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Fermentative Stability of Wine Yeast *Saccharomyces Sensu Stricto* Complex and Their Hybrids

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Summary

The objective of this paper is to investigate the technological usefulness of selected industrial wine yeasts *Saccharomyces cerevisiae* and *Saccharomyces bayanus* and their intra- and interspecific hybrids responsible for excessively acidic musts. The stability of yeast fermentation profiles in apple musts was assessed after 90–170 generations, following previous subculturing under aerobic or anaerobic conditions in media with or without L-malic acid. During this study, 35 apple wines produced by wild strains and their segregates were statistically evaluated according to 12 chemical parameters. Although the wines met the official standards for basic chemical parameters, their total acidity was too low. Both the yeasts and their segregates metabolized from 66.3 to 77.0 % of malic acid present in the must. The industrial wine yeasts and their hybrids exhibited marked polymorphism of fermentation profiles in apple must with elevated L-malic acid content. At the same time, the level of demalication activity made it possible to clearly differentiate segregates from the wild strains, which may suggest that malic acid is probably one of the principal factors in the adaptive evolution of yeasts. Our study proves that among industrial wine yeasts, there are both, strains expressing very high stability (*Saccharomyces cerevisiae* W-13) and labile ones (*S. cerevisiae* Syrena). The interspecific hybrids *S. cerevisiae* × *S. bayanus* showed low stability of technological features, while the intraspecific hybrid of *S. cerevisiae* preserved its fermentative capacity. The presented results indicate that fermentative stability assessment under environmental stress can help to select the yeast strains best suited for the fermentation of specific musts.

Key words: wine yeasts, yeast hybrids, *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, stability

Introduction

The role of yeasts in the creation of the organoleptic qualities of wine has been widely discussed (*1–5*). Yeasts are not only responsible for alcoholic fermentation, but also produce minor metabolites crucial for the unique taste and aroma of wine. During vinification, yeasts are subjected to constant changes of environmental conditions, which affect their physiological and genetic state (*6,7*). Wine yeasts of the *Saccharomyces sensu stricto* complex are known for their ability to respond quickly to a variety of environmental stresses, resulting in their better adaptability to vinification processes (*6,8*). Moreover, due to horizontal gene transfer within the *Saccharomyces* group, many wine yeasts are intra- or interspecific hybrids (*8*). Natural hybrids between *S. cerevisiae* and *S. bayanus* (*9*), *S. cerevisiae* and *S. bayanus* var. *uvarum* (*10*), *S. cerevisiae*, *S. bayanus* and *S. kudriavzevii* (*11*), *S. cerevisiae* and *S. kudriavzevii* (*12–15*) serve as good examples of yeasts involved in wine and cider production. Laboratory-constructed interspecific wine yeast hybrids are also interesting in oenological terms (for a review see *16*) and

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interspecific hybrids *S. cerevisiae* and *S. bayanus* were successfully used for fermentation at low temperatures and with acidity regulation (*17–19*). Phenotypes of interspecific hybrids within *Saccharomyces* were shown as homogeneous (*20*) with high fermentation competitiveness (heterosis) and intermediate production of secondary metabolic compounds compared to parental strains (*17*). Mitochondrion genomes of wine yeast hybrids are homoplasmic (for a review see *8*); however, the uniparental inheritance in artificial hybrids of *S. cerevisiae* and *S. uvarum* was also proved (*21,22*). The model of fast adaptive genome evolution (FAGE) suggested for wine yeasts indicates the possibility of inducing genotypic changes both during vegetative growth and at the sexual stage (*23*). Taking into account the plasticity of wine yeast genome, there arises the question of the stability of their technological features and their sensitivity to environmental stress during fermentation. Highly acidic musts are a problem in wineries of cold regions. Excessive acidity both adversely affects the wine yeasts (*24*) and leads to wines with an improper balance among sugar, acid and aroma components (*25–27*). Biological deacidification with the use of wine yeasts expressing accelerated acid metabolism is one of the solutions. Within the range of organic acids contributing to must acidity, L-malic acid is the only one metabolized by *Saccharomyces* yeasts during fermentation. Tartaric and malic acids are the main organic acids in grapes, as they account for up to 90 % of all organic acids present (*28*). In other fruits used in Polish wineries, malic acid content reaches 94–98 % of total acids present in apples and cherries and up to 65 % in pears (*29*). However, all wine yeasts belong to K(–) group, which are unable to metabolize intermediates of tricarboxylic acid (TCA) cycle as a sole carbon and energy source (*30*), unless in the presence of assimilable carbon sources. Their ability to degrade malate is strain-dependent and varies from 0 to 48 % (*26,31*). The weak degradation of malate is attributed to the efficiency of dicarboxylic acid transport and low substrate affinity of malic enzyme (*27,32,33*). Moreover, malic enzyme located in mitochondria is suppressed due to the reduced number of mitochondria and their dysfunction under vinification conditions (*27,31*). To meet the consumers' demand for high quality wines, there is a need for valuable wine yeast strains expressing high fermentative stability.

Previously, we had selected the industrial yeasts *S. cerevisiae* and *S. bayanus* with accelerated ability of L-malic acid decomposition (*34*), and produced their intra- and interspecific hybrids (*35*). The yeasts were then subcultured in standard media under aerobic and anaerobic conditions, simultaneously subjected to acidic stress. Both the wild yeasts (unpublished data) and their hybrids (*36*) expressed varied, strain-dependent physiological and genetic stability in the presence of malic acid. The objective of this paper is to investigate the technological usefulness of parental strains and their intra- and interspecific hybrids within the *Saccharomyces sensu stricto* complex under acidic stress. Even though grape cultivation in Poland has been intensively developed in the last four years, Polish wineries still rely on fruit juices. Apple musts are not only used in cider production, but also as a component of fruit wines. In the light of Polish law, according to the Act of Winemaking from 2011 (*37*), branded fruit wine may also be produced from fruits other than grapes. Moreover, Poland is one of the leading producers of apples in the EU, covering 24 % of the European production (*38*). Thus, the stability of their fermentation profiles in apple musts with elevated L-malic acid content was assessed. During this study, 35 apple wines produced by wild strains and their segregates were statistically evaluated according to 13 chemical parameters.

Materials and Methods

Microorganisms

The following wine yeasts were used: two strains of *Saccharomyces cerevisiae* (Syrena and W-13) and one *Saccharomyces bayanus* Cz-2. *S. cerevisiae* Syrena and W-13 are industrial strains commonly used in Poland and are deposited in the Collection of Pure Cultures of the Institute of Fermentation Technology and Microbiology (ŁOCK 105), Technical University of Łódź, Łódź, Poland. Strain Cz-2 was isolated from Italian dried wine yeast *S. bayanus* purchased from F.lli Marescalchi S.p.A. (Casale Monferrato, Italy).

Moreover, the following wine yeast hybrids were used: one intraspecies hybrid of *S. cerevisiae* Syrena and *S. cerevisiae* W-13 (HG3-2) and three interspecies hybrids of *S. cerevisiae* Syrena and *S. bayanus* Cz-2 (HW2-3, HW2-6 and HW2-7). Hybrids were produced by natural hybridization using 'mass-mating' technique where vegetative haploid cells of opposite mating types were used. Haploids had previously been obtained by parental strain ascus dissection and spore germination (*39*). The hybrids were deposited in the Collection of Pure Cultures of the Institute of Fermentation Technology and Microbiology (ŁOCK 105). The microorganisms were activated through double passaging in YGP liquid medium (in g/L: yeast extract 10, glucose 20 and peptone 10) at 28 °C for 48 h.

Yeast segregates

All yeasts were subcultured 20 times under aerobic or anaerobic conditions in both YGP and YG (in g/L: yeast extract 4, glucose 100, L-malic acid 7, KH_2PO_4 5 and $MgSO_4$ 0.4; at pH=3.0) media. Aerobic cultures were conducted in 50 mL of liquid YGP or YG medium in 100-mL flat-bottomed flasks at 28 °C for 48 h and constantly shaken (200 rpm). Anaerobically, yeasts were grown in 110 mL of YGP or YG medium in conical 250-mL flasks for 7 days at 25 °C. The media were inoculated with 1 % yeast cell suspensions in saline solution (γ(NaCl)=8.5 g/L) standardized to a density of 10^8 CFU/mL. Numbers of generations were estimated according to Mesa *et al*. (*40*). After completing the last passage, yeast cells were centrifuged, resuspended in YGP medium and frozen at –80 °C with glycerol added to 50 %. Streaks from the starting frozen samples were incubated at 28 °C for 48 h on YGP agar plates. Subsequently, 10 representative colonies were picked randomly from the plates, and subjected to macro- and micromorphological analyses. Because no differences in morphological features were observed, the biomass of frozen samples was used to prepare precultures for wine inoculation.

Apple musts and fermentations

Fermentations were carried out in triplicate in 2000 mL of apple musts with the addition of 190 g/L of sucrose (γ(total sucrose)=267.5 g/L) and 7 g/L of L-malic acid, pH=3.01, and incubated at 28 °C for 30 days. The musts were inoculated with precultures of yeasts to a final concentration of 5 % (by mass per volume). To avoid the acidic stress, the precultures were prepared in apple must with 4 g/L of L-malic acid and 245 g/L of sucrose, pH=3.50, and incubated at 28 °C for 2 days before inoculation.

Chemical analysis

L-malic acid and ethanol were determined enzymatically with specialized kits (Roche Diagnostics GmbH, Mannheim, Germany). Succinic, lactic and acetic acids, acetaldehyde, ethanol and glycerol were determined by the HPLC method (*41*). Reducing sugars, total extract, total acidity, pH, total SO_2 and free SO_2 were determined according to the official analytical methods of the International Organization of Vine and Wine (OIV) (*42*).

Statistical analysis

Results were presented as an arithmetic mean of six determinations and were analyzed using a 3-way analysis of variance (ANOVA) test at a confidence level of $p<0.05$. Calculations were conducted using STATISTICA v. 7.1. software (*43*). To discern patterns between variables and wine samples, statistical analysis of 13 wine parameters was conducted by principal component analysis using the same software.

Results and Discussion

The fermentative stability of three industrial wine yeasts and their four hybrids was assessed after approx. 90–170 generations (data not presented), depending on the strain and culture conditions. Under the assumption that changes occurring in microorganism populations over 20 generations reflect evolutionary processes (*44*), we tried to follow the physiological and genetic diversity of the yeasts subjected to acidic stress. To complete our research on the physiological and genetic changes of the studied yeasts (*36*), we investigated their technological features during apple must fermentation.

General wine parameters

Tables 1 and 2 show the mean values of the general parameters (N=6) of wines fermented by wild wine strains and their hybrids, respectively. Changes in the mean values of these parameters are presented for aerobic and anaerobic segregates, and statistically significant differences ($p<0.05$) in comparison with the nonsubcultured wild yeasts are given. The levels of reducing sugars and extract were strain dependent and comparable to *S. cerevisiae* industrial strains and all the hybrids tested. Generally, the high level of reducing sugars indicates incomplete must fermentation, which may be due to the absence of wine mellowing. The level of ethanol in apple wines, ranging from 10.26 to 15.73 % by volume, was comparable to wines manufactured from highly acidic musts (*31,45*). Ethanol content in wines produced with the hybrids was up to 3.5 % lower than that in wines produced with wild parental strains (Tables 1 and 2). Similarly, lower ethanol content in grape wines fermented by the interspecific hybrids of *S. cerevisiae* and *S. bayanus* was observed by Coloretti *et al.* (*46*). Statistically significant differences in ethanol production were found only for *S. cerevisiae* W-13 and the majority of the hybrid segregates. Substantial variation was found in glycerol content. The wine yeasts and their segregates produced from 3.45 to 7.19 g of glycerol per 1 L of wine, and strain-dependent differences reached 110 %. Among the aerobic segregates of the hybrids, only the interspecific strain HW2-3 changed its glycerol production; in contrast, nearly all anaerobic ones expressed notable differences in its level. Previous studies showed considerable variation among strains in terms of glycerol production (*47,48*), and its content in wines was determined at 2 to 11 g/L. The levels of glycerol content in the wines produced with the studied yeasts have led to their classification, pursuant to Grazia *et al.* (*47*), into strains producing small quantities of glycerol, *i.e.* 3.5–5.0 g/L (hybrids HW2-3 and HW2-6 and their segregates), and large quantities of glycerol, *i.e.* 5–7.5 g/L (*S. cerevisiae* Syrena, W-13, *S. bayanus* Cz-2, as well as hybrids HW2-7 and HG3-2 and their segregates).

Another significant influence on wine quality is exerted by reduced sulphur compounds (*49*). Our results reveal that total SO_2 content remains below half of the threshold values recommended for fruit wines (*50*), as well as for nonsulphited grape wines (*22*). Wines produced with wild yeast segregates usually showed an increase in total SO_2 content by 3.2–16.6 g/L, while segregates of the hybrids generated less SO_2 than the hybrids themselves (by up to 20 g/L).

Acetaldehyde and organic acids

Organic acid profiles were unique for all wine yeast strains and their segregates, which indicates individual yeast changes irrespective of subculturing conditions. According to literature data (*48*), the ability of yeasts to overproduce glycerol may lead to an increased content of acetaldehyde, acetic acid, and succinic acid in wine. Statistical analysis of our data confirms a relationship between glycerol content and acetic and succinic acid concentrations, with the correlation coefficients of 0.61 and 0.69, respectively, at the confidence level of $p<0.05$. However, glycerol and acetaldehyde levels were not found to be correlated.

Acetaldehyde is the main aldehyde normally produced during vinification and its amount can be influenced by the yeast strain (*4*), although some authors believe that its content in wine is mostly determined by environmental factors (*48*). Under the same fermentation conditions, the tested wine yeasts, their hybrids and all segregates produced various amounts of acetaldehyde, ranging from 11 to 56 mg/L of wine. Aldehyde content in the wines was significantly higher than that reported by Remize *et al.* (*48*), but consistent with the data published by other authors (*4,45*).

Another positive contribution to wine quality is made by lactic acid, which additionally has preservative properties. Due to the low activity of mitochondrial lactate dehydrogenase, *Saccharomyces* yeasts generate only small

Table 1. Chemical profiles of wines produced by *Saccharomyces sensu stricto* yeasts and their segregates

Yeasts	γ(reducing sugars) g/L	γ(total extract) g/L	φ(ethanol) %	γ(glycerol) g/L	γ(total acidity) g/L	pH	γ(L-malic acid) g/L	γ(succinic acid) g/L	γ(lactic acid) g/L	γ(acetic acid) g/L	γ(acet-aldehyde) mg/L	γ(total SO_2) mg/L	γ(free SO_2) mg/L
Wild strains													
Syrena	13.16 ±0.96	40.50 ±0.00	15.05 ±0.30	6.69 ±0.05	4.50 ±0.00	3.52 ±0.02	2.36 ±0.03	0.94 ±0.01	0.26 ±0.01	0.27 ±0.04	44 ±1	17.2 ±0.1	11.2 ±0.1
W-13	15.58 ±0.60	34.45 ±1.00	13.97 ±0.65	7.19 ±0.22	4.20 ±0.08	3.85 ±0.04	2.31 ±0.05	0.85 ±0.06	0.73 ±0.01	0.73 ±0.01	25 ±1	10.6 ±0.2	6.7 ±0.2
Cz-2	1.66 ±0.39	20.12 ±0.00	15.73 ±0.15	5.65 ±0.01	4.04 ±0.09	3.80 ±0.02	2.27 ±0.05	0.64 ±0	0.46 ±0.01	0.27 ±0.02	22 ±1	17.9 ±0.0	10.2 ±0.0
Aerobic segregates*													
Syrena/A	6.54[b]	5.21[b]	–1.30[b]	0.19[a]	–0.37[b]	0.10[b]	–0.23[b]	–0.12[b]	0.09[a]	0.12[b]	–16[b]	–1.5[b]	–2.6[b]
Syrena/AM	–5.05[b]	–10.22[b]	–0.15[a]	0.14[a]	0.19[a]	–0.04[a]	0.04[a]	0.03[a]	0.48[b]	0.25[b]	12[b]	0.1[a]	0[a]
W-13/A	–5.39[b]	–6.68[b]	–0.18[a]	–0.55[b]	–0.15[a]	0.03[a]	0.04[a]	0.04[a]	0.08[a]	–0.24[b]	–3[b]	3.2[b]	0.3[a]
W-13/AM	2.65[b]	13.91[b]	–0.53[a]	–0.70[b]	–0.04[a]	0[a]	0.04[a]	0.09[a]	0.01[a]	–0.34[b]	–4[b]	1.0[a]	–1.6[b]
Cz-2/A	0.86[b]	26.42[b]	–2.12[b]	0.08[a]	0.05[a]	0[a]	–0.29[b]	0.01[a]	0.18[b]	0.02[a]	6[b]	5.8[b]	4.8[b]
Cz-2/AM	0.20[a]	0[a]	–0.25[a]	0.42[b]	0.09[a]	0[a]	–0.64[b]	0.24[b]	0.12[b]	–0.04[b]	11[b]	1.0[a]	0[a]
Anaerobic segregates*													
Syrena/An	9.02[b]	12.99[b]	–2.20[b]	–1.69[b]	–0.83[b]	0.17[b]	–0.22[b]	–0.44[b]	0.03[a]	0.02[a]	–33[b]	–6.6[b]	–5.8[b]
Syrena/AnM	7.82[b]	5.21[b]	–2.25[b]	–0.77[b]	–0.34[b]	0.10[b]	–0.30[b]	–0.19[b]	0.03[a]	0.07[a]	–26[b]	–9.2[b]	–6.4[b]
W-13/An	0.64[a]	2.50[b]	–0.85[a]	–0.17[a]	0.03[a]	0[a]	0.03[a]	–0.10[a]	0.07[a]	–0.31[b]	–2[b]	0.3[a]	0.2[a]
W-13/AnM	1.47[b]	2.67[b]	–0.47[a]	–0.50[b]	0.13[a]	–0.03[a]	0[a]	–0.02[a]	–0.04[a]	–0.33[b]	–3[b]	3.6[b]	–0.7[a]
Cz-2/An	1.89[b]	20.47[b]	–1.46[b]	0.23[b]	0.24[b]	–0.05[b]	–0.49[b]	0.02[a]	0.15[b]	0.07[b]	9[b]	–2.0[b]	–0.9[a]
Cz-2/AnM	25.80[b]	44.81[b]	–3.15[b]	0.51[b]	–0.10[b]	0.02[b]	–0.25[b]	0.28[b]	–0.13[b]	0.16[b]	2[b]	16.6[b]	9.4[b]

A=aerobic segregates, An=anaerobic segregates, M=segregates grown under acidic stress
*changes expressed in appropriate units
[a]not statistically significant differences ($p<0.05$) compared to wild strains
[b]statistically significant differences ($p<0.05$) compared to wild strains

Table 2. Chemical profiles of wines produced by yeast hybrids and their segregates

Yeasts	γ(reducing sugars)	γ(total extract)	φ(ethanol)	γ(glycerol)	γ(total acidity)	pH	γ(L-malic acid)	γ(succinic acid)	γ(lactic acid)	γ(acetic acid)	γ(acet-aldehyde)	γ(total SO_2)	γ(free SO_2)
	g/L	g/L	%	g/L	g/L		g/L	g/L	g/L	g/L	mg/L	mg/L	mg/L
Hybrids													
HG3-2	25.20 ±0.70	38.45 ±0.75	12.77 ±0.03	6.34 ±0.16	3.68 ±0.15	3.95 ±0.04	2.15 ±0.06	0.69 ±0.01	0.23 ±0.04	0.53 ±0.03	17 ±0	14.1 ±0.6	8.3 ±0.6
HW2-3	21.46 ±0.26	56.10 ±1.40	13.63 ±0.52	4.41 ±0.07	3.71 ±0.04	3.98 ±0.05	2.07 ±0.12	0.54 ±0.03	0.39 ±0.02	0.21 ±0.01	24 ±1	29.4 ±0.6	15.0 ±0.3
HW2-6	19.48 ±0.68	50.10 ±0.90	11.53 ±0.12	5.42 ±0.03	3.11 ±0.19	4.20 ±0.07	1.82 ±0.04	0.62 ±0.02	0.23 ±0.01	0.35 ±0.01	24 ±3	12.2 ±0.6	12.2 ±0.6
HW2-7	15.70 ±1.10	40.60 ±0.40	11.51 ±0.18	5.29 ±0.22	3.11 ±0.11	4.20 ±0.07	1.61 ±0.08	0.71 ±0.07	0.18 ±0.00	0.30 ±0.06	15 ±3	17.3 ±0.6	12.2 ±0.6
Aerobic segregates*													
HG3-2/A	–0.79[a]	20.25[b]	–0.35[a]	–0.52[a]	0.22[a]	–0.04[a]	0.14[a]	0[a]	0.03[a]	–0.09[b]	–2[b]	–5.5[b]	–3.5[b]
HG3-2/AM	–3.70[b]	17.64[b]	–0.25[a]	0.03[a]	0.18[a]	–0.04[a]	–0.01[a]	0.03[a]	0.02[a]	0.04[a]	1[a]	–3.5[b]	–1.3[b]
HW2-3/A	–3.45[b]	–17.61[b]	–2.59[b]	–0.96[b]	–0.37[b]	0.10[b]	–0.03[a]	–0.25[b]	0.34[b]	0.03[a]	–13[b]	–3.5[b]	–4.8[b]
HW2-3/AM	–12.83[b]	5.17[b]	–0.04[a]	–0.50[b]	–0.33[b]	0.09[b]	–0.28[a]	0[a]	0[a]	0[a]	–6[b]	3.9[b]	5.2[b]
HW2-6/A	–1.26[b]	–4.35[b]	0.14[a]	0.09[a]	0.15[a]	–0.04[a]	0.05[a]	0.09[b]	–0.03[b]	–0.02[a]	5[a]	–5.1[b]	–5.8[b]
HW2-6/AM	–10.42[b]	–22.38[b]	0.24[a]	–0.02[a]	0.23[a]	–0.04[a]	0.22[b]	0.14[b]	–0.04[b]	–0.01[a]	–8[b]	–0.3[a]	–3.3[b]
HW2-7/A	–2.80[b]	–5.19[b]	–0.10[a]	–0.07[a]	0.34[b]	–0.10[b]	0.15[a]	0.06[a]	0.01[b]	–0.03[a]	6[a]	–1.6[b]	–1.6[b]
HW2-7/AM	–0.18[a]	–3.12[b]	0.21[a]	0.18[a]	0.53[b]	–0.11[b]	0.32[b]	0.05[a]	–0.02[b]	0.11[b]	11[b]	–1.6[b]	–2.0[b]
Anaerobic segregates*													
HG3-2/An	14.33[b]	36.00[b]	–1.91[b]	–0.65[b]	0.15[a]	–0.04[a]	–0.09[a]	–0.07[b]	0.10[b]	0.12[b]	2[b]	–2.3[b]	–1.3[b]
HG3-2/AnM	–5.22[b]	7.25[b]	0.23[a]	–0.10[a]	0.26[a]	–0.05[a]	–0.09[a]	0.05[a]	–0.06[b]	–0.04[a]	0[a]	–2.9[b]	–0.6[a]
HW2-3/An	9.34[b]	14.96[b]	–3.37[b]	–0.46[b]	–0.11[a]	0.02[a]	0.08[a]	–0.10[b]	–0.21[b]	–0.03[a]	–11[b]	–19.8[b]	–9.2[b]
HW2-3/AnM	0.56[a]	–5.21[b]	0.43[a]	0.68[b]	0.42[b]	–0.10[b]	0.11[a]	0.21[b]	–0.12[b]	0.02[a]	–5[b]	–13.4[b]	–6.4[b]
HW2-6/An	–6.33[b]	–4.38[b]	–0.26[a]	–1.58[b]	0.49[b]	–0.11[b]	0.36[b]	–0.18[b]	–0.08[b]	–0.02[a]	–1[b]	–7.3[b]	–7.1[b]
HW2-6/AnM	–3.26[b]	–4.38[b]	0.15[a]	–1.46[b]	0.72[b]	–0.15[b]	0.69[b]	–0.16[b]	–0.08[b]	–0.06[a]	–3[b]	–4.1[b]	–5.2[b]
HW2–7/An	12.88[b]	20.75[b]	0.45[a]	–0.79[b]	0.83[b]	–0.19[b]	0.58[b]	–0.04[a]	0.34[b]	–0.07[a]	5[a]	–3.2[b]	–4.5[b]
HW2-7/AnM	–0.40[a]	–1.33[a]	–0.18[a]	0.03[a]	0.88[b]	–0.21[b]	0.57[b]	0.04[a]	0.04[b]	–0.02[a]	7[a]	–2.5[b]	–2.7[b]

A=aerobic segregates, An=anaerobic segregates, M=segregates grown under acidic stress
*changes expressed in appropriate units
[a]not statistically significant differences ($p<0.05$) compared to wild strains
[b]statistically significant differences ($p<0.05$) compared to wild strains

amounts of lactic acid during alcoholic fermentation (*51*), and its levels in grape wines produced only with wine yeasts usually range from 0.01 to 0.4 g/L (*4,31,45*). The analyzed strains and their segregates generated lactic acid at a level similar to that of acidic grape musts (*31*). The content of lactic, malic, acetic and succinic acids contributes to the total acidity of wines, and the biodegradation of L-malic acid during fermentation leads to a considerable decrease in their acidity (*52*). The tested wine yeasts had previously been recognized as strains with elevated demalication activity (*34*). Both the wild yeasts and their segregates metabolized from 66.3 to 77.0 % of malic acid present in the must, depending on the strain. At the same time, their demalication activity was by 30–92 % higher than that reported in the literature (*18,46,52–54*). Malate utilization by all hybrids was 2.05–10.7 % higher, compared to their parental strains. However, the interspecific hybrids HW2-3 and HW2-7 expressed the highest activity in malate decomposition, but their segregates did not preserve this feature at the same level (Table 2). Statistically significant changes in the ability to decompose L-malic acid were found in 46 % of the segregates, but yeast subculturing conditions did not influence this feature in a consistent manner.

The level of succinic acid was similar (*46*) or lower (*31*) than that determined for white grape wines, and no correlation was found between malic and succinic acid content. Due to the high demalication activity during vinification, the total acidity of all the wines was significantly diminished, down to 3.11–4.69 g/L, depending on the strain. During must fermentation, a slight, continuous increase in volatile acidity was observed, resulting primarily from the presence of acetic acid. While the increase of acetic acid level in white wines up to 0.9 g/L is normally acceptable (*55*), its actual content usually ranges from 0.3 to 0.8 g/L (*48*) and depends on the yeast strain (*48,56*) and environmental conditions (*56*). Despite the substantial differences between particular yeast strains and their segregates, acetic acid content in the wines was low and varied from 0.21 to 0.73 g/L, which is consistent with the literature data for white wines (*46,48,55*).

The presented results confirm the polymorphism of the fermentation profiles of the yeasts, which change under environmental, and usually multidirectional, stress. The yeasts respond by genotypic and phenotypic alterations, which may persist in subsequent generations.

Assessment of fermentation stability

Wines produced with the studied industrial wine strains, hybrids, and their segregates met the official standards for basic chemical parameters. The only deviation was total acidity, which was too low and remained outside the accepted range for fruit wines. However, statistically significant differences among the chemical parameters of wines produced with the wild strains and their segregates indicate unstable fermentation profiles. Statistical comparison of the technological stability of the yeasts was conducted by means of principal component analysis using the twelve features presented in Tables 1 and 2. The three principal components used in the analysis together explained 72.78 % of the total variation of the features (the first component accounted for 36.40 % of the total variation, the second one for 25.24 %, and the third one for 11.14 %). The first component showed a strong, positive correlation with glycerol content, total acidity, and succinic acid content. In the scatter diagram, the above features distinguished the strain *S. cerevisiae* W-13 and its segregates as well as the strain *S. cerevisiae* Syrena and its two aerobic segregates from the interspecific hybrid HW2-3 and its three segregates (Fig. 1). At the same time, the parameters related to the first component indicated marked differences between the segregates and wild strains of the yeasts *S. cerevisiae* Syrena, the interspecific hybrid HW2-3 and, to a lesser degree, the interspecific hybrids HW2-6 and HW2-7.

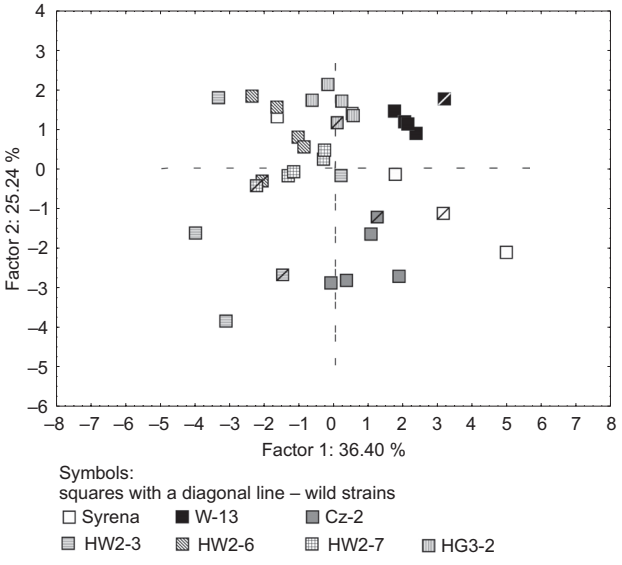

Fig. 1. Discriminant analysis of yeast fermentation profiles according to the yeast strain: scatter plot of principal component 1 (factor 1) *vs.* principal component 2 (factor 2)

The second component revealed a considerable negative correlation with sulphur dioxide content. Analysis based on the second component differentiated the strains *S. cerevisiae* Syrena, its aerobic segregate Syrena/AM, *S. bayanus* Cz-2 and its segregates, and hybrid HW2-3 and its aerobic segregate HW2-3/AM (Fig. 1). This set of features correlated with the second component confirmed the considerable differentiation between the segregates and wild strains for *S. cerevisiae* Syrena and hybrid HW2-3.

Only the content of L-malic acid showed a strong positive correlation with the third component, with substantial differentiation among the yeasts HW2-3, HW2-6, HW2-7 and their segregates (Fig. 2). At the same time, demalication ability, a feature related to the third component, more strongly differentiated the segregates from their wild strains than the features correlated with the first or second component. In particular, this concerned *S. cerevisiae* W-13, *S. bayanus* Cz-2 and hybrid *S. cerevisiae* HG3-2.

Principal component analysis made it possible to identify strains characterized by stable technological features. In respect of features correlated with the first and second component, *S. cerevisiae* W-13 and hybrid *S. cerevisiae* HG3-2 showed stable fermentation profiles. In contrast, the most pronounced changes in fermentation

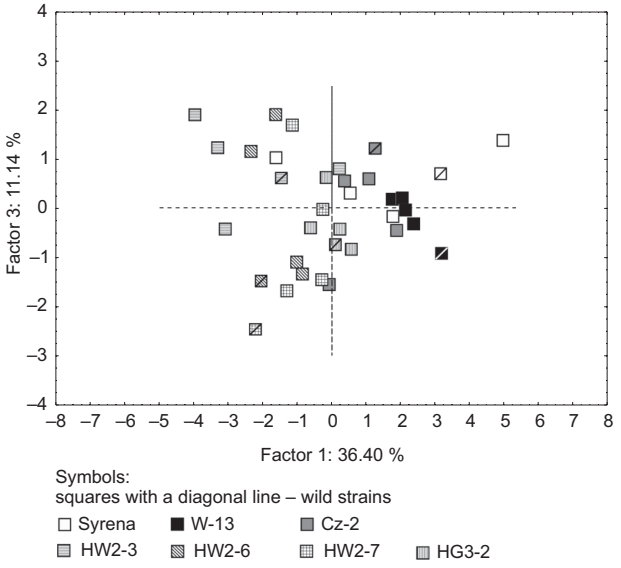

Fig. 2. Discriminant analysis of yeast fermentation profiles according to the yeast strain: scatter plot of principal component 1 (factor 1) *vs.* principal component 3 (factor 3)

activity in terms of the features correlated with the first, second, and third component were found for *S. cerevisiae* Syrena and the interspecies hybrid HW2-3. The low stability of technological features of interspecific hybrids was also reflected in the substantial rearrangements of their genomes, which had been shown in our previous study (*36*). The hybrids lost or acquired up to 5 bands in the karyotypes, but the recorded changes in their mtDNA patterns were even broader, reaching 12 missing and 6 additional bands. Despite some phenotypic changes, the intraspecific hybrid of *S. cerevisiae* was characterized by high genetic stability with any changes in chromosomal and mitochondrial DNA patterns (*36*), which was correlated with the preservation of its fermentative features.

Conclusions

Industrial wine yeasts and their hybrids reveal marked polymorphism of fermentation profiles in apple must with elevated L-malic acid content. At the same time, on the basis of the observed levels of demalication activity, it is possible to distinguish the segregates from the wild strains, which indicates that malic acid may probably be one of the principal factors in yeast adaptive evolution. However, in our previous research on the genotypic stability of these yeasts, we had not been able to find any direct relationship between the acidic stress and the changes in chromosomal and mitochondrial DNA patterns (*15*). Our study on the genetic and fermentative stability of yeasts typically used in winemaking was consistent and proved that there exist both some very stable strains (*S. cerevisiae* W-13) as well as labile ones (*S. cerevisiae* Syrena). Interspecific hybrids tend to change their fermentative features deeper than the intraspecific ones. The presented results indicate that the assessment of fermentative stability under environmental stress can help to select the yeast strains best suited for the fermentation of specific musts.

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