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Yeasts isolated from fermenting juice extracted from white-wine grape varieties in Zulia state, Venezuela

Aislamiento de levaduras de jugo fermentado extraído de variedades de uvas de vino blanco en el Estado Zulia, Venezuela

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Abstract

Wine making in Zulia State, Venezuela, has been steadily increasing for the past few years. One of the goals is to produce wine with regional characteristics, and one of the approaches is to use endogenous yeasts well adapted to the environment. A total of 191 isolated yeasts were obtained from fermenting juice extracted from the white-wine grape varieties *French colombard* and *Ugni blanc*, 43 of which were identified. Standard plate and broth techniques were used for morphology, colony aspect, pigment production and formation of pseudomycelium and true mycelium. Ascospore formation, together with sugar fermentation and carbon and nitrogen asimilation studies, were used for yeast identification. Genera found in both varieties were *Candida*, *Pichia*, *Brettanomyces*, *Zygosaccharomyces* and *Saccharomyces*. *Kloeckera* and *Schizosaccharomyces* were only isolated from *French colombard*. All isolated species are related to wine making, some of them with undesirable properties and others with desirable ones, among which Saccharomyces rouxii and S. cerevisiae species look the most promising.

Key words: grape must, *French colombard, Ugni blanc, Candida, Pichia, Brettanomyces Zygosaccharomyces, Saccharomyces, Kloeckera, Schizosaccharomyces*, yeast isolation, yeast identification.

Resumen

La producción de vinos en el Estado Zulia, Venezuela, se ha venido incrementando continuamente en los últimos años. Entre las metas a alcanzar está la de producir vinos con características regionales, y una de las alternativas es la de utilizar levaduras endógenas bien adaptadas al medio. Para ello se aislaron 191 levaduras de jugo fermentado extraído de uvas blancas de las

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variedades *French colombard* y *Ugni blanc*, 43 de las cuales fueron identificadas. Se utilizaron técnicas en placa y caldo para el estudio de la morfología, aspecto de colonia, producción de pigmento y formación de pseudomicelio y micelio verdadero. Para la identificación de levaduras se estudió la formación de ascosporas, junto con fermentación de azúcares y asimilación de sustratos carbonados y nitrogenados. Los géneros encontrados en ambas variadades fueron *Candida, Pichia, Brettanomyces, Zygosacharomyces* y *Saccharomyces. Kloeckera* y *Schizosaccharomyces* aparecieron solo en la variedad *French colombard*. Todas las especies de levaduras encontradas están relacionadas con la manufactura de vino, algunas con propiedades indeseables y otras deseables, entre las cuales las especies de *Saccharomyces rouxii* y *S. cerevisiae* lucen las más promisorias.
Palabras claves: mosto de uvas, *French colombard, Ugni blanc, Candida, Pichia, Brettanomyces Zygosaccharomyces, Saccharomyces, Kloeckera, Schizosaccharomyces,* aislamiento de levaduras, identificación de levaduras.

Introduction

Wine production is becoming an important industry in several South America countries such as Argentina, Chile, Brazil and Peru. Venezuela began growing grapes several years ago. Currently, about 700 ha are grown and more than 3,200 kg grapes/ha are produced per year in Zulia State. About 5,000 bottles of wine/year were produced in the Tropical Viticulture Development Center in a small-scale plant (6). Since then, the wine production in Zulia State has increased to 600,000 bottles/wine per year (7). Endogenous yeasts are required for producing wine in specific environments with regional wine characteristics (14,25). Selection of strains well-adapted to performance under winemaking conditions is of the utmost importance, because winemaking practice varies from one region to another, as does wine pH and temperature (9). The objective of this work is the isolation of endogenous yeasts, adapted to our environment, some of which might be candidates to produce wine with "native" enological characteristics, and that satisfy world and Venezuela tastes. Currently, commercial strains of *Saccharomyces cerevisiae* are used. This kind of yeast is the most used for wine production in the world (11).

Materials and methods

Sampling. 20 kg of each *French colombard* and *Ugni blanc* white-wine grape varieties, grown in Zulia State, were randomly and aseptically taken from 105 to 110 day old grapevines. Grape berries were crushed in a mortar. The resultant must was sieved and 50 mL aliquots were dispersed in 125 mL Erlenmeyer flasks with cotton caps. They were allowed to ferment at room temperature (25-28°C) for up to 21 days.

Yeast isolation and identification. Samples from different fermentation-time flasks were streaked on 0.025% chloramphenicol Malt Agar plates (8). The inverted plates were incubated at 25°C for 48 h. Morphologically-different colonies were picked, purified, transferred to Malt Extract Agar slants and kept at 4°C until used. Growth, colony aspect and pigment production were studied on Malt Extract Agar and cellular morphology, grouping, size and reproduction on Malt Extract Broth (18). The Dalmau's plate technique (18) was employed to study the formation of pseudomycelium and true mycelium. Ascospore formation was studied in Gorodkowa Agar (Difco) at 25°C for 8 weeks (3) and spores were visualized with the Schaffer-Fulton's modification of the Wirtz method (18). Fermentation of sugars (2% glucose, galactose, sucrose, maltose and lactose, and 4% raffinose) was studied in Yeast Extract Medium (Difco) test tubes containing Durham tubes at 25-28°C up to 15 days (18). Carbon and nitrogen assimilation was studied on Agar media (Merck) as auxonograms (3). Substrates used appear in Table 2. Fermentation and assimilation tests were carried out by triplicate and high grade sugars and positive and negative control yeasts were used. The keys reported by Lodder (19), Barnett & Pankhurst (2), Belin (5) and Kreger-Van Rij (18) were used for identification, and the taxonomic designations were updated according to Barnett *et al.* (3).

Results and discussion

A total of 78 colonies from *F. colombard* and 113 from *U. blanc* were isolated, 23 and 20 of which, respectively, were identified.

Polar, bipolar and multipolar budding were observed in all but for one strain of *Schizosaccharomyces*. Among the identified isolates, 40 formed sediment, 10 ring and 9 pellicle. 51.1% of the yeasts formed *pseudomycelium*, being 39.5 % *mycotorula* type and the rest (11.6%) *mycocandida*-type. *Pichia stipitis* strains formed both types of *pseudomycelium* and they were found in their asexual state *(Candida shehatae)*. Some yeasts required from 6 to 8 weeks to sporulate. 17 of the yeasts (39.53%) were sporogeneous and belonged to the genera *Pichia, Zygosaccharomyces, Saccharomyces* and *Schizosaccharomyces.*

Fermentation tests (table 1) revealed that yeasts that did not ferment glucose, did not ferment any other sugar and they were 23.3% of the total. Most of the yeasts (58.1%) fermented 3 or 4 sugars, being *Saccharomyces* and *Brettanomyces* strains among them. However, only the former are considered suitable for wine making. The latter produce undesirable acids (19). Glucose (table 2) was the most fermented sugar (76.7% of the yeasts fermented it). It was followed by galactose (60.5%), maltose (58.1%), sucrose (32.6%) and raffinose (9.3%). No yeast fermented lactose. Although

Table 1. Fermentative ability of the yeast.[1]

Number of Fermented sugars	*French colombard,*	*Ugni blanc,*	Yeasts (%)
0	2, 3, 4, 5, 6, 16, 22	17, 19, 20	23.3
1*	1, 15	14, 16	9.3
2	21, 23	-	4.6
3	7, 8, 10, 11, 12, 13, 18, 19	2, 5, 7, 8, 9, 10, 15	37.2
4	9,14,127,20	4,11,12,13,18	20.9
5	-	1,3	4.6

[1]:Numbers below *F. colombard* and *U. blanc* are isolation codes of the yeasts (1-23: *F. colombard*, 1-20: *U. blanc*). *: Glucose.

a lactose-fermenting strain usually ferments galactose (18), it did not occur on the contrary.

Table 3 shows the percentage of yeasts that metabolized each of the carbon and nitrogen substrates. As it was expected, glucose was used by all the strains. Sucrose, galactose, mannitol, maltose and xylose were assimilated by a great number of yeasts while erythritol, dulcitol, rhamnose and inulin, were the least assimilated. Peptone was used by 98% of the yeasts and nitrate by only 2%.

Table 4 shows the identification and the frequency of appearance of the yeasts. *Candida*, *Brettanomyces*, *Pichia*, *Zygosaccharomyces* and *Saccharomyces* were isolated from both grape varieties. *Kloeckera* and *Schizosaccharomyces* did not appear in the *U. blanc* variety. Non-identified yeasts account for 6,98%. All the genera found in this work, have been previously reported as related to wine making (18, 19, 24).

Amerine *et al.* (1) reported that *Kloeckera* and *Saccharomyces* genera are the most frequent in grapes all over the world. According to Heard & Fleet (17), *S. cerevisiae* was the dominant yeast in Australian musts. However, they found a significant growth of *K. apiculata (K. lindneri), C. stellata,* and *C. pulcherrima*. The first two species were also found in this work, although

Table 2. Percentage of yeasts that fermented the sugars.

Sugar	%
Glucose	76.7
Galactose	60.5
Maltose	58.1
Sucrose	32.6
Raffinose	9.3
Lactose	0

Table 3. Percentage of yeasts that assimilated the substrates.[1]

Substrate	%	Substrate	%
Glucose	100.00	L-Arabinose	20.93
Sucrose	81.40	Lactose	16.28
Galactose	76.74	Inositol	11.63
Mannitol	69.77	Erythritol	11.63
Maltose	67.44	Dulcitol	9.30
Xylose	65.11	Rhamnose	9.30
Trehalose	51.16	Inulin	9.30
Cellobiose	27.90		
Melibiose	27.90	Peptone	98.00
Raffinose	20.93	Nitrate	2.00

1: Peptone and nitrate were used for nitrogen assimilation; the others for carbon assimilation.

Candida sp. predominated. This genus also predominated in Majorca-wine grapes, specifically *C. stellata* (20). Yeasts belonging to this genus lack good enological properties, since they only grow well in low alcohol wines, are strongly oxidative and most of them do not ferment glucose. Therefore, they would not produce a good wine. On the other hand, *Saccharomyces,*

Table 4. Yeasts identification.[1]

Species	*French colombard*	*Ugni blanc*	Genus (%)
Kloeckera lindneri	1, 15		4.65
Candida graminis	2		
C. spp.	5,6,16		
C. albicans	11,12,13	6	27.91
C. stellata	7	13	
C. kruisii	8,10		
Brettanomyces intermedius		1, 3, 4	
B. lambicus 9	15	11, 63	
Pichia carsonii	3, 4	17	
P. stipitis	2, 7, 8, 9*		
P. fermentans		14	27.91
P. polymorpha		10	
P. membranaefaciens		16, 19, 20	
Zygosaccharomyces rouxii	14, 17, 21	18	9.30
Saccharomyces rouxii	18, 19		
S. cerevisiae	20	5	9.30
Schizosaccharomyces pombe	23		2.33
non-identified	22	11,22	6.98

1: Numbers are isolation codes. *: Found in its asexual state as *C. shehatae* (Barnett *et al.* 1990).

Debaryomyces, Kluyveromyces, Pichia, Hanseniospora, Saccharomycodes and *Torulopsis* (recently designated as *Candida*) have been found in France (16) *and Candida, Kloeckera, Pichia, Saccharomyces* and *Torulospora* were the predominant yeasts in fresh grape musts of the Utiel-Requena Region of Spain (22). Several of these genera may be observed in Table 4. A strain of *Schizosaccharomyces pombe* was also found in this work. This yeast was found in 40% of Sicilia grape musts studied by Florenzano *et al.* (12), and it is considered a typical component of grape microflora of that region. *Candida, Kloeckera, Pichia* and *Brettanomyces* yeasts have been related to wine souring (15,21) so that they should be avoided in the musts. In addition, Fleet (10) reported that *Kloeckera* and *Candida* species can grow with *Saccharomyces cerevisiae* and even exceed its population, mainly at cool fermentation temperatures, changing the organoleptic properties of the wine.

The genera isolated in grapevines grown in Zulia State (western Venezuela) reported in this work, have also been found in other regions of the country (8,13,14). Garassini (13) also reported that the best wines were produced with *Saccharomyces* sp. According to Peynaud (23), they produce higher ethanol concentrations, lower quantities of volatile acids and better flavor and aroma. In addition, most of commercial wine strains belong to this genus (4). Therefore, *S. rouxii* and *S. cerevisiae* strains isolated in this study (strains N° 18, 19, 20 from *F. colombard* and 5 from *U. blanc*) will be tested for wine production.

Conclusions

Yeasts found in this work are typical of grapes and fermenting musts. Most of them showed strong fermentation and assimilation ability. Glucose, followed by galactose, maltose and sucrose, were the most fermented sugars. Glucose was assimilated by all yeasts. Among the seven genera isolated, five were found in both grape varieties, although there were differences in species. Many yeasts present in the must have been related to wine souring. However, *Saccharomyces rouxii* and *S. cerevisiae* species look very promising and will be tested in wine making.

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