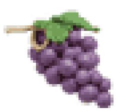
Section 4 - The Malolactic Fermentation
Lesson 12: Introduction

Malolactic Fermentation

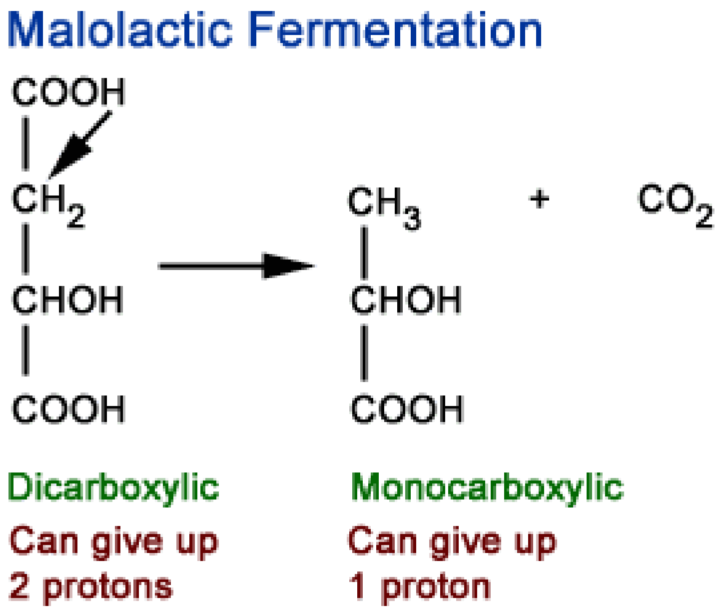
This section of the course will cover the Malolactic Fermentation, the conversion of malate to lactate conducted by members of the lactic acid bacteria family. In the first lecture we will discuss the biology of this important class of organisms and in the second cover the topic of management of this fermentation.

The lactic acid bacteria are gram positive organisms. They are found broadly in nature and are important in the production of many fermented foods and beverages. The malolactic fermentation refers to the conversion of malate to lactate, which can result in the generation of energy in the form of ATP.



The "maloctic fermentation" refers to the conversion of the grape acid malate to lactate conducted by members of the lactic acid bacteria.

Malate is a dicarboxylic acid, meaning that it contains two carboxyl groups. Lactate has a single carboxyl group and is monocarboxylic. Therefore the conversion of malate to lactate is a decarboxylation producing one molecule of CO_2 for every molecule of lactate.



Dicarboxylic acids have two acidic groups that can release protons while lactate contains only one proton that can be released. Note that the other terminal carbon in lactate has three hydrogen ions. One of the "free protons" in the system has been fixed in the conversion of malate to lactate. Thus the acidity is decreased and the pH of the wine is increased.

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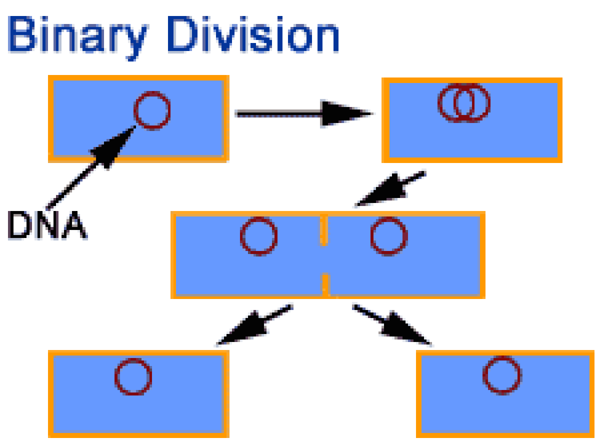
Lesson 12: The Lactic Acid Bacteria

Lactic Acid Bacteria: Characteristics

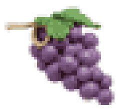
- **Prokaryotes: no membrane around nucleus**
- **Gram positive**
  - **Peptidoglycan**
  - **Teicholic acid**
- **Divide by binary fission**

The lactic acid bacteria are true prokaryotes: they do not contain a membrane-bound nucleus or other organelles found in eukaryotes like yeast. Their cell walls contain peptidoglycan a characteristic of gram positive organisms. Teichoic acid is another characteristic component of the gram positive cell wall. However some of the lactic acid bacteria do not contain this compound. The lactic acid bacteria may be rods or cocci. These bacteria divide by binary fission.



Cell division is initiated by the synthesis of a new copy of the single circular DNA molecule. The DNA molecules are attached to the surface of the cell. New growth occurs between the two DNA molecules separating them from each other. Once sufficient growth has occurred, the cell synthesizes a membrane and cell wall between the two DNA molecules, forming two new cells. In contrast to the yeast cell division, there are no mother or daughter cells just two daughters. Each daughter contains new cell material and old cell material.

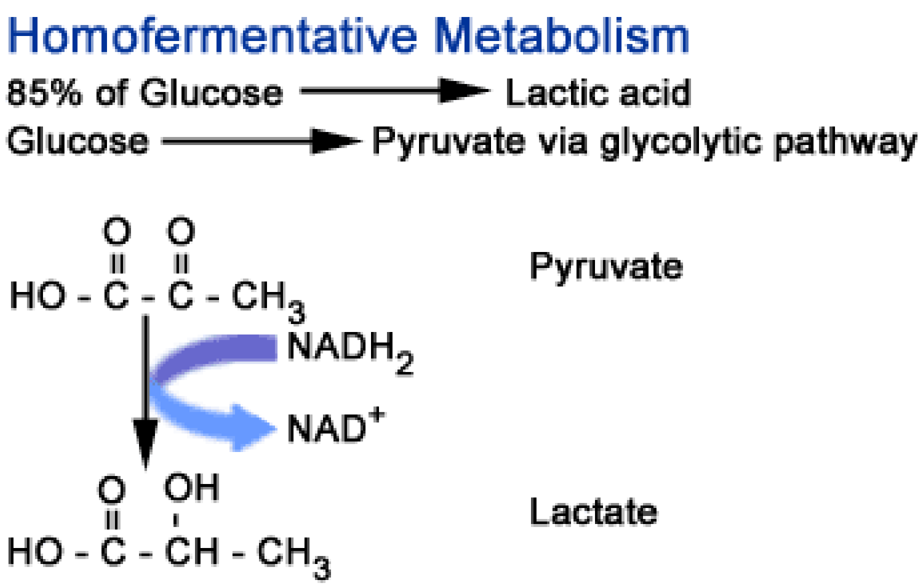
The lactic acid bacteria are chemotrophic meaning that they obtain energy from the oxidation of chemical compounds just as was the case with yeast. When one compound is oxidized it loses electrons. To balance metabolism a recipient compound that receives the electrons and is thereby more reduced must be formed. In the malolactic conversion, malate is the electron donor and the electron (proton) appears on lactate. These bacteria can also use pyruvate as an electron acceptor, which also yields lactate.



Lactic Acid Bacteria: Divisions

- **Group I: Strict homofermenters**
- **Group II: Facultative heterofermenters**
- **Group III: Strict heterofermenters**

The lactic acid bacteria have been divided into different groups based upon the spectrum of end products produced. There are strict homofermenters, which only produce lactic acid and are unable to grow on pentoses. The strict heterofermenters generate several other compounds in addition to lactic acid. The final class is the facultative heterofermentative (or facultative homofermentative) lactic acid bacteria. These organisms are the most versatile and can switch between hetero- and homofermentative modes of metabolism, depending upon the carbon source.



In homofermentative metabolism the majority of the sugar present in the medium is converted to lactic acid. Lactic acid is produced from the reduction of pyruvate. Pyruvate is formed via glycolysis from hexoses using the same enzymatic steps previously described for *Saccharomyces*. Two lactic acid molecules are formed from each hexose molecule catabolized.

Heterofermentative Metabolism

Organism smetabolize glucose via the pentose phosphate pathway.

End products can vary depending upon level of aeration and presence of other proton and electron acceptors.

Acetyl-phosphate can be converted to acetate and ATP or reduced to ethanol without ATP production.

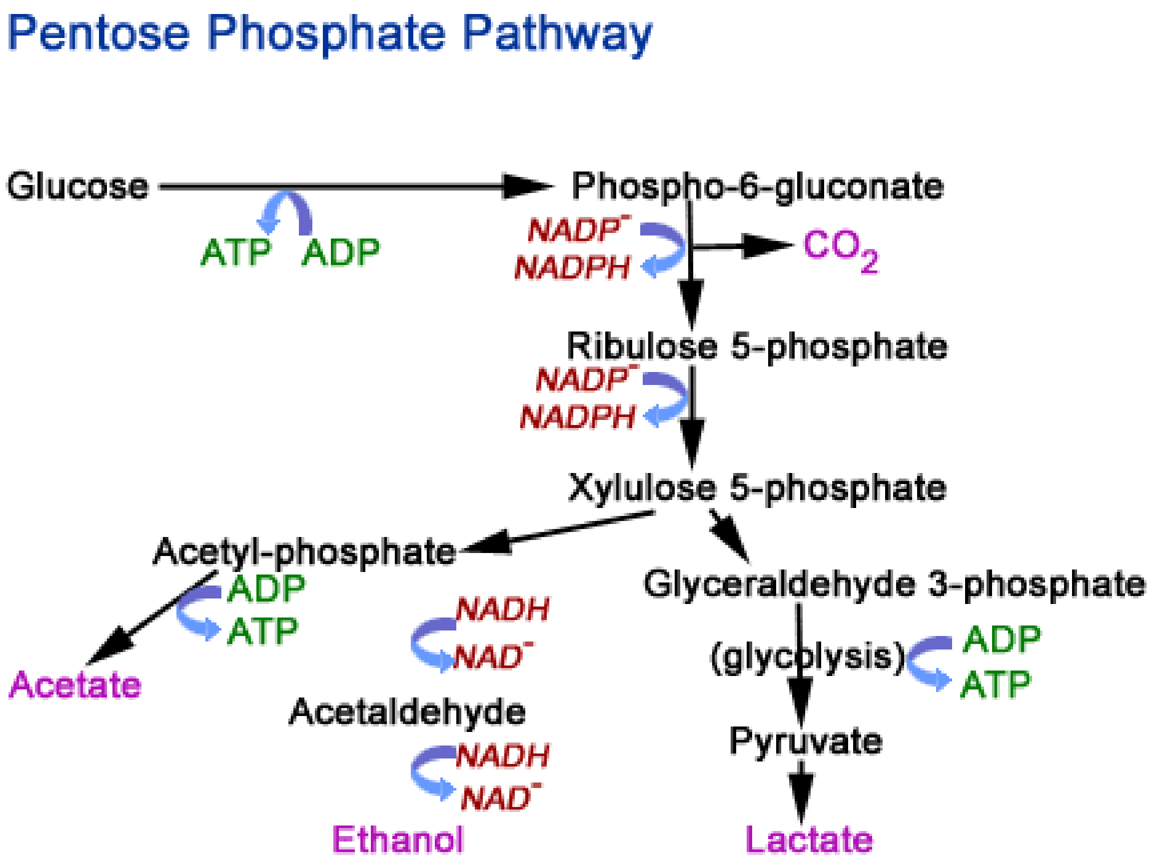
Heterofermentative organisms also produce significant amounts of lactate, but they use the pentose phosphate pathway for sugar catabolism rather than glycolysis. Five carbon sugars or pentoses can also be metabolized via this pathway. In this case the carbons appear as lactate and acetate.

Pentose Phosphate Pathway

Lactic Acid Bacteria can also metabolize pentose such as ribose, arabinose and xylose, via the pentose phosphate pathway.

Acetyl-phosphate leads to the generation of acetate and ATP exclusively in pentose metabolism.

The rest of end products produced depends upon other environmental and nutritional factors. The formation of acetate results in the generation of an ATP molecule so this will be favored, conditions permitting. The strict heterofermentors lack the fructose 1,6-diphosphate aldolase, a key reaction in glycolysis, and therefore only use the pentose phosphate pathway giving lactate, acetate, ethanol and CO_2. The strict homofermenters use the glycolytic pathway for hexose catabolism, but not the pentose phosphate pathway and produce two molecules of lactate from hexoses. These organisms do not ferment pentoses. The facultative heterofermenters can use both pathways. Glycolysis is the preferred mode of metabolism of hexoses but pentoses are fermented via the pentose phosphate pathway. However, if conditions (aeration) exist favoring generation of energy from the production of acetate, these organisms may shunt sugar carbon towards this pathway.



Acetate is also formed from six carbon hexoses in the heterofermentative bacteria. Six carbon compounds are decarboxylated to a five-carbon compound then continue through the pathway. If conditions are not permissive for acetate formation, ethanol may be produced instead. Ethanol production will occur if the cells need to transfer electrons to recipient compounds, that is, when oxidative catabolism is generating reduced cofactors that cannot be reoxidized any other way. If oxygen is present, the bacteria can transfer electrons to molecular oxygen forming water and in some cases hydrogen peroxide. If oxygen is present, this mode of metabolism will be favored since it yields more ATP.

Lactic Acid Bacteria: Genera

- ***Oenococcus***
- ***Pedicoccus***
- ***Lactobacillus***
- ***Leuconostoc***

There are four genera of lactic acid bacteria that are important in wine production. I have listed both *Oenococcus* and *Leuconostoc* since both terms appear in the literature. There is only one species of *Leuconostoc* found in wine, *Leuconostoc oenos*. This organism is quite different from other members of this genus so in 1995 taxonomists proposed a new genus and species name for this organism, *Oenococcus oeni*. The proposal was approved and *Oenococcus oeni* has now become the official designation. Both names can be found in the wine literature so it is important to remember that they are the same organism.

Oenococcus

- ***O. oeni***

O. oeni is a facultative heterofermentative lactic. It produces only lactic acid from hexoses and is therefore a homolactic hexose fermenter. There is only one species of *Oenococcus*. Several species of the *Pediococcus* and *Lactobacillus* genera are present in wine. Some are homolactic (producing only lactate from hexoses) and others are heterolactic (producing lactate, acetate, ethanol and CO_2).

Pediococcus

- ***P. damnosus***
- ***P. parvulus***
- ***P. pentosaceus***
- ***P. acidilactici***

P. damnosus is heterolactic while *P. pentosaceus* is homolactic.

In contrast to the typical gram positive bacteria, members of *Pediococcus* do not have teichoic acid in their cell walls. These species can be differentiated based upon the maximal temperature that will support growth.

Lactobacillus

Homolactic on hexoses	Heterolactic on hexoses
L. bavaricus	*L. fermentum*
L. casei	*L. brevis*
L. homohoichii	*L. buchneri*
L. curvatus	*L. fructovorans*
L. saki	*L. hilgardii*
L. plantarum	

Many more species of *Lactobacillus* can be found in wine. They are split between homo- and heterolactic fermenters, based on their behavior in the degradation of hexoses. The homolactic species listed above produce only lactic acid, but they are actually facultative heterofermenters. No strict homofermentative organisms have been isolated from wine to date. The heterolactic species listed above are strict heterofermenters, meaning that compounds in addition to lactate are made from hexose catabolism. Many of the *Lactobacillus* species will not persist in wine at low pH (3.5 or lower). The end products of the biological activities of the lactic acid bacteria will be dependent upon which species are present and which mode of metabolism, homolactic or heterolactic occurs.

Lactic Acid Bacteria: Prevalence in Wine

- **Only *O. oeni* is found at low (<3.5)pH**
- ***Pediococcus* and *Lactobacillus* grow at pH values above 3.5.**

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Lesson 12: The Malolactic Fermentation

The malolactic fermentation has many effects on the flavor and aroma of wines. As noted above, it is a deacidification reducing the net concentration of carboxyl groups and therefore acidity. This is important in regions where the fruit is high in acid at harvest.

Effects of Malolactic Fermentation

- **Deacidification**

Deacidification may not be desired in wines already low in acidity, but this conversion may occur whether desired on not if conditions support the growth of the organisms.

Deacidification

- **Decrease titratable acidity by 0.01 to 0.03 g/L because of H^+ fixation**
- **Increase pH by 0.1 to 0.3 units**
- **Important for high acid wines**
- **May be undesirable in low acid situations**

The fixation of hydrogen ions on lactate can reduce the titratable acidity by 0.01 to 0.03 g tartaric acid equivalents/ L. The pH is also increased by as much as 0.3 units. This is very important because if a wine is low in pH (below 3.5) the metabolic activity of the lactic acid bacteria can raise the pH to a level supporting the growth of many more species.

Effects of Malolactic Fermentation

- **Deacidification**
- **Bacterial stability**

Lesson 12: Bacterial Stability

The malolactic fermentation is also important because it confers bacterial stability to the wine, meaning that the growth of other organisms is inhibited. This is due to the consumption of nutrients so that conditions are not permissive for other microbes, but it may also be a consequence of the production of bacteriocins, compounds that are toxic to members of other species.

Bacterial Stability

- **Consume nutrients that would otherwise be available for other organisms**
- **Produce toxins (bacteriocins) that may inhibit growth of other bacteria**
- **Prevent malolactic fermentation from occurring in bottle**

Another important consideration is the timing of the malolactic fermentation. If it occurs prior to bottling it prevents microbial growth in the bottle. There are several reasons why growth in the bottle is undesirable.

Malolactic Fermentation in Bottle:

- **Increases turbidity due to cell growth**
- **Produces noticeable gas as CO_2**
- **May produce polysaccharides material**
  - **Haze**
  - **Ropiness**
- **May raise pH allowing growth of spoilage organisms**
- **Does not allow for control of flavor/aroma profile of wine**

Cloudiness or turbidity is objectionable in wine. Many consumers do not understand the source of the cloudiness so equate it with spoilage. The decarboxylation of malate yields carbon dioxide, which will produce noticeable bubbles in the wine. This is again undesired because many consumers do not understand the source of the CO_2, so equate it with an inferior or spoiled product. The bacteria may produce other unwanted products that are noticeable in the bottle. If these characters appear in the wine prior to bottling, they can be removed. But if they appear in the bottle, this may require recalling the production lot and uncorking, treating and re-bottling, an expensive process. Also if the reaction occurs in the bottle, the winemaker has no control over the process. As we will see in the next lecture, the malolactic fermentation can be managed and directed towards desired versus undesired compounds. If a problem arises, the winemaker can increase the amount of SO_2 or decrease the pH if it is high enough to be stimulatory to the "bad" lactics. None of this is possible if the wine is already in the bottle.

Effects of Malolactic Fermentation

- **Deacidification**
- **Bacterial stability**
- **Flavor changes**

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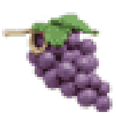
Lesson 12: Flavor Changes of the Malolactic Fermentation

There are several important flavor changes that occur in wine that has undergone a malolactic fermentation in addition to deacidification. If heterofermentative organisms are present, acetic acid can be produced.

Flavor Changes Associated with Malolactic Fermentation

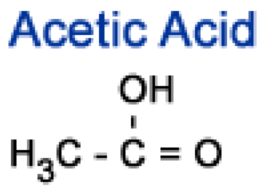
- **Acetic acid**
- **Diacetyl**
- **Acetoin**
- **2,3 Butantediol**
- **Ethyl lactate**
- **Diethyl succinate**
- **Acrolein**
- **Other compounds**

Acetic Acid



- **From sugar metabolism**
- **Amount formed versus ethanol depends upon aeration and presence of other electron acceptors**
- **Level produced can be significant**
- **Can also be produced from citrate metabolism**
- **Low levels can be made by *Saccharomyces***

Acetic acid itself is pungent but not offensively smelling. However its production can lead to the formation of other compounds that are objectionable.



Acetic acid is formed from sugar catabolism. As discussed above, the organism has the option of producing acetate or ethanol. Ethanol will be produced if other electron acceptors are not present. If molecular oxygen is available, acetic acid will be produced. This is one reason that extensive aeration of the wine during the ML fermentation is not recommended. However, oxygen stimulates growth of the malolactic bacteria much as it does in yeast. Limited oxygenation can actually stimulate the production of lactate, but it must be applied with caution. With limited aeration, *O. oeni* tends to produce lactate and ethanol and requires more full aeration for the production of acetate. However, other lactics may produce acetate under conditions where *O. oeni* will not.

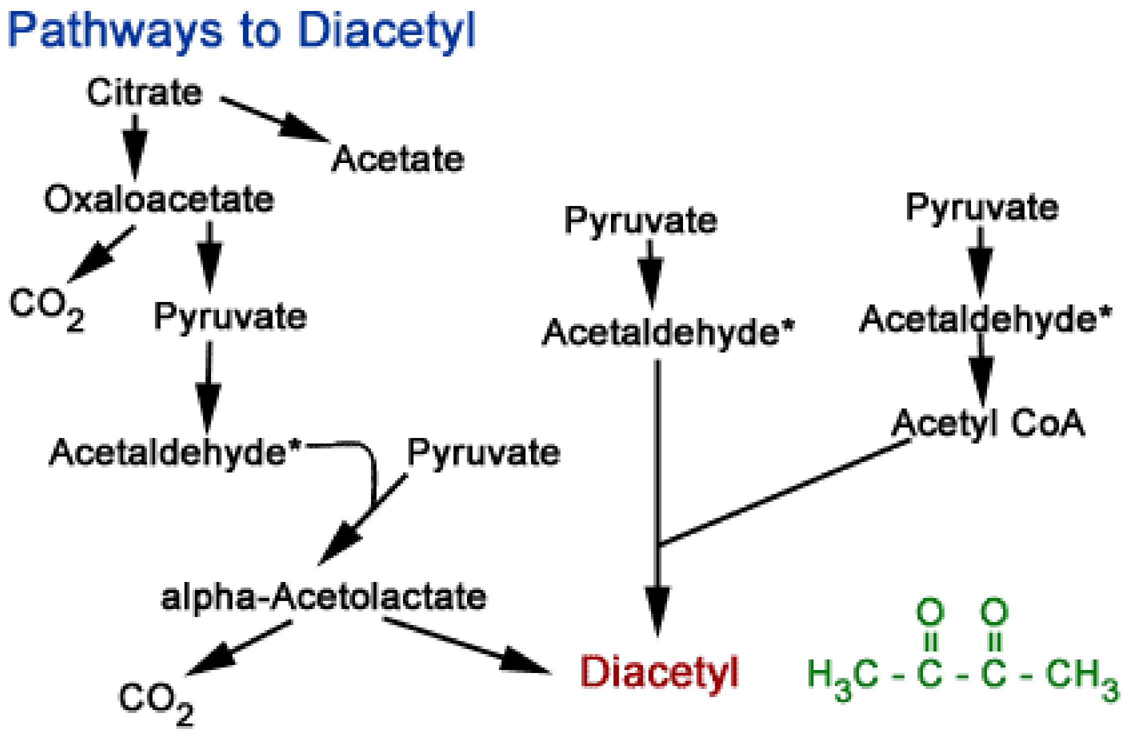
The amount of acetic acid produced can be significant and above the sensory threshold of detection. As discussed below acetate can also be formed from citrate metabolism by the lactics.

Diacetyl

- **Made from pyruvate**
- **Multiple pathways to pyruvate**
- **1-4 mg/L adds complexity "buttery"**
- **Over 4 mg/L dominates "movie popcorn butter/rancid"**
- **Low amounts can be produced by yeast**

Another very important compound produced by the lactic acid bacteria is diacetyl. This compound has a characteristic buttery note, which can become as strong as popcorn butter in high concentration, and may take on a rancid taint.

Diacetyl is formed from metabolites of pyruvate. There are multiple ways in which pyruvate can be formed.



Pyruvate can come from acid catabolism as well as from sugar catabolism. Its formation will therefore be dependent upon the amount of these precursors present. Diacetyl is formed from the reaction of two two carbon compounds, active acetaldehyde and acetyl CoA, or from the reaction of pyruvate and active acetaldehyde producing the 5 carbon acetolactate which is the converted to one four carbon diacetyl molecule plus one molecule of carbon dioxide.

Acetaldehyde* refers to "active acetaldehyde" which indicates the enzymatically bound form of acetaldehyde with the coenzyme thiamine pyrophosphate.

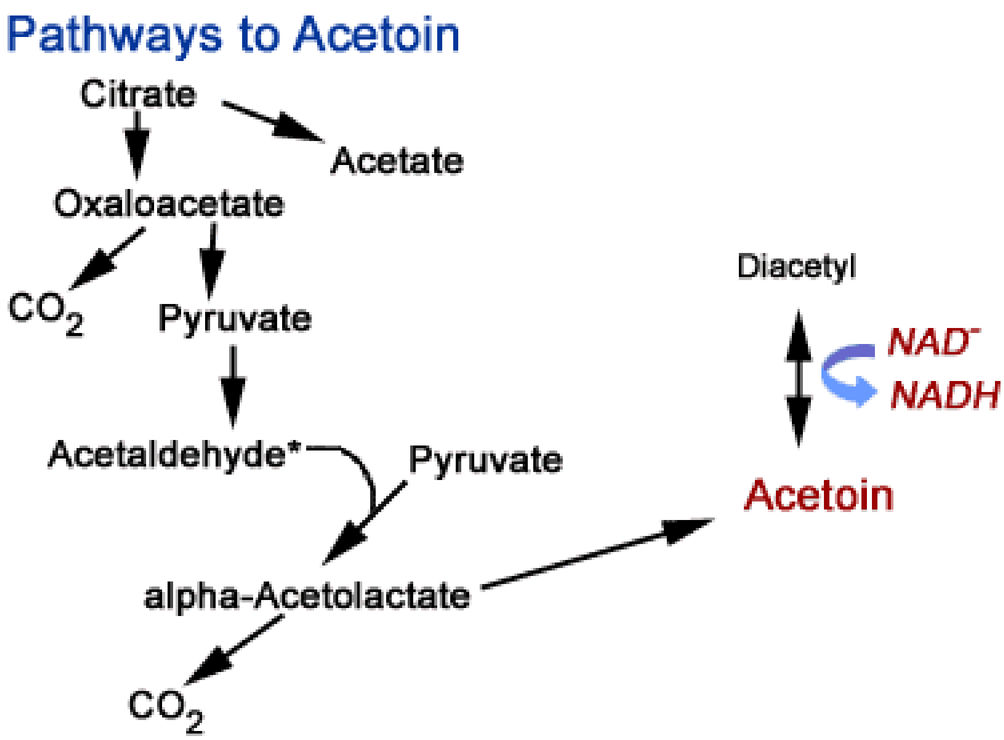
Diacetyl and other dicarbonyl compounds have recently been shown to interact with the sulfur-containing amino acids producing a spectrum of characters such as hazelnut, chocolate, cheese, potatoe, cabbage, popcorn and roasted nut. Whether these compounds are formed or not depends upon the amino acid composition of the juice or wine.

Acetoin

- **Also produced from pyruvate**
- **Can be derived from diacetyl**
- **Generally present below threshold of detection**

Another compound that can be produced by lactic acid bacteria is acetoin. It can be produced from pyruvate as well and it can also come from diacetyl.

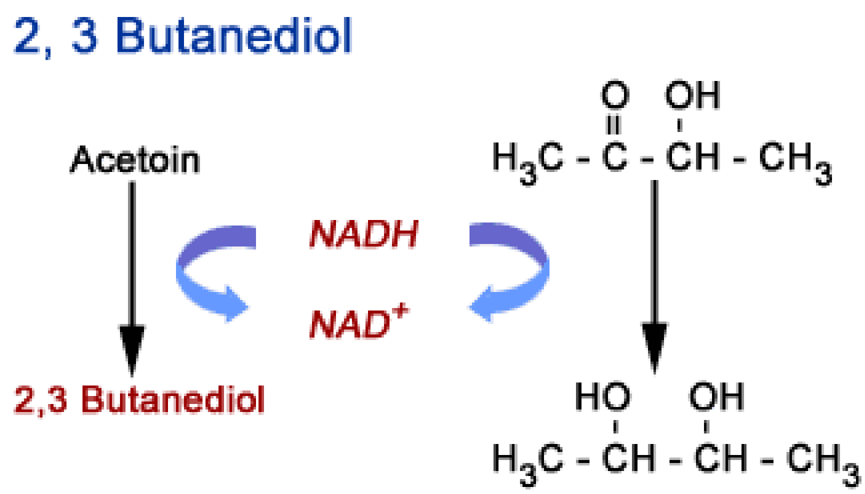
Acetoin is usually present below the threshold of detection in most wines.



2,3 Butantediol

- **Derived from acetoin**
- **Generally present below threshold of detection**
- **Mild alcohol flavor that borders on bitterness**
- **Can be produced by yeast**

Lactic acid bacteria also produce 2-3 butanediol. This compound comes from acetoin. It is generally below the threshold of detection, but it has a flavor that has been described as a mild alcohol that borders on a bitter finish.

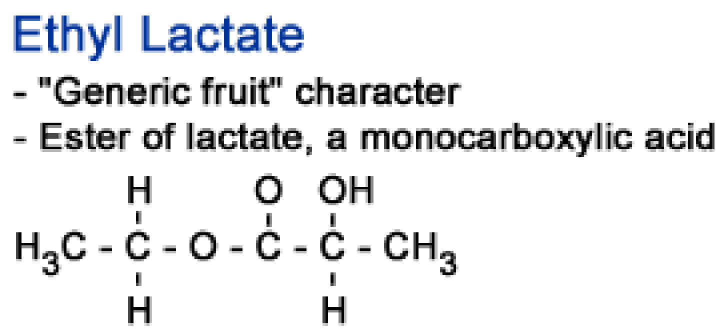


This compound can also be produced by yeast, but not in very high quantities. Note that the formation of these compounds is reductive, that is, electrons are fixed in the formation of both acetoin and 2,3 butanediol. They allow the organism to continue to catabolize substrates oxidatively and to produce acetic acid if oxygen is present by serving to recycle reduced cofactors.

The lactic acid bacteria can also produce some ester characters that contribute positively to the aroma profile of the wine.

Ethyl Lactate

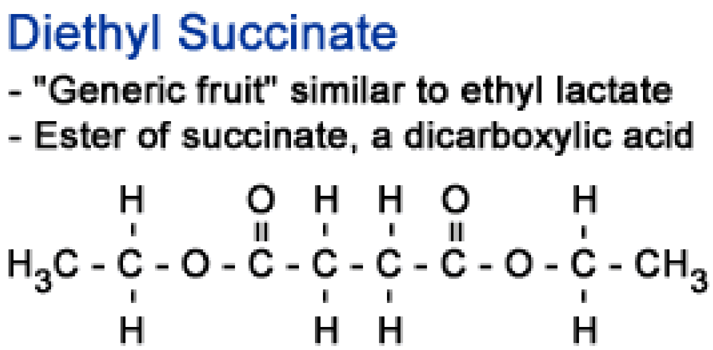
Ethyl lactate is described as "generic fruit". It is an ester formed from the reaction of the acid lactate and the alcohol ethanol. Since both of these compounds are present during active lactic bacterial metabolism, the formation of this ester is not surprising.



Generic fruit notes tend to increase the overall perception of the fruit character of the wine. That is, ethyl lactate may not be identifiable as such in a wine, the wine just appears to have more intense fruity and perhaps varietal character. However as we will see in the last section of the course, esters tend to hydrolyze under wine conditions so are not stable over time.

Diethyl Succinate

Diethyl succinate can also be produced by the lactic acid bacteria. This compound also imparts a generic fruit character.

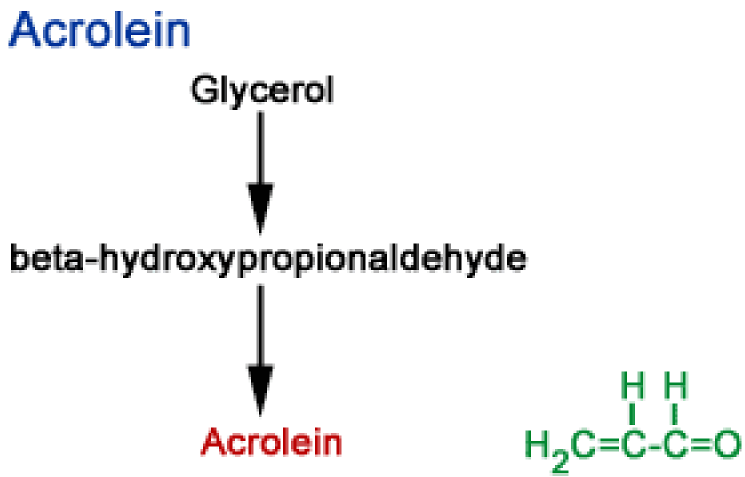


This compound is formed from the reaction of the dicarboxylic acid succinate with two ethanol molecules.

Acrolein

- **Made from glycerol**
- **Creates an intensely bitter taste when combined with phenolic compounds**

Acrolein can also be produced by lactic acid bacteria, but is fortunately for winemakers only rarely found. This compound is made from glycerol and is intensely bitter.



Not all strains can produce this compound, and glycerol levels in wine are generally low enough to prevent its formation. However the use of yeast strains releasing large quantities of glycerol to improve mouth feel should be used with caution if the wine is also subjected to a malolactic fermentation, especially under conditions leading to the presence of *Pediococcus* and *Lactobacillus*.

The lactic acid bacteria can produce many other characters, depending upon what energy sources are available in the medium. Some of these compounds are the degradation products of amino acids.

Other Compounds

The Lactic Acid Bacteria are capable of producing numerous other aroma compounds, especially from the degradation of amino acids. It is likely that some of these compounds are also being produced during growth in wine.

Some of the notes found in cheeses may be present in wine, including the characters that border on fecal. These characters are classically described as "pig feces".

In the laboratory section of the course we have had the dubious pleasure of seeing the fecal character that can be produced by these organisms. This has more commonly occurred in nitrogen rich juices inoculated early in the fermentation or pre-fermentation by the yeast consistent with derivitazation from the amino acids. Another nasty character that can be produced from amino acids is the mousy taint that comes from the degradation of lysine. Again, production of this compound requires the presence of lysine. Lysine is an important nutrient for *Saccharomyces* as we discussed in the yeast lectures, and supplementation with lysine can stimulate fermentation rate is some strains, but if over-supplementation occurs, the incidence of mousiness will increase. For those of you fortunate enough to be unfamiliar with this taint, it smells and tastes like a "used" mouse cage - a mixture of rodent and rodent urine. The character that we perceive is an oxidation product of the compound produced by the bacterium. It can be noticed by placing a drop of wine on one palm and rubbing palms together. When the palms are then smelled, it has the odor of a mouse cage. It is quite unpleasant by mouth as it is not initially detectable, but once oxidized imparts a nasty character that seems to originate in the back of the throat and is quite persistent.

Tartrate

> **Some strains of *L. plantarum* and *L. brevis* are capable of metabolizing tartrate to acetic acid, referred to as "*tourne* disease" by Pasteur. This is always undesirable.**

Some lactic strains can metabolize tartrate to acetate. This is also always undesirable in wines.

The spectrum of compounds produced by the lactic acid bacteria are dependent upon the species present and the composition of the wine, juice or must at the time of the malolactic conversion.

Flavor Changes Associated with the Malolactic Fermentation

> **Amounts produced are strain dependent and dependent upon the composition of the juice and level of aeration.**

In the next lecture we will discuss management of the malolactic fermentation and the options available to the winemaker.

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Lesson 13: Introduction

Managing the Malolactic Fermentation

The factors impacting the rate and progression of the malolactic fermentation and strategies for stimulation and inhibition of this biological process will be covered in this lecture. The timing of this fermentation with respect to the alcoholic fermentation is also important, and will be addressed. Some yeast strains are sensitive to the presence of the bacteria and will arrest fermentation upon inoculation of the must or fermenting medium with lactics. Others are resistant and still others are inhibitory. We will also survey the current state of knowledge on the possible microbial interactions occurring in wine as related to the malolactic conversion relevant to these phenomena of stimulation and inhibition.

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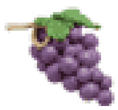
Lesson 13: The Biology of the Malolactic Fermentation

The conversion of malate to lactate occurs after the lactic acid bacteria have reached stationary phase. This reaction requires the cofactor NAD+ and manganese.

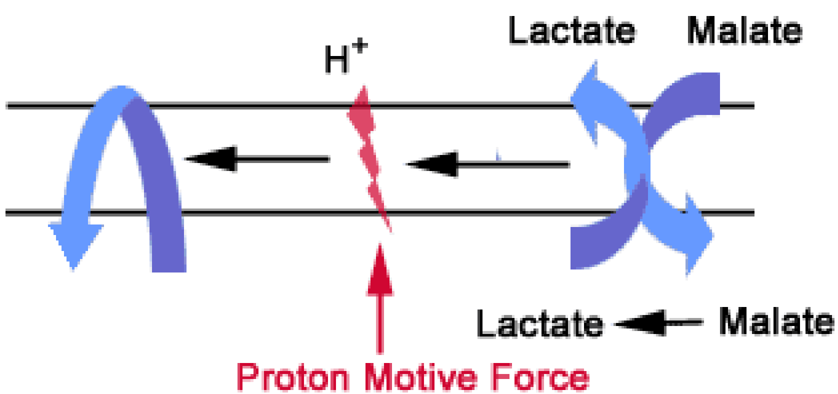


The Malolactic Fermentation

- **Requires NAD+, Mn++**
- **Occurs after exponential growth phase**
- **Used to genetate energy**

Malate is directly converted into lactic acid (L-lactic acid) by the malolactic enzyme. This is a direct decarboxylation that does not pass through pyruvate. Production of the malolactic enzyme is induced by malate and stimulated by sugars. This conversion is used by the cell to generate energy.

Energy Generation from the Malolactic Conversion



The conversion of malate to lactate and accompanying "fixing" of a proton decreases the proton content of the cytoplasm upon efflux of lactate thereby creating a "proton motive force" across the membrane; the energy of the proton movement can then be captured in ATP

The fixing of a proton on lactate creates what is called a "proton motive force" across the membrane. This force means that protons are in higher concentration on the outside of the cell and their entry into the cell is energetically favorable. The lactic acid bacteria are able to capture this energy and form ATP. However, it is not stoichiometric, meaning that one ATP is not produced for every lactate molecule generated. A plasma membrane ATPase is able to convert the energy from hydrogen ion movements into the biochemically active form of energy, ATP. However, not all organisms that conduct the malolactic conversion have been shown to obtain energy by this process. There may be other metabolic factors driving this conversion.

Lesson 13: Factors Influencing the Malolactic Fermentation

As noted above, the malolactic conversion involves generation of a proton motive force, which requires maintaining a strong proton gradient across the membrane.

Factors Affecting the Malolactic Fermentation

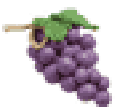
- **pH**
- **SO_2**
- **Nutrient composition**
- **Oxygen**
- **CO_2**
- **Alcohol**
- **Temperature**
- **Organic acids**
- **Phenolic compounds**
- **Presence of other lactic acid bacteria**
- **Bacteriophage**

pH

- **Affects which strains/species will grow**
- **Affects rate of growth**
- **Affects survival of organism**
- **Affects metabolic behavior of strains that are growing**

One of the most important factors affecting the progression of the malolactic fermentation is pH. The pH has many effects on the progression of the malolactic fermentation in addition to contributing to the proton gradient. The pH of the medium determines which lactic acid bacteria will be present. It also impacts growth rate and is inhibitory if too low (less than 2.9). The pH also affects viability in addition to the inhibition of growth. Finally, the pH will influence the metabolism of the organisms. Malate can be catabolized at pH values (3.2) below what is permissive for sugar catabolism (3.5) in many lactic acid bacteria. The malolactic conversion is actually faster at pH 3.8 than at lower pH values, however. The conversion will occur approximately 10 times slower at pH 3.2 as compared to 3.8. There is also considerable species and strain variation with respect to pH tolerance.

SO_2



- **Sulfur dioxide is inhibitory**
- **All genera/species/strains appear to be equally sensitive**
- **Even if SO_2 is not added, it may be produced by yeast at an inhibitory concentration**

Another factor that is very important in managing the malolactic fermentation is the use of sulfur dioxide. These organisms are very sensitive to sulfur dioxide, much more so than *Saccharomyces*. All strains appear to be equally sensitive and there are no SO_2 tolerant strains of *Oenococcus*. The molecular or free form of SO_2 is the inhibitory species. As discussed in an earlier lecture the amount of free SO_2 present is dependent upon the pH. The amount of free SO_2 is also a function of the presence of compounds that will bind sulfite. Bound SO_2 can also be inhibitory to the lactic acid bacteria, but is less so.

The SO_2 produced by *Saccharomyces* may be inhibitory to the lactic acid bacteria. Yeast produce on the order of 20 mg/L sulfite, which, if the pH conditions are appropriate, may be high enough to inhibit some malolactic strains.

The Nutrient Composition

- **Lactic acid bacteria are fastidious: numerous growth requirements**
- **Aging on yeast lees increases micronutrient content via autolysis**
- **Extended skin contact enhances malolactic fermentation**
- **Higher solids/less clarification enhances malolactic fermentation**

Nutrient availability is as important to the encouragement of the lactic acid bacteria and the malolactic conversion as it was for the yeast and the alcoholic fermentation. The lactic acid bacteria are more fastidious than the yeast, requiring many more micronutrients and growth factors.

This is one reason that the malolactic conversion is stimulated by autolysis of the yeast. Extended skin contact and higher solids levels are also stimulatory to the lactic acid bacteria. In contrast to the yeast, the bacteria require the presence of several amino acids. That means that they cannot synthesize all 20 amino acids from ammonia. Strains vary in which amino acids are required. Yeast release amino acids at the end of fermentation, so amino acid limitation is not usually a problem unless the bacteria are inoculated and the conversion expected before this release occurs. We have found that the malolactic conversion occurs more readily pre-yeast fermentation or after the release of the amino acids (post-fermentation), but is less likely to occur if inoculated during the active phase of the alcoholic fermentation. Several proprietary mixes of lactic acid nutrients are available, but if added early in the fermentation may also stimulate yeast growth and metabolism. Again it is important to time nutrient additions so that the desired population is the one that is fed.

Oxygen

- **Stimulatory to growth**
- **Affects spectrum of end products**
- **Can produce more energy (and acetic acid) in presence of oxygen**

Molecular oxygen is stimulatory to the malolactic fermentation, depending upon the organisms present. It stimulates the growth of some lactic acid bacteria, behaving as a growth factor just as in the case of yeast. This is not observed with all species however, and some appear to be inhibited by oxygen. Limited oxygenation generally appears to be stimulatory to the malolactic fermentation in wine production. However, if too much oxygen is applied, and if strict heterofermentative organisms present, acetic acid may be produced as discussed in the previous lecture.

Aeration regimes during the malolactic fermentation should be limited to avoid unwanted end products. Winemakers frequently report acetic acid accumulation when the malolactic conversion occurs prior to the onset of the alcoholic fermentation. This usually coincides with an aeration of the juice or must.

CO_2

- **Stimulatory to malolactic fermentation**
- **Mechanism unknown**

Carbon dioxide is also stimulatory to lactic acid bacteria. This may be because CO_2 is associated with much better mixing, rather than it providing some other sort of nutritional benefit.

Carbon dioxide might also impact the buffering capacity of the wine and therefore stimulate growth and metabolism, but the actual mechanism of stimulation is not known.

Alcohol

- **High alcohol slows malolactic fermentation**
- **Affects bacterial viability**
- **Affects which species/strains are present**

Ethanol also affects the malolactic bacteria. As in the case of the yeast, these organisms have an upper limit of ethanol that can be tolerated. The malolactic bacteria are generally inhibited above 14% ethanol, but some species display a much stronger sensitivity. If the malolactic conversion is desired in a late harvest or high Brix juice, it may be necessary to conduct this fermentation prior to the onset of the alcoholic fermentation.

In general, the higher the alcohol content the slower the malolactic fermentation.

Temperature

- **Growth of malolactic bacteria better at higher temperatures**
- **Malolactic fermentation faster at higher temperatures**

Temperature is an extremely important variable in the stimulation or inhibition of the malolactic fermentation. The optimum temperature for growth of the malolactic bacteria ranges from 20 to 37°C. Growth of the bacteria is inhibited below 15°C. Within the permissive range, the higher the temperature the faster the growth rate and the metabolic conversion of malate to lactate.

It is frequently necessary therefore to warm a fermentation before the malolactic fermentation will occur.

Organic Acids

- **Fumarate inhibitory at low concentrations**
- **Can be produced by yeast**
- **Fatty acids can also be inhibitory**
- **Malate stimulates growth prior to malolactic fermentation**

The presence of specific organic acids other than malate can influence the malolactic fermentation as well as other metabolic activities of the organisms. Malate will stimulate the growth of most of the bacteria prior to the onset of the conversion of malate to lactate. Interestingly, those that are not stimulated by malate are the same strains that do not appear to be able to generate ATP from the malolactic conversion.

Fumarate, which is produced in the tricarboxylic acid cycle by yeast, is inhibitory to the malolactic bacteria. It is not clear if one reason specific yeast strains tend to inhibit the lactic acid bacteria is due to the release of fumaric acid during the alcoholic fermentation. Citric acid can be metabolized by the lactic acid bacteria as described in the previous lecture. Certain fatty acids may be inhibitory to the lactic acid bacteria.

Phenolic Compounds

The bacteria are also affected by the phenolic compounds of the wine. They can be inhibited by seed phenolics just as was the case for *Saccharomyces*. Stimulation by anthocyanins and gallic acid has been reported in the literature.

Presence of Other Lactic Acid Bacteria

- **Mixed cultures may yield "better" complexity**
- **Can be stimulatory**
  - **Increase in pH**
- **Can be inhibitory**
  - **Bacteriocin production**
  - **Competition for nutrients**

The presence of other bacteria will influence the malolactic conversion. This can be stimulatory, such as would occur if *O. oeni* raised the pH to a level permitting growth of other lactics or could be inhibitory, competition for nutrients or production of bacteriocins. There is a belief in the California wine industry that mixed culture malolactic fermentations provide greater complexity than single culture conversions, but it is not clear how many species in these preparations actually contribute to the overall character of the wine versus impacting the metabolic activities of the dominant organisms.

Bacteriophages

- **Bacterial "viruses" that can be spread from one bacterium to another and that cause cell death**
- **Not known if this is a problem in wine production or not; it is a problem in other lactic acid bacteria fermentations**

Another factor that is mentioned as possibly problematic in lactic acid bacteria fermentations is the presence of bacteriophage. Bacterial phages are virus-like particles that infect and lyse target bacteria.

They spread from one bacterium to another due to release of mature virus particles and lysis of the host cell and can eliminate an entire population. This is certainly a problem in other types of lactic fermentations, but it is not clear if this is problematic in wine. Bacteriophages infecting wine lactic acid bacteria have been discovered, but they have not been associated with prevention of the ML fermentation.

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Lesson 13: Management of the Malolactic Fermentation

There are several considerations that must be taken into account by the winemaker with respect to the malolactic fermentation. The first question is whether or not this conversion is desired.

First Decision:

Do you want the MLF?

In some cases the malolactic fermentation is desired for the resulting increase in wine complexity; in other cases it will occur whether the winemaker wishes it to or not due to the composition of the wine. It is my experience that this fermentation demonstrates Mother Nature's sense of humor: it seems to go most readily under conditions where it is not desired and is more difficult to encourage when most wanted. Many winemakers contend that the best way to stimulate the ML fermentation is to bottle the wine!

Reasons MLF is Desirable

- **Acidity reduction**
- **Addition of flavors**
- **Bacterial stability of product**

There are three main reasons for desiring the malolactic fermentation: reduction in acidity, flavor addition and bacterial stability.

Reasons MLF is Undesirable

- **Acidity reduction**
- **Addition of flavors**

With the exception of bacterial stability, these are also the reasons the malolactic fermentation might not be desired!

If the malolactic fermentation is desired, there are several things that can be done to encourage it. Obviously little to no use of SO_2 should occur and the winemaker should use strains of *Saccharomyces* that do not produce high levels of sulfite.

MLF Stimulated By:

- **Low to no use of SO_2**
- **Warm temperatures**
- **Addition of nutrients**
- **Use of inocula**
- **Low ethanol (avoid late harvest wines)**
- **Delay racking off yeast lees**
- **Acid/pH adjustment**

The wine should be held at a permissive temperature for the bacterial conversion (above 15°C). Nutrients can be added, if needed, that is, if the wine has been racked off of the yeast lees prematurely or the wine was not fermented with a high solids content or with significant skin contact. As with *Saccharomyces*, an ML inoculum can be used rather than relying on a spontaneous initiation of the fermentation. The ethanol content of the wine should not be at an inhibitory value if the malolactic fermentation is desired. We have found that there are lactic bacteria that seem to grow in wines of high ethanol content, but they do not appear to conduct the malate to lactate conversion to completion, and they can produce objectionable characters in these wines. Finally, the pH should be adjusted upward if it is too low (below 3.2 for *O. oeni*). Doing the opposite of the above will be inhibitory to the malolactic conversion.

MLF Inhibited By:

- **Use of SO_2**
- **Early racking**
- **Downward pH adjustment**
- **Low temperature**
- **Filtration**
- **Addition of fumaric acid**

If inhibition is desired, the winemaker may want to filter the wine off of the yeast lees. But it is important to remember that the yeast tend to release amino acids prior to reaching true dryness, so this practice may be ineffective. One could use fumaric acid inhibition, but this acid will precipitate under wine conditions dropping its concentration in the wine, thus it is not a permanent guard against the ML bacteria. The same is true for SO_2, sulfite is volatile and reactive and its free concentration will decrease over time in the wine. If it drops below the inhibitory concentration, the ML bacteria may bloom.

The second major decision to be made by the winemaker is whether to inoculate with ML bacteria or to allow a native fermentation to occur.

Second Decision:

Inoculated *versus* Spontaneous Malolactic Fermentation

There are benefits and disadvantages to both, just as there was for the alcoholic fermentation.

Inoculated MLF

- **Better control over both timing and organisms present**
- **Difficult to maintain inocula**
- **Starter culture must be "pure"**
- **Percent inoculation: 1-50% depending upon vigor of culture**

Inoculation allows better control over the timing and the organisms (homolactic on hexoses (facultative heterofermenters)) versus the obligate or strict heterofermenters. That said, it is far more challenging to use a bacterial starter than a yeast starter culture. The bacteria are more fastidious so must be grown in a nutrient rich, part juice medium. This medium is impossible to fully sterilize, meaning that there is a certain loss of control over the purity of the species present. If a "bad" lactic becomes dominant, it will then be used to infect the entire production lot. It is imperative that all ML inocula be sensorially evaluated before being used as an inoculum. The best percent inoculum to use depends upon the vigor of the culture. Very high inocula tend to initiate the fermentation faster and it is thought that they are less problematic than low inocula. There are now some active dry ML bacteria products in the marketplace with which some winemakers have had excellent experience.

The basic steps of ML inoculum preparation are as follows:

Inoculum Preparation

1. **Start culture from slant in medium supporting good growth of organism**
2. **Inoculated "diluted" juice (with water) from starter with addition of nutrients**
3. **Use #2 to inoculate full strength wine or juice with addition of nutrients**

Again I cannot emphasize enough that the inoculum should be smelled at each step. Your nose is the best indicator of the presence of a problem microbe.

Spontaneous MLF

- **Uncontrolled timing of process**
- **Risk of unwanted species/strains**
- **Off-characters can be produced if MLF occurs when undesired**

Allowing a spontaneous ML fermentation to occur runs many of the same risks as a spontaneous yeast fermentation. There is no control over the timing of the process and whether it will occur, has just occurred, or will not occur. Given the fastidious nature of the bacteria, the timing is far less certain than for the alcoholic fermentation. Also, conditions that stimulate the desired ML bacteria (the facultative heterofermenters that basically function as homolactic hexose fermenters in wine) also stimulate the bad lactics (the strict heterofermenters). This can lead to the formation of objectionable characters that would not be present if the "good" lactics were preferentially encouraged and consumed nutrients and produced factors inhibiting growth of the "bad" lactics. Good and bad are far more distinct with respect to the lactic acid bacteria than was the case for the yeast.

The next decision for the winemaker is the timing of the malolactic fermentation.

Third Decision

Timing of Malolactic Fermentation

An inappropriate choice in timing may result in the inhibition of the alcoholic fermentation or alternately may greatly reduce the chance that the ML will occur at all. The options are to encourage the ML prior to the initiation of the alcoholic fermentation, fully post fermentation, to encourage simultaneous yeast and ML fermentations or to inoculate with the ML bacteria at some specific point in the ML fermentation. Every year in the laboratory section of VEN124 we conduct a trial on the timing of inoculation of the ML fermentation versus success in completion of the fermentation. Our experience is that if it is going to go at all it will most likely occur pre- or post yeast fermentation. In most years the simultaneous inoculation does not lead to a successful ML fermentation nor does a mid fermentation inoculation. There is a trend in the industry to inoculate the ferment with about 5°Brix of sugar remaining. We have not had much success with inoculation at this time. Sometimes the post-fermentation ML will not occur and other times the pre- yeast fermentation sample will not undergo an ML fermentation.

Timing of MLF: Options

- **Prior to yeast fermentation**
- **Simultaneous with yeast fermentation**
- **Mid-way throughout yeast fermentation**
- **After yeast fermentation**

The timing of the ML fermentation will obviously depend upon the conditions of the juice and whether or not the temperature, pH and nutrients are permissive for all organisms.

Timing of MLF: Pre-fermentation Inoculation

- **Decreases yeast nutrients**
    - **Stuck/sluggish fermentation**
    - **Production of off-characters**
- **May lead to production of inhibitory compounds (acetic acid) due to presence of oxygen**

Pre-fermentation inoculation with the ML bacteria can decrease the nutrients available for the yeast. This can lead to off-character production or arrest of fermentation. It has been well established that lactic acid bacteria produce compounds that are inhibitory to yeast growth and fermentation. This is an area currently under active investigation.

Other types of problems may be associated with a simultaneous inoculation.

Timing of MLF: Simultaneous with Yeast Inoculation

- **See increases in acetic acid**
- **See a decrease in viability of both yeast and bacteria**
- **Yeast "rebound" better than bacteria**

Winemakers frequently report an increase in acetic acid production with simultaneous inoculation. A decrease in the viable populations of both organisms can be noted as well. It appears that the yeast are able to rebound more readily than the bacteria under these conditions, but the culture may still be prone to arrest. There are important strain factors that impact the fate of the yeast and bacteria. We have found that the commercial yeast, Premier Cuvee, seems to be relatively unaffected by the activity of the ML bacteria, but may be quite inhibitory towards many strains of the bacteria. Other commercial strains are much more sensitive to fermentation arrest upon inoculation with the bacteria.

Timing of MLF: Mid-Fermentation

- **Nutrients left for bacteria**
- **Ethanol low and not inhibitory**
- **Yeast-produced SO_2 may be inhibitory**
- **May lead to arrest of yeast fermentation**

Inoculation mid-way or late in the alcoholic fermentation but before it is completed can be quite risky.

At this point there are hardly any nutrients left for the bacteria since the yeast have not yet released any. Ethanol may be low and not inhibitory at the point of inoculation, so it may seem like a great idea especially for high Brix musts and juices, but the yeast will continue to metabolize and raise the content of alcohol. Yeast-produced sulfur dioxide may also be highest at this time, especially if the rate of carbon dioxide production and therefore loss of SO_2 due to CO_2 evolution has slowed. While we have not seen this in our experiments, many winemakers report an arrest of the yeast fermentation upon inoculation with the ML bacteria mid or late into the alcoholic fermentation. Since high inoculum strengths are typically used, it may be that some inhibitor produced by the bacteria in the inoculum preparation is carried over into the yeast ferment. Alternately, at this point the yeast is most dependent upon available oxygen in the must and on fatty acids needed for ethanol tolerance the introduction of biomass at this point may rapidly deplete the fermentation of needed survival factors.

Timing of MLF: Post Fermentation

- **Nutrients have been depleted**
  - **Add nutrients**
  - **Encourages yeast autoysis**
- **Ethanol concentration high**
- **Concentration of other yeast inhibitory compounds also high**
- **Better temperature control**

Post-fermentation inoculation is another strategy. This prevents any inhibitory effect of the malolactic bacteria against the yeast, but will be problematic if the ethanol content is too high.

At this point other nutrients have been depleted and yeast autolysis requires on the order of six months to occur. Other inhibitory factors may have been produced by the yeast that may impact the ML bacteria. Post-fermentation inoculation allows better temperature control in that it permits the primary alcoholic fermentation to be conducted at a temperature leading to greater retention of grape volatile characters but too low for growth of the ML bacteria. Warming of the wine post alcoholic fermentation to make conditions permissive for the malate to lactate conversion does not lead to the same loss of characters that occurs if the wine is warm during the maximal rates of yeast catabolism and CO_2 evolution.

Another important choice for the winemaker is the selection of the ML strain to be used during the ML fermentation. It can be argued that this is more of an issue than it was with *Saccharomyces* because there is a greater diversity in the amounts and nature of sensorially detectable characters produced by different strains of lactics.

Fourth Decision:

Choice of Strain

This is complicated by the fact that the bacteria are so fastidious and the correct strain or pattern of metabolic activity may not actually be what is being encouraged to grow.

MLF: Choice of Strain

- **Compatible with yeast**
- **Production of desirable characters**
- **Ability to complete ML fermentation**
- **Vigor**
- **Availability as freeze-dried inoculum**

It is important that the ML strain selected be compatible with the yeast strain utilized. It is also important that the strain not have a tendency to produce off-characters and display reasonable vigor in growth as well as in conducting the ML conversion. Since the bacteria are fastidious in their requirements and numerous grape compositional factors impact the ML fermentation, it is not easy to be sure the strain will perform as expected. If the strain is to be prepared as a commercial inoculum it is important that the strain be able to survive the production process and display good viability upon rehydration. One other note, it has frequently been reported that "cultured" ML strains lose the vigor and aroma profile of the "native" organisms.

As with the yeast fermentation, the winery needs to develop a strategy for monitoring the ML fermentation.

Fifth Decision:

Method of monitoring MLF

Monitoring the MLF

- **By conversion of malate to lactate**
    - **Loss of malate not appearance of lactate***
    - **HPLC, Enzymatic, Paper chromatography**
- **By flavor changes**
    - **Tells you bacteria are active**
    - **Does not tell you when they are done**

*** Lactate can be produced from other sources**

The fermentation can be monitored by following the conversion of malate to lactate. This should be done by evaluating loss of malate, not the appearance of lactate. Recall that the yeast can make lactate and the bacteria can make this compound from hexoses so its appearance is not indicative of a malolactic fermentation. The loss of malate can be followed by simple paper chromatography or more quantitatively using an enzymatic assay method or HPLC or CE analysis. It is also important to note the appearance of other characteristic or "signature" ML compounds. This can frequently tell the winemaker that the bacteria are indeed present and metabolizing. However this will not tell you if the ML fermentation has been initiated or completed.

A final decision that can be made concerns the primary reason for conducting the malolactic fermentation.

Sixth Decision:

Alternative Method of Acid Reduction

If it is being conducted simply to reduce acidity, then alternative methods of acid reduction might be considered.

Alternative Methods of Acid Reduction

- **Immobilized enzyme**
- **Immobilized cells**
- **Yeast mediated conversion of malate to ethanol**
  - **Conducted by *S. pombe***
  - ***S. cerevisiae* has been genetically engineered to perform this conversion**
- **Expression of ML enzyme in *Saccharomyces***
- **Chemical precipitation**

The options noted above are mainly in the design stage or are undergoing testing and are not in commercial use as yet. It is possible to reduce acidity enzymatically by use of an immobilized enzyme system. The enzymes being evaluated convert malate to lactate, so the primary end product will not change. Immobilized cells can also be used. In theory it is easier to control and limit the biological activities of immobilized cells. The goal is a more controlled and guaranteed ML conversion. Dr. H. van Vuuren and colleagues have genetically engineered a yeast strain that will convert malate to ethanol. While this might not be desired in a high Brix juice or must, it may be an option for fruit low in sugar but high in acid at the time of harvest. The enzymes used were derived from the yeast *Schizosaccharomyces pombe*. Earlier Dr. Kunkee and colleagues were able to engineer a strain of *Saccharomyces* expressing the bacterial malolactic enzyme. These genetically engineered strains are not commercially available and are not being used in the production of wines except on a laboratory scale.

This concludes our section on the Malolactic bacteria. The most important goal of the winemaker is to make sure that this conversion occurs prior to bottling. If it has not, the only way to guarantee that it will not occur in the bottle is to sterilely filter and sterilely bottle the wine.

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