

Article

Fermentative Potential of Native Yeast *Candida famata* for Prokupac Grape Must Fermentation

Stojan Mančić ¹, Bojana Danilović ¹ , Marko Malićanin ², Sandra Stamenković Stojanović ¹ , Nada Nikolić ¹, Miodrag Lazić ¹ and Ivana Karabegović ^{1,*} 

¹ Faculty of Technology, University of Niš, Bulevar oslobođenja 124, 16000 Leskovac, Serbia; stojan.manciv@student.ni.ac.rs (S.M.); bojana.danilovic@junis.ni.ac.rs (B.D.); sandrastamenkovic@tf.ni.ac.rs (S.S.S.); nikolicnada@tf.ni.ac.rs (N.N.); lazic@tf.ni.ac.rs (M.L.)

² Faculty of Agriculture, University of Niš, Kosančićeva 4, 37000 Kruševac, Serbia; malicanin.marko@ni.ac.rs

* Correspondence: ivana.karabegovic@tf.ni.ac.rs

Abstract: The fermentative potential of native *Candida famata* isolates from wild and cultivated blackberries was evaluated for potential application in Prokupac grape must fermentation. 5 isolates, out of a total 22 isolated yeasts, were identified as *C. famata*. After the initial screening of fermentative performances, microfermentation was performed in a sterile grape must. Produced samples were analyzed using the HPLC technique. All isolates showed an ability to grow at lower temperatures, good tolerance to 7% ethanol and 300 ppm of SO₂. *C. famata* isolates WB-1, WB-2 and W-5 had similar fermentation performance, but WB-1 isolate was chosen for validation at a laboratory-scale level according to a pleasant, fruity aroma, highest fermentative vigor and power, good organic acid profile and the highest level of ethanol and glycerol produced in micro-vinification experiments. Good enological performance of selected *C. famata* WB-1 isolate is confirmed by higher level of glycerol, lower level of ethanol and acetic acid in wine samples produced in pure and sequential fermentation, when compared to the control sample. Throughout the selection of *C. famata* yeasts with good enological potential, this work gives a contribution in the area of precision enology, aiming to find a perfect match between non-exploited yeasts and “autochthonous” grape cultivar Prokupac.

Keywords: fermentative potential; *Candida famata*; Prokupac grape; native yeast; wine yeast



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1. Introduction

Modern wine consumers demand for authentic and outstanding wines with unique character, imposing the need for fundamental changes to current practices in vineyards and cellars. The use of new and emerging technologies in the field of viticulture and enology allows the winemaker to modify traditional approaches, tailoring them to real and specific needs. Utilization of non-*Saccharomyces* yeast represent a new approach in winemaking industry. It is well documented how non-*Saccharomyces* yeasts positively impact wine quality, where enhancement of glycerol production, ester formation, increased or decreased acidity and transformation of glycosylated flavorless precursors to its active volatile forms are just some of the mechanisms [1,2]. Thus, the application of non-*Saccharomyces* yeasts responds to a clear demand for wine wholesomeness, such as less ethanol, ethyl carbamate and biogenic amines formation or higher production of bioactive compounds with antioxidant activity [2].

Although the industrial production of non-*Saccharomyces* wine starter cultures is restricted mainly to the *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia kluyveri* and *Torulaspora delbueckii* strains [3–7], a search for non-exploited and non-conventional yeasts with good enological characteristics is an increasing trend in wine research [8–11]. Enological potential of wild non-*Saccharomyces* yeast required for the wine industry mainly refers to the viability of these yeasts, as well as the ability of substrate conversion to ethanol and an array of volatile aroma compounds without the production of undesirable

compounds. Additionally, one of the important criteria is to be cost-effective for commercial production and resistant to drying or storage without loss of its activity [2,12,13].

Candida spp. is one of the yeast genera commonly present in must at early stage of fermentation [12]. Yeasts from this genera are characterized as high glycerol and low acetic acid producers, with negative effects on the content of aldehydes and acetate esters in wines. Among them, *Candida famata* / *Debaryomyces hansenii* represent one of the frequent indigenous yeast located on grapes [1,13]. It has the ability to produce β -D-glucosidases which hydrolyze glycosides in grape must [14]. An isolated enzyme was efficiently used for the release of monoterpenols and the significant increase of nerol and linalool in Muscat grape must. Also, it was confirmed that β -D-glucosidase from *C. famata* remained active in up to 15% vol. of ethanol and cannot be inhibited by glucose [15]. The presence of this enzyme can contribute to the formation of aroma compounds in wine. Also, the production of protease can promote the growth of microorganisms during the fermentation due to the release of assimilable nutrient sources as peptides and amino acids [16]. *C. famata* can also produce exopolysaccharides from various sugar sources [17]. The composition and structure of produced polysaccharides have an influence on mouth-feel properties and the astringency of wines [18]. Moreover, according to the literature data, *Debaryomyces hansenii* H525 have the ability to degrade a broad spectrum of biogenic amines, compounds known for their harmful effects on human health when present in significant levels [19], which makes this yeast species a potential tool for reduction of these kind of compounds in wines. Considering attractive metabolic activity of this species and poor amounts of data available regarding its fermentation capacities, further research activities on this matter are raising interest in enology.

Prokupac is an autochthonous red grape variety characteristic for the central part of Serbia with long use in wine production [20]. Prokupac wine has unique phenolic profile with high content of anthocyanins, flavonols, flavon-3-ols monomers and hydroxybenzoic acid derivatives which contribute to expressive antioxidant activity [21,22]. Wine produced from this grape variety has been characterized as low in color intensity, but with a dominant red-berry and a discreet floral aroma described as linden, acacia and rose notes [20].

Knowing that wine flavor is a consequence of the vitivinicultural terroir and that native yeasts isolated from the plants of a specific region could be associated with the complete natural environment in which a particular wine is produced [23], it can be assumed that application of non-conventional native yeasts in the production of Prokupac wine can enhance flavor complexity, regional specificity of aroma profile and overall quality of wine. Although grapes and vinery equipment are still considered as a main source of new yeast strains with potentially good enological characteristics, we should bear in mind that yeast occur widespread in nature [24]. Therefore, the search for non-exploited yeast strains with good enological characteristics must go beyond vineyards and cellars, and focus on other starting materials and points, such as different fruits and fruit products that grow in a specific region. A recent study showed that yeast isolated from native berries have excellent enological properties [23]. Blackberry, also known as “super fruit” is traditionally used for the preparation of dessert blackberry wine thanks to the wealth of native yeasts living on its surface [25]. Blackberries are rich in minerals, vitamins and phenols, ranked highly among fruits for their antioxidant power, which can influence composition and properties of the yeast inhabiting its surface, due to the fact that the structure of yeast's cell wall may depend on their natural habitat [26,27].

The combination of autochthonous grape, such as Prokupac and native yeast from regionally grown fruit, such as blackberry, represent a concept of precision enology, which by definition, requires site-specific methodology in order to optimize cellar practices and management [28]. Wild yeast isolated from the region of grape's origin are indeed a site specific input with great, yet unexploited potential in the winemaking process [28,29].

Consequently, the aim of this work was to investigate fermentative capabilities of *C. famata* isolates from blackberries and their possible use in the fermentation of Prokupac grape must. Since blackberries are traditionally grown in Serbia containing wealth of

native yeast on its surface (including *C. famata*) that can be associated with a specific vitivinicultural terroir in which Prokupac is grown, application of *C. famata* on Prokupac contribute to precision enology in terms of a “specific grape-specific yeast” concept.

2. Materials and Methods

2.1. Isolation and Identification of Yeasts

A total number of six fruit samples of wild (3 samples) and cultivated (3 samples) black-berries were collected from the area of Southern Serbia in the period September–October 2020. Samples were transported in sterile plastic bags and yeasts were isolated within a period of 24 h. Fruit samples (10 g) were mashed and mixed with 90 mL of sterile saline water (0.8% *w/w* NaCl). Serial dilutions were made and plated on Sabouraud maltose agar (SMA) (Torlak, Belgrade, Serbia) plates with the addition of chloramphenicol 80 mg/L (Merck, Darmstadt, Germany) and gentamycin sulphate 5 mg/L (Merck, Darmstadt, Germany). Plates were incubated at 25 °C for 48 h and individual colonies were transferred to new SMA plates. Yeast isolates were purified by three consecutive transfers and the purity was checked out by microscopy. Isolates were identified by API 20 C AUX system (bioMérieux, Marcy l’Etoile, France) and *C. famata* isolates were selected and stored at −20 °C until further investigation.

2.2. Characterization and Fermentative Performances of Yeast Isolates

In order to characterize *C. famata* isolates, they were screened for growth at different temperature. Yeast isolates were plated on SMA plates and incubated at 4, 10, 15 and 20 °C, during 48 h. Detection of visible growth was stated as positive. The ability of sugar fermentation was analyzed by the addition of 0.2 mL of overnight yeast culture in 10 mL of fermentative broth containing: 10 g/L peptone 4 (Torlak, Belgrade, Serbia), 20 g/L glucose (Centrohem, Stara Pazova, Serbia) and 20 g/L fructose (Merck, Darmstadt, Germany). Each inoculated test tube contained Durham tube, and accumulation of CO₂ in Durham tubes after the incubation at 25 °C during 48 h was stated as positive reaction. Ability of yeast isolates to growth under different concentrations of ethanol was analyzed by inoculation of 0.2 mL of overnight yeast culture in 10 mL of fermentative broth supplemented with 3, 5, 7, 9, 11, 13 and 15% vol. ethanol. Samples were incubated for 24 h at 25 °C and growth was detected by UV/VIS spectrophotometric (Pye Unicam, Cambridge, UK) measurement of the absorbance at 620 nm. Tolerance to ethanol was expressed as a % of growth compared to the control without ethanol. Resistance to SO₂ was analyzed at fermentative agar plates supplemented with 50, 100, 200 and 300 ppm of potassium metabisulphite (Zorka Šabac, Serbia) [30]. After the inoculation with overnight culture of yeast isolates, plates were incubated at 25 °C for 48 h and visible colonies were stated as positive reaction.

2.3. Screening Method for Initial Aroma Production

The olfactory sniff test on plates was applied as a quick and fast method for the initial aroma screening [31]. Yeast isolates were inoculated on the surface of sterilized grape juice with the addition of 1.5% agar (Torlak, Belgrade, Serbia), incubated at room temperature up to seven days and subjected to sensory analysis (sniffed directly) by a panel of 9 people. Detected aroma was described as pleasant or unpleasant, with different levels of intensity (weak, intermediate and strong).

2.4. Microfermentation of Prokupac Grape Must

Microfermentations were performed according to the modified method of Romano et al. (2003). Prokupac grape was manually harvested at the end of October 2020 (approximately 100 kg of grapes) from the six-year-old vineyard (total vineyard area 1 hectare, Central Serbia wine region, Tri Morave wine subregion, 43°30′ N, 21°39′ E, continental climate, single Royat cordon vine training system). The characteristics of the must were: 22.6 Brix°, 7.4 g/L total acidity, pH 3.59. The grape must (100 mL) supplemented with 75 mg/L ammonium sulphate (Centrohem, Stara Pazova, Serbia) and 131 mg/L ammo-

nium dihydrogen phosphate (Merck, Darmstadt, Germany) was put in 300 mL Erlenmeyer flasks, autoclaved at 100 °C for 20 min and used for fermentation trials. Each sample was inoculated with 48 h old yeast culture, to the final cell number of 1×10^6 CFU/mL, while commercial *Saccharomyces cerevisiae* strain ICV D254 (1×10^6 CFU/mL, Lallemand, Montreal, QC, Canada) was used for control fermentation trial. Fermentations were carried out at 25 °C, and sampling was performed in one-day intervals. In order to simulate the real fermentation conditions, after 9 days of fermentation 1 mL of the nitrogen source solution (75 mg/L $(\text{NH}_4)_2\text{SO}_4$, 131 mg/L $\text{NH}_4\text{H}_2\text{PO}_4$) was added to the fermentation vessels. Determination of cell number was performed by measuring absorbance at 620 nm and comparing with the standard curve for each yeast. Parallel experiments were set out in order to determine the release of CO_2 by measuring the weight loss during the fermentation. The fermentations were considered complete when the weight loss in two consecutive days did not differ by more than 1%. All microfermentations were run in duplicate. In order to simulate conditions closer to those in real fermentation, the *C. famata* isolate with best fermentative performances and commercial *Saccharomyces cerevisiae* were used in laboratory-scale fermentation trials (3 L). Fresh Prokupac grape must was inoculated with selected *C. famata* isolate (final cell number was 1×10^6 CFU/mL) in pure and sequential fermentation with commercial *Saccharomyces cerevisiae*. Potassium-metabisulfite (2 mg/L, Centrohem, Stara Pazova, Serbia) and pectolytic enzyme EXV (2 mg/L, Lallemand, Canada) were added to the must. Fermentation temperature was maintained at 18 °C. Yeast nutrient Fermaid E (30 mg/L, Lallemand, Canada) was added in all fermentation trials when sugar content dropped below 7 Brix° (ATC Refractometer, Giorgio Bormac, Italy), while the fermentations were considered complete when the residual sugar content was below 4 g/L. Wine samples produced with selected *C. famata* isolate in pure (CF), sequential inoculation with commercial *Saccharomyces cerevisiae* (CFSC) and control sample inoculated with *Saccharomyces cerevisiae* (SC) were further subjected to the standard wine analysis according to The International Organisation of Vine and Wine (OIV, 2019) and to the HPLC analysis.

2.5. HPLC Analysis

Organic acids, fermentable sugars, ethanol and glycerol were determined by high-performance liquid chromatography (HPLC) on a device Agilent 1100 Series (Agilent Technologies, Santa Clara, CA, USA). Samples of fermented grape must were centrifuged (Th16B Centrifuge, Zhengzhou, China) at 6000 rpm for 15 min and filtered through a syringe filter 0.45. Detection of the compounds was performed on Aminex HPX-87H column with 5 mM H_2SO_4 used as eluent with following operating conditions: injection volume 20 μL , temperature 50 °C and eluent flow of 0.6 mL/min. The concentration of each compound was calculated according to the external standard and expressed as g/L.

2.6. Statistical Analysis

All experiments were conducted in triplicate and the results are expressed as the average value with standard deviation. Software IBM SPSS Statistics 21 was used for calculation of significant difference among the sample by One-Way ANOVA with Tukey's Post Hock multiple comparisons. The samples with the *p* value lower than 0.05 were considered significantly different.

3. Results

A total number of 22 yeast isolates has been isolated from the samples of wild and cultivated blackberries. Among 17 yeasts isolated from wild blackberries, 4 of them were identified as *C. famata* (24%). A much lower number of yeast isolates (5 isolates) was isolated from cultivated blackberries, and 1 of them was identified as *C. famata* (20%). These five *C. famata* isolates were selected for further analysis and characterized for the production of CO_2 , growth at different temperatures, tolerance to ethanol and SO_2 up to 15% and 50–300 ppm, respectively.

All *C. famata* isolates showed similar characteristics (Table 1). The absorbance at 620 nm after 24 h of growth was in the range 0.9–1. *C. famata* isolates showed good growth characteristics at low temperatures since they can all grow in the range 4–20 °C. All isolates were highly tolerant to 5% of ethanol with the reduction of growth for less than 30%. Also, isolates showed good tolerance to 7% ethanol maintaining the growth for 56–75%. The increase of the ethanol content to 9% significantly reduced the viability of *C. famata* isolates to the range of 21–37%. Further increase of ethanol content can be considered toxic for analysed isolates lowering the grow capability for less than 15%. Results of the analysis of tolerance to SO₂ indicated that all isolates were tolerant to up to 300 ppm of SO₂.

Table 1. Characteristics and fermentative performances of *C. famata* isolates and commercial *S. cerevisiae*.

Characteristics and Fermentative Performances		<i>C. famata</i> Isolate					<i>S. cerevisiae</i> ICV D254
		WB-1	WB-2	WB-4	WB-17	W-5	
A ₆₂₀		1.020 ± 0.00 a	0.902 ± 0.024 b	1.004 ± 0.003 ac	0.876 ± 0.004 b	1.016 ± 0.031 a	1.101 ± 0.070 ad
CO ₂ production		+	+	+	+	+	+
Growth at different temperature	4 °C	+	+	+	+	+	+
	10 °C	+	+	+	+	+	+
	15 °C	+	+	+	+	+	+
	20 °C	+	+	+	+	+	+
Tolerance to ethanol	3% vol.	100%	97%	99%	100%	100%	100%
	5% vol.	81%	89%	86%	88%	80%	100%
	7% vol.	65%	64%	63%	62%	52%	96%
	9% vol.	31%	29%	25%	37%	21%	88%
	11% vol.	12%	4%	13%	15%	4%	78%
	13% vol.	6%	-	6%	8%	2%	67%
Tolerance to SO ₂	15% vol.	-	-	-	-	-	47%
	50 ppm	+	+	+	+	+	+
	100 ppm	+	+	+	+	+	+
	200 ppm	+	+	+	+	+	+
Fermentative vigor ¹	300 ppm	+	+	+	+	+	+
		2.66 ± 0.05 a	2.44 ± 0.08 b	2.24 ± 0.02 c	2.51 ± 0.06 b	2.17 ± 0.06 c	5.18 ± 0.05 d
Fermentative power ²		7.41 ± 0.09 a	6.51 ± 0.22 b	6.07 ± 0.00 c	7.36 ± 0.10 a	6.62 ± 0.10 b	11.22 ± 0.07 d
Aroma production ³		P++	P+	U+	U+	P++	P+++

Different letters indicate significant difference between the samples, $p < 0.05$. ¹ Fermentation vigor—expressed as grams of CO₂ produced in 100 mL of must during the first 3 day of fermentation, ² Fermentation power—expressed as grams of CO₂ produced in 100 mL of must until the end of fermentation, ³ P—Pleasant aroma U—Unpleasant aroma, Aroma intensity level: (+) weak, (++) intermediate (+++) strong.

Three *C. famata* isolates (WB-1, WB-2, W-5) showed an ability to produce a pleasant aroma described as fruity, while WB-4 and WB-17 isolates give weak intensity of unpleasant aroma, mainly described as a solvent. All *C. famata* isolates are characterized by significantly lower fermentative vigor (2.17 to 2.66 g CO₂/100 mL) and power (6.07 to 7.41 g CO₂/100 mL) than *S. cerevisiae*.

In order to estimate the fermentative potential of *C. famata* isolates, selected yeasts were inoculated into sterile Prokupac grape must. During the microfermentations, sugar content, release of CO₂ (Figure 1) and yeast cell number (Figure 2) were monitored. The sugar content was reduced in all fermentation trials from the initial value of 247.40 g/L. In the control sample inoculated with *S. cerevisiae*, sugar content reduced rapidly during first 5 days of fermentation and the fermentation was completed in 10 days. Fermentation with different *C. famata* isolates required longer time until the end of the process, due to slower fermentation kinetics. Among these isolates, the fastest reduction was observed for the isolate WB-1, and the slowest for fermentation with WB-4.

The release of CO₂ during the first five days of fermentation with *S. cerevisiae* rapidly increased 10–20 g/L/day (Figure 1) and in the following four days the increase was approximately 3 g/L/day. Until the end of monitoring period the release of CO₂ was from 1 to 0.13 g/L/day and the total released amount was 112.2 ± 0.7 g/L. The daily CO₂ production by the activity of *C. famata* isolates didn't changed significantly during the fermentation period (Figure 1) and ranged about 4–7 g/L/day. According to the total produced CO₂, *C. famata* can be divided in two groups. The first group with the isolates

WB-4, WB-2 and W-5, that produced around 65 g/L total amount of CO₂, while the other three isolates WB-1 and WB-17 produced up to 74 g/L.

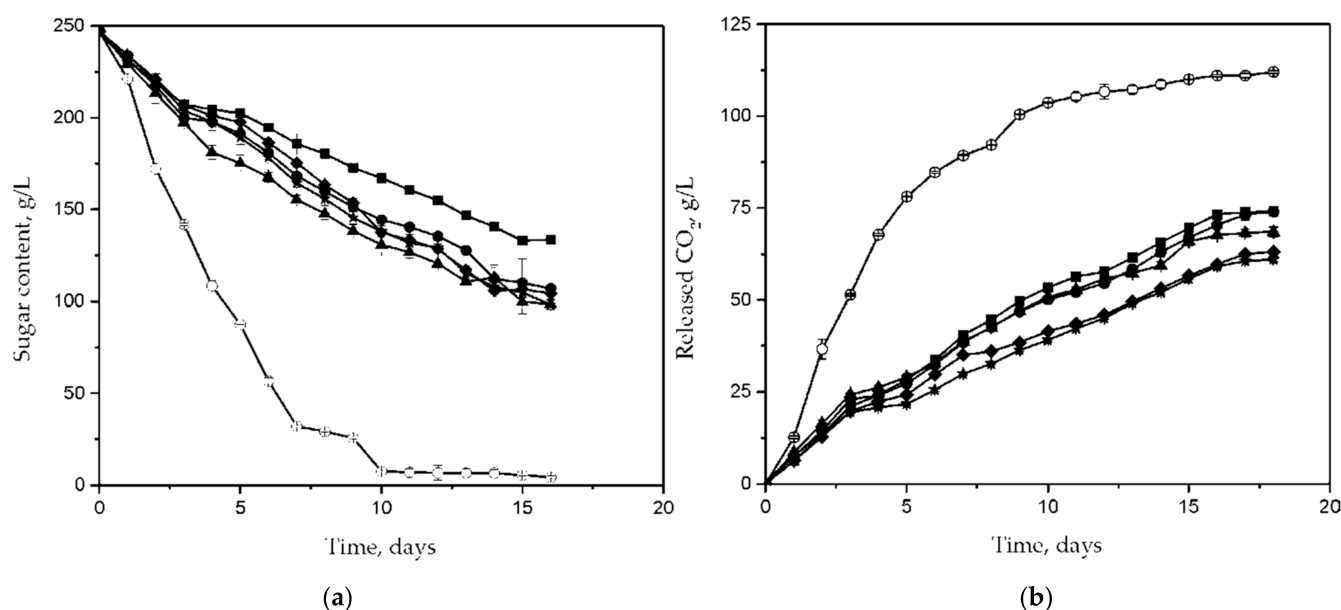


Figure 1. Total sugar consumption (a) and released amount of CO₂ (b) during microfermentation of Prokupac grape must with *C. famata* isolates WB-1 (●), WB-2 (▲), WB-4 (★), WB-17 (■), W-5 (◆) and *S. cerevisiae* ICV D254 (○). Error bars represent standard deviation.

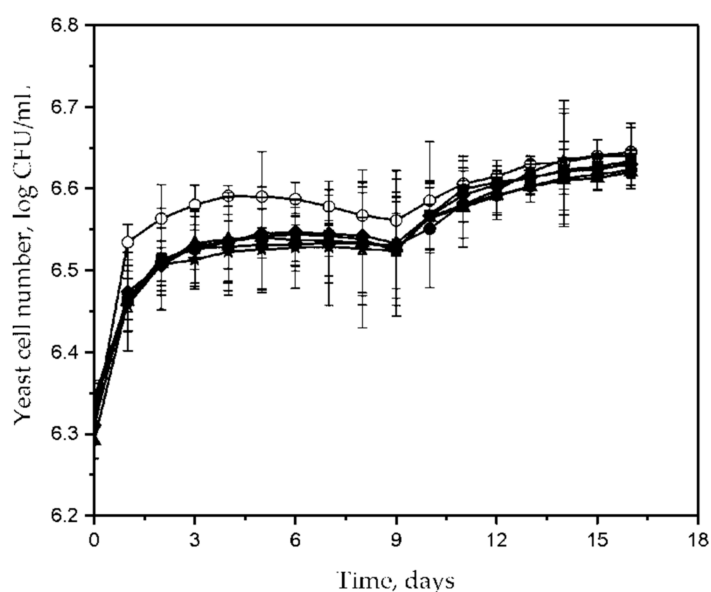


Figure 2. Cell number increase during micro-fermentation of Prokupac grape must with *C. famata* isolates WB-1 (●), WB-2 (▲), WB-4 (★), WB-17 (■), W-5 (◆) and *S. cerevisiae* ICV D254 (○). Error bars represent standard deviation.

The highest increase of yeast cell number during the fermentation was observed during the first two days, indicating that the cells were well adapted to the conditions in grape must (Figure 2).

The cell number measured after 2 days remained mostly stable until the eighth day of fermentation. This number was about 6.6 log CFU/mL for *S. cerevisiae*, and 6.5 log CFU/mL for *C. famata* isolates. No significant changes were observed for all analyzed *C. famata* isolates. After 9 days of fermentation the number of cells slightly reduced; hence, the

supplementation of nitrogen source was applied. As a result, the cell number increased and in the following period reached the value of 6.62–6.64 log CFU/mL for all analyzed yeasts.

Organic acids (tartaric, malic, lactic, succinic and acetic acid), sugars (glucose, fructose), ethanol and glycerol were detected by HPLC analysis (Table 2). Concentration of organic acids differed significantly for the samples inoculated with *C. famata* compared to the control. Tartaric acid was detected in the range 5.09–5.57 g/L, significantly higher than 4.50 g/L in the sample inoculated with *S. cerevisiae*. Malic acid was present in all samples, but was represented in a higher concentration in the samples inoculated by all analysed *C. famata* isolates. Lactic acid was also present in all samples, but in the concentration 1–1.8 g/L. The concentration significantly varied among the samples. Acetic acid was detected only in the control sample in the amount of 0.57 g/L. Analysed *C. famata* isolates didn't produce acetic acid. On the other hand, succinic acid was detected only in the fermentations with *C. famata* isolates in the range 0.6–0.9 g/L.

Table 2. Concentration reducing sugars, glycerol, ethanol and organic acid profile of grape must and wine samples at the end of microfermentations inoculated with *C. famata* isolates and *S. cerevisiae*.

Compound	Prokupac Grape Must	<i>C. famata</i> Isolate					<i>S. cerevisiae</i> ICV D254
		WB-1	WB-2	WB-4	WB-17	W-5	
Tartaric acid, g/L	5.85 ± 0.29 ^a	5.09 ± 0.06 ^b	5.17 ± 0.03 ^b	5.51 ± 0.02 ^c	5.35 ± 0.06 ^d	5.57 ± 0.07 ^a	4.80 ± 0.08 ^e
Malic acid, g/L	3.23 ± 0.01 ^a	2.86 ± 0.05 ^b	3.37 ± 0.06 ^c	4.56 ± 0.06 ^d	3.22 ± 0.06 ^a	3.03 ± 0.06 ^e	1.11 ± 0.04 ^f
Lactic acid, g/L	n.d	1.84 ± 0.08 ^a	0.98 ± 0.02 ^b	1.49 ± 0.01 ^c	1.17 ± 0.03 ^d	1.71 ± 0.06 ^e	1.64 ± 0.02 ^e
Succinic acid, g/L	n.d	0.62 ± 0.02 ^a	0.91 ± 0.04 ^b	0.56 ± 0.03 ^a	0.85 ± 0.01 ^c	0.81 ± 0.02 ^c	n.d ^d
Acetic acid, g/L	n.d	n.d	n.d	n.d	n.d	n.d	0.57 ± 0.02
Glucose, g/L	120.85 ± 3.24 ^a	25.94 ± 1.57 ^b	31.74 ± 3.11 ^{c,f}	51.22 ± 2.28 ^d	28.83 ± 1.51 ^{b,f}	28.82 ± 0.26 ^{b,f}	0.20 ± 0.11 ^e
Fructose, g/L	115.43 ± 2.78 ^a	64.31 ± 1.74 ^b	62.92 ± 2.35 ^b	79.73 ± 2.61 ^c	61.57 ± 1.50 ^b	62.11 ± 0.29 ^b	2.52 ± 0.09 ^d
Glycerol, g/L	n.d	4.98 ± 0.21 ^a	3.94 ± 0.25 ^b	3.53 ± 0.11 ^b	3.48 ± 0.22 ^b	4.70 ± 0.14 ^a	2.42 ± 0.11 ^c
Ethanol, % vol.	n.d	6.42 ± 0.26 ^a	6.12 ± 0.28 ^a	5.93 ± 0.18 ^{b,d}	6.52 ± 0.33 ^a	5.71 ± 0.05 ^{a,d}	11.03 ± 0.45 ^c

Different letters indicate significant difference between the samples, $p < 0.05$.

The total amount of glucose + fructose in the control sample was lower than 3 g/L, while in the must fermented with *C. famata* isolates WB-1, WB-2, WB-17 and W-5 the concentration of sugars was slightly higher than 90 g/L. The highest sugar concentration of 130 g/L was observed in the sample fermented with *C. famata* isolate WB-4. Analysed *C. famata* isolates produced a significantly higher amount of glycerol compared to the control sample. *C. famata* isolates WB-1 and WB-17 have shown that they are good producers with almost 5 g/L of glycerol, while other three isolates produced glycerol in the range 3.5–4 g/L. As expected, ethanol was produced in a much lower concentration compared to the control sample. The ethanol content was in the range 5.7–6.5% vol. for *C. famata* isolates, and 11% for *S. cerevisiae*.

In order to estimate the enological potential, *C. famata* isolate WB-1 was chosen for further study. Two wine samples were produced with *C. famata* isolate WB-1 in pure (CF) and sequential (CFSC) fermentation with commercial *S. cerevisiae* strain. Standard wine parameters (ethanol concentration, reducing sugar, volatile and total acidity), as well as the organic acid profile for both wines were compared with the control (SC) wine sample and given in the Table 3. Commercial *S. cerevisiae* strain produced wines with significantly higher ($p < 0.05$) ethanol content (13.0% vol.), lower volatile acidity (0.39 g/L) and reducing sugar content (1.39 g/L). However, the fermentation reached dryness in all fermentation trials, independently on the inoculated yeast and type of fermentation (pure or sequential). Several organic acids (tartaric, malic, lactic, succinic and acetic acid) were detected in wines. Content of detected organic acid in the Prokupac wine sample produced in pure and sequential fermentation with selected *C. famata* isolates were affected by the used yeast isolate.

Table 3. Characterization of Prokupac wines produced in pure and sequential fermentation with *C. famata* isolate WB-1 and *S. cerevisiae*.

Parameter	Prokupac Wine Sample		
	CF ¹	CFSC ²	SC ³
Ethanol, % vol.	11.3 ± 0.07 ^a	12.6 ± 0.02 ^b	13.0 ± 0.08 ^c
Reducing sugar, g/L	2.25 ± 0.06 ^a	2.21 ± 0.05 ^a	1.39 ± 0.02 ^b
Total acidity (as tartaric acid), g/L	6.53 ± 0.06 ^a	6.55 ± 0.07 ^a	6.48 ± 0.09 ^a
Volatile acidity (as acetic acid,) g/L	0.30 ± 0.015 ^a	0.32 ± 0.015 ^b	0.43 ± 0.02 ^c
HPLC analysis			
Tartaric acid, g/L	4.62 ± 0.12 ^a	5.47 ± 0.18 ^b	6.03 ± 0.01 ^c
Malic acid, g/L	1.32 ± 0.02 ^a	1.52 ± 0.01 ^b	3.02 ± 0.09 ^c
Lactic acid, g/L	0.67 ± 0.00 ^a	0.27 ± 0.02 ^b	0.29 ± 0.01 ^b
Succinic acid, g/L	0.75 ± 0.02 ^a	0.53 ± 0.05 ^b	0.37 ± 0.01 ^c
Acetic acid, g/L	0.06 ± 0.01 ^a	0.05 ± 0.01 ^a	0.33 ± 0.01 ^b
Glucose, g/L	0.19 ± 0.00 ^a	0.14 ± 0.00 ^b	0.12 ± 0.00 ^c
Fructose, g/L	1.56 ± 0.00 ^a	1.79 ± 0.02 ^b	1.60 ± 0.00 ^c
Glycerol, g/L	7.12 ± 0.13 ^a	7.45 ± 0.23 ^a	5.76 ± 0.09 ^b
Ethanol, % vol.	11.63 ± 0.08 ^a	12.05 ± 0.16 ^b	13.00 ± 0.42 ^c

Different letters indicate significant difference between the samples, $p < 0.05$. ¹ CF—pure fermentation with *C. famata* isolate WB-1, ² CFSC—fermentation with sequential inoculation of *C. famata* isolate WB-1 and *S. cerevisiae*, ³ SC control sample inoculated with *S. cerevisiae*.

4. Discussion

Despite the fact that *Candida* spp. along with other non-*Saccharomyces* wine yeasts positively contribute to the final quality of the wine [2], to the best of our knowledge, the fermentative capabilities of *C. famata* have not been tested. The possible use of *C. famata* in wine fermentation can also be justified by the production of enzymes, mainly protease and β -glucosidase, which is more expressed in non-*Saccharomyces* yeasts than in *S. cerevisiae* strains [16]. This enzyme can enhance the conversion of non-aromatic precursors from grape must to actively form of aromatic volatile compounds in the wine [15]. In order to assess the potential of the *C. famata* for the production of Prokupac wine, isolation, identification and fermentation ability of the *C. famata* isolated from blackberries were performed. Yeast isolates from wild and cultivated blackberries were identified. Among the yeast isolates, *C. famata* represented 24% of wild and 20% of cultivated blackberries microbiota. The total number of isolated yeasts from cultivated blackberries was significantly lower probably due to the application of fungicides.

Characterization of *C. famata* isolates indicated good tolerance to low temperatures and growth even at the refrigeration temperature. Sulfite resistance is required attribute for the wine yeasts because during winemaking SO₂ is almost always used as a microbial inhibitor and antioxidant. The tolerance to SO₂ was very good, since it reached the value of 300 ppm. Investigation of possible application of *Starmerella bacillaris* in wine production showed poor tolerance to SO₂ reaching the value of 50 ppm [16]. On the other hand, *Nakazawaea ishiwadae* and *Lodderomyces elongisporus* showed particularly good tolerance to SO₂ since they can growth in the presence of 400 ppm SO₂ [3]. Analysis of the ethanol tolerance indicated that growth reduction in the presence of 5% ethanol was up to 20%, while W-5 isolate showed the reduction of almost 50% with the ethanol concentration of 7%. Further increase of ethanol content significantly reduced the growth of *C. famata* isolates. The results are consistent with [13], which showed good ethanol tolerance (up to 6%) for the most isolates of *C. famata* from the Mavrodafni grapes and must solids, while a few isolates could tolerate ethanol concentrations up to 8%. The ability of *C. famata* isolates (WB-1, WB-2, WB-4 and WB-17) to grow (maintain more than 50% growth) at moderate concentrations of ethanol (up to 7%) indicate the possibility for this species to be present in

the middle phase of the fermentation. According to literature data [32], the initial phase of fermentation (the tumultuous stage) lasts until around 30% of the initial amount of sugar is consumed, while after this point, the middle phase of fermentation begins. Some authors have shown that various non-*Saccharomyces* species (*Metschnikowia*, *Candida*, *Pichia*) are present in the later stages of alcoholic fermentation [33] suggesting their relatively good tolerance to ethanol, but also indicating the potential to influence the aroma and complexity of the produced wines. Literature data related to other non-*Saccharomyces* yeast presented the tolerance to 10% ethanol for *Aureobasidium pullulans*, significant decrease of viability of *Torulaspora delbrueckii* with 6% ethanol, and very little growth of *Kazachstania aerobia* in the presence of ethanol concentrations higher than 2% (Lin et al., 2020). The growth of *N. ishiwadae* and *L. elongisporus* was reduced in the presence of 5% and 10% ethanol and no growth of *L. elongisporus* was observed with 15% of ethanol [3]. Fermentative vigor and power, as an important selection criteria for the wine yeast, indicate moderate fermentation capacity of tested *C. famata* isolates. However, WB-1 and WB-17 isolates had higher values, when compared to the other isolates. Results obtained in this work are in line with previously published for different *Candida* species (from 2 to 9.6% of ethanol) [34].

Results of the sugar content reduction indicated that fermentation with all tested *C. famata* isolates was much slower compared to the control, but during the monitoring period the fermentation was continuous. The rapid reduction of sugar content during the first 5 days of fermentation for a sample inoculated with *S. cerevisiae* resulted in the rapid release of great amount of CO₂ and indicated good fermentation ability of this strain. Similarly, Lin et al. [11] showed that the total amount of sugars in grape juice by *S. cerevisiae* EC1118 was depleted after 80 h of fermentation. Although, analyzed *C. famata* isolates showed slower consumption of sugars, they released a significant amount of CO₂ indicating relatively good fermentation capacity. Similar results for CO₂ release were reported for *Lachancea thermotolerans*, *Candida zemplinina* and *Metschnikowia* spp. in grape must [35] and for *L. thermotolerans* SOL13, *C. zemplinina* MALV45, *Metschnikowia* sp. FIANO12 and *S. cerevisiae* EC 1118 in pasteurized grape must [36]. Slower wine fermentation can be considered positive due to the better retention of volatiles and lower demand for energy during fermentation [37]. The cell number in the control sample differed significantly ($p < 0.05$) from other samples until the 13th day of fermentation. After that point, the difference was not significant ($p > 0.05$), and all analyzed isolates showed similar growth characteristics. The increase of the cell number in both fermentations with *C. famata* and *S. cerevisiae* was much lower than the increase of 1–2 log CFU/mL reported by Binati et al. [35] or *L. thermotolerans*, *C. zemplinina* and *Metschnikowia* spp. On the other hand, after 10 days of fermentation the cell number increase of *L. thermotolerans* SOL13, *C. zemplinina* MALV45 in pasteurized grape must was negligible [36].

C. famata isolates were subjected to the simple screening olfactory test useful for separating them into three categories: without growth, these that generate pleasant and unpleasant aroma [31]. According to the results, the most promising *C. famata* isolates were WB-1 and W-5 which were able to produce intermediate level of pleasant aroma characterized as fruity. An unpleasant aroma profile was detected on plates inoculated with *C. famata* isolates WB-4 and WB-17 which diminishes their importance for winemaking.

Organic acids are of the crucial importance for the sugar-acid balance, organoleptic characteristics and chemical stability of wines [38]. Total acidity, as well as the content of individual organic acids contribute to the overall quality and taste of wine. Tartaric, malic and citric acids present in wine originated from grapes, while succinic, lactic and acetic acids are formed during the fermentation process and mainly depend on the vinification conditions and the yeast strain [38,39]. After micro-vinification of sterile Prokupac gape must tartaric, malic, succinic, lactic and acetic acid were detected in the samples. Although the total acidity of wine is crucial for the sugar-acid balance and sensory character of wine, the type and content of each individual organic acid in the wine is very important because of sensory attributes and contribution to the organoleptic characteristics of wines. Yeast

strain and fermentation conditions were underlined as the main factor responsible for the organic acid profiles of red and white wine [39].

All analyzed samples, including the one fermented with *S. cerevisiae*, had significant amount of tartaric acid 4.5–5.6 g/L. Analysis of different types of red wine indicated the concentration of tartaric acid up to 4 g/L with tendency to decrease during fermentation [40]. Concentration of malic acid was significantly lower in the sample fermented with *S. cerevisiae* compared to *C. famata* isolates. Compared to other isolates, WB-4 isolate produced significantly higher quantity of malic acid, which are higher than a range detected in red Spanish and Brazilian wines [38]. The content of malic acid in Tempranilo red wines produced with *Saccharomyces* and non-*Saccharomyces* yeasts was in the range 1.5–2.5 [41] which is higher than the value obtained for sample inoculated with *S. cerevisiae* in this study. Higher concentration of malic acid was observed in the samples with incomplete conversation of sugar. This is in accordance with the decrease of the malic acid concentration during wine fermentation [40] and the fact that *C. famata* isolates were not able to conduct the fermentation till the end. This is often case with non-*Saccharomyces* yeasts and the best solution is the sequential fermentation with *S. cerevisiae*. The concentration of lactic acid varied among the samples, while the highest content of 1.8 g/L was detected in the fermentation trial with the isolate WB-1. Obtained results are in accordance with the literature data, suggesting normally level of lactic acid in wines, from 1 to 3 g/L [39]. Similar amount of lactic acid, up to 4 g/L was observed for non-*Saccharomyces* yeast *L. thermotolerans* in sequential fermentation with *S. cerevisiae* [35]. Acetic acid was produced only by *S. cerevisiae* in the concentration of 0.57 g/L which is in accordance with the research of fermentation capabilities of different *S. cerevisiae* strains for the production of Tokaj wine [6] and in the optimal range of 0.2–0.7 [9]. On the other hand, *S. cerevisiae* had no capability of producing succinic acid which was detected in all the samples fermented by *C. famata* isolates. Similar amount of succinic acid was detected in Pino Noir red wines [42]. The production of succinic acid can positively affect the organoleptic properties of wine, but as this acid has a “salt-bitter-acid” taste, its increased concentration can negatively affect the quality of wine [1].

During micro-vinification *C. famata* isolates produced significant amount of glycerol in the range 3.5–5 g/L. Isolates WB-1 and W-5 were significantly better glycerol producers compared to other analyzed isolates. The lowest amount of glycerol was produced by *S. cerevisiae*. As expected, the highest content of ethanol (11%) was detected in fermentation trial with *S. cerevisiae*, while none of the tested *C. famata* isolates did not ferment Prokupac grape must to dryness. In all fermentation trials with *C. famata* isolates, the ethanol content was similar and in the range from 5.71 to 6.52% vol. Results confirmed the earlier report where *Candida* yeast was characterized as low ethanol, and high glycerol producer [43]. Similar was observed for other non-*Saccharomyces* yeasts. *Hanseniaspora uvarum* during micro-vinification of Macabeo grape must produced 2.3 g/L glycerol and 3.1% vol. ethanol with 92 g/L residual glucose. *Metschnikowia pulcherrima* inoculated in the same type of must produced higher amount of ethanol, 5.37% and lower concentration of glycerol, 1.34 g/L, while residual glucose was 44 g/L. *Pichia fermentans* produced similar amount of ethanol 5.98% and 2.31 g/L glycerol with 39 g/L residual glucose. *S. cerevisiae* used in the same study, produced 14% ethanol and 0.57 g/L glycerol and consumed almost all amount of glucose, similar to the results obtained in present research [44]. Many strains of *T. delbrueckii* were able to produce glycerol in the amount 5–6 g/L, but produced significantly higher amount of ethanol [45]. Glycerol has no influence on the wine aromatic characteristics, but contributes to mouth-feel and sweetness and its concentration in wines is in the range 1.3–14.7 g/L [46].

Despite the fact that *C. famata* isolates WB-1, WB-2 and W-5 showed similar fermentation performance, *C. famata* WB-1 isolate was chosen for validation at laboratory-scale level based on pleasant fruity aroma, highest fermentative vigor and power, good organic acid profile and highest level of ethanol and glycerol produced in micro-vinification experiments.

Although the ethanol content in the wine sample produced in pure fermentation with *C. famata* WB-1 isolate (11.3% vol.) was lower compared to the control sample (13% vol.), low level of residual sugar indicate that the fermentation was complete. Since it has been shown that *C. famata* WB-1 isolate can produce about 6.4% vol. ethanol in sterile Prokupac must, it can be concluded that native *S. cerevisiae* strains from the grape indigenous microbiota took over and finished the fermentation. Further, it was shown that yeast strain used for the fermentation did not significantly influence total wine acidity, while significantly lower volatile acidity was detected in both samples fermented with *C. famata* (pure or sequential) compared to the control sample. The total (titratable) and volatile acidity for both samples produced with *C. famata* isolate were consistent with previously published results for the monovarietal Prokupac wines [20,22]. Beside the total acidity, it was proven that organic acid profile is strongly correlated with wine overall quality and sensory characteristics [38,39]. Content of grape acids (tartaric and malic acid) mainly stay unaffected by the *S. cerevisiae* yeast metabolism [47], however, in the line with the results in this study, significant reductions in the malic acid were detected earlier in the wine samples produced by some non-*Saccharomyces* strains [40,48]. Level of tartaric and malic acid, known as the grape organic acids, detected in produced Prokupac wines were in ranges found in wines from Syrah, Carmen, Merlot and Bordo grape varieties [39,49].

Independently on the *C. famata* inoculation, significantly higher glycerol content was detected in both samples produced in pure and sequential fermentation than in the control sample. Level of produced glycerol was consistent with previously published results for some *Candida* strains [43]. High level of glycerol production is one of the desirable characteristics in wine yeast selection. A negligible amount of acetic acid was produced in both samples inoculated with *C. famata*, however, the amount detected in the control sample (0.33 g/L) is still below the level considered undesirable [50]. Enhancement of glycerol production and low level of acetic acid production during fermentation has previously been confirmed for different *Candida* species [51–53]. According to low production of acetic acid and consequently low volatile acidity, the fermentation purities (ratio between total volatile acidity in g/L and ethanol in % vol.), were lower than 0.05 for the samples produced in pure and sequential fermentation of Prokupac grape must with selected *C. famata* isolate, revealing the good enological performance of this isolate.

5. Conclusions

The results presented in this study indicate a similarity of *C. famata* isolates (WB1, WB2 and W5) based on the tested fermentation performances. These isolates can potentially be used as a starter culture for the Prokupac wine fermentation, since they are able to ferment about 60% of sugar from the fermentative media, generate a pleasant fruity aroma, produce a satisfactory level of organic acids and a higher amount of glycerol compared to the commercial *S. cerevisiae* strain. Selected *C. famata* WB-1 isolate, in both pure and sequential fermentations, can produce dry wines with a lower level of ethanol which is in line with consumer demand of wine wholesomeness. Good enological performance of this isolate confirms high potential in glycerol production, good organic acid profile in produced samples and high fermentation purity.

Sensory evaluation and aroma profile of produced wine samples should be performed to complement the obtained results and confirm the ability of selected *C. famata* isolates to produce high quality Prokupac wines.

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