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Directed evolution of *Oenococcus oeni* strains for more efficient malolactic fermentation in a multi-stressor wine environment

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ABSTRACT

High concentrations of ethanol, low pH, the presence of sulfur dioxide and some polyphenols have been reported to inhibit *Oenococcus oeni* growth, thereby negatively affecting malolactic fermentation (MLF) of wine. In order to generate superior *O. oeni* strains that can conduct more efficient MLF, despite these multiple stressors, a continuous culture approach was designed to directly evolve an existing ethanol tolerant *O. oeni* strain, A90. The strain was grown for ~350 generations in a red wine-like environment with increasing levels of stressors. Three strains were selected from screening experiments based on their completion of fermentation in a synthetic wine/wine blend with 15.1% (v/v) ethanol, 26 mg/L SO_2 at pH 3.35 within 160 h, while the parent strain fermented no more than two thirds of L-malic acid in this medium. These superior strains also fermented faster and/or had a larger population in four different wines. A reduced or equivalent amount of the undesirable volatile, acetic acid, was produced by the optimised strains compared to a commercial strain in Mouvedre and Merlot wines. These findings demonstrate the feasibility of using directed evolution as a tool to generate more efficient MLF starters tailored for wines with multiple stressors.

1. Introduction

Malolactic fermentation (MLF), which involves the conversion of L-malic acid to L-lactic acid by lactic acid bacteria (LAB), is required in most red wines and some white wines (Davis et al., 1985). This process not only deacidifies the wine, to produce a softer mouth-feel, but also increases microbial stability of the wine by removing biologically active organic acids. The LAB strains that typically conduct MLF due to their better tolerance of wine-related stressors belong to the species *Oenococcus oeni* (Wibowo et al., 1985).

While MLF is desirable because of the benefits it confers to wine, it is often difficult for bacteria to complete due to poor growth or malolactic activity in wine. High ethanol, low pH, SO_2, medium chain fatty acids and some phenolic compounds are common inhibitors to bacterial growth. For example, Capucho and Romfio (1994) reported that an ethanol content between 8 and 12% (v/v) strongly inhibited *O. oeni* cell growth. Even though 8–12% (v/v) ethanol had a negative effect on bacterial growth, it had little effect on malolactic activity. When ethanol concentration was increased to 18% (v/v) and higher, the malolactic activity was also eliminated (Capucho and Romfio, 1994).

Ethanol-stressed cells of *O. oeni* exhibit increased membrane fluidity and a reduced production of ATP (da Silveira et al., 2003; da Silveira and Abee, 2009). The presence of SO_2, medium chain fatty acids and low pH also impairs the ATPase activity of *O. oeni*, resulting in a reduced population and prolonged fermentation (Tourdot-Maréchal et al., 1999; Carreté et al., 2002). Medium chain fatty acids can be produced by yeast during alcoholic fermentation. SO_2 is often added during winemaking to avoid oxidation and restrict the growth of unwanted microorganisms (Ough and Crowell, 1987; Takahashi et al., 2014). Phenolic compounds have a dual effect on bacterial growth. Some hydroxybenzoic acids and their derivatives, phenolic aldehydes, flavonoids and tannins inhibit *O. oeni* growth and viability (Figueiredo et al., 2008; Campos et al., 2009; García-Ruiz et al., 2011), while gallic acid and free anthocyanins activate cell growth (Vivas et al., 1997). The growth of *O. oeni* can also be restricted by nutrient limitation, with several amino acids such as cysteine being essential (Saguir and Manca de Nadra, 2002).

The combination of stressors, such as found in the complex

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matrix that is wine, can produce an even more severe inhibitory effect on *O. oeni* growth compared to a single stressor (reviewed by Betteridge et al., 2015). For example, a combination of pH at 3.2 and 26 mg/L of SO_2 was more inhibitory for MLF compared to the individual stressors of either pH or SO_2 (Nielsen et al., 1996). Exposure to 10% (v/v) ethanol at pH 3.5 for 30 min led to near total cell death whereas 75% and 70% cell viability was retained upon exposure to pH at 3.0 or 10% (v/v) ethanol, respectively, for the same time (Chu-Ky et al., 2005).

Given the multi-stressor environment of wine, and hence potential delays in completion of wine processing and stabilisation, more robust LAB strains with improved tolerance under such conditions are needed to improve the efficiency of MLF. Torriani et al. (2010) summarised the criteria for selection of improved LAB, which included that more efficient LAB starters must not only grow and ferment desirably in an environment with ethanol content (over 14% (v/v)), low pH (under 3.0), high SO_2, but also produce pleasant aromas to enhance the sensory profile of the wine.

Two main approaches can be used for strain improvement: recombinant and non-recombinant methods (Betteridge et al., 2015). Recombinant methods require a good knowledge of the genetic basis of the attribute in question, while non-recombinant methods do not necessarily require such knowledge (Hindré et al., 2012; Betteridge et al., 2015). A few transformation experiments have been conducted successfully, however, they either could not be repeated by other laboratories, or may have low transformation efficiency for *O. oeni* (Dicks, 1994; Assad-García et al., 2008). Recently, successful transformation experiments with *O. oeni* were reported, however, transformation efficiencies were not provided (Darsonval et al., 2016a; b). While recombinant methods are precise, some consumers may reject food or wine produced by genetically modified microorganisms (Pretorius and Bauer, 2002).

Non-recombinant methods, including mutagenesis, classical strain selection and directed evolution (DE), require no specific genetic knowledge, but need effective screening processes to select the best candidate(s). UV mutagenesis has been successfully applied to *O. oeni*, and a strain with greater malic acid utilisation and enhanced softness index as well as reduced wine decolouration was selected from treated isolates (Li et al., 2015). Even though mutagenesis is easy to perform, disadvantages relate to the fact that the random mutations might not generate the desired phenotype or produce undesired ones. Classical strain selection involves the isolation and screening of LAB from environments such as wineries. Many strains that show promise for carrying out MLF have been selected using this method (Capozzi et al., 2010; Berbegal et al., 2016; Iorizzo et al., 2016). However, strains with excellent combinations of technological traits (e.g. high performance in MLF and desired flavour attributes) are unlikely to be identified only by relying on natural diversity, otherwise such strains might have already been found (Betteridge et al., 2015).

Directed evolution (DE) can be straightforward to establish, however, it can be time consuming. Optimised strains are generated as the population struggles to adapt to the stressors applied in the DE system. Two approaches of DE, a sequential batch system or continuous culture, can be performed with LAB based on the time frame of DE and the generation time of the target bacteria. An acid-resistant mutant of *Lactobacillus casei* was obtained by sequentially transferring a culture into De Man, Rogosa and Sharpe medium (MRS; de Man et al., 1960) with decreasing pH levels over 70 days (Zhang et al., 2012). A sequential batch system has also been successfully applied to *Lb. plantarum* and *Lactococcus lactis* (Teusink et al., 2009; Bachmann et al., 2012).

In terms of *O. oeni*, we provided the first report of the successful application of DE to generate the more ethanol resistant strain, A90, from a commercially available strain (LACTOENOS SB3, henceforth SB3) (Betteridge et al., 2018). In that proof-of-concept study, SB3 was cultured continuously in MRSAJ (MRS with 20% (v/v) apple juice) rather than wine, and exposed to increasing ethanol concentrations (from 5 to 15% (v/v)). Individual isolates were in fact found to exhibit improved L-malic acid consumption in MRSAJ and to show greater tolerance to ethanol-supplemented media (22% (v/v)). Whilst confirming the effectiveness of DE in the development of a candidate strain with the potential for application in the wine industry, a thorough evaluation of this ethanol tolerant strain in the face of additional wine stresses had not been done.

Assessment of the multi-stress (cross) tolerance of A90 and the development of A90 to produce strains resistant to the many stresses found in wine were therefore the main aims of this study. Since it was possible to generate ethanol resistant *O. oeni* by DE, it was hypothesised that DE in the presence of multiple selective pressures would also yield strains more adapted to the winemaking environment, which incorporates a broader range of stress factors. Such strains should be better able to complete MLF in the typical wine environment, which features low pH and the presence of SO_2, as key stresses.

2. Materials and methods

2.1. Bacterial cultures

SB3 is a commercial *O. oeni* strain from Laffort Oenologie. *O. oeni* A90 (A90) was generated from SB3 via DE and has improved ethanol tolerance in MRSAJ (Betteridge et al., 2018). A90 was used as the parental strain for DE performed in this study.

2.2. Media and wine

MRS (AM 103, Amyl Media, Australia) supplemented with 20% (v/v) apple juice (MRSAJ) was used to culture bacteria. The medium was sterilised at 121 °C, 0.1 MPa for 20 min before use. A synthetic wine medium was prepared from a chemically defined grape juice medium (McBryde et al., 2006) supplemented with 5% (v/v) grape tannin extract (GSKINEX, Tarac Technologies, Australia) and fermented by *Saccharomyces cerevisiae* yeast to produce Red Fermented Chemically Defined Grape Juice Medium (RFCDGJM). This RFCDGJM was then supplemented with analytical reagent grade ethanol and L-malic acid and used as the DE feed and for the first and second round screening experiments (Table 1).

A Shiraz wine made in 2015 at the University of Adelaide's Waite Campus and in which MLF became stuck at 0.8 g/L malic acid was considered suitable as a medium for strain improvement. This wine was supplemented with L-malic acid and ethanol for DE feed and the third screening experiment (Table 1). Wines used to further characterise the best DE isolates were Mouvedre from McLaren Vale, as well as Shiraz and Merlot from the Waite Campus, Urrbrae and Pinot Noir from the Adelaide Hills, South Australia, from the 2016 vintage. All wines, except the Pinot Noir, had not undergone MLF. An extra 2.7 g/L of L-malic acid was added to the Pinot Noir to test the performance of evolved *O. oeni* strains under nutrient deficient conditions (Table 1). RFCDGJM and all wines were filter sterilised (0.22 μm) before use.

2.3. Preparation and inoculation of O. oeni strains

All strains were cultured in MRSAJ before inoculation into the experimental medium. Except for the micro-scale screening experiments, for which a simplified inoculation regime was used (see below), growth of bacterial starter cultures was monitored at 600 nm (OD_{600}) using a Helios Cuvette spectrophotometer (Thermo Scientific). Once OD_{600} reached 1.5, liquid cultures were diluted