Final Evaluation Report

Your Details	
Full Name	Sandra Victoria Flechas
Project Title	The role of the skin fungal community as a barrier defense against the pathogen Batrachochytrium dendrobatidis
Application ID	23052-2
Grant Amount	£4815
Email Address	vickyflechas@gmail.com
Date of this Report	November 12, 2019

1. Indicate the level of achievement of the project's original objectives and include any relevant comments on factors affecting this.

Objective	Not achieved	Partially achieved	Fully achieved	Comments
To confirm the presence of the fungus and to accurately quantify the number of Bd zoospores in the sample				To determine infection status, Bd presence/absence, as well as the intensity of infection (as zoospore loads), each animal was swabbed by running a synthetic rayon swab (MWE 100) 10 times over the ventral surface, the inner thigh area and the plantar surface of the hind feet webbing for a total of 50 times. Swabs were stored dry at -20 °C until processed. Presence of Bd was diagnosed and quantified using qPCR (Real-Time PCR). For the qPCR Bd assay, DNA was extracted using PrepMan Ultra. Extractions and qPCR's were performed following the methods described by Hyatt et al. (2007) and Boyle et al. (2004). Each reaction consists of 0.45 ml of each primer (50 mM), 5.975 ml of water, 12.5 ml of TaqMan, 0.625 ml of MGB Probe and 5 ml of the DNA template. To quantify infection intensity (as number of zoospores) standards of known concentrations and negative controls were used.
To describe the fungus microbiome, using culture-independent techniques.				In order to characterise the culturable portion of the skin microbiota and identify candidate fungi with anti-Bd properties, we took samples from 1 to five individuals per stage and species. In total we collected samples from 23 individuals (22 individuals of *Rheobates palmatus* and one of *Dendropsophus molitor*). Samples were preserved in 2 ml cryovials containing 1 ml saline solution (0.85%). We performed serial dilutions (until 10^{-3}) and plated it in PDA (Potato Dextrose Agar). Plates were incubated at room temperature for 8 days. Single colonies from each

				bacterial morphotype, discriminated by eye inspection, were streaked on fresh agar plates and subcultures were made until pure cultures were obtained. For strain identification, we used morphological and molecular approaches. 1) Characteristics such as texture, pigmentations and obverse and reverse colour of the colony were observed after 7 days of incubation on PDA (with rifampicin) plates. For microscopic characteristics we observe colour, septation, branching patterns, conidia and chlamydospores. 2) To identify each isolate, the ITS region was amplified and will be sequenced next month. Isolates were grown in Sabouraud´s dextrose with yeast (SDY) medium with glucose (2%), yeast extract (1%) and peptone (1%) in agitation for 7 days at 25°C. Then fungi were filtered using a 25 µm pore filter paper and lyophilised. DNA was extracted using phenol-chloroform method described by Goodwin, Drenth, & Fry (1992). DNA extractions were verified using a 1% agarose gel electrophoresis. The ITS region was amplified using ITS1F (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers. The PCR parameters were: denaturation step at 94°C for 5 min; 30 cycles of 94°C for 30s, 59°C for 30s and 72°C for 1 min; and a final extension step of 7 min at 72°C. The results were checked by electrophoresis in 1% agarose gels. The products are being sent to the sequencing lab at University of los Andes, Bogotá, Colombia. We hope to have the final results at the end of this year.
To perform antagonism assays where each isolated fungus morphotype will be tested against *Bd*.				To test if the skin fungi can inhibit or facilitate Bd growth, challenge assays were performed using each isolated morphotype. To perform the growth inhibition assays, Bd strain JEL423 was

				plated in TGhL agar (Tryptone 10 g, Agar 10 g, Gelatine hydrolisate 4 g, 1 l distilled water), and allow to grow at 23 ºC for at least 3 days or until maximum zoospores production. Bd was harvested from Petri dishes, re-suspended in TG broth and the solution was filtered in order to allow the passage of the zoospores and retain the zoosporangia. The filtered zoospores were count on a haemocytometer and re-suspend in sterile TGhL broth to a concentration of $1x10^6$ zoospores/mL. We ran two kinds of challenge assays: (1) Each isolated morphotype was tested against Bd in plates with TGh agar in order to determine if there is a mechanical response of fungi, for example under the microscope we observed hyphae surrounding Bd zoosporangia. (2) Each fungal morphotype was growth in petri plates with PDA agar, then each morphotype was grown in 1% tryptone broth for 3 days in sterile centrifuge tubes at room temperature and growth was verified by checking for turbidity. Following confirmation of growth, samples were centrifuged at 2,225 × g for 5 min to pellet the cells and the liquid was filtered through 0.22µm filters. Then in 96-well plates we added 50 µl of a zoospore solution and 50 µl of the secondary metabolites. The positive control plates containing Bd and 50 µl of sterile water, and the negative control plates containing 50 µl of heat killed Bd and 50 µl of sterile water. The plates were incubated for seven days at 23°C and measure at 490 nm absorbance the first day, third day and seventh day to registered changes in optical density.

2. Please explain any unforeseen difficulties that arose during the project and how these were tackled.

The first difficulty we had was finding individuals of one of the study species. We believe that the decrease in the abundance of amphibians in the study site is due to the presence of a fish that have never been reported for the pond. We think that this fish was fed on the frogs, mainly on *Dendropsophus molitor*. During our surveys we only found one individual of *D. molitor* and 22 of *R. palmatus.* We have been studied these two species in the same locality since 2007, and it was surprisingly sad that they are now very difficult to find, and we are pretty confident that Bd was not the responsible for the declines. Another problem we had was the re-activation of the Bd strain EV001, after we manage to activate the strain, the growth of this strain was very slow and the production of zoospores very low. After trying to work with this strain for a while, we had a contamination in most of the petri dishes, which is why we contacted Dr Joyce Longcore (University of Maine) to acquire a new Bd culture from a different strain (JEL423). Then, for a couple of months we were unable to grow Bd to the required zoospores concentration to perform the tests. Also, we consider that other difficulties in this kind of research projects are to standardise the protocols and procedures in the laboratory. First, we spend a couple of months trying to have all the extractions and PCR reactions in the proper conditions. Furthermore, we experienced unforeseen delays to run the challenge assays, especially obtaining the zoospores and the growth of Bd. In addition, it took several attempts until we standardised the protocol to measure inhibition. All these difficulties experienced during the project were almost always very worrying because we did not know if we would be able to find a solution. However, the situation confers ourselves with capacities to find various strategies in order to solve each problem we faced.

3. Briefly describe the three most important outcomes of your project.

a) Our goal was to confirm the presence of the fungus and to accurately quantify the number of Bd zoospores in the sample. We detected a higher prevalence of Bd in the juveniles (80% of juveniles were Bd positive, seven of nine individuals of *Rheobates palmatus* and one *Dendropsophus molitor*) then the tadpoles (50% of tadpoles were Bd positive, five of 10 individuals of *Rheobates palmatus*) and none of the adults were Bd positive (three *Rheobates palmatus*). Infection intensity ranges from 0 to 55714 ZE (Zoospore Equivalents).

b) To describe the fungal microbiota, we found that in general tadpoles, juveniles and adults cannot be separated in either host species based on their skin fungi. Except for some morphotypes that were found just in one stage. We describe in total 40 fungal morphotypes in all stages. We find nine yeast and 31 moulds, in the case of morphotype 17 (yeast) was found just in tadpoles of *Rheobates palmatus*, the morphotypes 35 and 36 (molts) were found just in the juvenile of *Dendropsophus molitor.*

c) In order to determine the potential antimicrobial activity against Bd, we performed antagonism assays to determine inhibition. In the first assays, each isolated morphotype was tested against Bd in plates with TGh agar to determine if

there is a mechanical inhibition of Bd. Here we found that just two isolates exhibited some kind of mechanical action on the zoosporangia. For example, we observed that the hyphae surrounded the zoosporangia, and appeared to avoid the growth of Bd, since the zoosporangia seemed to be not viable (they look broken). In the second assays, were just the metabolites were tested we found seven morphotypes with antifungal activity, one of these was just found in tadpoles (morphotype 17) and the one with the greatest antimicrobial potential was also the morphotype with the highest pigment production (morphotype 34).

4. Briefly describe the involvement of local communities and how they have benefitted from the project.

Our project did not involve the participation of local communities, but we involved an undergraduate student who helped us with most of the laboratory procedures. She will be co-author in any publication that result from this research project.

5. Are there any plans to continue this work?

Yes, we are very interested in continue our research on the ecology of amphibian diseases and to deeply understand the mechanisms that allow some species to survive a pathogen that has been considered the responsible for the decline of at least 500 species all over the world. First we want to involve another undergraduate student so we can try again at our study site to see if it is possible to find individuals from *D. molitor*, or to look for a second population in which we can examine the fungal community and its potential role as Bd inhibitors. We are planning to apply for funds that allow us to identify the secondary metabolites secreted by fungi with outstanding inhibiting capacities in order to come out with a more accurate approach for an effective therapy that helps to reduce the impact of Bd in wild populations, and to contribute in the conservation of amphibian species that are facing a high risk of extinction. In addition to evaluate Bd presence and to find mechanisms to counteract the impact of chytridiomycosis, we are now very interested in other diseases that affect amphibians all over the world but that have barely studied in our country, this is the case of the viral disease known as ranavirosis.

6. How do you plan to share the results of your work with others?

We are planning to present our results in the 8th Latin-American Congress of Herpetology that will take place in La Paz, Bolivia in October 2020. In addition, we will present our results in the BoMM (Bogotá Microbial Meeting) in August 2020. We are now working on a manuscript to be submitted to an indexed scientific journal next year.

7. Timescale: Over what period was the grant used? How does this compare to the anticipated or actual length of the project?

The funds from RSG were expected to be use for 1 year (from September 2018 to September 2018). However due to the difficulties experimented in the field and in the lab, we asked for an extension. At the end we spend almost 2 years working in the project, in order to complete the proposed goals.

8. Budget: Provide a breakdown of budgeted versus actual expenditure and the reasons for any differences. All figures should be in £ sterling, indicating the local exchange rate used. It is important that you retain the management accounts and all paid invoices relating to the project for at least 2 years as these may be required for inspection at our discretion.

Item	Budgeted Amount	Actual Amount	Difference	Comments
Challenge assays	400	564	+164	It took us lots of time to standardize the methodology, then, we have to buy extra materials and supplies to complete the assays. Supplies were also expensive, and since we had problems and delays growing the pathogen to run the tests, we expended more money than we ask for at the beginning.
Fungi isolation	700	717	+17	Since we had unexpected delays trying to reactivate the Bd strain we ended buying extra materials that we did not consider initially. In addition, we had to keep our cultures viable to perform the assays, so we had to buy more supplies for this.
qPCR analysis supplies	900	548	-353	In our budget we considered samples for two frog species, however we were unable to find frogs of *D. molitor.* Since we had few samples to analyze, we did not use all the money we asked for.
Fungi identification	2000	1224	-776	Our goal was to isolate, identify and test all the fungal morphotypes against Bd, however due to the impossibility to find one of our study species we did not have as many morphotypes as we expected, then did not use all the money we asked for.
Supplies for the field	400	62	-338	We only spent a £62 in supplies for the field. We use the material available in the lab, then, we did not use all the money.
Food in the field	200	109	-91	We provided food to each person who helped us during the fieldwork.

				However, we did not use all the money.
Transportation	215	121	-94	This amount indicated what we spent in gas and the toll tickets. Although we visited the site more times than we originally consider and based on our previous work, we did not spend all the money we asked for.
TOTAL	**4815**	**3345**	**-1470**	Unused funds returned. *We used the same exchange rate we used in our approved proposal. 1 sterling pound = 3645 COP.*

9. Looking ahead, what do you feel are the important next steps?

We consider the most important step is to try an innovative treatment where we can use secondary metabolites secreted by the fungi that exhibited inhibition abilities. At this time, and after many research groups tried probiotics as a potential treatment without success, we believe that is important to implement different / new ways to combat Bd. We hope that the mycobiome, which has been understudied as a potential tool to treat Bd can give us useful information to treat endangered tropical species. In addition, we still believe that species that can survive Bd infection are the source of information key for developing an effective strategy to fight the fungal pathogen.

10. Did you use The Rufford Foundation logo in any materials produced in relation to this project? Did the Foundation receive any publicity during the course of your work?

The logo will be displayed in every opportunity we have to present our results. Additionally, the RF will be mentioned in the acknowledgments of any manuscript derived from this project.

11. Please provide a full list of all the members of your team and briefly what was their role in the project.

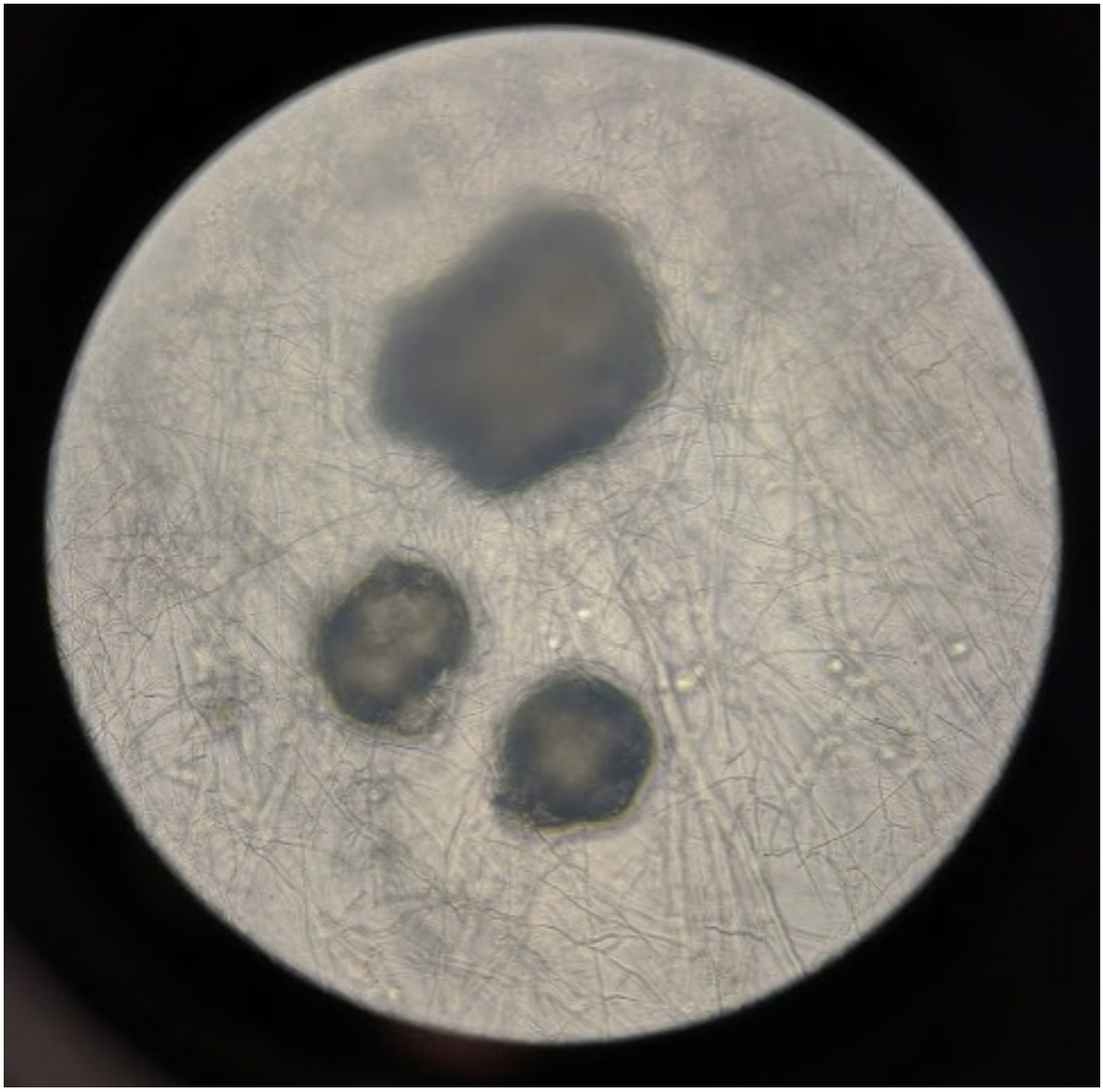
Melissa Hernández Poveda. She was responsible of the isolation of fungal morphotypes, morphological and molecular characterization. Also, she performs antagonism assays where each isolated fungus morphotype was tested against Bd.

Andrew J. Crawford, PhD. He was responsible for the experimental and sampling design as well as the statistical analyses of the data. He was also co-responsible for the field sampling and he will be key during the preparation of the scientific manuscript.

Alejandro Acosta-González, PhD. He was responsible of the standardization of the molecular characterization. He was also co-responsible for the field sampling. He will be involved in the preparation of the scientific manuscript.

12. Any other comments?

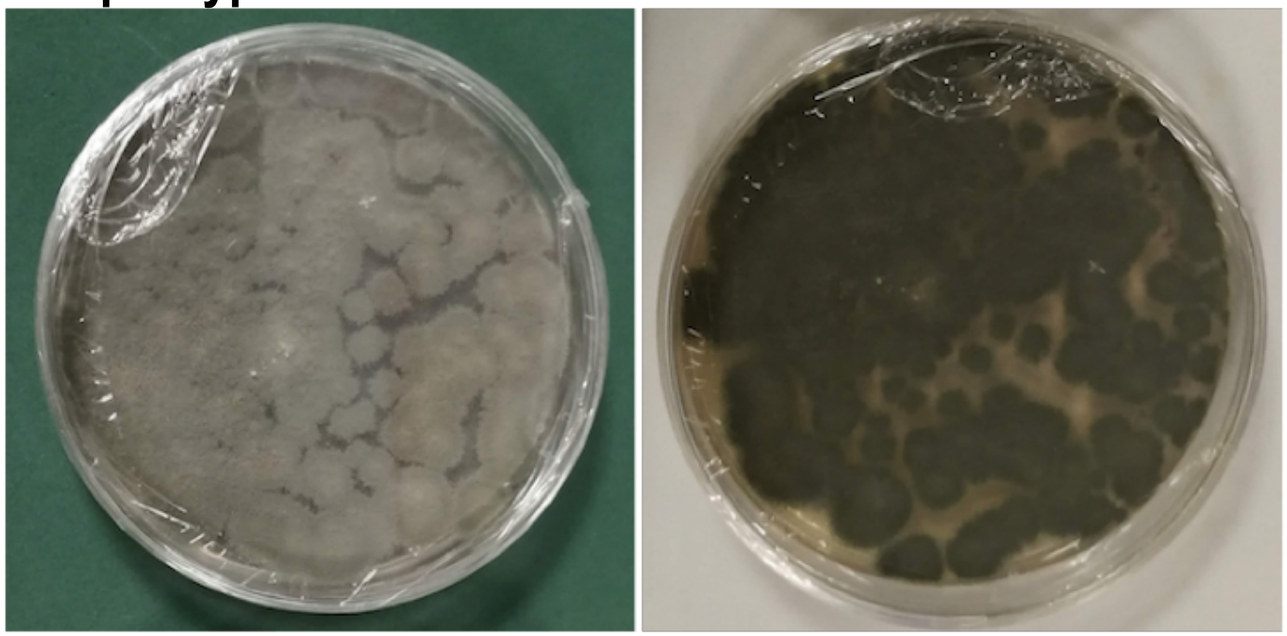
With financial support from Rufford Foundation we were able to determine the contribution of the fungal community as a barrier against Bd, however, we could not complete our goals as we stated in the initial proposal. Our main goal was to examine the mycobiota in the skin of two amphibian's species from the Andes of Colombia, however we could not find one of these species, and we suspect that a fish is responsible for its disappearance.



Inhibition assays

Rheobates palmatus

Morphotype 1



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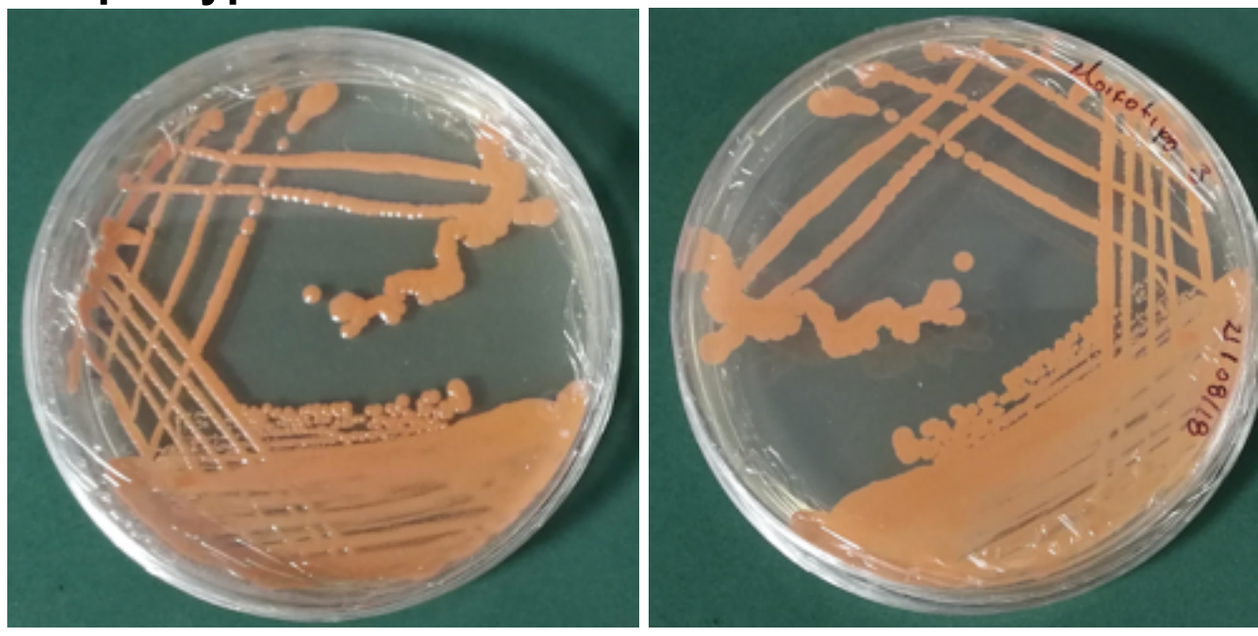

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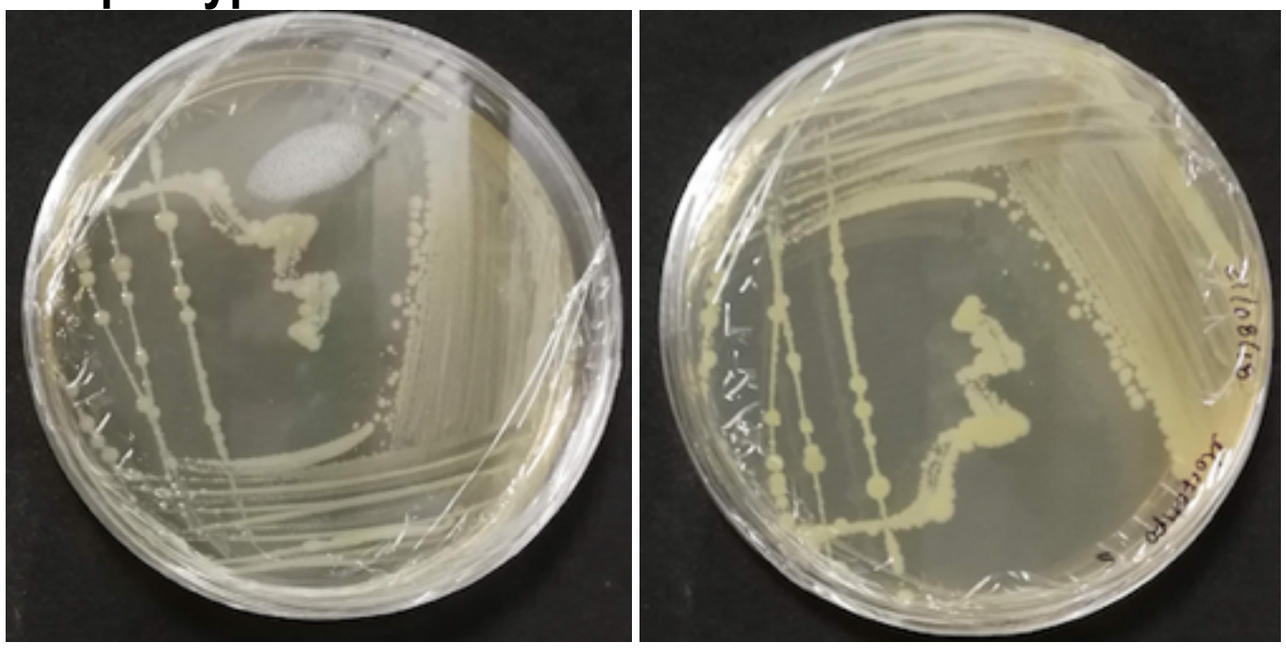
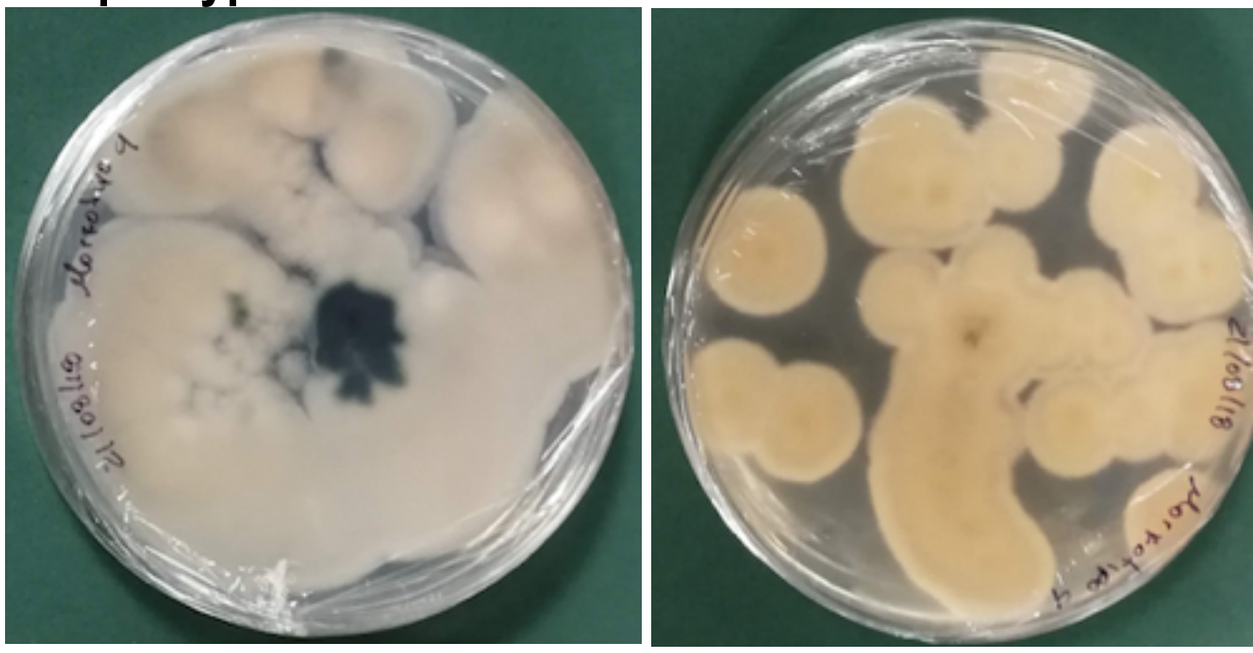


Morphotype 9



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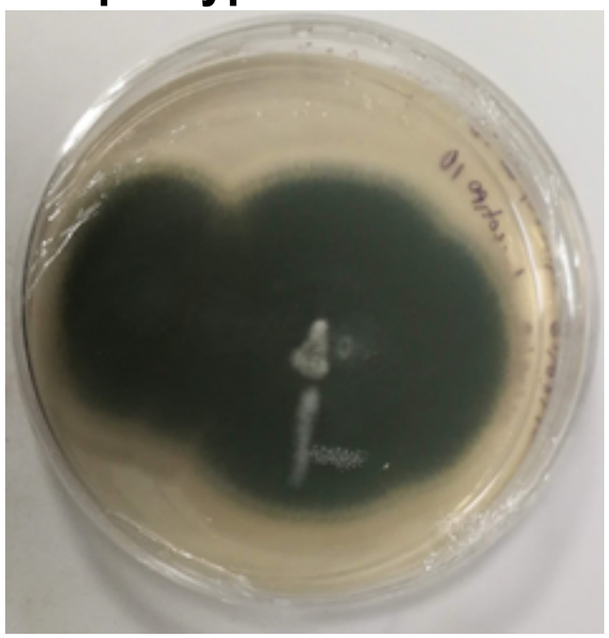

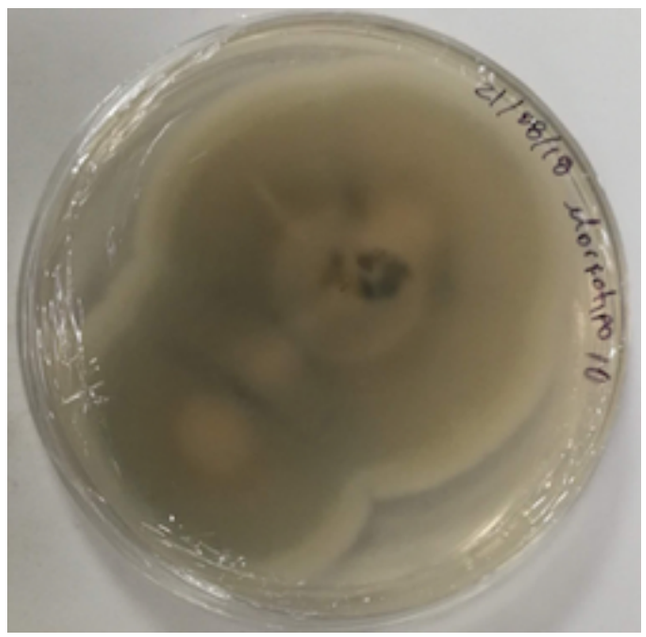

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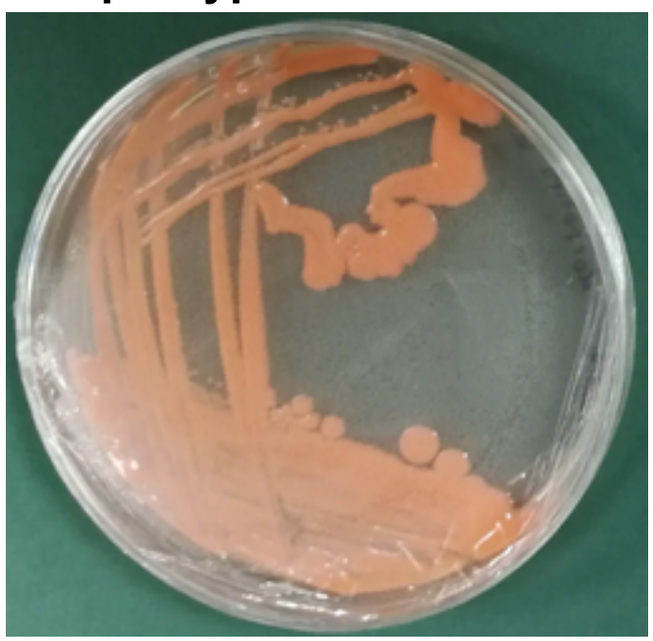

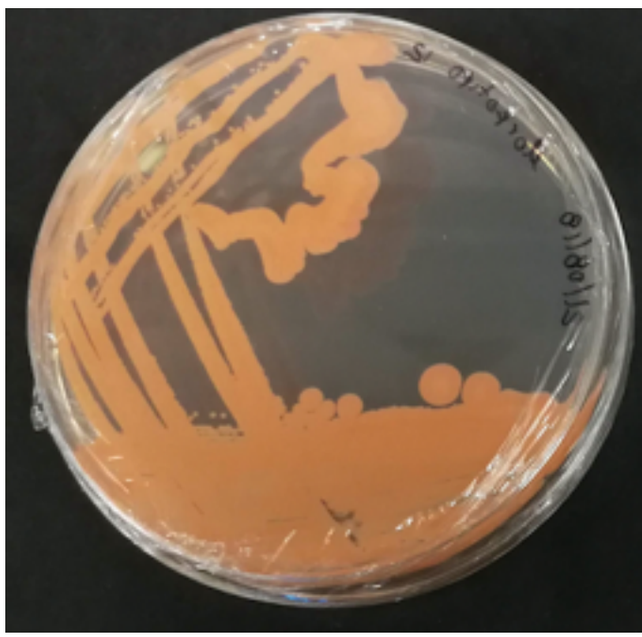

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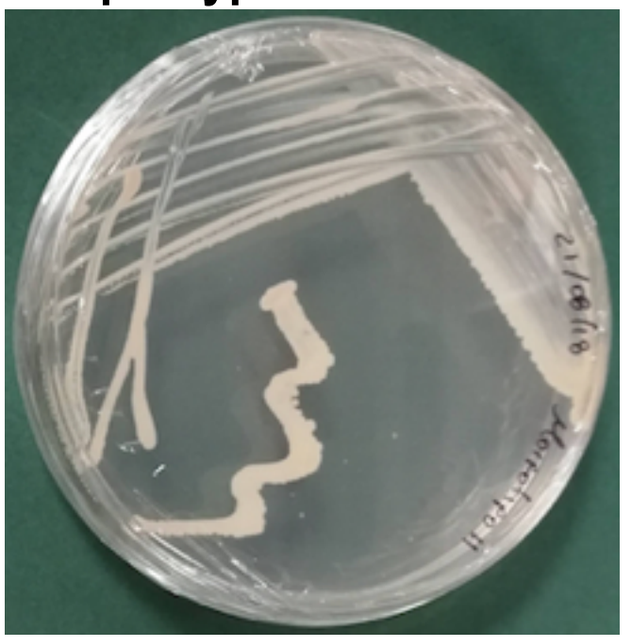

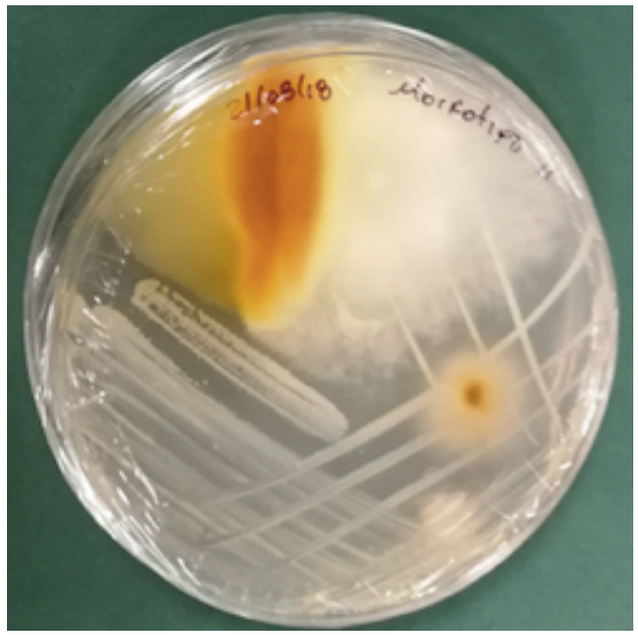

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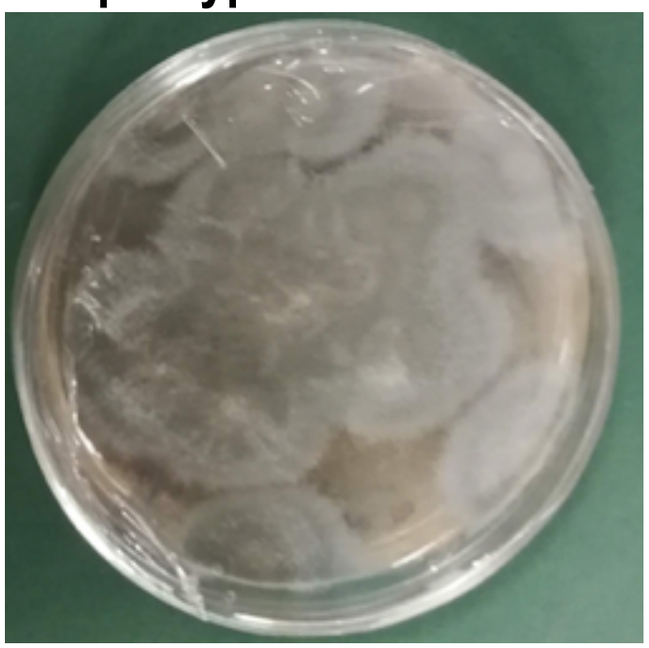

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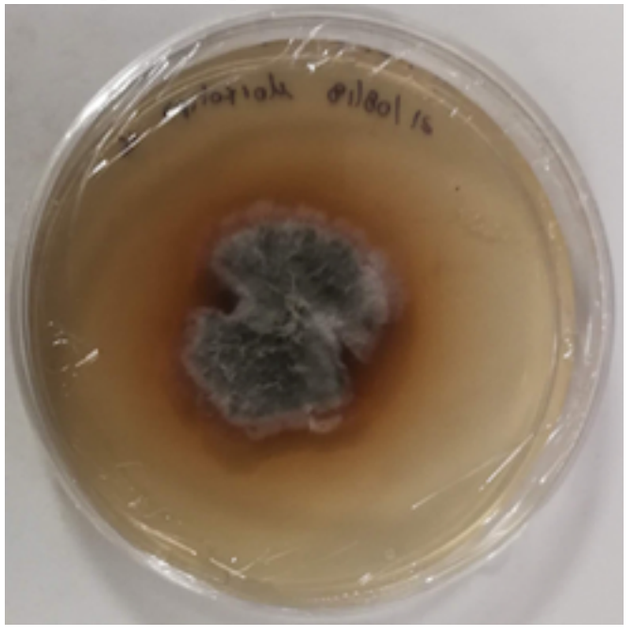

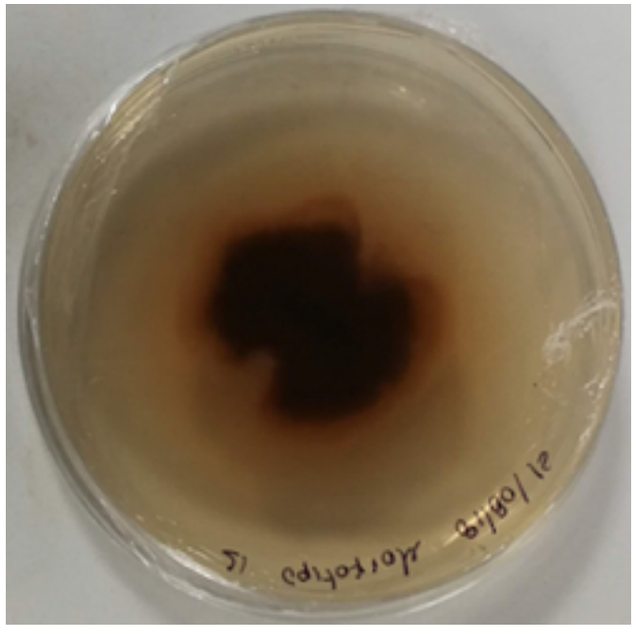

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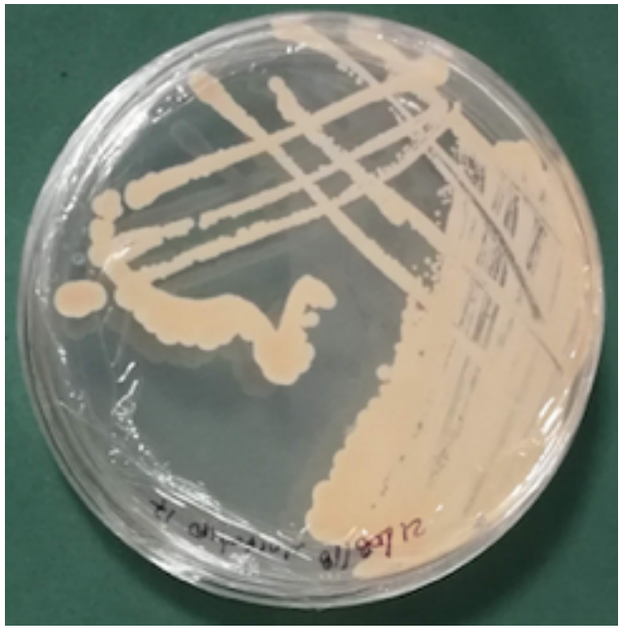

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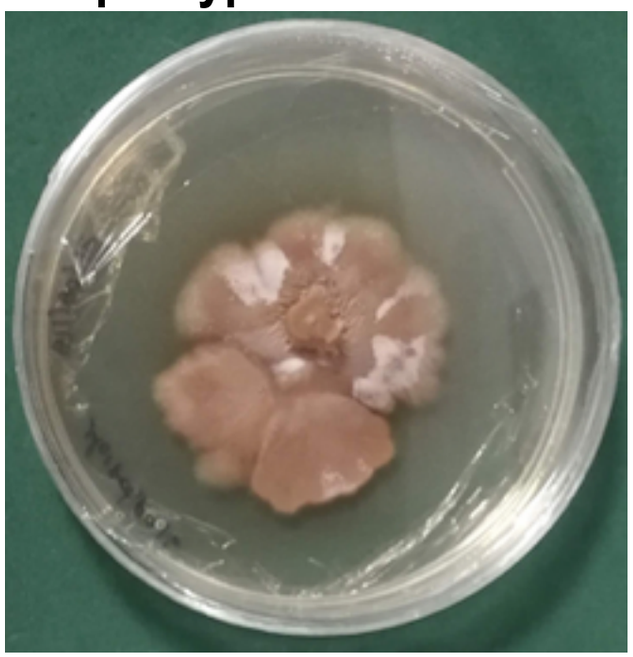

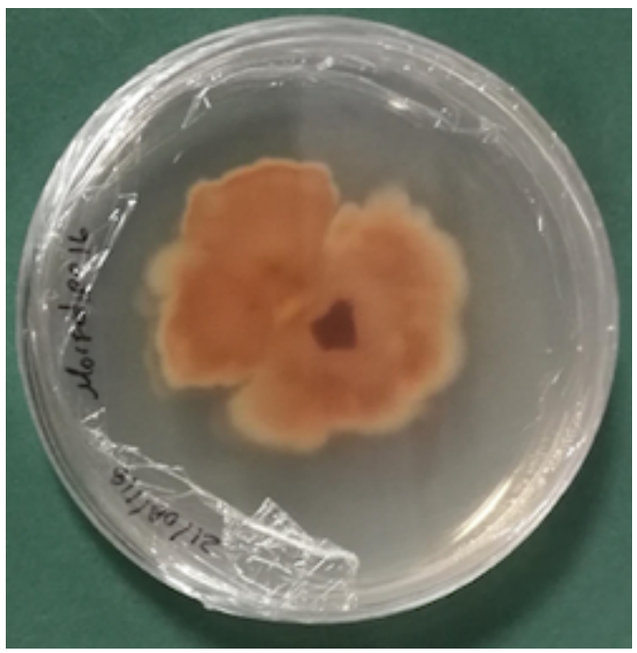

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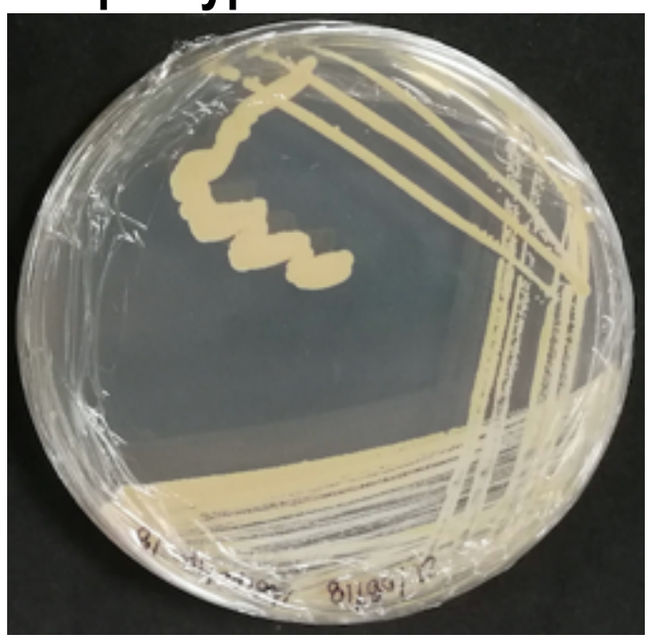

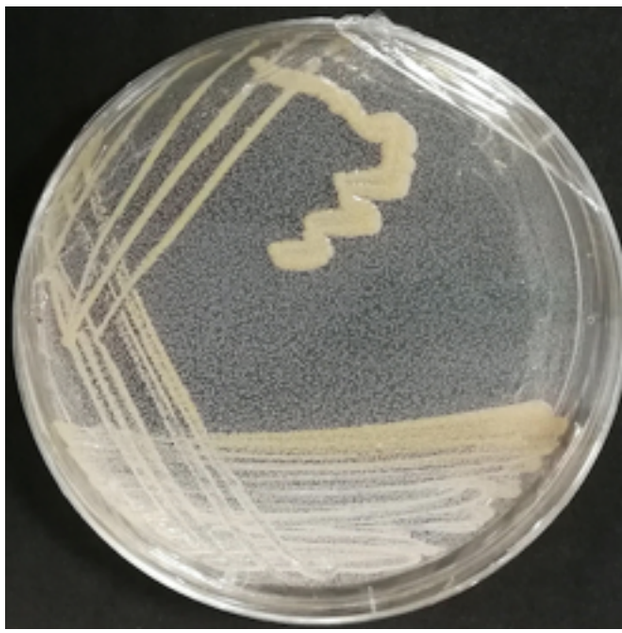

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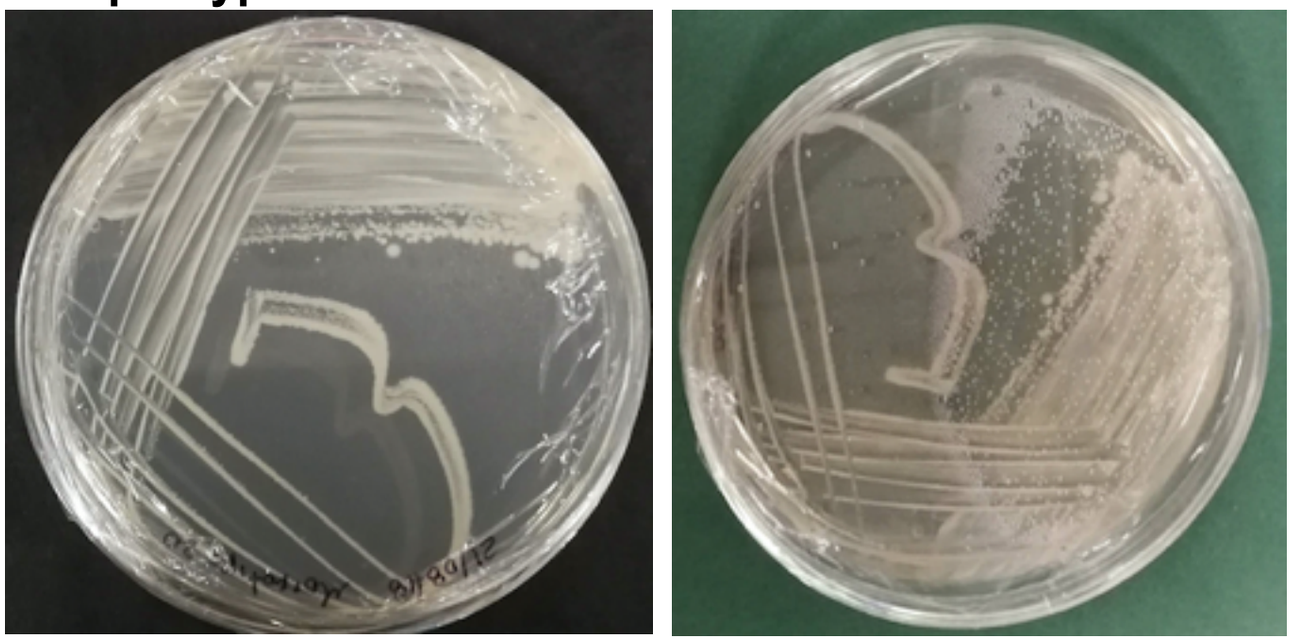

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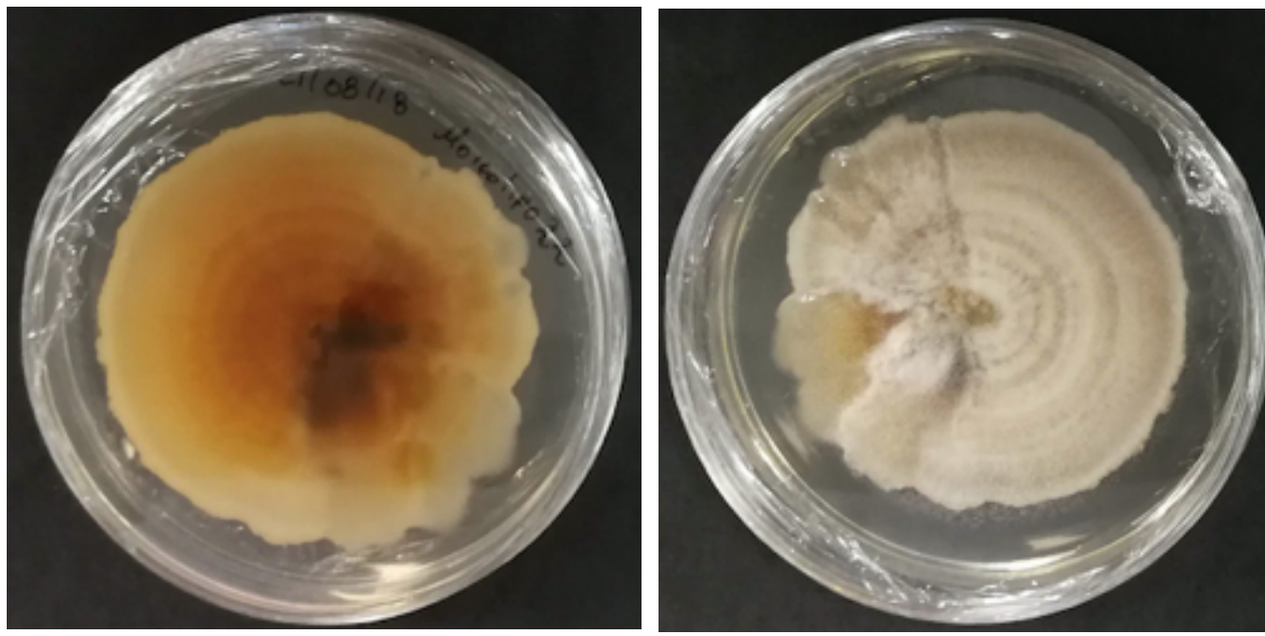

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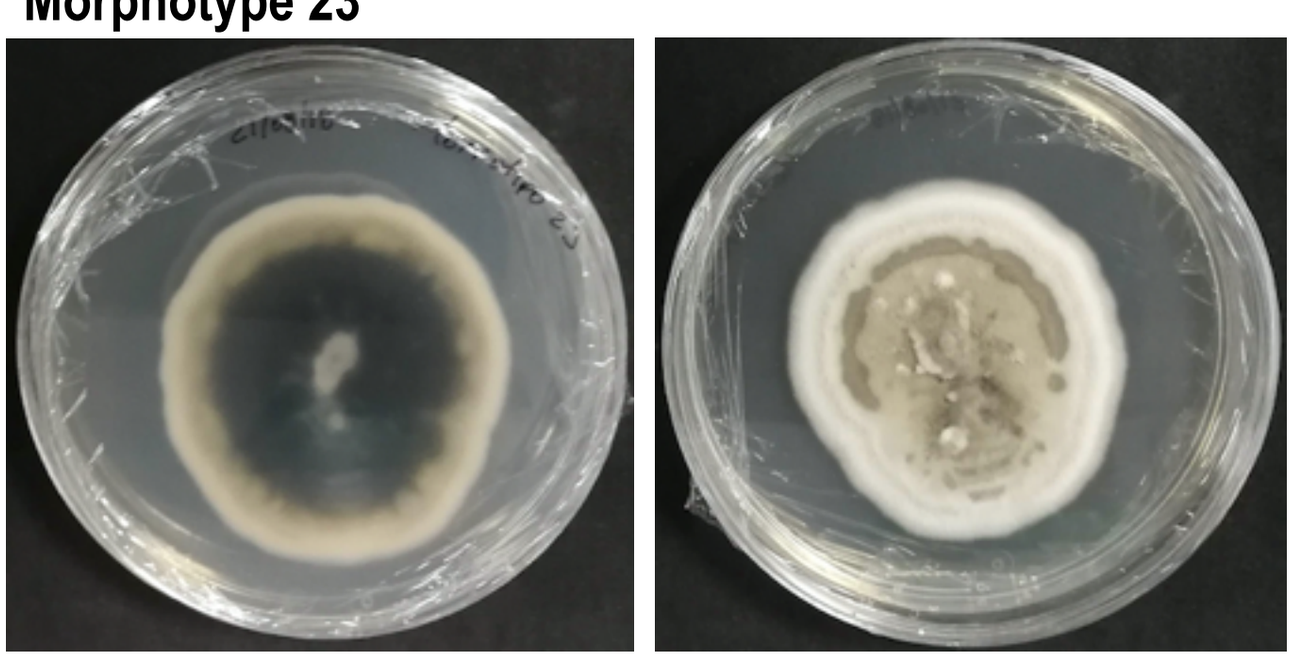

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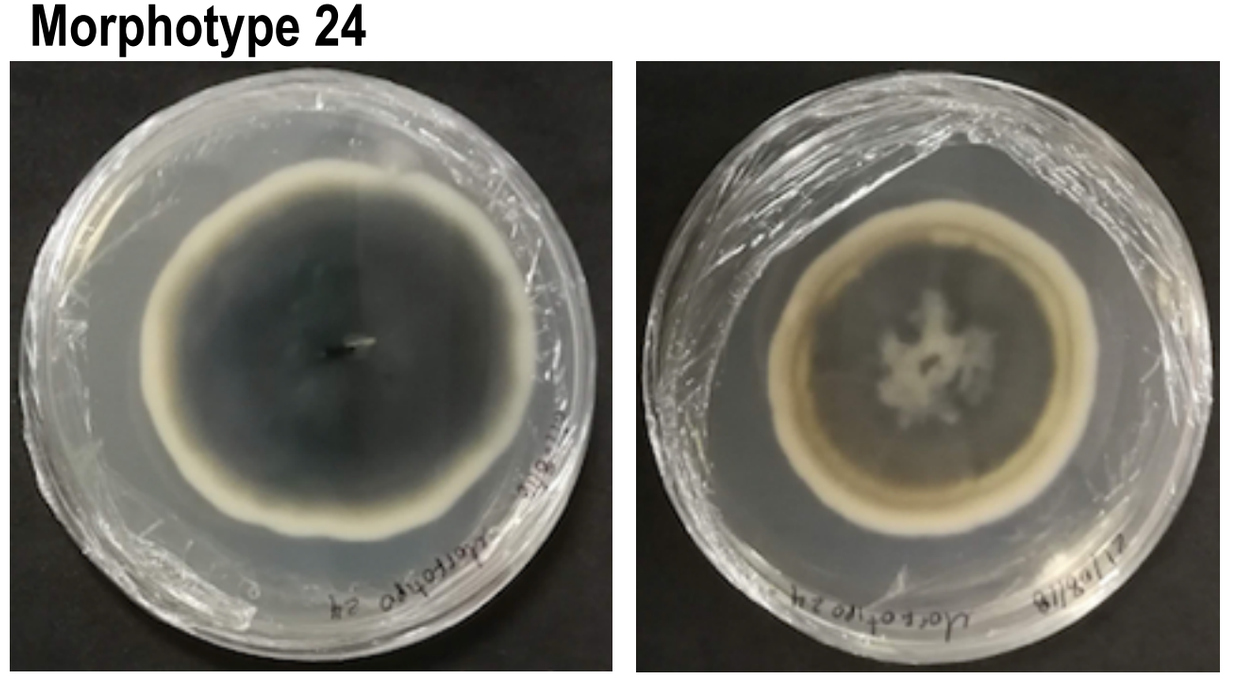

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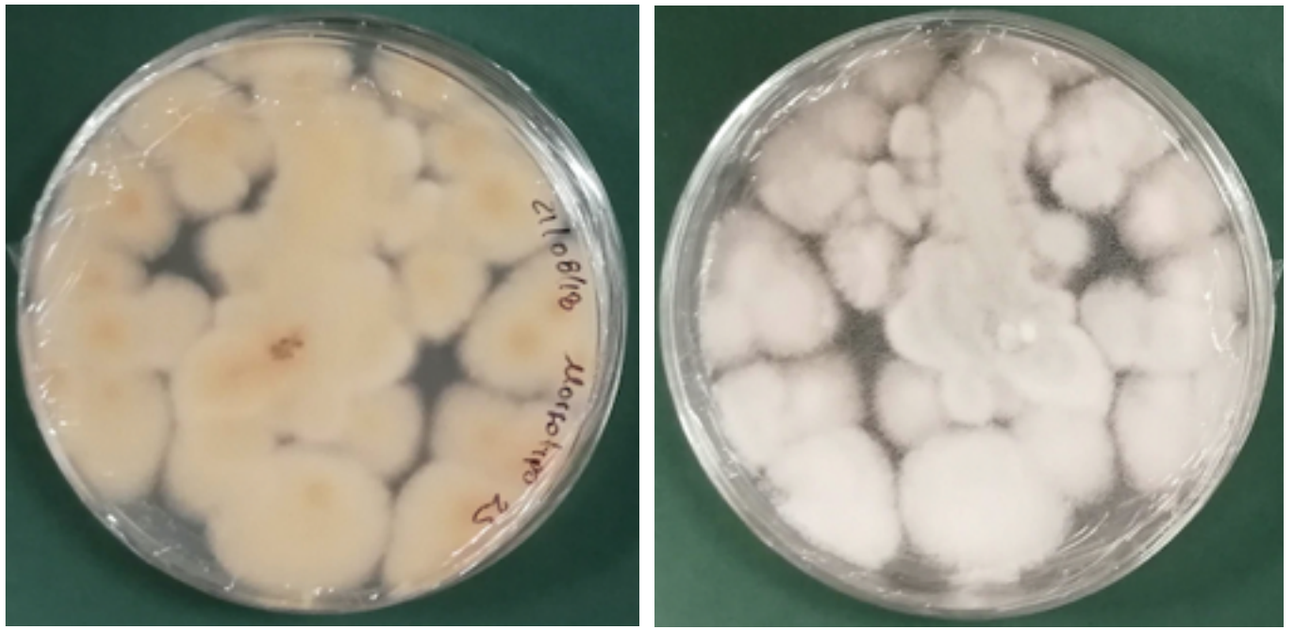

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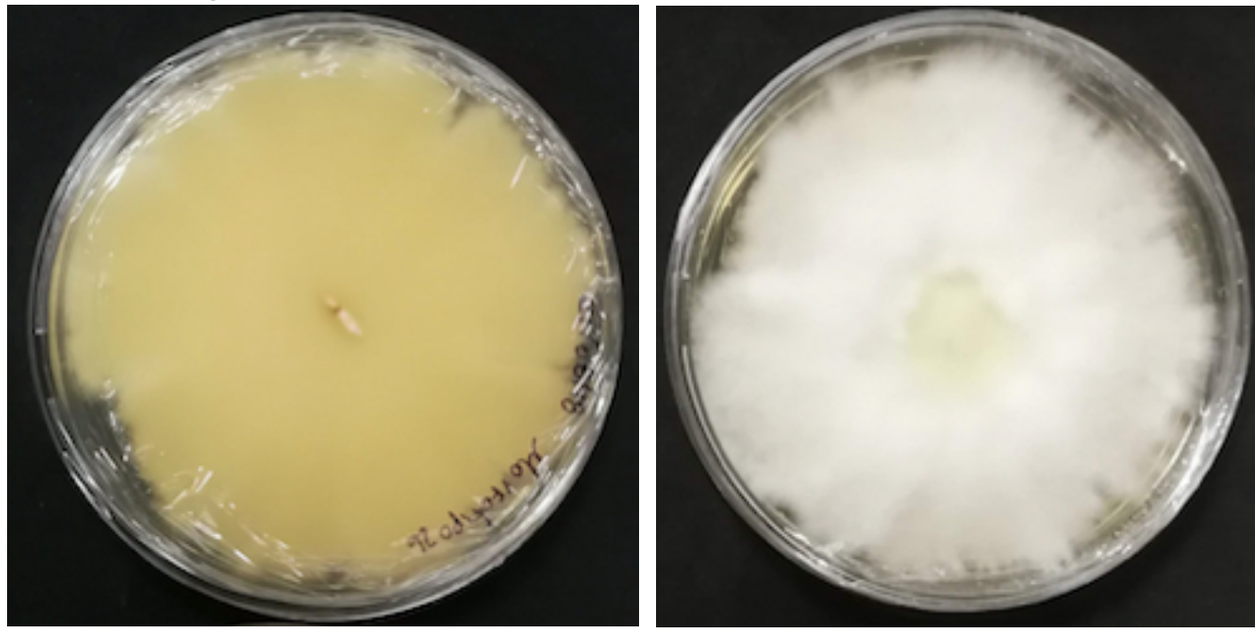

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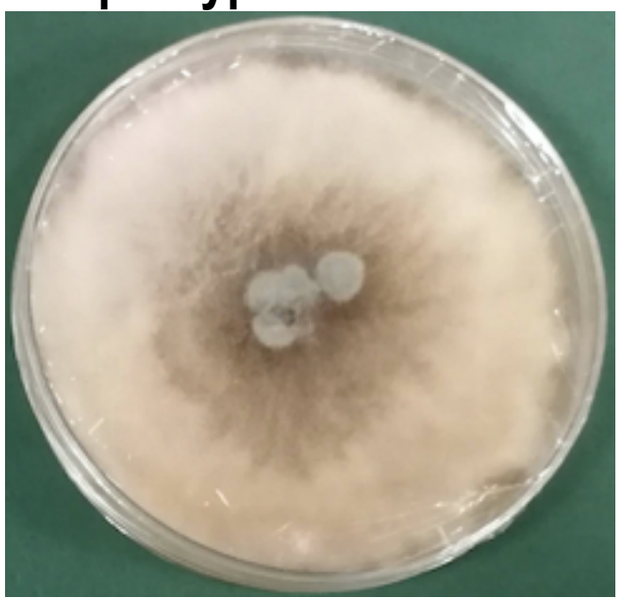

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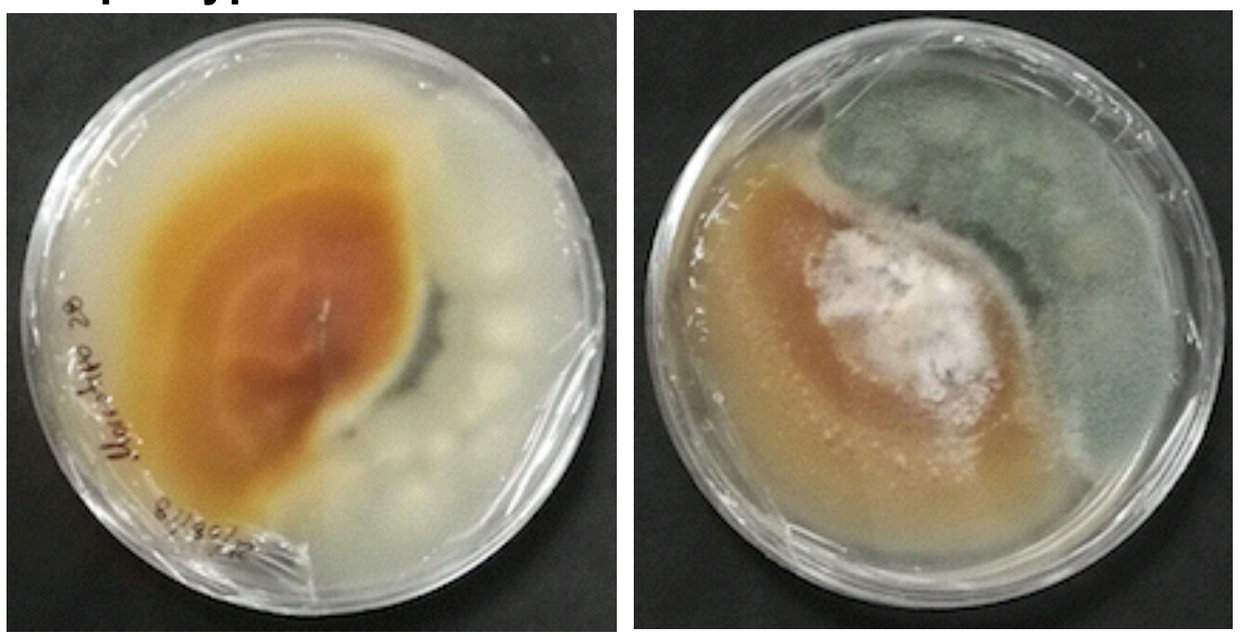

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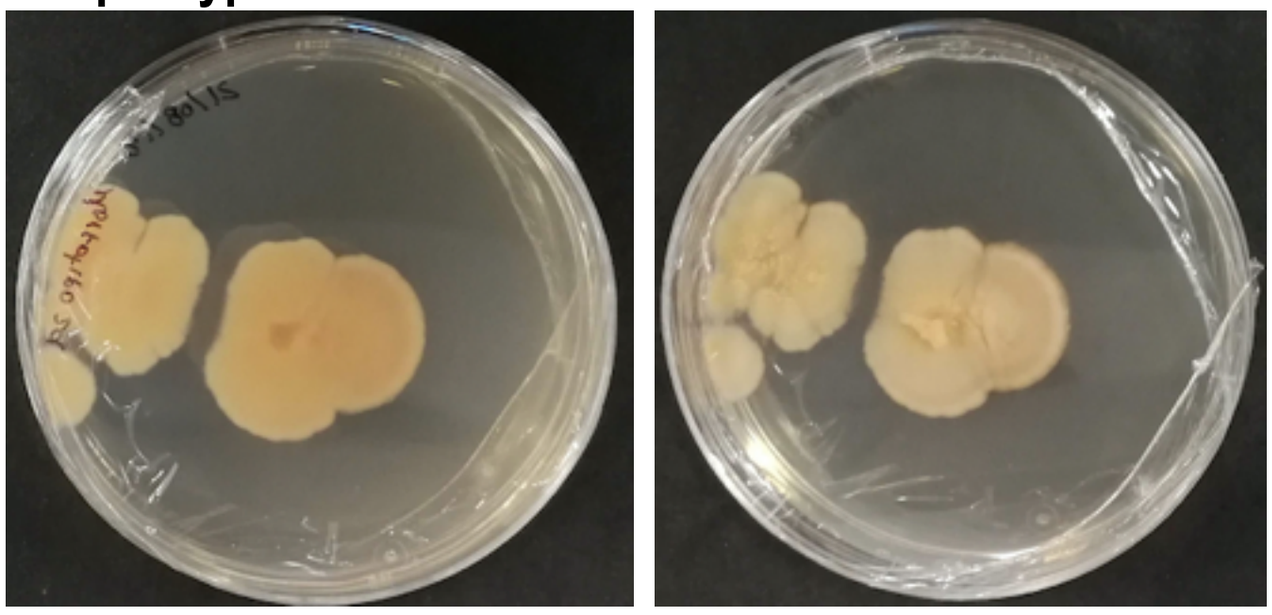

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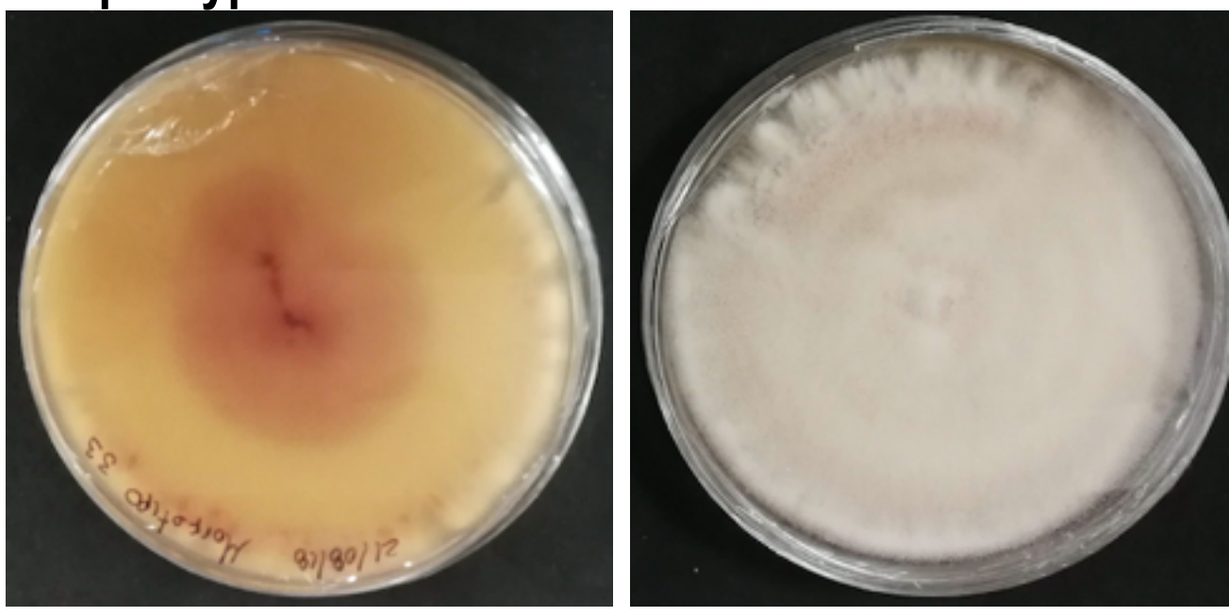

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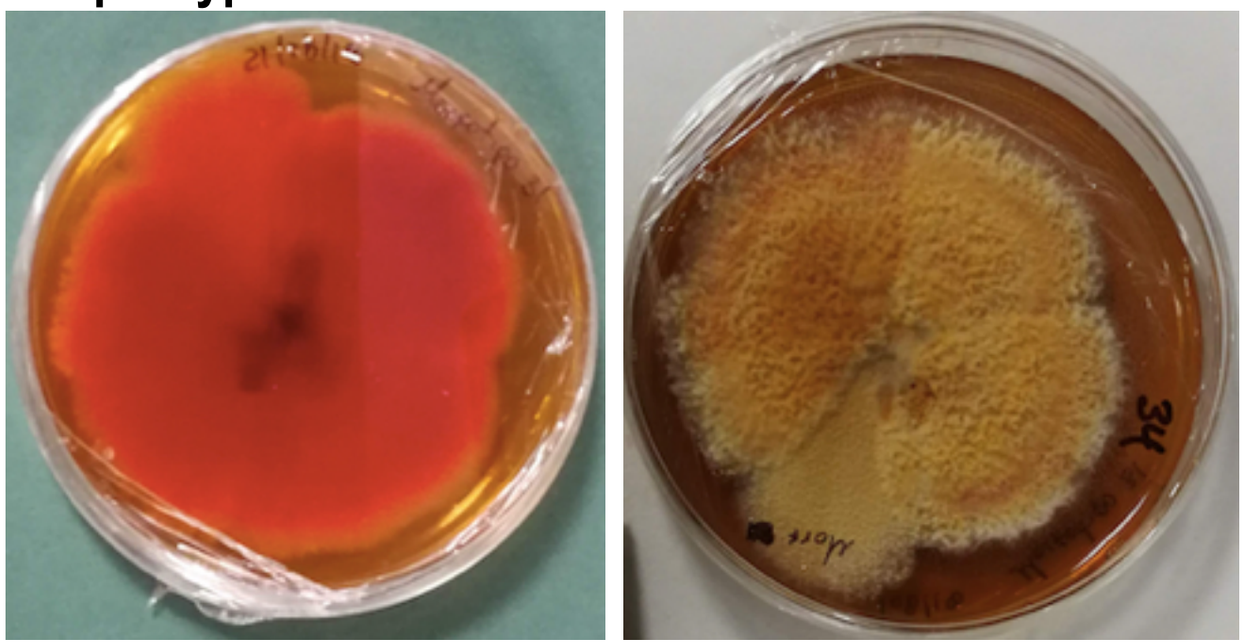

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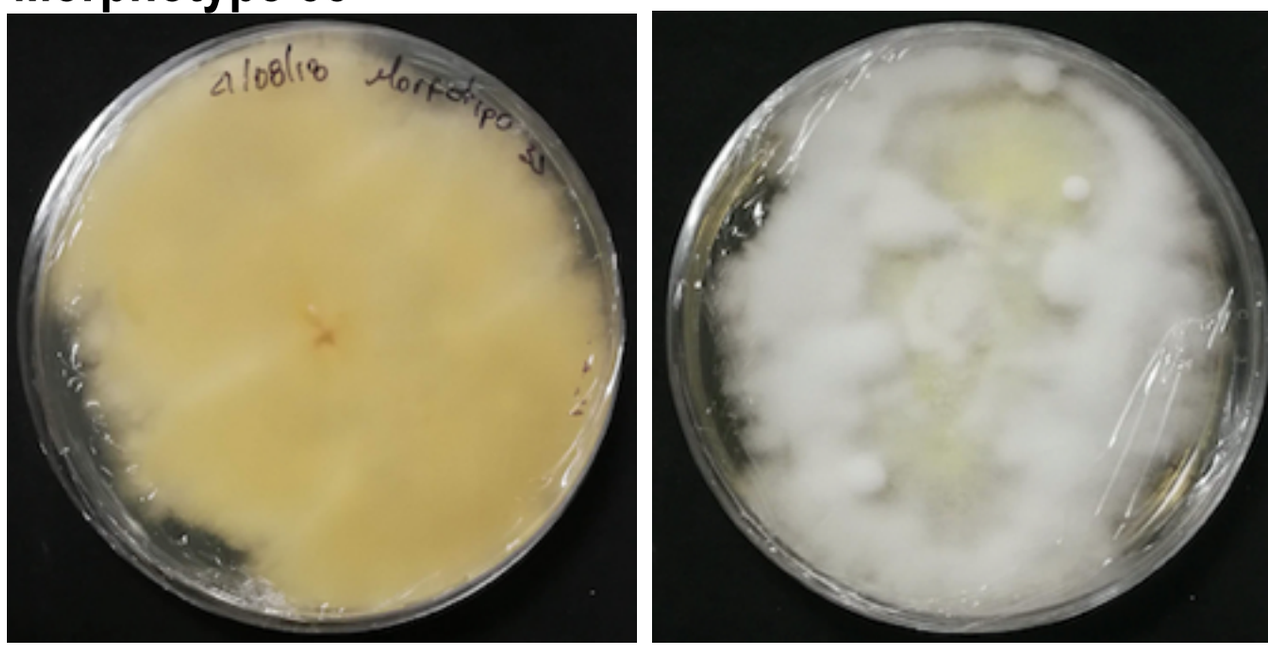

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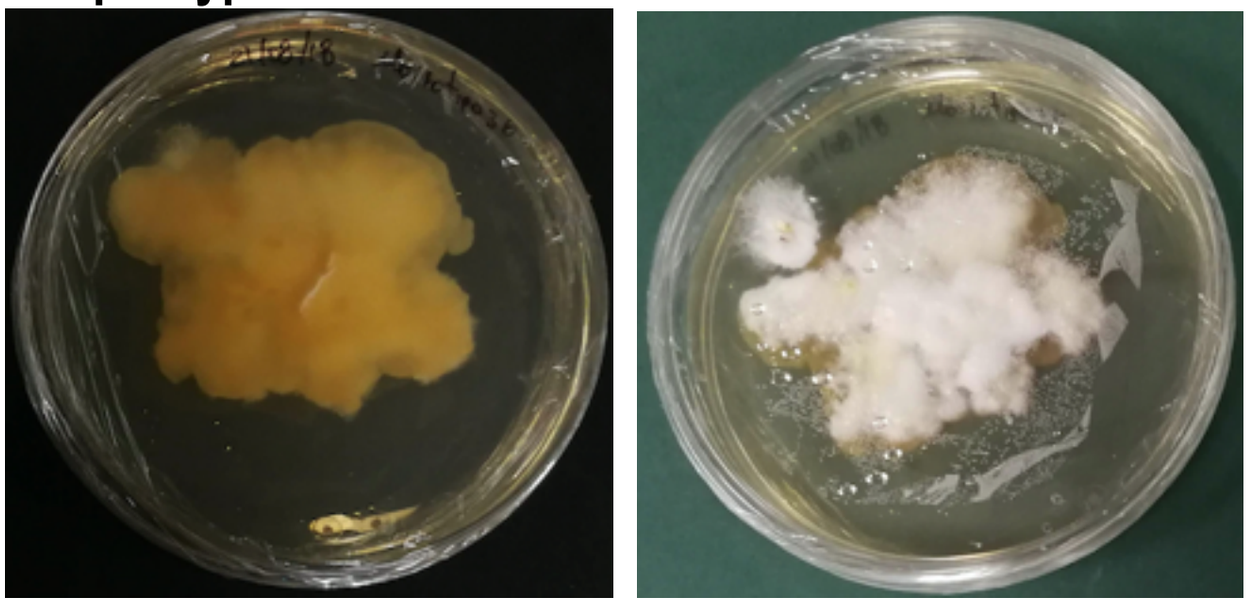
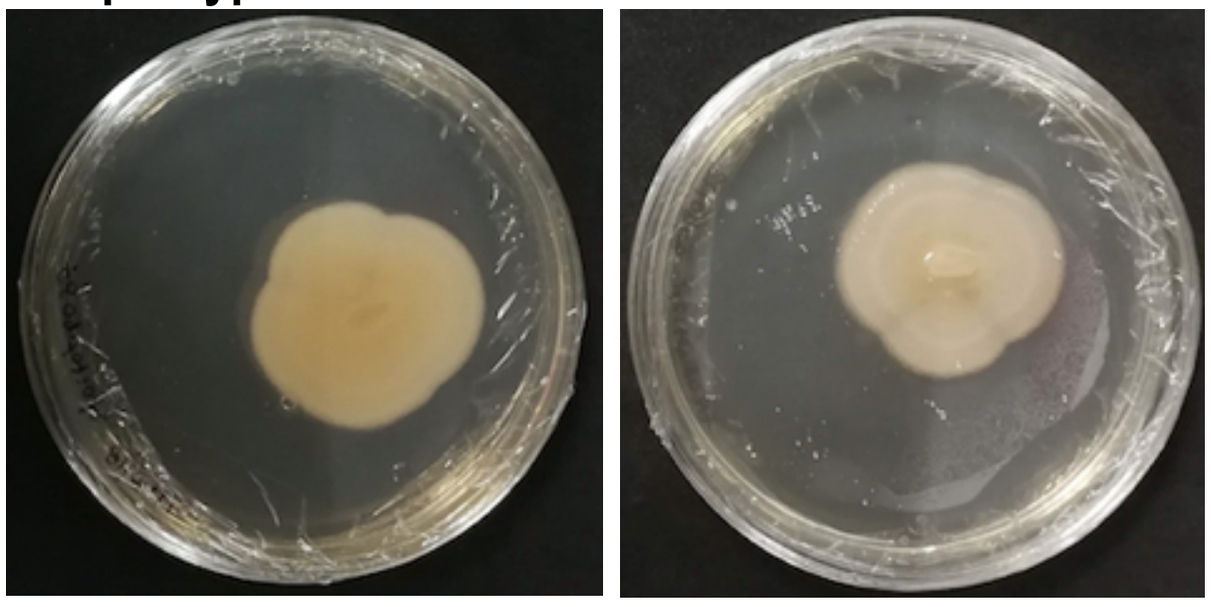
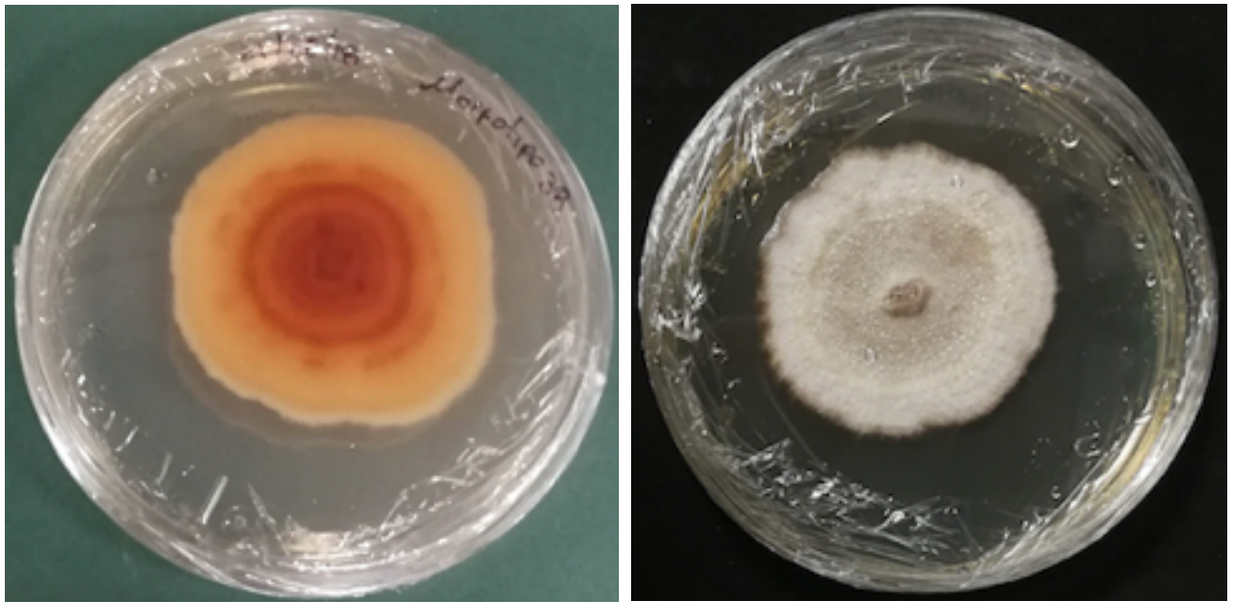


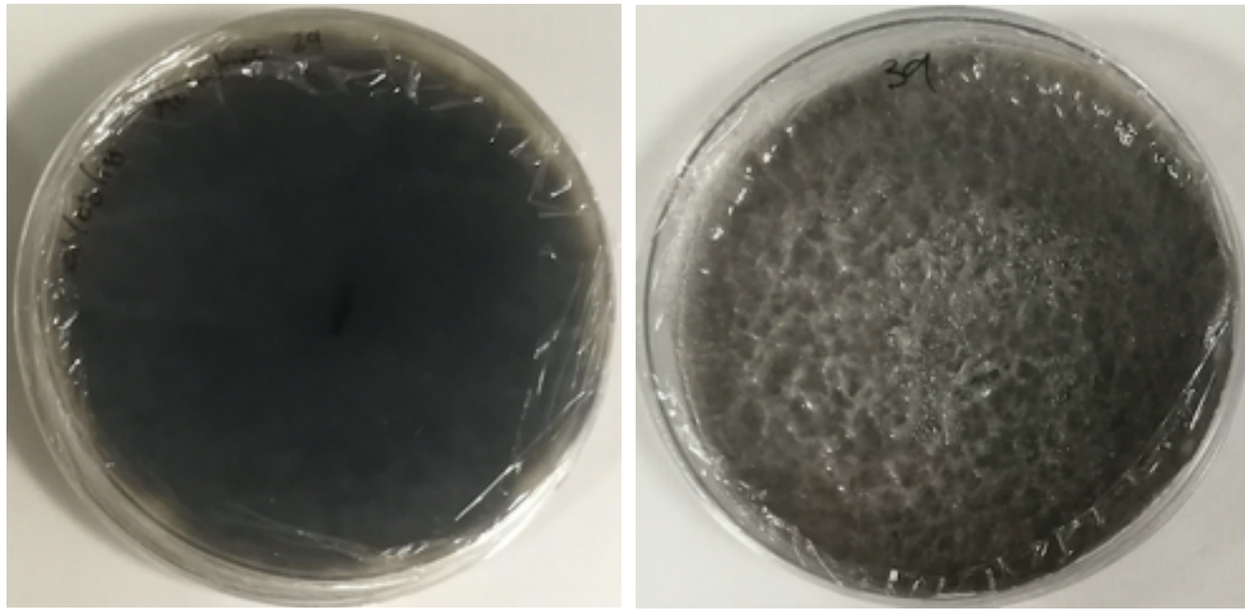
Morphotype 37



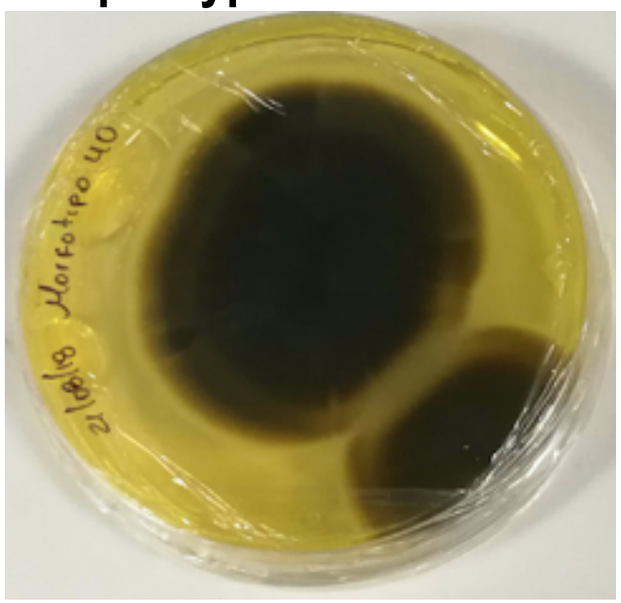
Morphotype 38

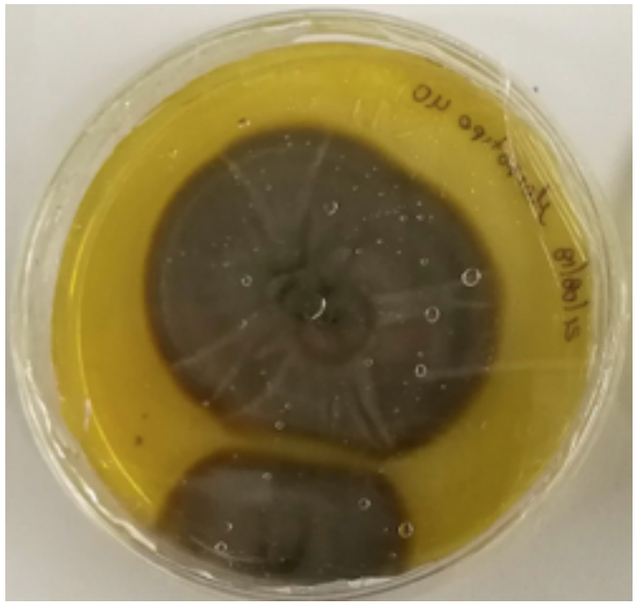


Morphotype 39

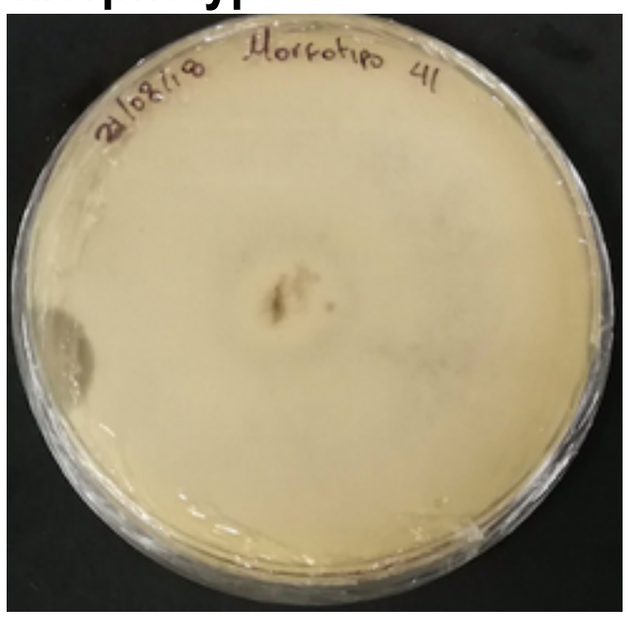


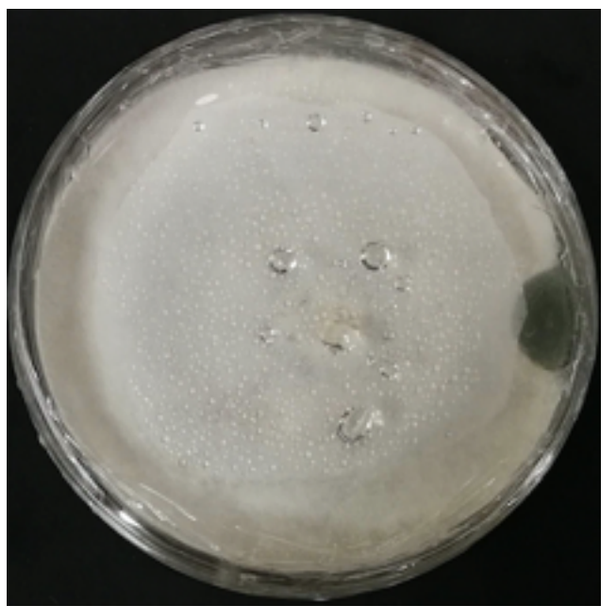
Morphotype 40



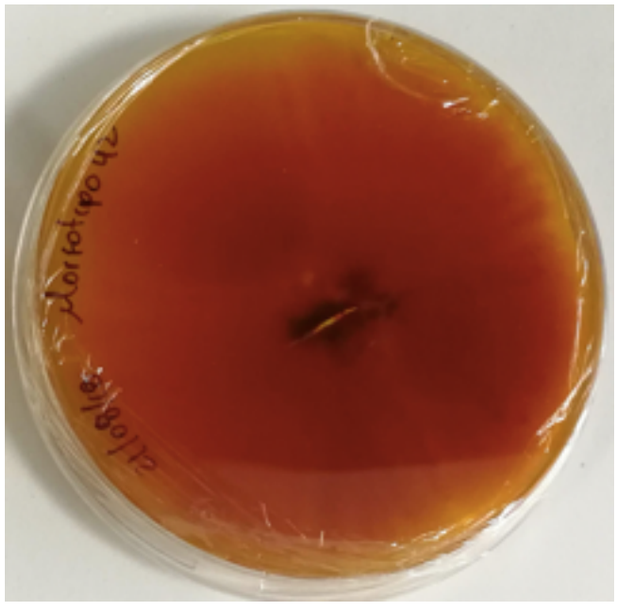


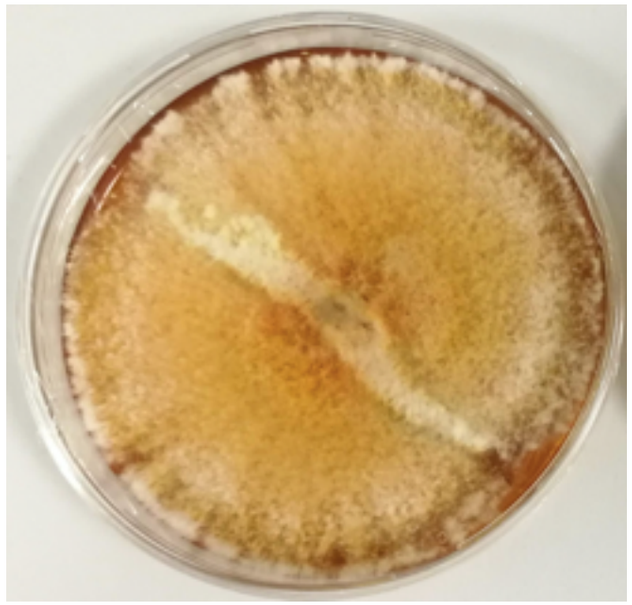
Morphotype 41



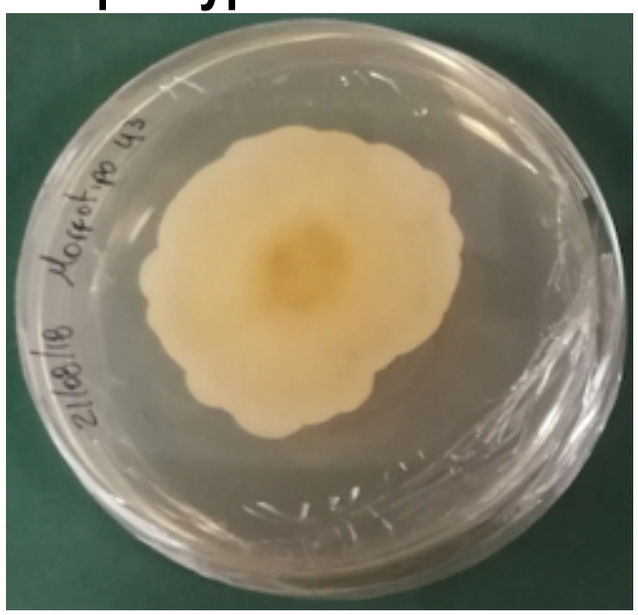


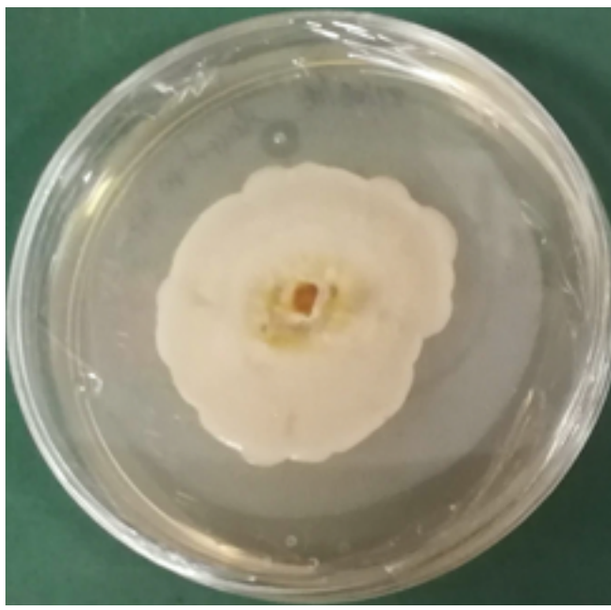
Morphotype 42



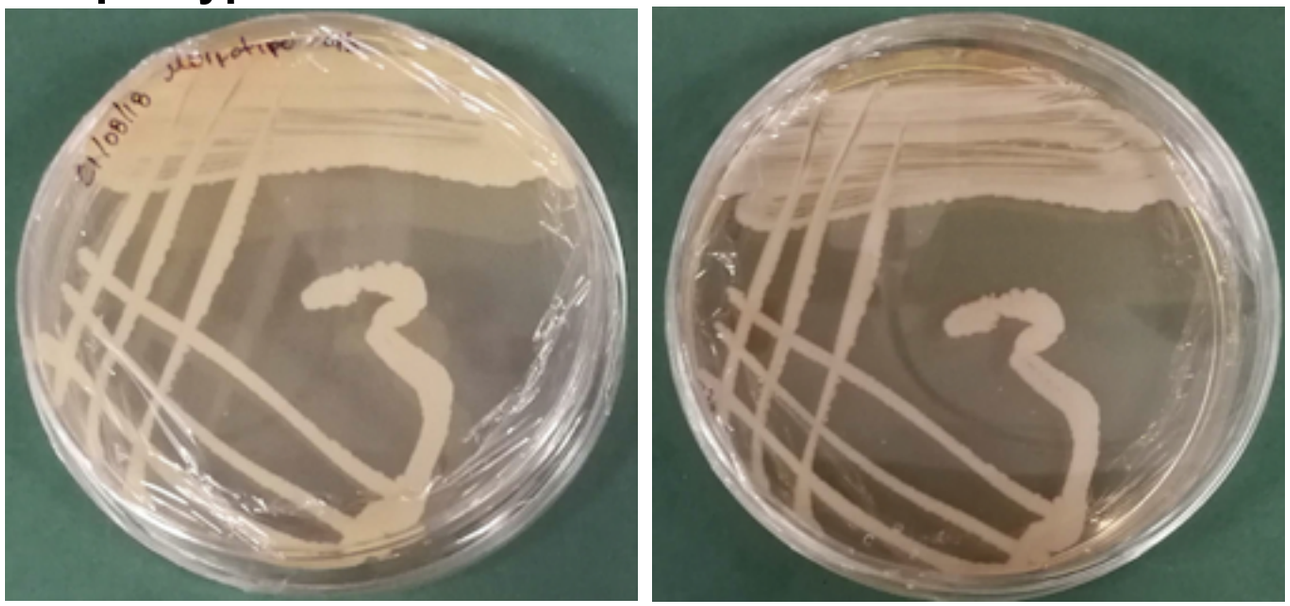


Morphotype 43





Morphotype 45



Morphotype 46