

## The antifungal effect of the biopreparation of *Xenorhabdus budapestensis* bacteria on some *Candida* species

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**Introduction:** The prevalence of invasive fungal diseases shows an increasing trend. Due to the frequent but unprofessional usage of antifungal medications, the fungi show decreasing susceptibility towards these agents and this trend may lead to the emergence of resistant pathogens. There is a great need to develop antifungal medications with new mechanisms. One of these options is to apply proteins with natural antifungal effects. The objective was to measure the antifungal efficacy of *Xenorhabdus budapestensis* in vitro on clinical *Candida* species (*Candida albicans*, *Candida lusitanae*, *Candida krusei*, *Candida kefyr*, *Candida tropicalis*, and *Candida glabrata*). **Materials and methods:** We defined the sensitivity of the *Candida* species towards antibiotics. We conducted agar diffusion tests with the cleaned biopreparation of *X. budapestensis* (100%) and its dilutions (80%, 60%, 40%, and 20%). Zones of inhibition were measured after 24, 48, and 96 hr. **Results:** Most of the tested *Candida* species have shown sensitivity to the biopreparation and its 40% dilution. The area of the zones of inhibition did not decrease after several days. The most sensitive species was *C. lusitanae* and the least sensitive was *C. krusei*. **Conclusion:** We assume that the proteins produced by *X. budapestensis* have antifungal effect, as the area of the zones of inhibition did not change.

**Keywords:** *Candida* species, antifungal effect, *Xenorhabdus budapestensis*, resistance

### INTRODUCTION

The number of diseases caused by fungi is gradually increasing. The most common species is *Candida albicans*, the other group consists of non-*albicans* *Candida* species as per its frequency [1].

*C. albicans* is part of the normal human microflora, but under certain circumstances, it may cause superficial or invasive fungal diseases. It may produce a biofilm on a biotic or abiotic surface and its adhesion is supported by adhesins [2]. Its hyphae may produce certain hydrolases, which promote their entry to the host cell. They are also capable of easily adapting to various pH levels and other stress factors within the human body [3].

*Candida glabrata* is the second most common species, it has been isolated from human faeces in 1917 [4]. Its growth temperature optimum is 37 °C, which is the core temperature of the healthy human body. Its tolerance towards different stress factors is very high and tolerates the absence of nutrients well. It is also resistant against the attacks of the immune system. It has shown high resistance against azole-type antifungal medications. A protein called adhesin ensures its adhesion to the cell membrane. It easily produces biofilm on medical devices, such as catheters [5].

*Candida lusitanae* occurs more rarely than the rest of the *Candida* species. The first reports of this species were presented in 1979. Its teleomorph form is called *Clavispora lusitanae*. It is resistant to Amphotericin B and produces haemolysin [6]. The sensitivity towards Amphotericin B can be increased if combined with fluconazole and 5-fluorocytosine [7]. One of the reasons behind the resistance is the change in the biosynthesis of ergosterol. Due to that, the ergosterol content of the cell membrane decreases [8].

*Candida kefyr* has been isolated from kefir in 1909, its former name is *Candida pseudotropicalis*. Its teleomorph form is the yeast of kefir, the *Kluyveromyces marxianus* [9]. Rarely, it may cause invasive infections, especially simultaneously with malignant diseases of the haematopoietic system [10]. It produces protease, haemolysin, and phospholipase [6].

The prevalence of *Candida krusei* is increasing similar to *C. glabrata*. It is fluconazole resistant due to the altered

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functioning of the P450 isoenzyme [11]. It also shows decreased resistance to Amphotericin B and flucytosine. It is likely to produce biofilm [12] and to produce phospholipase [6].

*Candida tropicalis* was first reported as *Oidium tropicale* in 1910. It is a frequently isolated *Candida* species especially in cancer patients. It has a strong adhesive feature and easily produces biofilm [6]. Its prevalence is greatly increased due to the use of broad-spectrum antibiotics or long-lasting catheters. The matrix of the biofilm differs from the ones produced by other species due to its hexosamine content. It is resistant to Amphotericin B and fluconazole. Its hypha-producing ability and enzyme production (phospholipase, protease, and haemolysin) only increase its virulence [6].

The number of *C. krusei*, *C. glabrata*, and *C. tropicalis* infections also increases gradually.

## OBJECTIVES AND METHODOLOGY

The objective of our work is to test the antimicrobial effects of the protein produced by the Gram-negative insect pathogen bacteria *Xenorhabdus budapestensis* on six *Candida* species (*C. albicans*, *C. krusei*, *C. tropicalis*, *C. lusitaniae*, *C. kefyr*, and *C. glabrata*). The isolated specimens are from clinical samples.

Our experiment has been motivated by two factors. One of them is the results originating from the previous testing of the cell-free culture media (CFCM) and its cleared biopreparation [purified fraction (PF)] of *X. budapestensis* on Gram-negative and Gram-positive bacteria originating from veterinary and human infections [13–16].

The other factor is the experiments conducted with *Xenorhabdus cabanillasii* bacteria. This bacterium was able to inhibit the growth of various filamentous fungus species, including *Fusarium oxysporum* and *Candida* as well [17]. It has to be noted that our team has also conducted experiments with filamentous fungi (*Aspergillus fumigatus*, *Aspergillus flavus*, and *Fusarium* spp.). However, with the clinical samples, we have not been able to achieve any antifungal effects that could be considered as lasting. These experiments prove that there are major differences between the antimicrobial proteins of the various *Xenorhabdus* species.

The antimicrobial agent of *X. budapestensis* is a hexapeptide protein, which was named as *bicornutin-A* back in 2009 [18]. Researchers in Frankfurt also noted the antimicrobial effects of *X. budapestensis*. MALDI spectrometry has proven that this hybrid molecule protein is rich in arginine and contains putrescine and phenylethylamine, and was named as *fabclavin*. Moreover, a 6,848-bp section of the *bicA* gene has been discovered, which is responsible for the production of bicornutin-A. The gene cluster has been recognised in *E. coli* *DH10B* bacteria with the heterologous expression of the *bicA* gene [19].

Our work was carried out in the microbiology laboratory of the Faculty of Health Sciences of Semmelweis University cooperating with the Research Institute of Bioengineering, Membrane Technology and Energetics of the University of Pannonia. We have received the tested *Candida* species from the clinical microbiology laboratory of Semmelweis University.

## The process of producing CFCM

The CFCM and the cleaned biopreparation were made from the *Xenorhabdus* antimicrobial peptide (XAMP)-producing primer variant of the DSM-16342<sup>T</sup> entomopathogenic bacteria strain of *X. budapestensis*, which was able to produce the substance with the highest antimicrobial effect in previous studies [20].

The bacterium, which produces the antimicrobial substance, was grown in lysogeny broth at 25 °C temperature, shaken at 200 rpm and at increasing levels in three steps. After reaching the stationary phase, we have centrifuged the cell culture (15,000 × g, 20 °C, 20 min) and filtered it with a 0.22-µm pore-diameter (Merck Millipore, Merck Kft., An affiliate of Merck KGaA, Darmstadt, Germany) filter. The CFCM was produced from this. A further 3 L of conditioned CFCM were cleaned, from which the fraction rich in cleaned peptides (PF) was prepared.

The prepared solutions were shaken for 48 hr on 150 rpm with 20 g/L Amberlite<sup>®</sup> XAD 1180<sup>R</sup> cation ion-exchange resin – previously activated in distilled water (121 °C, 30 min) with an autoclave – in order to better adsorb the bioactive substances. Then, the washing of the resin and extraction was carried out in multiple steps with applying methanol solutions of different concentrations in the following order: 1 L sterile distilled water, 500 ml of 25% MeOH, 3 × 300 ml of 50% MeOH, 3 × 300 ml of 80% MeOH, and finally 300 ml cc MeOH, which has been acidised with 3 ml 2 N HCl. The concentrated, acidic methanol fraction, which contains the components with antimicrobial effect, has been vaporised to an approximate volume of 50 ml with the help of rotational vacuum distillation on 35 °C, then it was neutralised to pH 6 with a 2N NH<sub>4</sub>OH solution. Then, the neutralised sample has been completely dried out. That is how the process ended with a substance with 47.7 mg/ml dry material content, which was stored in 10 ml distilled water in a sterile tube in a refrigerator until its application.

Testing of the biopreparation was conducted on six clinical samples of *Candida* species (*C. albicans*, *C. krusei*, *C. tropicalis*, *C. lusitaniae*, *C. kefyr*, and *C. glabrata*). We have transferred one unit from each pure culture of the yeast per species with a sterile inoculation loop to 10 ml *Sabouraud-dextrose* culture medium, which has been cultured in a thermostat at 28 °C under aerobic conditions after vortexing. The yeast cultures picked for testing were set to 0.5 McFarland standard on the following day.

We have transferred 300 µl of each of these cultures and mixed them with 2.7 ml of soft agar, which has been previously heated up to 54 °C, then poured them to the agar plates with a quick movement. After a 20-min wait, the soft agar settled and holes were made with a 9-mm pin drill, which were capable of holding substances up to 100 µl [21]. Each plate contained five holes (Figure 1).

The stock solution was available in 10 ml volumes (0.47 mg dissolved in 10 ml distilled water), which was our 100% solution. The distilled water made 80%, 60%, 40%, and 20% dilutions. The dilutions have been performed according to the macro dilution methods published by the *Clinical and Laboratory Standard Institute* [22]. The diluted solutions had the following dry material



Figure 1. Agar diffusion technique on Sabouraud agar

contents: 100% (0.47), 80% (0.38), 60% (0.28), 40% (0.19), and 20% (0.095) µg/ml. Three parallel series were made.

The sensitivity of tested *Candida* species to certain antifungal medications has been determined by paper disk diffusion method on Mueller–Hinton agar fungi (MHF) (Figure 2; Table 1). Based on the area of zones of inhibition, we may differentiate *susceptible* (S), *intermediate* (I), and



Figure 2. Determining the antifungal sensitivity of *C. krusei* with paper disk method

*resistant* (R) species. A certain yeast species can be considered susceptible, if the inhibition zone is  $\geq 20$  mm (or  $\geq 10$  mm in case of griseofulvin); intermediate, if the inhibition zone is between 12 and 19 mm; and resistant, if the inhibition zone is  $\leq 11$  mm in vitro [23].

Data analysis was carried out with IBM SPSS Statistics 20.0 software [IBM Corp. Released 2011, Armonk, NY; descriptive statistics, one-way analysis of variance (ANOVA)].

## RESULTS

The first evaluation of the plates was performed after 24 hr and the zones of inhibition were also measured (Figure 3). The plates were put in a thermostat for further incubation and the measurements were carried out again after 48 and 96 hr (Figures 4 and 5).

The *vertical* axis displays the area of the zones of inhibition in millimeters, whereas the *horizontal* axis displays the tested *Candida* species. The columns with different colors represent different dilutions. Certain fungi species displayed a minor increase in the average area of the zones of inhibition compared to the 24-hr values. However, the diameter of the zones remained the same at later tests.

The 96-hr average values of the three parallel zones of inhibition can be seen in Figure 5. A minor increase in the diameter can be observed with certain dilutions, but a decrease could never be observed, not even after 96 hr.

## DISCUSSION

We tested the efficiency of the antimicrobial proteins synthesised by *X. budapestensis* on six different *Candida* species isolated from clinical samples with agar diffusion method. We have worked with five different dilutions of the cleaned, protein-rich biopreparation. Based on the results of the zones of inhibition after a 24-hr incubation period, it can be observed that the tested protein had an immediate antifungal effect on the *Candida* species. Every tested *Candida* species showed sensitivity to the 100% dilution,

Table 1. The sensitivity of *Candida* species for antifungal medications with paper disk method

Tested active agents	<i>C. albicans</i>	<i>C. glabrata</i>	<i>C. lusitaniae</i>	<i>C. kefyr</i>	<i>C. tropicalis</i>	<i>C. krusei</i>
Origin of yeast from human culture	Haemo-culture	Cannula	Sputum	Sputum	Wound exudate	Trachea
ECO (10 µg)	S	S	S	S	I	I
ITC (50 µg)	R	R	R	I	R	R
FC (1 µg)	R	I	R	I	R	R
FLU (100 µg)	R	R	S	I	R	I
KTC (10 µg)	I	R	R	I	S	S
GRS (10 µg)	R	R	R	R	R	R
AMB (20 ut)	R	I	R	S	R	R
CLT (50 µg)	S	S	S	S	I	S
ECO (50 µg)	S	S	S	S	S	I
MCZ (10 µg)	R	R	R	R	R	R

Note. Abbreviation of antifungal disks according to the manufacturers. AMB: amphotericin B (20 ut); CLT: clotrimazole (50 µg); FLU: fluconazole(100 µg); ECO 10: econazole (10 µg); ECO 50: econazole (50 µg); ITC: itraconazol (50 µg); FC: flucytosine (1 µg); KTC: ketoconazole (10 µg); GRS: griseofulvin (10 µg), MCZ: miconazole (10 µg); S: susceptible; I: intermediate; R: resistant.

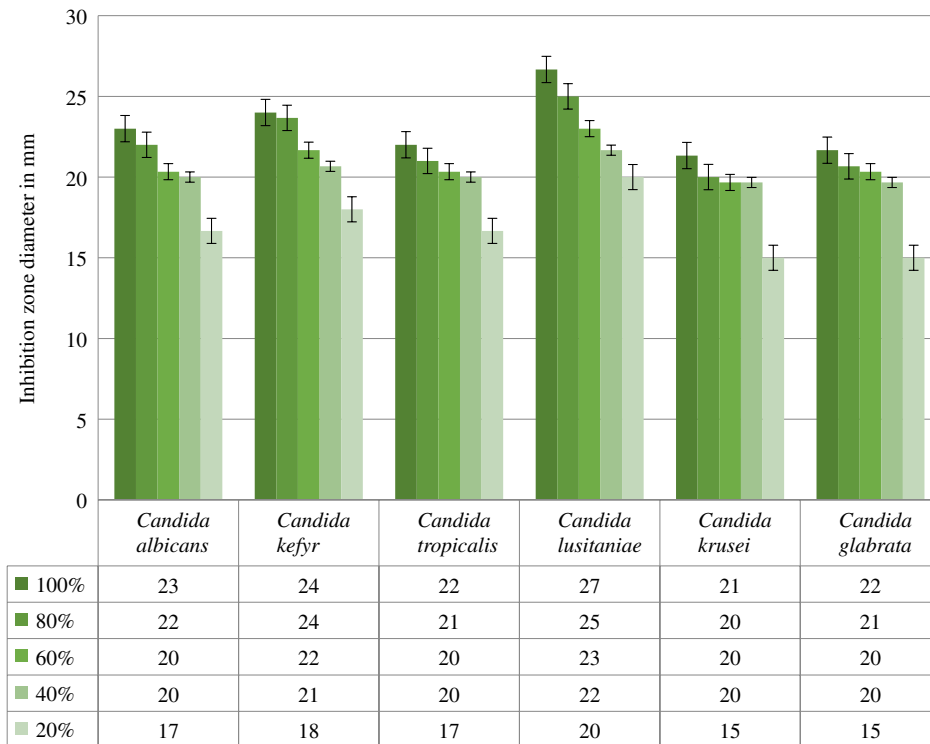


Figure 3. The average results of the antifungal effects of *X. budapestensis* at 24 hr

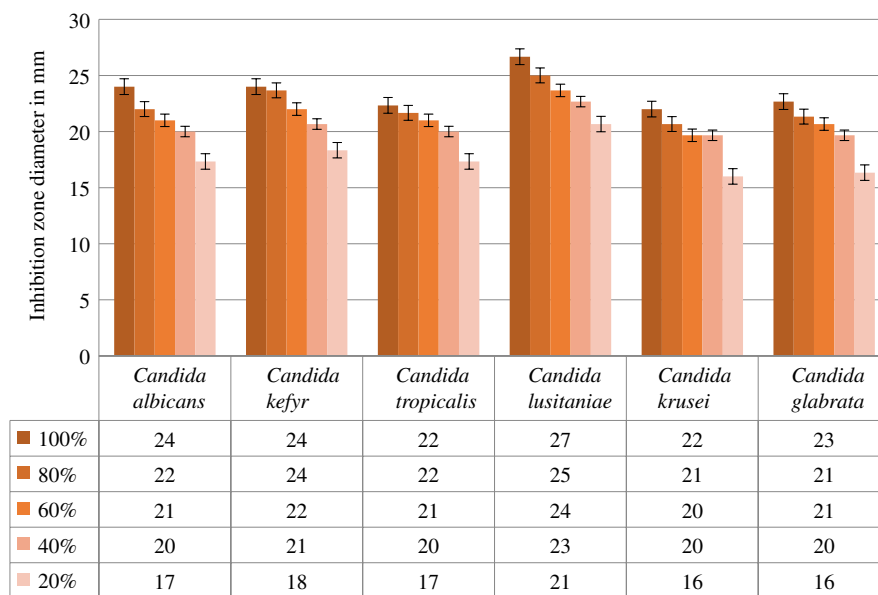


Figure 4. The average results of the antifungal effects of *X. budapestensis* at 48 hr

as the zones of inhibition have exceeded 20 mm in most of the cases. *C. lusitaniae* appeared to be the most sensitive, whereas *C. krusei* the least sensitive. The sensitivity has been confirmed with 80% dilutions as well, the zones of inhibition were all larger than 20 mm with every fungus. In case of 60% and 40% dilutions, the *Candida* species still showed sensitivity though with less intensity.

Based on our previous research, the 20% dilution was enough to produce the desired effect [15]. In a similar study mentioned at the methodology part, the protein produced by

*X. cabanillasi* called “cabanillasin” had a greater inhibitor effect on *C. krusei* and *C. lusitaniae* than on *C. albicans* and *C. glabrata* after the 24-hr measurement.

However, the activity of *cabanillasin* on the tested fungi has decreased after 48 hr in these experiments [17].

Every single tested species showed sensitivity in our case. After 48 hr, some of the *Candida* species showed an increase in the diameter of the zones of inhibition with certain dilutions, but none of the species showed a significant decrease. Similar to the 24-hr results, we have

### The antifungal effect of *Xenorhabdus budapestensis*

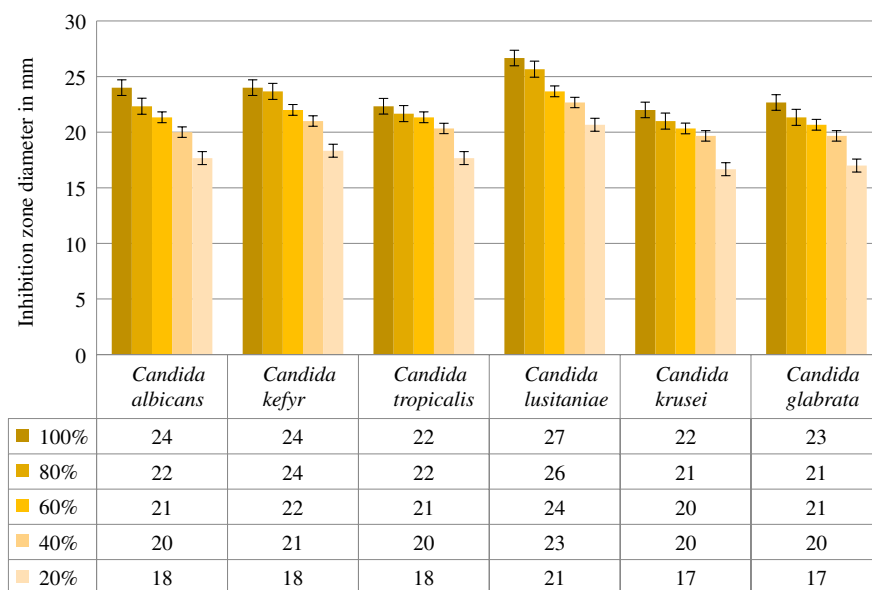


Figure 5. The average results of the antifungal effects of *X. budapestensis* at 96 hr

measured zones of inhibition greater than 20 mm in case of 100%, 80%, 60%, and 40% dilutions.

The last measurement of the zones of inhibition was carried out after 96 hr. It is observable from the results that there was no decrease in the diameter of the zones of inhibition with any of the *Candida* species. Therefore, it can be assumed that “fungicide” effect was reached in our experiment. The dry material content was 0.19 µg/ml, thus this value can be called the *Minimal Fungicidal Concentration* (MFC), which was created by the 40% dilution.

Dry material content was different for each dilution as they had different protein amounts. Solutions with higher concentration had more useful proteins with antimicrobial effect.

There was no relation between the antifungal and XAMP sensitivity of the *Candida* species. Despite a considerably high resistance against other antifungal compounds, *C. lusitanae* showed the highest sensitivity to the proteins in the biopreparation.

It was important to clarify in this study whether different concentrations and protein content have any effect on the intensity and to what degree if there is any. To prove the hypothesis, we used single-factor ANOVA on 5% significance level.

The result of the test is  $p < .001$ ; therefore, the null hypothesis, which claims that the generated effect depends on the dilution, can be confirmed. This proves that our hypothesis has been confirmed, meaning that significantly greater zones of inhibition have been measured at higher concentration levels and that there is correlation between the antimicrobial effect and the dilution of the biopreparation.

### CONCLUSIONS

Every tested *Candida* species has shown sensitivity to the proteins synthesised by *X. budapestensis* up to

40% dilution. The area of the zones of inhibition did not decrease as time passed during our agar diffusion experiment. We assume that the proteins in the biopreparation have a “fungicide” effect. The greatest sensitivity was shown by *C. Lusitanae*, whereas *C. krusei* was the least sensitive. Our results confirmed the previous experiments in which *C. albicans* showed sensitivity to the antimicrobial protein of *X. cabanillasi* [17].

The frequent and unprofessional usage of antifungal medications significantly decreases the sensitivity of fungi towards these products. Reasons behind the resistance mechanism may include: decreased amount of active agents get into the cell, increased amount of active agents are excreted through the efflux pumps, or the target molecule is modified [24]. There is an increasing need to develop new antifungal medications with new mechanisms and with different active agents.

During our previous studies, we have also confirmed the bactericide effect of the biopreparation of *X. budapestensis* on Gram-positive and Gram-negative bacteria. Gram-positive bacteria were more sensitive, but every tested bacterium showed sensitivity to even the 20% dilution [15].

### ABBREVIATIONS

<i>bicA</i>	: bicornutin-A gene
CFCM	: cell-free culture media
HCL	: hydrogen chloride
MALDI	: matrix-assisted laser desorption/ionisation
MeOH	: methyl alcohol
MFC	: minimal fungicidal concentration
MHF	: Mueller–Hinton agar fungi
NH <sub>4</sub> OH	: ammonium hydroxid
PF	: purified fraction
pH	: pondus hidrogenii

SD : Sabouraud dextrose agar  
 XAMP : Xenorhabdus antimicrobial peptide

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