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Characteristics of Killer Toxin of the Yeast *Cryptococcus pinus*

Ekaterina Kulakovskaya, Anton Zvonarev, Vasilina Farofonova

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Federal Research Center "Pushchino Research Center for Biological Studies", Russian Academy of Sciences, Pushchino, Russia
Email: Ekaterina.kulakovskaya@gmail.com

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Abstract

This work was aimed to study of fungicidal activity and mechanism of action of the killer toxin of *Cryptococcus pinus* VKM Y-2958. A killer-toxin preparation was obtained and partly purified from the culture liquid of the yeast *Cryptococcus pinus*. Its fungicidal activity against the pathogenic yeast *Filobasidiella neoformans* was assessed by assay of target cells growth inhibition and propidium iodide staining. The preparation contained three peptides with molecular masses of about 11 - 13 kDa, which did not show similarities in the mass spectrometric analysis. When frozen, the killer-toxin preparation remained active for several years. The killer toxin of *Cr. pinus* exhibits the high fungicidal activity against the known pathogen *F. neoformans*, is stable during long-term storage, and its mechanism of action is based on damaging target cell membranes. The killer toxin was obtained by a simple procedure and is of interest for the development of novel fungicidal preparations.

Keywords

Cryptococcus pinus, *Filobasidiella neoformans*, Killer Toxin, Fungicide, Membrane-Damaging Activity

1. Introduction

Many yeast species can produce killer toxins active, inter alia, against pathogenic yeasts [1] [2] [3]. Yeast killer toxins are proteins or glycoproteins causing cell death in representatives of the same species or genus, or in other phylogenetically related genera [1] [2] [3]. The molecular masses of killer toxins vary from 10 to 50 kDa. They have different physicochemical properties, in particular, thermostability, optimal pH value and sensitivity to proteinases, as well as the mechanisms of action on target cells.

The fungicidal activities of killer toxins can manifest themselves in damaging the cytoplasmic membrane and forming ion channels [4] [5] [6]. Such membrane-damaging effect leads to the intracellular pH decrease, the release of potassium ions, ATP, and other low-molecular compounds from target cells, and the suppression of amino acid transport to cells. In addition, some killer toxins arrest the cell cycle in different phases [7], being the triggering mechanisms of apoptosis [7] [8], and cause damage to transport RNA [9] and DNA [10] by exhibiting a specific anticodon nuclease activity and affecting yeast cell wall components, e.g., β-1,6-glucan [11].

Investigation of the killer phenomenon in yeasts is a source of valuable information about some fundamental aspects of biology in eukaryotes and the interaction between different eukaryotic organisms. In addition, killer toxins are of unquestionable practical interest, because many of them demonstrate a broad range of activity against microorganisms, including those causing food spoilage [12], as well as plant, animal and human pathogens [13].

Recently, killer toxin secretion has been found in the yeast *Cryptococcus pinus* using co-cultivation method [14]. The killer toxin produced by *Cr. pinus* VKM Y-2958 has a rather broad spectrum of activity, exhibits fungicidal activity at low pH values of the medium, is thermolabile (completely inactivated after 5-min heating to 100˚C) and proteinase-resistant. The study of this killer toxin is highly interesting in the context of its activity against *Filobasidiella neoformans* causing mycoses.

This work was aimed at characterizing the killer toxin produced by *Cr. Pinus* VKM Y-2958 and investigating its fungicidal activity and the mechanism of action on target cells.

2. Materials and Methods

2.1. Yeast Strains and Cultivation Conditions

The yeast strains were *Cryptococcus pinus* VKM Y-2958, *Cystofilobasidim bisporidii* VKM Y-2700 and *Cryptococcus terreus* VKM Y-2253 from the All-Russian Collection of Microorganisms, as well as the yeast *Filobasidiella neoformans* IGC 3957 from the Collection of the Gulbenkian Institute of Science (Portugal).

The yeast *Cr. pinus* was grown in a medium containing (g/L): glucose, 20; peptone (enzymatic, Sigma, USA), 10; yeast extract (Fluka, Germany), 5; $Na_2HPO_4 \times 12\ H_2O$, 16.3. The pH value was adjusted to 4.5 with citric acid. The cultivation was performed on a shaker at 25˚C for 2 weeks. The yeast *Cys. bisporidii* was grown in a medium containing (g/L): glucose, 5; peptone (enzymatic, Sigma, USA), 2.5; yeast extract (Fluka, Germany), 2; $Na_2HPO_4 \times 12\ H_2O$, 10.9. The pH value was adjusted to 3.5 with citric acid. The cultivation was performed on a shaker at 25˚C for 4 days. The yeasts *Cr. terreus* and *F. neoformans* were grown in YPD-P medium containing (g/L): glucose, 10; peptone (enzymatic, Sigma, USA), 5; yeast extract (Fluka, Germany), 4. The cultivation was performed on a shaker at 25˚C for 2 days.

2.2. Killer Toxin Preparation

The culture liquids of *Cr. pinus* and *Cys. bisporidii* yeasts were separated from biomass by 30-min centrifugation at 5000 g and concentrated by ultrafiltration using YM-10 membrane (Millipore, USA). The concentrates supplemented by ammonium sulfate to 70% saturation; the precipitates were separated by 60-min centrifugation at 12,000 g and dissolved in 0.04 M citrate phosphate buffer, pH 4.5. The resultant preparations were desalted by ultrafiltration using YM-10 membrane (Millipore, USA) with of 0.04 M citrate phosphate buffer, pH 4.5, or in Sephadex G-15.

The desalted killer-toxin preparation of *Cr. pinus* was exposed to chromatography with the following carriers: Butyl-Toyopearl 650 M (elution with $(NH_4)_2SO_4$, 45% saturation in 0.04 M citrate buffer, pH 4.5), CM-Toyopearl (elution with 0.05 M citrate buffer, pH 4.5, and then with 0.5 M NaCl in the same buffer), P23-cellulose (elution with 0.05 M citrate buffer, pH 4.5, and 1 M NaCl in the same buffer) and heparin agarose (elution with 0.02 M citrate buffer, pH 4.5, and then with 0.1 M and 1 M KCl in the same buffer).

The protein content in killer-toxin preparations was assayed according to Bradford [15] using Coomassie Plus (Thermo Fisher, United States) in standard microplates for immunoassays. The preparation (10 μL) and Coomassie Plus (300 μL) were introduced into each well. Optical density was measured with a microplate photometer (Sapphire, Russia) at 594 nm. Bovine serum albumin was used as a standard.

2.3. Electrophoresis of Killer-Toxin Preparation

The preparation containing the killer toxin of *Cr. pinus* was analyzed by electrophoresis according to Laemmli [16] in 15% polyacrylamide gel. In addition, the preparation was analyzed by electrophoresis in 16% polyacrylamide gel according to Schägger [17] for detecting the peptides. In both cases, the gel was stained with Coomassie R-250 (0.025%). For detecting glycoproteins, the gel was stained with Schiff reagent [18], with the modifications described on the website: https://studylib.net/doc/7779551/glycoprotein-staining.

2.4. Mass Spectrometric Analysis

Protein bands were cut out after electrophoresis and staining; peptide molecular masses were determined after trypsine treatment using MALDI TOF spectrometry with LCODECAXP (Thermo Finnigan, USA). Peptides were identified with Mascot (http://www/matrixscience.com/).

2.5. Antifungal Activity Assay on Agarized Medium

The antifungal activity of killer-toxin preparations was determined on a glucose peptone agar (GPA) containing (g/L): glucose, 5.0; peptone, 2.5; yeast extract, 2; agar, 20; $Na_2HPO_4 \times 12\ H_2O$, 8.15. The pH value was adjusted to 4.5 with citric acid. Sterile GF/A disks with applied aliquots of the tested preparations were

placed on the medium surface inoculated with test cultures. The Petri dishes were incubated for 2 days at 25˚C. The diameters of growth inhibition zones were measured.

2.6. Determination of Antifungal Activity in Immunoassay Microplates

The antifungal activity of killer toxins was tested in immunoassay microplates in YPD-P medium with 0.04 M citrate phosphate buffer, pH 4.5. The immunoassay microplate contained 0.03 mL of the medium, 0.01 mL of cell suspension (the initial optical density in a well was 0.09 - 0.12), and the killer-toxin preparation (1.5 to 22 μg protein per mL). After 2-day cultivation in a thermoshaker at 25˚C, optical density of the culture was measured with a microplate photometer (Sapphire, Russia) at 594 nm.

The dependence of test culture growth inhibition on incubation period was determined as follows: 0.1 ml of the *Cr. pinus* killer-toxin preparation in 0.04 M citrate phosphate buffer, pH 4.5, was added to 0.01 mL of the *Cr. Terreus* VKM Y-2253 yeast culture grown to stationary phase. The same buffer (0.1 m·Ll) was added to the control samples. The samples were incubated at 25˚C for 1 or 24 h. Then the cells were separated by centrifugation at 14,000 g for 5 min, washed with 0.1 M sorbitol solution in 0.04 M citrate phosphate buffer, pH 4.5, and each sample was suspended in 0.3 mL of the YPD-P medium. The resultant suspensions were placed into the wells of immunoassay microplate and incubated at 25˚C for 48 h, followed by optical density measurement.

2.7. ATP Release from Yeast Cells

For detecting the killer toxin-induced release of ATP from yeast cells, killer-toxin preparations (0.02 mg protein) were added to the samples containing each: 0.08 mL of 0.04 M citrate buffer, pH 4.5, and 0.04 mL of test culture cell suspension. The buffer (0.08 mL) was added to the control samples. The samples were incubated for 10 - 60 min. The level of ATP release was determined by the luciferin-luciferase method [19] with Luminometer 1250 (LKB, Sweden). The cuvette contained 2 mL of the buffer (25 mM Tris-HCl, 5 mM $MgSO_4$, 0.5 mM EDTA, and 0.5 mM dithiotreitol, pH 7.8), 0.03 mL of luciferin-luciferase, and 0.05 mL of the sample. ATP (Sigma, USA) of the known concentration was used as a standard.

2.8. Propidium Iodide Staining

0.1 mL of the *Cr. pinus* killer-toxin preparation (0.025 mg protein) was added to 0.01 mL of the *Cr. terreus* yeast culture grown to stationary phase. The 0.04 M citrate phosphate buffer, pH 4.5, was used as a control. The samples were incubated at 25˚C for 24 h. Then, 0.1 mL of 0.17 mM propidium iodide in PBS buffer per 0.01 mL of cell suspension was added to the samples. The staining was performed for 15 min at 28˚C - 30˚C under stirring. Microscopy was performed

with a Zeiss Filter Set 56 HE (at the excitation and emission wavelengths of 538 nm and 617 nm, respectively). The proportion of dead cells was calculated in 3 - 5 microphotographs.

All measurements were made in three replicates; the results were statistically processed with Microsoft Excel.

3. Results and Discussion

The killer-toxin preparation obtained from the *Cr. pinus* VKM Y-2958 culture liquid by precipitation with ammonium sulfate had a fungicidal activity against *Cr. terreus* and *F. neoformans* (Figure 1). The killer toxin of *Cr. pinus* inhibited the growth of *Cr. terreus* already in the amount of 0.58 μg protein per disk. The 5-fold higher quantitiy of preparation was needed to inhibit the growth of *F. neoformans* (2.9 μg protein per disk). The minimum inhibitory concentration of the *Cr. pinus* killer toxin for *F. neoformans* in liquid medium was 8.7 μg protein/mL (Figure 2).

The desalinated killer-toxin preparation was stored at −20˚C in 0.04 mM phosphate citrate buffer, pH 4.5. The preparation activity was tested once in several months on agar medium with the above-mentioned test cultures. The level of fungicidal activity was unchanged during 4 years of observation.

According to Laemmli gel electrophoresis, the killer-toxin preparation contained polypeptides with molecular masses below 14 kDa (data not shown). The polyacrylamide gel electrophoresis according to Schägger for separating proteins with low molecular masses and peptides showed the presence of three bands corresponding to proteins with molecular masses below 12,300 Da (the molecular mass of the protein marker—cytochrome *C*) (Figure 3).

The bands were well stained with Coomassie R-250 and were not stained with Schiff reagent, demonstrating that they were not glycoproteins.

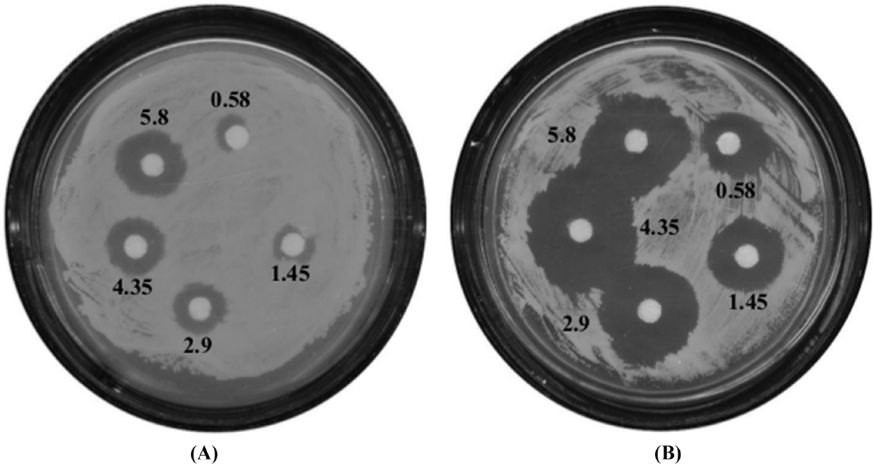

Figure 1. The growth inhibition of *Filobasidiella neoformans* IGC 3957 (A) and *Cryptococcus terreus* VKM Y-2253 (B) by the killer toxin of *Cryptococcus pinus* VKM Y-2958 on glucose peptone agar. Numbers indicate the amount of protein applied to the disks, μg.

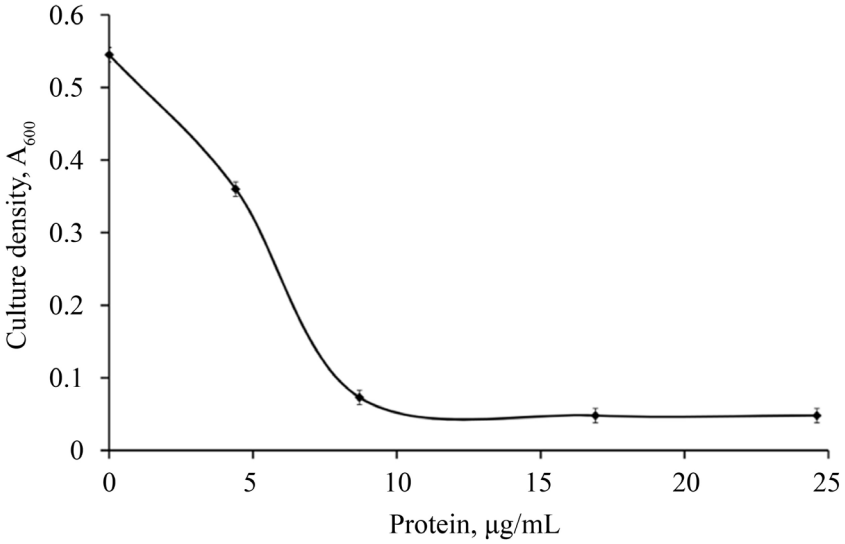

Figure 2. The growth inhibition of *Filobasidiella neoformans* IGC 3957 by the killer toxin of *Cryptococcus pinus* VKM Y-2958 in liquid medium.

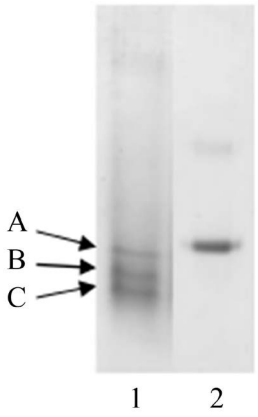

Figure 3. The electrophoregram of the killer-toxin preparation of *Cryptococcus pinus* VKM Y-2958 according to Schägger. 1—Killer-toxin preparation; 2—protein marker, cytochrome *C* from equine heart. (A-C)—Peptides analyzed by mass spectrometry.

The attempts were made to further purification of killer toxin. The fungicidal activities in the purification experiments were tested on agar medium against *Cr. terreus*. Killer toxin was not adsorbed on Butyl-Toyopearl, CM-Toyopearl and P23 cellulose. The killer toxin was adsorbed on heparin agarose; however, the binding was weak and the killer toxin was eluted with 0.02 M citrate buffer, pH 4.5. The fractions eluted with 0.1 and 1.0 M KCl had no fungicidal activity.

Electrophoresis of the preparation eluted with the buffer showed the presence of the same polypeptides as in the initial preparation. Since we failed to separate these polypeptides, the bands from polyacrylamide gel were analyzed by mass spectrometry. No similarities were observed in peptides composition between the tree protein bands under study (not shown). The results of searching for similar proteins in the databases are presented in **Table 1**. The proteins A, B, C contained in the killer-toxin preparation demonstrate no significant similarity to

Table 1. Fungal proteins showing sequence similarity to the proteins of the preparation of the killer toxin of *Cr. pinus*.

Proteins of *Cr. pinus* (Figure 3)	Proteins in databases				
	Mol. mass, kDa	% of overlapping	Function	Organism	Database
A	12,785	81	Mitochondrial cytochrome oxidase assembly factor COX23	*Ashbya gossypii*	SwissProt
	12,051	59	NADH-ubiquinone oxidoreductase subunit	*Sclerotinia borealis*	NCBIprot
B	10,068	82	Mitochondrial import inner membrane translocase subunit TIM9	*Cryptococcus neoformans var. neoformans serotype D*	SwissProt
	12,620	76	DUF1960 domain-containing protein	*Aspergillus taichungensis*	NCBIprot
C	13,798	98	Non-annotated protein	*Zymoseptoria tritici iST99CH_1E4*	NCBIprot
	11,286	47	Mitochondrial import inner membrane translocase subunit TIM14	*Gibberella zeae*	SwissProt

each other; however, low-molecular membrane proteins with similar sequences, including those involved in transport processes, were found for each of them. This fact suggests that the mechanism of action of this killer toxin is associated with the effect on target cell membranes.

It is known that many killer toxins causepermeabilization of the cytoplasmic membrane and release of low-molecular compounds and ions from the cell [4] [5] [6]. We used the ATP release methods for assessing the membrane damaging activity of killer toxins [20]. The release of ATP from *Cr. terreus* cells incubated with the *Cr. pinus* and *Cys. bisporidii* killer toxins was measured. The *Cys. bisporidii* killer toxin preparation was used as a control because its membrane-damaging effect is known [21]. The killer toxin of *Cys. bisporidii* caused the rapid release of ATP, reaching the maximum level already in 30 min of incubation. When the cells of *Cr. terreus* were treated with the killer toxin of *Cr. pinus*, the release of ATP was insignificant for even 60 min (**Figure 4**).

These data suggested that the killer toxin of *Cr. pinus* had no membrane-damaging activity.

An experiment was conducted to investigate the effect of treatment time on cell growth after toxin removal from the incubation medium. There was no growth inhibition in the microplates after 1-h treatment of *Cr. terreus* cells (data not shown), while 2-fold growth inhibition was observed after 24-h treatment (**Figure 5(A)**). The staining of killer toxin-treated cells of *Cr. terreus* with propidium iodide, the well-known fluorescent dye staining the cells with damaged plasma membrane, showed a 20-fold increase in the number of stained cells after 24-h incubation with the killer toxin (**Figure 5(B)**). The results demonstrate that

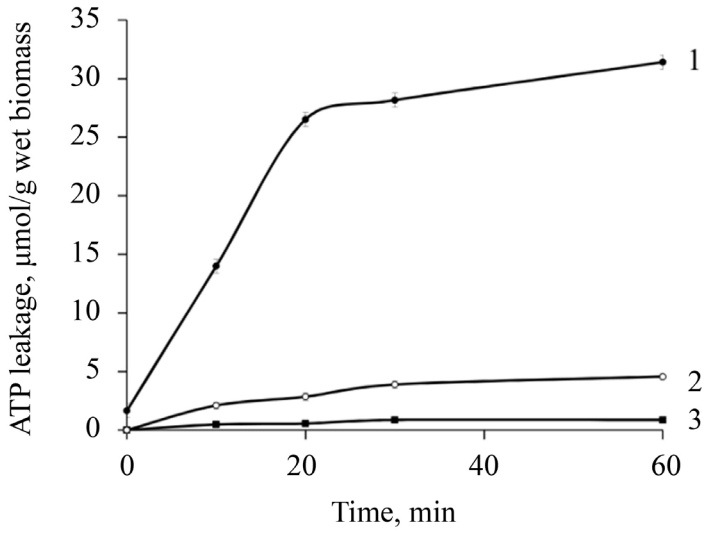

Figure 4. Time dependence of ATP release from *Cryptococcus terreus* VKM Y-2253 cells treated with killer toxins. 1—cells treated with the killer toxin of *Cystofilobasidium bisporidii* VKM Y-2700; 2—cells treated with the killer toxin of *Cryptococcus pinus* VKM Y-2958; 3—control cells.

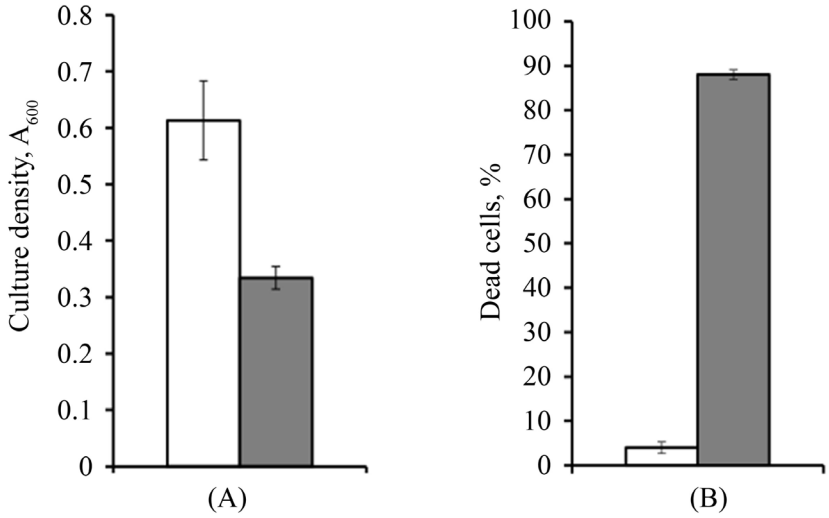

Figure 5. The effects of the killer toxin of *Cryptococcus pinus* VKM Y-2958 on *Cryptococcus terreus* VKM Y-2253 cells after 24-h incubation. (A)—Inhibition of culture growth in liquid medium; (B)—cell staining with propidium iodide. White columns – control cells; gray columns—cells treated with the killer toxin.

the killer toxin of *Cr. pinus* has a membrane-damaging activity; however, manifestation of this activity requires a long-term period of treatment.

4. Conclusion

The killer toxin of *Cr. pinus* exhibits the high fungicidal activity against the known pathogen *F. neoformans*, it is stable during long-term storage, and its mechanism of action is based on damaging target cell membranes. The killer toxin was obtained by a simple procedure and is of interest for the development

of novel fungicidal preparations.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Marquina, D., Santos, A. and Peinado, J.M. (2002) Biology of Killer Yeasts. *International Microbiology*, **5**, 65-71. https://doi.org/10.1007/s10123-002-0066-z

[2] Hatoum, R., Labrie, S. and Fliss, I. (2012) Antimicrobial and Probiotic Properties of Yeasts: From Fundamental to Novel Applications. *Frontiers of Microbiology*, **3**, 421. https://doi.org/10.3389/fmicb.2012.00421

[3] Liu, G.L., Chi, Z., Wang, G.Y., Wang, Z.P., Li, Y. and Chi, Z.M. (2015) Yeast Killer Toxins, Molecular Mechanisms of Their Action and Their Applications. *Critical Review of Biotechnology*, **35**, 222-234. https://doi.org/10.3109/07388551.2013.833582

[4] Gage, M.J., Rane, S.G., Hockerman, G.H. and Smith, T.J. (2002) The Virally Encoded Fungal Toxin KP4 Specifically Blocks L-Type Voltage-Gated Calcium Channels. *Molecular Pharmacology*, **61**, 936-944. https://doi.org/10.1124/mol.61.4.936

[5] Novotná, D., Flegelová, H. and Janderová, B. (2004) Different action of Killer Toxins K1 and K2 on the Plasma Membrane and the Cell Wall of *Saccharomyces cerevisiae*. *FEMS Yeast Research*, **4**, 803-813. https://doi.org/10.1016/j.femsyr.2004.04.007

[6] Orentaite, I., Poranen, M.M., Oksanen, H.M., Daugelavicius, R. and Bamford, D.H. (2016) K2 Killer Toxin-Induced Physiological Changes in the Yeast *Saccharomyces cerevisiae*. *FEMS Yeast Research*, **16**, fow003. https://doi.org/10.1093/femsyr/fow003

[7] Santos, A., Alonso, A., Belda, I. and Marquina, D. (2013) Cell Cycle Arrest and Apoptosis, Two Alternative Mechanisms for PMKT2 Killer Activity. *Fungal Genetics and Biology*, **50**, 44-54. https://doi.org/10.1016/j.fgb.2012.10.006

[8] Carmona-Gutierrez, D., Eisenberg, T., Büttner, S., Meisinger, C., Kroemer, G. and Madeo, F. (2010) Apoptosis in Yeast: Triggers, Pathways, Subroutines. *Cell Death and Differentiation*, **17**, 763-773. https://doi.org/10.1038/cdd.2009.219

[9] Kast, A., Klassen, R. and Meinhardt, F. (2014) RRNA Fragmentation Induced by a Yeast Killer Toxin. *Molecular Microbiology*, **91**, 606-617. https://doi.org/10.1111/mmi.12481

[10] Wemhoff, S., Klassen, R. and Meinhardt, F. (2016) DNA Damage Induced by the Anticodon Nuclease from a *Pichiaacaciae* Killer Strain Is Linked to Ribonucleotide Reductase Depletion. *Cellular Microbiology*, **18**, 211-222. https://doi.org/10.1111/cmi.12496

[11] Lukša, J., Podoliankaitė, M., Vepštaitė, I., Strazdaitė-Žielienė, Ž., Urbonavičius, J. and Servienė, E. (2015) Yeast β-1,6-Glucan Is a Primary Target for the *Saccharo-*

myces cerevisiae K2 Toxin. *Eukaryotic Cell*, **14**, 406-414. https://doi.org/10.1128/EC.00287-14

[12] Labbani, F.Z., Turchetti, B., Bennamoun, L., Dakhmouche, S., Roberti, R., Corazzi, L., Meraihi, Z. and Buzzini, P. (2015) A Novel Killer Protein from *Pichiakluyveri* Isolated from an Algerian Soil: Purification and Characterization of Its *in Vitro* Activity against Food and Beverage Spoilage Yeasts. *Antonie Van Leeuwenhoek*, **107**, 961-970. https://doi.org/10.1007/s10482-015-0388-4

[13] Muccilli, S. and Restuccia, C. (2015) Bioprotective Role of Yeasts. *Microorganisms*, **3**, 588-611. https://doi.org/10.3390/microorganisms3040588

[14] Golubev, W.I. (2009) Anti-Tremellomycetes Activity of *Cryptococcus pinus* Mycocin. *Microbiology*, **78**, 315-320. https://doi.org/10.1134/S0026261709030096

[15] Bradford, M.M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*, **72**, 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

[16] Laemmli, U.K. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, **227**, 680-685. https://doi.org/10.1038/227680a0

[17] Schägger, H. (2006) Tricine-SDS-PAGE. *Nature Protocols*, **1**, 16-22. https://doi.org/10.1038/nprot.2006.4

[18] Zacharius, R.M., Zell, T.E., Morrison, J.H. and Woodlock, J.J. (1969) Glycoprotein Staining Following Electrophoresis on Acrylamide Gels. *Analytical Biochemistry*, **30**, 148-152. https://doi.org/10.1016/0003-2697(69)90383-2

[19] Wulff, K. and Doppen, W. (1985) Luminometric Method. In: Bergmayer, H.U., Bergmayer, J. and Grasl, M., Eds., *Bergmayer Methods of Enzymatic Analysis*, VCH Verlagsgesellschaft, Weinheim, 357-364.

[20] Kulakovskaya, T.V., Kulakovskaya, E.V. and Golubev, W.I. (2003) ATP Leakage from Yeast Cells Treated by Extracellular Glycolipids of *Pseudozymafus iformata*. *FEMS Yeast Research*, **3**, 401-404. https://doi.org/10.1016/S1567-1356(02)00202-7

[21] Kulakovskaya, T.V., Karamysheva, Z.N., Andreeva, N.A. and Golubev, W.I. (1996) Some Characteristics of Mycocin Produced by *Cystofilobasidium bisporidii*. *Microbiology*, **65**, 673-677.