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Killer activity of *Pichia anomala* CMGB 88

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ABSTRACT

The killer activity of *P. anomala* is of high interest for biocontrol and biomedical applications as well as for food and feed processing. *P. anomala* CMGB 88 showed killer activity against *Saccharomyces cerevisiae* and different *Candida* potential pathogenic species. Best results were obtained on *S. cerevisiae* 17/17 at pH 6.2 at 37 and 28°C and, to a lower extent, on *C. parapsilosis* CBS 604 at 28°C and pH ranging from 4.4 to 6.2. The isolated killer toxin had good thermostability. When used in high concentrations at 37°C, the toxin determined important reduction of *S. cerevisiae* 17/17 growth kinetics during first hours at pH 4.4. On the contrary, at pH 6.2, growth inhibition was smoother, with rather close values for all concentrations. The impact of the killer toxin on *C. parapsilosis* CBS 604 cell viability augmented in time. The effect of *P. anomala* CMGB 88 toxin against *S. cerevisiae* recommends it for use in food industry in processes spanning over long periods of time.

Keywords: *killer, pH, temperature, Pichia, Saccharomyces, Candida*

1. INTRODUCTION

Strains belonging to *Pichia anomala* (E.C. Hansen) Kurtzman have been isolated from various habitats such as plants, soil, fermented foods or immunocompromised hosts, presenting important metabolic abilities and ability of resisting at stress conditions [1]. The species have been recently renamed *Wickerhamomyces anomalus* and the draft genome sequence revealed the genetic basis for a large range of biotechnological, biomedical and industrial practical applications: glucanase synthesis for biocontrol, production of volatile compounds (ethyl acetate) for food and feed processing and generation of killer toxins with antimicrobial and antifungal properties for biomedicine and food preservation [2].

The killer phenomenon represents a mechanism by which some yeast species and strains are competing for the environmental substrates, reducing or even eliminating the competitor microorganisms: bacteria, fungi or yeasts belonging to the same species or to a different species. The killer toxins can be codified by extrachromosomal virus-like particles with RNA genome, in the case of *Saccharomyces cerevisiae, Ustilago maydis* or *Hanseniaspora uvarum*, by linear DNA plasmids in *Kluyveromyces lactis* or by chromosomal genes in *Williopsis mrakii* and *Pichia anomala* [3, 4].

In present, different research groups reported the characterization of various *P. anomala* killer toxins (PaKT/PKT; Pickt) isolated from different strains, showing especially β-1-3-glucanase activity and molecular weights from 3 up to 85 kDa [5]. Besides their importance as biocontrol agent against fungi [6, 7], *P. anomala* killer toxins were successfully used for obtaining polyclonal, monoclonal and recombinant anti-idiotypic antibiobodies with antimicrobial action against pro- and eukaryotic pathogens [8].

The present work deals with the characterization of a new killer toxin from *P. anomala* CMGB 88. The influence of pH and temperature on the killer activity is also studied as well as the effect of the toxin on the growth kinetics of yeast species of industrial and medical interest.

2. EXPERIMENTAL SECTION

2. 1.Yeast strains. The yeast strain *Pichia anomala* CMGB 88 from the Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest, Romania, was maintained on yeast peptone glucose (YPG) medium (yeast extract 5 g/L, peptone 10 g/L, glucose 20 g/L) supplemented with 20% glycerol. Other yeast strains used during this study were: *Candida albicans* ATCC 10231, *Candida glabrata* CMGB 35, *Candida guilliermondii* CMGB 44, *Candida krusei* CMGB 94, *Candida parapsilosis* CBS 604, *Candida parapsilosis* CMGB 79, *Candida tropicalis* CMGB 165, *Candida zeylandoydes* CMGB 166 and *Saccharomyces cerevisiae* 17/17 (Kil-K0, a his K-R-).

2.2. Killer activity screening tests. The screening tests were performed by spotting colonies from an overnight (o/n) grown culture of *P. anomala* CMGB 88 on Petri plates with agar K medium (0.1 M phosphate citrate buffer pH 4.8, 20 g/L glucose, 10 g/L yeast extract, 20 g/L agar, 0.3 g/L methylene blue) inoculated with an overlay of 10^7 cells/mL of potential sensitive yeast strains. The killer activity was recorded during seven days of incubation at 22°C and considered positive when a blue halo or a zone with reduced growth of the potential sensitive strain appeared surrounding the *P. anomala* CMGB 88 colonies [9].

2.3. Production of killer toxin concentrate. The killer toxin concentrate was obtained as described previously [10]. On this purpose, an o/n grown culture of *P. anomala* CMGB 88 was centrifuged for 10 min at 8000 rpm at 4°C, the supernatant was filtered using 0.22 μm size pore filters (Millipore) and then

incubated for 1 hour at 4°C with ethanol 99% for the precipitation of the killer toxin. After a centrifugation for 20 min at 12000 rpm at 4°C, the sediment was re-suspended in citrate-phosphate buffer (pH: 4.4, 5.0, 5.6 and 6.2) for obtaining a 10-fold concentrated killer toxin which was stored at 4°C.

2.4. Influence of pH and temperature on killer toxin activity against *S. cerevisiae* 17/17 and *C. parapsilosis* CBS 604. *S. cerevisiae* 17/17 and *C. parapsilosis* CBS 604 cultures were mixed with agar K medium with different pH values (4.4, 5.0, 5.6 and 6.2) for a final inoculum of 10^7 cells/mL and then poured into Petri plates. The well test assays were performed by loading 8 mm-diameter wells cut in the agar with 80 µL of concentrated killer toxin. The plates were incubated for seven days at 22, 28 and 37°C.

The activity of the killer toxin was expressed in arbitrary units (AU), one unit representing the amount of toxin contained in 80 µL forming a growth inhibition halo of 8 mm [11, 12].

2. 5. Effect of killer toxin on growth kinetics of sensitive strains. The strains *S. cerevisiae* 17/17 and *C. parapsilosis* CBS 604 were grown for 20 hours in YPG medium and 10^7 cells/mL were mixed in microtitre wells with 10, 20 or 35 µL concentrated killer toxin (pH 6.2) in a final volume of 150 µL. The microplates were incubated at 28°C and the cell growth kinetics was evaluated by reading the OD 480 nm at 2-hours intervals for the first 8 hour using an automatic plate reader (Multi-mode reader Synergy HTK, BioTek) [13, 14]. Growth inhibition was expressed in percentage of viable cells reported to growth rates of the reference sample (without killer toxin).

3. RESULTS SECTION

The screening tests revealed the killer activity of *P. anomala* CMGB 88 after only three days of incubation in presence of *C. parapsilosis* CBS 604 and *S. cerevisiae* 17/17. After seven days, killer activity appeared against most of the tested yeast strains (Table 1). Two types of actions were recorded: zones of growth inhibition (marked in blue in Table 1) with the widest diameter for *C. parapsilosis* CBS 604 and clear halos for *S. cerevisiae* 17/17 and *C. tropicalis* CMGB 165 (Figure 1).

Table 1. Killer activity of *P. anomala* CMGB88 at 22°C.

Potential sensitive strain	Killer activity
C. albicans ATCC 10231	-
C. glabrata CMGB 35	-
C. guilliermondii CMGB 44	-
C. krusei CMGB 94	+
C. parapsilosis CBS 604	++++
C. parapsilosis CMGB 79	++
C. tropicalis CMGB 165	++
C. zeylandoydes CMGB 166	-
S. cerevisiae 17/17	++

- = no activity, + = medium, ++ = good; ++++ = high activity

Figure 1. Killer activity of *P. anomala* CMGB 88 after three days of incubation against: (a) *C. parapsilosis* CBS 604, (b) *S. cerevisiae* 17/17, (c) *C. tropicalis* CMGB 165.

Since best results were obtained in short time for *C. parapsilosis* CBS 604 and *S. cerevisiae* 17/17, further tests were performed using these sensitive strains and the *P. anomala* CMGB 88 concentrated killer toxin. In the case of *S. cerevisiae* 17/17, the pH and temperature seemed to have a complementary influence on the *P. anomala* CMGB 88 killer toxin activity. Thus, if at 22°C at pH 5.6 and pH 6.2, the AU values were 50, respectively 56 (Figure 2a, b), at 28°C even at lower pH values (5.0 and pH 5.6) the activity was higher (AU 75) (Figure 2c). Surprisingly, the lower activity was obtained at 37°C and pH 4.4 (AU 38) (Figure 2e). However, high pH values and temperatures enhanced the killer toxin activity in this case. Thus, best results were obtained against *S. cerevisiae* 17/17 at pH 6.2 at 37°C (AU 141) (Figure 2f), respectively, at 28°C (AU 88) (Figure 2d).

The killer toxin had lower activity against *C. parapsilosis* CBS 604. Thus, rather similar results were obtained at 22°C and pH 6.2 (AU 50), respectively, at 28°C and pH ranging from 4.4 to 6.2 (AU 54-55) (Figure 3). On the contrary, faint halos (AU 41) were observed at 37°C only at pH 6.2. As [15] observed, the *P. anomala* killer toxin was inactivated by incubation at 37°C and had no activity against *C. albicans* UCSC 10R. On the other hand, [16] obtained anti-*Candida* killer activity at 37°C using *P. anomala* killer toxin at pH 6.0. Moreover, [17] determined killer activity for *P. anomala* toxin at pH 4.5 and 37°C with a higher incidence against *S. cerevisiae* (87% of *S. cerevisiae* tested strains were sensitive) compared with *C. parapsilosis* (maximum 75% sensitive strains). This is in agreement with our results, under similar conditions, the killer activity of *P. anomala* CMGB 88 being more effective against *S. cerevisiae17 /17* compared with *C. parapsilosis* CBS 604.

In general, the killer toxin from *P. anomala* CMGB 88 showed good stability at all the pH and temperatures tested [12, 18]. The high activity of *P. anomala* toxin against *S. cerevisiae* in conditions similar to those used during our experiments, i.e. pH values above 4.4 and 30°C, was also described by [19]. The *P. anomala* toxin targets mainly the β-1-3-glucan from the cell wall of the sensitive yeast cell having a lower affinity towards β-1-6-glucan [19, 20]. In *C. albicans*, presenting high similar cell structure to *C. parapsilosis*, more than 50% of the cell wall glucans are β-1-6 linked [21]. This could provide a possible explanation for the lower activity of the *P. anomala* CMGB 88 killer toxin against *C. parapsilosis* CBS 604 compared to *S. cerevisiae* 17/17. Nevertheless, the activity of a killer toxin also varies depending on the sensitive strain tested.

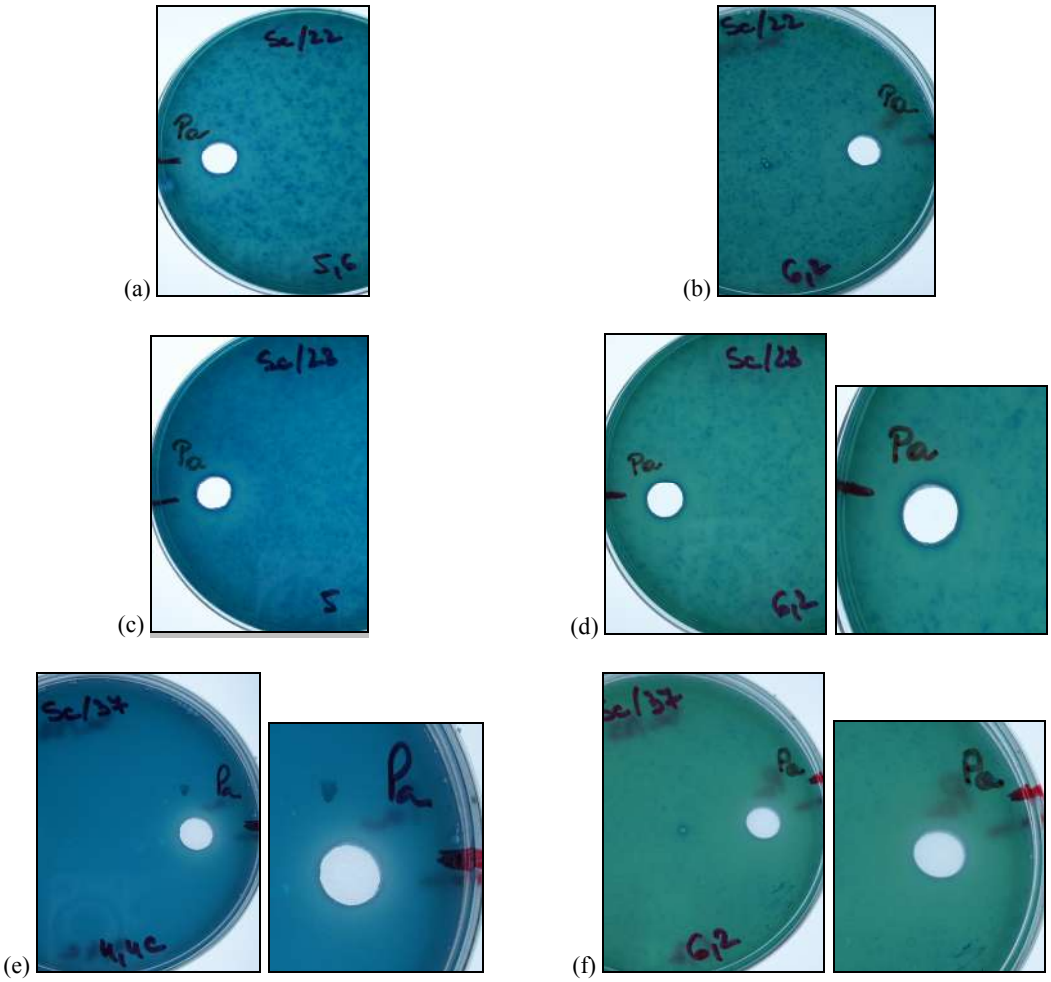

Figure 2. Killer activity of *P. anomala* CMGB 88 toxin concentrate against S. *cerevisiae* 17/17

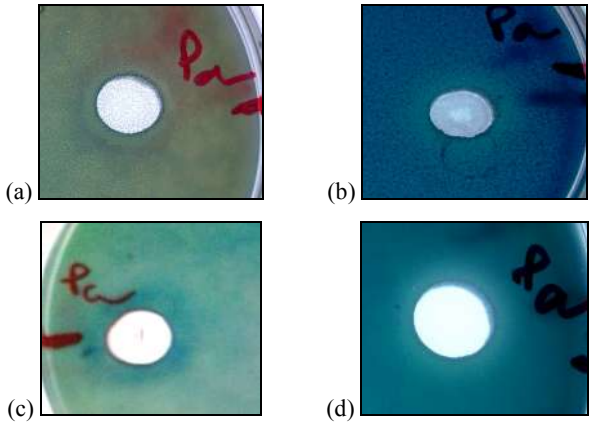

Figure 3. Killer activity of *P. anomala* CMGB 88 toxin concentrate against *C. parapsilosis* CBS 604
(a) 22°C – pH 6.2; (b) 28°C – pH 4.4; (c) 28°C – pH 6.2; (d) 37°C – pH 6.2.

Differences between the activity of the killer toxin on *S. cerevisiae* and *C. parapsilosis* strains at 28°C and pH 6.2 were recorded even from the first eight hours of incubation. Thus, the number of viable cells decreased in the case of *S. cerevisiae* 17/17, with a visible impact in presence of 35 µl toxin (K35, Figure 4a). In the case of *C. parapsilosis* CBS 604, after the first four hours, the effect diminished (Figure 4b). Moreover, the presence of toxin in different concentrations, i.e. 10 µl, respectively, 20 µl did not seem to have any impact on *C. parapsilosis* cell viability (K10 and K20, Figure 4b). The results suggest that the killer toxin activity augmented in time.

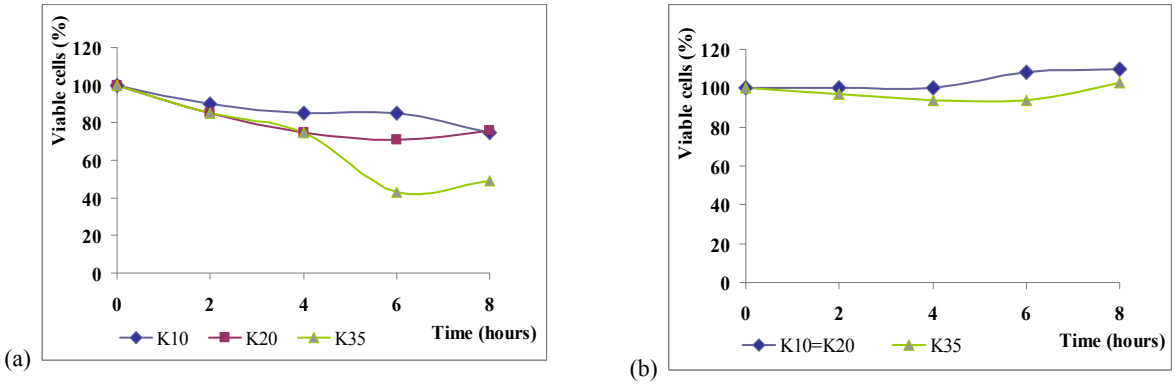

Figure 4. Effect of killer toxin concentrations at 28°C and pH 6.2, on the growth rate of: (a) *S. cerevisiae* 17/17, (b) *C. parapsilosis* CBS 604.

The growth reduction assays on *S. cerevisiae* 17/17 revealed that at 37°C the toxin was very active during first hours at pH 4.4 especially for high concentrations (20 and 35 µl) (K20 and K35, Figure 5a). Since previous experiments showed that after seven days the activity was very low (AU 38), the data imply that, most probably, under these conditions, the killer effect diminished after the first hours. On the contrary, at pH 6.2, growth inhibition was smoother, with rather close values for all three concentrations (Figure 5b). However, the best killer activity of the toxin concentrate against *S. cerevisiae* 17/17 was obtained at 37°C and pH 6.2 (AU 141). Similar with the case of *C. parapsilosis* CBS 604 at 28°C, the toxin activity at pH 6.2 proved to be highly influenced by a longer incubation (Figure 6).

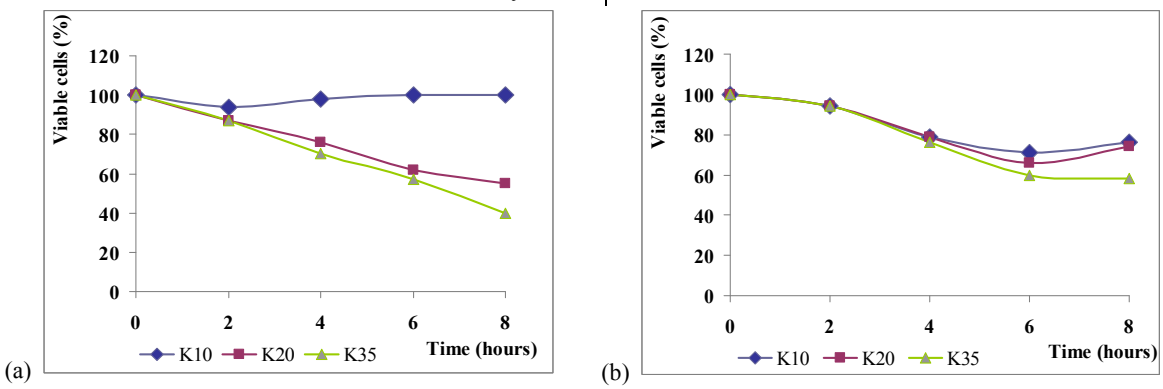

Figure 5. Effect of killer toxin concentrations on *S. cerevisiae* 17/17 growth at 37°C and (a) pH 4.4, (b) pH 6.2

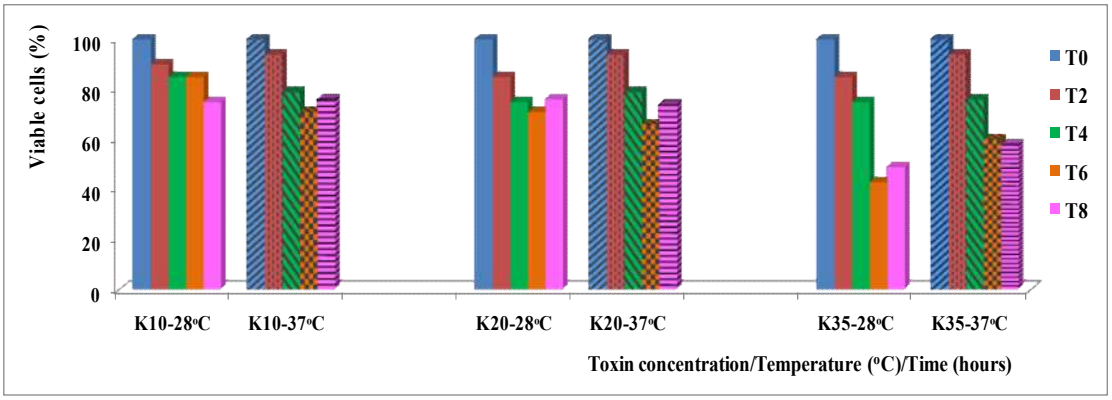

Figure 6. Effect of different concentrations of killer toxin concentrate at pH 6.2 on *S. cerevisiae* 17/17 growth kinetics.

4. CONCLUSIONS

The yeast *P. anomala* CMGB 88 showed killer activity against *Candida* potential pathogenic species and *S. cerevisiae*. The killer toxin had good thermostability and was active for a wide range of pH, from 4.4 to 6.2. The influence of pH and temperature values on the activity of the concentrated toxin was species specific, with a preference against the *S. cerevisiae* sensitive strain. The high killer activity of the *P. anomala* CMGB 88 toxin against *S. cerevisiae* during a week suggests its possible application in processes spanning over long periods of time, such as wine maturation.

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