Apple Wine Processing with Different Nitrogen Contents

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ABSTRACT

The aim of this work was to evaluate the nitrogen content in different varieties of apple musts and to study the effect of different nitrogen concentrations in apple wine fermentation. The average total nitrogen content in 51 different apples juices was 155.81 mg/L, with 86.28 % of the values above 100 mg/L. The apple must with 59.0, 122.0 and 163.0 mg/L of total nitrogen content showed the maximum population of 2.05×10^7; 4.42×10^7 and 8.66×10^7 cell/mL, respectively. Therefore, the maximum fermentation rates were dependent on the initial nitrogen level, corresponding to 1.4, 5.1 and 9.2 g/L.day, respectively. The nitrogen content in the apple musts was an important factor of growth and fermentation velocity.

Key words: Apple juice fermentated, fermentation rate, cider, yeast

INTRODUCTION

The apple juice fermentation process used to obtain a pleasant alcoholic beverage has been in practice in the Eastern Mediterranean areas since more than 2000 years (Laplace et al., 2001). Presently the fermented apple juice is used as the basis for manufacturing the cider, a sparkling and refreshing fruit flavored beverage, consumed in many countries in the world. Apple juice contains all necessary nutrients for the growth of yeast (Binnig and Possmann, 1993; Downing, 1989; Smock and Neubert, 1950). The main sources of carbon and energy are soluble sugars, and the concentration of 120 g/L is enough to allow the growth of the yeast to reach around 6.0×10^7 cell/mL (Nogueira and Wosiacki 2010). However, sluggish apple juice fermentations seem to be associated with raw material when there are not sufficient levels of nutrients such as available nitrogen, with technological factors such as low dissolved oxygen due to enzymatic browning reactions before the exponential growth phase or phytosterol and unsaturated fatty acid elimination by excessive must clarification. There could be non-*Saccharomyces* yeasts also in the medium which too would compete for the nutrients (Drilleau, 1990; Lea and Drilleau, 2003; Nogueira, 2003). The available nitrogen content of the apple juice, usually ranging from 27 up to 574 mg/L, is considered the main limiting factor for the growth of the yeast (Cruz et al., 2002; Drilleau, 1990). In the alcoholic fermentation, the available nitrogen content is needed in the stationary phase (corresponding to 80% of the alcoholic fermentation) to stimulate the protein synthesis

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particularly for the sugar transport systems (Bely et al., 1994), improving the microbial metabolism and reaching up to 10% of the yeast biomass on dry weight basis (Goñi and Azpilicueta, 1999; Julien et al., 2000; Julien et al., 2001; Ribereau-Gayon et al., 1998).Some factors such as the juice composition and the kind of the yeast strain can affect the assimilation of the nitrogenous compounds (Colombié et al., 2007; Julien et al., 2000; Manginot et al., 1998) as the metabolism of amino acids can affect the efficiency of the alcoholic fermentation and the quality of the product (Salmon and Barre, 1998).

The nitrogen fraction in apple juice comprehends the amino acids asparagine, glutamine, aspartic acid, glutamic acid and serine, representing together from 86 to 95 % of total amino acids, and they are rapidly assimilated by yeasts. The fruits harvested from the orchards with extremely high fertilizers can contain the nitrogen compounds in the juices up to five times higher (Lequere and Drilleau, 1998) than the usual. The amount of nitrogen in apple juices can be low (<75 mg/L) or high (>150 mg/L) depending mainly on the age of the orchards and the amount of the fertilizers used (Nogueira et al., 2003; Drilleau, 1993). These facts influence the growth of the yeast and affect the fermentation rate, kinetic of aroma production and microbiological instability in naturally gasified cider (Garde-Cerdán and AncÌn-Azpilicueta, 2008; Goñi and Azpilicueta, 1999; Ingledew and Kunkee, 1985; Nogueira and Wosiacki, 2010; Ough et al., 1991; Pérez-Zúñiga et al., 1997).

The nitrogen content in apples can vary from different orchards and also from crop to crop (Drilleau, 1996), because the values can be influenced by the area, amount and kind of nitrogen fertilizers used and by the season (Baron et al., 1977).

In Brazil, the addition of nitrogen, as ammonium phosphate or thiamine is a common practice in the apple juice fermentation industry to adjust the chemical composition of the juice before inoculation with the commercial dry yeast. This procedure prevents the sluggish fermentations and ensures a rapid fermentation (5-10 days) at 20-35 °C (Nogueira, and Wosiacki, 2010). However, since there is no analysis done to determine the initial concentration of nitrogen the nitrogen level can rise too much if the natural concentration is already high. This affects the whole process, including the growth of the yeast and a high production of ethyl carbamate and an excess of

higher alcohols (above 400 mg/L) which exert a negative effect on the quality of cider (Beltran et al., 2005; Goñi and Azpilicueta, 1999).

In Brazilian cider manufacturing industries, the effect of nitrogen in the alcoholic fermentation and cider quality has not been well studied. Hence, the aim of this work was to evaluate the influence of the nitrogen content in the apple must during the processing on apple juice fermentation process and product.

MATERIALS AND METHODS

Apple varieties
Samples containing around 10 kg of 51 apple varieties used for juice and fermented production, harvested from 2003 up to 2006 from different places and producers of Southern States of Brazil, were used to determine the total nitrogen content. Apple samples (40 kg) of Gala, Fuji and Joaquina cultivars from the Experimental Station in São Joaquim (Epagri/SC) were used for fermentation.

Apple juice processing
The fruits were washed and transferred to a laboratory microprocessor (Processing Metvisa, Type MPA) where a pressure of 3 kgf/cm^2 (Hydraulica Press Eureka, Hoppe Ind. Ltda, Brazil) was applied for five minutes . The resulting juice was depectinized with enzymes (Pectinex 3XL, Novozymes) in the proportion of 3 mL/hL (60 min at 45 °C) and after sedimentation and paper filtration, it was used in the fermentation trials.

Apple juice fermentation
The juice of each variety (450 mL) was conditioned in seven Erlenmeyer flasks (500 mL). All fermentation systems were equipped with a bung and had been previously sterilized (Vertical Sterilizer Phoenix, Model AV75) for 20 min at 1 bar (121 °C). The starter culture, a commercial yeast strain *Saccharomyces cerevisiae* (Uvaferm CK - Danstar Ferment GAC, Denmark) used in Brazil cider processing, stored in its active dry form, and re-hydrated during 20 min in a glucose solution 3.0 g/100mL, had the initial population of 2.0 x 10^6 cell/mL. The yeast cells were counted in a Neubauer (XB-K-25, SMIC, China) chamber (Lee et al., 1981). The contents of the seven micro-fermenters were analyzed after 12, 24, 36, 48, 200 and 360 h, with an initial control at the

beginning (0 h) after this time the fermented apple juice was transferred and bottled, and stored at 5ºC.

Kinetics of fermentation

The fermentation was monitored by the loss of mass from the system caused by the release of the CO_2 (Bely et al., 1990; Roger et al., 2002) and the weight was determined at every two hours at a sensibility of 0.001 g during 15 days (fermentation time) at room temperature (18-25 ºC). The CO_2 production rate (dCO_2/dt) was calculated by the polynomial smoothing.

Two other kinetic parameters were also determined by the loss of mass, maximum specific CO_2 production rate $[(1/X.dCO_2/dt)_{max}]$ and maximum acceleration $[(d^2CO_2/dt^{-2})_{max}]$. The fermentation was considered over $[T_{max}]$ when total sugar content was less than 1.0 g/L (Bely et al., 1990). All the fermentation experiments were run in duplicate.

Physicochemical Analysis

The reducing sugars were quantified by the method of Somogyi (1945) and Nelson. (1944); The total reducing sugars were obtained after hydrolysis of sucrose with 1N HCl (50 ºC/ 5 min). Glucose was quantified by the enzymatic method of glucose oxidase and sucrose and fructose were calculated by difference. All the sugars were expressed in g/100mL (Tanner and Brunner, 1985). The total acidity was determined by neutralization with 0.1N NaOH with phenolphthalein as an indicator and expressed as malic acid in g/100mL (IAL, 2008). The total phenolic compounds were quantified with the Folin Ciocalteau reagent using catechin as the standard, and the results were expressed as mg/L (Singleton and Rossi, 1965). The ethyl alcohol content was determined by ebuliometry. The total nitrogen content was determined by the method of Kjedhal (IAL, 2008) and that of N α-amine was calculated by the formol titration according to Julien et al. (2001).

RESULTS AND DISCUSSION

Nitrogen content in apple must

The average value found for the nitrogen content in 51 analyzed samples was 155.81 mg/L (Table 1) and the distribution frequency of the results (Fig. 1) showed that 86.28 % of the samples had more than 100 mg/L nitrogen. Since the average value is enough to drive to an unstable end product with high residual nitrogen content due to the fact that *Saccharomyces* sp. does not assimilate all the available nitrogen in the growth phase (Nogueira and Wosiacki, 2010). In this case, nitrogen is not a nutrient factor for alcoholic fermentation. From a practical point of view, as in Brazil the cider is made with the mixture of juices from different varieties, those with low nitrogen content (13.72 %) should be corrected by blending without any supplementation.

The same kind of analysis was made in industrial apple must in France in the 1960's and the average nitrogen content found was 80 mg/L. This value increased up to 130 mg/L 30 years later in the 1990's due to an extensive use nitrogen fertilizers in the soil and with the effect of keeping the color and the flavor of apples (Drilleau, 1990; EPAGRI, 2002). This increase of nitrogen causes some difficulty to control of the fermentation speed and the residual content of sugar in the final product, which show troubles as cloud or haze formation in the stored cider and also microbiological instability, leading to explosions of bottles due to the formation of gases. These problems led to the modifications in the processing technology in order to maintain the standard of quality of the French cider.

Table 1 - Statistic descriptive of 51 total nitrogen analysis of the apple must.

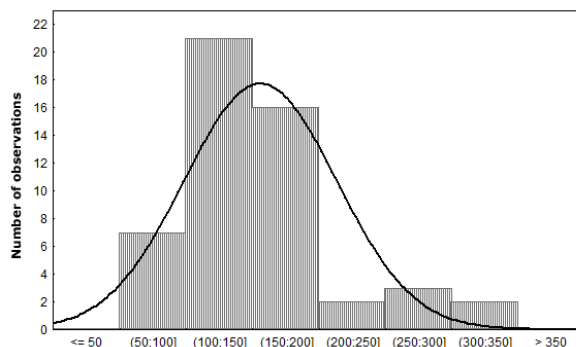| Statistic parameters	Values
Minimum, mg/L	59.00
Average, mg/L	155.81
Maximum, mg/L	330.12
Standard deviation,	57.43
Variation coefficient, %	36.86

Figure 1 - Frequency distribution of the nitrogen determinations (mg/L) in 51 apple must.

The number and the precision of the loss of mass determination allowed to determine the rate of gas formation accurately. The optimization of this system showed that the maximum fermentation rates $[(dCO_2/dt)_{max}]$ in five assays was 4.48 g/L.day with 0.072 g/L of standard deviation, leading to a low variation coefficient of 1.51 % (Fig. 2). The maximum fermentation rate was reached when 4.50 ± 0.10 g/L of CO_2 was released.
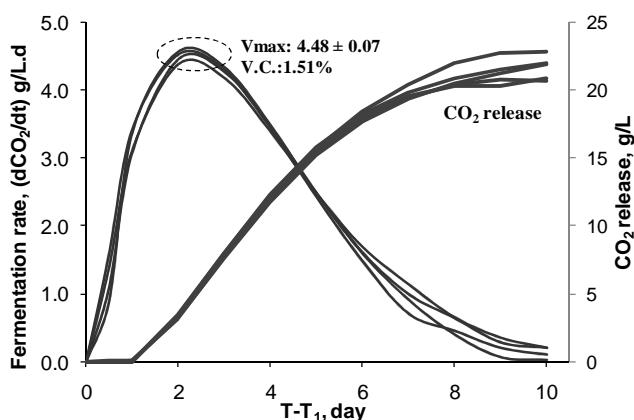

Figure 2 - Illustration of the precision of kinetic parameter in five 500 mL-flasks fermentation.

Nitrogen content and fermentation rate

For this assay three different apples juices were used. Table 2 depicted the results of the physicochemical parameters of the juices obtained from three apple cultivars, which showed similar values in all the analyses, except in the total nitrogen level, 163.64 ± 2.41, 122.39 ± 3.22 and 58.82 ± 6.18 mg/L, for Fuji, Gala and Joaquina cultivars, respectively, which were considered as high (>150 mg/L), normal (75 to 150 mg/L) and low (< 75 mg/L), respectively according to Nogueira and Wosiacki (2010). The amine nitrogen content was 53.0, 30.0 and 29.0 % of the total nitrogen for Joaquina, Gala and Fuji, respectively, which was in agreement with Burroughs (1957) who reported that the proportion of both nitrogen in apple must corresponded from 15.2 up to 61.2 %. Asparagine accounted for up to 50 % of the soluble nitrogen content of the high nitrogen juices but was relatively less important in those of low nitrogen; aspartic and glutamic acids were important constituents of all the juices (Beech, 1993; Drilleau, 1993).

Table 2 - Physicochemical composition of three apple juices.

Physicochemical composition,	Apple juices		
	Fuji	Gala	Joaquina
Total sugar*	135.9 ± 0.61	128.6 ± 0.51	131.2 ± 0.55
Reducing sugar*	114.2 ± 0.50	104.9 ± 0.60	104.1 ± 0.19
Sucrose*	21.6 ± 0.68	23.7 ± 1.09	27.1 ± 0.54
Glucose*	23.5 ± 0.80	16.1 ± 0.26	19.7 ± 0.72
Fructose*	90.7 ± 0.54	88.8 ± 0.77	84.4 ± 0.88
Malic acid*	2.0 ± 0.02	2.5 ± 0.01	2.6 ± 0.01
Volatile acidity*	0.04	0.04	0.04
Total phenols**	309 ± 30	392 ± 60	330 ± 65
Total nitrogen**	163.64 ± 2.41	122.39 ± 3.22	58.82 ± 3.18
Amine nitrogen**	46.6 ± 0.03	37.3 ± 0.03	31.1 ± 0.01

Note: * g/L; **mg/L.

Fig. 3A, shows the growth profile of *S. cerevisiae* Uvaferm CK in the three three varities of juices. Table 3 shows the kinetics values. In the fermentation medium of Fuji cv., the yeast reached a maximum population (X_{max}) of 8.66 x 10^7 cell/mL in 1.56 days, which was higher than the number usually found (2 to 4 x 10^7 cell/mL) (Lequeré and Drilleau, 1998; Drilleau, 1996; Nogueira et al., 2007a). In the case of cv. Gala, the maximum population was lower (4.42 x 10^7 cell/mL), although still close of the usual population level at the same time (1.6 days). The fermentation medium of cv. Joaquina supported a population of 2.05 x 10^7 cell/mL which was the at longer time (1.8 days). The differences in yeast development are due to the effect of the initial nitrogen levels. Fig. 3B showed the consumption of available nitrogen by the yeast in the three varities of the must during the fermentation. The nitrogen consumption was 103, 86 and 27 mg/L for Fuji, Gala and Joaquina apple juice, respectively. The consumption by the yeast was associated with the growth phase and the amount utilized was directly related to the maximum yeast population which was in agreement with Drilleau (1993) who found that 80 to 90 % of the total nitrogen was consumed during the exponential phase in cider fermentation.

The residual non-available nitrogen (lysine) or very low available nitrogen (leucine, phenylalanine, serine and tyrosine) by the yeasts in apple juice fermentation could vary between 20 to 35 mg/L (Nogueira et al., 2007b). If the residual concentrations (Fig. 3B) were higher than these, it is probable that there was still some available nitrogen in the residue, as was the fermented of Fuji juice variety, with around 60 mg/L of residual nitrogen (Fig. 3B).

There was a significant effect on the fermentation profile of the three varieties of the musts (Figs. 3C and 3D). When the sugar concentration was less than 2.0 g/L, the rate of fermentation reached very near to zero. The sugar consumption and alcohol production showed a straight relationship with assimilated nitrogen by the yeast. The fermentation period (T_{max}) determined with sugar content lower than 1.0 g/100mL was of 10.5 and 19.0 days for Fuji and Joaquina, respectively (Table 3). The nitrogen difference between these two varieties almost duplicated the fermentation time.

The fermentation rate (Fig. 3D) was calculated considering the loss of mass as measured every two hours. At the end of the process, the results showed a theoretical tendency expressed as a 6th order polynomial equation with high correlation coefficients: Fuji, R^2 = 0.998, Gala, R^2 = 0.999 and Joaquina, R^2 = 0.999. The kinetics parameters are shown in Table 3. Maximum fermentation rates [$(dCO_2/dt)_{max}$] were dependent on the initial nitrogen level, corresponding to 9.2, 5.1 and 1.4 g/(L.d), for the assays of cv. Fuji, Gala and Joaquina, respectively. The fermentation rates were coherent with the values of acceleration [$(d^2CO_2/dt^2)_{max}$] 7.9, 5.9 and 0.9 g/(L.d^2), respectively.

In Fuji cv. 7.3 g/L CO_2 was released during the growth phase with CO_2/N of 0.07 g/mg and the nitrogen content was enough for the production of the biomass and for starting the fermentation. In Joaquina cv, 0.8 g/L CO_2 was released during growth phase with CO_2/N (g/mg) of 0.03 g/mg and all the nitrogen was utilized only for biomass production, and even then it was not enough for starting the fermentation.
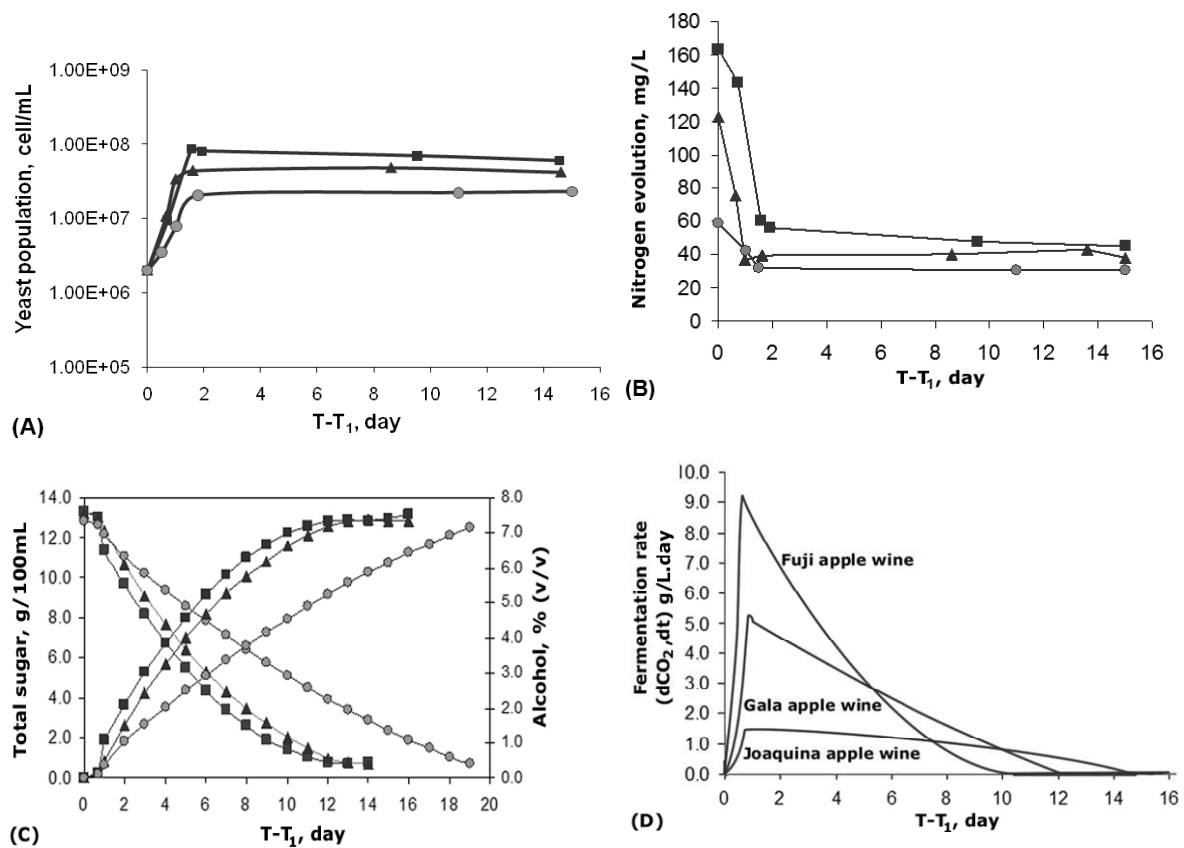
Figure 3 - Growth profile of *Saccharomyces cerevisiae* Uvaferm CK (A), nitrogen consumption (B), sugar consumption and alcohol production (C), fermentation rate (D) during cider fermentation in apple varietal must: Fuji (■); Gala (▲) and Joaquina (●).

Table 3 - Kinetic parameters of the three varietal apple juice fermentation.

Kinetic parameters	Varietal apple juice fermented		
	Fuji	Gala	Joaquina
$(dCO_2/dt)_{max}$ (g/L.day)	9.20	5.10	1.40
υ_{max} (g.10^{-7}/d)	1.06	1.15	0.56
X_{max} (10^6/mL)	86.60	44.20	25.00
T_{max} (day)	10.50	12.00	19.00
$(d^2CO_2/dt^{-2})_{max}$ (g/L.d^2)	7.90	5.90	0.90
(g CO_2 release / mg N)$_{max}$*	0.07	0.05	0.03

* end growth phase.

$(dCO_2/dt)_{max}$: maximum CO_2 production rate.

υ_{max} : $(1/X.dCO_2/dt)_{max}$: maximum specific CO_2 production rate.

X_{max}: maximum cell production.

T_{max}: fermentation duration (sugar < 1.0 g.L).

$(d^2CO_2/dt^{-2})_{max}$: maximum acceleration.

CONCLUSIONS

Brazilian apple must showed average nitrogen concentration above 100 mg/L. At this concentration the alcoholic fermentation occurs without any interruptions. The nitrogen content in apple juice affects directly the yeast growth and fermentation kinetics. However, although in must with low nitrogen content, the fermentation was slower but it occurred until complete exhaustion

of the fermentable sugars. The Brazilian apple must not be supplemented with nitrogen for alcoholic fermentation.

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