Special Project

The Study of Microbial Diversity in Aging Wine Produced from BT 3015 experiment in Biotech Pilot Plant at Assumption University

By

Ms. Numpetch Wanjaroen

ID.441-8895

A special project submitted to the School of Biotechnology, Assumption University in part fulfillment of the requirements of the degree of Bachelor of Science in Biotechnology

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School	:	Biotechnology
Academic Year	:	2006

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The Study of Microbial Diversity in Aging Wine Produced from BT 3015 experiment in Biotech Pilot Plant at Assumption University

Keywords: Wine microbial diversity, Wine microorganisms and Aging wine

By **Ms. Numpetch Wanjaroen**

Advisor **Dr. Viyada Kunathigan**

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Abstract

During aging of wine, wine flavor and aroma could be further modified by various yeasts and bacteria. Some of these microorganisms are considered spoilage organisms. Depending on the type of microorganisms and the extent of growth, desirable fruit flavors can be lost or masked by unpleasant aromas and taste. Under good aging conditions, without spoilage microorganisms the wine continues to change due to chemical and biochemical (enzymes still active in the wine) conversions. Depending on wine style and personal preference an optimum combination of 1) fresh fruit flavors, 2) fermentation flavors, and 3) aging flavors are reached after as little as half a year and more than 10 years. Since aging period is important for the quality of wine and microorganisms play an important role at this period but not much research have been done to assess the aging of wine produced in Thailand .This report aims to study the microbial diversity in wine produced by Assumption University in Biotech Pilot Plant during aging period and also to isolate the microorganisms that may responsible for quality development of this wine. The taxonomy using morphological characteristic and biochemical activity method was used to identify the genus of the isolated strain from four sample which were White wine 2004, White wine 2005, Red wine 2004, and Red wine 2005. White wine was produced from lychee and Red wine was produce from grape. The result showed that thirty one strains were isolated from all samples and twenty-six strains could be maintained which can separate into bacteria and yeast. From the morphological characteristic, nine strains were classified

as bacteria and seventeen strains were classified as yeast. Four from nine strains of bacteria could be identified at genus level, which two strains were classified in the genus of *Enterobacter Sp.* and the other two were classify in the genus of *Gluconobacter Sp.*. The five remaining bacteria strains could not be identified according to the result and need further test. From seventeen strains that were classified as yeast, fourteen strains classified in the family of *Saccharomycetaceae* and one strain classify in the family of *Candidaceae* and from the result of morphology and some biochemical test lead to *Brettanomyces* sp.. However, yeast also has two remaining strains that could not identify the group and it need further study in molecular identification for definite identify of all isolated strain.

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Numpetch Wanjaroen

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Introduction

Grape is an important fruit found all over the world, it is used for eating and for production of wine. The exact origin of wine is not cleared. However, used of the grape fruit for production of wine, was "domesticated" before 4,000 B.C. in Mesopotamia and Egypt. Any beverage derived from fermented fruit juice is considered wine. However, wine as we know it is still fermented grape juice from 6,000 years after its domestication (Rapp *et al.* 1986; Tue, 2001). By improving fermentation methods, the grape juice concentrate could allow fermentation of high alcohol (wines) which eliminate brandy fortification and provide an easier process to produce dessert wines. The strains of yeast (*Saccharomyces cerevisiae*) are mostly used for fermentations of sugar into alcohols (Buescher *et al* .2001). At the start of 2004, press services reported that the worldwide wine industry had grown as large as the global cosmetics industry. That achievement is outstanding for a beverage that dates back perhaps as far as 100,000 years and that can enhance one's beauty only in the spiritual sense. However, in Thailand wine consumption accounts for only one percent of the total figure of alcoholic beverage consumption. Regardless, though, the wine trade in Thailand remains lively, spirited and competitive with quite a few interesting trends developing. Those trends to keep an eye on during 2005 are: the effect of free trade agreements (FTAs) with wine-producing countries, the emergence of new wine distributors with high-quality products and the likelihood of an increase in the wine excise tax following the national election. (Anonymous, 2005a)

Total wine aging can be considered to be all the reactions and changes that occur after the first racking that lead to improvement at some stage rather than spoilage. Aging, like all groups of reaction, takes more or less time depending upon temperature and other conditions. In early time's malolactic fermentation, final clarification, and tartrate stabilization come about during the postfermentation period and were part of "aging". (Boulton, 1996).

Since aging period is important for the quality of wine and Assumption University has produced wine for a period of time, however nobody has studied about microbial diversity in this wine. So this report aims to study the microbial diversity in

wine produced by Assumption University in Biotech Pilot Plant during aging period and also to isolate the microorganisms that responsible for quality development of this wine.

Objectives

- To study the diversity of microbes in aging wine produced from BT 3015 in ABAC pilot plant.
- To isolate the microbe this may involve in the aging period.

Literature review

The principle of winemaking has changed very little since the early days of winemaking. Based on the early works of Antonie van Leeuwenhoek's microscopic examination of yeast cells, Louis Pastuer concluded that microbial activities were the catalyst in winemaking. With the knowledge that yeast was responsible for the biotransformation of grape sugars (mainly glucose and fructose) into alcohol and carbon dioxide, winemakers could control the process from the vineyard to bottling plant. Pasteur also able to destroy unwanted souring bacteria. As a result, the quality and quantity of wine production were vastly improved. (Pretorius, 2000)

Among the many fermented fruit beverages in the world, grape wine is perhaps the most economically important fruit juice alcohol. Commercialization of this beverage by industrialization has made grape wine a very important beverage in many South American and African countries. In Thailand, grapes are not of that important crop but her rich tropical fruits can also be made into wine and are available all year round. (Anonymous, 2006a)

Generally winemaking follows a set of simple standard procedure n which fruit juice is inoculated with a yeast starter culture. The fermentation usually takes around one week. During the week most of the sugar is converted to ethanol, yeast cells and carbon dioxide. Yeast cells are then removed. After that a slower fermentation is allowed to develop flavor. Sometimes sugar is added in the initial fermentation to acquire the desired alcohol content or to achieve the desired flavor. (Ought, 1987)

Fermentation

Grape juice or fruit juice must is converted into wine by may be lactic acid bacteria. It can be fermented spontaneously by inoculating yeast starter culture. But the microorganism is not just only yeast during the fermentation period because many winemakers also malolactic fermentation is as a second fermentation of wine, which follows or simultaneously couples the alcoholic (yeast) fermentation. Typically lactic

acid bacteria *Leuconostoc oenos* will be added in wine and convert malic acid to lactic acid and CO_2. This decarboxylation reduces the acidity of the wine. The bacteria also modify the fruit flavor of wine and add some flavor compounds from their metabolism. Malolactic fermentation is an example of how the detailed understanding of the biochemical pathway of the conversion of malic to lactic acid and CO_2 helps to produce starter cultures with desired activity and helps the winemaker to guide the fermentation. Temperature, pH, and availability of other sources of energy affect the rate of malic acid utilization. The malolactic activity of a starter culture is determined by strain characteristics, growth conditions, and the method of preservation. The majority of aromatic compounds found in grape and grape wine have simpler structures. The contents of these organic compounds are organic acids esters, hydroxybenzene and terpene etc (Schreier, 1979; Sften *et al.* 1993).Furthermore, the odor of wine is due to four esters (ethyl acetate, isoamyl acetate, ethyl hexanoate and octanoate) along with two alcohols, (isobutyl and isoamyl alcohol) and acetaldehyde, all of which are fermentation products (Ferreira *et al.* 1995b; Rapp and Mandery, 1986; Perez *et al.* 2003). The volatile components of wine are considered as the basic fragrance when supplemented to the wine can improve the quality of wine (Avakyants *et al.*1981; Falque *et al.*1995).

Wine aging

After fermentation is completed and wine is racked several times to remove the largest solids, the young wine is usually rough, raw and "green" and needs to settle for a period of time. This aging can be done in neutral containers such as stainless steel, cement lined vats, old large casks, etc. or it can be done in small relatively new wood barrels which are not neutral, but which will influence the developing wine. (Anonymous, 2006b)

During aging of wine in tanks, the wine flavor might be further modified by various yeasts and bacteria. Some of these microorganisms considered as spoilage microorganisms. Depending on the type of microorganism and on the growth, desirable fruit flavors can be lost or masked by unpleasant aromas and taste. Under

good aging conditions, without spoilage microorganisms the wine continues to change due to chemical and biochemical conversions. Depending on wine style and personal preference an optimum combination of 1) fresh fruit flavors, 2) fermentation flavors, and 3) aging flavors are reached after as little as half a year and more than ten years (Anonymous, 2006c)

The general objectives of aging fall into four groups that may be termed subtraction, addition, carry over, and multiplication. Aging is traditionally slow and for flavor to be changed relatively few molecules of key compounds need be changed.

a. Subtraction

Some characteristics and conditions present at first racking need to be removed or diminished. Among these may be removal of the gassiness of the carbon dioxide of fermentation and of yeast effects on flavor and appearance. Harsh or "green" flavors may be present and need modification. Young wines may be excessively tart or tannic (astringent) and require adjustments.

b. Addition

An important group of effects sought in maturation and aging is the addition of further characteristics. Extraction of flavors from oak, development of color and flavor from oxidation, and development of bottle bouquets are members of this group. As a rule, such additions must be limited and subtle so as to complement rather than overshadow the wine's underlying flavors. Excessive oakiness, for example, has been frequently seen in mismanaged wines. Oxidation that is appropriate in sherries and other modernized wines would be highly excessive and undesirable in white table wines.

c. Carry over

As much as possible of the attractive grapey fruitiness and especially the varietals aromas and flavors should be retained and carried forward during maturation and aging. The same is true of the desirable vinous flavors from the wine fermentation. Aging may strengthen some such flavors as, for example, acid hydrolysis of their glycosides can augment the volatile terpenes of Muscat family varieties.

d. Multiplication, Complexity

For the majority of wines whose flavors do not depend primarily upon processing, the character of a young well-made example can be quite attractive and its quality high. Nevertheless, appropriate maturation and aging can contribute breadth, depth, and complexity and increase its value without overthrowing its fundamental nature. The effect can be likened to adding instrument to an orchestra even through the same score is played. Another analogy is adding a pinch of several spices rather than none or too much of to an elegant food recipe. Complexity and increased interest will result if properly done. The concert, the meal, or the wine will be more intriguing, less rapidly tiring, and less likely to satiate if it is complex and displays many facets to the senses.

Aging gives wine time to develop flavors, bouquet and odor. It is one of the most complex and necessary processes of winemaking, yet requires little more than a suitably chosen container and time. Wines may be aged in wood, aluminum, stainless steel or plastic containers (Maynard, 1981). During aging of wine in tanks and bottles, the wine flavor is further modified by various yeasts and bacteria.

While emptying the tanks, the risk of acetic bacterial development is very high. Moreover, a well-filled tank, or a wine without sugar or malic acid, is not a wine protected from spoilages. If the temperature is favorable, and there is not a sufficient level of active SO_2 and if there has been contamination, the quantity of dangerous microorganisms can multiply

Brettanomyces is widely distributed in winery environments. They produce high concentrations of volatile acids, esters, and the volatile phenols 4-ethylphenol (4EP) and 4-ethylguaiacol (4EG). These volatile phenols are largely responsible for off-flavors or taint associated with *Brettanomyces*.

Zygosaccharomyces is spoilage yeast that is tolerant of high sugar concentrations and is resistant to sorbate. It is commonly found throughout the winery environment and is often associated with grape juice concentrates that are used to adjust color and sugar in final wine blends. The yeast can cause turbidity and CO_2 gas in bottled wines.

Pichia is wild yeast that is often present at high levels on incoming fruit. *Pichia* can initiate fermentation, resulting in production of high levels of volatile acids, including acetic acid and ethyl acetate. This yeast has been associated with films formed in barrels and tanks during storage.

Hanseniaspora *Hanseniaspora* is wild apiculate yeast that is often present at high levels on incoming fruit. *Hanseniaspora* can initiate fermentation in the must and produce high levels of volatile acids, including acetic acid and ethyl acetate. It has been associated with acid rot in grapes infected by *Botrytis cinerea*. Population levels usually decline as alcohol concentration increases.

Pediococcus is one of the common malolactic bacteria found in wine. They may produce polysaccharides that cause undesirable texture defects. *Pediococcus* are unusually adept at generating biogenic amines, such as histamine putrescine and cadaverine. . Although biogenic amines are not currently regulated in the United States, legislation in the European Union, Australia and Switzerland is a forewarning of future export hurdles.

Lactobacillus is another malolactic bacteria commonly found in wine. They may produce high concentrations of diacetyl often causing undesired buttery flavors. *Lactobacillus* is also notorious for producing acetic acid in a short period of time – often in a matter of a few days. This can occur readily during sluggish or stuck fermentations. These bacteria have also been implicated in the production of mousy flavor.

Acetic acid bacteria are commonly associated with grapes and the winery environment. The two groups of acetic acid bacteria detected are *Gluconobacter* and *Acetobacter*. These bacteria can generate acetic acid in the absence of SO 2 and in the presence of oxygen. These organisms can cause elevated volatile acidity in wines exposed to air. The presence of acetic acid bacteria indicates that wine conditions may support the growth and activity of other spoilage yeast and bacteria. (Anonymous, 2006c)

The above microbe can be produce milligrams of products, particularly sensorially negative, even before the evolution of volatile acidity confirms their activity. Depending on the type of microorganism and on the extent of growth, desirable fruit flavors can be lost or masked by unpleasant aromas and taste. Under good aging conditions, without spoilage microorganisms the wine continues to change due to chemical and biochemical (enzymes still active in the wine) conversions. Depending on wine style and personal preference an optimum combination of 1) fresh fruit flavors, 2) fermentation flavors, and 3) aging flavors are reached after as little as half a year and more than 10 years (Jackson, 2005). In this project, the group of microorganism during aging period that interesting is *Brettanomyces* yeast.

***Brettanomyces* yeast**

The desirable or otherwise of the wine character known as "Brett" is one of the most controversial issues of recent times. Arguments have been made for Brett character being a complexing and a legitimate expression of natural, uncomplicated winemaking, while others view it simply as an unattractive wine fault that results from poor winery hygiene and sloppy winemaking. The wine character described as "Bretty" comes in various forms. It is the combined result of the creation of a number of compounds by the yeast *Brettanomyces bruxellensis*, and its close relative, *Dekkara bryxulensis*. The three most important known aroma active compound are 1) 4-ethyl phenol (4-ep), which has been variously described as having the aromas of Band-aid, antiseptic and horse stable 2) 4-ethyl guaiacol (4-eg) which has a rather pleasant aroma of smoked bacon, spice or cloves and 3) isovaleric acid which has an unpleasant smell of sweaty animal, cheese and rancidity. Other characters associated with Brett with include wet

dog, creosote, brunt beans, rotting vegetation, plastic and (but not exclusively caused by Brett) mouse cage aroma and vinegar (Gawel, 2004).

Identification

Taxonomic key for identification of bacteria

Identification of microorganisms is a systemic process based on the morphological, cultural and physiological characteristics of the unknown microorganism. The Bergey's Manual of Systemic Bacteriology is the most widely accepted for the identification of bacteria. It was compiled by over 200 specialists from 19 countries. (Benson, 1998)

Taxonomic key for identification of Yeast

Yeast is unicellular fungi. The precise classification is a field that uses the characteristics of the cell, ascospore and colony. Physiological characteristics are also used to identify species. One of the more well known characteristics is the ability to ferment sugars for the production of ethanol. Budding yeasts are true fungi of the phylum *Ascomycetes*, class *Hemiascomycetes*. The true yeasts are separated into one main order *Saccharomycetales*.

Yeasts are characterized by a wide dispersion of natural habitats. They are commonly found on plant leaves and flowers, soil and salt water. Yeasts are also found on the skin surfaces and in the intestinal tracts of warm-blooded animals, where they may live symbiotically or as parasites. The common "yeast infection" is typically Candidiasis is caused by the yeast-like fungus *Candida albicans*.

Yeasts multiply as single cells that divide by budding (eg *Saccharomyces*) or direct division (fission, eg. *Schizosaccharomyces*), or they may grow as simple irregular filaments (mycelium). In sexual reproduction most yeasts form asci, which contain up to eight haploid ascospores. These ascospores may fuse with adjoining nuclei and multiply through vegetative division or, as with certain yeasts, fuse with other ascospores.

Yeast is a very well studied group of microoragnisms. A phenotype producing gene can be quickly mapped to a region of the S. cerevisiae genome. S cerevisiae has been a model system for the studies of molecular genetics, because the basic cellular mechanics of replication, recombination, cell division and metabolism are generally conserved between yeast and larger eukaryotes, including mammals.

Strains of *Saccharomyces cerevisiae* and related yeast are the most well known and commercially significant yeasts. These organisms have long been utilized to ferment the sugars of rice, wheat, barley, and corn to produce alcoholic beverages and in the baking industry to expand, or raise, dough. *Saccharomyces cerevisiae* is commonly used as baker's yeast and for some types of fermentation. Yeast is often taken as a vitamin supplement because it is 50 percent protein and is a rich source of B vitamins, niacin, and folic acid. In brewing, *Saccharomyces carlsbergensis*, named after the Carlsberg Brewery in Copenhagen, where it was first isolated in pure culture by Dr. Emil Christian Hansen in 1883, is used in the production of several types of beers including lagers. *S. carlsbergensis* is used for bottom fermentation. *S. cerevisiae* used for the production of ales and conducts top fermentation, in which the yeast rise to the surface of the brewing vessel. In modern brewing many of the original top fermentation strains have been modified to be bottom fermenters. Currently the *S. carlsbergensis* designation is not used; the *S. cerevisiae* classification is used instead. (Anonymous, 2005b)

Today's technology allows us to identify any living thing to any level of precision - by using a single cell. This technology uses molecular probes that bind only to the DNA of targeted organisms, is based on gene amplification that multiplies a million fold the DNA of interest, and involves comparing specific pieces of DNA between organisms. Molecular identification has two important advantages over conventional techniques of microscopic examination. Identification can be made using a very small amount of material, and is much more accurate than with previous methods a species, a population or even an individual can be identified. (Vossbrinck, 1991)

This research aims to study the microbial diversity in the fruit wine (from lychee and grape) produced from class BT 3015 in ABAC pilot plant. The diversity of microbes in

aging wine will be a good indicator for quality of wine. This project leads to the isolation of the microbes this may involve in the aging period and contribute to the quality of finished wine. Therefore this research will help us to understand more about wine produce in our pilot plant and enable the development of the process for the production of high quality wine.

Equipments and Reagents

I. Equipments

- Analytical balance (Ohause, Analytical plus AP 210S)
- Autoclave (Hirayama, Model HA 300 M II)
- Incubator (Jouan, EB 280)
- Laminar Flow (Dwyer Mark II, "Clean" Model H2)
- Microscope (Nikon, SMZ-1)
- Various glassware (Pyrex)

II. Reagents

- White wine, Year 2004 and 2005 (from BT 3015, Biotech Pilot Plant, Assumption University)
- Red wine, year 2004 and 2005 (from BT 3015, Biotech Pilot Plant, Assumption University)
- YM agar and YM broth (ingredient from Himedia Laboratories Pvt Limited)
- YPD agar and YPD broth (ingredient from Merck company)
- NA and NA broth (ingredient from Himedia Laboratories Pvt LImited)
- YNB medium
- WL agar (ingredient from Merck company)
- 1% Cycloheximide solution (Metha Tradding company)
- Phenol red
- MR-VP medium

- 3 % hydrogen peroxide
- Urea broth
- SIM medium
- Sporulation medium
- Clinitest reagent tablets (from Bayer Diagnostics Aust. Pty. Ltd.)

Procedure

I. Wine analysis

Wine samples were analysis for the following quality;

- pH
- Total soluble solid using refractometer
- % Reducing sugar using clinitest
- % Alcohol using ebulliometer

II. Media preparation

Preparation media according to procedure in Microbiology Media, Ronald M. Atlas, Lawrence C. Parks

III. Isolation of the microbes in wine

Wine sample (100 ml) were taken from Biotech Pilot Plant, Assumption University which are white wine, Year 2004 and 2005 and red wine, year 2004 and 2005. These samples were produced as small project in BT 3015 (industrial fermentation) class. Spread plate on five replicates of each agar which is 1) WL agar 2) WL agar + 1% cycloheximide 3) YM agar 4) YPD agar and 5) NA and incubated at room temperature for 24-48 hours. The single colonies from each sample were selected and moved to similar media in the slant type except the single colonies on WL Agar and WL agar + 1% cycloheximide were moved to YM slant instead. The isolated stains were kept in – 20 °C freezer by using glycerol.

III. Morphological Test

The isolated stains were observed under microscope by using two methods which are gram stain, wet mouth, spore stain for bacteria, and sporulation test for yeast (Benson, 1998) in order to classify the type of microbe.

IV. Biochemical Test

The biochemical test was separated into two parts which are biochemical test for bacteria and biochemical test for yeast.

- Biochemical test for bacteria (Benson, 1998)
  - Phenol red broth + carbon source (1% glucose, 1% sucrose, 1% maltose, 1% mannose and 1% lactose)
  - Durham Tube Sugar Fermentations
  - Carbon source (Glucose, Maltose, Mannitol, Lactose, and sucrose)
  - Citrate, Simmons
  - Vodes-Proskauer Test or VP test
  - Methyl Red Test or MR test
  - Catalase Production
  - Hydrogen Sulfide Production
  - Spore staining

- Biochemical test for Yeast
  - YNB + carbon source (1% glucose, 1% sucrose, 1% maltose, 1% mannose and 1% lactose)
  - Vary Temperature by using YNB+ 1% glucose as a medium
    - 4°C
    - Room temperature
    - 40°C - 45°C
  - YNB + 1% glucose + 1% cycloheximide
  - Urea hydrolysis (Benson, 1998)
  - Sporulation Test (Benson, 1998)

V. Identification

The sample was identify by using taxonomic key for both bacteria and yeast

Result and Discussion

I. Wine characteristic and strain isolation

There were four types of wine which were White wine 2004, White wine 2005, Red wine 2004, and Red wine 2005. White wine was produced as experiment in BT3015 class from lychee and Red wine from grape. The characteristics of wines samples are shown in table 1.

The pH of all four samples were not very much different (pH 4.6 – 5.1). Generally wine has pH values range from 2.9 to 4.2. Wine's chemical and biological stability are very dependent on pH value. Lower pH values are known to improve the stability, so winemakers usually prefer a pH range of 3.0 to 3.5. The wine is so stable in this range that many winemakers believe pH is a crucial guideline in wine making whereas the pH of these samples were higher than 4.2 which was higher than the usual range of pH in wine. The risk of contamination with other microorganisms was also higher which may led to the isolation of many microorganisms in these samples. The low pH was occurred by the present of lactic acid bacteria. Most winemakers maintain low pH in wine by adding lactic acid bacteria, so these wine samples might not have or have a little of lactic acid bacteria. Moreover, there are many advantages to low pH values in wine. Low pH inhibits bacteria, causes sugar fermentation to progress more evenly and makes malolactic fermentation easier to control. (Pandell, 1999)

The total soluble solid (TSS) of sample W2 was the highest (7.5 ^{o}Brix). Sample W1 and sample R2 had the same amount of TSS which was 5^{o}Brix as the least value and TSS of Sample R1 was 6.8 ^{o}Brix. The percentage of reducing sugar was also determined and the result showed that sample W2 had the highest percentage of sugar which was higher than 2 %(w/v). The percent sugar of sample W1 and sample R2 was equal at 0.25 %(w/v) while sample R1 has 0.75%(w/v) sugar. Therefore, the ^{o}Brix had given the correspond data with % reducing sugar. Three types of wine were classified according to reducing sugar; (1) Dry wines which reducing sugar is less than 1%, (2) Semi dry wines which reducing sugars is 2-5%,

and (3) Sweet wines which reducing sugars are higher than 5%. Therefore, the reducing sugars of sample W2 was over 2%w/v which was classified as semi dry wines, sample R1 ,sample W1 and sample R2 were dry wines because the reducing sugar was less than 1%. The higher numbers of microorganisms was isolated in high reducing sugar. The highest percent reducing sugar was from sample W2 which contained the highest number of isolated strain.

The number of isolated strains from sample W2 were 11 with all strains able to recovery after collected under -20°C with 15% (v/v) glycerol. Whereas the 33% lose was shown in sample W1 which 3 isolated strains was found but only 2 strains could be recovered. The 9 strains were isolated from sample R2 while 8 strains could be recovered so the percent loss was 11%. The percent loss of strain in sample R1 was the highest (25%) since 6 of 8 isolated strains could be maintained. As a result, some microorganisms loss may be because of the weakness of those microorganisms. After preserved microorganisms in freezer, those microorganisms could not survive further. They may require ethanol as a nutrient source while the isolation media was not composed of ethanol. Moreover, those microorganisms might be fastidious microorganisms which have unusual and/or complex nutritional needs and must be grown on enriched media (Anonymous, 2005c).

Table 1: The characteristic of wine, number of isolated strain and number of recovered cell in four types of wine which were White wine 2004 (W2), White wine 2005 (W1), Red wine 2004 (R2), and Red wine 2005 (R1)

Sample	pH	% Alcohol /volume	°Brix	% Reducing sugar (w/v)	No. of isolated strain	No. of recovered strain	% lose
(W2)	4.8	10.5	7.5	over 2	11	11	0
(W1)	4.6	13.9	5	0.25	3	2	33
(R2)	4.9	13.3	5	0.25	9	8	11
(R1)	5.1	10.3	6.8	0.75	8	6	25

Microorganisms in wine making can be either natural culture or pure culture. In the process produced from class in ABAC pilot plant, KMS was added into the fruit juice and only *Saccharomyces cerevisiae* was added for fermentation as the starter culture. After fermentation wine was clarify by using diatomite as filter aid in order to get rid of the small sediment before aging at 14°C in plastic vat with minimal headspace. Although during fermentation process only *Saccharomyces cerevisiae* was added but there were many types of microorganisms which could be found from these wines such as *Zygosaccharomyces*, *Pediococcus*, *Lactobacillus, Pichia* , and *Brettanomyces* (Anonymous, 2006c). These strains may be natural cultures which attached at the surface of fruit or stayed in the mature fruit. Therefore the presented microorganisms may tolerant to sulfur or, moreover, the unhygienic of winemaking process can also led to the contamination.

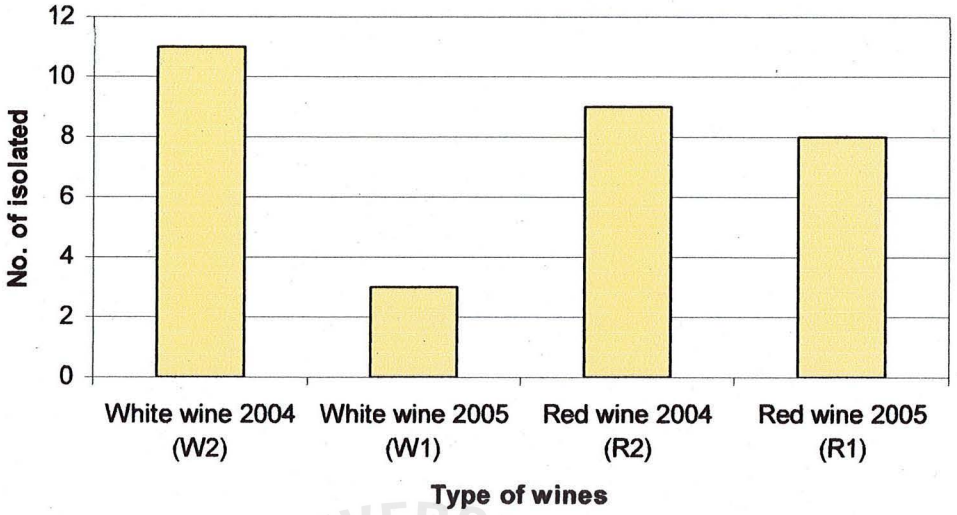


Figure 1: The number of isolated strains in each type of wine: White wine 2004, White wine 2005, Red wine 2004 and Red wine 2005

II. Identification of isolated stains from wine sample

2.1 Bacterial strains

In this experiment, the morphology and some biochemical test such as growth in different sugar were observed however, this only leads to the identification of genus of microorganisms. Usually two groups of bacteria play prominent roles in winemaking which are the acetic acid bacteria represented by *Gluconobacter* and *Acetobacter* may play an early role in grape quality and secondarily, in the case of *Acetobacter*, in stored wine stability. Growth in this case is clearly undesirable. The second major group of bacteria, the lactic acid bacteria (LAB), is represented by the three genera *Lactobacillus, Leuconostoc*, and *Pediococcus* sp. whether their growth is viewed as positive or negative depends on winemaker philosophy, wine chemistry, and the organism involved (Fugelsang, 1997).

The 9 strains of bacteria were isolated from four samples. Sample W1only had 1 strain isolated. There were 3 strains from Sample W2 whose strain number was 6.2, 8, and 25. Strains number 23, 24, and 28 were isolated from sample R1 and 2 strains, strain number 21 and 26, were isolated from sample R2. The biochemical testing was

also conducted in order to identify the genus of each bacterium by using Berkey's manual of Systematic Bacteriology and its results were shown in appendix.

From the morphology and biochemical test, it could classify the genus of all nine bacteria strains into three groups which were *Enterobacter*, *Gluconobacter* and unidentified genus. The gram characteristic of all isolated bacteria strain in white wine were shown in Figure 2 and red wine in Figure 3.

In the group that classified into genus *Enterobacter*, there were two strains. There common characteristic were facultative anaerobic gram-negative rod. Strain number 20 was spore-producing bacteria, and rod-shaped whose characteristic belonged in *Enterobacter*. Strain number 6.2 was also in *Enterobacter.* but it was not spore-producing bacteria and shorter rod. There were 2 species of *Enterobacter.* which were isolated from different samples, sample W1 and sample W2. It was identified by different morphology and biochemical testing which was shown in appendix. *Enterobacter.* is a common species in water and sewage as well as the intestinal tract of warm-blooded animals and is an occasional pathogen in urinary tract infections. Therefore *Enterobacter Sp.*, which was found in sample W1 and sample W2, might be contaminated by water that used to clean the container because some bacteria that belong to this genus can tolerant with KMS and also can live in high concentration of alcohol.

In the group that classified into genus *Gluconobacter*, there were also two strains. There common characteristic were gram-negative rod. *Gluconobacter*, the morphology of isolated bacteria of Sample R1 was rod. Strain number of 23 was specified as *Gluconobacter.* which was gram negative and spore-producing bacteria. Another sample could identify as *Gluconobacter.* was Strain number 26 from sample R2. It was spore-producing bacteria and its shape was medium rod. *Gluconobacter* is characterized by its incomplete oxidation of sugars or alcohols to acids, such as the oxidation of glucose to gluconic acid or ethanol to acetic acid. It lacks a complete citric acid cycle and it is unable to oxidize acetic acid. Therefore pH of wine should be low. According to table 1, pH of sample R1 and Sample R2 were not show lower in pH for sample R1 and sample R2 that the reason might be from during wine aging was processed under anaerobic condition which was not suitable to the growth of

Gluconobacter. *Gluconobacter* is aerobic bacteria so it is inactive under anaerobic condition which also affected the production of acetic acid which causes the decreasing of pH.

However, there were 5 strains which were number of 8, 24, 21 and 25 could not identify the genus. But both of strain number 8 and 25 were non-spored producing bacteria and short rod. The only difference between strain number 8 and 25 was gram's type. Stock number 8 was gram negative but strain number 25 was gram positive. The genus and sporulation of strain number 21 could not be identified but its morphology was rod. Stock number of 24 was gram positive rod and non spore-producing bacteria which could not identify as well.

After identification of bacteria in wines, the group of unidentified bacteria could not classified in the group of bacteria in wine or the results were not enough to specify those microorganisms. Therefore unidentified microorganisms might be contaminant and could cause spoilage in wine.

Table 2: The morphological characteristics and predicted genus of bacteria isolated from aging wine samples

Sample	Stock no.	Gram strain	Spore	Shape	Genus
W2	20	-	+	Rod	*Enterobacter Sp.*
W2	6.2	-	-	Short rod	*Enterobacter Sp.*
W2	8	-	-	Short rod	unknown
W2	25	+	-	Short rod	unknown
R1	23	-	+	Rod	*Gluconobacter Sp.*
R1	24	+	-	Rod	unknown
R1	28	-	-	Rod	unknown
R2	21	-		Rod	unknown
R2	26	-	+	Medium rod	*Gluconobacter Sp.*

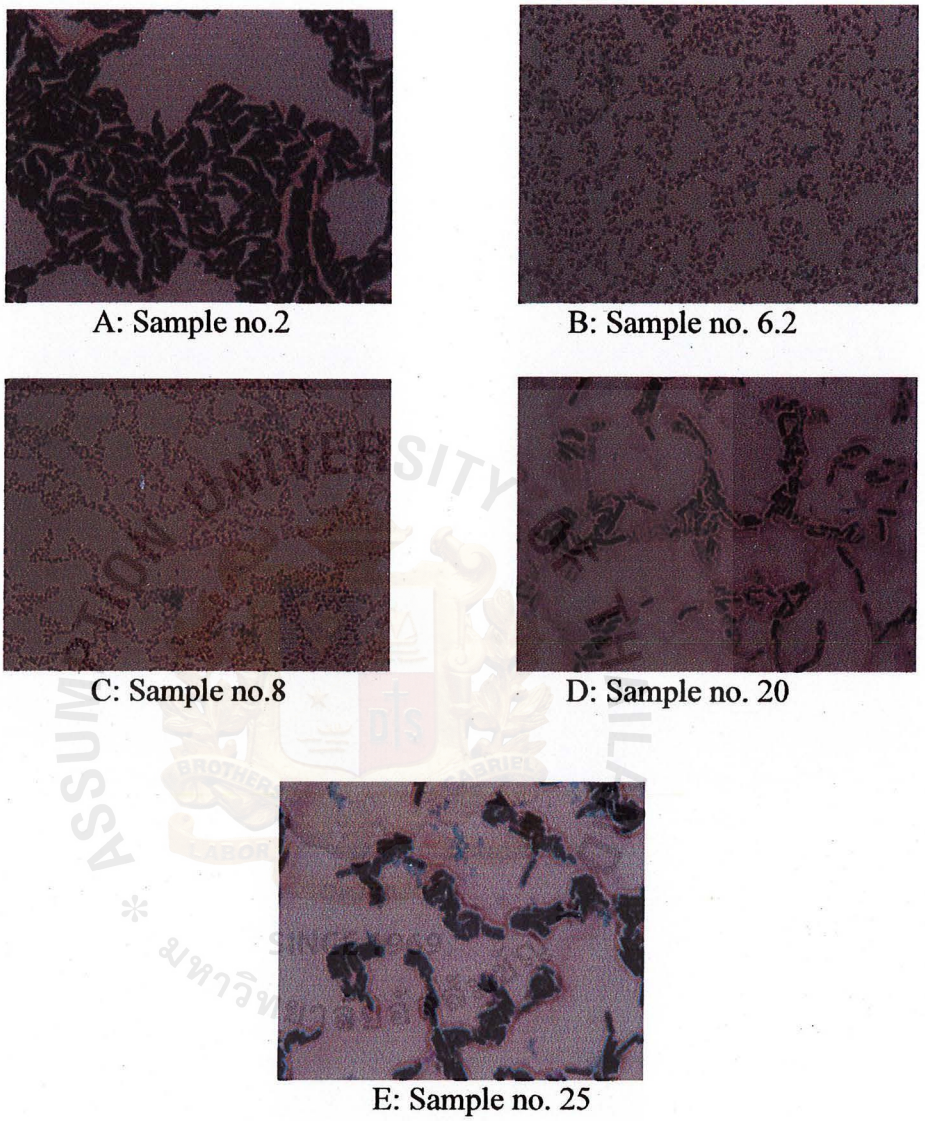


Figure 2: Gram characteristics of some bacteria from White wine (W2) 2004 and White wine (W1) 2005

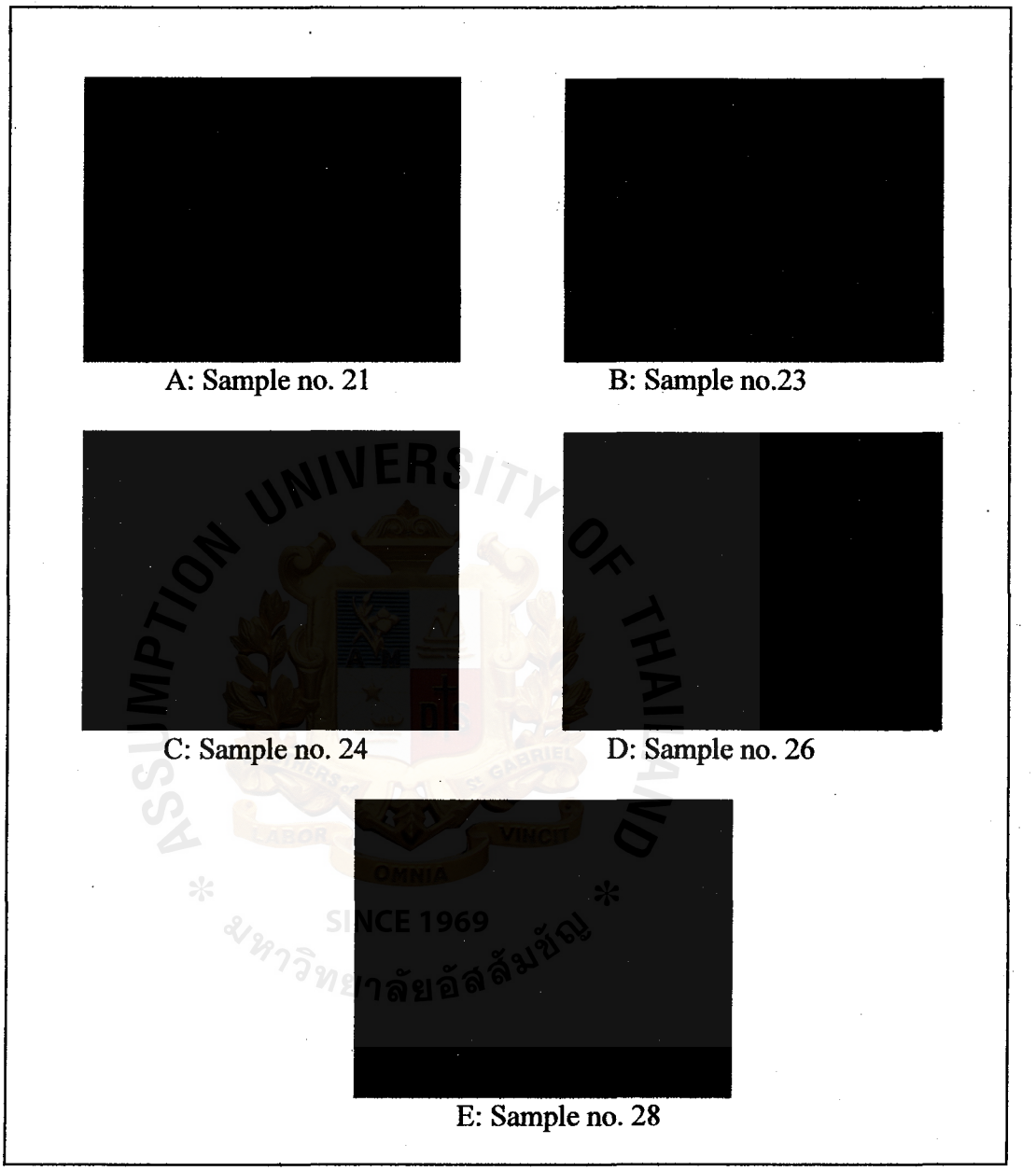


Figure 3: Gram characteristics of some bacteria from Red wine (R2) 2004 and Red wine (R1) 2005

2.2 Yeast strain

Several yeasts appear during the course of winemaking. These included the oxidative species, *Pichia* and *Candida*, which either do not ferment or are very weakly fermentative although capable of growth in musts and juice, they are important members of the film yeast community in stored wine. The weakly fermentative species *Hansenula anomala* and *Kloeckera apiculata* / *Hanseniaspora uvarum* are seem early in the course of fermentation and are levels of acetic acid and ethyl acetate. Fermentative species including *Brettanomyces* / *Dekkera, Schizosaccharomyces pombe, Saccharomyces, and Zygosaccharomyces,* are capable of complete, albeit in some cases, slow fermentation. Compared with already mentioned yeasts these species grow in sugar and alcohol rich environments and their documented presence in the vineyard is rare. *Saccharomyces cerevisiae* deserves specials mention amount of yeasts associated with fermentation. On the one hand, *Saccharomyces* plays a partnership role with the winemaker in transformation of sugar to alcohol during fermentation. But, on the other hand, it becomes a significant adversary where oxidative conditions permit its growth during cellar aging. In this letter role, *Saccharomyces* play an important role in the film yeast community (Fugelsang, 1997).

The table 3 showed the family of yeast which could be isolated from four types of wine. There were four types of media; WL, YPD, YM, and NA, which were used to grow yeast. The biochemical testing was also conducted in order to identify the class of each strain no. and its results were shown in appendix. Most of isolated yeast from strain number 1, 3, 5, 6.1, 7, 9, 10, 11, 12, 13, 14, 18, 19, and 22, was in the family of *Saccharomycetaceae*. Although *Saccharomyces* is in the family of *Saccharomycetaceae*, the biochemical test of them, which was shown in appendix was different which they could not be identified to be *Saccharomyces* at all. This class had to be found in wine because it was native yeast in wine for fermentation.

From the morphology and biochemical test, it could classify the genus of all seventeen yeast strains to be three groups which fourteen strains classify in the family of *Saccharomycetaceae,* one strain can classify in the family of *Candidaceae*, The

remaining two strains were unidetify. The wet mouth characteristic of all isolated yeasts in white wine were shown in Figure 4 and red wine in Figure 5.

Stock number of 30 from Sample W2 was *Brettanomyces* which could grow in WL media. Candidaceae is the genus which it could be identified rather confidently because the results of both biochemical and morphology were similar to *Brettanomyces*. *Brettanomyces* species of yeast are unique in that they are one of the new microorganisms that can grow in barrel aging or finished dry wines. Spoilage of such wines by these yeasts has become an increasing problem in recent years .It is one of the most complex and controversial yeast issues a winemaker encounters when making red wine. To some, even the slightest hint of *Brettanomyces* character is cause for a wine's rejection. To others, *Brettanomyces* is viewed as an integral part of red wine character, providing an essential dimension of complexity. (Chatonnet, *et al.* 1992). Areas which typically provide suitable niches for *Brettanomyces* are must lines, dirty crush equipment, wooden cooperage, or any tank or transfer line which is not cleaned effectively. There have also been suggestions that the fruit-fly can carry *Brettanomyces*.

There were two strain numbers, 15 and 16, which could not be identified. Both were non-spore forming and came from Sample R1 and grew on YPD medium. According to the result of the experiment, it was not enough information to indicate the group of those yeasts. All of unidentified yeast was non-spore producing yeast. In the experiment, the spore-forming was observed only 1 week while some spore-producing yeast takes more than 1 week for spore formation. Moreover, in the method of yeast's identification started with observing the capabilities of spore formation. If those yeasts were non-spore producing yeast, they were classified in the group of non-spore producing yeast. However, some groups of non-spore producing yeast can grow under the condition of wine process and aging.

As the identification of microorganisms in wine, most microorganisms, which were found in wine samples, did not give any benefit to wine but it could make wine to be a low quality wine because most identified microorganisms were contaminants. Therefore the quality of raw materials, processes, and wine makers have to be controlled in order to prevent the contamination. The sanitation has to be concerned

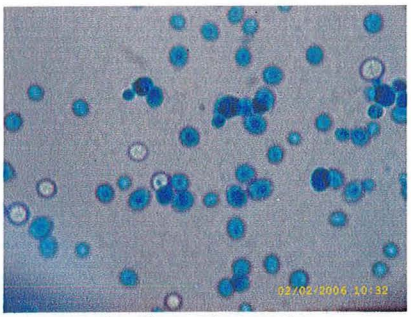
along the process of wine. Moreover, the condition of fermentation and aging should be controlled such as temperature. The causes of contamination can be that wine is lack of free sulfur dioxide; contamination is from fermentation tank.

However, these experiments were conducted to study the characteristics of isolated microorganisms in wine. These did not focused on the quantitative of microorganisms in wine and sensory evaluation was not involved in this project.

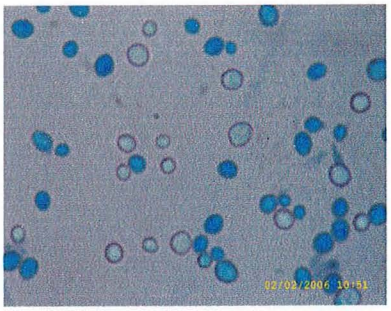
The further study of this experiment, the molecular taxonomic is required in order to identify the species of all isolated microorganism and this method can lead to identify the species of all isolated microorganisms.

Table 3: The morphological characteristics and predicted family of yeast isolated from aging wine samples.

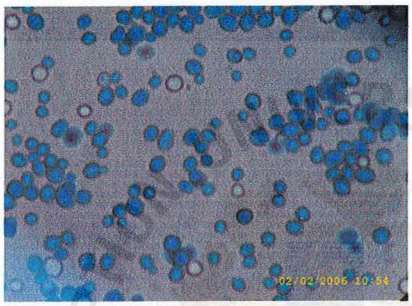
Stock no.	Sample	Agar	Type of microorganism	Spore	Group
1	R2	WL	Yeast	+	*Saccharomycetaceae*
3	W2	WL	Yeast	+	*Saccharomycetaceae*
5	W2	WL	Yeast	+	*Saccharomycetaceae*
6.1	W2	WL	Yeast	+	*Saccharomycetaceae*
7	W2	WL	Yeast	+	*Saccharomycetaceae*
9	R2	WL	Yeast	+	*Saccharomycetaceae*
10	R2	WL	Yeast	+	*Saccharomycetaceae*
11	R2	YPD	Yeast	+	*Saccharomycetaceae*
12	W2	YPD	Yeast	+	*Saccharomycetaceae*
13	W2	YPD	Yeast	+	*Saccharomycetaceae*
14	R2	YPD	Yeast	+	*Saccharomycetaceae*
15	R1	YPD	Yeast	-	unknown
16	R1	YPD	Yeast	-	unknown
18	R2	YM	Yeast	+	*Saccharomycetaceae*
19	W2	YM	Yeast	+	*Saccharomycetaceae*
22	R2	NA	Yeast	+	*Saccharomycetaceae*
30	W2	WL	Yeast	-	*Candidaceae* (*Brettanomyces sp.*)



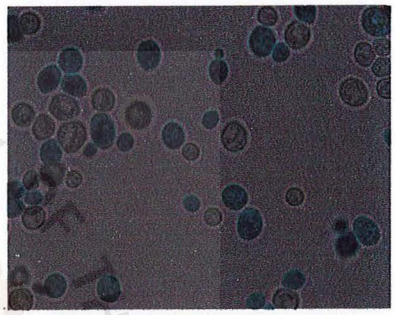
A: Sample no.3



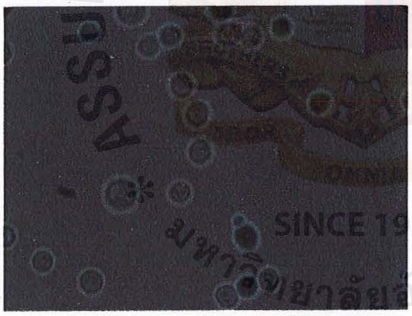
B: Sample no.5



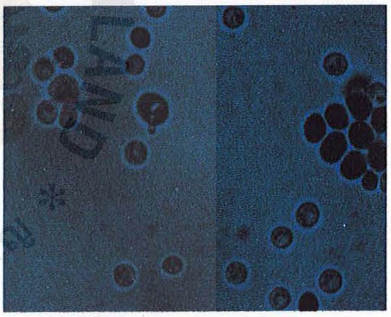
C: Sample no. 6.1



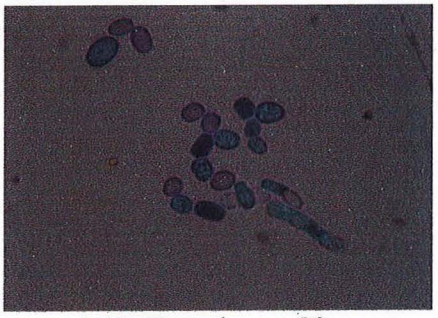
D: Sample no. 7



E: Sample no. 12



F: Sample no. 13



G: Sample no. 30

Figure 4: Wet mouth characteristic of some yeast in White wine (W2) 2004 and White wine (W1)2005

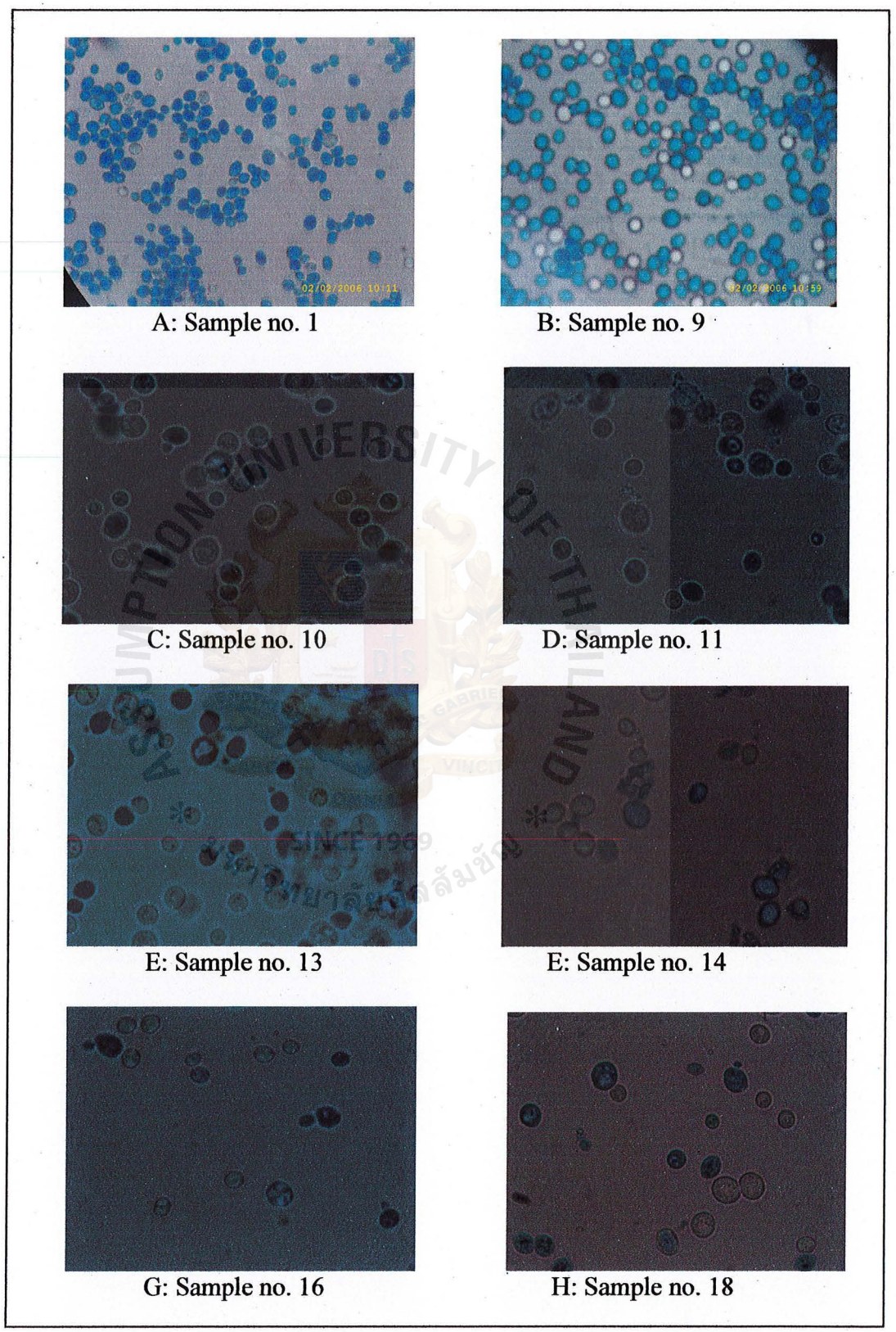


Figure 5: Wet mouth characteristic of some yeast in Red wine (R2)2004 and Red wine (R1) 2005

Conclusion

There were four types of wine which were White wine 2004, White wine 2005, Red wine 2004, and Red wine 2005. White wine was produced from lychee and Red wine was produce from grape. Thirty one strains were isolated from all sample and twenty-seven strains could maintain which can separate into two groups were bacteria and yeast. From the morphology characteristic, nine strains were classified as bacteria and seventeen strains were classified as yeast. Four from nine strains of bacteria could identify the genus which two strains were classified in the genus of *Enterobacter Sp.* and the other two were classify in the genus of *Gluconobacter Sp.*. Unfortunately, the five remaining bacteria strains could not identify the genus. Seventeen strains were classified as yeast and fourteen strains in the family of *Saccharomycetaceae* and one strain in the family of *Candidaceae*. The one strain that identified as member of Candidaceae was future analyzed using the result of morphology and some biochemical test, which lead to the prediction as member of *Brettanomyces* sp.. However, yeast also has two remaining strains that could not be identified and it need further study in molecular taxonomy for definite identify of all isolated strain.

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Appendixes

Appendix A:

Media

- WL Agar

WL Agar	g/liter
Yeast extract	4
Casitone	5
Dextrose	50
Monopotassium phosphate	0.55
Potassium Chloride	0.425
Calcium chloride	0.125
Magnesium sulphate	0.125
Ferric Chloride	0.0025
Manganese sulphate	0.0025
Bromcresol green	0.022
Agar	20

- YM agar

YM Agar	g/liter
Yeast extract	3
Malt extract	3
Peptone	5
Dextrose	10
Agar	20

Note: Autoclave at 121°C for 15 minutes.

Media

- NA

NA	g/liter
Yeast extract	2
Beef extract	1
Sodium chloride	5
Peptone	5
Agar	15

- YPD Agar

YPD Agar	g/liter
Yeast extract	10
Bacto Peptone	20
agar	20

- Make up volume to 950 mL and Autoclave
- Autoclave 40% Glucose(115°C, 10 minutes) for 2% YPD
- Add 40% Glucose to media

Note: Autoclave at 121°C for 15 minutes.

Appendix B:

Raw Data

Table 4: Number of isolated bacteria and yeast strains on four different agars from aging wine samples produced from BT 3015 class.

	No. isolated stain on WL			**No. isolated stain on YM**			**No. isolated stain on YPD**			**No. isolated stain on NA**		
Type of wine	Yeast	Bacteria	Total	Yeast	Bacteria	Total	Yeast	Bacteria	Total	Yeast	Bacteria	Total
White wine 2004 (W2)	5	2	7	1	-	1	2	-	2	-	1	1
White wine 2005 (W1)	1	1	2	-	1	1	-	-	-	-	-	-
Red wine 2004 (R2)	3	1	4	1	-	1	2	-	2	1	1	2
Red wine 2005 (R1)	1	2	3	1	-	1	2	-	2	-	2	2
	Total		16	Total			Total		6	Total		5

Table 5: The morphological characteristics and some of biochemical test of isolated bacterial strains

Stock no.	Sample	Agar	Type of m.o.	Gram strain	Shape	MR	VP	Citrate
2	W1	WL	Bacteria	-	Rod			
6.2	W2	WL	Bacteria	-	Short Rod	-	+	+
8	W2	WL	Bacteria	-	Short Rod	+	+	+
20	W1	YM	Bacteria	-	Rod	+	-	-
21	R2	NA	Bacteria	-	Rod	+	-	-
23	R1	NA	Bacteria	-	Rod	-	+	+
24	R1	NA	Bacteria	+	Rod	+	-	-
25	W2	NA	Bacteria	+	Short Rod	+	+	+
26	R2	WL	Bacteria	-	Rod	+	+	+
28	R1	WL	Bacteria	-	Medium Rod	+	-	-

Table 5 (continue)

Stock no.	sucrose		glucose		lactose		maltose		mannitol		Hydrogen sulfide	citrate	
	color	gas	color	gas	color	gas	color	gas	color	gas		slant	deep
2													
6.2	++	++	++	+	++	+++	++	++	++	+++	-	+	+
8	++	++	++	+++	++	+++	++	++	++	+++	+	+	+
20	+	-	+	-	+	-	++	++	+	-	-	-	+
21	++	-	+	+	++	++	+	-	-	-	-	+	+
23	++	++	++	+++	++	++	++	++	++	+++	-	+	-
24	++	++	++	+++	++	+	++	+++	++	++	-	+	+
25	++	-	++	-	++	-	++	-	++	-	-	+	+
26	++	-	++	-	++	-	++	-	++	-	-	+	-
28	++	-	++	-	++	-	++	-	++	++	-	-	-

Table 6: The morphological characteristics and some of biochemical test of isolated yeast strains

Strain no.	Sample	Agar	Type of m.o.	Room temp.	4 C	40-45 C	Cyclohexamide	Urea
1	R2	WL	Yeast	+	++	++	-	-
3	W2	WL	Yeast	+	++	++	-	-
5	W2	WL	Yeast	+	++	++	-	-
6.1	W2	WL	Yeast	+	++	-	-	-
7	W2	WL	Yeast	+	++	-	-	-
9	R2	WL	Yeast	+	++	-	-	-
10	R2	WL	Yeast	+	++	-	-	-
11	R2	YPD	Yeast	+	++	-	-	-
12	W2	YPD	Yeast	+	++	-	-	-
13	W2	YPD	Yeast	+	++	-	-	-
14	R2	YPD	Yeast	+	++	-	-	-
15	R1	YPD	Yeast	+	++	-	-	-
16	R1	YPD	Yeast	+	++	-	-	-
17	R1	YM						
18	R2	YM	Yeast	+	++	-	-	-
19	W2	YM	Yeast	+	++	-	-	-
22	R2	NA	Yeast	+	++	-	-	-
27	W1	WL	Yeast					
29	R1	WL	Yeast					
30	W2	WL	Yeast	- (film)	++	- (film)	+ (film)	+

Table 6 (continue)

Stock no.	glucose	sucose	maltose	mannitol	lactose	spore
1	+	++	+	-	+	+
3	+	++	+	-	+	+
5	+	++	+	-	+	+
6.1	+	++	+	-	+	+
7	+	++	+	-	+	+
9	+	++	+	-	+	+
10	+	++	+	-	+	+
11	+	++	+	-	+	+
12	+	++	++	+	+	+
13	+	++	++	+	+	+
14	+	++	++	-	-	+
15	+	+	+	-	-	-
16	+	++	++	+	+	-
17						
18	+	++	++	+	+	+
19	+	++	++	+	+	+
22	+	++	+	+	+	+
27						
29						
30	- (film)	- (film)	- (film)	- (film)	- (film)	-