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METABOLIC ACTIVITY OF WINE-RELATED YEAST SPECIES IN STRESS CONDITIONS, WITH SPECIAL REGARD TO SUGAR CONCENTRATION

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1. Background

Commercial yeast starter culture application in winemaking has a history of sixty years, though up to this day its usage is not exclusive. After the first starter cultures, it became a dynamically developing, diversified industry focusing on employing strains with outstanding properties as improved, new products, to serve the work of the winemakers and fulfil the consumer's expectations. Decades of research resulted in multiple hundred starter cultures, that are currently available on the market, which assortment is growing year after year.

Regarding the non-*Saccharomyces* yeasts, the earlier reputation of neutral or negative contributors to grape juice fermentation changed lately since some selected strains proved to be particularly valuable. The earlier monopol position of *Saccharomyces cerevisiae* (and to smaller extent *Saccharomyces bayanus var. uvarum,* abbreviated in this work as *S. uvarum*) is diversified considerably. Although from research point of view the selection work and the development of active dried yeast production have quite not finished yet. For example, *Starmerella bacillaris (syn. Candida zemplinina)* is a valuable starter candidate with some distinct properties for winemaking.

Employing non-*Saccharomyces* yeasts usually imply mixed culture, combined fermentation, thus the dynamics-, metabolites-, control of fermentation, and other aspects became more complex compared to monoculture fermentation.

Yeast selection consists of the screening of candidates for general microbiological, then oenology-related properties. First, the species is investigated as a whole, then the extent of intraspecies variability is assessed, which is well-known for many-many features of *S. cerevisiae.* Considerably less information is available about *S. uvarum*, whilst *S. bacillaris* is only in the focus for a decade.

Defining the stress tolerance limits of starter candidates and commercial starters could be useful in case of less usual intended application as natural sweet wines or botrytized wines like Tokaj Aszú. In these musts during fermentation extreme osmotic conditions should be tolerated by the fermenting yeasts. The average sugar concentration in the grape berry -for non-sweet wine production- has a slightly increasing tendency due to reasons like the climate change, consequently, osmotolerance is a highlighted character. The decreasing fermentation temperature trend underlines the cold tolerance as well. Moreover, other traits could be emphasized due to the also developing technology in viticulture and oenology.

In connection with the above-mentioned topics, in my doctoral research the influence of various stress conditions on cell growth and metabolic activity of *S. bacillaris* in comparison with the two most important wine yeasts *S. cerevisiae* and *S. uvarum* is discussed.

The comparative approach of these studies is supported by the fact, that during spontaneous fermentation of Tokaj Aszú, these species are main contributors, moreover, their combined application in mixed starter cultures could be feasible.

2. Objectives

The main focus of my doctoral research is to describe the valuable properties of *S. bacillaris* compared to *Saccharomyces* yeasts, to further specify its known tolerance in special winemaking situations and define its unknown character. However, I tried to elucidate the limitations of *S. bacillaris* application as well. My goals were:

- To compare selected strains of the above-mentioned species in oenologically relevant stress conditions, as increasing initial sugar concentration, decreasing temperature and their combinations. *S. bacillaris* was described earlier as an osmotolerant and psychrophilic species in 'general microbiological' term, that I wish to test in winemaking environment.

- To monitor the growth capabilities of *S. cerevisiae, S. bayanus var. uvarum, S. bacillaris* and *Zygosaccharomyces bailii* (as a highly tolerant, spoilage yeast) in the presence of non-endogenic medium-chain fatty acids (MCFA) as stress agents.

- To examine the main fermentative metabolites, non-volatile secondary metabolites and some aspects of nitrogen utilisation influenced by increasing initial sugar concentrations (high and extreme range) in case of selected *S. bacillaris*, *S. cerevisiae* and *S. uvarum* strains.

- In connection with the initial sugar-related stress, the intra- and extracellular glycerol management of the three species were compared, i.e. the role of glycerol as part of the osmotic stress response.

- Last but not least, in a pilot-scale fermentation *S. bacillaris*- *S. cerevisiae* combined inoculation strategies were compared in terms of the analytical parameters of the new wine as well as its sensory properties.

In this thesis research, there was no intention to investigate the molecular, genetic background of the chosen stress factors, but with a targeted metabolomic approach, the phenotypic traits and the change in metabolic activity was monitored, if applicable with mathematical-statistical evaluation.

3. Materials and methods

The employed yeast strains were commercial starter cultures, type strains, and strains from the Culture Collection of Department of Oenology (earlier identified by traditional and/or molecular analysis). The majority of the strains originated from winery surfaces, musts or wines. The strain set varied considering the particular experiment, sampling regime, time requirement, etc., though in each case *S. bacillaris* was compared with *S. cerevisiae*, *S. uvarum* and in one case with *Zygosaccharomyces bailii*.

The medium of the growth- and fermentation experiments, apart from the pilot-scale fermentation were sterilized natural musts (Bianca I-III. and Pinot gris) and model media mimicking natural musts (stuck model must based on Santos *et al*., 2008; model broth and agar completed with medium-chain fatty acids; modified YEPD agar; model must for fermentation metabolites based on Henschke és Jiranek, 2003). Certain experimental parameters were set to various levels. The initial sugar concentration represented a fully matured- (220-250 g/L), a considerably overripen- (270-320 g/L) and a fully botrytized grape juice (370-470 g/L). The temperature during fermentation at 20°C represented a general level, at 12°C a relevant cold fermentation was carried out, while 6°C was chosen to check the known criotolerance of *S. uvarum* and the potential criotolerance of *S. bacillaris*.

The inoculum preparation was similar for the experimental fermentations (preliminary experiments, cold stress, initial sugar influenced osmotic stress, intracellular glycerol monitoring, investigation of the fermentative metabolite profile, pilot-scale mixed culture fermentation) at various scale (15-60-180-200 mL and 60 L) and growth monitoring on solid agar surface (initial sugar × cold stress, medium-chain fatty acid stress).

The employed traditional analytical and microbiological methods (total soluble solids, ethanol, volatile acidity, titratable acidity, pH, reducing sugars,

live/dead cell ratio), enzymatic-spectrophotometric methods (L-malic acid, glycerol, yeast amino nitrogen, L-succinic acid, glucose/fructose ratio, soluble proteins and de-proteinisation, optical density) and sensory evaluation (profile analysis) were carried out according to the OIV descriptions and in case of kits manufacturer's recommendation. H^1 NMR method based on Godelmann *et al.* 2012 was performed on a Bruker AVANCE 400 spectrometer and on 400'54 ASCEND magnet system.

Drop test (based on Perez-Torrado *et al.*, 2016): plating on various solid agar surface in Petri dish took place. 6 or 4 cell dilutions of a 10-fold dilution series in 10/5 µL drops were placed to the agar sufrace, then after incubation images of the colonies were recorded in a fix parameter vision system with a Sony Exmor RS IMX315 12 MP camera. The raw images were processed to quantify the cell growth with Area Measurement function in ImageJ software (Schneider *et al*., 2012).

Intracellular glycerol level was monitored according to Perez-Torrado *et al*. (2016) modified method.

Natural isolates of *S. bacillaris* available in the Culture Collection of Department of Onenology, employed in further experiments were identified on species level with their 26 S rDNA D1-D2 domain.

The results were evaluated with various statistical probes after checking the assumptions. One-way-variance analysis (ANOVA), multivariate analysis of variance (MANOVA), cross-tabulation analysis (CROSSTAB), principal component analysis (PCA) assisted the interpretation of the results. IBM Corp. 2016 SPSS Statistics for Windows, Version 23.0. Armonk, NY (USA); Microsoft Corp. 2018 Office 365 Excel for Windows, Redmond, WA (USA) and Addinsoft Inc. XLSTAT, MS Excel Add-in, Version 2020.1.2., New York, NY (USA) softwares were employed.

4. Results

4.1. In **preliminary experiments**, the fermentation dynamics of various *S. cerevisiae* and *S. uvarum* strains were monitored. In this step, the set of *Saccharomyces* strains was selected for the further experiments, mainly for the metabolic activity studies. The goal was to preserve the original diversity so not only the most robust but also the moderate fermentation vigour was presented in the narrower strain set.

4.2. The growth of *S. bacillaris* was considerably influenced by the **decreasing fermentation temperature**. At 20°C, the moderate growth and fermentation ability of *S. bacillaris* was presented compared to the investigated *Saccharomyces* yeasts. At 12°C in case of *S. bacillaris* the decrease in specific growth rate and fermentation was considerable, while at 6°C the decline was more pronounced. The criotolerance of *S. bacillaris* in the investigated temperature range in real winemaking conditions is not comparable with *S. uvarum* (**Thesis 1**).

4.3. Evaluating **the** growth of *S. bacillaris* at **increasing initial sugar levels,** in the 220-320 g/L range the sugar content has considerable influence on the growth parameters of the investigated wine yeasts, but the extent of the decrease is species-dependent. The specific growth rate of *S. bacillaris* decreased considerably with the increasing sugar level, while the size of the population was influenced less markedly by the change in the osmotic conditions. At the end of the monitored period the *S. bacillaris*-fermented sugar amount was 50-60 g/L, mainly fructose, while the *Saccharomyces* yeasts utilised 140-150 g/L with strong glucose preference.

4.4. The growth of *S. bacillaris* in combined stress conditions of increasing sugar levels and decreasing fermentation temperature was compared with *Saccharomyces* yeasts. At 20°C and the lower part of the investigated sugar range (220-470 g/L), the growth of the three species were comparable, while

at the higher sugar region the growth of the two *Saccharomyces* was more suppressed than that of *S. bacillaris*. The decreasing temperature hardly influenced the criotolerant *S. uvarum*, while the growth of *S. cerevisiae* considerably decreased. The reduction in the growth of *S. bacillaris* was less pronounced, particularly at extreme sugar concentration (420 g/L × 12 °C) (**Thesis 1**).

4.5. The **growth inhibition of medium-chain fatty acids** was pronounced at 10 mg/L in case of *S. bacillaris*, whilst the two investigated *Saccharomyces* species were inhibited moderately by 20 and 40 mg/L MCFA with remarkable intraspecies variability, while at 80 mg/L the above-mentioned three species were incapable to grow. *Z. bailii* spoilage yeast, responsible for serious economic loss unfortunately, well-tolerated the highest inhibitor level as well. The intraspecies diversity in MCFA sensitivity did not seem to be in correlation with the geographical origin, the source of isolation or the fermentation robustness of the strains (**Thesis 2**).

4.6. During the combined stressor (4.4.) and MCFA (4.5.) experiments Drop-test, a fix vision system and image analysis was carried out, where the various levels of e.g. initial sugar in the medium caused a distortion in the evaluation of the raw growth percentages. To solve this problem a within-image, medium-dependent reference point must be created, that was carried out with a ’calibration cell’ i.e. the undiluted drops of the cell culture, as the full drop-growth at the certain parameters. Apart from that serial dilution of the cell culture was applied to the agar surface in triplicate as ’measuring cells’. The final growth percentage of a strain is normalized with both the control growth (25 g/L sugar or 0 mg/L inhibitor) and the certain agar conditions. Consequently, the comparison could be standard, quantifiable, precise, and distortion-free among considerably different conditions as 6-20°C

× 25-470 g/L sugar or 0-80 mg/L MCFA. I would like to look at the above-described method development as a novelty (**Thesis 5**).

4.7. The accumulated **intracellular glycerol** level of *S. bacillaris* during the first 72 hours of fermentation did not change considerably, though at the start point it was relatively high while regarding the investigated *Saccharomyces* yeasts a significant increase was observed. In case of *S. bacillaris* the total glycerol amount did not seem to be compensated by the extracellular glycerol concentration either, since at the start of the fermentation the three species were similar, then after 72 hours *S. bacillaris* produced considerably lower amount than the other species (**Thesis 3**).

4.8. During **the metabolite profile** experiment the main fermentative metabolites, some aspects of nitrogen utilisation, and non-volatile secondary metabolites and their yield was evaluated influenced by the increasing initial sugar concentration. The change in particular metabolites were considered one-by-one, where different reactions were observed in case of the three investigated species and their strains. For example, the sugar dependence of volatile acidity production was the least pronounced in *S. uvarum* strains, the *S. bacillaris* strains showed intraspecies variability, both sugar dependent and independent behaviour were found. While the *S. cerevisiae* strains responded to the sugar change with a considerable increase in the volatile acidity. With principal component analysis, multiple metabolites were evaluated in one dimension-reduced space, where at both sugar levels *S. bacillaris* was separable from the two *Saccharomyces* yeasts (**Thesis 4**).

4.9. Combined pilot-scale fermentation was carried out with sequenced and co-inoculation of a *S. bacillaris-S. cerevisiae* pair and compared to monoculture *S. cerevisiae* fermentation. There was a negligible difference in the course of the fermentation, while in the chemical analysis of the new wines were also similar. During the quantitative descriptive analysis, the two

combined inoculation regimes did not seem to be different, and compared to the monoculture fermentation no benefits, neither in odour nor in taste were acknowledged by the panelists. According to these results, the particular yeast pair failed to ferment successfully together, though in the literature there are multiple beneficial results available. Thus, no further species-generalized consequences should be drawn than to highlight the importance of the well-chosen yeasts for combined fermentations.

5. Novel scientific results

Thesis 1. The criotolerance of *S. bacillaris* – in normal winemaking environment – was not confirmed. In this property, an unexpectedly low intraspecific variability was observed. The growth of *S. bacillaris* were decreased considerably less at low temperature and high sugar concentration.

Thesis 2. *S. bacillaris* was found to be sensitive to the medium-chain fatty acids in the growth medium, while *S. cerevisiae* and *S. uvarum* showed moderate tolerance. The spoilage yeast, *Z. bailii* showed excellent tolerance.

Thesis 3. Although *S. bacillaris* is a high glycerol producer, defined at the end of fermentation, the intracellular glycerol production of *S. bacillaris* at the start of the fermentation was considerably different than that of *Saccharomyces* yeasts. *S. bacillaris* answered with significantly less intracellular glycerol accumulation to the high initial sugar concentration in the medium, which implies that in this species glycerol has a smaller role in the osmotic stress response.

Thesis 4. The change in the fermentative metabolite profile of *S. bacillaris* at extreme level of initial sugar concentration (e.g. decrease in YAN utilisation, slightly improved proline uptake, strain- and occasionally sugar level-dependent volatile acidity production) considerably different than in case of the investigated *S. cerevisiae* and *S. uvarum*.

Thesis 5. The Drop-test (e.g. in Perez-Torrado *et al.*, 2016) and the fix vision system with image analysis (e.g. in Schneider *et al*., 2012) were

connected from earlier works and the interpretation of the predefined cell conditions are regarded as method development. With the application of the above-mentioned steps, the growth comparison on considerably different media is available in a standard, quantified way.

6. Conclusions and suggestions

From the **preliminary experiments** - apart from the defining the strain-set- the following confirmatory conclusions could be drawn about the oenological traits of the two *Saccharomyces* yeasts. In musts with normal sugar range, the fermentation dynamics of *S. uvarum* strains, isolated from winemaking environment, barely different from that of *S. cerevisiae* strains. In high sugar-containing, nutrient-limited medium, the fermentation of *S. uvarum* strains was considerably slower. Significant fructose utilisation was found in case of both species without outstanding strains.

Regarding **the growth capability of *S. bacillaris* in various stress conditions**, the cold tolerance of the species is found to be weaker, than that of *S. uvarum* a known criotolerant yeast. *S. bacillaris* is able to grow at relatively low temperatures, but its rate is not acceptable during wine fermentation. The specific growth rate was influenced more by the low temperature than the maximum cell number. During combined application of this well-known moderate fermenter non-*Saccharomyces* the right temperature should be chosen carefully In mixed culture, low-temperature fermentations its application is not recommended moreover, its participation in the process can be influenced by temperature control (cold). The remarkable osmotolerance of *S. bacillaris* was confirmed in a wide sugar range (220-470 g/L). While investigating the combined osmotic- and cold stress it was found, that at 12°C and extremely high sugar level the growth of *S. bacillaris* exceeded the criotolerant *S. uvarum*. In case of *S. bacillaris*, the growth inhibition of the two stress factors did not seem to be additive, the excellent osmotolerance is more pronounced, than the limited col tolerance. This finding could partially explain the high occurrence of *S. bacillaris* in Tokaj wine specialties, where the sugar concentration is high or extremely high, while the fermentation temperature is low (~13 °C).

About **the inhibitory effect of medium-chain fatty acids**, there was very limited information available in the literature. The MCFA showed the most pronounced inhibition against *S. bacillaris*, while to a somewhat less extent it was found in case of *S. cerevisiae* and *S. uvarum* as well. *Z. bailii* was found to be the most tolerant out of the investigated four species. Based on beneficial properties of *S. bacillaris* as strong fructophilic behaviour, low ethanol production, but good ethanol tolerance, etc., this species could have been useful to re-start a stuck or sluggish fermentation, but due to the limited MCFA tolerance this expected application should be rejected. In the other hand, the extreme tolerance of *Z. bailii* might be a major limitation in the future industrial application of MCFA, furthermore, this must be kept in mind during the definition of the minimum effective concentration.

Monitoring **the intracellular glycerol management,** it can be concluded, that *S. bacillaris* accumulates glycerol within the cell with different dynamics and in lower concentration than *Saccharomyces* yeasts. It seems, that the same amount of sugar in the medium is equivalent with less stress in case of the remarkably osmotolerant species. Moreover, it is hypothesized, that glycerol could have different a role in the sugar tolerance of *S. bacillaris*, than in *Saccharomyces* yeasts and the activation of some other alternative stress responses cannot be excluded.

Concerning the **metabolic activity change due to increasing initial sugar concentration,** the following can be assumed. Fermenting 320 g/L sugar the dynamics of sugar consumption did not change compared to 220 g/L in the investigated *S. bacillaris* and *S. uvarum* strains. The volatile acid production of *S. bacillaris* was strain-dependent, which is further supported by the contradictory earlier findings of the species. The examined strains are not suitable for reduced volatile acidity production. The fructophilic behaviour, low ethanol production, and excellent glycerol production of *S.*

bacillaris was confirmed is this work. Due to the moderate net organic acid production, *S. bacillaris* is not particularly appropriate to balance acid harmony in certain vintages. The investigated *S. bacillaris* strains showed modest utilisation of assimilable nitrogen at the extremely high sugar concentration together with a small amount of proline uptake. These nitrogen-related features could be highly valuable in mixed culture fermentation of grape juices with high or extremely high initial sugar concentration. Regarding the complex change in the metabolic profile, some responses of *S. bacillaris* were found to be different than that of the investigated *Saccharomyces* yeasts.

From **the pilot-scale combined fermentation,** it can be concluded, that one key to the successful mixed culture fermentation is the compatibility of the sequential or co-inoculated *S. cerevisiae* and non-*Saccharomyces* strains, in this particular case *S. bacillaris* strain. Investigated at pilot-scale, the Y1756 *S. bacillaris* and Uvaferm®228 *S. cerevisiae* yeast strain pair is not suitable for ethanol management or balancing the harmony in acidity, regardless of inoculation methods. The application of the employed strains (Y1756 *S. bacillaris* and Uvaferm®228 *S. cerevisiae*) is not recommended, since there was only a minimal difference during fermentation while the analytical and sensory properties of the experimental wines changed negligibly compared to monoculture *S. cerevisiae* (Uvaferm®228) fermentation. However, from this result generalized consequences about different strains of the same species should not be drawn.

All in all, *S. bacillaris* is a promising starter candidate with multiple beneficial properties, though its limitations should also be kept in mind. In the near future, selected strains of this non-*Saccharomyces* species could have importance in mixed culture fermentations, since some excellent oenological

properties are highlighted in earlier works and in this thesis as well, moreover distinct stress responses could possibly compensate for various problems during wine fermentation. Based on my results, this species could be a suitable participant in mixed culture fermentation (after thorough compatibility check of the strains) of extremely high sugar containing or botrytized grape juice.

7. Publications in the field of the thesis

Articles in impact factor journals

Borbála Oláhné Horváth, Diána Nyitrainé Sárdy, Nikolett Kellner, Ildikó Magyar: Effects of the high sugar content on the fermentation dynamics and some metabolites of wine-related yeast species *Saccharomyces cerevisiae, S. uvarum* and *Starmerella bacillaris*. Food Technology and Biotechnology, 58(1):76-83.
https://doi.org/10.17113/ftb.58.01.20.6461 IF: 1,52 (2018); Q2.

Oláhné Horváth Borbála, Fazekas Eszter, Kellner Nikolett, Magyar Ildikó: Influence of Medium Chain Fatty Acids on Some Botrytized Wine-related Yeast Species and on spontaneous refermentation of Tokaj Essence. Acta Alimentaria. 49(3):339-347.
IF:0,547 (2018); Q3.

Articles in non-ipact factor journals

Oláhné Horváth Borbála, Kellner Nikolett, Csuka Bence, Magyar Ildikó: Újabb *Saccharomyces* és nem-*Saccharomyces* starterkultúrák értékelése fehérborok erjesztésében. Borászati Füzetek, 30(2): 32-35.

Conference abstracts in Hungarian

Ildikó Magyar, Miklós Kállay, **Borbála Oláhné Horváth**, Annamária Sólyom-Leskó, Nikolett Kellner, Diána Nyitrainé Sárdy: Phenotypic Characterization of *Starmerella bacillaris (syn. Candida zemplinina)* from Oenological Aspects; Magyar Mikrobiológiai Társaság 2018. évi Nagygyűlése és 13. Fermentációs Kollokvium Eger, Absztraktfüzet. pp. 38.

Ildikó Magyar, **Borbála Oláhné Horváth**: Újabb *Saccharomyces* és nem-*Saccharomyces* élesztő starterkultúrák értékelése. I. Borászati Szakmai

Tudományos Konferencia, 2018, Kivonat-kötet. pp.12-13. (ISBN 978-615-00-2176-8).

International conference abstracts in English

Borbála Oláhné Horváth, Fanni Lajszner, Anna Pápai, Ildikó Magyar: Combined, osmotic and temperature stress tolerance of wine-related strains of *Starmerella bacillaris (syn. Candida zemplinina)*. 3. International Food Conference, Budapest.
ISBN 978-963-269-794-9.

Borbála Oláhné Horváth, Vivien Kormos, Edina Nagy, Ildikó Magyar: Influence of the Osmotic Stress in Grape Must on the Growth Kinetics of Wine Related Strains of *Starmerella bacillaris (syn. Candida zemplinina)* 5 th Central European Forum For Microbiology – 2017. Acta Microbiologica et Immunologica Hungarica. 64(Suppl 1.): 156.

Borbála Oláhné Horváth, Nyitrai-Sárdy, Diána, Kellner, Nikolett, Magyar Ildikó: Change in metabolic footprint of some wine-related yeasts induced by extreme initial sugar content, 18th International Congress of the Hungarian Society for Microbiology, Budapest, 2019 július 3-5. Acta Microbiologica et Immunologica Hungarica. 66(Suppl. 1.) 39.

Borbála Oláhné Horváth, Zita Balogh, Rebeka Takács, Ildikó Magyar, Andrea Pomázi: Influence of non-*Saccharomyces* Yeast Cultures on the Yeast and Lactic Acid Bacteria Population During Prefermentative Cold Maceration of Red Grapes 18th International Congress of the Hungarian Society for Microbiology, Budapest, 2019 július 3-5. Acta Microbiologica et Immunologica Hungarica, 66(Suppl. 1.) 173.