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Optimization of fermentation-relevant factors: A strategy to reduce ethanol in red wine by sequential culture of native yeasts

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ABSTRACT

Current consumer preferences are determined by well-structured, full-bodied wines with a rich flavor and with reduced alcohol levels. One of the strategies for obtaining wines with reduced ethanol content is sequential inoculation of non-*Saccharomyces* and *Saccharomyces cerevisiae* yeasts. However, different factors affect the production of metabolites like ethanol, glycerol and acetic acid by inoculated yeasts. In order to obtain low alcohol wines without quality loss, the aims of our study were: i) to determine optimum conditions (fermentation temperature and time of permanence and initial inoculum size of the non-*Saccharomyces* population at the beginning of the process, prior to inoculation with *S. cerevisiae*); ii) to validate the optimized factors; and iii) to assess sensory quality of the wines obtained after validation. Two combinations of yeasts were used in this study: *Hanseniaspora uvarum* BHu9/*S. cerevisiae* BSc114 and *Candida membranaefaciens* BCm71/*S. cerevisiae* BSc114. Optimization of three fermentation factors that affect to non-*Saccharomyces* yeasts prior to *S. cerevisiae* inoculation was carried out using a Box-Behnken experimental design. Applying the models constructed by Response Surface Methodology, the lowest ethanol production by *H. uvarum* BHu9/*S. cerevisiae* BSc114 co-culture was obtained when *H. uvarum* BHu9 was inoculated 48 h 37 min prior to *S. cerevisiae* inoculation, at a fermentation temperature of 25 °C and at an initial inoculum size of 5×10^6 cells/mL. Lowest alcohol production with *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 was observed when *C. membranaefaciens* BCm71 was inoculated 24 h 15 min prior to *S. cerevisiae* at a fermentation temperature of 24.94 °C and at an initial inoculum size of 2.72×10^6 cells/mL. The optimized conditions of the two co-cultures were subsequently submitted to lab-scale validation. Both proposed strategies yielded ethanol levels that were significantly lower than control cultures (*S. cerevisiae*). Wines fermented with non-*Saccharomyces*/*Saccharomyces* co-cultures under optimized conditions were also associated with higher aromatic complexity characterized by the presence of red fruit aromas, whereas wines obtained with *S. cerevisiae* BSc114 were described by parameters linked with high ethanol levels.

1. Introduction

Spontaneous alcohol fermentation is a process carried out by a complex group of diverse yeast species associated with the grape and enological environment, and generally classified into two groups: non-*Saccharomyces* and *Saccharomyces* yeasts. The development of non-*Saccharomyces* yeasts during the early stage of winemaking is still considered an uncontrollable risk by some scientists and enologists, whereas for others, their participation is a way to enhance the wine quality (Albertin et al., 2017). Numerous researchers have reported exhaustive and appropriate screening methods to select non-*Saccharomyces* yeasts that positively affect the winemaking process (Comitini et al., 2011; Mestre et al., 2017; Varela, 2016; Viana et al., 2008).

Current consumer preferences are determined by well-structured, full-bodied wines with a rich flavor and with reduced alcohol levels. However, successful accomplishment of this set of traits is rather difficult due to imbalance between sugar accumulation and phenolic maturity of the berries (which require late harvest in order to guarantee proper aromatic and phenolic maturity) (Goold et al., 2017). Diverse strategies (viticultural, pre-fermentation, microbiological and post

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fermentation practices) have been proposed to obtain wine with reduced ethanol content, and the use of native non-*Saccharomyces* yeasts could be emphasized (Contreras et al., 2014). Some authors have reported a reduction in ethanol concentration using non-*Saccharomyces* yeasts in co-cultures with *S. cerevisiae* (compared to the ethanol concentration obtained with a single *S. cerevisiae* inoculum) (Ciani et al., 2014; Contreras et al., 2014, 2015; Englezos et al., 2016; Varela, 2016). In a previous study, we evaluated 114 non-*Saccharomyces* yeasts to determine their respiratory, fermentation and physiological characteristics in order to select yeast isolates as candidates for the design of sequential co-culture for production of wine with reduced alcohol content (Mestre et al., 2017). However, many factors are involved in the yeast-yeast interactions and metabolite production of the inoculated yeasts. Optimization of some factors to favor the desired metabolic activity of the selected non-*Saccharomyces* yeasts is very important to obtain good results.

It is well known that different factors can affect the course of the fermentation process, affecting the performance and adaptation of each specific yeast population. Temperature is one of the main variables that directly affects microorganism growth and membrane composition (Charoenchai et al., 1998), and consequently it defines the final wine composition (Torija et al., 2003). A number of researchers have emphasized that low fermentation temperatures (10–15 °C) increase tolerance of certain non-*Saccharomyces* yeasts to ethanol and high sugar concentrations, and therefore they can remain longer in the process (Gao and Fleet, 1988; Tofalo et al., 2012; Zott et al., 2008).

The use of different yeast starter species under co-culture conditions needs to be carefully monitored and analyzed prior to its application in winemaking.

In order to promote the impact of non-*Saccharomyces* populations on the final wine composition, several authors have shown that by increasing the inoculation ratio of the non-*Saccharomyces* (NS) population (like NS:*S. cerevisiae* 10:1, 100:1 or 1000:1) they could outgrow *S. cerevisiae* populations, which would prolong persistence of NS populations (Comitini et al., 2011; Domizio et al., 2011; Pérez-Nevado et al., 2006). Another strategy is the use of sequential inoculation, which delays *S. cerevisiae* starter development. This approach is supposed to mimic spontaneous fermentation, and it would allow more interaction between NS and *S. cerevisiae* yeast populations (Ciani et al., 2016). Therefore, the concentration and viability of non-*Saccharomyces* inocula at the beginning of the alcoholic fermentation as well as the period of time that non-*Saccharomyces* yeasts remain alone (in absence of *S. cerevisiae*) is crucial to their implantation and persistence in the process.

Response Surface Methodology (RSM) is a very useful statistical and mathematical tool to optimize a response (output variable) which is influenced by several independent variables (input variables) (Behera et al., 2018). Several researchers have studied optimization of factors that affect winemaking such as pH, temperature, ammonium and glucose concentrations, inoculum size, and inoculation strategy (Arroyo-López et al., 2009; D'amato et al., 2006; Englezos et al., 2016; Gosh et al., 2012). However, more information is necessary to better understand NS yeasts during winemaking in order to reduce ethanol in wines. In this context, we proposed optimizing three controllable factors that directly affect the performance of non-*Saccharomyces* yeasts during the fermentation using a Box-Behnken experimental design (BB). This method is suitable for exploration of quadratic response surfaces and it generates a second degree polynomial model, which is used to optimize a process using a small number of experimental runs (Amenaghawon et al., 2013). In order to obtain wines with reduced ethanol without loss of quality, the aims of our study were: i) to determine the optimum conditions of the time of permanence of non-*Saccharomyces*, fermentation temperature and the non-*Saccharomyces* inoculum size at the beginning of the process prior to *S. cerevisiae* inoculation; ii) to validate the optimized factors; and iii) to assess sensory quality of the wines obtained after validation.

2. Materials and methods

2.1. Microorganisms

Hanseniaspora uvarum BHu9, *Candida membranaefaciens* BCm71 and *Saccharomyces cerevisiae* BSc114 were used in this study. These yeasts have been previously isolated from oenological environments and molecularly identified by our research group (Maturano et al., 2015). Selection of the yeasts was based on their fermentative performance and respiratory characteristics to be employed in sequential inoculation to obtain wines with reduced ethanol content (Mestre et al., 2017). Strains were stored and cryogenically preserved at −80 °C in the Culture Collection of Autochthonous Microorganisms (Institute of Biotechnology, School of Engineering-UNSJ, San Juan, Argentina).

2.2. Yeast inoculum preparation

Each strain was grown on YEPD-agar (g/L): Yeast extract 10, peptone 20, glucose 20, agar-agar 20, and incubated at 25 °C. After 48 h, entire plates were collected and inoculated in YEPD-broth at 25 °C for 4 h under aerobic conditions (130 rpm). After that, the pre-inoculum was transferred to the grape must (13 °Brix, pH 3.8, supplemented with 0.1% yeast extract and 0.4% peptone) and kept at 25 °C during 12 h under aerobic conditions (130 rpm). Cell counts were carried out with an improved Neubauer counting chamber. The pre-adaptation in YEPD broth, was carried out in order to reduce the lag-stage in the grape must, which would mean that strains started growing exponentially in the grape juice.

2.3. Experimental design

A Box–Behnken (BB) experimental design was employed to assess the effects of three independent variables at three different levels for ethanol reduction (Table 1). The selection of the variables and their levels was established into a range of real conditions employed in winemaking. BB designs were independently assayed for the following yeast combinations: BHu9/BSc114 and BCm71/BSc114. Time of permanence of non-*Saccharomyces* (NS) prior to *S. cerevisiae* BSc114 inoculation (X_1), temperature of must prior to *S. cerevisiae* BSc114 inoculation (X_2) and initial inoculum size of NS yeasts (X_3) were determined. Minimum, medium and maximum time of permanence, temperature and inoculation size assayed were: 24, 48 and 72 h; 15, 20 and 25 °C; and 1, 3 and 5 × 10^6 cells/mL, respectively. Then, each experiment was inoculated with 2 × 10^6 cells/mL of *S. cerevisiae* BSc114 in order to complete alcoholic fermentation under static conditions at 25 °C ± 2.

Ethanol production was selected as response variable, while glycerol and acetic acid were used as quality parameters. A total of 15 experiments, including three replicates of the center point, were carried out in 75 mL of sterile grape must at 21 °Brix (pH was adjusted to 3.8 with tartaric acid, total acidity 5.5 g/L). All fermentations were conducted under static conditions and monitored through the release of CO_2 by measuring daily weight loss. Gentle manual agitation of the Erlenmeyer flask was performed every 24 h for 5 s to assure oxygen availability in the fermentation medium.

Table 1
Summary of the three parameters evaluated according to the Box-Behnken design for two yeast combinations: BHu9/BSc114 and BCm71/BSc114.

Levels	X_1 - time of permanence of NS yeasts (h)	X_2 - temperature (°C)	X_3 – inoculum size of NS yeasts (×10^6 cells/mL)
Minimum	24	15	1
Medium	48	20	3
Maximum	72	25	5