Effect Of *Candida zemplinina* oak chips biofilm on wine aroma profile

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Introduction

Candida zemplinina (synonym *Starmerella bacillaris*) is known to play relevant activities during winemaking, due to its fructophilic nature and low ethanol production rate (Masneuf-Pomarede et al., 2015). Moreover, wines fermented with *C. zemplinina* and *S. cerevisiae* showed higher amounts of glycerol and esters (Englezos et al., 2016; Tofalo et al., 2016). *Saccharomyces* and non-*Saccharomyces* yeasts' formulations are available in different forms including the Cream Yeast (CRY) preparation, Active Dry Yeast (ADY), Active Frozen Yeast (AFY) (Fracassetti et al., 2020). In all these cases yeasts develop as planktonic cells. However, in nature microbes prefer a sessile lifestyle and are often organized in biofilms. Biofilms are structures made up of single- and multiple- species attached on a biotic or an abiotic surface and embedded in a self-produced matrix (Lebleux et al., 2020). Sessile cells are more resistant to stressing conditions through metabolic cross-feeding, cell–cell interactions and especially chemical and physical resistance (Bastard et al., 2016). This growth strategy is exploited to improve some microbial activities, for instance it is used to increase ethanol production (Germec et al., 2016), and a recent study revealed that *Oenococcus oeni* cells organized in biofilms on oak showed a better malolactic activity, and improved the volatile composition of wines (Bastard et al., 2016). Despite the positive impact of *C. zemplinina* on wine characteristics the majority of studies are focused on planktonic cells.
Therefore, in this study, 10 strains of *C. zemplinina* were tested for their ability to form biofilms on winemaking material such as stainless steel and oak chips. The impact of oak surface-associated *C. zemplinina* cells on fermentation kinetics and volatile profile was also evaluated.

Materials and methods

Yeast Strains and Media

Candida zemplinina strains were isolated from Montepulciano d'Abruzzo organic must. *Saccharomyces cerevisiae* autochthonous strain (RT73) has been previously characterized for its oenological performances in Montepulciano d'Abruzzo microvinification trials (Suzzi et al., 2012 a,b). All strains belong to the Microbial Biotechnology Laboratory Collection (University of Teramo, Italy). Strains were routinely grown in YPD medium (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose) for 48 h under aerobic conditions. All strains were stored at −80◦C in YPD broth supplemented with glycerol (20% v/v final concentration; Sigma-Aldrich, Milan, Italy).

Adhesion to stainless stell and oak chips

Ten strains of *C. zemplinina* were tested for their ability to form biofilm on oak and stainless steel chips as previously described by Lebleux, et al. (2020) with some modifications. Briefly, pre adapted strains were inoculated in must at a final concentration of 6 log CFU/mL and chips were added. Samples were incubated for 15 days at 30°C. Non-adhered cells were eliminated washing with physiological solution (NaCl 0.85 w/v). Adhered cells on stainless chips were detached by sonication. The oak chips were rinsed twice with sterile saline solution, placed in 10 ml saline and scrubbed with a toothbrush (2 min per side). Sessile cells were numbered by plate count on YPD after serial dilutions. Plates were incubated at 30 °C for 2 days. The strain

able to better adhere on oak chips was used as inoculum for small lab scale fermentations together with a strain of *S. cerevisiae* (RT73).

Small scale fermentations

Triplicate fermentations were carried out using pasteurized Montepulciano d'Abruzzo must. Ninety-five mL of must were inoculated with 24 h pre-cultures grown in the same must at a final concentration of 6 log CFU/mL. 3 fermentation trials were carried out: trial A: co-inoculation of *S. cerevisiae* (RT73) and *C. zemplinina* (SB10); trial B: co-inoculation of RT73 and SB10 attached on oak chips; trial C: co-inoculation of RT73 and SB10 with oak chips. Fermentation kinetics were monitored daily determining the weight lost due to CO_2 release. When the weight was constant, the fermentation was considered finished.

Oenological parameters determination

The main wine analytical components (ethanol, residual sugar, pH, volatile acidity, total acidity and glycerol) were determined using the Winescan (FOSS, Hillerød, Denmark) calibrated following EEC 2676 standard procedure (European Commission 1990).

Volatile profile assessment

Aroma compounds were extracted by solid-phase microextraction and analyzed using gas chromatography/mass spectrometry (GC–MS) as previously described (Tofalo et al., 2016).

Results and discussion

***Candida zemplinina* can colonize stainless steel and oak chips**

Stainless steel tanks and oak barrels are widely used in winemaking, therefore the ability of *C. zemplinina* strains to adhere on these surfaces was evaluated. On stainless stell chips sessile cells ranged from 2.6 Log CFU/mL to 5 Log CFU/mL, while on oak chips were about 2 log higher ranging from 4.3 Log CFU/mL to 6.8 Log CFU/mL (Figure 1). This data could be due to the different characteristics of materials tested. Stainless steel is frequently used in food processing to limit the adhesion of microorganisms, while wood has micro-topographical features and chemical structures that enhance bacterial adhesion (Bastard et al., 2016). SB10 strain was characterized by the highest adhesion to oak chips and was used for small scale fermentations.

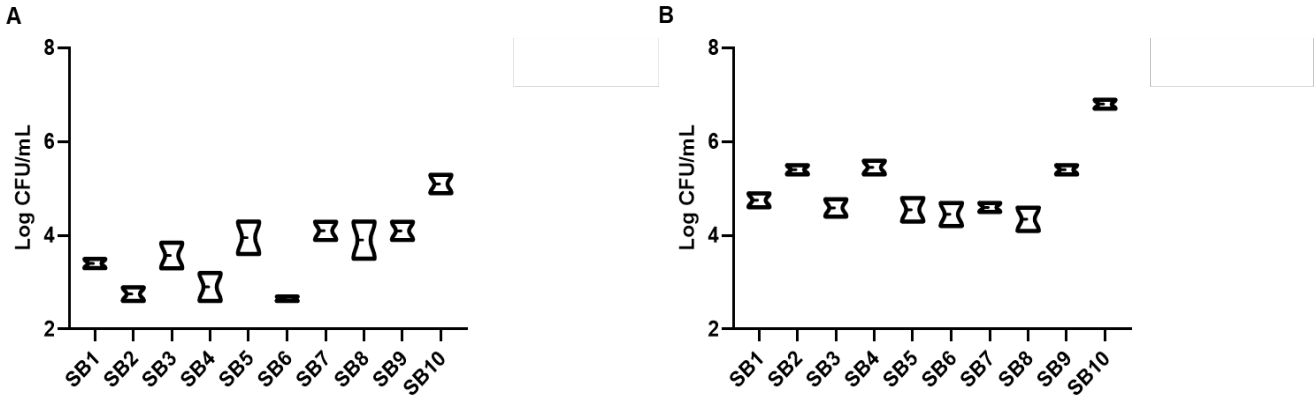

Figure 1. Candida zemplinina cells adhered on stainless steel (A) and oak (B) chips

Impact of oak surface-associated *C. zemplinina* cells on fermentation process

To evaluate the performance of cells in biofilms in the fermentation process, main enological analyses were performed. All trials were characterized by a good fermentation kinetics. All vinifications completed the alcoholic fermentation process in 15 days. However, fermentation started 2 days later when *C. zemplinina* was adhered on oak chips (trial B), but similar amounts of CO_2 were produced at the end of fermentation.

No significant differences were observed for the main oenological characteristics (data not shown). The only exception was the concentration of glycerol which was detected in higher concentration in trial B (11.92±0.17 mg/L). This data could be due to a major presence of proteins involved in glycerol metabolism in sessile cells and/or a different contribution of proteins under planktonic or sessile state (Moreno-García et al., 2015). This tendency to produce glycerol could be explained also as consequence of its slow growth and low fermentative rate when attached on oak chips.

Oak surface-associated *C. zemplinina* cells did not influence organic acids and higher alcohols. On the contrary, the content of esters underwent significant variations both from a qualitative and quantitative point of view (Figure 2). Wines fermented with *C. zemplinina* adhered on oak chips were characterized by a higher concentration of esters. Some compounds were detected only in these wines such as methyl vanillate (vanilla notes), ethyl isobutyrate (sweet, etherial and fruity with pungent, alcoholic, fusel and rummy nuances), ethyl isovalerate (sweet, fruity, spice, metallic and green with a pineapple and apple lift). These wines also showed the highest concentrations of some other compounds associated to fruity notes such as 3-Methyl-but-1-yl ethanoate (isoamyl acetate), ethyl ethanoate and ethyl phenylacetate. The production of methylvanillate has been described for other non-*Saccharomyces* yeast during wine aging but never for *C. zemplinina*, offering new ideas to exploit this yeast to improve wine quality and create new wine styles.

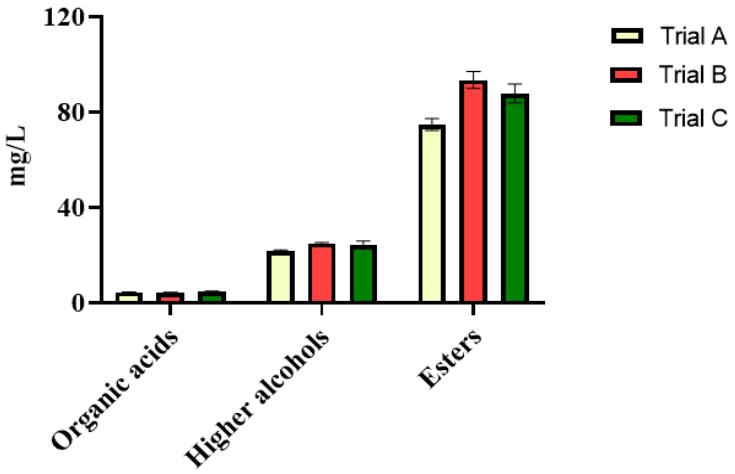

Figure 2. Total amounts of volatile compounds detected in the fermentation trials. A RT73+SB10; B RT73+SB10 on oak chips; C RT73+SB10+oak chips

Conclusions

Oak surface-associated *C. zemplinina* allows to modulate the content of esters and glycerol in wine. This approach could represent a new way to produce starter cultures and improve the impact of oak chips in red wine maceration to obtain effects comparable to wine aged in oak barrels. Further studies are necessary to characterize *C. zemplinina* biofilm lifestyles in different enological conditions using different grape varieties and malolactic bacteria to generate novel wine styles and validate their sensorial perceptions. On the other hand, a focus can be made on the nature of interactions between wine compounds and biofilm to assess possible biological activity specific to biofilm.

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