การคัดเลือกสายพันธุ์ยีสต์ที่มีเอนไซม์เบต้ากลูโคซิเดสเพื่อเพิ่มความหอมในไวน์

พัชราภรณ์ ศรีปัญญา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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SELECTION OF YEAST STRAINS CONTAINING β-D-GLUCOSIDASE FOR IMPROVING AROMA IN WINE

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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SELECTION OF YEAST STRAINS CONTAINING β-D-GLUCOSIDASE FOR IMPROVING AROMA IN WINE

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

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กลิ่นหอมเป็นคุณลักษณะที่สำคัญของไวน์ที่ได้จากสารประกอบขององุ่น กระบวนการหมัก และการบ่ม เอนไซม์เบต้ากลูโคซิเดสเป็นเอนไซม์ชนิดหนึ่งที่สามารถเพิ่มความหอมในไวน์องุ่นได้ ในการศึกษาครั้งนี้ได้ทำการวัดปริมาณของเอนไซม์เบต้ากลูโคซิเดสจากยีสต์จำนวน 17 สายพันธุ์ โดยผลการทดลองหาค่ากิจกรรมของเอนไซม์เบต้ากลูโคซิเดส พบว่า ยีสต์สายพันธุ์ 71B-1122 มีค่ากิจกรรมสูงสุดที่เวลา 72 ชั่วโมง จึงได้คัดเลือกยีสต์สายพันธุ์ 71B-1122 มาสกัดเอนไซม์ให้บริสุทธิ์ โดยพบว่า เอนไซม์มีน้ำหนักโมเลกุล 29.8 kDa และมีค่ากิจกรรมสูงสุดที่พีเอช 5.0 และที่อุณหภูมิ 50 องศาเซลเซียส โดยมีค่าจลพลศาสตร์ของเอนไซม์ K_m และ V_{max} สำหรับ *p*NPG เท่ากับ 2.44 mM และ 55.56 U/ml ตามลำดับ เอนไซม์สามารถทนอุณหภูมิ 20, 30 และ 40 องศาเซลเซียสเป็นเวลา 1 ชั่วโมง 30 นาที และที่อุณหภูมิ 50 และ 60 องศาเซลเซียส เอนไซม์มีกิจกรรมลดลงอย่างรวดเร็วภายในเวลา 30 นาที เอนไซม์ถูกยับยั้งได้ด้วยเอธานอลที่ความเข้มข้น 5 เปอร์เซ็นต์ จากนั้นนำเอนไซม์ที่แยกบริสุทธิ์ได้ไปใส่ในน้ำองุ่นพันธุ์มัสแคดแฮมเบรท แล้วทดสอบหาสารระเหยพบว่า สาร nerol และ geraniol เพิ่มขึ้นอย่างมีนัยสำคัญ นอกจากนี้เมื่อนำน้ำองุ่นที่ทำการหมักด้วยยีสต์สายพันธุ์ EC-1118 พบว่า ปริมาณของสารระเหยของไวน์ได้ให้สาร hexyl acetate และ nerol เพิ่มขึ้น ในขณะที่ปริมาณของสารระเหยของไวน์ที่เติมเอนไซม์แล้วหมักด้วยยีสต์สายพันธุ์ 71B-1122 พบว่าให้สาร hexyl acetate, linalool, phenethyl alcohol และ nerol เพิ่มขึ้นอย่างมีนัยสำคัญ และยีสต์สายพันธุ์ 71B-1122 ให้กลิ่นหอมได้มากกว่ายีสต์สายพันธุ์ EC-1118 ดังนั้น การคัดเลือกสายพันธุ์ยีสต์และการเพิ่มเอนไซม์เบต้ากลูโคซิเดสในกระบวนการผลิตจึงควรนำมาพิจารณาเพราะส่งปัจจัยดังกล่าวมีความสำคัญในการเพิ่มกลิ่นหอมในไวน์องุ่น

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2548 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

PATCHARAPORN SRIPUNYA : SELECTION OF YEAST STRAINS CONTAINING OF β-D-GLUCOSIDASE FOR IMPROVING AROMA IN WINE. THESIS ADVISOR : ASST. PROF. CHOKCHAI WANAPU, Ph.D., 110 PP. ISBN 974-533-483-9

AROMA/β-D-GLUCOSIDASE/SACCHAROMYCES CEREVISIAE/WINE

Aroma is an important component for characteristics of wine. Wine aroma is the outcome of complex interaction among the substances from the grape berry, those produced during fermentation and aging. β-D-Glucosidase is an enzyme for enhancing wine aroma. The aim of this study was to evaluate β-D-glucosidase activities from 17 yeast strains. It was found that strain 71B-1122 exhibited highest activity of β-D-glucosidase when incubated for 72 hrs. The enzyme of this strain was isolated and purified and found that it was smallest MW of 29.8 kDa. Its optimal pH was 5.0 and optimal temperature was 50°C. The values of K_m and V_{max} are 2.44 mM and 55.56 U/ml, respectively. A half life of this purified enzyme at 20°C, 30°C and 40°C were 1.30 hrs. When incubated at 50°C and 60°C caused a rapid decrease of its activity within 30 min. The enzyme was inhibited by ethanol concentration at 5% (v/v). Muscat Hamberg grape was used and the purified enzyme was added in this process. The must and wine were determined for volatile compounds and found that the nerol and geraniol compounds were significantly after treated with the enzyme. When the must was added with β-D-glucosidase and fermented with EC-1118 strain. The wine showed significantly increased in hexyl acetate and nerol compounds. It was interesting to find that when the yeast strain 71B-1122 was used volatile

compounds of wine were hexyl acetate, linalool, phenethyl alcohol and nerol and they were significantly increased. Moreover this yeast 71B-1122 strain was not only increased volatile compounds but also aroma in wine more than EC-1118 strain. Therefore, in producing wine yeast strain selection and addition of β-D-glucosidase enzyme should be considered because they were important factors affecting wine aroma.

School of Biotechnology

Academic Year 2005

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Patcharaporn Sripunya

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LIST OF ABBREVIATIONS

ANOVA	Analysis of varience
°C	Degree celsius
cm	Centimeter
GC	Gas chromatography
g	Gram
hr	Hour
HS	Head space
kDa	Kilodalton
K_m	Michaelis Menten constant
L	Liter
LC	Liquid chromatogryphy
M	Molarlity
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
MS	Mass Spectrometer
µL	Microliter
µM	Micromolar
µmol	Micromole

LIST OF ABBREVIATIONS (Continued)

MW	Molecular weight
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PDMS	Polydimethylsiloxane
pNPG	para-Nitrophenyl-β-D-glucopyranoside
ppm	Part per million
rpm	Round per minute
s	Second
SAS	Statistical analysis system
SDS	Sodium dodecyl sulfate
SPME	Solid phase micro-extraction
[S]	Concentration of substrate
V_{max}	Maximum velocity
U	Unit
%v/v	Percentage volume by volume
%w/v	Percentage weight by volume

CHAPTER I

INTRODUCTION

1.1 Significant of this study

The principal microorganisms in the alcoholic fermentation of wines are yeasts belonging to genus *Saccharomyces*, in particular *S. cerevisiae*, *S. cerevisiae* var. *ellipsoideus*, *S. bayanus* and *S. oviformis* (Steinkraus, 1992).

Wine making is a part of biotechnology which is centuries old. It has become a global enterprise, which significantly affects the economic well being of many countries. Yeast and certain beneficial bacteria play additional roles, in the development of the flavor and aroma of the wine following the maturation of the liquid after primary fermentation (Walker, 1999).

Wine flavor and aroma are the outcome of a complex interaction among the substances from the grapes, those are produced during fermentation and aging. The typical flavor of wines is mainly due to volatile compounds that come from grapes. It is now well established that apart from free flavor compounds, a significant part of several flavor compounds is accumulated in grape berry and several fruits as odorless non-volatile glycosides (Gunata *et al*., 1985a). Aglycone moieties of glycosides include monoterpenes, C_{13}-norisoprenoids, benzene derivatives and aliphatic alcohols which sugar part is represented by glucose or disaccharides (rhamnose-glucose, arabinose-glucose, and apiose-glucose). The volatile compounds

from glycosides can be released by acid or enzyme hydrolysis. The acid hydrolysis occurs quite slowly in wine making condition (Gunata *et al*., 1985b). Enzymatic hydrolysis of glycosides are carried out with various enzymes, which act sequentially according to two steps: firstly, α-L-rhamnosidase, α-L-arabinosidase or β-D-apiosidase cleavage glycoside linkage between aroma (aglycone) and terminal sugar such as rhamnose, arabinose, orapiose. And secondly, corresponding to the β-D-glucoside, which are released; subsequently liberation of flavor obtains after action of β-D-glucosidase (Mateo and Jimenez, 2000).

β-D-Glucosidase (E.C. 3.2.1.21) is an enzyme for enhancement of wine flavor and aroma. β-D-Glucosidase occurs ubiquitously in plants, fungi, animals and bacteria (Esen, 1993).

Therefore, the objective of this thesis was to determine the capability of specific species of wine yeast to release aroma compounds from the odorless glycoconjugated forms to increase the flavor of wine.

1.2 Research objectives

1.2.1 To determine the quantity of β-D-glucosidase activity of different strains of *S. cerevisiase*

1.2.2 To select the highest activity yeast for purification of β-D-glucosidase

1.2.3 To determine the capability of different species of *S. cerevisiae* of wine yeast to release aroma compounds in wine process

1.2.4 To improve flavor of wine by using purified β-D-glucosidase

1.3 Research hypothesis

The β-D-glucosidase enzyme from *S. cerevisiae* and *S. bayanus* could activate releasing aroma compounds from the odorless glycoconjugated.

1.4 Scope and limitation of the study

The activity of β-D-glucosidase enzyme was determined from seventeen yeast strains, YPD medium was used as fermentation medium for culture and selection of best strain based on high β-D-glucosidase activity. Extracellular β-D-glucosidase enzyme was purified and determined MW using SDS-PAGE. The purified enzyme was characterized and studied of kinetics. The application of purified enzyme was done in the final step for enhancing flavor in juice and wine. Quantification of volatile compounds were done by GC/MS.

1.5 Expected result

1.1.1. Wine yeast strains can produce β-D-glucosidase enzyme.

1.1.2. The β-D-glucosidase which produced from yeast can be used to improve aroma in grape wine.

CHAPTER II

LITERATURE REVIEW

2.1 Wine

2.1.1 Wine yeasts

Yeasts are eukaryotic unicellular microfungi that are widely distributed in the natural environment (Walker, 2000). Yeasts are taxonomically classified as follows (Vine *et al*., 1997):

Phylum	Thallophyta
Subphylum	Fungi
Class	Eumycetes
Subclass	Ascomycetes
Order	Endomycetales
Family	Saccharomycetaceae
Subfamily	Saccharomycoideae
Genera	*Saccharomyces*

Yeasts belonging to genus *Saccharomyces* in particular *S. cerevisiae*, *S. cerevisiae* var. *ellipsoideus, S. bayanus*, and *S. oviformis* are being used in wine production. Particularly in wine companies, to inoculate pure cultures of selected wine yeasts. These may be selected for flavor, production of less or no foam, ability to ferment at low temperatures or flocculation sedimentation behavior. They are

more often selected for absence of any undesirable flavors in wines.

2. 1.2 Wine making

Wine making is a biotechnology which is centuries old. It has become a global enterprise, which significantly affects the economic well being of many countries. The basic activities of modern wineries are fundamentally the same as those undertaken traditionally; sugar from grapes or other fruits are physically extracted, and then fermented by yeasts to produce alcoholic beverage. Yeasts and certain beneficial bacteria play additional roles, in the development of the flavour and aroma of the wine following the maturation of liquid after alcoholic fermentation (Walker, 1999).

2.1.3 Wine classification

Wines are classified according to color (white, red, or rosè) and ethanol content. Wines with 7% to 14% ethanol are described as table wines; more than 14% ethanol designates fortified wines such as port and sherry. Wines are dry (non-sweet), semi-dry, semi-sweet, or sweet. Even dry (non-sweet) wine contains small quantities of sugar. Wines can also be categorized according to texture (still or sparkling) (Steinkraus, 1992 and Walker, 1999).

2.1.4 Steps in manufacture of wine

2.1.4.1) Harvesting: Grapes should be harvested when they are at their peak of maturity with desired acidity and flavor with a high sugar content. They should be as intact as possible and be processed quickly (see Figure 2.1).

2.1.4.2) De-stemming: Stems are removed and the grapes are crushed. The skins are retained for red wines. Sulfite may be added to inhibit oxidation and growth of the natural microbial flora (see Figure 2.1).

2.1.4.3) Pressing: Pressing is separation of the juice from the skins, seeds, and pulp-the pomace. Pectinase may be added to facilitate juice extraction (see Figure 2.1).

2.1.4.4) Fermentation: Fermentation can proceed either through the action of the naturally present yeast or by inoculation of a selected pure yeast culture. Fermentation temperature is generally 10ºC to 15ºC for white wines and somewhat higher for red wines. The higher the temperature, up to about 30ºC, the more rapid the fermentation; however, the slower, lower temperature fermentations are considered to lead to higher quality wines. Less volatile aroma is lost. As the fermentation process, the actively metabolizing yeast produce heat and the temperature of the must tends to rise (see Figure 2.1).

2.1.4.5) Racking: As the fermentation nears completion, the yeast cells tend to settle, and after fermentation has been completed, the wine can be decanted or siphoned (racked) as the first step in clarification of the wine. Racking is repeated periodically until most or all the yeast cells have been separated from the wine. This may be combined with cold stabilization, which is accomplished by storing the wine at about -2ºC. Cold stabilization removes excess tartrates and other materials that might cause cloudiness in the bottle later (see Figure 2.1).

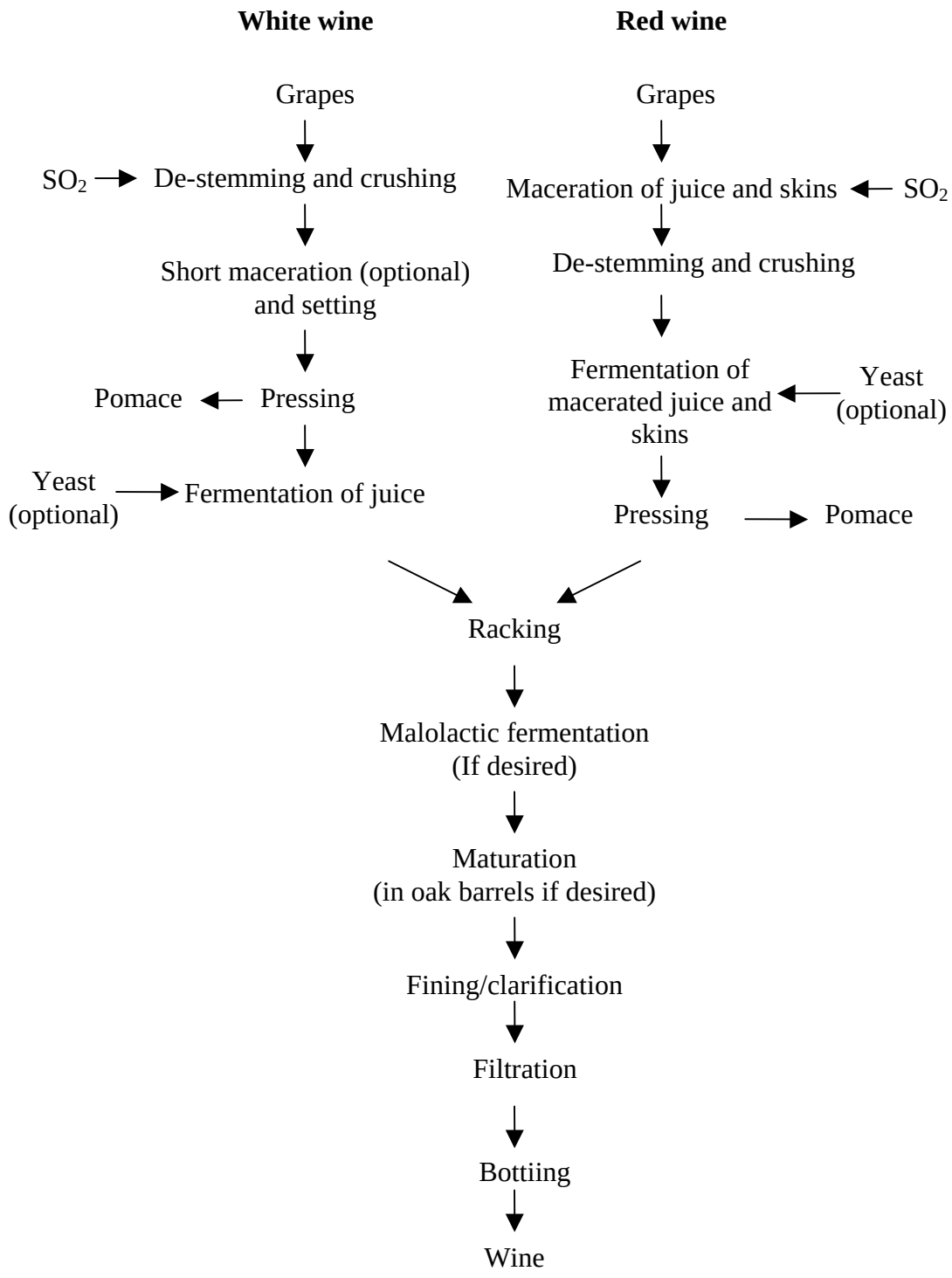


Figure 2.1 Wine production**.** Source: Walker (1999)

2.1.4.6) Clarification: Most wine consumers like a crystal clear wine with no haze or sediment. To accomplish this objective, gelatin, isinglass, or white egg may be added to the wine. These ingredients that removes some of the materials that might cause turbidity. White wines may not contain sufficient tannin. In that case, some tannin sufficient to react with the added protein may be necessary to produce the precipitate and stabilize the wine. Bentonite, clay, is also widely used for clarification. Ion exchange may be used to change potassium to sodium tartrate, thus increasing the solubility and reducing the likelihood of its precipitation later (see Figure 2.1).

2.1.4.7) Bottling: Wines are generally bottled in dark green or brown bottles to decrease deleterious effects on quality that can be caused by sunlight. Small amounts of sulfite may be added to inhibit oxidation. Sweet wines may be fortified with ethanol (18-20%) or the wines may be pasteurized or sterile filtered to prevent growth of contamination in the bottle (see Figure 2.1).

2.1.4.8) Aging: Aging occurs after fermentation. It can occur in tanks, in barrels, or in the bottle. The flavor may be improved through esterification reactions in which small portions of ethanol combine with organic acids in the wine (see Figure 2.1) (Steinkraus, 1992).

2.1.5 Wine fermentation

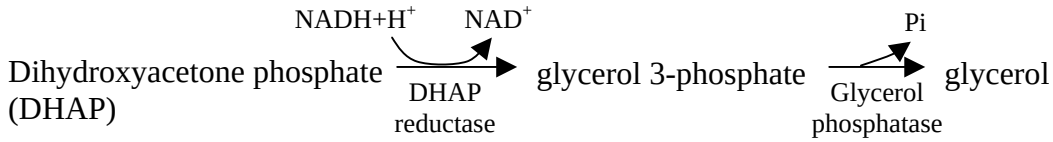
2.1.5.1 Alcoholic fermentation

The yeasts utilize glucose and fructose, the principle sugars in grape juice and metabolize them via the glycolytic pathway to pyruvate. This pathway furnishes the yeast cells with energy and with reducing power for

biosynthesis. Under anaerobic conditions, the yeasts pyruvate yield acetaldehyde and CO_2 by using pyruvate decarboxylase. The final step in alcoholic fermentation is catalysed by alcohol dehydrogenase and involves the reduced coenzyme NADH, and results in the reduction of acetaldehyde to ethanol. The conversion of glucose to ethanol by *S. cerevisiae* can be summarized as**:**

$$C_6H_{12}O_6 + 2Pi + 2ADP + 2H^+ \longrightarrow 2C_2H_5OH + 2CO_2 + 2ATP + 2H_2O$$

In addition to ethanol and CO_2, one of the quantitatively most important products of fermentation by wine yeasts is glycerol. Variable levels (generally in the rang 2-10 g l^{-1}) of glycerol are found in wine, depending on the yeast strains and the fermentation conditions. It is produced by the following reaction**:**



Glycolysis is the major mechanism for the catabolism of carbon compounds by yeasts, but other metabolic pathways also operate during grape juice fermentations including the pentose phosphate pathway and the citric acid cycle.

2.1.5.2 Malolactic fermentation

Malolactic fermentation is a secondary wine fermentation,

carried out by the lactic acid bacteria. The word malolactic comes from the conversion of L-malic acid into L-lactic acid by the activity of these bacteria. During this conversion, CO_2 is also produced. This reaction will decrease total acidity and enhance wine stability. Malolactic bacteria are capable of direct decarboxylation of malic acid to lactic acid by the enzyme malate carboxylase (EC. 1.1.1.38), which is present in various lactic acid bacteria, but particularly in three genera: *Lactobacillus, Leuconostoc* and *Pediococcus* (Bozoglu and Yurdugul, 1999).

2.1.6 Volatile compounds

The complexity of wine aromas are difficult to study due to the diversity of the mechanisms involved in their devolvement:

1. Grape mechanism, depending on the variety, as well as soil, climate, and vineyard management techniques
2. Biochemical phenomena (oxidation and hydrolysis) occurring prior to fermentation, triggered during extraction of the juice and maceration
3. The fermentation mechanisms of the microorganisms responsible for alcoholic and malolactic fermentations. The basic odor of wines to four ester (ethyl acetate, isoamyl acetate, ethyl hexanoate and actanoate), two alcohols (isobutyl and isoamyl alcohol) and cysteine; which is a common component in wine and its degradation can help lead to the formation of thiol (thiazole, Trimethloxazol, thiopheae-2-thiol) as showed in Figure 2.2 (Klingshirn, 2002; Rapp and Mandery, 1986).
4. Chemical or enzymatic reaction occurring after fermentation, during aging of the wine in vat, barrel or bottle. (Maujean *et al*.,

2000). Aging wine in oak also affects the flavour considerably, with the extraction of volatile phenolics including vanillin and eugenol (see in Figure 2.2), which produce vanilla and clove or spicy aromas in the finished wines (Ramey and Ough, 1980; Puech, 1987). The bouquets were produced from the transformation of the aroma during aging (Rapp and Mandery, 1986).

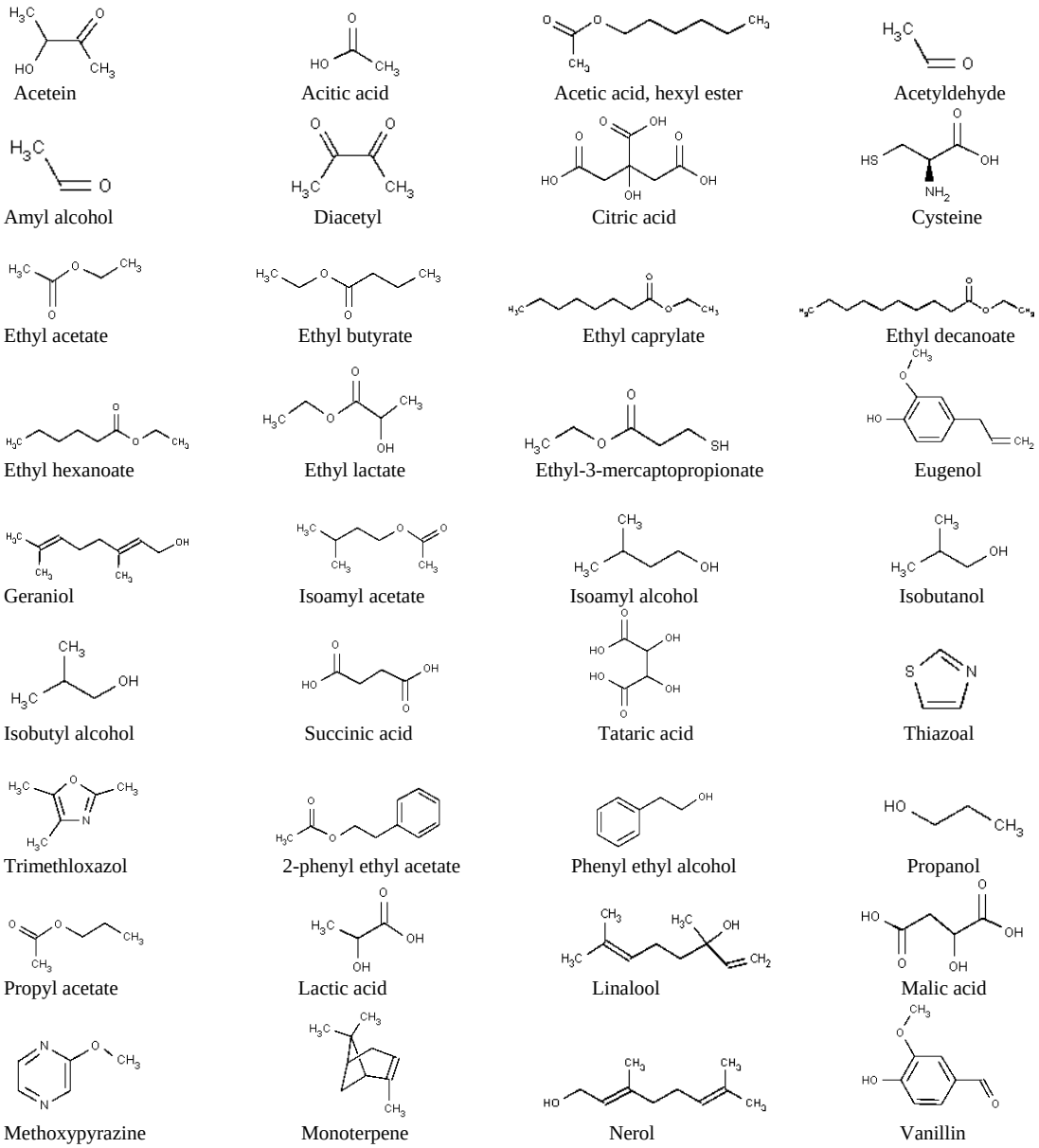


Figure 2.2 Chemical compounds in wine making process (Online; http://chem.sis.nlm.nih.gov/chemidplus/)

More than 800 components have been identified in the volatile fraction of wine by using gas chromatography, which is a primary analytical technique to detect the different volatile compounds (Gunata *et al*., 1985b; Heras *et al*., 2002).

The volatile compounds are one of the major grape that contribute to wine aroma. They are present in two forms: a free volatile form and non-volatile glycosides. The aglycone moieties of glycosides include monoterpenes, C_{13}-norisoprenoids, benzene derivatives and aliphatic alcohols; and a sugar part is represented by glucose or disaccharides (rhamnoses-glucose, arabinose-glucose, and apiose-glucose) (Gunata *et al*., 1985a).

The aroma compounds in *Vitis vinifera* grapes which have been studied in the greatest detail belong to the terpene family. These compounds are responsible for the characteristic aroma in Muscat grapes and wines. Both free forms and odorless, mainly glycosylated, precursors have been identified in wine and grapes.

Other compounds also contribute to varietal aroma. Norisoprenoids, not strictly considered terpenes, are produced by the chemical or enzymic breakdown of carotenoids in grapes. They also occur in the form of glycosylated precursors.

The role of methoxypyrazines in the herbaceous aroma of certain grape varieties, such as Cabernet Sauvignon, is now well-established. These compounds exist in a free state in grapes and no precusor forms have been identified. More recently some highly odoriferous sulfur compounds with thiol functions have been shown to participate in the aromas of certain grape varieties, especially Sauvignon Blanc. The compounds occur in grape in *S*-cysteine conjugate form (Maujean *et al*., 2000).

2.2 Terpene compounds

2.2.1 Odoriferous terpene

The large family of terpene compounds (approximately 4000 compounds) are very widespread in the plate kingdom. Compounds within this family likely to be odoriferous are monoterpenes (compound with 10 carbon atoms) and sesquiterpenes (15 carbon atoms), formed from two and three isoprene units, respectively as showed in Figure 2.3. Monoterpenes occur in the form of simple hydrocarbons (limonene, myrcene, etc.), aldehydes (linalal, geranial, etc.), alcohols (linalool, geraniol, etc.), acids (linalic and geranic acid, etc.), and even ester (linalyl acetate, etc.). About forty terpene compounds have been identified in grapes. Some of the monoterpene alcohols are among the odoriferous, especially linalool, α-terpineol nerol, geraniol, citronellol and Ho-trienol (see in Figure 2.3) has a floral aroma reminiscent of rose essence. The olfactory perception thresholds of there compounds are rather low, as little as a few hundred micrograms per liter. The most odoriferous are citronellol and linalool. Furthermore, the olfactory impact of terpene compounds is synergistic. They play a major role in the aromas of grapes and wine from the Muscat family, as concentrations are often well above the olfactory perception thresholds. Monoterpenol concentrations in wines made from grape varieties with simple flavors (Sauvignon Blanc, Syrah, Cabernet Sauvignon, Chardonnay Franc, Merlot, etc.) are generally below the perception threshold. There are, however, Chardonnay clones with the Muscat character. There are normally eliminated from clonal selections of vines, as their wines do not have typical varietals character (Maujean *et al*., 2000).

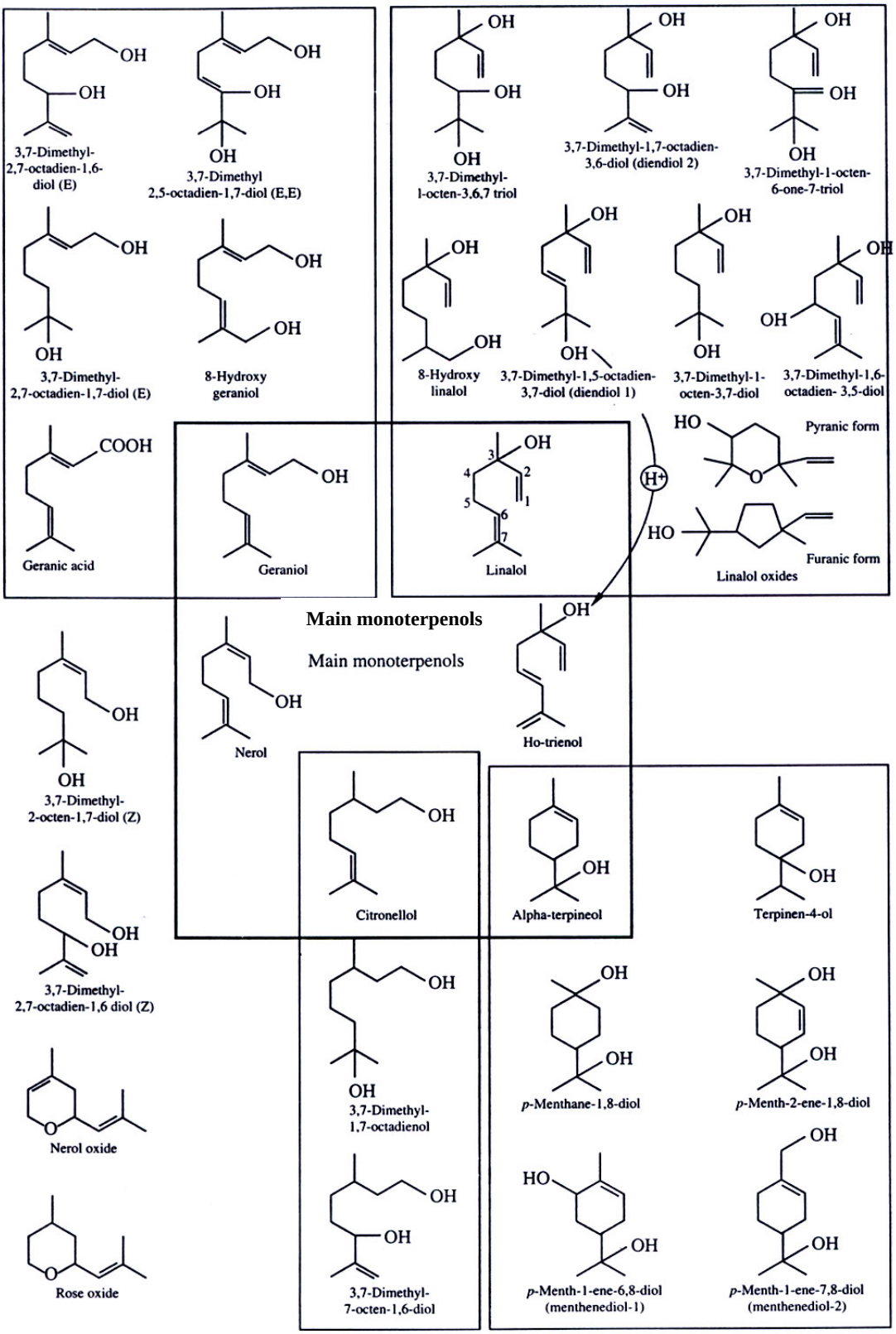


Figure 2.3 The main monoterpenes and theirs derivatives identified in grapes and wine (Maujean *et al*., 2000)

2.2.2 Glycosylated forms of volatile terpenols

The main monoterpenenols and terpene polyols are presented in grapes in glycoside form, including the basic 'sugar': glucose, arabinose, rahmnose and apiose. Four types of glycosides have thus been identified: three diglycisides (6-O-α-L-arabinofuranosyl-β-D-glucopyranoside, 6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside, 6-O-β-D-apiofuranosyl-β-D-glucopyranoside, and monoglucoside (β-D-glucopyranoside), as depicted in Figure 2.4.

All grape varieties contain similar glycosides, but the Muscat-flavored grape varieties have the highest concentrations. Glycosylated forms are frequently more common then free aromas. Among the glycosides corresponding to the most odoriferous aglycones, apisylglucosides and arabinosylglucosides are the most widespread, followed by ramnosidase and then β-D-glucosidase. Terpene glycoside are very common in plants. However, in vines, unlike other plants, monoglucosides are in the minorlity as compared to diglycosides (Maujean *et al.*, 2000).

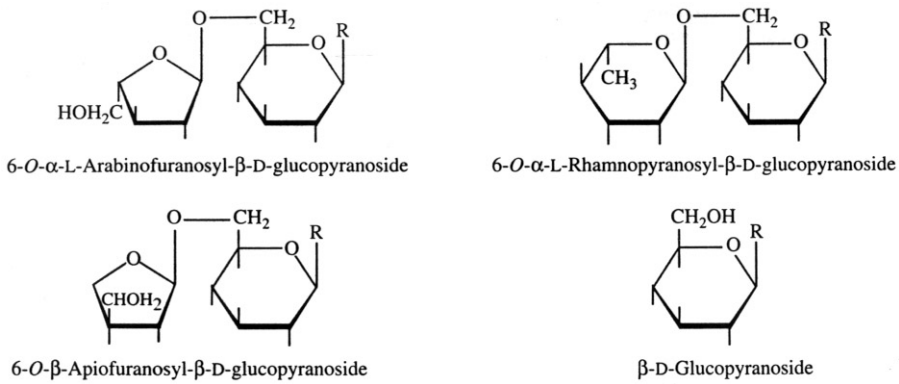


Figure 2.4 The various forms of terpene glycoside (or norisoprenoid) identified in grapes: R= terpene or C_{13}-noriziprenoid (Maujean *et al.*, 2000)

Grape skins have a higher concentration of free and glycosylated monoterpenes than the flesh or juice. The free terpenol composition varies a great deal in the different parts of grapes. Thus, geraniol and nerol are more common in the skin than in the flesh and juice. The proportions of the various bound terpenols are largely the same throughout the grape. The relative proportions of free and bonded compounds depend on the grape variety.

As glycosides are much more water soluble than aglycones, they are considered to be vectors for the transport and accumulation of monoterpenes in plants. Glycosides have been primarily identified in vines leaves and leaf stems. In these glycosylated derivatives, the aglycones are not exclusively alcohols or terpene polyols.

2.2.3 C_{13}-Norisoprenoid derivatives

2.2.3.1 Odoriferous C_{13}-Norisoprenoid derivatives.

The oxidative degradation of carotenoids terpenes with 40 carbon atoms (tetraterpenes), produces derivatives with 9, 10, 11 or 13 carbon atoms have interesting odoriferous properties. From a chemical point of view, these norisoprenoid derivatives are divided into two main forms: megastigmane and non-megastigmane. Each of these includes a large number of volatile compounds. The megastigmane skeleton is characterized by benzene cycle substituted on carbon 1, 5 and 6, and an unsaturated aliphatic chain with four carbon atoms attached to C_6. Megastigmane are oxygenated C_{13}-norisoprenoid, with skeletons oxygenated on carbon 7 (demascone series) or carbon 9 (ionone series) as showed in Figure 2.5. Non-megastigmane C_{13}-norisoprenold derivatives have also been identified,

including a few rather odoriferous compounds. The most important of these is TDN (1,1,6-trimethyl-1,2-dihydronaphtalene), which has a distinctive kerosene odor. It plays a major role in the 'petroleum' smell of old Riesling wines. TDN is generally absent in grapes and young wine, but may appear during bottle aging, reaching concentrations of 200 µg/l, whereas its perception threshold is on the order of 20 µg/l. However, C_{13}-norisoprenoids are mainly present in grapes in the form of non-volatile precursors (carotenoids and glucosides). Like monoterpenes, certain C_{13}-norisoprenoids (vomifoliol, 3-oxo-α-ionol, 3-hydroxydamascone) exist in glycoside form (Maujean *et al*., 2000).

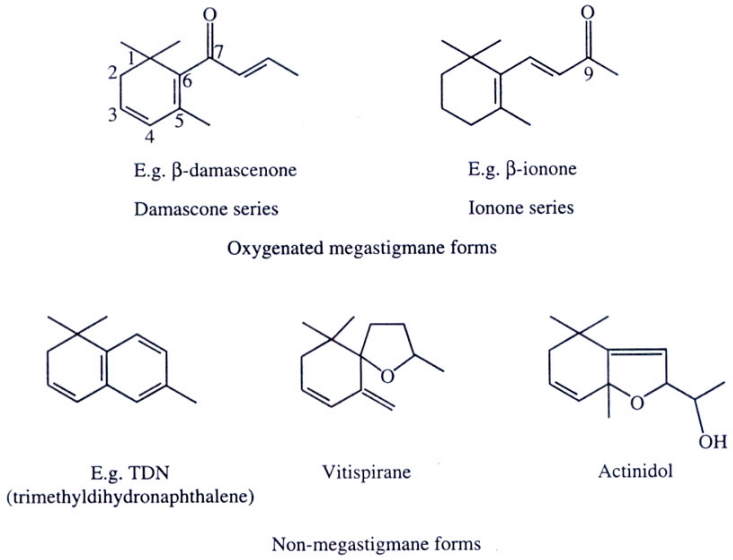
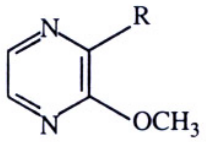


Figure 2.5 Main families of C_{13}-norisoprenoid derivatives in grapes (Maujean *et al*., 2000)

2.2.4 Methoxypyrazines

Methoxypyrazines are nitrogenated heterocycles produced by the metabolism of amino acid. The compound appeared in Figure 2.6 2-methoxy-3-isopropypyrazine, 2-methoxy-3-sec-butylpyrazine and 2-methoxy-3-

isobutylpyrazine, have odors reminiscent of green pepper, vegetative, herbaceous, bell pepper or earthy aroma to wines of some grape varieties. This was first identified in grapes (Cabernet Sauvignon). Since then, 2-methoxy-3-isobutylpyrazine and the other pyrazines have been identified in many grape varieties and their wines (Sauvignon Blanc, Carbernet Franc, Merlot, Pinot Noir, Chardonnay, and Riesling. However, concentrations of these compounds are only significantly above the recognition threshold in Sauvignon Blanc, Cabernet Sauvignon and Carernet Franc grapes and wines and some time Merlot (Maujean *et al*., 2000).



R: $CH_2CH(CH_3)_2$ 2-Methoxy-3-isobutylpyrazine
R: $CH(CH_3)_2$ 2-Methoxy-3-isopropylpyrazine
R: $CH(CH_3)CH_2CH_3$ 2-Methoxy-3-*sec*-butylpyrazine

Figure 2.6 The main methoxypyrazines (Maujean *et al*., 2000)

2.2.5 Sulfur compounds with a thiol function

2.2.5.1 Odoriferous volatile thiols involved in the varietal aromas of wines

Sulfur compounds in the thiol family (or mercaptans) are generally held responsible for olfactory defects. However, their major contribution to the aromas of certain fruits and aromatic plants has been clearly established. Thus, specific thiols are involved in the characteristic aromas of fruits such as black currant, grape fruit, passion fruit and guava. Two mercaptans, ethyl-3-mercaptopropionate) and ethyl-2-mercaptopionate (Figure 2.7-2.8), have been identified as components in the aroma of *V. labrusca* grapes. Several highly

odoriferous thiols have recently been identified in Sauvignon Blanc wines. These wines have marked characteristic aromas, featuring various herbaceous and fruity nuances, reminiscent of green pepper, box wood, broom, grape fruit, passion fruit and smoke (Maujean *et al*., 2000).

2.6.4.2 Precursors of volatile thaiols derivatived from cystine

Sauvignon Blanc musts, like those of many grape varieties with relatively simple aromas, are not highly odoriferous. The characteristic aroma of the grape variety appears during alcoholic fermentation. It has now been established that 3-mercaptohexanol, 4-methyl-4-mercaptopentan-2-one and 4- methyl-4-mercaptopentane-2-ol are present in *S*-cysteine conjugate form *S*-(3-hexan-1-ol)-cysteine, *S*-(4-methylpentan-2-one)-cysteine, *S*-(4-methylpentan-2-ol)-cysteine and *S*-(3-hexan-1-ol)–cysteine (Figure 2.7). These compounds are present in must in high quantities than that aromas they generate in wine. The corresponding aromas are revealed during alcoholic fermentation, probably due to the action of specific β-lyase (Maujean *et al*., 2000). Thus, fermentation by *S. cerevisiae* of a model medium supplemented with these compounds, ether extracted from must or obtained by chemical synthesis, is accompanied by releasing of the corresponding aroma. These aromas vary in intensity depending on the *S. cerevisiae* yeast strains (Maujean *et al*., 2000).

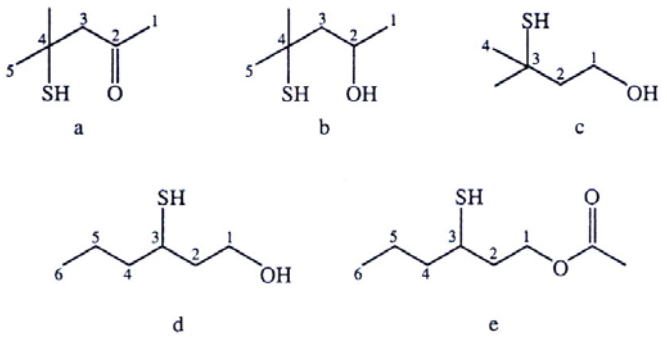


Figure 2.7 Volatile thiolsodoriferous identified in Sauvignon Blanc wine: a, 4-mercapto-4-methyl-pentane-2-one (4MMP); b, 4-mercapto-4-methyl-pentane-1-ol (4MMPOH); c, 3-mercapto-3-methyl-butan-1-ol (3MMB); d, 3-mercaptohexan-1-ol (3MH) and e, 3-mercaptohexanol acetate (A3MH) (Maujean *et al*., 2000)

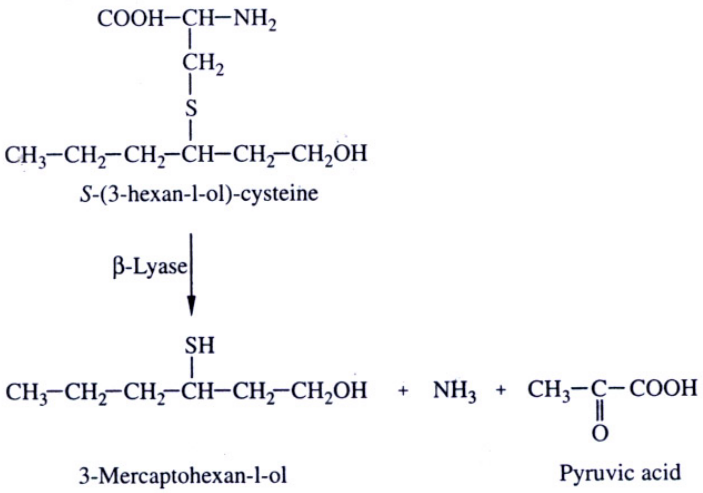


Figure 2.8 The cysteine conjugated form of 3-mercaptohexanol, revealed by a specific *β*-lyase (Maujean *et al*., 2000)

During fermentation wine yeasts can produce many compounds. The metabolism of nitrogen and sulphur compounds yield, together with the main metabolites, hundreds of volatile and non-volatile minor metabolites, which

collectively contribute to the flavour and aroma of wine. These include:

- higher alcohols: isoamyl alcohol, active amyl alcohol, isobutanol, propanol, 2-phenylethanol
- ester: ethyl acetate, ethyl lactate, phenylethyl acetate, isoamyl acetate, ethyl octanoate, ethyl hexanoate
- organic acid: succinic acid, tartaric acid, malic acid, lactic acid, acetic acid, citric acid
- aldehydes and ketones: acetaldehyde, diacetyl, acetoin
- sulphur compounds: hydrogen sulfide, sulfur dioxide, dimethyl sulphide.

The relative concentrations of these compounds depend on the strains of yeast employed and the fermentation conditions, especially temperature. White wine fermentations are generally conducted at 10-18 ºC (for 7-14 days or longer), are red wine fermentations at 20-30 ºC (for 7 days or longer) (Walker, 1999).

The volatile compounds are higher alcohol, ester and aldehyes which can contribute to wine aroma during fermentation.

2.2.6 Higher alcohols

Higher alcohols are produced from the carbon skeletons of amino acids. They may arise by the decarboxylation and deamination of amino acids present in the wort or by a biosynthetic route using the amino acid biosynthetic pathway of the yeast (Figure 2.9). Both of these routes may occur in the same fermentation with a switch from the degradative route to the biosynthetic route occurring when the amino acids in the wort have been metabolized. In the general,

conditions which flavor a high growth rate tend to stimulate the level of higher alcohol production. These parameters include an elevated temperature, high inoculums levels, and aeration of the medium and replacement of sugars such as maltose by glucose. These general effects will increase the levels of all higher alcohols present in the medium; however, the levels of individual higher alcohols can be manipulated by altering the amount of the corresponding amino acid in the worth or must, or by genetically manipulating the organism so that producers may control the amount of a given amino acid or higher alcohol. This can be advantageous since certain amino acids have a distinctive flavour; for example, phenylalanine stimulates phenyl ethanol production, a higher alcohol which gives a rose-like aroma (Lea and Piggott, 2003).

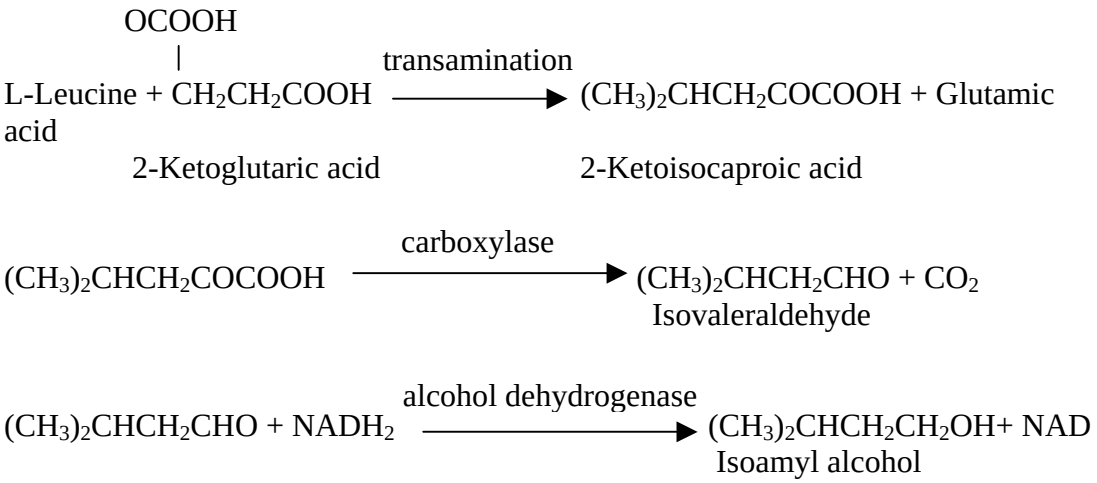


Figure 2.9 Basic routes by which yeasts form the major flavour groups during fermentation (Lea and Piggott, 2003)

2.2.7 Esters

Esters are produced during the fermentation by the yeast which acyl

CoA molecules are key intermediates in the production of free organic acids. The amount of esters is produced dependent on the relative abundance of the correspounding alcohol and acyl CoA produced by the yeast (see Figure 2.10). Since acetyl CoA and ethanol are the most abundant acids and alcohol present in the fermentation, ethyl acetate is normally the most abundant ester. However, if good analytical techniques are used, almost every combination of acyl CoA and alcohol can be detected as esters in the fermentation products (Lea and Piggott, 2003).

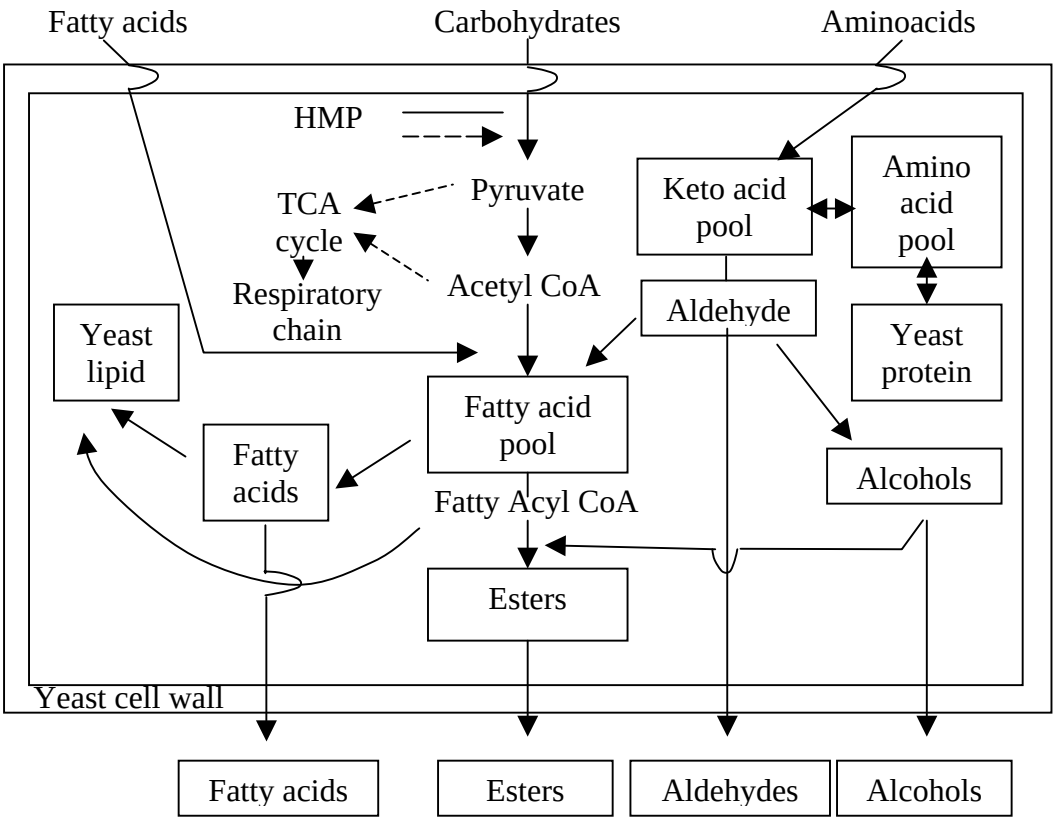


Figure 2.10 Basic routes by which yeasts form the major flavor groups during fermentation (Ramsay and Barry, 1982)

2.2.8 Aldehydes

Grapes contain a number of aldehydes, although the importance in the aroma of wine appears limited. Aldehydes are the second compound containing the carbonyl group (C=O).

Moreover, the volatile glycosides can be released by acid or enzyme hydrolysis (Mateo and Jimenez, 2000). The acid hydrolysis of grape glycosides occurs when protonated reagents break the glycosyl bond between D-glucose and the aglycone, producing one molecule of water. Acids naturally present in wine can cause such cleavage, but at normal wine pH (3.2-3.8) this reaction proceeds very slowly (Sefton, 1998). Enzymatic hydrolysis can released of free volatile compounds from natural glycoside precursors to improvement wine aroma (Cabaroglu *et al.*, 2003; Mateo and Jimenez, 2000).

2.3 Enzymatic hydrolysis

Enzymatic hydrolysis of diglycosides (Figure 2.11) general occurs two steps: first, the inter-sugar linkage is cleaved by either α-L-arabinofuranosidase, α-L-rhamnosidase or β-D-apiosidase regardless of the structure of aglycone moiety (and the corresponding monoterpenyl β-D-glucoside are released. The liberation of the aglycone moiety can only take place during the second step, which consists in the action of an β-D-glucosidase on the previous monoterpenyl β-D-glucoside (Gunata *et al.*, 1988; Mateo and Jimenez, 2000).

2.4 β-D-Glucosidase

β-D-Glucosidase or β-D-glucosidase glucohydrolase (E.C.3.2.1.21) is an enzyme catalyze the hydrolysis of glycosidic linkages in aryl and alkyl β-D-glucoside and cellobiose. A general scheme for the mechanisms of glycoside hydrolases is illustrated in Figure 2.12.

It is found in an astonishing number of organisms, as the hydrolysis of glycosids is an essential pathway of much metabolic process. Plants were found from sweet almond (Lalegerie, 1974), chick pea (Hosel *et al*., 1978) *V. vinifera* (Gunata *et al*., 1985a), raspberry (Pabst *et al*., 1991), pineapple (Wu *et al*., 1991). Fugals such as, *Aspergillus oryzae* (Riou *et al*., 1998). *A. niger* (Spagna *et al*., 1998). Yeasts such as, *Saccharomyces* (Zarzoso *et. al*., 1998; Gueguen *et al*., 1994; Hernández *et al*., 2002), *Candida wickerhanii* (Skory *et al*., 1996), and Bacteria such as *Agrobacterium* (Trimber *et al*., 1993), *Lactobacillus plantarum* (Spano *et al*., 2005).

β-D-glucosidase has been the subject of much recent research due to the key role these enzymes play in biological processes and potential biotechnological applications. Among them, plant β-D-glucosidase play role in defense against pests (Niemeyer, 1988; Poulton, 1990), phytohormone activation (Brzobohaty *et al*., 1993; Matsuzaki and Koiwai *et al*., 1986; Schliemann, 1984; Smith and Van Staden, 1978; Wiese and Grambow, 1986), lignification (Dharmawardhana *et al*., 1995), and cell wall catabolism (Simos *et al*., 1994).

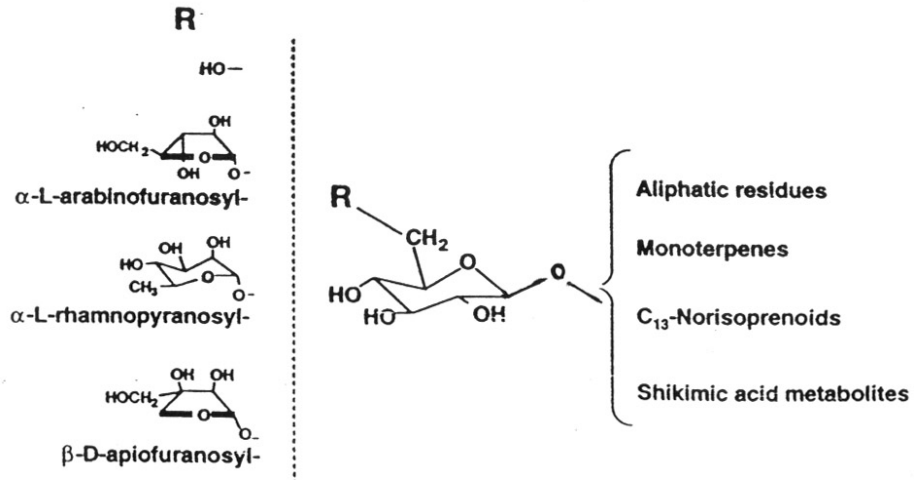


Figure 2.11 Types of glycoconjugates identified as flavor precursors in fruits (Williams, 1993)

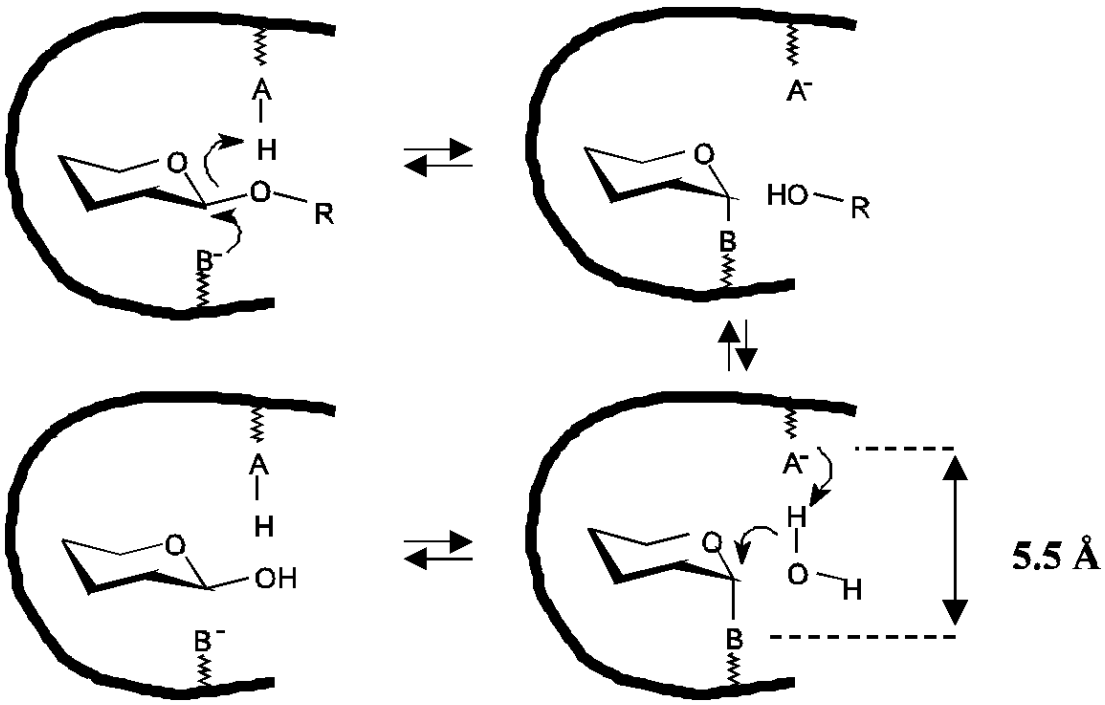


Figure 2.12 General glycoside hydrolysis mechanism for β-D-glucosidase-R, where R is an aglycone such as geraniol (Sinnott, 1991 and McCarter and Withers, 1994)

β-D-Glucosidase can have a high degree of substrate specificity, grape β-D-glucosidase was found to be quite specific to the aglycone (Esen, 1993). Furthermore, many β-D-glucosidase enzymes area studied so far in viticulture and enology have exoglucosidase activity and comparatively few have endoglucosidase activity.

2.11 Yeast β-D-glucosidase

β-D-Glucosidase in *Candida molischiana* and *C. wickeranii* showed a low sensitivity to glucose and are active on a rather non-specific range of aglycones (Sánchez-Palomo *et al*., 2005). Rosi *et al*. (1994) found a strain of *Debraomyces hansenii* capable of producing an exocellular β-D-glucosidase with activity uninhibited by high ethanol and glucose concentrations and whose activity was largely unaffected by acidic pH and low environmental temperatures. Laboratory strains of *S. cerevisiae* have been found to process β-D-glucosidase encoding genes, and do exhibit some hydrolytic activity, but non-*Saccharomyces* yeasts present higher hydrolytic activity (Zarzoso *et. al*., 1998). Isolation two genes for extracellular β-D-glucosidase, BGL1 and BGL2 from genomic library of the yeast *Sm. fibuligera*. Gene products (BGLI and BGLII) were purified from the culture fluids of recombinant *S. cerevisiae*. Molecular weights of BGLI and BGLII are 220 kDa and 200 kDa, respectively by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Both β-D-glucosidase enzymes showed the same enzymatic characteristics, such as thermo-denaturation kinetics and dependencies on pH and temperature, but quite different substrate specificities: BGLI hydrolyzed cellobiose efficiently, but BGLII did not. The observation that the *S. cerevisiae* transformant

carrying BGL1, fermented cellobiose to ethanol but the transformant carrying BGL2 did not (Machida *et al.*, 1988). A number of parameters affecting β-D-glucosidase activity were evaluated. Optimal pH and temperature were 4-6 and 40-60ºC, respectively (Hernández *et al.*, 2002; Gueguen *et al.*, 1994).

2.6 β-D-Glucosidase in wine making

β-D-Glucosidase activity contributes to aroma formation during the wine making process. Free and glycosidically bound volatile compounds were isolated and identified from Muscat grape juice. Most abundant in free and bound form was the Muscat character impact aroma compound 2,5-dimethyl-4-hydroxy-3(2H)-furanone (Furaneol). Other aroma compounds, such as *o*-aminoaacetophenone, 2-phenylethanol and *p*-vinylgualacol were in the glycoside form and many bound monoterpenes were identified (Baek and Cadwallader, 1999). β-D-Glucosidase of glycoside extracted from Muscat, Riesling, Semillon, Chardonnay, Sauvignon and Shiraz verities have provoked the liberation not only of terpenes, but also C_{13}-norisoprenoids, such as 3-oxo-α-ionol and 3-hydroxy-β-damascenona (Mateo and Jimenez, 2000) volatiles, benzene derivatives and attributes honey, lime and smoky (Cabaroglu *et al.*, 2003). Moreover, Gueguen *et al.*, (1997) used β-D-glucosidase in immobilized form to improve the aromatic quality of Muscat wine. The Gas chromatograpephy - Mass spectrophotometer (GC-MS) analysis of indicate significant increase in flavor compound e.g. nerol, geraniol, linalool, γ-terpinen, 2-phenylethanol, and benzyl alcohol.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All chemicals used for chemical reaction were analytical grades and chromatography grades for protein purification They were purchased from BDH, England; Fluka Chemika, Swizerland; Himedia, India; Merck, Germany; and Across, USA.

3.1.2 Microorganisms

Table 3.1 Yeast strains in this study

Yeast strains	Species	Source
1) DV-10	*S. bayanus*	The LALLEMAND Inc., Montreal Canada (LALVIN®)
2) D254	*S. cerevisiae*	”
3) K1-V1116	*S. cerevisiae*	”
4) EC-1118	*S. bayanus*	”
5) Bayanus	*S. bayanus*	”
6) ICV D47	*S. cerevisiae*	”
7) 71B-1122	*S. cerevisiae*	”
8) RC212	*S. cerevisiae*	”
9) Pasteur Red	*S. cerevisiae*	The Universal Food corporation Milwaukee (RED-STAR®)
10)Pasteur Champagne	*S. bayanus*	”
11)Montrachet	*S. cerevisiae*	”
12) Danstil 46 EDV	*S. cerevisiae*	”
13) Noble Ferm	*S. cerevisiae*	The Winetech Supply and Consult Co., Ltd

Yeast strains	Species	Source
14) Ruby Ferm	*S. cerevisiae*	”
15) CY-3079	*S. bayanus*	CP Co., Ltd., Thailand
16) L3109	*S. cerevisiae*	Suranaree University of Technology, Thailand
17) Beerkit	*S. cerevisiae*	Auckland New Zealand (BREWTEC®)

3.1.3 Grapes

Muscat Hambery, Viognier and Early Muscat (*V. vinifera*) were obtained from Suranaree University of Technology farm which was harvested in May 2005.

3.1.4 Equipment

Equipment used was as follows: Gas chromatography-Mass spectrophotometer: GC-MS (model CP 3800 coupled to a mass model 1200L Quadrupole MS/MS, Varian®), solid-phase microextraction (SPME) (Slupelco®), DEAE Sepharose column (Bio-Rad®), hydroxyapatite column (Bio-Rad®), Gel electrophoresis (Bio-Rad®), spectrophotometer, microcentrifuge, incubator, water bath, hot air oven, refrigerator (4°C), freezer (-20°C), laminar flow hood, pH meter, compound microscope and basic microbiological equipment. All equipments are located at the Center for Scientific and Technological Equipment, Suranaree University of Technology.

3.2 Methods

3.2.1 Quantification of β-D-glucosidase activity

The quantificative determination of β-D-glucosidase activities of 17 *Saccharomyces* strains cultured under aerobic condition was done as following; 6 ml of modified YPD medium from Hernández *et al*. (2002) (1% yeast extraction, 2%

peptone, 2% glucose, 0.05% $MgSO_4$, 0.03% $CaCl_2$) in 15 ml test tube was incubated at 30ºC. Growth was monitored by measuring absorbance at 600 nm. Supernatant were stored at 4ºC for analysis (Hernández *et al.*, 2002). The 900 µl of reaction mixture containing 3.3 mM of *p*-nitrophenol-β-D-glucopyranoside (*p*NPG), 100 mM acetic acid buffer, pH 5.0 and 100 µl of supernatant was incubated at 50°C for 25 min. The reaction was stopped by adding 2 ml of 1 M Na_2CO_3 and absorbance was measured at 400 nm. The β-D-glucosidase activity was evidenced by hydrolysis of the substrate (*p*NPG), resulting in the released of *p*-nitrophenol, a pigment substrate which can be measured by using spectrophotometer at 400 nm (Hernández *et al*., 2002; Mateo and Stefano, 1997).

3.2.2 β-D-Glucosidase purification

A. Preparation of crude enzyme

For purification of extracellular β-D-glucosidase**,** cells were grown in 6 L flasks containing 4 L of applied YPD medium. Supernatant solution was collected by centrifuge at 10,000 rpm (SORVALL®GSA ROTOR RC 5C Plus Super Speed Centrifuge) for 5 min. The supernatant obtained after centrifugation was saved at 4ºC as the crude enzyme for purification.

B. Purification of β-D-glucosidase

All purification steps were carried out at 4ºC. The crude enzyme was subjected to 80% ammonium sulfate precipitation and the precipitate was redissolved in minimal volume of 50 mM sodium phosphate buffer (pH 7.0). Dialyzed and concentrated enzyme was loaded on ion exchange chromatography column.

DEAE- Sepharose

Crude enzyme from a 4 L culture was applied to the first column (1x28 cm) of DEAE-Sepharose, which had been equilibrated with 50 mM phosphate buffer (pH 7.0). The flow rate was 25 ml/hr. The enzymes were eluted with a linear gradient of 0.1-0.5 M KCl in 50 mM phosphate buffer at the flow rate of 20 ml/hr, and fraction size of 2 ml was collected. The fractions were for analyzed β-D-glucosidase activity and protein by absorbance at 280 nm. The fractions of each activity peak were pooled and dialysis for 24 hrs at 4ºC. The protein peak containing high enzyme activity was transferred to the second column (Wallecha and Mishra, 2003).

Hydroxyapatite column

The enzyme mixtures separated from the first column were layered onto the second column (1x14 cm) of hydroxyapatite column, which equilibrated with 20 mM phosphate buffer (pH 7.0). The enzymes were eluted with a linear gradient of 20 to 500 mM phosphate buffer at flow rate of 10 ml/hr and 2 ml fractions were collected. Protein fractions were assayed for β-D-glucosidase activity. The fraction showing high specific anzyme activity was pooled and analyzed by gel electrophoresis (Wallecha and Mishra, 2003).

C. Quantification of protein concentration

Quantification of protein concentration was done by using Dye-binding Bradford method (Bradford, 1976 and Stoscheck, 1990). Bradford reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 85 % phosphoric acid, diluted with H_2O to 1 L and filtered through Whatman No. 1 paper

just before used. One ml of protein sample was added 250 µl of 1 M NaOH and vertex. Then, added 5 ml of dye reagent to protein sample containing 100-1500 mg of protein per ml. Mix and let stand at room temperature for 5 min and measured protein by spectrophotometer at 590 nm which compared with blank consisting of 1 ml of sample buffer.

Enzymes catalyze a specific reactions and amount of product formed per unit time. This is called the activity, was calculated in equation:

$$\text{Activity} = \text{Amount of product/Time}$$

Enzyme activity was defined as in micromoles of product formation or (released) per minute. This would be one unit of activity in equation:

$$1 \text{ Unit of activity (U)} = 1 \text{ µmol/min}$$

Specific activity is the activity in units divided by the number of milligrams of protein in the sample for observed concentration.

$$\text{Specific activity} = \text{U/mg protein} = \frac{\text{U/ml}}{\text{mg/ml}}$$

Other quantity is the fold purification. It is always a starting point in purification, and as purify, for compared the fractions to the starting point. The starting points in usually called the crude (Farrell and Ranallo, 2000).

$$\text{Fold purification} = \frac{\text{Specific activity of fraction}}{\text{Specific activity of crude}}$$

$$\text{Concentration factor (flod)} = \frac{\text{activity of fraction}}{\text{activity of crude}}$$

$$\text{Yield (\%)} = \frac{\text{Total activity of a given fraction}}{\text{Total activity of the starting material}} \times 100$$

3.2.3 Electrophoresis

The present of sodium dodecyl sulfate polyacrylamide was gel electrophoresis carried out according to the method of Leonard *et al*. (1994). After electrophoresis, the protein was stained with Coomassie Brillant Blue R-250. The molecular weight was determined by compare with protein marker: Myosin (215 kDa), Phosphorylase B (120 kDa), Bovine serum albumin (84 kDa), Ovalbumin (60 kDa), Carbonic Anhydrase (39.2 kDa), Trypsin Inhibitor (28 kDa) and Lysozyme (18.3 kDa) (BlueRanger@Prestained, PIERCE). The molecular weight was evaluated using the relationship between the log molecular weight and relative mobility of the standard protein.

3.2.4 Characterization of the purified enzyme

A. pH optimum

The optimal pH of β-D-glucosidase activity was measured as in 3.2.2 but different buffers were used instead. The pH ranges of 4 to 5 of 50 mM sodium acetate, 6 to 7 of 50 mM potassium phosphate buffer, 8 to 9 of 50 mM Tris-HCl buffer and 9 to 10 of 50 mM glycine-NaOH buffer (Thongma, 1997).

B. Optimum temperature

Optimun temperature of β-D-glucosidase activity was assayed as described in 3.2.1 but at the determined temperature 20, 30, 40, 45, 50, 55, 60, 65, 70, and 80ºC (Thongma, 1997).

C. Temperature stability

For the determination of heat stability of the purified enzyme, the enzyme solution was incubated at 20, 30, 40, 50 and 60ºC. After incubation at each temperature for the certain period 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hrs, the enzyme solution was rapidly cooled and centrifuged at 5,000 rpm for 5 min at 4ºC to removed the denatured protein. The enzyme assay was performed as described in 3.2.1 (Thongma, 1997).

D. Ethanol tolerant

For the determination of ethanol tolerance of the purified enzyme, the enzyme solution was added ethanol concentration (0, 5, 10, 15% v/v) and was assayed residual activity as described in 3.2.1.

E. Kinetics of the β-D-glucosidase reaction

The kinetics of β-D-glucosidase enzyme was determined K_m and V_{max} by using Lineweaver-Burk plot of enzyme activity against varied the concentration of the single substrate (*p*NPG).

3.2.5 Wine making process

A. Must preparation

Muscat Hamberg (*V. vinifera*) berries were harvested from vineyard. Grape bunches were destemed and crushed by crushing machine and kept frozen until used. Must was treated with 100 ppm of sulphur dioxide. The grape pomace was separated before clarification by centrifugation at 10,000 rpm (SORVALL®GSA ROTOR RC 5C Plus Super Speed Centrifuge) for 5 min at 4ºC. The supernatant was adjusted to pH 5.0 with citric acid and sugar concentration to 22 ºBrix with sucrose.

B. Starter preparation

Sterilized juice was inoculated with *S. cerevisiae (bayanus*) EC-1118 and *S. cerevisiae* 71B-1122, at 30ºC for overnight in aerobic condition. The yeast population was determined by counting of viable cell on hemocytometer and calculated the cell by used equation (Fugelsang, 1997):

$$\text{Cells/ml} = \text{cell counted} \times 10^4 \times \text{dilution}$$

C. Alcoholic fermentation

Must was fermented in 150 ml Erlenmeyer flask with volume of 100 ml and treated with 100 U/mg of β-D-glucosidase from purification then, incubated at 20ºC for overnight. Yeast (2×10^6 cells/ml) was inoculated into flask. The alcoholic fermentation was done in duplicates at 20ºC. The samples were taken of the end of alcoholic fermentation and centrifuged at 5,000 rpm

(SORVALL®GSA ROTOR RC 5C Plus Super Speed Centrifuge) for 5 min. and supernatant was kept at -20ºC until used.

D. Ethanol determination

Ethanol content was determined by using direct injection of 0.2 µl of centrifuged wine sample to Gas chromatography (GC). Analysis of volatile compounds was operated on flame-ionization detector and a PE-wax column (30 m x 0.32 mm. i.d., 0.5 µm film thickness, Autosample XL 3000, Perkin Elmer) and an inlet system using the splitless injection technique, injector and detector temperature were 220 and 250ºC, respectively. The analytical conditions were modified from Cabaroglu *et al.* (2003); Gueguen *et al.* (1997) and Mateo and Stefano (1997) by using thermal program instead of isothermal for oven temperature program. It was 35ºC for 5 min. The carrier gas was helium and adjusted to 14 psi. The volatile compounds were primarily identified by comparing the retention times of the gas chromatographic peaks with those of commercial standards.

E. Determination of volatile compound

The volatile compound standards shown in Table 3.2. The 30 µm Polydimethylsiloxane (PDMS) fiber was chosen for sample preparation. Solid phase microextraction (SPME) (Figure 3.1) is a very simple and efficient solventless sample preparation method, invented by Pawliszyn and Belardi (1989). There are two typical SPME applications, sampling gases headspace (HS) or sampling solutions.

This technique is composed of two steps (Figure 3.2). First is an extraction step, where analytes are absorbed onto the fiber and extracted from the

solution or the headspace. Then, a desorbtion step which analystes are thermally desorbed into a heated GC injection port.

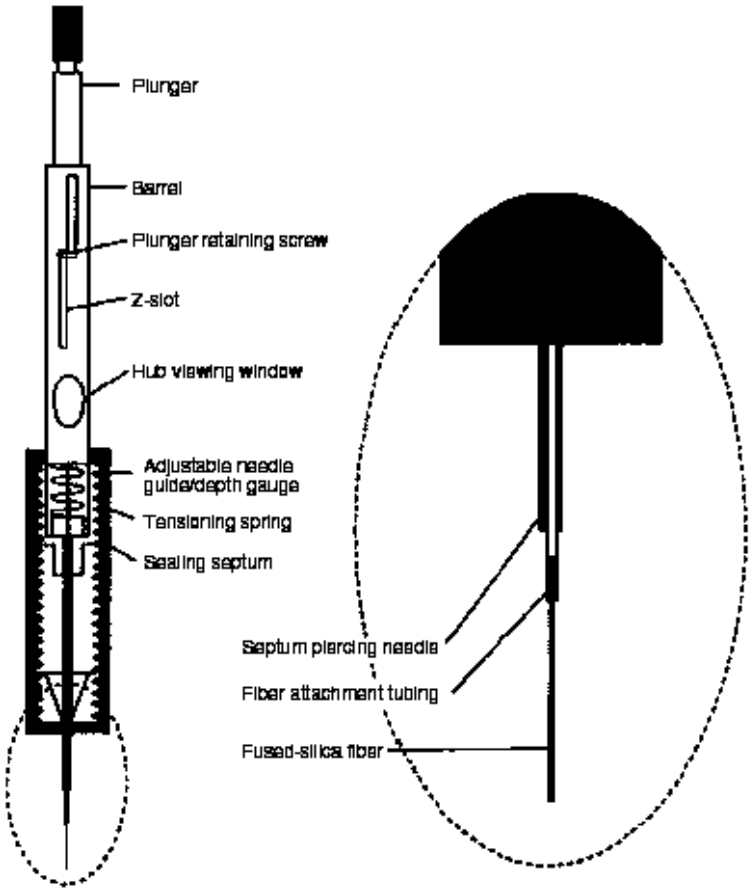


Figure 3.1 Schematic diagram of a commercial SPME device (Vas and Vékey, 2004)

Five ml of must and wine sample was taken into 25 ml vial and was added 12.5 % (w/v) of Na_2SO_4 for increasing of volatility of analytes (Zierler *et. al.*, 2004). Sample was heated at 70ºC for 20ºC min. (Sánchez-Palomo *et al.*, 2005).

An extraction program took place by piercing septum vial by a septum piercing needle. After that, the fiber was exposed to the headspace of sample (20 mm

above sample surface) for 20 min. This allowed the analytes from the solution to diffuse to the fiber. Then, the fiber was retreated inside the septum piercing needle and the fiber holder was removed from the sample. All analytes which were absorbed on the fiber was desorbed by piercing GC inlet septum for 5 min.

GC-MS condition

GC-MS analysis was performed on gas chromatography model CP 3800 coupled with to a mass detector model 1200L Quadrupole MS/MS (Varian). Compounds were separated on a VF-5ms column (30 m x 0.25 mm i.d.: 0.25 μm film thickness). Column temperature was 40ºC and held, then was ramped for 5 min., to 200ºC by rate 10ºC/min. and 250ºC by rate 5ºC/min. (running time 29 min.). Transfer line temperature was 250ºC. Mass detector conditions were electronic impact, EI0 mode at -70 eV. Source temperature was 220ºC, scanning rate 1 scan.s^{-1}. Mass acquisition range was 45-170. Carrier gas was helium at 0.5 ml.min^{-1} (Penton, 2005).

Identification of the volatile components of must and wine was based on comparison of their GC retention times and mass spectra with authentic standards from NIST Mass pectral seareh Program for the NIST/EPA/NIH Mass Spectral Library version 2.0.

Standard curves were prepared with deionized water and contain 40% (v/v) ethanol cause to standard dissolved in this concentration. All volatile compounds standard were of greater than 99% purity the standards were sampled by SPME in the same way with sample.

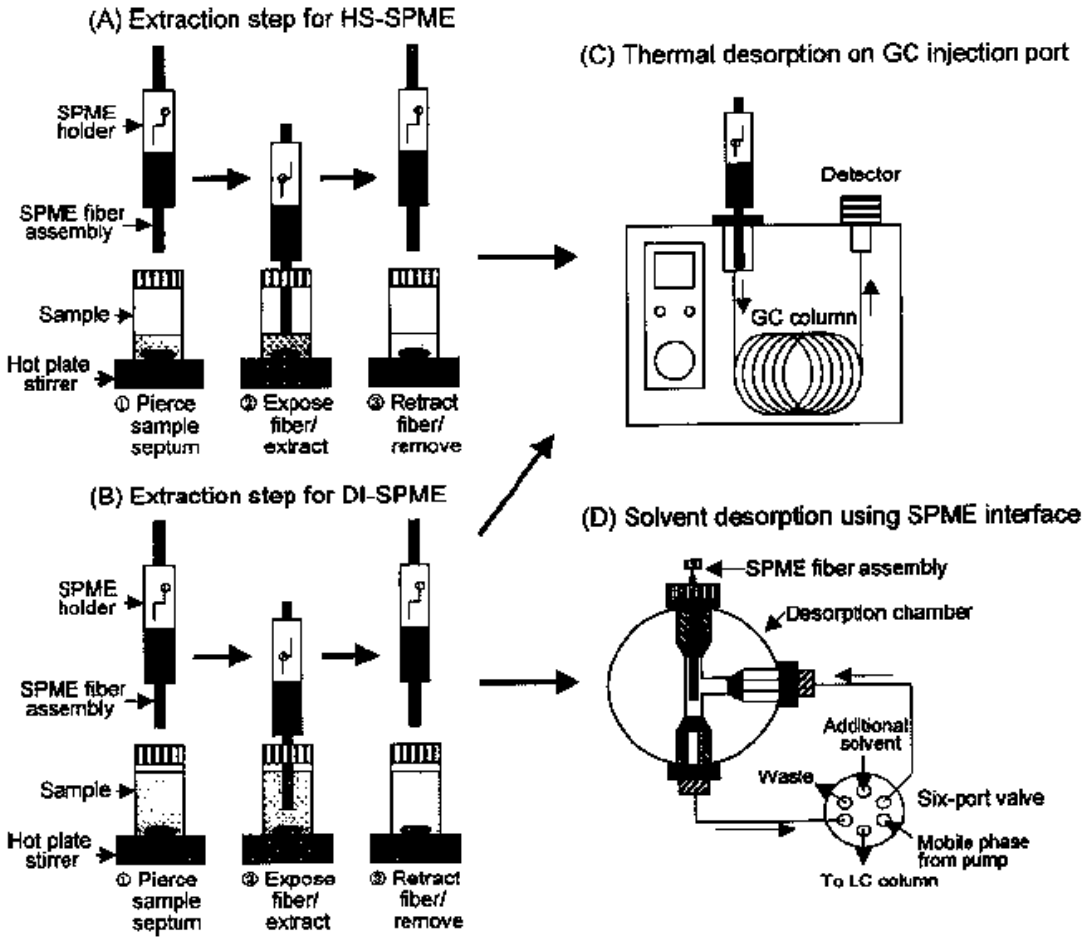


Figure 3.2 SPME procedures for Gas-chromatography and Liquid-chromatography (Vas and Vékey, 2004)

Table 3.2 Volatile compounds standard

Compounds	Carbon atom	Boiling point (°C)
propyl acetate	$C_5H_{10}O_2$	10
ethyl butyrate	$C_6H_{12}O_6$	26
isoamyl acetate	$C_7H_{14}O_2$	25
ethyl hexanoate	$C_8H_{16}O_2$	75
hexyl acetate	$C_8H_{16}O_2$	77
ethyl octanoate	$C_8H_{16}O_2$	54
Phenethyl alcohol	$C_8H_{10}O$	220
linalool	$C_{10}H_{18}O$	200
nerol	$C_{10}H_{18}O$	225
geraniol	$C_{10}H_{18}O$	229
2-phenylethyl acetate	$C_{10}H_{12}O_2$	221
ethyl decanoate	$C_{12}H_{24}O_2$	102

(Cabaroglu *et al.*, 2003; Gueguen *et al.*, 1997; Mateo and Stefano, 1997; Sánchez-Palomo *et al*., 2005).

3.2.6 Statistical analysis

Analysis of variance (ANOVA) was applied to the volatile data by using the SAS (Statistical Analysis System) program version 6.12 for windows (Copyright 1989-1996 by SAS Institute Inc., Cary, North Carolina, USA). Dancan's New Multiple Range was used for detection of differences. Differences were considered to be significant when $p<0.05$.

CHAPTER IV

RESULT AND DISCUSSION

4.1 Quntification of β-D-glucosidase of *Saccharomyces* 17 strains

The β-D-glucosidase activity was evidenced by hydrolysis of the substrate (*p*NPG) and released of *p*-nitrophenol which could be measured by using spectrophotometer at 400 nm. The amount of β-D-glucosidase activity was measured at time 0, 24, 48 and 72 hrs. Seventeen yeast strains showed activities of β-D-glucosidase. The strain 71B-1122 was founded to give the highest activity of β-D-glucosidase at 72 hrs. when compared to other strains (Figure 4.1).

4.2 β-D-Glucosidase purification

The extracellular extract obtained from a 4 L culture broth of *Saccharomyces cerevisiae* 71B-1122 was processed through a series of chromatography columns. The result of a typical purification procedure was summarized in Table 4.1. The enzyme was eluted from the exchanger by increasing the ionic strength of the elution buffer. The purification factor from step 2 and step 3 showed an approximately 24.90 and 22.05 fold, respectively. The final specific activity was 43,996.10 U/mg protein. Details of the chromatographic profiles of the purification steps were shown in (Figure 4.2 and 4.3). The degree of purification of the final preparation of enzyme was examined by SDS-PAGE (Figure 4.4).

4.2.1 DEAE-Sepharose

The β-D-glucosidase was adsorbed on the anion exchange DEAE-Sepharose column that had been equilibrated with 50 mM sodium phosphate buffer pH 7.0. The column was washed with the same buffer, proteins were eluted gradually using 0.1-0.5 M KCl linear gradient (Figure 4.2). The eluted enzyme peaks overlapped with the eluted proteins. The protein peak which showed high activity. The active fraction was pooled and dialysis and was loaded onto the next column.

4.2.2 Hydroxyapatite column

The elution profiled of β-D-glucosidase on Hydroxyapatile column was showed in Figure 4.3. The separation patterns show a protein peak with had high activity. The active fractions were pooled and molecular weight determined on SDS-PAGE (Figure 4.4).

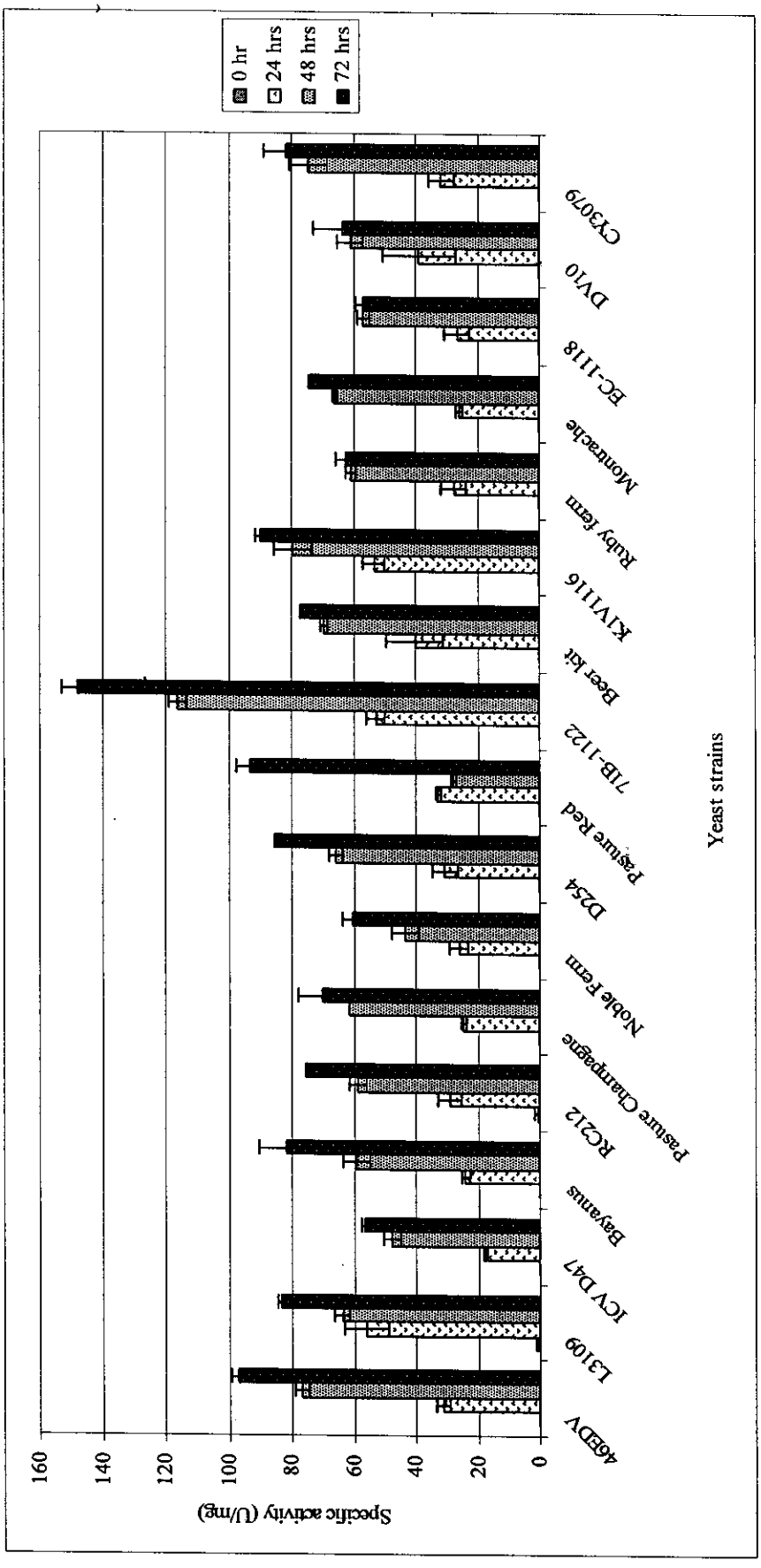


Figure 4.1 Quantification of β-D-glucosidase activity of the 17 yeast strains

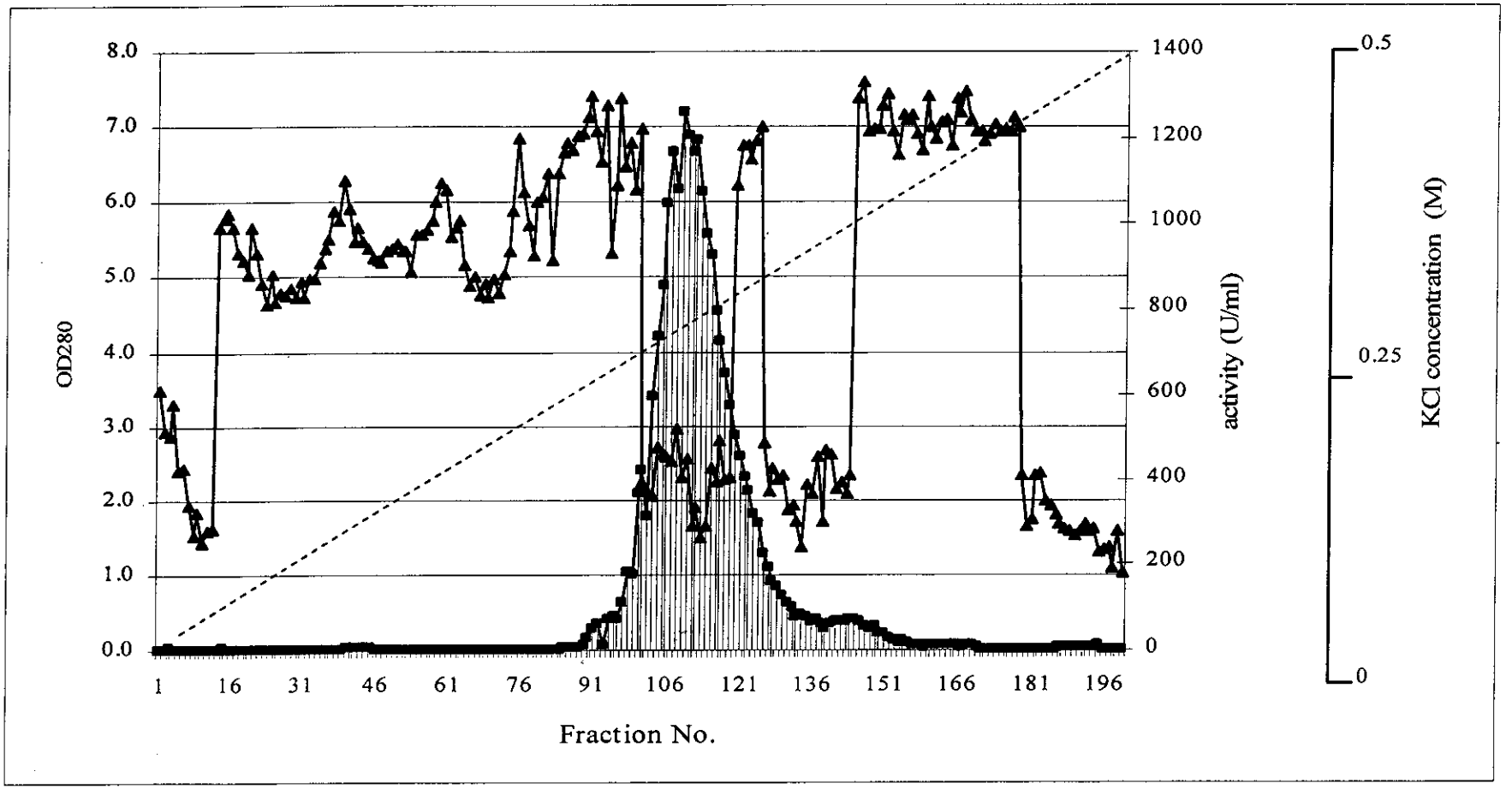


Figure 4.2 Chromatographic profile of extracellular β-D-glucosidase on DEAE-Sepharose column (■); OD 280 nm, (▲); activity (U/ml) and (---) KCl concentration (M)

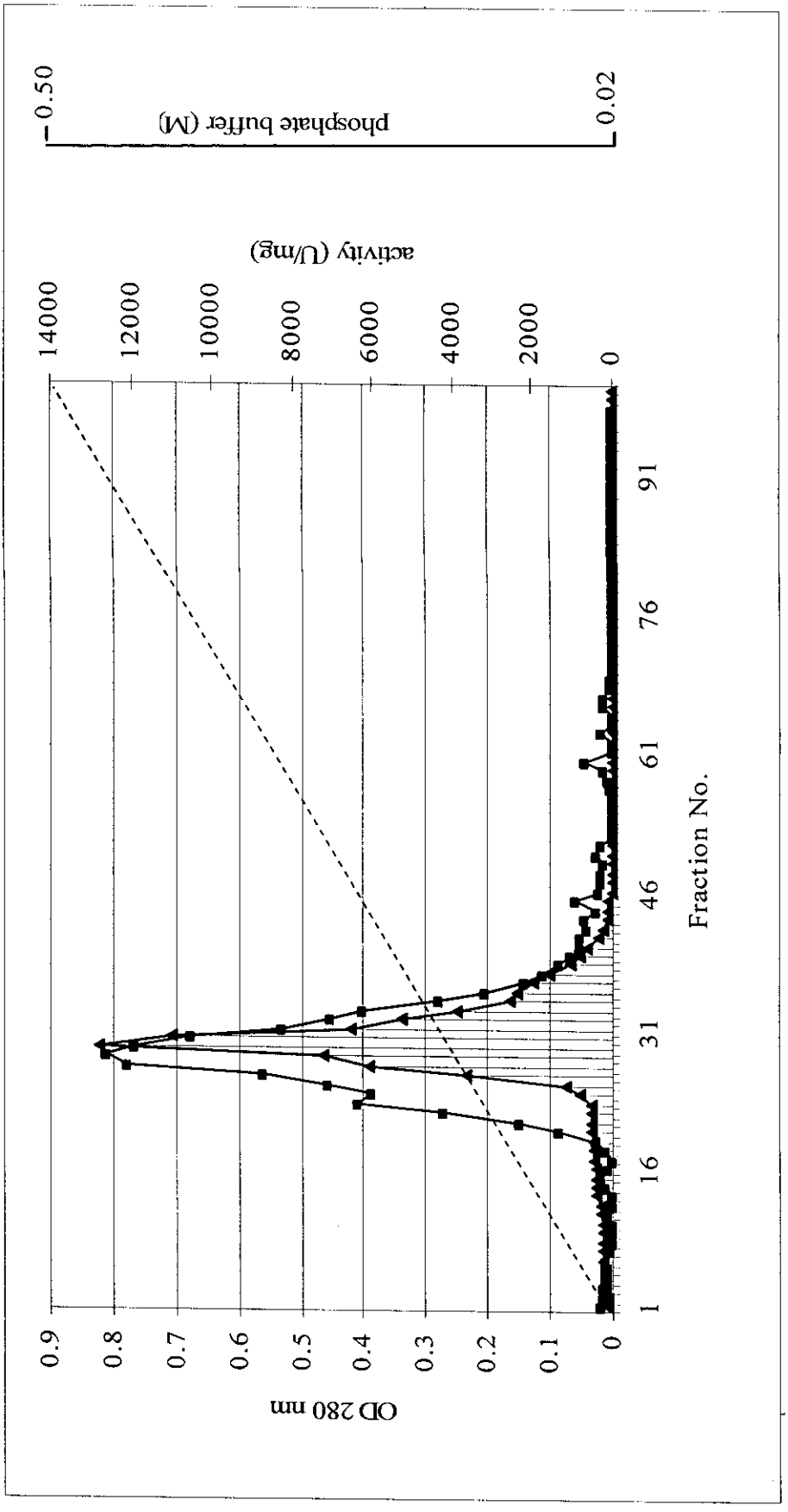


Figure 4.3 Chromatographic profile of extracellular β-D-glucosidase on Hydroxyapatite column (■); OD 280 nm, (▲); activity (U/ml) and (---) phosphate buffer (M)

Table 4.1 Purification of extracellular β-D-glucosidase from *Saccharomyces cerevisiae* 71B-1122

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Activity (U/ml)	Specific activity (U/mg)	Purification factor (fold)	Concentration factor (fold)	Yield (%)
Crude	4,000	370,280.00	0.046	92.57	1,995.06	1	1	100
Dialysis	245	147,279.30	0.041	601.14	14,626.35	7.33	6.49	39.78
DEAE Sepharose	15	87,942.90	0.118	5,862.86	49,685.23	24.90	63.33	23.75
Hydroxyapatite	25	48,285.75	0.044	1,931.43	43,996.10	22.05	20.86	13.04

One unit is μmol/min.

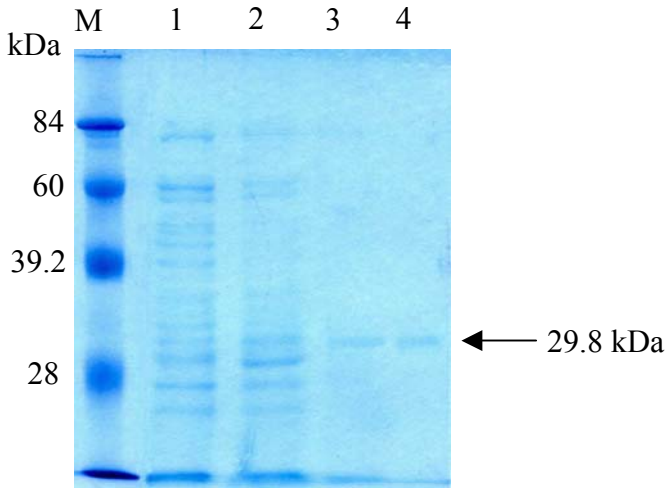


Figure 4.4 SDS-PAGE of variouse stages of proteins purification. Proteins were strained with coomassie brilliant blue. Lane 1, marker proteins; lane 2, crude enzyme, lane3, enzyme from DEAE Sepharose and lane 4, enzyme from Hydroxyapatile column. Arrow indicated the extracellular β-D-glucosidase from *Saccharomyces cerevisiae* 71B-1122 strain

4.3 Kinetics of the β-D-glucosidase reaction

The partially purified enzyme was determined with various concentrations of substrate in 50 mM sodium acetate buffer, pH 5.0. The Michaelis-Menten constant (K_m) and the maximum rate (V_{max}) of the enzyme derived from rate of catalysis measured at different substrate concentration. The results in Table 4.2 were tabulated and calculated to obtain in the K_m and V_{max} values for *p*NPG (Figure 4.5). A Line Weaver Burk plot of 1/V versus 1/[S] showed that K_m values for *p*NPG were 2.44 mM and V_{max} values were 55.56 U/ml mg protein, respectively. The K_m and V_{max} value for *p*NPG was showed differences when compared with β-D-glucosidases in Table 4.3.

Table 4.2 Kinetics of the purified β-D-glucosidase with pNPG as substrate

pNPG (mM)	**V (μmole/min)**	**1/[S] (mM^{-1})**	**1/V $(\mu mole/min)^{-1}$**
5	47.24	0.20	0.027
4	41.98	0.25	0.024
3	32.50	0.33	0.031
2	20.50	0.50	0.049
1	10.88	1	0.059

$$V = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

$$\frac{1}{V_{max}} = 0.018$$

$V_{max} = 55.56$ U/ml

$$\text{Slope} = \frac{K_m}{V_{max}} = 0.044$$

$K_m = 0.044 \cdot V_{max}$

$= 0.044 \times 55.56$

$= 2.44$ mM

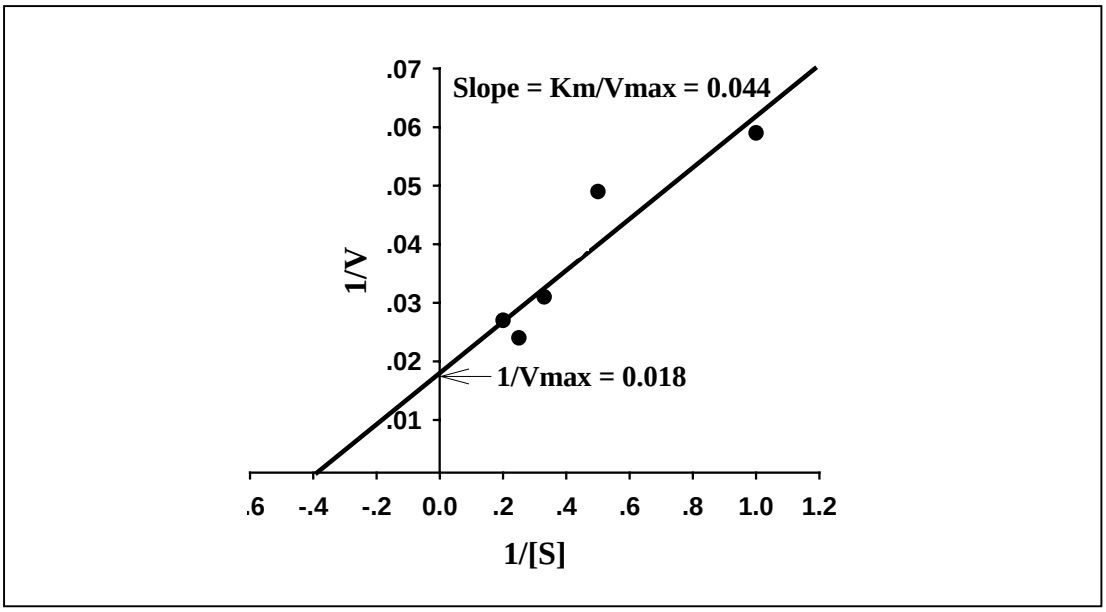


Figure 4.5 Kinetics of the purified β-D-glucosidase from *Saccharomyces cerevisiae* 71B-1122 strain with 50 mM pNPG as substrate

4.4 Characterization of β-D-glucosidase from purification

4.4.1 Molecular weight determination

From a plot of log molecular weight, the practically purified extracellular β-D-glucosidase and reference proteins were estimated by SDS-PAGE in Figure 4.4. Although after the SDS-PAGE, the electrophoretic mobilities of the extracellular β-D-glucosidase was eluted from DEAE and hydrxyapatite column were showed single subunit of enzyme in lane 3 and lane 4 of purification which the band has the MW of 29.8 kDa. The MW of this result was showed differences and smallest size than other strains (shown in Table 4.3).

4.4.2 Optimum pH

Enzyme activity was assayed at different pH ranging from pH 3.0 to pH 10.0. The result was showed the extracellular β-D-glucosidase has an optimal pH at the 5.0 (Figure 4.6). It is similar to the effect of pH on the extracellular β-D-glucosidase from *A. oryzae* (Riou *et al*., 1998). *A. sojae* (Kimura *et al*., 1999) *D. hansenii* (Riccio *et al*., 1999). *D. vanrijiae* (Belancic *et al*., 2003), *C. albicans* (Polacheck *et al*., 1987), *C. wickerhamii* (Freer, 1985), *C. petata* (Saha and Bothast, 1996), *Monilia* (Berry and Dekker, 1986), *Chalara paradoxa* CH 32 (Lucas *et al*., 2000) and endolular β-D-glucosidase of *C. entomophila* (Gueguen *et al*., 1994). Furthermore, the optimum activities of β-D-glucosidase was reported to be at a pH 6.0 from *T. lanuginosus* SSBP (Lin *et al*., 1999), *C. wickerhamii* (Skory *et al*., 1996) and *P. etchellsii* (Wallecha and Mishra, 2003). Optimal pH of β-D-glucosidase from *Pectobacterium carotovorium* (An *et al*., 2004) and *P. pastoris* (Turan and Zheng, 2004) were 7.0 and 7.3. Moreover, optimal pH β-D-glucosidase of *A. niger* (Spagna *et al*., 1998 and Barbagallo *et al*., 2004) and *C. sake* strain were 4.25-4.50 (Freer, 1985 and Gueguen *et al*., 2001). This result showed that the pH optimum for the enzyme activity was slightly acid. Moreover, Spagna *et al*. (2002a) found *S. cerevisiae* AL 41 extracellular β-D-glucosidase activity about 50-80% in the pH range 3.5-4.0 on must and wine condition (see in Table 4.3).

Table 4.3 Parameter of β-glucosidase from variety sources

Sources	MW (kDa)	K_m (mM)	V_{max} (U/mg protein)	Optimum pH	Optimal temperature (ºC)	References
intracellular β-glucosidase *C. sake*	240	6.6	ND	4.25	52	Gueguen *et al*. (2001)
intracellular β-glucosidase *P. pastoris*	275 and 70	0.12, 0.22, 0.096	6.19, 9.95, 1173	7.3	40	Turan and Zheng (2004)
intracellular β-glucosidase *Pectobacterium carotovorum*	53	ND	ND	7	40	An *et al*. (2004)
intracellular β-glucosidase *P. anomala* AL 112	ND	0.55	6.98	5.5	20	Spagna *et al*. (2002b).
intracellular β-glucosidase *C.wickerhamii*	94	0.28	525	6.0-6.25	35 (on wine model)	Skory *et al*. (1996)
extracellular β-glucosidase *A. oryzae*	130 and 43	0.55	1.066	5	50	Riou *et al*. (1998)
extracellular β *-glucosidase D. hansenii*	109 and 81	3.68	ND	5	ND	Riccio (1999)
extracellular β-*glucosidase C. albicans*	ND	ND	ND	5	ND	Polacheck (1987)
endocellular β-glucosidase *C. entomophila*	400	ND	ND	5	60	Gueguen *et al*. (1994)
β-glucosidase *T. lanuginosus* SSBP	200	0.075	12.12	6	65	Lin *et al*. (1999)
extracellular β-*glucosidase S. cerevisiae* AL 41	ND	2.55	1.71	3.5-4.0 (must and wine)	20-25 (on wine model)	Spagna *et al*. (2002a)
cell wall bound β-glucosidase *C. wickerhamii*	198	4.17	ND	4.0-5.0	ND	Freer (1985)
extracellular β-glucosidase *D.vanrijiae*	100	1.07	47.6	5	40	Belancic *et al*. (2003)
extracellular β-glucosidase *A. sojae*	118	0.14	16.7	5	60	Kimura *et al*. (1999)
extracellular β-glucosidase *C.peltata*	43	2.3, 66, 39, 35, 21, 18	ND	5	50	Saha and Bothast (1996)
extracellular β-glucosidase *Monilia*	46.6	ND	ND	4.0-5.0	50	Berry ane Dekker (1986)
β-glucosidase *A. niger*	ND	0.61-2.53	ND	4.5	20 (on wine model)	Barbagallo *et al*. (2004)
extracellular β-glucosidases *Chalara paradoxa* CH32	167	ND	ND	4.0-5.0	45	Lucas *et al*. (2000)
cell wall bound inducible β-glucosidases *P. etchellsii*	186 and 340	0.33	55, 25	6.0	50	Wallecha and Mishra (2003)
extracellular β-glucosidase *S. cerevisiae* 71B1122	**29.8**	**2.44**	**55.56**	**5.0**	**50**	**this study**

ND; not determined

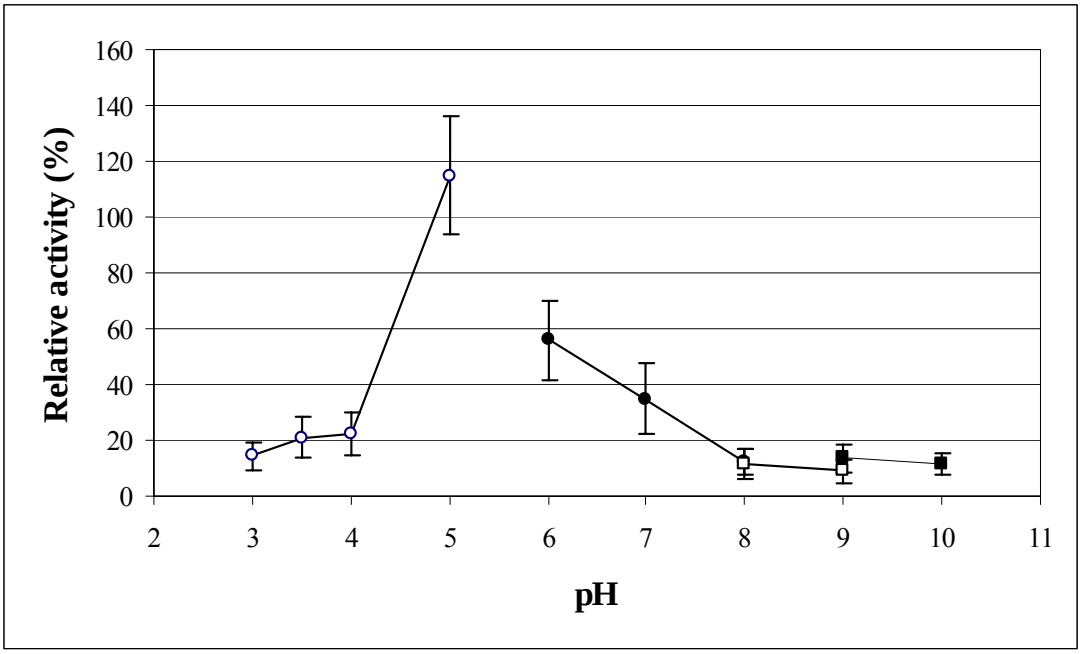


Figure 4.6 The optimum pH of the extracellular β-D-glucosidase assay

pH 3-5 = 50 mM sodium acetate; (○). Error bar showed pH 3.0, ±4.74; pH 3.5, ±7.28; pH 4.0, ±7.78 and pH 5.0, ±21.21

pH 6-8 = 50 mM potassium phosphate; (●).Error bar showed pH 6.0, ±14.14; pH 7.0, ±12.52 and pH 8.0, ± 4.55

pH 8-9 = 50 mM Tris-HCl; (□). Error bar showed pH 8.0, ±5.29 and pH 9.0,±4.17

pH 9-10 = 50 mM glycine-NaOH; (■). Error bar showed pH 9.0, ±5.05 and pH 10.0, ±3.74

4.4.3 Optimum temperature

The effect of temperature on the rate of the reaction was studied over the range of 20-80ºC under standard assay condition, and the result was showed in Figure 4.7. The optimal temperature which gave the highest activity was at 50ºC. At

temperature higher than 55ºC and lower than 40ºC, the enzyme activity dropped off rapidly. This result was in agreement with to the result of Berry and Dekker (1986), Saha and Bothast (1996) and Riou (1998). However, many optimal temperature of enzyme have been report, at 35°C from *C. wickerhamii* (Skory *et al*., 1996), at 40ºC from *D. vanrijiae* (Belancic *et al*., 2003), *P. pastoris* (Turan and Zheng, 2004), and *Pectobacterium carotovorum* (An *et al*., 2004), at 45ºC from *Chalara paradoxa* CH32 (Lucas *et al*., 2000), at 60ºC of endocellular β-D-glucosidase from *C. entomophila* (Gueguen *et al*., 1994), *A. niger* (Spagna, 1998) and *A. sojae* (Kimura *et al*., 1999). Spagna *et al*. (2002a) and Barbagallo *et al*. (2004) reported that optimal temperature of β-D-glucosidase from *A. niger* and *S. cerevisiae* AL 41 strain were 20-25ºC on wine model. Moreover, enzyme activity from mold was stable in wine model solution at 20ºC for 12 days and at least 35-45 days for that from yeast. Similarly the enzyme from *P. anomala* had an optimum temperature at 20ºC (Spagna *et al*., 2002b). The results suggested that β-D-glucosidase enzyme could work in range of temperature from 40-60ºC and could be stable in wine model solution at 20ºC (see in Table 4.3).

4.4.4 Thermal stability

The stability of the extracellular β-D-glucosidase from the purification at evaluated temperature is noteworthy (Figure 4.8). The thermal stability of this enzyme was rather high, when the enzyme was pre-incubated at 20, 30 and 40ºC. Pre-incubation at 50 and 60ºC caused a rapid decreases of activity to 40% and 0% after 0.5 hr, respectively.

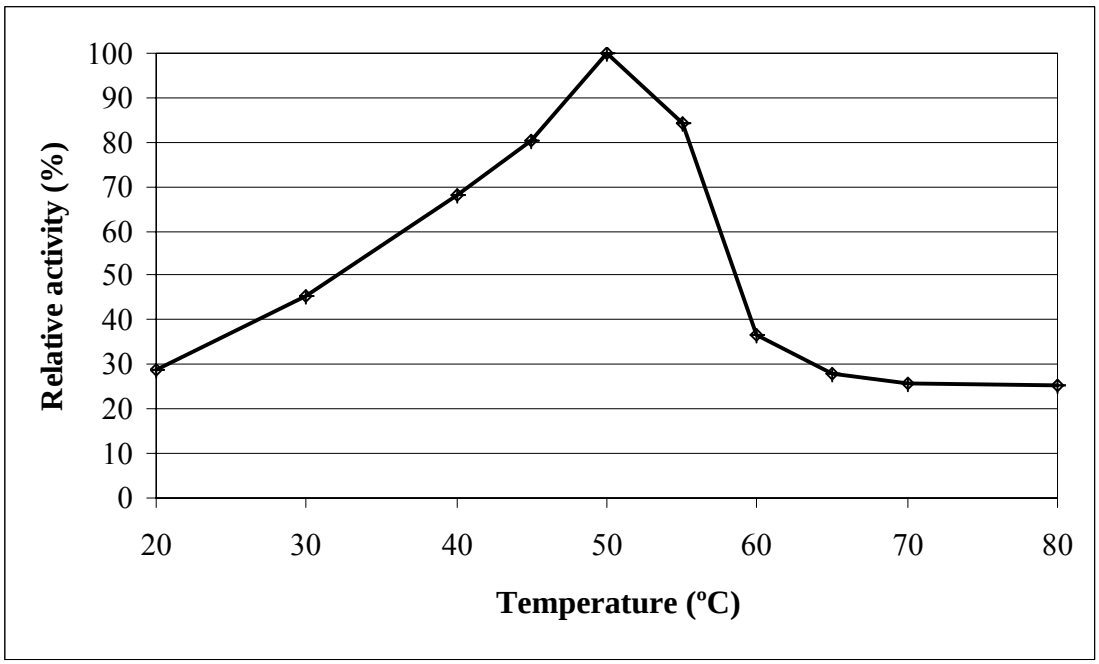


Figure 4.7 The optimal temperature of the extracellular β-D-glucosidase assay, error bar data were shown in appendix B.

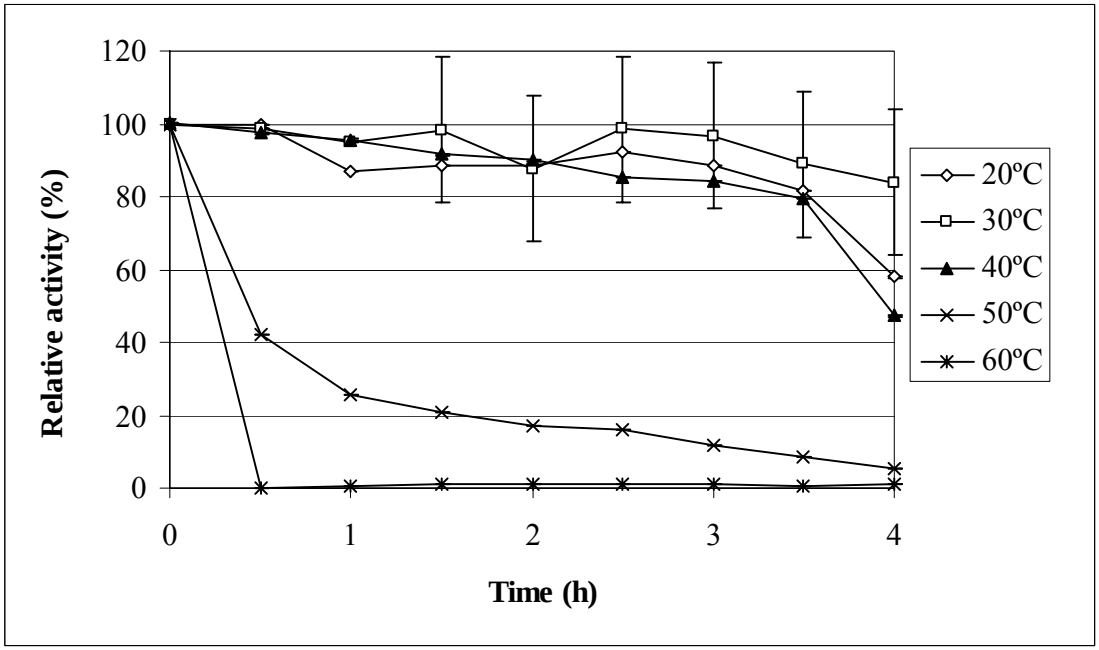


Figure 4.8 Thermal stability of the extracellular β-D-glucosidase assay. The enzyme was pre-incubated at 20ºC, 30ºC, 40ºC, 50ºC and 60ºC at various times and then assayed for extracellular β-D-glucosidase (Error bar data were shown in appendix B).

4.4.5 Ethanol inhibition

The effect of ethanol on extracellular β-D-glucosidase was studied and the result was depicted in Figure 4.9. The enzyme was inhibited to 60% inhibition at 5% (v/v) ethanol and rising to 80% inhibition at 10-15% (v/v) ethanol, similar to Hemingway *et al*. (1999) and Mateo and Stefano (1997) results. Moreover, the endocellular β-D-glucosidase from *C. entomophila* was inhibited by glucose and by D-gluconic acid lactone. The enzyme was constitutive and a glucosyltransferase activity was observed in the presence of ethanol (Gueguen *et al*. 1997).

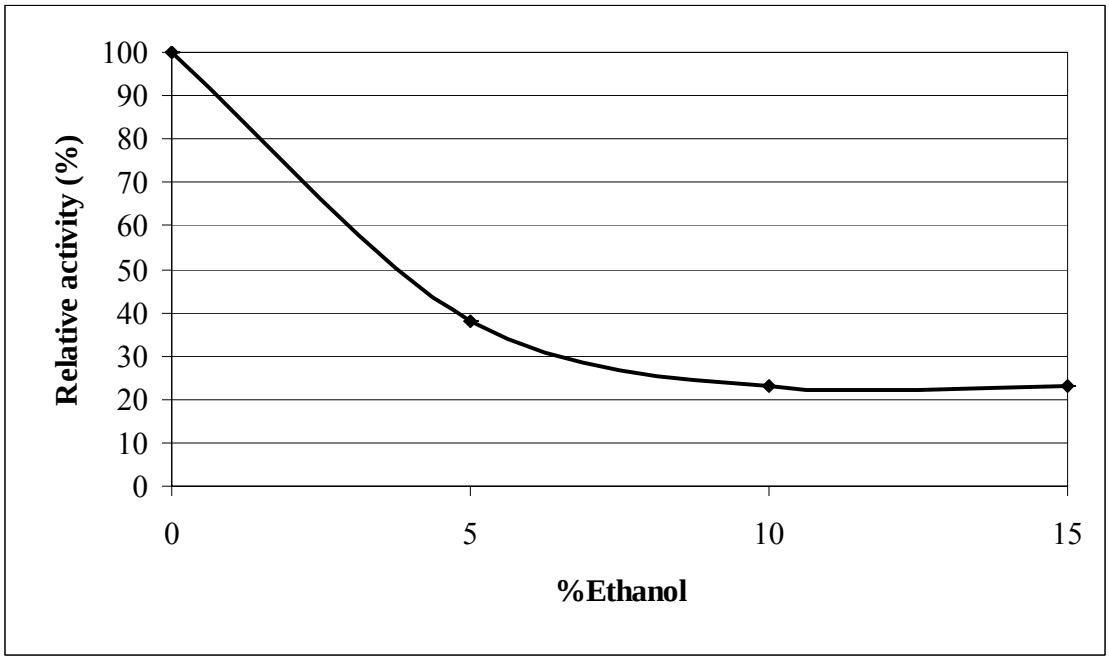


Figure 4.9 Effect of ethanol concentration on extracellular β-D-glucosidase from purification (Error bar data were showed in appendix B).

4.5 Wine making process

4.5.1 Must selection

Muscat Hambery, Viognier and Early Muscat (*Vitis vinifera*) must

were determined for volatile compounds using SPME/GC-MS. The measurement was described in methodology. More volatile compounds were detected in Muscat Hambery must when compared with other grapes (see in Appendix B). Thus, in wine making process Muscat Hambery must was selected for 100 U/mg protein of β-D-glucosidase treatment on must and incubated at 20ºC for overnight. This temperature was used for making wine and could protected volatile compounds more than optimal temperature of enzyme at 50°C for 1 hr. which the resulted of some volatile compounds lost during incubation at 50°C for 1 hr. (see in Appendix B). Moreover, many reports had suggested that high temperature vinification had lower concentrations of esters alcohols and total volatile compounds. Girard *et al*. (2000) described the effect of vinification method on ester alcohol and volatile compounds, the results showed cold vinificaion at 15ºC had the highest ester alcohols and total volatile compounds followed by ambient temperature at 20ºC. The range of fermentation temperatures (12ºC, 15.5ºC, 21ºC, and 27ºC) were examined for Pinot noir wines, furthermore, color were judged the best in both 21ºC and 27ºC treatments (Girard *et al*.,1997). Therefore, the temperature for incubation at 20ºC for overnight was selected for treated enzyme on must and wine.

4.5.2 Volatile compound determination

The effect of β-D-glucosidase on wine making process which was treated with the enzyme at 20ºC overnight before yeast inoculation. Must in wine making process was added with 50 mM sodium phosphate buffer (pH 7.0) control treatment. Moreover, *S. cerevisiae* EC-1118 and 71B-1122 strains were selected for wine making process because EC-1118 and 71B-1122 strains showed lowest and

highest activity of β-D-glucosidase, respectively (Figure 4.1).

β-D-glucosidase on must

In this study must was treated with β-D-glucosidase at 20ºC overnight. The volatile compounds were measured by headspace (HS)-SPME/GC-MS. The result in Table 4.4 showed increased in linalool, phenethyl alcohol, nerol, geraniol and 2-phenylethyl acetate when compare with control. This result showed significant increasing of nerol and geraniol compounds from 1.185 to 14.943 mg/l and 22.526 to 23.295 mg/l, respectively. Nerol compound was increased more than geraniol compound. Gunata *et al*. (1996) suggested that monoterpenyl (gerahyl, neryl, citronellyl, linalyl and α-terpinyl), benzyl and 2-phenylethyl-β-glucoside were released by β-D-glucosidase encode by *bglA* and *bglB* genes from *Bacillus polymyxa* to *Escherichia coli*. They found that (S)-citronellyl-β-D-glucoside was cleaved at a lower rate than geranyl and neryl-β-D- glucoside. And, nerol and geraniol are only differentiated by isomer position, with nerol being the *cis* isomer. However, there were reported that benzyl and 2-phenylethyl-β-D-glucoside were the substrates for *B. polymyxa* β-D-glucosidases. Benzyl-β-D-glucoside was hydrolyzed at a faster rate than 2-phenyl ethyl-β-D-glucoside. Therefore, linalool, nerol and geraniol compounds on must were released by β-D-glucosidase. Moreover, the quantity of linalool, nerol and geraniol compounds were more represented in must than that of other compounds.

Table 4.4 Qualities of volatile compounds of must and wine

Sample (mg/l)	Compounds										
	Isoamyl acetate	**Ethyl hexanoate**	**Hexyl acetate**	**Linalool**	**Phenethyl alcohol**	**Ethyl octanoate**	**Nerol**	**Geraniol**	**2-Phenylethyl acetate**	**Ethyl decanoate**	**Ethyl butyrate**
Control	0.320a**	0.003a	0.021a**	9.015a	0.030a	0.032a	1.185b	22.526b	0.011a	0.008a	0.000
Treated	0.268b	0.004a	0.000b	10.955a	0.041a	0.028a	14.943a**	23.295a*	0.027a	0.055a	0.000
WECcontrol	5.925a	5.589a	0.046b	7.653a	7.448a	5.400a	7.647b	2.684a	3.043a	6.075a	0.000
WECtreat	5.660a	4.557a	2.102a**	7.108a	6.813a	4.826a	12.280a**	3.917a	2.553a	5.510a	0.000
W71control	6.863a	2.495a	0.000b	3.935b	3.279b	3.702a	3.986b	3.051a	1.306a	4.503a	0.000
W71treat	6.807a	2.144a	4.543a*	7.708a**	7.944a*	4.465a	9.608a*	3.654a	2.363a	4.658a	0.000

The sign (*) is significantly difference and (**) is highly significantly difference. Mean in a column followed by the same letter are not significantly different ($p>0.05$).

GJ: grape juice, **Control**: must was treated with 50 mM sodium phosphate buffer pH 7.0, **Treat**: must was treated with 100 U/mg protein of β-glucosidase in 50 mM sodium phosphate buffer pH 7.0, **WECcontrol**: wine was treated with 50 mM sodium phosphate buffer pH 7.0 and fermented with EC-1188 yeast strain, **WECtreat**: wine was treated with 100 U/mg protein of β-glucosidase in 50 mM sodium phosphate buffer pH 7.0 and Fermented with EC-1188 yeast strain, **W71Bcontrol**: wine was treated with 50 mM sodium phosphate buffer pH 7.0 and fermented with 71B-1122 yeast strain, **W71Btreat**: wine was treated with 100 U/mg protein of β-glucosidase in 50 mM sodium phosphate buffer pH 7.0 and Fermented with 71B-1122 yeast strain.

β-D-Glucosidase on wine making

Must was inoculated with populations of yeast at $2x10^6$ cells/ml and the temperature of fermentation at 20ºC which stable for β-D-glucosidase during wine making (Spagna *et al*., 2002a and Barbagallo *et al*., 2004). Moreover, low temperatures wine fermentation resulted in improvement of wine quality and the produced wine had a distinctive aromatic profile (Kourkoutas *et al*., 2005).

The amount of volatile compounds of wine when treated with β-D-glucosidase and fermented with yeast EC-1118 strain. It was shown significant increasing of hexyl acetate and nerol compounds were 0.046 to 2.102 mg/l and 7.647 to 12.280 mg/l, respectively. Moreover, result of wine fermented from 71B-1122 strain was showed significant increasing of hexyl acetate, linalool, phenethyl alcohol and nerol compound were 0.000 to 4.543 mg/l, 3.935 to 7.708 mg/l, 3.279 to 7.944mg/l and 3.986 to 9.608 mg/l, respectively (see in Table 4.4).

The compounds of aroma characteristic are important in wine odor. They are mainly higher alcohol, ethyl ester, ethyl acetate. From this studied it was found that volatile compounds in aroma characteristics of wine, there are isoamyl alcohol, ethyl hexanoate, hexyl acetate, phenethyl alcohol, ethyl octanoate, 2-phenylethyl acetate and ethyl decanoate were increased after fermentation when compared with must and quantity of aroma characteristics, there are linalool, nerol and geraniol. Moreover, compounds giving aroma characteristic were not increased after wine fermentation. They were stable with β-D-glucosidase in wine.

Major volatile compounds in wines and alcoholic beverages are mainly higher alcohols. They have more carbon atoms than ethanol. The wine

alcohol 1-propanol, isobutanol, isoamyl alchol and 2-phenylethanol are those typically present at the highest concentration from 10 to 500 mg/l. Ethyl ester of fatty acid such as ethyl acetate, ethyl lactate and diethyl succinate (Peinado *et al*., 2004). Ethyl acetate concentrations were relatively high up to 113 mg/l with lower temperature fermentation. Shibomoto (2002) wine fermented with the 71B-1122 strain contained highest concentrations of 2-pheyl ethanol and 2-phenyl ethyl acetate in the spontaneously fermented wines. It showed higher concentrations of butyric and caproic acid and ethyl butyrate when compare to Herjavec *et al*. (2002). Moreover, other flavor precursors benzyl and 2-phenylethyl-β-D-glucoside, can contribute to the bitterness of citrus juice are also substrates of the enzyme (Riou *et al*., 1998; Gueguen *et al*., 1997; Fernádez *et al*., 2000; Baek and Cadwallader, 1999). In addition, Chassagne *et al*. (2005) suggested different *S. cerevisiae* strain to tested for ability to hydrolyse glucosyl-glucose (G-G) compounds present in Chardonnay must during alcoholic fermentation. The extents of hydrolysis vary form 17-57% of the initial glycoside concentration in order to confirm the decrease in G-G concentration during alcoholic fermentation is due to hydrolytic action. The effects of temperature, ethanol concentration and pH on the rate of hydrolysis of common volatile esters of wine (ethyl butanoate, ethyl hexanoate, ethyl octanote, ethyl decanoate, isobutyl acetate, hexyl acetate and 2-phenylethyl acetate) were investigated in model solutions. Furthermore, ethanol concentration differences in amounts of 10-14% v/v had esters. Several wines were also analyzed of changes in ester concentration with time at several different temperatures (Ramey and Ough, 1980). During the primary alcoholic fermentation of sugar, the wine yeast *Saccharomyces*, together with other indigenous non- *Saccharomyces* species produced ethanol, carbon dioxide and a

number of by-products of these yeast derived metabolites, the alcohols acetate and C_4-C_8 fatty acid ethyl ester are formed the highest concentration of wine. The volatile substance, by-products, contribute to the fermentation bouquet characteristic in young wines. The production levels of these metabolites are variable and yeast strain specific (Lambrechts and Pretorius, 2000). Therefore, yeasts influenced the volatile compound during alcoholic fermentation (Rankine, 1967). Also the persistence of these non or wild yeast during fermentation depends on many factors, such as the temperature of fermentation, nutrient availability, use an levels of SO_2 and the quantity and identity of organisms present on the grapes (Rapp and Versini, 1991). Herraiz *et al*. (1989), reported that higher alcohols increased with amino acid increase in the must. Furthermore, Lopez *et al*. (1996) found the amino acid of grape must were assimilated at a lower rate with temperature decrease these findings may offer an explanation for increased amounts of alcohols produced by immobilize and free cell at higher temperature. The total amount of acid decreased with temperature decreased (Mallouchos *et al*., 2003). Moreover, amyl alcohol was reduced by the decreased of the temperature below 20ºC. The percentages of amyl alcohol as functions of total volatiles were reduced and those of ethyl acetate were increased by the decreased of temperature indicating improvements in quality and nutritional value (Iconomopeulou *et al*., 2002). Furthermore, the carbonic maceration could improve the formation of benzyl alcohol and skin fermentation improves that of phenylethanol (Gomez *et al*., 1994). Important aroma compounds in wine one the ethyl ester as well as acetates. Ethyl ester and among them; 3-methylbutyl acetate and hexyl acetate are considered as the most powerful contributors to wine aroma. Other significant positive contributors detected in all wines are 2-phenylethyl acetate

and ethyl 2-hydroxy-propanoate (Etievant, 1991). Yeast formed ethyl ester from amino acid during the fermentation. Esters were mainly formed in the second half of the fermentation when the concentration of ethanol in the medium is high reference. The level of these compounds appeared to increased during the lysis. A possible mechanism involving the activation at a inside the cell by fermentation amino acyl acetate during the protein biosynthesis and further reaction with ethanol intracellular to synthesis the esters is suggested to explain the formation of ethyl esters of amino acid by yeast (Herraiz and Ough, 1993). Ethyl esters are formed enzymematically call during fermentation and contribute to the fruity and floral sensory properties of the wine (Nordstrom, 1964). Moreover, the production of alcohol acetates during fermentation is dependent on the activity of at least three acetyl transferase namely, alcohol acetyltransferase (AAT), ethanol acetyltransferase, and iso-amyl AAT. (Lilly *et al*., 2000). Lambrechts and Pretorious (2000) investigate the feasibility of improving the aroma of wine and distillates by overexpressing one of the endogenous yeast genes that controls acetate ester production during fermentation. However, the wine yeast produce smaller amounts of ethyl ester of fatty acid from acetic acid to octanolic acid under aerobic fermentation conditions than under strictly anerobic condition (Nykänen, 1985). During alcoholic fermentation, acetate ester was investigated in relation to alcohol acetyltransferase activity. Alcohol acetlytransferase activity influenced by the fatty acid composition of the yeast cell membreance (Yoshioka and Hashimoto, 1983a) and depending on the degree at affinity with various kinds of alcohols (Yoshioka and Hashimoto, 1981). Moreover, glucose gave more acetate ester with a higher activity of alcohol acetyl transferase (Yoshioka and Hashimoto, 1983b). Furthermore, yeast strains had influence to aroma

and flavor of wine which Majdak *et al*. (2002) determined the specific enological characteristics of *S. paradoxus* species potential difference in production of volatile compounds between *S. paradoxus* and *S. cerevisiae* strains and their influence on final wine quality. Wine chemical analyses showed statistically significant differences depending on the yeast strain used. Some compounds such as 2-phenylethanol, 2-phenethyl acetate, ethyl lactate, 3-ethoxypropanol and to a lesser extent, diethyl succinate and propionic and characterized examinaed *S. bayanus* yeasts. The other yeasts showed great differences, which are difficult to correlate with the strain (Antonelli *et al*., 1999 and Patel and Shibamoto, 2002). The fruity characters of young wines depend on the contents of terpenes present in the grape together with acetates mono- and dicarboxylic acid ethyl ester which appeared during fermentation process. The characteristic fruity odoures of fermentation bouquet are primary due to a mixture of hexyl acetate, ethyl caprilate and ethyl caprylate giving an apple-like aroma, isoamyl acetate giving a banana like aroma and 2-phenylethyl acetate giving a fruity and flowery flavoure with a honey note (Rapp and Mandery, 1986). In addition, isoamyl acetate, isobutyl acetate, ethyl butyrate, hexyl acetate were produced and retained at thee lower fermentation temperature (10ºC). The higher boiling more aromatic or "heady" esters (ethyl octanoate, 2-phenetyl acetate, ethyl decanoate) were produced and retained in the wine in greater amounts at the higher fermentation temperature (15-10ºC) (Kilian and Ough, 1979). Nevertheless, during aging this bouquet gradually decreased in intensity and finally disappeared. This change was accompanied by concurrent decrease in isoamyl acetate concentration. A similar reduction in bouquet intensity and isoamyl acetate concentration was observed when the win was expost to an excess of oxygen as well

as evaluated temperature (Wyk *et al*., 1979).

4.5.2 Ethanol concentration

Ethanol concentration (Table 4.5) was measured by Gas-chromatography. Ethanol concentration of wine from EC-1118 strain were 9.61 and 9.93% (v/v) for control and treated β-D-glucosidase, respectively. And ethanol concentration of wine from 71B-1122 strain were 12.86 and 12.70% (v/v) for control and treated β-D-glucosidase, respectively. β-D-glucosidase enzyme released glucose molecule an important substrate for ethanol production (Cho *et al*., 1999). The increasing of ethanol concentration during alcoholic fermentation could also explain the sequential growth of yeast. Mallouchous *et al*. (2003), reported that concentration of total alcohols increased when increase fermentation temperature. Moreover, many researches reported that β-D-glucosidase enzyme is one of cellulolytic hydrolysis enzyme released glucose from cellulose molecule and convert of cellulose to ethanol (Freer and Greene, 1990; Skory and freer, 1995; Cho *et al*., 1999; Fujita *et al*., 2002).

Table 4.5 Ethanol concentration of wine EC-1118 and 71-B1122

	WECcontrol	WECtreat	W71control	W71treat
% (v/v) ethanol	9.61a	9.93a	12.86a	12.70a

Mean in a column followed by the same letter are not significantly different ($p>0.05$).

WECcontrol: wine was treated with 50 mM sodium phosphate buffer pH 7.0 and fermented with EC-1188 yeast strain, **WECtreat**: wine was treated with 100 U/mg protein of β-D-glucosidase in 50 mM sodium phosphate buffer pH 7.0 and Fermented with EC-1188 yeast strain, **W71Bcontrol**: wine was treated with 50 mM sodium phosphate buffer pH 7.0 and fermented with 71B-1122 yeast strain, **W71Btreat**: wine was treated with 100 U/mg protein of β-D-glucosidase in 50 mM sodium phosphate buffer pH 7.0 and Fermented with 71B-1122 yeast strain.

CHAPTER V

CONCLUSION

Of seventeen *Saccharomyces cerevisiae* strains, the 71B-1122 strain show highest β-D-glucosidase activity. The β-D-glucosidase was purified from 71B-1122 strain. The K_m and V_{max} values for *p*NPG were 2.44 mM and 55.56 Uml^{-1} mg protein, respectively. The enzyme had molecular weight of 29.8 kDa on SDS-PAGE. It showed optimal pH at 5.0 and optimal temperature which gave the highest activity was at 50ºC. This enzyme is stable at low temperature of 20, 30 and 40 ºC for 1.30 hr. Ethanol could inhibit this enzyme at concentration 5% (v/v) ethanol.

In wine making process β-D-glucosidase have influence to the production of volatile compounds such as hexyl acetate, linalool, nerol and phenethyl alcohol. There compounds increased after β-D-glucosidase treat. The incubation temperature of the enzyme also affect the amount of the volatile compounds which decreased at high temperature. After fermentation, increased isoamyl acetate, hexyl acetate, phenethyl alcohol, ethyl octanoate, 2-phenylelthyl acetate and ethyl decanoate. Furthermore, yeast strains also influenced on volatile compounds production. The volatile compound increased when treated with β-D-glucosidase and fermented with 71B-1122 strain. Moreover, yeast strain also effect the ethanol production. Therefore, in producing wine yeast strain selection and addition of β-D-glucosidase enzyme should be considered because they were important factors affecting wine aroma.

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APPENDICES

APPENDIX A

METHOD

1. SDS-PAGE

Materials

1. 30% (w/v) arylamide: bisacrylamind (29: 1) solution:

acrylamide	145	g
bisacrylamide	5	g
H_2O	400	ml
Final volume	500	ml

2. 10% SDS

SDS	50	g
H_2O	500	ml

3. TEMED (N,N,N',N'-tetramethyl ethylendiamine)
4. 10% (w/v) ammonium persulfate

ammonium persulfate	0.5	g
H_2O	5	ml

5. 1.5 M Tris-Cl, pH 8.8

Tris base	90.75	g
1N HCl	100	ml
H_2O	300	ml
Adjust to pH 8.8		

	Final volume	500	ml
6.	0.5 M Tris-Cl, pH 6.8		
	Tris base	6	g
	1 N HCl	40	ml
	Adjust to pH 6.8		
	Final volume	100	ml
7.	0.025 M Tris-0.19 M glycine (pH 8.3) running buffer		
	Tris base	3	g
	Glycine	14.4	g
	H_2O	990	ml
	10% SDS	10	ml
	Final volume	1000	ml
8.	1% Bromophenol blue		
	Bromophenol blue	0.1	g
	H_2O	10	ml
9.	2x Protein sample buffer		
	0.5 M Tris-Cl, pH 6.8	2	ml
	10% SDS	4	ml
	1% Bromophenol blue	2	ml
	Glycerol	2	ml
	1 M DTT		
	Final volume	10	ml
10.	Acetic acid		

11. Methanol

12. 0.2% Coomassie blue staining solution

Coomassie brilliant blue-R250	1	g
Methanol	250	ml
Acetic acid	50	ml
H_2O	200	ml

13. Destaining solution

Methanol	300	ml
Glacial acetic acid	100	ml
H_2O	600	ml

Method

1. Thorough clean glass plates with detergen and H_2O, rinse with H_2O and ethanol and dry.
2. The slab gel (9x5x0.1 cm) with a 10% separating gel and 4% polyacrylamind stacking gel (9x2x0.1 cm) was used. The gel should polymerize fully in about 10 to 20 min.
3. The sample was prepared by resuspension in 2x sample buffer, include in 0.1 vol. of 1 M DTT. The samples are then boiled for 3 min in capped micro centrifuge tubes. The sample can be removed and left at room temperature until load on gel.
4. The comb is removed, and the wells are rinsed out with H_2O to remove excess unpolmerized acrylamide.

5. Running buffer is loaded to the bottom chamber and then the top chamber.
6. The samples (20 μl and up to 100 μl with the well comb) are loaded into the well.
7. Run gel at about 100 V until try entering the separating gel, and then increasing the power to 200 to 250 V.
8. When dry has reached the bottom of the gel turn of the power and remove gel from the gel box.
9. Add 200 ml of 0.2% Coomassie blue staining solution and incubate with agitation if possible for 3 hr. or overnight at room temperature.
10. Destain gel and remove which can be reused four or five time with little lose of effectiveness.
11. Transfers gel to a piece of plastic wrap and then lay a piece of Whatman paper on top of gel transfer to a gel drying apparatus according to manufacture's instructions and dry for several hours, or longer at 80°C.

Table 2A Final concentration of ammonium sulfate (% saturation at 0°C).

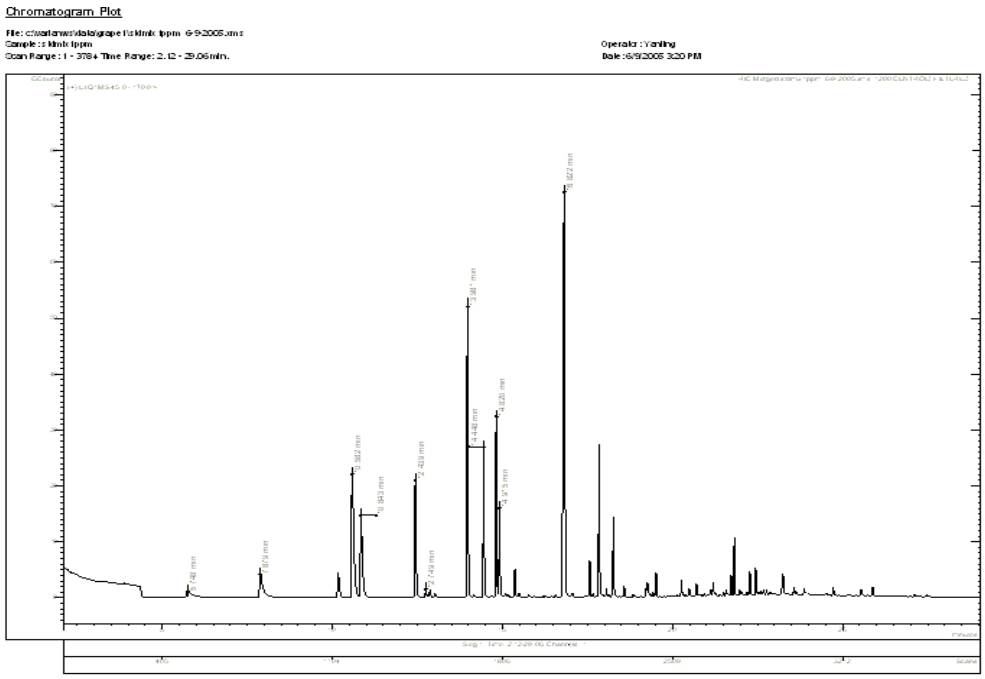
Initial concentration of ammonium sulfate (% saturation at 0°C)	*20*	*25*	*30*	*35*	*40*	*45*	*50*	*55*	*60*	*65*	*70*	*75*	*80*	*85*	*90*	*95*	*100*
	solid ammonium sulfate to add to 100 ml of solution																
0	10.6	13.4	16.4	19.4	22.6	25.8	29.1	32.6	36.1	39.8	43.6	47.6	51.6	55.9	60.3	65.0	69.7
5	7.9	10.8	13.7	16.6	19.7	22.9	26.2	29.6	33.1	36.8	40.5	44.4	48.4	52.6	57.0	61.5	66.2
10	5.3	8.1	10.9	13.9	16.9	20.0	23.3	26.6	30.1	33.7	37.4	41.2	45.2	49.3	53.6	58.1	62.7
15	2.6	5.4	8.2	11.1	14.1	17.2	20.4	23.7	27.1	30.6	34.3	38.1	42.0	46.0	50.3	54.7	59.2
20	0	2.7	5.5	8.3	11.3	14.3	17.5	20.7	24.1	27.6	31.2	34.9	38.7	42.7	46.9	51.2	55.7
25		0	2.7	5.6	8.4	11.5	14.6	17.9	21.1	24.5	28.0	31.7	35.5	39.5	43.6	47.8	52.2
30			0	2.8	5.6	8.6	11.7	14.8	18.1	21.4	24.9	28.5	32.3	36.2	40.2	44.5	48.8
35				0	2.8	5.7	8.7	11.8	15.1	18.4	21.8	25.4	29.1	32.9	36.9	41.0	45.3
40					0	2.9	5.8	8.9	12.0	15.3	18.7	22.2	25.8	29.6	33.5	37.6	41.8
45						0	2.9	5.9	9.0	12.3	15.6	19.0	22.6	26.3	30.2	34.2	38.3
50							0	3.0	6.0	9.2	12.5	15.9	19.4	23.0	26.8	30.8	34.8
55								0	3.0	6.1	9.3	12.7	16.1	19.7	23.5	27.3	31.3
60									0	3.1	6.2	9.5	12.9	16.4	20.1	23.9	27.9
65										0	3.1	6.3	9.7	13.2	16.8	20.5	24.4
70											0	3.2	6.5	9.9	13.4	17.1	20.9
75												0	3.2	6.6	10.1	13.7	17.4
80													0	3.3	6.7	10.3	13.9
85														0	3.4	6.8	10.5
90															0	3.4	7.0
95																0	3.5
100																	0

Note: The pH of the solution may decrease significantly on addition of ammonium sulfate.

APPENDIX B

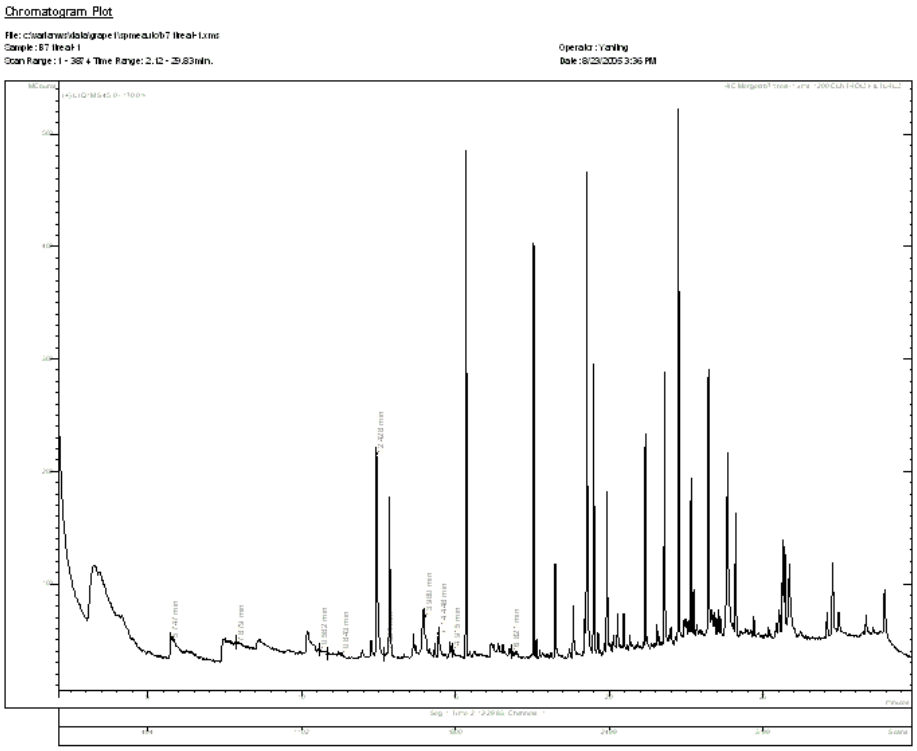
RESULTS

1. Chromatograms of volatile compounds analysis by GC/MS.



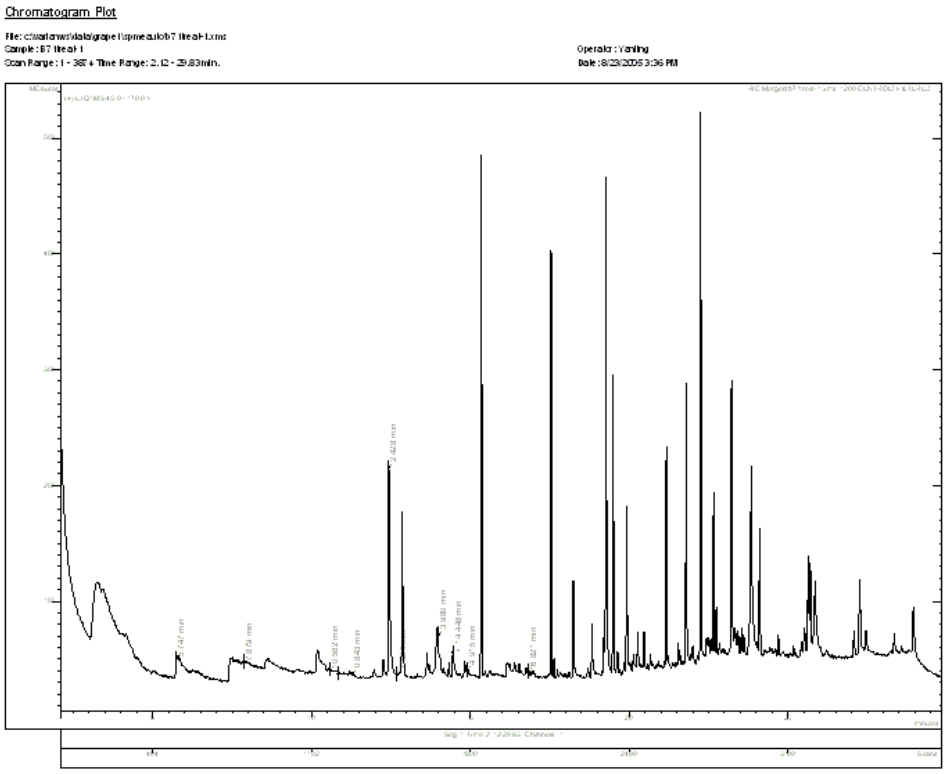
Compounds	Retention time (min)
Butanoic acid, ethyl ester (ethyl butyrate)	5.748
1-Butanol,3-methyl acetate (isoamyl acetate)	7.879
Hexanoic acid, ethyl ester (ethyl hexanoate)	10.617
Acetic acid, hexyl ester (hexyl acetate)	10.848
1, 6-Octadien-1-ol, 3,7-dimethyl (linalool)	12.431
Phenethyl alcohol	12.762
Octanoic acid, ethyl ester (ethyl octanoate)	13.976
2, 6-Octadien-1-ol, 3,7-dimethyl-, (Z) (nerol)	14.441
2, 6-Octadien-1-ol, 3,7-dimethyl-, (E) (geraniol)	14.483
Acetic acid, 2- phenylethyl ester (2-phenylethyl acetate)	14.922
Decanolic acid, ethyl ester (ethyl decanoate)	16.814

Figure 1B Chromatograms of standard volatile compounds



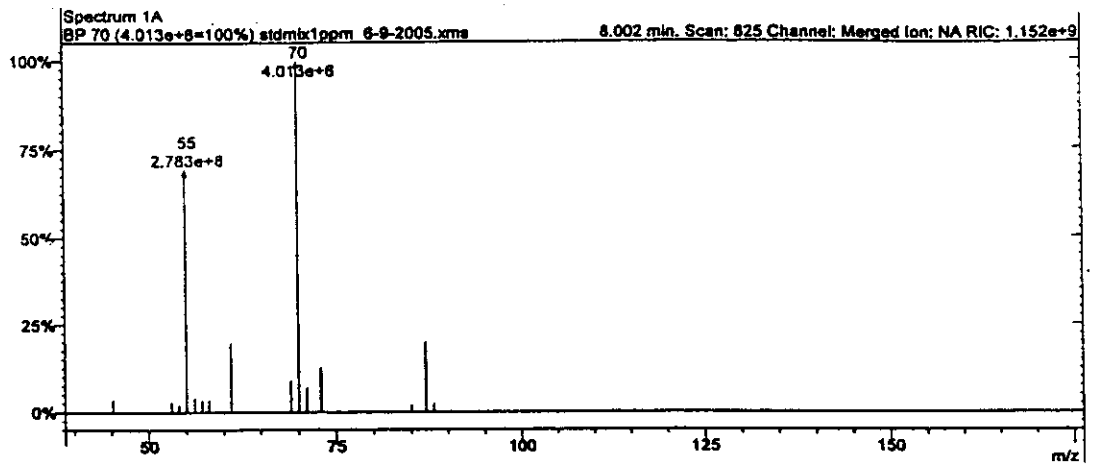
Compounds	Retention time (min)
Butanoic acid, ethyl ester (ethyl butyrate)	5.748
1-Butanol,3-methyl acetate (isoamyl acetate)	7.879
Hexanoic acid, ethyl ester (ethyl hexanoate)	10.617
Acetic acid, hexyl ester (hexyl acetate)	10.848
1, 6-Octadien-1-ol, 3,7-dimethyl (linalool)	12.431
Phenethyl alcohol	12.762
Octanoic acid, ethyl ester (ethyl octanoate)	13.976
2, 6-Octadien-1-ol, 3,7-dimethyl-, (Z) (nerol)	14.441
2, 6-Octadien-1-ol, 3,7-dimethyl-, (E) (geraniol)	14.483
Acetic acid, 2- phenylethyl ester (2-phenylethyl acetate)	14.922
Decanolic acid, ethyl ester (ethyl decanoate)	16.814

Figure 2B Chromatograms of volatile compound in must



Compounds	Retention time (min)
Butanoic acid, ethyl ester (ethyl butyrate)	5.748
1-Butanol,3-methyl acetate (isoamyl acetate)	7.879
Hexanoic acid, ethyl ester (ethyl hexanoate)	10.617
Acetic acid, hexyl ester (hexyl acetate)	10.848
1, 6-Octadien-1-ol, 3,7-dimethyl (linalool)	12.431
Phenethyl alcohol	12.762
Octanoic acid, ethyl ester (ethyl octanoate)	13.976
2, 6-Octadien-1-ol, 3,7-dimethyl-, (Z) (nerol)	14.441
2, 6-Octadien-1-ol, 3,7-dimethyl-, (E) (geraniol)	14.483
Acetic acid, 2- phenylethyl ester (2-phenylethyl acetate)	14.922
Decanolic acid, ethyl ester (ethyl decanoate)	16.814

Figure 3B Chromatogram of volatile compound in wine

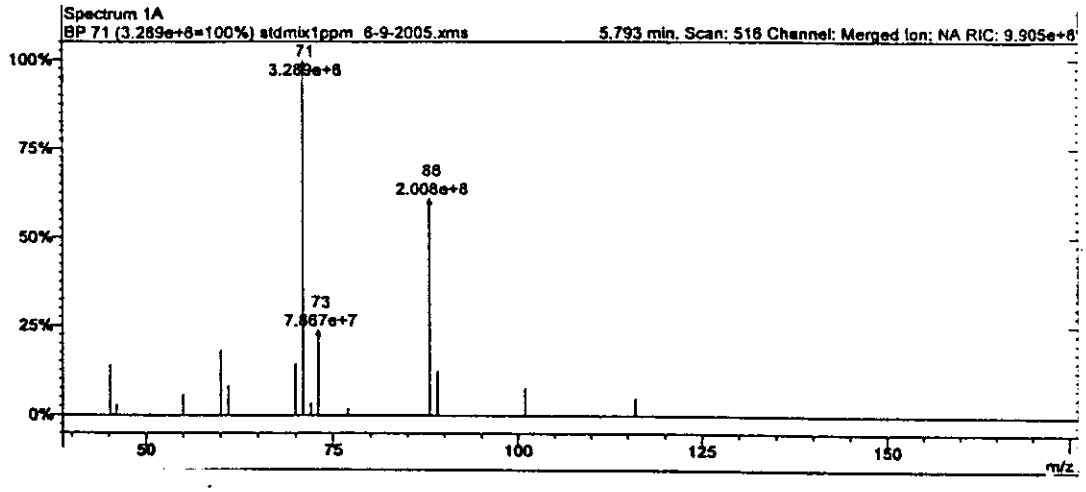


Spectrum from c:\varianws\data\grape1\stdmix1ppm 6-9-2005.xms
Scan No: 825, Time: 8.002 minutes
No averaging. Not background corrected.
Comment: 8.002 min. Scan: 825 Channel: Merged Ion: NA RIC: 1.152e+9
Pair Count: 15 MW: 0 Formula: None
CAS No: None Acquired Range: 45 - 170 m/z

Method Description: EI
Scan 1 Channel Description: 45.0 - 170.0 >
Scan Information: cp = 0.0 PSI
Precursor Mass Range: 45 - 170 m/z

Ion	Int	%BP	Ion	Int	%BP	Ion	Int	%BP
45	1.453e+7	3.6	57	1.425e+7	3.6	71	2.873e+7	7.2
53	1.262e+7	3.1	58	1.479e+7	3.7	73	5.177e+7	12.9
54	8.328e+6	2.1	61	8.027e+7	20.0	85	8.944e+6	2.2
55	2.783e+8	69.4	69	3.708e+7	9.2	87	8.101e+7	20.2
56	1.698e+7	4.2	70	4.013e+8	100.0	88	1.044e+7	2.6

Figure 4B Chromatogram of ethyl butyrate compound from MS detector



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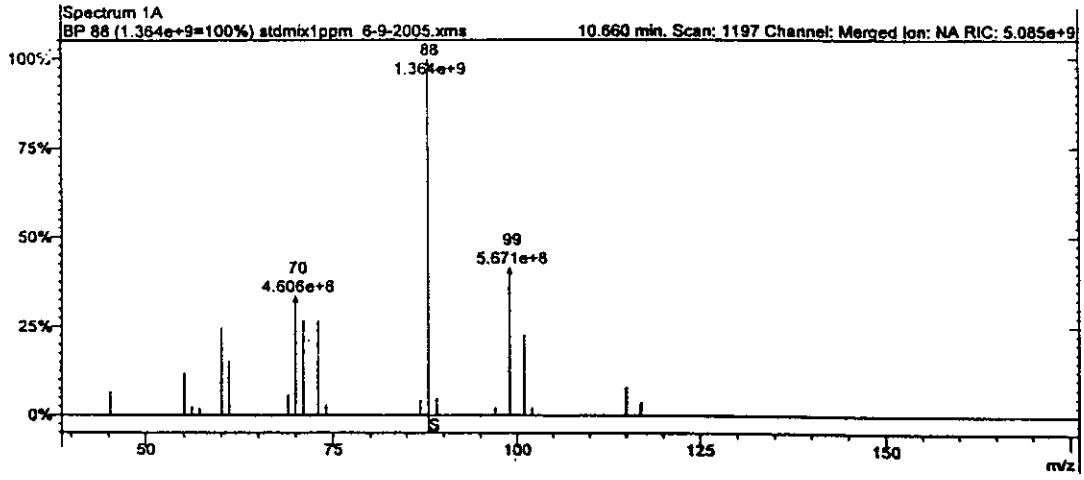
Pair Count: 14 MW: 0 Formula: None
CAS No: None Acquired Range: 45 - 170 m/z

Method Description: EI
Scan 1 Channel Description: 45.0 - 170.0 >
Scan Information: cp = 0.0 PSI
Precursor Mass Range: 45 - 170 m/z

Ion	Int	%BP	Ion	Int	%BP	Ion	Int	%BP
45	4.733e+7	14.4	70	4.774e+7	14.5	88	2.008e+8	61.0
46	1.097e+7	3.3	71	3.289e+8	100.0	89	4.163e+7	12.7
55	2.010e+7	6.1	72	1.254e+7	3.8	101	2.559e+7	7.8
60	6.042e+7	18.4	73	7.867e+7	23.9	116	1.638e+7	5.0
61	2.758e+7	8.4	77	7.210e+6	2.2			

Figure 5B Chromatogram of isoamyl acetate compound from MS detector

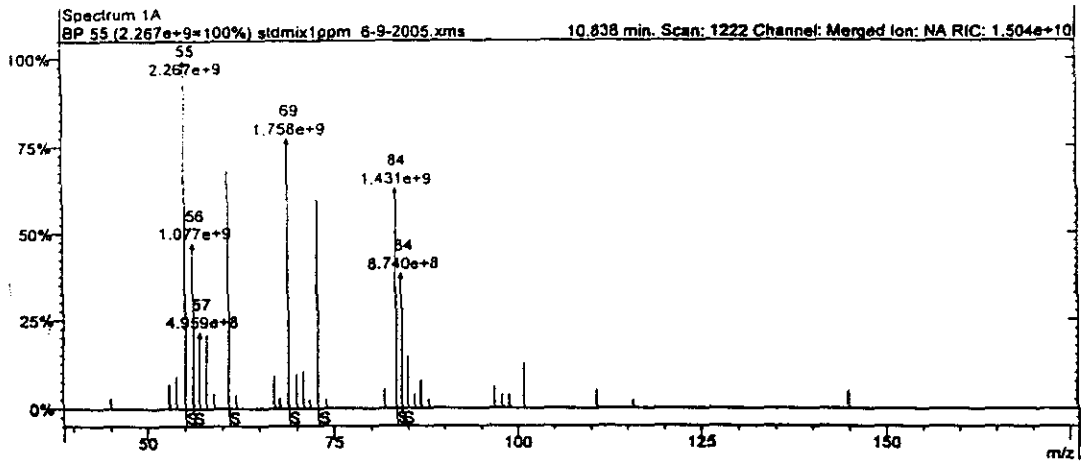


Spectrum from c:\varianws\data\grape1\stdmix1ppm 6-9-2005.xms
Scan No: 1197, Time: 10.660 minutes
No averaging. Not background corrected.
Comment: 10.660 min. Scan: 1197 Channel: Merged Ion: NA RIC: 5.085e+9
Pair Count: 20 MW: 0 Formula: None
CAS No: None Acquired Range: 45 - 170 m/z

Method Description: EI
Scan 1 Channel Description: 45.0 - 170.0 >
Scan Information: cp = 0.0 PSI
Precursor Mass Range: 45 - 170 m/z

Ion	Int	%BP	Ion	Int	%BP	Ion	Int	%BP
45	9.313e+7	6.8	70	4.606e+8	33.8	97	3.211e+7	2.4
55	1.666e+8	12.2	71	3.684e+8	27.0	99	5.671e+8	41.6
56	3.527e+7	2.6	73	3.655e+8	26.8	101	3.107e+8	22.8
57	2.966e+7	2.2	74	4.273e+7	3.1	102	3.139e+7	2.3
60	3.404e+8	25.0	87	6.032e+7	4.4	115	1.104e+8	8.1
61	2.125e+8	15.6	88	1.364e+9	100.0	117	5.116e+7	3.7
69	8.148e+7	6.0	89	6.655e+7	4.9			

Figure 6B Chromatogram of ethyl hexanoate compound from MS detector



Spectrum from c:\varianws\data\grape1\stdmix1ppm 6-9-2005.xms
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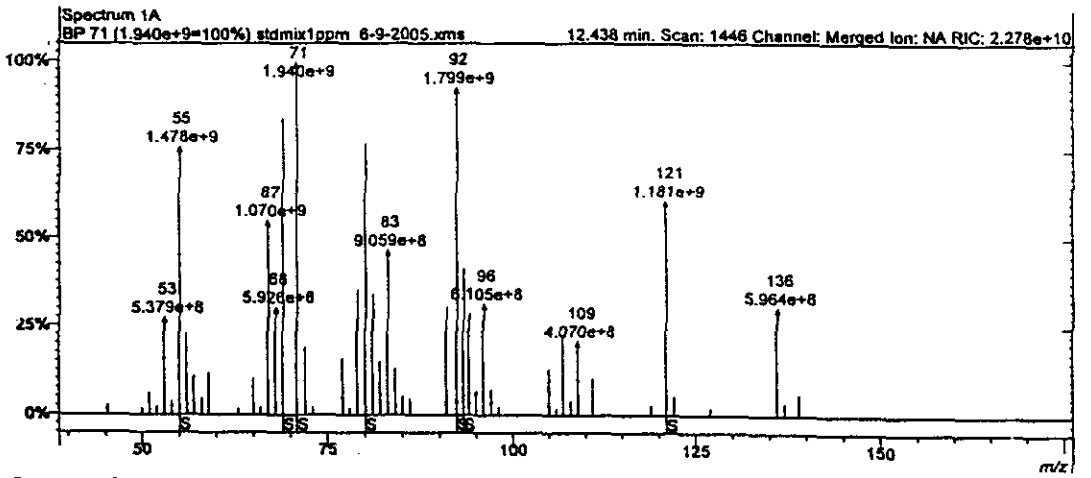
No averaging. Not background corrected.
Comment: 10.838 min. Scan: 1222 Channel: Merged Ion: NA RIC: 1.504e+10
Pair Count: 32 MW: 0 Formula: None
CAS No: None Acquired Range: 45 - 170 m/z

Method Description: EI
Scan 1 Channel Description: 45.0 - 170.0 >
Scan Information: cp = 0.0 PSI
Precursor Mass Range: 45 - 170 m/z

Ion	Int	%BP	Ion	Int	%BP	Ion	Int	%BP
45	7.514e+7	3.3	68	7.150e+7	3.2	86	9.435e+7	4.2
53	1.612e+8	7.1	69	1.758e+9	77.6	87	1.864e+8	8.2
54	2.107e+8	9.3	70	2.270e+8	10.0	88	6.576e+7	2.9
55	2.267e+9	100.0	71	2.476e+8	10.9	97	1.486e+8	6.6
56	1.077e+9	47.5	72	6.327e+7	2.8	98	9.088e+7	4.0
57	4.959e+8	21.9	73	1.370e+9	60.4	99	9.199e+7	4.1
58	4.815e+8	21.2	74	6.644e+7	2.9	101	2.906e+8	12.8
59	1.004e+8	4.4	82	1.266e+8	5.6	111	1.237e+8	5.5
61	1.556e+9	68.7	84	1.431e+9	63.2	116	5.756e+7	2.5
62	9.017e+7	4.0	84	8.740e+8	38.6	145	1.156e+8	5.1
67	2.119e+8	9.3	85	3.354e+8	14.8			

Figure 7B Chromatogram of hexyl acetate compound from MS detector

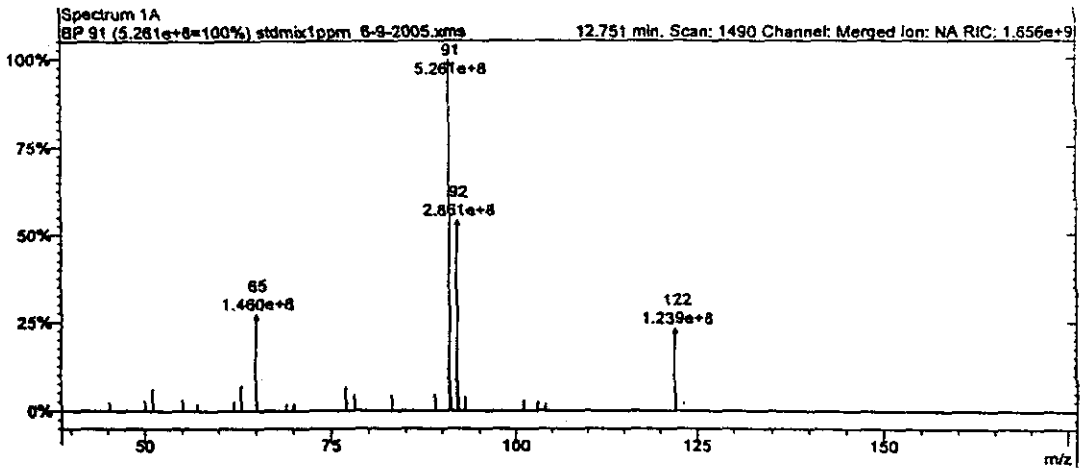


Spectrum from c:\varianws\data\grape1\stdmix1ppm 6-9-2005.xms
Scan No: 1446, Time: 12.438 minutes
No averaging. Not background corrected.
Comment: 12.438 min. Scan: 1446 Channel: Merged Ion: NA RIC: 2.278e+10
Pair Count: 51 MW: 0 Formula: None
CAS No: None Acquired Range: 45 - 170 *m/z*

Method Description: EI
Scan 1 Channel Description: 45.0 - 170.0 >
Scan Information: cp = 0.0 PSI
Precursor Mass Range: 45 - 170 m/z

Ion	Int	%BP	Ion	Int	%BP	Ion	Int	%BP
45	5.814e+7	3.0	71	1.940e+9	100.0	95	1.348e+8	6.9
50	3.895e+7	2.0	72	3.760e+8	19.4	96	6.105e+8	31.5
51	1.234e+8	6.4	73	4.972e+7	2.6	97	1.383e+8	7.1
52	4.725e+7	2.4	77	3.150e+8	16.2	98	4.697e+7	2.4
53	5.379e+8	27.7	78	4.015e+7	2.1	105	2.569e+8	13.2
54	7.654e+7	3.9	79	6.866e+8	35.4	106	4.265e+7	2.2
55	1.478e+9	76.2	80	1.495e+9	77.1	107	4.220e+8	21.8
56	4.493e+8	23.2	81	6.633e+8	34.2	108	8.026e+7	4.1
57	2.154e+8	11.1	82	2.946e+8	15.2	109	4.070e+8	21.0
58	9.952e+7	5.1	83	9.059e+8	46.7	111	2.077e+8	10.7
59	2.353e+8	12.1	84	2.564e+8	13.2	119	5.707e+7	2.9
63	3.992e+7	2.1	85	1.049e+8	5.4	121	1.181e+9	60.9
65	2.026e+8	10.4	86	9.592e+7	4.9	122	1.071e+8	5.5
66	4.536e+7	2.3	91	5.929e+8	30.6	127	4.518e+7	2.3
67	1.070e+9	55.2	92	1.799e+9	92.7	136	5.964e+8	30.8
68	5.926e+8	30.6	93	8.044e+8	41.5	137	7.127e+7	3.7
69	1.633e+9	84.2	94	5.546e+8	28.6	139	1.177e+8	6.1

Figure 8B Chromatogram of linalool compound from MS detector

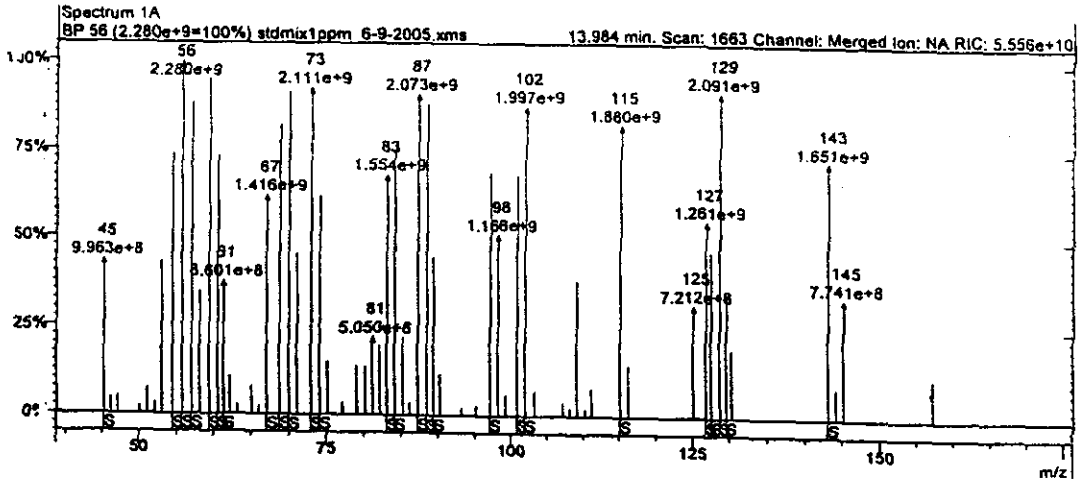


Spectrum from c:\varianws\data\grape1\stdmix1ppm 6-9-2005.xms
Scan No: 1490, Time: 12.751 minutes
No averaging. Not background corrected.
Comment: 12.751 min. Scan: 1490 Channel: Merged Ion: NA RIC: 1.656e+9
Pair Count: 21 MW: 0 Formula: None
CAS No: None Acquired Range: 45 - 170 m/z

Method Description: EI
Scan 1 Channel Description: 45.0 - 170.0 >
Scan Information: cp = 0.0 PSI
Precursor Mass Range: 45 - 170 m/z

Ion	Int	%BP	Ion	Int	%BP	Ion	Int	%BP
45	1.337e+7	2.5	65	1.460e+8	27.8	91	5.261e+8	100.0
50	1.609e+7	3.1	69	1.082e+7	2.1	92	2.861e+8	54.4
51	3.343e+7	6.4	70	1.175e+7	2.2	93	2.150e+7	4.1
55	1.688e+7	3.2	77	3.614e+7	6.9	101	1.622e+7	3.1
57	1.163e+7	2.2	78	2.478e+7	4.7	103	1.425e+7	2.7
62	1.517e+7	2.9	83	2.357e+7	4.5	104	1.159e+7	2.2
63	3.782e+7	7.2	89	2.452e+7	4.7	122	1.239e+8	23.6

Figure 9B Chromatogram of phenethyl alcohol compound from MS detector

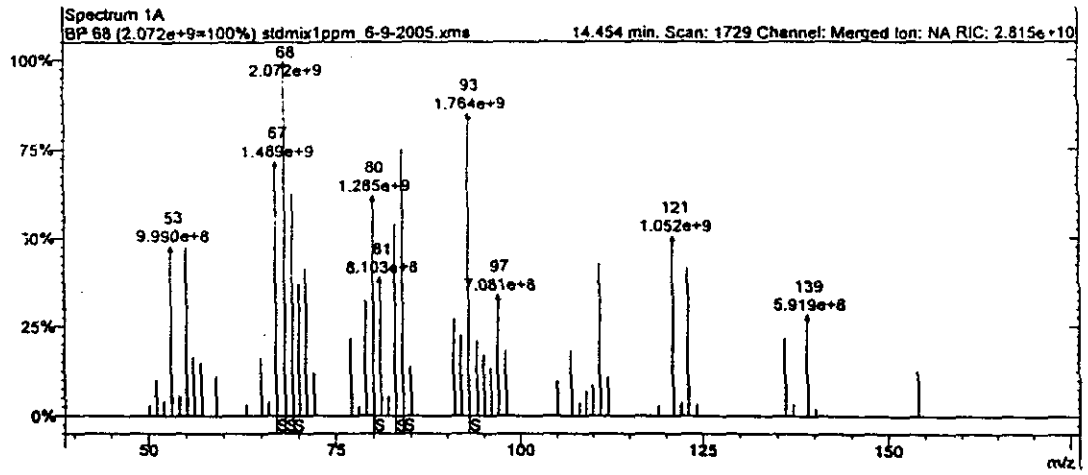


Spectrum from c:\varianws\data\grape1\stdmix1ppm 6-9-2005.xms
Scan No: 1663, Time: 13.984 minutes
No averaging. Not background corrected.
Comment: 13.984 min. Scan: 1663 Channel: Merged Ion: NA RIC: 5.556e+10
Pair Count: 63 MW: 0 Formula: None
CAS No: None Acquired Range: 45 - 170 m/z

Method Description: EI
Scan 1 Channel Description: 45.0 - 170.0 >
Scan Information: cp = 0.0 PSI
Precursor Mass Range: 45 - 170 m/z

Ion	Int	%BP	Ion	Int	%BP	Ion	Int	%BP
45	9.963e+8	43.7	71	1.042e+9	45.7	99	1.354e+8	5.9
46	1.121e+8	4.9	73	2.111e+9	92.6	101	1.551e+9	68.0
47	1.243e+8	5.5	74	1.411e+9	61.9	102	1.997e+9	87.6
50	5.613e+7	2.5	75	3.435e+8	15.1	103	1.647e+8	7.2
51	1.701e+8	7.5	77	8.471e+7	3.7	107	9.132e+7	4.0
52	8.447e+7	3.7	79	3.211e+8	14.1	108	5.817e+7	2.6
53	9.908e+8	43.5	80	3.101e+8	13.6	109	8.755e+8	38.4
54	1.694e+9	74.3	81	5.050e+8	22.1	110	5.214e+7	2.3
56	2.280e+9	100.0	82	4.475e+8	19.6	111	1.829e+8	8.0
57	2.012e+9	88.3	83	1.554e+9	68.1	115	1.880e+9	82.5
58	7.931e+8	34.8	84	1.717e+9	75.3	116	3.352e+8	14.7
59	2.167e+9	95.0	85	5.037e+8	22.1	125	7.212e+8	31.6
60	1.675e+9	73.5	86	8.523e+7	3.7	127	1.261e+9	55.3
61	8.601e+8	37.7	87	2.073e+9	90.9	127	1.061e+9	46.5
62	2.479e+8	10.9	88	2.010e+9	88.2	129	2.091e+9	91.7
63	6.614e+7	2.9	89	1.026e+9	45.0	129	7.895e+8	34.6
65	1.850e+8	8.1	90	2.722e+8	11.9	130	4.416e+8	19.4
66	5.825e+7	2.6	93	5.437e+7	2.4	143	1.651e+9	72.4
67	1.416e+9	62.1	95	6.987e+7	3.1	144	1.991e+8	8.7
69	1.878e+9	82.3	97	1.570e+9	68.8	145	7.741e+8	33.9
70	2.080e+9	91.2	98	1.166e+9	51.1	157	2.663e+8	11.7

Figure 10B Chromatogram of ethyl octanoate compound from MS detector

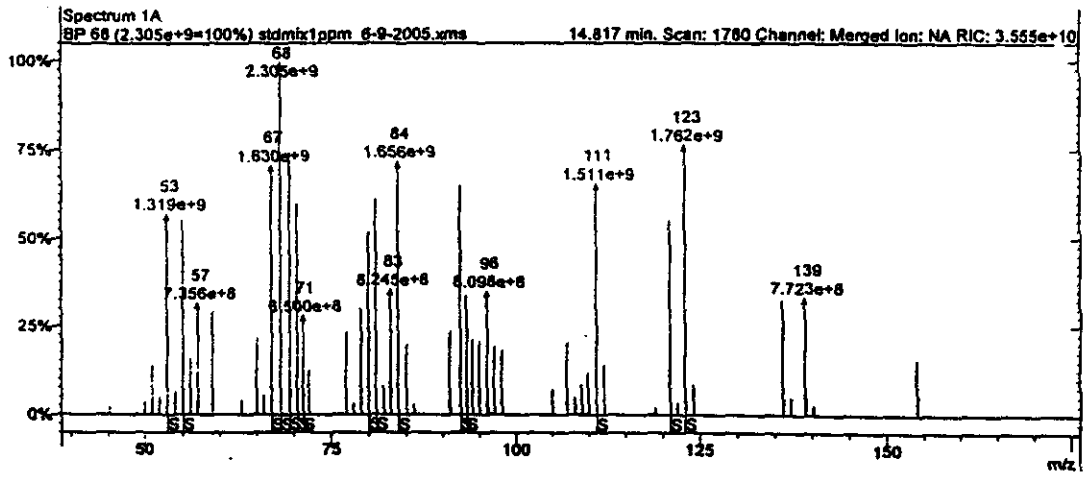


Spectrum from c:\varianws\data\grape1\stdmix1ppm 6-9-2005.xms
Scan No: 1729, Time: 14.454 minutes
No averaging. Not background corrected.
Comment: 14.454 min. Scan: 1729 Channel: Merged Ion: NA RIC: 2.815e+10
Pair Count: 52 MW: 0 Formula: None
CAS No: None Acquired Range: 45 - 170 m/z

Method Description: EI
Scan 1 Channel Description: 45.0 - 170.0 >
Scan Information: cp = 0.0 PSI
Precursor Mass Range: 45 - 170 m/z

Ion	Int	%BP	Ion	Int	%BP	Ion	Int	%BP
50	6.557e+7	3.2	77	4.574e+8	22.1	105	2.038e+8	9.8
51	2.148e+8	10.4	78	5.977e+7	2.9	107	3.794e+8	18.3
52	8.742e+7	4.2	79	6.739e+8	32.5	108	8.046e+7	3.9
53	9.990e+8	48.2	80	1.285e+9	62.0	109	1.503e+8	7.3
54	1.187e+8	5.7	81	8.103e+8	39.1	110	1.808e+8	8.7
55	9.877e+8	47.7	82	1.162e+8	5.6	111	8.909e+8	43.0
56	3.463e+8	16.7	83	1.122e+9	54.2	112	2.307e+8	11.1
57	3.125e+8	15.1	84	1.560e+9	75.3	119	6.110e+7	2.9
59	2.380e+8	11.5	85	2.902e+8	14.0	121	1.052e+9	50.8
63	6.783e+7	3.3	91	5.644e+8	27.2	122	7.948e+7	3.8
65	3.444e+8	16.6	92	4.711e+8	22.7	123	8.613e+8	41.6
66	8.510e+7	4.1	93	1.764e+9	85.1	124	7.051e+7	3.4
67	1.489e+9	71.9	94	4.374e+8	21.1	136	4.526e+8	21.8
68	2.072e+9	100.0	95	3.571e+8	17.2	137	7.115e+7	3.4
69	1.305e+9	63.0	96	2.803e+8	13.5	139	5.919e+8	28.6
70	7.744e+8	37.4	97	7.081e+8	34.2	140	4.763e+7	2.3
71	8.628e+8	41.6	98	3.820e+8	18.4	154	2.638e+8	12.7
72	2.496e+8	12.0						

Figure 11B Chromatogram of nerol compound from MS detector

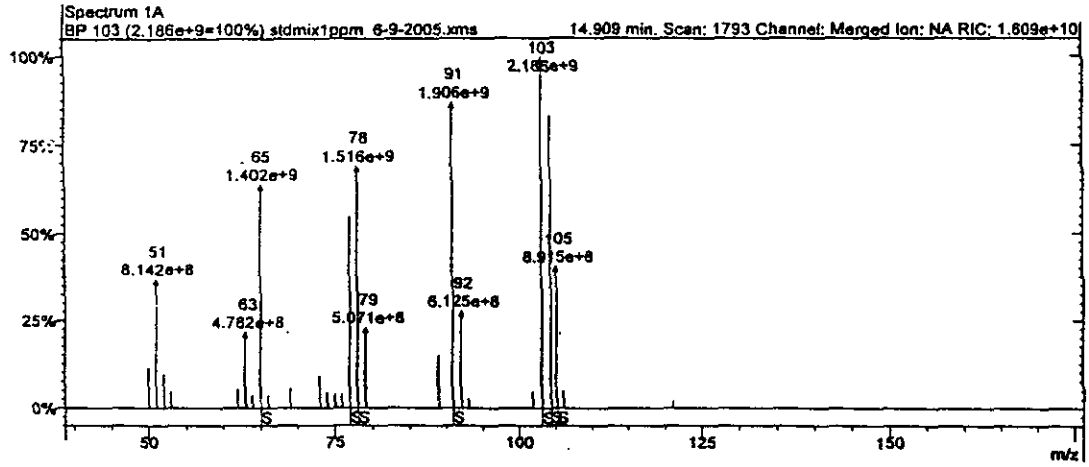


Spectrum from c:\varianws\data\grape1\stdmix1ppm 6-9-2005.xms
Scan No: 1780, Time: 14.817 minutes
No averaging. Not background corrected.
Comment: 14.817 min. Scan: 1780 Channel: Merged Ion: NA RIC: 3.555e+10
Pair Count: 54 MW: 0 Formula: None
CAS No: None Acquired Range: 45 - 170 m/z

Method Description: EI
Scan 1 Channel Description: 45.0 - 170.0 >
Scan Information: cp = 0.0 PSI
Precursor Mass Range: 45 - 170 m/z

Ion	Int	%BP	Ion	Int	%BP	Ion	Int	%BP
45	5.684e+7	2.5	72	2.909e+8	12.6	98	4.219e+8	18.3
50	9.099e+7	3.9	77	5.366e+8	23.3	105	1.685e+8	7.3
51	3.295e+8	14.3	78	7.683e+7	3.3	107	4.699e+8	20.4
52	1.234e+8	5.4	79	6.988e+8	30.3	108	1.168e+8	5.1
53	1.319e+9	57.2	80	1.199e+9	52.0	109	2.010e+8	8.7
54	1.523e+8	6.6	81	1.412e+9	61.2	110	2.759e+8	12.0
55	1.284e+9	55.7	82	1.899e+8	8.2	111	1.511e+9	65.5
56	3.664e+8	15.9	83	8.245e+8	35.8	112	3.294e+8	14.3
57	7.356e+8	31.9	84	1.656e+9	71.8	119	4.931e+7	2.1
59	6.817e+8	29.6	85	4.610e+8	20.0	121	1.274e+9	55.3
63	9.811e+7	4.3	86	7.332e+7	3.2	122	8.653e+7	3.8
65	5.044e+8	21.9	91	5.432e+8	23.6	123	1.762e+9	76.4
66	1.298e+8	5.6	92	1.503e+9	65.2	124	2.007e+8	8.7
67	1.630e+9	70.7	93	7.857e+8	34.1	136	7.586e+8	32.9
68	2.305e+9	100.0	94	4.885e+8	21.2	137	1.150e+8	5.0
69	1.723e+9	74.7	95	4.804e+8	20.8	139	7.723e+8	33.5
71	1.381e+9	59.9	96	8.098e+8	35.1	140	6.420e+7	2.8
71	6.500e+8	28.2	97	4.445e+8	19.3	154	3.662e+8	15.9

Figure 12B Chromatogram of geraniol compound from MS detector

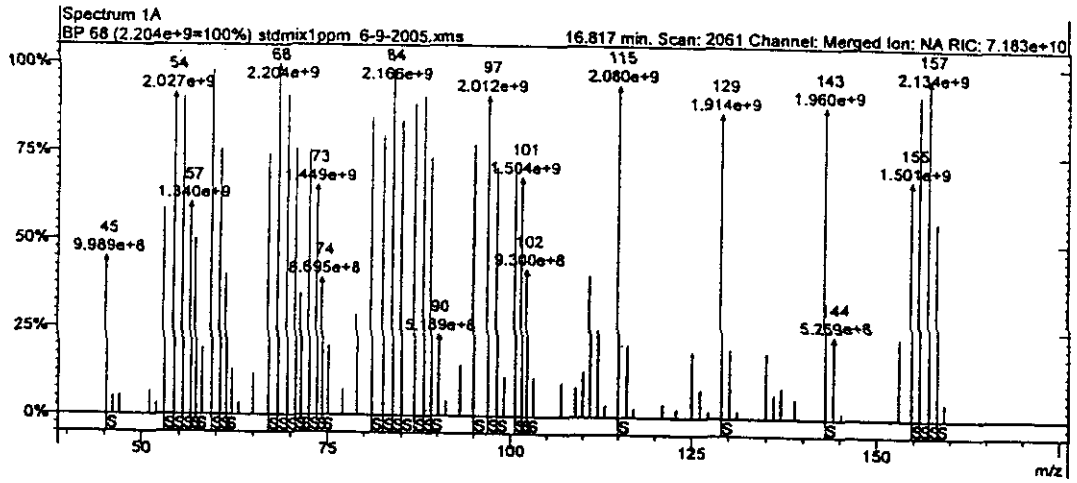


Spectrum from c:\varianws\data\grape1\stdmix1ppm 6-9-2005.xms
Scan No: 1793, Time: 14.909 minutes
No averaging. Not background corrected.
Comment: 14.909 min. Scan: 1793 Channel: Merged Ion: NA RIC: 1.609e+10
Pair Count: 27 MW: 0 Formula: None
CAS No: None Acquired Range: 45 - 170 m/z

Method Description: EI
Scan 1 Channel Description: 45.0 - 170.0 >
Scan Information: cp = 0.0 PSI
Precursor Mass Range: 45 - 170 m/z

Ion	Int	%BP	Ion	Int	%BP	Ion	Int	%BP
50	2.510e+8	11.5	69	1.288e+8	5.9	91	1.906e+9	87.2
51	8.142e+8	37.2	73	2.045e+8	9.4	92	6.125e+8	28.0
52	2.153e+8	9.8	74	1.024e+8	4.7	93	6.511e+7	3.0
53	1.088e+8	5.0	75	9.178e+7	4.2	102	1.061e+8	4.9
62	1.220e+8	5.6	76	9.040e+7	4.1	103	2.186e+9	100.0
63	4.782e+8	21.9	77	1.204e+9	55.0	104	1.827e+9	83.6
64	8.492e+7	3.9	78	1.516e+9	69.3	105	8.915e+8	40.8
65	1.402e+9	64.1	79	5.071e+8	23.2	106	1.115e+8	5.1
66	8.643e+7	4.0	89	3.319e+8	15.2	121	5.023e+7	2.3

Figure 13B Chromatogram of 2- phenelethyl acetate compound from MS detector



Spectrum from c:\varianws\data\grape1\stdmix1ppm 6-9-2005.xms
Scan No: 2061, Time: 16.817 minutes
No averaging. Not background corrected.
Comment: 16.817 min. Scan: 2061 Channel: Merged Ion: NA RIC: 7.183e+10
Pair Count: 76 MW: 0 Formula: None
CAS No: None Acquired Range: 45 - 170 m/z

Method Description: EI
Scan 1 Channel Description: 45.0 - 170.0 >
Scan Information: cp = 0.0 PSI
Precursor Mass Range: 45 - 170 m/z

Ion	Int	%BP	Ion	Int	%BP	Ion	Int	%BP
45	9.989e+8	45.3	77	1.677e+8	7.6	113	7.734e+7	3.5
46	1.197e+8	5.4	79	6.406e+8	29.1	115	2.080e+9	94.3
47	1.256e+8	5.7	81	1.876e+9	85.1	116	4.599e+8	20.9
51	1.518e+8	6.9	83	1.764e+9	80.0	117	6.283e+7	2.9
52	7.844e+7	3.6	84	2.166e+9	98.3	121	8.732e+7	4.0
53	1.303e+9	59.1	85	1.857e+9	84.3	123	5.609e+7	2.5
54	2.027e+9	92.0	87	1.967e+9	89.2	125	4.179e+8	19.0
56	2.004e+9	90.9	88	2.010e+9	91.2	126	1.811e+8	8.2
57	1.340e+9	60.8	89	1.621e+9	73.5	127	4.863e+7	2.2
57	1.109e+9	50.3	90	5.189e+8	23.5	129	1.914e+9	86.8
58	4.274e+8	19.4	91	9.870e+7	4.5	130	4.358e+8	19.8
59	2.163e+9	98.1	93	3.268e+8	14.8	131	4.911e+7	2.2
61	1.675e+9	76.0	95	1.710e+9	77.6	135	4.159e+8	18.9
61	8.907e+8	40.4	97	2.012e+9	91.3	136	1.521e+8	6.9
62	2.940e+8	13.3	98	1.564e+9	71.0	137	1.966e+8	8.9
63	7.675e+7	3.5	99	2.520e+8	11.4	139	1.290e+8	5.9
65	2.659e+8	12.1	101	1.597e+9	72.5	143	1.960e+9	88.9
67	1.649e+9	74.8	101	1.504e+9	68.2	144	5.259e+8	23.9
68	2.204e+9	100.0	102	9.300e+8	42.2	145	4.477e+7	2.0
70	2.010e+9	91.2	103	2.469e+8	11.2	153	5.099e+8	23.1
71	1.675e+9	76.0	107	2.186e+8	9.9	155	1.501e+9	68.1
71	7.690e+8	34.9	109	1.885e+8	8.6	156	2.034e+9	92.3
73	1.679e+9	76.2	110	2.903e+8	13.2	157	2.134e+9	96.8
73	1.449e+9	65.8	111	8.921e+8	40.5	158	1.246e+9	56.5
74	8.695e+8	39.4	112	5.543e+8	25.1	159	1.104e+8	5.0
75	4.433e+8	20.1						

Figure 14B Chromatogram of ethyl decanoate compound from MS detector

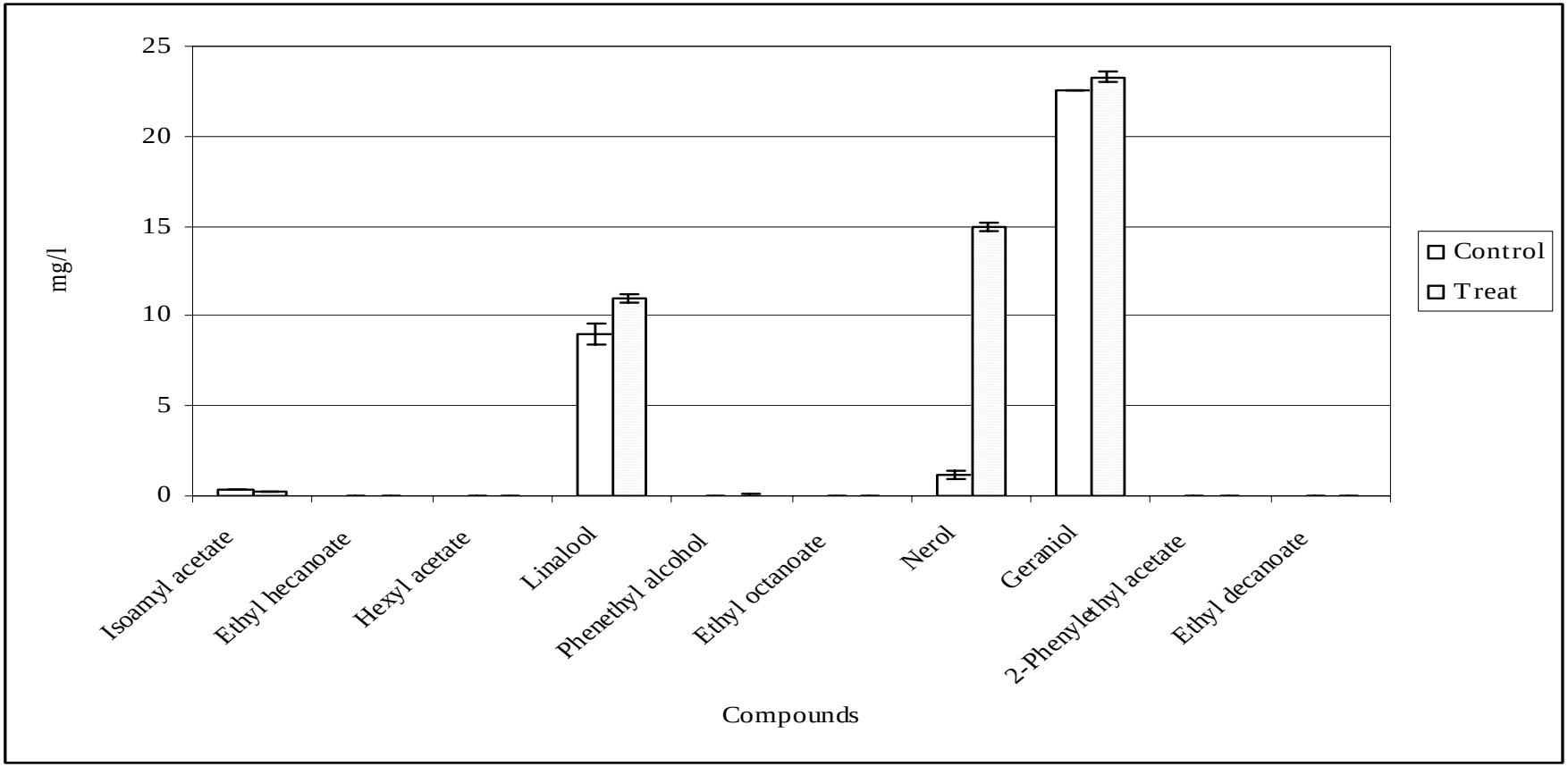


Figure 15B Volatile compound of must was treated with 100 U/mg protein of β-D-glucosidase and control

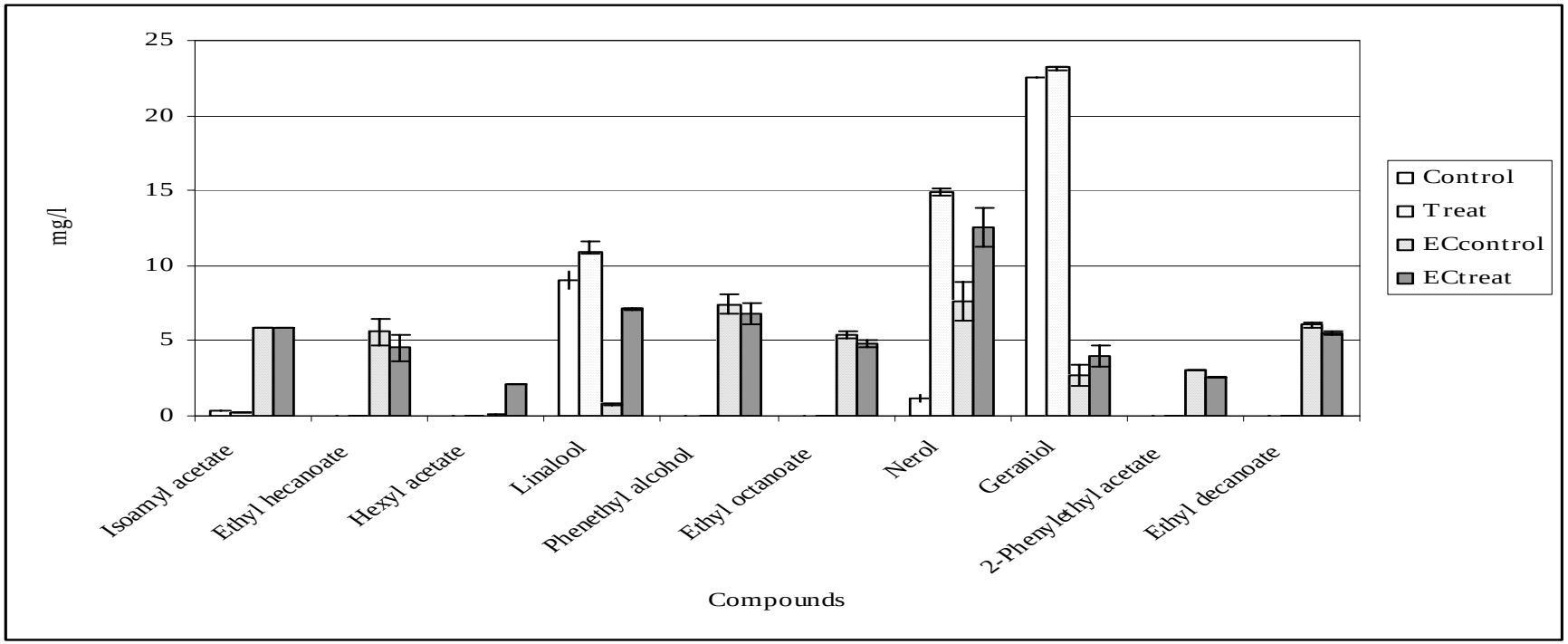


Figure 16B Volatile compound of wine fermented with yeast EC-1118 strain was treated with β-D-glucosidase and control (Control: must, Control treated: must was treated with 100 U/mg protein of β-D-glucosidase, WECcontrol: wine fermented with yeast EC-1118 strain and WECtreated: wine fermented with yeast EC-1118 strain and treated with 100 U/mg protein of β-D-glucosidase).

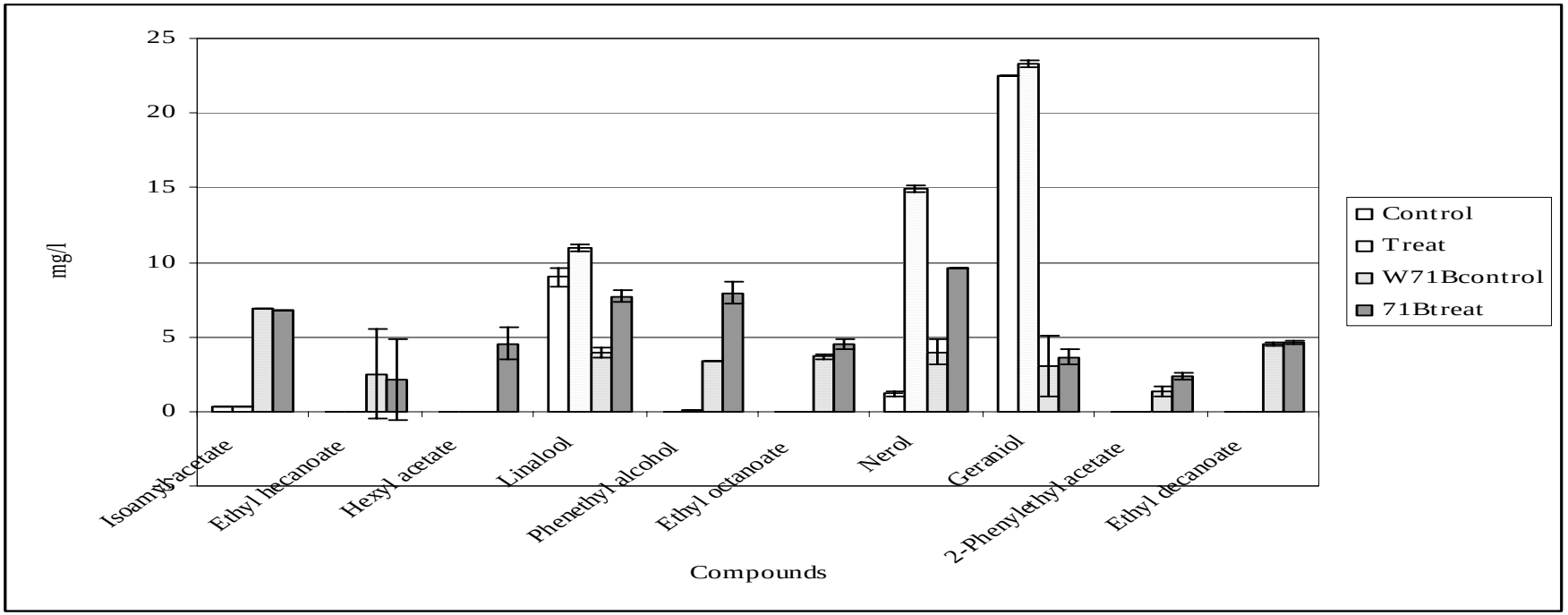


Figure 17B Volatile compound of wine fermented with yeast 71B-1122 strain was treated with β-D-glucosidase and control (Control: must, Control treated: must was treated with 100 U/mg protein of β-D-glucosidase, WECcontrol: wine fermented with yeast 71B-1122 strain and WECtreated: wine fermented with yeast 71B-1122 strain and treated with 100 U/mg protein of β-D-glucosidase)

Table 1B Error bar data of optimal temperature

Temperature (°C)	SD
20	±0.004
30	±0.006
40	±0.005
45	±0.014
50	±0.009
55	±0.007
60	±0.001
65	±0.002
70	±0.004
80	±0.003

Table 2B Error bar data of thermal stability of the extracellular β-D-glucosidase assay

Time	**Temperature**				
(hr)	**20ºC**	**30ºC**	**40ºC**	**50ºC**	**60ºC**
0.0	±0.089	±0.050	±0.004	±0.073	±0.047
0.5	±0.005	±0.052	±0.113	±0.021	±0.001
1.0	±0.029	±0.046	±0.064	±0.006	±0.001
1.5	±0.038	±19.939	±0.102	±0.023	±0.001
2.0	±0.100	±19.939	±0.060	±0.016	±0.001
2.5	±0.053	±19.955	±0.086	±0.015	±0.001
3.0	±0.026	±19.975	±0.045	±0.008	±0.001
3.5	±0.030	±19.995	±0.069	±0.001	±0.001
4.0	±0.095	±20.012	±0.134	±0.001	±0.004

Table 3B Error bar data of ethanol concentration inhibition

Ethanol (%)	SD
0.0	±0.038
5.0	±0.040
10.0	±0.019
15.0	±0.022

Table 4B Qualities of volatile compounds after pre-incubated at 20 and 50ºC for overnight and 1 hr, respectively.

Compounds	20ºC (mg/l)		50ºC (mg/l)	
	Control	**Treated**	**Control**	**Treated**
Butanoic acid, ethyl ester (ethyl butyrate)	0.00	0.00	0.84	0.65
1-Butanol,3-methyl acetate (isoamyl acetate)	0.00	0.00	0.06	0.01
Hexanoic acid, ethyl ester (ethyl hexanoate)	0.01	0.00	0.56	0.57
Acetic acid, hexyl ester (ethyl hexanoate)	2.87	2.69	0.86	0.71
1, 6-Octadien-1-ol, 3,7-dimethyl (linalool)	0.07	0.03	0.03	0.04
Phenethyl alcohol	0.56	0.43	0.15	0.14
Octanoic acid, ethyl ester (ethyl octanoate)	0.29	0.26	0.00	0.00
2, 6-Octadien-1-ol, 3,7-dimethyl-, (Z) (nerol)	0.75	0.63	0.00	0.00
2, 6-Octadien-1-ol, 3,7-dimethyl-, (E) (geraniol)	0.10	0.39	0.00	0.00
Acetic acid, 2- phenylethyl ester (2-phenylethyl acetate)	0.01	0.01	0.00	0.00
Decanolic acid, ethyl ester (ethyl decanoate)	0.00	0.00	0.00	0.00

BIOGRAPHY

Patcharaporn Sripunya was born in Khonkaen, Thailand on December 4, 1979. She studied in primary school and high school at Chumphaeseuksa shool. In 1998, she studied in School of Applied Biology, Rajabhat Institute Loei, Loei. She participated in the Co-operative Education Program to work as analysis quality department MITR PHOL Co. Ltd., Chaiyapum. She graduated the Bachelor's of Science in Applied Biology in 2002. After graduation, in 2002, she was Master's student in the School of Biotechnology at Suranaree University of Technology. In 2005, she got scholarship from Suranaree University of Technology for finanancial support. This supportive encouraged her research, which studied on selection of yeast strains containing of β-glucosidase for improving aroma in grape wine. During Master's student, she had an experience oral presentation in title "Effect of β-glucosidase enzyme in *Saccharomyces cerevisiae* strains on aroma production during mango (chok-anan) wine fermentation" at the 4th National Symposium on Graduate Research, August 10-11, 2004, Lotus Pang Suan Kaew Hotel, Chiangmai, Thailand. And Poster presentation in the title "Selection of yeast strains of β-glucosidase for improving aroma in grape wine" was presented in The 31st Congress on Science and Technology of Thailand, October 18-20, 2005, Technopolis, Suranaree University of Technology.

Publication and Award:

1. Sripunya, P., Wanapu, C., and Boonkard, N. (2004). **Effect of β-glucosidase enzyme in *Saccharomysis cerevisiae* strains on aroma production during mango (chok-anan) wine fermentation.** The 4th National sysposium on Graduate Research, August 10-11, 2004, Lotus Pang Suan Kaew Hotel, Chiangmai, Thailand. (Oral Presentation).
2. The out standing presenter award in title " Effect of β-glucosidase enzyme in *Saccharomyces cerevisiae* strains on aroma production during mango (chok-anan) wine fermentation" at the 4th National Symposium on Graduate Research, August 10-11, 2004, Lotus Pang Suan Kaew Hotel, Chiangmai, Thailand.
3. Sripunya, P., Wanapu, C., and Boonkerd, N. (2005). Effect of β-glucosidase enzyme in *Saccharomysis cerevisiae* strains on aroma production during mango (chok-anan) wine fermentation. **Thai Journal Biotechnology**, 6:50-56.
4. Sripunya, P.and Wanapu, C. (2005). **Selection of yeast strains of β-glucosidase for improving aroma in grape wine.** The 31st Congress on Science and Technology of Thailand, October 18-20, 2005, Technopolis, Suranaree University of Technology. (Poster Presentation).