Growth and fermentation patterns of *Saccharomyces cerevisiae* under different ammonium concentrations and its implications in winemaking industry

A. Mendes-Ferreira[1], A. Mendes-Faia[1] and C. Leão[2]

[1]*Centro de Genética e Biotecnologia, Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal, and* [2]*Escola de Ciências da Saúde, Universidade do Minho, Campus de Gualtar, Braga, Portugal*

ABSTRACT

A. MENDES-FERREIRA, A. MENDES-FAIA AND C. LEÃO. 2004.

Aims: To study the effects of assimilable nitrogen concentration on growth profile and on fermentation kinetics of *Saccharomyces cerevisiae*.

Methods and Results: *Saccharomyces cerevisiae* was grown in batch in a defined medium with glucose (200 g l^{-1}) as the only carbon and energy source, and nitrogen supplied as ammonium sulphate or phosphate forms under different concentrations. The initial nitrogen concentration in the media had no effect on specific growth rates of the yeast strain PYCC 4072. However, fermentation rate and the time required for completion of the alcoholic fermentation were strongly dependent on nitrogen availability. At the stationary phase, the addition of ammonium was effective in increasing cell population, fermentation rate and ethanol.

Conclusions: The yeast strain required a minimum of 267 mg N l^{-1} to attain complete dryness of media, within the time considered for the experiments. Lower levels were enough to support growth, although leading to sluggish or stuck fermentation.

Significance and Impact of the Study: The findings reported here contribute to elucidate the role of nitrogen on growth and fermentation performance of wine yeast. This information might be useful to the wine industry where excessive addition of nitrogen to prevent sluggish or stuck fermentation might have a negative impact on wine stability and quality.

Keywords: ammonium, fermentation, growth, oenological conditions, *Saccharomyces cerevisiae*.

INTRODUCTION

Sluggish or stuck fermentation (Salmon 1989; Kunkee 1991) as well as undesirable by-products such as S-off-flavours (Henschke and Jiranek 1993; Rauhut 1993) are still common problems in modern winemaking. Previous research has suggested that the major cause of these problems has been the limited nitrogen content of some natural grape juice (Vos and Gray 1979; Giudici and Kunkee 1994; Jiranek *et al.* 1995; 1996; Spayd *et al.* 1995;

Correspondence to: A. Mendes Faia, Centro de Genética e Biotecnologia, Universidade de Trás-os-Montes e Alto Douro, 5001-Vila Real, Portugal (e-mail: afaia@utad.pt).

Hallinan *et al.* 1999; Spiropoulos and Bisson 2000; Spiropoulos *et al.* 2000). Although nitrogen has been the most studied cause of sluggish and/or stuck fermentation, the basic mechanism responsible for sugar breakdown arrest is far from clear. Salmon (1989) suggested that sugar transport catabolite-inactivation because of protein synthesis arrest might partially explain the inhibition of alcoholic fermentation in nitrogen-deficient musts. Later on, Lagunas and co-workers (Lucero *et al.* 2002) confirmed the inactivation of sugar transporters were mainly because of the stimulation of protein turnover that follows nitrogen starvation. Indeed, they observed a very low inactivation in the presence of a nitrogen source.

In oenological conditions, mean values of 140 mg l^{-1} (Agenbach 1977) of amino nitrogen has been given as sufficient for complete fermentation of reasonably ripened grapes while six times more has been pointed out to be the optimum level (Kunkee 1991). One strategy used by winemakers for nitrogen-limited musts is the addition of nitrogen supplements, as inorganic forms such as diammonium phosphate, prior to grape juice fermentation. However, the addition of ammonium in order to overcome fermentation problems has not always been successful. Furthermore, legislation is pending to limit nitrogen addition in musts and wines because yeast can produce urea, the major precursor of ethyl carbamate, a suspected carcinogen. Hence, this study was designed to evaluate the effect of the initial nitrogen concentration on the growth pattern and alcoholic fermentation profile of *Saccharomyces cerevisiae*. The study also evaluated the effectiveness of nitrogen addition, to nitrogen-deficient media, at the end of exponential phase or at mid stationary phase, on cell viability and on fermentation rate.

MATERIAL AND METHODS

Strain and culture conditions

Saccharomyces cerevisiae PYCC 4072 originally isolated from a sample of Fermivin, industrial wine yeast distributed by Rapidase, was obtained from the Portuguese Yeast Culture Collection (New University of Lisbon, Lisbon, Portugal). The yeast culture was maintained at 4°C on slants of yeast peptone dextrose agar (YPD), containing: glucose 20 g l^{-1}, peptone 10 g l^{-1}, yeast extract 5 g l^{-1} and agar 20 g l^{-1}. Before use, it was transferred to a new slant of YPD for 24–48 h at 25°C.

Culture media

A defined grape juice medium (GJM) was used, similar in composition to typical grape juice as previously described by Henschke and Jiranek (1993) with some minor modifications. Briefly, glucose was the only carbon and energy source and nitrogen was supplied as indicated either as ammonium – $(NH_4)_2SO_4$ or $(NH_4)_2HPO_4$. In all media, pH was adjusted to 3·7.

Inocula and fermentation conditions

Inocula for all experiments were prepared by pregrowing the yeast in 100 ml shake flasks containing 70 ml of medium with the same composition of those used in all assays. Then the flasks were incubated overnight at 25°C in an orbital shaker at 150 rev min^{-1}. This preculture was used to inoculate experimental cultures with an initial viable population of *ca* 5×10^5 CFU ml^{-1}. Fermentations were carried out in 500 ml flasks filled to 2/3 of their volume, fitted with a fermentation lock and a septum seal for sampling, and maintained at 20°C under permanent magnetic stir-bar agitation. Yeast growth was followed by periodical measurement of absorbance at 640 nm in a spectrophotometer Schimadzu UV-2101 (Schimadzu Co, Kyoto, Japan).

Determination of culture dry weight

Culture dry weight was determined by filtering 25 ml of the culture media over predried and preweighed nitrocellulose filters (0·45 μm pore size; Millipore Corp., Billerica, MA, USA) which were rinsed twice with the same amount of demineralized water and desiccated at 80°C to constant weight. Duplicate determinations varied by <1%.

Monitoring of fermentation

The progress of alcoholic fermentation was monitored by periodical determination of glucose consumed and ethanol produced by HPLC, using a Gilson Chromatograph equipped with ion exclusion, cation exchange column Aminex HPX 87H (Bio-Rad Laboratories, Hercules, CA, USA) preceded by a precolumn, auto sampler and refractive index detector. The column was eluted with 6·5 mM sulphuric acid at a flow rate of 0·5 ml min^{-1} and maintained at 62°C. The sample injection volume was 6 μl. Quantitative measurements were performed by applying external standards.

Determination of ammonium

Ammonium calculation was performed in a continuous-flow analysis system equipped with sampler, pump, dialysis unit, ammonium unit, photometer and recorder. Ammonium calculation was based on the Berthelot reaction in which phenol derivative forms indophenol in the presence of ammonia and hypochloride under catalytic action of nitroprusside. In alkaline medium, the indophenol has a greenish-blue colour quantified at 660 nm.

Addition of nitrogen to N-limiting media

The effect of nitrogen addition during fermentation on yeast viability and on fermentation rate was studied in GJM containing an initial concentration of nitrogen of 66 mg l^{-1} supplied as diammonium phosphate or as a mixture of amino acids. In this case, the amino acids solution of the original recipe of GJM (Henschke and Jiranek (1993) was diluted to reach 66 mg N l^{-1}. Cells were grown in the two media to late exponential phase (24 h) or stationary phase

(72 h). At these times, each medium was subdivided into three other smaller flasks. Cycloheximide (Sigma, St Louis, MO, USA), at a concentration of 100 μg ml^{-1}, was added to one of the flasks, half an hour before the addition of nitrogen. Nitrogen was added at the final concentration of 66 mg l^{-1}, as diammonium phosphate, to both flasks, leaving the third as a control. Again, all the cultures were incubated at 20°C in a water bath, under permanent magnetic stir-bar agitation. The number of viable cells in each trial was periodically determined using serial dilutions of the sample and pour-plated in YPD. The number of colony-forming units was counted after 48 h at 25°C. Fermentation progress was evaluated by measurements of flasks weight loss every 24 h or by glucose determination by HPLC if indicated.

Reproducibility of the results

All experiments were run at least three times and all reported data are mean values.

RESULTS

The effect of initial nitrogen concentration on growth and alcoholic fermentation profile of *S. cerevisiae*

The effect of assimilable nitrogen on growth was evaluated in batch cultures of *S. cerevisiae* under different nitrogen concentrations, from 16·5 to 805 mg N l^{-1}, in well-controlled conditions. The results show that the yeast strain PYCC 4072 appear not to be affected by the initial nitrogen concentration, at least over the range of 267–805 mg N l^{-1}, normal values found in natural grape juice. Specific growth rates were *ca* 0·19–0·21 h^{-1} irrespective of initial nitrogen concentration observed in this study (Fig. 1). At nitrogen concentrations lower than 66 mg N l^{-1} an earlier slowdown of cell growth and a relevant decrease in cell biomass were observed. In fact, over a range of nitrogen from 16·5 to 66 mg l^{-1}, the strain produced 0·9–2·2 g of cell dry weight l^{-1}. At high nitrogen concentrations, 267–805 mg l^{-1}, the strain appears to be a poor N-responder as the highest biomass production (7·8 g l^{-1}) was observed in the media with 402 mg N l^{-1} (Table 1). The kinetics of glucose and nitrogen consumption, shown in Fig. 2a,b), indicate that the ability of the yeast strain to break down sugars is strongly increased with nitrogen availability in the media. In fact, complete dryness was attained after 96 and 144 h in the media with 805 mg and 267 mg N l^{-1}, respectively. Yet, as shown in the same figure (Fig. 2b), the extent of nitrogen consumption by the yeast strain increased in accordance with the increased availability of nitrogen in the media: total consumption of
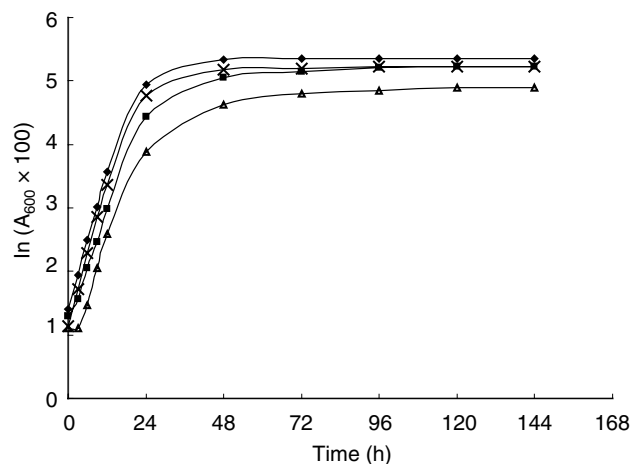

Fig. 1 Growth patterns of *Saccharomyces cerevisiae* PYCC 4072 in synthetic grape juice medium, under different nitrogen concentrations (◆, 805 mg l^{-1}; ✕, 66 mg l^{-1}; ■, 33 mg l^{-1}; △, 16·5 mg l^{-1}), supplied as ammonium sulphate, at 20°C and pH 3·7. Growth patterns of 267 and 402 mg l^{-1} are not displayed because they overlapped with 805 mg l^{-1}

nitrogen occurred after 24, 48 and 72 h from the media with 66, 267 and 402 mg N l^{-1}, respectively. In excess nitrogen media (805 mg l^{-1}), regardless of the nitrogen source *ca* 30% of ammonia remained at the end.

Efficiency of nitrogen addition, as diammonium phosphate, on viability and fermentation performance

This experiment was designed to evaluate the efficiency of nitrogen addition to N-limited musts, at the end of exponential phase (24 h) or at mid-stationary phase (72 h) on growth and fermentation activity. The yeast strain was inoculated in GJM with an initial concentration of 66 mg l^{-1} supplied as a mixture of amino acids for a more accurate extrapolation to wine fermentation conditions. The results show that supplementation with diammonium phosphate added during fermentation, at both times, increased cell population (Fig. 3), fermentation rate and ethanol increased from 3·0 to 6·3% by nitrogen addition as shown in Fig. 4. In order to have a more precise picture on the physiological effect of that addition, cycloheximide was simultaneously added with nitrogen, to prevent '*de novo*' protein synthesis. Under these conditions, as shown in Fig. 4, diammonium phosphate had no effect on yeast growth or fermentation activity, suggesting that protein synthesis is required to restore alcoholic fermentation. Similar behaviour was observed when other nitrogen source (diammonium phosphate or ammonium sulphate) was used, instead of amino acids (results not shown).

Table 1 Specific growth rate, total biomass, fermentation rate and ethanol produced by *Saccharomyces cerevisiae* PYCC 4072 in synthetic grape juice medium under different initial nitrogen concentration, supplied as ammonium sulphate, at 20°C and pH 3·7

Initial nitrogen concentration (mg l^{-1})	μ h^{-1}	Final biomass (mg ml^{-1})	Glucose consumption (g h^{-1})	Final ethanol concentration (% v/v)
805·0	0·19 ± 0·01	7·488 ± 0·085	2·78 ± 0·29	11·0 ± 0·31
402·5	0·19 ± 0·03	7·840 ± 0·069	2·05 ± 0·09	11·1 ± 0·20
267·0	0·20 ± 0·01	6·194 ± 0·010	2·02 ± 0·10	11·7 ± 0·06
66·0	0·19 ± 0·01	2·209 ± 0·054	–	3·06 ± 0·20
33·0	0·19 ± 0·03	1·155 ± 0·027	–	1·7 ± 0·36
16·5	0·21 ± 0·01	0·909 ± 0·030	–	0·5 ± 0·4

μ h^{-1} – specific growth rate per hour.
Average ± standard deviation based on the results obtained from three independent cultures.
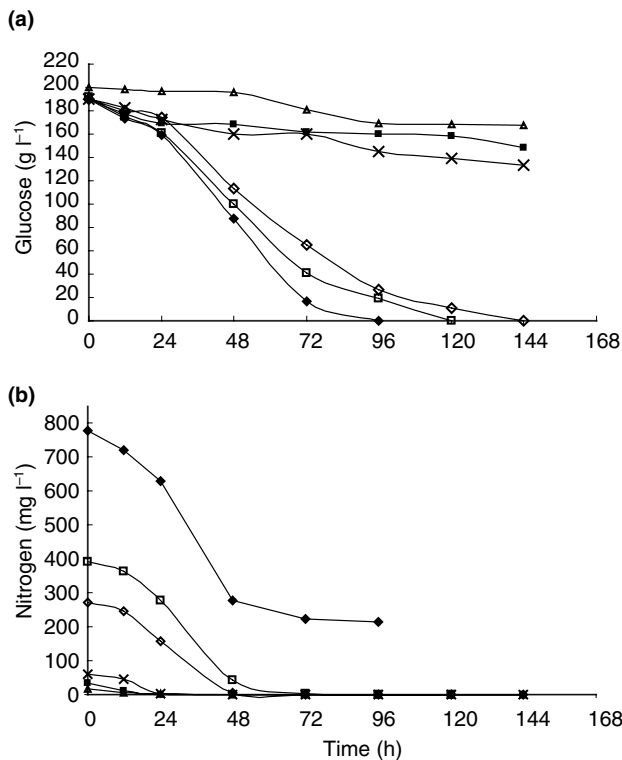

Fig. 2 Disappearance of glucose (a) and ammonium (b) from synthetic grape juice medium cultivated with *Saccharomyces cerevisiae* PYCC 4072, under different nitrogen concentrations (♦, 805 mg l^{-1}; □, 402 mg l^{-1}; ◇, 267 mg l^{-1} × 66 mg l^{-1}; ■, 33 mg l^{-1}; △, 16·5 mg l^{-1}), supplied as ammonium sulphate, at 20°C and pH 3·7

Fig. 3 Effect of nitrogen addition (66 mg N l^{-1}) on cell viability of *Saccharomyces cerevisiae* PYCC 4072, in synthetic grape juice media, at 20°C and pH 3·7. (●, without addition; ○, with addition at 24 h; ■, without addition; □, with addition at 72 h). The arrows indicate the time of addition

DISCUSSION

The transformation of grape juice to wine usually takes 7–10 days to be complete, depending on fermentation conditions. A slow or sluggish fermentation is one that requires a longer than the average time to attain dryness while a stuck fermentation is when residual sugar is higher than 2 g l$^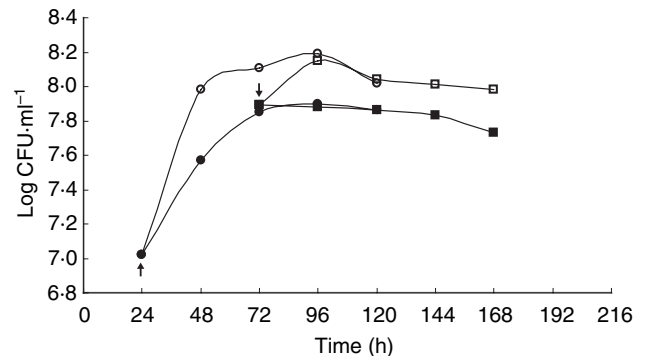{-1}$. Nitrogen has been reported as one of the major limiting factor on yeast growth and fermentation performance, leading to these fermentation problems. Several studies have been conducted in natural grape juice whose composition is dependent upon grape variety, year, climate as well as being correlated with the soil and with the fertilization applied to vineyards. In most experiments, ammonium instead of a mixture of amino acids plus ammonium found in grape musts was used because it has been considered a good nitrogen source as it directly enters into the intracellular pool of precursors biosynthesis (Bisson 1991; Henschke and Jiranek 1993). Low levels of this nutrient have been associated with low cellular activity (Bely *et al.* 1990, 1994) and low biomass yield (Bisson 1991; Spayd *et al.* 1995; Manginot *et al.* 1998). The results of this study agree with those previously reported, however, the strain PYCC 4072 seems to be a poor nitrogen-responder, at least at high nitrogen concentration, from 267 to 805 mg N l^{-1}, as increasing nitrogen did not significantly affect cell biomass nor specific growth rates. The kinetics of glucose and nitrogen consumption demonstrate that the higher the
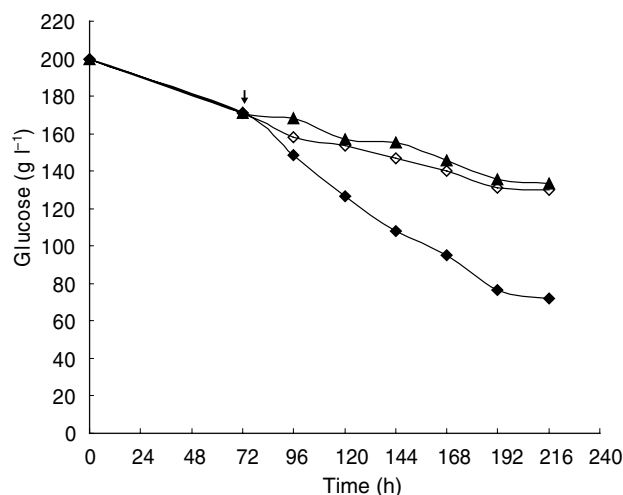
Fig. 4 Fermentative activity of *Saccharomyces cerevisiae* PYCC 4072, in synthetic grape juice media, with an initial nitrogen concentration of 66 mg N l^{-1}, at 20°C and pH 3·7. Control experiment was performed without any addition ($\diamond$); addition of 66 mg N l^{-1}, alone ($\blacklozenge$); addition of 66 mg N l^{-1}, plus cycloheximide (100 μg ml^{-1}) ($\blacktriangle$). The arrow indicates the time of the addition

nitrogen availability, the higher the fermentation rate, as previously shown by Ingledew and Kunkee (1985). The minimum nitrogen required by the yeast strain PYCC 4072 for completion of alcoholic fermentation, in a reasonable time, was above the values earlier reported in the literature (Agenbach 1977; Kunkee 1991). However, it is noteworthy that fermentations containing 66 mg l^{-1} also achieved dryness after 28 days and viable biomass remains high (results not shown). In this case, we are in the presence of a slow and not stuck fermentation suggesting that assimilable nitrogen could be accumulated as cellular reserve in the vacuole and used, later on, when needed (Bisson 1991).

Growth and fermentation activity of the strain PYCC 4072 seem quite different to that of V8-6 reported by Salmon (1989) under the initial nitrogen concentration from 24 to 390 mg l^{-1}. The differences between the estimates of nitrogen requirement observed in this study and those obtained by others might have numerous explanations. For example, growth conditions and strain differences may account for the discrepancy, which confirms a great variability in nitrogen demands of wine yeasts, and agree with the relevance of knowing their exigencies to select nitrogen-effective strains (Manginot *et al.* 1998) for the best control of fermentation process.

As some sluggish or stuck fermentation seem to be a consequence of nitrogen deficiency in grape juice, addition of diammonium phosphate to nitrogen-deficient musts is widely used by winemakers, largely as a precaution against fermentation problems (Henschke and Jiranek 1993). As it

has been claimed that nitrogen addition at stationary phase has no effects on cell population only reduce fermentation duration. Bely *et al.* (1990, 1994) only recommended the addition of nitrogen at mid-fermentation for saving energy required for temperature regulation in the fermentation process. On the contrary, our results show that supplementation with diammonium phosphate during fermentation, increased cell population, fermentation rate and ethanol increased from 3·0 to 6·3% (v/v). Furthermore, in presence of cycloheximide, an antibiotic known to inhibit protein synthesis, nitrogen was not effective to restart alcoholic fermentation suggesting that a '*de novo*' protein synthesis is required to restore fermentation activity. When 200 mg l^{-1} of nitrogen were added to nitrogen deficient media (66 mg l^{-1}), at stationary phase, the yeast strain required an additional 48 h to complete alcoholic fermentation than that with initial nitrogen concentration of 267 mg l^{-1} (results not shown). So, nitrogen should be added earlier before the end of cellular growth as already suggested by Salmon (1989) or even to the musts.

This study provides useful information about *S. cerevisiae* nitrogen demands for growth and fermentation performance under oenological conditions, particularly for the strain Fermivin often used as a starter culture in winemaking. This knowledge of the yeast behaviour under nutrient limitation might be a starting point for further studies to better understand the biology of *S. cerevisiae* during both normal and problem oenological fermentations.

ACKNOWLEDGEMENTS

REFERENCES

Agenbach, W.A. (1977) A study of must nitrogen content in relation to incomplete fermentations, yeast production and fermentation activity. In *Proceedings of the South African Society of Enology and Viticulture* (Cape Town, Nov 1977) pp. 66–87. Stellenbosch: South African Society of Enology and Viticulture.

Bely, M., Sablayrolles, J.M. and Barre, P. (1990) Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in enological conditions. *Journal of Fermentation and Bioengineering* **70**, 246–252.

Bely, M., Salmon, J.M. and Barre, P. (1994) Assimilable nitrogen addition and hexose transport system during alcoholic enological fermentation. *Journal of Institute Brewing* **100**, 279–282.

Bisson, L. (1991) Influence of nitrogen on yeast and fermentation of grapes. In *Proceedings of the International Symposium on Nitrogen in Grapes and Wine* (Seattle WA, 1991) ed. Rantz, J. pp. 78–89. Davis, CA: American Society for Enology and Viticulture.

Giudici, P. and Kunkee, R.E. (1994) The effect of nitrogen deficiency and sulfur-containing amino acids on the reduction of sulfate to hydrogen sulfide by wine yeasts. *American Journal of Enology and Viticulture* **45**, 107–112.

Hallinan, C.P., Saul, D. and Jiranek, V. (1999) Differential utilisation of sulfur compounds for sulphide liberation by nitrogen-starved wine yeasts. *Australian Journal Grape Wine Research* **5**, 82–90.

Henschke, P.A. and Jiranek, V. (1993) Yeasts – metabolism of nitrogen compounds. In *Wine Microbiology and Biotechnology* ed. Fleet, G.H. pp. 77–163. Poststrasse 22, Switzerland: Harwood Academic Publishers.

Ingledew, W.M. and Kunkee, R.E. (1985) Factors influencing sluggish fermentations of grape juice. *American Journal of Enology and Viticulture* **36**, 65–76.

Jiranek, V., Langridge, P. and Henschke, P.A. (1995) Regulation of hydrogen sulfide liberation in wine producing *S. cerevisiae* strains by assimilable nitrogen. *Applied and Environmental Microbiology* **61**, 461–467.

Jiranek, V., Langridge, P. and Henschke, P.A. (1996) Determination of sulphite reductase activity and its response to assimilable nitrogen in a commercial *S. cerevisiae* wine yeast. *Journal of Applied Bacteriology* **61**, 461–467.

Kunkee, R.E. (1991) Relationship between nitrogen content in must and sluggish fermentation. In *Proceedings of the International Symposium on Nitrogen in Grapes and Wine* ed. Rantz, J. pp. 148–155. Davis, CA: American Society for Enology and Viticulture.

Lucero, P., Moreno, E. and Lagunas, R. (2002) Catabolite inactivation of sugar transporters in *Saccharomyces cerevisiae* is inhibited by the presence of a nitrogen source. *FEMS Yeast Research* **1**, 307–314.

Manginot, C., Roustan, J.L. and Sablayrolles, J.M. (1998) Nitrogen demand of different yeast strains during alcoholic fermentation. Importance of the stationary phase. *Enzyme and Microbial Technology* **23**, 511–517.

Rauhut, D. (1993) Yeasts – production of sulfur compounds. In *Wine Microbiology and Biotechnology* ed. Fleet, G.H. pp. 183–223. Poststrasse 22, Switzerland: Harwood Academic Publishers.

Salmon, J.M. (1989) Effect of sugar transport inactivation in *Saccharomyces cerevisiae* on sluggish and stuck enological fermentations. *Applied and Environmental Microbiology* **55**, 933–958.

Spayd, S.E., Nagel, C.W. and Edwards, C.G. (1995) Yeast growth in Riesling juice as affected by vineyard nitrogen fertilization. *American Journal of Enology and Viticulture* **46**, 49–55.

Spiropoulos, A. and Bisson, L. (2000) MET17 and hydrogen sulfide formation in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **66**, 4421–4426.

Spiropoulos, A., Tanaka, J., Flerianos, I. and Bisson, L. (2000) Characterization of hydrogen sulfide in commercial and natural wine isolates of *Saccharomyces cerevisiae*. *American Journal of Enology and Viticulture* **51**, 233–248.

Vos, P.J.A. and Gray, R.S. (1979) The origin and control of H_2S during fermentation of grape must. *American Journal of Enology and Viticulture* **30**, 187–197.