Article

Selection of Wine *Saccharomyces cerevisiae* Strains and Their Screening for the Adsorption Activity of Pigments, Phenolics and Ochratoxin A

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Received: 22 July 2020; Accepted: 1 August 2020; Published: 5 August 2020

Abstract: Ochratoxin A is a dangerous mycotoxin present in wines and is considered the principal safety hazard in the winemaking process. Several authors have investigated the ochratoxin A adsorption ability of *Saccharomyces cerevisiae* yeasts, and specifically selected strains for this desired trait. In the present work, a huge selection of wine yeasts was done starting from Portuguese, Spanish and Italian fermenting musts of different cultivars. Firstly, 150 isolates were collected, and 99 non-redundant *S. cerevisiae* strains were identified. Then, the strains were screened following a multi-step approach in order to select those having primary oenological traits, mainly (a) good fermentation performance, (b) low production of H_2S and (c) low production of acetic acid. The preselected strains were further investigated for their adsorption activity of pigments, phenolic compounds and ochratoxin A. Finally, 10 strains showed the desired features. The goal of this work was to select the strains capable of absorbing ochratoxin A but not pigments and phenolic compounds in order to improve and valorise both the quality and safety of red wines. The selected strains are considered good candidates for wine starters, moreover, they can be exploited to obtain a further enhancement of the specific adsorption/non-adsorption activity by applying a yeast breeding approach.

Keywords: adsorption; ochratoxin A; phenolics; pigments; *Saccharomyces*; winemaking; yeasts

1. Introduction

Ochratoxin A (OTA) is a mycotoxin largely detected in several foods such as cereals, bread, coffee, dried wine fruits, as well as in beer, grape juice and wine [1–3].

OTA is composed of a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-R-methylisocoumarin (OTA α) moiety and an L-β-phenylalanine molecule linked through the 7-carboxy group by an amide bond [4] (Figure 1).

Figure 1. Chemical structure of Ochratoxin A.

The presence of OTA in wines and juices occurs due to the growth of a fungal contamination in grapes at pre- and post-harvest [5]. Moulds belonging to *Aspergillus* and *Penicillium* genera are mainly responsible for the contamination [6,7].

During the last few years, OTA has received a special focus as it is considered the principal safety hazard in the winemaking process [8,9]. OTA contamination is, in fact, very dangerous due to its carcinogenic, nephrotoxic and teratogenic effects [6]. The contamination of grapes seems to be mainly influenced by the different geographical and climatic zones. In Europe OTA is prevalent in wines originating from southern areas of the Mediterranean basin with typically warmer climates [10,11]. In fact, in these wine-growing regions, red wines are frequently more contaminated than white wines [12]. In order to protect consumers, the EU committee has established the maximum OTA levels for wines and musts at 2 μg/L [2]. Common strategies used for the containment of the toxin include good agricultural practices, selection of fungal resistant crop varieties, correct application of fungicides as well as proper storage of commodities. However, these individual measures could result in unsuccessful or be inadequate with the results of still having OTA in feed and products which require additional decontamination or detoxification procedures [13]. Inorganic adsorbents such as zeolites, bentonites and activated carbon can be used to control OTA but, in many cases, they decrease the nutritive value and organoleptic properties as well as affect the production cost. An alternative approach, which has received a growing interest, is the mycotoxin detoxification by microorganisms. Numerous studies reveal that some bacteria and yeasts species, such as *Lactobacillus acidophilus*, *Bifidobacterium animalis*, *Saccharomyces cerevisiae*, and *Kloeckera apiculata* are able to detoxify mycotoxins [2]. In particular, several authors have investigated the OTA adsorption ability of wine *S. cerevisiae* strains [4,14–16] and have proposed polyphasic approaches to select the strains with this desired trait in order to use them as a wine starter [17,18]. Although the exact mechanism of OTA-removal from contaminated grape must by yeasts is still not fully understood, it has been supposed a fundamental role of the cell wall and its primary components such as β-glucans and mannoproteins [4,19]. In particular, mannoproteins could be implicated in the OTA adsorption because of their common ability to bind mycotoxins, attributed to modified mannanoligosaccharide [14].

Unfortunately, yeast cell wall can adsorb not only OTA, but, at different levels of adsorption, also other components, such as phenolic compounds and pigments, causing colour loss in wines [14,19,20].

Therefore, the aim of this work was to select wine *S. cerevisiae* strains that are able to show an opposite adsorption activity of OTA, pigments and phenolic compounds. The selection was performed with a multi-step screening starting from the sampling of a large number of wine yeasts, which were preliminary selected for primary oenological traits. Only the best strains were then screened for the following traits of interest: (a) wine colour protection, (b) preservation of phenolics, and (c) removal of OTA. The final goal was to find strains with all the desired traits, to be used as wine starters able to improve and valorise both quality and safety of red wines.

2. Materials and Methods

2.1. Samples and Yeast Isolation

In this study, spontaneously fermenting musts were collected in three different Mediterranean areas (Italy, Spain and Portugal).

Each sample was diluted in physiological solution (9 g/L NaCl) by a tenfold dilution series. A volume of 0.1 mL of the highest dilution (10^{-7}) was spread onto plates of Yeast Peptone Dextrose Agar (YPDA) medium (1% *w/v* yeast extract, 1% *w/v* peptone, 2% *w/v* dextrose and 2% *w/v* agar). The plates were incubated at 27 °C for two days and the isolated colonies were purified by streaking on fresh YPDA medium. A total of 150 isolates were initially characterised according to the standard procedures of the Microbial Resource Research Infrastructure-Italian Joint Research Unit (MIRRI-IT) [21]. rDNA ITS-RFLP restriction analysis and interdelta regions typing [22] allowed us to identify the 99 non-redundant *S. cerevisiae* described in Table 1. Periodical transplants of the yeast cultures in a fresh YPDA were performed to ensure growth and viability of the microbial cells used in the different screenings. Strain sporulation activity was tested on acetate medium (1% *w/v* anhydrous sodium acetate, 2% *w/v* agar). The plates were incubated at 28 °C and asci formation was microscopically checked after 14 days.

Table 1. Origin of the 99 *S. cerevisiae* strains used in the present work.

Wine Sample Cultivar.	Region (Country)	Number of Non-Redundant *S. cerevisiae* Strains (Codes Range)
Nerello mascalese	Sicily (Italy)	21 (RE001–RE021)
Carricante		3 (RE022–RE024)
Grecanico		8 (RE025–RE032)
Grecanico	Penedès and La Rioja (Spain)	2 (RE033–RE034)
Tempranillo		33 (RE035–RE067)
Touriga		3 (RE068–RE070)
Touriga national	Porto (Portugal)	7 (RE071–RE077)
Touriga franca		12 (RE078–RE089)
Tinta rorizi		10 (RE090–RE099)

Culture copies cultivated on YPDA were preserved at −80 °C in cryovials supplemented with glycerol (Merck, Darmstadt, Germany) at 25% (*v/v*) final concentration and safe deposited at Unimore Microbial Culture Collection (UMCC).

2.2. Preliminary Screening

The strains were first screened by evaluating: (a) growth modality and foam production during grape must fermentation. Assays were performed in test tubes containing 10 mL of pasteurised (110 °C × 10 min) and filtered (through sterile gauze) must from white grape of the cultivar *Greco bianco.* The must was inoculated with each culture strain and tested for acetic acid production on chalk agar at 30 °C for 3 days according to Lemaresquier et al. [23]; H_2S production on BiGGY agar (Oxoid, Milan, Italy) at 25 °C for 48 h, according to Nickerson [24].

2.3. Fermentation Trials and Adsorption Activity of Pigments and Phenolics

Based on the results obtained with the preliminary screening, the resulting strains were further studied for their aptitude for adsorbing grape pigments. To this purpose, the strains were grown in Petri dishes with a chromogenic grape-skin agar medium following the procedure described by Caridi [20]. The dishes were put into plastic bags and hermetically closed after the insufflation of nitrogen gas for 1 min. After 10 days of anaerobic incubation at 28 °C, yeast biomass was subjected to the computer-assisted evaluation of the red, green, and blue component. The image was processed for colour using Adobe Photoshop CS for Windows XP. The region of interest was set to 5 × 5 pixels

taking four replicates for each strain. Photoshop's red-green-blue colour mode assigned an intensity value to each region. The same strains were tested in micro-winemaking trials to assess their aptitude for adsorbing grape pigments and phenolics during fermentation. The must was prepared starting from red grapes subjected to a pre-fermentative maceration to extract phenolic compounds from skins and seeds. They were destemmed, crushed and cold soaked at 0 °C for 3 days, performing a punch down twice per day. The must obtained after pressing (pH 3.50, °Brix 23) was divided in aliquots of 20 mL, suddenly inoculated at 5% in triplicate with the *S. cerevisiae* strains and incubated at 20 °C. After three days, the fermentation vigour was assessed as the weight loss caused by CO_2 production (g CO_2/100 mL). At the end of fermentation, wine samples were centrifuged at 2300× *g* for 5 min and diluted 1:5 (*v/v*) with a pH 3.5 buffer (citric acid monohydrate 0.1 M and Na_2HPO_4 0.2 M). The wine absorbance was read at 420, 520, and 620 nm; the intensity (I) was calculated with the following formula: I = A420 + A520 + A620 [25]. The total phenolic content was determined using the Folin-Ciocalteu index (FC index) according to Singleton and Rossi [26]. The strains which showed a high adsorption activity of grape pigments and phenolics were excluded from the final trial.

2.4. OTA Adsorption Activity

The remaining strains were studied for their aptitude for removing OTA from synthetic must (6.7 g/L yeast nitrogen base, 5.0 g/L tartaric acid, 5.0 g/L malic acid, 0.2 g/L citric acid, 110 g/L dextrose, 100 g/L fructose, and 7 g/L saccharose, pH 3.3) contaminated with the addition of 5 µg/L OTA (Sigma-Aldrich, St. Luis, MO, USA). Yeast precultures were prepared in YPD broth and cultivated at 28 °C for 48 h. Tests were performed inoculating in triplicate 10 mL of the synthetic must with 0.2 mL of the precultures (~10^6 cells/mL final concentration). The fermentations were carried out at 25 °C and after 28 days the OTA content of the samples was detected by HPLC applying the protocol described by Meca et al. [4].

2.5. Statistical Analysis

All experiments were carried out at least in triplicate. The results are expressed as mean value ± standard deviation (sd). One-way ANOVA followed by a Tukey HSD post hoc test was applied to the OTA adsorption activity and fermentative vigour data, showed by the selected strains, to establish significant differences between means ($p < 0.05$). For the statistical analysis, the software GraphPad Prism version 8 was used (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Preliminary Trials on the S. cerevisiae Strains

Firstly, the 99 *S. cerevisiae* strains were screened in order to choose those with desired primary oenological traits related to growth modality, foam production, acetic acid and H_2S production. In particular, the strains which exhibited a non-flocculant growth and null or low fleeting foam production during grape must fermentation overtook the trial. Concerning the acetic acid production on chalk agar, the strains producing null or little halos (<3 mm) in the used medium passed the screening. Regarding the H_2S production, only the strains showing white or light brown colony colours on BiGGY agar medium were chosen as low hydrogen sulphide-producing yeasts. On the basis of their different behaviours, 14 strains were excluded for one or more parameters. The remaining 85 strains shown in Table 2 were further screened.

Table 2. Fermentation vigour and assessment of pigments and phenolics adsorption activity showed by the 85 preselected *S. cerevisiae* strains.

Strain	Yeast Biomass Colour on Grape-Skin Agar			Fermentation Vigour after 3 Days ($g\ CO_2$/100 mL)	Wine Colour Intensity	Phenolics Content
	Red Component	Green Component	Blue Component		Absorbance (420 + 520 + 620) nm	Folin–Ciocalteu's Index (FC)
RE001	81 ± 4	40 ± 2	45 ± 2	8.58 ± 0.11	3.908 ± 0.181	11.08 ± 5.68
RE002	73 ± 3	37 ± 1	40 ± 2	8.45 ± 0.14	4.933 ± 0.392	11.98 ± 6.48
RE004	95 ± 2	52 ± 2	50 ± 1	8.65 ± 0.22	4.580 ± 1.052	10.98 ± 5.87
RE005	78 ± 3	39 ± 2	39 ± 2	9.14 ± 0.08	3.145 ± 0.818	8.83 ± 3.30
RE006	73 ± 3	37 ± 4	40 ± 4	8.56 ± 0.07	3.897 ± 0.161	11.33 ± 5.68
RE007	60 ± 5	28 ± 2	34 ± 2	8.58 ± 0.11	4.108 ± 0.590	10.22 ± 2.55
RE008	82 ± 3	38 ± 2	42 ± 2	8.12 ± 0.14	1.855 ± 0.398	9.18 ± 2.55
RE010	71 ± 2	36 ± 2	39 ± 2	8.14 ± 0.22	3.528 ± 0.763	12.08 ± 7.07
RE011	65 ± 1	32 ± 2	38 ± 2	9.15 ± 0.08	5.418 ± 0.460	22.85 ± 3.50
RE012	66 ± 2	31 ± 2	37 ± 1	8.41 ± 0.07	5.557 ± 0.333	10.25 ± 8.46
RE013	67 ± 4	33 ± 1	41 ± 1	8.25 ± 0.11	2.833 ± 0.855	9.12 ± 5.74
RE014	80 ± 2	43 ± 1	46 ± 2	7.45 ± 0.14	4.715 ± 0.654	12.82 ± 2.33
RE015	86 ± 2	48 ± 2	49 ± 3	7.12 ± 0.18	3.532 ± 0.488	12.25 ± 0.73
RE017	108 ± 5	65 ± 4	61 ± 4	8.51 ± 0.08	6.417 ± 0.336	21.55 ± 0.41
RE018	91 ± 3	49 ± 3	50 ± 1	9.56 ± 0.07	1.588 ± 0.172	9.65 ± 3.34
RE019	69 ± 3	34 ± 1	39 ± 2	7.75 ± 0.11	4.498 ± 0.595	11.00 ± 2.35
RE020	69 ± 2	36 ± 3	41 ± 3	9.12 ± 0.14	4.870 ± 0.441	12.85 ± 6.29
RE022	72 ± 4	39 ± 2	42 ± 2	8.90 ± 0.22	4.940 ± 0.440	18.42 ± 2.68
RE023	84 ± 2	45 ± 3	45 ± 2	7.52 ± 0.08	3.128 ± 0.418	8.38 ± 6.13
RE024	70 ± 5	35 ± 1	38 ± 2	7.75 ± 0.07	3.147 ± 0.511	12.97 ± 1.88
RE025	70 ± 4	34 ± 2	39 ± 4	8.42 ± 0.11	3.317 ± 0.587	8.62 ± 5.15
RE026	68 ± 4	34 ± 2	38 ± 3	8.21 ± 0.14	2.358 ± 0.660	9.62 ± 5.40
RE027	114 ± 3	69 ± 2	62 ± 3	7.21 ± 0.18	3.957 ± 0.305	12.07 ± 1.83
RE028	62 ± 1	30 ± 2	36 ± 1	8.05 ± 0.22	4.958 ± 0.398	12.77 ± 2.48
RE029	57 ± 3	33 ± 2	37 ± 3	8.74 ± 0.08	1.530 ± 0.411	9.43 ± 5.34
RE030	70 ± 2	36 ± 2	40 ± 3	7.78 ± 0.07	3.483 ± 0.700	10.17 ± 3.60
RE031	79 ± 2	38 ± 1	40 ± 2	7.89 ± 0.11	2.888 ± 0.935	12.17 ± 3.12
RE032	91 ± 3	50 ± 2	51 ± 2	7.56 ± 0.14	3.522 ± 0.713	15.08 ± 0.53
RE033	76 ± 4	36 ± 2	40 ± 3	9.78 ± 0.18	4.990 ± 0.156	15.45 ± 2.99
RE034	61 ± 3	28 ± 4	33 ± 2	8.95 ± 0.22	3.903 ± 0.582	9.72 ± 3.77
RE036	83 ± 3	44 ± 1	47 ± 3	8.45 ± 0.07	4.817 ± 0.189	16.43 ± 1.43

Table 2. *Cont.*

Strain	Yeast Biomass Colour on Grape-Skin Agar			Fermentation Vigour after 3 Days ($g\ CO_2$/100 mL)	Wine Colour Intensity	Phenolics Content
	Red Component	**Green Component**	**Blue Component**		**Absorbance (420 + 520 + 620) nm**	**Folin–Ciocalteu's Index (FC)**
RE037	76 ± 2	40 ± 2	40 ± 2	9.67 ± 0.11	2.580 ± 0.420	7.55 ± 6.37
RE038	76 ± 3	40 ± 4	41 ± 3	9.56 ± 0.14	4.462 ± 1.220	11.37 ± 2.02
RE039	74 ± 1	37 ± 2	42 ± 4	9.63 ± 0.18	4.235 ± 0.075	13.78 ± 0.88
RE040	62 ± 2	31 ± 3	35 ± 3	8.67 ± 0.22	4.922 ± 0.473	12.32 ± 3.07
RE041	85 ± 3	45 ± 3	48 ± 3	8.36 ± 0.08	2.383 ± 0.626	6.15 ± 4.90
RE042	76 ± 2	36 ± 2	41 ± 2	9.67 ± 0.07	3.532 ± 0.647	12.53 ± 1.46
RE043	76 ± 3	37 ± 2	42 ± 1	7.89 ± 0.11	2.517 ± 0.756	7.52 ± 5.95
RE044	112 ± 6	69 ± 4	64 ± 2	6.89 ± 0.14	4.397 ± 0.305	19.90 ± 0.97
RE045	81 ± 3	43 ± 1	43 ± 1	9.89 ± 0.18	4.912 ± 0.903	17.5 2 ± 11.23
RE046	94 ± 4	54 ± 1	54 ± 2	7.45 ± 0.22	4.435 ± 0.371	12.40 ± 2.35
RE048	112 ± 4	69 ± 2	64 ± 3	8.92 ± 0.07	5.008 ± 0.375	23.67 ± 0.13
RE049	102 ± 2	56 ± 1	57 ± 1	7.12 ± 0.11	5.845 ± 0.153	25.18 ± 0.65
RE050	93 ± 3	52 ± 3	53 ± 2	8.76 ± 0.14	4.653 ± 0.888	12.72 ± 2.15
RE051	64 ± 5	36 ± 3	41 ± 3	9.82 ± 0.18	4.372 ± 0.518	12.97 ± 0.95
RE052	86 ± 5	45 ± 2	49 ± 4	9.75 ± 0.22	3.150 ± 1.197	9.92 ± 3.03
RE053	93 ± 4	47 ± 3	47 ± 1	7.56 ± 0.08	5.072 ± 0.719	12.20 ± 2.68
RE054	97 ± 4	50 ± 1	53 ± 1	7.50 ± 0.07	2.010 ± 0.752	9.45 ± 2.84
RE055	106 ± 3	62 ± 2	59 ± 3	8.72 ± 0.11	5.284 ± 0.367	22.90 ± 0.80
RE056	75 ± 4	37 ± 1	39 ± 2	7.69 ± 0.14	1.968 ± 0.450	14.58 ± 4.82
RE057	102 ± 3	56 ± 2	59 ± 2	7.98 ± 0.18	2.873 ± 0.475	19.82 ± 0.60
RE058	67 ± 1	31 ± 1	34 ± 2	8.23 ± 0.22	5.375 ± 0.679	19.98 ± 5.57
RE059	73 ± 2	37 ± 3	43 ± 2	7.54 ± 0.08	1.900 ± 0.566	9.05 ± 4.93
RE060	96 ± 2	53 ± 1	52 ± 2	8.88 ± 0.07	3.485 ± 0.805	13.03 ± 2.99
RE061	110 ± 2	68 ± 3	63 ± 2	6.56 ± 0.11	3.343 ± 0.296	18.82 ± 0.60
RE062	82 ± 2	43 ± 2	46 ± 1	9.55 ± 0.14	2.197 ± 0.955	10.87 ± 2.62
RE063	73 ± 2	37 ± 3	41 ± 1	8.21 ± 0.18	6.210 ± 0.874	22.83 ± 7.61
RE064	107 ± 2	64 ± 1	60 ± 2	8.84 ± 0.22	4.056 ± 0.318	19.17 ± 0.23
RE065	64 ± 1	32 ± 2	36 ± 2	7.71 ± 0.08	4.640 ± 1.043	14.30 ± 3.36
RE066	100 ± 3	56 ± 2	54 ± 1	7.34 ± 0.07	5.620 ± 0.231	20.48 ± 0.21
RE068	107 ± 2	65 ± 1	57 ± 2	7.65 ± 0.14	3.220 ± 0.304	14.37 ± 0.25

Table 2. *Cont.*

Strain	Yeast Biomass Colour on Grape-Skin Agar			Fermentation Vigour after 3 Days ($g\ CO_2/100\ mL$)	Wine Colour Intensity	Phenolics Content
	Red Component	Green Component	Blue Component		Absorbance (420 + 520 + 620) nm	Folin–Ciocalteu's Index (FC)
RE069	127 ± 2	87 ± 1	78 ± 2	7.87 ± 0.18	3.473 ± 0.476	16.15 ± 0.52
RE070	107 ± 1	65 ± 1	59 ± 1	7.45 ± 0.22	2.943 ± 0.927	16.42 ± 0.40
RE071	111 ± 3	68 ± 3	66 ± 2	7.76 ± 0.08	5.093 ± 0.213	25.48 ± 1.52
RE072	96 ± 3	53 ± 2	51 ± 2	7.68 ± 0.07	5.803 ± 0.363	12.87 ± 3.91
RE073	68 ± 2	34 ± 2	36 ± 2	7.38 ± 0.11	1.670 ± 0.527	7.70 ± 4.45
RE074	89 ± 2	48 ± 2	48 ± 1	7.98 ± 0.14	1.452 ± 0.414	7.57 ± 1.76
RE075	87 ± 3	45 ± 2	46 ± 2	7.89 ± 0.18	6.125 ± 0.423	10.55 ± 8.71
RE076	129 ± 5	88 ± 1	82 ± 1	8.93 ± 0.22	4.543 ± 0.508	22.77 ± 1.27
RE077	112 ± 3	71 ± 2	67 ± 1	9.78 ± 0.08	2.628 ± 0.340	15.80 ± 0.18
RE079	51 ± 2	28 ± 1	31 ± 3	6.98 ± 0.11	3.823 ± 0.078	13.32 ± 1.70
RE080	82 ± 2	41 ± 1	43 ± 1	7.78 ± 0.14	1.715 ± 0.373	8.48 ± 1.04
RE081	68 ± 4	33 ± 2	36 ± 3	7.12 ± 0.18	6.253 ± 0.437	19.13 ± 1.01
RE083	87 ± 4	47 ± 4	49 ± 3	7.54 ± 0.08	2.453 ± 1.164	8.08 ± 1.81
RE084	68 ± 3	33 ± 2	39 ± 1	7.66 ± 0.07	2.108 ± 0.798	7.47 ± 3.18
RE085	69 ± 3	35 ± 1	39 ± 2	8.12 ± 0.11	2.013 ± 0.894	7.00 ± 3.43
RE086	65 ± 2	35 ± 1	39 ± 2	9.31 ± 0.14	2.038 ± 0.736	7.33 ± 2.38
RE087	64 ± 5	29 ± 1	35 ± 4	7.23 ± 0.18	2.365 ± 0.573	9.17 ± 1.37
RE088	96 ± 3	54 ± 2	54 ± 2	6.99 ± 0.22	2.198 ± 0.778	8.42 ± 1.52
RE089	85 ± 3	44 ± 4	45 ± 1	7.23 ± 0.08	2.132 ± 0.878	7.53 ± 3.96
RE090	106 ± 3	65 ± 1	62 ± 1	7.54 ± 0.07	5.588 ± 0.316	23.57 ± 1.25
RE092	80 ± 3	40 ± 2	43 ± 2	7.98 ± 0.14	2.295 ± 0.898	10,42 ± 2.93
RE093	82 ± 4	42 ± 3	48 ± 3	7.54 ± 0.18	5.325 ± 0.158	15.07 ± 3.66
RE097	59 ± 3	27 ± 2	33 ± 2	6.87 ± 0.18	4.222 ± 0.762	10.50 ± 3.50
RE098	82 ± 1	41 ± 1	42 ± 1	7.78 ± 0.22	5.570 ± 0.413	17.17 ± 3.27

Data highlighted in grey have caused the exclusion of the strains as they indicate a higher adsorption activity of pigments and phenolics. Bold font indicates the strain selected for further tests. The data are expressed as mean value ± standard deviation.

3.2. Assessment of the Fermentation Vigour and Adsorption Activity

The screening of the pigment's adsorption activity, assessed as biomass colour on grape-skin agar, underlined a different aptitude of the preselected strains (Table 2). According to Caridi [20], in a colour image, the intensity values ranged from zero (black) to 255 (white) for each of the red, green, and blue components, therefore, high grape pigment adsorption matched low values of these components.

In our screening, the lowest value of the red component was observed for the strain RE079 (51 ± 2) while the strain RE076 showed the highest value (129 ± 5) with a mean value of 83.

The green component of the strain biomass colour ranged from a minimum value of 27 ± 2 (strain RE097) to a maximum value of 88 ± 1 (strain RE076) with a mean value of 45. Regarding the blue component, data ranged from a minimum value of 31 ± 3 (strain RE079) to a maximum value of 82 ± 1 (strain RE076) with a mean value of 46.

The strains which exhibited values less or equal to the mean value (arbitrarily chosen as threshold) of a colour component were considered unsuitable for the final selection due to their greater adsorption activity.

Regarding the micro-winemaking trials, the fermentation vigour, detected for all the strains after 3 days, was generally considered satisfying and no exclusions were made for this parameter in the screening. At the end of the fermentation process, the wine colour and the phenolics content were also assessed. In particular, the colour intensity, obtained by adding the wine absorbance read at 420, 520, and 620 nm, ranged from a minimum value of 1.452 ± 0.414 (strain RE074) to a maximum value of 6.417 ± 0.336 (strain RE017) with a mean value of 3.798. Regarding the adsorption activity of phenolics, assessed on the basis of the FC index, it ranged from a minimum value of 6.15 ± 4.90 (strain RE041) to a maximum value of 25.48 ± 1.52 (strain RE071) with a mean value of 13.35.

Similar to what is described above, the mean values were chosen as threshold, therefore, the strains which exhibited values less or equal to the mean values were excluded due to an adsorption activity that was considered too high. In total, 75 strains were discarded at the end of the screening for one or more data highlighted in grey in Table 2.

3.3. Assessment of the OTA Adsorption Activity and Final Strain Selection

The strains, which passed the previous screening, were tested for the OTA adsorption activity (Figure 2). The percentage of OTA removed from the preselected strains ranged from a minimum value of 34.52 ± 1.80 (strain RE064) to a maximum value of 48.96 ± 4.93 (strain RE049). The ANOVA analysis of the OTA data revealed no significant difference among the strains with the only exception being that between RE049 and RE064 ($p < 0.05$). Interestingly, significant differences were observed among the fermentative vigour data of the 10 strains (Figure 3). In particular, the strains RE048 (8.92 ± 0.07) and RE076 (8.93 ± 0.22) showed the highest values. The latter is definitively the best strain as it combines all the desired traits, although the other nine strains can also be considered good candidates for winemaking.

The values (%) of OTA adsorption activity, showed by the selected strains, are summarised in Table 3. The strains have been safe deposited in UMCC and recorded in the database with a UMCC code.

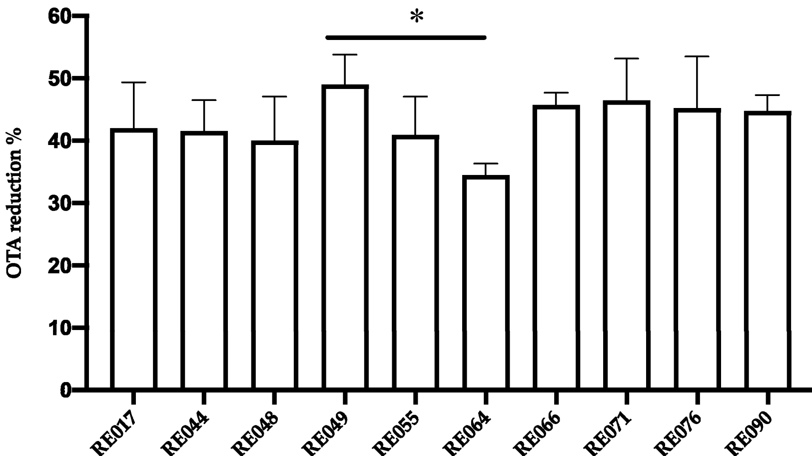

Figure 2. Ochratoxin A (OTA) reduction (%) obtained with the *S. cerevisiae* strains which passed the previous trials. The asterisk indicates significant differences at 95% of confidence level ($p < 0.05$) between strains RE049 and RE064. Bars indicate ± standard deviation.

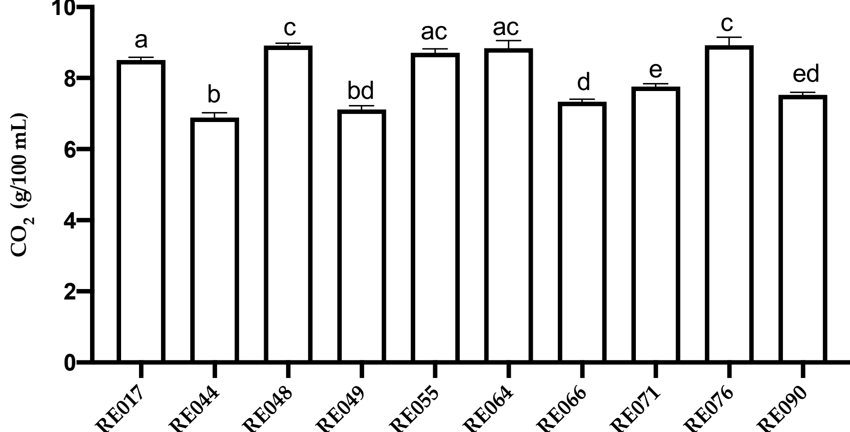

Figure 3. Fermentative vigour of the *S. cerevisiae* strains for which OTA reduction activity was detected. Different superscript letters within the column indicate significant differences in data set according to one-way ANOVA and Tukey's HSD post hoc test tests at $p < 0.05$. Bars indicate ± standard deviation.

Table 3. *S. cerevisiae* strains deposited in Unimore Microbial Culture (UMCC) and their OTA absorption activity.

UMCC Code	Original Code	OTA Absorption Activity (%)
UMCC 2954	RE017	42.03 ± 7.33
UMCC 2955	RE044	41.54 ± 4.98
UMCC 2956	RE048	40.01 ± 7.08
UMCC 2957	RE049	48.96 ± 4.83
UMCC 2958	RE055	40.90 ± 6.18
UMCC 2959	RE064	34.52 ± 1.80
UMCC 2960	RE066	45.72 ± 1.95
UMCC 2961	RE071	46.42 ± 6.75
UMCC 2962	RE076	45.23 ± 8.28
UMCC 2963	RE090	44.75 ± 2.55

4. Discussion

Nowadays, winemakers demand starter cultures with a whole range of specific properties that largely differ according to the type and style of wine to be made, as well as to the technical requirements

of the winery [18]. A new frontier goal for yeasts selection intended as a starter for wines is represented by the ability to remove OTA.

During the past years, significant correlations between yeast strains used for winemaking and phenolic content of wines have been reported, proving that yeast behaviour can somewhat modify chromatic properties, phenolic profile, antioxidant power [27,28] as well as the aroma profile of wine [29,30]. In fact, greater or lesser phenolic adsorption on a yeast cell wall influences the concentration and the composition of phenolics in wine [31–33]. Moreover, the parietal adsorption of pigments during alcoholic fermentation has important consequences because of the loss of wine colour [34,35].

Several authors [2,18,36,37] have proposed a specific selection of wine yeasts that are able to get a selective removal of OTA. However, strains which remove more OTA, also adsorb more colour and phenolics from wine. For this reason, in our selection, only the strains with pigments and phenolics adsorption activity below the mean observed values were chosen for the OTA screening. This was carried out after 28 days of fermentation, considering that OTA is mainly adsorbed during the yeast exponential growth phase and, in some cases, again released in wine, probably due to the premature autolysis of yeast cells [16].

The percentages of OTA adsorption assessed are considered good in accordance with the data reported by Petruzzi et al. [17] and Aponte and Blaiotta [16].

In the present work, the extensive yeasts selection, starting with the collection of oenological samples from three Mediterranean areas particularly interested in OTA contamination [10,11], allowed us to isolate a high number of strains that were potentially able to possess the ability to remove the toxin. Certainly, good wine yeasts must always feature both good primary traits, which are related to the overall fermentative fitness of the strain, and secondary traits, which provide accessory features of technological value [38,39]. Nevertheless, even if the naturally isolated strains do not meet all the desired traits for winemaking, they generate a biodiversity background, which is very useful for successive improvements [40]. In particular, the genetic improvement of yeast strains can be achieved in several ways by exploiting the suitable method according to the complexity of the targeted character and the knowledge of molecular and regulatory interactions, which lie behind a specific desired trait [41,42].

In the context of our work, the inheritable nature of the adsorption of wine colour and OTA was recently analysed on descendants derived from wine strains of *S. cerevisiae* [33,43,44]. Investigation on the progeny demonstrated that adsorption of wine colour and OTA are polygenic inheritable quantitative traits loci, partially and interdependently correlated to colour and phenolic content of wines. This may justify the further improvement of the 10 strains obtained from our selection. In fact, as all the selected strains were able to sporulate, a yeast breeding approach to obtain new strains with traits of interest could be performed through sexual recombination and hybridisation strategies.

Moreover, since indigenous strains are believed to be able to maintain the typical sensory properties and to enhance the peculiarities of a wine [45], the influence of the strains on the aroma profile could also be considered in a further study.

5. Conclusions

The sequential screening implemented in this study allowed a selective reduction in a high number of isolates by excluding those definitely unable to be candidates as wine starters.

The strains reported in Table 3 were specifically selected for the desired features and, among them, the strain RE076 stands out for its high fermentative vigour.

To our knowledge, this is the first work in which the selection of wine strains, exhibiting good fermentative performance and OTA removal activity, was achieved, taking into account the low adsorption activity of pigments and phenolics.

The natural variability of yeasts for the studied traits may be exploited to obtain a further enhancement of the definite adsorption activity of wine strains through specific genetic improvement

strategies in order to develop new strains that possess the desired features for winemakers above the high fermentative fitness.

Author Contributions: All authors participated in the design and discussion of the research. Specific contributions were conceptualisation and supervision, A.P. and A.C.; methodology and investigation, A.P., G.B., R.S., A.C.; data curation, writing—review and editing, L.D.V.; visualisation and editing, G.I.; review and editing, M.G. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Italian Ministry of Education, University and Research, PRIN (Projects of Relevant National Interest), title of the project: "Wine strain improvement strategies to enhance red wine safety based on parietal adsorption activity".

Conflicts of Interest: The authors declare no conflict of interest.

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