MICROBIOLOGICAL ASPECT AND LABORATORY DIAGNOSIS OF FUNGI OF THE GENUS *BRETTANOMYCES*

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Abstract. *Wine spoilage can be caused by different genera and types of wild yeast. One of the most harmful microorganisms is the yeast of the genus Brettanomyces/Dekkera. The timely detection and quantification of these microorganisms is essential to prevent wine spoilage. The detection of yeast in the raw wine materials was carried out by classical microbiological methods. The potential of microbiological for wine monitoring has been studied in order to optimize the analysis process. As a result of studies in raw wines produced in the microvinification section of the FTA's Department of Oenology and Chemistry, the advantages and disadvantages of the "gold standard" of microbiology, the cultural method for Brettanomyces/Dekkera yeast, were evaluated.*

Key words*: Brettanomyces, wine, cultivation, culture media.*

Introduction

The wine industry is traditionally considered the main and strategic sector of the economy of the Republic of Moldova, it is an important source of direct and indirect income for a significant part of the country's population. In the conditions of difficult economic situation in the country, the wineries of the Republic of Moldova are making significant efforts to reorient and diversify their exports. In this context, the main objective is the production of wines, competitive on foreign markets and also using modern analysis methods of wines quality. Wines are subject to chemical and microbiological spoilage and yeast of the genus *Brettanomyces* or its teleomorph *Dekkera* can be important causes of these problems, especially in red wines, with the formation of ethyl phenols and other compounds with an unpleasant odor, which are usually called phenolic, medicinal or animal odors of the farmyard (horse sweat, stable and skin) . Our study was aimed to determine of these yeasts in the red wines using the classical microbiological method, the "gold standard" of microbiology - microbial culture. *Brettanomyces / Dekkera* isolates were quantified by plating dry red wine samples on a selective culture medium.

I. Microbiological characteristics of the yeast of the genus *Brettanomyces*

1.1. Systematics and nomenclature

Currently, taxonomy includes five species of yeast within the genus *Brettanomyces: B. bruxellensis, B. anomalus, B. custersianus, B. naardenensis, and B. nanus.* They are characterized by asexual reproduction - a form of anamorph. For the first two species, there are also teleomorphs - sexually multiplicating strains: *Dekkera bruxellensis and Dekkera anomalus*.

Scientific classification

Domen: Procariotic
Kingdom: Fungi
Phylum: Ascomycota
Subphylum: Saccharomycotina
Class: Saccharomycetes
Order: Saccharomycetales
Family: Pichiaceae
Genus: Brettanomyces
Species *B. bruxellensis*

1.2. Morphobiological characteristics

Yeasts of the genus *Brettanomyces* have elliptical, cylindrical, elongated-oblong cells, often arrow-pointed at one end (fig.1). Some strains have elongated cells, often connected by 2 or more. Gram-positive, no sporulation. They are facultative anaerobes. In a favorable nutrient medium, especially in liquid media, most strains show a tendency to the formation of pseudomycelia [2]. Vacuoles and granulation are observed. Budding is bilateral and multiple [1]. Reproduction is very slow (the beginning of growth in a nutrient medium on the 5th day).

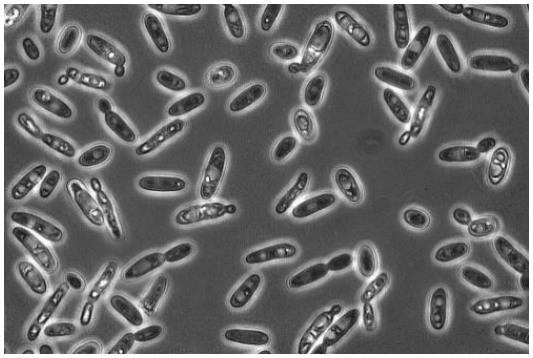


Figure 1. Yeasts of the genus *Brettanomyces* under phase-contrast microscopy

1.3. Cultural characteristics.

On liquid nutrient media, the yeast of the genus *Brettanomyces* forms a flocculent or viscous precipitate, and can form more or less thick surface films. On the surface of the wine, most strains form a thin, smooth, grayish-white film. Poorly fermented sugar. Many species are resistant to high concentrations of alcohol [2]. On dense nutrient media, whitish, domed colonies grow, often resembling drops of sour cream (fig.2), but there may be other descriptions, because their appearance depends on the age of the colony and on the nutrient medium on which they grew [5]. Usually they are moist and shiny; edges lobed, slightly expressed. They are able to thinn gelatin. In terms of needs for growth factors, all types of *Brettanomyces* are prototrophs, they are able to reproduce in the complete absence of vitamins. These yeasts can be re-infused for an indefinitely long time in an inorganic environment devoid of vitamins. But in such conditions, the rate of reproduction of yeast is very low and the introduction of individual growth factors from the outside into the medium has a positive effect on them. The optimum temperature for *Brettanomyces* yeast is fixed in the range from 25 to 32 °C. These yeast are thermophiles, at a temperature below 12 °C their growth in wine stops. In general, cell growth is possible in the range from 10 to 37 ° C. At the same time, some strains demonstrate a rapid decline at 35 °C in viability within 12 hours [4]. Oxygen. It was found that in a medium with an increased level of dissolved oxygen, *Brettanomyces* yeast can use both glucose and ethanol as a substrate, while synthesizing acetic acid [2]. Weak-aerobic conditions contribute to the greatest accumulation of biomass. Increased oxygen concentrations lead to a decrease in the rate of reproduction of yeast and an increase in the synthesis of acetic acid. In the anaerobic conditions, the *Brettanomyces* yeast synthesizes the largest amount of ethanol, and no significant amounts of acetic acid among the metabolic products [2].

Figure 2. Eleven day-culture of *Brettanomyces bruxellensis* on Sabouraud media

II. Materials and methods

Dry red wines made from two grades of Feteasca Neagra and Rara Neagra grapes in the section of microvinification of the Department of Enology and Chemistry of TUM were taken as the test material.

Methods for the detection and identification of yeast of the genus *Brettanomyces* can be divided into microbiological and molecular. Microbiological methods for detecting yeast are based on the cultivation of microorganisms on elective and differential diagnostic mediums with the subsequent determination of morphological, physiological and biochemical parameters.

2.1. Microscopic method: To assess the physiological state of the strain, its morphology is evaluated. Native and stained preparations are used to detect yeast cells. For native preparations are used phase-contrast microscop (fig.1). For this, a drop of the suspension of the test yeast culture is applied to the surface of a defatted laboratory glass slide with a microbiological loop, and if necessary, a drop of dye is added. Then a drop is carefully covered with a cover glass. The morphological features of each culture have to be studied: the shape and size of cells, the nature of reproduction, the presence of vacuoles and lipid inclusions [4]. Another method is immersion light microscopy: the smear - preparation is stained according to Gram: a drop of the test material is applied to the fat-free glass slide (culture suspension from the nutrient medium, supernatant, etc.) After drying on the open air, the smear has to be fixed in the burner flame and stained in 4 stages according to the instructions. As a result, gram-positive, round or oval cells, elongated and branched chains of pseudomycelia will be clearly visible in the field of view [5].

2.2. Bacteriological method: The following culture media were used to isolate *Brettanomyces*: Sabouraud 4% Glucose Agar , Malt Extract Agar Base and FastOrangeTM Yeast Agar. Test material was seeded on the surface of culture media in the Petri dishes. 1,0 ml of the analyzed sample was applied to agar, distributed evenly with a glass spatula, dried and incubated at the optimum temperature for 7 days. After incubation, phenotypic characterization and quantification of the colonies were carried out by direct counting, then microscopy was performed.

III. Results and discussions

On Sabouraud 4% Glucose Agar medium, two types of wine were cultured: Feteasca Neagra and Rara Neagra. In the first sample analyzed, 3 × 10 colonies of *Brettanomyces* yeast were detected. The grown colonies are white, irregular in shape with a rounded surface and a fringing. In the second sample, about 4 × 10 colonies of *Brettanomyces* yeast were found with a phenotype similar to the yeast in sample I: white, irregular in shape, with a glossy surface, rounded edges (fig.3).

Brettanomyces yeast also grew on Malt Extract Agar Base from Feteasca Neagra and Rara Neagra wines. 3 × 10 colonies were isolated from the first sample and 2 × 10 colonies from the second. The phenotypic characteristics of the isolated yeast were the same: white colonies of irregular shape, shiny surface and rounded edge (fig.4).

On Fast OrangeTM Yeast Agar. 3 × 10 colonies were found from the first analyzed sample, and 5 × 10 colonies from the second. The phenotypic characteristics of the yeast also corresponded to the genus *Brettanomyces / Dekkera* (fig.5).

Based on the results obtained, it was possible to detect that the agar Sabouraud 4% Glucose Agar is most suitable culture medium for the cultivation and microbiological detection wild yeast *Brettanomyces / Dekkera*. At the same time, it should be noted that the detection of wild *Brettanomyces / Dekkera* yeast by microbiological methods is a time-consuming process, it takes 10-14 days.

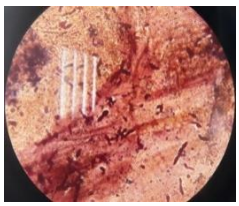
a)  b) 

Figure 3. a) colonies of *Brettanomyces* on the Sabouraud 4% Glucose Agar, b) microscopy, Gram stain

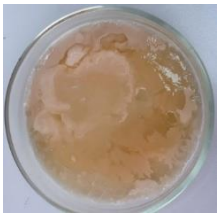
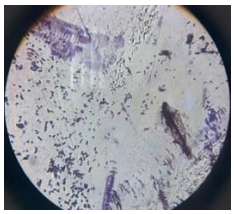
a)  b) 

Figure 4. a) colonies of *Brettanomyces* on the *Malt Extract Agar Base* b) microscopy, Gram stain

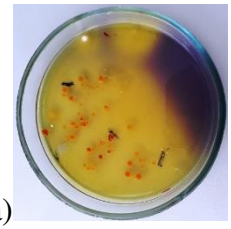
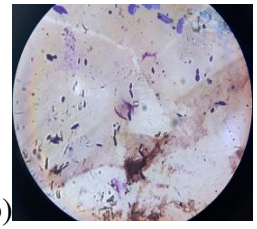
a)  b) 

Figure 5. a) colonies of *Brettanomyces* on the *Fast OrangeTM Yeast Agar*. b) microscopy, Gram stain

Conclusions

1. Wild *Brettanomyces/Dekkera* yeast is a very successful model for studying methods of detecting microorganisms in wine.
2. Saburo 4% Glucose Agar is the most suitable culture medium for the cultivation and microbiological detection of wild *Brettanomyces/Dekkera* yeast.
3. The detection of *Brettanomyces/Dekkera* by the culture method, along with the advantages associated with the availability of these methods, also has disadvantages: a long test time (from 7 days to 3 weeks). That won't allow us to react quickly and effectively in the case of necessary.

References

1. BARNETT, J. A., PAYNE, R. W., YARROW, D. In Yeast: characterization and identification. 2nd edition. Cambridge University Press (ed.). *Cambridge, New York,* Port Chester, Sydney. 1990
2. CIANNI, M., MACARELLI, F., FATICHENTI, F. Growth and fermentation behavior of Brettanomyces/Dekkera in winemaking. J *Sc of Food and Agricul*, 75 (4), pp. 489-495. 2003
3. PIKA WeihenstephanTM FastOrangeTM Yeast Agar – агар для определения дрожжей Disponibil: http://studydoc.ru/doc/2289670/agar-dlya-opredeleniya-drozhzhej
4. RODRIGUEZ, N., GONCALVES, G., PEREIRA-DA-SILVA, S. Development and use of a new medium to detect yeast of the genera Dekkera/Brettanomyces. J Appl Microbiol, 90, 2001. pp. 588-590.
5. The Brettanomyces Project. MYPG Media Agar http://brettanomycesproject.com/gallery/mypg-media-agar/