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ETHANOL, SUGAR AND POTASSIUM SORBATE EFFECTS ON *ZYGOSACHAROMYCES BAILII* GROWTH IN WHITE WINE USING RESPONSE SURFACE METHODOLOGY

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Abstract

Zygosaccharomyces bailii represents an important causative spoiling agent in sweet and dry wines. Its tolerance to osmotic stress, 10 - 18% v.v. ethanol, high acidic milieu conditions and to most preservatives, makes its control essential for improved overall quality of wines. The aim of present study was to evaluate the single and simultaneous effect of practical concentrations of invert sugar, ethanol, sulfur dioxide and potassium sorbate on the growth of *Zygosaccharomyces bailii* in white wine using Response Surface Methodology (RSM).

Spoilage yeast strain *Zygosaccharomyces bailii* isolated from white wine was maintained in peptone yeast extract agar medium. Single facture experiments were conducted to study individual influence of factors - invert sugar, ethanol, potassium sorbate and SO_2 concentrations on *Zygosaccharomyces bailii* growth. Optimal composite design (OCD) was designed to describe simultaneous influence of variables: sugar, ethanol and potassium sorbate on *Zygosaccharomyces bailii* growth.

As a result from single facture experiments, it has been observed that ethanol and invert sugar concentrations in the range respectively 10 - 14% v.v. and 3 - 50 g/L could not counteract development of infective *Zygosaccharomyces bailii* microflora. In studied conditions, effective weapon for wine prevention was the addition of preservatives - potassium sorbate and sulfur dioxide in concentrations up to 150 mg/L. Applied RSM methodology revealed that there was not significant interactive suppressive effect between factors and potassium sorbate concentration had most important impact on spoilage yeasts *Zygosaccharomyces bailii* growth. The strongest suppressive effect on *Zygosaccharomyces bailii* growth was observed in case with addition of 200 mg/L sorbic acid in medium containing 100 mg/L total sulfur dioxide, 14% (v.v.) ethanol and 3 g/L invert sugar.

Obtained mathematical models for simultaneous effects of sugar, ethanol and preservatives concentrations on *Zygosaccharomyces bailii* yeast development can be used in a prediction for risk of biological destabilization in wines.

Key words: *Zygosacharomyces bailii, wine, RSM, Medium conditions.*

1. Introduction

Beside Non-Saccharomyces yeasts, generally recognized as aggressive wine spoilage microorganisms, *Zygosaccharomyces* spices seem to be an important causative agent participating in the course of those undesirable processes. The *Zygosaccharomyces* genus consists of twelve species [1, 2, 3], of which *Z. bailii*, *Z. bisporus*, and *Z. rouxii* are the most relevant to the spoilage of foods and beverages [4] and can colonize and spoil high sugar, high salt and high acidic products [5, 6, 7]. Authors reported that most of detrimental impacts on wine quality are mainly associated with spice *Zygosaccharomyces bailii* which activity includes visible sediment formation, cloudiness or haziness in dry wines, fermentation in sweet wines, carbon dioxide production and variety of undesirable odor active metabolites [7, 8, 9]. Malfeito-Ferreira and Silva,

2019 [7] highlighted that given the visual nature of spoiling effect, *Zygosaccharomyces bailii* is a greater concern in white wines. Control and early detection are challenging, as one cell in a bottle may eventually cause a spoilage event [10, 11, 12].

Over recent decades, scientists largely have been studied *Zygosaccharomyces bailii*. Their experiences [11, 13] revealed that *Zygosaccharomyces bailii* demonstrate exceptional tolerance to osmotic stress, ethanol and a variety of common preservatives and organic acids. *Zygosaccharomyces bailii* grows well in sugar syrups and juice concentrates containing 50-60 % sugars [14]. Its ethanol tolerance may be a function of strain [15, 16]. However, authors noted that it can tolerate up to 10 % ethanol and even up to 18 % ethanol [13, 17, 18, 19]. *Zygosaccharomyces bailii* can counteract significant sulfite concentrations and demonstrates strong resistance to benzoic and sorbic acids [20, 21]. Stratford and Anslow, 1998 [22] revealed that its sorbic acid resistance demonstrated to be highly correlated to its ethanol resistance. During CCRD trials in red wine, yeast populations of *Zygosaccharomyces bailii* slowly decreased with time when the sulfur dioxide level was 20 mg/L or higher in presence of 12.5 % v.v. ethanol [23].

Basically, these experiences show that interactions between physicochemical factors and preservatives have an impact on *Zygosaccharomyces bailii* growth in wine and must be considered in different strategies to combat spoilage.

Microbial spoilage modeling can be effectively used for spoilage prevention [24]. Response Surface Methodology (RSM) is a set of mathematical and statistical approaches which are useful for analyzing and modeling of difficulties in which a response of interest is affected simultaneously by several factors (variables) [25]. RSM is widely used in engineering and manufacturing, microbiology, pharmacology and food chemistry where many variables can be involved in and the objective is to optimize the response.

Based on previous experiences and regarding the potential *Zygosaccharomyces* spoiling activities, the aim of present study was to evaluate the single and simultaneous effect of practical concentrations of invert sugar, ethanol, sulfur dioxide and potassium sorbate on the growth of *Zygosaccharomyces bailii* in white wine using RSM.

2. Materials and Methods

2.1 Yeast strain

Zygosacharomyces bailii strain isolated from white wine was maintained in Peptone Yeast extract Agar medium (Merck Millipore, Darmstadt, Germany).

2.2 Culture conditions

The culture was incubated in a liquid nutrient medium – sterile grape juice, containing 23.6% reducing sugars, 7.18 g/L titratable acids and pH 3.19, at 25° C for 72 - 96 h. Concentrations were determined by Burker hemocytometer counting chamber.

2.3 Single effect of ethanol, sugar, potassium sorbate and SO_2 concentrations on spoilage yeast development

Sterile white wines were inoculated to generate an initial population of approximately 5.10^3 cells/mL. Wines were incubated for 42 days (for sugar and ethanol samples) and 25 days (for potassium sorbate and SO_2 samples) at 25° C in 250 mL glass bottles capped with rubber plugs. During incubation, yeast concentration in wine samples was measured periodically as absorbance at 605 nm and counted with Burker chamber. A recalculation was performed to bring the absorbance in cells/mL. The results presented are the mean values of a three-fold repetition of each of samples.

The response of invert sugar (sugar concentration) was studied in the range of 3, 10, 20, 30, 50 g/L. Invert sugar was added to yield wine "sugar" concentrations. Sugar contents were determined by Luff Schoorl procedure [26].

The effect of ethanol was investigated in the range of 10 – 14 % (v/v). Fixed volumes with 96 % ethanol (Valerus, Sofia, Bulgaria) were added to yield wine ethanol concentrations. Ethanol concentrations were measured with ebulliometer.

The effect of potassium sorbate concentration was analyzed in the range of 0, 50, 100, 150, 200 mg/L by adding potassium sorbate (Valerus, Sofia, Bulgaria) directly in wine samples.

The response of SO_2 was determined in the range of 0, 50, 100, 150, 200 mg/L (total SO_2) by adding sulfuric acid (Valerus, Sofia, Bulgaria).

Experimental wines were sterilized through 0.25 µm membranes (Millipore) and analyzed for ethanol content, pH, volatile and total acidity, free, total sulfur dioxide [27] and sugar content [26]. Parameters of experimental wine are: ethanol content: 10% (v/v), sugar content: 3 g/L, pH: 3.3, titratable acids content: 6 g/L, volatile acids content: 0.25 g/L, free SO_2 content: 0 g/L.

2.4 Combined (simultaneous) effects of sugar, ethanol and potassium sorbate on spoilage yeast development

Sterile white wines were inoculated to generate an initial population of approximately 5.10^3 cells/mL.

Wines were incubated for 36 days at 25° C in 250 mL glass bottles capped with rubber plugs. After incubation, yeast concentration in wine samples was measured as absorbance at 605 nm.

RSM and 3^2 Optimal Composite Design (OCD) with "star points" around the center point were used to determine influence of factors (variables) sugar, ethanol and potassium sorbate on *Zygosaccharomyces bailii* growth. The quadratic regression models are one of the most widely used in practice. They allow description of the object in a comparatively wide area of input variable change [28, 29].

The distance from the design center to a factorial point was a = ±1. The quadratic regression model was expressed as follows:

$$\hat{Y} = b_0 + \sum_{i=1}^{m} b_i . x_i + \sum_{i=1, j=i+1}^{m} b_{ij} . x_i . x_j + \sum_{i=1}^{m} b_{ii} . x_i^2$$

Where: Y is the response variable (yeasts concentration), b the regression coefficients of the model and x the coded levels of the independent variables (sugar, ethanol, potassium sorbate).

Sygma Plot software from Systat Software, Inc. was used for regression and graphical analysis. The independent variables participating in the 3^2 OCD and their values are presented in Table 1.

Table 1. Levels of independent variables examined with the 3^2 optimal composite design

Independent variables	Interval of variation		
	-1	0	1
x_1- ethanol concentration, (% v/v)	10	12	14
x_2- sugar concentration, (g/L)	3	15	27
x_3- potassium sorbate concentration, (mg/L)	0	100	200

3. Results and Discussion

3.1 Effect of sugar concentration on *Zygosacharomyces bailii* growth

Increasing the invert sugar concentration in medium from 3 to 10, 20, 30 and 50 g/L, the growth rate of *Zygosacharomyces bailii* strain was not affected in specific way (Figure 1). The higher sugar concentration did not inhibit the microorganisims' growth activity and even in 50g/L sugar in medium, a maximum cells concentration was established. Obtained results fitted well with previous researches revealing the osmotolerant nature of *Zygosacharomyces bailii* [5, 6, 7, and 30].

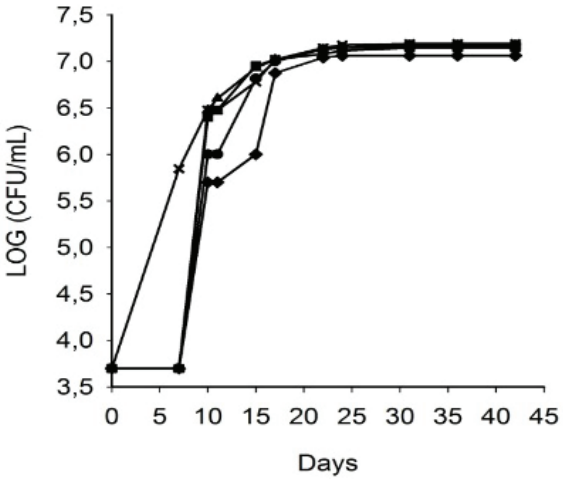

Figure 1. Effect of sugar concentration on *Zygosacharomyces bailii* growth, (g/L): (♦) 3, (■) 10, (▲) 20, (X) 30, (●) 50

3.2 Effect of ethanol concentration on *Zygosaccharomyces bailii* growth

The effect of ethanol concentration on *Zygosaccharomyces bailii* growth is presented on Figure 2.

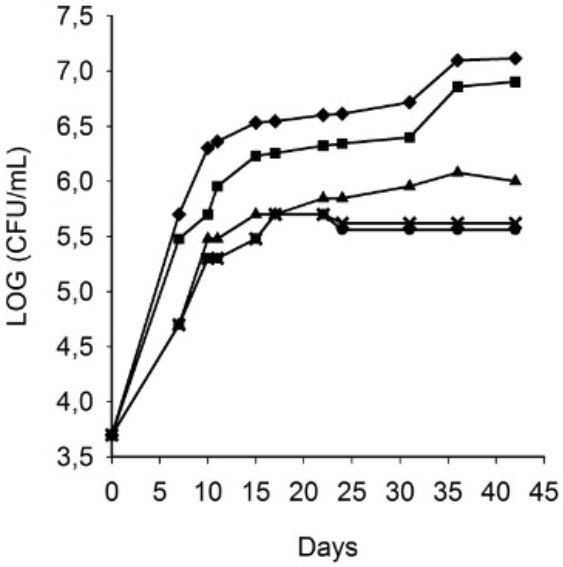

Figure 2. Effect of ethanol concentration on *Zygosacharomyces bailii* growth, (% v/v): (♦) 10, (■) 11, (▲) 12, (X) 13, (●) 14

Despite of powerful ethanol selection capacity for wine microflora, including spoilage yeasts, in studied conditions the ethanol did not exhibit a valuable suppressive effect on yeast growth. However, it must be mentioned that higher ethanol concentrations of 13 and 14 % v.v. lead to a weak reduction in yeast concentration.

3.3 Effect of potassium sorbate and SO_2 concentrations on *Zygosaccharomyces bailii* growth.

Important impact on yeasts growth rate was established in concentrations of 200 mg/L total SO_2 and 200 mg/l potassium sorbate (Figure 3 and Figure 4). In those conditions, none augmentation in yeast concentration have been observed.

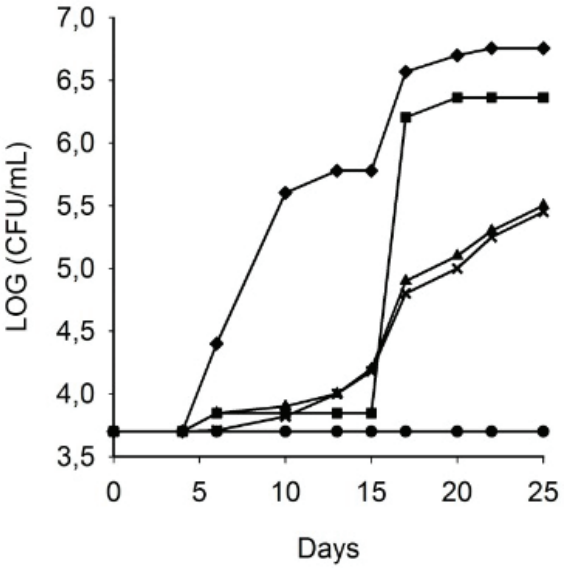

Figure 3. Effect of potassium sorbate concentration on *Zygosacharomyces bailii* growth, (mg/L): (♦) 0, (■) 50, (▲) 100, (X) 150, (●) 200

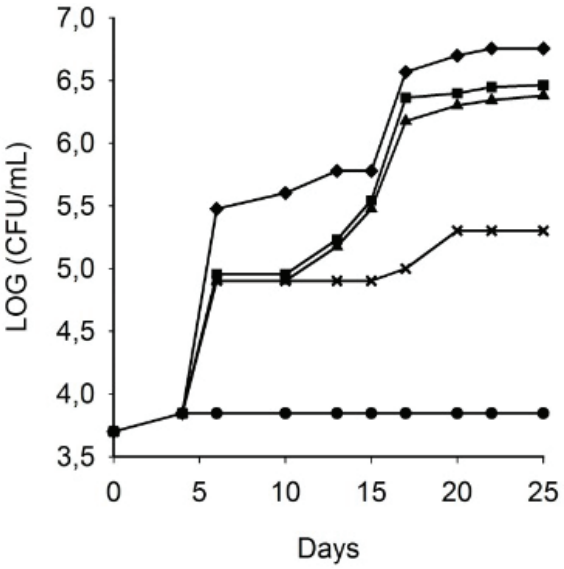

Figure 4. Effect of SO_2 concentration on *Zygosacharomyces bailii* growth, (mg/L): (♦) 0, (■) 50, (▲) 100, (X) 150, (●) 200

Potassium sorbate concentrations in order of 100-150 mg/L were insufficient to inhibit effectively the growth of *Zygosaccharomyces bailii*, which population reached 5.5 LOG (CFU/mL).

Like-minded effect was perceived in sulfur dioxide preservative case. Lower concentrations of 100 and 50 mg/l total SO_2 did not affect yeasts development in negative way, even in condition of relatively low concentrations of essential energy sources (sugar) in medium (3 g/L). In concentrations of 100 and 50 mg/L total SO_2 the yeast development (6.4 LOG (CFU/ml) almost reached the maximum cells concentration of control sample without preservatives - 6.8 LOG (CFU/ml). The addition of 150 mg/L total SO_2 lowered the infective yeasts concentration to 5.3 LOG (CFU/mL).

3.4 Optimization of spoilage yeast concentration by RSM

The full factor experiment was involved by fixing total SO_2 at concentration of 100 mg/L in all samples. The aim was to study concentrations of spoilage yeasts occurring, even the use of comparatively high total SO_2 concentration. This was motivated by suggestions of scientists that some *Zygosaccharomyces* developed well in presence of SO_2 because of various resistant mechanisms [9, 20] and by our suggestion that in presence of infectious microflora in bottled and bag-in-box wines, destabilization can occur after a certain period of time - several months during which the concentration of sulfur dioxide falls (especially its free form) and spoilage virulent cells develop.

From single factor experiment, sugar concentration, ethanol and potassium sorbat contents in medium had the most significant effect on spoilage yeasts growth. For these reasons, a mathematical experimental design was conducted to study the co-influence of both three factors on yeasts growth.

The matrix of 3^2 OCD with variables (sugar, ethanol and potassium sorbate) is shown in Table 2.

A mathematical model, describing the effect of sugar, ethanol and potassium sorbate contents on *Zygosaccharomyces bailii* was developed as follows:

$$\hat{Y}=6.342 - 0.338*x_1 - 1.447*x_3 - 0.337*x_1^2 + 0.106*x_2^2 - 1.011*x_3^2$$

Only significant regression coefficients (P value > 0.05) and quadratic members are included in the model. The model is characterized by a high correlation coefficient $R^2 = 0.95$ and it is adequate at a confidence level of 0.05 (Significance $F = 1.97*10^{-6}$).

Applied methodology for simultaneous effects estimation of input variables showed that in studied conditions there was not significant interactive impact between factors. Coefficients for ethanol and sugar factors are relatively low and only coefficient for potassium sorbate is higher. This is well aligned with our results from single-factor experiments for *Zygosaccharomyces bailii* and shows that their growth

Table 2. Optimal composite design 3^2

№	Coded levels			Cells concentration, log CFU	
	x_1	x_2	x_3	Y	Ŷ
1	-1	-1	-1	7.1	7.1
2	1	-1	-1	6.0	5.9
3	-1	1	-1	6.9	7.1
4	1	1	-1	6.4	6.2
5	-1	-1	1	3.7	3.9
6	1	-1	1	3.7	3.4
7	-1	1	1	3.7	3.7
8	1	1	1	3.7	3.6
9	-1	0	0	6.7	6.3
10	1	0	0	4.9	5.7
11	0	-1	0	6.1	6.4
12	0	1	0	6.4	6.5
13	0	0	-1	6.6	6.8
14	0	0	1	3.7	3.9
15	0	0	0	6.6	6.3
16	0	0	0	6.6	6.3
17	0	0	0	6.6	6.3

Legend: x_1 – ethanol concentration; x_2 – sugar concentration x_3 – potassium sorbate concentration; Y - cells concentration, log CFU; Ŷ – predicted cells concentration, log CFU.

rate was most strongly affected by the preservatives' concentration and lowers the concentration of sugars and ethanol.

Response surface fitted to experimental data points is presented on Figure 5.

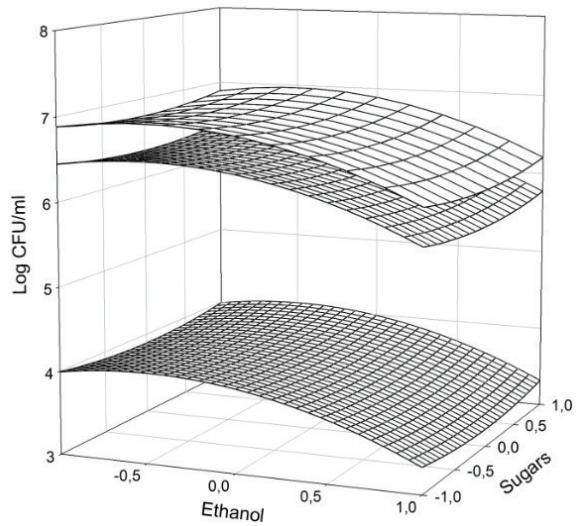

Figure 5. Effect of ethanol, sugars and potassium sorbate concentrations on *Zygosaccharomyces bailii* growth (from top to bottom): first grid line - 0 mg/L potassium sorbate, second grid line - 100 mg/L potassium sorbate, third grid line - 200 mg/L potassium sorbate

In absence of potassium sorbate, exposed to 100 mg/L SO_2 and ethanol 10-14 % (v/v) *Zygosaccharomyces bailii* growth wasn't suppressed, even in cases that the invert sugar concentration in medium was very low (3 g/L). The achieved maximum cell concentration was 6.8 LOG CFU/mL. Similar trend was observed in case with the addition of 100 mg/L potassium sorbate in medium, where maximum cell concentration was about 6.5 LOG CFU/mL. Potassium sorbate addition showed a valuable positive toxic effect on *Zygosaccharomyces bailii* growth in concentration of 200 mg/L and in this case even the high invert sugar concentrations (27 g/L) did not support the tolerance of yeasts.

Response surface confirmed the strong suppressive effect of potassium sorbate on *Zygosaccharomyces bailii* growth. Minimum spoilage yeast concentration (Ŷmin) was observed at the following coefficients of co-acting input factors: $x_1 = 1$ (ethanol concentration 14% v/v), $x_2 = -1$ (sugar concentration 3 g/L) and $x_3 = 1$ (concentration of potassium sorbate 200 mg/L).

4. Conclusions

- In both dry and sweet wines, yeast contamination by *Zygosaccharomyces bailii* can lead to detrimental impact on quality characteristics and hygienic status of products. In studied conditions ethanol and sugar concentrations in range respectively 10-14 % v.v. and 3-50 g/L could not counteract development of infective *Zygosaccharomyces bailii* microflora.

- It has been established that an effective weapon for wine prevention was the addition of preservatives - potassium sorbate and sulfur dioxide in concentrations up to 150 mg/L.

- Obtained mathematical models for simultaneous effects of sugar, ethanol and preservatives concentrations on *Zygosaccharomyces bailii* yeast development can be used in a prediction for risk of biological destabilization in wines.

- Applied RSM methodology revealed that there was not significant interactive suppressive effect between factors and potassium sorbate concentration had most important impact on spoilage yeasts *Zygosaccharomyces bailii* growth. The strongest suppressive effects on *Zygosaccharomyces bailii* growth were observed in cases with addition of 200 mg/L sorbic acid in medium containing 100 mg/L total sulfur dioxide, 10-14 % (v.v.) ethanol and 3-27 g/L invert sugar.

5. References

[1] Kuanyshev N., Adamo G., Porro D., Branduardi P. (2017). *The spoilage yeast Zygosaccharomyces bailii: Foe or friend?* Yeast, 34, pp. 359-370.

[2] James S. A., Stratford M. (2011). *Zygosaccharomyces Barker (1901)*. In: Kurtzman J. W. C., Boekhout T. F., (Eds.), The Yeasts, Elsevier, London, UK.

[3] Kurtzman C. P., Robnett C. J. (2003). *Phylogenetic relationships among yeasts of the 'Saccharomyces complex' determined from multigene sequence analyses*. FEMS Yeast Researh, 3, pp. 417-432.

[4] Petruzzi L., Corbo M.R., Sinigaglia M., Bevilacqua B. (Eds.). (2017). *The Microbiological Quality of Food,* Woodhead Publishing, Cambrige, UK.

[5] Blackburn W. de C. (Ed). (2006). *Managing microbial food spoilage*. Food Spoilage Microorganisms, Woodhead Publishing, Cambrige, UK.

[6] Kurtzman C. P., James S. A. (Eds.). (2006). *Zygosaccharomyces and related genera.* Food Spoilage Microorganisms, CRC Press, Boca Raton, USA.

[7] Malfeito-Fereira M., Silva A. C. (2019). *Spoilage Yeasts in Wine Production*. In: Romano P., Ciani M., Fleet H. G. (Eds.), Yeasts in the Production of Wine, Springer, New York, USA, pp. 375-395.

[8] Fugelsang K., Edwards C. (2007). *Wine Microbiology (Practical application and procedures)*. Springer, New York, USA.

[9] Zuehlke J. M., Petrova B., Edwards C. G. (20130. *Advances in the Control of Wine Spoilage by Zygosaccharomyces and Dekkera/Brettanomyces*. Annual review of Food Science and Technology, 4, (1), pp. 57-78.

[10] Deak T., Reichart O. (1986). *Unacceptable levels of yeasts in bottled wine, Methods for the Mycological Examination of Food*. Plenum Press, New York, USA.

[11] Zuehlke J. M. (2013). *Influence of Zygosaccharomyces and Brettanomyces on Wine Quality and their Control During Vinification*. PhD thesis, School of Food Science, Washington State University, Washington, USA.

[12] James S., Stratford M. (2003). *Spoilage yeasts with emphasis on the genus Zygosaccharomyces.* In: Boekhout T., Robert V. (Eds.), Yeasts in Food, Woodhead Publishing, Cambridge, UK.

[13] Thomas D. Davenport R. (1985). *Zygosaccharomyces bailii: A profile of characteristics and spoilage activities*. Food Microbiology, 2, pp. 157-169.

[14] Stratford M., Steels H., Nebe-von-Caron G., Novodvorska M., Hayer K., Archer D. B. (2013). *Extreme resistance to weak-acid preservatives in the spoilage yeast Zygosaccharomyces bailii.* International Journal of Food Microbiology, 166, pp. 126-134.

[15] Pilone D. A., Pilone G. S., Rankine B. C. (1973). *Influence of Yeast Strain, pH and Temperature on Degradation of Fumaric Acid in Grape Juice Fermentation*. American Journal of Enology and Viticulture, 24, pp. 97-102.

[16] Kalathenos P., Sutherland J. P., Roberts T. A. (1995). *Resistance of some wine spoilage yeasts to combinations of ethanol and acids present in wine.* Journal of Applied Bacteriology, 78, pp. 245-250.

[17] Romano P., Suzzi G. (1993). *Higher alcohol and acetoin production by Zygosaccharomyces wine yeast*. Journal of Applied Bacteriology, 75, pp. 541-545.

[18] Du Toit M., Pretorius I. S. (2000). *Microbial Spoilage and Preservation of wine: Using weapons from Nature's own arsenal - A review*. South African Journal for Enology and Viticulture, 21, pp. 74-96.

[19] Van Wyk S., Silva F. V. M. (2019). *Nonthermal preservation of wine*. In: Grumezescu M. A., Holban M. A. (Eds.), Preservatives and Preservation Approaches in Beverages, Academic Press, Cambridge, USA, pp. 203-235.

[20] Warth A. D. (1985). *Resistance of Yeast Spices to Bensoic and Sorbic Acids and to Sulfur Dioxide*. Journal of Food Protection, 48, (7), pp. 564-569.

[21] Steels H., James S. A., Roberts I. N., Stratford M. (2000). *Sorbic acid resistance: The inoculum effect*. Yeast, 16, pp. 1173-1183.

[22] Stratford M., Anslow P. A. (1998). *Evidence that sorbic acid does not inhibit yeast as a classic 'weak acid preservative'.* Letters of Applied Microbiology, 27, (4), pp. 203-206.

[23] Chandra M., Oro I., Ferreira-Dias S., Malfeito-Ferreira, M. (2015). *Effect of Ethanol, Sulfur Dioxide and Glucose on the Growth of Wine Spoilage Yeasts Using Response Surface Methodology*. PloS one, 10, (6), <URL: https://doi.org/10.1371/journal.pone.0128702. Accessed 10 June 2020.

[24] Doyle M E. (2007). *Microbial food spoilage - Losses and control strategies, (A brief review of the Literature)* FRI Briefings. <URL: www.wisc.edu/fri/. Accessed 15 June 2020.

[25] Abbasi B., Mahlooji H. (2012). *Improving response surface methodology by using artificial neural network and simulated annealing.* Expert Systems with Application, 39, (3), pp. 3461-3468.

[26] Mellet P., Lionnet G. R. E., Kimmeling Z. J., Bennett P. J. (1982). *Standards for the analytical precision of sugar and molasses analyses*. Proceedings of South African Sugarcane Technology Assessment, 56, pp. 55- 57.

[27] OIV. (2006). *International Compendium of Methods of Analysis of Wines and Must* (in French). OIV, Paris, France.

[28] Vuchkov I., Stoyanov S. (1980). *Mathematical modelling and optimization of technological objects*, Technics, Sofia, pp. 135-151.

[29] Dobrev G. T., Pishtiyski I. G., Stanchev V. S., Mircheva R. (2007). *Optimization of nutrient medium containing agricultural wastes for xylanase production by Aspergillus niger B03 using optimal composite experimental design*. Bioresource Technology, 98, (14), pp. 2671-2678.

[30] Martorell P., Stratford M., Steels H., Fernandez-Espinar M. T., Querol A. (2007). *Physiological characterization of spoilage strains of Zygosaccharomyces bailii and Zygosaccharomyces rouxii isolated from high sugar environments*. International Journal of Food Microbiology, 114, pp. 234-242.