Ann Microbiol (2011) 61:75–78
DOI 10.1007/s13213-010-0099-z

SHORT COMMUNICATION

Isolation and molecular identification of wine yeasts from a Brazilian vineyard

Milla Alves Baffi · Carolina dos Santos Bezerra · María Arévalo-Villena · Ana Isabel Briones-Pérez · Eleni Gomes · Roberto Da Silva

Received: 16 May 2010 / Accepted: 7 July 2010 / Published online: 21 July 2010

Abstract Traditional winemaking is carried out by spontaneous yeasts from the vineyard that persist throughout the alcoholic fermentation. This study examined the diversity of yeast species isolated from grape skin and musts of two varieties of *Vitis labrusca* from a vineyard in the southeast region of Brazil (Jales, São Paulo), which produces artisanal wines. Molecular identification was achieved by a combination of PCR-RFLP/sequencing of the internal transcribed spacers (ITS) and sequencing of the D1/D2 domain of ribosomal DNA. Eighty yeast samples were isolated from grapes and musts, and seven different species were identified. The diversity of species varied according to the grape variety. The most frequent species were *Hanseniaspora uvarum* with 28 isolates, followed by *Issatchenkia occientalis* with 19 isolates and *Issatchenkia orientalis* with 16 isolates. Other species with a lower number of isolates were: *Issatchenkia terricola*, *Saccharomyces cerevisiae*, *Aureobasidium pullulans* and *Sporidiobolus pararoseus*. Our results showed that molecular identification is a very powerful identification method in which natural isolates of ascomycetous or basidiomycetous yeasts can be rapidly and reliably identified with better reproducibility and higher throughput than conventional phenotypic methods. This is the first report of the diversity of indigenous yeast species from a vineyard in Brazil.

Keywords Yeasts · Non-*Saccharomyces* · Brazil · Wine aroma

This paper is part of the special issue "Wine microbiology and safety: from the vineyard to the bottle (Microsafetywine)", 19–20 November 2009, Martina Franca (Italy).

M. A. Baffi (✉) · C. dos Santos Bezerra · E. Gomes · R. Da Silva
Laboratory of Biochemistry and Applied Microbiology,
São Paulo State University, UNESP,
São José do Rio Preto, SP, Brazil
e-mail: millabaffi@yahoo.com.br

M. Arévalo-Villena · A. Isabel Briones-Pérez
Departamento de Química Analítica y Tecnología de Alimentos,
Universidad de Castilla La Mancha, UCLM,
Ciudad Real, Spain

Introduction

In traditional winemaking, alcoholic fermentation is carried out by spontaneous yeasts present in grape juice that commences in the vineyard and continues throughout the fermentation. Wine is the product of complex interactions between the yeasts that are responsible for the subtlety of the wine aroma. The great majority of studies on the microbiological aspects of winemaking have focussed mainly on yeasts belonging to the *Saccharomyces* genus that are usually employed in alcoholic fermentation (Barrajón et al. 2009). However, in grape skin, and during the early stages of fermentation, there is substantial growth of *non-Saccharomyces* yeasts, which can produce useful hydrolytic enzymes, such as β-D-glucosidases, which are able to develop aroma active compounds and enhance the final wine characteristics (Ubeda and Briones 2000; Arévalo-Villena et al. 2007).

Traditionally, yeast identification has been based on morphological and physiological traits. However, these methodologies are laborious and time consuming. In contrast, molecular techniques are faster than classical tests. A reliable identification can be achieved by restriction analysis (PCR-RFLP) of noncoding ribosomal DNA regions, which includes the internal transcribed spacers and the 5.8S rDNA (ITS-5.8S region). Sequence analysis of the ITS-5.8S region or the D1/D2 domains in the large 26S subunit of rDNA are also used in identification tests (Baleiras Couto et al. 2005; Martorell et al. 2006). These

molecular techniques are useful for rapid identification of yeasts in wine ecosystems (Capece et al. 2003; Sun et al. 2009) and also in other foods (Heras-Vazquez et al. 2003; Rómo-Sánchez et al. 2010).

Information about the yeast diversity in grapes is important for wine-makers in order to produce wine of high quality. Brazil is an important grape-producing country and is also currently becoming a wine-producer. The region of Jales (Sao Paulo State) is one of the most important centres of grape production in the southeast of the country, and has recently started to produce wine. Nevertheless, this region has no tradition in winemaking and the wine is produced based on spontaneous fermentation and without any commercial enzyme preparations. To our knowledge, there are no current reports concerning ecological studies about the yeast microbiota of local ecosystems. As a pioneering study in Brazil, this work reports the isolation and identification of autochthonous yeasts from a vineyard in the region of Jales.

Materials and methods

Samples of healthy and undamaged grape berry surfaces of two varieties of *Vitis labrusca* (Isabel and Bordeaux) were collected randomly and aseptically during harvest time from a vineyard in the region of Jales (Sao Paulo, Brazil). Samples of grape juice (must) were also taken immediately after the grapes of both varieties were crushed. Samples of grape skins and grape juice were diluted in saline solution (0.8% w/v NaCl) with dilutions from 10^{-1} to 10^{-5} of each sample. Aliquots of each dilution were spread (0.1 mL each) on plates of YPD agar (1% yeast extract, 1% peptone and 2% glucose) supplemented with choramphenicol (100 mg/L) + ampicillin (25 μL/mL) and sodium propionate (0.25 g/L) in order to inhibit bacteria and mould growth. Plates were incubated at 28°C for 48–72 h. Colonies that appeared were counted as colony forming unit (CFU/mL) and plates containing approximately 100 colonies (or 100 CFU) per plate were selected. A total of 80 yeast colonies were randomly isolated from the plates (30 from Isabel variety, 30 from Bordeaux variety and 20 from must). Selected colonies were sub-cultured on new plates and purified by repeated streaking. Yeast pure cultures were maintained on YPD agar slants at 4°C and in glycerol stock (20%) at −80°C for future identification.

Yeasts were assigned to species level by PCR-RFLP analysis of the 5.8S-ITS rDNA region. Isolated colonies were grown in 3 mL YPD medium for 24 h at 28°C and DNA was isolated (Esteve-Zarzoso et al. 1999). PCR fragments were generated using the primer pair ITS1 and ITS4 covering the internal transcribed spacers (ITS1 and ITS2) and 5.8S region. PCR was carried out in 50 μL final volumes containing 1 U *Taq* DNA polymerase (Fermentas, St. Leon Rot, Germany), 0.1 μM of each primer, 0.1 mM of each dNTP, 1 × PCR reaction buffer, 2 mM $MgCl_2$ and 10–50 ng genomic DNA as template. Amplification reaction was performed in a Perkin-Elmer model 2400 thermocycler. PCR conditions were: initial denaturation at 95°C for 5 min; 30 cycles of denaturing at 95°C for 1 min; annealing at 55°C for 2 min, an extension at 72°C for 2 min; and a final extension step of 5 min at 72°C. PCR products were electrophoresed in 1.5% agarose gels in 1x Tris-Borate-EDTA buffer, stained with ethidium bromide, and visualized under UV light. The amplified fragments were treated with endonucleases *Hinf*I, *Hae*III, *Hha*I (*Cfo*I) and *Hpa*II (Fermentas), according to the supplier's instructions. The restriction fragments were checked by electrophoresis in 1.5% agarose gel, stained with ethidium bromide. PCR and RFLP fragment lengths were used for identification of yeasts using the program Quidy (developed by Wine Yeasts Biotechnology Laboratory from Castilla La Mancha University, UCLM, Spain).

One strain of each distinct PCR-RFLP profile was sequenced in the 5.8S-ITS region. Amplicons were purified using GenElute PCR kit (Invitrogen, La Jolla, CA). After purification, PCR products were sequenced with the forward primer ITS1 and/or the reverse primer ITS4 using the ABI BigDye terminator cycle sequencing kit on an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA). The ITS1-5.8S-ITS2 sequences obtained were aligned using the program Megalign (DNAStar) and compared with sequences available in the GenBank

Table 1 Yeasts identification by PCR-RFLP analysis of the 5.8S-ITS region. Sizes in bp

Species	PCR product	Restriction fragments of 5.8S-ITS region			
		Cfo I	*Hae* III	*Hinf* I	*Hpa* I
Saccharomyces cerevisiae	850	360+310+135	310+240+180+140	370+360+120	720+130
Hanseniaspora uvarum	775	330+320+120	750	350+190+170	-
Sporidiobolus pararoseus	650	300+300	640	265+175+170	-
Aureobasidium pullulans	600	190+180+110	450+150	290+180+130	-
Issatchenkia orientalis	530	215+185+80+70	390+95	235+165+135	-
Issatchenkia occidentalis	500	220+100+80	310+100+80	260+120+100	-
Issatchenkia terricola	450	120+110+100+90	290+130	240+110+100	-

Table 2 Yeast species and their accession numbers in GenBank. Sizes in bp

Species	5.8S-ITS region			26S region		
	Accession number	Length	% identity	Accession number	Length	% identity
Hanseniaspora uvarum	GQ 254807	546	100	-	-	-
Issatchenkia occidentalis	GQ 254805	442	99	-	-	-
Issatchenkia orientalis	GQ 254804	462	98	GQ891885	548	99
Issatchenkia terrícola	GQ 254806	366	97	GQ891888	565	100
Aureobasidium pullulans	GQ 254803	531	98	GQ891886	413	99
Saccharomyces cerevisiae	GQ 254808	666	97	GQ891887	528	100
Sporidiobolus pararoseus	GQ 254802	542	98	GQ891889	534	100

database at the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sequences with 98% nucleotide identity or higher in the 5.8S-ITS rDNA region were considered to represent the same species. Samples whose sequences presented an identity score below 98% with 5.8S-ITS sequences described in GenBank data library were also sequenced in the D1/D2 domains of the 26S rDNA. Amplification and sequencing of the D1/D2 region followed the protocol of Baleiras Couto et al. (2005), using the primers NL1 and NL4. PCR was performed with an initial denaturation at 94°C for 3 min, followed by 36 cycles with a temperature profile of 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min and a final extension period of 5 min at 72°C. Sequences obtained were deposited in GenBank under accession numbers (GQ254802–08 for 5.8S-ITS region and GQ891885–89 for the D1/D2 region).

Results and discussion

A total of 80 yeast strains isolated randomly from grape surfaces and musts were analysed. Restriction analysis demonstrated that yeast species can be differentiated by combining the results for each restriction endonuclease. Seven different PCR-RFLP profiles of the ITS-5.8S region were obtained (Table 1), corresponding to seven different yeast species, as corroborated by sequence analysis of ITS-5.8S and/or D1/D2 regions (Table 2). Non-*Saccharomyces* yeasts were the dominant species on the grape surfaces, which is in agreement with previous reports (Sabate et al. 2002).

Hanseniaspora uvarum was the most frequent species (34%), isolated from all grape varieties and must (Table 3). This apiculate species is commonly isolated from grape surfaces and is considered one of the predominant yeasts during the first days of alcoholic fermentation (Sabate et al. 2002). Some authors have described its importance as a producer of esters and glycerol, and its contribution to the varietal aroma of wines through its capacity to secrete several enzymes (Jolly et al. 2006).

Issatchenkia occidentalis, the second most frequent species (24%), isolated from grape skin of Bordeaux variety, has also been described in wine ecosystems (Jolly et al. 2006). The third most frequent species was *Issatchenkia orientalis* (20%), isolated from grape skin and musts of Niagara and Bordeaux/Isabel varieties (Table 3). *I.*

Table 3 Frequency of yeast species isolated from grape surfaces and must

Isolation source	Species	Frequency of isolation (%)
Bordeaux grape variety[a]	*Issatchenkia occidentalis*	53
	Hanseniaspora uvarum	22
	Issatchenkia terricola	11
	Aureobasidium pullulans	8
	Sporidiobolus pararoseus	6
Isabel grape variety[a]	*Hanseniaspora uvarum*	55
	Issatchenkia orientalis	25
	Issatchenkia terricola	15
	Aureobasidium pullulans	5
Bordeaux/Isabel Must[b]	*Issatchenkia orientalis*	46
	Hanseniaspora uvarum	37
	Saccharomyces cerevisiae	17

[a] Thirty colonies were analysed

[b] Twenty colonies were analysed

orientalis is present in musts during all the stages of fermentation, and is important for the production of higher alcohols in table wines (Clemente-Jimenez et al. 2004).

The proportions of other species were relatively low (Table 3). *Issatchenkia terricola*, found with 9%, was isolated from grape skin of Bordeaux and Isabel varieties. *I. terricola* is well known for its occurrence in grapes of several different varieties throughout the world (Clemente-Jimenez et al. 2004). *Aureobasidium pullulans* (6%), *Saccharomyces cerevisiae* (5%) and *Sporidiobolus pararoseus* (2%) were species isolated in minor frequency. *A. pullulans*, isolated from grape skin of Bordeaux and Isabel varieties, was described as a potent agent for the biocontrol of spoilage fungi in grapes and wines (Dimakopoulou et al. 2008), and is a potential producer of β-glucosidases (Leite et al. 2008).

In spite of its important role in alcoholic fermentation, *Saccharomyces cerevisiae* was not detected on the grape surfaces, being isolated only from must (Table 3). This is in agreement with previous reports, which discussed that *S. cerevisiae* is not a dominant species at grape surfaces and in the initial of fermentation, but that it predominates from the middle to the end of fermentation (Nikolaou et al. 2006). Its frequency can also vary as a function of the geographic region and the grape variety (Tofalo et al. 2009). Finally, the yeast isolated with the lowest percentage (from grape skin of Bordeaux variety) was the red pigmented yeast *Sporidiobolus pararoseus* (Table 3). This species was recently isolated from grape ecosystems (Li et al. 2010). However, the authors did not attribute an oenological application to this species.

In conclusion, the current work provides the first report on the yeast flora associated with grape ecosystems in Brazil. This ecological study is an essential step towards the exploitation of the oenological potential of the Brazilian indigenous yeast microbiota and contributes to the development of the local wine industry. Investigations are currently in progress and further studies will be carried out on the participation of these wild yeasts in spontaneous fermentation.

Acknowledgements This research was funded by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors are also grateful to Mr. Sebastião Santin, the owner of "*Vinhos Santin"* winery (Jales, Brazil) for supplying grape samples.

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