Antimicrobial Potential of the Alkalophilic Fungus *Sodiomyces alkalinus* and Selection of Strains—Producers of New Antimicotic Compound

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Abstract—The ability of alkalophilic micromycetes of the species *Sodiomyces alkalinus* to produce antimicrobial compounds was studied. As a result of the determination of the spectrum and yield of antibiotic compounds, a promising producer of the antimycotics *Sodiomyces alkalinus* was selected from the most active strains 8KS17–10. The producer exhibited antifungal activity against opportunistic fungi, as well as pathogenic clinical isolates of molds and yeasts—pathogens of systemic mycoses. The isolated active compound can be attributed to the group of antimicrobial glycopeptides based on the totality of the identified structural features (molecular weight, absorption ratio at certain wavelengths).

Keywords: antimicrobial glycopeptides, *Sodiomyces*, alkaliphiles, micromycetes, antibiotics

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INTRODUCTION

The spread of antibiotic-resistant strains of pathogenic microorganisms is a global challenge to the health systems of all countries. The widespread use of antibiotics in recent decades has led to a situation in which 30% of cases of infectious diseases cannot be treated with known drugs, including the latest generations of antibiotics. Complex radical measures, including the search for fundamentally new compounds with antimicrobial activity, are needed to restrain the expansion of resistant strains observed around the world.

Fungi are one of the main groups of living organisms that are considered to be producers of antibiotics. However, only a small proportion of them has been studied to date for the synthesis of secondary antimicrobial products.

Traditionally, antibiotic-producing fungi have been isolated from soil samples. Nevertheless, this source has been exhausted for the most part, and unconventional biotopes with recently discovered organisms have come to the fore in the search for new and more effective antimicrobial compounds [1]. These biotopes include arid soils; caves; areas with high or low temperatures and high salinity and alkalinity; deep

seas and oceans; etc. Survival under such conditions is associated with the synthesis of various metabolites with structures that differ from those previously studied. Over the past 10–15 years, more than 20000 such compounds produced by extremophilic microorganisms have been isolated and characterized [2, 3]. Despite the difficulties in the detection and cultivation of extremophilic fungi, in particular, alkalophilic fungi, screening studies indicate their enormous potential as sources of new bioactive compounds [4].

Thus, the alkalotolerant fungus *Paecilomyces lilacinus* demonstrates the synthesis of peptide antibiotics 1907-II and 1907-VIII with antibacterial and antifungal activities [5]. The alkalophilic fungus *Aspergillus flavus* produces kojic acid and fomaligol A, which are active against gram-positive and gram-negative bacteria [6, 7]. A new lipopeptaibol, emericellipsin A, was discovered and described in strains of the alkalophilic fungus *Emericellopsis alkalina* isolated from alkaline saline soils. It has antifungal, antibacterial (including against gram-negative bacteria), and antitumor activities [8].

Most of the currently known alkalophilic and alkalotolerant fungal taxa belong to ascomycetes from the Plectosphaerellaceae. Members of the

recently described genus *Sodiomyces* (Ascomycota, Plectosphaerellaceae), for which the obligate-alkalophilic type of adaptation was confirmed, are especially interesting. Species of this genus serve as models in studies of the ecophysiology of fungi, the study of the biochemical basis of adaptation to the pH factor [9, 10], the transfer of bacterial genes into fungal genes, and the evolution of enzymes [11]. It was shown that the genome *S. alkalinus* contains sequences encoding the main enzymes necessary for the biosynthesis of beta-lactam antibiotics. It is also known that beta-lactams rapidly degrade at a high pH [12, 13]. It remains an open question whether the fungus synthesizes these substances in natural conditions, where the variety of various groups of prokaryotes is high and some alkali-resistant fungi are abundantly represented, or whether it produces other antimicrobial compounds under alkaline conditions [11].

The goal of this work is to assess the antimicrobial activity of alkalophilic isolates of the *Sodiomyces alkalinus*, to select producers of peptide antibiotics, and to identify the most active of them.

EXPERIMENTAL

The objects of study were 25 alkalophilic strains of the recently described species *Sodiomyces alkalinus* (Bilanenko & M.Ivanova) A.A. Grum-Grzhim., A.J.M. Debets & Bilanenko (<https://www.ncbi.nlm.nih.gov/nuccore/?term=Sodiomyces+alkalinus>), isolated from alkaline saline soils in different geographic regions [14]. The cultures were obtained from the Fungi of Extreme Conditions collection of the Department of Mycology and Algology, Biology Faculty, Lomonosov Moscow State University (Russia). Some isolates were deposited in the All-Russia collection of microorganisms (VKM, Pushchino, Russia) and the Fungal Biodiversity Center (CBS, Utrecht, Netherlands). The strain used in this work, F11 (= CBS 110278 = VKM F-3762), is type for this species, and its complete genome is annotated [11].

The antimicrobial activity of the strains was assessed in the first stages of work via diffusion in agar on test cultures of opportunistic microorganisms *Aspergillus niger* INA 00760 and *Bacillus subtilis* ATCC 6633. Cultures were considered to be highly active if the zone of growth retardation of the test organism was 25 mm or more; moderately active cultures had a growth retardation zone of 10–25 mm, and weakly active cultures had a zone of less than 10 mm.

The ability to synthesize antimicrobial substances was evaluated with growth on a specialized alkaline medium that, according to previously obtained data, is optimal for the growth and development of this fungus [11, 14].

For the selected eight strains, the formation of antimicrobial substances was studied upon cultivation in a liquid alkaline medium with the following composi-

tion (g/L): Na₂CO₃, 24; NaHCO₃, 12; NaCl, 6; KNO₃, 1; K₂HPO₄, 1; malt extract (15° Balling), 200 mL; yeast extract, 1; and distilled water, 800 mL [15]. Stationary fungi were grown in 500-mL flasks for 14 days. After cultivation, the culture liquid was separated via filtration through membrane filters on a Seitz funnel under a vacuum.

The antimicrobial activity for three strains was compared for different storage methods. We compared the same strains; some were stored in glycerin at –70°C in a kelvinator, and others were stored on an alkaline medium in test tubes in a refrigerator at 6°C. Both variants were stored under these conditions for at least 2 years.

To isolate antibiotic substances, the culture fluid (CF) of the producer was extracted with ethyl acetate in the ratio of organic solvent, CL 5 : 1. The obtained extracts were evaporated in a vacuum on a rotary evaporator Rotavapor-RBuchi (Büchi, Switzerland) at 42°C to a dry state; the dry residue was dissolved in aqueous 70% ethanol, and alcohol concentrates were obtained.

The antimicrobial activity was determined in the initial culture liquid and in alcoholic concentrates of extracts of CL and mycelium on sterile paper disks (filter paper F GOST 12026-76, Russia) soaked in extracts and dried under sterile conditions. The sensitivity of the test organism was controlled with standard discs containing amphotericin B for fungi (40 µg/disk) and ampicillin for bacteria (10 µg/disk) (Research Institute of Pasteur, Russia).

The spectrum of antimicrobial activity of the culture liquid, extracts, and individual compounds was determined on test cultures of mycelial and yeast microscopic fungi and on bacteria from the collection of cultures of the Gause Institute of New Antibiotics (Moscow, Russia). Opportunistic mold and yeast test cultures of the fungal species *Aspergillus fumigatus* KPB F-37, *A. niger* INA 00760, and *Candida albicans* ATCC 2091 and test cultures of gram-positive strains *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* FDA 209P and gram-negative bacteria – *Escherichia coli* ATCC 25922 were used.

The spectrum of antimycotic action of the antimicrobial peptide was also evaluated on clinical isolates of molds and yeasts—pathogens of opportunistic pneumomycosis of the bronchi and lungs—in tuberculosis patients with multiresistance to the antibiotics-azoles used in clinical practice, from the collection of the mycological laboratory of the Moscow City Scientific and Practical Center for Tuberculosis Control (Russia): *Candida albicans* 1582m 2016, *C. glabrata* 1402m 2016, *C. krusei* 1447m 2016, *C. parapsilopsis* 571m, *C. tropicalis* 156m 2017, *Cryptococcus neoformans* 297 m 2017, *Aspergillus fumigatus* 390m, and *A. niger* 219.

Further separation of the active fractions (after extraction) was carried out via analytical reversed-phase high-performance liquid chromatography (RP-HPLC) with an XBridge 5 µm 130 A column with a size of

250 × 4.6 mm (Waters, Ireland) in a growing linear gradient of the acetonitrile concentration as a mobile phase (eluent A, 0.1% trifluoroacetic acid (TFA) in water MQ; eluent B, 80% acetonitrile in 0.1% aqueous TFA) at a flow rate of 950 µL/min. Ultragradient acetonitrile (Panreac, Spain) and TFA (Sigma-Aldrich, United States) were used for RP-HPLC. The substances to be separated were determined at the wavelength of 214 nm in the concentration gradient of eluent B: 16–28% in 12 minutes; 28–55% in 27 minutes; 55–75% in 20 min; and 75–85% in 10 min. This was followed by isocratic elution for 25 min. In order to scale up the production of individual components of the alcoholic concentrate of the culture broth extract of the producer, a similar separation was carried out via semipreparative RP-HPLC on an XBridge 10 µm 130 A column (250×10 mm). The absorbance (D) was determined at a wavelength of 214 nm and a mobile phase flow rate of 4 mL/min. The fractions obtained during RP-HPLC, which correspond to individual peaks, were collected manually, and the excess of organic solvent (acetonitrile) was then removed via evaporation in a SpeedVac vacuum centrifuge (Savant, United States) and lyophilized (Labconco, United States) to remove residual amounts of TFA. The spectrum of antimicrobial action of the substances contained in the fractions was determined via disk diffusion, as described above.

The molecular weights of the active compounds in the isolated fraction were determined on an AutoSpeed matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Germany) equipped with a 355-nm UV laser (Nd: YAG) in positive-ion mode with a reflectron. On the target, 1 µL of a sample solution and 1 µL of a solution of 2,5-dihydroxybenzoic acid with a concentration of 10 mg/mL in 20% acetonitrile with 0.5% TFA acid were mixed. The resulting mixture was dried in air.

The absorption spectra were recorded with a UV-1800 spectrophotometer (Shimadzu, Japan) in 2-mL quartz cuvettes with an optical path length of 1 cm.

The minimum inhibitory concentration (MIC) was determined at 24 h for the yeast fungi *C. albicans* and at 48 hours for the molds *A. niger* and *A. fumigatus*. The MIC was defined as the minimum concentration of a substance that completely suppresses the growth of the test culture.

The experiments were carried out in three to five replicates. Statistical processing of the results and assessment of the reliability of differences in mean values were carried out according to the Student's t-test for a probability level of at least 95% with the Microsoft Excel 2007 and Statistica 10.0 software.

RESULTS AND DISCUSSION

An alkaline medium was used to study the antimicrobial activity of the fungal strains *Sodiomyces alkali-*

nus. Previous studies of the features of ecophysiology have shown that *S. alkalinus* is an obligate alkaliphile, i.e., it is incapable of growth at an acidic pH. At pH values of 6–7, its growth rate significantly decreases, asexual and sexual sporulation is poorly developed or absent, the aerial mycelium is poorly expressed, and the hyphae often have an ugly appearance with numerous swellings. An alkaline medium specially designed for alkalophilic fungi made it possible to maintain high pH values (10.5), thereby simulating the conditions of natural alkaline biotopes from which isolates of this unique fungus were isolated. The maximal *S. alkalinus* growth rate was noted on an alkaline medium (as compared to media with acidic and neutral pHs), and all its characteristic morphological and cultural features were fully manifested [14]. Optimal conditions for many *S. alkalinus* enzymes (cellulases, hemicellulases, proteases) were observed at pH levels of 8 and 10 [11]. For isolates of another alkalophilic fungus also isolated from biotopes with an alkaline medium, *Emericellopsis alkalina*, it was previously shown that the greatest antifungal activity was manifested in a medium with a high pH value [15, 16].

Evaluation of the antimicrobial activity of 25 *Sodiomyces alkalinus* strains in relation to test fungi and bacteria showed that about half of the studied strains had moderate or high activity against *A. niger* INA 00760 and *B. subtilis* ATCC 6633, while the proportion of highly active isolates with antifungal activity was 20% (Table 1). The revealed antimicrobial spectrum may be a reflection of the habitat in natural saline biotopes, where numerous bacteria developed, and several species of alkali-resistant micromycetes are abundant. The foremost of these is *E. alkalina*, the isolates of which also show pronounced antifungal activity [15, 16].

Based on the results of primary screening on solid media, eight active *S. alkalinus* strains were selected, including the type strain (F11) used in further studies with culturing on liquid media.

Of the eight selected strains, four (5KS17-8, 8KS17-10, 11KS17-1, F11) showed significant antifungal activity of the culture fluid and its extracts grown in liquid medium against the opportunistic fungus *A. niger* INA 00760, and one strain (1KS13-4) showed the same against the yeast *C. albicans* ATCC 2091 (Table 2). Two from the eight strains, 1KS13-4 and F11 (Table 2), were highly active against the gram-positive bacteria *B. subtilis* ATCC 6633. No activity was detected against the gram-positive bacteria *Staphylococcus aureus* FDA 209P and the gram-negative bacteria *Escherichia coli* ATCC 25922.

Three strains, 8KS17-10, 11KS17-1, and F11, were selected for further study of an antibiotic complex exhibiting antifungal activity; the activity of their ethyl acetate extracts of culture fluids was higher than that of amphotericin B at a concentration of 40 µg/disk. Analysis of the antibiotic activity of these strains showed that culture storage in a kelinator at –70°C in glycerin or in

Fig. 1. RP-HPLC profile of extracts of the culture liquid after affinity chromatography on a Heparin-Sepharose column; a dominant peak is marked by a star. Axis: X—time, min, Y—is a digital signal axis (mAU, milli Absorbance Units): (a) 8KS17-10, (b) 11KS17-1, (c) F11.

a refrigerator on agar medium in a test tube at 6°C did not have a significant effect on antibiotic synthesis.

Subsequently, a scheme was developed for the separation of an antibiotic complex of three strains via RP-HPLC. As a result, a similar profile of the compo-

nents of the active concentrate was obtained for all studied strains, with three prevailing fractions (Fig. 1) having varying degrees of hydrophobicity.

Testing of the obtained main individual compounds of the complex for the presence of antimicro-

Table 1. Ratio of the studied *Sodiomyces alkalinus* strains with antifungal and antibacterial activity

Test organism	<i>S. alkalinus</i> strains (25 strains, 100%)		
	weak	moderately active	highly active
<i>A. niger</i> INA 00760	12 (52%)	8 (28%)	5 (20%)
<i>B. subtilis</i> ATCC 6633	11 (44%)	11 (44%)	3 (12%)

Fig. 1. (Contd.)

bial properties found a pronounced activity in only one dominant peak, Sod 1 which eluted from the column at 34.2 min. The zone of growth inhibition of *A. niger* INA 00760 for the Sod 1 compound was 22 mm.

For the active compound, a spectrum was obtained absorption in the wavelength range of 210–340 nm. It has only one characteristic maximum in the region of short-wave ultraviolet radiation (about 205 nm), which indicates the presence of peptide bonds in its structure (Fig. 2).

The primary structural characteristics were carried out by MALDI-mass-spectrometric analysis. The average molecular weight of the compound is 7918.4 Da (Fig. 3). The nature of the distribution of m/z signals with a step of more than 100 Da indicates the fragmentation of the molecule through hydrolysis of glycosidic bonds, which determines the difference in molecular weight with aglycone (7586.34 Da) of approximately 332 Da, which presumably corresponds to the presence of sugar residues.

Fig. 1. (Contd.)

Table 2. Antimicrobial activity of culture liquid and extracts of the culture liquid of *Sodiomyces alkalinus* (in mm zones of inhibition of test-organism growth)

Strain, no.	Zone of inhibition, culture liquid, mm				Zone of inhibition, extracts of the culture liquid, mm			
	<i>B. subtilis</i> ATCC 6633	<i>A. niger</i> INA 00760	<i>C. albicans</i> ATCC 2091	<i>A. fumigatus</i> KBP F-37	<i>B. subtilis</i> ATCC 6633	<i>A. niger</i> INA 00760	<i>C. albicans</i> ATCC 2091	<i>A. fumigatus</i> KBP F-37
5KS17-8	18	14	0	0	0	17	8	8
8KS17-10	33	9	12	0	0	40	9	12
9KS17-1	15	11	0	0	0	12	0	9
11KS17-1	17	12	0	0	13	40	17	12
2KS10-1	23	16	0	0	13	8	0	8
3KS11-1	0	12	12	0	0	9	9	10
1KS13-4	30	13	0	0	25	10	18	8
F11 (type strain)	10	13	0	0	22	18	0	13

Fig. 2. UV absorption profile of the Sod 1 in the range 200–340 nm.

For the Sod 1 compound, the MIC was determined in relation to opportunistic–pathogenic mold and yeast fungi and bacteria, as well as clinical isolates—the causative agents of invasive aspergillosis, candidemia, and cryptococcosis (Table 3).

It was found that the MIC of the Sod 1 compound for *Cryptococcus neoformans* 297m 2017 is 1 µg/mL; at

a concentration of 16 µg/mL, it inhibits *C. albicans* 1582m, *A. niger* 219, and *A. fumigatus* 390 m.

CONCLUSIONS

Thus, the ability to produce antimycotic compounds with high and moderate activity was established in one third of all tested extremophilic isolates of *S. alkalinus*, which may indicate the prospects for the search for antimycotic producers in representatives of this species. Three strains of this species showing pronounced antifungal activity against yeast fungi, including clinical isolates (the causative agents of invasive mycoses) were preliminarily selected. They can be recommended in the future as producers of promising new drugs for the treatment of severe mycoses in cancer patients (presumably, the absence of undesirable phenomena and side toxic effects). The pronounced antifungal action against *C. neoformans* may also hold promise for the etiologic treatment of cryptococcosis, for which amphotericin B is now used in therapy with significant side effects.

Based on the set of revealed structural features (molecular weight, fragmentation pattern), the isolated active compound can be preliminarily assigned to the

Fig. 3. MALDI mass spectrometric analysis of the Sod 1 compound.

Table 3. Antifungal activity of the Sod 1 compound against clinical pathogenic molds and yeasts with multiple resistance to azoles

Test organism	Suppression zone diameter, mm			
	Sod 1 (40 mcg/disk)	Amphotericin B	Fluconasol	Vorikonasol
<i>C. albicans</i> 1582m 2016	18 ± 0.1	10 ± 0.6	0	0
<i>C. glabrata</i> 1402m 2016	16 ± 0.3	15 ± 0.1	0	0
<i>C. krusei</i> 1447m 2016	12.5 ± 0.2	0	0	0
<i>C. tropicalis</i> 156m 2017	14 ± 0.1	0	0	0
<i>C. parapsilosis</i> 571m	14 ± 0.2	18 ± 0.3	0	0
<i>Cryptococcus neoformans</i> 297m 2017	30 ± 0.1	18 ± 0.6	0	0
<i>A. fumigatus</i> 390m	12 ± 0.5	9 ± 0.6	0	0
<i>A. niger</i> 219	14 ± 0.1	15 ± 0.8	0	0

group of glycosylated antimicrobial peptides with strong antifungal activity. Further structural identification will be carried out with a combination of physicochemical methods: high resolution mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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