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Antimicrobial Activity of Actinomycetes Isolated from Soils in the Brazilian Amazon

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HIGHLIGHTS

- The genus *Streptomyces* is the most frequent actinomycete in samples of only Amazonia.
- The bioautography method reveal that actinomycete extracts possess an active substance against human pathogens.
- Active metabolites of actinomycetes are correlated to the stationary phase of biomass production.
- Actinomycetes from the Amazon show potential to produce new antibiotics.

Abstract: The objective of this work was to evaluate the antimicrobial activity of actinomycetes isolated from soils in the Brazilian Amazon. Soil samples were collected for the isolation of actinomycetes. The actinomycetes were grown in submerged culture and the metabolites extracted in ethyl acetate. The well diffusion method was used to verify the antimicrobial activity of isolated actinomycetes against test microorganisms. The microdilution method was used to determine the minimum inhibitory concentration (MIC) of the extracts against the test microorganisms. Bioautography was used to verify the bioactive compound of the extracts. The biomass and active metabolites were determined. 219 actinomycetes were isolated from Amazonian soils, organized into 153 morphospecies. The most frequent genus were *Streptomyces* (48.85%) and *Nocardia* (20.09%). Of the 153 extracts analyzed, 12 (7.84%) had antimicrobial activity, 9 against Gram positive bacteria and 5 against *Candida albicans*. The MICs of the extracts with activity ranged from 15 to 250 ug/mL. The bioautography method showed that the extracts from *Streptomyces* 3323 HEX, *Streptomyces* 3098 DCM, *Streptomyces* 3333 DCM and *Streptomyces* 3333 EtOAc generally had an active substance, and that they are different from commercial antimicrobials produced by

actinomycetes. Therefore, the actinomycetes from these areas can contribute to the discovery of new antibiotics and the fight against resistant pathogens.

Keywords: microorganisms; antibiotics; Acre; Streptomyces; Nocardia.

INTRODUCTION

Actinomycetes are gram-positive, spore-forming, cosmopolitan filamentous bacteria that colonize the most varied ecosystems, such as water, rocks, food, animals, plants, buildings, among others, but their main habitat is the soil [1, 46, 42].

Among the promising sources of new antibiotics, the greatest importance is in natural microbial products, especially actinomycetes, as they have a long history in the production of bioactive metabolites [2, 40, 45]. Since the beginning of antibiotic production in 1940, antibiotics have played an important role in antagonizing human pathogens, producing more than 50% of known antibiotics, mainly by species of the genus *Streptomyces* [3, 41, 43, 44].

Drug-resistant pathogenic bacteria are a worldwide problem that requires research and discovery of new products against pathogenic microorganisms. The incidence of serious infections related to opportunistic fungi has increased due to resistance to current antifungal agents, especially *Candida* spp., which is the fourth most isolated pathogen in blood infections [4].

Gram-positive bacteria, such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, and Gram-negative bacteria, such as *Escherichia coli* and *Klebsiella pneumoniae*, and the yeast *Candida albicans* are microorganisms of medical importance, as they are involved in urinary tract infections [5], endocarditis, pneumonia and septicemia [6], meningitis, otitis and conjunctivitis [7]. These pathogens are often capable of killing the patient, due to the ability to acquire resistance to frequently used antibiotics, a factor that motivated the need to discover new antibiotics [8].

In the Brazilian Amazon, there are few studies on actinomycetes isolated from plants and soil [9-10] demonstrating the importance of Amazonian actinomycetes to produce secondary bioactive metabolites. In Southwest Amazonia, there are no studies with actinomycetes, reinforcing the importance of researching this group of microorganisms that, in poorly explored environments, may reveal new antimicrobials.

Thus, this work aimed to evaluate the antimicrobial potential of secondary metabolites produced by actinomycetes isolated from soil in the Brazilian Amazon.

MATERIAL AND METHODS

Study area

The study area is located in the southwest of the Amazon region of Brazil, in the state of Acre. Twenty soil samples from Parque Zoobotânico of the Universidade Federal do Acre (UFAC), in Rio Branco (S 09º 57.429 'W 067º 52.412'), and 10 samples from Fazenda Experimental Catuaba (UFAC), in Senador Guimard (S 10º 04.002 ' W 067º36.306'), were randomly selected for the study. The collections were made from September to October 2016.

Isolation of Actinomycetes

Samples were collected in soil areas with waste disposal. During the study, 30 soil samples were collected aseptically at two sites, 10 cm from the ground, using standard methods [3]. The collected samples were transferred to the Laboratório de Microbiologia (UFAC). Soil samples were sieved through a sieve with a pore size of 250 µm. From each sample, 2 g of soil were added into Erlenmeyers containing 18 mL of saline solution (0.9 g/L NaCl) and stirred for 1 h at 120 rpm. These Erlenmeyers were considered as stock crops for different soil sampling sites.

From the stock cultures, a decimal dilution was made in a sterile 0.9% NaCl solution to a concentration of 10^{-2}, and 0.2 mL of this suspension was spread aseptically with a Drigalski spatula on the surface of plates containing starch-casein agar-AC (10g starch, 2g KNO_3, 0.3g casein, 2g NaCl, 2g K_2HPO_4, 0.05g $MgSO_4.7H_2O$, 0.02g $CaCO_3$, 0.01g $FeSO_4.7H_2O$, 15g agar to 1L) and chitosan agar-AQ (10g quitosana, 2g KNO_3, 2g NaCl, 2g K_2HPO_4, 0.05g $MgSO_4.7H_2O$, 0.01g $FeSO_4.7H_2O$, 15g agar to 1L) by the scattering technique. Cercobin (100 µg/mL) was added to both media to inhibit fungal contamination. The plates were incubated at 28 °C for up to 21 days and observed every day during the incubation [11]. After incubation, the

actinomycetes on the plates were identified based on color, texture, rough and convex colony. Colonies with these characteristics were purified by the stripping method by depletion in Potato-Dextrose-Agar-PDA (200g potato infusion, 20g dextrose, 15g agar for 1L). The isolates were kept in tubes with PDA medium at room temperature. For preservation, they were cultured in starch-casein broth and cryopreserved at -80 °C with 20% glycerol [12].

Morphological Identification

After the isolation of colonies, each colony was identified based on its colonial morphology, hyphae color, and presence or absence of aerial mycelium and substrate on Potato-Dextrose-Agar-PDA [13]. The micromorphological observation of the isolates was done in PDA and oat culture medium (30g oat, 15g agar to 1L) using Gram stain. Spore-bearing hyphae, spore arrangement, and spore chain structure were observed.

Primary Screening

Actinomycetes isolated and identified from different soil samples were selected for their antimicrobial spectrum. For the production of metabolites, isolated colonies of actinomycetes were transferred to test tubes containing 3 mL of AC broth and incubated under agitation at 120 rpm at 28 ± 1 °C for 14 days [14]. 2 mL of the AC broth was rotaevaporated by liquid-liquid division with a 1:1 ratio. (v/v) ethyl acetate [15]. The organic phase was collected, evaporated at 37 °C and solubilized in 400 µL of 99.9% dimethylsulfoxide (DMSO) for testing.

The test microorganisms used for primary screening were the bacteria *Staphylococcus aureus* ATCC25923, *Streptococcus pneumoniae* ATCC11733, *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* ATCC4952 and the fungus *Candida albicans* ATCC24433. The activities were evaluated using Muller Hinton agar (MH) for bacteria and Sabouraud-Dextrose agar (SDA) for fungi. A isolated colony of the test microorganism was collected and transferred to test tubes with Luria-Bertani (LB) broth for bacteria and Sabouraud-Dextrose (SD) broth for fungi. The tubes were incubated at 37 °C for 4-6 h until the turbidity was the standard 0.5 McFarland for bacteria and 1 McFarland for fungi. After adjusting for turbidity, a sterile swab was dipped into the suspension and applied to the surface of the culture medium. Wells of 5 mm in diameter were made in the medium and 20 µL of extract was placed. The plates were stored at 4 °C for 24 h, for diffusion of the extract, and incubated at 37 °C for 24 h. After incubation, the zone of inhibition was measured activity halos above 19 mm were considered as high. [6].

Secondary screening and molecular identification of actinomycetes

Based on the zone of inhibition in the primary screening, actinomycetes (*Streptomyces* 3098, *Streptomyces* 3323, *Actinobacterium* 3333) with potential antimicrobial activity were selected for submerged culture and extraction, and then the crude extracts were evaluated following agar diffusion methods as in the primary screening. As pre-inoculum, 15 plugs of promising cultures of actinomycetes grown in PDA (1 cm^2) were inoculated into AC broth (100 mL) and incubated at 28 °C for 48 h at 120 rpm. Finally, the culture broth was inoculated into Erlenmeyer flask containing AC broth (1000 mL) and incubated at 28 °C for 14 days at 120 rpm [16].

To concentrate the antimicrobial metabolite produced by the actinomycetes, medium were extracted using hexane (HEX), dichloromethane (DCM) and ethyl acetate (AcOEt) (1000 mL), in increasing order of polarity. Then, the organic phase, containing the active metabolite, was separated from the aqueous phase with a separatory funnel and the extracts were concentrated in a rotary evaporator at 37 °C [17]. Crude extracts obtained from the isolates were dissolved in DMSO and used as a stock solution to determine antimicrobial activity to the test pathogens, using DMSO as a control treatment.

The DNA from the *Streptomyces* 3098 and *Actinobacterium* 3333 was extracted by using the Quick-DNA Fungal/Bacterial miniprep kit (Zymo Research) following the manufacturer's instruction. The amplification of the gene encoding 16S rRNA region was done in a 50 µL reaction mixture, which included 2 µL DNA template (1-20 ng), 0.4 µM of each primer F243 (5′-GGATGAGCCCGCGGCCTA-3′) and R513GC (5′-CGGCCGCGGCTGCTGGCACGTA-3′) [18], 1.5 mM $MgCl_2$, 0.2 µM dNTPs, 5 µL of Taq buffer, and 1.25 U Taq DNA polymerase (Qiagen). The PCR amplification was done on a cycler PCR machine (Bio-Rad) with the initial denaturation at 95 °C for 2 min, followed by 35 cycles of amplification (95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 1 min) and an extension step of 72 °C for 7 min. The PCR products around 250 to 300 bp were purified using the QIAquick PCR Purification Kit (Qiagen) and quantified in 2% agarose gel. Forward and reverse sequencing reactions were done on a 7330xl DNA Analyzer (Applied Biosystems). Each isolate's

forward and reverse reads were paired to generate a consensus sequence. The consensus sequences had been blasted through the BLASTn in the National Center for Biotechnology Information (NCBI). The top hits results were used to identify the actinomycetes.

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of extracts of actinomycetes with antimicrobial activity was done by the microdilution technique, using sterile 96-well microplates. For assays with bacteria, MH broth was used and for assays with fungus, SD broth. 100 µL of broth was distributed in all wells of the plate and 100 µL of extract at a concentration of 2 mg/mL in DMSO was placed in the test well for serial dilution, resulting in an initial concentration of 1 mg/mL. The serial dilution was performed seven times, being homogenized and transferring 100 µL to the next well, resulting in a final concentration of 15.62 µg/mL. The control drug, chloramphenicol 2 mg/mL for bacteria and fluconazole 2 mg/mL for fungi, was diluted similarly to the extracts. 5 µL of inoculum from each strain tested were added, except for the negative control [19]. The negative control had 100 µL of broth and the positive control had 100 µL of broth and 5 µL of inoculum. The microplates were incubated at 37 °C for 24 h, then 20 µL of Resazurin (0.15 mg/mL) was placed in each well, which indicates microbial growth the color changes from blue to red [20].

In both tests, the effect of 10% DMSO on the microbial strain was evaluated, with serial dilutions under the same conditions as the extracts. The tests were done in triplicate.

TLC – Contact Bioautography Technique (Indirect)

20 µL of extract with antimicrobial activity were applied to thin layer chromatography (TLC) plates (Silica Gel 60) and eluted in different systems: HEX, HEX-AcOEt (1:1), AcOEt, AcOEt-Methanol (MeOH) (1:1) and analyzed under ultraviolet light (UV) 312 nm. The bioautography of the extracts was done by the indirect diffusion method on agar against the bacteria *S. aureus* and *S. pneumoniae* on MH and SDA agar for *C. albicans*. The extracts eluted in TLC were placed with their silicone face in uniform contact with the culture medium, kept at 4 °C for 12 h, for diffusion of the metabolites. The TLC plates were removed and then the test microorganisms were prepared as in the preliminary test and incubated for 24 h at 37 °C. The zone of inhibition was observed and compared to the Rf values of the TLC plate [21].

A comparison was also made between extracts of actinomycetes and standard antimicrobials (Erythromycin, Neomycin, Amoxicillin, Ciprofloxacin, Ketoconazole, Nystatin) by TLC, eluted in AcOEt and observed under UV 312 nm [22].

Production of biomass and bioactive metabolites

Actinomycetes with antimicrobial activity were inoculated on AC agar until spore formation, then NaCl (0.9%)/Tween 80 (0.1%) solution was added to the culture, and spore counts were done on a Neubauer chamber [23]. The spore suspension was inoculated to obtain a concentration of 10^6 spores/mL in 14 test tubes containing 2.7 mL of AC broth and incubated at 28 °C and 120 rpm for 14 days [24-25]. One tube from the system was removed every 24 h and the culture was transferred to a 2 mL microtube and centrifuged at 10,000 rpm for 15 min [26]. The centrifuge supernatant was separated to extract secondary metabolites, and 2 mL of distilled water was added to the centrifuge pellet and centrifuged again, this procedure being performed twice. The supernatant resulting from the second and third centrifugations was discarded and the pellet was dried at 40 °C to constant weight. The mass was determined by calculating the difference between the weight of the empty 2 mL tube and then the dry microbial mass, being mg/2.7 mL of the AC medium. The supernatant from the first centrifugation was extracted from secondary metabolites with AcOEt 1:1 (v/v) and the solvent was evaporated at 37 °C [2].

This procedure was done for 14 days for each morphospecies in triplicate. Extracts of actinomycetes metabolites were tested for antimicrobial activity using the agar diffusion test described in the primary screening.

Statistical analysis

The data obtained were examined through statistical analysis with the SPSS version 22.0 statistical package. Mean and standard deviation were analyzed and treatments compared to drug control using the paired Student's t test. The level of significance in the T test is assumed with p-value < 0.05 (5%).

RESULTS

Isolation and identification of actinomycetes

A total of 219 actinomycetes, 48.85% of the genus *Streptomyces*, 20.09% *Nocardia*, 4.1% *Thermomonospora*, 4.1% *Nocardiopsis*, 2.28% *Frankia* and 20.54% unidentified (NI) were identified in genus level, but all showed the macromorphological characteristics of actinomycetes. The isolates were obtained more frequently in the AC culture medium, with the exception of morphospecies of the genus *Nocardia*, which were more frequent in the AC medium (Figure 1).

The 219 actinomycetes were classified into 153 morphospecies, by macroscopic and microscopic characterization, and 111 (72.54%) were identified at the genus level. *Streptomyces* was the most frequent genus, followed by *Nocardia*, *Thermomonospora*, *Frankia* and *Nocardiopsis*.

The AC medium presented a higher frequency of isolates of the genera *Streptomyces* and *Nocardiopsis*, and in the ACh medium, the isolates of the genus *Nocardia* were more frequent. In the *Frankia* and *Thermomonospora* genera, the frequency was similar for both media used alone (Figure 1).

The partial 16S rDNA sequence of strain 3333 shared a maximum 100% sequence identity with *Actinobacterium* sp. KC758298. The strain 3098 was closely related with *Streptomyces albogriseolus* MN252550, showing 99.28% of identity (Table 1).

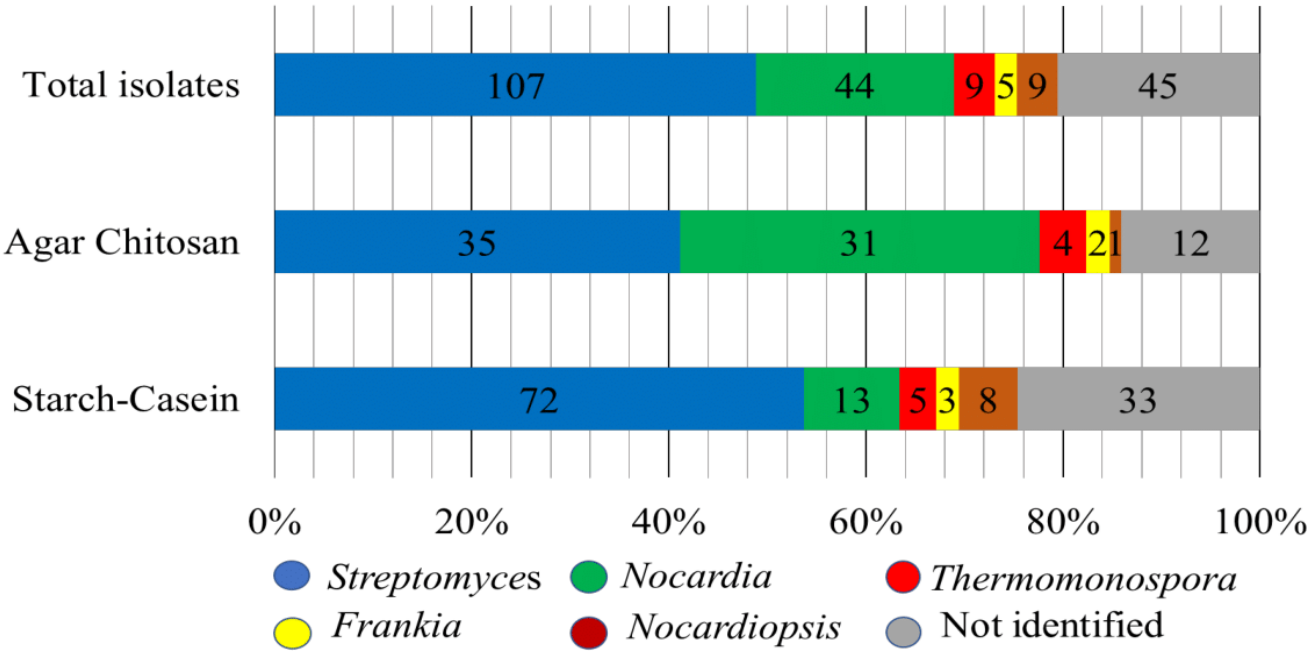

Figure 1. Quantitative analysis of actinomycetes isolated from Amazonian soils according to culture medium.

Table 1. Maximum nucleotide identity match for actinomycetes from Amazon soils based on the partial 16S rDNA sequences using BLASTn analysis.

Actinomycetes strain	GenBank Accession Nº	Closest match in GenBank	Percentage identity
3333	ON365507	*Actinobacterium* sp. (KC758298)	100
3098	ON365508	*Streptomyces albogriseolus* (MN252550)	99.28

Primary screening

Of the 153 extracts of actinomycetes, 12 (7.84%) showed antimicrobial activity. Nine morphospecies showed antibacterial activity against Gram-positive bacteria, five against *C. albicans* and none against Gram-negative bacteria. The most frequent genus of actinomycetes with bioactive metabolites was *Streptomyces* (Table 2).

The AcOEt extract of *Streptomyces* 3323 was the most efficient for both Gram-positive bacteria analyzed, followed by *Streptomyces* 3098, *Actinobacterium* 3333, *Streptomyces* 3170 (Table 2 and

Figure 2). Five extracts inhibited the growth of *C. albicans*, and two gram-positive bacteria and *C. albicans* (Table 2).

Table 2. Preliminary study of metabolite extracts of actinomycetes isolated from Amazonian soils with antimicrobial activity.

Actinomycete	Zone of inhibition (mm)		
	Staphylococcus aureus	***Streptococcus pneumoniae***	***Candida albicans***
Streptomyces 3098	26.0 ± 0.0*	25.3 ± 1.9*	-
Streptomyces 3141	11.3 ± 0.9*	11.3 ± 0.9*	-
Streptomyces 3170	22.0 ± 1.6*	22.0 ± 0.0*	-
Streptomyces 3223	10.6 ± 0.9*	10.0 ± 1.6*	-
Streptomyces 3273	14.0 ± 0.0*	13.0 ± 0.8*	-
Streptomyces 3317	10.6 ± 0.9*	12.0 ± 1.6*	-
Actinobacterium 3333	22.0 ± 1.6*	26.0 ± 1.6*	-
Streptomyces 3323	42.0 ± 0.0*	42.0 ± 0.0*	12.0 ± 0.0*
Actinomycete 3195	10.0 ± 1.6*	9.3 ± 0.9*	10.0 ± 1.6*
Streptomyces 3134	-	-	12.0 ± 0.0*
Streptomyces 3200	-	-	10.6 ± 1.6*
Actinomycete 3345	-	-	12.6 ± 0.9*
Chloramphenicol 2 mg/mL	44.0 ± 0.0	42.6 ± 0.9	-
Fluconazole 2 mg/mL	-	-	30.0 ± 0.0
Total	**9**	**9**	**5**

*Significant differences ($p < 0.05$) using Student's t test; Antimicrobial activity: low (halo 8-12 mm), medium (halo 13-19 mm) and high (halo >19 mm). [6]

Secondary Screening

Both DCM and EtOAc extracts from *Streptomyces* 3098 and *Actinobacterium* 3333 showed antibacterial activity at a concentration of 2mg/mL, and from *Actinobacterium* 3333 only the HEX extract showed activity (Table 3). For antifungal activity, only the extract of *Streptomyces* 3323 in DCM showed activity at 10 mg/mL (halo 22 ± 0.0 mm) and 50 mg/mL (30 ± 0.0 mm). The extracts of *Streptomyces* 3134 and *Actinomycete* 3345 did not show activity at any of the three concentrations analyzed (2, 10 and 50 mg/mL). The control drug Fluconazole 2 mg/mL had a halo of 30 ± 0.0 mm.

Minimum Inhibitory Concentration

The MIC ranged from 15.62 to 250 µg/mL for *S. aureus* and *S. pneumoniae* bacteria, and the *Actinobacterium* 3333 AcOEt extract showed the lowest MIC value for both bacteria tested. Three extracts showed MIC values equal to those of the control drug chloramphenicol against *S. pneumoniae*. The extract tested against *C. albicans* showed a MIC 2.5x higher than the control drug fluconazole (Table 4).

Table 3. Antibacterial activity of extracts of secondary metabolites produced by the Amazonian actinomycetes *Streptomyces* 3098, *Streptomyces* 3323 and *Actinobacterium* 3333.

Actinomycete	Extract 2 mg/mL	Zone of inhibition (mm)	
		Staphylococcus aureus	***Streptococcus pneumoniae***
Streptomyces 3098	HEX	-	-
	DCM	21.3 ± 0.9*	21.3 ± 0.9*
	AcOEt	12.6 ± 0.9*	12.6 ± 0.9*
Streptomyces 3323	HEX	30.0 ± 1.6*	28.6 ± 0.9*
	DCM	-	-
	AcOEt	-	-
Actinobacterium 3333	HEX	-	-
	DCM	28.6 ± 2.5*	28.0 ± 0.0*
	AcOEt	30.6 ± 2.5*	28.6 ± 0.9*
Chloramphenicol 2 mg/mL	-	44.0 ± 0.0	42.6 ± 0.9

*Significant differences ($p < 0.05$) using Student's t test; HEX=hexane; DCM=dichloromid e; AcOEt=ethyl acetate.

Table 4. Minimum Inhibitory Concentration of extracts of actinomycetes isolated from Amazonian soils with antimicrobial activity.

Extract	MIC (µg/mL)		
	Staphylococcus aureus	***Streptococcus pneumoniae***	***Candida albicans***
Streptomyces 3098 DCM	31.25	15.62	-
Streptomyces 3323 HEX	125	250	-
Actinobacterium 3333 DCM	62.5	15.62	-
Actinobacterium 3333 AcOEt	15.62	15.62	-
Streptomyces 3323 DCM	-	-	312.5
Cloranfenicol	7.81	15.62	-
Fluconazol	-	-	125

HEX=hexane; DCM=dichloromide; AcOEt=ethyl acetate.

TLC - Contact Bioautography Technique (Indirect)

It was possible to determine the antibacterial activity of compounds produced by actinomycetes separated by TLC. The antibacterial activity of *Streptomyces* 3098 extract in DCM against *S. aureus* and *S. pneumoniae* was demonstrated by compounds with Rf values of 0.7 and 0.75 in bioautography with AcOEt/MeOH (1:1) eluent, evidenced by the clear indicating inhibition (Figure 2a).

In the *Streptomyces* 3323 HEX extract, only one compound had activity against *S. aureus* and *S. pneumoniae*, with Rf of 0.73 in the HEX/AcOEt eluent (1:1) (Figure 2b). In the bioautography against *C. albicans*, the DCM *Streptomyces* 3323 extract inhibited growth over the entire range of TLC chromatography in contact with SDA, indicating that there are multiple bioactive substances or a substance with greater affinity for the fixed phase (silica) of TLC.

The extracts of *Actinobacterium* 3333 in DCM and AcOEt had a similar chromatographic profile in TLC, evidenced by bioautography, where the compound with activity against *S. aureus* and *S. pneumoniae* presented the same Rf in the different elution systems, indicating that it was probably the same compound in both extracts.

The analysis of four drugs used in antimicrobial therapy and extracts of actinomycetes showed that the bioactive substances found in the extracts have a different Rf than the drugs, with the exception of the AcOEt extract of *Actinobacterium* 3333, where the active compound remained in the base, similar to three of the four drugs antibacterials analyzed (Figure 3). The DCM extract of *Streptomyces* 3323, active against *C. albicans*, showed three substances with different Rf than the antifungals ketoconazole and nystatin (Figure 3). The bioactive substance was defined according to the data obtained in the bioautography with ethyl acetate.

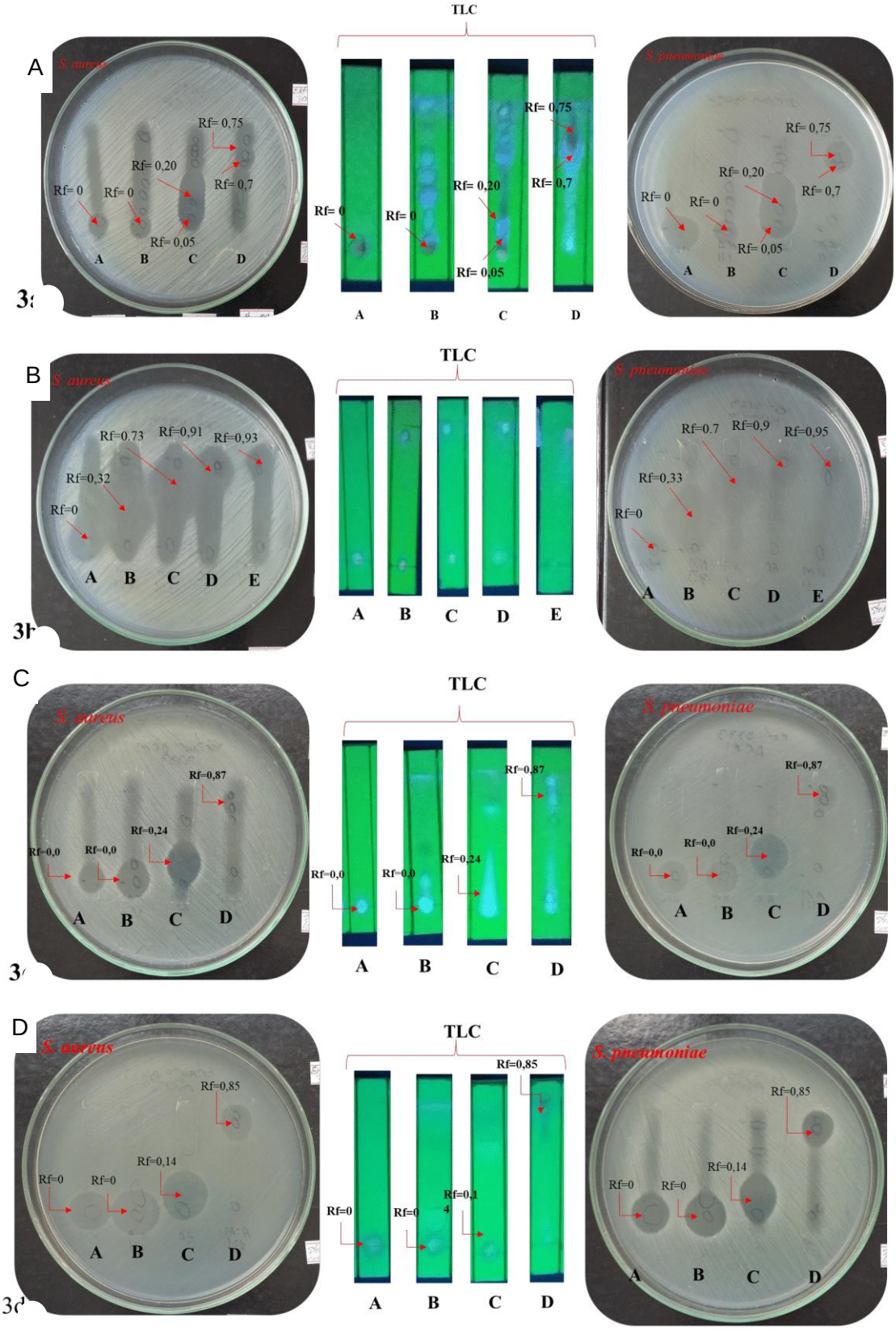

Figure 2. Bioautography of actinomycete metabolites with antibacterial activity. 2a. *Streptomyces* 3098 DCM Extract; 2b. *Streptomyces* 3323 HEX Extract; 2c. *Streptomyces* 3333 AcOEt Extract; 2d. *Streptomyces* 3333 DCM extract. (A) eluent system in HEX, (B) eluent system HEX/AcOEt (1:1), (C) eluent system in AcOEt and (D) eluent system in AcOET/MeOH (1:1), (Rf) retention factor, (TLC) thin layer chromatography, test against *Staphylococcus aureus* and *Streptococcus pneumoniae*.
HEX=hexane; DCM=dichloromide; AcOEt=ethyl acetate; MeOH=methanol.

Production of biomass and bioactive metabolites

The morphospecies *Streptomyces* 3098 had an initial biomass growth at 2 mg, growing until the 8th cultivation day. Of the 8th to 14th day of cultivation, the biomass remained in the stationary phase with approximately 4 mg. The production of bioactive metabolites was higher than the 4th to 9th day of cultivation, with greater production on the ninth day. The production of metabolites was estimated by measuring the inhibition zones against *S. aureus* and *S. pneumoniae* bacteria (Figure 4a).

The morphospecies *Streptomyces* 3323 had an exponential growth from the 1st to the 3rd day of cultivation with increased biomass. From the 3rd to the 5th day, there was a decrease in biomass, stabilizing from the 6th day of cultivation, with small variations until the 14th day. The bioactive metabolites showed antibacterial activity from the 1st day of growth, production remained stable until the 10th day, with similar inhibition halos, and from the 10th to 14th day of growth there was a decrease in the production of antibacterials. The 14th day showed the greatest inhibition halo (Figure 4b).

The morphospecies *Actinobacterium* 3333 showed high growth, approximately 100% of the initial biomass inoculated on the 1st day. From the second to the 4th day of cultivation, there was a decrease in biomass, and from the 6th day of cultivation to the 14th day, the biomass remained stable, close to 4 mg. The production of bioactive compounds started on the 2nd day of cultivation, decreasing production on the 3rd and 4th days, entering the exponential production phase from the 5th to the 6th day of growth, and stabilizing production on the 12th day. The 14th day showed the highest inhibition halo for bacterial tests (Figure 4c).

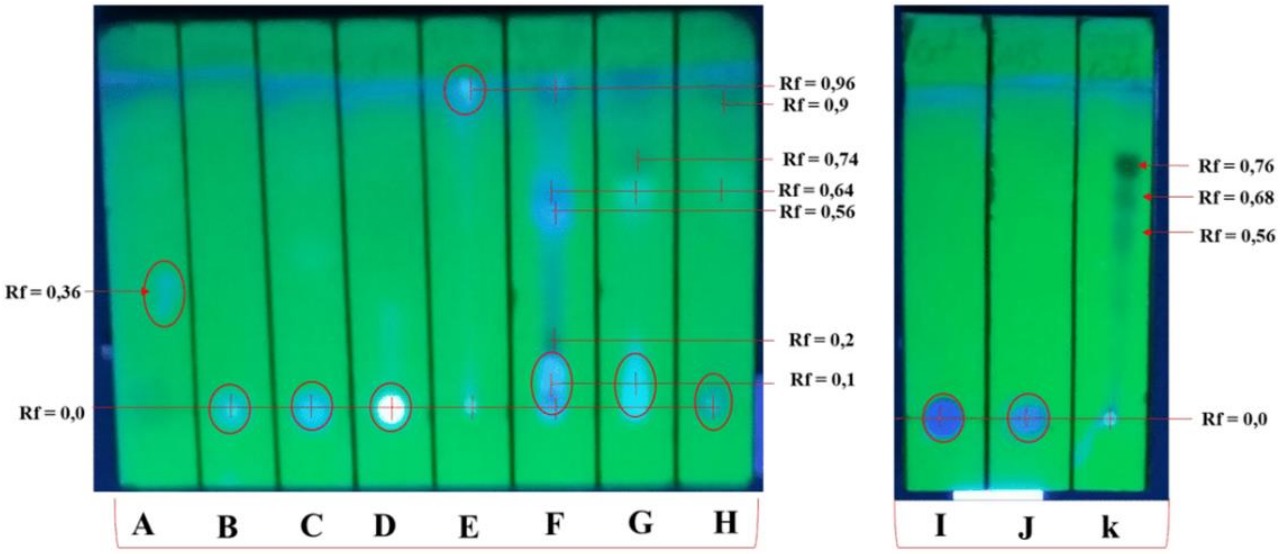

Figure 3. TLC chromatographic profile revealed at UV 312 nm, standard drugs and metabolite extracts of actinomycetes. (A) Erythromycin; (B) Neomycin; (C) Amoxicillin; (D) Ciprofloxacin; (E) *Streptomyces* 3323 HEX Extract; (F) *Streptomyces* 3098 DCM extract; (G) *Streptomyces* 3333 DCM extract; (H) *Streptomyces* 3333 EtOAc extract; (I) Ketoconazole; (J) Nystatin; (K) *Streptomyces* 3323 DCM extract. HEX=hexane; DCM=dichloromide; AcOEt=ethyl acetate.

The morphospecies *Streptomyces* 3134 showed higher biomass production on the 2nd day of growth with 3 mg, and from the 7th day, the biomass remained stable at approximately 2.2 mg. The production of metabolites showed a higher inhibition halo against *C. albicans* on the 3rd day of cultivation, when the biomass starts to decrease, and stabilizes the production of bioactive metabolites from the 6th day of growth, with a 12 mm halo (Figure 5a).

The morphospecies Actinomycete 3345 showed exponential growth from the beginning of inoculation to the 8th day of cultivation, with approximately 12 mg, and from the 8th day, the biomass showed small variations, stabilizing until the 14th day. The bioactive metabolites had two production peaks, one on the 8th and 9th day and the other on days 13th and 14th of the submerged culture (Figure 5b). The morphospecies *Streptomyces* 3098 and *Actinobacterium* 3333 showed similar biomass growth, acquiring 2x the initial mass (Figure 4a and 4c), different from the *Streptomyces* 3134 and *Streptomyces* 3323, presenting a growth curve between days 1-5 and day 6-14, maintaining approximately the same initial mass as the inoculum (Figures 5a and 4b). The morphospecies Actinomycete 3345 presented different biomass growth from the other four morphospecies, with exponential growth until the 8th day and biomass acquisition 6x the initial inoculated mass (Figure 5b).

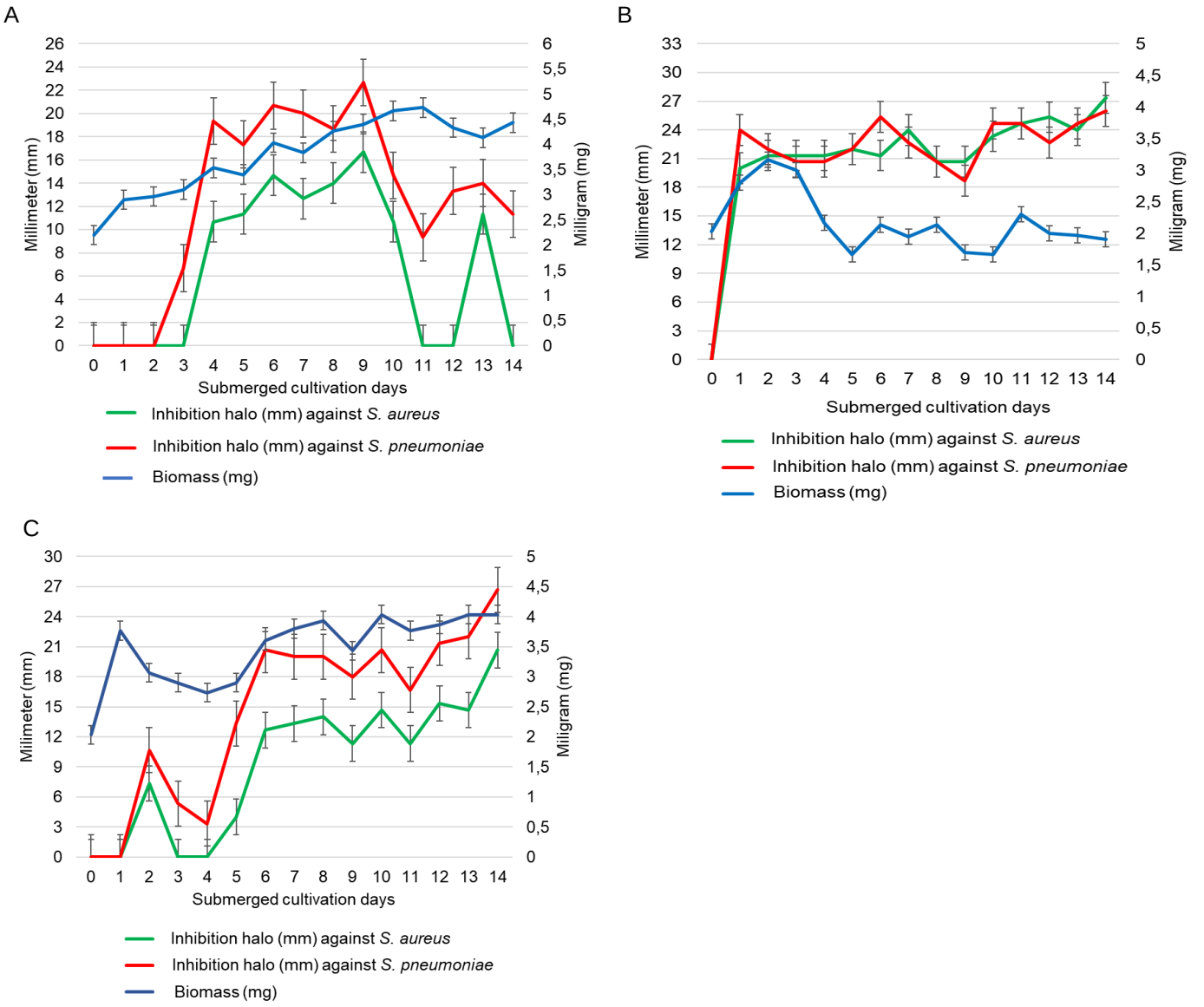

Figure 4. Production of Biomass and metabolites from Actinomycete. (A) *Streptomyces* 3098. (B) *Streptomyces* 3323. (C) *Streptomyces* 3333.

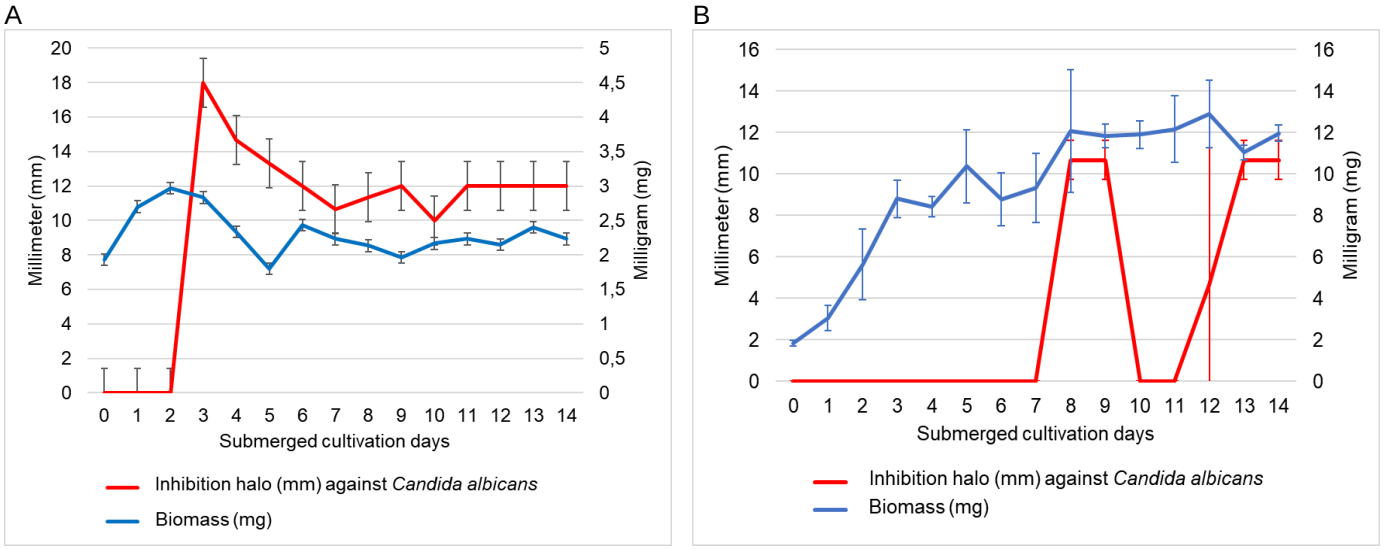

Figure 5. Production of Biomass and metabolites from Actinomycete. (A) *Streptomyces* 3134. (B) Actinomycete 3345.

DISCUSSION

Antibiotics are the most important bioactive compounds for the treatment of infectious diseases. However, due to emergencies arising from multidrug-resistant pathogens, challenges exist for the effective treatment of infectious diseases. Thus, due to the high frequency of multidrug-resistant pathogens in the world, there has been a growing interest in finding effective antibiotics from soil actinomycetes in different ecological niches [27].

In this study, randomly selected soil samples were collected from areas of primary and secondary forest in southwestern Amazon for the isolation of actinomycetes. Successful isolation of actinomycetes from environmental samples requires an understanding of potential soil sampling areas and the environmental factors that affect their growth [28].

A total of 219 actinomycetes were isolated from Amazonian soils, *Streptomyces* (48.85%), the most frequent, followed by *Nocardi*a (20.09%), *Thermomonospora* (4.1%), *Nocardiopsis* (4.1%) and unidentified (20,54%).

In Brazil, the genus *Streptomyces* is the most isolated genus [29-31], followed by the genus *Nocardia* (30, 32] as confirmed in this study.

The use of two culture media allowed greater quantity and diversity of actinomycetes (Figure 1). The importance of using different culture media is related to obtaining different microorganisms, as different nutritional sources allow greater isolation of the species, as they increase the chances of microorganism development [33].

It was not possible to identify all the morphospecies isolated by the methodology used, since the conventional methods defined by macroscopic and microscopic characterization are limited, not allowing a characterization by species, as well as some genera, since the morphology often does not allow a classification species level [34]. For a more detailed and complete analysis, it is necessary to done a molecular characterization of the isolates.

Of the 153 actinomycetes extracts analyzed, 12 (7.84%) had antimicrobial activity, 9 against Gram positive bacteria and 5 against *C. albicans*. This activity result is lower than the results previously observed for actinomycetes, 12.39% [9], and 41.93% [8]. Antimicrobial activity was only against Gram positive bacteria, corroborating results obtained in other studies with actinomycetes from different environments producing bioactive metabolites [3,9] (Table 1). The fact that there is no inhibition for Gram-negative bacteria may be due to their complex and naturally resistant outer membrane [35].

Differently from what was observed in this study, the actinomycetes isolated in Paraná were more active against *C. albicans* and also showed activity against Gram-negative bacteria [32]. The composition of the sample environment is related to the isolates and the production of secondary metabolites. Microorganisms produce bioactive molecules according to their needs and the environment is a relevant factor, competition with other organisms and substrate composition also influence the production of metabolites [33].

More than 50% of the actinomycetes in the Amazon region near Manaus were able to produce compounds antagonistic to *C. albicans* [31]. The reason for the lower frequency of actinomycetes with antifungal activity in this study may be related to the extraction solvent, with medium polarity, used to facilitate the isolation of bioactive metabolites. Of the 12 actinomycetes with antimicrobial activity in the primary screening, five best actinomycetes were selected, three for antibacterial activity and two for antifungal activity.

Extracts of *Streptomyces* 3098, *Streptomyces* 3323 and *Actinobacterium* 3333 demonstrated stability of the antimicrobial compound during the primary and secondary screening processes, showing promise for future research and isolation of compounds. *Streptomyces* 3134 and *Actinomycete* 3345 showed no antimicrobial activity in secondary screening. Possibly, the compounds were unstable and were degraded in the extract production process due to heating at 37 °C under reduced pressure (method used in the extract concentration process) using a rotary evaporator, which is the differential in the production of extracts between the screenings primary and secondary. According to [36], in a study comparing three different temperatures (25, 30 and 35 °C) in relation to antimicrobial activity, the cultivation temperature at 30 °C is the best for the production of bioactive metabolites, and the temperature of 35 °C, the compounds had no activity against *C. albicans*, indicating that they were degraded. Among the analyzed extracts, the *Actinobacterium* 3333 AcOEt extract presented MIC 2.5x higher than the control drug, chloramphenicol, for *S. aureus*. The extracts *Streptomyces* 3098 DCM, *Actinobacterium* 3333 DCM and *Actinobacterium* 3333 AcOEt showed MIC equal to the control drug (15.62 µg/mL) against *S. pneumoniae*, and the only extract where the antifungal activity was stable, *Streptomyces* 3323, showed activity in a concentration 2.5x higher than the control drug Fluconazole (Table 3). The MIC results of actinomycetes extracts demonstrate the potential for antibiotic production, as they have a relatively low MIC for the crude extract with mixtures of substances.

TLC bioautography is a simple and efficient technique for separating complex mixtures of compounds by locating the active constituents on the TLC plate, which is important for antimicrobial research [37]. TLC does not provide specific measurements, but it is an excellent method of identifying and locating compounds with antibacterial activity [21] and contact bioautography makes it possible to detect whether a single compound or multiple compounds are responsible for the activity antibacterial or antifungal. The use of this technique made it possible to verify that the bioactive compounds of the actinomycetes extracts in this study are, for the most part, isolated and not a mixture of substances. This feature facilitates the following steps in compound purification processes. However, other analyzes must be done in the future for the separation and structural elucidation of the biomolecules.

Actinomycetes are known as slow-growing microorganisms, with a notable increase in biomass production up to 4 days of incubation [38], similar to what was observed in this study, with the exception of *Actinomycete* 3345, which showed faster growth than the others. Actinomycetes generally produce secondary metabolites after obtaining the stationary phase, which is in agreement with three of the five morphospecies of this work, and also, the production of secondary bioactive metabolites occurs in different time periods depending on the species, which also observed in this work [39]. The efficiency of antimicrobial activity decreased considerably after an incubation period, which may be due to the degradation of bioactive compounds [39], a fact that was observed only with *Streptomyces* 3098 and *Streptomyces* 3134.

CONCLUSION

Amazonian soil proved to be a rich source of actinomycetes, enabling the identification of genera already described as producers of antimicrobial compounds. The bioautography method showed that Streptomyces extracts generally contain an active substance, and that they are different from commercial antimicrobials produced by actinomycetes. However, other analyzes must be carried out in the future for the separation and structural elucidation of biomolecules. Therefore, actinomycetes from these areas can contribute to the discovery of new antibiotics and the fight against resistant pathogens.

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