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INFLUENCE OF MEDIUM CHAIN FATTY ACIDS ON SOME BOTRYTISED WINE-RELATED YEAST SPECIES AND ON SPONTANEOUS REFERMENTATION OF TOKAJ ESSENCE

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Medium chain fatty acids are candidates of partial sulphur dioxide replacement in wine, as a solution to the growing consumer concerns about chemical additives. In botrytised sweet wine specialties, large amount of sulphur dioxide addition is one of the effective practices to stop alcoholic fermentation. Increasing medium chain fatty acid levels up to 80 mg l^{-1} was tested as a sole inhibitor on solid agar surface. *S. bacillaris* seemed to be the most sensitive, *S. cerevsisiae* and *S. bayanus* were more tolerant, while *Z. bailii* showed the highest tolerance. Then, increasing medium chain fatty acid levels up to 40 mg l^{-1} combined with 100 mg l^{-1} sulphur dioxide was introduced into a Tokaj Essence under refermentation. After 56 days, the highest dosage had pronounced effect on the yeast population, but the refermentation was not inhibited completely. Medium chain fatty acids have varying inhibitory effect on botrytised wine-related yeasts, moreover, it could be used effectively in media with high ethanol content, unlike Tokaj Essence.

Keywords: medium chain fatty acids, *Starmerella bacillaris, Zygosaccharomyces bailii, Saccharomyces cerevisiae, Saccharomyces uvarum*

Nowadays, consumers are particularly concerned about health aspects, connected with sulphite toxicity in wine, therefore, the current general tendency is to reduce the use of sulphite in winemaking. Sulphur dioxide is the most frequently used chemical additive in winemaking, employed for multiple benefits as antiseptic, antioxidant, colour-, fragrance-, and taste protector (Santos et al., 2012). Based on current knowledge, none of the studied alternatives can totally replace SO_2, which remains a useful, sometimes indispensable agent.

In Tokaj botrytised wine specialties, it is a widespread practice to stop alcoholic fermentation and save the residual sugars with a considerable SO_2 addition in combination with cooling, racking, and microfiltration (Magyar, 2011). In some cases, it is a challenge to meet the upper limit of total SO_2 concentration set by EU Commission legislation 607/2009/EC (EC, 2009). Consequently, any effective SO_2 replacement could facilitate botrytised winemaking.

Recent candidates for partial substitution of SO_2 in wine are medium chain fatty acids (MCFA). MCFA and their esters are common yeast secondary metabolites usually produced in small quantities (Balmaseda et al., 2018). Earlier studies revealed that artificially added MCFA could be used to stop an alcoholic fermentation carried out by *S. cerevisiae* (Babikova et al., 2012), inhibit refermentation (Baron, 2014), and consequently decrease the necessary SO_2 addition. MCFA application in wines is studied only by a few (e.g. Babikova et al., 2012;

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Baron et al., 2017) in normal winemaking environments. However, the inhibitory effect might be significantly different for various yeast species and in special winemaking environments, like botrytised winemaking.

In case of botrytised wine fermentation, the original yeast biota of the grape berry is altered considerably (reviewed by e.g. Sipiczki, 2019), the harsh fermentation conditions are tolerable only for the well-adapted species. Beside others, *Saccharomyces cerevisiae* has great importance, *Saccharomyces bayanus* is also well presented (Masneuf-Pomarede et al., 2010). *Starmerella bacillaris (*syn. *Candida zemplinina)* was originally described in Tokaj wine region and connected to botrytised, sweet wine fermentation (Sipiczki, 2003). Due to the significant amount of remaining sugars, refermentation of these wine specialties by the tolerant spoilage yeast *Zygosaccharomyces bailii* is a major threat (Alonso et al., 2015).

In this study, first we focused on the characterisation of the general tolerance of *S. cerevisiae, S. bayanus, S. bacillaris*, and *Z. bailii* against MCFA as a sole additive in the medium. Furthermore, various MCFA concentrations were tested in combination with SO_2 to inhibit spontaneous refermentation in a Tokaj Essence.

1. Materials and methods

1.1. Tolerance test

Yeast strains: Yeast strains are shown in Table 1. Natural isolates were previously identified by their rDNA and ITS regions (based on the methods of Zott and co-workers, 2010) except *Z. bailii* strains, which were formerly identified by classical methods, upon characteristic sporulation and physiological traits. Inoculum was prepared in YEPD broth (20 g l^{-1} glucose, 10 g l^{-1} peptone, and 10 g l^{-1} yeast extract), incubated (25 °C/48 h) without agitation.

Culture media: For preculturing the strains, fermentation was performed in a model medium (40 g l^{-1} glucose, 0.75 g l^{-1} $MgSO_4 \times 7\ H_2O$, 1.0 g l^{-1} $KHPO_4$, 5.0 g l^{-1} peptone, 3.0 g l^{-1} yeast extract, and 3 g l^{-1} DL-malic acid, pH=3.5). Agar plates used for drop test had a composition identical with the model medium except for 5 v/v% ethanol, 15 g l^{-1} agar-agar, and increasing MCFA concentrations.

Fermentation conditions: Fermentation (preculturing) was carried out under semi-anaerobic condition at 20 °C, in test tubes containing 5 ml aliquots of model media, without shaking. Tubes were inoculated to a level of 1×10^6 cell ml^{-1} with 48-hour-old yeast cultures grown in YEPD broth. Cell concentration was measured by Bürker chamber cell counting after 72 h of fermentation.

Drop test: After 72-hour fermentation without preservative, the inhibitory effect of MCFA was tested on solid agar surface. The test was carried out with 5 µl of serial dilutions from the cultures (10^{-1}, 10^{-3}, 10^{-5}), in triplicate, according to Perez-Torrado and co-workers' (2016) modified method. Into the agar 0, 10, 20, 40, and 80 mg l^{-1} MCFA mixture was introduced. Upon the results of Baron (2014), the MCFA mixture contained C_8:C_{10}:C_{12} in 2:7:1 ratio, solved in 70 v/v% ethanol. After 7 days of incubation at 20 °C, drop test images were recorded in a fix vision system with a Sony Exmor RSIMX315 camera (Sony Corp., Minato, Japan). Growth area analysis with ImageJ software (Schneider et al., 2012) was used to assess the capability of the strains to grow under various MCFA conditions. Growth values given as percentage are raw colony area-means of triplicate drop-tests, normalised with the control growth of each strain. Data were evaluated with ANOVA after checking the assumptions, using IBM SPSS 23.0. Armonk, NY, USA.

Table 1. Yeast strains used in this study

Species	Strain	Source	Origin
Saccharomyces cerevisiae	RA100	DO-SZIU	Tokaj Aszú, Tokaj wine region, HU
	PM321	DO-SZIU	Tokaj Aszú, Tokaj wine region, HU
	S701	DO-SZIU	Somló wine region, HU
	Uvaferm PM®	Lallemand Inc.	commercial starter culture, selected in FR
	Uvaferm 228®	Lallemand Inc.	commercial starter culture, selected in DE
Saccharomyces uvarum	CBS395^T	NCAIM	black current juice, NL
	TKH1	DO-SZIU	Tokaj Aszú, Tokaj wine region, HU
	SB42	DO-SZIU	Tokaj Aszú, Tokaj wine region, HU
	E105	DO-SZIU	Etyek-Buda wine region, HU
	S103	DO-SZIU	Somló wine region, HU
Starmerella bacillaris	Y1667^T	NCAIM	Tokaj Aszú, Tokaj wine region, HU
	Y1756	NCAIM	Aszú berry, Tokaj wine region, HU
	MLO	DO-SZIU	Etyek-Buda wine region, HU
	SJ1	DO-SZIU	Aszú berry, Tokaj wine region, HU
	R1	DO-SZIU	Aszú berry, Tokaj wine region, HU
Zygosaccharomyces bailii	Y954^T	NCAIM	sorghum brandy mash, JP
	Z6	DO-SZIU	SO_2 preserved must, HU
	Z22	DO-SZIU	Badacsony wine region, HU
	PM614	DO-SZIU	Tokaj Aszú, Tokaj wine region, HU
	DS3	DO-SZIU	Mátra wine region, HU

DO-SZIU: Department of Oenology, Szent István University; NCAIM: National Collection of Agricultural and Industrial Microorganisms; FR=France, DE=Germany; NL=the Netherlands; HU=Hungary; JP=Japan

1.2. Inhibition of refermentation in Tokaj Essence by MCFA

Culture media: A Tokaj Essence from Vintage 2005 was bottle aged by the producer at 12 °C in cellar conditions, then re-bottled in 2018 into Tokaj-shape 0.33 l bottles. After two weeks of bottling the Essence was sent to our laboratory in a spontaneous refermentation state with 1.86×10^5 CFU ml^{-1} initial cell concentration. Total yeast count was determined by culturing on DRBC agar (Sigma Aldrich), and the population was found heterogeneous upon colony and microscopic morphology (no further identification was performed). Basic parameters of the Tokaj Essence were determined according to the official OIV methods: 0/38 mg l^{-1} free/total SO_2 (OIV-MA-AS323-04B), 54.19 °Brix total soluble solids (OIV-MA-AS2-02), 2.32 v/v% ethanol (OIV-MA-AS312-01A).

Refermentation conditions: To stop the spoilage, 0, 10, 20, and 40 mg l^{-1} of MCFA mixture was applied to 150 ml of Tokaj Essence in 200 ml flasks, in duplicates, incubated at 15 °C. After 24 h of the MCFA dosage 100 mg l^{-1} SO_2 was added to each of these treatments, (except for an absolute control, where no SO_2 and no MCFA were used) according to the recommendation of an earlier study (Baron, 2014). Composition of the MCFA mixture was identical with the above described (see *drop test* section). Population dynamic changes of the

MCFA-treated Tokaj Essence was followed with traditional plating of serial dilutions on DRBC agar surface, sampling at day 0, 1, 2, 7, 14, 21, 28, and 56.

All chemicals were purchased from Sigma-Aldrich Chemie Gmbh (Munich, Germany).

2. Results and discussion

2.1. Tolerance test

Four botrytised wine-related yeast species were evaluated in terms of their tolerance in growth towards increasing amounts of MCFA. Considering the suggested practical application of MCFA, that is to stop fermentation (Baron, 2017), the conditions of the test included a small amount (5%) of ethanol present in the medium. All species under study showed some intraspecific variation between 5.8% and 25.6%, without correlation among better MCFA tolerance, better fermentation ability (e.g. Magyar & Tóth, 2011), and geographical origin (Table 1).

Due to the increasing MCFA levels, considerable differences were detected among the investigated species in their tolerance (Fig. 1). At 10 mg l^{-1} MCFA, all *S. bacillaris* strains were slightly inhibited, while the other three species were not influenced significantly (Fig. 2). In earlier studies, this MCFA level seemed to be effective in combination with SO_2 (100 mg l^{-1} total) and a higher concentration of ethanol (12 v/v%) in fermenting wine (Baron et al., 2017). From our results it could be seen that the MCFA mixture without SO_2 and with the presence of only 5 v/v% ethanol cannot inhibit the growth of the investigated strains at this low concentration.

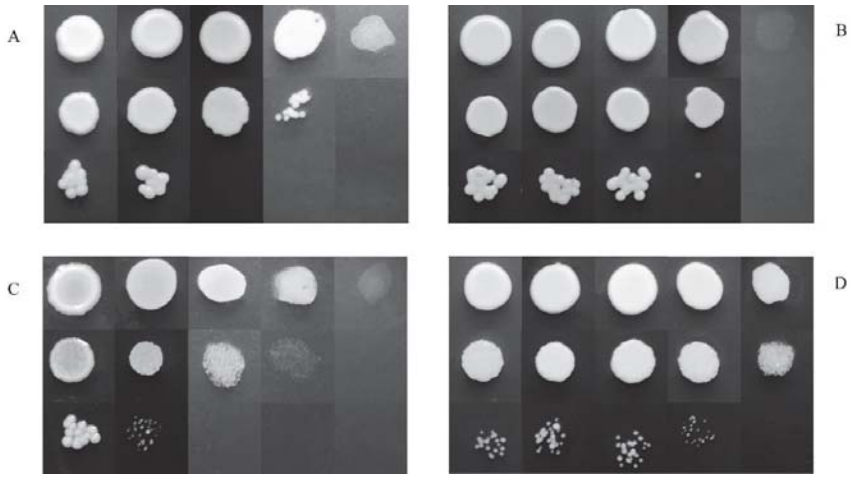

Fig. 1. Tolerance comparison of the investigated species towards MCFA. A: *S. cerevisiae* UVAFERM PM, B: *S. uvarum* TKH1, C: *S. bacillaris* Y1756, D: *Z. bailii* Z6. Vertical bands show serial dilutions of one culture (10^{-1}, 10^{-3}, 10^{-5}) plated on model media completed with increasing MCFA levels. Different bands from left to right display 0-10-20-40-80 mg l^{-1} MCFA. A representative image of the strains from the biological triplicates is presented

At 20 and 40 mg l^{-1} MCFA, *S. bacillaris* strains were further inhibited. *S. cerevisiae* strains were slightly reduced in growth at 20 mg l^{-1} MCFA, while the effect of the 40 mg l^{-1} MCFA was more pronounced. *S. uvarum* growth was not influenced considerably by 20 mg l^{-1} MCFA, while 40 mg l^{-1} MCFA resulted in noticeable intraspecific variance. E105, SB42, and TKH1 did not seem to be influenced, while S103 decreased moderately and CBS395 showed the highest sensitivity, comparable with that of *S. bacillaris* (Fig. 2). *Z. bailii* strains were able to tolerate these concentrations without significant reduction (Fig. 2)

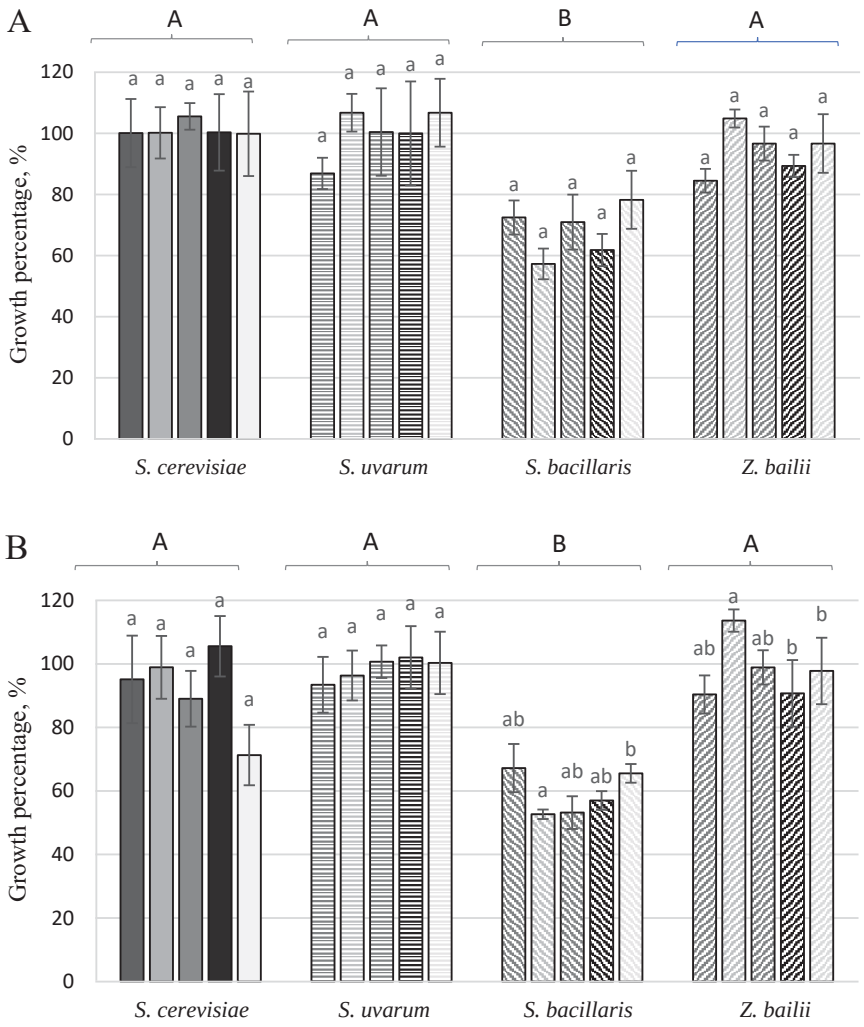

Fig. 2. Growth percentage of the investigated yeast strains: *S. cerevisiae* (■: PM321; ■: RA100; ■: S7101; ■: Uvaferm 228®; ■: Uvaferm PM®), *S. uvarum* (▤: CBS395, ▤: E105, ▤: S103, ▤: SB42, ▤: TKH1), *S. bacillaris* (▨: Y1756, ▨: R1, ▨: MLO, ▨: Y1667, ▨: SJ1), and *Z. bailii* (▨: DS3, ▨: PM614, ▨: Y954, ▨: Z22, ▨: Z6). A: 10 mg l^{-1} MCFA; B: 20 mg l^{-1} MCFA; C: 40 mg l^{-1} MCFA; D: 80 mg l^{-1} MCFA. Percentage values are calculated from the colony area, normalised by the area of control growth. Columns are means of triplicate drop-tests of each strain. Upon Games-Howell post hoc comparison, means are statistically different at a level $P<0.05$, indexed with different lower case letter between the strains within each species, with capital letter between species (species means are not displayed)

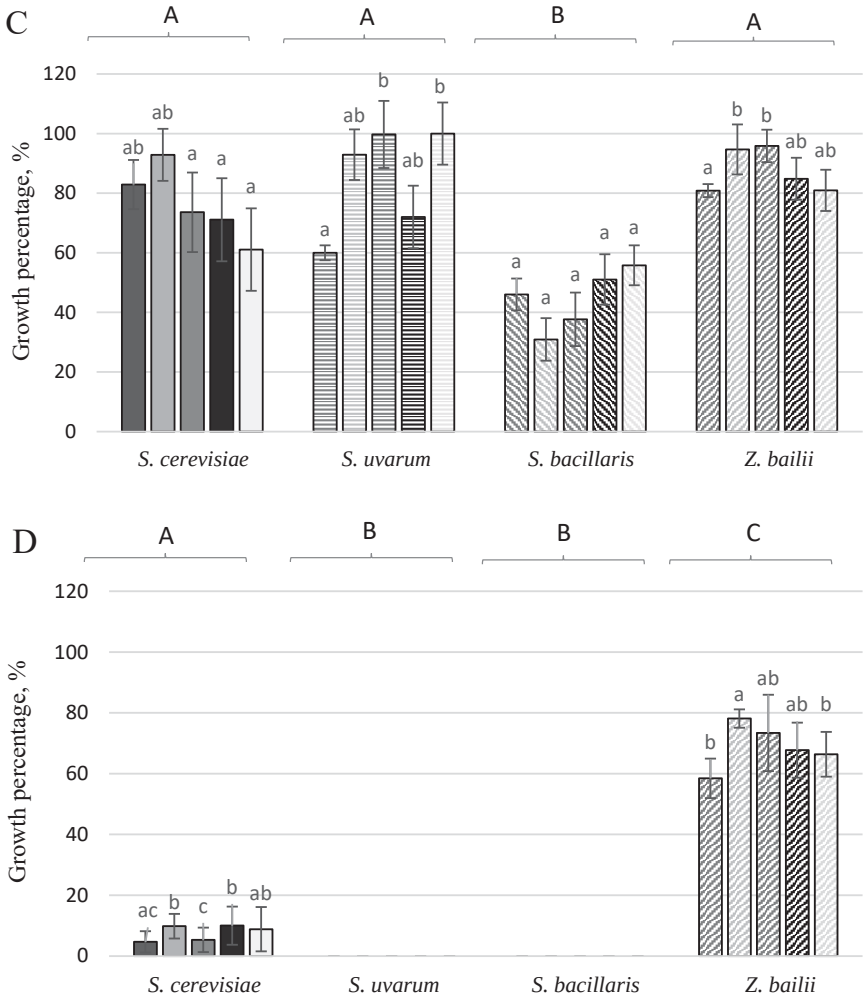

Fig. 2. (continued)

At 80 mg l^{-1} MCFA, all *S. uvarum* and *S. bacillaris* strains were inhibited completely, while some very limited growth was detected in the case of *S. cerevisiae* strains. *Z. bailii* strains showed still significant growth and excellent tolerance. This could be a limitation of industrial MCFA application against refermentation, since this wine yeast is often responsible for spoilage of sweet wines (Alonso et al., 2015). In this investigation, MCFA mixture as a sole yeast-inhibitor seemed to be effective only at considerably higher levels than in combinations used in earlier works (Babikova et al., 2012; Baron et al., 2017).

2.2. Stop of refermentation in Tokaj Essence by MCFA

At the start, the Tokaj Essence had a considerable yeast concentration of 1.86×10^5 CFU ml^{-1}, which heterogeneous yeast population presented an excellent overall tolerance towards low

cellar temperature and extreme amount of sugars (54.19 °Brix). In general, MCFA addition had a prompt effect on the population, since after 1 day, the cell concentration decreased by one order of magnitude in the case of 10 and 20 mg l^{-1} MCFA, and by two orders of magnitude in the case of 40 mg l^{-1} MCFA. The SO_2 addition in combination with the MCFA did not have additional short-term effect on the yeast population (Fig. 3). Comparing the absolute and sulphited control, it could be seen that this SO_2 concentration itself did not inhibit refermentation (Fig. 3), which is in accordance with the high sulphite binding capacity of botrytised wines and general yeast characterisation (Romano & Suzzi, 1993).

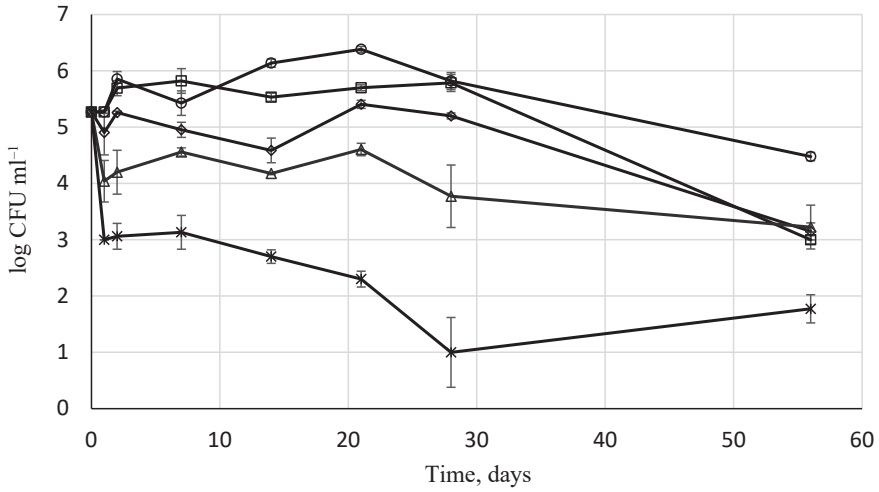

Fig. 3. Yeast population dynamic changes in Tokaj Essence in the presence of various MCFA concentrations in combination with 100 mg l^{-1} total SO_2. Values are means and standard deviations
—○—: 0 mg l^{-1} MCFA+ 0 mg l^{-1} SO2; —□—: 0 mg l^{-1} MCFA+ 100 mg l^{-1} SO2;
—◇—: 10 mg l^{-1} MCFA+ 100 mg l^{-1} SO2; —△—: 20 mg l^{-1} MCFA+ 100 mg l^{-1} SO2;
—✳—: 40 mg l^{-1} MCFA+ 100 mg l^{-1} SO2

The 10 mg l^{-1} MCFA+100 mg l^{-1} SO_2 had negligible inhibitory effect on the yeast population in the first 28 days, while in the case of 20 mg l^{-1} MCFA+100 mg l^{-1} SO_2 cell concentrations were lower than both controls, but still rather limited inhibition was noticed. The 40 mg l^{-1} MCFA+100 mg l^{-1} SO_2 had more pronounced effect on the refermentation, gradual decline was observed (Fig. 3). After 28 days, the cell concentration decreased to 10^1 CFU ml^{-1} level, but the Essence still cannot be regarded as stable, free from possible refermentation.

After 56 days, the 10 and 20 mg l^{-1} MCFA+100 mg l^{-1} SO_2 reduced the living yeast cell concentration with only two orders of magnitude (Fig. 3), which is a considerable decrease regarding the initial cell number, although the 100 mg l^{-1} SO_2 alone had the same inhibitory effect. In the case of the 40 mg l^{-1} MCFA+100 mg l^{-1} SO_2, the cell concentration remained in the 10^1 CFU ml^{-1} range, however, from an oenological point of view, the lowest remaining cell amount is still not acceptable in bottled wine. These results are difficult to compare with earlier works, since the parameters of the botrytised wine specialties, particularly Tokaj Essence, are considerably different from those in a normal wine. The limited inhibitory effect

of the MCFA dosage must be influenced by the reduced ethanol content of the Tokaj Essence, which is in accordance with an earlier finding about *S. cerevisiae* (Viegas et al., 1989).

3. Conclusions

Due to the increasing MCFA concentrations, considerable differences were detected among the investigated yeast species in growth. *S. bacillaris* seemed to be the most sensitive, *S. cerevsisiae* and *S. bayanus* were more tolerant, while *Z. bailii* showed the highest tolerance. It could be concluded that at low ethanol content (5%) and without SO_2, the MCFA mixture as a sole additive needs to be implied in considerably higher amounts than suggested for normal wines. The inhibitory effect of MCFA should be thoroughly tested in the future with a wider strain set of the currently investigated and other species.

The MCFA-SO_2 combinations had rather limited inhibitory effect on the Tokaj Essence refermentation, possibly due to the low alcohol content of the botrytised wine specialty, but the excellent general stress tolerance of the spoilage yeasts should not be excluded. Consequently, the future MCFA application should be reduced only to wine-media, where significant amount of ethanol is present to reach acceptable inhibition.

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