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Isolation, characterization, and identification of wild killer yeasts from sugarcane juice

Isolamento, caracterização e identificação de leveduras killer de caldo de cana de açúcar

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Abstract

Ethanol produced by the fermentation of sugarcane juice has emerged as an important renewable fuel. The yield of this fermentation is affected by undesirable microbial contaminants, but killer yeasts can be a promising strategy to reduce this problem. The present study aimed to isolate, characterize, and identify wild killer yeasts from sugarcane juice. Samples were inoculated in culture medium containing chloramphenicol, and 140 colonies with different characteristics were selected. These isolates were submitted to the killer phenotype assay, and the positive killers were characterized and identified according to the standard methods. Only two strains showed killer activity, identified as *Pichia anomala* CE025 and *P. membranaefaciens* CE088. At 25°C, both strains exhibited killer activity at pH 4.0, 4.3, and 4.5, but this activity was not detected at pH 3.0, 3.5, 5.0, and 6.0. The killer phenotype of *P. membranaefaciens* CE088 was inhibited above 30°C, while for *P. anomala*, CE025 inhibition occurred only at a higher temperature. Both strains were able to grow in 12% ethanol, and *P. anomala* CE025 was more tolerant than *P. membranaefaciens* CE 088. Further studies will be conducted to isolate, purify and identify the killer toxins produced by *Pichia anomala* and *Pichia membranaefaciens* species.
Key words: Alcoholic fermentation, antimicrobial activity, biocontrol, ethanol

Resumo

O etanol produzido a partir da fermentação do caldo de cana emergiu como um combustível renovável. O rendimento desta fermentação é afetado por micro-organismos indesejáveis e as leveduras killer se constituem uma alternativa promissora para combater essa contaminação. Nesta perspectiva, o presente trabalho teve como objetivo isolar, caracterizar e identificar leveduras killer de caldo de cana. As amostras foram inoculadas em meio de cultura contendo cloranfenicol e 140 colônias com diferentes características foram selecionadas. Esses isolados foram avaliados quanto à presença do fator killer e os isolados positivos caracterizados e identificados por métodos convencionais. Apenas dois isolados apresentaram atividade killer e foram identificados como *Pichia anomala* CE025 e *P. membranaefaciens* CE088. A 25°C as duas linhagens exibiram atividade killer em pH 4.0, 4.3 e 4.5, mas esta atividade foi inibida a pH 3.0, 3.5, 5.0 e 6.0. Para *P. membranaefaciens* CE088 o fenótipo killer foi inibido acima de 30°C, enquanto que a *P. anomala* CE025 exibiu essa característica acima deste valor. Ambas as linhagens foram capazes de crescer na presença de 12% de etanol, mas *P. anomala* CE025 foi mais tolerante do que *P. membranaefaciens* CE088. Estudos posteriores serão realizados para isolar, purificar e identificar as toxinas killer produzidas pelas espécies *Pichia anomala* e *Pichia membranaefaciens*.
Palavras-chave: Fermentação alcóolica, atividade antimicrobiana, biocontrole, etanol

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Introduction

Killer activity has been found to be widely distributed among yeasts. Ecological studies indicate that this characteristic confers a competitive edge to the producer strains by excluding other yeasts from its habits (SCHMITT; BREINIG, 2006). Killer yeasts can produce toxin proteins or glycoproteins that can be lethal to susceptible yeasts, molds, and bacteria strains, while the killer strains are resistant to their own toxins (MENEGHIN et al., 2010). The action of these yeasts is dependent on the environmental conditions, mainly pH and temperature (ARROYO-HELGUERA et al., 2012).

The increasing energy demand and depletion of oil reserves motivate the search for alternative energy resources. Sugarcane is one of the most important crops in the world, and Brazil is the largest producer of this plant and the second biggest producer of ethanol using sugarcane as raw material (FERREIRA et al., 2012). However, ethanol production is affected by undesirable yeasts from sugarcane and the environment. Killer yeasts may be a tool to reduce the effects caused by this microbial contaminant present in systems under non-sterile conditions (CECCATO-ANTONINI et al., 2004). Allied to the killer characteristic, ethanol tolerance is an important property for yeasts used in industrial ethanol production (TIKKA et al., 2013). Since sugarcane is a reservoir/natural habitat of yeasts and an important raw material for producing ethanol, this study aimed to isolate, characterize, and identify wild killer yeasts from sugarcane juice.

Materials and Methods

Sampling and isolation of yeasts

Samples of sugarcane juice were collected from an alcohol distillery located in Maranguape city Ceará, Brazil. The samples were placed in sterile flasks and transported on ice to the Environmental Microbiology Laboratory of the Biology Department of Federal University of Ceará. A total of 30 samples were taken, each composed of 300 mL of sugarcane juice. Samples were homogenized, and pH was determined by direct measurement in a pH-meter. Then, 10-fold serial dilutions were inoculated on YEPDA (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, dextrose 20 g L^{-1}, and agar 20 g L^{-1}) supplemented with chloramphenicol (2% w v^{-1}) (Merck, Darmstadt, Germany) for the isolation of yeasts. Petri dishes were incubated at 25°C and examined daily for three days (KURTZMAN; FELL, 2011). For each sample, three to five morphologically different colonies were selected and streaked onto YEPDA plates. The isolates were further purified by streaking on YEPD agar and maintained at 4°C in slants with the same medium covered with sterile mineral oil.

Yeast strains

Saccharomyces cerevisiae NCYC 1006 and *Candida glabrata* Y55 were used as sensitive controls, while *Saccharomyces cerevisiae* K1 NCYC 232 was used as a positive control. These strains were provided by the Culture Collection of Federal University of Minas Gerais (UFMG), Brazil, and maintained on YEPDA at 4°C.

Screening of the isolated yeasts for killer phenotype

The killer phenotype was evaluated by the cross-streak test. The isolates were inoculated in ~1cm diameter concentrated zones onto YEPDA supplemented with 0.003% methylene blue and buffered to pH 4.5 with 100 mM citrate/phosphate buffer, previously seeded with 5.0×10^5 cells mL^{-1} of the standard sensitive strains. Plates were incubated for 72 h at 25°C. The appearance of a growth inhibition zone encircled by blue margins representing killed cells around the streak indicated killer activity (ANTUNES; AGUIAR, 2012). The toxin activity was confirmed by the well test method. The positive killer isolates and the positive control *S. cerevisiae* K1 NCYC 232 were cultivated separately in YEPD broth at 25°C for 72 h. The cultures were incubated under agitation at 120 rpm

on a rotatory shaker. Then the cell suspensions were centrifuged at 5000 x g for 5 min at 4°C, and a volume of 100 µL of supernatant was placed into wells (6 mm diameter) cut in the YEPD agar plates that had previously been seeded with 10^5cells mL^{-1} of sensitive indicator strains. The killing activity was measured as the inhibition diameter around the well after incubation for 72 h at 25°C (CECCATO-ANTONINI et al., 2004).

Morphological and biochemical characterization of killer yeast isolates

The colonial morphology of the confirmed killer isolates was observed on YEPD agar. Simple staining was performed, and morphological characters such as cell shape, size, and budding were observed under a microscope. Biochemical characteristics (carbohydrate fermentation, carbohydrate assimilation, gelatinase, and urease) were determined. The killer isolates partially characterized were named CE025 and CE088. Final identification was completed in the Microbiology Department of Federal University of Minas Gerais according to conventional methods used in yeast taxonomy (KURTZMAN; FELL, 2011). CE025 and CE088 were identified as *P. anomala* and *P. membranaefaciens*, respectively, and are deposited in the Culture Collection of the Biology Department of Federal University of Ceará.

Effect of pH and temperature on killer activity

Positive killer isolates were activated in YEPD broth incubated at 25°C for 24 h. Then these isolates were inoculated in a well of 6 mm on the surface of plates containing YEPDA previously seeded with the yeast sensitive *S. cerevisisae* NCYC1006, set to pH values of 3.0, 3.5, 4.0, 4.3, 4.5, 5.0, and 6.0. Plates corresponding to each pH value were incubated in triplicate at 25°C, 30°C, 35°C, and 40°C for 72 h. The killing activity was measured as the inhibition diameter around the well.

Ethanol tolerance

Aliquots of 1 mL of killer yeast suspensions were inoculated in 10 mL of liquid YEPD broth supplemented with 1mL of ethanol in concentrations of 8%, 10%, 12%, 13%, 14%, and 16% (v/v). The initial optical density of each tube was adjusted in a spectrophotometer at 600 nm to 0.1 corresponding to 1 x 10^7 yeast cells. All cultures were incubated at 25°C for 48 h. The increase in optical density was recorded as evidence of growth. The concentration of alcohol at which the growth of yeasts was just inhibited was assessed as the ethanol tolerance of the yeast strains (TIKKA et al., 2013).

Results and Discussion

The pH of sugarcane juice samples ranged from 4.5 to 5.3, which allowed the development of acid-tolerant micro-organisms such as yeasts. Similar values were reported by Oliveira et al. (2006). A total of 140 yeast colonies were selected from 30 sugarcane juice samples. All the isolates were tested for their killer phenotype. Fifteen isolates (~11%) showed a blue precipitated zone, but in the well test method only two of these isolates showed inhibition zones. The killer isolates, designated CE025 and CE088, showed colonies and cells with different morphologies. In the carbohydrate fermentation, CE025 was capable of utilizing glucose, sucrose, and maltose, but not galactose, lactose, and xylose. CE088 was able to ferment only glucose. In the assimilation carbon source test, CE025 could make use of glucose, maltose, celobiose, and manitol. Arabinose, sucrose, lactose, and inositol were not assimilated. CE088 only assimilated glucose. Both strains were negative for gelatinase and urease tests.

In the well test method, CE025 presented inhibition zones of 20 mm and 15 mm against the sensitive yeasts *S. cerevisiae* NCYC 1006 and *C. glabrata* Y55, respectively. For CE088, these measurements were 16 mm and 10 mm, indicating the superiority of the CE025 isolate. The inhibition diameter of the positive control *S. cerevisiae* K1 NCYC 232 was

similar to that found for the CE025 isolate and was higher than that presented by CE088 isolate.

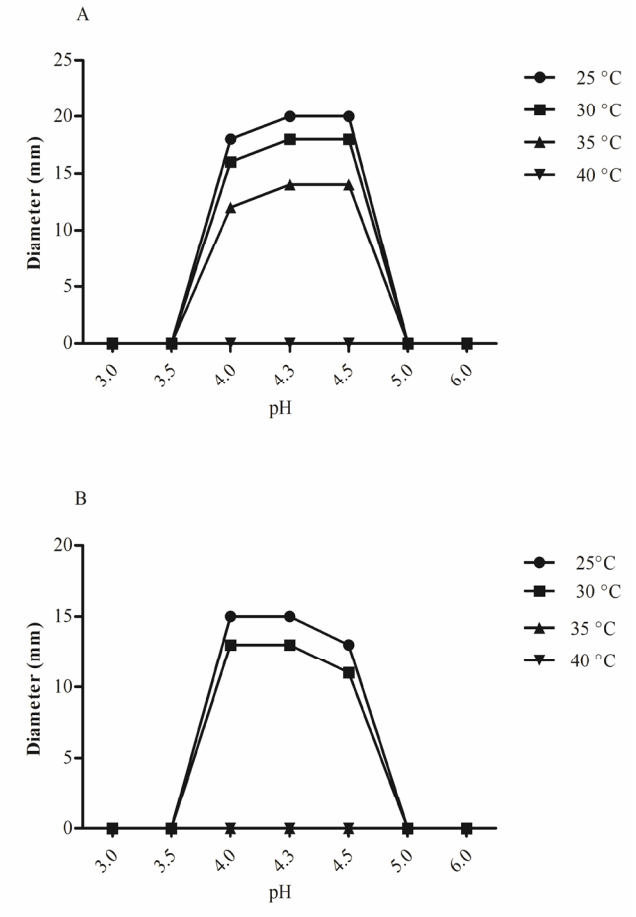
According to Chung et al. (2013), *S. cerevisiae* remains the world's most exploited organism in industrial bioprocesses, but other non-*Saccharomyces* yeasts like *Pichia* spp. have excellent potential for biotechnology applications.

The intergeneric killing spectrum of *Pichia* toxins was reported by Izgu et al. (2006). In this work, *in vitro* inhibition of *S. cerevisiae* NCYC 1006 and of *C. glabrata* Y55 by *P. anomala* CE025 and *P. membranaefaciens* CE088 confirms such a characteristic, which is very promising to combat contaminant yeasts in ethanol fermentation processes.

P. anomala CE025 was able to ferment sucrose, the main carbohydrate of sugarcane for production of ethanol. *P. membranaefaciens* CE088 was unable to ferment sucrose, so this strain is not a first option for fermentation using sugarcane juice as raw material. However, this species may have other applications, such as to control mold diseases (ANTUNES; AGUIAR, 2012).

The action of killer toxins on sensitive cells is influenced by the temperature and especially by the pH of the medium (ARROYO-HELGUERA et al., 2012). *P. anomala* CE025 showed killer activity at pH 4.0, 4.3, and 4.5 at 25°C, 30°C, 35°C, and 40°C, but at pH 3.5, 5.0, and 6.0 this activity was negative at all temperatures (Figure 1A). *P. membranaefaciens* CE088 exhibited killer activity at pH 4.0, 4.3, and 4.5, at 25ºC and 30ºC, but not at 35°C and 40°C (Figure 1B).

Figure 1. Effect of pH and temperature on "killer" activity of *Pichia anomala* CE025 (A) and *Pichia membranaefaciens* CE088 (B).



Under the same conditions of pH and temperature, *P. anomala* CE025 showed better performance than *P. membranaefaciens* CE088, since the inhibition diameter was also higher. Like in this study, Santos and Marquina (2004) found that the killer activity of *P. membranaefaciens* CYC 1106 was observed at pH 4 at temperatures up to 20°C. At pH 4.5, this activity decreased sharply and was barely noticeable at pH 6.

Studies of a variety of killer toxins have shown that they are stable and active only at acidic pH values (SANTOS et al., 2009). Wang et al. (2007) reported that the optimal pH and temperature of the killer *P. anomala* YF07b were 4.5 and 40°C, respectively. In the present work, killer activity of *P. anomala* CE025 was detected up to 35°C in a pH range of 4.0 to 4.5.

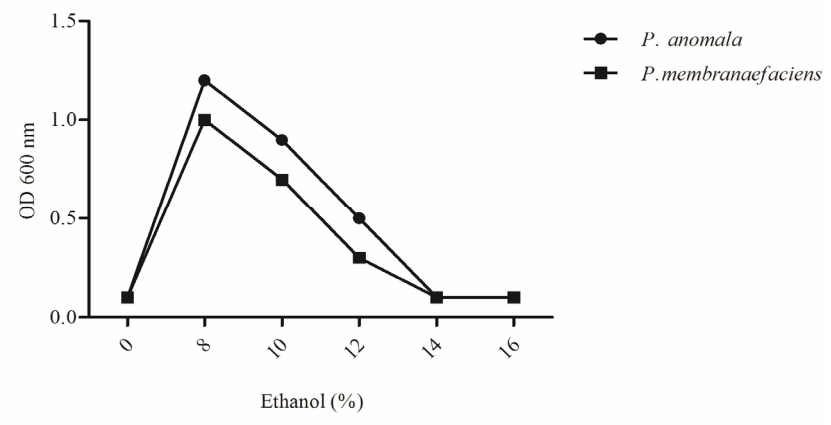
The parameters that favor toxin production of the killer isolates coincide with those found at the beginning of the ethanol fermentation process, at a temperature of about 25°C and pH of sugarcane juice in the range between 4.5 and 5.0. A limitation of ethanol fermentation is the capacity of yeasts to tolerate ethanol concentration. Thus, the ethanol tolerance is an advantage when a yeast is being considered for industrial use, especially where ethanol is being produced (TIKKA et al., 2013).

In this work, the effect of ethanol concentration on the cell growth of *P. anomala* CE025 and *P. membranaefaciens* CE088 was tested. Both strains could grow well in the presence of 8%, 10%, and 12% ethanol, but growth was inhibited in the presence of 14% and 16% ethanol. *P. anomala* CE025 showed better performance than *P. membranaefaciens* CE088 (Figure 2).

Mehdikhan et al. (2011), studying the screening of yeasts for ethanol fermentation, reported the ability of *P. kudriavzevii* to tolerate up to 15% v/v ethanol. Tao et al. (2011) characterized *P. anomala* Y-1 as an ethanol-tolerant strain, enduring ethanol concentrations of up to 14%.

Although the killer activity was detected in *P. anomala* CE025 and *P. membranaefaciens* CE088, in the first strain this characteristic was manifested at higher temperatures, and the diameters of the inhibition zones were larger than found for *P. membranaefaciens* CE088. *P. anomala* CE025 also showed greater ethanol tolerance and was able to ferment glucose, sucrose, and maltose, while *P. membranaefaciens* CE088 fermented only glucose.

Figure 2. Ethanol tolerance for *Pichia anomala* CE025 and *Pichia membranaefaciens* CE088.



Conclusions

P. anomala CE025 has the potential to be exploited in ethanol fermentation. Further research is necessary to isolate and identify the killer toxins produced by *P. anomala* CE025 and *P. membranaefaciens* CE088.

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