EFFECT OF ALCOHOLIC FERMENTATION TEMPERATURES ON RED WINE FLAVOR

Miss Ulaiwan Usansa

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ผลของอุณหภูมิการหมักแอลกอฮอล์ต่อกลิ่นรสของไวน์แดง

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master' s Degree.

Thesis Examining Committee

.....

(Asst. Prof. Dr. Mariena Ketudat-Cairns)
Chairperson

.....

(Prof. Dr. Nantakorn Boonkerd)
Member (Thesis Advisor)

.....

(Asst. Prof. Dr. Chokchai Wanapu)
Member

.....

(Assoc. Prof. Dr. Neung Teaumroong)
Member

.....

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อุณหภูมิในการหมักไวน์เป็นปัจจัยหนึ่งที่ส่งผลต่อคุณภาพของไวน์แดง งานวิจัยเรื่องนี้มีวัตถุประสงค์เพื่อเปรียบเทียบผลของอุณหภูมิการหมักแอลกอฮอล์ต่อกลิ่นรสของไวน์แดง โดยเฉพาะการหมักที่อุณหภูมิห้องของเมืองไทยคือช่วง 27-32 °ซ เปรียบเทียบกับ 5 อุณหภูมิ ดังนี้ 15 °ซ, 20 °ซ, 25 °ซ, 30 °ซ และ 35 °ซ องุ่นที่ใช้ในการศึกษา คือ พันธุ์ชีราซ โดยทำการศึกษาผลของอุณหภูมิการหมักต่อการเจริญของยีสต์สายพันธุ์ *Saccharomyces bayanus* EC1118 และผลของอุณหภูมิการหมักต่อสารให้กลิ่นรส ได้แก่ เอสเทอร์, แอลกอฮอล์หนักและสารฟีนอลในกลุ่มของคาทีชินและเอพิคาทีชิน จากการทดลองพบว่าอัตราการเจริญจำเพาะของยีสต์เพิ่มขึ้นเมื่ออุณหภูมิสูงขึ้นและสูงสุดที่อุณหภูมิ 25 °ซ สารกลุ่มเอสเทอร์ที่พบ ได้แก่ โพรพิลอะซิเทท, เอธิลออคทะโนเอท, ไอโซเอมิลอะซิเทท, เฮคซิลอะซิเทท, เอธิลเฮคซะโนเอท, เอธิลเดคคะโนเอท และฟีเนธิลอะซิเททพบปริมาณสูงสุดที่อุณหภูมิ 15 °ซ และเอธิลบิวธิเรทพบมากที่อุณหภูมิ 20 °ซ ในขณะที่สารกลุ่มแอลกอฮอล์หนัก ได้แก่ โพรพานอล, เพนธานอลและฟีเนธิลแอลกอฮอล์พบปริมาณสูงสุดที่อุณหภูมิ 35 °ซ สำหรับบิวทานอล, ไอโซเอมิลแอลกอฮอล์และเฮพทานอลพบปริมาณสูงสุดที่อุณหภูมิ 30 °ซ ยกเว้นเฮคซะนอลพบว่ามีปริมาณสูงสุดที่อุณหภูมิ 15 °ซ สารคาทีชินและอิพิคาทีชินในไวน์มีปริมาณเพิ่มขึ้นเมื่ออุณหภูมิสูงขึ้นและมีปริมาณสูงสุดที่อุณหภูมิห้องและอุณหภูมิ 25 °ซ ตามลำดับ คุณภาพทางประสาทสัมผัสของผลิตภัณฑ์สุดท้ายถูกทดสอบโดยผู้ชิม 12 คน พบ 5 คุณลักษณะที่มีความแตกต่างอย่างมีนัยสำคัญที่ระดับความเชื่อมั่น 95 % คือ สี, บอดี้, อะโรมา, ความสมดุลของรสชาติและความฝาด คุณลักษณะทางอะโรมาให้ผลสอดคล้องกับคุณภาพทางเคมี ซึ่งอุณหภูมิที่ได้คะแนนสูงสุดคือ 15 °ซ คุณลักษณะโดยรวมของไวน์ไม่มีความแตกต่างในทางสถิติ และไวน์ที่ได้รับคะแนนสูงสุดคือ ไวน์หมักที่อุณหภูมิ 25 °ซ

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2546

ลายมือชื่อนักศึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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The objective of this experiment was to study the effect of fermentation temperature on flavor compound in red wine. This study was aimed at comparing, the room temperature in Thailand which were around 27-32 °C with five temperatures, including of 15 °C, 20 °C, 25 °C, 30 °C and 35 °C. Wine fermentation was carried out by using commercial yeast *Sacchromyces bayanus* EC1118. The wine grape was Shiraz. It was found that the fermentation temperature affected on yeast growth kinetics. The specific growth rate increased with temperature and the maximum specific growth rate was found at 25 °C. The ester formation was found highest at low temperature. The acetate ester, *n*-propyl acetate, ethyl octanoate, isoamyl acetate, hexyl acetate, ethyl hexanoate, ethyl decanoate and 2-phenethyl acetate, were highest content at 15 °C and ethyl butyrate at 20 °C. Higher alcohols were found highest at high fermentation temperature. The 1-propanol, pentanol and 2-phenetyl alcohol were highest content at 35°C and 1-butanol, iso-amyl alcohol, heptanol were highest content at 30 °C, except hexanol was found highest content at 15 °C. The concentration of catechin and epicatechin in finished wine increased with temperature. The maximal concentrations were found at ambient temperature and 25 °C, respectively. Finished wines were evaluated by sensory test with twelve panelists. It was found that 5 wine characters were significantly different at 95 % confidential, including of color, body, aroma, balance, astringency and among them the aroma was related to the chemical quality of wine. However, the overall quality of wine was highly accepted in wine fermented at 25 °C.

School of Biotechnology
Academic Year 2003

Student's Signature.....
Advisor's Signature.....
Co-Advisor's Signature.....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

ALF	=	Alcoholic Fermentation
ANOVA	=	Analysis of Varience
A.M.	=	Ante Meridian
°C	=	Degree Celsius
cfu	=	Colony forming unit
d	=	Day
DVB	=	Divinylbenzene
FID	=	Flame Ionization Detecter
g	=	Gram
GAE	=	Gallic acid equivalent
GC	=	Gas Chromatograph
g_{ETH}/g_{GLU}	=	Gram of ethanol production per gram of glucose
h	=	Hour
HPLC	=	High Performance Liquid Chromatograph
l	=	Liter
M	=	Molarity
mg	=	Miligram
mg/l	=	Milligram per liter
min	=	Minute
ml	=	Milliliter
MLF	=	Malolactic Fermentation
N	=	Normality

LIST OF ABBREVIATIONS (continued)

NaOH	=	Sodium hydroxide
ODS	=	Octadecyl silane
P.M.	=	Post Meridian
ppm	=	Part per million
psi	=	Pound square inch
QDA	=	Quantitative Descriptive Analysis
rpm	=	Round per minute
Rt	=	Retention time
s	=	Second
SAS	=	Statistical Analysis System
SD	=	Standard Deviation
S-rDNA	=	Small riobosomal Deoxiribonucleic acid
t_d	=	Doubling time
T_{max}	=	Maximal temperature
UV	=	Ultra Violet
%v/v	=	Percentage volume by volume
%w/v	=	Percentage weight by volume
xg	=	Multiply by force of gravity
μ	=	Specific growth rate
μl	=	Microliter
μm	=	Micrometer

CHAPTER I

INTRODUCTION

Flavor is the summation of sensory attributes formed by complex interaction of the volatile and non-volatile compounds, which is the important factor for wine quality. Many oenological practices were conducted in the purpose of increasing favorite flavor into wine. Sources of wine flavor are grape and fermented products of winemaking. Some grape has specific aroma such as Semillon and Cabernet Savignon have vegetative aroma, that is methoxypyrazine (Waterhouse and Ebeler, 1999). Phenolic compounds in grape play a very important role in red wine sensory properties of color, aroma, astringency and bitterness (Gawel, 1998; Sun, Spranger, Roque-do-Val, Leandro and Belchior, 2001). Winemaking processes contribute flavor are fermentation and aging. The special attention on each step of winemaking brings about good flavor. Every step of winemaking is concerned with temperature since grape harvest until finished product and serving on the table. The main purpose of temperature control is to get strong good flavor. Alcoholic fermentation is the first important step in providing flavor into wine. Many investigators reported the modified temperature on wine fermentation (Gerbaux, Béatrice, and Alain, 2002; Killian and Ough, 1979; Reynolds, Cliff, Girard and Kopp, 2001). Increasing fermentation temperature from 15 °C to 30 °C trends to increase Black Currant flavor, reduce herbaceous aroma and increase wine color (Reynolds et al., 2001). Ough and Amerine (1960, quoted in Girard, Kopp, Reynolds, Cliff, 1997) found that increasing fermentation temperature caused increasing of total extraction of phenolic compound. The major compound responsible

for bitterness and astringency are catechin and epicatechin. Fischer and Noble (1994) found that increment of catechin concentration increased bitterness intensity. However, modification of temperature by using heat extraction follows by low fermentation temperature was studied. Girard et al. (1997) showed that thermovinification practiced by heat extraction followed by fermentation at 15 ºC increased ester concentration four-fold higher compared to normal practice, simulated alcoholic fermentation by ethanol supplementation increased diethyl succinate and ethyl succinate (Tensiéré, Baumes, Bayonove and Flanzy, 1989). Increasing fermentation temperatures (from 12 ºC to 27 ºC) increased both color and tannin in Pinot noir wines (Ough and Amerine, 1960). Production and retention of volatile wine components are additional areas in which temperature plays an extremely significant role (Kramer, www, 2001).

Rate of fermentation depends on fermentation condition, especially fermentation temperature, influence on yeast growth rate. Higher temperatures generally lead to increase in fermentation rate. Charoenchai, Fleet, and Henschke (1998) studied the effect of temperatures on the fermentation rate for 22 different strains of yeast. They found that higher temperatures increased growth rates. However, some different species of yeast have different temperature limitation. *Sacchromyces cerevisiae* and *S. bayanus* have the trend to be more ethanol tolerant at higher temperature than non-*Saccharomyces* yeast, such as *Klockera apiculata*. Eglinton et al. (2000) studied by using *S. bayanus* to modify the chemical and sensory profile of wine, the result indicated that *S. bayanus* produced higher concentration of some higher alcohol, particularly 2-phenylethanol than *S. cerevisae*. *S. bayanus* yeasts typically produce more 2-phenylethyl acetate than *S. cerevisae* (Antonelli, Castellari, Zambonelli, and Carnacini, 1999). Therefore, the objective of this reseach was focused on the effect of

temperatures on wine flavor purposes.

1.1 Objectives

The overall aim of this research was to find how fermentation temperatures affected the wine flavor. The specific objectives were:

1.1.1 To investigate the ester formation during fermentation time at different fermentation temperatures.

1.1.2 To determine the effect of fermentation temperature on catechin and epicatechin content in red wine.

1.2 Hypothesis

Production of good flavor wine under room temperature in Thailand is possible.

1.3 Basic agreement

This research was conducted by using Shiraz wine grape *Vitis vinifera* grown in Nakhon Ratchasima, Thailand. One hundred berries were taken from vineyard before harvesting for the measurement of sugar content in order to obtain high quality must. *S. bayanus* EC1118 (Lalermand co, Ltd.) was chosen because it grows well under severe condition and need low nitrogen for growth. All fermentation treatments were arranged in duplicates 6 l round flat bottom flasks, which were equiped with fermentation lock (air lock) and thermometers. The biological incubator was used for temperature control. The real-time temperature was recorded at each sampling time, every 12 h. Yeast kinetic and volatile esters were measured. Wine samples were taken by 120 ml for chemical analysis. Products of fermentation, ester and higher alcohol

were monitored by using Gas Chromatograph (GC). Besides, the phenolic compounds; catechin and epicatechin were determined in wine product by using High Performance Liquid Chromatograph (HPLC). Malolactic fermentation was done after alcoholic fermentation finished and then cold stabilization was conducted before aging for six months. The sensory evaluation was done with the trained panelists by Quantitative Descriptive Analysis (QDA). Statistical analysis of data was conducted by SAS (Statistical Analysis System) program.

1.4 Scope and limitation of the study

Kinetics of yeast was study because it might effect on fermentation products. The correlation between temperatures and flavors were focused on the volatile esters, higher alcohols and phenolic compounds.

1.5 Expected results

1.5.1 To obtain of the optimal alcoholic fermentation temperature for pleasant flavor formation.

1.5.2 Knowledge obtained from this research could be applied to the pilot scale and industrial scale.

CHAPTER II

REVIEW LITERATURE

2.1 The vine

Vitis is a deciduous plant that climbs by grasping supporting objects without growths of very special leaf-type organs called tendrils. There are many *vitis* grapes. *Vitis vinifera* is cultivated around the world as the true noble wine grape. For examples, Chadonnay, Sauvignon blanc, Cabernet sauvignon, Merlot, Pinot noir, and Shiraz. Another one species so-called fox grape is *V. labrusca*, such as Delaware, Niangara, Concord, Isabella. *V. riparia* species is referred to as the "post-oak" or "frost" grape, and other North American grape species are *V. aestivalis*, *V. berlandieri*, *V. cinerea*, and *V. rupestris*.

2.1.1 The development stages of grape

Grape is a berry plant. The berries are organized into a cluster. Grape develops from ovary to ripe fruit, generally divided into three phases by some parameters such as berry diameter, weight and volume. The initial rapid growth takes 45-65 days. Cellular growth begins about 2 weeks after fertilization. Chlorophyll is the predominant pigment. Berries have an intense metabolic activity and rapid accumulate of acids. Next step is slow growth phase or *vèraison.* Berries pigment has changed from colorless to colored. Growth substance is decreased but abcissic acid concentration is increased. The last phase is maturation. The respiratory intensity is decreased. This

period last 35 to 55 days. Grape accumulatesfree sugars, potassium, amino acid and phenolic compounds while malic acid and ammonium decrease (Ribèreau-Gayon, Glories, Maujean and Dubourdieu, 2000).

2.1.2 Grape berry morphology

Grape berry composed of a group of tissues or called "pericarp" surround the seed. The pericarp is divided into three types, exocarp or skin, mesocarp or grape pulp, and endocarp or tissue lines the seed receptacles containing the seed but is not distinguishable from the rest of the pulps (Ribèreau-Gayon, Dubourdieu, Donèche and Lonvaud, 2000).

2.1.3 Grape chemical constituents

A. Sugars

The principle grape sugars are glucose and fructose. Other sugars found in grape in relatively insignificant amounts. Sucrose is rarely found in *Vitis vinifera* grape. Grape sugar content varies depending on the species, variety, maturity, and health of the fruit. *V. vinifera* generally reach a sugar concentration of 20 % or more at maturity. Grape sugar content is critical to yeast growth and metabolism. It is important that most grape nutrients be in the form of glucose and fructose. Unfermented sugars are termed residual sugars. In dry wines, the residual sugar content consists primarily of pentose sugar such as arabinose, rhamnose, xylose, and small amount of unfermented glucose and fructose (1-2 g/l) (Jackson, 2000).

B. Acids

Grape contains appreciable amounts of various organic acids. Malic acid may

constitute about half of the total acidity of grapes and wine. The malic acid concentration decrease when grape mature, especially during hot period. Malic acid is one of the indicators for determining harvest date. This acid leads a flat taste into wine, the acid transformation is necessary for wine with high content of malic acid. Tartaric acid is the other major grape acid. This acid is not decrease during grape ripening but it is metabolized by few microorganism (Ough and Amerine, 1988). At wine process, tartaric acid formed crystal with potassium salt as potassium tartrate. Wines often are cooled near the end of maturation to enhance early tartrate precipitation and avoid crystal deposition in the bottle (Boulton, Singleton, Bisson and Kunkee, 1996).

C. Phenols and related phenols

Phenols are cyclic benzene compounds possessing one or more hydroxyl groups associated directly with the ring structure. There are two phenol groups occure in grape and wine, the flavonoids and the nonflavonoids (Jackson, 2000).

Flavonoids are characterized as molecules possessing two phenols joined by a pyran carbon-ring structure. The most common flavonoids in wine are flavonols, catechin (flavan-3-ol), and anthocyanin. Flavonol and anthocyanins commonly collect in cellular vacuoles of the skin. Flavan-3-ol production occures primarily in stems and seed. These tannins consist primarily of catechin, epicatechin, and gallate epicatechin subunits. Those tannins that do occur in the skin are characteriazed by a higher degree of polymerization. Flavonoids are 85% of red wine phenol content. The primary non-flavonoids are derivatives of hydroxycinamic acid and hydroxybenzoic acid. They are stored primarily in the cell vacuoles of grape cells and easily extracted on crushing. Phenolics have a number of important role to play in viticulture and enology including

UV protection, disease resistance, pollination, color, and defense against predation in plants, as well as haze formation, hue and taste in wines. Burns et al. (2000) found correlation of total phenolic compounds with antioxidant activity and vasodilation activity. In addition, the antioxidant activity was associated with gallic acid, total resveratrol, and total catechin. Red wine is a complex fluid whic contains water, sugars, acids, alcohol and a wide range of phenolic compounds. The extraction of the phenolic was influenced by vinification procedure, grape quality, and grape variety. Burns et al. (2001) indicated that wine after 9 days of vinification had antioxidant activity significantly lower than a finished wine. This suggests that the polymerlization during aging make a sizable contribution to the overall antioxidant activity of red wine.

2. 2 Wine process

Wine is fermented grape or grape juice. According to the definition, wine process starts from raw material and material preparation. Then fermentation takes place by using yeast governs the change of sugar to ethanol. It is quite so simple to make wine but human wants many things when drinks wine, such as wine balance or harmony, good of color and touch, good flavor, etc. So, they develop new method and use science and technology to increase wine quality.

2.2.1 Must preparation

There are three types of table wines, white, red and rosè wines. The name defines type of raw material. White wine is normally made from juice of white grape. Red wine made from red or black grape by using whole berry. Rosè wine is normally made from juice of red or black grape without pulp. When grape is colored, it is sampled from vineyard for sugar test every day or every week until sugar content is

high enough. Grape is harvested and destemed by hand or machine and then crushing in purpose of breaking grape berries and release juice. This helps yeast work easy and increase rate of fermentation.

A. Harvesting

Ripen grape are harvested from vineyard. It is important for wine maker to have knowledge of the composition of must because quality of finished wine depends largely on composition. Normally, grapes are sampled from wine yard before harvesting for testing sugar content.

The sampling of grape 9-14 boxes from loads of 180-200 boxes to ensure a deviation of less than 0.05 °Brix and sampling of 200-500 single berries from all parts of the cluster and from all parts of the vines to reduce the standard error of the mean to ± 0.25% was recommended (Ough and Amerine, 1988).

B. Crushing

A suitable crusher and destemmer are used for crush and destem all grapes in order to release the pulp and juice (Ribéreau-Gayon, Dubourdiéu, Donéche and Lonvaud, 2000). If producing of small amount of wine, it is possible to do by hand. Sulphur dioxide is added in this step, to prevent growth of bacteria and mold and inhibit oxidation, 50-100 ppm is recommended (Mayén, Mérida and Medina, 1995).

C. Pectic enzyme treatment

Recently, the enzyme technology offers enormous benefits to wine industry. Commercial enzyme preparations are available specifically for improving the maceration of grapes, color extraction, wine filtration and wine quality. There are three

main enzymes using in wine production. Pectinases, β-glucanases and hemicellulases have main benefits as mentioned above. The first microbial enzyme used in the wine industry was a commercial pectinase, which from *Aspergillus* contains varying amounts of pectin esterase, polygalacturonase, pectin lyase and small amount of hemicellulase. Gerbaux et al. (2002) found 16% increasing of polyphenols in Pinot noir must treated by pectinase. Currently, β-glucosidase has attracted in the wine industry because of its ability to improve the aroma of wine. Cabroglu, Selli, Canbas, Lepoutre and Günata (2003), studied of using pectinase enzyme possessing the glycosidases to enhance wine flavor. They found the enzyme treated wines were highly significantly different from non-treated wine. Especially, the volatiles, monoterpenes, C_{13}-norisoprenoids, and benzene derivatives were increased in enzyme treated wine. The use of pectolytic enzyme to facilitate extraction or clarification of the must may cause an increase in methanol as a result of the pectin esterase activity. However, grape has a relatively low pectin content, wine is the fermented beverage with the lowest methanol concentration, between 30-35 mg/l (Ribéreau-Gayon, Glories, Maujean and Dubourdieu, 2000).

D. Must adjustment

Acidity and pH are important for growth of yeast and to ensure the right balance of acidity to the finished wine. The optimal pH values for yeast growth range from pH 4-6 (Cantarelli and Lanzarini, 1989, quoted in Fleet, 1996). Some low acid must should be added with tartaric acid, which is naturally found in grape. Commercial standard dictate a grape juice acidity of about 0.6-0.9 % (as grams of tartaric acid per 100 ml of juice). Dry table wines should have in the range 3.1-3.6. Table wine usually has a pH not exceeding 3.6 and dessert wine more than 3.8 (Ough and Amerine, 1988).

Nitrogen content plays an important rule in fermentation. It is one of growth limiting factors for yeast. Must is frequently supplemented with assimilable nitrogen (Fleet, 1996). Diammonium phosphate is widely used for nitrogen and phosphorus addition in wine to ensure enough nitrogen content for yeast growing (Monk and Storer, 1986; Vianna and Ebeler, 2001). Urea and several commercial yeast foods are alternative nitrogen supplements.

2.2.2 Alcoholic fermentation

Saccharomyces metabolize glucose and fructose to pyruvate via the glycolytic pathway (Figure 2.1). One molecule of glucose or fructose yields two molecules each of ethanol and carbon dioxide. The theoretical conversion of 180 g of sugar into 88 g of carbon dioxide and 92 g of ethanol means that yield of ethanol is 51.1% on a weight basis. In model fermentation starting about 22 to 24 % sugar, 95 % of the sugar is converted into ethanol and carbon dioxide, 1% is converted into cellular material, and the remaining 4% is converted to other end products. This percentage may vary depending upon inoculum size, fermentation temperature and nutrient availability (Boulton et al., 1996).

Typical inoculum of $1x10^6$ cells/ml will occur to reach a final cell density of 1 to $2x10^8$ cells/ml. At present, the commercial yeast is available in dehydrated form. Prior to addition to must, yeast must be rehydrated and expanded to 2 to $5x10^6$ cells/ml (Zoecklein , Fugelsang , Gump and Nury, 1995). There is another one method of red wine making, carbonic maceration. Carbonic marceration may be as old as winemaking itself. It was initially purposed to refer to the anaerobic maceration of whole berries placed in an atmosphere of carbon dioxide. It happened by grape-cell alcoholic fermentation or flushing with carbon dioxide.

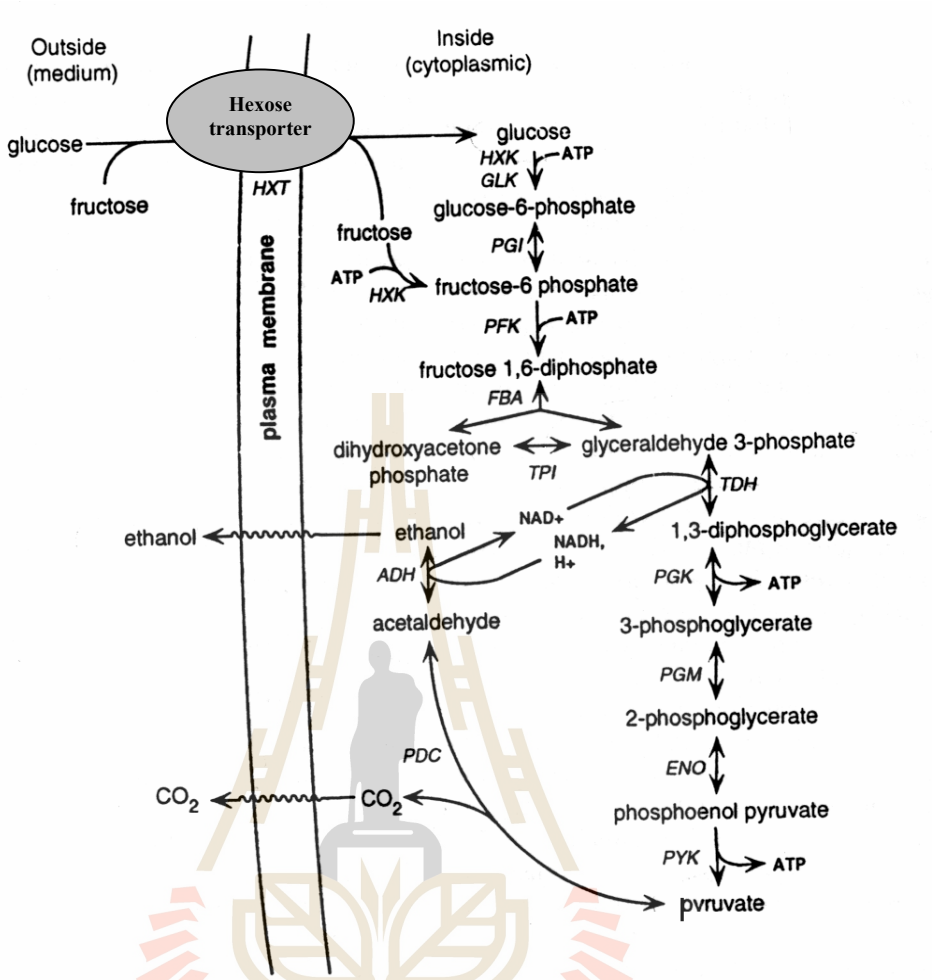


Figure 2.1 Enzymatic steps of the glycolytic pathway HXT (hexose transporter), HXK (hexokinase) GLK (glucokinase), PGI (phosphoglucose isomerase), PFK (phosphofructokinase), FBA (aldolase), TPI (triosephosphate isomerase), PGM (phosphoglycerate mutase), ENO (enolase), PYK (pyruvate kinase), PDC (pyruvate decarboxylase), ADH (alcohol dehydrogenase) (Boulton et al., 1996).

2.2.3 Malolactic fermentation

The principle effect of malolactic fermentaton is a reduction in acidity. Wine with excessive acidity, the reduction is desirable. The malolactic fermentation is analogy term with alcoholic fermentation, the metabolizing of L-malic acid (dicarboxylic acid)

to L- lactic acid (a monocarboxylic acid) is not a fermentative pathway, but decarboxylation. Malic acid decarboxylation is catalized by malolactic enzyme (Van-Vuuren and Dicks, 1993). Lactic acid bacteria are one of few bacterial groups capable of growing under acidic condition, pH below 5. During their growth, lactic acid bacteria ferment residual sugars, hexoses and pentoses left by yeast.

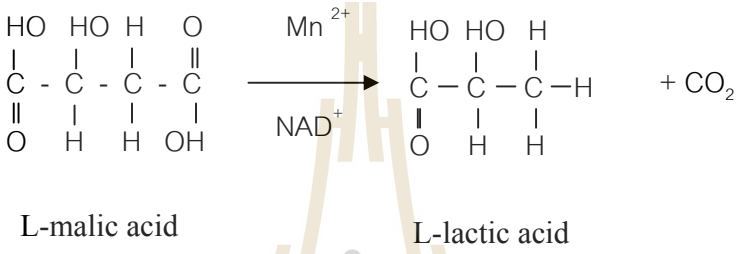


Figure 2.2 Transformation of malic acid to lactic acid.

Normally, bacterial population on grape berries is low. The population varies from 10^2 cfu/ml to 10^4 cfu/ml, depending on climatic condition during the final days of maturation. At this stage up to 8 or 9 species can be identified. Four genera are represented: *Lactobacillus, Pediococcus, Leuconostoc* and *Oenococcus*. Lactobacilli belongs to facultative (*Lactobacillus plantarum*, *L. casei*) and obligatory (*L. hilgardii, L. brevis, L. fructivorans*) heterofermentative species. The homofermentative cocci are mainly *Pediococcus damnosus and P. pentosaceus, P. parvulus*. Heterofermentative cocci of wines were classified in the *Leuconostoc* genus until 1995 in the species *Leucoconostoc mensenteroides* and *Leu. oenos*. The molecular approach to taxomomy, based on 16S-rDNA and 23S-rDNA sequencing, led to the creation of a new genus *Oenococcus*, with the species *Oenococcus oeni* (Dicks, Dellaglio, and Collins, 1995)

Numerous substances produced by bacteria are involved in the aroma changes of wine during malolactic fermentation. One of most significant is diacetyl,

which is buttery descriptor for sensory panelists and acetoin, which is also produced from citric acid metabolism by lactic acid bacteria (Lonvaud-Funnel, 1995).

2.3 Volatile compounds in wine

Wine color, aroma, and flavor depend on both the initial composition of the grapes and subsequent enological processes. During the primary (alcoholic) fermentation of grape must, the wine yeast produces ethanol, carbon dioxide, and some of by-products, including esters, aldehyde, higher alcohol. The volatile end products produced by yeast during fermentation can lead to increased complexity in wines (Fleet, 1996).

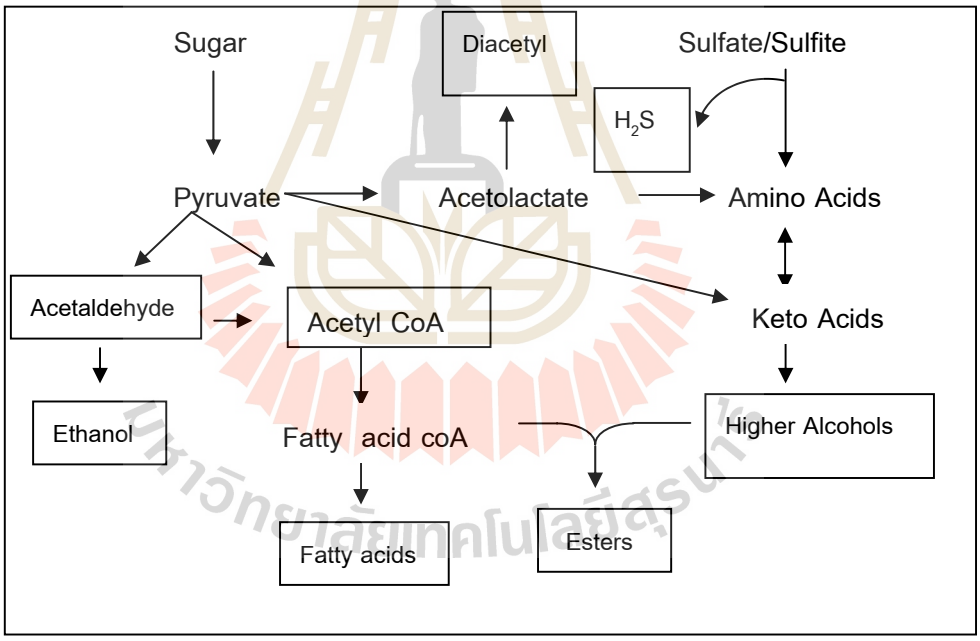


Figure 2.3 Derivation of flavor compounds from sugar, amino acids and sulfur metabolism by yeast (Fleet, 1996).

2.3.1 Ethyl alcohol

Ethanol in wine is mainly produced by the alcoholic fermentation of sugar in must. It has pungent odor and has multiple effects on taste and mouth-feel. Fisher

and Noble (1994) studied the effect of ethanol on sourness and bitterness of wine by using 20 judges to evaluated 3 levels of ethanol. Increasing of ethanol reduced sourness and increased bitterness because ethanol changed the ion active and passive influx and efflux of ions in the taste bud receptor cells. Besides, ethanol's solvent property is also useful for dissolving phenols from grape pomace and certainly contributes to the expression of aromas in wine (Ribéreau-Gayon, Glories, Maujean and Dubourdieu, 2000).

2.3.2 Higher alcohols

One important group, which contributes complexity to the bouquet is the higher (fusel) alcohol, the alcohol with more than two carbon atom. They commonly account for about 50% of the aromatic constituents of wine, excluding ethanol (Ron, 2000). The principal higher alcohols produced by yeast are the aliphatic alcohols n-propanol, isobutanol (2-methyl-1-propanol), active amyl alcohol (2-methyl-1-butanol), isoamyl alcohol (3-mthyl-1-butanol), and the aromatic alcohols hexanol and 2-phenethyl alcohol (Table 2.1). At low concentrations (less than 300 mg/l), they contribute to wine's aromatic complexity. At higher levels, their penetrating odors mask the wine's aromatic finesse (Ribéreau-Gayon, Glories, Maujean and Dubourdieu, 2000).

Higher alcohols are formed by yeast, directly from sugars or from grape amino acids by the Erlich reaction. Reaction is caused by the activity of a FAD^+ dehydrogenase, which oxidizes amino acids into amino acids. These are hydrolyzed into α-keto acid, then subjected to the action of a decarboxylase with thaiamin pyrophosphate coenzymes (TPP). The higher alcohol content of wine varies according to fermentation condition, especially the species of yeast. Although, they exhibit

unpleasant aroma, at the concentrations generally found in wine, below 300 mg/l, they are desirable (Fleet, 1996). They contribute to a wine's aromatic complexity.

Table 2.1 Metabolites of amino acids and related compounds.

Amino acids	**Keto acids**	**Aldehydes**	**Alcohols**
Alanine	2-ketopropionic acid	Acetaldehyde	Ethanol
Serine	3-Hydroxy-2-keto-propionic acid	Glyoxal	Glycol
2-Amino-butyric acid	2-ketobutyric acid	Propionaldehyde	1-propanol
-	-	Butylaldehyde	1-butanol
Valline	2-ketoisovaleric acid	isobutyraldehyde	2-methyl-1-propanol
Isoleucine	2-keto-3-Methyl-valeric acid	2-Methylbutyraldehyde	2-Methyl-1-butanol
Leucine	2-ketoisocaproic acid	Isovaleraldehyde	3-Methyl-1-butanol
-	-	Hexanal	1-hexanol
Phenylanine	3-phenyl-2-keto-propionic acid	-	Phenethyl alcohol

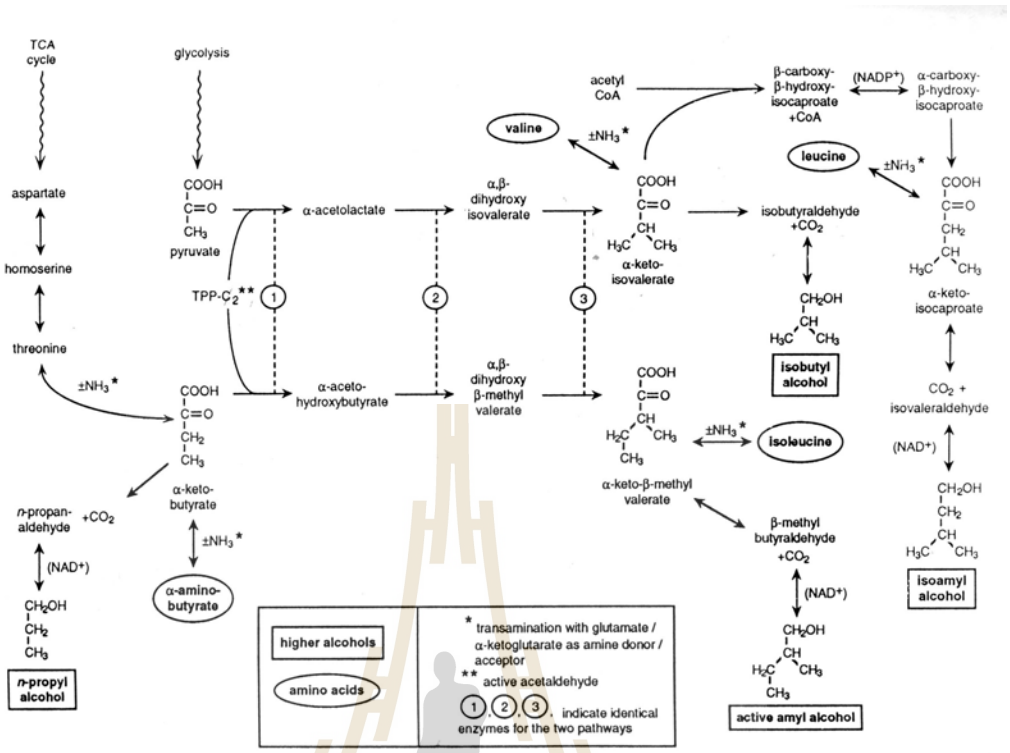


Figure 2.4 Pathway for formation of higher alcohol from glucose (Boulton, 1996).

2.3.3 Ester

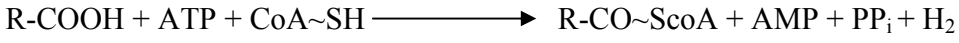
Among of secondary products of fermentation, ester plays an important role in wine odor, which impart pleasant smell. Ester is formed when an alcohol function reacts with an acid function and water molecule is eliminated. There are a lot of different alcohols and acids in wines, so the number of possible ester is also very large. Ester in wine have two distinct origins; enzymatic esterification during the fermentation process and chemical esterification during long term aging (Ribéreau, Glories, Maujean and Dubourdieu, 2000).

The enzymatic esterification is governed by yeast and influenced by many factors such as yeast strains (Antonelli et al., 1999; Vainna and Ebeler, 2001) and fermentation condition; temperatures, oxygen levels and unsaturated fatty acids (Killain

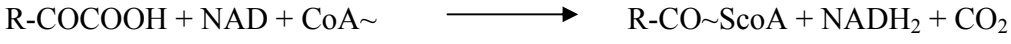
and Ough, 1979; Nykänen, 1986). Two important groups of ester are fatty acid ethyl ester and acetate ester, which contribute floral and fruity sensory properties to the wine. Fatty acid ethyl esters (e.g., ethyl butanoate, ethyl hexanoate, ethyl octanoate, etc.) are obtained from ethanolysis of acetyl coenzyme-A that is formed during fatty acid synthesis or degradation. According to the mechanism describes in Figure 2.5, acetyl CoA function as the key compound in the biosynthesis of esters. During fermentation, yeast produces acetyl-CoA in cells either through the activation of fatty acid or through oxidative decarboxylation of keto acid. These two mechanisms mainly differ in the capacity to utilize ATP, the activation of fatty acid requires ATP and the oxidative not.

Regarding the effect of temperatures on ester production, Killian and Ough (1979) found that at temperatures 15 °C and 20 °C promote the production of higher-molecular weight esters or some fatty acid ethyl ester, such as ethyl octanoate (apple-like aroma), ethyl decanoate. However, higher temperatures also tend to suppress ester accumulation by favoring hydrolysis, whereas, the low fermentation temperatures (~ 10°C) favor the synthesis of acetate esters, such as isoamyl acetate (banana-like aroma), isobutyl acetate and hexyl acetates. The pattern of ester formation is increased with alcohol concentration. Killain and Ough (1979); Vianna and Ebeler (2001) showed the similar pattern of isoamyl acetate and ethyl acetate concentration which were highest at the late of fermentation even they did with different white grape varieties and yeast strain. However, Vianna and Ebeler found that some esters increased again at the second week after fermentation finished in the Chardonnay wine, the reason for this is might be due to yeast cell is autolysed. Charpentier, Nguyen, Bonaly and Feuillat (1986) showed that there is a loss of both amino acids and glucans in the cells wall of yeast during autolysis which results in a structural loosening of these cell walls.

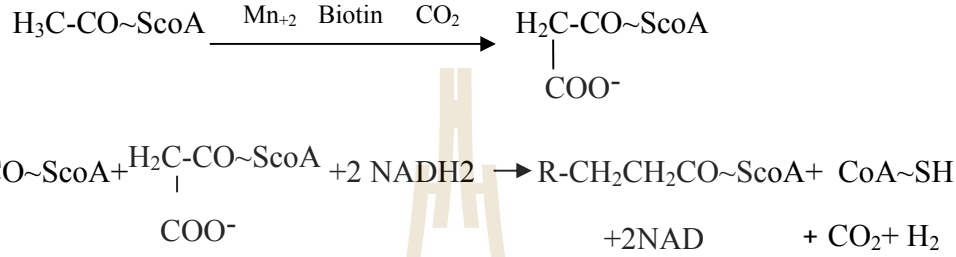
By activation of monocarboxylic acid :



From 2-oxo acid by oxidative decarboxylation :



From intermediates of long chain monocarboxylic acid synthesis



Erters are formed by alcoholysis of acyl – CoA compounds:

Figure 2.5 Path way of ester formation.

CHAPTER III

MATERIALS AND METHODS

3.1 Materails

3.1.1 Chemicals

All chemicals used for chemical reaction were of analytical grade and some chromatographic grade for chemical analysis by HPLC, and were purchased from Carlo Erba Reanenti, Fluka Chemika, Difco (Detroit, MI, USA) and Across.

3.1.2 Microoganisms

Microorganisms for wine fermentation were *Saccharomyces bayanus* EC1118 and *Leuconostoc oenos* (commercially as Vinifera).

3.1.3 Grape

Shiraz (*Vitis vinifera*) was obtained from vineyard at Park Chong, Nakhon Ratchasima. Grape was harvested on December, 2001.

3.1.4 Equipment

Equipment used were as follows: high-performance liquid chromatograph: HPLC (Thermo Seperation Products Inc., USA), gas chromatograph: GC (Autosample XL, Perkin Elmer), incubator, hot air oven, refrigerator (4 °), freezer (-20 °), larminar flow hood, pH-meter, analytical balance, Compound microscope, Sonicator, Vortex mixer and

basic microbiological equipment.

3.1 Methods

3.2.1 Winemaking

A. Must preparation

Shiraz (*Vitis vinifera*) grape berries were harvested from vineyard and cooled down with dry ice and kept frozen until used. Grape bunches were destemed, and crushed by crushing machine. Then, must was treated with 100 ppm sulphur dioxide prior to added 0.05 % (v/v) pectinase enzyme. The maceration was done for overnight at room temperature. The grape pomace was separated before clarification by centrifugation at 10,000 rpm for 5 min at 4 °C. Must acidity and total soluble was measured and adjusted to 22 °Brix (Ough, and Amerine, 1988).

B. Starter preparation

Sterilized juice was inoculated with *S. bayanus* EC1118 strain then incubated at 25 °C for 18 hours. The yeast cell population was determined by spread plate technique on YM media.

C. Alcoholic fermentation

Must was fermented in 6 l fermentation flask with volume of 3.6 l and inoculated with 10% inoculum size (400 ml of starter). The alcoholic fermentation was done in duplicates at five varied temperatures placing in biological incubator (15 ºC, 20 ºC, 25 ºC, 30 ºC and 35 ºC) and one treatment was placed at ambient temperature. The fermentation temperatures were recorded every sampling time. Wines were taken

every 12 hours until the end of alcoholic fermentation. Samples were centrifuged at 4,500 rpm for 5 minutes and supernatant was kept at –20 ºC until needed.

D. Malolactic fermentation

Malolactic fermentation was inoculated with 5 mg of *L. oenos* (Vinifera) in dried powder, it was done after alcoholic fermentation finished. The initial population was 2.16×10^6 cfu/ml. Sampling was done once a week for one month.

E. Cold stabilization

Juice was typically supersaturated with potassium bitartrate at crushing. As the alcohol content raised during fermentation, the solubility of bitartrate decreased. This practice was to induce the slow precipitation of potassium bitartrate. For cold stabilization, table wine was chilled near the wine's freezing point. The stabilization was estimated using the formula empirically established by Perin (Jackson, 2000).

Temperature (-°C) = (% alcohol ÷ 2) – 1

In this experiment, the expected alcohol content was 12%, thus cold stabilization was done at –5 °C for one month.

D. Aging

Wine was aged at 20 °C for six months.

3.2.2 Microbiological methods

A. Yeast viability

The active yeast cells were determined at each sampling time by spread plate technique on YM media (Golden and Beuchat, 1990). Yeast culture was incubated at 30 º C for 24 hours.

B. *Leuconostoc oenos* viability.

The viability of dried form bacteria (cell/mg) was determined by weighing 1 mg of dried powder and suspended in 1 ml of 0.9% NaCl with 0.1% peptone (Nielsen, Prahl and Lonvaud-Funel, 1996), followed by pour-plate seeding in *Leuconostoc oenos* medium (Deutsche Sammlung von Mikroorganismen und Zellkulturen, www, 1999). Viable count was obtained after incubation at 20 ºC for a week and viability was $4.32x10^7$ cells/mg.

3.2.3 Chemical Analysis

A. Total soluble solid, Acidity and pH of must

The total soluble solid was done by using hand refractometer. Titration technique for acidity determination was done by titration with 0.1 N NaOH and phenolpthalein as indicator. End point of titration at pH 8.2 was recognized by pH-meter (Ough, and Amerine, 1988). The pH value of juice was determined by pH-meter.

B. Total nitrogen determination

The total nitrogen content of wines is determined by using the Kjeldahl method. Sulfuric acid-salicylic acid reagent was prepared by dissolved 33 g of salicylic acid in 1 l of concentrated sulfuric acid used for sample digestion. The amino nitrogen was converted to ammonium bisulfate, and organic materials of sample were

destroyed by boiling with strong acid. Boiling mixture of 1 g of copper sulfate pentahydrate, 2 g of ferrous sulfate heptahydrate, and 20 g of sodium sulfate was used for rising the boiling temperature. Five ml of must and wine sample was used for this analysis. Digestion was done in 250 ml digestion tube by added 20 ml of sulfuric-salicylic acid, 3 g of boiling mixture, small amount of selenium and few drops of silicone as anti-foam. Digestion temperature was 420 °C until solution cleared. After the tube was cooled down, nitrogen distillation was done by automated distilator. The distillation program was set as 100 ml of distilled water added, 60 ml of 12 N NaOH added, and distillated for 6 mins. 30 ml of 4 % boric acid was used for ammonia collection. Then, titration with 0.1 M HCl was done by using mixture indicator of methyl red and bromocresol green (Ough and Amerine, 1988).

C. Determination of ester formation during fermentation time

Liguid-liquid extraction was chosen for this experiment. It is a separation process that takes advantage of the relative solubility of solutes in immiscible solvents. The solute dissolves more readily and become more concentrated in the solvent in which it has a higher solubility. Dichloromethane is one of the best solvent was used for ester extraction with the low boiling point, 40 °C. Frozen wine sample was thawed at 4 °C, then put in an ice bath. Dichloromethane (40 ml) was added and the mixture was stirred during 15 min at 500 rpm. The wine-solvent mixture was supplemented with 40 ml of dichloromethane and stirring was continued for 15 minutes. The organic phase was separated in a separating funnel, then was centrifuged for 5 minutes at 10,000xg (at 4 °C) for completely separated two phases and removed emulsion. The volatile extract was dried over sodium sulfate at 0 °C for 12 hours and then it was concentrated to 1 ml by evaporated in water bath 40 °C. The final

concentration factor was 100 (Kotseridis and Baumes, 2000). The volatile extract was frozen until ready for GC analysis.

GC condition; the extract was analyzed by Perkin-Elmer XL Autosample instrument with the PE-1 column (5% polydimethylxyloxane). The method was modified from Ramey and Ough, (1980) by using helium instead of nitrogen gas as mobile phase at 7 psi. The oven temperature program was 55 °C for 10 minutes, ramped to 155 °C with rate 3.0 °C/min, FID detector was 270 °C and injection temperature was 220 °C.

The following standards were used to construct standard curves; ethyl acetate, *n*-propyl acetate, ethyl butyrate, isoamyl acetate, hexyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and phenethyl acetate. The n-butyl acetate was used as an internal standard. All ester standards were of greater than 99% purity.

D. Determination of higher alcohol during fermentation time

The higher alcohol including, 1-propanol, 1-butanol, iso-amyl alcohol, pentanol, hexanol, heptanol and 2-phenetyl alcohol were extracted from wine by using solid phase microextraction (SPME). The 50/30 μm DVB-Carboxen-PDMS fiber was chosen for sample preparation. SPME is a recent technique and available commercially since 1994. A fiber was used to extract small amounts (ppm) of analytes from a solution. This technique is composed of two steps (Figure 3.1; 3.2). First is an extraction step, where analytes are sorbed onto the fiber and extracted from the solution or the headspace. Then, a desorption step, which analytes are thermally desorped into a heated GC injection port.

The 0.8 ml of wine sample was taken into 2 ml vial and was added 25 %w/v of NaCl for increasing of volatility of analytes. Sample was sonicated in purpose of molecule vibration and move into the headspace, this was done for 15 minutes and then, heated sample at 35 °C for 15 minutes. An extraction step took place by piercing septum vial by a septum piercing needle. After that, the fiber was exposed to the headspace of sample (2 mm above sample surface), for 5 minutes. This allowed the analytes from the solution to diffuse into the fiber. Then, the fiber was retracted inside the septum piercing needle and the fiber holder was removed from the sample. All analytes, which were absorbed on the fiber was desorped by piercing GC inlet septum. Then the fiber was exposed to the hot GC for all running time in order to allowed complete desorption and cleaning. Finally, fiber was retracted inside the septum piercing needle and the fiber holder was removed from the GC.

Standard curves were constructed by using model wine solutions. These were prepared with deionized water and contained 0.75 g/100 ml tartaric acid and 12 % ethanol. The pH was adjusted to 3.5 with 6 N NaOH (Vianna and Ebeler, 2001). All higher alcohol standards were of greater than 99% purity. The standards were sampled by SPME in the same way with sample.

GC condition; The PE-Wax column was used as stationary phase and helium gas as carrier. The GC condition was modified from Vianna and Ebeler, (2001) by adjusting injection port and FID detector temperature to 220 °C. An oven program was 30 °C for 5 minutes and ramped to 220 °C by rate 10 °C /min.

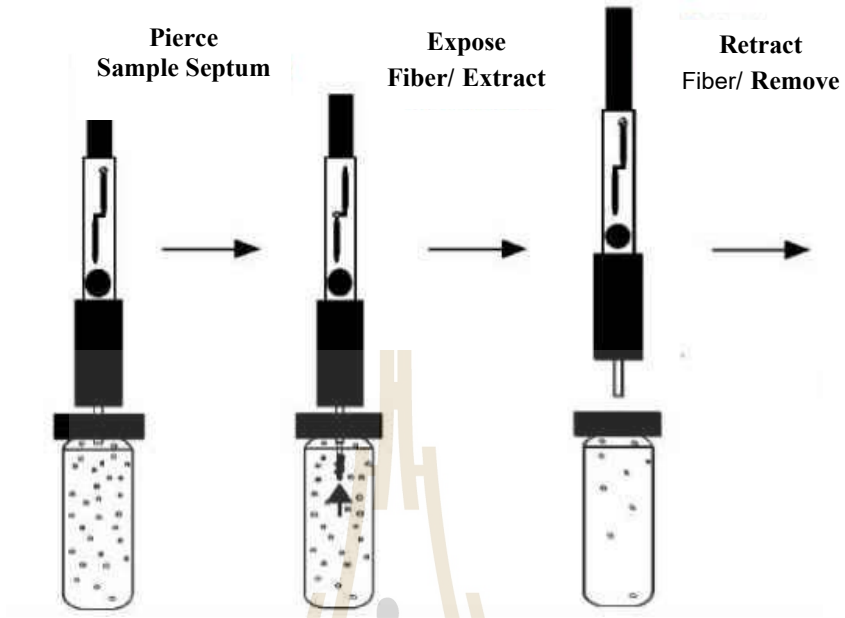


Figure 3.1 Extraction step of SPME.

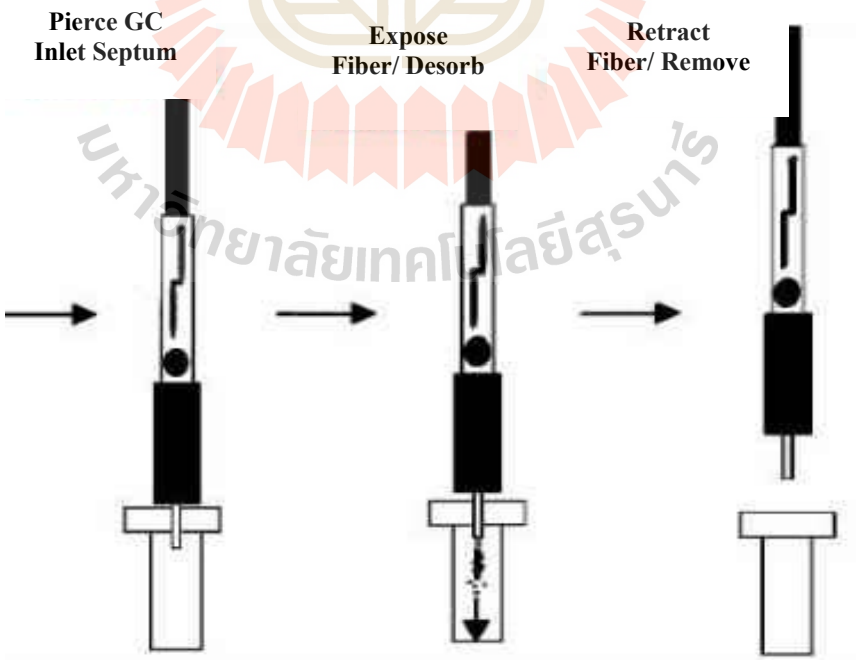


Figure 3.2 Desorption step of SPME

E. Ethanol determination

Ethanol content was determined by using direct injection of 1 µl of prepared wine sample (0.45 µm membrane-filtered) into Autosample XL GC, Perkin Elmer, with split ratio 100:1. The analytical conditions were modified from Martínez, Valcárcel, Pérez and Benítez (1998), by using thermal program instead of isothermal for oven temperature program. It was 40 °C for 2 minutes and ramped to 80 °C by rate 10°C /min, hold for 2 min, an injector temperature was 200 °C. PE-Wax column was used as stationary phase and helium was adjusted to 14 psi. FID was programmed temperature to 150 °C.

F. Glucose determination

Five µl of the prepared sample or standard solution (0.45µm memebrane-filtered) were injected into Thermo Separation Product liquid chromatograph equipped with refractive index detector and anlyzed using Rezex RPM-Monosaccharide with a guard column cartridge. The column was operated at 75 °C and eluted with deionized water (flow rate, 0.6 ml/min) (Phenomenex, www, 1999).

H. Detemination of epicatechin and catechin

High performance liquid chromatographic (HPLC) method was used to detecteded catechin and epicatechin. Wine sample was passed through 0.45 µm membrane filter. The five microliter of wine sample was injected. The water spherisorb ODS2 (octadecyl silane 2) column was used as stationary phase and 10% acetonitrile in water adjusted to pH 1.5 with trifluoroacetic acid as mobile phase UV-Visible detector was set at 280 nm (Burns et al., 2000).

I. Malolactic fermentation monitoring

Malic acid disappearing was monitored by paper chromatographic method. Wine acid partition themselves in a chromatographic system according to their relative affinities for the mobile solvent and stationary phase. The solvent mixture used contained the pH indicator bromocresol green, which undergoes a color change from yellow to blue in the pH range 3.8-5.4. The presence of an acid is indicated as a yellow spot on a blue background (Zoecklein, Fugelsang, Gump and Nury, 1995).

Wine acid including of malic acid, tartaric acid and lactic acid (0.3%w/v) were used as standard. The ten microliters of all acids and wines were spotted on Whatman No.1 chromatographic paper, then put into chromatography developing tank contained chromatographic solvent. The solvent prepared from 100 ml of n-butanol, 100 ml of deionized water, 10.7 ml stock formic acid and 15 ml of 1% bromocresol green indicator solution prepared by deionized water. Solvent mixture was taken to separatory funnel for discarded organic phase.

J. Total phenolic compound

Folin-Denis method was used for total phenolic compound determination. The standard curve was constructed by pipetted 0, 1, 3 , 5, and 10 ml aliquots of phenol stock solution into 100 ml volumetric flasks, and diluted each to volume with water. The phenol concentrations of these solutions (expressed as gallic acid equivalents, GAE) were 0, 50, 100, 150, 250, and 500 mg/l. From each solution, pipetted 1 ml into 100-ml volumetric flasks, each flask was added with 60 ml of water, mixed, and was added 5 ml of the Folin-Ciocalteu reagent and mixed, after 30s and before 8 min. After that, the mixture was added with 15 ml of the 20% sodium

carbonate solution, mixed, and brought to volume with water. Then, let the solutions stayed for 2h at 20 ºC, and determined the absorbance of each solution at 765 nm against the blank in 10-mm cells and plotted absorbance against concentration. Red wine sample was 10 times diluted with water (Ough and Anerine, 1988).

3.2.3 Sensory evaluation

Quantitative Descriptive Analysis (QDA) was used in this study. Wine samples were tasted by trained panelists. All panelists were the person who interested in wine tasting. Panelists were trained to identify volatile flavors and tastes of wine by using model substances (Gutierrez-Afonzo, Darias, Armas, Medina and Eugenio-Diaz, 1998). Then, all of them were qualified by using pair different test and triangle test. The panelists who had more than 75% total score were chosen to taste wine (Lawless and Heyman, 1998). The sensory evaluation system was set appropriately by follow the university wine course; a wine appreciation text and self-tutorial (Baldy et al., 1993). The sample was served at 20 °C and two sets of samples were served at eleven A.M. and two P.M.

3.2.4 Statistical analysis

Analysis of variance (ANOVA) was applied to the sensory data by using the SAS (Statistical Analysis System) program, version 6.08 for windows (Copyright 1989 by SAS Institute Inc., Cary, North Carolina, USA) (Girard et al., 1997).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Wine fermentation

4.1.1 Must characterization

The must characteristics were analyzed not only with the fresh must but also must after treated by pectinase enzyme and clarification. All characters were not different among each step of preparation, except for the total nitrogen, which increased after treated by pectinase, and was decreased after clarification. The results are summarized in Table 4.1.

Table 4.1 Must characteristics.

Characters	Fresh must	After treated by pectinase enzyme	After clarification
Sugar content (°Brix)	17.4	17.2	17.2
Acidity (g/l)	0.696	0.693	0.693
pH	3.61	3.64	3.64
Total Nitrogen (mg/l)	708.29	799.67	700.67

The sugar was added into must for adjustment of sugar content to twenty-two degree brix in order to get at least 12 % ethanol and treated with 100 ppm sulphur dioxide for microbial inhibitory and prevented must oxidation before fermentation took place. The pH of must after clarification step was 3.64 and range of optimal pH for yeast growth is 3.0 to 4.0 (Charoenchai et al., 1998). Therefore,

there was no need for pH adjustment. The total nitrogen was determined to ensure sufficient nitrogen content for yeast growth. Normally, total nitrogen content of grape juice ranges from 60-2400 mg N/l (Fleet, 1996). Cantarelli and Lanzarini (1989, quoted in Fleet, 1996) recommended that minimum of 200 mg N/l was necessary for adequate yeast growth and fermentation. Thus, total nitrogen of must in this experiment was enough for yeast growth.

4.1.2 Fermentation kinetics

Fermentation was done at various temperatures, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and ambient temperature by using biological incubators for temperature control. The temperature of fermentation flask was monitored every 12 hours (appendix A). The temperature of fermentation flask at ambient condition was fluctuated during day and night between 27 °C to 32 °C, which the averaged at 29.3 °C. The summarized results of fermentation kinetics are presented in Table 4.2 and yeast growth kinetics were shown in Figure 4.1-4.4. The temperature strongly affected to fermentation kinetics, the results showed that temperature effected on length of fermentation time. Fermentation finished time was considered when no residuals sugars were left (less than 2 g/l) (Torija, Rozés, Poblet, Guillamón and Mas, 2003). For this experiment, it was the time of glucose left less than 1 g/l. Low fermentation temperature took long length of fermentation time. These results related to the rate of glucose consumption, which was increased with increasing temperature. At 35 °C, glucose was rapidly consumed by yeast in the beginning and still after 84h of fermentation time (Figure 4.2). Yeast could not consume all sugar which, called stuck fermentation and there was glucose left 5.117 g/l at 180h. The result of yeast growth indicated that viable cell concentration at 35 °C was rapidly decreased before

fermentation completed (Figure 4.1). The temperatures lower than 35 °C caused yeast utilized most of sugar.

Table 4.2 Fermentation kinetics of yeast at various temperatures.

Parameters	Temperature (°C)					
	15	20	25	30	35	Ambient
Fermentation time (h)	168	144	120	96	84 [a]	84
Maximal population (cfu/ml)	$1.81x10^8$	$1.67x10^8$	$1.65.x10^8$	$1.02x10^8$	$4.75x10^7$	$1.19x10^8$
Specific growth rate (μ) (h^{-1})	0.1051	0.1312	0.1766	0.1593	0.1420	0.1571
T_d (h)	6.59	5.28	3.92	4.35	4.88	4.41
Rate of glucose consumption (g/l/ h)	0.747	0.855	1.043	1.280	1.369 [b]	1.427
Ethanol concentration (g/l)	111.96	112.05	112.77	108.57	93.47	103.64
Rate of ethanol production (g/l/h)	0.667	0.778	0.940	1.131	1.113 [b]	1.234
Yield of ethanol (g_{Eth}/g_{Glu})	0.875	0.875	0.881	0.848	0.730	0.810

[a] Stuck fermentation occurred. [b] Calculated by stuck fermentation time

The rate of yeast growth was increased with temperature according to the specific growth rate at 15 °C was lowest, 0.1051 h^{-1} and highest at 25 °C, 0.1766 h^{-1} (Figure 4.4). For the ambient condition, the fluctuated temperature activated yeast to consume sugar rapidly but maximal population was not high. Although, yeast could utilize all glucose, but ethanol concentration at this condition was low, 103.64 g/l, yield of ethanol was 0.813 g_{Eth}/g_{Glu}. The reason might be yeast used some sugar to

maintain cell to survived under severe condition, so viable cell concentration was not much, the maximal population was 1.19 x 10^8 cfu/ml at 36h. Moreover, the trend of glucose consumption and ethanol production in must fermented at 30 °C were similar to ambient temperature (Figure 4.2-4.3). Ethanol production and yield of ethanol were slightly different among 15 °C, 20°C and 25 °C, 111.96 g/l, 112.05 g/l, and 112.77 g/l, respectively.

The excessive temperature, for this experiment was 35 °C caused yeast could not completely utilize nutrients in wine and rapidly died before fermentation completed. It was found that, the maximal temperature for *S. bayanus* EC1118 was lower than 35 °C, which were similar to Van (1984), who studied about temperature profile of yeast and found that *S. cerevisiae* strains had T_{max} values ranged from 35-43 °C, whereas strain of *S. bayanus* and *S. pastorianus* failed to grow above 35 °C. Thus, the maximal temperature of *S. bayanus* EC1118, which was found in this experiment was the ambient temperature, 27-32 °C. The maximal temperature for growth is relatively constant within a species. Charoenchai et al. (1998) studied the effect of temperature on growth rate of twenty-two strains of wine yeasts. The different yeast strains showed different specific growth rate at different temperature. The maximal specific growth rate of *S. bayanus* EC1118 was found at 25 °C, 0.1766 (h^{-1}) and declined when temperature above 25 °C. Thermal damage to yeast cells result from disruption of hydrogen bonding and hydrophobic interactions leading to general denaturation of proteins and nucleic acids (Walker, 1998). Watson (1987) had review the influence of sub-optimal and supra-optimal growth temperatures on various morphological features of psychrophilic, mesophilic and thermophilic yeasts and mentioned that high temperature effected on plasma membrane structure and

function. The fluidity of plasma membrane is increased whereas the permeatability of essential nutrients is reduced. This might be the reason of low population found at high temperature because the essential nutrients could not easily pass trough cell membrane.

The malolactic fermentation was done after the end of alcoholic fermentation by inoculating 5 mg of dried form *L. oenos* in order to get $2.16x10^6$cfu/ml. The result of paper chromatography showed that malic acid conversed to lactic acid completely with in one month (data not shown). Then, all wines were stabilized at -5 ° C for one month and aged at 20 ° C for six months. Chemical analysis was done in each step and sensory evaluation was done with finished product, respectively.

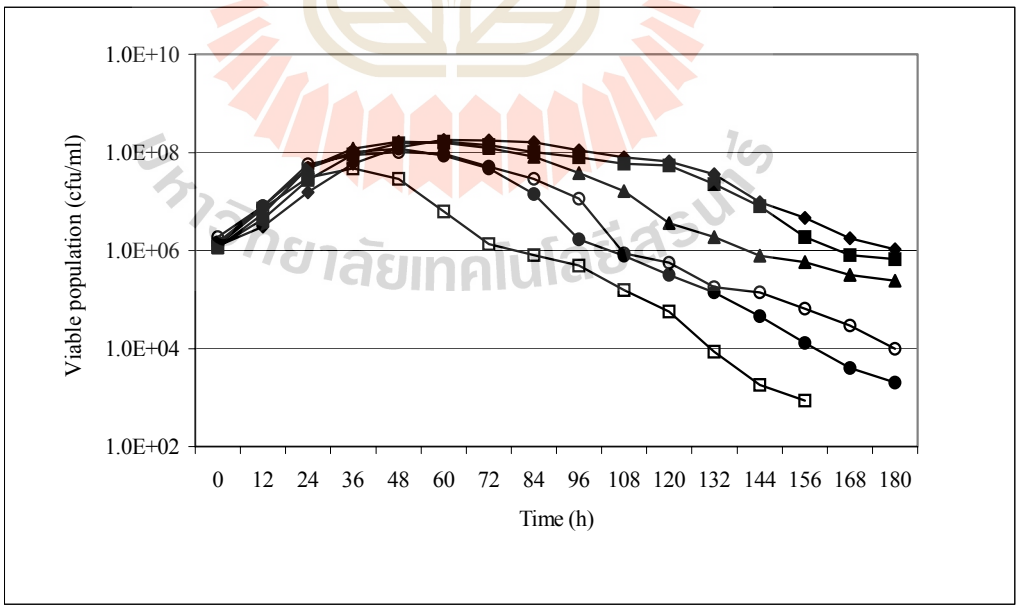


Figure 4.1 Growth pattern of *S. bayanus* EC1118 at various temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

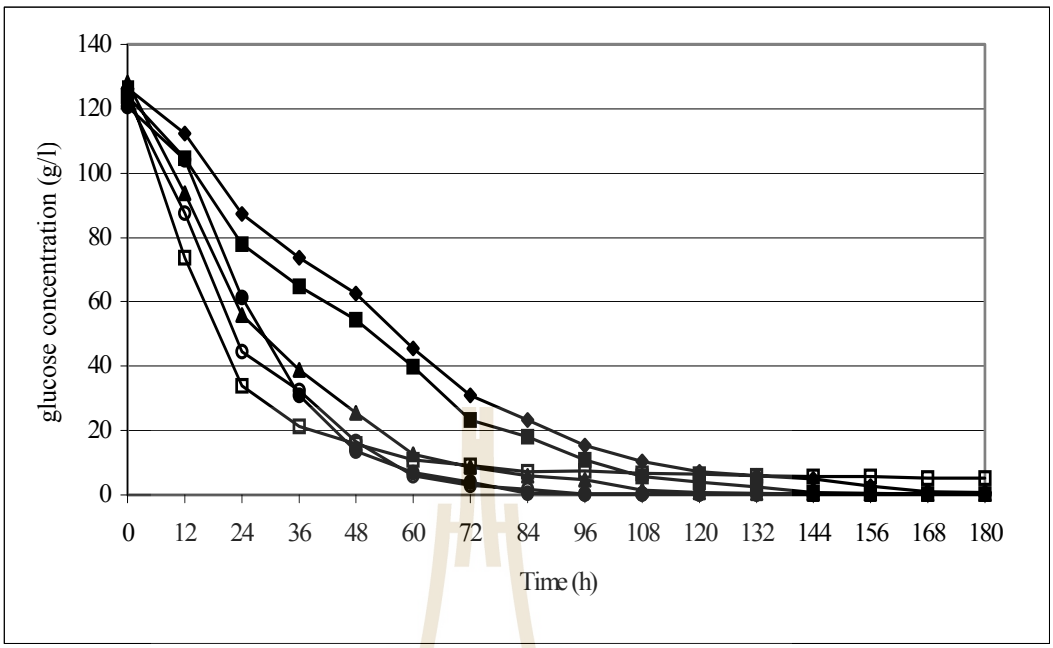


Figure 4.2 Glucose consumption of *S. bayanus* EC1118 at various temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

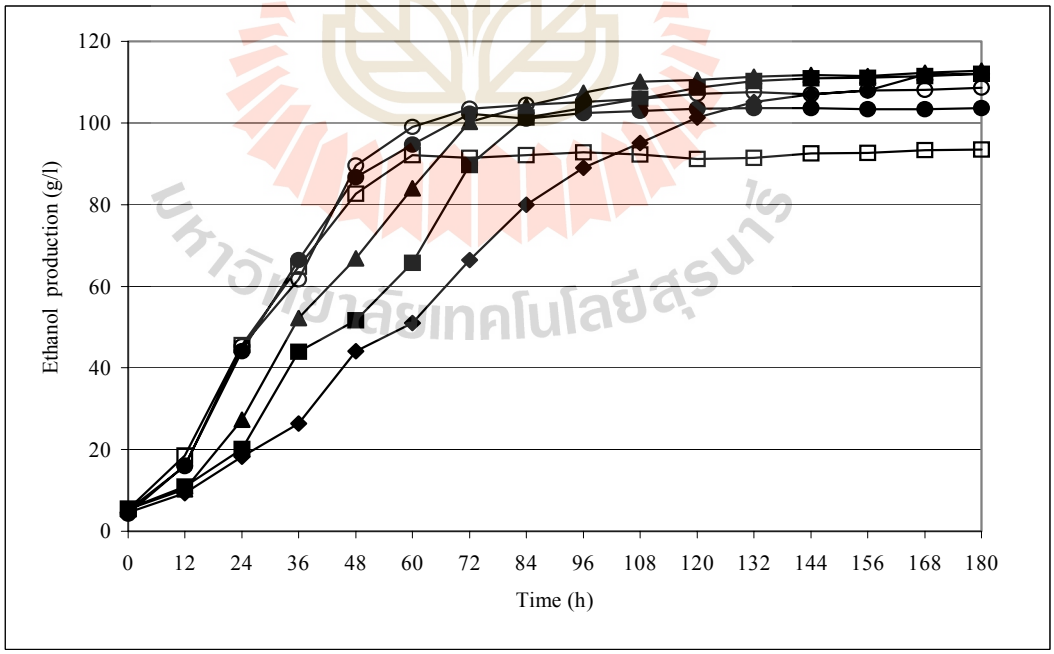


Figure 4.3 Ethanol production of *S. bayanus* EC1118 at various temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

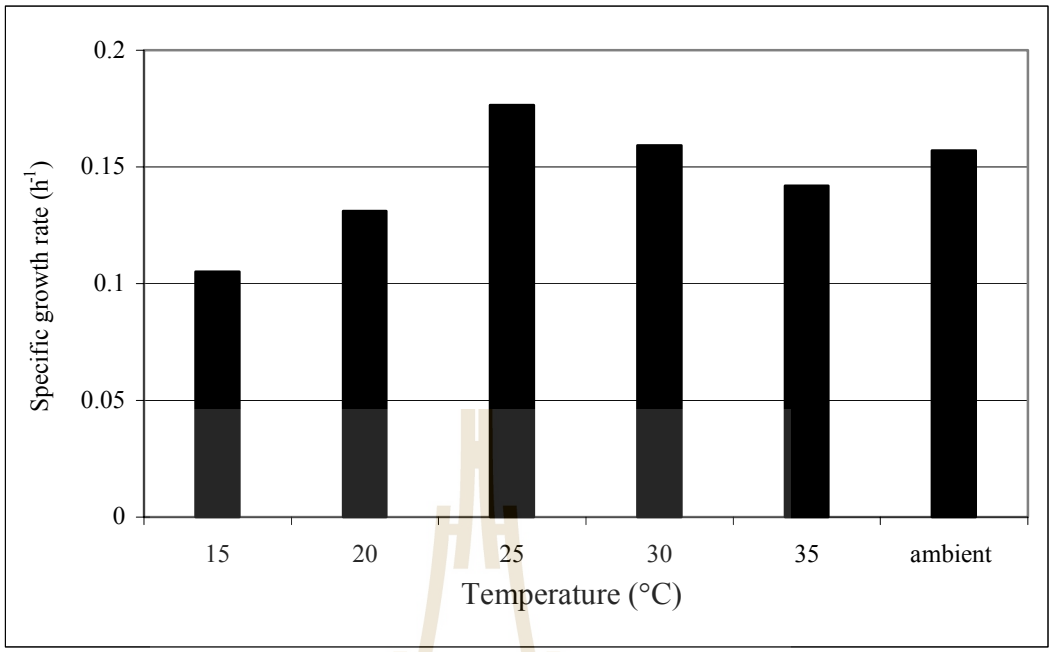


Figure 4.4 Specific growth rate of *S. bayanus* EC1118 at various temperatures.

4. 2 Effect of temperature on ester formation

The individual esters were determined in grape juice and wine samples during alcoholic fermentation took place. The chromatogram of grape juice showed that there was no ester found in juice so that most of ester in wine was the product from yeast metabolism. In the beginning of fermentation, yeast had short lag phase and rapidly went into exponential phase. The ester formation was occurred after the exponential phase and increased with fermentation time until the dead phase. The maximal amounts formed when ethanol concentration was greater than 9 to 10 % w/v in all temperatures (Figure 4.5-4.10). It has been reported that acetate esters are formed by reaction of alcohols and acids in wine and alcohol acetyltransferase enzyme (ATF), which is the important enzyme for acetate ester formation (Fujii, Kobayashi, Yoshimoto, Furukawa and Tamai, 1997; Lilly, Lambrechts and Pretorius, 2000; Lyness, Steele and Stewart, 1997; Gracia-Mauricio et al., 1993). Yoshioka and

Hashimoto (1983) indicated that this enzyme was localized in the cell membrane of brewer's yeast and enzyme activity was increased with fermentation time. The maximal activity showed at day 6 and 8, late of fermentation. After yeast growth was in stationary phase, some yeast might be injured from toxic substances such as ethanol and yeast autolysis occurred. Charpentier et al. (1986) showed that there is a loss of both amino acids and glucans in the cell wall of yeasts during autolysis, which results in structural loosening of yeast cell wall. Thus, the ester concentration showed maximal concentration after stationary phase may result from their released from the yeast cell.

The maximal concentration of each ester was shown in Table 4.2. It had been shown in this study that temperature was strongly affected to ester formation and retention, trend of ester formation was decreased with increased temperature (Figure 4.11-4.17). The major ester produced during fermentation was acetate ester, which termed ' fruit ester' because of their pleasing aroma. Iso-amyl acetate was the highest concentration ester, it is banana like aroma and hexyl acetate (apple like aroma) was the lowest. The maximal ester concentration was found at 15 °C (n-propyl acetate, ethyl butyrate, isoamyl acetate, hexyl acetate, ethyl hexanoate, ethyl decanoate and 2-phenethyl acetate) and at 20 °C (ethyl butyrate). The lowest concentration was found at 35 °C (isoamyl acetate, hexyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate) and ambient temperature (n-propyl acetate and ethyl butyrate and 2-phenethyl acetate). There are many investigators found high content of ester at low temperature(Killain and Ough (1979), Mallouchos, Komaitis, Koutina, Kanellaki, (2003), Kourkoutas et al., (2003)). Another ester is fatty acid ethyl ester the ester of

free fatty acid. Bardi, Crivelli, and Marzona (1998) found large amounts of medium chain fatty acid (MCFA) were released during anaerobic fermentation and related to ethyl ester formation. Torija et al. (2003) studied the effect of temperature and *Sachharomyces* species on cell fatty acid composition. They found that the concentration MCFA of *S. bayanus* at low fermentation temperature (13 °C) was higher than 25 °C and related to concentration of volatile compounds, which were higher in wine produced at lower temperature. This finding agreed with this experiment, which found high concentration of fatty acid ethyl ester at low temperature. Moreover, high growth rate which found at higher temperature would lead to an increased cellular need for Ac-Co-A, leaving less Ac-Co-A available for ester synthesis and alcohol acetyl transferase enzyme was repressed by disruption of essential messenger at high temperature (Verstrepen et al., 2003). The amount of ester formation might be related to yeast cell population. The maximal ester concentration was found at 15 °C, which had highest amount of population and ethanol concentration in the sample. The higher temperature had lower amount of yeast cells and ethanol. During fermentation, esters were produced, and then decreased. The rate of ester reduction was increased with temperatures. The high temperatures as 30 °C, 35 °C and ambient temperature (27-32 °C), showed higher rate of ester reduction. The ester hydrolysis was studied in model wine (Ramey and Ough, 1980). They found the increasing of temperature strongly effected on rate of ester hydrolysis. The ester formation and retention were monitored continuously until the end of six months aging. Total ester concentration at the end of alcoholic fermentation, malolactic fermentation and aging were shown in Figure 4.19. Ester was decreased with time. Therefore, the alcoholic fermentation is important step for ester formation and retention. Wine fermentation at 15 °C showed good impact on

wine flavor.

Table 4.2 Maximal concentration of individual ester during alcoholic fermentation.

Esters (mg/l)	Fermentation temperature (°C)					
	15	20	25	30	35	Ambient
n-propyl acetate	0.41	0.34	0.34	0.30	0.06	0.05
Ethyl butyrate	0.61	0.66	0.43	0.14	0.17	0.12
Isoamyl acetate	9.71	7.29	6.89	0.69	0.16	0.44
Ethyl octanoate	5.019	4.45	3.57	0.16	0.11	0.79
Hexyl acetate	0.49	0.33	0.25	0.06	0.02	0.06
Ethyl hexanoate	6.53	5.60	6.50	0.37	0.10	0.40
2-phenethyl acetate	2.67	2.03	2.47	0.14	0.14	0.10
Ethyl decanoate	5.29	3.44	1.92	0.16	0.11	0.21

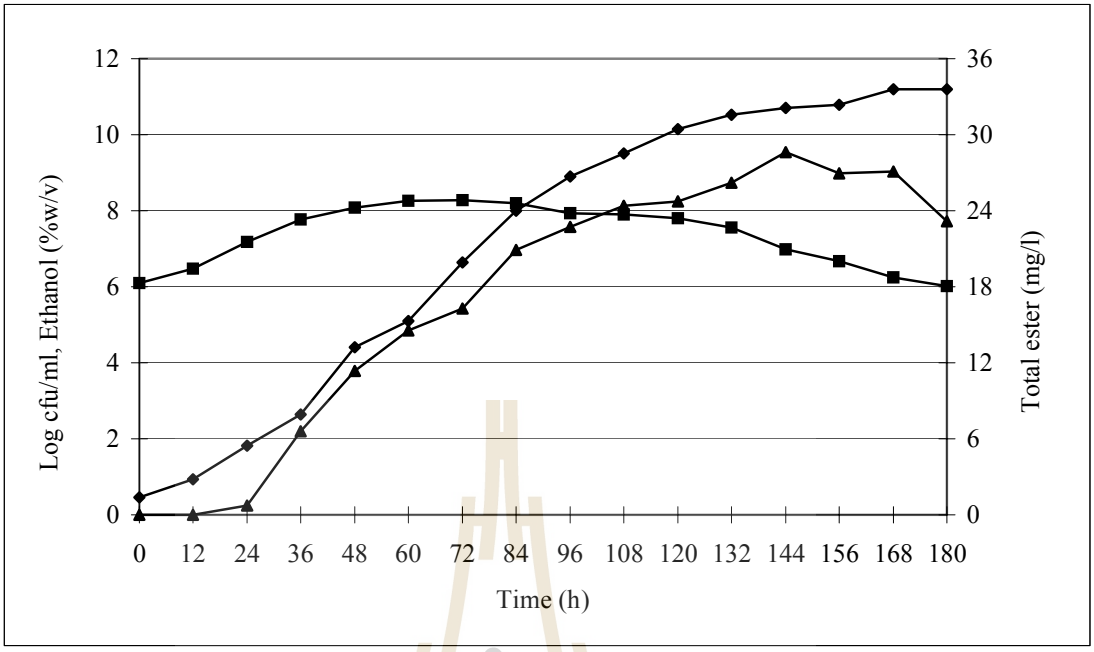


Figure 4.5 Time course of growth, ethanol and ester production during wine fermentation at 15 °C; log cfu/ml (■), ethanol (◆), total ester ( ).

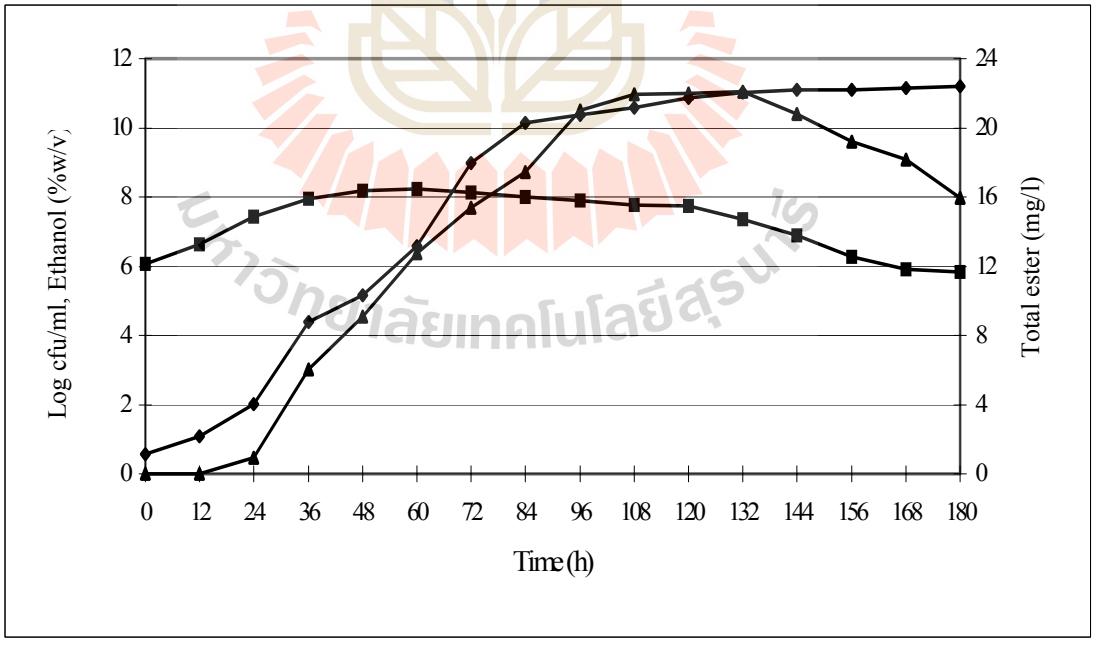


Figure 4.6 Time course of growth, ethanol and ester production during wine fermentation at 20 °C; log cfu/ml (■), ethanol (◆), total ester ( ).

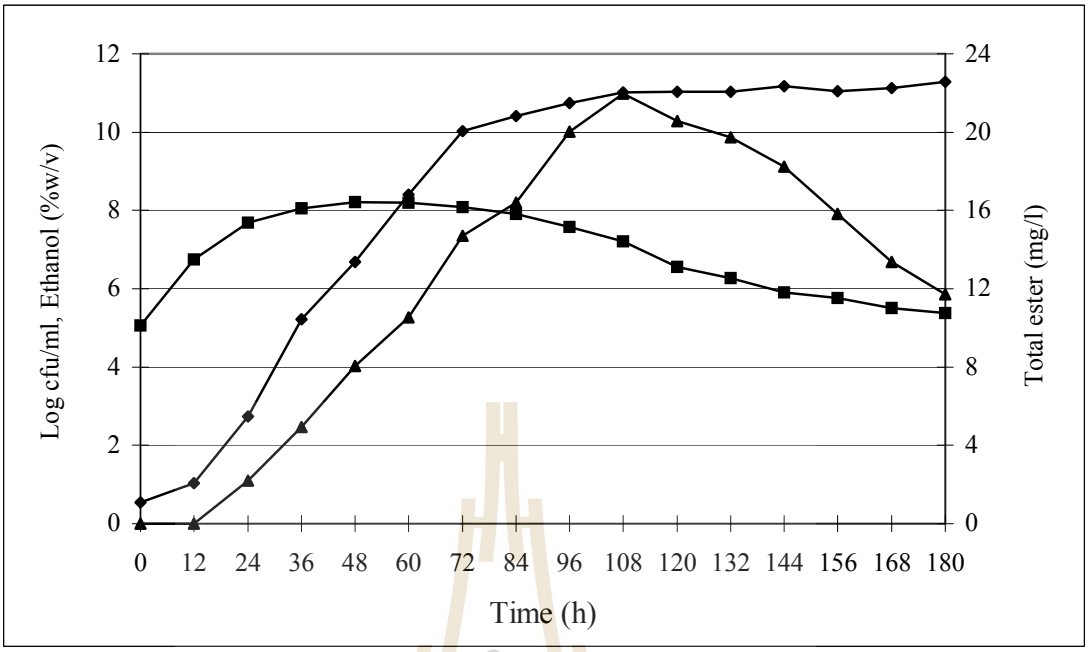


Figure 4.7 Time course of growth, ethanol and ester production during wine fermentation at 25 °C; log cfu/ml (■), ethanol (◆), total ester ( ).

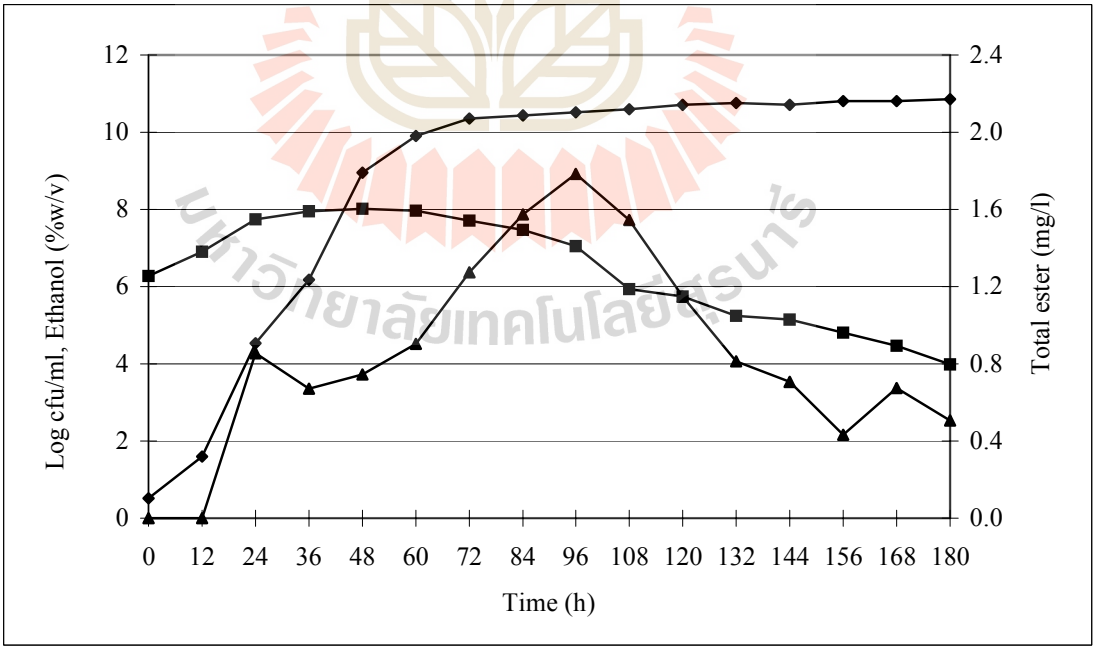


Figure 4.8 Time course of growth, ethanol and ester production during wine fermentation at 30 °C; log cfu/ml (■), ethanol (◆), total ester ( ).

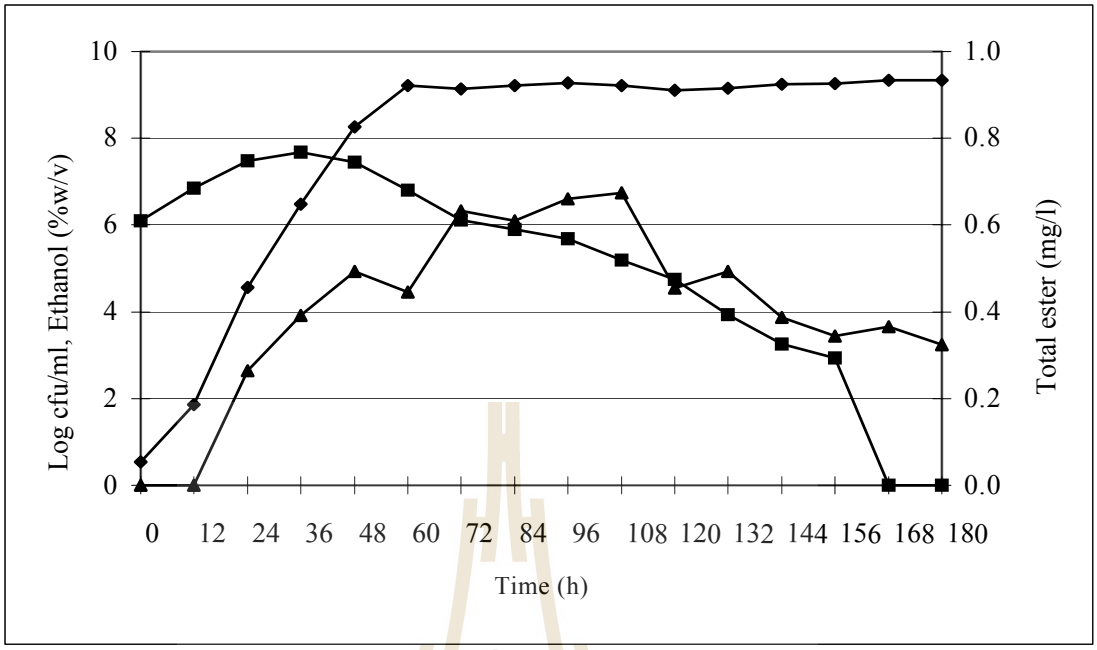


Figure 4.9 Time course of growth, ethanol and ester production during wine fermentation at 35 °C; log cfu/ml (■), ethanol (◆), total ester ( ).

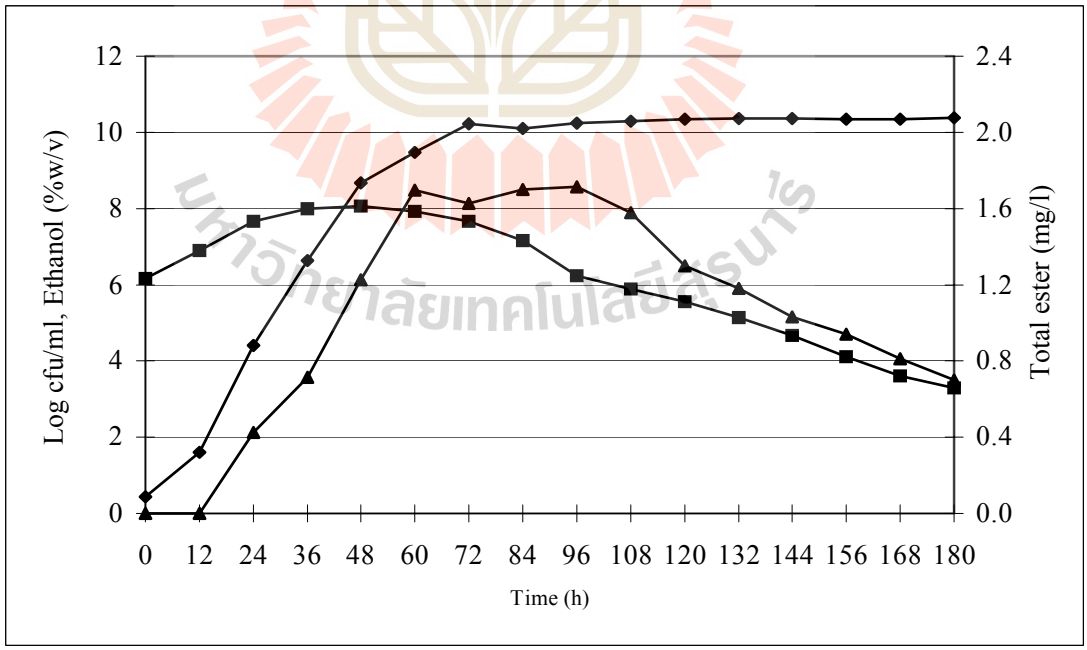


Figure 4.10 Time course of growth, ethanol and ester production during wine fermentation at ambient temperature; log cfu/ml (■), ethanol (◆), total ester ( ).

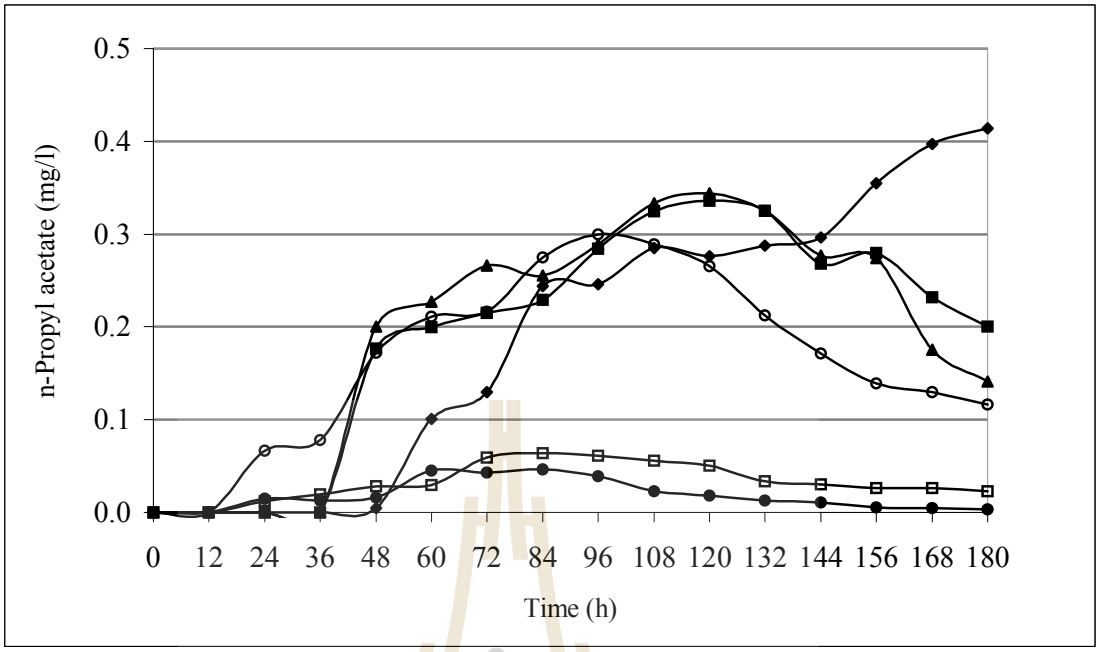


Figure 4.11 n-Propyl acetate formation during wine fermenation at various temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

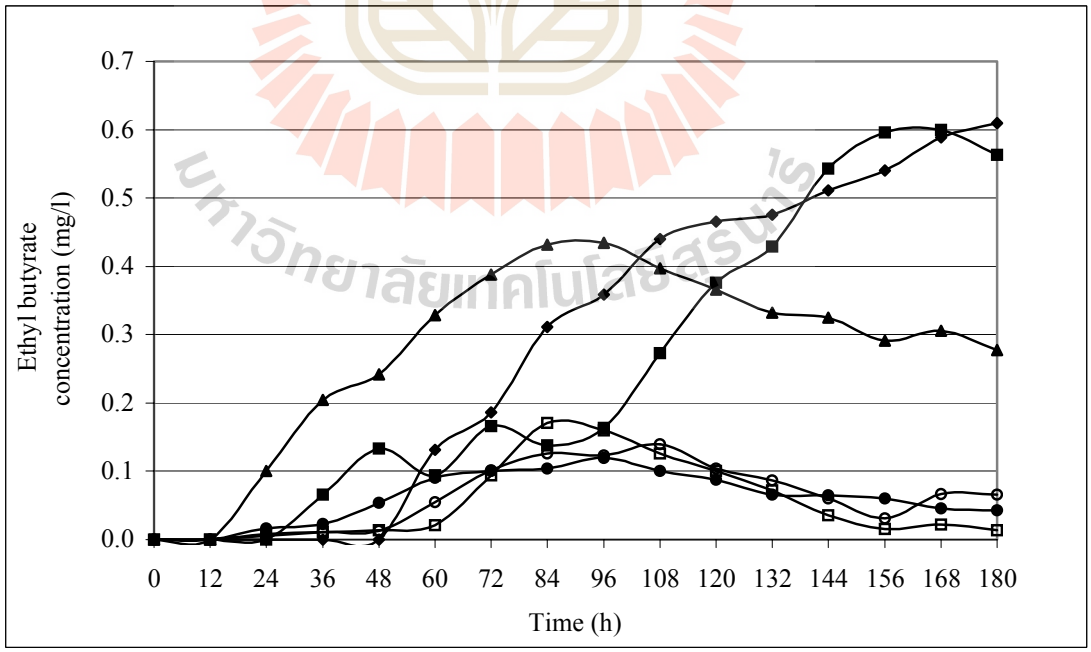


Figure 4.12 Ethyl butyrate formation during wine fermentation at various temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

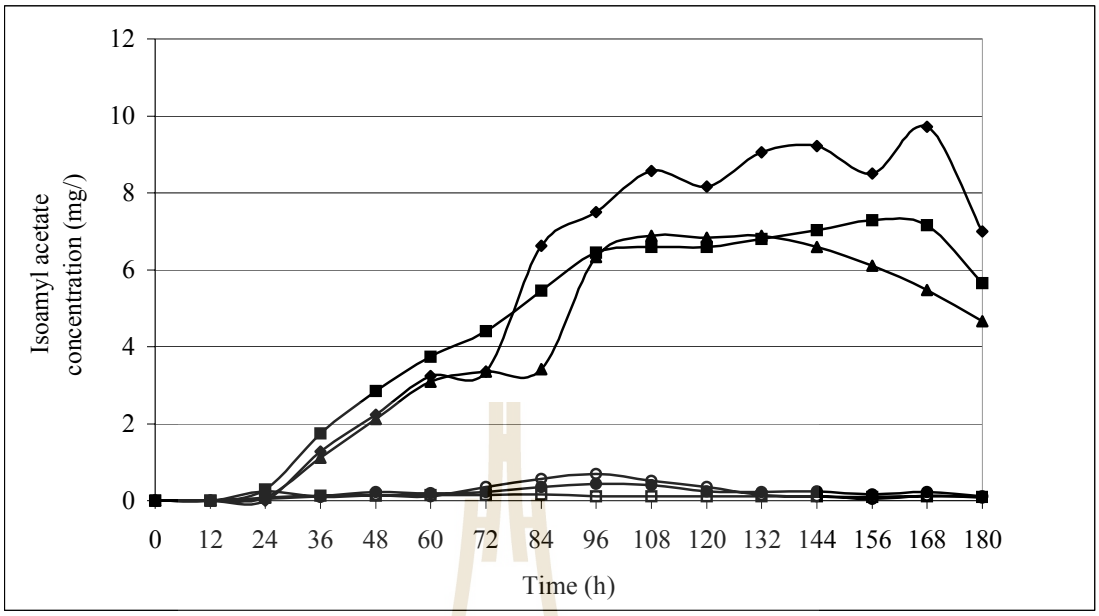


Figure 4.13 Isoamyl acetate formation during wine fermentation at various temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

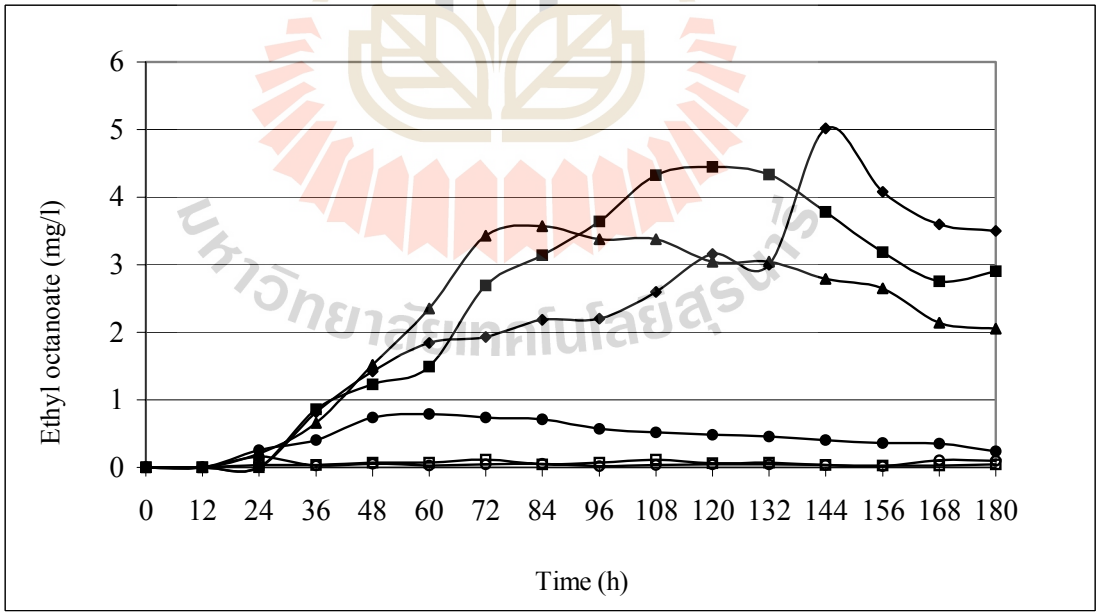


Figure 4.14 Ethyl octanoate formation during wine fermentation at various temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

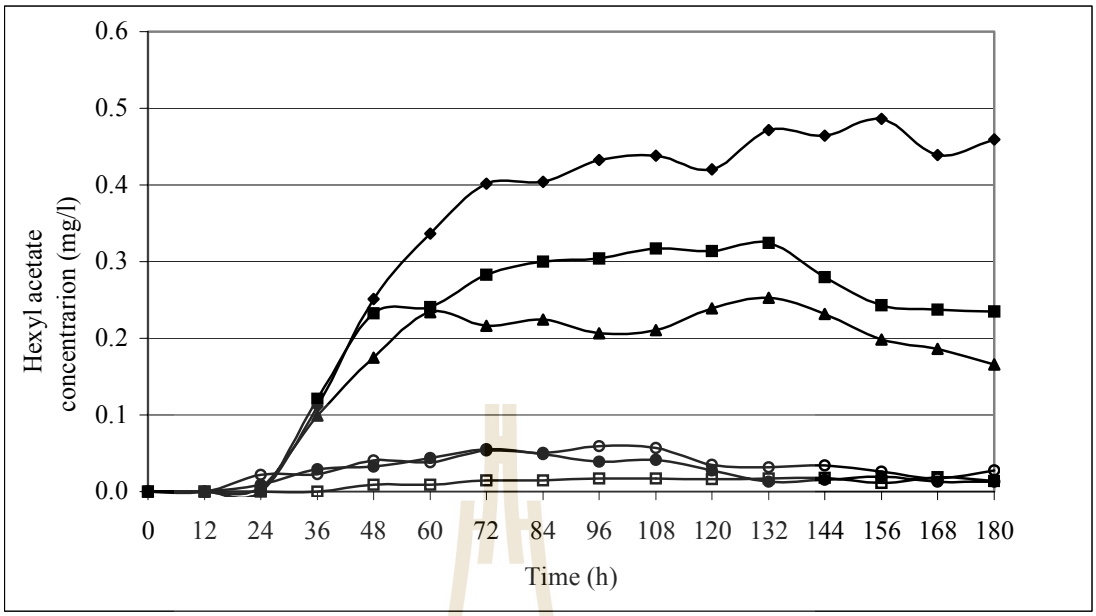


Figure 4.15 Hexyl acetate formation during wine fermentation at various temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

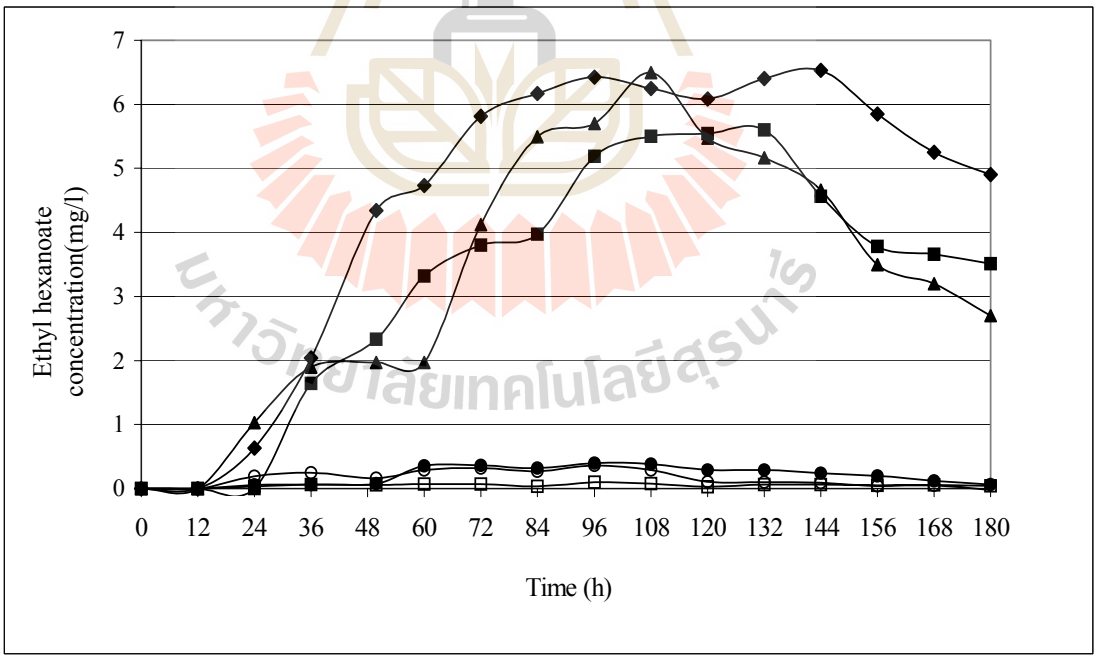


Figure 4.16 Ethyl hexanoate formation during wine fermentation at various temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

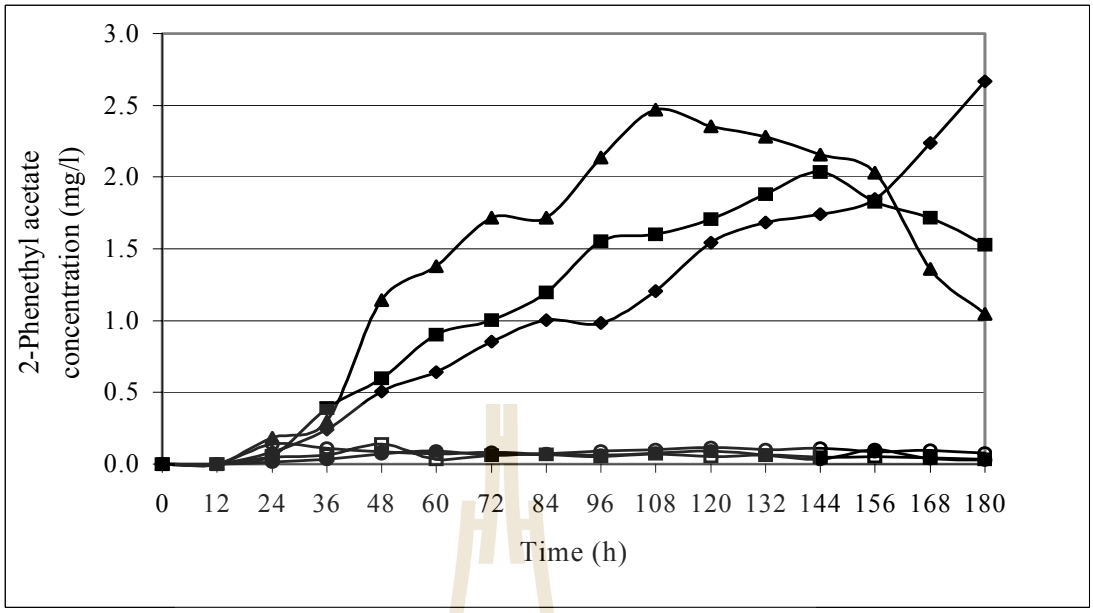
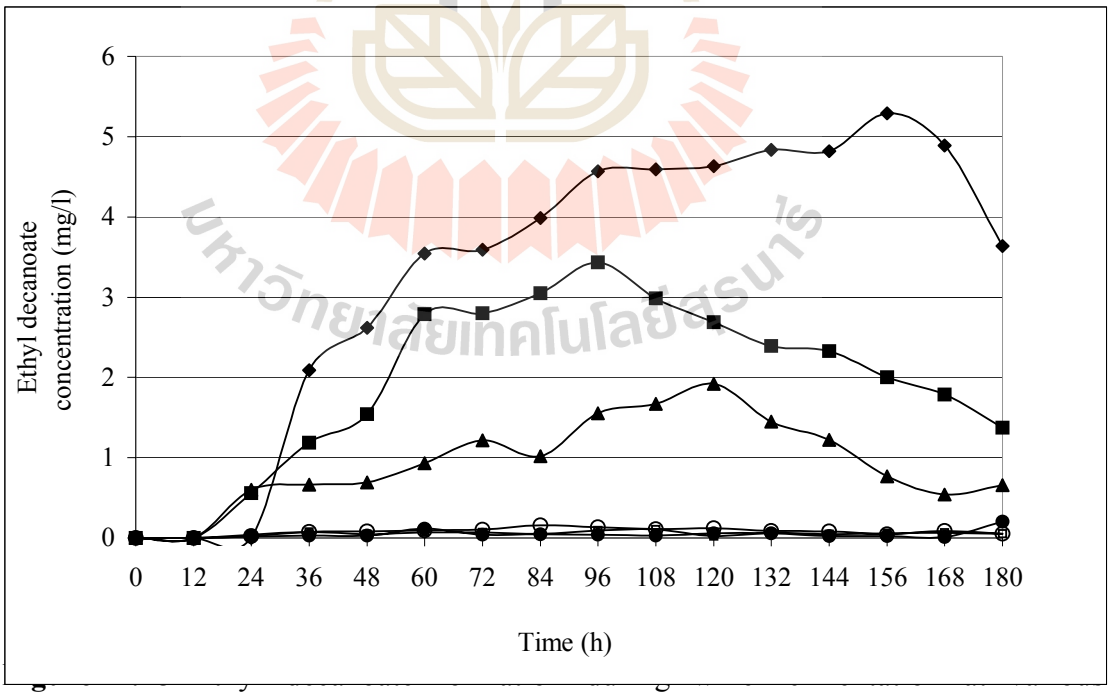


Figure 4.17 2-Phenethyl acetate formation during wine fermentation at various temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).



temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

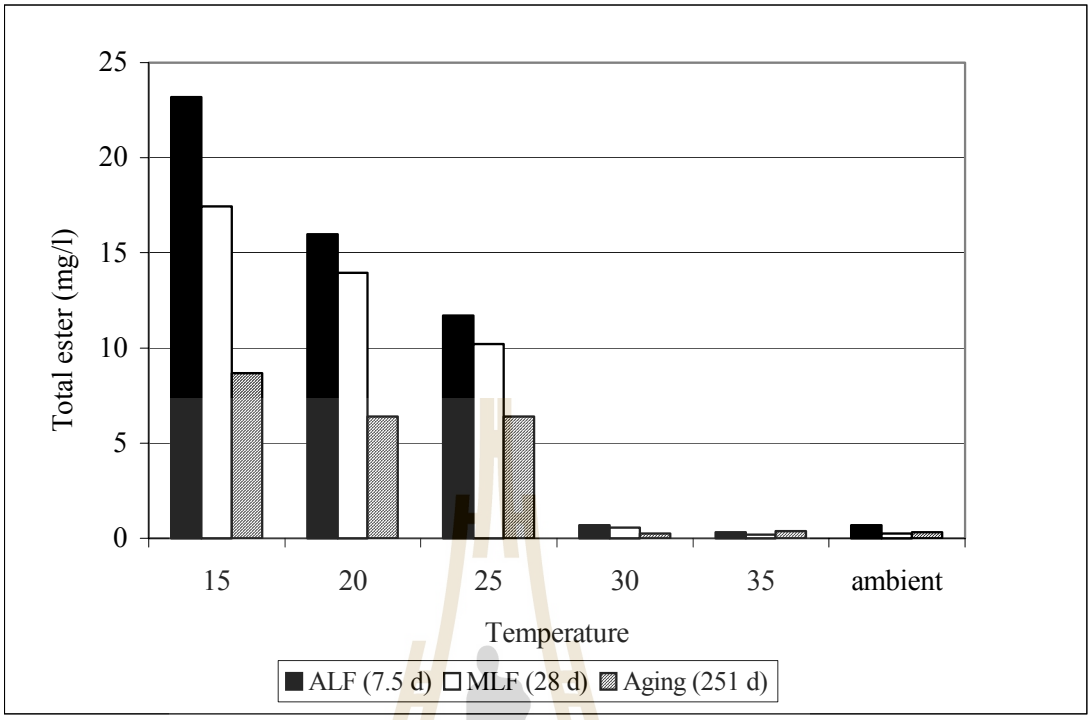


Figure 4.19 Ester reduction in wine process; ALF is alcoholic fermentation, MLF is malolactic fermentation and six months aging.

4.3 Effect of temperature on higher alcohol formation

Since higher alcohol is an important by-products from fermentation, therefore this experiment was conducted in order to investigate the effect of temperatures on higher alcohol formation. All higher alcohol formation occurred after exponential phase and showed maximal concentration after yeast reached stationary phase (Figure 4.20-4.25). The higher alcohol under high temperature, the greater amount was found under low temperatures (Figure 4.26-4.32). But only hexanol found in highest concentration at 15 °C and decreased with increasing temperature (Table 4.3). Hexanol is the major higher alcohol found in healthy grapes and also produce herbaceous odors in wines (Jacson, 2000). Since it was major found in grape, the temperature might be the reason of volatilized higher alcohol. Iso-amyl alcohol was the major higher alcohol, which had highest amount (151.34 mg/l at 30 °C) and heptanol was the lowest amount, the maximal concentration was found at 30 °C, 1.04 mg/l. Wine fermentation at 35°C was shown high content of 1-pentanol, 1-propanol and 2-phenethyl ethanol. Heptanol and 1-butanol were found highest at 30 °C and ambient temperature, respectively. After all higher alcohol reached maximal concentration at the end of alcoholic fermentation, the total of higher alcohol was slightly decreased in malolactic fermentation and six months of bottle aging reduced higher alcohol (Figure 4.33).

The alcohol is precursor of ester formation. Nykänen (1986), indicated that addition of isopentyl alcohol raised the formation of isopentyl acetate in sugar fermentation. Results obtained from this study indicated that, there was counter-correlation between higher alcohol and ester formation. High amount of ester was found at low temperatures whereas higher alcohol was found at high temperatures.

Substrate of higher alcohol formation was mainly considered in fermentation medium. Herraiz, Martin-Alvarez, Reglero, Herraiz and Cabexudo (1989, quoted in Fleet, 1996) reported that increasing of amino acid in the must increased higher alcohols. Furthermore, Lopez, Santamaria, Gutierrez and Iniguez (1996) reported that the amino acids in must were assimilated at a higher rate with temperature increased. Then a lot of Ac-Co-A was produced and run into pathway of higher alcohol formation. This finding agreed with Verstrepen et al. (2003), they indicated that high temperature would lead to an increased cellular needed for Ac-Co-A. Even though, ester synthesis also needed Ac-Co-A as precursor and Verstrepen et al. mentioned that alcohol acetyl transferase enzyme was repressed at high temperature by disrupted some essential messenger.

Table 4.3 Maximal concentration of higher alcohol at various fermentation temperatures.

Higher alcohols (mg/l)	**Temperature (°C)**					
	15	20	25	30	35	Ambient
1-Propanol	8.75	12.42	79.16	89.44	90.83	73.82
1-Butanol	4.55	5.30	9.64	45.66	34.69	71.02
Isoamyl alcohol	52.64	49.35	125.33	151.34	132.48	128.14
Pentanol	16.63	25.14	26.87	39.29	44.25	36.55
Heptanol	0.25	0.87	0.81	1.04	0.61	0.59
Hexanol	6.48	5.90	4.91	2.58	1.36	2.09
2-phenethyl alcohol	19.67	21.77	21.36	31.48	33.50	29.00

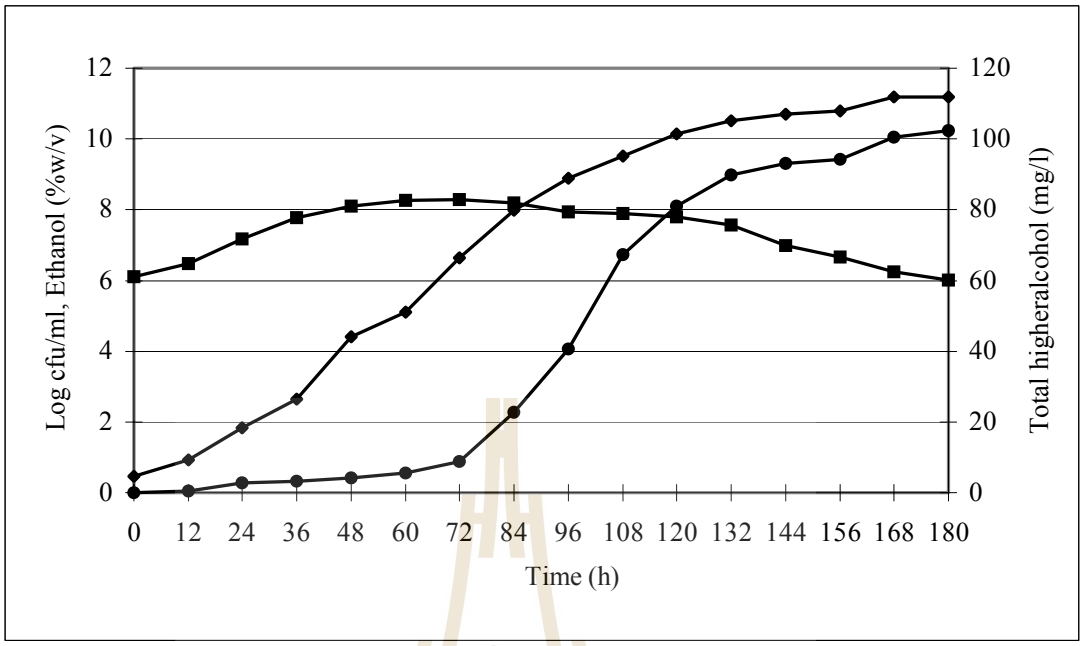


Figure 4.20 Time course of growth, ethanol production and higher alcohol formation during wine fermentation at 15 °C; log cfu/ml (■), ethanol (◆), total higher alcohol(●).

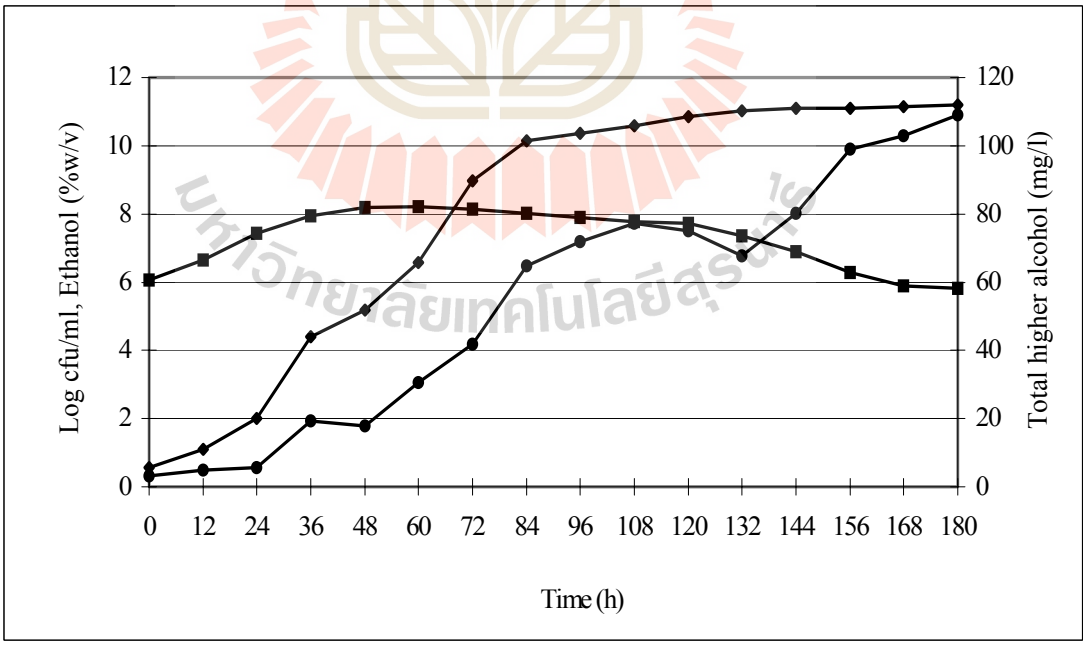


Figure 2.21 Time course of growth, ethanol production and higher alcohol formation during wine fermentation at 20 °C ; log cfu/ml (■), ethanol(◆), total higher alcohol(●).

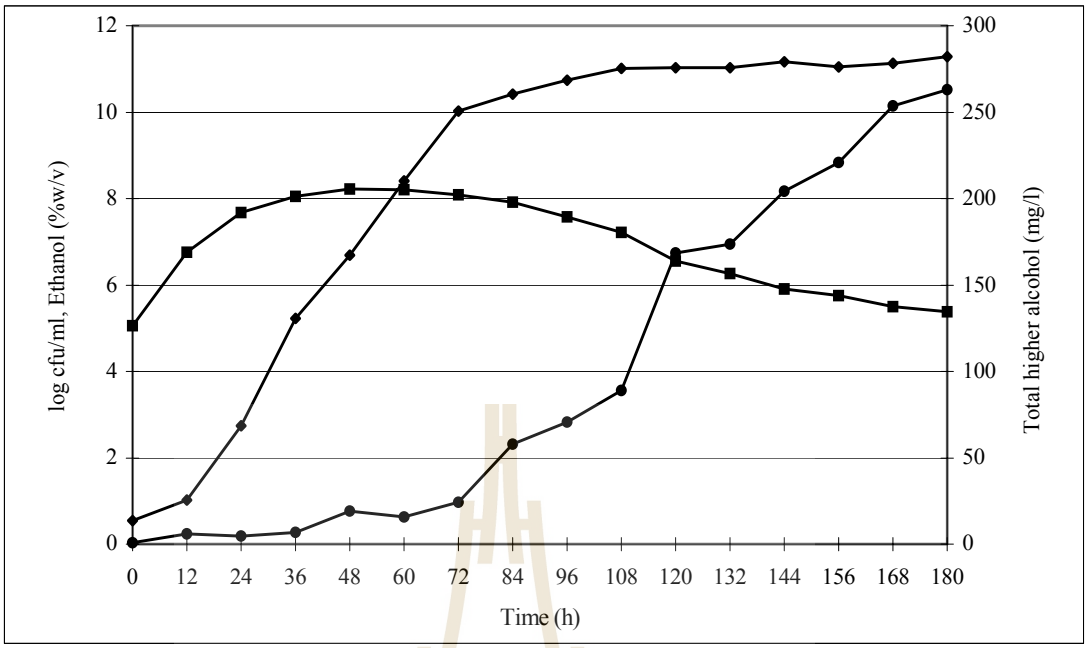


Figure 4.22 Time course of growth, ethanol production and higher alcohol formation during wine fermentation at 25 °C; log cfu/ml (■), ethanol(◆), total higher alcohol(●).

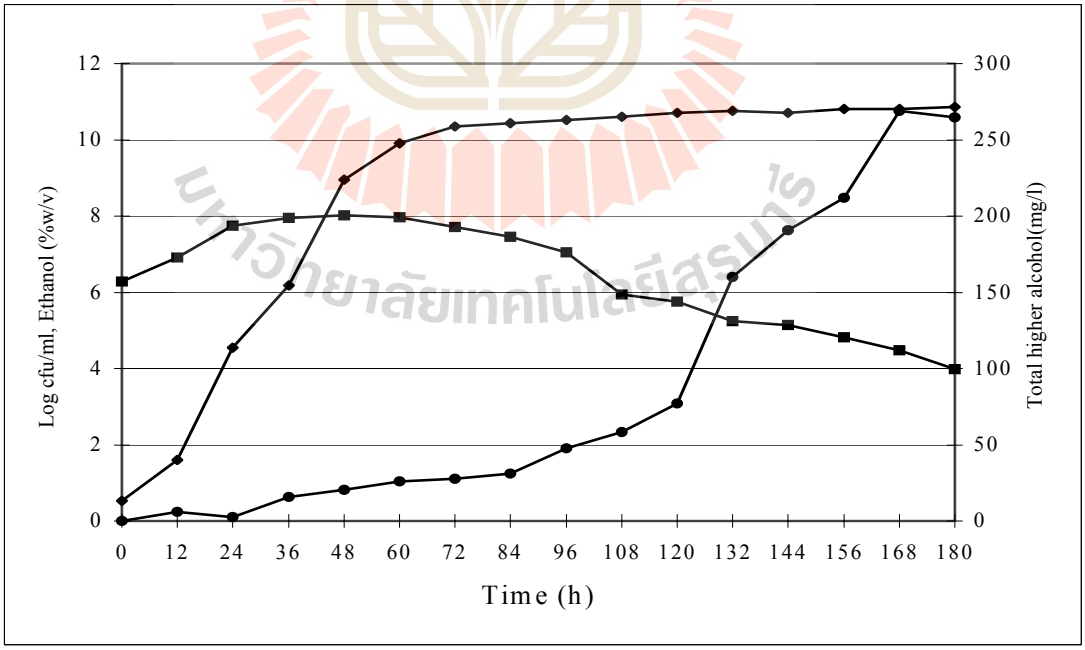


Figure 4.23 Time course of growth, ethanol production and higher alcohol formation during wine fermentation at 30 °C; log cfu/ml (■), ethanol(◆), total higher alcohol(●).

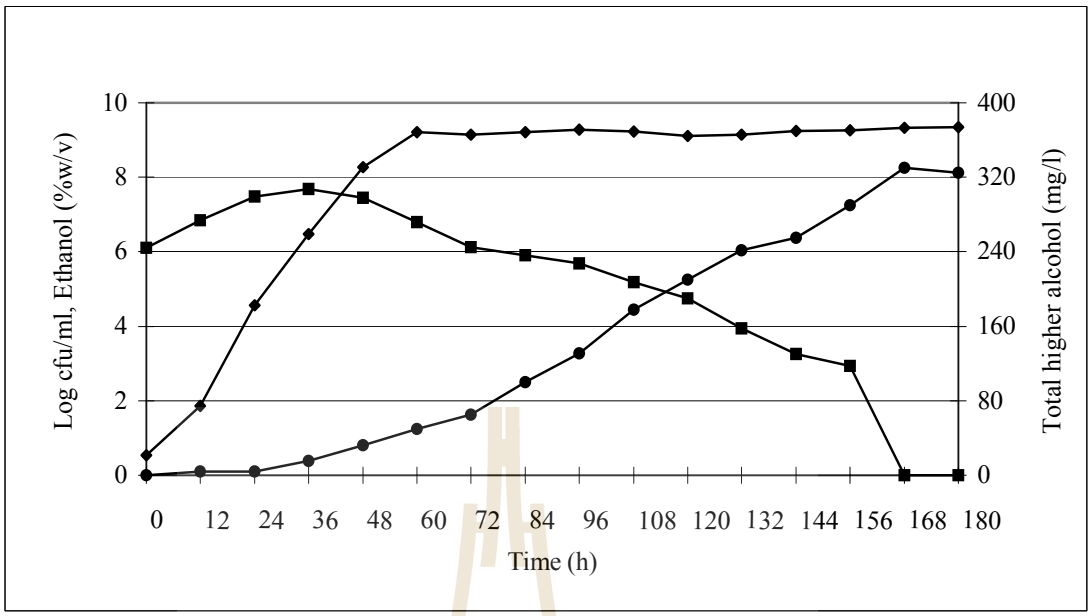


Figure 4.24 Time course of growth, ethanol production and higher alcohol formation during wine fermentation at 35 °C; log cfu/ml (■), ethanol(◆), total higher alcohol(●).

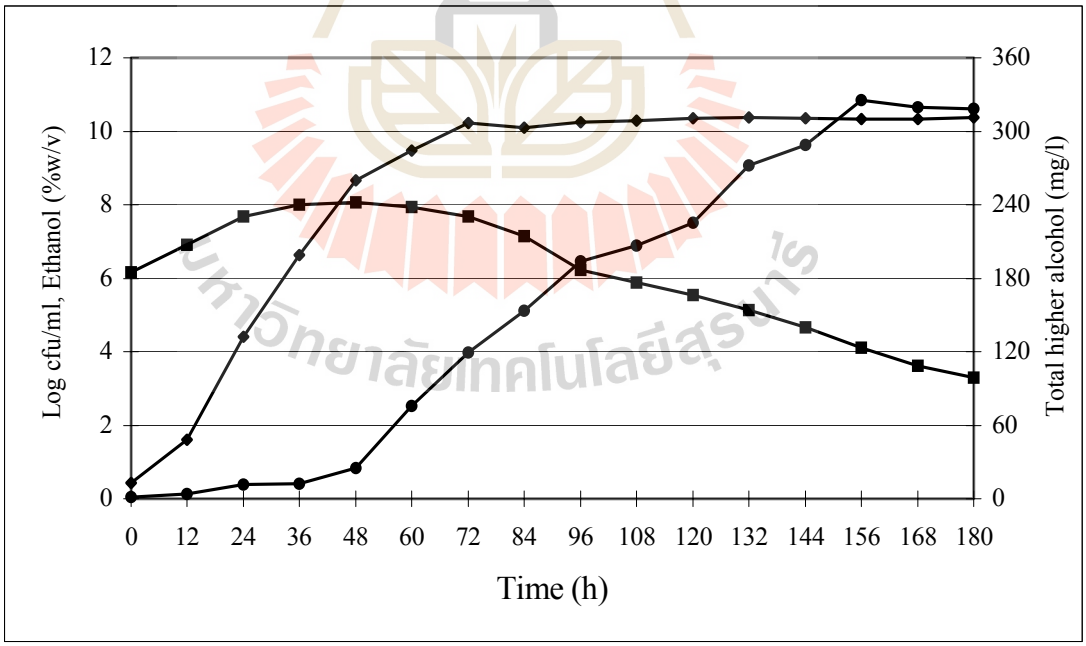


Figure 4.25 Time course of growth, ethanol production and higher alcohol formation during wine fermentation at ambient temperature (27-32 °C); log cfu/ml(■), ethanol (◆), total higher alcohol(●).

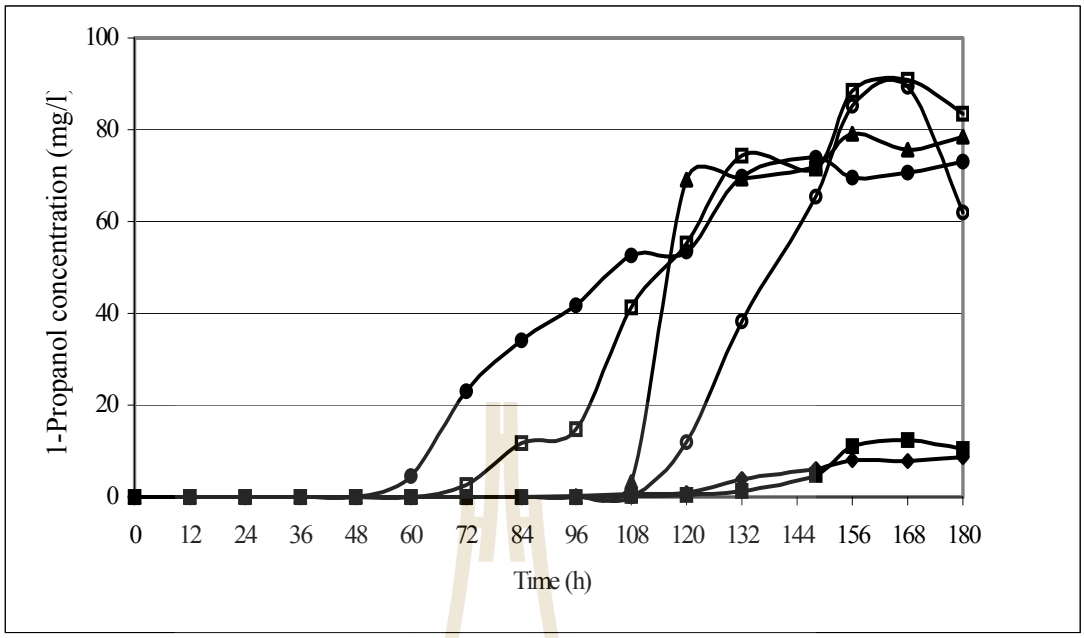


Figure 4.26 1-Propanol formation in various fermentation temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

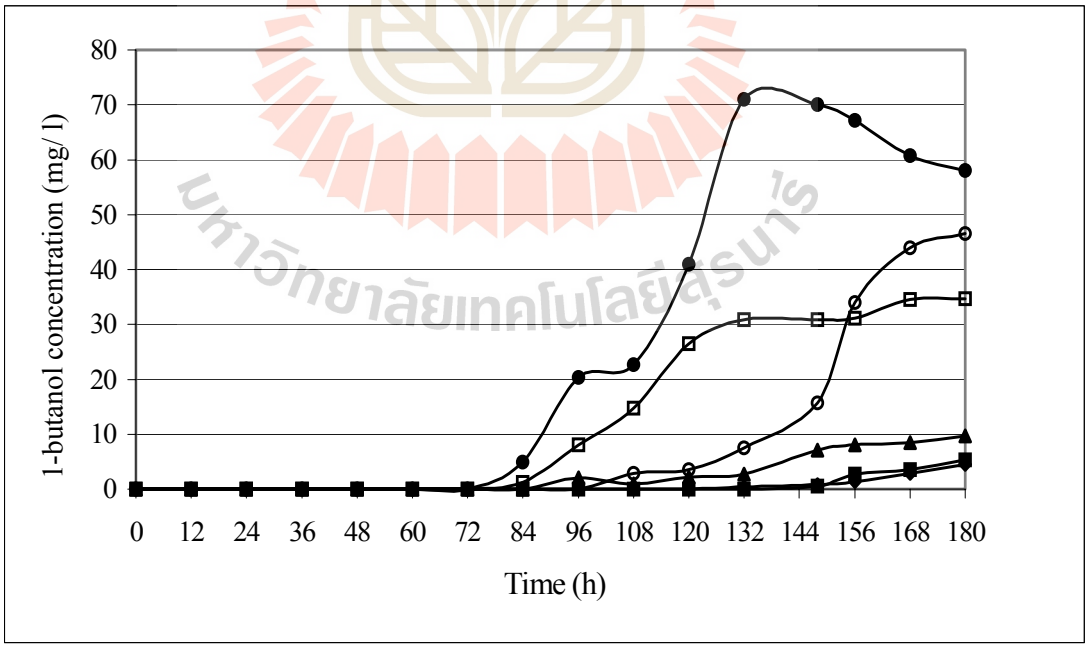


Figure 2.27 1-Butanol formation in various fermentation temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

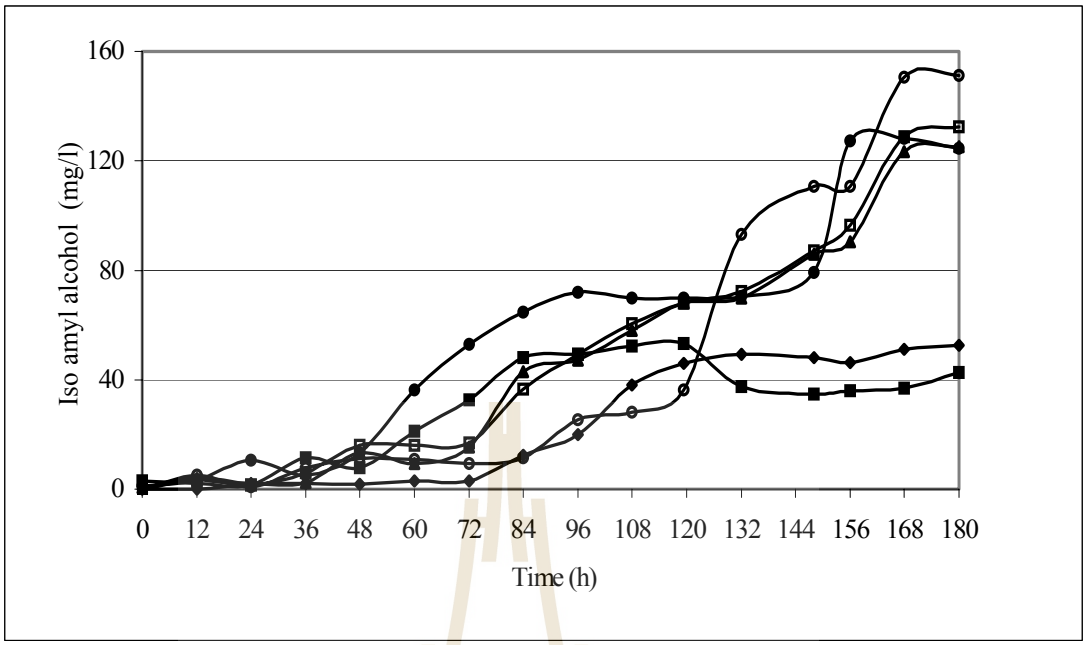


Figure 4.28 Isoamyl alcohol formation in various fermentation temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

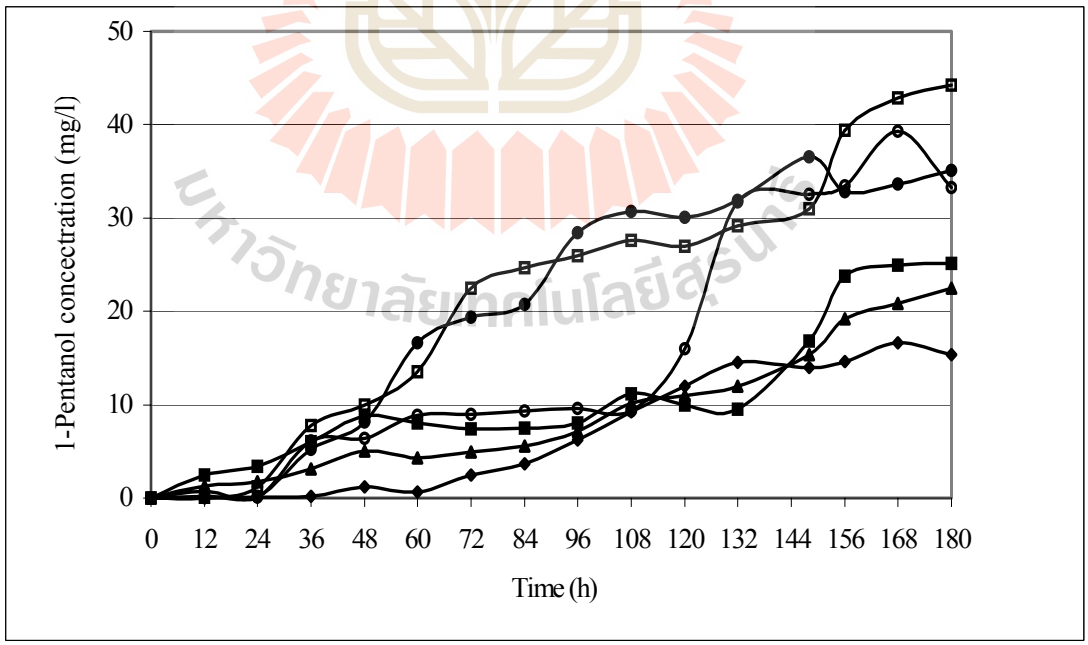


Figure 4.29 1-Pentanol formation in various fermentation temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□)and ambient temperature (27-32 ºC) ( ).

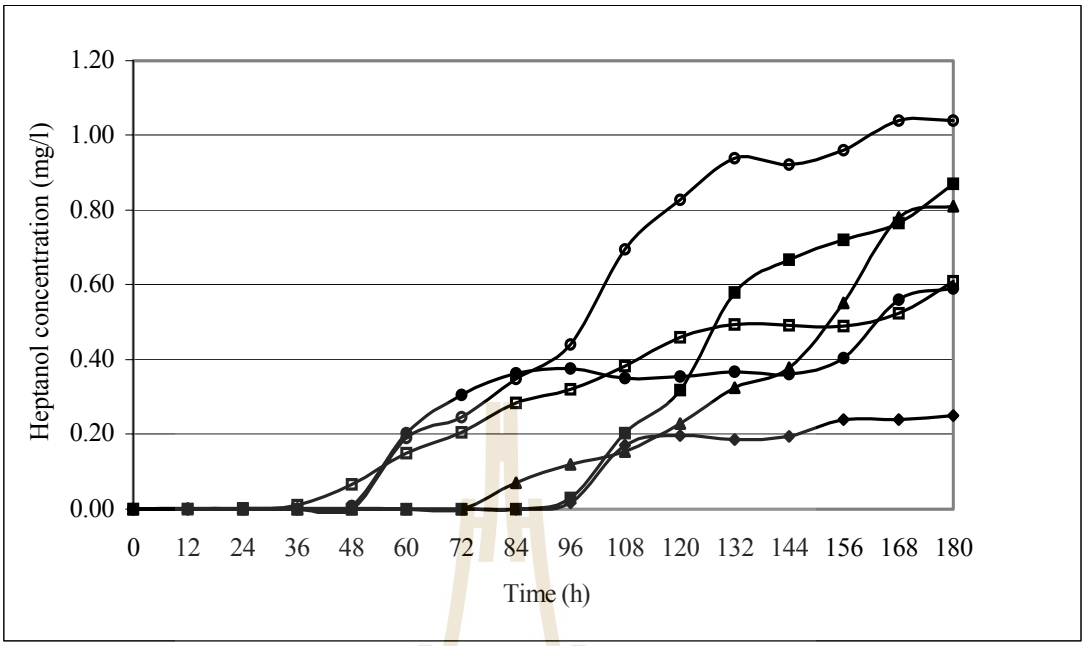


Figure 4.30 Heptanol formation in various fermentation temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC)( ).

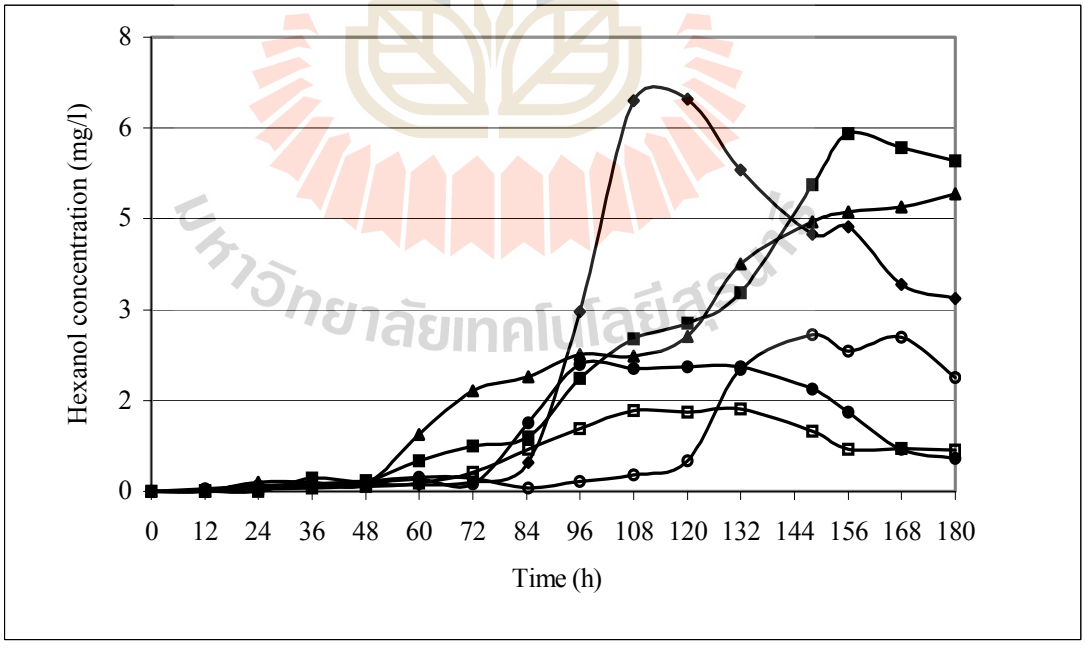


Figure 4.31 Hexanol formation in various fermentation temperatures;15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□) and ambient temperature (27-32 ºC) ( ).

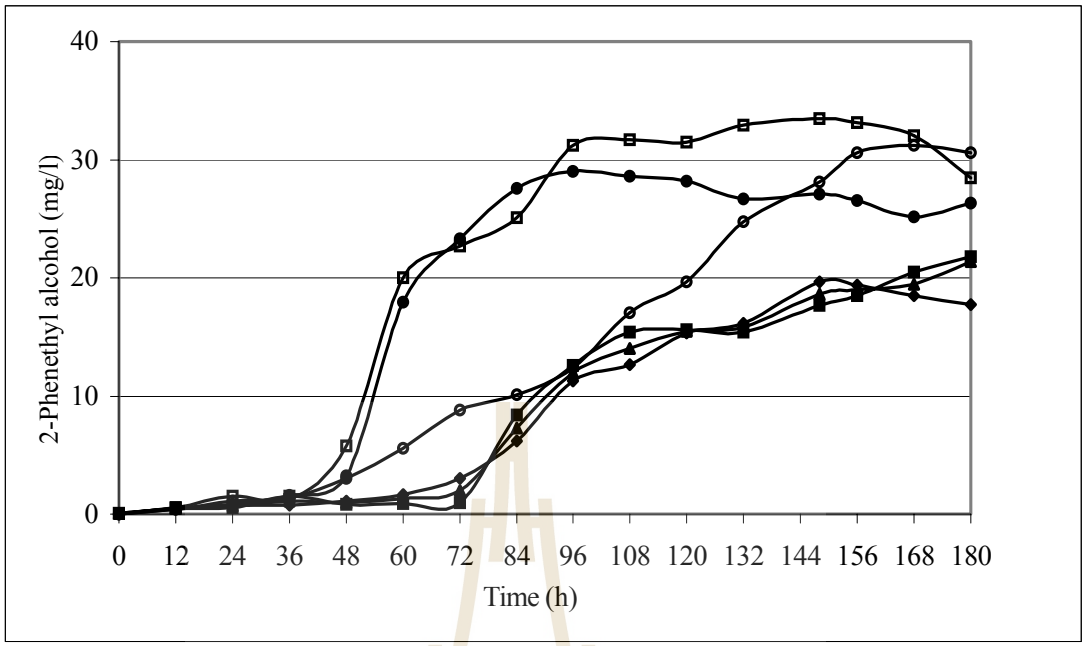


Figure 4.32 2-Phenethyl alcohol formation in various fermentation temperatures; 15 ºC (◆), 20 ºC (■), 25 ºC ( ), 30 ºC (○), 35 ºC (□)and ambient temperature (27-32 ºC)( ).

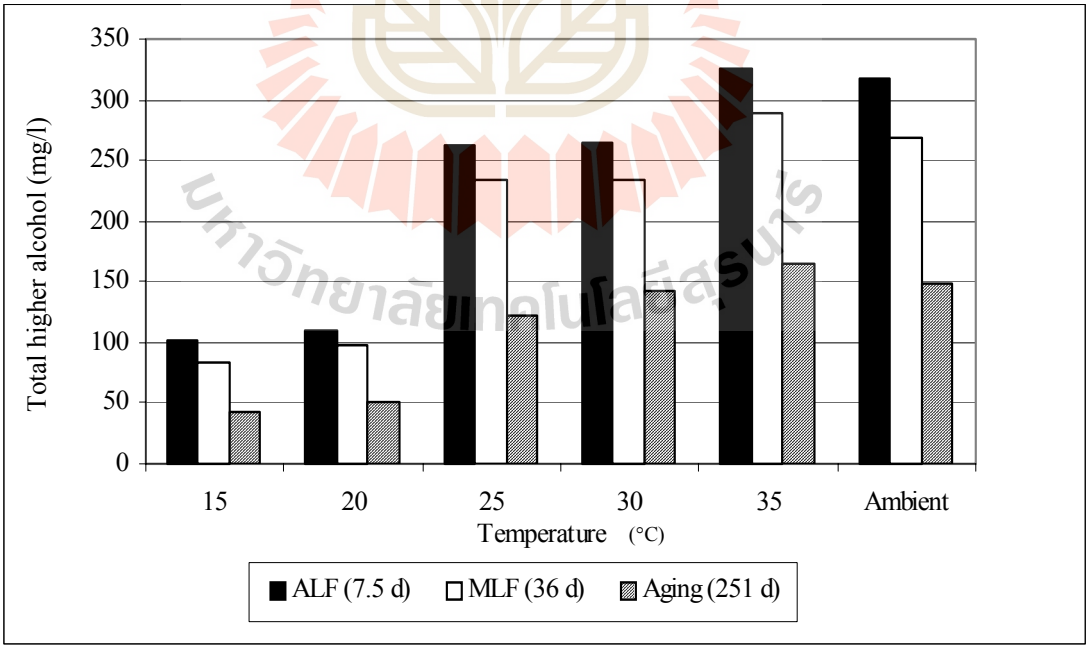


Figure 4.33 Higher alcohol reduction in wine process: ALF is alcoholic fermentation, MLF is malolactic fermentation, and six months aging.

4.4 Effect of temperatures on catechin, epicatechin and total phenolic compound.

The catechin and epicatechin were determined by HPLC and the results were shown in Figure 4.34. Catechin and epicatechin are known as flavan-3-ols, flavanols, or procyanidins. They are present as monomers in grapes and wine and are the primary components of polymeric phenols which are extraected from skin and seeds. The fermentation was done by using clarified must and without skin contact during fermentation, which was different from normal style of red wine making. Therefore, catechin and epicatechin in the beginning of fermentation were the same in every fermentation flasks. The comparison of catechin and epicatechin of must and wine, showed that these compounds in wine were higher than must. This finding indicated that monomeric compound could be released from polymeric compound by wine process. There was other observation, which indicated the increasing of ethanol percentages improved flavan-3-ols extraction into wine (González-Manzano, Rivas-Gonzalo and Santos-Buelga, 2004). The catechin and epicatechin content in wine of different fermentation temperatures were increased when temperature were increased to 25 °C, and decreased when temperature were increased from 25 to 35 °C, except for the ambient temperature was found highest content of catechin (308.81 mg/l) but lowest in epicatechin (10.71 mg/l). During wines were aged, the catechin reacted with anthocyanidin to form polymer in wine and acetaldehyde caused increasing the anthocyanin-tannin polymerization in red wine (Sims and Morris, 1986). At high fermentation temperature, the higher acetaldehyde formation (Ough and Amerin, 1988) and this compound enhanced tannin-anthocyanin polymerisation, then small amount of monomeric catechins were left in wine, this might be the reason of low catechin and

epicatechin detected in wine fermented at high temperature. Moreover, the results of total phenolic compounds of wine samples were lower than must. Thus, total phenolic compounds were reduced during wine process. The total phenolic compound in red wine usually ranges between 1,000 and 3,500 mg/l (Noble, 1990). The total phenolic compound of must was observed 1,659 mg/l and decreased in finished products. It was found lowest at 15 °C (1,299 mg/l) and highest content was found at ambient temperature (1,446 mg/l). It was similar pattern to catechin and epicatechin content in wine. The polymeric phenol precipitated during wine fermentation and stabilization, especially stabilization for one month at -5 °C could lead protein precipitated. Normally, tannin binding with protein in wine, once protein precipitated tannin was also precipitated.

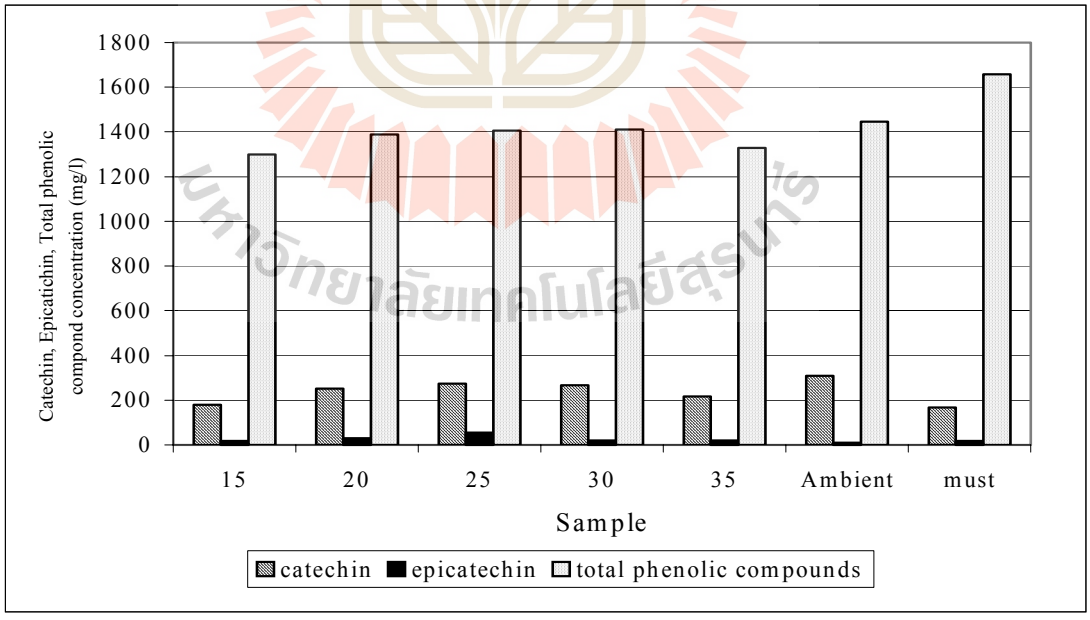


Figure 4.34 Concentration of catechin, epicatechin and total phenolic compound in wine sample fermented at various temperatures.

4.5 Effect of temperatures on wine characters evaluated by sensory test

Wine fermentation was conducted to the end of aging. The finished products were tasted by trained panelists and evaluated characters of wine. The objective of the sensory test was to determine weather temperatures effect on sensory profile of wine. Mean intensity ratings for the wine made by six temperatures were plotted on polar coordinate or radar graph, the center of the graph represented low intensity with respect to each character increasing to an intensity of 10 at the ends of axes. The results were shown on Figure 4.35. The graph showed five characters of wine, which were significantly different among six temperatures including color, aroma, body, balance, and astringency ($p< 0.05$). The Least Significant Different (LSD) was calculated to determined where the different occured and denoted by letters (Table 4.4). The panelists evaluated wine sample made at 15 °C to highest score in character of Color and Aroma. There were no significantly differences in sample of 20 °C, 25 °C, 30 °C 35 °C and ambient temperature in the term of color. Aroma of samples at 15 °C and 25 °C were stronger than ambient temperature, 20 °C, 30 °C and 35 °C respectively. This result was related to ester formation. The acetate ester produces fruit aroma, isoamyl acetate and floral aroma from 2-phenethylacetate were found high content at 15 °C. The lowest intensity rating of aroma was found at 35 °C and similar to ester content. Body is the term used for describing perception of weight in the mouth produced by the major organic constituents in wine, ethanol, sugars, glycerol and tannins (Jackson, 2000). Moreover, glycerol is non-volatile and has no direct impact on the aromatic characteristics on wine, it cause a favorable effect on wine quality by increasing viscosity and smoothness which are defined by the term 'body of wine. Balli et al.,

(2003) studied the effect of temperature on glyceral content in alcoholic fermentation by using synthetic medium and must. They found the glycerol content was decreased as temperature decreased for every fermentation medium. The highest intensity rating of body was found in wine fermented at 30°C, followed by 25°C, ambient temperature and 35°C, respectively. Therefore, the high body wine could be found in wines made under high temperatures. High body wine contained higher content of total phenolic compound and sugar left in finished wine. Astringency was significantly different in sample of ambient temperature and the rest samples were not significantly different. The astringency might related to the high content of total phenolic compound of ambient wine. Noble (1999), indicated that the smaller phenolic compound are described as more bitter and less astringent, while the larger phenolic compound more astringent and less bitter. The balanced wine is the index of pleasing proportions of acidity, sweetness and flavor of wine. Wine fermented in 25 °C showed highly significant different and higher than 15 °C, ambient, 30 °C, 35 °C, and 20 °C respectively. For bouguet, off-odor, flavor, bitterness, aftertaste and over-all were not significantly different among six temperatures ($p \geq 0.05$). However, the over-all, which is the term to defined conclusion of all quality even though there was no significantly differences found in this term but the mean rating score was the highest at 25 °C 30°C and 15°C, respectively and lowest score was found at ambient temperature.

Table 4.4 Mean rating and Least Significant Differences (LSD).

Characters	Temperature (°C)					
	15	20	25	30	35	Ambient
Color	6.93 a	3.15 b	3.41 b	3.51 b	3.05 b	3.59 b
Aroma	5.23 a	4.01 b	4.58 ab	3.89 bc	2.98 c	4.09 b
Off-odor	3.03 a	2.60 a	2.58 a	1.93 a	2.34 a	2.18 a
Bouguet	4.93 a	4.92 a	5.51 a	5.21 a	4.87 a	5.47 a
Flavor	4.92 a	4.92 a	5.41 a	4.82 a	5.05 a	5.31 a
Balance	4.74 ab	4.74 ab	4.89 a	4.04 b	4.06 b	4.61ab
Bitterness	3.56 a	3.75 a	3.57 a	3.48 a	3.66 a	3.43 a
Astringency	4.52 a	4.11 a	4.06 a	4.25 a	3.88 a	2.33 b
Body	3.12 b	3.27 b	4.01 ab	4.23 a	3.44 ab	3.48 ab
Aftertaste	4.23 a	4.42 a	4.75 a	4.58 a	4.41 a	4.20 a
Overall	4.70 a	4.43 a	5.02 a	4.74 a	4.43 a	4.37 a

Means in a column followed by the same letter are not significantly different ($p \geq 0.05$)

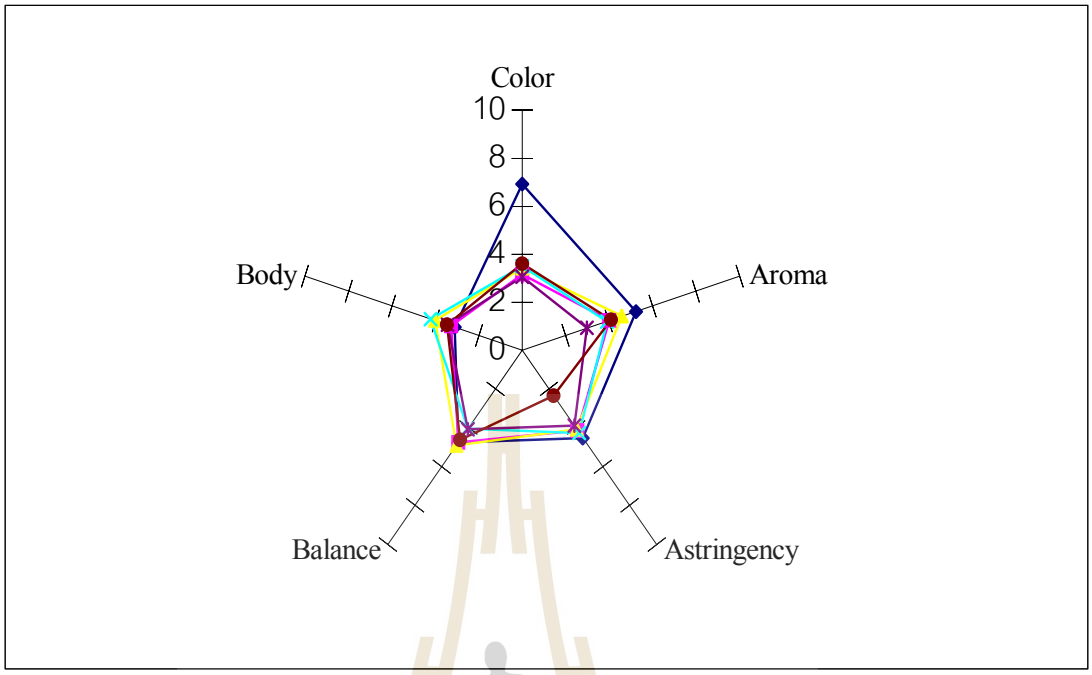


Figure 4.35 Polar coordinate graph of the mean intensity rating of six wine samples in term of color, astringency , balance, body and aroma ($p<0.05$) ; 15 ºC (blue line), 20 ºC (pink line), 25 ºC (yellow line), 30 ºC (light blue line), 35 ºC (purple line) and ambient temperature (27-32 °C) (blown line).

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CHAPTER V

CONCLUSION

It could be concluded that fermentation temperatures were strongly effected on fermentation kinetics, rate and length of fermentation. The temperatures were also affected yeast metabolism, which determined chemical composition of the wine. Fermentation at low temperatures enhanced the aromatics of wines, especially fruity ester, isoamyl acetate. Using *S. bayanus* enhanced floral aroma, 2- phenethylacetate. The maximum concentration of all esters were found at 15 °C and ethyl butyrate was found at 20 °C. Fermentation at high temperatures increased negative flavor of higher alchohol, but hexanol was highest amount at 15 °C. Wine produced under high temperatures contained higher content of phenolic compound than that produced under low temperatures. This compound could be developed into phenolic ester during aging of wine. Monomeric phenol of catechin and epicatechin were found highest concentration at ambient temperature and 25 °C, respectively. There were significantly differences found in five wine characters (color, balance, body, astringency and aroma)($p<0.05$). There was only aroma descriptors, were related to chemical analysis. From a practical point of view, fermentation temperatures effected on wine flavor and low temperature showed good impact to wine. The optimal temperature for yeast growth must be firstly considered and that temperature should not higher than 25 °C for greater retention of the volatile flavors. Results obtained from this research would be useful for winemaker to design an appropriate fermentation system in tropical country.

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APPENDIX

A. Methods

1. Sensory evaluation

The sensory evaluation of wines were done by using 10 volunteers and 2 experts of wine making. All ten volunteers participated in a round table discussion session. They were given representative wines, terminology of wine descriptors and tentative reference standards. These standards were prepared from attributes reported in the literature (Noble et al, 1987). After group discussion, the panelists were evaluated performance before taste wine. Panelist had score lower than 75% were rejected.

2. Evaluation of panelist performance by using Duo-Trio test and ranking test

The three cups of two flavors were constructed for Duo-Trio test. Panelists had to identify the cup, which had the same flavor to reference cup by smelling. For ranking test, four cups of different concentration of tartaric acid were used. Panelist had to consequence the sour taste in ascending order of acidity. Work sheet was shown here.

Duo-Trio Test

Panelist_____

In front of you are three samples, one marked **R** and the other two coded; Evaluate the samples starting from left to right. First **R** and then the other two coded. **Circle the code of the sample different from R**. You may retaste the samples. You must make a choice. Thank you.

R _____ _____

Figure 1A Work sheet of duo-trio test for the panelist's performance evaluation.

Ranking test

Panelist_____

Rank the sour taste solutions in the coded cup in ascending order of acidity.

	Least sour			Most sour
Code	_____	_____	_____	_____

Figure 2A Work sheet of ranking test for the panelist's performance evaluation.

QDA work sheet for wine tasting

Please evaluate the aroma, mouthfeel and flavor of the sample in sequence. Place a vertical line across the horizontal line at the horizontal line at the point that best describes each property in the sample.

A. Appearance
1.Color — light-red ... Dark-red

B. Odor
1. Aroma — weak ... moderate ... strong
2. Off-odor — weak ... moderate ... strong
3. Bouquet — weak ... moderate ... strong

C. Mouth impression
1. Flavor — weak ... moderate ... strong
2. Taste
-Balance — weak ... moderate ... strong
-Bitterness — weak ... moderate ... strong
3. Touch
-Astringency — weak ... moderate ... strong
-Body — weak ... moderate ... strong

D. Aftertaste — weak ... moderate ... strong

E. Overall-impression — Dislike ... Neither ... Like

Figure 3 A Work sheet of QDA for wine testing.

B. Results

1. Fermentation temperature

Table 1B. Real time temperature recording during fermentation.

Time (h)	Fermentation temperature(°C)											
	15		20		25		30		35		Ambient temperature	
	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
0	12	12.4	18.6	18.6	23.4	23.6	28.8	28.8	32.0	32.5	27.2	27.0
12	16.2	16.2	20.4	20.4	24.8	24.8	30.2	30.2	36.0	36.0	28.5	28.6
24	14.4	14.4	19.8	19.8	25.2	25.2	31.2	31.2	36	36	29.5	29.5
36	16.5	16.4	20.6	20.6	25.4	25.4	31.0	31.2	35.5	35.5	30.6	30.2
48	14.8	14.8	20.0	20.0	25.6	25.6	30.6	30.6	35.2	35.2	27.6	27.4
60	15.5	15.6	22.2	22.2	26.2	26.2	31.2	31.4	35.5	35.5	32.4	32.4
72	14.6	14.6	20.5	20.4	26.0	26.0	31.0	31.0	35.0	35.0	28.8	28.8
84	15.6	15.6	22.0	22.0	25.4	25.4	30.5	30.5	35.0	35.0	30.2	30.2
96	15.2	15.2	21.4	21.4	25.8	25.8	30.8	30.8	35.0	35.0	28.5	28.5
108	16.2	16.2	22.4	22.4	26.4	26.4	31.0	31.2	35.2	35.2	30.5	30.5
120	15.4	15.4	20.8	20.8	26.0	26.0	30.8	30.8	35.0	35.0	28.6	28.6
132	16.4	16.4	22.0	22.0	25.8	25.8	30.8	30.8	35.0	35.0	29.8	29.8
144	15.2	15.2	20.4	20.4	25.4	25.4	31.0	31.0	35.0	35.0	28.4	28.4
156	15.8	15.8	20.6	20.6	25.8	25.6	30.6	30.6	35.0	35.0	29.5	29.6
168	14.8	14.8	21.2	21.2	25.4	25.4	30.6	30.6	35.0	35.0	30.4	30.4
Avg	15.24	15.26	20.86	20.85	25.51	25.51	30.67	30.71	35.02	35.06	29.36	29.32

2. Chromatograms of ester extract by liquid-liquid extraction method

The chromatogram of must extract was found only 4 peaks of volatile compound. First is ethanol (1.20 min), 2-phenethylethanol (30.31 min) and two unknowns (38.7 and 40.5 min). The chromatogram of wine extract showed more peaks of volatile compound were produced during fermentation.

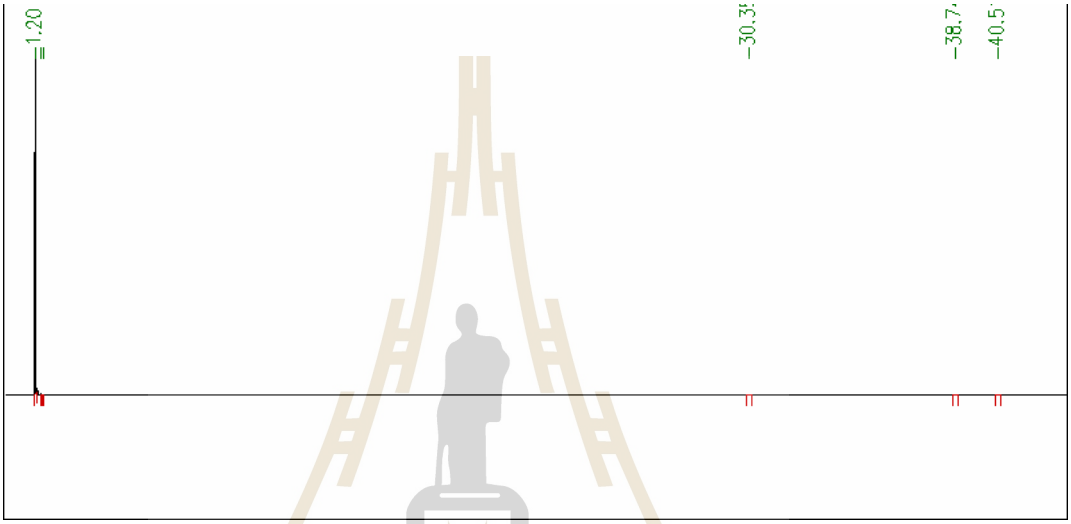

Figure 1B Chromatogram of dichloromethane extract of must.

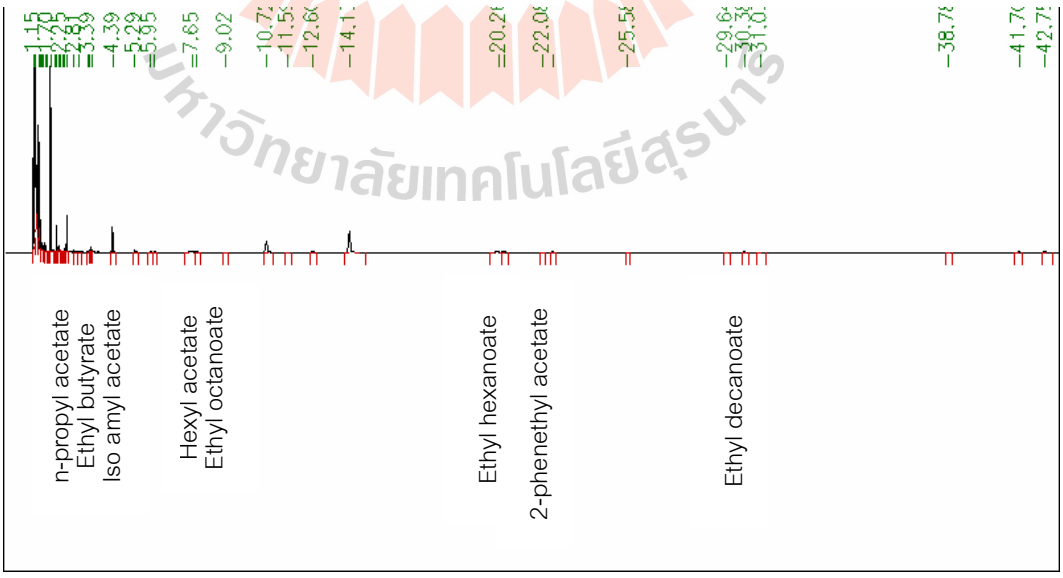

Figure 2B Chromatogram of dichloromethane extract of wine extract.

3. Efficiency of extraction method

The efficiency of extraction method was determined by tripicates extraction of standard ester 100 mg/ L in model wine and 12.5 mg/ L of n-butyl acetate as internal standard. The relative peak areas were compared with injection of standard ester without extraction with the same concentration.

Table 2B. Percentage recoveries of a gas chromatographic determination of esters in dichloromethane extract of a model wine.

Components	Rt	Concentration added (mg/l)	Recovery concentration (mg/l)			Average %recovery	%SD
			1	2	3		
n-Propyl acetate	1.68	100	74.63	77.55	65.61	72.60	6.22
Ethyl butyrate	2.37	100	82.41	90.08	95.55	89.35	6.60
Isoamyl acetate	3.53	100	88.25	79.32	95.66	87.74	8.18
Ethyl octanoate	9.02	100	86.61	80.14	83.32	83.36	3.24
Hexyl acetate	7.85	100	100.21	98.45	90.41	96.36	5.22
Ethyl hexanoate	20.21	100	102.32	99.41	96.71	99.48	2.81
2-phenethyl acetate	22.60	100	97.52	88.36	91.05	92.31	4.71
Ethyl decanoate	30.49	100	95.14	82.11	88.47	88.57	6.52
n-Butyl acetate	2.51	12.50	11.75	10.36	12.54	92.40	8.83

4. Chromatogram of Higher alcohol extract by SPME method

All experiments were seriously controlled extraction time in every step of extraction in order to get high accuracy. In SPME, the analytes establish equilibria among the sample matrix, the headspace above the samples, and length of polymer-coated fused silica fiber.

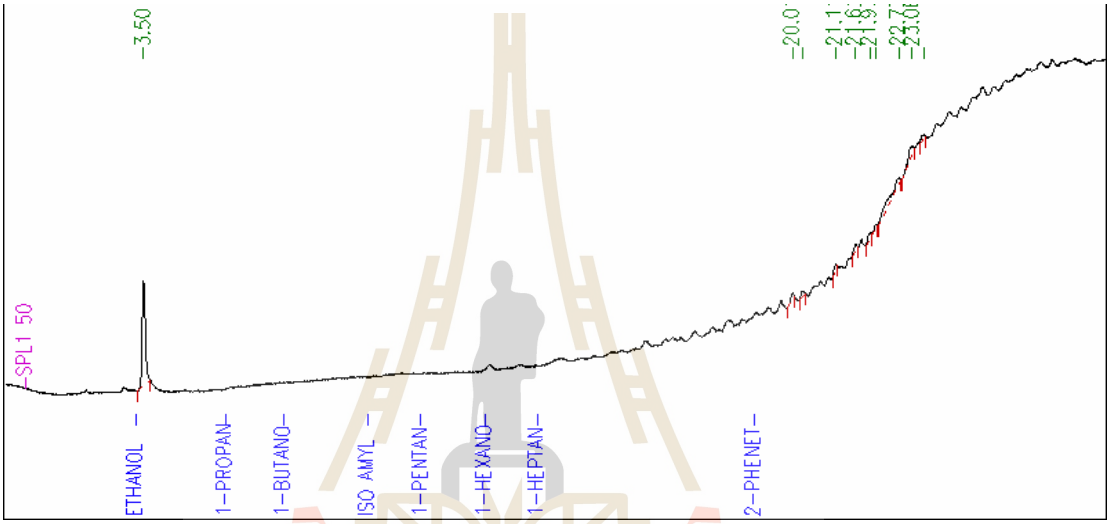

Figure 3B Chromatogram of higher alcohol extract of must.

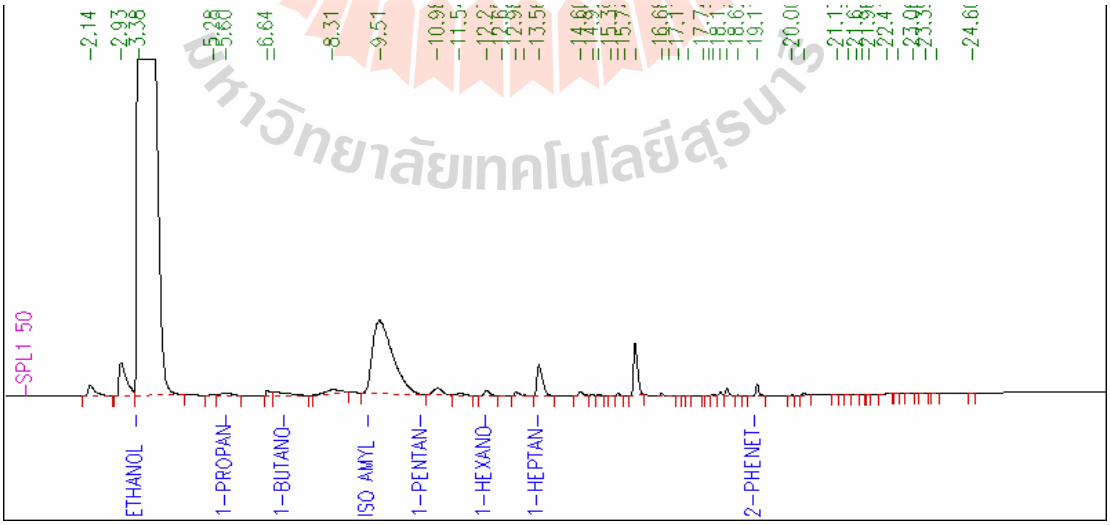

Figure 4B Chromatogram of higher alcohol extract of wine sample.

5. Chromatogram of sugar and phenolic compound analysis by HPLC

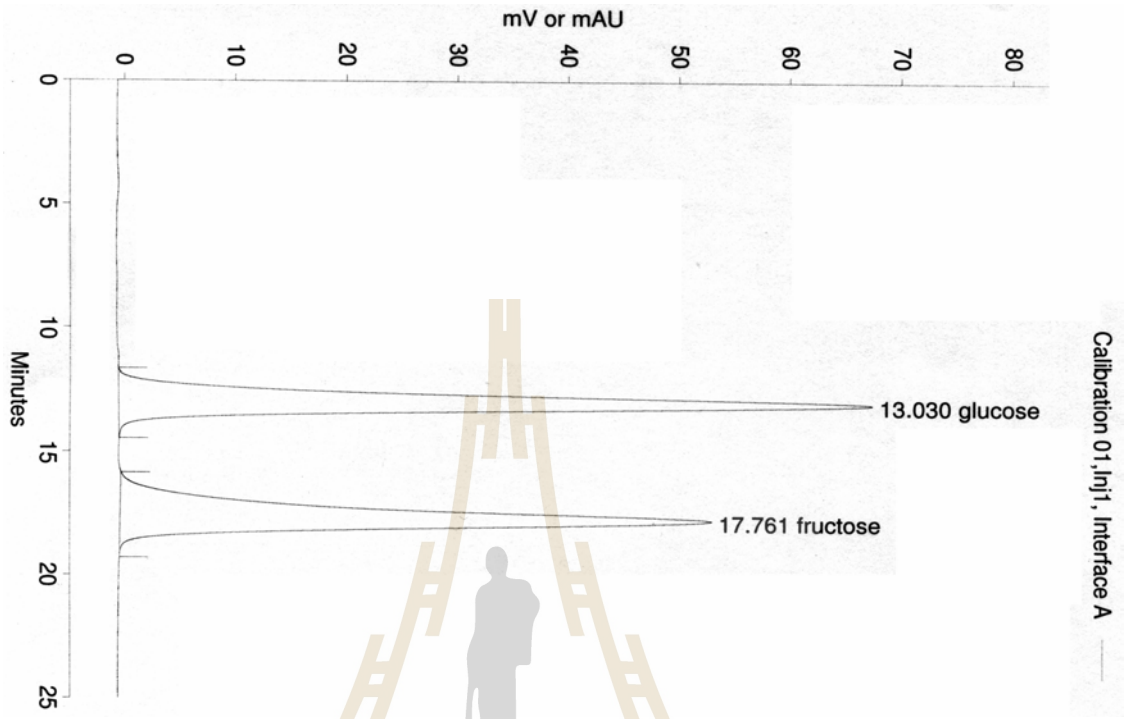

Figure 5B Chromatogram of standard glucose and fructos.

Figure 6B Chromatogram of catechin and epicatechin in wine sample

BIOGRAPHY

Ulaiwan Usansa was born in Nakhon Phanom, Thailand on September 20, 1974. She studied in primary school and high school at Piyamaharachalai school. In 1993, she studied in School of Food Technology, Suranaree University of Technology, Nakhon Ratchasima. She participated in the Co-operative Education Program to work as trainee at Siam Preserved Food Co. Ltd., Ratchaburi. She was trained in research and development department and quality assurance department. She graduated the Bachelor's of science in Food Technology in 1997. After graduation, she worked as teaching assistance for three years in School of Food Technology, Suranaree University of Technology. In 2000, she was Master's student in the field of Biotechnology at Suranaree University of Technology. In the second academic year, she got scholarship from The National Science and Technology Development Agency (NSTDA). This supportive encouraged her research, which studied on effect of alcoholic fermentation temperature on red wine flavor. Moreover, she had great opportunity to be international exchanged student of the French-Thai cooperation program. She worked at Ecole Nationale Supérieure Agronomique of Toulouse (ENSAT) and cooperated with Ecole Nationale Supérieure des Ingénieurs en Arts Cheimique et Technologiques (ENSIACET), Toulouse, France. That work was presented in 23rd International Specialised Symposium on Yeasts "interactions between yeasts and other organisms" 2003, Budapest, Hungary.