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ORIGINAL PAPER

Effect of low-temperature fermentation on yeast nitrogen metabolism

Gemma Beltran · Nicolas Rozès · Albert Mas · José M. Guillamón

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Abstract The aim of this study was to analyse the influence of low-temperature wine fermentation on nitrogen consumption and nitrogen regulation. Synthetic grape must was fermented at 25 and 13°C. Low-temperature decreased both the fermentation and the growth rates. Yeast cells growing at low-temperature consumed less nitrogen than at 25°C. Specifically, cells at 13°C consumed less ammonium and glutamine, and more tryptophan. Low-temperature seemed to relax the nitrogen catabolite repression (NCR) as deduced from the gene expression of ammonium and amino acid permeases (*MEP2* and *GAP1*) and the uptake of some amino acids subjected to NCR (i.e. arginine and glutamine). Low-temperature influences the quantity and the quality of yeast nitrogen requirements. Nitrogen-deficient grape musts and low temperature are two of the main prevalent causes of sluggish fermentations and, therefore, the effects of both growth conditions on yeast metabolism are of considerable interest for wine making.

Keywords Low-temperature stress · Nitrogen regulation · *S. cerevisiae* · Wine fermentation

Introduction

Low temperatures (10–15°C) are used in wine fermentations to enhance production and retain flavour volatiles. In this way, white and rosé wines of greater aromatic complexity can be produced at low temperatures (Lambrechts and Pretorius 2000; Beltran et al. 2002; Llauradó et al. 2002; Novo et al. 2003a, b; Torija et al. 2003). However, temperature affects both the rate of yeast growth and the fermentation rate, with lower temperatures giving longer fermentations. Changes in the fermentation rate may also modify the yeast and bacterial ecology, ethanol sensitivity and yeast metabolism (Fleet and Heard 1993).

Yeast metabolism mainly depends on the uptake of nutrients driven by permeases. Membrane permeases are highly temperature dependent, because changes in temperature can cause conformational changes to their structure (Entian and Barnett 1992). Several studies have shown that, for temperatures between 15 and 35°C, glucose transport and glycolytic flux increase steadily with temperature (Sa-Correia and van Uden 1983; Leao and van Uden 1985). The uptake of nitrogen by the cells is regulated by the mechanism known as Nitrogen Catabolite Repression (NCR). When good nitrogen sources are present in the medium, the transcription of some genes involved in the uptake and the utilization of poorer nitrogen sources is repressed, and their corresponding products are inactivated and degraded (reviews ter Schure et al. 2000; Magasanik and Kaiser 2002). *GAP1* and *MEP2*, which encode two important permeases in the transport of amino acids and ammonium, respectively, are a good example of genes that are down regulated in a nitrogen-rich medium by the NCR mechanism (Beltran et al. 2004).

In this study, we have analysed the patterns of assimilable nitrogen uptake (amino acid and ammonium) by yeasts and the transcriptional activity of the

G. Beltran · N. Rozès · A. Mas · J. M. Guillamón (✉)
Unitat d'Enologia del Centre de Referència de Tecnologia d'Aliments, Department Bioquímica i Biotecnologia, Facultat d'Enologia de Tarragona, Universitat Rovira i Virgili, Marcel·lí Domingo, s/n, 43007, Tarragona, Spain
e-mail: josemanuel.guillamon@urv.net

genes *GAP1* and *MEP2* in order to determine how fermentation temperature affects nitrogen consumption and metabolism. The quantity and quality of the nitrogen requirements of the yeast cells are not the same at optimum and low temperatures. This differential uptake of nitrogen may determine the biomass yield and the fermentation rate and also impact the production of volatile compounds, therefore affecting wine quality.

Materials and methods

Strain, fermentations and sampling

The commercial wine strain *Saccharomyces cerevisiae* QA23 (Lallemand S.A., Toulouse, France) was used in this study. Fermentations were carried out in a synthetic grape must (pH 3.3) as described by Riou et al. (1997) but with 200 g of reducing sugars l^{-1} (100 g glucose and 100 g fructose l^{-1}). The Yeast Assimilable Nitrogen (YAN) content was 300 mg N l^{-1}, ammoniacal nitrogen (NH_4Cl) 120 mg N l^{-1} and amino acids 180 mg N l^{-1}. Concentration of each individual amino acid (in mg l^{-1}) is shown in Fig. 1.

Fermentations took place at 13 and 25°C in laboratory-scale fermentors: 2-l bottles filled with 1.8 l of medium and fitted with closures that enabled the carbon dioxide to escape and the samples to be removed. Fermentations took place in semi-aerobic conditions, since limited aeration was necessary in order to harvest samples for the subsequent analysis. The population inoculated in every flask was 2×10^6 cells ml^{-1} from dry yeast rehydrated in water at 37°C.

Cell growth was determined by measuring the absorbance at 600 nm. Optical density (OD) values were corrected for the initial OD reading obtained for synthetic must. Every day the density was measured by weighing 5 ml of medium. In the latter stages of the fermentation, the sugar consumption was assayed by enzymatic kits (Roche Applied Science, Mannheim, Germany). Fermentation was considered to be complete when the residual sugars were below 2 g l^{-1}.

Cells were harvested at different points during the fermentation so that mRNA could be analysed. Flasks were magnetically stirred to resuspend the settled biomass, which was then transferred to centrifuge tubes and centrifuged at 5,000 rpm for 5 min at room temperature to prevent temperature shock. Cell pellets were transferred to 1.5-ml Eppendorf tubes and frozen immediately in liquid nitrogen. They were kept at –80°C until they were analysed. The supernatant of these samples was stored at –20°C for extracellular metabolites and nitrogen content analysis.

Nitrogen content analysis

The YAN was analysed by the formol index method (Aerny 1996), and the ammonium content was quantified using an enzymatic method (Roche Applied Science). The individual amino and imino acids were analysed by OPA and FMOC derivatizations, respectively, using the Agilent 1100 Series HPLC as

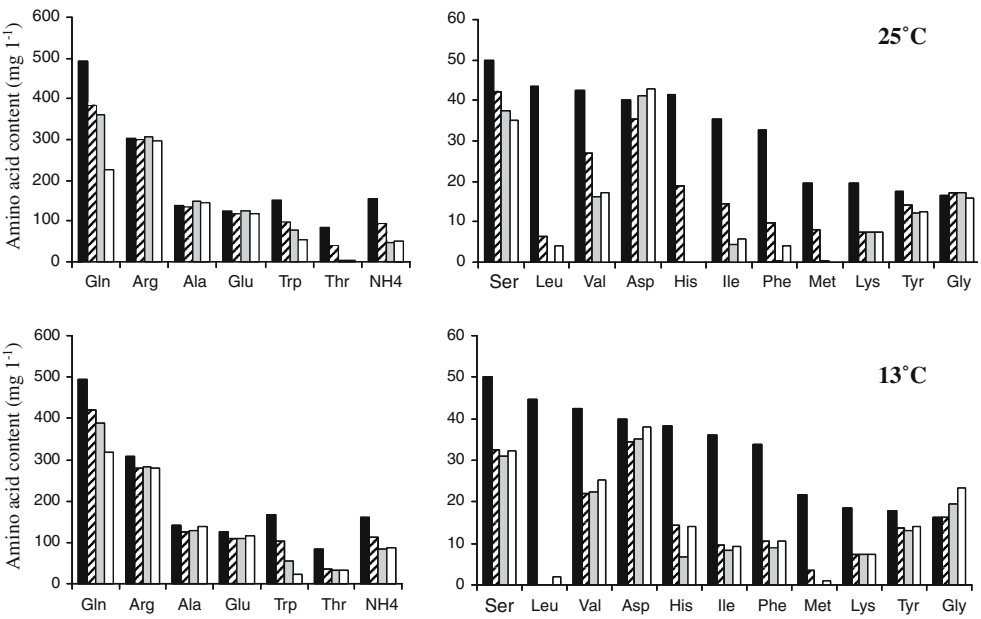

Fig. 1 Amino acid and ammonium concentration in the synthetic grape-must media at different stages of the fermentations at 25 and 13°C: Must (*filled rectangle*); Density 1,065 mg l^{-1} (*half-shaded rectangle*); Density 1,020 mg l^{-1} (*Shaded rectangle*) and at the end of fermentation (*opened rectangle*)

described in Beltran et al. (2004). Several dilutions of each sample were analysed and averaged using the analysis software. The concentration of each amino acid was calculated using external and internal standards and expressed as mg l^{-1}. The software used was Agilent ChemStation Plus (Agilent Technologies, Waldbronn, Germany).

RNA extraction and cDNA synthesis

Total RNA was isolated from yeast samples as described by Sierkstra et al. (1992) and resuspended in 50 µl of diethyl pyrocarbonate-treated water. Total RNA suspensions were purified using the High Pure Isolation kit (Roche Applied Science) following the protocol provided by the manufacturer. RNA concentrations were determined using a GenQuant spectrophotometer (Pharmacia, Ottawa, ON, Canada) and the quality of RNA was verified electrophoretically on 0.8% agarose gels. Solutions and equipment were treated so that they were RNase free, as outlined in Sambrook et al. (1989).

Total RNA was reverse-transcripted with Superscript™ II RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in a GenAmp PCR System 2700 (Applied Biosystem, Foster City, CA, USA). About 0.5 µg of Oligo $(dT)_{12–18}$ Primer (Invitrogen) was used with 0.8 µg of total RNA as template in a reaction volume of 20 µl. Following the protocol provided by the manufacturer, after denaturation at 70°C for 10 min, cDNA was synthesized at 42°C for 50 min. Finally, the reaction was inactivated at 70°C for 15 min.

Real-time quantitative PCR

The PCR primers used in this study are: *ACT-F*: TGGATTCCGGTGATGGTGTT and *ACT-R*: CGGC CAAATCGATTCTCAA (*ACT*, for actine gene); *GAP1-F*: CTGTGGATGCTGCTGCTTCA and *GAP 1-R*: CAACACTTGGCAAACCCTTGA (*GAP1*, for general amino acid permease gene); *MEP2-F*: GGT ATCATCGCTGGCCTAGTG and *MEP2-R*: ACAA CGGCTGACCAGATTGG (*MEP2*, for ammonium permease gene) (Beltran et al. 2004).

For each gene, a standard curve was made with yeast genomic DNA and the Real-Time Quantitative PCR reaction was performed in a PE Applied Biosystems 5700 thermal cycler as described in Beltran et al. (2004). All samples were analysed in duplicate and the expression values were averaged by the analysis software (Applied Biosystems). The coefficient of variation in all the samples analysed was less than 10%.

Results

Wine fermentations were carried out at 25 and 13°C on laboratory scale using synthetic media to estimate the effect of temperature on nitrogen consumption (ammonium and amino acid uptake), and on the NCR system (gene expression of permeases Gap1p and Mep2p).

Effect of temperature on nitrogen consumption

Figure 2 shows the fermentation kinetics and the biomass production measured as OD at both fermentation temperatures. As expected, low-temperature decreased the maximum fermentation rate (20.2 mg l^{-1} day^{-1} vs. 8.6 mg l^{-1} day^{-1}) and increased the length of fermentation (13 days vs. 29 days). Biomass production (or maximum population) was higher at 25°C than at 13°C. The duration of the exponential phase was shorter at 25°C than at 13°C (2 days vs. 7 days), although at the two temperatures, the non-proliferating or stationary phase was reached after ~30% of the sugars initially present in the medium had been consumed (at a density of *ca.* 1,050).

We observed different nitrogen requirements in yeasts fermenting at both temperatures (Table 1). The final nitrogen consumption by yeasts was higher at 25°C than at 13°C, which correlated with a higher

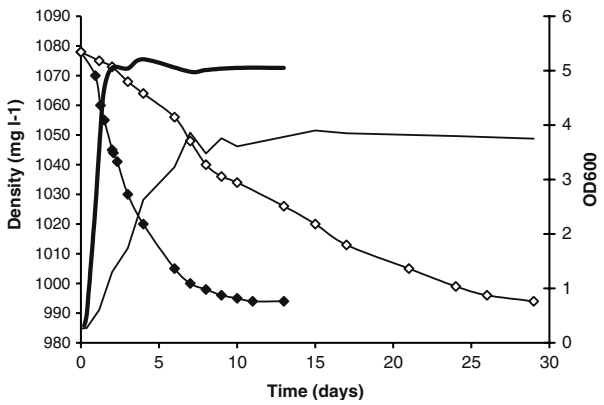

Fig. 2 Fermentation kinetics (measured as density reduction) at 13°C (*open triangle*) and 25°C (*filled triangle*) and cell growth (measured as OD600) throughout 13°C (*thin line*) and 25°C (*thick line*) fermentations

Table 1 Consumption of Yeast Assimilable Nitrogen (*YAN*), expressed as ammonia (YAN NH4) and amino acid (YAN aas) nitrogen, at different phases of 13 and 25°C fermentation

	Density (mg l^{-1})		
	1,065	1,020	990 (FF)
25°C			
YAN aas	38.3	50.6	66.8
YAN NH4	45.7	83.5	79.2
Total YAN	84.1	134.1	146.0
13°C			
YAN aas	44.1	53.5	59.4
YAN NH4	37.9	61.4	59.0
Total YAN	81.9	114.8	118.4

FF: final fermentation

Table 2 Final consumption of amino acids (mg l^{-1}) by yeast cells fermenting at 25 and 13°C

	25°C	13°C	Ratio
His	41.4	24.2	1.7
Ser	21.0	12.8	1.6
Gln	266.3	175.3	1.5
Thr	79.8	51.6	1.5
Val	25.4	17.4	1.5
Tyr	5.1	3.8	1.3
Phe	28.7	23.1	1.2
Ile	29.6	26.9	1.1
Lys	11.9	10.9	1.1
Leu	39.4	42.7	0.9
Met	19.2	20.7	0.9
Trp	96.7	144.9	0.7
Glu	4.8	11.1	0.4
Arg	7.8	28.5	0.3
Gly	0.9	0.0	
Ala	0.0	3.0	
Asp	0.0	4.1	
Suma	668.4	593.8	1.1

As the ratio between total amino acids consumed at 25 and 13°C was 1.1, amino acids with a ratio >1.1 were consumed more at 25°C and amino acids with a ratio <1.1 were more consumed at 13°C

Values are the average of two determinations and the coefficient of variation in all the compounds analysed was less than 10% with the exception of Phe and Ile, which ranged between 15 and 20%

biomass production. This difference between both temperatures was more significant in the consumption of ammonium (79 mg l^{-1} at 25°C, 59 mg l^{-1} at 13°C) than in the consumption of amino acids (67 mg l^{-1} at 25°C, 59 mg l^{-1} at 13°C). Cells at 13°C consumed most of the nitrogen in the first stages (density of 1,065), which coincided with biomass production during the exponential phase (Fig. 2). For this biomass production, cells at 13°C preferred amino acids to ammonium. Cells at 25°C, however, used nitrogen throughout and ammonium as preferred source.

Amino acid preferences were also different at both temperatures (Table 2). Interestingly, the uptake of tryptophan was greater at 13°C whereas glutamine uptake was higher at 25°C. The consumption of threonine, histidine, valine and serine was proportionally greater at 25°C. On the other hand, a set of amino acids (arginine, glutamate, alanine and aspartate) were hardly consumed at 25°C but proportionally much more so at 13°C.

Figure 1 shows the pattern of amino acids and ammonia consumption at different phases of fermentation. The major bulk of amino acids were consumed in the initial stages of fermentation at 13°C (density 1,065), during the growth phase. Only glutamine and tryptophan were consumed in the latter phases of 13°C fermentation, whereas at 25°C yeast cells consumed a greater variety of amino acids throughout the fermentation.

Effect of low-temperature fermentation on GAP1 and MEP2 expression

The expression of the nitrogen transporters *GAP1* and *MEP2* was analysed and quantified relative to the expression of the housekeeping actin gene. Time zero referred to the level of expression of these genes before yeast inoculation (and after rehydration, density 1,080).

Because of the presence of good nitrogen sources and, therefore, the effect of NCR (ter Schure et al. 2000; Beltran et al. 2004, 2005), both genes were repressed in the first hours after inoculation in the must-like medium (Fig. 3). As nitrogen was not completely consumed, its presence in the media repressed these genes throughout the fermentation. However, this NCR of the permeases seemed to be less efficient at the end of the 13°C fermentation, where the values of gene expression were higher.

Discussion

We recently studied nitrogen regulation by using this commercial wine *S. cerevisiae* strain and the same synthetic medium which mimics grape-must (Beltran et al. 2004). In the present study, our aim was to analyse the influence of low temperature on nitrogen regulation and, therefore, on nitrogen consumption. Our results suggest that nitrogen regulation is different at low temperature, which may affect the growth rate

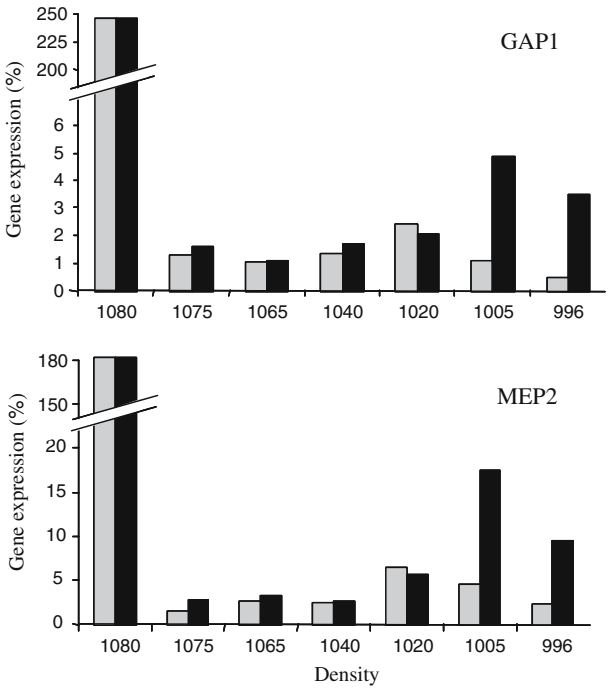

Fig. 3 Gene expression of ammonium permease (*MEP2)* and general amino acid permease (*GAP1)* at time zero (before inoculation) and at different points during the fermentations at 25°C (*Shaded rectangle*) and 13°C (*filled rectangle*). The data were quantified by calculating the ratio between the concentration of the studied genes normalized with the concentration of the housekeeping *ACT* gene, and expressed as a percentage (the quantity ratio 1 was set as 100%)

and nitrogen preferences. This is not unexpected since, in a global transcriptomic analysis of *S. cerevisiae*, genes associated with nitrogen transport and amino acid metabolism were considerably up-regulated when growing cultures were shifted from 30 to 10°C (Schade et al. 2004).

Somehow low-temperature fermentation produced similar metabolic effects to those obtained in nitrogen-limited fermentations: both conditions decreased biomass yield and fermentation rate. In this study, fermentations were not nitrogen-limited, but even so yeast cells consumed much less nitrogen at 13°C than at 25°C. Therefore, these data pointed to the hypothesis that low-temperatures impair the nitrogen uptake capacity of the cell. Low temperatures decrease the fluidity of the plasma membrane, which considerably reduces the molecular motion of phospholipids and membrane proteins (Macdonald 1987). This decrease in membrane fluidity might impair the activity of some permeases (Abe and Horikoshi 2000).

The nitrogen fraction in grape must consists of organic (amino acids) and inorganic (ammonium) nitrogen. In our previous study of fermentations at optimum temperature (Beltran et al. 2005), we proved that ammonium is the preferred nitrogen source for biomass production while amino acids are preferentially used during the stationary phase, during which most of the nitrogen compounds are devoted to cell maintenance. However, in the present study, amino acids were consumed most during the exponential phase in the low-temperature fermentation. One explanation for this different pattern of nitrogen consumption might be the slower flux of sugars into the glycolytic pathway (slow fermentation rate) at low temperature, which determines a lower availability of carbon skeletons for amino acid biosynthesis. A coordinated modulation between nitrogen and carbon metabolisms for ammonium assimilation has already been reported (DeLuna et al. 2001; Guillamon et al. 2001).

In the amino acid pool, we also detected differences in the pattern of amino acid consumption at both temperatures. Glutamine and tryptophan were the main amino acids consumed (more than 50%) in both fermentations. Glutamine was consumed much more at 25°C than at 13°C whereas tryptophan was consumed more at the low temperature. The uptake of tryptophan as a rate-limiting step in growth at low temperature has already been reported (Tokai et al. 2000). The overexpression of the gene encoding the high-affinity tryptophan permease Tat2p gave the yeast cells good growth capacity at low temperature (Abe and Horikoshi 2000; Nagayama et al. 2004). Other amino acids whose uptake increased at low temperature were arginine and glutamic acid. In our previous study (Beltran et al. 2004), we reported the inhibition of the uptake of these amino acids when the cells are subjected to a nitrogen-repressed situation.

Another important finding of this study is that the fermentation temperature affects the mechanism of NCR. This NCR enables the cell to select the best nitrogen sources by repressing the transcription of some genes involved in the utilization of poorer nitrogen sources (Wiame et al. 1985; Magasanik 1992). In a previous study, we also detected that the presence of ethanol also influenced the effectiveness of this NCR (Beltran et al. 2005). A common feature of ethanol toxicity and low temperature is that the composition of the cell's plasma membrane is modified, which can substantially impair its function (Alexandre et al. 1994). Therefore, the sensing system of nitrogen sources, mainly located in the plasma membrane (Gagiano et al. 2002), may be affected by both these conditions.

Nitrogen-deficient grape musts and low temperature are two of the main prevalent causes of sluggish

fermentations and, therefore, the effects of both growth conditions on yeast metabolism are of considerable interest for wine making. Varela et al. (2004) reported that the viable cell concentration governs fermentation rates in nitrogen-poor musts. They proposed the addition of viable biomass from other fermentation tanks as a possible solution for dealing with nitrogen-deficient musts. As far as we know, some winemakers already increase the viable biomass to decrease the lag phase and increase the fermentation rate at low temperature. The second and more usual alternative to the deficiency of nitrogen in musts is nitrogen supplementation. However, these additions do not overcome the fermentation problems at low temperature if the nitrogen uptake systems of yeast cells are impaired.

To sum up, the fermentation temperature influenced the nitrogen regulation of this wine strain. The cells growing at low temperature had a less effective NCR system, which determined the ratio of organic/inorganic nitrogen consumed and the patterns of amino acid consumption. The effects of a low-fermentation temperature on yeast cells were similar to the effects of nitrogen-limited grape musts. The most plausible explanation is that low temperature decreases membrane fluidity and this affects permease activity and therefore nitrogen uptake.

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