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New insights into the advantages of ammonium as a winemaking nutrient

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ABSTRACT

Nitrogen limitation is the most common cause for stuck or sluggish fermentation in winemaking, and it is usually dealt with by supplementing grape juice with either ammonium salts or organic nutrients. These practices have a direct impact on both fermentation kinetics and the sensorial features of the final product. The aim of this work is to provide a detailed characterization of yeast physiology in response to ammonium supplementation during alcoholic fermentation. This is done by determining changes in metabolic rates on a high frequency basis, as a sensitive way to detect the impact of fermentation conditions on yeast physiology. Our results indicate that the choice of supplementation strategy has an impact on several enological parameters like fermentation length, volatile acidity, final glycerol content, and aroma profile. Interestingly, a higher proportion of ammonium relates with improved glycerol and volatile acidity, for the same global yeast assimilable nitrogen content. However, ammonium over-supplementation has a negative impact on quality related parameters, notably on volatile acidity and aroma complexity. Production kinetics and final content of several volatile compounds are also differentially influenced by standard or excess ammonium supplementation.

1. Introduction

Availability of nitrogen sources is frequently reported as a limiting factor in winemaking (Carrau et al., 2008; Varela et al., 2004), and low nitrogen content in must is a major cause of sluggish or stuck fermentation (Bisson and Butzke, 2000). In order to prevent wine spoilage due to fermentation problems, a common practice in wineries is to supply must with nitrogen sources, usually in the form of ammonium salts at early stages of alcoholic fermentation (Jiranek et al., 1995). This is often done on a routine basis, and frequently without knowledge of the actual nitrogen content of grape juice, or the nitrogen requirements of the yeast strain in use. On occasions, this results in nitrogen over-supplementation, which can lead to the production of unwanted metabolites such as urea, ethyl carbamate or biogenic amines, by either fermenting yeast or spoilage microbiota (Ribéreau-Gayon et al., 2004).

During wine fermentation yeasts release many flavor active metabolites including acetic acid, fusel alcohols, esters or fatty acids (Rapp and Versini, 1991; Styger et al., 2011; Swiegers et al., 2005) whose formation is closely related to nitrogen metabolism (Bell and Henschke, 2005). Several studies about the effects of nitrogen supplementation on fermentation performance, production of aroma compounds, or gene expression have been recently published (Barbosa et al., 2009; Beltran et al., 2005; Garde-Cerdán and Ancín-Azpilicueta, 2008; González-Marco et al., 2010; Jiménez-Martí and del Olmo, 2008; Marks et al., 2003; Mendes-Ferreira et al., 2007; Torrea et al., 2011). The aim of this work is to quantify the short and long term metabolic responses of yeast cells, in response to ammonium supplementation and timing. By plotting short term changes in growth and metabolic rates, we identified previously unnoticed effects of nitrogen availability on yeast growth kinetics during the initial stages of fermentation. In addition, we unveiled a differential effect of ammonium and amino acids as nitrogen sources, concerning glycerol and volatile acidity production. Finally we analyzed the impact of ammonium supplementation, not only on the final levels of volatile compounds, but also on production kinetics.

2. Material and methods

2.1. Yeast strain

A single commercial wine yeast strain, *Saccharomyces cerevisiae* EC1118 (Lallemand Inc., Ontario, Canada), was used throughout this study. The strain was grown at 28 °C and routinely maintained at 4 °C on YPD plates (2% glucose, 2% peptone, 1% yeast extract, 2% agar) or in glycerol stocks at −80 °C.

2.2. Culture conditions

Two basal media, SM200C and SM100, modified from Bely et al. (1990) according to Quirós et al. (2013) and containing 200 and 100 mg/L YAN (yeast assimilable nitrogen) respectively, were used in this work (Table 1). According to previous studies, SM100 is nitrogen

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Table 1
Composition of the two basal media used in this work.

	SM200C	SM100
C sources (g/L)		
Glucose	120.0	120.0
Fructose	120.0	120.0
Malic acid	6.0	6.0
Citric acid	6.0	6.0
N sources (mg/L)		
Alanine	97.0	48.5
Arginine	245.0	122.5
Aspartate	29.0	14.5
Cysteine	14.0	7.0
Glycine	12.0	6.0
Glutamine	333.0	166.5
Glutamate	80.0	40.0
Histidine	23.0	11.5
Isoleucine	22.0	11.0
Leucine	32.0	16.0
Lysine	11.0	5.5
Methionine	21.0	10.5
Phenylalanine	25.0	12.5
Proline	400.0	200.0
Serine	52.0	26.0
Threonine	50.0	25.0
Tryptophan	116.0	58.0
Tyrosine	13.0	6.5
Valine	29.0	14.5
NH_4Cl	306.0	153.0
Micronutrients and other additives		
YNB[a] (g/L)	1.70	1.70
Myo-inositol (mg/L)	20.0	20.0
$K_2S_2O_5$ (mg/L)	60.0	60.0
Ergosterol (mg/L)	15.5	15.5
Oleic acid (mg/L)	5.5	5.5
Tween 80 (ml/L)	0.5	0.5

[a] Yeast Nitrogen Base w/o amino acids and w/o ammonium sulfate.

deficient (Martínez-Moreno et al., 2012), while SM200C is nitrogen sufficient (considering complete sugar consumption in less than five days at 28 C). Nitrogen supplementation assays were performed as described in Section 2.3.

All batch cultures (300 mL initial volume) were performed in triplicate in a DASGIP parallel fermentation platform (DASGIP AG, Jülich, Germany) equipped with four SR0400SS vessels. Agitation was maintained at 250 rpm using magnetic stirrers and the temperature was kept at 28 °C using a water bath. Medium pH was maintained at 3.5 by the automated addition of 2 N NaOH. Anaerobic conditions were maintained by gassing the headspace of the bioreactors with pure N_2 (4.5 sL/h) and the concentration of CO_2 in the off-gas measured with a GA4 gas analyzer (DASGIP AG). The off-gas was previously cooled in gas condensers (2 °C), to minimize stripping of volatile compounds, but it was not enough to completely prevent it. Consequently, ethanol yields are slightly underestimated. For inoculum preparation, yeast strain was grown in 25 mL of YPD and incubated at 28 °C and 150 rpm orbital shaking. After 48 h, cells were washed twice with sterile deionized water, resuspended in 5 mL of the appropriate synthetic must and inoculated to 0.2 initial OD_{600} (1–2 10^6 cells/mL).

2.3. Nitrogen supplementation

SM200C was supplemented with ammonium chloride to a final YAN content of 500 mg/L in order to mimic a nitrogen over-supplemented must (SM500). On the other hand, SM100 was supplemented with ammonium chloride to a final YAN content of 200 mg/L in order to mimic optimally supplemented must (SM200). Additions were performed at different time points: at inoculation time (E); and when nitrogen becomes a limiting nutrient by the end of yeast exponential growth phase (L). The exact moment for ammonium addition in the second case (L) was two hours after the first sample point showing total ammonium consumption (determined experimentally as described below). So, according to nitrogen content and supplementation, six different fermentation conditions were used in this work: SM200C, SM100, SM200E, SM200L, SM500E and SM500L.

2.4. Determination of cell growth and biomass dry weight

Cell growth was monitored by optical density (OD_{600}) using a Shimadzu UV-1800 spectrophotometer (Shimadzu Europe GmbH, Duisburg, Germany). When necessary, samples were diluted with deionized water to obtain OD_{600} values in the range of 0.1–0.4 units. OD_{600} data were then transformed to dry weight values using a calibration curve previously determined for *S. cerevisiae* EC1118 in SM200C. Experimental dry weight determination was made in triplicate by filtering 10 mL of the broth followed by 10 mL distilled water through a pre-dried and pre-weighed 25 mm nitrocellulose filter with 0.45 μm pore size (Millipore, Billerica, USA). Filters were dried at 70 °C until constant weight (12–24 h).

2.5. Analytical methods

2.5.1. HPLC and enzymatic analyses

The concentration of glucose, fructose, glycerol, ethanol and organic acids (acetic, lactic and succinic acids) was determined using a Surveyor Plus chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index and photodiode array detectors (Surveyor RI Plus and Surveyor PDA Plus, respectively) on a HyperREZ™ XP Carbohydrate H+ 8 μm column and guard (Thermo Fisher Scientific). The column was maintained at 50 °C, and 1.5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min. Prior to injection in duplicate, samples were filtered through 0.22 μm pore size nylon filters (Espectrocroma, Madrid, Spain) and diluted when necessary. The concentration of each amino acid was analyzed in duplicate according to the method described by Gómez-Alonso et al. (2007) using an Accela 600 chromatograph (Thermo Fisher Scientific) equipped with a PDA detector and a ACE 5 C18-HL 5 μm column and guard (ACE, Aberdeen, Scotland). Ammonium was assayed spectrophotometrically using a specific R-Biopharm assay kit (Darmstadt, Germany). YAN was calculated taking into account the number of assimilable atoms of nitrogen from each amino acid according to Martínez-Moreno et al. (2012).

2.5.2. Analysis of volatile compounds

Volatile compounds were analyzed in duplicate using headspace solid-phase micro extraction coupled with gas chromatography–mass spectrometry (HS-SPME/GC–MS) by a modification of the protocol described by Ortega et al. (2001) using a Thermo Scientific Trace GC Ultra gas chromatograph equipped with a Thermo Scientific TriPlus Autosampler and a Thermo Scientific ISQ mass detector.

The evolution of 22 volatile compounds directly related to yeast metabolism (butanoic acid, valeric acid, isovaleric acid, hexanoic acid, octanoic acid, decanoic acid, dodecanoic acid, propanol, isobutanol, 2-methylbutanol, 3-methylbutanol, 2-phenylethanol, ethyl acetate, ethyl butanoate, ethyl isobutanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate, isoamyl acetate, phenylethyl acetate, and methionol) was analyzed for each condition. One gram of sodium chloride was added to 20 mL headspace vials containing 2 mL of filtered fermentation broth and 500 μL of 25 ppm internal standard solution that consisted of heptanoic acid, 2-butanol, 2-ethylhexanol, 4-methyl-2-pentanol, 1-nonanol, ethyl heptanoate and ethyl nonanoate in 1.25% ethanol. Briefly, the vial was tightly capped with a PTFE/Silicone cap and then heated for 10 min at 70 °C. Then, a Supelco 50/30 μm DVB/CAR/PDMS fiber was exposed to the headspace of the sample vials for 30 min and desorbed in the GC inlet for 4 min. The GC temperature program was as follows: 40 °C (5 min hold), 3 °C/min up to 200 °C and 15 °C/min up to 240 °C (10 min hold). The 0.75 mm I.D. SPME