Original scientific paper
UDC 663.258:582.282.23

STUDY OF ETHANOL, SUGAR AND POTASSIUM SORBATE EFFECTS ON *BRETANOMYCES INTERMEDIUS* GROWTH IN WHITE WINE USING RESPONSE SURFACE METHODOLOGY

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Abstract

During winemaking and wine storage, microbial spoilage can occur, affecting quality and hygienic status of the end product. Authors highlighted that induced microbiological hazards are associated with release of mousy off-flavor compounds, turbidity, formation of sediment, and secondary fermentation of wine. Yeasts play a central role in spoilage of foods and beverages. *Bretanomyces* genera are referred as one of the most common and dangerous contaminating yeasts in wine. The increasing importance of *Bretanomyces* yeasts in wine spoilage is referred more frequently with their resistance at high sugar, ethanol and preservatives concentrations. The aim of this study was to determine single and simultaneous effects of practical concentrations of sugars, ethanol and potassium sorbate on *Bretanomyces* intermedius growth in white wine.

Response surface methodology (RSM) was utilized to investigate interactions among variables and their influence on spoilage yeasts development. Spoilage yeast strain *Brettanomyces intermedius* isolated from white wine was maintained in malt extract agar medium. Single facture experiments were conducted to study individual influence of factors: invert sugar, ethanol, potassium sorbate and SO_2 concentrations on *Brettanomyces intermedius* growth. Optimal composite design (OCD) was designed to describe simultaneous influence of variables: sugar, ethanol and potassium sorbate on *Bretanomyces intermedius* growth.

Overall results from single facture experiments showed that exposed to ethanol and sugar concentrations respectively 10 - 13 % (v/v) and 3 - 50 g/L, *Brettanomyces intermedius* yeasts demonstrated relatively high resistance. Significant suppressive effect on their growth was observed after adding preservatives in concentrations higher than 150 mg/L total SO_2 and 100 mg/L potassium sorbate. By RSM it was found that only potassium sorbate is the significant factor affecting in negative way *Brettanomyces intermedius* growth and cells can't counteract to levels of 200 mg/L.

Applied methodology revealed that there was not significant interactive suppressive effect between factors.

***Key words**: Brettanomyces intermedius, Wine, RSM, Medium conditions.*

1. Introduction

Yeasts play a central role in spoilage of foods and beverages [1]. In wine, spoilage can appear even before starting alcoholic fermentation or along with its early stages [2, 3, and 4]. In succeeding stages of winemaking, spoilage yeast genera, often found include: *Brettanomyces, Saccharomyces, Schizosaccharomyces* and *Zygosacharomyces* [5, 6]. Thus, monitoring of spoilage yeasts should include all phases of winemaking [1].

Brettanomyces genera are referred as one of the most common and dangerous contaminating yeasts in wine [7, 8]. The increasing importance of yeasts *Brettanomyces* in wine spoilage is related frequently with their resistance at high sugar, ethanol and preservatives concentrations. Different authors [9, 10] highlighted that yeasts *Brettanomyces* developed well with 13% and more ethanol concentration, and are resistant in permitted amounts of sulfur dioxide and sorbic acid. From 30 *Brettanomyces* strains examined, 23 exhibited growth up to 14.5% (v/v) ethanol in media and only two tolerated 15% (v/v) [11]. More recently, authors reported for two strains *Brettanomyces bruxellensis* exhibiting longer lag phases as the ethanol concentrations of the wine increased from 12 to 15% (v/v) over a range of temperatures (i.e., 15 to 21 ^{0}C), with a total loss of culturability noted for all wines at 16% (v/v) ethanol [12]. Our previous studies shown that some *Brettanomyces intermedius* strains can grow in dry and sweet wines, containing 100 mg/L SO_2 and 100 mg/L potassium sorbate, but others are not as resistant [13]. However, high resistance of *Brettanomyces bruxellensis* has been demonstrated even in 1000 mg/L of sorbic acid [14].

Basically, the experience of present authors confirms that growth of spoilage yeasts *Brettanomyces* in wine depends on specific strain characteristics of genera and on specific conditions of the medium as: concentration of sugars, ethanol and preservatives.

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) [15]. 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.

Aim of this study was to determine single and simultaneous effect of practical concentrations of sugars, ethanol and potassium sorbate on *Brettanomyces intermedius* growth in white wine and to develop mathematical models describing adequately their individual strain behavior as function of milieu variables by RSM.

2. Materials and methods

2.1 Yeast strain

Brettanomyces intermedius strain isolated from white wine was maintained in MEA medium (Merck, Darmstadt, Germany) was used in this research.

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 ^{0}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 x 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 ^{0}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/cm^3. 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, and 50 g/L. Invert sugar was added to yield wine "sugar" concentrations. Sugar contents were determined by Luff Schoorl procedure [16].

Effect of ethanol was investigated in the range of 10 - 14% (v/v). Fixed volumes with 96% ethanol (Merck, Darmstadt, Germany) were added to yield wine ethanol concentrations. Ethanol concentrations were measured with ebulliometer.

Effect of potassium sorbate concentration was analyzed in the range of: 0, 50, 100, 150, and 200 mg/L by adding potassium sorbate (Merck, Darmstadt, Germany) directly in wine samples.

Response of SO_2 was determined in the range of: 0, 50, 100, 150, and 200 mg/L (total SO_2) by adding sulfuric acid (Merck, Darmstadt, Germany).

Experimental wines were sterilized through 0.25 µm membranes (Millipore) and analyzed for: ethanol content, pH, volatile and total acidity, free, total sulfur dioxide [17], and sugar content [16]. 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, and 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 x 10^3 cells/mL. Wines were incubated for 36 days at 25 ^{0}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) as sugar, ethanol and potassium sorbate on *Brettanomyces intermedius* 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 [18, 19].

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 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 *Brettanomyces intermedius* growth

Obtained results for spoilage yeasts *Brettanomyces* in white wine, showed that sugar concentration in medium affects in specific way the strain growth (Figure 1).

Even in low sugar concentration in medium (3 g/L), spoilage yeasts exhibited substantial growth. In the range of 3 - 50 g/L sugars in medium, *Brettanomyces intermedius* demonstrated relatively long lag phase (up to 10 days) that could be explained with longer adaptation period of strain which have already been described as slow-growing yeast spice [1]. However, after 10th day, the strain showed significant growth.

When increasing invert sugar concentration in medium, the growth of *Brettanomyces intermedius* generally increased, except at 50 g/L sugar which exhibited weak suppressive effect.

3.2 Effect of ethanol concentration on *Bretanomyces intermedius* growth

Ethanol is a powerful selection factor for wine microflora including spoilage yeasts. The ability of ethanol to suppress their development is expressed in reducing the microbial enzymes activity, governing the cells respiration, fermentation and reproduction [20, 21]. However, several spoilage yeasts, including *Brettanomyces,* tolerate relatively high ethanol concentrations of 14% (v/v) [11, 12, and 22].

Effect of ethanol concentration on *Brettanomyces intermedius* growth is presented on Figure 2.

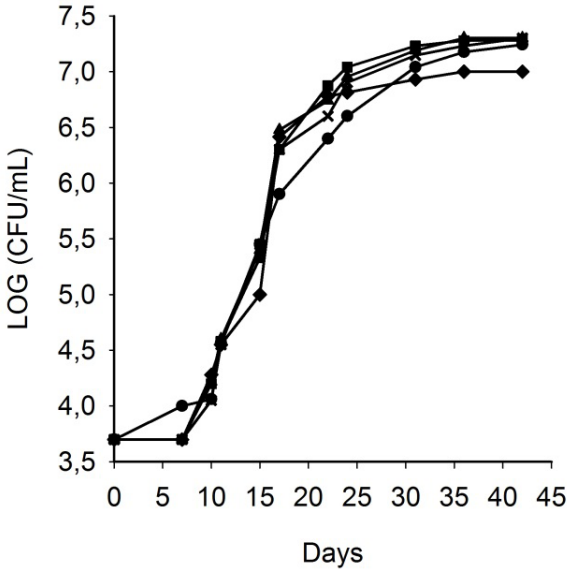

Figure 1. Effect of sugar concentration on *Brettanomyces intermedius* growth, (g/L): (♦) 3, (■) 10, (▲) 20, (X) 30, (●) 50

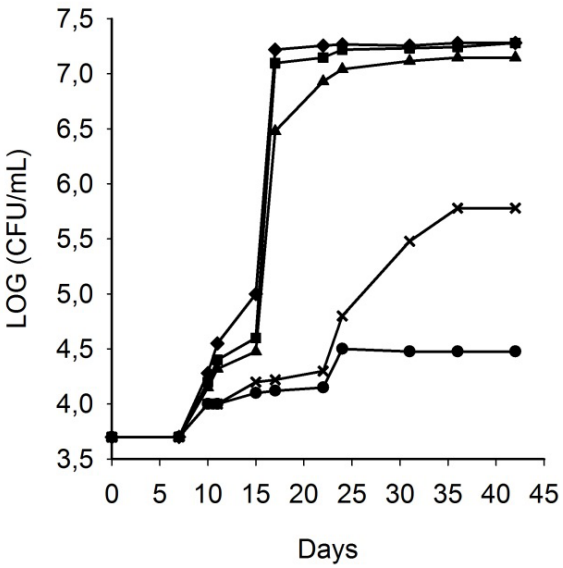

Figure 2. Effect of ethanol concentration on *Brettanomyces intermedius* growth, (% v/v): (♦) 10, (■) 11, (▲) 12, (X) 13, (●) 14

In studied conditions, our results supported the view of above mentioned opinions, and ethanol did not exhibit suppressive effect on *Brettanomyces intermedius* growth. *Brettanomyces* strain showed adaptation period to medium (10 days). At higher ethanol concentrations (13 - 14% v/v), a weak suppressive effect was observed on *Brettanomyces* growth. However, this effect can't be regarded as "safe" for wine quality because *Brettanomyces* yeasts can produce the characteristic "brett" flavors when growing at low cell density of several hundred to several thousand cells per mL [23].

3.3 Effect of potassium sorbate and SO_2 concentrations on *Brettanomyces intermedius* growth

Processing methods for spoilage yeasts control in wines basically consist in addition of preservatives like sorbic acid and SO_2. Sorbic acid is reported by researchers as non-effective preservative and *Brettanomyces* yeasts can stand up to 900 mg/L [24, 25]. However, at pH 3.5, 20 mg/L free SO_2 showed positive toxic effect on *Dekkera/Brettanomyces* cells which is a permitted wine additive, but the problem is that over time its concentration falls at a rate dependent on the pH. It should therefore be checked periodically, especially in wine ageing in wooden barrels, to ensure that protection against *Dekkera/Brettanomyces* is maintained [24].

Figures 3 and 4 illustrate spoilage yeasts growth as function of potassium sorbate and SO_2 concentrations in medium.

In concentrations up to 100 mg/L, potassium sorbate exhibited strong suppressive effect on *Brettanomyces intermedius* growth. Lower concentrations 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).

Like-minded effect on yeasts growth was observed in total SO_2 concentrations higher than 150 mg/L. Relatively high strain resistance of yeasts was in agreement with previous reported data [9, 10].

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. 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 *Brettanomyces* developed well with permitted amounts of SO_2 [9], 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 sulfate contents in medium had the most significant effect on spoilage yeasts

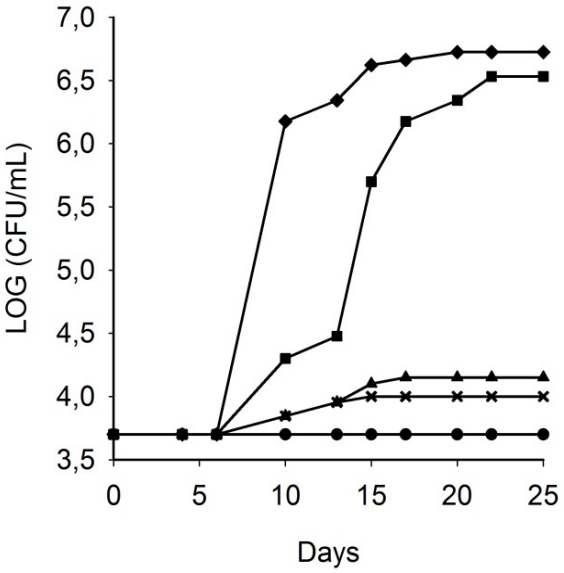

Figure 3. Effect of potassium sorbate concentration on *Brettanomyces intermedius* growth, (mg/L): (♦) 0, (■) 50, (▲) 100, (X) 150, (●) 200

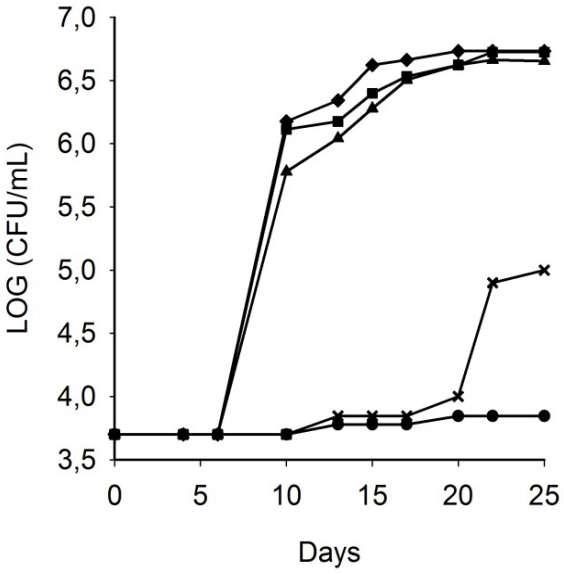

Figure 4. Effect of SO_2 concentration on *Brettanomyces intermedius* growth, (mg/L): (♦) 0, (■) 50, (▲) 100, (X) 150, (●) 200

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) affecting growth of *Brettanomyces intermedius* is shown in Table 2.

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	6.9
2	1	-1	-1	6.9	6.9
3	-1	1	-1	7.2	6.9
4	1	1	-1	7.1	6.9
5	-1	-1	1	3.7	3.9
6	1	-1	1	3.7	3.9
7	-1	1	1	3.7	3.9
8	1	1	1	3.7	3.9
9	-1	0	0	6.9	6.6
10	1	0	0	6.7	6.6
11	0	-1	0	6.6	6.6
12	0	1	0	6.9	6.6
13	0	0	-1	7.2	7.6
14	0	0	1	5.3	4.5
15	0	0	0	6.7	6.9
16	0	0	0	6.7	6.9
17	0	0	0	6.7	6.9

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.

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

$$\hat{Y}=6.897-1.547*x_3-0.306*x_1^2-0.315*x_2^2-0.833*x_3^2$$

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 $R^2 = 0.96$ and it is adequate at a confidence level of 0.05 (Significance $F = 4.22*10^{-8}$).

Applied methodology for estimation the simultaneous effects of input variables showed that there was not significant interactive impact between factors.

Values of factors' coefficients for ethanol and sugar are relatively low and only coefficient for potassium sorbate is higher. This is well aligned with the results of single-factor experiments for yeasts of this genus and shows that their development was most strongly affected by the preservative's concentration and lowers the concentration of sugars and ethanol. Ethanol, in the most common range is not a problem for their growth. This was in agreement with results obtained in single facture experiments and with reported data [11, 12].

In absence of potassium sorbate, exposed to 100 mg/L SO_2 and ethanol 10-14% (v/v) *Brettanomyces* growth wasn't suppressed, even in cases that the invert sugar concentration in medium was very low (3 g/L). Potassium sorbate addition showed positive toxic effect on *Brettanomyces* and high invert sugar concentrations did not support the tolerance of yeasts.

Response surface fitted to experimental data points is presented on Figure 9.

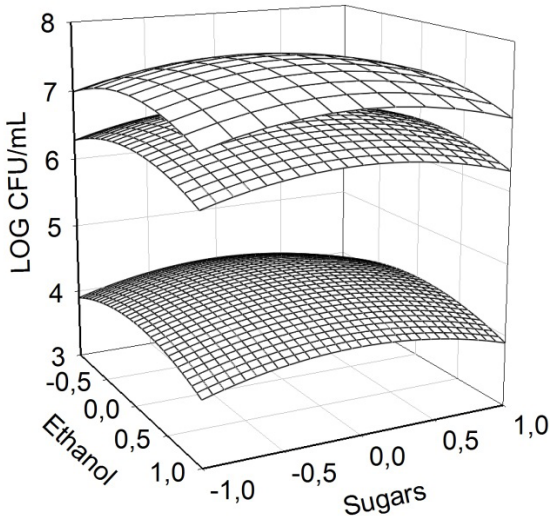

Figure 5. Effect of ethanol, sugars and potassium sorbate concentrations on *Brettanomyces intermedius* growth (from top to bottom): first grid line - 0 mg/L potassium sorbate, second grid line - 100 mg/L potassium sorbate, third grid line - 200 mg/L potassium sorbate

Response surface confirmed the strong suppressive effect of potassium sorbate on *Brettanomyces* growth. Minimum cells 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

- *Brettanomyces* yeasts can be a significant contamination factor both in dry wines and in wines with residual sugar. Ethanol concentrations in range 10 - 14% can't counteract development of infective *Brettanmyces* microflora.

- Effective way for wine prevention is the addition of chemical preservatives. Obtained mathematical models from simultaneous effects of sugar, ethanol and preservatives concentrations on *Brettanomyces intermedius* yeast development can be used in a prediction for risk of biological destabilization in packaged 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 *Brettanomyces intermedius* growth. This give grounds to claim that wines with parameters within the limits we use are susceptible to biological destabilization with sufficiently high initial contamination of spoilage microflora. Exception is wines with total SO_2 concentration in range of 100 mg/L and potassium sorbate in concentrations above 200 mg/L.

5. References

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