Section 3 - The Alcoholic Fermentation
Lesson 8: Introduction

Yeast Biology

In this section of the course we will cover the primary fermentation, the conversion of sugar to ethanol, which is the foundation of the transformation of grapes into wine. The first lecture will cover the basic biology of the yeast *Saccharomyces*. Subsequent lectures will cover all aspects of fermentation management, and the problems that can arise. Principle among these problems is off-character production and slow or incomplete fermentations.

The alcoholic fermentation is conducted by yeast of the genus *Saccharomyces*. The two common species involved are *S. cerevisiae* and *S. bayanus*. These two species are closely related, and the subject of a continuing debate among taxonomists as to whether they constitute separate species or races of the same species. *Saccharomyces* converts the glucose, fructose and sucrose found in grape must and juice into ethanol via the process of fermentation. In fermentation, an organic compound, in this case acetaldehyde, serves as terminal electron acceptor. This leads to the production of ethanol.

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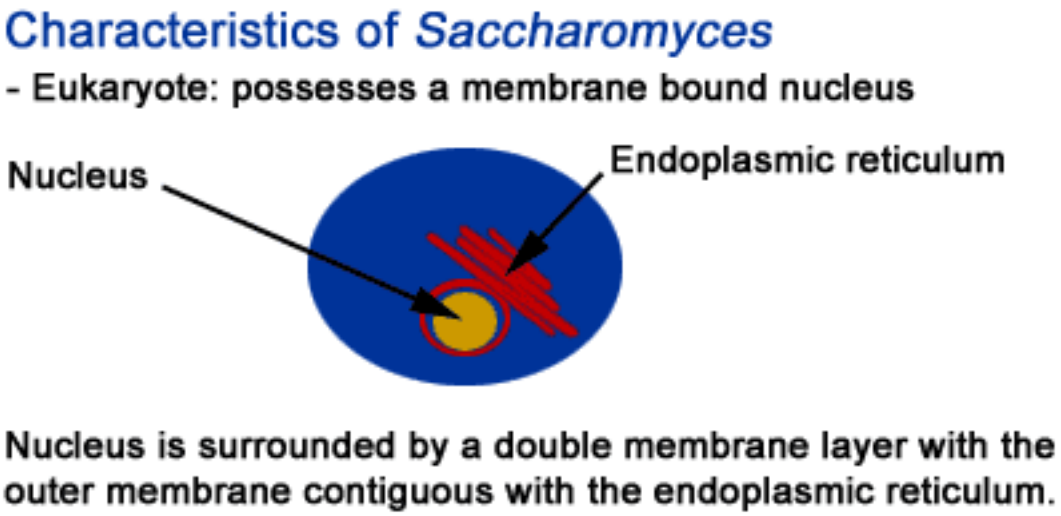
Lesson 8: Yeast Biology

Characteristics of *Saccharomyces*

- **Eukaryote: possesses a membrane bound nucleus**
- **Reproduces by budding**
- **Grows vegetatively as haploid (1N) or diploid (2N)**
- **Capable of conjugation (1N to 2N) and sporulation (2N to 1N)**
- **Non-motile**

Saccharomyces is a Eukaryote

Saccharomyces is a member of the kingdom of fungi. Fungi possess plant-like cell walls, but have other features more in common with animals. A significant amount of information is known about *Saccharomyces* due to the utility of this organism as an experimental system. Many of the fundamentals of genetic inheritance in eukaryotic cells were initially identified and studied in this yeast. The fungi are eukaryotic organisms meaning that they **possess a membrane bound nucleus**.

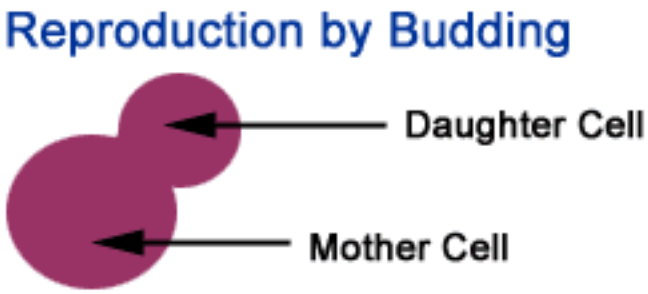


The nucleus has a double membrane structure. The outer membrane is contiguous with an organelle known as the endoplasmic reticulum. The endoplasmic reticulum

(ER) is involved in secretion of extracellular proteins and in *de novo* biosynthesis of the plasma membrane.

Saccharomyces Reproduces by Budding

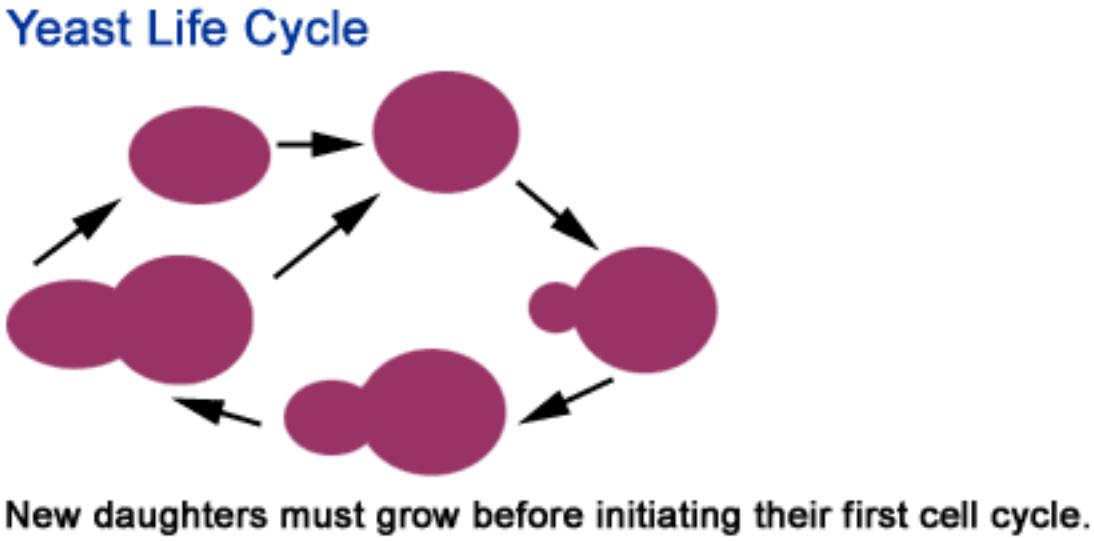
Saccharomyces reproduces by a process called budding. A mother cell initiates a new replication cycle by formation of an immature bud. This process is called bud emergence.



The emerging bud is referred to as the daughter cell, and it appears at one end of the mother cell. This yeast displays multilateral budding, meaning that the site of selection of a new bud is toward one of the poles of the cell, where the curvature is greatest, but is not restricted to the pole. Each time a new bud is produced, a circular scar, called a bud scar, is left at the site of bud emergence. Counting of the number of bud scars is an indication of the number of cell divisions a particular mother cell has undergone. Yeast cells are mortal, meaning a limited life span. On average, a cell can only undergo roughly 40 cell divisions. After this point, the cell is no longer able to divide.

Saccharomyces Grows Vegetatively as Haploid (1N) or Diploid (2N)

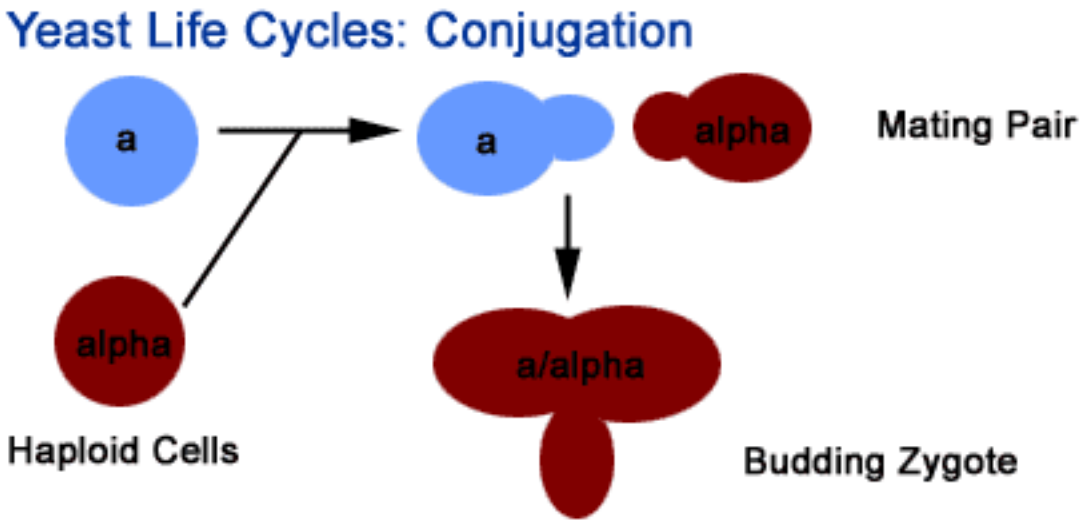
Saccharomyces can grow vegetatively as either a haploid or a diploid. Haploid cells have one set of chromosomes (1N) and diploid cells have two sets (2N). Many other organisms can only grow vegetatively as a haploid or a diploid, with the other state serving only for reproductive purposes.



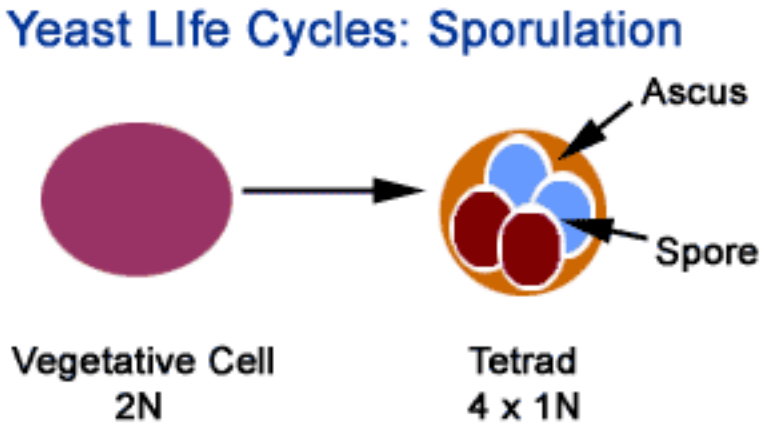
Budding is asymmetric, meaning that the daughter cell is typically smaller than the mother cell, depending upon growth conditions. Daughter cells must grow to a critical mass before initiating a new cell cycle, that is, before becoming a mother cell. This serves to make sure that sufficient nutrients are available for the next cycle to go to completion. Yeast cells divide only under conditions of nutrient sufficiency. Their resting state is as an unbudded cell. After separation from the mother cell, the bud assesses the nutrient composition of the medium before making the decision to enter a non-growing or stationary phase or to divide.

Saccharomyces is Capable of Conjugation (1N to 2N) and Sporulation (2N to 1N)

Haploid cells of *Saccharomyces* can mate and produce a diploid cell. There are two yeast sexes or mating types. These have been termed "a" and "α".



Each of the mating types produces a peptide mating factor or pheromone that serves to signal their presence to cells of the opposite mating type. When two cells of the opposite mating type are near each other, they respond to the presence of mating type factor by growing in the direction of each other. This process is called shmoo formation. When the surfaces of the two haploid cells contact, fusion of the cell walls and membranes occurs. This is followed by fusion of the two nuclei and formation of a zygote. The zygote gives rise to 2N or diploid buds.



Diploid cells can generate haploid cells via the process known as sporulation. Under appropriate environmental conditions the diploid cell decides that rather than undergo vegetative cell division, a reductive or meiotic cell cycle will occur. In this process, the replicated DNA is divided into four nuclei, two each of the a and &945; mating types. The nuclei are then surrounded by cytoplasm and a plasma membrane and cell wall. The four spores that result are still housed inside the mother cell, which has the appearance of a sac. This is why the yeast *Saccharomyces* is classified as an ascomycete. Ascomycete means that sexual spores are formed with in a sac or ascus.

Because four spores are formed, the ascus is called a tetrad. The purpose of alternating haploid and diploid life cycles is genetic reassortment. That is why these spores are called sexual spores. They should not be confused with the asexual spores produced by bacteria and other fungi that function as highly resistant cellular forms. The sole purpose of the haploid spores after germination is mating. *Saccharomyces* strains may be either heterothallic, not self fertile, or homothallic, self-fertile. Heterothallic yeast strains produce spores that need to find a spore of the opposite mating type in order to form a zygote. They can mate with a sister spore (spore from the same ascus) or a non-sister spore. In contrast, homothallic yeast strains can mate with their own vegetative progeny. That is, a mother cell gives rise to a bud of the same mating type as the mother, then the mother cell switches to the opposite mating type, and can then mate with the daughter.

Saccharomyces is Non-motile

Saccharomyces is not motile, meaning that the cells do not display chemotaxis and the ability to move toward or away from specific environmental conditions. *Saccharomyces* displays the sub-cellular organization of the typical eukaryote. The outer most surface of the cell is comprised of glucan and phosphomannan, forming a tough cell wall. The cell wall is therefore composed of carbohydrate and protein.

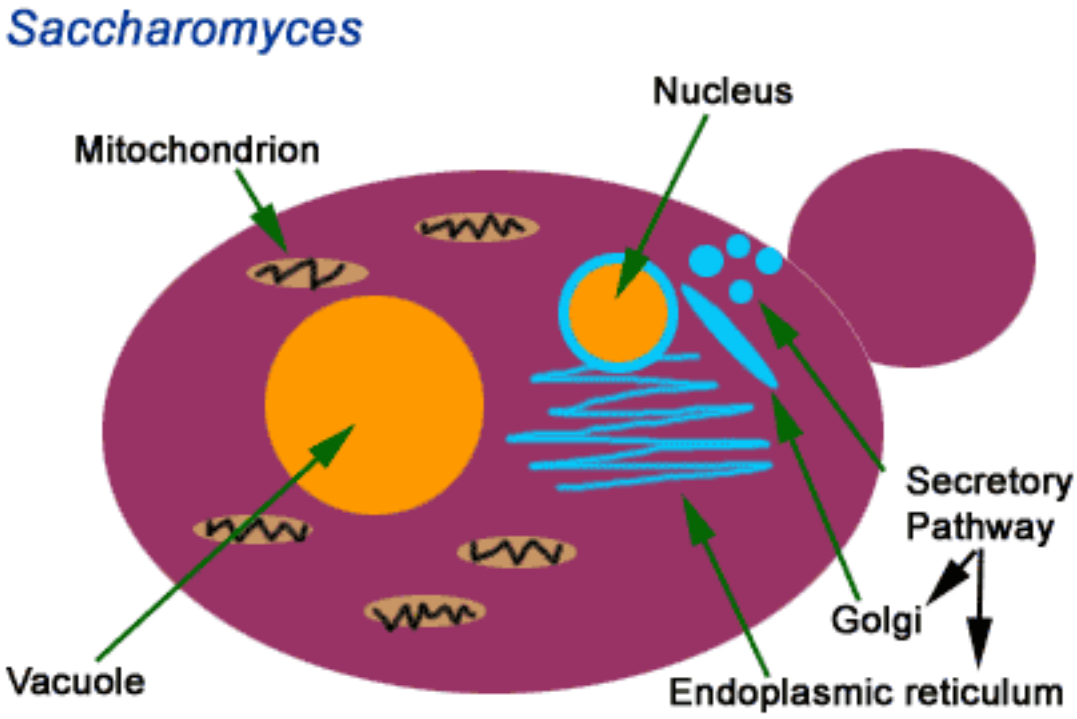
Characteristics of *Saccharomyces*: Sub-cellular Organization

- **Plant-like cell wall: comprised of carbohydrate (glucan, mannan) and glycosylated protein (phosphomanno protein)**
- **Mitochondria: site of oxidative reactions**
- **Vacuoles: site of storage and hydrolysis**
- **Secretory pathway**
- **Nucleus**

Saccharomyces cells possess **mitochondria**, the site of oxidative phosphorylation and respiration. *Saccharomyces* can generate energy via respiration, with oxygen as the terminal electron acceptor producing water, as well as via fermentation. Other key biological activities are also localized in the mitochondria. Oxidative biosynthetic (fatty acid biosynthesis) and degradative reactions (proline degradation) are confined to the mitochondrion as well. This serves to limit the potential damage to other cellular components of any errant oxygen radicals that might be produced as a consequence

of enzymatic reactions involving molecular oxygen.

When viewed under a microscope, yeast cells contain a darkly visible circular structure. This is frequently confused with the nucleus, but it is instead another organelle, the vacuole. The vacuole houses hydrolytic enzymes and is the site of degradation of cellular components that are no longer needed. The advantages of locating these damaging activities in an organelle are numerous. The **vacuole** is also the site of cell storage. Excess amino acids, phosphate and other compounds are stored in the vacuole. In this case, the vacuole serves the same purposes in both yeast and plant cells.



The chromosomal DNA is housed in the **nucleus**. *Saccharomyces* possess 16 chromosomes. The sequence of the entire *Saccharomyces* genome has been determined. All of the candidate genes are now known. Systematic studies are underway to determine the function of each gene.

Yeast also possess a typical eukaryotic **secretory pathway**. The secretory pathway is comprised of the endoplasmic reticulum, Golgi bodies and secretory vesicles. New cell wall and plasma membrane growth occurs at the junction between the mother cell and growing bud. Cytoskeletal elements target the fusion of secretory vesicles to the region of rapid growth. For proteins destined for the cell surface, protein synthesis is initiated in the cytoplasm. Ribosomes synthesizing surface proteins associate with specific receptor proteins on the surface of the endoplasmic reticulum. The nascent or growing

peptide chain is then inserted across the ER membrane into the lumen of the organelle. The protein is then processed through a series of organelles, the Golgi bodies and secretory vesicles, and arrives at the cell surface fully modified and adjacent to the proper proteins with which it interacts.

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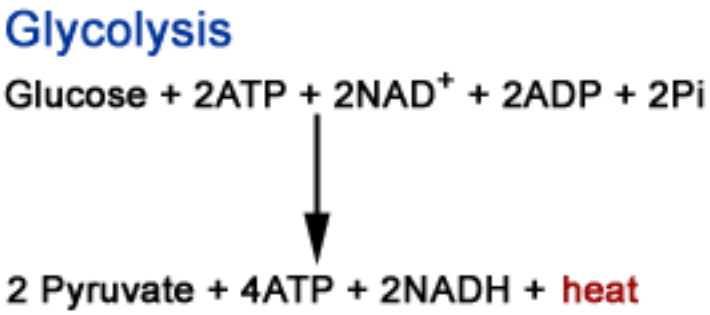
Lesson 8: Glycolytic Pathway

The universal biochemical pathway by which sugars are degraded in an energy-yielding process to the three carbon compound pyruvate is called glycolysis. This pathway is found throughout the plant, animal, fungal, bacterial and archae kingdoms. Energy is generated in the form of ATP via a process called substrate level phosphorylation.

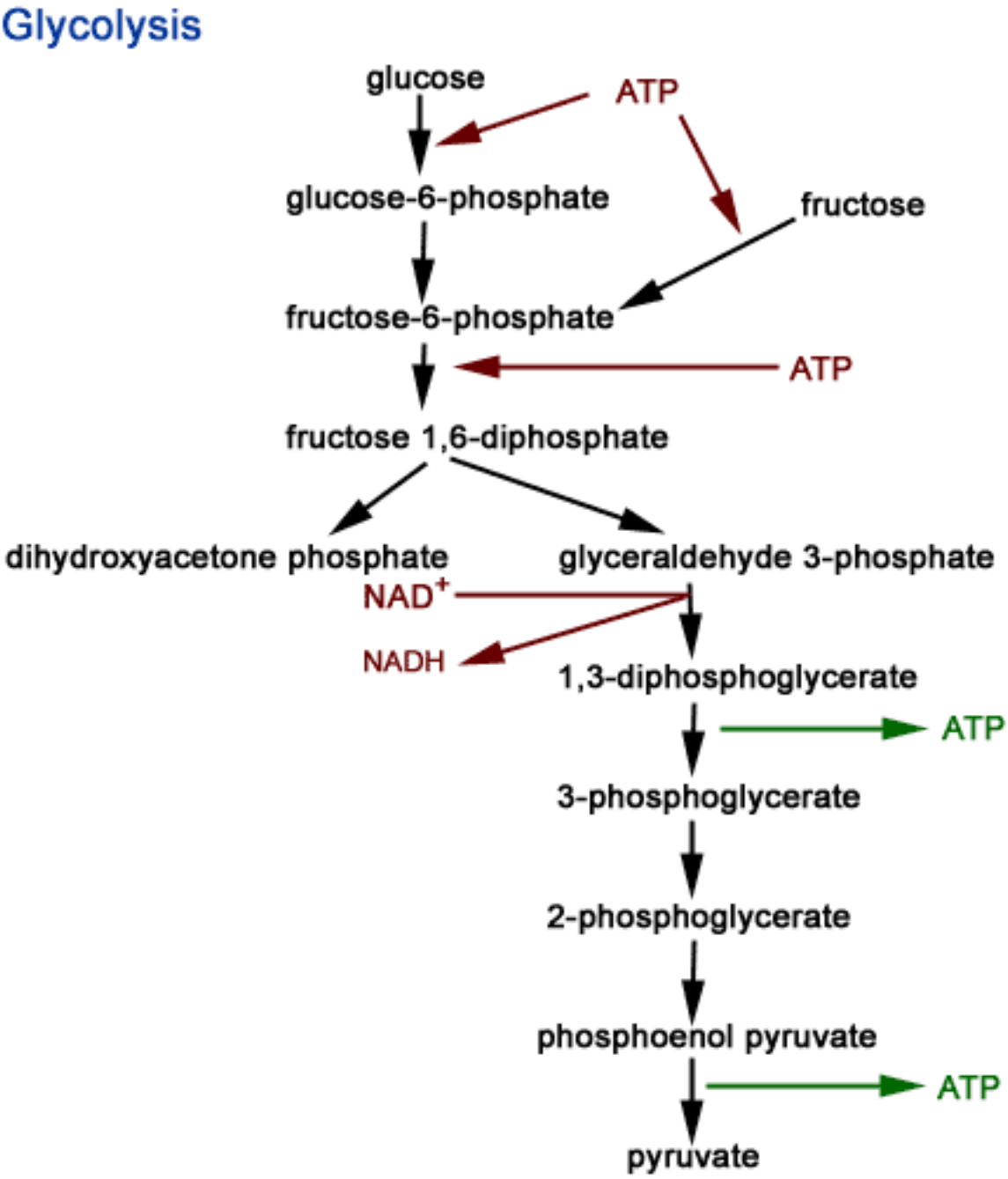
Glycolysis

- **The set of biochemical reactions converting hexose (6 carbon) sugars to two 3 carbon pyruvate molecules, during which energy is released and recaptured in the form of ATP.**

We can think of glycolysis as a process rearranging the energy in the bonds of a sugar molecule, so that a high-energy bond is formed that can then transfer that energy in a conservative manner to ADP generating ATP, the universal energy source. The energy in the ATP bond can be used to drive energetically unfavorable reactions.



This process requires the cofactor NAD+ that is converted to the reduced form NADH. Heat is also given off as an end product of glycolysis. One sugar molecule plus two ATP and 2ADP molecules are converted into 2 pyruvate and 4 ATP molecules. Early steps in the glycolytic pathway consume ATP. The first reaction is a phosphorylation of glucose (or fructose) at the six position.



With glucose as substrate, the second reaction is the isomerization of glucose-6-phosphate to fructose-6-phosphate. A second phosphorylation then occurs forming fructose 1,6-fructose diphosphate. Phosphorylation occurs to facilitate downstream rearrangement of bond energies. Fructose 1-6 diphosphate is cleaved into two three-carbon molecules, dihydroxyacetone phosphate and glyceraldehyde-3 phosphate. Triosephosphate isomerase interconverts dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Glyceraldehyde-3 phosphate is the entry molecule for rearranging bond energy. One ATP molecule is generated in the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate. The second ATP molecule is formed from phosphoenol pyruvate with pyruvate as the ultimate end product. At this point four

molecules of ATP have been produced, two replacing the initial ATP molecules used in phosphorylation of the sugars, and two net ATP molecules. Upper glycolysis refers to the ATP consuming steps and lower glycolysis refers to the steps that generate net ATP. It is important that upper and lower glycolysis be coordinated.

"Upper Glycolysis": consumes two molecules of ATP

"Lower Glycolysis": produces four molecules of ATP

NET PRODUCTION: TWO MOLECULES OF ATP

Sophisticated regulatory mechanisms exist to make sure that energy is not consumed in upper glycolysis unless lower glycolysis is fully functional.

Regulation of Glycolysis

- **Transport: site of global rate control**
- **Allosteric enzymatic steps: localized rate control**
  - **Hexokinase**
  - **Phosphofructokinase**
  - **Pyruvate kinase**
- **Effectors of regulation: ATP, ADP, AMP, fructose2, 6 bisphosphate, citrate, glucose**

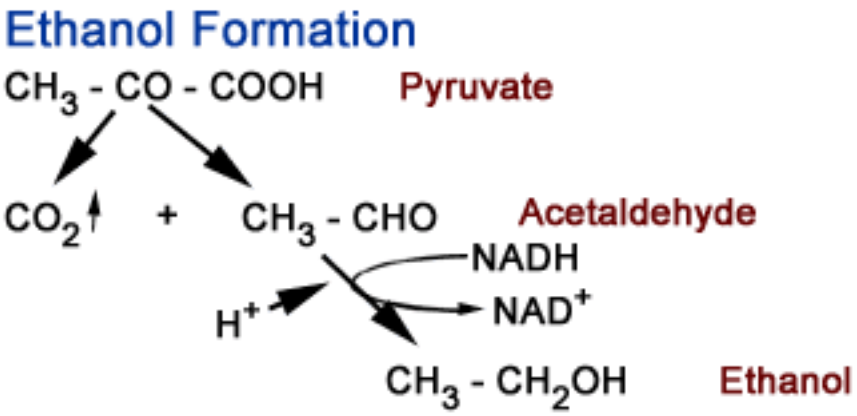
The most important site of control of the rate of carbon flux through glycolysis is transport of the sugar into the cell. If problems arise limiting the glycolytic pathway, the rate of uptake is adjusted to match the maximal rate of flux through the entire pathway. This is a global response to limiting metabolic conditions. Internal pathway controls also exist to adjust the rates of flux between upper and lower glycolysis. Three key enzymes, hexokinase, phosphofructokinase and pyruvate kinase are regulated by small molecule effectors. These enzymes catalyze important reactions in the pathway. Hexokinase is responsible for the phosphorylation of glucose and fructose and phosphofrucotkinase converts fructose-6-phosphate to fructose 1,6-diphosphate. These two enzymatic reactions are ATP consuming. Pyruvate kinase generates pyruvate from phosphoenol pyruvate, generating ATP. It is not surprising that these key ATP consuming and producing reactions are tightly coordinated with other metabolic activities of the cell.

The production of pyruvate serves the purpose of generation of chemical energy in the form of ATP. But this process converts two molecules of the cofactor NAD+ to two molecules of NADH. Continued operation of the pathway necessitates converting the NADH back to NAD+.

Where does ethanol come from?

- **The end products of glycolysis are pyruvate and 2 molecules of the reduced co-factor NADH.**
- **Yeast cells regenerate NAD+ by transferring the hydrogen molecule (electron) to an organic molecule: acetaldehyde**

In the alcoholic fermentation, pyruvate is decarboxylated to acetaldehyde, which is then reduced to ethanol with concomitant formation of NADH.



Carbon dioxide is also an end product of glycolytic metabolism. Thus, the 6-carbon sugars are converted into 2 one-carbon molecules of carbon dioxide and 2 of the two-carbon molecule ethanol. Other organisms use other means to regenerate NAD+. Some reduce pyruvate directly to lactic acid.

Other organisms use different strategies to generate NAD+.

Their presence in wine leads to a diversity of end products of sugar catabolism.

The majority of the sugar is used to generate ATP. Respiration generates a greater yield of ATP (36-38) per glucose molecule and glucose is converted to six molecules of carbon dioxide. This is more efficient in terms of optimization of energy production when sugar is limiting. However, if substrate is plentiful, respiration is not necessary as energy needs can be met solely from fermentation. Roughly 95% of the hexose consumed appears as carbon dioxide and ethanol.

Carbon Distribution at End of Fermentation

- **95% = Carbon Dioxide + Ethanol**
- **1% = New Cells**
- **4% = Other End Products**
  - **Pyruvate**
  - **Acetate**
  - **Acetaldehyde**
  - **Glycerol**
  - **Lactate**

Approximately 1% of the substrate is used as building blocks for new cells. About 4% of the initial sugar carbon will appear as other compounds, such as pyruvate, acetate, acetaldehyde, glycerol and lactate. The percentage varies, depending upon environmental conditions. Glycerol is produced from dihydroxyacetone phosphate. NADH is consumed in the process of making glycerol. Therefore glycerol production is increased under conditions limiting NAD^+ generation from pyruvate. For this reason one tends to find production of glycerol accompanied by pyruvate release.

The theoretical maximum yield of ethanol is roughly 0.6 times the initial Brix reading. In practice, the actual yield is around 0.55 times the initial Brix.

Ethanol Yield

1M glucose (fructose)

↓

2 M CO_2 + 2M ethanol

Theoretical Maximum:

180g 2(46g) = 92/180 = 51.1% w/w
= 63.9% v/w = ~0.6 original Brix value

One of the interesting properties of *Saccharomyces* is that given a high enough concentration of sugar, they will ferment even in the presence of oxygen.

Yeast will ferment even in the presence of oxygen. Why?

On the surface, this would seem kind of foolish since energy generation is more complete with respiration. If you can obtain between 36 to 38 ATP's per molecule of glucose, why would a cell settle for 2? It is actually more rapid to get ATP simply from glycolysis and fermentation than from respiration. Further, the phenolic compounds present in grapes may interfere with respiration. This is one of the principle classes of anti-fungal compounds produced by the grape. Ethanol is also inhibitory to respiration.

Fermentation vs. Respiration

- **Fermentation: 2 ATP/glucose (fructose)**
- **Respiration: 36-38 ATP/glucose (fructose)**

Efficiency of ATP yield is only an issue if sugar is limiting

Thus, *Saccharomyces* has developed biological mechanisms to assure that fermentation will occur in preference to respiration. Glucose itself regulates the switch between fermentation and respiration. High sugar concentrations repress the synthesis

of mRNA from genes that are involved in respiration and oxidative metabolism.

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Lesson 8: Choice of Yeast Strain

Individual yeast strains possess different physiological traits. There are several traits that are highly desired in wine strains of *Saccharomyces.*

Yeast Choice: Desirable Traits

- **Fermentation to dryness**
- **Reasonable rate of fermentation**
- **Predictable fermentation characteristics**
- **Good ethanol tolerance**
- **Good temperature tolerance**
- **Sulfur dioxide tolerance**
- **Little to no off-character production**
  - **Hydrogen sulfide**
  - **Acetic acid**
  - **Ethyl carbamate**
- **Little to no inhibition of other desirable microbes**
- **Killer factor resistant**
- **Production of desired aroma characters**

The most important characteristic is that the strain be able to complete the fermentation, **leaving little to no residual sugar**. It is also critical that the strain display a **reasonable rate of fermentation**. It is problematic if the rate is too fast as well as too slow. A slow rate of fermentation becomes difficult to distinguish from a problem fermentation. If the rate is too fast, the fermentation may reach too high of a temperature due to the rate of heat release from metabolism. Rapid fermentations may also lead to increased loss of volatile components. It is also valuable if the strain is **predictable in terms of its fermentation characteristics**. This also aids in identification of problematic fermentations and in diagnosis of the cause of the decrease in fermentation rate. It is also important to have a predictable fermentation as this allows for optimal use of fermenter space and planning of the harvest. The strain also needs to display a **reasonable tolerance to ethanol**. Wine strains of *Saccharomyces* are typically resistant to 16-17% ethanol. Many wild strains are not as tolerant, and may be resistant only to 12-14% ethanol. If the grapes are harvested at a high Brix level, these strains might not be able to complete the fermentation. Another parameter that can inhibit yeast metabolic activities is temperature. Since wine is

frequently fermented at extremes of **temperature** to which *Saccharomyces* is **tolerant**, it is critical that the strain used not be unduly inhibited by heat or by very cool conditions. White wines are generally fermented at as low of a temperature that the yeast can tolerate (12-14°C) while many red wines are fermented at temperatures as high as the yeast can tolerate (35-42°C) in order to facilitate extraction.

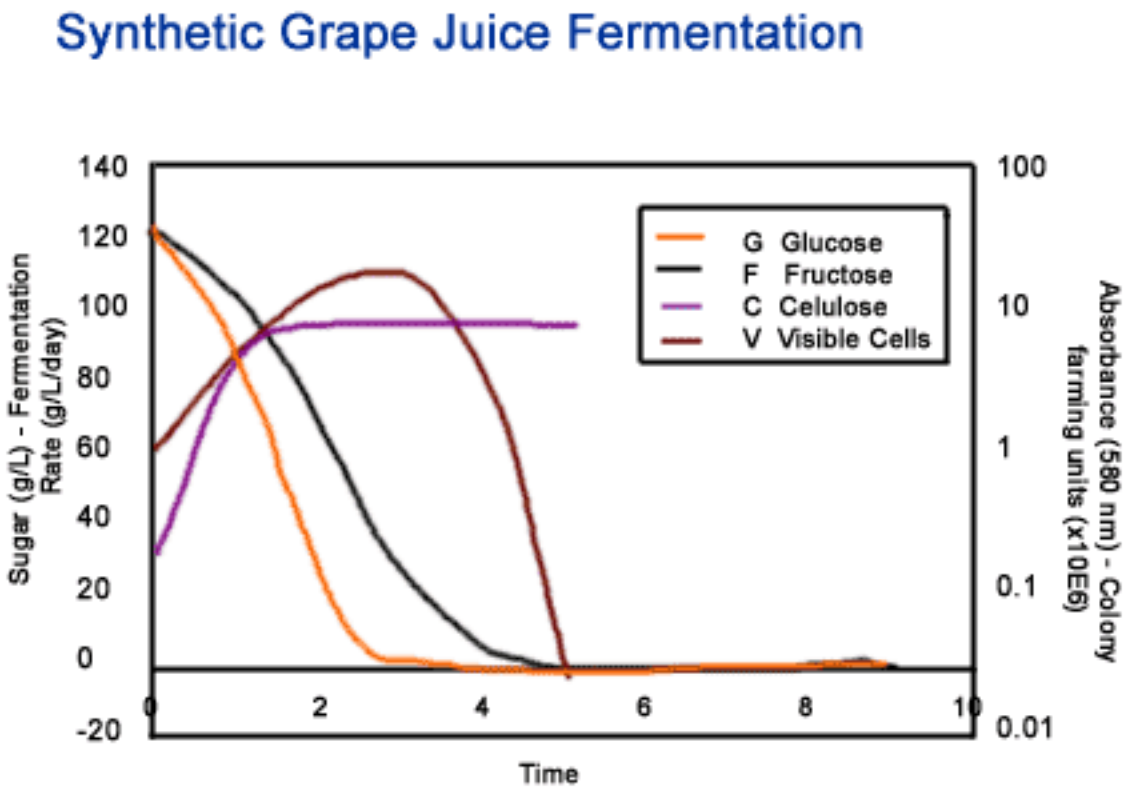
Since sulfur dioxide is used as an antioxidant, and has antimicrobial properties; therefore it is important that the yeast strain used be **tolerant of sulfur dioxide**. As mentioned in an earlier lecture, *Saccharomyces* detoxifies SO_2 via the formation of acetaldehyde adjuncts. It is important that the strain used be tolerant of the SO_2 levels used in the winemaking procedure. Sulfur dioxide tolerant strains are viable in fairly high concentrations of SO_2 (200-500 ppm).

It is desirable that the yeast stains present during fermentation not have any "bad habits", that is, produce **little to no off-characters**. This will be discussed in detail in a later lecture, but the most important class of off-characters is **hydrogen sulfide** "rotten egg" and other sulfur-containing volatile compounds. *Saccharomyces* also makes **acetic acid**, but generally not in a high enough concentration to be above the threshold of detection. Nevertheless, many commercial strains are available with little to no acetic acid production. It is also important that the yeast strain used be **compatible with any other microbes** that are desired in the fermentation. There are "good" and "bad" match ups of specific yeast strains and the lactic acid bacteria. While the exact reasons for the inhibition of one organism by the other are not known, it is likely due to competition for micronutrients or the production of inhibitory end products. Killer factor refers to a peptide produced by one strain of *Saccharomyces* that kills other strains of *Saccharomyces*. A virus like particle that yeast seem to have acquired at some point in their evolutionary life encodes killer factor. There are strains that both produce and are **resistant to killer factor**, strains that do not produce the factor but are resistant to it, and strains that are sensitive. Many wild strains of *Saccharomyces* either produce or are resistant to killer factor. The majority of the commercial strains are resistant, but may not produce killer factor. It is important to make sure that any novel strain selected by the winemaker is killer-factor resistant.

One final important property of yeast strains is their ability to confer the **desired organoleptic characters** to the wine. This is particularly important in sparkling wine production. However, even in table wines certain yeast strains may be preferred. The characters produced by yeast will be discussed in a later lecture.

Lesson 8: Yeast Nutrition

One of the most critical components of management of the yeast fermentations is to make sure that the yeast have all of the requisite nutrients to maintain fermentation rates and optimal ethanol and temperature tolerance. A typical yeast fermentation profile is shown below. Glucose is consumed at a faster rate than fructose, likely due to the different kinetic properties of glucose and fructose transport and metabolism. However, late in fermentation, the relative concentration of fructose will be higher than that of glucose.



The maximal rate of fermentation of both sugars coincides with the maximal viable cell biomass. Cells lose viability late in fermentation, once the usable sugar has been consumed. The maximal cell population for *Saccharomyces* is 1-2 x 10^8 cells/mL. Fermentations may attain this level, but are typically on the order of 2-5 x 10^7 cells/mL. The final cell concentration achieved is dependent upon the nutrients present in the must or juice. There are two principle classes of nutrients: macronutrients and micronutrients.

Yeast Nutrition

- **Macronutrients: Building blocks needed for new cell material**
- **Micronutrients: Catalysts needed to facilitate biochemical reactions**

Macronutrients are the compounds that supply the needs for cell division and energy generation. They are needed in high or stoichiometric amounts. In contrast, the micronutrient vitamin and minerals are required in much lower amounts and are catalysts involved in many enzymatic reactions.

Macronutrients

- **Carbon/Energy Sources: glucose, fructose, sucrose**
- **Nitrogen Sources: amino acids, ammonia, nucleotide bases, peptides**
- **Phosphate Sources: inorganic phosphate, organic phosphate compounds**
- **Sulfur Sources: inorganic sulfate, organic sulfur compounds**

The macronutrients are sources of carbon, nitrogen, phosphate and sulfate. These four elements are required for production of a new cell as well as for maintenance of a cell in stationary phase. In grape juice the carbon sources available for *Saccharomyces* are plentiful: glucose, fructose and sucrose. Grape juice contains other sugars and carbon compounds used by other organisms as carbon and energy sources, but the *Saccharomyces* repertoire is more limited. *Saccharomyces* is able to use the following compounds as energy sources. Only the sugars are fermented, the other compounds can only be used under conditions conducive to respiration.

Macronutrient Energy Sources

- **Monosaccharides: glucose, fructose, galactose, mannose**
- **Disaccharides: sucrose, maltose, melibiose**
- **Trisaccharides: raffinose**
- **Pentoses: None**
- **Oxidative Substrates: pyruvate, acetate, lactate, glycerol, ethanol**

The major nitrogen sources are ammonia, amino acids, nucleotide bases and small peptides. There are other nitrogen sources that *Saccharomyces* is not able to use, but that will support the nutritional needs of other microbes. Not all amino acids can be completely degraded by *Saccharomyces*, especially under anaerobic conditions. Amino acids can be classified into different categories based upon their ability to be utilized as sole nitrogen source.

Categories of Yeast Nitrogen Sources

- **Compound may be used as that compound for biosynthesis**
- **Compound may be converted to related compounds for biosynthesis**
- **Compound may be degraded with release of nitrogen**

All 20 amino acids can be transported and used directly as that amino acid in protein biosynthesis. Many amino acids may be converted to related amino acids, such as the interconversion between cysteine and methionine. A smaller subset of amino acids can be completely degraded releasing ammonia, which can then be used in biosynthesis. That is, can serve as sole nitrogen source. Sole nitrogen source means that the yeast can grow if that amino acid is the only nitrogen-containing compound in the medium. Amino acids, such as glycine, histidine and lysine, do not serve as sole nitrogen sources, but they can be important nutritional components of juice. Their presence means that they will not have to be synthesized and the available nitrogen sources can be mobilized to synthesize other needed compounds.

Yeast Nitrogen Sources

- **Degradation may depend upon availability of other components: vitamins and oxygen**
- **Utilization impacted by other environmental factors such as pH**

Whether a specific nitrogen compound can be used as a nitrogen source or not depends upon other factors. If specific micronutrients are required for the degradation and those nutrients are not available in the fermenting must or juice, the yeast will not be able to use that compound.

Saccharomyces can use inorganic or organic sources of phosphate, but is not permeable to compounds other than inorganic phosphate. The yeast secretes phosphatases that degrade the organic forms of phosphate external to the cell, allowing the inorganic phosphate that is released to then be taken up by the cell. Yeast sulfur sources are sulfate or the sulfur-containing amino acids.

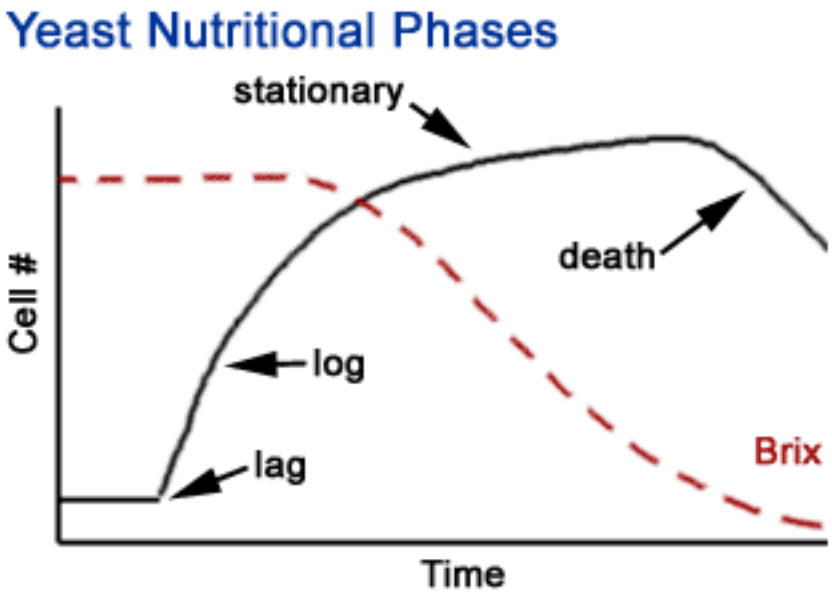
The micronutrient composition of juice is just as important as that of the macronutrients. Micronutrient deficiency might prevent synthesis of a single crucial compound the lack of which will lead to arrest of cell growth and perhaps of fermentation.

Micronutrients

- **Mineral and Trace Elements: Mg, Ca, Mn, K, Zn, Fe, Cu**
- **Vitamins: biotin is the only required vitamin, but others are stimulatory**

The essential minerals and trace elements are the cations required universally by eukaryotic organisms. Biotin is the only vitamin that yeast cannot synthesize *de novo*, and at least a precursor of this vitamin must be present in the grape juice. While other vitamins can be synthesized, yeast growth and fermentation is accelerated in the presence of these compounds.

Yeast require nutrients during active growth, but also require compounds during the non-proliferative phase of fermentation and to prevent loss of viability as ethanol accumulates in the medium.



The nutrients required for these phases differ somewhat.

Nutritional Requirements of Different Phases of Fermentation

- **Growth Phase: Building blocks and catalysts**
- **Stationary Phase: Survival factors**

Growth phase requires that the compounds needed for net synthesis of new cell material be present in sufficient quantities to encourage division. These are the building blocks and essential micronutrient catalysts. The compounds required for maintenance of fermentation rates and viability during stationary phase are called survival factors.

Most of the fermentation is conducted by stationary phase cells

- **Stationary phase:**
  1. **rate of growth = rate of death**
  2. **quiescent, no growth, no death**
- **Stationary Phase: Survival factors**

As seen from the typical yeast fermentation profile presented above, stationary phase cells conduct the bulk of the alcoholic fermentation. Stationary phase has two different definitions. It is characterized by the failure to detect an increase in cell number. This may arise because the rate of cell death equals the rate of cell division. In this case, the total number of cells present will remain the same. A second type of stationary phase is truly non-proliferative. That is, there is no cell division nor is there cell death. Cells are simply not dividing. There is evidence for both types of stationary phase in yeast strains during vinification. Survival factors are important for the maintenance of cell viability.

Role of Survival Factors

- **Maintain viability of cells**
- **Increase ethanol tolerance**
- **Maintain energy generation**

Survival factors maintain cell viability by providing the nutrients needed to repair cellular damage and support the limited synthesis of needed proteins and other cellular components. Survival factors also increase ethanol tolerance and help maintain fermentation rates and energy generation.

Survival Factors

- **Oxygen**
- **Fatty Acids**
- **Sterols**
- **Nutritional Factors**

The yeast survival factors are fatty acids and sterols. These compounds are needed for ethanol tolerance. This topic will be discussed in more detail in a subsequent lecture. Molecular oxygen is required for the synthesis of these cellular components, so if oxygen is present, there is no need for fatty acid and sterol supplementation. Nutritional factors such as nitrogen are also required. This is because protein synthesis is required to maintain the transport capacity of the yeast cells and therefore of fermentation.

How Does Ethanol Inhibit Yeast?

- **Displaces water of hydration changing the properties of protein-lipid interactions**
- **Denatures proteins**
- **Disrupts protein active sites**
- **Allows increased passage of protons from the medium into cell leading to acidification of the cytoplasm**
- **Removal of protons requires expenditure of energy**

Ethanol has several inhibitory effects on yeast cells. Ethanol disrupts protein-lipid interactions in the plasma membrane. It may also disrupt internal bond interactions in proteins leading to their denaturation and/or inactivation. Further, ethanol allows hydrogen ions to penetrate the yeast cell membrane at a higher rate than in the absence of ethanol. This can lead to acidification of the yeast cytoplasm and cell death if it exceeds the capacity of the cells to correct. Removal of protons that have leaked into the cell is an energy requiring process. This is why the cells quickly lose viability in the absence of an energy source late in fermentation. The required fatty acids and sterols are needed in order to synthesize an ethanol tolerant plasma membrane. The nitrogen is needed because different proteins, not as susceptible to damage by ethanol, must be synthesized.

Survival Factors

- **Needed to alter composition of the plasma membrane (sterols, fatty acids and proteins) so that it can withstand the perturbing effects of ethanol**
- **Both phospholipids and protein content must be adjusted**

The winemaker must make sure that the nutritional needs of the yeast are met during active growth as well as for maintenance of ethanol tolerance. Nutrients can be added to stimulate biomass production and prevent fermentation arrest.

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Lesson 9: Introduction

Fermentation Management

This lecture will describe the enological parameters impacting yeast fermentation performance and strategies for management of the fermentation. We will survey the tools and options available to the winemaker for manipulation of the metabolic activities of yeast. As with other operations, there are many decisions that must be made by the winemaker that will impact the microbial contribution to the composition of the finished wine. The first decision is whether or not the fermentation will be conducted spontaneously or by use of a commercial culture.

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Lesson 9: Native Flora versus Inoculated Fermentations

Saccharomyces is isolatable from vineyards in very low numbers, and can be found as part of the winery flora in very high numbers, especially once crush is underway. Spontaneous fermentations are conducted by vineyard and winery flora.

Sources of *Saccharomyces*

- **Vineyard flora**
- **Winery flora**
- **Inoculum**

The alternative is to use a commercial preparation as an inoculum. In this case, the characteristics of the primary strain conducting the fermentation are known, and potential fermentation problems can be minimized. Native flora or uninoculated fermentations are not necessarily problematic, but it is incumbent upon the winemaker to monitor them a bit more closely and to take appropriate action should a problem arise in the ferment. If a commercial strain has been used in a winery, it requires about 3 years of use of a different strain to displace the original strain in the winery.

Inoculated Fermentations

- **With active dry yeast (ADY)**
- **With a starter culture in juice**
- **With an already fermenting must/juice**

There are several ways in which a fermentation may be inoculated. One can use an active dry yeast culture, rehydrated according to instructions on the packet. Some yeast manufacturers recommend rehydration in the presence of a nutrient mix. If the winery has the capability, liquid starter cultures can be prepared in either juice or a defined medium, and used to inoculate juice or must. Not all strains are amenable to commercial production in a dehydrated form. If such a strain is desired, it will need to be prepared as a culture. Some yeast suppliers offer this service to wineries. An alternative method is to inoculate a juice with a juice that is already in the active phase of fermentation. The typical inoculum in all cases is between 10^5 and 10^6 cells/mL or 1

to 0.1% on a volume-to-volume basis. Higher strength inocula are also occasionally used. More yeast esters are produced with very high inocula, which may or may not be desirable depending upon the style of wine. Yeast cultures in stationary phase make a characteristic rose oil perfumey ester called phenethyl acetate in addition to characters commonly associated with bread. These two classes of characters are not necessarily harmonious, depending upon the rest of the composition of the wine and the goals of the winemaker.

Level of Inoculum

- **Typically 10^5 - 10^6 cells/ml or 1 to 0.1% on a volume/volume basis**

Inoculated fermentations are more predictable in terms of onset, duration and maximal rate of fermentation than are native flora fermentations. Because of the predictability problems arising in the fermentation, discussed in the next lecture, can be readily recognized and appropriate treatments performed. Many commercial strains are available that are quite neutral in the aroma characters produced. These are desired when the aim is to minimize the contribution of the yeast, such as in cases where the style emphasizes varietal character.

Inoculated Fermentations: The Benefits

- **Predictability**
- **Control of spoilage characteristics**
- **Neutrality: enhanced varietal characteristics**

With a clean start to the fermentation, the yeast is able to dominate quickly minimizing the organoleptic contribution of the indigenous microbial flora. Again, depending upon the style produced, this may be desirable or undesirable.

Inoculated Fermentations: The Negatives

- **Reduce overall complexity**
- **Fermentation rates too rapid**
- **Wine too "yeasty"**

The undesirable effects of inoculation are the overall reduced complexity of the wine due to the absence of microbial characters. In a sense, the wines are as predictable as the yeast strain used. In some segments of the marketplace, this is considered quite negative in and of itself. In other segments it is desired by the consumers. Inoculated fermentations, especially if overfed, may occur too rapidly which as we have discussed before, leads to heating of the ferment and the loss of volatile flavor and aroma characters from the juice or must. A final problem is that inoculated fermentations may have too strong of a yeast signature, that is, be too yeasty and bread-like.

Spontaneous or native flora fermentations in contrast are generally not predictable unless they are being taken over by a commercial strain that dominates the winery flora. This is frequently the case in many wineries. The level of yeast inoculum from winery equipment is on the order of 10^3 to 10^4 cells/mL, depending upon sanitation practices, the type of equipment used, the commercial strains used and their ability to form a **biofilm**. Lower initial biomass delays the onset of the fermentation and the domination of the must or juice by *Saccharomyces*. This allows the non-*Saccharomyces* flora to produce metabolites that will contribute to the aroma profile of the wine, but still assures that a strain capable of finishing the fermentation is present.

Native Flora Fermentations: The Benefits

- **Increased microbial complexity**
- **Slower fermentation rates**

Therefore, the primary benefit of a native flora fermentation is the increase complexity of microbial characters of the resulting wine. Some of these notes may be quite subtle, and enhance perception of varietal character. In other cases, the microbial contribution may be dominant over that of the varietal. There are many wines that are intensely microbial in the origin of the major aroma characters. A secondary benefit of native flora fermentations is their slow rate of fermentation. This minimizes heating of the

ferment and thus of loss of volatile varietal characters. This again results in an enhancement of varietal character of the wines.

Native Flora Fermentations: The Negatives

- **Off-character formation**
- **Lack of predictability**
- **Seasonal variation in microbial populations on fruit**

However, the native flora can produce undesirable characters in the wine that may detract from wine quality. They are the main producers of acetic acid and ethyl acetate, which are reminiscent of vinegar in low concentrations, but in high concentrations have the aroma of nail polish remover. These characters are volatile and may be lost later in the fermentation or, in the case of acetic acid, reconsumed by other organisms. This is not always guaranteed, of course, especially if a cool fermentation is being conducted. Another problem with native flora fermentations is lack of predictability. Since the size of the inoculum is not known nor are the fermentation characteristics of the dominating yeast strain, it is not possible to predict how quickly a fermentation should commence or if the fermentation rate is maximal or reflects a nutritional problem. Since the non-*Saccharomyces* flora make a spectrum of undesirable characters, especially from the degradation of amino acids, nutrient supplementation may not be a good option. Their unpredictability is also an advantage in a segment of the marketplace, as the composition of the wines will vary from vintage to vintage. When it works well, the wines can command a high price, but vintages of much lower quality are expected and therefore still marketable. This variability in characters formed is due in part to the variability in the chemical composition of the juice or must, but it is also due to seasonal variability in the numbers and kinds of flora present in the must or juice. What organisms are present is influenced by vineyard operations and practices as well as to changes in environmental conditions (humidity, by presence of insect vectors, disease pressure and berry infection, for example).

Many wineries that conduct native flora fermentations take out an "insurance policy". Roughly 10% of the juice or must is inoculated with a neutral commercial yeast. This can be used as an inoculum for the native flora fermentations if a problem (off-character, reduced fermentation rate) arises.

Lesson 9: The Role of Non-*Saccharomyces* Flora in Wine Production

Second Decision

Encourage or discourage grape berry microflora?

Grape Berry Microflora

- **Bacteria**
- **Molds**
- **Yeast**

Whether fermentations are inoculated with *Saccharomyces* or not, the winemaker must decide if the non-*Saccharomyces* flora is to be encouraged or discouraged. The berry flora is comprised of bacteria, molds and yeasts.

Bacteria

- ***Bacillus***
- ***Pseudomonas***
- ***Micrococcus***
- **Lactic Acid Bacteria**
- **Acetic Acid Bacteria**

The bacterial species most prevalent on the surface of grapes are members of the genera, ***Bacillus***, ***Pseudomonas*** and ***Micrococcus***. The **acetic acid bacteria**, principally *Acetobacter* are also present. Members of these genera are strict aerobes, so once the juice becomes anaerobic further growth and metabolism is inhibited. Many of them have mechanisms for surviving periods of anaerobicity, so one should not assume that they are completely eliminated in the ferment. In fact, they will be viable at the air interface at the top of the tank until that headspace is displaced by the carbon dioxide of active fermentation. In the case of red wine production, the process of

pumping over bathes the cap in ethanol, which is toxic to many of the aerobic organisms, but not to all.

The final class of organisms that are predominant on grape surfaces are the lactic acid bacteria. **The lactic acid bacteria** are facultative anaerobes, so they can persist under anaerobic conditions, depending upon the pH of the juice or must.

Minor species may also be found, some of which are not berry residents but are indigenous to the vineyard soil or other parts of the plant. Vineyard and harvesting conditions may lead to their inclusion in the must or juice. These organisms do not generally persist under juice or must fermentation conditions but can contaminate other winery surfaces. *Streptomyces* for example is a cellulose degrader and can infect winery filtration apparatus if appropriate sanitary practices are not employed. This organism is responsible for the characteristic odor of dry dirt, which is quite noticeable in the wine upon filtration. The diversity of *Bacillus* species present also depends upon the amount of soil contamination of the fruit. Members of this genus are particularly troublesome if they infect a winery because of the production of spores that are highly resistant to both heat and chemicals. They are therefore difficult to displace from the winery flora. *Bacillus* infection is not common in California, but is present in other wine growing regions of the world.

Molds

- ***Aspergillus***
- ***Penicillium***
- ***Rhizopus***
- ***Mucor***
- ***Botrytis***

Several genera of molds are also found. The molds and the yeast are classified together as fungi. To be called a "yeast" the organism must exist vegetatively primarily as single free-living cells. Molds are found primarily in the mycellial or multicellular form. The four most commonly isolated molds are members of the genera ***Aspergillus***, ***Penicillium***, ***Rhizopus*** and ***Mucor***. These molds are found just about everywhere in the environment. They function in the degradation of complex organic material. These fungi produce both sexual and asexual spores. The asexual spores largely function in dispersal of the colony. They can be carried great distances by wind

or deposited locally. Fungi are communal organisms due to their principle biological activity of degradation of biological matter. The fungi are prolific secretors of hydrolytic enzymes allowing digestion of material outside of the cell. This process is greatly accelerated by a community of cells working in concert. In this case a critical mass of catalytically active enzymes can be produced quickly and serve to feed the entire fungal population.

Some fungi are able to initiate the degradation process on living material. They possess characteristics that allow penetration of the cell walls of plants for access to plant nutrients. At some point these organisms cross the line between free-living and pathogenic. ***Botrytis*** is able to infect berries on the vine leading to deterioration of the fruit. As noted in an earlier lecture, wines made from Botrytized fruit contain many unique and distinctive characters as a consequence of the infection. Some of these notes derive from the plant and are produced in response to infection, others arise due to the impact of mold metabolites on the flora conducting the fermentation, and some may derive from *Botrytis* itself. Other genera of molds are opportunistic, that is, will participate in an infection once initiated by another organism, but are not capable of causing disease on their own.

The molds are all obligate aerobes and are not present during the active phase of fermentation. They also do not persist in the fermentation in contrast to the bacteria. Initial must platings are dominated by mold, but platings we have done from juice or must just hours after crushing display little to no mold. The aerobic bacteria are frequently still isolatable at this time.

Yeast

- ***Kloeckera/Hanseniaspora***
- ***Metschnikowia pulcherrima***
- ***Hansenula* species**
- ***Candida* species**
- ***Saccharomyces***

Four yeast genera are typically found on the surface of the grape berry: ***Hanseniaspora***, ***Metschnikowia***, ***Hansenula*** and ***Candida***. Taxonomists have historically divided the yeast into two categories, the ascomycetes and the basidiomycetes, based upon whether the sexual spores are formed inside of a

structure or sac (ascomycetes) or are borne on a stalk (basidiomycetes). This classification depends upon the ability to observe sexual spores in an isolate. There are other factors such as the cell wall structure and type of budding of vegetative cells that can also be used to classify yeast isolates as ascomycetes or basidiomycetes. However, the practice that has developed is to separately categorize isolates for which no sexual stage has been observed. These organisms are called the deuteromycetes. Many of the deuteromycetes are identical in all other properties to isolates classified as ascomycetes or basidiomycetes. When this is the case, they are referred to as the imperfect form of the organism for which a sexual cycle has been observed. The strain possessing the sexual stage is called the perfect form. Grape and wine isolates are members of the ascomycetes. Members of the genus *Hanseniaspora* are perfect forms, that is, possess documented sexual cycles. The imperfect forms of these yeasts are classified as *Kloeckera*. These yeasts are identical in all ways except the observation of a sexual cycle. One of the most commonly isolated strains from grape surfaces is *Kloeckera apiculata*, the imperfect form of *Hanseniaspora uvarum*. Another commonly isolated yeast is *Metschnikowia pulcherrima*, the perfect form of *Candida pulcherrima*. In this case the species names are the same making it easier for the novice to recognize that these designations represent the perfect and imperfect forms of the same organism. These names are used interchangeably in the wine literature for the most part. Comparative analysis of DNA sequences from each of these pairs of organisms clearly supports their identity to each other. Be that as it may, the convention is still to use the two different designations, with the "perfect" designation reserved for those isolates for which a sexual stage has been observed. The ability to compare DNA sequences is revolutionizing the field of taxonomy, and in the near future the deuteromycetes may disappear.

Hansenula and other members of the *Candida* genus are also frequently isolated from grape surfaces. More rarely isolated are *Pichia* and *Issachenkia* species, yeasts that are commonly found on fruit surfaces. ***Saccharomyces*** can also be isolated from grape surfaces but not in great numbers. Many yeast ecologists believe that *Saccharomyces* is not a normal resident of grape surfaces as it is not tolerant of UV light and is out-competed by other yeasts when a direct inoculation of the fruit is attempted. *Saccharomyces* is referred to as a domestic yeast, sort of the Cocker Spaniel of the fungal world. *Saccharomyces* is readily isolatable from humans and may be transferred to the vineyard by human contact. If the yeast lees are used in the vineyard as fertilizer, then *Saccharomyces* might also be transferred from the winery to the vineyard and back again. This cycling is thought to have selected for regionally specific strains of yeast.

Grape Berry Microflora

- **95-98% of total organisms are molds and bacteria**
- **2-5% are yeast, principally *Hanseniaspora* and *Metschnikowia***
- **Non-*Saccharomyces* yeasts present at levels of 10^5- 10^6 organisms/mL, *Saccharomyces* present at 10^{-2} - 10^{-3} cells/mL**

The majority of the microbial biomass of the grape surface is comprised of the molds and bacteria. The yeast represents around 2 to 5% of the isolatable organisms. Non-*Saccharomyces* yeasts are present at the levels of 10^5 to 10^6 in intact fruit, but can rise to 10^7 to 10^8 in later harvest fruit or in fruit with some berry damage. *Saccharomyces* is found at levels of 10^{-2} to 10^{-3} cells per mL, meaning that there is 1 cell in 100mL to 1 cell in 1 Liter. Numerous factors impact the types and numbers of microbes on the grape surface.

Factors Affecting Grape Berry Microflora

- **Rainfall / Humidity**
- **Insect vectors**
- **Altitude**
- **Temperature**
- **Vineyard fertilization practices**
- **Varietal factors: tightness of cluster**
- **Vineyard practices: inoculation of fruit with soil microbes**

Rainfall or **high humidity** favors growth of the molds, and accompanying damage to the berries then encourages the growth of bacteria and some of the yeasts as well. **Insect vectors** are also important. The molds produce air-borne spores that are able to disperse the culture. The bacteria but principally the yeast rely on insect vectors to be relocated. In fact, there are numerous examples of symbiotic relationships between yeasts and insects. The yeast produces esters that attract the insects to a fruit food source. The insects then feed and pick up the microbial flora on their bodies. As they visit other clusters, the yeast can then be deposited. This phenomenon can readily be observed in the winery. Fermentations producing high amounts of acetic acid and ethyl acetate are strongly attracting to the fruit fly *Drosophila*. *Drosophila* infestation of the

winery can quickly spread *Acetobacter* and the yeasts throughout the facility.

The **altitude** of the vineyard also influences the flora of the fruit. It is not clear if this is a direct effect of altitude or an indirect effect, that is, different altitudes support different insect populations and feeding activity. Interestingly, in one of the few comprehensive studies that was undertaken, *Hanseniaspora* was isolated from high altitudes while *Kloeckera* was found at lower altitudes. This suggests some selective advantage to maintaining the ability for sexual reproduction in some environments.

The mean **temperature** of the vineyard also impacts the flora present. At very high temperatures under dry conditions, such as those found in Davis and the Central Valley of California, *Saccharomyces* does not persist in the vineyards. This yeast does not display the range of temperature tolerance of the other organisms present, another indicator that the native environment of this yeast is likely not the berry surface. It is not clear if this is due to the heat alone or to dehydration or to inhibition by other microbes more tolerant of heat.

Vineyard fertilization practices are also important. What is being applied and how it is being applied can both have an impact. Foliar applications of nutrients favor the growth of those organisms that can utilize the compound being applied as a nutrient. Foliar application of urea is used in fruit crops to encourage the growth of the beneficial native flora. In the case of apples, it was shown to increase the numbers of bacteria. The increase in bacteria resulted in a decrease in mold, and reduces the incidence of mold infestation and spoilage. Urea also used to be used in vineyards, but this practice was terminated when it was discovered that urea in the presence of ethanol will react spontaneously to produce the carcinogen ethyl carbamate.

Varietal specific factors also impact the numbers and kinds of flora present. Varieties prone to grape damage, such as those with tight clusters, release more nutrients to the surface of the fruit, making them available to support microbial growth. The tendency of the fruit to produce antimicrobial substances will also impact the flora if any susceptible microbes are present.

Finally, **vineyard practices** also can affect the microbial flora of the fruit. If soil is stirred up so that dust can land on the fruit, soil microbes might be transiently associated with the grape surface. Time of harvesting is also important. We have seen greater numbers of both molds and bacteria on late harvest fruit that otherwise appears to be healthy and free of infection. Vineyard practices also impact the insect vectors that may be available and therefore the spread of microbes through the vineyard. Environmental factors such as the practice of leaf pulling and presence of

wind are also critical, as these will impact local cluster humidity. In addition to factors affecting the microflora of the fruit, must or juice composition and processing factors will impact the persistence of the flora in the fermentation.

Factors Affecting Persistence of Grape Berry Microflora in Must/Juice

- **pH**
- **Temperature**
- **Oxygen**
- **Nutrient Levels**
- **Presence of Inhibitors**
- **Microbial Interactions**
- **Inoculation Practices**
- **Winery Practices**

pH

- **Low pH (<3.5) inhibits many bacteria**
- **Yeast not pH sensitive at normal juice pH values (2.8-4.2)**

One of the most important factors is pH. The **low pH** of wine is inhibitory to many bacterial species. If the value is below pH 3.5, many of the lactic acid bacteria are unable to grow.

Temperature

- **Low temperatures inhibit bacteria**
- **Low temperature enriches for non-*Saccharomyces* yeasts**

Temperature is another important factor, just as it was in the vineyard. Holding the must at low temperatures is inhibitory to the bacteria. The yeast *Saccharomyces* is also inhibited at low temperature and will not initiate fermentation below about 12°C. *Hanseniaspora/Kloeckera* tolerates low temperatures well and will be dominant in musts following a cold soak. Thus, the processing decision to do a cold soak will impact the flora present. The longer the soak, the stronger the effect on the flora. If a procedure encouraging native flora has been performed, addition of nutrients may feed this population and not the intended *Saccharomyces*.

Oxygen

- **Lack of O_2 inhibits all molds**
- **Lack of O_2 inhibits aerobic bacteria**
- **Oxygen stimulatory to yeast: not clear how different species are affected**

The presence or absence of oxygen is likewise an important factor. As mentioned previously, many of the members of the berry flora are aerobes. If the juice receives an oxygen treatment (is aerated) before the development of *Saccharomyces*, spoilage may occur. It is also important to mention that oxygen is stimulatory to the facultative anaerobes as well. As we will see in the lecture on the lactic acid bacteria, these organisms can produce acetic acid as an end product of metabolism in an energy-generating pathway if molecular oxygen is available. *Saccharomyces* is an excellent competitor for oxygen, so if aeration is needed, it should occur after the yeast has become established.

Nutrient Levels

- **Must/Juice composition**
- **Supplementation**
- **Timing of addition**

Nutrient levels are clearly going to impact the organisms present and whether or not they are competing for nutrients or there are ample to go around. Nutrients arise in the

grape itself, but are obviously influenced by nutrient addition practices. The timing of addition is important as discussed above. The dominant species at the time of feeding will be the group that benefits the most from the addition. Again, if *Saccharomyces* is the intended beneficiary , nutrients should be added once this organism has become established or following inoculation with a commercial culture.

Presence of Inhibitors

- **Fungicide/pesticide residues**
- **Sulfur dioxide**

The use of pesticides and fungicides in the vineyard will impact the relative ratios of organisms on the fruit, but if applied too close to harvest, may also influence the microbial flora of the fermentation. It is important to remember in this regard that *Saccharomyces* is a fungus and may be inhibited by some antifungal agents. Many commercially available compounds have been tested for effects on the flora of the fermentation, but the vineyard manager should make sure these studies have been done before applying agents to the fruit.

Sulfur dioxide has been mentioned before with respect to its antimicrobial activity, and depending upon juice conditions may be quite inhibitory to the flora present.

Types of Microbial Interactions

- **Production of Inhibitors**
  - **Acetic acid**
  - **Ethanol**
  - **Fatty acids**
  - **Killer factors**
- **Competition for nutrients**
- **Stimulation**
  - **Removal of inhibitor**
  - **Release of micronutrients**

Interestingly, but not surprisingly, the persistence of microbes in the ferment is affected by the types of other microbes present. Microbes compete for nutrients and if nutrients are limiting, those that are more able to consume them will reduce the numbers of those that are not. In addition to competition, some **organisms produce end products that are inhibitory to other organisms**. For example, **ethanol** is inhibitory to most bacteria and molds and many yeasts. Those normally associated with ferments are more tolerant, but the non-*Saccharomyces* flora can be inhibited at concentrations as low as 7% ethanol. Cell division in *Saccharomyces* is reduced at this level and above as well. Acetic and other organic acids are also inhibitory. *Saccharomyces* is less tolerant of **acetic acid** than are other organisms, but many of the acetic acid producers will produce sufficient compounds to inhibit their own growth as well. Other kinds of inhibitory compounds can also be formed. We discussed killer factor, inhibitory peptides produced by some strains of *Saccharomyces* that affect other strains of the same yeast. Certain non-*Saccharomyces* yeasts produce broader spectrum **killer factors**. Their presence in a fermentation can be inhibitory to other yeasts. Bacteria make analogous compounds, called **bacteriocins** that are inhibitory to other bacteria. Molds, while not present in the fermentation, may have produced **mycotoxins** on the surface of the berry that can be inhibitory to members of the must or juice flora.

Alternately, the presence of one microbe may stimulate the persistence of others. A classic example of this is the detoxification of sulfur dioxide by *Saccharomyces*. Specific organisms may **remove other inhibitory substances** as well. This may be due to active degradation or to depletion of the inhibitor by the initial biomass. In this case, the initial population may decline due to the impact of the inhibitory compound, allowing other populations to then bloom.

Inoculation Practices

- **Early inoculation minimizes impact of flora**
- **Higher levels of inoculation limit impact of microbial flora**

Another obvious factor impacting population dynamics is the practice of inoculation, either with a commercial preparation or with a tank that is already undergoing active fermentation. The introduction of a new population of organisms will definitely impact the existing population and vice versa.

The earlier and the heavier the inoculation, the stronger the inhibitory affects against the indigenous flora.

Winery Practices Impacting Microbial Flora

- **Sanitation**
- **SO_2**
- **Cap management**
- **Nutrient additions/Juice adjustments**
- **Maceration strategy**
- **Temperature of fermentation**

Winery practices in addition to inoculation will also impact the numbers, kinds and persistence of individual members of the flora. Several of these factors have already been mentioned, SO_2 use, cold soak, temperature of fermentation, aeration practices, addition of nutrients. The **sanitation** procedures used in the winery are also important: how frequently the equipment is cleaned, the type of microbial reservoirs that can accumulate in the equipment, nature of the sanitation process. Are chemicals used? Hot water? Steam? Are hoses stored in a position allowing good drainage? All of these factors impact the numbers of organisms that can inoculate the juice or must. Also, are conditions established that encourage insect infestation of the winery? If so, organisms can then be spread from tank to tank. Just as it was important to walk through the vineyard, it is also important to take a thorough look at winery practices from the perspective of sanitation.

In addition to these factors, skin contact, cap management and maceration strategies also affect the flora. The berry flora usually occurs as a biofilm attached to the surface of the fruit. This biofilm is difficult to dislodge, but in nutrient sufficient liquid conditions such as a ferment, new cells produced from cell division will be released into the culture. Therefore the length of time the skins are in contact with the juice will affect the numbers of progeny cells released. Cap management practices, number and kinds of pumpovers performed, will affect the organisms present on the cap. The ethanol from the fermentation will inhibit many microbes on the surface as will the establishment of anaerobic conditions due to the formation of the carbon dioxide blanket. **Maceration strategies** directly influence the release of phenolic compounds (which may be inhibitory to microbes) and nutrients (which may be stimulatory). The effect of these practices can be difficult to predict. One winery in California began extensive

pumpovers of late harvest fruit before the yeast had become established in the tank. This practice strongly encouraged a bloom of lactic acid bacteria and production of volatile acidity. The levels produced were not as high as those obtained from *Acetobacter*, but were high enough to be inhibitory to the yeast during fermentation.

Several things can be done if the winemaker desires to encourage the berry flora. No or late inoculation with *Saccharomyces* will allow the other microbes more time to build up populations and to produce their spectrum of end products. Early **addition of nutrients** will also favor the non-*Saccharomyces* flora.

To Encourage Grape Berry Microflora

- **No or late inoculation with *Saccharomyces***
- **Add nutrients early (pre-inoculation)**
- **Hold must/juice at low temperature**
- **No to low SO_2**
- **Adjust pH**

Sulfur dioxide addition should be avoided, or added late. The pH should be adjusted upwards (above 3.5) if the goal is to encourage bacterial flora. Finally, the must or juice can be held under conditions stimulatory to microbes, but that limit the ability of *Saccharomyces* to dominate the fermentation. The opposite can be done to discourage the wild flora.

To Discourage Grape Berry Microflora

- **Early addition of SO_2, other antimicrobials**
- **Early inoculation with *Saccharomyces***
- **Use a high level of inoculum**
- **Add nutrient after *Saccharomyces* is established**
- **Avoid incubation at low temperature**

Lesson 9: Monitoring the Alcoholic Fermentation

The next decision to be made by the winemaker is to determine how will the fermentation be monitored and what exactly will be evaluated. Another important question: is what will be done with the information?

Third Decision:

How will fermentation be monitored?

Fermentation monitoring may be as simple as measuring Brix or sugar level, or may involve analysis of many other parameters.

Fermentation Monitoring

- **What will be monitored?**
- **How will it be measured?**
- **How frequently will measurement be taken?**

It is important to have a good understanding of how what is being evaluated relates to the information desired. It is equally important to know the reproducibility, precision and accuracy of the method and what types of factors will interfere in the measurement.

Fermentation Factors to Be Monitored

- **Sugar consumption**
- **Nitrogen availability/consumption**
- **Microbial flora**
- **Microbial activity**
- **Acidity changes**

Monitoring Sugar Consumption

- **Hydrometry (specific gravity/density)**
- **CO_2 evolution (weight/pressure change)**
- **Loss of glucose/fructose (HPLC, CE, enzyme assay)**
- **Ethanol evolution (GC, eubillometry)**
- **Temperature release**

Sugar levels can be monitored in one of several different ways. The most common is to use the Brix scale or a similar means to assess the **specific gravity or density** of the ferment. The amount of carbon dioxide liberated can also be used to determine the amount of sugar consumed. The advantage of measuring **CO_2** loss is that this can be evaluated continuously by the change in weight of the tank. This can be done automatically and downloaded to a computer spreadsheet, being instantly available to the winemaker. The levels of the two sugars, **glucose and fructose**, can also be evaluated, either using an enzymatic assay, which can now be automated, or by HPLC (High Performance Liquid Chromatography) or CE (Capillary Electrophoresis). These latter methods are more accurate and precise, but require sophisticated analytical equipment and someone competent to keep the equipment in proper working order.

Ethanol evolution can also be measured as a means to determine the amount of sugar consumed. Eubillometry is the most common method, but Gas Chromatography, CE and HPLC methods are also available. Fermentation activity can also be evaluated directly by measuring the heat released from fermentation and the change in **temperature** of the tank. Software programs exist to allow this to be done even in tanks that are refrigerated. If ethanol or carbon dioxide are used as parameters to monitor the fermentation, it is important to know the starting sugar concentration so that the winemaker will know when the fermentation is finished.

Monitoring Nitrogen Availability/Consumption

- **Amino acid analysis (HPLC)**
- **Free amino nitrogen (FAN)**
- **NOPA (nitrogen by OPA)**
- **Yeast Utilizable Nitrogen (Hefeverwertbarer Stickstoff)**

The nitrogen content of the must or juice can also be measured to provide the winemaker with information on the amount of supplementation required. An **amino acid analysis** can be performed using HPLC. This provides quantitative information on a variety of nitrogen containing compounds that can be used by yeast and bacteria, including ammonia. However, as with most HPLC analyses, it is a tad time consuming and may not provide information rapidly enough to make an informed decision regarding supplementation. Other chemical assays have been developed that serve to measure the compounds possessing a certain type of nitrogen moiety. The three most common are the **free amino nitrogen** or FAN analysis, the **NOPA analysis** developed at UC Davis and the **Yeast Utilizable Nitrogen** analysis developed by Geisenheim.

Monitoring Microbial Flora

- **Microscopic observation**
  - **Total counts**
  - **Qualitative assessment**
- **Plate counts**
  - **Total viable counts**
  - **Differential media**

The microbial flora of the wine can also be evaluated. Yeast and bacteria can be distinguished from each other under the **microscope** so the relative numbers of these two classes of organisms can be easily determined using a counting chamber. However, the yeasts are so similar to each other in appearance that it is not possible with any degree of accuracy to distinguish among the yeast genera microscopically. *Kloeckera* is an apiculate yeast, meaning that it has a point on the ends and is lemon shaped versus the ovoid *Saccharomyces*, but "newborn" *Kloeckera* cells develop the distinct pointed shape only after several rounds of replication. The same is true of the bacteria, it is not possible to distinguish genera solely using microscopic observation.

Qualitative estimates of relative numbers of microbial populations can also be made, but are less reliable. Microscopic observation can be modified by the use of vital dyes to distinguish between viable and non-viable organisms. However, we have found that the ability to take up some of these dyes is influenced by ethanol content and the number of non-viable organisms can be overestimated. Cultivatable or **viable organisms** can be monitored directly by plating a sample of the must or juice. General media can be used supporting the growth of a broad spectrum of organisms or more **selective media** can be employed that support the growth of a subset of the microbes. In this regard we have found the WL or Wallerstein Laboratories medium to be most useful. This medium supports the growth of a wide spectrum of organisms but slows the growth of all so that no one subset is able to dominate the plate. The colonies formed display quite distinctive morphologies allowing quantitation of subpopulations. For example, on this medium, colonies of *Hanseniaspora/Kloeckera* are an intense green, *Metschnikowia* forms colonies that appear red when viewed from the bottom of the plate, *Saccharomyces* forms colonies that are off-white to a pale green and *Brettanomyces* forms small colonies that are a distinctive olive green. The other yeasts commonly present also have quite distinct morphologies. It is possible to plate at a high density and see the unique morphologies over a lawn of the more common ones. Other media exist that contain inhibitors of yeast growth to enrich for the more fastidious bacteria. This type of analysis will allow the winemaker to profile the flora of the juice or must and determine the point at which *Saccharomyces* becomes dominant. These analyses require the availability of a laboratory equipped for microbiological analysis, which may be beyond the capability of many wineries. In California, there are several commercial services that can provide a full microbiological analysis of the ferment. These services are most commonly used to determine the source of off characters appearing in the wine, so that steps can be taken to eliminate the contaminant. It is important to define a contaminant or spoilage organism. A spoilage organism is simply one that is unwanted. One winemaker's spoilage problem may be another's critical contributor to style.

At this point it is important to again underscore the importance of a statistically valid sampling of the tank for microbial profiling. Tanks are not uniform in the flora present. For example, there may be localized high concentrations of some organisms near the surface or at the bottom of the tank or in relationship to the temperature distribution across the tank. Analysis of a lone sample taken from the racking valve might not provide an accurate picture of the distribution of the flora throughout the tank. For organisms present in low number, it may be necessary to collect the microbial flora from a large sample by sterile filtration and to then plate at more concentrated levels.

Monitoring Microbial Activity

- **Volatile acidity analysis**
- **Vinyl phenols**
- **Hydrogen sulfide**
- **"Sniff" test**
  - **By nose**
  - **Very accurate**

Rather than monitoring the organisms themselves, end products of metabolism can be evaluated. The amount of a specific end product present is an indication of the metabolic activity of the microbes not just their presence or absence. **Volatile acidity** or VA analysis can be used to measure acetic acid content. This is an indication of the presence of *Acetobacter* or of the lactic acid bacteria. One can then look for other compounds produced by one or the other class of organisms to determine which bacterium is responsible for the volatile acidity. The level of **vinyl phenols** is an index of the presence and metabolic activity of *Brettanomyces*. **Hydrogen sulfide** can be produced by many microbes, but in wine conditions, is most frequently associated with *Saccharomyces*. The factors affecting H_2S production will be discussed in a subsequent lecture. Finally one of the most important analytical tools available to the winemaker is their own **sense of smell**. Off-characters can readily be detected by nose and it is important that ferments be sniffed on a regular basis in order to detect problematic compounds and metabolic activities.

Another factor that can be evaluated during the fermentation is changes in acidity. This can be done by monitoring titratable acidity as discussed in one of the earlier lectures and by measurement of pH. Levels of malate and lactate are typically directly measured as this is correlated with the presence and level of activity of the lactic acid bacteria.

Monitoring Acidity Changes

- **Titratable acidity**
- **pH**
- **Enzymatic assay**
- **HPLC**
- **Paper chromatography**
    - **Malate**
    - **Tartrate**
    - **Lactate**

These acids can be measured by enzymatic assay, by HPLC or by paper chromatography. The former two methods are quantitative, while the latter gives a qualitative estimate of the levels of these acid species. The latter method is easy to perform and is the most common method used in wineries today because of its ease and simplicity, and because frequently qualitative information is all that is required.

Monitoring Strategy

- **Ease vs. Frequency**
- **Cost**
- **Skill level required/Difficulty of analysis**
- **Is information necessary?**

The monitoring strategy of the winery needs to take into consideration several factors. The first factor is **ease of the measurement versus the number of times** the measurement must be made. If it is a cumbersome and time consuming method, it may not be able to be performed in a timely fashion on a large scale, that is, on hundreds of barrels or tanks fermenting simultaneously. Or, it may be performed hastily and therefore inaccurately.

As always, **cost** versus benefit of the information is a critical factor. Many wineries forgo sophisticated nitrogen analysis because of the cost of commercial services, and instead adopt the practice of treating every fermentation as if it will stick. In this case, every must and juice receives nutrient additions. The **skill level required** of the laboratory technician is an equally important consideration. Methods improperly performed yield inaccurate information. The inaccuracy might not be immediately apparent and erroneous decisions may be made as a consequence. The final question

that needs to be answered is **"Is the information necessary?"** In many cases wineries are performing analyses out of habit more than for any other reason. The information is recorded, but not referred to again or used to guide winemaking practices.

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Lesson 9: Temperature of Fermentation

The next critical decision for management of the fermentation is the temperature of the fermentation. Will the temperature be controlled? If so, what is the ideal temperature? And, most importantly, why is that the ideal temperature?

Fourth Decision:

Temperature of Fermentation

We have discussed the numerous effects temperature can have on the extraction of skin and seed materials and on the growth and proliferation of microorganisms.

High Fermentation Temperatures

- **Speed fermentation rate**
- **Discourage diverse flora**
- **Enhance extraction (reds)**
- **Greater loss of volatile aroma characters**
- **May increase risk of stuck fermentation**

High temperatures of fermentation encourage rapid rates of metabolism speeding the production of ethanol, and lead to greater loss of volatile characters. They also discourage a diverse flora due to the stimulatory effect toward *Saccharomyces*. However, if the fermentation becomes too hot, further growth and metabolism of *Saccharomyces* will be inhibited. We have found experimentally that very hot ferments tend to arrest late in fermentation, once the juice has cooled. This appears to be an arrest at a specific ethanol concentration, as discussed in the next lecture. Thus, the impact of a poor choice of temperature during the most active phase of fermentation, may impact the finish of the fermentation and the ability of the yeast to consume all of the available sugar.

Low Fermentation Temperatures

- **Favor non-*Saccharomyces* flora**
- **Better retention of volatile aroma compounds**
- **Slow fermentation rates**

Lower temperatures result in slow rates of metabolism, allowing other non-*Saccharomyces* organisms to persist in the ferment. There is obviously better retention of volatile characters, the lower the temperature.

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Lesson 9: Type of Fermentation Tank

A final important consideration for fermentation management is the nature of the vessel in which the fermentation will occur.

Fifth Decision:

Fermentation vessel

The nature of the fermentation vessel is oftentimes dictated by stylistic considerations, and the need to control temperature.

Fermentation Vessel

- **Wooden cask**
  - **Size**
  - **Source of wood: Oak? Redwood?**
- **Stainless steel tank**
  - **Refrigeration**
  - **Size**
- **Barrel**
  - **Age**
  - **Type of oak**
- **Cement**

Wooden fermentation vessels may impart characters to the wine, depending upon the number of times the cask or barrel has been used. Wood supports higher microbial loads or biofilms than stainless steel and is harder to sanitize. It is easier to control the temperature of stainless steel than of wood. If wood is used, the nature of the wood must also be determined, redwood or oak are the options in California. Cement tanks are still used in many parts of the world and in some wineries in California, but are more difficult to maintain.

Lesson 10: Introduction

Problem Fermentations

In this lecture we will discuss the two main types of problems associated with *Saccharomyces* fermentations: fermentation arrest and off-character production. Occasionally a fermentation will slow down dramatically or even stop before all of the grape sugar is converted to ethanol. The former is called a "sluggish" and the latter a "stuck" fermentation. These types of fermentations are problematic for several reasons. First, high residual sugar may not be desired in the wine. Second, high residual sugar can be viewed as an open invitation to many classes of spoilage organisms that would otherwise not be able to infect the wine. Third, the winemaker must protect the wine that no longer enjoys the benefit of a carbon dioxide blanket from oxidation but not take any action that would further inhibit yeast metabolism or ethanol tolerance (such as restriction of oxygen). Fourth, the inability to predict if the fermentation is simply slow but will go dry or is actually stuck is difficult. Needed tank space may be occupied indefinitely by the arrested ferment while the winemaker waits to see what will happen. This limits the flexibility of tank usage.

Objectionable compounds or "off-characters" are likewise problematic. Conditions leading to slow or incomplete fermentations frequently also result in the production of undesirable yeast metabolites such as sulfur volatiles. These characters must be removed or wine quality will be diminished. In both cases, it is better to prevent the problem in the first place than to treat it after the fact.

Problem Fermentions

- **Slow**
- **Stuck**
- **Off-character production**
  - **Hydrogen sulfide**
  - **Sulfur volatiles**
  - **Acetic acid**
  - **Undesired Esters**

Treatments for particular off-characters may be available, but can have undesirable impacts on wine quality. Any time an attempt is made to remove chemical components

of a wine, the risk of unavoidably removing positive characters as well exists.

We will begin with a detailed examination of slow (sluggish) and incomplete (stuck or arrested) fermentations.

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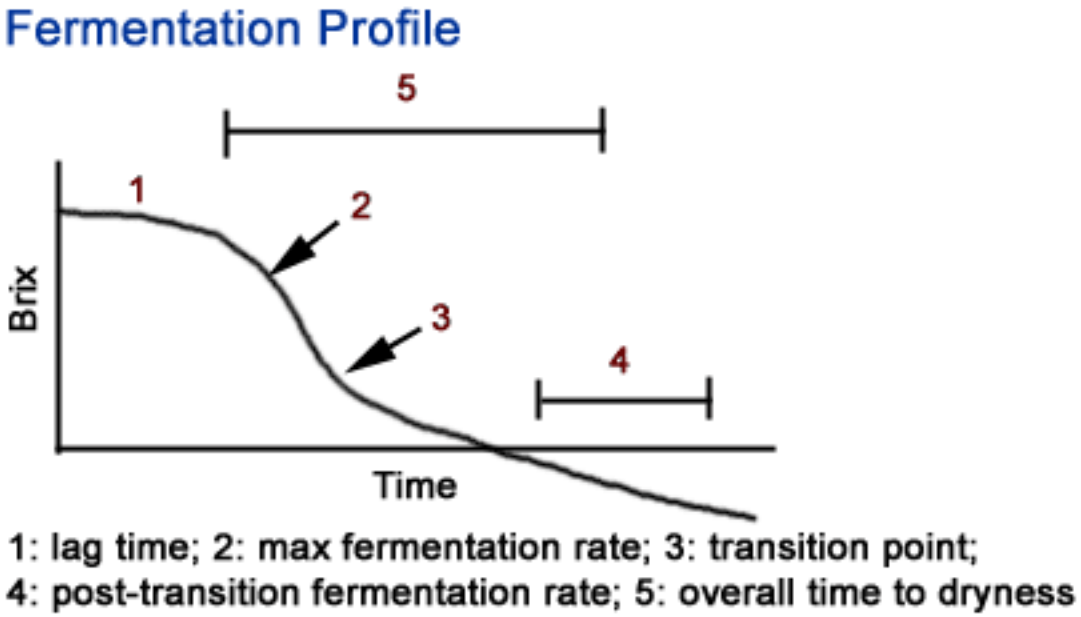
Lesson 10: Stuck and Sluggish Fermentations

A stuck fermentation is defined as a fermentation containing a high or undesired level of residual sugar. In a typical fermentation, residual sugar concentration is less than 0.2g/L. In some styles that tolerate a higher sugar level, 4 g/L may be considered dry.

Stuck and Sluggish Fermentations

- **Characterized by failure of yeast to consume sugar**
- **Multiple causes**
- **Difficult to treat**
- **Leads to reduced wine quality**

Stuck fermentations arise when the **yeast fails to consume the available sugar**. As we will see, there are **multiple causes** of cessation of yeast metabolism. Sluggish fermentations are defined as those that are progressing very slowly, requiring a period of several weeks to complete. Fermentations are generally complete in two to three weeks under typical California vinification conditions. This of course depends upon the temperature of fermentation, the nutritional content of the juice or must and the yeast strain used. Both stuck and sluggish fermentations are **challenging to treat** since the cause is often obscure. Identification of aberrant fermentation kinetics requires first understanding what a normal profile looks like. "Normal" may vary depending upon vinification conditions and profiles may differ for different strains.



A typical fermentation is presented in the graph above. There is an initial lag in sugar consumption. The lag phase comprises the time during which rapid proliferation of the yeast is occurring. The maximal fermentation rate coincides with the time of maximal biomass production. As ethanol increases, the fermentation rate slows and may change abruptly or more gradually, depending upon the conditions of the fermentation. The point at which there is a dramatic change in rate is called the transition point. This point may be very late in the fermentation or may not be observed at all, depending upon the strain and the nutritional conditions. If it occurs early, at a Brix value greater than 5, the fermentation frequently becomes sluggish and at risk of arrest. The Brix value at which the transition occurs therefore has diagnostic value. The difference between the maximal fermentation rate and the post transition fermentation rate likewise has predictive value for the occurrence of sluggish fermentations. The smaller the difference, the healthier the culture. Also, if there is a transition from one rate to another rate, and the second rate is steadily decreasing this may simply reflect a change in the dominating yeast strain present. If the rate post -transition is not steady, but instead continues to decline, the fermentation may be at risk of arrest. Finally, it is important to note the overall time required to achieve dryness. We define this as the time from the initiation of fermentation (exit from lag) to dryness, less than 0.2% sugar.

Fermentation Profile

- **Lag time**
  - **Duration?**
- **Maximum fermentation rate**
  - **Rate value?**
  - **Duration?**
- **Transition point**
  - **At what Brix level?**
  - **How sharp?**
- **Post-transition fermentation rate**
  - **Value relative to max fermentation rate?**
  - **Length of time?**
  - **Brix/ethanol/nitrogen level at which it occurs?**
- **Overall time to dryness**

What is normal for one strain may not be typical for another so it is important to know the characteristic traits of the strain used.

Fermentation Capacity Is a Function of:

- **Yeast Biomass Concentration**
- **Fermentative Ability of Individual Cells**

The maximal fermentation rate is related to both the total number of cells and the fermentative capacity of the cells. Not all cells in a culture are uniform. There may be distinct subpopulations differing in fermentative capacity. What is typically measured is the fermentation rate of a population, which is then averaged over the number of cells or biomass present. This is because it is experimentally difficult, if not impossible, to measure individual rates of fermentation or rates of sub-populations. However, it is not known if all of the cells at a given phase of fermentation are metabolically identical; indeed data suggests that they might not be and distinct subpopulations may be present. As noted in the lecture on yeast cell biology, yeast are mortal and new cell surface growth is restricted to the bud. Thus the bud may have a different protein complement than the mother cell. Since changes in the composition of both the cell wall and plasma membrane affect yeast cell viability and fermentative activity,

depending upon the time of "birth" of a cell it may have a completely different tolerance to ethanol, and a different capacity for fermentation.

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Lesson 10: Causes of Slow and Incomplete Fermentations

Several factors have been identified that impact fermentation rate or progression and that can lead to slow or incomplete fermentations.

Causes of Stuck/Sluggish Fermentations

- **Nutrient limitation**
- **Nutrient imbalance**
- **Substrate inhibition**
- **Ethanol toxicity**
- **Presence of toxic substances**
- **Poor adaptation of strain**
- **Low pH**
- **Temperature shock**

Nutrient Limitation as a Cause of Sluggish Fermentation

Nutrient limitation is frequently a cause of fermentation arrest. If insufficient nutrients are available to support the production of maximal yeast biomass, the maximal rate of fermentation will obviously be diminished. Nutrients are also needed to maintain the fermentation capacity of individual cells. That is, the fermentation may achieve maximal cell density of 10^8 cells/mL, but if the cells have diminished capacity for sugar catabolism, the rate may still not attain the maximum possible value. Typically there are between 10^7 and 10^8 cells/mL during fermentation. The cell number fluctuates, depending upon the strain used. This is partly due to settling of yeast to the bottom of the fermentation vessel. It seems that the culture must drop below a certain threshold before re-growth to 10^8 cells/mL. It is important to monitor the suspended cell count as well as the total cell count (obtained upon thorough mixing of the fermentation vessel - which may be difficult under commercial production conditions).

Nutrient Limitation: Nitrogen

- **Nitrogen: most often limiting**
- **Amino Acids**
  - **Can be degraded as N source via transamination**
  - **Can be interconverted with related amino acids**
  - **Can be used as that amino acid**
- **Ammonia**
  - **Mobilized by direct amination**

The **most frequently limiting nutrient is nitrogen**. There are several compounds that can serve as yeast nitrogen sources. Some of these compounds can serve as sole nitrogen sources, meaning that if the compound is the only nitrogen-containing compound in the medium yeast will still be able to grow and metabolize substrate. Lysine and cysteine were found to be incapable of supporting growth of any one of five yeast strains that were evaluated when present as sole nitrogen source in a defined medium. The ability to serve as sole nitrogen source means that all other nitrogen containing compounds necessary for cell growth and viability can be made from that compound. Two or more strains were not able to use glycine or histidine as sole nitrogen source. For the rest of the strains, growth was quite poor on these compounds. Similar and rapid growth rates were obtained with ammonia, glutamine, asparagine, arginine, glutamine, serine, alanine, aspartate, allantoin, urea, and ornithine. Other amino acids supported growth at intermediate doubling times. There is significant strain variation in the efficiency of use of amino acids.

Amino acids that cannot serve as sole nitrogen source may still be important to the cell. All amino acids can be used as that amino acid to support protein synthesis. Some amino acids can be inter-converted with other amino acids, meaning that they can be used to synthesize other nitrogen containing compounds without being completely degraded. In other words, the amino acid may feed multiple cellular pools. The fastest growth rates occur on mixtures of amino acids. In this case the yeast is saved the need of having to expend energy to synthesize amino acids and only needs to invest in their uptake into the cell.

Ammonia is the most versatile nitrogen source; it can be directly incorporated into carbon skeletons via direct amination. Both glutamate and glutamine can be produced enzymatically by amination.

Amination

NH_4 + alpha-ketoglutarate ⟶ glutamate

NH_4 + glutamate ⟶ glutamine

The compounds produced, glutamate and glutamine, can in turn be used in transamination reactions. In these reactions the amine group is transferred from one carbon skeleton to another.

Transamination

Glutamate + X ⟶ alpha - ketoglutarate + N - X
Glutamine + X' ⟶ glutamate + N - X'
Alanine + X" ⟶ pyruvate + N - X"

Where "X" is an intermediate in amino acid/nucleotide biosynthesis, and "N - X" is an amino acid or nucleotide base.

Alanine can also be a nitrogen donor in transamination reactions. Nitrogen moieties are transferred to carbon skeletons either via direct amination or transamination. Other nitrogen compounds can be degraded to generate free ammonia, alanine, glutamine or glutamate thereby becoming mobilized to synthesize other amino acids.

When presented with a mixture of amino acids, yeast first fills cytoplasmic amino acid pools. Once pools become depleted the yeast must start synthesizing amino acids from the remaining nitrogen sources. Yeast show a distinct preference for some amino acids over others. Preferred nitrogen sources are defined as those that are consumed from the medium first when the culture is presented with a mixture.

Preference for Nitrogen Sources

- **How readily can it be converted to NH_4, glutamate or glutamine?**
- **Expense of utilization (ATP, cofactor, oxygen requirement)**
- **Toxicity of C-skeleton**
- **What else is available?**

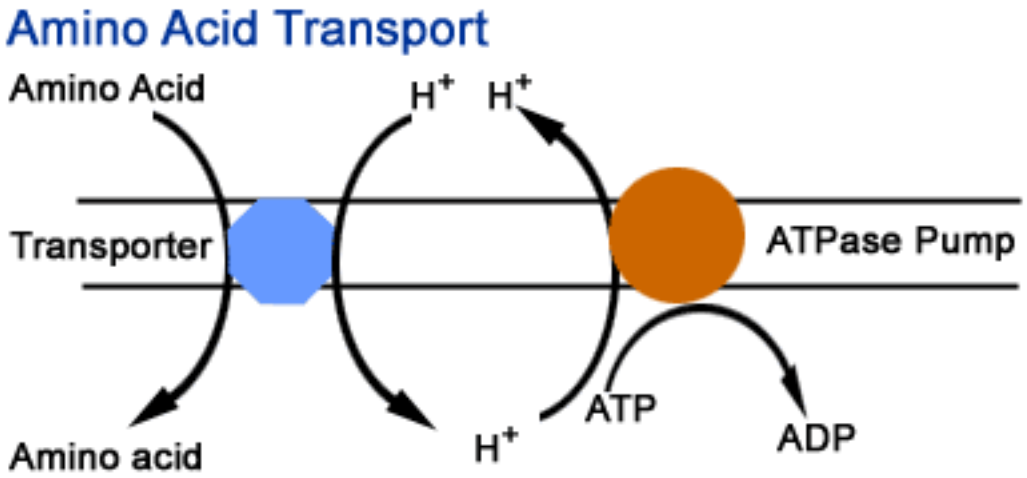
Nitrogen source preference is dependent upon several factors. Principal among these is how readily the compound can be used to **generate ammonia, glutamate or glutamine**, as these are the principle compounds used in biosynthesis. Amino acids that are "inexpensive" to degrade will be used in preference to those that require energy or micronutrients. Proline and some of the aromatic amino acids cannot be degraded under anaerobic conditions. This is because molecular oxygen is required for degradation. Another important factor concerns the possible toxicity of the **carbon skeleton** generated following deamination or removal of the amino group. For those amino acids that require micronutrient vitamins for degradation, the availability of those cofactors will impact whether or not the compound can be used. Therefore nitrogen source preference is influenced by the composition of the medium. Strain differences also exist. Some strains utilize certain amino acids more efficiently than others. The genetic basis of these differences are largely unknown in commercial strains, but, if supplementing a fermentation, it is important to understand the nutritional needs of the specific strain in question. All strains evaluated are able to use ammonia, therefore ammonia supplementation is the most widely effective.

Factors Affecting Nitrogen Compound Utilization and Preference

- **pH**
  - **Transport is coupled to H+ ion movements**
- **Ethanol**
  - **Inhibits amino acid transporter function (80%(decreases) at 5% ethanol for the general amino acid permease)**
  - **Increases passive proton flux**
- **Other N compounds**
  - **Competition for uptake**
  - **Nitrogen repression**
  - **Induction**

- **Yeast strain differences**

Other factors also affect nitrogen compound utilization. Amino acids need to be transported against their concentration gradient, meaning that the amino acid concentration inside of the cell is higher than that of the medium. Transport against a gradient is energetically unfavorable. *Saccharomyces* has solved this problem by coupling movement of amino acids to a component that displays a steep gradient across the cell membrane: hydrogen ions. The internal **pH** of a yeast cell is near neutrality (pH 6.5- 7.0), while the pH of the medium is 3.0 to 4.0. This creates a large gradient of protons, and entry of hydrogen ions into the cell is energetically favorable. Thus, movement of amino acids is coupled to that of protons. Amino acid transporters transfer both an amino acid molecule and a proton into the cell. The proton is then exported out of the cell via a proton pump. The pump extrudes protons in an energy dependent manner since excretion occurs against the proton gradient. Hydrolysis of ATP provides the energy for operation of the pump. Continued amino acid uptake requires efficient excretion of the protons co-transported with the amino acid; thus **amino acid uptake is an energy requiring process**. Since amino acid uptake is coupled to protons it is not surprising that medium pH affects amino acid uptake. If the pH is too low, below 2.7, protons tend to enter the cell due to passive proton flux. The proton pump must then be directed towards removal of these protons. The cell has a limited capacity for the removal of protons, thus at very low pH the cell is not able to sustain amino acid uptake due to the lack of capacity of the proton pump.



Ethanol also increases the tendency of protons to enter the cell. At high ethanol concentrations, protons tend to enter the cell again due to passive proton flux. Amino acid uptake is inhibited at high ethanol concentrations. Studies have shown that

addition of ethanol results in a decrease or inhibition of amino acid permeases. Some permeases or transporters may be inhibited up to 80% by the presence of ethanol. This regulatory mechanism assures that internalized protons will not swamp the cell and proton influx will not exceed the capacity to pump protons out of the cell. What is the consequence of failure to remove protons? The cytoplasm of the cell becomes acidified which denatures proteins leading to cell death.

Many amino acids share common transport mechanisms. There are general amino acid transporters and specific amino acid transport mechanisms. Amino acids that share common protein uptake systems compete with each other for uptake. The kinetics of uptake of an amino acid will therefore be affected by the presence of other amino acids in the medium. In addition to competition for uptake, nitrogen compound degradation is also regulated transcriptionally. Preferred **nitrogen sources** block the degradation of the less preferred compounds. Ammonia, for example, represses the synthesis of many of the enzymes required for degradation of amino acids. In effect this saves those amino acids for protein synthesis. Another regulatory process that occurs is called induction. This means that degradative enzymes will not be synthesized in the absence of their respective substrate. For example, it would not be prudent to synthesize asparaginase unless asparagine was actually present in the medium.

Different **yeast strains** vary in response to these factors. Some are supersensitive to proton influx and arrest amino acid uptake under conditions that are still permissive for other strains. Whether this is due to differences in proton influx or to the capacity of the proton ATPase pump is not known.

Sources of Nutrients

- **Grape**
- **Nutrient additions (winemaker)**
  - **Diammonium phosphate**
  - **Yeast extracts**
  - **Yeast "ghosts"**
  - **Proprietary yeast nutrient mix**
- **Yeast autolysis**

Nitrogen compounds come from the **grape** itself. The winemaker may also augment the nitrogen content of the fermentation by addition of diammonium phosphate or other

yeast **nutrient preparations**. At the end of fermentation, **yeast autolysis** may also result in the release of internal components including amino acids.

Ionic Imbalance as a Cause of Sluggish Fermentation

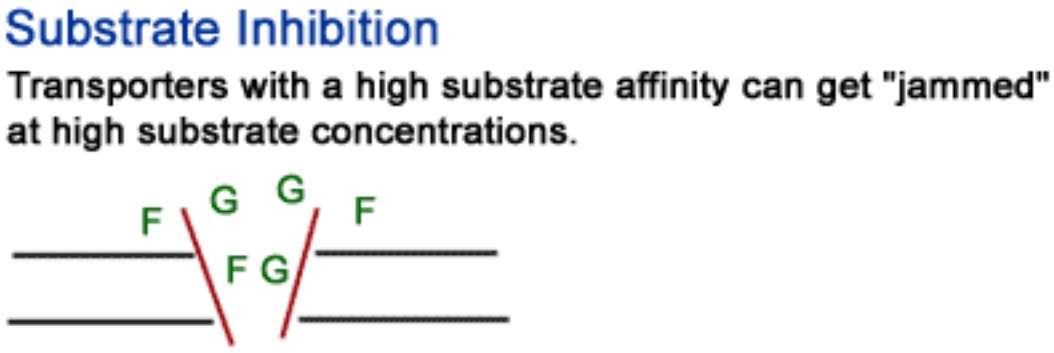
- **Ratio of K+:H+**
- **Must be at least 25:1**
- **Needs to be adjusted early in fermentation**
- **Probably important in building an ethanol tolerant membrane**

Imbalances in the relative concentrations of ions can also lead to arrest of fermentation. Yeast ferment better if there is an excess of potassium over protons. The function of the potassium is not known. Some researchers think that it blocks the disruptive effects of free protons on protein structure and function. Others believe that potassium is important in regulating proton efflux from the cell. In this model, protons can be exchanged for potassium in a process that does not require ATP. A third model suggests that potassium is incorporated into the cell wall and plasma membrane which makes the membrane better able to resist the disruptive effects of ethanol.

The **ratio of potassium to hydrogen ions** needs to be on the order of **25:1**, on a molar basis (not gram to gram). **Adjustment** of the ratio late in fermentation does not restore fermentation rate. This suggests that the last model, that potassium is required for synthesis of **ethanol tolerant cellular components** is more likely to be correct than the other models.

Substrate Inhibition as a Cause of Sluggish Fermentation

Another cause of a decrease in fermentation rate is substrate inhibition. This phenomenon appears to have two different causes. It has been well established that high substrate concentration can inhibit sugar uptake if the cells have adapted to a low substrate concentration. This would occur if one were using one fermentation to inoculate another. *Saccharomyces* possesses a large family of sugar transporters named the "HXT genes" for HeXose Transport. There are 18 HXT genes in laboratory strains.



Transporters that are designed to work at low substrate concentrations are said to have a high affinity for substrate. They have an open conformation allowing them to recognize the substrate regardless of the way in which the substrate approaches the transporter. In this case, the transporter has more than one recognition or binding site for the substrate. Binding triggers molecular movement of the transporter and the translocation of substrate to the inside of the cell. If substrate concentration is high then more than one substrate molecule binds to the transporter at the same time. Two sugars occupying the binding sites prevents the molecular movement of the protein from occurring and the transporter is jammed. This is only a problem when the cells shift from a low substrate concentration to a high concentration. This mechanism assures that the cell will have the opportunity to readjust metabolism to the new substrate level. This is important since as noted in the lecture on glycolysis, excessive sugar uptake leads to an imbalance in ATP consumption, which will arrest cell growth. This phenomenon is called substrate inhibition.

A different form of substrate inhibition occurs late in fermentation. Cells metabolize glucose with faster kinetics than fructose. This results in a change in the glucose to fructose ratio from the 1:1 of juice. Under certain conditions the yeast appear to arrest or slow utilization of fructose but not of glucose. In this case the medium accumulates fructose. This imbalance of fructose over glucose appears to inhibit re-initiation of the fermentation. The mechanism by which this occurs is not known.

Ethanol Toxicity as a Cause of Sluggish Fermentation

Ethanol:

- **Perturbs membrane structure at protein:lipid interface**
- **Leads to increased "passive proton flux" and acidification of cytoplasm**
- **Inhibits protein activity**
- **Affects membrane "fluidity"**

Another factor impacting fermentation rate is ethanol itself. Ethanol inhibits many cellular activities including transport of amino acids and other nutrients. Interestingly it does not impact sugar uptake unless at very high concentrations.

Ethanol Toxicity

Plasma membrane is the most ethanol-sensitive cell structure:

Composition:	Protein	50%
	Lipid	40%
	Other	10%

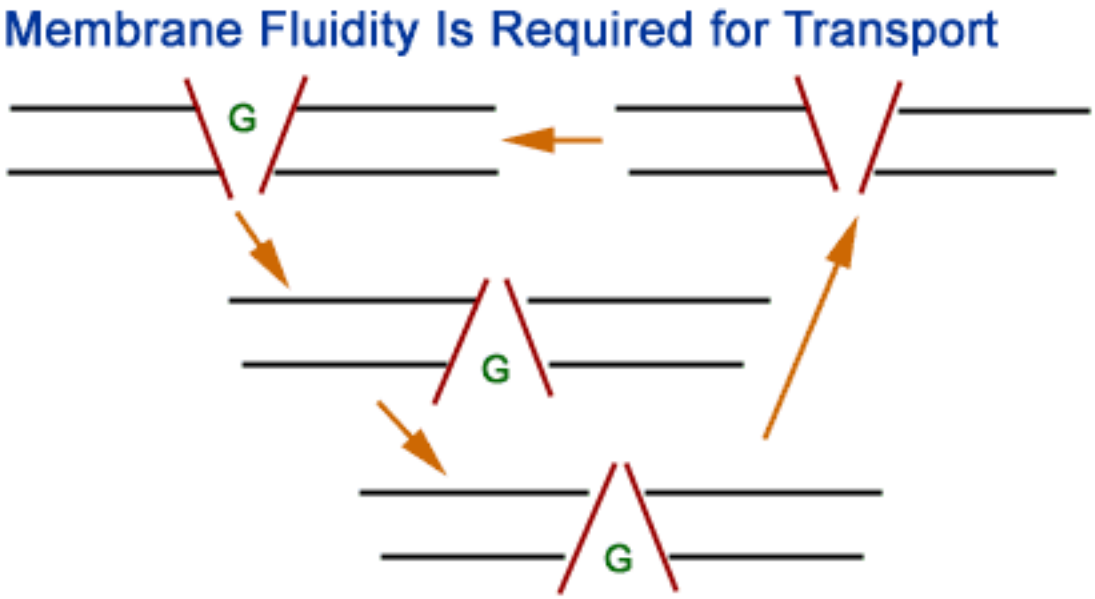
Functions:

- Permeability barrier
- Regulation of uptake
- Mediates response to environment
- Maintains electrochemical gradients
- Mediates cell - cell interactions

The main target of ethanol inhibition is the **plasma membrane**. The plasma membrane is comprised largely of protein and lipid. This cellular structure plays a vital role in regulation of uptake and excretion of cellular components. It also mediates the response of the cells to environmental stress and maintains cellular electrochemical gradients. In addition, the plasma membrane plays a key role in cell-cell interactions such as mating and flocculation. Ethanol impacts function of the membrane in several ways.

It **perturbs the interaction between lipids and proteins** in the membrane. This can lead to leakage or **increased passive flux of protons**, which in turn leads the acidification to the cytoplasm. Ethanol can disrupt the interaction between proteins and water thereby affecting protein activity. One of the principle responses of the cells is to

adjust the fluidity of the membrane, which has lead to the hypothesis that ethanol perturbs membrane fluidity.



To return to the model of glucose transport, interaction of a glucose molecule with the transporter causes a change from a conformation facing the outside of the cell to one facing the cytoplasm. The sugar molecule then dissociates from the transporter and is transferred to the cytoplasm. The inward facing transporter must then make a second conformational change to reface the outside of the cell to pick up another sugar molecule. The conformational change requires that the protein be able to move within the lipid matrix. The membrane must allow movement but at the same time restrict it to prevent the protein from denaturing. This elastic property of the membrane is called **membrane fluidity**. If the membrane is too stiff, the movement will not occur and there will be no net transport. If the membrane is too fluid, the protein will move too much, and it will be energetically difficult to realign the protein. Anything that alters membrane fluidity will affect transporter protein activity.

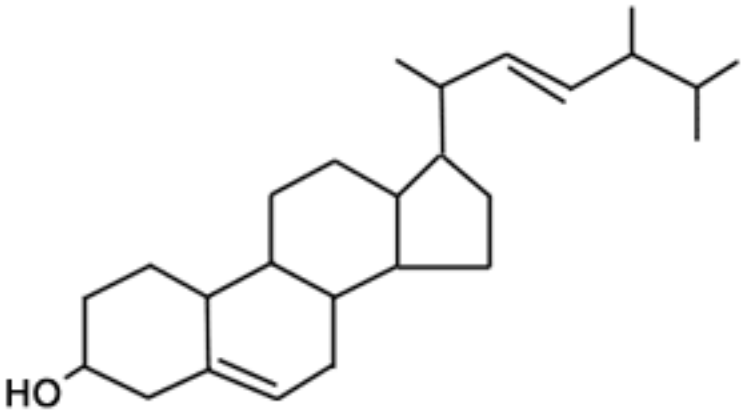
Ethanol Toxicity

Adaptation of membrane requires:

- **Increasing content of sterols**
- **Increasing relative content of proteins**
- **Increasing level of desaturation (number of double bonds) in fatty acid side chains**
- **Modification of phospholipid head groups?**

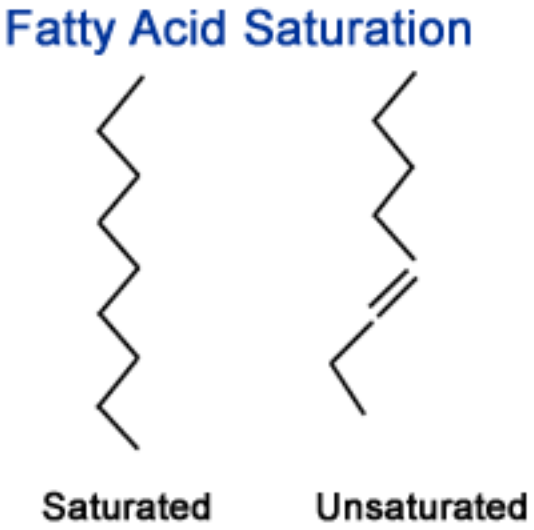
Plasma membrane composition can be altered in order to minimize the impact of ethanol produced during fermentation. Several studies have shown that membranes isolated from high ethanol growth conditions are higher in sterol content, have a higher ratio of protein to lipid. The yeast sterol is ergosterol, which has the following composition.

Ergosterol

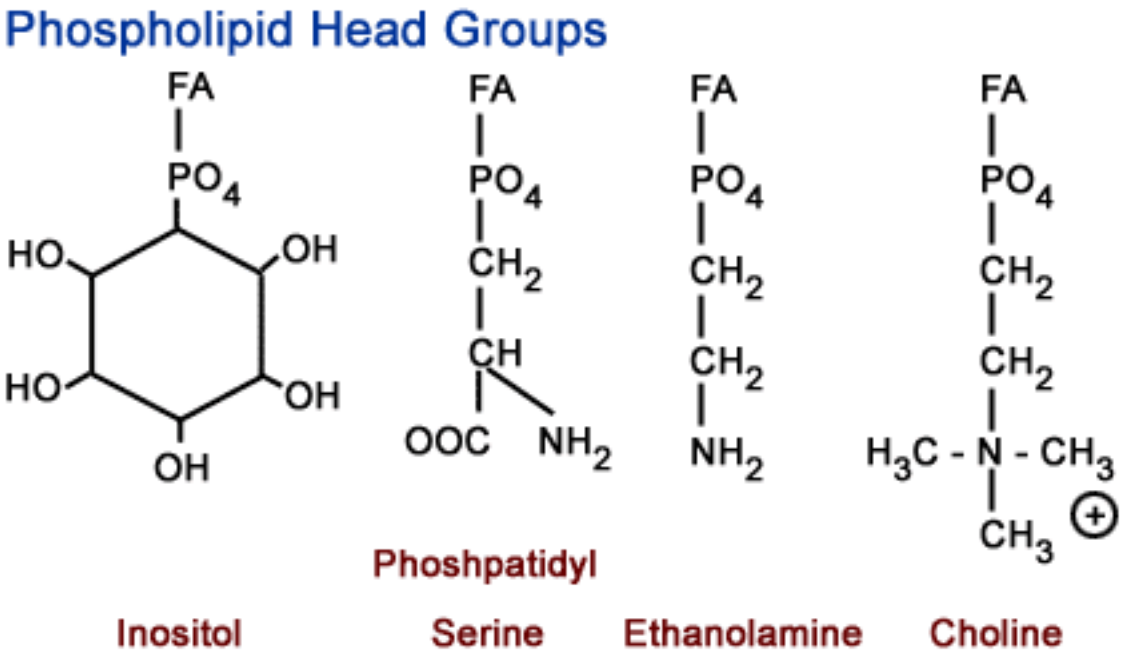


Plants also produce **sterols** that can be used by yeast as substitutes for ergosterol. The actual requirement for ergosterol is quite low, other sterols, including animal cholesterol, can provide what has been termed the "bulk" or structural function.

In addition the nature of the fatty acid chains of the lipid molecules appears to change. The number of double bonds (unsaturated lipids) increases.



There are conflicting reports in the direction of changes of the phospholipid head groups.



One study suggests that phosphatidyl inositol levels increase in ethanol tolerant membranes while in another study phosphatidyl choline levels increased relative to other phospholipid head groups. The differences in these conclusions likely reflect differences in the way the studies were conducted and what substrate molecules needed to continue to be translocated into the cell. It is important when reading the yeast cell literature to pay attention to the conditions under which the studies were conducted.

Ethanol Toxicity

- **Sterol and fatty acid desaturation are Oxygen-requiring processes**
- **New protein synthesis requires nitrogen be available**
- **Phospholipid head group synthesis requires cofactors (S-adenosylmethionine) be available**

Both sterol biosynthesis and fatty acid desaturation require the formation of double bonds in carbon chains. Enzymatically this occurs by transfer of hydrogen ions to molecular oxygen. This means that these reactions require that oxygen be present in the medium. They will not occur under strict anaerobic conditions such as occur in grape juice fermentation. The alteration of the protein content of the membrane requires that new proteins be synthesized. This of course will not occur in the absence of a nitrogen source. Phospholipid head group synthesis requires that key cofactors be available. If the yeast are deficient in any of these compounds, the change cannot occur. There is disagreement in the literature as to which factor, lipid or protein modification, is the most important. Part of this disagreement is likely due to the way in which these studies have been conducted. Some have been an "ethanol challenge" where ethanol is added to a culture with low to no ethanol. This leaves no opportunity for adaptation. Other studies use naturally accumulated ethanol, that is, initiate the experiment with a high concentration of sugar. In this case the yeast is able to make adaptive changes during the course of fermentation. It is not surprising that different conclusions would be reached under these conditions. Further, **acetaldehyde** may accompany natural ethanol accumulation and will compound the interpretation of the inhibitory effect of the ethanol. Acetaldehyde is itself quite toxic.

Presence of Toxic Substances

Substances that inhibit growth or lead to cell death will also impact fermentation rate and can lead to sluggish or stuck fermentations. Toxins can arise from several sources during wine production.

Presence of Toxic Substances

- **Toxins may arise from the metabolic activity of other microbes**
- **Toxins may arise from metabolic activity of *Saccharomyces***
- **Toxins may have arisen in vineyard, but are not inhibitory until ethanol has accumulated**

Toxins are most commonly thought of as **deriving from other microbes** that impact the biological activities of *Saccharomyces.* Inhibitory factors may also be **produced by strains of *Saccharomyces***. These compounds are called **killer factors**. They are small peptides that inhibit the growth of sensitive strains of *Saccharomyces*. With respect to killer factor there are three types of strains. Those that both produce it and are resistant to it, those that are resistant to it but do not produce it, and those that are sensitive to the killer factor. The killer factors produced by *Saccharomyces* only inhibit other strains of *Saccharomyces*. Other yeasts produce similar inhibitory peptides that have a broader spectrum, inhibiting many genera of yeast. Toxins may also arise from the vineyard. As noted before, *Saccharomyces* is a member of the kingdom of fungi. Fungicides used in the vineyard to inhibit mold infestation of the fruit may also inhibit *Saccharomyces* if present in high enough concentration at the time of harvest.

The most common toxins occurring in grape juice/must fermentation are listed below.

The Most Common Toxins

- **Acetic acid**
- **Higher organic acids C_2-C_4**
- **Medium chain fatty acids/fatty acid esters**
- **Acetaldehyde**
- **Fungicide/Pesticide residues**
- **Higher alcohols**
- **Higher aldehydes**
- **Killer factors**
- **Sulfur dioxide**

Bacteria commonly produce **acetic acid**, **higher organic acids** and **medium chain**

fatty acids and **fatty acid esters**. Some yeast strains, *Kloeckera/Hanseniaspora* for example, produce acetic acid as an end product of fermentation. Acetaldehyde and higher aldehydes can be produced by *Saccharomyces* during the course of fermentation and amino acid catabolism. It is thought that many of the inhibitory affects associated with ethanol are actually due to acetaldehyde, which is far more toxic at lower concentrations. Aldehydes are very reactive molecules that can interact with amino acid side chains inhibiting protein activity. Higher alcohols (greater than two carbons) are also inhibitory, and are more inhibitory than ethanol. They can be produced by *Saccharomyces*, but it is thought that if they are present in inhibitory concentrations they were produced by bacteria or other non-*Saccharomyces* yeasts. Sulfur dioxide can also be inhibitory. The levels produced by *Saccharomyces* are never in the inhibitory range; however, it is all too common for multiple SO_2 additions to accidentally be made in a winery that result in an inhibitory concentration being present.

Poor Adaptation of Strain

- **Strain may not display ethanol tolerance**
- **Strain may have high nitrogen/vitamin requirements**
- **Strain may be a poor fermentor, but capable of dominating the fermentation**
- **Temperature effects**

Another factor impacting strain fermentation performance is a poor adaptation of the strain to the fermentation conditions.

If the cell has insufficient oxygen available, it will not be able to make sterols and unsaturated fatty acids required for optimal ethanol tolerance. *Saccharomyces* can use fatty acids and sterols that are stored in internal membranes. That is, it can parasitize its own internal membranous organelles. However if it has been grown under poor or sub-optimal growth conditions it will not have anything to recruit fatty acid or sterol components from and is therefore poorly adapted to the growth conditions. Similarly, vitamins accumulate within yeast cells. They frequently have sufficient vitamin stores to undergo up to 40 cell divisions in the absence of vitamin supplementation. In an inoculated fermentation cells typically undergo five to seven generations, nowhere near 40. However, if the population has been undernourished, it will become vitamin deficient during fermentation. Some strains have higher vitamin or nitrogen

requirements than others and may be poorly adapted to fermentation conditions. Some strains are more sensitive to temperature extremes than others. If one of these strains is used in a "hot" or "cold" fermentation, it will arrest once ethanol accumulates in the medium. Thus the ability of strains to ferment is a function of intrinsic genetic characters as well as of medium composition.

Low pH

- **pH is reduced by metabolism of *Saccharomyces***
- **Low pH musts may drop to an inhibitory level**
- **Dependent upon K+ concentration**

Saccharomyces produces protons during fermentation. The resulting release of protons can decrease the pH of the fermenting juice by 0.1 to 0.3 pH units. If the pH of the starting wine is too low (3.0 or below) this decrease can lower the pH to an inhibitory level (less than 2.75). At these low pH values further fermentation will not occur.

Inhibition by hydrogen ion concentrations is a function of the potassium ion concentration. The lower the pH the higher the concentration of potassium needed to sustain fermentation rates.

Temperature Shock

- **Super-cooling/heating of tank due to equipment failure**
- **High temperature fermentations becoming too warm due to yeast metabolism**

Temperature swings during fermentation can also inhibit sugar catabolism. Temperature impacts membrane fluidity. The higher the temperature the more fluid the membrane. High temperatures may alter membrane fluidity impacting ethanol tolerance. At higher ethanol concentrations, the temperature maxima supporting growth is lowered while the minima is increased. In other words, the temperature range supporting growth and metabolism narrows. For example if the temperature range

supporting growth is from 12 to 35°C in the absence of ethanol, in its presence the range will be narrowed to 20 to 30°C. In general, it is more difficult to produce a membrane simultaneously tolerant to low temperature and to high ethanol as these parameters require opposing adaptations. It is easier to maintain growth at higher temperatures in the presence of ethanol, but this too has its limits. Temperature shock refers to a dramatic (greater than 5°C) change in the mean temperature of the tank. This may arise due to supercooling, which can occur as the fermentation slows and the heat release from yeast metabolism decreases.

High temperatures can occur when yeast metabolism generates much more heat than can be dissipated during the fermentation.

The factors leading to arrest of fermentation are interacting. Limitations for nutrients enhances the toxicity of ethanol as does the presence of other toxic substances.

In general if multiple inhibitory conditions are present, the effect on yeast metabolism may be synergistic rather than additive. Many inhibitory compounds are more deleterious at higher ethanol concentrations.

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Lesson 10: Off-Character Production

The next section of the lecture will cover the second class of problem fermentations, those that result in formation of off-characters.

The *Saccharomyces* Off Characters

- **Volatile Sulfur Compounds**
- **Acetic Acid**
- **Higher Alcohols**
- **Acetaldehyde/Higher Aldehydes**
- **Unwanted Esters**
- **Vinyl Phenols**

The principle class of off-characters is the volatile sulfur compounds. As a group, these compounds have objectionable odors and have low thresholds of detection by humans.

Volatile sulfur compounds derive from the degradation of sulfur containing amino acids. Hydrogen sulfide can arise from the reduction of sulfate. The following table lists the most common sulfur containing volatiles found in wine.

Volatile Sulfur Compounds

- **Hydrogen Sulfide: H_2S**
- **Methanethiol: CH_3-SH**
- **Ethanethiol: C_2H_5-SH**
- **Dimethyl sulfide: CH_3-S-CH_3**
- **Dimethyl disulfide: CH_3-S-S-CH_3**
- **Diethyl sulfide: C_2H_5-S-C_2H_5**
- **Diethyl disulfide: C_2H_5-S-S-C_2H_5**

Hydrogen sulfide may also come from the degradation of sulfur containing amino acids or from the reduction of organic sulfur used in the vineyard as a fungicide. If other sulfur containing fungicides or pesticides were used in the vineyard, they may also be degraded to a volatile sulfur compound. Some of these compounds may then undergo other spontaneous chemical reactions producing other types of molecules. Hydrogen sulfide formation from inorganic sulfur is thought to be a spontaneous chemical reaction requiring reducing conditions established by yeast metabolism but is not catalyzed directly by *Saccharomyces*. The term "higher sulfides" refers to any compound other than H_2S.

Sources of Sulfur Compounds

- **Sulfate reduction pathway**
- **Degradation of sulfur containing amino acids**
- **Inorganic sulfur**
  - **Non-enzymatic**
  - **Requires reducing conditions established by yeast**
- **Degradation of S-containing pesticides/fungicides**

Other factors also impact formation of volatile sulfur compounds. In most strains, limitation for nitrogen leads to the production of higher levels of hydrogen sulfide.

Higher Sulfides

- **Come from degradation of sulfur containing amino acids**
- **From reaction of reduced sulfur intermediates with other cellular metabolites?**
- **Formed chemically due to reduced conditions**

There are many sources of hydrogen sulfide, and just as many factors impact its formation during fermentation.

Hydrogen Sulfide Formation

- **Due to nitrogen limitation**
- **Sulfate reduction regulated by nitrogen availability**
- **Lack of nitrogenous reduced sulfur acceptors leads to excessive production of reduced sulfate and release as H_2S**
- **Strain variation**

Sulfate is reduced in order to synthesize the sulfur containing amino acids. Therefore it is not surprising that there is a connection between nitrogen availability and release of reduced sulfur in excess of the nitrogen-carbon acceptor molecules. There is also significant strain variation. Some commercial strains tend to produce high levels of H_2S while others produce relatively low levels.

Hydrogen sulfide formation is a chronic problem in the wine industry worldwide. Copper fining can remove sulfide via the formation of a CuS precipitate. Copper must then be removed by other treatments. While H_2S can be removed, most winemakers would prefer that it not be made in the first place. A significant amount of research has been conducted over the years to develop fermentation strategies to limit or eliminate production of H_2S.

Current Understanding of H_2S Formation

- **Nitrogen levels not well-correlated with H_2S**
- **Under complex genetic control**
- **Tremendous strain variation in H_2S production**

While nitrogen limitation clearly leads to higher levels of hydrogen sulfide in most strains, there is not a consistent relationship between nitrogen level in the medium and sulfide release. This is due in large part to the high degree of variability in H_2S release across strains. The process of sulfate reduction generates several toxic intermediates and the end product cysteine has also been shown to be toxic if it accumulates in yeast cells. Of these compounds, H_2S is the least toxic. Thus, if there is a problem in sulfate reduction, the cells would prefer to make H_2S. Other work suggests that hydrogen sulfide is inhibitory to respiration so it may only be non-toxic during anaerobic conditions.

Factors Impacting H_2S Formation

- **Level of total nitrogen**
- **Level of methionine relative to total nitrogen**
- **Fermentation rate**
- **Use of SO_2**
- **Vitamin deficiency**
- **Presence of metal ions**
- **Inorganic sulfur in vineyard**
- **Use of pesticides/fungicides**
- **Strain genetic background**

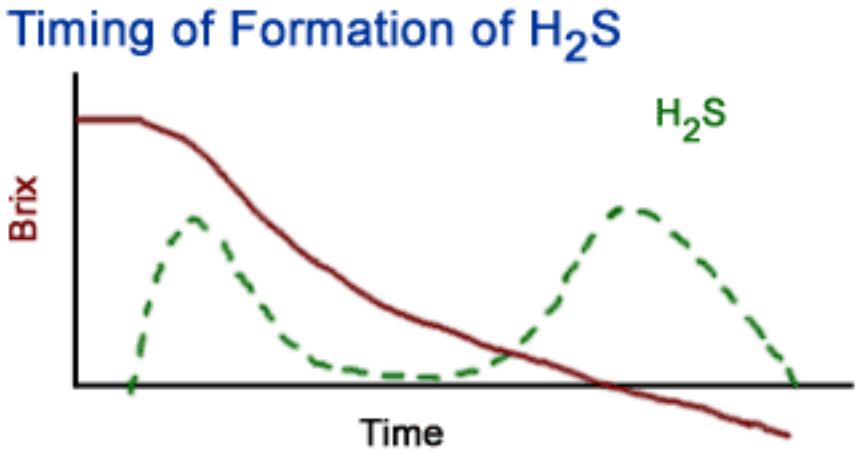
In addition to medium **nitrogen concentration**, other factors impact the appearance of H_2S. Obviously genetic background is a key factor although one poorly understood at this time. Inorganic sulfur and pesticide/fungicide residues impact sulfide as mentioned above. In addition, the sulfate reduction pathway is regulated by the concentrations of cysteine and **methionine** in the cell. Methionine represses expression of the genes encoding the enzymes of sulfate reduction, which reduces the amount of H_2S formed

by the pathway. Cysteine blocks formation of an inducer of expression of the sulfate reduction pathway. When both methionine and cysteine are present in high concentrations, expression of the pathway is at its lowest. However, as stated several times above, if methionine and cysteine are present in excess they can be degraded for their nitrogen content leading to release of hydrogen sulfide or production of higher sulfides.

Some strains show an impact of **fermentation rate** on sulfide formation, but this is not observed in all conditions, and may be an artifact of how the H_2S is measured as it is driven off of the fermentation by CO_2.

Synthesis of methionine requires the **vitamin** pantothenate. If pantothenate is limiting, sulfide levels increase because methionine and cysteine do not accumulate. Sulfide is also produced by yeast to detoxify metal ions in the environment. This is currently not a problem in the wine industry as juices and musts do not contain high enough concentrations of **metal ions**. Finally, **sulfur dioxide** can be correlated with H_2S formation in some strains. In the sulfate reduction sequence, sulfide is produced from the reduction of sulfur dioxide. Hydrogen sulfide can be produced in some strains from the reduction of external sulfite.

The pattern of formation of hydrogen sulfide during fermentation can vary. There are two points in the fermentation where high levels of sulfide might appear in the medium.



The early peak occurs usually around the time of maximal cell density. This is associated with the active phase of metabolism. The later peak occurs as the cells are finishing fermentation. It is thought to be due to degradation of sulfur containing amino acids. Some strains produce lower levels of sulfide throughout fermentation.

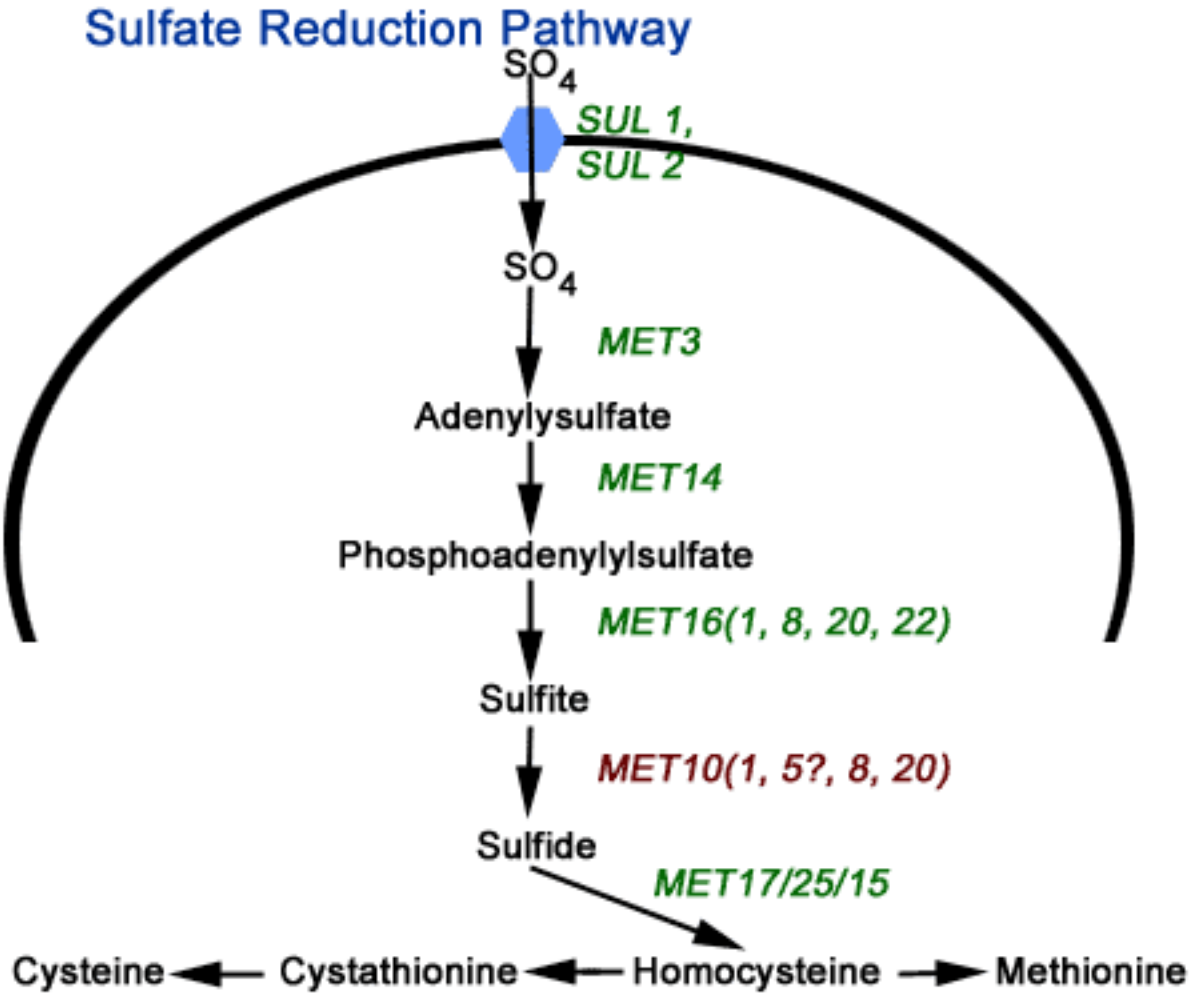
Timing of Formation of H_2S Early (first 2-4 days): due to N imbalance

Late (end of fermentation): due to autolysis, degradation of S-containing compounds

H_2S produced early can be driven off by carbon dioxide during active phase of fermentation

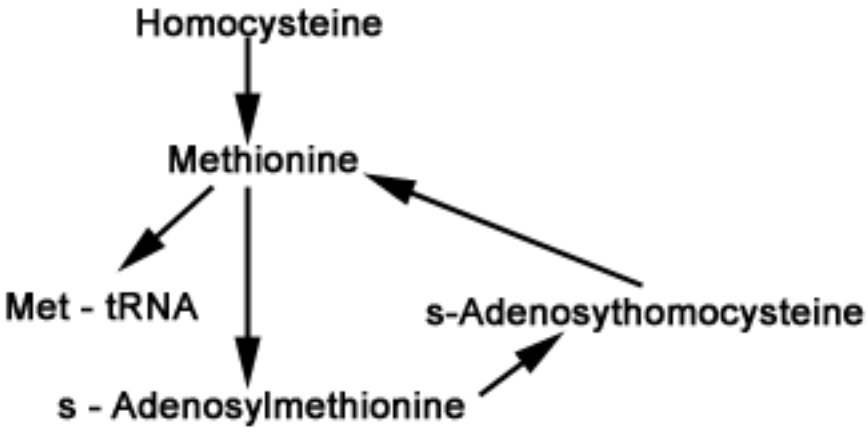
Hydrogen sulfide produced early in fermentation can be driven off by the carbon dioxide produced during fermentation, so it is not considered to be as problematic as H_2S produced after the fermentation rate slows.

The sulfate reduction pathway is presented in the following figure.

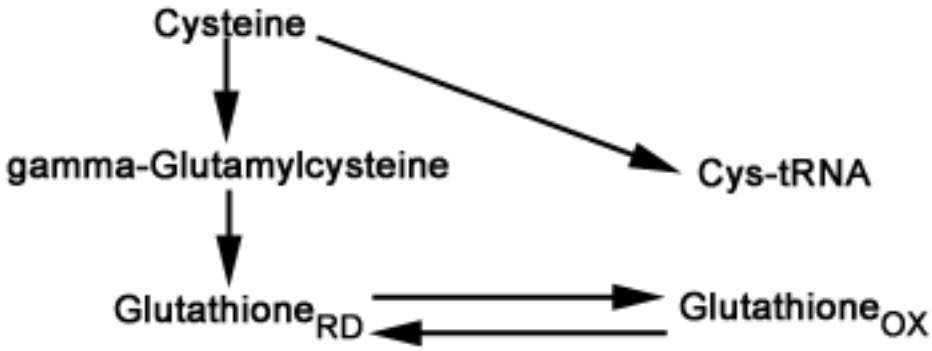


Sulfide is incorporated into homoserine yielding homocysteine. Homocysteine can be

converted into methionine or into cystathionine and then cysteine.



Methionine is used to synthesize Met-tRNA used in protein synthesis and S-Adenosylmethionine (SAM), which is required for one-carbon transfers used in many biosynthetic reactions. Transfer of the methyl group from SAM produces S-adenosylhomocysteine that can then be reconverted to homocysteine and methionine.



Cysteine is used to produce Cys-tRNA and to produce the tripeptide glutathione. Glutathione regulates the redox status of the cells and is a very important compound. Thus both methionine and cysteine are required to synthesize compounds other than proteins that are vital to the continued growth and metabolism of the cells.

Acetic Acid

Another off character arising during fermentation is acetic acid. *Saccharomyces* can produce acetic acid but this is generally at levels below the threshold of detection.

Other organisms are more prolific producers of acetic acid. Still, the amount produced by *Saccharomyces* can contribute to the overall level if other organisms are present

and making acetate. There are strain differences in the amount of acetic acid produced by *Saccharomyces*.

Acetic Acid Production by *Saccharomyces*

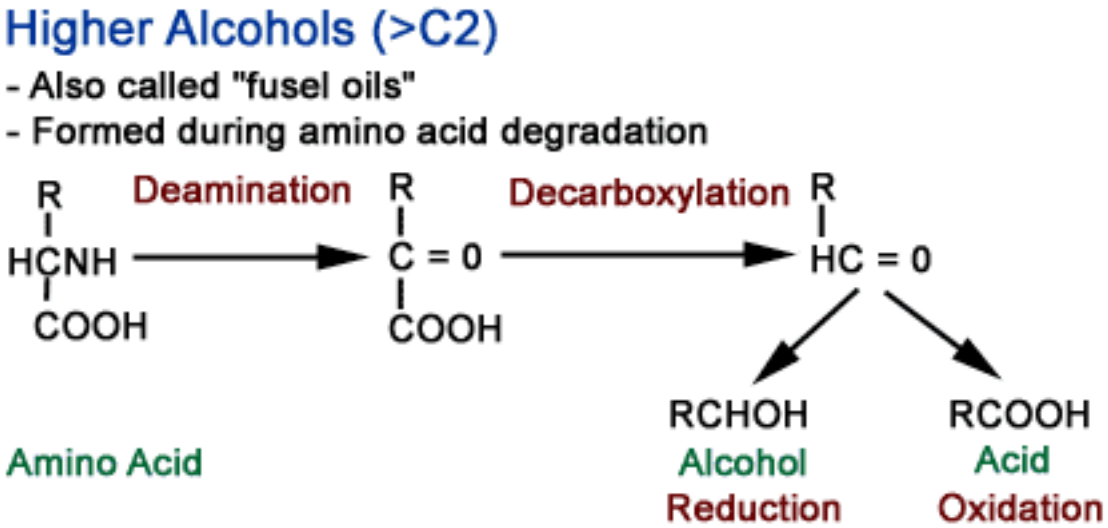
- **Levels made by *Saccharomyces* are low**
- **Strain differences in amount formed**
- **Derived from:**
  - **Fatty acid biosynthesis/degradation**
  - **Amino acid degradation**

Under anaerobic conditions, acetic acid is produced from fatty acid biosynthesis or degradation and from amino acid degradation. It can be produced from acetaldehyde under aerobic conditions.

Higher Alcohols

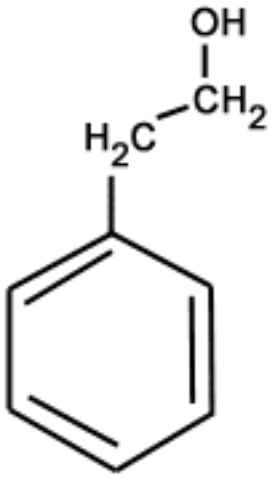
- **Fusel oils**
- **Phenethyl alcohol**

Higher alcohols, those with more than two carbons, may also be considered as off-characters depending upon the amount produced and the style of wine desired.



Higher alcohols are produced from amino acid degradation and are collectively called "fusel oils". The amino acid is first deaminated followed by a decarboxylation of the resulting carbon skeleton. This yields an aldehyde that can be reduced to an alcohol. This reduction is catalyzed by the same enzyme family that converts acetaldehyde to ethanol. Under some conditions, the aldehyde is oxidized to an acid rather than being reduced to an alcohol. Aldehydes are more toxic than alcohols or acids so the yeast would prefer to make one or the other of these compounds from the amino acid skeleton.

Phenethyl Alcohol



Generic "floral"
May be too intense for some wines

Alcohols can also be made from aromatic amino acids. One of the most common is phenethyl alcohol. This compound has been described as having a floral aroma, which, if present in high concentration may be too intense for some wines.

Acetaldehyde/Higher Aldehydes

Acetaldehyde and the higher aldehydes can be considered as off-characters if present in high concentration. These compounds are desired in some styles, such as sherry production, and are associated with wine age. Many students describe the aldehyde character in wine as "sherry". The formation of these compounds during aging will be discussed in a subsequent lecture.

Aldehyde Production

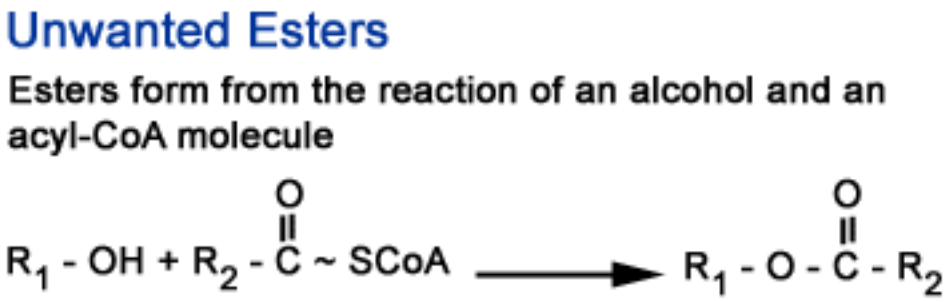
- **Acetaldehyde from glycolysis**
  - **Released when conversion to ethanol is blocked**
  - **Released as SO_2 adjunct**
- **Higher aldehydes from amino acid degradation**
  - **Released when formation of higher alcohols is blocked**

Acetaldehyde is formed during glycolysis. It is released under two conditions, when ethanol formation is blocked due to absence of alcohol dehydrogenase or when NADH is being used for some other purpose and does not need to be recycled during end product production. Acetaldehyde is also released as the detoxification mechanism for sulfites.

Unwanted Esters

- **Fatty acid metabolism**
- **Amino acid metabolism**
  - **Phenethyl Acetate**

Saccharomyces produces many esters during fermentation. Some wine styles are dependent upon the presence and spectrum of yeast esters produced. While in other wines they are undesired. Esters hydrolyze readily under acidic conditions so if the wine is to be aged they may not be present at the time of bottling.



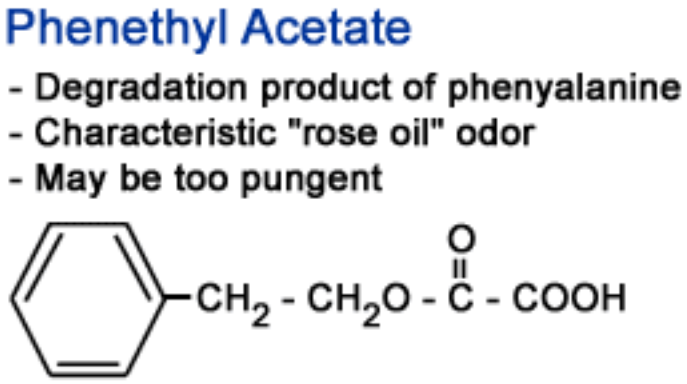
Esters are formed from the reaction between an alcohol and an acid species. The acid

species is first activated by attachment to coenzyme A. The most common ester is ethyl acetate formed from the reaction of acetyl-CoA and ethanol. These are the most common acyl-CoA and alcohol compounds, respectively.

Source of Esters

- **Most common ester is ethyl acetate made from the reaction of ethanol with acetyl-CoA**
- **Esters can derive from amino acid degradation and reaction of acids with ethanol or of alcohols with acetyl-CoA**
- **Esters can derive from fatty acid metabolism**

Esters are also produced during fatty acid degradation. If an acid species is produced it may react with ethanol. Higher alcohols may in turn react with acetyl-CoA. Fatty acid biosynthesis and degradation involves attachment of the growing (or shrinking) fatty acid chain to CoA. The acid species can react with ethanol, producing a long chain ester. Long chain esters are soapy and are more stable than short chain esters.



Phenethyl alcohol can react with acetyl-CoA producing phenethyl acetate, which has the character associated with rose oil. It has a perfumy rose character that again may be too intense for some wines. It tends to be produced by *Saccharomyces* late in fermentation.

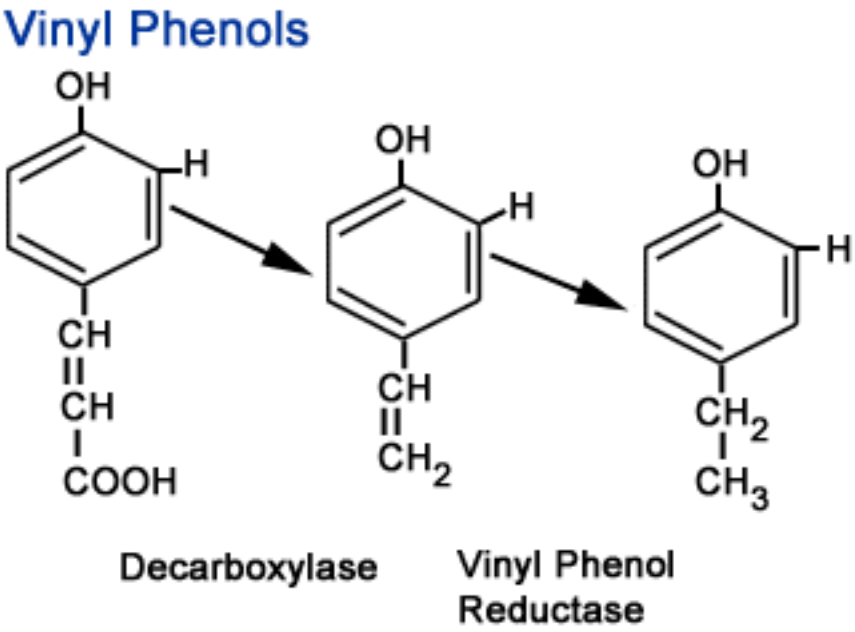
Many factors impact ester formation. In the wild, yeast make esters to attract insects to sites of yeast growth. The yeast can then be picked up by the insect and transferred to a new site. Yeasts tend to produce esters late in fermentation or under conditions of some types of stress. Under these circumstances the culture is running out of nutrients

and would like to be relocated. Esters are volatile and less toxic than either the alcohol or the acid moiety. Under conditions of high cell density, production of a volatile compound as a means to detoxify the environment may be advantageous.

Vinyl Phenols

- **Responsible for sweaty, horsy, stable off aromas**
- **Usually formed by *Brettanomyces***
- ***Saccharomyces* possesses the enzymes needed to make vinyl phenols and there are reports that it will make them under certain conditions**

The final class of off-characters we will consider here are the vinyl phenols. These compounds have very distinctive medicinal or pharmaceutical aromas and are responsible for the "animal" or barnyard characters found in wines.



Decarboxylated phenols are reduced to vinyl phenols by yeast enzymatic activity. The principle yeast producing vinyl phenols is *Brettanomyces*. However the enzymes catalyzing vinyl phenol production have been found in *Saccharomyces* so it is believed that this yeast could produce them as well. Other yeasts are also thought to be capable of producing these compounds, but *Brettanomyces* appears to do so most frequently.

Vinyl phenol formation is dependent upon the phenolic composition of the fruit, and what other compounds are available to be reduced. The reason *Brettanomyces* produced high levels of these compounds relates to its mode of metabolism of sugar substrates. *Brettanomyces* produces acetic acid as a primary end product of metabolism. This results in formation of extra NADH, as acetaldehyde is oxidized to acetate. NAD^+ can be regenerated via the production of vinyl phenols. Oxygen is the preferred acceptor of electrons, and oxygen will limit vinyl phenol formation but has the negative side effect of strongly encouraging acetic acid formation.

Moral:

Yeast needs are simple, but it can be challenging to keep them happy.

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Lesson 11: Introduction

Stuck Fermentation: Diagnosis and Rectification

In this lecture we will cover identification of the cause of a stuck fermentation and strategies that can be used to restart the fermentation. Successful re-initiation of an arrested ferment depends upon knowing why it arrested and what must be done to alleviate the biological or environmental stress to the yeast that is limiting sugar utilization.

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Lesson 11: The "Normal" Yeast Fermentation Profile

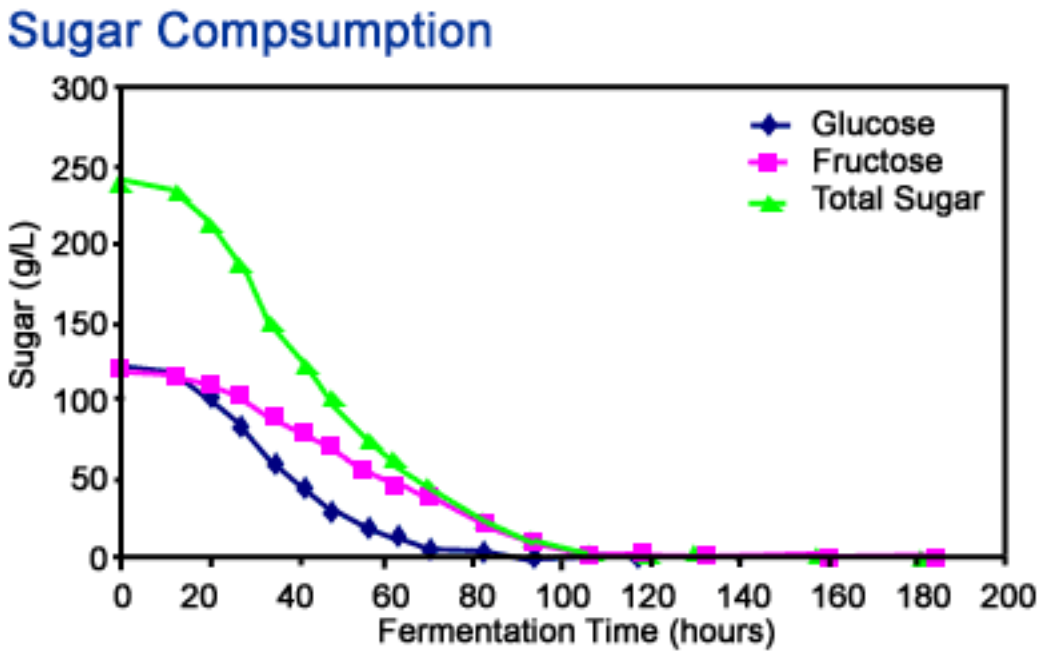
The ability to recognize a problem fermentation requires that the normal or typical course of fermentation be understood. Winemakers frequently monitor soluble solids or specific gravity of the fermentation rather than directly measuring grape sugars. Glucose and fructose are not consumed at the same rate by yeast cells. Glucose disappears with more rapid kinetics than does fructose. The hexose transporters that translocate each sugar into the cell display a higher affinity for glucose than fructose, thus, some of the difference in rate is due to the biochemical properties of the transporters themselves.

Normal Fermentation Profile

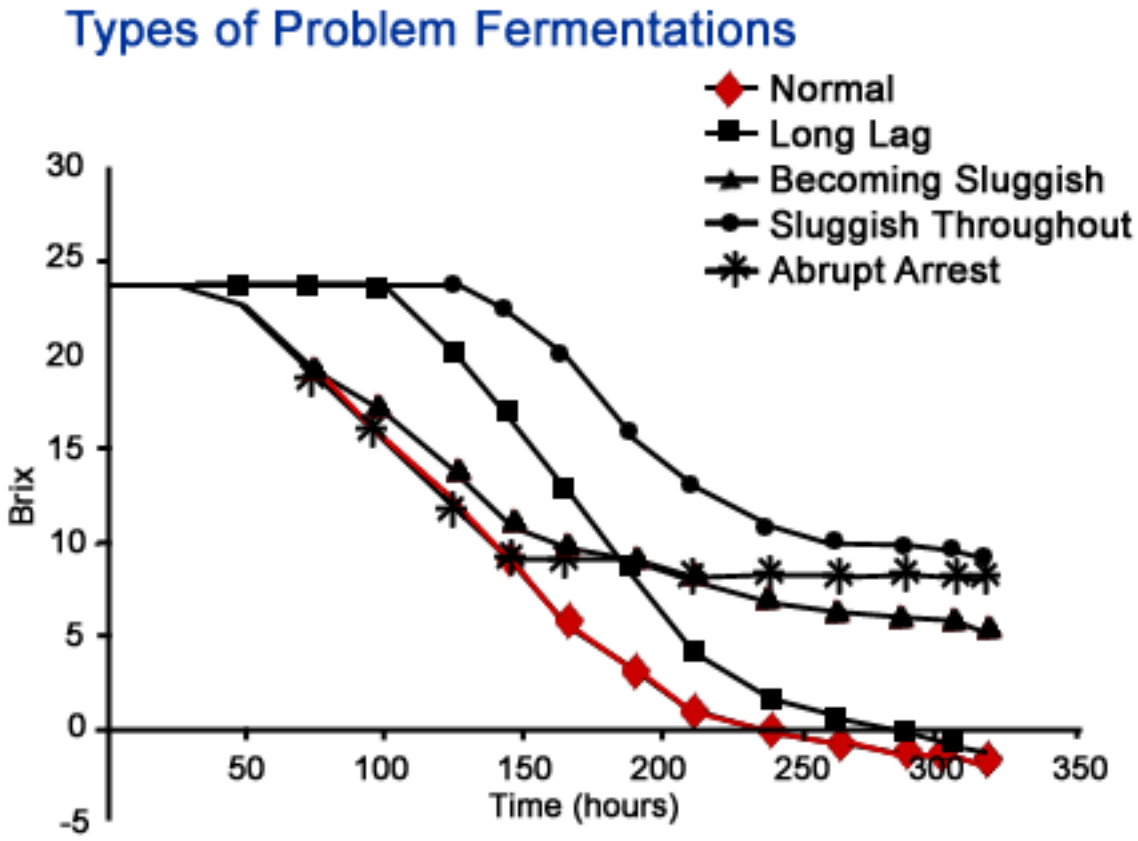
Glucose is consumed faster than fructose

Arrested fermentations will be high in fructose relative to glucose

As a consequence, fructose concentrations will typically be much higher than glucose late in fermentation. If an arrest occurs at this time, then the yeast used as a new inoculum to get the fermentation started will need to be able to ferment fructose efficiently. The following graph shows the profiles of glucose, fructose and total sugar accumulation in a typical Chardonnay fermentation.



Analysis of the ratio of glucose to fructose and how the ratio is changing over time can be used to determine the health of the fermentation. The pattern of total sugar consumption is also diagnostic. There are several types of deviations from the normal curve. The type of deviation from normal provides important information on the type of stress being experienced by the yeast.



This graph depicts the normal fermentation of a Chenin blanc juice highlighted in red. Fermentations may be slower than expected because of an unusually long lag before the onset of fermentation. They may start normally, but slow down during the courses of fermentation, or they may be sluggish throughout, never attaining a high rate of fermentation. Finally, a normal fermentation may display a rather abrupt arrest. These types of profiles are associated with different types of stress for the yeast.

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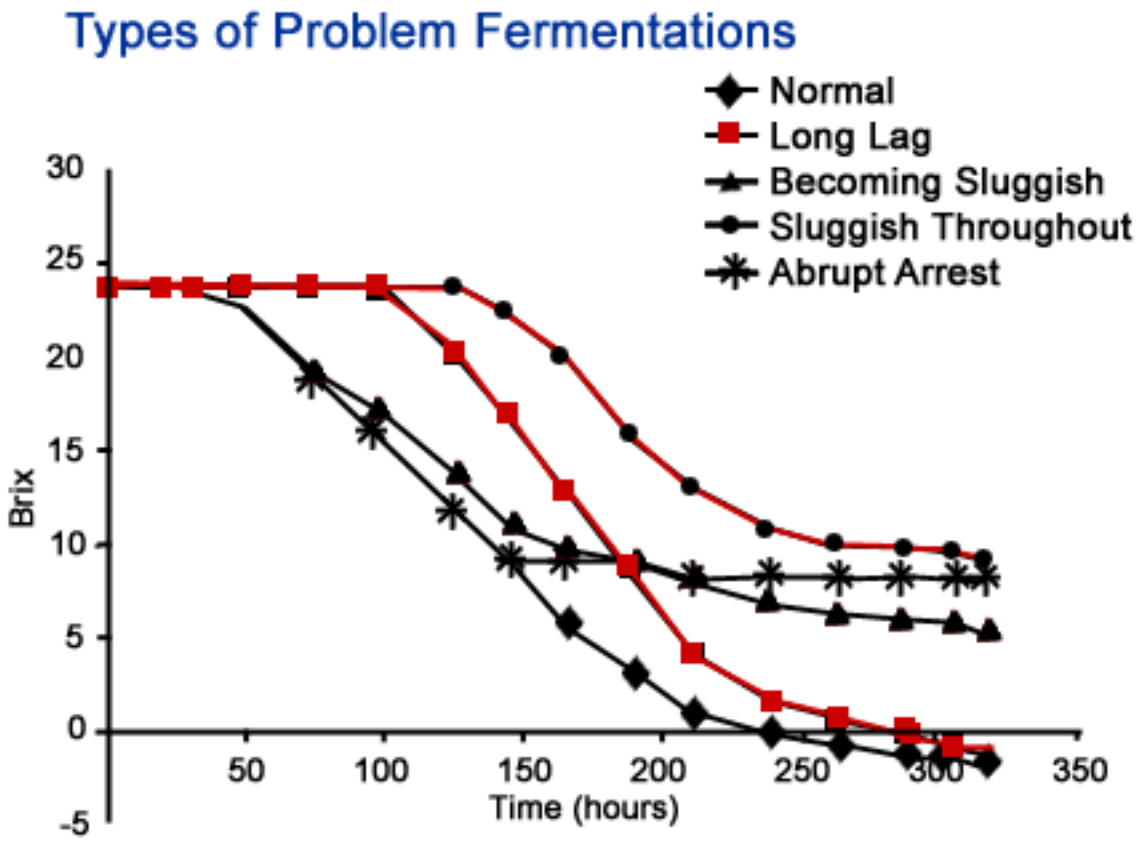
Lesson 11: Fermentations Displaying a Long Lag

Types of Sluggish Fermentations

- **Long Lag**

We will first consider the situation of a long lag. The long lag ferment is highlighted in red in the following graph.



A typical inoculated fermentation initiates, that is, shows a dramatic drop in sugar, starting between 24 and 72 hours. As shown here, initiation may not occur for 5 to 7 days or longer. Such fermentations display a lag in initiation of rapid sugar consumption. This is not necessarily undesirable, especially if the reason for the sluggish start is known. There are several causes of a long lag. As shown here, a long lag does not necessarily mean the fermentation will be sluggish or that it will arrest prior to dryness.

Causes of Long Lag

- **Poor health of starter culture**
- **Presence of inhibitors**
- **Grape quality**

Poor health of Starter Culture as a Cause of Long Lag

The most frequent cause of a long lag is **poor health** of the starter culture in inoculated fermentations. The final yeast cell density is roughly 10^8 cells/ml. An inoculum of 10^6 cells/ml must undergo seven generations to reach 10^8. The yeast doubling time is on the order of 3 to 5 hours in a nutritionally rich grape juice fermenting at 25°C. Seven generations under these conditions would require 24 to 35 hours. In most cases growth does not initiate immediately and there is a period of adaptation of the yeast to the juice or must. This may take 12 to 24 hours depending upon the condition of the inoculum (active dry yeast, fermenting juice or must at high ethanol) or may be much shorter if a mid-fermentation must or juice (less than 7% ethanol) is used as inoculum. This also assumes that the cells of the inoculum are fully viable. This may not be the case.

Improperly rehydrated yeast (too high or too low of a hydration temperature or rehydrated in wine rather than water) will rapidly lose viability. In this case the viable count may not be 10^6cells/ml post-inoculation. We have seen it as low as 10^2cells/ml. The yeast then simply requires more doubling times to attain maximal biomass and therefore the maximal fermentation rate. If the practice is to inoculate one juice with a previously fermenting juice rather than with active dry yeast viability can also be an issue. Late in the fermentation cells can lose viability due to the loss of sugar as an energy source. We generally see the post-fermentation population drop to 10^3 cells/ml. If this is used as a 20% inoculum, again the fermentation will be inoculated with roughly 10^2cells/ml final concentration. It requires on the order of 60 hours for 10^2 cells/ml to reach 10^6.

Poor Health of Starter Culture

- **Active Dry Yeast: Past expiration date**
  Not hydrated properly
  Not stored properly
- **Natural Fermentation: Yeast numbers low**
  Inhibitory microbes present
  Poor yeast strain present

Native flora fermentations frequently display long lags due to the low numbers of *Saccharomyces* present on the grapes at the time of harvest. Early during the vintage, the levels may be well below 10^2 cells/ml. However later in harvest the yeast populations build up on winery equipment and the level present in an uninoculated fermentation is more on the order of 10^4 cells/ml. Native flora fermentations may be difficult to predict for several reasons. The dominant yeast strains present at the start of fermentation may not be the ones best suited to conduct a fermentation to dryness. Proliferation of *Saccharomyces* may be inhibited by the presence of other organisms, and thus the generation time is significantly longer than 3-5 hours. If a low inoculum is being used then the winemaker knows to expect a sluggish start, so this is not problematic. The most frequent cause of unexpected sluggish starts is failure to follow manufacturer's instructions for the rehydration of the yeast. Yeast should never be exposed to extremes of temperature during rehydration. Likewise, rehydration in wine is not recommended, as the yeast does not maintain viability during the rehydration process in the presence of high ethanol concentrations.

In addition to a low inoculum level, long lags might also arise if there is some problem with juice composition particularly the presence of a toxin or toxic condition that the yeast must overcome before fermentation can initiate. We have already discussed how yeast adapts to sulfur dioxide by detoxifying it. The detoxification process has to be complete before growth will initiate.

Presence of Inhibitors as a Cause of Long Lag

- **Sulfur dioxide concentration too high**
- **Sulfur dioxide added improperly**
- **Microbial activity resulting in inhibition**
- **Pesticide/fungicide residues on grapes at harvest**
- **Temperature of must/juice too high/low**

The yeast will have a longer lag the higher the **SO_2 concentration** of the must or juice. Sometimes in a winery situation during crush communication may not be optimal and more than one person may sulfite the same tank. This leads to a very high level that must be detoxified. Also one must be careful in how the SO_2 is added. It should never be mixed in with the yeast inoculum, as the level present will be toxic. Nor should it be layered on to of a yeast inoculum, a practice that will again result in a localized high concentration for the yeast. The SO_2 should be well mixed into the fermentation before the yeast is added. The yeast inoculum should also be well mixed post addition.

It is also important that the inoculum be well dispersed, that is, not rehydrated into clumps, which will simply sink to the bottom of the tank. This requires **gentle** mixing, too vigorous mixing may break the cells leading to a drop in viability.

The non-*Saccharomyces* **flora may produce toxins limiting yeast growth**. The presence of toxins may be manifest as an inhibition of cell growth and therefore of the start of the fermentation. *Saccharomyces* is inhibited by high concentrations of acetic acid, which can be produced by wild yeasts and bacteria in the must or juice, especially if the juice is aerated significantly. As noted in the previous lecture, *Saccharomyces* is a fungus so incorrectly applied fungicides in the vineyard may wind up in the must or juice and prevent initiation of fermentation. Finally, if the yeast inoculum is exposed to extreme **temperature** cell viability will decrease. This would occur if the juice or must were too warm (greater than 40°C) as might happen following a heat treatment to eliminate polyphenol oxidase activity. Similarly, if the juice or must has been subjected to cold settling or cold maceration, it is best to warm the juice to ambient temperature before inoculation with the yeast.

Grape Quality

- **Infected grapes: loss of micronutrients**
- **Infected grapes: high microbial loads**

The condition of the grapes also impacts the rate of initiation of fermentation. If the grapes have heavy damage, especially while on the vine, the growth of non-*Saccharomyces* organisms will occur which may lead to the consumption of macro and micronutrients. If the deficiency is severe enough, maximal cell biomass will not be attained. Even if nutrients have not been depleted the simple presence of a high bioload of organisms may limit fermentation until ethanol accumulates to a high enough level to eliminate the competition.

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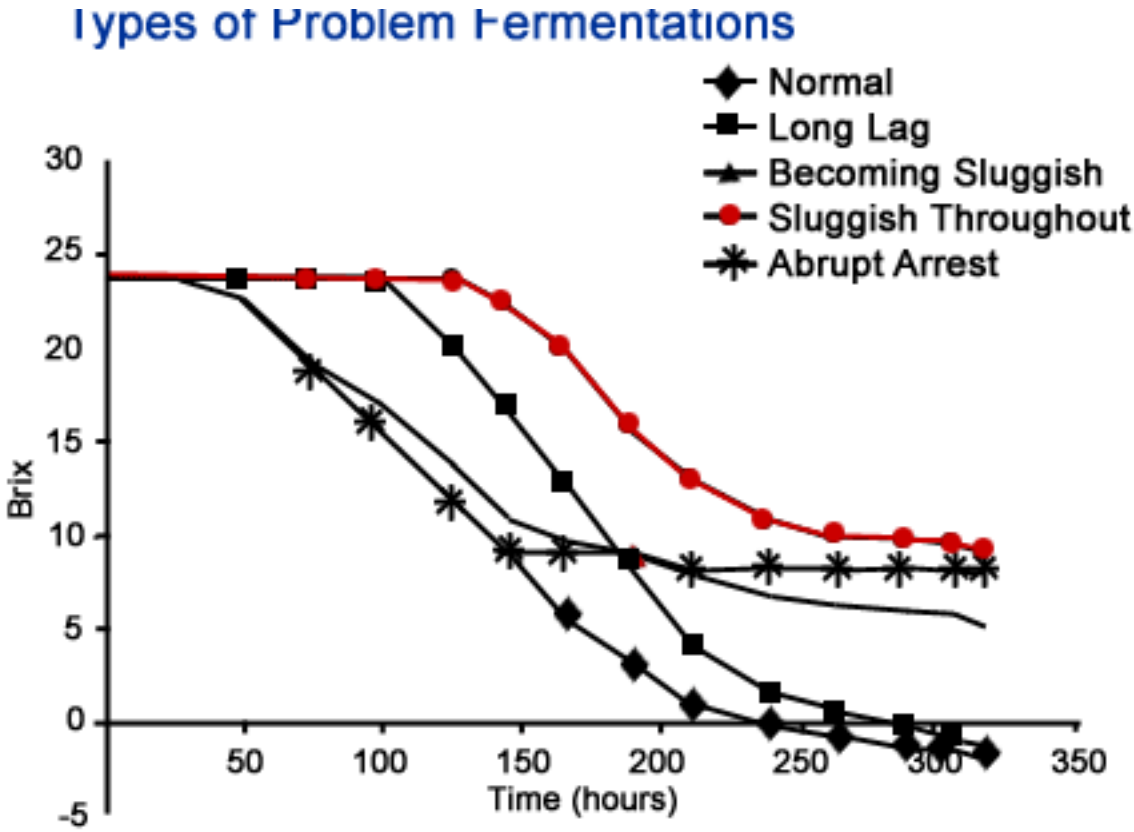
Lesson 11: Fermentations Displaying a Sluggish Rate Throughout

Types of Sluggish Fermentations

- **Long Lag**
- **Slow Rate Over Entire Course of Fermentation**

The following graph displays a fermentation that would be described as sluggish throughout highlighted in red.



In this particular juice there was a long lag followed by failure to attain maximal fermentation rate. In the previous long lag example, once maximal cell biomass had been achieved the fermentation progressed more or less normally.

Causes of Slow Rate Over Entire Time Course

- **Failure to reach maximum cell density**
- **Nutrient limitation**
- **Strain a poor choice for conditions**
- **Inhibitory fermentation conditions: temperature, pH, ionic imbalances**

Sluggish fermentations generally mean that the culture has **not been able to attain or maintain maximal biomass production** at 10^8 cells/ml. This may be due to **nutrient limitation**, that is, insufficient growth factors, or it may mean that the **strain is not well adapted to growth in a grape juice or must environment**. Slow fermentations may be associated with **inhibitory conditions** such as a low temperature of fermentation, low pH or some type of ionic imbalance. Slow fermentations may eventually arrest or they may complete, it depends upon the characteristics of the yeast strain. Strains can vary quite a bit in terms of maximal fermentation rate and responses to stress conditions. If the strain is "naturally" slow then a slow fermentation does not mean the fermentation will arrest. On the other hand, if the strain is typically a robust fermentor a drop in fermentation rate may indicate a problem in the fermentation. Yeast strains display differences in ethanol tolerance ranging from 10% to well over 19%. If the initial sugar content is high and a strain with poor ethanol tolerance is selected or present without having been selected, the fermentation may be sluggish and then arrest. This underscores the need to understand what the normal behavior of a given strain is during fermentation. Sluggish fermentations need not arrest, they may go to dryness if the strain is simply a slow fermentor, but is not being inhibited by any stress factors.

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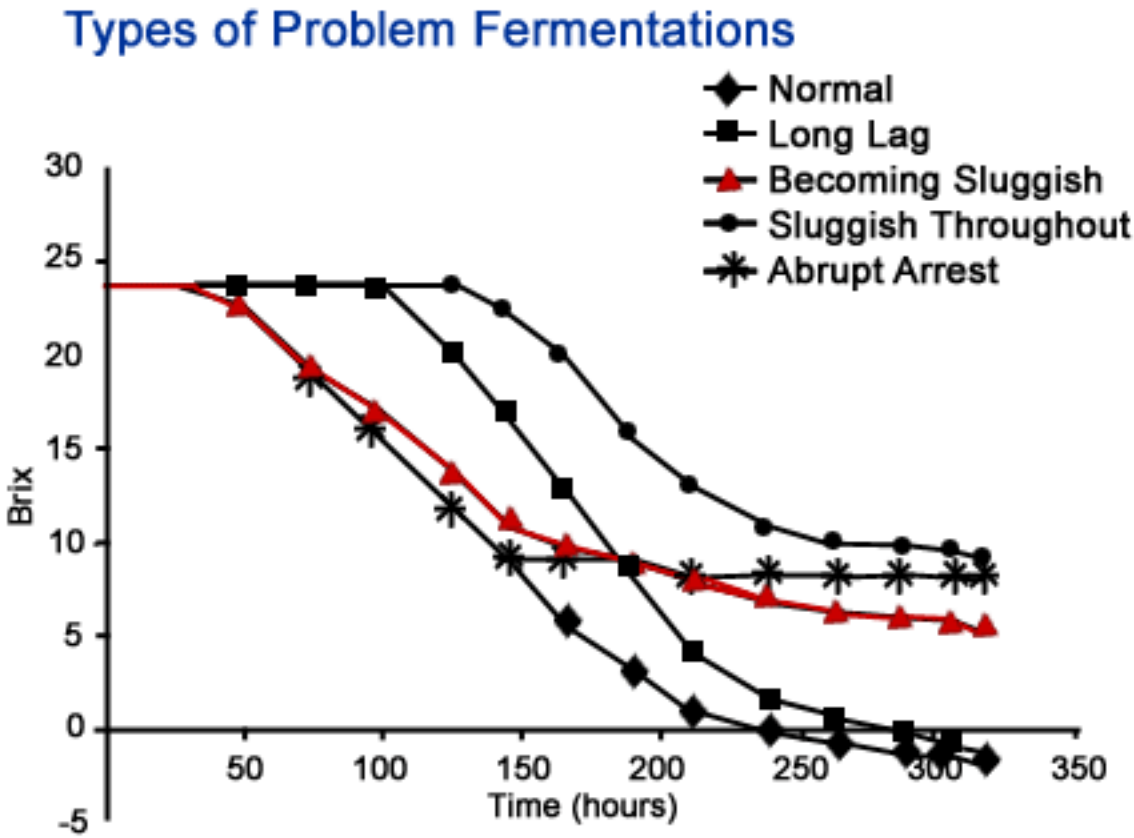
Lesson 11: Normal Initiation of Fermentation Becoming Slow

One of the most common types of fermentation problems in California is the seemingly normal fermentation that slows dramatically at a high sugar concentration. These types of fermentations often arrest with a high residual sugar content.

Types of Sluggish Fermentations

- **Long Lag**
- **Slow Rate Over Entire Course of Fermentation**
- **Rapid Rate Becoming Slow**

There are many known and suspected causes of this type of fermentation arrest. It is generally thought to reflect a problem with ethanol tolerance of the culture.



In this case, the stain either displays a poor native tolerance to ethanol or other

conditions in the fermentation impact and limit innate ethanol tolerance.

Causes of a Decrease in Rate

- **Poor ethanol tolerance**
- **Loss of viability**
- **Loss of fermentative capacity**
- **Nutrient limitation**
- **Poor strain**

Ethanol tolerance will be reduced if the cells do not have sufficient resources such as sterols and unsaturated fatty acids, to generate an ethanol resistant membrane. Tolerance will also be reduced if the cells have insufficient nitrogen to synthesize the new proteins needed that are more resistant to the inhibitory effects of cellular ethanol.

The ability to tolerate ethanol is a function of external pH, temperature and the presence of other inhibitors. The following compounds seem to synergistically impact ethanol tolerance: acetic and other organic acids, seed tannins, and inhibitory fatty acids. If these compounds are present, the maximal tolerable ethanol concentration decreases.

If there is some condition that **reduces cell viability** such that cell numbers drop below 10^8 cells/ml, the fermentation rate will be likewise reduced since it is a function of total biomass. It is also a function of the **rate of fermentation** of the individual cells. It is possible to have a normal maximal biomass but to still not observe the maximal fermentation rate because the cells have a "diminished capacity" to ferment. An example of this would be a culture that does not have enough nitrogen to make the hexose transporters with higher substrate affinity needed to optimize fermentation rate as the external concentration drops.

To review some biochemistry, sugar is transported in yeast by a process known as **facilitated diffusion**. In this case, substrate simply moves along its concentration gradient. This process **does not require energy** and is therefore the most beneficial process to use for uptake of an energy source. Facilitated diffusion systems have a limitation, however, not found with active energy-requiring transport systems. The only work well within a concentration range 10 fold higher and 10 fold lower than their Km (the concentration at which the transport process is "half-saturated" - that is, that yields one half of the maximal transport rate). Grape sugar spans the concentrations from

molar to milimolar, or a thousand-fold range. This is well beyond the capacity of a single transport protein. For this reason *Saccharomyces* has a family of hexose transporters spanning the Km range from 1 M to 1mM. As sugar concentration in the medium drops the yeast cells make different transporters so that the Km of the transporter is well matched to the sugar concentration of the fermenting juice. Since the transporters are proteins, this process requires nitrogen be available. If it is not, yeast will only be able to use the current transporters that will not work as efficiently. Therefore the rate per cell is less than it would be if they were able to make the transporters they needed. There seems to be strain differences in the ability to express the appropriate transporters under conditions of moderate nutritional stress. The biological reason this occurs is unknown.

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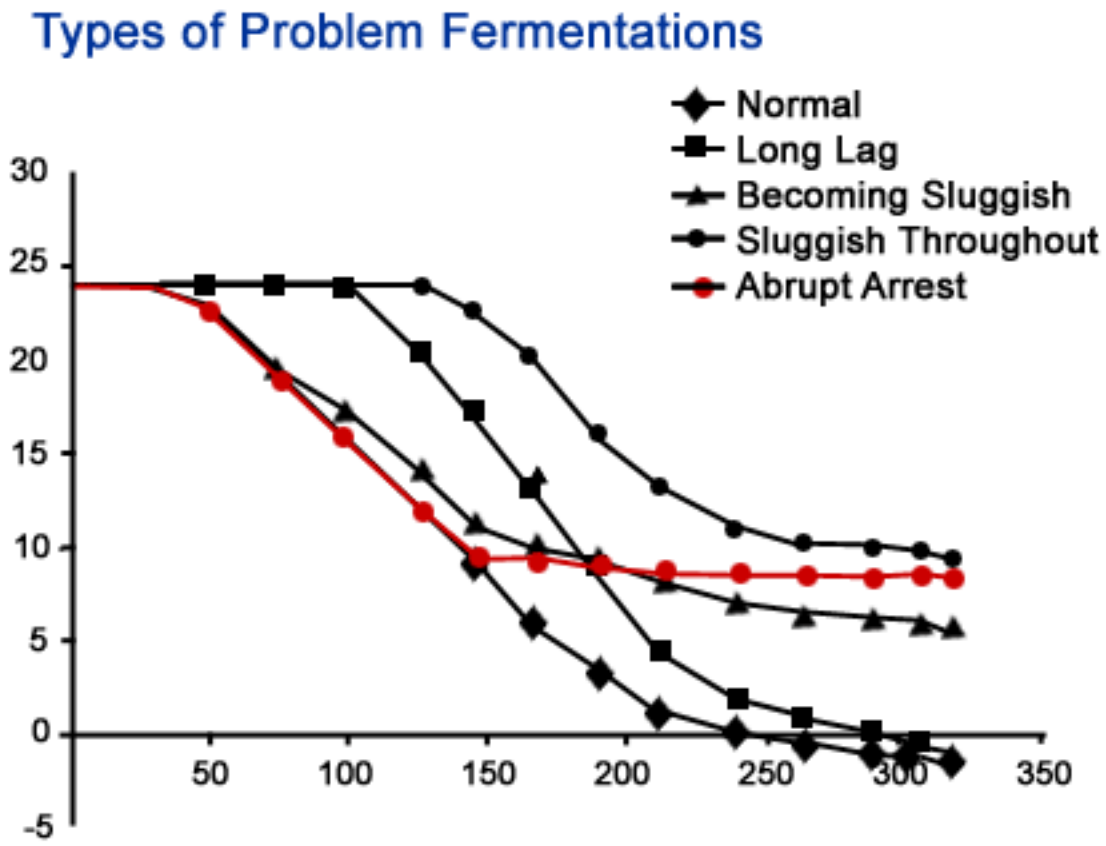
Lesson 11: Normal Fermentation Arresting Abruptly

In the example above, a normal fermentation becomes sluggish and then may or may not arrest. Occasionally, a fermentation arrests abruptly - there is no "sluggish becoming stuck" phase.

Types of Sluggish Fermentations

- **Long Lag**
- **Slow Rate Over Entire Course of Fermentation**
- **Rapid Rate Becoming Slow**
- **Abrupt Stop**

This is usually indicative of an environmental shock to the yeast rather than a nutritional problem.



These types of fermentations are among the most difficult to re-initiate. They may not be associated with loss of viability, but rather with the yeast entering a resting

stationary phase. Alternately, if some manipulation of the fermentation occurred leading to cell death, loss of viability may be a cause. An example of this would be a late addition of a high SO_2 level. This might occur if the winemaker became concerned that a spoilage organism might be present. Fermentations will also arrest abruptly if a temperature shock occurs at a high, but not quite finished, ethanol concentration, or if a change in pH has occurred. This would be the case if the pH of the juice were adjusted late in fermentation, as a means to control microbial flora for example.

Causes of Abrupt Stop

- **Temperature shock**
- **Build up of inhibitors: acetic/organic acids**
- **pH decreases too much**
- **Strain very ethanol sensitive**

An abrupt arrest may also occur if there is significant metabolic activity by other organisms producing an inhibitory end product. Some yeast strains are sensitive to co-inoculation with the malolactic bacteria. In this case, inoculation with a vigorous ML bacteria culture late in fermentation may abruptly arrest fermentation. If this is the practice at the winery, a yeast strain tolerant of lactic acid bacteria should be used. The fermentation will also abruptly stop once the yeast has reached its maximal ethanol tolerance level.

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Lesson 11: The Most Common Causes of Fermentation Arrest

Numerous possible causes of fermentation arrest have been described in the literature, but it is important to know what the probable causes are under specific winery conditions. The following slide lists the five most common causes of fermentation arrest under California production conditions.

Most Common Causes of Stuck/Sluggish Fermentation

- **Nutrient deficiency**
- **Temperature extreme**
- **Presence of a toxic substance**
- **Microbial incompatibility**
- **Deficient yeast strain**

The most common juice or must problem that can lead to arrest of fermentation is **nutrient limitation**. It is current California practice to treat every fermentation as if it is or will be deficient and to add nitrogen and phosphate in the form of diammonium phosphate at the start of fermentation. It could be argued that this practice eliminates nutrient deficiency as a cause of slow or incomplete fermentations. However, the winemaker must time nutrient additions so that the yeast conducting the bulk of the fermentation will benefit from them. Early additions may feed the wrong population. Yeast appears to arrest uptake of nitrogen compounds at high ethanol concentrations, so a late addition is not necessarily beneficial either.

A second common cause is **temperature shock** or exposing the fermentation to a temperature extreme. This is more common in red wine production because many reds tend to be fermented "hot" (30°C or warmer) and the maximal tolerable temperature for yeast is between 35 and 42°C. The heat given off during fermentation may raise the temperature to an inhibitory level. We have found that cooling the tank may lead to restart, but the culture is fundamentally changed and tends to arrest at high ethanol levels, around 11%. The culture arrests at this point and if sugar is still present, it will not be fermented. We have not noted the same types of problems with low temperature shock as would occur if supercooling of the tank (forgetting to turn off a refrigeration system as fermentation rate slows) took place. However, winemakers have reported this problem and it may be strain specific.

There are various types of **toxic substances** that may be present during fermentation. Fungi make compounds inhibitory to plants as a mechanism to enhance infection of the plant. Some of these **mycotoxins** are known to be inhibitory to yeast depending upon the composition of the must or juice.

Another common complaint is that fermentation seems to arrest following a "harmless" lactic acid bacterial bloom. It is not clear if this is due to **competition for limiting nutrients or production of a toxic substance by the bacteria** such as acetic or another organic acid. Nutrient supplementation does not appear to solve this problem, so if it is competition for a nutrient, the nutrient is something other than what is typically found in yeast nutritional supplements.

Winemakers frequently note that certain vineyards or sections of a vineyard tend to cause stuck fermentations more than fruit of the same varietal harvested from a geographically similar area. This may reflect consistent differences in the flora of one section of the vineyard versus another, but a more likely explanation is the presence of **inhibitory phenolic compounds**. We have found that seed phenolic preparations are inhibitory to fermentation. Plants take up phenolic compounds from the soil and concentrate them in the seed. From the lecture on viticulture we know that the purpose of the seed is to generate a new plant and it is important to make sure the seed contains antimicrobial compounds. Depending upon the "phenolic" history of one region of the vineyard versus another, seed composition may vary which would lead to differences in the presence of inhibitors in the seeds.

Finally, some popular commercial **strains** are more sensitive to certain types of stresses than others. It is important to match the strain to the fermentation conditions in order to avoid fermentation problem.

It is frequently challenging to restart an arrested fermentation. For this reason, winemakers would prefer to have the fermentation complete in a timely fashion.

Why are stuck fermentations difficult to treat?

- **Cells adapt to adverse conditions by reducing fermentation capacity**
- **Biological adaptation difficult to reverse**
- **Diagnosis of cause of fermentation problem difficult**
- **Conditions that cause stuck fermentations are also conducive to cell death**
- **New inocula respond to cell death by arresting activities**

Yeast cells **decrease fermentation rate under conditions of environmental or biological stress**. These conditions frequently lead to formation of a resting state, sometimes called a "deep" stationary phase. Resting stage cells have drastically reduced metabolic activity, but do not readily re-enter a metabolically active state unless environmental conditions drastically change for the better. **Biological adaptations are difficult to reverse** as they represent a survival mechanism. Further, if the exact cause of the arrest of fermentation is not known, it is **challenging to correct the cause of stress for the yeast**. Conditions of stress leading to entry to a resting state frequently are lethal or near-lethal situations. If uncorrected, a significant percentage of the **population may die**. Many microbes are social organisms, and respond to signals generated by other members of the same species in the environment. Massive cell death appears to lead to the **release of compounds that signal** conditions leading to loss of viability. New cells in the environment will respond to these signals, even if conditions are otherwise permissive for growth. The presence of these types of compounds during grape juice fermentation is hypothesized, but has not been directly demonstrated yet. However, it is likely that such compounds are produced and would lead to arrest of cells in the new inoculum.

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Lesson 11: Factors Most Impacting Fermentations Rate Under Enological Conditions

Several winery practices have a dramatic impact on yeast fermentation. Some of these practices may be employed with the intent of stimulating the yeast, while others are used for a different purpose with the stimulation of yeast being a secondary benefit.

Fermentation Variables Impacting Progression and Rate

- **Oxygenation**
- **Mixing: Natural or Assisted**
- **Type of Fermentation Vessel**
- **Inoculation Practices**
- **Temperature of Fermentation**
- **Supplementation/Juice Treatments**
- **Lees Contact**
- **Presence of Solids**

Oxygenation:

- **Oxygen is a micronutrient electron acceptor**
- **Oxygen is a survival factor**
- **Oxygen can lead to color changes (brown, pink, orange)**

Principle among these fermentation variables is oxygenation. Oxygen is very stimulatory to yeast growth and fermentation, largely because oxygen allows cells to form ethanol tolerant membranes.

While oxygen plays an important role in provision of survival factors needed for ethanol tolerance, it can also lead to oxidative changes in the wine. Sometimes these changes are desired and other times they are not as we have discussed at length in earlier lectures. If the yeast are actively metabolizing they are quite efficient at sequestration

of medium oxygen that would limit formation of oxidative off-characters.

Mixing (natural or assisted):

- **Separation of yeast from end products**
- **Brings yeast in contact with new nutrients**
- **Can facilitate skin extraction in reds and solids extraction in whites and reds**

Oxygenation may be deliberate, for the sole purpose of stimulating yeast or it may be a consequence of other activities such as mixing of the tank. Mixing leads to oxygen exposure unless done under a modified atmosphere, which is rare. Mixing will also occur with a vigorous fermentation due to the rapid formation of carbon dioxide. Mixing even in the absence of aeration can be stimulatory to yeast, perhaps because it distributes toxic end products more evenly preventing localized accumulation.

Mixing can also bring yeast in contact with nutrients again by distributing the yeast more uniformly in the tank. It can also increase extraction of components from the skins and solids during fermentation. This provision of nutrients may be stimulatory if nutrients are limiting.

Type of Fermentation Vessel

- **Stainless Steel**
  - **Can be cooled**
  - **Easier to sanitize**
  - **Inert**
- **Wood:**
  - **Can impart positive flavors and aromas**
  - **Difficult to clean; impossible to sterilize**
  - **Develops stable biofilm of microflora**

The type and design of the fermentation vessel may also impact fermentation performance.

These effects concern ease of temperature control of the vessel, and presence or absence of a biofilm of flora. A biofilm may be comprised of beneficial organisms, of spoilage organism or of both types. Sanitation is easier for stainless steel than for wood or cement fermenters.

Inoculation Practices

- **Spontaneous Fermentation**
- **Inoculated Fermentation**

One of the most important winemaking decisions that impact fermentation initiation and progression is inoculation practices.

The two options discussed at length in an earlier lecture are inoculated and spontaneous fermentations. Inoculation allows the winemaker to control the biomass level of *Saccharomyces* and to minimize the impact of non-*Saccharomyces* microbes. It is more difficult to predict the course of a spontaneous fermentation as the bioload of *Saccharomyces* and the fermentation behavior of the dominant strains are unknown.

Temperature of Fermentation

- **Affects presence and persistence of wild flora**
- **Affects fermentation and growth rates**
- **Extremes are inhibitory**
- **Impacts spontaneous chemical reactions**

Fermentation temperature is one of the most important variables impacting rate. Temperature affects growth as well as metabolic rates. Within permissive limits, the higher the temperature the higher the fermentation rate. However, winemakers need to be cautious in manipulation of temperature as yeast adapt to temperature using similar mechanisms as adaptation to ethanol. Great swings in temperature will result in arrest if the strain is intolerant.

In addition, temperature will affect both the types and numbers of non-*Saccharomyces*

flora. The effect of this will depend upon what microbes are present and whether they are inhibitory, stimulatory or neutral towards yeast metabolism. Temperature affects the rate of spontaneous chemical reactions and processes such as volatilization. We have discussed at length the impact of temperature on extraction. Thus, frequently the temperature selected is more influenced by winemaking style issues than by considerations for the comfort of the yeast.

Supplementation/Juice Treatments

- **Prevents nutritional deficiency**
- **May impact spectrum of yeast end products**
- **Residual nutrients encourage growth of spoilage organisms**
- **Unwanted byproducts may be made**

An obvious variable impacting yeast fermentation is nutrient addition strategies and juice/must manipulation practices (cold soak, cold settling, pH adjustment, SO_2 addition, etc). These factors have been discussed previously.

If fermentations are overfed, there may be high levels of residual nutrients at the end of yeast fermentation. These nutrients can then encourage the growth of other organisms, and may increase spoilage problems post-fermentation.

Juice/Must Supplements

- **Diammonium phosphate (0.96 g/L; 8 lbs/1000 gal)**
- **Yeast nutritional supplements (varies by producer)**
- **Yeast autolysates (3lb/1000 gal)**
- **Thiamin hydrochloride (0.005 lb/1000 gal)**

In addition to nutrient supplements summarized above, other juice treatments may have a strong impact on yeast fermentation.

Other Juice Treatments

- **Fining**
- **Centrifugation**
- **Aeration**
- **Clarification: settling/filtration**

Processes such as fining and centrifugation may impact fermentation performance as these techniques may strip the wine of nutrients or other growth stimulating conditions. Clarification, if carried to an extreme, may have a similar negative impact by stripping the wine of solids.

Lees Contact

- **Extraction of nutrients**
- **Extraction of grape characters**
- **Function as solids**

Lees contact, both grape and yeast, also affects yeast performance. Lees may contain nutrients that will be more slowly released during the course of fermentation. The slower release tends to make these compounds available late in fermentation when it is most critical to feed the dominant species present.

Grape lees can function as solids in stimulation of yeast growth and have been proposed as being able to sequester toxins, although this has not been definitively shown. Similarly, it has been proposed that yeast lees can remove toxins from the fermentation thereby allowing the remaining yeast to complete the fermentation. In addition, lees contact will affect the composition of the must/juice that may be desired independently of impacts on yeast biology.

Presence of Solids

- **Natural (grape material) or added (bentonite, yeast hulls)**
- **Stimulate fermentation**
- **Stimulate growth**
- **Source of nutrients**
- **Removal of inhibitory components**

We have noted above several times that solids are important stimulators of yeast fermentation. The exact effect of the solids is not known, but several hypotheses have been put forward.

Solids may be "natural" deriving from the grape or added such as the fining agent bentonite or yeast hulls. They are known to stimulate fermentation and growth. Cultures receiving solids treatment tend to have higher maximal cell numbers, which may explain the faster fermentation rate. Solids are proposed as providing nutrients or removing inhibitors. It has also been suggested that solids may help hold molecular oxygen in the juice or lead to faster nucleation and loss of carbon dioxide, which aids in mixing. Finally, it has been suggested that solids affect the non-*Saccharomyces* flora having an indirect effect on yeast metabolic activities.

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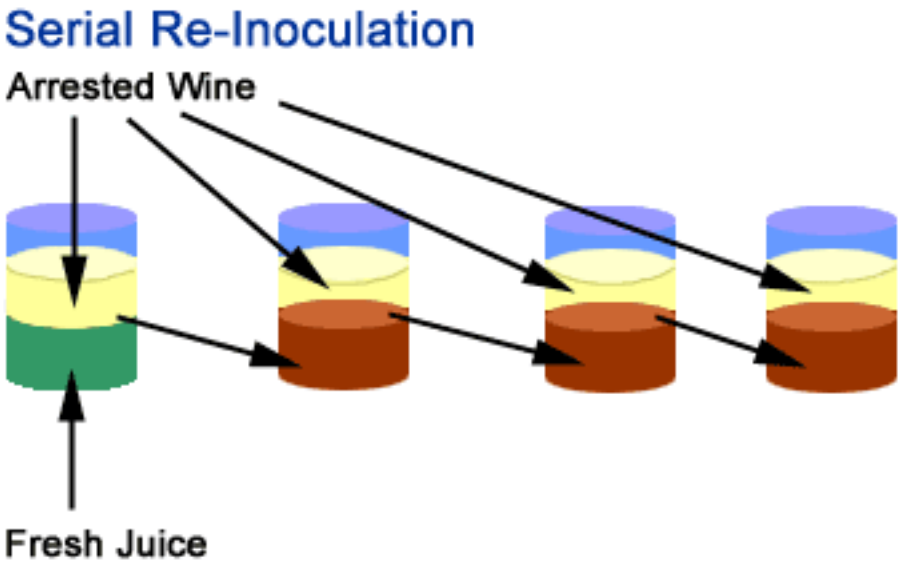
Lesson 11: Re-initiation of Stuck Fermentations

Our final topic for this lecture concerns the restart of arrested fermentations. There are some key guidelines for a successful restart. First, it is helpful if the cause of arrest is known, a determination which may not be as easy as it seems.

Re-initiation of Stuck Fermentations

- **Correct diagnosis of nature of the problem important**
- **If re-innoculating, make sure inoculum is adapted to conditions of stuck wine**
- **Serial re-innoculation**
- **May need to remove existing biomass**

It is also important to make sure that the new inoculum is adapted to the fermentative conditions (temperature, pH and ethanol content) of the arrested ferment. This will prevent shock of the inoculum upon addition to the ferment. The best way to achieve this is via the procedure called serial re-inoculation. In this case, the arrested wine is mixed with an equal volume of juice or actively fermenting must. This 50:50 mix is allowed to ferment to conditions near the arrested culture and a second 50:50 mix occurs of the total volume with an equal volume of arrested fermentation. This procedure is repeated until the arrested fermentation is restarted in total.



This procedure can be tricky to perform. It is important that the fermentation never be

allowed to go dry. If this happens fermentation will not occur in the downstream 50:50 mix.

Serial Re-Innoculation

Transfer initial 50:50 blend to second tank when it is 2-4 Brix above the arrested Brix level of the stuck fermentation

Do not let any of the intermediate steps in the series go dry, transfer them at the equivalent or slightly higher Brix than the arrested wine

This procedure or variations thereof work much of the time but not all of the time. It may be necessary to remove the existing yeast biomass through settling and racking or filtration.

This ends the series of lectures on the alcoholic fermentation and problems fermentations. In the next section we will consider the other principle microbial conversion that occurs during wine production, the malolactic fermentation.

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