GROWTH CHARACTERISTICS OF *DEKKERA BRUXELLENSIS* DURING GRAPE WINE FERMENTATION

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

Growth Characteristics of *Dekkera bruxellensis*

during Grape Wine Fermentation

Presented by Yike Chen

A candidate for the degree of Master of Science,

And hereby certify that, in their opinion, it is worthy of acceptance.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1 Introduction

Wine is an alcoholic beverage usually made from fermented fruit juice, especially from grapes. Theoretically, anything that contains sugar can be fermented into wine. Grapes can be fermented by different types of yeast after crushing and filtration. Now wine consumption has become much larger than beer around the world as reported by the World Health Organization. Two basic metabolic pathways, respiration and fermentation, can be used by the wine yeast, *Saccharomyces bayanus*, during the winemaking process. The fermentation of glucose, which is an anaerobic pathway, occurs primarily when the glucose concentration is high or when oxygen is not available. The stoichiometry of this reaction is: $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$.

The wine industry used to be craft that needed a lot of experience. Today, more and more wineries around the world are built to produce a huge amount of wine to meet its high consumption. Some of them use huge fermenters, which are very different from traditional winemaking, to get larger production from each batch of fermentation. It is not hard to imagine the great economic losses even if only a single batch is spoiled. Thus, these changes require modern winemakers to pay enough attention to the wine spoilage problem. Also, the large fermenters used in current wineries always have temperature, pH and dissolved oxygen sensors, which are helpful to monitor and control most of the factors online during the fermentation process. Therefore, the main purpose of this work, which is to control the wine spoilage problem, is justified.

The yeast species *Dekkera bruxellensis*, along with its anamorph *Brettanomyces bruxellensis*, is reported to be responsible for the mousy off-flavor in wine. Thus, this

research was aimed at minimizing the spoilage yeast population by controlling variables during fermentation. An unculturable state of the spoilage yeast was observed during this work. This viable but unculturable state also occurs with the presence of sulfur dioxide, which is the most widely used chemical preservative in wines. The wine spoilage problem could be mitigated if the unculturable state can be induced during fermentation.

This work aimed to investigate the influence of several factors that may affect the state of the spoilage yeast. There were two more specific objectives: 1) to develop a method to determine the individual yeast concentrations in a co-culture of wine and spoilage yeasts, and 2) to determine fermentation conditions that affect the culturability of the spoilage yeast.

Chapter 2 Literature Review

Winemaking has a long history dating back to almost at the beginning of the human society. During this process, raw materials and other environment conditions are of importance to making a good wine. Wine products have become the most popular alcoholic beverage all around the world not only because of its special flavor but also certain health benefits (Kirs-Etherton, et al. 2002). This unique combination is because of the coexistence of both alcohol and antioxidants. Biological antioxidants are believed to provide longevity that will have somehow influence on degenerative diseases, such as Parkinson's and Alzheimer's disease. In this chapter, the basic concepts of winemaking are reviewed. One of the major issues, the wine spoilage problem, is discussed in terms of its cause and influence.

2.1 Wine Fermentation Process

Wine fermentation, or vinification, is a very complex process involving various kinds of metabolic pathways. Generally speaking, microbes, usually fermentative yeasts, utilize sugar and other nutrition in grape must to produce a highly flavored alcoholic beverage. During this process, different enzymatic reactions are used by the yeasts to acquire energy and obtain precursor molecules and reducing energy for cell growth, preservation and propagation. In the meantime, ethanol is accumulated, as well as some volatile and non-volatile compounds, which will finally contribute to the wine sensory profile (Moreno and Polo 2009).

A lot of research related to the metabolic pathways of the yeasts of the genera *Saccharomyces* during wine fermentation has been conducted since it was realized that *S. cerevisiae* could be a very useful model eukaryotic microorganism in genetic engineering (Alexandre and Charpentier 1994, Beltran, et al. 2002, Barnett 2003). Three major pathways are involved in the wine fermentation process as discussed in the following paragraphs.

2.1.1 Glycolysis

One of the most important pathways in the process is the glycolytic pathway, which is also the main approach used for sugar metabolism. This pathway, first studied in 1940, can be seen as the beginning of the fermentation process (Kresge, Simoni and Hill 2005). Figure 2.1 shows all the eleven reactions involved in the pathway.

This biochemical pathway is the initial process to convert hexose into pyruvate, which can be transformed into ethanol and carbon dioxide as final products by fermentation. At the end of glycolysis, two molecules of pyruvate, four of ATP and one of NADH are produced per molecular hexose in total (Barnett 2003). Therefore, glycolysis only generates two ATPs since two molecules of ATP are consumed previously during the phosphorylation process (Moreno and Polo 2009).

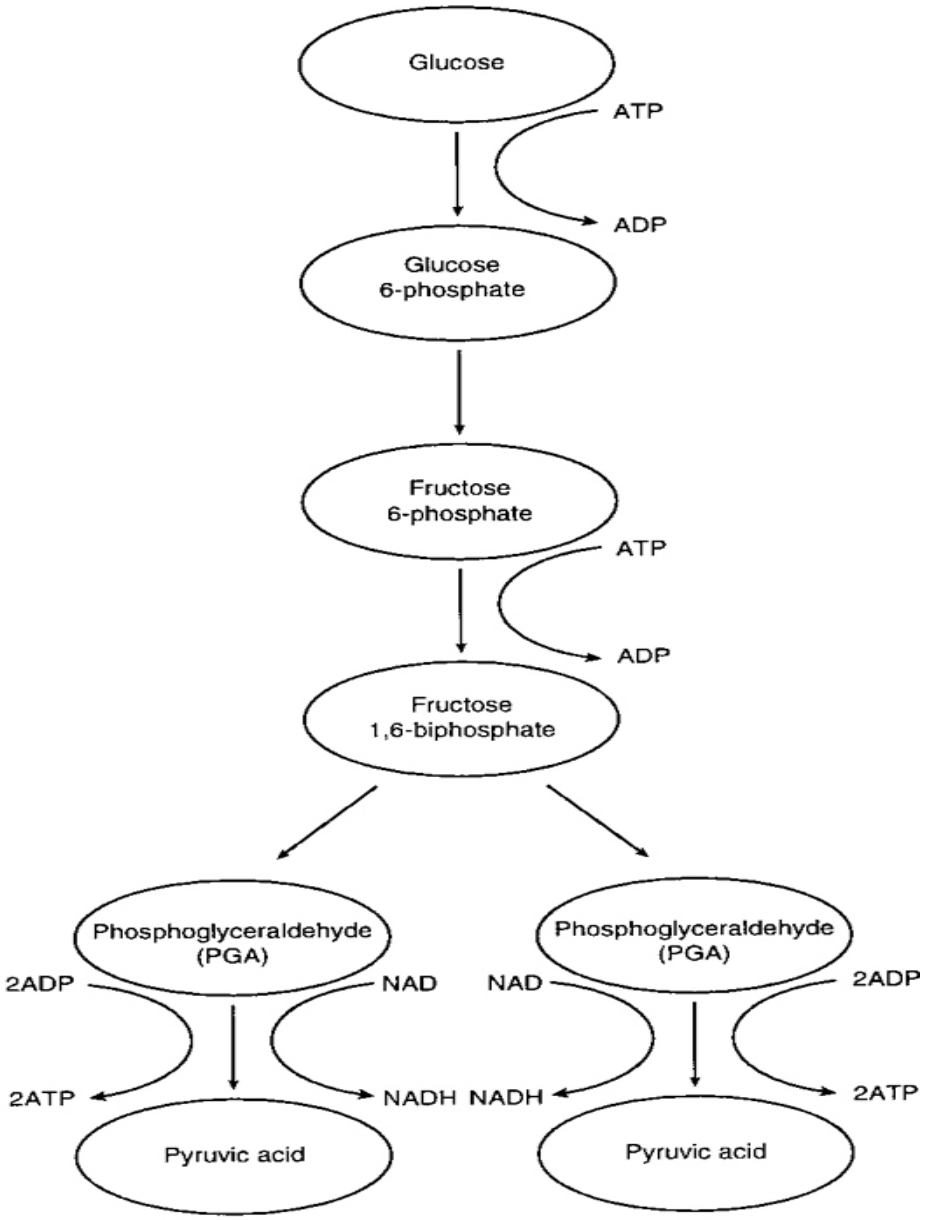


Figure 2.1 Biochemical mechanism of glycolysis (Kent 1998)

2.1.2 Respiration and fermentation

After glycolysis generates pyruvate, yeasts are able to metabolize it either aerobically or anaerobically, which are respiration and fermentation respectively (Boulton, et al.

1996). Figure 2.2 illustrates some main biochemical reactions in both metabolic pathways, and Table 2.1 lists some major differences between aerobic and anaerobic pathways.

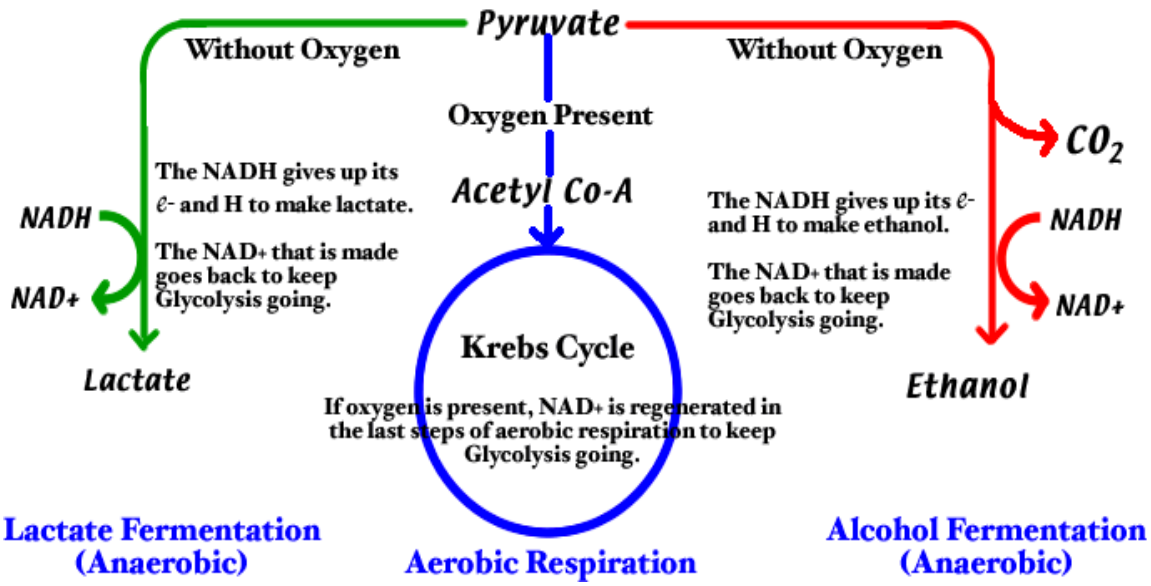


Figure 2.2 Fermentation and respiration (http://science.halleyhosting.com/)

Typically, the aerobic process can generate 36 or 38 ATP molecules per metabolized hexose, and consequently is more efficient than anaerobic fermentation, which gives only two ATP molecules in total. Therefore, the regulation, especially the enzyme for the reaction of pyruvate into ethanol or acetyl-coA, becomes a main concern for wine fermentation.

Table 2.1 Major differences between respiration and fermentation by *S. cerevisiae* (Moreno and Polo 2009)

Respiration	Fermentation
• efficient (38 ATP)	• inefficient (2 ATP)
• performed by eukaryotic cells	• performed by prokaryotic and eukaryotic cells
• goes from cytosol to mitochondria	• only in cytosol
• uses oxygen as electron acceptor	• does not use oxygen

Louis Pasteur first observed the regulation process in 1861 (Racker 1974). He found that aerobic process increases biomass production while decreasing ethanol formation. Therefore, he concluded that aeration inhibits alcoholic fermentation, which is later known as the Pasteur Effect. Along with the development of modern biochemistry, scientists have explained this phenomenon: the respiration process needs much more ADP as a receptor for oxidative phosphorylation than fermentation as calculated above, which consumes lots of ADP and inorganic phosphate in the cytoplasm. The unbalanced ATP-ADP concentration may decrease the sugar transportation inside the cell (Barnett and Entian 2005).

Carbon dioxide is created continuously once the yeast starts to consume sugar as shown in the Figure 2.2. Therefore, oxygen is displaced by carbon dioxide in grape juice and creates a semi-anaerobic environment, where alcoholic fermentation takes place (Moreno and Polo 2009).

Even in the presence of oxygen, however, *S. cerevisiae* will not metabolize sugar by respiration if the sugar concentration is higher than 9 g/L (Meijer, et al. 1998). This phenomenon was first presented by Crabtree in 1929 and was defined as “catabolic repression by glucose” or “the Pasteur contrary effect”. Usually, at the beginning of wine fermentation, the sugar concentration is much higher than the repression level, and oxygen is depleted quickly after the yeast begins to grow, *S. cerevisiae*, therefore, can only catabolize sugar by fermentation under wine conditions consequently. The real respiration process, which is under comparatively low sugar concentration and high oxygen content, is only used in producing selective dry wine yeast.

In conclusion, the picture of yeast metabolism pathways is relatively clear, although some parts are still less so. The generation of other by-products is still under investigation, including some volatile and non-volatile compounds. Under wine fermentation condition, fermentative microorganisms tend to utilize the alcoholic fermentation pathway to convert sugar into ethanol and carbon dioxide.

2.2 Wine Sensory Analysis

It is always hard to evaluate wine products. Even in some European countries that have quite long histories of wine fermentation, experts can only distinguish a few wine characteristics. The descriptors that are used to specify aroma and flavor of wines are listed in the aroma wheel in Figure 2.3. Problems may occur when wine products are consumed by individuals because of their different preferences. However, highly trained experts are still valuable to wineries because they can interpret detailed sensory properties and make sound judgments.

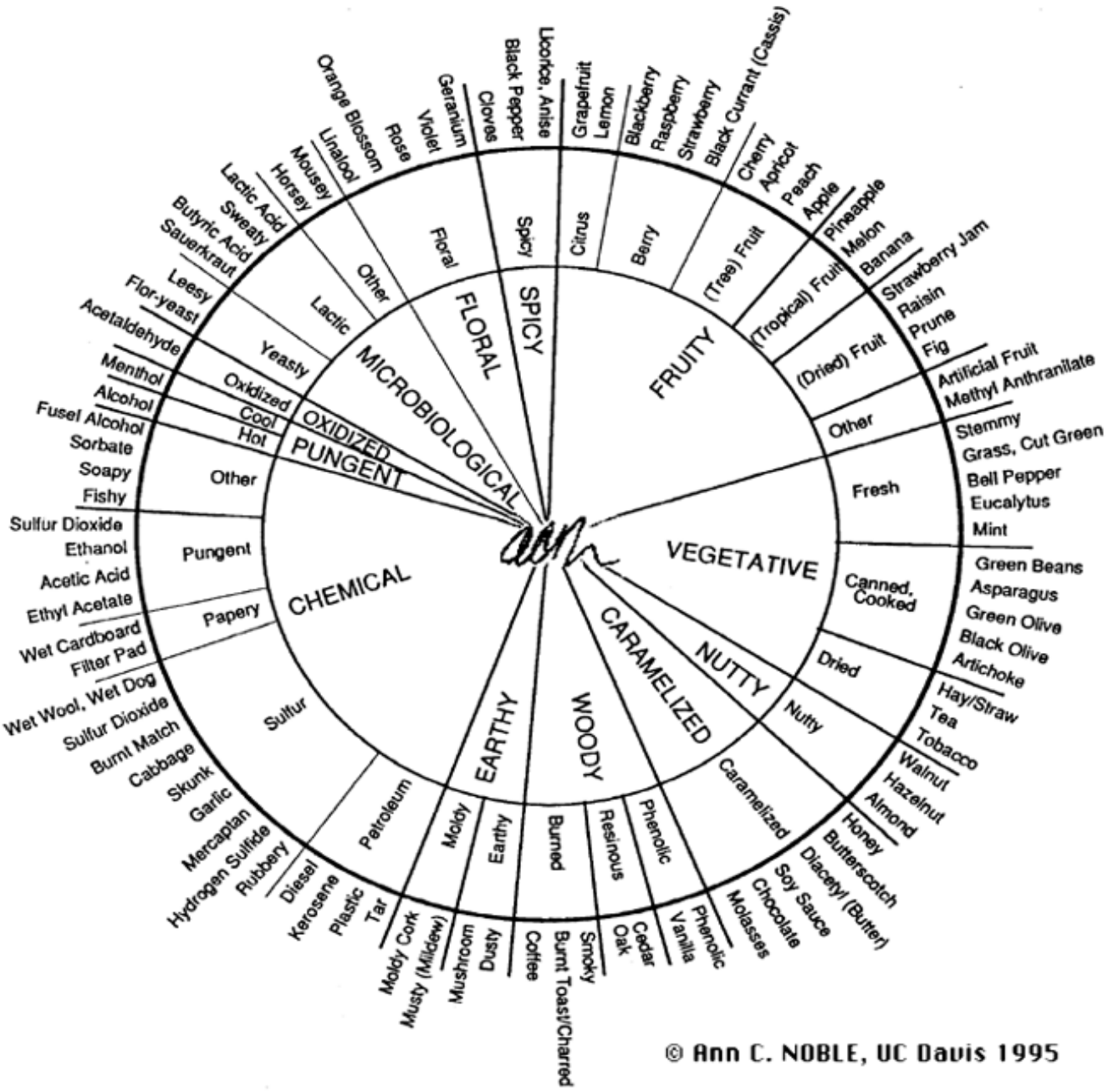


Figure 2.3 Aroma Wheel (Source: UC Davis 1995)

The application of selected dry yeast also increases the difficulty of sensory analysis. Wines used to be made with little intervention and usually depended on spontaneous fermentation in traditional wineries. Nevertheless, this situation has been completely

changed because of the quick development of genetic and biological engineering. The use of gene-manipulated species of *Saccharomyces* is widely accepted by today's winemakers, although a few wineries still consider natural fermentation as a better method for wine production. Besides, different species other than *cerevisiae* were isolated and applied in fermentation practice. Table 2.3 shows the characteristics of different selected dry yeast usually chosen by large wineries. Such selected strains are believed to be more tolerant to ambient environments changes and produce less off-flavor compounds. Recent studies on metabolic pathways and their regulations showed a clearer picture of the sources of key flavor compounds in wine. Still, quite a few ecologists are researching on wine flavor improvements and yeast species that can better adapt to grape must.

While wine sensory analysis is useful, expert taste analysis can only be conducted after the fermentation process ends and thus cannot be used for in-production quality control.

To resolve these issues above, analytical techniques are widely used in large wineries presently (Table 2.2). High performance liquid chromatography (HPLC) is one of the most common capabilities acquired by most of wine research facilities, which is used in this research to determine organic acids and sugar concentration during wine fermentation (Zoecklein 1995). This technology, which has been fully developed in the past 20 years, is extremely helpful when wineries want to monitor organic acids routinely. Take malolactic fermentation as an example. During red wine fermentation, the conversion of malic acid, which has a strong sour taste, to softer and milky lactic acid is recommended by manually adding lactic acid bacteria in the middle of fermentation

process (See Chapter 2.3). The result should be reflected by the peak in malic acid reduction after the inoculation, which can be monitored by using HPLC.

Table 2.2 Analytical techniques and current applications (Zoecklein 1995)

HPLC: acids, sugars, phenolics, microbial metabolites
Atomic Absorption (AA): Cu, Fe, Ca, K, other trace metals including Pb
Gas Chromatography (GC): ethanol, methanol, higher alcohols, esters, DEG
GC/MS: ethyl carbamate, procymibone*, sulfides, 2,4,6-TCA, pesticide residues, contamination, 4-ethyl phenol
Near Infrared spectroscopy (NIR): ethanol, residual sugar

*Procymibone is a fungicide widely used throughout the world on grapes

Gas chromatography/mass spectrometry (GC/MS) is another sophisticated technique used in some wineries and sensory laboratories. Currently, ethyl carbamate, procymidone, and many other compounds, as well as contaminations, can be detected by GC/MS. One of the most important applications of GC/MS is to measure the amount of pesticides, fungicides and other agricultural chemicals used on grapes. The other use is for the analysis of organic sulfides and other sensory compounds. For example, a recent application of GC/MS is to analyze 4-ethyl phenol, which is believed to be associated with wine off-flavor produced by wine spoilage yeast *Brettanomyces/Dekkera* (Vigentini, et al. 2013).

Overall, analytical technologies have become more and more important with the new hybrid grape varieties, the development of novel fermentation techniques and selected dry wine yeast, as well as increased demand from customers and regulation by the government.

2.3 Wine Quality

Quality of wine is a subjective and comparative judgment, which depends on the character of the grape, the species of microorganisms and also the fermentation conditions. By using sensory analysis methods, researches can make conclusions on the relationship between specific compounds with the quality of wine.

2.3.1 The importance of grapes

Grapes are of great importance to winemaking. Different grape varieties can produce numerous aromas. Wine styles, therefore, can change because of distinct differences in grapes and growing conditions. Ecologists did not begin to think about grape species until late 1980s, but quickly became very interested owing to the wide usage of HPLC and GC/MS technologies. These technologies can measure not only the sugar level and pH, but also potassium and other organic acids in the grape must. Furthermore, sugar concentration, initial pH and other grape characteristics will also affect the growth of microorganisms. Researchers at the University of Missouri in this study have also made a significant contribution to the wine industry (Thomas, et al. 2013). They have collected and analyzed various types of grapes from different vineyards and different treatments. Overall, grape variety and vinification techniques both influence the wine quality.

2.3.2 Microorganisms in wine making

Fermentative microorganisms, such as yeasts and molds, are considered to be the most significant factor in wine making because sugars are metabolized to ethanol and

other aroma compounds by them. The following paragraphs are intended to highlight microorganisms of importance in wine making.

2.3.2.1 Molds

Molds, which are multicellular and filamentous fungi, can grow in a dark, warm, and humid environment. They can grow on the surface of cooperages, on the walls and other porous surfaces as well. Molds are considered to be unpleasant microorganisms because of the potential leaching of their metabolites into fermenting wine, thus resulting in moldy odors. However, molds are aerobic organisms and cannot survive in alcohol media (Moreno and Polo 2009). Thus, proper sanitizing process can control mold contamination.

2.3.2.2 Yeasts

Like molds, yeasts reproduce asexually, usually by budding. Although budding is not the only way for yeast replication in nature and certain yeasts may multiply by a sexual cycle, this reproductive difference is hard for wine makers to use as a way to identify yeast species. The shape of yeasts also varies with age of the colony and media used for growing. Yeast species can be divided into two groups according to their performance during wine fermentation: film forming yeasts and fermentative yeasts. The previous group may grow on the surface of wine as a film if exposed to air improperly, while the latter one does not grow as film. The film-yeast community is also regarded as a kind of wine spoilage due to their production of a mixture of oxidized end products (Malfeito-Ferreira 2011).

The group of *Saccharomyces sp.* is spherical to ellipsoidal in shape and 8×7 μm in size depending on the growth media (Zoecklein 1995). This type of yeasts is added as wine fermentation yeast, which is able to produce as much as 18% alcohol and complex aroma. Prior to inoculation, dry yeast is always activated in growth media to a final concentration on the order of 2 to 5×10^5 cells/mL. Then the pre-culture is inoculated based on the initial sugar concentration, usually 1 to 3% vol/vol. In addition to the yeast starter preparation, the must should also be warmed up carefully, because cold shock may reduce the yeast viability by up to 60% (Zoecklein 1995).

Some U.S. wine makers are no longer satisfied with normal native yeast species, so selected or manipulated fermentative yeasts are transported from other places, such as European countries. For example, the yeast strain *Saccharomyces bayanus* EC1118 (Table 2.3), which is used in this experiment, was isolated from Champagne fermentations. Lower foam, volatile acid and hydrogen sulfide were measured during the whole process, which makes the strain a good choice for all types of wines, especially late harvest grapes. Table 2.4 shows some distinct characteristics of selected dry yeasts. Evidently, isolated yeast species would ensure the quality of wine and produce more complex and satisfactory sensory profiles (Serra, Strehaiano and Taillandier 2005).

Table 2.3 Basic characteristics of selective dry yeast (http://www.lalvinyeast.com/strains.asp)

	S. cerevisiae RC 212	*S. cerevisiae* 71B-1122	*S. cerevisiae* ICV K1V-1116	*S. bayanus* EC-1118
Temp. range (^{o}C)	20-30	15-30	10-35	10-30
Fermentation speed	Moderate	Moderate	Moderate	Very fast
Alcohol tolerance (%/vol.)	16%	14%	18%	18%
Nutritional requirements	High	Low	Low	Low

Table 2.4 Advantages of selected pure yeast cultures over native strains (Moreno-Arribas and Polo 2009)

- Rapid onset of active fermentation and predictable rate of sugar-to-alcohol conversion
- Complete utilization of fermentable sugars
- Improved ethanol tolerance
- Less SO_2 and H_2S production
- Reduced tendency to foam
- Reduced formation of acetic acid
- Higher clarification

2.3.2.3 Wine bacteria

Melolactic fermentation (MLF) is another critical catabolic pathway, in which L-malic acid is oxidized to L-lactic acid and carbon dioxide. The occurrence of MLF is commonly because of the existence of lactic acid bacteria (LAB). Its importance in wine fermentation is embodied in acid balance and its contribution to sensory properties. Studies in recent years have focused on the parameters affecting growth of LAB, timing of inoculation, and byproducts of MLF (Lasik 2013).

In summary, a huge amount of microorganisms can survive under wine fermentation conditions because of the nutrients contained in grapes, especially at the beginning of the process where there is low ethanol production. The growth of different microbes along with their metabolic products should be carefully considered depending on their contributions to wine quality.

2.3.2 Fermentation conditions

Many additional factors, such as initial sugar concentration, temperature, sulfur dioxide addition and even water quality, can be classified as fermentation conditions. These also include the fining and aging process. Related information can be found in many reference books (Zoecklein, Fugelsang, et al. 1995, Moreno and Polo 2009).

The quality of wine, or the wine sensory profile, is established by the grape quality, yeast species, and fermentation conditions. Wineries should consider all the possible elements that will potentially influence the wine quality. In particular, it is very important to keep the winery clean and neat, which helps winemakers avoid microbial disaster that will be discussed in the following section.

2.4 Wine Spoilage

The wine fermentation process is relatively simple, which allows a large number of wineries to be established in the past few decades. For new wineries to earn their reputation in the global competition, they must be able to fulfill quality standards, at least have no defects that may be caused by raw materials, grapes especially, and spoilage

microorganisms. It has been known that every stage during wine fermentation can be contaminated, even in the sealed bottles (Coulon, et al. 2010).

Wine spoilage is often a disaster to a winery, which will result in huge economic losses. To understand how it happens and how to avoid this situation is imperative to ensure good aromas in wines. As discussed above, wine fermentation is usually generated by selected wine yeast. Large wineries still inoculate a wide range of other yeast species to make different contributions to wine quality, such as *Lactobacillus* in MLF (Lonvaud-Funel 1999). This raises the risk of contamination by other microbial species, many of which can survive in the air or on the grape skin. The situation becomes even worse in red wines, because the red grape skin is fermented together with the juice at the first stage of wine fermentation to get enough anthocyan pigments. Some of the microorganisms will not affect the wine flavor profile too much, however, certain kinds of spoilage yeast are serious, due to their possible off-flavor production. The most common symptoms of wine spoilage are film formation in bulk wines, cloudiness, and off-flavor production (Zoecklein, Fugelsang, et al. 1995). These can happen during all fermentation process, storing and aging stages. Recent developments in sensory detection technologies have been helpful in spoilage analysis, especially off-flavor monitoring. Several indigenous yeasts have been isolated and studied to characterize their potential sensory contribution to wine spoilage. It has been proved that volatile phenols are the most common off-flavor worldwide, which is produced by one of the most common kinds of non-Saccharomyces yeast species, *Dekkera bruxellensis*. The next section describes the microorganism species and metabolic compounds that cause the wine spoilage.

2.4.1 Wine spoilage yeast

Non-Saccharomyces and Saccharomyces yeasts sometimes share common aroma characteristics, but non-Saccharomyces yeasts produce more distinctive aromas, some of which are considered to be positive to wine sensory quality, and others, however, are negative. This negative contaminated fermentation is called wine spoilage (Moreno and Polo 2009). Although, more and more wineries have accepted and utilized the new concepts of pure culture fermentation, grapes and facilities used in fermentation and other procedures, such as transportation and aging, still contain a variety of microorganisms. It is not hard to imagine the difficulty to sterilize all of the equipment in an industrial scale, and sometimes it is unnecessary if proper sanitizing process is carefully applied. These kinds of microorganisms, therefore, will grow whether the must is inoculated with a pure culture or not. Uncontrolled propagation of microorganisms will eventually lead to the production of off-flavors and other byproducts. Traditionally, sulfur dioxide is widely added at the beginning of the fermentation process. However, the application level has dropped down dramatically in recent years because of government regulations worldwide. There has been a trend to reduce the usage of sulfur dioxide in wine if we can find another feasible way to control wine spoilage (Zoecklein 1995).

The yeast of the species *Dekkera bruxellensis*, and its anamorph *Brettanomyces bruxellensis*, is reported to be responsible for the mousy off-flavor in wine, especially after storage for a long time (Barara, et al. 2008). The yeast was initially used to produce Belgian Lambic beer in a combination of yeasts and lactic acid bacteria. It was also isolated in the ethanol production industry, where it has outcompeted *S. cerevisiae* as a microorganism (Blomqvist, Nogue and Gorwa-Grauslaund, et al. 2012). Microscopically,

these yeasts are similar to *S. cerevisiae*, although somewhat smaller usually. They are also described as ogival in shape (Moreno-Arribas and Polo 2009).

Nowadays *Dekkera/Brettanomyces bruxellensis* were isolated in most wine-producing countries worldwide (Loureiro and Malfeito-Ferreira 2003). In the 1950s, ecologists first recorded that spoiled wines always turn turbid (Moreno-Arribas and Polo 2009). They did not realize that the spoilage yeast was the cause of off-flavor aromas until the 1990s. Historically, *Brettanomyces* has been studied as a principal problem in red wines, as well as white table wine (Wright and Parle 1974). The spoilage issue is particularly difficult to control because its presence is unnoticed until the wine is permanently spoiled. Also, the flavor cannot be removed using today's technology. Moreover, expensive oak barrels provide an appropriate environment for the reproduction of spoilage yeast during the wine aging process, which will affect high quality red wines. Therefore, the unpleasant flavors in wine will cause significant economic losses. A lot of research has been focused on the cause of off-flavors. It is widely believed that the undesirable odors and flavors come from the production of ethylphenols by the spoilage yeast (Loureiro and Malfeito-Ferreira 2003). Lactic acid bacteria, several *Candida* species, and *pichia guilliermondii* also produce ethylphenols, but researchers still consider that contamination of *D. bruxellensis* is the most frequent reason for this. Also, *D. bruxellensis* is the only type of yeast that produces great enough concentrations of these volatile phenols to have a sensory impact (Dias, et al. 2003).

2.4.2 Volatile phenols

Volatile phenols (VP) can be produced by yeasts, molds and bacteria as secondary metabolites, which potentially affect the flavor of wine and other fermented food products (Loureiro and Malfeito-Ferreira 2003). Although VP was studied first due to their off-flavor in red wines, their toxicological data still cannot state whether they have acute or long-term effects to human bodies (Moreno and Polo 2009). Volatile phenols are a group of molecules with a phenolic ring and different side chains. 4-vinylphenol (4-VP), 4-vinylguaiacol (4-VG), 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG), 4-ethylcathecol (4-EC) and 4-ethylsyringol (4-ES) are considered to be the most common off-flavor in wines. The precursors of VPs are hydroxycinnamic acids. Figure 2.4 shows the conversion of hydroxycinnamic acids to vinylphenols and ethylphenols. Even *S. cerevisiae* may produce vinylphenol during fermentation due to the presence of hydroxycinnamate decarboxylase enzymes (Moreno-Arribas and Polo 2009). However, after the decarboxylation step, only *D. bruxellensis* and *P. guilliermondii* have the enzymes to sequentially reduce vinylphenols to ethylphenols afterwards. Also, *D. bruxellensis* is the only yeast that appears to have the pathway to convert both p-coumaric acid and hydroxycinnamic acids into 4-EP. Further, the concentration of 4-EP is highly related to the yeast concentration, which also makes *D. bruxellensis* the only species that can produce noticeable off-flavors because the latter one cannot proliferate at high ethanol concentration. Studies have not mentioned the conversion of other hydroxycinnamic acids by yeasts, but the efficiency of utilizing other precursors may not be as high as p-coumaric acid and ferulic acid. These mechanisms also agree with the experimental results mentioned above.

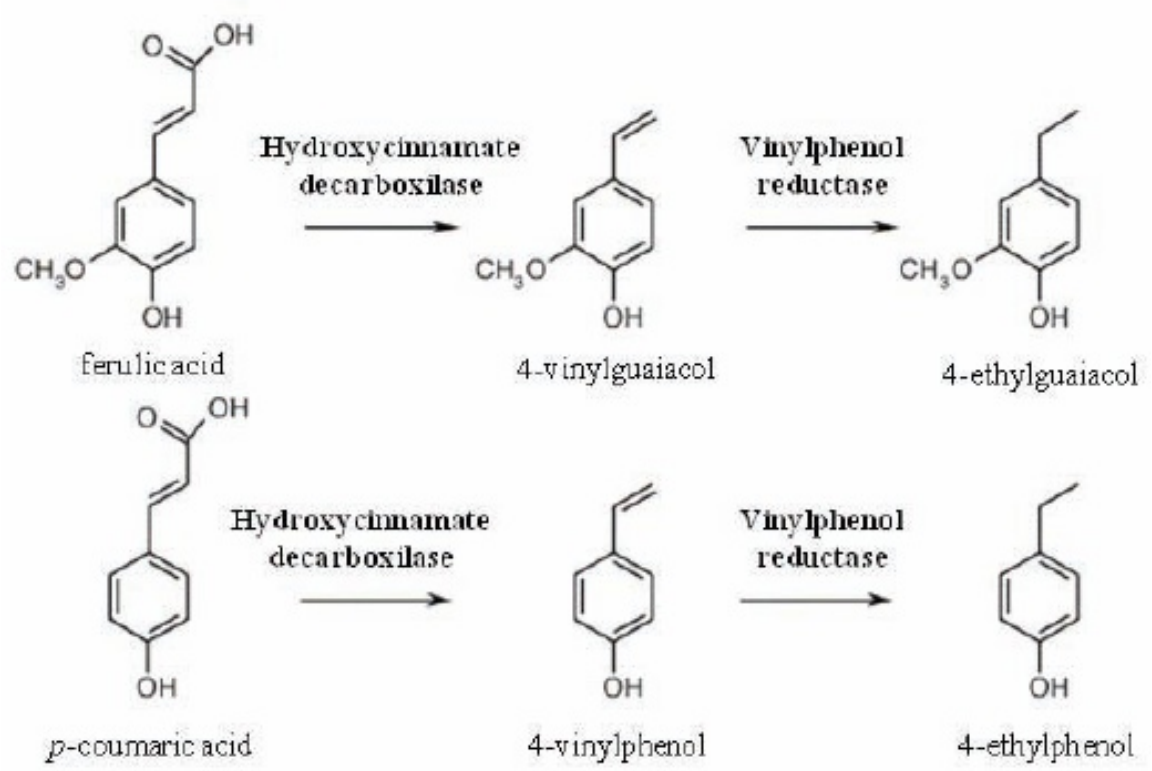


Figure 2.4 Formation of ethylphenols from their hydroxycinnamic precursors (Botelho, Valiau and Silva 2011)

2.4.3 Effect of volatile phenols on wine quality

The effect of volatile phenols could be positive or negative to the wine depending on the concentration and individual preference. It is difficult to determine the concentration limits of VPs because the odors are mixtures of different compounds rather than a single compound. Traditional sensory tests have been used to determine the threshold of VP concentration in wine to cause dissatisfaction. For instance, in a 70 person jury in one study, the preference threshold for 4-EP is about 620 μg/L (Chatonnet, Viala and Dubourdieu 1997). Below this concentration, 4-EP may be identified as a combination of complex wine aroma, while wines are completely substandard if 4-EP is over this level.

Volatile phenols above certain level are always described as spices, leather or smoke. However, the threshold is still closely related to grape varieties and wine styles.

In order to determine whether a wine is good or not, HPLC and GC/MS are usually used to directly detect the components in the wine. Wine fermentation, however, is a lengthy process that makes the measurement quite difficult and labor consuming. As *Dekkera/Brettanomyces bruxellensis* has been recognized as the main cause of the "peculiarly disagreeable" aroma, which is "closely resembling to the smell of a residence if mice" (Snowdon, et al. 2006) or "horse sweat taste" (Brandam, et al. 2008), the population of the spoilage yeast in wine may have a close relationship with the quality of wine. The population of the spoilage yeast in wine during the whole process can be measured by traditional microbiology techniques, so it may be used as an indicator of wine quality. Then the question is how to decrease the spoilage yeast concentration in wine fermentation process.

2.5 Present Control Methods

There is, at present, no effective way to "cure" wines affected by the spoilage yeast. In this situation, ecologists start to pretreat every single batch of juice in advance in order to control potential spoilage, which include grape juice pre-treatment and additional chemical preservatives. However, the crucial procedure wineries should consider is still to increase the awareness of contamination during the fermentation process, which requires wine makers to separate sound grapes from damaged ones, minimize residual nutrient and sugar, and handle oak barrel aging properly (Moreno-Arribas and Polo

2009). In addition to carefulness, some other traditional control methods are used as discussed in the follow paragraphs.

2.5.1 Physical operations

Clarification, fining, centrifugation and filtration can be classified into physical operations, the objective of which is not to directly kill microorganisms. All these processes must be simultaneously accompanied by proper sterilizing procedures. Nevertheless, sanitizing efficiency decreases due to increased surface roughness from stainless steel to rubber and wood. Strict awareness is particularly important to avoid contamination after physical processing (Malfeito-Ferreira 2011).

Filtration technology is used to separate skins and solids in the must at the beginning of fermentation. It is quite different after the end of fermentation when it is used to stabilize wines for storage. Potentially, a variety of spoilage yeast contaminations may occur without filtration, thus filtration can provide a relatively sterilized wine before bottling.

Selection of membrane pore size is a major problem. It was studied based on the requirement of throughput and the size of microorganisms. After considering the economic factors, 0.45 μm pore sizes are widely recommended and accepted. However, some winemakers select to use larger pore size filters. According to the report that 1.0 μm membrane is sufficient to remove normal spoilage yeasts and bacteria from wine (Renouf, et al. 2007). On the other hand, cell size may shrink after exposure to sulfur dioxide according to several different papers (Barara, et al. 2008, Toit, Pretorius and Lonvaud-Funel 2005). Therefore, some researchers reported that a 0.45 μm filter was not

enough to remove *D. bruxellensis* from wine when exposed to sulfur dioxide (Umiker, et al. 2013).

Although, the separation of spoilage yeast is quite important before long storage time, there is always a debate on the impact of aroma and color changes after filtration. Some wines, especially stylish red wines, might be affected by membrane filtration since certain compounds can react with the protein on the membrane (Malfeito-Ferreira 2011). There are also reports about wine quality changes after diatomaceous earth filtration or ultrafiltration (Gergely, Bekassy-Molnar and Vatai 2003).

2.5.2 Chemical preservatives

Sulfur dioxide is widely used as an inhibitor of wine spoilage and antioxidant in the wine and several related food industries, which was considered to be safe and efficient in the past few decades (Malfeito-Ferreira 2011). It is considered as the most effective method to control microbial population in wineries. In wines, an adequate level of sulfur dioxide was added either in the free or combined form. SO_2 can dissolve in water in equilibrium between molecular SO_2, bisulphite and sulphite forms, which most depends on pH (Sturm, Arroyo-Lopez, et al. 2014). Although the bisulphite form is dominant under wine fermentation conditions, in pH between 3 and 4, only molecular SO_2 is confirmed to be effective to control spoilage yeasts. It was reported that molecular sulfur dioxide might be more efficient and an extra 1 mg/L of PMB needs to be added to dry red wine to get the same result as molecular sulfur dioxide in laboratory conditions (Barara, et al. 2008). In the past, gaseous SO_2 was straightly added before wine fermentation. Instead, potassium metabisulphite (PMB) aqueous solution, which is easier to calculate,

is now more often used to produce about 57% of sulphite in recent winery practice. Research also showed that in oak barrels under winery conditions, the sulfur dioxide was helpful in preventing the growth of the spoilage yeast during the 4 months of storage. The efficient concentration of molecular SO_2 largely depends on pH, ethanol, temperature, and nutrient contents. 0.5-0.8 mg/L of molecular SO_2 is usually recommended in order to discourage most of the spoilage yeast (Malfeito-Ferreira 2011). However, a single strain of *B. bruxellensis* was found to be able to grow in an enrichment medium with 1.79 mg/L molecular SO_2 (Toit, Pretorius and Lonvaud-Funel 2005). The authors also invested the molecular and cellular level of the impact of sulfur dioxide and found that increase of ethanol and decrease of oxygen concentration would cause spoilage yeast to be more sensitive to molecular SO_2.

Unfortunately, the explanation of the inhibition is not clear yet. One of the possible reasons is that molecular SO_2 can diffuse across cell membrane, and ultimately reduce intracellular pH. It may also interact with ATP, NAD^+, and FAD and/or induce mutation in genetic material, which will inhibit spoilage yeast in microbial level (Moreno-Arribas and Polo 2009).

Molecular sulfur dioxide, however, has its own flavor that might also affect the wine quality, and it is hard to separate in aqueous solution. Also, individuals with sulfite-sensitivity or asthma would be affected as revealed by the International Organization of Vine and Wine (OIV). Considering the possible health problem to asthmatic individuals, the U.S. Food and Drug Administration (FDA) required the declaration of sulfite presence at a level of greater than 10 mg/L (ppm) in 1987. Besides this health concern, the industry is trying to avoid using sulfur dioxide for a better wine quality and to

enhance the melolactic fermentation, especially at the beginning of wine fermentation when the spoilage yeast has strong growth potential.

Recently, dimethyl dicarbonate (DMDC) was approved in the US and Europewith the maximum level of 200 mg/L (Fugelsang and Edwards 2007). Winemakers tried to prove DMDC as a good substitute of traditional sulfur dioxide. However, the efficiency of this chemical preservative depends on the microorganism species. Previous research showed that bacteria are more resistant to DMDC than yeasts. It should be not only used routinely under legal concentration, but also added with other antiseptics, such as SO_2 (Malfeito-Ferreira 2011).

2.5.3 Temperature

Temperature is important for wine fermentation. Generally speaking, spoilage yeasts grow slower than wine yeasts at low temperatures, which enables *Saccharomyces sp.* to dominate the fermentation. A lot of research has been done to improve wine quality by changing the environment temperature without considering the spoilage yeast. The growth kinetics of traditional wine yeasts of the genera *Saccharomyces* was well studied in the past few years. Similar methods have been applied to spoilage yeast studies. Research showed that 32^{o}C is the best temperature for ethanol production and 25^{o}C for acetic acid production (Brandam, Castro-Martínez, et al. 2008). Further, the metabolism and kinetics were also investigated. The reduction in membrane fluidity and affinity of the protein transport for substrates are the two possible reasons why the spoilage yeast cannot endure temperature over 35^{o}C (Dias, et al. 2003). By comparing different growth kinetics, temperature should be one of the desirable control methods that can be used to

inhibit the spoilage yeast. Contrary to other food industries, temperature control is always not a feasible way to practice in wineries, especially heating. The thermodynamics, including heat distribution, should be further studied in industrial scale. Also, temperature can affect the oxygen concentration and enzyme activity in fermented juice.

2.5.4 Sugar concentration

The concentration of glucose or fructose is also a good option to control the fermentation process because it is easy to measure and calculate. It is widely accepted that the sugar is used as carbon sources in the culture media. No production of ethylphenols was found if there were no carbon sources according to previous study (Dias, et al. 2003). That is why the sugar concentration plays an important role in the fermentation process. Study showed that the growth rate of *D. bruxellensis* was increased under low concentration of glucose and fructose from 0.2 to 20 g/L (Barata, et al. 2008). In high concentration of sugar, inhibition would occur. Both growth rate and ethanol production would decrease. In different types of grapes, the sugar concentration would vary tremendously. Adding pure sugar or diluting the grape juice might be technically practicable.

In conclusion, four types of spoilage control methods have been discussed. The purpose is to maintain the quality of wine in industrial wineries. The off-flavor produced by the spoilage yeast is the most common cause of economic losses, thus the population of the spoilage yeast in wine should be investigated routinely during the whole fermentation process.

Chapter 3 Materials and Methods

In the previous chapters, the wine spoilage problem was presented and the literature was reviewed to better understand the reason and consequence of this realistic problem. In this chapter, yeast cultures and other supplies are listed and the experimental designs, such as randomized block design, are introduced according to the characteristics of the co-culture system used in this work.

3.1 Yeast Strains and Pre-culture Condition

Both commercial wine yeast, *Saccharomyces bayanus* EC-1118 (Dnnstar Ferment AG, Bahnhof-strasse 7, 6300 ZUG Switzerland) and spoilage yeast strain, *Dekkera bruxellensis* (ATCC, Manassas, VA) were maintained on YM agar slants [0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1%dextrose, 2% agar (w/v)] at 4^{o}C with transferring every two months to keep the cultures active. Both of the yeasts were pre-cultured in 200 ml YM broth, obtained from Difco™ (Detroit, MI), at 32^{o}C for 2 days until the highest cell concentration was obtained.

3.2 Yeast Differentiation and Culturability

A total of five agar media was compared with to select for the best differential abilities. The compositions of the five media are summarized in Table 3.1.

Table 3.1 Composition of yeast selective media

YM (w/v)	0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1%dextrose, 2% agar
YM-CaCO3 (w/v)	0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1%dextrose, 2% agar, 0.5% $CaCO_3$
YM-BCG (w/v)	0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1%dextrose, 2% agar, 2.2×10^{-3} % bromocersol green
GYP (w/v)	2% glucose, 0.5% yeast extract, 1% peptone, 2% agar
YMC (w/v)	0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1%dextrose, 2% agar, 1×10^{-3} % cycloheximide

Serial dilution and plating techniques were carried out on two different agar plates, YM and YMC, to determine total viable yeast counts. YM agar is regarded as a perfect and accurate growing environment for both *S. bayanus* and *D. bruxellensis*. Previous studies showed that a high concentration of cycloheximide, which is necessary for the development of *D. bruxellensis*, prevented the growth of *Saccharomyces* during the first seven days (Curtin et al. 2007; Coulon et al. 2010; Morneau 2011). Therefore, to differentiate the yeasts in a co-culture system used in this experiment, 10mg/L cycloheximide was added to YMC agar, in addition to normal YM agar, to select against *S. bayanus* strains.

Samples were plated directly using a sterile glass spreader with 0.1 mL undiluted or diluted mixed culture. Both kinds of agar plates were then incubated at 32^{o}C for two days. After that, plates with between 15 and 150 CFU/plate were selected for population enumeration. Although *D. bruxellensis* is also supposed to grow on YM agar, its growth rate is much slower than that of *S. cerevisiae* at 32^{o}C. As a result, wine yeast colonies were comparatively larger than spoilage ones on YM agar. This phenomenon occurred

from the first day of culturing, whereby the spoilage yeast concentration never went above 10^3 CFU/mL, while wine yeast usually reached 10^5 CFU/mL after 36 h.

3.3 Fermentation Conditions

The minimal wine media for the growth kinetics tests were prepared as provided (Blomqvist, Nogue and Gorwa-Grauslund, et al. 2012): 50 g/L glucose, 5 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 1 g/L yeast extract, and 0.4 g/L $MgSO_4 \cdot 7H_2O$. The wine medium was then sterilized at 121^{o}C for 15 min and stored in a 4^{o}C refrigerator after cooling for later use. *S. bayanus* and *D. bruxellensis* were inoculated undiluted into shake-flasks each containing 800 mL minimal wine media described above. In order to illustrate different sugar concentration effects, different initial sugar concentrations were added in the sugar trial (See Table 3.2). Each of the experiments was duplicated.

Table 3.2 Three levels of glucose concentration and initial readings in sugar trial

	High sugar	Normal sugar	Low sugar
Initial glucose concentration (g/L)	100	50	25
Initial refractometer reading (^{o}Bx)	10.3 ± 0.1	5.7 ± 0.1	3.4 ± 0.1

During the fermentation process, dissolved oxygen and sugar concentration were measured by a Hanna HI9146 Dissolved Oxygen and Temperature Meter (Hanna® Instruments, Woonsocket, RI) and Digital Wine Refractometer WM-7 (ATAGOTM, Japan) respectively.

3.4 Ethanol and pH Tolerance Tests

In order to determine the effect of different ethanol concentration and pH on the growth of the spoilage yeast, five ethanol and two pH levels were tested by altering the initial wine media conditions. An ethanol tolerance test was prepared by mixing various proportions of 95% ethanol with minimal wine media described above. The proportions of ethanol and wine media volume are summarized in Table 3.3. Also, pH tolerance of the spoilage yeast was observed at normal pH = 5.3 and low pH = 4.0 by adjusting the minimal wine media with an 85% (v/v) orthophosphoric acid solution.

Table 3.3 Ethanol tolerance test (5 mL in total)

	0%	5%	10%	15%	20%
Ethanol (mL)	0.00	0.25	0.50	0.75	1.00
Wine media (mL)	5.00	4.75	4.50	4.25	4.00

3.5 Antibiotics Test

Some bacteria are found to produce antibiotics during their stationary growth phase. Similarly, wine yeast might also produce antibiotics, which can be used for its own growth advantage. In order to determine if the wine yeast does produce inhibitory substances that affect the growth of the spoilage yeast, the agar well test was conducted. Freshly grown, undiluted spoilage yeast (0.2 mL) was spread on YM agar plates to form a thick lawn of yeast colonies. Five wells were made using a sterile cork borer as shown in Figure 3.1. Samples were collected from a fermented minimal wine media, and unfiltered and filtered samples were inoculated into a1, a2 and b1, b2 respectively.

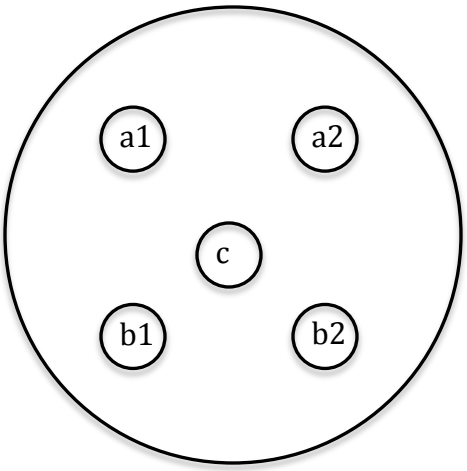


Figure 3.1 Antibiotics test
(a: unfiltered wine media; b: filtered wine media; c: 0.85% NaCl solution)

3.6 Yeast Viability Test

The yeast cell viability was determined by the exclusion of trypan blue (Sigma®). Trypan blue, as a dye, has many advantages in its cost and speed of performance as compared to other more complex methods, such as live/dead bacterial viability kit and RAMAN microscopy (Stoddart 2011). After mixing the same volume of wine samples with this dye, the live cells are impermeable to it, thus remaining clear, while the dead ones are dyed. The microscopic images were taken by the EVOS® XL Core Imaging System (AMG, WA).

3.7 Experimental Design

As mentioned in Chapter 2, spoilage yeast may cause serious economic losses if it is not properly controlled. Many efforts have been spent on different chemical or physical

inhibitors to control spoilage yeasts in the wine industry. In this work, several target variables were considered in a randomized block design (RBD). These include initial sugar concentration, dissolved oxygen (DO), initial yeast concentration, temperature, ethanol and pH, as well as nutrition, and humidity, which can be eliminated by RBD. Among these factors, temperature and pH were set as nuisance factors, which might affect the control system, but were not the primary interest, because pH and temperature are strictly controlled during wine fermentation in large wineries. Usually, wine fermentation is carried out at low temperatures and pH conditions (Nevoigt 2008). In addition, dissolved oxygen is hard to alter in batch fermentation without aeration. However, the results showed that dissolved oxygen still played a major role in the control process. Furthermore, the initial yeast concentration was also fixed if following the standard starter preparation (See Chapter 2). Nevertheless, the spoilage yeast concentration may vary depend on the winery locations. Thus, different wine yeast and spoilage yeast combinations were considered as one of the blocking factors.

Therefore, the final blocking primary variables were the initial sugar concentration and yeast combination. Each variable has three levels and each level was duplicated. Table 3.3 shows the Randomized block design. In total nine minimal wine media were fermented at the same time for seven days to create homogeneous blocks in which the nuisance factors were held constant. During this period of time, yeast concentrations, sugar level and dissolved oxygen were measured at least every 12 h.

Table 3.4 Randomized block design for the simulation of yeast population

		Sugar concentration*		
		low	normal	high
Yeast combination (*D. bruxellensis* to *S. bayanus*)	2:8	1	2	3
	5:5	4	5	6
	8:2	7	8	9

*Sugar concentration: low = 25 g/L; normal = 50 g/L; high = 100 g/L.

To determine the effect of oxygen on the growth of the spoilage yeast, anaerobic, aerobic, late inoculation and pure culture fermentations were conducted.

First of all, nitrogen was blown into minimal wine media for 20 min, and the head space was filled for another 10 min for a strictly anaerobic fermentation condition. After that, the whole tank was sealed in an anaerobic chamber with a gas generator envelope showed in Figure 3.2 below.

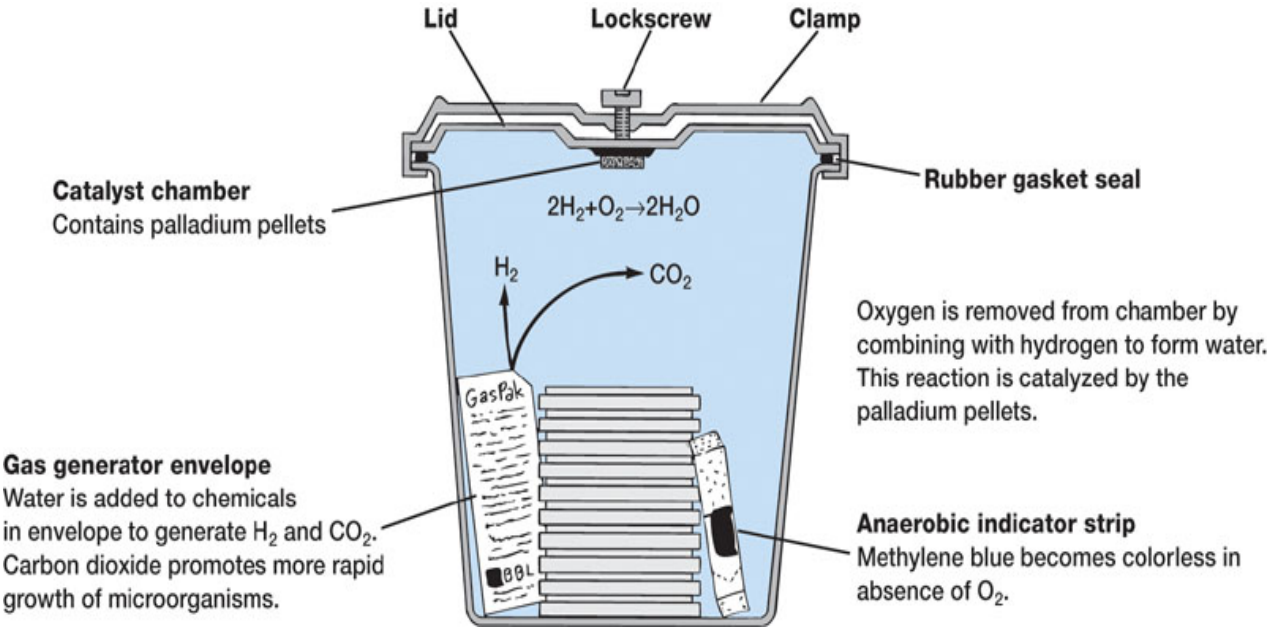


Figure 3.2 Strictly anaerobic fermentation system (http://intranet.tdmu.edu.ua/)

Secondly, aerobic fermentation was also observed to determine the growth kinetics of the spoilage yeast with high oxygen concentration. Filtered air was blown into the fermentation flask to ensure that enough oxygen transferred from the gas to the liquid phase.

Further, the same wine media was also used in late inoculation trials to determine the influence of the growth of wine yeast on the spoilage yeast population.

Moreover, pure spoilage yeast culture was inoculated undiluted to check the growth pattern and environmental conditions in a monoculture system.

In conclusion, different fermentation conditions were applied to directly or indirectly prove the limiting factor that can be used to control the wine spoilage problem.

3.8 Organic Acid Analysis

Chardonnay grape juice (Year 2013) from the University of Missouri-Columbia research winery was filtered through a pre-sterilized 0.45μm filter (Millipore), and then inoculated with both yeasts at the same initial concentration. Samples were collected twice a day to check the organic acid changes by HPLC.

All the HPLC determinations were performed using a Varian Pro Star HPLC module (Agilent Technologies, Inc., Santa Clara, CA) equipped with a pump, a variable wavelength (UV) detector, set at 220nm, and connected in series with a Varian 356-LC Refractive Index (RI) Detector. Wine samples were filtered through a 0.45 μm filter (Millipore), and injected with a 20 μl sampling loop using a Model 410 autosampler. The separation was performed with an Aminex HPX-87H column (5μm, 7.8mm × 300mm) preceded by a guard column. Column temperature was set to 65^{o}C by a column heater

Model 631 (Alltech). The conditions used were as follows: flow rate 0.5 ml/min, mobile phase 0.045N H_2SO_4 with 6% acetonitrile (v/v). Data acquisition and peak processing were performed with a Galaxie Chromatography software (Agilent). Samples were undiluted for organic acids (citric acid, tartaric acid, malic acid, succinic acid, lactic acid and acetic acid), and 1:50 diluted for sugars (glucose and fructose). All the concentrations were calculated by peak area and standard curves made before.

Chapter 4 Results and Discussion

The mystery of the growth pattern and metabolic pathways of the yeast genes of *Saccharomyces* as a eukaryote model have been studied for years. However, the topic of the biochemical and genetic mechanisms of the spoilage yeast, *Dekkera bruxellensis*, launched a heated debate more recently after the confirmation that this yeast species is the reason for most wine spoilage problems. Food scientists applied identical methods to gain reliable information about gene functions and metabolic pathways of this spoilage yeast species in order to avoid contamination by this spoilage yeast. Several pieces of literature introduced some innovative techniques to help us better understand the influence of the spoilage yeast in the food industry (Rodrigues, et al. 2001, Tofalo, et al. 2012, Aguilar-Uscanga, et al. 2011). However, results are still inconsistent, even contradictory sometimes. The reason might be the individual differences between yeast species. In this chapter, the experimental data from this research were analyzed for further discussion.

4.1 Selection of Differential Media

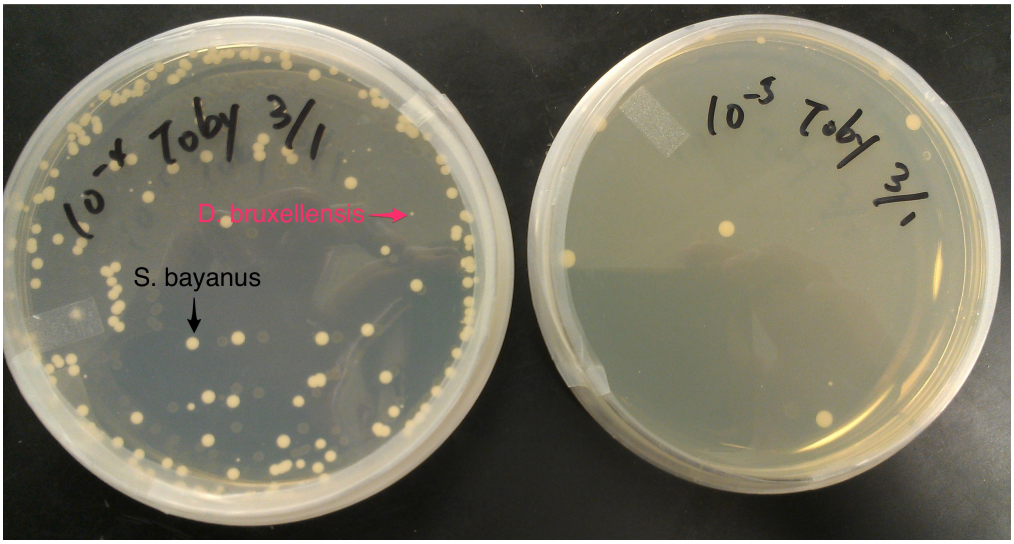
As discussed in the previous chapters, the wine spoilage problem can be controlled by minimizing the population of the spoilage yeast during the fermentation process. If the yeast growth in a co-culture system could be mimicked, some control methods can be designed and applied to encourage the growth of the wine yeast and discourage that of the spoilage one. Therefore, the first problem that needed to be solved was determining a

way to differentiate the two different yeasts in a co-culture system, in order to study both of the growth patterns separately, which is made more complicated by the slow growth of the spoilage yeast.

However, recent research has been more focused on the monoculture system, whereby only one kind of yeast species is studied at a time. This idealized simplification is of value for scientific research, such as genetic engineering, but it is not a true simulation of most wineries that have wine spoilage problems. Hence, the application of a co-culture system becomes more and more important to simulate the actual winery environment.

Some traditional methods are widely accepted by most research institutions. First of all, the spectrophotometer is one of the most reliable tools used to measure cell density according to the turbidity of the media. It is accurate under a monoculture condition. However, spoilage yeast cannot be distinguished by this simple method. Also, the Fuchs-Rosenthal Counting Chamber has been utilized for cell counting for several decades, which can be used in co-culture fermentation due to some morphological differences between two kinds of microorganisms. However, cell size and shape always change because of the temperature, nutrition and other possible external factors. Further, the Fuchs-Rosenthal Counting Chamber is not that accurate because human eyes are employed. Thus, serial dilution associated with the plating technique was applied in this work. Several selective culture media listed in Chapter 3 were compared as a preliminary experiment to better differentiate two kinds of yeast and guarantee the accuracy as well.

A



B

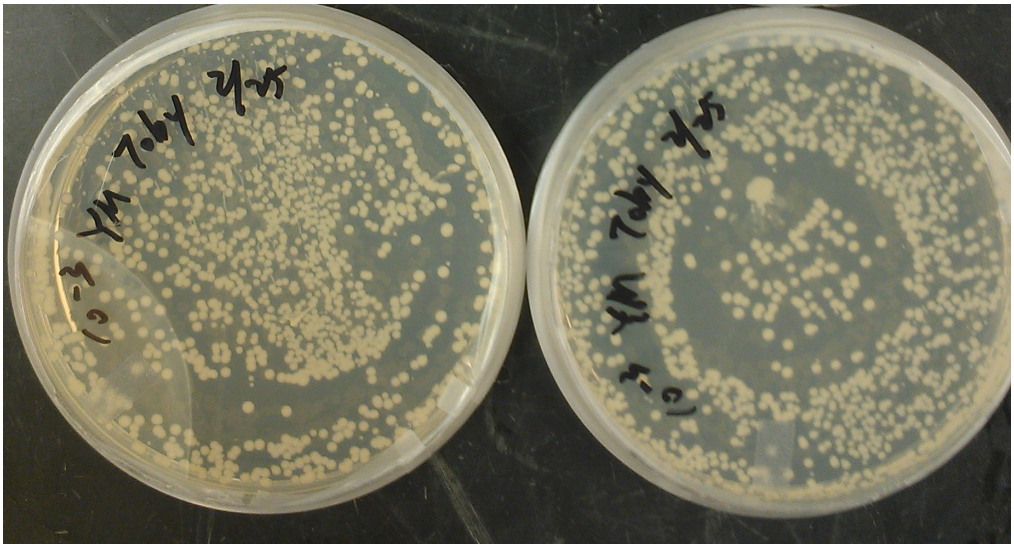


Figure 4.1 Colonies of yeast formed on YM agar plates with serial dilution. In the pictures, the smaller colonies are the spoilage yeast, *D. bruxellensis*, and the larger ones are the wine yeast, *S. bayanus*.

Figure 4.1 (A and B) shows the colonies grown on yeast malt (YM) agar plates. Both of the wine and spoilage yeasts can grow to form single colonies on this enriched agar plate at 32^{o}C within two days. The comparatively small colonies are the spoilage yeast *D.*

bruxellensis, while the larger ones are *S. bayanus*. This seems to be feasible at the beginning of the fermentation when the wine yeast has not led the process. Usually after 24 h, wine yeast colonies cannot be distinguished at a 10^{-3} dilution rate, while the concentration of spoilage yeast is still at around 10^{2} CFU/mL. As shown in Figure 4.1 B, the two yeast species cannot be clearly separated at a low dilution rate, however, colonies of the spoilage yeast are not visible at higher ones.

The next two kinds of agar plates were applied based on the fact that acetic acid, produced by the spoilage yeast, might decrease the pH. Thus, calcium carbonate and bromocresol green were added as pH indicators to differentiate these two yeast species.

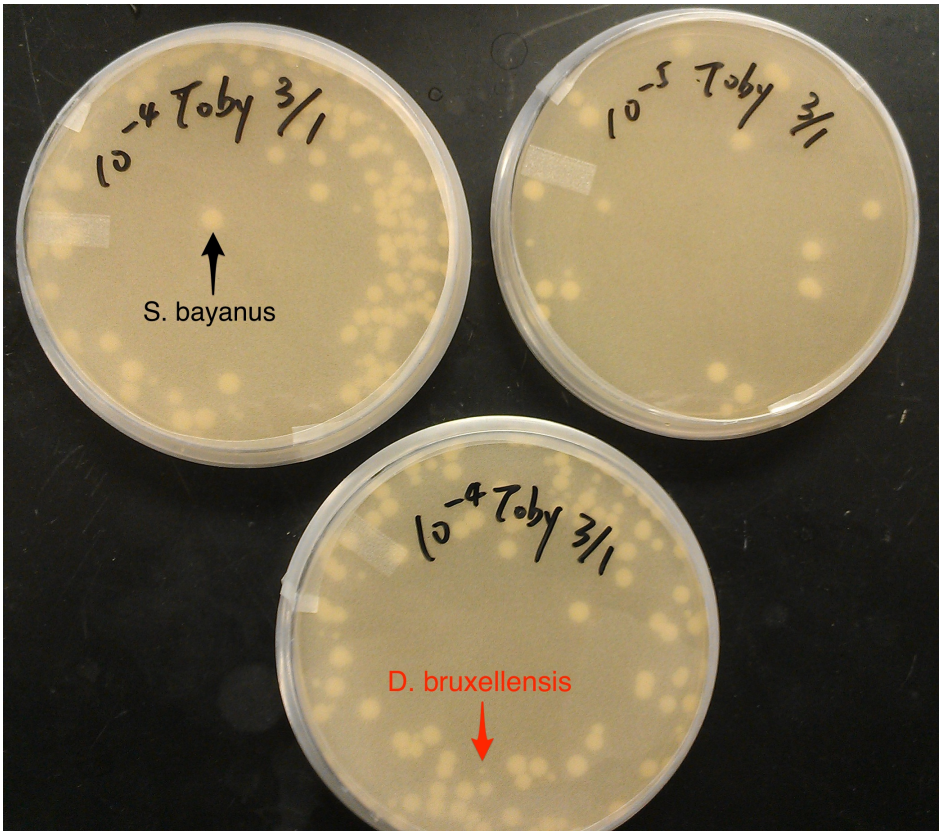


Figure 4.2 Colonies of both yeasts formed on YM-$CaCO_3$ agar plates

Figure 4.2 presents the addition of calcium carbonate ($CaCO_3$) into YM agar plates to differentiate the two kinds of yeasts. YM-Ca plates were turbid before plating. Theoretically, acetic acid produced by the spoilage yeast would dissolve calcium carbonate and then form a transparent circle around the spoilage yeast colonies (Rodrigues, et al. 2001). However, no transparent circles were observed on YM-Ca plates, although both of the yeasts could grow in the presence of $CaCO_3$.

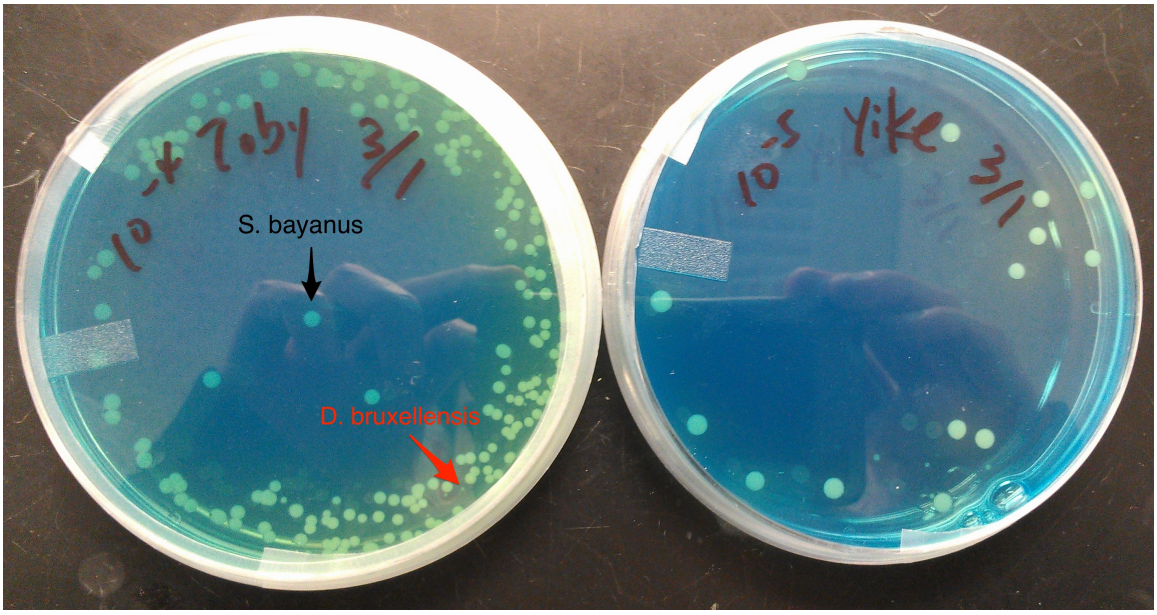


Figure 4.3 Colonies of both yeasts formed on YM-BCG agar plates

Figure 4.3 shows the result of the addition of bromocresol green (BCG) as a pH indicator, which is yellow below pH 3.8 and blue above pH 5.4. This ionic equilibrium can roughly reflect the acids produced by the spoilage yeast. BCG, as a dye, received research attention because the pKa of this chemical is 4.8, which is around the median pH in the wine industry. The periphery of the left plate turned yellow, but no color change occurred in the right one. Therefore, the pH did decrease because certain acids had been

produced, even though this might not be sufficient to dissolve the $CaCO_3$. But this still cannot be applied to differentiate two yeast species because the color only changed with a large population of yeast colonies, which makes cell counting difficult. Also, the color similarly changed around the wine yeast colonies as well.

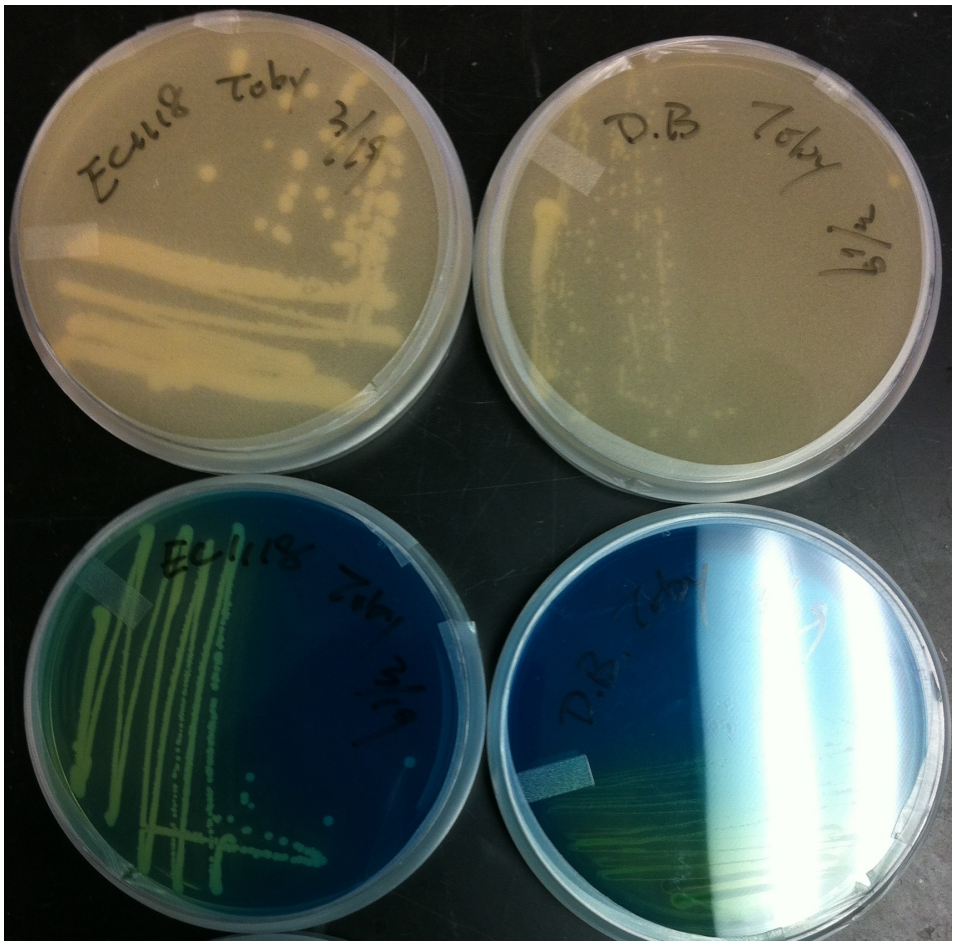


Figure 4.4 Comparison of the differentiation ability of different agar plates by streaking two kinds of yeast

Figure 4.4 summarizes the appearances of two different agar plates by streaking. Both yeast species may produce acids as one of the metabolites, which can reduce the pH. The reduction of pH can alter the ionic form of the pH indicator, BCG, but is not sufficient

enough to dissolve the $CaCO_3$ in the plates. However, neither of these two agar plates is feasible in this experiment because the yeasts cannot be clearly distinguished on such agar plates.

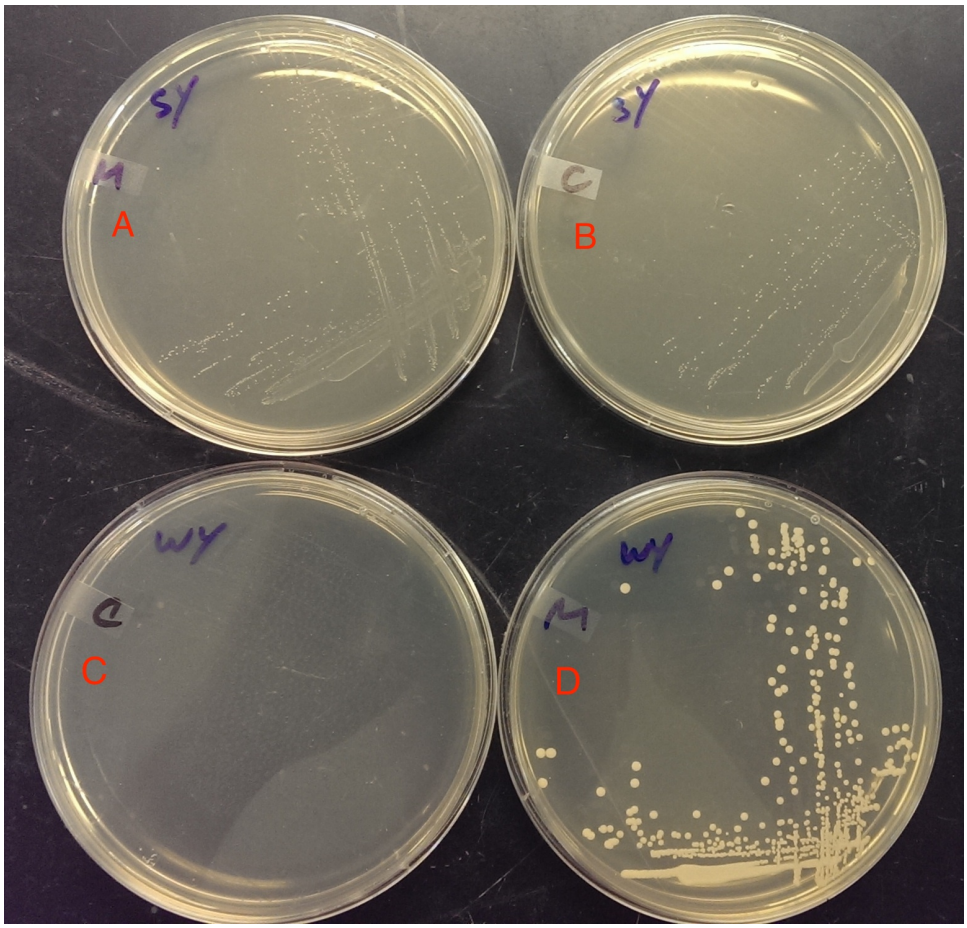


Figure 4.5 Colonies formed on YM and YMC agar plates to differentiate the two yeasts. (A: spoilage yeast-YM; B: spoilage yeast-YMC; C: wine yeast-YMC; D: wine yeast-YM) The wine yeast can only grow on YM agar (C and D), but the spoilage wine can grow on both YM and YMC agar (A and B).

After these trials, the expectation of using a single plate to differentiate the wine and spoilage yeasts did not seem to be feasible with pH indicators. Further experiments were conducted by two separate agar plates to distinguish the spoilage yeast with the antibiotic

cycloheximide (Figure 4.5). Cycloheximide (10 mg/L) has been proved, by restraining the growth of the wine yeast, to provide a well differentiating ability (Rodrigues, et al. 2001). Moreover, glucose yeast peptone medium (GYP) was also compared with YM for better selectivity. However, colonies were formed after five days on GYP agar instead of two days on YM agar at 32^{o}C (figures not shown). Therefore, YM and YM with cycloheximide (YMC) agar plates were used in this work for the purpose of both selectivity and fast growth.

4.2 Growth of Two Yeasts in a Co-culture System and HPLC Analysis

The selectivity of the differentiate agar plates has been confirmed from preliminary experiments, then both kinds of yeast were inoculated into minimal wine media to investigate differential ability of the agar plates in a co-culture system.

4.2.1 Growth curves of both yeasts and sugar consumption analysis

Figure 4.6 plots the results of the growth patterns of the two kinds of yeasts and their sugar consumption characteristics.

First of all, the wine yeast grew to approximately the same concentration as in monoculture systems (Figure 4.6 A), even though the growth rates were similar regardless of the initial sugar level and yeast strain combination at the beginning of fermentation. The growth of the wine yeast agreed with the typical growth curves of microorganisms, which started from the lag and exponential phases and followed by the stationary phase. However, it was surprising that the growth rates in different initial sugar concentrations were almost the same, while 2.5% initial glucose, which was 3.4^{o}Bx, was

extremely low compared to the regular sugar concentration, approximately 12^{o}Bx, in grape juice. This indicated that the sugar concentration was not the limiting factor in the minimal wine media fermentation process. Another experiment showed that this particular kind of wine yeast could even grow in the wine media without additional sugar (data not shown), which means the only carbon source in the media was 1 g/L yeast extract.

Secondly, the sugar level decreased as the yeast population increased and stayed constant after the wine yeast entered the stationary phase (Figure 4.6 B). Higher initial sugar concentrations ended up with higher final sugar concentrations. It is worth noting that the glucose concentration did not drop down to zero because the refractometer is designed to measure total dissolved solids concentration. The refractometer reading of plain minimal wine media was 1.0^{o}Bx, which means other compounds in the media, such as yeast extract, were also calculated into the readings. Although ^{o}Bx can only approximate the dissolved solid content, it is still useful in the wine industry due to the large proportion of sucrose in grape juice. A hydrometer might be more accurate for the measurement of sugar concentration. However, it could introduce contamination problems in laboratory conditions.

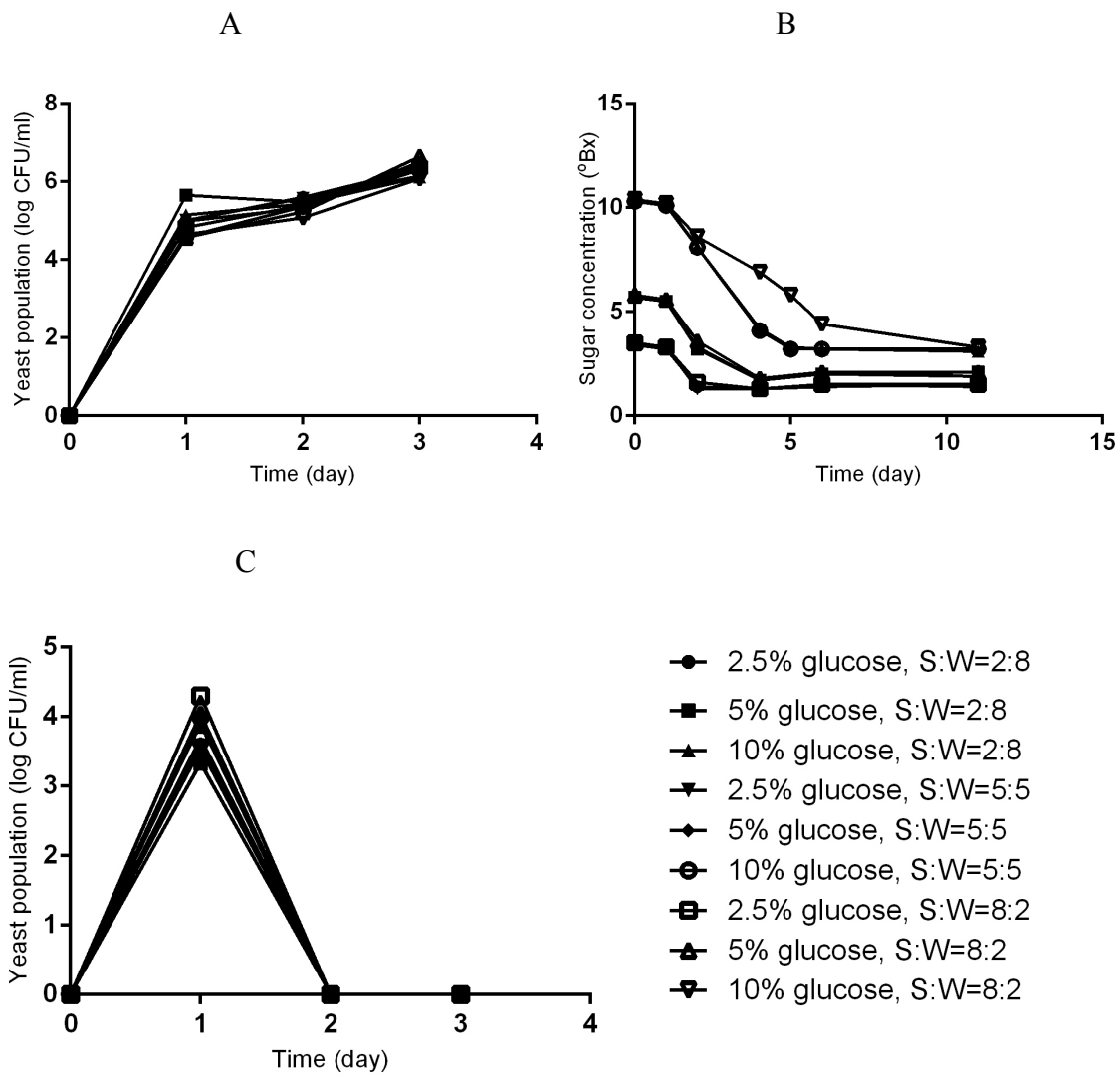


Figure 4.6 Effect of different initial glucose concentration and yeast combination on the growth curves of the yeast population. A: the growth patterns of the wine yeast; B: the consumption of glucose; C: the growth patterns of the spoilage yeast.

Further, the spoilage yeast population all went to zero on the second day of fermentation (Figure 4.6 C). This was unexpected because the wine yeast could grow normally like they do in the single culture fermentation, while the spoilage one could not. This discovery of the spoilage yeast entering the unculturable state immediately led to several other questions. In general, if we can identify the factor(s) which caused the

spoilage yeast to become unculturable, we might be able to propose a unique control prototype without any chemical additives.

Thus, our initial goal was altered from controlling the population of spoilage yeast to determining the cause of the spoilage yeast becoming unculturable. The viability of the spoilage yeast will be verified in Chapter 4.5, however, it was confirmed from Figure 4.6 that the spoilage yeast could no longer multiply on the agar plates. Even if the spoilage yeast became unculturable, it could be prevented from multiplying at a comparatively low concentration. Research showed that the spoilage yeast would produce fewer off-flavor compounds when they entered the viable but nonculturable state with the presence of SO_2 (Sturm, Arroyo-López, et al. 2014). Thus, the main objective of this work became to identify the factor(s) that led to the spoilage yeast becoming unculturable.

4.2.2 HPLC analysis

Figure 4.7 illustrates the concentration of six major organic acids in Chardonnay grape juice fermentation. As mentioned in Chapter 2, one of the most important features of wine spoilage is high concentration of acetic acid usually produced by the spoilage yeast. The average range of acetic acid varies from undetectable to 3 mg/mL, although the acetic acid in a new dry wine should be lower than 0.4 mg/mL. Apparently, the concentration of the acetic acid in this experiment was above the average level, which was around 5 mg/mL. It was a sign that a huge amount of acetic acid was produced by the spoilage yeast at the beginning of the fermentation. Also, the other two main organic acids, malic acid and lactic acid, stayed constant without the presence of MLF bacteria.

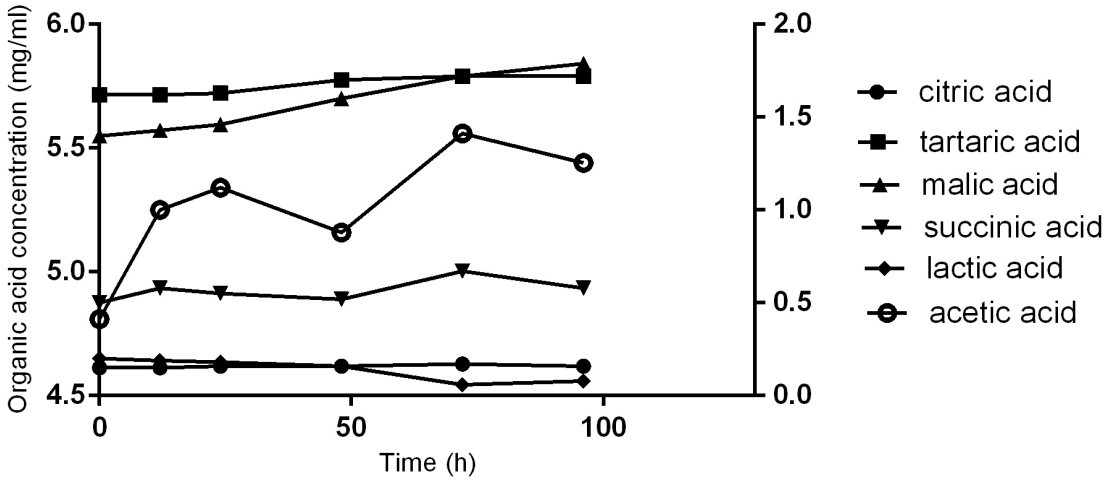


Figure 4.7 Organic acid concentration of Chardonnay wine
(Left axis: acetic acid; Right axis: citric acid, tartaric acid, malic acid, succinic acid and lactic acid)

However, the organic acid level did not change too much during the whole wine making process. There is no detectable organic acid in minimal wine media due to the simple recipe compared to the grape juice. Therefore, there is no need to consider the effect of organic acid in the following experiments using minimal wine media.

In conclusion, to inspect the factors that caused the spoilage yeast to become unculturable, more experiments were designed to check as many reasons as possible. In laboratory conditions, minimal wine media was used so that the available nutrients were just enough for yeast growth and maintenance. Five factors, ethanol concentration, pH, initial sugar concentration, dissolved oxygen and antibiotics, were verified in the following paragraphs.

4.3 Ethanol and pH Tolerance Tests

4.3.1 Ethanol concentration

Generally speaking, the spoilage yeast of *Dekkera bruxellensis* is more tolerant to high ethanol and low pH conditions than most wine yeast species. Table 4.1 shows the results of the ethanol tolerance of both yeasts measured in the same minimum wine media used in the fermentation process with different proportion of 95% ethanol.

Table 4.1 Results of the ethanol tolerance screening of both yeast species. *

Ethanol concentration (v/v)	0%	5%	10%	15%	20%
Wine yeast	1	2	3	4	5
	+	+	+	−	−
Spoilage yeast	a	b	c	d	e
	+	+	+	−	−

* +: media turned turbid after two days incubation at 32^{o}C, which means the yeast can survive in this ethanol concentration environment;
−: media did not become turbid after two days incubation at 32^{o}C, which means the yeast cannot survive in this ethanol concentration environment.

As shown in the table, both of the yeasts can survive in ethanol concentrations up to 10% (v/v). This is reasonable because normal wine contains approximately 12% ethanol. To determine if ethanol was the limiting factor that caused the spoilage yeast to stop growing, the final ethanol concentration was also measured by an ebulliometer at different sugar concentrations after 14 days of fermentation. Table 4.2 below summarizes the results.

Table 4.2 Maximum ethanol concentrations produced after different initial sugar concentration batches

	water	high sugar	normal sugar	low sugar
Temperature (^{o}C)	99.4	95.4	96.6	96.9
Ethanol concentration (%)	0	4.88	3.31	2.95

As seen from the table, the highest ethanol concentration was 4.88% (v/v), which was produced from the highest initial sugar concentration. It is understandable that ethanol is mainly converted from sugar, thus a higher sugar concentration should result in a wine with greater ethanol concentration. Also, ethanol as the limiting element can be ruled out because the final ethanol concentration of all three batches did not exceed the highest ethanol level both yeasts could tolerate.

4.3.2 pH

pH is also a very important factor to consider in most food related fields. Wineries, however, do not pay much attention to it because pH usually stays constant during the wine fermentation process. Researchers have already made conclusions that a lower pH can control the spoilage problem (Sturm, Arroyo-López, et al. 2014). In this work, two different pH levels were selected according to the regular pH range during wine fermentation to test the pH tolerance of the spoilage yeast.

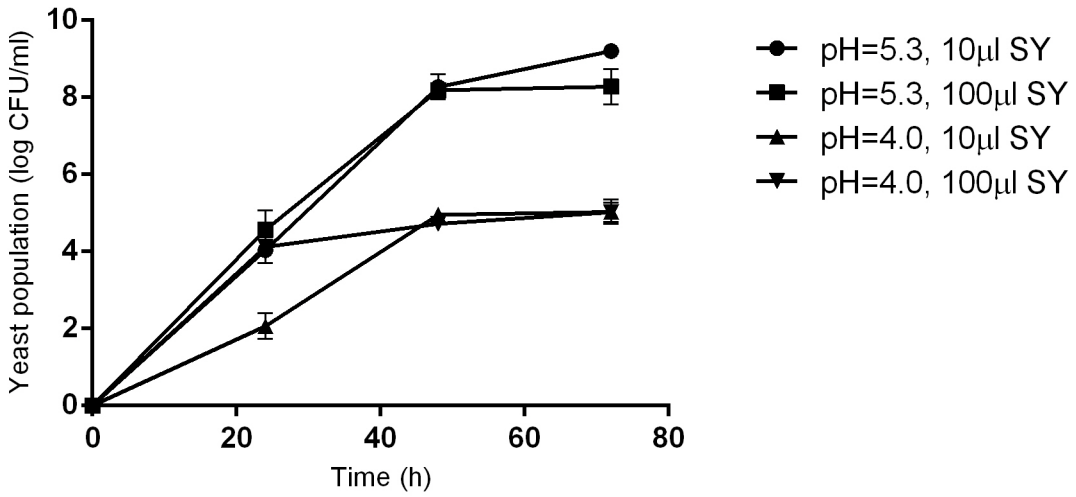


Figure 4.8 Effect of pH and inoculation volume on the growth of the spoilage yeast

Figure 4.8 shows the growth curves of the spoilage yeast at two different pH levels with different amounts of inoculation volumes. In agreement with other researches, a lower pH discouraged the population of the spoilage yeast, although they could still survive under a low pH condition. On the other side, the inoculation volume did not affect the growth patterns much. The spoilage yeast did grow slower at a lower pH and a low inoculation volume, but in regular wine media whereby the pH equals 5, the difference became negligible.

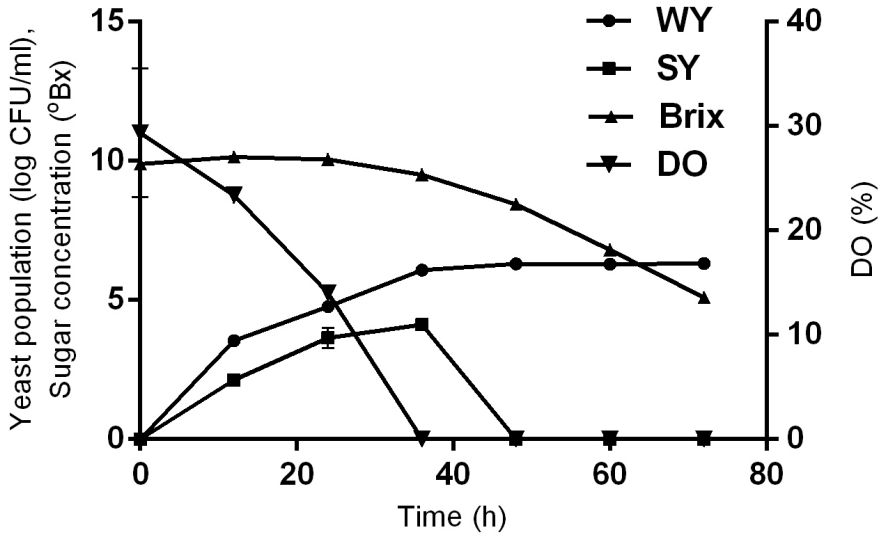
Therefore, pH can also be ruled out as one of the factors that made the spoilage yeast stop growing on agar plates.

4.4 Effect of Initial Sugar Concentration under Laboratory Conditions

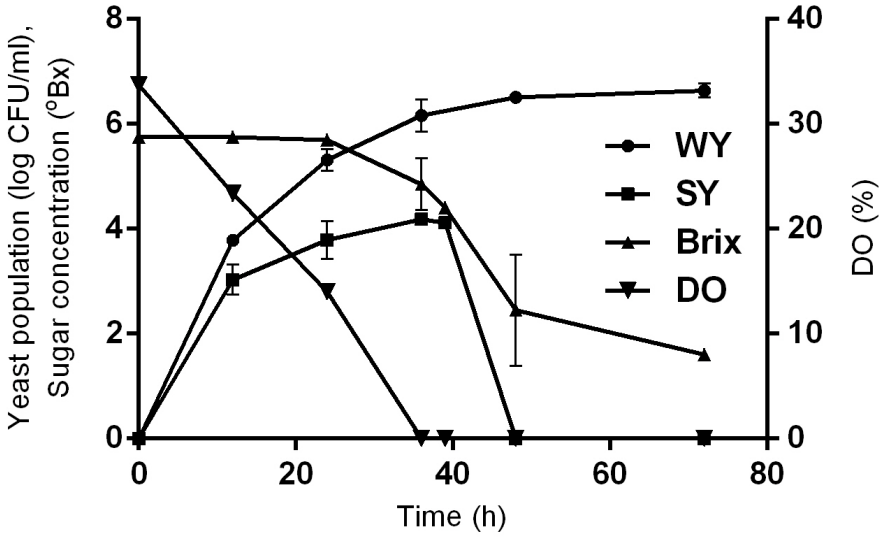
Initial sugar concentration is also worth studying in the wine industry because the sweetness of grapes varies year to year with environmental factors, such as sunshine

hours, difference in temperature between day and night, humidity, and human factors, like different chemical fertilizers, as well. In this section, three levels of initial sugar concentration, especially glucose concentration, on the growth curves of two kinds of yeasts were investigated as described in Chapter 3.

A



B



C

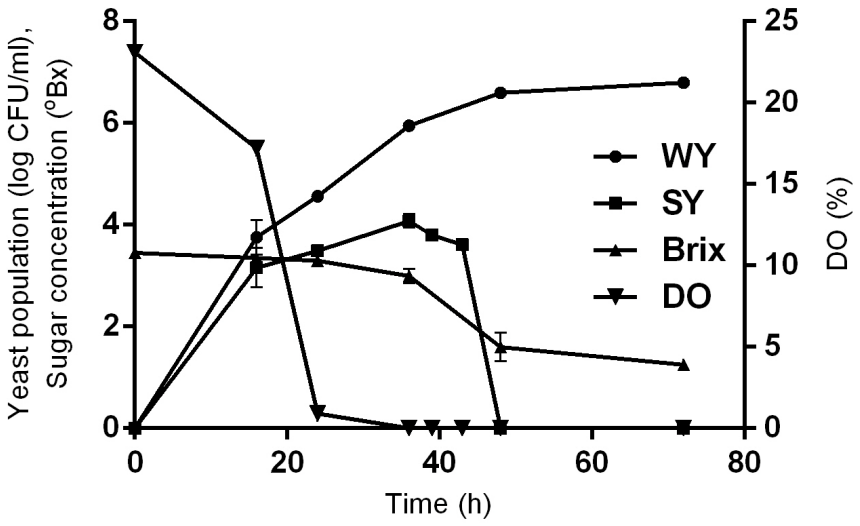


Figure 4.9 Growth curves of *Saccharomyces bayanus* and *Dekkera bruxellensis* inoculated in minimal wine media at different initial sugar levels
A: high sugar; B: normal sugar; C: low sugar

The growth patterns of two yeasts in minimal wine media at different initial sugar concentrations did not change too much compared to the previous data. At this time, dissolved oxygen was also measured in order to describe the oxygen changes in the whole picture.

First of all, the wine yeast can still grow very fast and enter the stationary state at around 48 h. With the growth of the wine yeast, oxygen was depleted and glucose was consumed. Secondly, the spoilage yeast could still multiply during the first 48 h, before the population suddenly dropping down to zero. Moreover, among these three conditions, the sugar concentration did not decrease until oxygen was depleted. It is understandable because the respiration process was applied by the wine yeast with the presence of oxygen, which creates much more ATP molecules, and in return requires less sugar, than fermentation. In other words, the strictly anaerobic environment made the wine yeast

converted their metabolic pathway from respiration to fermentation. As seen from the graphs, dissolved oxygen all went down to zero before sugar was consumed. Furthermore, it is worth noting that the spoilage yeast population always decreased to zero at around 24 h after the oxygen was depleted.

However, low sugar concentration may not be the factor that limited the spoilage yeast growth, although it was initially thought to be. As seen from Figure 4.9 C, the initial sugar concentration of low sugar levels was lower than the sugar concentration when the spoilage yeast became unculturable in the high sugar batch (Figure 4.9 A). Thus, there must be other reasons because it is not possible that the spoilage yeast can multiply at sugar concentration as low as 3.8^{o}Bx but quit growing at the concentration above 5^{o}Bx if sugar is the only limiting factor.

In conclusion, the wine yeast can always grow to a regular concentration despite the initial sugar concentration within two days. After the wine yeast entered the stationary growth phase, the spoilage yeast became unculturable with the depletion of oxygen and decrease of sugar concentration. In addition, all the spoilage yeast in three batches stopped forming colonies on agar plates at 24 h after the oxygen went down to zero regardless of the residue sugar concentration. Hence, sugar should not be the cause, at least the only cause, of the spoilage yeast becoming unculturable.

4.5 Yeast Viability Test

The viable but nonculturable (VBNC) state was first found in some kinds of bacteria, which is defined as an inability of microorganisms to multiply on growth media (Serpaggi, et al. 2012). Researchers found that this phenomenon also applied to spoilage

yeasts when they investigated the effect of the SO_2 as a preservative. The yeast cells were still viable in wine and could recover within 80 days after the removal of SO_2 (Sturm, Arroyo-López, et al. 2014). But even though the spoilage yeast was still viable, the production of off-flavor compounds was dramatically decreased according to the same research.

In order to confidently prove that the spoilage yeast did enter a VBNC state, a yeast viability test was conducted by microscopically observing the samples that were stained with trypan blue at 48 h after the inoculation and 48 h after the fermentation was ended.

Figures 4.10 A and B show the results of the cells viability test after 2 days of inoculation, where most of the wine yeast are viable in the minimal wine media, as well as the spoilage yeast. The spoilage yeast, nevertheless, cannot form colonies on agar plates anymore from the results in Chapter 4.4. Thus, the existence of the unculturable state can be positively proved while the spoilage yeast was still viable in the media. When the oxygen went down to zero, the wine yeast started to ferment sugar and produce ethanol. As the sugar was consumed or the ethanol became the limiting factor, the wine yeast entered the death phase. Therefore, the wine yeast cells were blue (Figure 4.10 C), which means they were dead, while the spoilage yeast cells stayed viable (Figure 4.10 D) at 2 days after the fermentation was over. It is worth noting that sharp images could not be rendered at the same time due to the huge differences in the size of these two yeast species. In other words, when a sharp image of the wine yeast cells was acquired, the objective lens was focused on the surface membrane of the spoilage yeast. This also explained why the color of the spoilage yeast cells was blue in Figure 4.10 C. The confirmation of the unculturability of the spoilage yeast also provided us a logical cause

of the wine spoilage problem, which would not occur if the spoilage yeast were always dead after the fermentation process.

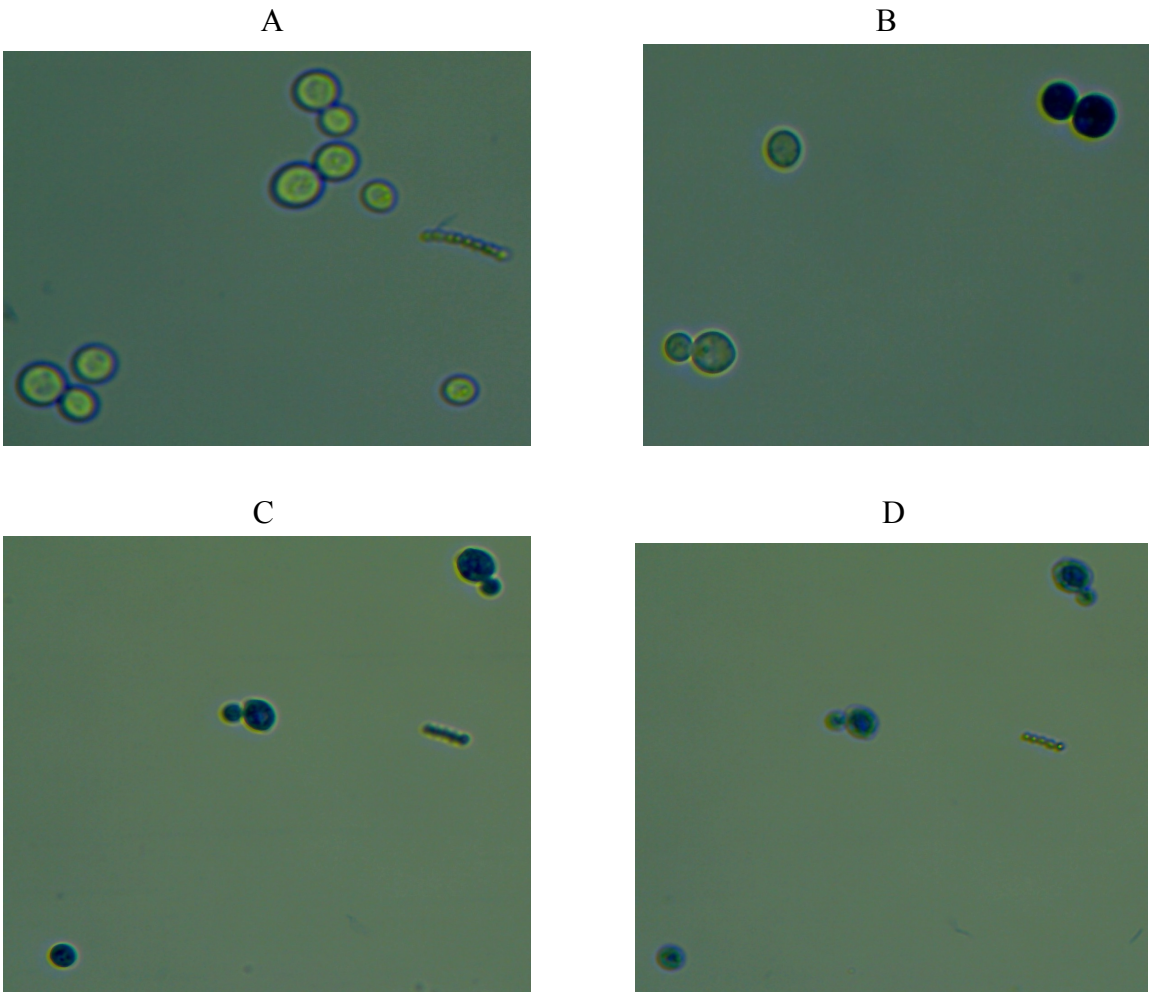


Figure 4.10 Microscopic images of both yeasts stained with trypan blue at 48 h after the inoculation (A and B) and 48 h after the fermentation ended (C and D)

4.6 Antibiotics Test

Some microorganisms can produce antibiotics to compete against others during the stationary phase because the nutrient is just enough for cell maintenance at that time. This phenomenon is also reasonable to expect here because sugar was consumed and oxygen

was depleted from the system, thus, in order to acquire as much food as possible, antibiotics might be produced by the wine yeast during stationary growth phase. The antibiotics tests were carried out in order to show the possibility of antibiotics production, which might be harmful to the spoilage yeast.

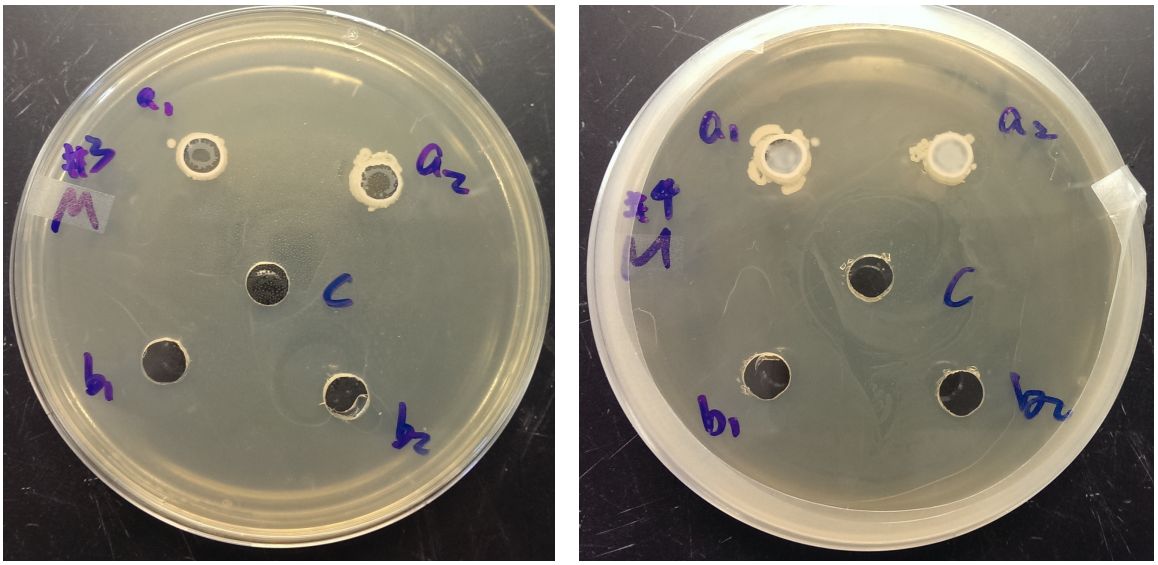


Figure 4.11 Results of the antibiotics test. Fermented wine media was added into each well on the YM agar plates with pre-cultured spoilage yeast colonies.

Figure 4.11 shows the results of the antibiotics test after 4 days of incubation. The spoilage yeast was spread to form thick colonies covering the whole agar plates. Unfiltered (a1, a2) and filtered (b1. b2) wine media taken from fermented minimal wine media was added in the wells. Unfiltered wine media contained viable wine yeast so that large white colonies were formed on the agar plates. Nevertheless, there was no transparent zones of clearing around each hole, which indicated that the wine yeast was not able to produce antibiotics to affect the growth of the spoilage one.

4.7 Effect of Oxygen on the Growth of the Spoilage Yeast

Custer's effect was first described in 1940 as the inhibition of a fermentation process in glucose ferment yeast (Wijsman, et al. 1984). In other words, the Custer's effect stated the phenomenon that yeast cells cannot ferment glucose without the presence of oxygen. If *D. bruxellensis* also follows this effect, then oxygen would become the most suspicious factor that turned the spoilage yeast unculturable. In this section, two sets of experiments were conducted in order to determine the influence of oxygen on the wine fermentation process.

4.7.1 Anaerobic fermentation

Figure 4.12 demonstrates the results of the sugar, dissolved oxygen and yeast population in anaerobic fermentation.

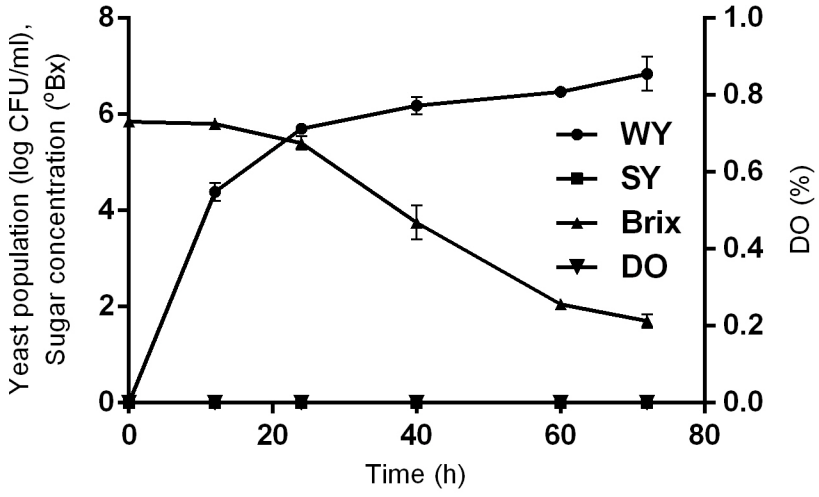


Figure 4.12 Growth patterns of yeast population and sugar consumption under strictly anaerobic condition

The wine yeast still grew normally under anaerobic conditions, while the spoilage yeast cannot multiply to a detectable concentration. The wine yeast may switch to an anaerobic pathway to ferment sugar instead of aerobic respiration. Thus, the sugar consumption was a little bit different from the previous batches, it started to decrease earlier than before. But still, sugar was consumed very fast after the wine yeast reached the steady state. Additionally, oxygen was strictly controlled in this batch. This figure gave us a strong proof that oxygen is the limiting factor for the growth of the spoilage yeast. However, it is not necessary if the spoilage yeast cannot be kept culturable under a high oxygen concentration environment. Similarly, another experiment with continuous airflow to ensure enough oxygen for the spoilage yeast to maintain their culturability could be tested.

4.7.2 Aerobic fermentation

After confirming that the spoilage yeast could not grow under anaerobic conditions, which indicated that oxygen might be the limiting factor, the aerobic trial with air blowing was monitored and summarized in Figure 4.13.

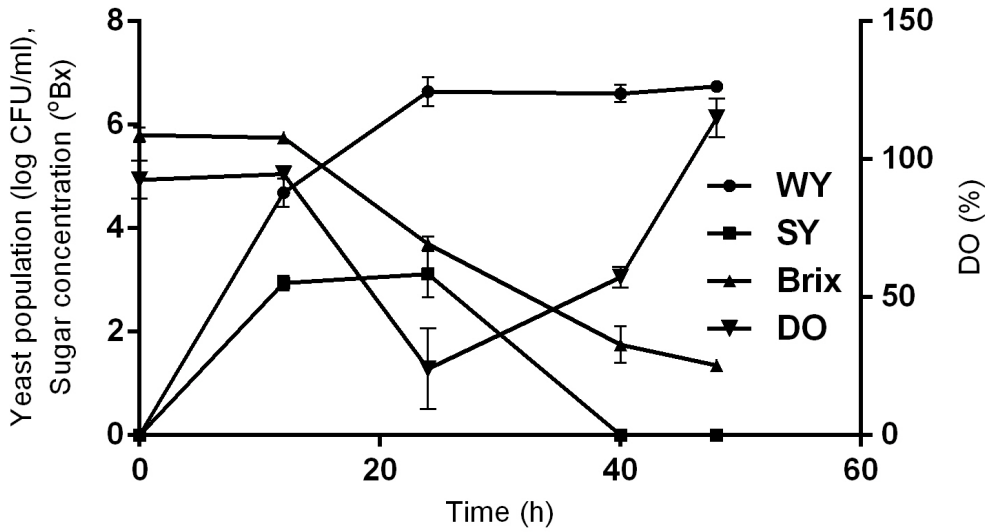


Figure 4.13 Growth patterns of yeast populations and sugar consumption under aerobic condition with filtered air blowing

If the hypothesis was correct, the spoilage yeast should grow and stay constant as the wine yeast, however, the population of the spoilage yeast still dropped to zero. As seen from the graph, the oxygen levels went down quickly as the wine yeast grew exponentially. After the wine yeast stopped growing, dissolved oxygen rose progressively, and then reached the initial concentration. This huge initial reduction of oxygen concentration might affect the growth of the spoilage yeast. Actually, the reduction is not avoidable due to the quick growth of the wine yeast. Respiration requires a large amount of oxygen for the reduction of energy. One glucose molecule needs six molecules of oxygen to produce carbon dioxide, water and energy. If the process of respiration in winemaking could be simulated, a better understanding of the balance of yeast growth and oxygen consumption could be achieved. On the other side, as the wine yeast grew fast, the media became more and more turbid, which made the shear force of breaking the

air bubbles greater. Therefore, unfortunately, an environment with constant oxygen in a co-culture system could not be created to maintain the spoilage yeast viability.

The wine media was then inoculated into a fresh minimal medium to check the recovery of the spoilage yeast. It was irreversible under laboratory conditions with minimum nutrient (data not shown).

In conclusion, oxygen became the most suspicious factor that caused the spoilage yeast to lose their budding abilities. Nevertheless, it cannot be strongly proven that it is sufficient and necessary by the current experimental instruments.

4.8 Late Inoculation and Pure Spoilage Yeast Trials

Because an aerobic environment could not be created for the spoilage yeast to maintain their culturability, different ways were tried to prove that the oxygen is the reason indirectly.

4.8.1 Late inoculation of the wine yeast

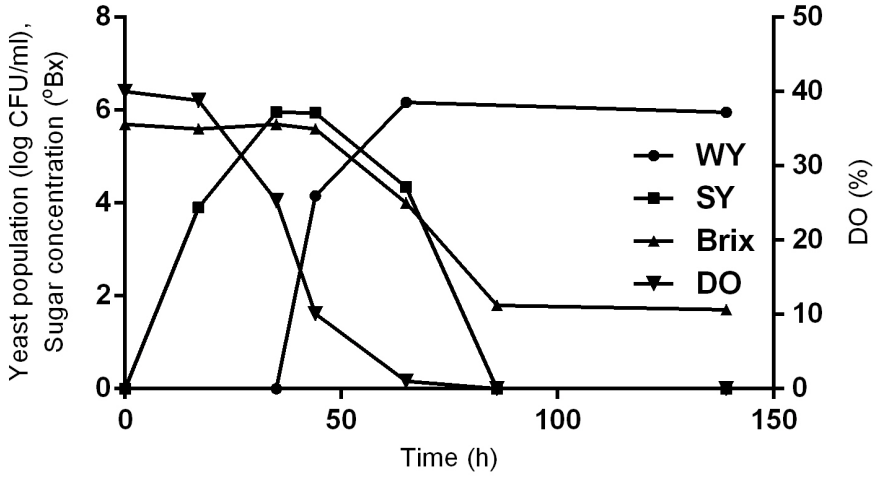


Figure 4.14 Effects of late inoculation of the wine yeast

Figure 4.14 shows the changes in several variables with late inoculation of wine yeast. This experiment was designed according to the concept of limiting the growth of the wine yeast to lower the oxygen consumption rate so that the gas-liquid mass transfer may be enough to keep the oxygen level constant. Like previous experiments, the sugar concentration was kept high before the wine yeast led the fermentation process. The wine yeast was inoculated at 48 h, and with the fast growth of the wine yeast, the sugar concentration dropped down to its lowest level within another 48 h. Secondly, the oxygen concentration went down as the wine yeast rapidly grew. Also within 24 h after the oxygen was consumed, the spoilage yeast became unculturable on agar plates. From this experiment, sugar content and oxygen concentration remained at high levels before the inoculation of the wine yeast.

4.8.2 Late inoculation of the spoilage yeast

This trial was also conducted by the concept of limiting the oxygen consumption rate. According to the aerobic fermentation trial, the oxygen level went back to the initial concentration after the wine yeast entered the stationary phase. Therefore, the spoilage yeast was inoculation after the wine yeast was stable.

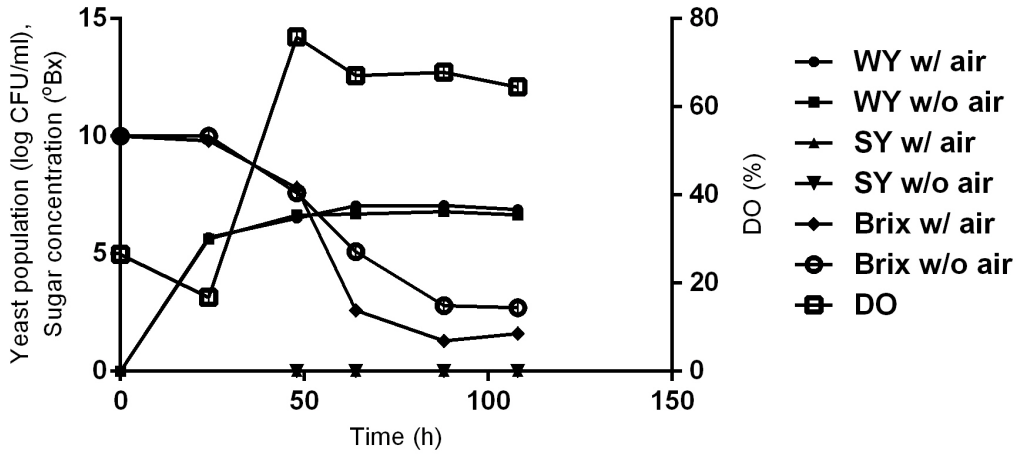


Figure 4.15 Effects of the late inoculation of the spoilage yeast

Figure 4.15 illustrates the curves of the wine yeast population, spoilage yeast population, sugar concentration and dissolved oxygen. Unfortunately, the environment that allowed the spoilage yeast to grow to a constant concentration was still unatainable. After the inoculation of the spoilage yeast, one of the two fermentation flasks was kept sealed while air was blown into the other one. The results showed that both of the batches were not able to support the growth of the spoilage yeast. The reason at this time may be the large sheer force introduced by bubbling.

Another interesting discovery in this trial was that the final sugar concentration with airflow was lower than that without airflow. Additional oxygen helped the wine yeast to ferment more sugar, which can be used in the dry wine production.

4.8.3 Pure culture of the spoilage yeast

Figure 4.16 summarizes the fermentation process of the pure culture of the spoilage yeast. The yeast population went up to 10^5 CFU/mL, which is the same as the wine yeast

population in a monoculture batch. It is clear that the discouragement of the spoilage yeast in a co-culture system is due to its slow growth rate. Also, the sugar concentration did not decrease as in the co-culture fermentation. This may be because the spoilage yeast utilized the respiration pathway during the process, which does not require as much sugar as the fermentation process. Further, the oxygen level dropped to approximately 10% and stayed constant, which is contradictory to the previous experiments. Therefore, the spoilage yeast can be classified as an aerobic microorganism according to this phenomenon. Besides, this trial indirectly pointed out that the anaerobic condition was the reason for the spoilage yeast to become unculturable.

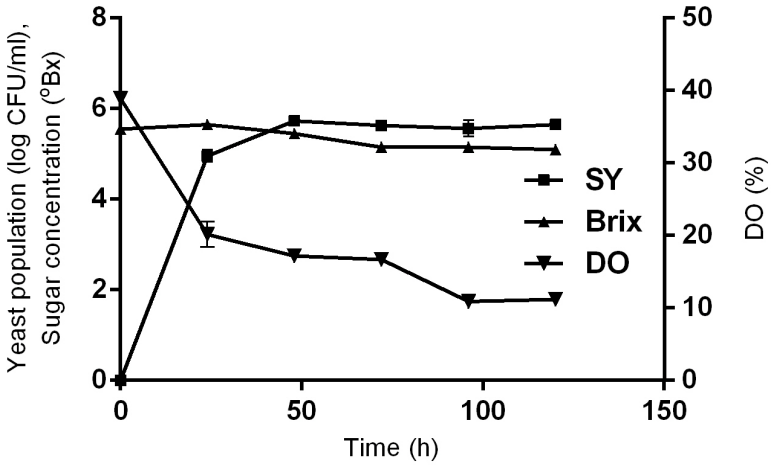


Figure 4.16 Pure spoilage yeast culture trial

In conclusion, from these additional experiments, the oxygen was still the most suspicious factor that limited the growth of the spoilage yeast. But with our current laboratory equipment, a fermentation condition that is favorable to both kinds of yeast could not be created. Usually, oxygen was quickly depleted with the reduction of sugar concentration in a co-culture system, which cannot be solved in a batch bioreactor.

Chapter 5 Conclusions and Future Work

In the previous chapters, the wine spoilage problem was stated, the experimental design was described and the results were analyzed and discussed as well. In this chapter, all the results will be summarized again and some future works will be derived from these conclusions.

5.1 Conclusions

This research focused on the growth curves of the wine yeast *S. bayanus* and the spoilage yeast *D. bruxellensis*, in order to propose a unique method to control the wine spoilage problem. At the beginning of the experiment, the unculturability of the spoilage yeast, which was shown as they always stopped multiplying on selective agar plates after 48 h, was observed. Therefore, the objective was changed from simulating the growth patterns of both yeasts to determining the factor that made the spoilage yeast to become unculturable. If we can artificially bring forward the unculturable state, the spoilage yeast population should stay low so that less off-flavor compounds would be produced. Therefore, at least five conclusions can be made from these experiments.

a. A new selective media was developed in this experiment to differentiate two kinds of yeast within 48 h. Experiments showed that these two agar plates, YM and YMC, were reliable in most wine spoilage conditions, because the antibiotic cycloheximide inhibited the growth of the wine yeast, but had no influence on the spoilage yeast.

b. Initial yeast concentration and different yeast combinations in a co-culture system did not affect the growth curves of both yeasts very much. As a fast growing yeast species, the wine yeast can always lead the fermentation process after 24 h according to the results. In a suitable growth condition, the growth rates of both yeasts were fixed, and would not be affected by the initial inoculation concentration. Thus, the initial yeast concentration is not a main consideration in the wine industry despite it being usually constant if the standard starter preparation protocols are followed.

c. Ethanol was produced after the wine yeast entered the stationary phase when the wine yeast started to ferment sugar. Both of the yeasts were tolerant to the ethanol concentration of up to 10% (v/v). However, pH did affect the growth of the spoilage yeast. Based on the experiment, the spoilage yeast population would be discouraged by the low pH, although it is not a sufficient way to control the wine spoilage problem because large amounts of acid solution would be used, which may also cause serious health problems.

d. The influence of different initial sugar concentrations was also evaluated in this work. Based on the experimental data, sugar concentration was not the limiting factor that caused the spoilage yeast to become unculturable. The experiments using three initial sugar levels showed that the spoilage yeast could grow at 3.8^{o}Bx sugar concentration but quit growing at a concentration above 5^{o}Bx.

e. Oxygen was the most suspicious factor that caused the spoilage yeast to become unculturable. The anaerobic fermentation confirmed the sufficiency of the low oxygen condition. However, the necessity still cannot be experimentally verified. At this point, the sugar reduction could also be argued to be the factor that made the

spoilage yeast unculturable. These two factors cannot be separated because the fermentation process occurred in a strictly anaerobic condition, which consumed much more sugar than respiration, and then was inevitably followed by a large sugar reduction. This issue might be solved by a continuous bioreactor, which will be discussed in the future work.

5.2 Suggestions for Future Work

First of all, a continuous bioreactor with pH and dissolved oxygen probes can be used in this work to monitor these factors during the whole fermentation process. Other than that, a continuous bioreactor is helpful because it can control the sugar and oxygen concentration in the tank by adding fresh media continuously. The total volume in the bioreactor can be controlled by a recycling system with a filter membrane to capture all the yeast cells.

Secondly, if the oxygen was proved to be the cause of the spoilage yeast becoming unculturable, and the oxygen level was constantly controlled in a bioreactor, then different oxygen level tests could be conducted to compare the final spoilage yeast population. According to these results, we would be able to confidently propose that an anaerobic condition could control the wine spoilage problem. Thinking widely, the winemakers might want to create an anaerobic condition at the beginning of fermentation to minimize the spoilage yeast population, then blow air to generate the growth of the wine yeast. This innovative prototype may change the whole wine fermentation process.

Furthermore, real juice could be used at the end to relate the spoilage yeast population with the off-flavor compounds by GC/MS. The bad aroma cannot be detected in the

minimal wine media because there were no precursor compounds in the media. Thus, the usage of real grape juice could help us better verify the relationship between the spoilage yeast concentration and the concentration of aroma compounds.

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