Gelatin Hydrolysis Test Protocol

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Information

History

In 1926, Frazier described the very first method to detect the ability of microorganisms to liquefy gelatin (2). In this plate method technique, the microorganisms grew on low-peptone agar medium supplemented with gelatin. The digestion of gelatin was detected by treating plates with acidic mercuric chloride or tannic acid. The method was tested against isolated intestinal Gram-negative bacilli by Barer and against aerobic spore-forming bacilli by Smith, Gordon, and Clarke in 1946 as mentioned by Clarke (2). The method for gelatin hydrolysis was then listed as one of the biochemical tests for bacteriology (1, 9).

In the 1950s, several published modifications to this gelatin hydrolysis method aimed at providing a quicker, simpler, and more accurate detection of gelatin hydrolysis. Clarke (1953) described a simplified plate method using 10% leaf gelatin and $HgCl_2$–HCl solution for the detection of gelatin-liquefying bacteria and compared it with the gelatin stab method and the Frazier's plate method (2). This comparison showed that hydrolysis of gelatin was generally more accurate and rapid in the simplified plate method (3 days) than in the stab method (up to 14 days) and Frazier's plate method (up to 4 days). The plate test, however, did not eliminate the use of acidic mercuric chloride to visualize the digestion of gelatin. Green and Larks (1955) also reported a quick method for the detection of gelatin-liquefying bacteria using formalin-denatured gelatin-charcoal (4). In this method, gelatin hydrolysis was observed when charcoal particles were liberated and settled to the bottom of the culture tube. This method was found to be quicker than the gelatin stab method.

The need for a rapid and more accurate biochemical method for bacterial identification compelled McDade and Weaver in 1959 to evaluate different methods for the detection of gelatin hydrolysis (8). All methods evaluated gave accurate results. The major difference was the amount of time required before the results were detected, i.e., from 1 hour to 9 weeks. In some of the tests that were evaluated, the addition of reagents or chemicals (e.g., Ninhydrin reagent, acidic mercuric chloride, ammonium sulfate, and manganese sulfate) onto the inoculated media after incubation resulted in varying durations of the tests. However, the incorporation of these compounds during the experiment brought with it the potential hazards associated with these chemicals. With the advent of commercially-available dehydrated culture media, including nutrient gelatin, gelatin hydrolysis tests could be done without the need for

potentially hazardous reagents, thus making the tests safe and easy to do without a compromise in accuracy and reproducibility. The API rapid test kit (BioMérieux, Durham, NC) also allowed rapid detection of gelatin hydrolysis with the diffusion of black pigment within the cupule.

Purpose

The gelatin hydrolysis test detects the ability of bacteria to produce gelatinases. This test aids in the identification of*Serratia*, *Pseudomonas*, *Flavobacterium*, and *Clostridium* (7, 11). It distinguishes the gelatinase-positive, pathogenic*Staphylococcus aureus* from the gelatinase-negative, nonpathogenic *Staphylococcus epidermidis* (5). Gram-positive, spore-forming, rod-shaped, aerobic or anaerobic bacteria such as *Bacillus anthracis*, *B. cereus*, *B. subtilis*,*Clostridium perfringens* and *C. tetani* are also positive for gelatin hydrolysis (6). The test can be used to differentiate genera of gelatinase-producing bacteria such as *Serratia* and *Proteus* from other members of the family*Enterobacteriaceae* (6).

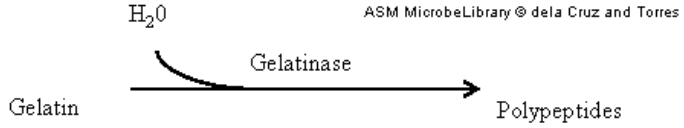
TABLE 1 List of common bacteria and their reactions to the gelatin hydrolysis test performed on nutrient gelatin (3, 6)

Species	Biosafety level	Growth	Liquefaction
Bacillus subtilis	1	+	+
Clostridium perfringens	1 or 2	+	+
Escherichia coli	1	+	-
Proteus vulgaris	1	+	+
Serratia liquefaciens	1	+	+
Staphylococcus aureus	1	+	+

Theory

Gelatin is a protein derived from the connective tissues of vertebrates, that is, collagen (6). It is produced when collagen is boiled in water (5). Gelatin hydrolysis detects the presence of gelatinases. Gelatinases are proteases secreted extracellularly by some bacteria which hydrolyze or digest gelatin (6). This process takes place in two sequential reactions (Fig. 1).

In the first reaction, gelatinases degrade gelatin to polypeptides (6).



Then, the polypeptides are further converted into amino acids (6).

FIG 1 Reactions involved in gelatin hydrolysis.
The bacterial cells can then take up these amino acids and use them in their metabolic processes.

Gelatin hydrolysis is detected using a nutrient gelatin medium (5, 6). This medium contains peptic digest of animal tissue (peptone), beef extract, and gelatin (3). Gelatin serves as both solidifying agent and substrate for gelatinase activity. When nutrient gelatin tubes are stab-inoculated with a gelatinase-positive bacterium, the secreted gelatinases will hydrolyze the gelatin resulting in the liquefaction of the medium (6). Since gelatin is digested and is no longer able to gel, the medium will remain liquid when placed inside a refrigerator or in an ice bath (5). A nutrient gelatin medium inoculated with a gelatinase-negative bacterium will remain solid after the cold treatment. The medium can be inoculated with both aerobic and anaerobic bacteria and incubated as appropriate.

RECIPE

Nutrient gelatin (3)

Peptone	5.0 g/liter
Beef extract	3.0 g/liter
Gelatin	120.0 g/liter

Final pH: 6.8 ± 0.2 at 25°C.

Prepare medium by mixing all ingredients in 1,000 ml of distilled or deionized water and heating gently to dissolve. Dispense 2 to 3 ml of medium into 13- by 100-mm culture tubes. Autoclave medium at 121°C (15 psi) for 15 minutes. Allow the tubed medium to cool in an upright position before use. Store the prepared medium at 2 to 8°C. Tubed medium stored at 2 to 8°C may be used until its expiration date. Do not use tubed medium if it shows signs of microbial contamination, discoloration, drying, or other signs of deterioration (3).

PROTOCOL

Nutrient gelatin stab method. There are several methods for determining gelatinase production, all of which make use of gelatin as the substrate. The standard and most commonly employed method is the nutrient gelatin stab method. This method offers an easy and accurate interpretation of results (medium liquefaction) and eliminates the use of reagents or chemicals that may be hazardous to health.

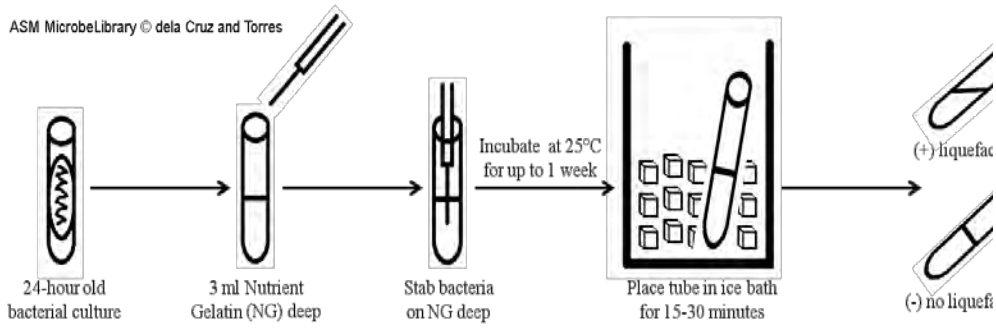
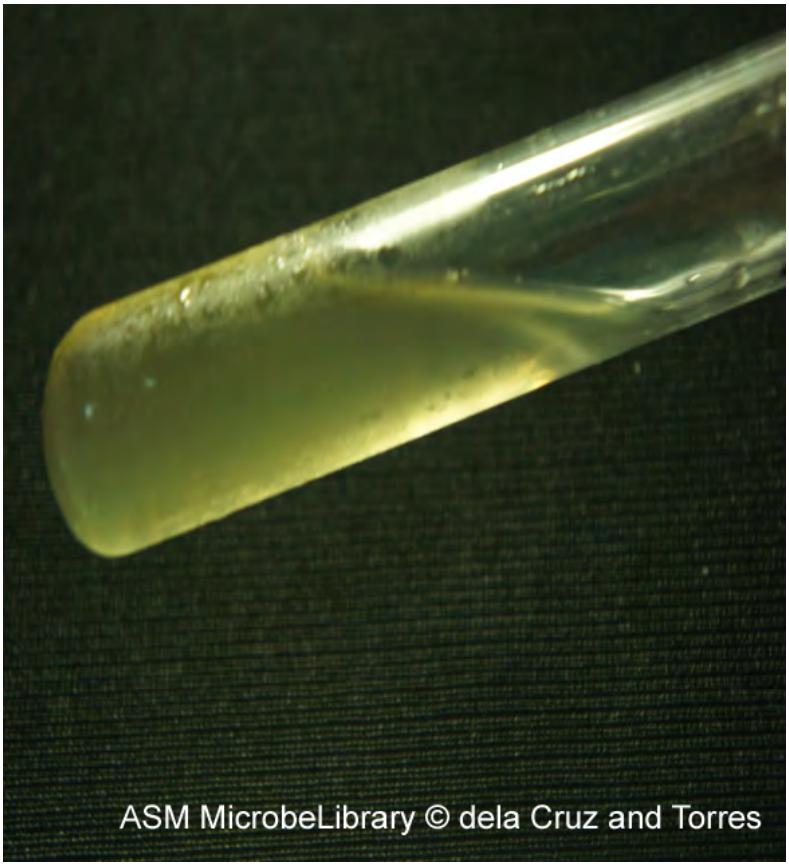
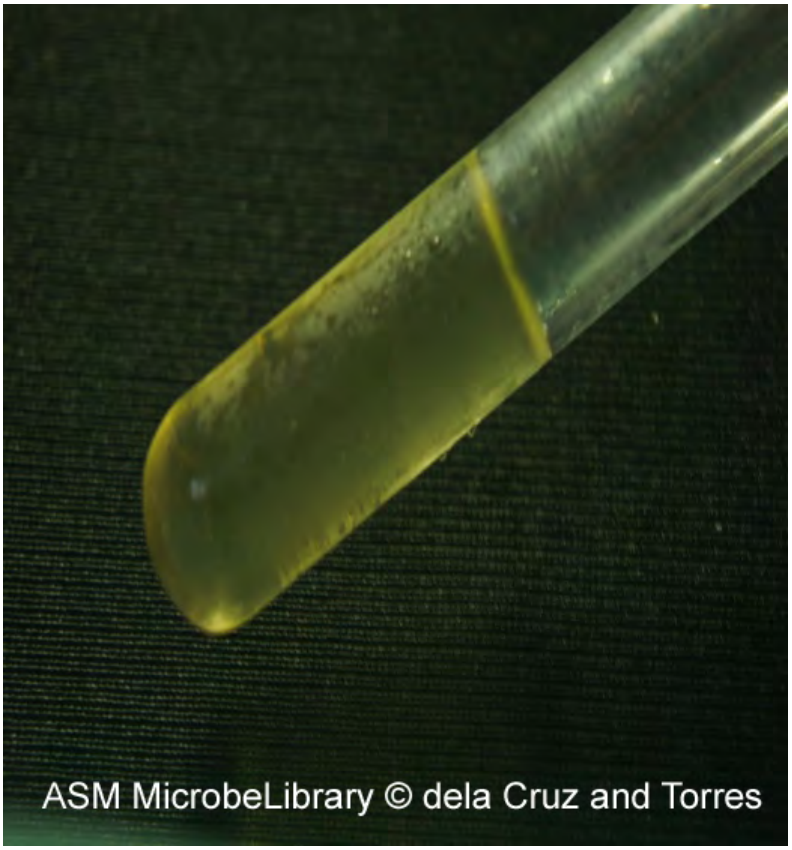


FIG 2 A schematic diagram of the gelatin hydrolysis test using the nutrient gelatin stab method. The use of screw-capped test tubes is recommended.

In this method, a heavy inoculum of an 18- to 24-hour-old test bacteria is stab-inoculated into tubes containing nutrient gelatin (Fig. 2). The inoculated tubes and an uninoculated control tube are incubated at 25°C, or at the test bacterium's optimal growth temperature, for up to 1 week, and checked everyday for gelatin liquefaction. Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes are immersed in an ice bath for 15 to 30 minutes. Afterwards, tubes are tilted to observe if gelatin has been hydrolyzed. Hydrolyzed gelatin will result in a liquid medium even after exposure to cold temperature (ice bath), while the uninoculated control medium will remain solid (Fig. 3). For weak positive results, incubate the inoculated nutrient gelatin tube longer until complete liquefaction is observed. The hydrolysis of gelatin indicates the secretion of gelatinase by the test organism into the medium. Note that some bacterial species, e.g., *Proteus vulgaris*, may require up to 14 days of incubation to yield a positive result. Aseptic techniques and appropriate biosafety protocols should always be strictly observed and practiced in the microbiology laboratory.



A



B

FIG 3 Gelatin hydrolysis test. After 30 minutes in an ice bath, a nutrient gelatin tube inoculated with *Bacillus subtilis*exhibited positive gelatin hydrolysis as shown by medium liquefaction (A) while the uninoculated control remained solid (B). Gelatin hydrolysis was observed after 3 days of incubation.

For the nutrient gelatin stab method, it should be noted that some gelatinase-positive organisms produce the enzyme at a slower rate. Thus, the following tips are recommended:

- Stab-inoculate nutrient gelatin tubes with a heavy inoculum of the test bacteria.
- Incubate nutrient gelatin tubes for a longer period (7 days up to 14 days).
- Use smaller test tubes for the nutrient gelatin medium (13 by 100 mm).
- Use a smaller volume of nutrient gelatin (2 to 3 ml) in the tubes.

Nutrient gelatin plate method. A suggested alternate test to detect gelatin hydrolysis is the nutrient gelatin plate method. In this method, a heavy inoculum of an 18- to 24-hour-old test bacteria is stab-inoculated onto culture plates prefilled with nutrient gelatin (23 g/liter nutrient agar, 8 g/liter gelatin). Inoculated nutrient gelatin plates are incubated at 35°C for 24 hours. Gelatin hydrolysis is indicated by clear zones around gelatinase-positive colonies (Fig. 4). In some cases, plates are flooded with saturated ammonium sulfate to precipitate unhydrolyzed gelatin,

making the clear zones easier to see. Results are often observed within 5 to 10 minutes after flooding with saturated ammonium sulfate (C. Hopper, University of Maine, personal communication).

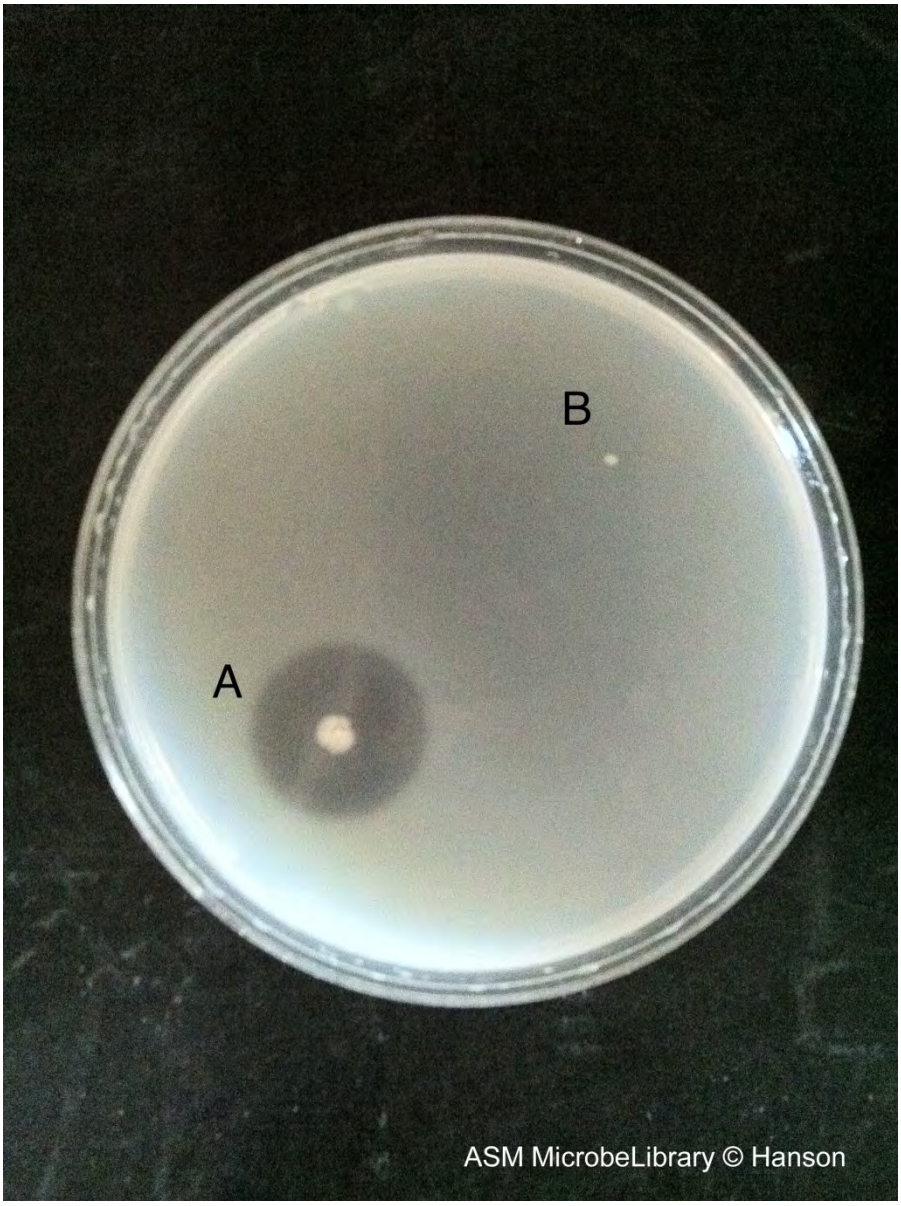


FIG 4 Gelatin hydrolysis test using nutrient gelatin plate method. (A) Positive gelatin hydrolysis exhibited by *Bacillus subtilis* indicated by the clear zone around the colony after the addition of saturated ammonium sulfate. (B) Negative gelatin hydrolysis exhibited by *Escherichia coli* indicated by the absence of a clear zone around the colony. Inoculated culture plates were incubated for 24 hours.

Another suggested medium for the nutrient gelatin plate method contains 4 g/liter of peptone, 1 g/liter of yeast extract, 12 g/liter of gelatin, and 15 g/liter of agar. In most cases, this medium gives a visible zone without treatmentby saturated ammonium sulfate (10).

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the ASM Curriculum Recommendations: Introductory Course in Microbiology and the Guidelines for Biosafety in Teaching Laboratories.

COMMENTS AND TIPS

- The gelatin hydrolysis test is a great way to highlight proteolysis by bacteria and introduce the idea that some tests for taxonomy can also demonstrate virulence mechanisms.
- Nutrient gelatin plates or tubes may need incubation periods longer than 24 hours. When this is the case, the plates or tubes should be checked every 24 hours for up to 14 days.

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