# Characterisation of *Hanseniaspora* Isolates with Potential Aroma-enhancing Properties in Muscat Wines

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**The sensorial characteristics of the wines produced with Muscat grapes are related to the level of terpene alcohols, so an improvement of such a level is expected as a result of hydrolytic processes conducted by *Hanseniaspora*. The aim of this work was to select and identify new strains for this purpose. In a second step, the strains were assayed to evaluate their oenological abilities. *H. uvarum* H107 and *H. vineae* G26 and P38 were proven to be good candidates to be used in commercial vinification processes to enhance wine properties. Wine inoculated with yeasts showed an increase in the level of aromatic compounds.**

## INTRODUCTION

*Hanseniaspora*, which are yeasts mainly found in soil, on fruits and trees and in spoiled foods and beverages, are characterised by apiculate cells and undergo vegetative reproduction by bipolar budding in basipetal succession. There are several species in the genus *Hanseniaspora* (Cadez *et al*., 2003; Jindamorakot *et al*., 2009; Kurtzman *et al*., 2011). They are physiologically very similar (Kurtzman & Robnett, 1998): they ferment glucose, assimilate a few carbon compounds (arbutin, cellobiose, glucose, glucono-***δ***-lactone and salicin), and require inositol for growth (Kurtzman *et al*., 2011). The identification of yeast species has traditionally been based on assimilation and fermentation tests and morphological traits. These studies must be accompanied by other assays to assess a correct and unambiguous identification of the yeast. Molecular methods have been used to explore this yeast biodiversity, conferring a high degree of accuracy in the final identification (Kurtzman *et al*., 1994, 2011; Guillamón *et al*., 1998; Esteve-Zarzoso *et al*., 1999; Arroyo-López *et al*., 2006).

From a biotechnological point of view, although these yeasts can produce spoilage of fruits (Arroyo-López *et al*., 2008), they also possess many interesting technological properties (Charoenchai *et al*., 1997; Strauss *et al*., 2001; Maicas & Mateo, 2005). In this way, exocellular enzymes of relative yeast species in different ecosystems have previously been reviewed (Mateo *et al*., 2011). Winemaking has been the process more deeply studied regarding the influence of *Hanseniaspora* (*H. guilliermondii, H. uvarum, H. osmophila* and *H. vineae*) and other non-*Saccharomyces* species. The modification of the characteristics of the wine is attributed to the capacity of certain non-*Saccharomyces* yeasts to produce and secrete hydrolytic enzymes that are able to transform grape compounds (Pando *et al*., 2012). These compounds are present in varying amounts as non-volatile flavour glycosylated precursors (Ugliano, 2009), mainly the disaccharides 6-O-α-L-arabinofuranosyl-β-D-glucopyranoside, 6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside,2-O-β-D-xylosyl-D-glucopyranoside and 6-O-β-L-apiofuranosil-β-D-glucopyranoside (Mateo *et al*., 2011). The action of enzymes produced by wine yeasts, *i.e.* β-glucosidase or β-xylosidases, can contribute to liberate flavour from these compounds (Romano *et al*., 2003). Several groups have already made trial fermentations to study the compounds generated by *Hanseniaspora* (Paraggio, 2004; Arévalo *et al*., 2007; Swangkeaw *et al*., 2011).

The aim of the present study was to select and correctly identify *Hanseniaspora* strains to be used further as a source of enzymes in the winemaking industry and in other biotechnological processes. These yeasts are non-pathogenic and are recognised as safe organisms (GRAS) that can be used for the production of a variety of enzymes. Finally, our assays proved the benefits of using these yeasts to increase the level of terpenes in Muscat wine.

## MATERIALS AND METHODS

### Yeast strains

Grape juice and wine samples were collected from cellars in the D.O. Utiel-Requena (Eastern Spain) over the last 22 years (Mateo *et al*., 1991, 1992, 2011; Gil *et al*., 1996). Samples were stored at -20ºC and routinely spread onto malt agar (20 g/L malt extract, 1 g/L peptone, 20 g/L glucose, 20 g/L agar) and grown at 28ºC for viability check, or in YPD (20 g/L yeast extract, 10 g/L peptone, 20 g/L glucose) for routine assays. A total of 31 strains of *Hanseniaspora* yeast from our collection that previously were selected by using lysine

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agar as differential medium (Heard & Fleet, 1986) were used in this study (Table 1). Isolates were identified according to their morphological and physiological characteristics as described by Kurtzman *et al.* (2011), and further verified by sequencing of the D1/D2 domain of 26S rDNA, as described by Kurtzman and Robnett (1998). Moreover, the Rapid ID Yeast Plus System (Remel, Lenexa, KS) was used to assess the strain-specific pattern of carbon compound assimilation, and other phenotypic assays (strain typing) according to the manufacturer's instructions.

**Qualitative screening of the biochemical activities**

***β-Glucosidase activity***

The basal medium consisted of 1.7 g/L yeast nitrogen base (Difco), 5 g/L ammonium sulphate, 5 g/L glucose and 20 g/L agar. After autoclaving, 2 mL of a sterile 1% (w/v) 4-methylumbelliferyl-β-D-glucopyranoside (Sigma-Aldrich, St. Louis, MO) was added to 100 mL of melted medium (Manzanares *et al*., 1999). The medium was then poured into Petri dishes and inoculated with 24 h-old yeast cultures. Plates were incubated at 28ºC for three days. The presence of enzymatic activity was visualised as a fluorescent halo surrounding yeast growth by plate exposure to UV light.

***Xylanase activity***

Xylanase production was determined according to Hernández *at al*. (2007) by spreading yeast colonies onto agar plates containing 5 g/L xylan, 5 g/L peptone and 5 g/L NaCl. Plates were incubated at 28ºC for seven days. A clear zone around the colony was indicative of xylanase activity.

***Pectinase and polygalacturonase activities***

The assays were carried out in the following medium: 1 g/L yeast extract, 1 g/L ammonium sulphate, 6 g/L $NaHPO_4$, 3 g/L $KH_2PO_4$, 5 g/L pectin (for pectinase activity) or 5 g/L polygalacturonic acid (for polygalacturonase activity), 15 g/L agar. After streaking 48 h-old yeast cultures onto the surface of the medium, the plates were incubated at 28ºC for five days and then revealed by the addition of a solution of hexadecyltrimethylammonium bromide (1 g/L), according to the procedure described by Oliveira *et al*. (2009). Both activities were evident from the presence of a clear halo around the colonies.

***Lipase and esterase activities***

Yeast isolates were used to determine esterase and lipase activity on tributyrin and rhodamine olive oil agar media respectively, according to the previously described procedures (Rodríguez *et al*., 2010; Madrigal *et al*. 2013). After 48 h of incubation in the media at 28ºC, the colonies were investigated. For the detection of lipase activity they were irradiated with UV light at 350 nm; lipase activity was detected by an orange fluorescent halo around colonies. Esterase activity was detected by the formation of a clear transparent halo around colonies.

*Saccharomyces cerevisiae* CECT11783 and *Candida molischiana* ATCC 2516 were used as a positive control for polygalacturonase and β-glucosidase production respectively. Lipase from *C. antarctica,* esterase from *S. cerevisiae* and pectinase from *Aspergillus niger* were purchased from Sigma-Aldrich, St. Louis, MO for the same purpose. All the biochemical activities previously described were assayed in triplicate.

**Quantitative spectrophotometric assays**

***β-Glucosidase and β-xylosidase activities***

β-Glucosidase and β-xylosidase activities were basically assayed using 4-nitrophenyl-β-D-glucopyranoside and 4-nitrophenyl-β-D-xylopyranoside as the substrates (Romero *et al*., 2012). The yeasts were centrifuged and resuspended in 750 µL of 0.2 M citrate-0.1 M phosphate buffer (pH 5.0). Then 250 µL of 5 mM *p*NP substrate in the same buffer were added and the mixture was incubated at 40°C for 90 min. The reaction was stopped by adding 1.0 mL of 0.2 M $Na_2CO_3$, and absorbance at 404 nm was measured. Activity was expressed as nanokatals (1 nkat = 1 nmol of *p*NP liberated in one hour by $10^6$ yeast cells). Assays were performed in triplicate.

**Effect of sugars and ethanol on β-glucosidase and β-xylosidase activities**

The effects of sugar on enzyme activities were conducted by using sugar concentrations over a range of 0 to 500 mM (glucose and fructose) or 0 to 50 mM (sucrose). The effect of ethanol (Merck, Darmstadt, Germany) on enzyme activity was determined by using ethanol concentration over a range of 0 to 20% (v/v). The enzyme assays were carried out as described previously.

**Effect of temperature and pH on β-glucosidase and β-xylosidase activities**

The pH optimum was determined in 0.2 M citrate-0.1 M phosphate buffer, covering a pH range from 3.0 to 8.0, at

TABLE 1
*Hanseniaspora* yeast isolates from the Utiel-Requena region.

| Yeast species | Number of isolates | Matching nucleotides (%)[a] | Collection number |
|---|---|---|---|
| *Hanseniaspora uvarum* | 18 | 99.4-99.6% | E18, E20, E44, F42, G09, G89, H03, H105, H106, H107, H156, H172, B21, B95, M45, M46, M47, M48 |
| *H. vineae* | 4 | 99.5-99.6% | E71, G26, P30, P38 |
| *H. guilliermondii* | 8 | 99.6-99.8% | A27, A29, B02, B04, C06, D55, D56, D57 |
| *H. osmophila* | 1 | 99.7% | C07 |
| Total | 31 | | |

[a]Sequence identity in the D1/D2 region of 26S ribosomal genes and closest relative species in the NCBI GenBank database.

40ºC for 90 min. The temperature optimum was measured from 4ºC to 60ºC for 90 min of incubation in the same buffer, at pH 5.0. The residual activities were determined.

**Winemaking**

Muscat juice (1 000 hL) was fermented using 30 g/hL commercial wine yeast strain *S. cerevisiae* QA23 (Lalvin, Lallemand, Montreal, Canada). Fermentation was carried out at 16 to 24ºC. Must sampling for the analysis of sugar concentrations was performed weekly. After 23 days, when less than 2 g/L residual sugar remained, the wine was separated from the gross lees. Samples were collected and stored at 4ºC for the second inoculation with *Hanseniaspora*. This wine, provided by the Baronía de Turís winery, was as follows: ethanol, 13.3% v/v; pH, 3.33; titratable acidity, 3.6 g/L; volatile acidity, 0.35 g/L; malic acid, 1.0 g/L. The wine was sterilised by 0.45 µm filtration and sterility was verified by spreading 100 µL wine on YPD plates.

**Determination of volatile compounds liberated from wine incubated with *Hanseniaspora* strains**

*H. uvarum* H107 and *H. vineae* G26 and P38 were grown in YPD, centrifuged, resuspended in distilled water, added to 400 mL Muscat wine at a final concentration of $1.0 \times 10^6$ cfu/mL, and incubated in separated 500 mL cotton-plugged flasks at 20ºC for 14 days, without shaking. Wine produced with *S. cerevisiae*, without added *Hanseniaspora* yeasts, was assayed in parallel to act as a control. The isolation of terpenes was then carried out on C18 SPE columns (Waters, Milford, MA). A total of 250 mL of each wine was eluted through the columns, previously activated with 30 mL of methanol (Merck, Darmstadt, Germany), and followed by 50 mL of water. The cartridge was washed with 100 mL of water and lipophilic compounds (terpenes) were eluted with 50 mL of dichloromethane (Merck). Volatile compounds were determined by using an Agilent 6890 N gas chromatograph-5973N mass detector system. The chromatograph was equipped with a HP-20M fused silica capillary column (50 m × 0.32 mm i.d., 0.3 µm film thickness). The splitless injector was maintained at 250ºC. Helium was used as carrier gas (inlet pressure 10.5 psi) and the interface temperature was 230ºC. The oven was held isothermally at 30ºC for 1 min and then increased to 60ºC at 30ºC/min. The oven temperature was programmed from 60ºC to 120ºC at 3ºC/min, and then to 220ºC at 2ºC/min. 1-Heptanol (1 mg/mL) was used as internal standard.

**Statistical analyses**

The volatile compounds were subjected to one factor analysis of variance (ANOVA, Statbox software). The results were considered significant if the associated *P* values were below 0.05.

## RESULTS

**Identification and physiological characterisation of strains**

The 218 non-*Saccharomyces* yeasts stored in our collection were investigated. A total of 31 isolates were presumptively identified as *Hanseniaspora* and used to carry out this work (Table 1). The ribosomal D1/D2 regions of these isolates were sequenced and, after screening for DNA homology using the BLAST program, yeast species identities were verified by comparing sequences of their ribosomal internal transcribed spacer regions (Cadez *et al*., 2002). We found isolates belonging to four oenological species of the genus *H. uvarum* (18), *H. vineae* (4), *H. osmophila* (1) and *H. guillermondii* (8) (Kurtzman & Robnett, 1998).

The strain-specific pattern of carbon compound assimilation and other phenotypic assays (strain typing) were carried out using Rapid ID Yeast Plus. The results obtained with this commercial kit (Table 2) preliminarily suggested that none of the *Hanseniaspora* species used in this study had αgalactosidase, β-galactosidase, phosphatase, phosphatidylcoliesterase or urease activities. Moreover, N-acetyl-glucosamine and fatty acid esters were not assimilated. Some other differences were detected among strains. More accurate qualitative assays were further carried out to achieve better characterisation of biochemical activities.

**Qualitative assays of enzymatic activities**

The final results for the 31 *Hanseniaspora* strains characterised in this work are shown in Table 3. Similar patterns of overall enzyme activity were observed. Almost all strains displayed moderated or no lipase, esterase, polygalacturonase or pectinase activity. Moreover, a total of 28 strains showed β-glucosidase activity.

**β-glucosidase and β-xylosidase activities**

We examined our isolates to determine β-glucosidase and β-xylosidase activities, the two more relevant glycosidic activities for non-*Saccharomyces* yeasts in wine (Mateo & Di Stefano, 1997; Fernández *et al*., 2000; Strauss *et al*., 2001; Mateo *et al*., 2011). *H. uvarum* H107 and *H. vineae* (G26 and P38) exhibited strong reactions in both the β-glucosidase and β-xylosidase quantitative assays (> 30 nkat) (Table 4). These isolates were selected to quantify the level of production of these activities under oenological stress conditions, i.e. high sugar or ethanol concentrations.

**Effect of sugars or ethanol on β-glucosidase and β-xylosidase activities**

The effects of sugar concentration (glucose, fructose and sucrose) on β-glucosidase and β-xylosidase were assayed. The β-glucosidase and β-xylosidase activities of the three strains were not affected by fructose (0 to 500 mM) and sucrose (0 to 50 mM) (data not shown). The β-glucosidase activity of the three *Hanseniaspora* strains was slightly inhibited by low glucose concentrations (Fig. 1A). The enzyme retained 70 to 80% and 40 to 60% of relative activity in the presence of 50 and 100 mM glucose respectively. Moreover, 30% remaining activity was detected in the presence of 500 mM glucose. Similar results were observed in the three assayed strains. The β-xylosidase activity results were similar (Fig. 1B). We also detected moderated inhibition at 50 mM glucose, but 30 to 50% activity was retained at higher glucose concentrations (100 to 500 mM).

The effect of ethanol concentration in the range 0 to 20% (v/v) on both glycolytic activities was also determined. The β-glucosidase activity from the three *Hanseniaspora*

TABLE 2
Results of the RapID yeast plus assays. (-) No assimilation, (+) assimilation

| Collection number | Species | Glucose | Maltose | Sucrose | Trehalose | Raffinose | Fatty acid ester | N-Acetyl-glucosamine | a-Glucoside | b-Glucoside | b-Galactoside | a-Galactoside | b-Fucoside | Phosphate | Phosphatidylcholine | Urea | Proline-β-naphthylamide | Histidine β-naphthylamide | Leucyl-glycine β-naphthylamide |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A27 | *H. guilliermondii* | + | + | + | + | + | - | - | + | + | - | - | + | - | - | - | - | + | + |
| A29 | *H. guilliermondii* | + | - | - | - | - | - | - | + | + | - | - | - | - | - | - | + | + | + |
| B02 | *H. guilliermondii* | + | + | + | + | + | - | - | + | + | - | - | + | - | - | - | - | + | + |
| B04 | *H. guilliermondii* | + | - | - | - | - | - | - | + | + | - | - | + | - | - | - | - | + | + |
| C06 | *H. guilliermondii* | + | + | + | - | + | - | - | + | + | - | - | + | - | - | - | - | + | + |
| D55 | *H. guilliermondii* | + | + | + | + | + | - | - | + | + | - | - | + | - | - | - | - | + | + |
| D56 | *H. guilliermondii* | + | - | - | - | - | - | - | + | + | - | - | + | - | - | - | - | + | + |
| D57 | *H. guilliermondii* | + | - | - | - | - | - | - | + | + | - | - | - | - | - | - | + | + | + |
| C07 | *H. osmophila* | + | + | + | + | - | - | - | + | + | - | - | - | - | - | - | - | + | + |
| E18 | *H. uvarum* | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - |
| E20 | *H. uvarum* | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - |
| E44 | *H. uvarum* | + | - | + | - | + | - | - | + | - | - | - | - | - | - | - | + | + | + |
| F42 | *H. uvarum* | + | + | + | + | + | - | - | - | + | - | - | - | - | - | - | - | + | - |
| G89 | *H. uvarum* | + | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - | + | - |
| G09 | *H. uvarum* | + | - | + | - | + | - | - | - | - | - | - | - | - | - | - | - | + | - |
| H03 | *H. uvarum* | + | + | - | - | + | - | - | - | - | - | - | - | - | - | - | + | + | + |
| H105 | *H. uvarum* | + | - | - | - | + | - | - | - | + | - | - | - | - | - | - | - | - | - |
| H106 | *H. uvarum* | + | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| H107 | *H. uvarum* | + | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | + | - |
| H156 | *H. uvarum* | + | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - | + | - |
| H172 | *H. uvarum* | + | - | + | - | + | - | - | + | - | - | - | - | - | - | - | + | + | + |
| B21 | *H. uvarum* | + | + | + | + | + | - | - | - | + | - | - | - | - | - | - | - | + | - |
| B95 | *H. uvarum* | + | - | - | - | + | - | - | - | + | - | - | - | - | - | - | - | - | - |
| M45 | *H. uvarum* | + | - | + | - | + | - | - | + | - | - | - | - | - | - | - | + | + | + |
| M46 | *H. uvarum* | + | + | - | - | + | - | - | - | - | - | - | - | - | - | - | + | + | + |
| M47 | *H. uvarum* | + | - | - | - | + | - | - | - | + | - | - | - | - | - | - | - | - | - |
| M48 | *H. uvarum* | + | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| E71 | *H. vineae* | + | + | + | - | + | - | - | + | + | - | - | + | - | - | - | + | + | + |
| G26 | *H. vineae* | + | - | - | - | - | - | - | + | + | - | - | + | - | - | - | - | + | + |
| P30 | *H. vineae* | + | + | + | - | + | - | - | + | + | - | - | + | - | - | - | - | + | + |
| P38 | *H. vineae* | + | + | + | - | + | - | - | + | + | - | - | + | - | - | - | + | + | + |

TABLE 3
Enzymatic activity of the *Hanseniaspora* strains.

| Yeast | Number isolates | Enzymes level of activity[a] | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Pectinase | | | Esterase | | | Lipase | | | β-glucosidase | | | Poly-galacturonase | | | Xylanase | | |
| | | − | + | ++ | − | + | ++ | − | + | ++ | − | + | ++ | − | + | ++ | − | + | ++ |
| *H. uvarum* | 18 | 12 | 6 | | 6 | 6 | | 8 | 10 | | | 6 | 12 | 14 | 4 | | 15 | 3 | |
| *H. vineae* | 4 | 3 | 1 | | 1 | 1 | | 1 | 3 | | | | 4 | 1 | 3 | | 2 | 2 | |
| *H. guilliermondii* | 8 | 6 | 2 | | 3 | 3 | | 8 | | | 3 | 1 | 3 | 5 | 3 | | 6 | 2 | |
| *H. osmophila* | 1 | 1 | | | 1 | 1 | | 1 | | | | | 1 | 1 | | | 1 | | |
| Total | 31 | 22 | 9 | | 11 | 20 | | 18 | 13 | | 3 | 8 | 20 | 21 | 10 | | 24 | 7 | |

[a] Activity: (-), no activity; (+), moderate activity; (++), strong activity. Data obtained from triplicate assays

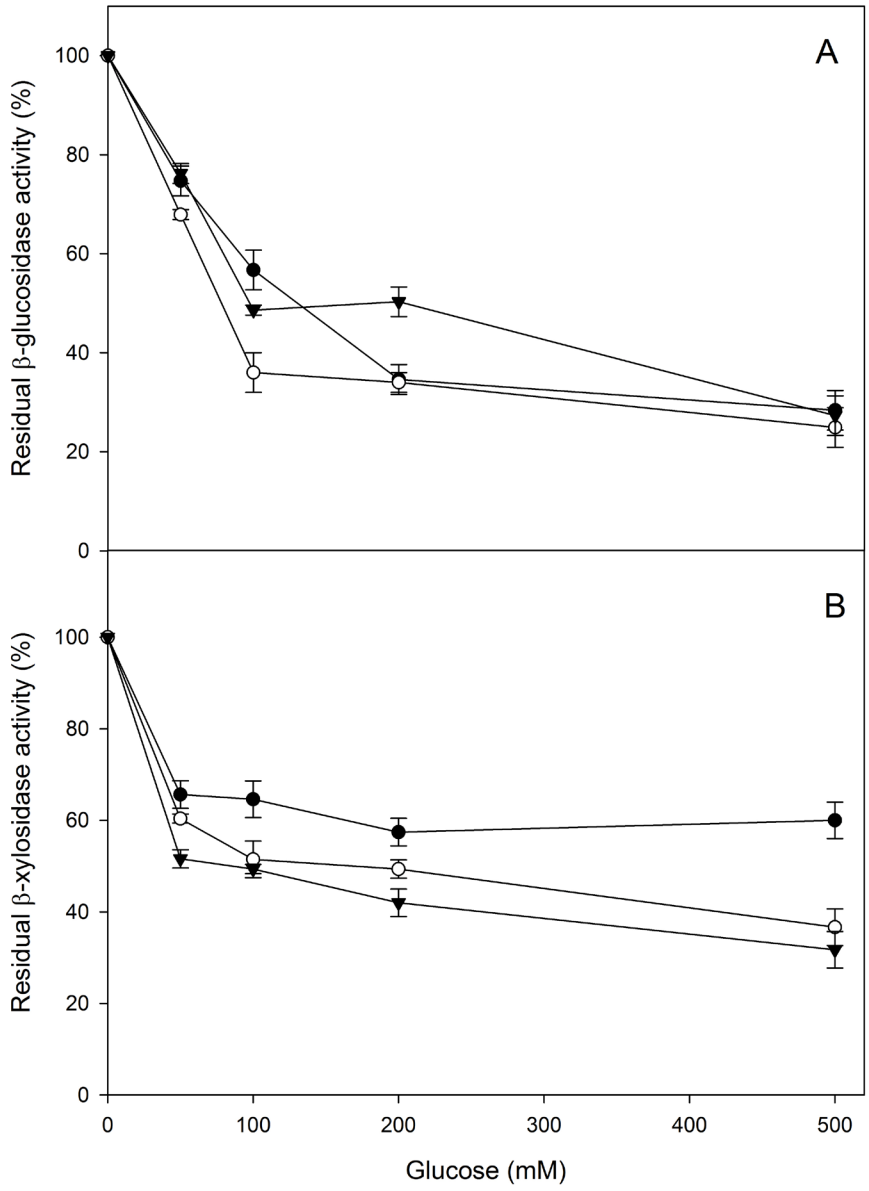


FIGURE 1
Effect of glucose concentration on glycolytic activities of different *Hanseniaspora* strains: (●) *H. uvarum* H107, (○) *H. vineae* G26, (▼) *H. vineae* P38. Enzyme activities were measured in the presence of various amounts of glucose under the standard assay conditions for A) **β-glucosidase and** B) **β-xylosidase activities.** Data represent means ± standard deviations from triplicate assays.

strains was slightly inhibited by low and medium ethanol concentrations (0 to 10 %, v/v) (Fig. 2A). In the presence of 20% (v/v) ethanol, 20 to 50% of activity remained. Similar results were expected from the β-xylosidases (Fig. 2B). The remaining activity of the three strains at 20% (v/v) was similar (around 40%).

**Effect of temperature and pH on β-glucosidase and β-xylosidase activities**

The β-glucosidase activity did not vary with temperature ranging from 4ºC to 60ºC (Fig. 3A), with an optimum of 30ºC to 40ºC. However, the β-xylosidase activity was highly influenced by temperature (Fig. 3B). Optimum temperature for the three strains was detected at 30ºC, showing percentages of activity lower than 40% at extreme values (4ºC to 60ºC).

The pH optimum was also evaluated in phosphate buffer ranging from pH 3.0 to 8.0. The β-glucosidase activity of the three *Hanseniaspora* strains was influenced slightly inside the interval assay, with residual activities between 80% and 100% (Fig. 4A). β-Xylosidase exhibited high pH stability between pH 5.0 and 8.0, retaining more than 80% of residual activity *in vitro*, with an optimum value of around pH 6.0 to 7.0 (Fig. 4B).

**Determination of volatile compounds liberated from wine treated with different *Hanseniaspora* yeasts**

Muscat juice was used for vinification with a commercial *S. cerevisiae* strain. Afterwards, *H. uvarum* H107 and *H. vineae* G26 and P38 were inoculated (in triplicate assays) and terpene compounds were determined (Table 5). We were not able to detect a significant increase in the level of nerol and geraniol after the addition of *Hanseniaspora* isolates. This can be attributed to the fact that the production of

TABLE 4
Glycolytic activities (nmol Pnp/h/$10^6$ yeasts = nkat) of the *Hanseniaspora* strains

| Isolate | Species | β-Xylosidase (nkat)[a] | β-Glucosidase (nkat)[a] |
|---|---|---|---|
| A27 | *H. guilliermondii* | 2.65 ± 0.08 | 1.70 ± 0.17 |
| A29 | *H. guilliermondii* | 2.18 ± 0.34 | 8.09 ± 0.22 |
| B02 | *H. guilliermondii* | 2.38 ± 0.07 | 21.07 ± 0.11 |
| B04 | *H. guilliermondii* | 2.24 ± 0.84 | 4.83 ± 0.23 |
| C06 | *H. guilliermondii* | 4.33 ± 0.08 | 5.27 ± 0.21 |
| D55 | *H. guilliermondii* | 3.60 ± 0.64 | 36.10 ± 0.13 |
| D56 | *H. guilliermondii* | 2.99 ± 0.14 | 20.80 ± 2.23 |
| D57 | *H. guilliermondii* | 2.72 ± 0.45 | 6.66 ± 0.26 |
| C07 | *H. osmophila* | 2.92 ± 0.23 | 5.91 ± 0.89 |
| E18 | *H. uvarum* | 2.31 ± 0.57 | 3.06 ± 0.84 |
| E20 | *H. uvarum* | 2.11 ± 0.17 | 42.49 ± 0.43 |
| E44 | *H. uvarum* | 2.99 ± 0.62 | 2.99 ± 0.11 |
| F42 | *H. uvarum* | 2.86 ± 0.11 | 3.26 ± 0.56 |
| G89 | *H. uvarum* | 1.90 ± 0.26 | 2.92 ± 0.19 |
| G09 | *H. uvarum* | 9.45 ± 0.23 | 40.38 ± 0.64 |
| H03 | *H. uvarum* | 1.90 ± 0.07 | 2.58 ± 0.05 |
| H105 | *H. uvarum* | 3.60 ± 0.71 | 5.64 ± 0.34 |
| H106 | *H. uvarum* | 15.83 ± 3.83 | 16.36 ± 3.16 |
| H107 | *H. uvarum* | 38.34 ± 0.14 | 48.74 ± 2.83 |
| H156 | *H. uvarum* | 2.58 ± 0.23 | 5.30 ± 0.23 |
| H172 | *H. uvarum* | 2.24 ± 0.78 | 21.89 ± 2.98 |
| B21 | *H. uvarum* | 12.11 ± 0.13 | 8.06 ± 0.32 |
| B95 | *H. uvarum* | 2.99 ± 0.64 | 20.08 ± 0.11 |
| M45 | *H. uvarum* | 22.86 ± 0.12 | 4.63 ± 0.24 |
| M46 | *H. uvarum* | 1.90 ± 0.29 | 3.26 ± 0.82 |
| M47 | *H. uvarum* | 18.45 ± 0.21 | 2.91 ± 0.39 |
| M48 | *H. uvarum* | 18.17 ± 0.14 | 5.24 ± 0.24 |
| E71 | *H. vineae* | 2.86 ± 0.19 | 26.85 ± 0.71 |
| G26 | *H. vineae* | 39.22 ± 0.84 | 48.81 ± 0.78 |
| P30 | *H. vineae* | 2.72 ± 0.51 | 13.53 ± 0.16 |
| P38 | *H. vineae* | 31.00 ± 0.71 | 51.26 ± 3.11 |

[a] Average of duplicates

these compounds could have been carried out previously by *Saccharomyces* strains during the alcoholic fermentation of Muscat wines (Mateo & Di Stefano, 1997; Restuccia *et al*., 2002). The concentration of *cis*-5-vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol was not increased, despite the addition of *Hanseniaspora*, while the other oxides (*trans*-5-vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol, *cis*-6-vinyltetrahy-dro-2,2,6-trimethyl-2H-pyran-3-ol and *trans*-6-vinyltetrahydro-2,2,6-trimethyl-2H-pyran-3-ol) were not detected. Moreover, 2,6-dimethyl-2,7-octadien-1,6-diol levels were not affected.

Linalool and derivative compounds and aromatic alcohols were detected at the same level of the control wine, after the addition of *Hanseniaspora* isolates. Terpineol increased only after the addition of *H. uvarum* H107 or *H. vineae* G26, but not when *H. vineae* P38 was added. The same results were recorded for 4-vinyl-phenol and 2-methoxy-4-vinylphenol. Linalool, 2-phenyl ethanol and 4-vinyl-phenol compounds are associated with fruity characteristics (Maicas & Mateo, 2005; López de Lerma & Peinado, 2011).

The analysis of the other compounds revealed an increase in concentration when yeasts were added, therefore assessing the effect of glycosidases. Wines treated with *Hanseniaspora* showed higher concentrations of 2-phenyl ethanol (Gunata *et al*., 1988; Dubourdieu *et al*., 1989). *H. uvarum* H107 provides improvements in 2-phenylethyl acetate production, as previously reported for other *Hanseniaspora* strains (Rojas *et al*., 2001; Moreira *et al*., 2005; Viana *et al*., 2009).

## DISCUSSION

The criteria for the discrimination of yeast species proposed by Kurtzman and Robnett (1998) using D1/D2 rDNA sequencing was used in this work: a match greater than 99% was required to discriminate among yeast species. The analysis of the miniaturised biochemical assay strips basically confirmed these results, although some other minor

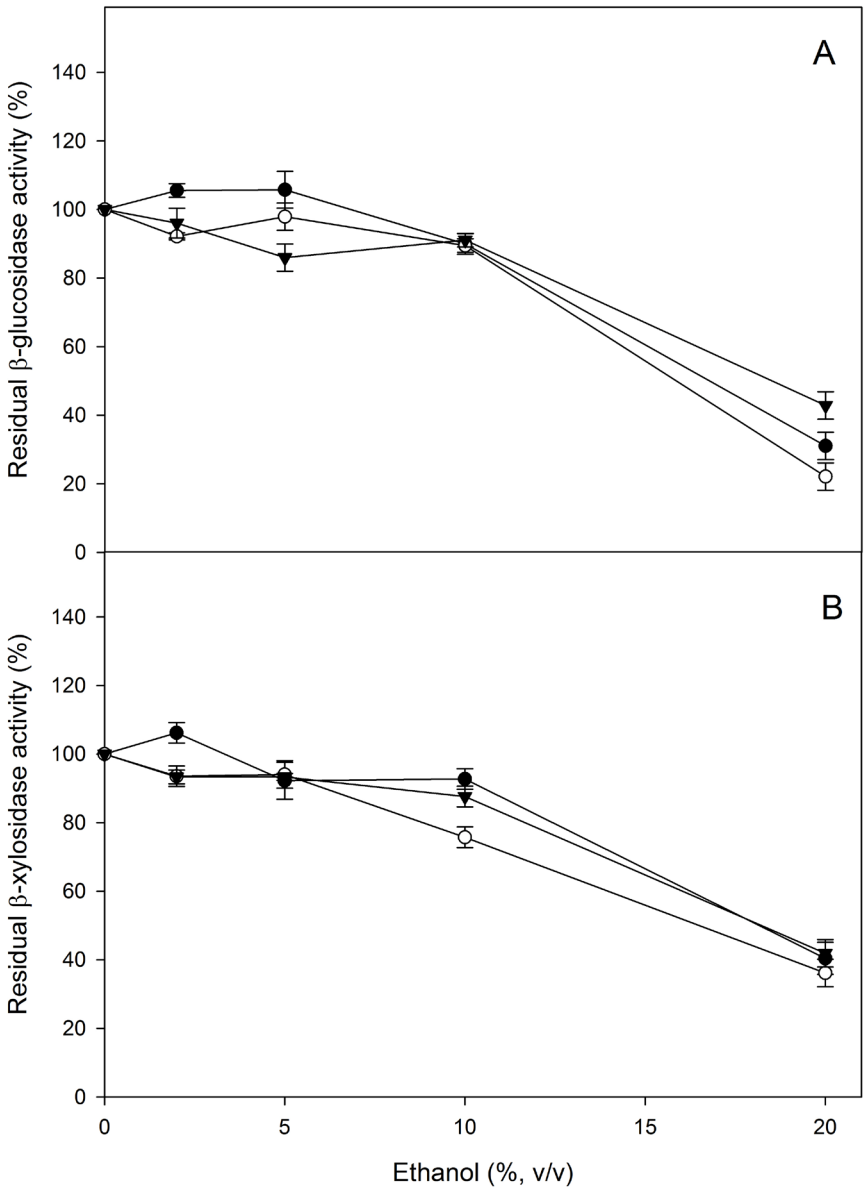


FIGURE 2
Effect of ethanol concentration on glycolytic activities of different *Hanseniaspora* strains. Legends are as shown in Fig. 1.

differences were observed – probably due to strain rather than intraspecific differences. The use of these yeast identification tests, although in some cases species specific, can be used for a preliminary characterisation of the assimilation of carbon compounds (strain typing), rather than for identification purposes.

The role of microbial glycosidases in the hydrolysis of glycosides during the winemaking process was detected. From a technological point of view, this activity justifies the utilisation of *Hanseniaspora* in the industrial production of aromatic wines (Fernández-González *et al*., 2003; Mateo *et al*., 2011; Swangkeaw *et al*., 2011). However, yeasts belonging to the *Hanseniaspora* genus have been considered as spoilage yeasts, particularly during the first stage of fermentation, due to the undesirable production of compounds such as acetic acid or ethyl acetate (Ciani & Comitini, 2011). The significance of glycosidases for the wine industry lies in their potential to release flavour compounds from glycosidically bound nonvolatile precursors in wine (Gueguen *et al*., 1997; Ubeda & Briones, 2000; Ferreira *et al*., 2001; Strauss *et al*., 2001; Maicas & Mateo, 2005; Arévalo *et al*., 2007). Some aromatic precursors (terpenes) found in wine are not volatile and therefore cannot contribute to wine aroma. The *Hanseniaspora* strains included in this work showed β-glucosidase and β-xylosidase activities (remarkable *H. uvarum* H107 and *H. vineae* G26 and P38). To elucidate if wine conditions could affect their activity, as suggested by Grimaldi *et al*. (2005), we also studied the influence of sugar and ethanol addition to simulate native conditions in the wine. Our results were in agreement with Strauss *et al*. (2001), detecting a significant negative influence on the activities of β-glucosidase and β-xylosidase at high glucose concentration. This negative influence was greater than that exerted by the ethanol on the same enzymes, suggesting that they may be less effective in wine. These results also confirm data previously reported by our group in other non-*Saccharomyces* strains (Mateo *et al*., 2011; Romero *et al*., 2012). Our report is surprising, as *Hanseniaspora* is a genus

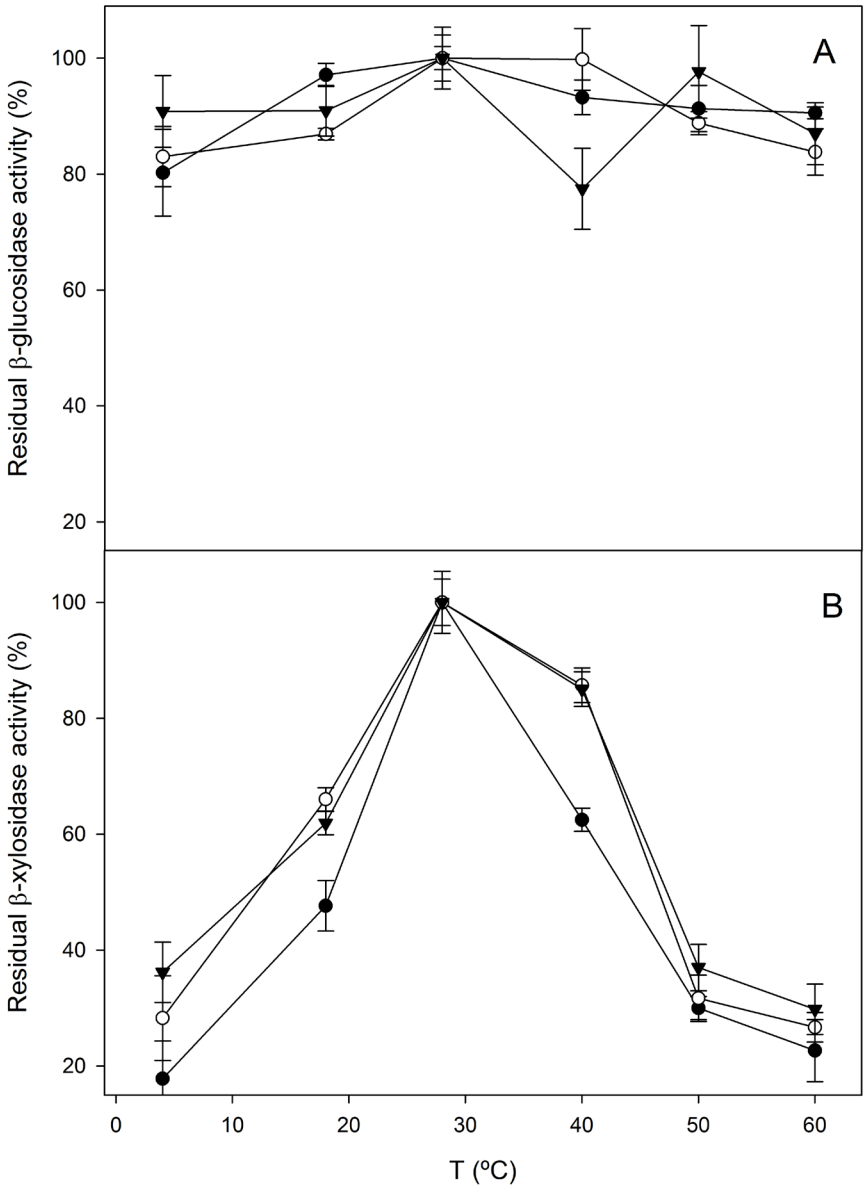


FIGURE 3
Effect of temperature on glycolytic activities of different *Hanseniaspora* strains. Legends are as shown in Fig. 1.

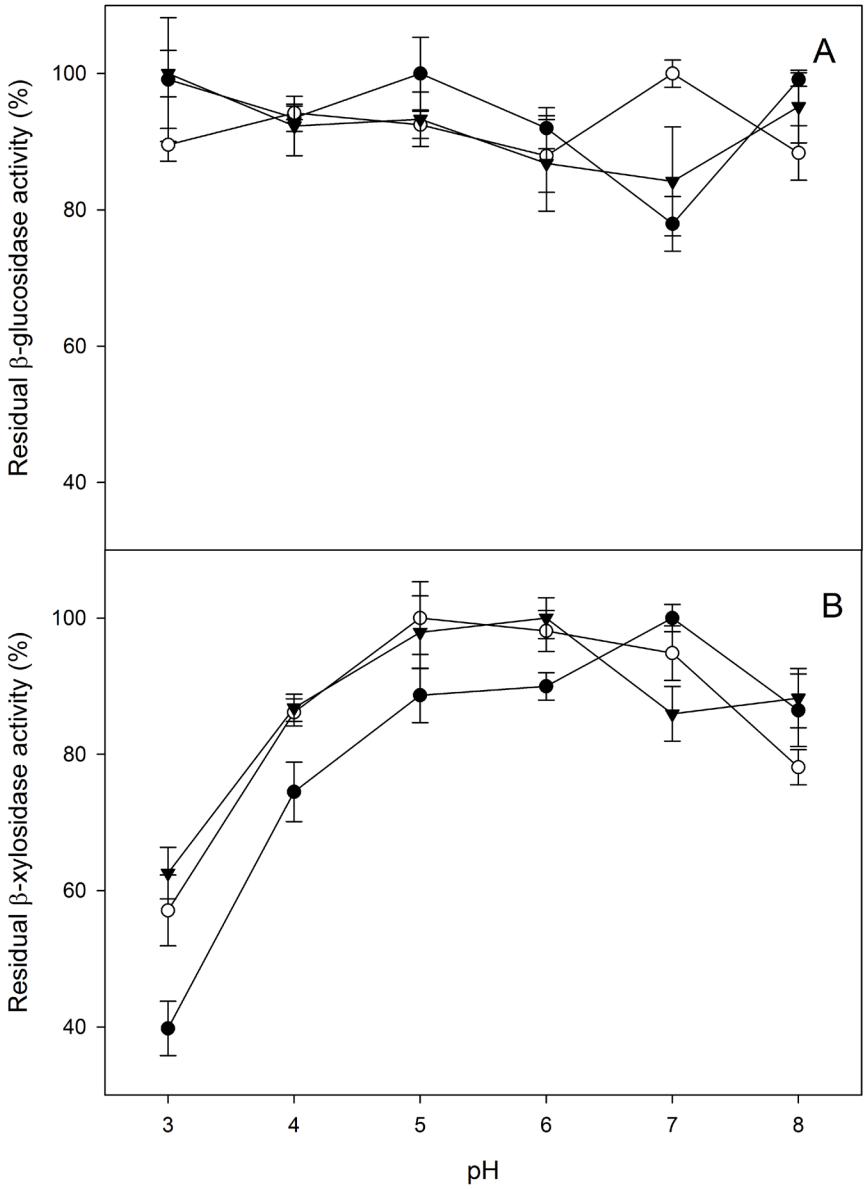


FIGURE 4
Effect of pH on glycolytic activities of different *Hanseniaspora* strains. Legends are as shown in Fig. 1.

described as ethanol inhibited *in vivo* (Branco *et al*., 2012). It has previously been reported that low quantities of alcohol stimulate β-glucosidase activity (Swangkeav *et al*., 2011). These results are similar to our findings for β-glucosidase, but mainly for β-xylosidase. Romero *et al*. (2012) argued that the glycosyltransferase activity of the enzyme could be conditioning this fact.

The β-glucosidase activity was not affected by temperature, while β-xylosidase activity was highly influenced. The first result is very important, because winemaking is now conducted at low temperatures, hence it is important to obtain enzymes with high activity under these conditions. The high activity of β-glucosidase at different values of pH also permits a broad interval of use. However, the β-xylosidase optimal range of use was at pH 5.0 to 8.0. The residual activity, even at low pH (3.0 to 5.0), was good enough to permit use in wines at different pH values.

Monoterpenes, norisoprenoids, benzene derivatives and aliphatic components are traditionally involved in Muscat grape juice and wine. These compounds have been detected in the glycosidically bound form: therefore, their liberation could enhance wine aroma. In order to confirm our previous laboratory results, assays were also carried out in Muscat wine, and volatile compounds were analysed by GC/MS. Muscat wine (13%, v/v, initial alcohol) showed only a moderate overall increase in terpene (1.1- to 1.3-fold) when treated with these strains. These results are conditioned by the effect of ethanol on glycolytic enzymes. Going into detail, the use of these strains offered an increase in the levels of ho-trienol, 2-phenylethanol and 2,6-dimethyl-3,7-octadien-2,6-diol in the wine. The sum of ho-trienol, linalool and terpineol seems to play an important role in the aromatic definition of wines from the Loureiro and Alvarinho varieties (Oliveira *et al*., 2008). 2-Phenylethanol also participates to confer fruity and floral notes to these wines, and its presence is related to the metabolic activity of the non-*Saccharomyces*

TABLE 5
Terpene compounds in Muscat wine. Concentration expressed as µg/L[a].

| | Control | *Hanseniaspora* inoculated | | |
|---|---|---|---|---|
| | | *H. uvarum* H107 | *H. vineae* G26 | *H. vineae* P38 |
| Oxide A[b] | 29.7 (1.2) | 30.4 (2.1) | 33.7 (3.2) | 26.9 (3.4) |
| Oxide B[c] | nd | nd | nd | nd |
| Linalool | 20.0 (0.9) | 40.4*(3.9) | 47.4*(3.4) | 38.2*(5.3) |
| Ho-trienol | 24.0 (3.2) | 51.3*(5.3) | 35.1*(4.2) | 24.9*(0.6) |
| 2-Phenylethanol | 1890.2 (43.4) | 3057.5*(39.8) | 2747.8*(26.8) | 2568.5*(45.6) |
| Oxide C[d] | nd | nd | nd | nd |
| Oxide D[e] | nd | nd | nd | nd |
| Terpineol | 53.3 (3.4) | 67.2*(4.7) | 65.1*(1.2) | 54.5 (3.9) |
| Nerol | 24.6 (2.8) | 25.8 (1.1) | 23.4 (3.1) | 26.3 (1.2) |
| Geraniol | 59.8 (5.0) | 61.3 (3.7) | 56.9 (1.7) | 62.8 (1.7) |
| Diol 1[f] | 43.2 (4.7) | 87.9*(2.1) | 80.2*(2.1) | 81.2*(3.2) |
| 4-Vinylphenol | 63.2 (1.2) | 89.7*(2.4) | 75.7*(5.8) | 62.1 (0.9) |
| Endiol[g] | nd | 58.8*(2.1) | 52.0*(3.4) | 34.1*(4.2) |
| Diol 2[h] | 12.0 (0.6) | 13.4 (0.9) | 7.8 (2.6) | 10.1 (0.9) |
| 2-Phenylethyl acetate | 28.0 (4.1) | 56.2*(7.2) | 23.3 (1.2) | 25.8 (4.7) |
| 2-Methoxy-4-vinylphenol | 89.0 (6.1) | 103.0*(5.3) | 105.4*(6.5) | 94.1 (2.9) |

[a] Values in brackets represent standard deviation (n = 3). ANOVA one factor, significant difference is indicated as * ($p < 0.05$)
[b] *cis*-5-vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol
[c] *trans*-5-vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol
[d] *cis*-6-vinyltetrahydro-2,2,6-trimethyl-2H-pyran-3-ol
[e] *trans*-6-vinyltetrahydro-2,2,6-trimethyl-2H-pyran-3-ol
[f] 2,6-Dimethyl-3,7-octadien-2,6-diol
[g] 2,6-Dimethyl-7-octene-2,6-diol
[h] 2,6-Dimethyl-2,7-octadien-1,6-diol
nd: not detected

yeasts (Swiegers *et al*., 2005). Our findings are similar to the observations of Fernández-González *et al.* (2003), who showed the ability of several wine yeasts to hydrolyse terpenoids, norisoprenoids and benzenoids glycosides. Among wine yeasts, *H. uvarum* was able to hydrolyse both glycoconjugated forms of pyranic and furanic oxides of linalool. Our results open the possibility for the use of these strains to improve the aromatic characteristics of the wines with regard to the liberation of terpenes. The production of wines with the addition of non-*Saccharomyces* strains has traditionally been related to high concentrations of vinyl-phenols (4-vinyl-phenol, 4-vinyl-guayacol), reaching concentrations of up to 1 mg/L (Sefton & Williams, 1991; Gunata, 1993). The concentration of 4-vinyl-phenol in the tested wines was under 90 µg/L, which enables the use of our selected strains in winemaking.

## CONCLUSIONS

In the present study we characterised different *Hanseniaspora* strains previously isolated and stored in our laboratory. The strains were classified according to the molecular results and also were assayed to determine some biochemical activities of oenological interest. The yeasts basically showed moderated or no lipase, esterase, polygalacturonase, pectinase or xylanase activity but, interestingly, glycolitic (β-xylosidase and β-glucosidase) activities were detected. These enzymatic activities can be used to enhance the quality of wine produced with *S. cerevisiae* strains.

This study contributes to the research on the role and potential exploitation of *Hanseniaspora* strains to release volatile terpenes in wines. The potential enzymatic activities shown by these isolates could be used profitably in specific vinification processes by consuming the remaining precursors of aromatic compounds. The two wineries supporting this study have decided to evaluate the use of the selected strains in further campaigns.

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