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Screening of *Hanseniaspora* Strains for the Production of Enzymes with Potential Interest for Winemaking

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Abstract: Some non-*Saccharomyces* yeasts, including *Hanseniaspora*, participate in the first stages of wine fermentation. Besides their importance in the wine production process some of these yeasts have been described to be potential producers of hydrolytic enzymes to industrial level. In this work, we pretend to evaluate the technological abilities of the *Hanseniaspora* strains deposited in the Spanish Type Culture Collection (CECT). First of all, we considered verification of the correct identification of the strains using several miniaturized biochemical systems and molecular techniques (PCR, RFLP and sequencing of the ribosomal D1/D2 region). The results allowed us to verify the correct adscription of the 26 strains included in this study, which exhibited concordant profiles of restriction with one of the three species described in previous studies (*H. occidentalis*, *H. osmophila* and *H. valbyensis*). Some other strains assigned to the species (*H. uvarum*, *H. vineae* and *H. guilliermondii*) showed at least two different profiles. The other objective of this study was to perform an initial screening to characterize both at quantitative and qualitative levels, the ability of these yeasts to produce valuable enzymes for wine fermentation (increase of aroma) and other applications. The more important enzymatic activities detected were β-glucosidase, β-xylosidase, and protease. The HU7, HU8, HV1, HV3, HO2 and HOC1 strains showed high levels of β-glucosidase and β-xylosidase activity, whereas some strains (HG1, HG3, HVA1, HOC 3 and HOC4 were useful for protease production.

Keywords: *Hanseniaspora*; β-glucosidase; β-xylosidase; proteases

1. Introduction

Hanseniaspora is an *Ascomycete* characterized to own bipolar budding, which confers its typical apiculate form under microscopic observation [1]. Traditionally, this genus is included within the denominated no-*Saccharomyces* yeasts, frequently isolated during the first stages of fermented beverage production, as in the case of wine or cider. They can also be found at the surface of the raw material (grapes or apples) as well as in the industries and the machinery of harvesting and processing of these fruits [2].

Nowadays, there are available diverse methodologies to correctly identify the different species from *Hanseniaspora*. Traditionally, the identification of yeast species has been based on the accomplishment of different tests to discriminate among them centered in the analysis of morphologic, physiological and biochemical traits [3]. Different miniaturized systems useful for yeast identification are commercially available, generally based on several tests of assimilation or fermentation of substrates that, although are not conclusive, can be used to complement other studies. The progress in the molecular techniques has allowed the development of other methods of yeast identification, like 5.8S rDNA RFLPs, to typify different *Hanseniaspora* strains; or the amplification

and sequencing of D1/D2 region of the 26S rDNA gene that allows comparing the results with the existing data bases [4].

The non-*Saccharomyces* yeasts predominate during the first 3–4 days of alcoholic fermentation, until the concentration of ethanol and its ability to capture sugars favors the implantation of *Saccharomyces cerevisiae* [5]. *Hanseniaspora* participates in this process, and also in the production of some other compounds, such as alcohols, esters or organic acids that can modify the organoleptic characteristics of the wine [6]. The production of these compounds depends on the conditions of fermentation, the species and even on the implied yeast strains [5]. Exocellular enzymes of related yeast species in different ecosystems have been previously reviewed [7]. 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 able to transform grape compounds [8]. These compounds are present in varying amounts as non-volatile flavor glycosylated precursors [9], mainly 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 [10]. The action of enzymes produced by wine yeasts *i.e.*, β-glucosidase or β-xylosidases can contribute to liberate flavor from these compounds [10]. Several research groups have already made trial fermentations to study the compounds generated by *Hanseniaspora* [11–13]. Yeast proteases contribute to the reduction of turbidity in wine and other fermented beverages [2]. The use of these and other enzymes from non-*Saccharomyces* yeasts has been previously reported [2,8].

In this study, we screened several *Hanseniaspora* strains, regarding their ability to produce enzymes (mainly β-glucosidases, β-xylosidases, proteases) with potential for commercial application either in winemaking or in other biotechnological processes. We also assessed the effect of adding those *Hanseniaspora* strains to fermented grape musts regarding the increase of some volatile compounds such as terpenes that might have a positive impact on the final flavor of wines.

2. Experimental Section

2.1. Yeast Strains

A total of 26 yeast strains were obtained from the Spanish Type Culture Collection (CECT). These strains were catalogued in the collection and assigned to six *Hanseniaspora* species: *H. guilliermondii*, *H. occidentalis*, *H. osmophila*, *H. uvarum*, *H. valbyensis* and *H. vineae*. The lyophiles were recovered according to the procedure recommended by the CECT and the yeasts 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 28 °C for viability check, or in YPD (20 g/L yeast extract, 10 g/L peptone, 20 g/L glucose) for routinely assays, basically as previously described [7].

2.2. Molecular Characterization

2.2.1. DNA Extraction

Pure cultures of yeasts were grown on 10 mL of YEPD medium (1% yeast extract, 2% mycological peptone, 2% glucose) at 28 °C for 24–48 h on an orbital shaker. Small-scale preparation of chromosomal DNA was performed by using Ultraclean Microbial DNA isolation Kit (MoBio, Carlsbad, CA, USA). The quality of the extracted DNA was checked by electrophoresis in 0.8% (*w*/*v*) agarose mini-gels using TBE (45 mM Tris borate, 1 mM EDTA, pH 8.0) buffer with added ethidium bromide at a final concentration of 0.5 µg/mL. The size of the DNA was compared with the standard molecular weights of phage X174-*Hinc*II (Takara, Shiga, Japan). The bands were observed and photographed under UV light (Gelbase Analysis Software, Upland, CA, USA).

2.2.2. PCR Amplification and Restriction Profiles

PCR amplification was carried out using Internal Transcribed Spacers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [11]. Subsequently, PCR products were digested with the following restriction enzymes: *Hae*III, *Cfo*I and *Hinf*I. The restriction fragments were checked by electrophoresis in 2% (*w*/*v*) agarose gel. The restriction fragment sizes measured as base pairs were calculated in comparison with a 100 bp DNA ladder commercial standard (Takara, Shiga, Japan). Profiles were compared with the Yeast-id.org database (CECT, Paterna, Spain).

Isolates sharing the same Restriction Fragment Length Polymorphism (RFLP) were grouped and a type was assigned for each group.

2.2.3. Sequencing

Direct sequencing of the purified PCR products was performed by ABI Prism BigDye Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems, Stafford, TX, USA) in the SCSIE service (Universitat de València). For sequence analysis of the D1/D2 domains of the 26S rDNA gene, PCR amplification was basically performed according to Kurtzman and Robnett [14]. The PCR product was purified using UltraClean PCR Clean Up kit (MoBio) according to the manufacturer's instructions. Direct sequencing of the purified PCR products was performed by ABI Prism BigDye Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems, Stafford, TX, USA).The sequences were aligned, by using the BLAST search program, with complete or nearly complete 26S rDNA gene sequences retrieved from the EMBL nucleotide sequence data libraries [15].

2.3. Physiological Characterization

Miniaturized identification systems API 20C AUX and Rapid Yeast Plus ID were used. The API 20C AUX (Bio Merieux, Marcy l'Etoile, France) system comprises 20 domes with dehydrated substrates prepared to test the assimilation of 19 carbon sources. The Rapid Yeast ID Plus (Remel, Lenexa, KS, USA) is based on a panel of 18 wells, each containing a conventional or chromogenic substrate designed to detect the assimilation of carbohydrates, organic acids or amino acids and the hydrolysis of urea and fatty acids. All procedures were carried out following the manufacturer's instructions.

2.4. Qualitative Tests of Enzymatic Activities

2.4.1. β-Glucosidase Activity

The basal medium consisted on 1.7 g/L Yeast Nitrogen Base (Difco), 5 g/L ammonium sulfate, 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, USA) were added to 100 mL of melted medium [16]. The medium was then poured into Petri dishes and inoculated with 24 h old yeast cultures. Plates were incubated at 28 °C for three days. The presence of the enzymatic activity was visualized as a fluorescent halo surrounding yeast growth by plate exposition to UV light.

2.4.2. Protease Activity

Exocellular protease production was determined by spreading yeast colonies onto YPD agar plates containing 20 g/L casein. Plates were incubated at 28 °C for 7 days. Protease activity was determined by the presence of a clear zone around the colony.

2.4.3. Lipase and Esterase Activities

Yeast isolates were used to determine esterase and lipase activities on tributyrin and rhodamine olive-oil agar media, respectively, according to the previously described procedures [17,18]. After 48 h of incubation at 28 °C in the media, colonies were investigated. For 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.

2.4.4. Pectinase and Polygalacturonase Activities

The assays were carried out in the following medium: 1 g/L yeast extract, 1 g/L ammonium sulfate, 6 g/L NaH_2PO_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 28 °C for five days and then revealed by addition of a solution of hexadecyltrimethylammonium bromide (1 g/L), according to the procedure described by Oliveira *et al.* [19]. Both activities were evidenced by the presence of a clear halo around the 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, USA for the same purpose. All the biochemical activities previously described were assayed in triplicate.

2.5. Quantitative Tests of Enzymatic Activities

2.5.1. Glycosidase Activities

α-Arabinosidase, α-rhamnosidase, β-glucosidase and β-xylosidase activities were determined by quantification of *p*-nitrophenol released from the chromogenic substrates corresponding to each test [7]. Activities were determined by adding 10^6 cells/mL of 24 h-old cultures of the different isolates to an induction medium (0.1% yeast extract, 1% mycological peptone, 4% xylan). After incubating 2 days at 28 °C, 2 mL of yeast cultures were centrifuged and pellet resuspended in 750 µL 0.1 M pH 5.0 citrate phosphate buffer. 250 µL of the corresponding *p*-nitrophenyl-µ-D-pyranoside (1 mg/mL) solution were added and the mixture was incubated at 40 °C for 90 min. To stop the reaction, 1.0 mL of 0.2 M Na_2CO_3 was added. The result of the activities was expressed in nanokatals (1 nkat = 1 nmol of *p*NP released in 1 min by 10^6 yeasts). 1 U = 16.67 nkat.

2.5.2. Lipase and Esterase Activities

The assay of these activities was conducted by quantifying the *p*-nitrophenol released from the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP) and *p*-nitrophenyl butyrate (*p*-NPB), chromogenic substrates for lipase and esterase enzymes respectively [17]. Activities were determined by using 10^6 cells/mL of 24 h-old cultures in YEPD. After centrifugation, cells were resuspended in 900 µL 0.1 M Tris HCl pH 8.0 buffer and then added with 100 µL of *p*-nitrophenyl palmitate (for lipase determination) or 1 mg/mL *p*-nitrophenyl butyrate (for esterase assay) solution. The mixture was incubated at 37 °C for 60 min and then stopped with addition of 1.0 mL of 0.2 M Na_2CO_3. Yellow color released from the substrate was measured at 404 nm. The result of the activities expressed in nanokatals.

2.5.3. Protease Activity

The protease assay procedure was carried out as described by Tremacoldi and Carmona [20] with modifications. Briefly, yeasts were inoculated in YPD agar plates containing 20 g/L casein and

incubated at 26 °C for 6 days to induce protease activity. After adding 10^6 cells/mL to a solution containing 500 µL 0.1 M citrate phosphate buffer pH 6.0 and 500 µL 1% (*w*/*v*) BSA in the same buffer, mixture was incubated 1 h at 50 °C and then, 1 mL 3% (*w*/*v*) TCA was added. Tubes were maintained on ice for 30 min and centrifugated. Liberated tyrosine was determined by absorbance at 280 nm. The protease activity was calculated as nanokatals (1 nkat = 1 nmol of tyrosine liberated in 1 h by 10^6 yeasts).

2.6. Effect of Glucose and Ethanol on Glycosidase Activities

Effects of glucose on enzymes activities were conducted by using sugar concentrations over a range of 0–500 mM. Effect of ethanol (Merck, Darmstadt, Germany) on enzyme activity was conducted by using ethanol concentration over a range of 0%–20% (*v*/*v*). The enzyme assays were carried out as previously described.

2.7. Winemaking and Determination of Volatile Compounds

Muscat juice (1000 hL) was fermented at 20 °C using 25 g/hL commercial wine yeast strain *S. cerevisiae* QA23 (Lalvin, Lallemand, Montreal, QC, Canada). Must sampling for analysis of sugar concentrations were performed weekly. After 21 days, when less than 1.8 g/L residual sugar remained, the wine was separated from the gross lees. This wine, provided by Baronía de Turís winery was as follows: ethanol, 13.2% *v*/*v*; pH, 3.36; titratable acidity, 3.4 g/L; volatile acidity, 0.32 g/L; malic acid, 0.85 g/L; free sulphur dioxide, 47 mg/L; total sulphur dioxide, 111 mg/L. Wine was sterilized by 0.45-µm filtration and sterility was verified by 100-µL wine spreading on YPD plates. Samples were then inoculated again with the four selected yeasts (1×10^6 cfu/mL), separately. The concentration of volatile compounds was determined by using an Agilent 6890 N gas chromatograph-5973N mass detector system (GC/MS) as described by López *et al.* [21]. 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.

3. Results

3.1. Molecular Analysis

Amplification of D1/D2 rDNA domain of the studied strains was sequenced and compared, obtaining a sequence homology with the D1/D2 fragments deposited in database >98%. The size of the amplicons ranged between 372 and 855 bp. The analysis of the restrictions of each one of these fragments, carried out with the above described enzymes showed great inter- and intraspecific variability, which allowed us to propose different groups to include the *Hanseniaspora* strains (Table 1).

Most of the formed groups displayed an amplificon size between 750 and 800 bp. The greater intraspecific variability occurs within species *H. uvarum*, with 5 different groups (IV). The size of amplicons of both groups of *H. vineae* (III) was very different from the one from the rest of the species. *H. guilliermondii* shows three groups (I–III). *H. valbyensis*, *H. osmophila* and *H. occidentalis*, displaying a characteristic restriction profile for each species.

3.2. Physiological Characterization

Characterization of physiological traits of the 26 strains *Hanseniaspora* yeasts was carried out using miniaturized systems API 20C AUX (Table 2) and RapID Yeast Extra (Table 3).

Briefly, the API 20C AUX strips assay revealed that genus *Hanseniaspora* was not able to assimilate L-arabinose, inositol, lactose nor maltose. *H. osmophila* strains were not able to assimilate any of the other substrates included in the strips, under the assayed conditions while *H. vineae* strains were only able to assimilate cellobiose. All *H. guilliermondii* strains were able to assimilate this saccharide and also 2-keto-glutarate and sucrose. Upon this global profile, there are strain specific differences for the other substrates, attributed to individual strain characteristics. According to RapID

Yeast Plus strips (Table 2), none of *Hanseniaspora* species used in this study had lipase, α-galactosidase, β-galactosidase, phosphatase, phosphatidylcoliesterase nor urease. Moreover, *N*-acetyl-glucosamine was not assimilated. As has been reported above some other minor differences were observed, probably due to strain rather than intraspecific differences. The use of these yeast identification tests, although in some cases is species-specific, has been used for a preliminary characterization of assimilation of carbon compounds.

Table 1. Restriction profiles of *Hanseniaspora* strains amplified with primers ITS1 and ITS4.

Group	Species and Laboratory Code	N° CECT	Amplified rDNA Size (bp)	Size of Restriction Fragments (bp)		
				*Hae*III	*Hinf*I	*Cfo*I
	H. uvarum					
I	HU1	1444	748	748, 500, 413, 156	320	295
II	HU2-HU4-HU7-HU8	10387, 10509, 11106, 11107	786	335, 234, 136	372	335, 303
III	HU3-HU9	10389, 11156	748	748	345, 201, 177	321, 132
IV	HU5	10603	855	307, 233, 176	345	337, 307
V	HU6	11105	372	372, 273, 120	206, 190	136, 129
	H. vineae					
I	HV1-HV3	11326, 11338	577	577	293	520
II	HV2	11330	673	288, 194, 104	319	300, 270, 96
	H. guilliermondii					
I	HG1-HG3	11102, 11104	489	489	265	480
II	HG2	11103	363	363, 269	194, 183	200
III	HG4-HG5	11027, 11029	755	755	356, 212, 190	336, 147
	H. valbyensis					
	HVA1-HVA2-HVA3	1445, 10122, 11339	763	763	239, 211, 165, 123	646, 123
	H. osmophila					
	HO1-HO2-HO3	1119,11206, 11207	769	434, 170, 153	361	279, 184, 157
	H. occidentalis					
	HOC1-HOC3-HOC4	11341, 11472, 11329	795	673, 128	276, 239, 134	340, 125

The relationship between the code and the numbering laboratory CECT is used throughout the study.

In a second stage, qualitative analyses were carried out. The most relevant enzymatic assays were tested in individual plates, obtaining the results shown in Table 4.

β-Glucosidase activity was detected in all *Hanseniaspora* strains, with strong dark halos in HVA3, HO2, HOC3 and HOC4 strains. Another interesting activity is the protease, since it was detected in the majority of the strains. Protease we detected in *H. guilliermondii* HG2. Lipase and esterase activities were not detected in any strain, agreeing with the data previously collected in the RapID Yeast Extra strips. Lipase activity was only detected in some *H. vineae* and *H. uvarum* strains, whereas the esterase activity was only detected in some *H. guilliermondii* and *H. occidentalis* strains. Polygalacturonase and pectinase activities were detected, although with low intensity, in a limited number of strains; a moderated activity in 6 of the 26 analyzed strains was observed.

Quantitative analysis of the glycosidase enzymatic activities is shown in Figure 1. No remarkable levels were detected in the α-arabinoside and α-rhamnosidase activities in any of the 26 strains. The HU7, HU8, HV1, HV3, HO2 and HOC1 strains showed high levels of β-glucosidase and β-xylosidase activity, while other strains as HU4, HO1 and HVA3 exhibited high levels of β-glucosidase.

Table 2. Substrate assimilation of *Hanseniaspora* strains incubated in the API 20C AUX system.

Strain	Substrate																		
	Glucose	Glycerol	2-Ketoglutarate	Arabinose	Xylose	Adonitol	Xylitol	Galactose	Inositol	Sorbitol	Methyl-α-D-glucopyranoside	*N*-acethyl-glucosamine	Cellobiose	Lactose	Maltose	Sucrose	Trehalose	Melezitose	Raffinose
HU1	+	+	+	-	-	-	-	+	-	-	-	+	+	-	-	+	-	-	-
HU2	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	+	-	+	+
HU3	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+
HU4	+	+	-	-	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-
HU5	+	+	-	-	+	-	-	+	-	-	-	-	+	-	-	+	-	+	+
HU6	+	+	+	-	-	+	+	+	-	-	-	-	+	-	-	+	+	+	+
HU7	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+
HU8	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+
HU9	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
HV1	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
HV2	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
HV3	+	+	-	-	-	-	-	-	-	+	+	-	+	-	-	+	-	+	+
HG1	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+
HG2	+	+	+	-	-	+	+	+	-	+	+	+	+	-	-	+	-	+	-
HG3	+	+	+	-	-	+	+	-	-	+	+	+	+	-	-	+	-	+	+
HG4	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-
HG5	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+
HVA1	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+
HVA2	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
HVA3	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
HO1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HO2	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
HO3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HOC1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HOC3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HOC4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(+) positive result; (−) negative result.

Table 3. Results of *Hanseniaspora* strains incubated in the RapID Yeast Plus system.

Strain	Substrate																	
	Glucose	Maltose	Sucrose	Trehalose	Raffinose	Fatty acid ester	*N*-Acetyl-glucosamine	α-Glucoside	β-Glucoside	β-Galactoside	α-Galactoside	β-Fucoside	Phosphate	Phosphatidylcholine	Urea	Proline-β-naphthylamide	Histidine β-naphthylamide	Leucyl-glycine β-naphthylamide
HU1	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
HU2	+	−	+	−	+	−	−	+	−	−	−	−	−	−	−	+	+	+
HU3	+	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−	+	−
HU4	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	−
HU5	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−
HU6	+	+	−	−	+	−	−	+	−	−	−	−	−	−	−	+	+	+
HU7	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
HU8	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
HU9	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−
HV1	+	+	+	−	+	−	−	+	+	−	−	+	−	−	−	+	+	+
HV2	+	−	−	−	−	−	−	+	+	−	−	+	−	−	−	−	+	+
HV3	+	+	+	−	+	−	−	+	+	−	−	+	−	−	−	−	+	+
HG1	+	+	+	+	+	−	−	+	+	−	−	+	−	−	−	−	+	+
HG2	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	+	+	+
HG3	+	+	+	+	+	−	−	+	+	−	−	+	−	−	−	−	+	+
HG4	+	+	+	+	−	−	−	+	+	−	−	+	−	−	−	−	+	−
HG5	+	+	+	+	+	−	−	−	+	−	−	+	−	−	−	−	−	−
HVA1	+	−	−	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−
HVA2	+	−	−	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−
HVA3	+	+	+	+	−	−	−	−	+	−	−	+	−	−	−	−	−	−
HO1	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+
HO2	+	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+
HO3	+	+	+	+	−	−	−	+	+	−	−	−	−	−	−	−	+	+
HOC1	+	−	+	−	−	−	−	+	+	−	−	+	−	−	−	−	+	+
HOC3	+	−	+	−	−	−	−	+	+	−	−	+	−	−	−	−	+	+
HOC4	+	+	+	+	+	−	−	−	+	−	−	+	−	−	−	−	+	+

(+) positive result; (−) negative result.

Table 4. Qualitative detection of enzymes in *Hanseniaspora* strains.

	Protease	Esterase	Lipase	β-Glucosidase	Polygalacturonase	Pectinase
HU1	−	+	−	+	−	+
HU2	−	+	+	++	−	−
HU3	+	−	−	+	−	−
HU4	+	+	+	++	−	+
HU5	−	+	+	+	−	−
HU6	−	−	−	++	+	−
HU7	+	+	+	++	+	+
HU8	+	+	+	++	−	−
HU9	+	−	−	++	−	−
HV1	+	+	+	++	+	−
HV2	−	−	−	++	−	−
HV3	+	+	+	++	+	+
HG1	−	+	−	+	−	−
HG2	++	+	−	++	−	−
HG3	+	+	−	++	+	+
HG4	+	−	−	++	−	−
HG5	+	−	−	++	+	−
HVA1	+	−	−	+	−	−
HVA2	−	−	−	++	−	−
HVA3	−	−	−	+++	−	−
HO1	+	−	−	+	−	−
HO2	−	−	−	+++	−	−
HO3	+	−	−	+	−	−
HOC1	+	−	−	+	−	−
HOC3	+	+	−	+++	−	+
HOC4	−	−	−	+++	−	−

+++ = strong activity; ++ = moderated media activity; + = weak activity; − = no activity.

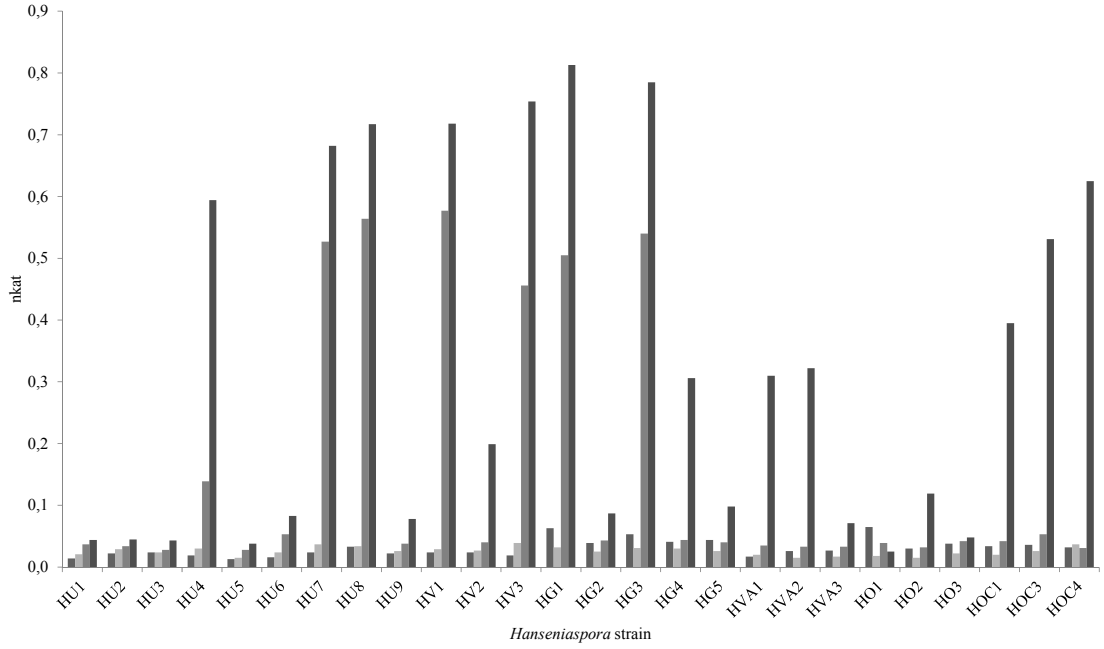

Figure 1. Quantitative enzymatic activities in *Hanseniaspora* strains. From left to right, for each strain: α-rhamnosidase, α-arabinosidase, β-xylosidase and β-glucosidase. Units: nkat (nmol *p*NP released min^{-1} 10^6 $yeast^{-1}$).

The tests of the lipase and esterase activities showed low levels in all the strains, which allowed us to determine that *Hanseniaspora* genus is not a good producer of this enzyme.

Once all isolates were identified, they were inoculated on YPD casein agar plates to detect the production of an exocellular protease; only five *Hanseniaspora* isolates showed an interesting level of this enzymatic activity and were selected for quantitative determination (Table 5).

Table 5. Protease activity (nkat) in selected *Hanseniaspora* isolates.

Hanseniaspora Isolate	Activity (nkat)
HG1	20.7 ± 2.2
HG3	37.9 ± 1.4
HVA1	35.8 ± 1.1
HOC3	40.5 ± 1.8
HOC4	31.7 ± 2.1

In four of the strains (HG3, HVA1, HOC3 and HOC4), protease levels were greater than 30 nkat.

3.3. Effect of Sugars and Ethanol on Glycosidase Activities

Hanseniaspora strains HU7, HU8, HV1 and HV3 were selected on the basis of their higher levels of activity in β-glucosidase and β-xylosidase quantitative analysis. β-Glucosidase (Figure 2a) and β-xylosidase (Figure 2c) maintained 80% of their activities up to concentrations of 10% (*v*/*v*) ethanol. In the presence of different amounts of glucose, β-glucosidase (Figure 2b) and β-xylosidase (Figure 2d) reached a stable residual activity at concentrations close to 100 mM. However, strain HU7 exhibited a 60% of residual β-xylosidase activity even in the presence of 500 mM glucose (a concentration higher than the typically found in wine).

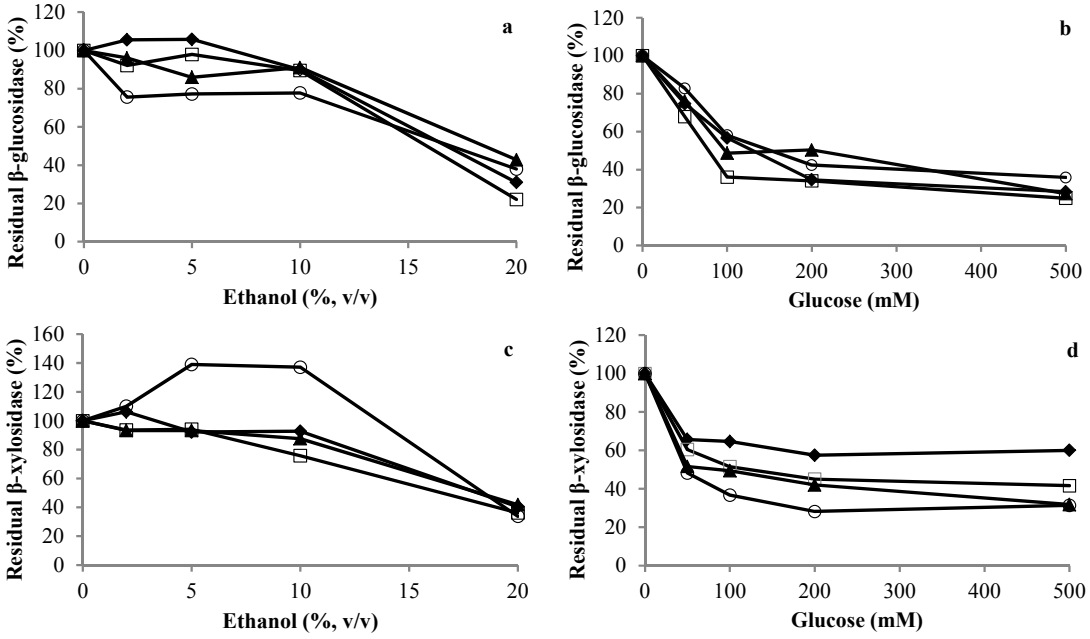

Figure 2. Influence of ethanol (**a**,**c**); and glucose (**b**,**d**) on the β-glucosidase (**a**,**b**); and β-xylosidase (**c**,**d**) activities in (♦) HU7, (▫) HU8, (▲) HV1 and (○) HV3 strains.

3.4. Determination of Volatile Compounds Liberated from Wine

Muscat juice was used for vinification with a commercial *S. cerevisiae* strain. Subsequently, the four non-*Saccharomyces* isolates were individually inoculated (in triplicate assays) and volatile compounds were determined (Table 6).

Table 6. Terpene and other volatile compounds in Muscat wine.

Compound	Control [b]	Non-*Saccharomyces* Yeast Inoculated [a]			
		***H. uvarum* HU8**	***H. uvarum* HU7**	***H. vineae* HV1**	***H. vineae* HV3**
TERPENES					
cis-5-Vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol	29.7 (1.2)	30.3 (3.1)	33.6 (3.3)	33.9 (3.3)	35.9 (3.3)
trans-5-Vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol	nd	nd	nd	3.8 * (1.3)	6.3 * (3.3)
Linalool	20.0 (0.9)	30.3 * (3.9)	36.3 * (3.3)	38.8 * (3.6)	33.6 * (3.9)
Ho-trienol	24.0 (3.2)	51.3 * (5.3)	35.1 * (3.3)	38.0 (3.3)	30.0 (3.6)
cis-6-Vinyltetrahydro-2,2,6-trimethyl-2*H*-pyran-3-ol	nd	36.1 * (3.6)	nd	3.9 (3.6)	nd
trans-6-Vinyltetrahydro-2,2,6-trimethyl-2*H*-pyran-3-ol	nd	36.3 * (1.3)	nd	3.9 (0.9)	nd
Terpineol	53.3 (3.4)	66.3 * (3.6)	65.1 * (1.3)	51.1 (5.6)	36.3 (3.3)
Nerol	24.6 (2.8)	35.8 (1.1)	33.3 (3.1)	35.1 (3.5)	36.1 (1.3)
Geraniol	59.8 (5.0)	61.3 (3.6)	56.9 (1.6)	59.1 (3.3)	58.3 (3.3)
2,6-Dimethyl-3,7-octadien-2,6-diol	43.2 (4.7)	86.9 * (3.1)	80.3 * (3.1)	68.3 * (3.3)	69.0 * (3.8)
2,6-Dimethyl-7-octene-2,6-diol	nd	58.8 * (3.1)	53.0 * (3.3)	35.6 * (3.6)	39.6 * (3.3)
2,6-Dimethyl-2,7-octadien-1,6-diol	12.0 (0.6)	13.3 (0.9)	6.8 (3.6)	10.3 (3.6)	5.8 (1.5)
OTHER VOLATILE COMPOUNDS					
4-Vinylphenol	63.2 (1.2)	89.6 * (3.3)	65.6 * (5.8)	56.9 (3.5)	59.3 (3.1)
2-Methoxy-4-vinylphenol	89.0 (6.1)	103.0 * (5.3)	105.3 * (6.5)	88.6 (3.9)	83.6 (3.3)
2-Phenylethanol	1890.2 (43.4)	3056.5 * (39.8)	3636.8 * (36.8)	3308.8 * (36.6)	3333.3 * (33.6)
2-Phenylethyl acetate	28.0 (4.1)	56.3 * (6.3)	33.3 (1.3)	33.6 (3.3)	16.3 (3.5)

[a] Concentration expressed as μg/L. Values in brackets represent standard deviation (n = 3). ANOVA one factor, significant difference is indicated as * ($p < 0.05$); [b] Wine produced only with *Saccharomyces cerevisiae*; nd: not detected.

We were not able to detect significant increase in the level of nerol and geraniol (sweet rose) after the addition of non-*Saccharomyces* strains. The concentration of *cis*-5-vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol was not increased despite the addition of any of the four *non-Saccharomyces* yeasts, while the other oxides (*trans*-5-vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol, *cis*-6-vinyltetrahydro-2,2,6-trimethyl-2*H*-pyran-3-ol and *trans*-6-vinyltetrahydro-2,2,6-trimethyl-2*H*-pyran-3-ol) were not detected in controls and in inoculations. *Trans*-5-vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol (faint burning odor) was detected in the HV1 and HV3 inoculation tests while *cis*-6-vinyltetrahydro-2,2,6-trimethyl-2*H*-pyran-3-ol and *trans*-6-vinyltetrahydro-2,2,6-trimethyl-2*H*-pyran-3-ol (floral, fresh) only were detected in HU7 and HU8 ones. Moreover, 2,6-dimethyl-2,7-octadien-1,6-diol levels were not affected.

Linalool (floral, with a touch of spiciness) and derivative compounds and aromatic alcohols were detected at the same level of control wine, after the addition of any of the four isolates. Terpineol (odor similar to lilac) increased only after the addition of *H. uvarum* isolates, but not when *H. vineae* strains were added. The same results were recorded for 4-vinylphenol (barnyard, medicinal and mousy) and 2-methoxy-4-vinylphenol (wine-like and curry). Linalool, 2-phenyl ethanol and 4-vinylphenol compounds are associated with fruity and medicinal characteristics, respectively [22].

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* strains produced more 2-phenyl ethanol as previously described [23,24]. *H. uvarum* HU8 provided improvements in 2-phenylethyl acetate production as previously reported for other *Hanseniaspora* strains [25–27].

4. Discussion

The genus *Hanseniaspora* is one of the most abundant among the non-*Saccharomyces* yeasts present on the surface of intact grapes [22], and it is also considered to be one of the main producers of glycolytic [16] and other enzymatic activities [28]. Some authors consider substrate assimilation miniaturized systems not to be appropriate tools for the identification of non-*Saccharomyces* yeasts isolated from natural ecosystems, although they can complement RFLP analysis [3]. The results of our work show that in some of the studied species (*H. valbyensis*, *H. osmophila* and *H. occidentalis*), the amplification of the ITS1–ITS2 region generates fragments of similar size, corresponding with the physiological profiles. In the other three species, the results of the two methods are not comparable. This may be because the substrate assimilation miniaturized identification methods are not fully precise for the identification process of the six species of yeasts analyzed [29]. The miniaturized systems help to characterize the strains, but as they are strain dependant, they vary among isolates included in the same species (by molecular typing).

Previous studies pointed to the existence of a unique restriction profile for all strains of the genus *Hanseniaspora*, after the digestion of a standard amplicon of approximately 750–775 bp [3,29,30]. Our results show some differences, as *H. osmophila*, *H. occidentalis*, and *H. valbyensis* showed a unique restriction profile for all of the assayed strains. However, in the other three species, several restriction profiles within a single species can be observed. Among the *H. guilliermondii* strains, only the group HG4–HG5 showed a similar profile to that previously reported [30]. The restriction profiles of the *H. vineae* strains were different from those described by other authors [3,29]. In *H. uvarum* we find greater diversity, obtaining a larger number of profiles. Only the group formed by HU3 and HU9 strains showed a profile similar to that described in other publications. Moreover, amplicons from HU6 and HG2 strains were different in size from those of other strains (372 bp). Some of the strains included in this study were further used for amplification of the region D1/D2 [4] and subsequent sequencing, yielding coincident homologies in some of the strains. According to these data, we conclude that there is a dispute in the identification of this genus of yeasts, and further studies are needed in order to clarify this situation.

The search for enzymatic activities in non-*Saccharomyces* yeast is a crucial point due to the contribution of these enzymes to the improvement of the aroma of wines. Interesting studies have been reported that demonstrate the role of glycosidases on microbial hydrolysis of glycosides during the winemaking process [32]. Some of the aromatic precursors found in wine, such as some terpenes, are in the glycosylated form, which is not volatile and cannot contribute to the aroma of wine. The *Hanseniaspora* strains analyzed in this study have no relevant α-rhamnosidase or α-arabinosidase activities, but β-glucosidase and β-xylosidase were frequently detected; strains with increased activity (HU7, HU8, HV3, HO2 and HOC1) could be selected for further study, similar to those described above [33,34].

Previous studies suggest the importance of the proteolytic activity of yeasts in relation to the reduction of turbidity in wine and other fermented beverages [2]. Our work confirms that some strains are capable of producing *Hanseniaspora* proteolytic enzymes. These strains belong to the species *H. guilliermondii*, *H. occidentalis* and *H. valbyensis*. This activity has already been described by other authors in some strains of *Kloeckera apiculata* (*H. uvarum*) [2], although increased activity has also been reported in other yeasts, such as *Candida pulcherrima* and *Pichia anomala* [28]. Yeast proteases may liberate amino acids and peptides from grape protein during fermentation, which can benefit the growth of microorganisms during or after alcoholic fermentation. Another aspect is that yeast

cells may release nitrogen-containing metabolites into the media. The composition of amino acids, peptides and proteins in wine is based on grape-related compounds transferred and transformed during the winemaking process and breakdown products through protease activity from yeasts and compounds released by yeasts [35]. Results obtained in our laboratory in previous work allow us to conclude that protease activity in *Pichia* and *Wickerhamomyces* isolates was very low [18], in accordance with results obtained by other authors [2,28]. These authors suggested that *Hanseniaspora* isolates could be a more interesting group of yeasts to obtain this enzymatic activity, but some contradictory data have been obtained. Many of these studies have been conducted with *H. uvarum* (*K. apiculata*) isolates and, on the basis of the results obtained in our work, the exocellular protease of this species has a very low activity. On the other hand, assays performed by these authors have used acidic buffers and we have shown that protease from *Hanseniaspora* yeasts is pH dependent, showing maximum activity at pH 6.0.

The discovery of pectinase in yeast is an asset to winemakers, because of its importance in winemaking. However, studies related to the search for pectinase activities are often contradictory [2,28]. Our results point to a low production of pectinase and polygalacturonase by *Hanseniaspora* strains, as already described [8,36]. Remarkable lipase and esterase activities were not found in these yeasts and, in fact, some studies support the absence of lipolytic enzymes in the genus *Hanseniaspora* [28].

Both glycosidases and proteases have direct applications in the wine industry, but in wine some conditions are present that may affect their activity. The high concentration of sugars in the must as well as the ethanol concentration in the wine may inhibit glycosidase enzymes [37]. In this work, we have studied the influence of both factors on the stimulation of the enzymatic conditions in must and wine. Our results are in agreement with Strauss *et al.* [2] who detected a significant negative influence of glucose on β-glucosidase and β-xylosidase activities. This negative influence was greater than that exerted on ethanol enzymes, suggesting that they may be less effective in wine.

Monoterpenes, benzene derivatives, aliphatic components and norisoprenoids are habitually involved in Muscat grape wine and juice. These compounds have been identified in the glycosidically bound form: consequently, their release could enhance wine aroma. Volatile compounds in Muscat wine were analyzed by GC/MS. Muscat wine (13.2% *v*/*v* initial alcohol) exhibited only a moderated overall terpene increase (1.1 to 1.3-fold) when inoculated with these yeasts. These results are conditioned by the effect of ethanol on glycolytic enzymes. Basically, 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 wine. The sum of ho-trienol, linalool and terpineol seems to play a central role in the aromatic definition of the wines of Alvarinho and Loureiro cultivars [38]. 2-Phenylethanol also contributes by adding floral and fruity notes to these wines, and its presence is connected to the metabolic activity of the non-*Saccharomyces* yeasts [39]. Our results are similar to the interpretations of Fernandez-González *et al.* [40], who have shown the ability of several wine yeasts to hydrolyze norisoprenoids, benzenoids glycosides and terpenoids; among wine yeasts *H. uvarum* was able to hydrolyse both glycoconjugated forms of furanic and pyranic oxides of linalool. Our results open the opportunity to the use of these strains for improving the aromatic characteristics of wines, in regard to the liberation of terpenes. The production of wines with the addition of non-*Saccharomyces* strains has been habitually related to high concentrations in vinyl-phenols (4-vinyl-phenol, 4-vinyl-guayacol) reaching concentrations of up to 1 mg/L [41,42]. The concentration of 4-vinyl-phenol in the studied wines was under 90 μg/L, which enables the use of our selected strains in winemaking.

5. Conclusions

Miniaturized systems are not adequate for proper identification of the different species of *Hanseniaspora* yeasts, although in some cases, they complement molecular studies. The use of molecular techniques (RFLP analysis) allows differentiation between species within the genus *Hanseniaspora*, although the species *H. uvarum*, *H. vineae* and *H. guilliermondii* display multiple

profiles. These discrepancies reveal the need for further studies on the molecular and physiological characterization of these strains. Enzymatic assays show that the enzymes β-glucosidase, β-xylosidase and protease are the activities of greatest interest for the development of further studies. Strains HG1, HG3, HV1, HV3, HU7 and HU8 are those with higher levels of glycosidase activity. Strains HVA1 and HG3 have been selected for the production of proteases, especially given their strong activity at typical pH values of fermented beverages. β-Glucosidase and β-xylosidase enzymes showed high levels of tolerance to ethanol, which was greater than the resistance to glucose, thus these enzymes may be more effective in wine than in must. This work contributes to research on the role and potential commercial use of *Hanseniaspora* strains to the liberation of volatile terpenes in wines. The potential enzymatic activities exhibited by these yeasts could be used in the vinification processes by consuming the residual precursors of aromatic compounds.

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Conflicts of Interest: The authors declare no conflict of interest.

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