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Selection of Native Non-*Saccharomyces* Yeasts with Biocontrol Activity against Spoilage Yeasts in Order to Produce Healthy Regional Wines

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Received: 6 June 2019; Accepted: 4 July 2019; Published: 8 July 2019

Abstract: Two major spoilage yeasts in the wine industry, *Brettanomyces bruxellensis* and *Zygosaccharomyces rouxii*, produce off-flavors and gas, causing considerable economic losses. Traditionally, SO_2 has been used in winemaking to prevent spoilage, but strict regulations are in place regarding its use due to its toxic and allergenic effects. To reduce its usage researchers have been searching for alternative techniques. One alternative is biocontrol, which can be used either independently or in a complementary way to chemical control (SO_2). The present study analyzed 122 native non-*Saccharomyces* yeasts for their biocontrol activity and their ability to be employed under fermentation conditions, as well as certain enological traits. After the native non-*Saccharomyces* yeasts were assayed for their biocontrol activity, 10 biocontroller yeasts were selected and assayed for their ability to prevail in the fermentation medium, as well as with respect to their corresponding positive/negative contribution to the wine. Two yeasts that satisfy these characteristics were *Wickerhamomyces anomalus* BWa156 and *Metschnikowia pulcherrima* BMp29, which were selected for further research in application to mixed fermentations.

Keywords: biocontrol application; non-*Saccharomyces* screening; SO_2 reduction

1. Introduction

Wine is the product of complex microbial interactions that start on the grape surface and continue throughout the fermentation [1]. Some yeasts generate metabolites that lead to wine faults that affect flavor, haze or CO_2 production in the final product. One of the major spoilage yeasts is *Brettanomyces bruxellensis* [2]. Wines contaminated with this yeast are characterized by the presence of off-flavors [3]. Other spoilage yeasts frequently described in the food industry belong to the *Zygosaccharomyces* genus. They produce gas in food and beverages [4], and they are difficult to control chemically [5]. Spoilage resulting from this yeast is widespread and causes considerable economic losses in the food industry [6,7].

Traditionally, SO_2 has been used in winemaking during non-fermentation stages to control microbial proliferation such as bacteria, yeasts and fungi. Nevertheless, there are strict regulations regarding its use due to its toxic and allergenic effects on human health [8]. International organizations such as the *Organisation Internationale de la vigne et du vin* encourage SO_2 reduction [9]. Moreover,

modern consumers prefer more natural and healthy foods and beverages that are minimally processed and free of preservatives [4,10].

Biocontrol is an alternative proposal that can be used either independently or in a complementary way to chemical control (SO_2). Some *Saccharomyces* and non-*Saccharomyces* yeasts have the ability to biosuppress other yeasts through different mechanisms such as the production of toxic compounds [2], competition for limiting substrates [11] and/or cell to cell contact [1].

At present, a re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking and their use as selected starters in mixed fermentations with *Saccharomyces cerevisiae* is being carried out [12,13]. Non-*Saccharomyces* yeasts are supposed to enhance the wine quality [14]. Nowadays there is a special interest in yeast strains associated with specific geographical locations as they may introduce a regional character or 'terroir' to the winemaking process [12,15].

Yeast growth parameters such as specific growth rate, lag phase duration, product yield and metabolic rates of substrates and products may provide useful information to understand their biocontrol mechanisms and how to use them during the fermentation process. Taking into account that yeast bio-suppression can be associated with substrate competition and secretion of toxic substances, it is important to understand the growth parameters of non-*Saccharomyces* yeasts during fermentation, in order to plan co-inoculation or sequential mixed inoculation with *Saccharomyces* [16–19].

Several authors have analyzed indirect values like "fermentation rate" (CO_2 release) [20,21]. However, there are no reports related to selection of non-*Saccharomyces* yeast for vinification that have studied the prevalence of yeasts with clearly defined kinetic parameters. The aim of the present study was to analyze the biocontrol ability of 122 native non-*Saccharomyces* yeasts against two of the most relevant wine spoilage yeast species, *Z. rouxii* and *B. bruxellensis*. Subsequently, biocontrolling yeasts were characterized for their ability to be employed under fermentation conditions and their capacity to generate positive or negative enological traits, in order to reduce SO_2 and improve the quality of regional wines.

2. Materials and Methods

2.1. Microorganisms

One hundred and twenty-two non-*Saccharomyces* yeasts (Table 1), previously isolated from enological environments from San Juan and Mendoza, Argentina (Cuyo region), were obtained from the Culture Collection of Autochthonous Microorganisms of the Institute of Biotechnology, School of Engineering, UNSJ, San Juan, Argentina, and used in the present study. The yeasts had been used in previous studies by our research group [22,23].

Table 1. Non-*Saccharomyces* yeast isolates assayed.

Species	N° of Isolates	Strain Nomenclature
Candida apis	1	BCa80
Candida cantarelli	1	BCca78
Candida catenulata	1	BCct79
Candida diversa	1	BCd75
Candida famata	4	BCf84
Candida intermedia	1	BCi85
Candida membranifaciens	3	BCm69, BCm70, BCm71
Candida pararugosa	1	BCp73
Candida rugosa	1	BCr81
Candida sake	6	BCsa74, BCsa82, BCsa83, BCsa86, BCsa88, BCsa95
Candida steatolytica	1	BCse76

Table 1. *Cont.*

Species	N° of Isolates	Strain Nomenclature
Candida stellata	1	BCst68
Clavispora lusitaniae	1	BCl157
Cryptococcus albidus	1	BCra158
Debaryomyces hansenii	5	BDb150, BDb152, BDb153, BDb154, BDb155
Debaryomycesvanrijiae	1	BDv151
Hanseniaspora guilliermondii	6	BHg42, BHg44, BHg45, BHg46, BHg47, BHg48
Hanseniaspora osmophila	1	BHo51
Hanseniaspora uvarum	27	BHu1, BHu2, BHu3, BHu5, BHu8, BHu9, BHu10, BHu11, BHu12, BHu13, BHu17, BHu18, BHu19, BHu20, BHu21, BHu23, BHu24, BHu26, BHu27, BHu28, BHu30, BHu31, BHu32, BHu38, BHu40, BHu41
Hanseniaspora vineae	2	BHv43, BHv50
Issatchenkia orientalis	1	BIo160
Metschnikowia pulcherrima	6	BMp4, BMp29, BMp49, BMp151, BMp144, BMp145
Pichia fabianii	1	BPf127
Pichia guilliermondii	1	BPg138
Pichia kluyveri	3	BPkl130, BPkl131, BPkl133
Pichia kudriavzevii	5	BPku128, BPku129, BPku132, BPku134, BPku135
Pichia manshurica	1	BPm125
Pichia membranifaciens	1	BPm136
Pichia occidentalis	21	BPo96, BPo97, BPo98, BPo100, BPo101, BPo102, BPo104, BPo108, BPo110, BPo111, BPo112, BPo113, BPo114, BPo115, BPo116, BPo117, BPo120, BPo121, BPo122, BPo123, BPo124
Starmerella bacillaris	12	BSb52, BSb53, BSb54, BSb55, BSb56, BSb57, BSb58, BSb59, BSb62, BSb63, BSb66, BSb67
Torulaspora delbrueckii	3	BTd147, BTd148, BTd149
Wickerhamomyces anomalus	1	BWa156
TOTAL	122	

Eight spoilage yeasts (4 *Brettanomyces bruxellensis* isolates and 4 *Zygosaccharomyces rouxii* isolates) were obtained from the EEA INTA culture collection, Lujan, Mendoza, Argentina, and used in the study [4,24]. *Saccharomyces cerevisiae* BSc114 [23] was used as positive control with regard to fermentative performance. Isolates were identified through biochemical, physiological and morphological methods [6] as well as molecular methods [25].

2.2. Culture Media

Propagation was carried out in YEPD broth (g/L): Yeast extract 10, peptone, glucose 20, pH: 4.5 adjusted with HCl 1N.

Viable yeast counting was carried out on YEPD-agar (g/L): Yeast extract 10, peptone 20, glucose 20, agar-agar 20, pH: 4.5.

Biocontrol was carried out on YMB-agar supplemented with 0.2 M citrate-phosphate buffer, pH 4.5 (g/L): Glucose 10, yeast extract 3, malt extract 3, peptone 5, NaCl 30, methylene blue 0.030, glycerol 10% *v*/*v* [26] (modified).

Kinetics and tolerance assays were carried out with concentrated grape must (65 °Brix), diluted at 21 °Brix with 1 g/L of yeast extract added, pH: 4.

Inocula for biocontrol and plate assays were obtained with YEPD broth pH: 4.5, 24 h incubation period.

Inocula for kinetic and tolerances assays were obtained with concentrated grape must (65 °Brix), diluted at 21 °Brix with 1 g/L of yeast extract added, pH: 4, 24 h incubation period.

Complex media was sterilized at 121 °C for 20 min and grape must media at 111 °C for 20 min.

2.3. Screening for Biocontrol Ability of Yeasts

Each spoilage yeast was incorporated at a concentration of 10^6 cells/mL in liquid YMB-agar biocontrol medium at 45 °C, mixed to uniform, and immediately poured into sterile petri dishes. Potential biocontrolling non-*Saccharomyces* yeasts were inoculated as a drop (20 µL) on the agar surface, and plates were incubated at 25 °C until a well-developed lawn. Killer activity was visualized as zone of growth inhibition (more than 1 mm) around the spotted killer yeast colony on plates [2].

Biocontrolling activity against the spoilage species was calculated in 2 ways: (a) Intraspecific inhibition: the proportion at which one biocontrolling strain inhibited the spoilage isolates belonging to one species. In addition, (b) Total inhibition: the proportion at which one biocontroller yeast strain inhibited the spoilage isolates belonging to both species.

2.4. Fermentative Performance

2.4.1. Growth Kinetics during Fermentation

Yeasts were separately cultured in Erlenmeyer flasks (250 mL) containing 200 mL of growth medium. Each isolate was seeded at a concentration of 10^6 cells/mL and incubated at 25 °C for 21 days under static conditions, according to [27] for growth determination by viable cell count. Samples were taken on days 1, 2, 3, 6, 15 and 21. Plates were used for viable cell counts and the experimental data was used to construct a growth curve, which was used to determine kinetic parameters. µmax was calculated as described Monod [28] and the lag phase as described Lodge and Hinshelwood [29], which are the most widespread methods according to [16,17].

2.4.2. Tolerance to Different Stress

Low temperature (15 °C), High concentrations of reducing sugar (30 °Brix), and different Ethanol concentrations (8, 10, 12 and 14% *v*/*v*) and different molecular SO_2 concentrations (0.1, 0.15, 0.2, 0.3 and 0.4 ppm) were carried out according to Vazquez et al. [27] in tolerance medium for each strain. Growth was monitored with Durham tubes (CO_2 production). Gas production in the Durham tubes was monitored one day after the positive control of each strain. Molecular SO_2 was calculated according to [30,31]. Control was performed at 25 °C, 21 °Brix, 0% *v*/*v* ethanol and 0 ppm of SO_2.

2.4.3. Plate Assays

SH_2 production: Yeasts were spot-inoculated and evaluated as semi-quantitative over BigGy-agar (BBL™, Becton, Dickinson and Company, Sparks, USA, Le Pont de Claix, France) following elaboration instructions. Incubation: 3 days at 25 °C. An arbitrary scale was used for the color of the colony from 1, white color (no production); 2, light brown; 3, brown; 4, dark brown; 5, dark brown/black (high production) [20].

β– glucosidase activity: was performed according to Strauss et al. [32]. Medium containing (g/L): yeast nitrogen base 6.7 (YNB, Difco™, Becton, Dickinson and Company, Sparks, USA), Arbutin 5 (Sigma™, Sigma-Aldrich, Saint Louis, USA) and agar-agar 2, pH: 5, then autoclaved (121 ºC, 20 min). 2 mL of filtered 1% ammonium ferric citrate solution was added to 100 mL media before pouring into plates. Yeast was spot-inoculated. Incubation: 5 days at 30 °C. Activity was positive when a discolored halo of hydrolysis was observed.

Protease activity: was performed according to Comitini et al. [20]. The medium contained (g/L): yeast extract 3, malt extract 3, peptone 5, glucose 10, NaCl 5 and agar-agar 15. In a separate vessel, an

equal volume of skimmed milk was prepared with sterile water at 10% p/v. After sterilization both solutions were mixed and poured into sterile petri dishes. Yeast was spot-inoculated. Incubation: 3 days at 25 °C. Activity was observed as a clear halo of hydrolysis.

Pectinase activity: was performed according to Fernandez-Salomäo et al. [33]. The medium contained (g/L): citrus pectin 2, yeast extract 1, KH_2PO_4 0.2, $CaCl_2$ 0.05, $(NH_4)_2SO_4$ 1, $MgSO_4.7H_2O$ 0.8, $MnSO_4$ 0.05, agar-agar 20, pH: 4.5. After sterilization (121 °C, 20 min), it was poured into sterile petri dishes and yeast was spot-inoculated. Incubation: 3 days at 30 °C. After incubation, Lugol solution was added and pectin degradation was observed as a clear halo of hydrolysis.

Pathogenicity: hemolysin production of yeasts was performed according to Manns et al. [34] and Menezes et al. [35], which used Blood agar medium in petri dishes (Britania™, CABA, Argentina) for this purpose. Yeast was spot-inoculated. Incubation: 2 days at 37 °C. Positive activity was observed as a clear zone of hydrolysis.

All assays were carried out using *Saccharomyces cerevisiae* BSc114 as a positive control for biocontrol, sensitivity to inhibition of selected isolates, tolerance to low temperature, and high concentrations of reducing sugars, ethanol and SO_2, and as a negative control for H_2S, β–glucosidase, protease and pectinase activity [23]. The prokaryote *Pseudomonas aeruginosa* BPa987 was used as positive control for hemolysin production of the yeasts [36,37].

2.5. Data Analysis

Each assay was performed independently in triplicate and results are represented as the average of three determinations with the corresponding standard deviation (±SD). Data were tested for normality, homoscedasticity and independence. Parametrical data and significant differences were analyzed by Fisher test. Principal components analysis (PCA) was used to simplify interpretation of the yeast behavior data and is presented in a biplot graph. InfoStat™ -Professional software version 1.5 was used for data analysis.

3. Results and Discussion

To be used as co-inocula together with *Saccharomyces cerevisiae* in wine fermentations, biocontroller yeasts must possess a good specific growth rate and a short lag phase during anaerobiosis to predominate in the medium [18]. In addition, they should not produce any negative attributes to wine, but instead, they should contribute with positive attributes.

3.1. Biocontrol Screening

Non-*Saccharomyces* yeasts are considered to improve the wine complexity and enhance positive traits of regional wines. Several authors have reported that a rational selection of non-*Saccharomyces* yeasts as *S. cerevisiae* co-inoculum improves the quality of wines [21,23,38]. In the present study, 122 non-*Saccharomyces* yeasts belonging to 10 genera and isolated from different enological environments were screened to assess their ability to biocontrol wine spoilage yeasts belonging to *Zygosaccharomyces rouxii* (4 isolates) and *Brettanomyces bruxellensis* (4 isolates) species.

Bioassaying showed that 23 non-*Saccharomyces* yeasts belonging to 6 genera inhibited growth of at least one isolate of either *Brettanomyces bruxellensis* or *Zygosaccharomyces rouxii* (Table 2).

None of the selected biocontrollers inhibited the control (BSc114) lawn development. This fact would allow the application of these yeast isolations in co-inocula with this strain of *S. cerevisiae*. Some of the species used in this work have already been used as biocontrollers of non-*Saccharomyces* yeasts and did not inhibit the development of *S.cerevisiae* [14].

Table 2. Biocontrol of spoilage yeasts by non-*Saccharomyces* yeast isolates.

Yeast specie	Isolate	BBb1	BBb11	BBb20	BBb29	BZr4	BZr6	BZr9	BZr10	BSc114	Intraspecific *B. bruxellensis* inhibition	Intraspecific *Z. rouxii* inhibition	Total Inhibition
Candida intermedia	BCi85	−	−	+	−	+	−	−	−	−	0.25	0.25	0.25
Candida membranifaciens	BCm70	−	−	−	+	−	−	−	−	−	0.25	0	0.13
Candida sake	BCs88	−	−	−	−	+	+	−	−	−	0	0.5	0.25
Candida sake	BCs95	−	−	+	−	+	+	−	−	−	0.25	0.5	0.38
Hanseniaspora uvarum	BHu5	+	−	+	+	+	+	−	−	−	0.75	0.5	0.63
Hanseniaspora uvarum	BHu18	−	−	−	−	+	+	+	−	−	0	0.75	0.38
Hanseniaspora uvarum	BHu23	−	−	−	−	+	+	+	+	−	0	1	0.5
Hanseniaspora uvarum	BHu27	+	+	−	−	−	−	+	−	−	0.5	0.25	0.38
Hanseniaspora uvarum	BHu31	−	−	−	+	−	+	−	+	−	0.25	0.5	0.38
Hanseniaspora uvarum	BHu32	−	−	+	−	−	+	−	+	−	0.25	0.5	0.38
Issatchenkia orientalis	BIo160	−	−	−	−	−	+	−	−	−	0	0.25	0.13
Metschnikowia pulcherrima	BMp4	−	+	+	+	+	+	+	−	−	0.75	0.75	0.75
Metschnikowia pulcherrima	BMp29	+	−	+	−	−	+	+	−	−	0.5	0.5	0.5
Metschnikowia pulcherrima	BMp49	+	+	+	+	+	+	+	−	−	1	0.75	0.88
Metschnikowia pulcherrima	BMp145	−	−	+	−	+	+	+	+	−	0.25	1	0.63
Metschnikowia pulcherrima	BMp151	−	−	−	−	+	−	−	−	−	0	0.25	0.13
Pichia occidentalis	BPo104	−	−	+	−	+	+	+	−	−	0.25	0.75	0.5
Pichia occidentalis	BPo108	−	−	+	−	+	+	+	+	−	0.25	1	0.63
Pichia occidentalis	BPo120	−	−	−	−	−	−	−	+	−	0	0.25	0.13
Pichia guilliermondii	BPg138	+	+	+	+	+	−	−	−	−	1	0.25	0.63
Starmerella bacillaris	BSb57	−	−	+	−	+	+	−	−	−	0.25	0.5	0.38
Starmerella bacillaris	BSb58	−	−	+	−	−	−	−	−	−	0.25	0	0.13
Wickerhamomyces anomalus	BWa156	−	−	+	+	+	+	−	−	−	0.5	0.5	0.5

(+): inhibition of the spoilage yeast, (−): no inhibition of the spoilage yeast (n = 3).

Yeast species that showed biocontrol activity in our laboratories have also been cited in other studies as antagonists of different spoilage yeasts, with different mechanisms being involved. *Pichia guilliermondii*, associated with killer toxin production, has been proven to interact with *Penicillium expansum* [39]. *Wickerhamomyces anomalus* has been cited as a *B. bruxellensis* biocontroller, confirming the observations in the present study [40]. Different *W. anomalus* strains have been associated with three killer toxins [39]. This species has also been found to kill a broad range of organisms, including bacteria, hyphomycetes and yeasts [41].

Metschnikowia pulcherrima has been commented on by Oro et al. [14] because of its biocontrol capacity to a wide spectrum of genera like *Hanseniaspora*, *Pichia*, *Torulaspora*, *Zygosaccharomyces*, *Saccharomycodes*, *Candida*, *Issatchenkia*, *Brettanomyces* and *Schizosaccharomyces*, which also confirms our results. The biocontrol mechanism for *M. pulcherrima* would be iron depletion from the medium through binding to pulcherrimic acid [14].

These results can be analyzed from two perspectives: from the spoilage yeast or the antagonistic yeast point of view. Considering spoilage yeasts, the *B. bruxellensis* and *Z. rouxii* isolates analyzed in our study showed different sensitivity to *Candida sake*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Pichia occidentalis* and *Starmerella bacillaris* species. Oro et al. [14] reported a similar behavior of spoilage yeasts with different sensitivities to *M. pulcherrima* strains.

Regarding biocontrol isolates, intraspecific biocontrol was observed for BMp49 and BPg138 against all *B. bruxellensis* strains assayed. In the case of *Z. rouxii*, all 4 strains assayed were inhibited by BHu23, BMp145 and BPo108. BHu5, BHu27, BMp4, BMp29, BMp49, BPg138 and BWa156 showed an intraspecific inhibition higher than 0.5 against *B. bruxellensis*, whereas Cs88, Cs95, BHu5, BHu18, BHu23, BHu31, BHu32, BMp4, BMp29, BMp49, BMp145, BPo104, BPo108 and BWa156 demonstrated the same inhibition against *Z. rouxii*. The relevance of wide intraspecific inhibition is the possibility of avoiding adaptation of the spoilage yeast to a particular action mechanism by the antagonistic yeast [42]. In addition, wide interspecific/intergeneric inhibition is also considered a positive factor, because it may control other potential spoilage yeasts not detected in the spoilage analysis [43]. Interspecific/intergeneric biocontrol behavior against *B. bruxellensis* and *Z. rouxii* species was observed for BCi85, BCs95, BHu5, BHu27, BHu31, BHu32, BMp4, BMp29, BMp49, BMp145, BPo104, BPo108, BPg138, BSb57 and BWa156 yeasts; they biocontrolled at least one isolate from each species. Most of the yeasts with interspecific/intergeneric biocontrol showed an intraspecific inhibition of 0.5 or higher. This could be related to a common site of action of the killer toxin [42] or a common biocontrol mode of action affecting yeasts in general, like substrate competition [1].

Hanseniaspora uvarum isolates BHu5 and BHu23, *Metschnikowia pulcherrima* isolates BMp4, BMp29, BMp49 and BMp145, *Pichia guilliermondii* BPg138, *Pichia occidentalis* isolates BPo104 and BPo108 and *Wickerhamomyces anomalus* BWa156 were selected because they showed a total inhibition of 0.50 or more. Except for BHu23, all biocontroller yeasts inhibited at least one strain of both spoilage yeasts. In addition, the 10 isolates were evaluated for their enological characteristics.

3.2. Behavior of the Antagonistic Yeasts

3.2.1. Kinetic Parameters

When selecting non-*Saccharomyces* yeasts for oenological fermentations as a co-inoculum with *S. cerevisiae*, special attention should be paid to their beneficial characteristics to enhance wine quality besides their biocontrolling properties.

To achieve these goals, predominance of the selected non-*Saccharomyces* yeasts in the medium during the first stage of the fermentation is very important. Anaerobic growth kinetics of non-*Saccharomyces* yeasts possess important parameters to elucidate such predominance. Duration of the lag phase (or adaptation) and maximal growth rate are two relevant anaerobiosis parameters, which are described below [16–19].

The present study examined the kinetic parameters of each individual yeast. Nevertheless, in mixed fermentations with grape must, when limiting substrate availability is more prominent compared

with the saturation constant, each species will growth at its maximum rate. This is the main parameter to ensure predominance [16], but only when the previous state of the culture (growth stage, age and size of the inoculum) is homogeneous for all experiments [44]. Moreover, this will be governed by the chemical and physical characteristics of the environment unless one of the interacting species produces inhibitory agents against the other [16]. It is also known that the yeast complexes behave differently because of competition, antagonism or cooperation and this could result in the predominance of different yeasts [45,46].

A fermentation growth curve of each antagonist yeast was performed. Viable cell data were recorded to calculate the specific maximal growth rate (μmax) and lag phase. Most of the non-*Saccharomyces* yeasts assayed reached a specific maximal growth rate near 0.04 h^{-1} (Figure 1). BMp29, BMp49 and BMp145 showed a higher μmax which was significantly different. BPg138 and BPo104 displayed a lower μmax which was also significantly different. High specific growth rates are desired because they are a relevant factor in the prevalence of an organism during fermentation [18]. *M. pulcherrima* isolates presented the highest specific growth rates. This behavior could be related to the fact that the mode of action of this species is through the competition of limited substrates and not through a killer factor [14]. The killer factor has been found to consume metabolic energy, reducing the fitness of the yeast that possesses the factor [47], and hence it could decrease the fitness of the other non-*Saccharomyces* yeasts. Growth rates of 0.29 h^{-1} [48], 0.31 h^{-1} [49] and even 0.5 $h^{-}1$ have been found for *Saccharomyces cerevisiae* during anaerobiosis [50]. Therefore, prevalence of the non-*Saccharomyces* in the medium at the start of the fermentation should be considered for sequential co-inoculation with *S. cerevisiae*.

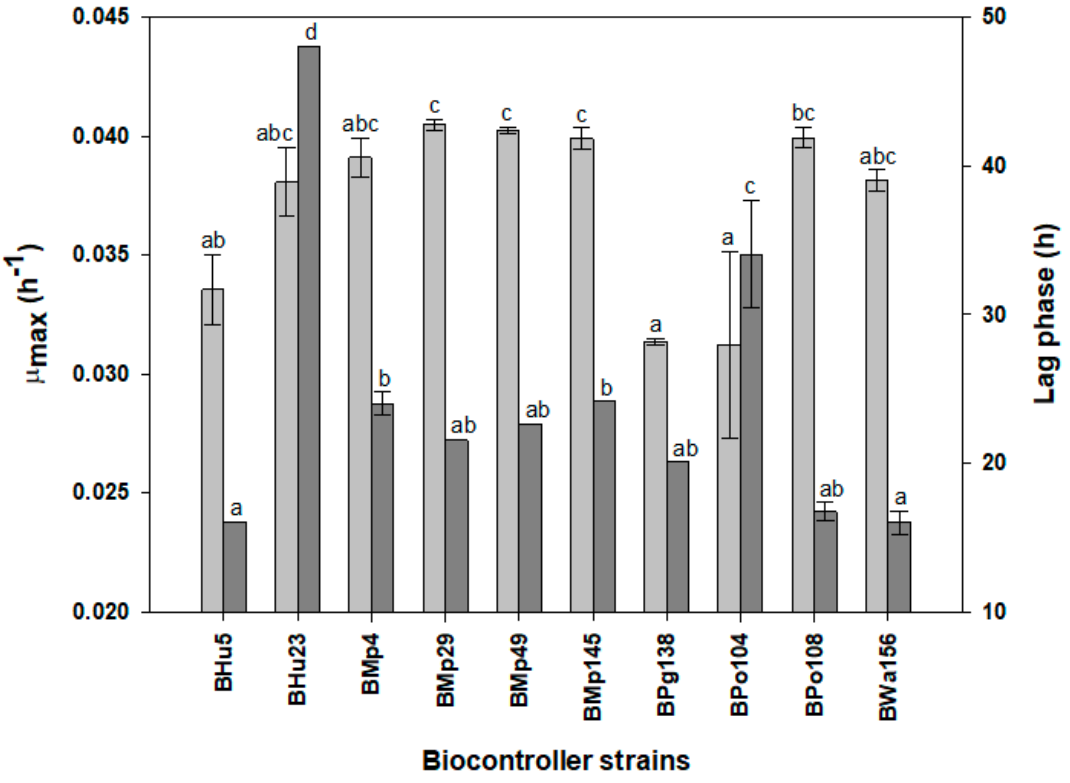

Figure 1. Specific growth rate (light grey) and lag phase (dark grey) of the non-*Saccharomyces* yeasts assayed. Rates are means with standard deviation (n = 3). Values with different letters are significantly different.

A successful predominance of the biocontroller during the fermentation start should demonstrate a short lag phase [10]. Most strains showed a lag time of about 20 h (Figure 1). BHu5 and BWa156 showed the shortest lag phases, about 15 h, and they were significantly different. BPo104 and BHu23 showed a significant longer lag phase of about 35 h and 50 h, respectively. A reduced lag phase

increments the possibility of non-*Saccharomyces* to predominate the medium, since the lag phase is defined as the period prior of reaching the specific growth rate [16]. The lag phase is also relevant for the non-*Saccharomyces* strains to achieve a constant number for a determined period of time prior to inoculation of *S. cerevisiae* in a sequential mixed fermentation.

3.2.2. Enological Characterization of Yeasts: Tolerance to Molecular SO_2, Ethanol, High Reducing Sugar Concentrations and Low Temperature

The control yeast, *S. cerevisiae* BSc114, was able to ferment grape must at 30 °Brix and 15 °C and tolerated 14% *v/v* of ethanol and 0.4 ppm of molecular SO_2 (Table 3). With respect to SO_2, the non-*Saccharomyces* yeasts BMp29, BMp49, BMp145, BPg138, BPo104, BPo108 and BWa156 showed higher tolerance to SO_2 (0.4 ppm) (Table 3). Although a reduction in SO_2 is a goal of this study, it is relevant to evaluate resistance of the selected isolates to typical SO_2 concentrations used in wineries at the beginning of the process. The chemical could be present after yeast production, but never more than 100 ppm of total SO_2 [51]. Additionally, when non-*Saccharomyces* yeasts are used in integrated management (biocontrol yeasts—SO_2 application) they should be able to tolerate certain SO_2 concentrations. Typical winemaking generally uses at least 0.5 ppm of molecular SO_2 and in order to avoid any microbial contamination, this can increase to a final molecular SO_2 concentration of 0.8 ppm [52]. This means that BMp4, which showed the lowest tolerance and did not show any growth at molecular SO_2 concentrations above 0.1 ppm, would not be suitable for integrated management. Typically, non-*Saccharomyces* yeasts have been cited to be low SO_2 tolerant, but this sensitivity could also be linked to the combination of several factors such as ethanol, SO_2 and temperature [53].

Table 3. Tolerance of individual strains to high sugar concentration and low temperature, and different concentrations of molecular SO_2 and ethanol.

Molecular SO_2 (ppm)	BHu5	BHu23	BMp4	BMp29	BMp49	BMp145	BPg138	BPo104	BPo108	BWa156	BSc114
0 (Control)	+	+	+	+	+	+	+	+	+	+	+
0.1	+	+	−	+	+	+	+	+	+	+	+
0.15	+	+	−	+	+	+	+	+	+	+	+
0.2	+	+	−	+	+	+	+	+	+	+	+
0.3	+	+	−	+	+	+	+	+	+	+	+
0.4	−	−	−	+	+	+	+	+	+	+	+
Ethanol (% *v/v*)											
0 (Control)	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	−	+	+	+
12	−	−	−	+	+	−	−	−	−	−	+
14	−	−	−	−	−	−	−	−	−	−	+
High Sugar concentration (°Brix)											
21 (Control)	+	+	+	+	+	+	+	+	+	+	+
30	−	−	−	+	−	+	−	−	−	+	+
Low Temperature (°C)											
25 (Control)	+	+	+	+	+	+	+	+	+	+	+
15	−	+	+	+	+	+	+	+	+	+	+

Tolerance of yeast strains to different SO_2 and Ethanol concentrations, and to High Reducing Sugar Concentration and Low Temperature. CO_2 production: (+) gas production in Durham tubes and (–) no gas production in Durham tubes. Fermentation results for each strain and treatment were taken one day after the start of the control fermentation. Tubes with SO_2 = 0 ppm, Ethanol = 0% *v/v*, Sugars = 21 °Brix and Temperature = 25 °C were used as controls.

Regarding ethanol tolerance, BPo104 was the least tolerant strain and did not present growth above 10% *v/v* ethanol (Table 3). The most tolerant strains were BMp29 and BMp49 (12% *v/v* of ethanol), and the remaining isolates tolerated 10% *v/v*. None of the isolates were able to grow at 14% *v/v*. Tolerance of the non-*Saccharomyces* yeasts to ethanol is especially important with increasing permanence in the fermentation medium, as the growing *Saccharomyces sp.* population produces high amounts of ethanol [1]. All isolates seemed to tolerate 8% *v/v* during the first fermentation stages [45]. However, high ethanol tolerance could be a problem, because *S. cerevisiae* uses this method to biocontrol other native microbiota [1,54]. The presence of some non-*Saccharomyces* species like

killer yeasts for prolonged periods of time could negatively modify the sensory quality of wine and cause stuck fermentation [41]. Even, the effect of metabolic interactions between non-*Saccharomyces* and *S. cerevisiae* wine yeasts could affect the growth and fermentation behavior of *S. cerevisiae* during fermentation [22]. Despite the fact that the high ethanol tolerance and wide biocontrol spectrum described for *M. pulcherrima* could be a potential risk for the normal fermentation process of *S. cerevisiae*, Oro et al. [14] mentioned that *Metschnikowia pulcherrima* does not biocontrol *S. cerevisiae*.

BMp29, BMp145 and BWa156 were able to carry out fermentation at high sugar concentrations (Table 3) whereas the remaining isolates were not. Tolerance to high sugar concentrations is relevant, because must from the Cuyo region (San Juan and Mendoza provinces) usually possesses a high sugar concentration [13]. As *Z. rouxii* yeasts are highly osmotolerant, it is extra important that *Z. rouxii* antagonists develop well under similar conditions [4,55].

With regard to tolerance to low temperature, BHu5 was the only isolate that did not grow (-). The remaining isolates were considered tolerant to low temperatures at the start of the fermentation (+). This is also a relevant factor when the biocontroller yeast is used during white wine fermentations or fermentations carried out at low temperature to preserve aromas [56].

3.2.3. Enzyme and H_2S Production

Control strain BSc114 reported low H_2S production and did not present any of the desired enzymatic activities assayed (Table 4). All non-*Saccharomyces* isolates evaluated except for BMp4 demonstrated desired protease activity (Table 4). This activity contributes to the degradation of proteins that could cause haze in the wine, thus facilitating the process of clarification and filtration [2]. Only BWa156 showed pectinase activity. This activity is another positive attribute that enables degradation of structural grape polysaccharides, increasing juice extraction and improving wine clarification and filtration [57]. It facilitates the release of aromatic precursors from the cells of the skin, seeds and flesh of the grape to the must [22,58]. Pectinase activity could be linked to a substrate colonization role or a trophic role [59]. Regarding yeast development and sugar consumption, firstly, BWa156 could be able to obtain sugars from the intracellular matrix of plant cells. In red wine fermentations with BWa156, this could generate a competitive advantage of the strain in the grape skin layer. Secondly, the yeast could consume galacturonic acid [59] as an alternative to glucose, which is quickly consumed by *S. cerevisiae* [60]. This would extend the time of this energy source for BWa156 and therefore result in a long-term competitive advantage. Although the activity is strain-dependent [22], pectinase production has already been associated with *W. anomalus* [61,62]. Nevertheless, more research is needed. None of the assayed yeasts showed β- glucosidase activity [22].

Table 4. Non-*Saccharomyces* attributes.

Isolate	Positive Traits			Negative Trait
	Protease	*Pectinase*	*β-Glucosidase*	*H_2S production*
BHu5	+	−	−	3
BHu23	+	−	−	3
BMp4	−	−	−	3
BMp29	+	−	−	3
BMp49	+	−	−	3
BMp145	+	−	−	3
BPg138	+	−	−	4
BPo104	+	−	−	3
BPo108	+	−	−	4
BWa156	+	+	−	2
BSc114	−	−	−	2

Means (n = 3). Arbitrary H_2S production scale [20]: 1: no production, 5: high production. Enzymes: (+): activity; (−): no activity.

Regarding the possible contribution to negative wine characteristics, most of the assayed yeasts showed medium H_2S production (3 or less on the scale in Table 3). Only BPg138 and BPo108 showed a higher production, 4, which is not desirable. Lowest production was produced by BWa156 (2 on the scale). H_2S production is highly relevant in winemaking and thus very important for the yeast selection because it is associated with the negative persistent odor described as "rotten egg" [27,38].

None of the isolates displayed hemolysin production. This is an important phenotypic characteristic of pathogenicity because it is related to lysis of erythrocytes [34].

Principal components analysis explained 62% of the variation among components (Figure 2). Desirable and undesirable characteristics can be clearly differentiated on the main axis (explaining 36.4%). Desirable characteristics observed were: high growth rate, tolerance to low temperature and high concentrations of ethanol, SO_2 and reducing sugars, and production of positive enzymes such as protease and pectinase. Prolonged adaptation time (Lag phase) and high H_2S production were undesirable characteristics.

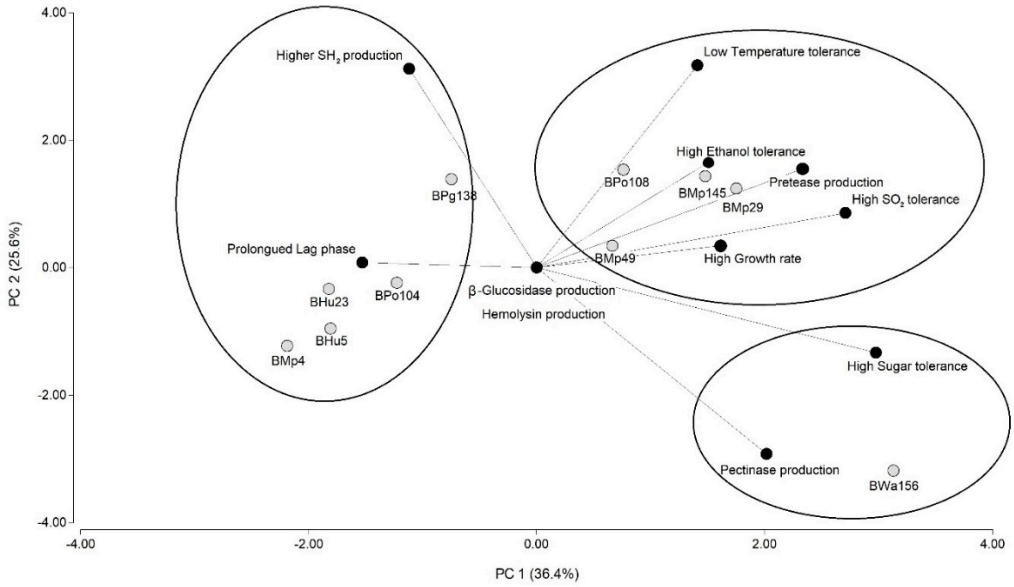

Figure 2. Principal Components Analysis (PCA) of yeast characteristics. References: Ellipses represent clusters obtained from hierarchical cluster analysis (HCA).

In the biplot it can be observed that BWa156 has the ability to prevail in the medium during the early stage of the fermentation. Compared with the other non-*Saccharomyces* assayed, this yeast possesses a short lag phase and high growth rate. The latter characteristic is related to the cellular multiplication and enables the release of killer toxins that may be constitutive [63], incrementing the possibilities of the biocontrol yeast. BWa156 also releases enzymes that could allow utilization of alternative energy sources. Our study also showed its capacity to grow in adverse must conditions such as high sugar and high SO_2 concentrations and the ability to grow at low temperature. In addition, it should be highlighted that the strain may positively contribute to the wine quality through the release of grape compounds because of its protease and pectinase production; these enzymes are not produced by BSc114. As a consequence, it could help intensify the color and enhance aromatic characteristics of the wine. Another advantage of the strain is the low H_2S production.

The biplot demonstrates that BMp29 presents more possibilities to prevail in the medium compared with the other non-*Saccharomyces* isolates assayed, because of its high growth rate and short lag phase. The strain is also able to grow under adverse conditions of grape must such as high sugar and high SO_2 concentrations and low temperature. Its high ethanol tolerance facilitates its growth and possible biocontrol during the fermentation. In the cluster, BMp49 presented a similar behavior to that of BMp29, but it did not develop at high reducing sugar concentrations. BMp145 and BPo108 also showed

similar characteristics, but the first had a prolonged lag phase and the second strain the disadvantage of a higher potential to produce H_2S.

BWa156 and BMp29 demonstrated a wide biocontrol spectrum. *Wickerhamomyces anomalus* and *Metchnikowia pulcherrima* have already been used in co-inocula with *Saccharomyces cerevisiae* by Comitini et al. [11] and Oro et al. [14]. Albertin et al. [19] described positive flavor attributes related to *M. pulcherrima*, which supports the possibility of using such species as co-inocula. However, further research is necessary to determine the biocontrol application of the two selected strains [41].

4. Conclusions

The selected non-*Saccharomyces* yeasts BWa156 and BMp29 are highly applicable antagonistic yeasts that positively contribute to the wine process. They are active against relevant spoilage yeasts in the wine industry and can be used to produce wines with reduced SO_2 concentration. The present study is part of a comprehensive research project focusing on the application of non-*Saccharomyces* biocontroller yeasts. The biocontrolling sources and the conditions of implantation, prevalence and biocontrol kinetics is the projection of future research.

Author Contributions: Conceptualization, B.K., F.V., M.E.T., Y.P.M. and M.C.; methodology and experimental design, F.V., M.C. and Y.P.M.; software, B.K. and M.V.M.; validation, F.V. and M.E.T.; formal analysis, B.K., M.V.M. and F.V.; investigation, B.K., Y.P.M., M.V.V., M.C., M.E.T. and F.V.; data curation, B.K., writing—original draft preparation, B.K.; writing—review and editing, B.K., F.V., M.E.T. and Y.P.M.; visualization, B.K.; supervision, F.V., M.E.T. and M.C.; project administration, Y.P.M.; funding acquisition, Y.P.M. All authors discussed the results of the experiments and contributed to the final manuscript.

Funding: This work was supported by the Research Project IDeA 1400- SECITI 0041-2014.

Acknowledgments: The authors want to thank the Chemical Engineer Martha Dina Vallejo for the suggested corrections, the chemical donations and the accompaniment.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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