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OPTIMIZATION OF THE ALCOHOLIC FERMENTATION BY CORRELATING THE INITIAL SUGAR CONCENTRATION WITH THE INOCULUM SIZE OF YEASTS AND ASSIMILABLE NITROGEN REQUIREMENTS

George A. COJOCARU, Arina Oana ANTOCE

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Horticulture, Department of Bioengineering of Horti-Viticultural Systems, 59 Mărăşti, District 1, 011464 Bucharest, Romania, cojocaru.george@ymail.com

Corresponding author email: aantoce@yahoo.com

Abstract

It is known from the literature that the yeasts require assimilable nitrogen (YAN) in a certain dosage in order to ferment sugar and for the exact calculation of the nitrogen can be done by applying a certain equation derived from the Bisson and Butzke tables. This equation however does not take into account the yeast strain and its requirements for assimilable nitrogen, nor the possibilities of yeasts to ferment all the available sugar. Taking into account the high concentration of sugar which is more and more found in our grape musts in the later years the selection of yeast and the inoculum size is also important. We have used a 'Feteasca regala' must with various amounts of initial sugar and we have corrected the YAN in accordance with the Bisson and Butzke table for each sample. In order to optimize the fermentation, we tested 3 different yeast strains, used in quantities proportional to the initial sugar concentration. In this way the yeast quantity used is optimized in accordance to sugar fermentation requirements. We have observed that not all the yeasts are able to totally ferment the sugar in samples with 26-28° Brix, leaving in the same time unconsumed YAN. Therefore, the correction of nitrogen should not by applied only in accordance to the calculations, but should be also adapted and limited in the case of high sugar concentration musts. The moment of nitrogen correction, the yeast strain and the inoculum size are evaluated and discussed.

Key words: *fermentation, inoculum, optimization, YAN*

INTRODUCTION

Musts composition and the progress of alcoholic fermentation are essential for the quality of wine, therefore the amount of nutrients for yeast growth found in musts should be in good correlation with the yeast strain and sugar level. To help oenologists make the corrections, Bisson and Butzke (2000), proposed a table with the amount of YAN required for the yeast growth in musts with various sugar concentrations.

Underestimating the YAN requirement and the selection of an inappropriate yeast strain can lead to low rates of fermentation and, consequently, to stuck or sluggish fermentations possibly accompanied by hydrogen sulfide production. On the other hand, an overestimation of nutrient needs, can lead to high rates of fermentations and fast increase of medium temperature due to the rapid yeast multiplication, while the remaining amounts of YAN in wines can lead to microbial spoilage and again the possibility of hydrogen sulfide formation, due this time to a different mechanism than the one involved in the cases of low YAN content. Several authors shows that high concentrations of YAN do not necessarily protect against elevated H_2S formation (Butzke et al., 2011; Sea et al., 1997; Ugliano et al., 2009) and likewise a higher production of total H_2S was observed in musts with high initial concentration of ammonium ions, as compared to non-supplemented musts (Butzke et al., 2011).

Anywise, was confirmed that a typical must require a minimum 140-150 mg N/l YAN to successfully complete the alcoholic fermentation (Henschke et al., 1993; Bell et al., 2005).

Another important factor is the yeast rehydration process that should be done in accordance with producer's specifications. Moreover, the yeast dosage should be well correlated with the sugar concentration of the inoculated must.
This fact was also obverved by Kontkanen et al. (2004) during a research on icewine, that is a higher dosage of yeast forms a higher ethanol concentration and allows for a better rate of fermentation, rather than a sluggish fermentation that usually occurs in wines with high sugar concentrations. In these conditions, for an optimal alcoholic fermentation, oenologists should create the optimal equilibrium between sugar concentration, YAN, yeast inoculum, yeast strain and adequate temperature control.

MATHERIALS AND METHODS

Raw material. White must of 'Feteasca regala' from the experimental vineyard of UASVM Bucharest was used for the study. The physico-chemical parameters of the must are presented in Table 1.

Table 1. Must parameters of the must of 'Feteasca regala'

Harvesting date	09.09.2013
Brix, %	23.75
Titratable acidity, g/l acid tartric	4.58
pH	3.54
YAN, mg/l	68.05
Turbidity, NTU	620

Methods of analyses and equipments: *pH* was determined with a Hanna pH 112 (OIV, 2009b). *Total titratable acidity* (TA) was determined with TitroLine easy Schott Instruments until the end point of titration at pH 7.0 (OIV, 2009a), while the *YAN* was determined using the same titrator and the modified Sørensen method, in which titration with NaOH 0.1 N is performed until reaching the pH = 8.0, after the addition in the must of a solution of 38% formaldehyde with pH = 8.0, so that the amine basic function groups are blocked and the carboxylic acid functions are released (Filipe-Ribeiro et al., 2007; Gump et al., 2002; Shively et al., 2001). *Turbidity* of must was determined with a MRC, model TU-2016 portable turbidimeter by using an official method of OIV (OIV, 2009c). *Degrees brix* were measured with a digital probe refractometer Misco DFR123 by directly immersion of optical sensor in musts at 20°C. *Density* and *temperature* measurements were determined using physical methods. *Reducing sugar* was determined by Luff-Schoorl method (OIV, 2009d). *Alcoholic strength by volume* was determined by distillation and density measurement with a pycnometer (OIV, 2009e).
The growth of the yeasts in the musts was followed by recording the heat evolved in the medium by using an isothermal calorimeter working on the principle of the heat conduction (Antoce, 1998).
This calorimeter consists of 25 calorimetric units, in which 24 microbial cultures can be monitored in several inhibition conditions, while the last one is being used as a reference. A thermopile plate located on the bottom of each unit measures the amount of heat generated in the unit during the microbial growth, as it is transferred to the surrounding aluminum block, which is kept at a constant temperature by circulating water through copper pipes located around it. The heat flux established between the calorimetric unit and surroundings is detected by the thermopile plates (Melcore CF-70.1, New Jersey, SUA) and the difference between each sample and a reference cell is recorded as a voltage signal. The voltage signal is measured for each sample at a fixed time interval by using a Keithley digital voltmeter and a channel scanner. All 24 signals are thus digitalized and stored into a computer database. The specialized software for the data analysis works under Origin General Scientific 2.8v platform and is of in-house design (Antoce et al., 2011).
The recorded growth thermograms were used to calculate *growth rate constants* and times of *growth retardation* of yeast for each experimental culture.
Treatments. Preparation of the must consisted firstly in a pectolytic enzymatic treatment with 3 g/hl, followed by sedimentation of must at 15°C. The concentration of enzymatic product used was of 15200 U/g enzymatic activity including: 10000 U/g pectin lyase, 650 U/g pectin

methyl esterase and 4550 U/g polygalacturonase.

After the sedimentation the must was racked from the lees and corrections of sugar, titratable acidity, pH, turbidity and YAN were performed. Sugar correction was done with inverted sucrose solution prepared, from 2 g/l tartaric acid to 1 kg of sucrose, boiled for 20 minutes.

In order to optimize and correlate the YAN corrections with respect to sugar concentration, experiment was conducted on musts with 18, 20, 22, 24, 26 and 28 Brix, obtained by correction from the initial raw must of 'Feteasca regala' with the parameters presented previously in Table 1.

Titratable acidity and pH were harshly corrected with tartaric acid to create a supplementary stress factor during alcoholic fermentation in order to make our evaluation for the worst case scenario winemakers can encounter in practice and subsequently correct. It is well known that fermentation of musts with very low pH and / or high sugar concentration leads to increased volatile acidity production by yeasts due to the passive ion influx stress and effect of osmotic pressure. Furthermore, the resulted acetic acid can inhibit the growth of yeasts and this situation can lead to a stuck or sluggish fermentation.

YAN adjustment / ***monitoring*** were made in correlation to the sugar concentration in the must samples, based on the correction table of Bisson et al. (2000), but using an equation devrived from it: $YAN\,(minimal), mg/l = 25 \times \%Brix - 350$.

For the YAN adjustment a commercial nutrient was used, consisting of a mixture of a 5 : 3 ammonium sulphate to diammonium hydrogen phosphate. The turbidity of musts was reduced to 100 NTU by using a cellulose filter aid. The composition of the musts resulted after these adjustments were re-analyzed and the physico-chemicals parameters included in Table 4.

Inoculum size. For the yeast inoculation we tried to obey the recommendations found in literature (table 2), which range from 10^5 to 10^7 cells/ml. In order to optimize the inoculum size and correlated it with sugar concentration, for this experiment we created a simple mathematical model, and devising the following equation: $i = v \times b \times 10^6$

where: i – correlated inoculum with % Brix, expressed in cells/ml; v - constant to correlate the inoculum size with the sugar content in must (v = 0.2); b – brix, % determined by refractometry;

Table 2. Recommended startup inoculum sizes

Recommended inoculum, cells/ml	Reference
10^5-10^6	Bisson, 2001
10^5-10^6	Jackson, 2008
1 x 10^6 - 3 x 10^6	Fugelsang, 2007
10^6-10^7	Jacobson, 2006
10^6	Ribéreau-Gayon *et al.*, 2006
10^6	Boulton *et al.*, 1996
*3,8 x 10^6 - 1 x 10^7	Kontkanen *et al.*, 2004
3 x 10^6 - 5 x 10^6	Monk, 1986; Monk, 1997
5 x 10^6	O'Brien *et al.*, 1990

*inoculums tested for icewine production;

Yeast strains. The selection of yeast strains for the experminent was based on availability and yeast oenological traits (Table 3).

Table 3. Oenological characteristics of yeasts

Strain	Bayanus PC	Epernay 2	Premium Blanc 12V
Species	*S. bayanus*	*S. cerevisiae*	*S. cerevisiae*
Origin	-	-	Alsazia region
Alcohol tolerance, % vol.	15	15	13
Alcohol yield (% vol./g of sugar)	0.057	0.058	0.058
Optimum temperature	11-30	12-30	10-35
SO_2 production	medium	low	medium
Action on malic acid (-%)	20-30	35-45	25-35
Glycerol production	medium	high	medium
Aromatic features	Crust bread	Fruity and fresh notes	Varietal expression

Experimental design. The experimental fermentations were conducted in 6 musts with 6 different sugar concentrations (18, 20, 22, 24, 26, 28%), each must being separately inoculated with one of the 3 yeast strains (Bayanus PC, Epernay 2 and Premium Blanc 12V).

Table 4. Quality parameters of 'Feteasca regala' musts with sugar level adjustments, inoculum sizes and yeast strains used in the experiment

Parameter	Sample with 18% sugar	Sample with 20% sugar	Sample with 22% sugar	Sample with 24% sugar	Sample with 26% sugar	Sample with 28% sugar
Brix, %	18.15	20.25	22.20	24.15	26.40	28.20
Titratable acidity, g/l tartaric acid	9.41	9.41	9.09	9.03	8.87	8.83
pH	2.96	3.06	3.11	3.13	3.16	3.15
[1]YAN_i, mg/l	50.84	57.06	63.42	67.39	65.07	62.80
[2]YAN_f, mg/l	120.29	154.18	194.28	254.71	303.28	348.46
[3]NTU_i	48.92	48.67	69.00	42.09	53.67	61.00
[4]NTU_f	100	102	101	102	100	102
Inoculum size, cells/ml	3.63 x 10^6	4.05 x 10^6	4.44 x 10^6	4.83 x 10^6	5.28 x 10^6	5.64 x 10^6
Yeast strains	Bayanus PC / Epernay 2 / Premium Blanc 12V;					

[1]YAN_i - yeast assimilabile nitrogen prior correction; [2]YAN_f - yeast assimilabile nitrogen after correction; [3]NTU_i – turbidity of musts prior correction; [4]NTU_f – turbidity of musts after correction;

The experiment was run in triplicate for each sugar concentration and yeast. In each must YAN was adjusted and yeast inoculated in accordance to the sugar level, as described in Table 4.

RESULTS AND DISCUSIONS

After the completion of fermentation, the alcoholic concentration (Figure 1) and the residual reducing sugar in each sample (Figure 2) were determined.

It can be observed that all yeast strains used in our experimental conditions produced in the samples with high content of sugar more alcohol than the level the producer said they would normally tolerate (Table 3). This may be explained by the optimization of YAN and inoculum size with the the sugar concentration in each sample.

Kontkanen et al. (2004) found similar results regarding the alcoholic strength on icewines with high sugar concentrations when the yeast dosage was increased.

For reason of stability, in winemaking the interest is to produce wines with low sugar remaining after the completion of fermentation, of a maximum of 4 g/l (the limit between dry and semi-dry wines) or even less than 2 g/l, for the prevention of *Brettanomyces* infections (Antoce, 2005). This means that a better tolerance and a higher transformation yield of sugar into ethanol, will ensure the oenologists that most of the musts will ferment to dryness. To achieve this goal, a good correlation of YAN and yeast inoculum size would give satisfactory results in wine production.

From figure 2, it can be observed that only the musts with very high sugar concentrations cannot be fermented to dryness, especially in the case of using regular yeast strains, which are moderately resistant to alcohol. Even more, among the yeast we have used, the one more sensible to alcohol, Premium Blanc 12V, may be more prone to lead to sluggish or stuck fermentations. This behavior can be easily observed in figure 2, for the must samples containing sugar levels of 26 and 28% Brix and fermented with this yeast strain. Aside of these extreme cases, even using this less tolerant yeast strain would not generate any fementation inconveniences, provided the YAN and inoculum size is optimized in accordance with the sugar level.

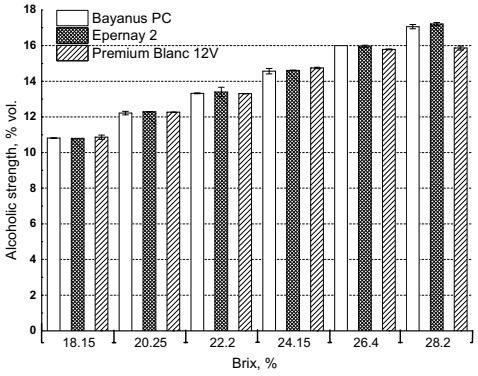


Figure 1. Alcoholic strength of the wines obtained form musts with various levels of sugar content and fermented with 3 different yeast strains

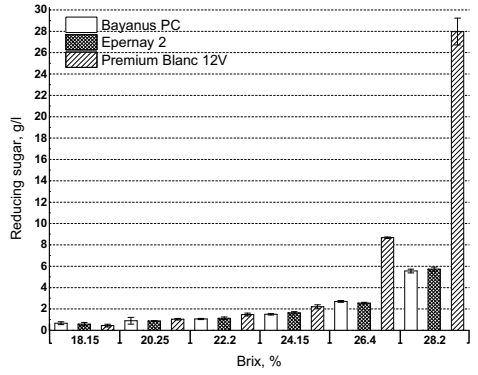


Figure 2. Residual sugar of wines obtained form musts with various levels of sugar content and fermented with 3 different yeast strains

Another crucial parameter for the wine quality is the volatile acidity, which was analyzed after fermentation in each sample, the values being presented in Fig. 3. In accordance to the knowledge in this field, we also observed a trend towards the increase in volatile acidity with the initial sugar concentration of must.

The values of volatile acidity are slightly higher in our experimental case, than would normally be in production conditions, due to the harsh changes of pHs which we artificially induced in the must samples, to create a supplementary stress for the yeasts and have,

accordingly, a worst-case scenario. Even so, the legal EU limit of volatile acidity, which is 1.08 g/l acetic acid for white wines, was not exceeded.

By comparing the volatile acidity produced by each strain in all the must samples it can be observed that Premium Blanc 12V (Figure 4a) is the most productive, while Epernay 2 (Figure 4b) and Bayanus PC (Figure 4c) give similar results in our experimental conditions.

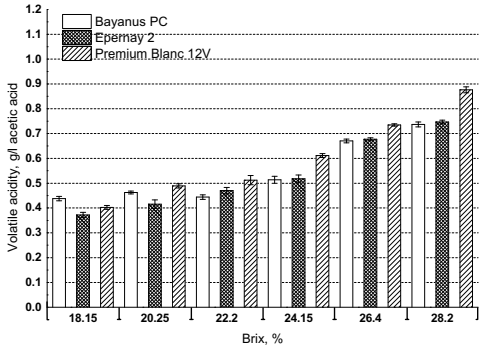
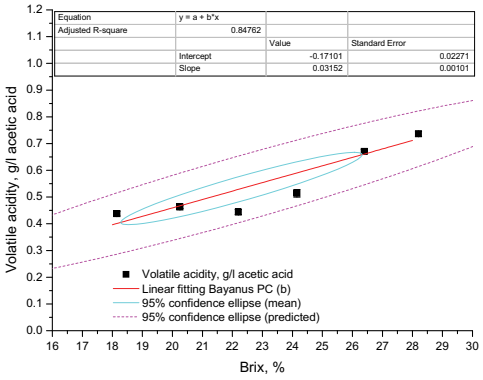
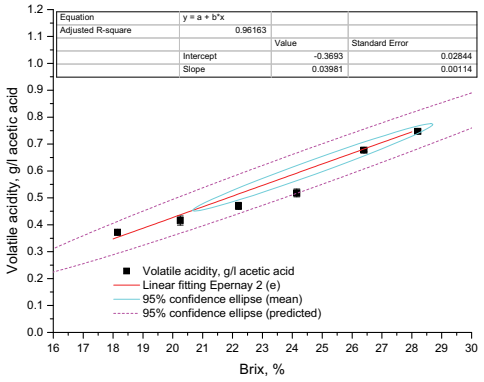


Figure 3. Volatile acidity of wines obtained form musts with various levels of sugar content and fermented with 3 different yeast strains





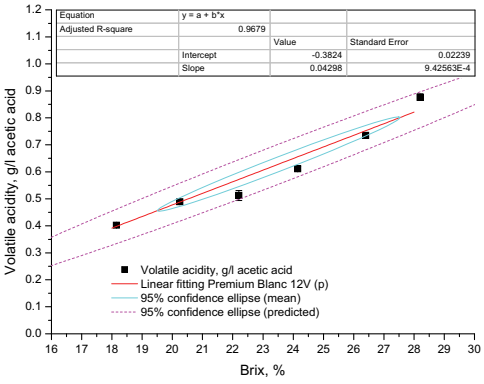


Figure 4. Volatile acidity production trend in wines obtained form musts with various levels of sugar content and fermented with Bayanus PC (a), Epernay 2 (b) and Premium Blanc 12V (c) yeast strains

The production of volatile acidity is very well linearly correlated (R^2 of 0.85-0.97) to the sugar concentration in the initial must, irrespective of the yeast strain used (Fig. 4a-c). This fact proves that the fermentation took place in optimal nutritional conditions even in the case of the samples with 26 and 28 % Brix, which usually lead to much higher volatile acidity (a surplus of 0.3-0.6 g/l), as the yeast struggles to survive in unfavourable conditions.

The growth thermograms obtained for each yeast culture introduced in the calorimeter showed that in our experimental conditions for each yeast strain the growth rates were similar for the samples with initial sugar concentration of 18, 24, 26, 28 % Brix, even though the inoculum size was different for each sugar level (Figure 5). This fact suggests a good correlation of the the inoculum size,

sugar and YAN concentrations in the samples of these musts. It could be concluded that these growth rates with growth rate constants in the range of 0.1-0.2 min^{-1} are optimal and to slow down the growth in the samples of 20 and 22% Brix we should, for example, decrease the inoculum size. As it can be seen in Figure 5, for the Bayanus PC and Epernay 2 no further adjustments of inoculum size is needed, as the growth rate constant is between 0.1-0.2 min^{-1} irrespective of the initial sugar concentration. However, Premium Blanc 12V is a fastidious yeast strain, growing much faster in samples with medium sugar level (20-22% Brix) and high YAN concentrations. This fact is also confirmed by the yeast growth retardation chart (Figure 6), where we can see that Premium Blanc 12V yeast strain starts growing faster at 20-22% Brix, within 25-30 hours after inoculation, as compared to Epernay 2 and Bayanus PC, who need 33-53 hours for the same growth level. A faster growing can be an advantage in achieving the necessary number of yeast cells for the fermentation, but is not anymore if the rate of fermentation is also increased, because in a fast fermentation more wine aroma compounds are negatively affected or lost. Therefore, more studies are needed to decide if it is acceptable to apply the same equation for the adjustments in must composition for all the yeasts or the fast growing ones should be treated differently.

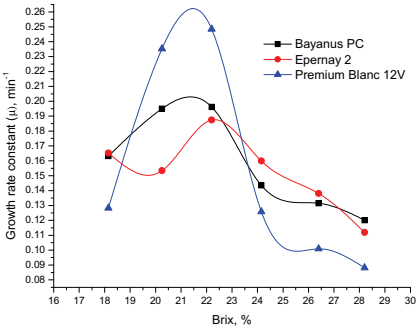


Figure 5. Growth rate constants of the 3 yeast strains in musts with different sugar concentrations

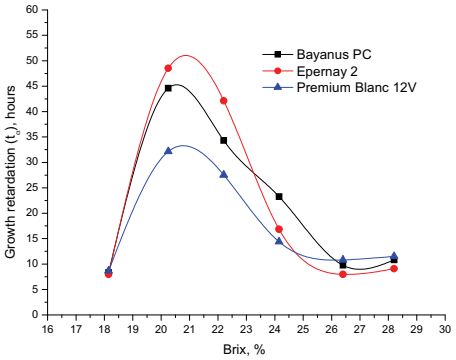


Figure 6. Growth retardation of the 3 yeast strains in musts with different sugar concentrations

The growth retardation of yeasts (Fig. 6) determined by calorimetry in musts with various sugar concentrations and adjusted YAN levels is a good indication of the optimization achieved in the culture conditions. The growth retardation represents the time passed until a calorimetric signal of a certain level (in our case 10 mV) is reached on the growth thermograms mathematically processed (Antoce et al., 1996, 1997) to depict the real growth from the recorded calorimetric data.

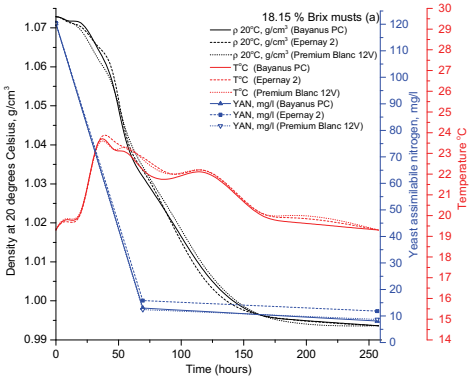
The fact that the lowest retardation (10-25 hours) is obtained in samples with 18 and 24-28 % Brix show that the inoculum size is best selected for these samples. The growth retardation of 35-53 hours observed in the samples with 22-24 % Brix suggests that for these musts the inoculum size was underestimated. However, because the growth rates for the musts with 22-24 % Brix are still in the normally expected ranges (Figure 5) and there is no risk for a sluggish fermentation, no need for further adjustment seems in order.

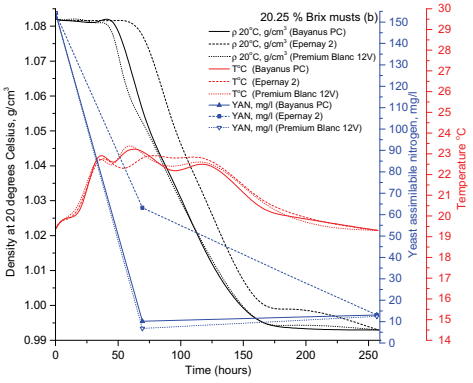
The proposed model for the adjustments in yeast dosage and YAN levels is particularly useful in the case of high sugar concentrations. This was proved by the rapid growth (10 hours) after the yeast inoculation in all samples with 26 and 28% Brix. Here, the high inoculum size and nutrient level, compensated the osmotic stress induced by high sugar concentrations. This type of approach is also supported by the wine yeast producing companies, which recommend in their technical sheets an increase in yeast dosage from the normal 10-20 g/hl to 30-40

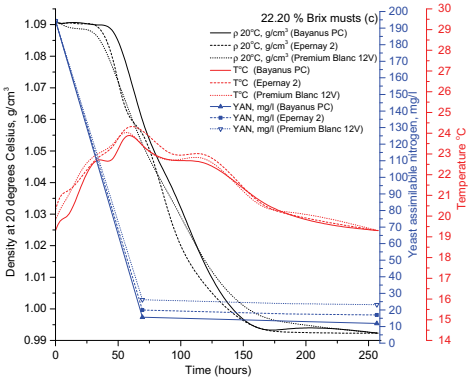
g/hl in the case of high sugar content musts and a further yeast nutrient supplementation. In our work we were able to make more precise recommendations regarding the inoculum size and YAN levels required for a must with a certain initial sugar concentration. The fermentation process was monitored for each sample by following the evolution of density, temperature and YAN. Useful information was thus obtained regarding the consumption period of YAN, residual YAN and progress of alcohol accumulation.

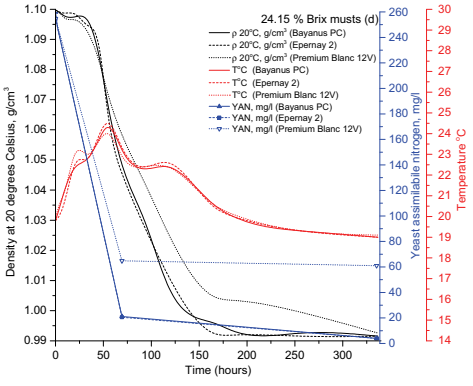
Normally, irrespective of the sugar concentration, YAN concentration should not be excesive, so that yeasts should be able to consume it down to a level of 10 to 20 mg N/l. Higher residual YAN may lead to bacterial spoilage in wines. As it can be seen in Figure 7, the available YAN is consumed within the first 70 hours of fermentation, which corresponds mostly with the multiplication of yeast cells period and with the consumption of a 1/3 of the total sugar content. Another study shows similar results on YAN consumption period (Bely et al., 2003).

The administration of subsequent doses of YAN should be avoided, due to the risk of remaining unconsumed.









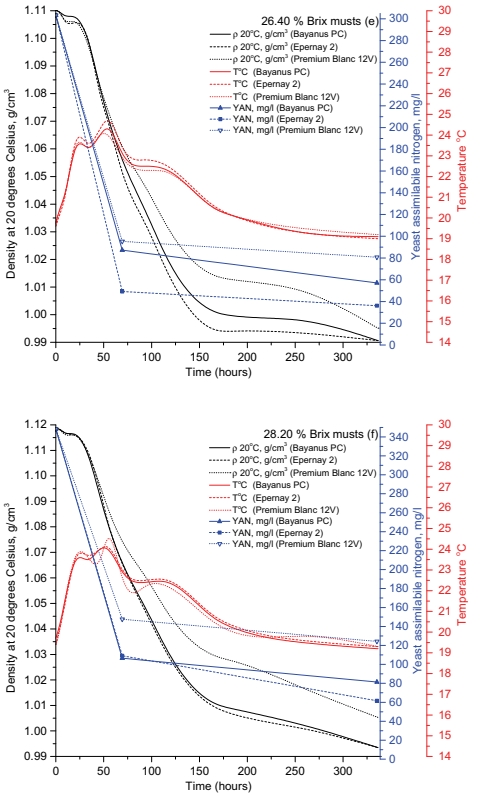


Figure 7. Evolution of density, temperature and YAN during alcoholic fermentation of musts with different level of sugar with yeasts with correlated inoculum sizes

Figure 7a-d, shows that for must with 18-24% Brix all the fermentations progressed normally, leading to dry wines (Figure 2), with residual YAN of no more than 20 mg/l. The musts with 26 and 28% Brix (Fig. 7e and 7f), for which the calculated and added YAN was in excess of 250 mg N/l, led to wines with high residual YAN, of 40-80 mg/l and 60-120 mg/l, respectively. The differences in the final YAN concentration for these high sugar samples were due to the yeast strain used for the fermentation, Premium blanc leaving the highest levels of nitrogen in the final wines.

In some cases, when the fermentation starts sluggishly (longer lag phase), YAN can be consumed even after the first 70 hours of fermentation. Such event happened for the fermentation of 20 % Brix musts with Premium Blanc 12V yeast strain (Figure 7b) and can frequently happen in musts with high sugar concentration, where the sugar consumption is also retarded and only less than 1/3 of its quantity is used in the first 70 hours of fermentation.

To avoid bacterial spoilage, in the case of sweet wine production, the oenologists should choose low alcohol tolerant yeast strains with low nutrient requirements and limit the YAN level in must, so that it will not remain unconsumed in wines.

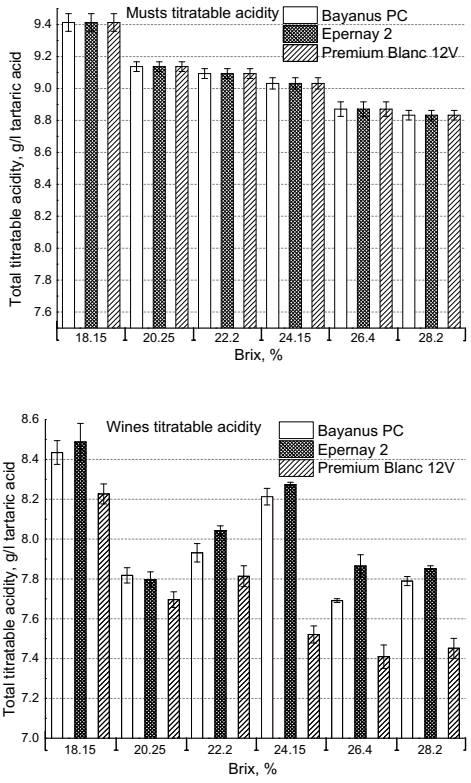


Figure 8. Titratable acidity of the musts (a) and the resulted wines (b) produced with 3 different yeast strains

The titratable acidity, another important parameter for the wine quality, generally dropped more in the wines obtained from musts with high sugar content (Figure 8b). This behaviour was not surprising, since the higher alcohol content produced in wines made from musts with high sugar concentration decreased the solubility of the potassium hydrogen tartrate, which precipitated in larger amounts. The small varations in the titratable acidity of the wines (Figure 8b) produced from the same must (Figure 8a) with various yeast strains can be

accounted for the different metabolic mechanisms for some acid formation (eg. succinic acid) ore depletion (eg. malic acid). Generally, the wines resulted from musts inoculated with Premium Blanc 12V yeast strain had less titratable acids than the wines of the other strains (Figure 8b).

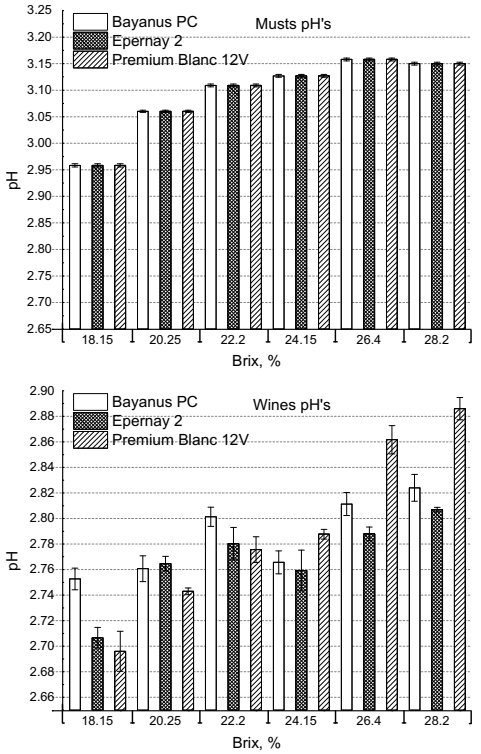


Figure 9. pH of the musts (a) and the resulted wines (b) produced with 3 different yeast strains

In Figure 9a and b the pH of the musts and the resulted wines produced with 3 different yeast strains are shown. As in the case of titratable acidity, pH was dependent on the metabolic pathways of yeasts and on the solubility of the tartaric acid salts and the precipitations of potassium hydrogen tartrate, but also on the added ammonium salts used as nutrients. As we can see, the adjustment of YAN in increasing amounts correlated to the sugar concentration, increased the pH of those musts accordingly (Figure 9a). A lower pH is good for the microbiological wine stability (Figure 9.b), but in must a pH below 2.9 is a stress factor for the yeast, especially when the alcohol also starts to accumulate. That is why, the YAN adjustments with ammonium salts are not only good for yeasts as nutrients, but are also good for the pH regulation in musts, increasing their fermentability.

CONCLUSIONS

Adjustments of YAN levels and inoculum sizes correlated with the sugar concentration of musts should be applied in wine technology to a certain extent, that is, to a maximum YAN concentration of 250 mg N/l. For a good management of fermentation YAN level should be at least 150 mg N/l, value reported in the literature by many authors as the minimal necessary to complete fermentation. This correction should be done prior to inoculation, while and a second correction should be applied only when necessary, 48 hours after the inoculation or, better, when sugar depletion is 1/3 of initial sugar concentration.

A recommended YAN level, correlated with the sugar concentration would be defined by the following equation: $YAN, mg/l = 10 \times \%Brix - 30$.

The second correction to be applied when 1/3 of initial sugar concentration is consumed may be calculated by substracting from the calculated YAN level the minimal recommended YAN level, that is 150 mg/l.

Aside of YAN corrections, the optimization of alcoholic fermentation implies also the adequate yeast strain selection and the inoculation of a optimum number of cell/ml. The formula we used for the inoculum size calculation in this experiment seems to provide good practical results and for this reason we recommend it to be applied in wine production sector.

If sweet wines are desired, a strain of yeast with low to medium tolerance to alcohol should be used and YAN should not be supplemented to more than 150 mg N/l.

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