

THE USE OF PCR-RFLP TECHNIQUE FOR THE ANALYSIS OF YEAST CONTAMINANTS IN WINEMAKING

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Abstract: During the production of wine may occur infections of microbial, which can negatively affects the quality and characteristics sensory of product. In order to control the quality of given product, molecular techniques are used. They allow to quick and accurate identify of spoilage microorganism. PCR-RFLP method is very useful for study of the yeasts. It is based on amplification of rDNA gene repeat unit, which includes 5,8 rRNA gene and two non-coding internal transcribed spacers ITS1 and ITS2. The PCR products are then subjected to restriction enzyme digestion and electrophoretic separation. During experiments used *HaeIII* enzyme was also used and identification of yeast strains was made by comparing the length of restriction patterns in agarose gel. This allowed for quick and easy classification of the studied microorganisms. Distinguish between strains of the same species with a single restriction enzyme, proved to be virtually impossible, because of the similar length of the restriction fragments.

Keywords: 5.8-ITS region, microbiological contamination, PCR-RFLP, yeasts

INTRODUCTION

During the productions of wine may occur infections of microbial that negatively influence on the quality and stability of the finished products. The sources of microbial contamination are microorganisms derived from fruits, appearing during alcoholic fermentation and the aging of wine. The uncontrolled growth of microorganisms in the three phases, can change the chemical composition of wine and negatively influence its flavor and aroma (Satora and Tuszyński, 2004). Contaminant microflora may also be present on the surface insufficiently disinfected tanks and equipments (Wzorek and Pogorzelski, 1995).

Yeast that contaminate of wine belong to the know wild yeasts: *Rhodotorula*, *Aureobasidium*, *Hanseniaspora* (*Kloeckera*), *Candida* or *Cryptococcus*. They are killed during the fermentation, however, can produce a variety of compounds, which have a negative impact on the quality and sensory properties of wine, and the yeast of the *Saccharomyces* genus (Bonin, 2005). The yeasts of *Candida*, *Metschnikowia*, *Pichia* and *Hansenula* can create a film on the surface of the wine. The yeast *Zygosaccharomyces* may carry out the re-fermentation in storage of wine (Fugelsang and Edwards, 2007). A *Brettanomyces* species may contaminate the finished products of bottled and stored in cellars (Satora and Tuszyński, 2004).

Therefore, it is important to quickly identify the different species of microorganisms during the winemaking process and define their positive or negative role. For this purpose, a molecular techniques are used, for example PCR-RFLP. The RFLP method is to analyze 5.8S rRNA gene and internal non-coding regions (ITS1 i ITS2). Usually the products of PCR are digested by three different restriction enzymes: *HaeIII*, *HinfI* and *CfoI*. Using this method, 33 species of yeast in wine were identified (Guillamón et al., 1998).

The aim of this study was the identification of yeast strains from the collection of pure cultures of the Department of Fermentation Technology and Technical Microbiology UR,

using PCR-RFLP method and restriction enzyme *HaeIII*. These strains can be a potential source of contamination of wine.

MATERIALS AND METHODS

The study were used microorganisms derived from the collection of pure cultures of Department of Fermentation Technology and Technical Microbiology UR (*Candida stellata* CBS 157, *Hanseniaspora uvarum* DSM2768, *Metschnikowia pulcherrima* DSM 70321, *Saccharomyces bayanus* DSM 3774, *S. cerevisiae* var. *ellipsoideus*, *S. carlsbengensis*, *Rhodotorula graminis*, *R. mucilaginosa*, *Pichia anomala* CBS 5759, *S. paradoxus* CBS 7302, *Schizosaccharomyces pombe* var. *pombe* DSM 70576, *C. vini* DSM 70187, *Dekkera bruxellensis* CBS 3429 oraz *Zygosacchomyces bailii* CBS 749). All yeasts were grown on medium Sabouraud Dextrose with chloramphenicol LAB-AGAR (24 h, 28°C). From each strains of yeast was isolated DNA using kit for isolation and purification of yeast genomic DNA (Rapid Yeast Genomic DNA Extraction Kit, Bio Basic) according to the protocol. DNA of yeasts was stored at -20°C for further analysis. The reaction mixture for PCR containing: EconoTaq PLUS GREEN 2X Master Mix (LUCIGEN), primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and primer ITS4 (5'-TCCTCCGCTTATTGATA TGC-3') (OLIGO.PL), template DNA and DNA-ase free water. Carry out DNA amplification in an thermocycler (MultiGene Mini, Labnet Inter.). PCR conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturing at 94°C for 1 min, annealing at 55,5°C for 2 min and extension at 72°C for 2 min; and a final extension at 72°C for 10 min. PCR products (1µg/ml) were digested with the restriction endonuclease *HaeIII* (Promega) according to the supplier's instructions. The PCR products and their restriction fragments were separated on 2% agarose gels stained with ethidium bromide, with 1 x TAE buffer (100 V, for 1.10 h). After electrophoresis, gels were visualized under UV light and photographed. Sizes were estimated by comparison against a DNA length standard (100 bp ladder, Promega).

RESULTS AND DISCUSSION

Analyzing the length of the amplified DNA fragments, we observed variations in the range of 400 bp to 1050 bp (Fig. 1). Between strains of *Candida* species were slight differences. After PCR, the ITS region of yeast *Candida stellata* had a length of 475 bp, while *C. vini* 465 bp. This fragment of DNA was slightly less than expected. Esteve-Zarzozo et al. (1999) obtained pattern of 500 bp. A similar fragment of DNA has *Dekkera bruxellensis* (485 bp). Microorganisms belong to *Saccharomyces* show a very comparable length of patterns: *S. bayanus* – 880 bp, *S. cerevisiae* var. *ellipsoideus* – 860 bp, *S. carlsbengensis* – 840 bp, and *S. paradoxus* – 880 bp (Fig. 1). On the basis of the electrophoretic separation of PCR products, distinguish these strains would be practically impossible. The differences between the *Rhodotorula graminis* (660 bp) and *R. mucilaginosa* (640 bp) were also low. A similar size was pattern of *Pichia anomala* (650 bp). The shortest fragment of after the amplification reaction had a *Metschnikowia pulcherrima* (400 bp). The yeasts, which had patterns of 750-800 bp, that contains 5,8S rRNA gene and parts of non-coding ITS1 and ITS2, were *Hanseniaspora uvarum* – 750 bp and *Zygosaccharomyces billi* – 790 bp. The longest amplicons 1050 bp had *Schizosaccharomyces pombe* var. *pombe*.

The analysis of the length of fragments after digestion with the restriction enzyme *HaeIII*, shows that not all of the amplified fragments of DNA yeast strains has a restriction site for the enzyme. *C. stellata*, *C. vini*, *S. pombe* var. *pombe* and *H. uvarum* do not digested and length of their amplicons were unchanged (Fig. 2).

Strains of *Saccharomyces* species: *S. cerevisiae* var. *ellipsoideus* and *S. carlsbengensis* had a similar distribution of patterns. The homology occurred in fragments of length of 340,

230 i 150 bp. They differ only a fragment with a length of 120 bp which was in *S. paradoxus*. Probably, the similarity between these yeast results from the presence of the same sequence recognized by the enzyme *Hae*III. According to the model given by the Esteve-Zarzoso et al. (1999), *S. carlsbergensis* strains should have an additional pattern of 150 bp, and *S. paradoxus* in the fragment of 180 bp. The fourth strain of *Saccharomyces* species, *S. bayanus*, shows similar patterns to the previously described microorganisms (145 bp and 210 bp), but has also a larger fragment of DNA – 490 bp. Other investigators have not described of patterns in this strain of yeast, and this can facilitate its identify.

Differences between strains of *Rhodotorula* were slight. Their amplicons were larger than given in the literature (Esteve-Zarzoso et al., 1999). Both strains of *Rhodotorula* have the fragments of approximately 300 bp. Additional *R. graminis* had a pattern of length 480 bp, and *R. mucilaginosa* had a pattern of length 500 bp. Analysis of electrophoresis of *M. pulcherrima* revealed two patterns of length 280 and 100 bp. *Hae*III restriction enzyme cut the DNA of *P. anomala* on two amplicons with a length of approximately 600 bp and 50 bp. The latter two strains of yeast have been digested similarly as in publication of Esteve-Zarzoso et al. (1999). *D. bruxellensis* had fragments of 375 and 95 bp, and *Z. bailii* has been degraded to parts 690 and 90 bp.

PCR-RFLP method turned out to be useful for quicker and easier qualifying of all species enclosed in this article. This method is characterized by high repeatability (Esteve-Zarzoso et al., 1999). It can be used for identifying species of yeast that occur during the fermentation of grape in wine production. Also it gives information, especially about the initial phase of fermentation, such as the quantity and quality of yeasts, and the dynamics of yeast population. This can affect the organoleptic properties of the final product (Granchi et al., 1999). PCR-RFLP is based on amplification of rDNA, which contains the 5,8S rRNA gene and two non-coding internal transcribed spacers ITS1 and ITS2. ITS regions show a much greater variation than rDNA genes encoding and these sequences are looking for genetic variation between species of yeast (Guillamón et al., 1997).

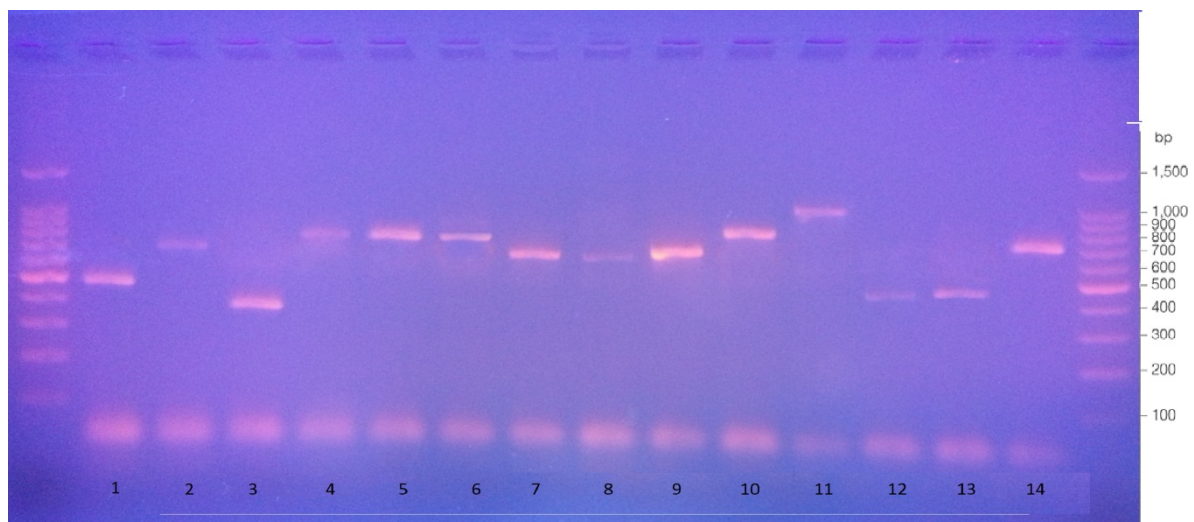


Fig. 1. The length of DNA fragments after PCR. Respectively: 1-*Candida stellata*, 2-*Hanseniaspora uvarum*, 3-*Metschnikowia pulcherrima*, 4-*Saccharomyces bayanus*, 5-*S. cerevisiae* var. *ellipsoideus*, 6-*S. carlsbergensis*, 7-*Rhodotorula graminis*, 8-*R. mucilaginosa*, 9-*Pichia anomala*, 10-*S. paradoxus*, 11-*Schizosaccharomyces pombe* var. *pomb*, 12-*C. vini*, 13-*Dekkera bruxellensis*, 14-*Zygosacchomyces bailii*

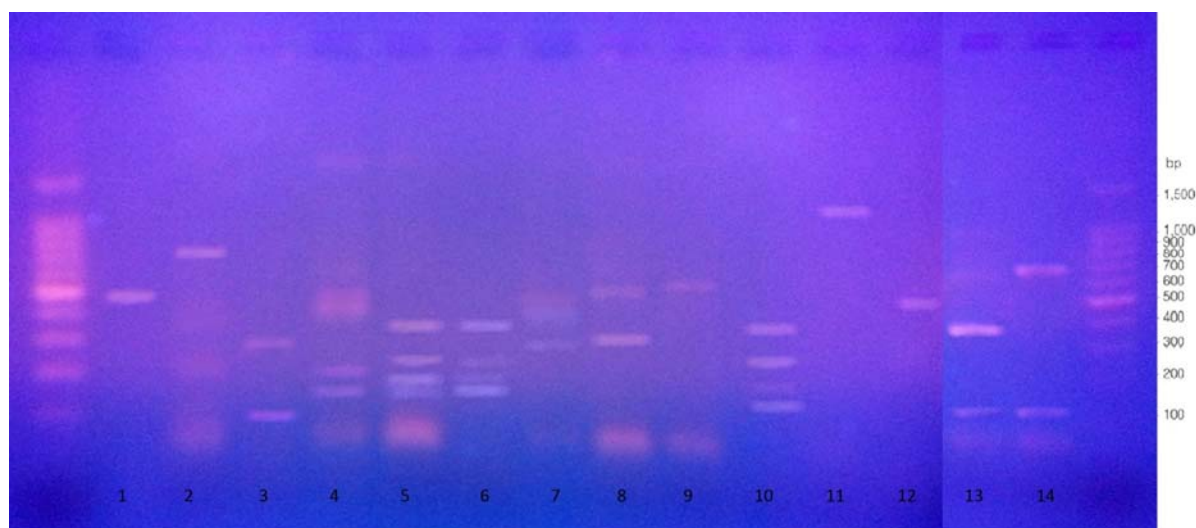


Fig. 2. The fragment length after restriction enzyme digestion *Hae*III. Respectively: 1-*Candida stellata*, 2-*Hanseniospora uvarum*, 3-*Metschnikowia pulcherrima*, 4-*Saccharomyces bayanus*, 5-*S. cerevisiae* var. *ellipsoideus*, 6-*S. carlsbengensis*, 7-*Rhodotorula graminis*, 8-*R. mucilaginosa*, 9-*Pichia anomala*, 10-*S. paradoxus*, 11- *Schizosaccharomyces pombe* var. *pombe*, 12-*C. vini*, 13-*Dekkera bruxellensis*. 14-*Zygosacchromyces bailii*

CONCLUSIONS

1. The PCR-RFLP method allows rapid and easy identification and classification of the different species of yeast.
2. Distinguishing between strains of yeast within the same species using only one restriction enzyme is practically impossible.
3. Strains of *C. stellata*, *C. vini*, *S. pombe* var. *pombe* and *H. uvarum* not have a restriction site for the enzyme *Hae*III.

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