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Arginine addition in the stationary phase influences the fermentation rate and synthesis of aroma compounds in a synthetic must fermented by three commercial wine strains

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ABSTRACT

During fermentation, the nature and amount of nitrogen added into grape must affect the fermentation rate and secondary aroma production. Timing of addition is also relevant for the resultant wine. The wine industry uses different nitrogen preparations to avoid nitrogen deficiency in wine fermentations. However, it is important to know the proper way to carry out additions. In this work, we studied the effect of nitrogen addition to three commercial wine strains in the stationary phase of synthetic must fermentations. We analyzed the impact of the concentration and source of nitrogen on fermentation activity and aroma production. Nitrogen addition stimulates the fermentation rate, mainly in N-deficient medium. Moreover, aroma synthesis changes with nitrogen additions. The use of ammonium and arginine as additives produces wines with lower higher alcohol content and higher ester concentration respectively. The results indicate that different wine strains and nitrogen sources can be used to produce wines with divergent aroma profiles to meet consumer demands for diversified products.

1. Introduction

Nitrogen deficiency is commonly observed in many viticultural regions and is often considered a growth-limiting factor (Pretorius, 2000). The importance of nitrogen for growth and fermentation activity is well-established (Bisson, 1999). Low nitrogen in grape must leads to low yeast populations and poor fermentation vigor, increased risk of sluggish or stuck fermentations, and undesirable compounds, such as hydrogen sulfide. Conversely, a high nitrogen concentration leads to increased biomass and stimulates the sugar utilization rate. Furthermore, nitrogen availability can also affect many yeast metabolism aspects, including the formation of volatile and non volatile compounds that are important for the organoleptic quality of wine.

Currently, addition of nitrogen to must is a usual practice in winemaking to avoid nitrogen limitation-related problems and it has a significant effect on wine flavor. Inorganic supplements, such as diammonium phosphate (DAP), are widely used to prevent these problems. Ammonium is the preferred source of nitrogen by yeast and is firstly consumed by repressing the assimilation of other nitrogen compounds (Beltran, Novo, Rozes, Mas, & Guillamon, 2004). However, this rapid assimilation has been recently questioned by Crépin, Nidelet, Sanchez, Dequin, & Camarasa (2012), who ranked ammonium as a late consumed nitrogen source. Discrepancies between studies may be the result of a complex pattern assimilation that depends on the different study conditions used. More recently, organic nitrogen sources, such as commercial preparations containing inactivated yeast or yeast products, have become commercially available and their use as a nitrogen supplement is becoming a common practice during wine fermentation.

Nitrogen supplementation results in many changes in the course of fermentation, which may differ depending on the yeast strain used, the grape juice composition, the timing of the addition and the nitrogen source type (Bell & Henschke, 2005; Clement et al., 2013; Ugliano et al., 2009). Nitrogen supplementation has been seen to stimulate the production of some volatile compounds, such as ethyl and acetate esters, and it reduces others, such as higher alcohols (Bell & Henschke, 2005). The type of nitrogen has also been reported to result in quantitative differences for most yeast aroma and flavor compounds (Torrea et al., 2011). Amino acid and ammonium supplementation, as compared with ammonium as unique nitrogen source, has been seen to favor the production of

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ethyl and acetate esters, whereas ammonium nitrogen favors acetic acid and its ester ethyl acetate. Ammonium addition in the stationary phase has also resulted in a larger increase in glycerol than the same nitrogen addition, but in the form of valine (Clement et al., 2013). Yet despite the concentration and source of nitrogen determined the synthesis of aroma compounds, this synthesis can be modulated by the wine strain used in fermentation (Barbosa, Falco, Mendes-Faia, & Mendes-Ferreira, 2009; Barbosa, Mendes-Faia, & Mendes-Ferreira, 2012; Carrau et al., 2008; Hernández-Orte, Bely, Cacho, & Ferreira, 2006; Miller, Wolff, Bisson, & Ebeler, 2007; Torrea, Fraile, Garde, & Ancín, 2003; Vilanova, Siebert, Varela, Pretorius, & Henschke, 2012).

Winemakers sometimes proactively add nitrogen to must, even without knowing its initial nitrogen status and the consequences related with specific yeast strains used in the process. The addition of nitrogen compounds to must should follow certain criteria to prevent a large quantity of residual nitrogen, which may have negative consequences, such as microbial contamination and formation of ethyl carbamate (Araque, Bordons, & Reguant, 2013; Ough, 1991). Determination of optimum supplementation timing has also been studied (Bely, Sablayrolles, & Barre, 1990; Hernández-Orte et al., 2006; Manginot, Roustan, & Sablayrolles, 1998; Manginot, Sablayrolles, Roustan, & Barre, 1997). Nitrogen addition in the yeast growth phase increases the size of the yeast population and, conversely, additions in later fermentation stages have little effect on population size. Regarding the fermentation rate, nitrogen addition is equally effective throughout fermentation, with response kinetics decreasing in later stages (Beltran, Esteve-Zarzoso, Rozes, Mas, & Guillamon, 2005). Winemaking is an industrial process during which the growth phase covers only the first hours until nitrogen exhaustion. Thereafter, most of the sugar is consumed by resting cells in the stationary phase.

As mentioned above, most nitrogen additions are done empirically in winemaking, and they do not take into account the yeast strain's different nitrogen requirements during wine fermentation, the proper timing of these additions or the nitrogen source added. Nowadays, research is being conducted into new supplements; therefore, it is of interest to know how nitrogen source addition affects the fermentation rate, metabolite synthesis, and the production of higher alcohols and their corresponding esters.

In this context, this study aims to determine the effect of the addition of different nitrogen sources in the stationary phase in three commercial wine yeasts, which are widely used in the Spanish wine industry. Nitrogen addition is performed in the stationary phase to distinguish the impact on fermentation rate with no growth cell factor. Ammonium and arginine were used as nitrogen supplements. Although the effect of ammonium and a mixture of amino acids have been well-studied (Ancín-Azpilicueta, González-Marco, & Jiménez-Moreno, 2010; Barbosa et al., 2009, 2012; Beltran et al., 2005; Carrau et al., 2008; Hernández-Orte et al., 2006; Hernández-Orte, Ibartz, Cacho, & Ferreira, 2005; Jiménez-Martí, Aranda, Mendes-Ferreira, Mendes-Faia, & del Olmo, 2007; Mendes-Ferreira, Barbosa, Falco, Leão, & Mendes-Faia, 2009; Miller et al., 2007; Torrea et al., 2011; Vilanova et al., 2012), we aimed to focus on arginine as a sole source, which is one of the most common nitrogenous compounds in grape juice. In our previous work (Gutiérrez et al., 2012), we observed the positive effect of this amino acid on biomass production and fermentation activity. Our new approach was to determine whether the effect is also shown when the addition is carried out in the stationary phase or if it occurs only when cells are proliferated. Moreover, our objective was to analyze the impact on volatiles and non volatiles metabolites which contribute to wine quality.

2. Materials and methods

2.1. Yeast strains and inoculum preparation

The yeast strains used in this study were the following: ARM, RVA and TTA, all of which were provided by the Agrovin Company (Ciudad Real, Spain). The oenological features of these strains can be obtained from the company web page (http://www.agrovin.com). A taxonomic description of these strains was carried out by the RFLPs of the ITS/5.8S region (Guillamón, Sabaté, Barrio, Cano, & Querol, 1998). Strains RVA and TTA belonged to the species *Saccharomyces cerevisiae*, while strain ARM was identified as a hybrid between *S. cerevisiae* and *S. kudriavzevii*, following the procedure proposed by González, Barrio, & Querol (2008). This latter strain is commercialized by Maurivin as EP2 and its hybrid nature has been confirmed by Dunn, Richter, Kvitek, Pugh, & Sherlock (2012). These wine strains were used at an initial population of 2×10^6 cell/mL of active dry yeast (ADY) rehydrated in warm water prior to inoculation, according to the manufacturer's instructions (37 °C for 30 min).

2.2. Fermentation media

The synthetic grape must (SM) was prepared according to Riou, Nicaud, Barre, & Gaillardin (1997), but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) (Panreac, Spain), and with no anaerobic factors (Beltran et al., 2004). The following organic acids were used: malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L (Panreac, Spain). The following mineral salts were utilized: KH_2PO_4 750 mg/L, K_2SO_4 500 mg/L, $MgSO_4$ 250 mg/L, $CaCl_2$ 155 mg/L, NaCl 200 mg/L, $MnSO_4$ 4 mg/L, $ZnSO_4$ 4 mg/L, $CuSO_4$ 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH4)_6Mo_7O_{24}$ 1 mg/L (Panreac, Spain). The following vitamins were employed: myo-inositol 20 mg/L, calcium panthothenate 1.5 mg/L, nicotinic acid 2 mg/L, chlorohydrate thiamine 0.25 mg/L, chlorohydrate pyridoxine 0.25 mg/L and biotine 0.003 mg/L (Sigma–Aldrich, Spain). The yeast assimilable nitrogen (YAN) content in the synthetic grape must was 140 mg N/L: 42 mg N/L as ammonium nitrogen (NH_4Cl) and 98 mg N/L as the amino acids form (Sigma–Aldrich, Spain). The proportion of each amino acid was administered as previously proposed by Riou et al. (1997). This amount of YAN was established as the minimum concentration required to obtain maximum growth in the strains used in this experiment (Gutiérrez et al., 2012). The final pH of the SM was adjusted to 3.3 with NaOH.

2.3. Fermentation conditions

The first stage of the fermentation (exponential phase) was carried out at 28 °C with continuous orbital shaking (150 rpm). They were performed in 1 L glass bottles containing 900 mL of the SM under semi-anaerobic conditions since limited aeration was required to harvest samples for monitoring fermentation evolution. These samples were stored at −20 °C to analyze sugar and nitrogen consumption. When cell growth (measured by OD at 600 nm) was stopped, the fermentation medium was divided into other smaller flasks (250 mL bottles filled with 150 mL) and supplemented with different ammonium or arginine concentrations (40, 80 and 200 mg N/L). Only the control condition was not supplemented with nitrogen. After these additions, the ANKOM^{RF} Gas Production System (ANKOM Technology, NY, USA) was used to monitor CO_2 production. The wireless system consisted of a Radio-Frequency (RF) pressure sensor modules connected with the bottles (fermentors), a zero remote module that measured ambient pressure, a computer interface base coordinator and operational software. The