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# Stress Resistance and Adhesive Properties of Commercial Flor and Wine Strains, and Environmental Isolates of *Saccharomyces cerevisiae*

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**Abstract:** Flor strains of *Saccharomyces cerevisiae* represent a special group of yeasts used for producing biologically aged wines. We analyzed the collection of commercial wine and flor yeast strains, as well as environmental strains isolated from the surface of grapes growing in vineyards, for resistance to abiotic stresses, adhesive properties, and the ability to form a floating flor. The degree of resistance of commercial strains to ethanol, acetaldehyde, and hydrogen peroxide was generally not higher than that of environmental isolates, some of which had high resistance to the tested stress agents. The relatively low degree of stress resistance of flor strains can be explained both by the peculiarities of their adaptive mechanisms and by differences in the nature of their exposure to various types of stress in the course of biological wine aging and under the experimental conditions we used. The hydrophobicity and adhesive properties of cells were determined by the efficiency of adsorption to polystyrene and the distribution of cells between the aqueous and organic phases. Flor strains were distinguished by a higher degree of hydrophobicity of the cell surface and an increased ability to adhere to polystyrene. A clear correlation between biofilm formation and adhesive properties was also observed for environmental yeast isolates. The overall results of this study indicate that relatively simple tests for cell hydrophobicity can be used for the rapid screening of new candidate flor strains in yeast culture collections and among environmental isolates.

**Keywords:** flor yeast; wine; abiotic stress; adhesive properties; oenology

## 1. Introduction

Flor strains of *Saccharomyces cerevisiae* yeasts represent a special group of wine-making microbiota and are used in a number of traditional technologies for producing biologically aged wines [1,2]. Methods of obtaining such wines as Jerez (Spain), Vin Jaune (Jura, France), Vernaccia di Oristano (Sardinia, Italy), and Native dry tokay (Hungary) differ, but they all involve a long biological aging under a yeast biofilm (flor) formed on the surface of fortified fermented wine materials [3,4]. Genetic, biochemical, and physiological features of flor yeast associated with their adaptation to the specific conditions of making sherry-type wines have been studied in detail [1,2,5]. It has been shown that the change in yeast metabolism from fermentative to oxidative in the flor leads to the production of acetaldehyde, higher alcohols, and other compounds, causing profound changes in the biochemical and sensory properties of wine with the formation of organoleptic characteristics

unique for this wine style [3,6–11]. In addition to *S. cerevisiae*, other yeast species (*Wickerhamomyces anomalus*, *Pichia membranaefaciens*, *Pichia kudriavzevii*, and *Pichia manshurica*) were found in industrial biofilms in minor amounts, but their role in the formation of a biofilm and wine aging has not been studied [12].

Resistance to environmental stresses is a key factor for the biotechnological success of *Saccharomyces* yeasts [13]. During alcoholic fermentation, yeast cells are subjected to various stress factors, the most important being osmotic and ethanol stresses [14,15]. Osmotic stress is induced by the high content of sugars in the grape must (about 20% *w*/*v*) at the beginning of vinification, and the yeasts must resist it in order to start the alcoholic fermentation. As fermentation progresses, yeast encounters an increasing concentration of ethanol, which is extremely toxic to cells, primarily damaging cell membranes [15–17]. In the process of biological wine ageing, flor yeast encounters high concentrations of ethanol (up to 15.5%) and acetaldehyde, together with deficiency of sugars and nitrogen sources, and high oxygen concentrations [13]. Resistance to ethanol is particularly important and the strains that better tolerate ethanol were more abundant in a sherry cellar [18].

Comparative genomic studies revealed that flor yeast represents a distinct lineage that separated from the wine clade through a relatively recent bottleneck event [2,19]. Microsatellite analysis revealed overall low genetic diversity of flor strains [20,21], although genetic differences between strains from different wineries were observed [12]. The important role of particular genes—in particular, the surface adhesin FLO11—in determining the ability of flor yeast to biofilm formation has been established by genetic engineering methods [22,23]. Comparative genomic analysis revealed numerous flor yeast-specific genetic differences in various pathways of metabolism and cell signaling, such as oxidative metabolism, cell wall biogenesis, stress resistance, lipid biosynthesis, and ion transport with a potential adaptive value [19,24,25]. The events of gene loss and acquisition, specific for flor strains, and specific genomic loci distinguishing between flor and usual wine strains, probably selected through positive selection, were identified; changes in signaling pathways that control the expression of the *FLO11* gene were revealed [19]. Comparative proteomic analysis revealed that at the stage of biofilm formation in flor strains, there is an overexpression of a number of proteins involved in oxidative metabolism of non-fermentable carbon sources, cell wall biogenesis, stress resistance, etc. [26,27].

In comparison with the rather intensive application of "omics" technologies to the study of flor yeast, information about the origin and evolution of these strains is a relatively limited. One of the recent studies demonstrated that very few flor yeasts could be isolated from wine following alcoholic fermentation, suggesting that flor yeast development results from the colonization of yeast present in the aging cellar [28]. However, the origin of flor yeasts is still unclear, i.e., whether their source is the natural microbiota of grapes. The search for potential flor yeast progenitor strains among isolates from natural environments is an important task both for understanding the evolutionary history of flor strains and for obtaining new microbial agents to improve sherry wine production technologies, for example, by creating various compositions with industrial yeast strains.

Earlier, we isolated yeast strains from grape samples taken in different climatic zones of the Crimea and Rostov region of Russia (hereinafter referred to as "environmental strains") and determined their oenological and molecular genetic characteristics [29]. In order to select candidate strains that are promising for the production of sherry-like wines, we screened a collection of environmental strains for the presence of molecular markers characteristic of flor strains, determined such parameters of strains as fermentation activity, ability to induce flor formation, synthesis of volatile acids, acetaldehyde, etc. It was shown that the presence of "flor" alleles can serve as a convenient criterion for the selection of strains that are promising for further breeding work [29].

Obviously, obtaining new flor strains suitable for commercial winemaking is a complex and lengthy process that involves a multi-stage selection procedure for a number of key winemaking parameters. At the same time, we suppose that a number of relatively simple criteria can be applied already at the first stages of screening. These criteria include

such important characteristics for flor yeast as the ability of adhesion; flor formation; and resistance to oxidative stress, high concentrations of ethanol, and acetaldehyde. A quantitative assessment of these parameters can be easily carried out in the laboratory using standard tests that are often used in phenotyping environmental, industrial, and clinical yeast isolates [30–32].

This work is devoted to the analysis of the phenotypic variability of *S. cerevisiae* strains from the previously obtained collection, including commercial flor and wine strains as well as environmental isolates, in order to identify possible correlations between the phenotypic, genetic, and wine-making characteristics of strains in order to simplify the selection of candidate flor yeast strains for the production of sherry-type wines. The strains were evaluated for growth characteristics; resistance to ethanol, acetaldehyde, and oxidative stress; as well as the adhesion properties of cells and their ability to form a biofilm. The yeast strains with hydrophobic properties and molecular markers characteristic of flor yeast can be used for new flor candidate strains.

## 2. Materials and Methods

### *2.1. Yeast Strains*

We used *S. cerevisiae* strains from the collection of microorganisms of winemaking of the All-Russian Research Institute of Viticulture and Winemaking "Magarach" of the Russian Academy of Sciences used for making sherry-like ("flor strains") and ordinary wines ("wine strains"), as well as *S. cerevisiae* yeast strains isolated in the winemaking seasons of 2016 and 2018 from the surface of grapes growing in vineyards of different climatic zones of Crimea and Rostov area ("environmental strains"). All strains were previously characterized using microbiological, biochemical, oenological, and genetic methods [6,29].

### *2.2. Cultivation Methods*

Yeast cultures were grown at room temperature (18–20 °C) in rich YP media (1% yeast extract, 2% peptone) or on minimal synthetic medium SC (0.67% yeast nitrogen base). As a carbon source, we used glucose at a final concentration of 2% or 0.1%, or a mixture of ethanol and glycerol at final concentrations of 5% and 1%, respectively.

When analyzing the growth characteristics of strains under micro-winemaking conditions, the strains were cultivated on pasteurized (at 114 °C for 30 min) grape juice in 100 mL flasks. Yeast cultures were added to the medium at a rate of $2 \times 10^6$ cells per ml, closed with fermentation locks and incubated at 18 ± 0.5 °C, weighing daily. Sixty days after the end of the fermentation process (no weight change), the concentrations of sugars, alcohol, volatile acids, and aldehydes were determined in the wine materials. Fermentations were performed in triplicates. The capacity of strains for flor formation was determined according to the flor growth on the surface of the fermented must. The flor growth was estimated visually daily after the end of fermentation for 60 days: it begins with the formation of individual "islands"; later, the flor covers the entire surface of the flask.

Evaluation of wine samples for the presence of "sherry" tones in the aroma and taste was carried by a panel of five researchers from the Magarach Institute with experience in sensory evaluation. Odor and flavor were evaluated. A positive assessment was given in case of consent of at least 4 out of 5 experts.

### *2.3. Analysis of Sensitivity for Strains to Abiotic Stresses and Toxic Compounds*

Tests to determine the degree of sensitivity of strains to ethanol, hydrogen peroxide, and acetaldehyde were carried out by culturing the strains in the presence of the toxicant for a standard period of time (2 h), followed by a comparative analysis of subsequent cell growth when culturing an aliquot for 16–18 h in YP liquid medium with 2% glucose.

To determine the degree of ethanol resistance and resistance to acetaldehyde, the strains were grown overnight on agarized YP medium with 2% glucose. An aliquot of grown cells was taken, washed in water, and suspended in SC medium (1% yeast nitrogen

base, BD Difco, NY, USA) with 2% glucose to $OD_{600}$ of 1.0 in Eppendorf tubes. For each analyzed strain, three such samples were prepared and analyzed as biological replicas. The samples were incubated in a TS-100 benchtop shaker (BioSan, Riga, Latvia) for 2 h at 25 °C at 600 rpm, after which each sample was divided into two parts of equal volume. An equal amount of SC medium with 2% glucose (control) was added to the first part; to determine alcohol resistance, the SC medium with 2% glucose and ethanol was added in equal amounts to the second part to a final concentration of the latter of 10%. To determine the resistance to acetaldehyde, SC medium with 2% glucose and with acetaldehyde was added in equal amounts to a final concentration of the latter 2% (2% acetaldehyde was obtained by preliminarily diluting it in ethanol in a ratio of 1:10). The resulting mixtures were left at room temperature for 2 h. Cell viability was checked after culturing an aliquot (300 µL was added to 2700 µL of YP medium, either experiment or control—the same culture without addition of ethanol or acetaldehyde) for 24 h in liquid YP medium with 2% glucose by measuring $OD_{600}$. A quantitative assessment of the degree of resistance to the toxicant was the ratio of the $OD_{600}$ of the experimental and control cultures. The average ratio for the three replicate samples has been determined.

To determine resistance to oxidative stress, the strains were prepared in a similar way, with the difference being that the samples were incubated in a TS-100 tabletop shaker (BioSan, Latvia) for 1 h under the same conditions as in the determination of ethanol resistance. Three samples were analyzed for each strain. An equal amount of SC medium with 2% glucose and hydrogen peroxide was added to the test samples to a final concentration of the latter 3 mM. Cell viability was checked by adding an aliquot of the cell suspension (5 µL) to 1 mL of liquid medium, followed by incubation for 16–20 h at room temperature without stirring, and $OD_{600}$ was measured. The degree of resistance was expressed as the ratio of $OD_{600}$ of cultures in the experimental sample to the control (the same culture without the addition of hydrogen peroxide).

### *2.4. Analysis of Hydrophobicity for Yeast Cells (Isooctane Test)*

The test of microbial adhesion to hydrocarbons was carried out according to the MATH (microbial adhesion to hydrocarbon) test, described by Rosenberg et al. [30], with modifications. Cells grown in a wide streak for 24 h on YP agar with 5% ethanol and 1% glycerol were washed in water and suspended in 50 mM sodium acetate buffer (pH 4.5) to $OD_{600}$ equal to 1.0 ($A_0$). Three 1 mL samples were taken from the resulting suspension, and 200 µL of isooctane was added to each of them. The tubes were vigorously shaken three times for 1 min with intervals of 5 min, and left for 10 min. Next, the $OD_{600}$ of the aqueous phase ($A_t$) was determined. The degree of hydrophobicity (the proportion of cells bound to the organic phase) was calculated using the formula: $Adh = (1 - A_t/A_0) \times 100$. The higher degree of adhesion to isooctane, the higher degree of hydrophobicity of the strains is.

### *2.5. Determination of Ability to Adhere to Polystyrene for Yeast Cells*

Testing for the adhesion of yeast cells to polystyrene was carried out according to the method described in [33], with modifications. The cells of strains grown on YP agar with 5% ethanol and 1% glycerol were suspended in YP medium with 0.1% glucose to $OD_{600}$ of 1.0. Then, 100 µL of the cell suspension (in five replications) was transferred into the wells of a sterile 96-well polystyrene plate (Lenpolimer, Saint Petersburg, Russia) and incubated for 1 h at 30 °C. The cell suspension was removed; the cells adsorbed on the walls of polystyrene plate wells were stained by adding 100 µL of 1% crystal violet dye for 15 min. The wells were washed twice with distilled water and then an equal volume (100 µL) of 33% acetic acid was added to desorb the dye. The absorbance of the dye bound to the cells in the well of the plate was determined at 600 nm. The color intensity was used to evaluate the degree of cell adhesion to polystyrene.

### 2.6. Determination of the Growth Rate of Yeast Strains

The cultivation of the strains was carried out in a liquid medium YPD (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose). To prepare YPD plates, 20 g/L agar was added to the medium. The strains were grown on YPD plates for 24 h at 26 °C to obtain separate colonies. The cells were then inoculated into 50 mL tubes with 2 mL of YPD medium. The tubes were incubated in an orbital shaker–incubator (Multitron Infors, Bottmingen, Switzerland) for 18 h at 26 °C with a stirring speed of 200 rpm and an amplitude of 50 mm. At the end of the cultivation, the $OD_{600}$ of the culture was determined.

Strain growth curves were plotted using an incubator shaker with the function of measuring the optical density of cells in real time (TVS062CA Advantec, Tokyo, Japan). The culture was inoculated into L-shaped tubes with 5 mL of YPD medium to $OD_{600}$ of 0.001. The cells were cultured for 36–40 h at 26 °C with a stirring speed of 70 rpm. The $OD_{600}$ was recorded every 30 min. At the end of the cultivation, the specific growth rate of cells, μ ($h^{-1}$), and the generation time g (h) were calculated.

### 2.7. Statistical Analysis

The difference between the groups of strains in the hydrophobicity tests was assessed using a two-sample *t*-test. *p* values were calculated using t test function in R version 3.6.0 programming language (https://www.r-project.org/). The difference was considered significant if the *p* value was less than 0.05.

## 3. Results

### 3.1. Growth Characteristics of Strains

In our previous studies, detailed biochemical, microbiological, genetic, and oenological characteristics of flor and wine strains from the collection of microorganisms for winemaking "Magarach" as well as yeast strains isolated during the winemaking seasons of 2016 and 2018 from grapes growing in the vineyards of the Crimea and the Rostov region were determined [6,29,34]. In particular, strains 229, 285, 329, and 566 have all the characteristics of flor strains; i.e., they are able to grow in a biofilm on the surface of fermented fortified must, conferring a characteristic sherry-like aroma to the wine. Based on the combination of molecular genetic (presence of "flor" alleles of loci *YDC387C-A*, *FLO11*, *ITS1–4*) and oenological characteristics, the most promising strains containing 1, 2, or 3 "flor" alleles were chosen (Table 1). At the first stage, the proliferative and fermentative capacity of strains was assessed under conditions of "micro-winemaking" (see Materials and Methods). Three groups of strains (flor, wine, and environmental) had certain differences.

**Table 1.** Genetic and oenological characteristics of *S. cerevisiae* strains.

| Strain ID | Genotype * | | | Oenological Parameters | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Concentration | | | | Flor Growth *** | Presence of "Sherry" Tones in Aroma and Taste ** |
| | FLO11 | ITS1-4 | YDR 379 C-A | Sugars, g/L | Volatile Acids, g/L | Aldehydes, mg/L | Alcohol, % vol. | | |
| **Flor Strains** | | | | | | | | | |
| 229 | F | F | F | 2.5 ± 0.06 | 0.66 ± 0.01 | 286.0 ± 5.9 | 11.3 ± 0.1 | +++ | + |
| 285 | F | F | - | 1.3 ± 0.06 | 0.48 ± 0.03 | 211.0 ± 9.4 | 11.3 ± 0.1 | +++ | + |
| 329 | F | F | F | 1.2 ± 0.06 | 0.52 ± 0.02 | 179.6 ± 2.6 | 11.4 ± 0.1 | +++ | + |
| 566 | W | F | F | 0.8 ± 0.15 | 0.60 ± 0.02 | 352.0 ± 8.4 | 11.1 ± 0.1 | +++ | + |
| **Wine Strains** | | | | | | | | | |
| 271 | W | F | F | 3.7 ± 0.15 | 0.10 ± 0.01 | 343.2 ± 20.8 | 9.5 ± 0.1 | +++ | + |
| 328 | W | F | - | 4.2 ± 0.10 | 0.46 ± 0.01 | 132 ± 6.8 | 11.4 ± 0.1 | +++ | + |
| 525 | W | F | F | 2.7 ± 0.06 | 0.59 ± 0.05 | 149.6 ± 9.1 | 11.7 ± 0.1 | + | + |
| **Environmental Strains** | | | | | | | | | |
| 3 | F/W | F | F | 1.9 ± 0.12 | 0.78 ± 0.04 | 65.9 ± 4.0 | 12.3 ± 0.1 | +++ | + |
| 23 | F | F | F | 2.3 ± 0.15 | 0.46 ± 0.03 | 105.6 ± 6.4 | 11.9 ± 0.1 | +++ | + |

**Table 1.** *Cont.*

| Strain ID | Genotype * | | | Oenological Parameters | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | FLO11 | ITS1-4 | YDR 379 C-A | Concentration | | | | Flor Growth *** | Presence of "Sherry" Tones in Aroma and Taste ** |
| | | | | Sugars, g/L | Volatile Acids, g/L | Aldehydes, mg/L | Alcohol, % vol. | | |
| 28 | W | F | W | 0.9 ± 0.06 | 0.35 ± 0.02 | 73.0 ± 4.4 | 12.8 ± 0.2 | - | - |
| 45 | W | W | F | 1.9 ± 0.06 | 0.64 ± 0.02 | 84.5 ± 5.1 | 12.2 ± 0.1 | - | - |
| 53 | F/W | W | W | 1.1 ± 0.10 | 0.21 ± 0.01 | 34.3 ± 2.1 | 12.9 ± 0.1 | + | - |
| 54 | F/W | W | F | 0.1 ± 0.06 | 0.59 ± 0.02 | 148.7 ± 9.0 | 12.7 ± 0.2 | +++ | + |
| 78 | W | W | F | 0.6 ± 0.06 | 0.75 ± 0.03 | 154.9 ± 9.4 | 12.6 ± 0.1 | + | + |
| 79 | W | W | F | 0.2 ± 0.10 | 0.66 ± 0.02 | 91.5 ± 5.5 | 12.3 ± 0.0 | - | - |
| 90 | F | W | W | 1.3 ± 0.06 | 0.30 ± 0.02 | 74.5 ± 4.5 | 12.7 ± 0.1 | - | - |
| 98 | W | W | W | 1.3 ± 0.06 | 1.10 ± 0.10 | 183.9 ± 6.6 | 13.0 ± 0.1 | - | - |
| 109 | F/W | F | F | 0.3 ± 0.06 | 0.72 ± 0.03 | 283.4 ± 17.2 | 12.7 ± 0.2 | +++ | + |
| 110 | F | W | F | 0.7 ± 0.06 | 0.90 ± 0.04 | 253.4 ± 15.4 | 12.5 ± 0.1 | + | + |
| 111 | F/W | F | F | 0.6 ± 0.12 | 0.66 ± 0.02 | 279.8 ± 17.0 | 11.9 ± 0.1 | +++ | + |
| 112 | W | W | F | 1.6 ± 0.06 | 0.54 ± 0.03 | 176.0 ± 10.7 | 12.3 ± 0.1 | - | - |
| 113 | F/W | F | F | 1.2 ± 0.10 | 0.87 ± 0.03 | 176.0 ± 10.7 | 12.6 ± 0.1 | +++ | + |

* The distribution of alleles characteristic of wine (W) and flor (F) strains is shown; F/W—heterozygote. Mean values and standard deviations are shown. ** Expert opinion based on the results of the tasting. *** Flor grown on the surface of fermented must: (-) no growth, (+) formation of few islands, (+++) flor covering the entire surface of the flask.

To measure the growth kinetics, fresh inoculum was used, and the cultivation conditions were unified. The duration of the lag phase was approximately the same for all strains, averaging about 5 h (Table 2). Exceptions were environmental strains 54 and 109, and flor strains 285 and 566, in which the lag phase was longer. After the transition to the exponential phase, the growth rates of environmental strains became nearly equal. On average, the duration of the logarithmic growth phase for them was from 13 to 18 h, with the exceptions being strains 23, 79, and 113. For flor strains, this period was somewhat longer, 20–30 h. To characterize the intensity of cell growth, their specific rate μ ($h^{-1}$) was calculated. However, a more indicative parameter appeared to be the generation time. Thus, for environmental strains, this indicator turned out to be approximately two times less (1 h 46 min) than for flor strains (2 h 51 min). The exceptions were strains 23, 54, 78, 79, 109, and 113, growing slower than other environmental strains (Table 2).

**Table 2.** Growth characteristics of strains.

| Strain ID | Lag Phase Duration, h | Exponential Growth (from–to, h) | Exponential Growth Phase Duration, h | Specific Growth Rate, μ, $h^{-1}$ | Generation Time, h:min | Generation Time, h:min |
|---|---|---|---|---|---|---|
| **Flor Strains** | | | | | | |
| 229 | 7 | 7–26 | 19 | 0.315 | 2:19 | 2:11 |
| 285 | 15 | 15–35 | 20 | 0.266 | 2:59 | 2:35 |
| 329 | 5 | 5–35 | 30 | 0.219 | 3:15 | 3:09 |
| 566 | 9 | 9–22 | 13 | 0.363 | 1:91 | 1:55 |
| **Wine Strains** | | | | | | |
| 271 | 5 | 5–29 | 24 | 0.266 | 2:59 | 2:35 |
| 328 | 4 | 4–19.5 | 16 | 0.424 | 1:63 | 1:37 |
| 525 | 5 | 4.5–25 | 21 | 0.338 | 2:04 | 2:02 |
| **Environmental Strains** | | | | | | |
| 3 | 7 | 7–20 | 13 | 0.425 | 1:63 | 1:38 |
| 23 | 6 | 6–24 | 18 | 0.348 | 1:98 | 1:59 |
| 28 | 5 | 5–20 | 15 | 0.463 | 1:49 | 1:29 |
| 45 | 5 | 5–20 | 15 | 0.462 | 1:49 | 1:29 |
| 53 | 4 | 4–18 | 14 | 0.483 | 1:43 | 1:25 |
| 54 | 9 | 9–24 | 15 | 0.401 | 1:72 | 1:43 |

**Table 2.** *Cont.*

| Strain ID | Lag Phase Duration, h | Exponential Growth (from–to, h) | Exponential Growth Phase Duration, h | Specific Growth Rate, μ, $h^{-1}$ | Generation Time, h:min | Generation Time, h:min |
|---|---|---|---|---|---|---|
| 78 | 6 | 6–24 | 18 | 0.393 | 1:76 | 1:45 |
| 79 | 5 | 5–25 | 20 | 0.362 | 1:91 | 1:54 |
| 90 | 7 | 7–20 | 13 | 0.489 | 1:41 | 1:24 |
| 98 | 2 | 2–17 | 15 | 0.506 | 1:36 | 1:21 |
| 109 | 8 | 8–24 | 16 | 0.383 | 1:80 | 1:48 |
| 110 | 3 | 3–21 | 18 | 0.464 | 1:49 | 1:29 |
| 112 | 4 | 3.5–20.5 | 17 | 0.442 | 1:56 | 1:33 |
| 113 | 4 | 4–24 | 20 | 0.389 | 1:78 | 1:46 |

In general, despite a small sample size, when comparing the wine/environmental and flor yeast strains groups, the first group tends to have a shorter lag phase. These data are consistent with the results of Ferreira et al. [31], who showed that wine strains seem to have a tendency to exhibit shorter lag phases than flor strains. Perhaps this is due to the ability of flor yeast to proliferate only at the end of the fermentation process. Such features can be explained by physiological and biochemical characteristics specific to flor strains associated with their adaptation to oxidative, rather than fermentative, metabolism. The industrial flor strain 329 has a distinctive feature—the duration of its exponential phase was 30 h (generation time: 3 h 15 min). However, the wine strain 271 has similar characteristics—the duration of this phase was 24 h and the generation time was 2 h 59 min. Environmental strains 23, 54, 78, 79, 109, and 113 also had a long exponential phase.

### *3.2. Stress Tolerance*

The options for setting up tests to determine the degree of resistance of microorganisms to certain stressful influences are quite diverse, ranging from high-throughput screening systems manufactured by Singer Instruments and ending with the classic plate test using serial dilutions [35,36]. A recently proposed express method for assessing the degree of resistance of yeast cells to various chemical toxicants is based on the cultivation of yeast cells in 96-well microplates followed by OD measurement using a microplate reader [37]. However, an attempt to adapt this method for the strains used in this work was unsuccessful, primarily due to the high degree of adhesion of the cells of the flor strains to the wells of polystyrene plates. We found that the most reliable and reproducible was the cultivation of strains in 1.5 mL microcentrifuge tubes. The parameters of exposure time and concentration of growth inhibitors (ethanol, hydrogen peroxide, acetaldehyde) were selected on the basis of literature data and preliminary experiments.

The results of the analysis of the degree of resistance of the strains to the stress agents used are presented in Figure 1. Contrary to expectations, it turned out that flor yeast strains were not "leaders" in any of the tests, yielding in the degree of resistance to wine strains and environmental isolates. To select strains with cross-resistance to different toxic agents, the following criteria were chosen: ethanol resistance >63%, hydrogen peroxide resistance >42%, acetaldehyde resistance >50%. With this grouping, 6 strains were simultaneously resistant to ethanol and peroxide stresses, 5 strains were resistant to ethanol and acetaldehyde, and 4 strains were resistant to all three toxicants (Figure 2). Flor strains from the collection were present only in the first set. At the same time, only some environmental isolates that were previously selected using a combination of genetic markers with subsequent assessment of oenological characteristics [29] turned out to be resistant to all three stress agents.

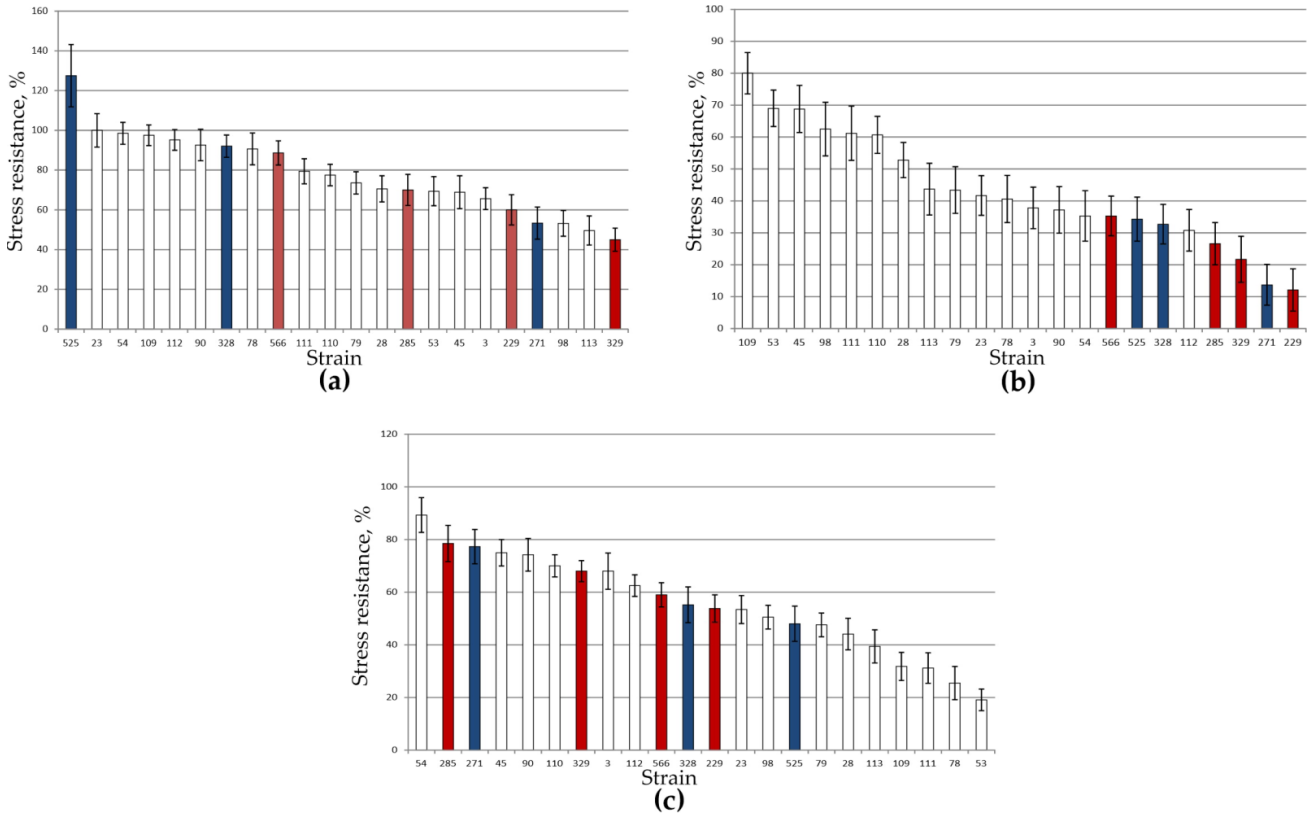


**Figure 1.** Resistance of yeast strains to (**a**) ethanol, (**b**) acetoaldehyde, and (**c**) hydrogen peroxide. Industrial flor strains are shown in red, wine strains in blue, and environmental isolates in open bars. On top bars show standard deviation.

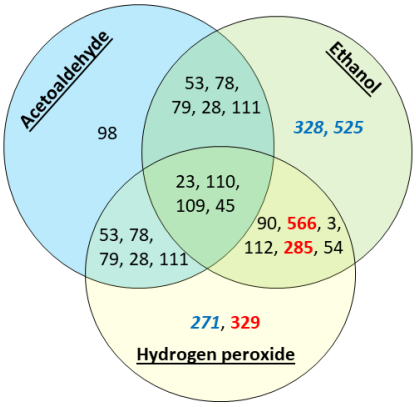


**Figure 2.** Venn diagram illustrating resistance to various stress agents. Industrial flor strains are shown in red font, wine strains in blue font, and environmental isolates in black font.

### *3.3. Adhesive Properties*

Figure 3 shows the results of the analysis of the adhesive properties of the strains using two tests. The first analyzes the distribution of cells between the aqueous phase and a hydrophobic organic solvent, isooctane. The higher the degree of hydrophobicity of cells, the greater their fraction will be present in the organic phase. The second test measured the ability of cells to bind to polystyrene, which also positively correlates with adhesion properties. The high hydrophobicity of the cell surface in the test with isooctane (>50%) was demonstrated by environmental strains 3, 23, 78, 54, 109, 28, 79, 111, 113, flor strains 329, 229, and 285, and wine strains 271 and 525. Most of these strains were also among the leaders in the test for adhesion to polystyrene (Figure 3b).

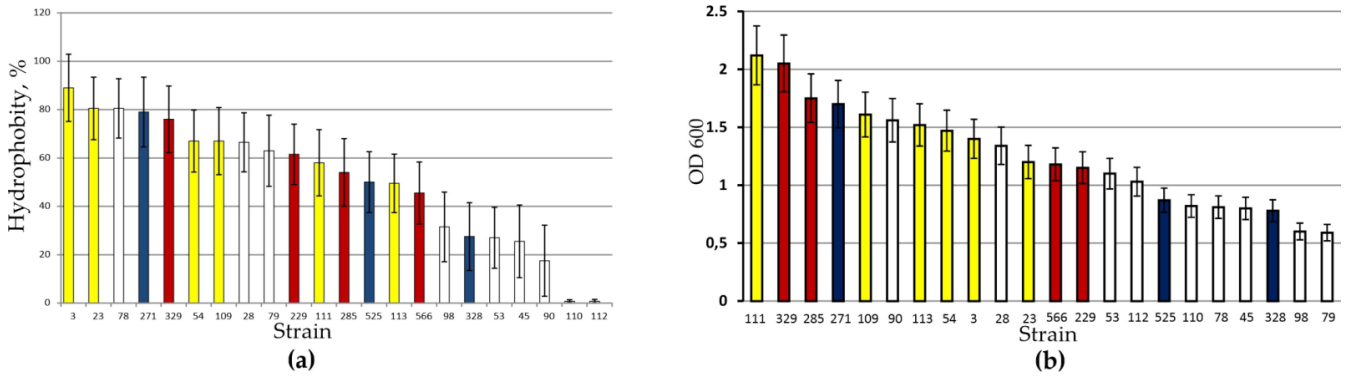


**Figure 3.** Adhesive properties of yeast strains. (**a**) Isooctane test; (**b**) adhesion to polystyrene. Flor strains are shown in red, wine strains—in blue, environmental isolates able of forming a flor (indicated by +++ in Table 1) in yellow, other environmental isolates—in open bars. On top bars show standard deviation.

Interestingly, most of the environmental strains that were able to form biofilms under micro-winemaking conditions (highlighted in yellow in Figure 3) showed high hydrophobicity in these tests. Comparison of flor strains and biofilm-forming environmental strains (10 strains, group 1) with the rest of the strains (12 strains, group 2) revealed statistically significant differences between these groups both in the isooctane test ($p$ value 0.016) and in the test with polystyrene ($p$ value 0.0018).

## 4. Discussion

Stress tolerance is one of the important characteristics that are taken into account for the selection of strains promising for winemaking [38]. High resistance to stresses has been found to correlate with good fermentative capacity [39] and used as a good criterion for selecting promising wine strains [40]. Recently, polyphasic approaches have begun to be carried out to select new candidate strains, including both genetic technologies and physiological characteristics of the strains [41,42]. In this work, commercial wine and flor yeast strains, as well as environmental isolates were tested for resistance to various types of abiotic stress, the adhesion properties of cells and their ability to form a biofilm.

The analysis revealed a fairly high variability of proliferative and adhesive characteristics, and the degree of stress resistance of the studied strains. Contrary to expectations, the level of resistance to ethanol and acetaldehyde, determined in our experiments, in collection flor yeast strains was, in general, lower than in environmental isolates (Figure 1). This was especially noticeable with regard to acetaldehyde resistance. In terms of resistance to oxidative stress, flor strains appeared in the leading group, although they were again inferior to some environmental isolates. We are inclined to explain this phenomenon both by the peculiarities of the adaptive mechanisms of flor strains and by differences in the nature of their exposure to various types of stress in the course of biological wine aging and under the experimental conditions used in this work. In our experiments, the strains were exposed to a short-term (1–2 h) action of toxic agents ("acute stress") with subsequent measurement of cell proliferative responses. This approach is widespread and is widely used to study the physiological and transcriptomic responses of yeast strains to certain external influences or genetic changes [43]. Under the conditions of biological wine aging, yeast is exposed to another type of stress. This is not a short-term acute but a long-term chronic stress, with a duration under micro-winemaking conditions up to 90 days, during which the concentrations of acetaldehyde and ethanol can change gradually and rather smoothly. Under such conditions, initially low concentrations of toxicants (for example, acetaldehyde) can act as inducers of the general adaptive stress response of yeast cells to higher concentrations of this and other toxicants. The genetic and biochemical mechanisms of this "environment stress response" have been well studied in yeast [44] and, as shown in our recent analysis, genes for the "response to external stress" are induced during flor for-

mation and maturation [45]. The evaluation of the effects of such induced cross-resistance to various types of stress in yeast strains is the subject of our further research. In contrast to the collection of flor and wine strains, some environmental yeast isolates had a fairly high resistance to ethanol and acetaldehyde. It is noteworthy that among these isolates there were strains that were promising for making sherry-like wines, previously selected using a combination of genetic and oenological methods. It should be noted, however, that while ethanol resistance is an important factor for wine yeast, the degree of resistance varies greatly between different industrial wine strains [14]. For example, among 14 commercial wine strains, some were more sensitive to ethanol even than the laboratory strain of *S. cerevisiae*, and only one strain was able to grow on plates containing 15% (*v*/*v*) ethanol [14].

The ability to form biofilms and adhere to surfaces is widespread in various yeast species related to winemaking and production of other fermented foods [46–48]. Laboratory methods for assessing the adhesive properties of yeast cells, such as the ability to sorption on polystyrene, distribution of cells between aqueous and organic phases, and others can be used as express tests for biofilm-forming ability [30,49,50].

Biofilm formation and efficient adherence to surfaces is one of the strategies for the dispersion of wine spoilage yeasts and their survival in the presence of various disinfectants used in winemaking, which has been shown for *Brettanomyces bruxellensis* [51,52], *Pichia pastoris* [53], *Pichia manshurica* [50], *Pichia kudriavzevii*, *Pichia membranifaciens* [54].

In the case of commercial wine yeasts, their ability to form biofilms and adhere to surfaces in the wine environment allow such yeasts to colonize and survive in the winery environment and may explain the observations that commercial strains contribute to the yeast population in spontaneous fermentations [47]. Moreover, commercial wine yeast could adhere and even invade grapes for colonization [47], which can contribute to their spread not only in wineries, but also in vineyards.

The ability to form a floating biofilm (velum) is a key adaptive and technological characteristic of flor strains, necessary for survival under stressful conditions of biological wine aging and for switching to oxidative metabolism, which ensures the formation of a unique aroma and bouquet of sherry-like wines [1,2,55]. It is likely that the ability to form biofilms contribute to the spread of such yeast strains in vineyards, from which we isolated potential flor strains. All of the collection of flor yeast strains showed a high level of cell hydrophobicity in the iso-octane test and the ability to effectively adhere to polystyrene. A strong correlation between efficient flor formation and adhesive properties was also observed in environmental yeast isolates. Thus, all 5 environmental strains (3, 23, 54, 109, and 111) that formed a flor under conditions of micro-winemaking and imparted a specific sherry-like aroma to wine showed good adhesive properties in both tests. Note that for testing, the strains were grown on YP agar medium with 5% ethanol and 1% glycerol, rather than in the form of a biofilm on the surface of fermented wine materials. Apparently, even under these conditions, the cells of strains capable of flor formation have a sufficiently high hydrophobicity, which makes it possible to use a simple test for adhesiveness for rapid screening of candidate flor strains among environmental yeast isolates.

## 5. Conclusions

The proposed methodological approach complements the previously developed methods of primary selection of yeast strains for breeding work well, and the results obtained expand our understanding of stress resistance and adhesive properties of environmental and commercial yeast strains. Flor strains were distinguished by a higher degree of hydrophobicity of the cell surface and an increased ability to adhere to polystyrene. A clear correlation between biofilm formation and adhesive properties was observed for environmental yeast isolates. Overall, our results indicate that relatively simple tests for cell hydrophobicity, together with the identification of molecular markers characteristic for flor yeasts, can be used for the rapid screening of new candidate flor strains in yeast culture collections and among environmental isolates. Four environmental strains (3, 23,

111, and 113) showing high hydrophobicity of the cell surface in the isooctane test and good adhesion to polystyrene could be a promising flor strains for the production of sherry-type wines.

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