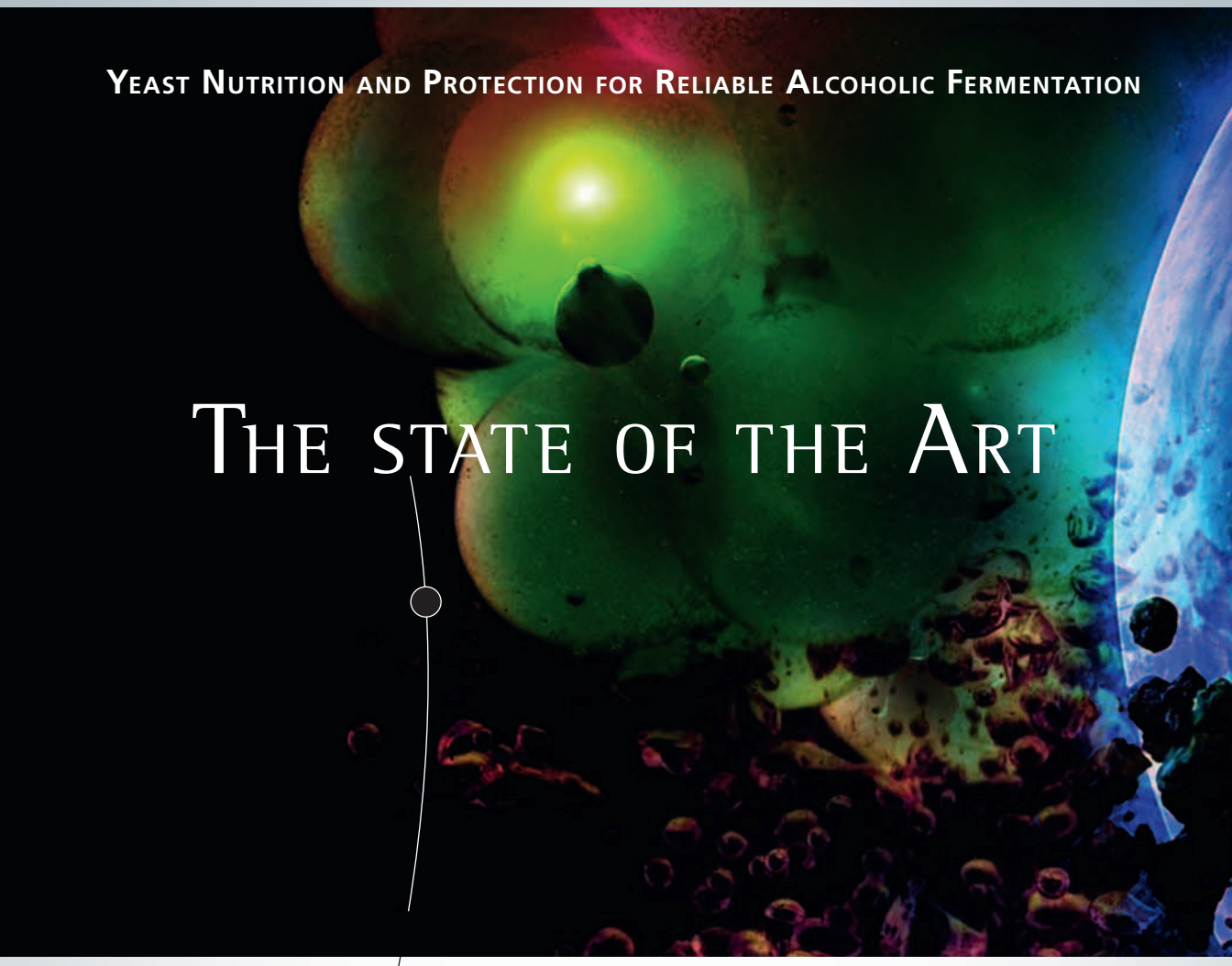
YEAST NUTRITION AND PROTECTION FOR RELIABLE ALCOHOLIC FERMENTATION



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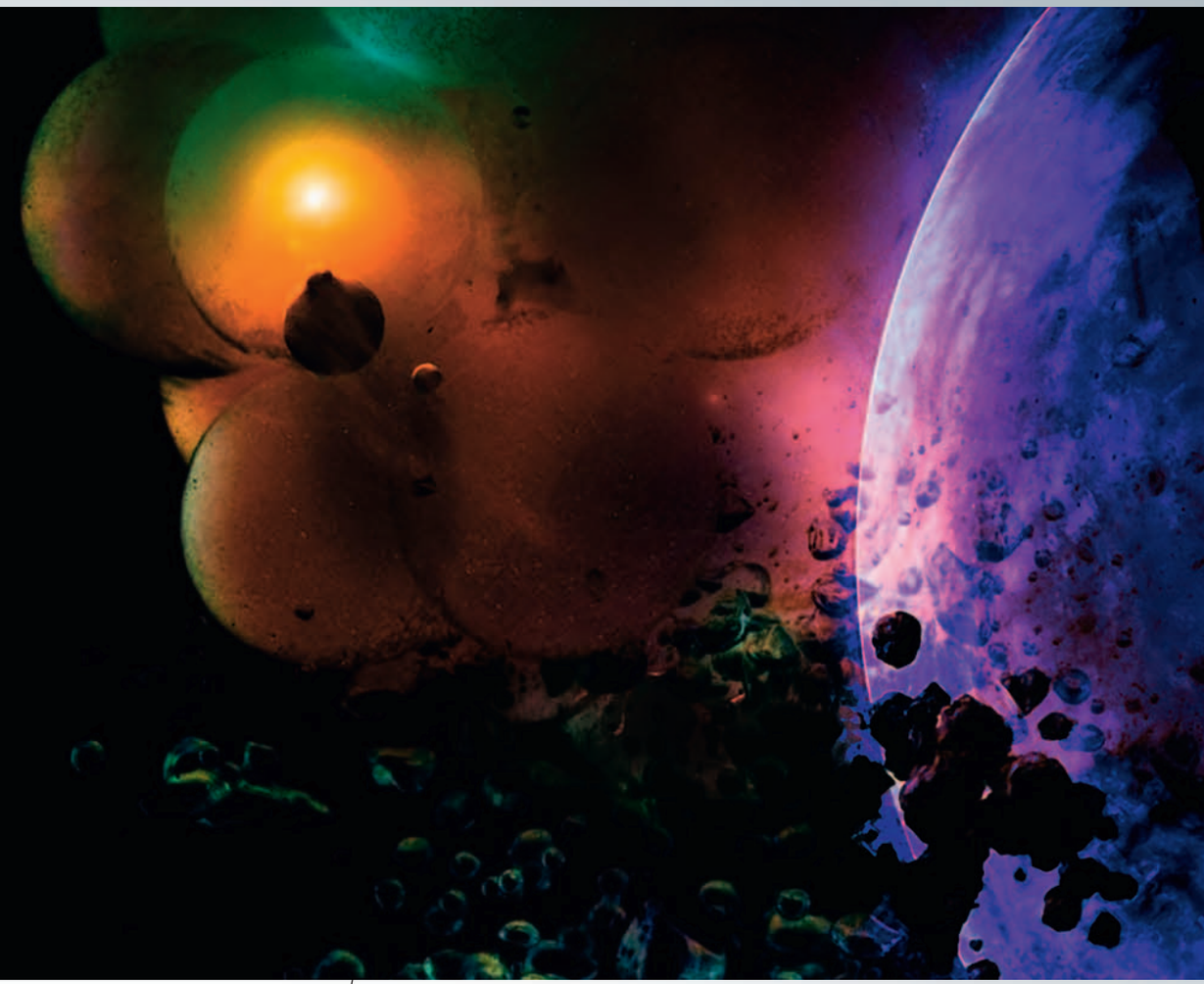
LALLEMAND

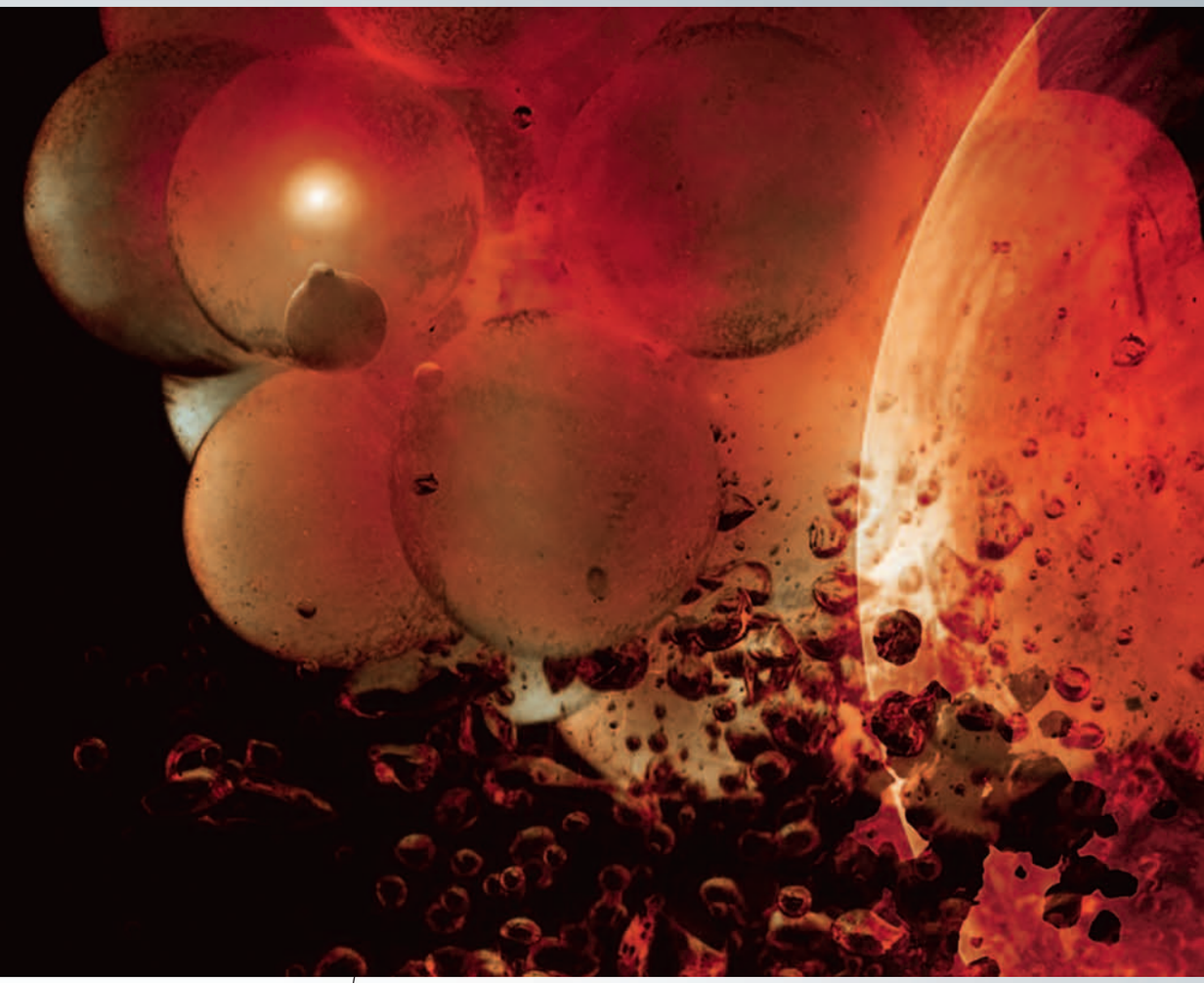
Yeast Nutrition and Protection for Reliable Alcoholic Fermentation



Lallemand, a leading producer of Oenological yeasts and bacteria and their fermentation nutrients, is a privately owned Canadian corporation. The Oenology Group, based in Toulouse (France), is focused on research and development, both in-house and in collaboration with renowned research institutes. The purpose of this document is to provide winemakers and oenologists with a description of the current scientific understanding on Yeast Nutrition and protection for reliable Alcoholic fermentation management.

Document prepared by Anne Julien (Lallemand SAS, France), Didier Theodore (Lallemand SAS, France), Gordon Specht (Lallemand, North America), Ann Dumont (Lallemand Inc., Canada) and Cecile Brung (Lallemand SAS, France).

Acknowledgements:
Dr. Charles Edwards - Washington State University (USA)
Pr. Jean-Marie Sablayrolles - UMR Sciences pour l'Œnologie - INRA - Montpellier (France)
Dr. Jean-Michel Salmon - UMR Sciences pour l'Œnologie - INRA - Montpellier (France)



1. Introduction

To assure a complete and regular fermentation, it is necessary for the grape must to have a reasonable balance of nutritional, physical and chemical environment that will allow the yeast to multiply and thrive. It is essential to have an optimum development of healthy yeast cells in order to reduce the risk of sluggish and stuck fermentations.

The following factors are the most likely causes of stuck and sluggish fermentations:

- Nitrogen deficiency
- Lack of oxygen
- Lack of temperature management
- Improper yeast rehydration and handling
- Lack of growth factors such as vitamins and minerals
- Highly clarified musts
- Inhibitory metabolites produced by yeasts
- Excessive sulphur dioxide or agrochemical residues

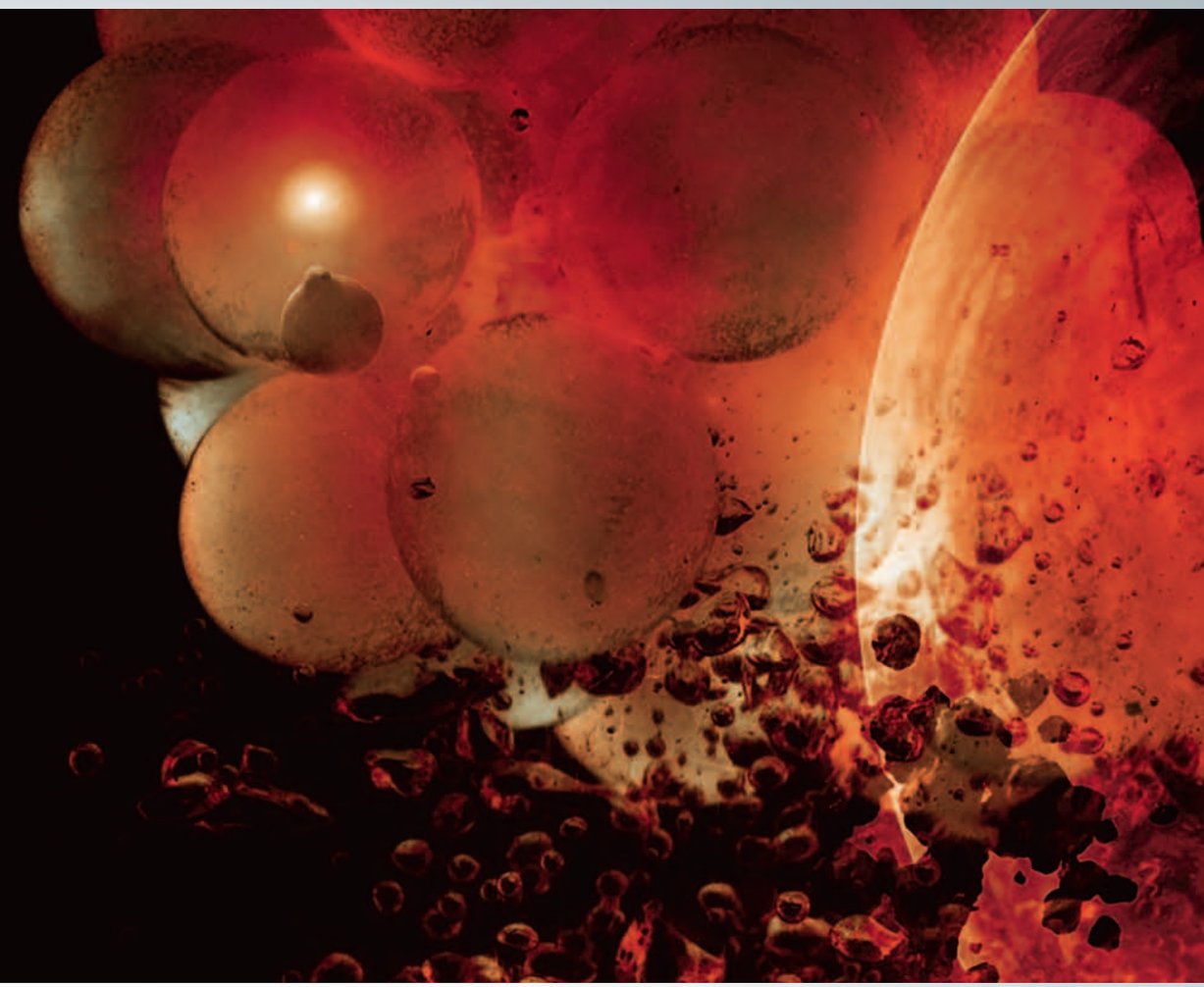
Some viticultural factors can affect the condition of must fermentation. Among these factors, it is important to remember:

- The high degree of maturity resulting in a high concentration of fermentable sugars in the grape must is associated with limited levels of available nitrogen increasing the risk of slow or stuck fermentations.
- A poor sanitary condition of the grapes resulting in the presence of large populations of undesirable microorganisms (bacteria and yeasts, *Botrytis*) reduces the availability of nitrogen, preventing desired yeast growth and their activity.
- Agrochemical residues are also implicated in fermentation issues. Some of them are responsible for a longer lag phase delaying the start of alcoholic fermentation (Suarez et *al.*, 2000).

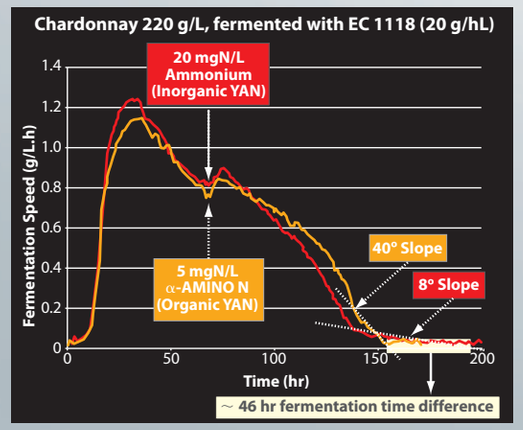
These factors are used to assess the potential risk of stuck fermentation, but when a particular wine style is desired, the intervention in the vineyard is not always possible. However, winemakers can compensate the situation by being proactive during fermentations.

1.1 Nitrogen

Nitrogen, the most important yeast nutrient, is a key factor that has a significant impact on wine fermentation. This nutrient influences both fermentation kinetics and the resulting wine quality (Agenbach, 1977; Bezenger and Navarro, 1987). In general, nitrogen must deficiency limits yeast growth and fermentation speed (Bely et *al.*, 1990).



Nitrogen content in grape juice is variable and ranges from 60 to 500 mg nitrogen/L (J.M Sablayrolles and J.M Salmon, 1996) depending on grape variety, vintage and microclimate. Grape musts contain nitrogen under different forms: proteins, peptides, ammonium ions (NH_4^+) and alpha amino acids. The yeast assimilable nitrogen (YAN) is composed of ammonium and alpha amino acids except proline which is not assimilable by yeast. Sometimes, YAN levels in must are not measured even though they are frequently deficient.



Efficiency of organic versus inorganic nitrogen on EC1118 wine yeast fermentation kinetics as measured by CO_2 release. The sluggish fermentation with inorganic nitrogen added had 4 times the YAN level than the organic nitrogen addition which finished alcohol fermentation ~46 hours earlier.

Nitrogen assimilation by yeast

Nitrogen is essential for yeast protein synthesis throughout fermentation. However, the different types of nitrogen are assimilated all differently. Organic nitrogen in the form of amino acids enters the cell through an active transport and can be accumulated inside the yeast cells (Bisson, *1991).* The main system is the GAP (General Amino Permease) which is able to transport all alpha amino acids. Different groups have been determined as a function of their assimilation order in a must from "very fast" to "partial" (Henschke et *al., 1993).* Once inside the yeast cell, these alpha amino acids can be integrated directly into proteins, degraded into ammonium (NH_4^+), or converted into glutamate. Their assimilation by the yeast is more gradual and efficient during fermentation compared to inorganic nitrogen. Ammonium nitrogen, the other type of assimilable nitrogen referred to as inorganic nitrogen, is rapidly consumed by yeast but is less beneficial.

Nitrogen impact on alcoholic fermentation

YAN content has the most influence on the fermentation speed; it impacts the yeast biomass at the beginning of fermentation, as well as the sugar transport kinetics during fermentation. As soon as a must is nitrogen depleted at the end of the growth phase, there is a decrease in protein synthesis and sugar transport activity (Bely et *al., 1994)*. YAN addition to nitrogen deficient must leads to a significant decrease in fermentation length. After a YAN addition, there is a reactivation of the protein synthesis and the sugar transport speed, which results in an increase in the fermentation rate.

Nitrogen and aromatic profile of wine

Amino acids composition of the must is quite important for the wine's aromatic profile. Higher alcohols and esters (ester acetates and ethyl esters) production is influenced by the amino acids must content. Sulfur compounds production is also linked with the nitrogen metabolism. When assimilable nitrogen is depleted and/or deficiency in methionine and its derivates appears, H_2S synthesis is favoured.

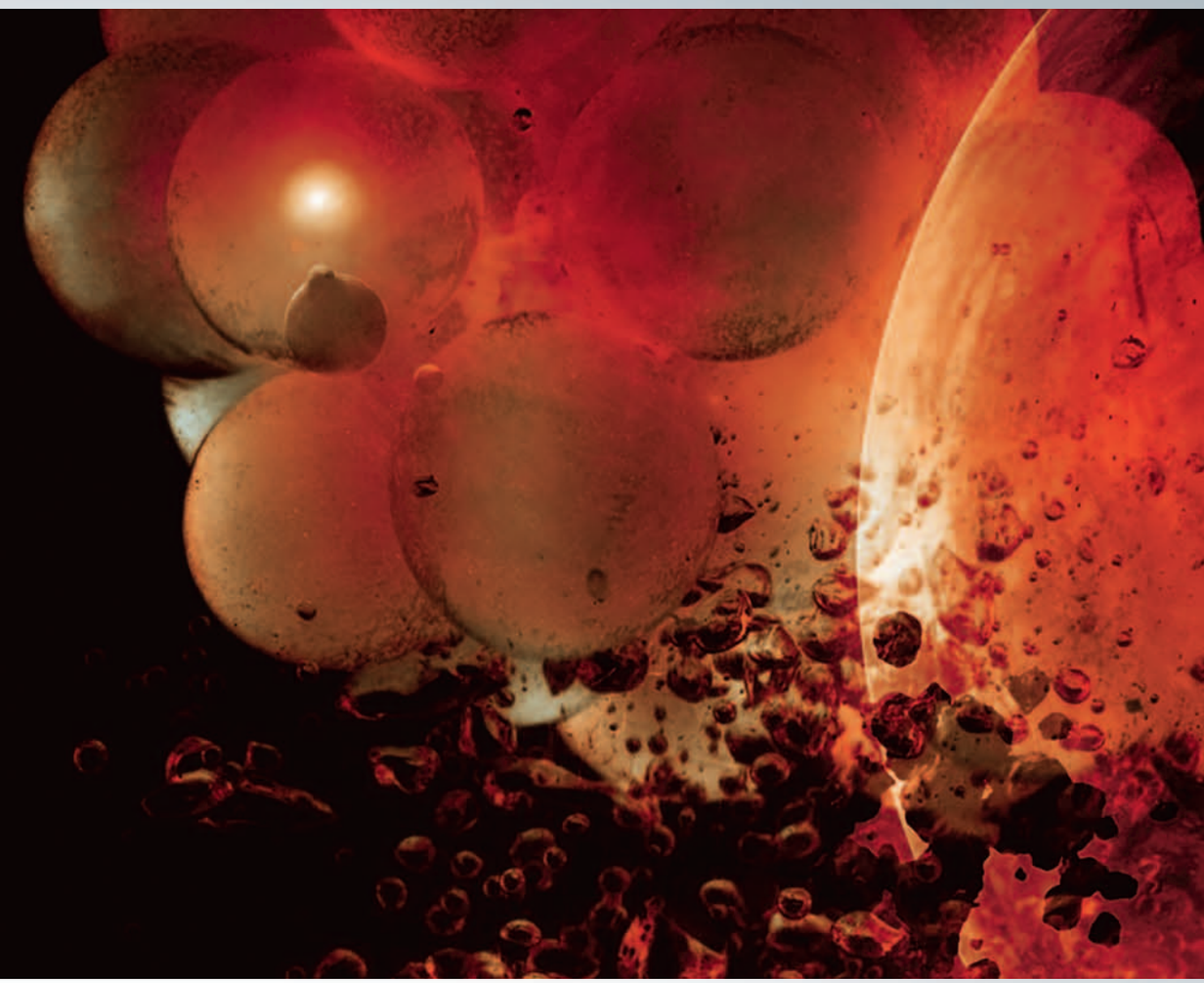
How much YAN is needed?

The minimum quantity of YAN is 150 to 200mg/L and lower levels in the must are considered as nitrogen deficient. This minimal level varies based on different parameters:

- Temperature: an increase in temperature stimulates the growth of yeast and fermentation is faster requiring increased amounts of nitrogen.
- Oxygen: when adding more O_2 to the must, nitrogen is captured faster and more is needed in comparison to fermentations taking place under anaerobic conditions (white wine).
- Initial sugar content: the higher the initial concentration, the more YAN is required.
- Turbidity: when musts are over clarified, many nutritional factors for yeast are removed, therefore it is necessary to supplement them with the addition of complete and balanced nutrients.

1.2 Oxygen/Survival Factors (Sterols)

Oxygen plays an essential role in alcoholic fermentation helping the development of an adequate yeast population and maintaining their vitality. Oxygen is required for the synthesis of survival factors such as sterols and unsaturated fatty acids (Kirsop, 1973; Kirsop, 1978; Alexandre et *al.*, 1994; Fornairon-Bonnefond et *al.*, 2002), which are components of the yeast cell membrane. They play a key role on the membrane structure helping to maintain the membrane fluidity, cell integrity and viability.



Oxygen and stuck fermentations

At the beginning of the alcoholic fermentation, oxygen is rapidly consumed by the yeasts and by the oxidases naturally present in the must. This decrease in oxygen availability is responsible for inhibition of sterols and fatty acids biosynthesis by yeasts.
The main consequences are:

- a decrease of the yeast growth.
- a decrease of the membrane protein activity.
- a decrease of the yeast viability at the end of fermentation.
- an increase of the short and medium chain fatty acids levels.

During the yeast growth phase, each multiplication cycle dilutes the lipid content of the yeast cell. When lipids become insufficient, the yeast cell membrane does not function properly under limiting oxygen conditions. Sterols, located around the membrane proteins responsible for the flux selectivity between the interior and the exterior of the cell, are no longer synthesised.
During fermentation, as the ethanol level increases, the yeast mortality increases. Without an adequate yeast sterol concentration, the yeast cell membrane permease activity suffers. When these proteins do not function adequately under increased ethanol concentration, there is an accumulation of H+ ion in the intracellular medium and the yeast requires more energy to expulse them outside the cell. This will lead rapidly to the acidification of the cell (drop of intracellular pH) resulting in cell death and stuck fermentation. So the more sterols are synthesized in the membrane, the better the resistance to ethanol is.

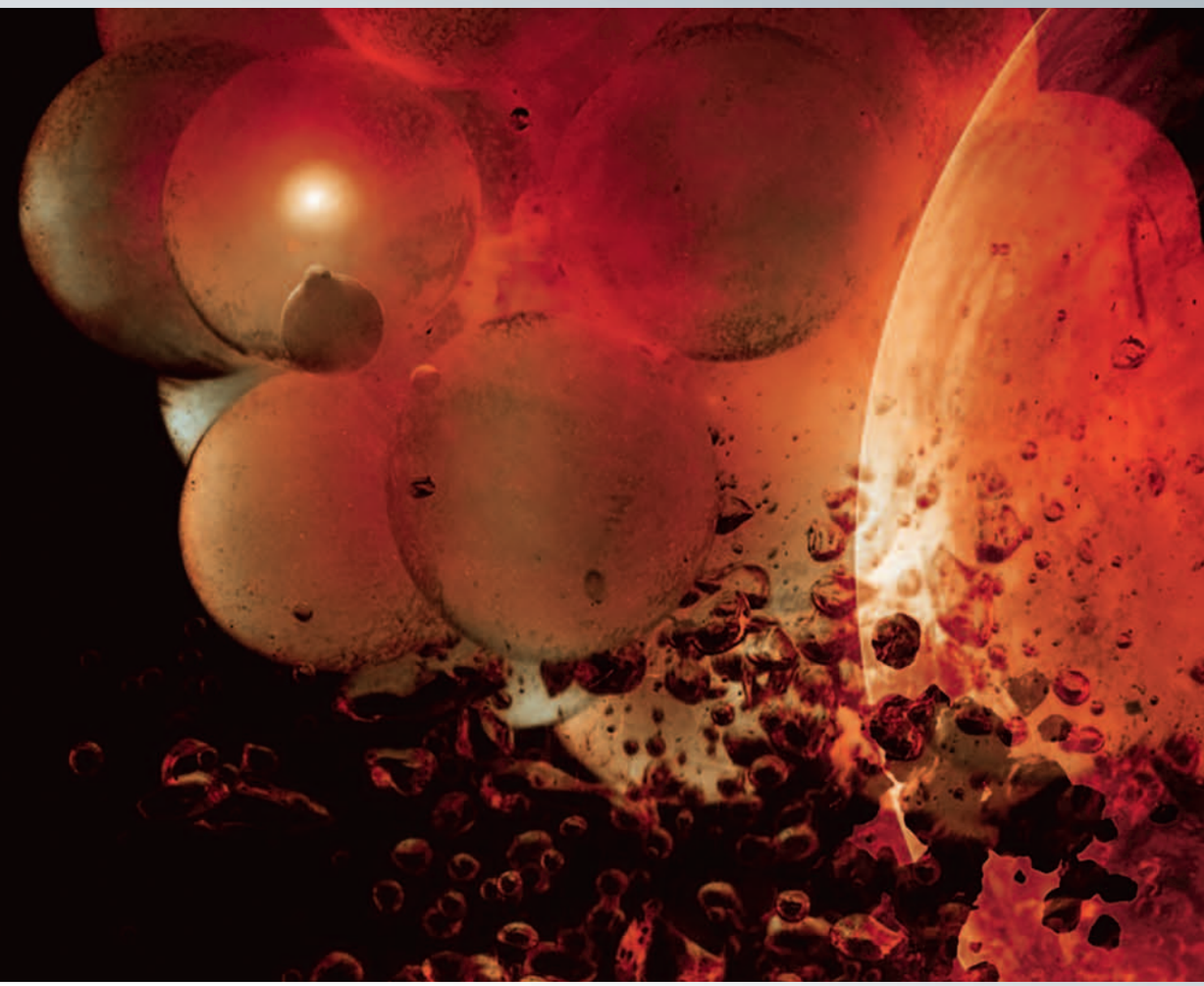
Oxygen addition

Sablayrolles and Barre *(1986)* have shown the efficiency of an 5 to 10 mg/L oxygen addition on the fermentation when O_2 is added at the end of the yeast cell growth phase. Most of the yeasts are still viable at the end of the growth phase and when oxygen is supplied after cell division and lipid dilution, oxygen is used efficiently for further sterols synthesis (Kirsop, *1982)*. Blateyron et *al.*, *(1998)* demonstrated that fermentations were always completed when O_2 was added at this moment.

Oxygen can be incorporated efficiently into the must in different ways, such as micro oxygenation systems that allow for very precise amounts and very small bubbles. Pumping juice over the must or splash racking with a venturi can be also used. However, these last two methods can be less efficient since musts are saturated with CO_2. As O_2 addition is not always controlled in wineries, and to avoid the negative effect of excessive O_2 addition which can lead to sterols oxidation, complex nutrients containing inactivated yeast may be used as yeast is a natural source of sterols. The best moment to add these complex nutrients is at roughly 1/3 of the way through alcoholic fermentation (AF), which corresponds to the end of the yeast growth phase.

1.3 Minerals

For many years, not enough attention was given to the role of minerals in the physiology and fermentation performance of the yeast. Nevertheless, minerals such as magnesium are absolutely essential for the growth and metabolism of the yeast, as well as zinc and potassium.



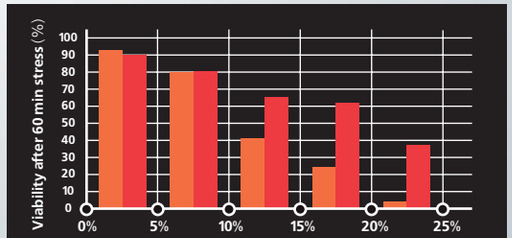
In must, the bioavailability of these minerals is low, because they are often bound to different types of must components such as proteins and polyphenols. Also their level is lower in overripe grapes and in those attacked by fungi that consume large quantities of minerals.
The type of the soil and the viticulture practices play a role as well on the mineral content. However, minerals can be supplemented in a balanced ratio since an overdose of calcium can result in the inhibition of the effects of magnesium. The magnesium to calcium ratio should always be 1 or more. Walker *(1994)* demonstrated that yeast cells have a very high magnesium demand for growth which is indispensable for the glycolytic pathway since it is required for hexokinase and phosphofructokinase activity. Magnesium is involved as well in the activation of some enzymes, and this element stabilizes membrane structure which can explain its central role in ethanol production. Limited magnesium availability is responsible for the decrease of yeast growth and fermentative activity (Dombek, *1986)*.

Kunkee and Bisson *(1993)* noted that 5mg/L was the optimum concentration of magnesium ions necessary to restore viability in yeast.

Other authors (Kudo et *al., 1998)* showed that the ratio of potassium to hydrogen ions doesn't affect the rate of yeast growth or maximal cell biomass, but it does have a big impact on the maintenance of cell viability as potassium deficiency can lead to stuck fermentation especially at low pH.

The role of minerals:

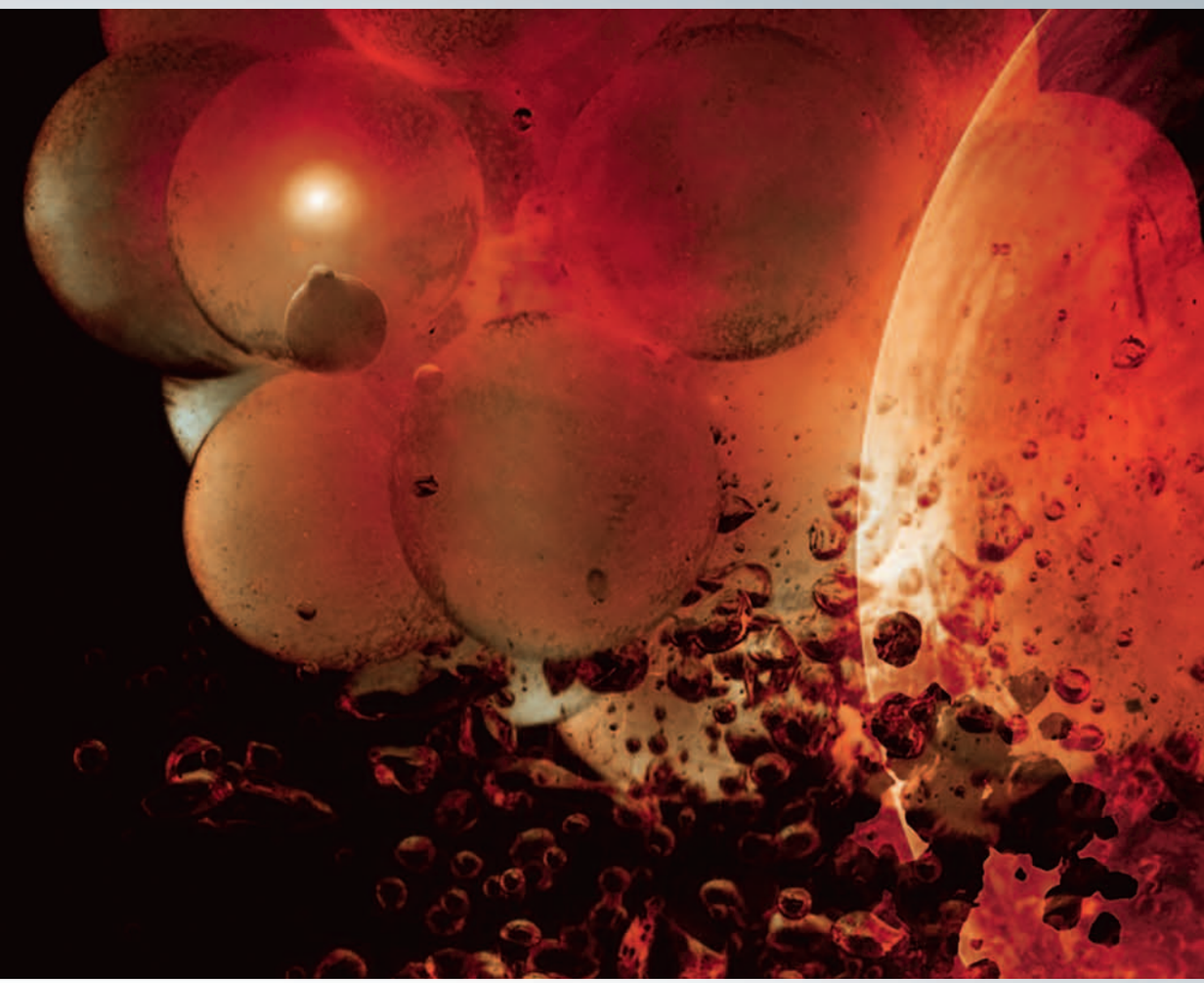
- Cofactors of several glycolytic enzymes.
- Increase ATPase activity and the pumping of the compounds across the yeast membrane.
- Increase yeast tolerance to ethanol and temperature.
- Antagonistic effect on the toxicity of heavy metals.
- Regulation of cellular growth
- Regulation of the formation of alcohol and esters



Positive effect of Magnesium concentration on yeast cells viability under increasing alcohol concentration (0-20% vol)
(Birch & Walker, 2000)

1.4 Vitamins

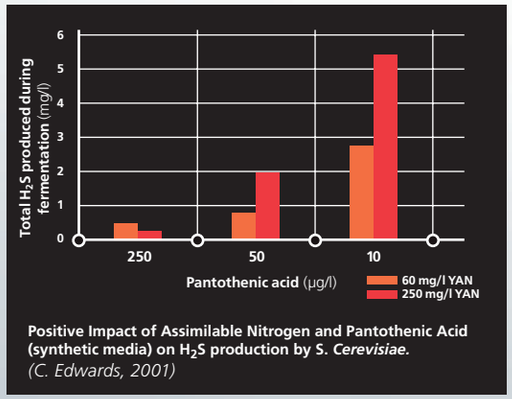
Vitamins are organic compounds essential for the optimum growth of yeasts cells and for their influence to survive under stressful conditions. A deficiency in vitamins can induce sudden changes in the fermentation kinetics. The majority of them act as enzymatic cofactors, they can also intervene in energy transfers or in supporting the membrane integrity.



Biotin, for example, favours the production of esters and allows a better cellular viability for the fermentation. When biotin is deficient, the cellular growth is affected significantly.

Pantothenic acid (Vitamin B5) helps avoid the production of H_2S, as well as volatile acidity.

Finally, the function of thiamine has been the subject of numerous studies. It is another vitamin that can be limited and when deficient can cause stuck fermentation. The concentration of thiamine has a large influence on the fermentation kinetics; its presence increases the production of yeast biomass and the speed of fermentation (Bataillon et *al., 1996).*

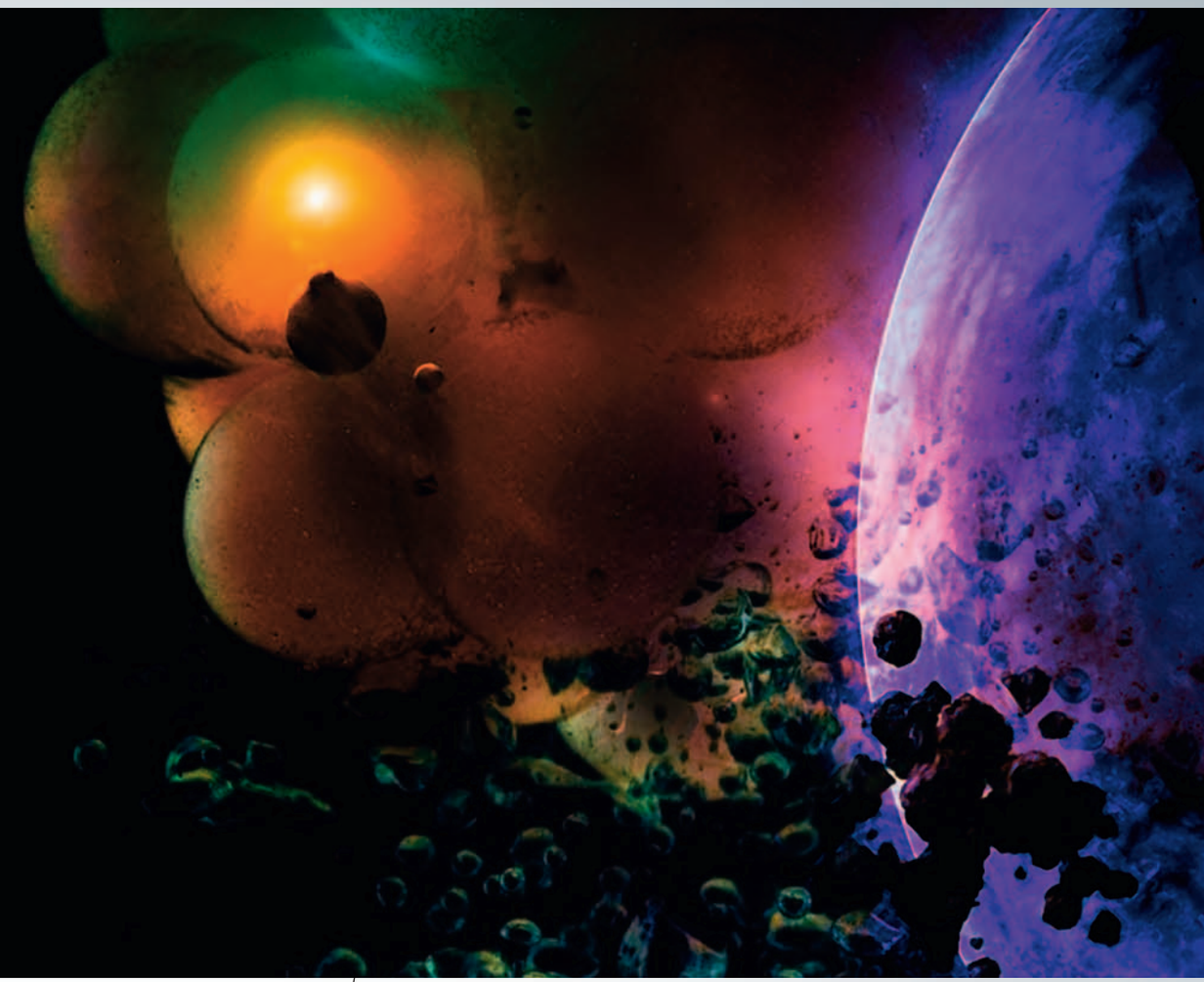


Positive Impact of Assimilable Nitrogen and Pantothenic Acid (synthetic media) on H_2S production by S. *Cerevisiae.*
(C. Edwards, 2001)

Availability of vitamins in the must

Most of the musts initially contain the principal vitamins needed by yeasts but their concentrations change significantly. Some of them such as thiamine and biotin are consumed during the first phase of the fermentation, whereas others, such as pyridoxine, pantothenic acid, nicotinic acid, and riboflavin, remain present during the stationary phase. Thiamine can combine with SO_2 and therefore it will not be available to the yeast. Also, it can disappear rapidly from the medium. It has been demonstrated that in the presence of 10 x10^6 cell/mL of *S. cerevisiae*, practically all of the thiamine can disappear within a few hours or even faster in the presence of indigenous flora (ex. *Kloeckera apiculata*). During the pre-fermentation stage, the indigenous microorganisms consume a big part of the principal vitamins, hence the importance of adding key vitamins. Also, some deficiency is often linked to certain pre-fermentative treatments along with uncontrolled SO_2 additions and temperatures.

In conclusion for Chapter 1, the nutrient balance in a must is influenced by the health status of the grapes, the yield, the level of ripeness, the soil, the climate and other viticulture practices.
Every grape harvest contributes its own variability so it is very important to reduce the risk of sluggish, stuck or sensory deviations in the fermentations. One of the keys for proper fermentation management is a appropriate nutritional strategy.



11. Selection and Proper Handling of Yeast

Winemakers now can select the appropriate specialty yeast according to their objectives and the characteristics of the must (i.e. potential alcohol, assimilable nitrogen content, turbidity, temperature, etc...). These specialty yeasts should ferment well under normal winemaking demands including their reliability to finish the fermentation as well as their ability to reveal the grapes potential.

11.1 YSEO® Yeast

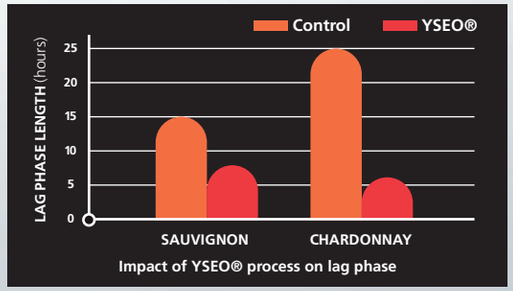
In recent years, the use of selected yeasts in winemaking has become widespread, allowing greater influence of the alcoholic fermentation and reducing the risk of unwanted sensory faults that result from the growth and metabolism of contaminating indigenous yeast. Most of the indignous yeasts are not able to finish fermentation so the choice of the yeast is absolutely fundamental: it is an additional tool to achieve a particular wine style.

Selecting the most appropriate yeast strains based on the must conditions and the type of wine style desired is an important winemaking decision. Lallemand's know-how is focused on fermentation. Since the 1970's, this expertise has been successfully applied to oenology via the selection, characterization and production of natural yeast strains. In partnership with national and regional research institutes, these selected yeasts meet the needs voiced by winemakers for yeasts that will allow them to control alcoholic fermentation, promote the expression of a particular style of wine as well as respect terroir specificities. Today, Lallemand produces over 150 oenological yeasts trying to keep up with the winemaking demands of having reliable fermentations while revealing some varietal or fermentation aromas, impacting the texture, preserving the colour potential as well as other specific winemaking objectives. However, winemaking conditions today are more and more difficult for yeasts due to climate changes, higher levels of sugar resulting in higher alcohol potentials, increased must pHs. This led Lallemand to find new ways to improve alcoholic fermentation and the yeast's ability to thrive under the more difficult conditions. YSEO® (Yeast Security Optimization) is a fully optimized production process from the multiplication through the drying step, with a particular focus on how to feed the yeasts to make them more resilient to more difficult oenological conditions. Simply described, this process optimizes the timing for the addition of nutrients (i.e. nitrogen containing molecules, vitamins and trace minerals) during yeast growth. This new process optimizes the reliability of alcoholic fermentation with a shorter lag phase and lowers the risk of sulfur like off aromas due to better yeast implantation in the must (Bohlscheid, J. et *al, 2007)*. Laboratory and field trials over the previous years have demonstrated that under variable must nutrient conditions, yeasts made by the YSEO® process resulted in steady and complete fermentations along with decreased H_2S and volatile acidity production. In addition, the YSEO® process appears to lower antagonisms against malolactic bacteria (MLB) resulting in a more reliable malolactic fermentation (MLF).

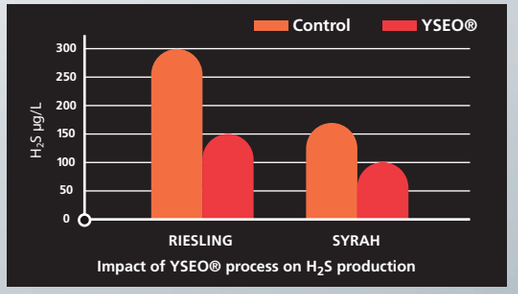
Testimony | *Dr Charles Edwards*

Dr. Charles Edwards - Washington State University: "Our role in the YSEO® yeast project has been validation. In the first years, maybe two years, we did a number of research trials in our laboratory looking at YSEO® under various conditions and what we found overall was that the YSEO® process seemed to improve the quality of the yeast performance during fermentation, very specifically we saw overall enhancements in fermentation rate but also in such key parameters as H_2S production. Yeasts prepared using the YSEO® process yielded less H_2S as compared to the same strain processed by conventional means. To conduct the commercial scale fermentations, we began with two fermentors for the red wines. The winemakers did like the quality of the wines produced by the YSEO® process so we had the opportunity to continue the research in year 2 and 3 with each of those years increasing the volume of the wines fermented with YSEO® prepared yeasts. Overall we did see a reduction in the fermentation time to complete fermentation but in addition the winemakers did note that in the standard yeast preparations, they would occasionally smell hydrogen sulphide whereas this was an observation never made with the YSEO® process."

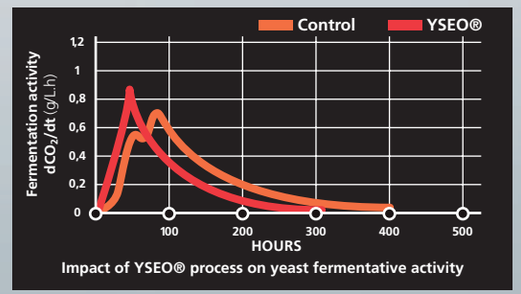
Note: YSEO® does not replace a good yeast nutrition strategy, proper yeast protection during rehydration and good fermentation practices such as temperature management which are all essential to optimize fermentation management especially under difficult conditions.



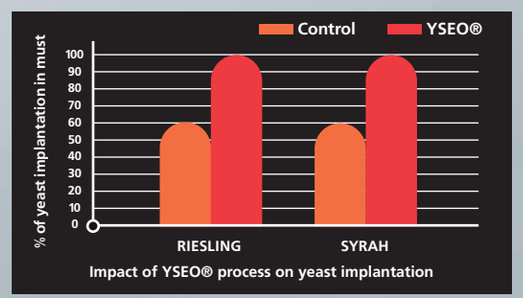
Impact of YSEO® process on lag phase



Impact of YSEO® process on H_2S production



Impact of YSEO® process on yeast fermentative activity



Impact of YSEO® process on yeast implantation

11.2 Specific Selected Yeast: Characterization of Nitrogen Requirements

Lallemand oenological research is driven by the various criteria to be considered during yeast selection and its adaptation to difficult fermentation conditions. The study of yeast resilience to the different stresses encountered during fermentations such as high ethanol levels, low pH, unbalanced nutrient pool and extreme temperatures is especially important. Different yeasts fermentative performance under different winemaking conditions is highly variable from one yeast to another (Jiranek et *al.*, 1991; Manginot et *al.*, 1998; Julien et *al.*, 2000).

Since 2001, a laboratory scale system for monitoring the CO_2 released during the alcoholic fermentation is used. This powerful tool allows Lallemand to evaluate selected yeast performance under variable conditions such as in nitrogen deficient must. Yeast nitrogen requirements are estimated individually during the stationary fermentation phase where nitrogen additions are most effective (Bely et *al., 1991)*. By constant tracking of fermentation kinetics by the CO_2 released, it is possible to compare the amount of nitrogen required by different yeasts in order to ferment a nitrogen deficient synthetic must at the same fermentation rate. One hundred and fifty different commercial wine yeasts are now categorized for their relative nitrogen requirements and, under the same conditions, some strains required three times more nitrogen than others to ferment at the same speed nitrogen deficient must. Knowing that only 1 gram of CO_2 produced by yeast represents 2.17 g of sugar consumed by yeast, we expressed all results as mg of nitrogen required for the yeast to consume 1 g of sugar. The variability of the nitrogen requirements by yeasts tested is shown in figure 1. These nitrogen requirements have been grouped according to their relative nitrogen requirements on a scale of 1 to 6:

- Group 1: very low nitrogen requirements
- Group 2: low nitrogen requirements
- Group 3: low to medium nitrogen requirements

These 2 groups (2 & 3) represent more than 50% of the selected yeasts studied.

- Group 4: medium to high nitrogen requirements
- Group 5: high nitrogen requirements
- Group 6: extreme nitrogen requirements

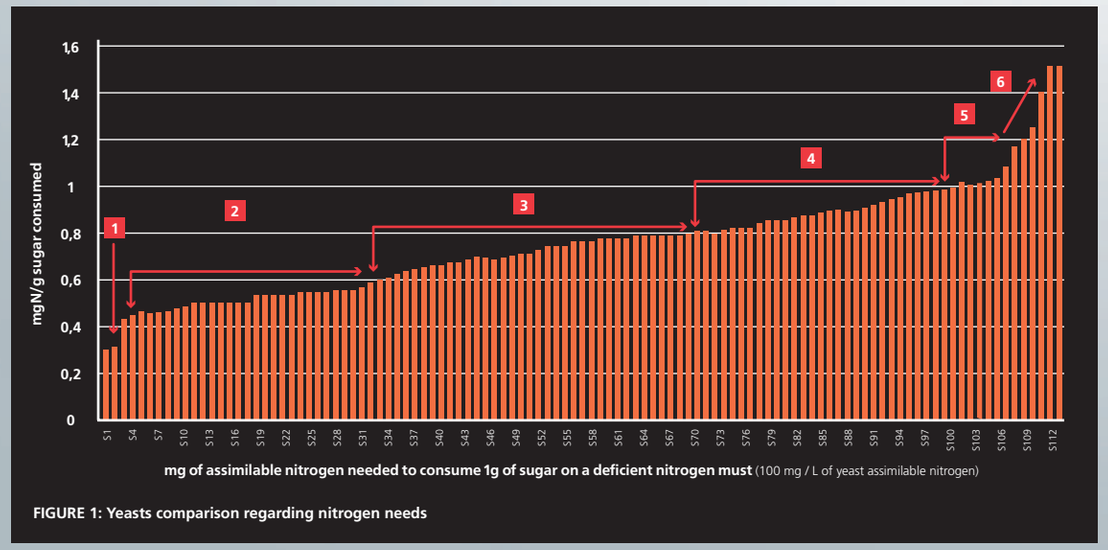


FIGURE 1: Yeasts comparison regarding nitrogen needs

To assess the fermentation performance of the different Lallemand yeast strains and the efficiency of nutrients, a system for online monitoring of fermentation has been developed in close collaboration with INRA Montpellier (thanks to the expertise of the Team UMR-SPO).

11.3 Yeast Rehydration Optimization: NATSTEP® Process

(PATENT WO/2006/053994)

Rehydration is a crucial phase for the survival and efficiency of the selected yeast: it often happens that more than half of the yeasts population will die after a poor rehydration.

What happens during rehydration?

During yeast rehydration, the active dry yeast will absorb water and recover their original form. The organs inside the cell continue rehydrating and a part of them disperse in the rehydration water. This loss can represent between 20-30 % of the dry weight of the yeast and can result in a micronutrient deficit.

Like any living organism, the yeast cell is separated from the extracellular medium by a cytoplasmic membrane. Under oenological conditions, this membrane separates the interior of the cell from grape must, then from wine. The cytoplasmic membrane is made up of a double-layered lamellate, mainly made of lipids that create an extremely hydrophobic barrier between the interior of the cell and the external fermenting must.

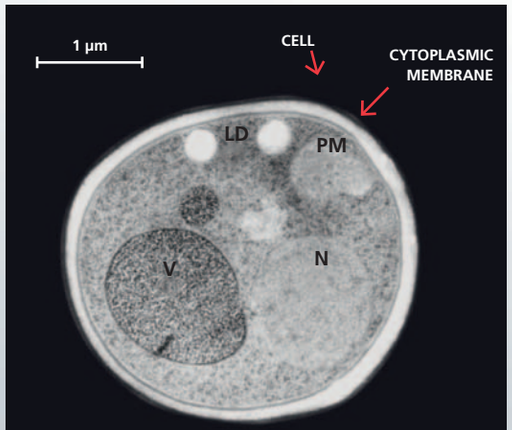
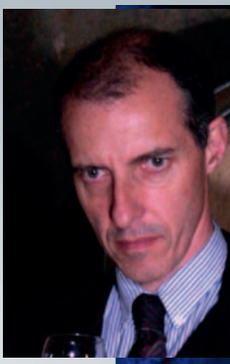


FIGURE 1 : Electronic microscopy of a yeast *Saccharomyces cerevisiae* during alcoholic fermentation.
PM: promitochondrie, N: nucleus, V: vacuole, LD: lipid inclusions. Plasma membrane is presented in a double-layer lamellate of lipid compounds.

Testimony | *Dr Jean-Michel Salmon*



Dr. Jean-Michel Salmon - UMR Sciences pour l'œnologie - Microbiology and Fermentation Technology Research Director - Institut National de Recherche Agronomique (INRA - Montpellier -France): Yeast survival in wine conditions is a constant challenge. The grape and wine are extremely hostile environments and difficult to ferment for yeast: very strong acidity (one of the strongest of the vegetable world), high alcohol content during the fermentation, environments with relatively low nitrogen but rich in sugars (the highest in the plant world). Yeast is the best micro-organism to carry out the fermentation, because it has adapted to its environment. The plasma membrane, consisting mainly of lipids, including sterols which are the first barrier, plays a key role against adverse environmental conditions. Strengthening the yeast sterol reserves during rehydration helps to develop early «protectors» and the yeast has more chance to resist a hostile environment and complete the fermentation of sugars while maintaining the sensory properties of the wine. The research results from our collaboration with Lallemand resulted in a joint patent on the NATSTEP® process.

However, the cell must be able to multiply to ensure its growth: this cytoplasmic membrane also has proteins for nutrient transport necessary to maintain an active fermentative metabolism. This cytoplasmic membrane represents an extremely effective barrier to the entry of many external molecules in the cell other than small hydrophobic or lipophilic molecules and protons (H+). To fight against the passive diffusion of these protons from the external medium towards the interior of the cell, the cytoplasmic membrane also has a very important enzymatic activity for the survival of the cell during oenological fermentation: the ATPase proton pump activity. Like all living organisms, the yeast cell must maintain its level of intracellular acidity close to neutral (pH 7: low level of protons) whereas the fermenting must has a very strong acidity (pH 3: critically high level of protons).

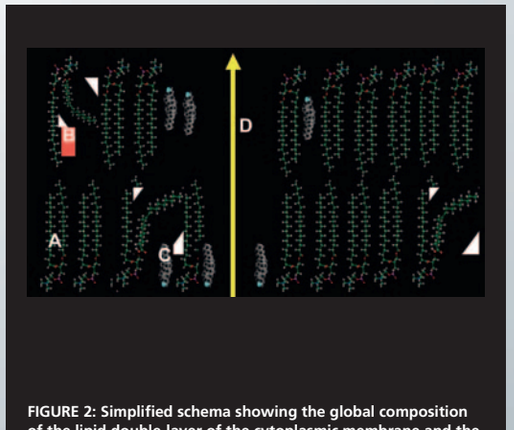
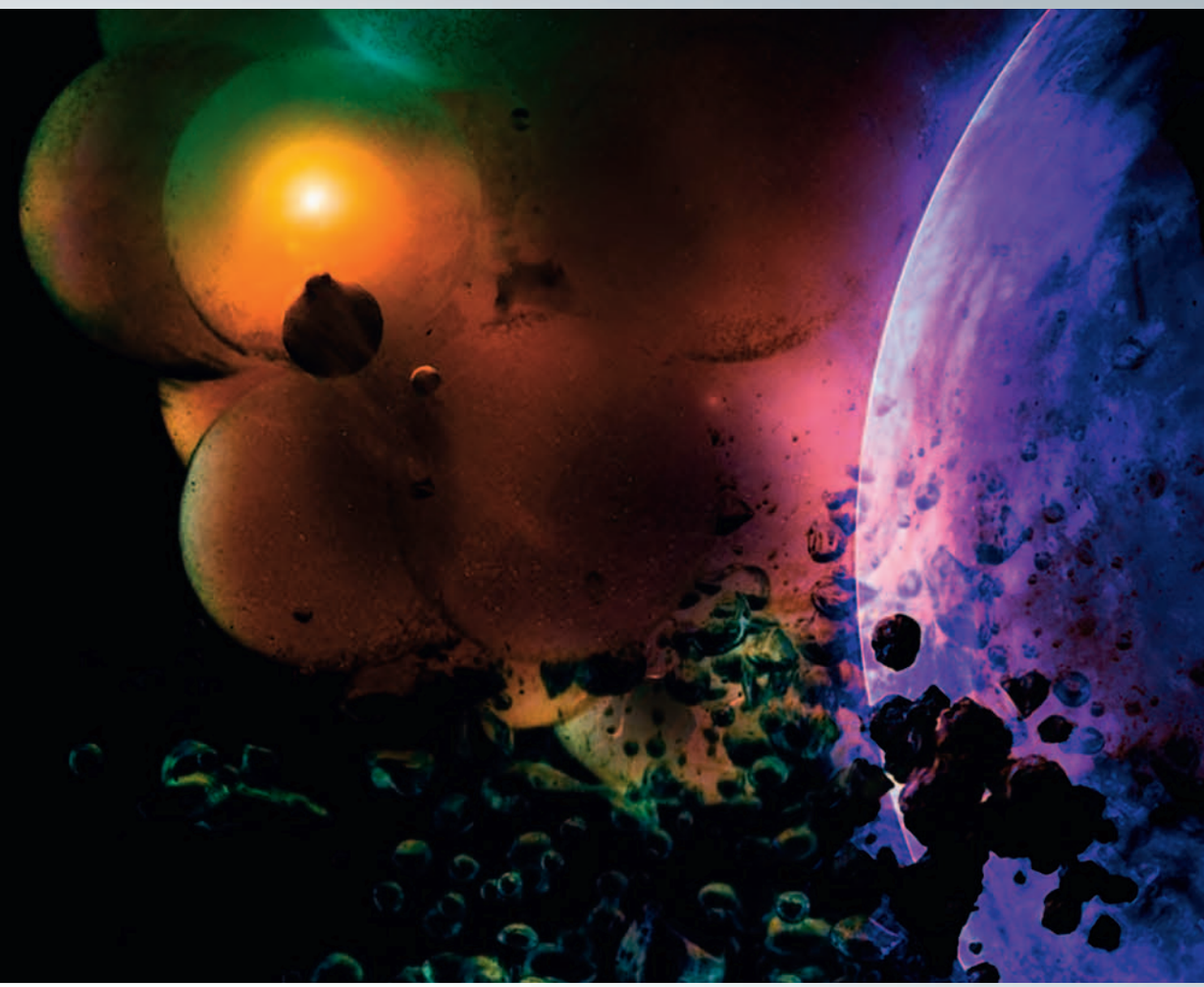


FIGURE 2: Simplified schema showing the global composition of the lipid double-layer of the cytoplasmic membrane and the role of the sterols in the membrane stabilisation.
A: phospholipids of saturated fatty acids chain
B: phospholipids of unsaturated fatty acids chain
C: sterols
D: transport protein integrated in the cytoplasmic membrane

11.3 .../...

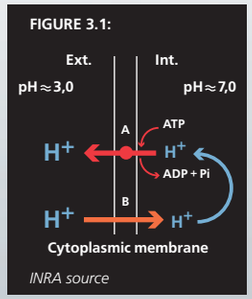


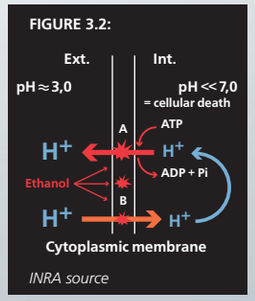
Sterols: membrane survival factors

The cytoplasmic membrane is mainly composed of a double-layered of phospholipids whose absorbent heads are oriented towards the outside of the membrane while their tails are directed towards the interior of this membrane in order to form a hydrophobic zone *(Figure 2)*. This architecture because of it's mainly lipid nature, remains very fluid and subjected to many physical and/or physicochemical aggressions of the external medium. It is stabilized consequently by small molecules whose absorbent heads are also exposed towards the outside of the membrane. These sterols are distributed in an uneven way throughout the membrane and present zones of stronger density around the majority of transport proteins integrated within the cytoplasmic membrane. From their rigid structure, these sterols offer the proteins that they surround an environment more structured and less prone to strong variations of fluidity, which helps them to properly function.

Ethanol molecules exert a toxic effect on the cytoplasmic membrane

During alcoholic fermentation, fermentable sugars are transformed into carbon dioxide and ethanol by the fermentative metabolism of yeast. The ethanol is a very small molecule with the chemical properties very similar to those of water and thus likely to replace this water in many enzymatic reactions of the metabolism. This molecule, from its physicochemical properties, diffuses extremely easily and freely in the cytoplasmic membrane, where it comes to replace water molecules and thus to interfere with the interactions existing between proteins and phospholipids within this membrane. The insertion of ethanol in high quantities in the cytoplasmic membrane tends to make the membrane more permeable to protons and reduces the activity of transport proteins such as the ATPase proton pump activity *(Figure 3)*. The cell, to compensate this increasing entry of protons, must spend more energy in the form of ATP to maintain a good viability. When the balance between the protons passive entry (favoured by alcohol) and the active excretion of protons (under dependence of the cellular metabolism) is not maintained any more, the cell ends up dying by intracellular acidification *(Figure 3.2)*. Ethanol toxicity has a progressive and direct impact on yeast metabolism and at extremely high or low temperatures, this impact is more severe.

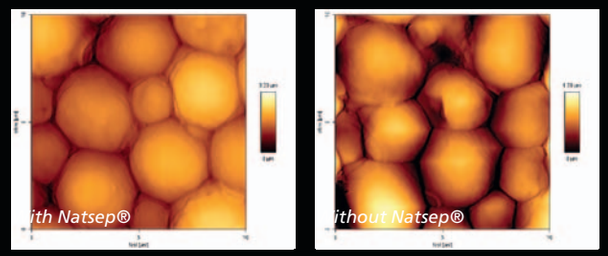




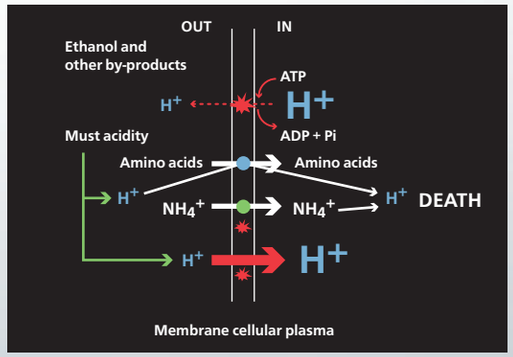
Can we protect the yeast, and when should it be done?

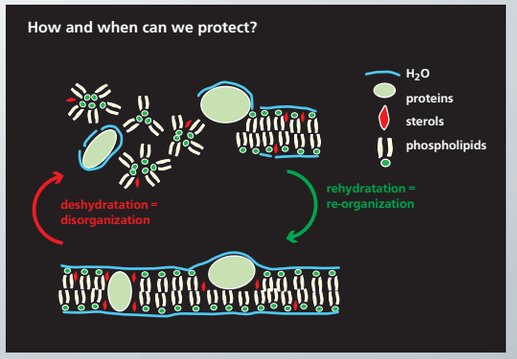
Yeast sterols added during the active dry yeast (ADY) rehydration are particularly efficient at stimulating fermentation. The polysaccharides, sterols and phospholipids found in inactive dry yeast take a specific micelle form allowing the incorporation of these sterols into their cell membrane. These emulsifying micelles are found at around half way through the rehydration procedure and at about a concentration of 100 mg/L (emulsifying micelles). At this concentration, they interact rapidly with the cellular membrane of the active dry yeast, modifying the membrane cellular plasma, therefore increasing the sterols content in the rehydrated yeast (Soubeyrand V et *al.* 2005; Luparia V et *al.* 2004).

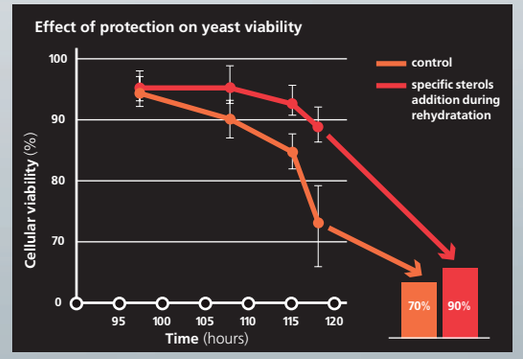
11.3 .../...

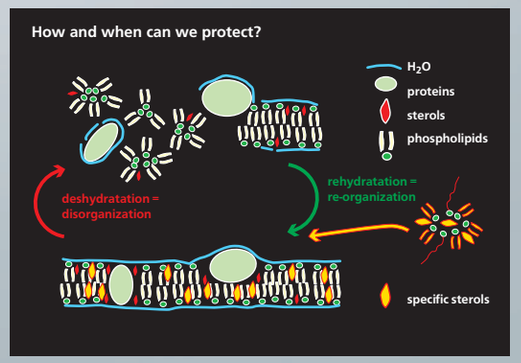


Introducing yeast into an aggressive must and consequently running the risk of seeing them perish, represents a qualitative and economic loss. Protecting the yeast by supplementing with sterols and polyunsaturated fatty acids increases their chances of survival while optimising their sensory impact. During the rehydration phase, protected yeast maintains a very high viability rate. Its membrane is ultimately able to withstand high concentrations of alcohol and prevent the alcohol from entering the cells. With this protection, the yeast can finish consuming all the sugars in the must. Healthy and stress-free yeast cells do not produce excessive amounts of undesirable compounds or volatile acidity. The micronutrients (vitamins and minerals) are absorbed by the yeast cells from the rehydration water, with the dry yeast cells acting like sponges absorbing the water, causing their cell volume to increase 1.5 times. This allows the yeast cells to readily reactivate their internal metabolism. The micro-protectors, specific sterols and polyunsaturated fatty acids (PUFA), gradually integrate into the yeast's cellular membrane, strengthening it and facilitating exchanges with the exterior, thereby preventing the loss of internal cell material. During incorporation into the must, the yeast suffers osmotic shock due to the high sugar concentration in the must. The specific micro-protectors protect the yeast, helping it resist osmotic shock during incorporation into the must: the yeast can acclimatize better. As a result, the yeast produces less volatile acidity and sulfur-like compounds that influence the final wine quality. During the yeast multiplication phase, the inoculated yeast will transfer part of its own cellular material to the following generations of daughter cells, gradually reducing the thickness of their cell membrane in each successive generation. To protect yeast during fermentation, Lallemand in collaboration with INRA have developed and patented the NATSTEP® process (WO/2006/053994). This process consists of adding specific inactivated yeast enriched in sterols during the rehydration of the selected yeast. This protection gives the yeast a higher rate of survival, increases its capacity to resist alcohol and ensures a reliable fermentation finish while limiting sensory deviations, especially in high potential alcohol or low turbidity musts. Protecting the yeasts will reduce their stress at the end of fermentation and allow them to better compete against potential contaminating flora and subsequent risk of *Brettanomyces* and other spoilage organisms.









III. Yeast Nutrition Management

Testimony | *Dr Jean-Marie Sablayrolles*

Dr. Jean-Marie Sablayrolles - UMR Sciences pour l'œnologie - Œnology Director – Institut National de Recherche Agronomique (INRA - Montpellier -France): "In fermentation, the nitrogen content of the must affects the yeast population but also the kinetics of sugars transport by yeasts. But this rate of transport is often the limiting step of the fermentation kinetics. Thus, the nitrogen content directly affects the speed of fermentation. When fermenting, the available nitrogen is consumed very quickly and the rate of protein synthesis and the rate of sugars transport fall rapidly.

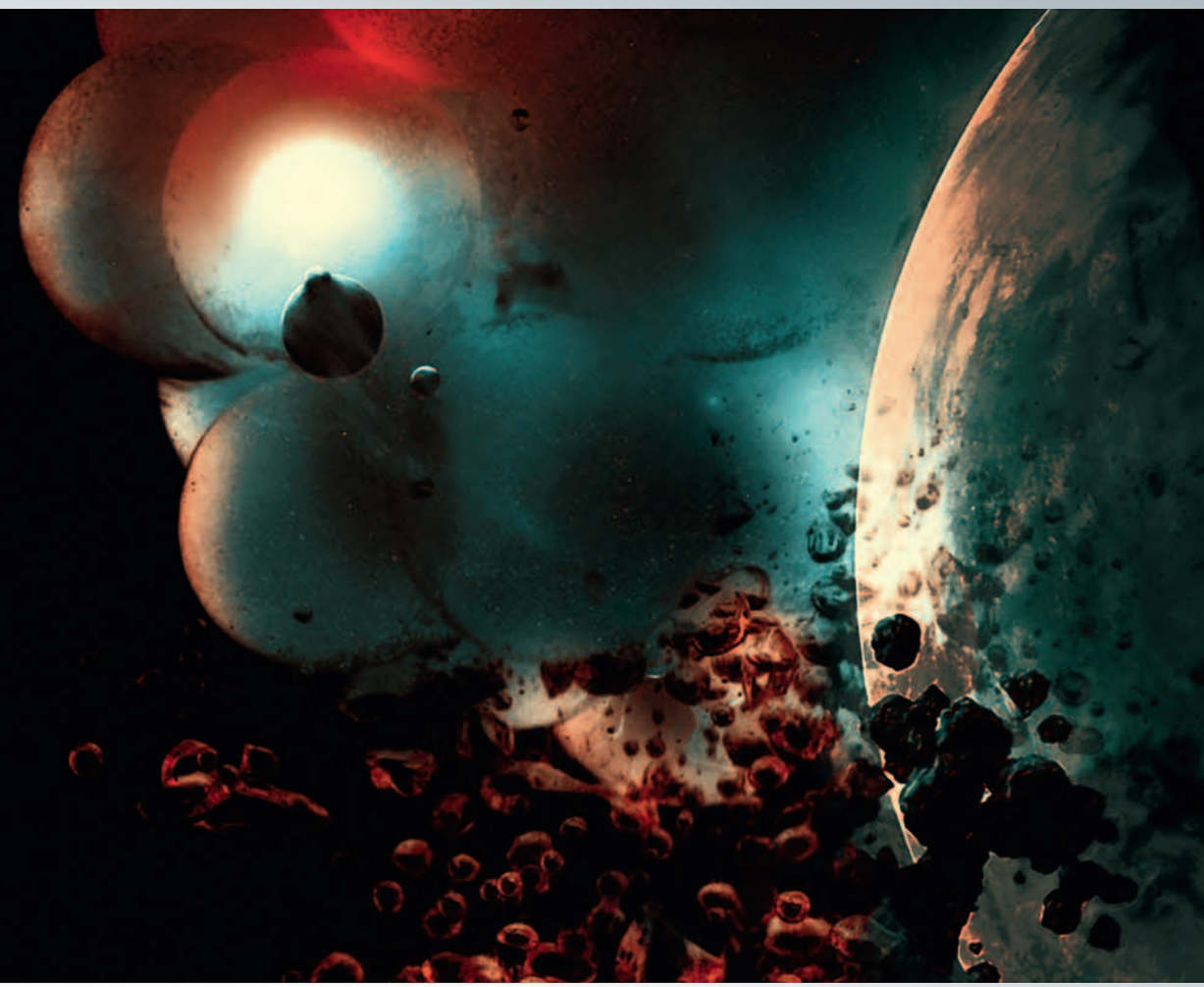
To overcome these deficiencies, additions of nitrogen or nitrogen sources (such as complex nutrients) can result in very significant shortening of the duration of fermentation. The addition of nitrogen increases the speed rapidly with a reactivation of protein synthesis and particularly sugar transporters. It is important to note that only an addition of nitrogen made at the beginning of the fermentation causes an increase in yeast population.

From a technological standpoint, the best time to perform this addition is approximately 1/3 of the way through the alcoholic fermentation: this time is also optimal for adding oxygen or survival factors. In cases of extreme deficiency in available nitrogen, adding half at the beginning of the AF and the balance at 1/3 of the way through the AF is an effective solution.

Finally, it is important to distinguish two related problems of fermentation: sluggish fermentation and stuck fermentation. Indeed, sluggish is the result of a deficiency in available nitrogen resulting in a low rate throughout the fermentation. Stuck fermentations occur when yeasts are dead, not due to a nitrogen deficiency, but to a deficiency of survival factors such as sterols and oxygen."

III.1 The Importance of Complex Nutrients such as Fermaid®

A complex nutrient is a blend of ammonium salts, thiamine, vitamins, minerals and specific inactivated yeast, which are a natural source of amino acids. Lallemand as a yeast producer tests a large range of yeasts that can be inactivated in order to select the most appropriate ones able to supplement naturally these compounds to the must during alcoholic fermentation.



The objective of these complex nutrients is to add this integrated nutrition to the yeast in case of must YAN deficiencies. The assimilable nitrogen is supplied by two sources: alpha amino acids and ammonium. Depending on the YAN content and sugar (table page 29), one or two additions is recommended.

- If only one addition, it should be at 1/3 of the way through the alcoholic fermentation.
- If two additions, it should be at the beginning, and then at 1/3 of the way through the alcoholic fermentation.

When one addition is used, the timing of addition will provide the selected yeast with growth factors necessary to assure the reactivation of transport proteins synthesis and activity when the must is depleted in assimilable nitrogen. Complex nutrients containing high levels of amino acids will help the yeast growth from the beginning of the alcoholic fermentation and also to maintain a good fermentative activity, when added at roughly 1/3 of the way through fermentation. By minimizing the yeasts exposure to ethanol stress towards the end of fermentation, winemakers are decreasing the risk of stuck and sluggish fermentation and preventing sulfur like compounds formation.
To summarize, complex nutrients such as Fermaid® will have an effect on cellular growth and fermentative activity, improving yeast multiplication, sugar consumption and aroma expression.

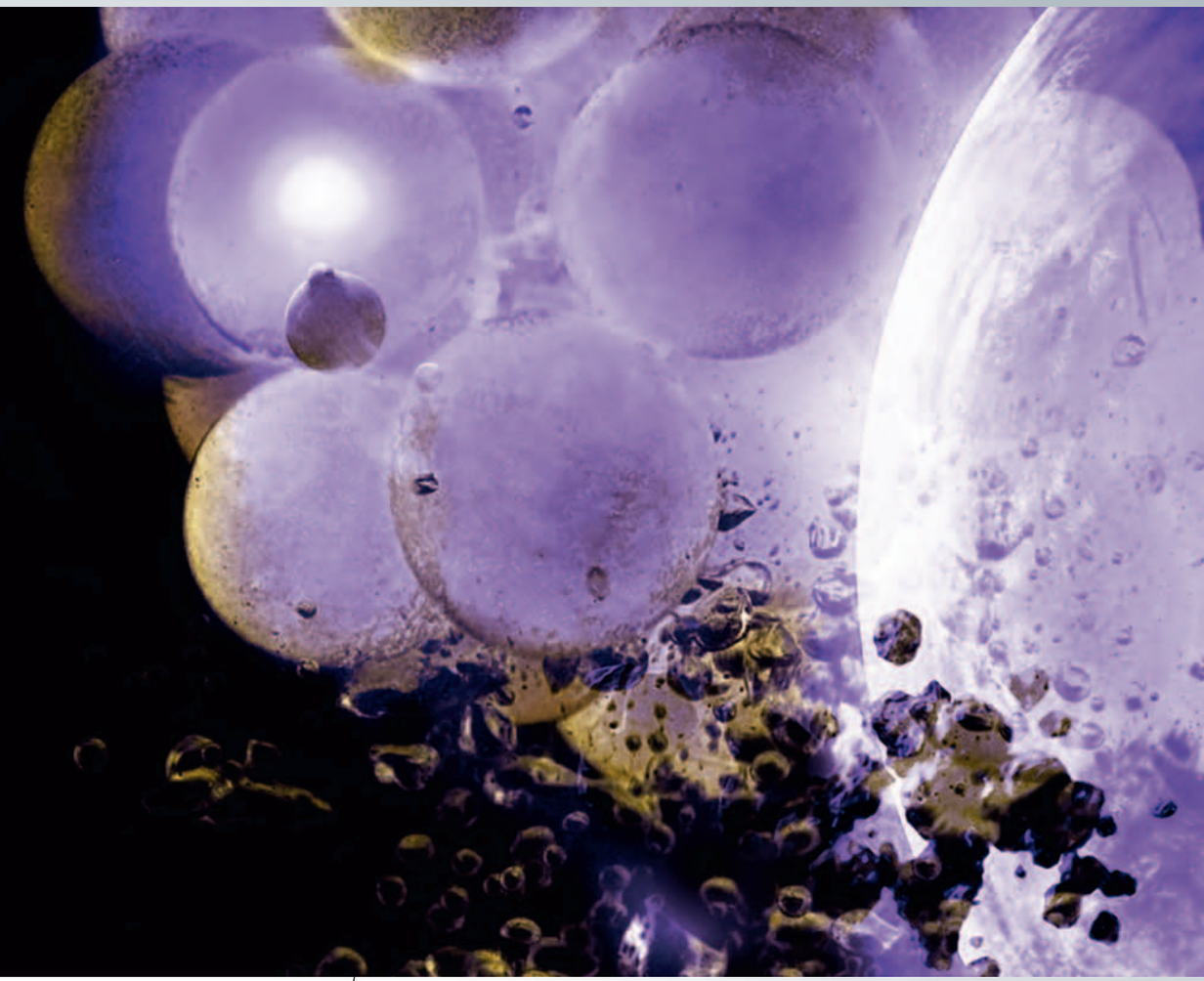
Protection and Nutrition Comparison Chart

30G/HL	NATSTEP PROTECTION	FERMAID COMPLEX NUTRITION	FERMAID ORGANIC NUTRITION	DAP INORGANIC NUTRITION
YAN	8-10 mg/L	25-42 mg/L	12 mg/L	63 mg/L
Amino acids	+	+	+++	-
N_2 Inorganic	-	++	-	+++
Sterols	+++	+	+	-
Minerals	++	++	+	-
Vitamins	++	+++	+	-
Addition time	Rehydration	Beginning and/or 1/3 AF	Beginning & 1/3 AF	1/3 AF
Sensorial impact	+++	+++	+++	H_2S, VA

- None *++ Moderate*
+ Low *+++ High*

IV. Stuck Fermentation

Dr. Paul Monk used to say, "The best solution for a stuck fermentation is prevention," but it seems like no matter what preventive steps you take, problem ferments still occur. For difficult conditions, use the traditional method that involves the winemaker gradually adapting the yeast to the antagonistic wine environment. Whichever restart method you decide to use, reacting quickly is very important once the stuck fermentation is discovered.



IV.1 Prepare the Stuck wine

Take the necessary precautions to avoid growth of spoilage bacteria by adding SO_2 and/or Lallzyme Lysozyme™.

The addition of Nutrient Vit end™ at 40 g/hL (3.2 lb/1000 gal) helps remove potential inhibitory substances in the wine. Suspend Nutrient Vit end™ in warm water, gently stir the suspension into the stuck wine, and allow Nutrient Vit end™ to settle for 48 hours, then rack or filter.

IV.2 Prepare the rescue yeast

Select a "rescue" yeast that is both alcohol tolerant and a vigorous fermenter, such as a highly fructophilic yeast.

Calculate the amount of yeast required for the total volume of stuck wine at 50 g/hL (4 lb/1000 gal). Use twice this amount, 100 g/hL, if you lack good temperature control in the cellar. Calculate the amount of NATSTEP® protectant required (1.25 times the weight of yeast). Suspend the NATSTEP® in 20 times its weight of 43°C (110°F) clean water (approximately 2 litres water for each 100 g NATSTEP®). Mix gently and allow the NATSTEP® solution to cool to 35-40°C (95-104°F). When the temperature has cooled, sprinkle the rescue yeast on the NATSTEP®/water suspension. Stir very gently to mix and avoid clumping. Let suspension stand for 15 to 30 minutes before adding to initial wine/water/sugar mixture.

IV.3 Activate the prepared rescue yeast with nutrients and sugar

The nutrient content of the stuck fermentation will most likely be quite low and unable to support adequate yeast growth. In addition, the rescue yeast culture will require adaptation to the alcohol content of the wine.

Prepare the following initial starter mixture and adjust to 25–30°C (77–86°F):

A. 2.5% of volume of stuck wine (25 hL/1000 gal)
B. 2.5% of volume as water (25 hL/1000 gal)
C. 50 g complex nutrition/hL wine and water mix (4 lb/1000 gal)
D. Adjust sugar level of this mixture to 5°Brix with juice, concentrate or sugar.

IV.4 Restart the fermentation and sequential addition of the stuck wine

A. Slowly add the NATSTEP®/rehydrated rescue yeast suspension to this wine/water/sugar mix and maintain the temperature at 20-24°C (68-75°F).
B. Monitor the sugar level of the starter. When the sugar level has dropped by half (approximately 2.5°Brix), begin to add the stuck wine to the starter and maintain between 20-24°C (68-75°F).
C. Add 25 g/hL (2 lb/1000 gal) of Nutrient Vit end™ to each batch prior to adding to the starter. The correct time to add a new batch is when the sugar from the previous addition has decreased by half. Only at the last batch of added stuck wine should the sugar be allowed to completely deplete.

Note: When starting stuck fermentations in barrels, the initial starter mixture can be apportioned to 20% of the barrels, expanding the number of barrels at each stage.

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Glossary

AA: Amino Acids
ADY: Active Dry Yeast
AF: Alcoholic Fermentation
DAP: Diammonium Phosphate
DAS: Diammonium Sulphate
GAP: General Amino Permease
MCFA: Medium-Chain Fatty-Acids
MLB: Malolactic Bacteria
MLF: Malolactic Fermentation
NTU: Nephelometric Turbidity Unit
PUFA: Polyunsaturated Fatty Acids
VA: Volatile Acidity
YAN: Yeast Assimilable Nitrogen

Lallemand SAS
19, rue des Briquetiers - BP 59 - 31702 Blagnac Cedex
FRANCE

Lallemand Inc. Succ. Italiana
Via Rossini 14/B - 37060 Castel D'Azzano - Verona
ITALY

Lallemand Península Ibérica
C/Zurbano 71, Oficina 6 - 28010 Madrid
SPAIN

Lallemand Fermented Beverages
Zeiselberg 18 - 3550 Langlois
AUSTRIA

Ferment Zagreb
Vincenta iz Kastva 17 - 10 000 Zagreb
CROATIA

Lallemand North America
PO Box 5512 - Petaluma - California 94955
USA

Lallferm S.A.
Pedro Molina 433 - Primer Piso, Oficina 2 - Mendoza 5501
ARGENTINA

Lallemand Inc. Chile
Camino Publico Los Siete Puentes s/n - Bodega N°6
Los Lirios - Requinoa - Rancagua
CHILE

Lallemand Australia
149 Holbrooks Road - Underdale SA 5032
AUSTRALIA

Lallemand South Africa
31 Blousuikerbos Street - Proteavalley - BELLVILLE - 7530
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