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Impacts of timing of nitrogen addition on Chardonnay fermentations sequentially inoculated with *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae*

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ABSTRACT

Sequential inoculation of *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* has been shown to produce wines containing less alcohol. However, the inoculation creates competition between these yeasts for scarce nutrients such as amino acids and ammonia. This study examined impacts of the timing of nitrogen addition (1, 3, or 5 days after *M. pulcherrima* inoculation) on fermentation kinetics, yeast assimilable nitrogen (YAN) consumption, and formation of by-products, including ethanol. After adjusting 28 °Brix, *M. pulcherrima* P01A016 on day 0 and *S. cerevisiae* Lalvin NBC™ on day 4 were inoculated into Chardonnay must with or without YAN supplementation. Most wines reached dryness by day 31, except for those without the supplementation. YAN addition on day 5 significantly reduced fermentation duration by 20 % while producing higher concentrations of glycerol (10.5 g/L). Wines that reached dryness with sequential inoculation had similar alcohol content to those inoculated with only *S. cerevisiae* (15.5–16.1 % versus 16.3 % *v/v*). Regardless of timing, YAN supplementation supported sequential inoculation for fermenting Chardonnay wines to dryness. Furthermore, the timing of nitrogen addition had a significant impact on fermentation duration and glycerol synthesis, but it did not significantly affect alcohol reduction. The timing of nitrogen addition may be used to shorten fermentation time and enhance glycerol production in the wine industry.

KEYWORDS: non-*Saccharomyces* yeasts, nitrogen addition, wine, ethanol, yeast metabolism

INTRODUCTION

Recent studies to reduce the amount of alcohol produced in wines during fermentation have focused on the use of non-*Saccharomyces* species. While *Saccharomyces cerevisiae* converts sugar from must into ethanol and CO_2, non-*Saccharomyces* yeasts metabolize sugar to by-products other than ethanol such as biomass, glycerol, or succinic acid. Among the species examined, *Metschnikowia pulcherrima* has been reported to not only produce wines containing less alcohol but also increase glycerol and decrease acetic acid (Comitini *et al.,* 2011; Contreras *et al.,* 2014; González-Royo *et al.,* 2015; Quirós *et al.,* 2014). In fact, researchers have observed that sequential inoculation of *M. pulcherrima* with *S. cerevisiae* reduces ethanol by 0.20 % to 3.0 % (*v/v*) depending on conditions, yeast species and strains, and medium (Aplin & Edwards, 2021; Carbon *et al.,* 2023; Contreras *et al.,* 2014; Edwards & Aplin, 2022; Garciá *et al.,* 2020; Hranilovic *et al.,* 2020; Varela, 2016). More recently, Aplin *et al.* (2019) noted that *M. pulcherrima* provided 0.9 % (*v/v*) ethanol reduction in Syrah wines having an initial sugar (glucose and fructose) concentration of 301 g/L. Carbon *et al.* (2023) also reported that using *M. pulcherrima* in a synthetic grape juice medium with 240 g/L of sugar led to a reduction of up to 3 % (*v/v*) in alcohol content while reaching dryness.

Like *S. cerevisiae*, non-*Saccharomyces* yeasts require specific nutrients for growth and metabolism, with a preference for nitrogen-containing compounds such as amino acids and ammonium. Effective nitrogen management is therefore crucial when applying sequential inoculation. Most nitrogen additions are applied practically, without considering the cell's nitrogen needs or the timing throughout wine fermentation (Beltran *et al.,* 2005). Early nitrogen supplementation may result in excessive biomass production, whereas delayed addition can potentially lead to fermentation challenges such as sluggish fermentations and/or the production of off-odors/flavors (Beltran *et al.,* 2005; Bisson, 1999; Gobert *et al.,* 2019; Varela *et al.,* 2004; Vilanova *et al.,* 2007). In addition, nitrogen starvation causes changes in the kinetics of yeast metabolism and intracellular protein content, specifically RNA, glycogen, and trehalose (Schulze *et al.,* 1996; Walker, 1998). Fermentation kinetics are also affected by the timing of nitrogen supplementation (Bely *et al.,* 1990a; Godillot *et al.,* 2022). Therefore, the objective of this study was to evaluate the impact of nitrogen addition timing during sequential inoculation of *M. pulcherrima* and a commercial strain of *S. cerevisiae* in the fermentation of Chardonnay grape must, with the aim of optimizing yeast assimilable nitrogen (YAN) management strategies for winemakers.

MATERIALS AND METHODS

1. Preparation of Starter Cultures

M. pulcherrima P01A016 was previously isolated from vineyards located at the Irrigated Research and Extension Center, Washington State University (Prosser, WA, USA) as described by Bourret *et al.* (2013). *S. cerevisiae* (Lalvin NBC™) was obtained from Lallemand Inc. (Montréal, Quebec, Canada). Both yeasts were cultured using yeast extract-peptone-glucose (YPG) broth/agar (Zott *et al.,* 2010) which contained 10 g/L yeast extract (Becton, Dickinson, and Company, Sparks, MD, USA), 10 g/L peptone and 20 g/L glucose (VWR International, Radnor, PA, USA), as well as 20 g/L agar (Acros Organics, Morris, NJ, USA).

Single colonies of *M. pulcherrima* were aseptically removed from solidified media and transferred to 10 mL YPG broth. After the incubation at 28 ˚C for 24 h, the culture was transferred to 100 mL. After reaching the end of exponential growth phase, cells were harvested by centrifugation at 2500 × g for 15 min, washed twice with 0.2 M sodium hydrogen phosphate buffer (Na_2HPO_4, pH 7), and resuspended in the buffer before inoculation into grape must. Active dried cultures of *S. cerevisiae* were prepared according to the manufacturer's instructions.

2. Vinification

Chardonnay grape juice (25.6 ˚Brix, pH 3.65, 48.4 mg N/L YAN) was obtained from Columbia Crest Winery (Paterson, WA, USA). Soluble solids (˚Brix), the populations of total yeasts and non-*Saccharomyces* yeasts, and concentrations of SO_2 and YAN were measured as described in the following section. An additional 30 mg/L total SO_2 was added as $K_2S_2O_5$ while the concentrations of glucose and fructose (1:1) were adjusted to yield 28 ˚Brix before transfer into 3 L Celstir fermentation vessels (Wheaton Science Products, Millville, NJ, USA). Duplicate fermentations were conducted at 20 °C based on a completely randomized design with four treatments; no YAN addition (Treatment A) or YAN added on day 1 (Treatment B), day 3 (Treatment C), or day 5 (Treatment D) (Table 1). Treatment A was established as a control to assess the effects of nitrogen addition. In terms of fermentation progress, day 1 corresponded to the lag phase of the alcoholic fermentation (27.7 ˚Brix), day 3 to exponential growth (26.9 ˚Brix), day 5 to stationary phase (17.2 ˚Brix), and day 8 to middle of the fermentation (9.2 ˚Brix). All experiments were conducted with two independent replicates to ensure reproducibility. According to Scott Laboratories (2022) (https://scottlab.com/fermentation-nutrition-planning), theoretically, a must with 28 °Brix should contain 260 mg N/L YAN to complete fermentation. Therefore, the must, which initially contained 48.4 mg N/L YAN, was supplemented with two separate Fermaid K™ additions, containing inorganic nitrogen (DAP), organic nitrogen (alpha amino nitrogen), essential nutrients (biotin, magnesium sulfate, folic acid, thiamine, niacin, and calcium pantothenate), and inactivated yeast (Lallemand Inc., 2025), of a calculated 105.8 mg N/L YAN to reduce the risk of sluggish or stuck fermentation. Table 1 shows the timing of additions and inoculation for *M. pulcherrima* and *S. cerevisiae*. Musts were inoculated with *M. pulcherrima* at 10^5 CFU/mL on day 0, with *S. cerevisiae* added at 10^6 CFU/mL on day 4. For the control, *S. cerevisiae* was only inoculated on day 4. For control fermentations with only *S. cerevisiae*

TABLE 1. Experimental design.

Treatment/ Days	A	B	C	D	E
0	Initial:25.6 °Brix, 48.4 mg N/L YAN Adjust 28°Brix *M. pulcherrima* inoculation	Initial:25.6 °Brix, 48.4 mg N/L YAN Adjust 28°Brix *M. pulcherrima* inoculation	Initial:25.6 °Brix, 48.4 mg N/L YAN Adjust 28°Brix *M. pulcherrima* inoculation	Initial:25.6 °Brix, 48.4 mg N/L YAN Adjust 28°Brix *M. pulcherrima* inoculation	-
1	-	105.8 mg N/L YAN addition	-	-	-
2	-	-		-	-
3			105.8 mg N/L YAN addition		-
4	*S. cerevisiae* inoculation	*S. cerevisiae* inoculation	*S. cerevisiae* inoculation	*S. cerevisiae* inoculation	Initial:25.6 °Brix, 48.4 mg N/L YAN Adjust 28°Brix *S. cerevisiae* inoculation
5	-	-	-	105.8 mg N/L YAN addition	105.8 mg N/L YAN addition
6	-	-	-	-	-
7	-	-	-	-	-
8	-	105.8 mg N/L YAN addition	105.8 mg N/L YAN addition	105.8 mg N/L YAN addition	105.8 mg N/L YAN addition

inoculated (Treatment E), YAN was added on days 1 and 4 after *S. cerevisiae* inoculation, and this fermentation was carried out concurrently with the other treatments through inoculation with *S. cerevisiae* on day 4. Treatment E served as an additional control to evaluate alcohol reduction through treatments.

3. Analytical Methods

A spiral plater (Autoplate 4000, Spiral Biotech, Bethesda, MD) was used to monitor yeast culturability using lysine agar (Oxoid, Hampshire, England) for non-*Saccharomyces* yeasts and Wallenstein Laboratory agar (WL, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for total yeast populations. Prior to colony counting, all plates were incubated for three days at 28 °C.

Fermentation progress was tracked by measuring soluble solids (°Brix) with a portable density meter (DMA35, Anton-Paar, Graz, Austria). However, °Brix does not directly indicate dryness since it is affected by ethanol and other dissolved compounds. Therefore, fermentation was considered complete when residual sugar fell below 4 g/L, which was confirmed using AimTab™ reducing sugar tablets (Germaine Laboratories Inc., TX, USA) and then HPLC. Molecular SO_2 concentrations were calculated according to Buechsenstein and Ough (1978).

Biomass was measured triplicate using the method described by Schnierda *et al.* (2014) and used to inform the effects of timing of nitrogen addition. To monitor YAN levels throughout fermentation, amino acids were quantified using the NOPA method (Dukes & Butzke, 1998), and ammonium levels were measured with an ion-selective probe (Denver Instruments, Orville, NY, USA), following Zoecklein *et al.* (1995). YAN was calculated as the sum of free amino acids and ammonium concentrations.

Residual sugars (glucose and fructose), glycerol, ethanol, and organic acids (acetic, and succinic acids) were measured using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) fitted with a refractive index detector with some modifications to Eyéghé-Bickong *et al.* (2012) in triplicate. Samples were centrifuged and filtered through 0.45 µm polyethersulfone membranes (MilliporeSigma) into crimp-top vials. An Aminex HPX-87H column (300 × 7.8 mm, BIO-RAD, Hercules, CA, USA) maintained at 60 °C was used for separation using a mobile phase of 0.005 M H_2SO_4 and a flow rate of 0.6 mL/min. Instrument controls were performed using ChemStation software (version C.01.10) based on external calibration curves ($r^2 \geq 0.99$).

4. Statistical Methods

Data analyses were carried out using Excel 365 (Microsoft Corporation, Richmond, WA, USA). Data related to yeast population, ammonium, amino acids, soluble solids, and biomass were reported as mean values accompanied by standard deviation from duplicate fermentations. In addition,

statistical analyses about biomass, glucose, fructose, glycerol, ethanol, acetic acid, and succinic acid were conducted with ANOVA with mean separations performed by Fisher's least significant difference at $p \leq 0.05$ using XLSTAT (Lumivero, Denver, CO, USA).

RESULTS

1. Changes in yeast population, YAN, soluble solids, and biomass

The Chardonnay must initially contained 48.4 mg N/L YAN and before the inoculation, the indigenous non-*Saccharomyces* and total yeast populations were found as $(1.0 \pm 0.6) \times 10^4$ CFU/mL, and $(1.4 \pm 0.6) \times 10^4$ CFU/mL, respectively (mean ± standard deviation). Changes in populations, YAN, and soluble solids in Chardonnay ferments inoculated with *M. pulcherrima* and *S. cerevisiae* but without additional nitrogen (Treatment A) are presented in Figure 1. Here, non-*Saccharomyces* populations were initially $4.0 \pm 2.8 \times 10^5$ CFU/mL on day 1 but reached $1.9 \pm 0.0 \times 10^7$ CFU/mL on day 4. However, the population of non-*Saccharomyces* yeasts decreased to $7.0 \pm 0.5 \times 10^6$ CFU/mL on day 7, whereas total yeast population mainly *S. cerevisiae* was maintained at 10^7 to 10^8 CFU/mL until day 25. The grape must initially contained 48.4 mg N/L YAN which were mostly exhausted by day 4 (Figure 1B). Finally, the fermentation did not reach dryness, even after 34 days.

When YAN was added one day after *M. pulcherrima* inoculation in the lag phase (Treatment B), the population trends of non-*Saccharomyces* and total yeast followed a similar pattern to those of Treatment A during the first four days, but YAN consumption was higher (Figure 2). Yeasts in Treatment B depleted nearly all of the YAN, including both preexisting and added sources. Despite this, not all of the ammonium and amino acid sources were consumed following YAN addition. Rather, concentrations decreased to 1.15 mg N/L. From days 4 to 8, the YAN level fluctuated between 1 to 2 mg N/L, while the non-*Saccharomyces* population decreased from 10^7 to 10^6 CFU/mL. YAN consumption was much lower after the second YAN addition. As evidence, on day 9, YAN levels measured 89.2 mg N/L, while YAN measured 79.1 mg N/L on day 19. After day 19, YAN concentration increased by 35.2 mg N/L during the final fermentation stage. Unlike Treatment A, Treatment B reached dryness by day 31 (Figure 2B).

The yeast populations and fermentation characteristics were similar for treatments B and C, but there was a slight difference in nitrogen consumption (Figure 3). While the majority of YAN was consumed after three days in Treatment B, this same level of depletion required only one day for Treatment C. As evidence, nearly all YAN that was added on day 3 in the exponential growth phase was consumed by day 4, leaving only 2.38 mg N/L of YAN (Figure 3B). Following the second addition of YAN, YAN consumption closely resembled treatment B. On day 9, YAN levels measured 89 mg N/L, as compared to 81.9 mg N/L on day 19. After day 19, YAN concentration

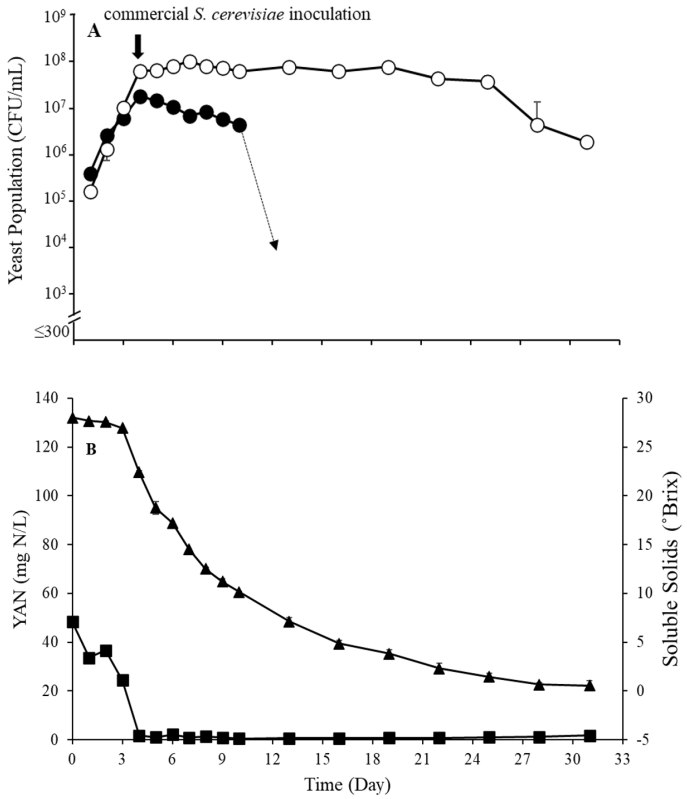


FIGURE 1. Chardonnay fermentations sequentially inoculated with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4) for Treatment A. Changes in (A) non-*Saccharomyces* (●) and total yeast (○) populations, (B) YAN (■) and soluble solids (▲) over time without YAN addition. Data are presented as mean values and error bars indicate standard deviation.

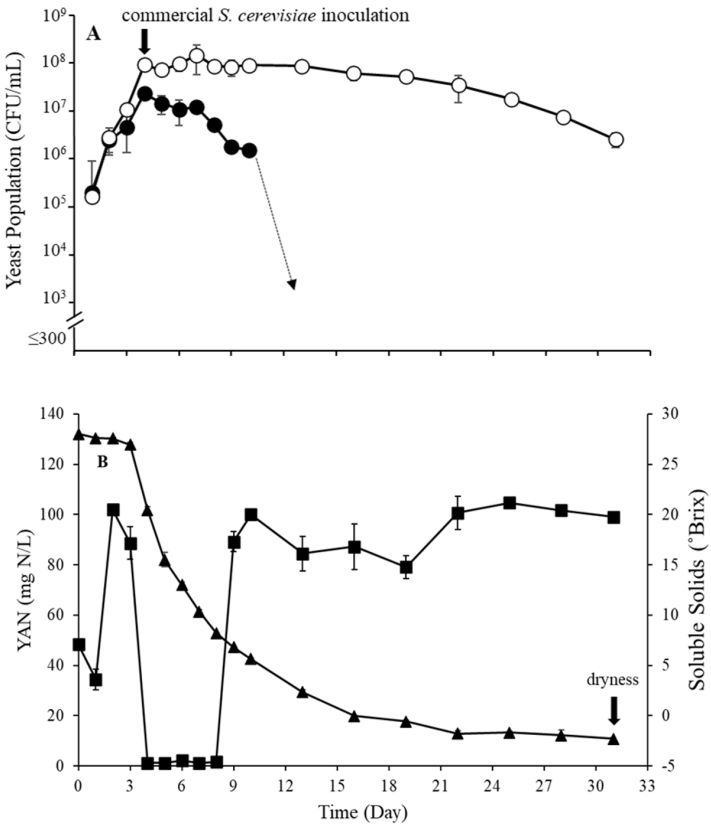


FIGURE 2. Chardonnay fermentations sequentially inoculated with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4) for Treatment B. Changes in (A) non-*Saccharomyces* (●) and total yeast (○) populations, (B) YAN (■) and soluble solids (▲) over time. YAN was added to the treatment after sampling on days 1 and 8. Data are presented as mean values and error bars indicate standard deviation.

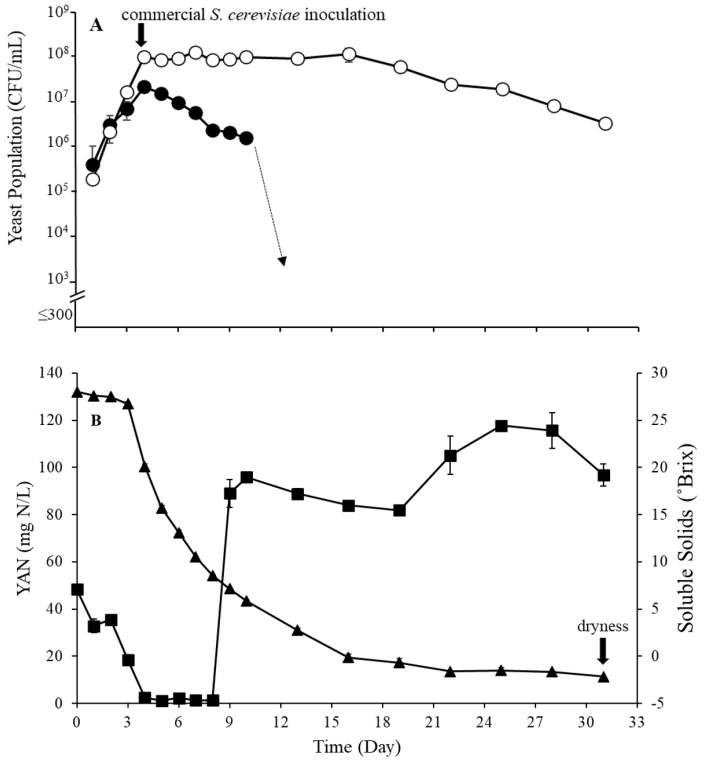


FIGURE 3. Chardonnay fermentations sequentially inoculated with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4) for Treatment C. Changes in (A) non-*Saccharomyces* (●) and total yeast (○) populations, (B) YAN (■) and soluble solids (▲) over time. YAN was added to the treatment after sampling on days 3 and 8. Data are presented as mean values and error bars indicate standard deviation.

(primarily ammonium) increased by 33.7 mg N/L towards the final stage of fermentation. Similar to Treatment B, dryness was reached in 31 days.

While the first addition of YAN on day 5 towards the stationary phase did not affect the yeast population in treatment D, it reduced fermentation time compared to treatment A (Figure 4). The non-*Saccharomyces* yeast population peaked at 10^7 CFU/mL on day 4 and then declined to 10^6 CFU/mL on day 7 (Figure 4A). Nearly all of the naturally occurring YAN was metabolized before nitrogen was added, with 1.34 mg N/L measured on day 4 (Figure 4B). Following the addition on day 5, YAN was rapidly metabolized and only 1.32 mg N/L remained on day 7. Since the YAN additions in this treatment were closer together by time, the second added YAN was consumed more slowly than other treatments. However, a greater quantity of YAN was metabolized in the time following the second addition in treatment D compared to other treatments. Following day 19, YAN concentration (predominantly ammonium) rose by 30.0 mg N/L as fermentation reached its final stage, similar to those in treatments B and C. Fermentation in treatment D reached dryness on day 25, six days earlier than treatments B and C.

As previously mentioned, before the commercial *S. cerevisiae* inoculation, the indigenous non-*Saccharomyces* and total yeast populations were counted as 1.0×10^4 CFU/mL, and 1.4×10^4 CFU/mL, respectively. After the commercial *S. cerevisiae* inoculation in Treatment E, total yeast population increased 6.5×10^6 CFU/mL 1 day later and then up to 2.8×10^8 CFU/mL within 24 hours (Figure 5A). Until reaching dryness, total yeast population was around $3.0 \pm 1.0 \times 10^8$ CFU/mL, after that point, it decreased to 10^7 CFU/mL. On the other hand, the indigenous non-*Saccharomyces* yeast population were counted as 5.0×10^4 CFU/mL 1 day later, 1.5×10^5 CFU/mL 2 days later and reached to 7.0×10^5 CFU/mL 4 days later after commercial *S. cerevisiae* inoculation and then it was not detected after that point. Yeasts consumed nearly all YAN in the must within two days. After the second YAN addition on day 9, YAN was measured at 95 mg N/L but decreased to 89 mg N/L by day 19. Despite reaching dryness on day 19, the YAN concentration increased by an additional 27 mg N/L when the final measurements were taken on day 31 (Figure 5B).

Prior to *S. cerevisiae* inoculation, treatment C produced 3.33 g/L of biomass, significantly higher than the other treatments on day 4 (data not shown). In contrast, the other treatments produced similar biomass levels, ranging from 2.50 g/L to 2.67 g/L (Figure 6).

2. Wine composition

The impact of nitrogen addition during the first 10 days of fermentation was tracked by monitoring residual sugar, alcohol, glycerol, and organic acid production. Until day 7, ethanol, glycerol production, and sugar consumption were greater in treatments B and C compared to treatments A and D (Figure 7 and 8). After day 7, ethanol and glycerol production in treatment D increased, surpassing the other treatments. For organic acids,

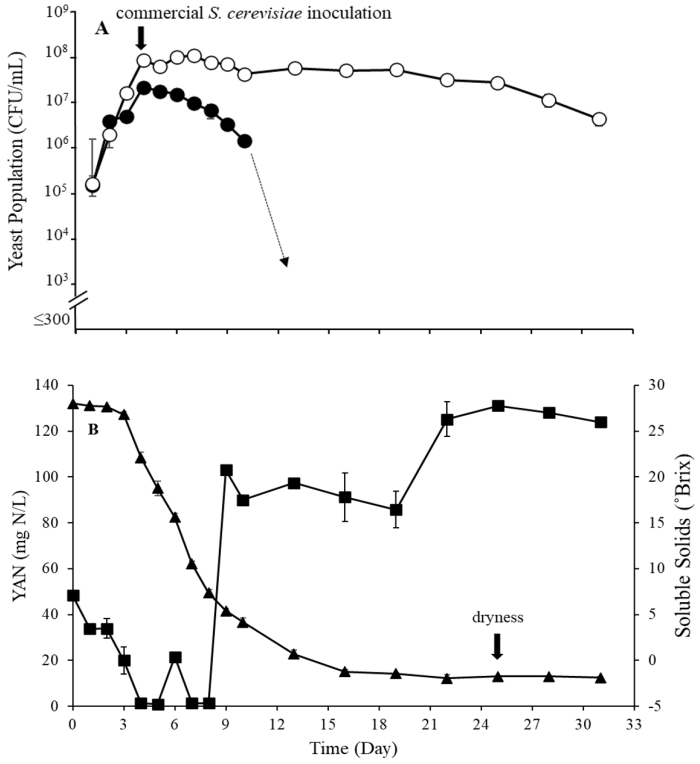


FIGURE 4. Chardonnay fermentations sequentially inoculated with *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4) for Treatment D. Changes in (A) non-*Saccharomyces* (●) and total yeast (○) populations, (B) YAN (■) and soluble solids (▲) over time. YAN was added to the treatment after sampling on days 5 and 8. Data are presented as mean values and error bars indicate standard deviation.

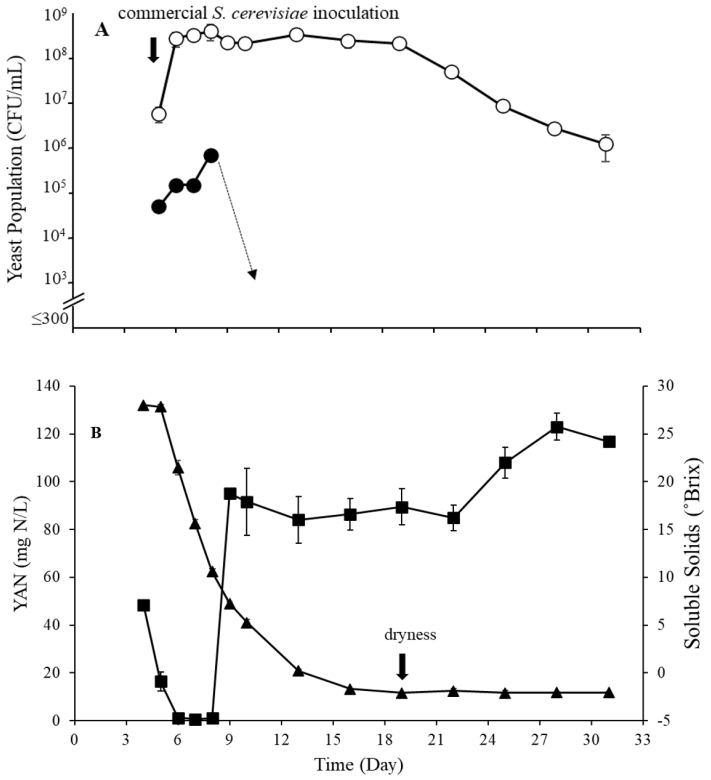


FIGURE 5. Chardonnay fermentations only inoculated with *S. cerevisiae* (day 4) for Treatment E. Changes in (A) non-*Saccharomyces* (●) and total yeast (○) populations, (B) YAN (■) and soluble solids (▲) over time. YAN was added to the treatment after sampling on days 5 and 8, which means day 1 and 4 after *S. cerevisiae* inoculation. This treatment was started on day 4 when concurrently *S. cerevisiae* inoculation in all treatments. Data are presented as mean values and error bars indicate standard deviation.

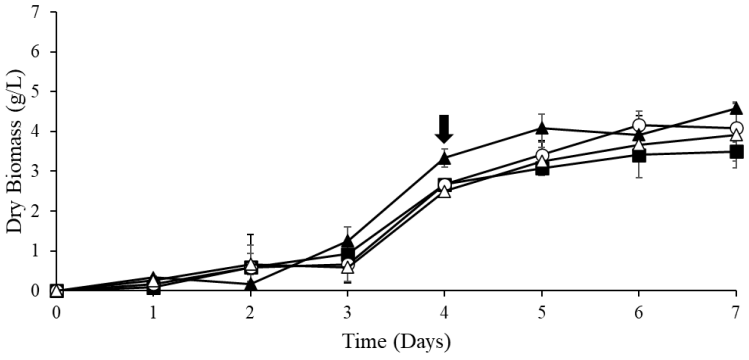


FIGURE 6. Changes in biomass during fermentation of Chardonnay grape must sequentially inoculated *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4) for treatments A (■), B (○), C (▲), or D (Δ). The time of inoculation of *S. cerevisiae* was indicated with an arrow. Data are presented as mean values and error bars indicate standard deviation.

the acetic acid level in treatment B was higher than in the other treatments until day 7. However, all treatments produced similar amounts of succinic acid over the first 10 days.

There were few differences in the chemical composition of treatments that received supplemental nitrogen (Table 2). In contrast, treatment A, without YAN addition, differed significantly from the other treatments in concentrations of ethanol, glycerol, fructose, and succinic acid. For Treatments A, B, C, and D, wines containing *M. pulcherrima* had alcohol contents of 14.2, 16.1, 15.5, and 15.9 % (*v/v*), respectively, whereas the wine inoculated with only *S. cerevisiae* as a control fermenter had 16.3 % (*v/v*) alcohol content. Treatment A produced significantly less ethanol but had a high residual fructose concentration. Furthermore, glycerol production was significantly highest in treatment D (10.5 g/L), compared to treatments A (8.65 g/L), B (9.64 g/L), C (9.78 g/L), and E (10.1 g/L). In addition, acetic acid levels in treatment D (0.786 g/L) were significantly higher than in treatment E (0.627 g/L), while treatments B (0.739 g/L) and C (0.672 g/L) showed no significant difference. Treatment A had a significantly higher succinic acid concentration (0.569 g/L) than treatments B, C, D, and E (0.277, 0.326, 0.371, and 0.379 g/L, respectively).

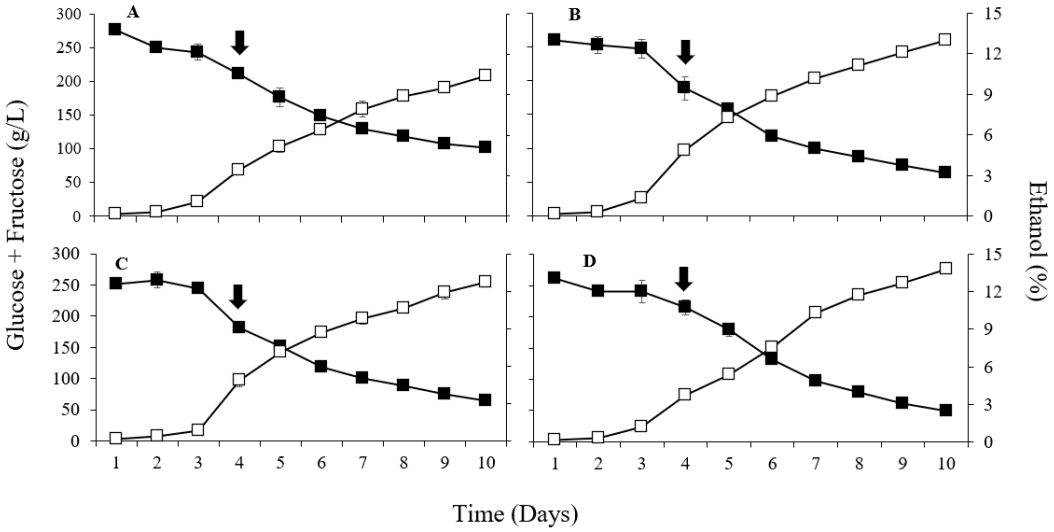


FIGURE 7. Changes in residual sugars (■) and ethanol levels (□) during Chardonnay fermentation for treatments A (A), B (B), C (C), or D (D). The time of inoculation of *S. cerevisiae* was indicated with an arrow. Data are presented as mean values and error bars indicate standard deviation.

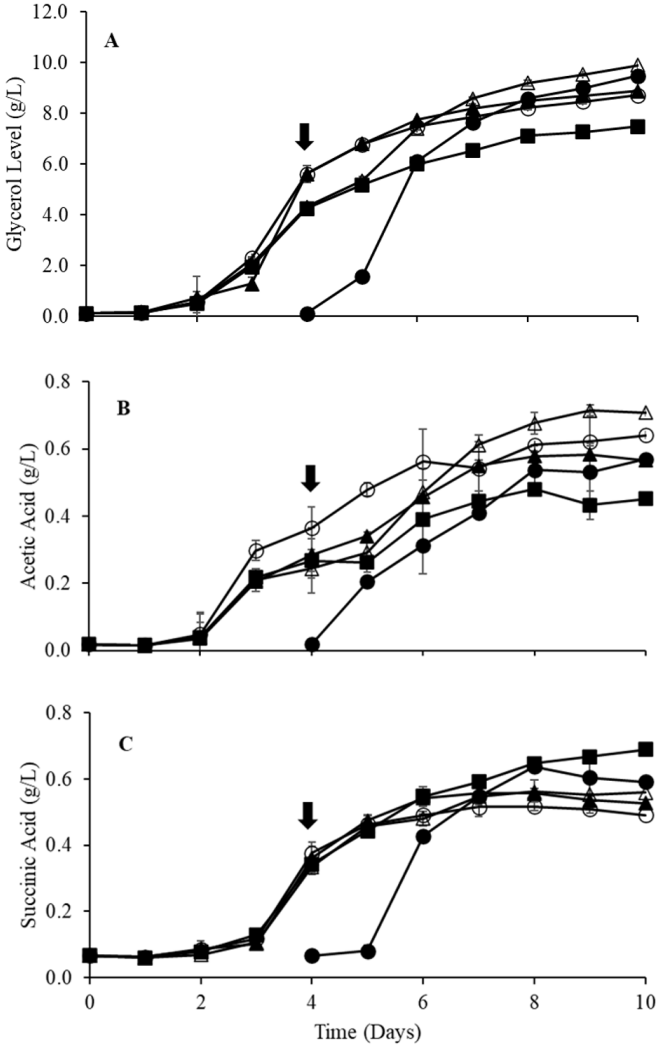


FIGURE 8. Changes in glycerol (A), acetic acid (B), succinic acid (C) level during fermentation of Chardonnay grapes over 10 days for treatments A (■), B (○), C (▲), D (Δ), or D (●). The time of inoculation of *S. cerevisiae* was indicated with an arrow. Data are presented as mean values and error bars indicate standard deviation.

TABLE 2. Concentration of ethanol, glycerol, glucose, and organic acids with different additions time of YAN at the end of the fermentation.

Treatments	Ethanol (% v/v)	Glycerol (g/L)	Glucose (g/L)	Fructose (g/L)	Acetic Acid (g/L)	Succinic Acid (g/L)
A*	$14.2^{b} \pm 0.706$	$8.65^{d} \pm 0.118$	$1.79^{a} \pm 0.172$	$22.9^{a} \pm 2.35$	$0.575^{c} \pm 0.100$	$0.569^{a} \pm 0.100$
B*	$16.1^{a} \pm 0.301$	$9.64^{c} \pm 0.144$	$1.47^{b} \pm 0.013$	$0.685^{b} \pm 0.270$	$0.739^{a,b} \pm 0.060$	$0.277^{b} \pm 0.023$
C*	$15.5^{a,b} \pm 0.034$	$9.78^{b,c} \pm 0.189$	$1.61^{a,b} \pm 0.021$	$1.26^{b} \pm 1.09$	$0.672^{a,b,c} \pm 0.011$	$0.326^{b} \pm 0.087$
D*	$15.9^{a} \pm 0.191$	$10.5^{a} \pm 0.090$	$1.68^{a,b} \pm 0.130$	$0.118^{b} \pm 0.167$	$0.786^{a} \pm 0.006$	$0.371^{b} \pm 0.050$
E**	$16.3^{a} \pm 0.916$	$10.1^{b} \pm 0.016$	$1.62^{a,b} \pm 0.038$	NF	$0.627^{b,c} \pm 0.012$	$0.379^{b} \pm 0.029$

The means within a column with different superscript letters indicate significant differences at $p \leq 0.05$. NF: not found. * *M. pulcherrima* (day 0) and *S. cerevisiae* (day 4) were inoculated on this treatment. ** This treatment contained only *S. cerevisiae*, and the fermentation started on day 4.

DISCUSSION

The timing of nitrogen addition is crucial for yeast assimilation. In this study, nitrogen was supplemented with Fermaid K™, a complex nutrient containing inorganic nitrogen (ammonium), amino acids, and other micronutrients. Treatment C consumed the supplemented nitrogen within 24 hours, aligning with the findings of Beltran *et al.* (2005) and Su *et al.* (2021). In Treatments B and D, the yeasts utilized nitrogen more slowly. By day 4, yeasts had consumed nearly all measurable ammonium in the must. At that point, amino acids were more abundant than ammonium in all treatments but remained below 2.15 mg N/L. Our results confirm that ammonium was preferentially assimilated from the available nitrogen sources, as supported by previous studies (Bell & Henschke, 2005; Brice *et al.,* 2018; Jiranek *et al.,* 1995; Torrea *et al.,* 2011). The addition of Fermaid K™ enhanced overall nitrogen assimilation, as yeasts in Treatments B, C, and D consumed both preexisting and added sources, consistent with Rollero *et al.* (2021).

Non-*Saccharomyces* yeasts assimilated nearly all measured YAN sources (ammonium and amino acids) in Treatments A, B, C, and D before *S. cerevisiae* inoculation. Gobert *et al.* (2017) showed that depending on species, these yeasts can metabolize between 66 to 215 mg N/L of YAN. This rapid YAN consumption by non-*Saccharomyces* yeasts can deplete nitrogen levels, potentially hindering *S. cerevisiae* growth and fermentation efficiency (Kemsawasd *et al.,* 2015; Medina *et al.,* 2012).

In our study, an initial YAN level of 260 mg N/L was applied to meet the moderate–high nitrogen requirements of the commercial *S. cerevisiae* strain under high-sugar conditions and to minimize the risk of sluggish or stuck fermentations. This is consistent with the guideline of Bisson and Butzke (2000), who recommend approximately 25 mg N/L per 1 °Brix increase. Although elevated YAN could theoretically support the growth of spoilage organisms such as *Brettanomyces bruxellensis*, Childs *et al.* (2015) demonstrated that at similar YAN levels (250 mg N/L) in a high-sugar medium, *B. bruxellensis* was not detected due to the inhibitory effect of high ethanol concentrations (15.6–15.9 % *v/v*). Likewise, the wines in our study reached 15.5–16.3 % (*v/v*) ethanol, which likely limited the risk of microbial instability despite the relatively high YAN.

To avoid sluggish or stuck fermentation, a second YAN addition was made on day 8 using Fermaid K™. YAN was only partially consumed, in contrast to the efficient uptake observed with earlier additions. The possible reasons of that might decrease cell viability, elevated ethanol concentrations (Varela *et al.,* 2004; Tesnière *et al.,* 2013). In addition, contrary to expectations, the measured YAN levels, particularly ammonium, increased towards the end of fermentation. This rise could be due to yeast autolysis, where nitrogen is released as cells die in the final stage of the stationary phase (Alexandre & Guilloux-Benatier, 2006). During autolysis, intracellular nitrogen is released into the surrounding environment, increasing ammonium and amino acid levels. This rise is consistent with both the findings of this study and those of previous studies (Beltran *et al.,* 2004; Martínez-Rodríguez & Polo, 2000; Martínez-Rodríguez *et al.,* 2001).

The timing of nitrogen addition can significantly influence fermentation duration. In Treatment D, where YAN was supplemented with Fermaid K™ after *S. cerevisiae* inoculation, fermentation reached dryness six days faster than in Treatments B and C, where YAN was added before *S. cerevisiae* inoculation, contrasting with Medina *et al.* (2012). Godillot *et al.* (2022) also noted that adding nitrogen during the stationary phase can accelerate fermentation kinetics. Similarly, Su *et al.* (2021) reported that yeast strains with high nitrogen needs, such as *S. cerevisiae* Lalvin NBC™ strain, completed fermentation in less time when YAN additions were made. Supplementation after *S. cerevisiae* inoculation proved most successful in enhancing both fermentation activity and nitrogen assimilation.

In this study, Chardonnay grape musts with an initial sugar content of 28 °Brix reached dryness with YAN addition. In contrast, Treatment A (no YAN addition) did not achieve dryness. This may be attributed to the inactivation of sugar transporters. Nitrogen starvation hindered protein synthesis, likely affecting sugar transport activation during fermentation (Lucero *et al.,* 2002; Mendes-Ferreira *et al.,* 2004; Salmon, 1989). The addition of nitrogen in treatments B, C, D, and E likely supported sugar transport and resulted in greater sugar metabolism.

Treatment C produced significantly more biomass than the other treatments prior to the addition of *S. cerevisiae*. Treatment B did not show a similar increase in biomass. During the exponential growth phase at the onset of alcoholic fermentation, nitrogen availability is directly related to biomass output (Martínez-Moreno *et al.,* 2012; Varela *et al.,* 2004). Treatment C supports this relationship, while Treatment B does not exhibit the same effect. In addition, as noted by Bely *et al.* (1990b) and Varela *et al.* (2004), the late addition of YAN can accelerate fermentation without impacting biomass, as seen in Treatment D.

Wines with YAN addition showed no significant reduction in alcohol content. Treatments B, C, and D reached dryness and produced 15.5 to 16.1 % (*v/v*) alcohol. The control treatment (Treatment E) containing only *S. cerevisiae* produced 16.3 % (*v/v*) alcohol which is not significantly different from the other treatments. The lack of alcohol reduction in Treatments B, C, and D may be due to the high initial soluble solids (28 °Brix) and insufficient aeration. Similarly, Hranilovic *et al.* (2017) observed no significant alcohol reduction in Shiraz wine with 29 °Brix, sequentially inoculated with *M. pulcherrima* and *S. cerevisiae*. On the other hand, Aplin *et al.* (2019) reported a 0.9 % (*v/v*) alcohol reduction in Syrah wines (301 g/L initial sugar and 253 mg N/L YAN) with sequential inoculation of *M. pulcherrima* and *S. cerevisiae*.

YAN supplementation before or after *S. cerevisiae* inoculation impacted glycerol production across treatments B, C, and D. Treatment D produced the highest level of glycerol, 10.5 g/L. This may be due to the differential expression of 17 % of yeast genes, as observed in a study on the timing of nitrogen addition and its effect on gene expression such as GPD1 and ATF2 genes, which are roles in glycerol and acetate ester synthesis, respectively (Du *et al.,* 2012; Godillot *et al.,* 2022).

Succinic acid production can influence assimilable nitrogen and amino acids although its relationship with the timing of nitrogen addition remains unclear (de Klerk, 2010; Torres-Guardado *et al.,* 2024). A previous study indicates that nitrogen availability has an adverse impact on succinic acid production, where lower YAN typically results in increased concentrations (Su *et al.,* 2021). Our study found that treatment A, which had the lowest YAN, produced the highest levels of succinic acid. In contrast, YAN addition in treatments B through E decreased succinic acid production, with no significant impact observed from the timing of supplementation. All treatments except Treatment A had succinic acid levels below 0.5 g/L. In literature, the concentration of succinic acid in wine typically falls within the range of 0.5 to 1.5 g/L depending on the yeast species, and it contributes to salty-bitter notes (Chidi *et al.,* 2015; Contreras *et al.,* 2015; de Klerk, 2010). Therefore, succinic acid was dosed to comparatively evaluate the potential effects of YAN addition and its timing on its production.

Nitrogen addition can also decrease acetic acid production depending on timing of the addition and the amount of nitrogen (Barbosa *et al.,* 2009; Su *et al.,* 2021). Barbosa *et al.* (2009) reported that nitrogen addition on day 3 decreased acetic acid production, consistent with previous studies showing an inverse relationship between nitrogen availability and acetic acid formation under low to moderate nitrogen conditions but direct effect at high nitrogen level (Barbosa *et al.,* 2009; Hernandez-Orte *et al.,* 2006). In our study, treatment C (YAN supplementation on day 3) produced similar acetic acid levels comparable to the other treatments, contrary to the aforementioned studies. The timing of YAN addition did not significantly affect acetic acid production; however, a significant difference was observed between treatments D and E, which may be attributed to the inoculation with *M. pulcherrima*. Although a significant difference was observed between treatments D and E, acetic acid concentrations in all treatments remained above the detection threshold of 0.28 g/L but below 0.9 g/L, the level at which it typically imparts sour and bitter flavours (Contreras *et al.,* 2015; Lambrechts & Pretorius, 2000; Ribereau-Gayon *et al.,* 2006).

Overall, YAN supplementation is a key factor in completing alcoholic fermentation, especially in musts with high sugar content. In this study, by day 4, YAN levels were nearly depleted in all treatments, including the control group without YAN addition (Treatment A), as well as in the groups supplemented on day 1 (Treatment B) and day 3 (Treatment C), indicating early and intense nitrogen uptake by yeast. Therefore, a second YAN supplementation is recommended after day 4 to support fermentation. Although two separate YAN additions enabled fermentation completion, the timing of these additions produced distinct effects: addition on day 3 in the exponential growth phase (treatment C) promoted biomass formation, whereas addition on day 5 in the stationary phase (treatment D) shortened fermentation duration and increased glycerol production. These findings highlight the importance of optimizing both the amount and timing of YAN addition to align with specific fermentation objectives, such as optimizing yeast growth, decreasing fermentation duration or modulating metabolite production.

CONCLUSIONS

This study evaluated the effects of the timing of YAN addition on Chardonnay fermentation sequentially inoculated with *M. pulcherrima* and *S. cerevisiae*. Treatments with YAN addition reached dryness, though fermentation durations varied significantly. After the *S. cerevisiae* inoculation (treatment D), the addition of YAN significantly reduced the duration of fermentation (20 %) and produced noticeably more glycerol (10.5 g/L). However, there were no significant differences in alcohol reduction across the YAN supplemented treatments, possibly due to osmotic stress encountered by *M. pulcherrima* at the beginning of fermentation. In summary, while YAN addition did not affect alcohol reduction, it significantly influenced fermentation duration and glycerol production. Future studies could explore how nitrogen timing affects sensory and chemical profiles of wine.

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REFERENCES

Alexandre, H., & Guilloux-Benatier, M. (2006). Yeast autolysis in sparkling wine – a review. *Australian Journal of Grape and Wine Research*, *12*(2), 119–127. https://doi.org/10.1111/j.1755-0238.2006.tb00051.x

Aplin, J. J., & Edwards, C. G. (2021). Impacts of non-*Saccharomyces* species and aeration on sequential inoculation with *Saccharomyces cerevisiae* to produce lower alcohol Merlot wines from Washington state. *Journal of the Science of Food and Agriculture*, *101*(4), 1715–1719. https://doi.org/10.1002/JSFA.10769

Aplin, J. J., White, K. P., & Edwards, C. G. (2019). Growth and metabolism of non-*Saccharomyces* yeasts isolated from Washington state vineyards in media and high sugar grape musts. *Food Microbiology*, *77*, 158–165. https://doi.org/10.1016/J.FM.2018.09.004

Barbosa, C., Falco, V., Mendes-Faia, A., & Mendes-Ferreira, A. (2009). Nitrogen addition influences formation of aroma compounds, volatile acidity and ethanol in nitrogen –deficient media fermented by *Saccharomyces cerevisiae* wine strains. *Journal of Bioscience and Bioengineering*, *108*(2), 99–104. https://doi.org/10.1016/j.jbiosc.2009.02.017

Bell, S. J., & Henschke, P. A. (2005). Implications of nitrogen nutrition for grapes, fermentation and wine. *Australian Journal of Grape and Wine Research*, *11*(3), 242–295. https://doi.org/10.1111/J.1755-0238.2005.TB00028.X

Beltran, G., Esteve-Zarzoso, B., Rozes, N., Mas, A., & Guillamon, J. M. (2005). Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *Journal of Agricultural and Food Chemistry*, *53*(4), 996–1002. https://doi.org/10.1021/jf0487001

Beltran, G., Novo, M., Rozes, N., Mas, A., & Guillamon, J. M. (2004). Nitrogen catabolite repression in *Saccharomyces cerevisiae* during wine fermentations. *FEMS Yeast Research*, *4*(6), 625–632. https://doi.org/10.1016/j.femsyr.2003.12.004

Bely, M., Sablayrolles, J. M., & Barre, P. (1990a). Description of alcoholic fermentation kinetics–its variability and significance. *American Journal of Enology and Viticulture, 41*, 319–324. https://doi.org/10.5344/ajev.1990.41.4.319

Bely, M., Sablayrolles, J. M., & Barre, P. (1990b). Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. *Journal of Fermentation and Bioengineering, 70*(4), 246–252. https://doi.org/10.1016/0922-338X(90)90057-4

Bisson, L. F. (1999). Stuck and sluggish fermentations. *American Journal of Enology and Viticulture*, *50*(1), 107–119. https://doi.org/10.5344/AJEV.1999.50.1.107

Bisson, L. F., & Butzke, C. E. (2000). Diagnosis and rectification of stuck and sluggish fermentations. *American Journal of Enology and Viticulture*, *51*, 168–177. https://doi.org/10.5344/ajev.2000.51.2.168

Bourret, T. B., Grove, G. G., Vandemark, G. J., Henick-Kling, T., & Glawe, D. A. (2013). Diversity and molecular determination of wild yeasts in a central Washington State vineyard. *North American Fungi*, *8*(15), 1–32. https://doi.org/10.2509/naf2013.008.015

Brice, C., Cubillos, F. A., Dequin, S., Camarasa, C., & Martínez, C. (2018). Adaptability of the *Saccharomyces cerevisiae* yeasts to wine fermentation conditions relies on their strong ability to consume nitrogen. *PLoS ONE*, *13*(2), e0192383. https://doi.org/10.1371/journal.pone.0192383

Buechsenstein, J. W., & Ough, C. S. (1978). SO_2 determination by aeration-oxidation: A comparison with Ripper. *American Journal of Enology and Viticulture*, *29*(3), 161–164. https://doi.org/10.5344/ajev.1978.29.3.161

Carbon, H. N., Aplin, J. J., Jiang, G. Z., Gibney, P. A., & Edwards, C. G. (2023). Fate of carbon in synthetic media fermentations containing *Metschnikowia pulcherrima* or *Meyerozyma guilliermondii* in the presence and absence of *Saccharomyces cerevisiae*. *Food Microbiology*, *114*, 104308. https://doi.org/10.1016/J.FM.2023.104308

Chidi, B. S., Rossouw, D., Buica, A. S., & Bauer F. F. (2015). Determining the impact of industrial wine yeast strains on organic acid production under white and red wine like fermentation conditions. *South African Journal of Enology & Viticulture, 36*(3), 316–327. https://doi.org/10.21548/36-3-965

Childs, B. C., Bohlscheid, J. C., & Edwards, C. G. (2015). Impact of available nitrogen and sugar concentration in musts on alcoholic fermentation and subsequent wine spoilage by *Brettanomyces bruxellensis*. *Food Microbiology*, *46*, 604–609. https://doi.org/10.1016/j.fm.2014.10.006

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., & Ciani, M. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiology*, *28*(5), 873–882. https://doi.org/10.1016/j.fm.2010.12.001

Contreras, A., Hidalgo, C., Henschke, P. A., Chambers, P. J., Curtin, C., & Varela, C. (2014). Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Applied and Environmental Microbiology*, *80*(5), 1670–1678. https://doi.org/10.1128/AEM.03780-13

Contreras, A., Hidalgo, C., Schmidt, S., Henschke, P. A., Curtin, C., & Varela, C. (2015). The application of non-*Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content. *International Journal of Food Microbiology*, *205*, 7–15. https://doi.org/10.1016/j.ijfoodmicro.2015.03.027

de Klerk, J. L. (2010). *Succinic acid production by wine yeasts* [Master's thesis, Stellenbosch University]. https://hdl.handle.net/10019.1/4228

Du, G., Zhan, J., Li, J., You, Y., Zhao, Y., & Huang, W. (2012). Effect of fermentation temperature and culture medium on glycerol and ethanol during wine fermentation. *American Journal of Enology and Viticulture*, *63*, 132–138. https://doi.org/10.5344/ajev.2011.11067

Dukes, B. C., & Butzke, C. E. (1998). Rapid determination of primary amino acids in grape juice using an o-Phthaldialdehyde/N-Acetyl-L-Cysteine spectrophotometric assay. *American Journal of Enology and Viticulture*, *49*, 125–134. https://doi.org/10.5344/ajev.1998.49.2.125

Edwards, C. G., & Aplin, J. J. (2022). Application of cool fermentation temperatures to encourage non-*Saccharomyces* yeasts to yield lower ethanol concentrations in wines. *Fermentation*, *8*(9), 421. https://doi.org/10.3390/FERMENTATION8090421

Eyéghé-Bickong, H. A., Alexandersson, E. O., Gouws, L. M., Young, P. R., & Vivier, M. A. (2012). Optimisation of an HPLC method for the simultaneous quantification of the major sugars and organic acids in grapevine berries. *Journal of Chromatography B*, *885–886*, 43–49. https://doi.org/10.1016/j.jchromb.2011.12.011

García, M., Esteve-Zarzoso, B., Cabellos, J. M., & Arroyo, T. (2020). Sequential non-*Saccharomyces* and *Saccharomyces cerevisiae* fermentations to reduce the alcohol content in wine. *Fermentation, 6*(2), 60. https://doi.org/10.3390/fermentation6020060

Gobert, A., Tourdot-Maréchal, R., Morge, C., Sparrow, C., Liu, Y., Quintanilla-Casas, B., Vichi, S., & Alexandre, H. (2017). Non-*Saccharomyces* yeasts nitrogen source preferences: Impact on sequential fermentation and wine volatile compounds profile. *Frontiers in Microbiology*, *8*, 2175. https://doi.org/10.3389/fmicb.2017.02175

Gobert, A., Tourdot-Maréchal, R., Sparrow, C., Morge, C., & Alexandre, H. (2019). Influence of nitrogen status in wine alcoholic fermentation. *Food Microbiology*, *83*, 71–85. https://doi.org/10.1016/j.fm.2019.04.008

Godillot, J., Sanchez, I., Perez, M., Picou, C., Galeote, V., Sablayrolles, J. M., Farines, V., & Mouret, J. R. (2022). The timing of nitrogen addition impacts yeast genes expression and the production of aroma compounds during wine fermentation. *Frontiers in Microbiology*, *13*, 829786. https://doi.org/10.3389/fmicb.2022.829786

González-Royo, E., Pascual, O., Kontoudakis, N., Esteruelas, M., Esteve-Zarzoso, B., Mas, A., Canals, J. M., & Zamora, F. (2015). Oenological consequences of sequential inoculation with non-*Saccharomyces* yeasts (*Torulaspora delbrueckii* or *Metschnikowia pulcherrima*) and *Saccharomyces cerevisiae* in base wine for sparkling wine production. *European Food Research and Technology*, *240*, 999–1012. https://doi.org/10.1007/s00217-014-2404-8

Hernandez-Orte, P., Bely, M., Cacho, J., & Ferreira, V. (2006). Impact of ammonium additions on volatile acidity, ethanol, and aromatic compound production by different *Saccharomyces cerevisiae* strains during fermentation in controlled synthetic media. *Australian Journal of Grape and Wine Research, 12*(2), 150–160. https://doi.org/10.1111/j.1755-0238.2006.tb00055.x

Hranilovic, A., Gambetta, J. M., Jeffery, D. W., Grbin, P. R., & Jiranek, V. (2020). Lower alcohol wines produced by *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* co- fermentations: The effect of sequential inoculation timing. *International Journal of Food Microbiology*, *329*, 108651. https://doi.org/10.1016/j.ijfoodmicro.2020.108651

Hranilovic, A., Li, S., Boss, P. K., Bindon, K., Ristic, R., Grbin, P. R., Van der Westhuizen, T., & Jiranek, V. (2017). Chemical and sensory profiling of Shiraz wines co-fermented with commercial non-*Saccharomyces* inocula. *Australian Journal of Grape and Wine Research*, *24*(2), 166–180. https://doi.org/10.1111/ajgw.12320

Jiranek, V., Langridge, P., & Henschke, P. A. (1995). Amino acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. *American Journal of Enology and Viticulture*, *46*, 75–83. https://doi.org/10.5344/ajev.1995.46.1.75

Kemsawasd, V., Viana, T., Ardö, Y., & Arneborg, N. (2015). Influence of nitrogen sources on growth and fermentation performance of different wine yeast species during alcoholic fermentation. *Applied Microbiology and Biotechnology*, *99*, 10191–10207. https://doi.org/10.1007/s00253-015-6835-3

Lambrechts, M., & Pretorius, I. S. (2000). Yeast and its importance to wine aroma - a review. *South African Journal of Enology & Viticulture*, *21*, 97–129. https://doi.org/10.21548/21-1-3560

Lallemand Inc. (2025) *Fermaid K*™. Lallemand Wine. https://www.lallemandwine.com/en/unitedstates/products/nutrients-and-protectors/fermaid-k/

Lucero, P., Moreno, E., & Lagunas, R. (2002). Catabolite inactivation of the sugar transporters in *Saccharomyces cerevisiae* is inhibited by the presence of a nitrogen source. *FEMS Yeast Research*, *1*(4), 307–314. https://doi.org/10.1111/j.1567-1364.2002.tb00049.x

Martínez-Moreno, R., Morales, P., Gonzalez, R., Mas, A., & Beltran, G. (2012). Biomass production and alcoholic fermentation performance of *Saccharomyces cerevisiae* as a function of nitrogen source. *FEMS Yeast Research*, *12*(4), 477–485. https://doi.org/10.1111/j.1567-1364.2012.00802.x

Martínez-Rodriguez, A. J., Carrascosa, A. V., & Polo, M. C. (2001). Release of nitrogen compounds to the extracellular medium by three strains of *Saccharomyces cerevisiae* during induced autolysis in a model wine system. *International Journal of Food Microbiology*, *68*(1–2), 155–160. https://doi.org/10.1016/s0168-1605(01)00486-x

Martínez-Rodríguez, A. J., & Polo, M. C. (2000). Characterization of the nitrogen compounds released during yeast autolysis in a model wine system. *Journal of Agricultural and Food Chemistry*, *48*(4), 1081–1085. https://doi.org/10.1021/jf991047a

Medina, K., Boido, E., Dellacassa, E., & Carrau, F. (2012). Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *International Journal of Food Microbiology*, *157*(2), 245–250. https://doi.org/10.1016/j.ijfoodmicro.2012.05.012

Mendes-Ferreira, A., Mendes-Faia, A., & Leao, C. (2004). Growth and fermentation patterns of *Saccharomyces cerevisiae* under different ammonium concentrations and its implications in winemaking industry. *Journal of Applied Microbiology*, *97*(3), 540–545. https://doi.org/10.1111/j.1365-2672.2004.02331.x

Quirós, M., Rojas, V., Gonzalez, R., & Morales, P. (2014). Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. *International Journal of Food Microbiology*, *181*, 85–91. https://doi.org/10.1016/j.ijfoodmicro.2014.04.024

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., & Lonvaud, A. (2006). *Handbook of Enology, Volume 1: The Microbiology of Wine and Vinifications*. John Wiley & Sons. https://doi.org/10.1002/0470010363

Rollero, S., Bloem, A., Brand, J., Ortiz-Julien, A., Camarasa, C., & Divol, B. (2021). Nitrogen metabolism in three non-conventional wine yeast species: A tool to modulate wine aroma profiles. *Food Microbiology*, *94*, 103650, 1–11. https://doi.org/10.1016/j.fm.2020.103650

Salmon, J. M. (1989). Effect of sugar transport inactivation in *Saccharomyces cerevisiae* on sluggish and stuck enological fermentations. *Applied and Environmental Microbiology*, *55*, 953–958. https://doi.org/10.1128/aem.55.4.953-958.1989

Schnierda, T., Bauer, F. F., Divol, B., van Rensburg, E., & Görgens, J. F. (2014). Optimization of carbon and nitrogen medium components for biomass production using non-*Saccharomyces* wine yeasts. *Letters in Applied Microbiology*, *58*(5), 478–485. https://doi.org/10.1111/lam.12217

Schulze, U., Lidén, G., Nielsen, J., & Villadsen, J. (1996). Physiological effects of nitrogen starvation in an anaerobic batch culture of *Saccharomyces cerevisiae*. *Microbiology*, *142*(8), 2299–2310. https://doi.org/10.1099/13500872-142-8-2299

Scott Laboratories. (2022). *Fermentation Nutrition Planner*. https://scottlab.com/fermentation-nutritionplanning

Su, Y., Heras, J. M., Gamero, A., Querol, A., & Guillamón, J. M. (2021). Impact of nitrogen addition on wine fermentation by *S. cerevisiae* strains with different nitrogen requirements. *Journal of Agricultural and Food Chemistry*, *69*(21), 6022–6031. https://doi.org/10.1021/acs.jafc.1c01266

Tesnière, C., Delobel, P., Pradal, M., & Blondin, B. (2013). Impact of nutrient imbalance on wine alcoholic fermentations: Nitrogen excess enhances yeast cell death in lipid- limited must. *PLoS ONE*, *8*(4), e61645. https://doi.org/10.1371/journal.pone.0061645

Torrea, D., Varela, C., Ugliano, M., Ancin-Azpilicueta, C., Leigh Francis, I., & Henschke, P. A. (2011). Comparison of inorganic and organic nitrogen supplementation of grape juice - Effect on volatile composition and aroma profile of a Chardonnay wine fermented with *Saccharomyces cerevisiae* yeast. *Food Chemistry*, *127*(3), 1072–1083. https://doi.org/10.1016/j.foodchem.2011.01.092

Torres-Guardado, R., Rozès, N., Esteve-Zarzoso, B., Reguant, C., & Bordons, A. (2024). Succinic acid production by wine yeasts and the influence of GABA and glutamic acid. *International Microbiology*, *27*, 505–512. https://doi.org/10.1007/s10123-023-00410-9

Varela, C. (2016). The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. *Applied Microbiology and Biotechnology*, *100*, 9861–9874. https://doi.org/10.1007/s00253-016-7941-6

Varela, C., Pizarro, F., & Agosin, E. (2004). Biomass content governs fermentation rate in nitrogen-deficient wine musts. *Applied and Environmental Microbiology*, *70*, 3392– 3400. https://doi.org/10.1128/AEM.70.6.3392-3400.2004

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I. S., & Henschke, P. A. (2007). Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. *Applied Microbiology and Biotechnology*, *77*, 145–157. https://doi.org/10.1007/s00253-007-1145-z

Walker, G. M. (1998). Yeast nutrients. In *Yeast physiology and biotechnology* (pp. 51–101). John Wiley & Sons.

Zoecklein, B. W., Fugelsang, K. C., Gump, B. H., & Nury, F. S. (1995). *Wine analysis and production* (1st ed.). Springer. https://doi.org/10.1007/978-1-4757-6978-4

Zott, K., Claisse, O., Lucas, P., Coulon, J., Lonvaud-Funel, A., & Masneuf-Pomarede, I. (2010). Characterization of the yeast ecosystem in grape must and wine using real-time PCR. *Food Microbiology*, *27*(5), 559–567. https://doi.org/10.1016/j.fm.2010.01.006