THE DETECTION OF SPOILAGE YEASTS IN RAW WINES PRODUCED AT UNIVERSITY MICRO-WINERY

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Abstract: Yeasts that are present during winemaking play an essential role during fermentation, but the growth of wild yeasts may also lead to wine spoilage, reduce wine quality and value and are bearing an immense damage potential. One of the aims of winemaking is to minimize potential for yeast and bacterial spoilage of wine. This review focuses on the yeast contamination of raw wines produced at Oenology department of the Technical University of Moldova and on methods for detection of wild yeasts.

Key words: microbiological contamination, spoilage yeasts, Saccharomyces cerevisiae var. diastaticus, PIKA Weihenstephan protocols, quantification of DNA.

Introduction

Fermentation yeasts usually grow together with wild yeasts: *Saccharomyces cerevisiae var. diastaticus (S. diastaticus), Saccharomyces ludwigii, Zygosaccharomyces bailii Brettanomyces*, *Pichia*, *Candida*, *Hansenula*… Some wild strains of *Saccharomyces cerevisiae* can produce excessive amounts of acetic acid, sulphur compounds, SO_2, urea and volatile substances which might be detrimental to wine quality and must be considered as spoilage microorganisms [6].

S. diastaticus is facultative anaerobic yeast and can be found in a variety of places: bottling lines, pipework, pitching yeast, the brewhouse, fermentation cellar [3], further break down the more complex carbohydrates like starches and dextrins [12]. Damage ability of *S. diastaticus* has been linked to the presence of STA genes, which encode for the exoenzyme glucoamylase, also referred to as amyloglucosidase [5]. This amylolytic activity can lead to hyperattenuation, and/or secondary fermentation which can cause excess carbon dioxide formation in bottles, cans or kegs. Wines that contain residual sugars after packaging may undergo refermentation and may cause swelling and explosion of the container.

Wild yeasts can cause wine spoilage during alcoholic fermentation, storage and after bottling [2]. These particular strains of yeasts tolerate the very hostile conditions including high ethanol concentration (even more than 15%), high residual sugar concentration (up to 85g/L), acidity and SO_2 (more than 300mg/L total) [*1*]. That's why is very important to establish the origin of wine spoilage yeasts, their routes of contamination, critical points of yeast infection, and of course, their control.

The main goal of this research was to study yeast contamination of raw wines produced at Oenology department of the Technical University of Moldova.

The objectives of our research were: to study raw wines using microbiological, cytological, genetic and physical-chemical methods of analysis; to screen the presence of the spoilage yeasts in wines, like *S. diastaticus*; to evaluate the efficiency of different methods in screening spoilage yeasts; to implement efficient methods for testing the presence of spoilage microorganisms in wines at Oenology department.

Materials and methods

Raw wines studied at Oenology department has been obtained using general technologies of winemaking [4]. We have carried out the analysis of six red and white wine samples produced from following grapes varieties: 1 – Cabernet Sauvignon; 2 – Saperavi; 3 – Chardonnay; 4 – Sauvignon Blanc; 5 – Feteasca Neagră; 6 – Merlot. These samples were a model object for studying the biological stability of wine and have been not treated.

For microbiological detection and identification of yeasts in wine samples have been used a enrichment culture medium PIKA Weihenstephan™ FastOrange™ Yeast Agar [9].

The biological microscope Motic B1 ADVANCED have been used for citological identification of yeasts in wine.

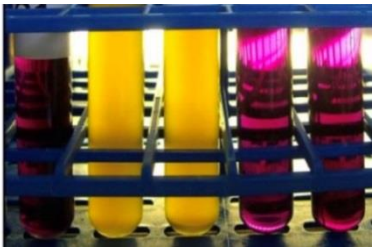
Isolation of DNA from yeasts have been done according PIKA Weihenstephan™ S0 Detection Kit H S. diastaticus protocol [10]. Material, equipment and consumables for DNA isolation were washing and lysis buffers, laboratory microcentrifuge EBA 21, thermoincubator CH-100, Vortex V-1 Plus, micropipettes, Eppendorf reaction tubes 1,5 ml.

Quantification of DNA have been performed using Jenway Genova Nano micro-volume spectrophotometer [13].

Results and discussion

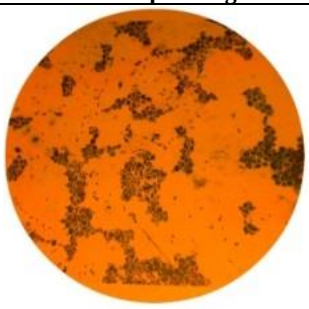
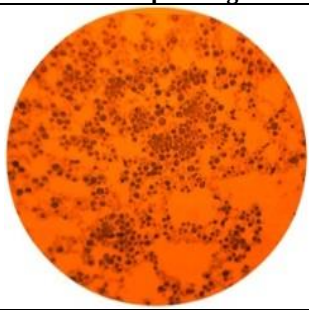
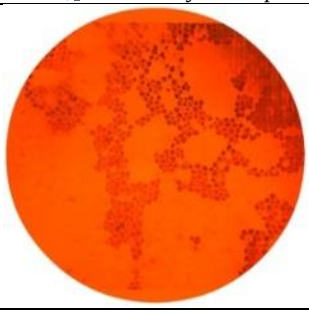
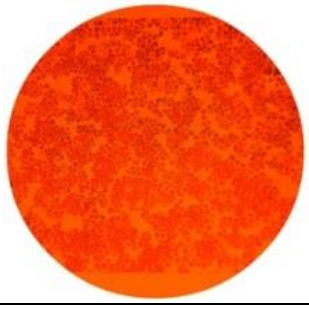
The presence of spoilage yeasts in raw wines produced at small winery has been determined using microbiological, cytological, genetic and physical-chemical methods of analysis.

At the first stage of our research was used microbiological method for detection of wild yeasts. Several microbiological techniques can be used for yeasts detection, such as $CuSO_4$ based media [12], starch agar plates, as well as growth in certain enrichment broths [11]. The choice of the enrichment medium always influences the growth rates of the wild and cultured yeast. We used PIKA Weihenstephan™, FastOrange™ Yeast Agar, a medium which was developed to detect contaminations by yeasts and molds. Yeasts and molds can be enriched with this medium, while bacteria are usually not able to grow. Besides turbidity and sediment formation, the presence of acid-producing yeasts is detected by a violet-to-yellow color change of the medium in all wine samples. The results of the our microbiological study showed that yeasts were present in all wine samples. It should be noted that **traditional plating** is semi-specific, requires healthy cells for presumptive yeast identification. Incubation and confirmation of results can take up to 14 days [8].



At the second phase of our research was done microscopic analysis of isolated wild yeasts from enrichment medium PIKA Weihenstephan™ FastOrange™ Yeast Agar and from wine samples. In all wine samples have been detected *S. diastaticus* yeasts (table 1).

Table 1. *Microscopic images of yeasts*

Nr.	Microscopic images	Nr.	Microscopic images
1		**4**	
	Saccharomyces cerevisiae var. diastaticus; Saccharomyces ellipsoideus		*Saccharomyces cerevisiae var. diastaticus; Saccharomycodes ludwigii*
2		**5**	
	Saccharomyces cerevisiae var. diastaticus; Saccharomyces oviformis		*Saccharomyces cerevisiae var. diastaticus; Saccharomyces ellipsoideus*
3		**6**	
	Saccharomyces cerevisiae var. diastaticus; Saccharomyces oviformis		*Saccharomyces cerevisiae var. diastaticus*

1 – Cabernet Sauvignon; 2 – Saperavi; 3 – Chardonnay; 4 – Sauvignon Blanc; 5 – Feteasca Neagră; 6 – Merlot.

Its morphology is comparable to the cultured wine yeasts like *Saccharomyces oviformis* or to the bottom fermenting brewer's yeast like *Saccharomyces pastorianus*. Cells are oval to egg-shaped, mostly single or in pairs. Microscopy analysis of yeasts requires 100,000 cells/mL for presumptive identification [8], but is not specific because ambiguous morphological characteristics of cells can easily cause false identification.

At the third stage of our experiment, we isolated yeasts DNA according to PIKA Weihenstephan™ S0 Detection Kit H S. diastaticus protocol [10]. We have transfered the wine samples containing yeasts into a sterile, 1.5-mL reaction tubes. Wine samples have been centrifuged several times at microcentrifuge EBA 21 for 3 minutes at 14,000 rpm. The liquid phase has been removed carefully from reaction tubes. The obtained pellet of yeast containing sample has been washed by adding 200 µL of washing buffer A. The samples have been resuspended briefly at Vortex V-1 Plus and have been centrifugated again. After that we have added 200 µL of lysis buffer B to the pellet and have resuspended the pellet by mixing briefly. The samples have been incubated at 80±5°C for 10 minutes in a thermoincubator CH-100 for lysis of cell wall of yeasts. The samples have been have been centrifuged again and 100 µL of the liquid phase containing the DNA have been transferred into a new reaction tubes for spectrophotometric analysis.

The quantification of DNA is a necessary procedure that allow to check the success of DNA isolation [10] and to show the presence of yeasts DNA in the samples. The results of the spectrofotometric DNA quantification is shown in the table 2.

Table 2. *DNA concentration, µg/ml*

Wine samples	Concentration,
Cabernet-Sauvignon	13,489
Saperavi	48,08
Chardonnay	5,52
Sauvignon-Blanc	88,39
Feteasca Neagră	81,85
Merlot	152,89

DNA concentration can be assessed using absorbance or optical density. The wavelength of *maximum absorption* for DNA is *260 nm [7]*. The absorbance at 260 nm is used to calculate the concentration of nucleic acids. The determination of absorbance has been done using a 1cm path length cuvette. The results of the spectrophotometric analysis show that all wine samples contain yeasts DNA. This kind of analysis is non-specific and do not indicate the type of yeast: cultured or wild.

The most reliable and fastest analysis today to get knowledge about the identity of a wild yeast is PCR testing, especially when testing for *S. diastaticus*, as there is no selective medium available for its detection. The sensitivity and specificity of PCR analysis is unreached by any other method – you can detect 1 spoiler cell within 400,000 yeast cells [12].

Conclusions

The presence of spoilage yeasts in raw wines produced at small winery has been determined using microbiological, cytological, genetic and physical-chemical methods of analysis. The obtained data bring new contribution in implementation the modern methods for monitoring yeast contamination at department of Oenology.

In all raw wine samples have been detected wild yeasts *S. diastaticus*, which were an object model in our research.

PIKA Weihenstephan™ FastOrange™ Yeast Agar is one of available enrichment medium for yeasts and molds and might be used for microbiological detection of wild yeasts. At the same time wild yeast detection, using traditional plating, generally can take a long time, as these species might grow slowly.

Analysis of contamination with *S. diastaticus* by microscopy might be done, but conventional analysis will not always detect it during the production process, as these wild yeasts looks just as cultured yeasts.

The advantages of spectrophotometry usage are that the process of obtaining resultis is rapid, is relatively inexpensive, and the obtained results are very reliable. The machinery is also easy to operate as it is automatable. The results of the spectrophotometric analysis cannot indicate the type of yeasts, but might be used to detect the presence of DNA yeasts in wine before bottling.

All presented methods for testing the presence of spoilage microorganisms in wines have been implemented successfully at Oenology department.

To test for *S. diastaticus*, we are recommending a combination of the following methods covering enrichment and specific detection of yeasts using PCR testing.

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