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Evaluation of different nitrogen sources on growth and fermentation performance for enhancing ethanol production by wine yeasts

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ABSTRACT

The utilization of grape juice from low oenological value grape varieties for bioethanol production represent an alternative for diversification and value addition in viticulture. Optimizing Very High Gravity (VHG) fermentation can significantly increase ethanol productivity while reducing water and energy consumption. In this study, the impact of different nitrogen sources on growth and fermentative performance of locally selected yeast strains was investigated. Five yeast strains of species *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii* were cultured in both synthetic culture media and natural grape juice supplemented with ammonium sulfate (NH), yeast extract (YE), Fermaid K (FERM), and urea (U) at varying concentrations. Due to the very low fermentation rate, the *Z. rouxii* strain was excluded from the selection. The results obtained in synthetic medium showed that nitrogen sources that promoted growth (NH and YE) had minimal effects on fermentative performance and were highly dependent on the specific yeast strain. However, the combination of urea and ammonium favored the rate of sugar consumption. When validated in natural grape juice, urea combined with ammonium (U + NH 300 + 75 mg/L) improved both growth parameters and ethanol yield. Doubling the concentration (U + NH 600 + 150 mg/L) further enhanced sugar consumption and ethanol production while reducing unwanted by-products. The combined use of urea and ammonium exhibited a synergistic effect, making it a cost-effective nitrogen supplement for VHG fermentations.

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1. Introduction

Winemaking production in Argentina is primarily concentrated in the Cuyo Region, with Mendoza and San Juan being the main provinces for grape and wine production. This activity plays a vital role in the regional economy, with approximately 193,000 ha of vineyards, accounting for 91 % of the country's total vineyard area [1]. The viticulture region exhibits a blend of technological and commercial innovations alongside traditional wineries, catering to both domestic and international markets [2].

The wine production industry in Argentina faces cyclic challenges due to market saturation caused by a high volume of grapes with low oenological value. These grapes, primarily represented by the "Criollas" grape varieties, account for 50 % of the total grape production in Mendoza and San Juan provinces. They are characterized by a high yield per hectare (up to 45,000 kg/ha), resulting in an annual production of 1,000,000 tons of grapes [2]. The imbalance between grape supply and demand has historically hindered wine production. Oversupply crises negatively affect all actors in the production chain, leading to reduced profitability [2,3]. To address this issue, redirecting low-value grapes towards bioethanol production could help alleviate the surplus. Additionally, this strategy focuses on productive diversification rather than energy production. Grapes, due to their high sugar content (200–240 g/L), are highly suitable for the production of ethanol at high concentrations. Ethanol derived from plant sources is currently in high demand and offers economic rewards.

Ethanol production relies on a fermentation process in which sucrose (glucose and fructose) is converted into ethanol by yeast, primarily belonging to the *Saccharomyces cerevisiae* species [4]. Research has demonstrated that yeast strains locally isolated may be well-suited for the industrial environment, displaying favorable fermentation yields and productivity [5,6].

Very High Gravity (VHG) fermentation is a strategy that significantly increases both fermentation productivity and ethanol concentration while minimizing water and energy consumption [7,8]. VHG fermentation involves the preparation and complete fermentation of mashes containing 27 g or more dissolved solids per 100 g mash [8,9]. The production of a high final ethanol concentration in VHG fermentation imposes ethanol stress on yeast, highlighting the importance of selecting well-adapted yeast strains that exhibit both high ethanol yields and tolerance to ethanol. Consequently, the selection and characterization of autochthonous yeasts that are specifically adapted to the fermentation processes becomes indispensable in achieving an optimal and profitable product.

Nutrient availability is essential for yeast growth and fermentation. The composition of the medium strongly influences the physiological state and fermentation performance of yeast in bioethanol production [10]. Nitrogen, as a critical component, plays a vital role in supporting yeast growth and fermentation [11,12]. While peptone and yeast extract are often used as nitrogen sources in laboratory-scale culture media, they are cost-prohibitive for industrial-scale production [13]. Furthermore, the nitrogen requirements vary among yeast species and strains, and the optimal growth conditions may differ from those for optimal fermentative performance [14]. To make bioethanol production economically viable, the process must be optimized in terms of both yield and ethanol production rate [12,15–17].

The objective of this study was to assess the impact of different nitrogen supplementations in synthetic medium and natural grape juice on the growth and fermentation performance of five different yeast strains, with the aim of increasing ethanol production.

2. Materials and methods

2.1. Yeasts

Five yeasts were evaluated in the study: 3 wine locally selected strains of *S. cerevisiae* (M, G, and C strains), 1 commercial strain of *Saccharomyces cerevisiae* Lalvin EC1118 from Lallemand Inc (E), and 1 isolate of *Zygosaccharomcyes rouxii* MC10 (Z). Autochthonous yeast strains were previously isolated from spontaneous fermentation of grapes from Luján de Cuyo and Maipú vineyards located Mendoza province and selected due to their high ethanol tolerance (data not shown). The yeast isolates were molecularly identified as *S. cerevisiae* by sequencing of the D1/D2 domain of the 26S ribosomal gene [18] and deposited in the Microorganism Collection of the INTA Mendoza (CoMIM-Mendoza, Argentina). The commercial *S. cerevisiae* strain EC1118 (E) was included as a reference since it is widely used to ferment high sugar grape juices. Finally, *Z. rouxii* yeast (Z) is a locally isolate from spoiled concentrated grape juice, identified (GenBank Access number KJ909203) and characterized as an osmophilic yeast with high ethanol tolerance.

2.2. Nitrogen sources

The nitrogen (N) sources were chosen among the most commonly used and the lowest cost in the market. Four N-sources were evaluated: YE: yeast extract (~15 % N) (Oxoid Inc. England), NH: ammonium phosphate dibasic (18 % N) (Biopack Co.), FERM: Fermaid K (~18 % N) (Lallemand Inc.), and U: urea (46 % N) (Certfied ACS, Profertil Co.)

2.3. Experimental design in synthetic medium

The effect of the different N-sources on yeast growth and fermentative parameters was carried out in Yeast Carbon Base (YCB) medium modified to reach a sugar concentration of 300 g/L (150 g/L glucose, 150 g/L fructose, 1 g/L potassium phosphate, and 0.5 g/L magnesium sulfate).

The experimental design included the evaluation of 3 N-sources (YE, NH, and FERM) in 4 different concentrations (N-levels: 150,

300, 450, and 600 mg/L). An additional condition was also included, combining urea (U 600 mg/L) with low concentration of diammonium phosphate (NH 150 mg/L) (U + NH 600 + 150). Treatments were independently evaluated in triplicates for the 5 yeasts previously mentioned (3 N-sources x 4 N-levels + 1 Urea test x 5 yeast x triplicate = 195 experimental units). The assay was simultaneously carried out in two-laboratory scale: i) 300 μL microtitter plates to assess growth parameters by optical density (OD_{600}) measurement, and ii) 15 mL falcon tubes to assess to fermentative parameters by measuring residual sugar an ethanol determination by HPLC as described below.

Yeasts growth was monitored by measuring the OD_{600} changes using an automatic spectrophotometer model Bioscreen C (Labsystem, Finland). For inoculum preparation, the 5 yeasts were grown overnight on 5 mL YPD broth medium (20 g/L glucose, 10 g/L bacteriological peptone and 10 g/L yeast extract) at 25 °C and 150 rpm. Triplicate wells were filled with 290 μL of each treatment medium and 10 μL of each yeast culture to reach an initial OD_{600} of 0.1 (approximately 10^6 CFU/mL). The microplates were incubated at 28 °C for 15 days. OD_{600} measurements were performed after a pre-shaking of 8 s every 2 h.

2.4. Validation in natural grape juice

The N-treatment (N-source and N-level) selected for each yeast as previously described, were validated in natural grape juice. The concentrated grape juice was diluted to achieve a sugar concentration of 390 g/L, which corresponds to 36.6 °Brix and a potential ethanol production of 20 % v/v. Additionally, this juice was supplemented with 1 g/L potassium phosphate and 0.5 g/L magnesium sulfate. The N-treatments selected for each yeast were: 300 and 450 mg/L YE for the strains C and G, 450 and 600 mg/L YE for the strain M, and 450 mg/L YE for the reference strain E. Additionally, urea (U) supplementation both alone or in combination with diammonium phosphate (NH) was included for all the yeast strains. Urea treatments were: U (300 mg/L), high U + NH (600 + 150 mg/L), and low U + NH (300 + 75 mg/L). Assays were conducted in 300 mL medium at 28 °C for 40 days. Fermentations were monitored by weight loss daily measurements. The fermented juices were centrifuged, and the clear supernatant was analyzed for ethanol and residual sugar determinations as described below.

2.5. Growth and fermentative parameters

Yeast growth parameters were calculated from each growth curve directly by fitting OD_{600} measurements vs time to the re-parameterized Gompertz primary model equation (1) developed by Zwietering et al. [19]:

$$y = A \times \exp\{-\exp[(((\mu\_max \times e))/A(\lambda - t))+1]\} \quad \text{[equation 1]}$$

where $y = \ln(OD_t/OD_0)$, being OD_0 the initial OD and OD_t is the OD at time t, A is the maximum asymptotic, equivalent to $\ln(OD_{max}/OD_0)$, μ_{max} is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h). These parameters were obtained by a nonlinear regression procedure, minimizing the sum of squares of the difference between the experimental data and the fitted model, i.e., loss function (observed–predicted), using the nonlinear module of the Statistica 7.1 software package (StatSoft Inc, USA) and its Quasi-Newton option. Fit adequacy was checked by the proportion of total variance explained by the model (R^2) with respect to experimental data.

Also, ethanol and residual sugars concentrations were determined at the end of assays at 15 mL-scale synthetic medium and in natural grape juice. Residual sugars (sucrose, glucose, fructose) and ethanol were determined by HPLC according to the protocols described by Rodríguez-Gómez et al. [20]. For the analysis of residual sugars in each liquid, 0.9 mL of liquid and 1.5 mL of internal standard (sorbitol at 0,5 % w/v) were put into contact with 1 g IRA 120 (H + -form, 16–45 mesh, Fluka) and 1 g IRA 96 (free base, 20–50 mesh, Fluka) resins. After 1 h of contact, 1.0 mL was centrifuged. A 20 μL aliquot of the clarified liquid was injected into the chromatograph. Using a Rezex RCM- Monosaccharide Ca+ (8 %) column (300 × 7.8 mm i.d., Phenomenex) held at 85 °C. Deionized water was used as eluent at 0.6mLmin-1. Quantification of sugars was made by using commercials standard. For ethanol, Samples (0.5 mL) were diluted (1:1) with deionized water, centrifuged at 11,600 g. A 20 μL aliquot of supernatant was injected into the chromatograph. Using a Spherisorb ODS-2 (5 mm, 250 × 4.6 mm, Waters Inc.) column with deionized water (pH adjusted to 2.3 with phosphoric acid) as mobile phase at 1.2 mL/min flow rate. The quantification was performed by comparing the areas obtained with the standard area of ethanol. Both determinations were carried out in a chromatographic system composed by a chromatograph Water 2690 Alliance (which includes a pump, heater and auto-sampler modules). The detections were achieved using a Water 410 Differential Refractometer Fermentative parameters: Yield (Yp/s: ethanol produced/sugar consumed) and percentage of sugar consumed (% SC) were calculated from each treatment.

Additionally, acetic acid (g/L) and glycerol (g/L) was determined by infrared spectrophotometric analysis with the Alpha-FT-IR WineAnalyzer (Bruker Co).

2.6. Statistical analyses

Statistical differences between growth and fermentative parameters from different treatments were assessed by Fisher LSD *Post-Hoc* comparison test using the multivariate ANOVA (MANOVA) and Principal Component Analysis were carried out using InfoStat Statistical Software [21].

3. Results

3.1. Effects of nitrogen sources on growth and fermentation parameters in synthetic medium

Three growth parameters (λ: lag phase, A: maximum biomass, and μ_{max}: growth rate) and two fermentation parameters (Yp/s: Ethanol yield and %SC: percentage of sugar consumed) were calculated in synthetic medium supplemented with different nitrogen sources and levels fermented by 5 yeasts. The fitted growth curves showed the absence of lag phase (0 h), indicating that all yeasts were well-adapted for growing at these sugar concentrations (300 g/L). Therefore, lag phase was not included in the subsequent statistical analysis. The fit of the growth curves was good, with an R^2 ranging from 0.97 to 1.

The overall impact of nitrogen sources (N-sources) on growth and fermentation parameters was initially evaluated, regardless of the yeast strains and levels. Statistical MANOVA analysis revealed that the different N-sources affected growth parameters, with NH addition resulting in higher biomass (A) and growth rate (μ_{max}). Additionally, YE addition favored growth rate (Table 1). In terms of fermentative parameters (Yp/s and %SC), non-statistical differences among the N-sources, except for the U + NH treatment were observed. The combination of urea and a low concentration of diammonium phosphate (U + NH) led to a higher rate of sugar consumption, as indicated by the %SC parameter (Table 1).

Secondly, the impact of different N-sources on each yeast strain was analyzed. Fig. 1 shows the differences in growth parameters based on the N-sources evaluated, being NH addition which produced a higher biomass (A) and growth rate (μ_{max}) in most yeast strains (Fig. 1 A and B). Notably, yeast strains C and G exhibited significantly higher biomass (A) with NH supplementation compared to other N-sources (Table S1). No statistical differences were observed in ethanol yield (Yp/s) among yeast strains with different N-source supplementation (Fig. 1C–Table S1). However, the addition of urea led to a higher rate of sugar consumption (%SC) in yeast strains M and G (Fig. 1D–Table S1). *Zygosaccharomyces rouxii* (Z) displayed the lowest values for both fermentative parameters across all N-sources, and thus, it was excluded from subsequent analyses.

To identify the optimal nitrogen conditions (N-source and level) to improve growth and fermentative parameters in each yeast strain, Principal Component Analysis (PCA) was performed to visualize the relationship between N-treatments and fermentation parameters (Fig. 2) exhibited similar patterns across N-treatments for all strains, with their coordinate vectors localized in the same quadrant (Fig. 2). Conversely, the two fermentative parameters (Yp/s and %SC) were differentially influenced by N-treatments depending on the strain. For example, for yeast strain M, sugar consumption (%SC) and ethanol yield (Yp/s) exhibited associated vectors, indicating that the same N-treatments had a similar effect on both variables. In contrast, for yeast strain C, the fermentative parameters were positioned in opposite quadrants, indicating that N-treatments had distinct effects (positive or negative) on each fermentative parameter (Fig. 2). Generally, NH treatments were associated with growth parameters across all strains, while other N-treatments were linked to fermentative parameters depending on the strain and level assessed (Fig. 2). Furthermore, the urea treatment (U + NH) led to rapid sugar consumption in three out of four yeast strains evaluated (Fig. 2).

Statistical analysis was conducted to determine the best N-treatments for each yeast strain (Table 2). For yeast strain C, the highest ethanol yield (Yp/s) was achieved with YE, FERM, and NH, with no statistical difference between the different concentrations/levels (Table 2). Addition of urea (U + NH) resulted in the highest %SC, which was used as an indirect measurement of fermentative rate, along with intermediate concentrations of YE and NH (300 and 450), and the maximum concentration of FERM (Table 2). Moreover, the highest growth rate (μ_{max}) was observed with YE450 and YE600, as well as U + NH, for yeast strain C (Table 2).

For yeast strain M, there were no significant differences in ethanol yield (Yp/s) among the N-sources at different levels. However, significant differences were observed in the %SC parameter, with YE600 and U + NH displaying the highest values (Table 2). Both growth parameters (A and μ_{max}) were favored YE300, YE450, FERM600, and all levels of NH (except NH450), which formed a statistically homogeneous group for this yeast strain (Table 2).

In general, for yeast strain G, no statistical differences were found in ethanol yield (Yp/s) among all N-treatments. However, U + NH and NH450 resulted in the highest rate of sugar consumption (%SC) (Table 2). NH-treatments led to the highest biomass (A) and growth rate (μ_{max}) for this strain. Similarly, YE300, YE450, FERM450, and FERM600 treatments produced the highest growth rate values (μmax) in yeast strain G (Table 2).

In yeast strain E, several N-treatments favored the fermentative parameters (Yp/s and %SC), including NH300, NH450, the three major levels of YE (300, 450, and 600), intermediate levels of FERM (300 and 450), and the Urea treatment (U + NH) (Table 2). On the

Table 1
Global effect of nitrogen source on growth (A: maximum biomass and μ_{max}: growth rate) and fermentative parameters (Yp/s: Ethanol yield and %SC: percentage of sugar consumed) in synthetic medium regardless of the yeast strains and nitrogen levels.

N-source	Growth parameters		Fermentative parameters	
	A±SD	μ_{max}±SD	Yp/s±SD	%SC±SD
NH	2.61 ± 0.37a	0.13 ± 0.02a	0.30 ± 0.06a	94.38 ± 4.17ab
YE	2.28 ± 0.24b	0.12 ± 0.02ab	0.33 ± 0.07a	92.79 ± 3.62b
FERM	2.19 ± 0.24b	0.11 ± 0.02c	0.32 ± 0.07a	91.15 ± 2.74b
U + NH	2.28 ± 0.17b	0.11 ± 0.01bce	0.33 ± 0.06a	99.54 ± 0.19a

Values expressed are mean and standard deviation from triplicate experiments. Different letters mean significant statistical difference ($p \leq 0.05$) in the same row according to the Fisher-LSD comparison test. NH: diammonium phosphate, YE: yeast extract, FERM: Fermaid K (Lallemand Inc.) U + NH: urea + diammonium phosphate.

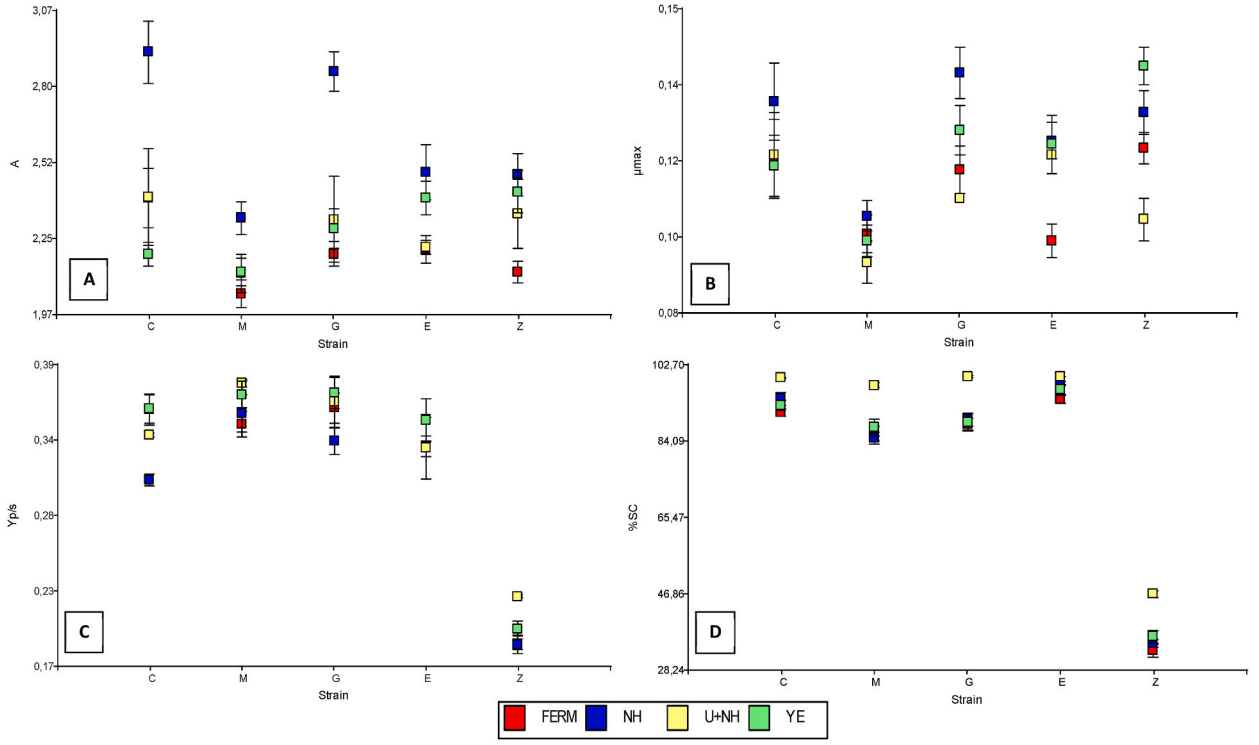

Fig. 1. Yeast strain dependent effect of nitrogen sources on growth (A: A: maximum biomass and B: μ_{max}: growth rate) and fermentative (C: Yp/s: Ethanol yield and D: %SC: percentage of sugar consumed) parameters. Vertical bars denote Standard Deviation according to Fisher LSD Test ($p \leq 0.05$).

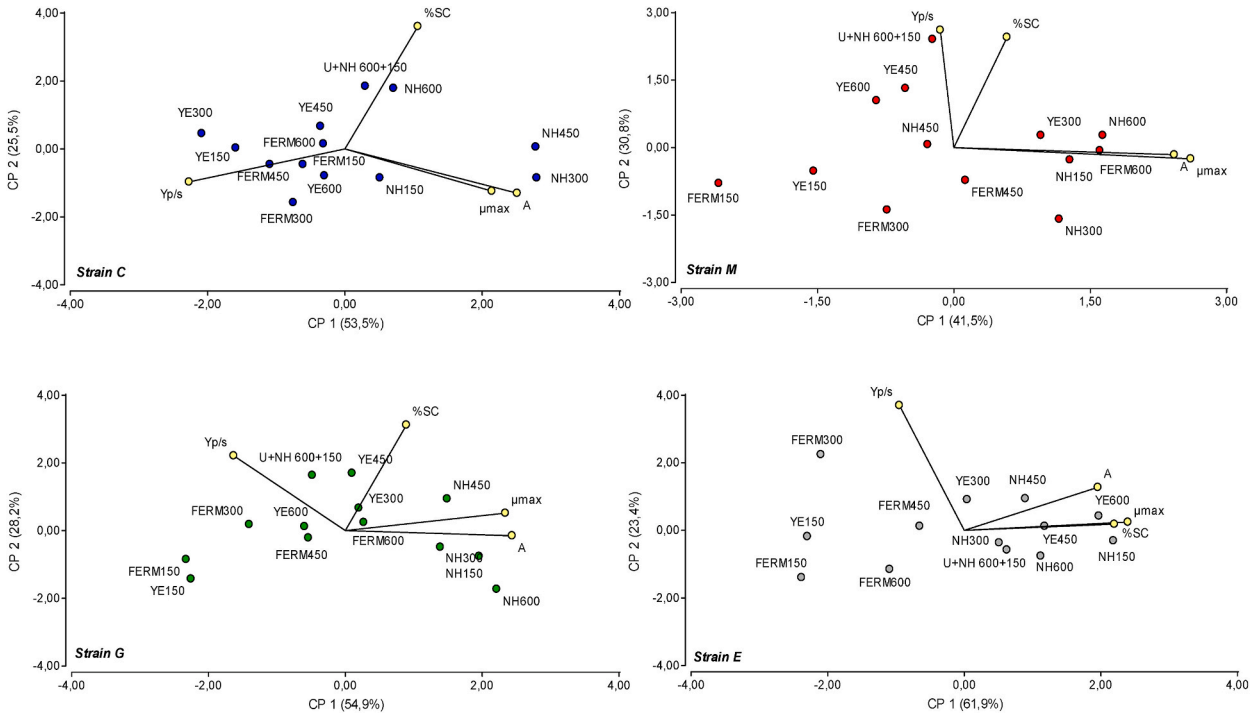

Fig. 2. Principal component analysis (PCA) of growth (A: maximum biomass and μ_{max}: growth rate) and fermentative (Yp/s: Ethanol yield and %SC: percentage of sugar consumed) parameters with different nitrogen treatments for each strain (C, M, G and E) in synthetic medium. NH: diammonium phosphate, YE: yeast extract, FERM: Fermaid K (Lallemand Inc.) U + NH: urea + diammonium phosphate.

Table 2
Growth (A: maximum biomass and μ_{max}: growth rate) and fermentative parameters (Yp/s: Ethanol yield and %SC: percentage of sugar consumed) of different nitrogen treatments in synthetic medium.

YeastStrain	Nitrogen source	Level (mg/L)	A±SD	μ_{max}±SD	Yp/s±SD	%SC±SD
C	NH	150	2.91 ± 0.32ab	0.11 ± 0.02abcd	0.32 ± 0.01cd	90.27 ± 7.08bce
		300	3.21 ± 0.21a	0.16 ± 0.01a	0.31 ± 0.01d	93.64 ± 2.36abc
		450	3.14 ± 0.26a	0.15 ± 0.05ab	0.30 ± 0.01d	95.89 ± 1.73abc
		600	2.44 ± 0.29bce	0.11 ± 0.01abcd	0.30 ± 0.02d	97.71 ± 1.10ab
	YE	150	2.18 ± 0.01c	0.09 ± 0.01d	0.34 ± 0.01abcd	90.65 ± 1.38bce
		300	2.13 ± 0.19c	0.10 ± 0.02cd	0.39 ± 0.05a	94.07 ± 2.35abc
		450	2.17 ± 0.12c	0.13 ± 0.01abcd	0.35 ± 0.02abcd	95.40 ± 1.16abc
		600	2.26 ± 0.23c	0.14 ± 0.02abc	0.35 ± 0.01abcd	91.03 ± 7.12bce
	FERM	150	2.36 ± 0.18c	0.11 ± 0.01cd	0.33 ± 0.02abcd	90.20 ± 5.69c
		300	2.57 ± 0.73bce	0.13 ± 0.06abcd	0.38 ± 0.02ab	89.38 ± 1.13c
		450	2.29 ± 0.13c	0.12 ± 0.01abcd	0.37 ± 0.04abc	91.75 ± 0.57bce
		600	2.34 ± 0.32c	0.12 ± 0.03abcd	0.34 ± 0.03abcd	93.27 ± 0.77abc
	U + NH	600 + 150	2.40 ± 0.24bce	0.12 ± 0.02abcd	0.34 ± 0.01bcd	99.54 ± 0.19a
M	NH	150	2.38 ± 0.25ab	0.11 ± 0.01ab	0.37 ± 0.05a	81.13 ± 2.20fg
		300	2.33 ± 0.16abc	0.11 ± 0.01ab	0.34 ± 0.07a	81.95 ± 0.92efg
		450	2.17 ± 0.13abcde	0.10 ± 0.01bce	0.36 ± 0.03a	86.73 ± 0.87cd
		600	2.40 ± 0.29a	0.11 ± 0.02ab	0.36 ± 0.02a	89.46 ± 3.59cd
	YE	150	2.11 ± 0.03bcde	0.09 ± 0.01bce	0.36 ± 0.04a	81.11 ± 0.24fg
		300	2.28 ± 0.21abcd	0.11 ± 0.01ab	0.37 ± 0.01a	85.32 ± 2.83def
		450	2.11 ± 0.19abcde	0.10 ± 0.01abc	0.38 ± 0.03a	88.96 ± 3.74cd
		600	1.99 ± 0.13de	0.10 ± 0.02abc	0.36 ± 0.04a	94.07 ± 3.51ab
	FERM	150	1.89 ± 0.19e	0.09 ± 0.01c	0.36 ± 0.04a	78.33 ± 0.85g
		300	2.05 ± 0.14cde	0.10 ± 0.02bce	0.33 ± 0.02a	86.22 ± 1.18cde
		450	2.05 ± 0.18cde	0.11 ± 0.01abc	0.34 ± 0.02a	88.40 ± 0.10cd
		600	2.19 ± 0.09abcd	0.12 ± 0.01a	0.35 ± 0.03a	90.73 ± 0.59bce
	U + NH	600 + 150	2.12 ± 0.10abcde	0.10 ± 0.01bce	0.38 ± 0.01a	97.71 ± 0.77a
G	NH	150	2.88 ± 0.33a	0.15 ± 0.03a	0.34 ± 0.02ab	86.18 ± 4.73cd
		300	2.76 ± 0.33ab	0.13 ± 0.03ab	0.33 ± 0.01ab	90.86 ± 4.30bce
		450	2.87 ± 0.29a	0.14 ± 0.02ab	0.36 ± 0.04ab	94.08 ± 0.91ab
		600	2.88 ± 0.10a	0.14 ± 0.01ab	0.31 ± 0.03b	86.05 ± 0.71cd
	YE	150	2.05 ± 0.18e	0.10 ± 0.01cd	0.36 ± 0.02ab	80.13 ± 2.65e
		300	2.44 ± 0.04bcd	0.13 ± 0.01abc	0.36 ± 0.06ab	92.53 ± 2.08b
		450	2.50 ± 0.03bce	0.14 ± 0.01a	0.39 ± 0.01a	93.29 ± 3.36b
		600	2.13 ± 0.27de	0.13 ± 0.02ab	0.36 ± 0.03ab	88.81 ± 5.07bce
	FERM	150	2.08 ± 0.05e	0.10 ± 0.01d	0.37 ± 0.05ab	81.99 ± 1.36de
		300	2.21 ± 0.25cde	0.11 ± 0.02bcd	0.37 ± 0.01ab	88.37 ± 0.77bce
		450	2.23 ± 0.13cde	0.12 ± 0.01abcd	0.35 ± 0.05ab	89.06 ± 0.48bce
		600	2.25 ± 0.17cde	0.13 ± 0.01ab	0.34 ± 0.01ab	93.33 ± 1.09b
	U + NH	600 + 150	2.32 ± 0.22cde	0.11 ± 0.01bcd	0.36 ± 0.03ab	99.84 ± 0.23a
E	NH	150	2.79 ± 0.07a	0.13 ± 0.01abc	0.31 ± 0.01b	97.61 ± 1.53abc
		300	2.37 ± 0.35bce	0.12 ± 0.02bce	0.33 ± 0.02ab	97.49 ± 1.99abc
		450	2.46 ± 0.46abc	0.13 ± 0.02abc	0.36 ± 0.01ab	97.89 ± 1.54abc
		600	2.31 ± 0.36bce	0.13 ± 0.02ab	0.32 ± 0.01b	99.06 ± 0.59ab
	YE	150	2.16 ± 0.10bce	0.09 ± 0.01ef	0.35 ± 0.04ab	92.62 ± 3.80c
		300	2.49 ± 0.11abc	0.12 ± 0.01bce	0.36 ± 0.04ab	95.16 ± 5.20abc
		450	2.39 ± 0.05bce	0.13 ± 0.01abc	0.34 ± 0.02ab	99.14 ± 0.06ab
		600	2.54 ± 0.31ab	0.14 ± 0.02a	0.34 ± 0.09ab	99.43 ± 0.24ab
	FERM	150	2.10 ± 0.17c	0.08 ± 0.01f	0.32 ± 0.01b	93.22 ± 1.93bce
		300	2.28 ± 0.14bce	0.09 ± 0.01def	0.41 ± 0.04a	94.63 ± 4.58abc
		450	2.27 ± 0.12bce	0.11 ± 0.01cde	0.35 ± 0.02ab	95.76 ± 5.42abc
		600	2.18 ± 0.26bce	0.11 ± 0.01bcd	0.32 ± 0.03b	93.64 ± 2.14abc
	U + NH	600 + 150	2.22 ± 0.04bce	0.12 ± 0.01abc	0.33 ± 0.03ab	99.81 ± 0.14a

Values expressed are mean and standard deviation from triplicate experiments. Different letters mean significant statistical difference ($p \leq 0.05$) in the same row according to the Fisher-LSD comparison test. NH: diammonium phosphate, YE: yeast extract, FERM: Fermaid K (Lallemand Inc.) U + NH: urea + diammonium phosphate. N-treatment selected are grey shaded.

other hand, the growth parameters (A and μ_{max}) were favored by NH150, NH450, and YE600 for yeast strain E (Table 2).

Consistent with the findings in Fig. 2 and Table 2, the statistical analysis revealed that nitrogen sources that favored the growth parameters were not always the same that improved the fermentative parameters. This relationship depended on the yeast strain and the levels of each N-source evaluated. Therefore, N-treatments that statistically favored/improved fermentation parameters without a negative impact on growth parameters were selected for further testing in natural grape juice. In this regard, the YE-treatments met the criteria, as they improved the fermentation parameters in all yeast strains without adverse effects on the growth parameters. Specifically, YE300 and YE450 were selected for yeast strains C and G, YE450 and YE600 were chosen for yeast strain M, and only YE450 was selected for the commercial yeast strain E (Table 2, shaded in grey). Additionally, urea (U + NH) supplementation also showed improvement in the fermentative parameters of all yeast strains. Therefore, this N-treatment was included in the subsequent trial,

where different urea levels, added alone or in combination with ammonium sulfate, were evaluated.

3.2. Validation of N-treatments on growth and fermentation parameters in natural grape juice

Selected N-treatments for each yeast strain were evaluated in natural grape juice to assess their impact on growth and fermentation parameters. Furthermore, ethanol, acetic acid and glycerol concentration was determined. Acetic acid and glycerol were considered as unfavorable by-products due to their divergence from the primary fermentative metabolism aimed at ethanol production.

The influence of N-treatments on the production of undesirable fermentation by-products, such as glycerol and acetic acid, is clearly depicted in Fig. 3 (PCA plot). YE-treatments, along with U300 (urea), were associated with higher levels of these unwanted compounds (Fig. 3).

N-treatments that promoted both growth parameters (A and μ_{max}) and the fermentative parameter ethanol yield (Yp/s) were grouped in the upper-right quadrant of the PCA plot. For the native yeast strains G, C, and M, the U + NH 300 + 75 treatment showed improvements in these parameters (Fig. 3). Conversely, in the lower-right quadrant of the plot, the N-treatments that favored a higher percentage of sugar consumption (%SC) and ethanol concentration were clustered. In this case, all yeast strains benefitted from the U + NH 600 + 150 treatment (Fig. 3). It is important to note that a higher ethanol concentration was achieved at the expense of lower ethanol yield. However, in a high-sugar substrate, rapid and complete sugar consumption with a high ethanol concentration can be advantageous, despite the lower yield observed in this nitrogen condition.

Statistical analysis of the results further supported PCA plot observations (Fig. 3 and Table 3). The U + NH 600 + 150 treatment showed the best statistically significant values for the three autochthonous yeast strains. This treatment led to rapid sugar consumption, higher ethanol concentration, and lower concentrations of undesirable by-products like acetic acid and glycerol (Table 3). Conversely, the same nitrogen sources at lower concentrations (U + NH 300 + 75) had a different effect, favoring growth and yield parameters but resulting in slower sugar consumption and higher concentrations of unwanted by-products (Table 3). The commercial yeast strain E displayed a different response compared to the autochthonous yeast strains, with the YE450 treatment yielding the best values for both growth and fermentation parameters. Additionally, the addition of urea alone (U300) also improved the growth parameters and ethanol yield for this yeast strain (Table 3).

These results highlight that nitrogen treatments produce effects on the growth and fermentation performance of native yeast strains that are strain-specific. In addition, these effects are dependent on the nitrogen source and concentration.

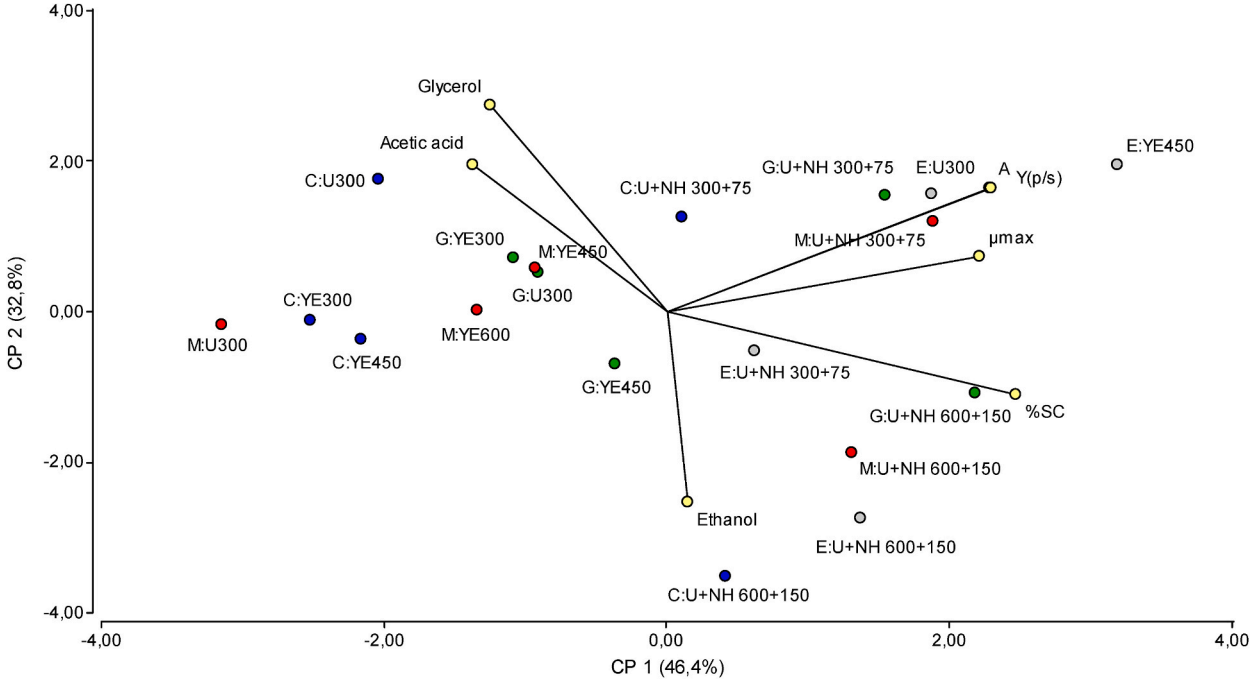

Fig. 3. Principal component analysis (PCA) of growth (A: maximum biomass and μ_{max}: growth rate) and fermentative (Yp/s: Ethanol yield and %SC: percentage of sugar consumed) parameters and wine chemical products of different nitrogen treatments for different yeast strains (C: blue point, M: red point, G: green point and E: grey point) in natural grape juice. NH: diammonium phosphate, YE: yeast extract, FERM: Fermaid K (Lallemand Inc.) U + NH: urea + diammonium phosphate.

Table 3
Growth (A: maximum biomass and μ_{max}: growth rate) and fermentative parameters (Yp/s: Ethanol yield and %SC: percentage of sugar consumed) and wine chemical composition of different nitrogen treatments for yeast strains (C, M, G and E) in natural grape juice.

Yeast	N-source	Level (mg/L)	Growth and fermentative parameters				Wine chemical composition		
			A±SD	μ_{max}±SD	Yp/s±SD	%SC±SD	Ethanol±SD (%v/v)	Acetic acid ±SD (g/L)	Glycerol±SD (g/L)
	YE	300	10.21 ± 1.65e	0.47 ± 0.01bcde	0.26 ± 0.04e	78.56 ± 0.94e	11.45 ± 1.06abcd	1.46 ± 0.35ef	14.65 ± 0.07ef
	YE	450	11.09 ± 0.93de	0.46 ± 0.02cde	0.28 ± 0.02de	79.35 ± 0.24e	11.55 ± 0.35abcd	1.45 ± 0.01ef	13.70 ± 0.42bcde
C	U + NH	600 + 150	10.98 ± 0.34de	0.51 ± 0.00abcd	0.28 ± 0.01de	95.69 ± 0.22ab	13.40 ± 0.14a	1.09 ± 0.01ab	9.85 ± 0.35a
	U + NH	300 + 75	16.01 ± 4.74bcd	0.48 ± 0.08abcde	0.41 ± 0.12bcd	85.29 ± 3.72cd	9.70 ± 2.97cd	1.37 ± 0.13cdef	13.30 ± 0.85bcd
	U	300	12.97 ± 2.63cde	0.45 ± 0.05cde	0.33 ± 0.07cde	78.82 ± 1.45e	9.35 ± 2.19d	1.51 ± 0.06fg	15.35 ± 1.06f
	YE	450	15.27 ± 2.00bcde	0.45 ± 0.04cdef	0.39 ± 0.05bcde	82.63 ± 1.76cde	11.85 ± 0.49abcd	1.69 ± 0.09h	13.15 ± 0.92bce
	YE	600	14.27 ± 0.93bcde	0.41 ± 0.01ef	0.37 ± 0.02bcde	85.94 ± 0.45c	12.10 ± 0.28abcd	1.71 ± 0.01h	13.00 ± 0.14bd
M	U + NH	600 + 150	15.51 ± 2.16bcd	0.47 ± 0.04bcde	0.40 ± 0.06bcd	98.21 ± 0.18a	12.50 ± 1.27abc	1.30 ± 0.05cde	9.90 ± 0.01a
	U + NH	300 + 75	18.84 ± 3.20ab	0.51 ± 0.06abcd	0.48 ± 0.08ab	96.54 ± 1.74ab	11.60 ± 0.71abcd	1.68 ± 0.12h	14.00 ± 0.99bcde
	U	300	11.61 ± 0.22de	0.37 ± 0.00f	0.30 ± 0.01de	79.72 ± 2.50e	12.15 ± 0.64abcd	1.65 ± 0.08gh	14.50 ± 0.28def
	YE	300	13.94 ± 0.90bcde	0.46 ± 0.00bcde	0.36 ± 0.02bcde	81.00 ± 0.76de	10.40 ± 0.42bcd	1.38 ± 0.04def	13.85 ± 0.21bcde
	YE	450	13.87 ± 2.68bcde	0.47 ± 0.00bcde	0.36 ± 0.07bcde	84.38 ± 0.36cd	11.80 ± 0.71abcd	1.22 ± 0.08bce	12.75 ± 0.07b
G	U + NH	600 + 150	16.00 ± 4.21bcd	0.53 ± 0.04abc	0.41 ± 0.11bcd	97.90 ± 0.07a	11.05 ± 2.33abcd	1.04 ± 0.10a	10.75 ± 0.92a
	U + NH	300 + 75	18.16 ± 2.48abc	0.54 ± 0.08ab	0.47 ± 0.06abc	92.36 ± 3.95b	9.70 ± 0.99cd	1.27 ± 0.09cd	13.45 ± 0.92bcde
	U	300	13.67 ± 0.25bcde	0.47 ± 0.01bcde	0.35 ± 0.01bcde	85.62 ± 5.26c	10.95 ± 0.49abcd	1.37 ± 0.05cdef	14.65 ± 0.07ef
	YE	450	21.64 ± 1.03a	0.56 ± 0.03a	0.56 ± 0.03a	95.78 ± 0.27ab	11.25 ± 1.06abcd	1.40 ± 0.11def	13.85 ± 0.49bcde
E	U + NH	600 + 150	14.59 ± 1.57bcde	0.46 ± 0.01bcde	0.37 ± 0.04bcde	98.41 ± 0.11a	12.95 ± 0.64ab	1.00 ± 0.03a	10.25 ± 0.07a
	U + NH	300 + 75	15.65 ± 4.23bcd	0.44 ± 0.02def	0.40 ± 0.11bcd	96.50 ± 1.00ab	11.50 ± 2.69abcd	1.25 ± 0.10bcd	12.85 ± 1.34b
	U	300	18.09 ± 3.40abc	0.56 ± 0.08a	0.46 ± 0.09abc	92.87 ± 4.13b	11.55 ± 1.63abcd	1.38 ± 0.06cdef	14.25 ± 0.07cdef

Values are mean and standard deviation from triplicate experiments. Different letters mean significant statistical difference ($p \leq 0.05$) in the same row according to the Fisher-LSD post-hoc comparison test. NH: diammonium phosphate, YE: yeast extract, U: urea.

4. Discussion

The use of grape juices of low oenological value in bioethanol production could be a strategy for adding value and productive diversification to the industry. This approach entails enriching the grape musts with concentrated grape juice to enhance the final ethanol yield. Concentrated grape juice is commonly regarded as a low-value by-product and is mainly exported as a commodity, thus potentially representing a profitable additive for the industry. The very high gravity (VHG) fermentation of these juices is strategically advantageous from an industrial standpoint for obtaining bioethanol, as it reduces costs due to lower energy consumption [22]. However, these fermentations are rarely rapid and complete, as they require molecular responses and physiological changes in microbial cells to adapt to the initial osmotic stress and the final ethanol stress [23]. In this sense, the understanding of the impact of a given nitrogen source on growth and fermentation parameters becomes critical to promote and improve stress tolerance in fermenting yeast cells.

Nitrogen is a critical nutrient that directly affects yeast growth, fermentation performance, and the development of organoleptic qualities [24–26]. Specifically, it plays a key role in the entire fermentation process, as low levels of nitrogen in musts are the main cause of sluggish and stuck fermentation [27–29]. Although phenotypic diversity among yeast strains has been well characterized, particularly in relation to central carbon metabolism, certain aspects of the differences in their ability to efficiently metabolize nitrogen compounds remain unclear [30].

Yeast species can use a wide variety of nitrogen sources to grow, including ammonium, urea, peptone, yeast extract, free amino acids, and other nitrogen compounds [12,28,31,32]. These nitrogen sources have been classified as preferred (such as ammonium, glutamine, glutamate, and asparagine) or non-preferred (such as urea, proline, and allantoin) [12,33]. When a pool of these

nitrogen-containing compounds is present in the medium, they are sequentially consumed during growth phase. Interestingly, studies in *S. cerevisiae* have shown that this sequence is slightly dependent on the availability of other substrates and is likely the result of differential regulation of the permeases involved in the uptake of these molecules [34].

S. cerevisiae strains are genetically diverse, largely due to the development of strains specifically adapted to various fermentation processes. Adaptive pressures from various ecological niches have generated differences among these strains, particularly in terms of their nitrogen uptake capacity [35]. Previous studies have demonstrated the substantial diversity of nitrogen uptake capacities within *S. cerevisiae* species [15,34,36,37]. In our study, we compared the growth and fermentation performance of five yeast strains in synthetic medium and natural grape juice using different nitrogen compounds and levels, confirming these differential behaviors.

Specifically, we observed that when ammonium was added as the sole nitrogen source in synthetic medium, both growth parameters (A and μ_{max}) were favored, while fermentative parameters (Yp/s and %SC) were less affected. However, the addition of urea combined with ammonium increased sugar consumption rate in two of the evaluated *S. cerevisiae* strains. These findings align with those of Gutiérrez et al. [15], who evaluated the specific nitrogen demands of four commercial *S. cerevisiae* strains widely used in the wine industry. Their study analyzed different nitrogen source concentrations, including ammonium, arginine, and glutamine, and examined their impact on maximum growth rate, biomass, and fermentative activity. They demonstrated that nitrogen sources that enhanced growth did not necessarily improve fermentation performance, as the strain with the worst growth behavior exhibited the best fermentation activity [15]. Moreover, all the strains showed the worst fermentation performances with ammonium. In contrast, Varela et al. [29] studying metabolic flux balancing and biomass at different nitrogen concentrations concluded that the increase in viable cell biomass positively correlated with the increase in fermentation rates. Additionally, other studies have shown that yeast extract has positive effects on growth and fermentation by stimulating growth rate and ethanol production [38,39]. Yeast extract supplements also enhance sugar utilization, which may contribute to improve ethanol yield in supplemented substrates. The presence of important cofactors like biotin and riboflavin in yeast extract may explain the enhanced ethanol production observed with its supplementation since they are growth factors [39]. Bafrncová et al. [38] demonstrated that ethanol productivity is increased by more than 50 % when the medium is supplemented with yeast extract in very high gravity (VHG) ethanolic fermentations. Our results align with these observations, as yeast extract supplementation was associated with improved ethanol yield. However, it should be noted that yeast extract supplements can be expensive which could be a limitation for its industrial application.

In our study, urea supplementation combined with ammonium (U + NH) improved fermentative parameters in synthetic medium in all native yeast strains evaluated. Therefore, this nitrogen source was validated in the subsequent trial using natural grape juice, where we evaluated different levels of urea added individually or in combination with ammonium sulfate. Strain-dependent differences were also observed, although most of the tested yeasts exhibited the best and worst fermentation performances with the combination of urea and ammonium, and urea alone, respectively. Amino nitrogen is the primary component of the urea. The beneficial impact of an increased concentration of free amino nitrogen has been frequently documented during the optimization of VHG fermentation mediums [38]. However, in our study, it was necessary to combine low concentrations of ammonium with high concentrations of urea to achieve a high ethanol concentration. This could be attributed to the potential of ammonium to stimulate initial growth, leading to more efficient utilization of urea compared to using urea alone.

Pereira et al. [22] conducted a study to identify critical nutrients for optimizing a medium for ethanol production in VHG. They included different nitrogen sources (such as sweet corn liquor, urea, and ammonium sulfate) and other mineral salts. In agreement with our results in natural grape juice, these authors found that urea supplementation had a positive effect on fermentation rate and ethanol concentration. Consistent with these findings, our study also observed an improvement in the presence of urea supplementation, which was dependent on the level used. The addition of urea combined with ammonium (U + NH 300 + 75) resulted in improved growth parameters and ethanol yield for the three autochthonous strains. When these concentrations were doubled (U + NH 600 + 150), sugar consumption rate and ethanol concentration was also improved. Additionally, this treatment led to a decrease in the concentration of unwanted by-products such as acetic acid and glycerol, which is advantageous from an industrial perspective. The commercial strain (E) exhibited a different behavior, where growth and fermentation parameters were generally favored by the presence of yeast extract, but it showed lower ethanol concentrations (although not statistically significant) and higher acetic acid and glycerol concentrations.

Urea metabolism in *S. cerevisiae* has been well studied. This species can utilize alternative nitrogen sources like urea when nitrogen-rich sources such as glutamine, asparagine, or ammonium are unavailable in the environment. The absence of nitrogen-rich sources results in the alleviation of nitrogen repression at the transcriptional level of nitrogen-poor catabolic pathways, a phenomenon known as nitrogen catabolite repression (NCR9) [40]. Our results with urea, particularly the synergistic effect observed when it was added in combination with ammonium, suggest that the sequential use of these nitrogen sources (preferred and non-preferred) could be advantageous for ethanol production under VHG conditions. Urea has a higher nitrogen content compared to ammonium sulfate, making it more effective in terms of weight. Since urea is less expensive than ammonium sulfate on a nitrogen equivalent basis, this could bring significant cost benefits. The benefits of replacing ammonium sulfate with urea for the growth and biomass accumulation of other yeast species as *Yarrowia lipolytica* have been previously demonstrated by Ref. [41].

In our study, the use of urea as a nitrogen source combined with ammonium not only promoted yeast growth but also favored fermentative parameters and reduced the formation of unwanted by-products compared to other evaluated nitrogen sources.

The difference in the results obtained in different experimental substrates (synthetic medium and natural grape juice) with similar nitrogen treatments suggest a dependence on other components of the medium. Further research is required to better understand the mechanisms underlying this lack of correlation. Additionally, the different behaviors observed among the yeast strains confirm the intra-specific variability in the use of nitrogenous compounds within *S. cerevisiae* and emphasize the need to optimize nitrogen nutrition according to the substrate and strain to be used.

5. Conclusion

This study contributes to expand our understanding of the role of nitrogen sources on yeast growth and fermentation performance for bioethanol production. The findings presented in this study support the use of urea combined with ammonium (600 + 150 mg/L) as an effective nitrogen supplementation to improve ethanol production, as well as to reduce unwanted by-products in VHG fermentations of high-sugar grape juice using autochthonous *S. cerevisiae* strains.

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Data availability Statement

Data associated with our study were included in article, supplemental material and referenced in article and there has not been deposited into a publicly available repository.

CRediT authorship contribution statement

María Cecilia Rojo: Writing – original draft, Methodology, Investigation, Conceptualization. **Paola Mónica Talia:** Resources, Funding acquisition, Formal analysis, Conceptualization. **María Cecilia Lerena:** Writing – review & editing, Investigation, Formal analysis. **María Lorena Ponsone:** Formal analysis. **Magalí Lucía Gonzalez:** Formal analysis. **Lucía Maribel Becerra:** Investigation. **Laura Analía Mercado:** Writing – review & editing, Resources, Methodology. **Virginia Martín-Arranz:** Methodology, Investigation. **Fancisco Rodríguez-Gómez:** Methodology, Conceptualization. **Francisco Noé Arroyo-López:** Writing – review & editing, Resources, Formal analysis, Conceptualization. **Mariana Combina:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22608.

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