CHAPTER VI

CONCLUSIONS

This thesis has reported investigation of yeasts in spontaneous pineapple juice fermentations to find appropriate yeasts which could be applied as starter cultures for quality pineapple wine fermentations. The first experimental chapter involved an investigation of the indigenous yeasts associated with pineapple fruits and with freshly crushed pineapple juices allowing natural fermentation to occur. A combination of cultural and molecular methods were used to analyze the yeasts isolates and the competence of the methods in yeasts identification was evaluated prior to use in this study (Chapter 3). The yeasts isolates obtained from this study were selected and subjected to an evaluation of their alcoholic fermentation properties in pineapple juices (Chapter 4). Subsequently, the yeasts performing appropriate fermentations were selected and applied as multi-starter cultures in pineapple wine fermentations. The fermentation characteristics of these multi-starter cultures were investigated (Chapter 5). Overall, this study represents the pioneer investigation of yeasts in the system of spontaneous pineapple juice fermentations and the potentiality of the yeast isolates form these systems for applying as species specific starter culture for pineapple wine fermentations. Some major conclusions of this study are outlined in the following paragraphs.

1. Sequencing of D1/D2 region of 26S rDNA and ITS regions, RFLP analysis and ID 32 C system were evaluated prior to use for yeasts identification. According to the results, approximately 50% of yeast isolates identification was achieved by used

of 26S rDNA in combination with ITS sequencing analysis. Whereas, yeast identification results by RFLP analysis and ID 32 C were frequently different from the two sequencing methods. Therefore, two sequencing analysis were selected as the main methods for the identification of the yeast isolates throughout this study. However, where there was no matching of these two methods, another identified method was performed by subjecting to ID 32 C system or RFLP analysis. The yeast isolates would be assigned as unidentified species, if there were some yeast isolates identified by three methods that did not give similar identification results.

2. For the investigation of the indigenous yeasts associated with pineapple fruits, the pineapples at various stages of maturity throughout cultivation in the fields of Thailand including damaged fruits were examined for their populations and species of yeasts. Yeast associated on the pineapple fruits were determined by performing the isolation of yeast by rinsing and enrichment methods. With rinse-plating method, *A. pullulans* was found as a common yeast on intact fruits skin at every cultivation stage, whereas only fermentative yeast, *P. guilliermondii*, was found as the prevalent species on mature, intact fruits. The population of individual yeast increased in parallel with maturity development of pineapple fruits. Their populations were quite low, and varied between 3-5 log cfu g^{-1}. On damaged fruits surfaces, osmophillic yeast only *Z. bailii* was found and its population was 6.23 log cfu g^{-1}.

Grape juice and pineapple juice and these juices added with 6% ethanol were used as enrichment cultures to detect yeast species present in very low populations and not detectable by rinse-plating methods. With the enrichment methods, *P. guilliermondii* was consistently found on intact pineapple skins at every

cultivation stage enriched in all 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*. *I. orientalis*, it was another yeast consistently found on intact fruits throughout cultivation stages when isolated by enrichment culture containing 6%(v/v) alcohol. For damaged fruits, only *Z. bailii* was consistently isolated from damaged skin enriched in all cultures. *P. fermentans* and *Candida* sp. were also found on damaged skin, but not isolated as frequently as *Z. bailii*. Not many yeast species were additionally found on pineapple skins, even when isolated by enrichment cultures. Apart from unidentified yeast, there were just four yeast species, *C. tropicalis*, *Candida* sp., *E. hasegawianum* and *S'codes ludwigii*, which were found on a few occasions. Surprisingly, *S. cerevisiae* and *S. bayanus* were not isolated from the pineapple examined in this study even by enrichment cultures with 6% alcohol added.

3. For the investigation of the yeasts in spontaneous pineapple juices fermentation, the yeasts in the natural fermentation of pineapple samples from Thailand and Australia were studied to investigate the influence of climate and region on the yeasts associated with these systems. The yeasts associated with the fermentations were determined by cultural isolation and by PCR-DGGE analysis of extracted DNA. Based on the data obtained from the cultural plating and PCR-DGGE, it was found that *H. uvarum* and *P. guilliermondii* were the main species similarly 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 to 8 log cfu ml^{-1} through to the end of fermentation. Ethanol generated in the system of these natural fermentations were varied between 1-4 %

(v/v). Based on the population number of individual species, yeasts during the day of fermentation, *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. Therefore, it seems that *P. guilliermondii* initiated the fermentation, then *H. uvarum* became more prevalent and persisted to play role in the fermentation system. This could confirm that the occurrences of *H. uvarum* and *P. guilliermondii* in freshly crushed juice did not depend on climate and region. In addition, it seems that the condition of juices could be selective for these yeasts.

4. Yeast isolates obtained from chapter 3 were primarily screened for their ability with alcohol production characteristics by performing micro-fermentation tests using pasteurized pineapple juice as a substrate. The yeasts produced alcohol higher than 5% (v/v) were selected to investigation of their fermentation characteristics in sterilized pineapple juices. There were *S'codes ludwigii* (Sl; 8.0% (v/v)), *Candida* sp.1 (Cs1; 6.7% (v/v)), *Candida* sp.2 (Cs2; 5.7% (v/v)), *Z. bailii* (Zb; 5.7% (v/v)), *H. uvarum* (Hu1; 5.2% (v/v)) and *H. uvarum* (Hu2; 5.0% (v/v)). The alcoholic fermentation properties of these yeasts were determined compared to a commercial *S. cerevisiae*. The yeasts having proper fermentation characteristics were selected to application as starter cultures for pineapple wine fermentation. Fermentation characteristics as criteria for the yeast selection were investigated in term of alcohol, organic acids and volatile compound productions. Based on these criteria, *S'codes ludwigii* (Sl) was selected since it could produce the highest amount of ethanol content relative to the other yeasts isolates including a commercial *S. cerevisiae*. In addition, it showed the other fermentation characteristics, which were similar to the commercial *S. cerevisiae*. *H. uvarum*1 (Hu1) was also selected since it could generate

2-phenylethyl acetate, which reported as a volatile compound giving rose and flowery odors. Therefore, these two yeast isolates were selected to apply as multi-starter cultures for pineapple wine fermentation.

5. The mixed cultures of yeasts isolates selected from chapter 4 and the commercial *S. cerevisiae* were applied as starter cultures for pasteurized pineapple wine fermentations. The fermentation characteristics of the mix starter cultures, a combination of two yeasts (*S. cerevisiae* and *H. uvarum*, *S. cerevisiae* and *S'codes ludwigii*, and *S'codes ludwigii* and *H. uvarum*), and a combination of three yeasts were determined. It was found that *S'codes ludwigii* could be used as the main yeasts to produce the alcohol and complete the pineapple wine fermentation same as *S. cerevisiae.* Interestingly, the chemical constituents and volatile compounds of pineapple wine produced by mixed starter culture were dependant on the fermentative behavior of the main starter culture. *H. uvarum* could not show its main volatile compounds in wine product when *S. cerevisiae* was used to be combined as main starter culture. Conversely, *S'codes ludwigii,* could extend the survival of *H. uvarum* during the fermentation allowing the *H. uvarum* to carry out the fermentation and produce the 2-phenylethyl acetate in wine before it started the decline phase. Therefore, the volatile compounds generated in pineapple wine by the mixed starter cultures of *S'codes ludwigii* and *H. uvarum* was more complex. Therefore, based on their positive interaction, multi-starter from these two yeast isolates could be a potential starter culture for quality pineapple wine fermentation.

RECOMMENDATIONS

This study has vastly extended the knowledge of yeasts associated with pineapple fruits and its spontaneous fermentation. The following are recommended aspects that will be useful for future study.

New scientific findings

This study represents a pioneering study of yeasts in spontaneous pineapple juice fermentations and the potential of using yeast isolates from these systems as novel starter cultures to conduct more control fermentation. Some new scientific findings and novel observations were obtained from this research.

The spontaneous fermentation systems of the fruit juices, the yeast species and strain present in these systems were normally diverse. In freshly crushed pineapple juices, the condition 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 two yeasts. In addition, it was found that the occurrence of *H. uvarum* and *P. guilliermondii* in freshly crushed juice does not depend on climate and region. This observation demonstrated that the pineapple wine making could not be achieved by using traditional process (natural fermentation), some inhibitory substance for the wine yeasts such as protease should be eliminated from pineapple before use as base for fermentation.

The novelty of research observations

This research firstly reported the alcoholic characteristic of *S'codes ludwigii* isolates in pineapple wine fermentation. It was found that *S'codes ludwigii* could be used as the main yeasts to produce the alcohol and complete the pineapple wine fermentation same as *S. cerevisiae*. It also had distinguishing properties when used as mixed culture *S'codes ludwigii.* Since it slightly generate alcohol during the initial stage of fermentation, it could extend the survival of other yeasts during the fermentation allowing them to carry out the fermentation and produce the desirable compounds in wine. Therefore, the volatile compounds generated in pineapple wine by the mix starter, having *S'codes ludwigii* as the main culture, could be more complex.

Commercial value of the findings

Saccharomycodes ludwigii (Sl) and *H. uvarum*1 (H1) isolates are obtained from this research. The use of mixed culture of these as starter culture for pineapple wine fermentation has resulted in delicate pineapple wine with high ethanol content and desirable volatile compounds. If this produced pineapple wine has acceptable sensory quality to consumers, this combination of yeasts as starter culture could be developed as a commercial concept and product. This concept of starter could also be used in the production of other types of fruit wine (this finding could convince wine manufacturer to develop quality pineapple wine and expand this products in to the larger market).

Additional Interesting finding

During the course of this investigation, lactic acid bacteria were sometime observed in natural pineapple juice fermentation. These bacteria could enhance wine flavor by utilizing or producing of organic acids and producing other desirable compounds or they could contribute spoilage properties. Therefore, further research is recommended to study ecology and significance of lactic acid bacteria in pineapple juice fermentation.