A REVIEW OF INORGANIC AND ORGANIC NITROGEN CONSUMPTION BY *SACCHAROMYCES CEREVISIAE* DURING ALCOHOLIC FERMENTATION IN WINE

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ABSTRACT

Nitrogen is an essential nutrient for all microbial growth. Yeast cells require nitrogen to grow and complete fermentation. While both organic and inorganic nitrogen are present in grape must, it is common practice in the wine industry to add nitrogen to fermentations. Available products include an inorganic nitrogen source, di-ammonium phosphate (DAP), and an organic nitrogen source, amino acid mixtures. Although both inorganic and organic nitrogen additions to wine fermentations are common practice in the wine industry, it is not well known how the type of nitrogen added impacts the fermentation. This review examines current research to determine what impact, if any, nitrogen source has on alcoholic fermentations in wine and highlights the differences between inorganic and organic sources of nitrogen in alcoholic fermentations in wine.

BIOGRAPHICAL SKETCH

Riley Sanchez was born in San Diego, California. She attended the University of Oregon as an undergraduate, where she earned her Bachelor of Science in Biology and Chemistry. Upon graduation, she took a harvest internship at a winery in Edna Valley, California. This internship sparked her passion for winemaking, blending together her two academic passions perfectly. She continued to work in the wine industry, working in several regions of California and in the Marlborough region of New Zealand. In 2020, Riley began her Master's study in Food Science and Technology, specializing in Enology under the supervision of Dr. Patrick Gibney at Cornell University. She is an executive member of the Graduate Wine Society and a researcher in the Gibney Lab, which focuses on wine microbiology. The following research project reflects her scientific enthusiasm for wine microbiology and her desire to improve the wine industry's understanding of the importance of microbiology in winemaking.

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INTRODUCTION

Wine is an alcoholic beverage that is produced from grape juice. Yeast cells consume sugars present in the juice and produce ethanol and carbon dioxide as byproducts. Additionally, yeast cells release volatile aroma compounds from non-volatile precursors produced by grapes, influencing the aromatic qualities of wine (Bell and Henschke 2005). *Saccharomyces cerevisiae* is the most common yeast used during wine fermentations. Grape juice is a complex matrix containing many compounds that can be metabolized by yeast. During fermentation, yeast cells metabolize these compounds in the juice that we then perceive as positive aroma and sensory characteristics (Bell and Henschke 2005). In addition to the *S. cerevisiae* cells that perform this activity, there are many other yeast and bacterial species present in the juice that can spoil wine. All of these microbes need nitrogen as a nutrient source to grow.

Nitrogen:

Nitrogen is an essential nutrient for all microbial growth. For yeast growth in wine fermentations, nitrogen is an essential component of the nitrogenous bases which comprise the information-containing code in nucleic acids of DNA and RNA, is present in all amino acids, and is necessary for yeast to synthesize these molecules (Bell and Henschke 2005). This means that for a yeast cell to grow and divide, nitrogen must be available for consumption. Grape juice or must typically starts with a low concentration of usable nitrogen for yeast growth. Thus, it is common practice to add a nitrogen nutrient source at the onset of fermentation to the juice or must, and again at one-third's consumption of available nitrogen. In addition to influencing the yeasts ability to grow, nitrogen availability impacts the production of enzymes and nucleic acids, with lower nitrogen availability corresponding to slower fermentations (Salmon *et al*. 1996) and higher nitrogen availability leading to microbial instability (e.g. excess nitrogen levels can support the growth of spoilage microorganisms after alcoholic fermentation is completed). Both slower fermentations caused by limited nitrogen and microbial instability caused by an overabundance of nitrogen can lead to off flavor and aroma formation in the final wine (Waterhouse *et al*. 2016). Because of the impact nitrogen has on fermentation kinetics and sensory characteristics, it is common practice in the wine industry for winemakers to add nitrogen to their fermentations.

Nitrogen in Fermentation:

There are two main categories of nitrogen formulation routinely used in the wine industry to supplement a fermentation: organic nitrogen and inorganic nitrogen. Organic nitrogen is comprised of amino acids, proteins, and peptides (Santamaria *et al*. 2020). GoFerm and yeast extracts are commonly used to supplement organic nitrogen into fermentation. Using an organic nitrogen source to supplement fermentation allows amino acids to induce their own uptake within yeast cells (Ljungdahl *et al*. 2009). Inorganic nitrogen is often supplied as ammonium ions (NH^{4+}) and is in the form of di-ammonium phosphate (DAP). While there can be a small amount of NH^{4+} present in grape juice, most inorganic nitrogen found in a fermentation is added using DAP. The yeast cell membranes have general amino acid permeases (Gap1p) that actively select for specific nitrogen sources (Feldman *et al*. 2010). These permeases will have different affinities based on the concentration of the nitrogen source.

Nitrogen Catabolite Repression:

Nitrogen catabolite repression (NCR) occurs when a present nitrogen source reduces transmembrane protein uptake of a differing source, increasing the metabolism for the present source. Glutamate and glutamine are important in the nitrogen metabolic pathway because they allow for the synthesis of necessary amino acids that lead to the growth of yeast cells. Both glutamate and glutamine cause strong nitrogen catabolite repression effects because they are major nitrogen donors to amino acid synthesis. Their structures enable them to be continuously recycled in amino acid synthesis. NCR is used to determine whether or not a nitrogen source is preferred by yeast in alcoholic fermentation. Most of what is known about nitrogen sources and their NCR effects is based on lab strains of yeast. There is not a good amount of information available on wild yeast strains and their NCR effects. A nitrogen source's ability to reduce the uptake of a different nitrogen source and increase the yeast cell's affinity for its own uptake defines what a preferred source versus non-preferred source is during alcoholic fermentation.

Yeast Assimilable Nitrogen:

Yeast assimilable nitrogen (YAN) is the fraction of nitrogen that yeast can utilize for growth. In winemaking, YAN refers to the fraction of nitrogen that yeast can utilize in alcoholic fermentation conditions. In some industries, such as the brewing industry, the usable nitrogen by yeast is known as free amino nitrogen (FAN). YAN is a combination of both organic and inorganic nitrogen sources. For organic nitrogen sources, L-amino acids except for lysine, histidine, proline, and cysteine, can be utilized by wine yeasts in fermentation conditions for their nitrogen source (Waterhouse *et al*. 2016). Lysine, histidine, proline and cysteine are considered yeast non-assimilable nitrogen because their higher molecular weight coupled with *Saccharomyces cerevisiae* lack of significant proteolytic activity in fermentation conditions makes these amino acids unusable during fermentation (Bell and Henschke 2005). *Saccharomyces cerevisiae*, specifically, can utilize ammonium ions and free alpha amino acids (Bell and Henschke 2005). While different yeasts have different YAN requirements, typically a juice or must is considered to have a low level of YAN if the concentration is <100 mg/L, a moderate level if the concentration is 150-350 mg/L, and a high level if the concentration is >600 mg/L (Bisson 1991, Ough 1991, Henschke 1993, Jiranek 1995, Beltran 2004). Yeast cells are immobile, forcing them to interact with the source of nitrogen that is closest to their cell membrane. However, yeast can select different nitrogen sources preferentially through the use of permeases, which are protein transporters encoded by multiple different genes in the yeast genome. General amino acid permeases, such as Gap1p, are used to bring amino acid nitrogen sources into the cell, while permeases such as Mep1p, Mep2p, and Mep3p are used to select for NH^{4+} (Waterhouse *et al*. 2016).

Amino Acids:

Amino acids are an organic source of nitrogen that is present in must. Some amino acids are preferentially utilized regardless of an inorganic source's presence (Prior *et al*. 2019). While some amino acids are preferentially selected, there are several amino acids that are not utilized at all during fermentation, such as proline. If an abundance of amino acids are present in must, yeast will not turn on the pathway to synthesize their own amino acids, but rather will import the existing amino acids (Prior *et al*. 2019).

DAP:

DAP is an inorganic nitrogen source containing two ammonium ions per molecule. It is well documented that an addition of DAP at growth phase increases population size of yeast (Bely *et al*. 1990a). Increased population size is often correlated to increased fermentation kinetics and decreased off-aroma production (Bely *et al*. 1990a). In a study by Arias-Gil et al 2007, ammonium addition decreased consumption of aspartic acid, alanine, and arginine while addition of amino acids increased the consumption of aspartic acid, alanine, and arginine. This study suggests that DAP may have NCR effects in yeast cells when present, displaying a preference by yeast for inorganic sources of nitrogen. Lab strain experiments have supported the idea that ammonium is a preferred nitrogen source in fermentation conditions (Ljungdahl and Bertrand 2012).

Nitrogen Source Impact on Fermentation Kinetics:

Current research suggests that the nitrogen source alone does not impact fermentation kinetics, but rather the nitrogen concentration of the source has a large role in determining fermentation kinetics. In research conducted by P. Seguinot *et al*. 2018, nitrogen source did not impact fermentation kinetics, with inorganic and organic fermentations progressing at the same rate. They found that the timing of nitrogen addition in combination with concentration of nitrogen added had the most impact on fermentation kinetics. Similarly, a 2021 study by Santamaria *et al*. revealed that there are no significant differences in fermentations based on type of nitrogen added. Instead, both studies point to the yeast strain's specific nitrogen requirements as the determining factor for having high vigor fermentations. In addition, a study by Roca-Mesa *et al*. found that at the right concentration, source did not matter. They also found that at too low of a concentration (under 120 mg N/L) or too high of concentration (in excess of double the required amount by the specific yeast strain) of nitrogen impacted fermentation kinetics negatively, resulting in undesirable fermentation speeds and undesirable characteristics in the wine. Overall, fermentation kinetics are less dependent on the type of preferred source added and are more impacted by the concentration of that source.

Higher Alcohol Formation:

Higher alcohols are defined as alcohols that have more than two carbons. In wine fermentations, amino acids are metabolized through the Elrich pathway and converted into their higher alcohol form (Hazelwood *et al*. 2008). Higher alcohol formation is an energetically favorable pathway and can be attributed to fermentations where the sole source of nitrogen was amino acids. Low concentration of amino acids creates an imbalance to the equilibrium where higher alcohol formation is favored. Formation of higher alcohols is favored in anaerobic conditions, such as fermentation. Higher alcohols form because the yeast cell is taking the amine group from the amino acids to use to make other amino acids. In doing so, it leaves behind the carbon skeleton of the amino acid, which becomes a higher alcohol.

Acetate Ester Formation:

It is commonly accepted that the easiest way for a winemaker to increase or decrease acetate ester formation is through yeast selection or manipulating fermentation conditions. For example, anaerobic conditions favor acetate ester formation, while aerobic conditions inhibit acetate formation. However, some research indicates that in addition to yeast selection and fermentation conditions, nitrogen source can impact acetate ester formation. Amino acid rich juice leads to increased Aft1p activity and decreased Atf2p activity (Seguinot *et al*. 2018). Aft1p is responsible for acetate ester production to a larger extent than available substrates. This increase in Atf1p expression, in conjunction with other fermentation factors, can increase the total acetate esters formed.

Nitrogen Source Impact on Wine Sensory Parameters:

Higher alcohol formation occurs in fermentations where amino acids are the abundant nitrogen source available to yeast. In some cases, higher alcohol formation can lead to positive sensory characteristics, such as higher alcohols transformation into acetate esters that give Sauvignon Blanc its key aroma characteristics. However, higher alcohols are mostly known as fusel alcohols, with aroma descriptors such as solvent, fusel, and boiled potato. Depending on the type of wine being made, nitrogen source can be used as a way to limit the negative aroma characteristics in the final wine.

Hydrogen sulfide is a negative aroma characteristic that is produced when either an organic source or an inorganic source of nitrogen is at low concentrations. Hydrogen sulfide production diminishes when nitrogen is in excess. This is supported by Wang *et al*. 2003, who observed a decrease in hydrogen sulfide production in musts with increasing YAN concentrations up to 250 mg/L. Thus, a combination of nitrogen in slight excess and a limited sugar supply reduces this aroma fault (Jinarek *et al*.1995). Acetate ester form when the proper concentration of amino acids is available. Too low of a YAN concentration leads to a decrease in the production of acetate esters. Acetate esters are responsible for the fruity, floral, and tropical aromas found in many wines. Acetate esters are desirable in young wines, as their concentration diminishes over time as they are converted into their higher alcohol conjugate. Overall, moderate levels of nitrogen yield the most desirable aroma characteristics (Torrea *et al*. 2011).

FUTURE DIRECTION

There is a lot of available research on nitrogen's importance as a nutrient in yeast growth and metabolism, the impacts of nitrogen additions on fermentation kinetics, and the impacts of nitrogen in producing pleasant sensory characteristics. However, there is a lack of information on the impacts different types of nitrogen sources have on a variety of juices in alcoholic fermentation. We do not know whether the nitrogen source, organic or inorganic, is more important for determining fermentation kinetics or impacting final wine sensory parameters positively in different varietal fermentations. There are several experiments that could provide more information on this. For example, an experiment could be to measure both fermentation kinetics and wine sensory parameters of juice fermentations that have organic, inorganic, and mixed nitrogen sources. This could be done for several common wine grapes, such as Chardonnay, Sauvignon Blanc, and Pinot Noir. These results can then be compared to see if there is any change between the different juices. Not only will this provide information on the different needs of juices/musts, this will also provide clear results as to whether or not the nitrogen source specifically impacts fermentation. Overall, the importance of nitrogen to wine fermentations is clear and there is still more to learn about how to utilize nitrogen source to positively impact fermentations.

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