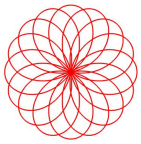
BioLumen Reactor Laboratory Manual



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Introduction

The goal of the BioLumen Reactor project is to design and build a bioreactor to grow the bioluminescent bacterium *Vibrio fischeri*. This has required the establishment of multiple laboratory and cell culture protocols that are adaptable to non-standardized working environments, and that are understandable to those with little to no prior laboratory experience. These protocols have been written with reference to prior literature, and also from hands-on experience working with *Vibrio fischeri* in cell culture. They are also written such that all materials required are non-restricted, and can be freely purchased from sites like eBay, Amazon, and other online retailers.

It is important to note that *Vibrio fischeri* is a BSL-1 (Biosafety Level 1) organism, meaning it is generally recognized as safe for research, and that it is non-pathogenic to humans. This bacterium exhibits multiple beneficial properties for cell culture, as it is tolerant to a wide range of pH, temperature, and shear conditions in culture. This allows flexibility for culturing at room temperature, with no pH fixing, and with different forms of agitation. It is also commonly used as an educational tool, and so is freely available for purchase at an inexpensive price - in comparison to research-grade strains of bacteria.

This manual represents a year's worth of laboratory work and literature research by undergraduates at the University of Minnesota - Twin Cities. May it provide you a deeper understanding of cell culture and serve as a future resource for open source science.

David Wang

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Basic Aseptic Technique for Cell Culture

Purpose

To remove possible routes of contamination when manipulating cell cultures, and to promote a safe working environment for the cell culturist.

Materials

- Nitrile/latex gloves
- Lab coat (*optional*)
- Safety glasses (*optional*)
- Biosafety cabinet (*optional*)
- Bunsen burner
- 70% ethanol or isopropanol

Procedure

1. Before beginning work, tie long hair up and wash hands thoroughly with soap and water
2. Gown to the appropriate level of personal protective equipment (PPE) for the organism being cultured
    a. Gloves are the minimum requirement for PPE
    b. Depending on the infection risk of the organism or the necessity for maximum sterility, lab coats, safety glasses or even more stringent PPE may be worn
3. Sterilize the work surface by wiping down thoroughly with 70% ethanol
    a. Re-sterilize gloved hands periodically while working with 70% ethanol
    b. Similar to **Step 2b**, a laminar flow hood or biosafety cabinet may be required as an additional layer of protection against contamination and adverse exposure events
    c. A non-porous, easily-sterilized work surface in a well-ventilated space is the minimum requirement
4. If working outside of a laminar flow hood or biosafety cabinet, a Bunsen burner can be used to promote a sterile working area (air convection due to flame)
    a. Ethanol lamps or butane torches/camp stoves can be used as an alternative to Bunsen burners
    b. Avoid rapid gestures when working near a Bunsen burner, and direct coughs or sneezes away from the sterile working area
5. Sterilize any containers or tools that are brought into the sterile area
    a. For containers, spray or wipe down openings before use with 70% ethanol
    b. For containers, can also ‘flame’ openings before use by slowly moving opening over a Bunsen burner
    c. For tools made to be sterilized (glass/steel), can flame sterilize by pouring 95% ethanol over and burning vapors off over a Bunsen burner

    d. For inoculation loops (nickel/chrome), can flame sterilize directly in a Bunsen burner until glowing orange
    e. Let 10 seconds pass after flame sterilizing a tool before use, and do not bring tool into contact with non-sterile surfaces in the interim
6. When finished working, re-sterilize the work surface with 70% ethanol and wash hands thoroughly with soap and water

References

1. Coté, R. Aseptic Technique For Cell Culture. *Current Protocols in Cell Biology* 1998, *00* (1), 1.3.1-1.3.10.
2. Cell Culture Protocol 1: Proper Aseptic Technique and Sterile Handling of Cells. https://www.sigmaaldrich.com/technical-documents/protocols/biology/aseptic-technique.html (accessed Aug 17, 2020).

Alternative Method for Glassware and Reagent Sterilization

Purpose

To ensure that all glassware, media, and other components that come into contact with a culture are properly sterilized before use.

Materials

- Pressure Cooker or Autoclave (*recommended*)
- Liquid Medium/Agar/Glassware to sterilize

Procedure

1. If using an autoclave, see manufacturer instructions for most accurate guidance.
    a. Generally use a 'liquid cycle' if available for liquid media
    b. For agar/agarose gels, cycle times > 20 minutes are recommended
    c. Do not fill your container more than half of its total volume with liquid, to prevent over-pressurization
    d. Ensure a layer of secondary containment when autoclaving, i.e. an autoclave-safe tray or bin
    e. For vessels or bottles filled with liquid, make sure caps are screwed on loosely, to prevent over-pressuring the container
    f. It is recommended to use autoclave tape on the vessel of interest, which changes color to ensure that the autoclave reached the proper temperature
2. If using a pressure cooker, set so as to maintain 15 psi for > 15 minutes
    a. An inch or two of water in the pressure cooker vessel is necessary for steam pressurization
    b. Set containers offset from the bottom of the pressure cooker vessel, to prevent overheating
    c. Do not fill your container more than half of its total volume with liquid, to prevent overpressurization
    d. For vessels or bottles filled with liquid, make sure caps are screwed on loosely, to prevent over-pressuring the container
    e. It is recommended to let steam vent naturally, rather than directly releasing steam via a pressure cooker's depressurization valve after a pressure cycle
    f. A foil cap, taped over the container can be substituted if a vessel does not have a built-in cap
3. After sterilizing a container, do not open or expose its contents unless in a sterile environment
    a. Either a biosafety cabinet, laminar flow hood, or sterile field provided by a Bunsen burner

References

1. Teach.Genetics. 2015. *Sterilizing Liquids*. [online] Available at: <https://teach.genetics.utah.edu/content/microbiology/liquids/> [Accessed 19 August 2020].
2. Swenson, V.; Stacy, A.; Gaylor, M.; Ushijima, B.; Philmus, B.; Cozy, L.; Videau, N.; Videau, P. Assessment And Verification Of Commercially Available Pressure Cookers For Laboratory Sterilization. *PLOS ONE* 2018, *13* (12), e0208769.

Preparation of Photobacterium Liquid and Solid Medium

Purpose

To prepare both liquid and solid growth medium for the culture of *Vibrio fischeri* or other photobacterium.

Materials

- Seawater aquarium salt (33 g/L)
- Yeast extract (5 g/L)
- Tryptone (5 g/L)
- Glycerol (3 g/L, ~2.5–3 mL)
- Tris (6 g/L)
- NH_4Cl (5 g/L)
- Distilled water
- CaCO3 (1 g/L, *for solid medium*)
- Agar (15–20 g/L, *for solid medium*)
- Erlenmeyer flask/media bottle
- Plastic cell culture plates or sterile glass petri dishes (*for solid medium*)

Procedure

1. Measure the required solids for liquid medium, and transfer to a suitable container for autoclaving and pouring (Erlenmeyer flask/media bottle)
    a. If preparing solid medium, measure the additional solids, $CaCO_3$ and agar, to supplement the liquid recipe
2. Dissolve the solids in the desired volume of distilled water, and add liquid glycerol
    a. Small amount of solids may not completely dissolve at room temperature, can move forward if solution is nearly clear
3. Autoclave or pressure sterilize, a cycle time > 20 minutes is recommended
    a. See 'Alternative Method for Glassware and Reagent Sterilization'
4. Allow solution to cool to a manageable temperature, ~15-20 minutes
    a. If preparing liquid medium, once cooled can store refrigerated for 3-4 weeks or longer if kept sterile
5. If preparing solid medium, set up a sterile work area to pour agar plates
    a. See 'Basic Aseptic Technique for Cell Culture', **Steps 1-4**
6. Pour plates within the sterile work area
    a. Set plates on sterile work surface and pour agar down from container
    b. Keep one hand free to move plate lids, and use the other to pour liquid agar from the container
    c. Only need to pour enough to cover the bottom of the plate
    d. Replace place lid, slightly askew, after pouring agar
    e. Allow excess steam to be released, ~15-20 minutes, before properly closing plate lids and storing plates

f. Store plates upside down, agar-side up, to prevent condensation forming on the agar surface
g. Can store plates either in plastic-wrapped stacks or in the plastic sleeves that they originally came in
h. Plates can be stored refrigerator for 3-4 weeks or longer if kept sterile, and if the agar has not dried out

7. Re-sterilize the work area and work tools; clear the work area and put away items used for the procedure

References

1. Madden, D. and Lidesten, B., 2001. *Bacterial Illumination*. [online] Available at: <https://bioenv.gu.se/digitalAssets/1566/1566430_photoen.pdf> [Accessed 24 August 2020].
2. Making Agar Plates. https://teach.genetics.utah.edu/content/microbiology/plates/ (accessed Aug 24, 2020).

Subculture of *Vibrio Fischeri* on Solid Medium

Purpose

To promote the aseptic subculturing of *Vibrio fischeri* from a starter culture on solid medium to another solid medium.

Materials

- Inoculation loop
- Starter culture, on agar slant/plate
- Bunsen burner or alternative
- 70% ethanol
- Photobacterium agar plate

Procedure

1. Prepare a sterile work area with the Bunsen burner and by wiping down work surfaces with 70% ethanol
    a. See Basic Aseptic Technique for Cell Culture, **Steps 1-4**
2. Move all containers and working items into the sterile work area
    a. See Basic Aseptic Technique for Cell Culture, **Step 5**
3. Flame sterilize inoculation loop in Bunsen burner before contact with any culture
    a. See Basic Aseptic Technique for Cell Culture, **Step 5**
4. Work carefully, using one hand to manipulate the inoculation loop, and one hand to manipulate the cell cultures
5. Rub inoculation loop over an area of the starter culture surface with visible bacteria growth
    a. Can look like white or yellow ‘plaques’ on the agar
    b. Can confirm if the visible areas are *Vibrio fischeri* growth by viewing them in a dark room to check bioluminescence - should glow blue-green
6. Without touching the inoculated loop to any other surface, rub the loop onto the surface of the fresh agar plate
    a. Recommended technique is to ‘streak’ from center to rim of the plate
    b. Very little force is necessary to streak the plate with the inoculation loop, be careful not to tear the agar
    c. See **Reference 1** for more information on plating techniques
7. Label plate lid with a Sharpie or other indelible marker, noting the date of subculture, the organism name, the type of medium, and the name of the cell culturist
8. Let plate incubate at the appropriate temperature, light intensity, and humidity
    a. For *Vibrio fischeri*, average room temperature (~22°C) or slightly below is an appropriate temperature, and store in a dark, dry space to incubate
9. Re-sterilize the work area and work tools; clear the work area and put away items used for the procedure

10. Depending on how effective the inoculation from the starter culture was, bioluminescence of the culture on solid medium can be observed after ~24 hours
    a. Observe in a dark room, and give time for eyesight to adjust to the dark before viewing

References

1. Sanders, E. Aseptic Laboratory Techniques: Plating Methods. *Journal of Visualized Experiments* 2012, No. 63.

Initiation of *Vibrio Fischeri* Culture in Liquid Medium

Purpose

To promote the aseptic subculturing of *Vibrio fischeri* from a starter culture on solid medium to liquid medium.

Materials

- Inoculation loop
- Starter culture, on agar slant/plate
- Bunsen burner or alternative
- 70% ethanol
- Photobacterium liquid medium
- Cell culture flask (Shake flask/Erlenmeyer flask)
- Magnetic stir plate/shaker platform
- Magnetic stir bar

Procedure

1. Prepare a sterile work area with the Bunsen burner and by wiping down work surfaces with 70% ethanol
    a. See Basic Aseptic Technique for Cell Culture, **Steps 1-4**
2. Move all containers and working items into the sterile work area
    a. See Basic Aseptic Technique for Cell Culture, **Step 5**
3. Flame sterilize inoculation loop in Bunsen burner before contact with any culture
    a. See Basic Aseptic Technique for Cell Culture, **Step 5**
4. Prepare the cell culture flask (sterilized shake/Erlenmeyer flask) by filling with liquid medium
    a. If using a magnetic stir plate, insert a magnetic stir bar into the cell culture flask
    b. If using an Erlenmeyer flask without a lid, use a metal foil cap (secured with tape) to cover the opening before taking the flask out of the sterile region
    c. Do not fill the cell culture flask beyond half of its indicated volume, to ensure excess oxygen headspace for the culture
    d. The smaller the volume of media, the faster it will take for the seed culture of *Vibrio fischeri* to deplete the media of nutrients and begin to die off; 15-20 mL of liquid media for the initiation is recommended
5. Work carefully, using one hand to manipulate the inoculation loop, and one hand to manipulate the cell cultures
6. Rub inoculation loop over an area of the starter culture surface with visible bacteria growth
    a. Can look like white or yellow ‘plaques’ on the agar
    b. Can confirm if the visible areas are *Vibrio fischeri* growth by viewing them in a dark room to check bioluminescence - should glow blue-green

    c. To ensure enough transfer of cells, scrape enough cells to notice visually from the surface of the plate
7. Without touching the inoculated loop to any other surface, insert the loop below the liquid surface of the cell culture flask and swirl/shake the loop to inoculate the medium with seed cells
    a. Swirling the inoculation loop in the liquid medu only requires ~15 seconds
    b. If a visible cell mass was scraped from the starter culture, shake until dislodged from the inoculation loop
8. Reseal the cell culture flask, and incubate at the appropriate temperature, light intensity, and humidity on either a magnetic stir plate or shaker platform
    a. For *Vibrio fischeri*, average room temperature (~22°C) or slightly below is an appropriate temperature, and store in a dark, dry space to incubate
    b. To determine a proper agitation rate for shaker or stir plate in the case that the culture was inoculated with a visible cell mass, choose a high enough rate to visibly rotate the cell mass at > 1/second.
9. Re-sterilize the work area and work tools; clear the work area and put away items used for the procedure
10. Depending on the volume of media in the cell culture flask and how large the inoculation mass was, bioluminescence of the liquid culture can be observed after ~24 hours, and can be induced by shaking the culture flask, assuming excess oxygen headspace for the culture
    a. Observe in a dark room, and give time for eyesight to adjust to the dark before viewing

References

1. Christensen, D. and Visick, K., 2020. Vibrio fischeri : Laboratory Cultivation, Storage, and Common Phenotypic Assays. *Current Protocols in Microbiology*, 57(1).
2. Living Organism Care Guide: Vibrio fischeri. https://www.carolina.com/teacher-resources/Interactive/living-organism-care-guide-vibrio-fischeri/tr23902.tr (accessed Aug 29, 2020).

Subculture of *Vibrio Fischeri* in Liquid Medium

Purpose

To promote the aseptic subculturing of *Vibrio fischeri* from a starter culture in liquid medium to another liquid medium.

Materials

- Serological pipette/pipette gun
- Starter culture, in liquid medium
- Bunsen burner or alternative
- 70% ethanol
- Photobacterium liquid medium
- Cell culture flask (Shake flask/Erlenmeyer flask)
- Magnetic stir plate/shaker platform
- Magnetic stir bar (if using stir plate)

Procedure

1. Prepare a sterile work area with the Bunsen burner and by wiping down work surfaces with 70% ethanol
    a. See Basic Aseptic Technique for Cell Culture, **Steps 1-4**
2. Move all containers and working items into the sterile work area
    a. See Basic Aseptic Technique for Cell Culture, **Step 5**
3. Prepare the cell culture flask (sterilized shake/Erlenmeyer flask) by filling with liquid medium
    a. If using a magnetic stir plate, insert a magnetic stir bar into the cell culture flask
    b. If using an Erlenmeyer flask without a lid, use a metal foil cap (secured with tape) to cover the opening before taking the flask out of the sterile region
    c. Do not fill the cell culture flask beyond half of its indicated volume, to ensure excess oxygen headspace for the culture
    d. The chosen volume of liquid medium for the subculture vessel depends on multiple factors:
        i. If expansion of the cell culture is desired, the entire starter culture can be transferred into a much larger volume of fresh cell culture media
        ii. If the total volume of the culture is meant to be conserved, a fractional volume of the starter culture can be transferred into a proportional volume of fresh cell culture media
        iii. A volume ratio of 1:100 starter culture to fresh media is recommended for cell culture expansion, to maintain ~3 day growth cycle
4. Using a serological pipette and pipette gun, transfer the intended volume of active culture from the starter culture flask to the fresh medium flask
    a. Before transferring liquid from the starter culture flask to the serological pipette, swirl the flask to resuspend and homogenize the cells in the culture medium

    b. Do not contaminate the outer surface of the serological pipette by touching to any other surface while transferring the cell culture liquid
    c. If a serological pipette and pipette gun is not available, it is possible to pour starter culture liquid from one flask to another; however, this increases the risk of contamination by the surrounding air, and increases the risks of spills
5. Reseal the new cell culture flask, and incubate at the appropriate temperature, light intensity, and humidity on either a magnetic stir plate or shaker platform
    a. For *Vibrio fischeri*, average room temperature (~22°C) or slightly below is an appropriate temperature, and store in a dark, dry space to incubate
    b. To determine a proper agitation rate for shaker or stir plate in the case that the culture was inoculated with a visible cell mass, choose a high enough rate to visibly rotate the cell mass at > 1/second.
6. Re-sterilize the work area and work tools; clear the work area and put away items used for the procedure
7. Depending on the volume of media in the cell culture flask and how large the inoculation volume was, bioluminescence of the liquid culture can be observed after ~24 hours, and can be induced by shaking the culture flask, assuming excess oxygen headspace for the culture
    a. Observe in a dark room, and give time for eyesight to adjust to the dark before viewing

References

1. Christensen, D. and Visick, K., 2020. Vibrio fischeri : Laboratory Cultivation, Storage, and Common Phenotypic Assays. *Current Protocols in Microbiology*, 57(1).
2. Living Organism Care Guide: Vibrio fischeri. https://www.carolina.com/teacher-resources/Interactive/living-organism-care-guide-vibrio-fischeri/tr23902.tr (accessed Aug 29, 2020).

Decontamination of Cell Culture Waste

Purpose

To safely decontaminate and dispose of cell culture wastes - most importantly, spent cell culture medium used for the growth of *Vibrio fischeri*.

Materials

- Clorox Bleach (NaOCl)
- Cell Culture Waste
- Autoclave (or equivalent)

Procedure

1. For decontamination of cell cultures in liquid medium with bleach, dilute until flask contains 10% bleach by volume
    a. If possible, perform decontamination procedure within a fume hood or other well-vented area
    b. Allow at least 20 minutes of contact time for bleach in solution to inactivate any cells left in the culture
    c. Can dispose of decontaminated solution via drain disposal, flushed down with copious amounts of water
2. For decontamination of cell cultures on solid medium with bleach, can prepare a solution of 10% bleach in water in a large bin
    a. Submerge cell culture plates, or other solid growth media fully under bleach solution
    b. Allow at least 20 minutes of contact time for bleach in solution to inactivate any cells left in the culture
    c. Can dispose of decontaminated solids as biohazard waste in accordance with institutional or local guidelines
3. For decontamination of cell cultures in liquid or solid medium in an autoclave or equivalent:
    a. Place cell culture flasks/plates in a leakproof, autoclave-safe bin
    b. Ensure that any containers with liquid are no more than half full
    c. Autoclave as recommended by machine instructions; see Alternative Method for Glassware and Reagent Sterilization for more information
    d. Can dispose of decontaminated liquids via drain disposal, flushed down with copious amounts of water
    e. Can dispose of decontaminated solids as biohazard waste in accordance with institutional or local guidelines

References

1. COMS Policy on Suitable Methods of Liquid Decontamination and Disposal. https://hms.harvard.edu/departments/committee-microbiological-safety/registering-coms/coms-policies/coms-policy-suitable-methods-liquid-decontamination-disposal (accessed Aug 31, 2020).
2. COMS Policy on Suitable Methods of Liquid Decontamination and Disposal. https://hms.harvard.edu/departments/committee-microbiological-safety/registering-coms/coms-policies/coms-policy-suitable-methods-liquid-decontamination-disposal (accessed Aug 31, 2020).