Study of the antifungal potential of actinomycetes strains against the pathogen *Aspergillus fumigatus*

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Introduction

Antimicrobial resistance is one of the major health and social emergencies our society is currently facing. However, resistance to antibiotics against bacterial pathogens has overshadowed other resistances, such as resistance to antifungal agents [1] In recent years, drug-resistant fungi have emerged including resistance to azole antifungals. *Aspergillus fumigatus* causes invasive pulmonary aspergillosis, one of the deadliest lung infections with a high mortality rate, particularly among patients who are infected with azole-resistant strains [2]. Therefore, the discovery of new antimicrobial compounds and the improvement of their production is a priority [3].

One of the natural sources of antimicrobial compounds is the group of actinomycetes. Within this group, the genus *Streptomyces* stands out for the production of bioactive compounds. These bacteria have large genomes in which there may be 25-70 biosynthetic gene clusters (BGCs) [4]. These clusters are responsible for their high production of secondary metabolites. Many of these clusters are cryptic, so they could produce novel compounds with antimicrobial potential.

Objectives

1. Search for actinomycetes strains with antimicrobial potential in natural sources.
2. Identification of biosynthetic gene clusters (BGCs) with antifungal potential from the sequenced genome of the strains of interest.
3. Study of the antifungal potential of *Streptomyces* strains against the pathogenic fungus *Aspergillus fumigatus*.

Materials and methods

1. **Isolation of potentially antimicrobial-producing strains of actinomycetes from natural sources.** Through citizen science projects such as Tiny Earth [5] and other natural sources (compost, alkaline soils, trees, insects, etc.) we isolated a few strains of actinomycetes, mainly from the genus *Streptomyces* [6, 7], that produced antimicrobial compounds.

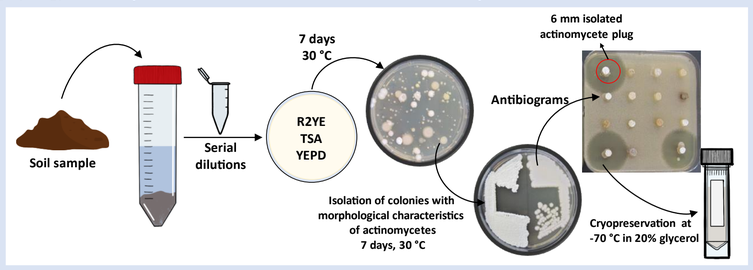

Figure 1. Method for isolating potentially antimicrobial producing strains of actinomycetes from natural sources. Antibiograms were performed against several control microorganisms: *Escherichia coli*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Saccharomyces cerevisiae.*

For the present work, we have used two *Streptomyces* strains from our isolate collections: ***Streptomyces syringium*** and ***Streptomyces kitasatoensis***.

2. **Identification of biosynthetic gene clusters (BGCs) with AntiSMASH and genome annotation.** We annotated *S. syringium* genoma with Prokka [8]. We performed a bioinformatics study with AntiSMASH 7.0 [9] on the annotated genome in order to analyse the presence of BGCs.

3. **Study of the effect of culture extracts from *Streptomyces kitasatoensis* and *Streptomyces syringium* against *Aspergillus fumigatus*.** The first step was to optimise the culture medium. For this purpose, we tested eight media: HT, ISP2, PDA, R2YE, R5, SFM, TBO and YEPD. An antibiogram against *Aspergillus fumigatus* AF14 (wild type strain) [10] was carried out.

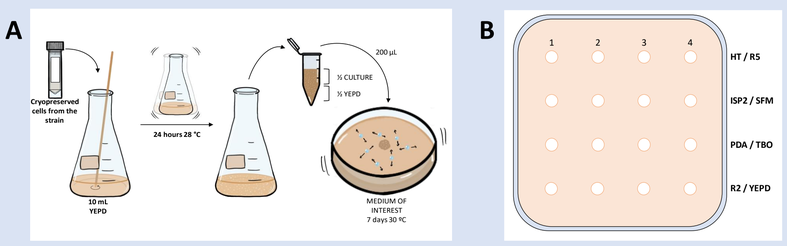

Figure 2. A. Protocol for inoculation of our *Streptomyces* strains. **B**. Antibiogram against *Aspergillus fumigatus*. 10^6 spores of *A. fumigatus* were inoculated into 70 mL of AMM medium and poured into a 12 cm square Petri dish. 6 mm plugs of test cultures *Streptomyces* strains are placed onto the medium

Extractions were carried out from the medium with the highest antifungal production and tested against *Aspergillus fumigatus*. First, we selected the best solvent to extract the compounds of interest among different solvents (water, acidified ethyl acetate and methanol). Then, we analysed whether the compound was inside the cells or diffused into the medium. For this purpose, the strain of interest was inoculated onto sterile cellophane membranes placed on the top of plates of the selected medium. Extraction was performed according to the protocol.

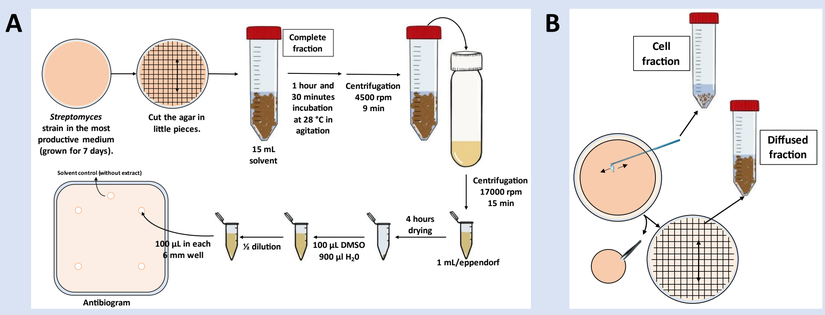

Figure 3. Protocol for the extraction of active compounds from *Streptomyces* strains. A. Normal extraction. **B**. Extraction from sterile cellophane membrane plates. The cells are harvested from the membrane into a tube and the agar is cut into little pieces into another tube. Once the fractions are separated, the same protocol as for normal extraction is followed. Extraction was performed with methanol.

Results

1. **Isolation of potentially antimicrobial-producing strains of actinomycetes from natural sources.** Twenty-six actinomycetes with antimicrobial potential were isolated from the various studies carried out during the period of the thesis.

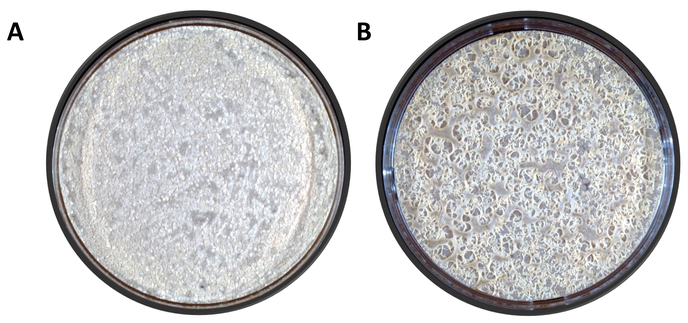

Figure 4. *Streptomyces* strains selected for the work.
A. Streptomyces syringium.
B. Streptomyces kitasatoensis.

2. **Identification of biosynthetic gene clusters (BGCs) with AntiSMASH and genome annotation.**

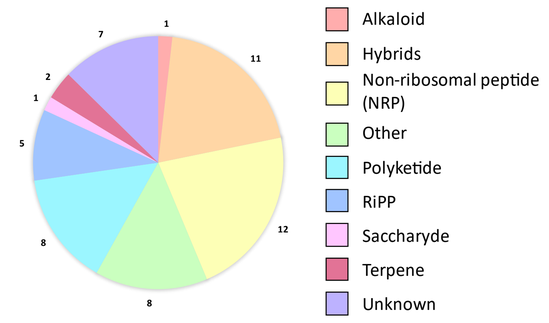

Figure 5. Bioinformatic analysis of *Streptomyces syringium* using AntiSMASH 7.0. The figure shows the types of BGCs corresponding to the clusters of secondary metabolites most similar to those predicted for the strain under study. For *S. syringium*, **55 BGCs were predicted**, of which 16 seem to correspond to putative antimicrobial compounds, **4 of them antifungal**, based on the literature consulted.

3. **Study of *Streptomyces kitasatoensis* and *Streptomyces syringium* against *Aspergillus fumigatus*.**

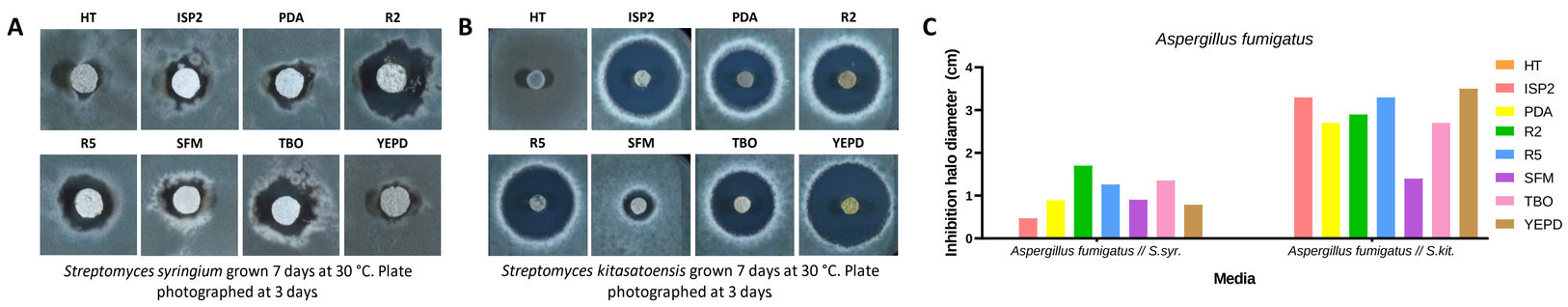

Figure 6. Determination of the optimal medium for the production of antifungal compounds against *Aspergillus fumigatus*. Figures **A** and **B** show the inhibition halo of each strain in each medium against the pathogenic fungi. Figure **C** shows a graphical representation of the inhibition halo measurement of both strains (*S. syringium* as *S. syr.* and *S. kitasatoensis* as *S. kit.*). The medium that induced the production of the most efficient antifungal activity in ***S. syringium*** was **R2**, whereas in ***S. kitasatoensis*** the higher antifungal activity was observed similarly in the media **ISP2**, **R5** and **YEPD**.

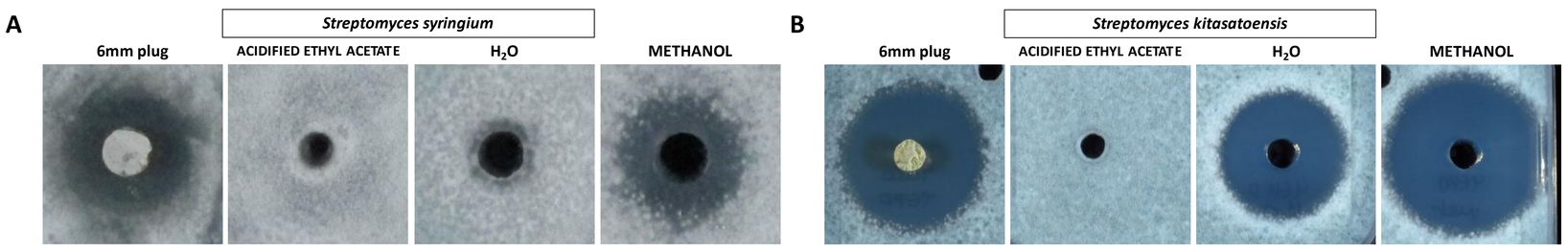

Figure 7. Selection of the best solvent for the extraction of active antifungal compounds. A. In the case of *S. syringium*, extraction was performed using R2 medium. **B**. For *S. kitasatoensis*, YEPD medium was used. The most suitable solvent for the extraction of active compounds in both strains was **methanol**.

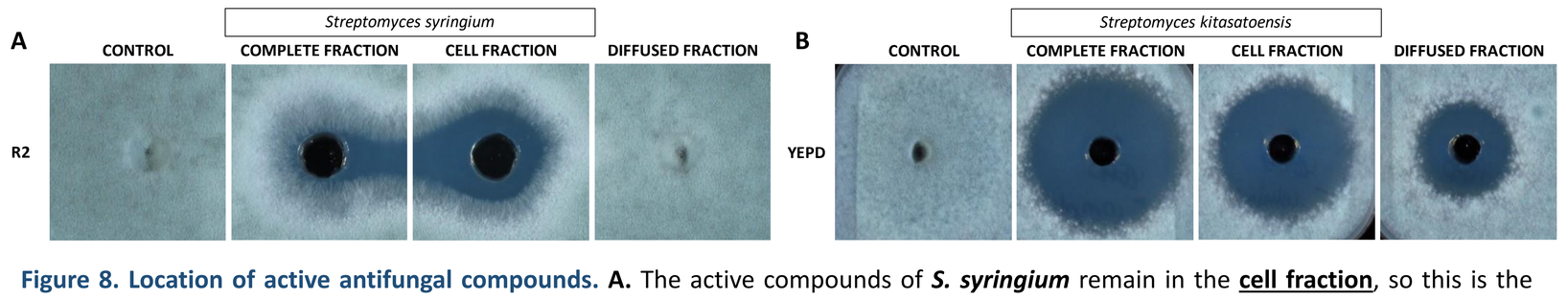

Figure 8. Location of active antifungal compounds. A. The active compounds of ***S. syringium*** remain in the **cell fraction**, so this is the fraction we will use for further studies. **B**. In ***S. kitasatoensis***, the antifungal activity was distributed in both the cellular and agar diffusion fractions. Therefore, for the following studies with this strain, the **complete fraction** will be used.

Conclusions

1. **Screening of soils, insects and other sources** is a valid approach for searching new antimicrobial compounds.
2. The bioinformatic approach using **Prokka** and **AntiSMASH** seems to be a good way to annotate and **identify BGCs of antifungal interest**.
3. Both *Streptomyces* strains show activity against *Aspergillus*, with ***S. kitasatoensis*** being the most prominent. It produces the highest activity in **ISP2**, **R5** and **YEPD** and in both cellular and diffused fractions.

References

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