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ORIGINAL PAPER

Monitoring of killer yeast populations in mixed cultures: influence of incubation temperature of microvinifications samples

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Abstract Killer yeasts are frequently used to combat and prevent contamination by wild-type yeasts during wine production and they can even dominate the wine fermentation. Stuck and sluggish fermentations can be caused by an unbalanced ratio of killer to sensitive yeasts in the bioreactor, and therefore it is important to determine the proportion of both populations. The aim of this study was to provide a simple tool to monitor killer yeast populations during controlled mixed microvinifications of killer and sensitive *Saccharomyces cerevisiae*. Samples were periodically extracted during vinification, seeded on Petri dishes and incubated at 25 and 37 °C; the latter temperature was assayed for possible inactivation of killer toxin production. Colonies developed under the described conditions were randomly transferred to killer phenotype detection medium. Significant differences in the killer/sensitive ratio were observed between both incubation temperatures in all microvinifications. These results suggest that 37 °C seems a better option to determine the biomass of sensitive yeasts, in order to avoid underestimation of sensitive cells in the presence of killer yeasts during fermentations. Incubation at a toxin-inhibiting temperature clearly showed the real ratio of killer to sensitive cells in fermentation systems.

Keywords Mixed cultures · Killer and sensitive yeasts · Samples incubation temperature

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Introduction

Killer yeasts are immune to the antimicrobial compounds they produce. Killer/sensitive (K/S) yeast interactions have been detected in a variety of genera obtained from different sources (e.g. *Saccharomyces, Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Kluyveromyces, Pichia, Williopsis* and *Zygosaccharomyces*) (Abranches et al. 1997; Magliani et al. 1997; Buzzini and Martini 2001; Ciani and Fatichenti 2001; Pérez-Nevado et al. 2006; Liu and Tsao 2009; Santos et al. 2009). Toxins produced by killer yeasts exclude other species from their habitats, and killer yeasts have several applications in the food and fermentation industries. It is important to determine the degree of real killer yeast dominance under different fermentation conditions because it has been found that stuck

and sluggish wine fermentations depend on the ratio of killer/sensitive cells (Shimizu 1993; Magliani et al. 1997; Medina et al. 1997; Musmanno et al. 1999).

Detection of killer-sensitive yeast interactions strongly depends on the strains used. Killing ability of yeasts may be underestimated or even remain unnoticed depending on the selection of the appropriate sensitive strain and the assay conditions applied (Llorente et al. 1997). Susceptibility of indigenous microbiota to killer strains should be assessed prior to selecting a yeast starter strain for fermentation (Magliani et al. 1997; Hernández et al. 2008). In this context, killer and sensitive fermentation experiments have become relevant. However, contradictory results have been reported regarding the adequate initial killer/sensitive ratio that allows killer yeasts to predominate fermentation environments (Petering et al. 1991; Musmanno et al. 1999; Pérez et al. 2001; Pérez-Nevado et al. 2006). It is not only important to detect killer yeasts in mixed cultures but also resistant and sensitive strains. Methods used to identify killer and sensitive strains are based on the growth rates of the strains, production of H_2S, use of auxotrophic and respiratory-deficient mutants and fluorescent staining (Rosini 1985; Jacobs et al. 1988; Kurzweilová and Sigler 1993). Assaying of killer activity has been thoroughly described by several authors (Longo et al. 1990; Jacobs and van Vuuren 1991; Sangorrín et al. 2002). However, evaluation of killer-sensitive biomass dynamics in experiments is limited because of the difficulty of monitoring different strains when growing in mixed cultures. This is due to the different conditions of genuine fermentation environments and experimental cultures. Some of the methodologies used to determine biomass dynamics are evaluation of randomly picked representative colonies, the use of differential media, and antibiotic resistance (da Silva 1996; Ramon-Portugal et al. 1998; Pérez et al. 2001; Vadasz et al. 2002). The incubation temperature in these studies ranged from 18 to 28 °C, which allows expression of the *Saccharomyces cerevisiae* killer toxin. Several authors reported that toxins from *Saccharomyces* and other killer genera normally lose their activity at higher temperatures (>35 or 40 °C) (Soares and Sato 2000; Ciani and Fatichenti 2001).

The aim of the present work was to provide a simple and economical tool to monitor killer and sensitive yeast populations during controlled mixed fermentations and obtain an accurate K/S ratio by growing samples at a toxin-inhibiting temperature.

Materials and methods

Microorganisms

Wild and mutant yeasts were obtained from the Biotechnology Institute Culture Collection (National University of San Juan, Argentina): BSc400 and BSc411, two *S. cerevisiae* killer phenotypes, and BSc377, a *S. cerevisiae* sensitive phenotype.

Wild yeasts were isolated from enological environments in the San Juan province, Argentina, and identified by conventional morphological, physiological and biochemical procedures according to Kurtzman and Fell (1998). Molecular identification was carried out by PCR RFLP amplification of the rRNA ITS1-5.8S-ITS2 region and nuclear complex genetics and polymorphism analysis of the restriction fragments (ITS1-5.8S-ITS2 PCR RFLP), according to Esteve-Zarzoso et al. (1999).

BScIM41, an isogenic mutant of BSc411 (sensitive phenotype) obtained after acridine orange treatment (Cansado et al. 1989), and ATCC 38636 were also employed in the fermentation experiments.

Culture media

YEPD broth was used as propagation medium (g/l): Yeast Extract (Difco) 10, Peptone (Difco) 20, Glucose (Difco) 20, pH 4.6.

Experimental fermentations (microvinifications) were carried out as follows. Commercial concentrated Pedro Jiménez grape must was diluted to 21°Brix by addition of distilled water and then supplemented with 1 g/l yeast extract (Medina et al. 1997). The final pH was adjusted to 4.6, a common pH at the start of wine fermentation in Argentina. The mixture was heated at 70 °C for 20 min and cooled down to ambient temperature. This operation was repeated on three consecutive days to eliminate natural microbiota (Toro and Vazquez 2002).

Citrate-buffered YEPD broth was used to grow strains prior to killer assays (g/l): Yeast Extract 10, Peptone 20, Glucose 20, 0.1 M citrate-phosphate buffer; pH 4.6.

YEPD Agar-MB, containing 0.003 % (w/v) methylene blue (MB) and 0.1 M citrate-phosphate buffer, pH 4.6, was used for killer phenotype assaying (da Silva 1996; Buzzini and Martini 2001; Pérez et al. 2001).

Fermentation conditions

Yeast strains were inoculated in 250-ml Erlenmeyer flasks containing 50 ml of propagation medium for 12 h (exponential growth phase) under agitation (200 rpm) at 25 °C.

To compare the fermentation behavior of the strains in mixed K/S cultures the following yeast proportions were used: BSc400-BSc377 (1–99 %; 10–90 %; 50–50 %), BSc411-BScIM41 (1–99 %; 10–90 %; 50–50 %) and BSc411-ATCC38636 (1–99 %; 10–90 %; 50–50 %).

Microvinifications were carried out in 250-ml Erlenmeyer flasks with 150 ml of diluted commercial concentrated Pedro Jiménez grape must at a final inoculum

concentration (100 %) of 2×10^6 cells/ml. Flasks were aseptically sealed with a Müller valve (a glass device which contains 50 % sulphuric acid that allows only CO_2 to escape from the system) and statically incubated at 25 °C. Fermentations were controlled measuring the weight loss of the flasks due to CO_2 production until constant weight (completed fermentation) (Vazquez et al. 2001; Kurtzman and Fell 1998).

Samples of approximately 400 µl were periodically taken to determine killer biomass dynamics.

Killer yeast viability and phenotype stability

BSc411 and BSc400 were assayed at 37 °C for inactivation of the toxin production, yeast survival rate and mutagenesis, regarding killer factor repair (Nally et al. 2005). The well test was performed with culture supernatants of both killer yeasts (Ciani and Fatichenti 2001; Ramón-Portugal et al. 1994). Plates were examined for inhibition halos on sensitive lawns at 25 and 37 °C; the killer toxin is considered to be inactivated at the latter temperature (Wickner 1974; Soares and Sato 2000).

Killer phenotype assaying at 25 and 37 °C

Mixed K/S microvinification samples were diluted, seeded onto YEPD Agar and incubated at both 25 and 37 °C for 2–3 days to develop 200–300 colonies. Developed colonies (250) were randomly transferred to buffered YEPD Agar-MB to determine the K/S cell ratio as described by Bussey et al. (1973). The sensitive yeast corresponding to each mixed microvinification was used as lawn.

Data analysis

All assays were carried out in triplicate and repeated measures analysis of variance (ANOVA) and one-way ANOVA ($p \leq 0.05$) were performed using SPSS Base Version 17.0 statistical software (SPSS Inc. Chicago, IL, USA). The percentages of yeasts were arcsine-square-root transformed before analysis of variance in order to satisfy the assumptions of the ANOVA that data be normally distributed.

Results

Killer yeast assays showed inhibition halos at 25 °C but not at 37 °C. No significant differences were observed in survival rate or killer factor repair at the temperatures assayed: 25 or 37 °C.

Statistical analysis of the development of killer yeast populations (Table 1; Figs. 1, 2, 3) showed a significant difference between microvinifications with the same inoculum proportions incubated at 25 or at 37 °C. This difference was observed throughout the fermentation process (repeated measures ANOVA) and in most of the samples from each experimental condition (one-way ANOVA).

1 % killer/99 % sensitive inoculum ratio

Mixed cultures of BSc400 and BSc377 showed a significant difference in killer biomass between incubation at 25 and 37 °C throughout the experiment (Table 1).

Distinct prevalence patterns became evident when BSc411 was co-cultured with different sensitive yeasts. After 288 h, the killer population dominated (99.2 %) the mixed culture medium with BScIM41 when samples were incubated at 25 °C (Fig. 1; Table 1). Nevertheless, when the same samples were grown at 37 °C, the killer population was significantly lower: 90.03 % (Table 1). After 336 and 384 h the killer yeast population had completely outgrown the sensitive strain when samples were incubated at 25 °C, whereas at 37 °C, the sensitive population was still present with 4.2 and 1.6 %, respectively (Table 1).

BSc411 outgrew (51.3 %) ATCC 38636 after 120 h, when samples were incubated at 25 °C (Fig. 1). At 37 °C, BSc411 represented 58.8 % of the total population after 216 h (Table 1). At both incubation temperatures killer yeasts did not completely dominate the medium (86.7 % after 528 h and 75.85 % after 480 h at 25 and 37 °C, respectively). Furthermore, a decrease in killer yeast prevalence was observed at both sample incubation temperatures (Fig. 1). Almost all microvinification sample times showed a higher number of killer yeasts after incubation at 25 °C than at 37 °C. Significant differences were found after 16 out of 19 sample times with one-way ANOVA (Table 1).

10 % killer/90 % sensitive inoculum ratio

The killer population oscillated during microvinifications with 10 % killer/90 % sensitive strains, and this fluctuation was more evident when samples were incubated at 37 °C (Fig. 2). Biomass showed significant differences between both incubation temperatures in 11 of the 19 samples statistically analyzed. A higher presence of sensitive cells could be observed at 37 °C, which inhibited killer activity (Table 1). After 12 h of incubation, BSc400 prevailed over BSc377 until the end of the experiment at this temperature.

BScIM41 was almost eliminated after 24 h of incubation in mixed microvinifications with BSc411 (Fig. 2). The percentage of sensitive yeasts in samples incubated at 25 and 37 °C was significantly different at this stage of the fermentation with 16.13 and 36.34 %, respectively (Fig. 2; Table 1).

Table 1 Dominance of killer yeasts and significant differences (ANOVA) between killer (K) and sensitive (S) populations after different fermentation times (h) under all experimental conditions assayed

	BSc400 (K)/BSc377 (S)						BSc411 (K)/BScIM41 (S)						BSc411 (K)/ATCC38636 (S)					
	A		B		C		A		B		C		A		B		C	
Time (h)	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C
0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	O	–	–	–	–	–	–	–	O	–	O	–	–	–	O	–	O	–
24	O	–	O	–	–	–	–	–	O	–	O	–	O	–	O	–	O	–
48	O	–	O	–	O	–	O	–	–	–	–	O	–	–	O	–	X O	–
72	O	–	O	–	O	–	–	O	O	–	–	X O	O	–	O	–	X O	–
96	O	–	–	–	O	–	–	–	x	x	x	x	O	–	O	–	X O	–
120	O	–	O	–	O	–	–	O	x	x	x	–	O	–	O	–	x	–
144	O	–	O	–	O	–	–	O	–	–	x	x	O	–	O	–	x	x
168	O	–	O	–	O	–	–	O	X O	–	x	x	O	–	O	–	x	–
192	O	–	O	–	O	–	–	O	X O	–	x	x	O	–	O	–	x	x
216	O	–	–	–	–	–	–	O	–	–	x	x	O	–	–	–	x	–
240	O	–	O	–	O	–	–	O	x	–	x	x	O	–	O	–	x	–
288	O	–	–	O	O	–	O	–	X O	–	x	x	O	–	–	–	X O	–
336	O	–	–	–	O	–	X O	–	x	x	x	x	O	–	–	–	X O	–
384	O	–	O	–	–	O	X O	–	x	x	x	x	O	–	X O	–	X O	–
432	O	–	–	O	O	–	x	x	x	x	x	x	O	–	–	–	X O	–
480	O	–	–	–	O	–	x	x	x	x	x	x	O	–	x	–	x	–
528	O	–	O	–	O	–	x	x	x	x	x	x	O	–	x	–	x	x
576	O	–	O	–	O	–	x	x	x	x	x	x	O	–	x	–	x	–

A: 1 % Killer/99 % Sensitive, B: 10 % Killer/90 % Sensitive and **C**: 50 % Killer/50 % Sensitive; x: 100 % killer yeasts (complete dominance); –: no dominance of killer yeasts; O: Significant difference observed between samples incubated at 25 and 37 °C, indicating a higher number of killer yeast colonies; X O: 100 % dominance of killers and significant differences in number of killer yeasts colonies detected at both incubation temperatures (25 and 37 °C)

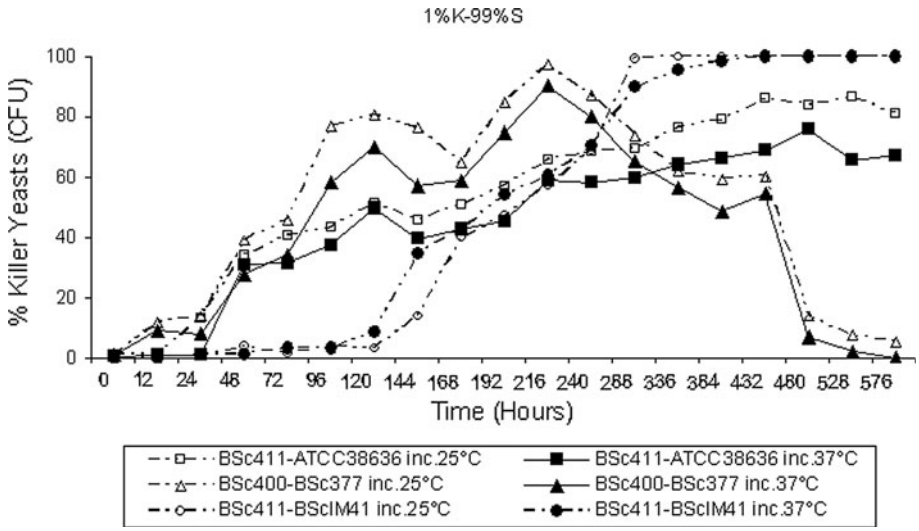

Fig. 1 Killer yeast proportion after different fermentation times in mixed microvinifications of 1 % killer/99 % sensitive strains. Bioreactor samples were incubated at 25° and 37 °C before cell counts. Repeated measures ANOVA showed significant differences throughout the experiment ($p = 0.0002$; F = 54.56)

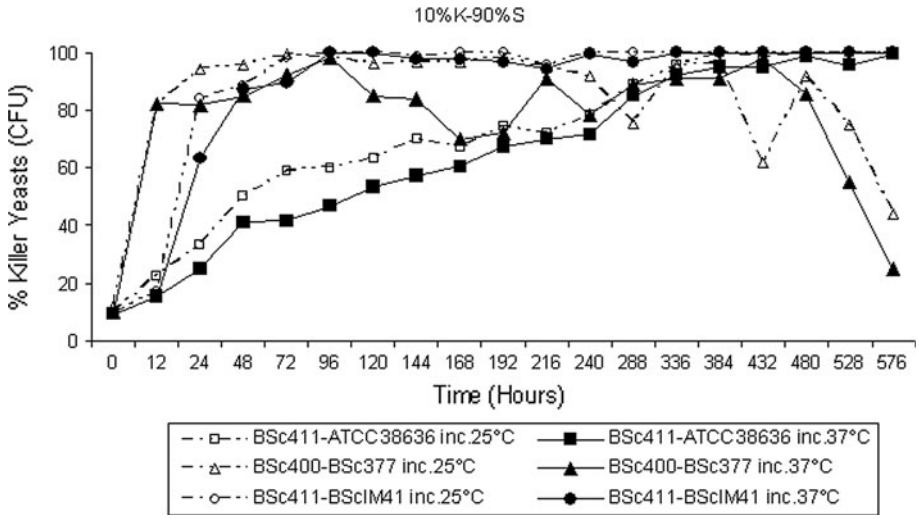

Fig. 2 Killer yeast proportion after different fermentation times in mixed microvinifications of 10 % killer/90 % sensitive strains. Bioreactor samples were incubated at 25 and 37 °C before cell counts. Repeated measures ANOVA showed significant differences throughout the experiment ($p = 0.0001$; F = 56.78)

In microvinifications carried out with BSc411 and ATCC 38636, the killer population represented 100 % of the biomass after 384 h and incubation at 25 °C. The proportion of killer yeasts in the same sample but incubated at 37 °C was significantly lower: 94.85 %. Killer yeast cell counts never reached 100 % in samples incubated at 37 °C (Fig. 2; Table 1).

50 % killer/50 % sensitive inoculum ratio

Killer biomass in mixed microvinifications with 50 % killer (BSc400) and 50 % sensitive (BSc377) oscillated too. This phenomenon was more evident when samples were incubated at 37 °C than at 25 °C. From 48 h onward, significant differences were observed between the two sample incubation temperatures (Table 1). It is remarkable that killer yeasts prevailed (i.e. 90 % or more) in samples taken between 48 and 192 h and incubated at 25 °C, whereas killer populations from the same samples but incubated at 37 °C ranged between 38.09 and 86.04 % (Fig. 3).

BSc411 outgrew BScIM41 after 12 h of the microvinification under the experimental conditions (Fig. 3). After 24 h the killer yeast proportion ranged between 94 % (samples incubated at 37 °C) and 99 % (samples incubated at 25 °C) (Table 1).

In mixed microvinifications carried out with BSc411 and ATCC38636, the killer yeast biomass in samples taken

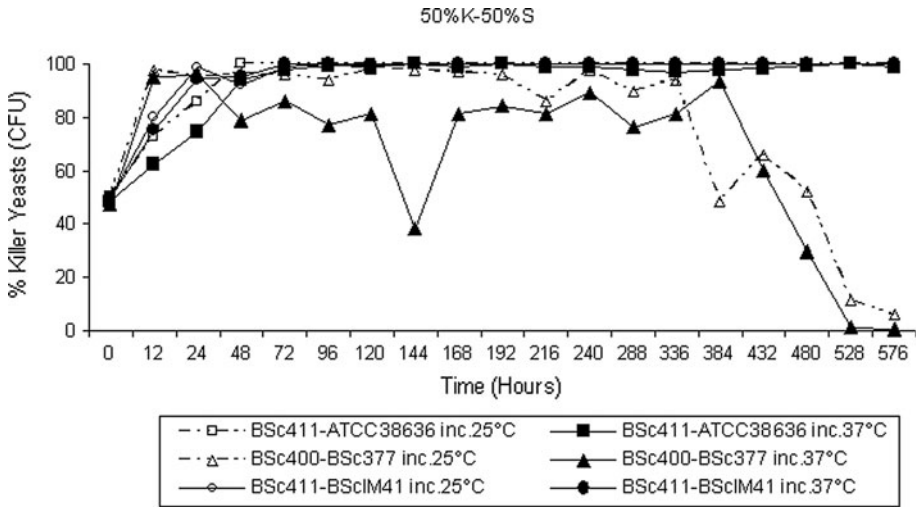

Fig. 3 Killer yeast proportion after different fermentation times in mixed microvinifications of 50 % killer/50 % sensitive strains. Bioreactor samples were incubated at 25 and 37 °C before cell counts. Repeated measures ANOVA showed significant differences throughout the experiment ($p = 0.0009$, $F = 44.5$)

after 12 to 48 h and incubated at 25 °C was higher than that grown at 37 °C (Fig. 3; Table 1). Samples incubated at 25 °C showed that killer yeasts completely dominated the medium (100 %) two days after the beginning of the experiment (Table 1). A different scenario is observed when samples were incubated at 37 °C. After 48 h, sensitive yeasts represented 6.23 % of the total population (Fig. 3). Killer yeast cell counts revealed significant differences compared with sensitive yeasts after 72, 96, 288, 336, 384 and 432 h for samples incubated at 37 °C (Table 1).

Discussion

There are several compounds and interactions produced by yeasts or environmental conditions during alcoholic fermentations that may become inhibitory to many yeast species or strains (Ludovico et al. 2001; Fleet 2003; Károlyi et al. 2005; Pérez-Nevado et al. 2006). Even so, between populations with otherwise similar environmental requirements, the advantage of toxin release may be decisive for the outcome of competition.

In this work, statistical analysis revealed that the fermentation dynamics of the killer yeast biomass of samples incubated at 25 °C were significantly different from those incubated at 37 °C. Sensitive yeasts were more present in samples incubated at 37 °C than at 25 °C, which means that inhibition of the killer toxin at 37 °C allowed a better development of sensitive cells. The findings of this work are in accordance with the loss of killer toxin production at temperatures during yeasts growth between 30 and 40 °C. This effect in both killer toxin stability and killer activity were verified in *Saccharomyces* and non-*Saccharomyces* yeasts as *Pichia membranifaciens* (Santos and Marquina 2004; Santos et al. 2009) and *Schwanniomyces occidentalis* (Chen et al. 2000). The loss or significant decrease of killer activity of *Saccharomyces* killer toxin was verified at temperatures mentioned above in solid and liquid medium.

Incubation at 37 °C of mixed K/S fermentation samples could be useful when sensitive yeasts seem to be eliminated from the medium in samples assayed at 25 °C. Our findings show several situations in which sensitive yeasts were completely eliminated (100 % killer yeasts) in samples incubated at 25 °C (Table 1), and this killer yeast prevalence was not observed when the same samples were incubated at 37 °C. In a mixed culture of 10 % BSc411 and 90 % BScIM41 (Fig. 2), sensitive yeasts were almost eliminated after 96 h of the microvinification. Samples incubated at 37 °C revealed sensitive yeasts after 168, 192 and 288 h of fermentation, whereas incubation of the same samples at 25 °C showed a killer biomass of 100 %. Similar results were observed under the given assay conditions with 1 % Bc411/99 % BScIM41 and 50 % BSc411/50 % ATCC 38636 (Figs. 1, 3). Even though previous studies reported complete elimination of sensitive *S. cerevisiae* yeasts by killer strains of the same species, it is important to mention that these studies did not take into account the incubation temperature of the samples extracted from the bioreactors (Longo et al. 1990; Pérez et al. 2001).

Soares and Sato (2000) studied the influence of the temperature on toxin activity of a *S. cerevisiae* strain. When this strain was incubated at 22–25 °C, the activity of the toxin was maximum. After incubation for 2.5 h at 30 °C, activity dropped 50 % and incubation for 1 h at 40 °C showed a complete loss of activity. Similar results were found with *Kluyveromyces phaffii* (Ciani and Fatichenti 2001).

Prior to detection of killer yeasts in samples incubated at 25 °C, yeast cell proximity may induce elimination of sensitive yeasts by killers. Sensitive cells, however,

develop at a higher incubation temperature (37 °C) and may compete in the bioreactor due to different environmental conditions or mixing effects (Károlyi et al. 2005). Hence, incubation of samples at killer detection temperature (20–25 °C) may lead to an underestimation of sensitive cells, and consequently, the proportion of killer and sensitive biomass may not reflect the real situation in the bioreactor.

The current study has shown the coexistence of sensitive and killer yeasts and an oscillating pattern of the prevalence of the killers under different assay conditions (Figs. 1, 2, 3). Vadasz et al. (2002) confirmed this behavior theoretically and empirically, pointing out the importance of a reliable method to determine the K/S ratio in fermentation systems. Sensitive populations in bioreactors can easily be underestimated, misinterpreting the purity of the fermentation. Similarly, the oscillating K/S behavior may be clearly misunderstood if the sensitive biomass population is not correctly determined.

The agar-plate techniques currently used for killer-sensitive detection including those using specific media (Musmanno et al. 1999; Pataro et al. 2000; Comitini and Ciani 2010) can be complemented with the methodology proposed in this work.

The simplicity and inexpensiveness of the methodology developed in the present study may be very useful to wineries.

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