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Assay Development for Isolation and Characterization of Anticancer Properties of Marine Fungi

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Abstract

Metabolites from fungi have potential use in the drug discovery process and have been used in the past to develop therapeutic agents for human use. Initial characterization of potential therapeutic properties of fungi is thus an important first step in identifying novel therapeutic compounds. In the present study, marine fungi were isolated from Myrtle Beach and assayed for anticancer properties using the soft agar colony formation assay in a 3D *in vitro* cellular environment. Many species were isolated from environmental samples, displaying unique morphologies and growth patterns. To optimize the soft agar assay in a six-well cell culture plate for incubation with the isolated fungal samples, a 3D printed tray was designed. This tray allowed for structural support of fungal growth media while allowing for layer separation after incubation for visualization of the cell-containing layer. Completion of this work was impacted by the COVID-19 pandemic, and further optimization of the protocol is required for its use.

Introduction

Metabolites from marine fungi have been found to contain antiviral, antibacterial, and anticancer properties that hold potential developmental insights for the drug discovery process[1]. Previously, fungi have been the source of important drugs such as penicillin (derived from *Penicillium* molds), and studies of naturally derived compounds from marine fungal samples have demonstrated potential for use as therapeutic agents[1–3]. Thus, characterization of marine fungi, their metabolites, and their biochemical mechanisms has potential to be a source of therapeutic agents for human and animal use.

A rich variety of marine fungal species can be found in and around oceans, beaches, sea foams, sea ices, mangroves, and other marine locations[4]. Even areas that are geographically close and topographically similar may vary greatly in their marine fungal species. A previous exploratory study from our lab examined the marine fungi found at two different sites in northeastern South Carolina. Myrtle Beach is a commercial beach which maintains high human traffic during its peak season in the summer, while Waties Island is a comparatively low-traffic beach used exclusively by Coastal Carolina University for marine research. Various fungal species were found in both locations and were identified using micro- and macroscopic observation in conjunction with DNA sequencing. Both sites yielded fungal species distinct from one another without identifiable overlap (unpublished data).

Analysis of the extracted fungi revealed *Aspergillus* sp. in samples from Myrtle Beach, but not in samples from Waties Island (unpublished data). The genus *Aspergillus* contains species of opportunistic human pathogens which can be responsible for invasive pulmonary infections that have a very high fatality rate in immunocompromised

patients[5,6]. However, compounds from *Aspergillus* sp. also hold therapeutic potential, and previous work has demonstrated their anticancer properties in the laboratory setting[1]. Because these human pathogenic fungi were found in the area of high human traffic and not in the area of low human traffic at two beaches in close proximity with comparable climates, it is possible that the presence of humans influences the biological composition of the marine mycobiome.

To find fungal species with anticancer properties, samples for the present study were taken from the Myrtle Beach site only in order to increase the chances of finding *Aspergillus* sp. or other human pathogenic fungi that may contain metabolites which act as anticancer agents. This work seeks to isolate and identify marine fungi and assess their effectiveness in managing the proliferation of human cancer cells in an *in vitro* 3D cellular environment.

Materials and Methods

Field Collection

In mid-January, environmental samples were collected from Myrtle Beach, a commercial beach in northeastern South Carolina (33°41'29.7"N 78°52'44.7"W). A metal hand auger was used to core the sand 58cm below the surface to collect the samples. Two cores were taken, one at the backshore (dry sand, 40ft from shoreline) and one at the foreshore (wet sand, 20ft from shoreline). Seawater was then collected by entering the water and sampling from approximately 50cm below the surface. Finally, two samples of seafoam were collected, one from foam on the surface of the water (wet foam) and one

from foam that had washed up on shore (dry foam). Samples were collected one hour before peak high tide.

Sample Purification

Samples were filtered using Nalgene vacuum filter units with a 0.45 μm clearance obtained from Fischer Scientific. 3g of each sand sample (backshore and foreshore) were suspended in 20mL sterile water and vortexed for 30 seconds. The dry seafoam sample was also suspended in 20mL sterile water and vortexed for 30 seconds. Once all dry samples were suspended in sterile water, they were poured onto the filter units and vacuumed. Both the seawater sample and the wet seafoam sample were poured directly onto the filter and vacuumed. Due to manufacturer error, the filter unit used for the wet seafoam sample lacked a filter membrane; a second filtration was performed with a new unit and both the original paper lacking a membrane and the second filter membrane were saved for use. The filter papers from all units were removed using tweezers and placed on fresh Sabouraud Dextrose Agar (SDA), a selective media commonly used for isolation of fungi and yeasts[8]. Samples were left to incubate at 27°C for 2-5 days until fungi had grown to cover the majority of the plate on and around the filter paper.

Plates were photographed following fungal growth and each unique species present was subcultured by streaking on a new SDA plate using a microbiological loop. Plates were again left to incubate at 27°C for 2-5 days. This process was repeated as necessary until all unique species were isolated.

DNA Extraction and PCR Amplification

DNA was extracted from six unique species for sequencing identification. DNA extraction was performed using the QIAamp© DNA extraction protocol (QIAGEN). Samples were frozen until use in a Polymerase Chain Reaction (PCR).

15µL master mix (Primers, RNase-free water, and Taq polymerase) was added to 5uL DNA sample and PCR was run according to the cycle in Table 1. Samples were stored in the freezer until they are able to be sent out for sequencing.

Cell Culture

Human rhabdomyosarcoma (RD) cells obtained from the American Type Culture Collection (ATCC) were cultured in a cell culture incubator at 37°C in plastic cell culture bottles with Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% fetal bovine serum (FBS) and antibiotics, according to standard protocol[9]. Cells were split into new plastic cell culture bottles approximately once a week, or after cells reached 100% confluence. Cell culture media was replaced with fresh media 1-2 times per week.

Soft Agar Colony Formation Assay

RD cells were suspended in an agarose gel enriched with DMEM in a 6-well cell culture plate according to the protocol by Horibata et al[7]. In this assay, cells are grown in a soft agarose gel matrix containing cell culture media. This assay contains several key properties which make it highly useful for *in vitro* cell proliferation studies. First, this assay measures the ability of cells to proliferate in the 3D environment of semi-solid matrices, which is more akin to the cellular environment that is seen *in vivo* as opposed to 2D cultivation that is commonly used in the conventional monolayer. Second, it allows for

ease of testing for the effects of novel compounds such as metabolites. Finally, this assay allows for quantitative assessment of cell proliferation through measurement of colony size.

A 3D-printed 6-well plate tray was designed for use in the present study (Dremel 3D45 printer). The tray was designed to provide structural support for the SDA growth media with direct contact between the fungal layer and the soft agar cell-containing layer. Direct contact between layers allowed for diffusion of fungal metabolites to the cell-containing layer. Perhaps the most important characteristic of the design was ease of separation between the fungal layer and the cell-containing layer. This characteristic was essential for use in the soft agar assay because after incubation with the potential anticancer agent, the cell-containing layer must be uncovered for microscopic visualization of cell colony size. The round tray had an open base which was able to be filled with SDA agar and placed inside a well on a 6-well plate (Figure 1).

After growing an isolated fungal species on an individual tray, it was placed on top of the soft agar cell layer along with a control tray containing SDA but no fungus. The plate was then left to incubate in a bacterial incubator at 37°C for one week. Following incubation, the fungal tray and control tray were removed from the cell layer, which was then visualized under the microscope.

Protein Extraction

The cell wall of the fungal samples was broken in order to extract the proteins and cellular material present within the samples. Fungal samples were picked from culture plates using a toothpick and were suspended in 2mL PBS in a 50mL tube. Samples were

sonicated on high for 3x 10 second pulses. This was repeated a second time after resting on ice for 6 minutes, resulting in a total sonication time of 1 minute. Samples were then centrifuged at 6,500 RPM for 15 minutes at 4°C and the supernatant was collected in a 15mL tube. Samples were stored in the fridge.

Results

Fungal Isolation

Fungal growth was visible on all plates following incubation of filtered environmental samples. All but one sample contained a filamentous mold which dominated most of the area of growth surrounding the filter paper, though the morphology of these dominant fungi differed between different samples. The sample isolated from the backshore appeared to contain four unique fungal species with one dominant white and grey filamentous fungus surrounding the filter paper and three additional species growing directly on top of the filter paper (Figure 2A). The sample isolated from the foreshore was dominated both around and on top of the filter paper by one fuzzy white fungal species with an additional green filamentous species in the center of the filter paper (Figure 2B). The sample collected from seawater appeared to contain at least seven morphologically unique fungi, including a black and grey filamentous species surrounding the filter paper and six small colonies on the filter paper (Figure 2C). The sample isolated from wet seafoam without a filter had the most rapid growth and contained at least six unique fungi, including a black and grey filamentous species surrounding the non-filter paper and five smaller colonies on the paper (Figure 2D). The sample isolated from wet seafoam after a

second round of filtration including the filter paper contained five distinct species, none of which appeared to be unique from the morphologies found on other plates (Figure 2E). Finally, the sample isolated from dry seafoam contained two filamentous fungi dominating the area surrounding the filter paper, one white and one black and grey. Three additional species were present on the filter paper (Figure 2F).

Several rounds of subculturing were performed in order to obtain single species isolates. Ultimately, six unique isolates were selected for identification. While other unique species were found, these species were selected for their visually distinct morphologies and ease of isolation into individual colonies. Species 1 was isolated from the backshore and had small, round colonies with a tan slimy appearance similar to that of bacterial colonies (Figure 3A). Species 2 was isolated from seawater and had white circular colonies which matured into asymmetric teal colonies after several days of growth (Figure 3B). Species 3 was isolated from the foreshore and had large green colonies with a long, fuzzy appearance (Figure 3C). Species 4, 5, and 6 were all isolated from the unfiltered wet seafoam sample. Species 4 had small white colonies with a fuzzy appearance which grew closely together (Figure 3D). Species 5 had larger white colonies which grew to dominate the plate in one large fuzzy mass if left to incubate for several days (Figure 3E). Species 6 had round, dome-shaped colonies which were black on the bottom and grey on the top with a felt-like texture (Figure 3F). DNA from each of these six isolates was obtained for future species determination via sequencing.

Soft Agar Colony Formation Assay

Cells suspended in soft agar were able to be visualized in multiple layers of the 3D environment following the soft agar protocol for a 6-well plate (Figure 4). Cell colonies

could be visualized under the microscope using both a fluorescent bulb and white light (Figures 4B, 4C).

A 3D printed tray for the 6-well cell culture plate on which fungi could be grown was designed specifically for use in this soft agar assay (Figure 1). When placed on top, the tray appeared to sink slightly into the soft agar cell-containing layer (Figure 5A). Following one week of incubation in a 37°C incubator, there was a change in media color in both the fungal and the control wells (Figure 5B). After removal of the trays, it was clear that the cell-containing and feeder layers of agar had shrunk in size (Figure 5C). Cell colonies were unable to be visualized microscopically after removal of the trays.

Discussion

Fungal isolation

Substantial fungal growth was seen from every environmental sample with multiple species present in each. Samples were collected in the winter, which one study has demonstrated is the time of year in which diversity of marine fungi is highest in temperate environments such as the east coast of the United States[10]. While only six species were chosen for identification and experimentation for initial development of this assay based on their distinct morphology and ease of isolation, many other unique species were found in the samples. Most of these were filamentous or fuzzy molds which could not be isolated into single colonies, but instead grew to blanket the plate even at low concentrations.

Once the assay has been optimized, a larger volume of samples can be tested and sequenced to develop a library of species present in the area at different locations

(backshore, foreshore, seafoam, etc.) at different times of year. DNA and bioinformatic analysis can be performed on fungal samples isolated in these different conditions in order to determine how closely related they are to one another and to marine fungal species which have previously demonstrated potential use as therapeutic agents.

Soft Agar Colony Formation Assay

The original soft agar colony formation assay from Horibata et al. was developed using the MCF10DCIS cell line from human breast cancer tissue[7]. Here, we show that the method is viable for other cancer cell types, namely human rhabdomyosarcoma (RD). These cells were able to proliferate in the 3D media and be visualized microscopically. Colony growth could be determined by directly measuring the size of the colony over time, showing that this method is effective for use in this setting.

Limitations and Future Work

A campus-wide evacuation due to the COVID-19 pandemic interrupted the completion of this work. Here, the current limitations and future plans for optimization which would have been carried out in the original timeline are outlined.

In the presence of the fungal layer, incubation of the soft agar assay could not take place in the cell culture incubator due to risk of contamination of other cell samples by the fungus. Because of this, the assay was incubated in a bacterial incubator at optimal temperature for cell proliferation. However, after one week in the incubator the soft agar layers were clearly desiccated, and the cells were unable to be visualized. It is likely that the interior of the bacterial incubator was too dry for proper incubation since the soft agar showed no sign of desiccation in the cell culture incubator before addition of the fungal

layer. Despite this initial setback, the assay should not be abandoned altogether. Future work in optimization of this method should include proper incubation in a cell culture incubator separate from other cell samples. If such an incubator is unavailable, another option would be the addition of a container sterilized water to the bacterial incubator to maintain the necessary moisture levels comparable to the cell culture incubator to prevent desiccation.

As an alternative to directly incubating the fungal layer with the cell layer, the proteins and metabolites of the fungal samples were extracted for use in the cell culture incubator without risk of contamination of other samples. Fungi were lysed via ultrasonication in order to extract the cellular contents. This extract was intended to be mixed directly with the soft agar cell-containing layer in order to measure its potential effects on cancer cell colony proliferation. The samples were successfully sonicated and prepped, but the experiment was unable to be performed prior to the evacuation of campus. When the project resumes, this assay must be optimized to determine the ideal ratio of fungal cell extract to RD cells. Once this is achieved, the cell colonies can be visualized and measured.

Conclusions

In this study, a diverse selection of marine fungal species was isolated when collected from one beach site. Many other species are likely present in this area and surrounding areas, representing a valuable source of biodiversity which has the potential to lead to discoveries of novel marine fungal compounds.

With further optimization, the soft agar assay will be a useful tool for future studies of the anticancer properties of marine fungi. Cell colonies demonstrated measurable growth which will allow for quantification of cancer proliferation. Without interruption from the pandemic, the project aim of optimizing this assay for use with marine fungi would have been completed in the given timeline. Optimization of this assay to screen for potential anticancer agents remains a current aim of this lab.

Cycle Step	**Temperature**	**Time**	**Number of Cycles**
Initial denaturation	95ºC	3 minutes	1
Denaturation	95ºC	30 seconds	
Annealing	55ºC	1 minute	30
Extension	72ºC	1 minute	
Final Extension	72ºC	10 minutes	1
Hold	10ºC	N/A	1

Table 1. PCR cycle for amplification of fungal DNA.

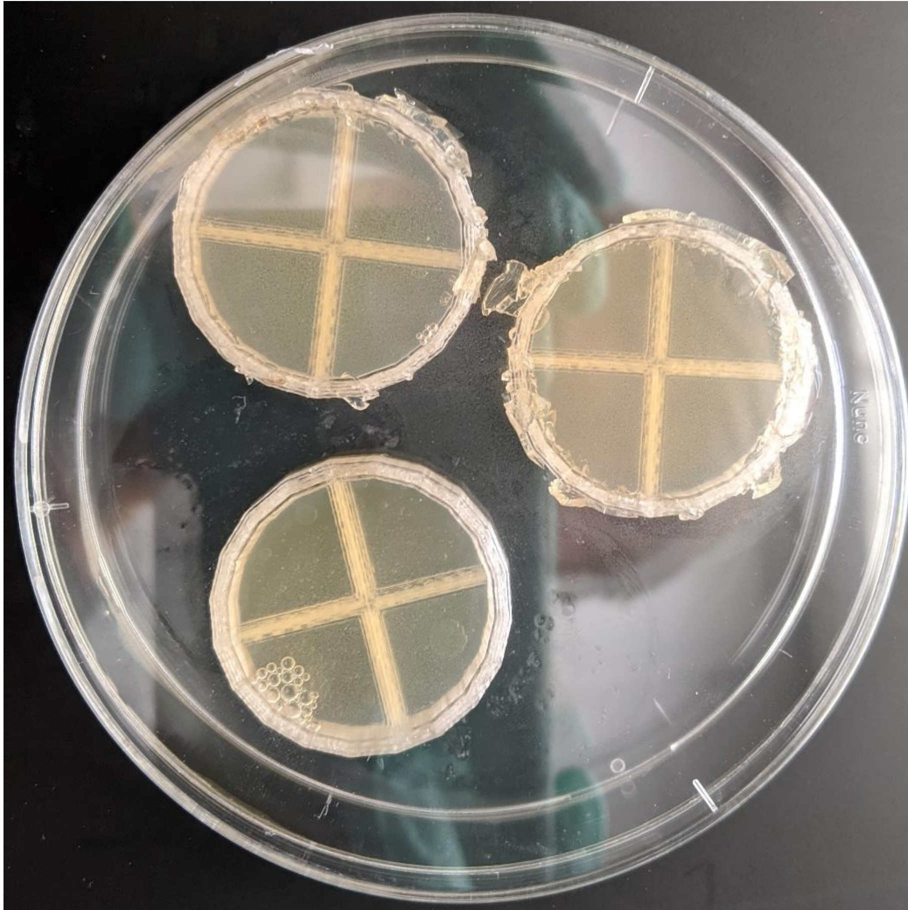

Figure 1. 3D printed tray developed for use in the soft agar assay. After trays were printed, SDA agar was poured and allowed to solidify.

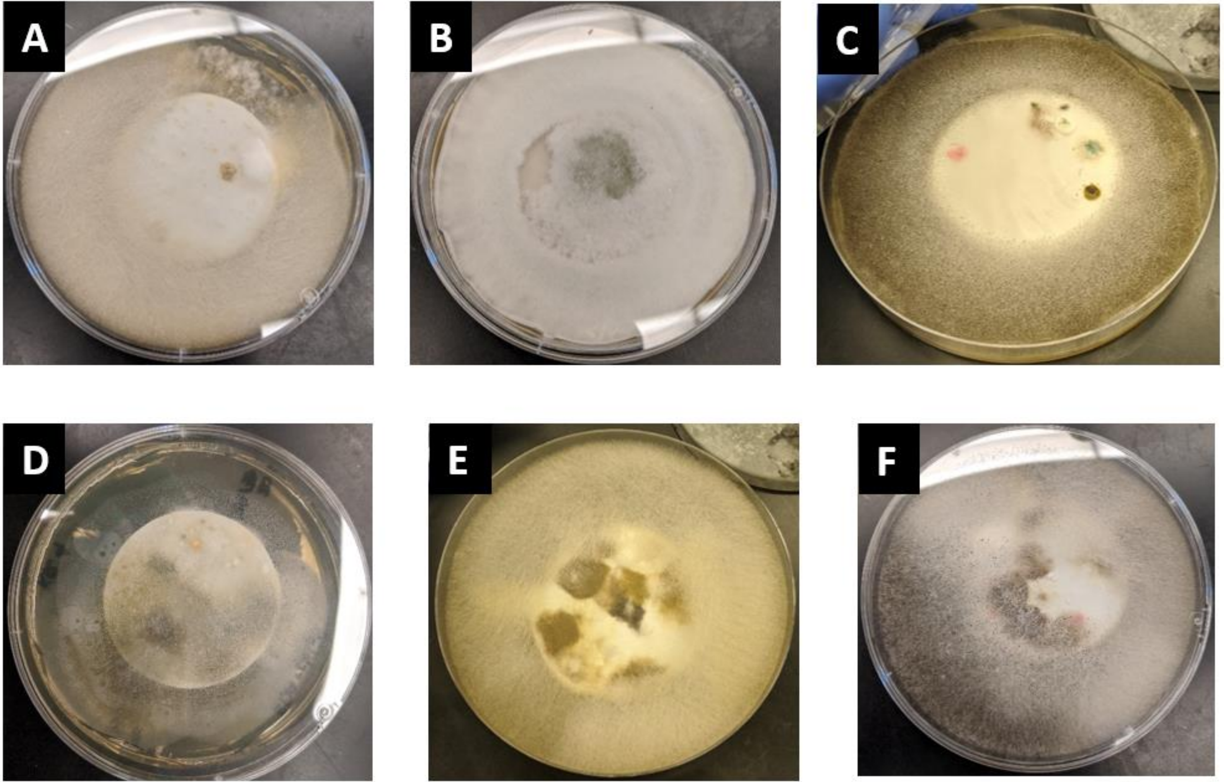

Figure 2. Initial fungal growth from environmental samples on SDA agar. **A.** Culture from backshore sand. **B.** Culture from foreshore sand. **C.** Culture from seawater. **D.** Culture from wet seafoam, unfiltered. **E.** Culture from wet seafoam after second filtration. **F.** Culture from dry seafoam.

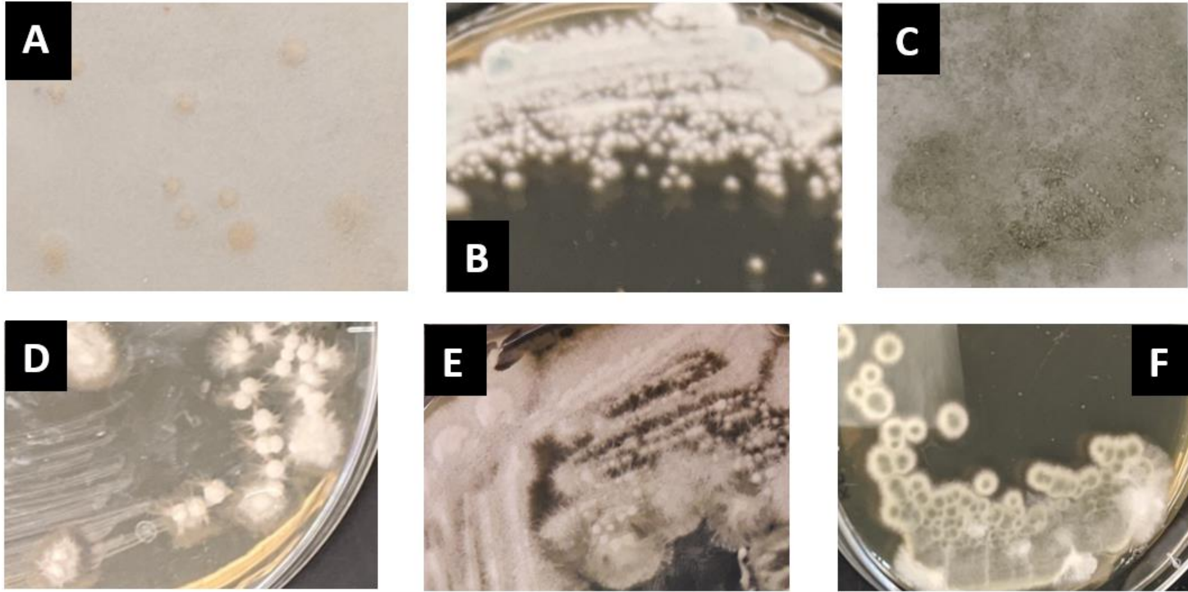

Figure 3. Six unique isolated fungal species. (A) Species 1 isolated from backshore. (B) Species 2 isolated from seawater. (C) Species 3 isolated from foreshore. (D) Species 4 isolated from wet seafoam, unfiltered. (E) Species 5 isolated from wet seafoam, unfiltered. (F) Species 6 isolated from wet seafoam, unfiltered.

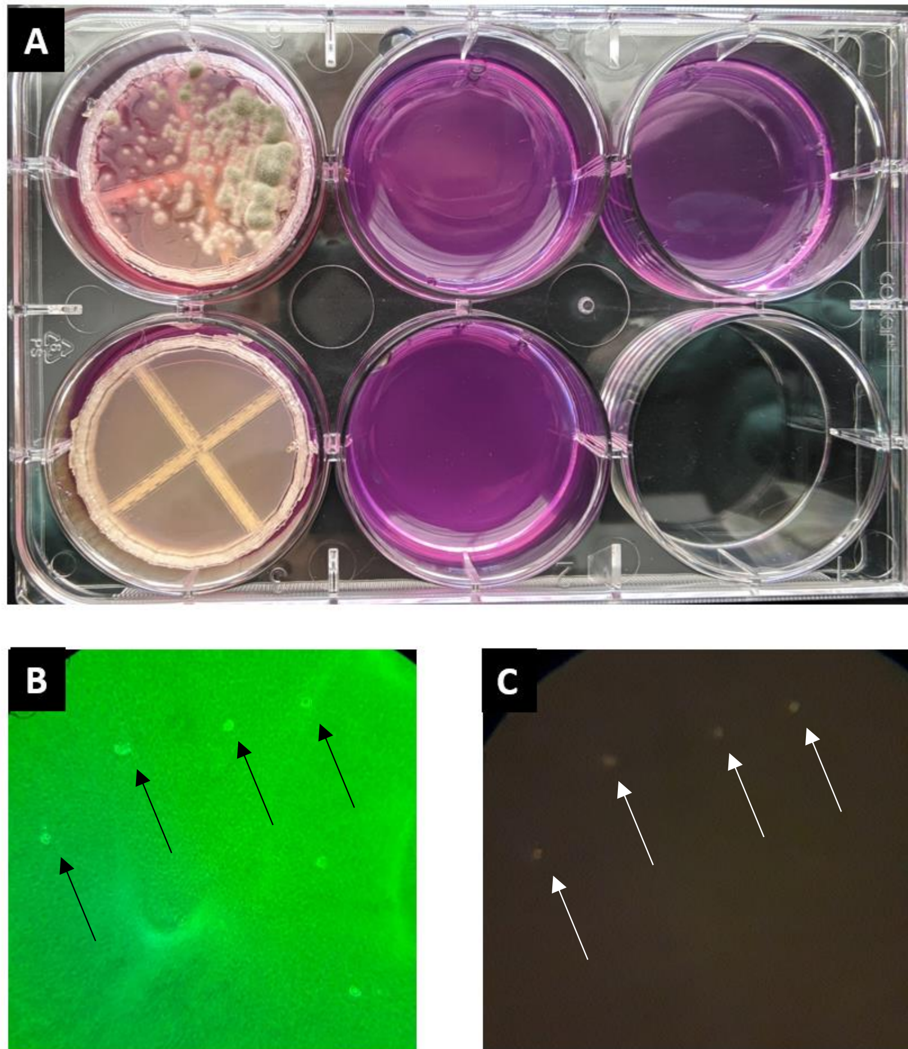

Figure 4. Human rhabdomyosarcoma cells suspended in DMEM-enriched soft agar in a 6-well cell culture plate. **A.** Soft agar preparation in a 6-well plate. Each well contained a bottom feeder layer of agarose enriched with DMEM beneath a top soft agar layer containing DMEM and RD cells. Well 1 contains a fungal sample grown on a 3D printed tray set directly on top of the soft agar cell-containing later. Well 4 contains a control 3D printed tray with no fungal sample. **B.** Visualization of cell colonies within the soft agar layer under an inverted microscope (with green filter). **C.** Visualization of cell colonies within the soft agar layer under an inverted microscope (darkfield illumination).

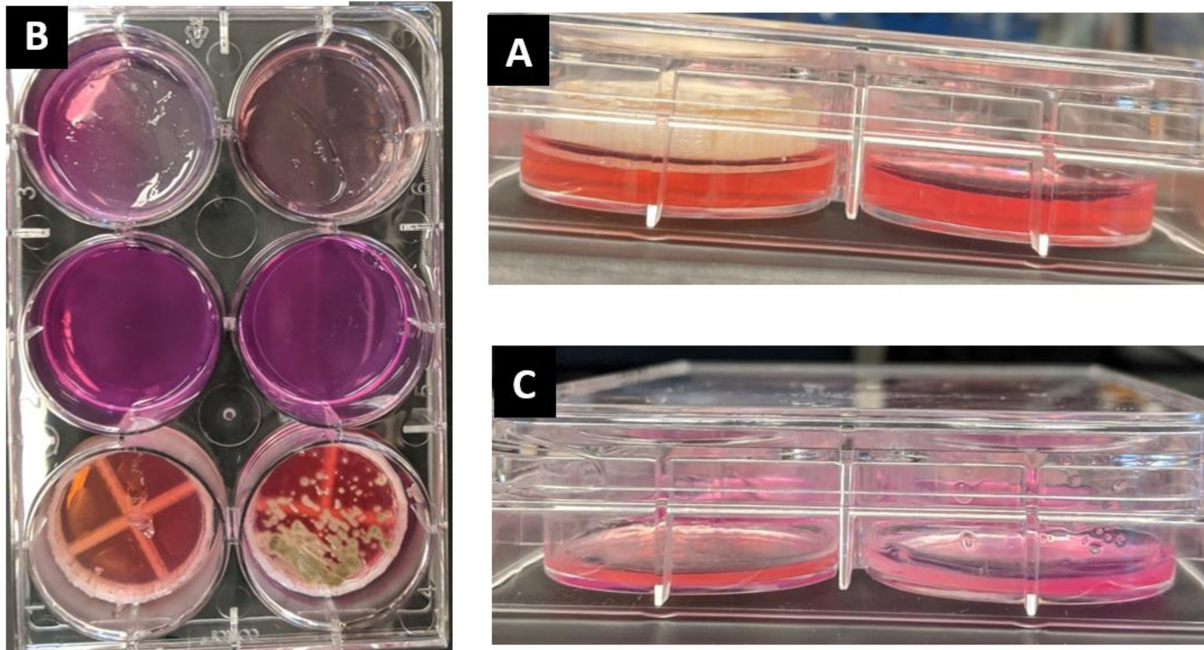

Figure 5. Soft agar assay in a 6-well plate following incubation with fungal sample. **A.** Side view of well containing fungal sample in 3D printed tray placed directly on top of soft agar layer prior to incubation. **B.** Top view of the 6-well plate following one week incubation with fungal sample and control blank tray. **C.** Side view of wells after removal of fungal and control trays following one week incubation.

References

1. Deshmukh, S. K., Prakash, V. & Ranjan, N. Marine Fungi: A Source of Potential Anticancer Compounds. *Front. Microbiol.* **8**, 2536 (2018).
2. Saleem, M. *et al.* Marine natural products of fungal origin. *Nat. Prod. Rep.* **24**, 1142 (2007).
3. Imhoff, J. F. Natural Products from Marine Fungi--Still an Underrepresented Resource. *Mar Drugs* **14**, 19 (2016).
4. Gladfelter, A. S., James, T. Y. & Amend, A. S. Marine fungi. *Current Biology* **29**, R191–R195 (2019).
5. Lin, S.-J., Schranz, J. & Teutsch, S. M. Aspergillosis Case-Fatality Rate: Systematic Review of the Literature. *Clinical Infectious Diseases* **32**, 358–366 (2001).
6. Sugui, J. A., Kwon-Chung, K. J., Juvvadi, P. R., Latge, J.-P. & Steinbach, W. J. Aspergillus fumigatus and Related Species. *Cold Spring Harbor Perspectives in Medicine* **5**, a019786–a019786 (2015).
7. Horibata, S., Vo, T. V., Subramanian, V., Thompson, P. R. & Coonrod, S. A. Utilization of the Soft Agar Colony Formation Assay to Identify Inhibitors of Tumorigenicity in Breast Cancer Cells. *JoVE* 52727 (2015) doi:10.3791/52727.
8. Scognamiglio, T., Zinchuk, R., Gumpeni, P. & Larone, D. H. Comparison of inhibitory mold agar to Sabouraud dextrose agar as a primary medium for isolation of fungi. *J. Clin. Microbiol.* **48**, 1924–1925 (2010).
9. McAllister, R. M., Melnyk, J., Finkelstein, J. Z., Adams, E. C. & Gardner, M. B. Cultivation in vitro of cells derived from a human rhabdomyosarcoma. *Cancer* **24**, 520–526 (1969).

10. Duan, Y. *et al.* A High-Resolution Time Series Reveals Distinct Seasonal Patterns of Planktonic Fungi at a Temperate Coastal Ocean Site (Beaufort, North Carolina, USA). *Appl. Environ. Microbiol.* **84**, (2018).