarum* *C. tropicalis*	*I. orientalis*
5	*Candida* sp. Unidentified yeast	*I. orientalis*	*H. uvarum* *C. tropicalis* Unidentified yeast	*I. orientalis*
6	*H. uvarum* *P. guilliermondii*	*I. orientalis*	*H. uvarum* *C. tropicalis* *H. opuntiae*	*I. orientalis*
7	*H. uvarum* *I. orientalis* *C. tropicalis*	*I. orientalis*	*H. uvarum* *C. tropicalis*	*E. hasegawianum*

Table 3.8 Yeasts isolated from skin of damaged fruits by enrichment methods

Time (Days)	Yeast species isolated			
	Pineapple juice	Pineapple juice with 6% ethanol	Grape juice	Grape juice with 6% ethanol
1	*Candida* sp.	*Z. bailii* Unidentified yeast	*Z. bailii* Unidentified yeast	*S'codes ludwigii* Unidentified yeast
2	*I. orientalis* *P. fermentans*	*Z. bailii* *P. fermentans* Unidentified yeast Unidentified yeast	*Z. bailii* Unidentified yeast Unidentified yeast	*S'codes ludwigii* Unidentified yeast
3	*P. fermentans* Unidentified yeast	*Z. bailii* Unidentified yeast	*Z. bailii* Unidentified yeast	*S'codes ludwigii*
4	*Z. bailii* *P. fermentans* Unidentified yeast Unidentified yeast	*Z. bailii* Unidentified yeast	*Z. bailii* *S'codes ludwigii*	*S'codes ludwigii* Unidentified yeast Unidentified yeast
5	*P. fermentans* Unidentified yeast	*Z. bailii*	*Z. bailii* *P. fermentans* *Candida* sp. Unidentified yeast	*I. orientalis* *Z. bailii* *Candida* sp.
6	*P. fermentans*	*Z. bailii*	*Z. bailii*	*C. tropicalis*
7	*P. fermentans*	*Z. bailii*	*Z. bailii*	*Z. bailii*

Yeast species isolated from pineapple fruit skins at different cultivation stages in enriched cultures are also summarized in Table 3.9. The number in the parenthesis in the last column was the number of enrichment cultures in which the yeasts were isolated from pineapple skin at each stage of cultivation. The data indicate that *P. guilliermondii* was consistently found on intact pineapple skins at every cultivation stage of enriched cultures. *H. uvarum* was also found as a main yeast on the intact pineapple skin throughout cultivation stages, but it was not isolated as frequently as *P. guilliermondii*. Surprisingly, *I. orientalis* was another yeast consistently found on intact fruits throughout cultivation stages and it was isolated as frequently as *P. guilliermondii*. In addition, this yeast was also isolated from damaged fruits. *C. tropicalis* and *E. hasegawianum* was found on the fruits after 3 months throughout maturity development. The other yeasts, such as *A. pullulans*, *Candida* sp. and *H. opuntiae*, were also found on skin on a few occasions. For damaged fruits, only *Z. bailii* was consistently isolated from enriched cultures. *P. fermentans* and *Candida* sp. were also found on damaged skin, but not isolated as frequently as *Z. bailii*.

Table 3.9 Summary of yeast species isolated by enrichment methods from skin of pineapples at different maturity stages, including damaged fruits

Pineapple cultivation stage	Pineapple juice	Pineapple juice with 6% ethanol	Grape juice	Grape juice with 6% ethanol	Frequency of isolation*
3 months before harvest	*P. guilliermondii* *H. uvarum* *Candida* sp. *Z. bailii*	*P. guilliermondii* *H. uvarum* *I. orientalis* *Candida* sp.	*A. pullulans* *P. guilliermondii* *H. uvarum*	*A. pullulans* *P. guilliermondii* *I. orientalis* *Candida* sp.	*P. guilliermondii* (4) *H. uvarum* (3) *Candida* sp. (3) *A. pullulans* (2) *I. orientalis* (2) *Z. bailii* (1)
2 months before harvest	*P. guilliermondii* *H. uvarum* *I. orientalis* *Candida* sp. *C. tropicalis*	*P. guilliermondii* *H. uvarum* *H. opuntiae* *I. orientalis* *R. mucilaginasa* *E. hasegawianum*	*P. guilliermondii* *H. uvarum* *H. opuntiae* *I. orientalis*	*P. guilliermondii* *H. uvarum* *H. opuntiae* *I. orientalis* *Candida* sp. *E. hasegawianum* *S'codes ludwigii*	*P. guilliermondii* (4) *H. uvarum* (4) *I. orientalis* (4) *H. opuntiae* (3) *Candida* sp. (2) *E. hasegawianum* (2) *C. tropicalis* (1) *S'codes ludwigii* (1) *R. mucilaginasa* (1)
1 month before harvest	*P. guilliermondii* *I. orientalis* *Candida* sp. *E. hasegawianum*	*P. guilliermondii* *I. orientalis* *E. hasegawianum*	*P. guilliermondii* *H. uvarum* *I. orientalis* *C. tropicalis* *E. hasegawianum*	*P. guilliermondii* *H. uvarum* *I. orientalis* *C. tropicalis* *C. stellata* *E. hasegawianum*	*P. guilliermondii* (4) *I. o rientalis* (4) *E. hasegawianum* (4) *H. uvarum* (2) *C. tropicalis* (2) *Candida* sp. (1) *C. stellata* (1)
Harvesting stage	*A. pullulans* *P. guilliermondii* *H. uvarum* *I. orientalis* *C. tropicalis* *Candida* sp. *R. mucillaginosa*	*I. orientalis*	*P. guilliermondii* *H. uvarum* *H. opuntiae* *I. orientalis* *C. tropicalis* *Candida* sp.	*P. guilliermondii* *I. orientalis* *Cryptocuccus* sp. *E. hasegawianum*	*I. orientalis* (4) *P. guilliermondii* (3) *H. uvarum* (2) *Candida* sp. (2) *C. tropicalis* (2) *H. opuntiae* (1) *A. pullulans* (1) *E. hasegawianum* (1) *R. mucilaginasa* (1) *Cryptocuccus* sp. (1)
Damaged fruits	*Z. bailii* *I. orientalis* *Candida* sp. *P. fermentans*	*Z. bailii* *P. fermentans*	*Z. bailii* *Candida* sp. *P. fermentans* *S'codes ludwigii*	*Z. bailii* *I. orientalis* *C. tropicalis* *Candida* sp. *S'codes ludwigii*	*Z. bailii* (4) *P. fermentans* (3) *Candida* sp. (3) *S'codes ludwigii* (2) *I. orientalis* (2) *C. tropicalis* (1)

* the number in the parenthesis is the number of enrichment culture in which the yeasts were found

Two methodologies, rinsing and enrichment, were used to determine the diversity of yeast species associated with pineapple fruits. The rinse-plating method was performed to isolate normal flora, and population levels for individual species were counted. Grape juice and pineapple juice were used as enrichment cultures to detect yeast species present in very low populations and not detectable by rinse-plating methods. This approach was used to increases the prospects of detecting fermentative species (Mrak and McClung, 1940; Martini, Ciani and Scorzetti, 1996; Mortimer and Polsinelli, 1999; Khan et al., 2000). Moreover, 6% ethanol was added into the juices to make the conditions more selective for wine yeasts, in particular, *S. cerevisiae*.

Through rinse-plating method, *A. pullulans* was a common organism associated on the intact pineapple fruits examined. The prevalence of *A. pullulans* might be explained by the fact as mentioned in Section 3.3.2.1 that it is widely associated with plants and fruits as a phyllospheric organism, and has possibly evolved to tolerate the undesirable environmental conditions (Fleet, 2003b). Also, it showed notable antagonistic activities against the growth of other yeasts and fungi (Castoria et al., 2001). This was inhibitory to many other yeasts and could account for the absence of the other species on the pineapple surface. In contrast, through enrichment method, *A. pullulans* was isolated from pineapple skin enriched in the juice cultures on a few occasions since it was dominated by *P. guilliermondii* and *H. uvarum.* It is known that *A. pullulans* is the yeast-like fungi which generally prefers to grow under an aerobic condition and at pH range 6-7, depending on its strain (Leathers, 2005). Therefore, the acid condition of grape and pineapple juices used as enrichment cultures, including the oxygen limit in the cultures could possibly inhibit

the growth of *A. pullulans*. In contrast, under this condition, fermentative yeasts *P. guilliermondii* and *H. uvarum* could grow well and became prevalent as found in this study. *P. guilliermondii* also found to be a common yeast on the pineapple surfaces throughout cultivation stages, but it was present as a minor population due to the influence of *A. pullulans*, as mentioned (section 3.3.2.1). Interestingly, it became more prevalent when maturity of the fruit developed. This reflected that this species could survive on pineapple surface condition and grow by utilizing nutrients that the fruit give as exudates. Therefore, it could be still more active and show better growth than the other yeasts when exposed to the enrichment cultures. Because of its fermentable property, this yeast became more prevalent in the fruit juices with high sugar and low pH as enrichment cultures (Kurtzman and Fell, 1998; Barnett et al., 2000). Consequently, its predominance could account for the absence of the other yeast species in enrichment cultural systems. Although *P. guilliermondii* has been reported as wildly present in natural habitats and in water resources (Barnett et al., 2000), it is not frequently reported on fruit surfaces, even on wine grape surfaces determined by enrichment isolation that are reported in the review of Fleet et al. (2002) and in the report of Stringini et al. (2008). Therefore, the consistent prevalence of *P. guilliermondii* on pineapples as found by both enrichment cultures and rinse–plating cultures at every cultivation stage indicates that the intact pineapple skin could provide some conditions selective for its survival and growth.

H. uvarum was another yeast found prevalently in enrichment cultures of the pineapple skins throughout the cultivation stages, although it was not detectable by the rinse-plating method. Generally, *K. apiculata* / *H. uvarum* was most frequently reported as the dominant species on grapes, and could account for up to 70% of the

total yeast flora on grape surfaces (Martini, Ciani and Scorzetti, 1996; Sabate et al., 2002). It was also frequently found as a prevalent species on the other types of fruits, such as cider apples (Morrissey et al., 2004; Coton et al., 2006) and papaya (Stringini et al., 2008). However, in research that used enrichment methodologies to isolate the yeasts associated with fruits such as grape berries (Martini, Ciani and Scorzetti, 1996; Mortimer and Polsinelli, 1999; Khan et al., 2000; Mrak and McClung, 1940; Martini, Ciani and Scorzetti, 1996 and van der Westhuizen et al., 2000a; 2000b), banana and papaya (Stringini et al., 2008), some differences in finding *H. uvarum* were evident. It appeared that *H. uvarum* was not reported as a main yeast species in these systems as determined by enrichment culture. This yeast is mainly found on these fruits by rinse-plating culture. Therefore, the finding of this yeast on pineapple skins in this study was opposite to these studies. The reason for this observation could not be given in this section. However, as mentioned above, *H. uvarum* was mainly found in enrichment cultures of pineapple skin throughout cultivation stages, although it was not observed by rinse-plating methods at any stage. This observation demonstrated that *H. uvarum* could naturally exist and survive on the pineapple fruits, but it could not reproduce itself on this environments as well as the *P. guilliermondii*. Nevertheless, the prevalence of this yeast on pineapple skins in all enriched cultures suggests that *H. uvarum* might be a significant indigenous yeast of pineapples. However, further research is needed to understand and explain these observations.

The isolation of *I. orientalis* from pineapples was also an interesting observation. This yeast was not observed on pineapple fruits at any cultivation stage by rinse-plating isolation, but it was consistently isolated from all enrichment cultures containing 6% alcohol. It is known that *I. orientalis* is an ethanol tolerant species and

can utilize ethanol as a carbon source for it growth (Barnett et al., 2000). In addition, its predominance in the alcoholic conditions could account for the absences of the other species, in particular *S. cerevisiae* which was expected to be found under these conditions. The presence of this yeast in the alcoholic enrichment cultures of pineapple skin suggests that it could naturally exist and survive on pineapple fruits similar to *H. uvarum*.

Apart from the three species just mentioned, only four other yeast species, *C. tropicalis*, *Candida* sp., *E. hasegawianum* and *S'codes ludwigii*, were found on a few occasions. Surprisingly, *S. cerevisiae* and *S. bayanus* were not isolated from the pineapples examined in this study, even by enrichment cultures with 6% alcohol added. If it is not an influence a regional influence, it seems that pineapple is not a natural habitat for these species.

Damaged fruits harbour a much higher population of yeasts, but less diversity of yeasts species was found. This observation was inconsistent with the finding of yeasts on the other damaged fruit surfaces. Generally, damaged fruit surfaces are likely be a significant source of the typical fermentative yeasts such as *Kloeckera / Hanseniaspora*, *Metschnikowia*, *Candida*, *Saccharomyces* and *Kluyveromyces spp.* (Davenport, 1976; Guerzoni and Marchetti, 1987; Yanagida et al., 1992). In contrast, on the surfaces of damaged pineapple skins, *Z. bailii* was the only prevalent species consistently found. In addition, as stated by Fleet et al. (2002), fruit surfaces generally harbour large diversity of yeasts species since they provide preferable conditions for yeasts to grow. But, as found in this study, the yeast species observed was not diverse on both intact and damaged pineapple surfaces, which did

not correspond to those statements. Interestingly, pineapple surfaces might have the specific conditions, and in particular, substances, in the fruit exudates might be selective for some yeasts to survive and grow; in particular, *P. guilliermondii*, *H. uvarum*, *I. orientalis* and *Z. bailii,* as found in this study. However, other influences of variables such as regions, climates, seasons and cultivation environment should be considered and studied to maximize the reliability of the data obtained and to prove this assumption (Fleet, 2003a). A systematic, controlled investigation of these variables was beyond the time frame and scope of this study, but tentative observations and conclusions can be advanced.

In conclusion, from this study of yeasts associated with pineapple fruits by rinse-plating and enrichment methods, some new information has been added. It was known that there was less diversity of yeast species associated with pineapple fruits throughout their maturation. *P. guilliermondii* and *H. uvarum* consistently occurred on intact pineapple fruits, and *Z. bailii* was a significant yeast associated with damaged fruits.

3.3.3 Yeasts in spontaneous fermentation of freshly crushed pineapple juices

In this study, the populations and identification of yeast species associated with freshly crushed juice allowed to undergo natural fermentation are reported. The yeasts associated with the fermentations were determined by cultural isolation and by PCR-DGGE analysis of extracted DNA. The populations of individual species were counted, and alcohol, sugars and organic acids including pH that changed during the fermentation were also determined. In addition, in order to

study the influence of regions and climates on the yeasts associated with the pineapple fruits, the experiments were done in Thailand and Australia. The experimental results of both countries were then compared. Two samples of freshly crushed juice squeezed from pineapple fruits grown in Thailand were named TH1 and TH2, and two samples from Australia were named as AUS1 and AUS2.

3.3.3.1 Spontaneous fermentation of freshly crushed pineapple juices from pineapple in Thailand

In Thailand, pineapple sample named TH1 was taken from the same field as reported in Section 3.2.1, at the harvest stage. After crushing, the juices were allowed to ferment for 6 days. In freshly crushed juice, the main species found were *H. uvarum, P. guilliermondii* and *I. occidentalis* (Figure 3.1, Table 3.10). Only *H. uvarum* was mainly observed throughout the fermentations, while *P. guilliermondii* was occasionally absent. Population of *H. uvarum* at the initial day of fermentation was about 6 log cfu ml^{-1} and relatively stable through day 2. In day 4, its population increased to 8 log cfu ml^{-1} and was stable through day 5 and then reduced to 6 log cfu ml^{-1} in the last day of fermentation. For *P. guilliermondii,* at the initial day of fermentation, its population was approximately 7 log cfu ml^{-1} and gradually increase to 8 log cfu ml^{-1} through day 2. This yeast was absent in day 3 and 4 of fermentation then it was observed in day 5 and day 6 in which its population was below 6 log cfu ml^{-1}. The isolation of *I. occidentalis* was found in only the last two days of the fermentation and its population was approximately 6 log cfu ml^{-1} (Figure 3.1, Table 3.10). These species were also detected by PCR-DGGE (Figure 3.2). With

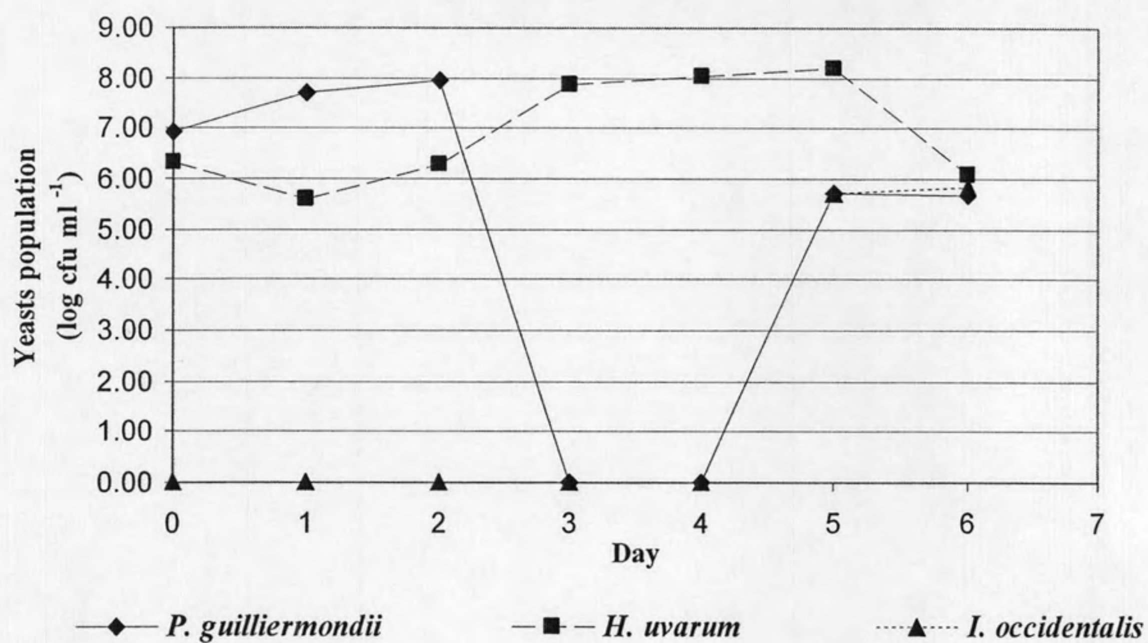

Figure 3.1 Yeasts population during natural fermentation of freshly crushed pineapple juice sample 1 of fruits grown in Thailand (TH1)

Table 3.10 Yeasts population during natural fermentation of freshly crushed pineapple juice sample 1 of fruits grown in Thailand (TH1)

Fermentation time (days)	Yeast species		DGGE analysis
	Cultural method	Population number (log cfu ml^{-1})	
0	*P. guilliermondii*	6.94	*A. pullulans*
	H. uvarum	6.31	*P. guilliermondii*
			H. uvarum
			I. orientalis
			C. sorboxylosa
1	*P. guilliermondii*	7.71	*A. pullulans*
	H. uvarum	5.60	*P. guilliermondii*
			H. uvarum
			I. orientalis
			C. sorboxylosa
2	*P. guilliermondii*	7.97	*P. guilliermondii*
	H. uvarum	6.28	*H. uvarum*
			I. occidentalis
3	*H. uvarum*	7.88	*P. guilliermondii*
			H. uvarum
			I. occidentalis
4	*H. uvarum*	8.01	*P. guilliermondii*
			H. uvarum
			I. occidentalis
5	*P. guilliermondii*	5.70	*P. guilliermondii*
	H. uvarum	8.19	*H. uvarum*
	I. occidentalis	5.70	*I. occidentalis*
6	*P. guilliermondii*	5.68	*P. guilliermondii*
	H. uvarum	6.10	*H. uvarum*
	I. occidentalis	5.85	*I. occidentalis*

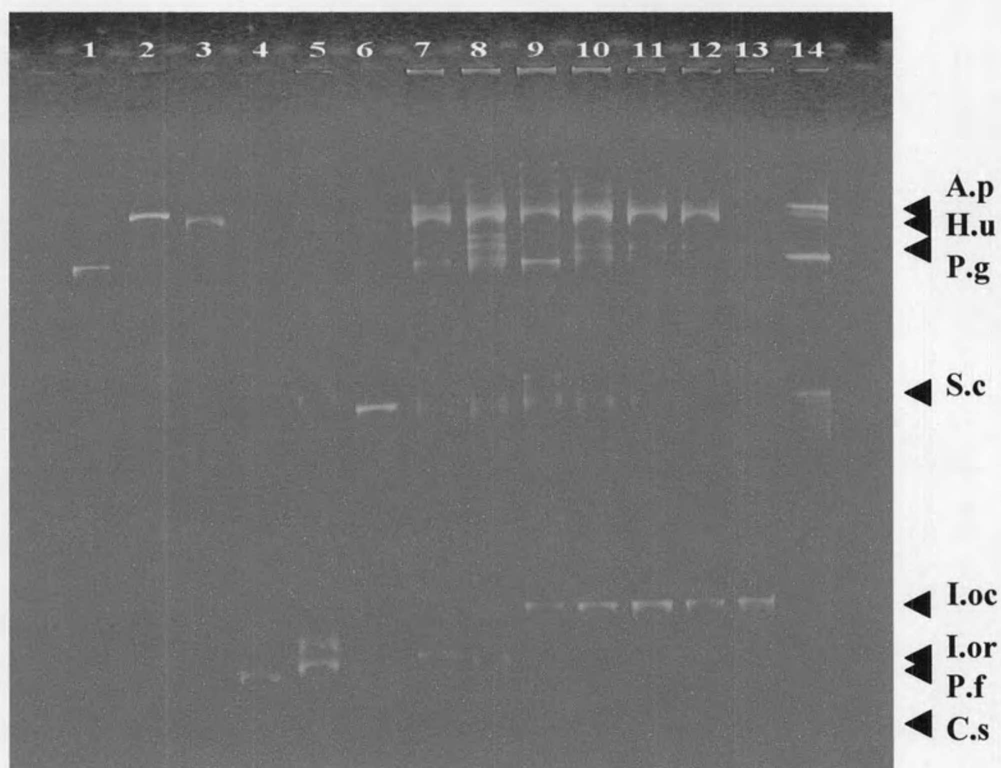

Figure 3.2 PCR-DGGE analysis of yeasts associated with natural fermentation of freshly crushed pineapple juice, sample 1 of fruits grown in Thailand (TH1); *P. guilliermondii* (P.g, lane 1), *A. pullulans* (A.p, lane 2), *H. uvarum* (H.u, lane 3), *P. fermentans* (P.f, lane 4), *I. orientalis* (I.or, lane 5), *S. cerevisiae* (S.c, lane 6), *I. occidentalis* (I.oc), *Candida sorboxylosa* (C.s), Yeasts in fermenting juice at 0, 1, 2, 3, 4, 5 and 6 days, respectively (lane 7-13); a mixture of reference cultures (lane 14)

an occasional exception, the yeasts at each day of fermentation was relatively consistent with those found by cultural plating. *A. pullulans* and *C. sorboxylosa* were additionally observed in the fermented juice of Day 1 and 2 (Lane 8). They were not readily isolated by plate culture, but they were detected by PCR-DGGE.

For the chemical analysis (Figure 3.3), ethanol was maximally generated to 1.98 % (v/v) at day 2, then its concentration gradually reduced throughout until the last day. Sucrose was the main sugar in the juice, having an initial concentration of about 73.3 g l^{-1}. It was used during fermentation but not completely and its concentration decreased to about 52.6 g l^{-1} by the end of fermentation. Fructose has an initial concentration of about 23.7 g l^{-1}. It was used during fermentation but not completely and its concentration decreased to about 11.3 g l^{-1} by the end of fermentation. Glucose was an another sugar in the juice, having an initial concentration of about 19.2 g l^{-1}. It was used during fermentation and its concentration decreased to about 2.7 g l^{-1} by the end of fermentation.

The initial pH of the juice was about 3.9 and this decreased to 3.6 by the end of fermentation. During fermentation, sucrose was decreased (20.7 g l^{-1}) more than the glucose (16.5 g l^{-1}) and fructose (12.4 g l^{-1}). pH value was gradually reduced through the fermentation.

Citric acid was the main acid available in the freshly pineapple juice (Figure 3.4). During fermentation, it's concentration decreased from 5.3 to 2.6 g l^{-1}. Lactic acid was the only organic acid generated in large amounts during fermentation. Its contents increased from 2.0 to 5.8 g l^{-1} during day 0 to day 4, then

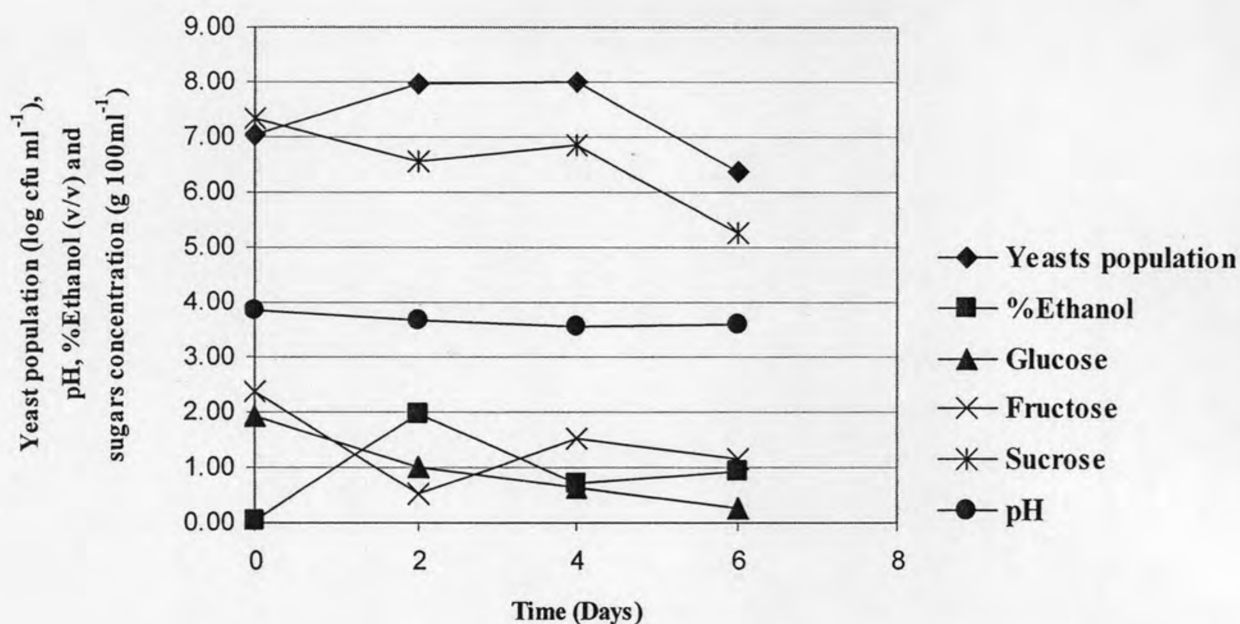

Figure 3.3 The changes of yeasts population, % ethanol, sugar concentrations and pH during natural fermentation of freshly crushed pineapple juice of sample 1 fruits grown in Thailand (TH1)

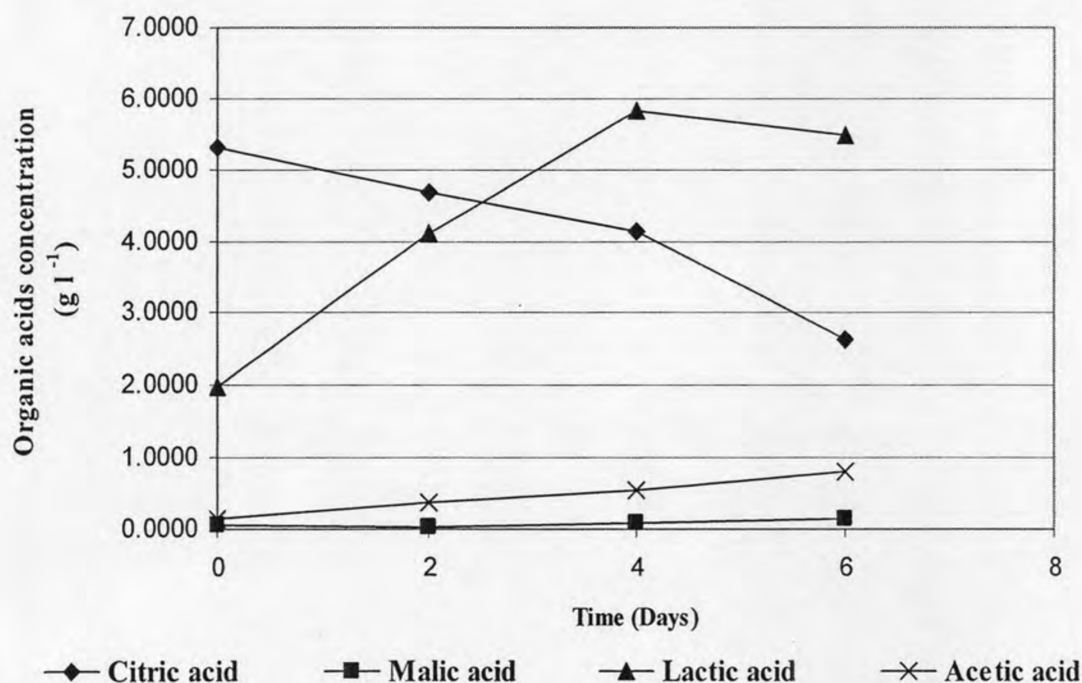

Figure 3.4 Change in concentration of organic acids during natural fermentation of freshly crushed pineapple juice, sample 1 of fruits grown in Thailand (TH1)

slightly decreased throughout day 6. Trace amounts of malic and acetic acids were also generated and detected in the fermentation system.

For the TH2 fermentation, the pineapple sample was taken from a market located in Patumwan, Bangkok. Both microbiological and chemical analytical results during fermentation were relatively similar to the TH1 sample. *H. uvarum*, *P. guilliermondii* and *I. orientalis* were still the main species observed during the fermentations. The presence of individual species in each day of the fermentation was also similar to TH1 sample. *P. guilliermondii* was absent in day 3 and 4 of fermentation, which was consistent with the experiment of TH1. The populations of each species also ranged from 6 to 8 log cfu ml^{-1} (Figure 3.5, Table 3.11).

With an occasional exception, these yeasts were similarly detected during the fermentation by plate culture and PCR-DGGE (Figure 3.6, Table 3.11). *A. pullulans*, *C. sorboxyloza* and *C. apicola*, which were not readily detected in TH1 sample, were occasionally detected by only PCR-DGGE.

From the chemical analysis (Figure 3.7), the similar trend of ethanol generation, sugars reduction and organic acids profile during the juice fermentation of TH1 were observed. Ethanol was maximally produced to 1.93 % (v/v) in day 2, and its content fluctuated. Sucrose has an initial concentration of about 78.7 g l^{-1}. It was used during fermentation but not completely and its concentration decreased to about 59.3 g l^{-1} by the end of fermentation. Fructose has an initial concentration of about 30.2 g l^{-1}. It was used during fermentation but not completely and its concentration decreased to about 25.9 g l^{-1} by the end of fermentation. Glucose

was an another sugar in the juice, having an initial concentration of about 16.0 g l^{-1}. It was used during fermentation and its concentration decreased to about 2.0 g l^{-1} by the end of fermentation.

The initial pH of the juice was about 4.0 and this decreased to 3.5 by the end of fermentation. During fermentation, sucrose was decreased (19.4 g l^{-1}) more than the glucose (14.0 g l^{-1}) and fructose (4.3 g l^{-1}). pH value was gradually reduced through the fermentation.

The significant decrease of citric acid and increase of lactic acid were observed throughout the fermentation day of this sample, as well as the TH1 sample. Small amounts of malic and acetic acids were also detected in this fermentation system (Figure 3.8).

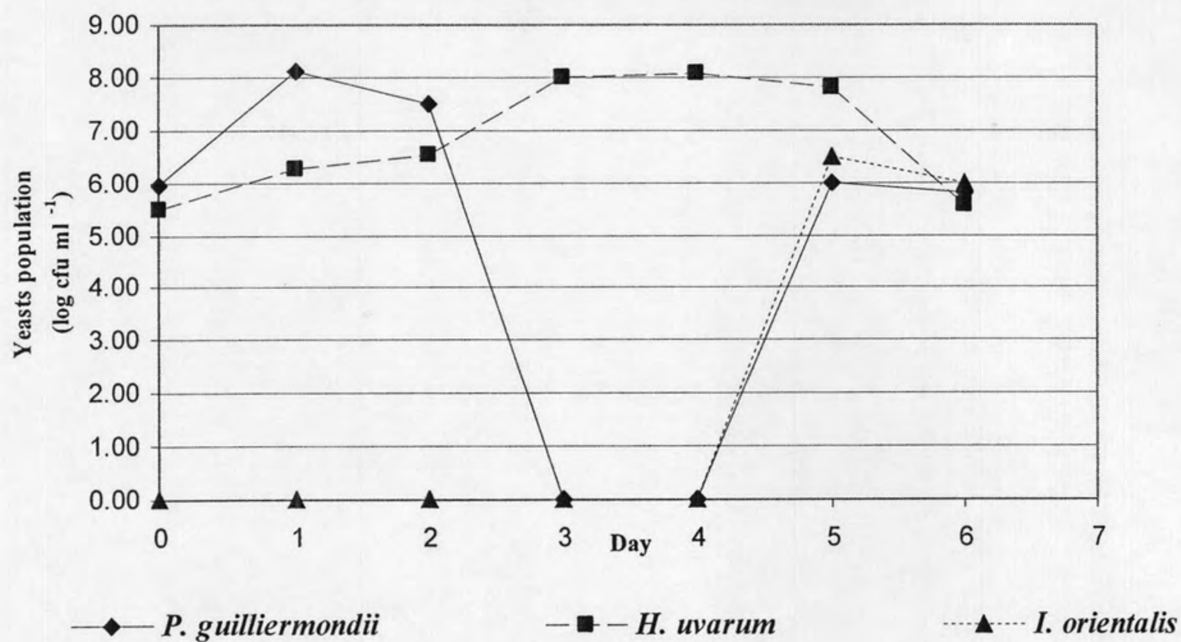

Figure 3.5 Yeasts population during natural fermentation of freshly crushed pineapple juice sample 2 of fruits grown in Thailand (TH2)

Table 3.11 Yeasts population during natural fermentation of freshly crushed pineapple juice sample 2 of fruits grown in Thailand (TH2)

Fermentation time (days)	Yeast species		DGGE analysis
	Cultural method	Population number (log cfu ml^{-1})	
0	*P. guilliermondii*	5.95	*A. pullulans*
	H. uvarum	5.49	*P. guilliermondii*
			H. uvarum
			I. orientalis
1	*P. guilliermondii*	8.10	*H. uvarum*
	H. uvarum	6.26	*C. apicola*
2	*P. guilliermondii*	7.51	*P. guilliermondii*
	H. uvarum	6.53	*H. uvarum*
			I. orientalis
3	*H. uvarum*	7.98	*P. guilliermondii*
			H. uvarum
			I. orientalis
4	*H. uvarum*	8.06	*P. guilliermondii*
			H. uvarum
			I. orientalis
			C. sorboxylosa
5	*P. guilliermondii*	6.00	*P. guilliermondii*
	H. uvarum	7.79	*H. uvarum*
	I. orientalis	6.48	*I. orientalis*
			C. sorboxylosa
6	*P. guilliermondii*	5.79	*P. guilliermondii*
	H. uvarum	5.57	*H. uvarum*
	I. orientalis	6.00	*I. orientalis*

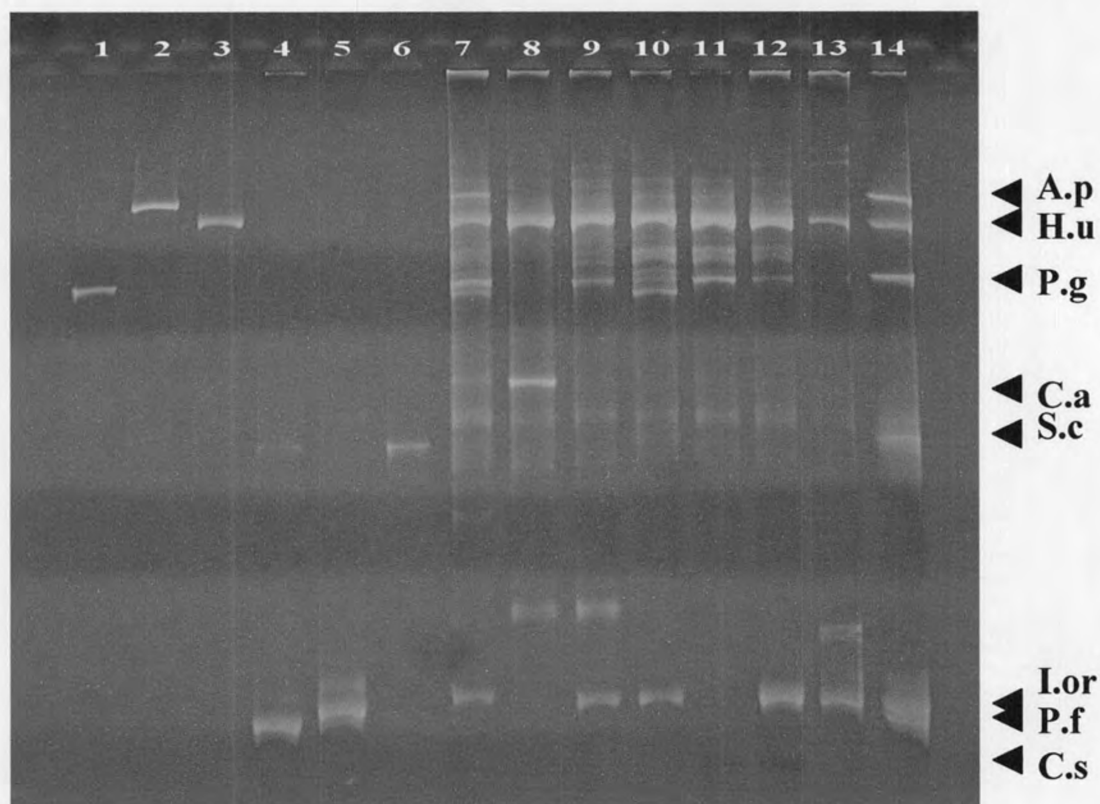

Figure 3.6 PCR-DGGE analysis of yeasts associated with natural fermentation of freshly crushed pineapple juice, sample 2 of fruits grown in Thailand (TH2); *P. guilliermondii* (P.g, lane 1), *A. pullulans* (A.p, lane 2), *H. uvarum* (H.u, lane 3), *P. fermentans* (P.f, lane 4), *I. orientalis* (I.or, lane 5), *S. cerevisiae* (S.c, lane 6), *C. apicola* (C.a) *C. sorboxylosa* (C.s), Yeasts in fermenting juice at 0,1, 2, 3, 4, 5 and 6 days, respectively (lane 7-13); a mixture of reference cultures (lane 14)

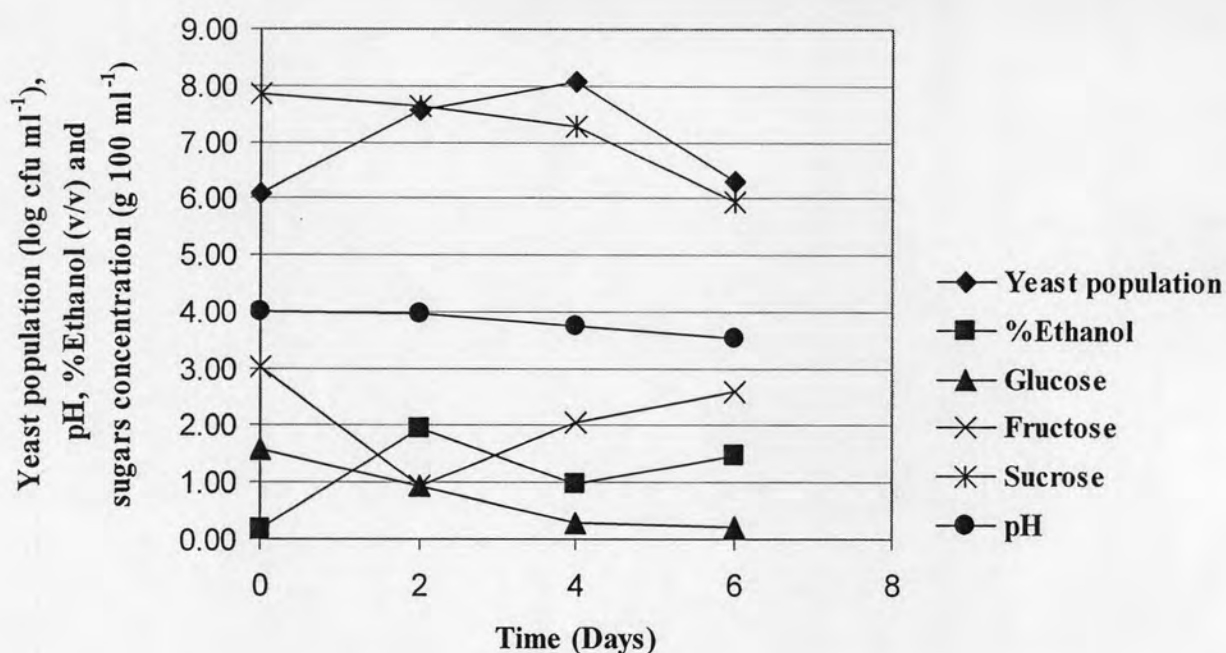

Figure 3.7 The changes of yeasts population, % ethanol, sugar concentrations and pH during natural fermentation of freshly crushed pineapple juice of sample 2 fruits grown in Thailand (TH2)

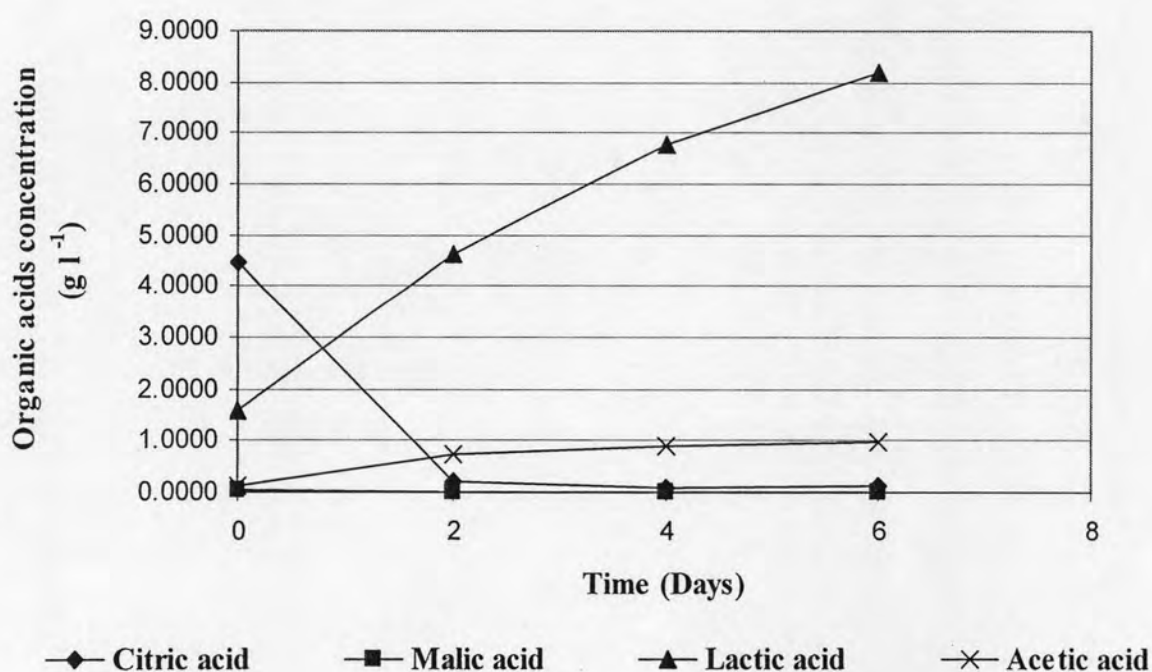

Figure 3.8 Change in concentration of organic acids during natural fermentation of freshly crushed pineapple juice, sample 2 of fruits grown in Thailand (TH2)

3.3.3.2 Spontaneous fermentation of freshly crushed pineapple juices from pineapples in Australia

In Australia, pineapple sample at the harvest stage named AUS1 was taken from a market located in Kingsford, Sydney. The same methodologies as conducted in Thailand were used to monitor the yeasts and chemical changes during the spontaneous fermentation of the freshly crushed pineapple juices. The results are shown in Figure 3.9 and Table 3.12. The main yeasts found during the fermentation were similar to those found in fermentations of pineapple juice in Thailand. These were *H. uvarum* and *P. guilliermondii*. *H. uvarum* were more prevalent than the other yeasts throughout the fermentations, while *P. guilliermondii* was occasionally absent. Interestingly, *P. guilliermondii* was absent in day 3 and 4 of fermentation, as occurred in the samples of Thailand. At the initial day of fermentation *H. uvarum* was not observed. When it was detected in day 1, its population was about 6 log cfu ml^{-1}, then increased to 8 log cfu ml^{-1} in day 2 and was stable throughout the day of fermentations. For *P. guilliermondii,* at the initial day of fermentation, its population was approximately 5 log cfu ml^{-1} and gradually increase to 6 log cfu ml^{-1} through day 2. This yeast was absent in day 3 and 4 of fermentation then it was observed in day 5 and day 6 in which its population was approximately 6 log cfu ml^{-1}. The isolation of *I. orientalis***,** which was readily detected in the sample of Thailand, was found in day 5 of fermentation only. *Tr. globispora* was found in this sample in day 1 only. The populations of these both species were below 6 log cfu ml^{-1} (Figure 3.9, Table 3.12). The observation of the main yeasts in each day of fermentation by DGGE corresponded relatively to cultural plating. With an occasional

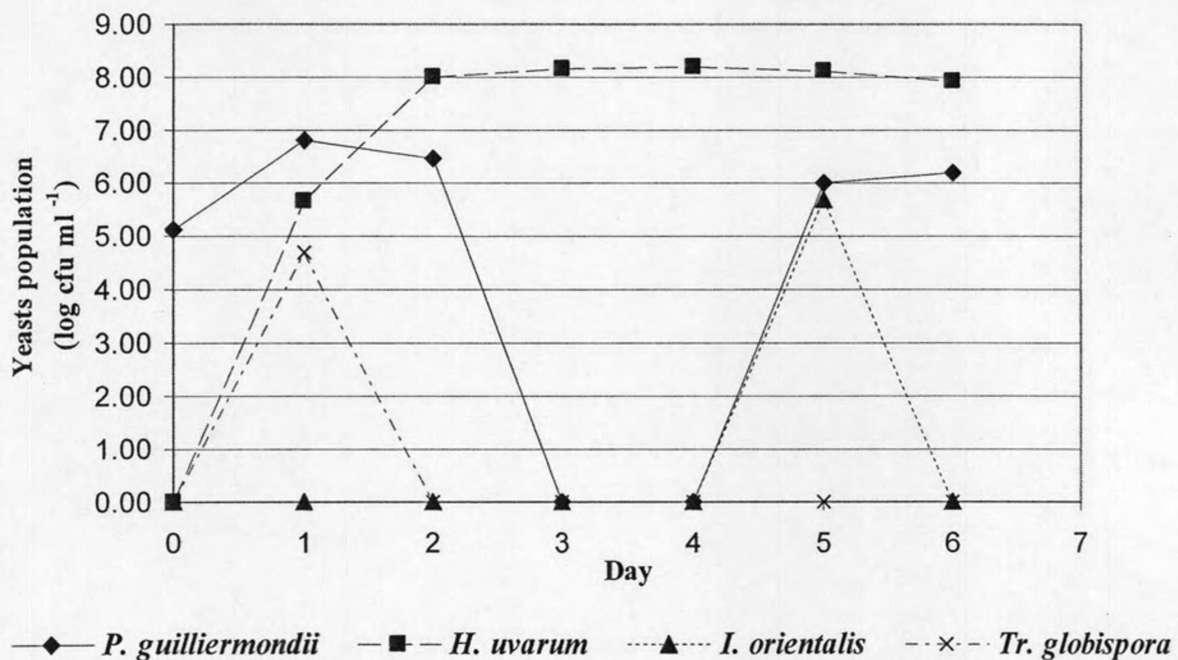

Figure 3.9 Yeasts population during natural fermentation of freshly crushed pineapple juice sample 1 of fruits grown in Australia (AUS1)

Table 3.12 Yeasts population during natural fermentation of freshly crushed pineapple juice sample 1 of fruits grown in Australia (AUS1)

Fermentation time (days)	Yeast species		DGGE analysis
	Cultural method	Population number (log cfu ml^{-1})	
0	*P. guilliermondii*	5.11	*A. pullulans* *P. guilliermondii*
1	*P. guilliermondii* *H. uvarum* *Tr. globispora*	6.80 5.65 4.70	*A. pullulans* *P. guilliermondii* *H. uvarum* *I. orientalis*
2	*P. guilliermondii* *H. uvarum*	6.48 8.01	*A. pullulans* *H. uvarum* *I. orientalis*
3	*H. uvarum*	8.14	*A. pullulans* *H. uvarum* *I. orientalis*
4	*H. uvarum*	8.17	*A. pullulans* *H. uvarum*
5	*P. guilliermondii* *H. uvarum* *I. orientalis*	6.00 8.10 5.70	*H. uvarum*
6	*P. guilliermondii* *H. uvarum*	6.18 7.91	*P. guilliermondii* *H. uvarum* *I. orientalis*

exception, *A. pullulans* was observed in the fermented juice from day 1 to day 4 (Lane 8) by PCR-DGGE, but was not observed by plate culture (Figure 3.10).

For chemical analysis (Figure 3.11), ethanol was gradually generated throughout the fermentation. The ethanol content was increased to a maximum of 3.13 % (v/v) in the final day of fermentation. Sucrose has an initial concentration of about 29.7 g l^{-1}. It was used during fermentation but not completely and its concentration decreased to about 16.7 g l^{-1} by the end of fermentation. Fructose has an initial concentration of about 20.9 g l^{-1}. It was used during fermentation and its concentration decreased to about 4.1 g l^{-1} by the end of fermentation. Glucose has an initial concentration of about 19.1 g l^{-1}. It was used during fermentation and its concentration decreased to about 7.1 g l^{-1} by the end of fermentation.

The pH value of the juices was stable in range 3.2-3.4 throughout the fermentation. During fermentation, fructose was decreased (16.8 g l^{-1}) more than the sucrose (13.0 g l^{-1}) and glucose (12.0 g l^{-1}).

The pattern of the changes in organic acids during the fermentation of freshly crushed pineapple juice of AUS1 sample was significantly different from both samples from Thailand (Figure 3.12). Citric acid, the main acid found in the juice, was relatively stable throughout the fermentation. Malic acid content was higher than the lactic acid and their content fluctuated during the fermentation. Lactic acid was detected in day 4 of the fermentation, and its content was much lower than the content observed in TH1 and TH2 samples. Acetic acid was not significantly observed in this system.

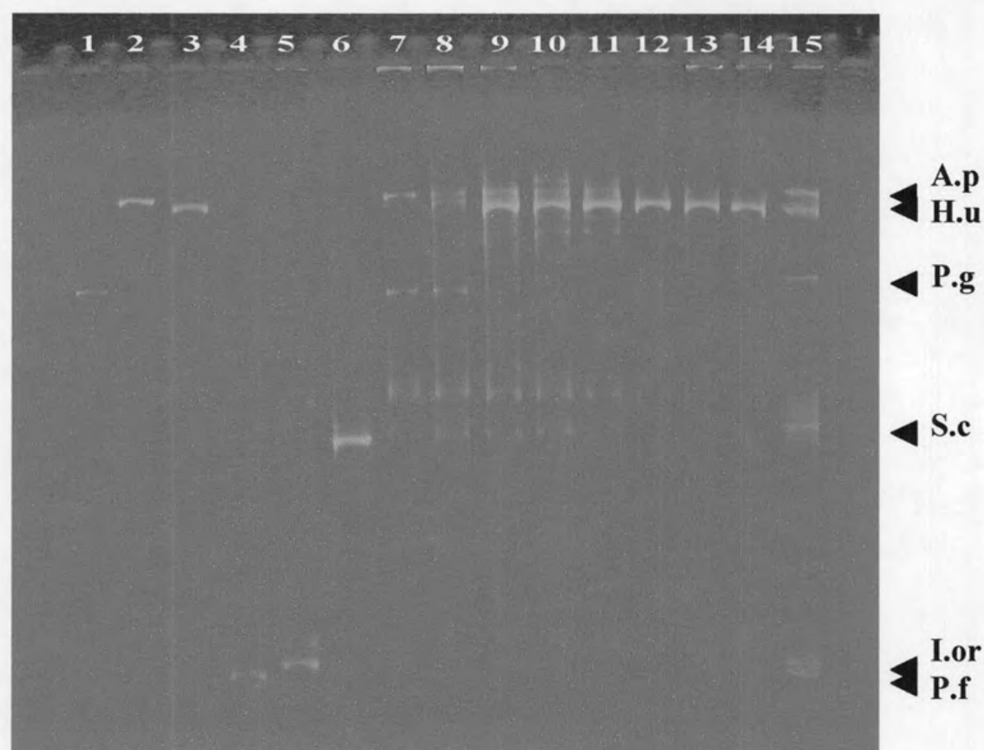

Figure 3.10 PCR-DGGE analysis of yeasts associated with natural fermentation of freshly crushed pineapple juice, sample 1 of fruits grown in Australia (AUS1); *P. guilliermondii* (P.g, lane 1), *A. pullulans* (A.p, lane 2), *H. uvarum* (H.u, lane 3), *P. fermentans* (P.f, lane 4), *I. orientalis* (I.or, lane 5), *S. cerevisiae* (S.c, lane 6), Yeasts in fermenting juice at 0, 1 , 2, 3, 4, 5, 6 and 7 days, respectively (lane 7-14); a mixture of reference cultures (lane 15)

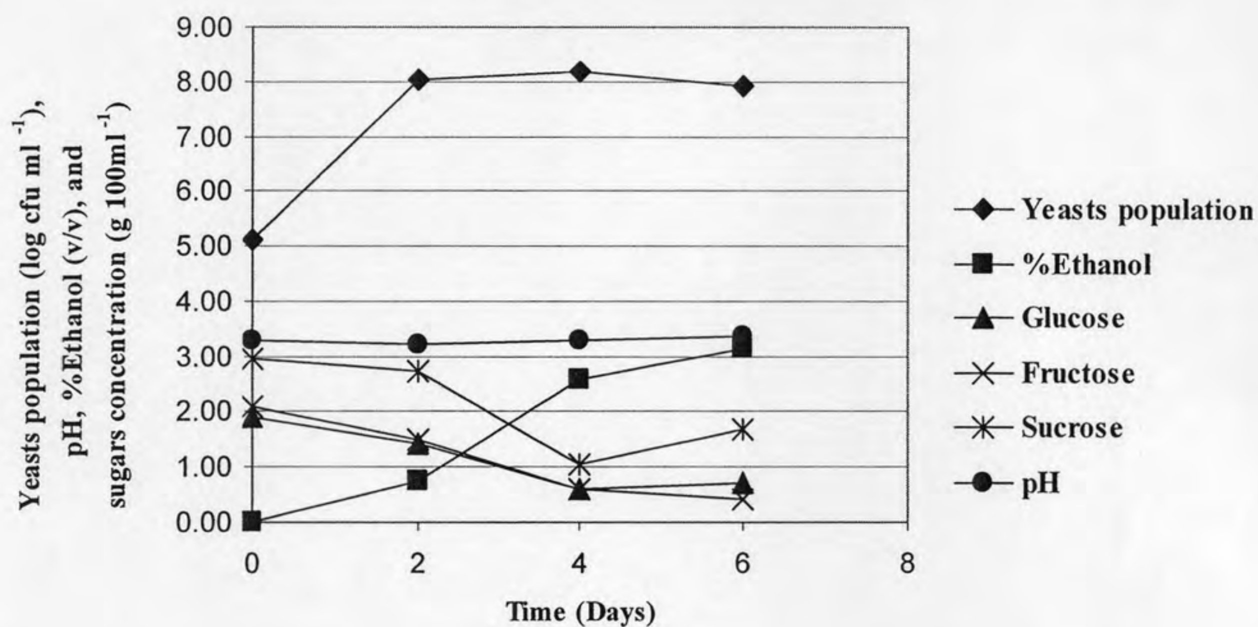

Figure 3.11 The changes of yeasts population, % ethanol, sugar concentrations and pH during natural fermentation of freshly crushed pineapple juice of sample 1 fruits grown in Australia (AUS1)

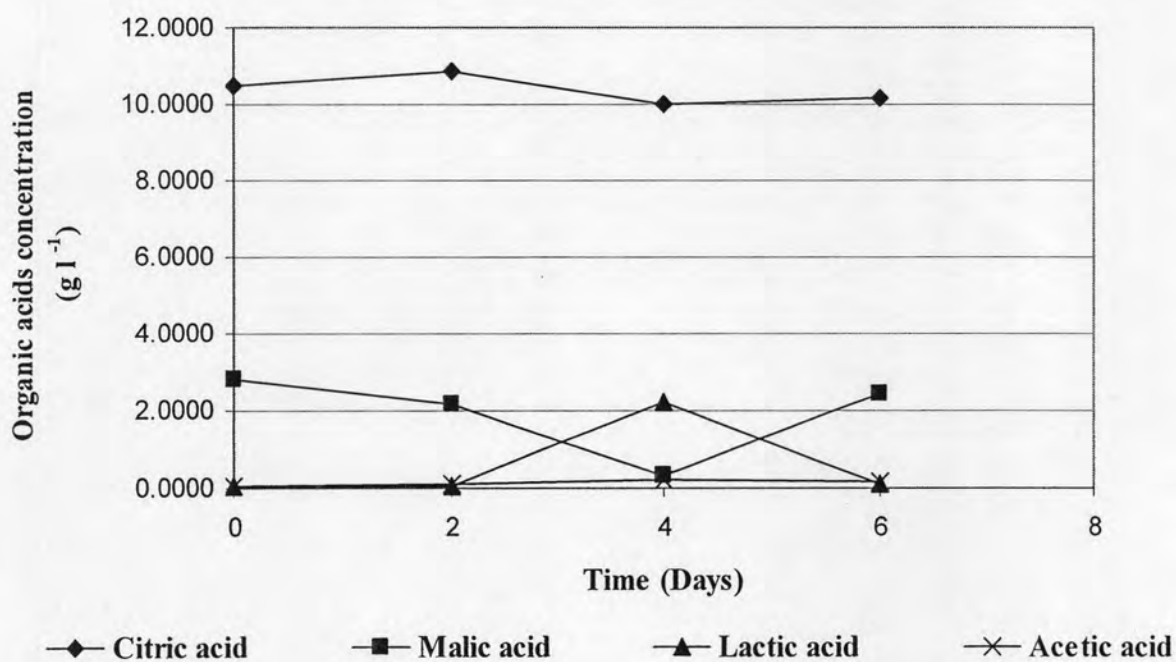

Figure 3.12 Change in concentration of organic acids during natural fermentation of freshly crushed pineapple juice, sample 1 of fruits grown in Australia (AUS1)

The AUS2 sample was pineapple samples taken from the market located in Cronulla, Sydney. During fermentation of freshly crushed juices of this sample, there was a notable presence of *P. guilliermondii* and *H. uvarum* in the AUS2 fermentation in system, as with AUS1 (Figure 3.13). The population number of *H. uvarum* observed was totally larger than the other yeasts throughout the fermentation and its growth dynamic was same as in spontaneous fermentation of both Thai samples and AUS1 sample. In contrast with the other samples, *P. guilliermondii* was observed everyday in the spontaneous fermentation of this sample. Its population was approximately 6 log cfu ml^{-1}, which was stable throughout the day of fermentation. *Candida sp. and Y. lipolytica* were observed in this test, but detected on few occasions. The yeasts profile observed by DGGE analysis results was also consistent with the cultural plating (Figure 3.14, Table 3.13).

For the chemical analysis, when compared to AUS1 sample, the consistent trend of the chemical determined was observed (Figure 3.15). The ethanol was maximally generated to 2.74 %(v/v) during the final day of fermentation. Sucrose has an initial concentration of about 31.7 g l^{-1}. It was used during fermentation and its concentration decreased to about 3.6 g l^{-1} by the end of fermentation. Fructose has an initial concentration of about 21.3 g l^{-1}. It was used during fermentation and its concentration decreased to about 4.4 g l^{-1} by the end of fermentation. Glucose has an initial concentration of about 22.6 g l^{-1}. It was used during fermentation and its concentration decreased to about 4.5 g l^{-1} by the end of fermentation.

The pH value of the juices was stable in range 3.6-3.7 throughout the fermentation. During fermentation, sucrose was decreased (28.1 g l^{-1}) more than the glucose (18.1 g l^{-1}) and fructose (16.9 g l^{-1}).

The organic acids profile of AUS2 during natural fermentation (Figure 3.16) was relatively similar to AUS1 sample. Citric acid was relatively stable throughout the fermentation, but the reduction of this acid was observed in the last day. Malic and lactic acids content ranges were similar to AUS1 sample, but their content fluctuated during the fermentation. Acetic acid was also not significantly observed in this system.

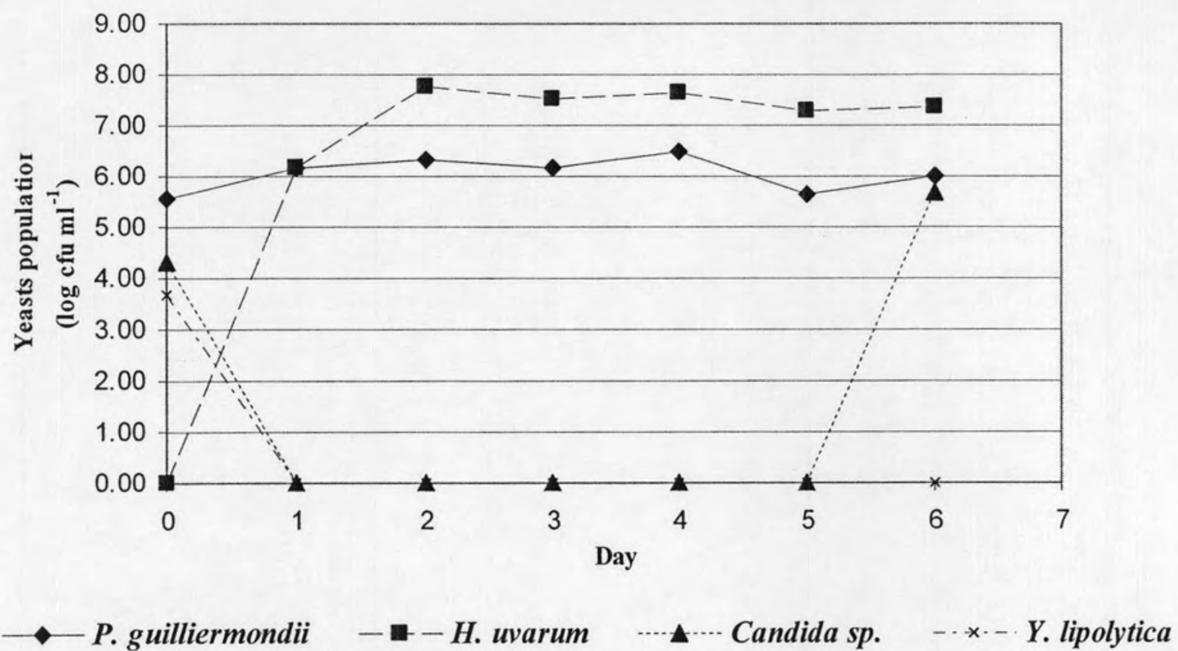

Figure 3.13 Yeasts population during natural fermentation of freshly crushed pineapple juice sample 2 of fruits grown in Australia (AUS2)

Table 3.13 Yeasts population during natural fermentation of freshly crushed pineapple juice sample 2 of fruits grown in Australia (AUS2)

Fermentation time (days)	Yeast species		DGGE analysis
	Cultural method	Population number (log cfu ml^{-1})	
0	*P. guilliermondii*	5.57	*A. pullulans*
	Candida sp.	4.30	*P. guilliermondii*[a]
	Y. lipolytica	3.70	*I. orientalis*
1	*P. guilliermondii*	6.15	*P. guilliermondii*[a]
	H. uvarum	6.18	*H. uvarum*[a]
			I. orientalis
2	*P. guilliermondii*	6.30	*P. guilliermondii*[a]
	H. uvarum	7.76	*H. uvarum*[a]
			I. orientalis
3	*P. guilliermondii*	6.18	*P. guilliermondii*
	H. uvarum	7.52	*H. uvarum*
			Candida sp.
			I. orientalis
4	*P. guilliermondii*	6.48	*P. guilliermondii*
	H. uvarum	7.63	*H. uvarum*
			Candida sp.
			I. orientalis
5	*P. guilliermondii*	5.65	*H. uvarum*
	H. uvarum	7.29	*Candida* sp.
			I. orientalis
6	*P. guilliermondii*	6.00	*H. uvarum*
	H. uvarum	7.36	*I. orientalis*
	Candida sp.	5.70	

[a] indicated the intensity of DNA bands

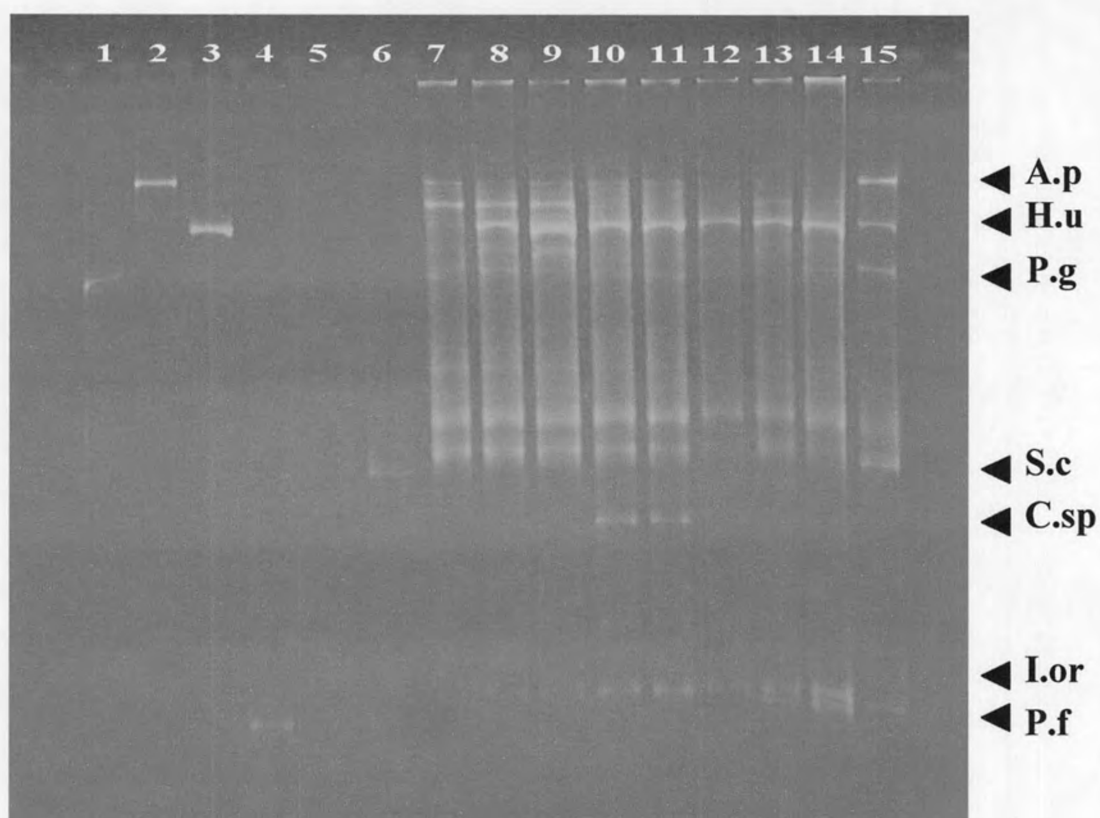

Figure 3.14 PCR-DGGE analysis of yeasts associated with natural fermentation of freshly crushed pineapple juice, sample 2 of fruits grown in Australia (AUS2); *P. guilliermondii* (P.g, lane 1), *A. pullulans* (A.p, lane 2), *H. uvarum* (H.u, lane 3), *P. fermentans* (P.f, lane 4), *I. orientalis* (I.or, lane 5), *S. cerevisiae* (S.c, lane 6), *Candida* sp. (C.sp), Yeasts in fermenting juice at 0, 1 , 2, 3, 4, 5, 6 and 7 days, respectively (lane 7-14); a mixture of reference cultures (lane 15)

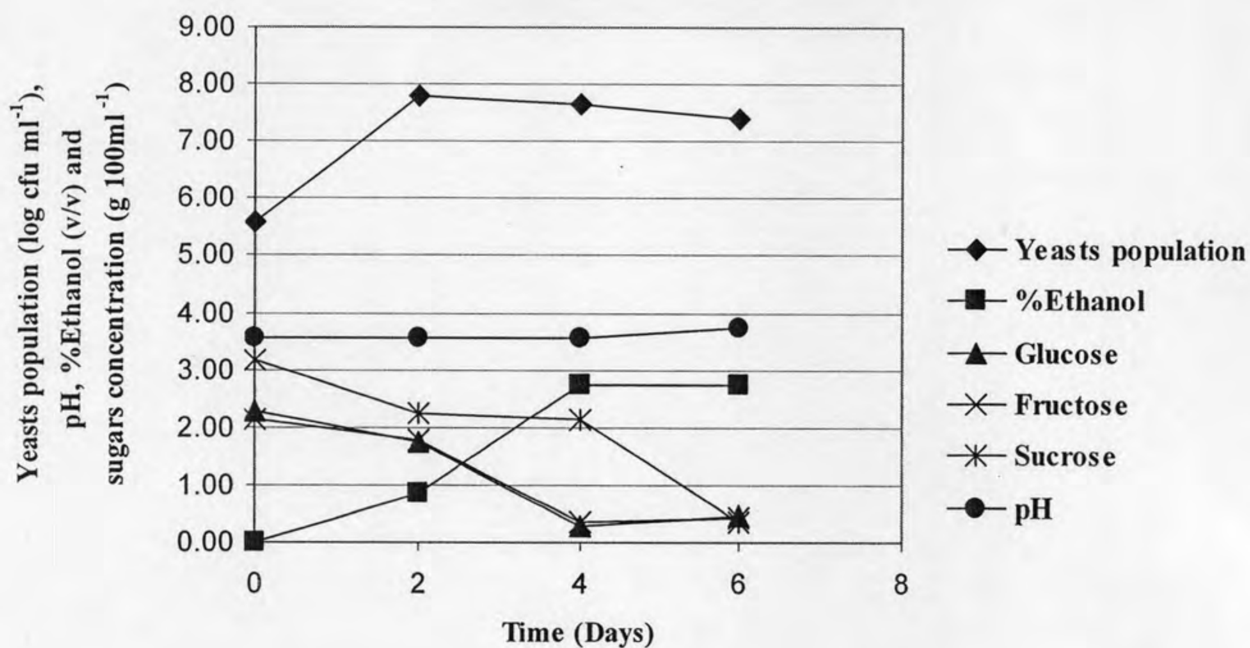

Figure 3.15 The changes of yeasts population, % ethanol, sugar concentrations and pH during natural fermentation of freshly crushed pineapple juice of sample 2 fruits grown in Australia (AUS2)

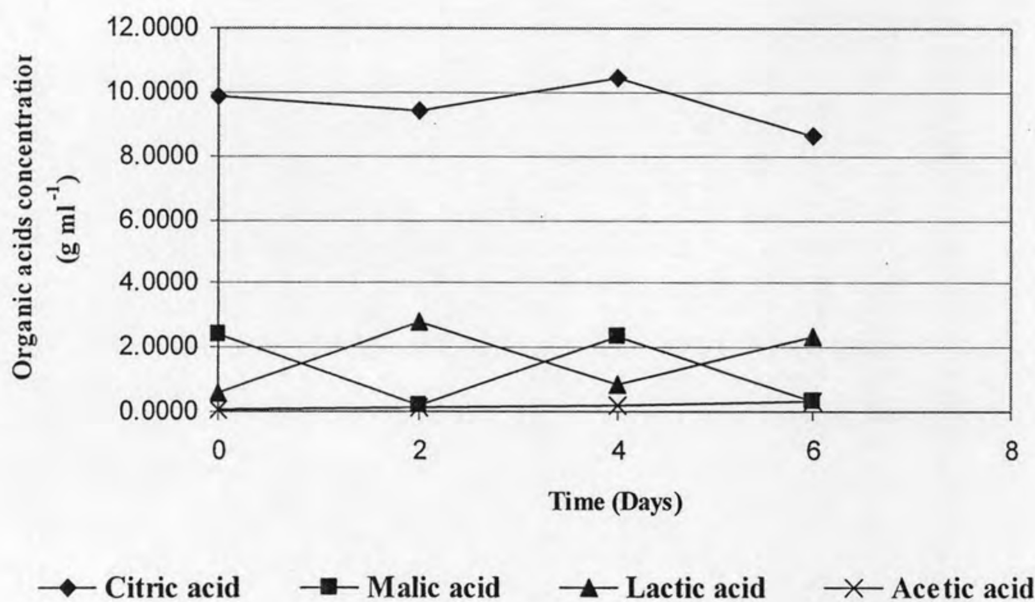

Figure 3.16 Change in concentration of organic acids during natural fermentation of freshly crushed pineapple juice, sample 2 of fruits grown in Australia (AUS2)

3.3.4 Yeast flora of fermentations

The procedures used to determine the diversity of yeast species associated with natural pineapple juice fermentations were plating on agar media and analysis of DNA extracts by PCR-denaturing gradient gel electrophoresis (PCR-DGGE). In this study, molecular analytical methods based on PCR-DGGE was applied to determine yeast community in natural fermented pineapple juices in order to maximize the reliability of the data obtained, since in addition to detecting species normally found by cultural methods, it offers the prospect of detecting species that may be present in the fermentation system as viable but non-culturable states (Rappe and Giovannini, 2003, Prakitchaiwattana, Fleet and Heard, 2004). Therefore, the yeast species observed during natural pineapple juiced fermentation was concluded from the data obtained from cultural isolation on plates of MEA and PCR-DGGE analysis throughout this study.

Based on the data obtained from the cultural plating and PCR-DGGE, *H. uvarum* and *P. guilliermondii* were the main species isolated from the natural fermentation systems of freshly crushed pineapple juice in all samples from Thailand and Australia. Their populations increased from initial approximately 5 log cfu ml^{-1} to 8 log cfu ml^{-1} through the end of fermentation. Based on the population number of individual species, *P. guilliermondii* was present as a dominant species during the early stage of the fermentation, then *H. uvarum* became more prevalent until the final day of fermentation. From the study of Section 3.3.2.1, *P. guilliermondii* was found as a common species on pineapple fruits collected from the same farm as TH1 sample when isolated by rising and enrichment methods. However, *H. uvarum* was only

found as a prevalent species on the pineapple surfaces when isolated by enrichment method. As expected, these yeasts were prevalent in the natural fermentation system of freshly crushed juices. Interestingly, this observation was also found in the spontaneous fermentation of freshly crushed pineapples juices of TH2 sample and even in AUS1 and AUS2 samples, despite these pineapple samples being grown in totally different climates and locations. This confirms that the occurrence of *H. uvarum* and *P. guilliermondii* in freshly crushed juice does not depend on climate and region. In addition, it seems that the condition of juices could be selective for these yeasts. Many reports demonstrated that in the spontaneous fermentation systems of the fruit juices, the yeast species and strain present in these systems were normally diverse. In the spontaneous system of grape juices, the diverse species of *Hanseniaspora*, *Pichia* and many other non-*Saccharomyces* yeasts are found during the early stage of spontaneous fermentation, then are replaced by *Saccharomyces* yeasts which becomes the predominant yeasts and continues the fermentation until its completion (Heard and Fleet, 1985; Pretorius, 2000; Fleet et al., 2002 and Fleet, 2003a). A similar community of yeasts associated with spontaneous fermentation of cider was also reported (Morrissey et al., 2004; Coton et al., 2006). In natural palm juice fermentation, as reported by Thammarat (1978), *K. apiculata*, *P. membranaefaciens*, *Cadida spp.*, *S. chevalieri* and *S. cerevisiae* were found as yeasts involved in the fermentation. Mangosteen paste allowing natural fermentation to occur, diverse species of yeasts, in particular, *Saccharomyces* were also found (Deeraksa et al., 2005). Interestingly, in the freshly crushed orange juices, the yeast diverse species of *Hanseniaspora*, *Pichia, Candida*, *Saccharomyces*, *Clavispora*, *Rhodotorula*, *Saccharomycopsis*, *Torulaspora*, *Metschnikowia*, *Issatchenkia* and

Geotrichum were significantly present as the main species (Okunowo, Okotore and Osuntoki, 2005).

However, there were a few occasions where the presence of yeast species in the fermented juice was detected by PCR-DGGE, but not found by culture on agar media. The DNA from *Candida* species, *C. sorboxylosa*, *Candida* sp., *C. apicola*, and *Y. lipolytica* (asexual stage *C. lipolytica*), were occasionally detected in some samples. It is known that *Candida* yeasts are normally present in natural habitats such as soil, plants and water (Barnett et al., 2000). In fermented fruit juice, these yeasts were not detected on the cultural plate. Therefore, the observation of these yeasts on some occasions by PCR-DGGE might be just the detection of their DNA trace left in the juices. The presence of *A. pullulans* in the fermented juice was also detected by PCR-DGGE, but not found by culture on agar media. As the results show in section 3.3.2.1 (table 3.3), it was known that this yeast was a common yeast on pineapple fruit surfaces, but that it was not prevalent (active) in pineapple juice when used as an enrichment culture. Therefore, due to an influence of pineapple juices, it could be present as a non-culturable state cell and have no role in the spontaneous fermentation. Interestingly, the isolation of *I. orientalis* was another yeast frequently isolated from fermented freshly crushed pineapple juices of both samples from Thailand, particularly during the last day of fermentation. This yeast was detected by both PCR-DGGE and cultural methods. This was not surprising, since this yeast was prevalently found on pineapple surfaces isolated by enriched pineapple and grape juices containing 6% alcohol. This yeast was reported as a yeast that could utilize alcohol as a carbon source (Barnett et al., 2000). It seems that the occurrence of this

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yeast could have an influence on the spontaneous fermentation system of the pineapple juices and will be discussed later.

In the natural fermentation system of pineapple juices as observed in this study, only two species of yeasts were significantly present. It seems that *P. guilliermondii* initiated the fermentation, then *H. uvarum* became more prevalent and persisted to play role in the fermentation system. It is interesting that other wine yeasts, in particular, *Saccharomyces* yeasts, were not found in any fermentation systems of this study. As discussed in the literature, climatic effects and region have the potential to influence the occurrence of yeasts on fruits surfaces which are associated with spontaneous fermentations of the fruit juices. However, the results obtained from this study revealed that these factors are not associated with the occurrences of *P. guilliermondii* and *H. uvarum* in the spontaneous pineapple juice fermentations. Thus, from a microbiological perspective, only broad conclusions can be drawn about influences of freshly crushed pineapple juices properties. Importantly, among fruits as reported above, pineapple is the only fruit which its juice containing abundant protease known as bromelain (Bartholomew, Paull and Rohrbach, 2003). This protease was produced in the edible portion of pineapple fruits during maturity development (Singleton and Gortner, 1965; Bartholomew, Paull and Rohrbach, 2003). It is known that protease enzymes have property in hydrolyzing microbial cell walls (Uhlig and Linsmaier-Bednar, 1998). Therefore, possibly, in freshly crushed pineapple juices, the protease could be inhibitory to many other yeasts, in particular wine yeast, but not to *P. guilliermondii* and *H. uvarum*. This indicates that the freshly crushed juice or even pineapple fruits exudates could be a selective condition for these yeasts. This is a relatively novel observation that requires further investigation

to address these concepts, as this enzyme will have an important influence on the yeast species that will occur in juice and contribute to fermentation.

3.3.5 Chemical changes during fermentations - sugars, ethanol production and organic acids

To better understand the fermentation profile of the freshly crushed pineapple juices, chemical determinations were done and the relationship between yeasts and the chemical changes during the fermentation were investigated. As mentioned above, it seems that *P. guilliermondii* initiate the fermentation, then *H. uvarum* became more prevalent and played role in the spontaneous freshly crushed pineapple juice fermentation system. The maximum alcohol levels generated in the spontaneous fermentations were in the range of 1-3 % (v/v). In the spontaneous fermentation of samples from Australia, the alcohol generation was different from the samples from Thailand. For the samples from Australia, the alcohol generated was higher and increased throughout the fermentation day. All sugars were gradually utilized, while all organic acids, even pH, were relatively stable throughout the fermentation. Citric acid is the major organic acid in pineapple juice and influences on pH of pineapple juice (Singleton and Gortner, 1965; Dull, 1971; Camara et al., 1994; Bartolome, Rupbrez and Carmen, 1995; Bartholomew, Paull and Rohrbach, 2003). From organic acid analysis, it was found that the concentrations of citric acid of fruits grown in Australia were higher than the fruits grown in Thailand. It is known that cultivation conditions such as soil composition, rainfall and temperature affect the chemical composition of the fruits and its juice. In addition, the temperature known as a key factor normally influences on acid accumulation in the fruit. The fruits grown in

colder tend to have higher acids. Therefore, citric acid contents in pineapple fruit grown in Australia where the temperature is generally lower than in Thailand, are normally higher than those of the fruits grown in Thailand (Bartholomew, Paull and Rohrbach, 2003). The stable citric acid content during the spontaneous fermentations, demonstrated that citric acid could not be utilized by indigenous yeasts. Consequently, pH value could be kept steadily throughout fermentation. In contrast, in the samples from Thailand, the alcohol slightly increased and the alcohol generation fluctuated (Figure 3.2, 3.5, 3.8 and 3.11), while sugar, in particular glucose, was rapidly utilized. The significant reduction of citric acid and the generation of lactic acid were also observed, which resulted in the decrease of pH value in the fermented juices of these samples. These resulted from the growth of lactic acid bacteria which was found in the freshly crushed pineapple juices (data not shown). These bacteria completely utilized glucose and also used citric acid as a carbon source to produce lactic acid, resulting in retarding of the fermentation of the yeast (Amerine and Kunkee, 1968). This observation is relatively interesting. From the results observed in spontaneous fermentation of sample from Australia as mentioned above, it indicated that the yeast *P. guilliermondii* and *H. uvarum* could not breakdown or utilize citric acid. Therefore, it seems that the reduction of citric acid was mainly responded by the lactic acid bacteria. It is a well-established fact that this organism attacks citric acid and produces lactic and acetic acids. The presence of acetic acid in wine is considered as spoilage. Generally, spoilage of wines by lactic acid bacteria takes the following forms. (i) The production of excess acidity from sugars, due to producing lactic acid, acetic acid, CO_2, and mannitol from fructose; (ii) the same excess acidity, accompanied by a mousey flavor (acetamide); (iii) the decomposition of tartaric acid, giving acetic acid and CO_2; (iv) the decomposition of

glycerol, with the production of acetic, lactic and propionic acids, and (v) the decomposition of malic acid giving lactic acid and CO_2. The last is not generally considered as spoilage (Carr, 1958). Interestingly, in this study, acetic acid occurs only in traces in the spontaneous fermentation of pineapple juices. The reaction of this bacteria in spontaneous pineapples juice fermentations is similar to lactic acid bacteria resident in the grape wine, which are responsible for the malolactic fermentation (MLF). The main reaction of MLF is decarboxylation of L-malic acid to L-lactic acid, giving a decrease in acidity of the wine. A decrease in acidity by malolactic fermentation gives a wine with softer, mellower taste. Also, growth of malolactic bacteria in wine contributes additional metabolites that may confer complex and interesting flavor characteristics. A decrease in acidity by malolactic fermentation gives a wine with softer, mellower taste. Also, growth of malolactic bacteria in wine contributes additional metabolites that may confer complex and interesting flavor characteristics (Fleet, 1998; 2001). Therefore, based on the fermentation properties of lactic acid bacteria as found in spontaneous pineapples juices fermentations, it could be alternative for development of pineapples wine flavor in term of acid taste reduction, possibly including aroma compounds. This observation is fundamental for the fermentation of lactic acid bacteria in pineapple juice that requires further investigation to prove these concepts.

Issatchenkia orientalis was another yeast frequently isolated from fermented freshly crushed pineapple juices of both samples from Thailand. The occurrence of this yeast could have an influence on the spontaneous fermentation system of these pineapple juices, since it could utilize ethanol as a carbon source (Barnett et al., 2000). Therefore, the alcohol fluctuation in these systems could affect

this yeast. It seems that this yeast significantly interfered in the spontaneous pineapple fermentation systems. Importantly, it was consistently found in both pineapple samples from Thailand, which were grown in different areas. Therefore, this is an interesting observation that requires further investigation to determine if it is a regional or more general phenomenon.