APPLICATION REPORT

Yeast nutrients

Can nitrogen in the form of ammonium work?

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Requirements of modern oenology

Vines and other higher plants absorb inorganic nitrogen from the soil primarily as nitrate. This nitrate can be absorbed as fertilizer, for example in the form of ammonium nitrate or potassium nitrate. Organic nitrogen such as from humus, dead plant fragments, and organic fertilizer are also initially converted by bacteria to nitrate via an intermediate ammonium stage and then absorbed by the vine roots.

Theoretically, ammonium – from ammonium-containing fertilizer or from decomposing organic matter – can be absorbed directly. However, in practice, it oxidizes (nitrifies) in the ground before it is absorbed. The vine forms amino acids from the nitrate. Some of these amino acids exist in free form in the plant; some are linked to form shorter or longer chains. Shorter chains are called peptides; longer chains are proteins that include all enzymes. In must, the amino acid content is higher than the protein content. Nitrate is only present in low concentrations of a few milligrams per liter (mg/l).

Some of the nitrogen in the must is present in the form of protein, some in the form of protein decomposition products such as peptides, amino acids or in the form of ammonium. During ripening of the grapes, the nitrogen content increases continuously, so that concentrations of 1 gram per liter (g/l) can be reached in the must. In rare cases, the concentration drops again slightly once a maximum value has been reached. Of great importance is the amino acid proline, since it cannot be metabolized by the yeast during alcoholic fermentation. The quantitative increase in proline depends on the grape variety. This applies in particular to grape ripening in warmer vintages and climates. Interestingly, the ammonium content continuously decreases during the ripening phase, while at the same time the amino acid content (peptides and proteins) increases [1].

Nitrogen compounds in must and their effect on alcoholic fermentation

At the beginning of the alcoholic fermentation, the yeast predominately absorbs ammonium. This inhibits the absorption of amino acids [1,2,6]. Once the ammonium has been used up after approximately 20 to 30 hours of fermentation, the yeast cells absorb alpha-amino acids and metabolize them during the ongoing alcoholic fermentation. Since proline is a secondary amino acid, the yeast cells can neither absorb it nor utilize it.

The key parameter for yeast development and fermentation is therefore not the total nitrogen content, but the proportion that can be utilized by the yeast cells. Various terms and abbreviations are used in the literature [1,2] for the proportion of nitrogen that can be utilized by the yeast. Particularly noteworthy are the terms YAN (yeast assimilable nitrogen) and APA (azeto prontalmente assimilablile). Put simply: It is the sum of ammonium and alpha-amino acids minus proline and hydroxyproline. Other unusable substances are peptides with

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high molecular weight, proteins, and biogenic amines. The ratio of utilizable and non-utilizable nitrogen depends on ratio of arginine and proline, which are the most common amino acids in grapes. For any given total amino acid content the ratio between the two amino acids can vary to such an extent that a sufficient YAN supply must be available (higher concentration of arginine than proline), while another may have insufficient YAN supply (higher concentration of proline than arginine) [9]. To ensure trouble-free fermentation, a YAN ratio of 120 to 140 mg/l is required [1]. In order to prevent the undesirable development of undersupplied musts during fermentation, legislation has approved the application of certain yeast nutrients. These include thiamine (vitamin B_1), yeast cell walls, and ammonium in the form of diammonium hydrogen phosphate (DAP) or ammonium sulphate (DAS).

Primarily the addition of ammonium increases the number of yeast cells and the fermentation speed. This shortens the fermentation time and makes successful and full alcoholic fermentation more likely without delayed fermentation.

How do the yeast nutrients (nitrogen) reach the yeast cells?

Three transport mechanisms are known for substance absorption in yeast cells: diffusion, simple diffusion, and active transport.

During diffusion, substance transport takes place without energy consumption through a concentration gradient. The substance diffuses from the higher concentration to the lower concentration until it reaches complete equalization. CO_2 transport from the cell most likely occurs through diffusion.

Simple diffusion is based on the same principle and is additionally supported by a protein structure. Sucrose, a must sugar, is transported into the yeast cell via this route. The sucrose is first hydrolyzed in the outer cell wall through the protein structure of the cell and then transported into the cell in the form of the monosaccharides glucose and fructose. Since the only substance absorption mechanism during diffusion and simple diffusion is the concentration gradient, the yeast cell absorbs no substances by any other means. For sugar absorption, for example, this means that the sucrose is split and transported into the yeast cell [2,4].

Active transport is based on the transport protein permease (P) in the yeast cell membrane and requires energy, which is supplied by adenosine triphosphate (ATP), the cell's energy source. Through active transport, a substance can be concentrated in the yeast cell against the concentration gradient. Most nitrogen compounds reach the yeast cell via active transport. As a result, the nitrogen content within the yeast cell is higher than outside the cell.

The general amino acid transporter system GAP (general amino acid permease) is an example for active transport of a number of amino acids. The yeast cell has other amino acid-specific transporter systems, which are coupled to proton intake. In other words, an amino acid molecule enters the yeast cell together with a proton (H^+). The proton intake causes an intracellular problem since it disturbs the balance between the pH of the must and the cell.

The difference between the must pH the yeast cell pH is about three units. In other words, the H^+ concentration in the must is 1000 times higher than in the cytoplasm inside the cell[4]. If an H^+ ion is now imported, the pH inside the cell is reduced. To avoid acidification, the cell has to dispose of the protons. This proton export is handled by the enzyme adenosine triphosphatase (ATPase), which serves as a hydrogen ion pump and releases energy in the process (see Fig. 1).

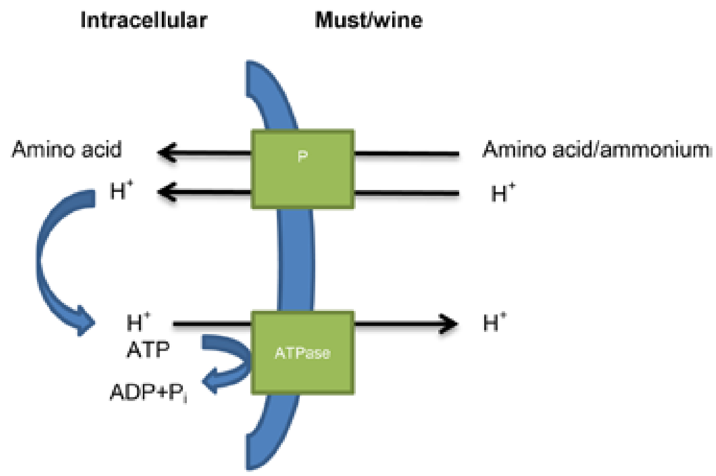


Fig. 1: Intake of amino acids/ammonium in the yeast cell at a low alcohol concentration (low H^+ concentration)

The further the alcoholic fermentation progresses, i.e. the more alcohol is formed, the less ammonium and amino acids the yeast cell can absorb. The cell membrane becomes more and more permeable with the increase in alcohol content and allows high intake of H^+ ions in the cell. To prevent overloading of the H^+ ion exchange, the yeast cell protects itself by blocking the H^+ ion import and therefore the intake of ammonium and amino acids. This mechanism explains why alcohol formation limits the nitrogen absorption (see Fig. 2). It also explains why nitrogen addition at the start of the alcoholic fermentation is more effective than addition at a later stage. In addition, it demonstrates that during active transport the intake of amino acids is highest at the beginning of the alcoholic fermentation. The amino acids can be concentrated in the yeast cell and metabolized later [5,6].

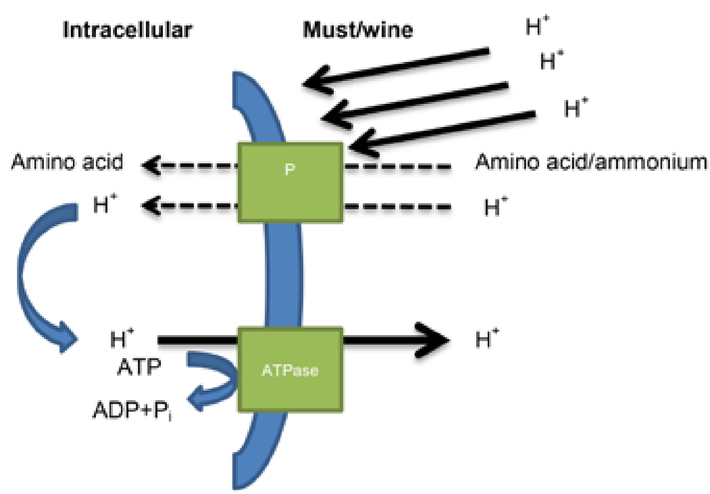


Fig. 2: Intake of amino acids/ammonium in the yeast cell at high alcohol concentration (high H^+ concentration)

Inhibition of amino acid absorption through ammonium

The yeast cell absorbs amino acids through various membrane proteins (transporter systems); a distinction is made between two main routes.

One route is transport through general permease (GAP), which absorbs amino acids in an unspecific manner [4,5]. It is inhibited

The new classification of yeast-based nutrients provides further information about their composition:

	Inactive yeasts (OIV/Oeno 459/2013)	Yeast cell walls/yeast rind preparation (OIV/Oeno 497/2013)	Yeast autolysate (OIV/Oeno 496/2013)	Mannoproteins from yeast extract (OIV/Oeno 26/2004)
Composition	Total N content: < 10% dry matter	Dry matter: ≥ 94% m/m	Total N content: < 12 % dry matter	Total N content: 5 – 75 g/kg
	Ammonium N content: < 0.5% dry matter	Carbohydrates: > 40% m/m	Ammonium N content: < 0.5% dry matter	Rotary capacity: $[\alpha]_D^{20\,°C}$ of the mannoproteins 80° and 150°
	Amino acids + small peptides < 10% dry matter	Total glucan and mannan content: > 60% total carbohydrate content Solubility: < 10% m/v	Amino acids: 1.9 – 3.7% dry matter	
Product examples	SIHA® PROFERM® Fit SIHA PROFERM Red SIHA SpeedFerm®	SIHA PROFERM Bio SIHA PROFERM Plus	SIHA PROFERM Plus SIHA PROFERM Fit	

Table 1: Classification of yeast-based nutrients according to OIV resolutions

by ammonium [5]. GAP therefore only becomes active after a third of the alcoholic fermentation, once the must contains no more free ammonium.

The other amino acid absorption route is via a number of specific permeases [4,5]. Each permease can transport a certain type of amino acid or group of amino acids. Ammonium cannot inhibit these specific permeases. They enable the yeast cell to absorb amino acids from the must during the latent phase at the beginning of fermentation. Since arginine has the highest percentage share of all amino acids in the must [7,8], the intake of this amino acid is highest. The intake of the majority of amino acids is complete when the first 30 g/l of must sugar have been metabolized during the alcoholic fermentation. Within this narrow timeframe, the yeast cell absorbs amino acids, provided energy is available, but the alcohol content is still low. It stores them in the vacuole and only metabolizes them when they are needed [3]. Overall, the yeast metabolizes 1 to 2 g/l of amino acids [7,8].

Why is nitrogen absorption important for the yeast cell?

The yeast can utilize ammonium and free amino acids for multiplication and metabolism processes. Not all amino acids are equally beneficial for the yeast cell. The most important by far is arginine. Arginine provides up to two-thirds of the YAN originating from amino acids. An arginine molecule contains no fewer than four utilizable nitrogen atoms; most other amino acids contain only one.

Which yeast nutrients are approved?

Details of the oenological processes and treatment agents approved in the EU are specified in Regulation (EC) no. 606/2009 of 1 August 2009, which can be accessed free of charge under http://eur-lex.europa.eu.

The following yeast nutrients are permitted: Diammonium phosphate (DAP), ammonium disulphate (DAS) or a combination of both salts up to a limit of 1 g/l (100 g/hl). During sparkling wine production up to 0.3 g/l (30 g/hl) of DAP or DAS may be added for the second fermentation, even if they were already added to the must. Ammonium bisulfite is also permitted up to a limit of 0.2 g/l (20 g/hl), thiamine (vitamin B_1) up to a maximum of 0.6 mg/l (60 mg/hl), and yeast rind preparation up to 0.4 g/l (40 g/hl).

What happens in practice?

All yeast nutrients are used, whereas ammonium compounds in the form of DAP as a single nutrient are more common. Mixtures of DAP and DAS and/or vitamin B_1 (thiamine) can additionally be used as combination compounds, or mixtures of the previously mentioned nutrients and yeast-based nutrients (see Table 1).

The maximum quantity of 1 g/l of DAP delivers 212 mg/l of ammonium. Strictly adhere to the mixing ratio for mixture preparations comprised of DAP, DAS, and thiamine. In some instances, the permitted quantity of 0.6 mg/l (60 mg/hl) of thiamine may be reached by adding as little as 0.5 g/l of a mixed compound. Add additional ammonium as a single nutrient (DAP, DAS) to enrich the must to the upper limit of 100 grams per hectoliter (g/hl).

Adding thiamine is sensible mainly for musts from grapes affected by botrytis since the botrytis fungus will have used up most of the vitamin B_1 contained in the grapes for its metabolism.

Another option is to use ammonium bisulfite. In this case, the must is enriched with ammonium and sulphurated. Regardless of whether liquid sulfur dioxide (SO_2), potassium disulphite ($K_2S_2O_5$), or ammonium bisulfite ($(NH_4)HSO_3$) is used for the sulfurization, the must will subsequently primarily contain hydrogen sulfite or bisulfite, due to its modified pH. The maximum dosage (according to the relevant EU Regulation) of 0.2 g/l of ammonium bisulfite is equal to a dosage of 129 mg/l of SO_2. This includes 28 mg/l of ammonium, which equals an equivalent DAP dosage of approximately 13 g/hl. This indicates that ammonium bisulfite is not effective as the sole yeast nutrient. It should also be noted that alcoholic fermentation is inhibited by adding 0.2 g/l of ammonium bisulfite and 100 mg/l of SO_2.

The addition of yeast-based nutrients such as an inactive yeast product, yeast cell wall, or yeast autolysate means that complex nutrition covers the YAN range of amino acids, minerals, lipids, and sterols. Depending on the selected product, the yeast cell is supplied with a complex, wide range of nutrients. In contrast to DAP/DAS nutrients, yeast-based nutrients can be expected to result in an improved aroma formation (amino acid → higher alcohols) and an increased formation of fruit esters.

How much yeast nutrient (nitrogen) does the yeast cell actually need for alcoholic fermentation?

Based on the assumption that a yeast cell weighs ≈ 10^{-10} g and that ≈ 25% of dry matter with ≈ 8% of nitrogen (N) are available during the fermentation, yeast contains $2*10^{-9}$ mg nitrogen per cell. During optimum and well-supplied alcoholic fermentation, up to 60 million cells/ml can be formed. This equals 60,000,000 cells*($2*10^{-9}$ mg/l of N) = 120 mg/l of N. In other words, optimum supply of 60 million cells/ml requires 120 mg/l of nitrogen (12 g/hl).

The calculation should also take into account that grape must contains nitrogen compounds with a concentration of about 1 g/l. This equals an average ammonium concentration of 80 to 150 mg/l. Adding DAP/DAS nutrients such as 100 g/hl of DAP (comprised of about 50% of P_2O_5 → 460 mg/l of P_2O_5 and approximately 20% of N → 212 mg/l of ammonium), results in a further 212 mg/l of ammonium and a total ammonium concentration of 280 to 350 mg/l in the must. As a consequence, more than twice the quantity of ammonium the yeast cell needs is available. This overdosing inhibits the intake of amino acids, especially the GAP transporter system and in the first few days of fermentation the yeast cell will metabolize mainly ammonium. The yeast cell absorbs the amino acids required for aroma formation only very slowly via the permeases. Another aspect is that adding DAP nutrients increases the phosphate content in the must and the finished wine. A DAP dosage of 1 g/l increases the total phosphate content in the wine by 460 mg/l. This phosphate increase also increases the pH and in conjunction with iron, it can lead to iron-phosphate hazing in the bottle. A DAS dosage of 1 g/l increases the sulphate content in the wine possibly leading to off-flavors, depending on the yeast strain.

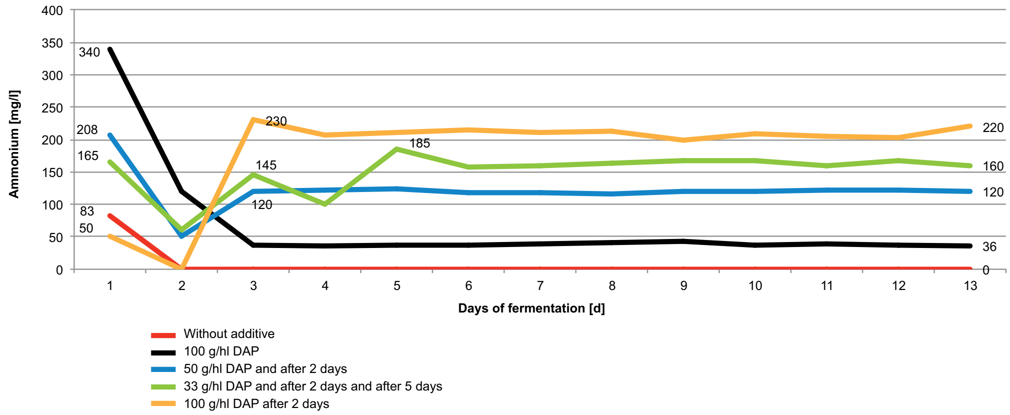


Fig. 3: Decrease of ammonium during alcoholic fermentation – grape variety Pinot Blanc

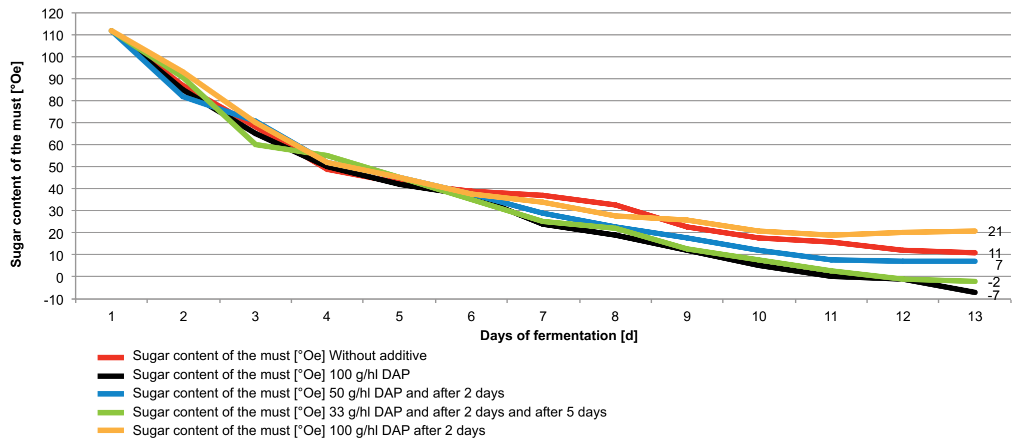


Fig. 4: Decrease of sugar content of the must during alcoholic fermentation – grape variety Pinot Blanc

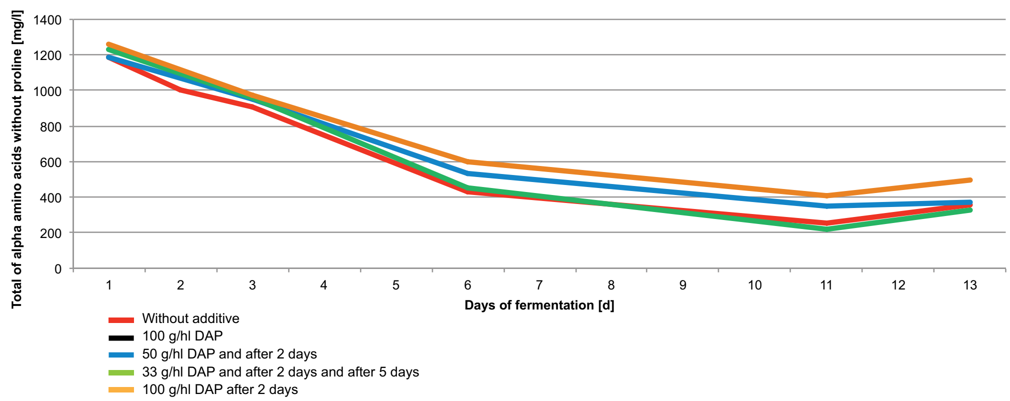


Fig. 5: Decrease in total alpha-amino acid content (without proline) during alcoholic fermentation – grape variety Pinot Blanc

Results encountered in practice

In practice, it is rare to add a single DAP dosage of 100 g/hl at the beginning of the alcoholic fermentation; it's typically added progressively. One option is to halve the quantity and add DAP dosages of 50 g/hl on the first and third day of fermentation. Another option is to add DAP dosages in three stages of 33 g/hl each on the first, third, and fifth day of fermentation (see Fig. 3). The ammonium reduction in Figure 3 indicates ammonium that is added later is no longer metabolized, particularly if DAP nutrients are added in stages. Residual ammonium concentrations of 120 mg/l have been found in fermented wine with the DAP dosing option of 50 g/hl at the beginning of fermentation and after two days. Concentrations of 160 mg/l of ammonium with the option of 33 g/hl of DAP at the beginning of fermentation and after two and five days of fermentation indicate that the ammonium was neither absorbed nor metabolized. The highest concentration of 220 mg/l of non-metabolized ammonium was found with a single dosage of 100 g/hl of DAP after two days of fermentation.

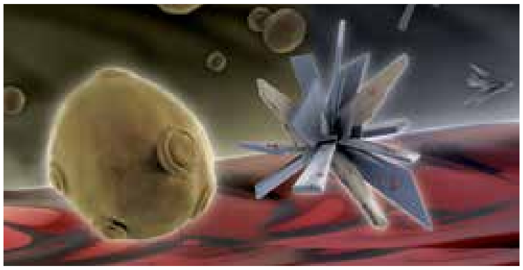
The results for a reduction of sugar content of the must (see Fig. 4) for the individual options correlate with the results for the decrease in ammonium concentrations. The option with the highest ammonium surplus (220 mg/l) showed the highest residual sugar concentration (21 °Oe) at the end of alcoholic fermentation. This corresponds to approx. 52 g/l of residual sugar. The option with 160 mg/l of ammonium contained a residual sugar concentration of 7 °Oe, and thus about 24 g/l of residual sugar. It is remarkable that the option with a DAP dosage of 100 g/hl at the beginning of alcoholic fermentation, the must sugar was fully fermented.

Fig. 5 shows the reduction in total alpha-amino acid content (without proline) during alcoholic fermentation. It is clear that subsequent addition of DAP results in delayed intake of amino acids. This is particularly noticeable with a single dosage of 100 g/hl of DAP after two days of fermentation.

Conclusion

The results of the fermentation trials with musts from the grape variety Pinot Blanc presented in this paper confirm that yeast cells metabolize ammonium during the first 72 hours of alcoholic fermentation [1,9]. Any ammonium that is progressively added later is only absorbed and utilized to a limited extent, depending on the yeast strain and the fermentation conditions. Any ammonium that is not absorbed by the yeast cell remains in the wine at the end of the alcoholic fermentation. Ammonium progressively added later cannot be metabolized effectively and may result in the wine becoming stuck.

It is almost impossible for the yeast cell to absorb amino acids in the presence of high ammonium content (progressive addition) since the transporter systems for ammonium and amino acids are not complementary, but inhibit each other competitively. As indicated in Fig. 3 and 5, this means that the alpha-amino acid intake is only delayed if DAP is added on the second day of fermentation or later. The inhibition of the active amino acid transport results in reduced intake and accumulation of amino acids in the yeast cell. In addition, the increasing alcohol content during the alcoholic fermentation inhibits the amino acid import, since the alcohol formation limits the nitrogen absorption (see Fig. 2). Because the yeast cell requires amino acids for the formation of fruity aromas and the corresponding esters, it is essential that it takes in sufficient quantities of nitrogen compounds to be able to perform the metabolism processes required for aroma formation.



Yeast cell (left) and yeast nutrient (right)

The answer to the question: 'Can nitrogen in the form of ammonium work?' can be clearly answered with 'no.' Balanced, complex nutrition optimally supports the yeast during fermentation of the musts into clean and aromatic wines. This is achieved through the application of ammonium- and yeast-based nutrients such as amino acids, vitamins, minerals, and sterols. The quantity of yeast-based nutrients should be twice that of ammonium-based nutrients.

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