

Establishing a Biofilm Co-culture of *Pseudomonas* and *Aspergillus* for Metabolite Extraction

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[Abstract] Filamentous fungi and bacteria form mixed-species biofilms in nature and diverse clinical contexts (Frey-Klett *et al.*, 2011; Peleg *et al.*, 2010). The interactions between fungi and bacteria, often mediated by secreted metabolites, have important ramifications for the biology of the interacting partners (Frey-Klett *et al.*, 2011). This is particularly true for the bacterium *Pseudomonas aeruginosa* (*P. aeruginosa*) and the fungus *Aspergillus fumigatus* (*A. fumigatus*) which often reside in the same niche such as lungs of cystic fibrosis (CF) patients. Some studies have reported that co-infection with *P. aeruginosa* and *A. fumigatus* could lead to a decrease in lung function relative to their respective single species infection (Amin *et al.*, 2010; Peleg *et al.*, 2010). Metabolite extraction and analysis allow for the characterization of specific microbial metabolites in the polymicrobial biofilm. This protocol describes how to prepare the *Pseudomonas-Aspergillus* co-culture biofilm on solid medium in preparation for metabolite extraction.

Materials and Reagents

1. Plastic spreader (Excel Scientific, catalog number: 5PR-L-510)
2. Falcon tubes (15 ml) (Corning Inc., catalog number: 352196)
3. Petri dish (100 x 15 mm) (Corning Inc., catalog number: 351029)
4. Petri dish (60 x 15 mm) (Corning Inc., catalog number: 351007)
5. Inoculating needle (VWR International, catalog number: 12000-816)
6. Glass vial (10 ml) (VWR International, catalog number: SC66022-300)
7. Aluminum foil
8. *Pseudomonas aeruginosa* culture
9. *Aspergillus fumigatus* culture
10. D-glucose (Thermo Fisher Scientific, Alfa Aesar, catalog number: A1682836)
11. Anhydrous dextrose (VWR International, BDH, catalog number: BDH9230500G)
12. Yeast extract (BD, Bacto™, catalog number: 288620)
13. Agar-agar (Merck Millipore Corporation, catalog number: EM1.01614.0500)
14. Peptone (BD, Bacto™, catalog number: 212120)
15. Luria-Bertani (LB) dehydrated medium (Merck Millipore Corporation, catalog number:

1.10285.5000)

16. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: S5881)
17. GR ACS Sodium Nitrate (NaNO_3) (Merck Millipore Corporation, catalog number: SX06551)
18. NaCl
19. Potassium Chloride (KCl) (VWR International, BDH, catalog number: 0395VBD500G)
20. Potassium dihydrogen phosphate (KH_2PO_4) (Thermo Fisher Scientific, Alfa Aesar, catalog number: 1159436)
21. Boric acid (H_3BO_3) (Sigma-Aldrich, catalog number: B6768)
22. Magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: M1880)
23. Copper(II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: C8027)
24. Zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: Z0251)
25. Ammonium molybdate tetrahydrate $[(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}]$ (Sigma-Aldrich, catalog number: 431346)
26. Manganese(II) sulfate monohydrate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: M7634)
27. Iron(II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: F8633)
28. Ethylenediaminetetraacetic acid disodium salt dehydrate ($\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: E4884)
29. Cobalt(II) chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: 255599)
30. Tween 20 solution (Sigma-Aldrich, catalog number: P2287)
31. Chloroform (VWR International, BDH, catalog number: PL049ZA4)
32. Acetonitrile (Honeywell International Inc., catalog number: AH015-4)
33. PBS-0.01 % Tween 20 buffer (see Recipes)
34. 20x sodium nitrate salts solution (see Recipes)
35. Trace elements (see Recipes)
36. Medium recipes (see Recipes)
 - a. Solid Luria-Bertani (LB) dehydrated medium (see Recipes)
 - b. Solid yeast extract-peptone-dextrose medium (YPD) (see Recipes)

Equipment

1. Incubator (VWR International, model: 1535)
2. Orbital shaker (Eppendorf AG, New Brunswick Scientific, model: I26)
3. Centrifuge (Beckman, model: J20) with rotor (Beckman, model: JLA-10.500)
4. Microscope (Celestron, model: 44345)
5. Hemocytometer (Hausser Scientific, catalog number: 3500)

6. Steel corer (38.1 mm in diameter) (Figure 1)



Figure 1. Steel corer

7. Steel blade (VWR International, catalog number: 55411-050)
8. Steel pestle (VWR International, catalog number: 62400-336)
9. 0.2 μ m-pore-size hydrophilic filter (VWR International, catalog number: 28145-477)
10. Glass culture tube (15 ml) (VWR International, catalog number: 47729-583)
11. Glass centrifuge tube (15 ml) (SP Industries, Wilmad-LabGlass, catalog number: C-8060-15)
12. Nitrogen gas cylinder

Procedure

A. Biofilm co-culture of *Pseudomonas* and *Aspergillus*

1. *Pseudomonas aeruginosa* culture preparation
 - a. Streak-inoculate *Pseudomonas aeruginosa* PA14 strain from -80 °C glycerol stock onto solid LB medium in a 100 x 15 mm Petri dish and incubate at 37 °C for 24 h.
 - b. Immediately prior to use for co-culture experiments, inoculate 5 ml of liquid LB medium in a 15 ml glass culture tube with a single colony from PA14 LB solid medium culture in step A1a and incubate at 37 °C, 225 rpm for 14 h to early stationary phase.
2. *Aspergillus fumigatus* conidia preparation
 - a. Take a ~10 μ l plug from the -80 °C glycerol stock of *Aspergillus fumigatus* AF293 using a sterile inoculating needle and streak onto solid glucose minimal medium (GMM) in a 100 x 15 mm Petri dish and incubate at 37 °C for 5 days (Figure 2).

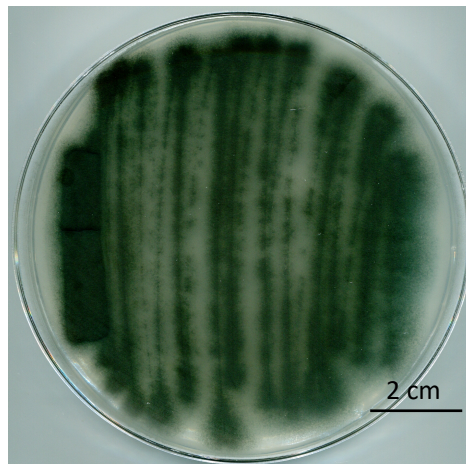


Figure 2. Example of *A. fumigatus* AF293 streak culture after 5 days of incubation at 37 °C

- b. Immediately prior to use, add 5 ml of phosphate buffered saline (PBS) -0.01% Tween 20 buffer (pH 7.2) to the fungal plate and gently scrap the surface of fungal colony with a sterile, plastic spreader.
- c. Collect the conidia suspension from Petri dish and transfer into a 15 ml Falcon tube.
- d. Centrifuge the conidia suspension at 5,000 x *g* for 5 min. Discard supernatant and re-suspend conidia pellet with 5 ml of PBS-0.01% Tween 20 buffer.
- e. Dilute the conidia suspension with PBS-0.01% Tween 20 buffer to a final concentration between 1 to 2 x 10⁶ conidia/ml. Quantify the number of conidia using a hemocytometer (Figure 3).

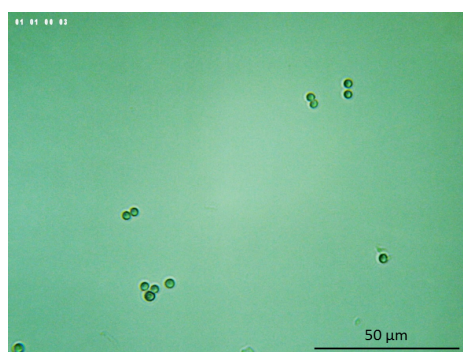


Figure 3. Example of *A. fumigatus* conidia under microscope

3. *Pseudomonas* and *Aspergillus* co-culture biofilm preparation

- a. Pour 10 ml of autoclaved YPD solid medium (1.6% agar) (bottom layer) into a 60 x 15 mm Petri dish and solidify at room temperature.

Note: Prepare multiple YPD agar plates for different co-culture time points and

replicates.

- b. Autoclave YPD solid medium (0.5% agar) (top layer) and cool in 50 °C water bath for 15 min.
- c. After YPD top layer is cooled, inoculate with freshly prepared and vortex-mixed *Aspergillus* conidia suspension to a final concentration of 3.33×10^5 conidia/ml.
- d. Mix the YPD top layer containing conidia thoroughly and pour 3 ml of the mixed top layer onto the solidified bottom layer.

Note: High temperature of autoclaved agar will kill Aspergillus conidia. Thus, it is critical to ensure that the temperature of YPD top layer is cooled enough to the extent that medium bottle can be held with hands. In the meanwhile, agar will solidify if the temperature drops too much. Steps A3c-d should be done as quickly as possible.

- e. Once the top layer is solidified, incubate the plate (fungal biofilm facing up) at 25 °C for 12 h.

Note: A. fumigatus grows slower than P. aeruginosa. 12 h pre-culture is necessary to avoid overwhelming bacterial growth.

- f. Spot-inoculate 10 µl aliquots of freshly prepared early stationary phase *P. aeruginosa* PA14 LB liquid culture in the center of the performed fungal lawn.
- g. Incubate the co-culture biofilm (co-culture biofilm facing up) at 25 °C for an additional 7 days.

Notes:

- i. See Figure 5 for an example of the co-culture biofilm.
- ii. It is critical to synchronize the growth of *P. aeruginosa* (24 h) and *A. fumigatus* (5 d) prior to the preparation of co-culture biofilm.

B. Metabolite extraction

1. After different periods of growth (for example, every two days after inoculation), cut the co-culture biofilm with a sterile steel corer (38.1 mm in diameter), which encompasses the co-culture biofilm.
2. Cut the 38.1 mm diameter extraction core into four, equal quarters using a sterile blade.
3. Transfer one quarter (about 285 mm² of surface) to a glass culture tube.

Note: Transfer both co-culture biofilm and supporting agar (Figure 4).

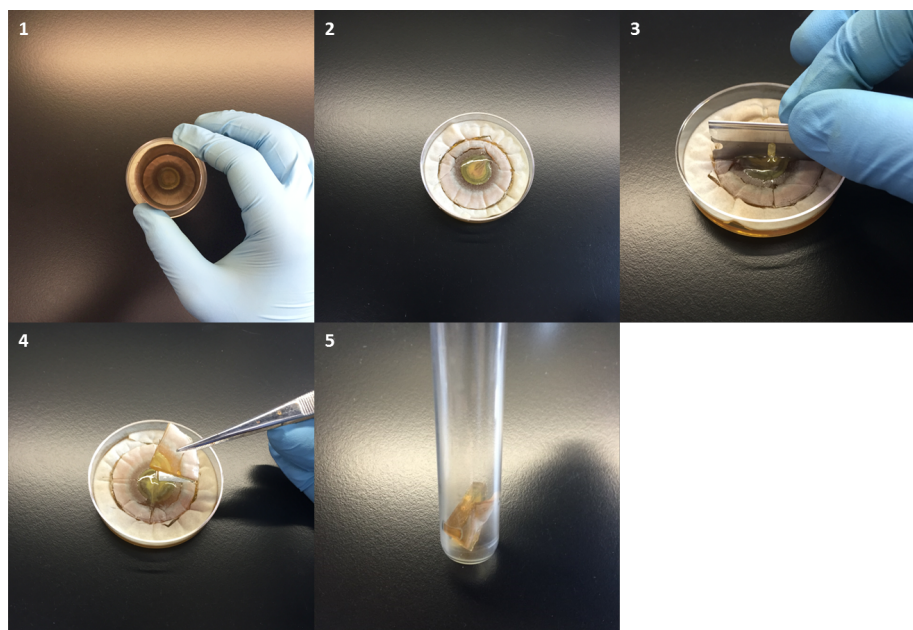


Figure 4. Example of cutting co-culture plate for extraction

4. Add 4 ml milli-Q water and homogenize using a pestle.
5. Add 4 ml chloroform, cap the tube, and vortex vigorously for 15 min.
6. Transfer the mixture to a 15 ml glass centrifuge tube.
7. Centrifuge at 500 x g for 10 min.
8. Collect water phase supernatant and filter through a 0.2 µm-pore-size hydrophilic filter.
9. Collect 1.5 ml of chloroform phase to a 10 ml glass vial.
10. Evaporate chloroform to dryness by using a gentle flow of nitrogen.
11. Re-dissolve the dried extract with 1.5 ml mixture of 50/50% acetonitrile/water solution.
Note: This dissolution solvent can be used for the assessment of metabolites that have similar chemical property as Pseudomonas phenazines, which were analyzed in Zheng et al. (2015).
12. If extracted sample contains metabolites that are unstable in acetonitrile such as 5-methoxy-phenazine-1-carboxylic acid (5-Me-PCA), collect another 1.5 ml aliquot of chloroform phase after step B9, evaporate to dryness under a gentle flow of nitrogen and re-dissolve in 1.5 ml milli-Q water.
Note: Most bacterial and fungal metabolites are light sensitive, thus, it is strongly suggested to protect extracts from light by wrapping all containers with aluminum foil.

Representative data

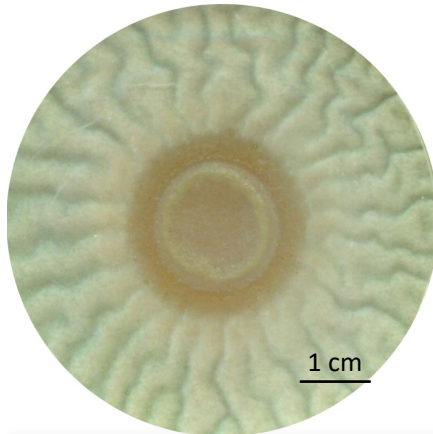


Figure 5. Representative image of the co-culture biofilm of *Pseudomonas aeruginosa* PA14 wild-type (center colony) and *Aspergillus fumigatus* AF293 wild-type (wrinkled biofilm surrounding bacterial colony) after 6 days of incubation at 25 °C

Notes

1. It is critical to keep the same pre-culture condition for both bacteria and fungi before co-culture inoculation. For fungi, you need to ensure the same pre-culture time. For bacteria, early stationary phase culture is recommended in this experiment. OD₆₀₀ can be measured to check bacteria growth. If you use different fungal and/or bacterial strains, it is strongly recommended to do a series of co-cultures with different pre-culture time to determine a condition that gives the best result.
2. Co-culture biofilms can grow in various Petri dishes of different sizes. You can scale up or down the dimension of co-cultures based on your research need. Note that the co-culture biofilm growth may vary in different types of Petri dishes.

Recipes

1. PBS-0.01% Tween 20 buffer (1 L)

NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄	1.42 g
KH ₂ PO ₄	0.24 g
Tween 20 solution	0.1 ml

Mix in about 900 ml milli-Q water and adjust pH to 7.2 with 5 M NaOH solution

Adjust volume with milli-Q water to 1 L and autoclave

2. 20x sodium nitrate salts solution

NaNO ₃	120 g
KCl	10.4 g
MgSO ₄ ·7H ₂ O	10.4 g
KH ₂ PO ₄	30.4 g

Add milli-Q water up to 1 L

Autoclave and store at room temperature

3. Trace elements (100 ml)

ZnSO ₄ ·7H ₂ O	2.2 g
H ₃ BO ₃	1.1 g
MnSO ₄ ·H ₂ O	0.43 g
FeSO ₄ ·7H ₂ O	0.5 g
CoCl ₂ ·6H ₂ O	0.17 g
CuSO ₄ ·5H ₂ O	0.16 g
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.11g
Na ₄ EDTA·2H ₂ O	4.9 g

Add solids into 80 ml milli-Q water, dissolving each completely before adding the next

Add 20 ml of 1 M NaOH solution to adjust the pH to 6.5

Mix solution until solids are completely dissolved

4. Medium recipes

a. Solid glucose minimal medium (GMM) (1 L)

20x sodium nitrate salts	50 ml
Trace elements (mix before using)	1 ml
D-glucose	10 g
Agar-agar	16 g

Mix in 900 ml milli-Q water and adjust pH to 6.5 with 5 M NaOH solution

Adjust volume with Milli-Q water to 1 L

b. Solid yeast extract-peptone-dextrose medium (YPD) (1 L)

Anhydrate dextrose	20 g
Yeast extract	10 g
Bacto peptone	20 g
Agar-agar	16 g
Trace elements (mix before using)	1 ml

Mix in 900 ml milli-Q water, adjust volume with milli-Q water to 1 L, and autoclave

c. Solid Luria-Bertani (LB) dehydrated medium (1 L)

Luria-Bertani medium powder	25 g
*Agar-agar	16 g

Mix in 900 ml milli-Q water, adjust volume with milli-Q water to 1 L, and autoclave

*Note: *Do not add agar when preparing liquid LB medium.*

Acknowledgments

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