Assessing the enological potential of Sicilian representatives of the yeast-like fungus *Aureobasidium pullulans*

T. Fasciana[(1)], G. Polizzotto[(2)], G. Amore [(3)], D. Oliva[(4*)]

Istituto Regionale del Vino e dell'Olio. Via Libertà 66, 90143. Palermo Italy.
1-teresa.fasciana@virgilio.it; 2-gambit1978@libero.it;
3-gabriele.amore@libero.it; 4-daniele.oliva@regione.sicilia.it
[*]corresponding author

ASTRACT

In this study we assessed the enological potential of representatives of the pseudo-yeast species *Aureobasidium pullulans*, isolated from Sicilian grapes and musts. This microorganisms can express β-glucosidase and β-1-3 glucanase activities, which might be useful during the first phases of wine fermentation, and/or during wine fining *sur lies*.
19 *Aureobasidium pullulans var. pullulans* and seven *A. pullulans var. melanogenum* isolates were assayed. The enzymatic activities of all isolates were measured by way of plate assays: arbutin plates (β-glucosidase activity); carboxymethyl pachyman (CMP) plates (β-1-3 glucanase activity). All isolates were able to hydrolyze arbutin, but this activity was inhibited at sugar concentrations of 200 g/L (comparable to those of the fermenting musts). Furthermore all isolates possessed β-1-3 glucanase activities but the alcohol concentration (12%) at which wine fining *sur lies* would occur, would not be compatible with the survival of the microorganisms. Finally a viability test was performed under different conditions of temperature, at ethanol concentrations close to those of wine fining *sur lies*. Based on our results, we concluded that the use of these *A. pullulans* strains is not possible during these phases of wine making.

RIASSUNTO

Abbiamo valutato il potenziale enologico di isolati dello pseudo-lievito *Aureobasidium pullulans*, ritrovati in mosti e in uve siciliani. Questo in virtù della capacità di questo microrganismo di produrre attività β-glucosidasica e β-1-3 glucanasica, che potrebbero essere utili nelle prime fasi di fermentazione, e/o durante l'affinamento *sur lies*.
Sono stati presi in considerazione 19 isolati di *Aureobasidium pullulans var. pullulans* e sette isolati di *A. pullulans var. melanogenum*. Le attività enzimatiche di tutti gli isolati sono state misurate in saggi su piastra: sono state usate piastre con arbutina per misurare l'attività β-glucosidasica e piastre con carbossimetil pachyman (CMP) per l'attività β-1-3 glucanasica.
Tutti gli isolati erano capaci di idrolizzare l'arbutina, ma non a concentrazioni di zucchero di 200 g/L (paragonabili a quelle dei mosti in fermentazione). Inoltre, tutti gli isolati possedevano attività β-1-3 glucanasica, ma i valori di concentrazione dell'alcol (12%) durante l'affinamento *sur lies* si sono rivelati incompatibili con la sopravvivenza dei microrganismi. Infine, è stato eseguito un test di vitalità, a diverse temperature e a concentrazioni di etanolo vicine a quelle dell'affinamento dei vini *sur lies*. Sulla base dei nostri risultati, abbiamo concluso che non è possibile utilizzare questi ceppi di *A. pullulans* in ambito enologico.

INTRODUCTION

Non-*Saccharomyces* yeast species have recently enjoyed much attention due to their ability of producing enzymes of enological interest (*e.g.* (González-Pombo et al., 2008; Maturano et al., 2012; Strauss et al., 2001; Viana et al., 2008)) such as glycosidase, esterases, proteases,

which can play an important role for the definition of wines' sensory aspects (Charoenchai et al., 1997). The introduction of these microorganisms during must fermentation represent a natural and attractive way to improve wine quality and increase their appeal. The relevance of these microorganisms lies in the properties of the lytic enzymes they produce and on the ability of secreting them in an active form in the must (Cordero Otero et al., 2003; Fernández et al., 2000; Fia et al., 2005). Glucosidases allow the liberation of aromatic compounds from their glycolsylated precursors; β-1-3 glucanases enrich wines with complex polysaccharides and nitrogen compounds which come from the lysis of cell walls and which increase smoothness, richness and aromatic persistence of wines (Villettaz et al., 1984; Zoecklein et al., 1997). The presence of these enzymes in non-*Saccharomyces* species has been documented in a limited number of reports (*e.g.*(Blättel et al., 2011; Cordero Otero et al., 2003, 2003; Fia et al., 2005; Mateo and Di Stefano, 1997)). *Aureobasidium pullulans* is a yeast-like fungus, which is able to produce and secrete these enzymes (Dake et al., 2003; Saha et al., 1994). It is present in spontaneous wine fermentations (Pardo et al., 1989; van Keulen et al., 2003) and although a number of variants exist, the *A. pullulans* var *pullulans* (with pink, light brown or yellow colonies) and the *A. pullulans* var *melanogenum* (with black or green-black colonies) are the most common (Takeo and de Hoog, 1991).
During a biodiversity survey on the fermentation yeast species present in musts from Sicily (Romancino et al., 2008) and from the Linosa Island (Polizzotto et al., in preparation), 19 *A. pullulans* var *pullulans* and seven *A. pullulans* var *melanogenum* isolates were recovered. Here we assessed the ability of these isolates to produce β-glucosidases and β-1-3-glucanases, active in the presence of sugar concentrations and at alcohol levels similar to those of pre-fermentation must and of wine respectively. We also performed test to assess the viability of these isolates under different conditions of temperature and ethanol concentration.

MATERIALS AND METHODS

Isolates.

Aureobasidium pullulans isolates (19 var *pullulans* and seven var *melanogenum*) from musts of the Linosa Island and from a previously established collection of yeasts from Sicilian musts (Romancino et al., 2008), were identified based on morphology on WL Nutrient Agar (Oxoid) plates. Membership of each isolate to the *A. pullulans* species was confirmed by sequencing of the D1/D2 Mt-DNA region (Groenewald et al., 2011).

Plate tests.

The β-glucosidase activity of each isolate was assessed following the protocol of (Kersters and De Ley, 1971). Arbutin agar plates (0.67% Yeast Nitrogen base; 2% Agar; 0.5% Arbutin; pH5.0; filter sterilized and added with 2 ml/100ml of a filter sterilized 1% ferric citrate solution) were incubated at 30^{o}C for 5 days. The *S. cerevisiae* strain L404 was used as negative control. Tests were performed in duplicate. The same test was performed in the presence of sugars (glucose and fructose), which were added to the plates at a final concentration of 200g/L, similar to the one normally found in musts (Caridi et al., 2005). Isolates were also plated on YAPD with 2.5% glucose to exclude false positives (producing pigment in the absence of arbutin). In both tests β-glucosidase activity was scored from 0 to 3, based on the intensity of the color change of the colonies (from no change to dark brown).

The β-1-3 glucanase activity of the isolates was assessed on YPDA agar plates containing 0.1% Carboxymethyl pachyman (CMP: β(1→3) D-glucan; Megazyme) (Teather and Wood, 1982); similar tests were performed to assess the β-1-4 glucanase activity of the isolates, on YPDA plates containing 0.2% Carboxymethyl Cellulose (CMC: β(1→4) D-glucan; Sigma) (Strauss et al., 2001). Zymolase was used as positive control on CMP plates (5 μl of a 1mg/mL solution were spotted on the plates). CMP tests were also performed as described above after adding 12% ethanol to the plates. All tests were performed at 18^{o}C

Viability tests

Tests in which the viability of the isolates was assessed under different conditions of temperature and ethanol concentration, were performed as follows: 0,5 mL of three-days liquid culture (WL Nutrient Agar, OXOID) were inoculated in 5mL of YPD broth and let overnight at 30^{o}C. 15 mL of YPD were then added, the culture was transferred in a 250 mL bottle and let 48h at 30^{o}C with stirring (100 rpm). An aliquot of 1 mL was taken, and serial dilutions (form 10^{-1} down to 10^{-6}) were seeded on WL Agar plates in duplicate. The liquid culture was divided into two aliquots: one was added with distilled water, the other with ethanol (11% final concentration). Both aliquots were incubated at 8^{o}C or at 30^{o}C. After 1h, 4h, 24h and 48h of incubation, 1 mL of culture were taken and serially diluted (from 10^{-1} down to 10^{-6}). Each dilution was seeded on WL Agar plates in duplicate and incubated at 30^{o}C. Colony growth was monitored once every 24h and colonies were counted the fifth day.

RESULTS AND DISCUSSION

All the *Aureobasidium pullulans* isolates we analyzed were able to hydrolyze arbutin. This result is shown in table 1 were the β-glucosidase activity of each isolate is scored from 0 to 3, depending on the browning of the colonies (left column). While a difference in the 19 *var pullulans* was noted (p1-p19), no difference was found in the activity of the 7 *var melanogenum* (m1-m7). However when plates contained 200 g/L of sugars, the activity of all isolates was completely inhibited (central column). No false positives were observed.

Table 1

β-glucosidase activity of 19 *A. pullulans var pullulans* and 7 isolates *A. pullulans var melanogenum.* P, *pullulans* variants; M, *melanogenum* variants; 0, no color change; 1, light brown, 2, brown, 3, dark brown.

	Arbutin 0,5% (w/v)	Arbutin+ 200g/L sugars	Glucose 2.5% (w/v)		Arbutin 0,5% (w/v)	Arbutin+ 200g/L sugars	Glucose 2.5% (w/v)		Arbutin 0,5% (w/v)	Arbutin+ 200g/L sugars	Glucose 2.5% (w/v)
P1	2	0	0	P10	2	0	0	P19	2	0	0
P2	2	0	0	P11	3	0	0	M1	1	0	0
P3	2	0	0	P12	2	0	0	M2	1	0	0
P4	2	0	0	P13	2	0	0	M3	1	0	0
P5	2	0	0	P14	3	0	0	M4	1	0	0
P6	2	0	0	P15	2	0	0	M5	1	0	0

P7	3	0	0	P16	3	0	0	M6	1	0	0
P8	3	0	0	P17	2	0	0	M7	1	0	0
P9	3	0	0	P18	2	0	0	L404	0	0	0

β-1-3 are by far the most abundant sugar bonds within the yeast cell wall (Manners et al., 1973). Therefore we tested the β-1-3 glucanase activity of the isolates by assessing the lysis area around each colony in CMP plates. All isolates possessed β-1-3 glucanase activity. However the presence of 12% ethanol did not allow survival of the microorganisms. Therefore no enzymatic activity could be displayed by the isolates in these conditions (table 2, below).

Table 2

β-glucanase activity of 19 *A. pullulans var pullulans* and 7 *A. pullulans var melanogenum* isolates. The activity is measured by assessing the size (in mm) of the hydrolysis halos produced in the presence of CMP, and CMP+ 12% ethanol

	CMP 0,1% (w/v)	CMP 0,1% +12% ethanol		CMP 0,1% (w/v)	CMP 0,1% +12% ethanol		CMP 0,1% (w/v)	CMP 0,1% +12% ethanol
P1	10	0	P10	15	0	P19	20	0
P2	10	0	P11	15	0	M1	10	0
P3	10	0	P12	20	0	M2	10	0
P4	10	0	P13	15	0	M3	15	0
P5	10	0	P14	15	0	M4	10	0
P6	10	0	P15	15	0	M5	15	0
P7	20	0	P16	15	0	M6	15	0
P8	15	0	P17	12	0	M7	20	0
P9	15	0	P18	15	0	Zymolase	40	0

Finally to understand if our isolates would remain viable under the conditions occurring in the musts and in the wine during fining, we assessed the effect of combining different temperatures and alcohol concentrations. While 11% ethanol and 8^{o}C allowed survival of the micro-organims, raising the temperature to 30^{o}C resulted to be lethal.

CONCLUSIONS

In our survey we were unable to find *A. pullulans* isolates that would be suitable for utilization in the conditions we operated. This because the presence of sugar concentrations similar to those found in the musts would inhibit β-glucosidase activities; also, the presence of 12% ethanol (that could be found toward the end of alcoholic fermentation) would be lethal to the isolates we tested. However one possibility that remains to be tested is to utilize lees at the end of fermentation. These could be washed to remove ethanol and used as a substrate for the

activity of live *A. pullulans* yeast strains. This could be a way to provide enzymatic activities, greatly reducing the costs of the procedure.

ACKOWLEDGEMENTS
Funds for this work were provided by IRVO

BIBLIOGRAPHY

Blättel, V., Larisika, M., Pfeiffer, P., Nowak, C., Eich, A., Eckelt, J., König, H., 2011. Beta-1,3-glucanase from *Delftia tsuruhatensis* strain MV01 and its potential application in vinification. Appl. Environ. Microbiol. 77, 983–990.

Caridi, A., Pulvirenti, A., Restuccia, C., Sidari, R., 2005. Screening for yeasts able to hydrolize arbutin in the presence of glucose and ethanol. Ann. Microbiol. 55, 43–46.

Charoenchai, C., Fleet, G.H., Henschke, P.A., Todd, B., 1997. Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. Aust. J. Grape Wine Res. 3, 2–8.

Cordero Otero, R.R., Ubeda Iranzo, J.F., Briones-Perez, A.I., Potgieter, N., Villena, M.A., Pretorius, I.S., Rensburg, P. van, 2003. Characterization of the β-Glucosidase Activity Produced by Enological Strains of Non-*Saccharomyces* Yeasts. J. Food Sci. 68, 2564–2569.

Dake, M.S., Jadhav, J.P., Patil, N.B., 2003. Induction and properties of (1→3)-b-D-glucanase from *Aureobasidium pullulans*. Indian J. Biotechnol. 3, 58–64.

Fernández, M., Ubeda, J.F., Briones, A.I., 2000. Typing of non-*Saccharomyces* yeasts with enzymatic activities of interest in wine-making. Int. J. Food Microbiol. 59, 29–36.

Fia, G., Giovani, G., Rosi, I., 2005. Study of beta-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. J. Appl. Microbiol. 99, 509–517.

González-Pombo, P., Pérez, G., Carrau, F., Guisán, J.M., Batista-Viera, F., Brena, B.M., 2008. One-step purification and characterization of an intracellular beta-glucosidase from *Metschnikowia pulcherrima*. Biotechnol. Lett. 30, 1469–1475.

Groenewald, M., Robert, V., Smith, M.T., 2011. The value of the D1/D2 and internal transcribed spacers (ITS) domains for the identification of yeast species belonging to the genus *Yamadazyma. Persoonia* Mol. Phylogeny Evol. Fungi 26, 40–46.

Kersters, K., De Ley, J., 1971. Enzymic tests with resting cells and cell-free extracts, in: Methods in Microbiology. Academic Press, London and New York, p. 42.

Manners, D.J., Masson, A.J., Patterson, J.C., 1973. The structure of a beta-(1→3)-D-glucan from yeast cell walls. Biochem. J. 135, 19–30.

Mateo, J.J., Di Stefano, R., 1997. Description of β-glucosidase activity of wine yeasts. Food Microbiol. 14, 583–591.

Maturano, Y.P., Rodríguez Assaf, L.A., Toro, M.E., Nally, M.C., Vallejo, M., Castellanos de Figueroa, L.I., Combina, M., Vazquez, F., 2012. Multi-enzyme production by pure and mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts during wine fermentation. Int. J. Food Microbiol. 155, 43–50.

Pardo, I., García, M.J., Zúñiga, M., Uruburu, F., 1989. Dynamics of Microbial Populations during Fermentation of Wines from the Utiel-Requena Region of Spain. Appl. Environ. Microbiol. 55, 539–541.

Romancino, D.P., Di Maio, S., Muriella, R., Oliva, D., 2008. Analysis of non-*Saccharomyces* yeast populations isolated from grape musts from Sicily (Italy). J. Appl. Microbiol. 105, 2248–54.

Saha, B.C., Freer, S.N., Bothast, R.J., 1994. Production, Purification, and Properties of a Thermostable beta-Glucosidase from a Color Variant Strain of *Aureobasidium pullulans*. Appl. Environ. Microbiol. 60, 3774–3780.

Strauss, M.L., Jolly, N.P., Lambrechts, M.G., van Rensburg, P., 2001. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. J Appl Microbiol 91, 182–90.

Takeo, K., de Hoog, G.S., 1991. Karyology and hyphal characters as taxonomic criteria in ascomycetous black yeasts and related fungi. Antonie Van Leeuwenhoek 60, 35–42.

Teather, R.M., Wood, P.J., 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. 43, 777–780.

Van Keulen, H., Lindmark, D.G., Zeman, K.E., Gerlosky, W., 2003. Yeasts present during spontaneous fermentation of Lake Erie Chardonnay, Pinot Gris and Riesling. Antonie Van Leeuwenhoek 83, 149–154.

Viana, F., Gil, J.V., Genovés, S., Vallés, S., Manzanares, P., 2008. Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. Food Microbiol. 25, 778–785.

Villettaz, J.-C., Steiner, D., Trogus, H., 1984. The Use of a Beta Glucanase as an Enzyme in Wine Clarification and Filtration. Am. J. Enol. Vitic. 35, 253–256.

Zoecklein, B.W., Marcy, J.E., Williams, J.M., Jassinski, Y., 1997. Effect of Native Yeasts and Selected Strains of *Saccharomyces cerevisiae* on Glycosyl Glucose, Potential Volatile Terpenes, and Selected Aglycones of White Riesling (Vitis vinifera L.) Wines. J. Food Compos. Anal. 10, 55–65.