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A novel culture medium for *Oenococcus oeni* malolactic starter production

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ABSTRACT

Malolactic fermentation is essential in wine quality. One of the strategies used to control this fermentation involves the inoculation of selected lactic acid bacteria, mainly *Oenococcus oeni*. Laboratory media usually produce large amounts of biomass, but with little or no adaptability to wine. We propose a culture medium to grow and pre-adapt *O. oeni* cells, and the steps to scale-up production. To achieve this objective, 27 different media were tested. All contained grape must and wine, and nutritional supplements in order to benefit bacterial growth. Those media contained different ethanol levels, pH values, and grape must concentrations. The optimized culture medium named *Oenococcus* Production Medium (OPM) contained diluted commercial 4X concentrate white must (1:6), $AS_v = 4\%$, and a pH of 3.8. The total time to obtain 80 L of an *O. oeni* liquid starter culture from the stock culture at the laboratory, with a final population of $CFU = 1 \times 10^9$ mL^{-1}, was 22 d. The starter culture was efficiently scaled-up, and preserved at 4 °C, −20 °C, or freeze-dried. This new culture medium also allowed adaptation of bacteria to the wine conditions, consuming all malic acid (3 g/L) in 7 d with an inoculum of $CFU = 1 \times 10^6$ mL^{-1}.

1. Introduction

The main value of malolactic fermentation (MLF) in vinification is biological de-acidification, which results from the transformation of L-malic acid into L-lactic acid by lactic acid bacteria (LAB), mainly by *Oenococcus oeni* (Lonvaud-Funel, 1999; Versari, Parpinello, & Cattaneo, 1999; Wibowo, Eschenbruch, Davis, Fleet, & Lee, 1985). MLF induces an increase in pH, contributes to microbiological stability, and changes wine taste (Davis, Wibowo, Fleet, & Lee, 1988; Kunkee, 1991; Maicas, Gil, Pardo, & Ferrer, 1999). MLF can be produced by two strategies: spontaneously or by inoculation with starter cultures. Traditionally, spontaneous and indigenous LAB carried out a natural MLF process. But this MLF method can take weeks or months and is uncontrolled (Zhang & Lovitt, 2006). The induction of MLF by using a starter culture ensures faster fermentation, reduces potential spoilage by other LAB, and allows the improvement of wine quality when using selected bacterial strains (Mira de Orduña, Patchett, Liu, & Pilone, 2001; Pozo-Bayón et al., 2005). Nevertheless, successful inoculation of the starter into wine depends not only on the suitable strain of *O. oeni*, but on the preparation and use of the cultures. For the production of a suitably large biomass, selected strains of *O. oeni* are grown under conditions that permit rapid growth and result in a high cell yields, but these conditions are very different to those present in wine. Consequently, when the starter culture is inoculated directly into wine, it loses much viability (Henick-Kling, 1988). *O. oeni* is usually grown in the laboratory in complex culture media such as MRS (De Man, Rogosa, & Sharpe, 1960), MLO (Zúñiga, Ferrer, & Pardo, 1994), or AGB (Dicks & van Vuuren, 1990). Most of them frequently contain grape juice (Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985) or apple juice (Champagne, Gardner, & Lafond, 1989). We prefer to use tomato juice instead, as it is a source of pantothenic acid, commonly used as a growth factor for wine bacteria (Amachi, Imamoto, & Yoshizumi, 1971; Richter, Vlad, & Unden, 2001; Terrade & Mira de Orduña, 2009). The culture media are generally supplemented with other nutrients like yeast extract, peptone, and Tween 80, to increase biomass production (Champagne, Gardner, & Doyon, 1989; Guerrini, Bastianini, Granchi, & Vincenzini, 2002; Krieger, Hammes, & Henick-Kling, 1990; Kunkee, 1974; Pilone & Kunkee, 1970). The growth of *O. oeni* has been also investigated in single sugars and their mixtures. The best growth was obtained with sugar mixtures (glucose–fructose) rather than growth on a single sugar (Maicas,

Abbreviations: AUC, area under the curve; LAB, lactic acid bacteria; MLF, malolactic fermentation; OPM, *Oenococcus* production medium.

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Ferrer, & Pardo, 2002; Maicas, González-Cabo, Ferrer, & Pardo, 1999; Zhang & Lovitt, 2005a). Other components like manganese or yeast mannoproteins and nutrient requirements have been studied (Stamer, Albury, & Pederson, 1964; Theobald, Pfeiffer, & König, 2005). Results revealed that the essential bacterial nutrients were strain-specific and *O. oeni* strains showed a large number of auxotrophies (Diez, Guadalupe, Ayestarán, & Ruiz-Larrea, 2010; Terrade & Mira de Orduña, 2009; Terrade, Noel, Couillaud, & De Mira Orduña, 2009; Theobald et al., 2005). Hayman and Monk (1982) evaluated the effect of adding wine to a medium for the production of *O. oeni* biomass, and found that a content of 40–80% wine in the medium improved LAB survival and malolactic activity. Nevertheless, further studies are necessary to find a medium for biomass production with a cheap easy recipe that allows any *O. oeni* strain to grow and adapt before being inoculated into wine.

The objectives of this work were to develop a liquid *O. oeni* production medium (OPM) that would permit high levels of biomass production, but also an adequate pre-adaptation to wine conditions, and evaluate cell viability and malolactic activity of the MLF starter culture in wine. Preservation conditions for the liquid starter culture were also studied.

2. Material and methods

2.1. Microorganisms

O. oeni strains E5003, E5067, E5259, and E5245 were taken from the Enolab culture collection of the University of Valencia (Spain). All strains had been previously isolated from spontaneous mid-MLF in Tempranillo wines from the Ribera del Duero region (Spain).

2.2. Growth in biomass production media

O. oeni strains were pre-grown in MLO broth (Zúñiga, Pardo, & Ferrer, 1993) to early stationary phase. Cells were then centrifuged (rotational speed of 8000 min^{-1}, 10 min), washed and transferred to the 27 culture media (Table 1) at a final concentration of $CFU = 1 \times 10^6$ mL^{-1}. The base of all these media contained: yeast extract (5 g/L), commercial tomato juice with no preservatives (23 mL/L), Tween 80 (0.5 mL/L), L-malic acid (3 g/L) and concentrate reconstituted white wine (glucose 0.13 g/L, fructose 0.14 g/L, L-malic acid 0.22 g/L, free SO_2 1 mg/L, ethanol $AS_v = 0\%$) from Agrovin S.A. (Spain) (400 mL/L). This basal medium was supplemented with 4X concentrated white grape must (glucose 360 g/L, fructose 360 g/L, L-malic acid 5.3 g/L, free SO_2 1.4 mg/L) from Agrovin S.A. (Spain), which was diluted 4, 6 and 8 times (576.5 mL/L) and the pH was adjusted to 3.8, 4 and 4.5. Media were sterilized by autoclave at 115 °C, 30 min. After sterilization, ethanol degree was adjusted at $AS_v = 4\%$, $AS_v = 6\%$ and $AS_v = 8\%$. All growth studies were carried out in duplicate, in 10 mL screw cap tubes and incubated at 28 °C, 7 d. Growth was monitored by measuring optical density at 600 nm (OD_{600}) with a spectrophotometer (CECIL, CE 373). The bacterial growth curve was transformed into a value of area under the curve (AUC) (Gagnon & Peterson, 1998).

2.3. Malolactic fermentation in red wine

The cultures were inoculated into red wine at a final concentration of $CFU = 1 \times 10^6$ mL^{-1}, and MLF was monitorized for 15 d. All fermentations were carried out in duplicate. The red wine was made at the laboratory using a red Tempranillo grape must, inoculated with *S. cerevisiae* Viniferm B4 (Agrovin S.A.) at a final concentration of $CFU = 2 \times 10^6$ mL^{-1}, and was incubated at 28 °C, 10 d, until the AF was finished. When the sugar concentration was lower than 1 g/L, the ethanol was adjusted to $AS_v = 11\%$, $AS_v = 12\%$, $AS_v = 13\%$ and $AS_v = 14\%$, 3.0 g/L of malic were added, and the pH was adjusted to 3.5. Then, it was sterilized by filtering through 0.22 μm pore filter and stored at 15 °C until use.

Table 1
Combination of diluted 4X grape must (1/4, 1/6 or 1/8), ethanol content ($AS_v = 4\%$, $AS_v = 6\%$ or $AS_v = 8\%$) and pH level (3.8, 4 or 4.5) resulted in 27 different media for the *O. oeni* biomass production.

Medium	Diluted must	Ethanol %	pH
1	1/4	4	3.8
2	1/4	4	4
3	1/4	4	4.5
4	1/4	6	3.8
5	1/4	6	4
6	1/4	6	4.5
7	1/4	8	3.8
8	1/4	8	4
9	1/4	8	4.5
10	1/6	4	3.8
11	1/6	4	4
12	1/6	4	4.5
13	1/6	6	3.8
14	1/6	6	4
15	1/6	6	4.5
16	1/6	8	3.8
17	1/6	8	4
18	1/6	8	4.5
19	1/8	4	3.8
20	1/8	4	4
21	1/8	4	4.5
22	1/8	6	3.8
23	1/8	6	4
24	1/8	6	4.5
25	1/8	8	3.8
26	1/8	8	4
27	1/8	8	4.5

2.4. Viable cell counts

Cell viability in red wine was studied by plate counting. The volume of 0.1 mL of decimal serial dilutions in sterile saline solution were spread in duplicate on MLO agar plates (Zúñiga et al., 1993) and were incubated at 28 °C for 7 d and then the colonies were counted.

2.5. Culture preservation

Freeze-drying was performed after growing the bacteria until the end of the exponential phase in 50 mL of OPM medium. Cells were recovered by centrifugation at rotational speed of 6000 min^{-1}, 15 min in a Heraeus Multifuge 1 S-R centrifuge. Then, cells were washed twice with glutamic acid 0.98%, recovered with the same above centrifugation conditions, and resuspended in 2 mL of 0.98% glutamic acid. The bacterial solution was distributed in aliquots of 400 μL per tube. Tubes were frozen at −20 °C, 1 h. Freeze-dying was performed at −60 °C for 18 h under vacuum (Virtis Sentry). Tubes were vacuum sealed and stored at 4 °C under dark. Refrigeration at 4 °C and freezing at −20 °C were performed after growing *O. oeni* until the exponential phase in OPM medium and later transferring aliquots to the respective temperature. Bacterial cultures were preserved and checked for viability up to 250 d.

2.6. Analytical methods

Glucose, fructose, malic acid and ethanol were quantified by high-pressure liquid chromatography (HPLC) (Agilent series 1200) with an isocratic pump (Agilent G1310A) following the procedure described by Frayne (1986) with minor modifications. The mobile phase consisted of a solution of 0.75 mL of 85% H_3PO_4 per litre of