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Use of a submerged yeast culture and lysozyme for the treatment of bacterial contamination during biological aging of sherry wines

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ABSTRACT

The aim of this study was to determine the effect of a submerged culture of "flor" velum yeast and lysozyme as treatments against bacterial contamination by lactic acid and acetic acid bacteria during the biological aging of sherry wine. Different submerged culture treatments with lysozyme were performed. To assess these treatments, we performed laboratory assays with wine spoiled by lactic acid bacteria (LAB) and/or acetic acid bacteria (AAB) selected from the "solera" sherry wine cellar system. The results indicate that lysozyme should be added to submerged culture conditions after the start of the yeast growth phase. The application of lysozyme at a dose of 12.5 g/hL and submerged culture conditions was very effective for the treatment of advanced heterolactic fermentation during the biological aging of wines, thereby inhibiting the LAB population and reducing the volatile acidity of the wines (<0.1 g/L). Similarly, the submerged culture was a very effective tool for the treatment of AAB contamination of recovering wines with high volatile acidity and the removal of AAB without alcohol correction of the wine. The combined lysozyme and submerged culture treatment was very effective at removing bacteria from the wine, reducing volatile acidity and preventing acetification.

1. Introduction

The biological aging of sherry wine ("fino o manzanilla") is a microbiological process that occurs under a biofilm of a *Saccharomyces* yeast races called the "flor velum yeast" (Martínez, Codón, Pérez, & Benítez, 1995; Pozo-Bayón & Moreno-Arribas, 2011). The "flor" velum develops spontaneously on the wine surface after alcoholic fermentation is completed and the young wine is fortified with at least 15% v/v wine alcohol of (Martínez de la Ossa, Caro, Bonat, Pérez & Domecq, 1987). The oxidative metabolism of the yeast reduces the ethanol, acetic acid, ethyl acetate, glycerol, amino acid and organic acid concentrations (Martínez de la Ossa, Pérez & Caro, 1987; Martínez, Varcárcel, Pérez & Benítez, 1998). Ethanol is the main source of energy for the cell and is transformed into acetaldehyde by alcohol dehydrogenase (Plata, Mauricio, Millán, & Ortega, 1998).

Yeast biofilms develop under difficult conditions, such as low oxygen concentrations, high ethanol concentrations (from 15% v/v to 16% v/v) and low pH (Alexandre, 2013; Pérez, 2005). Consequently, more than 95% of the biofilm microorganisms may be composed of film-forming *Saccharomyces cerevisiae* races (Alexandre, 2013; Martínez, Pérez, & Benítez, 1997; Mesa, Infante, Rebordinos, & Cantoral, 1999; Pozo-Bayón & Moreno-Arribas, 2011). However, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and non-*Saccharomyces* yeast can coexist with the velum yeast (Ibeas, Lozano, Perdigones, & Jiménez, 1996; Moreno-Arribas & Polo, 2008; Suárez-Lepe & Iñigo-Leal, 2004). Anaerobic conditions (anoxia) due to biological aging and the low concentration of free sulfur dioxide can facilitate LAB proliferation in the wine. Furthermore, the development of this bacteria is influenced by the gluconic acid content of the grapes used to produce this wine. Therefore, the high gluconic acid content (>600 mg/L) produced by *Botrytis cinerea* infection leads to heterolactic fermentation that results in high lactic acid and volatile acidity levels (Pérez, Valcárcel, González, & Domecq, 1991). In some cases, the development of lactic acid bacteria can lead to the "ropy" phenomenon (Carreté, Vidal, Bordons, & Constanti, 2002; Suárez & Agudelo, 1993). "Ropy" wine is characterized by an increase in the viscosity of the wine due to the production of extracellular polysaccharides by LAB in addition to high levels of lactic acid and volatile acidity and the production of biogenic amines (Moreno-

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Arribas & Polo, 2008; Suárez & Agudelo, 1993; Suárez, Callejo, & Colomo, 1994). Moreno-Arribas and Polo (2008) studied the occurrence of lactic acid bacterial populations during different stages of biological aging and showed that the populations of these bacteria remained low. *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus brevis*, and *Lactobacillus zeae* are the most commonly isolated bacterial species from biologically aged wines (Moreno-Arribas & Polo, 2008; Suárez et al., 1994). Lasanta, Roldán, Caro, Pérez, and Palacios (2010) identified *Lactobacillus* from 94% of the lactic acid bacteria isolated from oak barrels with advanced heterolactic fermentation (37% *Lactobacillus hilgardii*, 31% *L. plantarum* and 26% *L. brevis*). These species also showed good adaptation to high alcohol contents and temperature and pH variations (Vila, Rodríguez, Fernández, & Hernanz, 2010).

Acetic acid bacteria (AAB) are considered one of the most common wine spoilage microorganisms and are a threat for oenologists. Acetic acid bacteria have been widely associated with the occurrence of high volatile acidity in wines (Guillamón & Mas, 2011). These bacteria are taxonomically classified as obligate aerobic organisms and aerobic conditions are required for their development in the wine. However, some authors indicate that these bacteria may survive and even multiply under semi-anaerobic conditions, such as those found in wine stored in tanks and barrels (Drysdale & Fleet, 1988; Joyeux, Lafon-Lafourcade, & Ribereau-Gayon, 1984).

AAB are very rarely found during biological aging when the "flor" velum yeast is active and covers the entire surface of the wine (Casas, 2008). However, if the velum sinks during aging due to the high temperatures and/or volatile acidity that are often caused by lactic bacteria or *Brettanomyces* (Ibeas et al., 1996), acetic acid bacteria can develop and significantly increase the volatile acidity of the wine (Alexandre, 2013; Lasanta et al., 2010). Under these conditions, the regeneration and growth of the "flor" yeast is very difficult.

Control of LAB and AAB growth typically relies on the concentration of SO_2 used; however, a concentration of at least 100 mg/L of total SO_2 appears to be required for control (Joyeux et al., 1984; Watanabe & Lino, 1984). The use of these doses of SO_2 in sherry wine can control bacterial contamination; however, high SO_2 levels delay velum formation (Suárez & Agudelo, 1993). Moreover, practically all of the SO_2 is found in combination in this type of wine due to the high acetaldehyde concentration (Casas, 2008).

Therefore, it is necessary to develop new alternatives for the control and elimination of lactic bacteria and acetic acid bacteria in biological aging wines. One current alternative for lactic acid bacteria is the use of lysozyme (Liburdi, Benucci, & Esti, 2014). Lysozyme has the ability to hydrolyze the b (1,4) glycosidic bond between the N-acetylmuramic acid and the N-acetyl-D-glucosamine of peptidoglycan, the major component of the cell walls of Gram-positive bacteria (Losso, Nakai, & Charter, 2000). Lasanta et al. (2010) studied the use of lysozyme as an alternative tool for the prevention and treatment of spoilage by lactic acid bacteria in biological aging wines. These authors showed that the addition of lysozyme was effective at correcting and preventing heterolactic fermentation in this type of wine, with an optimal dose of 12.5 g/hL. However, velum development, the yeast metabolism and the wine characteristics were influenced by the yeast inoculation protocol and the methodology used for lysozyme addition (Roldán, Lasanta, Caro, & Palacios, 2012). The application of lysozyme together with inoculation of the "flor" velum yeast was very effective for the treatment of heterolactic fermentation in biological aging wines because lysozyme reduced the LAB population and the "flor" velum yeast reduced the acetic acid content of the wine. However, this treatment was not effective when the volatile acidity was above 1 g/L. Under these conditions, the growth of the "flor" was inhibited and the acetic bacteria began to appear and produce volatile acidity due to their ethanol metabolism (Lasanta et al., 2010).

Some authors have shown that "flor" yeast in submerged cultures exhibited rapid metabolism of acetaldehyde production and consumption of acetic acid (Ough & Amerine, 1958; Ter-Karapetian, 1953). Submerged cultures of *Saccharomyces cerevisiae* var. *capensis* can consume gluconic acid and accelerate aging compared with traditional biological aging under "flor" yeast velum without modifying the wine's sensory profile (Peinado, Mauricio, Ortega, Medina, & Moreno, 2003). However, there are no studies on the ability of "flor" yeast to resist high levels of volatile acidity under submerged culture conditions and their specific role in the reduction of volatile acidity. Additionally, the influence of lysozyme on yeast in submerged cultures is unknown.

The objective of this study was to investigate the use of lysozyme and submerged culture conditions with "flor" yeast as an alternative tool for the treatment of spoilage produced by lactic acid bacteria and acetic acid bacteria during the biological aging of wines.

2. Material and methods

2.1. Velum yeast

The velum yeast was obtained from the biological aging system of a sherry wine cellar and was identified as *Saccharomyces cerevisiae beticus* (strain codified as "B16" by Martínez et al., 1995). The velum yeast was disaggregated with an ultrasonic bath and homogenized in a small volume of wine for subsequent inoculation at a known concentration using a submerged protocol (Roldán et al., 2012).

2.2. "Flor" yeast submerged culture

To perform the assay under submerged culture conditions, we used 5 L tanks with a cooling jacket to maintain the temperature between 20 and 22 °C. Aeration was conducted through diffusers that allowed micro-aeration of the wine at a rate of 0.05 L/min. Continuous shaking at 400 rpm was performed during the experiments. In all assays, the oxygen concentration was kept below 1 ppm. All experiments were performed in triplicate.

In all assays, a starter of the submerged "flor" yeast culture was used ($>10^{12}$ viable cells/mL). The starter cultures were prepared using "sobretablas" wine previously filtered with a membrane filter with a 0.22 μm pore size. The starter culture composition is provided in Table 1. The "sobretablas" wine is a young wine fortified with ethanol (from wine distillation) to a 15% v/v alcoholic content once fermentation is finished. This wine is used in the first stage of the aging process ("solera" system) of sherry wine. The starters were prepared in Erlenmeyer flasks with 500 mL of wine and a population of "flor" yeast > 10^6 cells/mL at 20–22 °C with aeration provided by micro-oxygenation diffusers.

2.3. Lysozyme

Lysozyme (hydrochloride form) used was Enovinlyso (Agrovin, S.A., Ciudad Real, Spain). According to the commercial instructions, this lysozyme was extracted from a hen egg and had a protein purity of 100% and a FIP activity >35,000 IU/mg. A FIP unit is the measurement of enzyme activity according to the test methods of the Federation Internationale Pharmaceutique. Lysozyme was used at a dose of 12.5 g/hL based on the effective treatment of advanced heterolactic fermentation (Lasanta et al., 2010).