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Inhibition of Bacteria Contaminating Alcoholic Fermentations by Killer Yeasts

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ABSTRACT

The aim of this work was to study the in vitro *antibacterial activity possessed by killer yeast strains against bacteria contaminating alcoholic fermentation (*Bacillus subtilis, Lactobacillus plantarum, Lactobacillus fermentum *and* Leuconostoc mesenteroides*), in cell X cell and cell X crude toxin preparations. The bacteria were not inhibited by any* S. cerevisiae *killer strains (5 out of 11). The inhibition caused by two crude toxin preparations (*Trichosporon figueirae *and* Candida *sp) against* L. plantarum *was surprisingly high but not in the same extent for* B. subtilis, *especially with three killer strains* (Candida glabrata, Pichia anomala *and* Candida *sp).* L. mesenteroides *and* L. fermentum *strains were neither inhibited in cell X cell nor crude toxin X cell tests. The results suggested that killer activity of yeasts might operate over bacteria and it could be used for the biocontrol of contaminating bacteria from alcoholic fermentation if additional tests on toxin application in fermentation shown to be successful. A wider panel of* S. cerevisiae *killer strains should be used to confirm that they were really unable to control the growth of these Gram-positive bacteria.*

Key words: *Bacteria, killer yeasts, alcohol, fermentation*

INTRODUCTION

The industrial process for bioethanol production is a nonsterile open activity that uses sugar cane juice and/or molasses as substrate for yeast fermentation with cell recycle (Wheals et al., 1990). The use of crude cane must promote a continuous input of microorganisms into the process, demanding chemicals and causing operational problems in centrifuges and heat exchangers, due mainly to flocculation in the yeast suspension caused by contaminating bacteria (Yokoya and Oliva-Neto, 1991; Rodini, 1985; Nobre et al., 2007). The secretion of antimicrobial factors by yeasts involving killer strains, which produce a toxin that is lethal to sensitive microorganisms, is an interesting approach to the biocontrol of contaminant bacteria from fermentation process.

Killer toxins (mycocins) were first observed in strains of *Saccharomyces cerevisiae* by Bevan and Makower (1963) but yeast strains belonging to species of the genera *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Schwanniomyces*, *Williopsis*, *Cryptococcus*, *Metschnikowia* and *Candida* have been also found to be toxin-producers (Young and Yagiu, 1978; Sawant et al., 1989; Golubev, 1998).

Killer yeasts are immune to the activity of their own killer toxins. According to Buzzini et al. (2007), all killer toxins studied so far have been found to be protein or glycoproteins with

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molecular weights ranging from 5000-10,000 to 100,000 Da or even greater in a few cases. The mechanism of action of killer toxins varies from cell cycle arrest in G1 phase, increased membrane permeability to ions, formation of ion channel, plasma membrane damage or inhibition of glucan synthesis (Kagan, 1983; Izgu and Altinbay, 2004; Buzzini et al., 2007).

Initial observations had limited the activity of killer toxins to yeasts, but this action could be displayed against a great variety of unrelated eukaryotic and prokaryotic microorganisms (Morace et al., 1989; Provost et al., 1995). Some potential uses of the killer phenomenon have been proposed as biocontrol against spoilage yeasts and moulds in fermentation processes and food preservation (Palpacelli et al., 1991; Michalcakova et al., 1993; Coelho et al., 2007); biotyping of yeasts (Buzzini and Martini, 2001; Buzzini et al., 2004, 2007; Tosta, 2004) with special interest in biomedical and industrial applications; and as novel antimycotic agents (Sawant et al., 1989; Buzzini et al., 2004). An extensive review on yeast killer systems is found in Magliani et al. (1997). The yeast killer system was also applied for differentiation of etiological agents of nocardiosis and Gram-positive pathogenic bacteria (Morace et al., 1989; Izgu and Altinbay, 1997; Fuentefria et al., 2008).

In this context, this work aimed to study the *in vitro* antibacterial activity possessed by killer yeast strains against Gram-positive bacteria contaminating alcoholic fermentation, especially *Lactobacillus plantarum*¸ in cell X cell and cell X crude toxin preparations.

MATERIALS AND METHODS

Microorganisms

The strains of killer yeasts used are listed in Table 1. These were maintained in YEPD Agar medium (yeast extract 1%; bacteriological peptone 2%; glucose 2%; agar 2%) at 4ºC. The killer activity was determined by the production of an inhibition or blue zone around the killer strain when inoculated with the sensitive strains CCA003 (NCYC1006, *Saccharomyces cerevisiae*) or CCA039 (ATCC15126, *Torulopsis glabrata*), onto buffered-YEPD-methylene blue medium (citrate-phosphate buffer, pH 4.5-4.7, 2 mL/L of 1.5% methylene blue solution).

The following bacterial strains were used in experiments: *Bacillus subtilis* ATCC6051 (CCT 2471); *Lactobacillus fermentum* ATCC9338 (CCT0559); *Leuconostoc mesenteroides* ATCC19255 (CCT5852); *Lactobacillus plantarum* ATCC8014 (CCT0580). They were inoculated in appropriate culture media as MRS (dextrose 2%; bacteriological peptone 1%; meat extract 1%; yeast extract 0.5%; sodium acetate 0.5%; ammonium citrate 0.2%; potassium phosphate 0.2%; magnesium sulphate 0.2%; manganese sulphate 0.005%; Tween 80 0.001%; agar 2%) for *Lactobacillus* species at 37ºC, and nutrient agar (bacteriological peptone 0.5%; meat extract 0.3%; sodium chloride 0.1%; agar 2%) for the others at 30ºC. After growth, they were kept in the same medium at 4ºC.

Killer activity in cell X cell tests

The sensitivity tests were performed in Nutrient Agar or MRS media, spreading 100 µL of the bacterial suspension and inoculating the killer yeast strains with sterile toothpicks on the medium surface, in triplicate, following incubation at 30ºC for two days. An inhibition zone around the killer yeast colony characterized bacterial sensitivity (positive result).

Killer activity in cell X crude toxin tests

Initially, the growth curves of four killer yeast strains (CCA194, CCA199, CCA417 and CCA630) were determined as following. Two loops of each strain was inoculated in 20 mL of buffered-YEPD broth (citrate-phosphate buffer, pH 4.5-4.7) and incubated overnight at 30ºC and 160 rpm. This was transferred to a 1000-ml Erlenmeyer containing 180 ml of buffered-YEPD broth (10% inoculum vol/vol), which was incubated at 30ºC at 40 rpm for 48 hours. Samples were periodically taken to determine the optical density at 600 nm in a Thermo® Biomate-3 spectrophotometer. The sample was filtered through 0.45-µm membrane to obtain a crude toxin preparation. Values of pH were determined in a digital pH-meter and filtrates were kept in freezer.

The killer activity of crude toxin preparations was confirmed by spreading 100 µL of suspension of the sensitive strains (CCA003 and CCA039) on buffered-YEPD-methylene blue medium and after drying, 10-mm wells onto the medium were filled with 50 µL of crude toxin preparations. Petri dishes were incubated at 25ºC for two days and

inhibition and/or blue zone around the wells were measured.

The killer activity using crude toxin preparations and bacterial strains were performed as above; however, Nutrient Agar medium was used for *L. mesenteroides* and *B. subtilis*, while for *Lactobacillus*, MRS medium was utilized. All the experiments were incubated at 25ºC, with inhibition zone measurements after two days.

RESULTS AND DISCUSSION

The sensitivity tests of bacterial strains against killer yeasts are presented in Table 2. The bacteria were not inhibited by any *S. cerevisiae* killer strains. Izgu and Altinbay (1997) found that toxins of killer *Saccharomyces cerevisiae* were not active against bacterial strains. Polonelli and Morace (1986) had described the killer effect of 36 strains of *Hansenula*, *Pichia*, *Saccharomyces* and *Candida* on bacterial isolates, but *Saccharomyces* was only capable to inhibit Gram-negative bacteria.

The nature of the interaction(s) between the killer yeasts and strains of other microbial groups still remains to be elucidated. Buzzini et al. (2007) considered that different cell wall composition occurs in microbial groups other than fungi and it appeared likely that the secretion of nonproteinaceous inhibitory molecules was the most probable mechanism. Killer toxin labels for *S. cerevisiae* were K1, K2 and K28, whose genetic basis were dsRNA, all exhibiting β-(1,6)-glucan and mannoproteins as primary receptors at the cell wall level.

Table 1 - Killer yeast strains.

Code	Yeast characteristics and identification
CCA004	*Saccharomyces cerevisiae*, NCYC 738, K2 toxin
CCA008	*Saccharomyces cerevisiae*
CCA015	*Saccharomyces cerevisiae*, isolated from alcoholic fermentation
CCA176	*Saccharomyces cerevisiae*, strain Σ1278b, mat a/mat α
CCA194	*Candida glabrata*, NCYC388 (CCT2369), K4 toxin
CCA199	*Pichia anomala*, NCYC435 (CCT4373), K8 toxin
CCA231	*Saccharomyces cerevisiae*, isolated from alcoholic fermentation
CCA369	*Candida tropicalis*, isolated from alcoholic fermentation (inoculum)
CCA417	*Candida* sp., isolated from alcoholic fermentation (inoculum)
CCA510	*Kluyveromyces marxianus*, isolated from alcoholic fermentation (fermented must)
CCA630	*Trichosporon figueirae*, isolated from alcoholic fermentation (fermented must)

NCYC=National Collection of Yeast Cultures; CCT=Coleção de Culturas Tropical

Table 2 - Bacterial sensitivity to killer yeast strains in cell X cell tests, at 30ºC. LF = *Lactobacillus fermentum;* LM = *Leuconostoc mesenteroides;* LP = *Lactobacillus plantarum;* BS = *Bacillus subtilis.*

Bacteria	Killer strains										
	004	008	015	176	194	199	231	369	417	510	630
LF	-	-	-	-	-	-	-	-	-	-	-
LM	-	-	-	-	-	-	-	-	-	-	-
LP	-	-	-	-	+	+	-	-	+	-	+
BS	-	-	-	-	+	+	-	-	+	-	+

Legend: (-) no inhibition zone (+) inhibition zone

Antibacterial products secreted by yeasts are not common, especially in *S. cerevisiae*. Sasaki et al. (1984) screened 150 strains of *Saccharomyces* for inhibitory activity against Gram-positive and Gram-negative bacteria and found only eleven yeasts inhibiting all tested bacteria. In contrast, when surveying nearly 400 yeast strains belonging to 31 genera for their antibacterial activity, Bilinski et al. (1985) found only two species (*Kluyveromyces thermotolerans* and *Kloeckera*

apiculata) to possess activity against *L. plantarum* and *Bacillus cereus*. The authors demonstrated no adverse effects against Gram-negative bacteria.

Oliva-Neto et al. (2004) described a *Saccharomyces* strain (M26) that presented an inhibitory halo against *L. fermentum* culture and significant reduction in the culture turbidity and specific growth rate. However, they did not attribute this inhibition to the killer activity, but to complex substances from yeast secondary metabolism and succinic acid released in large quantities, as already observed by other authors (Bilinski et al., 1985; Basso et al., 1996).

The Gram-positive bacteria *L. plantarum, L. fermentum, L.mesenteroides* and *B. subtilis* were chosen mainly due to their importance as contaminants in alcoholic fermentations (Amorim and Oliveira, 1982; Rodini, 1985; Silva, 1988). According to Rodini (1985), Gram-positive bacteria account for 65% of total number in alcoholic fermentations. Proportions of 38, 12 and 3% were found, respectively, for *Lactobacillus, Leuconostoc* and *Bacillus* in sugar cane juice after clarification, pasteurization and cooling processes (Silva, 1988). Among the yeast strains, CCA194, CCA199, CCA417 and CCA630 showed inhibition potential against bacteria, so they were also selected for additional tests.

The killer strains were grown in buffered-YEPD medium for 48 h (Fig. 1) and free-cell filtrates were tested against the sensitive yeasts (Fig. 2) and bacterial strains (Fig. 3). Results in Fig. 2 have confirmed the killer activity of yeast filtrates from 6 h of cultivation, with high stability of toxicity during 48 h. The inhibition caused by the killer yeasts CCA417 and CCA630 against *L. plantarum* was surprisingly high (Fig. 3).

Crude toxin preparation from CCA194 (*C. glabrata)* inhibited *L. plantarum* after 24 h of killer yeast growth; with CCA199 (*P. anomala*), there was a small clear inhibition zone from the beginning of cultivation, which might be explained either by the fast growth of the yeast (log phase between 4-12 h) or by the low medium pH (4.5-4.7). In spite of using a buffered medium, which potentializes the killer toxin production, pH measurements were taken along the cultivation to assure that the inhibition could not be attributed to the pH decrease. In fact, this could be observed at the initial crude toxin preparations against bacteria, but the inhibition zone grew significantly although medium pH did not vary at all. With CCA417 (*Candida* sp), the same effect was observed. The last killer yeast and CCA630 (*T. figueirae*) were found to be great *L. plantarum* inhibitors (Fig. 3).

The killer phenomenon has already been reported for several species of the genus *Trichosporon* (Golubev, 2006), shown to possess a killer profile against 100 isolates of *Cryptococcus neoformans / Cryptococcus gattii* (Fuentefria et al., 2007) and was able to inhibit and discriminate sensitive strains of *Staphylococcus epidermidis* (Fuentefria et al., 2008).

Lactobacillus is highly adapted to the nutritional conditions and alcohol concentration in tanks, causing serious yeast flocculation problems, resulting in viability decrease of *S. cerevisiae* during fermentation (Yokoya and Oliva-Neto, 1991; Cherubin, 2003). *L. plantarum* was the most resistant bacterial strain to chlorine dioxide (125 ppm) proposed for the control of bacterial contamination in fermentation (Meneghin et al., 2008). Yeast killer toxin could be a new approach to this control.

In respect to *B. subtilis*, higher resistance to crude toxin preparations was observed. The killer yeasts CCA194, 199 and 417 were more efficient to inhibit this bacterial strain, however, not in the same extent as *L. plantarum* (Fig. 3). In any case, greater bacterial inhibition was found after yeast log phase. Buzzini et al. (2004) observed a constant increase in toxin production during exponential growth with a peak during early stationary phase for two killer strains. No inhibition zones were found for killer yeasts against *L. mesenteroides* and *L. fermentum* in cell X cell and crude toxin X cell tests.

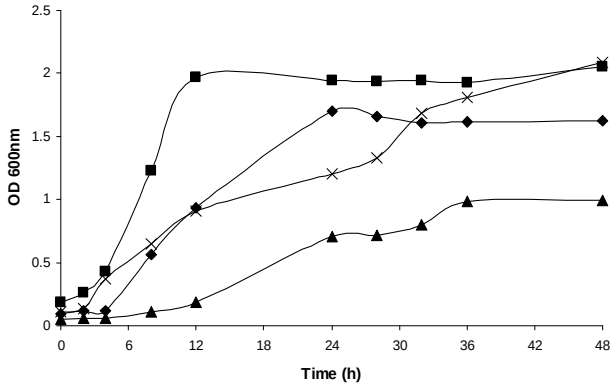

Figure 1 - Growth curves of yeast killer strains (♦CCA194; ■CCA199; ▲CCA417; ×CCA630), in buffered-YEPD medium, at 30ºC, 40 rpm, for two days.

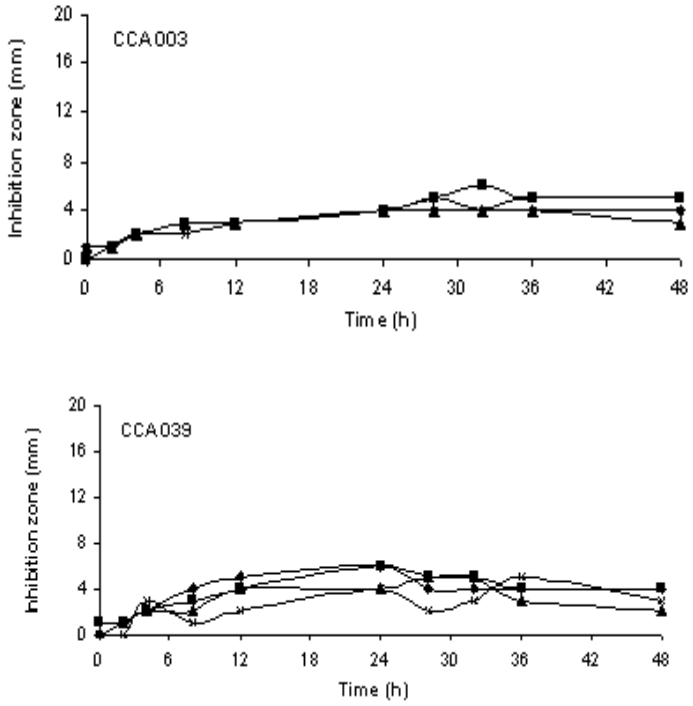

Figure 2 - Inhibition zone (mm) of killer yeast filtrates (♦CCA194; ■CCA199; ▲CCA417; ×CCA630) against the sensitive strains CCA003 (NCYC1006, *Saccharomyces cerevisiae*) or CCA039 (ATCC15126, *Torulopsis glabrata*), in buffered-YEPD-methylene blue medium, at 25ºC, for two days.

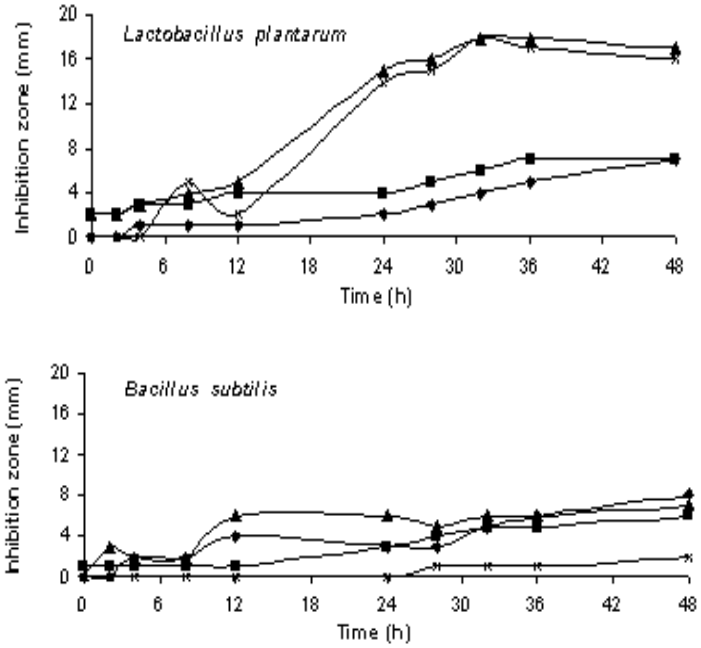

Figure 3 - Inhibition zone (mm) of yeast killer filtrates (♦CCA194; ■CCA199; ▲CCA417; ×CCA630) against *Lactobacillus plantarum* and *Bacillus subtilis* strains, at 25ºC, for two days.

The results suggested that killer activity of yeasts might operate over bacteria and it could be used for the biocontrol of contaminating bacteria from alcoholic fermentation. Additional studies are necessary to evaluate the application of killer toxin preparations into fermentation tanks to control the growth of these microorganisms. Other toxins could be assayed because in this study only toxins from *Candida* sp, *C. glabrata, P. anomala* and *T. figueirae* yeast species were found to be active. Neither dsRNA plasmids nor cure of the killer activity by actidione were detected for these strains (Reis and Ceccato-Antonini, unpublished results), showing a good perspective of stability for purification processes.

Although the inhibition observed here might not necessarily be due to killer toxins because they were not purified but used in crude preparations, the fact that recognized killer yeasts showed toxic activity suggested the involvement of such factor as a promising agent in the bacterial control.

A wider panel of *S. cerevisiae* killer yeasts might be used to confirm that they would be really unable to biocontrol these Gram-positive bacteria. This is an undesirable result since these yeasts, as fermentation agents, could be also screened for killer toxin production.

RESUMO

Este estudo mostrou a atividade antibacteriana *in vitro* de linhagens de leveduras *killer* contra bactérias contaminantes da fermentação alcoólica (*Bacillus subtilis, Lactobacillus plantarum, Lactobacillus fermentum* and *Leuconostoc mesenteroides*), em testes célula X célula e célula X toxina bruta. As bactérias não foram inibidas por linhagens *killer* de *Saccharomyces cerevisiae* (5 dentre 11). Os preparados brutos de toxina de duas leveduras (*Trichosporon figueirae* e *Candida* sp) causaram uma alta inibição no crescimento de *L. plantarum*, mas não na mesma extensão para *B. subtilis*, especialmente para três leveduras *killer* (*Candida glabrata, Pichia anomala* e *Candida* sp). Linhagens de *L. mesenteroides* e *L. fermentum* não foram inibidas em nenhum dos testes. Os resultados obtidos neste estudo sugerem a ação de toxinas *killer* de leveduras contra bactérias, a qual poderia ser utilizada para o biocontrole de

bactérias contaminantes da fermentação alcoólica se testes posteriores de aplicação da toxina dentro das dornas de fermentação se mostrarem eficientes. Um número maior de linhagens *killer* de *S. cerevisiae* deveria ser utilizado para confirmar se elas realmente são incapazes de controlar o crescimento destas bactérias Gram-positivas.

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