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IDENTIFICATION BY PCR ITS-RFLP TECHNIQUE OF NEW YEAST ISOLATES FROM PIETROASA VINEYARD

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Abstract

The biotechnological potential of local yeast is far to be completely investigated. Our work was focused on isolating and identifying by molecular tools different yeast isolated during the winemaking process (2017 and 2018), from the different varieties of grape, grape pomace and residual biomass (Burgund Mare, Busuioaca de Bohotin, Feteasca Neagra, Italian Riesling, Tamaioasa Romaneasca) originated in Pietroasa vineyard. A total of 14 different colonies were isolated and cultivated under specific conditions. In the next step, these isolates were subject to molecular identification by PCR-ITS-RFLP technique. The 5.8S-ITS region was amplified using the universal primers ITS1 and ITS4, followed by digestion with HhaI, HinfI, HaeIII. As expected, most frequent species was found to be Saccharomyces cerevisiae. Other non-Saccharomyces strains were identified as Dekkera anomala or Metschnikowia pulcherrima. The new isolated Saccharomyces and non-Saccharomyces strains will be subject of further test for the biomass production of different industrial application.

Key words*: local yeast, PCR-ITS-RFLP, grape pomace, biomass, Pietroasa vineyard.*

INTRODUCTION

Numerous studies have been reported about yeast frequency and diversity (classified in *Saccharomyces* and the non-*Saccharomyces*) associated with grape berries (vineyards) or wine production (must, during the fermentation process and wine, wineries) (Pretorius, 2000; Fleet et al., 2002; Prakitchaiwattana et al., 2004; Burcea & Radoi, 2005; Doaré-Lebrun, 2005; Renouf et al., 2005, 2007; Matei et al., 2011, 2014; Nemcová et al., 2015; Grangeteau et al., 2017; Abdo et al., 2020).

Yeast frequency is low, ranging from 10^2-10^3 CFU.g^{-1} on immature grapes and increase to 10^3-10^5 CFU.g^{-1} on grapes during harvest, with an increase in diversity of genera and species encountered (Prakitchaiwattana et al., 2004; Renouf et al., 2005, 2007; Barata et al., 2008; Grangeteau et al., 2017). Yeast populations present on grapes damaged or affected by rot are higher, ranging between 10^5-10^7 CFU.g^{-1} (Nisiotou and Nychas, 2007; Barata et al., 2008; Guzzon et al., 2014). Yeast diversity associated with grape berries has been reported in different vineyards from several countries: Argentina (Combina et al., 2005); Australia (Prakitchaiwattana et al., 2004), Brazil (Baffi et al., 2011); China (Li et al., 2010); France (Doaré-Lebrun, 2005; Renouf et al., 2005, 2007; Grangeteau et al., 2017); Germany (Brysch-Herzberg and Seidel, 2015), Greece (Nisiotou and Nychas, 2007); Slovakia (Nemcová et al., 2015); Spain (Sabate et al., 2002; Clavijo et al., 2010); Portugal (Barata et al., 2008).

According to the International Organisation of Vine and Wine (OIV), Romania is situated in top grape-producing countries in the world, alongside traditional wine-making countries (France, Italy, Spain, etc.) or more recent producers as United States, Chile Argentina or Australia (OIV, 2019). Several studies have been conducted on the isolation and characterization of indigenous yeasts from different Romanian vineyards and it was proven an important potential for industrial application of the autochthonous isolates (Burcea & Radoi, 2005; Viziteu et al., 2008;

Antoce et al., 2011; Matei and Găgeanu, 2011; Matei Radoi et al., 2011; Matei et al., 2014).

In the winemaking process, the wide use of commercial starter cultures of active dry yeast has become a routine practice in order to lead the controlled alcoholic fermentation, to produce of desirable compounds, as well to reduce the risk of spoilage and unpredictable changes of wine flavour (Lu et al., 2016; Belda et al., 2017; Berbegal et al., 2018). On the other hand, trends of wine market have been focused toward the production of wines by indigenous yeasts (*Saccharomyces* and non-*Saccharomyces*) isolated from grape berries (Grangeteau et al., 2017) or winery (Abdo et al., 2020), which can contribute to the specific aromatic fingerprints of wines.

The yeast identification based on cultural, morphological, and biochemical traits are laborious and time-consuming. On the other hand, various molecular methods based on DNA analysis have been developed enabling rapid and reliable identification of a large number of yeast at specie level in a much shorter period: Restriction Fragment Length Polymorphism (RFLP) based on the ITS-5.8S region (Esteve-Zarzoso et al., 1999; Sabate et al., 2002; Baffi et al., 2011; Csutak et al., 2016); Real-time quantitative PCR (qPCR) (Hierro et al., 2006; Zott et al., 2010) PCR-denaturing gel gradient electrophoresis (DGGE) (Prakitchaiwattana et al., 2004; Renouf et al., 2007), pyrosequencing (Grangeteau et al., 2017).

The present work is part of a larger national project focused on the yeast biodiversity of grape pomace after the winemaking process, as well on the selection of valuable indigenous yeast for added valued products.

MATERIALS AND METHODS

Grape samples and yeast isolation

Grape samples (grape or grape pomace or residual biomass) were picked up from vineyards owned by USAMV of Bucharest at Pietroasa Station, during the harvests 2017 and 2018. The samples originate from different grape varieties: (Burgund Mare, Busuioaca de Bohotin, Feteasca Neagra, Italian Riesling, Tamaioasa Romaneasca). A grape sample of 1 g was suspended in 9 ml distilled water for decimal dilution; 0.1 ml suspended solution was spread in the Petri dish with Malt Extract Agar (MEA) (VWR, USA) supplemented with colaramphenicol (to inhibit bacterial growth). The plates were incubated at 25°C, for 72 hours. Different colonies of the representative yeasts were purified until obtaining a pure culture and cryopreserved at -20°C in culture medium containing Difco™ Malt Extract Broth (Vwr, USA) with 25% (v/v) glycerol until further identification (Begea et al., 2012). All isolated yeasts were deposited in the culture collection of the UASVM Bucharest.

Morphological characterization

Isolated yeasts were grown on MEA medium at 25°C for 72 h and grouped based on their colony aspect and microscopically observations according to Lodder (1974), Pitt and Hocking (2009).

Extraction of the genomic DNA

Each isolate was cultivated in MEA medium for 72 hours. Yeasts cells were washed with sterile distilled water, transferred to a 1.5 ml Eppendorf tube and centrifuged at 5,000 *g* for 10 minutes 4°C to sediment the yeast cells. DNA was extracted according to procedures described by Ausubel et al. (2002) with some modifications. Cell pellets were resuspended in 200 µL lyse buffer (1% SDS, 2% Triton X-100, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, pH 8.0), 200 µL phenol:chloroform:isoamyl alcohol (25:24:1) solution, and homogenized with 0.3 g of glass beads (0.5 mm in diameter) in a Vortex agitator. After, TE buffer (200 µl) (10 mM Tris, 1 mM EDTA, pH 8), was added and centrifuged at 12,000 *g* for 5 min at 4°C. The supernatant was transferred to a new tube and treated with 2 µl RNase (10 mg/mL) (Thermo Scientific, USA) for 30 minutes at 37°C. DNA was precipitated with sodium acetate (3M) and cold absolute ethanol and kept in -20°C for 30 minutes. A new centrifugation was performed at 12,000 *g* for 5 min at 4°C, washed with 70% ethanol and centrifuged again at 12,000 *g* for 5 min at 4°C. DNA pellets were air dried at room temperature and solubilized in 50 µL ultrapure water. The concentration and purity of DNA were measured spectrophotometrically (SpectraMax® QuickDrop™ (Molecular Devices, USA). Finally, the DNA was stored at -20°C until use.

PCR-ITS

Molecular identification of the ITS1-5.8S-ITS2 region of each yeast isolate was performed using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers. The mixture of PCR (50 µL) consisted of 10X DreamTaq Green Buffer (contains 20 mM $MgCl_2$), 0.2 mM dNTPs, 0.5 mM of each primer, 0.025 U of DreamTaq DNA Polymerase (Thermo Scientific, USA) and 10 ng of yeast DNA. PCR was carried out in MultiGene thermal cycler (Labnet International, Inc., Cambridge, United Kingdom) with an initial denaturation cycle for 2 min at 94°C, followed by 34 cycles of amplification (with 1 min at 94°C, 1 min at 55°C, 2 min at 72°C) and a final extension cycle for 7 min at 72°C.

Restriction digestion

Digestion of ITS products was performed using *Hha*I, *Hinf*I, *Hae*III (Thermo Scientific, USA), according to ITS-RFLP method developed by Esteve-Zarzoso et al. (1999). The digestion reaction of each enzyme was performed in a volume of 20 µl containing 2 µL 10X digestion buffer, 1 U enzyme, 10 µL PCR product and ultrapure water to complete the volume. The digestions were performed at 37°C for 90 min.

PCR-RFLP analyses

PCR products and restriction fragments were checked by electrophoresis on 2% (w/v) agarose gel respectively, added with 0.05 µg/L of ethidium bromide in 1× TBE buffer (Tris-Borate-EDTA). The migration had lead at 90V for 1h. After electrophoresis, gels were visualized under UV light using GelDoc-It Imaging System (Analytik Jena, USA). All fragment sizes were approximated using standard molecular weight markers (GeneRuler 100bp Plus DNA Ladder, Thermo Scientific, USA). RFLP profile obtained with each enzyme was analysed and compared with the data of Esteve-Zarzoso et al. (1999), in order to identify at specie level.

RESULTS AND DISCUSSIONS

Fourteen strains have been isolated during the winemaking process (2017 and 2018), from the different varieties of grape (5 yeast isolates), grape pomace (8 yeasts isolates) and residual biomass (1 yeast isolate) originated in Pietroasa vineyard (Table 1).

Table 1. Classical identification based on macroscopic and microscopic characteristics of yeasts isolates

Yeast code	Origine	Colony aspect (shape, colour, aspect of the margin and texture)	Microscopic observations
PS1	grape pomace - Tămăioasă Românească	Round, white to cream, smooth, butyrous	globose to ovoidal
PM1	grape - Fetească Neagră	Round, white to cream, smooth, butyrous	globose to ovoidal
PC1	grape pomace- Busuioacă de Bohotin	Round, white to cream, smooth, butyrous	globose to ovoidal
PM2	grape pomace - Burgund Mare	Round, white to cream, smooth, butyrous	globose to ovoidal
PM3	grape pomace - Burgund Mare	Cream with red-brown from bottom, convex; smooth, opaque.	ovoid to ellipsoidal
PS2	residual biomass- Tămăioasă Românească	Round, white to cream, smooth, butyrous	globose to ovoidal
PM4	grape pomace - Fetească Neagră	Round, white to cream, smooth, butyrous	globose to ovoidal
PC2	grape pomace- Busuioacă de Bohotin	Cream with red-brown from bottom, convex; smooth, opaque.	ovoid to ellipsoidal
PC3	grape pomace- Busuioacă de Bohotin	Round, white to cream, smooth, butyrous	globose to ovoidal
PC4	grape pomace - Riesling Italian	Cream with red-brown from bottom, convex; smooth, opaque.	ovoid to ellipsoidal
PC5	grape - Busuioacă de Bohotin	Irregular, white to cream, smooth, butyrous to membranous	spheroidal to ellipsoidal
PM5	grape	Irregular, white to cream, smooth, butyrous	spheroidal to ellipsoidal
PC6	grape - Riesling Italian	Round, white to cream, smooth, butyrous	globose to ovoidal
PC7	grape	Round, white to cream, smooth, butyrous	globose to ovoidal

Initially, the strain identification of the yeast isolates was carried out on the basis of their microscopic and macroscopic observations (Table 1). 9 yeasts isolates showed round, white to cream, smooth, butyrous colonies and globose to ovoidal cells. Three yeasts isolates

were grouped by cream with red-brown colour on the bottom, convex; smooth, opaque aspects with ovoid to ellipsoidal cell morphology. Spheroidal to ellipsoidal cells were observed in two other yeasts isolates with irregular, white to cream, smooth, butyrous to membranous colonial aspects.

The identification of yeast isolates on specie level was performed by molecular methods. The internal transcribed spacer (ITS) region consisting of the 5.8S rRNA gene and two variable flanking is considered genetic markers (White et al., 1990). Polymerase chain reaction (PCR) methods based on this targeted region are developed to identify and differentiate between fungi species (Esteve-Zarzoso et al., 1999; Sabate et al., 2002; Prakitchaiwattana et al., 2004; Baffi et al., 2011; Zott et al., 2010). In our study, based on the amplification of the 5.8S-ITS region using primers ITS1 and ITS4, the amplicons were estimated to be approximately 400 bp (for 3 yeasts isolates), 800 bp (for 2 yeasts isolates) and 880 bp (for 9 yeasts isolates) (Table 2). Esteve-Zarzoso et al (1999) developed a PCR-RFLP method of the 5.8S-ITS region to discriminate 132 yeast species belonging to 25 genera using a combination of three endonucleease such as *Cfo*l, *Hae*lll and *Hinf*l. The PCR products were digested separately with three different restriction endonucleases, *Hha*I, *Hae*III and *Hinf*I and the restriction fragments obtained are presented in the Figure 1. Digestion with each restriction enzyme yielded three distinct restriction profiles (Table 2). The isolates were grouped according to distinct restriction patterns: nine yeasts isolates generated a restriction pattern corresponding to *Saccharomyces cerevisiae*, three yeasts isolates corresponding to *Metschnikowia pulcherrima* and two yeasts isolates corresponding to *Dekkera anomala* (Tabel 2).

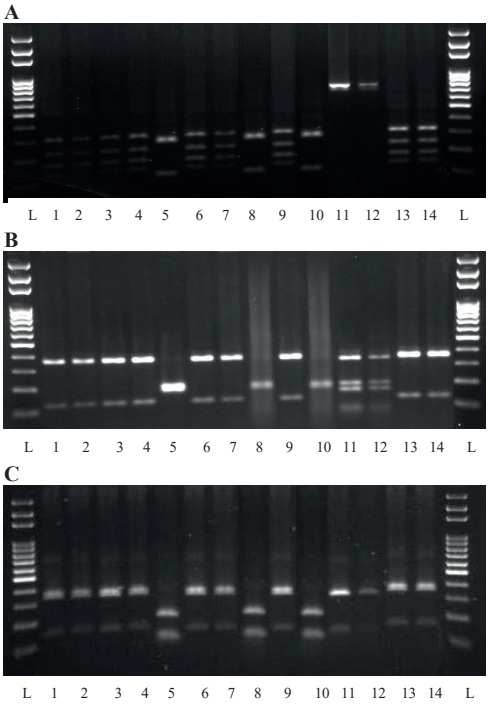

Figure 1. The restriction fragments of the PCR-ITS products of yeast isolates with *Hae*III (**A**); *Hinf*I (**B**); *Hha*I (**C**)
1- PS1; **2**- PM1; **3**- PC1; **4**- PM2; **5**- PM3; **6**- PS2; **7**- PM4; **8**- PC2; **9**- PC3; **10**- PC4; **11**- PC5; **12**- PM5; **13**- PC6; **14**- PC7

Table 2. Identification of yeasts isolates by RFLP analysis of 5.8S-ITS region

Yeast species	**Yeast isolate**	**PCR-ITS (pb)**	**Restriction fragments (bp)**		
			***Hae*III**	***Hinf*I**	***Hha*I**
Metschnikowia pulcherrima	PC6; PC2; PC4	400	280 + 120	200	210 + 110
Dekkera anomala	PC4; PM5	800	800	350+200+170+80	340+130
Saccharomyces cerevisiae	PS1; PM1; PC1; PM3; PS2; PM4; PC3; PC6; PC7	880	320+230+180+150	370+ 120	380+ 360+140

Saccharomyces cerevisiae, the main yeast involved in alcoholic fermentation, was detected on grapes and grape pomace. For some authors it is even undetectable on grapes (Combina et al., 2005) or present in a very low proportion of about 1-10 CFU.mL^{-1} (Prakitchaiwattana et al., 2004; Renouf et al. 2007) or its presence increased on spoilage grapes.

Metschnikowia is considered as a component of non- *Saccharomyces* yeasts in mature grape berries and during the first stages of grape

musts (Pretorius, 2000; Fleet et al., 2002; Prakitchaiwattana et al., 2004; Combina et al., 2005). *Metschnikowia* strains have been investigated as biocontrol agent, against filamentous fungi, yeasts, bacteria and for the high enzymatic activities to release aromatic precursors from grapes which enhance aromatic profiles of wines (Sipiczki, 2006; Csutak et al., 2013; Oro et al., 2014; Morata et al., 2019).

Among non-*Saccharomyces* yeast, two isolates have been identified as *Dekkera anomala*. Yeasts species belonging to the genus *Dekkera* (anamorph *Brettanomyces*) are associated with the off-flavours, due to the production of ethyl phenols, which lead to spoilage of wine (Pitt and Hocking, 2009; Oelofse et al., 2008; Berbegal et al., 2018). *Dekkera anomala* was detected as yeast spoilage of spoil beer, cider and soft drinks (Gray et al., 2011), but is not linkage to wine spoilage (Loureiro and Malfeito-Ferreira, 2003). For this reason, future investigations will be performed on these yeast isolates focussed on their ability to wine spoilage.

CONCLUSIONS

In the present study, 14 yeasts colonies were isolated, purified and grouped on the basis of appearance of colony and cell morphology. RFLP analysis of the ITS-5.8S region was completed the identification of yeast isolates at the species level: *Saccharomyces cerevisiae* (9 yeasts of all total isolates), followed by *Metschnikowia pulcherrima* (three of the total isolates) and *Dekkera anomala* (two of the total isolates). Future researches will lie on the use of indigenous *Saccharomyces cerevisiae* isolates mixed with *Metschnikowia pulcherrima* isolates and others non-*Saccharmomyces* as starter cultures to obtain and promote new wines with lower alcoholic degree and strong indigenous aromatic profiles. Also, the use of the *Saccharomyces* and non-*Saccharomyces* strains as biomass for other biotechnological application will be investigated (e.g. as potential feed source).

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