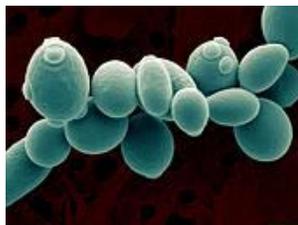


Basic Winemaking and Enology - 4

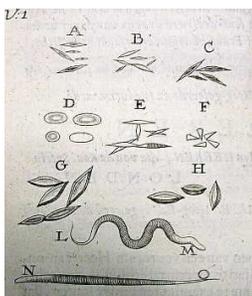


Saccharomyces cerevisiae var. *ellipsoideus*

Microorganisms and Fermentation. Wine yeasts are saprophytes. They are fungi living on dead or decaying organic matter. "Sapro" is from "sapro" (*rotten*) spoken by people of Greece starting about 1000 B.C. A saprophyte is any plant that depends on other dead plant or animal tissue for a source of nutrition and metabolic energy, e.g., most fungi (molds) and a few flowering plants, such as Indian pipe and some orchids. Most saprophytes do not produce chlorophyll and therefore do not photosynthesize; they are thus dependent on the food energy they absorb from the decaying tissues, which they help to break down.

The controlled fermentation of grape juice seems to be as ancient as Man's written records. Anton van Leeuwenhoek of Delft, the first to use the microscope in rigorous scientific studies, made his most important discovery early in his career. In 1674, he recognized the true nature of microorganisms. He began to observe bacteria and protozoa, or, as he called them his "very little animalcules", which he was able to isolate from different sources.

In 1687 he made drawings of what he titled "Crystals in Vinegar". The ellipses under D, are thought to be yeast cells.



Even though van Leeuwenhoek seems to have seen yeast, the role of yeast in winemaking was not understood until Louis Pasteur published "Etudes sur le Vin" in 1866. At the time of Pasteur, a large portion of the wines of Europe spoiled readily. He showed the basic understanding of fermentation and proposed the methods for controlling wines soundness. Pasteur's studies showed the microbiological basis for much of the spoiled wine at the time. His work was the foundation of present day microbiology. Soon the ecology of "native" yeast in the vineyards and winery, the use of single cell yeast starter culture in wine and beer production and the methods and source of malolactic fermentation were understood.

Although Pasteur showed the value of pure yeast cultures, it was not until the early 20th century that the Germans and French started isolating and using pure cultures. Most of this work was done in university laboratories. The commercial availability of yeast strains did not occur in the U.S. until the 1960's.

Up until this time, most American wine was in the dessert/appetizer class and because of their high alcohol and high SO₂, microbial spoilage was not a major problem. Soon, table wines become popular.

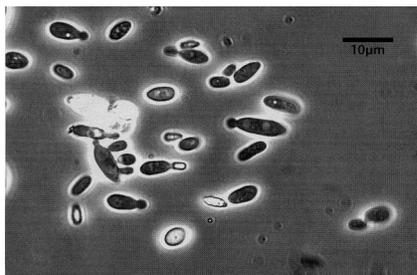
In the 1970's wine production doubled in the U.S. The production went from 80% dessert/appetizer to 80% table wine in the decade. Winemaking got much more complicated. The table wines most popular were not dry, but generally had a small amount of residual sugar. So the wines had lower alcohols and potentially fermentable sugar in the cellar tanks and bottle on the grocer's shelf.

By the 1980's the winery technologies had grown and most microbial concerns were controllable. The concept of sterilization became well advanced. Wines were made with varietal character as their calling card. At that time, wines were generally between 11% and 12.5% alcohol and pH's ranged from 3.2 to 3.5.

In the nineties, wine styles began to change. Winemakers were allowing grapes to remain on the vines to attain "fruit-forward" wine styles and higher pH's, new and native yeasts and bacteria were being used, new methods of maceration employed, *sur lie* aging was widespread, fermentation protocols varied and fermentations without SO₂ were being used. Some new microbiological spoilage problems began to take hold.

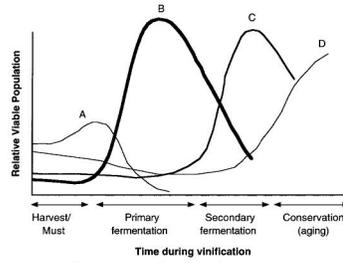
Wild yeast lives in the vineyards and wineries. The most important wine yeast is *Saccharomyces cerevisiae* var. *ellipsoideus*. At the beginning of 'natural' fermentation, the native yeast species are most active. These include yeast of the genera *Kloeckera*, *Metschnikowia*, *Hansenula*, *Candida* and *Hanseniaspora*. In cooler growing regions, *Kloeckera apiculata* seems to be the dominating yeast species on grapes, whereas in warmer regions it is *Hanseniaspora uvarum*. Many other strains exist.

In coastal California, *Kloeckera* normally represents the dominant native yeast on grapes at harvest.



Kloeckera apiculata-lemon shaped yeast

Like other non-*Saccharomyces* yeast, it possesses lower EtOH tolerance than *Saccharomyces* yeast. Even in non-inoculated fermentation, *Kloeckera* generally dies off when alcohol levels reach 5% to 6%. As the non-*Saccharomyces* yeast population fades, *Saccharomyces* will dominate and complete the fermentation.



- A. Growth of non-Saccharomyces yeast
 - B. Growth of Saccharomyces yeast
 - C. Growth of Oenococcus oeni
 - D. Growth of spoilage bacteria and yeast
- Wine Microbiology K. Fugelsang, et al

Today, commercial yeasts are available in dry or liquid form. Freeze dried yeast is easiest to use. Yeasts are grown in sterile media until vigorously fermenting. For inoculation, the optimum time to transfer yeast to another fermenter is when the inoculum is at 15° Brix. An inoculum of 1% to 5% (by volume) is added to the grape must or juice.

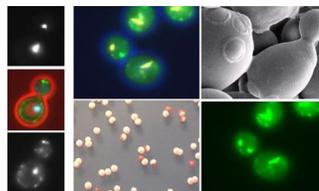
(See [Yeast rehydration.pdf](#))

In order to have a clean and completed fermentation, the following should be considered:

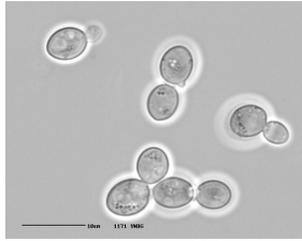
1. Yeast strain history
2. Lack of competition from undesirable yeasts
3. Inoculation volume
4. Yeast nutrients
5. Presence of O₂
6. Temperature
7. Additives in juice
8. Other wine components



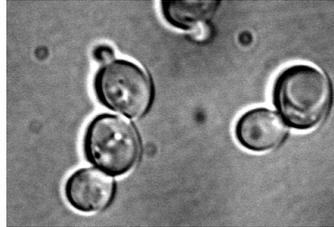
Saccharomyces Cerevisiae cells observed at the microscope



Saccharomyces cerevisiae* var. *ellipsoideus



Saccharomyces cerevisiae var. *ellipsoideus*

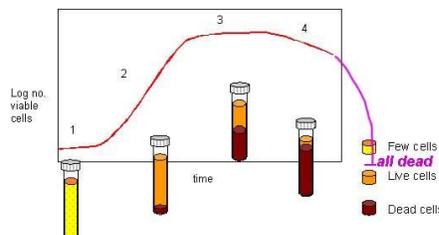


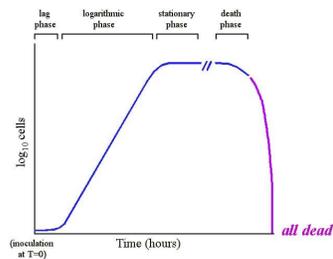
Saccharomyces cerevisiae var. *ellipsoideus*

After inoculation, the onset of fermentation in a batch of juice can occur in one day and is easily observed. In standard winemaking, yeast ferments sugar without O₂ (anaerobic) and largely convert it into EtOH and CO₂. In the presence of O₂ (aerobic), yeast convert sugar to CO₂ and H₂O and gains much more energy for growth.

Rapid multiplication of yeast cells at the start of fermentation is possible because of dissolved O₂ in the must or juice. After inoculation with yeast, enough O₂ is available in the juice to allow the yeast cells to multiply from 1 to 200 million cells/cc. This is the necessary growth phase. Then, when the dissolved oxygen is depleted, yeast growth slows and conversion of sugar to alcohol becomes the main reaction.

A yeast growth curve is a graph depicting stages of population growth and decline in a closed environment: First, there is exponential or logarithmic growth until carrying capacity of environment is approached; Second, a gradual increase and then leveling off or stationary phase at the peak; and Finally, a very rapid population decline to zero or death phase as the population is poisoned by its accumulated waste (EtOH) in a closed environment. .





Reds ferment quicker than whites because of higher fermentation temperature and oxygen additions during pumpovers. During fermentation, yeasts are influenced by temperature and must components and they also add both heat and other components to wine. Different yeast strains adapt to various temperatures. A good rule of thumb is, without heat loss, the temperature of fermenting must goes up 2.3 °F for each °Brix going down.

What conditions do yeast like and dislike? Al, Cu and Fe can all inhibit fermentation. At 65° to 75° F, most fermentation is completed in less than a week. At 40° to 50° F, most fermentation takes 2 to 6 weeks. Some yeast strains can ferment to 18% alcohol in making Sherries. Most yeast ferment glucose faster than fructose. Since fructose is sweeter, stopping fermentation before dryness gives higher fructose/glucose ratio and a sweeter taste than a wine sweetened with sucrose or grape concentrate. Without alcohol, yeast works best when sugars are 1% to 2% and are retarded at sugars >25%. German late harvests can have sugars of 40% to 65%. They ferment very slowly and yield alcohols of <9%.

H₂S production has been attributed to certain yeast strains, notably Montrachet. The problem is Montrachet gives incredibly aromatic, fruity wines. As mentioned, retained S from the vineyard, high Cu levels and lack of free amino nitrogen contribute to sulfide production. Bentonite added to juice prior to fermentation (reducing protein and foam) can also increase sulfides. Dirty and bentonited musts both ferment faster than settled, fined, centrifuged or filtered must. Juice solids <0.5% (v/v) are very slow fermenters and those with >2.5% are very fast.

Other Saccharomyces. The yeast used for sparkling wine production is usually *Saccharomyces bayanus*. It gives coarse heavy sediment that settles rapidly during fermentation. This aids in later disgorging.

In Sauternes (with high sugar because of Botrytis) *Saccharomyces bailii* ferments fructose faster than glucose giving less sweet and not cloying wines.

Spanish flor yeasts and those of the Jura of France use *S. bayanus* or *S. capensis*. California flor yeast is *S. fermentati*. Flor yeast forms a film on the surface of the fermenting wine containing 12% to 16% alcohol and oxidizes alcohol to acetaldehyde and other products that give flor sherry their unique character. In table wine, this character is considered to be spoilage.

In white wine fermentation the growth of yeast can sometimes stop at the point where ½ to 1/3 of the sugar still remains in the fermenting juice. The nongrowing, but viable, yeast cells complete the fermentation. Wine fermentation is a fermentation in which there are contributions from both cell growth and the resting phase activity.

Antimicrobial effects of sulfur dioxide. The addition of SO₂ to juice leads to the rapid killing of natural bacteria and yeast that had been present on the grape skins. Few studies exist as to the sensitivity and rate of death of specific yeast strains. It is generally assumed that the levels required for enzyme inhibition provides significant reduction in microflora viability or growth.

Levels of 25 to 75 mg/l of SO₂ in juice can lead to 75% to 95% inhibition in phenol oxidase activity. Enzyme inhibition can aid in reducing yeast viability.

The level of SO₂ sensitivity of wine yeast varies throughout the growth phase. The yeasts are more resistant as the stationary phase is approached. The existence of different survival patterns of log-phase growing cells and stationary-phase cells is apparent. The mechanisms of the yeast killing action are not clear, but a few theories are proposed.

Studies have shown that sulfur dioxide added to a solution of *Saccharomyces cerevisiae* was rapidly removed. It was suggested the sulfur dioxide was transported and bound to the yeast cell outer membrane with the aid of enzymes.

Most yeasts produce SO₂ by reducing sulfate in grape must. Acetaldehyde formed during fermentation binds bisulfite in wine, leaving little FSO₂. Wine generally needs SO₂ at dryness.

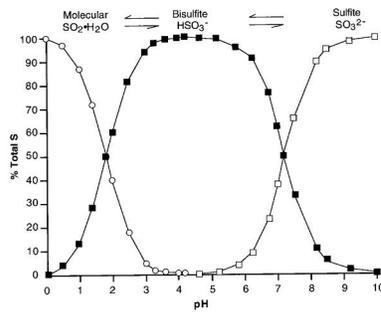
In fermentation, a carbon compound serves as a terminal acceptor of the electrons that are generated in the pathway in the course of converting the sugar metabolites to energy in the form of ATP. Cellular ATP is Adenosine triphosphate. It is an ester of adenosine that contains three phosphate groups; supplies energy for many biochemical cellular processes by undergoing enzymatic hydrolysis.

Some studies have shown that sulfur dioxide was binding to a membrane-bound compound causing uncontrolled loss of cellular ATP and hence cell viability. Cell membrane binding is suggested by some, others suggest that the molecular form of SO₂ enters the yeast cell by diffusion.

Little data exists to support any of the above proposals.

The toxicity of acidic forms of sulfite solutions has been studied for some time. Work has shown that the molecular form was several hundred times more toxic than the bisulfite form.





Relative abundance of molecular SO₂, Bisulfite and Sulfites vs. pH
Wine Microbiology K. Fugelsang, et al

Sulfur dioxide is a gas under normal conditions. It is very soluble in water. Approximately 40 volumes of SO₂ gas are soluble for each volume of water at 20 °C (68°F) and 55 volumes of SO₂ gas are soluble for each volume of water at 10 °C (50°F). The influence of temperature on solubility is important. It must be noted that higher percentages of SO₂ gas solution can be created at lower temperature, but that a saturated SO₂ gas solution at 50°F, if warmed to 68°F has the potential to release approximately 50 g/l (or 15 volumes) of SO₂ gas. So, be careful.

SO₂ gas, when dissolved in water, is a moderately strong acid. It has pK_a values of 1.77 and 7.20 while in wine or juice.

PK_a: Negative log of the dissociation constant (K_a) of an acid species.

Equilibrium Constants: Many chemical and biochemical reactions do not proceed to completion but instead reach an equilibrium condition at which point the forward and reverse reactions occur at the same rate such that there is no net change in product(s) concentrations. There are many factors that influence the establishment of equilibrium. The Principle of Le Chatelier states that equilibrium will shift in the direction that counteracts the applied force. For example, increasing temperature will favor the reaction that absorbs heat. There are many types of chemical and biochemical equilibria. Each type has an equilibrium constant, K, which describes numerically the relationship between the forward and reverse reactions. A large K value means the reaction is favored in the direction of that reaction while a low K value indicates that the opposite reaction is favored.

Type of Equilibrium	Reaction	Equilibrium Constant Expression	Equilibrium Constant
Water			
Dissociation	$2\text{H}_2\text{O} \rightleftharpoons \text{H}_3\text{O}^+ + \text{OH}^-$	$K_w = [\text{H}_3\text{O}^+] [\text{OH}^-]$	K _w : Ion product Constant
Acid-Base	$\text{HA} \rightleftharpoons \text{H}^+ + \text{A}^-$	$K_a = \frac{[\text{H}^+] [\text{A}^-]}{[\text{HA}]}$	K _d , K _a , K _b : Acidity (dissociation) Constant



The hydrated SO₂, or molecular form, is the major form below pH 1.86.

The molecular form seems also the most effective in reducing yeast viability. What level of molecular sulfur dioxide is needed to prevent growth of typical wine yeast and bacteria? The various studies on molecular sulfur dioxide requirements for yeast control are outlined below:

Medium	Molecular SO ₂ (mg/l)
Medium	1.3
Juice	6.6
Model wine	0.83
Medium	1.56
Wine	1.5

As reported in Principles and Practices of Winemaking

Winemakers are familiar with the concept of F/TSO₂. As sulfur dioxide is a very reactive substance, it binds with many compounds; acetaldehyde, sugars, anthocyanins and other carbonyl groups. This is the bound SO₂. As stated before, the FSO₂ is not bound and is the most effective part. The free and bound portions combine to give the TSO₂.



Because of its properties, FSO₂ is more significant. Remember, depending on the pH, various forms of sulfur dioxide are in a wine solution. From the studies available, the real effectiveness of sulfur dioxide as an antimicrobial and antioxidant agent is due to the molecular sulfur dioxide present in the wine. The problem is the molecular sulfur dioxide is only present in small percentages.

pH of wine	% of free SO ₂ in molecular form
3.0	6.0
3.2	4.0
3.4	2.5
3.6	1.5
3.8	1.0
4.0	0.6

A couple examples:

Wine X has FSO₂ of 20 ppm and pH of 3.0.

The molecular SO₂ level would be 6% of 20 ppm = 1.2 ppm

Wine Y has FSO₂ of 20 ppm and pH of 3.8.

The molecular SO₂ level would be 1% of 20 ppm = 0.2 ppm

What is the significance of this difference?

The level of molecular sulfur dioxide needed to stop bacterial growth and prevent any oxidation is believed to be at least 0.6 ppm. So, in the examples above, Wine x would be protected but Wine Y would not, even though it has 20 ppm FSO₂. The usefulness of molecular sulfur dioxide is also related to alcohol level. Understandably, the higher the alcohol, the less molecular sulfur dioxide needed.

The guidelines recommended are:

With 12% alcohol, a wine needs a minimum of 0.8 ppm molecular sulfur dioxide.

With 14% alcohol, a wine needs a minimum of 0.6 ppm molecular sulfur dioxide.

Estimating molecular sulfur dioxide. One molecular sulfur dioxide level will not satisfy the needs of all wines. Different levels are needed depending on the pH and alcohol. To get 0.8 ppm molecular sulfur dioxide, the following table can be used:

<u>pH</u>	<u>FSO₂</u>
3.0	13
3.1	16
3.2	21
3.3	26
3.4	32
3.5	40
3.6	50
3.7	60

The table can be handy. The level of molecular sulfur dioxide can also be calculated with a formula. It can easily be programmed into an Excel spread sheet.

$$\text{Molecular SO}_2 \text{ (ppm)} = \text{FSO}_2 / (1 + 10^{(\text{pH} - 1.83)})$$

This formula is not alcohol dependent. With the file Free SO₂ pH Distribution, FSO₂ levels can be found vs. pH to attain 0.8 ppm molecular SO₂.

An important note must be added. The Inter Winery Analysis Group did a recent survey of US winery lab accuracy. In 2002 they published results showing a coefficient of variation for both pH and sulfur dioxide in a laboratory proficiency program was between 19% and 39%. Any errors of this magnitude in lab analysis make all of the above of little value.

Finally, to achieve a level of 0.8 ppm molecular sulfur dioxide the following amounts of FSO₂ have been widely recommended:

White wines: pH 3.0 – 3.2 10-20 ppm FSO₂
pH 3.2 – 3.4 20-30 ppm FSO₂
pH 3.4 – 3.5 30-50 ppm FSO₂

Red wines: pH 3.4 – 3.6 10-20 ppm apparent FSO₂, or
50-150 ppm TSO₂

Sulfur dioxide behaves differently in red wine than white. The pigment in red wines also binds with sulfur dioxide. Therefore the FSO₂ measured in red wines (apparent FSO₂) is not the same as in white wine. The level of molecular sulfur dioxide is not changed, but the FSO₂ amount is different.

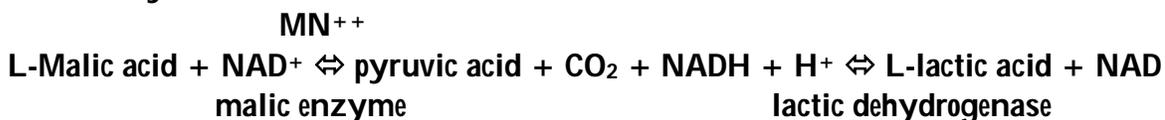
What is suggested is, if you want to stay clear of microbial troubles, don't make white wine at pH of 3.5 and above or red wines at pH of 3.6 and above.

Bacteria. The antibacterial properties of sulfur dioxide are not any more straight forward. Studies have shown both the free and bound form of sulfur dioxide can inhibit bacterial growth. The pH is also very important. Wine yeasts are quite tolerant of pH. Yeast growth does not change significantly over the normal range of wine pH values, and overall fermentation characteristics are little affected by pH. On the other hand, wine bacteria do not tolerate low pH values, and wine pH strongly influences both bacterial growth rate and bacterial fermentation characteristics. This is why malolactic fermentation is not likely to occur in wines with pH values lower than 3.3. Bacterial activity is reduced in low pH wines, and many of the bacterial problems become insignificant when wine pH is low.

Some forms of bacteria have been shown to bind with acetaldehyde. The bacteria consumed some of the acetaldehyde present, releasing free sulfur dioxide that prevented further bacterial growth. Some forms of bacteria were not hampered. One study found effects on both growth and malolactic fermentation due to both free and bound sulfur dioxide at pH 4.8. Under wine conditions (pH 3.5), it was found that 10 mg/l of acetaldehyde-bound sulfur dioxide reduced growth significantly, but not completely. At 30 mg/l, loss of all viable cells occurred.

More importantly the primary MLF bacteria, *Oenococcus oeni* (*Leuconostoc oenos*) has been found to be especially sensitive to levels of acetaldehyde-bound sulfur dioxide in the 20 mg/l to 60 mg/l range. It is thought that this is due to the release of free sulfur dioxide on the consumption of acetaldehyde or its sensitivity to its bound form.

Malolactic bacteria and malic fermenting yeast. Cool region grapes are high in acidity and have high ratios of malic acid to other acids. A malolactic fermentation (MLF) converts L-malic (a diacid) to L-lactic (a monoacid) acid plus CO₂, and other products. NAD-Nicotinamide-adenine dinucleotide plays a central role in yeast metabolism. NAD/NADH: Nicotinamide adenine dinucleotide is an important coenzyme in oxidation reduction reactions. Enzymes and salts aid in the reaction.

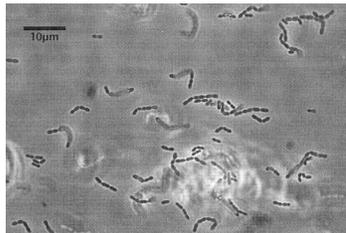


A molecule not superimposable on its mirror image is referred to as "chiral". Achiral is superimposable. Chiral molecules possess optical activity; they rotate the plane of plane-polarized light. Achiral do not. Some molecules rotate light clockwise (+) and some counterclockwise (-). They are called (+) d-dextrorotary and (-) l-levorotary.

After MLF, the acidity is halved. It can reduce T.A. by 1/3. This bacterial fermentation generally follows sugar fermentation. It increases wine complexity, but decreases fruitiness. It is done to reduce acidity but also to microbiologically stabilize the wines, even in warm regions like California. In warm regions, winemakers then must acidulate wines with tartaric, citric or d-malic acid. MLF gives reduced acidity and produces lactic ester, acetoin, and diacetyl (like melted butter) which account for aroma and taste changes. During MLF the wine gives off odors which go away or can be removed at MLF completion with racking. Few German producers use MLF since delicate white wines show "off" odor easily.

To prevent MLF from occurring, keep the wine clean, have good levels of molecular SO₂ and store in containers with untainted history. Once MLF has occurred in a wooden container, it is almost impossible to prevent future residents from doing the same. New wines can undergo MLF when kept on lees, *ala sur lie*, but not always. ML bacteria thrive in the presence of autolyzed yeast. The important thing is to maintain the molecular sulfur dioxide level at 0.8 ppm. It also helps if storage temperature is < 60 °F.

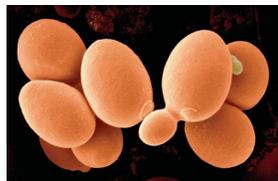
To encourage MLF, add the culture before the yeast fermentation is completed, at about 5 °Brix. After completion of alcohol fermentation, leave the wines (whites) *sur lie*, keep the temperature > 65 °F and add no SO₂. The primary MLF strain is *Oenococcus oeni* (ML 34, PSU 1, among others).



Oenococcus oeni (*Leuconostoc oenos*)

They seem to grow better in red wines containing grape skins. A good culture medium is apple juice diluted with 4 parts water. ML culture can be added to reds at about 5 °Brix and should be at 1% to 5% (v/v) of juice. In whites, skin contact helps ML culture. Inoculate after yeast fermentation has started when there is no FSO₂, but before alcohol gets too high. If you have no MLF bacteria, it is also been possible to add 5% wine that has undergone MLF or putting wine into barrels which contained wine that had finished MLF. Be careful that the previous MLF had been clean. Tracking MLF can be easily done with paper chromatography or more costly enzymatic methods. Measuring the T.A. and pH is not a reliable tracking method. All you care to know is if all the malic acid is gone. Bacteria can produce lactic acid from carbohydrates in wine and KHT can ppt. during fermentation and reduce acidity. ML bacteria can be removed through 0.45 μ (micron) membrane filters. MLF in the bottle can produce haze, gas, acid reduction and a sauerkraut aroma.

Yeast and bacteria that causes spoilage. *Candida vini* and *C. valida* are film yeasts that grow on low alcohol wines. They oxidize alcohol to CO₂ in the presence of O₂. To prevent, keep containers full. This is a very common problem. *Candida stellata* can produce a mousy odor in wine.



Candida stellata CBS 157

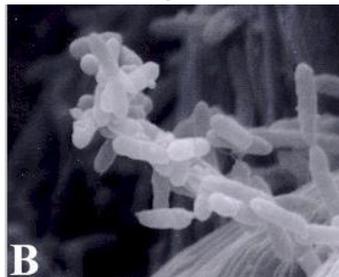
Dekkera/Brettanomyces is a spore forming yeast that produces a "horsey" or "barnyard" nose. Most winemakers consider "Brett" and its sporulating equivalent to threaten wine quality. The sensitivity of the spoilage yeast *Brettanomyces* to sulfur dioxide is a major concern to present

day winemakers. It is generally accepted to have 0.8 mg/l of molecular sulfur dioxide to control *Brettanomyces* and any other spoilage organism.



The lactic bacteria can cause problems. They grow best without O₂. These include *Lactobacillus*, *Leuconostoc* and *Pediococcus*. They produce haze, sediment and sour "sour milk" nose.

Acetobacter aceti, *A. pasteurianus* and *A. peroxydans* are found in wine.



Electron micrograph of *Acetobacter diazotrophicus* cells held together by a mucilage-type material found inside the sugarcane tissue that was colonized by this bacterium



Bacteria which turn alcohol and some sugars into acetic acid or vinegar; *Acetobacter aceti*.

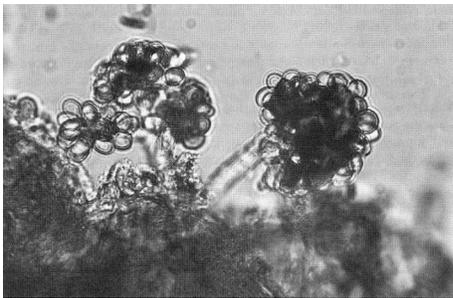
They produce EtAc which smells like nail polish remover. They form a very thin surface film. Alcohols >15%, pH <3.2 and FSO₂ > 100 ppm discourage acetic bacteria. Stuck fermentation wines are susceptible to spoilage. Without fermentation, there is no CO₂ protection. Pomace rapidly acetifies and fruit flies (*Drosophila melanogaster*) carry acetic bacteria.



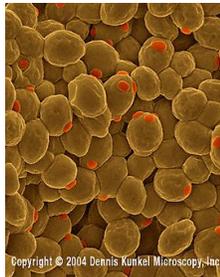
or for more spoilage odors



Lactobacillus trichiodes grow on fortified wines up to 21% alcohol and forms Mannitol (a hexa-alcohol, common in late harvest German wines) from fructose and also CO₂, EtOH, HAc and lactic spoilage. The bacteria form a protective coating of polysaccharides. The wine becomes so thick, a finger dipped in the wine can lift a "rope"; hence "ropiness" is used to describe the condition.



Botrytis cinerea (Gray mold)



Saccharomyces cerevisiae var. *ellipsoideus*



Saccharomyces cerevisiae var. *ellipsoideus*

All photos were given by Isak S. Pretorius, The Australian Wine Research Institute