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Research Article

ANTIMICROBIAL POTENTIAL OF FUNGI AND ACTINOBACTERIA ISOLATED FROM SANDY SEDIMENTS OF INTERTIDAL REGIONS

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ABSTRACT

The spread of drug resistant microorganisms is an important clinical issue, increasing the need of new compounds research with the potential to control them. These compounds can be sought in microorganisms from different niches, like the intertidal regions, where extreme environmental conditions cause the development of microorganisms with particular physiological and metabolic processes. This work aimed at the bioprospection of microorganisms isolated from the intertidal regions of Ilha do Mel, Paraná, Brazil. About 60 fungi from the genera *Aspergillus*, *Penicillium*, *Acrmonium*, *Trichoderma*, *Paecilomyces* e *Dreschslera* and 116 actinobacteria from the genera *Nocardia* and *Streptomyces* had their inhibitory activity evaluated against the pathogenic strains *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231). Among isolated fungi, 57.14% inhibited two or more pathogenic strains and 72% of actinobacteria yielded the same result. In order to characterize the substances with antimicrobial activity present in the extracts, the bioautography method was used. *Penicillium sp.* had an extract isolated that showed activity against *C. albicans*. The *Streptomyces* extract results were positive against *S. aureus* and MRSA. The isolates with antimicrobial potential were identified by the sequencing of the ITS1-5.8S-ITS2 of rDNA (fungi) and 16S rDNA (actinobacteria). These results highlight the importance of the study of the biotechnological potential of the microorganisms isolated from intertidal regions. This is the first work investigating the bioactivity of actinobacteria and fungi isolated from Ilha do Mel, Paraná, Brazil.

Keywords: Marine sediment, antimicrobials, fungi, actinobacteria.

INTRODUCTION

The intertidal microorganism communities perform essential processes that contribute to the balance of coastal regions, such as the carbon cycle, metal transferences and removal of organic pollutants to higher trophic levels. The environmental conditions of intertidal regions are extreme due to tide cycles, resulting in high gradients of moisture, temperature, wave action, UV radiation, nutrients and salinity. Such conditions lead to the development and the dissemination of microorganisms with specific metabolic and physiological processes[1,2].

Microorganisms are able to produce secondary metabolites to adapt and compete with other organisms in the same

environment. The conditions of growth media are key elements to the discovery and production of secondary metabolites[3]. The amount of carbon, nitrogen and trace elements (iron, manganese, and zinc), temperature and the pH of the growth medium are essential to the production of these metabolites[4,5]. Currently, more than 1000 substances were isolated from marine microorganisms and approximately 200 novel substances were described in recent years[6].

Fungi and actinobacteria are relevant sources of new bioactive compounds, such as antibiotics produced by marine environment species, which are totally novel and unique when compared to the ones produced by terrestrial organisms[7]. These microorganisms are recognized as novel sources for bioactive secondary metabolites, including antitumor, antibacterial, antiviral, antifungal and anti-inflammatory substances[8, 9].

The spread of drug-resistant pathogenic microorganisms is an alarming clinical issue, which can lead to morbidity and mortality caused by treatment failures. This relates directly to the extensive use of these drugs, which are often taken without prescription, on under dosages or during an insufficient treatment. Besides raising healthcare costs, the treatment and cure of infectious diseases become inefficient due to generalized antimicrobial use[3, 10].

The discovery and development of novel and efficient therapeutic agents are essential to combat resistant pathogens[11]. New antibiotics from fungi and actinobacteria are constantly being discovered and provide alternatives[12, 13]. This research aimed bioprospecting antimicrobial producing microorganisms. This is the first work to investigate the bioactivity of actinobacteria and fungi isolated from the intertidal region of Ilha do Mel, Paraná, Brazil.

MATERIALS AND METHODS

Biologic material

Fungi and actinobacteria were isolated from marine sediments of the intertidal region of Ilha do Mel, located at Paranaguá Bay (25°20'S – 48°20'W and 25°35' – 48°35'W), at the shore of Paraná, Brazil.

Isolates were selected, 60 fungi from the genera *Aspergillus, Acremonium, Penicillium, Paecilomyces, Trichoderma* e *Drechslera*, and 116 actinobacteria from the genera *Streptomyces* and *Nocardia*. The isolates are stored in the Biological Bank of Laboratório de Microbiologia e Biologia Molecular (LabMicro), at Universidade Federal do Paraná, Curitiba, Brazil.

The antimicrobial activity tests were made using the reference strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* 25922, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231 and the wild strains methicillin resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumoniae* Carbapenemase (KPC) from University Hospital of Curitiba, Brazil.

Evaluation of antimicrobial activity

The evaluation of antimicrobial activity was made using qualitative bioassays, in triplicate, using the well method[14]. The 60 fungi isolates and the 116 actinobacteria isolates were grown on Czapeck Dox (Himedia®)[5] and Sabouraud (Himedia®) growth media, during 7 and 14 days at 28ºC., depending on the growth rate of the microorganism.

To determine the difference between the media and the isolates, the initial experiment used the mentioned isolates against two important pathogen strains, *Staphylococcus aureus* and *Candida albicans*. The obtained data were transformed using logx+1 and analyzed using Analysis of Variance (ANOVA), followed by a factorial delineation. When needed, the Scot-Knott average test ($p<0,005$) was used. All the analyses were made using the software Assistat 7.6 version 2012[15].

Production of secondary metabolites in liquid growth medium

The fungi and actinobacteria isolates were selected and grown in Petri dishes containing the solid medium Czapeck Dox. After the growth during 7 and 14 days at 28ºC, the isolates were transferred to 50 mL liquid growth medium containing malt extract (Himedia®), with a concentration of $3x10^8$ spores/mL. Then, the isolates were incubated under 150 rpm agitation at 28ºC for 7 days, for analytic scale analysis[16].

Bioautography

To evaluate the antimicrobial activity of obtained secondary metabolites, a thin-layer chromatography (CCD) bioautography method was used[17]. The pathogenic strains were inoculated in 10 mL of Mueller-Hinton broth (Himedia®), homogenized with vortex and incubated at 35ºC for 24 hours. A 10^6 UFC dilution was prepared from this tube. Then, 100 µL were transferred to a test tube containing 10 ml of Mueller-Hinton broth, vortex agitated and incubated at 35ºC for 48 hours.

The obtained extracts from the fermentative liquid with ethyl acetate were tested on silicagel plates 60F254 (Merck®), CCD plates (20x20) cut in 6X6 squares. The extracts solubilized in methanol were applied in duplicate in 5 and 10 µL in each row, and the last row of the CCD plate received the positive control. This control consisted of 200 µg/mL gentamicin for *S. aureus*, *P. aeruginosa* and *E. coli*, vancomicin 200 µg/mL for MRSA and nistatin (100000 UI/mL) for yeasts, using the mobile phases chloroform:methanol (95:5 e 90:10). Control CCD plates were made with only extracts that were revealed with vanillin sulfuric.

Microorganism identification

The microorganisms were identified using macro and micro morphological characteristics[18-26] and Gram staining[27]. After the selection of isolates, those with greater potential to produce antimicrobial substances were identified at genus and species. The sequencing of region ITS1-5.8S-ITS2 of rDNA was used to identify fungi and the 16S region of rDNA was used to identify actinobacteria[28, 29].

Molecular characterization of isolates

DNA extraction

To extract the DNA[30], the isolates were grown in Czapeck Dox solid medium, at 28ºC for 72 hours. About 1 cm^2 of the cell culture was transferred to a 1.5 mL Eppendorf tube containing silicon and celite (2:1). The pellet was resuspended in 100 µL of ultrapure water and left overnight at room temperature. The DNA was stored in a -20ºC freezer and quantified using a spectrophotometer (Thermo Fisher Scientific Launches New NanoDrop 2000).

Amplification of the ITS1 - 5.8S - ITS2 region from fungus DNA

The PCR reaction was made using the primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') to amplify the ITS1, 5,8S and ITS2 region of rDNA, respectively[29]. The reaction was assembled using 50 ng of DNA, PCR 1x buffer, 0.5 µL Taq polimerase; 0.14 µM of primers (3.5 pmoles); 0.2 mM of each dNTP; 1.5 mM of $MgCl_2$ at the final volume of 25 µL.

The amplification was made in a *Mastercycler Gradient Eppendorf*® thermal cycler. The denaturation cycle was at 95ºC for 2 minutes, 35 cycles of 1 minute at 92ºC, 1 minute at 50ºC, 1 minute at 72ºC, followed by the final extension of 3 minutes at 72ºC.

After the amplification, the products were visualized in a 1.5% agarose gel, using the mass marker *Low DNA Mass Ladder* (1 kb *Ludwig Biotec*).

Amplification of 16S rDNA PCR actinobacteria

The PCR was performed with primers 9F (5 'GAGTTTGATCCTGGCTCAG3') and Sm5R (5 'GAACTGAGACCGGCTTTTTGA3') specific to the family Streptomycetaceae[28]. Amplification of actinobacteria followed the conditions described below: 50ng DNA extracted, 1X reaction buffer, 1.5 U Taq polymerase, 0.5 µM primers (25pmol/reaction), 0.2 mM each dNTP, 1.5 mM $MgCl_2$ in a final volume of 50µL. The amplification was carried out in thermocycler Eppendorf ® Model Mastercycler Gradient with initial denaturation at 95 ° C for 5 minutes, 30 cycles of 45 seconds at 94 ° C, 45 seconds at 65°C and 1 minute at 72 ° C, and final extension of 10 minutes at 72 °C for primers 9F and Sm5R. The amplification products were visualized on 1.5% agarose gel using the mass marker Low Mass DNA Ladder (1 kb Ludwig Biotec).

Sequencing reaction

The sequencing of regions ITS1, 5,8S e ITS2 from fungi rDNA and the 16S rDNA from actinobacteria was made using the chain-termination method[31], using the incorporation of fluorescent dideoxinucleotides in an automatic DNA sequencer.

The sequencing reactions contained 1 to 3 µL of the purified PCR product, 0.5 µL of buffer; 0.5 µL of each primer; 0.5 µL of Big-Dye; completing the final volume of 10 µL with ultrapure water. The amplification conditions were 96ºC for a minute, 35 cycles at 96ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 4 minutes. The samples were sequenced using ABI 3130 sequencer (Applied Biosystems).

Sequence editing and analysis

The obtained sequences were edited using the Staden package software version 1.6[32], aligned by the MEGA software version 4.0[33] and compared with other sequences at the NCBI database using the software BLAST[34]. This allowed the detection of similarity of these biological sequences with others. For further analyses of the obtained sequences, the BLASTn software was used.

RESULTS AND DISCUSSION

Evaluation of antibacterial activity

The screening of microbial natural products continues to represent an important route to the discovery of novel chemicals for the development of new therapeutic agents[35].

The fungi and actinobacteria isolates were grown in Czapeck Dox and Sabouraud growth media. The first has salts in its formula, like sodium nitrate, dipotassium phosphate, magnesium sulfate, potassium chloride and iron (II) sulfate, which according to Vogel[36] are essential minerals for the growth and metabolism of microorganisms. The second medium, according to Sharma[37], is considered a good growth and sporulation medium for soil isolated fungi. Many growth media and incubation periods are used for the development of actinobacteria and fungi. However, the choice of an appropriate medium is essential for the successful growth of the microorganism. The chosen medium should supply the necessary nutrients for the cell synthesis, specially carbon, nitrogen, and energy sources[38].

The Czapeck Dox and Sabouraud media have shown efficiency to the obtainment of secondary metabolites. The fungi isolates (Fig. 1a) grown in Sabouraud medium have inhibited *S. aureus* and *C. albicans* (22 and 7 isolates respectively). The same isolates, grown in Czapeck Dox medium also inhibited *S. aureus* and *C. albicans* (26 and 12 isolates, respectively). A greater number of actinobacteria grown in Sabouraud medium was observed to have activity against the pathogenic strains of *S. aureus* (56 isolates) and C. albicans (63 isolates), when compared to the same isolates grown in Czapeck Dox medium (11 and 20 isolates, respectively) (Fig. 1b).

The factorial experiments were made separately for fungi and actinobacteria. In both cases, two factors were evaluated: isolates (60 fungi and 116 actinobacteria) and growth media (Czapeck Dox and Sabouraud). First, these factors were analyzed against the pathogen *S. aureus*, followed by *C. albicans*, leading to two different statistical analysis for each microorganism (fungi and actinobacteria).

The F values were significant at 1% probability ($p<0,01$), showing that the halos from different fungi and bacteria had significant variance between samples. A significant difference between culture media was also observed. The Scott-Knott test was used to evaluate the activity against *S. aureus* as well as *C. albicans* (Fig. 1). The best results for fungi were observed using the Czapeck Dox growth medium and Sabouraud was the best for actinobacteria.

Lin[39] demonstrated that the mycelium growth and the antimicrobial activity against *S. aureus* ATCC 25925 were observed in different media. The three growth media used in the experiment were GPDA (200 g potato; 10 g glucose; 4 g peptone; 1000 mL distilled water), GYP (10 g glucose; 4 g peptone; 1g yeast extract; 1000 mL distilled water) and GS (20 g starch; 1 g KNO_3; 0,5 g K_2HPO_4; 0,5 g $MgSO_4$; 0,001 g $FeSO_4$; 1000 mL sterile distilled water). The most efficient medium for antimicrobial compound production was GPDA. Comparing the formula of the GPDA medium with the Sabouraud medium (10 g

peptone; 40 g glucose; 1000 mL sterile distilled water), it's observed both are similar regarding to the carbon and nitrogen sources. The same is observed between the GS and Czapeck Dox (30 g sucrose; 3 g $NaNO_3$; 1 g K_2HPO_4; 0,5 g $MgSO_4$; 0,5 g KCl; 0,01 g $FeSO_4$; 1000 mL sterile distilled water) medium regarding the salt concentrations. These data confirm the present work.

In total, 60 fungi were tested against pathogenic strains and 35 isolates (58%) showed inhibition against at least one strain. The strain *S. aureus* was the most sensitive to the metabolites produced by the fungi, but these organisms also inhibited simultaneously other strains, among them methicillin-resitant *S. aureus* (MRSA) and *C. albicans* (Fig. 2a). A similar result was found by Vanderlinde[40] in *S. aureus* was observed to be more sensitive to *Pycnoporus sanguineus* metabolites.

In this work, from 116 actinobacteria isolates, 79 (68%) have shown activity against pathogenic strains (Fig 2b). This number may be considered high in comparison to the research conducted by Penesyan[41] in Australia, in which 325 actinobacteria samples were isolated from the surface of marine algae and only 38 (12%) of isolates showed antimicrobial activity against at least one pathogenic strain.

The actinobacteria isolate AD 3B 17 (*Streptomyces longwoodensis*) had remarkable activity agains *S. aureus* and *Escherichia coli*. The fungus isolate AS G17 3B 21 (*Penicillium dipodomycola*) also had remarkable activity against S. aureus and *C. albicans* (Table 1)

Studies show that actinobacteria isolates have more activity against gram-positive bacteria than gram-negative. González[42] worked with 337 actinobacteria isolates and found antimicrobial activity in only 27%. Activity was most frequently found against gram-positive bacterias (23%), *C. albicans* (10,7%). The lowest activity rate was found against gram-negative bacteria (6,5%).

The observed results (Fig. 2a) show that 88,5% fungi isolates inhibited *S. aureus* ATCC 25923, *C. albicans* ATCC 10231 was inhibited by 45,7%; methicillin-resistant *S. aureus* was inhibited by 37,15%; *E. coli* ATCC 25922 was inhibited by 5,7%; *P. aeruginosa* ATCC 27853 was inhibited by 2,8%. Among these fungi, 57,14% inhibited two or more strains and 42,86% inhibited at least one pathogenic strain.

A wide marine fungi bioprospection made by Christophersen[43] showed results that confirm this work. From 755 species isolated from animal, plants and marine sediments at Mochima National Park and Paria Bay, Venezuela, 27% showed activity against the bacteria *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *Vibrio paraha-emolytivus* ATCC 17802. The 227 isolates were identified as *Eupenicillium*, *Penicillium, Aspergillus, Eurotium, Fusarium, Emericella, Alternaria* and *Gliocladium*. Another study was made using fungi isolated from the south to east coast of Tamil Nadu, India. Samuel[44] observed the raw extracts from the fungal species *Aspergillus flavus*, *Geotrichum candidum*, *Peniciliium leuteum*, *Penicillium granulatum* and *Acremonium sp*. These isolates showed antimicrobial activity against five human pathogenic bacteria, *Escherichia coli*, *Vibrio cholerae*, *Pseudomonas putida, Bacillus subtilis* and *Micrococcus* sp.

The fungi with promising results against *S. aureus*, *C. albicans*, methicillin-resistant *S. aureus* (MRSA), *E. coli* and *P. aeruginosa* belong to the genera *Aspergillus, Acremonium, Penicillium, Paecilomyces, Trichoderma* e *Drechslera*. Between these genera, *Aspergillus* had remarkable results, inhibiting approximately 76.5% of tested strains.

Studies using extracts from the sclerotium of *Penicillium raistrickii* have led to the isolation of many metabolites, and two of them resulted in promising results. The metabolite 3,3"-di-hidroxi-6'-desmetilterfenilin was active against *S. aureus* and metil-3,4,6,8-tetra-hidroxixanton was active against *Bacillus subtilis*[3,45].

The obtained results in this work evidence that from the 116 actinobacteria isolates, 87% inhibited *C. albicans*, 77% inhibited *S. aureus*, 18% inhibited *P. aeruginosa* and 4% inhibited *E. coli*. Approximately 72% of

tested samples have shown activity against two or more pathogenic strains (Fig. 2b).
The genus Streptomyces is known for its bioactivity against pathogenic fungi, as well as against gram-positive and gram negative bacteria[46]. Melo[47] observed that 41 actinobacteria isolated from soil had antimicrobial activity, 56% inhibited gram-positive bacteria, 49% inhibited gram-negative bacteria and 71% had inhibition halos against fungi. This work is confirmed by these data, as most isolates from this research were able to inhibit gram-positive bacteria (77%) and yeasts (87%).

Bioautography

From the analysis made using the well method, two fungi and actinobacteria isolates were selected for the bioautography test (Table 2). For this method, it was necessary to obtain an extract from each selected isolate.
The bioautography method was used to discover the group of the substances with antimicrobial activity present in the extract from the fungi and actinobacteria isolates. The aim of this technique was to identify the fraction with antimicrobial activity. The fungi isolate with remarkable yield raw extract and antimicrobial activity was AS G17 3B 21, from the *Penicillium* genus. The extracts 12 and 12'were isolated and the second was active against the strain C. albicans ATCC 10231 (Fig. 3A). Through bioautography, it was also observed that the extracts G6 and G9 had positive results against *S. aureus* ATCC 25923 and MRSA (Fig. 3c). The extract G6 was obtained from the actinobacteria AD 3B 17 and the extract G9 was obtained from AD G34 12B 82, both from the *Streptomyces* genus. It is possible to confirm that this assay is simple, reliable and sensitive, as at least 0.05 mg of bioactive substance have led to inhibition halos.
The extracts present at Table 1 were analyzed through thin-layer chromatography (CCD), using different solvents in the attempt to separate the present substances. From all CCD assays, the best solvent was the mixture of eluents dichloromethane/methanol (95:5 and 90:10) (Fig. 3b and 3d). The eluent used for the development of chromatograms was chosen according to the chemical properties of the extracts. The stains were visualized under ultraviolet light (365 nm) and application of chemical developer (sulfuric vanillin).
The highlights in Fig. 3 are the group of substances with antimicrobial activity present in extracts 12', G6 and G9 from actinobacteria and fungi isolates.
In a similar research, Bugni[8] isolated 9 fungi from marine environment in Fiji. These isolates were selected for fermentation assays. The extracts obtained from *Penicillium brocae* were active against methicillin resistant *S. aureus* (MRSA) and metivillin sensitive *S. aureus* (MSSA). However, Silva[48] tested extracts from 60 *Penicillium* strains from many species through the bioautography method. The antibacterial activity results revealed that 57.1% had and inhibition halo against *S. aureus* CCT 1352 and *C. albicans* DPUA 1240.
In this work, the Rf value (plate retention factor) found was 0.5, which translates as the value of the distance traveled by the extract divided by the distance traveled by the solvent. The 12'extract (from the fungus isolate AS G17 3B 21) had two active fractions for *C. albicans*. These active substances have non-polar character. Jaim[49] observed that the *Streptomyces* isolate VRY-1 was active against contra *Salmonella typhimurium* at the bioautography test with 0.39 Rf value. This antimicrobial substance produced by this actinobacteria was soluble in water and ethyl acetate.
The compound isolated from the G6 raw extract (*Streptomyces longwoodensis* – Table 3), is orangish, amorphous and chloroform-soluble. Lin[39] described a novel streptomycete, which was isolated from marine mud in China. The secondary metabolism of the L0804 strain produced two active compounds with antimicrobial and antitumor activity. One of them is compound A, an orangish-red alkaloid that is soluble in chloroform, acetone, ethanol, methanol, ethyl acetate and dimethyl sulfoxide. However, the compound is insoluble in cyclohexane, petroleum ether, ethyl ether and water. The above-described characteristics from compound A are

similar to the G6 compound isolated in this work.

Streptomyces spp. produces many classes of secondary metabolites with great biofunctional diversity. The extract of *S. albogriseolus* A1, a strain isolated from Red Sea coast sediments, showed potent antimicrobial activity against a set of diverse pathogenic microorganisms and seven compounds were identified. Structures of the isolated compounds were characterized as Butane-2,3-diol, *N*-acetyltyramine, (3*R*,4*R*)-3,4-dihydroxy-3-methyl-pentan-2-one, (3*S*,4*R*)-3,4-dihydroxy-3-methyl-pentan-2-one, benzoic acid, *cis*-cyclo-(Ph, Prol), indolyl-3-acetic acid and N^B -acetyl tryptamine[50]. Ammosamide D, an oxidized analog of the ammosamide family, was isolated from a marine-derived *S. variabilis*. Pyrroloquinoline alkaloids are a growing class of natural products, being the first example of an oxidized analog resulting in a 5,6-dioxo-5,6-dihydroquinoline ring system. Attempts at chemical conversion of ammosamide B to ammosamide D revealed that a strong chemical oxidant is required. Ammosamide D has modest cytotoxicity to the MIA PaCa-2 pancreatic cancer cell line[51, 52].

Zhong[53] discovered a novel complex bioactive oligosaccharide from the *S. luteogriseus* strain 670. This complex has showed remarkable activity against several saccharide hydrolases.

Yuanyuan[54] isolated and evaluated new anthraquinone glycosides (strepnoneside A and B, and chromomycin A_3) produced by *Streptomyces sp*. These isolates showed cytotoxic activity against human colon carcinoma cells (HCT116).

Cary[55] evaluated ilamycin B1, a cyclic heptapeptide consisted of only L-amino acid residues (L-*N*-methyl leucine, L-tryptophan derivative, L-2-amino-trans-4-hexenoic acid, L-leucine, L-*N*-methyl leucine, L-alanine and L3-nitrotyrosine). This compound is produced by *S. islandicus* and is an analogue of ilamycin, an antibiotic that inhibits the growth of mycobacteria.

Tuntiwachwuttikul[56] isolated and elucidated four novel compounds, lansai A-D, from *Streptomyces sp.* SUC1, an endophyte. The antifungal (activity against *Colletotrichum musae*) and anticancer activities (BC and KB cell lines)of these compounds were tested.

Terekhova[57] evaluated the antibiotic INA 2770 (cineromicin) produced by a *Streptomyces* strain. The compound was characterized and demonstrated activity against a wide range of gram-positive bacteria. Two minor components designated mycotrienols I and II which were concurrently produced with mycotrienins I and II by *S. rishiriensis* T-23 were isolated. The mycotrienols showed no antimicrobial activity over a dose range between 10 to 100 µg/ml, while mycotrienins showed antifungal and antiyeast activities (MIC= 4.0-12.5 µg/ml)[58].

Identification of fungi and actinobacteria isolates with potential antimicrobial substance production

The fungi identified as *Aspergillus, Acremonium, Penicillium, Paecilomyces, Trichoderma* and *Drechslera* were active in antimicrobial tests (Fig. 4).

The identified actinobacteria genera were *Streptomyces*[59] e *Nocardia*[60].

Molecular characterization of isolates

The most remarkable fungus regarding to antimicrobial activity and raw extract production was AS G17 3B 21, which was amplified using ITS1 and ITS4 primers. These oligonucleotides amplify the ITS1-5.8S-ITS2 of ribosomal DNA, producing a sequence of about 550 pb. According to analysis using the softwares Mega and Blastn, the species found is *Penicillium dipodomyicola* (Table 3).

Frisvad[61] selected about 100 isolates of each *Penicillium* species. These isolates were inoculated in Czapeck Dox agar with yeast extract addition and incubated for 7 dias at 25ºC for future metabolite extraction. Among these species, *P. dipodomycola* produced compounds of pharmacological interest, like the antifungal griseofulvin, which is active against dermatophyte fungi.

In this work, *P. Dipodomycola* was active against the yeast *C. albicans*, which may worsen the clinical presentation of immunocompromised patients[62].

The primers used to amplify the 16S region of actinobacteria rDNA were 9F and SM6R, specific to the Streptomycetaceae family. The sequences are about 1000pb long. Seven different species were identified as belonging to the *Streptomyces* genus (Table 3).

The 16S rDNA sequences indicate 97-100% similarity to the genus *Streptomyces* according to the GenBank database. This genus has been previously identified in the work of Wu[63].

The actinobacteria *Streptomyces seoulensis* has a Korean geographic origin and was first described by Chun[64]. This species is aerobic, gram positive, makes a yellow mycelium and grey spores on glycerol asparagine agar base. The lipoamide dehydrogenase (LPD) was purified from the raw extract of *Streptomyces seoulensis*[65]. This enzyme is an essential compound of the pyruvate dehydrogenase complex, which has a crucial role in the main metabolism of aerobic organisms. This enzyme is an enabling agent of the redox cycling of quinone compounds and has been widely studied, as many antitumor drugs have a quinone nucleus[66].

The species *Streptomyces parvus* HCCB 10043 produces arylomicin compounds, which are peptidase inhibitors and have broad-spectrum antibiotic potential against gram-positive bacteria as well as gram-negative, such as *Helicobacter pylori* and *Staphylococcus epidermidis* [67, 68].

In a study made in the Antarctic continent, it was possible to isolate actinobacteria from marine sponges, one of which is *Streptomyces bacillaris*. This species had genes that code to polyketide synthases (PKS) with potential to produce active compounds[69].

The research, sorting and identification of endophytic actinobacteria from medicinal plants has showed that 12 samples and 65 strains were able to inhibit penicillin resistant *Staphylococcus aureus*. These actinobacteria had their 16S rRNA sequences compared and the SC-N040902 was 99.9% similar to *Streptomyces cavourensis* NRRLB-8030T[70].

The species *Streptomyces longwoodensis*, which was described by Prosser[71], produces the antibiotics ionophore polyether, lysocline. It is characterized by the grey color of its spiral-shaped grey spores. Generally, ionophores are highly effective against gram-positive bacteria, but have little or no activity against gram-negative, which have an external membrane containing porines (protein channels) sized about 600 daltons (Da). Most ionophores are larger than 600 Da and lysocline has 660 Da[72].

Continuous efforts to seek natural products of marine actinobacteria with selective cytotoxicity against cancer cell strains, many fractions derived from the actinobacteria *Streptomyces variabilis* (strain SNA-020), which had activity against pancreatic MiaPaca-2 cancer cell strain. The active fraction analysis detected the presence of amosamide D[51, 52].

The review work made by Kalakoutskii[73] shows communities of algae and bacteria in mixed cultures, with the prevalence of actinobacteria. These organisms were found in algae in carbonate rock formations in temperate climate regions. The review emphasizes the formation of lichens and the association behavior of actinobacteria with algae cells and other organisms, such as *Streptomyces malachitospinus* INMI 217. This organism produces a physiologically active substance that stimulates the zygote formation of the filamentous fungus *Phycomyces blakesleeanus*. This actinobacteria has catenulate spiral spores and grey aerial mycelium.

Different scientific researchers were carried out on the antibiotic producing actinomycetes and fungi in various ecosystems, but this study gives the first hand information on the antimicrobial activity of *Streptomyces* spp. and *Penicillium dipodomyicola* isolated from sandy sediment of intertidal region of Ilha do Mel, Paraná, Brazil.

In conclusion, the microorganisms isolated from the intertidal region and tested for antimicrobial activity presented promising results for the obtainment of bioactive substances, suggesting these organisms may be sources for antibiotic and antifungal production. As such, it is important to search for compounds that may have a role as novel drugs to fight infection caused by microorganisms, as well as pathogens with

resistance to the drugs currently in use. The work of characterizing the bioactive metabolites of the potent fungal and actinobacteria strains is under way.

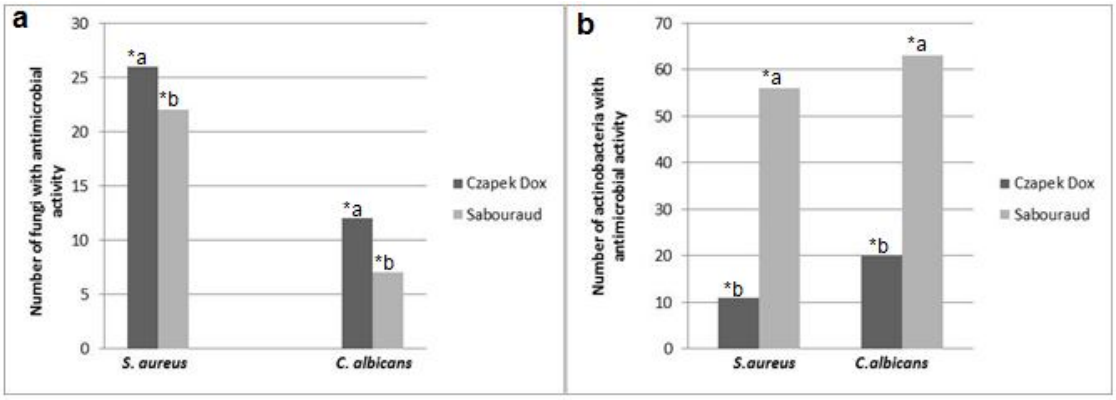

Fig. 1: Graphic representation of the microorganisms with antimicrobial activity in different culture media. The incubation ranged from 7 to 14 days at 28°C. a Fungi isolates. b Actinobacteria isolates *Averages followed by different letters are statistically different from one another Scott-Knott test with 5% probability.

Table 1: Evaluation of antimicrobial activity through well method against the following pathogenic strains: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231)

Isolate	Identification	Reference strains			
		E. coli	*S. aureus*	*P.aeruginosa*	*C.albicans*
AS G17 3B 21	*Penicillium dipodomyicola*	-	+++	-	+
AD G27 12B 83	*Streptomyces parvus*	-	+	-	+
AS G31 5A 43	*Streptomyces bacillaris*	-	+++	-	++
AD G32 11A 60	*Streptomyces seoulensis*	-	-	-	++
AS G34 3B 18	*Streptomyces variabilis*	-	++	-	+++
AD 7A 41	*Streptomyces variabilis*	+	+++	+	+++
AS G35 3A 43	*Streptomyces cavourensis*	-	+	++	++
AD 11B 76	*Streptomyces cavourensis*	+	++	+	-
AS 3A 26	*Streptomyces cavourensis*	+	-	-	+++
AD 3B 17	*Streptomyces longwoodensis*	-	+++	+	-
AD G34 12B 82	*Streptomyces malachitospinus*	+	+	+	+++

NOTE: +: inhibition halo <15mm diameter; ++: inhibition halo >or = 15 mm diameter; +++: inhibition halo >or = 20 mm diameter.

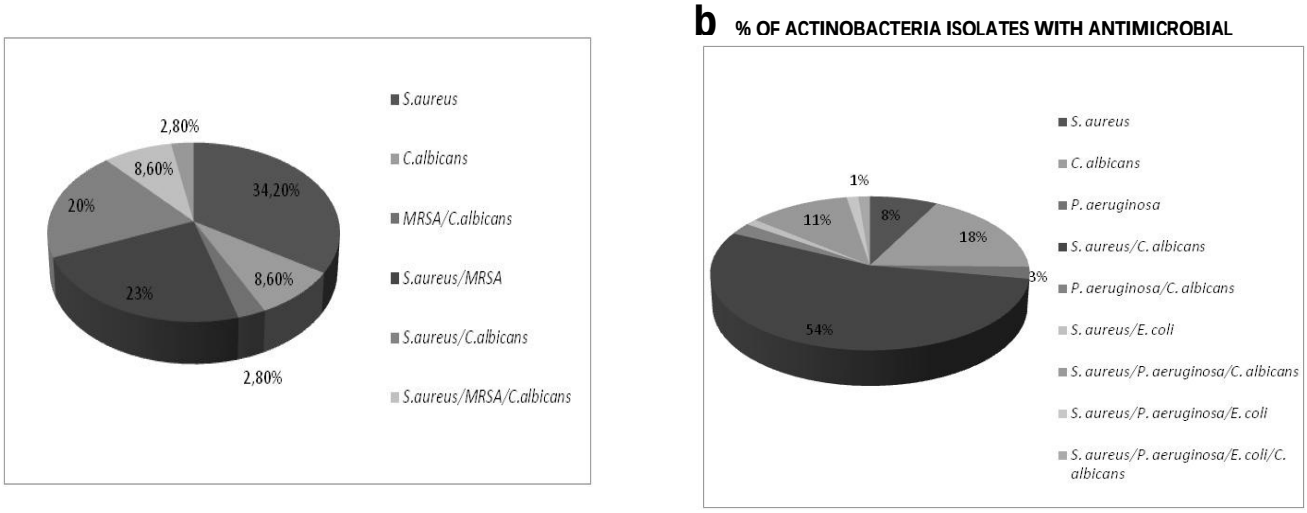

Fig. 2: Graphic representation of the percentage of microorganisms with antimicrobial activity. a Fungi isolates. b Actinobacteria isolates. Actinobacteria and fungi were grown in Czapeck Dox and Sabouraud media for 7 to 14 days at 28ºC

Table 2: Selected fungi and actinobacteria strains for analytic metabolite extraction and for the bioautography test

Pathogenic strains inhibited by well method	Extracts	Fungi and actinobacteria isolates	Yield extract (mg)
S. aureus/MRSA/*C. albicans*	12 e 12'	AS G17 3B 21*	11.6 e 9.0
E. coli/ S. aureus/ C. albicans	43	AS G1 1A 07 *	24.3
E.coli/P. aeruginosa/S. aureus/ MRSA/*C. albicans*	76	AS G2 1A 06*	≤7.0
C. albicans	G2	AD G32 11A 60**	≤.7.0
S. aureus/C. albicans	G4	AD G27 12B 83** AS 3A 26**	≤7.0
S. aureus/E. coli	G6	AD 3B 17**	16.0
S.aures/P. aeruginosa/C. albicans	G8	AD 11B 76**	≤7.0
S.aures/P. aeruginosa/C. albicans /E. coli	G9	AD G34 12B 82**	16.0

Legenda: *Fungi isolates and **Actinobacteria isolates
NOTE: The positive control used was gentamicin (0.2 mg/mL) and vancomicin (0.2mg/mL).

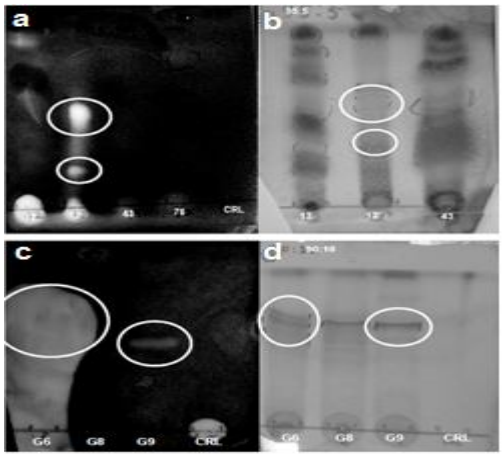

Fig. 3: Bioautography test of fungi and actinobacteria isolates with antimicrobial activity. Growth in Mueller-Hinton medium. Incubated for 36 to 48h at 37ºC. **a** CCD plate (6X6 cm) testing *C. albicans* ATCC 10231, mobile phases 95:5 ($CHCl_3$:MeOH), extracts (10 mg/mL) 12 12', 43, 76, nystatin control (CRL) (1000 UI); **b** CCD plate (6X6 cm), mobile phase 95:5 ($CHCl_3$:MeOH), extracts (10 mg/mL) 12, 12', 43, 76; **c** CCD plate (6X6 cm) pathogenic strain *S. aureus* ATCC 25923, mobile phase 90:10 ($CHCl_3$:MeOH), extracts (10 mg/mL) G6, G8, G9, gentamicin control (CRL) (0,2 mg/mL); **d** CCD plate (6X6 cm), mobile phase 90:10 ($CHCl_3$:MeOH), extracts (10 mg/mL) G6, G8, G9, gentamicin control (CRL) (0,2 mg/ml). The circled areas highlight the substances with antimicrobial activity from the fungi and actinobacteria extracts

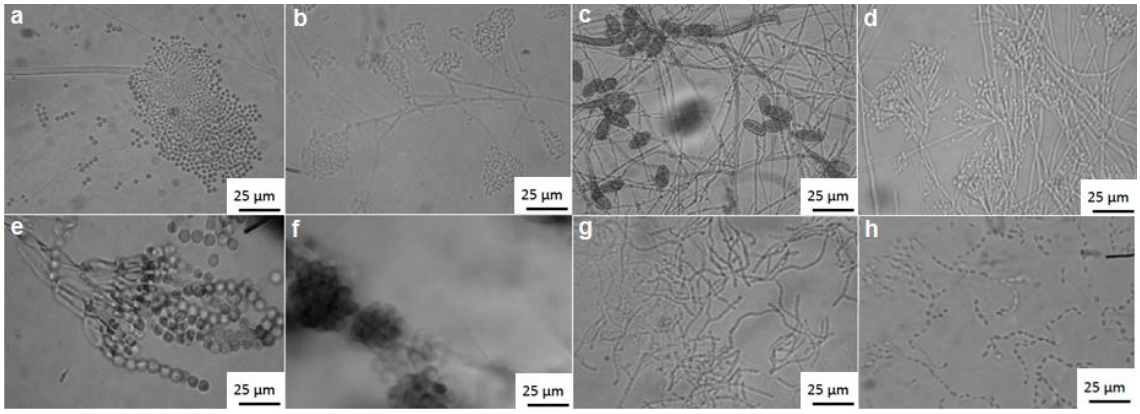

Fig. 4: Micromorphology of microorganisms isolated from superficial sediments from the intertidal region of Ilha do Mel, Paraná, grown for 7 days in Czapeck Dox medium at 28ºC. a *Aspergillus* sp. b *Acremonium* sp. c *Drechslera* sp. d *Paecilomyces* sp. e *Penicillium* sp. f *Trichoderma* sp. g *Streptomyces* sp. h *Nocardia* sp. (enlarged 400X: a, b, c, d, e, f; enlarged 1000X: g e h)

Table 3: Identification using morphological characteristics and comparison of sequences ITS1-5.8s-ITS2 do rDNA of fungi and 16S of actinobacteria rRNA.

GrenBank access number	Isolate	Morphological identification	Related microorganisms	GenBank access number	E-Value	Identity %
KC736977	AS G17 3B 21	*Penicillium* sp.	*Penicillium dipodomyicola*	GQ161752.1	0.0	99
JX997139	AD G27 12B 83	*Streptomyces* sp.	*Streptomyces parvus*	EU741180.1	0.0	99
JX997140	AS G31 5A 43	*Streptomyces* sp.	*Streptomyces bacillaris*	FJ792250.1	0.0	100
JX997141	AD G32 11A 60	*Streptomyces* sp.	*Streptomyces seoulensis*	EU841610	0.0	99
JX997143 JX997144	AS G34 3B 18 AD 7A 41	*Streptomyces* sp.	*Streptomyces variabilis*	JN180216.1	0.0	100
JX997146 JX997147	AS G35 3A 43 AD 11B 76	*Streptomyces* sp.	*Streptomyces cavourensis*	NR_043851.1	0.0	100
JX997145	AD 3B 17	*Streptomyces* sp.	*Streptomyces longwoodensis*	EU240414.1	0.0	97
JX997142	AD G34 12B 82	*Streptomyces* sp.	*Streptomyces malachitospinus*	NR041423.1	0.0	99

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