CHAPTER II

LITERATURE REVIEW

This thesis is concerned with the autochthonous yeasts associated with pineapple fruits and spontaneous pineapple juice fermentation including the application of these yeasts as the alternative strategies for the development of pineapple wine flavors. The molecular methods were applied for yeast identification. Key studies associated with these topics will be presented in the introduction and discussion sections of subsequent experimental chapters. This chapter will provide a brief background to the role of yeasts in wine making followed by a more detailed review of the literature describing the wine flavors, flavors analysis and also fruit wine fermentation. A final section of this chapter will provide some background information of the novel strategies for the development of pineapple wine fermentation.

2.1 Wine fermentation

The variety of wine making process depends on the types of produced wine and the technological innovations applied in that region or country where was the origin of grapes. However, there are some common principles and operations, which are outlined as background to understand the role of yeasts in wine production (Fleet, 1998; 2001). The main steps in red and white wines production are showed in Figure 2.1.

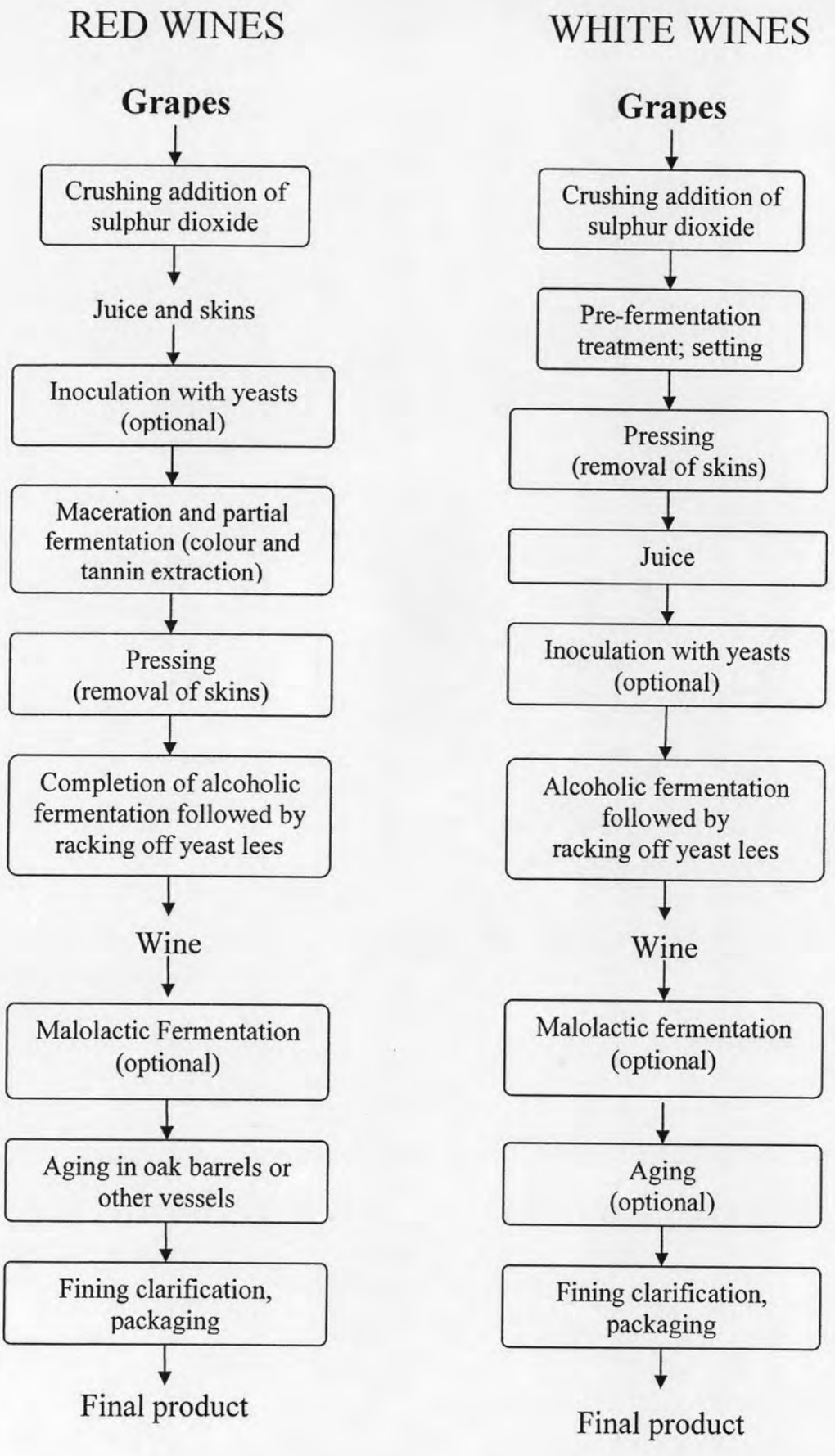


Figure 2.1 Processes diagram of red and white wine production (Fleet, 2001)

2.1.1 Wine grapes

The maturity stage of grapes determines the chemical composition of the juice extracted; sugar and acids in particular. Sugars and acids are the major elements for juice and are important factors of its fermentation properties (Fleet, 1998; 2001). The main compositions of grape juice are illustrated in Table 2.1.

Table 2.1 Major compositions of grape juice (Fleet, 2001)

Substances	Concentration (mg ml^{-1})
Glucose	75-150
Fructose	75-150
Pentose sugars	0.8-2
Pectin	0.1-1
Tartaric acid	2.10
Malic acid	1-8
Citric acid	0.1-0.5
Ammonia	5-150 $\mu g\ ml^{-1}$
Amino acid (Total)	150-2,500 $\mu g\ ml^{-1}$
Protein	10-100 $\mu g\ ml^{-1}$
Vitamin (varies with vitamin)	$\mu g\ ml^{-1}$
Anthocyanins	0.5
Flavonoid, non-flavonoids	0.1-1.0

2.1.2 Crushing and pre-fermentation treatments

In white wines production, the grapes are destemmed and crushed, and the juice is drained away from the skins. Fruit juices can be clarified using many ways, such as cold settling, filtration, centrifugation, or combinations of these

methods. Cold settling of the juice is normally processed approximately at 5 to 10°C for 24 to 48 hr. Pectolytic enzymes are added to degrade grape material during clarification. The juice is then transferred to the fermentation tank, where the fermentation begins naturally or the fermentation may be initiated by inoculation with starter culture (Ewart, 1995; Fleet, 1998; 2001).

For production of red wines, red grapes are destemmed and crushed, and then the juice and skins (must) are directly transferred to the fermentation tank. Fermentation can begin either naturally or by inoculation. During the first few days, the skins rise to the top of juice to form a cap. In this early stage, often described as maceration, juice is regularly pumped over the cap. The purpose of this step is to extract purple and red anthocyanin pigments, as well as other phenolic substances, from grape skins to give colour, tannic, and astringent character to the wine. The extraction process is assisted by the production of ethanol during this preliminary fermentation. When sufficient extraction has been achieved, the partially fermented wine is drained and pressed from the skins to another tank for completion of fermentation (Boulton, 1995; Boulton et al. 1997).

2.1.3 Alchoholic fermentation

White wines are generally fermented at 10 to 18°C for 7 to 14 days or more, where the lower temperature and slower fermentation rate favor the retention of desirable volatile flavor compounds. Red wines are fermented for about 7 days at 20 to 30°C, where the higher temperature is necessary to extract color from the grape skins. When the fermentable sugars, glucose and fructose, of the juice are completely utilized, the wine is then drained or pumped (racked) from the sediment of yeast and

grape material (lees). Then, it is transferred to stainless steel tank or wooden barrels for malolactic fermentation, if desired, and aging. Clarification by filtration or centrifugation may be done during this stage. Leaving the wine in contact with the lees for long periods is not encouraged because the yeast cells autolyze, with the potential of adversely affecting wine flavor and providing nutrients for the subsequent growth of spoilage bacteria (Fleet, 1998; 2001).

2.1.4 Malolactic fermentation

Malolactic fermentation is the bioconversion of L-malic acid in wine to L-lactic and carbon dioxide. The malolactic fermentation commences naturally by lactic acid bacteria. It takes about 2 to 3 weeks after completion of the alcoholic fermentation, and lasts for 2 to 4 weeks. However, nowadays, this reaction is widely encouraged using the inoculation of commercial cultures of *Oenococcus oeni* (*Leuconostoc oenos*) (Dicks, Dellaglio and Collins, 1995). The main reaction is decarboxylation of L-malic acid to L-lactic acid, giving a decrease in acidity of the wine and increases in its pH by about 0.3 to 0.5 units. A decrease in acidity by malolactic fermentation gives a softer, mellower taste to wine. Also, growth of malolactic bacteria in wine contributes additional metabolites that may confer complex and interesting flavor characteristics (Davis, Lee and Fleet, 1986; Henick-Kling, 1995; Fleet, 1998; 2001).

2.1.5 Post fermentation process

Most wines are not stored for lengthy periods after completion of the alcoholic fermentation or malolactic fermentation. If storage is necessary, it is

generally done in stainless steel tanks. Some white wines (e.g. Chardonnay) may be aged in wooden barrels. Most red wines are aged for periods of 1 to 2 years by storage in wooden (generally oak) barrels. The chemical reactions that contribute to flavor development occur between wine constituents and components extracted from the wood of the barrels during aging, (Rankine, 1989; Boulton, 1995). Critical points for control during storage and aging are exclusion of oxygen and addition of sulfur dioxide to free levels of 20 to 25 $\mu g\ ml^{-1}$. These controls are important to prevent the growth of spoilage bacteria and yeasts and to prevent unwanted oxidation reactions.

Before bottling, wine may be cold stored at 5 to 10°C to precipitate excess tartarate and then clarified by application of one or more processes, such as the addition of fining agents (bentonite, albumen, isinglass, gelatin), centrifugation, pad filtration, and membrane filtration. For some white wine with residual sugar, potassium sorbate up to 100 to 200 $\mu g\ ml^{-1}$ may be added to control yeast growth (Rankine, 1989; Ewart, 1995).

2.2 Fruit wine

Wines could be produced from the other types of fruit that are usually called fruit wine. Several fruits can be used to produce wine since they contain several nutrients for yeast growth and fermentation. The fruit wines making process is similar to the grape wine. However, the specific art or technology used to improve fruit wine quality is still at the initial stage of the development. There are some reports about fruit wine fermentation. Soufleros et al. (2001) studied the chemical constituents and volatile compounds of kiwi wine. The results indicated that kiwi could be a potential fruits used for wine fermentation. The dominant organic acids of kiwifruit wine were

citric, galacturonic, lactic and malic acid. The alcohol content of wines produced was in range 6.1-11.4% (v/v). The main volatile compounds found in kiwifruit wine were methanol, methyl-2-propan-1-ol, methyl-2-butan-1-ol, methyl-3-butan-1-ol, acetoin, ethyl acetate and butan-2,3-diol. Mingorance-Cazorla et al. (2003) applied 12 natural yeasts isolated from oranges to orange juice fermentation in comparison to grape must fermentation. Then, the fermented orange and grape juices were evaluated for their aroma formation. The results showed that *P. fermentans* CECT 11773, *R. mucilaginosa* OJ2 and *H. uvarum* CECT 10885 produced a good beverage with low alcoholic level from orange juice. *P. fermentans* CECT 11773 increased the presence of higher alcohols and ethyl acetate in both orange and grape musts. Deeraksa et al. (2005) used natural yeast isolates obtained from fermented mangosteen paste to make mangosteen wine compared to *S. bayanus*. The results demonstrated that the natural isolates provided significantly higher acceptance of sensory test than the pure culture of *S. bayanus* in mangosteen wine.

There were some researchers developed a novel fermented beverage similar to wine from cashew apple. Cashew apple juice is a very good raw material for alcoholic fermentation because of its rich in sugar and mineral contents. Garrutti et al. (2006) developed the alcoholic beverage from cashew apple and determined the significant volatile compounds and their role in the product aroma. This finding revealed that several ethyl esters, as well as some non-detected compounds were important to the sweet, fruity and cashew-like aroma of this alcoholic beverage.

2.3 Pineapple wine

2.3.1 Pineapple fruit

Pineapple is a significant economic farm-plant widely grown in every parts of Thailand. Thailand is also one of the largest exporter of pineapple fruits and pineapple products of the world. There are several varieties of pineapple grown in Thailand. Among these species, Smooth cayenne is the most popular because it can be used for fresh fruit and processing. Mostly, it is grown in the western and eastern parts of Thailand because of the appropriate geographic and climate condition. The period from the end of flowering to fully ripe pineapple fruit was approximately four months (Singelton, 1965). During fruit development, there were the progressively accumulation of the sugars, vitamins and minerals in fruitlets (eyes) along with the larger of fruit size. It ripens progressively, turning yellow from the base to the top which is reflected in a strong internal maturity gradient. The pineapple flesh is pale yellow, soft and juicy with considerable variation in sugar and acidity (Bartholomew, Paull and Rohrbach, 2003).

The general composition of ripe pineapple fruit flesh is demonstrated in Table 2.2. The range of chemical constituents of ripe pineapple fruit depends on the stage of fruit ripeness, and on agronomic and environmental conditions (Gortner, Dull and Krauss, 1967; Bartholomew, Paull and Rohrbach, 2003). The major carbohydrate constituents in pineapple fruit are sucrose, glucose and fructose. There is no starch accumulation in pineapple fruit. The major acids in ripe pineapple fruits are citric and malic acid (Singleton and Gortner, 1965; Gortner, Dull and Krauss, 1967; Dull, 1971; Camara et

Table 2.2 The general composition of ripe pineapple flesh (Akamine, 1976)

Constituients	Quantity (% fresh weight basis)
Brix	10.8-17.5
Sucrose	5.9-12.0
Glucose	1.0-3.2
Fructose	0.6-2.3
Cellulose	0.43-0.54
Pectin	0.06-0.16
Titratable acid (as citric acid)	0.6-1.62
Citric acid	0.32-1.22
Malic acid	0.1-0.47
Oxalic acid	0.005
Ash	0.30-0.42
Water	81.2-86.2
Fiber	0.30-0.61
Nitrogen	0.045-0.115
Ether extract	0.2
Pigments (ppm of carotene)	0.2-2.5
Carotene (mg)	0.13-0.29
Xanthophyll (mg)	0.03
Esters (ppm)	0.2-2.5
Vitamin (μg/100g) fresh weight	
Aminobenzoic acid	17-22
Folic acid	2.5-4.8
Niacin	200-280
Pantothenic acid	75-163
Thiamine	69-125
Riboflavin	20-88
Vitamin B6	10-140
Vitamin A	0.02-0.04
Ascorbic acid	10-25

al., 1994; Bartolome, Rupbrez and Carmen, 1995; Bartholomew, Paull and Rohrbach, 2003). In addition, the ripened fruit contains higher levels of glycine, alanine, methionine and leucine, whereas lysine, proline, histidine and arginine are present at relatively low levels (Gortner, Dull and Krauss, 1967). In addition, Elss et al. (2005) reported that several volatile compounds were identified in fresh pineapple juice from several countries. The major volatile constituents of fresh pineapple juice were indicated in Table 2.3.

2.3.2 Pineapple wine making

Pineapple fruit could be a good raw material for wine production because its juice has unique flavor, sufficient sugars, acids, nitrogen source, vitamins and minerals which could support the growth of yeasts during fermentation without adding yeasts nutrients which is similar to the nature of grape juice. The traditional pineapple wine fermentation in Thailand was demonstrated in Figure 2.2. These processes are similar to general wine fermentation. However, there is water adding into the pineapple juice in preparation of pineapple must (Chanrittisen, 2001; Kuruwanna, 2003). Probably, this process could be explained for the low flavor quality of the pineapple wine.

Table 2.3 Major volatile compounds of fresh pineapple juice (Elss et al., 2005)

Volatile compounds	Range ($\mu g\ l^{-1}$)	Mean ($\mu g\ l^{-1}$)	Olfactory description
methyl 2-methylbutanoate	160-6000	1500	Apple-like[a]
methyl 3-(methylthio)-propanoate	40-7100	1500	-
methyl butanoate	10-1800	490	Fruity[a,b]
methyl hexanoate	15-3800	1300	Fruity[a,b]
ethyl hexanoate	0-3500	500	Fruity[a,b]
Ethyl 3-(methylthio)-propanoate	15-2700	470	-
2.5-dimethyl-4-methoxy-3(2H)-furanone (mesifurane)	20-9200	1500	-
2.5-dimethyl-4-hydroxy-3(2H)-furanone (furaneol)	5-4700	960	-

a = Rychlik, Schieberle and Grosch (1998); b = Clarke and Bakker (2004)

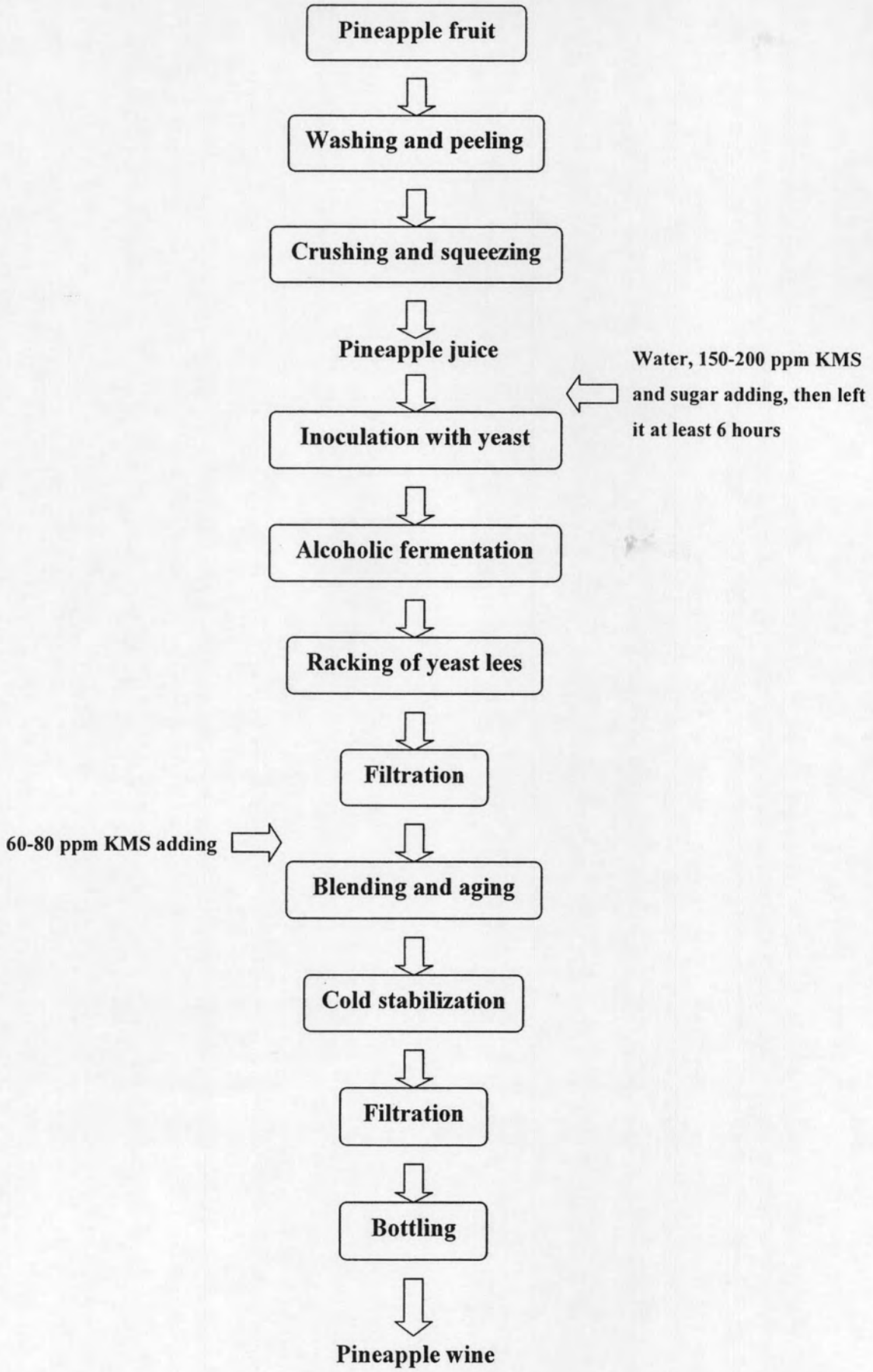


Figure 2.2 Traditional pineapple wine fermentation processes (Kuruwanna, 2003)

Some researchers studied the fermentation of pineapple wine. Ezeronye (2004) studied the batch fermentation of tropical fruits: Pineapple (*Anana comosus* Merr.), Mango (*Mangifera indica* L.), Cashew (*Anacarduim occidentals*) and Papaw (*Carica papaya* L.) using *S. cerevisiae* OW-11 previously isolated from palm wine. The results indicated pineapple have a high appeal in tropical wine making and have been used for successful production of wines. Ayogu (1999) also evaluated the performance of *S. cerevisiae* isolated from fermenting sap of palm wine (*Elaesis guineansis*) for wine making from pineapple fruits. This study demonstrated that Nigerian palm wine of the *E. guineansis* type could be an excellent alternative source of *S. cerevisiae* for commercial wine making from fruits, especially pineapple fruits.

In Thailand, most of the previous researchers studied the fermentation of pineapple juice for ethanol production (Wongwantanee, 1996) or vinegar fermentation from pineapple wine (Prasopwatana, 1988; Srisajjalertwaja; 1991) because pineapple is available throughout the year and not seasonal like other agricultural products. Some of previous literatures about the fermentation of pineapple wine were reported. Ruengrongpanya (1996) compared seven *S. cerevisiae* yeast strains in pineapple wine fermentation for 14 days. There were Berries industry wine (BIW), Industrial production of champagne (ChI), French wine (FW), Industrial alcohol fermentation (Ialc), Molasses alcohol fermentation (MAF), White wine (WW) and *S. cerevisiae* PRA (PRA). This study showed that pineapple juice could be a potential substrate for wine production. The highest ethanol content of pineapple wine fermented by FM was 13.42% (v/v). However, the pineapple wine fermented by ChI, BIW and MAF were accepted with the highest scores of taste and fruity flavors in sensory evaluation. Callens and De Smet (1991) developed the fermentation techniques for pineapple

liqueur wine production. The results showed that the stopping of fermentation at alcohol content around 13-14% (v/v) by means of adding ethanol to the wine to reach a level of 18% (v/v) alcohol could serve stabilization of wine from microbial spoilage. Consequently, the use of low dose of chemical preservatives and low temperature storage of wine after processing was omissible. Chuaychusri et al. (2005) studied the fermentation of pineapple juice and other local fruits in the upper-northern part of Thailand by Swedish yeast in a twenty liters polyethylene tank for 1 month. This study demonstrated that pineapple juice could be an alternative source for wine making.

2.4 Yeasts and wine fermentation

Microorganisms are fundamental to the wine making process. To understand their contribution, it is necessary to know (i) the taxonomic identity of each species associated with the process, (ii) the kinetics of growth and survival of each species throughout the process, (iii) the biochemical activities of these species and how such activities determine the physiochemical properties of the wine, (iv) the influence of wine making practices upon microbial growth and activity, and (v) the ultimate impact of microbial action upon sensory quality and consumer acceptability of the wine (Fleet, 1999).

It has been known that yeasts play a key role in wine production. Primarily, they conduct the alcoholic fermentation, but they also cause spoilage of wine fermentation. Generally, yeasts impact on wine constituents and flavor by: (i) affecting grape quality before harvest; (ii) conducting the alcoholic fermentation of grape juice into wine; (iii) biocatalysing the transformation of flavor neutral, grape

components into flavor active components; (iv) impacting on wine flavor and other properties through their autolysis; (v) bioadsorbtion of components of grape juice; (vi) causing spoilage during bulk storage of wine in the cellar and after packaging; and (viii) by influencing the growth of malolactic and spoilage bacteria (Henschke, 1997; Fleet, 2003a).

These influences depend upon the species and strains of yeasts which grow throughout the process. Hence, the individual characteristic of wine and quality depends very much on the yeast ecology of grape juice fermentation. Thus, it is very important to understand this ecology and, in particular, how these yeasts access the winery environment. The role of yeasts in wine production has been studied and reviewed in the literatures (Kunkee and Amerine, 1977; Benda, 1981; Kunkee and Bisson, 1993; Du-Toit and Pretorius, 2000; Pretorius, 2000; Fleet, 2003a)

Yeasts occur as natural flora on the surfaces of grapes. Consequently, grapes are a primary source of yeasts associated with wine fermentation. In the process of fermentation, yeasts species and strains within the genera *Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia*, and sometimes *Kluyveromyces* grow during the early stages of fermentation but eventually die off due to toxicity of the increasing concentration of ethanol, leaving *S. cerevisiae* or *S. bayanus* as the dominant species to complete the fermentation (Benda, 1981; Lafon-Lafourcade and Ribéreau-Gayon, 1984; Fleet, 1990). *S. cerevisiae* and *S. bayanus* have become universally accepted as the principal wine yeasts. Particular strains of these yeasts have been commercialized and are now sold to winemakers as active dry cultures for inoculation into freshly crushed juice. It is assumed that the inoculated *S. cerevisiae* and *S. bayanus* will overwhelm and diminish the growth of the indigenous flora, and dominate the fermentation.

However, even in these inoculated ferments, indigenous flora will always be present and will make variable contributions to the process, depending on the competitive success of the inoculated *S. cerevisiae* or *S. bayanus*. Even though indigenous non-*Saccharomyces* yeasts die off in the early stage of the fermentation, they will have previously grown to significant populations and put their imprint on wine character (Heard and Fleet, 1985). Also, there will be contributions from indigenous strains of *S. cerevisiae* and *S. bayanus* (Fleet, 2001).

Ecological studies of wine fermentations conducted in different regions around the world have revealed the vast biodiversity of yeast species and strains associated with this process. Whereas winemakers once saw this biodiversity in (largely) a negative context, they now have a clearer understanding of its significance and seek innovation in using this knowledge to enhance wine value. Such innovation includes more strategically managed indigenous fermentations and the use of novel species and strains in controlled inoculated fermentations. The link to such innovations, as well as better understanding and management of existing technology, is the yeast ecology of grapes. As indicated already, grapes are a primary source of yeasts for wine fermentation, but little quantitative detail is known about the populations and yeast species principally associated with grapes. It is noted that some of the finest wines produced in Europe originate from indigenous fermentations (Fleet, 1990; 1998; 2001; Fleet and Heard, 1993; Lambrechts and Pretorius, 2000).

2.5 Autochthonous yeasts

Autochthonous or indigenous yeasts are yeasts naturally resident on grapes or cellar environment which lead to the wine fermentation. In the following 100 years

ecological studies were mainly directed towards the evaluation of these yeasts. Several researchers investigated on the density and biodiversity of the yeast flora associated with vineyards and wineries. Yeast flora presenting on the grape surfaces were studied by different approaches, such as scanning electro microscopy, direct isolation and enumeration which revealed that the density of wild yeasts on grape berries is limited to 4-5 log cfu ml^{-1}. In addition, it was showed that cell density on grape varies depending upon grape variety, maturation stage, climate, geographic location and viticultural practices applied to the wineyard. (Mannazzu, Clementi and Ciani, 2002)

Mostly, the autochthonous yeasts of mature grapes are the low alcoholic tolerant apiculate yeasts belonging to the genera *Kloeckera* and *Hanseniaspora* which account for 50-75% of the total yeast population. Other yeasts were occasionally found are *Candida*, *Brettanomyces*, *Cryptococcus*, *Kluyveromyces*, *Metschnikowia*, *Pichia* and *Rhodotorula* (Fleet, 2001; Fleet et al., 2002; Mannazzu, Clementi and Ciani, 2002; Fleet, 2003a). However, yeasts belonging to the genera *Saccharomyces* were rarely present in this ecological system. The low population of *Saccharomyces* on grapes might be that they do not associate with the vineyard. Martini (1993) reported that the vineyard is not the primary source of this yeast and suggested that the origin of *Saccharomyces* is strictly associated with wineries and fermentation plants.

Apart from grape berries, the autochthonous yeasts associated with other fruits and agricultural products were studied by some researchers as indicated in Table 2.4.

2.6 Wine flavors

The quality of wine is determined by appearance (color, clarity, viscosity, spritz and tears) and flavor (odor and taste) (Jackson, 2002). However, when assessing the sensory properties of wine, the word "tasting" is used to indicate that the flavor of the wine is being judged (Clarke and Bakker, 2004).

Table 2.4 The autochthonous yeasts associated with fruits and other agricultural products

Sources of yeasts	Autochthonous yeasts
Grape berries (Fleet, 2001; Fleet et al., 2002; Fleet, 2003a; Mannazzu, Clementi and Ciani, 2002)	*Kloeckera*, *Hanseniaspora*, *Candida*, *Brettanomyces*, *Cryptococcus*, *Kluyveromyces*, *Metschnikowia*, *Pichia* *Rhodotorula*
Cider apple (Morrissey et al., 2004; Coton et al., 2006)	*Hanseniaspora, Candida, Brettanomyces, Saccharomyces*, *Dekkera*, *Kluyveromyces*, *Lachancea*, *Metschnikowia*
Orange (Okunowo, Okotore and Osuntoki, 2005)	*Hanseniaspora*, *Pichia*, *Candida*, *Saccharomyces* *Rhodotorula, Metschnikowia*
Palm juice (Thammarat, 1978; Ezeronye and Okerentugba, 2000)	*Saccharomyces*, *Candida*, *Kluyveromyces*, *Schizosaccharomyces*
Olive (Coton et al., 2006)	*Candida, Pichia*, *Debaryomyces*, *Saccharomycopsis*, *Zygoascus*, *Citeromyces*
Coconut palm sap (Atputharajah, Widanapathiranat and Samarajeewa, 1986)	*Candida*, *Pichia*, *Saccharomyces*, *Kloeckera*, *Rhodotorula*, *Schizosaccharomyces*, *Sporobolomyces*, *Torulopsis*

Wine flavor are composed of varietal, fermentation and aging flavors. However, extensive studies by various research teams have shown that the flavor

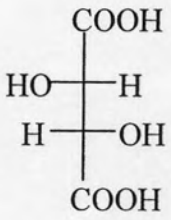
components are substantially responsible for the bouquet and the individual characteristics of a wine and most of these compounds are products of yeast metabolism (Clarke and Bakker, 2004). Yeasts metabolize grape juice constituents, principally the sugars, to a wide range of volatile and non-volatile end products. More than 400 volatile components are produced during yeast fermentation. The amounts of these components are considerably fluctuated due to the influence of various factors, such as the cultivar, soil, grape condition and ripeness, fermentation temperature, and in particular, due to the species of yeasts that grow during the fermentation (Fleet, 2002). According to various researches, the greatest differences in the nature and concentrations of these by-products are determined by the yeast species which can exert a marked effect on the production of secondary metabolizes of fermentation. The differences of comparison in wine made from different yeast species appear to be usually identical, but the relative amounts of the various compounds differ (Romano, 2002). The wine flavor compounds have been the subject of extensive studies which are well reviewed in the literatures (Schreier, 1979; Ribéreau-Gayon et al., 2000; Clarke and Bakker, 2004).

2.6.1 Wine flavors originated from grapes and pre-fermentation

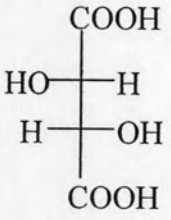
The grapes contribute trace amount of many volatile components that give the wine its distinctive varietal and fruity characters. In addition, they contribute nonvolatile acid which affect flavor and tannins that give bitterness and astringency (Fleet, 2001). The quantitatively dominating acids of grapes are malic, tartaric and citric acids, the first two of which account for over 90% of the total acid content of

grapes. Apart from these compounds, further nonvolatile carboxylic acids have been detected in grape. Figure 2.3 shows the main organic acids in grapes.

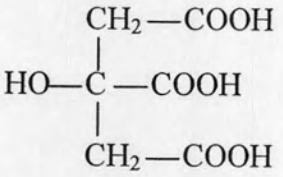
About forty terpene compounds have been identified in grapes. Some of the monoterpene alcohols are among the most odoriferous, especially linalol, α-terpineol, nerol, geraniol, citronellol and ho-trienol, which has a floral aroma reminiscent of rose essence as demonstrated in Figure 2.4. The olfactory perception threshold of these compounds are rather low, as little as a few hundreds micrograms per lit (Table 2.5). The most odiferous terpenes are citronellol and linalol. In addition, other alcohols, hydrocarbons, aldehydes, ketones, and volatile miscellaneous compounds have been identified in grapes which are also reviewed in literature (Schreier, 1979).



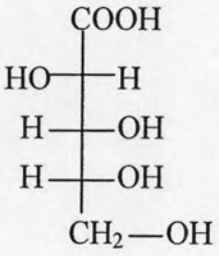
L(+)-Tartaric acid



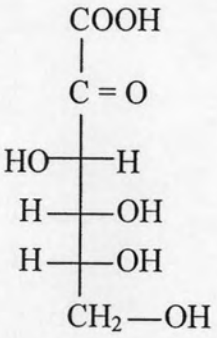
L(–)-Malic acid



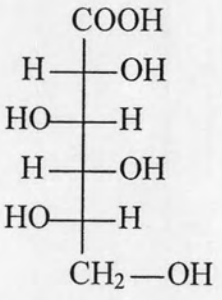
Citric acid



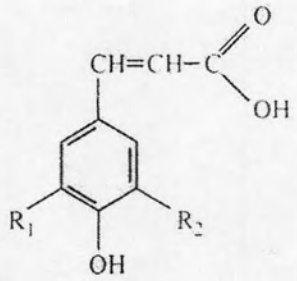
D-Gluconic acid



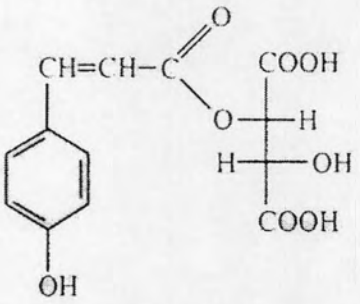
2-keto D-Gluconic acid



Mucic acid



Coumaric acid
($R_1 = R_2 = H$)
Caffeic acid
($R_1 = OH$; $R_2 = H$)



Coumaryl tartaric acid

Figure 2.3 The main organic acids in grapes (Ribéreau-Gayon et al., 2000)

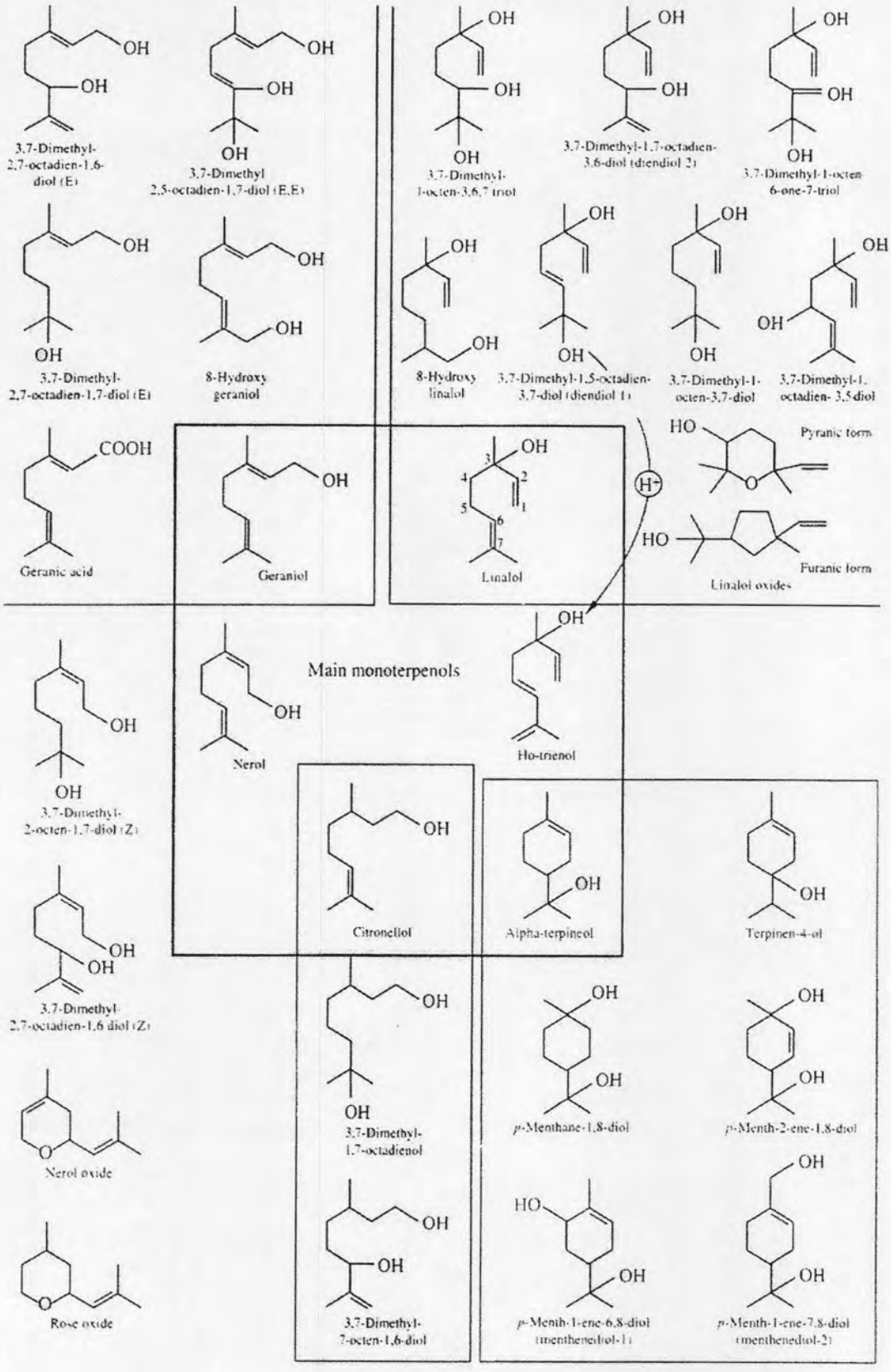


Figure 2.4 The main monoterpenes and derivatives identified in grape and wine (Ribéreau-Gayon et al., 2000)

Table 2.5 The main monoterpenols in grapes (Ribéreau-Gayon et al., 2000, modified)

Monoterpenols	Olfactory perception threshold ($\mu g\ l^{-1}$)	Content ($\mu g\ l^{-1}$) in wines							Olfactory dercription
		MA	MF	G	A	R	M	SB	
Linalol	50	455	473	6	80	40	50	17	Rose/ floral[a,b]
α-Terpineol	400	78	87	3	37	25	12	9	Lily of the valley/ peach like[a]
Citronellol	18	ND	ND	12	ND	4	3	2	Citronella/ rose[a]
Nerol	400	94	135	43	97	23	4	5	Rose/ floral[a]
Geraniol	130	506	327	218	58	35	16	5	Rose/ floral[a,b]
Ho-trienol	110	ND	ND	ND	127	25	ND	ND	Linden

a = Rychlik, Schieberle and Grosch (1998); b = Clarke and Bakker (2004)
MA = Muscat of Alexandria, MF = Muscat de Frontignan, G = Gewürztraminer
A = Albariño, R = Riesling, M = Muscadelle, SB = Sauvignon Blanc
ND = not detected

2.6.2 Wine flavors originated from fermentation

The alcoholic fermentation increase the chemical and flavor complexity by extraction of compounds from the grapes, modifying some grape derived substances, and producing a vast array of volatile and nonvolatile metabolic end products (Fleet, 2001). These secondary compounds formation by yeast during fermentation were showed in Figure 2.5.

Organic acids make major contributions to the composition, stability and organoleptic qualities of wines. These organic acid compounds are formed or modified due to yeast fermentation. Generally, organic acids in wine are divided into two categories, volatile and fixed acids. Volatile acidity refers to acids that can be readily removed by steam distillation, whereas fixed acidity describes those that are

poorly volatile. Total acidity in wine is the combination of both categories which are expressed in term of tartaric acid. The major organic acids produced during fermentation were shown in Figure 2.6.

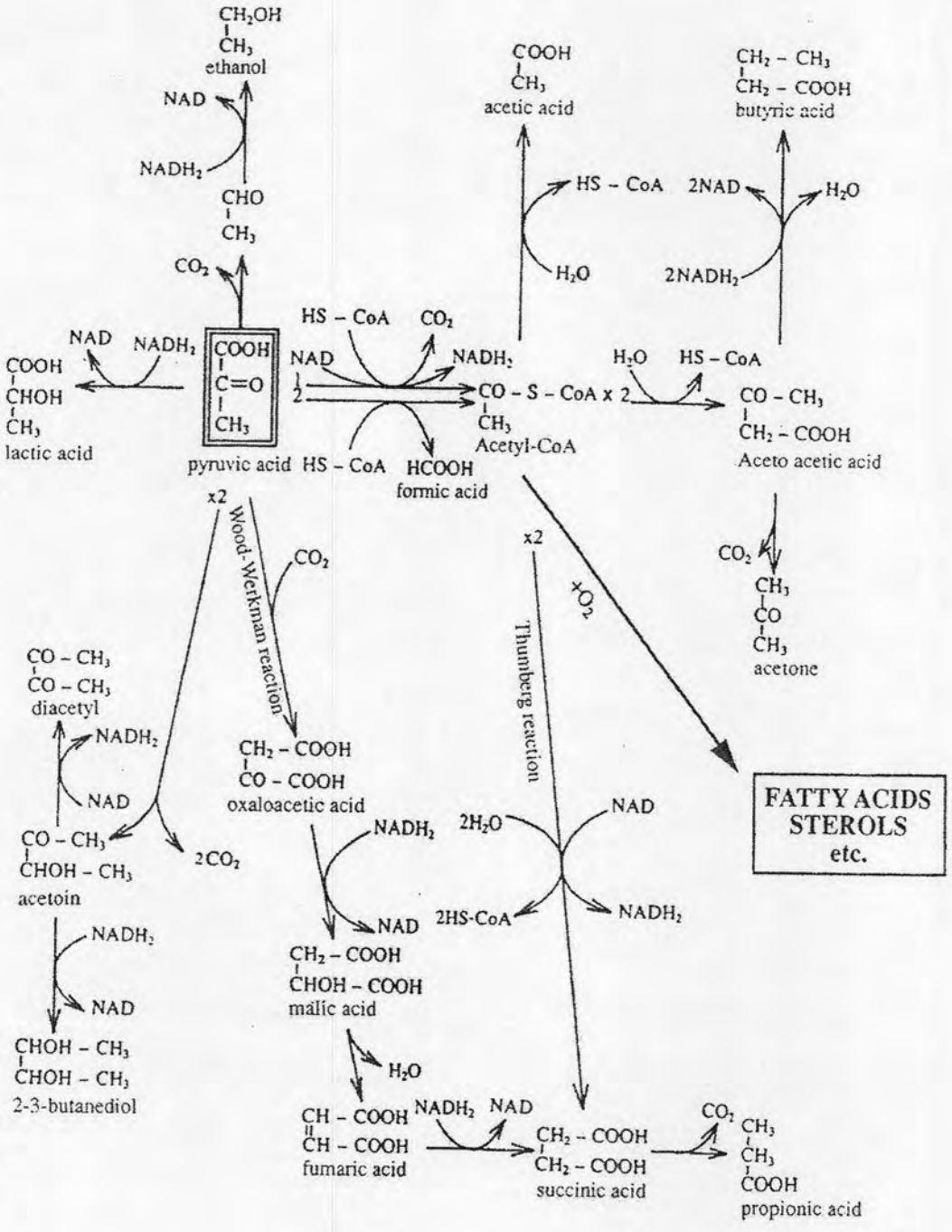
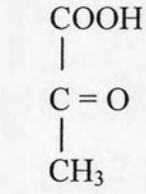
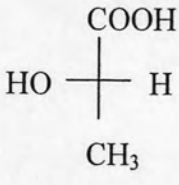


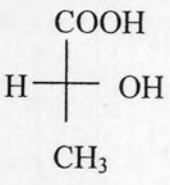
Figure 2.5 Secondary compounds formation by yeast during fermentation (Delfini and Formica, 2001)



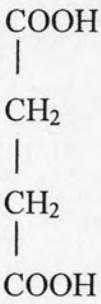
Pyruvic acid



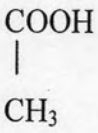
L(+)-Lactic acid



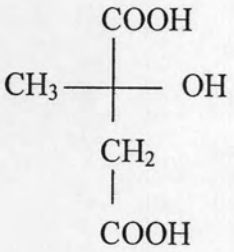
D(–)-Lactic acid



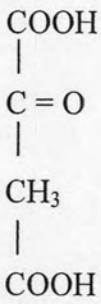
Succinic acid



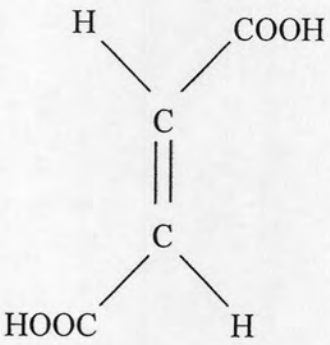
Acetic acid



Citramalic acid



Oxaloacetic acid



Fumaric acid

Figure 2.6 The major organic acids produced during fermentation (Ribéreau-Gayon et al., 2000)

2.6.2.1 Succinic acid .

Of the numerous organic acids produced in wine by yeasts, succinic acid is a common by-product of alcoholic fermentation (Radler, 1993). It is

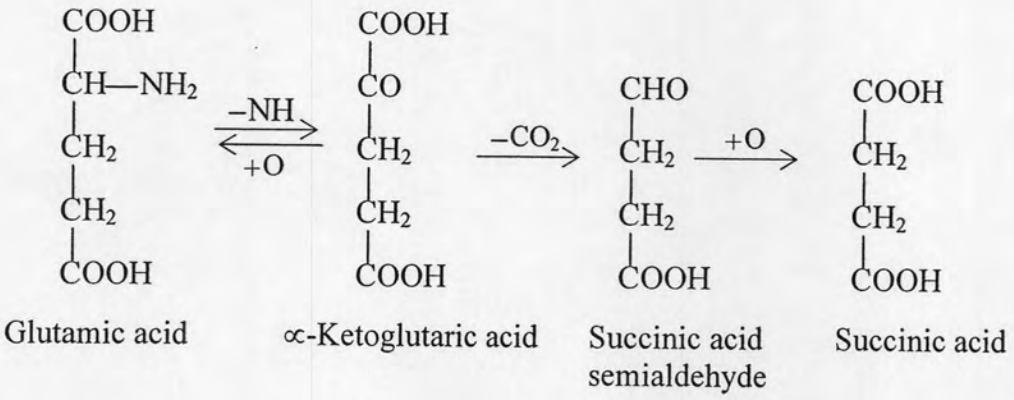
resistant to microbial attack under anaerobic conditions and is particularly stable in wine (Jackson, 2000). Succinic acid has a bitter salty taste and is produced by *S. cerevisiae* at concentrations up to 2.0 mg ml^{-1}., depending on the strain. Lower concentrations are produced by non-*Saccharomyces* species (Shimazu and Watanabe, 1981). The production of this acid is not associated with any major defects in wine quality (Fleet, 2001). Succinic acid is formed as a by-product of alcoholic fermentation of grape must. There are three views as to its formation; (i) double oxidation process of glutamic acid, (ii) degradation of sugars and (iii) Wood-Werkman reaction. These reactions were demonstrated in Figure 2.7

2.6.2.2 Acetic acid

Acetic acid is a main volatile acid which is formed by yeast during fermentation. At normal levels in wine (<300 mg l^{-1}), it can be a desirable flavorant, adding to the complexity of taste and odor (Jackson, 2000). Farkaš (1988) reported that the concentration of acetic or volatile acids in wine is 0.3-0.6 g l^{-1}. Acetic acid becomes detrimental to wine quality at concentration exceeding 1.5 mg ml^{-1} which progressively gives the wine a sour taste and taints its fragrance. These high levels of acetic acid are usually associated with contamination of grapes, juice or wine with acetic or lactic acid bacteria. The most strains of *S. cerevisiae* produce only small amounts (<0.75 mg ml^{-1}) of this acid, but some can produce greater than 1.0 mg ml^{-1} and are unsuitable for winemaking. Factors which limit yeast growth such as low temperature, high sugar concentration, low pH, deficiency in available nitrogen and excessive clarification cause increased acetic acid production by *S. cerevisiae* (Delfini and Costa, 1993; Moruno et al., 1993; Shimazu and Watanabe, 1981; Fleet,

2001). Figure 2.8 shows the formation of acetic acid during yeasts fermentation processes and the oxidation of ethanol to acetic acid by acetic acid bacteria.

(i)



(ii)

$$5C_6H_{12}O_6 \rightarrow 5C_3H_5(OH)_3 + 5CH_3—CHO + 5C_2O$$

Hexose — Glycerol — Acetadehyde

$$5CH_3—COH + 2H_2O \rightarrow COOH–CH_2–CH_2–COOH + 3C_2H_5OH$$

Acetadehyde — Succinic acid — Ethanol

(iii)

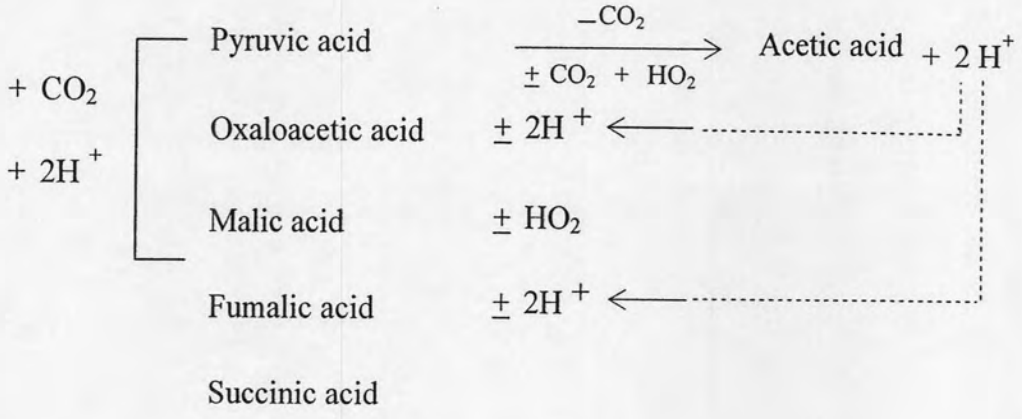
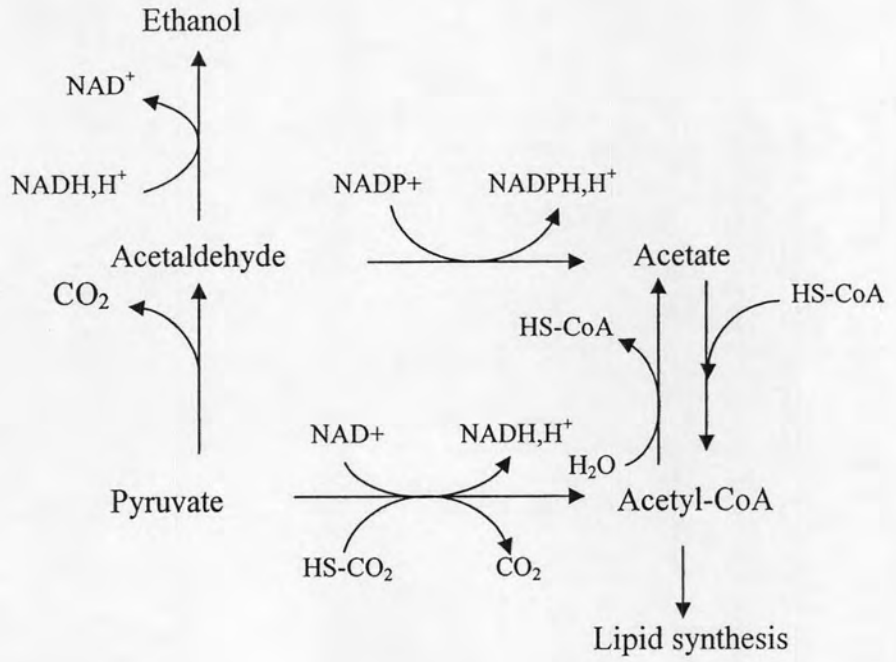


Figure 2.7 Formations of succinic acid (Farkaš, 1988)

(i)



(ii)

CH_3CH_2OH ⟶ CH_3CHO ⟶ CH_3COOH

Ethanol — Acetaldehyde — Acetic acid

Figure 2.8 Acetic acid formation by yeast fermentation (i) and acetic acid bacteria (ii) (Farkaš, 1988; Ribéreau-Gayon et al., 2006)

2.6.2.3 Fumaric acid

Fumaric acid is rarely found during wine fermentation. It was produced in the early stage of fermentation (Whiting, 1976), Panchal (1990) suggested that fumaric acid served the dual purposes of controlling growth of lactic acid bacteria and as an acidulant in wine. The level of fumaric acid for control LAB ranged from 0.7-1.5 g l^{-1}. However, the higher level of fumaric acid could impact to the sensory characteristics of wine.

2.6.2.4 Malic acid

Alcoholic fermentation is the principle pathway degrading malic acid. During alcoholic fermentation, malic acid is partially (5 to 50%) metabolized by *S. cerevisiae* and other wine yeasts. Different strains degrade varying amounts of this acid, and degradation is more significant when the pH is low. Malic acid completely degraded by some species of *Schizosaccharomyces* and some strain of *Z. bailii* (Radler, 1993; Gao and Fleet, 1995). The pyruvic acid resulting from this transformation is decarboxylated into ethanal, which is then converted to ethanol. The malic enzyme is responsible for the transformation of malic acid into pyruvic acid. The decomposition of malic acid by yeast as demonstrated in Figure 2.9.

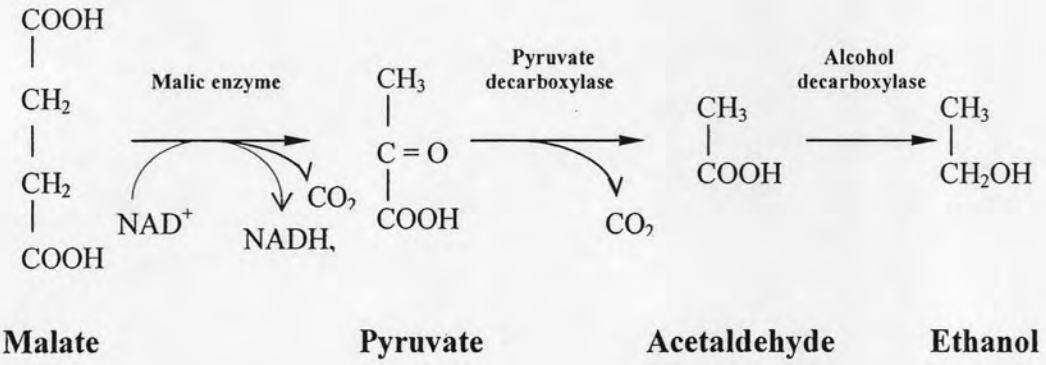


Figure 2.9 The decomposition of malic acid by yeast fermentation (Ribéreau-Gayon et al., 2006)

2.6.2.5 Lactic acid

The production of lactic acid by wine yeast is considered insignificant (<0.1 mg ml^{-1}) (Fleet, 2001). The mode of lactic acid formation can be

differentiated by the optical activity of the product: D(-)-lactic acid is formed by yeasts under anaerobic condition, and L(+)-lactic acid is produced by the action of bacteria (malolactic fermentation). The amount of D(-)-lactic acid depends very much upon yeast species (Schreier, 1979). The decarboxylation of L(-)-malic acid to L(-)-lactic acid is one of the most significant metabolic reactions conducted by lactic acid bacteria in wines. These bacteria possess mechanisms for the transport of malic acid into the cell, efflux of lactic acid, and the malolactic enzyme for decarboxylation (Fleet, 2001). Figure 2.10 demonstrates the malolactic fermentation.

The concentration of citric acid can be completely or partially metabolized during malolactic fermentation, depending on the wine pH and species of lactic acid bacteria. Its degradation is frequently correlated with small increases in the concentrations of acetic acid and diacetyl, the concentrations of fumaric, gluconic and pyruvic acids can decrease during malolactic fermentation (Fleet, 2001).

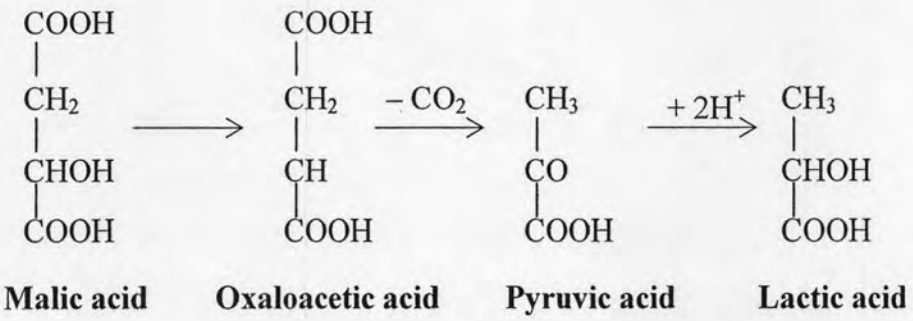


Figure 2.10 Malolactic fermentation (Farkaš, 1988)

2.6.2.6 Tartaric acid

Tartaric acid is a prevalent in grape juice and wine along with malic acid. Normally, tartaric acid is not metabolized by wine yeasts (Radler, 1993). Therefore, the decomposition of tartaric acid is considered the contamination in a wine. Tartaric acid is decomposed by some lactic acid bacteria (Radler and Yanissis, 1972).

The mechanism of degradation of tartaric acid by various lactic acid bacteria species was indicated in Figure 2.11. There are two possible degradation pathways. According to the mechanism (i), three molecules of carbon dioxide, one molecule of acetic acid and one molecule of lactic acid are formed from two molecule of tartaric acid under the effect of *L. plantarum*. Whereas the mechanism (ii), *L. brevis* species convert three molecules of tartaric acid to four molecules of carbon dioxide, two molecules of acetic acid and one molecules of succinic acid (Radler and Yanissis, 1972; Farkaš, 1988).

The volatile flavors compositions are responsible for the bouquet of wine which was decisively influenced during winemaking by yeast fermentation. Besides the quantitatively dominating compounds like ethanol and glycerol, a number of substances, including esters, carbonyls, sulfur-containing compounds, and other metabolites emerge, although some occur only in trace amounts (Schreier, 1979).

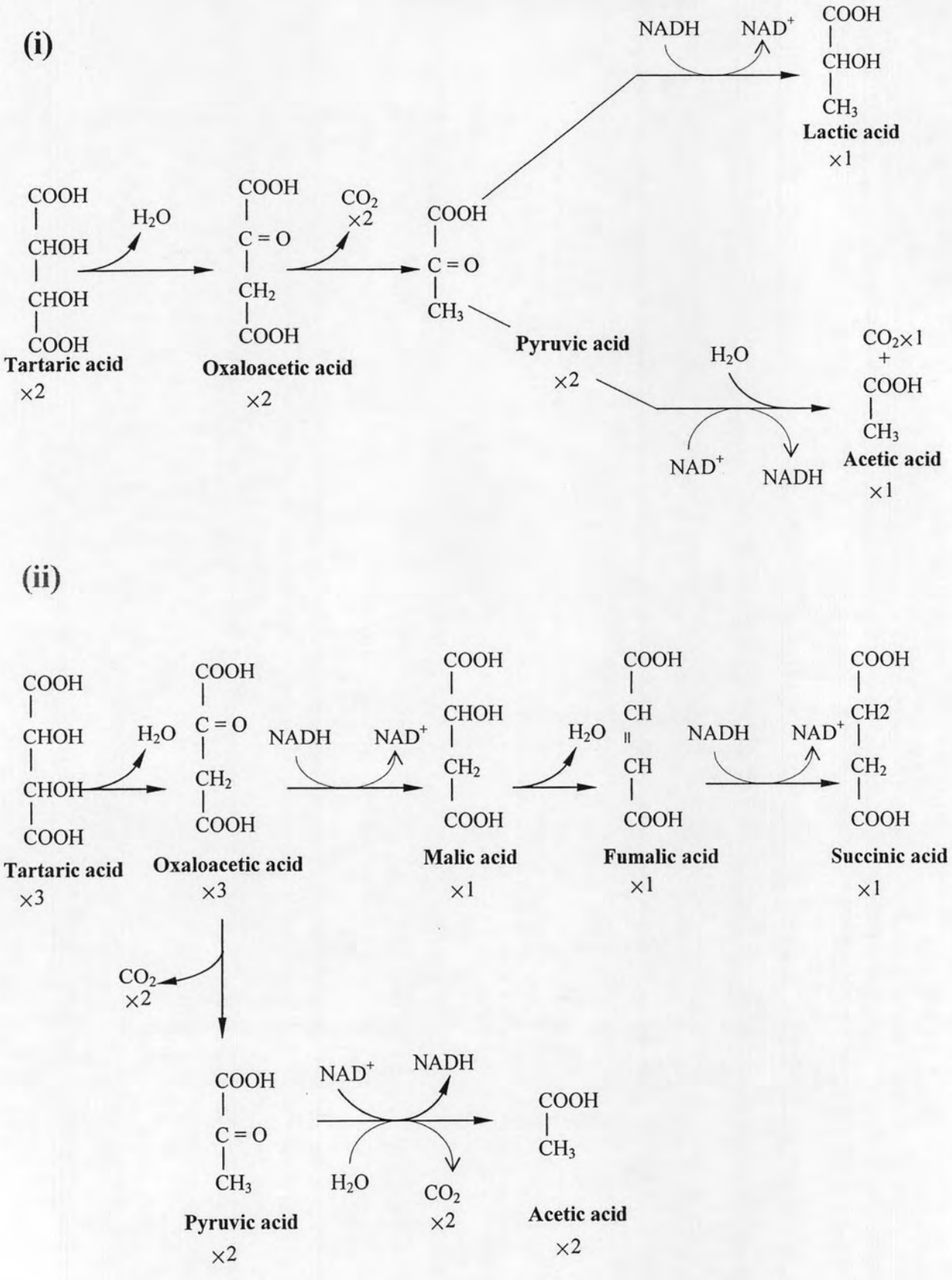


Figure 2.11 Decomposition of tartaric acid by lactic acid bacteria (Farkaš, 1988)

2.6.2.7 Esters

Esters form as condensation products between the carboxyl group of an organic acid and the hydroxyl group of an alcohol or phenol (esterification) (Figure 2.12). Of all functional group of wine, esters are the most frequently encountered. Over 160 specific esters have been identified. The most prevalent ester in wine is ethyl acetate. The olfactory perception threshold of ethyl acetate is approximately 160 mg l^{-1}. It may spoil the bouquet with an unpleasant, pungent tang of a sour-vinegar. However, ethyl acetate contributes the complexity to the fragrance when it is very low doses (50-80 mg l^{-1}) (Ribéreau-Gayon et al., 2000). Ester formation is influenced by many factors. Esterase activity in different yeast strains is an important factor. Jackon (2000) reported that low fermentation temperature (about 10°C) promotes the synthesis of fruity esters, such as isoamyl, isobutyl and hexyl acetates, whereas higher temperatures (15-20°C) promote the production of higher molecular weight esters, such as ethyl octanoate, ethyl decanoate, and phenethyl acetate.

Besides the esterification, the ethyl acetate of fatty acids and acetic esters of higher alcohols are synthesized from the condensation of acetyl coenzyme A originated from pyruvic acid during fermentation (Figure 2.13). These esters have more interesting aromas than the others. The main ethyl acetate of fatty acids are produced during alcoholic fermentation are ethyl hexanoate (caproate) and ethyl octanoate (caprylate) which serve fruity characteristic in wine. The main acetates of higher alcohols are isoamyl acetate (banana aroma) and phenylethyl acetate (rose aroma) (Ribéreau-Gayon et al., 2000).

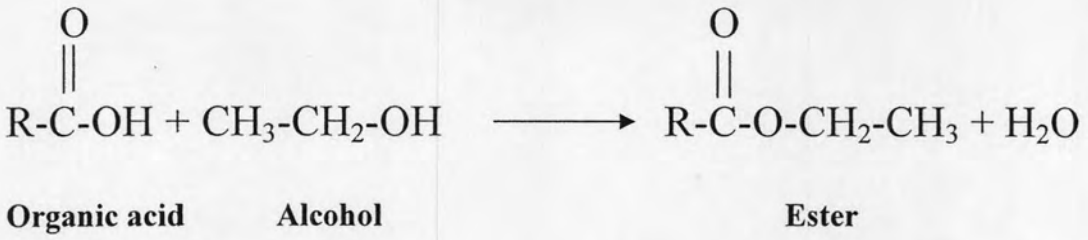


Figure 2.12 Esterification balance of an alcohol (Ribéreau-Gayon et al., 2000)

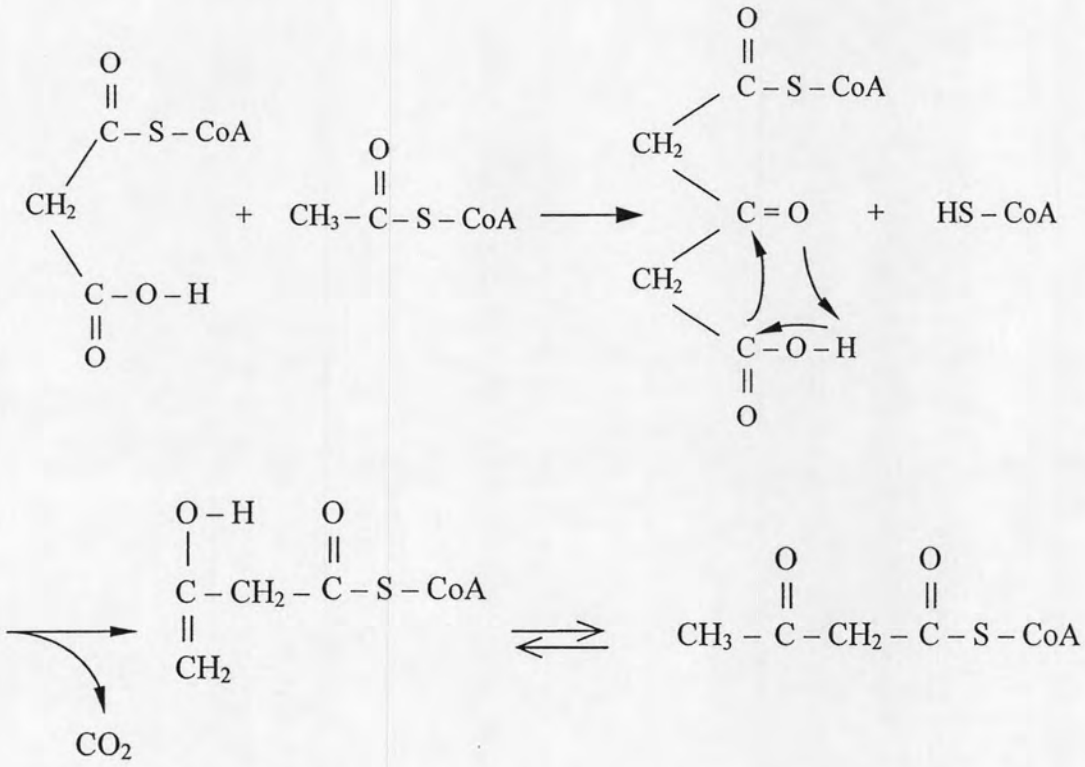


Figure 2.13 Biosynthesis mechanism of fatty acids (Ribéreau-Gayon et al., 2000)

2.6.2.8 Higher Alcohols

During alcoholic fermentation, yeasts can excrete ketonic acids originating from the deamination of amino acids only after their decarboxylation into aldehyde and reduction into alcohol (Figure 2.14) This mechanism known as the

Ehrlich reaction, explains in part the formation of higher alcohols in wine. The principle higher alcohols and their amino acids precursor in wine as indicated in Table 2.6. The higher alcohol production by yeasts appears to be linked not only to the catabolism of amino acids but also to their synthesis via the corresponding ketonic acids. These acids are derived from the metabolism of sugars. Consequently, the most higher alcohol in wine can also be formed by the metabolism of glucose without the involvement of amino acid (Ribéreau-Gayon et al., 2006).

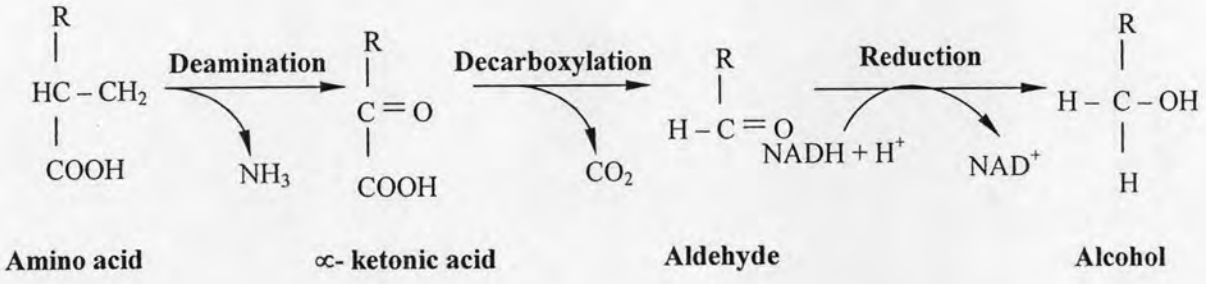


Figure 2.14 Formation of higher alcohol from amino acids (Ehrlich reactions) (Ribéreau-Gayon et al., 2006)

2.6.2.9 Aldehydes and ketones

The major aldehyde produced during alcoholic fermentation by yeasts is acetaldehyde (Schreier, 1979, Jackson, 2000). It often constitutes more than 90% of the aldehyde content of wine. Above threshold values, it usually is considered an off-odor. Combined with other oxidized compounds, it contributes to the fragrance of sherries and other oxidized wines. Acetaldehyde is one of the early metabolic by-products of fermentation. As fermentation approaches completion, acetaldehyde is

Table 2.6 The principle higher alcohols and their amino acids precursor in wine (Ribéreau-Gayon et al., 2006)

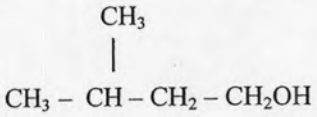
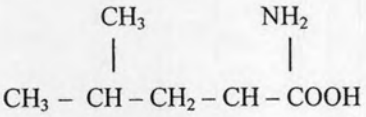
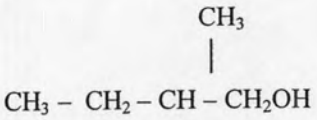
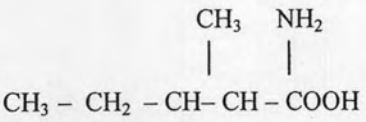
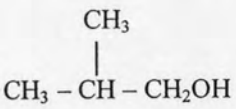
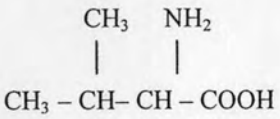
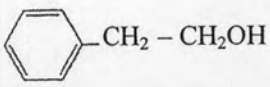
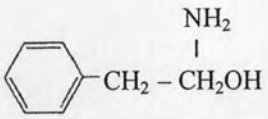
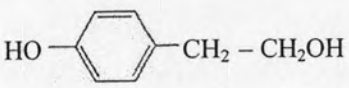
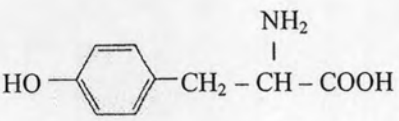
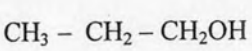
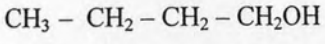
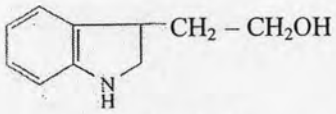
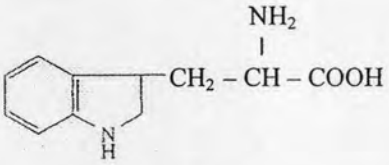
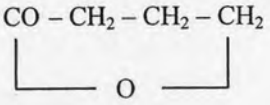
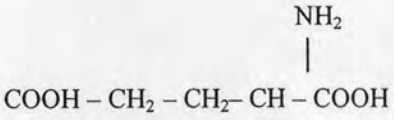
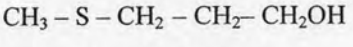
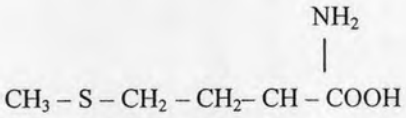
Higher Alcohol	Concentration in Wine (mg/l)	Amino Acid Precursor
 ***3-methyl-butan-1-ol or isoamyl alcohol***	80 – 300	 ***Leucine***
 ***2-methyl-butan-2-ol or active amyl alcohol***	30 – 100	 ***Isoleucine***
 ***2-methyl-propan-1-ol or isobutyl alcohol***	50 – 150	 ***Valine***
 ***Phenylethanol***	10 – 100	 ***Phenylalanine***
 ***Tyrosol***	20 – 50	 ***Tyrosine***
 ***Propan-1-ol***	10 – 50	*?*
 ***Butan-1-ol***	1 – 10	*?*

Table 2.6 The principle higher alcohols and their amino acids precursor in wine (Ribéreau-Gayon et al., 2006) (Cont.)

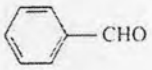
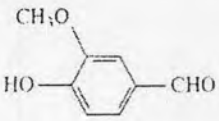
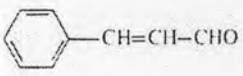
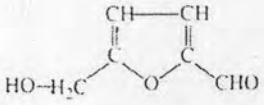
Higher Alcohol	Concentration in Wine (mg/l)	Amino Acid Precursor
 ***Thyptophol***	0 – 1	 ***Tryptophane***
 ***γ-Butyrolatone***	0 – 5	 ***Glutamic acid***
 ***Thyptophol***	0 – 5	 ***Tryptophane***

transported back into yeast cells and reduced to ethanol. Thus, the acetaldehyde content usually falls to a low level by the end of fermentation. In addition, the other aldehydes occasionally having a sensory impact on wine are furfural and 5-(hydroxymethyl)-2-furaldehyde. Because furfural synthesis from sugars is accelerated by high temperature, furfurals primarily occur in wine heated during processing. They add to the baked fragrance of such wines (Jackson, 2000).

Many ketones are produced during fermentation, but few appear to have sensory significance. The major exception is diacetyl (2,3

butanedione). At low concentrations, diacetyl donates a buttery, nutty or toasty flavor. However, at much above its sensory threshold, diacetyl generates a buttery, lactic-off odor. This commonly occurs in association with spoilage induced by certain strains of lactic acid bacteria. Diacetyl also produced by yeasts, especially at high fermentation temperatures. In addition, acetoin (3-hydroxy-2-butanone) possessing a sugary, butter-like character is also produced during fermentation (Jackson, 2000). The aldehydes and ketones were observed in wine as shown Table 2.8

Table 2.7 Aldehydes and ketones in wine (Ribéreau-Gayon et al., 2000)

Formula	Name	Boiling Point (C°)	Concentration (g/l)	Comments
$H-CHO$	Methanal	21	?	Formic aldehyde
CH_3-CHO	Ethanal	21	0.1	In combined sate with SO_2 Only Oxidized wines (Rancio, Sherry, etc) contain free ethanal
CH_3-CH_2-CHO	Propanal	49	Traces	
$CH_3-CH_2-CH_2-CHO$	Butanal	76	?	Valerianic aldehyde
$CH_3-CH(CH_3)-CHO$	Methyl-2-propanal	92	Traces	Isovalerianic aldehyde
$CH_3-CH_2-CH_2-CH_2-CHO$	Pentanal	102	?	Valerianic aldehyde
$CH_3-CH(CH_3)-CH_2-CHO$	Methyl-3-butanal	92	Traces	Isovalerianic aldehyde
$CH_3-CH_2-CH_2-CH_2-CH_2-CHO$	Hexanal	128	Traces	Caproic aldehyde
$CH_3-CH_2-CH_2-CH=CH-CHO$	Hexene-2-al		?	Only present in grapes
$CH_3-(CH_2)_5-CHO$	Heptanal	155	Traces	Oenanthic aldehyde
$CH_3-(CH_2)_6-CHO$	Octanal	167	?	Caprylic aldehyde
$CH_3-(CH_2)_7-CHO$	Nonanal	185	?	Pelargonic aldehyde
$CH_3-(CH_2)_8-CHO$	Decanal	208	?	Capric aldehyde
$CH_3-(CH_2)_{10}-CHO$	Dodecanal		?	Lauric aldehyde
$CH_3-CO-CH_3$	Propanone	56	Traces	Acetone
$CH_3-CH_2-CO-CH_3$	Butanone	80	?	Methylethyl ketone
$CH_3-CH_2-CH_2-CO-CH_3$	Pentanone-2	102	?	
$CH_3-CHOH-CO-CH_3$	Acetylmethyl carbinol	143	0.01	Acetone
$CH_3-CO-CO-CH_3$	Diacetyl	87	Traces	
$CH_3-C(SH)-CH_2-C(=O)-CH_3$	Mercaptopentanone			Sauvignon Blanc aroma
	Benzoic aldehyde	178	?	
	Vanillin	285	?	
	Cinnamic aldehyde	253	?	
	Hydroxymethyl Furfunal			Grape juice or wine subjected to heat treatment

2.6.2.10 Lactones

Lactones are formed by an internal esterification reaction between an acid function an alcohol function in the same molecule. This reaction produces an oxygen heterocycle. Volatile lactones produced during fermentation are likely to contribute to wine aroma. The best known is γ-butyrolactone, present in wine at concentrations on the order of a mg ml^{-1}. This compound results from the lactonization of γ-hydroxybutyric acid, an unstable molecule produced by deamination and decarboxylation of glutamic acid (Figure 2.15) (Schreier, 1979; Ribéreau-Gayon et al., 2000).

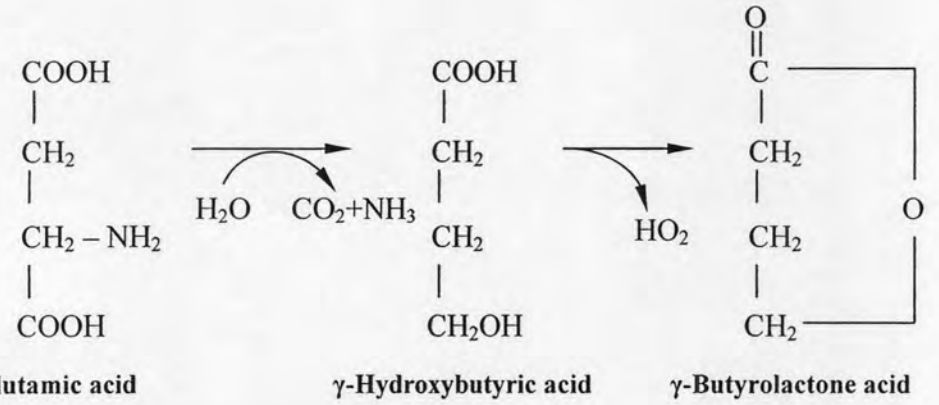


Figure 2.15 Formation of γ -butyrolactone (Ribéreau-Gayon et al., 2000)

2.6.2.11 Nitrogen-containing compounds

Many nitrogen-containing compounds are found in grapes and wine. These include inorganic forms, such as ammonia and nitrates, and diverse organic forms including amines, amides, amino acids, pyrazines, nitrogen bases, pyrimidines, proteins and nucleic acids. Although complex organic nitrogen

compounds (pyrimidines, proteins and nucleic acids) are essential for the growth and metabolism of grape and yeast cells, they seldom are involved directly in the sensory properties of wine. Occasionally, colloidal proteins cause haziness in wine (Jackson, 2000).

2.6.2.12 Sulfur-containing compounds

Organic sulfur compounds found in wine consist of a wide variety of straight-chain and cyclic molecules. They may be produced during the metabolism of sulfur-containing amino acids, peptides, and proteins. The autolysis of dead and dying yeast cells has also been implicated in the production of organosulfur off-odors. Exposure to light also may activate the production of organosulfur compounds. Structurally, the simplest organosulfur compounds are mercaptans. They are hydrocarbon chains attached to a sulfhydryl (-SH) group. A significant member of this group is ethanethiol (ethyl mercaptan). It produces a rotten onion, burnt-rubber odor at threshold levels. At higher levels, it has a shunky, fecal odor. Of the related thiol, 2-mercaptoethanol is involved in the production of a barnyard-like odor (Rapp, Güntert and Almy, 1985). Methanethiol (methyl mercaptan) can generate a rotten cabbage odor (Jackson, 2000).

Ethanedithiol is another compound occasionally producing sulfur-rubber off-odor in wine. Ethanedithiol is generated in the presence of hydrogen sulfide and acetaldehyde, and can itself combine with other wine constituents to cause other off-odors. The others organosulfur compounds are thioethers, thiolane, thiazoles, and thioesters. Most volatile sulfur compounds in wines are derived from

the microbial transformation of sulfur-containing amino acids or occasionary from sulfur added as a fungicide. However, several sulfur off-odors are generated by the breakdown of sulfur-containing organic pesticides (Jackson, 2000).

2.6.3 Wine flavors originated from aging

Further chemical alterations occur during aging; also, enzymes derived from the grapes and excreted by yeasts and malolactic bacteria, as well we, those added at prefermentation, might be expected to participate in chemical flavor transformation (Fleet, 2001). The additional aromas developing after fermentation are derived from two sources. Firstly, there is a slight oxidation of some existing components, either primary or secondary, by the dissolved oxygen present and absorbed by wine during storage (Clarke and Bakker, 2004). During aging, a decreasing in the amounts of free fatty acids and tannin and increasing in the concentration of acetaldehyde and different higher aldehyde iso-C4 up to C7 was noted. Secondly, a substantial importance is also attributed to the compounds originating from oak wood casks, some of which may be extracted into the wine. They are furfural, 5-methy-furfural and 4-hydroxy-3-methyloctanoic acid γ-lactone (Guymon and Crowell, 1972; Schreier, 1979). In addition, phenolic compounds also belong to the category of compounds originating from wooden casks which can be extracted into wine during the aging process. The volatile compounds produced during aging as demonstrated in Table 2.9.

Table 2.8 Some volatile compounds originated during wine aging (Schreier, 1979, modified)

Volatile compounds	Source	Olfactory description
Furfural	Oak wood casks	Sweet[a]
5-Methy-furfural	Oak wood casks	-
4-Hydroxy-3-methyloctanoic γ-lactone	Oak wood casks	Coconut[a,b]
Furane	Oak wood casks	-
Phenolic compounds		
Vanillin	American oak wood	Vanilla like[a]
Syringaldehyde	American oak wood	-
Coniferaldehyde	American oak wood	-
Sinapaldehyde	American oak wood	-

a = Rychlik, Schieberle and Grosch (1998); b = Clarke and Bakker (2004)

2.7 Autochthonous yeasts and their roles in wine flavors

Traditional wine has been produced for years by the natural fermentation of grape juice caused by yeast that originate from the grapes and winery equipment without deliberate inoculation to start the process (Ribéreau-Gayon et al., 2000; Fleet et al., 2002; Di Maro, Ercolini and Coppola, 2007). Spontaneous alcoholic fermentation of grape must is a complex biochemical process characterized by the presence of a large number of different yeast genera and species. These indigenous yeasts can significantly enhance desirable wine quality, unique contributions to flavors and produce reputable quality wine (Fleet et al., 2002; Fleet, 2003; Romano et al., 2003).

Among alcoholic beverages, wine is a natural product resulting from a number of the biochemical reactions which begin during ripening of the grapes and continue to yeasts fermentation. The organoleptic compounds produced by yeasts can be largely divided harvesting, throughout the alcoholic fermentation, clarification and after bottling (Romano et al., 2003).

Yeasts have a profound impact on wine quality and value. They conduct the alcoholic fermentation on grape juice, thereby contributing to the basic structure and individuality of wine flavor and aroma. The dimensions of this contribution vary with the species and strain of yeast. After alcoholic fermentation, various yeasts can cause wine spoilage and loss of value. The yeasts of wine making originate from several sources: the microflora that occur on the surface of grape berries, the microflora associated with the surfaces of wine processing equipment (originally come from grapes) and the microflora as starter or inoculum cultures of yeasts used to induce and conduct the alcoholic fermentation.

The natural wine fermentation evolves from the autochthonous or wild microflora that originated from the grapes and winery equipment. The dominant yeasts of mature grapes are apiculate yeasts belonging to the genera *Kloeckera* and *Hanseniaspora* which account for 50-75% of the total yeast population. Other yeasts belonging to the genera *Candida*, *Brettanomyces*, *Cryptococcus*, *Kluyveromyces*, *Metschnikowia*, *Pichia* and *Rhodotorula* are presented at a lower level. The spontaneous fermentation is carried out by the sequential action of autochthonous yeasts divided into 2 stages. The early stage of the alcoholic fermentation is dominated by these yeasts characterized by a low fermentative power (Fleet et al.,

2002). Their growth is significant and can influence the chemical composition of the wine. Nevertheless, the growth of these yeasts are limited to the first 2-4 days of fermentation and their growth declined rapidly at ethanol concentrations above 5-6% (w/v) (Romano, 2002). Under this condition, strains of *S. cerevisiae* and related species which are more tolerant to ethanol and more competitive for growth in media with high sugar concentration become the dominant yeasts and complete the fermentation process. (Querol, Jimenez and Huerta, 1990; Fleet and Heard, 1993). While spontaneous fermentations can produce wine of exceptional quality, the outcomes in terms of process efficiency and product quality are often inconsistent. In contrast, starter fermentations offer the advantages of a rapid, successfully process, and wine with more consistent and predictable quality. Generally, the commercial strain of *S. cerevisiae* is used in winemaking. In these fermentations, the inoculated strain of yeast overcomes and suppresses the influence of autochthonous flora. Consequently, the incident of microbiological faults in winemaking has been significantly decreased. However, the use of pure yeasts culture could also reduce the production of some desired metabolites because of the lack of the autochthonous yeasts contributing the positive and unique flavors to wine. Thus, these yeasts influence positively and negatively the sensory quality of the wine (Mannazzu, Clementi and Ciani, 2002).

Traditional wine fermentation, the indigenous yeasts were reported to be wild yeasts producing wines of unique flavors and exceptional quality. These yeasts were isolated from grape and winery equipment. There were a lot of previous researchers (Povhe Jemec et al., 2001; Rementeria et al., 2003; Combina et al., 2005; Di Maro, Ercolini and Coppola, 2007; Domizio et al., 2007) studied dynamic of indigenous

yeasts during the spontaneous fermentation of grape must grown in variety areas of vineyard to isolate the specific yeasts in that location. Then, their fermentation properties were studied and these yeasts could be developed to use as species specific starter cultures for wine fermentation in each area.

Apart from wine fermentation, some researches studied the spontaneous fermentation of other alcoholic beverages and fermented products. Atputharajah, Widanapathiranat and Samarajeewa (1986) investigated indigenous microorganisms associated with natural fermentation of coconut palm sap to produce a popular alcoholic beverage known as fermented toddy in Sri Lanka. Morissey et al. (2004) studied the role of the indigenous yeast flora in traditional Irish cider fermentations. Coton et al. (2006) studied yeasts biodiversity and dynamics of naturally fermented French ciders and black olives to isolate indigenous yeasts involving in cider aroma development and contributing to Nyons black olive typicity, respectively.

2.8 Specific yeasts

Nowadays, a number of viticultural and winemaking practices are being investigated to improve wine quality. There is a growing demand for new and improved wine yeast strains adapted to different types and styles of wines. Generally, industrial wine fermentations are currently conducted by starters of selected wine yeast strains of *S. cerevisiae* in contrast to traditional spontaneous fermentations conducted by the flora present on the grapes and in the winery. The advantages of using pure cultures of *S. cerevisiae* are with regard to the easy control and homogeneity of fermentations. However, wine produced with pure yeast

monocultures lacks the complexity of flavor, stylistic distinction and vintage variability caused by indigenous yeasts. (Lambrechts and Pretorius, 2000; Romano et al., 2003). Therefore, the specific properties of yeasts are used in the production of wines. Especially, the specific requirements for the production of a desired flavor in the final product are required in wine fermentations. This significantly depends on the species and/or strain of yeasts associated in the fermentation (Reed and Nagodawithana, 1991).

Some researchers reported that the flavors produced from different yeast species and strains were significantly different. Romano et al. (1997) investigated capacity of acetaldehyde and ethyl acetate production by 48 strains of *H. guilliermondii* and 48 strains of *K. apiculata* in basal synthetic medium. Acetaldehyde and ethyl acetate were analyzed by direct injection to gas chromatography. The results showed that *K. apiculata* produced more acetaldehyde than *H. guilliermondii.* However, the production of ethyl acetate was similar in both species. Romano et al. (1999) examined 19 strains *S'codes ludwigii* for the production of secondary products in grape must fermentation. The grape juice was inoculated with the selected *S'codes ludwigii* strains in comparison to a control of *S. cerevisiae* and evaluation of the fermented products were analyzed their by-products. Romano et al. (2003) studied by-products formation in a large number of natural wine yeasts: 127 isolates of *S. cerevisiae*, 28 isolates of *H. uvarum*, 25 isolates of *C. stellata*, 25 isolates of *Z. fermentati* and 25 isolates of *S'codes ludwigii* in grape must. The results showed that the metabolic profiles of *H. uvarum* and *C. stellata* was quite similar, being characterized by a high production of acetoin and ethyl acetate (higher in *C. stellata* than in *H. uvarum*) and a general low production of higher alcohols, whereas *S. cerevisiae* is characterized by high production of isoamyl alcohol and 2,3-butanediol

and low production of acetoin. The profile of *Z. fermentati* was characterized by a general low production of the compounds considered, with only a high 2,3-butanediol production. *S'codes ludwigii* was a high producer of acetoin, ethyl acetate, isoamyl alcohol and isobutanol.

2.9 Wine flavors improvement

In modern winemaking, consumer olfactory profiling is increasingly important since this could be used as a guide to production decision. The combination of chemical and sensory analyses of wine now makes it possible to understand the subtle nuances associated with varietal wine flavor and fermentation bouquet. The organoleptic quality (appearance, aroma and flavor) of wine depends upon the presence of absolute amounts and specific ratios of many desirable and inactive compounds, and the absence of negative ones. Subtle combinations of trace grape-derived components usually elicit the characteristic flavor and aroma notes of wine; these components include accumulated secondary metabolites such as terpenes in the aromatic varieties and alkoxypyrazines in the vegetative or herbaceous cultivars. The products of yeast fermentation, such as esters, alcohols, aldehydes, diacetyl fatty acids, phenolic acids, sulfites and sulfides, etc. contribute to the generic background flavor and aroma, as well as to the complexity and intensity of the aroma and taste of the final products. The components formed during fermentation dominate the volatile profile of wines, as these compounds are present in the highest concentrations. There is a clear need for the development of wine yeasts that could impart specific desirable characteristics to a wine (Pretorius, 2003). A lot of methodologies can be used for improvement wine flavor. Among these methodologies, multi-starter culture

fermentation is well known and used to apply for wine fermentation (Ciani, Fatichenti and Mannazzu, 2002).

As mentioned already, the most important yeast of alcoholic fermentation is *S. cerevisiae*. However, natural fermentation of grape must is usually started by low alcohol-tolerant apiculate yeasts (*Kloeckera* or *Hanseniaspora*) that predominant the first stages of fermentation. When the concentration of alcohol reaches 3-4% v/v, they are replaces by elliptical yeast (*S. cerevisiae*) that carry out and finish the fermentation process. In addition, during the various stages of fermentation, it is possible to isolate also other yeast species such as *C. stellata*, *T. delbrueckii* and *M. pulcherrima*. There has been a re-evaluation of the role of these non-*Saccharomyces* yeasts in winemaking. The presence and permanence of non-*Saccharomyces* yeasts throughout inoculated and non inoculated fermentations are well documented as well as their contribution to the analytical composition and sensorial characteristics of wine. Therefore, the contribution of non-*Saccharomyces* yeasts seems to lead to a more complex aroma and improve wine quality (Ciani, Fatichenti and Mannazzu, 2002). It is possible to promote the activity of non-*Saccharomyces* yeasts in winemaking by limiting or delaying the use of selected cultures. However, in this situation the fermentation remains an uncontrolled process, therefore the mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts should be used under more defined conditions.

The use of multi-starter cultures was proposed several years ago. The few techniques used in multi-starter cultures fermentation process are mixed cultures, sequential cultures and immobilized cells. The advantages of this strategy in wine

fermentation process are to improve the quality of wine in several ways, such as enhancement of volatile compounds, biological deacidification and enhancement of glycerol in wine. The examples of winemaking process by using controlled multi-starter cultures of wine yeasts as demonstrated in Table 2.10.

2.10 Wine yeast determination

The general methods to study the yeasts associated with wine grapes has been to rinse the surfaces of grapes, and then determine yeasts population number in the rinses by plating onto appropriate agar media. To examine the low population yeasts, grape samples have been incubated in liquid enrichment media, then plate onto agar medium. After that, the yeast colony isolated from rinses or enrichment medium will be further identified by conventional method combined with other identification methods.

Table 2.9 Multi-starter cultures used for wine fermentation

Starter cultures	Objective	Process	References
S. cerevisiae *T. delbrueckii*	Reduction of volatile acidity and enhancement of aroma complexity	Mixed cultures Mixed or sequential cultures Sequential cultures	Bely et al. (2008) Ciani, Beco and Comitini (2006) Herraiz et al. (1990)
S. cerevisiae *Sh. Pombe*	Deacidification	Immobilized cells	Magyar and Panyik (1989) Ciani (1995)
S. cerevisiae *C. stellata*	Enhancement of glycerol content	Immobilized cells	Ciani and Ferraro (1996) Ciani and Maccarelli, (1998) Ferraro, Fatichenti and Ciani (2000)
S. cerevisiae *H. uvarum*	Enhancement of aroma complexity	Mixed cultures Mixed or sequential cultures	Pérez-Nevado et al. (2006) Moreira et al. (2008) Zironi et al. (1993) Moreira et al. (2005) Ciani, Beco and Comitini (2006)
S. cerevisiae *K. thermotolerans*	Enhancement of aroma complexity	Mixed or sequential cultures Sequential cultures	Ciani, Beco and Comitini (2006) Mora, Barbas and Mulet (1990)
S. cerevisiae *H. guilliermondii*	Enhancement of aroma complexity	Mixed cultures	Rojas et al. (2003) Moreira et al. (2005) Pérez-Nevado et al. (2006) Moreira et al. (2008)
S. cerevisiae *P. anomala*	Enhancement of aroma complexity	Mixed cultures	Rojas et al. (2003)

2.10.1 Conventional method

The traditional method for yeast identification has been based on cultural, phenotypic analyses. The yeast isolate is examined for its morphological, biochemical and physiological properties, which are systematically compared with standard descriptions to give genus and species identity. Normally, it is necessary to conduct approximately 75-100 individual tests to obtain a reasonably reliable identification (Kurtzman and Fell, 1998; Barnett, Payne and Yarrow, 2000). Consequently, the entire process is very labour-intensive, lengthy and costly. Although various technical and diagnostic innovations have been developed to facilitate testing, they are not universal in their application and the data generated are not always equivalent (Querol et al., 1992).

2.10.2 Commercial kits

To improve conventional methods, various rapid and accurate manual and automated commercial kits have been developed as shown in Table 2.11. However, the commercial kits were designed for the needs of clinical yeast diagnosis, and the databases are restricted to 40 to 60 yeast species of clinical importance. Ramani et al. (1998) suggested that geographical differences in the prevalence of yeast species accounted for the different findings with identification kit. Among these commercial kits, the ID 32 C system (Biomerieux, France) is a biochemical identification system widely used and highly regarded by most European mycologists with reported identification accuracy above 94% (Buchaille et al., 1988; Gutierrez et al., 1994). It consist of a single-use disposable plastic strip with 32 well containing

substrates for 29 assimilation tests (carbohydrates, organic acids and amino acids), one of susceptibility test (cyclohexamide), one colorimetric test (esculin), and a negative control. A portion of growth from well-isolated colonies of each isolate was aseptically transferred from a freshly inoculated stock culture to sterile distilled water to prepare a suspension with final turbidity equivalent to McFarland standard #2. This suspension was then dispensed to an ampule of C medium provided by the manufacturer and homogenized to prepare an even dispersion of inoculum. After homogenizing, the inoculum suspension was used to inoculate the wells in the strip, the lid of the strip was replaced, and the system was incubated at 30°C for 48 hours. The strips were then visually examined and growth was determined to be positive or negative based on the presence or absence of turbidity in the wells. The results were transformed into the numerical biocodes, and the isolates were identified through the use of the ID 32C Analytical Profile Index (Ramani et al., 1998).

2.10.3 Molecular method

Molecular methods based on DNA analysis are now being used to quickly identify yeasts to genus and species level. The workload is minimal and, usually, reliable data can be obtained within 1-2 days. Various molecular methods based on analysis of rDNA have been developed for faster, more definitive yeast identification (Kurtzman and Robnett, 1998). Ribosomal DNA/RNA occurs in several size classes in eukaryotes. The genes coding for large (25S to 28S), small (18S) and 5.8S ribosomal RNAs, and the ribosomal ITS region occur as tandem repeats with as many as 100 to 200 copies per cell. Each of the ribosomal RNA size classes has been examined for their extent of phylogenetic information (Garber et al., 1998). Interest in

ribosomal RNA/DNA sequences comes from two important properties; (i) ribosomes are present in all cellular organisms and appear to share a common evolutionary origin, thus providing a molecular history shared by all organisms; and (ii) some ribosomal RNA/DNA sequences are sufficiently conserved that they are homologous for all organisms and serve as reference points that enable alignment of the less conserved areas to measure evolutionary relationships. The discovery of these properties has lead to the widespread use of ribosomal DNA sequences to develop yeast phylogeny and taxonomy (Kurtzman and Robnett, 1998).

Table 2.10 Different kits and systems used to identify foodborne yeasts

Systems	References
YeastsIdent	Salkin et al. (1987)
Uni-Yeast-Tek	Bowman and Ahearn (1975); Salkin et al. (1987)
Quantum II	Salkin et al. (1985); Pfaller et al. (1988)
Minitek	Gilliland and Speck (1977); Lin, Fung and Cox (1987)
MicroScan	Hussain et al. (1986); Land et al. (1991); St Germain and Beauchesne (1991)
Microring YT	Shankland et al. (1990); McGowan and Mortensen (1993)
AutoMicrobic	Pfaller et al. (1988); El-Zaatari et al. (1990)
ATB 32 ID	Looney, Gallusser and Modde (1990); Rohm, Lechner and Lehner (1990)
API 20C	El-Zaatari et al. (1990); Land et al. (1991); St-Germain and Beauchesne (1991); Ramani et al. (1998)
VITEK-2	Graf et al. (2000); Ling et al. (2001), Cárdenes-Perera et al. (2004)
ID 32C	Fricker-Hidalgo et al. (1995); Fricker-Hidalgo et al. (1996); Muir and Pritchard (1997); Ramani et al. (1998); Cárdenes-Perera et al. (2004)

There are many molecular methods based on DNA/RNA extraction used for yeast identification. Of the various molecular approaches available, sequencing of the D1/D2 domain of the 26S ribosomal DNA, sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA, and Restriction Fragment Length Polymorphism (RFLP) analysis of the ITS ribosomal DNA are frequently applied for yeast identification (Kurtzman and Robnett, 1998).

2.10.3.1 Restriction Fragment Length Polymorphisms (RFLP) analysis

Restriction fragment length polymorphism (RFLP) analysis of ribosomal DNA segments is emerging as one of the most useful methods for rapidly identifying food and beverage yeasts. The preferred region for analysis represents the ITS-5.8S-ITS2 segment. Using particular primers ITS1 and ITS4, this region is amplified by PCR. The PCR amplicon is then hydrolyzed with specific restriction endonucleases, the product or fragments of which are separated by gel electrophoresis. The number (usually 1-4) and size (base pairs) of the DNA fragments, as determined by banding patterns on gel electrophoresis are the principles used to discriminate between yeast species as. Generally, more than one restriction endonuclease is used in order to obtain unequivocal discrimination. Some restriction endonucleases commonly used are *cfo I*, *Hae* III, *Hinf* I, *Hpa* II, *Scr* FI, *Taq* I, *Nde* and *Dde* I (Granchi, Bosco and Vicenzini, 1999).

2.10.3.2 Sequencing method

DNA sequencing analysis is the process of determining the nucleotide order of a DNA fragment. The sequencing of specific genes is also being used in the development of yeast phylogeny, and to distinguish between closely related species. The majority of studies has focused on the D1/D2 domain of the 26S subunit of the ribosomal DNA consisting of about 600 nucleotides (Figure 2.16) and has been sequenced for all known yeast species. To identify yeasts on the basis of ribosomal DNA sequences, specific regions of ribosomal DNA need to be amplified by the polymerase chain reaction (PCR) using particular primers. The 26S rDNA coding region is amplified by the primer pair NL1 and NL4. The internal transcribed spacer region is amplified by the primer pair ITS1 and ITS4. The PCR amplicons are then used for sequence analysis.

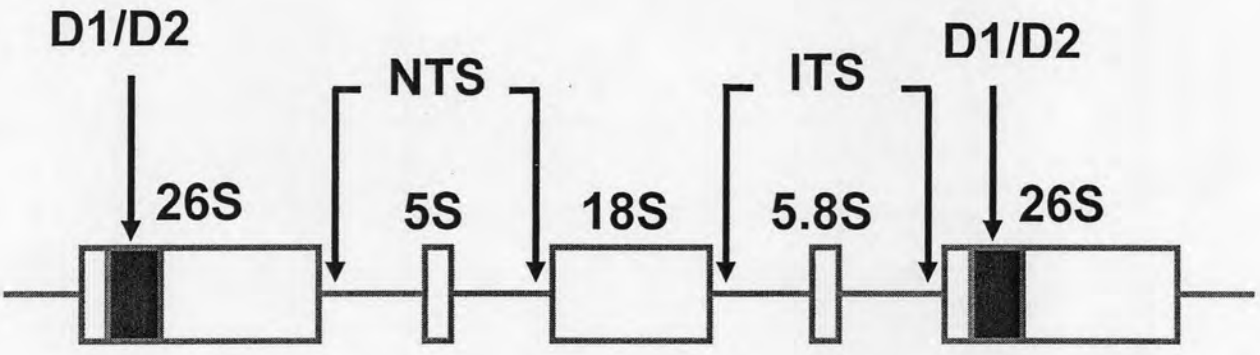


Figure 2.16 Diagram of the D1/D2 domain and ITS region of ribosomal DNA (Giudici and Pulvirenti, 2002)

The basis protocol for sequencing ribosomal DNA segments is preparation of a pure culture of the yeast isolate, extraction and purification of DNA, PCR amplification of the target DNA region to be sequenced using defined primer separation and isolation of the amplified product, sequencing of product and comparison of sequences with published data. Procedures for conducting these operations are well established but there are many variables that are not standardized and vary from one laboratory to another (Kurtzman and Robnett, 1998).

In this study, the application of RFLP of ITS region of yeast rDNA and sequencing analysis of D1/D2 domain of the 26S rDNA and ITS region of yeast rDNA for identification of the yeast community associated with pineapple fruits and natural fermentation of fresh crushed pineapple juice. Some studies on combinations of RFLP and sequencing analysis to identify yeasts in food and beverages as indicated in Table 2.12

Table 2.11 Some studies on combinations of RFLP and sequencing analysis to identify yeasts in food and beverages

Yeasts and sources of isolate	Identification methods	References
Yeasts isolated from orange juices	Yeasts (99 strains, 69 species, 14 genus) isolated from orange juices. 23 isolates yeast could not be identified by RFLP analysis and sequencing of D1/D2 region	Arias et al. (2002)
Yeasts isolated from orange fruit and juice	A combination of RFLP analysis and sequencing of ITS region gave reliable identification of new yeast strains. They could be identified correctly 98% of the isolates from orange juices	Heras-Vázquez et al. (2003)
Yeasts in wine	A combination of RFLP analysis and sequencing of ITS region gave reliable identification of yeasts isolated during fermentation	Clemente-Jimenez et al. (2004)
Yeasts in a manufacturing plant of candied fruits and nougats	A combination of RFLP analysis of ITS region and sequencing of D1/D2 region to confirmed the reliability of yeast identification	Martorell, Fernandez-Espinar and Querol (2005)
Yeasts associated with grape berries	A combination of RFLP analysis and sequencing of D1/D2 and ITS region gave reliable identification of yeasts isolated	Prakitchaiwattana (2005)
Yeasts in traditional balsamic vinegar fermentation	A combination of RFLP analysis of ITS region and sequencing of D1/D2 region to confirmed the reliability of yeast identification at species level	Solieri et al. (2006)
Yeasts in a cold maceration and alcoholic fermentation of grape must	A combination of RFLP analysis and sequencing of ITS region gave reliable identification of yeasts isolated during fermentation	Zott et al. (2008)
Yeasts in a spontaneous fermentation of a traditional high sugar must	A combination of RFLP analysis of ITS region and sequencing of D1/D2 region gave reliable identification of yeasts isolated during fermentation	Tofalo et al. (2009)

Although RFLP and sequencing analysis are rapid and reliable molecular methods, they are culture-dependent means which are drawback for the investigation of unculturable yeasts in a system. Hence, a culture-independent method, DGGE, is frequently applied as a tool to investigate yeast diversity in several aspects of researches, especially ecological investigation of food and beverages.

2.10.3.3 Denaturing Gradient Gel Electrophoresis (DGGE) analysis

Presently, to understand the fermentation profile of microorganisms in ecosystem of food and beverages, the new methodology has been used to monitor the changes of the microorganism during fermentation process which is a culture-independent methods based on denaturing gradient gel electrophoresis (DGGE). This method is now increasingly being used to determine the ecological profile of microorganisms in natural habitats, including use for monitoring yeasts during wine fermentation process (Muyzer and Smalla, 1998; Head, Saunders and Pickup, 1997).

PCR-DGGE analysis is a molecular analytical method for investigation of microorganism profile in microbial ecology without the need for agar culture. Therefore, its analysis results could serve all information of both culturable and unculturable microorganism in sample or ecosystem. It believed that this molecular approach overcomes the bias and limitation of culture methods, and reveals species that might fail to produce colonies on agar media. Consequently, a more accurate representation of the product/substrate ecology is obtained (Muyzer and Smalla, 1998; Head et al., 1997). So far, there were a lot of researchers used DGGE to

determine the ecological profile of microorganism during fermentation or ripening of foods and beverages, such as wine (Di Maro, Ercolini and Coppola, 2007), cocoa (Nielsen and Arneborg, 2007), sausage (Fontana, Vignolo and Cocconcelli, 2005), salami (Aquilanti et al., 2007; Silvestri et al., 2007) and cheese (Randazzo, Vaughan and Caggia, 2006; Bonetta et al., 2008). In this study, the application of DGGE in profiling the yeast community of natural fermentation of fresh crushed pineapple juice were compared with cultural isolation on Malt Extract Agar (MEA) plate. Furthermore, their fermentation profiles were also investigated during the fermentation.

The basic protocol for DGGE is performed as following procedures. Total DNA is extracted from samples of the product using universal primers. Yeast ribosomal DNA within the extract is specifically amplified with PCR. Generally, the D1/D2 domain of the 26S subunit is targeted, but other regions such as the 18S subunit may be used (Figure 2.16). The amplicons produced by PCR are next separated by DGGE, which resolve the different DNA amplicons on the basis of their sequence. DGGE uses a polyacrylamide gel containing a linear gradient of the denaturant (mixture of urea and formamide) to denature (melt) the strands of the DNA amplicons (Fischer and Lerman, 1979; Myers, Maniatis and Lerman, 1987). Usually, each DNA band found in the gel corresponds to a yeast species. The band is excised from the gel and reamplified to increase its concentration, and then sequenced to give the species identity. Thus, a profile of the species associated with the ecosystem is obtained, without the need for agar culture. It is believed that this molecular approach overcomes the bias and limitations of agar media. Consequently, a more accurate representation of the product / substrate ecology is obtained (Muyzer and Smalla,

1998; Head et al., 1997). Using reverse transcriptase-PCR to target extracted RNA, a profile of metabolically active species, as opposed to the dead or non-viable flora, can be obtained.

2.11 Wine flavor analysis

2.11.1 Non-volatile compounds

The non-volatile constituents of wine have significant influences on the organoleptic quality of wine products because they are responsible for the taste of wine. Nowadays, enzymatic and HPLC methods are used to determine these compounds during winemaking. However, the most important drawback of the enzymatic methods is necessary to perform specific and individual determinations for each compounds analyzed, which make them expensive (López-Tamames et al., 1996). High Performance Liquid Chromatography (HPLC) is the altered techniques to separate and quantify these chemical compounds. This method separates substances of different natures accurately and simultaneously. Hence, it is frequently exploited to investigate chemical compounds generated by yeasts during wine fermentation.

HPLC is an analytical technique for the separation and identification of both organic and inorganic compounds. The sample solution is injected into the mobile phase of the assay through the injector port. As the sample solution flows with the mobile phase through the stationary phase, the components of that solution will migrate according to the non-covalent interactions of the compounds with the stationary phase. The chemical interactions of the stationary phase and the sample

with the mobile phase, determines the degree of migration and separation of the components contained in the sample. The samples which have stronger interactions with the stationary phase than with the mobile phase will elute from the column less quickly, thus it have a longer retention time and vice versa. Then, the components eluted from column are detected as a series of peak on a recorder by detector. The detector sends information to a computer that records all of the data produced, converts the electrical impulses into visual displays.

Columns containing various types of stationary phases are commercially available. Among of these columns, Bio-Rad Aminex® HPX-87H ion exclusion column is frequently used for wine analysis because of its convenience. This column separates sugars, organic acids, alcohols, phenols and organic bases on a single column (Cazes, 2005). In addition, several researchers exploited this column for other application. Some studied of the application of Bio-Rad Aminex® HPX-87H ion exclusion column equipped with HPLC are demonstrated in Table 2.13

Table 2.12 Aminex® HPX-87H ion exclusion column equipped with HPLC used for chemical analysis

Chemical analysis	Samples	References
Ethanol, sugars, organic acids and glycerol	Wine	Pfeffer and Radler (1985)
Organic acids	Grape juice	Bissell, Ewart and Sangtippawan (1989)
Ethanol, sugars and organic acids	Extracts of fermenting cocoa beans	Tomlins, Baker and McDowell (1990)
Ethanol, sugars and organic acids	Cucumber juice and fermented cucumber brine	McFeeters (1993)
Ethanol, sugars and organic acids	Wine	Maicas et al. (1999) Walker et al. (2002)
Organic acids	Wine	Herjavec et al. (2003) de Villiers et al. (2004) Jeromel et al. (2008)
Organic acids	Strawberry puree	Sturm, Koron and Stampar (2003)
Ethanol, glycerol and organic acids	Wine	Urtubia et al. (2004)
Ethanol and organic acids	Vinegar	Aguiar et al. (2005)
Organic acids and sugars	Fruit juices	Chinnici et al. (2005)
Ethanol	Wine	Moreira et al. (2005)
Organic acids	Rinsing water of grape berries	Prakitchaiwattana (2005)
Organic acids	Orange juice	Shaw, Wilson and Hansen (1987)
Ethanol, sugars and organic acids	Wine	Berovič et al. (2007)
Ethanol, sugars	Wine	Coleman, Fish and Block (2007)
Organic acids	Pineapple fruits	Saradhuldhat and Paull (2007)
Ethanol, sugars and glycerol	Wine	Gonzalez-Ramos, Cebollero and Gonzalez (2008)

2.11.2 Volatile compounds

The nature of compounds giving flavor in wine is mostly volatile compounds which could be evaporated at low temperature, such as higher alcohols, esters and acetaldehyde. These compounds are responsible for the bouquet of wine. The identification of these compounds can be done by Gas Chromatography (GC). However, Gas Chromatography-Mass Spectrometry (GC-MS) is a more powerful tool since it could be used to identify and characterize the components that are present in wine. This tool is frequently exploited to investigate these volatile compounds contributed by yeasts in wine fermentation both in qualitative and quantitative.

Gas chromatography-mass spectrometry (GC-MS) is an instrumental technique comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS) by which complex mixtures of organic compounds may be separated, identified and quantified (Budde and Eichelberger, 1979). In order for a compound to be analyzed by GC-MS, it must be sufficiently volatile and thermally stable. In addition, functionalized compounds may require chemical modification prior to analysis, to eliminate undesirable adsorption effects that would otherwise affect the quality of the data obtained. The sample solution is injected into the GC inlet where it is vaporized and swept onto a chromatographic column by the carrier gas. The sample flows through the column and the compounds comprising the mixture of interest are separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase). The latter part of the column passes through a heated transfer line and ends at the entrance to ion source where compounds eluting from the column are converted to ions. The most frequently used

ion production method is electron ionization (EI) and the occasionally used alternative is chemical ionization (CI). After the ions are separated, they enter a detector the output from which is amplified to boost the signal. The detector sends information to a computer that records all of the data produced, converts the electrical impulses into visual displays.

There are several extraction techniques coupled with GC-MS used for volatile compounds analysis, such as simultaneous distillation, closed-loop stripping, purge and trap, and headspace-solid phase microextraction (HS-SPME). Among these techniques, HS-SPME is frequently used for analysis of volatile compounds because of its fast, simple and solvent-free technique. Thus, SPME has been used in a range of fields including studies of flavors and taints especially for quick screening of the volatile compounds of a wide range of products. The different types of fiber with a wide range of polarity can isolate trace compounds of different substrates. The different types of fiber and their application were showed in Table 2.14. Nowadays, there was a number of researchers have used HS-SPME coupled with GC-MS for volatile compounds analysis as shown in Table 2.15.

Table 2.13 Types of SPME Fibers (Supelco, USA)

Fibers	Application
Polydimethylsiloxane (PDMS)	Volatile compounds
Carboxen / Polydimethylsiloxane (CAR/PDMS)	Gases and low molecular weight compounds (MW 30-225)
Polydimethylsiloxane / Divinylbenzene (PDMS/DVB)	Volatile, amines, and nitroaromatic compounds
Divinylbenzene / Carboxen / Polydimethylsiloxane (DVB/CAR/PDMS)	Flavor compounds
Carbowax / Divinylbenzene (CW/DVB)	Volatile free acids, polar oxygenates compounds
Polyacylate (PA)	Polar Semivolatile compounds
Polyethylene glycol (PEG)	Polar compounds
Carbowax/Templated Resin (CW/TPR)	Surfactants

Table 2.14 HS-SPME coupled with GC-MS used for volatile compounds analysis

SPME fibers	Samples	References
PDMS	Wine	Vas et al. (1998)
	GPYM medium	Rojas et al. (2001)
	White wine	Demyttenaere et al. (2003)
	Fruit juices and nectars	Riu-Aumatell et al. (2004)
	Sparkling wine	Riu-Aumatell et al. (2006)
	Aniseed-flavoured spirit drinks	Jurado et al. (2007)
	Olive oil	Ribeiro et al. (2008)
	White wine, beer, whisky	Rodrigues, Caldeira and Câmara (2008)
PDMS/DVB	Grape skin and pulp	Sánchez-Palomo, Diaz-Maroto and Perez-Coello (2005)
	Aniseed-flavoured spirit drinks	Jurado et al. (2007)
	White wine, beer, whisky	Rodrigues, Caldeira and Câmara (2008)
	Arabica coffee	Ribeiro et al. (2009)
CAR/PDMS	Wine	López et al. (2007)
	Aniseed-flavoured spirit drinks	Jurado et al. (2007)
	White wine, beer, whisky	Rodrigues, Caldeira and Câmara (2008)
	Tomato	Serrano, Beltrán and Hernández (2009)
DVB/CAR/PDMS	White wine	Demyttenaere et al. (2003)
	Grape skin and pulp	Sánchez-Palomo, Diaz-Maroto and Perez-Coello (2005)
	Wine	López et al. (2007)
	Sparkling wine	Riu-Aumatell et al. (2006)
	Aniseed-flavoured spirit drinks	Jurado et al. (2007)
	White wine, beer, whisky	Rodrigues, Caldeira and Câmara (2008)
CW/DVB	Wine	Ferraro, Fatichenti and Ciani (2000)
	White wine	Demyttenaere et al. (2003)
	Red and white wine	Seibert et al. (2005)
PA	Grape skin and pulp	Sánchez-Palomo, Diaz-Maroto and Perez-Coello (2005)
	White wine	Siebert et al. (2005)
	Olive oil	Ribeiro et al. (2008)
	White wine, beer, whisky	Rodrigues, Caldeira and Câmara (2008)