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Chapter 4

Techniques for isolation of pure microbial colonies: Principles, methods, and applications

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1. Introduction:

Microbial isolation through plating is a fundamental method in microbiology, crucial for obtaining pure cultures of microorganisms from complex samples. This technique involves spreading or streaking a sample onto the surface of an agar plate, where the solid medium provides an ideal environment for microbial growth (Atlas, 2010; Tripathi et al 2013a, b).

1.1. Key Steps:

1. **Nutrient Agar Plate Preparation:** The process commences with the preparation of agar plates. Agar, derived from seaweed, forms a gel-like solid medium suitable for bacterial growth. Nutrient agar, offering essential nutrients, is commonly utilized.
2. **Inoculation:** A minute quantity of the amalgamated bacterial culture is uniformly distributed over the surface of the agar plate using aseptic procedures. This can be achieved using a sterile inoculating loop or spreader.
3. **Sterilization**: To prevent cross-contamination and ensure only the bacteria from the original inoculum are spread, the inoculating loop or spreader is sterilized between each streaking motion by passing it through a flame.
4. **Incubation:** After being inoculated, the agar plates are placed in an incubator at the ideal temperature for the target bacteria to thrive. The incubation settings differ depending on the particular bacterial species being studied.
5. **Colony Formation:** As bacteria multiply, they form discernible colonies on the surface of the agar plate. Every colony is derived from a solitary bacterial cell or a small group of similar cells found in the initial inoculum.
6. **Isolation and Enumeration:** The spread plate technique facilitates the isolation of individual bacterial colonies. By appropriately diluting the original sample before spreading, researchers ensure each colony originates from a single bacterial cell, simplifying subsequent analysis. Moreover, counting the colonies

enables estimation of the viable bacterial population in the original sample, providing valuable insights into microbial load.

2. Spread Plating Technique:

The spread plate technique is a fundamental method used in microbiology to isolate and enumerate bacterial colonies from a mixed culture. It's a simple yet powerful procedure that allows researchers to obtain pure cultures of individual bacterial species present in a sample (Prescott et al., 2005).

2.1. Procedure:

1. Using a wax pencil, make sure to label the bottom of the agar medium plates with the name of the bacterium to be inoculated, your name, and the date.
2. Transfer 0.1 ml of the bacterial culture onto the center of an agar plate by using a micropipette.
3. Immerse the L-shaped glass rod into a beaker of ethanol and remove any remaining alcohol by burning it off. Then, let it cool inside the lid of a sterile Petri plate.
4. Ensure that the bacterial sample is distributed evenly across the agar surface using the sterilized spreader.
5. Submerge the spreader in ethanol, gently tap the side of the beaker to eliminate any extra ethanol, and ignite it again.
6. Flip the plates and let them incubate for 24 to 48 hours at room temperature or 30°C.
7. After incubation, measure a few representative colonies and closely observe their morphology.

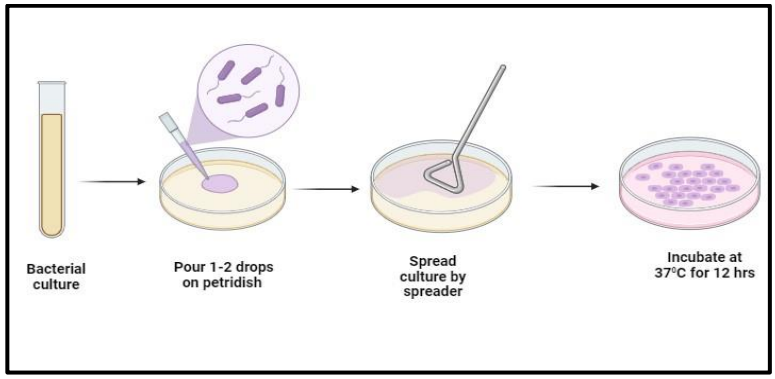

Figure 1: Schematic representation of spread plate technique

2.2. Precautions:

1. Work in a laminar flow hood or a clean, sterile workspace.
2. Wear sterile gloves, lab coats, and face masks to minimize the risk of contamination.

3. Sterilize all tools (inoculating loops or spreaders) by passing them through a flame until they are red hot before and after each use.
4. Dilute the bacterial suspension properly to ensure that individual colonies can be counted and isolated on the agar plate without overcrowding.
5. Ensure an even distribution of the bacterial suspension across the agar plate surface.
6. Use a calibrated spreader or the three-point streak method to spread the sample evenly over the plate without damaging the agar surface.
7. If isolating bacteria from a mixed sample, consider adding antibiotics to the agar to inhibit the growth of unwanted organisms while allowing the growth of target bacteria.
8. To encourage the growth of bacterial colonies, incubate the plates for the right amount of time and at a suitable temperature.
9. Maintain optimal humidity and avoid stacking plates to ensure uniform incubation conditions.
10. Regularly monitor plates during incubation for signs of contamination, such as mold growth or non-target bacterial colonies.
11. Discard contaminated plates and repeat the isolation process if necessary.
12. Label plates clearly with essential information such as date, sample source, dilution factor, and any additives (e.g., antibiotics).
13. Dispose of used plates and contaminated materials according to institutional guidelines and biohazard safety protocols to prevent accidental exposure to pathogens.
14. Clean and disinfect laboratory equipment, including spreaders, flame sterilizers, and work surfaces, regularly to minimize the risk of contamination during subsequent experiments.

3. Streak Plating Technique:

3.1. Introduction:

The streak plate method is a microbiological technique used to separate and purify bacterial colonies from a mixture of different bacteria. A minute quantity of the sample is applied in a specific arrangement on the surface of an agar plate using a sterile inoculating loop. After every consecutive occurrence, the loop undergoes sterilization in order to prevent any form of contamination. After incubation, individual bacterial colonies arise, each originating from a single cell in the original inoculum. This method enables the isolation of pure cultures for further study and enumeration of colony-forming units. It's a fundamental tool in microbiology, aiding in microbial identification, characterization, and research (Koch, 2001; Yadav et al., 2016; Tripathi et al.,2018).

3.2. Procedure:

1. The goal of this technique is to achieve single, isolated pure colonies.
2. Hold the loop in your right hand and gently heat the loop with a flame. Afterward, let it cool down.
3. Remove the lid or cotton wool plug from the test tube or plate and cautiously apply heat to the neck of the tube.
4. Immerse the loop in the culture broth and then extract it. Carefully lift the lid of the Petri dish containing the solid substrate.
5. Make sure that the charged loop is held in a parallel position to the surface of the agar.
6. Distribute the inoculum in a back-and-forth motion across a tiny portion of the medium.
7. Rotate the plate approximately 60 degrees and distribute the bacteria from the initial streak into a separate region.
8. Ensure the loop is properly sterilized once more using the identical procedure.
9. Rotate the plate approximately 60 degrees and distribute the bacteria from the second streak into a fresh area using the same pattern. Please sterilize the loop once more. Let the plate incubates overnight at 37 degrees Celsius.

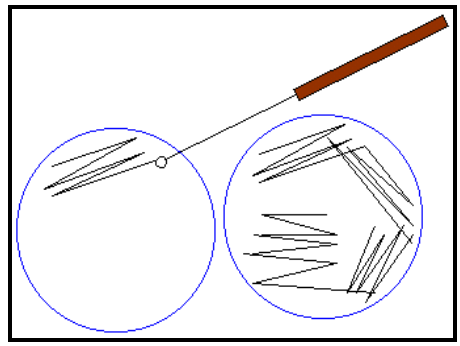

Figure 2: Isolation of pure colonies by streaking method

3.3. Precautions:

1. Practice strict aseptic technique throughout the entire procedure. Work in a laminar flow hood or a clean, sterile workspace.
2. Wear sterile gloves, lab coats, and face masks to prevent contamination.
3. To prevent cross-contamination between streaking areas on the plate sterilize the inoculating loop or needle by passing it through a flame until it is red hot before and after each use.
4. Use a fresh section of the agar plate for each streak to isolate individual bacterial colonies effectively.

5. Gradually dilute the bacterial sample on the loop or needle as you streak across the agar surface.
6. Avoid Excessive Pressure while streaking to avoid damaging the agar surface.
7. Maintain accurate records of the procedure and results for future reference.
8. Ensure optimal humidity and avoid stacking plates to maintain uniform incubation conditions.
9. Discard contaminated plates and repeat the streak plating process if necessary.
10. Dispose of used plates and contaminated materials according to institutional guidelines and biohazard safety protocols to prevent accidental exposure to pathogens.
11. Clean and disinfect laboratory equipment, including inoculating loops, flame sterilizers, and work surfaces, regularly to minimize the risk of contamination during subsequent experiments.

4. Isolation of microbial colonies by serial dilution method:

4.1. Introduction:

The serial dilution process includes the progressive dilution of a material within a solution. Generally, the concentration exhibits a logarithmic trend because of the consistent decrease in concentration at each successive step. A serial dilution can be performed by decreasing the concentration of a solution in a ten-fold manner, resulting in concentrations of 1 M, 0.1 M, 0.01 M, and 0.001 M. Serial dilutions are essential for creating precise and highly diluted solutions. Conducting a tenfold dilution for each stage is generally known as a logarithmic dilution or log-dilution. Serial dilutions are frequently used in diverse scientific disciplines, including biochemistry, pharmacology, and microbiology (Benson, 2001; Reddy et al., 2019).

All three bacterial plate count methods necessitate the use of serially diluted samples. Counting plates with over 300 CFU can be quite challenging. Plates containing fewer than 30 CFU lack statistical reliability. If a broth culture contains millions of bacteria and is plated directly from this turbid broth, all the CFU would grow together into a dense mass. Serial dilutions are typically carried out using 1mL pipettes and 9 mL sterile dilution bottles, as these devices are known for their high level of accuracy. Ensuring proper mixing of dilutions is crucial for both methods. Another option is to use phosphate buffered saline (Holt et al., 1994).

4.2. Procedure:

1. Number five tubes as 10^{-1} to 10^{-5}: 1:10, 1:100, 1:1000, 1:10,000, and 1:100,000.
2. Using a sterile 1ml pipette, carefully transfer 1ml of the broth culture into the 10^{-1} tube.
3. Each tube is filled with 9 ml of sterile water.

4. The initial tube contains a dilution of the original broth culture in a ratio of 1:10.
5. Using a fresh sterile 1mL pipette, carefully move 1mL of the broth from the initial tube (1:10 or $10^{-1)}$ and place it into a separate tube labeled 1:100 (10^{-2}).
6. Repeat the procedure multiple times, continuing until reaching a very small value.
7. Spread the 100 µl inoculums from dilutions of 10^{-5}, 10^{-6}, and 10^{-7}.
8. Use a sterile L-shaped spreader to evenly spread the inoculums across the plate's surface.
9. Ensure that the plates are incubated for the necessary duration and at the correct temperature.
10. Typically, this is maintained for duration of 24 hours at a temperature of 37° C.

4.3. Observation: After incubation, carefully observe the plate for the growth of bacterial colonies and proceed to count them applying the provided formula-

$$\text{CFU/ml} = \frac{\text{No. of colony x dilution factor}}{\text{Amount of sample}}$$

Dilution factor is reciprocal of dilution.

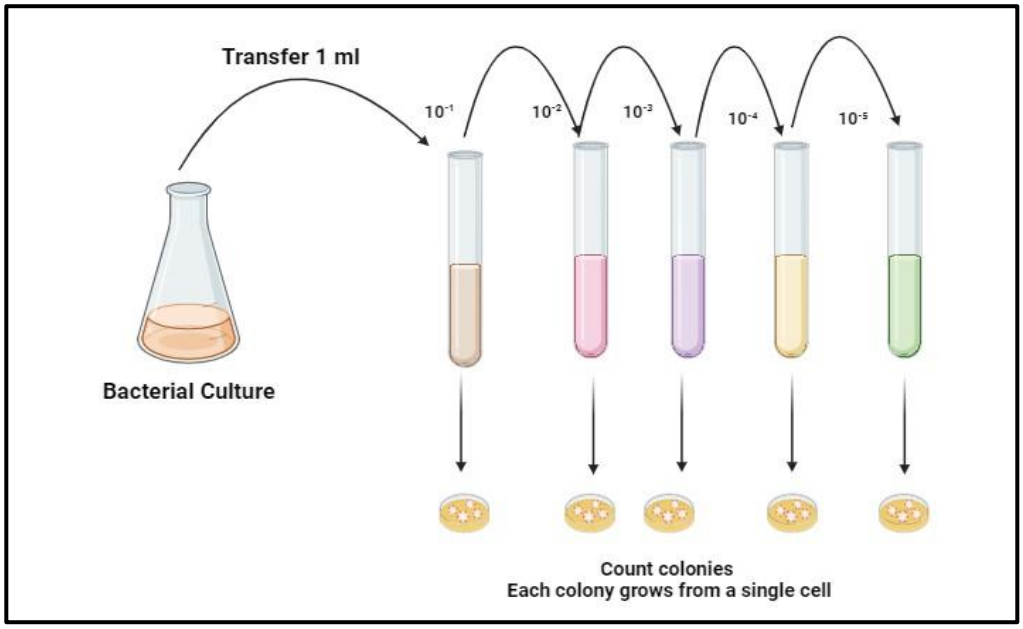

Figure 3: Isolation of microbial colonies by serial dilution method

4.4. Precautions:

1. Use sterile growth media and diluents prepared according to standard protocols.
2. Prepare a proper dilution series of the microbial sample to achieve dilutions that will allow for the isolation of individual colonies.

3. Include appropriate control experiments such as negative controls (sterile diluent) and positive controls (known cultures) to validate the serial dilution technique and detect any potential contamination.
4. To enable the formation of microbial colonies, incubate plates or tubes for the suggested period of time and at a suitable temperature.
5. Regularly monitor plates or tubes during incubation for signs of contamination, such as mold growth or unusual colony morphology.
6. Discard contaminated plates or tubes and repeat the isolation process if necessary.

5. References:

1. Atlas, R. M. (2010). *Handbook of Microbiological Media* (4th ed.). CRC Press.
2. Benson, H. J. (2001). *Microbiological Applications: A Laboratory Manual in General Microbiology* (8th ed.). McGraw-Hill.
3. Prescott, L. M., Harley, J. P., & Klein, D. A. (2005). *Microbiology* (6th ed.). McGraw-Hill.
4. Koch, A. L. (2001). Growth measurement and isolation of microorganisms using plating techniques. *Microbiology Reviews, 56*(3), 295–312.
5. Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., & Williams, S. T. (1994). *Bergey's Manual of Determinative Bacteriology* (9th ed.). Williams & Wilkins.
6. Tripathi, K., Sharma, N. K., Rai, V., & Rai, A. K. (2013a). Low cellular P-quota and poor metabolic adaptations of the freshwater cyanobacterium *Anabaena fertilissima* Rao during Pi-limitation. *Antonie van Leeuwenhoek, 103*, 277–291.
7. Tripathi, K., Sharma, N. K., Kageyama, H., Takabe, T., & Rai, A. K. (2013b). Physiological, biochemical, and molecular responses of the halophilic cyanobacterium *Aphanothece halophytica* to Pi-deficiency. *European Journal of Phycology, 48*(4), 461–473
8. Tripathi, K., Kumar, N., & Abraham, G. (Eds.). (2018). *The role of photosynthetic microbes in agriculture and industry*. Nova Science Publisher, USA.
9. Yadav, R. K., Thagela, P., Tripathi, K., & Abraham, G. (2016). Physiological and proteomic analysis of salinity tolerance of the halotolerant cyanobacterium Anabaena sp. *World Journal of Microbiology and Biotechnology, 32*, 1-11.
10. Reddy, Y. P., Yadav, R. K., Tripathi, K., & Abraham, G. (2019). Isolation and characterization of high temperature tolerant mutant from the cyanobacterium Anabaena doliolum. *Journal of basic microbiology, 59*(3), 314-322.