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Toward application of biocontrol to inhibit wine spoilage yeasts: The use of statistical designs for screening and optimisation

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ABSTRACT

Spoilage yeasts generate considerable economic losses in the wine industry, and although sulphur dioxide (SO_2) is traditionally used for control, its use has become controversial because of its negative effects on health. Biocontrol has emerged as a partial alternative to SO_2, and most research has focused on the selection of biocontrol yeasts and/or the mechanisms involved, while little research has been directed to the environmental conditions that make biocontrol effective for application. When there are two or more interacting yeasts, the physicochemical factors that affect their antagonism are many and therefore the application of biocontrol is complex. To reduce SO_2, the present study aimed to elucidate biocontrol mechanisms of two yeast interactions and to establish optimal physicochemical conditions for biocontrol of the spoilage yeast during grape must fermentation. Through the use of statistical design, it was possible to find relevant physicochemical factors and optimise them. *Wickerhamomyces anomalus* "BWa156" developed an active supernatant against *Zygosaccharomyces rouxii* "BZr6" while supernatant from *Metschnikowia pulcherrima* "BMp29" was ineffective. In mixed must fermentations, the first interaction (BWa156 *vs.* BZr6) showed fewer physicochemical factors impacting biocontrol compared to the second interaction (BMp29 *vs.* BZr6). However, the fewer factors of the first interaction had a stronger effect on the decline in the spoilage population. Validations showed that the optimal conditions for biocontrol with the first interaction could be predicted. Analysis of the results with BWa156 *vs.* BZr6 and BMp29 *vs.* BZr6 suggests that the first interaction is a competition that includes a killer toxin, while the second interaction involves competition for iron resources. Response surface methodology (RSM) allowed a reduction in the number of experiments and permitted to find the optimal biocontrol conditions (SO_2: 0 mg mL^{-1}; pH: 3.7; Reducing sugars: 23 °Brix) for the interaction between BWa156 and BZr6.

KEYWORDS

Zygosaccharomyces rouxii, *Brettanomyces bruxellensis*, *Metschnikowia pulcherrima*, *Wickerhamomyces anomalus*, Grape must fermentation, SO_2 reduction, Healthy wines.

Supplementary data can be downloaded through: https://oeno-one.eu/article/view/4510

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INTRODUCTION

Wine spoilage yeasts generate considerable economic losses because they produce unfavourable organoleptic characteristics (Rojo *et al.,* 2015). Traditionally, SO_2 has been used to counteract these yeasts, but its use has become controversial because of its negative effects on human health, and international organizations such as the World Health Organization and *Organisation Internationale de la vigne et du vin* encourage a reduction of its use (Comitini and Ciani, 2010; Ferrer-Gallego *et al.,* 2018).

Several authors have hypothesised about mixed co-inoculations during wine fermentation so that yeast populations that positively affect the fermentation process could control wine spoilage yeasts in the initial fermentation stages. This would result in a reduction in the use of chemical compounds such as SO_2 (Oro *et al.,* 2014; Berbegal *et al.,* 2017; Simonin *et al.,* 2020). These studies have focused on the selection of biocontrol yeasts and/or characterization of the biocontrol mechanisms. Nevertheless, there is little information about the conditions under which biocontrol is effective in the real environment, the wine fermentation (Boynton, 2019). This may have its origin in the fact that controlled fermentations for the application of certain yeast strains are characterised by complex interactions between the members of the consortium inoculated in the grape must and the yeast microbiota in the original must (Ciani and Comitini, 2015).

Ecologists classify the competitive strategies of organisms into two categories: exploitation and interference competition. Exploitation competition occurs when organisms sequester or consume resources and thereby prevent other organisms from having access to these resources. Interference competition occurs when organisms directly inhibit one another by interfering with viability or growth (*e.g.,* killer toxins) (Boynton, 2019). The ecologist classification is clearer in that biocontrol involves competition for the medium's resources. The individual yeast fitness and competitiveness between populations in a fermentation environment are influenced by a number of abiotic factors like pH, ethanol, osmotic pressure, SO_2 supplement, temperature, oxygen, and nutrients (Ciani *et al.,* 2016; Avbelj *et al.,* 2016) and biotic factors such as cell-cell contact and bioactive metabolites (Comitini *et al.,* 2004; Nissen *et al.,* 2003; Renault *et al.,* 2013; Avbelj *et al.,* 2016; Ciani *et al.,* 2016; Bordet *et al.,* 2020). However, the degree of influence of biotic and abiotic factors is difficult to determine individually, since the impact of one factor is generally affected by that of other factors (Fleet, 2011; Rojo *et al.,* 2015). Therefore, the performance of controlled mixed fermentations requires more knowledge about environmental factors and metabolic activities that influence yeast interactions (Ciani and Comitini, 2015).

The need for efficient methods to select among factors involved in a certain process has led to the adoption of experimental designs. The methodology based on the Plackett–Burman design (Plackett and Burman, 1946) provides an efficient way to reduce experimental times to select the most important factors from those cited in the literature, and it can help find relevant factors regarding interactions between antagonistic yeasts during the fermentation process. This would allow a reduction in the number of factors to focus on only a small set, which can be properly optimised (Montgomery, 1990). These designs have been used in many microbiological and some enological research projects (Dopico-García *et al.,* 2007; Mazzucco *et al.,* 2019).

Although screening allows differentiation between the most important reasons for a problem, it does not provide information on the quantitative effect of the response, so optimisation is necessary (Goicochea, 2016). On the other hand, the winemaking practice involves many traditional techniques (Ribéreau-Gayon *et al.,* 2006) and therefore some of the relevant factors of biocontrol should not be modified whereas others are more or less modifiable (tunable). Consequently, a meticulous selection of factors based on the objectives to be achieved is necessary (Goicochea, 2016). To optimise the interactions studied in a short period of time, the Response Surface Methodology (RSM) and statistical designs such as Box–Behnken are presented as powerful tools in the response analysis and the reduction in the number of trials, improving the analysis times (Leardi, 2009; Candioti *et al.,* 2014; Maturano *et al.,* 2019).

Recently, our working group selected two biocontrol yeasts, *Wickerhamomyces anomalus* (BWa156) and *Metschnikowia pulcherrima* (BMp29), because of their ability to biocontrol important wine spoilage yeasts like *Zygosaccharomyces rouxii* and *Brettanomyces bruxellensis*, due to be competitive in the fermentation medium (compared with other non-*Saccharomyces* isolates), tolerant to different fermentation stress factors and add positive enological traits (Kuchen *et al.,* 2019).

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The same biocontrol yeasts have recently been reported as outstanding in biocontrol by Berbegal *et al.* (2019) and Nardi (2020). The present study has reduced the complexity from a populations' point of view; mixed cultures with interactions of two non-*Saccharomyces* populations (BWa156 *vs. Zygosaccharomyces rouxii "*BZr6" and BMp29 *vs.* BZr6) were carried out under controlled conditions in sterile grape musts. This was done so, to be able to focus on the influence of the many physicochemical factors of these interactions prior to inoculation of *Saccharomyces* sp. *Z. rouxii* was used because it is one of the main spoilage yeasts in the wine industry (Berbegal *et al.,* 2019), particularly in naturally sweet or artificially sweetened wines (Ribéreau-Gayon *et al.,* 2006, Alonso *et al.,* 2015). Sweet wines tend to have higher SO_2 concentrations (Ribéreau-Gayon *et al.,* 2006) and therefore a reduction in SO_2 could be more significant. Moreover, efficient methods to control *Z. rouxii* are particularly necessary because the yeast is highly tolerant of SO_2 (Alonso *et al.,* 2015).

Up to now, there are no studies regarding the effect of physicochemical factors of the fermentation medium and optimization over the interactions of antagonistic yeasts. The present study aimed to elucidate biocontrol mechanisms of two interactions of antagonistic yeasts and to establish optimal physicochemical conditions for biocontrol in genuine experimental wine, to project a reduction in SO_2.

MATERIALS AND METHODS

1. Yeast strains

The following antagonistic interactions were examined: *Wickerhamomyces anomalus "*BWa156" *vs. Zygosaccharomyces rouxii* "BZr6" and *Metschnikowia pulcherrima* "BMp29" *vs.* "BZr6". Yeasts strains had previously been used by our research group (Kuchen *et al.,* 2019) and were obtained from the Culture Collection of Autochthonous Microorganisms of the Institute of Biotechnology, School of Engineering, UNSJ, San Juan, Argentina. Isolates were identified through molecular methods (Esteve-Zarzoso *et al.,* 1999).

2. Culture media and microvinifications

The following culture media were used: YPD Broth (%): Yeast Extract 1, Peptone 2, Dextrose 2, pH: 4.5. YPD-agar: Yeast extract 1, Peptone 2, Dextrose 2, Agar 2, pH: 4.5. YMB Broth (%): Yeast extract 0.3, Malt extract 0.3, Peptone 0.5, Dextrose 1, NaCl 3, Glycerol 10 % (v/v) in 0.2 M citrate phosphate buffer pH: 4.5 (Santos *et al.,* 2009). YMB-agar (%): Yeast extract 0.3, Malt extract 0.3, Peptone 0.5, Dextrose 1, NaCl 3, Glycerol 10 % (v/v), Agar 2, Methylene blue 0.003 % (v/v) in 0.2 M citrate phosphate buffer pH: 4.5 (Santos *et al.,* 2009). WLN: Wallerstein Laboratory Nutrient (Oxoid, Hampshire, UK) differential medium (Pallmann *et al.,* 2001). Media were sterilised at 1 atm. for 20 min.

Pre-inocula of microvinifications were carried out in 250 mL Erlenmeyer flasks with 200 mL of grape must (21 °Brix; initial pH of 4), which was prepared from concentrated must (65 °Brix) and supplemented with 0.1 % yeast extract. Microvinifications were carried out in 250 mL Erlenmeyer flasks with 200 mL of concentrated grape must of 65 °Brix, which was diluted and adjusted according to the assay requirements proposed by the experimental design. Grape must media were sterilised at 0.5 atm. for 20 min.

3. Supernatants

3.1. Production

Pre-inocula of the biocontrol yeasts (BWa156 and BMp29) were developed in YMB broth using an orbital shaker (110 rpm) at 23 °C for 24 h. Each pre-inoculum (1×10^6 cells mL^{-1}) was seeded in 500 mL YMB broth at 23 °C and 110 rpm. Supernatants were collected after 72 h by centrifugation at 5000 rpm at 4 °C for 10 min. The supernatant was filtered through 0.45 µm polyethersulfone (PES) membrane filters (Pall Life Sciences, Ann Arbor, MI, USA) (Mehlomakulu *et al.,* 2014). Sterile YMB broth was used as control. Filtrates were lyophilised for 72 h and reconstituted at ×20 (concentrated 20 times) with YMB broth. Hereinafter these solutions are called "Supernatants", in particular, BWa156 supernatant is called: "S156" and BMp29 supernatant: "S29". Proteinase K was added to some BWa156 supernatants: "S156 + P" and BMp29 supernatants: "S29 + P" at a final concentration of 10 mg mL^{-1} at 25 °C during 48 h to evaluate a possible proteinaceous nature of the mechanism (Banjara *et al.,* 2016).

3.2. Inhibition nature

YPD broth was seeded with 1×10^5 cells mL^{-1} of BZr6 and 200 µL of this mixture were poured into each well of a microplate (96 wells). Sterile YPD broth was used as control. Fifty µL of each supernatant (BWa156 and BMp29) with proteinase K (S156 + P and S29 + P) and without (S156 and S29) were added to the microplate (n = 3).

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The microplate was then incubated at 23 °C and 110 rpm for 5 days (Banjara *et al.,* 2016). Cell density was measured by optical density (OD) at 620 nm with a microplate reader, and the OD was correlated to a cellular concentration through a calibration curve. Specific growth rate and adaptation time (lag phase) were calculated according to Pirt (1975). Samples were taken after 9, 13, 19, 24, 34, 38, 49, 57 and 65 h.

4. Mixed fermentations

Mixed fermentations were developed for screening, optimization of physicochemical factors and validation of the optimization. Fermentations were carried out under static conditions in 250 mL Erlenmeyer flasks with 200 mL grape must. Each assay was performed with mixed cultures containing one biocontrol strain and one spoilage strain. Different proportions of pre-inocula of each strain were used to inoculate a total of 2×10^6 cells mL^{-1} at the ratio required by the assay and proposed by the experimental design (Plackett–Burman or Box–Behnken) or validation points. During the screening, optimization and validation assays, physicochemical conditions were fixed at the start of the experiment (T = 0); results were obtained after 120 h of fermentation, except for optimization of BMp29 *vs.* BZr6 in which time was an assay variable, and validations when fermentation was monitored for 360 h. One hundred and twenty (120) hours was considered the time limit to inoculate *Saccharomyces cerevisiae*.

Experimental assays, the number of samples and repetitions were performed as suggested in the experimental design, except for validation (see below). Fermentation samples were plated onto WLN differential medium for cell counts at a dilution that allowed to observe between 50 and 250 colonies of both strains. In cases where the population differences did not allow a simultaneous count, the populations were counted from different dilutions from the same sample with the same objective (50 and 250 colonies). A daily review of the development of the colonies was made. In previous works, it was observed that the colonies of strains were differentiable by colour and development time (data not shown).

TABLE 1. Possible factors involved in antagonism

Factor	Source used	Relation to process	Justification	Reference
SO_2	$K_2O_5S_2$	Intrinsic manipulatable	Traditional practice to inhibit spoilage yeast	(Ribéreau-Gayon *et al.,* 2006)
Cl_3Fe	Cl_3Fe	Added manipulatable	Competence mechanism through Fe^{3+} uptake	(Oro *et al.,* 2014)
Agitation	Orbital agitator	Added manipulatable	Increase of oxygen and contact between molecules	(Károlyi *et al.,* 2005)
Reducing sugars	Grape must (65 °Brix)	Intrinsic non-manipulatable	Variable yeast osmotolerance	(Combina *et al.,* 2005)
pH	HCl and NaOH (1M)	Intrinsic manipulatable	General effect on enzymatic activities	(Kurtzman *et al.,* 2011)
Nitrogen	$(NH_4)_2HPO_4$	Added manipulatable	Variable requirements by yeasts	(Ciani *et al.,* 2016)
Ethanol	C_2H_5OH Absolute	Intrinsic non-manipulatable	Variable toxicity in yeasts	(Pretorius, 2000)
Inoculation Relation Biocontroller/ Spoilage yeast		Added manipulatable	Influence on competition capacity	(Ciani *et al.,* 2016)
Temperature		Intrinsic manipulatable	Influence on competition capacity	(Ciani *et al.,* 2016)
Glycerol	$C_3H_8O_3$	Added manipulatable	Decrease in protein inactivation	(Santos *et al.,* 2009)
Dummy			Pure error	(Goicochea, 2016)

Factors termed "manipulatable" means that their level can be modified if necessary during the fermentation process.

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A stereoscope was used for detailed counting if necessary. Moreover, other authors support morphological differences between the strains (Kurtzman *et al.,* 2011; Kántor *et al.,* 2015; Fu *et al.,* 2018).

4.1. Screening of relevant physicochemical biocontrol factors

Factors and sources to evaluate were considered from the supernatant analysis and literature related to interaction mechanisms and wine environment (Table 1). Factors termed "manipulatable" means that their levels can be modified if necessary, during the fermentation process.

The screening was performed through microvinifications as previously described in mixed fermentations (see above) assaying physicochemical conditions and levels within the interval given in Table 2, using the Plackett–Burman experimental design (Plackett and Burman, 1946) (Table 3). Molecular SO_2 was independent of the effect of pH with the Henderson–Hasselbalch formula (Waterhouse *et al.,* 2016).

Model construction and data analysis were carried out with software for statistical analysis (see below). Significant model construction with high R^2 was obtained through the selection of relevant factors from Pareto charts. This analysis shows relevant factors from a mathematical model construction for a better response explanation, but it does not give a quantitative value of the effect, and therefore optimization is necessary. Factors that were not selected for model construction explain the experimental error (Goicochea, 2016).

TABLE 2. Factor levels used for screening

		level	
Factor	Unit	+	-
Cl_3Fe	mg L^{-1}	20	0
Agitation		YES	NO
Nitrogen (YAN)	mg L^{-1}	300	200
Reducing sugars	°Brix	26	23
Ethanol	% v/v	5	0
B/S ratio		1	0.02
Temperature	°C	25	20
pH		4	3.5
Glycerol	g L^{-1}	5	0
Molecular SO_2	mg L^{-1}	0.6	0
Dummy			

B/S ratio: Inoculation ratio between Biocontroller and Spoilage yeast. Tested conditions in Supplementary data

4.2. Optimisation of relevant physicochemical biocontrol factors

Relevant factors were considered with the consulted literature; some relevant factors were selected to observe their influence on biocontrol whereas others were chosen to manipulate the biocontrol mechanism. The selection process was an integrated decision for each interaction in relation to biocontrol application.

Optimisation was obtained through microvinifications as previously described in mixed fermentations. The sources are the same as described in 4.1 "Screening of relevant physicochemical biocontrol factors" (Table 1).

TABLE 3. Plackett–Burman Matrix

Factor	1	2	3	4	5	6	7	8	9	10	11	
Experiment												Response
1	+	+	+	+	+	+	+	+	+	+	+	
2	−	+	−	+	+	+	−	−	−	+	−	
3	−	−	+	−	+	+	+	−	−	−	+	
4	+	−	−	+	−	+	+	+	−	−	−	
5	−	+	−	−	+	−	+	+	+	−	−	
6	−	−	+	−	−	+	−	+	+	+	−	
7	−	−	−	+	−	−	+	−	+	+	+	
8	+	−	−	−	+	−	−	+	−	+	+	
9	+	+	−	−	−	+	−	−	+	−	+	
10	+	+	+	−	−	−	+	−	−	+	−	
11	−	+	+	+	−	−	−	+	−	−	+	
12	+	−	+	+	+	−	−	−	+	−	−	
Factor effects												

Combination of levels of the factors in the experiments

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Physicochemical conditions in microvinifications varied according to the Box–Behnken experimental design (Fisher, 1935) (Figure 1), B/S ratio for BWa156 *vs.* BZr6 interaction was fixed at 0.51. Factor levels to create a mathematical model explaining the response curvature for each factor were as follows: minimum (–1), medium (0) and maximum (+1) (Figure 1 and Table 4). The matrix was also developed to explain interactions between factors. The Box–Behnken design was used because it is highly efficient: more results can be obtained carrying out fewer experiments (Goicochea, 2016).

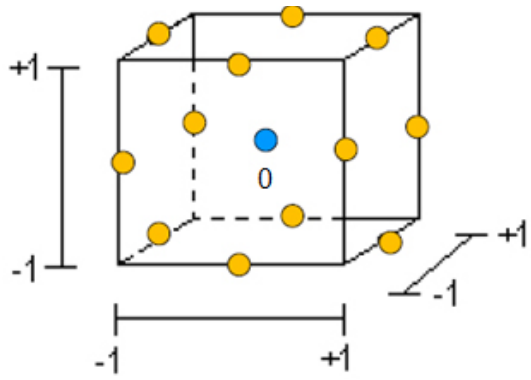


FIGURE 1. Box–Behnken experimental design

Experimental design and evaluation points of Box–Behnken design for 3 factors involved.

TABLE 4. Factor levels used for optimisation

Factor	Unit	Level		
		Minimum (–1)	Medium (0)	Maximum (+1)
BWa156 *vs.* BZr6				
Molecular SO_2	mg L^{-1}	0	2.5	5
Reducing sugars	°Brix	23	24.5	26
pH		3.2	3.7	4.2
BMp29 *vs.* BZr6				
Ethanol	% v/v	0	2.5	5
pH		3.2	3.7	4.2
Temperature	°C	17	21	25
Time	days	0	2.5	5
B/S ratio		0.02	0.51	1

Tested conditions in Supplementary data.
Fermentations' initial conditions.

Mathematical model construction and data analysis were performed using statistical analysis software (see below). Models were developed by manual elimination of insignificant factors to obtain 2nd order interaction models with a high R^2 and insignificant lack of fit (supplementary data). Errors were calculated through repetitions and software (Leardi, 2009) and results are displayed using "Response Surface Methodology" (Box and Wilson, 1951).

4.3. Validation of the response surface models

The validation conditions (validation points) were made as described in mixed fermentation apart and established according to the predictions of the response surface models (Table 5). The physicochemical conditions for the interaction between BWa156 and BZr6 were termed as "favourable", "intermediate" and "unfavourable" after the response surface results and consulting the literature in the optimization section. In the case of BMp29 *vs.* BZr6, model prediction points for BZr6 = 0 cells mL^{-1} within the design space used during optimization were not found. Therefore, the model obtained for BZr6 during optimization was used to predict BZr6 = 0 cells mL^{-1} allowing the B/S ratio (which is not a physicochemical factor for wine) to vary while the remaining physicochemical factors were fixed as favourable, intermediate and unfavourable conditions after consulting the literature during optimization. Controls of both interactions were obtained with single-strain cultures inoculated at 1×10^6 cells mL^{-1}. The population at each sampling time was monitored for 15 days (n = 3). Sampling times (h) for BWa156 *vs.* BZr6: 0, 10, 20, 32, 60, 69, 81, 93, 129, 216 and 360, and for BMp29 *vs.* BZr6: 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 216 and 360. Sampling times for controls: 0, 24, 60, 72, 132, 216 and 360 h. Empirical models were adjusted to validation data.

5. Statistical analysis

Data and standard error (SE) from spoilage yeast growth kinetics and their interactions with supernatants were statistically analysed with ANOVA and Fisher's LSD test when parametric data and Kruskal–Wallis when not, using InfoStat/P 2017. Design-Expert™ 7.0.0 Stat-Ease Inc. was employed to analyse the effect of the physicochemical factors in the screening through Pareto charts, to optimise the relevant factors through response surface methodology and to analyse data and errors obtained. The data points obtained in the validation were used to fit the kinetic curves of empirical pre-established models with Sigma-Plot™ 10.

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TABLE 5. Validations points within the design space

Factor	Unit	Favourable	Intermediate	Unfavourable
		BWa156 *vs.* BZr6		
SO_2	mg L^{-1}	0	0	0
Reducing sugars	°Brix	23	26	24.5
pH		3.7	4.2	4
		BMp29 *vs.* BZr6		
Ethanol	% (v/v)	0	2.5	2.5
pH		3.2	4	3.7
Temperature	°C	17	20	20
	days	5	5	5
Biocontroller/ Spoilage Yeast Relation		1.93	5.07	5.25

Fermentations' initial conditions

TABLE 6. Statistical analysis of kinetic parameters of BZr6 after supernatants treatments

	Lag phase			Specific growth rate		
Strain + Supernatant	Average (h)	Standard Error	*	Average (h^{-1})	Ranks	*
BZr6	18.29	1.34	A	0.07	10.5	AB
BZr6 + S 156 + P	21.97	1.52	AB	0.08	15	B
BZr6 + S 29 + P	22.35	1.52	AB	0.06	4.67	A
BZr6 + S 29	22.4	1.52	AB	0.06	5.67	A
BZr6 + S 156	24.91	1.52	B	0.07	6	A

Values with different letters are significantly different. The lag phase was evaluated with ANOVA (parametric data) and Fisher's test and Specific growth rate (non-parametric) with Kruskal–Wallis.

RESULTS

1. Supernatant inhibition

Growth kinetics of the individual spoilage yeast *Zygosaccharomyces rouxii* «BZr6» (Control) and in mixed cultures (treatments) with the two biocontrollers (BWa156 and BMp29) are shown in Figure 2. Lag phase and specific growth rate were analysed statistically (Table 6).

Lag phase analysis showed a significant difference between the acclimatization time of individual BZr6 (control) and treatment with S 156 (BZr6 + S 156), indicating inhibition activity by the BWa156 supernatant. However, BZr6 + S 156 + P (after Proteinase K action) did not show a significant difference compared with the control, suggesting a possible inhibition through protein action. BZr6 + S BMp29 and BZr6 + S BMp29 + P did not show significant differences, which means that the BMp29 supernatant did not affect BZr6. The results did not demonstrate any effect of the supernatants on the specific growth rates of BZr6 compared with the control.

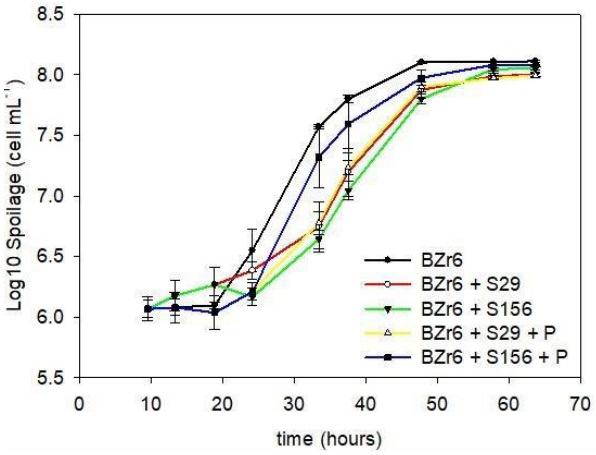


FIGURE 2. Growth kinetics of the individual spoilage yeast BZr6 and together with biocontroller supernatants

BZr6 (Control) and treatments: BZr6 + S 29 (BZr6 with BMp29 supernatant); BZr6 + S 29 + P (BZr6 with BMp29 supernatant previously treated with proteinase K); BZr6 + S 156 (BZr6 with BWa156 supernatant); BZr6 + S 156 + P (BZr6 with BWa156 supernatant previously treated with proteinase K).

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2. Mixed fermentations

2.1. Screening of relevant physicochemical biocontrol factors

2.1.1. BWa156 *vs.* BZr6 interaction

Results obtained with mixed fermentations of BWa156 and BZr6 were statistically analysed and used to construct significant models with a high R^2 (BWa156: 0.9, BZr6: 0.99), including relevant factors for each strain in the interaction (supplementary data).

In the Pareto charts given in Figure 3, it can be observed that only one factor significantly affected both yeasts (exceeding the Bonferroni limit in both cases): molecular SO_2. Four factors, Cl_3Fe, agitation, reducing sugars and pH, probably affected the yeasts (exceeding the *t* limit). An increase in SO_2 negatively affected both strains. An increase in Cl_3Fe positively affected the spoilage yeast, BZr6, and negatively BWa156, the biocontrol yeast. Agitation negatively affected BWa156, while reducing sugars positively affected BZr6, and BWa156 was positively influenced by an increase in pH.

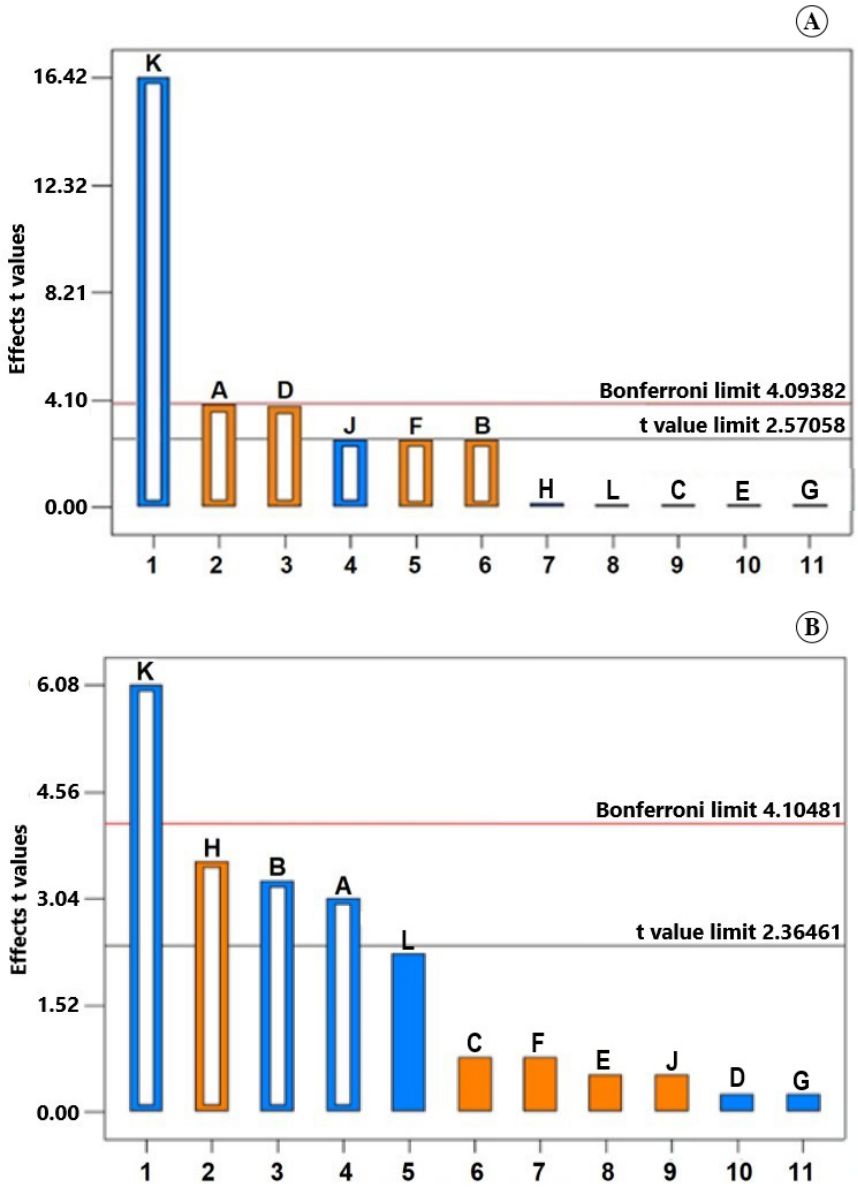


FIGURE 3. Screening for relevant factors: BWa156 *vs.* BZr6

Pareto charts: a: BZr6. b: BWa156. Factors involved: A: Cl_3Fe; B: Agitation; C: Nitrogen (FAN); D: Reducing sugars; E: Ethanol; F: Biocontroller/Spoilage yeast Relation; G: Temperature; H: pH; J: Glycerol; K: Molecular SO_2; L: Dummy. Bars with white core: Factors chosen for the model. Positive effects: Orange, Negative effects: Blue. Bars above the Bonferroni limit: Significant factors. Bars above the t value limit: Factors of probable significance.

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2.1.2. BMp29 *vs.* BZr6 interaction

Similarly, results from the BMp29 *vs.* BZr6 interaction were analysed for model construction. The results (BZr6 R^2: 0.96, BMp29 R^2: 0.98) are shown in Figure 4.

Figure 4 shows 2 factors, ethanol and glycerol, that significantly affected both the spoilage yeast (BZr6; positively) and the biocontroller (BWa156; negatively). Temperature and Cl_3Fe probably negatively affected the biocontrol yeast, whereas the B/S ratio was probably positive. On the other hand, the spoilage yeast was possibly positively affected by ethanol, glycerol, temperature and Cl_3Fe and negatively by the pH.

2.2. Optimisation of relevant physicochemical biocontrol factors

The selection criteria for the optimisation assays are summarised in Table 6.

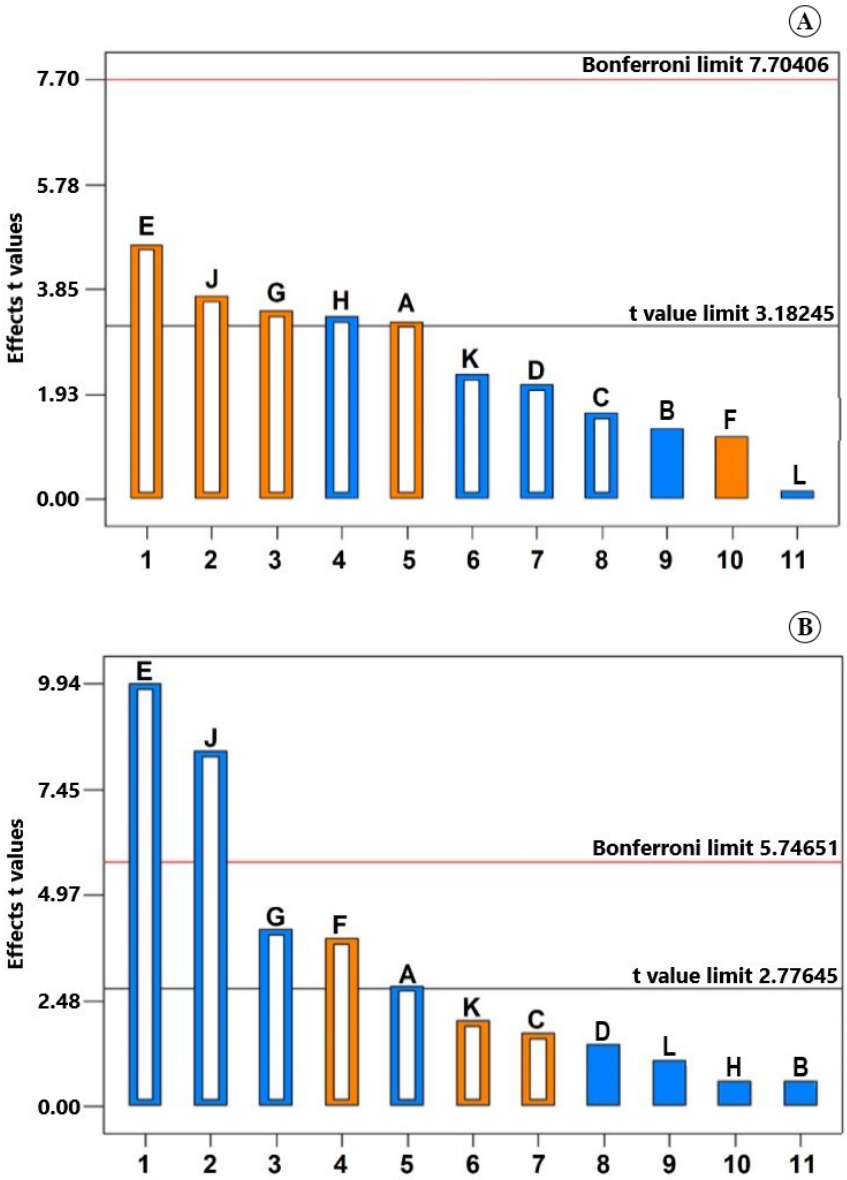


FIGURE 4. Screening for relevant factors: BMp29 *vs.* BZr6

Pareto charts: a: BZr6. b: BMp29. Factors involved: A: Cl3Fe; B: Agitation; C: Nitrogen (FAN); D: Reducing sugars; E: Ethanol; F: Biocontroller/Spoilage yeast Relation; G: Temperature; H: pH; J: Glycerol; K: Molecular SO2; L: Dummy. Bars with white core: Factors chosen for the model. Positive effects: Orange, Negative effects: Blue. Bars above the Bonferroni limit: Significant factors. Bars above the t value limit: Factors of probable significance.

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TABLE 7. Selection of factors to be optimised

		Interaction: BWa156 *vs.* BZr6				Interaction: BMp29 *vs.* BZr6			
Factor	Type	BWa156	BZr6	Biocontrol	Use	BWa156	BZr6	Biocontrol	Use
Cl_3Fe	Added manipulatable	Significant	Significant	Negative	NO	Significant	Significant	Negative	NO
Agitation	Added manipulatable	Significant		Negative	NO				NO
Nitrogen (YAN)	Added manipulatable				NO				NO
Reducing sugars	Intrinsic non-manipulatable		Significant	Negative	YES				NO
Ethanol	Intrinsic non-manipulatable				NO	Significant	Significant	Negative	YES
B/S ratio	Added manipulatable				NO	Significant		Positive	YES
Temperature	Intrinsic manipulatable				NO	Significant	Significant	Negative	YES
pH	Intrinsic manipulatable	Significant		Positive	YES		Significant	Positive	YES
Glycerol	Added manipulatable					Significant	Significant	Negative	NO
Molecular SO_2	Intrinsic manipulatable	Significant	Significant	Positive	YES				NO

2.2.1. BWa156 *vs.* BZr6 interaction

Results obtained with mixed fermentations of BWa156 and BZr6 were statistically analysed and used to construct significant models (Supplementary data) without a significant lack of fit and with a high R^2 (BZr6: 0.89, BWa156: 0.99). Optimisation of two factors (pH and reducing sugar concentration) involved in the interaction and at given SO_2 concentrations (0, 0.25 and 0.5 mg mL^{-1}) is shown in Figure 5.

At an SO_2 concentration of 0 mg mL^{-1}, viability of the spoilage yeast reached its maximum at intermediate reducing sugar concentrations assayed, but it decreased at higher sugar concentrations. The model shows beneficial conditions for the biocontrol yeast at lower reducing sugar concentrations and at medium to high pH. At SO_2 = 0.5 mg mL^{-1} and at higher pH values biocontrol was almost total (BZr6 = 0 cells mL^{-1}) and comprised all reducing sugar concentrations assayed. Biocontroller response surfaces for BWa156 (Figure 5b) demonstrated a model only affected by reducing sugars and not by the remaining factors.

2.2.2. BMp29 *vs.* BZr6 interaction

Optimization results with mixed fermentations of BMp29 and BZr6 and their statistical analysis were also developed as shown in supplementary data. The corresponding models were significant, without a significant lack of fit and with high R^2 (BZr6: 0.77 and BMp29: 0.93). Optimisation of several factors (ethanol, B/S ratio, temperature and time) involved in the interaction is given in Figure 6.

The most representative combinations of factors to describe the interaction are given in Figure 6. The yeast populations did not show complete biocontrol (BZr6 = 0) during the experimental assays. Populations varied from 1×10^6 cells mL^{-1} to 1×10^7 cells mL^{-1}.

2.3. Validation of the response surface models

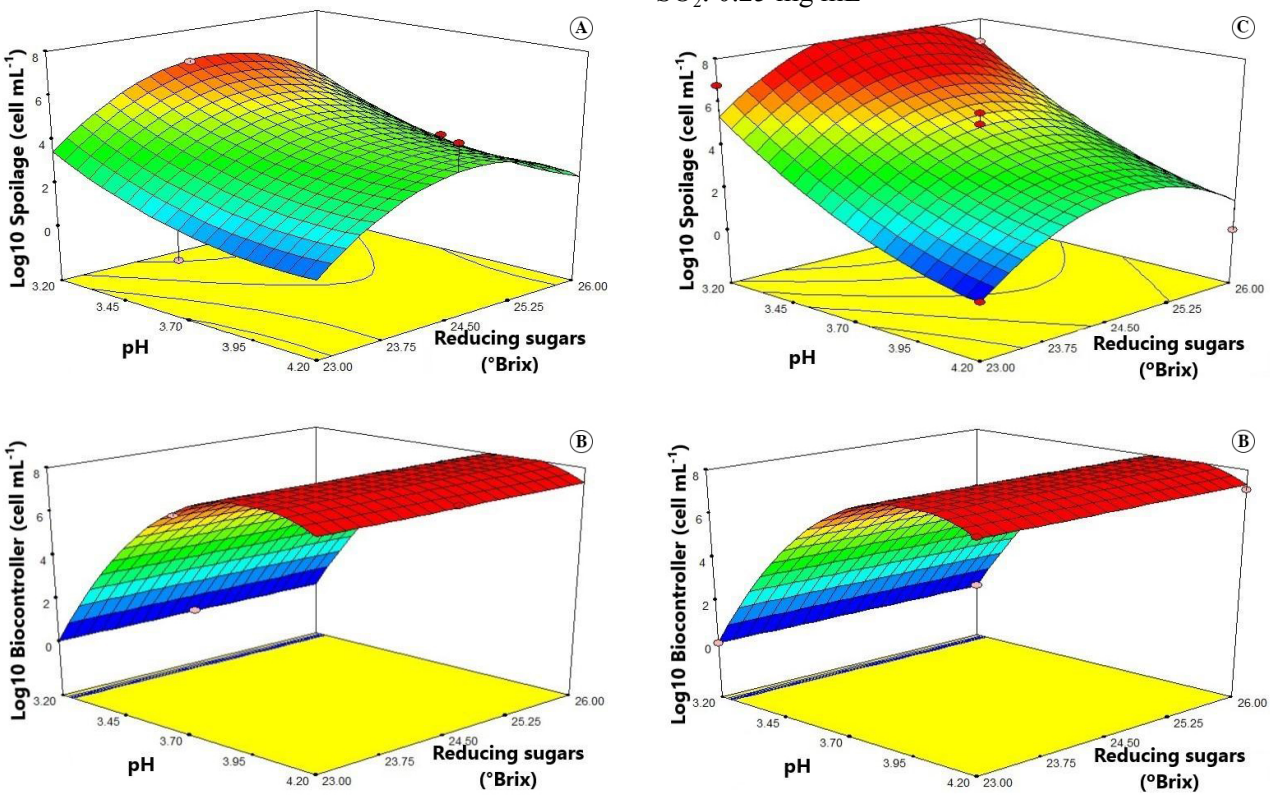
2.3.1. BWa156 *vs.* BZr6 interaction

"Favourable" conditions for BZr6 growth kinetics (Figure 7a) presented a peak (Gaussian peak) at the beginning, reaching a maximum population of 8.9×10^6 cells mL^{-1}, which was lower than that observed during the treatments. After reaching its maximum the population declined until it stabilised at 1.1×10^6 cells mL^{-1}.

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(1) SO2: 0 mg mL-1

SO_2: 0.25 mg mL^{-1}



(3) SO_2: 0.5 mg mL^{-1}

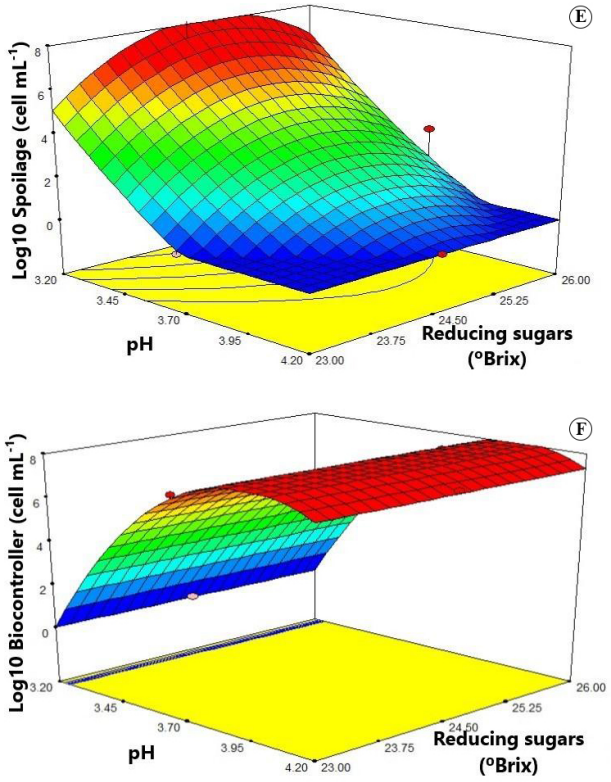


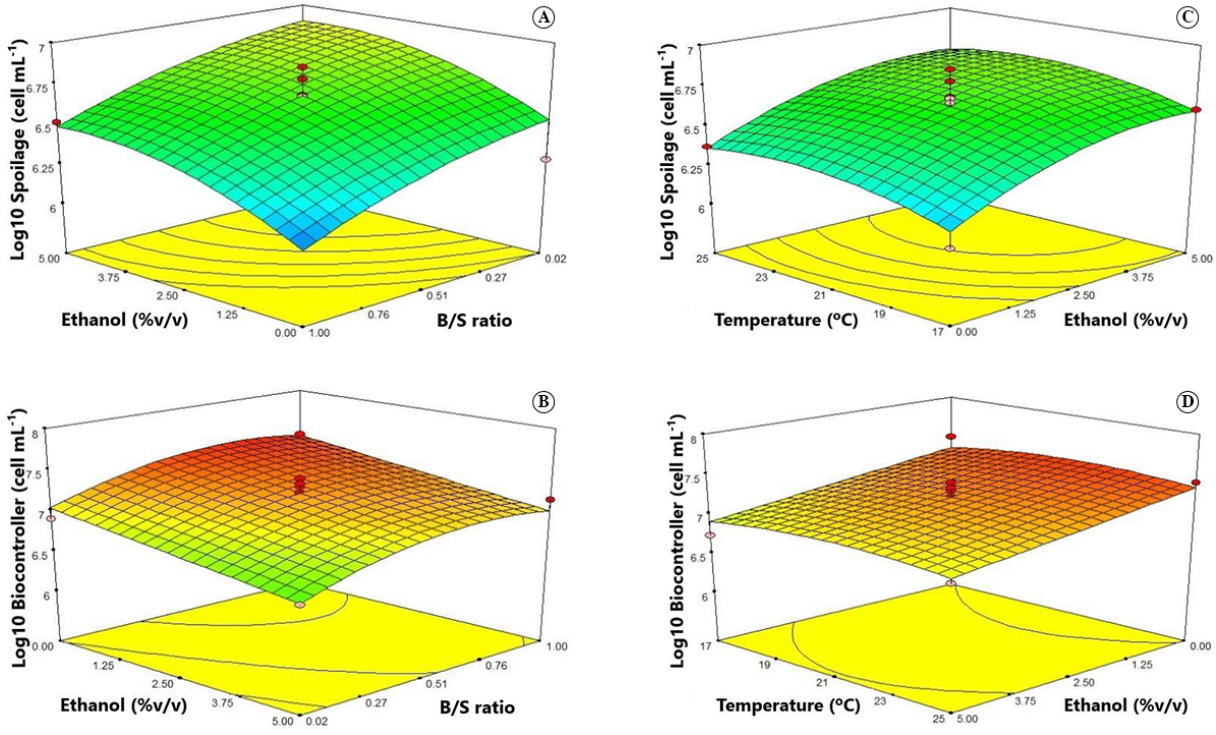
FIGURE 5. Factor optimisation for BWa156 *vs.* BZr6 interaction.

a, c, e: Spoilage yeast (BZr6), b, d, f: Biocontrol yeast (BWa156). Axes: pH and reducing sugar concentration.
1: molecular SO_2: 0 mg mL^{-1}, 2: molecular SO_2: 0.25 mg mL^{-1} and 3: molecular SO_2: 0.5 mg mL^{-1}

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(1) pH: 3.7; Temperature: 21 °C; Time: 2.5 days

(2) pH: 3.7; Time: 2.5 days;
Biocontroller/Spoilage yeast Relation: 0.51



(3) pH: 3.7; Ethanol: 2.5 % (v/v); Temperature: 21 °C

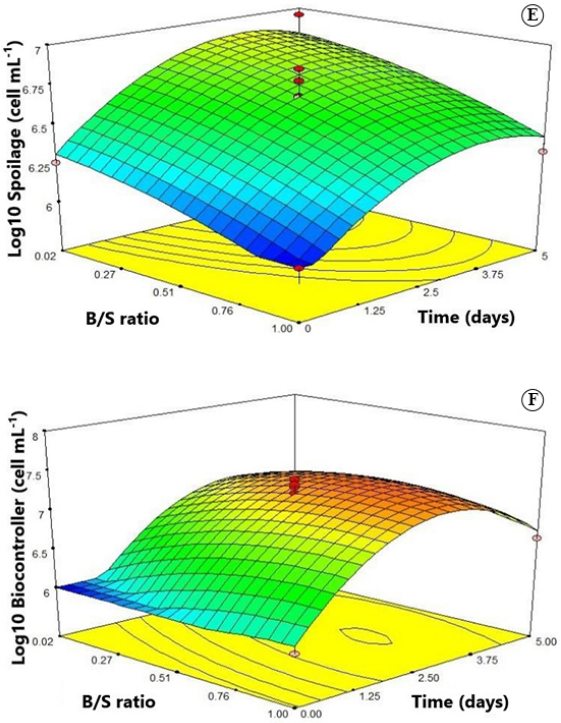


FIGURE 6. Factor optimisation for BMp29 *vs.* BZr6 interaction.

a, c, e: Spoilage yeast (BZr6); b, d, f: Biocontrol yeast (BMp29). 1. Fixed parameters: pH: 3.7; Temperature: 21 °C; Time: 2.5 days, axes: Ethanol and B/S ratio. 2. Fixed parameters: pH: 3.7; Time: 2.5 days; B/S ratio: 0.51, axes: Temperature and Ethanol. 3. Fixed parameters: pH: 3.7; Ethanol: 2.5 % (v/v); Temperature: 21 °C, axes: B/S ratio and Time

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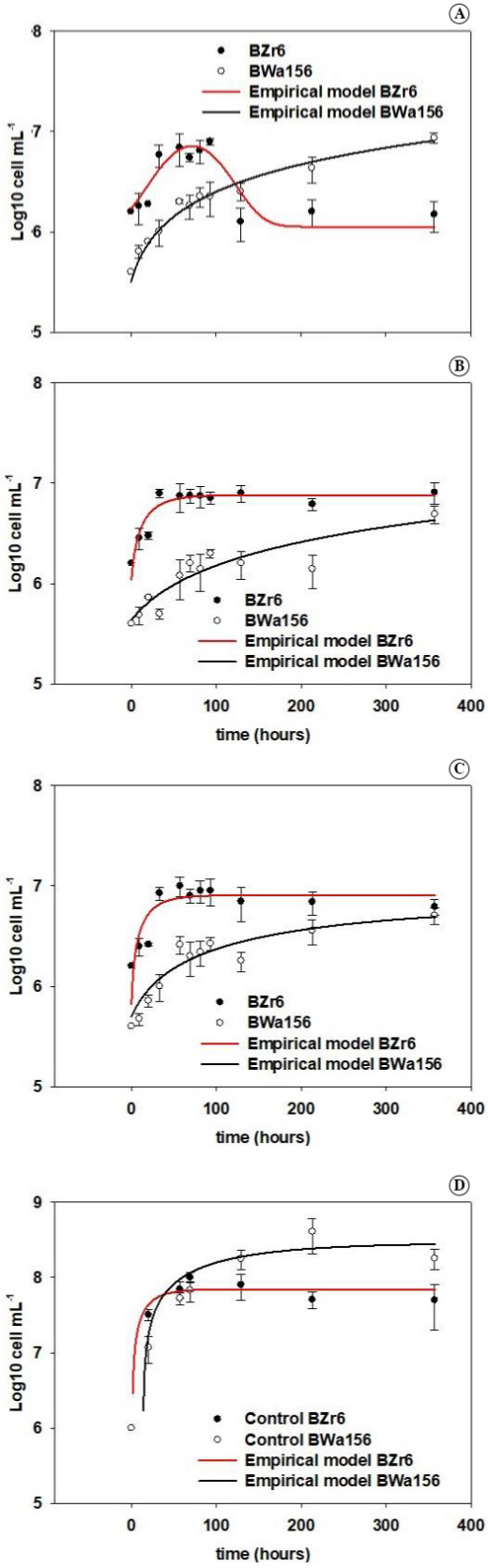


FIGURE 7. Validation of the response surface models of BWa156 *vs.* BZr6

Different initial culture conditions were assayed and empirical models adjusted. Initial conditions: a - Favourable (SO_2: 0 mg mL^{-1}; pH: 3.7; Reducing sugars: 23 ° Brix); b - Intermediate (SO_2: 0 mg mL^{-1}; pH: 4.2; Reducing sugars: 26 ° Brix); c - Unfavourable (SO_2: 0 mg mL^{-1}; pH: 4.2; Reducing sugars: 24.5 ° Brix); d - BWa156 and BZr6 controls under the different conditions assayed.

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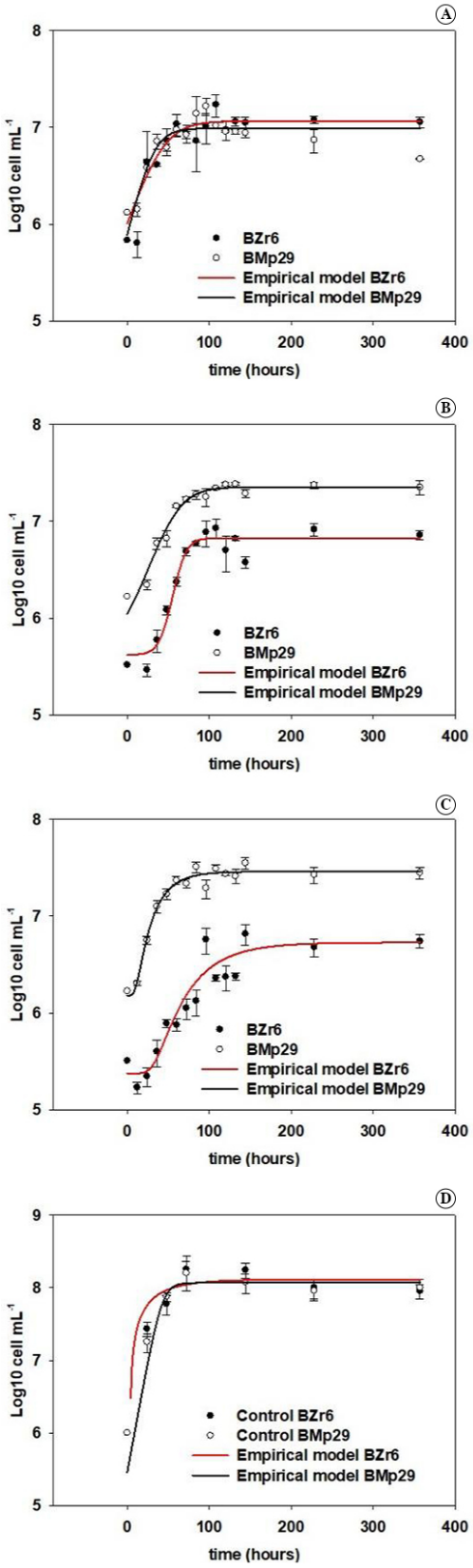


FIGURE 8. Validation of the response surface models of BMp29 *vs.* BZr6

Different initial culture conditions were assayed and empirical models adjusted. Initial conditions: a - Favourable (Ethanol: 0 % (v/v), pH: 3.2, Temperature: 17 °C, Time: 5 days, B/S ratio: 1.93). b - Intermediate (Ethanol: 2.5 % (v/v), pH: 4, Temperature: 20 °C, Time: 5 days, B/S ratio: 5.07). c - Unfavourable (Ethanol: 2.5 % (v/v), pH: 3.7, Temperature: 20 °C, Time: 5 days, B/S ratio: 5.25). d - BMp29 and BZr6 controls under the different conditions assayed.

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The growth kinetic of BWa156 demonstrated exponential growth until its maximum, which was higher than observed under the other conditions (intermediate and unfavourable).

Under intermediate and unfavourable conditions, the growth kinetics of BZr6 were similar (exponential growth until maximum). The strain demonstrated a steeper slope during the exponential phase and reached a higher maximum population than the biocontroller, dominating the fermentation throughout the experiment. On the other hand, BWa156 registered steeper slopes (exponential growth until maximum) under unfavourable conditions compared with intermediate conditions. Maximum populations of BZr6 under the assay conditions were as follows: favourable: 1.1×10^6 cells mL^{-1}, intermediate: 7.6×10^6 cells mL^{-1} and unfavourable: 8×10^6 cells mL^{-1}. Total population levels were around 1.1×10^7 cells mL^{-1}.

Under the different physicochemical conditions studied, controls of both strains showed similar growth kinetics (exponential growth until maximum) and maximum populations. Therefore, was adjusted one curve for each strain BWa156 and BZr6 (Figure 7d). The maximum population for BZr6 was 8.9×10^7 cells mL^{-1} and for BWa156 2×10^8 cells mL^{-1}.

2.3.2. BMp29 *vs.* BZr6 interaction

Under favourable conditions, growth kinetics (Figure 8a) demonstrated a similar behaviour for the spoilage yeast and the biocontrol population (exponential growth until maximum). Under intermediate and unfavourable conditions it was observed that the BZr6 population registered a longer lag phase, reaching an inferior total population than BMp29, which dominated the fermentation; both strains developed sigmoidal curves. BZr6 reached approximately 1.1×10^7 cells mL^{-1} (favourable conditions), 6.7×10^6 cells mL^{-1} (intermediate) and 5.3×10^6 cells mL^{-1} (unfavourable). Total populations of both strains reached 2×10^7 cells mL^{-1} under favourable conditions, 2.8×10^7 cells mL^{-1} under intermediate conditions and 3.2×10^7 cells mL^{-1} under unfavourable conditions.

Controls of both strains showed similar behaviour (exponential growth until maximum) and maximum populations under the different conditions evaluated and therefore was adjusted one curve for each strain (Figure 8). The maximum population for BZr6 was 1×10^8 cells mL^{-1} and for BMp29 1.2×10^8 cells mL^{-1}.

DISCUSSION

1. Biocontrol capacity of culture supernatants

Supernatant from *W. anomalus* (S156) produced significant changes in BZr6 kinetics (during the lag phase) while the supernatants from *M. pulcherrima* (S29) not. Moreover, when the BWa156 supernatant was treated with Proteinase K (S156 + P), the inhibitory effect of the supernatant was neutralised. This allows us to infer that the inhibition was related to a protein, probably a killer protein (Mehlomakulu *et al.,* 2014; Banjara *et al.,* 2016). This hypothesis is reinforced through the expression of three protein or killer toxins by *W. anomalus* as previously mentioned by Liu *et al.* (2013).

2. Screening of mixed cultures

Screening allowed a faster and more in-depth analysis of the interaction mechanisms with both yeast cultures present in the fermentation medium and the relevance of each factor in the success of the biocontrol treatment. Regarding the BWa156 *vs.* BZr6 interaction, Cl_3Fe probably played an important role in exerting a positive effect on *Z. rouxii* and a negative on *W. anomalus*. On the other hand, agitation was found to have a possible negative effect on *W. anomalus*, and hence it was hypothesised than iron and agitation could be involved in oxygen contact and cell respiration and *Z. Rouxii* is probably more efficient in oxygen utilization (Montes *et al.,* 1999; Hagman *et al.,* 2013; Krause *et al.,* 2018; Bizzarri *et al.,* 2019). In the present study, it was decided not to optimise Cl_3Fe and agitation, because these factors negatively affected *W. anomalus*. The increase in molecular SO_2 was also significant and negatively affected *W. anomalus* and *Z. rouxii* populations. This is a very important factor because it is traditionally used for spoilage yeasts control, among others, in the wine industry (Ribéreau-Gayon *et al.,* 2006) and could be used in conjunction with biocontrol, also most non-*Saccharomyces* yeasts are more sensitive to SO_2 than *Saccharomyces* sp. (Jolly *et al.,* 2006). Moreover, as SO_2 is a manipulatable factor (its concentration can be adjusted), it was decided to optimise this compound. An increase in reducing sugar concentration positively affected the spoilage yeast (BZr6). This is in accordance with the high osmotic tolerance observed for *Zygosaccharomyces* sp. (Leandro *et al.,* 2011; Stratford *et al.,* 2013). The sugar concentration in must is an intrinsic factor and in high-quality grape must it should not be modified using currently available methods (Ribéreau-Gayon *et al.,* 2006).

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Therefore, it was included in the optimization to examine its response. During the screening assays the pH probably positively affected the biocontrol yeast. Comitini *et al.* (2004) reported optimal activity of the *W. anomalus* killer toxin within a pH range between 4.2 and 4.4. Because this factor is variable in grape must but manipulatable through organic acids (Ribéreau-Gayon *et al.,* 2006), it was also included in the optimisation assays.

In the second interaction between BMp29 and BZr6, biocontrol would not be related to extracellular molecules (see the previous section). Hence, the inhibitory mechanism observed for BMp29 with plate assays in previous studies (Kuchen *et al.,* 2019) is most likely related to a method that involves the presence of both yeasts. During the screening of the BMp29 *vs.* BZr6 interaction, ethanol benefited the spoilage yeast compared with the biocontroller. This result can be explained by the high ethanol tolerance previously observed for *Zygosaccharomyces* sp. (Stratford *et al.,* 2013; Alonso *et al.,* 2015) in contrast with the low tolerance found for *M. pulcherrima* (Oro *et al.,* 2014). Because during early fermentation stages considerable alcohol concentrations (for most non-*Saccharomyces*) can occur as a result of complex yeast populations, ethanol was included in the optimization assays. Hypothetically, an increase in temperature would benefit BZr6 more than BWa156. Among environmental factors, temperature plays an important role in yeast interactions and the dominance of the fermentation medium. However, it is difficult to predict its influence because of the complexity of the mechanisms like membrane fluidity and enzyme reaction energy involved (Manayay and Ibarz, 2010; Ciani *et al.,* 2016). Consequently, this factor was also included in the optimisation experiments. An increase in glycerol concentration favoured the spoilage yeast compared with the biocontroller population. Casas (1999) described the ability of *Zygosaccharomyces* sp. to incorporate glycerol from the medium into the intracellular environment and, as a result, to increase cell osmotolerance, which favoured the competitive abilities of the spoilage yeast. Because Cl_3Fe supplemented and negatively affected the biocontrol, it was decided not to optimise this factor. Probably it affected both yeast populations and benefited the spoilage yeast. In contrast to the BWa156 *vs.* BZr6 interaction, agitation was not significant during the BMp29 *vs.* BZr6 interaction. The results suggest that oxygen would not affect the interaction, and the influence registered after the addition of Cl_3Fe could be linked to the biocontrol mechanism by *M. pulcherrima*, resource exploitation by iron consumption, already mentioned by different authors (Oro *et al.,* 2014; Sipiczki, 2020). A high pH possibly had a negative impact on the spoilage yeast (BZr6). This behaviour could be influenced by the already mentioned high tolerance of *Z. rouxii* to low pH values and optimum growth of *M. pulcherrima* at higher pH values (Martorell *et al.,* 2007, Stratford *et al.,* 2013, Wang *et al.,* 2016). The pH is an intrinsic factor in the wine industry, which is relatively easy manipulatable, affect the organoleptic quality and yeast fitness, and has shown a positive effect on biocontrol, and therefore it was decided to optimise the pH too. An increase in the inoculation ratio (Biocontroller/Spoilage yeast relation—B/S ratio) was the only factor that positively affected the Biocontroller (BMp29). This methodology of increasing inoculation ratio has been used by several authors to increase the persistence and influence of certain interesting yeast strains (Comitini *et al.,* 2011; Domizio *et al.,* 2011; Maturano *et al.,* 2019). Because the inoculation ratio is manipulatable and positive for biocontrol, it was also optimised.

3. Optimisation of mixed cultures

Optimisation enables one to find the most suitable physicochemical combination linked to the biocontrol mechanism in each interaction, to biocontrol spoilage yeasts in a genuine medium (must fermentation with mixed cultures). Winemaking involves several traditional practices (Ribéreau-Gayon *et al.,* 2006), and certain factors relevant to the success of biocontrol cannot be modified whereas others can be changed to a certain extent (manipulatable). Therefore, during the optimisation process, a combination of non-manipulatable (only observable) and manipulatable factors were selected to meet the objectives within the wine space design.

RSM of a mixed fermentation of BWa156 and BZr6 showed that an increase in SO_2 negatively affected *Z. rouxii* while *W. anomalus* remained unaffected (Figure 5), which is in contrast to the high SO_2 tolerance observed for *Zygosaccharomyces* sp. under individual culture conditions (Stratford *et al.,* 2013, Torres Palazzolo, 2015). Nonetheless, it should be noted that tolerance to SO_2 has been cited as strain-dependent (Gutiérrez *et al.,* 2015). The SO_2 effect in the present study was stronger at a lower pH range (3.7–4.2) when BWa156 reached higher populations, which could be attributed

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to the competence method involved. Because SO_2 tolerance is linked to efflux mechanisms of this compound (Park *et al.,* 1999) and because killer toxins can introduce permeability and functionality changes to the cell membrane (Liu *et al.,* 2013), hypothetically, SO_2 transport can be affected resulting in a negative influence on the cell fitness. The pH factor was highly significant, and *Z. rouxii* reached higher populations at a lower pH. A possible explanation for these results may be the acidophilic character for the genus *Zygosaccharomyces* (Martorell *et al.,* 2007). Furthermore, this was the only significant factor for BWa156, and an increasing pH positively affected the strain. This could be linked to the efficiency of killer toxins at optimum pH: 4.2-4.4 (Comitini *et al.,* 2004; Liu *et al.,* 2013). An increasing reducing sugar concentration displayed a larger BZr6 population, which agrees with Leandro *et al.* (2011) and Stratford *et al.* (2013). Osmotolerance of *Zygosaccharomyces* is generally higher in interactions with lower pH. It should be noted that an increase in sugar concentration within the range assayed, did not seem to affect the BWa156 population.

Regarding BMp29 *vs.* BZr6, an increase in both temperature and ethanol had a positive effect on BZr6 and a negative effect on BMp29. It has been mentioned that ethanol is related to temperature. For example, at 35 ºC ethanol accumulation inside *S. cerevisiae* cells augmented because the membrane fluidity increased and consequently its functionality decreased (Ciani *et al.,* 2016). This fact could be quite significant because the non-*Saccharomyces* species used in the present study are less tolerant to ethanol (Combina *et al.,* 2005, Kuchen *et al.,* 2019). Moreover, like previously discussed in the screening procedure, Oro *et al.* (2014) and Alonso *et al.* (2015) demonstrated that *Z. rouxii* is ethanol tolerant in contrast to *M. pulcherrima*, which is in agreement with our results. The inoculation ratio (Biocontroller/Spoilage yeast) had a higher impact on the interaction compared with the remaining factors assayed. BZr6 developed a peak at a low B/S ratio, whereas BMp29 developed maximum growth at a higher B/S ratio. Furthermore, the B/S ratio affected the dominance of the strains in the medium and their time of persistence, similar results have been obtained by Domizio *et al.* (2011) and Maturano *et al.* (2019) despite having different objectives. A population of BZr6 = 0 cells mL^{-1} was not detected throughout the study, even though the model predicted this desired response at a B/S ratio beyond the assay range. Therefore, validation of the B/S ratio was carried out differently, according to the methodology developed by Kostić *et al.* (2015). Besides, this factor is no intrinsic property of the fermentation but it is added, so the model was still representative of the desired objectives.

As can be observed in RSMs, biocontrol can be effective or not depending on the different physicochemical conditions. In the interference competition, the interaction greatly varies with the toxin efficiency, which depends on 1 - Toxin production: cell viability and multiplication of the biocontroller (Pommier *et al.,* 2005; Sinclair, 2014). 2 - Stability: narrow pH and temperature range (Comitini *et al.,* 2004). 3 - Toxin's adherence: receptors and sensibility, distances and cell densities, and other abiotic conditions (Károlyi *et al.,* 2005; Boynton, 2019). On the other hand, in the resource exploitation competition, physicochemical factors such as pH, temperature, nitrogen and molecular SO_2 can affect the competitiveness of yeast species to take advantage of the resources (Ciani *et al.,* 2016). The change in physicochemical factors throughout the fermentation should also be considered (Combina *et al.,* 2005; Ribéreau-Gayon *et al.,* 2006; Jolly *et al.,* 2006). General analysis of the data suggests that, in terms of substrate competition, several of the factors assayed were involved at different proportions with respect to the biocontrol progress, whereas in biocontrol directed by competition with toxin interference, fewer factors modulated biocontrol, but each of them contributed at a higher proportion.

4. Validation of the optimisation

Response surface models for the *W. anomalus* (BWa156) *vs. Z. rouxii* (BZr6) interaction were validated by establishing SO_2 at 0 mg mL^{-1} to achieve the main objective, a reduction in SO_2. Predictions of the response surface where BZr6 was observed at a minimum, intermediate and maximum population were defined as favourable, intermediate and unfavourable validation conditions, respectively. The results observed in the interference competition between BWa156 and BZr6 showed that different kinetic behaviours had been developed. In the «favourable» condition, the biocontroller dominated the medium after the spoilage population BZr6 declined; the spoilage yeast population showed a peak whereas the biocontroller, BWa156, grew exponentially. Under "intermediate" and "unfavourable" conditions the biocontrol and the

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spoilage population grew exponentially until they reached their maximum, and BZr6 dominated under both conditions. Several researchers have studied the kinetics of interference competitions with diverse results (Musmanno *et al.,* 1999; Vadasz *et al.,* 2003; Ramon-Portugal *et al.,* 1997; Pommier *et al.,* 2005) Consequently with these previous works, it can be assumed that dominance of the biocontroller in the medium implies biocontrol of the spoilage population. Pommier *et al.* (2005) found similar results to those described under the "favourable" condition of the BWa156 *vs.* BZr6 interaction, the only kinetic of this interaction were biocontrol occurs. A remarkable fact was that during the "favourable" condition of the BWa156 *vs.* BZr6 interaction a fraction of the spoilage yeast population remained stable and constant after its population decline by the biocontrol. Several authors describe the yeast coexistence (killer/sensible) and the emission of killer toxins as a predation mechanism to release nutrients into the medium (Wloch-Salamon *et al.,* 2008). On the other hand, it is also possible that certain inhibition factors for one or both yeasts generate an equilibrium between toxin release (biocontroller yeast multiplication) and toxin's adherence to spoilage yeast, influencing spoilage yeast multiplication (Pommier *et al.,* 2005, Sinclair, 2014). Other authors hypothesised about toxin consumption by sensitive yeast and the distance through the existence of patches (Czárán and Hoekstra, 2003; Sinclair, 2014). This can be explained through the absence of agitation in the fermentation medium (Károlyi *et al.,* 2005). The calculated validations responded to kinetics following the "favourable" predictions with more biocontrol activity, whereas during "intermediate" and "unfavourable" conditions, with lower biocontrol activity, the predicted values were not observed. This is possibly related to the substantial reduction in experimental data for the response surface, which could underestimate or overestimate the yeast population (Goicochea, 2016).

The results observed in the interaction between BMp29 and BZr6 generated sigmoidal curves with similar behaviour under the different assay conditions. However, dominance and total populations in the medium under "intermediate" and "unfavourable" conditions were different, when the biocontrol population prevailed. There exist no reports on the kinetics of biocontrol interactions between two non-*Saccharomyces* species involving resource exploitation. However, in this non- *Saccharomyces* interaction with competition for the same substrate, the result should be determined by the maximum growth rates once the resource is exhausted and the stationary phase begins (Pirt, 1975; Stanbury *et al.,* 2017), a behaviour that is reflected in sigmoid kinetics. Therefore, it is possible that in kinetics with larger amounts of *M. pulcherrima* ("intermediate" and "unfavourable" conditions), growth of *Z. rouxii* was impeded due to the absence of iron, and BMp29 continued to grow because it can produce, transport and use the pulcherrima-iron complex (Oro *et al.,* 2014; Sipiczki, 2020). Analysing the growth kinetics results, response surface predictions of higher and lower biocontrol activity were not observed, but biocontrol occurred in kinetics with increased B/S ratio. The model may underestimate the effect of the Biocontroller/ Spoilage yeast ratio and hence did not detect biocontrol optimal zones. Even when the model makes reasonable adjustments for R^2 (*e.g.*, an adjusted value of 0.75), and considering the low homogeneity of the system due to the absence of agitation under the given fermentation conditions (Ariyajaroenwong *et al.,* 2016), the prediction problems could also be linked to the fact that the B/S ratio is outside the original design space used.

CONCLUSIONS

The results obtained with *Wickerhamomyces anomalus* (BWa156, biocontroller) supernatants to inhibit *Zygosaccharomyces rouxii* (BZr6, spoilage yeast) suggest the development of an interference competition type interaction, probably the result of a proteinaceous toxin. Meanwhile, the screening results revealed an interaction of exploitation competition for iron resources between *Metschnikowia pulcherrima* (BMp29, biocontroller) and *Zygosaccharomyces rouxii* (BZr6, spoilage yeast).

The screening methodology used in the present study reduced the number of experiments and proposed only a small number of physicochemical factors that became relevant during optimization of the biocontrol process. The use of response surface methodology resulted in a reduction in the number of experiments and allowed us to find optimum biocontrol conditions (SO_2: 0 mg mL^{-1}; pH: 3.7; Reducing sugars: 23 °Brix) for the interaction between BWa156 and BZr6. RSM also suggested that interference competition through toxins is more efficient to reduce the spoilage yeast population but this mechanism is more sensitive to variations in physicochemical factors than exploitation competition for resources.

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