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ORIGINAL PAPER

# Polyhexamethyl biguanide can eliminate contaminant yeasts from fuel-ethanol fermentation process

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**Abstract** Industrial ethanol fermentation is a non-sterile process and contaminant microorganisms can lead to a decrease in industrial productivity and significant economic loss. Nowadays, some distilleries in Northeastern Brazil deal with bacterial contamination by decreasing must pH and adding bactericides. Alternatively, contamination can be challenged by adding a pure batch of *Saccharomyces cerevisiae*—a time-consuming and costly process. A better strategy might involve the development of a fungicide that kills contaminant yeasts while preserving *S. cerevisiae* cells. Here, we show that polyhexamethyl biguanide (PHMB) inhibits and kills the most important contaminant yeasts detected in the distilleries of Northeastern Brazil without affecting the cell viability and fermentation capacity of *S. cerevisiae*. Moreover, some physiological data suggest that PHMB acts through interaction with the yeast membrane. These results support the development of a new strategy for controlling contaminant yeast population whilst keeping industrial yields high.

**Keywords** Contaminant yeast · *Dekkera bruxellensis* · Ethanol fermentation · Fungicide · PHMB

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## Introduction

Industrial ethanol fermentation is normally performed without sterilization of the sugarcane or molasses feeding must. In the Northeast region of Brazil, an important bioethanol production area, most fermentation processes are called "continuous", characterized by the recycling of the yeast biomass throughout the harvesting period that can last for over 6 months [1]. This favors the appearance and fixation of wild *Saccharomyces cerevisiae* strains as well non-*S. cerevisiae* contaminants yeasts [1, 2] which at high cell counts can cause a decrease in ethanol productivity and other operational problems. Recently, we have shown that a number of adventitious or contaminant yeast species can inhibit industrial fermentation [2]. In total, at least 23 other contaminant species have been observed so far. Of these, *Dekkera bruxellensis*, *Pichia galeiformes* and *Candida tropicalis* are the species that most commonly cause severe episodes of contamination, and can contribute more than 30% of the yeast biomass in the fermentation tanks, resulting in reduced productivity [2, 3]. From those three important species, *D. bruxellensis* was involved in the most severe contamination episodes in different distilleries in Northeast Brazil [2, 3] and in bioethanol distilleries in Canada and USA [4].

The increasing demand for bioethanol as an important alternative renewable non-polluting fuel will require better microbiological control of the process to ensure high productivities. Bacterial infections are well-controlled by the normal low pH of the process and by use of industrial antibiotics, a strategy that could be applied also to control contaminant yeasts and to keep the quality of the yeast population. However, *S. cerevisiae* is very sensitive to fungicides and there is currently no agent known that affects non-*S. cerevisiae* contaminants specifically [5, 6]. Sulfite

treatment is normally used in wine industry to kill spoiling yeasts, especially due to the intrinsic resistance of *S. cerevisiae* to this compound [6]. It can be used in different stages of wine production process, from the sulfitation of grape juice to sanitation of barrels by sulfur burning [6]. Treatment with sulfite is mostly used to eliminate *D. bruxellensis*, which is in the top list of wine spoilage yeasts, although sulfitation alone might not eliminate completely this yeast from the process [6]. In the case of fuel-ethanol fermentation process, sulfitation does not seem possible due to operational limitations, like damages to vessel structures that are usually made of iron. Therefore, the search for new fungicides or for new application of currently used biocides is of interest for this industrial process.

In the present work we examined the fungicidal activity of polyhexamethyl biguanide (PHMB), a commonly-used disinfectant of hospitals with a broad spectrum of activities against Gram-negative, Gram-positive bacteria, fungi and protozoa [7–9]. It has for long been used at concentration of 200 mg $l^{-1}$ to treat infective keratitis caused by *Acanthamoeba* spp followed by secondary fungal infection [7, 10]. On the basis of growth inhibition and the killing effects of PHMB, both in laboratory cultures and industrial samples, we propose that this compound could be used to manage the main fuel-ethanol contaminants on an industrial scale.

## Materials and methods

### Yeast strains, cultivation media and PHMB

Industrial strains of *S. cerevisiae* JP-1 and PE-2 [1] and the industrial isolates of *D. bruxellensis*, *P. galeiformes* and *C. tropicalis* have been described previously [2]. All strains are maintained at the GDB Industrial Yeast Collection of Genetech Bioproductivity Ltd (http://www.genetech.com.br). Yeast cells were cultivated in YPD medium (1% yeast extract, 2% peptone and 2% glucose). Solid medium contained 2% agar. Commercial preparation of the *S. cerevisiae* strain JP1 and the biocide PHMB were provided by AEB Bioquímica Latino Americana S.A. (Brazil). Commercial preparation of PHMB provided at 80 g $l^{-1}$ is a mixture of polymeric biguanides, whose monomeric structure is presented in Fig. 1, that contains molecules from 400 Da ($n = 2$) to 8,000 Da ($n = 40$) [9].

### Growth inhibition and fungicidal assays

Yeast cells were flask pre-cultivated overnight in YPD at 30 °C (180 rpm) and transferred to fresh medium at $5 \times 10^6$ cells $ml^{-1}$ for a second phase of exponential growth. Cells were washed with sterile saline, suspended to $2 \times 10^5$ cells $ml^{-1}$ and 5 µl dropped on to YPD plates containing PHMB at different concentrations. Growth inhibition was evaluated after 72 h of incubation at 30 °C, in duplicated plates.

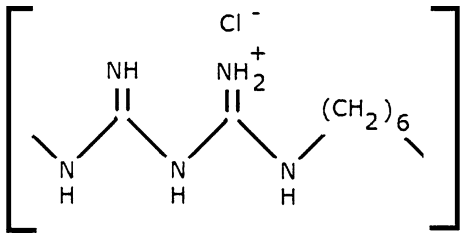


**Fig. 1** Molecular structure of PHMB monomer (adapted from Ref. [9])

To assay for cell toxicity, exponentially growing cells were washed and suspended in saline to $2 \times 10^7$ cell $ml^{-1}$ and the suspensions incubated for 10 min with different concentrations of PHMB. Control experiments used distilled water. After incubation, appropriate cell dilutions were performed and the cells were spread onto YPD medium plates. After incubation for 72 h at 30 °C, the number of yeast colonies was determined in order to evaluate the percentage of cell survivors after PHMB treatment. When necessary, pre-treatments were performed by incubating yeasts cells for 30 min in the presence of 5% threalose prior to adding PHMB. Cells were also cultivated in YP medium containing 1% ethanol as carbon source. Stationary growth phase cells were prepared by further incubation of exponential growing cells up to carbon depletion (48 h for glucose and 72 h for ethanol). The results are the average of three experiments with triplicates for each dose.

### *D. bruxellensis* detection and industrial sample treatments

Industrial must samples were collected from two distilleries in Northeastern Brazil and the numbers of *S. cerevisiae* and *D. bruxellensis* cells evaluated microscopically. Yeast cell numbers were confirmed by plating diluted samples onto YPD, collecting yeast colonies after 5 days and identifying them by specific DNA typing [3]. Mixed cell population from industrial samples were collected, washed in saline and suspended to $2 \times 10^7$ cell $ml^{-1}$ and treated with PHMB as above. Yeast suspensions were plated in parallel onto WLN medium either supplemented with bromocresol green, in which both *S. cerevisiae* and *D. bruxellensis* colonies can be discriminated by dying assimilation and colony morphology [3], or with 0.1% cyclohexamide, in which only *D. bruxellensis* cells could form colonies [3]. Cells for both morphotypes were PCR-typed using species-specific primers [3]. The lethal dose that promoted the killing of 50% of the yeast cells ($LD_{50}$) was determined from the survival curves. The results are the average of two experiments with triplicates for each dose.

Fermentation assays were performed with pure cultures of commercial JP1 cells (Fermolplus® Distiller, AEB

Bioquímica Latino Americana SA). Twenty grams of yeast cells were suspended in 100 ml sterile water and incubated for 30 min for cell activation and diluted 1:1 in filtered sugarcane juice to 10% cells (w/v) and 140 g sucrose $l^{-1}$ final concentrations. PHMB was added to 200 mg $l^{-1}$. Samples were incubated at 33 °C and samples were withdrawn at indicated periods, centrifuged and the supernatants were used for ethanol determination by gas chromatography (Varian 3600 GC device) [3]. Fermentation assays were also performed with industrial mixed yeast population. Samples collected from low contaminated distillery were centrifuged and the cells were suspended to the same initial volume with sugarcane juice (140 g sucrose $l^{-1}$ final concentrations) and PHMB was used to 200 mg $l^{-1}$. Samples were incubated at 33 °C and samples were withdrawn at indicated periods to determine the total number of yeast cells (cells $ml^{-1}$), the percentage of budded cells and the number of *D. bruxellensis* cells by direct microscope observation. Ethanol content in the worth (% v/v) was determined by GC analysis and medium acidity (as mg acetic acid per 100 ml) was determined by titration assay. Medium pH was directly determined using potentiometer. The results are the average of two experiments with triplicates for each dose.

## Results

### Fungicidal activity

The biocide PHMB was evaluated for its fungiostatic activity, by cell growth inhibition on plates, and for its fungicides activity, by its killing effect on yeast cell suspension. The fungiostatic activity of PHMB was tested using cells of *S. cerevisiae* industrial strains and three main contaminant yeasts of the fuel-ethanol fermentation process. Both *S. cerevisiae* JP1 and PE-2 and *C. tropicalis* cells grew in the presence of PHMB at 40 mg $l^{-1}$ whereas *D. bruxellensis* and *P. galeiformis* cell growth was impaired at that concentration (data not shown). Following this result, the fungicidal effect of PHMB were tested using cells collected at exponential and stationary growth phases. In the exponential growth phase, cells of *S. cerevisiae* JP1 strain were resistant to the killing effect of PHMB, whereas *D. bruxellensis* cells were moderately sensitive, *S. cerevisiae* PE-2 and *C. tropicalis* cells were sensitive and *P. galeiformis* cells were highly sensitive to this compound (Fig. 2a). The fungicide activity toward *D. bruxellensis* was only observed at high concentrations. Cells at stationary phase of growth behaved differently (Fig. 2b). Contaminant yeasts presented higher resistance while *S. cerevisiae* strains showed higher sensibility, mainly the PE-2 strain, compared to cells in exponential growth phase. The sensitivity of PE-2 cells to PHMB represents impairment for the use of PHMB in distilleries that uses PE-2 as fermenting strain. On the other hand, this high sensitivity of PE-2 cells makes this strain an interesting platform to study the mechanism of resistance to PHMB in *S. cerevisiae*.

The influence of both carbon sources on the killing effect of PHMB was tested by comparing yeast cells grown on glucose or ethanol. The experiments were performed at concentration 12 mg $l^{-1}$ that was sub-lethal to yeast cells (see Fig. 2). The results showed that ethanol-growing JP1 and PE-2 cells were extremely sensitive to PHMB in both exponential (Table 1) and stationary phase of growth (data not shown) comparing to glucose-growing cells. The yeast *D. bruxellensis* was also more sensitive to PHMB when grown in ethanol (Table 1). This ethanol-induced sensitivity is important when considering the stage of the fermentative process where PHMB can be used. Those results

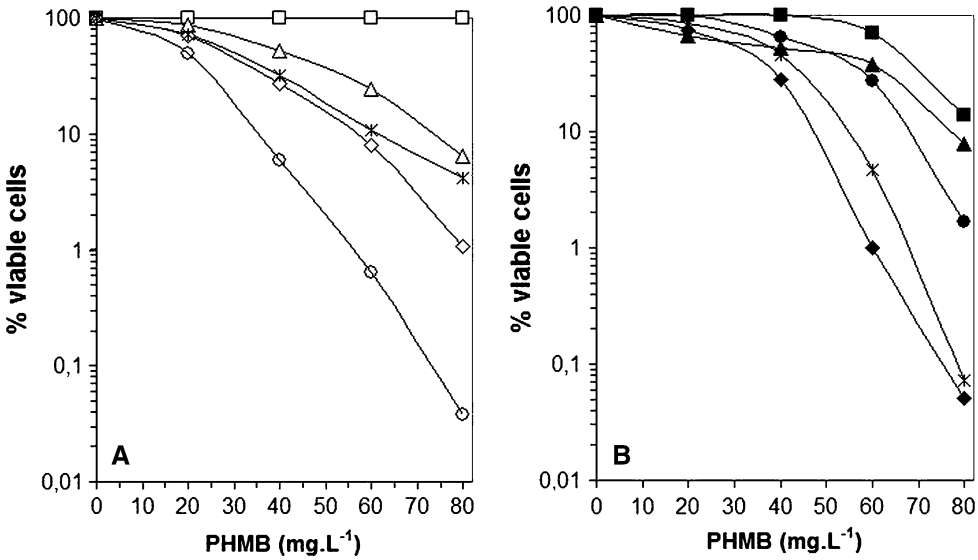


**Fig. 2** Cell survival curves of *S. cerevisiae* strains JP1 (*open square*) and PE-2 (*open diamond*), *D. bruxellensis* C1 (*open triangle*), *P. galeiformes* (*open circle*) and *C. tropicalis* (*star*) in different concentrations of PHMB. Yeast cells were treated at exponential (*panel A*) or stationary (*panel B*) phase of growth

**Table 1** Effect of carbon source on the resistance of exponential yeast cells exposed to PHMB

| Yeast | Glucose | | | Ethanol | |
|---|---|---|---|---|---|
| | PHMB (mg $l^{-1}$) | −Threalose | +Threalose | −Threalose | +Threalose |
| *S. cerevisiae* JP1 | 0 | 100 | 100 | 100 | 100 |
| | 12 | 100 | 100 | 15 | 83 |
| *S. cerevisiae* PE-2 | 0 | 100 | 100 | 100 | 100 |
| | 12 | 78 | 100 | 7 | 82 |
| *D. bruxellensis* | 0 | 100 | 100 | 100 | 100 |
| | 12 | 85 | 100 | 8 | 82 |

suggest that the increased content of phospholipids in cell membrane of ethanol-growing cells might rend yeast cells much sensitive to PHMB. To test this hypothesis, yeast cells were pre-treated with threalose, which is supposed to protect cell surface against the toxic effect of ethanol. The results showed that pre-incubation of *S. cerevisiae* and *D. bruxellensis* cells to threalose increased their survival to PHMB exposure for, irrespectively of the growth medium composition (Table 1).

### Industrial samples treatments

The fungicide effect of PHMB was further tested on cells collected directly from the industrial processes. The fermentation process carried in Northeast Brazil is so-called a "continuous process", such as yeast cells are re-pitched to the first tank where the substrate is added (high-sugar low-ethanol stage). Following the fermentation process, ethanol reaches the highest level in the last tank (no-sugar high-ethanol stage), from which yeast cells are recovered. Following that effect of ethanol on the yeast sensitivity to PHMB, industrial samples were collected from the first vat in two distilleries presenting different levels of infections. After exposure to PHMB, yeast cell viability assays revealed that *D. bruxellensis* cells were selectively killed by PHMB irrespective to its initial cell count in the population (Fig. 3). However, higher killing rate was observed when the number of *D. bruxellensis* cells was lower than *S. cerevisiae* cells ($LD_{50}$ of 20 mg $l^{-1}$) when compared to highly contaminated samples ($LD_{50}$ of 60 mg $l^{-1}$).

Other microbiological and physicochemical parameters were evaluated when treating industrial contaminated samples with PHMB (Table 2). Budding index for *S. cerevisiae* cells, as indicative of cell viability, was not altered by the presence of PHMB. It ensured normal cell growth and ethanol production. Not only final ethanol content in the medium was unaffected by the presence of PHMB, but also its production kinetics by the *S. cerevisiae* cells (data not shown). No change was also observed for medium pH and acidity upon treatment with PHMB. However, it effectively decreased the contamination level of the sample by reducing 44% the population of *D. bruxellensis* in the sample (Table 2). The exposure to PHMB was less effective after 6 h, probably due to degradation of PHMB at low pH or by interaction with ethanol.

## Discussion

We recently reported that *D. bruxellensis* cells at 2% of yeast population in the bioethanol fermentation process can overcome the *S. cerevisiae* cell population in intervals

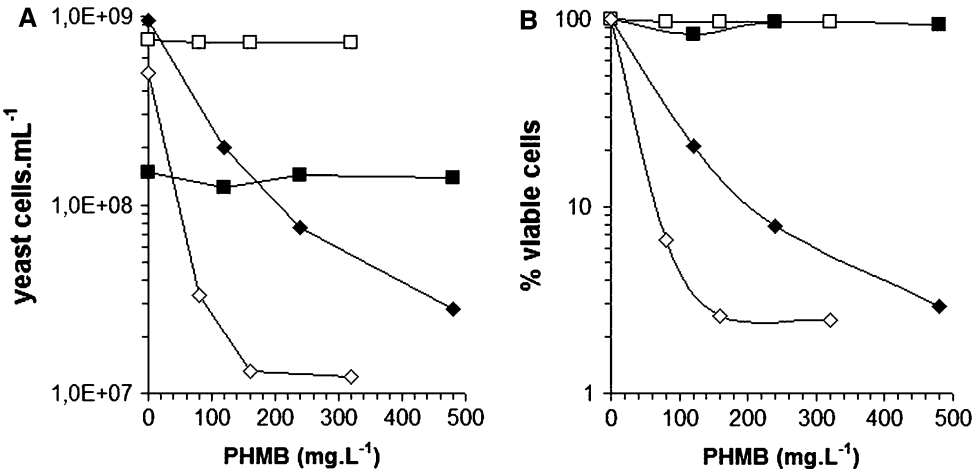


**Fig. 3** Killing effect of PHMB to *S. cerevisiae* (*square*) and *D. bruxellensis* (*diamond*) present in high-cell-density industrial samples collected during two episodes of process contamination:middle contamination (1 Sc: 1 Db) (*open symbols*) and severe contamination (1 Sc: 3 Db) (*closed symbols*). **a** Number of viable yeast cells in the worth before and after exposure to PHMB. **b** Survival curve of yeast cells in the worth representative of *panel A*

**Table 2** Microbiological and physicochemical parameters measured upon treatment with PHMB of infected industrial sample, compared to infected untreated samples

| | Control | +PHMB (200 mg $l^{-1}$) | | −PHMB |
|---|---|---|---|---|
| | 0 h | 6 h | 24 h | 24 h |
| *S. cerevisiae* cell count ($\times 10^7$ cell $ml^{-1}$) | 28 | 35 | 36 | 37 |
| *S. cerevisiae* cell budding (%) | 13.1 | 14.4 | 13.6 | 13.9 |
| *D. bruxellensis* cell count ($\times 10^7$ cell $ml^{-1}$) | 7.9 | 4.9 | 4.4 | 7.8 |
| Yeast contamination (Db/Sc, %) | 28 | 14 | 12 | 21 |
| Ethanol content (%, v/v) | 0.5 | 7.2 | 7.6 | 7.6 |
| Medium acidity (mg acetic acid $l^{-1}$) | 25 | 26 | 26 | 26 |
| Medium pH | 3.8 | 3.7 | 3.6 | 3.6 |

as short as 15 days [3]. The industrial filling assumes that *D. bruxellensis* cells up to 10% of the yeast population do not produce any drop in ethanol productivity. It is explained by the high ethanol yield reported for this yeast [3, 11]. However, *D. bruxellensis* present lower productivity and its count at high level lead to a decrease in the overall industrial production [3]. Thus, decreasing *D. bruxellensis* to safety levels may be of major industrial interest. This can afforded by combining the use of fast approaches for detection and quantification of contaminant yeasts, such as PCR based methods [3, 12] with periodical treatment of the yeast biomass with some specific fungicide may help to keep *D. bruxellensis* cell population at safety levels [3].

To date there is no such specific agent that can affect non-*S. cerevisiae* yeasts solely, given that *S. cerevisiae* is very sensitive to all fungicides available [5]. In wine fermentation, sulfur dioxide ($SO_2$) is mostly used to avoid contamination by spoiling yeasts [6]. However, its use in ethanol fermentation is not advisable due to its corrosive effect on the iron-made tanks. Other compounds have been used to kill wine spoilage yeasts, such as chitosan [13], hydroxycinnamates and organic acids [14], membrane-active antimicrobial peptides [15], killer toxins [16], natamycin [17], lactoferrin-derivatives [18]. Additionally, a group of synthetic D-hexapeptides have been successfully used to eliminate fungal phytopathogens [19]. Chitosan has also been reported to have a fungistatic effect on *D. bruxellensis*—the compound increases the lag period of *D. bruxellensis* cell growth to more than 60 h of cultivation at concentration above one gram per liter [13]. This compound effectively inhibited growth of *D. bruxellensis* cells in mixed cultures with *S. cerevisiae* only at high concentration, without affecting *S. cerevisiae* growth [11]. Recently, Enrique et al. [18] showed that synthetic D-hexapeptides promoted differential growth inhibition to *D. bruxellensis* cells and others wine contaminants, while hardly affecting *S. cerevisiae* growth. In the present work, we show that PHMB also affects specifically *D. bruxellensis* cells in mixed population with *S. cerevisiae*. However, this biocide effect not only impaired *D. bruxellensis* growth but killed their cells as well. Therefore, PHMB seems to be more effective than those compounds above in preventing and combating contamination by *D. bruxellensis*. Although the concentration required to effectively kill *D. bruxellensis* was 20 times higher than that used to inhibit clinical isolates of *C. albicans* and *Fusarium solani* and eight times higher than that observed for clinical isolates of *Aspergillus niger* [5], it was five times lower than that used to treat *Acanthamoeba* spp infection [10].

The biological effect of PHMB has been previously demonstrated to *Acanthamoeba castellanii* by causing structural changes in the cell membrane and inducing granulation of the cell cytoplasm [10]. Those morphological changes are accompanied by intracellular accumulation of $Cl^-$ anions and leakage of $K^+$ cations [20]. It was proposed that PHMB may interact with phospholipids in the parasite cell membrane causing changes in cell permeability [10, 20], which was further supported by work in *E. coli* [9]. The external layer of *S. cerevisiae* plasma membrane is enriched in phosphatidylcholine, ergosterol and sphingolipids [21]. Based on the cationic nature of PHMB (Fig. 1), its toxic effect may be mediated by its link to the negative phospholipids on the yeast cell surface. Some reports in the literature support this hypothesis. First, glycerophospholipids and sphingophospholipids constitute the most abundant class of negatively charged components of the fungal plasma membrane and may serve as anchor for the attachment of many cationic plant defensins [21]. Second, peptide-derivative lactoferrins that showed selective activity against contaminant yeasts seem to have a broad spectrum of action, from interfering in the membrane permeability to inhibiting protein synthesis [18, 19]. Therefore, differential constitution of cell surface may be the main cause of selective action of cationic compounds with fungicidal activity.

The results herein for PHMB suggest its mode of action. First, yeast cells grown on ethanol showed high sensitivity to PHMB. In the presence of this carbon source, yeasts protects themselves by increasing the content of mono-unsaturated fatty acids, mainly palmitoleic (16:1) and oleic (18:1)

acids, ergosterol and phosphatidylcholine at the cell surface [22, 23]. All those changes contribute to decrease fluidity and increase of $H^+$-ATPase activity of the cell membrane to counteract the increased proton influx across the membrane induced by the presence of ethanol [22, 23]. Thus, if the phospholipid content of the cell membrane is increased by the presence of ethanol, there should have more targets for PHMB molecules interactions at the cells surface, increasing their fungicidal activity as observed in Table 1. Second, this effect was reverted by pre-incubation of the cells with threalose prior PHMB exposure. This sugar acts as stress protectant by reducing membrane permeability, keeping the cell osmotic equilibrium and protecting cell proteins from denaturation [24]. Moreover, threalose is thought to stabilize yeast cell membranes by interaction to the polar groups of phospholipids in the yeast cell surface [21] and it protects the yeast cell membrane from oxidative damages by decreasing the level of lipid peroxidation [24]. Therefore, the interaction of threalose with the phospholipids at the cell membrane should prevent the interaction of phospholipids and PHMB, decreasing its biocidal activity as showed in Table 1.

Studies are now in course to identify the exact cell mechanism involved in the yeast resistance to PHMB. It has many implications for the correct use of PHMB in the fermentation process. The stage of the process to which PHMB may be added is of major concern. Using this biocide in the pre-fermentation tanks, where yeast biomass is aerated and fed with diluted cane juice should not affect *S. cerevisiae* cells as they are exposed to sucrose and to low amount of ethanol. Another concern is related to the *S. cerevisiae* strain that is being used by the distillery. It was clearly shown that PE-2 strain is sensitive to PHMB. This strain is one of the most commercially used distilling strains in Brazil, especially in distilleries that ferment molasses [1]. However, our previous analysis showed that this kind of distilleries is hardly contaminated by *D. bruxellensis* [2, 3], so the use of PHMB may not be necessary in those cases. Indeed, *D. bruxellensis* is the most important contaminant yeast so far detected in industrial plants that ferment sugar cane juice [3], and JP1 is nowadays the main *S. cerevisiae* fermenting strain used in those distilleries in Northeast Brazil [1], where the use of PHMB is advisable. From those results, it can be concluded that the use of PHMB at 200 mg $l^{-1}$ in combination with a high-fermenting PHMB-resistant strain may prevent the establishment of contamination episodes in the bioethanol fermentation process.

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