

Feb 01, 2025

# Characterization of extracellular polymeric substances (EPS) in co-culture of fungi and bacteria

DOI

<https://dx.doi.org/10.17504/protocols.io.4r3l294rpv1y/v1>

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**Protocol Citation:** Rishi Prasad 2025. Characterization of extracellular polymeric substances (EPS) in co-culture of fungi and bacteria. protocols.io <https://dx.doi.org/10.17504/protocols.io.4r3l294rpv1y/v1>

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**Protocol status:** Working

**We use this protocol and it's working**

**Created:** November 26, 2024

**Last Modified:** February 01, 2025

**Protocol Integer ID:** 112767

**Keywords:** bacterial-fungal interactions, microbiology, co-culture, EPS, extracellular DNA, fungal-bacterial interactions, BFIs, characterization of extracellular polymeric substance, extracellular polymeric substance, fungal interaction, fungal interactions in the context, fungal, fungi, polysaccharide, bacterial isolate, bacteria, bacteria this protocol, purification,

**Funders Acknowledgements:**

**DOE Office of Science, Office of Biological and Environmental Research (BER)**

**Grant ID:** DE-SC0023106

## Abstract

This protocol is intended to be a reference for studying bacterial-fungal interactions in the context of EPS (protein, polysaccharide, and DNA) production in liquid co-cultures of fungal and bacterial isolates. The experimental design, inoculation, sample processing, purification, and quantification steps are described, with measurements.

## Materials

### General supplies

*Micropipettes and tips (10, 200, 1000 $\mu$ L)*

*Microcentrifuge tubes (1.5, 2.0mL)*

*Microcentrifuge tube racks*

*Serological pipets (5, 10, 25mL)*

*Printable labels*

*50mL centrifuge tubes including high-performance tubes*

*Large centrifuge with temperature control*

*High-speed micro-centrifuge*

*Mini centrifuge*

*pH probe*

*Autoclave*

*Freezers (-20C, -80C)*

*Refrigerator*

*Paper towels*

*Kimwipes*

*Microplate reader*

*Lab tape*

*Writing utensils (pens, fine-tip permanent markers, pencils)*

*Deionized water*

*Double-distilled or MilliQ water*

*Volumetric flasks*

*Graduated cylinders*

*Biohazard and normal sharps disposal*

*General biohazard waste*

*Nitrile lab gloves*

*Reagent-grade ethanol*

### Culture work

*Standard culture reagents*

*Petri plates*

*Test tubes*

*Test tube racks*

*Test tube caps*

*250mL flat top Erlenmeyer flasks/caps*

*Test tube shaker/incubator*

*Large flask shaker/incubator*

*Laminar flow hood/standard supplies (spatulas, 7mm agar punch, scalpel, flamer, 70% ethanol spray, etc.)*

### Sample processing/dialysis/lyophilization

*Vacuum filtration apparatus (Buchner funnels, filter flasks, tubing)*

*Whatman 1 filter papers*

*Buckets (5-gallon)*

*SnakeSkin™ Dialysis Tubing (35mm I.D., 10kDa)*

<https://www.thermofisher.com/order/catalog/product/88245>

*Dialysis clips*

*Nylon string*

*Walk-in refrigerator*

*60mL syringes*

*Syringe filters (0.2 µm)*

*Lyophilizer*

*Cylindrical flasks and supplies for attaching to lyophilizer*

*Acid-wash container*

### **Protein, carbohydrate, DNA assays/qPCR**

*Pierce™ BCA Protein Assay Kits*

<https://www.thermofisher.com/order/catalog/product/232>

*Qubit 4 Fluorometer | Thermo Fisher Scientific - US*

<https://www.thermofisher.com/us/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit/models/qubit-4.html>

*Qubit™ dsDNA Quantification Assay Kits (High-Sensitivity)*

<https://www.thermofisher.com/order/catalog/product/Q32851>

*D -(+)-Glucose analytical standard 50-99-7*

<https://www.sigmaaldrich.com/US/en/product/supelco/47249?srsId=AfmBOooPqPMS7K8CDLQ-bnKOdniYRAfwP-8mnJ-yldQsEL7gVh6WletX>

*96-well microplates*

*Tube organization/storage boxes*

*Fume hood*

*1000µL micropipette filter tips*

*Hazardous waste storage vessels*

*Butyl rubber gloves*

*Safety goggles*

*Lab coat*

*Phenol*

*Concentrated sulfuric acid*

*Chloroform:Isoamyl Alcohol 24:1*

*Isopropanol*

*CTAB buffer*

*PVP (polyvinylpyrrolidone)*

*Tris buffer*

*NanoDrop One Microvolume UV-Vis Spectrophotometer | Thermo Fisher Scientific - US*

<https://www.thermofisher.com/us/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/uv-vis-spectrophotometry/instruments/nanodrop/instruments/nanodro-one.html>



## Preparation of fungal inoculum

- 1 Isolate fungal specimens from environmental samples and/or select available strains from cold storage and subculture on yeast-extract malt agar (YMA). Once cultures are isolated, store at 4 °C until ready for use (up to ~3 weeks), re-plating as necessary.

### Note

All organisms should be assigned taxonomy. Sanger sequencing is a relatively quick and easy way to do this.

- 2 When ready, subculture a 7 mm diameter agar plug of each isolate from step 1 to fresh agar plates.
- 3 Allow growth for four days (may vary depending on the fungus, but choose a length of time that allows all fungi sufficient growth time to collect a plug from).
- 4 After this period, transfer a 7 mm agar plug of the freshly grown fungus to the appropriate sample flask.

### Note

Hyphae at the edges of colonies are freshest. This protocol is designed for growth in 250 mL straight-neck Erlenmeyer flasks for sufficient EPS yield but can easily be modified for test tube, microcentrifuge tube, or microplate culture depending on the requirements of the researcher. In general, the sample should not exceed 20% of the volume of the vessel (except in smaller volumes) to ensure adequate air exchange and proper mixing.

## Preparation of bacterial/yeast inoculum

- 5 Obtain bacterial culture and/or yeast from cold storage.
- 6 Streak on LB agar plates, then allow growth for three days.

### Note

Single colony streaking will ensure that you are working with a pure culture.



- 7 Autoclave test tubes containing 5 mL of basal Modified Melin-Norkrans (MMN) liquid media.

#### Note

When preparing MMN media, omit adding malt or yeast extract, replacing it with 5g/L tryptone (malt or yeast extract may contain trace DNA, polysaccharides, or protein which are difficult to purify later; tryptone generally contains peptides small enough to purify). Also, omit agar and reduce glucose concentration to <1 g/L (this may encourage competitive/mutualistic interactions by constraining resources). Other modifications can be made as needed.

- 8 After three days, use a sterile loop to scoop a single bacterial colony and submerge the loop in an autoclaved test tube with MMN medium for one second.

- 9 Incubate test tubes for 24 hours in the test tube shaker at 25 °C and 100 RPM.

- 10 Vortex the contents of the test tubes. In a sterile manner, transfer aliquots of yeast and bacterial solution from test tubes into the appropriate sample flasks.

#### Note

The aliquot volumes will depend on the bacterial/yeast strain and should be pre-determined using OD/CFU assays. These aliquots can be added to the sample flask concurrently with the fungal culture, or afterward. Adding the aliquot after the fungus (e.g. 2-3 days) may allow the fungus to establish better if the bacteria selected rapidly replicate. Let "aliquot A" refer to the volume of bacterial liquid culture to use and "aliquot B" refer to the volume of yeast liquid culture to use for steps 13-16.

## Co-culture experimental procedure

- 11 Determine which samples to run, particularly which ones contain fungi only, bacteria only, both, or neither (negative control). For pairwise co-culture experiments, each fungus and bacterium should be paired, run individually, and with a negative control, in replicates (triplicates at the very least). Determine how many sample flasks can be incubated at once depending on the capacity of the shaker setup; if there are more samples than the shaker can hold (before replicates), the samples in each batch should be randomized.



## Note

For example, if there are three fungi and three bacteria in the experiment, there will be at least 48 co-culture flasks total ([9 co-cultures + 3 bacterial isolates + 3 fungal isolates + 1 negative control] \* 3 replicates)

- 12 Pre-heat a shaking incubator to 25°C and set the shaking speed to 50 RPM.

## Note

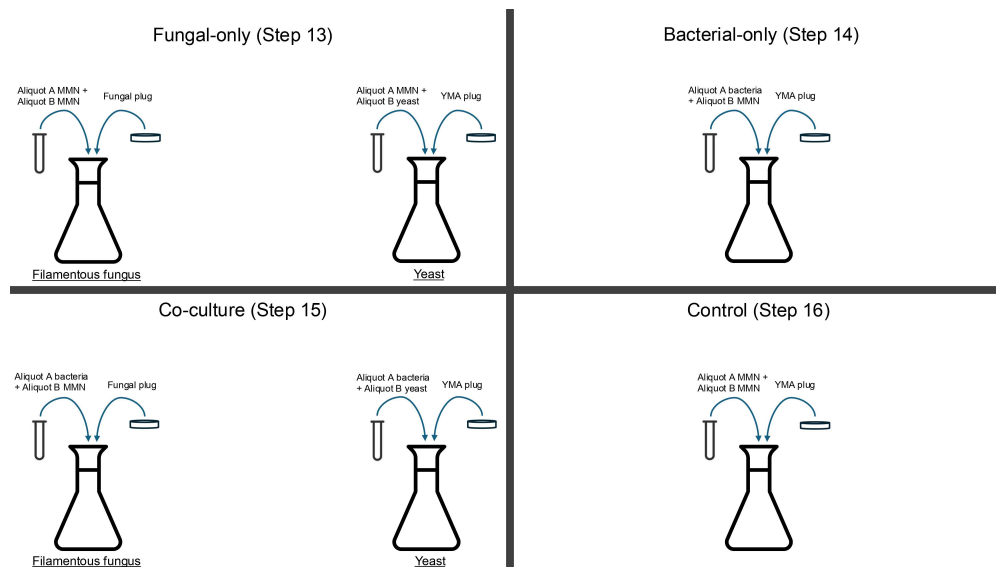
The shaking speed and temperature can be modified to suit the researcher's needs. Increasing the shaking speed may promote biofilm formation due to shear stress, but excessive speed may degrade biofilms or disrupt fungal growth.

- 13 To the *fungi-only* experiment 250 mL Erlenmeyer flasks, add a 7 mm diameter core of tissue from actively growing hyphae from the appropriate YMA plate. Then, add aliquots A and B (  [go to step #10](#) ) of sterile MMN liquid media. Immediately seal with the flask cap and label the flask.
- 13.1 If the fungus is a yeast then add aliquot A of sterile MMN, aliquot B of the yeast solution, and a 7 mm diameter core of sterile YMA media).
- 14 To the *bacteria-only* experimental 250 mL Erlenmeyer flasks, add a 7 mm diameter core of fresh, sterile YMA agar. Then add aliquot A (  [go to step #10](#) ) of the appropriate bacteria and aliquot B of sterile MMN liquid media. Immediately seal with the flask cap and label the flask.
- 15 To the experimental 250 mL Erlenmeyer flasks containing *both fungi and bacteria*, add a 7 mm diameter core of tissue from actively growing hyphae from the appropriate fungal culture. Then, aliquot A (  [go to step #10](#) ) of the appropriate bacteria and aliquot B of sterile MMN liquid media. Immediately seal with the flask cap and label the flask.
- 15.1 If the fungus is a yeast then add aliquot A of the appropriate bacteria, aliquot B of the yeast, and a 7 mm diameter core of sterile MMN media.
- 16 To the experimental 250 mL Erlenmeyer flasks containing the control (no inoculation), add a 7 mm diameter core of fresh, sterile YMA agar. Then add aliquots A and B (  [go to step #10](#) )

of sterile MMN liquid media. Immediately seal with the flask cap and label the flask.

#### Note

Steps 11-16 should all be performed using proper aseptic technique in a laminar flow hood. Make sure to prepare sterilized pipette tips in advance. Once flask caps are secured, flasks can be removed from the hood but should be additionally sealed with Parafilm, as airflow in the shaking incubator will contaminate flasks with only the lid on.



**Sample schematic of inoculation of isolates, co-cultures, and controls.** Aliquot A refers to the previously determined volume of bacterial liquid culture to be transferred and aliquot B refers to the previously determined volume of yeast liquid culture to be transferred. This is important for ensuring that the volume and composition of all sample flasks are as close as possible.

- 17 Secure sealed sample flasks to the shaking incubator, ensure temperature and shaking speed are at the target setting, and allow to incubate for seven days.
- 18 During the wait period, label four 50 mL centrifuge tubes for each sample including at least one high-performance tube that can withstand freezing at  $-80^{\circ}\text{C}$ .



- 19 After seven days, document any visual observations about the flasks and take pictures of each flask.

## Removal of microbial biomass

- 20 Set the centrifuge to 4 °C and 2500xg for 60 minutes.
- 21 Carefully pour the contents from the sample flasks into individually labeled, pre-weighed, 50 mL centrifuge tubes.
- 22 Centrifuge all samples at previously configured settings.
- 23 Decant the supernatant solutions into a new set of individually labeled 50 mL centrifuge tubes and store at 4 °C.
- 24 Add 5 mL 1X Tris-EDTA (TE) buffer to the first set of tubes containing the microbial pellet. Shake the pellets vigorously by hand to re-suspend the pellet and incubate overnight at 4°C (these tubes can be stored directly in the centrifuge if desired).

### Note

If the researcher is only interested in extracting diffusible EPS, skip this step and the next two steps. This secondary extraction is used to extract capsular EPS, or EPS that is bound to microbial cells.

- 25 Centrifuge at 2500xg for another 60 minutes at 4°C.
- 26 Decant the supernatant solutions into the second set of individually labeled 50 mL centrifuge tubes containing the previous supernatant solutions. Use a third set of tubes if necessary.
- 27 Vacuum filter all samples containing filamentous fungi through a Whatman 1 filter paper, thoroughly washing or replacing filter papers between samples, and filtering into a new or acid-washed centrifuge tube. Store all tubes on ice during this process.

**Note**

Samples not containing filamentous fungi do not need to be vacuum filtered.

- 28 Carefully transfer each solution to a syringe fitted with a 0.22  $\mu\text{m}$  filter, and filter into new individually labeled high-performance centrifuge tubes. Ensure that pellets are not disturbed when transferring the solutions, or filtration will be excessively arduous.

**Note**

If it is difficult to pass these samples through the filter, there may still be a lot of cells in solution. Centrifuge all samples at 2500xg for 60 minutes at 4°C. Ensure that no opacity remains in each sample. Then, retry the filtering step.

**Note**

It is essential to work with the EPS solutions on ice or at 4°C whenever possible because exposure to room temperature for an extended period can lead to contamination or sample degradation.

- 29 Store supernatant solutions at -80 °C until ready for purification.
- 30 Place the first tubes containing the microbial pellets in an oven set to 70 °C to dry them.
- 31 After drying, collect the mass of the tube. Subtract the original mass of the tube from this mass to calculate the dry microbial biomass produced. Save these tubes for qPCR analysis.

## Purification

- 32 Remove up to 16 tubes from the -80 °C freezer. Place in 4 °C refrigerator to thaw slowly over ~24 hours.

**Note**

Processing up to 16 samples at a time is appropriate for the volume of dialysis fluid used. Any more samples may lead to unsatisfactory removal of impurities.

- 33 Fill two 5-gallon buckets with tap water, label them, and chill them at 4°C for at least 12 hours.

**Note**

A continuous flow system is the most efficient and effective method to perform dialysis on a large sample volume. However, the methods listed here are useful if such a system is not feasible.

- 34 Prepare 16 ~10cm sections of dialysis membrane (SnakeSkin™ Dialysis Tubing, 10K MWCO, 35mm dry I.D.) and place them in ddH<sub>2</sub>O to hydrate. Ensure that two membrane clips are available per section of membrane. Also have ready an acid-washed, small funnel for each section.
- 35 Retrieve one of the 5-gallon buckets filled with tap water, and assemble all supplies for preparing dialysis membrane.
- 36 Carefully retrieve a section of dialysis tubing, straighten it out, and pat dry on a paper towel. Then attach one clip on one end. Open the other end of the membrane and place a funnel into the opening.
- 37 Slowly pour the EPS extract into each section of dialysis tubing through the funnel. Remove the funnel, consolidate as much solution as possible toward the sealed end of the membrane, then secure a second clip on the unsealed end of the membrane. Tie a nylon string with a tag marked with the corresponding sample number to one of the clips, then place it into the bucket containing chilled tap water. Repeat for remaining samples.

**Note**

It is best to mark the tags with a pencil to prevent bleeding.

- 38 Chill this bucket at 4 °C for 8-12 hours. After this time, transfer these samples into the second bucket. Empty the water from the first bucket and replace with deionized water.



- 39 Repeat step 38  [go to step #38](#) , except transfer samples to the first bucket containing deionized water and replace the second bucket also with deionized water.
- 40 Repeat step 38  [go to step #38](#) , except replace the first bucket with double distilled water.
- 41 Repeat step 38  [go to step #38](#) , except transfer samples into the first bucket containing double distilled water and replace the second bucket also with double distilled water.
- 42 Repeat step 38  [go to step #38](#) , except replace the first bucket with tap water if another batch of samples will be dialyzed soon.
- 43 Retrieve the bucket containing the samples and using acid-washed funnels, then carefully transfer the retentate into acid-washed or new centrifuge tubes.
- 44 Using a serological pipette, transfer a precise volume (it is easiest to do this by dividing the total volume of the original liquid culture plus the added 5 mL TE buffer by 2 for later calculations since different samples will have different volumes of EPS extract by this point) of each retentate into new, labeled, and pre-weighed high-performance centrifuge tubes.
- Note**
- Note: Centrifuge tubes need to be high-performance, otherwise they can crack under -80°C storage and result in sample loss during lyophilization.
- 45 Wrap each unlidded centrifuge tube opening securely with parafilm, then poke a few small holes through with a fine micropipette tip or needle.
- Note**
- This step is to prepare the tubes for lyophilization.
- 46 Store tubes on freeze-safe centrifuge tube racks at -80°C, at an angle if possible (freezing the solution along the sides of the tube will allow lyophilization to occur faster).



#### Note

Some centrifuge tube racks will crack tubes or make them impossible to remove without breaking the rack when stored at this temperature. It is worth testing, because this can result in sample loss.

## Lyophilization

- 47 Remove the tubes from storage at -80 °C and place in a cooler.
- 48 Quickly transport the tubes to a facility with a lyophilizer and immediately place back into a -80 °C freezer.
- 49 Load the sample tubes and allow complete sublimation of the water from samples. This can take 24-48 hours depending on sample volume and how the samples were frozen. Periodically check the machine to make sure there are no vacuum leaks, as this can adversely affect the samples by leading to premature melting.

#### Note

It is useful to try lyophilizing a set of test samples prior to actual experimental samples because the machine itself takes some practice to use properly and sample loss can easily occur if the machine is not set up correctly.

- 50 Once dry, remove the samples from the apparatus, remove the parafilm (tap the parafilm gently to release any particulates accumulating on the material), and recap the sample tubes. Turn off and clean the machine.
- 51 Weigh each of the dried samples and subtract the original tube mass to get the mass of EPS collected.
- 52 Add 2 mL of 1X Phosphate Buffered Saline (PBS) to each tube to redissolve the lyophilizate. It may be difficult to fully dissolve the material but agitate vigorously by hand and/or vortexer until fully homogeneous. If these solutions are rich in polysaccharides, they may become too viscous to homogeneously mix, in which case more PBS may be required for complete dissolution. Quickly centrifuge the tubes to spin down the solution.

**Note**

The volume of PBS added to redissolve the lyophilizate is necessary for later calculations so make sure this volume is precise and documented.

- 53 Transfer this solution to 2.0 mL microcentrifuge tubes. Organize these tubes in a PCR box, then freeze at -20 °C.

**Note**

If there is enough solution to fill up the microcentrifuge tube more than about 80%, the tubes can pop open in the freezer. To prevent this, two or more tubes can be used instead.

## Bicinchoninic Acid (BCA) Protein Assay

- 54 Obtain samples and allow to thaw. When thawed, vortex thoroughly until homogeneous.
- 55 Add 12.5 µL of each sample to 1.5 mL microcentrifuge tubes. Add 87.5 µL of 1X PBS solution to each microcentrifuge tube, then vortex and spin down. Store in the refrigerator immediately.

**Note**

This represents an 8x dilution; it may be appropriate to use a 4x or 6x dilution if more PBS was used to dissolve the lyophilizate.

- 56 Obtain a *Pierce BCA Protein Assay Kit*. Prepare the standards according to the recommended instructions in the manual for the microplate procedure and use PBS as the diluent.
- 57 Prepare an additional set of 1.5 mL microcentrifuge tubes for each sample and pipette 100 µL of each standard into these tubes.
- 58 Prepare an appropriate amount of working reagent for the assay according to the instructions in the manual. Each sample and standard will require 800 µL of working reagent; prepare enough working reagent for at least two additional samples.
- 59 Pipette 800 µL of working reagent into each sample and standard tube. Mix well, then pipette 200 µL of each sample and standard into an appropriate number of microplates in triplicate.



- 60 Incubate the plates at 37° C for 30 minutes.
- 61 Read the plate(s) at 562 nm on a plate reader and save the data to Excel.

#### Safety information

Store used BCA assay reagents and plastic waste to be disposed of properly, as they are toxic to aquatic life.



## Protein data processing

- 62 Consolidate the Excel data into one spreadsheet. Transfer the absorbance data corresponding with the standards to one column and the known concentration in another column.
- 63 Average the technical replicates for each standard in another column. Subtract the average absorbance value for the blank standard from all other standards, including itself. Then plot with the x-axis as the absorbance and the y-axis as the concentration. Use a 2nd order polynomial model to fit the data (using the LINEST function) and use the FALSE argument to force the y-intercept to 0.
- 64 Now, transfer all the sample absorbance values to a new column and use a second column to designate sample IDs. Subtract the average blank standard absorbance from all samples in a third column.
- 65 Using the polynomial equation from step 63 [↗ go to step #63](#) , plug the blank-corrected sample absorbances in for x to solve for y, the BSA-equivalent protein concentration. Multiply the dilution factor and the volume used to dissolve the lyophilizate with the protein concentration and divide by the volume of EPS extract that was lyophilized to obtain the estimated protein concentration in the EPS extract. This final concentration should be used for all further data analysis and is expressed in µg/mL.

## Phenol sulfuric acid carbohydrate assay

- 66

**Safety information**

Review SDS for proper PPE and precautions to take when handling sulfuric acid and phenol. It is advisable to work in a fume hood and imperative to contain liquid and plastic/glass waste for proper disposal.

67 Obtain 500 mg analytical grade d-glucose standard.

67.1 Dissolve in 1 mL PBS ([d-glucose]=500 mg/mL) by adding PBS directly to the vial containing the solid standard and agitating carefully until fully dissolved.

67.2 Transfer a 10  $\mu$ L aliquot from step 2 to 990  $\mu$ L PBS ([d-glucose]=5mg/mL) in a 1.5mL microcentrifuge tube and vortex thoroughly. Immediately store the stock solution at -20 °C and re-use for future assays.

67.3 Transfer a 180  $\mu$ L aliquot from step 3 to 1320  $\mu$ L PBS ([d-glucose]=0.6mg/mL) in a 2.0mL microcentrifuge tube and vortex to mix. This is the stock solution for producing dilutions.

68 Prepare nine 1.5 mL microcentrifuge tubes for standards. Follow dilution scheme below for preparation of standards and vortex mix/spin down standards sequentially.

| Vial | Volume PBS ( $\mu$ L) | Volume + Source of Stock ( $\mu$ L) | Final [d-glucose] ( $\mu$ g/mL) |
|------|-----------------------|-------------------------------------|---------------------------------|
| A    | 0                     | 100 (stock)                         | 600                             |
| B    | 100                   | 200 (stock)                         | 400                             |
| C    | 300                   | 300 (stock)                         | 300                             |
| D    | 150                   | 150 (vial B)                        | 200                             |
| E    | 300                   | 300 (vial C)                        | 150                             |
| F    | 300                   | 300 (vial E)                        | 75                              |
| G    | 300                   | 100 (vial D)                        | 50                              |
| H    | 100                   | 100 (vial G)                        | 25                              |
| I    | 100                   | 0                                   | 0                               |

**Example dilution scheme for standards in phenol-sulfuric acid assay**

**Note**

Steps 67-68 should be performed quantitatively so pipetting and mixing needs to be precise to ensure downstream analysis is accurate and reproducible

- 69 Add 12.5  $\mu$ L of each sample to 1.5mL microcentrifuge tubes. Add 87.5  $\mu$ L of PBS to each microcentrifuge tube, then vortex and spin down. Store in the refrigerator.

**Note**

This represents an 8x dilution; it may be appropriate to use a 4x or 6x dilution if more PBS was used to dissolve the lyophilizate.

- 70 Add 100  $\mu$ L of each standard to microcentrifuge tubes. Store in the refrigerator.
- 71 Transfer concentrated sulfuric acid to a 50mL centrifuge tube and keep at hand. Calculate the volume needed by multiplying the number of samples and standards by 0.5mL.
- 72 Prepare the 5% aqueous phenol solution by diluting concentrated phenol (~90%) with the appropriate volume of water in a 50 mL centrifuge tube. Calculate the total volume of 5% phenol solution needed by multiplying the number of samples and standards by 0.100 mL and add an additional 3-5 mL. Then back-calculate the volume of water and concentrated phenol to add to reach the total volume and dispense it in the centrifuge tube. Vortex well and wrap the tube in foil as phenol is light-sensitive and highly susceptible to oxidation.
- 73 Pipette 100  $\mu$ L 5% phenol into each microcentrifuge tube, then vortex.
- 74 Carefully, but promptly pipette 500  $\mu$ L concentrated sulfuric acid into each tube, then vortex. Gradually dispense the sulfuric acid; if it is dispensed too quickly, it can splash and contaminate other samples.
- 75 Start a timer for 30 minutes. During this time, pipette 200  $\mu$ L of each solution into three wells in a 96-well plate, according to a pre-determined plate layout.
- 76 After at least 30 minutes, read the plate(s) at 490 nm on a plate reader and save the data to Excel.

- 77 Determine that no absorbances from any samples exceed those of the most concentrated standard, otherwise, these will surpass the limit of quantitation and are not valid. If this is the case, samples must be diluted by a higher factor and the entire assay must be repeated with all such samples.

#### Note

If some samples are within range, these do not have to be repeated but it must be ensured that sample concentrations are calculated using a standard curve from the specific assay that those samples are from. In short, if a repeat assay is performed, two standard curves must be prepared for samples in each of the two assays.

## Polysaccharide data processing

- 78 Consolidate the Excel data into one spreadsheet. Transfer the absorbance data corresponding with the standards to one column and the known concentration in another column.
- 79 Average the technical replicates for each standard in another column. Subtract the average absorbance value for the blank standard from all other standards, including itself. Then plot with the y-axis as the absorbance and the x-axis as the concentration. Use a linear model to fit the data (using the LINEST function) and use the FALSE argument to force the y-intercept to 0.

#### Note

An indicator of a precise and accurate standard curve is the  $R^2$  value of the linear fit and 0.99 is a good target. Anything below this may indicate issues either with the assay or with standard preparation. Individual standards can be removed from the curve if it improves the linearity, but it may be necessary to repeat the assay.

- 80 Now, transfer all the sample absorbance values to a new column and use a second column to designate sample IDs. Subtract the average blank standard absorbance from all samples in a third column.
- 81 Using the linear equation from step 2, plug the blank-corrected sample absorbances in for  $y$  to solve for  $x$ , the BSA-equivalent protein concentration. Multiply the dilution factor and the volume used to dissolve the lyophilizate with the protein concentration and divide by the volume of EPS extract that was lyophilized to obtain the estimated protein concentration in the EPS extract. This final concentration should be used for all further data analysis and is represented in  $\mu\text{g/mL}$ .



## Qubit DNA Assay

- 82 Obtain a Qubit high-sensitivity (HS) kit for dsDNA quantitation and prepare standards and buffers according to the instructions in the manual.
- 83 Use the maximum sample volume for all samples initially (20  $\mu$ L) and run the assay. Make note of any samples that exceed the range of quantitation and record all concentrations for samples that are within the range.
- 84 If any samples exceeded the range of quantitation, perform the assay again but only use 10  $\mu$ L of sample and input this new value into the instrument.
- 85 Because the instrument already accounts for dilution and calculates the concentration of DNA in the original sample, the concentration of DNA in the lyophilizate diluted in buffer is given. Calculate the concentration of DNA in the original EPS extract by multiplying by the volume of the resuspended lyophilizate and dividing by the volume of EPS extract that was lyophilized.

## qPCR analysis

- 86 Perform DNA extractions on both the biomass pellets from the original co-culture flasks and the EPS extracts. Extractions are only necessary for samples that were inoculated with both a fungus and bacterium (i.e., single isolate and negative control samples do not need to undergo this analysis). For the biomass samples, re-suspend them in 10 mL of CTAB buffer and homogenize them with a mortar and pestle. Add 100  $\mu$ L of this suspension to 200  $\mu$ L CTAB + 2% PVP, and add 300  $\mu$ L CIA. For the EPS samples, use 200  $\mu$ L of the extract mixed with 100  $\mu$ L of CTAB + 2% PVP, then add 300  $\mu$ L CIA. Adjust the isopropanol precipitation step as needed if there is too much DNA lost or too much polysaccharide precipitation.

### Note

The standard DNA extraction protocol was used here, but other protocols could work better.

**Note**

The EPS samples will have much lower DNA than the biomass samples and may be more challenging to produce a high-quality extract from. Additionally, some bacteria may produce a high quantity of polysaccharides that co-precipitate with the DNA. In this case, try adjusting the amount of ice-cold isopropanol used and if there is still a large chunk of polysaccharide at the bottom of the tube, flick gently for it to slide out (some DNA will be lost but it will be a lot cleaner).

- 87 Check the purity of these samples, and troubleshoot as necessary to get DNA samples as pure as possible.

**Note**

Biomass samples are relatively straightforward to extract pure DNA from.

- 88 Normalize each sample to 100 ng/ $\mu$ L in PCR tubes or a 96-well plate, then amplify each sample using both 16S and ITS primers with replicates (duplicates at the very least).

**Note**

It is important to try to remove as many polysaccharides as possible in the DNA extraction because they are known as PCR inhibitors. However, as long as the reaction proceeds, this does not matter assuming the amplification of ITS and 16S in each sample are inhibited to the same degree, because the ratio of bacteria to fungi is of interest, not differences between samples.

**Note**

The purpose of this analysis is both to compare the amount of biomass produced by the fungal and bacterial organisms in the co-culture and to compare the amount of EPS-DNA produced by each of these organisms.

## Protocol references

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## Acknowledgements

Thank you to Dr. Nhu Nguyen, Soil Microbial Ecology Laboratory, University of Hawai'i at Mānoa who facilitated this research and review of this written protocol.