INQUIRY & INVESTIGATION

An Inexpensive and Convenient Method to Culture Facultative Anaerobic Microorganisms from Yogurt

NABT BioClub RECOMMENDATION

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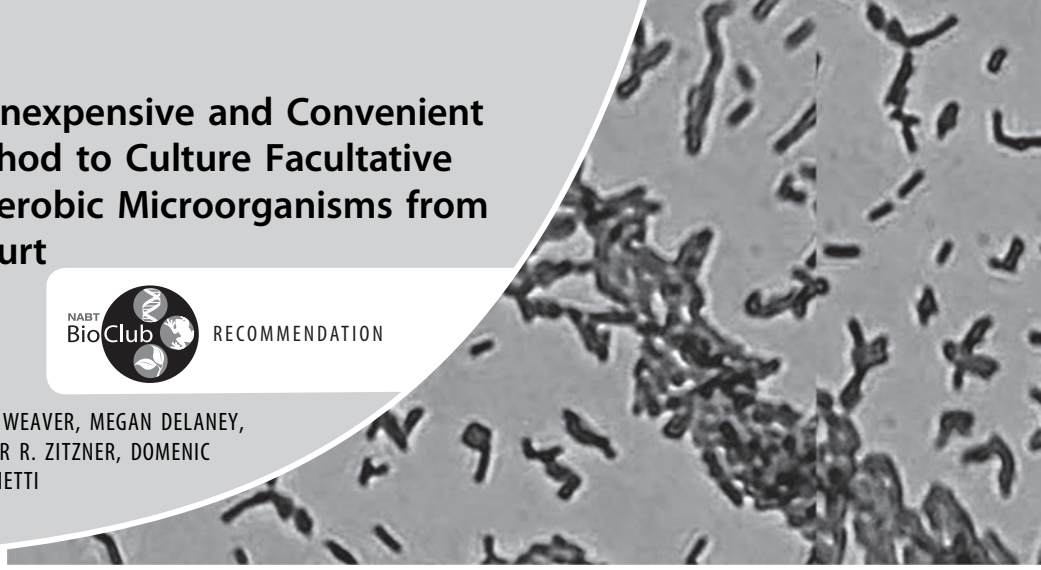

Abstract

It is often difficult for students grasp the concept that some organisms can grow in the absence of oxygen and that oxygen can be toxic to organisms. However, as educators usually describe in the classroom, the importance of the anaerobic growth of microorganisms can be seen in many areas of industry, health, and the creation of several categories of food ranging from fermented alcohols to breads and cheeses. To demonstrate that microorganisms can grow and thrive in the absence of oxygen, we have developed an inexpensive and easy technique to culture facultative anaerobic bacteria from yogurt in high school or undergraduate biology laboratories. Using the method discussed, which uses readily available and low-cost equipment, students can culture and visually identify facultative anaerobic bacteria that they may consume on a regular basis, demonstrating the utility of organisms that thrive in an anaerobic environment.

Key Words: *anaerobic culture; facultative anaerobes; yogurt bacteria; microbiology.*

○ Introduction

Microorganisms can be categorized in several ways including aerobes (require oxygen to survive), facultative anaerobes (able to use oxygen for aerobic respiration, but may also use fermentation or anaerobic respiration when oxygen is not present, or use fermentation in the presence of oxygen), or obligate anaerobes (cannot survive in the presence of oxygen). The concept that microorganisms can and sometimes require the absence of oxygen to grow is difficult to demonstrate in high school biology or undergraduate laboratories. Several microbiology laboratory demonstrations can be readily performed in these environments to learn microbiology culture and identification techniques. Culturing anaerobic microorganisms, however, poses a challenge in many biology classrooms because the equipment to grow, isolate, and identify anaerobic microorganisms can be costly. Culturing anaerobic organisms routinely uses anaerobic chambers or glove boxes that are often not available in non-research laboratories such as those found in the high school or collegiate teaching settings. Therefore, we developed an inexpensive and convenient method to demonstrate that microorganisms can grow in an anaerobic environment.

A low-cost and easily obtainable method to culture anaerobic or facultative anaerobic microorganisms.

Anaerobic and facultative anaerobic microorganisms are excellent examples of microorganisms that are responsible for the qualities of many food products. Yogurt contains several anaerobic or facultative anaerobic bacteria that contribute to its qualities. For our investigation, we utilized a readily available Greek yogurt that listed *Lactobacillus casei* as one of the live cultures. This rod-shaped, Gram-positive, facultative anaerobic bacterium is found as a member of human intestinal bacteria and also in food products such as fermented cheeses, olives, and yogurts (Guarner & Malagelada, 2003; Holzapfel et al., 2001). *L. casei* is a facultative anaerobe that grows readily under anaerobic conditions and can be visualized by morphology and Gram staining, processes often readily available in classroom laboratories. Utilizing readily available and commercially inexpensive anaerobic chambers and indicators, we cultured dilutions of the Greek yogurt, isolating the resultant bacteria and confirming the presence of *L. casei* by morphology, Gram staining, and nucleotide sequencing of part of the rRNA gene (an optional exercise not required but used in this case for confirmation).

○ Materials & Methods

A tightly sealed container can be readily found in many commercial department stores. We confirmed the method with the use of the two types of containers listed in Table 1, although other tightly sealed containers may be sufficient. To ensure a complete seal, we successfully used either silicon high-vacuum grease or petroleum jelly with the containers listed. Instead of using the more costly oxygen reduction packages available

The American Biology Teacher, Vol. 80, No 3, pages. 198–202, ISSN 0002-7685, electronic ISSN 1938-4211. DOI: https://doi.org/10.1525/abt.2018.80.3.198.

from scientific supply firms (e.g., GasPak™ EZ anaerobe container system, BD Diagnostics, Sparks, MD), we utilized Hot Hands® Hand Warmers as the oxygen consumer to create an anaerobic environment for the bacteria. These warmers use ferrous ion as the reductant to reduce, and consume, oxygen gas. Other ferrous ion oxygen reducers may be sufficient as well, however, the number of packets needed to consume the oxygen in the container described in this paper are with the use of the Hot Hands Hand Warmers.

Two Hot Hands Hand Warmers were used in a single chamber as the oxygen consumer. An oxygen indicator (consisting of 0.02% methylene blue indicator, 60% tris(hydroxymethyl)aminomethane, and 4% dextrose) was prepared as described (Brewer et al., 1966), with the following modifications: the solution was kept as a liquid and not put into packets, and the liquid indicator solution was placed into loosely capped, screw-capped test tubes and sterilized via autoclaving. If autoclaving is unavailable, loosely capping the solution in screw-capped test tubes and placing into a gently boiling water bath for 10–15 minutes is sufficient for preparing the indicator. The indicator thus prepared, in our trials, worked for at least one week. After the addition of the plated cultures (see below), Hot Hands packets and the methylene blue indicator, in an uncapped test tube, were added to the chamber, which was then sealed. If available, one may use parafilm to further seal the lids of the containers. The indicator color changed from green-blue to yellow-orange in the course of several hours, demonstrating that the oxygen was consumed. As anaerobiosis was established in the chamber, the formation of water was often noted as a condensate on the interior of the chamber.

As a control to our alternative system, we grew and characterized the yogurt bacteria via morphology and Gram staining in conventional equipment (2.4 L GasPak Anaerobic Chamber with a GasPak EZ anaerobe container system to remove oxygen). All experiments were repeated two times on two different occasions.

Our anaerobic chamber method was tried using both chambers listed, and the complete anaerobic chamber set up can be seen in Figure 1. To make an anaerobic chamber, several components were needed.

Dilutions of yogurt were done as described by Brown and Smith (2015) and were accomplished by diluting 1 g of the Greek yogurt in PBS and then plating the dilutions onto Brain Heart Infusion plates as described below. Cultures were placed in the anaerobic chambers and allowed to grow for 2–4 days. As the chambers are transparent, colony growth on the BHI plates could be observed. Upon removal of plates from the anaerobic chambers, single isolates were transferred and streaked onto new plates, re-incubated in the chambers once again made anaerobic as described above, and allowed to grow for 2–4 days. After this time, Gram staining was performed, and the individual isolates' cellular morphology was observed and recorded.

Table 1. Materials for the anaerobic culturing of yogurt microorganisms.

Anaerobic chambers (both were successfully used) • OXO Pop Container, 5.5 L, airtight seal (OXO International, New York, NY) • Cracker Jar (glass), 3.8 L (Anchor Hocking Company, Lancaster, OH)
Petroleum jelly or Dow Corning High Vacuum Grease
Hot Hands Hand Warmers (HeatMax Inc., Dalton, GA)
Methylene Blue Indicator for Anaerobiosis (Brewer et al., 1966)
• 0.02% methylene blue indicator
• 60% tris(hydroxymethyl)aminomethane
• 4% dextrose
Greek yogurt, raspberry (Friendly Farms, Aldi, Batavia, IL)
Note: Other types of yogurt may be suitable for this process although not all types of yogurt have been tested. It is recommended to check the ingredients of the yogurt to see what microorganisms are present and expected to be cultured from a particular yogurt.
Phosphate-buffered saline (PBS), pH 7.4 (NaCl 8 g, KCl 0.2 g, $Na_2HPO_4{\cdot}7H_2O$ 1.44 g, KH_2PO_4 0.24 g; distilled, deionized water, 1 L, bring to pH 7.4 with HCl). If the chemicals are not readily available, PBS can be ordered as a powder for preparation from Sigma-Aldrich (St. Louis, MO), P3813-1PAK or P3813-10PAK. If sterilizing the PBS via autoclaving is not possible, sterile PBS may be obtained from ABI-American BioInnovations, available from Amazon as Phosphate Buffered Saline, PBS (1X), Sterile, 500 mL. Although we did not utilize it, sterile saline (0.9% NaCl) should also function to make dilutions of the yogurt to be plated.
Brain Heart Infusion (BHI) Agar (Carolina Biological Supply Company, Burlington, NC, catalogue #781781) supplemented with 3 g/L agar for firmness. If the preparation of plates is not possible due to the lack of the appropriate facilities (e.g., an autoclave), prepared BHI plates, in packs of 10, may be obtained from Carolina Biological Supply Company, item #821282. BHI is a suitable medium as it contains the readily fermented substrate glucose, and although other media (Luria-Bertani broth, Trypticase Soy Agar, nutrient agar) have been suggested, we have not tested their suitability for the growth of the lactobacilli and for use with our system.

Figure 1. Low-cost anaerobic chamber system. (Left) Anaerobic chamber using Cracker Jar (glass), 3.8L. (Right) Anaerobic chamber using OXO Pop Container, 5.5L, Airtight Seal. Petri plates, anaerobic indicator, and Hot Hands packets are visible in the Cracker Jar (glass container). HotHand packets are present in the OXO Pop Container on the right, but have been moved behind the petri plates to better show the anaerobic indicator.

○ Methods

Procedure for Anaerobic Culturing & Isolate Characterization

A. Using sterile pipettes and glassware, prepare yogurt cultures dilutions (serial dilutions: 10^{-3} to 10^{-6} all mixed thoroughly):
   1. Suspend 1.0 g of yogurt in 99.0 mL of autoclaved (sterilized) PBS (1:100).
   2. Transfer 1 mL of 1:100 dilution to 9 mL of autoclaved PBS (1:1,000).
   3. Transfer 1 mL of 1:1,000 dilution to 9 mL of autoclaved PBS (1:10,000).
   4. Transfer 1 mL of 1:10,000 dilution to 9 mL of autoclaved PBS (1:100,000).

B. Plating of yogurt culture dilutions:
   1. Mix 52 g of Brain Heart Infusion (BHI) Agar (Catalogue #C5121, Hardy Diagnostics, Santa Maria, CA) and 3 g of agar (to provide a firmer agar) with 1 L of purified water, and place into a 2 L Erlenmeyer flask. Stir over heat until gently boiling and the agar is dissolved completely. Take care not to boil too vigorously as the medium will bubble out of the flask. Autoclave at 121°C for 15 minutes.
   2. Pour cooled (~50°C) agar into sterile petri plates and let solidify overnight.
   3. Transfer 0.1 mL of each culture solution to an appropriately labeled plate, and using a sterilized spreader (a bent glass rod shaped into a right angle that has been flamed after immersing in ethanol or isopropanol), evenly distribute the culture dilution on the plate. Each 0.1 mL plated represents the next highest dilution. For example, 0.1 mL of the 1/10,000 PBS preparation is a 1/100,000 (1×10^{-5}) dilution.
   4. Place BHI plates into either the OXO Pop or glass Cracker Jar containing 2 Hot Hands individual packets and the methylene blue aerobic indicator, seal the lids and let incubate.

C. Incubation and identification of cultures:
   1. Place each of these containers in a 37°C incubator for 2 to 4 days. Incubation at 30°C or room temperature will work as well but may require additional incubation time.
   2. After the bacteria have grown on each of the plates, flame an inoculating loop and pick a single colony off of a BHI plate. If possible, try to choose a few different colony morphology types to ensure that representative bacteria are isolated.
   3. Transfer the colony onto a new BHI plate by spreading the loop using the spread plate method.
   4. Repeat with additional colonies on new plates.
   5. Transfer plates back to containers and return to a 37°C incubator for 2 to 4 days. Repeat steps 2–5 until well-isolated single colonies of the same colony morphology are obtained.
   6. Observe and record the colonies for colony morphology. Prepare microscope slides using the isolated colonies, Gram stain the slides, and record cellular morphology and Gram staining.

D. Molecular identification of isolates:
   (*Optional:* may be beyond the scope and equipment of many teaching laboratories.)
   1. Grow isolated colonies in 100 mL Brain Heart Infusion Broth (Catalogue #C5121, Hardy Diagnostics, Santa Maria, CA) using either the Oxo or Anchor Hocking chamber as described above.
   2. Isolate genomic DNA from each isolated bacterium using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI).
   3. Using the 8F (AGAGTTTGATCCTGGCTCAG, Dicksved et al., 2007) and 926R (CCGTCAATTCCTTTRAGTTT, Liu et al., 1997) primers (these are primers designed to amplify the 16S rRNA gene of *Bacteria*; primers were obtained from Eurofins Genomics, Louisville, KY), prepare the genomic DNA-polymerase chain reaction (PCR) master mix according to the manufacturer's instructions (GeneMateTaq Master Mix 2X, Bioexpress-VWR, Batavia, IL).
   4. Perform a PCR protocol using the Techne TC412 (Keison Products, Grants Pass, OR), or similar, thermocycler and the following paramaters:
      a. 94°C for 4 min
      b. 33 cycles of 94°C for 45 s, 56.4°C for 60 s, 72°C for 90 s
      c. Final extension at 72°C for 7 min
      d. Final hold at 15°C

5. Isolate the amplicons generated using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) as directed by the manufacturer.
6. Send the isolated amplicons and the primer sets 8F and 926R, prepared as directed, to ACGT (Wheeling, IL), or other suitable facility, to sequence the amplicons.

Using the sequences obtained from the sequencing facility, perform a nucleotide BLAST using the NCBI site https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome

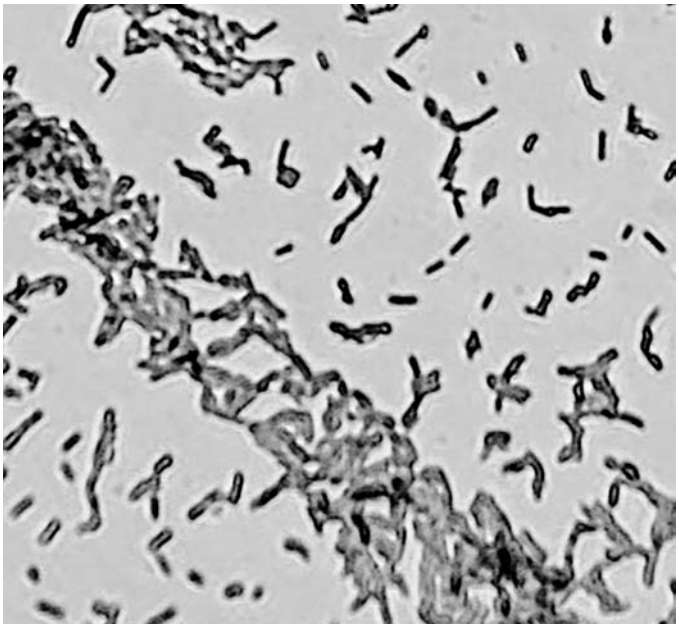

Figure 2. Cellular morphology of cultures following transfer and regrowth on new plates. Photomicrograph taken at 1000X and enlarged for detail. Cells are approximately 1–2 μm in length.

Results

The transfer of single colonies from the initial dilution of yogurt of 10^{-4} resulted in clearly separated colonies, which were transferred to new plates for growth. Morphology of the resulting cultures showed many rod-shaped bacteria (Figure 2) that stained Gram-positive. To ensure that the bacteria that we had grown were the *Lactobacilli* listed on the yogurt container, we sequenced part of the 16S rRNA gene from five isolates. The results of subjecting the sequence data to NCBI BLAST analyses identified the isolates as *L. casei* and *Lactobacillus paracasei*, which are genotypically and phenotypically indistinguishable (Table 2). This final step, although not essential, could be performed if funds and supplies are available and was done as a "proof of concept" that the two chambers used (OXO Pop Container and the Cracker Jar) allowed the culturing of the microbes in the yogurt.

Discussion

The investigation of anaerobic microorganisms in high school and many undergraduate laboratories is often limited because of the supplies and cost of growing microorganisms in an oxygen-free environment. However, anaerobic and facultative anerobic microorganisms are responsible for many of the characteristics of food products (Holzapfel et al., 2001). To illustrate the concept of the ability for microorganisms to grow in the absence of oxygen for undergraduate or high school biology courses, we have designed a simple and low-cost alternative to more expensive conventional anaerobic chambers.

Using the chambers described, we were able to easily culture and observe the morphology and Gram staining of *L. casei* from yogurt. These steps would be achievable for most high school and undergraduate laboratories. Further investigation by 16S rRNA gene sequencing confirmed that the colonies observed were *L. casei*. Although all laboratories may not have the equipment and funding to perform the confirmation by 16S rRNA gene sequencing, it is an optional additional step for students to learn more laboratory techniques such as PCR and understanding sequencing data. For those

Table 2. Abbreviated results of NCBI BLAST analyses of five yogurt isolates.

Isolate	Description	Query coverage	E value*	Identity
I	*Lactobacillus paracasei*			
	strain IIA	96%	0.0	99%
II	*Lactobacillus paracasei*			
	strain IIA	99%	0.0	99%
III	*Lactobacillus paracasei*			
	strain BCH-5	99%	0.0	99%
IV	*Lactobacillus casei*			
	strain MSJ1	100%	0.0	99%
V	*Lactobacillus casei*			
	strain HH7	99%	0.0	99%

*The E value represents the probability that the match was obtained by chance.

laboratories with such facilities, it would further enhance the inquiry of the process as both microbe culturing and modern species identification could be performed.

The initial cost of the anaerobic chamber was limited to the chamber, petroleum jelly, Hot Hands Hand Warmers, and anaerobic indicator. The cost of each of the two chambers used (Oxo and Anchor Hocking) was less than $20. We estimate the cost of the anaerobic indicator, made from common laboratory chemicals, to be less than $10, and a significant amount can be made and sterilized by boiling or autoclaving in loosely capped screw-capped test tubes. Once cooled, these can be firmly capped and stored at room temperature. We have used the indicator on more than one occasion and have observed a valid aerobic/anaerobic color change each time.

As to the mechanism of how Hot Hands warmers consume O_2, the company considers this proprietary information. Hot Hands packets, however, list "iron powder, water, salt, activated charcoal and vermiculite" as contents. From the literature (Singer & Stumm, 1969; Stumm & Lee, 1961), the oxidation of ferrous ion is complicated but may be summarized as (Stumm & Lee, 1961):

- $Fe^{2+} + O_2 \leftrightarrow Fe^{3+} + HO_2\cdot$
- $Fe^{2+} + HO_2\cdot \leftrightarrow Fe^{3+} + H_2O_2$
- $Fe^{2+} + H_2O_2 \leftrightarrow Fe^{3+} + HO\cdot + H_2O$
- $Fe^{2+} + HO\cdot \leftrightarrow Fe^{3+} + H_2O$

As noted by Stumm and Lee (1961), these reactions are not balanced but serve to suggest how the oxidation of ferrous ion by oxygen gas proceeds. Also note that water is generated, which may explain the condensation of moisture noted in our chambers.

If one considers the oxidation of iron pyrite (FeS_2), the situation is also complex but involves the consumption of oxygen gas and water with the ultimate generation of water, protons (H^+), ferric ion (Fe^{3+}) and sulfate (SO_4^{2-}) ion (Singer & Stumm, 1969). Given the components listed on the Hot Hands packet, an interesting observation is that materials such as clay (vermiculite), activated carbon, and cupric salts (Singer & Stumm, 1969, Stumm & Lee, 1961), all accelerate oxygen consumption and the conversion of Fe^{2+} to Fe^{3+}.

Although we utilized an oxygen indicator to show that the oxygen was consumed in the container, we also tested the container's ability to grow obligate anaerobes. The same procedure described was used to culture *Clostridium butyricum* (grown anaerobically with an added source of carbon dioxide) and *Clostridium sporogenes* (grown anaerobically without added carbon dioxide). Both are spore-forming obligate anaerobes. *Cupriavidus metallodurans*, an obligate aerobe, and *Streptococcus mutans* strains GS5 and UA159, a facultative anaerobe that can ferment in the presence oxygen but prefers anaerobic conditions with 5% (or more) CO_2, were also examined. As a control, *C. butyricum* was inoculated onto Plate Count Agar plates at the same time and incubated aerobically in a 37°C incubator. Although all of the plates cultured anaerobically in our chambers, except the obligate aerobe *C. metallodurans*, had growth within 3–4 days, none of these plates cultured aerobically had growth on them, again excepting the *C. metallodurans*, confirming that our chamber's configuration established anaerobic conditions. We chose to focus on the culturing of the yogurt bacteria, and not the clostridia (*C. butyricum* or *C. sporogenes*), however, as neither is readily available in most laboratories and the cost of obtaining either of them (American Type Culture Collection, Manassas, VA) ranges from $50 to $185.

We have established a low-cost and easily obtainable method to culture anaerobic or facultative anaerobic microorganisms that can be isolated from readily available food sources. Although in this method we have used a facultative anaerobe that can grow in the presence or absence of oxygen, if a more experimental design is desired, an obligate anaerobe may be purchased to compare growth in varying oxygen conditions. This method can be used to allow high school and undergraduate students to participate in an inquiry exercise that will explore the growth and identification of microorganisms that do not require oxygen to grow, and will reinforce the importance of anaerobic and facultative anaerobic microorganisms in foods such as yogurt and cheese. Further applications of this method can be used to investigate anaerobic processes such as glycolysis and lactic acid fermentation.

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