Effect of ammonium concentration on alcoholic fermentation kinetics by wine yeasts for high sugar content

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Running title: Effect of ammonium on wine fermentation

Abstract

Kinetics of alcoholic fermentation by *Saccharomyces cerevisiae* wine strains in a synthetic medium with high sugar content were established for different nitrogen initial content and are presented for 4 strains. The composition of the medium was close to grape must except that the nitrogen source consisted mainly in ammonium and was varied from 120 to 290 mg N/L assimilable nitrogen. The overall nitrogen consumed was also estimated in order to determine nitrogen requirement variability.

The effect of assimilable nitrogen was in general greater on sugar consumption rates than on growth and 3 kinds of effect on sugar consumption rates were observed: i) existence of an optimal initial nitrogen level for a maximal sugar consumption rate (inhibition if excess), ii) no effect of nitrogen beyond the intermediary level (saturation), iii) sugar consumption rate proportional to the initial nitrogen level (activation).

In all cases, the amount of consumed nitrogen increased with its initial concentration and so did the fructophilic capacity of the strains. The optimal requirement varied from 0.62 to 0.91 mg N per g of sugars according to the different strains. There was no general correlation between the sugar assimilation rates and the nitrogen requirement.

Key words: wine yeast, ammonium, fermentation, *Saccharomyces,* assimilable nitrogen

INTRODUCTION

In wine-making the problem of adequate nitrogen level in the grape musts for a good achievement of alcoholic fermentation is not yet totally solved. If the yeast suffers from nitrogen deficiency it can lead to stuck or sluggish fermentation (Salmon 1989, Bisson and Butzke 2000). On the opposite, if excessive ammonium addition is done, there could be a risk that the wine had modified characteristics for higher alcohols (Beltran et al. 2005), acetic acid (Bely at al. 2003), ethyl carbamate (Ough et al. 1988) or in some conditions hydrogen sulphide (Wang et al. 2003) content. Despite numerous studies carried out on this topic, the results are not always in agreement nor the conclusions very clear. For some authors addition of a nitrogen source to the must increases biomass concentration for *Saccharomyces cerevisiae* and sugars utilization rates (Henschke and Jiranek 1993, Bely et al. 2003, Beltran et al. 2005). This last effect could be explained by the stimulation of enzymes de-novo synthesis during sugar assimilation even during the stationary phase (Medeis-Ferreira 2004), some of this enzymes being sugar permease (Salmon et al. 1993). For others authors, the use of ammonium salts to increase the nitrogen content of grape must induces a repression of amino-acids consumption by the yeasts and could reduce the fermentation efficiency (Beltran et al. 2005). Thomas et al. (1996) even stated that the flux of carbon trough the glycolytic pathway was greater under nitrogen limitation. In fact it seems that nitrogen requirement of *Saccharomyces cerevisiae* could depend on the strain (Jiranek et al. 1995) and on the conditions of fermentation (sugars concentration, temperature, presence of oxygen…) (Valero et al. 2003). In most of the studies sugars concentration not greater than 200 g/L were used. For these reasons, we studied the nitrogen requirement using ammonium salts for eleven commercial wine strains from different origin in a synthetic grape must with high level of sugars (240 g/L). The results for the 4 most representative strains are presented in this paper. The kinetics of growth, glucose and fructose assimilation, as well as the total consumed

assimilable nitrogen amount were established for various initial assimilable nitrogen levels in the must. We chose a synthetic medium in order to well define and control its nitrogen content mainly consisting of ammonium. Moreover, ammonium salts are an excellent nitrogen source for *S. cerevisiae* (Torija et al. 2003) and are widely used by winemakers to increase nitrogen content in the legal limit of 1000 mg/L in Europe and 950 mg/L in USA (Henschke and Jiranek 1993) of ammonium phosphate or sulphate (21% of nitrogen).

MATERIALS AND METHODS

Yeast strains:

Eleven commercial *Saccharomyces cerevisiae* wine strains provided by Lamothe-Abiet (Bordeaux, France), Lallemand Inc. (Montréal Canada) and Anchor Yeast (Cape Town, South Africa) were used in this study. The results are shown for four of these strains: A, B, C and D. They were maintained on agar slants (peptone 10 g/L, glucose 5 g/L, yeast extract 10 g/L, agar 20 g/L) at 4°C.

Fermentations conditions:

Prior to inoculation, yeasts were propagated for 15 hours at 30°C in a synthetic medium containing (g/L): glucose, 50; yeast extract, 1; $(NH_4)_2SO_4$, 2; $MgSO_4$, 0.4; KH_2PO_4, 5. Before inoculation the yeast cells were washed with sterile water.

The volume of inoculum was calculated in order to get 3 millions of cells at the beginning of the fermentations in a medium containing (g/L): glucose, 120; fructose, 120; yeast extract, 0.75, citric acid, 0.3; malic acid, 4; tartaric acid, 4; $MgSO_4$, 0.4; KH_2PO_4, 5; sodium oleate, 0.005. The yeast extract (L21, Oxoid) was a vitamins source and at the same time provided 40 mg/L assimilable nitrogen mostly consisting in amino acids. For each strain 3 levels of total yeast assimilable nitrogen (YAN) were performed by varying the concentration of $(NH_4)_2SO_4$ in order to get 120 mg N/L, 190 mg N/L, and 290 mg N/L (table 1). The pH was adjusted at

3.3 with NaOH (5M). Fermentations were carried at 18°C, in Erlenmeyer flasks of 500 mL containing at the beginning 450 mL. They were shaken only 2 minutes before taking sample once a day. For one strain all fermentations were inoculated with the same inoculum and carried out at the same time. Fermentations were stopped when sugars were exhausted or their concentration remained constant.

Yeast biomass concentration and viability

Cells were counted under microscope (magnification 400) using a Thoma hematocymeter. The experimental error (coefficient variation) was always inferior to 10% (Lange et al. 1993). Yeast viability was assessed by methylene blue staining (Bonora and Mares 1982).

Sugars analysis

Glucose and fructose concentrations were determined by enzymatic analysis using Boeringher test reference EZS862 (R-Biopharm, France) with an experimental error less than 4%.

Nitrogen content analysis

Ammoniacal nitrogen was determined by enzymatic analysis using Boeringher test reference E1112732 (R-Biopharm, France) and expressed as mg N/L. Total assimilable nitrogen was determined by the Sorensen method (Zoecklein et al. 1995). The coefficient of variation was at maxium 5%.

Calculation of specific rates

Specific rates for sugars assimilation, ammonium assimilation and growth were calculated in the same way. First, experimental data for kinetics (sugars, ammonium and growth versus time) were adjusted to a mathematical model using Microsoft® Excel™ with stepwise cubic spline function. Secondly, this mathematical model was derivated and then divided by the model fitting growth (expressed in millions of cells per L) in order to obtain the specific rates versus time (expressed in g or mg N/millions of cells/day**).**

RESULTS AND DISCUSSION

In Enology, the Yeast Assimilable Nitrogen (YAN) is usually evaluated by the Sorensen or formol titration method (Zoecklein et al. 1995) and consists in ammonia and alpha-amino nitrogen. According to grape variety and maturity, climate, and fertilization of vineyard, grape must content in YAN mostly ranges from 50 to 500 mg N/L (Bely et al. 2003), with an average value of 120 to 140 mg N/L. This average value is usually considered as non-limiting to achieve alcoholic fermentation (Butzke 1990). Taking into account that the initial concentration of sugars in the medium was high (240 g/L) we varied the YAN from 120 to 290 mg/L with an intermediary value of 190 mg N/L to evaluate the requirement of the wine yeasts coming from various origins: various suppliers and different nitrogen requirement according to suppliers' indications. The results are presented for 4 strains representative of different behaviours: strain A, B, C, D. B and D have very similar behaviours but with a more significant impact of nitrogen on strain D.

1- Fermentation kinetics

Yeast growth

Figure 1 shows that 3 kinds of effect of nitrogen supplementation on growth could be observed. For the strain A, the nitrogen concentration had very little effect on yeast growth: only for the lowest level a slight decrease in the biomass maximal concentration was observed. For the strains B and D, the formation of biomass was correlated to the nitrogen initial content of the medium. For the strain C an optimal concentration (190 mg/L) was revealed for a maximal biomass concentration: for the lowest and the highest nitrogen concentration the final yeast concentration was more or less the same. In all cases yeast viability was greater than 95% until the end of fermentation. It is worth noting that during the first 5 to 8 days of fermentation the growth rates are similar for a given strain whatever the nitrogen content. In fact, the specific growth rates did not vary for a given strain according to

YAN content but growth did not cease at the same time, indicating that nitrogen poorly affected the yeast ability to multiply itself during the growth phase but controlled the transition to the stationary phase. So, contrary to some results reported in literature (Henschke and Jiranek 1993, Bely et al. 2003) assimilable nitrogen does not always enhance growth.

Sugar consumption

Figure 1 shows that sugars (glucose+fructose) were not totally consumed and the medium could not be considered as dry for 3 strains in some conditions: B and D for lowest level and, A whatever the YAN level. Regarding the sugars consumption profiles the YAN level had a weak effect on strains A and C with a positive correlation for the first one and an optimal concentration (190 mg/L) for the second one. Nevertheless for strain A the fermentations could be considered as sluggish. For the others 2 strains the effect was more pronounced: positive correlation for D and sugar consumption rates not increased over 190 mg/L for strain B.

We can note that the effect of nitrogen content is not always the same or with the same intensity for growth and sugars assimilation. This can be explained by the fact that during alcoholic fermentation in wine-making conditions most of the sugars are consumed during the stationary phase. Moreover divergent pathways are used for catabolism and anabolism. For our experiments despite different amount of biomass accumulated in the medium at the end of the growth phase the effect on sugar consumption rate is less important and the specific consumption rates remained constant whatever the nitrogen level (data not shown).

Nitrogen consumption

Figure 2 shows that for all strains the ammoniacal nitrogen was depleted for the smallest level at about 5 days, so before the growth stopped. For the other two levels the consumption of ammoniacal nitrogen ceased at about 10 days (end of the growth phase) and during the stationary phase the concentration remained constant even when it was still abundant in the

medium (higher level). However all the strains consumed more assimilable nitrogen when its concentration in the medium was higher, consumed amount being multiplied by 2.2 to 3 for highest initial level compared to smallest initial level. Previous works have shown that nitrogen sources can be accumulated in intracellular vacuoles (Henschke and Jiranek 1993, Torija et al. 2003). Reserves of nitrogen could then be used by the yeast during the stationary phase for new protein synthesis following protein turnover as suggested by Mendes-Ferreira et al (2003).

At the end of fermentation a slight increase of the YAN was observed (data not shown) due to release of amino acids when ammoniacal nitrogen has been exhausted and corresponding to residual ammonium plus amino-acids in the other cases. A release of amino-acids during the stationary phase was always reported in conditions of wine-making (Ancin et al. 1996, Valero et al. 2003, Torija et al. 2003) and probably in order to maintain a normal redox balance (Valero et al. 2003).

The specific consumption rates for ammoniacal nitrogen were calculated and are shown in figure 3. These rates decreased during the growth probably due to inhibition of up-take by ethanol (Ferreira Monteiro and Bisson 1992). For all strains excepting A these rates were the same for levels 190 and 290 mg/L and lower for level 120 mg/L indicating in this condition a limitation in nitrogen feeding compared to the yeast capacity.

2- Final and global characteristics of the fermented medium

Residual sugars concentration can be seen in Table 2 for each experiment. It is confirmed that except for strain C, sugars were not totally exhausted for the lowest nitrogen concentration (120 mg/L) indicating a deficit for this nutriment in these conditions. For strain A, the residual sugars were proportional to the nitrogen level. For strains D and B, the results can be considered as similar: no difference between 190 and 290 mg/L and more sugar left for 120 mg/L initial level, indicating also a limitation in nitrogen but only for the smallest

concentration. Regarding the average sugars consumption rate, which represents the efficiency of fermentation, the same classification can be done: for strain C there was an optimal concentration of YAN (190 mg/L), strain A exhibited a rate proportional to the nitrogen content and B and D consumed sugars at the same rate for 190 and 290 mg/L, and at a lower rate for 120 mg/L. This classification is concordant with the effect on growth only for strain C showing different influences of nitrogen on yeast growth and fermentation rate for the other strains. Generally speaking the average sugar consumption rate depends more on the strain than on the nitrogen content of the must as already reported by Hernandez-Orte et al. (2005): some strains are quicker than others whatever the nitrogen level. According to this criterion the eleven strains we studied can be classified as follows: activation of alcoholic fermentation by YAN: 5 strains among them strain A; the intermediary YAN level was sufficient (saturation): 2 strains B and D; existence of an optimal YAN concentration and decrease of the fermentation rate above this level: 4 strains among them strain C. This could be explained by a repression phenomenon for higher levels as reported by ter Shure et al. (2000) or by a decreased synthesis of phosphofructokinase, (a key regulatory enzyme of glycolysis) in excess of nitrogen as reported by Thomas et al. (1996) for another *S. cerevisiae* strain.

We then calculated the ratio consumed fructose/consumed glucose (g/g) which illustrates the fructophilic capacity of the strains (Berthels et al. 2004). In most of the cases, this characteristic was enhanced by nitrogen supplementation (table 2). The observed effect was as for residual sugars: no effect on strain C, the most fructophilic, correlation between fructophilic capacity and nitrogen for yeast A and, for yeasts B and D no increase of the capacity beyond 190 mg/L. According to Berthels et al. (2004) ammonium addition could counteract turnover of high fructose affinity transporters and also activate phosphofructokinase, first common enzyme of the fermentation pathway. However this has

not yet been demonstrated at the molecular level although Mendes-Ferreira et al. (2004) demonstrated that nitrogen addition during stationary phase could allow protein de-novo synthesis.

At least, we calculated the amount of consumed YAN per g of consumed sugar (mg N/g). In all cases this amount was highly increased when the medium content in nitrogen was higher. That is to say the more abundant is available nitrogen, the more the yeast consumes it. Strain A is not the greatest nitrogen consumer and never exhausted sugars even when consuming 0.91 mg N/g. On the opposite, strain C can ferment the medium to dryness even for the smallest level, and has the weakest nitrogen need (0.81 mg N/g of consumed sugar). In reality, this strain needs only 0.62 mg N per g of sugar for maximal fermentation efficiency.

The strains B and D seemed to be close from each other for some characteristics, but, strain B needed more nitrogen per g of sugar consumed (0.66 and 0.63 mg N/g respectively needed for exhaustion of sugars with the highest average rate). So the different strains had different nitrogen requirement varying from 0.62 to more than 0.91 mg N/g. There was no correlation between the sugar assimilation rates and the nitrogen requirement.

As a conclusion, wine yeasts exhibited different behaviour towards YAN supplementation that is not always relevant and efficient. In wine-making the adequate nitrogen requirement must be evaluated for each strain in order to avoid excessive preventive supplementation that can lead to repression phenomena and in some cases decrease the efficiency of fermentation. It can also lead to a lower synthesis of higher alcohols and formation of ethyl carbamate or microbial instability if the residual concentration of ammonium is too high. The assessment of optimal requirement should not be done in musts with excessive nitrogen content because of the accumulation capacities of the *Saccharomyces cerevisiae* in the vacuoles.

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Tables

Table 1: Variable composition in nitrogen of the synthetic medium

Assimilable nitrogen initial level YAN (mg/L)	$(NH_4)_2SO_4$ (g/L)	Assimilable nitrogen from ammonium (mg/L)
120	0.38	80
190	0.714	150
290	1.2	250

Table 2: Final and global characteristics of the fermented medium in function of the strain and initial nitrogen level (YAN in mg/L)

Experiment	Residual sugars (g/L)	Average sugar consumption rate ($g.L^{-1}.day^{-1}$)	Consumed YAN/consumed sugars (mg N/g)	Consumed fructose/consumed glucose (g/g)
Strain A				
120 mg/L	7.5	6.24	0.91	0.94
190 mg/L	18.5	6	0.75	0.85
290 mg/L	20.4	5.76	0.30	0.83
Strain B				
120 mg/L	1.2	8.64	1	0.99
190 mg/L	1.4	8.64	0.66	0.99
290 mg/L	4.6	8.4	0.37	0.96
Strain C				
120 mg/L	0	8.64	0.81	1
190 mg/L	0	9.12	0.62	1
290 mg/L	0	8.4	0.36	1
Strain D				
120 mg/L	0	6.96	0.93	1
190 mg/L	0	6.96	0.63	1
290 mg/L	10.7	6.24	0.39	0.91

Legends to figures:

Figure 1: Kinetics of alcoholic fermentations: growth (black symbols) and sugar consumption (open symbols) for the 4 strains in all conditions: (▲, ∆) 120 mg/L initial YAN, (■, □) 190 mg/L initial YAN, (●, ○) 290 mg/L initial YAN

Figure 2: Ammoniacal nitrogen profiles for all experiments: (▲) 120 mg/L initial YAN, (■) 190 mg/L initial YAN, (●) 290 mg/L initial YAN

Figure 3: Specific ammoniacal nitrogen assimilation rates as a function of fermentation time for all experiments: (▲) 120 mg/L initial YAN, (■) 190 mg/L initial YAN, (●) 290 mg/L initial YAN

Figure 1:

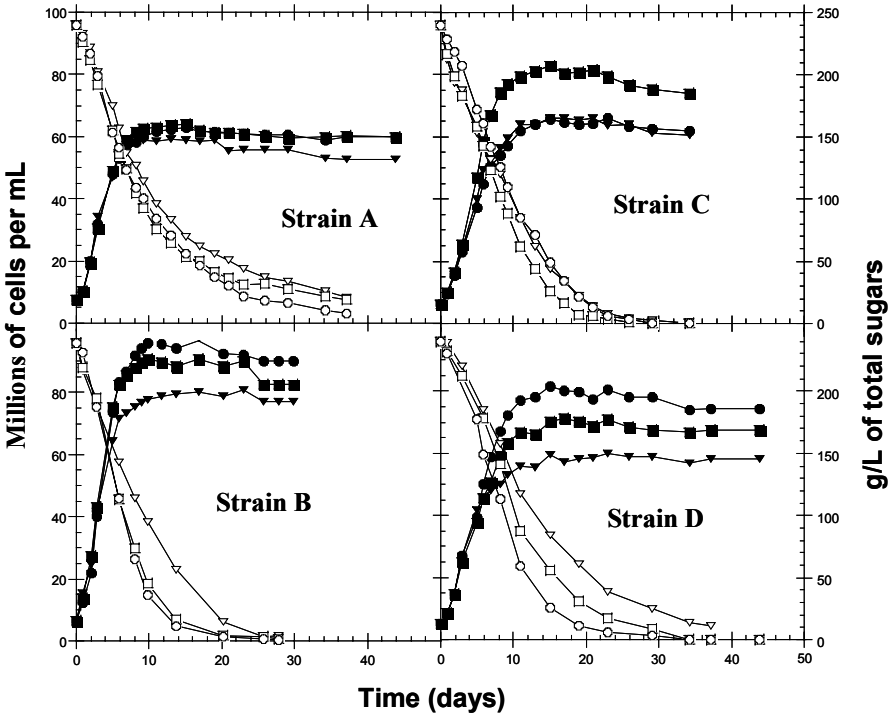

Strain A
Strain C
Strain B
Strain D
Millions of cells per mL
g/L of total sugars
Time (days)

Figure 2:

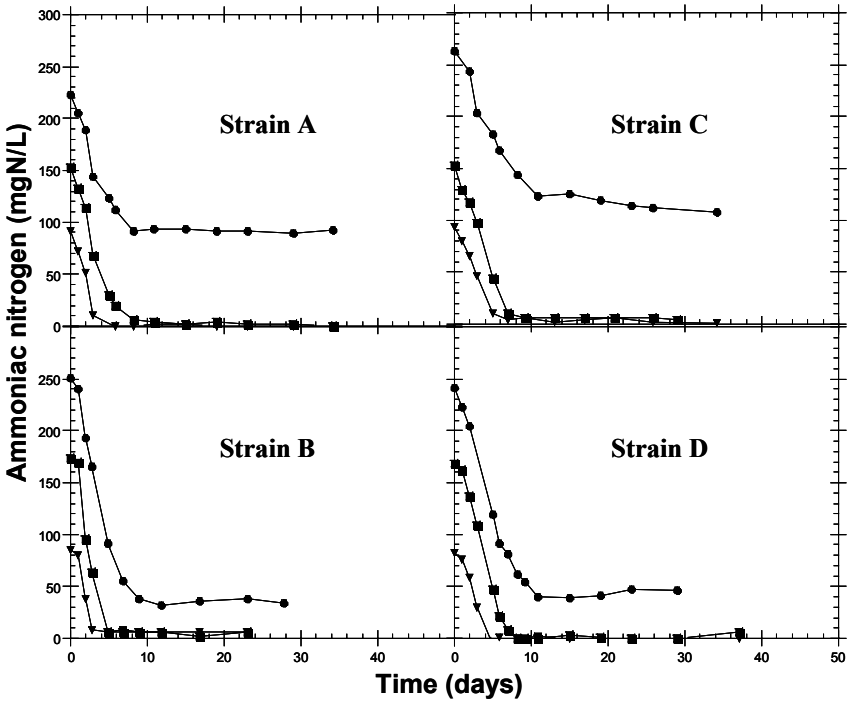
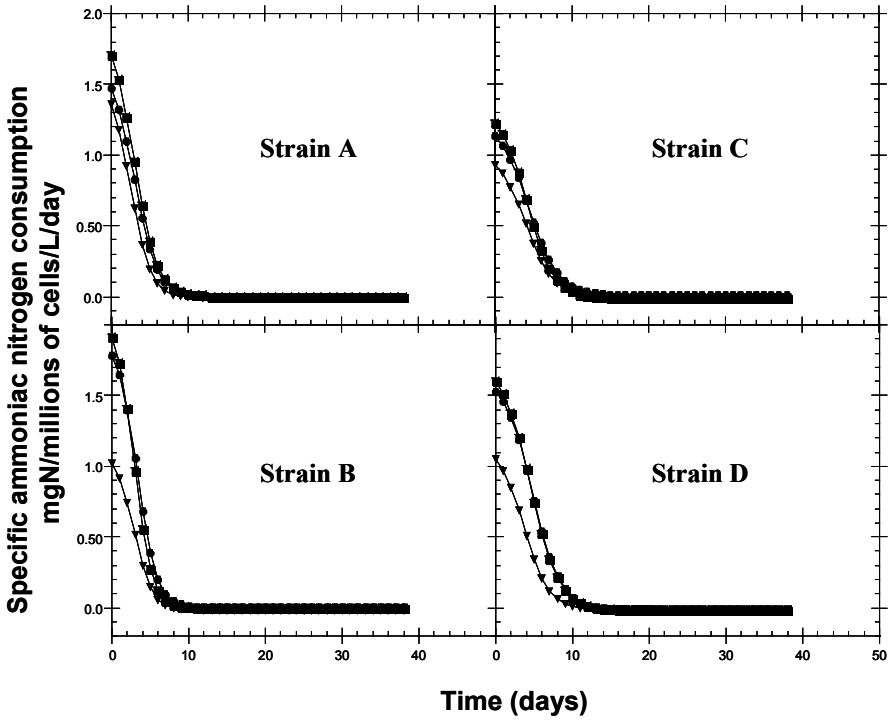

Strain A
Strain C
Strain B
Strain D
Ammoniac nitrogen (mgN/L)
Time (days)
0
50
100
150
200
250
300
0
10
20
30
40
50

Figure 3:


Specific ammoniac nitrogen consumption
mgN/millions of cells/L/day
Strain A
Strain C
Strain B
Strain D
Time (days)
2.0
1.5
1.0
0.50
0.0
0
10
20
30
40
50