# Isolation and Culture of Bioluminescent Bacteria

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**Before You Continue..**

This guide assumes that you are able to make sterile culture media in which to grow your target bacteria. If you have not yet made your own sterile media, it's not that hard. For instructions and recipes on homebrew culture media, see here: http://letters.cunningprojects.com/?p=97

**A Note on Equipment**

This experiment, and others like it, can be done without equipment. Particularly as the bacteria involved grow comfortably without incubation. However, to take microbiology on as a serious hobby, you may need to buy specialised equipment.

Although this will hopefully soon change, science is expensive, and not for fair reasons. Patents, monopolies, price fixing and a lack of consumer effort on the part of labs worldwide has led to a situation where even the simplest of lab equipment items costs thousands and does even less than a microwave.

With the advent of DIYbio and microbiology-as-a-hobby, small suppliers are appearing that cater to the needs of individuals and hobby groups at reasonable prices. Some suppliers or sources of equipment are listed at the end of the document. However, where possible in this document, I will attempt to work around the need for specialised equipment so that you can do this without a big investment.

**Microbiology as a Hobby**

Microbiology is the study of living things at a scale smaller than the human eye can see. This includes almost all bacteria, yeasts and molds, most of a fungus' life cycle, and many unique and strange organisms besides. The skills of a microbiologist can also be adapted and turned toward the study of larger organisms at a cellular level, for example the culture of animal or plant tissues.

Microbiology is not traditionally seen as a hobby, and in some respects this is justified. After all, the best known microbes are disease-causing pathogens or food poisoning agents. However, thesed microbes are by far the minority, and responsible behaviour and practise can make microbiology a very rewarding, stimulating and safe activity. Microbiology can be used to culture bacteria from the kitchen, workplace or bedroom so as to better understand the unseen aspect of the world you inhabit. It can be used to culture bacteria that dwell on the skin, to show how many bacteria live peacefully with us, and how others lie waiting for an opportunity to infect or poison us. It can even be used to culture natural curiosities, such as bacteria that glow softly in the dark. It is this latter exercise that this guide will demonstrate, but the key skills involved are easily adapted to other applications.

“Citizen Science” is increasingly recognised as a requirement for the stable advancement of knowledge in our society. Citizen Science is not merely public awareness, but public involvement; a Citizen Scientist doesn't passively wait for answers, but actively seeks them and shares them to help advance our understanding of the world. When science becomes a field only for specialists, and public awareness of the key skills and facts fade, then scientific advancement may well come to a standstill. Science was, at the outset, a hobby for the curious. It is high time the curious returned to science, as it becomes more and more important to our daily lives!

This guide to culturing bacteria will illustrate several key skills in microbiology. Streaking agar to isolate colonies can allow you to take a “mixed bag” of unknown bacteria from the wild and select only one species to grow and study. Identifying species by their appearance on an agar plate is the most basic way to tell them apart, though further tests are always needed to determine for certain what species you have found. Perhaps most importantly, developing a respect for the potential hazards, and a habit of keeping things sterile and tidy, will go a long way towards making your hobby safer.

With some practise and further study, Microbiology may become a much loved and useful skill as well as a hobby for you. If not, then it is hoped this guide will at least entertain. Enjoy!

**Your First Project: Culturing Bioluminescent Bacteria**

Bioluminescence is a property of some naturally occurring bacteria found in the benthic depths of the Oceans worldwide. It is a natural ability to produce visible levels of light, which benefits bacteria or their symbiotes in a variety of ways, from camoflage to warning to predation.

While individual bacteria do not produce enough light to be seen on their own, when grown in high densities they can put out enough to illuminate a small area; enough to see by or even read by with effort. If grown creatively on a solid or semisolid substrate and with enough oxygen, they can maintain a sustained glow for quite some time without care or maintainance.

Bioluminescence makes a great curio with which to demonstrate micriobiology, as well as presenting a few interesting applications to try out. As a dim night light, a culture of bioluminescent bacteria is inexpensive, long lasting and indifferent to power-cuts or brownouts. As a pure curio, a culture could be placed in a bubbling “lava lamp” instead of water. As part of an art project, they present an interesting live medium and provide a sustained glow as long as they have air and medium. They are under study as a potential source of light for rural areas in developing countries, where electricity is not available.

Culturing these bacteria is also pretty easy in comparison to some alternative projects centered on bacteria encountered in daily life. Because bioluminescent bacteria usually are found in the ocean, they grow comfortably in medium with a high salt content, which helps to keep down contamination by other bacteria accustomed to the land or freshwater. Finding the bacteria is as simple as leaving some fresh shrimp or squid in a bucket of salty water or clear brine in a cool room, and waiting for them to grow and start glowing. They are comfortable with cold temperatures and grow at only a little below room temperature (most grow at 18-20 degrees Celsius). They don't tend to exhaust their medium too quickly, so they can be kept stably without too much work for a while. In short, they make a great first project.

**General Hygiene and Caution**

Bacteria live everywhere, and can colonise almost anything. They can be pathogenic (disease causing), food spoiling or totally harmless. They can also be beneficial for agriculture (root bacteria, for example), digestion, protection from pathogens and a host of other things. In a way, they are just like anything else living; either a friend or a foe, and either helpful, useful or best avoided.

However, unlike most things you encounter daily, you are unable to see or identify by the normal range of your senses which bacteria you are dealing with. It is important to protect whatever species you are working with from contaminating strains found on your skin. It is more important to take precautions to protect your health and the health of others. Thankfully, these precautions are common sense measures that shouldn't be any different from what you are used to from cooking or cleaning a house.

Always wear gloves when working with microbiology. These can be disposable latex gloves, or re-usable rubber gloves, but what is important is that your hands be covered from the objects and materials you are using. I recommend buying a comfortably fitted pair of thin rubber gloves, and keeping them in good condition. Not only does this save money and the environment, but it cuts down on the number of waste items you may have to sterilise if you start experimenting with riskier strains later on. Remember to always wash your gloves while they are still on with an antibacterial detergent, before removing them and putting them away.

Never perform your microbiology experiments, transfers or cultures in a kitchen or near where food is prepared. Many bacteria that are generally considered safe because they do not cause disease can still be harmful if they grow to large numbers in food and are consumed. This is because of the way that the immune system reacts to some components of the bacterial cell membrane, and accounts for many cases of food poisoning yearly. Keep your bacteria out of the kitchen, and wash your gloves, and then your hands after every session of bacterial care!

Clearly label what you are working with, so you know which strains you put where, and if they present any hazards be sure to write these clearly on the outside to remind yourself and warn others. For that matter, good labelling, book-keeping and records are critical skills in any field; keep a little journal where you record your work and update it with your results.

Sterilise everything before you work with it, so that you can discount your tools or vessels as sources of contamination. Sterilisation is usually accomplished with an “autoclave”, which is a fancy form of pressure cooker. For a home lab, it is most sensible to learn how to use a pressure cooker safely, and to sterilise your equipment and culture materials with a 15-20 minute sterilisation cycle once full steam pressure is reached in the cooker. Remember to add enough water to prevent boil-off in the vessel, try to keep your equipment raised from the floor of the vessel, and remember to let steam force out the air in the cooker before allowing it to come to pressure. If liquid is to be sterilised, let it come down as slowly as possible from the heat and pressures of the sterilisation cycle, or the liquid will boil over and make a mess (see Boyle's Law on wikipedia for why this happens).

To sterilise your gloves, and for sterilisation of surfaces, you can use a mild bleach, an alcohol wipe/spray/gel or a surface cleaner with ample antibacterial action. Bleach and alcohol are staples of lab sterility, and are best used in combination, perhaps bleach first and alcohol after. Isopropyl (“rubbing”) alcohol or ethanol are appropriate for this task at about 70% concentration in deionised water. If you haven't anything special on hand, use one of the hand sterilising creams or gels that became ubiquitous around the advent of Swine Flu.

Finally, when you are finished with a strain, a culture vessel or if your strains die off, you must make sure to dispose of the bacteria responsibly. This means sterilising them before disposal! Put all of your vessels and culture media back in the cooker, and sterilise them for 20 minutes. Assuming no hazardous chemicals were in the vessels as well, they should be safe to dispose of normally afterwards. Throw away your agar, pour away your liquid medium, wash your hands and scrub the vessels with detergent. Don't mix agar and liquid or you'll have a mess that can't be thrown in the bin, and might clog your sink! If you do this by accident, you could try filtering the agar out of the mix with a sieve.

**Growth on Seafood and Isolation**

You will need several things, and some other things are preferable extras.

Needed tools and consumables:

- Fresh, unwashed, unfrozen squid or shrimp. As fresh as possible, kept on ice.
- Artificial “Brine”: 30g Sea Salt or Table Salt dissolved in bottled or deionised water.
- Luminescence Agar Medium: Sterilised artificial brine with added nutrients and antacid buffer.
  - Detailed fully in another document: http://letters.cunningprojects.com/?p=97
  - It may also be helpful to have Luminescent broth
- Wood Toothpicks, or a metal wire loop
- A Craft Knife

Bioluminescent bacteria of several types are found living freely in the water at benthic depths, and also live either as harmless commensals on the skin of marine wildlife, or instead may actually form a full symbiotic partnership with a fish or arthropod.

As many or most squid species that are commonly found at market will have lived their truncated lives at or about the benthic depths, they make an ideal source of bioluminescent bacteria. All you need to do is let the bacteria grow!

This part requires a container big enough for your squid carcass. After placing the squid carefully inside with minimal disruption or contact with the open areas of the skin, add the artificial “brine” to the container. Drop the liquid from a slight height so that it bubbles on impact with the surface; this helps to aerate the artificial seawater, which improves the visibility of glowing colonies.

Because many bioluminescent bacteria are able to grow at 4C, we can start selecting for our target species right away by keeping the squid (covered for hygiene!) in a fridge or cold room for 24-36 hours. However, some species will not glow effectively at these cold temperatures. Therefore after this, leave the carcass in a cool room (between 15-20C is ok) to encourage faster growth and visible glowing. You will need to check the carcass hourly, and to prepare a clean and tidy way to dispose of the squid once finished.

Each hour, darken the room as much as you can and let your eyes adjust for a few minutes, then scrutinise the carcass for spots of brightness. Bear in mind that the fovea of the eye, where the center of vision is focused, cannot see in the dark. Therefore if you look directly at something, you won't see it. Instead, focus slightly to the left or right to observe something close-up.

If you can buy or build a red lamp, flashlight or bulb then it may help at this stage. Deep shades of red light

do not interfere with night vision, so it can be used to see what you are doing without hindering your ability to see glowing colonies develop. You can often find red LED headlamps in DIY or camping shops.

It may take between six and ten hours before you start to see glowing colonies forming. The colonies glow in response to oxygen remember, so you may have to pour away the artificial brine and add freshly shaken and oxygenated medium now and then. Don't pour it right on the carcass in case it smears your bacteria. Cycling this liquid medium will incidentally help to rinse away squid ink, which can obviously interfere with seeing your colonies also. It is possible that colonies will be more abundant on the underside of the squid; some species use softly glowing bacteria to obscure their shadow from predators underneath.

When colonies start to grow, resist the urge to work right away. If you leave them grow for another hour or two, they will become even larger and brighter; not only does this present an interesting natural light show, but it makes it easier to pick your bacteria.

**Picking and Streaking Colonies**

Isolating a single species or even a single bacterium from a mix of cells is actually fairly simple. “Streaking” cells across a plate, so that they are spread out progressively thinner and thinner, is the normal way to spread cells so sparsely that any given colony can be assumed to have developed from a single cell.

When you feel ready, take a fresh pick, wire loop or craft knife. Turn it under a lighter for a second or two to sterilise it, and let it cool for a while so you don't burn your cells. Use the tip of the tool to scrape gently at the surface of the glowing colony you want to isolate.

Then, open a sterile plate/dish/ramekin/shallow jar of Luminescent Agar (detailed in the document linked above), and gently rub the tip across the surface of only one quarter of the plate. This inoculates that quarter with whatever bacteria were on the tip, which almost certainly contains more than just your desired bacteria.

To spread them more thinly, dry your pick and sterilise it again with the lighter. Give it a little while to cool down. Then drag it through one or three of the streaks you placed at first and drag from there into another quarter. Zigzag streak it in this other quarter also. Sterilise the tool and repeat for the other two quarters, each time dragging from the previous quarter. You should then have four quarters that will have progressively fewer cells in each; at least one of these should be suitable for isolating a pure strain later. If not, you can streak a likely colony from the plate on a fresh plate to further purify your strains until you can get a single colony.

Repeat this whole procedure of picking a colony, streaking it and thinning it over a plate, for at least three and preferably more agar plates. It is best to have wide, shallow containers like the petri dishes used in labs (and available from ebay), but if only small containers are available (jam jar lids, perhaps) you may want to streak a larger number to be sure.

You migh want to pick a colony or even excise a little glowing patch of the squid carcass and immerse it in liquid medium as an alternate method of growing your cells. If you can keep the liquid medium agitated as the bacteria grow, then the liquid will remain fairly aerated and the bacteria will grow happily in suspension. You will still have a mixed culture, and it is possible that non-target species will grow in the medium faster than your desired cells. If the medium starts to glow even slightly, then it's a good time to take a small liquid sample from the medium and streak that on agar to isolate cells.

When you have finished picking colonies, dispose of the carcass immediately and safely. It might be best to skip the bin in your house and place it straight in an outdoor bin for collection, bagged by itself. It is, after all, spoiled meat. Wash your gloves and hands after handling it.

Keep your cells at between 15C and 20C for optimum growth; most bioluminescent bacteria will grow at these temperatures. Some bacteria that grow at lower temperatures will not glow much until they have spent an hour or so growing at 15C-20C. You could consider incubating your bacteria at 4C to favour these “psychrophilic” (cold-loving) bacteria; they include Vibrio phosphoreum, the most vigourously glowing bacterium commonly isolated. Remove the bacteria for an hour before checking them for glowing in the dark.

Depending on the species and the temperature, it could be between 24 and 48 hours before you see evidence of growth. You will be able to see bacteria growing on a culture vessel with your eyes; single colonies are

usually circular or dome-shaped, but some bacteria form flattened smudges and some fungi will spread hyphae across the medium and form furry patches. Some will grow as a slowly spreading slime or crust on the medium. They may be coloured or uncoloured. When you take the plates into the dark, it shouldn't require outstanding nightsight to see a pure colony of bioluminescent bacteria growing, as long as they are aerated.

To isolate the single colony, simply take a sterile toothpick, metal pick, loop or craft knife and “pick up” the colony, being careful not to touch anything else on the plate that might be growing. You could streak it again just to be really sure you've separated it from possible contaminants.

Once isolated, you can start to study the bacteria themselves to determine what species or family you have. The methods that can be employed to study your bacteria are sometimes a matter of trial and error, with cross-referencing against a set of traits known to be possessed by a family or species. For example, it is known that Vibrio phosphoreum grows at 4C whereas many other bioluminescent bacteria do not. To further determine which of these 4C-growing bacteria you have, you might look up which “carbon sources” they can use (i.e. Molecules can they break down for energy and raw building material, whether a sugar, glycerol, or something more exotic), and try making up some batches of alternate medium offering these carbon sources instead of glycerol. If your bacteria grow well on a medium with an alternate carbon source then you can take that as a clue based on what is known of the species it might be. Recall that you can find more information on making homebrew bacterial media here: http://letters.cunningprojects.com/?p=97

If you can determine your species through these clues, or if you can at least determine what it is *not*, you may be able to comfortably say whether your species is a hazard. It is moderately unlikely that it is; marine bacteria tend not to pose a health risk (however, many or most are “gram negative” bacteria, so beware of allowing them to grow on your food or you may get ill). This can then open the door to more entertaining experimentation.

**Further Play and Study**

Bacteria make a great experimental test bed. They grow quickly, they tend to respond predictably to the same stimuli if applied repeatedly (essential for scientific merit) and their conditions of growth and culture can be adapted with ease.

The enzyme used by these bacteria to glow is known as a “luciferase”, and it generates light by digesting a substrate molecule known as a “luciferin”. These systems are highly sensitive to contamination with heavy metal ions such as lead or mercury. In fact, a team in Korea developed a system using Vibrio phosphoreum in a system of continuous, circulating culture to test water purity for this reason! You can perhaps attempt something similar, or discover whether certain forms of toxin are less effective at inhibiting bioluminescence:

- You could try making up a culture medium containing the pour-off from some canned foods or fish, to see if metal ions have leached into the food. Try this with a dented can and an undented one, see if you can notice any differences. Remember to have a “control” culture of bacterial in otherwise normal medium, to compare against.
- Liquidise and filter a small piece of oceanic fish to get a clear fluid. Grow up some glowing bacteria, and then add a little bit of this liquid to see how it impacts their glowing strength: results should be pretty fast if something happens. Many fish, particularly predators such as swordfish, accumulate toxic levels of mercury due to pollution and bioaccumulation.
- If you know someone who smokes, spare them the damage of a cigarette and use it for science! Drop a cigarette into your medium before sterilising, so that toxins can leach into the medium. Then see if your cells glow any differently.

You can use the bacteria as part of a light producing project or art project as well, provided people don't touch them:

- Try making a culture vessel with a small amount of liquid medium and some kitchen paper or card inside, so that the medium soaks up into the paper/card to make a large growth surface with a lot of air exposure. Results can be very satisfying! Can you create an even denser growth surface, perhaps even a clear or translucent one?
- If you have an old aquarium with bubble-stone, you could fill it with sterile medium and inoculate with bacteria. The Bubble-Stone will oxygenate the medium constantly, possibly providing you with a brightly glowing container!

Can you find or induce mutant strains? Remember that streaking your cells on a plate of agar will let you

isolate individual cells for observation. Perhaps by exposing cells to mutagenic substances, you can encourage brighter colonies to develop! Beware with this project area; many of the substances that induce mutagenesis in bacteria can be carcinogenic in humans, and vice versa.

- "Carcinogenic" often means "mutagenic". Pick something that can't harm you as a side effect of handling it, such as barbecue ash, a cigarette or a lump of cling film and let it leach into the medium. Remember, your cells will probably not glow well while growing in this medium due to toxic side effects, but streaking them may reveal mutants. "Dark" mutants are likely to be a more common occurrance than bright ones.
- If you can find a UV lamp, try exposing a culture to UV for a little while. Beware: too long and you'll kill them all and sterilise the culture! Just the right amount, and you'll induce all sorts of mutations. Most won't be visible, and most won't be useful or beneficial. If you are very lucky, one might affect glowing in an interesting way.

**Culture Maintainance**

Keeping your cells alive and happy is the only recurring difficulty that this hobby imposes. Without access to very expensive equipment, you mightn't be able to store your cells long-term without constantly changing their medium and moving them to new culture vessels. However, some methods of maintaining your bacteria are fairly long-lasting:

- *Agar Stab:* Add some luminescent agar to a thin jar or test tube, and then use a sterile pipette tip, pencil or other conical method to take a sample of your culture or a healthy colony and drive it down through the agar. These "stabs" have large surface area for growth, and if kept in a cold environment (perhaps 4C in a fridge) the bacteria should remain viable for some time. Check it now and then at room temperature for glowing (keep it a little aerated to prevent oxygen from being depleted)
- *Cold broth:* For many lab bacteria, growth at 4C is so slow as to be negligable for weeks or months. E.coli can be stored for a long time as a 4C broth culture, because it is so used to growth at 37C only. For the bioluminescent bacteria you may have isolated, it is possible that C presents a comfortable growth environment. Some, however, will not grow at 4C and may be stocked simply by keeping them in the fridge and reculturing occasionally.
- *Frozen Stock:* Although unlikely to work very well in a home -20C freezer (labs typically use -80C), you could try making a frozen stock of cells. This requires adding glycerol as a cryoprotectant, usually about 20% but sometimes as high as 40%. To do this, sterilise some glycerol of as high a concentration as you can find, and then simply add some bacteria grown in liquid medium until you have about 25% glycerol in the final mix. Put them directly in the coldest part of the freezer, and do not defrost thereafter. If you need to remove cells later for culture, scrape some cells from the surface of the frozen medium to inoculate your culture, then put the stocks immediately back in the freezer. If your glycerol stock has remained liquid, you may need to try adding less glycerol for a -20C freezer.
- *Continuous Culture:* This is very much more involved than the other options, really here more as an academic exercise. Still, if you want to establish continuous culture, it would make a great project to document online! Continuous culture is the creation of a vessel that has a certain volume of cells in suspension at all times, but slowly adds fresh medium constantly, displacing a like quantity into a drainage system or overflow container. Continuous culture must be carefully balanced to the growth rate of the cells, or they will be diluted out of the culture by an overly active feed or allowed to overgrow by an anaemic feed.
- Lyophilisation: Similar to the above, this is more a suggestion for an excellent DIY project. Lyophilisation is the process of freeze-drying a sample by keeping it frozen while applying a strong vacuum. The ice sublimes directly into water vapour and is removed. While most cells will not survive dessication, or drying by traditional evaporation, it is usually possible to lyophilise cells if an appropriate lyoprotectant chemical is chosen.

As a final note on care of bioluminescent bacteria, it may occasionally be necessary to re-streak your cells to isolate a brightly glowing colony if you notice that your cultures are getting darker. The so called "dark mutants" can emerge in liquid culture easily, because by removing the costs of producing luciferin and spending oxygen on luciferase, they can increase their growth rate and out-compete the glowing "wild-type" bacteria. Pay attention to the brightness of your cultures and do not ignore an inability to glow as vigorously as before.

**Suppliers of Affordable Labware**

Although you can perform experiments using ware found or available for kitchens, it is best to have a set of glassware specially for your lab to make things easier.

- Glass test tubes with caps, erlenmyer (conical) flasks and glass petri dishes are great culture vessels.

- Lab equipment such as incubators, centrifuges and sterile airflow cabinets are generally quite expensive but are essential for a professional lab. A cheap centrifuge is available from LabsFromFabs, and great gloved sterile inflatable bag is available from Applied Micro Science. They also stock an agitating incubator but it is not cheap.
    - Centrifuges can be used to "spin down" bacteria growing in a liquid broth for transfer to a fresh culture or for use in other experiments. They are also used for a host of other lab protocols.
    - Sterile flow hoods or gloved inflated bags allow you to work in a sterile space, preventing contamination of your media and cultures.
    - Incubators are essential to grow most bacteria effectively.
- Manual equipment like micropipettes can be found on ebay; search for "eppendorf pipette", or "pipetman" and work from there. You should be able to get second hand tools for £30 or less if you shop around, and new for about £50-70 depending on brand and quality.
- Lab consumables like pipettes, micropipettes tips, plastic ware and special tapes or films can make life easier. Re-usable glass pipettes can cut down on waste but are difficult to sterilise.

Suppliers besides those on ebay include:

- LabsFromFabs - http://www.shapeways.com/shops/labsfromfabs – Supplier of lab equipment produced by rapid prototyping machines. Particularly in this case for an inexpensive centrifuge rotor that attaches to a Dremel rotary tool. Sold as an ornament, as it carries some risks in use compared to a commercial centrifuge. Disclosure: this shop belongs to the author of this document.
- Applied Micro Labs - http://www.micro-science.co.uk – Supplier of equipment, media and culture seeds for fungal culture (edible mushrooms, for example) and plant tissue culture. Also supplies equipment needed to culture most microbes, and supplies ready-made media for many applications.
- Lab In A Box - http://labinabox.co.uk – Will soon be supplying kits and instructions for many home biology or school project experiments.

**References and Further Information**


- http://www.disknet.com/indiana_biolab/b203.htm - The excellent Indiana biolab disknet archive contains a host of information on bacterial culture, and contained the article that largely informed and inspired this work. It is very worth reading through the available pages on disknet.
- http://has100ideas.com/idea/culturing-bioluminescent-microbes-part-1 - Mackenzie Cowell's success in getting glowing bacteria to grow on a piece of seafood, and his subsequent linking of the original disknet article, was the original inspiration to perform this experiment and document it.
- http://microbewiki.kenyon.edu/index.php/Photobacterium_phosphoreum - The Microbewiki article on Photobacterium phosphoreum (old name for Vibrio phosphoreum) is highly informative, and links to several interesting articles on potential applications and significances of the bacterium. This wiki also contains information on other bacteria that can be of use in identifying your culture strains.