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Laboratory Maintenance of *Streptomyces* species

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Abstract

This unit includes general protocols for the laboratory maintenance of *Streptomyces* species, including growth in liquid media, growth on solid agar, and short and long-term storage. Considerations for the handling of *Streptomyces* species and the morphology of the bacteria are also reviewed.

Keywords

Streptomyces; media; strain storage; spore; mycelium

INTRODUCTION

Streptomyces are gram-positive soil bacteria, make up the largest genus of actinomycetes, and play an important role in natural product discovery. These bacteria produce a wide range of structurally diverse compounds with various pharmaceutical applications. Approximately 75% of commercially used antibiotics and more than half of the naturally occurring antibiotics have been isolated from *Streptomyces* (Miyadoh et al. 1993).

The protocols presented in this unit describe culture conditions, media recipes and storage conditions for common *Streptomyces* maintenance. Growth media for *Streptomyces* varies greatly. The most commonly used recipes are described herein but the list is hardly comprehensive.

CAUTION

Streptomyces are Biosafety Level 1 (BS1-1) organisms. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

BASIC PROTOCOL 1

Growth of *Streptomyces* in Liquid Media

Growth of *Streptomyces* in liquid media is very important for the production of secondary metabolites and for various genetic manipulation protocols including electroporation, protoplast transformation, conjugation and isolation of genomic/plasmid DNA. A variety of liquid media have been developed for the growth of different *Streptomyces* species, and the selection of a specific liquid media is largely dependent on the purpose of the experiment. The genus *Streptomyces* covers a large number of species with huge diversity in culture conditions, carbon source utilization and secondary metabolite production. It is extremely difficult to cover specific protocols in a single article. Therefore, general protocols to grow

these organisms in liquid media for the production of secondary metabolites and genetic manipulation purposes are summarized here.

Materials—250 mL Erlenmeyer baffled flasks

Agar plate of a *Streptomyces* species

28°C shaking incubator

Cotton gauge

Aluminum foil

Autoclave

Sterile disposable inoculation loops

Sterile liquid media (Tryptic Soy-Broth (TSB), R2YE and YEME media)

Sterile toothpicks

Biological safety cabinet

1. Prepare liquid culture media by following appropriate recipe (TSB, R2YE or YEME).
2. Partition the liquid into 250 mL Erlenmeyer baffled flasks (glass or plastic), adding 100 mL of liquid media per 250 mL flask.
3. Fill the flask end with cotton gauge to allow airflow but prevent contamination and wrap the cotton gauge with aluminum foil.
4. Sterilize 250 mL Erlenmeyer baffled flasks (glass or plastic) containing 100 mL of liquid media by heating to 121 °C for at least 45 minutes.
5. Take a flask of 100 mL sterilized liquid media and transfer into a biological safety cabinet.
6. Remove the aluminum foil wrap and the cotton gauge.
7. Use a sterile toothpick to scrape spores/substrate mycelia of the *Streptomyces* from an agar plate.
8. Partially dip the toothpick into the liquid media to transfer the spores/substrate mycelia. Remove any remaining spores/substrate mycelia into the liquid by spinning the toothpick against the side of the flask while submerged in liquid. Alternatively, 100 µL of spore/mycelia suspension from glycerol stock may be used. *Tilting the flask at a 45° angle facilitates the inoculation process. Streptomyces cultures often get contaminated through poor inoculation skill. Therefore, it is highly recommended to maintain a sterile experimental working environment.*
9. Close the flask with the cotton gauge and wrap the gauge with aluminum foil.
10. Incubate the culture flask in a shaker at 28°C with 200 rpm for 72 hours. *Growth rate of different Streptomyces species vary greatly. Therefore, it is recommended to monitor the growth of organism following 24 hours of culture. OD₆₀₀ of a culture in logarithmic growth will double every 4–6 hours. OD₆₀₀ readings above 1.5 are not reliable, as the mycelia of Streptomyces easily form aggregates.*

BASIC PROTOCOL 2

Growth of *Streptomyces* on Solid Media

Growth and maintenance of *Streptomyces* on agar media is very important for several reasons such as production of secondary metabolites and various genetic manipulation protocols including electroporation and conjugal transfer of DNA. *Streptomyces* can be maintained on agar plates for 2–8 weeks. Agar plates are used to conduct routine experiments. During the early growth stage, *Streptomyces* grow in the form of mycelia. As the growth of many *Streptomyces* species progress, sporulation begins in coordination with depletion of the strain's carbon source from the media. Figure 1 shows fully sporulated *Streptomyces lividans* TK64 on solid oatmeal agar media. Spores are relatively more stable and can be stored for several years as a glycerol stock (see **Storage of *Streptomyces* Spores as Glycerol Stocks**).

Materials

Petri dishes

Media components (see recipe)

Sterile toothpicks

Sterile plastic spreader

Biological safety cabinet

Microscope

1. Take 200 μ L of a fully grown liquid culture and transfer to solid media. Alternatively, 100–200 μ L of spore suspension can be used in the place of the fully grown liquid culture.
2. Use a sterile spreader to spread the mycelia (from liquid growth) or spores (from spore suspension) on the plate.
3. Allow the plate to dry uncovered in a biological safety cabinet for 30 minutes or until all remaining water has evaporated.
4. Incubate the plate in a 28°C incubator for 4–6 days. *The incubation period largely varies from species to species.*
5. Growth of the *Streptomyces* will become visible as mycelia. *A sterile toothpick can be used to scrape off a small portion of mycelia from the agar plate and can be visualized on the microscope as filamentous structures.*
6. If the *Streptomyces* species sporulates, white/brown spores will start to form. *A sterile toothpick can be used to scrape off a small portion of mycelia from the agar plate to visualize on the microscope. As they grow out of the mycelia, they appear segmented and when fully formed, they separate from the mycelia as circular structures.*
7. After 4–6 days or after sufficient growth and sporulation, wrap the culture plate with parafilm and store at 4°C for 2–8 weeks.

Note: Routine strain growth can be maintained on several solid media such as R2YE and YEME. The choice of media for sporulation of *Streptomyces*, however, varies largely from species to species. Most *Streptomyces* species sporulate very well on Oatmeal-agar, MYM-agar and ISP4-agar media.

BASIC PROTOCOL 3

Storage of *Streptomyces* on Solid Media

Short term storage of *Streptomyces* mycelia is useful for quickly culturing strains for various genetic and strain production purposes. Storage of mycelia on solid media is only recommended for 2–8 weeks, after which the viability of the strain may decrease dramatically.

Materials—Fresh agar plate of the *Streptomyces* strain

Petri dish

Refrigerator (4°C)

Biological safety cabinet

1. Prepare fresh mycelia on solid media (see **Growth of *Streptomyces* on Solid Media**).
2. Wrap the circumference of the plate with parafilm and store at 4°C for 2–8 weeks.

BASIC PROTOCOL 4

Storage of *Streptomyces* Mycelium as Glycerol Stocks

Long term storage of *Streptomyces* mycelia is useful for quickly culturing strains for various genetic and strain production purposes. Unlike storage on solid media, glycerol stocks will stay viable for several years. Mycelia glycerol stocks will tend to lose viability after continual freeze thaw cycles. To ensure the preservation of any important strain it is highly recommended to store the strain as spores (see **Storage of *Streptomyces* Spores as Glycerol Stocks**).

Materials—Fresh agar plate or liquid culture of the *Streptomyces* strain

Freezer (−80°C)

Sterile loop or sterile toothpick

Sterile water

Pipette

Sterile 2 mL screw top vials or 1.5 mL microcentrifuge tubes (for mycelia storage)

50% glycerol in water (sterilized by autoclaving)

1. Prepare fresh mycelia on solid or liquid media (see **Growth of *Streptomyces* in Liquid Media** or **Growth of *Streptomyces* on Solid Media**).
2. Place mycelia into 2 mL screw top vials.
 - a. If using solid media remove one quarter of the mycelia lawn and place into a 2 mL screw top vial containing 500 µL of sterile water.
 - b. If using liquid media remove 500 µL of the grown liquid culture to a 2 mL screw top vial. *Cut the end of 1 mL pipette tip to allow large mycelia fragments to be transferred.*
3. Add 500 µL of 50% glycerol to each vial (final glycerol concentration is 25%).

4. Freeze and store the mycelia glycerol stocks at -80°C .

BASIC PROTOCOL 5

Storage of *Streptomyces* Spores as Glycerol Stocks

Glycerol stocks of *Streptomyces* spore suspensions are useful for many purposes including reliable long-term storage of strains, conjugation protocols, and various genetic and strain production purposes. Strains stored at -80°C as spore glycerol stocks are viable for several years even after multiple freeze thaw cycles. The protocol presented here is modified from Kieser et al. (2000).

Materials—Fresh sporulating agar plate (or slant) of the strain

Sterile water

50% glycerol in water (sterilized by autoclaving)

Sterile spreader or sterile loop

Sterile pipette

Heavy-duty aluminum foil

Sterile 50 mL screw top vials (for spore collection and preparation)

Sterile 2 mL screw top vials or 1.5 mL microcentrifuge tubes (for spore storage)

Vortex mixer

Centrifuge

Freezer (-80°C)

Sterile cotton filter (Puncture the bottom of a 50 mL conical screw top vial and gently pack roughly 2 cm of cotton to the bottom of the vial. After packing the cotton re-attach the screw top and wrap the entire vial with aluminum foil and autoclave.)

1. Prepare fresh spores (see **Growth of *Streptomyces* on Solid Media**).
2. Add 3 mL of sterilized water to the freshly prepared plate.
3. Utilizing a sterile spreader or sterile loop rub the plate surface, first gently and then more vigorously, suspending the spores into the water. *Try to avoid scraping agar pieces while dislodging the spores, however; in some cases this is inescapable and the resulting agar pieces will be removed in later steps.*
4. Continue until you have completely suspended all spores. *Add additional water when needed as in some cases this may take more than the suggested 3 mL of sterile water.*
5. Using a sterile pipette transfer the suspended spores to a sterile 50 mL screw top vial containing 20 mL of sterile water. *Depending on the density of spores and the amount of plates you want to harvest, you can use additional screw top vials when needed.*
6. After transferring all spores to the 50 mL screw top vial, make the final volume up to 35 mL using sterile water. *Do not fill the entire tube as this does not allow proper mixing during the subsequent vortexing steps.*

7. Break up the spore chains by vigorously mixing on a vortex mixer as violently as possible until the spores become homogeneous throughout.
8. Pass the resulting suspension through a sterile cotton filter (see Materials) and collect the filtered spores in a sterile 50 mL screw top vial. Wash any remaining spores attached to the cotton with an additional 10 mL of sterile water. *Some protocols suggest using non-absorbent cotton for filtering spores however we prefer absorbent cotton for this task.*
9. Centrifuge the filtered spores for 10 minutes ($2000 \times g$).
10. Immediately after centrifugation remove the supernatant and suspend the spores in 1 mL of sterile water.
11. Using sterile 50% glycerol, prepare a 1:1 (v/v) mixture with the suspended spores and aliquot to screw top vials or 1.5 mL microcentrifuge tubes.
12. Freeze and store the spores at -80°C .

Media Recipes

Liquid Media Recipes—All liquid media should be placed into 250 mL baffled flasks with appropriate breathable tops and then autoclaved (121°C for 45 minutes) and cooled (25°C) or stored (4°C) before use. Baffled 250 mL Pyrex Erlenmeyer flasks with cotton or foam stoppers are preferred. The components for R2YE and YEME are modified from Kieser et al. (2000).

TSB media (1 L)

Tryptic Soy Broth media: 30 g

Water: 1 L

Transfer 100 mL into 10 baffled flasks (250 mL) and autoclave.

R2YE Media (1 L)

Media A (Prepare in a single autoclavable 1L bottle with a magnetic stirring bar)

Sucrose: 103 g

K_2SO_4 : 0.25 g

$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$: 10.12 g

Glucose: 10 g

Difco casamino acids: 0.1 g

Distilled water: 800 mL

Difco yeast extract: 5 g

Media B (Each component in a separate bottle, sterilized by autoclaving)

Trace element solution: 2 mL

TES buffer (5.73 %, w/v): 100 mL

KH_2PO_4 (0.5%, w/v): 10 mL

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (3.68%, w/v): 80 mL

L-proline (20%, w/v): 15 mL

1M NaOH: 5 mL

Trace element solution (1 L)

ZnCl_2 : 40 mg

$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$: 200 mg

$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$: 10 mg

$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$: 10 mg

$\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$: 10 mg

$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$: 10 mg

Autoclave the bottle containing Media A. Allow it to cool to at least 50°C before adding Media B while in a biological safety cabinet. Place on stirrer to mix evenly and then transfer 100 mL to 10–250 mL baffled flasks. Cool or store prior to use.

Modified YEME media (1L)

Difco yeast extract: 3 g

Difco bacto-peptone: 5 g

Oxoin malt extract: 3 g

Glucose: 10 g

Sucrose: 170 g

Transfer 100 mL to 10 baffled flasks and autoclave.

Solid Media Recipes—All solid media should be prepared as follows and then autoclaved. After sterilization, the solid media should be poured into petri dishes and allowed to cool and solidify. Plates should be poured in a sterile environment, preferably a biological safety cabinet. After solidifying, the plates can be stored at 4°C for several weeks.

R2YE Media (1 L)

Media A (Add to one autoclavable 1L bottle with a magnetic stirring bar)

Sucrose: 103 g

K_2SO_4 : 0.25 g

$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$: 10.12 g

Glucose: 10 g

Difco casamino acids: 0.1 g

Distilled water: 800 mL

Difco yeast extract: 5 g

Bacto-agar: 15 g for solid agar media

Media B (each component in a separate bottle, sterilized by autoclaving)

Trace element solution: 2 mL

TES buffer (5.73 %, w/v): 100 mL

KH₂PO₄ (0.5%, w/v): 10 mL

CaCl₂·2H₂O (3.68%, w/v): 80 mL

L-proline (20%, w/v): 15 mL

1M NaOH: 5 mL

Trace element solution (1 L)

ZnCl₂: 40 mg

FeCl₃·6H₂O: 200 mg

CuCl₂·2H₂O: 10 mg

MnCl₂·4H₂O: 10 mg

Na₂B₄O₇·10H₂O: 10 mg

(NH₄)₆Mo₇O₂₄·4H₂O: 10 mg

Autoclave the bottle containing Media A. Allow it to cool to at least 50°C before adding Media B while in a biological safety cabinet. Place on stirrer to mix evenly and then transfer 100 mL to 10–250 mL baffled flasks. Cool or store prior to use.

Modified YEME media (1 L)

Difco yeast extract: 3 g

Difco bacto-peptone: 5 g

Oxoin malt extract: 3 g

Glucose: 10 g

Sucrose: 170 g

Bacto-agar: 15 g for solid agar media

ISP4-agar (1 L)

Soluble starch: 10 g

Dipotassium phosphate: 1.0 g

Magnesium sulfate: 1.0 g

Ammonium sulfate: 2.0 g

Calcium carbonate: 1.0 g

Ferrous sulfate: 1 mg

Magnesium chloride: 1 mg

Zinc sulfate: 1 mg

Adjust to pH. 7.2

Bacto agar: 18 g

Oatmeal-agar (1 L)

Oatmeal: 60 g

Bacto agar: 12.5 g

Final pH: 6.0

MYM-agar (1 L)

Maltose: 4 g

Yeast extract: 4 g

Malt extract: 10 g

Bacto agar: 18 g

Commentary

Cultivation of *Streptomyces* on either liquid or solid media is common practice for further genetic manipulation procedures. Culture conditions for *Streptomyces* are highly similar and only the media used varies from species to species. Short and long term storage conditions are suggested for ease of use.

Critical Parameters and Troubleshooting

The greatest difficulty in handling *Streptomyces* is contamination. Doubling time for *Streptomyces* requires 4–6 hours whereas many other common contaminants such as *E. coli* and *Bacillus* species have relatively shorter doubling times. As a result, careless handling of *Streptomyces* often leads to contamination. Therefore, use of sterile conditions during manipulation of *Streptomyces* strains is highly recommended. Contamination predominantly occurs during the early growth phase.

Literature Cited

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Figure 1.