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SO_2 protects the amino nitrogen metabolism of *Saccharomyces cerevisiae* under thermal stress

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Summary

Thermal stress conditions during alcoholic fermentation modify yeasts' plasma membrane since they become more hyperfluid, which results in a loss of bilayer integrity. In this study, the influence of elevated temperatures on nitrogen metabolism of a *Saccharomyces cerevisiae* strain was studied, as well as the effect of different concentrations of SO_2 on nitrogen metabolism under thermal stress conditions. The results obtained revealed that amino nitrogen consumption was lower in the fermentation sample subjected to thermal stress than in the control, and differences in amino acid consumption preferences were also detected, especially at the beginning of the fermentation. Under thermal stress conditions, among the three doses of SO_2 studied (0, 35, 70 mg l^{-1} SO_2), the highest dose was observed to favour amino acid utilization during the fermentative process, whereas sugar consumption presented higher rates at medium doses.

Introduction

Changes in environmental conditions such as temperature, aeration and nutrients can significantly affect yeast performance during alcoholic fermentation. The application of high temperatures during alcoholic fermentation is useful in order to extract the greatest quantity of tannins and polyphenols in red wine making and to obtain wines with low alcohol content, in accordance with current consumer tastes. However, yeasts cannot regulate their internal temperature and thermal stress causes cellular damage, leading to adverse effects on yeast cell physiology. Thermal stress produces an increased fluidity and membrane permeability to protons and other ions (Bischof *et al*., 1995; Piper *et al*., 1997), denaturation of membrane proteins (Lepock *et al*., 1993), membrane blebbing (Martínez de Marañón *et al*., 1999) or cell lysis (Gershfeld and Murayama, 1988). Hazel (1995) and Martínez de Marañón and colleagues (1999) found that when temperature exceeds the physiological range, membranes become hyperfluid and destabilization of the lamellar phase occurs, which results in a loss of bilayer integrity. Moreover, yeast metabolism mainly depends on the uptake of nutrients driven by permeases and amino acid transport in yeast is also influenced by fatty acid composition (Ayestarán *et al*., 1995; 1998) and membrane fluidity (Mishra and Prasad, 1989). Ethanol concentration also affects the composition, structure and permeability of the plasma membrane, and consequently, the activity of glucose, ammonium and amino acids transport systems is altered (Leáo and van Uden, 1982; 1983). Furthermore, ethanol accelerates the passive entrance of protons from the medium to the cytoplasmic matrix (Ingram *et al*., 1986), and dissipates the proton motive force necessary for amino acid transport into the cell (Leáo and van Uden, 1984; Cartwright *et al*., 1986). Guerzoni and colleagues (1999) found that, at fermentation temperatures above 30°C, ethanol toxic action towards yeasts is enhanced.

SO_2 has been used as a preservative agent because of its several functions in wine conservation. *Saccharomyces cerevisiae* is relatively resistant to sulphite. The main protein involved in sulphite resistance in this yeast is the sulphite pump Ssulp (Avram and Bakalinsky, 1997; Park and Bakalinsky, 2000) that mediates sulphite efflux. On the other hand, SO_2 could influence the utilization of amino acids by yeast although it is not clear whether it negatively alters nitrogen metabolism or whether SO_2 is a protective agent for yeasts against stress conditions during fermentation. Maier and colleagues (1986) found that SO_2 inside the cell would induce changes in enzymatic 3D-conformations and would cause depletion in the yeast's cellular ATP content due to its effects on glycolysis and respiratory chain phosphorylation. Caridi (2002; 2003) suggested that it is possible that SO_2, inositol and catechin act as protectors of *S. cerevisiae*, minimizing the adverse effects produced in this yeast under stress conditions, thus improving wine quality. His results showed significant correlations between the addition of these protectants and the change in metabolic behaviour of yeasts

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under concomitant thermal and osmotic stress. He suggested that some strains increased fermentation vigour and produced more normal metabolite profiles. However, whether those conditions stimulated nitrogen accumulation was not reported.

For these reasons, the aims of this work were: (i) to study the influence of high temperatures on nitrogen metabolism of a *S. cerevisiae* strain and (ii) to observe the effect of different concentrations of SO_2 on the nitrogen metabolism of this yeast when subjected to heat stress.

Results and discussion

Fermentation kinetics and general parameters

Sugar consumption (%) of control fermentation is compared with that of thermal stress fermentation (F35) in Fig. 1A; these two fermentations were performed with the same initial level of SO_2 (35 mg l^{-1}). In this figure, it is observed that the initial sugar consumption rate was similar in both cases; however, after 4 days, the control sample presented a higher rate than the one subjected to thermal stress. In the control sample, 99% of must sugar was consumed, whereas in the sample subjected to thermal stress yeasts consumed 90% of sugar. In cells subjected to thermal stress the passive proton influx towards the cytoplasm is increased, so the intracellular pH decline and this can be a major factor contributing to the inhibition of fermentation rate (Neves and François, 1992). Besides, at the plasma membrane, the increase of the passive proton influx due to thermal stress will act to dissipate the electrochemical potential gradient maintained across this membrane by the action of plasma membrane H^+-ATPase (Serrano, 1991). The electrochemical potential gradient is essential for vital functions such as the maintenance of potassium balance and the regulation of intracellular pH.

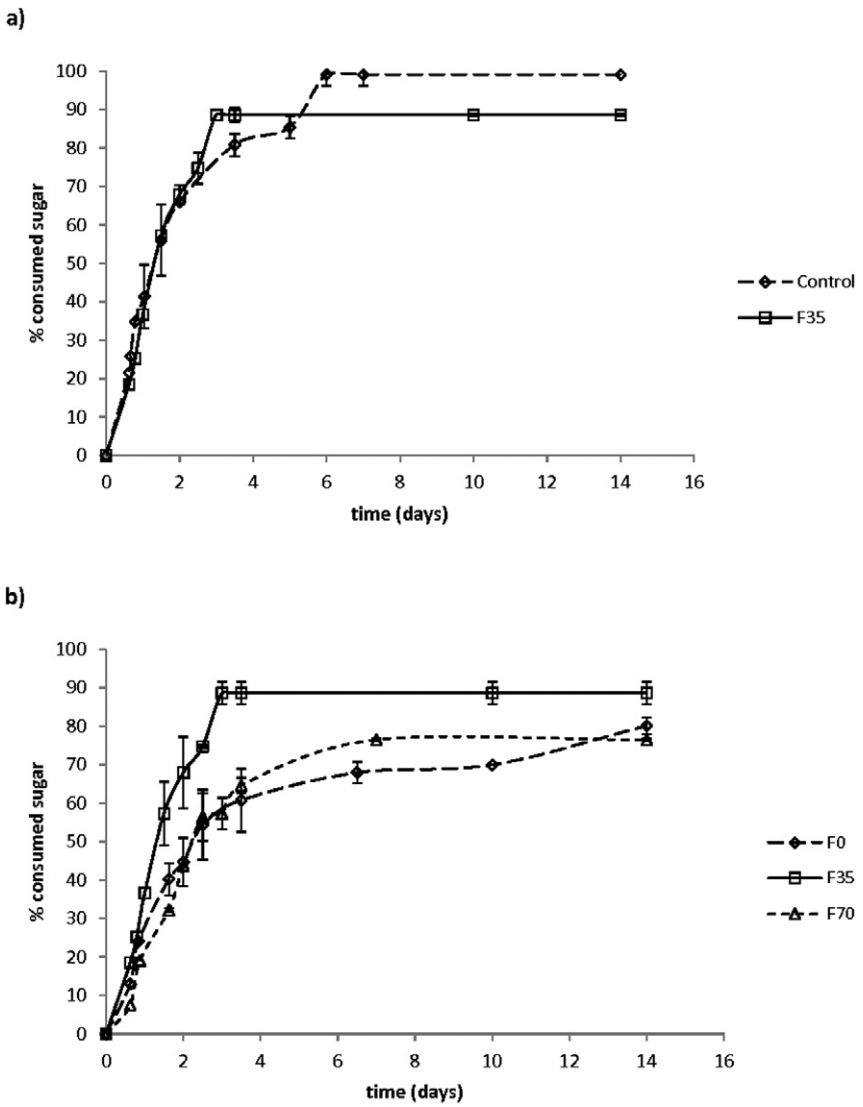

Fig. 1. Fermentation kinetics (A) at two different temperatures (29°C and 35°C) in the presence of 35 mg l^{-1} of SO_2 (B) thermal stress (35°C) in the presence of 0, 35, 70 mg l^{-1} of SO_2.

Table 1. Enological parameters of wines.

	Control (29°C, 35 mg l^{-1} SO_2)	F35 (35°C, 35 mg l^{-1} SO_2)	F0 (35°C, 0 mg l^{-1} SO_2)	F70 (35°C, 70 mg l^{-1} SO_2)
pH	3.09 ± 0.01	3.20 ± 0.01	3.12 ± 0.03	3.11 ± 0.02
Alcohol content (% v/v)	10.91 ± 0.02	10.28 ± 0.08	9.38 ± 0.01	9.24 ± 0.12
Total acidity (g l^{-1})[a]	7.4 ± 0.0	6.7 ± 0.2	7.5 ± 0.3	7.3 ± 0.1
Volatile acidity (g l^{-1})[b]	0.33 ± 0.02	0.46 ± 0.05	0.41 ± 0.01	0.42 ± 0.01
Reducing sugar (g l^{-1})	1.6 ± 0.4	20.5 ± 4.3	35.9 ± 3.2	42.5 ± 0.4
Glycerol (g l^{-1})	7.8 ± 0.1	7.1 ± 0.1	6.9 ± 0.1	6.4 ± 0.1

All parameters listed with standard deviation.
a. Expressed as tartaric acid.
b. Expressed as acetic acid.

In Fig. 1B it may be observed that the three fermentations subjected to thermal stress presented a high sugar residue. Comparing the three fermentations with different levels of SO_2, the fastest was the one with 35 mg l^{-1} of SO_2; fermentation without SO_2 was the slowest and the fermentation with 70 mg l^{-1} of SO_2 presented an intermediate rate. It has been suggested that the reason why SO_2 stimulates the fermentation by *S. cerevisiae* lies in the inhibition on the competing polyphenol-oxidase, making oxygen more available for *S. cerevisiae* (Boulton *et al*., 1996). Caridi (2002) studied the action of protective agents (catechin, inositol, SO_2) to reverse the metabolic changes induced in wine yeast by thermal stress. In this study, it was observed that the addition of 100 mg l^{-1} of SO_2 produced an increase in the fermentation rate in two of the six yeast strains studied. However, SO_2 can also act both in glycolysis and in respiratory chain phosphorylation in yeast, causing ATP depletion. Sulphite may react with certain molecules inducing changes in enzyme conformation, which produces inhibition of glyceraldehyde-3-phosphate dehydrogenase. This enzyme participate in the conversion of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate, so glycolytic pathway and ethanol formation are affected (Maier *et al*., 1986), as glyceraldehyde-3-phosphate is the molecule ultimately converted to pyruvate, which gives rise to ethanol (Boulton *et al*., 1996).

In Table 1 general parameters of the wines are presented. It may be observed that volatile acidity was greater in the wines obtained at high temperatures than in the control sample. This can be attributed to the lower fermentation rate of thermal stress fermentation. Volatile acidity of wines was inferior in all cases to the threshold found by Peynaud (1993) as undesirable for wine aroma (0.6 g HAc l^{-1}). Wines coming from fermentations subjected to thermal stress presented lower ethanol content than the control wine because there was an unfermented sugar residue. Caridi and colleagues (1999) also found that yeasts subjected to stress modified their metabolic behaviour and, probably as a defence mechanism, gave low ethanol yield and abnormal high acetic acid production. F35 sample showed a pH value slightly higher than the other samples. pH values in all wines were appropriate to their later stabilization and conservation, and they were in all cases between the range (3.0–3.67) that Amerine and Ough (1976) found as optimal for wine conservation. Total acidity, as occurs with pH, was slightly lower in F35 sample than in the other samples. In all wines, total acidity values were optimal for wine conservation. Glycerol content in F70 sample was lower than in the other samples. It seems that the higher dose of SO_2 (70 mg l^{-1}) acts as a protectant for yeasts.

Utilization of amino nitrogen during fermentation

Amino nitrogen, which represents the most important nitrogen fraction of the assimilable nitrogen by yeasts during the growing phase, was consumed both in the control sample and in F35 sample, mainly at the beginning of the fermentation (Table 2), before the appearance of appreciable quantities of ethanol, which difficult the nutrient transport inside the cell (O'Connor-Cox and Ingledew, 1989). However, comparing both fermentations, it can be observed that, at the beginning of fermentation, amino nitrogen consumption was greater ($P < 0.05$) in the control sample than in the F35 sample. Llauradó and colleagues (2005) also observed that yeast metabolism is affected by fermentation temperature. Previous physiological studies (Pizarro *et al*., 2008) have revealed that protein translation rates, cell membrane fluidity, RNA secondary structure stability, enzymatic activity, protein

Table 2. Consumption of amino nitrogen (mg N l^{-1}) during different stages of the fermentation.

	25% reducing sugar	50% reducing sugar	Wine
Control	93 ± 3 a	27 ± 1 a	7 ± 2 a
F35	80 ± 2 b	22 ± 2 a	10 ± 1 b
F0	76 ± 11 b	51 ± 7 b	6 ± 1 ac
F70	94 ± 4 a	45 ± 6 b	4 ± 1 c

Means within the same column followed by different letters are significantly different ($P < 0.05$).

folding rates and heat shock protein regulation are significantly affected by temperature. This would explain the different kinetics of amino nitrogen accumulation between the two samples. The consumption of amino nitrogen from 25% to 50% of consumed sugars was similar in both fermentations (Table 2). This is probably due to the fact that the cellular mechanisms for controlling stress conditions involves the rapid synthesis of protective molecules and the activation of signal transduction systems that induce the activation of enzyme activities and the transcription of genes encoding factors with protective functions (Novo *et al*., 2004). During the second half of fermentation (from 50% consumed sugar to wine), there were negligible levels of amino nitrogen consumption because high ethanol concentrations affected the composition and permeability of the plasma membrane and thus the amino acid transport systems were also altered.

Comparing the three fermentations subjected to heat stress with different SO_2 concentrations (F0, F35, F70), it may be observed that, at the beginning, greater levels of amino nitrogen were consumed in F70 sample than in F0 and F35 samples, whose consumption was similar (Table 2). Therefore, high concentrations of SO_2 seem to have favoured the amino nitrogen consumption. Under normal physiological conditions, degeneration and scavenging of ROS (Reactive Oxygen Species) keep balance to avoid molecular damage (Herrero *et al*., 2008). This balance is disturbed when cells are exposed to diverse environmental stress conditions, such as thermal stress. The increased level of ROS damages proteins and cell membranes, and therefore, a number of cellular processes are affected, such as nitrogen compounds transport through the plasma membrane. As SO_2 is an antioxidant, it could, at high concentrations (70 mg l^{-1}), act as a protector against ROS, improving membrane transporters behaviour. In the exponential phase of fermentation (25–50% consumed sugars), amino nitrogen consumption was greater ($P < 0.05$) in the F70 and F0 samples than in F35 sample. Therefore, both temperature and SO_2 concentration have an effect on nitrogen metabolism, which may affect the growth rate and amino nitrogen preferences.

Influence of fermentation temperature on the amino acids utilization

In both fermentations (control and F35) the greater bulk of amino acids were consumed in the initial stage of fermentation (25% consumed sugar) (Table 3). Throughout all the fermentation, total amino acids consumption was greater in the control sample than in the sample subjected to heat stress. According to the ratio obtained, there was greater consumption of glutamic acid, asparagine, glycine, proline, isoleucine and tryptophan in the control sample than in the sample subjected to heat stress (F35). In this sample, only aspartic acid and alanine were more consumed throughout the whole process. Beltrán and colleagues (2004) found that low-temperature fermentation produced similar metabolic effects to those obtained in nitrogen-limited fermentation. These authors observed

Table 3. Consumption (mg l^{-1}) of amino acids in control sample and in F35 sample during fermentation.

	25% consumed sugar		50% consumed sugar		Wine		Ratio[a]
	Control	F35	Control	F35	Control	F35	Control/F35
PSER	2.1 a	2.1 a	–4.2 a	–3.6 a	–7.6 a	–3.9 b	–
ASP	0.4 a	5.8 a	6.9 a	1.4 b	–1.9 a	–0.3 b	0.8
GLU	16.3 a	15.8 a	5.3 a	8.0 b	5.0 a	–4.7 b	1.4
PEA	1.3 a	1.3 a	–1.4 a	–1.3 a	1.4 a	–0.3 b	–
SER	8.9 a	8.9 a	0.5 a	0.5 a	–0.3 a	0.0 a	1.0
ASN	3.5 a	3.4 a	0.1 a	0.3 a	1.2 a	–0.7 b	1.6
GLY	0.1 a	2.1 b	0.3 a	2.5 a	0.0 a	–0.2 a	1.5
GABA	14.0 a	14.0 a	–1.1 a	–1.5 a	–0.3 a	–0.4 a	1.0
ALA	26.5 a	33.0 a	17.5 a	11.1 a	–2.0 a	1.2 a	0.9
ARG	245.7 a	197.5 b	10.1 a	58.1 b	–6.0 a	–2.9 b	1.0
PRO	–146.9 a	–143.9 a	152.4 a	64.3 b	49.3 a	85.8 b	11.0
VAL	8.2 a	7.7 b	1.5 a	1.9 b	–1.2 a	–1.0 a	1.0
MET	0.4 a	2.2 b	3.6 a	1.1 b	–0.6 a	–0.5 a	1.2
ILE	3.6 a	2.4 b	0.4 a	1.4 b	–0.5 a	–1.1 b	1.3
LEU	4.9 a	5.0 b	1.1 a	0.9 b	–1.6 a	–1.6 a	1.0
PHE	4.4 a	3.3 a	1.7 a	1.8 a	–1.3 a	–0.3 a	1.0
TRP	12.4 a	15.2 b	5.0 a	2.1 b	–0.5 a	–9.7 b	2.2
LYS	0.07 a	0.85 b	1.7 a	–0.6 a	1.4 a	–0.9 a	–
Aa consumed (mg l^{-1})	352.8 a	316.3 b	208.3 a	155.7 b	58.3 a	86.7 a	1.2
Amino-N consumed (mg N l^{-1})	75.6 a	61.3 b	26.7 a	30.4 a	4.9 a	7.4 a	–

Means within the same row followed by different letters are significantly different ($P < 0.05$).
a. As the ratio between total amino acids consumed in control and F35 samples was 1.2, amino acids with a ratio > 1.2 were more consumed in control sample.

that in fermentations that were not nitrogen-limited, yeasts cells consumed much less nitrogen at 13°C than at 25°C; this is due to a decrease in the fluidity of the plasma membrane at low temperatures, which considerably reduces the molecular motion of phospholipids and membrane proteins (McDonald, 1987). On the other hand, when temperature is too high, membrane become hyperfluid and destabilization of the lamellar phase occurs, which results in a loss of bilayer integrity (Hazel, 1995; Martínez de Marañón *et al*., 1999). This alteration in membrane fluidity might impair the activity of some permeases (Abe and Horikoshi, 2000). Therefore, at extreme temperatures, both above or below the physiological range, similar effects can be found in plasma membrane, although by means of different mechanisms. This is because membrane permeases are highly temperature-dependent because changes in temperature can cause conformational changes to their structure (Entian and Barnett, 1992).

Amino acid preferences were also different at both temperatures during the different stages of the fermentation (Table 3). In the first stage of the alcoholic fermentation (25% consumed sugars) the uptake of glycine, methionine, tryptophan and lysine was higher ($P < 0.05$) in the fermentation subjected to thermal stress than in the control sample. These amino acids are not preferential nitrogen sources for the yeasts (Boulton *et al*., 1996). On the other hand, arginine, valine and isoleucine, for which yeasts show more preference, were mainly consumed in the control sample. The uptake of nitrogen by the cells is regulated by the mechanism known as Nitrogen Catabolite Repression (NCR). This NCR enables the cell to select the best nitrogen sources by repressing the transcription of some genes involved in the utilization of poor nitrogen sources (Magasanik, 1992). High temperatures of fermentation would probably affect this regulator mechanism due to the changes produced in plasma membrane, where amino acids transporters are located. The lower consumption of arginine in yeasts subjected to heat stress may be underlined, as this is contrary to what was found in the work of Beltrán and colleagues (2004), where this amino acid was consumed more at low temperatures (13°C). Proline was excreted at the beginning of the fermentation in a similar way in both samples. This amino acid is important in response to stress, because it is accumulated in many bacteria and plant cells as a protectant. Poole and colleagues (2009) observed that the increase in proline accumulation was associated with increased cell viability in conditions of high temperature and osmotic stress. However, it has been shown that proline levels are not increased under various stress conditions in *S. cerevisiae* cells (Takagi, 2008). Our results showed higher proline consumption in the control fermentation (ratio C/F35 = 11) than in the samples subjected to thermal stress.

From 25% to 50% consumed sugars, total consumption of amino acids was also higher in control sample than in F35 sample (Table 3). In this stage, arginine uptake was higher in F35 sample than in the control sample. In both samples proline uptake was observed, although more in the control sample. In this sample there was also a greater consumption of asparagine, methionine, leucine, triptophan and lysine. At the end of the fermentation, most of the amino acids were excreted and only proline was consumed, although this consumption was higher in F35 sample than in the control sample.

Influence of SO_2 in the utilization of amino acids in samples subjected to thermal stress

Sulphite acts both on glycolysis and respiratory chain phosphorylation causing ATP depletion. Due to this, cells verify an energy deficiency status, which may require the modifications of some metabolic pathways to gain a more efficient utilization of the energy. At the beginning of the fermentation (25% consumed sugars), the sample that presented the higher total of amino acid consumption was F70 (Table 4); while between the other two samples, consumption was higher in F35 sample than in F0 sample. Arginine uptake was significantly higher ($P < 0.05$) in F70 sample than in the other ones, which presented similar results. This amino acid fulfils around 30–50% of the nitrogen requirements of yeasts because it is a major amino acid and its degradation provides three nitrogen atoms from each molecule in anaerobic or fermentative conditions, due to the fact that one atom of nitrogen is usually released as proline (Martin *et al*., 2003). Lysine and tryptophan, with more than one amino group in the molecule, were consumed equally in all the samples irrespective of the SO_2 quantity added. Alanine consumption did not show any significant differences with regards to the SO_2 treatment. Contrary, in previous studies (Garde-Cerdán *et al*., 2007; Cejudo-Bastante *et al*., 2010), it was observed that, in normal temperature conditions, this amino acid was highly consumed in fermentations with SO_2 than in those ones without this additive. The quantity of glutamic acid consumed was significantly higher ($P < 0.05$) in the sample with 70 mg l^{-1} of SO_2 than in the other ones (Table 4). As regards proline, an important excretion was produced in all three samples at the beginning of the fermentation, although it was greater in F70 sample. Although proline protects cells against many stress conditions including freezing, desiccation, oxidation and ethanol (Takagi, 2008), it does not present a heat-stress-protective activity, so instead of being accumulated in the cytoplasm; it is released outwards as a consequence of arginine metabolism. The rest of amino acids were consumed similarly in all three samples.

Table 4. Consumption (mg l^{-1}) of amino acids in control sample, F35 and F70 sample during fermentation.

	25% consumed sugar			50% consumed sugar			wine		
	Control	F35	F70	Control	F35	F70	Control	F35	F70
PSER	2.1 a	2.1 a	2.1 a	–6.3 a	–3.6 a	–3.6 a	–0.7 a	–3.9 a	–3.0 a
ASP	6.7 a	5.8 b	7.3 a	1.0 ab	1.4 a	0.6 b	0.6 a	–0.3 b	–1.4 c
GLU	15.5 a	15.8 a	24.8 b	6.8 a	8.0 a	–1.2 b	0.0 a	–4.7 b	3.4 c
PEA	1.3 a	1.3 a	1.3 a	–1.9 a	–1.3 b	–1.3 b	0.0 a	–0.3 b	0.7 c
SER	9.1 ab	8.9 a	9.3 b	0.4 a	0.5 a	0.2 a	–0.3 a	0.0 a	–0.7 b
ASN	3.4 a	3.4 a	3.8 b	0.4 a	0.3 ab	0.0 b	–0.1 a	–0.7 b	1.0 c
GLY	–1.2 ab	–2.1 a	–0.8 b	1.6 a	2.5 b	1.2 a	0.1 a	–0.2 b	0.2 a
GABA	10.0 a	14.0 b	14.0 b	3.6 a	–1.5 b	–5.4 c	–0.9 a	–0.4 a	4.9 b
ALA	33.2 a	33.0 a	35.0 a	5.6 a	11.1 b	10.4 b	6.5 a	1.2 b	0.4 b
ARG	188.5 a	197.5 a	240.0 b	66.0 a	58.1 a	13.8 b	0.4 a	–2.9 b	–3.5 b
PRO	–205.1 a	–143.9 a	–283.9 b	222.8 a	64.4 b	308.8 c	33.6 a	85.8 b	18.5 a
VAL	8.0 ab	7.7 bc	7.6 c	1.6 a	1.9 b	1.8 ab	–0.2 a	–1.0 b	–0.7 b
MET	2.3 a	2.2 a	2.1 a	1.0 a	1.1 b	1.4 a	–0.5 a	–0.5 a	–1.7 b
ILE	2.8 ab	2.4 a	3.1 b	1.2 bc	1.4 c	0.9 ab	0.1 a	–1.1 b	–2.5 c
LEU	4.9 ab	5.0 a	4.8 b	1.1 ab	0.9 b	1.2 a	–2.9 a	–1.6 a	–2.7 a
PHE	4.3 a	3.3 b	2.7 c	1.3 a	1.8 ab	2.4 b	–0.6 a	–0.3 a	–0.2 a
TRP	14.2 a	15.2 a	15.6 a	2.2 a	2.1 a	1.8 a	–0.7 a	–9.7 b	–2.6 a
LYS	0.9 a	0.85 a	1.0 a	–4.2 a	–0.6 b	0.0 b	0.7 a	–0.9 a	–0.2 a
Aa consumed (mg l^{-1})	306.4 a	318.4 b	374.5 c	316.8 a	155.7 b	344.3 a	42.1 a	86.7 b	31.5 a
Amino-N consumed (mg N l^{-1})	51.0 a	61.3 b	69.9 c	50.9 a	30.4 b	43.8 a	4.7 a	7.4 b	1.2 c

Means within the same row followed by different letters are significantly different ($P < 0.05$).

From 25% to 50% of fermented sugars, a higher total amino acids were consumed in F70 and F0 samples than in F35 sample (Table 4). Differences regarding total consumption of amino acids during this fermentative phase were mainly due to the high uptake of proline in F70 and F0 samples. During the early stages of wine fermentation, when oxygen may be present, high levels of preferred nitrogen sources result in repression of transporters synthesis and inactivation of existing general amino acid permease and proline specific permease (Soetens *et al.*, 2001). Arginine consumption was higher in F0 and in F35 samples than in F70 sample, so it seems that a high concentration of SO_2 difficulted this amino acid transport and, despite being a preferential nitrogen source, its consumption decreased. At the end of fermentation, excretion of most amino acids in the three samples may be underlined, although proline continued to be consumed, especially in F35 sample.

Experimental procedures

Samples and vinification

The grape variety used for this study was *Vitis vinifera* var. Mazuelo. The grapes were destemmed and crushed and afterwards they underwent pressing and filtering. The must obtained was sterilized by thermal treatment. Juices are usually processed with higher treatment times because they have more carbohydrates and less alcohol content than wines. Thus, this research grape juice was thermally treated using a bench scale continuous pasteurizer made of two stainless steel tubular heat exchangers, a peristaltic pump model D-21V (Dinko, Barcelona, Spain), and a stainless steel tubing system. The first heat exchanger raised and maintained grape juice temperature at 90°C whereas the second one refrigerated the processed juice below 5°C. Both heat exchangers were submerged in separate water baths where water temperatures were 90°C and 0°C respectively. Grape juice flow was adjusted to 0.67 ml s^{-1} to obtain a total thermal treatment of 1 min.

After that, the must was divided into eight aliquots (400 ml each one). The aliquots were inoculated with active dry yeasts *S. cerevisiae* VRB mesophilic strain commercialized by Lallemand (Madrid, Spain). The strains were inoculated in the must in a proportion of 0.25 g l^{-1}. To do this, 1.25 g of dry yeast was rehydrated in a sterile flask in 12.5 ml of distilled water with 0.125 g of sucrose (number of viable cells per gram $\geq 2 \times 10^9$). It was kept in this medium for 30 min at 35°C. The must was inoculated with mixing, in order to get a homogeneous distribution. Two fermentations were carried out at 29°C and 35 mg l^{-1} of SO_2 (the control samples). The other fermentations were subjected to heat stress at 35°C; two fermentations were performed with 0 mg l^{-1} of SO_2 (F0 samples), two fermentation were performed with 35 mg l^{-1} of SO_2 (F35 samples) and two with 70 mg l^{-1} of SO_2 (F70 samples). Fermentations took place in 0.5 l round-bottom flasks with a burnished lid with two outlets, one for sample extraction and the other with a CO_2 trap to allow its exit and prevent the entrance of air during fermentation. The orifice for sample extraction was covered with a septum during the fermentation. The fermentors were placed over magnetic stirrers (Framo-Gerätetechnik M21/1, Eisenbach, Germany) at 700 r.p.m., to ensure a homogenous fermentation. The fermentations were carried out in a hot–cold incubator (Selecta, Barcelona, Spain) at a controlled temperature. The fermentations were measured daily for sugar concentration through refraction index at 20°C, using a refractometer ABBE (Misco, Cleveland, USA). Samples were taken before the beginning

Table 5. Concentration (mg l^{-1}) of amino acids in must.

Phosphoserine (PSER)	2.1 ± 0.5
Aspartic acid (ASP)	8.6 ± 0.5
Glutamic acid (GLU)	28.6 ± 0.5
Phosphoethanolamine (PEA)	1.3 ± 0.5
Serine (SER)	9.8 ± 0.5
Asparagine (ASN)	4.8 ± 0.5
Glycine (GLY)	1.1 ± 0.5
Gamma aminobutyric acid (GABA)	14.0 ± 0.5
Alanine (ALA)	46.4 ± 0.5
Arginine (ARG)	256.7 ± 0.5
Proline (PRO)	148.0 ± 0.5
Valine (VAL)	10.0 ± 0.5
Methionine (MET)	4.0 ± 0.5
Isoleucine (ILE)	4.1 ± 0.5
Leucine (LEU)	6.0 ± 0.5
Phenylalanine (PHE)	6.1 ± 0.5
Tryptophan (TRP)	17.4 ± 0.5
Lysine (LYS)	3.2 ± 0.5
Total amino acids	572 ± 53
Total amino nitrogen	122 ± 6

of the fermentation, at 25% of fermented sugars, at 50% of fermented sugars and at the end of fermentation. The concentration of amino acids in the must is presented in Table 5.

Preparation of sample and HPLC analysis of free amino acids

Analyses were performed with a Waters high-pressure liquid chromatograph (Milford, MA, USA) equipped with two 510 pumps, a 717 Plus Autosampler, and a 996 Photodiode Array Detector used at 254 nm. Pico·Tag reverse phase column (300 mm × 3.9 mm i.d.), with a stationary phase of dimethyloctadecylsilyl bonded to amorphous silica, was used. The Pico·Tag method used for amino acid analysis is described by Ayestarán and colleagues (1995). Samples were cleaned by ultrafiltration with a Millipore Ultrafree MC cartridge (Billerica, MA, USA), and then L-norleucine and L-methionine sulfone (Aldrich, Gillingham, England) were added as internal standards. Afterwards, a precolumn derivatization was carried out with phenylisothiocyanate (Pierce Biotechnology, Rockford, IL, USA).

Empower 2.0 software was employed for chromatographic control. The amount of sample injected was 10 µl. The column was set at 46°C. Mobile phase A: solution of 2.5% (v/v) of acetonitrile (Scharlau, Barcelona, Spain) and 97.5% (v/v) of a solution of sodium acetate (70 mM), with pH adjusted to 6.55 with acetic acid (10%) (Merck, Darmstadt, Germany); mobile phase B: acetonitrile, water and methanol (Scharlau) (45:40:15, v/v/v). The mobile phases used were filtered through a 0.45 µm Millipore filter. Amino acids were eluted under the following conditions: 1 ml min^{-1} flow rate, elution with linear gradients from 0% to 3% B in 13.5 min, from 3% to 6% B in 10.5 min, from 6% to 9% B in 6 min, from 9% to 34% B in 20 min, maintained during 12 min, from 34% to 100% B in 0.5 min, maintained during 4 min, followed by washing and reconditioning of the column.

Amino acids determinations were performed in quadruplicate on representative samples of the musts. The coefficient of variation for amino acid data obtained by the method described was between 1% and 15%.

Nitrogen fractions and general enological parameters

Amino nitrogen in must (122.5 mg l^{-1}) and general parameters of wine were measured using WineScan 79000 Auto (Foss Analytical, Denmark) with Fourier Transform Infrared Spectroscopy technology. The wavelength range of 240–1295 nm was used for these analyses. Nitrogen fractions and oenological parameters were made in duplicate on the must and wine samples.

Statistical analysis

The statistical study was carried out with analysis of variance (ANOVA), and the means were compared with the Scheffé test. The probability level was 0.05. For data analysis, the software SPSS v.17.0 was used (Chicago, Illinois, USA).

Conclusions

Thermal stress (35°C) produced lower fermentation rates than control fermentation (29°C), and gave rise to wines with higher sugar residue. Amino nitrogen and amino acids consumption was lower in fermentation subjected to thermal stress than in control fermentation. It may be concluded that high fermentation temperatures generated metabolic effects similar to the ones obtained in nitrogen-limited fermentations. Thermal stress also caused differences in amino acids preferences, especially at the beginning of the fermentation. Regarding the effect of SO_2 in fermentations subjected to heat stress, it was found that high doses of SO_2 (70 mg l^{-1}) enhanced the total consumption of amino acids throughout the fermentative process, although these samples did not present the highest fermentative rate. Therefore, it can be stated that high doses of SO_2 protected transport systems of nitrogen better than those of sugars.

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