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ORIGINAL PAPER

Comparative study regarding the ethanol resistance of some yeast strains isolated from Romanian vineyards

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ARINA OANA ANTOCE[1(*)], IOAN CONSTANTIN NĂMOLOŞANU[1], FLORENTINA MATEI RADOI[2]

[1] University of Agronomical Sciences and Veterinary Medicine of Bucharest, Faculty of Horticulture and Romanian Authorized Winetasters Association, 59, Marasti Ave., Bucharest, 010464 Romania, ph. +40-21-318.02.97, fax +40-21-31828.88, E-mail: aantoce@yahoo.com

[2] University of Agronomical Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnology 59, Marasti Ave., Bucharest, 010464 Romania

[(*)] Corresponding Author

Abstract

Recently there is increased interest in the identification and characterization of new valuable yeast strains to be used for the production of quality wines with specific traits. Such new strains need to be tested extensively regarding their behavior in relation to various factors. The paper presents results on the resistance to ethanol of ten yeast strains from the Saccharomyces genus selected from Romanian vineyards. The behavior of yeasts in the presence of ethanol was investigated using a calorimetric technique which allows for the determination of the growth rate as well as quantitative parameters that describe the inhibition of microbial growth by ethanol, such as the Minimum Inhibitory Concentration (MIC) and the concentration of ethanol that reduces microbial activity by 50%. It can also indicate if the inhibition mechanism is of a bactericidal or of a bacteriostatic nature. The technique allows for a comparison of the various yeast strains from the viewpoint of ethanol tolerance, with a view to selecting the most suitable strain for certain winemaking processes.

Keywords: ethanol inhibition, wine yeast strains, isothermal calorimetry

Introduction

Studies in the field of yeast selection for wine production have identified in vineyards (in the soil, on grape skins and on the leaves, stems and vines) over 700 different species of yeasts belonging to more than 100 genera, of which at least 15 genera are directly involved in the winemaking process (Antoce, 2007). Yeasts located on the grape skins depend on grape variety, vintage and degree of grape maturation. These are the main microorganisms transferred in the grape must after crushing and also the ones who develop at least during the first stages of the must fermentation. Of all genera, *Sacharomyces* is recognized as being the most important for winemaking (Boulton *et al.*, 1995), as it is more ethanol tolerant then most others and thus able to finalize the fermentation and produce a dry wine. However, some non-*Saccharomyces* yeasts may also show good oenological properties (Ciani and Maccarelli, 1998) and their fermentation characteristics and parameters were studied in association with winemaking (Ciani and Picciotti, 1995; Ciani *et al.*, 2009).

Therefore, in order to produce a particular type of wine, isolating and selecting the most suitable yeasts that work particularly well in certain conditions is imperative (Mauricio *et al.*, 1997, Romano *et al.*, 1997). At present, winemakers can choose from a wide variety of yeasts provided by several large companies, yeasts that display a wide range of special characteristics adapted to specific needs. There are yeasts that can

ferment at high or low temperatures (Gao and Fleet, 1988), with slower or faster fermentation rates, foaming or non-foaming yeasts, strains that impart a particular aroma to the wine or, on the contrary, preserve the natural varietal flavor of the grapes (Pretorius *et al*., 2007), yeasts that do not produce compounds responsible for off-flavors and so on.

In line with the evolution of the consumers' preferences and even with climate changes that bring about a higher than before sugar concentration, finding yeasts with special traits is a continuous work (Pretorius *et al*., 2007). In this research our team tried to identify some local yeasts which can preserve the typical flavor of the local wines, at the same time being robust enough to ferment musts with high concentration of sugar and tolerate the ethanol produced as a result of fermentation. Tolerance to ethanol was a parameter considered to be one of the first to test, because during fermentation, aside of the osmotic pressure stress which the yeast cells encounter due to high sugar presence in the medium, ethanol inhibition is one of the most important adverse conditions (Ingram and Buttke, 1984; Birch and Walker, 2000; Querol *et al*., 2003; Xue *et al*., 2008).

In order to test the yeast species and strains isolated from Romanian vineyards in the framework of Project PNCDII 51065, a rapid method for testing yeast resistance to ethanol based on the principle of isothermal calorimetry was used (Antoce and Năмoloşanu, 2011).

The yeasts were classified in three groups on the basis of their ethanol resistance and used in accordance to their potential.

Materials and methods

Ten *Saccharomyces* strains identified and selected from Romanian vineyards were tested for their tolerance to ethanol. The yeasts, belonging to the collection of the University of Agronomical Sciences and Veterinary Medicine of Bucharest, were the following: *Torulaspora delbrueckii B19*, *Saccharomyces bayanus B3*, *S. cerevisiae B37*, *S. cerevisiae B116*, *S. bayanus B156*, *S. cerevisiae MB6b*, *S. cerevisiae B10*, *S. cerevisiae B193*, *S. cerevisiae B196* and *S. cerevisiae B20*.

The method used for the evaluation of the ethanol tolerance of the yeasts is based on microbial isothermal calorimetry (Nunomura and Fujita, 1981, Ashby and Beezer, 1996; Winkelmann *et al.*, 2004, Kaletun, 2009) and our particular apparatus and its application was in detail described elsewhere (Antoce and Namolosanu, 2011). This method has the advantage of providing a complete description of the inhibition, allowing the determination of both minimum inhibitory concentration parameters (MIC_{μ} and MIC_{θ}) and the inhibitor concentration necessary to halve the number of viable microorganisms (K_{μ}) or, similarly, to double the duration required for the microbial population to reach a certain level in the presence of an inhibitor (K_{θ}). The determination of both types of parameters (MIC and *K*) is important in inhibition characterization because, due to the different degree of cooperativeness in the inhibitor-microbial cell interaction, there are cases in which strains with the same minimum inhibitory concentration have very different values for *K*, or the other way around. Moreover, the method can discriminate between the bactericidal and bacteriostatic effect of an inhibitor on a certain strain.

The tests were performed in sterile Yeast Extract-Glucose-Peptone (YPG) broth containing 20 g/l glucose, 10 g/l yeast extract and 20 g/l peptone. For each test, sets of 25 calorimetric vials containing 5 ml of broth received various quantities of ethanol and then were inoculated with the yeast strain to be evaluated. The concentrations of ethanol added in each experiment varied from 0 to 8.73% v/v and the inoculum size in each vial was of the order of 10^6 cells/ml.

The vials were then incubated in the calorimeter at 30°C and the growth thermograms were recorded and analyzed with a special software. The microbial calorimeter and the principle of its construction are presented extensively in a previous paper (Antoce and Năməloşanu, 2011).

Results and discussions

All the strains tested showed good growth in the range of ethanol concentration applied and the growth thermograms or the $g(t)$ curves are shown in Fig. 1.

By analyzing these $g(t)$ curves in accordance with the methodology presented by Antoce and Năməloşanu (2011) the growth rates of each yeast strain in the absence of ethanol and the inhibition parameters in the presence of ethanol were calculated.

Table 1 shows the growth rate constants of the yeasts at the beginning of the fermentation, when no ethanol is present in the medium. The values are reported as a mean of 4 repetitions, and the yeast strains with similar growth rates, established on the basis of one-way ANOVA (analysis of variance) and Tukey test, are also shown. The yeast strains with growth rates that do not significantly differ from one another are noted with the same letter.

Table 1. Growth rate of the isolated yeast strains in YPG broth, at 30°C in the absence of ethanol

No.	Yeast strain	Growth rate constants in the absence of ethanol, μ	Strain similarity in growth rate (ANOVA)
1.	*Torulaspora delbrueckii B19*	0.3552 ± 0.0195	
2.	*Saccharomyces bayanus B3*	0.2150 ± 0.0363	
3.	*Saccharomyces cerevisiae B37*	0.1804 ± 0.0427	
4.	*Saccharomyces cerevisiae B116*	0.4685 ± 0.0201	b,d,e,f
5.	*Saccharomyces cerevisiae B156*	0.4683 ± 0.0164	c,f
6.	*Saccharomyces cerevisiae MB6b*	0.5180 ± 0.0133	a,c,d
7.	*Saccharomyces cerevisiae B10*	0.3503 ± 0.0267	
8.	*Saccharomyces cerevisiae B193*	0.4660 ± 0.0081	a,e
9.	*Saccharomyces cerevisiae B196*	0.4416 ± 0.0022	b
10.	*Saccharomyces cerevisiae B20*	0.3089 ± 0.0116	

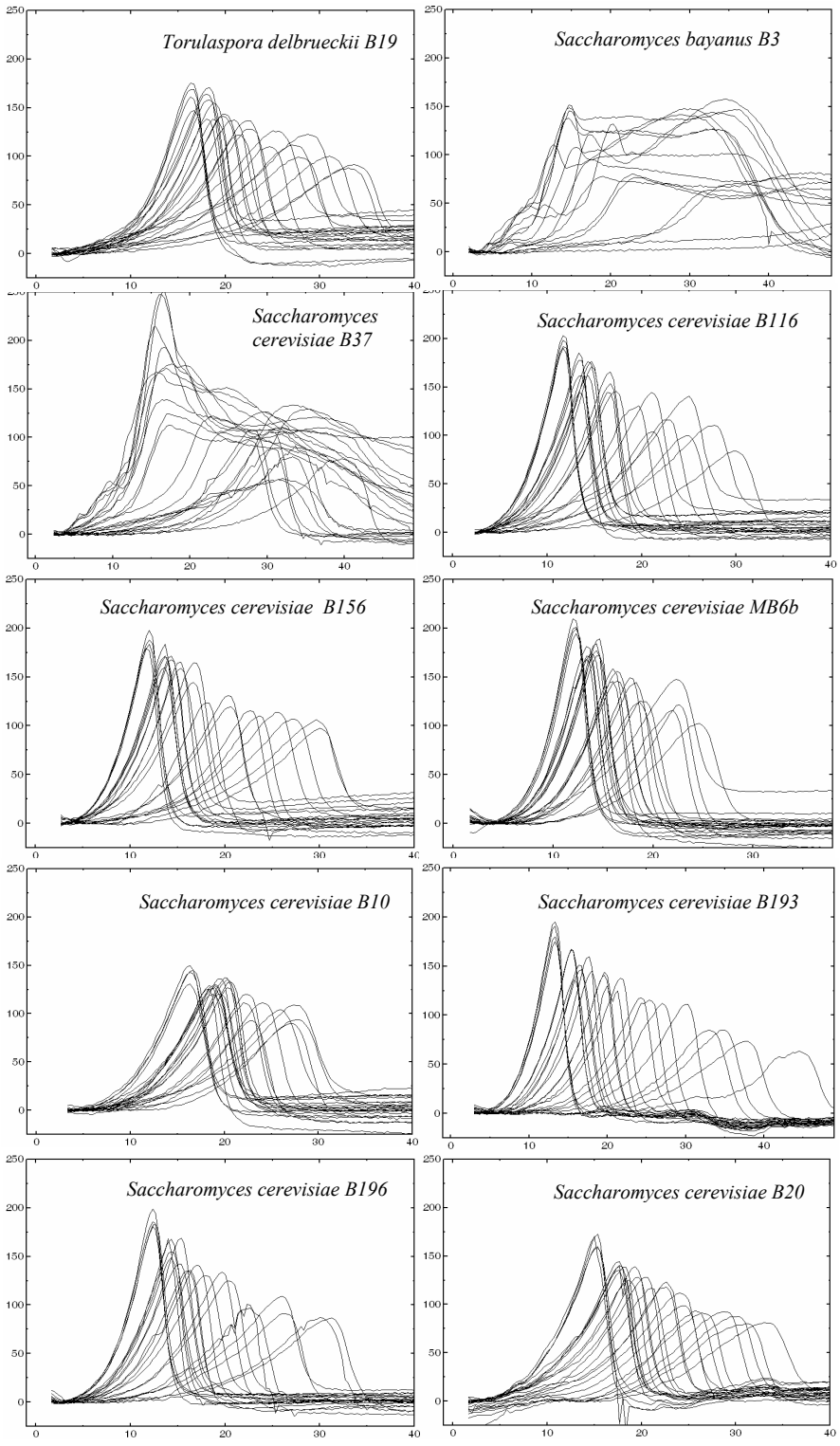

Fig. 1. Growth thermograms or $g(t)$ curves recorded in the presence of various concentrations of ethanol for ten strains of *Saccharomyces* yeasts selected in Romanian vineyards

It can be observed that *S. cerevisiae B116, B193 and B196* strains show similar growth rate constants, fact which confirms their classification in the same group. The rest of the yeast strains do not seem to belong to a certain group as far as the growth rate is concerned.

The growth rate is related to the ability of yeast strain to adapt to the growth conditions and is correlated to the rate of grape must fermentation. Taking into consideration

that the faster the growth of an inoculated yeast in must, the faster is the inhibition of the other wild yeast strains transferred in the must from the grapes, leaves and equipments, the selected yeasts with higher growth rate constants are more desirable for the obtainment of quality wines.

The inhibition parameters for all tested yeast strains, calculated from both growth rate constant reduction method (K_μ and MIC_μ) and growth retardation (K_θ and MIC_θ) method are included in Table 2. The values were calculated as a mean for three calorimetric experiments for each strain and are expressed in % v/v ethanol.

The parameter *m*, which represent the degree of cooperativeness in the inhibitor-yeast cell interaction, is non-dimensional and is correlated to the steepness of the drug potency curves (exemplified in Fig. 2) derived by analyzing the recorded signals from the calorimeter.

In the case of *S. cerevisiae B37* and *S. cerevisiae B20* the inhibition parameters could not be determined from the growth rate constant reduction, because for these strains the ethanol showed a bactericidal effect, killing from the beginning a number of yeast cells, but not affecting the growth rate of the viable cells, hence, the growth rate remained constant irrespective of the amount of ethanol added.

Table 2. Quantitative parameters that describe the resistance to ethanol of the ten yeast strains isolated from Romanian vineyards.

Yeast strain	m_μ	K_μ % v./v. EtOH	MIC_μ % v./v. EtOH	m_θ	K_θ % v./v. EtOH	MIC_θ % v./v. EtOH
T. delbrueckii B19	1.39 ± 0.20	5.88 ± 0.42	14.18 ± 1.78	1.84 ± 0.29	9.02 ± 0.68	14.44 ± 1.66
S. bayanus B3	7.65 ± 2.75	4.58 ± 0.20	5.66 ± 0.33	3.33 ± 0.68	3.11 ± 0.20	5.63 ± 0.47
S. cerevisiae B37	***	***	***	1.49 ± 0.12	2.97 ± 0.13	11.33 ± 1.20
S. cerevisiae B116	1.38 ± 0.21	7.20 ± 0.55	15.06 ± 1.87	1.52 ± 0.12	5.07 ± 0.19	12.52 ± 0.73
S. cerevisiae B156	1.75 ± 0.22	7.30 ± 0.40	13.50 ± 1.21	1.74 ± 0.08	4.93 ± 0.10	11.43 ± 0.31
S. cerevisiae MB6b	1.52 ± 0.22	8.95 ± 0.80	15.55 ± 2.25	1.46 ± 0.12	8.43 ± 0.38	16.13 ± 1.15
S. cerevisiae B10	2.99 ± 0.64	8.67 ± 0.55	11.73 ± 1.02	1.05 ± 0.11	7.87 ± 0.55	20.66 ± 2.65
S. cerevisiae B193	1.62 ± 0.18	7.40 ± 0.37	13.75 ± 0.95	1.59 ± 0.09	4.11 ± 0.11	11.06 ± 0.21
S. cerevisiae B196	1.95 ± 0.16	7.47 ± 0.23	11.96 ± 0.56	1.75 ± 0.10	5.72 ± 0.13	11.31 ± 0.25
S. cerevisiae B20	***	***	***	1.64 ± 0.06	6.13 ± 0.10	13.06 ± 0.43

*** these values could not be determined due to a bactericidal effect of ethanol

The drug potency curves and the MIC curves obtained from the processing of the recorded calorimetric signals and fitting the specific growth activity data points with specific equations (Antoce and Nămoloşanu, 2011) are exemplified in Fig. 2, for 5 of the 10 yeast strains, in continuous and discontinuous line, respectively. From these curves the quantitative inhibition parameters are derived: the MIC is the value where the MIC curve intersects the ethanol concentration axis, while the *K* value is obtained as the ethanol concentration at which the specific growth activity is reduced by 50%. In this figure on the left side there are the drug potency curves and MIC curves derived from the growth rate constants obtained in the presence of various ethanol concentrations, while on the right side there are the curves derived from the data points representing the relative retardation of growth due to the presence of ethanol in a certain concentration.

At this point it must be emphasized that both types of results, calculated from the specific growth rate activity or from the specific retardation of growth actually describe the same inhibition phenomenon. In most cases, the values taken into consideration as inhibition parameters are those derived from the specific growth activity data point, μ_i/μ_m, that is the parameters K_μ and MIC_μ. This is the most common case of inhibition encountered in nature – the bacteriostatic inhibition. Hence, in the case of a perfect bacteriostatic inhibition, the values determined for K_θ and MIC_θ do not differ much from the values of K_μ and MIC_μ, respectively.

And indeed, in 8 out of 10 of our studied yeast strains, because the action of the ethanol was bacteriostatic, the values determined in both ways did not differ too much.

However, there are also cases, such as the one of *S. cerevisiae B37* (Fig. 2, the fourth left diagram) in which the ethanol acts as a bactericidal inhibitor. As it can be seen, although a significant number of yeast cells are killed from the beginning in the media containing ethanol, the cells that survive are capable of growing with a rate μ_i almost similar with the growth rate attained in the absence of ethanol μ_m. This fact leads to values of the parameter μ_i/μ_m that remain approximately constant and equal to 1, which in turn makes impossible the fitting of the data points and the calculation of the inhibitory parameters K_μ and MIC_μ. For a case like this, the analysis based on the specific growth retardation $t_\alpha(0)/t_\alpha(i)$ provides an alternative for the calculation of the quantitative inhibition parameters.

Another situation is that of *S. cerevisiae B20*, in which the effect of ethanol is also bacteriostatic, but, as it can be seen in Fig. 2 (the fifth left diagram of the group), the values of the parameter μ_i/μ_m are constant only at small concentrations of ethanol; at higher concentrations the effect is becoming bacteriostatic and the values of μ_i/μ_m start decreasing.

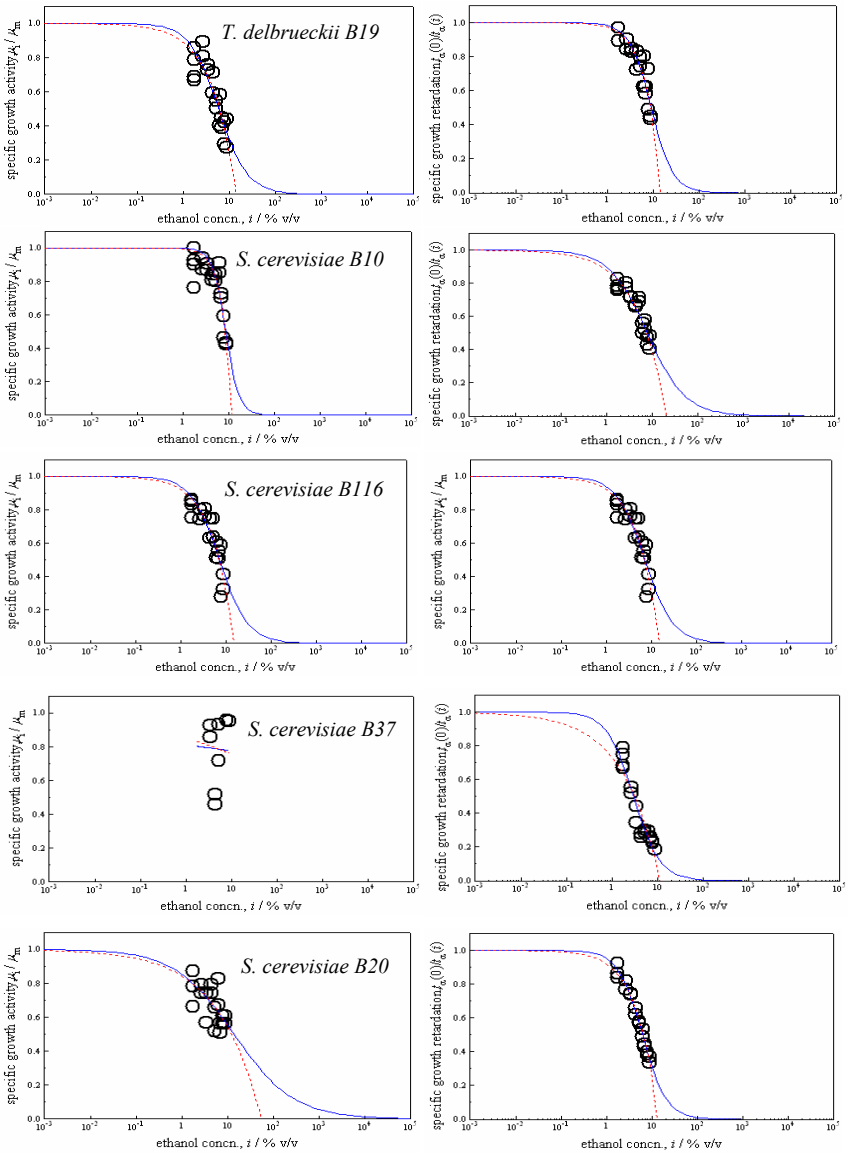

Fig. 2. Examples of drug potency curves (continue lines) and the MIC curves (discontinued line) obtained by processing the calorimetric data recorded for 5 yeast strains.

Conclusions

Examination of the quantitative inhibition parameters determined for the 10 selected yeast strains isolated in Romanian vineyards leads to some interesting conclusions. The yeast strain *S. bayanus B3* proved to be the least resistant to ethanol, both from the viewpoint of the reduction of the growth rate constant in the presence of ethanol and from the specific retardation of growth. Moreover, the degree of cooperativeness for ethanol (Antoce and Năməloşanu 2011) in its case ($m_{\mu,}$ but also m_{θ}) has the highest value determined for this parameter, which means the inhibition with ethanol occurs abruptly, even when the increase of ethanol in the medium is still in small aliquots. This is an unexpected behavior for a strain of *S. bayanus,* an yeast famous among the commercially available yeasts for its ethanol tolerance, being the first selection for the sparkling wine production. However, *S. bayanus* is a cryophilic yeast and the temperature of 30°C at which the tests were conducted may also have acted as an inhibiting factor.

Another interesting behavior is manifested by the *S. cerevisiae B20*, on which the ethanol has a clear bactericidal effect, as compared to all the rest yeast strains on which the ethanol acts in a bacteriostatic way.

Saccharomyces rosei, also classified as *Torulaspora delbrueckii* (Jolly *et al.*, 2006) is another strain with an interesting behavior as far as its inhibition by ethanol is concerned. This strains has an MIC of 14-14,5% v/v (in accordance with both determination methods), while the *K* parameters are different: the decrease in the growth rate constant in the presence of ethanol by 50% leads to a K_{μ} value around 6% v/v ethanol, while the doubling of the time necessary for the growth to reach a certain level leads to K_{θ} value 9% v./v. Strains of this yeast are often encountered in fermenting musts, even in the final stages of the fermentation (Dragomir-Tutulescu and Popa., 2009) and found to produce less volatile acidity then other *Saccharomyces* strains (Moreno *et al.,* 1991).

The most resistant to ethanol inhibition were *S. cerevisiae B116* and *S. cerevisiae MB6b*. The group of strains tolerant to ethanol is completed with *S. cerevisiae B10*, which has the value of MIC_{μ} in the normal range of 11-12% ethanol, but, seems to be much more resistant if we rely on the MIC_{θ} value calculated to be of 18-20% ethanol. Such differences are explicable when we look at the data points distribution of specific activities μ_i/μ_m and $t_{\theta}(0)/t_{\theta}(i)$ which leads to a difference in the steepness of the fitted curves. In these rare cases, the reliable calculated parameters are those obtained on the basis of the growth rate constant, K_{μ} and MIC_{μ}.

The ethanol inhibition parameters obtained for our selected strains are, generally, in accordance with the levels reported in literature (Pina *et al.*, 2004). However, for *S. bayanus B3* the inhibitory parameters were unexpectedly low, fact that could be explained by the influence of the test temperature which was unfavorable for a cryophilic strain such as this.

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