Bruce Zoecklein
Ken Fugelsang
Barry Bump

UNDERSTANDING AND MANAGING THE NUTRITIONAL STATUS OF JUICE AND WINE

Learning Outcomes: *Nitrogen availability to the vine can be considered a terrior factor. Nitrogen is one of the most important elements impacting vine growth, fruit chemistry, and therefore ultimate fruit and wine potential. Wines made from fruit with adequate nitrogen generally have superior aroma and overall quality. Evidence suggests that supplement additions fail to enhance the fermentation as much as natural, grape-produced nitrogen.*

Uninformed use of DAP bears the risk that a low to moderate must nitrogen can be instantly converted to a must with a high concentration, to produce a wine of very different, and possibly less preferred, style. The following highlights the need not only to monitor nitrogen in the vineyard, but to monitor must nitrogen so that appropriate additions can be made when required.

Nitrogen application in the vineyard can be used to manipulate the must YAN to a large degree, but the fine tuning can be more easily achieved in the winery through the use of nitrogen supplements. Forms of nitrogen other than ammonium (DAP), including amino acids, are important for the formation of a number of favorable wine components, and may enhance aromatic character.

Low must yeast assimilable nitrogen (YAN) has the potential to lead to reduced yeast populations and fermentation vigor, increased risk of slow and sluggish/stuck fermentations, increased production of "reductive" volatile thiols (e.g., H_2S, and mercaptans, etc.), and higher alcohols and decreased production of esters and long-chain fatty acids. High must YAN has the potential to lead to increased biomass and high heat output due to high fermentation vigor, and increased formation of ethyl acetate, acetic acid, and volatile acidity. There is also an increased risk of microbial instability, potential taint from Botrytis*-infected fruit and, possibly, atypical aging character with high YAN.*

Information on the importance of timing and addition rate of complex nutrients and DAP to must are discussed. Optimizing nitrogen management for red wine fermentations is not well understood, and likely differs from that for white wine fermentations. Optimization of vineyard and YAN nitrogen can contribute to quality factors in wine and, hence, affect its commercial value.

Bruce Zoecklein
Ken Fugelsang
Barry Bump

Chapter Outline

- **Plant Nitrogen**
  - **Nitrogen and Red vs. White Grapes**
  - **Nitrogen and ATA (atypical aging)**
- **Yeast Assimilable Nitrogen (YAN)**
  - **FAN Nitrogen**
- **Practical Considerations Influencing YAN**
  - **Vineyard Considerations**
  - **Fruit Rot and Nitrogen**
- **Yeast Strains, Microbial Ecology, Nutrient Desert and YAN**
  - **Yeast Starter Population Density**
  - **Temperature Shock**
- **Processing and YAN**
- **Nitrogen Impact on Aroma/Flavor Compounds**
- **Nutrient and Supplement Addition Products**
  - **Timing of Nutrient Additions**
  - **Vitamin Addition**
  - **Oxygen/Sulfur dioxide**
  - **Hydrogen ion concentration (pH)**
  - **Non-soluble solids**
  - **Fermentation temperature**
  - **Carbon dioxide toxicity**
  - **Sugar toxicity**
  - **Glucose/fructose**
  - **Alcohol toxicity**
  - **Native yeast/bacterial fermentations**
  - **Pesticides and fungicides**
- **Co-Inoculation, Multiple Yeast Strains, and Malolactic Fermentations (MLF)**
- **Analytical Methodology for Nitrogen Nutrition**
- **Ethyl Carbamate Formation**
- **Untypical/Atypical Aging**

Bruce Zoecklein
Ken Fugelsang
Barry Bump

Practical Summary of Winemaking Issues

Section 1.

Plant Nitrogen

In the Old World the conviction has been held for centuries that wine is, at its core, the reflection of a place. While there is no single-word translation for *terroir* into English, the French often use this one word to explain why a wine tastes the way it does, as a result of its place. Nitrogen availability to the vine can be considered a *terroir* factor. Nitrogen is one of the most important elements impacting vine growth, fruit chemistry, and therefore ultimate fruit and wine potential.

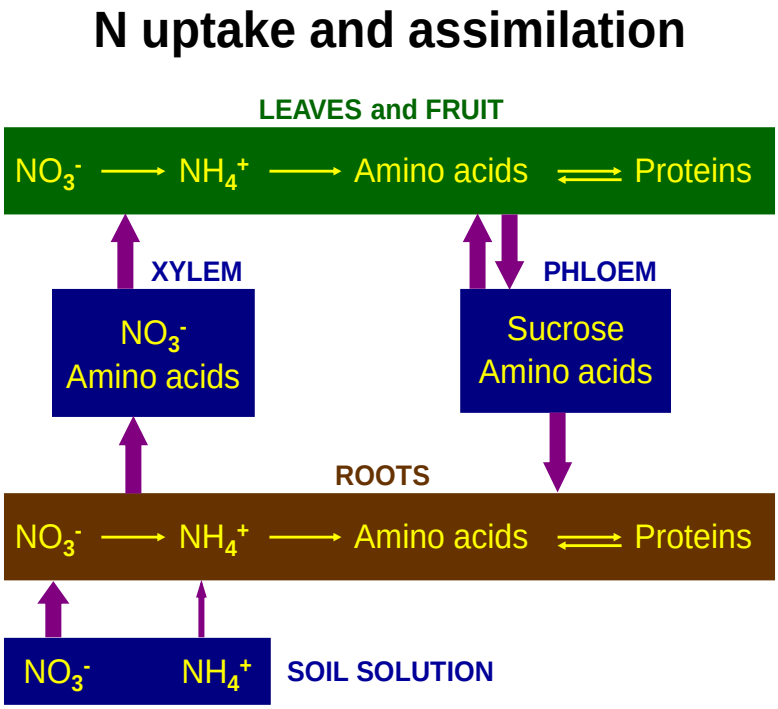
Wines made from fruit with adequate nitrogen generally have superior aroma and overall quality (Sinton et al 1978, Bell and Henschke 2005). While it is a universal New World practice to add supplemental nitrogen to the fermentor, there is ample evidence to suggest that such additions fail to enhance the fermentation as much as natural, grape-produced nitrogen (Sinton et al 1978, Treeby et al. 1996).

Nitrogen is the mineral nutrient for which the plant has the highest demand and which most limits growth. As outlined by Keller (2012) nitrogen in the plant is essential for the following:

- Nucleic acids > DNA, genes
- Amino acids> proteins, enzymes
- Chlorophyll > light interception
- Hormones > communication
- Secondary metabolites> aroma, flavor and phenolic compounds including color

Although N makes up 80% of the atmosphere grapevines cannot directly use N_2. Additionally, nitrogen is not contained in the mineral component of the soil. Nitrogen is released during the breakdown of organic matter in the soil. Vines rely on the uptake by roots, mostly in the form of nitrate (NO_3^-) dissolved in the soil. Nitrate ions are reduced by the nitrate reductase system to ammonium (NH_4^+), transported and stored as amino acids (Sponholz, 1991).

Bruce Zoecklein
Ken Fugelsang
Barry Bump



(Source Keller, 2013)

Soil nitrate levels fluctuate base on the composition of the organic matter, soil temperature, moisture and microorganisms. A high carbon to nitrogen ratio (greater than 20:1 favors the uptake of nitrogen by microorganisms. This process, known as mineralization, lowers the available nitrate pool in the soil. A low C/N ratio (less than 20:1) aids in the release of nitrate from the organic matter and increase the available nitrate in the soil. As such, the nitrate level available for the plant is from the balance between mineralization and nitrification and is influence naturally by fertilization practices.

Even in soils with limited nitrogen concentrations there can be large differences in the amount of N taken up by the vine due to soil type and composition, depth, moisture microbiological content, etc. Shallow soils are often reported to be superior in wine potential than deeper soils, due to lower water holding capacity and possibly lower N, both contributing to a reduction in vigor. Availability increases with the organic content

and organic turnover. Organic matter turnover is increased under the following conditions (van Leeuwen 2013):

- Low carbon/nitrogen ratio
- High soil pH
- Low temperature
- High soil moisture

Nitrogen and Red vs. White Grapes

Many calculate an annual nitrogen budget for their vines. While grape composition varies, it is understood that approximately 4 pounds of nitrogen are removed with each ton of fruit harvested (Greenspan 2014). As such, a 5 ton crop would require approximately 20 pounds of replace N per year.

Nitrogen status of the vine is most commonly evaluated by bloom-time petiole analysis. Generally, 0.9 to 1.1 percent total N is what is desired (Greenspan 2014). An important concept is that the plant N status is a *terroir* feature that can influence fruit composition and therefore wine composition.

Red grape potential for quality wines has been correlated to vine nitrogen status, particularly when water is not limiting (van Leeuwen 2013). Low vine N reduces vine vigor in general and increases tannins and anthocyanins (Chone et al. 2001). Thus, in many respects red grape quality is increased by limited vine N status (van Leeuwen et al 2013). Vegitatively vigours varieties can need less nitrogen and more moisture stress to attain vine balance (Gladstone 2011).

In white grape production, the considerations for the N status of the vine may be quite different. In whites, low nitrogen reduces the concentration of important aroma/flavor precursors. Additionally, low N can produce a low concentration of glutathione, an important antioxidant in white wine production (See Enology Notes Index for a discussion of glutathione).

Keller (2012) listed some important regarding controlled nitrogen levels commonly referred as regulated deficit nutrition (RDN). He also notes that post-veraison berries remain responsive to N:

- Berry size ⇘ (unless fruit set ⇘)
- Sugar (⇗-⇘)
- Malate ⇘ (less production)
- Tartrate (?)
- K^+ ⇘
- pH ⇗ (?)
- Amino acids (arginine) ⇘
- Phenolics (anthocyanins, flavonols...) ⇗
- Flavors (⇗-⇘)

Nitrogen and ATA (atypical aging)

ATA or untypical ageing (UTA), known as *untypischer Alterungsnote* in Germany, where it was first documented in the late 1980s, is a term used to identify a phenomenon found in white grapes in winegrowing regions world-wide. Heat and dry conditions immediately before and after véraison resulting in extreme moisture stress can lead to the development of this aroma/flavor defect. Wines from hot and dry growing seasons and sites are more prone to developing ATA. Additionally, vine nitrogen deficiency may also be contributing factor (Henick-Kling, 2008). Affected wines lose varietal character very early, develop atypical aromas and flavors described as naphthalene (moth balls), wet towel, or old furniture varnish, and may show an increase in bitterness. These characteristics are not the same as premature aging where some varietal typicity remains. Methods to minimize the development of ATA include avoiding extreme moisture stress around véraison, adequate plant nitrogen and avoiding over-cropping which could delay maturity. Ascorbic acid additions (100 – 150 mg/L) in conjunction with proper sulfur dioxide levels in the wine may help to limit the extent of this oxidative phenomenon.

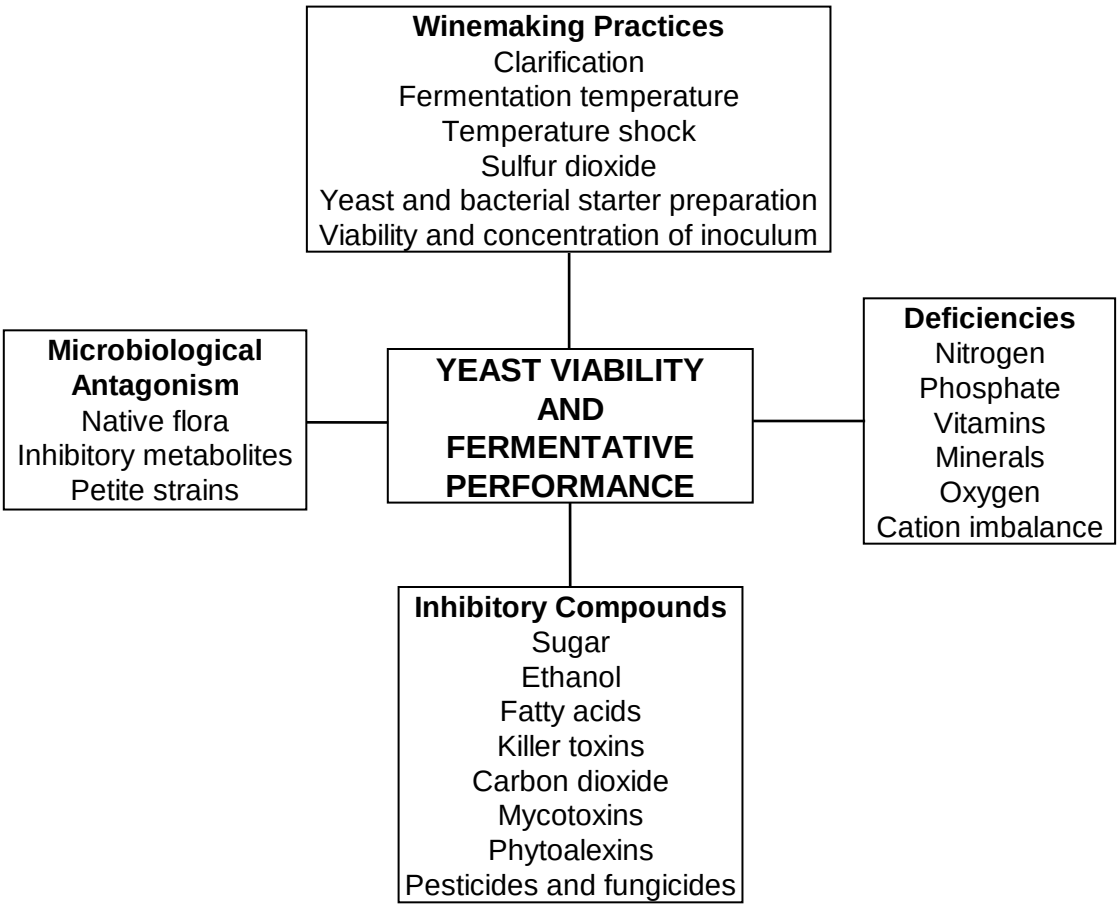
Yeast Assimilable Nitrogen (YAN)

Bruce Zoecklein
Ken Fugelsang
Barry Bump

The physical and chemical environment during grape juice fermentation, coupled with competition from indigenous yeast and bacteria, can present significant challenges to the growth of *Saccharomyces cerevisiae*, the typical wine yeast. These environmental factors may impact yeast growth, the conversion rate of sugar to alcohol, the production of off-odors, and reduction in varietal aroma intensity. Table wines containing biologically-available levels of sugar (>0.2% w/v glucose + fructose) risk undergoing spontaneous post-bottling re-yeast fermentation.

Although the underlying causes of fermentation difficulties may vary (see Figure 1), failure ultimately results from diminished, and eventually blocked, capacity of the yeast cell membrane to transport glucose and fructose into the cell (Lagunas, 1993). Fermentative growth, coupled with decreasing availability of critical nutrients, and increasing concentrations of ethanol and other inhibitory metabolites, may significantly alter membrane fluidity, thereby reducing the activity of glucose and fructose.

Figure 1. Environmental and Processing Factors Influencing Viability and Fermentative Performance of Wine Yeasts (Gump et al., 2001)



Yeast assimilable nitrogen concentration (YAN) is one of the underlying causes of problem fermentations. However, as suggested by Figure 1, there are many factors that may interactively contribute to protracted and/or interrupted fermentation.

Nitrogen compounds in grapes play important roles as nutrients for microorganisms involved in winemaking and wine spoilage, and as aroma substances and precursors (Swiegers et al., 2005). Nitrogen, taken up by the vine roots as nitrate, is reduced by the nitrate reductase system to ammonia, transported, and stored as amino acids (Sponholz, 1991). Total nitrogen ranges from 0.006 to 0.24%, of which only 0.0021-0.08% is biologically available to fermenting yeasts.

Assuming other factors are not limiting, the fermentation rate (conversion of sugars to alcohol and carbon dioxide) is directly related to yeast concentration (biomass).

- Yeasts follow a classic growth profile, beginning with a lag phase, followed by a period of rapid growth, culminating in a stationary phase where the population density remains relatively high.
- Yeasts in the stationary phase are responsible for the majority of alcoholic fermentation.
- Depletion of carbon and nitrogen, coupled with accumulation of toxic metabolites, leads to the cell death and decline phase.

During the stationary phase of growth, nitrogen uptake and utilization are directed toward cell maintenance. For example, transporter proteins have a high turnover rate during this stage of growth, and thus require continued re-synthesis (Lafon-Lafourcade and Ribéreau-Gayon, 1984).

Nitrogen may become an important growth-limiting constraint for microorganisms. The nitrogen content of juice and wine is made up of protein and non-protein fractions. Protein nitrogen comprises 1-13% of the total N (Correa et al., 1988). Polypeptides may account for more than 21% of proteins.

Although some native, non-*Saccharomyces* yeasts and bacteria are capable of producing proteases (Lagace and Bisson, 1990), *Saccharomyces* spp. lack both the extracellular proteases and transport enzymes necessary for incorporation (Rosi et al., 1987) and, thus, neither fraction plays a significant nutritional role in the growth cycle. Whether native non-*Saccharomyces* species have sufficient enzyme activity to yield utilizable forms of nitrogen is not clear. The nitrogenous components of grapes and juice that are metabolically available to yeasts include organic (amino) acids and inorganic ammonium salts (NH_4^+).

- Ammonium nitrogen and the utilizable amino acids (free *alpha*-amino acids, or FAN) are referred to as YAN (yeast assimilable nitrogen).
- YAN analysis, therefore, requires measurement of ammonium nitrogen and the utilizable amino acids.
- In grapes, the YAN ranges from near 30 to more than 400 mg/L.
- Ammonia is used by yeasts prior to amino acids.
- The minimum level of YAN required for successful completion of alcoholic fermentation is 120-140 mg/L, for musts with sugar concentrations of up to 22°Brix.
- A low YAN level may signal a low level of the micronutrients that are needed for yeast growth.
- The presence of NH_4^+ delays both the timing and extent of amino acid incorporation into the yeast cell.
- NH_4^+ is not only incorporated preferentially to *alpha*-amino acids, but also alters the established pattern of amino acid uptake (Jiranek et al., 1990).
- Timing of nitrogen supplementation (and the form of supplement) may play a crucial role in successful completion of alcoholic fermentation and volatiles produced.

FAN Nitrogen

All of the 20 commonly occurring *alpha*-amino acids are found in grapes and wine. Their total concentration is 0.4-6.5 g/L (Wurdig and Woller, 1989). The important directly-assimilable primary or free *alpha*-amino acids (FAN) fraction, as demonstrated by their uptake coinciding with cell growth, includes the following: arginine, glutamate, valine, iso-leucine, leucine, histidine, aspartate, tryptophan, phenylalanine and methionine.

Of the FAN amino acids, arginine is typically present at levels 5-10 times that of the other amino acids, and represents 30-50% of the total nitrogen utilization (Henschke and Jiranek, 1993). During growth, yeast utilize 1-2 g/L amino acids (Dittrich, 1987). Unlike bacteria, *Saccharomyces* can accumulate large intracellular concentrations of amino acids.

Depending upon the particular amino acid, the yeast's stage of growth, and the presence and activity of necessary transport enzymes, amino acids may be directly incorporated into proteins, degraded for either their nitrogen or carbon components, or stored in vacuoles for later utilization (Fugelsang, 1996).

Amino acid uptake by *Saccharomyces cerevisiae* requires two amino acid transport systems (Henschke and Jiranek, 1993). In the absence of NH_4^+, the first group of amino acids incorporated includes arginine, asparagine, aspartic acid, glutamine, serine, threonine, methionine, isoleucine, leucine, and lysine.

Not all amino acids are directly utilizable by yeast during fermentation. Proline is present in relatively high concentrations (700-800 mg/L), but is not biologically available to *Saccharomyces* spp. before or during alcoholic fermentation. Other amino acids that cannot be directly utilized as exclusive nitrogen sources include lysine, cysteine, histidine, and glycine (Cooper, 1982; Large, 1986).

Non-*Saccharomyces* yeasts are capable of utilizing lysine as a sole carbon source, thus making the technique a useful tool for identification in mixed cultures.

Bruce Zoecklein
Ken Fugelsang
Barry Bump

Bruce Zoecklein
Ken Fugelsang
Barry Bump

UNDERSTANDING AND MANAGING THE NUTRITIONAL STATUS OF JUICE AND WINE

Section 2.

Practical Considerations Influencing YAN

Fermentation anomalies may arise from numerous sources shown in Figure 1, including the following:

- deficiencies in the fruit
- inhibitory compounds
- microbiological antagonism
- winemaking practices

Factors may act individually or collectively to impact fermentations. Most grape growing and winemaking decisions can influence the relative proportion of the FAN/NH_4^+ fraction and total YAN, including the following:

- climate
- season
- grape variety
- rootstock selection
- soil type
- soil moisture
- irrigation practices
- cover crops, mulch
- fertilization
- vine diseases
- nutrient and/or mineral deficiencies
- maturity
- processing methodology

Vineyard Considerations

(See section titled plant nitrogen) Fermentation problems are often vineyard-specific. Nitrogen deficiency in apparently healthy grapes can be severe.

- Drought, grapevine nutrient deficiencies, high incidences of fungal degradation, and level of fruit maturity all influence must nitrogen and vitamins.
- Cultivar, rootstock, crop load, and growing season may also influence must nitrogen.
- Some varietals have a greater tendency towards nitrogen deficiency.
- Higher total nitrogen may also be associated with certain rootstocks. For example, grapes grown on St. George are higher in total nitrogen than those on AXR1.

Nitrogen application in the vineyard can increase the concentration of major nitrogenous compounds, such as total amino acids, arginine, proline, ammonium, and total nitrogen. Nitrogen application in the vineyard should be considered in the context all other factors including, vine vigor, canopy density, leaf color, and petiole nitrogen analysis, etc.

“Macro” tuning of berry nitrogen status can be achieved in the vineyard primarily in terms of the amount and composition of the potential juice YAN, given genetic constraints. At low nitrogen sites, this can be achieved by the judicious application of nitrogen supplements such as inorganic and, to a lesser extent, organic nitrogen supplements. Maintenance of adequate nitrogen status at moderate nitrogen sites can be achieved in the same way, but nitrogen application should be avoided at sites that are high in nitrogen.

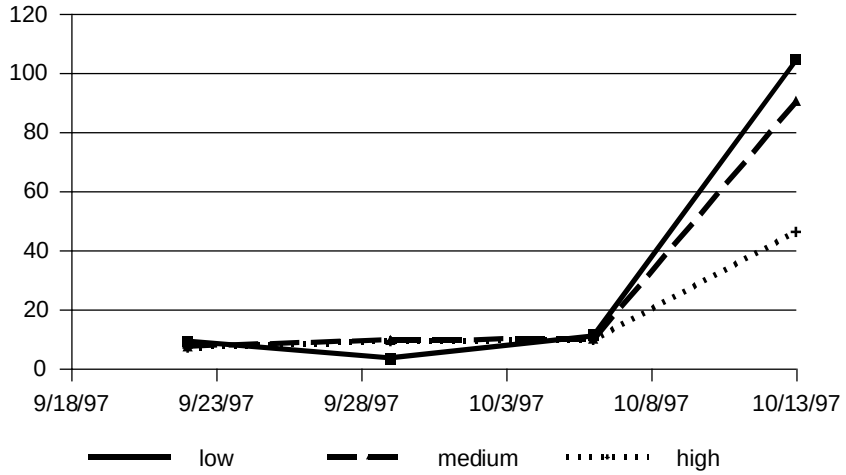
Arginine and proline are the main amino acids in the fruit if vine fertilization is low. With higher nitrogen fertilization (> 3g N/plant), the amino acid, glutamine, increases dramatically (Sponholz, 1991). Therefore, nitrogen available for yeast fermentation can be totally different among the wine-growing regions (Sponholz, 1991).

Grape maturity is an important issue influencing the concentration of YAN, in that under- and over-ripe fruit may be low in nitrogen (Dittrich, 1987). Butzke (1998) evaluated the yeast assimilable nitrogen status of *Vitis vinifera* musts from the western U.S. in 1996 and reported the following:

- YAN 40-559 mg N/L, average 213 mg N/L
- FAN 29-370 mg N/L, average 135 mg N/L
- NH_4^+ 5-325 mg N/L, average 70 mg N

As seen in Figure 2, the concentration of *alpha*-amino nitrogen in Cabernet Sauvignon grapes changes as a function of maturity and crop load.

Figure 2. *Alpha*-amino nitrogen (mM) in Cabernet Sauvignon grape clusters thinned to low (2.6 kg/vine), medium (4.9 kg/vine) and high (5.3 kg/vine) crop level in 1997 (de Bordenave and Zoecklein, 1999)



Henick-Kling et al. (1996) compared the concentrations of the two important sources of YAN (FAN and NH_4^+) among six New York-grown cultivars at harvest over two seasons (Table 1).

Table 1. Survey results from 1993 and 1994, showing mean content of free ammonia and free amino nitrogen (less free ammonia) (Henick-Kling et al., 1996)

	Free Ammonia (mg/L)		Free Amino Nitrogen (mg/L)	
Cultivar	**1993**	**1994**	**1993**	**1994**
Cayuga White	68	32	74	197
Chardonnay	46	55	151	177
Riesling	52	56	102	123
Seyval blanc	19	14	82	156
Pinot noir	52	88	135	116
Cabernet Sauvignon	49	69	74	142
Mean (all cultivars)	**48**	**52**	**103**	**152**

This study illustrated large variations from one season to the next, in both free ammonia and *alpha*-amino nitrogen, and significant differences in the concentration of both sources of nitrogen among cultivars.

Fruit Rot and Nitrogen

Microbiological deterioration of fruit can influence fruit FAN concentrations (Dittrich, 1987). Growth of *Botrytis* can consume 41% of the total amino acid concentration in the fruit (Sponholz, 1991). Mold growth on fruit is also known to cause fermentation problems due to formation of inhibitory metabolites (Dittrich, 1987; Donèche, 1993). Several mycotoxins, including ochratoxin, patulin, and botryticine, may also be present on mold-damaged fruit.

Botrytis cinerea produces botryticine, a heteropolysaccharide known to have an impact not only on the growth and fermentative properties of *Saccharomyces*, but on post-fermentation clarification and stability, as well (Dubourdieu et al., 1978). Botryticine is reported to stimulate *Saccharomyces* spp. to produce high and inhibitory levels of acetic acid at the onset and during the latter stages of alcoholic fermentation (Donèche, 1993).

Aside from formation of mycotoxins, fruit from fungal-diseased vines may also contain inhibitory levels of phytoalexins, produced by the plant in response to the parasite (Smith and Banks, 1986). These may be inhibitory towards *Saccharomyces* spp. Native yeasts, particularly *Kloeckera apiculata,* are known to deplete important vitamins, such as thiamine.

Yeast Strains, Microbial Ecology, Nutrient Desert and YAN

The requirement among cultured strains of *Saccharomyces* spp can vary significantly and include the following:

- nitrogen requirements
- oxygen requirements
- time of uptake and release of specific amino acids during fermentation
- ability to ferment to dryness
- concentration of H_2S and other sulfur-like off odor compounds produced
- magnitude of response to environmental conditions

Yeasts and bacteria are part of a complex series of interactions where competition, equilibrium and collaboration form a dynamic ecosystem contributing to the concept of microbial ecology. Even with the addition of cultured yeasts and sulfur dioxide to a red

must, for example, a portion of a fermentation can be conducted by other, native organisms (Bokulich et al. 2012). There can be a substantial difference in microbial populations among different wines produced at the same facility with the same inoculated yeast. Microbial ecology can be a source of *terroir* variation. See Table 3 below for a list of common native yeast involved in winemaking.

After alcoholic fermentation finishes, the *S. cerevisiae* population decreases. If, by this stage, there is no carbon source and nutrient supplies are exhausted, there is a greater likelihood that the wine will be stable with regard to *Brettanomyces* growth. If these conditions are not met, an opportunity for Brett growth remains. Brett lacks the genetic capacity to synthesize many of the micronutrients required for growth, a reason why blooms or excessive growth can follow alcoholic fermentation. If there is an excess of YAN (yeast assimilable nitrogen) in the fruit or must, there will be an excess remaining in the wine post-alcoholic fermentation. This can help Brett, if present, to flourish.

Yeast Starter Population Density

Yeast populations should be large enough to overwhelm indigenous microflora. Starter preparation/inoculation at 1-3% (vol/vol) yields an active *Saccharomyces* population of 2 - 5 x 10^6 viable cells/mL of juice. This is generally equal to 24 grams dry yeast/hL (2 lbs/1000 gal). Increases in inoculation levels maybe applied when the Brix level is above 24, the pH is below 3.1, the temperature less than 13°C/55°F and/or the fruit rot increases the potential for completing microorganisms.

Yeast Starter Preparation

The water temperature used for rehydration is crucial to maximize viable cells. Viability and vigor decrease as rehydration temperatures vary above or below those recommended.

- The yeast should be added to the juice/must within 20 minutes of rehydration.
- Where temperature acclimation or longer periods of time between rehydration and inoculation are necessary, ‘clean’ (optimally sterile) unsulfited juice should be added to the starter to prevent depletion of carbon/nitrogen levels, leading to low viable cell density.

Temperature Shock

Significant yeast cell death occurs when temperature differentials between starter and juice/must are more than 5-7°C. Monk (1986) reported that the addition of rehydrated yeast (40°C /104°F) directly to a must at 15°C/60°F resulted in a 50% reduction in viable cell density. Temperature shock can lead to formation of mutant strains ("petites") which do not grow or ferment as well as the original strain.

Processing and YAN

Interest in pre-fermentation maceration and native fermentations (both yeasts and lactic acid bacteria) has led to increased concern regarding depletion of FAN and vitamins required by *Saccharomyces* spp.

- Native yeast and bacteria, present initially at relatively low population densities, require significant amounts of YAN and vitamins to build biomass.
- By the time *Saccharomyces* spp. populations become established, levels of available nitrogen may be too low for complete fermentation or optimize desirable aroma production.

Winemaking practices coupled with juice clarification may impact nutritionally important substrates (Guitart et al., 1998; Houtman and duPleissis, 1981). Important processing conditions that impact YAN include the following:

- whole cluster pressing vs. crush and drain
- saignée or bleeding (removal of a portion of the red juice after a short exposure to the skins) vs. non-dejuiced
- short vatting vs. extended post-fermentation maceration

Amino acids are not equally distributed in the grape berry. For example, with mature Cabernet Sauvignon, about 8.5% of the total is in the seeds, 15% in the skins, and 77% in the pulp. Separation of the pulp juice from the skins, as occurs with bleeding, has a significant qualitative influence and quantitative impact on FAN.

The two amino acids present in the greatest concentration in the fruit are usually proline and arginine. Proline cannot generally be used by the yeast, while arginine can. The amino acid arginine has four nitrogen molecules. Although Ingledew (1996) suggests

that arginine hydrolyzes providing two assimilable nitrogens to the yeast, there have been other studies that suggest the provision of three assimilable nitrogens. Either way, the amino acid arginine is an a very important FAN component.

In the case of Cabernet Sauvignon (and likely most other red varieties), the ratio of arginine to proline is much greater in the skins than the pulp. Pulp juice, which is removed during bleeding or saignée, has a relatively high concentration of proline (approximately 55%) which cannot be used by the yeast, and a small concentration of the more potent amino acid arginine and others needed to carry out a healthy fermentation. The lower incidence of incomplete fermentation in red, compared with white, musts supports the concept that the slow release of nitrogen from grape skins during fermentation is important.

- Wines produced by bleeding generally require a higher concentration of supplemental N due to the relatively high proline versus arginine in the pulp juice.
- Nitrogen concentrations in white juice can be reduced by 10-15% following cold settling.
- Fining agents may serve to further deplete vital nitrogen sources.

Guitart et al. (1998) evaluated several commonly utilized pre-fermentation fining agents, with regard to amino acid reduction. They reported that silica gel additions removed the highest concentration of amino acids, followed by enzyme treatment, cold clarification, bentonite, and centrifugation.

Where pre-fermentation bentonite additions were made, reductions in total nitrogen of over 50% can occur. Rapp (1977) reported 15-30% reduction in amino acids, following bentonite additions of 1 g/L. Therefore, those conducting fermentations in the presence of bentonite, as a means of aiding post-fermentation protein stability, should make nitrogen supplementations accordingly.

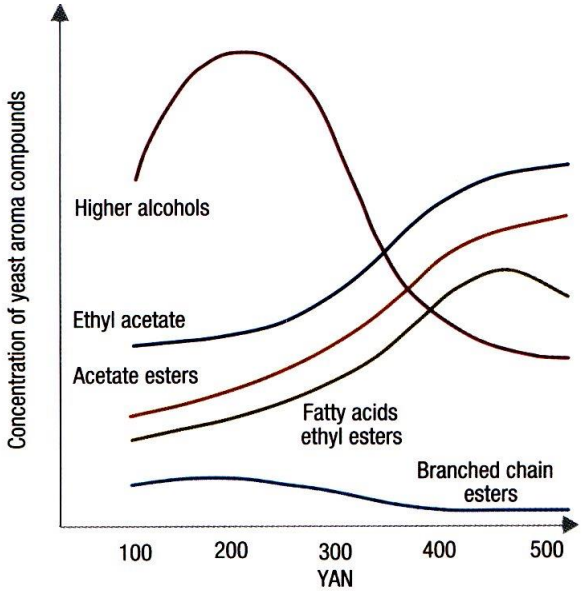
Section 3.

Nitrogen Impact on Aroma/Flavor Compounds

(See section titled plant nitrogen) Sugar metabolism by yeast results in the production of by-products which contribute to the aroma and flavor of a wine. Due to the importance of fermentation-derived volatiles in the aroma character of a wine, yeast metabolic pathways that are influenced by YAN levels are of particular interest and importance (Ugliano et al., 2007).

Various studies have demonstrated that the amount of available nitrogen can affect the production of different groups of volatile compounds. Figure 3 (Ugliano et al., 2007) shows a summary of general trends. These trends are conditional on yeast strain and fermentation conditions, but do show potential consequences of indiscriminate supplementation of a must particularly with DAP.

Figure 3. Relationship between initial YAN concentration and final concentration of volatile compounds after fermentation (Ugliano et al., 2007)



The following summarizes the relationships between YAN and aroma compounds:

- Higher alcohols have fusel oil-like characters that may contribute to complexity, but may also mask fruity character at higher levels.
- Fatty acid ethyl and acetate esters generally contribute to fruity character.
- High levels of ethyl acetate can give nail-polish-remover or solvent characters.
- Branched chain esters can be significant contributors to the red berry fruit character of some red wines.
- Assimilable nitrogen availability can contribute significantly to wine aroma and flavor, and, as a result, supplementation should be done with caution after having determined YAN concentrations.

Nutrient and Supplement Addition Products

Although some have suggested that a minimum of 120-140 mg/L yeast assimilable nitrogen is required by yeasts, others recommend 250 mg N/L, or more. Nutritional supplementation may be carried out using either the directly available ammonium salt, diammonium phosphate (DAP, 25.8% ammonia, 74.2% phosphate), or proprietary blends containing amino acids, minerals, and vitamins. In the U.S., the legal limit of DAP is 960 mg/L, which corresponds to 203 mg N/L.

Most advocate the use of balanced nutritional formulations, in addition to or in lieu of DAP. In addition to a readily-available form of nitrogen, many propriety products may

contain amino acids, minerals, vitamins, and/or other ingredients important for yeast growth, including some or all of the following:

- inorganic N (DAP)
- organic N (*alpha*-amino acids)
- unsaturated fatty acids
- sterols, thiamine, folic acid, niacin, biotin, and calcium pantothenate
- magnesium sulfate
- inactive yeast cell walls
- peptides
- micro-crystalline cellulose
- other yeast autolysis products

Inactivated yeast is whole yeast cells that have been killed by heat. It contains the cell wall, cell membrane, and the entire cellular contents of the yeast.

Yeast autolysates are the whole yeast cell that has been killed and then exposed to glucanase enzymes at 45°C for a certain time period. The result is that the cell wall, that contains glucans, is partially degraded, and the cell membrane and the “soluble inside” of the yeast are more exposed.

Yeast hulls/ghosts are the insoluble yeast cell wall fraction of yeast autolysate after centrifugation. Depending on the washing process used during the manufacturing, they may or may not contain parts of the cell membrane.

Yeast extract is the supernatant of yeast autolysate, or the soluble insides of yeast cells once the insoluble cell walls and cell membranes have been removed by centrifugation.

Specific yeast fractions usually contain mannoproteins. Mannoproteins are a specific cell wall constituent, and production thereof requires further processing of yeast cell walls.

Yeast hulls can have very good adsorbing capacities, depending on how they were produced. Their main role during fermentation is to bind to toxic medium-chain fatty acids secreted by the fermenting yeasts, thereby detoxifying the environment and allowing the fermenting yeast to ferment to dryness. If yeast cell walls contain parts of the cell membrane, they can also be a source of sterols and lipids.

Yeast cell walls may be added to enhance fermentation rates, as a source of nutrients, and to restart stuck fermentations.

- Yeast hulls stimulate yeast populations by providing a source of C_{16} and C_{18} unsaturated fatty acids, which act as oxygen substitutes under long-term fermentative conditions (Ingledew, 1996).
- Hulls may provide a source for some amino acids, as well as surface area to facilitate release of potentially inhibitory levels of CO_2.
- Because yeast hull preparations contain lipids (fats) that oxidize upon exposure to oxygen, they may degrade and develop a “rancid” character upon extended storage. They should be evaluated sensorilly prior to each use.

There are inactivated yeast-based products recommended for enhancing white wine longevity. These are not really nutrients, but rather are a source of glutathione. Glutathione is a thiol (sulfur-containing organic compound) that has antioxidative capacities. It is normally recommended for white wines made from grape varieties that contain volatile thiols such as Petite Manseng, Sauvignon blanc, etc. Such addition products are made from inactivated yeast that was glutathione-enriched during its production process.

Yeast-derived mouthfeel-enhancing products are usually specific yeast fractions (such as mannoproteins) that have mouthfeel enhancing capabilities. These products also do not serve as yeast nutrients.

Table 2. Some Traditional Yeast Nutrient YAN Contribution Products

NUTRIENT	DOSE 25 g/hL (2 lb/1000 gal)	DOSE 30 g/hL (2.5 lb/1000 gal)	YAN SOURCE
DAP	50 mg N/L	60 mg N/L	Inorganic nitrogen
FERMAID K	25 mg N/L	*Exceeds TTB limits of thiamin	Inorganic nitrogen (from DAP) and organic nitrogen from autolyzed yeast
FERMAID D	10 mg N/L	12 mg N/L	Organic nitrogen from autolyaed yeast
GO-FERM	7.5 mg N/L	10 mg N/L	Organic nitrogen from

			autolyzed yeast
GO-FERM PROTECT	7.5 mg N/L	10 mg N/L	Organic nitrogen from autolyzed yeast
NUTRIENT VIT END	7 mg N/L	8.5 mg N/L	Organic nitrogen from autolyzed yeast
SIY 33 (FERMAID 2133)	8 mg N/L	10 mg N/L	Organic nitrogen from autolyzed yeast

*Alcohol and Tobacco Tax and Trade Bureau (TTB) limits thiamin addition to wine or juice to 0.60 mg/L (0.005 lb/1000 gal): 21 CFR 184.1875.

Because some of the ingredients in these formulations may not be currently approved for use in the United States, winemakers must petition the U.S. Alcohol and Tobacco Tax and Trade Bureau under section 27 CFR 24.250 to use material not specifically authorized.

Timing of Nutrient Additions

There are several steps during the winemaking process where nutrient addition may be appropriate, including the following:

- during yeast rehydration
- at yeast inoculation
- during the first third of fermentation
- possibly if sulfur-like off odors are detected
- when re-inoculating stuck wines

Amino acids are not incorporated equally by yeast, and preferences may vary significantly among yeast strains (Manginot et al., 1998). Some are utilized at the beginning of the growth cycle, some later, and some not at all.

Ammonia, on the other hand, is consumed preferentially to amino acids in growing populations. Stationary phase yeast also vary significantly in terms of the order of amino acid incorporation (Manginot et al., 1998).

- Timing of nutrient additions is important.
- A single addition of DAP at the beginning, while convenient, may encourage the growth of native non-*Saccharomyces* species, as well as potentially leading to an excessive fermentation rate and an imbalance in the uptake and usage of amino acids.

- Additions at 16°Brix and 10°Brix are preferred. Where only a single addition is planned, research has shown that supplementation midway through fermentation was as effective as a single addition at the start, while avoiding the problems noted above (Sablayrolles and Dubois, 1996).

- Addition of ammonium (DAP) late in fermentation should be avoided, since this compound is not consumed by *Saccharomyces* at this stage of fermentation (Beltran et al., 2005). Furthermore, excessive ammonium in a wine may be enough to support the growth of spoilage microorganisms, including *Brettanomyces* spp., after fermentation is complete.

While addition of ammonium salts may not significantly benefit stationary-phase yeast (Lafon-Lafourcade and Ribéreau-Gayon, 1984), the addition of specific amino acids may have a stimulatory effect and extend fermentative activity (Manginot and Sablayrolles, 1997). Single amino acids may be quickly utilized to re-synthesize transporter proteins that are rapidly "turned over" during accelerated growth.

- Supplements added after about half the fermentation is complete may not be used by the yeast, because alcohol prevents their uptake.
- Simply adding nutrients to a stuck fermentation is seldom effective.

With increasing ethanol concentrations, the permeability of the cell membrane to hydrogen ions increases. This requires intracellular enzymes and ATPases to pump protons back out of the cell, in order to balance the internal pH of the yeast cell against the external pH of the juice/must.

Due to the competing nature of these coupled transport systems, nitrogen is picked up by the cell only in the early stages of fermentation, when it is stored in vacuoles and used upon demand. Nitrogen added late in the fermentation cannot be transported into the cell (Bisson, 1996). Once stopped due to nutrient stress, the fermentation may require significant effort to complete.

Vitamin Addition

Nutritional stress arising from deficiencies in YAN may be exacerbated by vitamin deficiency. Most species/strains of yeasts require various vitamins, such as thiamine, riboflavin, pantothenic acid, pyridoxine, nicotinamide, biotin, and inositol, depending on species and specific growing conditions (Monk, 1994; Ough et al., 1989). Most strains of

Saccharomyces require biotin and pantothenic acid, while some also need inositol and/or thiamine (Walker, 1998).

Vitamin deficiency may be a particular concern in cases where microbial growth on the fruit has occurred prior to harvest and/or to the onset of the *Saccharomyces* growth phase in native or un-inoculated fermentations.

- Growth of *Kloeckera apiculata* (a common yeast species found on grapes) has been reported to rapidly reduce thiamine levels below those required by *Saccharomyces* spp. (Bataillon and Rico, 1996).
- Addition of SO_2 may lead to reductions in levels of thiamine (Lafon-Lafourcade and Ribéreau-Gayon, 1984).

Vitamin supplementation has been demonstrated to exert a stimulatory effect on yeast growth during fermentation (Fleet and Heard, 1993). The use of "balanced" formulations (that include vitamins), versus DAP by itself, may be justified.

Oxygen/SO_2

Oxygen demands vary considerably among commercial wine yeasts (Julien et al., 2000). Although not directly stimulatory to fermentation, oxygen is required by yeasts for synthesis of cell membrane precursors, including ergosterol. Collectively, these are referred to as "survival factors." Without initial exposure to oxygen, yeast replication is usually restricted to four to five generations, as each budding cycle reduces the sterol content of the membrane by approximately half.

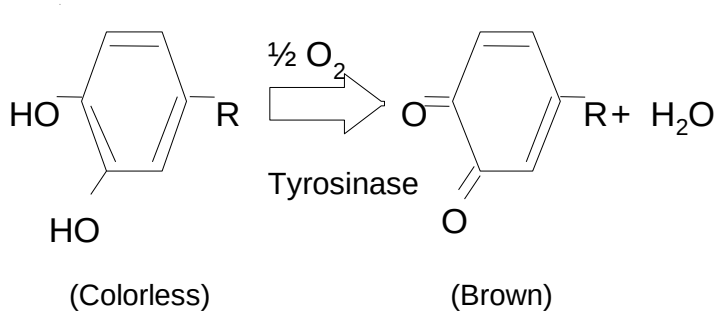
- When the sterol level reaches a critical low level, replication stops and fermentation must continue with the population present at that point.
- The grape itself may supply at least a portion of the lipids needed by yeast during anaerobic growth. Up to two-thirds of the cuticle waxes in some grape varieties are composed of oleanolic acid. This fatty acid has been found to replace the yeast's requirement for ergosterol supplementation under anaerobic conditions (Brechot et al., 1971).
- Pomace contact, either prior to pressing in white wine production, or extended during red wine fermentation, extracts lipids and other essential components from the grape cuticle.

Aeration of yeast starters plays an important role in subsequent fermentative performance. Wahlstrom and Fugelsang (1988) reported increased cell density and

more rapid fermentations when aerated starters were used, compared with non-aerated starters.

In the absence of sulfur dioxide, grape-derived oxidative enzymes (tyrosinases) catalyze conversion of nonflavonoid phenols to their corresponding quinones (see Figure 4).

Figure 4. Enzymatic Oxidation of Nonflavonoid Phenols (Zoecklein et al., 1999)



- The reaction brings about rapid (but reversible) browning of the juice, while consuming oxygen required by yeasts during the early stages of growth.
- Grape tyrosinase is easily inactivated by addition of SO_2 to the juice/must. However, excessive sulfur dioxide addition (greater than 70 mg/L) may inactivate important B-complex vitamins including thiamine.
- If additions of more than 70 mg/L SO_2 are made, thiamine (in the form of nutritional supplements) should be added to the fermentor.

Bruce Zoecklein
Ken Fugelsang
Barry Bump

UNDERSTANDING AND MANAGING THE NUTRITIONAL STATUS OF JUICE AND WINE

Section 4.

Hydrogen Ion Concentration (pH)

Yeast growth occurs over the pH range from 2.8 to 8.0 (Fleet and Heard, 1993). However, yeast do not function equally well throughout this wide range. Biomass is produced best above pH 4.0, and slows as pH goes down.

- Low pH reduces the tolerance of *Saccharomyces* spp. to ethanol.
- As pH drops below 3.2, the increase in H^+ raises the risk of premature arrest of fermentation.
- The added stress placed on yeast at lower pHs is compounded by low nutrient concentrations, temperature extremes, high sugar, and/or high alcohol.
- Highly chaptalized juice (that to which a large amount of sugar has been added to increase later ethanol levels) may have a limited buffering capacity. As a result, the organic acid and CO_2 production during the initial stage of fermentation can drop the pH.
- Juice/musts with pH <3.1 should receive an increased yeast inoculum from 3% v/v to approximately 5%.

Additionally, Kudo et al. (1998) demonstrated a relationship between the concentrations of K^+ and H^+, and the completion of alcoholic fermentation. They suggested that a minimum K^+/ H^+ ratio of 25:1 is required.

Nonsoluble Solids

Nonsoluble grape solids serve as nutritionally-important substrates and as oxygen reservoirs during the early stages of fermentation. Additionally, solids hold yeasts (native and inoculated strains) in suspension during the early stages of fermentation, and before

the evolution of large amounts of carbon dioxide. In general, yeasts do not grow well in highly-clarified musts, leading to increased likelihood of slow or stuck fermentations.

Conventional white juice processing calls for some level of suspended solids reduction prior to inoculation. This is an important stylistic tool. The clarity of juice is measured in nephelos turbidity units (NTUs). The range goes from about 350 down to below 100. However, reduction below this level (0.5%) can result in nutrient deficiencies and promote premature sedimentation of yeast.

Guitart et al. (1998) evaluated several commonly-utilized pre-fermentation fining agents with regard to amino acid reduction, and reported that silica gel additions removed the highest concentration of amino acids, followed by enzyme treatment, cold clarification, bentonite, and centrifugation. Since bentonite additions can also reduce must nitrogen, they should be done in conjunction with supplemental nutrient additions.

Aside from its impact on available nitrogen, over-clarification may also reduce sterols which are essential for yeast cell membrane integrity. Changes in the fatty acid composition of musts have also been reported (Varela et al., 1999).

Fermentation Temperature

Fermentation rates vary with temperature. The integrity and operation of the cell membranes are impacted when yeasts are grown at either the upper or lower limits of the recommended temperature range.

- Growth at higher temperature brings about inactivation/denaturation of cell membrane-associated transporter proteins and other enzymes.
- Lower temperatures compromise fluidity/pliability, thus impeding movement of substrate across the membrane (Stanier et al., 1976).
- Cell membrane function is also affected by the presence of increasing concentrations of ethanol, which accumulates faster at higher fermentation temperatures, thereby narrowing the temperature tolerance range.
- For low (<10°C/50°F) temperature fermentations, increased inoculum levels and nutrient additions are recommended.
- Within their growth range, temperature also affects the population balance between *Saccharomyces* and non-*Saccharomyces* yeasts. In red wine fermentations (20°C/68°F to 30°C/86°F), *Saccharomyces cerevisiae* usually represents the dominant species (Sharf and Margalith, 1983). At lower

fermentation temperatures, such as those used in white wine production, non-*Saccharomyces* yeasts can proliferate at the expense of desired wine strains.

CO_2 Toxicity

Carbon dioxide, at concentrations up to 0.2 atm, stimulates yeast growth. Above this level, carbon dioxide becomes inhibitory. Pekur et al. (1981) reported that, at increased pressures, carbon dioxide reduces the yeast's uptake of amino acids. (This is one of the reasons why pressure can be used to produce a muté-that is juice prevented from fermentation and used for blending). Agitation can be used to help prevent supersaturation with CO_2. Some yeast nutrient supplements contain particulates, such as micro-crystalline cellulose, to help change the carbon dioxide equilibrium and lower the dissolved carbon dioxide.

The role of carbon dioxide in fermentation has gained importance and is the reason why many stir tanks and barrels during fermentation. Additionally, newer tank configurations, such as egg shaped vessels, are designed to use convection currents to help mix the tank during fermentation, helping to lower the carbon dioxide concentration.

Sugar Toxicity

Increased osmotic pressure associated with high sugar concentrations can also inhibit yeast growth.

- Although *Saccharomyces* spp. are among the most tolerant species to high sugar concentrations, such environments are often nitrogen deficient.
- High Brix fermentations can begin slowly, and may stick prior to completion. In Brix ranges from 25 to 30°Brix, yeast starters should be prepared at greater concentrations, or more than 24 g/hL (2 lbs/1000 gal) or 5 x 10^6 yeast cells/mL.
- For >30°Brix musts, an addition of more than 24 g/hL .
- Ice wines, and some late harvest wines, require substantially more yeast inoculum, up to 20 x 10^6 yeast/mL (Cone, personal communication, 1996).
- High sugar musts require additional concentrations of YAN

Glucose/Fructose

Grape juice usually contains approximately equivalent concentrations of glucose and fructose sugars, depending on the variety and maturity. However, glucose is fermented

preferentially to fructose. Stress can affect the yeast's ability to metabolize the last residual fructose.

This problem seems to occur more with the *S. bayanus* strains, which are more glucophilic and, therefore, unable to ferment fructose as efficiently as glucose (Schultz and Gafner, 1993). Thus, fructose syrup would be the last choice among winemakers making a selection of chaptalization/amelioration agents.

Alcohol Toxicity

Alcohol and its precursor, acetaldehyde, are toxic to all yeasts, including *Saccharomyces* spp. Alcohol has a profound effect on all aspects of yeast metabolism, ranging from membrane integrity, to nitrogen uptake and sugar transport. There are a number of environmental factors that act in synergy with alcohol to inhibit yeast growth, including low pH, high temperature, acetic acid, sugar, short-chain fatty acids, nitrogen depletion, and deficiency of sterols and vitamins.

Acetaldehyde has also been reported to play a significant inhibitory role in survival of *Saccharomyces* spp. during fermentation (Stanley and Douglas, 1993), and may increase yeast sensitivity to increasing concentrations of ethanol (Jones, 1989).

- Light aeration during the growth phase stimulates synthesis of cell membrane precursors, which help maintain cell integrity.
- During fermentation, nitrogen supplementation may be helpful in mitigating the antagonistic affects of alcohol.

Native Yeast/Bacterial Fermentations

Usually, non-*Saccharomyces* species from the vineyard, and winery-associated *Saccharomyces* spp., dominate the initial and early stages of fermentation of un-inoculated musts. Their growth may result in significant depletion of nitrogen and vitamins, such as thiamine. Various indigenous yeast found on grapes are listed in Table 3. Several of these, and most notably *Torulospora* spp., are being used to co-ferment with traditional wine yeast.

Among vineyard-related native species, *Kloeckera/Hanseniaspora* is typically found at highest population densities. *Kloeckera* spp. are tolerant of both low temperature and the presence of sulfur dioxide and, thus, may have a selective advantage during cold soak (reds) or extended clarification of white must.

- Rapid proliferation of *Kloeckera* spp. can result in high levels of ethyl acetate prior to onset of alcoholic fermentation. Additionally, uncontrolled growth may also deplete nutrients to levels insufficient to support *Saccharomyces* growth.
- Acetic and lactic acid bacteria, in addition to native yeast, can produce potent inhibitors, as well as depleting must nitrogen and vitamin levels. Acetic acid is a strong inhibitor of *Saccharomyces* spp., especially when combined with other antagonistic factors, like high alcohol.

Table 3. Yeast Species used in Association with *S. cerevisiae* for Fermentation

Species	Purpose	References
Candida canterellii	Increase glycerol content	Toro and Vasquez (2002)
C. pulcherrima	Aromatic modulation	Jolly et al. (2003); Zohre and Erten (2002)
C. stellata	Increase acetic acid production	Ciani and Ferraro (1995, 1998); Ferraro et al. (2000)
	Aromatic modulation	Soden et al. (2000)
Debaromyces vanniji	Increase geraniol content	Garcia et al. (2002)
Hanseniaspora guilliermondii	Aromatic modulation	Zironi et al. (1993)
H. uvarum (*Kloeckera apiculata*)	Aromatic modulation	Ciani et al. (2006); Herraiz et al. (1990); Mendoza et al. (2007); Moreira (2005); Moreira et al. (2008); Zironi et al. (1993); Zohre and Erten (2002)
Issatchenkia orientalis	Reduce malic acid content	Kim et al. (2008)
Kluyveromyces thermotolerans	Reduce acetic acid production	Ciani et al. (2006); Mora et al. (1990)
	Increase total acid	Kapsopoulou et al. (2007)
Pichia fermentans	Aromatic modulation	Clemente-Jimenez et al. (2005)
P. kluyveri	Increase volatile thiol content	Anfang et al. (2009)
P. anomala	Aromatic modulation	Kurita et al. (2008)
Schizosaccharomyces pombe	Degradation of malic acid	Ciani (1995); Magyar and Panic (1989); Snow and Gallender (1979); Yokotsuka et al. (1993)
Torulaspora delbrueckii	Reduce production of acetic acid	Bely et al. (2008); Ciani et al. (2006); Lafon-

		Lafourcade (1981); Salmon et al. (2007)
	Aromatic modulation	Herraiz et al. (1990)

- Acetic acid levels of >0.8 g/L in stuck wine may need to be reduced before attempting re-fermentation (Rasmussen et al., 1995). The technology to accomplish this goal is commercially available, including reverse osmosis (RO).
- Some *Saccharomyces* species and strains, and some non-*Saccharomyces* yeasts, can produce killer toxins that inhibit other sensitive strains, and may play a role in stuck fermentations.
- Vigorous strains may be used for high-risk fermentations. Increasing the level of yeast inoculum, along with nitrogen supplementation, may also be helpful.

Pesticides and Fungicides

Pesticides and fungicides can influence fermentation by producing stress metabolites, and by inhibiting and/or preventing fermentation. Not all yeasts and bacteria are affected in the same way. For example, there is a significant difference between systemic and contact fungicides, with regard to residues.

Vinification practices influence residue concentrations. Pre-fermentation clarification, and utilization of bentonite, can affect the final concentration of contact fungicides in white wine fermentation. Close adherence to spray schedules, use of minimal applications, and avoidance of late season applications are recommended, as is the implementation of a viticultural HACCP plan. Actually, rinsing fruit with water prior to fermentation, and fermenting that lot separately, has been suggested as a means of evaluating the presence and possible impact of contact fungicides.

Bruce Zoecklein
Ken Fugelsang
Barry Bump

UNDERSTANDING AND MANAGING THE NUTRITIONAL STATUS OF JUICE AND WINE

Section 5.

Co-Inoculation, Multiple Yeast Strains, and Malolactic Fermentations (MLF)

Until recently, the use of more than one wine yeast strain to conduct alcoholic fermentations has not been common. It is clear that certain wines made from native fermentations (where more than one strain of yeast normally takes part) sometimes exhibit more interesting character than wines made with a single inoculated strain. The problem for winemakers, however, is to achieve such results reliably. Some issues in multiple yeast strain co-inoculation are listed below:

- Fermentation speed – Faster yeast strains out-complete slower yeast strains. For equal contribution in a co-inoculation, one has to choose two yeasts with the same speed, or use a higher ratio of the slower yeast.

- Temperature range – This is particularly important in white wine fermentations. In general, *Saccharomyces cerevisiae, S. bayanus*, and hybrids between *S. bayanus* and *S. cerevisiae* can ferment cold (55-59°F/13-15°C). Co-inoculation of these yeasts with *Saccharomyces cerevisiae* is usually best fermented above 59°F (15°C) to obtain contribution from the *S. cerevisiae* strain. A higher dose of *S. cerevisiae* is also required if an equal contribution is desired.

- Nitrogen demand – Generally, the yeast strains with lowest demand will out-compete the yeast strains with higher demand.

- Complex nutrient demand – Some yeast strains require additional vitamins, minerals, and sterols to enhance fermentation, or possibly to simply complete

fermentation. The yeast with the lower demand will ferment faster than yeast with a higher demand.

- Killer factor – A killer-positive yeast strain will show a much greater viability at the end of fermentation than will a killer-negative strain.

- Higher alcohol and ester production – In the right concentrations, esters and higher alcohols are desirable for the role they play in fruity aromas. Some esters, however, are not typical for certain wine styles. Co-inoculation with high ester producers in low-temperature fermentations can be problematic. With neutral grape varieties or mediocre quality grapes, it can be a viable tool. In other circumstances, however, it can result in atypical aromas for grape or style.

- Alcohol tolerance – The yeast least affected by alcohol will out-compete the more sensitive ones.

- Effect on volatile compounds – Yeast strains differ in their ability to form aromatic components from non-aromatic precursors during fermentation. Yeast strains also differ in their ability to convert these into aromatic esters. This is a principal justification for multiple yeast strain fermentation.

There are mixed culture propriety yeast blends on the market that have been prepared to help minimize most of the problems listed above.

MLF

Malolactic fermentation (MLF) is important due to the sensory impact, both directly and indirectly, by lowering a wine's acidity and modifying the taste profile. Not all bacteria, however, have positive effects. Malolactic fermentation is not always easy, even when conditions seem favourable. Classical parameters, such as pH, ethanol content, SO_2, and temperature influence the development of bacteria (see section titled Controlling Microbiological Growth in Wine).

Some *Oenococcus oeni* strains can produce excess volatile acidity, biogenic amines, and/or ethyl carbamate. The use of malolactic starter cultures can reduce bacterial deviation since the strains selected have been tested to avoid these undesirable metabolisms. Commercial cultures also reduce the potential for spoilage by indigenous strains.

Some microbial spoilages of wine are the result of yeast metabolisms. The production of volatile phenols, such as 4-ethyphenol and 4-ethylguaiacol, by the yeast species *Brettanomyces bruxellensis* is one of the most problematic. Smoked, animal, spicy, and the other “Brett” characters can lead to a loss of freshness and fruitiness, which may be detrimental.

The latency period between the primary and ML fermentations is a key stage for *Brettanomyces* development. *Brettanomyces* is opportunistic and is more resistant to ethanol and to nutrient deprivation than *S. cerevisiae*.

This explains why a sluggish alcoholic fermentation often leads to *Brettanomyces* development (Renouf et al., 2006). At the close of the alcoholic fermentation, *S. cerevisiae* populations naturally drop, leaving a void in the microbial ecosystem which other organisms then try to fill. In particular, *Brettanomyces* and *O. oeni* begin competition to occupy the available ecological niche. This period is extremely favorable for the growth of *B. bruxellensis*.

Brettanomyces is naturally more tolerant than *O. oeni* to low pH and high SO_2. MLF involving indigenous strains may take longer to complete. This repressive effect on the indigenous bacteria favors *Brettanomyces* in the race to occupy the ecosystem.

Malolactic starters can be used as a tool to reduce the risk of *Brettanomyces* contamination. Malolactic starter cultures have large populations ($1x\ 10^{6}$ cells per mL) of very efficient *O. oeni* strains which can reduce the time required to bring on MLF.

Using starter cultures can provide a competitive advantage for the MLF and concurrently suppress the competitive development of *Brettanomyces*. The inoculated MLF strain colonizes the wine ecosystem to the detriment of *Brettanomyces.* When MLF is very fast, and/or conducted during the alcoholic fermentation, *Brettanomyces* does not have the opportunity to grow and the volatile phenol production stays very low.

Bruce Zoecklein
Ken Fugelsang
Barry Bump

Yeast and MLF Co-Inoculation

It has long been thought that malolactic fermentation (MLF) should take place only after the completion of the alcoholic fermentation (AF). The principal rationales were to avoid yeast stress and stuck fermentations, and to reduce the potential for opportunistic bacterial infections. Such infections can lead to elevated levels of volatile acidity (VA).

Co-inoculation allows the MLF to adjust over time to conditions of rising alcohol, and to complete the fermentation prior to indigenous bacteria getting a chance to dominate and form biogenic amines.

It is now established that early growth of lactic acid bacteria does not necessarily lead to an increased acetic acid production, yeast antagonism to MLF, or stuck fermentation.

MLF in the presence of fermentable sugars does not necessarily lead to the production of excessive amounts of acetic acid by the bacteria, if the yeast fermentation starts promptly and goes to completion.

The presence of rapidly growing yeast can be antagonistic toward MLF development. This inhibition was attributed to the presence of yeast metabolites and/or the removal of substances important to bacterial nutrition.

The point at which bacteria transition from lag to logarithmic phase of growth in mixed cultures with yeast coincides with the death phase of the yeast. The death and autolysis of the yeast may result in the return of essential bacterial nutrients into the system. When comparing yeast growth curves in pure and mixed cultures, it is found that yeast growth through the stationary phase is unaffected by the presence of bacteria.

MLF is characterized by three phases. In Phase 1, bacterial growth reaches critical biomass (10^6 bacteria/mL). Critical biomass is necessary for MLF induction. During Phase 2, malic acid degradation takes place. Finally, in Phase 3, the degradation of citric and other organic acids occurs, accompanied by a slight increase in acetic acid.

Note that malic acid is always consumed first, followed by citric, fumaric, and other organic acids. Afterwards, depending on the pH, the bacteria may also start to consume sugars (pH > 3.5). Sugar degradation at this point will result in a significant volatile acid increase.

Acetic acid will not be produced during the growth phase of malolactic bacteria in an inoculated MLF. Simultaneous bacterial and yeast inoculation, versus bacterial inoculation at the end of alcoholic fermentation, showed no difference in the final acetic acid concentration. There was, however, a direct relationship between citric acid degradation and an increase in acetic acid.

Citric acid can also be a source of diacetyl formation. This mechanism depends on oxygen availability. In the presence of lees, MLF will produce lower diacetyl levels due to the reductive power of viable yeast. The yeast convert diacetyl (buttery flavor) to less-flavorful compounds. Co-inoculation of wine with yeast and bacteria results in less lactic and buttery flavors, and a more fruit-driven wine.

Early inoculation of bacterial starter cultures results in a faster MLF. Timing of co-fermentation has an important impact on the sensory profile of the wine. Simultaneous inoculations allow for a rapid onset of MLF, allowing for malic acid degradation under the reductive power of the still-active yeast. This reductive environment prevents the formation of buttery or lactic aromas, and retains more typical varietal aromas and flavors. Wines inoculated after alcoholic fermentation show the buttery and nutty flavors typical of an MLF, with limited varietal aromas and flavors.

Bacterial cells introduced at the end of alcoholic fermentation find harsh conditions. The high alcohol content becomes the most limiting factor, particularly in some New World style reds.

The progress of MLF is inhibited by medium-chain fatty acids (octanoic [C8] and decanoic [C10] acids) produced by yeast. MLF has difficulty completing when octanoic

acid content is over 25 mg/L and/or decanoic acid exceeds 5 mg/L. As such, the co-fermentation of yeast and bacteria must be done with an understanding of yeast stain and MLF strain compatibility. Such information is available from suppliers.

Analytical Methodology for Nitrogen Nutrition

Rapid, accurate, and precise analytical methods for assimilable nitrogen involving simple sample preparation, minimal waste products, and minimal instrumentation would be valuable tools for winemakers. Currently, several analytical methods are in common use.

The Formol titration is a simple and rapid determination of assimilable nitrogen. This method involves the addition of neutralized formaldehyde to liberate protons that are titrated directly with NaOH to a pH 8.0 endpoint (Gump et al., 2000). The analysis provides an approximate, but useful, index of the nutritional status of juice.

The NOPA procedure (Dukes and Butzke, 1998) has been used to obtain FAN nitrogen by derivatization of primary amino groups with *ortho*-phthaldialdehyde. Ammonium ion, the second main source of assimilable nitrogen, is not measured by the NOPA procedure.

Regardless of the methodology, it is essential that winemakers determine the YAN content of their juice prior to fermentation. The simple addition of complex yeast nutrients and/or DAP without an understanding of YAN can cause significant loss of potential wine quality.

Ethyl Carbamate Formation

Ethyl carbamate, or urethane, is a carcinogen (Zimmerli and Schlatter, 1991) that occurs naturally in fermented foods, including wine, as a result of the fermentative and assimilative activities of microorganisms.

- Even though ethyl carbamate is present in microgram quantities in wine, the metabolite is subject to international regulation and, therefore, must be carefully managed.
- At present, the U.S. wine industry has established a voluntary target level of <15 µg/L (ppb) for table wines, and <60 µg/L for dessert wines.

Ethyl carbamate is produced from the reaction of urea and ethanol. Although several precursors of urea and, subsequently, ethyl carbamate have been identified (Monteiro et al., 1989), quantitatively, the most important source is the amino acid arginine.

Upon incorporation into yeasts, arginine is converted to ornithine, citrulline, carbamyl phosphate, and urea, which is directly utilizable by fermenting yeasts (Ingledew, 1996). In the presence of urea and ethanol, formation of ethyl carbamate increases exponentially as a function of temperature (Stevens and Ough, 1993; Ough, 1993).

Formation of urea occurs during the early- to mid-phases of fermentation. This corresponds to the point at which the fermentation of dessert-style wines (such as Port) is typically arrested by additions of alcohol. Yeast strains exhibit variability in terms of urea uptake and excretion during fermentation (An and Ough, 1993).

Grapes from high-vigor vines and/or heavily fertilized vineyards have high levels of arginine (>400 mg/L). Modifying vineyard fertilization practices, utilizing yeast strains that release less urea, and timing the fortification of dessert wines when urea concentrations are low, may reduce ethyl carbamate formation (Ough, 1993). Commercial ureases produced from the lactic acid bacterium, *Lactobacillus fermentatum*, are available for post-fermentation treatment of wines (Yoshizawa and Takahashi, 1988).

Untypical/Atypical Aging

Grape nitrogen appears to be related to a sensory phenomenon known as untypical (UTA) or atypical (ATA) aging. Wines with this taint lose varietal character very early, and develop atypical aromas and flavors, described as naphthalene (moth balls), dirty

dish rag, wet towel, linden, floor polish, etc., and are characterized by an increase in a metallic-like bitterness. ATA can also be associated with an increased production of post-bottling reductive-odor defect.

First reported in Germany, ATA has been identified in other European wine-producing regions, the Pacific Northwest, California, and the eastern US. This sensory problem has been associated with vine stress impacting nitrogen metabolism.

Nitrogen application and cultivation of vineyard floors reduces ATA development. Moisture stress conditions, in general, may not allow for optimum aroma/flavor compound formation, by slowing the rate of fruit maturation. This, coupled with metabolites that appear to be unique to ATA, results in limited varietal intensity and the production of detrimental sensory features.

The characteristic ATA odor was originally thought to be caused by certain metabolites (*o*-aminoacetophenone, skatole, or indole, but more recent studies suggest otherwise. For a simple screening test for ATA in wines, see the *Enology Notes* index at www.vtwines.info.

Practical Summary of Winemaking Issues

- The nitrogenous components of grapes and juice that are metabolically available to yeasts are present as primary, or "free *alpha*-amino acids" (FAN), and ammonium salts (NH_4^+). The combination of these two groups is referred to as "yeast assimilable nitrogenous compounds," or YAN.
- Most vineyard management decisions, including fruit maturity, and winemaking decisions can influence the relative proportion of, and the relative impact on, fermentable nitrogen.
- Nitrogen can be considered a *terrior* factor.
- The desirable concentration of fruit nitrogen differs between red and white grapes.
- Native nitrogen concentration is preferable to the use of winemaking supplements.
- Microbiological deterioration of fruit can influence initial FAN and vitamin levels.
- Minimum levels of FAN required for successful completion of alcoholic fermentation range from 120 to 140 mg/L for musts with sugar concentrations of 16-22°Brix.
- Oxygen is required by yeasts for synthesis of cell membrane precursors.
- Once yeast fermentative vigor and vitality have diminished, revitalization may be difficult, if not impossible.
- Fermentation problems are often vineyard-specific; nitrogen deficiency in apparently-healthy grapes can be severe.
- Strain differences among *Saccharomyces* spp. may be significant, in terms of nitrogen requirements, the time frame for uptake and release of specific amino acids during fermentation, and the ability to ferment to dryness.
- The yeast supplier's rehydration protocol, including recommended temperatures, should be strictly followed to assure maximum yeast viability.
- The type of, and timing of, yeast nutrient additions is important. A single large addition of nutrients at the beginning may lead to an excessive fermentation rate, and an imbalance in the uptake and usage of amino acids. Complex yeast nutrients should be added first, and DAP used only to make up the deficiency afterward.
- Adding nutrients to a stuck fermentation is seldom effective.

- Non-soluble solids reduction below 0.5% can result in nutrient deficiencies, and promote premature sedimentation of yeast.
- Increased osmotic pressure associated with high sugar concentrations can inhibit yeast growth. Such environments are often nitrogen deficient, as well.
- Alcohol has a profound effect on all aspects of yeast metabolism, ranging from membrane integrity, to nitrogen uptake and sugar transport.
- Excessive nitrogen in juice and wines can impact wine aroma, fermentation rate, and yeast biomass, and support the growth of spoilage organisms and the formation of ethyl carbamate.
- Wines with untypical (UTA) or atypical (ATA) aging may lose varietal character very early, and develop atypical aromas and flavors.
- YAN levels have both a quantitative and qualitative impact on aroma volatiles.
- YAN levels can impact the rate of fermentation. Excessive fermentation rate should be avoided to maximize volatile retention in whites and cap extraction, including color in reds.

Study Questions

1. Oxygen is considered a yeast nutrient. Why?

2. List the factors to consider in selecting the correct YAN concentration to optimize a fermentation.

3. List the reasons why seasonal variations can impact YAN.

4. What are the nutritional concerns when fermenting rot-compromised fruit?

5. What are the practical steps to be taken to help minimize problems?

6. Deficiencies in YAN are frequently vineyard specific. Speculate on the advantages and possible disadvantages of changing vineyard management to abate deficiencies, versus making adjustments at the winery.

7. Many fermentation supplements contain DAP. Why, then, is it advantageous to add supplements?

8. What are possible advantages of yeast and MLF co-fermentations? Are there disadvantages?

9. A main goal in multiple yeast strain and species fermentation is sensorial diversity. What are the parameters to consider in selecting yeast to co-ferment?

References

An, D., and C.S. Ough. 1993. Urea excretion and uptake by various wine yeasts as affected by various factors. Am. J. Enol. Vitic. 44:35-40.

Bataillon, M., and A. Rico. 1996. Early thiamine assimilation by yeasts under enological conditions: impact on alcoholic fermentation kinetics. J. Ferm. Bioeng. 82:145-50.

Bell, S.J. and Henschke, P.A. 2005. Implications of nitrogen nutrition for grape fermentation and wine. Australian Journal of Grape and Wine Research 11, 242-295.

Beltran, G., B. Esteve-Zarzoso, N. Rozès, A. Mas, and J.M. Guillamón. 2005. Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. J. Agric. Food Chem. 53:996-1002.

Bisson, L.F. 1996. Yeast and biochemistry of ethanol formation. *In* Principles and Practices of Winemaking. R.B. Boulton, V.L. Singleton, L.F. Bisson, and R.E. Kunkee, (eds.), p. 140. Chapman & Hall, New York.

Brechot, P., J. Chauvet, P. Dupuy, M. Croson, and A. Rabatu. 1971. Acide oleanoique facteur de aroissance anaerobic de la levure du vin. C.R. Acad. Sci. 272:890-93.

Butzke, C.E. 1998. Survey of yeast assimilable nitrogen status in musts from California, Oregon and Washington. Am. J. Enol. Vitic. 49:220-224.

Chone, X.,van Leeuwen, C.Chery,P., and Ribereau-Gayon,P. 2001. Terrior influences on water status and nitrogen status of non-irrigated Cabernet Sauvignon . South African Journal of Enology and Viticulture 22: 8-15.

Cooper, T.G. 1982. Nitrogen metabolism in *Saccharomyces cerevisiae*. *In* The Molecular Biology of the Yeast Saccharomyces. J.N. Strathern, E.W. Jones, and J.B. Broach (eds.), pp. 39-99. Cold Spring Harbor Laboratory, New York.

Correa, I., M.C. Polo, L. Amigo, and M. Ramos. 1988. Separation des proteines des mouts de raison au moyen de techniques electrophoretiques. Bull. O.I.V. 39: 1475-89.

de Bordenave, C. and B.W. Zoecklein, 1999. Enology Notes. Virginia Tech electronic technical briefs. www.vtwines.info.

Dittrich, H.H. 1987. Die Garbeeinflussung 5.5 Stickstoff. *In* Mikrobiologie des Weines. Handbuch der Lebensmitteltechnologie, 2nd ed. H.H. Ditrick (ed.). Ulmer, Stuttgart.

Donèche, B.J. 1993. Botrytized wines. *In* Wine Microbiology and Biotechnology. G.H. Fleet (ed.). Harwood Academic Publishers.

Dubourdieu, D., B. Pucheu-Plante, M. Mercier, and P. Ribéreau-Gayon. 1978. Structure, role et localisation d'un glucane secrete par Botrytis cinerea dans la baie de raisin. Comptes Rendus Academie des Sciences, Paris, 287D:571-73.

Dukes, B.C., and C.E. Butzke. 1998. Rapid determination of primary amino acids in grape juice using an o-phthaldiadehyde/N-acetyl-L-cysteine spectrophotometric assay. Am. J. Enol. Vitic. 49:125-34.

Fleet, G.H., and G.M. Heard. 1993. Yeasts - growth during fermentation. *In* Wine Microbiology and Biotechnology. G.H. Fleet (ed.), pp. 27-54. Harwood Academic Pubs, Australia.

Fugelsang, K.C. 1996. Wine Microbiology. Chapman and Hall, New York, NY.

Gladstone, J. 2011. Wine, terrior and climate change. Wakefield press, Adelaide, SA. pp279.

Guitart, A., P.H. Orte, and J. Cacho. 1998. Effect of different clarification treatments on the amino acid content of Chardonnay musts and wines. Am. J. Enol. Vitic. 49:389-396.

Gump, B. H., B. W. Zoecklein, and K. C. Fugelsang. 2000. Prediction of prefermentation nutritional status of grape juice. *In* Methods in Biotechnology, Vol. 14: Food Microbiology Protocols. J.F.T. Spencer and A.L. Ragout de Spencer (eds.), pp. 283-296. Humana Press, Inc., Totowa, NJ.

Gump, B.H.; B.W. Zoecklein, and K. Fugelsang 2001. Prediction of nutritional status of grape juice. In: Food Microbiology Protocols. J. F.T. Spencer and A. > Rogout de Spencer (eds.) Humana Press. P. 283-296.

Henick-Kling, T. 2008. Studies on the origin and sensory aspects of atypical aging in white wines. Proc. 15th Int'l Enol. Sym., Trier, Germany.

Henick-Kling, T., W.D. Edinger, and I.-M. Larsson-Kovach. 1996. Survey of available nitrogen for yeast growth in New York grape musts. Die Wein-Wissenschaft 51:169-74.

Henschke, P.A., and V. Jiranek. 1993. Metabolism of nitrogen compounds. *In* Wine Microbiology and Biotechnology. G.H. Fleet (ed.), pp.27-54. Harwood Academic Pubs., Australia.

Houtman, A.C., and C.S. duPleissis. 1981. The effect of juice clarity and several conditions promoting yeast growth on fermentation rate, the production of aroma components and wine quality*. S. African J. Enol. Vitic.* 2:71-81.

Ingledew, W.M. 1996. Nutrients, yeast hulls and proline in wine fermentation. Die Wein-Wissenschaft 51:141-46.

Jiranek, V., P. Langridge, and P.A. Henschke. 1990. Nitrogen requirement of yeast during wine fermentation. *In* Proceedings of the Seventh Australian Wine Industry Technical Conference. P.J. Williams, D.M. Davidson, and T.H. Lee, (eds.), pp. 166-71. Australian Industrial Publishers, Adelaide, S.A.

Jones, R.P. 1989. Biological principles for the effects of ethanol. Enzyme Microbiol. Biotechnol. 11:30-53.

Julien, A., J.-L. Roustan, L. Dulau, and J.-M. Sablayrolles. 2000. Comparison of nitrogen and oxygen demands of enological yeasts: technological consequences. Am. J. Enol. Vitic. 51:215-222.

Koch, J. 1963. Proteines des vins blancs. Traitements des precipitations proteiques par chauffage et a l'aide de la bentonite. Ann. Technol. Agric. 12:297-313.

Kudo, M., P. Vagnoli, and L.F. Bisson. 1998. Imbalance of potassium and hydrogen ion concentrations as a cause for stuck enological fermentations. Am. J. Enol. Vitic. 49:296-301.

Lafon-Lafourcade, S., and P. Ribéreau-Gayon. 1984. Developments in the microbiology of wine production. Prog. Indust. Microbiol. 19:1-45.

Lagace, L.S., and L.F. Bisson. 1990. Survey of yeast acid proteases for effectiveness of wine haze reduction. Am. J. Enol. Vitic. 41:1246-49.

Lagunas, R. 1993. Sugar transport in *Saccharomyces cerevisiae.* FEMS Microbiol. Rev. 104:229-42.

Large, P.J. 1986. Degradation of organic nitrogen compounds by yeasts. *Yeast* 2:1-34.

Manginot, C. and J-M. Sablayrolles. 1997. Use of constant rate alcoholic fermentations to compare the effectiveness of different nitrogen sources added during the stationary phase. Enzyme Microbial Tech. 20:373-380.

Manginot, C., J.L. Roustan, and J-M. Sablayrolles. 1998. Nitrogen demand of different yeast strains during alcoholic fermentation. Importance of stationary phase. Enzyme Microbial Tech. 23:511-17.

Monk, P.R. 1986. Rehydration and propagation of active dry wine yeasts. Austral. Wine Ind. J. 1:3-5.

Monk, P.R. 1994. Nutrient requirements of wine yeast. Pract. Winery Vineyard Jul/Aug:24-60.

Monteiro, F.F., E.K. Trousdale, and L.F. Bisson. 1989. Ethyl carbamate formation in wine: Use of radioactively labeled precursors to demonstrate the involvement of urea. Am. J. Enol. Vitic. 40:1-8.

Ough, C.S. 1993. Report on ethyl carbamate for the Wine Institute. Ethyl carbamate/urease enzyme preparation. A compendium from June, 1993 seminars.

Ough, C.S., D. Stevens, and J. Almy. 1989. Preliminary comments on effects of grape vineyard nitrogen fertilization on the subsequent ethyl carbamate formation in wines. Am. J. Enol. Vitic. 40:219-220.

Pekur, G.N., N.I. Bur'yan, and N.M. Pavlenko. 1981. Characteristics of nitrogen metabolism in wine yeasts under different fermentation conditions. Appl. Biochem. Microbiol. 17:248-52.

Rapp, A. 1977. Uber den Gehalt der Aminosauren in Weinbeeren, Traubenmost und Wein, pp 136-51. Bundesausschu fur Weinforschung.

Rasmussen, J.E., E. Schultz, R.E. Snyder, R.S. Jones, and C.R. Smith. 1995. Acetic acid as a causative agent in producing stuck fermentations. Am. J. Enol. Vitic. 46:278-80.

Renouf V., M. Falcou, C. Miot-Sertier, M.C. Perello, G. De Revel, and A. Lonvaud-Funel. 2006. Interactions between Brettanomyces bruxellensis and other yeast species during the initial stages of winemaking. J. Appl. Microbiol. 100, 1208-1219.

Renouf, V., A. Lonvaud-Funel, and J. Coulon. 2007. The origin of Brettanomyces bruxellensis in wines: a review. J. Int. Sc. Vigne Vin 41:161-173.

Renouf V., M.C. Perello, G. De Revel, and A. Lonvaud-Funel. 2007. Microbiology of bottled wines. Am. J. Enol. Vitic. 58:379-386.

Rosi, I., L. Costamagna, and M. Bertuccioli. 1987. Screening for extracellular acid protease(s) production by wine yeasts. J. Instit. Brew. 93:322-24.

Sablayrolles, J.-M., and C. Dubois. 1996. Effectiveness of combined ammoniacal nitrogen and oxygen additions for completion of sluggish and stuck fermentations. J. Ferm. Bioeng. 92:377-381.

Salmon, J.M. 1989. Effect of sugar transportation inactivation in *Saccharomyces cerevisiae* on sluggish and stuck enological fermentations. Appl. Environ. Microbiol. 55:9536-38.

Schultz, M., and J. Gafner. 1993. Sluggish alcoholic fermentation in relation to alterations of the glucose-fructose ratio. Chem. Mikrobiol. Technol. Lebenom. 15:73-78.

Sharf, R., and P. Margalith. 1983. The effect of temperature on spontaneous wine fermentation. Appl. Microbiol. Biotechnol. 17:311-313.

Smith, D.A., and S.W. Banks. 1986. Biosynthesis, elicitation and biological activity of isoflavonoid phytoalexins. Phytochem. 25:979-95.

Smith, C.R. 1999. New Tools for an Ancient Craft. Vinovation, Inc., Sebastopol, CA.

Sponholz, W.R. 1991. Nitrogen compounds in grapes, must, and wine. *In* International Symposium on Nitrogen in Grapes and Wine. J. Rantz (ed.), pp.67-77. American Society for Enology and Viticulture, Davis, CA.

Stanier, R.Y., E.A. Adelberg, and J.L. Ingraham. 1976. Introduction to the Microbial World. Prentice-Hall, Englewood Cliffs, NJ.

Stanley, G.A., and N.G. Douglas. 1993. Inhibition and stimulation of yeast growth by acetaldehyde. Biotech. Letters 15:1199-1204.

Stevens, D.F., and C.S. Ough. 1993. Ethyl carbamate formation: reaction of urea and citrulline with ethanol in wine under low to normal temperature conditions. Am. J. Enol. Vitic. 44:309-312.

Sinton, T.H., Ough, C.S., Kussker, J.J. and Kasimatis, A.N. 1978. Grape juice indicator for prediction of potential wine quality. Am J. Enol.Vitic. 29: 267-271.

Swiegers, J.H., E.J. Bartowsky. P.A. Henschke, and I.S. Pretorius. 2005. Yeast and bacterial modulation of wine aroma and flavour. Aust. J. Grape Wine Res. 11:139-173.

Treeby, M.Holzapfel, B.P. and Friedrich, C.J. 1996. Managing vine nitrogen supply to improve wine grape composition. Proceedings Quality Management in Viticulture. Australian Society of Viticulture and Oenology, Adelaide.

Ugliano, M., P.A. Henschke, M.J. Herderich, and I.S. Pretorius. 2007. Nitrogen management is critical for wine style and flavor. Aust. N.Z. Wine Industry J. 22(6):25-30.

Varela, F., F. Calderón, M.C. González, B. Colomo, and J.A. Suárez. 1999. Effect of clarification on the fatty acid composition of grape must and the fermentation kinetics of white wines. Eur. Food Res. Technol. 209:439-444.

Wahlstrom, V.L. and K.C. Fugelsang. 1988. Utilization of yeast hulls in winemaking. Calif. Agric. Tech. Inst. Bull. 880103. California State University, Fresno.

Walker, G. 1998. Yeast physiology and biotechnology. John Wiley & Sons, Chichester, United Kingdom.

Wurdig, G., and R. Woller. 1989. Chemie des Weines. Handbuch der Lebensmittel-technologie. Ulmer, Stuttgart.

Yoshizawa, K., and K. Takahashi. 1988. Utilization of urease for decomposition of urea in sake. J. Brew. Soc. Japan 83:142-44.

Zimmerli, B., and J. Schlatter. 1991. Ethyl carbamate: analytical methodology, occurrence, formation, biological activity and risk assessment. Mutation Res. 259:325-350.

Zoecklein, B.W, K. Fugelsang, B.H.Gump 1999. Wine Analysis and Production. Kluwer Academic/Plendum Publishers, New York, NY. 621pp.