INDEPENDENT VALIDATION OF DIFFERING NITROGEN REQUIREMENTS AMONG WINE YEAST STRAINS

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ABSTRACT

Nitrogen is an essential nutrient for all life. For the budding yeast *Saccharomyces cerevisiae*, nitrogen is required for amino acid, nucleotide, and biomass synthesis, which in turn influence metabolic processes in the cell. Winemakers consistently cite nitrogen limitation as a leading cause of both "stuck or sluggish" fermentations – in which yeast do not reach "dryness," meaning they fail to convert glucose and fructose to ethanol – and the accumulation of undesirable flavor and aroma compounds such as hydrogen sulfide (H_2S). Commercial yeast suppliers include nitrogen requirements among a longer list of characteristics which winemakers may use to ensure a chosen yeast is supplied with appropriate nutrition. While crucial details about nitrogen metabolism in both laboratory and winemaking yeast strains are known – including the genes responsible for the sensing and signaling of environmental nitrogen sources, and the cell's response to nitrogen limitation under various conditions of stress – open questions about strain-specific requirements for nitrogen remain. Studies that show variation in nitrogen utilization do so using a steady-state growth apparatus to measure nitrogen consumed during stationary phase, once cells have reached maximum density and are no longer forming biomass. Other studies suggest that nitrogen availability during the exponential growth phase constitutes the most important period for wine yeast. Furthermore, the combination of stressors that are typical of wine fermentations – high levels of ethanol, low pH, and low nutrient availability – make it difficult to isolate the impact of a single condition. This work aims to independently validate existing knowledge about yeast strain-specific nitrogen utilization by comparing the fermentation kinetics, cell density, and sugar and nitrogen metabolism of 6 commercial wine strains under typical winemaking conditions.

BIOGRAPHICAL SKETCH

Ian Wayne completed his Bachelor of Arts degree from Hamilton College in 2012 with a focus in American Studies. After years working in restaurants in New York City as a waiter, bartender and wine steward, he returned to study in order to deepen his understanding of wine chemistry and microbiology.

ACKNOWLEDGMENTS

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INTRODUCTION

Uses within the cell

Nitrogen is an essential nutrient for all life. The budding yeast *Saccharomyces cerevisiae* uses nitrogen for amino acid biosynthesis. Nitrogen is used to make purines and pyrimidines, the bases for both DNA and RNA nucleotides which encode sequences of amino acids and the resulting vast array of proteins that are required for cellular functionality. Proteins constitute 15-30% of the cell wall, which contribute to roughly a third of total *S. cerevisiae* biomass (Cooper 1982).

Metabolism

While environmental amino acids may be utilized directly, they are more commonly deanimated to form ammonia or catabolized and used as substrates for enzymes (transanimases) that transfer amino acid groups to **α**-ketoglutaric acid and form glutamate (Magasanik and Kaiser 2002). Cells grown on glucose – the dominant sugar in grape juice – use ammonia in separate pathways to anabolize glutamate and glutamine (Wiame et al. 1985), which account for 85% and 15% of amino acid biosynthesis respectively (Cooper 1982). The internal pool of synthesized amino acids within the cell has been shown to vary as a function of growth rate and nitrogen source (Watson 1976), as well as sugar source and concentration, nitrogen limitation, and phosphate limitation (Kumar et al. 2021). These data shed light on the central role of nitrogen metabolism in yeasts' ability to adapt to changing environments.

Starvation

When yeast sense limitations of natural nutrients like nitrogen, sulfate and phosphate, cells adjust transcription, metabolism, and growth rate to match their

environment. Broadly, cells limited for natural nutrients will induce a similar pattern of gene expression that correlates with the level of stress and, importantly for winemaking, starving cells halt glucose consumption (Saldanha et al. 2004). Growth rate, cell cycle, metabolic activity and stress response are coordinated for cells limited for nitrogen, among other key nutrients. Growth rate slows as nitrogen limitation becomes more severe, and cells completely starved for nitrogen will arrest their cell cycle as unbudded cells (Brauer et al. 2008). Metabolite production seems to correlate with nutrient limitation; for instance, nitrogen limitation resulted in low intracellular amino acids in one study (Boer et al. 2010). Autophagy allows cells to continuously recycle internal amino acids to maintain homeostasis (Onodera and Ohsumi 2005), which contributes to differing rates of quiescence observed for cells starved of key nutrients (Klosinska et al. 2011). Furthermore, stress responses in yeast have been observed to differ among strains, suggesting that many of the parameters induced by nutrient limitation – including growth rate and autophagy – will vary depending on the provenance or phenotype of a given strain (Kvitek et al. 2008).

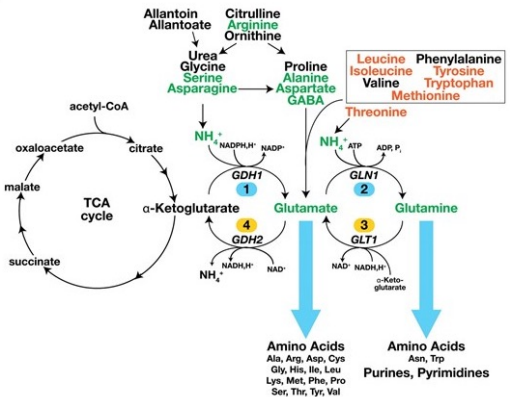

Figure 1. Main pathways of nitrogen metabolism.
(Ljungdahl and Daignan-Fornier 2012).

Nitrogen in grapes

A range of nitrogenous sources may be found in yeasts' environment. Typical nitrogen species in grapes include amino acids and ammonium salts. Their concentrations may vary significantly between 40-700 mg N/L, determined by grape variety differences and viticultural factors. A smaller portion are the **α**-amino acids which yeast can metabolize during anaerobic fermentation. Proline – a nonpreferred nitrogen source which requires oxidative metabolism – constitutes a substantial fraction (Bell and Henschke 2005). Significant variations in both the concentration and composition of nitrogen sources in grape musts are common; viticultural practices, weather, and grape variety all impact the nitrogen content of grapes at harvest (Bell and Henschke 2005; Huang and Ough 1991). While grapes contain a variety of proteins including oxidases, pectinases, glucosides, chitinases, esterases and glucanases (Vincenzi et al. 2012), they are typically found at very low concentrations and have a low impact on yeast metabolism (Waterhouse et al. 2016a). Given the variability of nitrogen found in grapes and the importance of nitrogen to successful fermentations, supplementation with ammonium salts such as diammonium phosphate (DAP) and amino acids is commonplace (Waterhouse 2016a). The relative benefits of supplementing a must with either or both nitrogen sources have been explored and discussed (Torrea et al. 2011).

Nitrogen catabolite repression (NCR)

Yeast cells rely on a regulatory system of sensing and signaling pathways that is generally described as nitrogen catabolite repression (NCR). In the presence of environmental nitrogen, yeast activate broad spectrum transport permeases for

preferred sources and represses transporters of nonpreferred ones (Ljungdahl and Daignan-Fornier 2012). The NCR is said to be active when permeases for nonpreferred sources are repressed; in the absence of preferred sources, permeases for nonpreferred amino acids are derepressed. A system for sensing specific amino acids in the environment (SPS) involves the known proteins Ssy1p, Ptr3p, and Ssy5p, whose gene regulation changes rapidly depending on the extracellular prevalence of their target amino acids (Forsberg and Ljungdahl 2001).

Laboratory assessments of NCR

Two criteria have been used to assess preferred nitrogen sources in laboratory studies. Growth rate is the first; preferred sources are those which confer greater growth rates in experimental analysis. Specific amino acids and ammonium have been shown to induce distinct growth rates when used as the only available nitrogen source (Cooper 1982). The preference of glutamate to glutamine and the nonpreference of proline have also been well-documented in this regime (Watson 1977). However, differences in growth rates can be quite small, making this criterion somewhat imprecise (Magasanik and Kaiser 2002). More recent work on the NCR has monitored the gene regulation of transport proteins. While these metrics often correlate, these two regimes do not always suggest identical profiles of nitrogen preference.

Strain-dependent preferences

Classification of nitrogen sources as preferred or nonpreferred in the NCR is not universal among *Saccharomyces cerevisiae* strains. Preferential distinctions exist between laboratory strains. Given the influence of other factors like temperature, nutrient

availability, and stress on cell growth, much of the strain-specific functionality of the NCR outside of optimal conditions remains unknown.

Fermentation kinetics

Fermentation kinetics, which have been shown to correlate with biomass and nitrogen availability (Salmon 1989), are of crucial importance to both small- and large-scale winemakers. Even in ideal fermentative conditions, wineries generally hope for predictable fermentation rates so they can adhere to grape harvest schedules. However, deviations from optimal kinetics are unfortunately common.

Stuck and sluggish fermentations involve the arrest of hexose degradation before a wine reaches "dryness," which in the United States generally describes wines with less than 3 g/L of hexose sugars. Stuck and sluggish fermentations are a perennial concern for winemakers, as they are difficult to rectify and may be caused by a host of conditions including those that are intrinsic to the winemaking process (like ethanol toxicity). Nitrogen deficiency has been described as a key factor leading to these undesirable outcomes (Alexandre and Charpentier 1998, Bisson 1999). Limiting nitrogen at different concentrations was shown to correlate with biomass production and fermentation kinetics (with low concentrations inducing stuck or sluggish fermentation) (Salmon 1989) long before these observations about growth rate and nutrient limitation were confirmed by genetic analysis (Saldanha et al. 2004; Brauer et al. 2008; Boer et al. 2010). The requirement for wine yeast to ferment under high ethanoic stress presents a unique challenge regarding nitrogen utilization. It has been suggested that, under ethanol stress, translocation of amino acids and other nitrogen sources is inhibited, halting synthesis of hexose transporter proteins (such as *Hxt6)*

which must be resynthesized regularly throughout stationary phase for fermentation to complete (Bisson 1999). With this in mind, some groups argue that supplementation should take place during stationary phase, but before significant ethanol has accumulated (Manginot et al. 1998). Others suggest adding nitrogen prior to or early in fermentation (Crépin et al. 2012).

The impacts of nitrogen utilization on flavor and aroma compounds are also of great importance to winemakers. Low nitrogen availability has been explored as a factor contributing to off-aroma compounds such as hydrogen sulfide (Ugliano et al. 2011) and higher alcohols (Vilanova et al. 2007). While excess nitrogen at the end of fermentation may increase the risk of microbial spoilage and formation of the higher alcohols of specific residual amino acids (Dickinson et al. 1998), the risks of low nitrogen content are generally considered greater than those of high nitrogen content (Malcorps et al. 1991; Waterhouse et al. 2016a).

Nitrogen preferences among wine strains

Broadly, high levels of ethanol stress differentiate wine fermentations from most laboratory conditions exploring nitrogen utilization. Nonetheless, the NCR of yeast in enological conditions – with high sugar concentrations, ethanoic stress, and low temperature and pH – has been explored. Like laboratory strains, enological yeast have preferred nitrogen sources. Jiranek et al. (1995) found that arginine was utilized to a much greater extent than other amino acids across all strains studied and that variations among strains were observed. Enological experiments have confirmed that proline is not a preferred source, as oxygen is required for its catabolism (Ingledew et al. 1987). 14 wine strains were found to assimilate nitrogen sources in a similar sequence, first

importing specific amino acids (Asp, Thr, Glu, Leu, His, Met, Ile, Ser, Gln, and Phe) associated with the SPS, and later consuming a second group (ammonium, Val, Arg, Ala, Trp, and Tyr) presumed to be linked to the NCR (Crépin et al. 2012). The NCR was shown to be active throughout a wine-like fermentation when nitrogen was highly available but was inactivated part way through fermentation when nitrogen was limited (Beltran et al. 2004). These studies confirm in the enological context that nitrogen utilization relies upon the sensing, signaling, and transport mechanisms that have been described among laboratory strains (Wiame et al. 1985; Magasanik and Kaiser 2002).

"Nitrogen requirements" and the importance of biomass for sugar metabolism

Industry has made an effort to consolidate the state of knowledge about nitrogen utilization in yeast into a practical system for winemakers. Over time, authors have used the term "nitrogen requirements" in the literature based on different criteria. Total required nitrogen describes the nitrogen required to achieve maximal growth or sugar catabolism (Jiranek et al. 1995); minimum required nitrogen corresponds to concentrations below which "time for completion of fermentation is unsatisfactory" (Jiranek et al. 1995). Experiments in winemaking conditions show that increased nitrogen concentrations and increased biomass correlate with shorter fermentation times (Bely et al. 1990; Monteiro and Bisson 1991; Mendes-Ferreira et al. 2004; Varela et al. 2004). 120 to 140 mg N/L is commonly cited as the threshold at which biomass no longer increases by increasing the nitrogen content of the must (Jiranek et al. 1995; Bely et al. 1990).

A series of papers asked if commercially available wine strains required differing concentrations during fermentation (Manginot et al. 1998; Julien et al. 2000). While

nitrogen utilization during growth phase was insignificant between strains, authors found that, by modulating the rate of nitrogen addition to chemostat fermenters, yeast could be made to produce carbon dioxide at a specific rate (Manginot et al. 1998). Thus, the nitrogen requirements suggested by this group are quantified as the amount of nitrogen required to maintain a constant rate of carbon dioxide production over time. These data contribute to the hypothesis that nitrogen may be utilized at differing rates among strains during stationary phase, perhaps relating to expression of *HXT* genes, which encode hexose transporter proteins (Manginot et al. 1998; Bisson 1999; Julien et al. 2000). However, this mechanism has not been directly investigated, leaving open questions about nitrogen use during stationary phase – and more generally, under the ethanoic stress.

Nonetheless, strain to strain variation in nitrogen preferences (ter Schure et al. 2000; Ljungdahl and Daignan-Fornier 2012) and fermentation "performance" in the winemaking context have been observed. Jiranek et al. (1995) found differences of nitrogen utilization by different strains when all nitrogen sources were in excess and sugar was limited. Members of this group later confirmed different fermentation kinetics, nitrogen utilization, and H_2S formation by two strains from their previous work by conducting fermentations with 125, 250, and 313 mg N/L of assimilable nitrogen (Gardner et al. 2002).

This work aims to independently validate existing knowledge about yeast strain-specific nitrogen utilization by comparing the fermentation kinetics, cell density, and sugar and nitrogen metabolism of 6 commercial wine strains with differing published requirements for nitrogen under typical winemaking conditions. Furthermore, we assess the relationship between published nitrogen requirements and two commonly used metrics for nitrogen uptake in yeast: biomass formation and the impact of

nitrogen limitation on cell density. This preliminary work may lead to more focused assessments of nitrogen utilization by yeast in the winemaking context.

METHODS

Yeast strains and Growth Media

All yeast strains used in this study (listed in Table 1) are commercially available wine yeasts provided by E & J Gallo Winery (Richter et al. 2013). Strain selections were based on nitrogen requirements listed in the 2023 Scott Labs Fermentation Handbook. Colonies were struck out on rich media YPD agar plates (1% yeast extract, 2% bacto peptone, 2% dextrose; 2% agar; pH~6.5) and grown in YPD liquid medium (same as above without agar).

Cell Size and Biomass Quantification

Single colonies of eight strains (Table 1) were grown overnight in duplicate in liquid YPD media. Cell size was measured with Beckman Coulter Z2 Coulter Particle Count and Size Analyzer with a 100-micron aperture. To determine biomass, empty microcentrifuge tubes were labeled with codes associated with each strain and weighed. Cells from 3 mL samples after overnight growth were collected by centrifugation and the supernatant was discarded. Cells were then washed once in sterile milliQ water, pelleted, and incubated overnight at 80°C to evaporate any remaining water. Biomass of each 3 mL sample was determined by weighing the dried cells and subtracting the initial mass of the corresponding microcentrifuge tubes.

Fermentation Experiments

Grape juice

Chardonnay juice (19.5° Brix, 9.9 g/L TA, pH 3.09, 87.3 mg N/L YAN) was harvested from Leonard Oakes Estate Winery on 9/24/2020. The juice was sterilized by

Cornell Craft Beverage Analytical Lab, stored at -20° C, and thawed before use. The juice was supplemented with diammonium phosphate (DAP) so that the initial YAN concentration of the must was 350 mg N/L.

Cultivation of cells and explanation of replicates

Four single colonies of each stain were grown overnight in liquid YPD media. Initial cell concentrations were measured using a Beckman Coulter Z2 Coulter Particle Count and Size Analyzer. Then, $3x10^6$ cells were washed with sterile milliQ water before being inoculated into the Chardonnay juice.

Washed cells were then inoculated into 250 mL Erlenmeyer flasks containing 150 mL of sterilized Chardonnay juice, mixed well, and fitted with sterilized airlocks. Among the four replicates for each strain, two were used to monitor fermentation progress through periodic assessment of flask weight, and two were used to measure cell concentration, cell size, sugar and YAN concentrations.

Assessment of fermentation kinetics

At each timepoint, the mass of the weight flasks were recorded as proxies for fermentation progress; weight loss during fermentation was assumed to correspond with the release of carbon dioxide, and thus the catabolism of hexose sugars and production of ethanol described by the Gay-Lussac equation (Figure 2). This calculation was based on measured initial sugar concentration (g/L) rather than soluble solids (°Brix). Experimentally, ethanol production typically reflects ~90% of the maximum predicted by stoichiometric conversion (Waterhouse et al. 2016b).

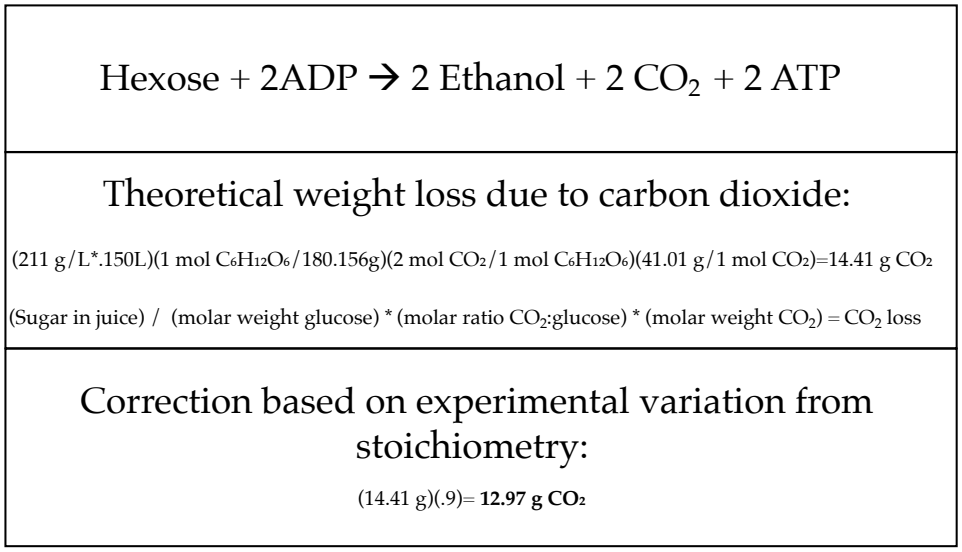

Figure 2. Calculation of carbon dioxide loss from initial sugar concentration. The Gay-Lussac Equation (A) describes the catabolism of hexose sugars during fermentation. Many eukaryotes favor respiration – which requires an external electron acceptor (O_2) and generates 36 ATP per hexose sugar – under aerobic conditions. Fermentation occurs without an external electron acceptor and often occurs in anaerobic conditions. The tendency of *Saccharomyces cerevisiae* to ferment hexose sugars even under aerobic conditions – thus accumulating ethanol – is termed the Crabtree effect (Waterhouse et al. 2016b). Theoretical weight loss (B) for a fermentation can be determined from the Gay-Lussac equation if the initial sugar concentration of a must is known. *S. cerevisiae* diverts a portion of glucose for biomass formation, and in practice 90% of ethanol and carbon dioxide predicted by the Gay-Lussac maximum is produced (C).

Determination of nitrogen and sugar content in juice and wine

At each time point, each sample flask was swirled vigorously for 10 seconds, or until all visible cells that had settled to the bottom of the flask were resuspended. Cell concentration and cell size were measured as described above. The remaining samples were centrifuged for 2 minutes at 4000 RPM, and 3 mL of supernatant were frozen for metabolite analysis. Enzymatic analyses for glucose and fructose and YAN were performed by the Cornell Craft Beverage Analytical Laboratory in Geneva, NY.

High glucose fermentation

Three single colonies for each strain were grown overnight in 5 mL of liquid YPD media. Sterilized Chardonnay juice was supplemented with glucose up to 24 °Brix. The juice was inoculated using the method described above into 150 mL Erlenmeyer flasks with 100 mL of Chardonnay juice, mixed well, and fitted with sterilized airlocks. Weight changes due to carbon dioxide loss was measured.

Assessment of growth-limiting nitrogen concentration

To evaluate the impact of nitrogen limitation on cell density, EC-1118 and CY3079 were grown overnight in YPD on a roller wheel at 30°C in triplicate. Cells were then washed twice in sterile water to discard any residual overnight media. Cell concentration was estimated by measuring optical density at 600 nm (OD600), and the cells were diluted to an OD600=0.22. Minimal media composed of 1X YNB (*y*east *n*itrogen *b*ase) with 20% glucose and no nitrogen source was aspirated across the middle 6 rows of a sterile, round bottom 96-well plate. The first column of the plate supplemented with diammonium phosphate (DAP) to 1000 mg N/L, and two-fold serial dilutions were performed so that nitrogen content varied between 1000 mg/L to 0.49 mg/L. 10 μL of diluted cells from each replicate were then added across a row, so that the final well volume was 220 μL and each row was inoculated with a different replicate. The plate was then covered with a breathable sealing tape and incubated and shaken at 30°C and 200 RPM for 5 days. After growth, OD600 of the plate was measured using a microplate spectrophotometer, shaking in a double orbital pattern. Finally, the microplate OD values were converted to single sample spectrophotometer ODs using a polynomial equation described by Lewis and Gibney (2023).

Table 1: Yeast strains used in this study

Yeast Strain	Nitrogen Requirement*	Catalog Name	Supplier	Origin
EC-1118	Low	Lalvin EC-1118	Lallemand	Champagne
ICV-D47	Low	Enoferm ICV-D47	Lallemand	Cotes du Rhone
43	Low	Uvaferm 43	Lallemand	Inter Rhone
IOC 18-2007	Low	IOC 18-2007	Epernay	-
NT112	Medium	NT 112	Anchor	Stellenbosch, South Africa
ICV-GRE	Medium	Lalvin ICV-GRE	Lallemand	Rhone Valley
CY3079	High	Lalvin CY3079	Lallemand	Bourgogne
AWRI R2	High	Maurivin R2	Maurivin	Bordeaux
BA11	High	BA11	Lallemand	Estacao Vitiviniccola de Baraida
BM45	High	Lalvin BM45 Brunello	Lallemand	University of Siena

* "Nitrogen requirements" for strains are designations provided by Scott Labs Winemaking Handbook 2023.

RESULTS

Cell volume and biomass

Initially, we set out to investigate the relationship between nitrogen requirements and cell volume or biomass. 8 strains were selected based on their reported nitrogen requirements: 4 low and 4 high nitrogen requiring strains. Independent colonies of each strain in duplicate were grown overnight in YPD, then cell volume and biomass were measured (Figure 3). Though differences in cell volume were observed, there was no observable trend relating to nitrogen requirement.

We then collected 3mL of cells from the same overnight growth to measure biomass. The cells were collected using centrifugation, washed once with sterile water, and pelleted using centrifugation. Residual water was evaporated during overnight incubation at 80°C before the cells were weighed. Again, differences in biomass among high and low nitrogen requiring strains was not significant. While a much larger sample size would be needed to conclusively support our results, we found no significant correlation between nitrogen requirement and either biomass or cell size.

Identifying growth-limiting concentrations of nitrogen

Fermentation kinetics correlate with biomass formation and nitrogen availability (Salmon 1989). With this in mind, we compared the impact of nitrogen limitation in minimal media on cell concentration with two commercial wine yeasts – one with high requirements, CY3079, and one with low requirements, EC-1118. By varying DAP concentrations between 1000 mg/L and 0.49 mg/L (as nitrogen), we expected to see cell

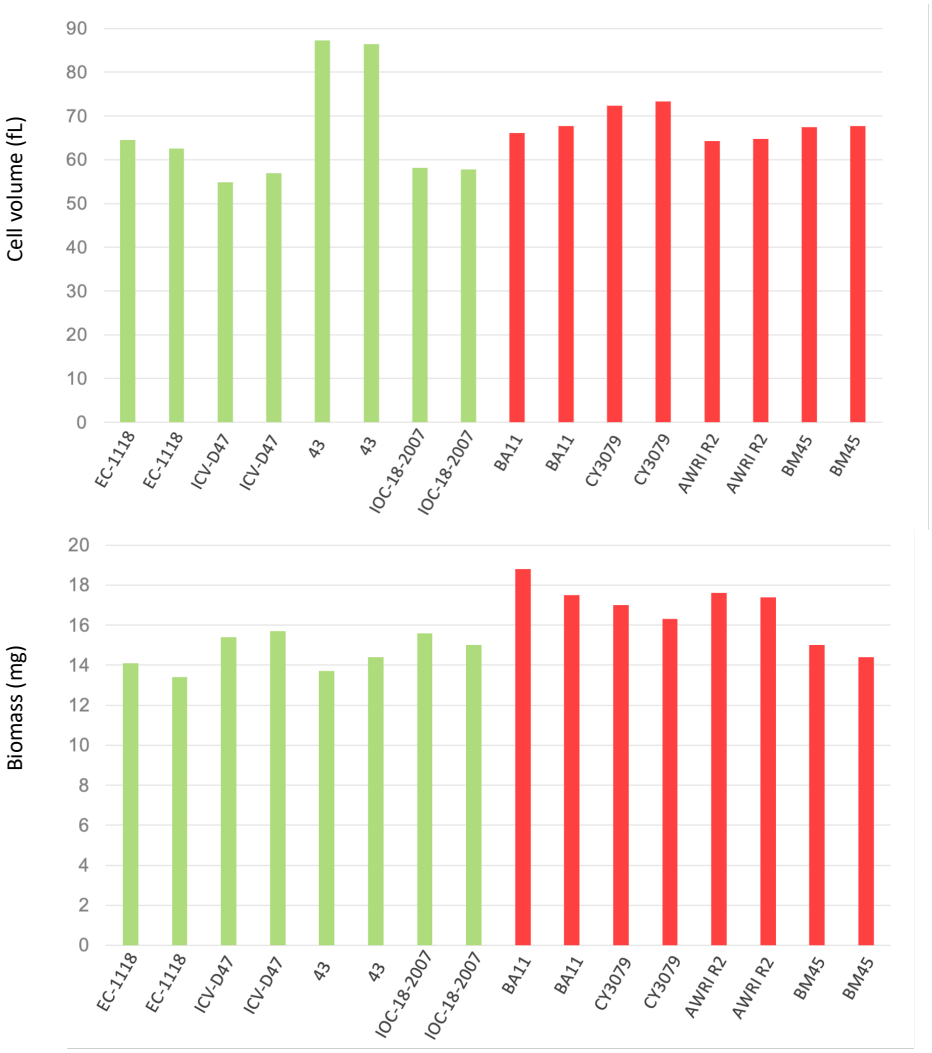

Figure 3. Comparing cell volume and biomass with nitrogen requirements. 8 strains were selected based on their published nitrogen requirements; 4 low (green) and 4 high (red). After overnight growth, cell volume was measured by Coulter counter. Biomass was determined after drying 3 mL of cells of washed cells overnight.

density increase with nitrogen content. It did (Figure 4). Above the upper limit for nitrogen limitation, cell density plateaued, and we assumed that either cells had reached maximum cell density, or that they were limited for another nutrient (Lewis and Gibney, 2023). We asked if nitrogen requirements correspond with growth-limiting concentrations of the nutrient – if so, cells with high reported nitrogen requirements might achieve less cell density than low requiring strains under the same nitrogen limitation. Interestingly, we found that both strains responded very similarly to limitation (Figure 4). Cell density (OD600) increased linearly with increasing nitrogen between 31.25 and 250 mg/L, with both strains achieving similar cell densities at the four concentrations within the linear range. Above the growth-limiting range, both strains also maintained similar cell densities. These data suggest that, in minimal media and under laboratory conditions, biomass formation when nitrogen is limited for both EC-1118 and CY3079 is nearly identical.

The results of our initial observations of cell volume, biomass formation, and the impacts of nitrogen limitation on growth validate findings by the group who first characterized nitrogen requirements. Manginot et al. (1998) observed no significant differences among strains in nitrogen consumed during growth, though strains required differing amounts of nitrogen to maintain a constant rate of carbon dioxide production during stationary phase. Our next experiment sought to observe glucose and nitrogen consumption during a typical wine fermentation to assess the comparative importance of the nutrient at different timepoints.

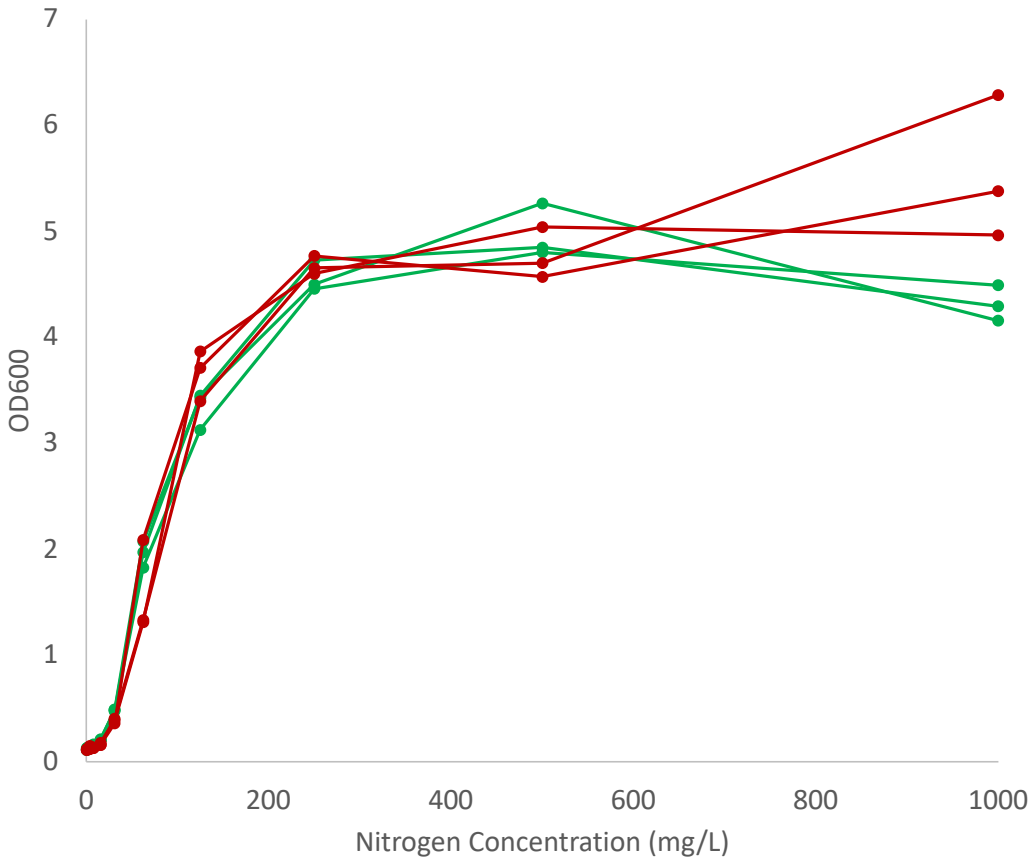

Figure 4. Identifying growth-limiting concentrations of nitrogen for strains with differing nitrogen requirements. OD600 of strains – one with a low nitrogen requirement (EC-1118, green) and one with high a requirement (CY3079, red). Cells were grown on a round bottomed microplate reader plate in minimal media (YNB, *y*east *n*itrogen *b*ase) with 20% glucose and nitrogen (as DAP) varying with two-fold dilutions between 1000 mg/L – 0.49 mg/L. The plate was incubated for 5 days at 30° C shaking at 200 RPM. Microplate readings were converted to single sample OD600 values using a polynomial equation from Lewis and Gibney (2023). When cells are growth-limited for a given nutrient, OD600 measurements increase with concentrations of the limiting nutrient (here, nitrogen). For both strains, cells were no longer growth-limited above 250 mg/L nitrogen; above that concentration, cells are assumed to be limited by another factor.

Fermentation kinetics, sugar consumption, and nitrogen utilization in typical winemaking conditions

6 strains (with 4 replicates each) were inoculated into sterile Chardonnay juice which was supplemented with DAP up to 350 mg/L yeast assimilable nitrogen. Weight changes due to carbon dioxide loss, cell concentration, glucose, and YAN were measured daily between the first and the eighth day of fermentation, and a final timepoint was taken on day 14. These data are compiled and illustrated in Figure 5.

Fermentation kinetics

Based on the initial sugar concentration of the must, we expected 12.97 g of weight loss due to carbon dioxide volatilization at the end of fermentation. By day 14, all replicates had lost between 13.52-14.68 g. Excess losses are likely explained by evaporation. We observed two trends for kinetics: Three strains (AWRI R2, ICV-GRE, and EC-1118) produced carbon dioxide to a greater extent throughout fermentation than the other strains studied. However, each strain represents a different category of reported nitrogen requirements. We found no correlation between fermentation kinetics and nitrogen requirement.

Cell concentration

Cell concentrations were measured by Coulter counter. All strains achieved and maintained a maximum cell density by day 4 except one replicate of CY3079, which increased from ~ $8x10^7$ to ~ $9x10^7$ cells between day 4 and 14. Medium-nitrogen-requiring strains (NT-112 and ICV-GRE) were the most tightly clustered, achieving between $4.01x10^7$ and $5.26x10^7$ cells. Variation in cell density was present between

replicates of high requiring strains ($5.19x10^7$ - $8.96x10^7$ on day 8) and low requiring strains ($4.88x10^7$ - $7.74x10^7$ on day 6), but no pattern emerged that corresponded to nitrogen requirement.

The strains that achieved the highest cell density also had among the quickest fermentation kinetics (CY3079, EC-1118), though fermentation kinetics by medium-nitrogen-requiring strains aren't explained by this correlation. The relationship between biomass and fermentation rate has been observed before (Salmon 1989; Mendes-Ferreira et al. 2004; Varela et al. 2004); here, biomass seems to correlate with fermentation kinetics, despite the lack of connection to nitrogen requirements.

Glucose consumption and nitrogen utilization

All strains had fermented to "dryness," meaning <3 g/L glucose, by day 14. NT-112 was the only strain whose two replicates failed to metabolize glucose below 1 g/L (1.3 and 1.5 g/L remained), and 2.7 g/L glucose remained for a single replicate of EC-1118. As anticipated, glucose metabolism corresponded with carbon dioxide loss (Figure 5). The three strains that produced carbon dioxide more quickly also consumed glucose at a quicker rate.

Variations in nitrogen consumption follow the trend observed for glucose consumption and carbon dioxide loss. The three strains that achieved quicker fermentation kinetics also consumed nitrogen to a greater extent than the others. AWRI R2, ICV-GRE, and EC-1118 utilized more than 300 mg/L of the 350 mg/L available nitrogen, while the three strains with slower fermentation kinetics consumed between 220 mg/L and 250 mg/L available nitrogen. Again, these three strains represent each of

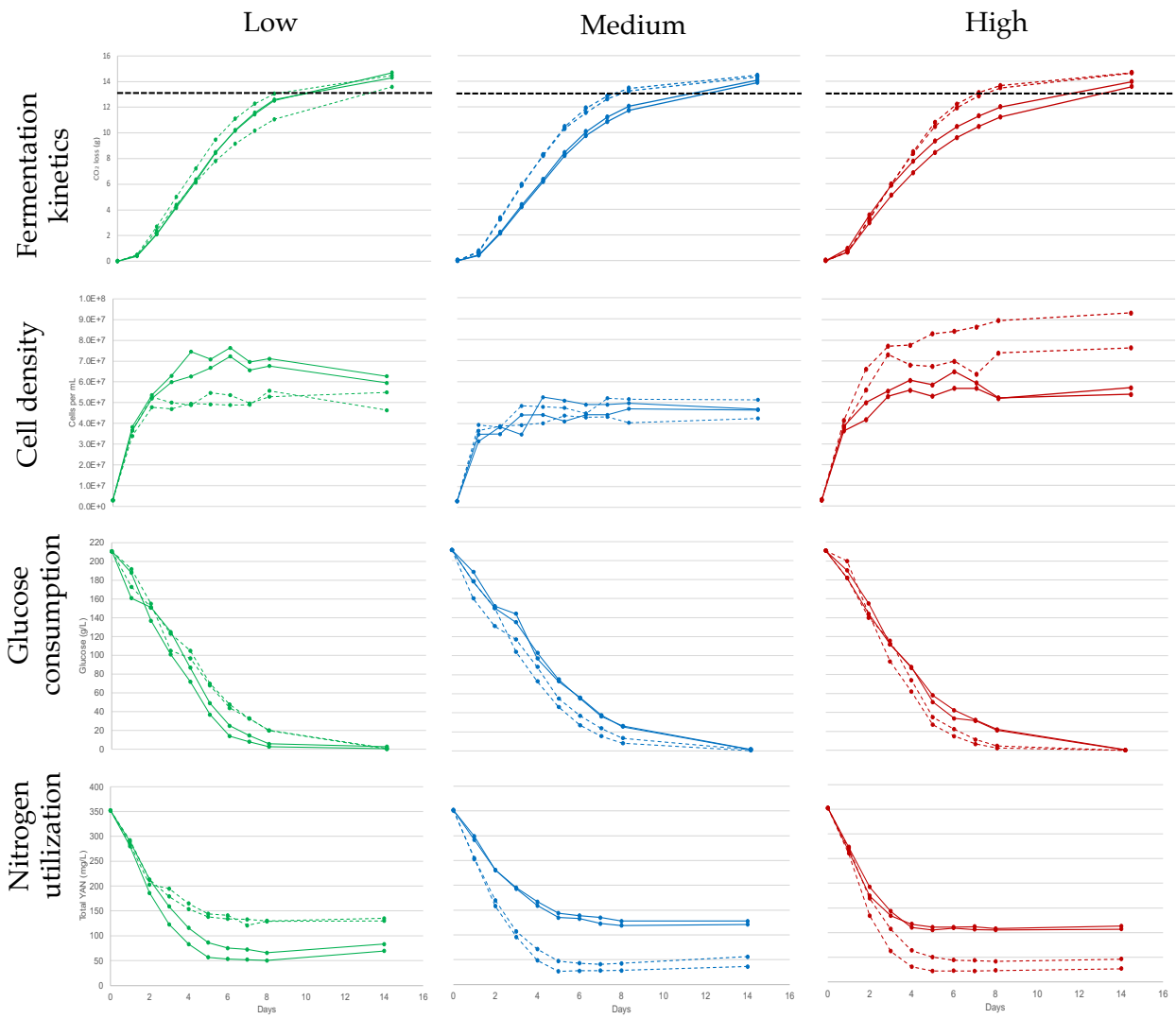

Figure 5. Assessing the impact of nitrogen requirement on fermentation kinetics, cell density, glucose consumption, and nitrogen utilization. 6 strains (two replicates each) were selected based on their published nitrogen requirements; 2 low (green), 2 medium (blue), and 2 high (red). Fermentations occurred in sterile Chardonnay juice (19.5 °Brix) supplemented to 350 mg N/L with diammonium phosphate (DAP). Among the four replicates for each strain, two were used to monitor fermentation progress through periodic assessment of flask weight (CO_2 loss), and two were used to measure cell concentration, cell size, sugar and YAN concentrations.

the reported nitrogen requirement categories; we find no correlation between overall nitrogen utilization and published requirement.

Interestingly, all strains utilized similar concentrations of free-amino nitrogen (FAN) but varied in terms of ammonia consumption (Figure 6). It is possible that variation in ammonia consumption represents differences in nitrogen preference between strains. NCR has been observed in wine fermentations (Beltran et al. 2004), and strain to strain variation in nitrogen preferences are expected (ter Schure et al. 2000; Ljungdahl and Daignan-Fornier 2012). Deeper investigations into the importance of nitrogen source, either in terms of strain preference or its impact on fermentation kinetics, are nonetheless outside the scope of this work.

On day 14, FAN increased for two strains, ICV-GRE and EC-1118. Release of amino nitrogen at this stage, when cells are experiencing ethanol stress, suggests that cells from these fermentations may have lysed. However, this observation would require additional work to establish a cause.

Impact of nitrogen consumption during stationary phase

Given that the regime used by previous groups to quantify nitrogen requirements focused on nitrogen utilization during stationary phase, (Manginot et al. 1998; Julien et al. 2000) we sought to assess the relative importance of nitrogen to biomass formation early in fermentation versus nitrogen use during stationary phase. We calculated the ratios of nitrogen consumed to glucose consumed during the first half of glucose metabolism (0-50%) and the second half (51%-end of fermentation) (Figure 7). Per unit of glucose consumed, nitrogen was utilized at 3- to 5-fold higher rates during the first half of glucose metabolism than the second half. AWRI R2, ICV-GRE

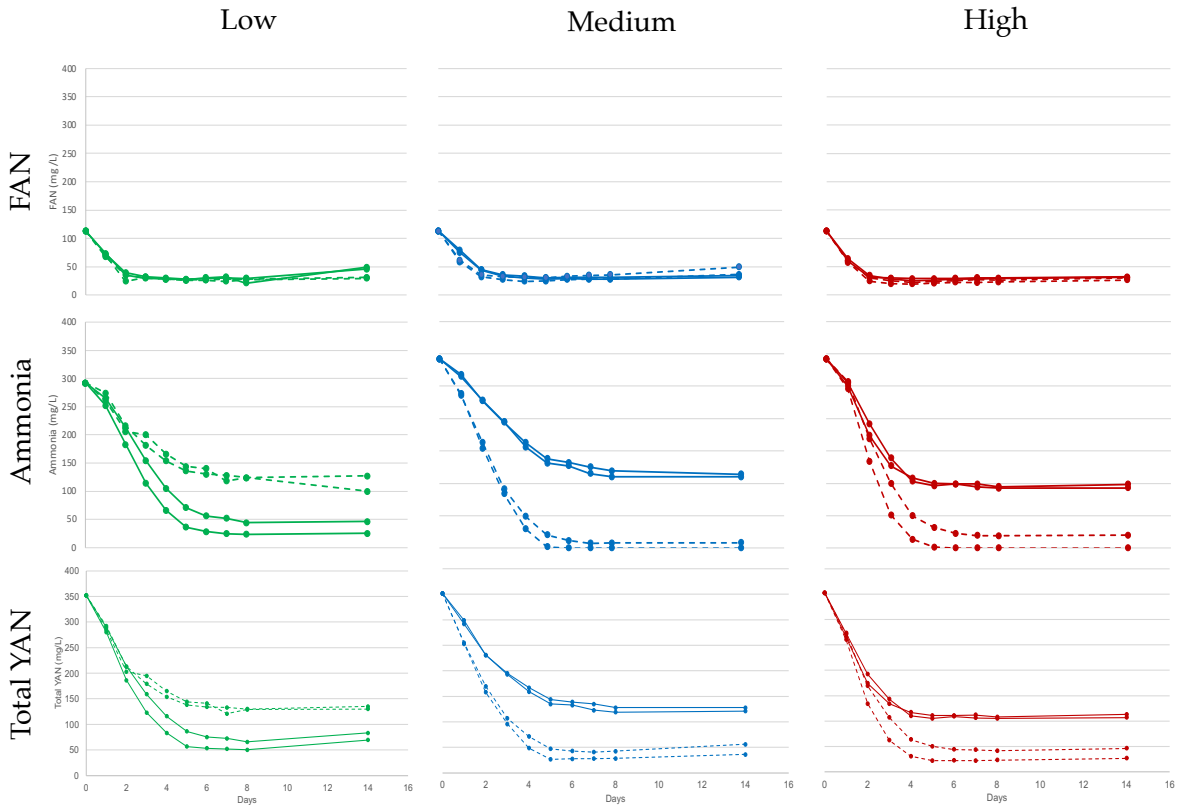

Figure 6. Illustration of nitrogen consumption based on nitrogen source. Nitrogen utilization during a typical white wine fermentation by six strains (two replicates each) with differing published nitrogen requirements: 2 low- (green), 2 medium- (blue) and 2 high- requiring (red) strains. The sum of FAN (*f*ree-*a*mino *n*itrogen) and ammonia constitute the total YAN (*y*east *a*ssimilable *n*itrogen) in each fermentation.

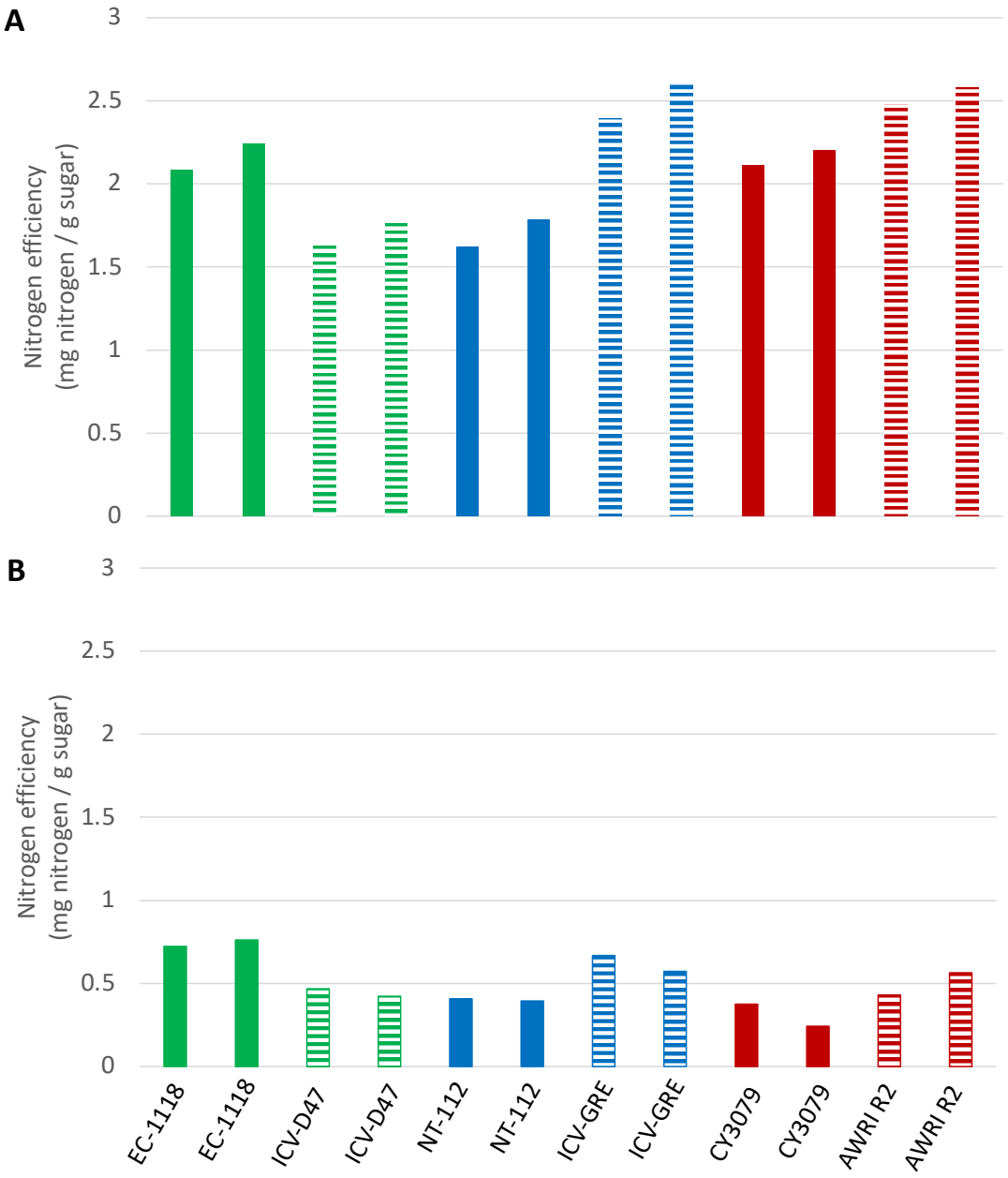

Figure 7. Relative nitrogen efficiency of low-, medium-, and high-requiring strains. Ratios of nitrogen consumed to glucose consumed by two timepoints: when glucose metabolism was 50% complete (A), and 51% to the end of fermentation (B).

and EC-1118, the strains with the fastest kinetics and greatest nitrogen consumption, also had the highest ratios of nitrogen : glucose consumed (Figure 7a). This metric illustrates the relative importance of nitrogen to glucose metabolism; our data suggest that nitrogen used for biomass formation is more important for wine fermentation than nitrogen consumed during the stationary phase.

Fermentation kinetics with a high Brix must

Finally, we wanted to see if fermenting the same six strains in the same grape juice with an increased sugar concentration would produce differing fermentation kinetics. High glucose concentrations confer a) increased initial osmotic stress and, as fermentation progresses, b) increased ethanol stress (Waterhouse et al. 2016c). We asked if strains behaved differently under these conditions, compared to a low sugar must, and if any differences correspond with their published nitrogen requirements. A comparison of the fermentation kinetics for all strains in the low- and high-sugar juice appears in Figure 8. Here, we report estimated percent ethanol rather than carbon dioxide loss to normalize weight differences between the two experiments. For all strains, ethanol production plateaued at roughly the same concentration in both the high and low Brix musts. Ethanol production in the high Brix juice appeared to take place at a slightly faster rate than the low Brix juice, but no other differences were observed. No strains fermented this juice to "dryness;" additional attempts to complete fermentation under these conditions would be helpful to validate our findings. Nonetheless, for our purposes, these data offer a valuable point of comparison. We saw no differences in the fermentation kinetics between strains with high, medium, and low nitrogen requirements in a high Brix grape juice.

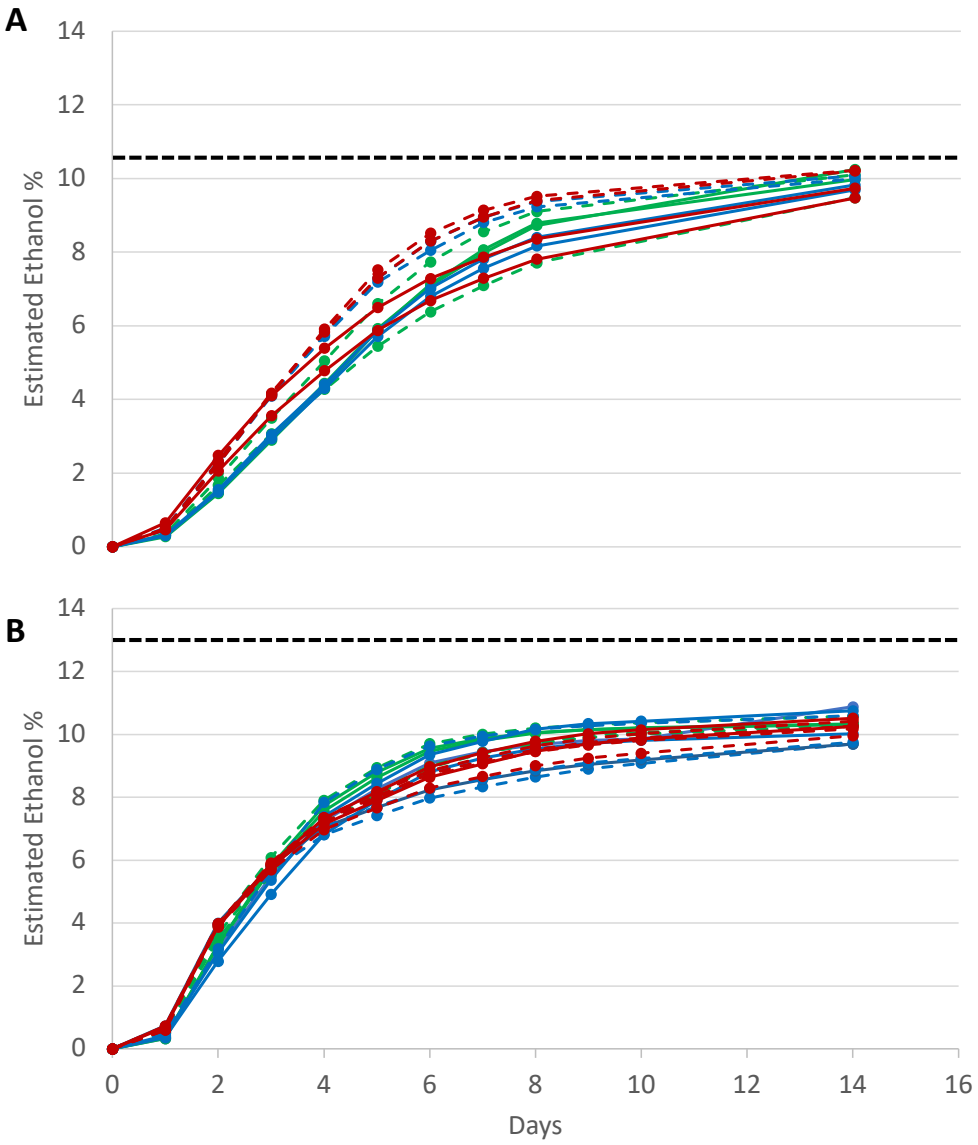

Figure 8. Comparison of fermentation kinetics in low and high sugar grape must. Estimated ethanol production in a low (A) and high (B) Brix must. Dotted lines represent the theoretical ethanol production based on initial sugar concentration. Estimations were determined by converting weight loss due to carbon dioxide and knowledge of the molar ratios of glucose, CO_2, and ethanol in alcoholic fermentations.

Conclusions

This study finds no correlation between reported nitrogen requirements and nitrogen consumption under typical winemaking conditions. Our exploration of cell size, biomass, and nitrogen limitation found insignificant differences between reported high- and low-nitrogen-requiring strains. During a typical white wine fermentation, faster fermentation kinetics and glucose consumption occurred for strains that utilized more nitrogen during the first half of fermentation, mostly for biomass accumulation. Under high sugar conditions, we also observed nearly identical fermentation kinetics. Among the six strains studied, increased nitrogen utilization did not correlate with a strain's published nitrogen requirement. In fact, increased nitrogen use was evenly distributed between the three nitrogen requirement designations. All strains utilized roughly the same concentration of free-amino nitrogen but varied in their use of ammonia. Strains that utilized more ammonia were also those with faster rates of fermentation kinetics and glucose consumption. Finally, we demonstrated that between 3- and 5-fold more nitrogen was required during the first half of glucose consumption compared to the second half, further emphasizing the well-established importance of nitrogen to biomass formation, and biomass formation to fermentation rate (Salmon et al. 1989; Mendes-Ferreira et al. 2004; Varela et al. 2004). Future work may follow the thread pulled here relating to free-amino versus ammoniacal nitrogen use in ecological strains or will continue to investigate the importance of nitrogen to non-dividing yeast under the ethanolic stresses typical in winemaking.

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