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COMPARISON OF THE YEAST MICROBIOTA OF DIFFERENT VARIETIES OF COOL-CLIMATE GRAPES BY PCR-RAPD

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ABSTRACT

The yeast microbiota occurring on different varieties of grapes grown in cool-climate is not completely researched. Therefore, its identification is important to research. On the other hand, yeasts occurring in these fruits can be potentially used as starter cultures to obtain particularly demanded features in the production of wine. In addition, rapid methods for yeast identification allow to eliminate the contamination with pathogenic yeasts, which could cause the loss of wine production. The aim of the study was to isolate and identify the yeasts occurring on the surface of the different varieties of white and red grapes, grown in cool-climate of Poland. Also, the aim was to compare the qualitative and quantitative composition of yeasts on the tested grapes. The 84 cultures of yeasts were isolated, that were initially macroscopic and microscopic analyzed and the purity of cultures was rated on the WL medium. Identification of yeasts by PCR-RAPD was carried using the M13 primer. In the PCR-RFLP method ITS1 and ITS4 primers, as well as restriction enzymes *HhaI*, *HinfI*, *HaeIII*, were used. Preliminary identification of yeasts by standard methods produced results very different from the results obtained by molecular methods. Among the isolated microorganisms yeasts were dominating, but bacteria and molds were also present. Using the PCR-RAPD method most strains of yeasts were identified. Yeast microflora of different varieties of white and red grapes was very similar as the same species of yeasts were identified. Yeasts of the genus *Saccharomyces* were present in all varieties of grapes. The *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, *Metschnikowia pulcherrima*, *Rhodotorula minuta*, *Pichia kluyveri*, *Hanseniaspora uvarum* and *Rhodotorula mucilaginosa* were identified by PCR-RAPD. 4 of the 33 tested strains of yeasts were identified by PCR-RFLP. By PCR-RAPD only *Hanseniaspora uvarum* was identified. The quantity and quality of microorganisms living on the surface of grape fruits is very important for the process of winemaking. Yeasts influence the course of alcoholic fermentation, the flavor, aroma, and thus the quality of the produced wine. To a large extent their presence depends on the condition of the surface of the fruit. Many researchers reported significant differences between yeast microflora in grapes of Mediterranean and cool-climate vineyards. As they are expected to affect the final wine properties precise researching of the microflora of cool-climate grapes may lead to the isolation of new species of yeasts and thus the wines with unique characteristics can be obtained.

Keywords: grape; yeast; PCR-RAPD

INTRODUCTION

The grapes on the surface have a very rich microflora, including yeasts, bacteria and molds. Yeasts, on the surface of matured grapes are from 10^3 to 10^6 cfu/g. Yeasts of the genus *Hanseniaspora/Kloeckera* constitute 50-75% of the total yeast population. Other types of yeasts occurring in grapes are: *Aureobasidium*, *Brettanomyces*, *Bulleromyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Issatchenka*, *Kluyveromyces*, *Lipomyces*, *Metschnikowia*, *Pichia*, *Rhodosporidium*, *Rhodotorula*, *Saccharomyces*, *Sporidiobolus*, *Sporobolomyces*, *Torulaspora*, *Yarrowia*, *Zygoascus*, *Zygosaccharomyces* **(Fleet et al., 2002; Renouf et al., 2007)**.

Many genera and species of yeasts during the wine production were found. The low pH and high sugar content quickly produce anaerobic conditions while the presence of phenolic compounds creates an ideal environment for yeast growth. Metabolic activity of microorganisms has a great influence on the composition of wine, and therefore its taste and aroma properties **(Gil et al., 1996; Lema et al., 1996; Romano et al., 2003; Fleet, 2003)**. In fact, the types of wine could depend on the specific metabolites of yeasts. Microorganisms that affect the taste of the wine can be derived from a vineyard or be carried by insect vectors of fruit flies, bees and wasps **(Fleet et al., 2002)**. The presence of different types of yeasts depends on regional and climate factors, grape varieties, atmospheric pressure, damage to grapes and vineyard practices **(Prakitchaiwattana et al., 2004)**.

Wine yeasts have specific metabolic characteristics that make it possible to produce wines of exceptional bouquet and interesting organoleptic properties. Small vineyards choose the production of wines with a unique taste, corresponding to the region and consumers. In such vineyards the spontaneous fermentation is used to produce wine. Yeast strains used for the production of wine should be characterized by a period of rapid adaptation to the environment of the must, which leads to rapid

fermentation. During fermentation, the yeasts should be stable and reproduce. In addition, the by-products of fermentation produced by yeasts should affect the aroma and taste of the wine. Moreover, the yeasts should be characterized: production of a specific quantity of ethanol, the ability to the spontaneous flocculation and sedimentation after the fermentation process, and the ability to metabolize malic acid, which is the main cause of the excessive acidity of grapes. On the other hand, the yeasts may contaminate the wine and cause their diseases. Yeasts of *Candida* and *Pichia* could lead to a significant reduction of alcoholic fermentation. This could result the organoleptic change of wines **(Ribereau-Gayon et al., 2006)**.

Microorganisms living at the surface of the grapes are an important element affecting the taste, aroma, and thus the quality of the finished wine. Their presence depends to a large extent on the condition of the fruit surface. Previous studies show that are differences between the species of microorganisms occurring on grapes in cool-climate and Mediterranean. Through evolution, new types of microorganisms were created that are dependent on the climatic conditions in which they live. Nowadays, the new test methods for the identification and analysis of the microflora have been established which are based on molecular biology techniques. Particularly noteworthy is PCR analysis, which has given rise to other methods. Identification methods are continually improved, which makes the obtained results reliable and fast. This is very important because different types and species of yeasts on the grapes of cool climate can identify. Such yeasts can be used to production of wines, as starter cultures about a particularly important characteristics. On the other hand, rapid methods of yeast identification allow to eliminate harmful or pathogenic microflora. The aim of this study was to isolate yeast microflora present on the surface of the different varieties of white and red grapes grown in Poland, as well as identification of yeasts by PCR-RAPD and RFLP-PCR methods.

MATERIAL AND METHODOLOGY

The experiments were performed on white grape varieties, i.e.: Seyval blanc, Hibernal, Johanniter, Jutrzenka, Solaris, as well as red grapes: Rondo, Regent, Cabernet cortis and Dornfelder, from vineyard Srebrna Góra (Krakow). All the grapes were mature, without mechanical and microbiological damages.

Grape samples from each variety (10 g), were placed in the sterile stomacher bags. For each of bags the 90 mL of sterile 0.85% NaCl were added. Samples were homogenized using a stomacher (Bad Mixer). From each sample decimal dilutions to 10^{-2} were performed. For the isolation of microorganisms the nutrient agar for total bacteria count (incubation at 32 °C for 24 h), DRBC agar for yeast (28 °C for 2 – 3 days), and Czapek-Dox agar for molds (28 °C for 2 – 3 days) were used. After incubation, the colonies were counted by a colony counter.

Pure cultures of yeasts were grown at 28 °C and stored at 4 °C for further analysis. Analysis of yeasts was carried out macro- and microscopically. The ability to sporulation (acetate agar, 28 °C, 72 h), as well as the growth on WL medium were assessed.

The isolation of the genomic DNA of yeasts was started from the propagation of yeasts on the Sabouraud Agar medium (28 °C, 48 h) and Sabouraud Broth (10 mL, 28 °C, 24 h). Cultures were centrifuged four-times (5000 rpm for 5 min). The density of the yeasts was determined by the McFarland densitometer (DEN-1B BIOSAN).

From each yeast strain DNA was isolated using kit for isolation and purification of yeasts genomic DNA (Genomie Mini AX Yeast Spin, A&A Biotechnology) according to the protocol. The reaction mixture (50 μL) for PCR containing: One Taq Standard Buffer (BioLabs), dNTPs (Gene DireX), M13 (5'GAGGGTGGCGGTTCT3'; Genomed) primer, One Taq Polymerase (BioLabs), DNA-ase free water (Sigma) and DNA template. DNA amplification was carried out in a thermocycler (MultiGene Mini, Labnet). PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturing at 95 °C for 1 min, annealing at 36 °C for 1 min and extension at 68 °C for 2 min; and a final extension at 68 °C for 7 min. PCR products was stored at -20 °C for further analysis. The PCR products were separated on 1,5% agarose gels stained (Lab Empire) with ethidium bromide (Sigma), with 1xTAE buffer. Separation was performed at a flow of 100 V for 70 min in the apparatus for electrophoresis (Labnet). After electrophoresis, gels were visualized under UV light and photographed (InGenius, IG-LHR). Sizes were estimated by comparison against a DNA length standard 100-1000 bp ladder (A&A Biotechnology).

The reaction mixture for PCR-RFLP containing: One Taq Standard Buffer, One Taq Polymerase, dNTPs, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') primer and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer (Genomed), DNA template and DNA-ase free water. PCR conditions were as follows: initial denaturation at 94 °C for 1 min; 30 cycles of denaturing at 94 °C for 30 s, annealing at 55.5 °C for 1 min and extension at 68 °C for 3 min; and a final extension at 68 °C for 5 min. PCR products (1 $\mu g.mL^{-1}$) were digested with the restriction endonucleases *HhaI*, *HinfI*, *HaeIII* (EURx) according to the supplier's instructions. Electrophoresis and its analysis was carried out as for PCR-RAPD.

Data obtained were expressed as the mean ± standard deviation (SD). A single-factor Analysis of Variance test (ANOVA) with a *post hoc* Tukey's test were applied to perform a statistical analysis. Distribution of normality was determined by Kołgmogorov-Smirnov test using the program InStat3.

RESULTS AND DISCUSSION

Determination of the amount of microorganisms is the primary method of microbiological analysis **(Walczak et al., 2013)**. Table 1 shows the amount of bacteria, yeasts and molds occurring in the surface of the grapes. The least number of microorganisms was found in Seyval blanc and Rondo grapes, the average for the Regent and Solaris grapes, and the largest for Hibernal and Dornfelder grapes. Numerical range varied between 345 cfu/g and 845 cfu/g. A significant impact on the populations of microorganisms have weather, climate, habitat and the varieties of grapes **(Ribereau-Gayon et al., 2006)**.

Table 1 Number of bacteria, yeasts and molds occurring in the tested varieties of grapes.

Vine varieties	Bacteria cfu/g grape	Molds cfu/g grape	Yeasts cfu/g grape
Seyval blanc	2 ±1 [a]	6 ±2 [a]	385 ±5 [a]
Solaris	16 ±2 [b,c]	43 ±3 [b,c]	615 ±6 [b,c]
Hibernal	31 ±4 [d,e]	112 ±8 [d,e]	729 ±9 [b,d,e]
Johanniter	9 ±1 [b,d,f,g]	69 ±3 [b,d,f,g]	440 ±6 [b,d,f,g]
Jutrzenka	10 ±2 [b,f,h]	92 ±3 [b,d,f,h]	412 ±4 [b,d,f,h]
Rondo	8,5 ±2 [a]	100 ±3 [a]	345 ±4 [a]
Regent	28 ±2 [b,c]	52 ±2 [b,c]	610 ±7 [b,c]
Cabernet cortis	36 ±4 [b,d,e]	29 ±2 [b,d,e]	625 ±6 [b,d,e]
Dornfelder	19 ±3 [b,d,f]	10 ±1 [b,d,f]	845 ±4 [b,d,f]

Note: Mean value ±standard deviation; n = 3; a - h – values in columns and denoted by different letters differ statistically significantly at p <0.05.

The amounts of yeasts in the varieties of white and red grapes were very similar. On the varieties of white grapes were from 385 to 729 cfu/g, and varieties of red grapes were from 345 to 606 cfu/g of yeasts. On the white and red grapes were much less bacteria and molds. Thus, for white grapes 14 cfu/g of bacteria and 64 cfu/g of molds were observed, whereas varieties of red grapes contained 23 cfu/g of bacteria and 48 cfu/g of molds. The quantitative composition of yeasts depends on many factors. The number of microorganisms is influenced by the variety of grapes, maturity and chemical composition of grapes, the climatic conditions and the treatments used. The grapes were harvested at full maturity, where there has been modification of the composition of organic compounds in berries. These changes take place at the final stage of grape ripening **(Ribereau-Gayon et al., 2006)**. The microflora of the vineyard has a large impact on the production of wines. Biofilm is readily formed on different surfaces. It may be on slippery stainless steel tanks and wooden barrels of rough, difficult to disinfect **(Joseph et al., 2007)**. The results of the quantitative analysis showed that the yeasts were the largest group of microorganisms present on the fruit surface.

Before identification of yeasts by molecular methods, it was important to obtain pure cultures of these microorganisms. The pure cultures allow to investigate the morphological, physiological and genetic characteristics of microorganisms. Using the classical methods were tested the purity of 84 isolated strains of yeasts. Such evaluation allows to select cultures before molecular studies.

By PCR-RAPD 84 strains of yeasts and by PCR-RFLP

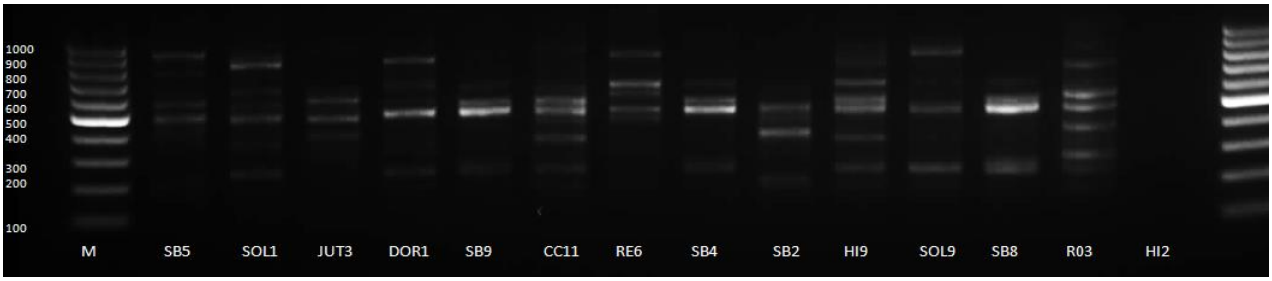

Figure 1 The separation of genetic material of yeasts in a 1.5% agarose gel by PCR-RAPD, M – is a marker, abbreviations of yeast strains described in table 2.

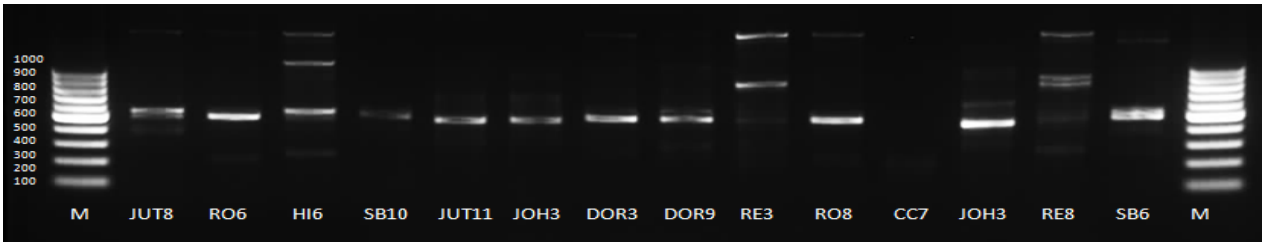

Figure 2 The separation of genetic material of yeasts in a 1.5% agarose gel by PCR-RAPD, M – is a marker, abbreviations of yeast strains described in Table 3.

33 strains of yeasts were identified. We were unable to replicate 7 samples that did not contain enough DNA. The RFLP technique allows to detect the restriction sites, the presence of which makes possible to distinguish the different strains of a single species. In both methods size of the PCR-amplified DNA region of the yeast strains was obtained which was compared with the image reference strains **(Łojkowska and Śledź, 2012)**. Figures 1 and 2 show examples of electrophoretic pattern of the genetic material of yeasts isolated from grapes after PCR-RAPD. Tables 2 and 3 show the size in bp of the PCR products the selected yeast species and their name, identified on the basis of literature **(Hierro et al., 2004; Cordero-Bueso et al., 2010)**.

The PCR-RFLP method allows to distinguish the yeast strains after treatment with the restriction enzymes. Because the genetic material of yeasts were degraded, by PCR-RFLP method only one strain of the yeast: *Hanseniaspora uvarum* was identified. Thus, 29 of strains of yeasts were not identified. Although the PCR-RFLP method gives reliable and precise results, it is more laborious and needs more genetic material than a PCR-RAPD.

The studied varieties of grapes grown in cool-climate, did not exhibit a significant diversity in the yeast population. Six species of yeasts were isolated, which belong to five genera, i.e.: *Rhodotorula, Saccharomyces, Pichia, Metschnikowia* and *Hanseniaspora*. Among the *Rhodotorula* genus two different species were determined: *Rh. mucilaginosa* and *Rh. minuta*. The grapes of Jutrzenka variety were characterized by the highest yeast species diversity as following species were identified: *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Rhodotorula mucilaginosa* and *Rhodotorula sp*. On the each varieties of grape has been identified an average of 3-4 types of yeasts.

As already stated, the surfaces of the grape berries were inhabited by a variety of microorganisms. Microorganisms have specific physiological characteristics which affect the final wine properties. The presence of these microorganisms is dependent on the stage of ripening of the grapes and availability of nutrients. A significant impact on the occurrence of microorganisms has the condition of fruit surface. Untouched skin of grapes may have traces of microcracks and dents, thereby changing the availability of nutrients. In this case, the population of yeasts with a low of fermentation activity is decreased.Such yeasts include *Candida sp*., *Hanseniaspora sp*., *Metschnikowia sp*. and *Pichia sp*. When the grape skin is significantly damaged, automatically the availability of sugars at high concentrations increases. This favors the growth of yeasts having a higher fermentation activity such as *Pichia sp*. and *Zygoascus hellenicus*. Well-fermenting sugars yeasts, such as *Saccharomyces cerevisiae* are rarely found on the undamaged grapes. Condition of fruit is a major factor influencing on ecology of microorganisms, their numbers and variety **(Barata et al., 2012)**.

Identified species of *Hanseniaspora*, *Metschnikowia* and *Pichia* were found on the undamaged surface of the grape **(Barata et al., 2012)**. The studied grapes did not have mechanical and microbiological defects. The *Saccharomyces* yeasts are rare in undamaged fruit. Number of the identified *Saccharomyces* could indicate the specific conditions in the vineyards **(Milanović et al., 2013)**. The *Saccharomyces* are highly desirable in the wine technology, because of the good ability to ferment sugars **(Ribereau-Gayon et al., 2006)**. This yeast can be used as a starter culture in the fermentation of Polish wines. Yeasts isolated from the vineyards of southern Europe could be

Table 2 Size in bp of the PCR product and identified yeasts.

Symbol of sample	Size of PCR product (bp)	Identified yeast species
SB5	500, 600, 950	*Rhodotorula mucilaginosa*
SOL1	250, 500, 600, 700, 900	*Saccharomyces cerevisiae*
JUT3	400, 500, 600	-
DOR1	250, 500, 900	*Metschnikowia pulcherrima*
SB9	250, 500, 600	*Saccharomyces cerevisiae*
CC11	250, 350, 500, 600	-
RE6	500, 700, 900	*Rhodotorula minuta*
SB4	250, 500, 600, 700	*Saccharomyces cerevisiae*
SB2	200, 400, 500	*Metschnikowia pulcherrima*
HI9	250, 350, 500, 600, 700	*Saccharomyces cerevisiae*
SOL9	250, 500, 850	*Saccharomyces cerevisiae*
SB8	600, 500, 250	*Saccharomyces cerevisiae*
RO3	300, 400, 500, 600, 800	-
HI2	-	absence of DNA

Table 3 Size in bp of the PCR product and identified yeasts.

Symbol of sample	Size of PCR product (bp)	Identified yeast species
JUT8	400, 500, 600	*Rhodotorula sp.*
RO6	300, 500	-
HI6	300, 600, 1000	*Pichia kluyveri*
SB10	600	*Metschnikowia pulcherrima*
JUT11	500, 700	*Hanseniaspora uvarum*
JOH3	500, 700, 800	*Rhodotorula minuta*
DOR3	500, 600, 900	*Saccharomyces cerevisiae*
DOR9	250, 500, 600, 900	*Saccharomyces cerevisiae*
RE3	500, 800	*Hanseniaspora uvarum*
RO8	150, 500, 1000	-
CC7	150	-
speciesJOH3	500, 650, 900	*Saccharomyces cerevisiae*
RE8	250, 500, 800, 900	*Saccharomyces cerevisiae*
SB6	500	*Metschnikowia pulcherrima*

different from those isolated in Poland. These differences are primarily affected by the total annual precipitation in each month, the average daily temperature, solar radiation and atmospheric fronts **(Gawęcki and Libudzisz, 2011)**.

Knowledge about the microflora of fruits from the Polish vineyards is still too small, so it is necessary to carry out series of studies using modern research methods. In the case of PCR-RAPD the identification of the DNA fragment length is problematic. This is associated with lack of complete database for analysis of microorganisms by PCR-RAPD. It is important to perform as much research on these organisms to be able to make up database, which could be widely available for all interested in the subject. The computer analysis allows to nominate fragments, which are located in the database **(Łojkowska and Śledź 2012)**. The second technique, PCR-RFLP analyzes the restriction fragment length polymorphism. It is used to identify closely related species of bacteria and fungi. Both methods are useful in the study of the identification and differentiation within a species of yeast. The RAPD-PCR method is faster and cheaper. Furthermore, the analysis requires a smaller amount of DNA template than for PCR-RFLP.

CONCLUSION

On different cool-climate varieties of grapes similar yeast species were identified. The *Saccharomyces* species on red and white grapes were identified. *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, *Metschnikowia pulcherrima*, *Rhodotorula minuta*, *Pichia kluyveri*, *Hanseniaspora uvarum* and *Rhodotorula mucilaginosa* were identified by PCR-RAPD methods, whereas PCR-RFLP allowed to identify *Hanseniaspora uvarum*. Among the isolated microorganisms yeasts were dominant, but bacteria and molds were also present. The quantity and quality of the yeasts on the surface of grapes are very important parameters for the production of wines. Therefore the obtained results require further investigation. Yeasts from different varieties of grapes, in different vineyards and within a few years need to be identified. Moreover, isolated yeasts shall be tested for use in the production of wine.

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