Potential biodiversity and antifungal activities of Amazonian actinomycetes isolated from rhizosphere of Inga edulis plants

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Abstract

Actinobacteria are major producers of antibacterial and antifungal metabolites and are targets of increasing research in the search for substances of biotechnological interest, especially for use in medicine and agriculture. The Amazon is potentially rich in actinobacteria, in turn poorly studied. Thus, we prospected actinobacteria from the rhizosphere of the South America native plant *Inga edulis*, which produces edible fruits and is economically useful in the whole Amazon region. Among all 64 strains of actinobacteria isolated, 20 strains representing 16 morphogroups were subjected to morphological analysis, genotyping using the *16S rRNA* gene, and dual-culture assays against pathogenic fungi of important agricultural plants in the region that also cause annual losses of millions of dollars to world agriculture. Using GenBank-NCBI and EzBioCloud data, 90% of the strains were identified as *Streptomyces*and 10% as *Kitasatospora*, indicating at least 16 specific species and possibly new species. All strains showed antagonisms for two or more different phytopathogens as *Corysnepora cassiicola*, *Colletotrichum* sp., *Colletotrichum guaranicola*, *Pestalotiopsis* sp., and *Sclerotium coffeicola*. The strains *Streptomyces* spp. LaBMicrA B270 and B280 stood out mainly against phytopathogens of the guarana plant (*Paullinia cupana*), an important crop for the regional economy and one of the best sources of caffeine in the world, whose powder is used in the food industry and traditional medicine. Furthermore, the acetate/isopropanol extract from the 10-day LaBMicrA B280 cultured medium presents fungistatic or fungicidal for all phytopathogens tested with a minimum inhibitory concentration (MIC) of 125 µg/mL.

Introdution

In the last decade, prospection of new isolates of actinomycetes that produce natural products (NPs) has been carried out in virtually every ecosystem on the planet. The screening of strains isolated from these environments, mainly by paired culture methods and biological assays with extracts, have revealed many of them to be promising sources of NPs of biotechnological interest (Alkhalifah 2021; Chevrette et al. 2019; Elshafie and Camele 2022; Liu et al. 2019 (a); Pavan et al. 2018; Rego et al. 2019; Xie and Pathom-Aree 2021). These prospections are a result of the scarcity of medicinal and agricultural resources to stop the spread of multidrug-resistant human pathogens and plant pests that ravage plantations in all parts of the planet. These pathogens compromise agricultural production and are due to the large-scale use of biocides that are employed to meet the growing food needs in the 20th and 21st centuries (Butler et al. 2023; Djebaili et al. 2021; Oyedoh et al. 2023; Priyashantha and Tibpromma 2023; Saeed et al.2021; Strobel et al. 2004).

The cosmopolitanism of actinomycetes is strictly related to their essential ecological activities for the planet and other living beings, such as nitrogen fixation, recycling of organic matter, and the secretions of phytohormones, antibiotics, lytic enzymes and biocontrol agents of phytoprages, among others. These activities make them great allies of plants through endophytic-epiphytic relationships and as rhizobacteria (Liu et al. 2019 (a); Liu et al. 2019 (b); Liu et al. 2019 (c)). In fact, actinomycetes maintain symbiotic systems with plants, secreting protective antimicrobials against phytopathogens and hormones that induce plant growth and feeding on nutrients released by plants, which makes them strong candidates for biocontrol agents and natural fertilizers (Djebaili et al. 2021; Saeed et al.2021). The potential of these microorganisms against fungal phytopathogens, which have attracted the attention of many researchers seeking new antifungals and more sustainable methods for agriculture, should be highlighted (Oyedoh et al. 2023).

Among the microorganisms that produce NPs, actinomycetes stand out for being responsible for the production of 70% of the antimicrobials available to medicine and agriculture (Bérdy 2012; Sharma and Thakur 2020). The genus *Streptomyces* is the best known and largest producer of NPs, with about 7,500 of the more than 10,000 NPs assigned to the more than 200 genera of the phylum *Actinobacteria* (Barka et al. 2016; Bérdy 2005; Law et al. 2019). Other genera, among them *Micromonospora*, *Kitasatospora*, and *Nocardia*, however, are gaining more and more interest in the search for new NPs (Takahashi 2017; Yan et al. 2022), especially the antifungal compounds whose best-known class for this group of microorganisms is that of macrolides (Kim et al. 2012; Nara et al. 2017; Wang et al. 2017).

Regarding biodiversity, the largest currently recognized genera are *Streptomyces*, with approximately 700 species (Komaki 2023), *Micromonospora*, with 110 species (Yan et al. 2022), and *Kitasatospora*, with 23 species (Takahashi 2017). In general, phenotypically, these bacteria are gram-positive, have aerial mycelia or an absence of mycelia, have segments with cylindrical, spherical, and ovoid shapes and spore chains that are spiral (or partially) and linear (Shirling and Gottlieb 1972). Their genomes are rich in guanine-cytosine, with sizes ranging between 8 and 10 million base pairs (BP) of nucleotides, and hold dozens of clusters of biosynthetic genes (BGCs) related to the synthesis of NPs (Van Bergeijk et al. 2020). Whole genome sequencing and DNA-DNA hybridization are the most-used techniques for taxonomic definitions at the species level. In addition to the traditional *16S rRNA* marker, currently, other markers are used to increase reliability in taxonomic definitions in the absence of the total genome sequence, for example, *atpD, gyrB, recA, rpoB* and *trpB* (Komaki 2023).

In this perspective, we investigated the diversity of a sample of actinomycetes isolated from the rhizospheres of three individuals of Inga edulis, a legume belonging to the Fabaceae family which is of agroecological and economic importance for the Amazon region (Rollo etal 2020; Piato et al. 2021; Piato et al. 2022), present on the campus of the Federal University of Amazonas (UFAM), the largest urban forest fragment in the Amazon and, at 670 hectares, one of the largest in the world, which potentially houses an incalculable and almost unexplored microbial diversity. The antifungal potential of these actinomycetes against phytopathogens of regional and global interest was also evaluated, among which stand out phytopathogens of the guarana plant (Paullinia cupana), one of the largest commercial sources of caffeine, and Sclerotium coffeicola, a phytopathogen recognized worldwide for affecting hundreds of plants of agricultural importance. This study is part of a larger project that seeks to collect, preserve, identify, and understand the biodiversity and potential applications of microorganisms from the Amazon, particularly from the campus at UFAM.

Materials and methods

Soil sample collections

Three collections were carried out at different points on the UFAM campus (Point 1–3°05'19.4" S 59°58'00.5" W, Point 2–3°05'20.8" S 59°57'57.5" W and Point 3–3° 5'19.06"S 59°57'56.26"W) (Supplementary Information - SI1), in which there were individuals of *I. edulis* that were not the period of seedpod production. At each point, three rhizosphere samples were collected at distances of 40 cm from the collar of the plant and 20 cm below in piliferous regions of the roots. Sterilized materials were used in all sample collections: 50 mL Falcon™ tubes, metal spatulas and plastic bags to transport the samples. The samples were collected and taken to the Laboratory of Bioassays and Microorganisms of the Amazon (LaBMicrA) - UFAM, where they were stored at -20 ºC.

Isolation and purification of actinobacterium strains

All the manipulations of the microorganisms in this study were performed in a biological safety cabin (Filterflux® Class II A1). Twenty-four hours after the collections, 1 g of soil from a sample of each of the points was dissolved in 5 mL of sterilized distilled water containing 0.02% Tween-20. One mL of each suspension was subjected to serial decimal dilution in test tubes containing 9 mL of 0.8% saline. From the 5th, 4th and 3rd dilutions, 50 µL were spread in triplicate in 90 x 15 mm Petri dishes containing the oat-based culture medium (for 1 L: 10 g of oats, 14 g of agar, 10 g of malt, 4 g of yeast extract, and 4 g of glucose) and ISP2 culture medium (for 1 L: 10 g of starch, 14 g of agar, 10 g of malt, 4 g of yeast extract, and 4 g of glucose), to which 100 µg/mL of the antifungals nystatin and tetracycline were added. This was followed by incubation in a BOD chamber at 28 ± 2 ºC for a period of 30 days, during which the strains of actinomycetes that grew were isolated and purified.

2.3. Microculture and microscope analysis

The isolated and purified actinobacteria were subjected to microcultures (adapted from Shirling and Gottlieb 1972) and were stained using the Gram method (LB Laborclin®). In summary, the spores of the purified strains were sown in 40 x 15 mm Petri dishes containing their respective isolation media; 18 x 18 mm coverslips were tilted at 45º over the streaks. After 96 hours of incubation at 28 ± 2 ºC in a BOD chamber, the coverslips were removed and attached with plastic tape to 26 x 76 mm slides; then they were subjected to the Gram staining method. All the stained slides were observed under an optical microscope with the 100x objective (Carl Zeiss optical microscope, coupled to Zen AxioCamER - Zen lite 2012 photo documenter). For the electronic microscopy of the *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701) strains, culture-media discs were transferred to aluminum stubs and subjected to room temperature for dehydration. After drying, the stubs containing the culture discs were viewed using a scanning electron microscope (SEM) model JSM IT500HR (JEOL Ltd. Japan). The images were obtained at voltages of 10 and 15 kV.

Conservation, selection, and morphological description of the strains of actinobacteria

For the conservation, the strains of actinobacteria were cultured in a solid medium (as described in Section 2.2) for 10 days. Five fragments of each colony were preserved in triplicates in 1.5 mL microtubes containing 20% glycerol. All the microtubes were stored in a freezer at -80 ºC and are preserved in the LaBMicrA microorganism collection at the UFAM's Analytical Center. As selection criteria and for the definition of the group of biotechnological studies, the macromorphological aspects of isolates grown in ISP2 were observed. The strains were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the number AC1746C. After cultivation in ISP2 culture medium, the selected strains were described morphologically according to the manuals of Shirling and Gottlieb (1972). In the macromorphological aspects, we observed the patterns of the pigmentation of the pseudohyphae, dorsal-dishes and types of edges of the colonies – the colors of the pseudohyphae and dorsal dishes were determined using the ISCC/NBS color system (available at https://www.w3schools.com/colors/colors_nbs.asp).

Molecular characterization (DNA extraction, PCR, sequencing) and phylogenetic analysis

For DNA extraction, the selected strains of actinobacteria were cultured in 125 mL Erlenmeyer flasks containing 30 mL of potato, dextrose and yeast extract (PDY) medium (Souza et al. 2004), at 28 ± 2°C and 120 rpm, for 72 h. The extractions followed the protocol of the Zymo Research Fungal/Bacterial DNA MicroPrep™kit. The DNAs were quantified in a spectrophotometer (NanoDrop, Thermo Scientific) and their integrities were recorded using electrophoresis with 0.8% agarose gel. The polymerase chain reaction (PCR) was performed using 20 ng of DNA, the PCR kit Illustratm PuReTaq Ready-to-GoTM PCR beads (GE Healthcare) and the primers 27F AGAGTTTGATCMTGGCTCAG and 1492R CGGTTACCTTGTTACGACTT (Haygood & Davidson 1997), with a final volume of 25 µL, in a 96-well thermal cycler (Applied Biosystems) (Ebrahimi-Zarandi et al. 2021). The amplified product was visualized in electrophoresis with 1% agarose gel, stained with GelRed™, using the Invitrogen® 1 Kb Plus DNA Ladder base pair marker, and photo-documented using the L-Pix Touch Loccus® Photo-documentation system. Amplicons were purified with the enzyme ExoSAP-IT (GE Healthcare). The samples were sequenced using the Sanger method and the BigDye Terminator kit (Applied Biosystems).

The products of the partial sequencing of the *16S rRNA* gene were compared with the sequences retrieved from the GenBank databases (NCBI) using the BLASTn alignment (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) and EzBioCloud (https://www.ezbiocloud.net/). The alignment of the sequencing products was performed using CLUSTAL W (MEGA 11 (Molecular Evolutionary Genetics Analysis). Subsequently, a phylogenetic tree was constructed using the

evolutionary analysis by the maximum likelihood method using the neighbor-joining and BioNJ (Tamura-Nei, 1993) algorithms and the bootstrap test with a maximum of 1,000 replications ((MEGA 11, available at www.megasoftware.net) (Felsenstein 1985; Tamura et al. 2021). Accession numbers generated by NCBI are available in the Supplementary Information (SI2).

Phytopathogens selected to evaluate the antifungal potential of the actinobacteria

To evaluate the antifungal potential of the actinobacteria, five phytopathogens were chosen, which were kindly provided by the Laboratory of Microbiology and Phytopathology of the Faculty of Agricultural Sciences (FCA) at UFAM: *Colletotrichum* sp. (habanero-type pepper-ISO01) isolated from leaf tissue lesions of habanero-type pepper plants (*Capsicum chinense*); *C. guaranicola* (guarana plant-P01) and *Pestalotiopsis* sp. (guarana plant-3002R2) isolated from leaf tissue lesions of different guarana plants (*Paullinia cupana*); *Corynespora cassicola* (tomato plant-ISO079) isolated from leaf tissue lesions of tomato plants (*Solanum lycopersicum*); and *Sclerotium coffeicola* (Mango tree-M01) isolated from leaf tissue lesions of mango trees (*Mangifera indica*). These plants, especially guarana, are of economic importance to the Amazon region and to rest of the world.

Screening using the paired cultures method

After being reactivated in ISP2, the actinomycete strains were inoculated linearly, from one end to the other, passing through the center, in new Petri dishes containing ISP2, and incubated at 28 ± 2 ºC in a BOD chamber. Two days previously, the pathogen lines were inoculated in PDA (Souza et al. 2004). On the fifth day of growth of the pathogens and third of the actinomycetes, discs of agar of 5 mm in diameter containing the phytopathogens were removed from the culture in PDA and inoculated at one centimeter from the edges, perpendicularly in relation to the line containing the bacterial inocula, in the dishes with ISP2. At one centimeter from one of these inocula, a cavity was made by removing a 10 mm wide strip of the culture medium from one edge to the other, parallel to the bacterial line, in order to verify the possible production of antifungal volatile substances (Fig. 5). Similar control dishes were also made with ISP2, which contained only each phytopathogen at two opposite poles and separated by a 10 mm cavity. All paired cultures were made in triplicate and incubated for 10 days, in order to observe the possible inhibition of pathogens by the actinomycetes. All paired cultures were done in triplicate and incubated for 10 days to observe possible inhibition of pathogens by actinomycetes. For each test triplicate, the average of the zones of inhibition of mycelial growth of fungal phytopathogens and the standard deviation (SI3) were calculated. Edington's formula was applied to this average (I= [(CFC-CFT)/CFC] x 100, where: I = percentage of inhibition; CFC = fungal growth in control; CFT = fungal growth in treatment), and the GraphPad Prism program (version 8.0.1) was used to construct a heatmap graph with the percentage of mycelial inhibition.

Production curves of the extracts

The strains *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701), which were selected from the bioassay of paired cultures, were cultured for 96 h in Petri dishes with ISP2 and 1.5% microbiological agar, from which two fragments of 5 x 5 mm were inoculated in an Erlenmeyer flask of 250 mL containing 120 mL of ISP2, with a total of 36 flasks. The strains were grown at 120 rpm and 28 ± 2 ºC. During incubation, three Erlenmeyer flasks of each strain were collected at scheduled intervals, totaling 12 samples in triplicate at intervals of 12 h, 24 h, 36 h, 48 h, 72 h, 120 h, 168 h, 216 h, 264 h, 312 h, 360 h, and 408 h. At each interval, glucose consumption and pH were measured, and intra- and extracellular extracts were obtained, the masses of which were used for the construction of the production curves of the extracts and biological assays.

To obtain the extracts at each interval, the biomass and cultured medium of each flask were separated via centrifugation (Eppendorf® centrifuge) at 4,000 rpm for 15 min, and then the biomass was washed three times with 10 mL of distilled water. The biomasses were then vortexed and macerated for 24 h with 5 mL of 1:1 MeOH/AcOEt. The cultured medium was partitioned with successive volumes of 40, 30, and 30 mL of AcOEt/2-propanol 9:1, which were then pooled. After evaporation of the solvents, the extracts were weighed, dried in a desiccator with activated silica, and weighed again, until they reached a constant weight, in order to establish the average values of the triplicate of each point. After this, the three extracts from each point were collected, concentrated, weighed, and stored at 4 ºC until the day of the bioassays.

Assays of the extracts from the points of the curve against phytopathogens

Only the extracts collected from the cultured medium of each of the twelve points of the growth curve of the *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701) strains were tested against the target phytopathogens. To the flasks containing the dried and weighed extracts, 200 µL of DMSO were added and, after being dissolved, 800 µL of autoclaved distilled water were added. From these solutions, stock solutions were made by diluting aliquots corresponding to 20 mg of each dry extract in sterilized distilled water, reaching a final volume of 1 mL. As a positive control, 20 mg of nystatin was dissolved in 1 mL of water. Phytopathogens were reactivated in Petri dishes containing potato, dextrose, agar and yeast extract (PDAY) ulture medium (Souza et al. 2004) at 28 ± 2 ºC for 96 h, in a BOD chamber. The extract and control assays were performed in triplicate in cell culture plates with 12 wells, containing: test: 0.950 mL PDAY, 50 µL extract + phytopathogen; negative control: 1 mL PDAY + phytopathogen; positive control: 0.950 mL PDAY, 50 µL nystatin solution + phytopathogen. The solutions of the extract or control with the culture medium were homogenized at approximately 40 ºC. For the inoculation of the pathogens in the wells, discs (5 mm in diameter) of the culture in PDAY were used in each of them. The assays were observed for 10 days.

Production of the extracts and minimum inhibitory concentration assay

From the results of the growth curves and bioassays, new extracts of LaBMicrA B270 (OR724732) and B280 (OR724701) strains were prepared, which were cultivated for 10 days using cultivation and extract production methods similar to those described above (Section 2.8.). In the production of the extracts, the only difference was the lyophilization of the cultured medium after partition with AcOEt/2-propanol 9:1. The concentrated extracts and lyophilized material were stored at -18 ºC. To determine the minimum inhibitory concentration (MIC), the AcOEt/2-propanol 9:1 and MeOH/AcOEt 1:1 extracts and the aqueous lyophilized material were successively diluted to the concentrations of: 1,000, 500, 250, 125, 62.5, 31.25, 15.63 and 7.81 µg/mL. The extracts were tested with the phytopathogens in a similar way to what is described in the previous item.

Results

Morphological and genotypic diversity

In this study, a total of 64 actinomycetes were obtained, with P2 standing out as the highest contributor, yielding 30 isolates (46.9%). It was followed by P1, which accounted for 19 isolates (29.7%), and P3 with 15 isolates (23.4%). The observations of the macromorphology and the pigmentation of the culture media in the Petri dishes (front and back) allowed us to preliminarily group the 64 isolated actinobacteria into 16 morphogroups. The 20 strains selected, one from each smaller group and two from each of the larger group of morphologically similar individuals, and the respective macro- and micromorphological descriptions according to the criteria established in Section 2.4, were tabulated

according to the collection point; these being: seven from P1, ten from P2 and three from P3 (Table 1) and Supplementary Material (SM2).

Table 1

Phenotypic and genotypic characteristics of actinomycetes isolated from the rhizospheres of three individuals of *Inga edulis* from the campus of the Federal University of Amazonas.

Origins of the isolates	Isolated strains	Macromophology (colony)	Micromorphology (spore chain)	GenBank/NCBI	EzBioCloud
P1	*Streptomyces* sp. LaBMicrA B267	Aerial mycelium light gray; deep orange-yellow back; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*S. pulveraceus* NBRC 3855 (99.51%)	*S. silvae* For3 (99.34%)
	Streptomyces sp. LaBMicrA B270	Aerial mycelium light gray; back deep yellow-brown; edge rough	Long/flexible partially spiral and cylindrical segments	*S. bullii* C2 (96.41%)	*S. murinus* NBRC 12799 (98.36%)
				S. murinus NRRL B-2286 (96.41%)	
				S. lanatus ISP 5090 (96.41%)	*S. lanatus* NBRC 12787 (98.36%)
				S. fractus MV32 (96.41%)	*S. fractus* MV32 (98.36%)
				S. graminearus NBRC 15420 (96.41%)	*S. graminearus* NBRC 15420 (98.36%)
				S. griseofuscus NBRC 12870 (96.41%)	
	Streptomyces sp. LaBMicrA B271	Aerial mycelium light gray-brown; back bright yellow-orange; edge rough	Long/flexible partially spiralled and ovoid segments	*S. graminearus* NBRC 15420 (97.53%)	*S. murinus* NBRC 12799 (96.66%)
				S. murinus NBRC 14802 (97.53%)	
				S. griseofuscus NBRC 12870 (97.53%)	*S. graminearus* NBRC 15420 (96.66%)
				S. murinus NBRC 12799 (97.53%)	
	Streptomyces sp. LaBMicrA B272	Aerial mycelium light gray; back deep yellowish-brown; edge rough	Long/flexible partially spiralled, cylindrical and ovoid segments	*S. acidicola* K1PN6 (88.60%)	*S. sundarbansensis* MS1/7 (78.35%)
	Streptomyces sp. LaBMicrA B278	Aerial mycelium light gray-brown; back moderate brown; edge rough	Partially long/flexible spirals, with spherical and ovoid clusters and segments	*S. misionensis* JCM 4497 (93.78%)	*S. misionensis* DSM 40306 (94.51%)
	Streptomyces sp. LaBMicrA B280	Aerial mycelium yellowish-gray-brown; back deep yellow-orange; edge rough	Long/flexible partially spiralled, cylindrical following	*S. graminearus* NBRC 15420 (99.09%)	*S. graminearus* NBRC 15420 (98.83%)
					S. murinus NBRC 12799 (98.83%)

Origins of the isolates	Isolated strains	Macromophology (colony)	Micromorphology (spore chain)	GenBank/NCBI	EzBioCloud
	Kitasatospora sp. LaBMicrA B282	Aerial mycelium white; back deep brownish-yellow; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*K. acidiphila* MMS16-CNU292 (92.45%)	*K. acidiphila* MMS16-CNU292 (90.98%)
P2	*Streptomyces* sp. LaBMicrA B301	Aerial mycelium bluish-white; back moderate olive-brown; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*S. pulveraceus* NBRC 3855 (96.65%)	S. *durocortorensis* RHZ10 (96.93%)
	Streptomyces sp. LaBMicrA B304	Aerial mycelium light grey-brown; back deep yellowish-brown; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*S. pulveraceus* NBRC 3855 (97.68%)	*S. pulveraceus* LMG 20322 (98.70)
	Streptomyces sp. LaBMicrA B310	Aerial mycelium light grey-brown; back deep yellowish-brown; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*S. pulveraceus* NBRC 3855 (98.96%)	*S. durocortorensis* RHZ10 (99.07%)
	Kitasatospora sp. LaBMicrA B313	Aerial mycelium yellowish-gray; back light olive-brown; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*K. arboriphila* HKI 0189 (98.44%)	*K. arboriphila* HKI 0189 (98.70%)
				K. aureofaciens KACC 20180 (98.44%)	
	Streptomyces sp. LaBMicrA B314	Aerial mycelium white; back deep yellow-orange; edge rough	Partially spiral long/flexible long, spherical and ovoid segments	*S. olivaceus* NBRC 12805 (99.61%)	*S. olivaceus* NRRL B-3009 (99.74%)
	Streptomyces sp. LaBMicrA B317	Aerial mycelium pinkish-gray; back deep yellow-orange; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*S. jiujiangensis* JXJ 0074 (97.11%)	*S. barringtoniae* JA03 (96.79)
	Streptomyces sp. LaBMicrA B318	Aerial mycelium white; back deep yellow-orange; edge rough	Long/flexible partially spiral and cylindrical segments	*S. similanensis* KC-106 (95.10%)	*S. similanensis* KC-106 (95.45%)
	Streptomyces sp. LaBMicrA B319	Aerial mycelium light bluish-gray; back deep yellowish-brown; edge rough	Long/flexible, short/compact partially spiral and cylindrical segments	*S. acrimycini* NBRC 12736 (97.93%)	*S. anandii* NRRL B-3590 (97.72%)
				S. roietensis WES2 (97.93%)	*S. pimonensis* TRM75549 (97.72%)
	Streptomyces sp. LaBMicrA B322	Aerial mycelium light grayish-brown; back deep yellowish-brown; edge rough	Long/flexible partially spiralled, spherical and ovoid segments	*S. enissocaesilis* NRRL B-16365 (96.76%)	*S. rochei* NRRL B-2410 (100%)
				S. rochei NRRL B-1559 (96.76%)	
				S. vinaceusdrappus NBRC 13099 (96.76%)	

Origins of the isolates	Isolated strains	Macromophology (colony)	Micromorphology (spore chain)	GenBank/NCBI	EzBioCloud
				S. plicatus NBRC 13071 (96.76%)	
				S. vinaceusdrappus NRRL 2363 (96.76%)	
	Streptomyces sp. LaBMicrA B325	Aerial mycelium vivid yellowish-pink; back deep orange; rough edge	Long/flexible partially spiral and cylindrical segments	*S. katrae* NRRL B-3093 (99.19%)	*S. katrae* NRRL ISP 5550 (97.66%)
				S. racemochromogenes NBRC 12906 (99.19%)	
				S. polychromogenes NRRL B-3032 (99.19%)	*S. racemochromogenes* NRRL B-5430 (97.66%)
				S. polychromogenes NBRC 13072 (99.19%)	
P3	*Streptomyces* sp. LaBMicrA B289	Aerial mycelium white; back deep orangey-yellow; rough edge	Long/flexible partially spiral and cylindrical segments	*S. sennicomposti* RCPT1-4 (99.16%)	*S. venetus* CMUAB225 (82.22%)
	Streptomyces sp. LaBMicrA B290	Aerial mycelium brownish-white; back bright yellow-orange; rough edge	Partially long/flexible spirals, with bunches, spherical and ovoid segment	*S. graminearus* NBRC 15420 (99.09%)	*S. murinus* NBRC 12799 (99.22%)
					S. graminearus NBRC 15420 (99.22%)
	Streptomyces sp. LaBMicrA B299	Aerial mycelium moderate brown; back bright yellow-orange; rough edge	Long/flexible partially spiralled, spherical and ovoid segments	*S. jiujiangensis* strain JXJ 0074 (87.36%)	*S. panaciradicis* 1MR8 (92.88%)

Molecular identification based on the 16S region revealed that 18 strains belong to the genus *Streptomyces* (90%), while two belong to the genus *Kitasatospora* (10%) (Table 1). Additionally, several strains showed identities exceeding 99% with a single type strain in one or both of the databases employed: Streptomyces spp. LaBMicrA B280, B289, and B290 in GenBank-NCBI; *Streptomyces* spp. LaBMicrA B310 and B322 in EzBioCloud; and *Streptomyces* spp. LaBMicrA B267 and B314 in both databases. Among them, *Streptomyces* sp. LaBMicrA B314 was the only one to share the same suggested species, *S. olivaceus* (NBRC 12805, 99.61% in GenBank-NCBI and NRRL B-3009, 99.74% in EzBioCloud). Additionally, four other strains, all with identities above 90% and below 99%, had a single suggested species in both databases: *Streptomyces* spp. LaBMicrA B278 (*S. misionensis*), B304 *(S. pulveraceus*), and B318 (*S. similanensis*), and *Kitasatospora* sp. LaBMicrA B282 (*K. acidiphila*). Furthermore, three strains exhibited identities below 90% in at least one database: *Streptomyces* spp. LaBMicrA B272 (in both), B289 (EzBioCloud), and B299 (GenBank). Eight strains, six of *Streptomyces* and two of *Kitasatospora*, presented more than one suggestion of species with equal weight: four in both databases, two in GenBank and two in EzBioCloud. More than 50% of the 20 strains present similarity values lower than 98% in both databases (Table 1).

Phylogeny

In the phylogenetic analysis using the 20 strains of this study and 28 type species, foram formados pelo menos quatro grupos considering only the values of the nodes from 50% of the bootstrap (Fig. 1). Among the groups formed, one is exclusively composed of strains of *Kitasatospora*, including the two in this study. Lineages B272, B301 and B267 are phylogenetically related to *S. acidicola* and *S. pulveraceus* and form a small group. The largest group formed contains 12 lineages, some of them positioned in subgroups with high bootstrap support such as B314 and B299, which is closely related to *S. olivaceus* and B317, B318 with *S. jiujianguensis*. Based on the 16S region, it was not possible to indicate a phylogenetic proximity relationship for most lineages given the low resolving power of this barcode in differentiating species close to Streptomyces.

Antifungal activities of the 20 strains against phytopathogens

In the screening using the paired cultures method, all the 20 strains showed antagonism against the phytopathogens *Corysnepora cassiicola* (ISO079) and *Colletotrichum* sp. (ISO01) (Fig. 2). Thirteen were active against *Pestalotiopsis* sp. (3002R2), the same amount was antagonistic against *Colletotrichum guaranicola* (P01). Only the strains *Streptomyces* sp. LaBMicrA B267, B280, and B301 showed antagonism against *S. coffeicola* (M01) and were also the only ones active against the five phytopathogens. The strains *Streptomyces* spp. LaBMicrA B270 and B280 (Fig. 3-A) stood out for their strong antagonism against *Pestalotiopsis* sp. (3002R2) and *Colletotrichum guaranicola* (P01), respectively, both phytopathogens of the guarana plant (*P. cupana*) (Fig. 3-B). No consistent signs of volatile metabolite activities of any actinobacterium against the phytopathogens were observed.

Production curves of the extracts and evolution of antifungal activities of the strains Streptomyces sp. LaBMicrA B270 and B280

The strains *Streptomyces* spp. LaBMicrA B270 and B280, selected due to their promising results in the previous tests, presented production curves of intra- and extracellular extracts that were compatible with the traditional bacterial growth curves (Fig. 4). Behavior that was also compatible was the glucose concentration that reached a level close to zero coincident with the beginning of the period of maximum production of the extracts. Interestingly, the behavior of the pH was quite distinct for the two strains: the first reaching a maximum of eight in this period and only falling again in the period of decline, while the second maintained pH 6.5 during all the stages. The decline phases in both curves began between 240 and 288 h of culture (10 and 12 days). The extract yields in the ISP2 medium cultured on the tenth day were respectively 542 and 720 mg/L for *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701).

In the assays against the five phytopathogens, the AcOEt/2-propanol 9:1 fractions of the cultured medium of the two strains from each point sampled showed antifungal activities after 36 hours of growth (Point 3), with the exception that the strain LaBMicrA B270 was confirmed as not being active against *S. coffeicola* (M01). The activities were confirmed for the other sampling points, until the beginning of the decline phases, which were observed in the respective production curves of the extracts.

Minimum inhibitory dosage assays

In minimum inhibitory dosage assays of extracts with 10 days of growth of the strains *Streptomyces* spp. LaBMicrA B270 and B280, the best results were obtained with the acetate/isopropanol fractions of the partition of the cultured media (Table 2). Confirming the previous assays, B280 showed activity against all the phytopathogens and B270 did not show activity for *S. coffeicola* (M01). Except in this case, the acetate/isopropanol fractions showed fungicidal activity at the minimum dosages of 250 or 125 µg/mL and, only below that, did they become fungistatic or inactive. In turn, the extracts of the bacterial biomasses in acetate/methanol showed much weaker activity and to only two or three strains. Finally, no activity was observed in the aqueous fractions of the cultured medium after partitioning with acetate/isopropanol.

Table 2
Minimum inhibitory dosage of extracts of the strains *Streptomyces* sp. LaBMicrA B270 and B280.

Strains tested	Concentrations and activities					Phytopathogens
	1,000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL	062 µg/mL	
	Acetate /isopropanol fraction (culture medium)					
Streptomyces sp. LaBMicrA B270	+	+	+	+	±	*Colletotrichum* sp. (ISO01)
	+	+	+	±	–	*C. guaranicola* (P01)
	+	+	+	±	–	*Corysnepora cassiicola* (ISO079)
	+	+	+	+	±	*Pestalotiopsis* sp. (3002R2)
	–	–	–	–	–	*Sclerotium coffeicola* (M01)
Streptomyces sp. LaBMicrA B280	+	+	+	+	±	*Colletotrichum* sp. (ISO01)
	+	+	+	+	±	*C. guaranicola* (P01)
	+	+	+	+	±	*Corysnepora cassiicola* (ISO079)
	+	+	+	+	±	*Pestalotiopsis* sp. (3002R2)
	+	+	+	±	–	*Sclerotium coffeicola* (M01)
Acetate/methanol fraction (cell biomass)						
Streptomyces sp. LaBMicrA B270	–	–	–	–	–	*Colletotrichum* sp. (ISO01)
	–	–	–	–	–	*C. guaranicola* (P01)
	+	+	±	–	–	*Corysnepora cassiicola* (ISO079)
	+	±	–	–	–	*Pestalotiopsis* sp. (3002R2)
	–	–	–	–	–	*Sclerotium coffeicola* (M01)
Streptomyces sp. LaBMicrA B280	+	+	±	–	–	*Colletotrichum* sp. (ISO01)
	+	±	–	–	–	*C. guaranicola* (P01)
	+	±	–	–	–	*Corysnepora cassiicola* (ISO079)
	–	–	–	–	–	*Pestalotiopsis* sp. (3002R2)
	–	–	–	–	–	*Sclerotium coffeicola* (M01)

Discussion

I. edulis has been presenting itself as a relevant agroecological vector in the cultivation of vegetables of economic interest, for example, Coffea canephora var. Robusta (robusta coffee), Theobroma cacao and Terminalia amazonia (Nichols and Carpenter, 2006; Piato et al. 2021; Piato et al. 2022; Sobanski and Marques, 2014; Iglesias et al. 2011). The success of a plant in any ecosystem depends, mainly, their relationships with microorganisms – which can be beneficial or harmful (Elshafie and Camele, 2022; Van Bergeijk et al. 2020). In this study, a small sample of actinomycetes isolated from the rhizospheres of three individuals of *I. edulis* present in the campus of UFAM, which is the largest urban fragment of the Amazon rainforest, was evaluated for its antifungal potential. The initial macromorphological analysis that resulted in 16 groups in the universe of the 64 strains of actinomycetes collected. Similarly, although the sequences of the *16S rRNA* gene revealed a low diversity of genera, only *Streptomyces* (90%) and *Kitasatospora* (10%), the diversity of species seems to be very high, if one considers the suggestions of the GenBank-NCBI and EzBioCloud databases: seven different species for the eight strains with sequences over 99% similar to at least one of these databases and nine species different from each other and from the previous ones for the 12 strains with similarities below 99%. Thus, only four strains had suggestions of species already suggested for others 20 sequenced strains. Considering that these 20 strains are representatives of the 16 groups of the 64 strains, there is the possibility of at least one different species for every four strains, which should be confirmed in the future by complete sequencing of the genomic DNA.

The phylogenetic analysis not only confirms the diversity of the strains of this study, among them and also in relation to those deposited in GenBank, but also reveals cases of high phylogenetic distance, mainly represented by the five strains with 50% or less possibility of clustering. Conversely, four other strains had high phylogenetic indices among themselves or with strains from this database, which reveals the high degree of preservation of the 16S rRNA gene inherited from their common ancestors. Despite the 16S rRNA marker (27F and 1492R (Haygood & Davidson (1997)) not being the gold standard in the taxonomic identification of this group of microorganisms, the possibility of new species can be considered due to this gene having the most evolutionarily conserved region among bacteria (Komaki 2023; Takahashi 2017). Thus, this possibility is greater among the more than 50% of the strains with similarities below 98% to those of GenBank and EzBioCloud and especially among the three with similarity below 90% in at least one of these databases.

Added to the rich biodiversity in this sample of actinomycetes is the potential for their application, which is particularly observed in the antifungal assays of the 20 strains against phytopathogens of agricultural importance, namely *Colletotrichum* sp. ISO01, *C. guaranicola* P01, *Pestalotiopsis* sp. 3002R2, *Corynespora cassiicola* ISO079 and *Sclerotium coffeicola* M01. These phytopathogens are reported to cause problems that generate millions of dollars in losses for agriculture. *Corynespora cassiicola* is widely cited as a disease-causing agent in more than 530 plant species of 380 different genera (Ma et al. 2018) and is a mycosis-causing infectious agent in humans working with vegetable crops (Huang et al. 2010). According to O'Connell et al. (2012), the genus *Colletotrichum* is responsible for diseases in more than 3,200 plant species. The genus *Pestalotiopsis* harbors several species of biotechnological interest (Maharachchikumbura et al. 2011; Sun et al. 2014), but some species are reported to be phytopathogens (Deng et al. 2013; Liotti et al. 2018; Marimuthu et al. 2020). *S. coffeicola* has a high impact on agricultural production and is responsible for lesions in Indian mulberry (*Morinda citrifolia* L.) leaves (Gasparotto et al. 2019) and mango trees (*Mangifera* sp.), which are important plants for traditional medicine and in the food and cosmetic industries (Gupta et al. 2022; Ribeiro and Schieber 2010).

The antagonistic results achieved in the *in vitro* assays, which are a prerequisite in methodologies that are currently under development within a more sustainable agricultural model (Oyedoh et al. 2023), confirm the broad capacity of *Streptomyces* species to fight other microorganisms, particularly fungi, and indicate that all 20 strains can be sources of antifungal substances. The production curve assays and concomitant evolution of antifungal activities of periodic extracts from the strains *Streptomyces* spp. LaBMicrA B270 and B280, as well as the minimum inhibitory dosage tests,

ratified their potential and suggest highly effective antifungal substances, considering their probable low concentration in these extracts. Chemical studies are being conducted to reveal the active ingredients of the extracts of these strains, which potentially have a broad antifungal spectrum and are strong candidates to combat the phytopathogens *Colletotrichum guaranicola* (P01) and *Pestalotiopsis* sp. (3002R2), which threaten the guarana plant (*P. cupana*), a very important crop in the economy of Amazonian communities. It is one of the best sources of caffeine in the world, and the powdered form is used in the production of soft drinks, as a food supplement and in traditional medicine (Marques et al. 2019). In addition, B280 was also active against *Sclerotium coffeicola* M01, a phytopathogen that is recognized worldwide for affecting hundreds of plants of agricultural importance.

The development of less harmful, more ecologically correct, and more sustainable methods for agriculture depends on the availability of microorganisms that promote biocontrol and fertilization. For example: Cao et al. (2020) demonstrated the biocontrol activity of a consortium of actinomycetes prevalent in the endosphere of healthy cucumber plants against *Fusarium oxysporum* f. sp. *cucumerinum* that causes wilt - the strain *Streptomyces* sp. NBRC 100767 showed the strongest activity; Ge et al. (2023) report the strain *Streptomyces* sp. JKTJ-3 with a biocontrol spectrum capable of inhibiting 12 phytopathogens - including *Pythium aphanidermatum* responsible for damping-off watermelon seedlings; Wang et al (2023), studying a consortium of actinomycetes isolated from insect intestines, discovered promising strains for the biocontrol of *Bipolaris maydis*, which causes rust on wheat leaves - *Streptomyces* sp. SN5431 was the most successful in biological assays with extracts; and Mattei et al. (2022) report that wheat seeds reverted with spores of *Streptomyces* sp. DEF39 produced 49% of wheat stalks with resistance against *Fusarium graminearum*. These studies also report significant improvements in plant products inoculated with actinomycetes, namely: improved mass and juiciness. Actinomycetes are cited in several studies as being excellent fertilizers, nitrogen fixers, and producers of various lytic enzymes, phytohormones and antimicrobials; as well as siderophores (Liotti et al. 2019; Liu et al. 2019 (a); Liu et al. 2019 (b); Strobel et al. 2004; Oyedoh et al. 2023).

In this context, according to the results obtained in the present work, actinomycetes from the rhizosphere of *I. edulis* represent a viable alternative for sustainable agriculture. Furthermore, the qualities mentioned above for actinomycetes and the results in this work may be related to the success of *I. edulis* as an ecological vector, which is used in South America in mixed crops and forest management that favor plants of economic importance (Nichols and Carpenter 2006; Piato et al. 2021; Piato et al. 2022; Sobanski and Marques 2014; Iglesias et al. 2011). It is possible that this success is due, in part, to rhizotopic actinomycetes transported via seeds or seedlings from one soil to another and that I. *edulis* plants, when growing, also spread them through the litter that they commonly generate in abundance (AbdElgawad et al. 2020; Hardoim et al. 2015; Jayasinghe andParkinson, 2008; Shahzad et al. 2018). This possibility, however, needs to be investigated.

Conclusion

In this study, it was revealed that the rhizospheres of three individuals of *I. edulis* harbor a rich diversity of actinomycetes with great potential against phytopathogenic fungi that commonly ravage plantations of numerous crops of global economic importance. Twenty strains showed antifungal activities against at least two phytopathogens, especially the two strains *Streptomyces* spp. LaBMicrA B270 (OR724732) and B280 (OR724701), whose extracts showed fungicidal activities at low concentrations, revealing them to be sources of metabolites with potent antifungal activities. These results are an encouraging example of the richness of actinomycetes that are associated with *I. edulis* in the Amazon, and they should be explored as potential producers of bioactive metabolites, especially against phytopathogenic fungi, and for the development of microbial products to be used in agriculture around the world. Moreover, the possibility of new species was signaled by the genotyping results, and these will be subsequently investigated. This study is thus further evidence of the vast biodiversity in the Amazon and reinforces the need to preserve it.

Declarations

Data availability

Data are contained in the article or Supplementary Material MS 1.

Interest conflicts

The authors declare no conflict of interest.

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Figures

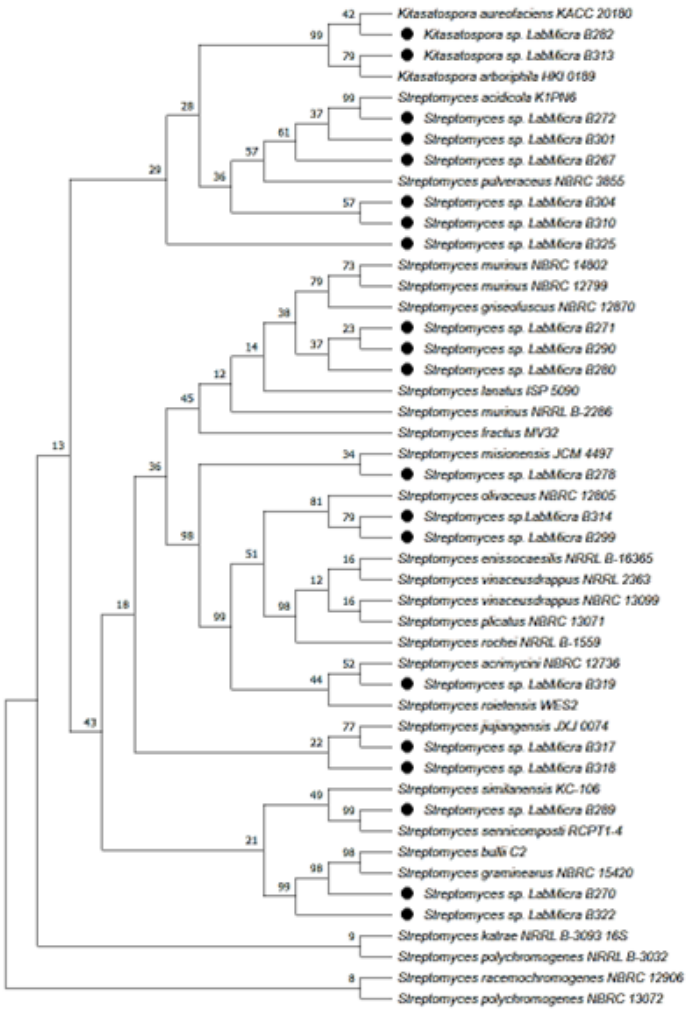

Figure 1

Phylogenetic tree of partial sequences of the *16S rRNA* by the Maximum Likelihood of actinobacteria from the rhizospheres of three individuals of *Inga edulis* (green rhombus) and type Species. Bootstrap values ≥ 50% only, with a maximum of 1,000 randomizations and 0.05 substitutions per nucleotide position (bootstrap method).

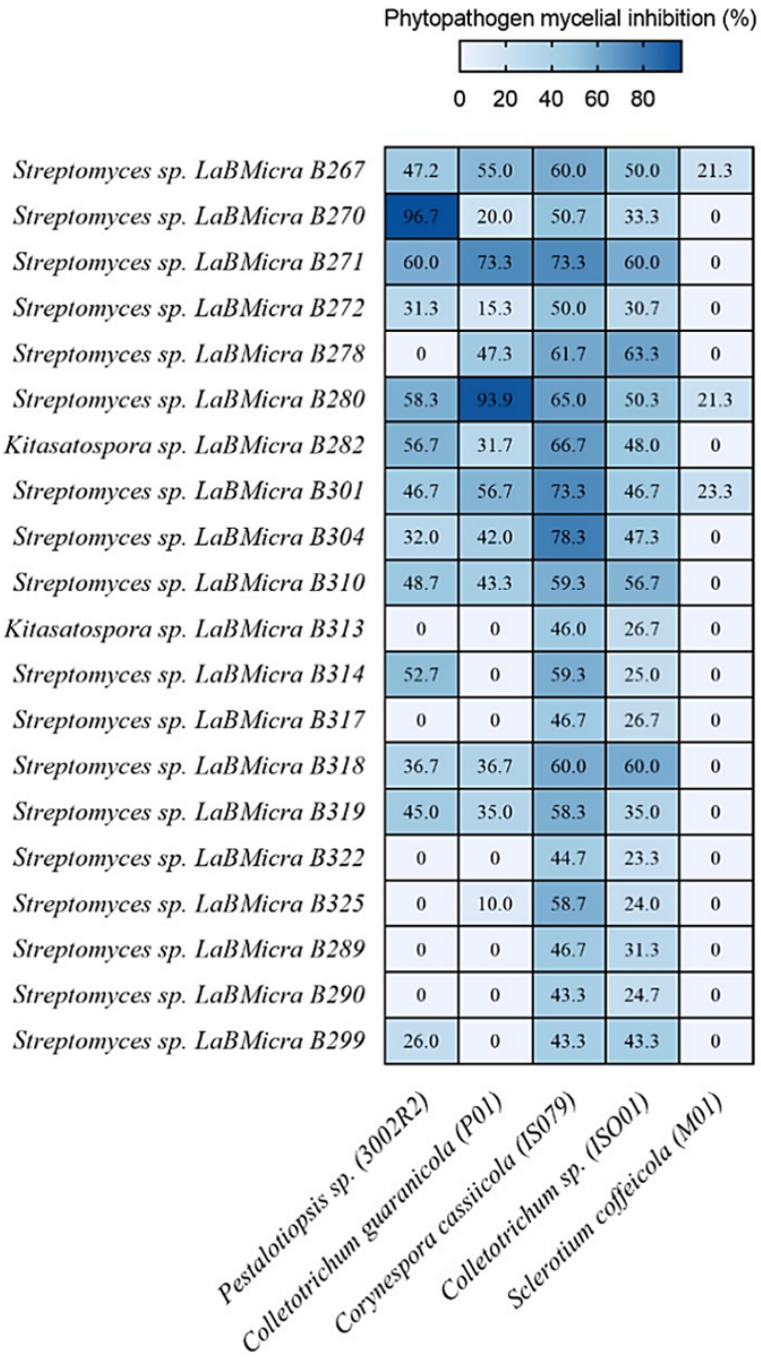

Figure 2

Heatmap graph showing the average percentage values of inhibition zones of mycelial growth of phytopathogens by actinomycetes (GraphPad Prism version 8.0.1).

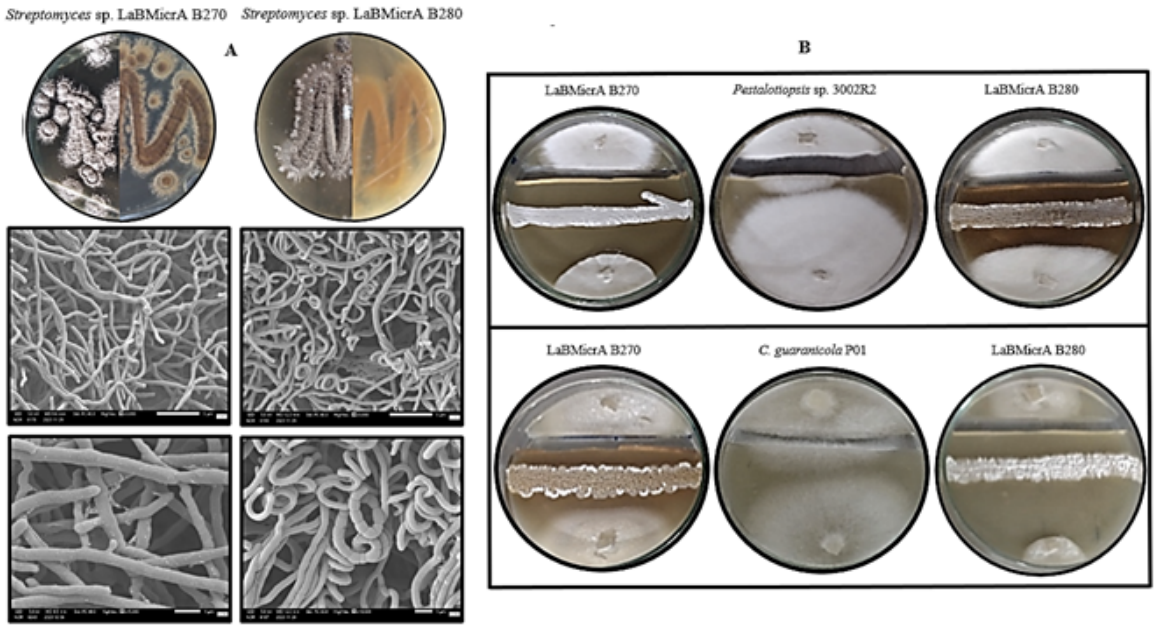

Figure 3

(A) Macro and micromorphological characteristics of strains LaBMicrA B270 and B280. Above: front and back of colonies on the twentieth day of cultivation; Below: mycelial ornamentations - scanning electron microscope (SEM) images. (B) Paired cultures of the strains LaBMicrA B270 and B280 (centered) with the phytopathogens Pestalotiopsis sp. (3002R2) and Colletotrichum guaranicola (P01) (extremities). In the middle: the control containing only the phytopathogens after the tenth day of culture.

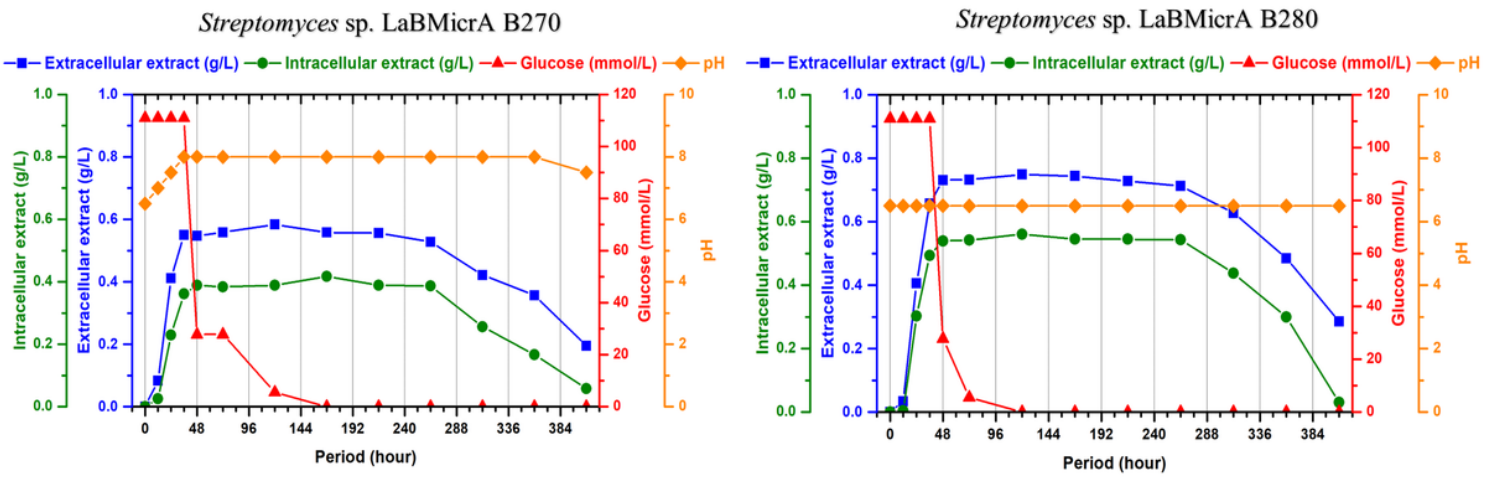

Figure 4

Yield curves of metabolic extracts of the strains selected for biological studies.

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