International Journal of Food Microbiology 207 (2015) 40–48

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Increase of fruity aroma during mixed *T. delbrueckii*/*S. cerevisiae* wine fermentation is linked to specific esters enhancement

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ARTICLE INFO

Article history:
Received 23 February 2015
Received in revised form 17 April 2015
Accepted 24 April 2015
Available online 2 May 2015

Keywords:
Non-*Saccharomyces*
Torulaspora delbrueckii
Wine
Fermentation
Mixed inoculation
Esters

ABSTRACT

The aim of this work was to study ester formation and the aromatic impact of *Torulaspora delbrueckii* when used in association with *Saccharomyces cerevisiae* during the alcoholic fermentation of must. In order to evaluate the influence of the inoculation procedure, sequential and simultaneous mixed cultures were carried out and compared to pure cultures of *T. delbrueckii* and *S. cerevisiae*.

Our results showed that mixed inoculations allowed the increase, in comparison to *S. cerevisiae* pure culture, of some esters specifically produced by *T. delbrueckii* and significantly correlated to the maximal *T. delbrueckii* population reached in mixed cultures. Thus, ethyl propanoate, ethyl isobutanoate and ethyl dihydrocinnamate were considered as activity markers of *T. delbrueckii*. On the other hand, isobutyl acetate and isoamyl acetate concentrations were systematically increased during mixed inoculations although not correlated with the development of either species but were rather due to positive interactions between these species.

Favoring *T. delbrueckii* development when performing sequential inoculation enhanced the concentration of esters linked to *T. delbrueckii* activity. On the contrary, simultaneous inoculation restricted the growth of *T. delbrueckii*, limiting the production of its activity markers, but involved a very important production of numerous esters due to more important positive interactions between species. These results suggest that the ester concentrations enhancement via interactions during mixed modalities was due to *S. cerevisiae* production in response to the presence of *T. delbrueckii*.

Finally, sensory analyses showed that mixed inoculations between *T. delbrueckii* and *S. cerevisiae* allowed to enhance the complexity and fruity notes of wine in comparison to *S. cerevisiae* pure culture. Furthermore, the higher levels of ethyl propanoate, ethyl isobutanoate, ethyl dihydrocinnamate and isobutyl acetate in mixed wines were found responsible for the increase of fruitiness and complexity.

1. Introduction

The fermentation of grape must is a complex microbial process, involving sequential development of various yeast communities including *Saccharomyces* and non-*Saccharomyces* species. The nutrient competition and the increasing ethanol content gradually eliminate the less tolerant species, thus favoring the development of *Saccharomyces cerevisiae* which then completes the fermentation (Heard and Fleet, 1985, 1986). The dominance of non-*Saccharomyces* yeasts during the early stages of the reaction has a major impact on the aromatic composition and sensory properties of wine (Ciani et al., 2010; Domizio et al., 2007; Fleet, 2008; Jolly et al., 2014; Renouf et al., 2007; Romano et al., 2003; Swiegers et al., 2005). Consequently, many researchers have investigated the specific metabolisms of the various non-*Saccharomyces* yeast species and their potential applications in the wine industry (Andorra et al., 2011; Capece et al., 2011; Comitini et al., 2011; Domizio et al., 2011; Jolly et al., 2003; Moreira et al., 2011; Tofalo et al., 2012).

In this context, *Torulaspora delbrueckii*, one of the few non-*Saccharomyces* yeast species currently commercialized, has been described as having a positive impact on the organoleptic quality of wines. Firstly, its low production of compounds like acetic acid, ethyl acetate, acetaldehyde, acetoin, hydrogen sulfide, and volatile phenols minimizes off-flavors (Cabrera et al., 1988; Ciani and Maccarelli, 1998; Ciani and Picciotti, 1995; Herraiz et al., 1990; Martinez et al., 1990; Plata et al., 2003; Renault et al., 2009; Shinohara et al., 2000).

Secondly, numerous authors (Azzolini et al., 2012; Comitini et al., 2011; King and Dickinson, 2000; Hernandez-Orte et al., 2008) showed that the strong β-glucosidase activity of this species enhanced wine aroma by modulating the levels of nor-isoprenoids, terpenols, and lactones by hydrolysing their respective precursors.

This species also releases 2-phenylethanol at higher levels than *S. cerevisiae* (Herraiz et al., 1990; Moreno et al., 1991; Renault et al., 2009). However, concerning esters, a report from Viana et al. (2008)

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.04.037
0168-1605/

indicates that in synthetic media, *T. delbrueckii* produces less ester acetates and ethyl hexanoate than *S. cerevisiae*. In similar conditions, other authors confirmed the low production capacities of *T. delbrueckii* towards isoamyl acetate, and as well as ethyl butanoate, hexanoate and octanoate (Hernandez-Orte et al., 2008; Plata et al., 2003; Renault et al., 2009). Sadoudi et al. (2012) showed a lower production than *S. cerevisiae* for isoamyl, hexyl and 2-phenylethyl acetate, but a higher production of ethyl 4-hydroxybutanoate, ethyl 3-hexanoate and diethyl succinate. It is important to note that ester production by this species is strain dependent (Renault et al., 2009) and that results are different when *T. delbrueckii* is associated to *S. cerevisiae* in mixed cultures.

Despite a good ethanol production for a non-*Saccharomyces* yeast, between 7 and 12% (v/v) (Cabrera et al., 1988; Ciani and Maccarelli, 1998; Ciani and Picciotti, 1995; Herraiz et al., 1990; Renault et al., 2009), the use of *T. delbrueckii* in pure culture leads to stuck fermentations. Mixed inoculations of selected strains of this species with *S. cerevisiae* have thus been proposed to modulate wine flavor and ensure complete alcoholic fermentation. In these conditions, authors have demonstrated that besides the reduction of off-flavors compounds like volatile acidity, acetaldehyde and acetoin (Bely et al., 2008; Ciani et al., 2006; Herraiz et al., 1990), the mixed inoculation of these two yeast species gives the systematic increase of 2-phenylethanol, terpenols and lactones (Azzolini et al., 2012; Comitini et al., 2011; Herraiz et al., 1990; Sadoudi et al., 2012). Results concerning esters production by *T. delbrueckii*/*S. cerevisiae* mixed inoculations remain confusing. According to Herraiz et al. (1990), mixed inoculations allow an increase, in comparison to pure cultures, of the total ester concentration and in particular the levels of isoamyl acetate and ethyl methanoate, octanoate, hexanoate and 3-hydroxybutanoate. On the contrary, Sadoudi et al. (2012) and Comitini et al. (2011) showed that total ester concentration of mixed inoculations was lower than that of a pure *S. cerevisiae* culture with a significant reduction in acetate esters and in particular isoamyl acetate. Azzolini et al. (2012) found no difference in the overall ester concentration between mixed *T. delbrueckii*/*S. cerevisiae* and pure *S. cerevisiae* cultures, but the level of some of them in mixed culture was higher (such as ethyl 3-hydroxybutanoate) while others were lower (such as isoamyl acetate). These overall contradicting results concerning ester concentrations in mixed inoculations may be due to the fact that their production depends on the development of each species during fermentation which has so far not been taken into account by authors in their discussion. The growth of the 2 yeasts may be different as a result of difference in must composition, fermentation temperature and initial concentration of each species. Furthermore, different interactions between yeasts (Albergaria et al., 2010; Fleet, 2003; Nissen et al., 2003; Renault et al., 2013) may be involved and modify the metabolic behavior of the 2 species.

Hence, the aim of this work was to study ester formation and the aromatic impact of *T. delbrueckii* when used in association with *S. cerevisiae* during the alcoholic fermentation of must. In these conditions, esters were evaluated at 40% of fermentations and at the end of fermentations conducted with pure and mixed cultures of the two species. To gain more insights on aroma release, the growth of the 2 species as well as the fermentation kinetics were also monitored throughout the fermentation.

2. Materials and methods

2.1. Microorganisms

In this study, 3 commercial strains from Laffort company (France) were used. The 2 *S. cerevisiae* strains were Zymaflore® X5 and Zymaflore® FX10 for laboratory and winery scale experiments respectively. *T. delbrueckii* Zymaflore® Alpha$^{TD\ N.\ SACCH.}$ was used in both types of experiment. Yeasts were grown at 24 °C on complete YPDA medium (1% yeast extract, 1% peptone, 2% dextrose) solidified with 2% agar and adjusted to pH 4.8.

2.2. Fermentation medium

2.2.1. Laboratory scale

The medium used in this study was a Sauvignon blanc must from Bordeaux area, pH: 3.15, with a sugar concentration of 203 g/L and an available nitrogen concentration adjusted to 210 mg/L (i.e. amino acids: 114 mg/L and ammonia: 96 mg/L). The total and free sulfur dioxide concentrations were respectively 60 and 19 mg/L.

Before yeast inoculation, the must was sterilized by filtration (0.45 μm nitrate cellulose membrane, Millipore, Molsheim, France).

2.2.2. Winery scale

The medium used in winery scale was a Merlot must from Bordeaux area, pH: 3.54, with a sugar concentration of 258 g/L and an available nitrogen concentration adjusted to 210 mg/L (i.e. amino acids: 119 mg/L and ammonia: 91 mg/L). The total and free sulfur dioxide concentrations were respectively 29 and 17 mg/L.

2.3. Fermentation conditions

2.3.1. Laboratory scale

Fermentation kinetics were monitored by CO_2 release (Bely et al., 1990 a, b). The amount of CO_2 release (g/L) was determined by automatic measurement of fermentor weight loss every 20 min. The CO_2 production rate (g/L/h) was obtained by polynomial smoothing of the last 11 CO_2 measurements. Weight loss due to evaporation was under 2%.

Yeasts were pre-cultured in Erlenmeyer flasks filled with must at 24 °C for 24 or 48 h for *S. cerevisiae* and *T. delbrueckii*, respectively. Fermentations were carried out at 24 °C with agitation in 1.2 L fermentors locked to maintain anaerobiosis throughout alcoholic fermentation (CO_2 was released through a sterile air outlet condenser). Four different fermentations were conducted: two with pure cultures and two with mixed cultures. Two types of mixed cultures were carried out: simultaneous mixed modality where *T. delbrueckii* and *S. cerevisiae* were inoculated at the same time and sequential mixed modality where *T. delbrueckii* was inoculated 24 h before *S. cerevisiae* yeast. Single and mixed cultures were inoculated with 1×10^7 viable cells/mL for *T. delbrueckii* and 2×10^6 viable cells/mL for *S. cerevisiae*. All experiments were performed in triplicate.

2.3.2. Winery scale

Fermentations were carried out in 200 L tanks at temperatures between 18 and 22 °C. Fermentation kinetics were evaluated by monitoring the density. Two different fermentations were carried out: a pure culture of *S. cerevisiae* and a sequential mixed culture where *T. delbrueckii* was inoculated 24 h before *S. cerevisiae* yeast. Single and mixed cultures were inoculated with 1×10^7 viable cells/mL for *T. delbrueckii* and 2×10^6 viable cells/mL for *S. cerevisiae*. All experiments were performed in duplicate.

2.4. Population dynamics

In mixed cultures, yeast growth was determined by plate counting on 2 different agar media. Samples were withdrawn throughout fermentation and diluted appropriately. Non-*Saccharomyces* cells were counted using a specific agar medium (NS): YPDA (1% yeast extract, 1% peptone, 2% dextrose, 2% agar; pH 4.8) supplemented with 1 μg/mL cycloheximide to promote the growth of *T. delbrueckii* Alpha and inhibit that of *S. cerevisiae* X5. The number of *S. cerevisiae* was given as the difference between the total plate count using YPDA medium and the plate count using NS medium. Yeast growth in single cultures was determined using only the YPDA medium. Plates were incubated at 24 °C for 4 days before counting.

The level of yeast population was only measured for experiments carried out in laboratory.