Saccharomyces cerevisiae vineyard strains have different nitrogen requirements that affect their fermentation performances

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RUNNING TITLE: *S. cerevisiae* strains nitrogen requirements

ABSTRACT

Aims

In this work fermentation performances of seven vineyard strains, together with the industrial strain EC1118, have been investigated at three differing yeast assimilable nitrogen concentrations (300 mg N l^{-1}, 150 mg N l^{-1} and 70 mg N l^{-1}) in synthetic musts.

Methods and results

Most of the strains showed a dramatic decrease of the fermentation at 70 mg N l^{-1} but no significant differences in CO_2 production were found when fermentations at 300 mg N l^{-1} and 150 mg N l^{-1} were compared. Only one among the vineyard strains showed a decrease of the fermentation when 150 mg N l^{-1} were present in the must.

Conclusions

Results indicate that the response to different nitrogen levels is strain dependent. These results contribute to shed light on strain nitrogen requirements and offer new perspectives to manage the fermentation process during winemaking.

Significance and impact of the study

Selected vineyard *S. cerevisiae* strains can improve the quality and the complexity of local wines. Wine quality is also influenced by nitrogen availability that modulates yeast fermentation activity. In this work, yeast nitrogen assimilation was evaluated to clarify the nitrogen requirements of vineyard strains. Most of the strains needed high nitrogen levels to express the best fermentation performances. The results obtained indicate the critical nitrogen levels. When the nitrogen concentration was above the critical level, the fermentation process increased, but if the level of nitrogen was further increased no effect on the fermentation was found.

KEYWORDS: Fermentation biotechnology, Fermentation, Beverages, Biotechnology, Environmental

INTRODUCTION

Wine fermentation is a process involving the sequential development of different yeast species (Fleet 2003).The process can be conducted by leaving the native microbiota present in the grapes to ferment the must or by adding selected commercial strains. In both cases, yeast strains are responsible for the production of several aromatic compounds whose quantities are species dependent (Combina *et al.* 2005). Therefore, they strongly contribute to the specific bouquet of wines. In particular, during spontaneous vinifications the variability of the vineyard species and strains contributes to the "terroir" of the local wine (Fleet 2008). Methods such as genome sequencing have been used to investigate how genetic variations influence gene expression during fermentation and to clarify the evolutionary relationship between vineyard wine isolates and industrial strains (Borneman *et al.* 2011; Treu *et al.* 2014b). In particular vineyard strain genome analysis demonstrated that in those strains gene sequences involved in nitrogen metabolism are more variable than those present in industrial strains, suggesting a different aptitude in nitrogen uptake and management (Treu *et al.* 2014a).

Besides yeast genetic aptitude, grape must composition, in particular nitrogen availability, has been demonstrated to influence yeast fermentation activity and wine quality and stability (Gutiérrez *et al.* 2012). Backhus *et al.* (2003) found that at low nitrogen concentration *S. cerevisiae* strains showed high expression levels of genes involved in oxidative carbon metabolism. On the contrary, high nitrogen concentration in the must leads the enhancement of the expression level of genes involved in biosynthesis of macromolecules. Nitrogen content of grape must, which is very often increased by the addition of ammonium salts at the beginning of the fermentation, influences the kinetics of the fermentation and the production of secondary compounds in particular esters and higher alcohols (Vilanova *et al.* 2007; Carrau *et al.* 2008). Indeed, some amino acids, manly threonine, valine, isoleucine and leucine, can be precursors of

higher alcohols such as 1-propanol, isobutanol, 2-methylbutanol and 3-methylbutanol whose concentrations can be modulated by the presence of non-*Saccharomyces* species (Comitini *et al.* 2011; Benito *et al.* 2016).

One of the problems in modern winemaking is the limited nitrogen content of natural must causing sluggish or stuck fermentations with the production of undesirable secondary compounds that negatively affect wine organoleptic characteristics (Carrau *et al.* 2008). One strategy used by winemakers to overcome the problem of nitrogen-limited musts is to select vineyard yeasts that have good fermentation performances in these extreme conditions (Gutiérrez *et al.* 2012). Therefore, understanding strain nitrogen requirement is of great interest.

In the present work seven strains of *Saccharomyces cerevisiae* isolated from Italian vineyard were chosen to evaluate the effect of different initial nitrogen concentrations on the fermentation performances in synthetic must. Ammonia and primary amino nitrogen consumption was monitored, as well.

RESULTS AND DISCUSSION

Strain fermentation performances at standard condition and H_2S production

In previous work a pool of *S. cerevisiae* strains was isolated from the vineyards and significant differences in terms of fermentation and nitrogen utilization were evidenced among the strains (Treu *et al.* 2014b). Seven vineyard yeasts were chosen, from the pool, on the basis of the isolation origins, the technological characteristics and the mtDNA profiles that allowed yeast identification at strain level. In particular, four strains (P293.8, P301.4, P301.9, P304.1) were isolated from a white grape variety, Glera, and three (R8.3, R103.1 and R133.5) from a red grape variety, Raboso Piave. All the strains showed different mtDNA profiles. Moreover the strains showed different fermentation abilities in terms of days of fermentation and sugar residue at standard condition. Days of fermentation are the days needed to consume all the sugars or to reach a plateau in the total CO_2 production. To confirm that those strains were phenotypically different, some important technological traits were tested: fermentation performances at standard conditions and H_2S production by means of plate assay. The production of sulphide is deleterious in winemaking as it is responsible for a specific off-flavour similar to that of rotten eggs (Thouki and Stern 1962; Silva Ferreira *et al.* 2003). The fermentation performances of seven vineyard *S. cerevisiae* strains were determined in standard synthetic must MS300 at 25°C. To assess strain fermentation performances, the fermentation vigor, in terms of total CO_2 production (g 100^{-1} ml^{-1}) after 48 h of incubation, was considered in order to evaluate the adaptation ability to the must conditions. Total CO_2 production after 139 h was considered, as at these fermentation step the widest range of CO_2 production was found between strains. As regards the fermentation vigor, no significant differences were observed, suggesting a similar adaptation ability to an environment rich in sugar such as the grape must (Table 1). On the contrary after 139 hours significant differences were

detected probably due to different strain ethanol tolerance or intrinsic growth rate. Indeed with the progress of the fermentation the strains showed different fermentation abilities. Strains P301.4, R133.5, P301.9 and R103.1 showed the highest CO_2 production, but the strain fermentation times were different: the first two completed the fermentation after 15 days and the others after 23 days. P293.8 and P304.1 that produced low CO_2 level after 139 h, completed the fermentation after 15 and 19 days, respectively. R8.3 was the slowest fermenting strain, completing the fermentation in 29 days. The foam production at the end of fermentation was evaluated and strains were divided into two groups: low producers (P304.1, R8.3, R103.1, R133.5), with the foam thickness ranging from 0 to 8 mm and high producers (P293.8, P301.4, P 301) that produced 10 to 18 mm of foam. Except for P293.8, that showed a very low H_2S production, all the strains produced an intermediate level of H_2S.

Fermentation performance at three nitrogen concentrations

In order to understand how the nitrogen content influences the fermentation performances of the seven *S. cerevisiae* vineyard strains, three concentrations of YAN were assayed in synthetic must. Above 140 mg l^{-1} of YAN is often quoted to be necessary to ferment filtered grape must containing moderate sugar level (20%) (Bell and Henschke, 2005). Therefore the following nitrogen concentrations were added to the synthetic must: 300 mg l^{-1} YAN (MS300 with high nitrogen level), 150 mg l^{-1} YAN (MS150 with intermediate nitrogen level) and 70 mg l^{-1} (MS70 with low nitrogen level) .The commercial strain EC1118 was tested as control. Results suggest that the nitrogen concentration affected the days of fermentation. Indeed, when 300 mg N l^{-1} were present in the must (MS300), fermentations were completed between 14 and 21 days, in MS150 between 14 to 24 days and in MS70 between 17 and 31 days. In order to evaluate differences among strains at each nitrogen concentration, the total CO_2 production (g 100^{-1} ml^{-1}) after 95 hours was reported (Figure 1). At this fermentation time the widest range of variability was found. Strain R8.3 showed no difference in CO2 production at the three nitrogen levels. In MS300 and MS150 R8.3 produced a significantly lower level of CO_2 than the other strains tested. In MS70 the level of CO_2 produced by strain R8.3 was comparable with that found at 150 and 300 mg N l^{-1}. On the contrary, at 70 mg N l^{-1}, the other strains decreased the CO_2 production level and strain P304.1 was found to be the slowest. In order to evaluate the effect of different nitrogen concentrations on each strain, the total CO_2 production (g 100^{-1} ml^{-1}) after 48 hours (fermentation vigor) and 139 hours were evaluated (Figure 2). All the strains showed no significant differences between MS300 and MS150 in the fermentation vigor (Figure 2). Strain R8.3 and the industrial strain EC1118 showed no significant differences in all the conditions tested. After 139 hours, the daily increment of CO_2 production decreased very rapidly indicating that the strains reached the stationary phase. Most of the strains showed a clear reduction in the CO_2 production in MS70, while no significant differences were found in MS300 and MS150. Only R8.3 and R133.5 fermentations were not influenced by the nitrogen

concentration present in the synthetic must as no significant differences were found at the three nitrogen levels. Strain R103.1 was the only yeast that showed a clear reduction of CO_2 production when YAN concentration decreased from 300 to 150 mg N l^{-1}. No significant difference was found in CO_2 production at 150 and 70 mg N l^{-1}.

As reported in Table 2A all the strains showed a very limited sugar residues at the end of the fermentation process. The concentration of the remaining sugar generally was higher when 150 and 70 mg N l^{-1} were present. Considering glycerol production (Table 2B) differences in glycerol concentrations at the three YAN level were very limited and seems not to be related to nitrogen content. In fact, it is well-known that this compound is manly related to sugar content. Glycerol is synthesized by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate (G3P) by NAD-dependent glycerol 3-phosphate dehydrogenase (GPDH) followed by dephosphorylation of G3P to glycerol by means of a specific glycerol 3-phosphatase (G3Pase). During alcoholic fermentation, the roles of glycerol formation are to counteract the osmotic pressure, due to the presence of high sugar in the must, and to maintain the redox balance. The former is related to glycerol as compatible solute. Excess NADH is produced during biomass formation, therefore the surplus of NADH is oxidized by the glycerol biosynthetic pathways (Van Dijken andScheffers, 1986).

Moreover from a sensory point of view glycerol contributes to palate fullness ("body") of wine (Gawel *et al.*, 2007).

The wine commercial strain EC1118 was included as control. This strain was classified as low nitrogen requirement yeast in comparison with other seven wine commercial yeasts (Brice *et al.* 2014).

Our results suggested that nitrogen level influenced both the early and the late stage of the fermentation. Only strain R8.3, together with the control strain EC1118, seemed to be poorly affected by the tested nitrogen levels, as no significant differences in CO_2 production were detected at the three nitrogen concentrations. This suggested that among the vineyard strains only R8.3, the worst fermenting strain in terms of days of fermentation, showed low nitrogen requirement, as well as strain EC1118. Due to the fact that the latter possess excellent fermentation performance, our results suggested that yeast nitrogen requirement can modulate fermentation performances, that are determined by strain-specific genetic features. Generally, all the other strains showed no differences in fermentation at 300 and 150 mg N l^{-1}, but a clear delay in the fermentation process when nitrogen concentration dropped from 150 to 70 mg N l^{-1} was observed. These fermentation difficulties were confirmed by the presence of sugar residue at 70 mg N l^{-1}. Only strain R103.1 evidenced a clear delay when YAN concentration dropped from 300 to 150 mg l^{-1}. No differences were found between 150 and 70 mg N l^{-1}.

Pilot-scale fermentation and nitrogen consumption

S. cerevisiae P304.1 was chosen to be tested in pilot-scale fermentation. Indeed, P304.1 fermentation performances at different nitrogen level depicted the fermentation trend of most of the tested strains. They showed no differences in CO_2

153 production at 300 and 150 mg N l^{-1}; when YAN concentration dropped from 150 to 70 mg N l^{-1} a clear delay in the
154 fermentation process was reported.
155 During pilot-scale fermentation, the composition of the synthetic musts was the same of the previous trial (MS300,
156 MS150, and MS70). To monitor the fermentation process, the fermentation rate (expressed as CO_2 g l^{-1} per hour) was
157 measured instead of total CO_2 production (g l^{-1}). Due to the method for CO_2 detection (by means of a mass flowmeter)
158 must agitation was needed. Agitation influenced cell growth, as the cells remain suspended in the medium for longer,
159 increasing their ability to assimilate nutrients. Therefore it speeded up the fermentation process that was, in any
160 condition tested, faster than that without agitation. In these conditions P304.1 was confirmed to be affected by nitrogen
161 concentrations particularly when 70 mg N l^{-1} were present as the fermentation peak value was considerably lower than
162 those at 300 and 150 mg N l^{-1} (Figure. 3). Strain R304.1 completed the fermentation after six days when 300 mg N l^{-1}
163 were present, and after eight days when 150 mg N l^{-1} were present, whereas at 70 mg N l^{-1} after eight days of
164 fermentation 1.73 g l^{-1} of sugar were still present in the must.
165 The analysis of both ammonium and α-amino nitrogen consumption during the first two days of fermentation at the
166 three nitrogen conditions are reported in Figure. 3. Ammonium was rapidly consumed by the strain in all the conditions
167 tested. In MS300 it was depleted in 26 hours, in MS150 and MS70 in 12 and 8 hours, respectively. As expected, the α-
168 amino acid consumption was slower than ammonium. In MS300 after 26 hours 103 mg l^{-1} α-amino nitrogen were still
169 present and constituted the residual nitrogen at the end of the fermentation. As MS300 is internationally considered a
170 gold standard, the assimilation trend noted in this study agreed with those from previous works (Beltran *et al.* 2004,
171 Gutiérrez *et al.* 2012). In MS150 and MS70 the α-amino nitrogen was depleted after 14 and 10 hours, respectively.
172 In conclusion vineyard strains showed different fermentation performances in response to the nitrogen level present in
173 the must. Both 150 and 70 mg l^{-1} YAN can be considered critical nitrogen levels as strain fermentation performances
174 were different at higher nitrogen concentration (300 and 150 mg l^{-1} N, respectively).
175 When the nitrogen concentration was at the strain critical level a delay in the fermentation process was observed. When
176 the nitrogen concentration was above the critical level, the fermentation process, in terms of total CO_2 produced (g 100^{-1}
177 ml^{-1}) at specific sampling points, increase, but if the level of nitrogen was further increased no effect on the
178 fermentation was found. Regarding the nitrogen critical level, its concentration is strain dependent. In our conditions,
179 most of the tested strains showed a critical nitrogen level at 70 mg l^{-1} YAN, one strain at 150mg l^{-1} YAN. As strain R8.3
180 and the industrial strain EC1118 used as control were not affected by the three nitrogen concentrations, it can be
181 suggested that the critical nitrogen level of these two strains is below 70mg l^{-1} YAN.
182
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MATERIALS AND METHODS

Yeast strains

Seven *S. cerevisiae* strains were used in this study. Four strains (P293.8, P301.4, P301.9, P304.1) were chosen from a pool of 354 isolates from Glera vineyards located in Conegliano–Valdobbiadene Prosecco superior Appellation of Origin area. Three strains (R8.3, R103.1, R133.5) were chosen from a pool of 78 isolates from Raboso Piave vineyards in the Piave Appellation of origin area. All yeasts were identified at species level by conventional methods, genus specific amplification and PCR-RFLP analysis of the ITS1 region of 18S rDNA (Guillamón, J. M at al. 1998; Nardi *et al.* 2006; Bovo *et al.* 2009); Characterization at strain level was performed by means of mt-DNA analysis (Treu *et al.* 2014b). Strain physiological characterization was performed for all the yeasts to evaluate the presence of technological trait interesting for winemaking (Treu *et al.* 2014b). The strains were chosen by taking into account the origin (isolation area), the differences in the mt-DNA profiles and in the physiological characteristics. Commercial strain EC1118 was used as a control.

Fermentation trials in standard synthetic must, at different nitrogen concentrations and H_2S production

Fermentations were performed in standard synthetic must (MS300), containing glucose (200 g l^{-1}), malic acid (6 g l^{-1}) at pH 3.2, prepared as described by Bely *et al.* (1990). Its amino acid composition simulates the nitrogen content of a standard grape juice, containing 300 mg l^{-1}/ (YAN). Two modified synthetic musts with different YAN concentrations were prepared, as well: MS150 (150 mg N l^{-1}) and MS70 (70 mg N l^{-1}). The musts were inoculated with yeast cultures prepared as followed. A loopful of a 3-day-old culture of each strain grown on YPD agar plate (yeast extract 10 g l^{-1}, peptone 10 g l^{-1}, and dextrose 20 g l^{-1}) was used. A suitable volume of each yeast culture, corresponding to a final cell concentration of 1.5×10^6 cells mL^{-1}, was inoculated in the must. All the fermentation were performed containing 100 ml of must and incubated at 25°C as described by Bovo *et al.* (2016). The fermentation process was followed by measuring twice a day the weight loss of each culture. When the weight loss was lower than 0.05 g per day the fermentations were considered concluded and the foam level was measured as described by Lemos Junior *et al.* (2016). All the fermentation trials were performed in triplicate.

The production of hydrogen sulphide was evaluated, as well, by streaking each strain on Biggy agar (Oxoid Ltd, UK).

Fermentation trials in bioreactor and nitrogen determination

MS300, MS150 and MS70 were used to perform one-litre fermentations in Multifors fermenter (INFORS-HT Multifors, Switzerland), equipped with 0-20 ml scale Read-y massflowmeter (CO_2 calibrated). CO_2 efflux (ml/min) was

measured every 10 minutes. The strain inoculum was performed as described by (Nadai *et al.*, 2016). Kinetic results are reported as the mean of three fermentations.

Aminic and ammoniacal nitrogen were measured at the moment of the inoculum and after 4, 8,10,12,16, 26 hours by means of a specific enzymatic kit (Steroglass, Italy).

Chemical analysis

At the end of the fermentation process an aliquot of each yeast culture was centrifuged, filtered and 10 µl were analyzed by Waters 1525 HPLC binary pump with a 300×7.8 mm stainless steel column packed with Aminex HPX_87H 300×7.8 mm was used. Waters 2414 Refractive Index Detector, set at 600 nm wavelength for the determination of glucose and glycerol was used as described by Nadai *et al.* (2016). For quantitative determination, calibration curves of glucose and glycerol were made in water and peaks area was determined by Breeze (Waters) program.

Statistical treatment of data

The statistical data analysis was performed with XLSTAT software, vers.7.5.2 (Addinsoft, Paris, France) using the one-way analysis of variance (ANOVA) at 95 % accuracy level. Tukey's test was used as comparison test when samples were significantly different after ANOVA ($p < 0.05$).

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Conflicts of interest

The authors declare no conflicts of interest.

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Table 1. Grape variety, CO_2 production during fermentation, days of fermentation and foam at the end of the fermentation of *S. cerevisiae* strains in MS300 and production of hydrogen sulphide. Data are expressed as the average of three replicates ± standard deviations.

Grape variety	Strain	CO_2 production at 48h	CO_2 production at 139h	Days of fermentation	Foam (mm)	H_2S*
Glera	P293.8	2.31^{a}±0.39	6.19^{cd}±0.19	15	10	1
Glera	P301.4	2.90^{a}±0.27	6.29^{bc}±0.29	15	12	3
Glera	P301.9	3.02^{a}±0.158	7.17^{abc}±0.16	23	18	3
Glera	P304.1	2.25^{a}±0.29	7.46^{ab}±0.54	19	8	3
Raboso Piave	R8.3	2.68^{a}±0.85	5.8^{cd}±0.12	29	4	3
Raboso Piave	R103.1	2.97^{a}±0.70	7.47^{ab}±1.34	23	0	2
Raboso Piave	R133.5	3.05^{a}±0.17	7.81^{a}±0.26	15	4	3

* The production of hydrogen sulphide was determined on BIGGY agar (DIFCO), scoring the browning degree of the yeast streak according to the following scale: 0= white, 2 = light brown, 3 = dark-brown, 4= black. Within the column, mean values followed by the same letter are not significantly different according to Tukey's test ($p \leq 0.05$).

Table 2. Residual glucose (g l^{-1}) (A) and glycerol (g l^{-1}) (B) concentrations at the end of the fermentation of *S. cerevisiae* strains in MS300, MS150 and MS70. Data are expressed as the average of three replicates ± standard deviations. Within the column, mean values followed by the same letter are not significantly different according to Tukey's test ($p \leq 0.05$). Latin letters represents comparison of all the strains at same nitrogen concentration. Greek letters represents comparison for each strain at different nitrogen concentrations.

A

Strains	**P293.8**	**P301.4**	**P301.9**	**P304.1**	**R103.1**	**R133.5**	**R8.3**	**EC1118**
MS300	$2.22^{a\alpha}$ ± 0.53	$1.97^{a\alpha}$ ± 0.13	$1.55^{a\alpha}$ ± 0.05	$1.55^{a\alpha}$ ± 0.03	$1.49^{a\alpha}$ ± 0.08	$3.35^{b\alpha}$ ± 0.68	$1.64^{a\alpha}$ ± 0.18	$1.46^{a\alpha}$ ± 0.12
MS150	$2.57^{b\alpha}$ ± 0.55	$1.80^{a\alpha}$ ± 0.01	$1.56^{a\alpha}$ ± 0.06	$1.48^{a\alpha}$ ± 0.01	$1.62^{a\alpha}$ ± 0.15	$5.39^{c\beta}$ ± 0.29	$1.73^{a\alpha\beta}$ ± 0.22	$1.80^{a\alpha}$ ± 0.03
MS70	$4.17^{b\beta}$ ± 0.30	$2.58^{a\beta}$ ± 0.24	$1.64^{a\alpha}$ ± 0.07	$1.94^{a\beta}$ ± 0.11	$1.56^{a\alpha}$ ± 0.04	$4.09^{b\alpha\beta}$ ± 0.83	$2.26^{a\beta}$ ± 0.29	$2.22^{a\alpha}$ ± 0.51

B

Strains	**P293.8**	**P301.4**	**P301.9**	**P304.1**	**R103.1**	**R133.5**	**R8.3**	**EC1118**
MS300	$7.71^{bc\alpha}$ ± 0.14	$6.98^{ad\alpha}$ ± 0.01	$6.73^{de\alpha}$ ± 0.22	$7.41^{ab\alpha\beta}$ ± 0.17	$6.34^{e\alpha}$ ± 0.06	$8.02^{c\alpha}$ ± 0.30	$7.24^{a\alpha}$ ± 0.11	$7.37^{ab\alpha}$ ± 0.17
MS150	$7.60^{ab\alpha\beta}$ ± 0.10	$6.57^{a\alpha}$ ± 0.20	$7.44^{ab\beta}$ ± 0.02	$7.90^{a\alpha}$ ± 0.09	$4.66^{c\beta}$ ± 1.14	$7.67^{ab\alpha}$ ± 0.43	$7.11^{ab\alpha}$ ± 0.14	$7.16^{ab\alpha}$ ± 0.14
MS70	$7.36^{bc\beta}$ ± 0.10	$6.32^{a\alpha}$ ± 0.46	$7.50^{bc\beta}$ ± 0.18	$6.94^{a\beta}$ ± 0.54	$6.22^{a\alpha}$ ± 0.59	$8.22^{c\alpha}$ ± 0.16	$7.50^{bc\alpha}$ ± 0.32	$6.82^{ab\beta}$ ± 0.01