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Effect of yeast assimilable nitrogen on the synthesis of phenolic aroma compounds by *Hanseniaspora vineae* strains

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Abstract

In several grape varieties, the dominating aryl alkyl alcohols found are the volatile group of phenylpropanoid-related compounds, such as glycosylated benzyl and 2-phenylethyl alcohol, which contribute to wine with floral and fruity aromas after being hydrolysed during fermentation. *Saccharomyces cerevisiae* is largely recognized as the main agent in grape must fermentation, but yeast strains belonging to other genera, including *Hanseniaspora*, are known to predominate during the first stages of alcoholic fermentation. Although non-*Saccharomyces* yeast strains have a well-recognized genetic diversity, understanding of their impact on wine flavour richness is still emerging. In this study, 11 *Hansenisapora vineae* strains were used to ferment a chemically defined simil-grape fermentation medium, resembling the nutrient composition of grape juice but devoid of grape-derived secondary metabolites. GC–MS analysis was performed to determine volatile compounds in the produced wines. Our results showed that benzyl alcohol, benzyl acetate and 2-phenylethyl acetate are significantly synthesized by *H. vineae* strains. Levels of these compounds found in fermentations with 11 *H. vineae* different strains were one or two orders of magnitude higher than those measured in fermentations with a known *S. cerevisiae* wine strain. The implications for winemaking in response to the negative correlation of benzyl alcohol, benzyl acetate and 2-phenylethyl acetate production with yeast assimilable nitrogen concentrations are discussed. Copyright © 2016 John Wiley & Sons, Ltd.

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Introduction

Nowadays, some winemakers and brewers are rediscovering the value of using mixed cultures or spontaneous fermentation in order to increase flavour complexity (Carrau, 2006; Carrau, *et al*., 2015). It is believed that a selected and inoculated strain of *S. cerevisiae* will suppress any native non-*Saccharomyces* species and dominate the fermentation process. Although this expectation has been widely accepted by winemakers, several studies have revealed that non-*Saccharomyces* yeasts can indeed persist during the various stages of fermentations that are inoculated with pure cultures of *S. cerevisiae* (Mora *et al*., 1990; Ciani *et al.*, 2010; Andorrá *et al.*, 2010; De Benedictis *et al*., 2011; Garavaglia *et al.*, 2015). Several studies evaluated the involvement of non-S*accharomyces* yeasts during alcoholic fermentation and their role in the metabolic impact and aroma complexity of the final product (Jolly *et al*., 2014; Carrau *et al*., 2015). Moreover, even though non-*Saccharomyces* yeast

strains, which account for > 99% of the grape native flora, have a well-recognized genetic diversity, understanding of their impact on wine flavour richness is still emerging (Steensels *et al.*, 2014; Ugliano and Henschke, 2009). The morphological apiculate yeast genus *Hanseniaspora* accounts for about 60% of this non-*Saccharomyces* grape natural flora (Carrau *et al.*, 2015). Besides, some key aroma compounds, levels such as 2-phenylethyl acetate, increased when *H. vineae* was compared to *Saccharomyces* single-strain fermentations (Carrau, 2006; Medina *et al*., 2013; Viana *et al*., 2011).

Benzyl alcohol has been shown to contribute to wine aroma descriptors, such as fig, tobacco and chocolate, in varieties such as Cabernet Sauvignon (Francis *et al.*, 1998), while 2-phenylethyl acetate has been reported as contributing to flowery and fruity descriptors (Boido, 2002).

This study contributes to the valuable application of *H. vineae* strains to synthesize benzyl alcohol, benzyl acetate, 2-phenylethyl alcohol and 2-phenylethyl acetate in higher concentrations than those usually found in grapes, thus contributing to increasing yeast flavour diversity after fermentation. Ammonium levels modifications were tested using a chemically defined simil-grape fermentation medium in order to evaluate its effect as potential regulator for the synthesis of phenylpropanoids.

Materials and methods

Yeast strains

The *S. cerevisiae* strain used was Montrachet UCD 522 (University of California, Davis), referred to as M522 in this work, a commercial *S. cerevisiae* strain used in wine production of *H. vineae* strains were isolated from Uruguayan vineyards. The identification codes used in this work correspond to those assigned for the Sección Enología (Facultad de Química-UdelaR, Montevideo, Uruguay) yeast culture collection. The 11 *H. vineae* isolates used in this work were identified by sequencing the variable D1/D2 region from the *26S* rDNA gene and differentiated as different strains by Tandem repeat tRNA PCR analysis (Barquet *et al*., 2012).

Fermentation conditions

Chemically defined synthetic grape fermentation medium (resembling the nutrient composition of grape juice but devoid of grape-derived secondary metabolites) was used, and is referred to as 'CDG medium' in this work. It was prepared as previously described (Carrau *et al.*, 2008) but modified as follows: the total nitrogen content was adjusted to a basic amount of yeast-assimilable nitrogen value (YAN level; sum of amino acids and ammonium without proline) of 50 mg N/l and up to 250 mg N/l for the experiments with diammonium phosphate additions. The nitrogen composition, expressed in mg N/l, of the CDG medium was as follows for a YAN level of 100 mg N/l: ammonium phosphate (60.3), arginine (90.4), glutamate (60.3), proline (60.3), serine (48.2), aspartate (42.2), threonine (42.2), leucine (36.2), lysine (30.1), glutamine (24.1), isoleucine (24.1), valine (24.1), asparagine (18.1), histidine (18.1), methionine (18.1), phenylalanine (18.1), alanine (12.1), tryptophan (12.1), glycine (6) and tyrosine (2.4). The final pH for each medium was adjusted to 3.5 with concentrated HCl. Equimolar concentrations of glucose and fructose were added to reach 200 g/l and the mixed vitamins and salts used were as previously described (Henschke and Jiranek, 1993). Under our experimental conditions, Tween 80 was excluded from the medium because it was found to have a negative impact on the sensory characteristics of the resultant wines. Ergosterol was added as the only lipid supplement, at a final concentration of 10 mg/l. Inocula were prepared in the same CDG medium at 100 mg N/l YAN by incubation for 12 h in a rotary shaker at 150 rpm and 25 °C. Inoculum size for all strains was 1×10^5 cells/ml in the final medium. Fermentations were carried out in 250 ml Erlenmeyer flasks containing 125 ml medium, closed with cotton plugs to simulate microaerobic conditions (Fariña *et al*., 2012). Static batch fermentation were conducted at 20 °C in triplicate, simulating wine-making conditions. Fermentation activity was measured as CO_2 weight loss and expressed in g/100 ml. Samples were taken once a day to measure cell growth in an improved Neubauer chamber. Samples for GC–MS analysis were taken 1 day after the end of the fermentation process and filtered through 0.45 mm pore membranes (Sartorius); SO_2 was added as sodium metabisulphite (50 mg/l).

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GC–MS analysis

Aroma volatile compounds

Extraction of aroma compounds was performed using adsorption and separate elution from an Isolute (IST Ltd, Mid-Glamorgan, UK) ENV1 cartridge packed with 1 g highly crosslinked styrene–divinyl benzene (SDVB) polymer. The sample preparation and GC–MS analysis was performed as described (Boido *et al.*, 2003), using a Shimadzu QP 2010 ULTRA (Tokyo, Japan) mass spectrometer equipped with a Stabilwax (30 m × 0.25 mm i.d., 0.25 μm film thickness; Restek) capillary column.

Identification and quantification

The wine aroma components were identified by comparison of their linear retention indices, with pure standards for benzyl alcohol, benzyl acetate, 2-phenylethyl alcohol and 2-phenylethyl acetate (Aldrich, Milwaukee, WI, USA). Comparison of fragmentation patterns in the MS with those stored on the GC–MS databases was also performed (McLafferty and Stauffer, 1991; Adams 2007). GC–MS instrumental procedures using an internal standard (1-heptanol) were applied for quantitative purposes, as previously described (Versini *et al*., 1994) and evaluated (Carlin, 1998; Boido *et al*., 2003).

Statistical analysis

2-Phenylethyl acetate levels produced in wine by using 12 yeast strains in the CDG fermentation medium were evaluated by analysis of variance (ANOVA), as also was the effect of diammonium phosphate addition on phenylpropanoids production by *H. vineae* at four YAN levels (50, 75, 100 and 250 mg N/l). Differences between mean values were determinate by LSD test. All the ANOVA analyses were performed with Statistica v. 7.0 software.

Results and discussion

Eleven *H. vineae* strains from our native yeast collection (Sección Enología, Facultad de Química-UdelaR), including Hv025, already applied by our group in wine making (Medina *et al*., 2013), were selected in order to compare, within the species, their ability to produce 2-phenylethyl acetate in a defined synthetic medium. A commercial *S. cerevisiae* wine strain (M522) was compared to the 11 *H. vineae* strains. The production of 2-phenylethyl acetate by wine yeasts was measured at the end of fermentation in CDG medium containing 150 mg N/l YAN (the usual YAN level found in industrial grape juice (see Materials and methods and Figure 1). As expected, the results found

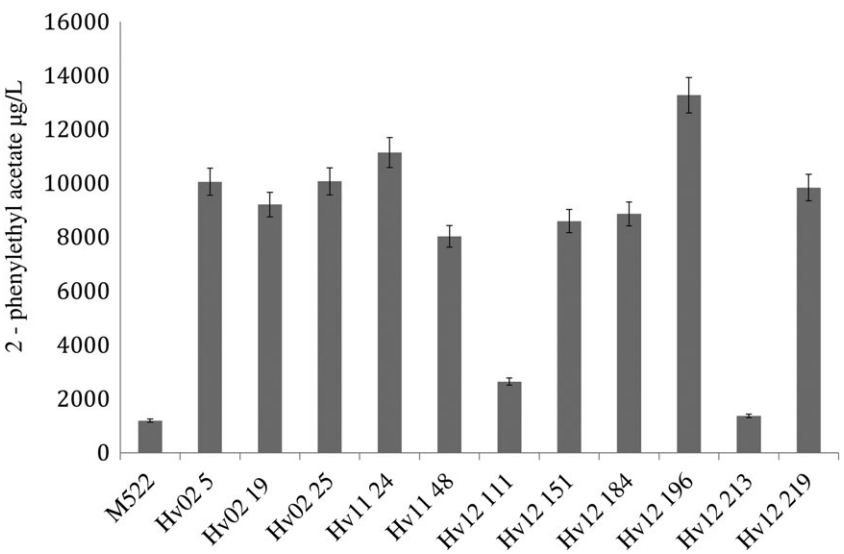

Figure 1. 2-Phenethyl acetate production by *H. vineae* strains compared to *Saccharomyces cerevisiae* strain wine yeasts (M522)

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showed that the production of 2-phenylethyl acetate by the *S. cerevisiae* strain is very limited in the CDG medium compared to most of the *H. vineae* strains tested. These results are in agreement with previous reports of mixed cultures of *H. vineae–S. cerevisiae* in real grape juice fermentations of Chardonnay (Carrau, 2006; Medina *et al.*, 2013) and Tempranillo (Viana *et al.*, 2011). Nevertheless, as seen in Figure 1, the wide strain diversity within this species of 2-phenylethyl acetate accumulation, which accounts for 1800–13 000 µg/l in our model medium, can be appreciated. All strains clearly produce above the threshold reported for this ester (250 µg/l) (Carrau *et al.*, 2008). No correlation was found between the production of 2-phenylethyl acetate and the fermentation rates (data not shown) of the different *H. vineae* strains, although the slowest-growing strain (Hv12213) showed the lowest 2-phenylethyl acetate production. Figure 2 shows the formation of benzyl and 2-phenylethyl alcohols and the corresponding acetates at the end of fermentation. When production and accumulation of these compounds were compared for the two yeast species, only 2-phenylethyl alcohol was produced at higher levels by *S. cerevisiae*. However, the sum of 2-phenylethyl alcohol and its acetate was greater in *H. vineae*; this result clearly showed that the difference between these two species will be at the acetylation step (56% of the alcohol is acetylated in *H. vineae* compared to $< 2\%$ in *S. cerevisiae*). Interestingly, benzyl alcohol acetylation showed a very low formation rate for both species (about 2% of the alcohol was acetylated).

Effect of ammonium on phenylpropanoid compounds

Feeding experiments, changing the ammonium concentration of the artificial medium, were designed to investigate its effect as a potential pathway regulator for the formation of phenylpropanoids using the main *H. vineae* producer, strain Hv12196. Figure 3 shows the ammonium effect, where a consistent negative correlation was observed in the formation of benzyl alcohol, 2-phenylethyl alcohol and their acetates with increased diammonium phosphate levels. A significant inhibition to the production of benzyl alcohol and 2-phenylethyl alcohol was observed at a YAN level of 250 mg N/l (78 and 5 µg/l, respectively). This behaviour in both alcohols could suggest an inhibitory effect from inorganic nitrogen at some level in the biosynthetic pathway, as was shown for the biosynthesis of other higher alcohols in *S. cerevisiae* (Carrau *et al.*, 2008). These results clearly showed that the increased use of diammonium phosphate in wine making, mainly applied for increasing ester production, will decrease the production of phenylpropanoid compounds, compromising the final flavour complexity of the wine. Further studies with other yeast-assimilable nitrogen

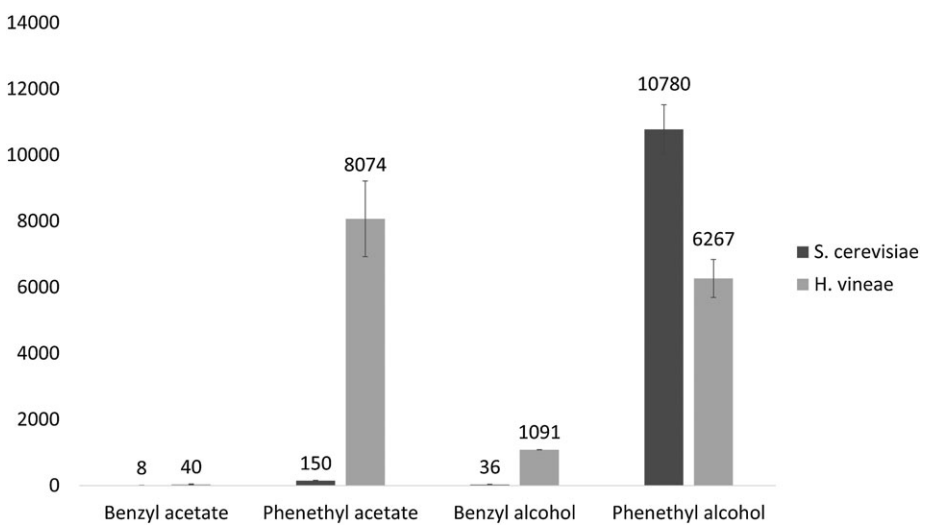

Figure 2. Production of benzyl alcohol, 2-phenylethyl alcohol and their acetates by *H. vineae* Hv12196 strain and *S. cerevisiae* M522

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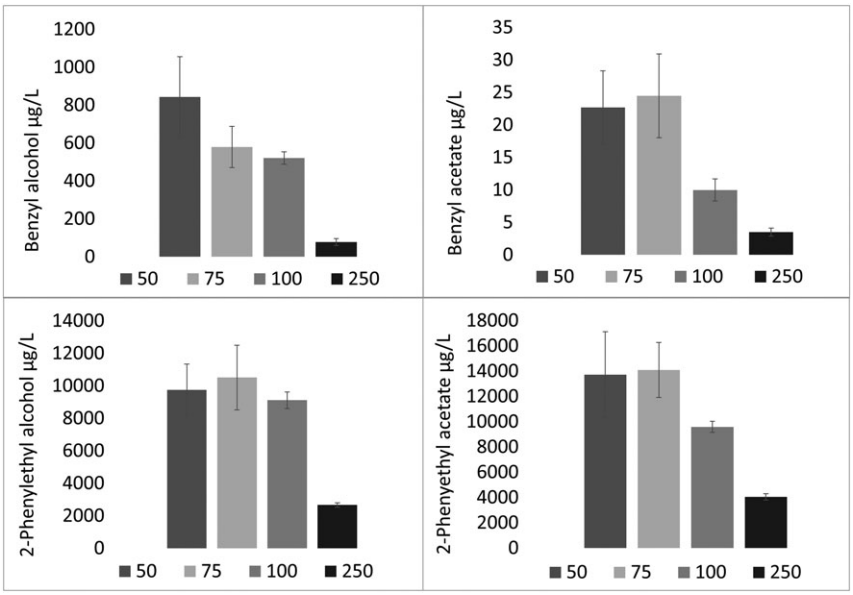

Figure 3. Production of benzyl alcohol, 2-phenylethyl alcohol and their acetates at different concentrations of YAN adjusted with diammonium phosphate with *H. vineae* Hv12196

compounds, such as the aromatic amino acids, will improve our understanding of the metabolic pathways of phenylpropanoids.

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