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ORIGINAL RESEARCH ARTICLE

Evaluation of the application of *Wickerhamomyces anomalus* supernatant for the control of relevant spoilage yeasts in wines

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ABSTRACT

SO_2 is traditionally used to limit or nullify the proliferation of spoilage yeasts in must/wine, but its use has become controversial due to its negative effects on human health. An alternative strategy for the control of spoilage yeasts is the use of biocontrol yeasts. *Wickerhamomyces anomalus* is an outstanding biocontroller yeast that has been examined by our group and that is a good biocontrol agent against *Brettanomyces bruxellensis* and *Zygosaccharomyces rouxii*. The challenge regarding the application of the results of microbial biocontrol studies lies in exploring technologically simple and economical ways to make use of microbial biocontrol activity. The introduction of foreign microbiota adds complexity to the fermentation medium and can cause technological difficulties and have oenological consequences inherent to the implementation of mixed cultures. Therefore, the objective of this study was to characterise the concentrated culture supernatant of *W. anomalus* and evaluate its biocontrol action in liquid medium on two main wine spoilage yeasts. *W. anomalus* BWa156 supernatant demonstrated inhibitory killer protein characteristics. The production of the inhibitory supernatant by the biocontroller yeast was independent of the presence or absence of the spoilage yeast. Supernatant can be produced faster under aerobic conditions than in traditional fermentation (i.e., with around 24 h), thus increasing its potential for technological development. The treatment with the supernatant of BWa156 was effective against the two spoilage populations *Zygosaccharomyces rouxii* and *Brettanomyces bruxellensis*, which are considered problematic for the wine industry. The supernatant of the biocontrol yeast can be considered to be a relevant additional stress factor for the spoilage population, which together with other factors such as ethanol, competition for nutrients, oxygen and pH, contributes to the elimination of the polluting population. This technology would allow a simple future application, through its production in a bioreactor parallel to the fermentation and subsequent inoculation in the must/wine.

KEYWORDS: biocontrol yeasts, model conditions, wine spoilage yeasts, *Wickerhamomyces anomalus*, *Brettanomyces bruxellensis*, *Zygosaccharomyces rouxii*

INTRODUCTION

Spoilage yeasts generate economic losses in the wine industry, because they produce deviations in the organoleptic characteristics of wine (Rojo *et al*., 2015). Traditionally, SO_2 is used to limit or nullify the proliferation of these yeasts, but its use has become controversial due to its negative effects on human health. Therefore, international organisations such as the World Health Organization (WHO) and the International Organisation of Vine and Wine (OIV) have encouraged a reduction in its use (Cejudo-Bastante *et al*., 2010; Comitini and Ciani, 2010; Ferrer-Gallego *et al*., 2018). An alternative strategy for the control of spoilage yeasts that could progressively replace SO_2 is the use of biocontrol yeasts (Fernández de Ullivarri *et al*., 2018; Kuchen *et al*., 2021). Most studies have focused on the selection of biocontrol yeasts, the characterisation of the biocontrol mechanism, the characterisation of killer toxins and the conditions for maximum activity (Liu *et al*., 2013; Oro *et al*., 2014; Berbegal *et al*., 2017; Fernández de Ullivarri *et al*., 2018).

Our research group has made advances in the study of the application of yeast/yeast biocontrol in fermenting must under static conditions and have examined optimal physicochemical conditions for the success of biocontrol and the kinetic progress of yeasts for the development of mathematical models. One of the most prominent biocontrol yeasts belongs to the *Wickerhamomyces anomalus* species, (Kuchen *et al*., 2022, Vergara Alvarez *et al*., 2023) in particular the Bwa156 strain. It has proven to be a good biocontrol agent against *Brettanomyces bruxellensis* and *Zygosaccharomyces rouxii* isolates in the must/wine environment. This means it is active within the range of physicochemical wine conditions and it also positively contributes to the organoleptic quality of wine (Kuchen *et al*., 2019; Kuchen *et al*., 2021; Kuchen *et al*., 2022).

However, the challenge regarding the application of the results of studies on microbial biocontrol lies in finding technologically simple and economical ways to make use of microbial biocontrol activity. Must fermentation consists of a complex process that involves different types of interactions between microorganisms and the environment. The kinetic progress of these microorganisms varies depending on their interactions with inhibitory molecules (Fernández de Ullivarri *et al*., 2018) or the interactions during substrate competition (Oro *et al*., 2014). The introduction of foreign microbiota adds complexity to the fermentation medium, and it can cause some technological difficulties and have negative oenological consequences inherent to the use of mixed cultures (González-Royo *et al*., 2015, Onetto *et al*., 2021; *Padilla et al*., 2018); for example, it can influence the kinetics of the fermentation strain of *Saccharomyces cerevisiae* itself. It should be highlighted that in several studies the non- *Saccharomyces* killer yeasts (including BWa156, which we used in the present study) did not inhibit *S. cerevisiae* (in our particular case tested in plate with the Bsc114 strain) (Oro *et al*., 2014; Kuchen *et al*., 2019), in other cases there was only influence at high inoculation ratios between non-*Saccharomyces*/ *Saccharomyces cerevisiae* (Comitini *et al*., 2011).

To avoid the introduction of foreign microbiota, some authors have tried to use purified toxins in wine -like environments to control spoilage yeasts (Villalba *et al*., 2016; Comitini *et al*., 2021). However, killer toxins usually have a limited antagonistic spectrum (Ciani and Comitini, 2011; Nardi, 2020) and the production cost of the toxin and its purification can be high. On the other hand, other yeast species have demonstrated the existence of different active inhibition components in culture supernatants, which can include proteaceous compounds (Comitini and Ciani, 2010; Albergaria and Arneborg, 2016), antimicrobial peptides (Branco *et al*., 2014), medium chain fatty acids (Viegas *et al*., 1989) and glycolipids (Kulakovskaya *et al*., 2009). This means that the biocontrol action of yeast supernatants can involve more than one inhibition mechanism (Kemsawasd *et al*., 2015). Therefore, they can be produced in the same yeast and lose spectrum of action when the culture supernatant is being purified. Depending on the industrial applicability, the use of crude supernatants with biocontrol activity is advantageous, as they are simple to obtain and can have a broad inhibition spectrum. The potential to use a crude extract or supernatant from yeasts with killer phenotype increases the likelihood of inhibition. In this study, a complex culture supernatant obtained through lyophilisation is used, since lyophilisation is one of the most efficient methods for the conservation of protein stability and for *W. anomalus* several killer toxins with different molecular weights have been cited (Liu *et al.*, 2013; Comitini *et al.*, 2021), moreover, the process is carried out in controlled conditions (relevant in experimentation repeatability). Nevertheless, it should be noted that this is a costly method from the energy point of view and comprises a batch process. Hence, it will be necessary research to find the most efficient production methods for conserving protein stabillity (e.g., atmospheric freeze drying or use of spray dryers) (Claussen *et al.,* 2007; Seghiri *et al.,* 2021). Therefore, the objective of this study was to characterise the inhibitory nature of the culture supernatant of *W. anomalus* and evaluate its biocontrol action in a model liquid medium against two main wine spoilage yeasts.

MATERIALS AND METHODS

1. Culture Supernatant Production

1.1. Strains and culture medium

The following isolates were obtained from the culture collection at the Institute of Biotechnology, Faculty of Engineering, San Juan National University (UNSJ), Argentina: *Wickerhamomyces anomalus* (BWa156), previously determined as a biocontroller with Killer phenotype (Kuchen *et al*., 2021), and *Zygosaccharomyces rouxii* (BZr6) and *Brettanomyces bruxellensis* (BBb1) as spoilage yeasts of wine. The latter two strains were molecularly identified by amplification, sequencing and BLAST analysis according to Linton *et al*. (2007).

Two culture media were used:

YPD (g/L): Yeast extract 10, peptone, glucose 20, pH of 4.5, adjusted with 1N HCl. Solid medium was obtained by adding 2 g/L agar-agar.

YMB supplemented with 0.2M citrate-phosphate buffer, had a pH of 4.5 and contained (g/L): Glucose 10, yeast extract 3, malt extract 3, peptone 5, NaCl 30, methylene blue 0.030 and 10 % (v/v) glycerol (Santos *et al*., 2009). Solid medium was obtained by adding 2 g/L agar-agar.

1.2. Preparation of the supernatant

A pure culture of BWa156 (1 * 106 cells/mL) and a co-inoculum of BWa156 (4 * 10^5 cells/mL) and BZr6 (1.6 * 10^6 cells/mL) were developed separately in 500 mL of YMB broth at 110 rpm and 25 °C. Samples were taken after 24 and 48 h. Each culture was centrifuged at 5,000 rpm at 4 °C for 10 min and then filtered through a 0.45 µm polyethersulfone membrane (Mehlomakulu *et al*., 2014). Each obtained culture supernatant was lyophilised for 72 h until it had reached room temperature. Later, each obtained lyophilisate was reconstituted with YMB broth to obtain a volume 20 times minor (X20) (hereafter refered to as the culture supernatant) then stored at -20 °C (Banjara *et al*., 2016).

1.3. Inhibition evaluation during supernatant production

The effect of the culture time on the inhibition and presence of the spoilage yeast in both supernatants was assayed using two methods: 1) Oxford cup method using a Petri dish with YMB agar medium and a contaminant lawn; the culture supernatant was poured into Oxford cups and the inhibition halo was measured with a micrometer, 2) Bradford Protein Assay: the total protein in the supernatant was measured spectrophotometrically at 595 nm (Kruger, 2009).

2. Characterisation of the supernatant

2.1. Thin layer chromatography (TLC)

Chromatography was performed in order to assess any lipid content with inhibitory effect in the supernatant. The supernatant from a pure BWa156 culture was used and positive (olive oil) and negative (YMB broth) controls were carried out with a capillary pipette (1 µL) on the lower part of a chromatography paper sheet (n = 4) on TLC cellulose F aluminium sheets (20 × 20 cm; 0.1 mm thickness, Merck 5574). A flow of hot air was used for solvent evaporation between applications. The mobile phase used was hexane:ethyl acetate:acetic acid (90:10:1) for lipid samples (Regner *et al*., 2019). Lipids were revealed with an ultraviolet lamp (254 nm).

2.2. Native polyacrylamide gel electrophoresis (native PAGE)

Buffered polyacrylamide gel without sodium dodecyl sulphate (SDS) was used for electrophoresis to avoid protein denaturation and maintain possible inhibitory activity. The concentration gel (4 mL) consisted of 0.6 mL of acrylamide-bis acrylamide (30:0.8), 1 mL of 0.5 M Tris-HCl buffer with a pH of 6.5, 2.4 mL of double distilled water, 20 µL of 0.1 g/mL ammonium persulfate and 6 µL of TEMED. The separating gel (10 mL) consisted of 3.3 mL of acrylamide-bis acrylamide (30:0.8), 2.5 mL of 1.5 M Tris-HCl buffer with a pH of 8.8, 4.2 mL of double distilled water, 60 µL of 0.1 g/mL ammonium persulfate and 12 µL of TEMED. The running buffer (1 L) consisted of double distilled water with 3 g Tris and 18 g glycine. The native loading buffer (25 mL) comprised 0.5M Tris-HCl buffer with a pH of 6.5, 20 % glycerol and 0.05 % bromophenol blue. For the polymerisation, 1 mm thick Biorad-Protean® was used. The *W. anomalus* SBWa156 supernatant was placed in a single lane for 1.5 h at 100 V. Subsequently, the gel was removed, cut into 1 cm wide bands, revealed with Coomassie Blue and decolorised with 50 % methanol and 12 % acetic acid (Wittig *et al*., 2006). The inhibition bioassay was carried out as follows: the residual gel was placed in a sterile Petri dish, covered with YMB-agar and the spoilage yeast BZr6 incorporated in the solid medium (inoculum: 2 * 10^5 cells/mL) and incubated at 25 °C for 4 d.

3. Evaluation of the supernatant inhibition kinetics in liquid medium

3.1. Proteinase K treatment

A 96-well microplate was filled with 200 µL of YPD broth, which was inoculated with BZr6 (1 * 10^6 cells/mL). Then, were added 40 µL of pure BWa156 supernatant (SBWa156) or the same volume of SBWa156 previously treated with "proteinase K" for 48 h for protein denaturation (hereafter referred to as "SBWa156 + P") (Banjara *et al*., 2016). Concentrated sterile YMB X20 (n = 3) was used as the control. The microplate was incubated at 23 °C and at 110 rpm for 84 h. Readings were performed by optical density at 620 nm in a Thermofisher microplate reader, and the optical density was correlated with the concentration of the cells using a calibration curve. The µmax of the growth kinetics was calculated according to Monod (1949) and the lag phase as described by Lodge and Hinshelwood (1943). The control lag phase was shortened to emphasise the differences between the treatments.

3.2. Effect of increasing supernatant concentration

A 96-well microplate was filled with 200 and 190 µL of YPD broth, which was inoculated with BZr6 or BBb1 (1 * 10^6 cells/mL). Then 50 and 60 µL of BWa156 supernatant were added respectively. Concentrated sterile YMB X20 (n = 3) was used as the control. The microplate was incubated at 23 °C and at 110 rpm for 84 h. Readings were performed by optical density at 620 nm in a Thermofisher microplate reader, and the optical density was correlated with the cell concentration using a calibration curve. The µmax of the growth kinetics was calculated according to Monod (1949) and the lag phase as described by Lodge and Hinshelwood (1943).

4. Statistical analysis

All the assays were performed in triplicate and repeated measures analysis of variance (ANOVA; $p < 0.05$) was performed using InfoStat statistical software, professional version 2016, Córdoba, Argentina. The "yeast" response was log-transformed prior to analysis of variance to satisfy ANOVA assumptions.

RESULTS

1. Supernatant production: effect of supernatant production time and spoilage yeast presence on supernatant inhibition capacity

In order to determine if the inhibitory capacity of the culture supernatant is produced by *W. anomalus per se* or if it is affected by the presence of *Z. rouxii*, two supernatants were examined: one from a pure culture of *W. anomalus* BWa156 (SBWa156) and the other obtained from a mixed culture of BWa156 and BZr6 (SBWa156+BZr6). Samples were taken after 24 and 48 h. The effect of the presence of the spoilage yeast *Zygosaccharomyces rouxii* BZr6 and the culture time on the inhibitory capacity of the *W. anomalus* supernatant over the BZr6 lawn was studied. It could be seen in the statistical analysis (data not shown), that neither of the factors, presence nor time, induced any significant changes in the inhibitory effect of the supernatant in the Oxford cups placed on a Petri dish with a BZr6 lawn. Moreover, the treatments could not be differentiated when examining the supernatants based on the protein concentration in the medium (which would be influential in the case of a protein toxin).

2. Characterisation of the supernatant

2.1. Thin layer chromatography of the BWa156 supernatant (TLC)

Figure 1, the thin layer chromatography of the supernatant does not show any displacement of lipid compounds of medium polarity at the wavelengths assayed.

2.2. Polyacrylamide gel electrophoresis of the supernatant (Native PAGE)

In order to visualise the presence of proteins and avoid their denaturation, native PAGE was performed. Figure 2 shows the native polyacrylamide gel electrophoresis (native PAGE) method and assay and their use in two parts: one part to identify the protein concentration zone using Coomassie blue and the other to carry out the inhibition bioassay with native PAGE, incorporating it into the culture medium and with the BZr6 lawn.

3. Evaluation of the supernatant biocontrol kinetics in liquid medium

3.1. Proteinase k kinetics

Figure 3 shows the growth kinetics of the spoilage yeast *Z. rouxii* BZr6 without supplements (control) and with supplements of supernatant of a pure culture of BWa156 (SBWa156) and SBWa156 with prior proteinase K treatment (SBWa156 + Prot. K). The results of the lag phase and specific growth rate were statistically analysed (Table 1).

In terms of the lag phase, there are significant differences between the control population (BZr6) and BZr6 supplemented with *W. anomalus* supernatant (BZr6 + SBWa156), indicating that the supernatant had an inhibitory effect. However, no significant differences were found between the control and the BZr6 population supplemented with supernatant previously treated with proteinase K for its degradation (BZr6 + SBWa156 + Prot. K).

3.2. Effect of the concentration of the supernatant on the kinetics of *Zygosaccharomyces rouxii* and *Brettanomyces bruxellensis*

Figure 4A and 4B show the growth kinetics of the spoilage yeasts *Z. rouxii* (BZr6) and *B. bruxellensis* (BBb1) respectively, and the effect of two concentrations of the *W. anomalus* supernatant (50 and 60 µL) on the multiplication rate.

The statistical analysis of the lag phase (Table 2) revealed significant differences between the growth of the spoilage yeast *Z. rouxii* BZr6 (control) and the treatments of the spoilage yeast with BWa156 supernatant. There were also differences between the treatments: a higher supernatant concentration corresponded to a longer duration of the lag phase. Regarding the maximum specific growth rate, µmax, the only treatment that was significantly lower was "BZr6 + SWa156 50". In the case of *B. bruxellensis* BBb1, the control showed a higher dormancy phase than BBb1 with treatments, but the latter had a significantly lower specific growth rate and its total population values were lower.

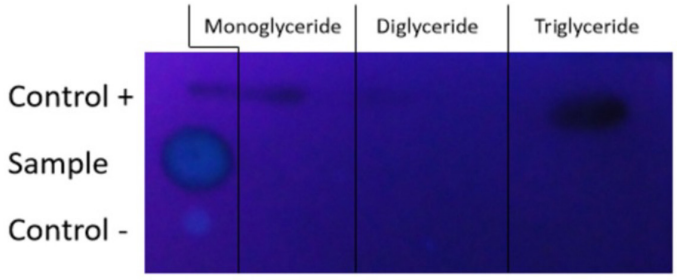

FIGURE 1. Thin layer chromatography (TLC) illuminated with 254 nm UV. Control (+) = sterile olive oil, Sample = supernatant sample of *W. anomalus* SBWa156, and Control (-) = sterile concentrated YMB.

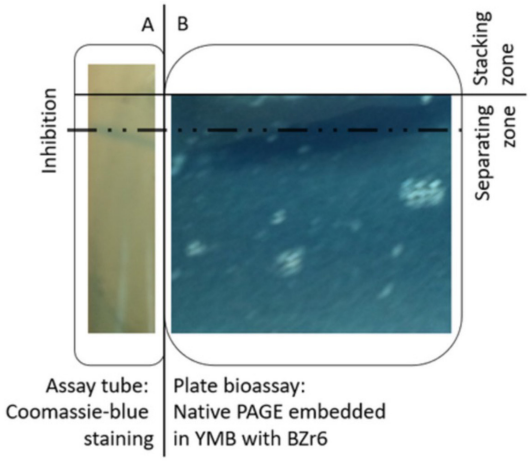

FIGURE 2. Polyacrylamide gel scheme and assay for the evaluation of the protein concentration zone of *W. anomalus* BWa156 supernatant (Coomassie blue staining) and the inhibition zone of BWa156 using a plate bioassay with BZr6 as a lawn.

Section A in Figure 2 shows a band stained with Coomassie blue in a test tube and Section B shows an inhibition zone coinciding with the band in the bioassay on a Petri dish. Native PAGE, with the displacement of proteins, is incorporated below the solidified YMB culture medium, which in turn contains the spoilage yeast BZr6.

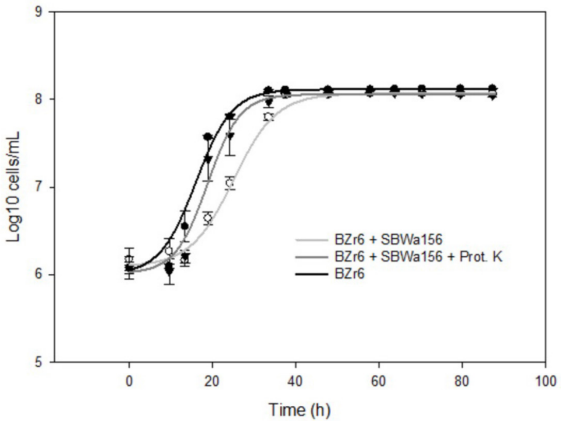

FIGURE 3. Microplate growth kinetics of the spoilage yeast *Zygosaccharomyces rouxii* BZr6 (control) and inhibition treatments: BZr6 + SBWa156 = *Z. rouxii* with supernatant of *W. anomalus* BWa156, BZr6 + SBWa156 + Prot. K = *Z. rouxii* with BWa156 supernatant previously treated with proteinase K.

DISCUSSION

1. Effect of the supernatant production method

In terms of the halo diameter obtained on the BZr6 lawn, no significant differences were found between the supernatant of the co-culture of the BWa156 (biocontrol) and BZr6 (spoilage) yeasts and the supernatant of the pure culture of BWa156. No differences in the protein concentration of the supernatants assayed using the Bradford method were observed either. While several authors, such as Schmidt *et al.* (2013), suggest that the production of inhibitory molecules by certain yeast species are promoted/induced by the presence of the antagonistic yeast, others suggest that toxin production is independent of the presence of the antagonistic yeast (İzgü *et al.*, 2006; Pommier *et al.*, 2005). From our results, it could be inferred that the production of the BZr6 inhibitor by BWa156 was not stimulated by the presence of the spoilage yeast. The biocontrol yeast BWa156 reached the end of its exponential growth phase during both treatments after 24 h of aerobic growth (kinetics not shown). Neither the pure nor the mixed supernatants obtained after 24 and 48 h of culture showed significant differences in terms of their ability to inhibit the spoilage lawn. This suggests that the production of the inhibitor may have occurred during the exponential phase. This is consistent with previous data obtained with the supernatant of *W. anomalus*, ex *Pichia anomala*, under aerobic conditions, which, when plate-assayed, demonstrated an increasing inhibitory activity during the exponential phase of the biocontrol yeast and maximum activity at the end of this phase (Comitini *et al.*, 2004; Mazzucco *et al.*, 2019; Villalba, 2022). On the other hand, although it is necessary to

TABLE 1. Statistical analysis of the lag phase and specific growth rate of BZr6 (control) and BZr6 with BWa156 supernatant with or without addition of proteinase K.

	Lag phase			Specific growth rate			
Treatment	Means (h)	Standard Error		Means (h^{-1})	Standard Deviation	Ranks	
BZr6	0.29	1.34	A	0.07	$1.40*10^{-4}$	10.5	AB
BZr6 + SBWa156 + Prot. K	3.97	1.52	AB	0.08	$5.30*10^{-4}$	15	B
BZr6 + SBWa156	6.91	1.52	B	0.07	$5.00*10^{-4}$	6	A

*BZr6 + SBWa156 = Growth kinetics of BZr6 with BWa156 supernatant. BZr6 + SBWa156 + Prot. K = growth kinetics of BZr6 with BWa156 supernatant previously treated with proteinase K for 48 h. BZr6 = growth kinetics control. Fisher's test: different letters indicate significant differences.

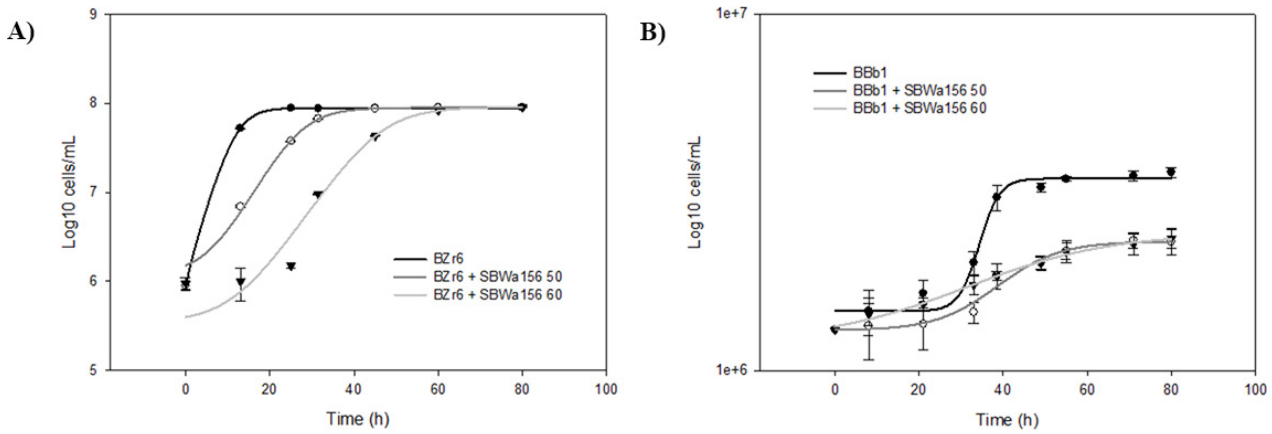

FIGURE 4. Microplate growth kinetics of the spoilage yeasts. A- *Zygosaccharomyces rouxii* BZr6 (Control) and treatments: BZr6 + SBWa156 50 = *Z. rouxii* with 50 µL of *W. anomalus* BWa156 supernatant, BZr6 + SBWa156 60 = *Z. rouxii* with 60 µL of BWa156 supernatant. B- *Brettanomyces bruxellensis* BBb1 (Control) and treatments: BBb1 + SBWa156 50 = *B. bruxellensis* with 50 µL of BWa156 supernatant, BBb1 + SBWa156 60 = *B. bruxellensis* with 60 µL of BWa156 supernatant.

obtain quantitative data from the analysis undertaken, it can be inferred that the supernatant can be stored as a lyophilised powder or as a lyophilisate reconstituted with YMB at -20 °C, as suggested by Carboni *et al*. (2020).

2. Characterisation of the supernatant

The *W. anomalus* supernatant was examined by thin layer chromatography (TLC) (Figure 1) to evaluate the possible presence of inhibitory lipid molecules (Regner *et al*., 2019). Although glycolipids have mainly been described as being biocontrol molecules in Basidiomycetes (Kulakovskaya *et al*., 2009; Abdel-Mawgoud and Stephanopoulos, 2018), several Ascomycete genera can also produce glycolipids (Sanches *et al*., 2021). Even *W. anomalus* has been studied as a producer of various types of glycolipids with biocontrol capacity (Morita *et al*., 2007; Farkas *et al*., 2012; Dejwatthanakomola *et al*., 2016). However, according to our results no compound displacement of the BWa156 supernatant occurred on the chromatography plate at the wavelengths assayed. On the other hand, the native polyacrylamide gel electrophoresis (PAGE) of SBWa156 showed a protein band when stained with Coomassie blue, and in the plate bioassay with a *Z. rouxii* BZr6 spoilage yeast lawn, an inhibition zone coinciding with the protein band. This indicates that the stained protein may be responsible for the inhibition. The assay conditions of native PAGE (100 V - 1.5 h) and the relative progress of the possible inhibitory proteins allow us to infer that, without denaturation, the proteins would have a molecular weight close to 100 kDa, similar to that found for *W. anomalus* strains by Liu *et al*. (2013). However, results vary when trying to estimate the molecular weight of a protein under native conditions by displacement in agarose gel (Wittig *et al*., 2006), but such estimations are beyond the scope of the objectives of the present study. In general, *W. anomalus* killer toxins, which are able to degrade the cell walls of other yeasts, can be related to β-1,3-D-glucanase activity (Cecarini *et al*., 2019). The lyophilisation method used for the culture supernatant concentration could be efficient for preserving the inhibitory activity of the proteins. Although lyophilisation is one of the most efficient methods and entails controlled conditions, it is also energy expensive and comprises a batch process. In the search for technological applications, other efficient methods in terms of inhibition activity conservation should be reviewed; i.e., continuous methods, such as atmospheric

TABLE 2. Statistical analysis of lag phase and specific growth rate of BZr6 and BBb1 with two different volumes of BWa156 supernatant.

Treatment	Lag phase			Specific growth rate		
	Means (h)	Standard Error		Means (h^{-1})	Standard Error	
BZr6	0.16	2.18	A	0.11	$2.30*10^{-3}$	B
BZr6 + SBWa156 50	14.91	2.18	B	0.05	$2.30*10^{-3}$	A
BZr6 + SBWa156 60	27.86	1.89	C	0.11	$2.00*10^{-3}$	B
BBb1	32.06	2.24	A	0.07	$1.85*10^{-3}$	A
BBb1 + SBWa156 50	23.24	3.1	B	0.02	$3.00*10^{-3}$	B
BBb1 + SBWa156 60	11.51	3.5	C	0.01	$5.00*10^{-3}$	B

*BZr6 + SBWa156 50 = Growth kinetics of BZr6 with 50 µL of BWa156 supernatant, BZr6 + SBWa156 60 = growth kinetics of BZr6 with 60 µL of BWa156 supernatant, BZr6 = growth kinetics control of *Z. rouxii*, BBb1 + SBWa156 50 = growth kinetics of BBb1 with 50 µL of BWa156 supernatant, BBb1 + SBWa156 60 = growth kinetics of BBb1 with 60 µL of BWa156 supernatant, BBb1 = growth kinetics control of *B. bruxellensis*. Fisher's test: different letters indicate significant differences.

freeze drying, spray drying and convection drying with invention patents that treat liquids with proteins to obtain wettable powders on an industrial scale (Claussen *et al*., 2007; Serghiri *et al*., 2021).

3. Evaluation of the supernatant's biocontrol kinetics in liquid medium

The inhibitory activity of the supernatant on certain microbial kinetics parameters of BZr6 was examined in a liquid medium in a microplate assay following the protocol by Banjara *et al*. (2016). This assay revealed differences in terms of lag phase between the kinetics of the pure population of the spoilage yeast BZr6 (control) and the kinetics of BZr6 supplemented with *W. anomalus* supernatant, however, no differences were found between the kinetics of the control and that of BZr6 with *W. anomalus* supernatant previously treated with Proteinase K. This can be explained by the loss in inhibitory activity of the supernatant in the presence of Proteinase K, which is in agreement with the results of experiments carried out by Banjara *et al*. (2016) and, together with the PAGE data, indicates that the inhibitory activity was produced by a protein. The fact that inhibition is a delay that does not occur for an indefinite period of time is supported by the fact that killer toxins (proteins) adhere to the cell wall of *Z. rouxii* inducing its death (Liu *et al*., 2013). As the biocontrol yeast is not present, there is no "de novo" production of killer toxins and the number of molecules decreases due to the adherence to the spoilage yeast's cell walls. When *Z. rouxii* overcomes this situation, the survivors multiply and develop normal kinetics after a prolonged lag phase (Banjara *et al*., 2016). It is also possible that there are components secreted by biocontrol cells that degrade or inhibit the toxin (e.g., proteases or other enzymes) resulting in the reactivation of spoilage yeast multiplication (Comitini *et al*., 2004; Bajaj *et al*., 2012). Regarding the degradation of the toxin, on the other hand, when it was stored at 4 °C or -20 °C and used after a week or a month respectively, no significant changes were observed when compared with its immediate use (data not shown). In addition, this inference is also supported by other studies with at least 4 protein killer toxins emitted by *W. anomalus* (Liu *et al*., 2013; Fernández de Ullivarri *et al*., 2018; Villalba, 2022).

Comitini *et al*. (2004) described a correlation between the increase in toxin concentration and the inhibition halo on solid medium. The present study examined different supernatant concentrations and the corresponding biocontrol action in liquid medium against two of the most harmful yeasts in the wine industry (Combina *et al*., 2008; Rojo *et al*., 2015; Ciani *et al*., 2016). The results of the *W. anomalus* SBWa156 supernatant used against *Z. rouxii* BZr6 indicate a direct relationship between the increase in supernatant concentration and a longer lag phase. Although the lag phase of the spoilage yeast *B. bruxellensis* BBb1 was longer for the control, in treatments with BWa156 supernatant the maximum specific growth rate and final population were significantly lower. This confirms an inhibitory effect of the toxin on the growth kinetics of *B. bruxellensis*.

CONCLUSIONS

Wickerhamomyces anomalus BWa156 supernatant demonstrated protein-like inhibitory characteristics. Production of the inhibitory supernatant was independent of the presence of the spoilage yeast. The supernatant can be produced faster under aerobic conditions than in traditional fermentation: within around 24 h, which would allow its technological application.

Treatment with the supernatant of *W. anomalus* is effective against the two spoilage populations, *Zygosaccharomyces rouxii* and *Brettanomyces bruxellensis*, which are considered problematic for the wine industry.

In order to apply this technology, it would be necessary to take into account the reactivation of the polluting population after a certain period of time, as observed in the assays. Therefore, the supernatant of the biocontrol yeast should be considered as a relevant additional stress factor for the

spoilage population, which, together with other factors, such as ethanol, competition for nutrients, oxygen and pH, contributes to the elimination of the polluting population.

There is potential for the simple future application of this technology in a bioreactor parallel to fermentation and subsequent inoculation in the must/wine.

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