Comparative Evaluation of the Dynamics of Alcohol Production of Wine Yeast Strains Isolated in Tokaj Region

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Comparative Evaluation of the Dynamics of Alcohol Production of Wine Yeast Strains Isolated in Tokaj Region

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Abstract - The dynamics of ethanol production of wine yeasts were examined in model experiments as well as in the winery. The ethanol concentration in young wines fermented by local strains of Saccharomyces cerevisiae, S. uvarum or Stammerella bacillaris (21, 2 and 2, respectively) did not vary considerably (c.v. 1.9 %). All of them produced significantly higher amount of ethanol than the type strain [ATCC 26108] of S. cerevisiae. However, their performance during the fermentation process diverged significantly. Thus the lag phase varied between 33 and 123 hours, while the time requested to produce half of the final ethanol concentration varied between 67 and 294 hours.

The dynamics of ethanol production differed at high degree between S. cerevisiae strains isolated of several vintages of local wines (c.v. 25 %), where the intensity of specific ethanol production (ISEP) varied between 0.81-4.56 % ethanol per day. Reverse relationship was revealed between the Lag phase and the ISEP ($r^2=0.858$, $p>0.01$), and the circumstances of fermentation did affect this trend. Based on their properties, S. uvarum and St. bacillaris strains applied nowadays in wine making have been positioned in the ranges of S. cerevisiae strains.

Baule-Mitherlich, Gompertz, hyperbolic, logistic, logarithmic, polynomial, and probit functions were applied to analyze the dynamics of fermentation. All functions fitted well to experimentally measured values at the range of 2 to 9 % of ethanol, that means, the half time could be approached by any of them at $p<0.05$ level. However, the predictive power of these functions differed significantly; both Lag phase and End point of fermentation could be calculated with requested precision ($p<0.001$) only with a polynomial function. The constant and secondary coefficients of this function counteracted to the primary one strictly in strain dependent manner, and the role of these three factors groups also varied in strain-dependent manners during the vinification process. Keywords: yeast, wine, fermentation dynamics, saccharomyces, starmerella, tokaj.

I. Introduction

The wine is an alcoholic drink usually fermented from grape juice by yeasts, and it is the result of the transformation of sugars into ethanol and carbon dioxide. This process has been well studied since the pioneer works of Pasteur, and numerous papers have focused on the dynamics of yeasts during the wine fermentation elucidating the role of Saccharomyces species. Among them, S. cerevisiae is considered to be primarily responsible for ethanol production metabolizing sugars via the fermentative pathway when the sugar concentration is high, and this species is widely preferred for initiating fermentation.

More than 40 of the 1500 known yeast species were isolated from grape must [1]. Nevertheless, some species of diverse microbiota presented in the vineyards [2, 3] and musts [4, 5] are also involved into the fermentation during the first stages of winemaking [6-9]. Still, studies comparing yeast ecology in vineyards and cellars clearly showed that the yeasts present on grapes are subject to natural phenomenons as grape maturity and weather, as well as to human interventions and the phytosanitary treatments carried out [10, 11]. Thus, in oenological conditions, these species due to their low capacity to multiply and their particular needs for micronutrients and oxygen [12, 13] have limited fermentation capacities compared to Saccharomyces yeasts, which are adaptable to hostile conditions[14]. Consequently, the populations of residual indigenous yeasts quickly decrease [15], and most of them disappear when the ethanol concentration increases over 4–5% (v/v) [16].

Nowadays, S. cerevisiae and S. uvarum are the leading species of alcohol fermentation. Still, St. bacillaris and Torulaspora delbrueckii also able to complete the alcohol fermentation [17], and these yeast species became a concern of interest in modern winemaking. Moreover, due to the consumer oriented wine markets, there is an ever-growing quest for specialized wine yeast strains possessing a wide range of optimized, improved or novel oenological properties [18], and winemakers have started to believe in the synergic effect of some non-Saccharomyces species in matters such as aroma intensity and complexity [19] (Table 1), as the incidences of non-selected
Saccharomyces or non-Saccharomyces opportunistic yeasts during fermentations were usually related to off flavors improving the overall quality of the wines [20, 21]. Nevertheless, their secreted enzymes could be detected throughout the fermentation process [22, 23], impacting the wine fruitiness and complexity [24].

In present times, due to fears towards GMO technologies and legal regulations, researchers have turned their attention to the Saccharomyces sensu stricto group. It is well known that members of this group can hybridize with each other in the nature. Under laboratory conditions any of the Saccharomyces species can form hybrids with any other species of the genus [25-43]. Hybridization brings all alleles of all relevant genes of different strains together and recomposes them during segregation/chimerization of the hybrid genomes. The hybrids and their chimera derivatives can outperform the parental strains in technologically relevant properties including stress response [41]. There are countless possibilities in this mechanism that can be exploited to design and create strains optimized for industrial tasks. For instance, comparative genomic analyses revealed that the thiol-releasing wine yeast, VIN7, has an allotriploid hybrid genome with S. cerevisiae and S. kudriavzevii origins [44] that explained the genetic basis of this VIN7's unique capacity to produce wines with a distinctive guava-like aroma [18].

By adopting and authorizing GMO technologies, the creation of engineered industrial strains can be accelerated, and strains optimized for a given task, expected aroma production, or even specific vintage conditions can be created. With the confluence of modern-day biomolecular sciences, information technology, and engineering, the DNA of yeasts can now be redesigned, reinvented, rewritten, and edited with astounding precision [45-48]. Engineering the biology of a model and non-model yeast strains (including clonal variants of natural isolates, mutants, hybrids, and genetically-engineered GM strains) with laser-sharp accuracy can stretch the realms of possibility in yeast research and wine yeast innovation [49]. Some attempts have already come to light; for example, the haploid wine strain (AWR1631) of S. cerevisiae was equipped with a biosynthetic pathway, which consists of four separate enzymatic activities required for the production of the raspberry ketone [50].

Over a thousand papers have been published on the use of sequential yeast mixtures in wine fermentation. The aim of these efforts was to regulate the vinification process as well as to direct the ethanol production from organic acids [14, 51-53]. Moreover, much interest has been developed to the low alcohol content and in the use of different wine yeast species to improve sensory impacts of vine grape varieties for wine utilizing the aromatic potential of some non-Saccharomyces yeasts [54]. The population dynamics of various strains alone or in mixtures as well as the kinetics of sugar consumption and carbon dioxide production have been examined in details with diverse methods, and selection of appropriate parameters of kinetics for comparative studies has been discussed [55-60]. We focused our attention on the kinetics of alcohol production with regard to varietal differences in the time course of the process. In the present study, we performed fermentations in laboratory models and semi-industrial scale to compare St. bacillaris and S. uvarum strains to S. cerevisiae, all isolated from the Tokaj (Hungary) region. The experimental data were analyzed with Baule-Mithrelich, Gompertz, hyperboloid, logistic, logarithmic, polynomial, and probit functions, and manual fitting in Descartes plots to reveal the usefulness of kinetic parameters of alcohol production in comparative studies.

II. Materials and Methods

Data on maintenance, origin, and methods of authentication of wine yeast strains used in model experiments (Table 2) were reported in detail by Kállai et al. [61].

**Microvinification:** 50 mL autoclaved Yellow Muscat must (204.3 g L⁻¹ sugar, pH 3.38) prepared from grapes harvested in Tarcal was inoculated with cells of an overnight culture to obtain 5x10⁶ cell mL⁻¹ concentration and it was incubated at 12°C without shaking for 30 days. The tests were carried out in two series.

**Semi-industrial fermentation in the winery:** The Furmint grape must (204.3 g L⁻¹ sugar, pH 3.2) was cleaned with a vacuum drum filter and equalized. The inoculation concentration was 5x10⁶ cells/mL of the must. The fermentations were carried out in 100 L steel tanks for 30 days. Samples were taken by time course given in Figure 1 to observe the dynamics of the fermentation.

**Analytics:** The alcohol, glucose, fructose, total sugar, and acetic acid concentration was measured with a Bruker Alpha FTIR spectrometer (Bruker Optic GmbH, Germany) and the results were processed with the Bruker OPUS software.

**Data analysis:** Fisher’s test was applied to evaluate the significance of differences between variants at p = 0.05 level. The average values of ethanol concentrations determined in samples were used to construct two data matrices; the first comprised data of S. cerevisiae strains fermenting Yellow Muscat juice (20×12; strain×time), while data of strains of three species (S. cerevisiae, S. uvarum and St. bacillaris) fermenting Furmint juice (3×2×12; species×strain×time) were put into the second one.

Both data matrices comprising time-dependent percentage values were subsequently analyzed by percent of ethanol versus log time regression applying Baule-Mithrelich, Gompertz, hyperbolic, logistic,
logarithmic, polynomial and probit functions to elucidate the character of dynamic changes in ethanol production during the fermentation following models described by Sváb [62]. The kinetic parameters (Lag phase, Half time of alcohol production, and End point) were also extrapolated by manual fitting on Descartes’s plot, and the values calculated by the above functions were correlated to these values by linear regression. Box & Whiskers plots were used to demonstrate differences both in the fitness of regression and predictive power of equations to evaluate the applicability of the examined regression models.

Statistical functions of Microsoft Office Excel 2003 (Microsoft, Redmondton, USA) and Statistica5 programs (StatSoft, Tusla, USA) were used for multivariate analysis of data. Graphical presentations of the results of data analysis were edited uniformly in MS Office PowerPoint 2003.

III. Results and Discussion

The levels of ethanol in the medium were determined with high accuracy (F\text{repl}=1.37 > F_{0.1}=2.88). The ethanol production of strains was different and varied between 11.65 and 12.95 % v/v (F\text{str}=2.281 > F_{0.05}=2.11; p<0.05). All strains isolated in the Tokaj region [61] performed better than the type strain (ATCC 26108) of S. cerevisiae (Table 2).

Plotting the actual ethanol concentrations determined analytically versus time of sampling (Fig. 1) revealed significant strain-dependent dynamics of ethanol accumulation (F\text{exp}=18.2 > F_{0.01}=15.38) during the vinification process, where four periods could be significantly distinguished (F\text{time}=1532.9, p<0.001) in each case. Thus, the detectable amount of ethanol got out after lag phase (P1) succeeded first with rapid then descending increase (P2 and P3), and the process terminated with slow changes (P4) up to the ethanol level characteristic to the strain concerned. The length of these stages can be determined manually, plotting the experimental data. This easy to handle method allows the assessment of the character of changes of alcohol concentration versus time as well as the crude approach of several kinetic parameters (Lag phase, Half time, the Specific rate of alcohol production). However, the fitness of correlation can not be precisely evaluated. The high variations in measured values both in parallel batches and performance of strains during the process (Fig. 2) indicate changes in the roles of influencing factors in vinification process of grape juice, first of all in the start of ethanol production (period P1), but parallel to the increase of ethanol concentration (over 5 % v/v) this variation decreases. Its varietal difference rapidly diminishes (c.v. < 1%).

Analyzing the time-dependent changes in ethanol concentrations on manually fitted scatterplot (Fig. 1) the use of sigmoid (logistic and Gompertz functions) and saturation (Baule-Mitscherlich, hyperbolic and logarithmic functions) models seemed to be plausible. Moreover, the applicability of the square approach (polynomial function) and linearization via probit transformation were also tested.

Comparing the fitness of various approaches, the sigmoid type models proved to be applicable to our set of data with limitations, because of the strong asymmetry of the fermentation dynamics (Figs. 1 and 2). Although, both logistic [63, 64] and Gompertz [56, 65, 66] functions were proposed for the analysis of dynamics of must fermentation, in our case these seem to be useful with care, because of the lack of data at the start of the process (stages P1 and P2 in Fig. 1). Thus some related results of calculations have been omitted of the comparative analysis of models. The determination coefficients showed high and strain-dependent variability of the fitness of regressions (Fig. 3) in the case of both logistic (symmetric sigmoid, r²=0.74-0.99) and Gompertz (asymmetric sigmoid, r²=0.70-0.99) functions. Due to extremely high variation after inoculation of grape juice, - as it was mentioned above, - we assume that the more frequent sampling in this period could not improve the exactitude of the extrapolations based on the sigmoid functions.

The analysis of a manually fitted scatter plot (Fig. 1) corroborates the suggestion of the use of saturation models as well. Meanwhile, both logarithmic and hyperbolic functions can be directly applied using experimental data, the Baule-Mitscherlich (BM) model, like the sigmoid one, requests a limit that can be determined by either iteration or giving a fixed value. In our case the iterated limit of BM function resulted in irrationally high ethanol concentrations (14-17 v/v % depending on the strain concerned). Thus we fixed the limit of this model in maximum ethanol concentration measured analytically in new wine produced by the actual strain. The determination coefficients in the case of BM model varied in strain-dependent manner (r²=0.85-0.98) but to a lesser extent than in the case of sigmoid functions (see Fig. 3). The fitness of logarithmic regression varied in strain-dependent manner (r²=0.87-0.99) at a lesser extent than BM one. The hyperbolic function proved to be much better (r²=0.94-0.99). However, the median was less than r²=0.98 in each saturation model.

The linear relationship was also used for studies the kinetics of fermentation [67]. Still, in our case the linearization of experimental data with probit function did not improve the fitness of time-dependent regression as compared to other models (Fig. 3), the variation of determination coefficient varied within wide limits (r²=0.84-0.99) in this case too.

The second-order polynomial function proposed by several authors [68, 69] was applied to test the square approach. This model surpassed all others involved; the determination coefficients were over 0.95 in each case, and the median was over 0.99, which means...
this function showed less strain-dependent variation in fitting the regression than other models tested (Fig. 3). The result of calculations based on the square approach was demonstrated in Figure 4 using strains of S. cerevisiae and St. baycai. The calculated curves fit excellently to analytically determined values of alcohol concentration of the start to the endpoint of fermentation. The expected alcohol content of new wines extrapolated applying polynomial function fitted well to the analytically measured values, contrary to the other models, where the extrapolations resulted in high and strain-dependent alterations (Fig. 5). Thus, this model was applied for calculation of specific ethanol production (Table 2), which intensity varied between 0.81-4.56 % EtOH per day (0.797-1.396 mM per hour).

All local strains surpassed the type strain [ATCC 26108], which produced 0.81% ethanol per day (0.517 mM per hour). Reverse relationship was revealed between the Lag phase and the ISEP (r²=0.81, p>0.01), and the circumstances of fermentation did not affect this trend. No relationship could be elucidated among other known properties of strains and intensity of their specific ethanol production rate. As it was demonstrated in Figure 6, the ISEP is not connected to oenological properties of strains, and can not be linked to their taxonomic position either (Fig. 7). The other kinetic parameters, calculated applying various functions, showed large variation in strain-dependent manner as well (Fig. 8). The continuance of both lag phase and half time extrapolated using polynomial function were more similar to experimental values than those computed by any other functions (Table 3).

The polynomial function permits to weigh the role of constant, primary, and secondary effects as well as to analyze their relationships in strain-dependent manner (Table 4). The actual ethanol concentration (Y) is a product of working cell factories and might be extrapolated applying polynomial function (Y=A+b₁×X+b₂×X²), where X is the actual time counted of the start of fermentation. At the same time, we can conceptualize the coefficients [A], [b₁] and [b₂] as vectors, i.e., sums of various factors influencing the alcohol-producing capacity of yeast cells in the vinification process. The [A] is a time-independent constant, which might be related to a group of properties of yeast strains that take part in ethanol production in a time-independent manners as well as not related to responses of cells to the changing environment in the fermentation tank. The influence of both [b₁] and [b₂] manifests in time-dependent mode, and can be considered to be vectors of primary and secondary factor groups, respectively, and these factors most probably take part in the regulation of the responses of cells to changes in environmental conditions. Our set of data allowed us to weigh their role in the regulation of dynamics of alcohol production. Their relationships in regulating the strain-dependent ethanol production during the time course of the vinification process shown in Figure 9. Surprisingly, strict trends (p<0.01) were elucidated in the manifestation of the simultaneous regulatory effect of these groups of factors. The [b₁] group of factors counteracted to both constant [A] and [b₂] groups. Moreover, the influence of factors [b₁] and [b₂] counteracting synchronously in time dependent manner was about two times stronger than the constant ones, meanwhile, the strength of [b₁] group surpasses that of the [b₂] one about five times. The yeast strains fit precisely to trend lines independently on their taxonomic position or other known properties. The weight of these factor groups changes during the vinification in a strain-dependent manner (Table 4). None of them dominated either P₁ or P₂ stages. The constant and secondary factors [A and b₂] counteracted to EtOH production in the second phase (P₂) contrary to primary one [b₁], and the strain-dependent ISEP negatively correlated to the effect of the primary factor group.

The changes of sugar and acid levels in vinification batches of our strains were checked during fermentation earlier [61], and these data were used for the multiple regression analysis (Table 5). The strain-dependent glucose utilization was connected to strain-dependent dynamics of alcohol production only in the start (period P₁) then the differences between strains in this respect became negligible. Meanwhile, the strain-dependent intensity of fructose utilization took place after the half time of EtOH production (period P₂) then the variation ceased. Contrarily, the acidity remained strain-dependent and connected to variations of alcohol production during the whole process.

The coefficients of polynomial function (Y=A+[b₁×X]+[b₂×X²]), where Y is the actual alcohol concentration measured at X hours after initiation of the fermentation) describing the dynamics of ethanol formation during vinification process can be connected to both extracellular and intracellular factors regulating the performance of proper strains, as these coefficients are most probably vector sums of a group of factors. Thus the Constant [A] is a time-independent variable [or group of factors influencing in time-independent manner], and the Primary [b₁] and Secondary [b₂] coefficients [or] and their role in the regulation of alcohol formations can be weighed correlating the strain-dependent coefficients with actual concentration of the components of fermented grape juice as well as the connection between actual concentration of the components and the produced ethanol can be revealed (Table 6). The Constant [A], acting in time-independent manner was not related significantly to strain dependent changes of components of fermented grape juice during the time course of vinification but fructose level (first block of determination coefficients in Table 6). The factors determining the strength of the Primary coefficient [b₁] strongly influenced the utilization of
fructose at the start of ethanol production. In contrast, while these factors took a role in the regulation of actual levels of acetic acid up to the end of fermentation. Altering the formers, the factor group determining the weight of the Secondary coefficient [b$_2$] strongly influenced the level of all components in the first period (P$_1$) of fermentation (see Fig. 1). It remained determinative in regulation of the strain-dependent acidity up to the end. We suspect that this group of factors [b$_2$] was responsible for highly expressed strain-dependent variation in the start of the vinification process.

The identification of these factor groups is a task of the future, and particular experiments should be designed to clarify their nature.

a) Prospects

The wine producers are facing more and more challenges due to the market demands and also the climate change. Recently, there has been an increased demand for wines with a more complex aroma composition spontaneously fermented by natural wild yeast populations. In order to meet this need safely and cost-effectively by wineries, the research of starter cultures has started to focus on the development of non-Saccharomyces starter cultures. Today, a number of non-Saccharomyces starter cultures are available to allow wineries to model the positive effects of spontaneous fermentation on aroma composition with a safe and controlled method. However, we have little knowledge of how the different species and their mixture affect the process of fermentation, its dynamics. Especially more data requested on their interactions when applied via co- and sequential inoculation. The extreme, unpredictable weather in the last few years has significantly changed the date of harvest compared to the usual times and made it difficult to predict it accurately. These anomalies caused by climate change are new challenges that request appropriate developments in vine cultivation.

In the future, we have to expect the rapid development of synthetic biology. Since cracking the genetic code of the first wine yeast strain (AWRI1631) in 2008, the genomes of several other widely used commercial wine yeast strains – including AWRI1796, EC1118, QA23, VIN7, VIN13, and VL3 – were sequenced and compared with the genomes of laboratory strains of S. cerevisiae (S288c and Sigma 1278b) as well as genomes of commercial Saccharomyces strains. [49, 70, 71]. The functions of several genes have already been elucidated. With these results, we can equip the yeasts with advantageous and valuable properties for industrial use. For example, the FSY1 and MPR1/2 genes are thought to convey fermentation robustness and performance; the IRC7 gene might be associated with aroma enhancement in wine. IRC7-expressing strains seem to release more volatile thiols during fermentation, thereby increasing the fruitiness of the wine. [49, 72].

We would facilitate the planned scheduling of the grape processing and winemaking with our ongoing work. If we know the analytical parameters of our raw material and we know the fermentation ability of the starter culture we want to apply well, we can predict the duration of fermentation as accurately as possible. After further experiments, it is necessary to develop new models to be able to predict the dynamics of fermentation more and more accurately, calculated with the effect of more sophisticated winemaking methods. The different inoculation methods, the interaction of different yeast species and their mixtures, the supply of nutrients, and the regulation of fermentation cycles at different temperatures affect the whole fermentation, including the time of its duration.

IV. Conclusions

The strain-dependent variations of the dynamics of ethanol production during the vinification process can be reliably characterized with second-order polynomial function (Table 3) that has significant predictive power (p<0.05) for calculation of parameters such as Lag-phase, Half time, Endpoint and Specific Intensity of ethanol production. A further advantage of this function is the possibility to weigh the role of constant, primary and secondary effects as well as to analyze their relationships in a strain-dependent manner (Table 4).

Although some quantitative differences manifested between Saccharomyces and Starmerella bacillaris strains, more non-Saccharomyces strains should be involved in studies to make satisfying conclusions in this respect.

Most probably, the toxic effect of ethanol produced also affects the strain-dependent dynamics of fermentation, primarily in the last phase nearing the End point (P$_4$, Fig 1), and this sensitivity response may influence the interaction of factors regulating both the ethanol production and the composition of the new wine; however, this assumption needs further studies. Nevertheless, the strain-dependent counteractions of constant [A] and time-dependent factors ([b$_1$] and [b$_3$]) play a seemingly more intensive role in the regulation of ethanol production in the first half of the vinification process (P$_1$ and P$_2$ stages, see Fig 1). In this period, the alcohol concentration is lower than 6 percent, and we can assume that the possible autocidal effect is not playing a role yet contrary to later phases (P$_3$ and P$_4$ stages, see Fig 1).

Near linear trend was manifested as well as the position of strains fits well independently on their age and taxonomic position when interactions of regulating factor groups ([A], [b$_1$] and [b$_3$]) were compared (Fig 9). Further studies are requested for an explanation of this finding.
Today we are not living in a time when we can be satisfied with routinely applied technologies if we want to run our winery successfully and economically on a market with a constant oversupply. We need to equip ourselves with the latest and most in-depth knowledge to gain an advantage. With the development of gene technologies, the range of possibilities can only be limited by our imagination.

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Table 1: Potential of non-Saccharomyces yeast strains to improve wine quality

| Strains                        | Reference | Saccharomyces cerevisiae (Desm.) Meyen |
|--------------------------------|-----------|---------------------------------------|
| Candida tropicalis             | [73]      | Lachancea lanzarotensis               |
| Candida stellata              | [75]      | Lachancea thermotolerans              |
| Candida zeylanoides           | [76]      | Metschnikowia pulcherrima             |
| Candida zemplinina            | [17]      | Pichia fermentis                      |
| Debaryomyces vanrijii         | [79, 80]  | Pichia guilliermondii                 |
| Hanseniaspora guilliermondii | [82]      | Pichia membranifaciens                |
| Hanseniaspora oshimaya       | [82]      | Torulaspora delbrueckii               |
| Hanseniaspora uvarum          | [82]      | Wickerhamomyces anomalus              |
| Kloeckera apiculata           | [86, 87]  | Williopsis pratenis                   |
| Kluyveromyces marxianus       | [81]      | Williopsis saturnus                   |
| Lachancea fermentis           | [91]      | Zygosaccharomyces bailii              |

Strains of listed species have been involved in experiments in the past two decades and their effects on wine quality have been elucidated in cited publications.

- Appropriate for carrying out the alcoholic fermentation [17]; β-glucosidase activity; concurrent; H2S production; malic acid conversion to ethanol; cider fermentation; syn: Candida famata.

Table 2: Kinetic parameters of the fermentation dynamics of wine yeast strains

| Code | Source | Type | L.P. | H.T. | H-L | SEP | DC | EtOH |
|------|--------|------|------|------|-----|-----|----|------|
| 10-157 | type strain of S. c. | B | 142 | 301 | 160 | 0.517 | 0.998 | 11.65 |
| 10-1390 | com. starter culture | E | 115 | 171 | 97 | 0.911 | 0.995 | 12.25 |
| 10-1343 | Young wine | A | 151 | 276 | 125 | 0.797 | 0.974 | 12.60 |
| 10-1346 | Young wine | A | 100 | 187 | 87 | 0.965 | 0.997 | 12.85 |
| 10-1352 | Young wine | D | 74 | 191 | 116 | 0.686 | 0.989 | 12.25 |
| 10-1345 | Young wine | A | 115 | 234 | 119 | 1.396 | 0.987 | 12.45 |
| 10-1347 | Young wine | A | 105 | 204 | 98 | 1.022 | 0.971 | 12.85 |
| 10-1350 | Young wine | C | 96 | 191 | 95 | 0.941 | 0.978 | 12.75 |
| 10-1357 | Montal Lulon wine | D | 85 | 169 | 85 | 0.799 | 0.955 | 12.55 |
| 10-1358 | Young Furmint wine | B | 114 | 261 | 147 | 0.662 | 0.999 | 12.20 |
| 10-1348 | Wine sediment | A | 174 | 282 | 108 | 0.777 | 0.950 | 12.15 |
| 10-1349 | Wine sediment | A | 151 | 258 | 107 | 0.971 | 0.971 | 12.70 |
| 10-1355 | Wine sediment | A | 120 | 259 | 139 | 0.897 | 0.990 | 12.90 |
| 10-1351 | Wine sediment | A | 110 | 226 | 116 | 0.902 | 0.987 | 12.65 |
| 10-1352 | Furmint sediment | C | 104 | 182 | 78 | 1.059 | 0.997 | 12.85 |
| 10-1344 | Furmint sediment | D | 95 | 166 | 71 | 0.975 | 0.996 | 12.45 |
| 10-1353 | Furmint sediment | A | 101 | 198 | 97 | 0.939 | 0.995 | 12.45 |
| 10-1356 | 5-year old aszú wine | B | 98 | 199 | 101 | 0.875 | 0.994 | 12.95 |
| 10-1359 | 5-year old aszú wine | C | 102 | 187 | 85 | 0.805 | 0.968 | 12.60 |
| 10-489 | sweet botrytized must | n.d. | 79 | 108 | 29 | 2.001 | 0.993 | 12.69 |
| 10-493. | sweet botrytized must | n.d. | 57 | 107 | 49 | 1.305 | 0.999 | 12.89 |

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Table 3: Similarity of kinetic parameters calculated by various functions

| Variable (D) | Importance of factor groups | Parameters of the equation |
|--------------|----------------------------|-----------------------------|
|              | $\beta_C$ | $\beta_P$ | $\beta_S$ | Chi-sq. | R-sqr. | p | $\lambda_{\text{Prime}}$ |
| EtOH conc.   | 0.2217    | -0.2762   | 0.3048    | 11.75   | 0.4210 | 0.0083 | 0.5790 |
| Lag phase    | 0.0044    | 0.1517    | -0.2633   | 26.54   | 0.7090 | 7.4E-06 | 0.2910 |
| Half time    | -0.2875   | 0.4331    | -0.5323   | 62.51   | 0.9454 | 1.8E-13 | 0.0546 |
| H-L          | -0.5188   | 0.6451    | -0.7267   | 58.18   | 0.9332 | 1.5E-12 | 0.0668 |
| ISEP         | 0.4147    | -0.5527   | 0.6448    | 28.26   | 0.7314 | 3.2E-06 | 0.2686 |

$^a$=See Fig. 1. $^b$= Coefficients ($\beta_C$, $\beta_P$, $\beta_S$) of the functions $D_{1-25} = [\text{CSQ}_{1-25} + \text{PSQ}_{1-25} + \text{SSQ}_{1-25}]$, where $D_{1-25}$ = dependent variable; CSQ$_{1-25}$=Constant; PSQ$_{1-25}$=Primary; SSQ$_{1-25}$=Secondary coefficients of the polynomial functions of 25 strains describing the dynamics of their ethanol production, respectively.

$^c$= Parameters of the multiple linear regression function: $D = f(X_1, X_2, X_3)$, where $D$ is a dependent variable of the first column.

$^d$= Ethanol concentrations in new wines fermented by proper strains listed in Table 1. Data imported of Kállai et al. [61]. $^e$= Strain dependent Lag phases (hours) of EtOH production. $^f$= Strain dependent time (hours) requested to reach the 50% of the final EtOH concentration produced by proper strains as measured of the start of fermentation. $^g$= Specific rate of alcohol production (mol EtOH/hour).
Table 5: Time dependent influence of strain characters on dynamic changes in composition of the fermented grape juice

| Parameters     | Time course (days) | F repl |
|----------------|-------------------|--------|
|                | 5                 | 10     | 15     | 20     | 25     | 30     |
| Glucose        | 0.9216***         | 0.5680+| 0.0704-| 0.0005-| 0.0002-| 0.0189-| 0.53   |
| Fructose       | 0.9742***         | 0.9047***| 0.6837+| 0.1304-| 0.0031-| 0.0084-| 0.04   |
| TS-TF-TG       | 0.8520**          | 0.0535-| 0.0046-| 0.0606-| 0.4820-| 0.3654-|        |
| Acetic acid    | 0.8179**          | 0.7715* | 0.9105***| 0.8591* | 0.8545**| 0.8866**| 1.88   |
| TA-AA          | 0.9920***         | 0.9913***| 0.9823***| 0.9808***| 0.9769***| 0.9894***|        |
| pH             | 0.9961***         | 0.9974***| 0.9959***| 0.9948***| 0.9985***| 0.9972***| 0.03   |

The concentrations of components measured (parameter) by the given time course were imported from Kállai et al. [61], and used for calculations applying multiple regression analysis to reveal the connection between dynamics of ethanol production and changes in composition of the fermented grape juice. TS-TF-TG=[total sugar]-[glucose]-[fructose], TA-AA=[total acids]-[acetic acid].

The $R^2$ is the determination coefficient of the function $P_{(1-21)}=[C_{(1-21)}+b1_{(1-21)}+b2_{(1-21)}]$ where $P_{(1-21)}$ is the parameter measured at the time of sampling and the $[C_{(1-21)}+b1_{(1-21)}+b2_{(1-21)}]$ are coefficients of proper functions describing the dynamics of alcohol production of each strain $[n=21]$ $\text{EtOH}_{(0-30)}=C+b1X+b2X^2$ describing the dynamics of ethanol production (Figure 3). We call the cases strain dependent where the coefficients were labelled with symbols + (p=0.05-0.1), *(p=0.01-0.05), **(p=0.001-0.01), ****(p<0.001) and strain independent with - (p>0.1). The F values show the exactitude of the measurement of the parameter concerned $F_{(1,=3.18)}$.

The values of DCs are proportional to dependence of strain properties related to dynamics of ethanol production, and values lower than 0.5 might be considered as low importance of proper strain characters in this respect. For example, changes in glucose level were strain dependent only in first steps of vinification ($P_1$ and $P_2$ in Fig. 1), and the number of stars marks the strength of effect.

Table 6: The influence of factors regulating strain dependent dynamics of ethanol production on the actual level of components in fermented grape juice.

| Factors   | Components | Time course (days) |
|-----------|------------|--------------------|
|           |            | 5      | 10     | 15     | 20     | 25     | 30     |
| Constant  | Glucose    | 0.181  | 0.018  | 0.014  | >0.001 | >0.001 | >0.001 |
| (A)       | Fructose   | 0.682  | 0.172  | 0.016  | 0.003  | 0.001  | 0.003  |
|           | TS-TF-TG   | 0.029  | 0.038  | 0.019  | 0.005  | 0.050  | 0.076  |
|           | Acetic acid| 0.459  | 0.332  | 0.395  | 0.312  | 0.274  | 0.447  |
| Primary   | Glucose    | 0.416  | 0.001  | 0.005  | >0.001 | >0.001 | >0.001 |
| (b1)      | Fructose   | 0.813  | 0.427  | 0.094  | 0.017  | 0.002  | 0.004  |
|           | TS-TF-TG   | 0.175  | 0.065  | 0.027  | 0.000  | 0.131  | 0.123  |
|           | Acetic acid| 0.548  | 0.402  | 0.545  | 0.444  | 0.438  | 0.575  |
| Secondary | Glucose    | 0.610  | 0.049  | >0.001 | 0.001  | 0.002  | 0.001  |
| (b2)      | Fructose   | 0.880  | 0.639  | 0.237  | 0.049  | 0.002  | 0.004  |
|           | TS-TF-TG   | 0.388  | 0.101  | 0.034  | 0.014  | 0.254  | 0.171  |
|           | Acetic acid| 0.603  | 0.428  | 0.645  | 0.541  | 0.575  | 0.661  |

Determination coefficient (DC) of multiple regression (time changes in the level of component given versus proper coefficient) higher than 0.5 mark selective and significant effect (p<0.05) of the strain dependent factor group (underlined). The values of DCs are proportional to dependence of strain properties related to dynamics of ethanol production, and values lower than 0.5 might be considered as low importance of proper strain characters in this respect. For example, changes in glucose level were strain dependent only in first steps of vinification ($P_1$ an $P_2$ in Fig. 1).
The amounts of ethanol produced by reference strain [ATTC 26108] were marked with opened circles, while those of \textit{S. cerevisiae} strains described by Kállai et al. [61] were marked with short lines. Full lines drawn manually show kinetics of changes in ethanol production (AC) of less and most potent strains (48 and 52), respectively, while the stripped curve was fitted to plotted experimental data of reference strain with function \(AC(\%) = -0.0167[\text{Time}]^2 + 1.0361[\text{Time}] - 4.57 \) (\(R^2 = 0.9964\)).

The process of alcoholic fermentation can be divided into four periods; \(P_1\) – no measurable amount in the medium (lag phase), \(P_2\) – accelerating growth of concentration, \(P_3\) – near monotonous growth, \(P_4\) – retarding growth. The arrow \(L\) marks the interval between lag phases of less to most rapid strains, while the arrow \(H\) marks the interval of the time requested to rich the half of produced ethanol concentration of less to most potent producers in the set of strains examined, and correspondingly; \(max\) is the range between lowest and highest ethanol concentrations in new wines, meanwhile \(T_h\) is the half of former values.

\textbf{Figure 1:} Time dependent changes of ethanol concentration during the fermentation of grape juice
Figure 2: Changes of varietal differences in production of ethanol during fermentation

The broken line marks time dependent average values of actual ethanol concentration in the medium (coordinate at right side). The time dependent variation of ethanol production (diamonds, coordinate at left side) relates to dissimilarity of fermentation capacity of S. cerevisiae strains (n=19) at the given sampling time. Abbreviation: P₁, P₂, P₃ and P₄ at the top of graph are intervals distinguished in Figure 1.

Arrows L, H and EP imported from Figure 1 show the strain dependent variation (minimum to maximum) of lag phase, half time and end point. Tₜ = The average half time of the set of S. cerevisiae strains isolated in Tokaj region.
Figure 3: Box & whiskers plot of determination coefficients of functions applied for extrapolation of kinetic parameters of the alcohol fermentation by S. cerevisiae strains

Box and whiskers plots were constructed of the determination coefficient values of the curve fittings based on experimentally measured ethanol concentration in samples taken during the fermentation in the medium of 21 S. cerevisiae strains, applying logistic (SIG), Gompertz (GOM), Baule-Mitcherlich (SAT), probit (PRO), logarithmic (LOG), hyperbolic (HYP) and polynomial (SQU) functions.

The higher coefficients show the higher significance of similarity between calculated and experimentally determined ethanol concentrations in the medium. Numbers at the right side of boxes in vertical arrows show cases that could be fitted at p>0.1, 0.1>p>0.05 and p<0.05 probability levels.

Abbreviations: maximum (max) and minimum (min) values, lower (LQ) and upper (UQ) quartiles, the white line is the median in the black box that shows the size of the middle two quartiles.
The curves were calculated of experimentally determined ethanol concentrations (AC) in the medium (measured values at subsequent samplings of *S. cerevisiae* [10-486] and *St. bacillaris* [10-374] are marked with full circles and squares, respectively):

**S. cerevisiae**; \[AC(\%) = -0.0167\text{[Time]}^2 + 1.0361\text{[Time]} - 4.57 \quad (R^2 = 0.9964)\]

**St. bacillaris**; \[AC(\%) = -0.0167\text{[Time]}^2 + 1.0361\text{[Time]} - 4.57 \quad (R^2 = 0.9964)\]

Abbreviations: LP=Lag phase, HT=Half time, E.P.= End point, D= time requested to produce half of the final ethanol content by strains, \(C_{\text{max}}\)=the maximum ethanol concentration calculated, \(S.c_{\exp}\) and \(S.b_{\exp}\)=ethanol concentration measured at the end (E.P.) of fermentation, \(S.c_{\max}\) and \(S.b_{\max}\)= ethanol concentration measured at the end (E.P.) of fermentation, \(S.b_{\exp}^1\)= time requested to produce ethanol concentration measured at the end point (this value for *S. cerevisiae* equal with calculated one).

The bold arrows indicate points of the end of lag phase (LP) and the half time (HT) of *S. cerevisiae* (full line) and *St. bacillaris* (stretched line); the values were compiled in Table 1.
The concentration of ethanol measured in new wine (EXP) was taken as a standard for comparison of concentration values extrapolated applying logistic (SIG), Gompertz (GOM), Baule-Mitscherlich (SAT), probit (PRO), logarithmic (LOG), hyperbolic (HYP) and polynomial (SQU) functions. Box and whiskers plots were constructed of the measured values (EXP) or extrapolated ethanol concentration at the end of fermentation in the medium of 21 S. cerevisiae strains. The dotted line shows the median of analytically measured values.

Abbreviations: maximum (max) and minimum (min) values, lower (LQ) and upper (UQ) quartiles, the white line is the median in the black box that shows the size of the middle two quartiles.

Figure 5: Box & whiskers plot of either measured or extrapolated ethanol concentrations of new wines fermented by S. cerevisiae strains of Yellow Muscat must.
The number of strains correspond to that in Table 2. The specific rate of ethanol production (mM h⁻¹) was calculated in half time applying polynomial function (see Table 2).

The clustergram was computed of data published by Kállai et al. [61]. The strains comprised in subclusters differ in their oenological properties at p<0.05.

**Figure 6:** Relationships between specific rate of ethanol production and similarities of oenological properties of *S. cerevisiae* strains

The number of strains correspond to that in Table 2. The specific rate of ethanol production (mM h⁻¹) was calculated in half time applying polynomial function (see Table 2).

The clustergram was computed of data published by Kállai et al. [61]. The strains comprised in subclusters differ in their oenological properties at p<0.05.
Figure 7: Relationships between specific ethanol production rates of strains and their genetic variability established on the basis of molecular diagnostics

The clusterograms have been imported from our earlier work Kállai et al. [61], either interdelta (left side) or RAPD (right side) method was applied for elucidating the molecular diversity of strains. The number of strains corresponds to the last two numerals in codes of strains given in Table 1. Columns assigned to the left graph indicate time intervals of the inoculation to the evolution of detectable ethanol concentration (Lag phase, white prism) and subsequent period to produce 50% of the final alcohol content in new wine fermented by the given strain (black prism), while the others at the right side are proportional to specific rate of ethanol production calculated at $T_h$ of the strain concerned, with polynomial function ($p<0.05$).

Abbreviations: AT=type strain [ATCC 26105] and UV=commercial starter strain [UVA43]; L = Lag phase, $T_h$ = half time; Source: YW = young wine, WS = wine sediment, FS = Furmint sediment, MLW = Muscat Lunel wine, YFW = young Furmint wine, 5YAW = 5 years old aszu wine; Types: A → D = Subclusters (See Fig. 7); Bars: the genetic distances according to Kállai et al. [61].
The lag phase (white box) and half time (black box) of ethanol production extrapolated either manually (EXP) or applying logistic (SIG), Gompertz (GOM), saturation (SAT), probit (PRO), logarithmic (LOG), hyperbolic (HYP) and polynomial (SQU) functions. Abbreviations: maximum (max) and minimum (min) values, lower (LQ) and upper (UQ) quartiles, black or white lines are the median in white or black boxes, respectively. The boxes show the size of the middle two quartiles. The horizontal dotted lines indicate either the median of manually fitted values of lag phases or half times.

Figure 8: Turn points in alcohol production
The regression lines $P (\pi_i = -0.8585c_i - 9.7445, r^2=0.98)$ and $S (s_i = 0.1795c_i + 5.0738, r^2=0.94)$ mark relationship between time dependent primary and secondary factors versus time independent constant factors influencing intensity of actual ethanol production of strains (i).

The labels correspond to the last two numerals of strains in codes given in Table 2. Abbreviations: T=type stain [ATCC 26105] and C=commercial starter strain (opened circles) of $S.\ cerevisiae$ (closed circles) fermented Yellow Muscat must in laboratory models, while $S.\ cerevisiae$ and $S.\ uvarum$ strains (opened squares and triangles, respectively), and Z1[93] and Z2[30] strains of $St.\ bacillaris$ (full squares) fermented Furmint grape must in a winery.

**Figure 9:** Relationships between constant and time dependent factors influencing the strain specific ethanol production