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ORIGINAL PAPER

A quick screening method to identify β-glucosidase activity in native wine yeast strains: application of Esculin Glycerol Agar (EGA) medium

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Abstract One hundred and fifty-four yeast strains were isolated from grapes and musts of Uruguayan vineyards and wineries. Only thirty strains showed β-glucosidase activity in Esculin Glycerol Agar (EGA) solid medium. Twenty-one were non-*Saccharomyces* and nine were *Saccharomyces cerevisiae* strains. The objective of this study was to evaluate the suitability of Esculin Glycerol Agar (EGA) solid medium for screening β-glucosidase activity in native yeasts strains. Halo sizes measured in the EGA solid medium were correlated to the Glycosyl-Glucose (GG) indexes measured after fermentation of grape musts with each strain. The two *S. cerevisiae* strains with the best performance were selected for further fermentations on a Muscat Miel grape must, rich in bound monoterpenes. The levels of free linalool, hodiol I and geraniol increased significantly as compared to fermentation with a commercial wine yeast strain. These results show the suitability of this simple and economic medium to identify *S. cerevisiae* glucosidase producers with a potential impact on real winemaking conditions. On the other hand, great variability was found for the non-*Saccharomyces* strains, and this would demand further studies for each species. In conclusion, the use of EGA solid medium shows that the screening method is suitable for exploring the glucosidase activity of native strains of *S. cerevisiae* and shows good correlation with its real impact on free aroma compounds in the final wine.

Keywords Yeast β-glucosidase · Esculin agar · Wine aroma compounds · *Saccharomyces cerevisiae* · Non-*Saccharomyces*

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Introduction

It is well established that a significant fraction of the available flavor compounds remain as odorless non-volatile glycosides after wine fermentation (Swiegers et al. 2005; Carrau et al. 2008a). In Uruguay, the most widely planted wine grape variety is Tannat (Carrau, 1997; Medina et al. 2005). The presence in this red variety of high concentrations of glycosides bound to aromatic aglycones, such as C-13 norisoprenoids, benzene derivatives, volatile phenols and aliphatic alcohols (Boido et al. 2002, 2003), prompted the selection of yeast and lactic bacteria strains with potential glucosidase activity.

In order to enhance the sensory attributes of the wines, it is important to explore the potential of native yeasts biodiversity and perform screenings for β-glucosidase producer strains from specific enological ecosystems. As only few strains of *S. cerevisiae* possess β-glucosidase activity, recent studies have focused on indigenous species of non-Saccharomyces strains isolated from grapes

(*Candida*, *Hanseniaspora*, *Metschnikowia* and *Pichia*) to impart special characteristics to the wines (Romano et al. 1997; Charoenchai et al. 1997; Soden et al. 2000; Fleet 2003; Jolly et al. 2003; Cordero et al. 2003; Fernández et al. 2003, see review by Jolly et al. (2006) and Ciani et al. (2010). The growth of these non-*Saccharomyces* yeasts is generally limited to the first 3 or 4 days of fermentation, after which they begin to die off, giving way to strains of *S. cerevisiae* which conduct the fermentation exclusively until completion (Goddard 2008).

Although a great diversity of *S. cerevisiae* strains are present in grapes and wines (Jubany et al. 2008), the glucosidases have not been studied extensively and their role in wine flavor is still unclear (Mateo and Di Stefano 1997; Swiegers et al. 2005). The lack of screening methods for glucosidase activity able to correlate with real wine conditions is one of the limitations for identifying these strains. In the majority of the literature reports, β-glucosidase activity was assayed by using β-glucoside analogues such as *p*-nitrophenyl-β-D-glucopyranoside (p-NPG) (Swiegers et al. 2005). Hernández et al. (2003) and Fia et al. (2005) proposed 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG) as a simple method using fluorescence detection. These substrates are usually expensive and their use is laborious, and they have not been shown to have a direct correlation to glucosidase activity in grape must or wines. McMahon et al. (1999) determined the β-glucosidase production in 32 yeast strains, but only one *S. cerevisiae* exhibited activity with the glucoside analogues, and although several of the non-*Saccharomyces* yeast species showed activity, only *Aureobasidium pullulans* was able to hydrolyse glucosides found in grapes. Strauss et al. (2001) suggested that the most reliable of these β-glucoside analogues were the arbutine or esculine derivatives. When these compounds are included in solid media, colonies showing glucosidase activity can be easily identified by their brown colour. If glucosidase activity is produced by the tested strain, the natural β-glucoside esculin is split into esculetin (6,7-dihydroxycoumarin) and glucose, and then the free esculetin reacts with the ferric ions present in the medium resulting in a brown precipitate. However there are no studies showing a correlation of this test with the natural grape glucoside hydrolysis capacity by yeasts.

In the present work, application of an esculin solid medium containing glycerol as carbon source previously used for studying *Neurospora crassa* glucosidase activity (Eberhart et al. 1964) was evaluated. The correlation of this simple plate method with the glucosidase quantification method by the GG index determination in grape musts and wines was evaluated. Two of the selected native strains were then used for the winemaking of a white aromatic grape must of Muscat Miel and compared to wines made with a conventional commercial strain.

Materials and methods

Yeast strains

Twenty-seven strains from yeast culture collections were used as controls and are shown in Table 1, including *S. cerevisiae* (21), *Kluyveromyces fragilis* (3), *Pachysolen tannophilus* (1), *Pichia stipitis* (1) and *Schizosaccharomyces pombe* (1). Of the *S. cerevisiae* strains, the widely studied wine yeast strain Montrachet UCD 522 (University of California, Davis) (M522 in this work) was used for microvinifications of grape juice and as a negative control for glucosidase activity. WL Nutrient agar medium was used as a rapid differentiation of strains from grapes and musts (Pallmann et al. 2001), and Lysine Agar (Fowell 1965) was used to differentiate *Saccharomyces* from non-*Saccharomyces*. All strains were maintained in Malt Extract Agar medium at 4°C. For the fermentation tests in grape juice or synthetic grape juice, inocula were prepared in the same medium by incubation for 12 h in a rotary shaker at 150 rev/min and 25°C. Inoculum size was 1×10^5 cells/ml of medium.

DNA isolation

Isolated fresh colonies from each strain were inoculated and grown on YPD [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] medium at 30°C for DNA isolation. The quick method described by González-Techera et al. (2001), was followed. Briefly, the pellet corresponding to around 10^9 cells (early stationary phase) was washed with sterile water and resuspended in 0.4 ml breaking buffer (2% Triton X-100, 1% sodium dodecylsulphate, 100 mM NaCl, 10 mM Tris pH 8 and 1 mM EDTA pH 8). The cells were homogenized by vortexing for 5 min with 0.3 g glass beads (Sigma G9268) in the presence of 0.4 ml phenol, pH 8. After centrifugation at 4°C, the aqueous phase was carefully removed and after ethanol precipitation and resuspension in TE (10 mM Tris pH 8 and 1 mM EDTA pH 8), DNA quality and concentration was determined in an agarose gel to adjust the appropriate dilution for the PCR reaction.

PCR and DNA sequencing reactions

Following Kurtzman and Robnett (1998), domains 1 and 2 from 26S rDNA gene were amplified using the primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG) (F) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) (R). Amplifications were performed with an initial denaturation at 94°C for 5 min, followed by 30 PCR cycles (denaturation at 94°C for 1 min, annealing at 52°C, and extension at 72°C for 2 min), with a final extension at 72°C for 10 min.

Table 1 Laboratory and commercial yeast collections strains and native isolates from grape and wine fermentation screened for β-glucosidase activity in the EGA solid medium

Spice	Collection or isolate identity[+]	Level of glucosidase activity in EGA medium	Sensory quality acceptance
Saccharomyces cerevisiae	M522, KU1 882,881,863, NCYC:1006,190,235,738,761; ATCC 46276, ICAITI: 169,180,181. ALG804,09CMB,09CMC,09CMD,09CEG,09CEI,09CEJ	−−	ND
Kluyveromyces fragilis	NRRLY: 1109, 2415	++	ND
Kluyveromyces fragilis	ATCC 12424	−−	ND
Pachysolen tannophilus	ATCC 32691	++	ND
Pichia stipitis	NRRL Y7124	++	ND
Schizosaccharomyces pombe	UCS	−−	ND
Hanseniaspora vineae	M00/10G	+	Yes
Hanseniaspora vineae	M00/11G	++	Yes
Hanseniaspora vineae	T00/20G, T 00/01F	+++	Yes
Hanseniaspora uvarum	T00/04F, M00/26G, M00/29F	+++	No
Hanseniaspora uvarum	T00/18F, M00/27G, T00/24G	++	No
Hanseniaspora uvarum	M00/31F, M00/36F	+	No
Metschnikowia pulcherrima	M00/08G	+	No
Metschnikowia pulcherrima	T00/19F, T00/03F	++	No
Metschnikowia pulcherrima	T00/21G	+++	No
Metschnikowia pulcherrima	T00/23G	+++	Yes
Metschnikowia pulcherrima	M00/09G	+	Yes
Candida railenensis	T00/22G	+	Yes
Metschnikowia aff fructicola	T00/25G	+++	Yes
Cryptococcus flavescens	M00/07G	+	Yes
Saccharomyces cerevisiae	M00/12F	+	Yes
Saccharomyces cerevisiae	M00/13F	+	Yes
Saccharomyces cerevisiae	M00/14F, T00/16F	++	No
Saccharomyces cerevisiae	T00/15F	++	Yes
Saccharomyces cerevisiae	T00/17F	++	Yes
Saccharomyces cerevisiae	M00/30F	++	Yes
Saccharomyces cerevisiae	M00/05G	+++	Yes
Saccharomyces cerevisiae	M00/35F	+++	Yes

Acceptable sensory attributes are indicated as yes. ND: not determined for the case of laboratory strains. The letters in the isolate identity means: the variety of origin of the isolation: *M* Merlot, *T* Tannat, and from where: G grapes; F must fermentation

Amplicons from five independent reactions were combined, purified and sequenced by Macrogen, Korea.

Culture media and fermentation conditions

Chemically-defined fermentation medium (nutrient components of grape juice) was prepared as described previously (Carrau et al. 2008b), containing Yeast Assimilable Nitrogen (YAN) at 100 mgN/l. The final pH was adjusted to 3.5 with HCl. Equimolar concentrations of glucose and fructose were added to reach 120 g/l, and media with YAN concentration of 100 mgN/l were made increasing the basic concentration of the 50 mgN/l amino acid mix by supplementation with diammonium phosphate (DAP) (Carrau et al. 2008b). On the other hand, a white grape juice of the aromatic variety Muscat Miel (Versini et al. 1999) was used for the production of wines with a high content of glycosylated monoterpenes. This was the grape juice fermentation medium utilized for experiments of Tables 2 and 3. All fermentations were carried out in 125 ml of medium in 250-ml Erlenmeyer flasks, closed with Muller valves filled with pure sulfuric acid. Static batch fermentation conditions were conducted at 20°C in duplicate, simulating wine making conditions. Fermentation activity was measured as CO_2 weight loss and expressed in grams per 100 ml and total residual sugars were analysed by the Fehling method (Zoecklein et al. 1995). Once a day samples were taken to measure cell

Table 2 Production of β-glucosidase comparing the halo diameter in EGA medium with the GG indexes in grape must of Muscat Miel

Strain	Species	Total CO_2 loss (g/100 ml of must)	Halo diameter in EGA medium (mm)	Glucosidase activity	G–G Index (μM/ml) (*)
Muscat Alexandria Juice					26.08 ± 0.41
M522	*S. cerevisiae*	3.35	0	Negative	11.07 ± 0.48
M00/12F	*S. cerevisiae*	3.18	15	Weak	3.17 ± 0
M00/13F	*S. cerevisiae*	3.31	17	Weak	14.4 ± 1.24
T00/17F	*S. cerevisiae*	3.59	18	Medium	2.75 ± 0.41
T00/15F	*S. cerevisiae*	3.33	20	Medium	3.6 ± 0.41
M00/30F	*S. cerevisiae*	3.33	22	Medium	1.48 ± 0.82
M00/35F	*S. cerevisiae*	3.82	23	Strong	1.08 ± 0.42
M00/05G	*S. cerevisiae*	3.45	21	Strong	1.58 ± 0.42
M00/10G	*H. vineae*	1.45	15	Weak	3.58 ± 0.42
T00/20G	*H. vineae*	1.91	27	Strong	11.08 ± 0.42
T00/23G	*M. pulcherrima*	1.58	23	Strong	10.66 ± 0.01

Strain M522 was used as *S. cerevisiae* negative control, and *H. vinea* and *M. pulcherrima* as non-*Saccharomyces* glucosidase producers

(*) Average of duplicates

Table 3 Wines analysis obtained with the two selected glucosidase active *S. cerevisiae* strains, M00/30F and M00/35F in Muscat Miel white wines compared to the control strain M 522

Strain identity	Growth biomass mg/ml	Final cell counts × 10^6	Final pH of wines	Final G-G index	Residual sugar g/l	Total evolved CO_2 g	Final alcohol % by vol.
M00/30F	1.63 ±0.03	13±1	3.40±0	20.55±1.55	5.62±0.62	13.7±0,15	11.15±0.25
M00/35F	1.76±0.03	97.5±12	3.35±0	23.7±1	1.76±0.15	14.55±0.05	11.85±0.05
M522	1.7±0.09	68.5±3.5	3.35±0.005	55.65±0.85	1.31±0.035	14.48±0.005	11.85±0.05
Grape Must Muscat Miel			3.3	98.7	208		

growth in an improved Neubauer chamber. Samples for sensory and GC–MS analysis were taken 2 days after the end of fermentation, filtered through 0.22 μm pore membranes and SO_2 added as 50 mg of sodium metabisulphite/l.

Screening method for β-glucosidase activity with Esculin Glycerol Agar (EGA) medium

Screening was carried out on agar plates with esculin as substrate. The medium consisted of esculin 1 g/l, ferric chloride 0.3 g/l, casein hydrolysate 1 g/l, yeast extract 25 g/l, glycerol 8 ml/l, agar 20 g/l. The final pH of the medium was 6.0. The medium was autoclaved at 121°C for 15 min and poured into Petri dishes (20 ml per plate). After solidifying, different yeast strains were inoculated as radial streaks from 48 h cultures in malt extract agar. Each plate was inoculated with four cultures, incubated at 25°C and examined after 2, 4, 6 and 8 days. A non inoculated plate served as control. Strains with β-glucosidase activity hydrolysed the substrate and a dark brown colour developed in the agar. The diameter of the brown halo was measured in millimeters.

Glycosyl-Glucose method for measuring glycosylated compounds

This method was developed as a direct measurement of the glycosylated aroma compounds in white grape must fermentations. Grape juices and wines were filtered through 0.22 μm pore filter disks and analysed for the glycoside content using the Glycosyl-glucose (GG) method proposed by Williams et al. (1995) before the fermentation and 2 days after the end of the fermentations,. A C18 Bakerbond SPE, 500 mg in a 3 ml column (J. T. Baker. USA. Cod. 7020-03) was used, and the liberated glucose was measured with the HK/G-6-PDH enzymatic kit (Wiener Laboratorios S.A.I.C. Rosario, Argentina. Cod. 1400051). The GG indexes obtained are based on the measurement of the glucose bound to aroma compounds; the lower values indicate higher enzyme activity. The results obtained with

the different strains were used to compare with the EGA medium halo sizes.

Analysis of free monoterpenes and bound aroma compounds

Extraction of terpene compounds was performed by adsorption and elution from an Isolute cartridge (IST Ltd., Mid Glamorgan, U.K. ENV+) packed with 1 g of highly cross-linked styrene-divinyl benzene (SDVB) polymer. A sample of 50 ml of wine diluted with 50 ml of distilled water and containing 0.1 ml of internal standard (1-heptanol at 230 ppm in a 50% hydroalcoholic solution) was applied (4–5 ml/min) to the cartridge, and finally residual was washed with 15 ml of distilled water (Carrau et al., 2005). The terpenes were eluted with 30 ml of dichloromethane; the eluate was dried with Na_2SO_4 and concentrated to 1.5 ml on a Vigreux column, and stored at −10°C. Immediately prior to GC–MS analysis, the samples were further concentrated to 100 µl under a gentle nitrogen stream.

The bound forms were eluted with 30 ml of methanol, and treated as described previously with Cytolase PCL5 (Gist-brocades, Lille Cedex, France) (Boido et al. 2003).

For quantitative results, each sample was analysed by GC-FID, on a Shimadzu GC 14 B gas chromatograph equipped with a Shimadzu data processor software EZ-Chrom, using a column Carbowax 20 M (Ohio Valley, Marietta, Ohio) bonded fused silica capillary column (25 m × 0.32 mm i.d.), coated with poly-(ethylene glycol) (0.25 mm phase thickness), column temperature, 40°C for 8 min, rising to 180°C at 3°C/min, then to 230°C at 20°C/min; injector temperature, 250°C; detector temperature, 250°C; injection mode, split; split ratio, 1:30; volume injected, 1.0 ml; carrier gas, hydrogen, 30 kPa. For identification proposes GC–MS analysis was performed using a Shimadzu QP 5050 mass spectrometer equipped with reference libraries (McLafferty and Stauffer 1991; Adams 2001) using a column and conditions as above described. Quantification using an internal standard (1-heptanol) was as previously described (Boido et al. 2003).

Sensory Analysis

Fermentation products were subjected to sensory analysis through a paired preference test. Samples fermented with selected strains in the chemically defined medium containing 100 mgN/l of YAN were presented to a group of five winemakers so as to evaluate aroma defects and positive characteristics. A free description of desirability and aroma characteristics was offered as the tasting sheet. Only when none of the tasters could identify an aroma defect, samples were considered acceptable and are shown in Table 1.

Statistical Analysis

ANOVA was performed to establish significant differences among strains when evaluating EGA halo sizes, G-G indexes and free and bound monoterpene formation. Statistica 5.1 software was utilized for this analysis.

Results

The isolation of 154 native yeast strains from grapes and musts was initially performed using WL Nutrient agar medium, and strain colonies were differentiated based on their morphology, color and texture (Pallmann et al. 2001). Native strains were then classified as non-*Saccharomyces* or *Saccharomyces* by the lysine agar medium test. Strains showing glucosidase activity were classified to the level of species by molecular methods as described in Materials and Methods. The ca. 600-nucleotide domain D1/D2 of 26S rDNA was sequenced and compared with the sequences previously deposited in GenBank using BLASTN (http://blast.ncbi.nlm.nih.gov/). According to Kurtzman & Robnett (1998), strains showing 99–100% sequence identities within this domain are considered to be members of the same species.

Screening for β-glucosidase activity using EGA medium

From the 154 native strains isolated, only 30 showed glucosidase activity using the EGA medium. Figure 1 shows the brown halo sizes for four strains with different levels of glucosidase activity. The halo diameter sizes were measured after 4–5 days, resulting in a semi-quantitative classification of glucosidase activity levels as: weak (between 14–17 mm, 10 strains), medium (18–22 mm, 10 strains) and strong (≥23 mm, 10 strains). A control group of 27 well known laboratory and commercial wine yeast strains was used to evaluate this EGA medium, and only eight showed glucosidase activity (Table 1). It should be noted that out of the 21 *S. cerevisiae* strains tested, none showed glucosidase activity. However, we found nine *S cerevisiae* native strains showing glucosidase activity in this selection.

Sensory analysis was carried out with wines produced at laboratory scale with the native yeast strains showing glucosidase activity so as to establish their suitability for winemaking production. From the selected 30 strains, sixteen resulted in wines acceptable in the sensory

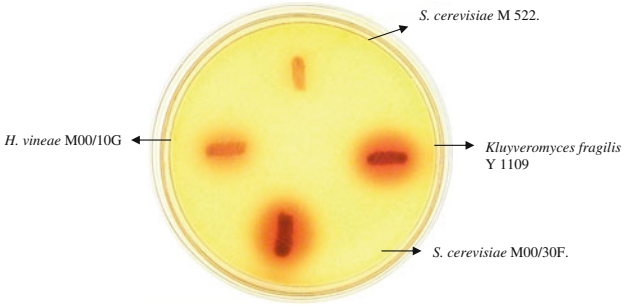

Fig. 1 Screening for β-glucosidase activity on EGA medium. Each strain to be tested on EGA medium was inoculated as a streak of 1 mm × 4 mm. A brown halo around a streak evidenced β-glucosidase production. The positive control was a strain of *Kluyveromyces fragilis* Y1109 and the negative control was *S. cerevisiae* (M 522). Examples of strains showing weak (MOO/10G) and strong (M00/30F) β-glucosidase activity levels are shown

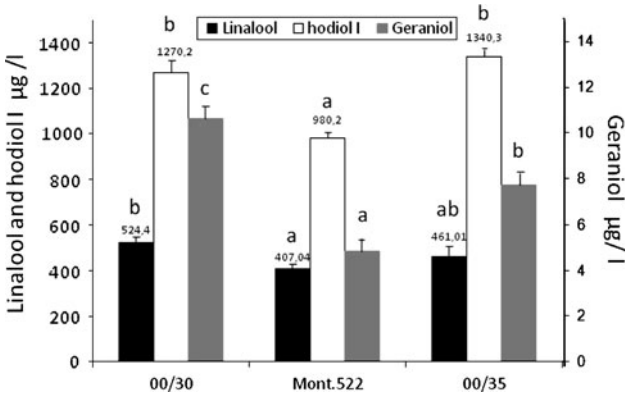

Fig. 2 Main free monoterpenes that show significant differences were linalool, hodiol I and geraniol determined by GC–MS analysis in the experimental wines produced with the three yeasts strains as indicated in Table 3. Letters at each column indicate the level of significant difference ($P < 0.05$) according to an LSD test of ANOVA calculated for each compound. Error bars indicate SD of the mean value

preference analysis. Only two *S. cerevisiae* strains were discarded based on the sensory analysis results (Table 1). From the non-*Saccharomyces* strains, interesting results were obtained with *Hanseniaspora vineae* and *Metschnikowia pulcherrima* strains.

Determination of the Glycosyl—Glucose index in grape juice

Complete fermentations of a grape juice were carried out with the native yeasts collection as shown in Table 2. The GG indexes for each of the eight *S. cerevisiae* strains with good sensory characteristics were measured, using M522 as negative control. The non-*Saccharomyces* strains were evaluated separately, as they needed co-inoculation with *Saccharomyces* to obtain complete fermentations. We included 3 strains as controls, two with strong glucosidase activity according to the EGA medium test (*H. vineae* T00/20G and *M. pulcherrima* T00/23G) and one with weak activity (*H. vineae* M00/10G). As can be seen, the GG index showed that the lower the value, the higher the enzyme activity as less glycosylated compounds remained in the wine.

White grape wine fermentations with selected glucosidase producers of *Saccharomyces cerevisiae*

For further studies on wine fermentations, we selected *S. cerevisiae* strains 00/30 and 00/35, which had shown a better performance in relation to fermentation kinetics and lower G-G indexes (Table 2). A wine variety rich in bound monoterpenes, Muscat Miel (Muscat Giallo), was selected to measure the potential of *S. cerevisiae* strains 00/30 and 00/35. This variety contains a high concentration of bound linalool as its main monoterpene compound (Versini et al. 1999). *S. cerevisiae* UCDavis M522 was used as a control industrial strain. Results for these experimental wines are shown in Table 3, demonstrating that both native strains are suitable for wine production. Strain 00/35 has a very similar behavior to M522 in relation to CO_2 production, low residual sugars and alcohol content. Relative to the GG index, both strains show a significant decrease compared to M522. Although *S. cerevisiae* glucosidase appears not to be extracellular, and is present in the periplasmic space of yeast cells (Darriet et al. 1988), our results show that the glucosidase is really active during wine fermentation. Analyses of free monoterpenes present in Muscat Miel with GS-MS were carried out to see the effect in real wine conditions (Fig. 2). The results clearly showed a significant increase of free linalool for strain 00/30 and of free geraniol and hodiol I (*trans*-3,7-dimethyl-1,5-octadiene 3,7-diol) for both strains 00/30 and 00/35, in comparison with M522. In addition, linalool levels increased by the use of these strains are also significant from the Active Odour Values (AOV) point of view. The sensory threshold value for this compound in simil-wine is 10 µg/l (Guth 1997). AOVs are calculated by dividing an aroma compound concentration of a wine sample by the aroma threshold value of this compound. In this case linalool resulted in a significant difference of more than 10 aromatic units of AOV between strain 00/30 and M522. Although geraniol levels are below the threshold value and hodiol I lacks a reported threshold value in the literature, results for these compounds are also significantly different between both native strains and M522 (Fig. 2). On the other hand, no significant differences were found in other free

monoterpenes analysed: nerol, citronellol, hodiol II (3,7-dimethyl-1,7-octadiene-3,6-diol), endiol (3,7-dimethyl-1-octen-3,7 diol), ho-trienol, and α-terpineol. However,

Table 4 Determination of bound aroma compounds by GC–MS analysis in the experimental wines produced with the three yeasts strains as indicated in Table 3

Strain identity	Bound aroma compounds μg/l						
	Linalool	Hodiol I	Geraniol	Nerol	Benzyl alcohol	2-phenylethanol	4-vinylguaiacol
M00/30F	204 ±13 a	221±15 a	260±19 ab	194±5 ab	166±12 ab	94±5 a	17.6±2 a
M00/35F	247±21 a	330±12 b	316±11 b	138±11 a	141±5 a	90±8 a	45.6±6 b
M522	335±9 b	297±35 b	279±15 ab	250±16 b	177±13 ab	117±12 b	51±5 b

Letters at each value indicate the level of significant difference ($P < 0.05$) according to LSD test of ANOVA calculated for each compound. Standard Deviation is indicated

interesting results were obtained with some bound aroma compounds that are shown in Table 4. Some glucosidase activity in other non-terpenoid compounds such as benzyl alcohol (described as almond aroma), 2-phenylethanol (rose and honey) and 4-vinylguaiacol (spicy notes) was also detected (see Table 4). Although is difficult to make a balance between free and bound monoterpenes due to successive biotransformations and/or chemical modifications that occur during wine fermentation, the differences in linalool free levels after fermentation with these native strains vs a commercial strain confirm the presence of active glucosidase in winemaking conditions.

Discussion

Although low pH, the presence of sugars and temperature could account for a limited glucosidase action in wines (Delcroix et al. 1994; Rosi et al. 1994), other authors support the notion that there is a real contribution of *S. cerevisiae* enzymes in wine glycosylated aroma compounds (Darriet et al. 1988; Mateo and Di Stefano 1997; Zoecklein et al. 1997; Spagna et al. 2002; Chassagne et al. 2005). On the other hand, Dubourdieu et al. (1988) had reported excretion of β-glucosidase into the medium, but only after autolysis of the cells. Some β-glucosidases of *S. cerevisiae* have been reported not to be inhibited by glucose and are not affected by 10% ethanol (Canal-Llauberes 1993), in contrast to the β-glucosidase enzymes isolated from grape or filamentous fungi. However, very few *S. cerevisiae* strains with glucosidase activity have been identified, and this fact is limiting the development of knowledge about their winemaking potential. Esculin has traditionally been used for bacterial identification (Miskin and Edberg 1978), for identifying glucosidase activity in zymography following PAGE (Kwon et al. 1994), and more recently for screening of enzyme chromatographic fractions (Saqib and Whitney 2006). Our results confirmed that an esculin glycerol solid medium (EGA) could help to identify and create a bigger collection of active native strains of this species. In contrast to what is normally observed with most commercial and laboratory strains, in this first screening with the EGA medium we could identify 9 glucosidase-active strains of this species. Relative to the GG index values, the selected *S. cerevisiae* strains showed a consistent correlation between the glucosidase activity measured by the EGA medium and the final GG indexes obtained in grape must (Table 2).

On the other hand, the non-*Saccharomyces* strains showed a different behavior between EGA measurements and GG indexes that would demand further studies. Interestingly, although strain *H. vineae* 00/10 showed a weak halo in the EGA medium, it appeared to have a very effective glucosidase activity in grape juice expressed as GG index (Table 2). Although glucosidase enzyme complexes of *Metschnikowia* and *Hanseniaspora* were reported as not active at low pH (*M. pulcherrima* strain T00/23G of Table 2 by González-Pombo et al. (2008) and a strain of *H. vineae* by Vasserot et al. (1989)), further studies of these species are needed to understand the good performance obtained here in grape juice by M00/10G. Yanai and Sato (2000) support the idea that other aspects rather than strong or weak glucosidase activity are essential for the stability of the enzyme under wine fermentation conditions and its action on glycosylated aroma compounds. Although other solid media for screening glucosidase activity in yeasts have been proposed, to our knowledge no correlation with the natural grape glucoside hydrolysis has been shown before.

Conclusion

In summary, the EGA solid medium can be used for the screening of glucosidase activity in native yeast strain collections. It is particularly suitable for performing large scale screenings and detecting the low proportion of *S. cerevisiae* strains that show this activity. The semiquantitative measurement of glucosidase activity in this medium showed good correlation to the potential action in real wine conditions, particularly for *Saccharomyces* strains. Using the G-G test method, the *Saccharomyces* strains selected in

this study showed significant glucosidase activity in Muscat Miel, an aromatic white wine. These results were confirmed by monoterpene GC analysis, showing significant increases of free linalool, hodiol I and geraniol after fermentation.

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