Effect of *Wickerhamomyces anomalus* and *Pichia membranifaciens* killer toxins on fermentation and chemical composition of apple wines produced from high-sugar juices

URSZULA BŁASZCZYK – PAWEŁ SROKA – PAWEŁ SATORA – ROBERT DULIŃSKI

Summary

Killer toxins are proteinaceous compounds that could be considered as a biological alternative to sulphur dioxide for the prevention of wine spoilage by undesirable wild yeasts. The current study investigated the influence of crude killer toxins secreted by *Wickerhamomyces anomalus* and *Pichia membranifaciens* strains on the fermentation process and chemical composition of apple wines. The main oenological parameters (ethanol, extract, total sugars, reducing sugars, titratable acidity) of obtained apple wines as well as selected volatile compounds and organic acids were analysed. It was revealed that the application of crude killer toxins to apple juices inoculated with *Saccharomyces* strains did not significantly change the fermentation kinetics, however, in most of the cases, the apple wines were distinguished by a slightly higher concentration of ethanol compared to the control samples fermented without killer toxins. The volatile acidity of the wines depended on yeast strain used in fermentation rather than on the type of killer toxin. It was also found that the addition of crude toxins slightly changed levels of several aroma components, however, the yeast strains used for the fermentation process contributed considerably to variations in profiles and concentrations of volatile compounds.

Keywords

killer toxin; fermentation; apple wine; volatile compounds; *Saccharomyces cerevisiae*

Uncontrolled growth of undesirable microorganisms can change chemical composition of wine, disrupting the taste and aroma. Low pH, high ethanol concentration and oxygen deficiency during fermentation process lead to the reduction or elimination of a population of certain microorganisms [1, 2]. However, the growth inhibition of undesirable spoilage microflora is mainly achieved by the addition of SO_2 to grape or apple musts. High doses of sulfur dioxide should be limited primarily for health reasons, but also because of their effect on the taste and aroma of wine. On the other hand, low levels of SO_2 do not ensure the stability of wine [3]. Excessive oxidation and microbial growth may adversely influence the quality of wine. Application of killer yeasts and their toxins seems to be one of possible ways to prevent the development of undesired microorganisms [4, 5]. Killer yeasts secrete toxins (usually proteins or glycoproteins), which could substitute SO_2 in wine production. The use of killer toxins during the pre-fermentation step could reduce SO_2 addition during fermentation and thus limit the content of this compound in the final product. Killer strains have been regarded useful in biological control of spoilage yeasts and in food preservation [4]. Starter cultures with killer activity could be used to combat contaminating wild-type yeasts and filamentous fungi during the production of wine, beer and bread [4].

Killer toxins of *Pichia* spp. have a broad spectrum of antifungal activity against wine spoilage yeasts such as *Brettanomyces bruxellensis*, *Hanseniaspora uvarum* and *Zygosaccharomyces* spp. [6–9]. In this context, it is interesting to investigate whether the use of killer toxins against wild-type yeasts can negatively influence alcoholic fermentation and metabolic activity of killer-resistant

Urszula Błaszczyk, Paweł Sroka, Department of Fermentation Technology and Technical Microbiology, University of Agriculture, ul. Balicka 122, 30-149 Krakow, Poland.
Paweł Satora, Department of Fermentation Technology and Technical Microbiology, University of Agriculture, ul. Balicka 122, 30-149 Krakow, Poland; Malopolska Centre for Biotechnology, Jagiellonian University, ul. Gronostajowa 7A, 30-387 Krakow, Poland.
Robert Duliński, Department of Food Biotechnology, University of Agriculture, ul. Balicka 122, 30-149 Krakow, Poland.

Correspondence author:
Urszula Błaszczyk, e-mail: u.blaszczyk@ur.krakow.pl, tel.: +48 12 6624790, fax: +48 12 6624798

starter cultures. The aim of this study was to determine the impact of *Wickerhamomyces anomalus* (formerly *Pichia anomala*) and *Pichia membranifaciens* crude toxin preparations on killer-resistant strains of *Saccharomyces cerevisiae* during the fermentation of high-sugar apple juice and on the chemical composition of obtained apple wines.

MATERIALS AND METHODS

Microorganisms and media

Killer yeast strains of *W. anomalus* CBS 1982, *W. anomalus* CBS 5759 and *P. membranifaciens* CBS 7373 were obtained from CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands). *Hanseniaspora uvarum* DSM 2768 was obtained from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Yeast cultures were maintained at 4 °C on yeast extract-peptone-dextrose (YEPD) agar containing 1% yeast extract, 2% peptone, 2% glucose and 2% agar. Dry active wine yeasts Challenge Aroma White, ES 181 and Challenge Vintage White supplied by Enartis (Novara, Italy) were used for the fermentation process. Yeasts were weighed and suspended in ten volumes of sterile water, and then the suspension was added to the apple juice.

Preparations of crude extracts of killer toxins

Killer yeast strains were cultured on YEPD agar for 24 h at 25 °C. Next, they were transferred into 50 ml of YEPD liquid medium and incubated at 25 °C for 24 h. At the next stage, 125 ml of YEPD liquid medium adjusted to pH 4.5 with 100 mmol·l^{-1} citrate-phosphate buffer was inoculated with the killer strain to the final yeast cell concentration (dry matter) of 2 g·l^{-1}. Killer cultures were cultured as described previously [10, 11] at 20–22 °C with shaking at 2 Hz on a laboratory shaker (Elpin Plus 358A, Lubawa, Poland). When the yeast culture reached the stationary phase, yeast cells were removed by centrifugation (1400 ×*g*, 15 min, 4 °C) and the culture supernatant was filtered through cellulose acetate membrane filter (pore size 0.2 μm; Sartorius, Goettingen, Germany). The filtrate was concentrated in two steps approximately 100-fold by using the Centricon Plus-70 centrifugal filter device (Merck Millipore, Darmstadt, Germany) with a molecular weight cutoff 10 kDa and 30 kDa.

Killer activity assay

Killer activity of crude toxin preparations was checked by the agar diffusion well method. Sensitive yeast strain was grown for 24 h at 25 °C on YEPD agar slants. Next, the suspension of sensitive yeast in sterile water was mixed with molten YEPD-MB medium containing 1% yeast extract, 2% peptone, 2% glucose, 3% sodium chloride, 2% agar, 0.003% methylene blue, and adjusted to pH 4.5 with 100 mmol·l^{-1} citrate-phosphate buffer. The plates were seeded with the sensitive yeast strain to a final concentration of approximately 2×10^5 cells per millilitre of the assay medium. Toxin samples of 70 μl were put into wells (diameter 5 mm) and the inoculated plates were incubated at 20 °C for 72 h. The appearance of a clear zone of growth inhibition bounded by bluish-stained cells was recorded as the presence of killer activity.

Determination of protein concentration

Protein concentration was determined according to the method of BRADFORD [12] using bovine serum albumin as standard.

Fermentation conditions

Prior to fermentation, apple juice concentrate (Apkon, Przemyśl, Poland) was diluted with distilled water and sweetened with saccharose to the desired sugar concentration (300 g·l^{-1}). The basic parameters of the reconstituted apple juice used for fermentations were as follows: total acidity 7.4 g·l^{-1}, extract 324 g·l^{-1}, total sugar concentration 300 g·l^{-1}, reducing sugars 125 g·l^{-1}, and sugar-free extract 24 g·l^{-1}.

Medium was pasteurized at 80 °C for 15 min, 0.4 l of pasteurized solution was transferred under sterile conditions to 0.7 l bottles and killer toxin preparations were added (15 mg protein per litre of apple juice, the volume of killer toxin preparations was 7.5 - 10 ml·l^{-1}). Each fermentation trial was inoculated with a precisely defined amount of starter culture (0.3 g·l^{-1}).

Commercial strains of *Saccharomyces* yeasts used in the experiment were selected on the basis of their resistance to killer toxins. The resistance to killer activity was checked by the agar diffusion well method. The plates were seeded with the yeast culture tested for sensitivity to crude killer toxins at a final concentration of approximately 1×10^5 cells per millilitre of the assay medium (YEPD-MB adjusted to pH 4.5 with 100 mmol·l^{-1} citrate-phosphate buffer). *Saccharomyces* strains were taken as resistant to tested killer toxin preparations when no zone of growth inhibition of the seeded strain was observed after 72 h of incubation at 20 °C.

During fermentation, the weight loss of samples was followed three times a week until the end

of the process characterized by a constant weight of two consecutive measurements. The fermentation was carried out in static conditions for 32 days at 20 °C. After this period, the young wines were separated from lees and stored in bottles for 30 days at 10 °C. Clarified young wines were a subject of further analysis. All fermentation experiments were conducted in triplicate.

Analysis of oenological parameters

Ethanol concentration, pH, extract (total dry matter) and sugar-free extract, titratable and volatile acidity were determined according to official analytical methods (OIV-MA-AS312-01A, OIV-MA-AS313-15, OIV-MA-AS2-03B, OIV-MA-AS313-01 and OIV-MA-AS313-02) [13]. Titratable acidity was calculated from the volume of NaOH used for titration (TitroLine alpha plus, Schott Instruments, Mainz, Germany) and expressed as grams of malic acid per litre. Reducing and total sugars were measured using the method with 3,5-dinitrosalicylic acid [14].

Organic acids and glycerol analysis

Wine samples were centrifuged (10 min, 6000 ×*g*, 20 °C), filtered through a Millipore membrane filter (pore size 0.45 μm) and then diluted 10-fold with deionized water. A Perkin Elmer Flexar high-performance liquid chromatography (HPLC) system equipped with a pump system, ultraviolet and refractive index detectors, was applied for the analysis. Tartaric, malic, lactic, citric and succinic acids, and glycerol (Sigma-Aldrich, St. Louis, Missouri, USA) were separated on a Rezex ROA-organic acid H^+ column (8%, 300 mm × 7.8 mm; Phenomenex, Torrance, California, USA) at a flow rate of 0.3 ml·min^{-1}. An isocratic method was employed as described by the supplier, using 2.5 mmol·l^{-1} H_2SO_4 as the solvent.

Analysis of volatile aroma compounds by gas chromatography

A volume of 1 ml of wine sample was transferred to a 15 ml screw-capped vial. Subsequently, 1 ml of deionized water and 0.1 ml of a mixture of internal standards solution (4-methyl-2-pentanol and ethyl nonanoate) were added. Solid-phase microextraction (SPME) was carried out with polydimethylsiloxan (PDMS) fibre (100 μm; Supelco, Bellefonte, Pennsylvania, USA) under the following conditions: extraction temperature 40 °C; extraction time 30 min; equilibration time 5 min; desorption temperature 250 °C; desorption time 3 min.

Gas chromatography (GC) with flame ionization detection (FID) analysis was performed on a Clarus 580 (Perkin Elmer, Baesweiler, Germany) chromatograph system. The volatile compounds were separated on a capillary column Elite-Wax ETR (length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm, PerkinElmer). The temperature of the detector and injector was set to 250 °C, and the column was heated using the following temperature program: 40 °C for 5 min at an increment of 5 °C·min^{-1} to 110 °C, then 20 °C·min^{-1} to 160 °C and maintaining a constant temperature for 5 min. Helium was used as the carrier gas and the flow rate was set at 20 ml·min^{-1}.

Gas chromatography–time-of-flight mass spectrometry (GC-TOF MS) analysis was conducted on a 7890B gas chromatograph (Agilent, Santa Clara, California, USA) interfaced with a Pegasus time-of-flight mass spectrometry (TOF MS) detector (LECO, St. Joseph, Michigan, USA) operated in electron ionization mode. Chromatographic separation was performed on a Restek Stabilwax (Crossbond Carbowax, polyethylene glycol) capillary column (length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm; Restek, Santa Clara, California, USA). Gerstel multipurpose sampler (MPS) possessing the functionality for automated SPME was used in the analysis. Volatile compounds adsorbed on the SPME fibre were desorbed at 260 °C (1 min). The carrier gas was helium at a constant flow rate of 1 ml·min^{-1} held by electronic pressure control. The cooled injection system (CIS) was operated at a temperature of 260 °C, and splitless injector mode was used. The gas chromatograph oven temperature program consisted of the following steps: 35 °C for 5 min, 35–110 °C at 5 °C·min^{-1}, 110–230 °C at 40 °C·min^{-1}, stable at 230 °C for 5 min. The transfer line and ion source temperatures were set at 250 °C, and ion source voltage was 70 eV. The mass spectrometric data were acquired in scan mode over *m/z* range of 30–300 at a rate of 20 spectra per second. Automatic peak detection and calculation of the peak area of specific compounds were done by ChromaTOF v. 4.51.6.0 software (LECO).

Qualitative identification and quantitative determination of volatile compounds were based on the comparison of retention times and peak areas read from sample and standard chromatograms. Each experiment was performed in triplicate.

Statistical analysis

InStat software, version 3.01 (GraphPad Software, San Diego, California, USA) was applied for statistical analyses of results. Statistically significant differences between results ($p < 0.05$) were evaluated using a single-factor analysis of variance (ANOVA) with a post hoc Tukey-Kramer's test.

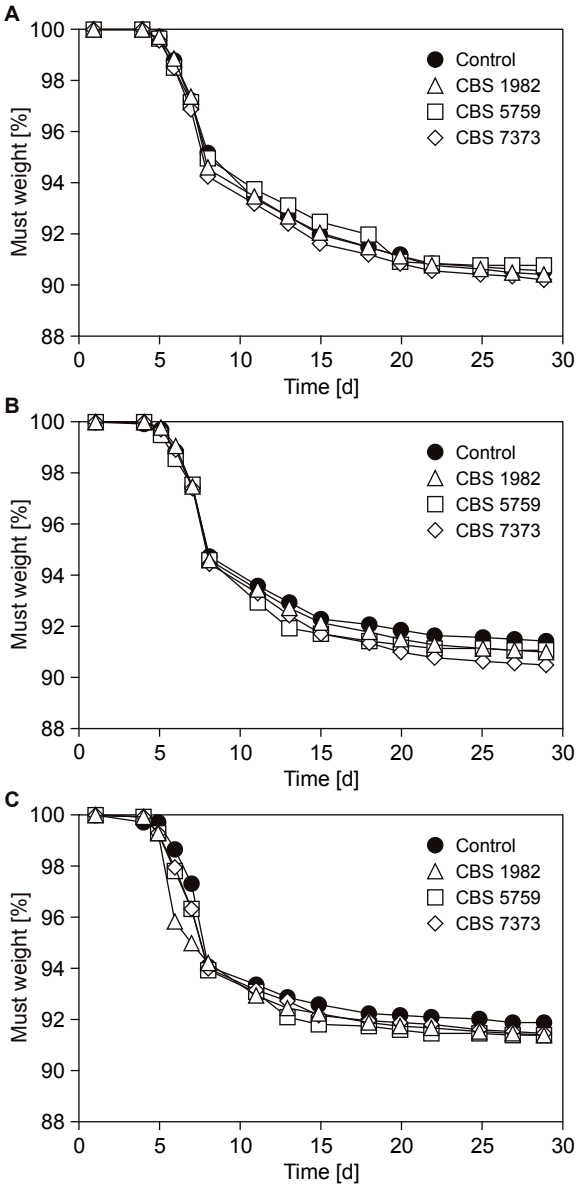

Fig. 1. Fermentation kinetics of apple juices.

Starter cultures: A – Challenge Aroma White, B – ES 181, C – Challenge Vintage White.

RESULTS AND DISCUSSION

Influence of killer toxin preparations on apple wine fermentation

Competition for nutrients has been frequently cited as a mechanism of biocontrol by antagonistic yeasts such as *Pichia* and *Wickerhamomyces* (formerly assigned to the genus *Pichia*) [15]. However, at a low yeast cell density, competition for space or nutrients may be reduced, increasing the relative contribution of other mechanisms of antagonistic action of these yeasts such as production and secretion of killer toxins or hydrolytic enzymes that degrade fungal cell walls (endo- and exo-β-1,3-glucanases) or antifungal volatile organic compounds (VOCs) [16]. Many killer toxins of *W. anomalus* possess β-1,3-glucanase activity (for example killer proteins produced by *W. anomalus NCYC 432, W. anomalus NCYC 434, W. anomalus strain K, W. anomalus* YF07b). Some killer toxins of *W. anomalus* and *P. membranifaciens* have only the killer activity and no β-1,3-glucanase activity [9, 17]. Sometimes more than one killer toxin is produced by the same species or even strain (for example *W. anomalus* YF07b) [17]. Under competitive conditions, the killer phenomenon offers a considerable advantage to killer strains against other sensitive microbial cells in their ecological niches [4, 9].

It is well known that killer yeasts can affect the fermentation kinetics of musts inoculated with starter cultures of *S. cerevisiae* [18–20]. The presence of killer yeasts may have an adverse effect on wine fermentation if the process is conducted by inoculated killer-sensitive *S. cerevisiae* strains. Similarly, in the case of spontaneous fermentation, replacement of a dominant population by killer strains may result in nutrient limitation, leading to fermentation problems such as decreased wine quality, sluggish or stuck wine fermentation [18, 21–23]. On the other hand, results of many studies revealed the potential of application of killer yeasts and their toxins in winemaking to avoid the development of spoilage yeasts [6, 7, 24].

In order to investigate the influence of preparations of killer toxins secreted by *W. anomalus* CBS 5759, *W. anomalus* CBS 1982 and *P. membranifaciens* CBS 7373 on fermentation kinetics of apple juices inoculated with three different starter cultures, measurements of the weight loss of fermenting samples were done regularly (Fig. 1). Killer activity of crude extracts of the killer toxins was determined by the agar diffusion bioassay. It was found that crude toxins were active against the strain of apiculate yeast *Hanseniaspora uvarum*.

In the case of fermentations in which Challenge Aroma White and ES 181 starter cultures were employed, the killer toxins did not influence significantly the fermentation rate (Fig. 1A, 1B). A relatively long adaptation phase lasting 4 days could be the result of the high concentration of sugars in samples. After 30 days of the experiment, the final amount of liberated carbon dioxide was highest in samples containing killer toxin of *P. membranifaciens* CBS 7373 (Fig. 1B). The fermentation kinetics of the samples inoculated with Challenge Vintage White starter culture are presented in Fig. 1C. After 5 days of fermentation,

the samples containing killer toxin of *W. anomalus* CBS 1982 were distinguished by the highest weight losses associated with the liberation of CO_2. The addition of above-mentioned killer toxin intensified the fermentation between 5th and 8th day. However, enhancement of fermentation process was not associated with significant changes in main oenological parameters or organic acids composition of the obtained apple wine (Tab. 1).

In a previous study, it was reported that *W. anomalus* CBS 1982 and CBS 5759 killer strains did not influence significantly the fermentation kinetics of apple musts inoculated with *S. cerevisiae* [19]. Only pasteurized apple musts fermented by mixed cultures of *S. cerevisiae* and *W. anomalus* were characterized by a faster fermentation rate compared to samples fermented by pure *S. cerevisiae* strain. The results obtained in the current study demonstrate that the used killer toxins did not affect significantly the fermentation kinetics of high-sugar apple juices.

Effect of killer toxins on the basic oenological parameters of apple wines

Ethanol concentration in the analysed samples ranged from 92.9 $g \cdot l^{-1}$ to 104.5 $g \cdot l^{-1}$ (Tab. 1–3). The

Tab. 1. Principal oenological parameters and organic acids composition of apple wines fermented with Challenge Vintage White starter culture in the presence of killer toxins.

Concentration [$g \cdot l^{-1}$]	Killer yeast				
	Control	CBS 1982	CBS 5759	CBS 7373	*LSD*
Ethanol	95.0 ± 1.4^{a}	97.1 ± 1.3^{a}	100.2 ± 1.3^{b}	92.9 ± 1.1^{c}	1 %
Extract	107.2 ± 4.3^{a}	101.1 ± 0.1^{b}	102.9 ± 1.6^{b}	112.5 ± 2.8^{c}	0.5 %
Total sugars	85.55 ± 4.62^{a}	78.08 ± 0.01^{b}	81.41 ± 0.22^{b}	91.11 ± 2.59^{c}	0.5 %
Reducing sugars	84.4 ± 3.4^{a}	75.8 ± 1.4^{b}	80.4 ± 0.8^{c}	90.4 ± 2.9^{d}	0.5 %
Sugar-free extract	21.7 ± 0.3^{a}	23.0 ± 0.1^{b}	21.5 ± 1.5^{ab}	21.4 ± 0.7^{a}	1 %
Glycerol	4.20 ± 0.27^{a}	3.94 ± 0.20^{a}	4.04 ± 0.08^{a}	4.70 ± 0.53^{b}	5 %
Volatile acidity	1.10 ± 0.12^{a}	1.21 ± 0.12^{b}	1.11 ± 0.01^{a}	1.21 ± 0.02^{ab}	5 %
Titratable acidity	8.07 ± 0.18^{ab}	8.01 ± 0.37^{ab}	8.26 ± 0.02^{a}	7.94 ± 0.15^{b}	5 %
Tartaric acid	0.56 ± 0.05	0.60 ± 0.05	0.62 ± 0.02	0.56 ± 0.02	ns
Malic acid	2.12 ± 0.18	2.14 ± 0.16	2.19 ± 0.05	2.18 ± 0.06	ns
Citric acid	0.94 ± 0.08	1.00 ± 0.08	1.02 ± 0.03	0.94 ± 0.03	ns
Lactic acid	0.50 ± 0.13^{ab}	0.46 ± 0.11^{ab}	0.60 ± 0.02^{a}	0.45 ± 0.03^{b}	5 %
Succinic acid	0.46 ± 0.07	0.47 ± 0.06	0.49 ± 0.02	0.44 ± 0.02	ns

Values are expressed as mean ± standard deviation. Values with different superscript letters (a–d) in the same row are significantly different ($p < 0.05$). Titratable acidity is expressed as grams of malic acid per litre.
LSD – least significant difference, ns – not significant.

Tab. 2. Principal oenological parameters and organic acids composition of apple wines fermented with ES 181 starter culture in the presence of killer toxins.

Concentration [$g \cdot l^{-1}$]	Killer yeast				
	Control	CBS 1982	CBS 5759	CBS 7373	*LSD*
Ethanol	95.1 ± 1.0^{a}	95.6 ± 0.6^{a}	99.0 ± 3.2^{b}	99.4 ± 2.4^{b}	5 %
Extract	119.1 ± 2.7^{a}	107.5 ± 1.6^{a}	98.7 ± 4.4^{b}	99.9 ± 2.3^{b}	0.5 %
Total sugars	86.08 ± 1.60^{a}	85.01 ± 0.92^{a}	75.67 ± 4.18^{b}	76.54 ± 1.60^{b}	0.5 %
Reducing sugars	84.5 ± 0.5^{a}	83.8 ± 1.1^{a}	75.0 ± 4.3^{b}	74.7 ± 4.1^{b}	0.5 %
Sugar-free extract	33.0 ± 2.2^{a}	22.5 ± 0.8^{b}	23.0 ± 0.3^{b}	23.4 ± 1.1^{b}	0.5 %
Glycerol	4.27 ± 0.16	4.30 ± 0.10	4.21 ± 0.08	4.12 ± 0.17	ns
Volatile acidity	1.29 ± 0.03^{a}	1.16 ± 0.10^{b}	1.26 ± 0.06^{ab}	1.24 ± 0.05^{ab}	1 %
Titratable acidity	7.82 ± 0.11^{a}	7.68 ± 0.04^{b}	7.96 ± 0.13^{a}	7.87 ± 0.21^{a}	0.5 %
Tartaric acid	0.51 ± 0.08	0.54 ± 0.07	0.58 ± 0.04	0.56 ± 0.02	ns
Malic acid	2.04 ± 0.26	2.11 ± 0.17	2.11 ± 0.13	2.12 ± 0.05	ns
Citric acid	0.86 ± 0.12	0.91 ± 0.11	0.98 ± 0.06	0.95 ± 0.02	ns
Lactic acid	0.59 ± 0.08	0.58 ± 0.04	0.59 ± 0.08	0.53 ± 0.05	ns
Succinic acid	0.50 ± 0.03	0.49 ± 0.02	0.47 ± 0.04	0.49 ± 0.03	ns

Values are expressed as mean ± standard deviation. Values with different superscript letters (a, b) in the same row are significantly different ($p < 0.05$). Titratable acidity is expressed as grams of malic acid per litre.
LSD – least significant difference, ns – not significant.

Tab. 3. Principal oenological parameters and organic acids composition of apple wines fermented with Challenge Aroma White starter culture in the presence of killer toxins.

Concentration [g·l^{-1}]	Killer yeast				
	Control	CBS 1982	CBS 5759	CBS 7373	*LSD*
Ethanol	100.6 ± 0.6^{a}	97.2 ± 1.0^{b}	102.8 ± 0.5^{c}	104.5 ± 2.4^{c}	1 %
Extract	94.0 ± 4.0^{a}	103.1 ± 1.5^{b}	86.6 ± 1.6^{c}	82.0 ± 4.4^{c}	0.5 %
Total sugars	71.15 ± 1.85^{a}	80.21 ± 1.85^{b}	63.68 ± 1.60^{c}	66.88 ± 3.20^{c}	0.5 %
Reducing sugars	70.0 ± 2.2^{a}	76.1 ± 1.0^{b}	62.5 ± 1.7^{c}	63.9 ± 1.3^{c}	0.5 %
Sugar-free extract	22.5 ± 2.1^{a}	22.9 ± 0.5^{a}	22.9 ± 0.7^{a}	15.1 ± 2.8^{b}	0.5 %
Glycerol	3.84 ± 0.54^{a}	5.91 ± 0.53^{b}	4.18 ± 0.08^{a}	4.02 ± 0.04^{a}	0.5 %
Volatile acidity	1.46 ± 0.14^{a}	1.50 ± 0.03^{a}	1.43 ± 0.08^{a}	1.71 ± 0.03^{b}	0.5 %
Titratable acidity	7.78 ± 0.13^{a}	7.78 ± 0.16^{a}	7.97 ± 0.02^{b}	7.44 ± 0.07^{c}	0.5 %
Tartaric acid	0.55 ± 0.02^{a}	0.62 ± 0.03^{b}	0.61 ± 0.01^{b}	0.58 ± 0.01^{a}	0.5 %
Malic acid	1.99 ± 0.04^{ab}	2.18 ± 0.07^{a}	1.80 ± 0.32^{ab}	1.75 ± 0.29^{b}	5 %
Citric acid	0.93 ± 0.02^{a}	1.04 ± 0.05^{b}	1.02 ± 0.01^{b}	0.96 ± 0.01^{a}	0.5 %
Lactic acid	0.59 ± 0.01^{a}	0.63 ± 0.02^{b}	0.68 ± 0.01^{c}	0.58 ± 0.01^{a}	0.5 %
Succinic acid	0.44 ± 0.01^{a}	0.47 ± 0.01^{b}	0.47 ± 0.01^{b}	0.46 ± 0.01^{ab}	5 %

Values are expressed as mean ± standard deviation. Values with different superscript letters (a–c) in the same row are significantly different ($p < 0.05$). Titratable acidity is expressed as grams of malic acid per litre.
LSD – least significant difference, ns – not significant.

apple wines fermented in the presence of killer toxins were characterized by differentiated ethanol concentration. Most of them contained a slightly higher concentration of ethanol as compared to the control samples without toxins. Killer proteins and other nitrogenous components present in crude extracts could be a source of nitrogen necessary for yeast growth and fermentation activity. Initial low concentration of nitrogen in grape or apple musts has been associated with the reduction of yeast development and as a result of slow and stuck fermentations [25]. Moreover, nitrogen compounds have a considerable impact on the formation of by-products (e.g. H_2S, higher alcohols, fatty acids and esters), which influence the wine composition and sensory properties [26].

A slightly lower ethanol concentration was observed in wines with killer toxins of *W. anomalus* CBS 1982 (Challenge Aroma White starter culture, Tab. 3) and *P. membranifaciens* CBS 7373 (Challenge Vintage White starter culture, Tab. 1). A reduction of ethanol yield correlated with increased levels of extract, total sugars, reducing sugars and enhanced glycerol formation. Presumably, killer toxins or other proteins and components contained in these two crude extracts negatively affected yeast growth and fermentation ability.

The extract in apple wines ranged from 82.0 g·l^{-1} to 119.1 g·l^{-1}, whereas reducing sugars after inversion ranged from 62.5 g·l^{-1} to 90.4 g·l^{-1} (Tab. 1–3). A decrease in the extract was proportional to the amount of ethanol produced during the process of fermentation. Similar tendency was observed for total and reducing sugars.

Glycerol concentration in the analysed wines varied between 3.8 g·l^{-1} and 5.9 g·l^{-1} (Tab. 1–3). The samples fermented with Challenge Aroma White starter culture in the presence of killer toxin of *W. anomalus* CBS 1982 were characterized by significantly higher glycerol concentration (Tab. 3). An increased level of glycerol may add a favourable attribute to wine. The compound is thought to improve the sensory qualities of the wine due to contribution to its smoothness and viscosity [27]. In *S. cerevisiae*, glycerol is a by-product of the fermentation of glucose to ethanol, and plays an essential role in osmotic stress resistance and maintenance of the oxidation-reduction balance [27]. It was reported that many factors can affect the amount of glycerol produced by yeast in wine, especially yeast strain, sugar concentration, pH, fermentation temperature, aeration, and the available nitrogen source [28].

Organic acid composition of analysed apple wines

It is known that acidity influences the sensory quality and stability of wine [29]. Acid composition can influence wine aroma and flavour in both positive and negative manner, depending on acid concentration as well as the type and style of wine [29]. The analysis of selected organic acids by HPLC showed that concentrations of tartaric, malic, citric, lactic and succinic acids were 0.51–0.62 g·l^{-1}, 1.75–2.19 g·l^{-1}, 0.86–1.04 g·l^{-1}, 0.45–0.68 g·l^{-1} and 0.44–0.50 g·l^{-1}, respectively (Tab. 1–3). Addition of killer toxins had an impact on the concentration of the analysed acids when apple wine sam-

ples were obtained with Challenge Aroma White starter culture. Killer toxins secreted by *W. anomalus* CBS 1982 and CBS 5759 caused the increase in concentration of citric, tartaric, succinic and lactic acids (Tab. 3). In the case of the other two starter cultures, no significant effects of toxins on the concentrations of the aforementioned compounds were observed (Tab. 1, 2).

Slight differences in titratable acidity of the analysed wines were observed (Tab. 1–3). Depending on the yeast strain and the type of killer toxin used, the values of titratable acidity were in the range from 7.44 $g \cdot l^{-1}$ to 8.26 $g \cdot l^{-1}$. The addition of toxin preparations changed titratable acidity to a small extent.

Volatile acidity of analysed apple wines ranged from 1.10 $g \cdot l^{-1}$ to 1.71 $g \cdot l^{-1}$ (Tab. 1–3). Elevated values of the above-mentioned parameter in the samples inoculated with Challenge Aroma White culture (Tab. 3) could be the result of the high concentration of sugars and/or other factors associated with the activation of cellular stress response. The volatile acidity of the control and the samples fermented in the presence of crude extracts of *W. anomalus* CBS 1982 and CBS 5759 killer toxins was approx. 1.5 $g \cdot l^{-1}$. The increase in the volatile acidity (0.2 $g \cdot l^{-1}$) in relation to the control was observed in the apple juice supplemented with *P. membranifaciens* CBS 7373 killer toxin. Samples inoculated with Vintage White and ES 181 starter cultures were distinguished by lower volatile acidity (Tab. 1, 2) than those fermented with Challenge Aroma White (Tab. 3). Our study revealed that supplementation of the samples with killer toxin preparations did not cause considerable changes in volatile acidity of the obtained wines compared to the controls (Tab. 1–3). All wine samples were characterized by an increased volatile acidity, and yeast strains used for fermentation affected the value of the volatile acidity more than the type of added toxins. Elevated level of volatile acidity in all fermentation trials could be attributed to the high concentration of sugar in the apple juice. In samples with high concentrations of sugar, the fermentation rate is reduced due to the action of the high osmotic pressure on yeast cells and, as a result, an increase in volatile acidity can be observed [30]. The high values of volatile acidity may also result from a low concentration of nitrogen compounds in apple juice. It was reported that volatile acidity was inversely correlated with the assimilable nitrogen concentration in high-sugar musts, while the higher the nitrogen concentration, the less volatile acidity was produced [31]. The time of assimilable nitrogen addition was the second factor that affected volatile acidity. Early addition of assimilable nitrogen, at the beginning of the culture growth, resulted in a decrease in volatile acidity production. Later addition, at the beginning of the stationary phase, had less effect on volatile acidity [31].

Volatile aroma compounds in apple wines

A headspace-solid-phase microextraction (HS-SPME) method coupled to GC-TOF MS and GC-FID was applied to analyse the volatile compounds. An exemplary GC-MS chromatogram of volatile compounds in obtained apple wines is presented in Fig. 2. The volatile composition of analysed samples is shown in Tab. 4–6.

The apple wines obtained with Challenge Aroma White starter culture (Tab. 4) were cha-

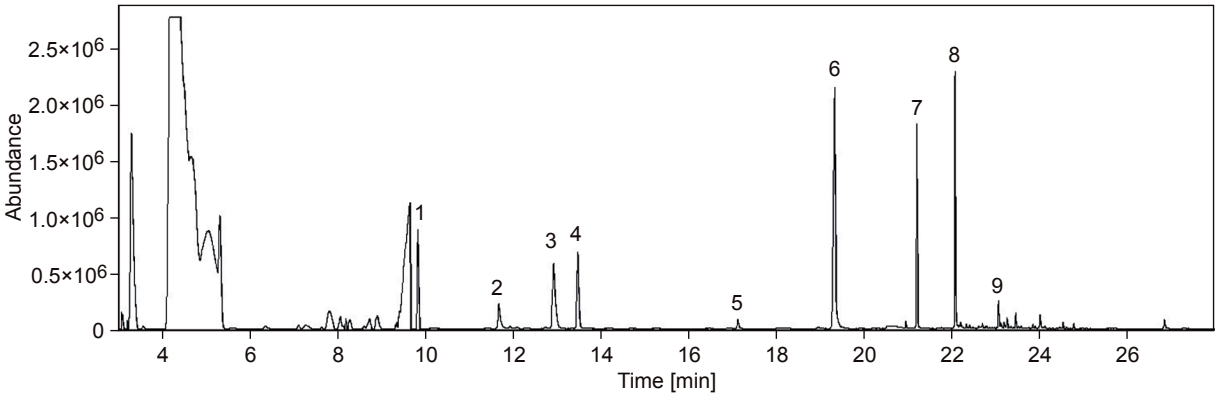

Fig. 2. Analytical ion chromatogram of volatile compounds in the apple wine sample fermented with Challenge Aroma White in the presence of *W. anomalus* CBS 1982 killer toxin.

Peak identitfication: 1 – 3-methylbutyl acetate; 2 – 4-methyl-2-pentanol; 3 – 2-methyl-1-butanol and 3-methyl-1-butanol (amyl alcohols); 4 – ethyl hexanoate; 5 – ethyl lactate; 6 – ethyl octanoate; 7 – ethyl nonanoate; 8 – ethyl decanoate; 9 – 2-phenylethyl acetate.

racterized by significantly differentiated concentration of amyl alcohols, isobutanol and some esters (3-methylbutyl acetate, ethyl octanoate, ethyl lactate, diethyl butanedioate). The samples fermented in the presence of *W. anomalus* CBS 5759 and *P. membranifaciens* CBS 7373 killer proteins were distinguished by slightly increased concentrations of amyl alcohols. An opposite effect was observed for the third killer toxin preparation. The addition of crude extract of killer toxin secreted by *W. anomalus* CBS 1982 caused lower production of amyl alcohols compared to control. Moreover, a slightly decreased concentration of isobutanol was noted. Similar tendency was found in the case of killer protein produced by *P. membranifaciens* CBS 7373 in samples inoculated with Challenge Vintage White starter culture (Tab. 6). Decreased concentrations of amyl alcohols and isobutanol were correlated with a slightly lower ethanol production (Tab. 1).

2-Methyl-1-butanol (active amyl alcohol), 3-methyl-1-butanol (isoamyl alcohol), isobutanol and 2-phenylethanol belong to the most important fusel alcohols produced during must fermentation [32]. At levels below 300 mg·l^{-1}, the higher alcohols usually contribute to the desirable complexity of wine aroma [33]. These compounds could be synthesized by yeasts through either the catabolic pathway from corresponding amino acids (isoleucine, leucine, valine and phenylalanine), or

Tab. 4. Volatile aroma compounds of analysed apple wines fermented with Challenge Aroma White starter culture in the presence of killer toxins.

Concentration [mg·l^{-1}]	Killer yeast				
	Control	CBS 1982	CBS 5759	CBS 7373	*LSD*
Amyl alcohols	115.4 ± 1.5^{a}	109.4 ± 1.8^{b}	120.3 ± 2.6^{c}	119.5 ± 0.9^{c}	0.5 %
Isobutanol	46.6 ± 2.1^{a}	43.6 ± 0.4^{b}	43.1 ± 2.2^{b}	54.8 ± 1.2^{c}	0.5 %
2-phenylethanol	24.3 ± 0.6^{a}	24.2 ± 0.2^{a}	24.9 ± 0.6^{ac}	25.1 ±0.2^{bc}	5 %
Ethyl acetate	61.8 ± 4.1^{a}	57.7 ± 2.2^{ac}	55.1 ± 5.3^{c}	61.5 ± 2.9^{a}	5 %
2-methylpropyl acetate	2.03 ± 0.04^{a}	2.23 ± 0.35^{a}	2.44 ± 0.21^{ab}	2.98 ± 0.73^{b}	5 %
3-methylbutyl acetate	17.94 ± 1.53^{a}	15.61 ± 0.84^{b}	28.45 ± 1.54^{c}	20.69 ± 0.71^{d}	0.5 %
2-phenylethyl acetate	0.18 ± 0.02^{a}	0.10 ± 0.05^{bc}	0.14 ± 0.05^{ac}	0.07 ± 0.01^{b}	1 %
Ethyl lactate	2.63 ± 0.08^{a}	2.90 ± 0.57^{a}	1.69 ± 0.20^{b}	2.49 ± 0.5^{a}	1 %
Ethyl hexanoate	4.38 ± 0.58^{a}	5.03 ± 0.36^{b}	5.52 ± 0.18^{b}	5.11 ± 0.23^{b}	1 %
Ethyl octanoate	1.97 ± 0.29^{a}	1.13 ± 0.11^{b}	2.22 ± 0.86^{a}	1.87± 0.45^{ab}	5 %
Ethyl decanoate	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	ns
Diethyl butanedioate	8.54 ± 0.43^{a}	9.30 ± 0.21^{ab}	8.81 ± 1.07^{a}	10.70 ± 0.79^{b}	1 %

Values are expressed as mean ± standard deviation. Values with different superscript letters (a–d) in the same row are significantly different ($p < 0.05$).
LSD – least significant difference, ns – not significant.

Tab. 5. Volatile aroma compounds of analysed apple wines fermented with ES 181 starter culture in the presence of killer toxins.

Concentration [mg·l^{-1}]	Killer yeast				
	Control	CBS 1982	CBS 5759	CBS 7373	*LSD*
Amyl alcohols	103.5 ± 1.6	105.5 ± 1.7	108.1 ± 5.8	106.7 ± 0.5	ns
Isobutanol	33.1 ± 0.3	30.7 ± 3.7	31.4 ± 6.4	30.2 ± 4.9	ns
2-phenylethanol	24.9 ± 0.3	24.8 ± 0.4	24.5 ± 0.4	25.1 ± 1.0	ns
Ethyl acetate	65.4 ± 0.8^{a}	64.5 ± 3.7^{a}	66.7 ± 1.5^{a}	56.2 ± 6.2^{b}	1 %
2-methylpropyl acetate	2.37 ± 0.42^{a}	4.15 ±0.76^{b}	3.23 ± 0.03^{ab}	4.23 ± 1.15^{b}	1 %
3-methylbutyl acetate	22.91 ± 7.15^{a}	52.63 ± 6.05^{b}	45.18±0.72^{bc}	43.18 ± 3.36^{c}	0.5 %
2-phenylethyl acetate	0.07 ± 0.01^{a}	0.13 ± 0.04^{b}	0.14 ± 0.03^{b}	0.14 ± 0.04^{b}	1 %
Ethyl lactate	2.62 ± 0.42	1.94 ± 0.20	2.10 ± 0.57	2.59 ± 0.59	ns
Ethyl hexanoate	6.84 ± 1.87^{a}	12.25 ±1.75^{b}	10.05 ± 0.75^{b}	12.44 ± 2.48^{b}	1 %
Ethyl octanoate	1.69 ± 0.56^{a}	2.97 ± 0.67^{b}	4.20 ± 0.80^{b}	4.46 ± 1.00^{c}	1 %
Ethyl decanoate	0.02 ± 0.01^{a}	0.06 ± 0.02^{b}	0.06 ± 0.01^{b}	0.06 ± 0.02^{b}	5 %
Diethyl butanedioate	24.11 ± 5.48^{a}	35.08 ± 5.46^{b}	34.19 ± 7.03^{b}	37.11 ± 3.16^{b}	1 %

Values are expressed as mean ± standard deviation. Values with different superscript letters (a–c) in the same row are significantly different ($p < 0.05$).
LSD – least significant difference, ns – not significant.

Tab. 6. Volatile aroma compounds of analysed apple wines fermented with Challenge Vintage White starter culture in the presence of killer toxins.

Concentration [mg·l^{-1}]	Killer yeast				
	Control	CBS 1982	CBS 5759	CBS 7373	*LSD*
Amyl alcohols	130.5 ± 2.3^{a}	133.6 ± 4.3^{a}	134.2 ± 2.4^{a}	120.0 ± 3.2^{b}	0.5 %
Isobutanol	40.9 ± 2.5^{a}	41.3 ± 3.0^{a}	41.5 ± 2.5^{a}	36.8 ± 2.7^{b}	5 %
2-phenylethanol	29.5 ± 1.3	29.6 ± 1.4	31.8 ± 0.5	29.3 ± 1.5	ns
Ethyl acetate	58.4 ± 5.5	59.3 ± 6.0	54.3 ± 4.2	52.9 ± 3.4	ns
2-methylpropyl acetate	1.44 ± 0.02	1.47 ± 0.03	1.44 ± 0.02	1.46 ± 0.07	ns
3-methylbutyl acetate	4.62 ± 0.61	6.28 ± 1.27	5.82 ± 0.21	5.57 ± 3.19	ns
2-phenylethyl acetate	0.12 ± 0.02	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	ns
Ethyl lactate	4.05 ± 0.55	4.08 ± 1.08	3.79 ± 1.21	3.54 ± 1.06	ns
Ethyl hexanoate	5.23 ± 0.29	5.23 ± 0.13	4.35 ± 0.96	4.66 ± 0.42	ns
Ethyl octanoate	1.87 ± 0.46^{a}	2.73 ± 0.80^{b}	3.61 ± 0.43^{c}	0.74 ± 0.26^{d}	0.5 %
Ethyl decanoate	0.03 ± 0.01^{ab}	0.03 ± 0.01^{ab}	0.04 ± 0.02^{a}	0.02 ± 0.01^{b}	5 %
Diethyl butanedioate	6.49 ± 0.72^{a}	6.01 ± 0.85^{a}	5.26 ± 0.40^{a}	8.56 ± 1.36^{b}	0.5 %

Values are expressed as mean ± standard deviation. Values with different superscript letters (a–d) in the same row are significantly different ($p < 0.05$).
LSD – least significant difference, ns – not significant.

through the anabolic route from sugar substrate [33, 34]. The nitrogen level (especially amino acid concentration) in must is among the most important factors influencing fusel alcohol production [33]. At low levels of assimilable nitrogen, the anabolic pathway predominates, whereas at high concentration the catabolic conversion (Ehrlich pathway) becomes prominent as a result of feedback and/or repression of key enzymes in the biosynthetic pathway [26]. The amount of higher alcohols produced during fermentation is also significantly dependent on the yeast strain [33]. In our study, the lowest amount of fusel alcohols was formed in apple wines fermented with ES 181 starter culture (Tab. 5). In the case of ES 181 starter culture it was observed that the samples fermented in the presence of killer toxins were distinguished by slightly higher concentrations of fusel alcohol acetates and ethyl esters (Tab. 5). Statistically significant differences were detected for 2-methylpropyl acetate, 3-methylbutyl acetate, 2-phenylethyl acetate, ethyl hexanoate, ethyl octanoate and diethyl butanedioate.

The amount of esters produced by yeasts during fermentation primarily depends on the yeast strain. Among other factors influencing ester formation are fermentation temperature, higher alcohol concentration, inoculum size, must pH, oxygen and nitrogen availability [26, 33]. The increased levels of compounds such as ethyl hexanoate (apple-like aroma), ethyl octanoate (apple-like aroma), 3-methylbutyl acetate (banana-like aroma), and 2-phenylethyl acetate (fruity, rose, flowery flavour with a honey note) in apple wines could contribute to a desirable fruity aroma of the fermentation bouquet.

Killer proteins and other nitrogenous compounds present in crude toxin preparations could be a source of nitrogen, and therefore play a role in formation of some volatile compounds. It is possible that some components of these crude extracts could affect the yeast metabolism and, as a result, impaired or enhanced production of some compounds was observed. However, the relationship between the type of killer toxin preparation used in fermentation process and volatile compounds formation by wine yeasts appears to be quite complex.

The addition of crude toxins slightly changed volatile composition of analysed apple wines, but wine yeast strains used in fermentation also contributed considerably to variations in profiles and concentrations of volatile compound.

CONCLUSION

In conclusion, the results of this study demonstrated that the addition of crude killer toxin preparations to apple juices inoculated with *Saccharomyces* strains did not significantly change the fermentation kinetics. However, the obtained apple wines were in most cases characterized by slightly higher concentrations of ethanol compared to the control samples. Only crude extracts of toxins secreted by *W. anomalus* CBS 1982 and *P. membranifaciens* CBS 7373 reduced the ethanol yield in two wine samples. A decrease in ethanol concentration was associated with increased levels of extract, total sugars, reducing sugars and

increased glycerol formation. Furthermore, GC analyses demonstrated slightly lower concentrations of amyl alcohols and isobutanol in those wine samples.

Killer toxin preparations influenced the concentration of acids in apple juice samples inoculated with one of the starter cultures (*Challenge Aroma White*). The values of volatile acidity depended on yeast strain used in fermentation rather than on the type of added killer toxin. It was also revealed that the addition of crude toxins influenced the concentration of several aroma components, however, wine yeast strains used in fermentation contributed considerably to variations in profiles and concentrations of volatile compounds.

It should be noted that crude toxin preparations could be used in apple wines fermentation because, when added to musts, they did not shown any significantly adverse effect on growth and metabolic activity of fermenting *Saccharomyces* strains used in the study. However, further investigations are required to fully establish their impact on *Saccharomyces* and spoilage yeast strains in the winemaking environment.

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