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# OENOLOGICAL CHARACTERIZATION OF SOME YEAST STRAINS ISOLATED FROM THE IAŞI VINEYARD ROMANIA

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## ABSTRACT

**This study investigated the oenological potential of indigenous*Saccharomyces* and non-*Saccharomyces* yeasts isolated from different stages of the natural must fermentation process. Screening of extracellular enzymatic activities was performed on agarized media in which the following substrates were added: arbutin, cellobiose, Tween 80, tributyrin, casein and citrus pectin, to highlight the activity of enzymes: ß-glucosidase, esterase, lipase, protease and pectinase. Among the 30 *Saccharomyces cerevisae* strains tested, 37% showed very low β-glucosidase activity, 100% showed esterase activity, 40% lipase activity, 90% protease activity and 53% pectinase activity.The non-*Saccharomyces* strain tested showed β-glucosidase, esteraseand protease activity. Tolerance to ethanol was assessed in YPD medium with ethanol concentrations of 5, 10 and 15% (v/v) by yeast culture development index.At 15% ethanol, development of all tested strains were inhibited. In the case of $SO_2$ tolerance, decrease in strains development was inversely correlated with the increase in potassium metabisulphite concentration, up to 200 mg/L. Only four *Saccharomyces* strains showed specific oenological characteristics and were selected to be tested in mixed and/or sequential cultures to obtain wines with improved sensory features.**

**Keywords***:* wineyeasts, *Saccharomyces*, non-*Saccharomyces*, extracellularenzymes, ethanol tolerance.

## INTRODUCTION

The use in winemaking of the starter cultures of *Saccharomyces cerevisiae* had the advantage of a controlled alcoholic fermentation process, but also the disadvantage of obtaining wines with deficiencies in terms of aromatic profile. Flavours are produced during the complex biochemical process of fermentation by extracellular enzymes secreted by yeasts. In the case of spontaneous fermentation, the range of extracellular enzymes is much larger and varied due to the participation of many yeast species both *Saccharomyces* and non-*Saccharomyces*. This explains why the wines obtained in natural alcoholic fermentation have a much more complex aromatic profile, compared to the wines obtained in monoculture with *Saccharomyces cerevisiae* starter yeast. The current practice of inhibiting the *non-Saccharomyces* yeast population which predominates at the beginning of alcoholic fermentation, by inoculation of *Saccharomyces* starter cultures, need to be reconsidered, due to the fact that research conducted by different authors revealed that some of these yeast strains showed superior oenological characteristics, with positive effects on the aromatic profile of wines.Soden et al. (2000) showed that a positive impact on the sensory

characteristics of wine is ensured by the involvement of non-*Saccharomyces* yeasts in the alcoholic fermentation, due to their potential to secrete extracellular enzymes (β-glucosidases, esterases and lipases). Thus, in the last years, the research focused on the study of extracellular enzymes of *Saccharomyces* and non-*Saccharomyces* yeasts, finding that the presence or absence of the enzymes influences the wine aromas (Strauss et al., 2001; Buzzini and Martini, 2002; Rodriguez et al., 2004; Ciani et al., 2006; Mendoza et al., 2007; Gaensly et al., 2015; López et al., 2015). The surveys carried out in recent years allowed the selection of new strains of wine yeast both *Saccharomyces*, but especially non-*Saccharomyces*, in order to obtain and use them in mixed cultures, to improve the flavours complex of thewine and toenhance regional identity of wines.Thus, the research opened a way to increase the sensory attributes of wines, in new winemaking technologies that involvesthe management of alcoholic fermentation in similar conditions to the natural process, but initiated with both *Saccharomyces* and non-*Saccharomyces*yeasts starter cultures. In this context, oenological characterization of indigenous *Saccharomyces* and non-*Saccharomyces* yeasts was initiated in order to identify and select performant strains in terms of extracellular enzyme activity, which by biochemical transformations of must compounds ensure the variety and the complexity of the desired sensory characteristics of wines. Also, by testing them in mixed and/or sequential cultures, important steps will be taken in increasing the quality and tipicity of wines.

**MATERIALS AND METHODS**

The evaluation of the oenological characteristics of the indigenous yeast strains was focused on the determination of the extracellular enzymatic potential, the tolerance to ethanol and $SO_2$. In this context, 30 *Saccharomyces cerevisiae* strains and one non-*Saccharomyces* strain, respectively *Torulospora delbrueckii*, were studied.

Screening for extracellular activities of *Saccharomyces* and non-*Saccharomyces* yeasts was performed on agar YPD medium(containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar), inoculated with suspensions of $10^6$ cells/mL from yeast cultures reactivated 24 hours at 25°C.

β-glucosidase activitywas determined on agar media containing 6.7 g/L Yeast Nitrogen Base - YNB (Sigma-Aldrich, Germany), 5 g/L arbutin (Fluka, Switzerland), 20 g agar(VWR, Belgium),pH 5.5. After autoclaving were added to 100 mL medium: 2 mL of 1% ferric ammonium citrate solution (Rossi et al., 1994). Yeasts that hydrolyzed the substrate produce β-glucosidase and a brown halo appears around the colonies. The strains which developed on cellobiose agar (10 g/L cellobiose, pH5.5) and hydrolysed at least one other substrate (either arbutin or esculin) were positive toβ-glucosidase production (Gaensly et al., 2015). Esterase activitywas tested on medium with the following composition: 10 g/L peptone, 5 g/L NaCl, 0.1 g/L $CaCl_2 \cdot 2\ H_2O$, 10 g/L Tween 80, 20 g/L agar, pH 6.8 (Slifkin, 2000). Yeasts with esterase activity hydrolysed the substrate and an opaquehalo appeared around the colonies. The assay of the protease activity was performed on an agar medium with the following composition: 3 g/L yeast extract (Scharlau,Spain), 3 g/L malt extract (Merck, Germany), 5 g/L peptone, 10 g/L glucose(VWR, Belgium), 5 g/L NaCl(Chemical Company, Romania) and15 g agar (Comitini et al., 2011).Separately, was prepared a solution containing an equal volume of pasteurized skimmed milk (0.1% fat) and sterile distilled water.After sterilising the medium, both solutionswere homogenised and brought to 45°C.The medium was distributed in Petri dishes and then was inoculated with the yeast suspension. Proteolytic yeasts hydrolyzemilk casein and a clear halo appears around the inoculum. Lipase activitywas tested on tributyrin agar medium with the following composition: 5 g/L peptone, 3 g/L yeast extract, 10 g/L tributyrin(Fluka, Switzerland), 15

g/L agar, pH 6.0 (Buzzini and Martini, 2002). After incubating the plates for 48 hours at 28°C, the detection of lipase activity was performed by UV irradiation at 350 nm. In the case of yeasts with lipolytic activity, a fluorescent halo was observed. Highlighting the pectinase (polygalacturonase) activity was performed according to McKay (1990) with some modifications. The medium contains: 10 g/L of citrus pectin (Sigma-Aldrich, Germany), 6.8 g/L $KH_2PO_4$, 6.7 g/L YNB (Sigma-Aldrich, Germany), 6.7 g D-glucose (VWR, Belgium) and20 g/L agar.Yeast suspensions were inoculated on the surface of the agar medium. The plates were incubated for 3-5 days at 30°C and then flooded with a 10 g/L hexadecyltrimethy-lammonium bromide solution (Sigma-Aldrich, Germany). The development of a clear halo around the colonies indicates the pectinase activity.

$SO_2$ tolerance was performed on sterilized YPD medium with potassium metabisulphite in concentrations of 50, 100, 150 and 200 mg/L. Control was represented by the same medium without $K_2S_2O_5$addition. From the reactivated yeast cultures on YPD medium, were obtained suspensions of $10^4$cells/mL. After inoculation and incubation at 26°C for 48 hours, the development of yeasts (positive or negative) was examined compared to the control medium. Resistance degree of yeast to sulphur dioxide is reported as the maximum dose at which the yeast exhibits a significant growth. Evaluation of ethanol tolerance of indigenous yeast strains was performed on 5 mL of YPD medium with ethanol concentrations of 5, 10 and 15% (v/v).Each ethanol concentration was inoculated with 100 µL of a 24-hour culture. Yeast strain development was monitored for 72 hours by measuring the optical density of the cultures at 600 nm, using YPD medium as the blank sample (Analytik Jena Specord 200 plus UV-vis spectrophotometer).The control sample consisted in 5 mL YPD medium without ethanol inoculated with 100 µL of each yeast culture. Tolerance to ethanol was determined based on the growth index (GI), calculated according to the following formula proposed by Bevilacquaet al. (2012): GI% = (Abs sample/Abs control) × 100. The degree of inhibition was established as follows: GI<25% = very high degree of inhibition; 25 <GI<75% = partial inhibition; IG>75% increase similar to the control sample (without ethanol).The values of the growth index represent the average of three determinations, standard deviation being calculated (±).

## RESULTS AND DISCUSSIONS

The purpose of the research was to select indigenous yeast strains with superior oenological characteristics for use in the process of alcoholic fermentation in mixed and/or sequential cultures, in order to improve the aromatic profile of wines and to enhance their regional identity. Yeast strains were isolated in different stages of natural (spontaneous) alcoholic fermentation and are stored in the collection of microorganisms of Research and Development Station for Viticulture and Oenology Iaşi, Romania, on YPD medium, at 6°C.

The screening of extracellular enzyme activities was performed on agarized media in which specific substrates were added: arbutin, cellobiose, Tween 80, tributyrin, casein and citrus pectin, to highlight the enzymes β-glucosidase, esterase, lipase, protease and pectinase.Depending on the number of extracellular enzymes, 5 groups of tested yeasts were distinguished: three strains with five extracellular enzymes (group I); six strains with four extracellular enzymes (group II); 13 strains with three extracellular enzymes (group III); six strains with two extracellular enzymes (group IV) and one strain with a single enzyme (group V).The data presented in table 1 highlight the following aspects: all yeast strains tested show different extracellular enzymatic activities; β-glucosidase activity was not detected on the arbut in substrate, instead, on the cellobiose substrate 11 strains were positive; the esterase activity was positive in all strains, and the enzymatic activities of lipase, protease and pectinase were highlighted in 12 strains, 27 strains, respectively 16

strains. Another aspect regarding the screening of *Saccharomyces cerevisiae* yeasts emerged from the assessment of the intensity of extracellular enzyme activities (Figures 1 - 5).

Table 1. Extracellular enzymatic activity of the tested yeast strains

| No. | Yeast strain (code) | Extracellular enzymatic activity | | | | | |
|---|---|---|---|---|---|---|---|
| | | β-glucosidase | | Esterase | Lipase | Protease | Pectinase |
| | | arbutin | cellobiose | Tween 80 | tributyrin | casein | pectin |
| *Saccharomyces cerevisiae* | | | | | | | |
| 1 | 1 | - | - | + | - | - | - |
| 2 | 3 | - | + | ++ | + | + | ++ |
| 3 | 4-1 | - | - | + | + | ++ | - |
| 4 | 4-1-11 | - | + | ++ | + | +++ | - |
| 5 | 4-3 | - | + | ++ | + | + | ++ |
| 6 | 4-5 | - | - | + | - | + | +++ |
| 7 | 4-6 | - | + | + | + | + | ++++ |
| 8 | 4-7 | - | - | + | - | + | ++++ |
| 9 | 4-8 | - | - | + | - | + | +++ |
| 10 | 4-10 | - | + | ++ | + | - | +++ |
| 11 | 4-12 | - | - | + | - | + | +++ |
| 12 | 4-13 | - | - | + | - | +++ | + |
| 13 | 4-14 | - | - | ++ | - | + | - |
| 14 | 4-15 | - | + | ++ | - | + | +++ |
| 15 | 4-16 | - | - | ++ | + | ++ | - |
| 16 | 4-17 | - | + | + | - | ++ | - |
| 17 | 4-17-341 | - | - | + | + | +++ | - |
| 18 | 4-19 | - | - | ++ | - | ++ | - |
| 19 | 4-20 | - | - | + | - | ++ | - |
| 20 | 4-21 | - | - | + | + | + | - |
| 21 | 5-1 | - | + | + | - | + | + |
| 22 | 5-2 | - | - | + | - | + | + |
| 23 | 6-1 | - | + | + | - | + | +++ |
| 24 | 6-2 | - | + | + | + | + | + |
| 25 | 6-3 | - | + | ++ | + | +++ | - |
| 26 | 6-6 | - | - | ++ | - | +++ | - |
| 27 | 7-2 | - | - | ++ | - | + | ++ |
| 28 | 7-3 | - | - | ++ | - | - | ++ |
| 29 | 8-1 | - | - | ++ | - | +++ | - |
| 30 | 8-2 | - | - | ++ | + | +++ | - |
| *Torulospora delbrueckii* | | | | | | | |
| 1 | 10 | + | + | - | +++ | - | + |

Note:−no activity; + very low activity; ++ low activity; +++ high activity; ++++ very high activity.

The importance of β-glucosidase in winemaking is determined by its potential to release flavor compounds. In wine must, some secondary metabolites are free or bound, the latter can be hydrolysed under the action of yeast enzymes. The enzymes involved in the hydrolysis of flavor precursors are glycosidases, respectively β-glycosidase which releases monoterpenes from the glycosylated form (Maicas and Mateo, 2005). Volatile compounds released from glycoside complexes play an important role in obtaining varietal aromas. Although some strains of *Saccharomyces cerevisiae* produce β-glucosidase, several studies have shown that some non-*Saccharomyces* yeasts have higher activity.The intensity of β-glucosidase activitywas identified in 11 strains of the 30 *Saccharomyces cerevisiae* yeasts tested, with a very low intensity (+) on the cellobiose substrate (figure 1).The difference was clear between the Petri dishes inoculated with the negative strains compared to the positive ones, for example strain 7-2 versus strain 4-10 (figure 1).The results obtained are in agreement with previous research. Rosi et al. (1994), Rodríguez et al. (2004) and Comitini et al. (2011), using arbutin as a substrate, among a high number of strains found β-glucosidase activity only for a single *Saccharomyces cerevisiae* strain.

The esterase activity was highlighted on agar medium added with Tween 80.Hydrolysis of the substrate by esterases led to the development of a visible opaque halo by precipitation of calcium ions. Among the *Saccharomyces cerevisiae* yeast strains, 16 showed very low activity (+)(e.g. strain 4-15) and 14 showed low activity (++) (e.g. strain 4-1-11) (figure 2).

Although lipase activity was found in yeasts, there are few references to its intensity in *Saccharomyces cerevisiae* strains. From the total of 30 strains tested, only in the case of 12 strains was determined a very low activity (+). The intensity of enzyme activity was assessed both by the presence of an opaque halo and by the appreciation of yeast development on the surface of agar media.The lack of activity was proven by the absence of inoculum development (figure 3).

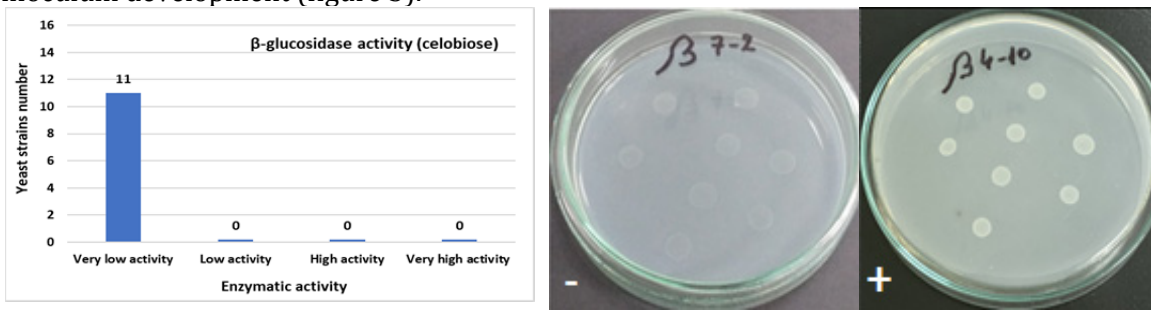


Figure 1. Number of yeast strains that showed β-glucosidase activity and the intensity of growth

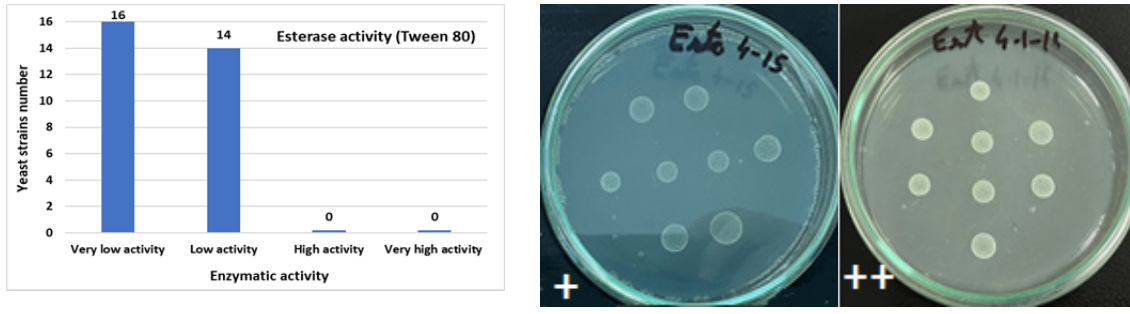


Figure 2. Number of yeast strains that showed esterase activity and intensity of the enzymatic activity

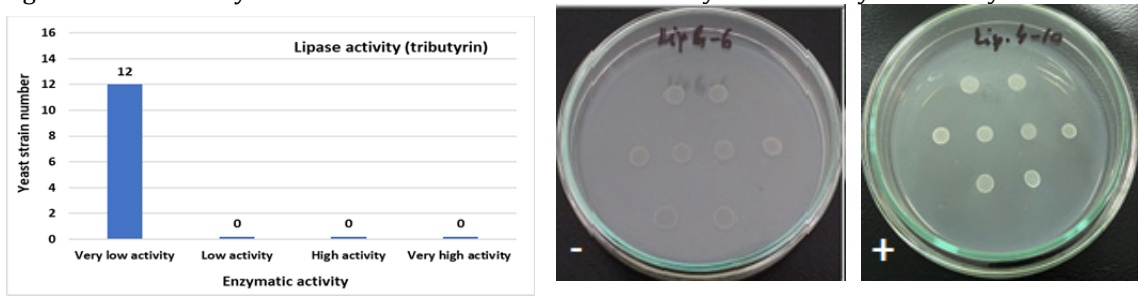


Figure 3. Number of yeast strains that showed lipase activity and intensity of the enzymatic activity

Lipase hydrolyses the long chains of triglycerides in the lipids present in the must and those resulting from the autolysis of yeasts. The enzymatic activity of lipase favours the extraction of colour from the skin of grapes and also the release of volatile compounds, such as ethyl esters and ethyl acetates that influence the aroma of wines.

Proteolytic activity has been studied for the stabilization and prevention of protein haze, where proteins are hydrolysed into amino acids and peptides. Protein haze is a problem in many white wines and the use of bentonite to stabilize it has disadvantages, both by losing wine volume and sensory properties (Maicas and Mateo, 2005). Information on the proteolytic activity of *Saccharomyces cerevisiae* yeasts is very scarce, but numerous in the case of *non-Saccharomyces*yeasts. The intensity of protease activity of the tested *Saccharomyces cerevisiae* strains was different on casein agarized medium (figure 4). Thus, 15 strains showed very low proteolytic activity (+), with a clear halo of 1 mm (strain 5-1), 5 strains showed low activity (++), with a clear halo of 3 mm and 7 strains with intense activity (+++) with a clear halo of 4-5 mm (strain 4-13).

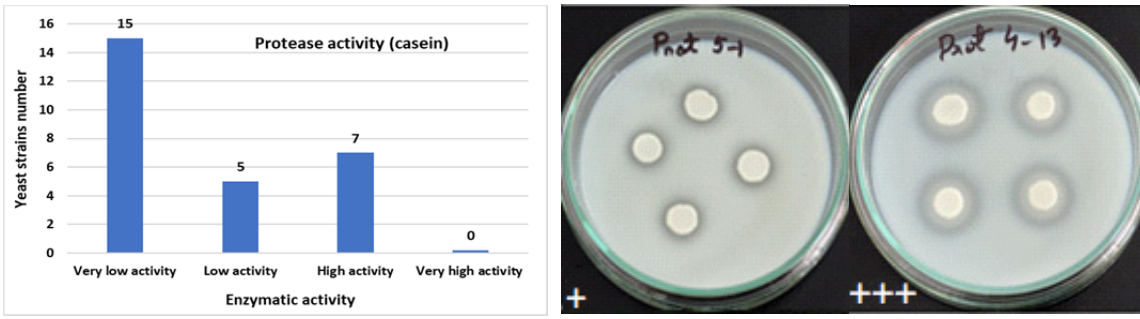


Figure 4. Number of yeast strains that showed protease activity and intensity of enzymatic activity

Among the pectinase enzymes group, this study determined the extracellular poly-galacturonase activity. Tested yeast strains showed variable intensity of polygalacturonase activity. The enzymatic intensity on an agarized medium was highlighted by a clear halo with different sizes from 1 to 5 mm.Depending on this parameter, the 16 positive strains were grouped as follows: 4 strains with very low activity (+), with a clear halo of 1 mm (strain 5.1), 4 strains with low activity (++), with a clear halo of 2 mm, 6 strains with intense activity (+++), with a clear halo of 4 mm and 2 strains with very intense activity (++++), with a halo of 5 mm (strain 4-7) (figure 5).

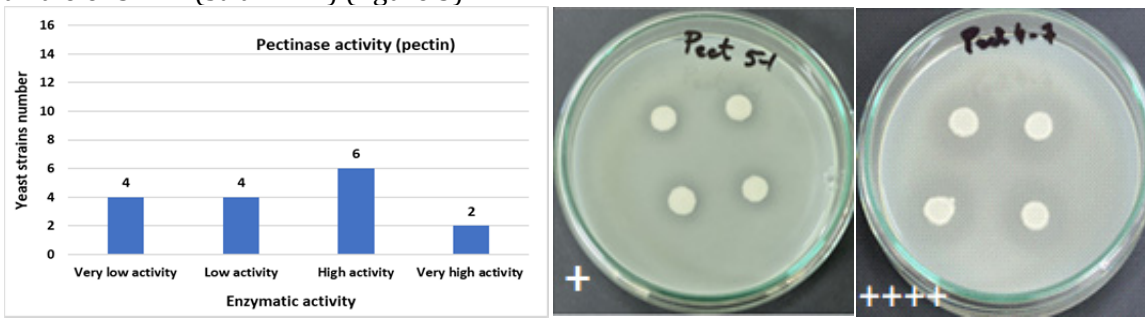


Figure 5. Number of yeast strains withpectinase activity and intensity of the enzymatic activity

Among extracellular enzymes, pectinases play an important role in oenology. These enzymes, mainly the depolymerizing ones, have a substrate specificity to cleave the oxide bonds 1-4 from the ends of the polygacturonic chain (exoenzymes) or from its middle (endoenzymes).By the action of these enzymes, the viscosity of the must decreases. The main wine yeast *Saccharomyces cerevisiae* has not been appreciated as a major producer of pectinase extracellular enzymes, although several strains have been shown to produce polygalacturonases (McKay, 1990).

Due to the small number of recent references on the extracellular enzymatic activity of *Saccharomyces cerevisiae* wine yeasts, the study has a double importance, in the way that the enzymatic potential of the wine yeasts was studied for the first time in Romania and, also, the study contributes to updating and completing data on this issue at international level.

In current study was also assessed the intensity of the extracellular enzymes activity foranon-*Saccharomyces* strain*, Torulospora delbrueckii*.The non-*Saccharomyces* strain showed low β-glucosidase and pectinase activity (+),no esterase orprotease activity, but the highest lipase activity (Table 1).Buzzini and Martini (2002) tested five*Torulospora delbrueckii*strains, of which only one strain showed esterase and lipase activity. Also, Rodriguez et al. (2004) tested four strains on arbutin medium, all strains being β-glucosidase negative. Comitini et al.(2011) tested nine *Torulospora delbrueckii* strains of which only two showed β-glucosidase activity and five strains showed esterase activity.

From a total of 30 *Saccharomyces* yeast strains whose enzymatic potential was tested, 14 yeast strains were selected from the groups with 3, 4 and 5 secreted extracellular enzymes, in order to test the tolerance to ethanol and $SO_2$.
Yeast tolerance to ethanol was assessed based on the values of the growth index (GI), the results obtained being presented in Table 2.

Table 2. Tolerance to ethanol and $SO_2$ of the tested *Saccharomyces cerevisiae* yeast strains

| Nr. | Strain code | Growth index (GI %) | | | $SO_2$ concentration | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 5% ethanol | 10% ethanol | 15% ethanol | Control | 50 mg/L $K_2S_2O_5$ | 100 mg/L $K_2S_2O_5$ | 150 mg/L $K_2S_2O_5$ | 200 mg/L $K_2S_2O_5$ |
| 1 | 3 | 92.0±1.2 | 77.9±0.2 | 0.0 | ++++ | +++ | +++ | ++ | - |
| 2 | 4-1-11 | 89.8±0.8 | 85.3±1.0 | 0.0 | ++++ | +++ | +++ | ++ | - |
| 3 | 4-3 | 87.9±1.3 | 83.3±0.5 | 0.0 | ++++ | +++ | +++ | ++ | - |
| 4 | 4-5 | 90.0±1.1 | 82.0±1.8 | 0.0 | ++++ | +++ | +++ | + | + |
| 5 | 4-6 | 86.9±1.4 | 86.0±1.0 | 0.0 | ++++ | +++ | +++ | + | - |
| 6 | 4-7 | 85.0±0.6 | 84.0±1.1 | 0.0 | ++++ | +++ | ++ | ++ | - |
| 7 | 4-8 | 90.0±1.0 | 86.0±1.4 | 0.0 | ++++ | +++ | +++ | + | - |
| 8 | 4-10 | 86.7±1.0 | 85.6±1.4 | 0.0 | ++++ | +++ | +++ | + | - |
| 9 | 4-12 | 89.1±1.2 | 79.4±0.9 | 0.0 | ++++ | +++ | ++ | ++ | + |
| 10 | 4-15 | 93.0±0.8 | 76.0±1.0 | 0.0 | ++++ | +++ | +++ | ++ | + |
| 11 | 5-2 | 72.0±1.6 | 48.9±1.2 | 0.0 | ++++ | +++ | +++ | + | - |
| 12 | 6-1 | 43.6±0.4 | 25.0±1.0 | 0.0 | ++++ | +++ | +++ | + | - |
| 13 | 6-3 | 91.9±1.1 | 75.3±1.0 | 0.0 | ++++ | +++ | +++ | ++ | - |
| 14 | 7-2 | 68.0±1.0 | 46.1±0.6 | 0.0 | ++++ | +++ | ++ | + | - |

Note: −no growth; + very weak growth; ++ weak growth; +++ intense growth; ++++ very intense growth.

According to the data presented in Table 2, it was found that at 5% ethanol (v/v), GI% values were < 75% for three strains, which corresponds to a partial inhibition (5.2, 6.1, 7.2). 11 strains showed GI values ≥75%,considered as similar in development to the control sample. At 10% (v/v) ethanol concentrations, one strain showed a high degree of inhibition (6.1) with GI of 25%, 2 strains a degree of partial inhibition (5.2, 7.2), respectively GI values <75% and 11 strains with GI values ≥75% indicating a development similar to that of the ethanol-free medium (control). At concentration of 15% ethanol (v/v), no tested strain has developed on the culture medium.The assessment of $SO_2$ tolerance was made according to the ability of the tested strains to develop at the concentrations of potassium metabisulphite used.The results obtained are presented in Table 2.

On the control medium ($SO_2$-free)all strains have developed very intensely (++++). At concentrations of 50 mg/L of potassium metabisulphite ($K_2S_2O_5$), no differences in culture development were observed between the tested strains, but, compared to the control, all strains showed a slightly attenuated development. Differences were reported between strains starting at concentration of 100 mg/L $K_2S_2O_5$, thus, 11 yeast strains had an intense development (+++) and 3 strains a low development (++).

With the increase of the concentration to 150 mg/L $K_2S_2O_5$, among the 14 *Saccharomyces*strains, nine were appreciated as having a low development (++) and five strains showing a very low development (+). At 200 mg/L $K_2S_2O_5$the inhibition of strain development was very high, 11 strains did not develop, only three strains showing a very low development.

## CONCLUSIONS

In the screening of the extracellular enzymatic activity, the obtained results led to the differentiation of the *Saccharomyces cerevisiae* yeast strains according to the complexity of

the enzymatic potential represented by the number of secreted enzymes.Thus, the strains that produced at least four enzymes were considered important for the proposed purpose. The intensity of the enzymes activity was very different, varying depending on the tested yeast strain. Very low activity was found forβ-glucosidase enzyme in 11 strains and for lipase in 12 strains. In the case of esterase, 16 yeast strains showed very low activity and 14 strains low activity. In contrast, seven *Saccharomyces cerevisiae* strains presented intense protease activity. Tested yeast strains showed high tolerance to ethanol concentrations up to 10% (v/v), confirmed by high values of the growth index (> 75%). In the case of $SO_2$ tolerance, the decrease of strains development was correlated with the increase of the potassium metabisulphite concentration. Among the tested yeastsonly four*Saccharomyces* strains, showingspecific oenological characteristics were selected to be tested in mixed and/or sequential cultures to obtain wines with improved sensory features.

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