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Nitrogen modulation of yeast fitness and viability during sparkling wine production

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ABSTRACT

In the production of sparkling wine by the traditional method a second fermentation inside the bottle is required. To survive in such conditions of high ethanol concentration and high pressure, yeast cells must previously undergo an acclimation process. In this study, we investigated the role of the nitrogen composition during the acclimation phase by measuring growth and fermentative parameters through the second fermentation process. We used eight *Saccharomyces cerevisiae* strains of different origin to determine the impact of yeast genetic background on the efficiency of the acclimation process. The nitrogen source used in the acclimation media had a strong impact on yeast growth during this phase, but also affected significantly fermentation kinetics during the second fermentation. The yeast strain origin mostly affected the second fermentation kinetics. Surprisingly, the use of a medium rich in amino acids that are precursors of fusel alcohols, although triggered slow growth during the acclimation phase, it increased yeast viability and fitness through the second fermentation. Overall, we demonstrated how the nitrogen composition of the acclimation media impacts on yeast fitness and viability. The modification of the nitrogen composition during this phase is proposed as a tool to optimize yeast performance during the second fermentation.

1. Introduction

Microbial acclimation occurs naturally and allows the microbial community to adjust its metabolism to the ever-changing environment. Furthermore, microorganism acclimation is used in several biotechnological processes in the food industry that require an inoculum adapted to a given stress, in order to maintain its metabolic activity in the stressful conditions, e.g. to optimize the leavening activity of the yeast in the bakery industry (Zamani et al., 2008) or to ensure the successful fermentation of ice wines (Kontkanen et al., 2004). In enology, sparkling wine production involves the use of an acclimated inoculum (pied-de-cuve) to ensure the successful completion of the process. Its production involves two fermentation steps. The first alcoholic fermentation transforms the grape must into the base wine. The second step consists of the transformation of the base wine into sparkling wine due to a second fermentation that, in the traditional method, takes place inside the bottle. This second fermentation begins after bottling the base wine and the "liqueur de tirage", which contains sucrose (20–24 g/l), adjuvant (usually bentonite, to promote the flocculation and later removal of the yeast cells) and an acclimated yeast inoculum (Buxaderas and López-Tamames, 2003; Carrascosa et al., 2011). In the bottle, the yeast population must face several environmental stresses, including the increasing CO_2 pressure, the high ethanol content of the base wine and the low temperatures of fermentation. To overcome these restrictive conditions, the yeast population must be properly acclimated before being inoculated into the base wine. The acclimation process usually involves the culture of the desired yeasts in diluted wine that contains increasing concentrations of ethanol and sugar. Kunkee and Ough (1966) analyzed the effect of the acclimation process on the growth and fermentation capability of *Saccharomyces cerevisiae* during the second fermentation. These authors reported that the acclimation of yeast in a base wine, either under or without CO_2 pressure, is essential for a quick and efficient completion of the second fermentation. Other studies analyzed the effect of aeration during the pied-de-cuve, the use of wine as the adaptation medium and the quantity of inoculum on the fermentation development

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(Juroszek et al., 1987; Laurent and Valade, 2007; Monk and Storer, 1986).

The role of nitrogen in wine production has been extensively studied, and it is known to affect growth and biomass production, fermentation kinetics and even the organoleptic characteristics of the final product (reviewed by Bell and Henschke (2005)). The role of nitrogen composition in the ability of yeast to overcome stressful conditions, such as fermenting high sugar musts, has also been recently analyzed (Martínez-Moreno et al., 2012). However, nitrogen preferences and utilization diverge among different yeast strains (Brice et al., 2014b; Gutiérrez et al., 2012), partly due to their genetic variations (Brice et al., 2014a; Ibstedt et al., 2014; Jara et al., 2014). The selected yeast strain has also a clear effect on both the fermentation kinetics (Martí-Raga et al., 2015) and the organoleptic characteristics of the final product, mostly due to its autolytic capacity (reviewed by Alexandre and Guilloux-Benatier (2006)).

The nitrogen concentration of the base wine will strictly depend on both the grape must nitrogen content and how much is consumed by yeast during the first fermentation. Our recent work (Martí-Raga et al., 2015) demonstrated that although the nitrogen content of the base wines is highly variable (17–75 mg N/l), a correlation between second fermentation kinetics and the nitrogen content could only be established for those base wines containing less than 30 mg N/l. Furthermore, we demonstrated that the nitrogen taken up during the pied-de-cuve is enough to cover the yeast nitrogen requirements during the second fermentation, independently of the nitrogen content of the base wine, and clearly affects the development of the fermentation, with strain-dependent nutrient preferences. Thus, the aim of this study is to understand how different sources of nitrogen in the pied-de-cuve can modulate yeast strain viability and fitness during the second fermentation. In order to determine also strain-specific requirements for given forms of nitrogen sources, several nitrogen mixtures, as well as several yeast strains of different natural origin, were used in the whole process. We propose variations on the nitrogen composition of the acclimation media as a tool to optimize yeast fitness for a given biotechnological process, such as sparkling wine production.

2. Materials and methods

2.1. Yeast strains used and propagation media

Eight *S. cerevisiae* yeast strains were used in this study, most of them being wine strains. However, to better capture the phenotypic variability of *S. cerevisiae* species two strains of distillery and one isolated from oak bark were also included. These strains were either wine starters supplied by the Laffort company (F10 and Spark) or diploid monosporic clones derived from different origins, such as wine (VL3, GN and SB), distillery (A24 and 294) or oak bark (OS104) (Supplementay Table S1). Before the experiments, yeast cells were cultivated 24 h at 25 °C in YNB medium (2% glucose, 0.17% YNB *w/o* amino acids and ammonium) supplemented with 140 mg N/l of ammonium phosphate.

2.2. Media for the pied-de-cuve and second fermentation

The experiment consisted of two successive steps: the adaptation (48 h) and the proliferation phases (96 h). Both steps were performed in a chemically defined media simulating base wine. This synthetic base wine contained sucrose 50 g/l, tartaric acid 4 g/l, L-citric acid 0.5 g/l, malic acid 0.5 g/l, sodium acetate 0.134 g/l, YNB *w/o* amino acids and ammonium 1.7 g/l and glycerol 4.0 g/l. The pH was adjusted to 3.3 with potassium hydroxide. The ethanol concentration was adjusted to 6 and 8% (v/v) for the adaptation and proliferation stages, respectively. In the adaptation phase, the nitrogen source used was 20 mg N/l diammonium phosphate. For the proliferation phase, six different nitrogen mixtures were used with a final concentration of 60 mg N/l (Table 1). The first three mixtures (I, Mu, and BW) attempted to emulate the common industrial practices, containing diammonium phosphate (I) or the nitrogen content of grape must (Mu) or base wine (BW). The three other nitrogen mixtures (O, Ar and AG) were experimental compositions used for investigating the effect of several organic nitrogen sources. The mixture O contained the average organic nitrogen content found in nitrogen-rich fermentation activators. The mixture Ar contained five amino acids that are precursors of aromatic compounds and showed endurance properties of yeast during the alcoholic fermentation when used as the sole source of nitrogen (Martínez-Moreno et al., 2012). The mixture AG combined the amino acids present in Ar with amino acids of quick uptake (glutamine/glutamate). This mixture was only used for the experiment on yeast viability in the second fermentation.

The second fermentation was conducted using natural base wine (ethanol 10.11% (v/v), pH = 3.1, YAN = 30.8 mg N/l), kindly donated by Juve & Camps (Penedés, traditional mixture of Macabeu, Xarel·lo and Parellada grape varieties) supplemented with 22 g/l sucrose.

2.3. Growth parameters for the pied-de-cuve

At the end of the adaptation step of pied-de-cuve (48 h, 25 °C), yeast growth was measured (OD_{600} readings) and yeast strains were inoculated (OD_{600} = 0.2) into a fresh synthetic base wine for the proliferation phase (96 h, 25 °C). During the proliferation stage of pied-de-cuve cell growth was monitored in 96-well microcultivation plates using a POLARstar Omega (BMG Labtech, Offenburg, Germany). Culture plates were shaken every 30 min for 60 s prior to the OD_{600} measurement. The well position on the microplate was randomized, and six replicates were run for each condition. Data from the microplate reader were transformed with the polynomial curve $y = -0.0018^{*}x^3 + 0.1464^{*}x^2 + 0.7757^{*}x + 0.0386$ to correct the nonlinearity of the optical recording at higher cell densities. The polynomial curve was obtained following the protocol described by (Warringer and Blomberg, 2003).

Growth kinetic data were fitted using a modified 3PL model to include an evaporation coefficient. The incorporation of the evaporation coefficient into the model allowed us to rectify the steady increase of the OD_{600} value due to the evaporation of the media that occurs once the stationary plateau is reached. The final equation was as follows,

$$N_t = \frac{KN_0 e^{rt}}{K + N_0(e^{rt} - 1)} + \text{evap}^{*}t$$

Here, *Nt* is the OD_{600} at time *t*, *K* is the maximum population (maximum OD_{600} reached), N_0 is the initial OD_{600} value, *r* is the growth rate and *evap* is the evaporation coefficient. The fitting of the data allowed us to extract *K* (OD units) and *r* (OD/h). We also calculated time-related parameters, such the lag phase (*lag*, h) (Fig. 1A).

2.4. Second fermentation in bottles: measurement of the fermentation kinetics

The kinetics of CO_2 production during the second fermentation were measured in a separate experiment. Yeasts were adapted following the previously described pied-de-cuve protocol. To obtain sufficient volume to inoculate the base wine, the pied-de-cuve was conducted using sterile vessels of 100 ml. To confirm