Innovative Food Science and Emerging Technologies 35 (2016) 21–28

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Post-harvest control of wine-grape mycobiota using electrolyzed water

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ARTICLE INFO

Article history:
Received 24 January 2016
Received in revised form 22 March 2016
Accepted 23 March 2016
Available online 29 March 2016

Keywords:
Electrolyzed water
Grape
Wine
Yeast dynamics
Sanitization
Innovative treatment

ABSTRACT

Electrolyzed water (EW) has recently attracted much attention due to its efficacy against a broad spectrum of microorganisms. In this study, we investigated the impact of two EW treatments (40 and 400 mg/L free chlorine) on grape mycobiota using culture-dependent and -independent approaches. Moreover, the effect of yeast inoculation on treated and non-treated grapes was also considered. At the end of the fermentation, the wines produced were subjected to chemical and aroma analyses. The results revealed a decrease of about 0.5 log CFU/mL of the total yeast population on grapes surface independently of the dose of EW applied. Yeast inoculation and EW treatments shortened the time needed by *Saccharomyces cerevisiae* to dominate apiculate yeasts, particularly, 2 days for inoculated and 7 days for spontaneous fermentations. A decrease of acetic acid (about 55%) was also observed compared to untreated spontaneous fermentation. In addition, aroma analysis highlighted a positive contribution of inoculated yeast on the wine aromas, since they had approximately 50 % higher pleasant esters compared to spontaneous fermented wines.
Industrial Relevance: Sulfur dioxide is widely used in crushed grapes prior to fermentation due to its antimicrobial and antioxidant activity. However, legislative rules, health risks and negative consumer perception related to its presence and use have resulted in a need to find new sanitizers able to reduce its use. The effectiveness of EW to reduce yeast species able to produce high levels of undesirable compounds was demonstrated. This research introduced an innovative antimicrobial agent, which could assist in the first step of wine production to reduce the use of SO_2.

1. Introduction

In wine, like in other fermented beverages, fermentations occur under conditions in which microbial activities, either from inoculated or environmental yeasts and bacteria, have a substantial role in the quality characteristics of the final product (Bokulich, Ohta, Richardson, & Mills, 2013). The adoption of fermentation practices, which limit spoilage by controlling the growth of desirable microorganisms is fundamental in order to enhance wine quality and safety (Du Toit & Pretorius, 2000). Sulfur dioxide (SO_2) is an antimicrobial agent commonly used in crushed grapes to inhibit the growth of spoilage microorganisms, including apiculate yeasts, acetic and lactic acid bacteria, and to minimize the oxidation of phenolic compounds (Boulton, Singleton, Bisson, & Kunkee, 1996).

In spite of these advantages, the resulting sulfites from the addition of SO_2 have been related to headaches, allergic reactions and breathing difficulties in asthma patients (Santos, Nunes, Saraiva, & Coimbra, 2012; Vally, Misso, & Madan, 2009). This negative impact of SO_2 led the International Organization of Vine and Wine (OIV) to reduce the maximum concentration limit to 150 mg/L and 200 mg/L (European Union Regulation: No 606/2009) for the red and white wines, respectively. In Europe, wine producers must indicate the presence of sulfites on the bottle when this exceeds 10 mg/L, due to restrictions applied by law (European Union Regulation: No 1991/2004). In addition to these legislative rules, mainstream consumers have become more health-conscious in the last decade, and focus their attention on healthy and natural products free of substances that are considered negative, such as chemical preservatives (Bech-Larsen & Scholderer, 2007).

The addition of SO_2 in winemaking industry is a complex subject, because many compounds bound with SO_2 by reducing its effectiveness against microbial proliferation and oxidation. In this context, the use of moderate levels of SO_2 prior to fermentation does not ensure an antiseptic protection, since the added SO_2 binds rapidly with the abundant grape sugars and as a consequence the percentage of free SO_2 declines (Ribéreau Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). Thus, there is an increasing interest in the search of innovative technologies able to reduce the levels of SO_2 in this stage of vinification. Further, the chance of a possible replacement of this additive could be particularly important in 'sulphite free' wines production (i.e. without SO_2 addition).

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http://dx.doi.org/10.1016/j.ifset.2016.03.010
1466-8564/

To this regard, the use of EW as sanitization agent is growing in popularity in the last decades due to the high antimicrobial activity against a wide spectrum of microorganisms (Hricova, Stephan, & Zweifel, 2008) and its simple generation by electrolysis from potable water and a salt (KCl) solution only (Buck, Iersel, Oetting, & Hung, 2002). EW can be produced on site with low production costs, while the treated water could be recycled during the harvest season by adding pure EW, favouring a wider implementation of this technology on an industrial scale. Concerning these positive aspects, in 2011 the Food and Drug Administration (FDA) declared EW to be considered as *Generally Recognized As Safe* (GRAS) substance to wash or to assist in peeling of fruit and vegetables, since it meets the requirements specified in 21CFR173.315 (FDA 2011).

Since that time, the application of EW in food industry has increased significantly (Jermann, Koutchma, Margas, Leadley, & Ros-Polski, 2015). Several studies investigated the antimicrobial effect of electrolyzed water in a wide variety of post-harvest fruits and vegetables. Despite this extensive use of EW in food industry, little is known about the application of EW in winemaking industry, except for few studies about the decay of *Botrytis cinerea* and the treatment effectiveness during the storage of post-harvest table grapes (Guentzel, Lam, Callan, Emmons, & Dunham, 2010; Kim, Chung, Kang, Chung, & Choi, 2003).

Information regarding the efficiency of EW to reduce or replace SO_2 in the first steps of the fermentation process against spoilage yeasts is needed to aid the development of alternative products with minimal environmental and health impact. Thus, the impact of grape EW treatments and yeast inoculation on wine fermentations was studied. Culture-dependent (traditional plate counts) and culture-independent (PCR-denaturing gradient gel electrophoresis [DGGE] and reverse transcription PCR [RT-PCR]-DGGE) techniques were used to depict yeast dynamics over the course of fermentation. Furthermore, two series of fermentations (spontaneous and inoculated with *Saccharomyces cerevisiae*) were investigated to assess the cumulative effects of inoculation and EW sanitization on yeast population dynamics and wine aroma profile.

2. Materials and methods

2.1. Grape samples

Wine grapes (*Vitis vinifera* L. Cultivar Barbera), grown in Asti province (Piedmont, Italy), were harvested in good phytosanitary conditions. Immediately after harvesting, about 36 kg of grapes were transported to the laboratory. The main stalk was removed and the berries were kept in clusters of 3 to 5 berries with the pedicel attached.

2.2. Preparation of EW solutions and grapes treatment

Concentrated EW solution was generated by using EVA SYSTEM® 100 equipment (Industrie De Nora S.p.A, Milan, Italy), following the manufacturer's instructions. An aqueous solution of40 g/L of potassium chloride (KCl) was prepared to obtain by electrolysis an EW solution of approximately 4000 mg/L of free chlorine, pH 9.0. This stock solution of EW was diluted with sterile deionized water (to avoid external contamination) to obtain the two working solutions with concentrations of 40 and 400 mg/L of free chlorine (pH 9.0 and 1% residual KCl). All EW solutions were freshly prepared before use. The amount of free chlorine, as well as the pH were verified prior to use according to the methods described by Laureano et al. (2016). About 2 kg (±100 g) of berries were placed in a single layer into perforated boxes (50 × 30 × 15 cm) and subsequently sprayed with 100 mL of working EW solution, using a hand spray bottle according to the following treatments, in six plicate: A, not treated with EW (Control); B, treated with EW containing 40 mg/L of free chlorine; and C, with EW containing 400 mg/L of free chlorine. After treatment each lot of grapes were crushed originating must, which was subjected to fermentation according the experimental plan (Fig. 1). For each treatment applied (A, B and C) two different sets of laboratory fermentations were performed: one trial was conducted by indigenous yeasts present on grape berries (sample codes: Control SA, treatment SB and SC), while in the second trial a commercial active dry yeast was inoculated (sample codes: Control IA, treatment IB and IC). Each fermentation was performed in triplicate.

2.3. Grape sampling

A set of about 30 berries, before and after treatments from each perforated box were sampled randomly and placed in a stomacher bag. After manual crushing, the resulting juice was subjected to microbiological analysis. Aliquots of 1 mL each, in duplicate, were centrifuged for 10 min at 14,000 rpm and the supernatant was removed. Pellets to be used for DNA extraction were immediately frozen at −20 °C, while those destined to RNA analysis were covered with 200 μL of RNA later (Ambion, Milan, Italy) prior to freezing.

2.4. Must fermentations

After each treatment, berries from each perforated box (about 2 kg ± 100 g) with the pedicel attached were aseptically collected in sterile plastic bags, immediately crushed and the juice with skins was transferred to sterile 2.5 L glass bottles contained approximately 1.7 L of grape must. The mean values of standard chemical parameters of the musts obtained were: 21.9 °Brix, pH 3.14 and titratable acidity of 9.51 g/L (expressed as tartaric acid). Inoculated fermentations were performed inoculating *S. cerevisiae* (Lalvin EC1118®, Lallemand, Montreal, Canada), according to manufacturer's instructions, at an initial cell concentration of 2.0×10^6 cells/mL. The bottles were closed with a sterile Müller valve containing sterile vaseline oil, in order to allow the CO_2 formed during the fermentation progress to escape from the system. Fermentations were carried out for 14 days, under static conditions at 25 ± 1 °C. Samples of the fermented musts were collected aseptically at the beginning (immediately following crushing), and after 2, 5, 7 and 14 days of fermentation. Aliquots for DNA and RNA extractions were taken only from the spontaneously fermented musts and stored at −20 °C until further processing.

2.5. Microbiological analyses

Samples were serially diluted in quarter strength Ringer's solution (Oxoid, Milan, Italy), then plated for cultivation and subsequent enumeration in two different microbiological media: the non-selective Wallerstein laboratory nutrient medium agar (WLN) (Biogenetics, Milan, Italy) and the selective medium Lysine medium agar (Oxoid, Milan, Italy). The latter was used to count the non-*Saccharomyces* yeast species, since it is a medium containing glucose, vitamins, inorganic salts, and L-lysine as the sole nitrogen source, which cannot be assimilated by the *Saccharomyces* spp. (Angelo & Siebert, 1987). Plates were incubated for 5 days at 30 °C and colonies were counted on the basis of the colour and morphology as described previously by Urso et al. (2008). Five isolates of each colony morphotype were picked and purified by streaking on WLN medium. All of them were stored in YPD broth (10 g/L yeast extract, 20 g/L bacteriological peptone and 20 g/L dextrose; all from Biogenetics, Milan, Italy) with glycerol (30%) (Sigma, Milan, Italy) at −20 °C for further analysis.

2.6. Molecular analysis

2.6.1. DNA extraction from pure cultures

Genomic DNA of each isolate was extracted from one-millilitre of an overnight culture in YPD broth, following the protocols described by Alessandria et al. (2015). Extracted DNA was quantified by using a Nanodrop Spectrophotometer (ND-100, Thermo Fisher Scientific, Milan, Italy) and standardized at 50 ng/μL. The isolates were identified