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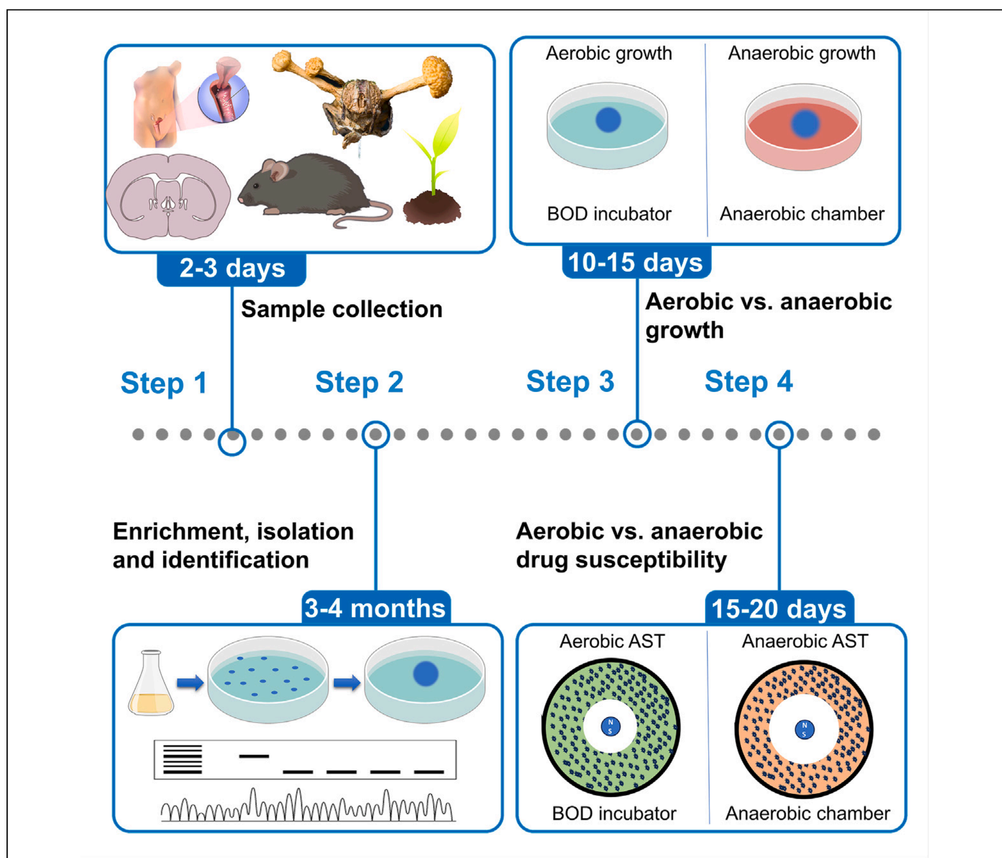
Protocol for enriching, isolating, and testing drug susceptibility of facultative anaerobic fungi



Research on fungi under anaerobic conditions is limited but crucial for understanding their ecological and pathological impacts. Here, we present a protocol for enriching, isolating, and characterizing anaerobic fungi from environmental and clinical samples. We describe steps for techniques for evaluating the anaerobic growth potential and drug susceptibility of fungal pathogens. This protocol can contribute to the need for initiating effective antifungal therapy to address and manage fungal infections or mycosis in oxygen-limited environments.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for enriching, isolating, and characterizing anaerobic fungi

Instructions on preparing pre-reduced or anaerobic media for anaerobic fungi

Steps for facultative anaerobic fungi growth in different conditions

Steps to test drug sensitivity for facultative anaerobic fungi in different conditions

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Protocol for enriching, isolating, and testing drug susceptibility of facultative anaerobic fungi

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SUMMARY

Research on fungi under anaerobic conditions is limited but crucial for understanding their ecological and pathological impacts. Here, we present a protocol for enriching, isolating, and characterizing anaerobic fungi from environmental and clinical samples. We also describe steps for evaluating the anaerobic growth potential and drug susceptibility of fungal pathogens. This protocol can contribute to the need for initiating effective antifungal therapy to address and manage fungal infections or mycosis in oxygen-limited environments.
For complete details on the use and execution of this protocol, please refer to Yadav et al.[1]

BEFORE YOU BEGIN

Fungal pathogens causing severe invasive infections encounter drastic changes and hostile conditions inside infected hosts.[2] One of the most common changes experienced by pathogens inside an infected host is infection-induced site-specific hypoxia or anoxia.[3,4] Since cases of invasive fungal infections of different hypoxic and anoxic body parts are relatively common in immunocompromised patients, it is evident that fungal pathogens can thrive under such conditions.[5–7] It was once considered that fungi, being eukaryotes, could not grow without oxygen, leading to limited research on their anaerobic growth capabilities.[8] Recent findings, however, show that many fungal species can thrive in low or no-oxygen environments by adapting their metabolism.[1,9–11] These altered metabolic pathways caused by drastic changes in the dynamics of the microenvironment of an infected area could also alter the response of fungi to antifungal drugs.[8,12] Thus; it is crucial to explore the anaerobic growth capabilities and drug susceptibility of fungal pathogens in both aerobic and anaerobic conditions for better management of fungal infections of anaerobic or hypoxic nature.

Therefore, here we present a detailed protocol for enrichment, isolation, and drug susceptibility of fungi from hypoxic or anoxic niches like landfills, gut, and deep internal organs like abscesses of the brain and lung. We utilized this protocol to isolate fifteen strains of facultatively anaerobic

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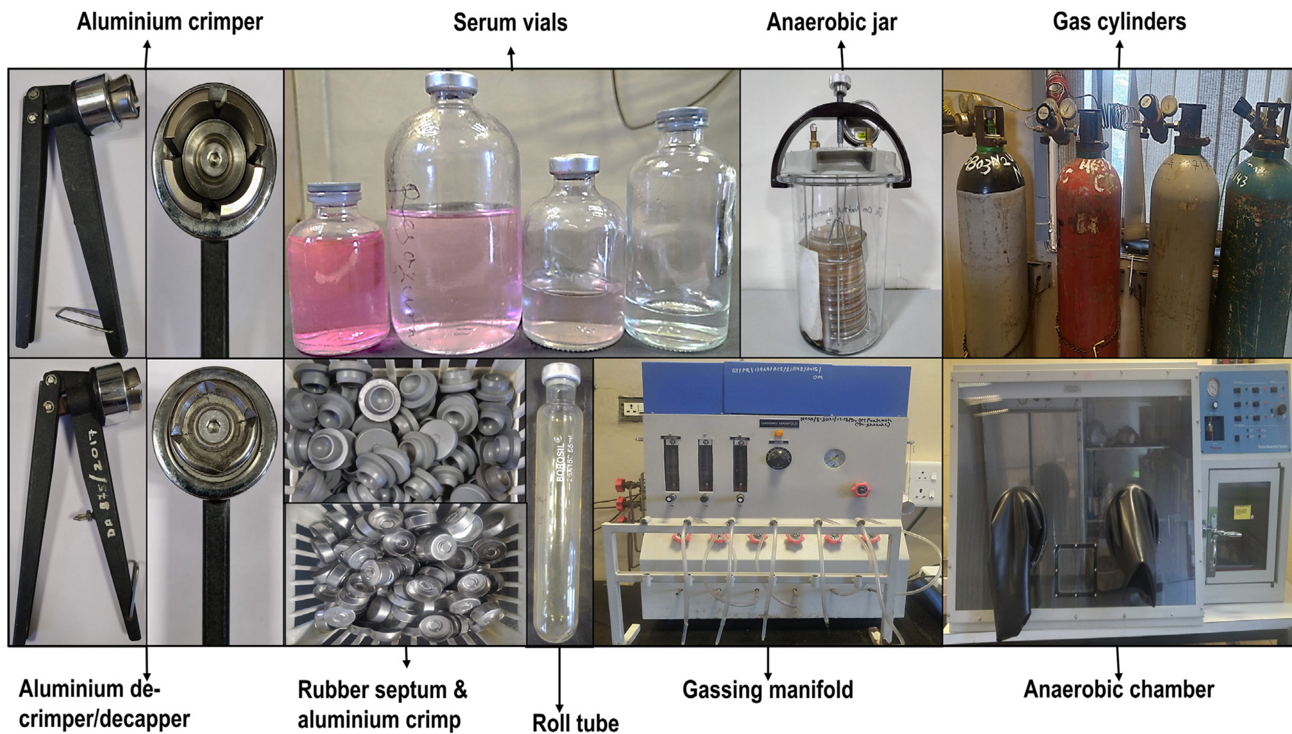

Figure 1. Basic instruments and equipment required in anaerobic lab to enrich and isolate anaerobic microorganisms

fungal pathogens, *S. apiospermum*, from soil or refuse samples collected from the Okhla landfill in Delhi, India. Later, we compared their drug susceptibility under aerobic and anaerobic conditions.[1] This protocol can be adapted to grow facultative or obligate anaerobic fungi from various environments to assess anaerobic growth potential and drug susceptibilities. To isolate fungi with anaerobic growth potential and assess their drug susceptibility under anaerobic conditions, it is crucial to have a well-equipped anaerobic lab. Essential equipment includes gassing manifolds, gas cylinders, and anaerobic chambers. Additionally, serum vials are needed for media preparation and enrichment, while a combination of rubber septum and aluminum crimp is used to ensure anaerobic conditions and seal the vials. To introduce inoculum and gases into these tightly sealed serum vials, syringes of various volumes and needles of differing gauges are indispensable (Figure 1).

Institutional permissions

The protocol outlines the cultivation of anaerobic fungi from various sample types. Researchers need permission and ethical clearance from the institutional ethics committee or local authorities before sampling, depending on the nature of the sample (animal/human studies). Environmental samples may not require this, but local guidelines should be followed.

Preparation for sample collection from landfill

Timing: 2–3 days

1. Before sampling, prepare sterile autoclavable high-density polyethene (HDPE) ziplock bags (1–2 kg capacity) and serum vials containing anoxic minimal media (100–120 mL) for sample collection. The size and capacity of the sample container vary depending on the needs.
2. Keep a clean and sterilized soil core sampler to collect the samples from lower hypoxic and anoxic zones (below 30–150 cm of the surface).

3. Keep a sterile spatula, rubber septum, aluminum crimp, and a crimper and de-crimper (decapper) for opening, inoculating, and sealing serum vials containing minimal medium with fresh samples from landfills or any other sites depending on objective of study.
4. Collect the sample from the anoxic zone of the landfill using a core sampler.
   a. Keep in a sterile ziplock polythene bag.
   b. Flush the bag with sterile nitrogen (N_2) or any other inert anoxic gas to replace the traces of oxygen.
   c. Close tightly to prevent environmental oxygen exposure.
   d. Transport the samples to the laboratory in a blue ice thermal box.
   e. Store inside the cold room till the beginning of the experiment.

Note: Minimum exposure to environmental oxygen is imperative to cultivate more anaerobic taxa. Strict anoxic and aseptic practices are essential for better results. Use fresh sample for better result.

Materials preparation for inoculation and enrichment

⏲ Timing: 24–72 h

5. Before starting the work, keep gas cylinders containing different gases like N_2 (for flushing out oxygen from media) and mix gas (N_2:H_2:CO_2-85:10:5) (for filling up enrichment vials necessary for the growth of anaerobic fungi) ready at gas station.

Note: If N_2 is not available, ultrapure argon (Ar) or a mixture of N_2 and carbon dioxide (CO_2) can be used to flush out oxygen from the medium and anaerobic chamber to create and maintain anaerobic conditions.

6. Test the anaerobic chamber to confirm it maintains anaerobic condition adequately and do not have any leak.
7. Autoclave and keep the sterile syringes, needles, septum, serum vials, crimper, and decapper, and other materials 48–72 h before inside the anaerobic chamber to remove the traces of oxygen and make it for anaerobic sample inoculation, dilution series preparation, and injecting and removing headspace gasses from enrichment vials.
8. Verify the anoxic condition of the anaerobic chamber using an oxygen detector or indicator dye.

Note: Maintaining the anaerobic conditions of the anaerobic chamber is crucial to avoid exposure to environmental oxygen during inoculation and incubation.

Preparation for aerobic vs. anaerobic drug susceptibility

⏲ Timing: 25–30 days

9. Inoculate culture or isolated fungal strain on Sabouraud dextrose agar medium plates for sporulation (duration mainly depends on the growth rate of isolates. *S. apiospermum* requires at least 15–20 days for enough sporulation).
10. Get Mueller Hinton Agar plates ready with 2% glucose and methylene blue. Glucose provides energy for the rapid growth of fungi and methylene blue increases the visibility of the inhibition zone.
11. Keep sterile cotton swabs ready to spread the spore solution.
12. Clean the anaerobic chamber, maintain strict anaerobic conditions using N_2 and mixed gasses, and maintain the temperature of the inbuilt incubator at 35°C.
13. Keep a clean BOD (Biochemical Oxygen Demand) incubator ready if the culture is facultatively anaerobic, and plan to compare aerobic vs. anaerobic drug susceptibility.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
HM itraconazole	HiMedia	Cat#EM073
HM ketoconazole	HiMedia	Cat#EM074
HM fluconazole	HiMedia	Cat#EM072
HM clotrimazole	HiMedia	Cat#EM144
HM miconazole	HiMedia	Cat#EM146
HM voriconazole	HiMedia	Cat#EM086
HM posaconazole	HiMedia	Cat#EM120
HM nystatin	HiMedia	Cat#EM145
HM amphotericin-B	HiMedia	Cat#EM071
HM micafungin	HiMedia	Cat#EM121
HM anidulafungin	HiMedia	Cat#EM122
HM caspofungin	HiMedia	Cat#EM119
HM flucytosine	HiMedia	Cat#EM118
HM griseofulvin	HiMedia	Cat#EM143
HM terbinafine	HiMedia	Cat#EM142
HM fluconazole	HiMedia	Cat#SD232
HM voriconazole	HiMedia	Cat#SD277
HM amphotericin-B	HiMedia	Cat#SD270
HM clotrimazole	HiMedia	Cat#SD115
HM itraconazole	HiMedia	Cat#SD276
HM ketoconazole	HiMedia	Cat#SD274
HM miconazole	HiMedia	Cat#SD272
HM nystatin	HiMedia	Cat#SD271
Mueller Hinton agar with 2% glucose with methylene blue	HiMedia	Cat#M1825
Potato dextrose broth, granulated	HiMedia	Cat#GM403
Sabouraud dextrose broth, granulated	HiMedia	Cat#GM033
Oat meal powder	HiMedia	Cat#RM2565
Malt extract powder	HiMedia	Cat#RM004
Czapek Dox broth, granulated	HiMedia	Cat#GM076
Other		
WHEATON serum, vial (125 mL)	DWK Life Sciences	Cat#223748
WHEATON E-Z Crimper (20 mm)	DWK Life Sciences	Cat#W225303
WHEATON E-Z Decapper (20 mm)	DWK Life Sciences	Cat#W225353
Anaerobic System Mark III	HiMedia	Cat# LE003
Anaerobic jar	Don Whitley Scientific	Cat#A05077

MATERIALS AND EQUIPMENT

ATCC medium: 1306 Nitrate mineral salts medium (NMS)		
Reagent	Final concentration	Amount
$MgSO_4.7H_2O$	0.1% (w/v)	1 g
$CaCl_2.6H_2O$	0.02% (w/v)	0.2 g
Chelated Iron Solution (see below)	0.2% (v/v)	2 mL
KNO_3	0.1% (w/v)	1 g
Trace Element Solution (see below)	0.05% (w/v)	0.5 mL
KH_2PO_4	0.0272% (w/v)	0.272 g
$Na_2HPO_4.12H_2O$	0.0717% (w/v)	0.717 g
Distilled deionized water	N/A	1000 mL
Add 20 g purified bacteriological agar for preparing Nitrate mineral salt agar medium.		

Note: Make fresh NMS media before commencing the experiment. Autoclaved and sterile NMS broth in serum vials or screw-capped glass bottles can be stored at 20°C for over 30 days. Sterile NMS agar plates can be stored for 10–15 days inside an anaerobic chamber.

Chelated Iron Solution

Reagent	Final concentration	Amount
Ferric (III) ammonium citrate*	0.1% (w/v)	0.1 g
EDTA, sodium salt	0.2% (w/v)	0.2 g
HCl (concentrated)	0.3% (v/v)	0.3 mL
Distilled deionized water	N/A	100 mL

*0.05 g of Ferric (III) chloride may be substituted. Autoclave and store the chelated iron solution at 4°C, ensuring no precipitation for repeated use (3–4 months).

Trace Element Solution

Reagent	Final concentration	Amount
EDTA	0.05% (w/v)	500 mg
$FeSO_4.7H_2O$	0.02% (w/v)	200 mg
$ZnSO_4.7H_2O$	0.001% (v/v)	10 mg
$MnCl_2.4H_2O$	0.0003% (w/v)	3 mg
H_3BO_3	0.003% (w/v)	30 mg
$CoCl_2.6H_2O$	0.002% (w/v)	20 mg
$CaCl_2.2H_2O$	0.0001% (w/v)	1 mg
$NiCl_2.6H_2O$	0.0002% (w/v)	2 mg
$Na_2MoO_4.2H_2O$	0.0003% (w/v)	3 mg
Distilled water	N/A	1000 mL

Autoclave and store the trace element solution at 4°C, ensuring no precipitation for repeated use (3–4 months).

STEP-BY-STEP METHOD DETAILS

Pre-reduced (anaerobic) media preparation

Timing: 2 days

Below are the steps for preparing anaerobic pre-reduced media. The purpose of preparing pre-reduced anaerobic media is to create strict anoxic conditions and provide the necessary nutrients for the growth and proliferation of anaerobic fungi. While we are outlining the preparation of an anaerobic NMS medium, this protocol can also be used to prepare anaerobic media with different compositions.

1. Prepare nitrate mineral salt (NMS) medium or any other minimal medium as per requirement using normal medium preparation practices devoid of carbon sources (ATCC medium: 1306 Nitrate mineral salts medium (NMS).
   a. To prepare one liter of anaerobic NMS media, dissolve the media components in 600 mL of double distilled water, excluding the phosphate buffer components.
   b. Dissolve phosphate buffer components (KH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$) in separate flasks with 100 mL double distilled water.
   c. Add phosphate buffer solution to the NMS media.
   d. Adjust the pH of the medium to 6.8 using HCl and NaOH solutions.
2. After preparation, heat the medium up to 70°C–90°C using a heating mantle or any other source of heating like a hot-plate, water bath, etc., to expel the dissolved oxygen.
3. To prevent the entry of environmental oxygen, the medium should be flushed with ultrapure N_2 gas using a gassing manifold.

4. Add resazurin dye (1.0 ppm), as an anoxic indicator to test the redox potential of the medium.
   a. Keep flushing it until the pink color of the media becomes completely colorless.
   b. Initially, the medium turns pink and finally colorless if the redox potential of the medium becomes sufficiently low (approximately −110 mV).

   Note: Resazurin is a commonly used redox indicator dye in anaerobic cultivation. The oxidized stage of the dye is blue, but in the reduced stage, it forms resorufin, which is pink. Further reduction led to the formation of colorless hydroresorfin, which indicates that media Redox Potential (Eh) reached approximately −110 mV.

5. Once the medium becomes colorless without precipitation, dispense 60 mL of pre-reduced media into empty 120 mL serum vials, continuously flushing with inert N_2 gas.
6. After media becomes colorless, close the serum vials with a rubber septum, seal with an aluminum crimp using a crimper, and then autoclave them at 121°C for 20 min.
7. Keep autoclaved media at room temperature for another 24 h to ensure serum vials are not leaking and maintain anoxic condition.

   Note: Add medium components one by one to avoid precipitation. Cysteine-HCl can be supplemented to the media to remove oxygen and get the right redox potential, but it depends on the researcher's choice. Some anaerobes and facultative anaerobes can grow in hypoxic conditions with traces of oxygen and no need to add any reducing agents in cultivation medium.

Enrichment and isolation of anaerobic fungi

Timing: 3–4 months

The text outlines steps for enriching anaerobic fungi in pre-reduced NMS media to increase their numbers before isolation. The process involves using minimal or fungal-specific media, with or without antibacterial agents, to suppress undesired microbes and promote the growth of anaerobic fungi. Though focused on anaerobic fungi, this approach can be adapted for other anaerobic microorganisms by selecting media with nutrients targeted at the desired microorganism.

8. Before beginning the enrichment process, it is important to check the color of the pre-reduced NMS media.

   Note: The autoclaved pre-reduced anaerobic media in tightly sealed serum vials should be colorless or retain its original color. Pink coloration indicates oxygen contamination.

9. Label vials to inoculate samples from different dilutions, including 10^0 to 10^{-8}.
10. Transfer the samples collected in ziplock bags inside an anaerobic chamber.
    a. Homogenize them to remove bulky materials and stones.
    b. Weigh 1-gram sample using a portable weighing balance.

    Note: Use a stainless-steel strainer filter with a 3 mm hole to remove debris from samples and then gently grind them using a mortar and pestle to obtain a fine consistency.

11. Prepare dilution series using the standard method in sterile anaerobic NMS media inside the chamber.
    a. Open serum vials (20 mL volume) containing (9 mL) sterile anaerobic NMS media with the help of a decapper.
    b. Add one gram of sample and close it again with the blue butyl rubber septum
    c. Seal it with an aluminum crimp using a crimper.

12. Take out enrichment vials from the anaerobic chamber and vigorously vortex it for 15–20 min to break the lumps and detach microbes from attached soil particles.
13. Take this sample suspension inside an anaerobic chamber and serially dilute it up to 10^{-7} dilutions with the help of sterile needles and syringes. Serial dilutions can also be prepared outside an anaerobic chamber using a gas station.
14. Inoculate the serum vials containing enrichment media from different dilutions with 10% inoculum (v/v) using a hypodermic (20 gauge) needle and syringes.
15. Supply the desired electron donor-acceptor combination or substrate in minimal medium (NMS) as per requirement and maintain the headspace pressure (Positive) of the vessels with desired gases by adding the sterile gas with the help of needle and syringes.
16. Incubate enrichment vials at 30°C inside an anaerobic chamber at the desired temperature in the dark and mix two to three times daily to homogenize the content.

Note: Alternatively, one can also incubate the enrichment vials at 30°C inside BOD incubators outside the anaerobic chamber, as they are airtight and tightly sealed with a blue-butyl septum and aluminum crimp.

17. Check vials for growth at regular intervals.
18. After 10–15 days, flush it again with a mixed or required gas to maintain the pressure and concentration of gasses inside serum vials necessary for the growth of microbes.
19. Observe mycelial or clumpy growth inside enrichment vials.
20. Shake it vigorously to confirm if the growth is mycelial or bacterial clumps.
21. Once mycelial growth is observed, prepare an anaerobic NMS agar medium or any other respective medium for isolation and purification purposes.

Note: If the sample is liquid, add 1 mL sample in serum vials with the help of a sterile needle and syringe for dilution series preparation. Maintain positive pressure inside serum vials to prevent diffusion of external oxygen. Successive transfer of inoculum in fresh medium is also a good way of enrichment and can be used.

Isolation and purification of enriched culture

Timing: 10–15 days

Here are the steps for isolating and purifying anaerobic fungi from the enrichment. For isolation, it is recommended to use the same medium that was used for the enrichment. Additionally, it is advisable to utilize different fungal-specific media alongside the enrichment media to improve the isolation, purification, and differentiation of fungal colonies. Following the steps, one can successfully isolate anaerobic fungi from the enrichment.

22. In case NMS medium is used for enrichment, prepare NMS-agar using 2% bacteriological agar supplemented with 1 ppm resazurin, as usual, autoclave it and pour the medium outside in a biosafety chamber in a sterile Petri plate.
23. Once the media has solidified outside, transfer the plate inside the anaerobic chamber for 24–48 h to make it anoxic prior to inoculation. A colorless plate indicates that medium turns anoxic.
24. Vigorously vortex enrichment vials showing mycelial or clumpy growth to break the clumps and make serial dilution series inside anaerobic chamber.
25. Spread 100 μL of diluted enrichment on NMS agar plates.
26. Incubate plates inside the anaerobic chamber at 30°C.
27. Observe plates for fungal growth for 10–15 days.
28. Using the hyphal tip method, isolate and purify colonies on anaerobic NMS and potato dextrose agar (PDA) plates.

Note: Prepare anaerobic PDA or any other fungal-specific media using the same method described for anaerobic NMS media.

Determining the facultative anaerobic nature of fungal isolates

Timing: 10–15 days

Here, we outline the steps for assessing the facultative anaerobic nature of fungal isolates isolated from strict anaerobic conditions. Although isolated from strictly anaerobic conditions, there is still a possibility that isolated fungi can be facultatively anaerobic and thrive under both aerobic and anaerobic conditions. Hence, it is necessary to confirm their facultative nature before conducting further experiments.

29. Prepare the plates of NMS, PDA, or any other available fungal specific medium aerobically that support the growth of fungi.
30. Inoculate a 5 mm fungal disc from a pure culture at the center of media plates in a biosafety cabinet under aerobic conditions.
31. After inoculation, incubate plates aerobically at 30°C inside the BOD incubator.
32. Observe plates for mycelial growth for 10–15 days (Figure 3).

Screening of the media and growth conditions

Timing: 20–30 days

Finding the best media for comparative growth experiments under aerobic and anaerobic conditions for facultatively anaerobic fungi is necessary. This section focuses on how to screen the best medium that provides almost equal growth in anaerobic and aerobic conditions for comparative physiology and drug susceptibility testing.

33. Prepare different fungal media plates including but not limited to Oatmeal Agar (OA), Malt Extract Agar (MEA), Czapek Dox Agar (CDA), Sabouraud Dextrose Agar (SDA), and PDA aerobically.
34. After media preparation, keep half of the plates inside the anaerobic chamber and incubate them for 24–48 h before inoculation to make them anaerobic.
35. Inoculate fungal disc (5 mm) of freshly grown pure culture in the center of media plates in triplicates (two sets).
    Incubate one set under aerobic conditions at 30°C inside the BOD incubator and leave another set under anaerobic conditions inside an anaerobic chamber at the same temperature conditions.
36. Observe plates for mycelial growth for 10–15 days.
37. After fifteen days, measure the diameter of the fungal growth.
38. Also, observe which media supports fast growth with considerable biomass under aerobic and anaerobic conditions (Figure 2).
39. For optimum growth temperature, prepare media on which fungal strains show the best growth under both conditions (Figure 2).
40. For aerobic media, follow the manufacturer's instructions.
41. For anaerobic media, follow the instructions given for NMS media.
42. Inoculate fungal disc (8 mm) of freshly grown pure culture in the center of media plates and incubate under aerobic (BOD incubator) and anaerobic conditions (anaerobic chamber) at different temperatures (15°C–40°C) with 5°C increments.
43. Observe plates for mycelial growth for 10–15 days and measure the diameter of the fungal growth after 15 days.

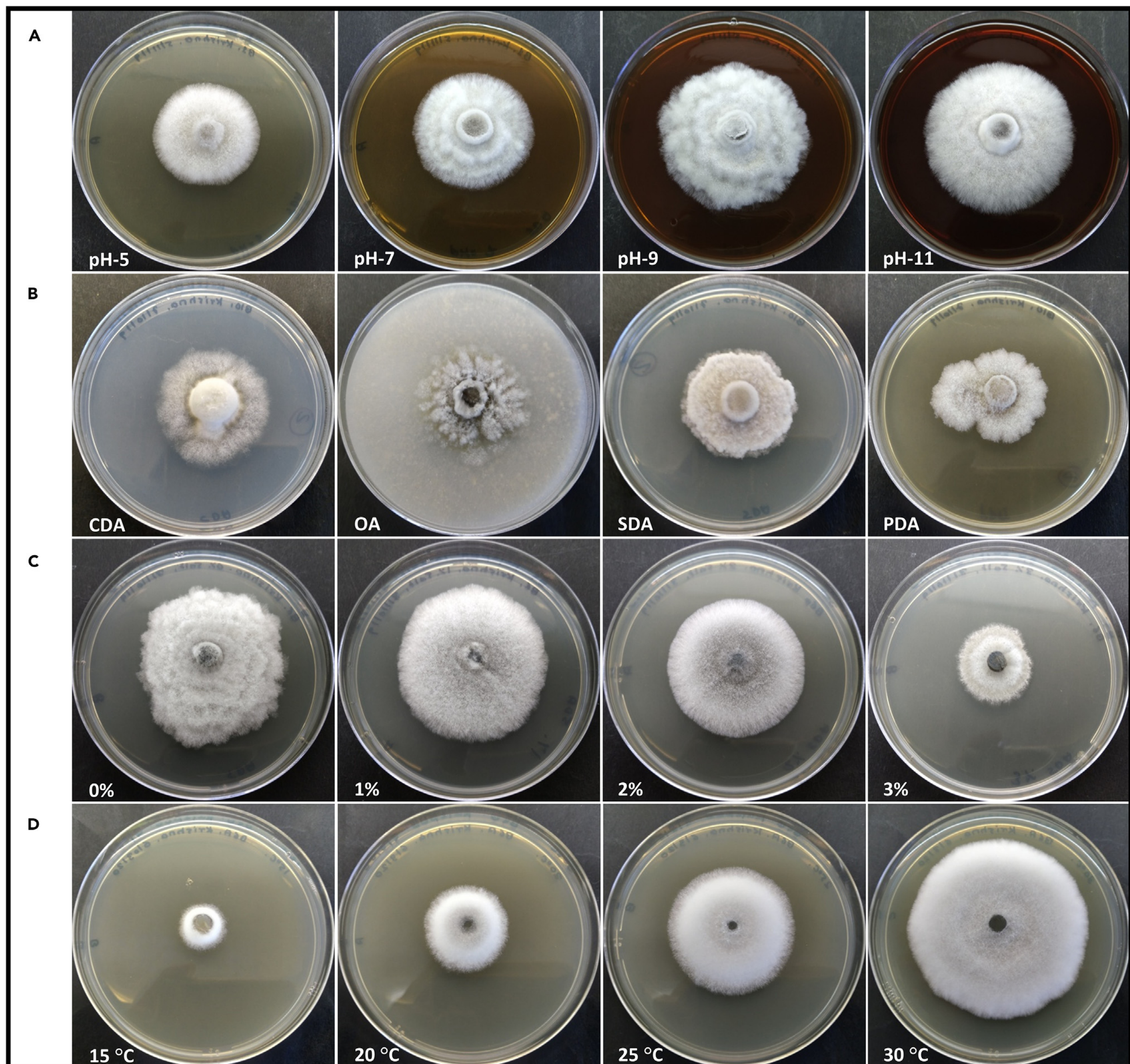

Figure 2. Representative images of physiological studies conducted to determine optimum growth conditions of *S. apiospermum* under aerobic conditions

(A) pH (5–11) (B) Media (C) Salt (NaCl) (0–3%) (D) Temperatures (15°C–30°C).

44. After confirmation of the optimum temperature and best medium optimum conditions of pH and salinity can also be obtained using a similar approach (Figure 2).

Note: Growth pattern observation and measurement days can be varied based on the growth rate of isolates.

Note: Conducting a temperature optimization experiment inside an anaerobic chamber is tedious due to the single fixed temperature; hence, use an anaerobic jar filled with mixed gas. After the detection of optimum condition aerobically, we can select the same temperature for anaerobic conditions, too.

45. Either take anaerobic jars inside the anaerobic chamber, create strict anaerobic conditions, place culture plates inside the jar, and close it tightly.
46. Alternatively, remove culture plates from the anaerobic chamber, keep them inside a jar, close it tightly, and then use a jar gassing system to create strict anaerobic conditions inside the anaerobic jar and fill it with mixed gas.
47. Now, incubate this anaerobic jar with strict anaerobic conditions and culture plates inside different BOD incubators at different temperatures (15°C–40°C) (Figure 2).

Aerobic vs. anaerobic growth

Timing: 15–20 days

This section delineates the procedures for comparing the development of facultatively anaerobic fungi in aerobic and anaerobic conditions. These comparisons provide valuable insights into the growth rate, growth patterns, morphological traits, and behavior of fungal isolates under both conditions. This information aids in understanding the adaptation process of fungal pathogens that allows them to thrive in anaerobic environments.

48. After finding the best media and optimum growth temperature, prepare media on which fungal isolates show the best growth under both conditions.
49. Prepare aerobic and anaerobic media using the method described above.
50. Inoculate disc (5 mm) of freshly grown culture (10–15 days old, if slow-growing) (3–4 days old, if fast-growing) on aerobic and anaerobic media, strictly maintaining required conditions.
51. Incubate aerobic and anaerobic culture plates at optimum growth temperature under aerobic and anaerobic conditions.
52. Observe plates for mycelial growth for 10–15 days and note morphological characteristics, pigmentation, sporulation patterns, and other characteristics like exudate formation under both conditions (Figure 3).
53. Also, measure the diameter of the fungal growth.
54. To study sporulation patterns and mycelial growth characteristics, prepare slides of cultures grown under both conditions by staining them with lactophenol cotton blue and observing them using a phase contrast light microscope.

Note: Growth pattern observation and measurement days can be varied based on the growth rate of isolates.

Study of drug susceptibility and minimum inhibitory concentration (MIC) in aerobic vs. anaerobic conditions

Timing: 30–45 days

The following section provides a detailed protocol for assessing the susceptibility of facultatively anaerobic fungi with pathogenic potential to various antifungal drugs under aerobic and anaerobic conditions. This evaluation is crucial for understanding the behavior of these fungal pathogens under different environmental conditions, which is essential for devising effective infection management strategies.

Note: The required time can vary based on the number of antifungal drugs tested. This test shows the physiological behaviour of fungi against different classes of antifungal drugs under aerobic vs anaerobic conditions.

55. Perform drug susceptibility tests and minimum inhibitory concentration studies under both conditions using standard guidelines suggested by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical & Laboratory Standards Institute (CLSI).[13]

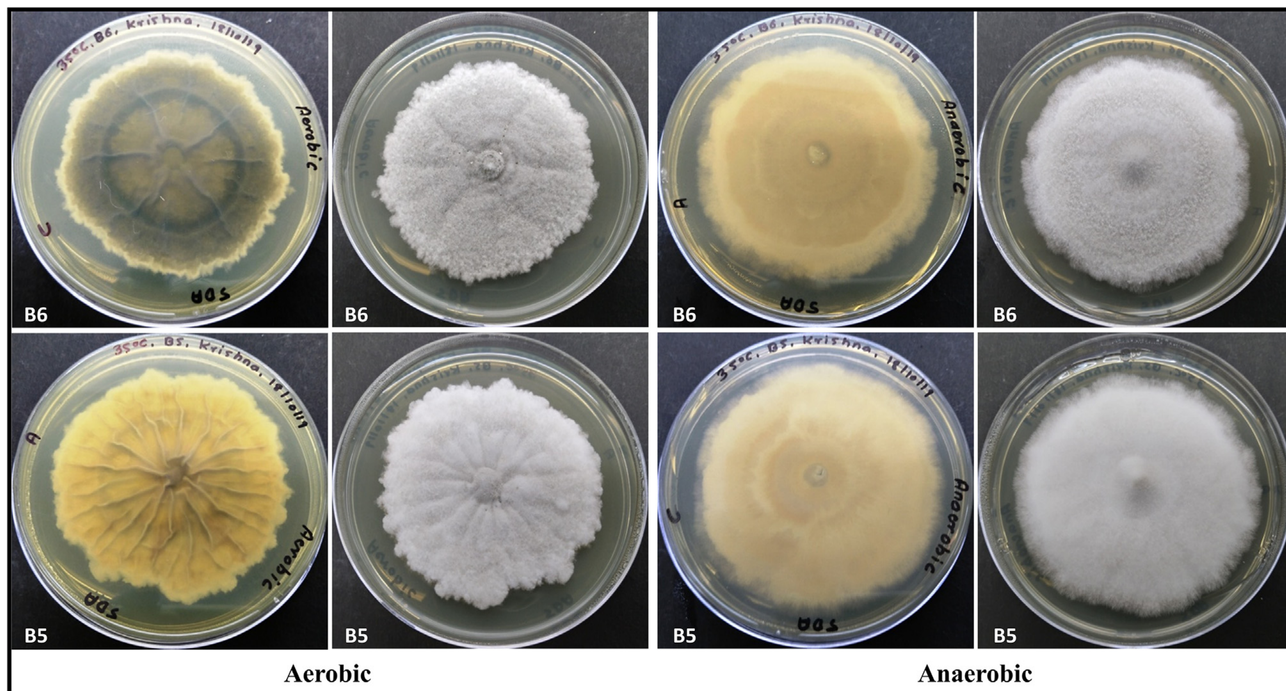

Figure 3. Representative images of growth and morphological characteristics of facultatively anaerobic strains of *S. apiospermum* under aerobic and anaerobic conditions at 35°C after 30 days of incubation

56. Prepare the required number of modified Mueller Hinton Agar plates with 2% glucose + 0.5 μg mL^{-1} methylene blue (as per manufacturer's instructions) under aerobic conditions.
57. After 48 h, the media is ready for the drug susceptibility test and minimum inhibitory concentration assay.
58. Prepare spore suspension in normal saline or phosphate buffer saline (pH - 7 $\pm$ 0.2) and adjust the spore numbers to 1×10^6 to 5×10^6 per milliliter using the Neubauer chamber or turbidity to 0.5 McFarland standards.
59. Spread 200 μL of spore suspension onto modified Mueller Hinton Agar plates with the help of a sterile cotton swab. Inoculate two sets (aerobic + anaerobic) of plates in three biological replicates
60. Dry plates for at least 10 min inside a laminar airflow or biosafety cabinet; place an antifungal disc in the center of the plates after drying for drug susceptibility test and MIC strips containing antifungal drugs for MIC assay.
61. Incubate aerobic and anaerobic drug susceptibility and MIC assay plates same time inside the aerobic and anaerobic incubators (anaerobic chamber) set at 35°C for 24–48 h.
62. Observe plates after 24 h, 36 h, and 48 h. Measure the diameter of the zone of inhibition and note down MIC values under both aerobic and anaerobic conditions (Figure 4).

Note: It is important to note that we have not yet investigated the specific effects of cysteine-HCl and resazurin on drug response, so we are unable to recommend their use at this time. The inclusion of cysteine-HCl might disturb the pH of the media. Additionally, the modified Mueller Hinton agar media employed in this study already includes methylene blue dye (which serves as an alternative anaerobic indicator to resazurin), and it has the potential to interact with resazurin, leading to inconsistent results.

⚠ CRITICAL: Fungal spores are necessary for conducting drug susceptibility tests. According to our observation with *S. apiospermum*, fungi do not produce spores in large numbers

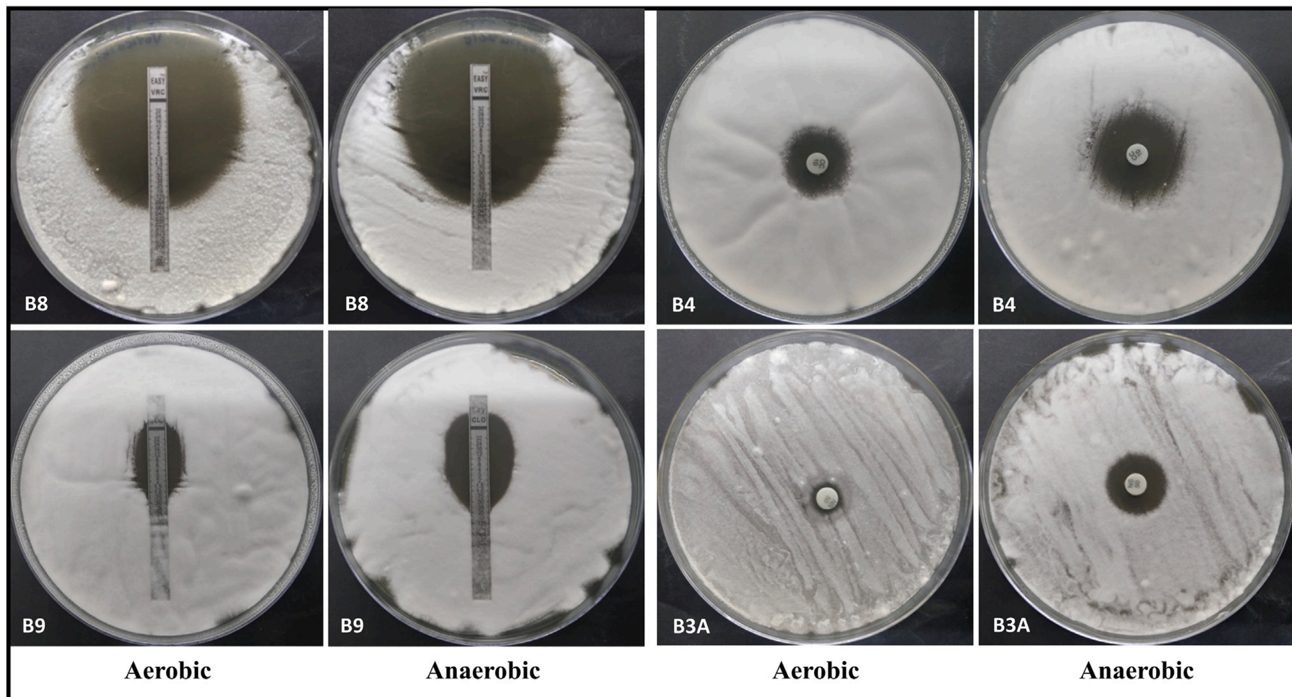

Figure 4. Representative images of *S. apiospermum* longitudinal antibiotic sensitivity tests (AST) and minimum inhibitory concentration (MIC) assays performed with Clotrimazole (CC), Nystatin (NS), and Voriconazole (VR) under aerobic and anaerobic conditions

under strict anaerobic conditions. Hence, grow facultatively anaerobic fungal strains under strict aerobic conditions inside BOD incubator on SDA plates to ensure the required sporulation. Also, the duration of getting the required sporulation can vary based on the growth rate of the isolate. Vigorously vortex spore suspension for at least 15–20 min to make uniform spore suspension.

Note: To smoothly and equally spread spore suspension, dip the cotton swab in sterile saline and wet it before dipping in spore suspension.

Note: Usually, the result can be observed after 24 h, but if fungal strains are slow-growing, it sometimes takes 36–48 h. Perform all the above experiments in triplicate to avoid errors.

EXPECTED OUTCOMES

The above-mentioned protocol can be used for cultivation, purification, and physiological studies of anaerobic fungal strains from diverse sample types, including environmental, clinical, and industrial, with minor modifications. It will also assist in studying the metabolic behaviors and antimicrobial drug susceptibility of facultative anaerobic pathogens isolated from deep-seated anaerobic infection sites.

QUANTIFICATION AND STATISTICAL ANALYSIS

A minimum of 3–5 biological replicates should be taken to test the reproducibility of the used method.

LIMITATIONS

The current protocol discussed a single medium and conditions for enrichment, which can lead to enrichment bias or suitable for a specific taxon. The cultivation conditions like pH, buffering

condition, nutritional requirement, redox potential, temperature, and incubation time can be modified according to the nature of the sample and site geochemistry. The discussed protocols are not universal for all the physiological groups and can suppress the growth of certain taxa. Knowledge of the field, appropriate modification in procedure, and the right judgment of the researcher are imperative to get the appropriate results.

TROUBLESHOOTING

Problem 1

The first step involves preparing anaerobic or pre-reduced NMS media. During this process, precipitation is a common issue that can occur. This happens when magnesium sulfate ($MgSO_4$) and calcium chloride ($CaCl_2$) salts are added together. These poorly dissolved salts then react with essential components such as Potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4), which are necessary for maintaining the neutral pH of the medium.

Potential solution

- Prepare stock solutions of different media components like macro-nutrients, micro-nutrients, and chelate iron solution. Keep them at room temperature overnight.
- While preparing media, sequentially add all media components except KH_2PO_4 and Na_2HPO_4. After adding each element of media, mix it well to dissolve it completely (until the last grains of salt dissolve).
- If $CaCl_2$ granules are still visible after mixing for a long time, filter media using 0.25-micron filter paper.
- Add a micronutrient or trace element salt solution and a chelate iron solution to the media. Mix it well.
- Now, you can add Cysteine-HCl (optional) in the medium.
- After adding cysteine-HCl, adjust the pH of the medium to 7 ± 0.2 (since cysteine-HCl significantly decreases the pH of the medium).
- Mix KH_2PO_4 and Na_2HPO_4 separately in sterile distilled water in a beaker or flask. Add sequentially and mix them well to dissolve completely. Add this buffer solution to the media and mix it well.

Problem 2

In step 7, bacterial contamination of antifungal discs is common during drug susceptibility testing due to handling errors.

Potential solution

- Bacterial contamination of antifungal discs and strips can be avoided by handling them properly under sterilized conditions (inside laminar airflow or biosafety cabinet).
- Open containers containing antifungal discs or strips inside laminar airflow or biosafety cabinets only.
- Keep at least 2–3 sterile forceps inside the biosafety cabinet dipped in alcohol.
- Use sterile (autoclaved) forceps to transfer the antifungal disc from the container to the experimental media plates.
- After each transfer, flame, sterilize forceps, and keep them dipped in alcohol to cool them down.
- Use separate forces if they touch the media surface while transferring antifungal discs and strips.
- Alternatively, one can add chloramphenicol (100 mg/L) in Mueller Hinton agar media to altogether avoid bacterial contamination.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Om Prakash (prakas1974@gmail.com, dyhead_scccs@siu.edu.in).

Technical contact

Further information and requests for technical issues should be directed to and will be fulfilled by the technical contact, Krishna K Yadav (yadavkrishna9881@gmail.com).

Materials availability

This research did not produce any novel reagents.

Data and code availability

This study did not generate any new datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, O.P.; methodology, O.P. and K.K.Y.; sampling and data curation, K.K.Y.; investigation, O.P. and K.K.Y.; writing – original draft, O.P. and K.K.Y.; writing – review and editing, O.P.; funding acquisition, O.P.; resources, O.P.; supervision, O.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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