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ORIGINAL PAPER

The production of hydrogen sulphide and other aroma compounds by wine strains of *Saccharomyces cerevisiae* in synthetic media with different nitrogen concentrations

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Abstract The aim of this study was to evaluate the combined effect of initial nitrogen content on the production of hydrogen sulphide and other volatile compounds during alcoholic fermentation. For that propose, three commercial wine strains of *Saccharomyces cerevisiae* were used to ferment synthetic grape juice media under different nitrogen concentrations. H_2S was measured throughout fermentations and other aroma compounds were analyzed at the end of the experiments. The trigger levels at which an inverse relationship between the initial nitrogen present in media and total H_2S production varied among the three strains tested. For UCD522 and PYCC4072, the highest H_2S levels were produced in media with 267 mg N l^{-1} of initial nitrogen, whereas the lowest levels were detected with nitrogen limitation/starvation conditions (66 mg N l^{-1}). Moreover, 21 other aroma compounds belonging to different chemical classes were identified and quantified by solid phase microextraction (SPME) coupled to gas chromatography–mass spectrometry (GC–MS). The initial nitrogen concentration more than yeast strain had a decisive effect on the final aroma composition, suggesting that modulation of nutrients emerges as a useful tool for producing desired flavour and odour compounds.

Keywords Yeast · Nitrogen · Hydrogen sulphide · Wine · Aroma compounds

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Introduction

Hydrogen sulphide (H_2S) production is of major concern in winemaking, since it has a profound negative effect on the sensory quality of wines due to its rotten egg odour detectable at very low concentrations. In *Saccharomyces cerevisiae*, H_2S is the product of the sulphate reduction sequence (SRS) pathway and acts as an intermediate in the biosynthesis of sulphur-containing amino acids [35]. The biosynthesis of sulphur amino acids requires nitrogen-containing carbon precursors derived from the intracellular nitrogen pool and sulphide from the sulphate reduction pathway. Thus, the rate of H_2S formation seems to be regulated by cellular demands for sulphur amino acids and maintenance of intracellular nitrogen pools [12]. Several environmental and nutritional factors have been associated with H_2S production under winemaking conditions, namely (1) levels of elemental sulphur [29, 34] naturally available as sulphate at an average concentration of 200 mg l^{-1} [24], (2) presence of sulphur dioxide [1, 32] commonly added (50–200 mg l^{-1}) to grape must prior to wine fermentation, (3) presence of organic compounds containing sulphur [2], and (4) vitamin deficiency [5, 36, 42, 43]. Yet, poor correlations between H_2S formation and assimilable nitrogen have been reported [30, 41]. Nevertheless, the effects of nitrogen as well as those factors mentioned before are highly dependent on yeast genetic background [12, 16, 31], making it difficult to

devise strategies to prevent H_2S formation during winemaking.

It is widely accepted that the nature and the availability of yeast assimilable nitrogen play a significant role in wine sensorial traits. Organic acids, higher alcohols, aldehydes, ketones, and sulphur-compounds have been considered the most significant sensorial components of wine and constitute the main group of compounds that form the ''fermentation bouquet'' [23]. A few organic acids are volatile enough to contribute to its odour, acetic (vinegary), propanoic (goaty) and butanoic acid (spoiled butter). In wine, only acetic acid can appear in concentrations above its perception threshold [23], and its production seems to be inversely correlated with initial nitrogen levels [3, 4, 37]. Higher alcohols such as isoamyl alcohol, amyl alcohol, and isobutyl alcohol are produced during fermentation by similar biochemical reactions as a result of decarboxylation of α-keto acids derived by either transamination of amino acids (α-ketoisocaproic acid from leucine, α-ketoisovaleric acid from valine, and α-keto-β-methylvaleric acid from isoleucine) via the Ehrlich pathway [44] or through sugar catabolism [8]. These alcohols, together with acids form esters such as ethyl acetate, hexyl acetate, isoamyl acetate (banana-like aroma) ethyl caproate, ethyl caprylate (apple-like aroma), and 2-phenylethyl acetate (fruity and flowery like aroma) during fermentation, which significantly contribute to the pleasant fruity aroma of wines, beer, and other alcoholic beverages. At low levels, all these compounds contribute to perceived wine aroma complexity. However they have also been considered as responsible for off-flavours when present in too high amounts [26]. Among other factors, nitrogen content affects the pattern of both higher alcohols and esters formed during fermentation, via regulation of the Erhlich, fatty acid, and ester synthesis pathways [33]. Furthermore, the quantities of these various compounds that are produced during fermentation have been shown to vary significantly within wine yeast species and strain [26]. Only recently a few studies have been conducted for determining the effect of nitrogen on aroma compounds formation in wine [7, 40]. Therefore, the aim of the present work was to assess H_2S production and to determine the potential differences in volatile aroma compounds formation by three wine yeast strains during high sugar alcoholic fermentations under different nitrogen conditions. In this way, we studied three widely used commercial *S. cerevisiae* strains grown in synthetic grape juice media and batch culture, mimicking winemaking conditions, under different initial nitrogen concentration 66, 267 and 402 mg N l^{-1}. The knowledge of growth and fermentative behaviour, volatile compound production and other oenological characteristics which impact wine properties could be useful for selecting the most appropriate strain to produce a wine with the desired flavour and odour qualities.

Materials and methods

Strains and maintenance conditions

Three strains of *S. cerevisiae* were used in this study. *S. cerevisiae* UCD522 was kindly supplied by the Enology Culture Collection, Department of Viticulture and Enology, University of California, Davis, USA. *S. cerevisiae* PYCC4072 was originally isolated from a sample of Fermivin, industrial wine yeast distributed by Rapidase, which was obtained from the Portuguese Yeast Culture Collection (PYCC), New University of Lisbon, Portugal, and the commercial strains *S. cerevisiae* EC1118 was obtained from the market as active dried yeast. The strains tested were chosen based on their potential ability to produce H_2S in solid media [18]: (1) UCD522 (a high producer), (2) PYCC4072 (an average producer) and (3) EC1118 (a poor producer). The yeast cultures were 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 h at 25°C.

Culture media

A chemically defined grape juice medium (GJM) similar in composition to typical grape juice, as previously described by Henschke and Jiranek [12] of the Australian Wine Research Institute, was used with minor modifications. Glucose (200 g l^{-1}) was used as the only carbon and energy source, and nitrogen was added at different concentrations (66, 267, and 402 mg N l^{-1}) supplied as diammonium phosphate (DAP), to facilitate monitoring nitrogen consumption profile. Moreover, ammonium, as ammonium phosphate or sulphate salts, up to the legal limit of 1,000 mg l^{-1} are widely used by winemakers to increase nitrogen content of grape musts according to OIV regulation. The pH was adjusted to 3.7 with NaOH prior to sterile filtration of the media.

Inocula and fermentation conditions

For all experiments, starter cultures were prepared by pre-growing the yeast overnight in 100 ml shake flasks, containing 70 ml of the same medium with the same composition used in all assays. Incubation was done at 25°C in an orbital shaker at 150 rpm. This pre-culture was used to inoculate experimental cultures with an initial population of 5×10^5 CFU ml^{-1}.

Fermentations were done in 250 ml flasks filled to 2/3 of their volume, fitted with a side-arm port sealed with a rubber septum for anaerobic sampling and maintained at 20°C in an orbital shaker at 120 rpm. Fermentations were

monitored using weight loss as an estimate of CO_2 production. Aseptic sampling was accomplished using a syringe-type system. To avoid medium accumulation in the system, a stylet was inserted in the needle holder. Each flask was closed with a rubber stopper, allowing fermentation gasses to escape through a glass tube connected to a two-way valve by Teflon tubing; the other end of the tubing was connected to a tube with 10 ml of trapping solution to create a fermentation lock and a trap for H_2S. Every 24 h, each flask was removed from the shaker, disconnected from the trap, weighed, and connected to a new trap. Samples were collected daily for assessing fermentation and growth parameters.

Prior to sampling, the flasks were stirred for homogeneity. The end of alcoholic fermentation was confirmed using Clinitest tablets (Roche). At that time, samples were taken from different fermented media to be screened for aroma compounds production by solid phase micro-extraction (SPME) coupled to gas chromatography–mass spectrometry (GC–MS). Additionally, viable cell number, culture dry weight and residual glucose and nitrogen levels were determined in all experiments.

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

Determination of viable cell number and culture dry weight

Optical density (660 nm) of appropriately diluted culture samples was used as an estimate of yeast cell growth. Samples of 2 × 50 and/or 3 × 15 ml were centrifuged in pre-weighed tubes for 10 min at 2,300*g*, washed twice with deionised water, dried for 24 h at 100°C, and stored in a desiccator before weighing. Duplicate and triplicate determinations varied by less than 1%.

Determination of sugar concentration

Glucose was quantified by the 2,4-dinitrosalicylic acid (DNS) method [21].

Determination of ammonium

Ammonium calculations were done using a continuous-flow analysis system (sampler, pump, dialysis unit, ammonium unit, photometer, and recorder). Ammonium calculations were based on the Berthelot reaction as previously described [19].

Determination of oenological parameters

Total SO_2, volatile acidity and ethanol production were determined as reported by the Office International de la Vigne et du Vin [22].

Hydrogen sulphide determination

The amount of H_2S liberated by yeast cultures was determined colorimetrically following selective collection of fermentation gases with a modified fermentation lock and sulphide-trapping system as described by Jiranek et al. [16]. A suspension of cadmium hydroxide was used as trapping solution, containing 5.59 mmol l^{-1} $3CdSO_4$ $8H_2O$ and 15 mmol l^{-1} NaOH. Amine reagent contained 37.5 mmol l^{-1} *N*,*N*-diethyl-*p*-phenylene diamine. HCl (Sigma, St. Louis, USA) in 9.12 mmol l^{-1} H_2SO_4, and $FeCl_3$ solution (60% w/w) was added immediately prior to use. This mixture (325 µl) was added to 10 ml of trapping solution in 30 ml screw-cap bottles, which were immediately closed and shaken vigorously for 30 s. After 1 h of incubation at room temperature, absorbance of samples was measured at 672 nm. H_2S content was calculated using a calibration curve prepared with known amounts of sulphide in the range of 0–12 µg, according to the methodology described by Acree et al. [1] and Rees et al. [25].

SPME procedure and GC–MS analysis

SPME fibers

The fibers used in this study were coated with polydimethylsiloxane (PDMS), 100 µm and were supplied by Supelco (Bellefonte, PA, USA). Also the holder used for manual injection was from the same supplier (Supelco, Bellefonte, PA, USA).

Chromatographic conditions

Chromatographic analyses were performed using an Agilent 6890 N gas chromatograph equipped with a 5973 N mass spectrometer. The target analytes were separated using a Innowax capillary column, 30 m × 0.25 mm with 0.5 µm film thickness (Agilent, Santa Clara, CA, USA). The column was maintained at 40°C for 5 min after desorption, ramped at 4°C min^{-1} up to 200°C, and then ramped at 10°C min^{-1} up to 240°C, where it was held for 15 min Helium was used as the carrier gas at 34 cm s^{-1} average linear velocity. A 0.75-mm liner was used and analysis performed in the splitless mode. All mass spectra were acquired in electron impact (EI) mode at 70 eV, using full scan with a scan range of 26–250 atomic mass units, at a rate of 6.12 scans s^{-1}. Identification of all compounds were confirmed by comparing mass spectra and retention indices with those of authentic standards. Ethyl propionate (99%), ethyl 2-methylbutyrate (95%), isoamyl alcohol (98.5%), ethyl hexanoate (99%), linalool (97%), 1-octanol (99.5%), butyric acid (99%), ethyl decanoate (99%), isovaleric acid (98%), diethyl succinate (99%), hexanoic acid (98%), octanoic

acid (98%), decanoic acid (99.5%), and 2-octanol (99.5%) were supplied by Fluka (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Ethyl acetate (99.5%), ethyl butyrate (98%), isoamyl acetate (98%), ethyl octanoate (98%), 2-phenylethyl acetate (99%), and 2-phenylethanol (99%) were from Merck (Merck, Damstadt, Germany). Ethyl isobutyrate (99%) and ethyl isovalerate (98%) were from Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

The compounds were quantified in selected ion monitoring (SIM) mode, by selecting for each compound their most characteristic ions as follows: ethyl acetate (43), ethyl propionate (57), ethyl isobutyrate (43 + 71), ethyl butyrate (71), ethyl 2-methylbutyrate (57 + 102), ethyl isovalerate (88), isoamyl acetate (43), isoamyl alcohol (55), ethyl hexanoate (88), ethyl octanoate (88), linalool (71 + 93), 1-octanol (55 + 56), butyric acid (60), ethyl decanoate (88), isovaleric acid (60), diethyl succinate (101), 2-phenylethyl acetate (104), hexanoic acid (60), 2-phenylethanol (91), octanoic acid (60), and decanoic acid (73). In order to eliminate variations in extraction efficiency caused by small differences in the sample matrix (particularly ethanol content), internal standardization using 2-octanol as the internal standard was applied to quantify the analytes.

SPME extraction procedure

SPME extraction was performed according to the methodology previously described [17], with minor modifications. For sample preparation, to 10 ml of sample (synthetic wine solution), 10 ml of internal standard solution (2-octanol, 200 μg l^{-1}) plus 4 g of NaCl were transferred to 40 ml vials (Supelco P/N 27181) and hermetically sealed with PTFE-faced silicone septum. Before the extraction step, samples were equilibrated for 10 min at 20 ± 1°C. Then the PDMS fibre was inserted through the vial septum and exposed to sample headspace for 60 min under constant temperature and permanent magnetic stirring (300 rpm) to perform the extraction, under the same conditions of temperature and agitation. Then the fibre was removed from the sample headspace and inserted into the injection port of the gas chromatograph, for thermal desorption (splitless mode) at 270°C for 10 min and subsequent chromatographic analysis was performed.

Preparation of standards and SPME fiber calibration

Single standard stock solutions (1% v/v) of the volatile compounds were prepared by spiking each compound in pure ethanol (Merck LiChrosolv). Working solutions, prepared just before use, were made from the stock solutions by spiking and mixing them with a hydro-alcoholic solution (11.5% v), containing tartaric acid (3 g l^{-1}) and potassium hydrogen tartrate (3 g l^{-1}), and adjusted to pH 3.2 with 6 M NaOH. The concentrations of the analytes in the working solution was as follows: ethyl acetate (50 mg l^{-1}), ethyl propionate (500 μg l^{-1}), ethyl isobutyrate (200 μg l^{-1}), ethyl butyrate (350 μg l^{-1}), ethyl 2-methylbutyrate (25 μg l^{-1}), ethyl isovalerate (25 μg l^{-1}), isoamyl acetate (3.5 mg l^{-1}), isoamyl alcohol (400 mg l^{-1}), ethyl hexanoate (400 μg l^{-1}), ethyl octanoate (300 μg l^{-1}), linalool (50 μg l^{-1}), 1-octanol (50 μg l^{-1}), butyric acid (500 μg l^{-1}), ethyl decanoate (200 μg l^{-1}), isovaleric acid (2 mg l^{-1}), diethyl succinate (50 μg l^{-1}), 2-phenylethyl acetate (2 mg l^{-1}), hexanoic acid (5 mg l^{-1}), 2-phenylethanol (150 mg l^{-1}), octanoic acid (6 mg l^{-1}), and decanoic acid (5 mg l^{-1}). This solution was used as the higher concentration calibration standard, and six more calibration solutions were prepared by serially diluting the working solution by twofold with the hydro-alcoholic solution. For fiber calibration, all calibration solutions were analyzed in triplicate by GC–MS using the same SPME extraction procedure as for the fermented GJM samples.

Statistical analysis

Factorial design experiments were used to analyze the influence of grape juice initial nitrogen concentration (three levels) and yeast strain (three strains) on aroma compounds formation. Statistical analyses were done using Statistica 7.0 software (StatSoft Inc., 2004). For paired comparisons, the Tukey honestly significant difference (HSD) test was used.

Results

Effects of nitrogen concentration on hydrogen sulphide production and its relation to growth and fermentation profiles

The formation of H_2S was evaluated during alcoholic fermentation in three wine yeast strains of *S. cerevisiae* grown in batch cultures, in synthetic grape juice medium, mimicking the conditions of natural wine fermentation. The amounts of assimilable nitrogen added into the media were selected according to previous data obtained in our laboratory to determine limiting nitrogen concentrations in growth and fermentation pattern using *S. cerevisiae* PYCC4072 [19]. Accordingly, three fermentation conditions have been established by manipulating nitrogen concentration in the media: (1) 66 mg l^{-1} is a N-limiting concentration that leads to sluggish fermentation (as complete dryness has been obtained after 28–29 days), (2) 267 mg l^{-1} is the concentration of nitrogen required for completion of alcoholic fermentation, in a reasonable time,

and (3) 402 mg l^{-1} is an excess nitrogen concentration, since some nitrogen remains in the fermented media at the end of alcoholic fermentation. To correctly analyze the effect of nitrogen availability on H_2S liberation, sulphur level was maintained constant in all media as $MgSO_4$ $7H_2O$ (1.23 g l^{-1}) and no sulphite was added prior to fermentation.

A global view of the fermentation, growth profiles and hydrogen sulphide production of each of the strains associated to the three test conditions are presented in Figs. 1, 2, 3 and in Table 1. The results show that in media containing 66 mg l^{-1} (limiting nitrogen concentration that leads to sluggish fermentation), the strain UCD522 was able to complete alcoholic fermentation after 576 h (Fig. 1a), while PYCC4072 and EC1118 (Figs. 2a, 3a) failed to attain total sugar degradation, leaving at 696 h (Table 1), approximately 13 and 11 g of residual glucose, respectively. Nitrogen was exhausted from the medium in the first 24 h, except for the strain EC1118, and glucose consumption followed for nearly 24–29 days, even in the absence of nitrogen in the medium (Figs. 1a, 2a, 3a). In addition, cell viability remained high (around 97%) until the end of the fermentation (data not shown). In media with 267 mg N l^{-1}, the strains UCD522 and PYCC4072 behaved similarly (Figs. 1b, 2b). All nitrogen available was consumed after 48 h and the values obtained for growth and fermentation parameters were approximately the same in both strains (Table 1). The EC1118 strain behaved quite differently, consuming nitrogen more slowly than the two others, which was correlated with the extent of fermentation

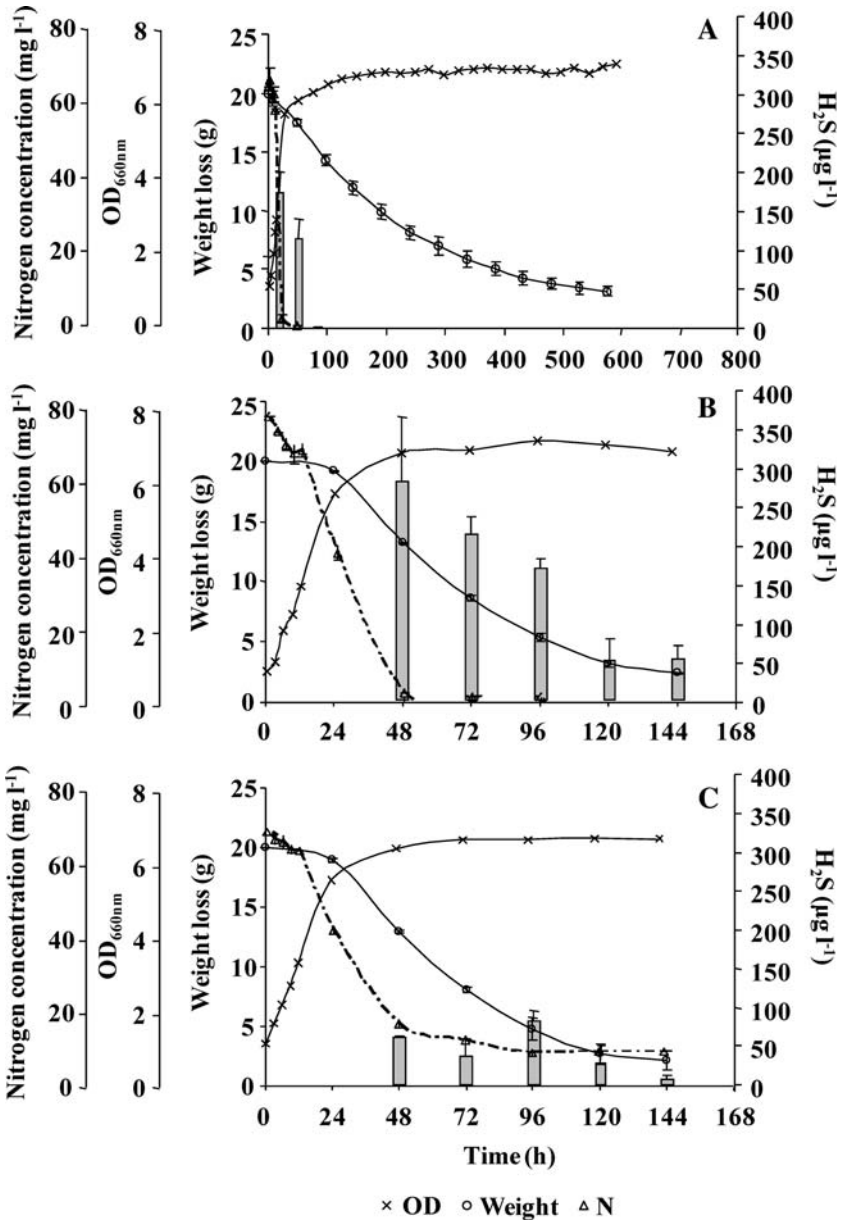

Fig. 1 Fermentation profiles and hydrogen sulphide liberation (*histograms*) in synthetic grape juice medium at 20°C, containing an initial nitrogen concentration of 66 (**a**), 267 (**b**) and 402 mg N l^{-1} (**c**) of yeast strain UCD522. Data points are the mean from triplicate fermentations ± SD (*smaller error bars* are hidden behind the data symbols)

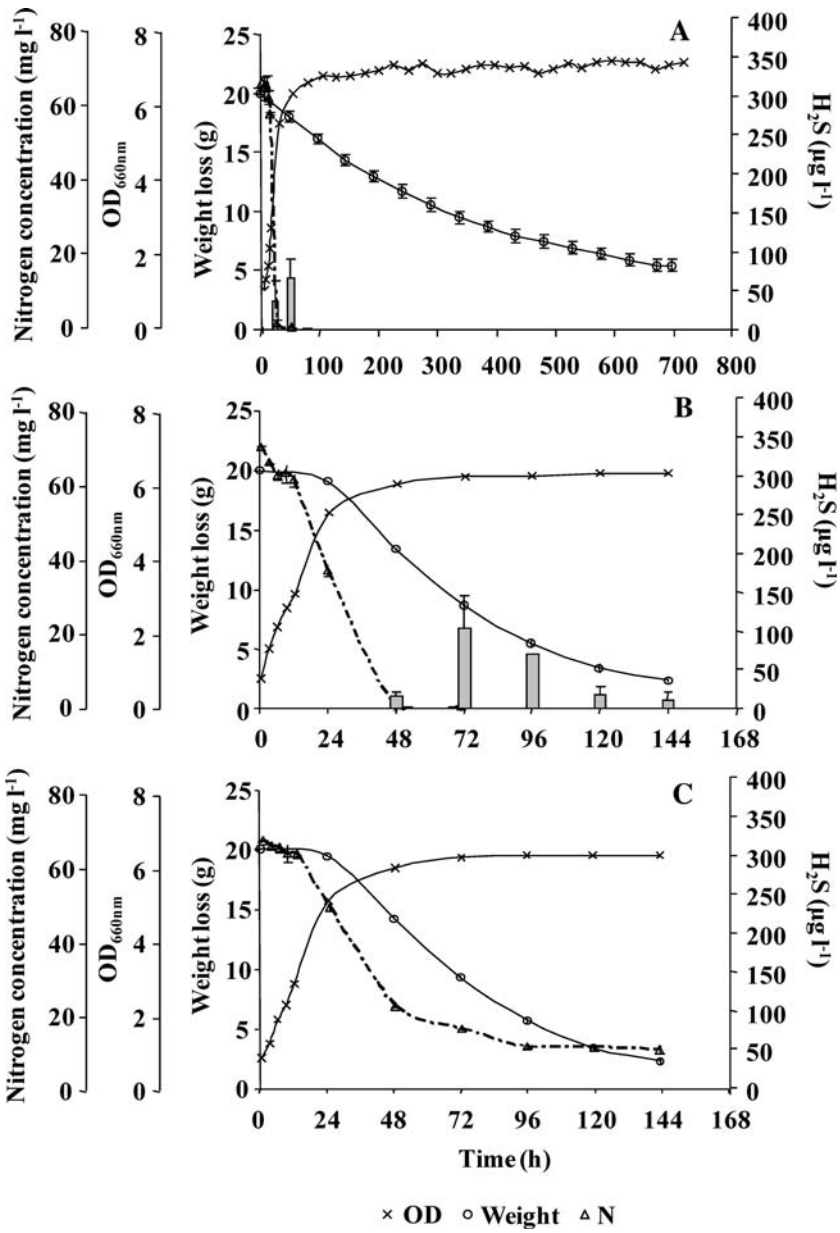

Fig. 2 Fermentation profiles and hydrogen sulphide liberation (*histograms*) in synthetic grape juice medium at 20°C, containing an initial nitrogen concentration of 66 (**a**), 267 (**b**) and 402 mg N l^{-1} (**c**) of yeast strain PYCC4072 Data points are the mean from triplicate fermentations ± SD (*smaller error bars* are hidden behind the data symbols)

(Fig. 3b; Table 1). Elevating initial nitrogen levels from 267 to 402 mg l^{-1} had no significant effect on UCD522 and PYCC4072 growth and fermentation patterns (Figs. 1b, c, 2b, c), yet for yeast strain EC1118 an increase in growth and fermentation rates was observed (Table 1). The fermentative patterns of all the yeasts tested were very similar, despite the differences in final biomass formation particularly lower for EC1118 (Figs. 1c, 2c, 3c). All fermentations were completed within 144 h without depleting nitrogen from the media, and no significant differences in the amount of nitrogen used by the three strains were observed.

With regards to the H_2S evolution during the fermentation, the results show that a strain-dependent variation in the H_2S formation in response to nitrogen availability occurs (Figs. 1, 2, 3). In opposition to what has been seen for wine yeast strains UCD522 and PYCC4072, for the strain EC1118 (poor H_2S producer) it was possible to observe an inverse relationship between the amounts of initial nitrogen present in the media and total H_2S production (Table 1). Nevertheless, for all the concentrations studied, the levels of sulphide produced by this strain were almost negligible (Fig. 3a–c). Regarding PYCC4072 (Fig. 2a–c) in the fermentations conducted with low and sufficient nitrogen levels, 66 and 267 mg l^{-1}, respectively, the peak of sulphide liberation occurred after nitrogen depletion. In media supplemented with 402 mg l^{-1}, where approximately 70 mg N l^{-1} remained in the medium (Table 1), there was not a notable H_2S production (Fig. 2c). On the contrary, for UCD522 even when nitrogen was in excess (Fig. 1c) a

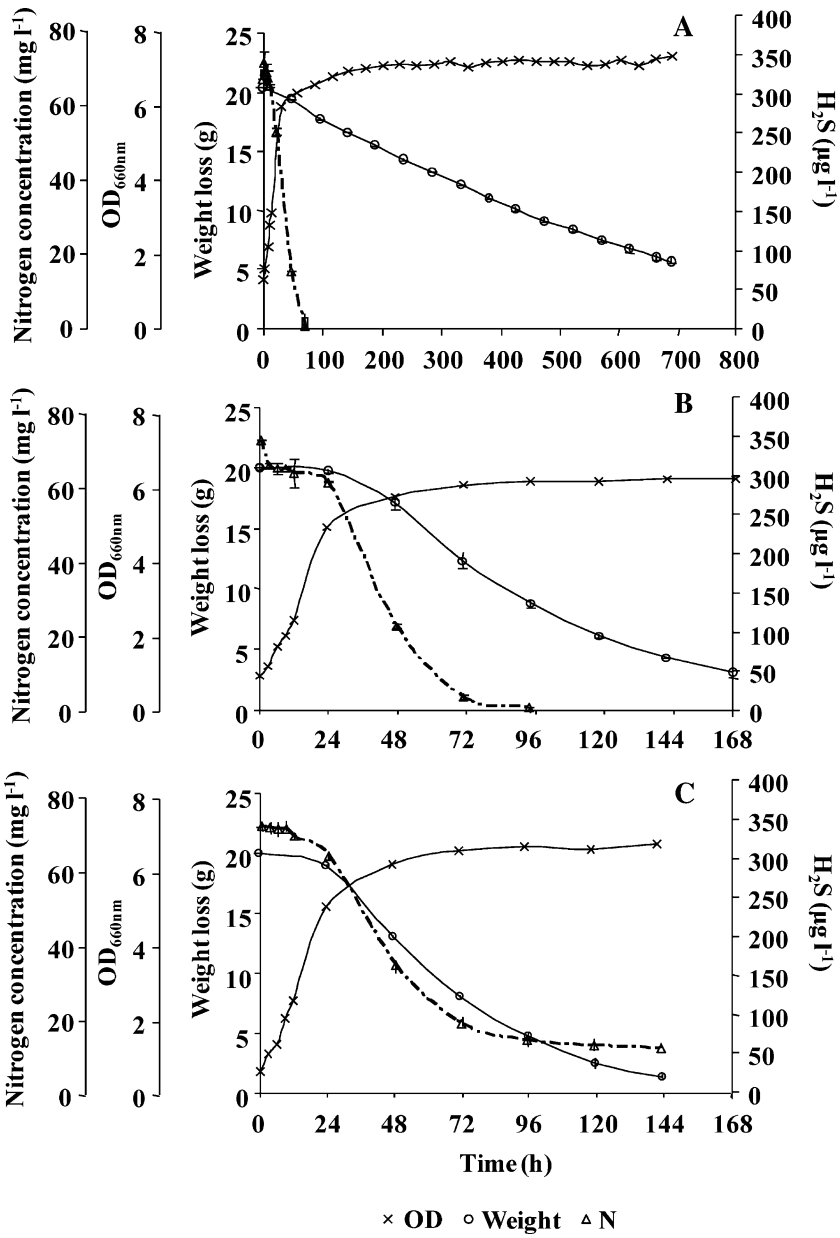

Fig. 3 Fermentation profiles and hydrogen sulphide liberation (*histograms*) in synthetic grape juice medium at 20°C, containing an initial nitrogen concentration of 66 (**a**), 267 (**b**) and 402 mg N l^{-1} (**c**) of yeast strain EC1118. Data points are the mean from triplicate fermentations ± SD (*smaller error bars* are hidden behind the data symbols)

noteworthy H_2S release was detected. As for PYCC4072 in the media with initial nitrogen levels of 66 and 267 mg l^{-1}, sulphide production was induced following complete nitrogen consumption by yeast cells, after 24 and 48 h, respectively (Fig. 1a, b).

Effect of yeast strain and initial nitrogen concentration on aroma compounds formation

To evaluate how nitrogen concentration in media affected aroma formation, samples were taken for GC–MS analysis of volatile compounds at the end of fermentations. The results of volatile compounds produced by the tested strains under different nitrogen conditions are presented in Figs. 4, 5, 6 and the results of a two-way ANOVA are presented in Table 2. Global analyses of results revealed that yeast strains differed only quantitatively, but not qualitatively with regard to the 21 aroma compounds analyzed in this study. The wine strain EC1118 produced significantly higher levels of total organic acids, specifically hexanoic, octanoic, and decanoic acids, whereas no significant differences between UCD522 and PYCC4072 were detected; only isovaleric acid was produced at higher levels by these two strains. However, the amounts of each one of the acids analyzed was significantly ($p < 0.001$) affected by nitrogen concentration. As the nitrogen concentration increased up to 267 mg l^{-1}, higher hexanoic acid production was observed for the three strains, being particularly evident in

Table 1 Overview of fermentation parameters, specific growth rates, final dry weight, nutrient concentrations and total hydrogen sulphide production from the fermentation of chemically defined grape juice media at 20°C, with different initial nitrogen levels by yeast strains UCD522, PYCC4072 and EC1118

Yeast strain	Initial nitrogen (mg l^{-1})	Time to reach dryness (h)	Overall fermentation rate (g h^{-1})	Maximum fermentation rate[a] (g h^{-1})	Final nitrogen (mg l^{-1})	Specific growth rate (μ h^{-1})	Final biomass (g l^{-1})	Total H_2S (µg l^{-1})
UCD522	66	576	0.029 ± 0.001	0.063 ± 0.003	0.0 ± 0.0	0.198 ± 0.001	3.46 ± 0.29	281.3 ± 2.8
	267	144	0.143 ± 0.002	0.219 ± 0.005	0.0 ± 0.0	0.235 ± 0.007	5.51 ± 0.37	778.5 ± 34.6
	402	144	0.146 ± 0.007	0.226 ± 0.003	55.0 ± 2.6	0.219 ± 0.009	5.39 ± 0.14	217.3 ± 43.6
PYCC4072	66	>696	0.020 ± 0.001	0.041 ± 0.004	0.0 ± 0.0	0.185 ± 0.004	3.17 ± 0.16	103.1 ± 3.6
	267	144	0.142 ± 0.002	0.217 ± 0.002	0.0 ± 0.0	0.226 ± 0.003	5.03 ± 0.04	222.7 ± 43.7
	402	144	0.141 ± 0.003	0.210 ± 0.004	71.2 ± 6.7	0.221 ± 0.001	5.71 ± 0.22	2.0 ± 1.7
EC1118	66	>696	0.021 ± 0.001	0.036 ± 0.001	0.0 ± 0.0	0.162 ± 0.001	2.38 ± 0.03	11.2 ± 4.1
	267	168	0.114 ± 0.002	0.174 ± 0.005	0.0 ± 0.0	0.184 ± 0.009	4.58 ± 0.07	7.5 ± 4.5
	402	144	0.135 ± 0.001	0.184 ± 0.001	67.9 ± 6.9	0.208 ± 0.004	4.76 ± 0.07	2.1 ± 0.7

Results are mean values from triplicate experiments with their standard deviation

[a] The maximum fermentation rate was determined using time points corresponding to the steepest decline in weight

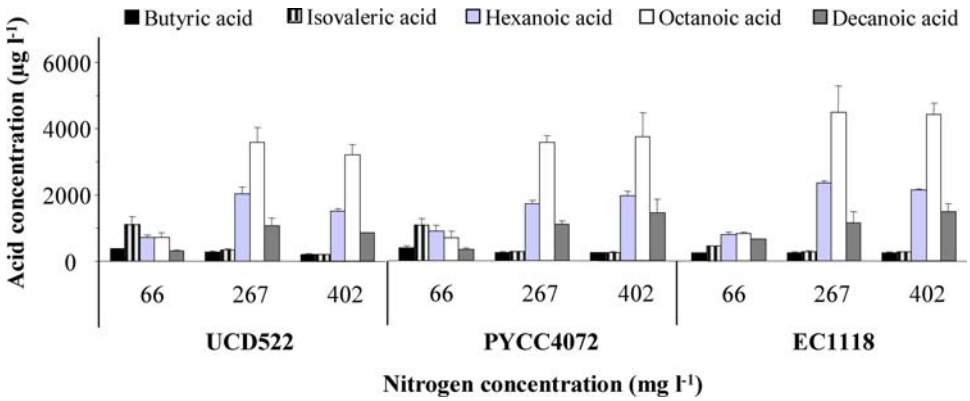

Fig. 4 Concentrations of acids produced by *Saccharomyces cerevisiae* UCD522, PYCC4072 and EC1118 grown in synthetic grape juice medium with 200 gl^{-1} of glucose and different nitrogen concentrations (66, 267, and 402 mg N l^{-1}) supplied as diammonium phosphate (DAP). Data are the mean from triplicate fermentations ± SD

EC1118 strain. In high nitrogen media (402 mg l^{-1}), hexanoic acid concentrations significantly decreased in EC1118 and UCD522 and increased in PYCC4072. In contrast, the amounts of isovaleric acid decreased with increasing nitrogen concentration, mainly in UCD522 and PYCC4072.

In respect to ethyl acetate formation, the tested strains responded differently to nitrogen variation (Table 2). The strain PYCC4072 produced less ethyl acetate at high levels of nitrogen, while the strains EC1118 and UCD522, in less extent, produced high ethyl acetate at high levels of nitrogen.

The amounts of ethyl butyrate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate were significantly ($p < 0.001$) variable within the strains and their highest concentrations were found in the media with 402 mg N l^{-1}, except for UCD522 for ethyl hexanoate and ethyl octanoate. Conversely, the amounts of diethyl succinate, ethyl isovalerate, ethyl propionate, ethyl isobutyrate, and ethyl 2-methylbutyrate were significantly found in the media with 66 mg N l^{-1}. In respect to the alcohols, there were significant differences in their final concentrations due to nitrogen ($p < 0.001$) as well as to the yeast strain, except for isoamyl alcohol as shown in Table 2. The strains PYCC4072 and in particular UCD522 were found to produce more 2-phenylethanol and linalool than EC1118. All strains produced high levels of isoamyl alcohol and 2-phenylethanol in low nitrogen media. Increasing nitrogen in the media resulted in a decrease in fusel alcohols produced by the three wine strains. A similar trend was observed for 2-phenylethyl acetate levels in all strains, while isoamyl acetate increased at high nitrogen concentrations. All strains were found to produce more linalool and 1-octanol in high nitrogen media. Nevertheless, the amounts of linalool produced by EC1118 almost doubled from low to high nitrogen concentration, while in the other two strains small (PYCC4072) or no variation was found (UCD522).

Oenological parameters

At the end of all fermentations, other parameters that could have an impact on wine character, such as alcohol production, volatile acidity, total sulphite (SO_2) and residual sugar

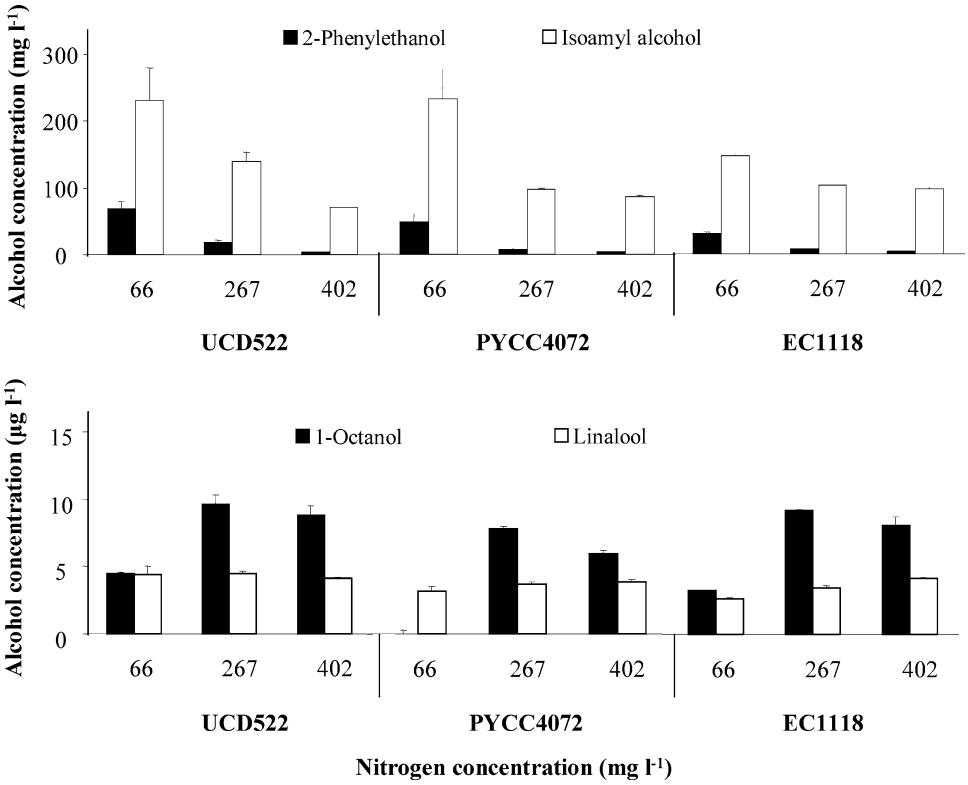

Fig. 5 Concentrations of alcohols produced by *Saccharomyces cerevisiae* UCD522, PYCC4072 and EC1118 grown in synthetic grape juice medium with 200 gl^{-1} of glucose and different nitrogen concentrations (66, 267, and 402 mg N l^{-1}) supplied as diammonium phosphate (DAP). Data are the mean from triplicate fermentations ± SD

were determined and the results are presented in Table 3. For all strains tested, the acetic acid content of final media, represented by volatile acidity, was lower in media with 66 mg l^{-1} of initial nitrogen. There were no major differences in the final ethanol concentration though being somewhat higher under 402 mg N l^{-1} in the strain EC1118.

The pH value fell from its initial value of 3.7 during fermentation in all cases with an inverse correlation with initial nitrogen content of the media. There was a rapid decrease in pH in fermentation course during the two initial days, but after this time, remained almost constant (results not shown). The yeast strains UCD522 and PYCC4072 produced more sulphite than EC1118, except under low nitrogen fermentation.

Discussion

In the present study, the influence of nitrogen levels on growth and fermentation kinetics as well as on the production of important yeast-derived wine aroma compounds was assessed. The initial nitrogen concentration affected fermentation length, maximum fermentation rate and final biomass which are consistent with previous findings [10, 12, 19, 39]. From the analysis of the yeast performance challenged by nitrogen deficiency, it can be highlighted that all three yeast strains tested were able to consume glucose for nearly a month in a nitrogen depleted media, suggesting that cells are able to sustain basal glucose uptake capacity during long periods of nitrogen deprivation. Using a transposon mutagenesis approach, Gardner et al. [11] have identified two genes *NGR1* and *GID7*, whose disruption improved sugar catabolism throughout nitrogen limited fermentations. The authors suggested that the absence of these genes could prevent deactivation of Hxt7p enhancing the ability to maintain its catabolic activity under conditions of nitrogen starvation. This result could explain why high numbers of nitrogen starved cells from sluggish cultures are able to reduce the time it takes to complete a nitrogen problematic fermentation, as observed by Varela et al. [39].

The association between a shortage of assimilable nitrogen and H_2S production during wine fermentations was first reported by Vos and Gray [41]. In this report, a strain-dependent variation in the H_2S formation in response to nitrogen availability was found, in agreement with previous findings [10, 12, 16, 31]. The wine strain EC1118 seems to be a poor-nitrogen responder in a range of 66–402 mg N l^{-1}, as no remarkable H_2S was detected under all experimental conditions. This strain has shown the ability to produce higher amounts of H_2S in response to nitrogen status during alcoholic fermentation using synthetic grape juice media [5, 43]. The discrepancy between H_2S liberation observed in this work compared with earlier studies could have various explanations. The nitrogen sources differed between the studies: a mixture of amino acids in the earlier studies as opposed to ammonium in the present

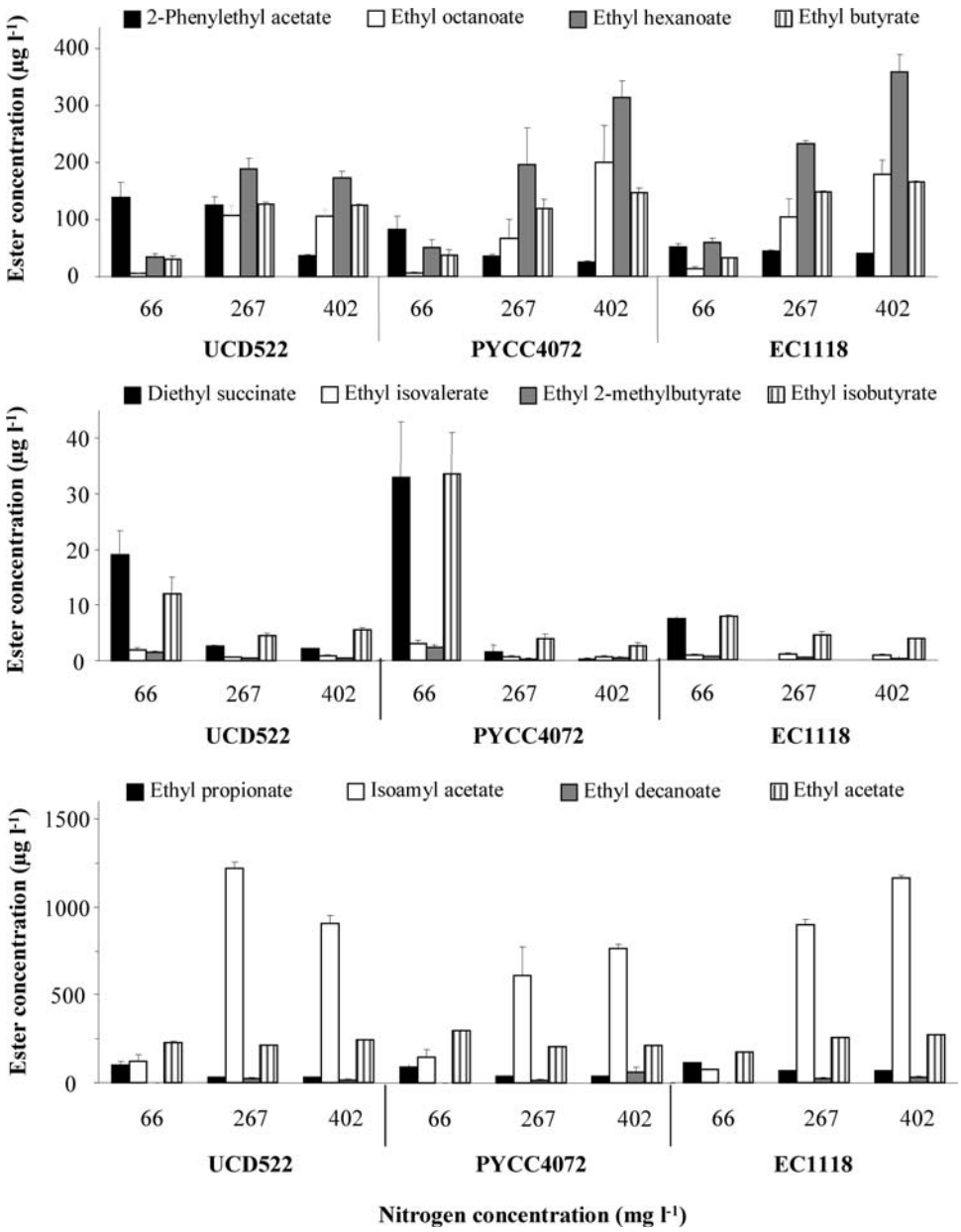

Fig. 6 Concentrations of esters produced by *Saccharomyces cerevisiae* UCD522, PYCC4072 and EC1118 grown in synthetic grape juice medium with 200 gl^{-1} of glucose and different nitrogen concentrations (66, 267, and 402 mg N l^{-1}) supplied as diammonium phosphate (DAP). Concentrations of ethyl acetate are divided by 100. Data are the mean from triplicate fermentations ± SD

work. Additionally, the former studies were performed under vitamin deficiency conditions [5, 43]. The amounts of pantothenic acid (10, 50 and 250 µg l^{-1}) [43] and biotin (0, 1 and 10 µg l^{-1}) [5] used in those studies could have stimulated H_2S liberation. These effects were overlooked in the current study as the initial levels of these vitamins (125 µg l^{-1} of biotin and 1,000 µg l^{-1} of pantothenic acid) in all media were largely in excess [12]. Taken together ours and theirs results, it seems that for wine strain EC1118, nitrogen source and vitamin deficiency could have a greater impact on H_2S liberation than nitrogen content.

For yeast strains UCD522 and PYCC4072, nitrogen depletion triggered H_2S liberation in the media with initial 66 and 267 mg N l^{-1}, although to different extents. Surprisingly, it was in the media with 66 mg N l^{-1}, that lower sulphide liberation was observed, being restricted to the first 48 h of fermentation. As far as we know, this is the first time that such behaviour has been pinpointed. It should be underlined that the inferior amounts of sulphide detected in the low nitrogen fermentations (66 mg N l^{-1}) could not be due to the reduced fermentation rate observed as in our experiments, anaerobic conditions were established in the first hours of fermentation and every 24 h, the CO_2 was forced to be released into the sulphide trap by vigorous agitation. This finding could be accounted for by the fact that sulphide production constitute a very energy-demanding process and under nitrogen starvation conditions, yeast cells must slow down some metabolic pathways to enable

Table 2 *F* values and significant differences for yeast-derived volatile compounds obtained by a two-way ANOVA

Compound	Source of variation			
	Nitrogen level (N)	Replication (R)	Yeast (Y)	N × Y
Ethyl acetate	0.78	0.12	0.16	11.01***
Ethyl propionate	139.99***	0.75	30.29***	2.78
Ethyl isobutyrate	57.47***	0.17	16.58***	22.54***
Ethyl butyrate	528.42***	0.34	14.79***	6.99**
Ethyl 2-methylbutyrate	61.71***	0.47	8.88**	11.87***
Ethyl isovalerate	31.85***	0.22	4.53*	10.00***
Isoamyl acetate	418.11***	0.02	37.88***	29.28***
Isoamyl alcohol	47.35***	0.70	3.33	4.67*
Ethyl hexanoate	136.37***	0.51	16.40***	7.51**
Ethyl octanoate	55.69***	1.60	1.43	4.07*
Linalool	12.94***	0.59	30.82***	7.64**
1-octanol	248.54***	0.10	29.17***	12.42***
Butyric acid	19.09***	0.34	0.07	4.58*
Ethyl decanoate	21.25***	1.58	3.29	5.49**
Isovaleric acid	72.97***	0.48	4.53*	8.45***
Diethyl succinate	61.95***	0.48	10.38**	8.84***
2-Phenylethyl acetate	37.21***	0.24	45.98***	11.88***
Hexanoic acid	238.20***	0.66	50.75***	14.03***
2-phenylethanol	144.49***	0.47	16.12***	7.84**
Octanoic acid	156.41***	1.35	19.28***	3.40*
Decanoic acid	33.50***	1.67	10.15**	2.06
df	2	2	2	4

Lack of a superscript indicates no significant difference; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Table 3 General oenological parameters of wines obtained from the fermentation of chemically defined grape juice media at 20°C, with different initial nitrogen levels by yeast strains UCD522, PYCC4072 and EC1118

Yeast strain	Initial nitrogen (mg l^{-1})	Final ethanol (% v/v)	Volatile acidity (g l^{-1})	Total SO_2 (mg l^{-1})	pH
UCD522	66	11.6 ± 0.1	0.35 ± 0.03	3.6 ± 0.9	3.61 ± 0.02
	267	11.7 ± 0.1	0.54 ± 0.03	15.7 ± 0.6	3.25 ± 0.02
	402	11.6 ± 0.0	0.63 ± 0.11	14.2 ± 0.6	3.07 ± 0.01
PYCC4072	66	10.7 ± 0.2	0.41 ± 0.05	3.4 ± 0.8	3.41 ± 0.02
	267	11.6 ± 0.1	0.71 ± 0.06	16.5 ± 1.3	3.27 ± 0.06
	402	11.5 ± 0.0	0.74 ± 0.02	14.4 ± 1.0	3.15 ± 0.01
EC1118	66	10.3 ± 0.3	0.23 ± 0.02	6.0 ± 0.1	3.45 ± 0.01
	267	11.6 ± 0.1	0.49 ± 0.06	6.9 ± 1.5	3.35 ± 0.01
	402	11.9 ± 0.0	0.56 ± 0.02	6.9 ± 0.3	3.20 ± 0.02

Results are mean values from triplicate experiments with their standard deviation

cell survival. These unexpected results are in line with those previously obtained for the strain PYCC4072 in which genes involved in the SRS, and thus in H_2S formation, are among the list of genes that are specifically down-regulated (unpublished results) under conditions of nitrogen deficiency (66 mg l^{-1}) [20], where in this study was found a lower level of H_2S liberated.

For both wine strains, UCD522 and PYCC4072, an inverse relationship between the amounts of initial nitrogen present in culture media and total H_2S production was seen with the higher levels of nitrogen (267 and 402 mg l^{-1}), where cells displayed higher fermentative activity. However, in this study under conditions of nitrogen excess (402 mg l^{-1}), H_2S liberation was almost undetectable in the strain PYCC4072, whereas UCD522 still produced considerable amounts of this off-flavour. This finding as already been seen by Gardner et al. [10] with the low nitrogen-requiring strain AWRI 835, which produced H_2S even

when nitrogen remained in the medium. These observations suggest a complex regulation of yeast sulphur and nitrogen metabolism highly dependent on the yeast strain genetic background and could be either due to differences in activity level of enzymes involved in incorporating sulphide into amino acids [31] or to differences in the ability to retain a greater amount of H_2S within the yeast cells [28].

Although, in the last 20 years, extensive research have been made on the effect of grape juice nitrogen status on H_2S production [10, 12, 16, 31], only recently, studies have focus on the impact of nitrogen on the formation of aroma compounds by yeast. In this study, a variety of volatile aroma compounds were found at the end of fermentations, confirming the contribution of yeast to the diversity of wine "bouquet". Moreover, this study provides good evidence that nitrogen concentration in grape juice strongly influences volatile compounds formed by the yeasts eliciting sensorial differences in the final product. Our results combined with those obtained in other laboratories conducted in natural grape juice [14, 38] or in model solutions [6, 40] show that, aside quantitative variation for each aroma compound found within different studies, most of them displayed the same trend in response to nitrogen level at the beginning of fermentation. That is at increasing nitrogen levels, a set of aroma compounds are elevated, including ethyl butyrate, isoamyl acetate, as well as the medium chain fatty acids, hexanoic, octanoic and decanoic, and their respective ethyl esters—ethyl hexanoate, ethyl octanoate, and ethyl decanoate. On the contrary, 2-phenylethanol, isoamyl alcohol, ethyl 2-methylbutyrate, and isovaleric acid are more produced under lower nitrogen concentrations, irrespective of the nitrogen source, yeast strain, or the fermentations conditions (static batch fermentation or mild agitation) used. A few compounds such as ethyl acetate, linalool and 2-phenyl-acetate seem to be more dependent on yeast strain. In fact, the levels of linalool reported in this study for UCD522 are of a similar order of magnitude of those obtained by Carrau et al. [6] for the same strain grown under different vinification conditions. Up to now, the role of yeasts in terpene formation in wines has been considered irrelevant or even ignored, since the increase of terpenoid compounds found in the must during fermentation has only been attributed to β-glucosidase activity, which releases free monoterpenes from glycosidically bound precursors [9, 13, 27]. Recently, Herrero et al. [15] were able to achieve the production of free linalool at a concentration above its aroma threshold (4–10 ppb) by engineering the pathway for monoterpene production in wine yeasts. However, the authors underline the fact that the commercial application of genetically modified industrial microorganisms is problematic due to public concern. Thus modulation of linalool formation by nitrogen concentration could be of sensory interest for winemakers.

In conclusion, the results obtained within the present work demonstrated that a complex interaction between initial nitrogen concentration and yeast strain exist in respect to H_2S and other aroma compounds formation. From the findings here reported, the wine strains respond to nitrogen limitation/starvation conditions by producing (1) less sulphide, (2) less sulphite, (3) less acetic acid, (4) less medium chain fatty acids, and (5) more isoamyl alcohol, ethyl-2-methyl-butyrate and 2-phenylethanol. These results are particularly relevant, considering that in winemaking is simultaneously desirable to achieve high levels of compounds that impart pleasant fruity and floral aromas, low levels of off-flavours and low levels of toxic compounds that could compromise yeast fermentative activity. It is worthwhile pointing out that while this is an interesting new observation, it might be difficult to achieve under industrial conditions and might have the disadvantage of leading to sluggish or stuck fermentation.

The results in this study also indicate that nutrient management in growth media could be an interesting option for producing wines with specific characters and styles. Further studies are underway in our laboratory to identify and understand how nitrogen levels affect the expression of genes involved on aroma compounds formation.

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