Influence of Media Composition and Temperature on Volatile Aroma Production by Various Wine Yeast Strains

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Abstract

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Volatile aroma compounds production by two autochthonous *Saccharomyces cerevisiae* strains, isolated from Istria region, and three other yeast strains (*Saccharomyces bayanus* and two commercial *Saccharomyces cerevisiae* wine yeasts) was investigated on a small scale using synthetic VP4 medium and Graševina must at 12 and 20°C. The results obtained by gas chromatography analyses were compared with the aroma production properties of the native microflora, remaining after Graševina must sulphiting. In both media and at both temperatures, the wine yeasts investigated showed different metabolic profiles regarding the tested volatile aroma compounds, which should be taken in consideration for autochthonous wine production. Although the synthetic medium proved to be appropriate for the investigation of the fermentative properties, the determination of secondary aroma production by wine yeasts has to be conducted by must fermentation or possibly by fermentation of another synthetic medium whose composition would be more similar to must.

Keywords: fermentation; *Saccharomyces bayanus*; *Saccharomyces cerevisiae*; volatile aroma compounds; wine yeast

The selection of a good yeast strain having desirable properties is a prerequisite for the quality wine production (Degree *et al*. 1993). Enological traits of *S. cerevisiae* have been divided into two groups, i.e. technological and qualitative, and both groups have to be considered in the selection of wine yeasts. The technological ones influence the fermentation efficiency, and the qualitative ones determine the chemical composition and sensorial characteristics of wines. Yeast should have a corresponding metabolic profile, i.e. they should be chosen according to aroma and flavour that is typical for each wine (Reed & Nagodavithana 1991; Romano *et al*. 1998; Raineri & Pretorius 2000).

In our previous paper, two autochthonous *Saccharomyces cerevisiae* yeast strains, isolated from Istria region, proved to have good fermentative properties which corresponded well both in the chemically defined VP4 medium (Petravić Tominac *et al*. 2005a) and in Graševina must (Petravić Tominac *et al*. 2005b) fermented at two temperatures. Because of the differences in the composition of

the two fermentation media, the aim of this work was to evaluate the metabolic profiles of the yeast strains in both media using a new gas chromatographic method developed in our laboratory.

MATERIAL AND METHODS

Microorganisms. Two *Saccharomyces cerevisiae* yeast strains (ZIM 1899 and ZIM 1900) were isolated from Istria region in our previous work (Petravić Tominac *et al*. 2005a). Strain Z-2 (*Saccharomyces bayanus*) and two commercial wine yeast strains (both *Saccharomyces cerevisiae*), denominated as A and B, were used to compare the wine yeast strains properties. Yeast strains were previously identified by sequencing D1/D2 region of the 26 S rDNA, as described in "Yeast of the world. Morphology, physiology, sequences and identification, Version 2.0" (software) (Boeckout *et al*. 2002). The native microflora, remaining after Graševina must sulphiting, was used as well for spontaneous fermentation.

Media. Synthetic VP4 medium was used for inocula cultivation and microfermentation. The composition of VP4 medium, containing yeast extract and peptone, is partially in agreement with the must composition as concerns sugar and nitrogen contents, pH value and the buffer capacity (Petravić Tominac *et al*. 2005a). Graševina must from Križevci area, Croatia, was used for microvinification (Petravić Tominac *et al*. 2005b).

Fermentation. The fermentation inocula preparation, as well as microfermentation of VP4 medium and microvinifications at 12 and 20°C, were done in triplicates as published previously (Petravić Tominac *et al*. 2005a, b). Yeast inocula used in both experiments was of 2×10^6 cells/ml. The fermented synthetic media and the produced wines were centrifuged and the supernatants were frozen for later analysis.

Headspace gas chromatography (HSS-GC). The concentrations of nine volatile aroma compounds (acetaldehyde, diacetyl, isoamyl alcohol, ethyl acetate, isoamyl acetate, ethyl propionate, ethyl hexanoate, ethyl octanoate, and ethyl lactate) were determined by gas chromatography using a Varian 3300 gas chromatograph with a split/splitless injector and a flame ionisation detector (FID). For the headspace analyses, Hewlett Packard headspace sampler HP 7694 was used. The method developed in our laboratory involved the injection of the standard and the analysed compounds, the determination of their retention times, and optimisation of carrier gas flow and working temperatures. The compounds of interest were resolved on a DB-624 capillary column (30 m × 0.25 mm i.d.× df = 0.25 μm) with the following parameters: the initial oven temperature of 35°C was kept for 2 min, then it was raised by 10°C/min to 90°C followed by 15°C /min to 150°C and kept for 7 min at 150°C. The injection port temperature was kept at 120°C, the carrier gas (nitrogen) pressure flow was 3 ml/min. The detector temperature was 200°C. The headspace sampler was equipped with a standard 1 ml loop. The carrier gas pressure was 4.4 Psi, vial pressure 7 Psi and injection time 0.2 min. The samples were equilibrated by heating at 80°C for10 minutes. Qualitative analysis was carried out by comparison of the retention times of the standards and the corresponding peaks obtained with the samples. The quantification was carried out by comparison of the areas of the peaks to those of the internal standard.

Statistical analysis. Statistical analyses of the experimental data were done by ANOVA using Excel 2000 (Anonymous 2002) and Duncan's multiple range test (Montgomery 1984).

RESULTS AND DISCUSSION

The basic fermentative properties of the tested yeast strains in VP4 medium and Graševina must fermented at two temperatures were published in our previous papers (Petravić Tominac *et al*. 2005a, b). Alcohol contents obtained in the final products and fermentation times are shown in

Table 1. Alcohol contents and fermentation times for the fermentations performed in VP4 medium (Petravić Tominac *et al.* 2005a) and in Graševina must (Petravić Tominac *et al.* 2005b)

Fermentation temperature (°C)	Fermented VP4 medium		Graševina must	
	φ(ethanol) (%)	*t* (days)	φ(ethanol) (%)	*t* (days)
12	9.25–9.88	59	11.41–12.05	29
20	8-79–9.22	38	11.21–11.81	21

Table 2. Concentrations of volatile aroma compounds (mg/l) in sterile synthetic VP4 medium and sulphited Graševina must

Volatile compound γ (mg/l)	Fermentation medium	
	VP4 medium	Graševina must
Acetaldehyde	1.08	9.00
Diacetyl	0	0.62
Ethyl acetate	4.57	0.20
Ethyl propionate	0	0
Isoamyl alcohol	1.68	1.65
Ethyl hexanoate	0.44	0
Ethyl lactate	13.45	0
Ethyl octanoate	0.57	0
Isoamyl acetate	1.12	0.01

Table 1. The aim of this work was to compare the production of volatile aroma compounds by the wine yeasts investigated in synthetic medium and in must fermented at two temperatures. The most pronounced differences between the sterilised unfermented VP4 medium and sulphited Graševina must regarding the volatile compounds were found with ethyl octanoate, following by acetaldehyde and ethyl acetate (Table 2).

The levels of the volatile aroma compounds determined in fermented VP4 medium are shown in Table 3 shows the concentrations of the same compounds in produced wines.

Lower fermentation temperature

The concentrations of the analysed volatile aroma compounds in synthetic VP4 medium and in must fermented at 12°C are shown in Tables 3 and 4, respectively.

Diacetyl concentrations present in all samples of both fermented media were higher than the usual literature values for wine, possibly because the samples were taken immediately after the end of fermentation, without maturation during which these concentrations would have been lowered (Bartowsky & Henschke 2004). The levels of diacetyl produced in must fermentation using different yeast strains were approximately 3 times higher than its levels in synthetic VP4 medium. The only exceptions were fermentations performed by commercial yeast starters A and B.

Although the increased diacetyl concentrations in wine can be produced by lactic acid bacteria that are able to produce much higher diacetyl levels than yeasts (Lambrechts & Pretorius 2000), in our work this was not the probable reason. Lactic acid bacteria could be still present in sulphited must, but the presence of bound SO_2 of 50–100 mg/l or free SO_2 of 1–10 mg/l is sufficient to inhibit their growth in wine (Romano & Suzzi 1993). Since the must was initially sulphited with 100 mg/l of SO_2 and at the moment of inoculation it contained 74.88 mg/l of total SO_2 (10.24 mg/l of free SO_2 and 64.64 mg/l of bound SO_2) (Petravić Tominac *et al.* 2005b), the growth of lactic acid bacteria was considered to be repressed and their number was not determined in this work.

The more probable reason for the increased diacetyl levels was that yeast physiological activity was negatively affected by sulphite added into must, which could favour diacetyl formation, similar to the well documented brewing of beer wort (Bartowsky & Henschke 2004). This presumption could be confirmed as well by diacetyl levels formed in spontaneously fermented wine (Table 4).

Acetaldehyde levels in both media and at both temperatures (Tables 3 and 4) were in agreement with the data of Romano *et al.* (1998), who reported that almost 90% of *Saccharomyces cerevisiae* wine strains produced less than 50 mg/l acetaldehyde. Among the investigated yeasts, ZIM 1899 and commercial yeast B produced the lowest but about equal acetaldehyde concentrations in synthetic VP4 medium (Table 3). Most of the investigated yeasts produced higher acetaldehyde concentrations in must than in synthetic medium fermentation. Apart from the differences in the fermentation media composition, it could be also due to sulphite added to must. It is known that acetaldehyde combines with sulphur dioxide in wines made from healthy grapes and acetaldehyde production can be a yeast self-defence mechanism. The formation of acetaldehyde is known as a strain-dependent property. Acetaldehyde values determined in our work referred to free acetaldehyde, not to its fraction bound to sulphite.

The levels of isoamyl alcohol were similar in both fermented media but about 30–50% higher in wines, depending on the yeast strain.

Generally, ethyl acetate formation was higher during must fermentation than during fermentation of the synthetic medium, because ethanol and acetic acid are most abundant in wine. Commercial

Table 3. Concentrations of volatile aroma compounds (mg/l) in synthetic VP4 medium fermented at 12°C and 20°C

Volatile compound γ (mg/l)	Inoculated yeast strain				
	ZIM 1900	ZIM 1899	Z-2	A	B
Fermented at 12°C					
Acetaldehyde	42.26 ± 7.721	16.50 ± 3.106	45.80 ± 12.848	23.47 ± 18.678	10.60 ± 5.792
Diacetyl	6.42 ± 0.972	6.09 ± 1.259	6.84 ± 2.249	5.95 ± 0.245	6.35 ± 2.546
Ethyl acetate	30.12 ± 2.698	35.40 ± 6.556	30.47 ± 8.892	47.35 ± 3.562	37.06 ± 15.756
Ethyl propionate	0.12 ± 0.003	0.08 ± 0.022	0.17 ± 0.059	0.07 ± 0.050	0.04 ± 0.021
Isoamyl alcohol	53.68 ± 4.548	57.42 ± 10.135	51.87 ± 15.183	74.00 ± 3.644	50.90 ± 20.763
Ethyl hexanoate	0.44 ± 0.086	0.26 ± 0.080	0.39 ± 0.158	0.24 ± 0.020	0.23 ± 0.180
Ethyl lactate	13.45 ± 3.667	10.85 ± 2.940	13.95 ± 2.872	11.72 ± 1.104	11.15 ± 0.873
Ethyl octanoate	0.57 ± 0.086	0.30 ± 0.135	0.57 ± 0.286	0.30 ± 0.068	0.32 ± 0.319
Isoamyl acetate	1.12 ± 0.115	0.89 ± 0.405	0.76 ± 0.313	1.88 ± 0.323	0.89 ± 0.849
Fermented at 20°C					
Acetaldehyde	9.61 ± 1.751	6.80 ± 0.977	13.55 ± 0.248	8.96 ± 0.301	10.01 ± 5.849
Diacetyl	7.54 ± 1.438	6.10 ± 0.294	9.105 ± 1.500	8.27 ± 0.063	11.23 ± 2.017
Ethyl acetate	45.37 ± 5.837	40.83 ± 3.845	44.41 ± 7.357	58.03 ± 0.704	108.12 ± 22.961
Ethyl propionate	0.04 ± 0.008	0.04 ± 0.003	0.06 ± 0.004	0.03 ± 0.0059	0.04 ± 0.012
Isoamyl alcohol	57.02 ± 7.246	48.33 ± 3.169	59.37 ± 9.479	65.23 ± 0.391	103.12 ± 23.292
Ethyl hexanoate	0.41 ± 0.129	0.19 ± 0.045	0.46 ± 0.076	0.31 ± 0.013	0.32 ± 0.047
Ethyl lactate	14.13 ± 1.010	11.52 ± 1.489	13.30 ± 0.630	12.28 ± 1.204	11.60 ± 4.797
Ethyl octanoate	0.41 ± 0.163	0.23 ± 0.037	0.49 ± 0.089	0.45 ± 0.073	0.47 ± 0.174
Isoamyl acetate	0.75 ± 0.172	0.49 ± 0.063	0.72 ± 0.036	1.31 ± 0.021	0.97 ± 0.145

yeast strain A was an exception, producing less ethyl acetate in must fermentation.

For most strains and at both temperatures, the concentrations of ethyl hexanoate and ethyl octanoate were lower in the fermented (Table 3) than in the non-fermented synthetic VP4 medium (Table 2). It could be concluded that the activity of ethyl hexanoate-synthesising enzyme, ethanol hexanoyl transferase Eht 1p, was lower in VP4 medium than was the activity of ethyl hexanoate-hydrolysing enzymes (Verstrepen *et al.* 2003). Similarly, if there was any ethyl octanoate-synthesising activity in VP4 medium, it was in most cases lower than its hydrolysing activity (Lambrechts & Pretorius 2000).

The concentrations of ethyl lactate produced by all yeast strains were similar, but slightly higher in wines (Table 4). Ethyl lactate levels formed in spontaneous fermentations were not significantly different from those obtained with inoculated fermentations. Differences also occurred between ethyl propionate levels in wine and VP4 medium. Spontaneous must fermentation gave less ethyl hexanoate than inoculated fermentations. Although ethyl hexanoate, ethyl octanoate, and ethyl lactate were present in non-fermented synthetic medium and absent from Graševina must (Table 2), their concentrations were higher in wines (Table 4) than in VP4 fermented medium (Table 3), supposedly because the enzyme activity responsible for their synthesis was higher in must than in VP4 medium (Verstrepen *et al.* 2003).

Fermentation at higher temperature

Generally, higher concentrations of diacetyl in both media were produced at 20°C (Tables 3 and 4). On the contrary, acetaldehyde levels produced at 20°C were smaller than its levels at 12°C but much higher in wine than in the fermented synthetic

Table 4. Concentrations of volatile aroma compounds (mg/l)) in Graševina wine produced at 12°C and 20°C

Volatile compound γ (mg/l)	Inoculated yeast strain					Spontaneous fermentation
	ZIM 1900	ZIM 1899	Z-2	A	B	
Fermented at 12°C						
Acetaldehyde	43.63 ± 7.939	41.07 ± 10.606	62.11 ±12.965	34.56 ± 12.139	35.45 ± 15.738	40.90 ± 20.195
Diacetyl	17.98 ± 3.332	16.16 ± 4.0315	22.99 ± 1.542	8.30 ± 5.677	13.82 ± 6.283	15.65 ± 3.717
Ethyl acetate	47.12 ± 6.381	44.78 ± 13.044	61.36 ± 2.957	39.19 ± 14.888	42.90 ± 21.092	46.11 ± 9.399
Ethyl propionate	0.09 ± 0.029	0.086 ± 0.0336	0.12 ± 0.029	0.07 ± 0.031	0.08 ± 0.042	0.10 ± 0.033
Isoamyl alcohol	73.93 ± 10.727	68.27 ± 18.921	92.67 ± 4.230	59.77 ± 23.250	64.69 ± 31.831	66.50 ± 13.760
Ethyl hexanoate	0.68 ± 0.151	0.65 ± 0.254	0.89 ± 0.115	0.53 ± 0.165	0.58 ± 0.377	0.46 ± 0.083
Ethyl lactate	15.61 ± 1.877	15.64 ± 0.915	16.26 ± 2.358	15.71 ± 0.833	13.90 ± 2.728	16.04 ± 1.150
Ethyl octanoate	1.05 ± 0.378	0.98 ± 0.475	1.33 ± 0.248	0.83 ± 0.218	0.87 ± 0.644	0.94 ± 0.208
Isoamyl acetate	3.06 ± 0.470	2.60 ± 0.856	3.48 ± 0.575	2.49 ± 0.853	2.53 ± 1.484	1.92 ± 0.410
Fermented at 20°C						
Acetaldehyde	40.39 ± 5.833	40.39 ± 5.833	34.59 ± 7.410	27.84 ± 4.967	35.94 ± 3.959	30.05 ± 3.284
Diacetyl	15.77 ± 2.856	15.77 ± 2.856	13.76 ± 2.870	10.74 ± 2.375	14.26 ± 2.945	12.01 ± 2.113
Ethyl acetate	112.04 ± 13.935	112.04 ± 13.935	83.62 ± 16.219	80.47 ± 20.569	109.06 ± 22.734	88.20 ± 16.037
Ethyl propionate	0.08 ± 0.012	0.08 ± 0.012	0.07 ± 0.018	0.06 ± 0.017	0.07 ± 0.032	0.06 ± 0.011
Isoamyl alcohol	123.55 ± 16.043	123.55 ± 16.043	100.04 ± 19.606	92.45 ± 24.884	117.54 ± 31.248	96.56 ± 18.653
Ethyl hexanoate	0.65 ± 0.076	0.65 ± 0.076	0.56 ± 0.161	0.49 ± 0.081	0.55 ± 0.086	0.27 ± 0.067
Ethyl lactate	16.59 ± 2.399	16.59 ± 2.399	15.84 ± 2.176	15.59 ± 1.073	17.03 ± 2.216	16.30 ± 1.663
Ethyl octanoate	0.90 ± 0.249	0.90 ± 0.249	0.80 ± 0.279	0.75 ± 0.209	0.93 ± 0.080	0.19 ± 0.048
Isoamyl acetate	3.44 ± 0.501	3.44 ± 0.501	2.77 ± 0.746	2.76 ± 0.537	2.95 ± 0.569	2.15 ± 0.451

medium. Partial reason for this was a significantly higher concentration of this compound in sulphited must than in the non-fermented synthetic medium (Table 2).

Wines produced at 20°C contained higher concentrations of isoamyl alcohol (Table 4). Its concentration was almost 2 times higher than in VP4 medium fermented under the same conditions (Table 3). Most of the yeasts starters used for microvinification resulted in higher levels of isoamyl alcohol in comparison to those that were produced in spontaneous fermentations.

Higher ethyl acetate levels were produced at the higher temperature in both media (Tables 3 and 4), which is in agreement with the literature (Verstrepen *et al*. 2003).

Generally, VP4 medium fermented at the higher temperature contained higher amounts of isoamyl acetate (Table 3). Its concentrations in the wine samples were significantly higher than in the fermented VP4 medium (Table 4). Spontaneous must fermentation gave considerably less isoamyl acetate than inoculated fermentations.

The concentrations of ethyl lactate in wine were similar to those produced at 12°C. The difference caused by different temperatures was most pronounced with commercial yeast strain B. Ethyl lactate levels formed in spontaneous fermentations were not significantly different from those obtained after inoculated fermentations. In both media, ethyl propionate concentrations were mostly smaller than that at 12°C. Differences also occurred between ethyl propionate levels in wine and those in VP4 medium.

Decreased levels of ethyl hexanoate and ethyl octanoate were observed in VP4 medium at 20°C, as well as at the lower temperature. Spontaneous must fermentation gave less ethyl hexanoate than inoculated fermentations, as found at 12°C, as well. Regarding ethyl hexanoate, ethyl octanoate, and ethyl lactate, their concentrations were higher in wines as already noticed at 12°C.

Only acetaldehyde, isoamyl alcohol, and ethyl lactate, produced by fermentation of both media at both temperatures, were below their taste thresholds (Etiévant 1991; Lambrechts & Pretorius 2000; Margalit 2004; Francis & Newton 2005; Swiegers & Pretorius 2005).

Statistic

In synthetic VP4 medium, yeast strains showed no significant differences regarding acetaldehyde, diacetyl, isoamyl alcohol, ethyl propionate, ethyl acetate, and ethyl octanoate ($P > 0.05$), while ethyl lactate, isoamyl acetate, and ethyl hexanoate were influenced by the yeast strains ($P < 0.05$). The fermentation temperature influenced acetaldehyde and ethyl propionate levels in VP4 medium ($P < 0.05$) which were higher at 12°C. The differences of diacetyl, isoamyl alcohol, ethyl acetate, ethyl octanoate, ethyl lactate, isoamyl acetate, and ethyl hexanoate concentrations at 12°C and at 20°C were not significant ($P > 0.05$).

In the Graševina wines produced, the fermentation temperature had a statistically significant influence only on isoamyl alcohol and ethyl acetate concentrations ($P < 0.05$), while the differences between other analysed volatile compounds at 12°C and at 20°C were not significant ($P > 0.05$).

At 12°C, the influence of the medium composition on ethyl acetate and isoamyl alcohol levels was not significant ($P > 0.05$), while at 20°C the medium showed significant differences regarding the concentrations of these two aroma compounds ($P < 0.05$). Acetaldehyde, diacetyl, isoamyl acetate, ethyl propionate, ethyl hexanoate, ethyl octanoate, and ethyl lactate were influenced by the medium used for fermentation at both temperatures ($P < 0.05$).

CONCLUSIONS

Fermentation of both VP4 synthetic medium and Graševina must resulted in different metabolic profiles of the yeast strains regarding nine tested volatile aroma compounds. Although VP4 medium in the previous tests proved to be a suitable medium for fermentative properties investigation, it exhibited slightly different secondary aroma production by wine yeasts compared to sulphited must. Therefore, metabolic profiles investigation has to be conducted by must fermentation or possibly by of fermentation such synthetic medium whose composition would be more similar to must.

Statistical methods (ANOVA, Duncan test) applied for the must fermentation performed at 20°C showed statistically significant differences between the tested yeast strains ($P < 0.05$) concerning the production of the analysed volatile aroma compounds with the exception of the yeast strain A and spontaneous fermentation ($P > 0.05$) (Anonymous 2002; Montgomery 1984). The yeast strain ZIM 1900 showed the highest production of the volatile compounds tested, followed by ZIM 1889, B, Z-2,

spontaneous fermentation, and the yeast strain A which was the smallest producer.

The influence of the yeast strain used for Graševina fermentation was smaller at 12°C. No statistically significant differences ($P > 0.05$) occurred in the production of volatile compounds between the following yeasts: ZIM 1899, ZIM 1900, and spontaneous fermentation; ZIM 1899, spontaneous fermentation and B; B and A. Other yeast combinations gave statistically significant differences in the levels of the aroma compounds analysed ($P < 0.05$). The strain Z-2 showed the highest production of volatile compounds, followed by ZIM 1990, ZIM 1899, spontaneous fermentation, B, and A.

Autochthonous wine yeasts characterisation, achieved in this work, might have a practical value for the wine industry. The wine yeasts investigated showed different metabolic profiles which should be taken in to consideration for particular wine production to obtain and stress the important characteristics of local wines.

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