Chapter 4

Overview of biochemical methods for the identification of bacteria

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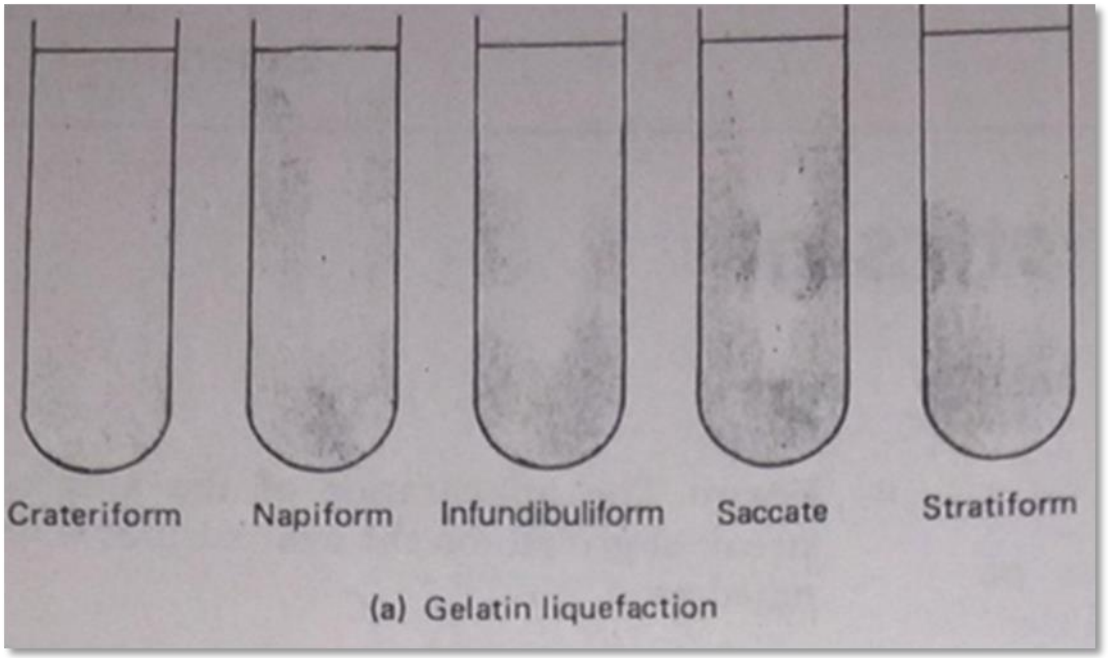
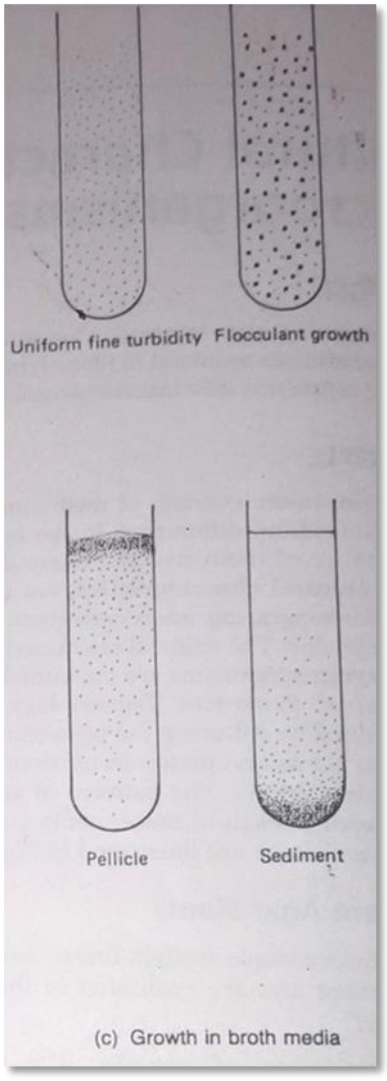
Bacteria are identified up to genus or species level using set of streamlined protocols. They are categorized as polyphasic taxonomy or systematic taxonomy which involves different methods starting from grams reaction, other macromolecular structure characterization, biochemical characterization, characterization for the fatty acid profile and molecularly based on 16s rDNA sequencing analysis, and DNA-DNA hybridization.

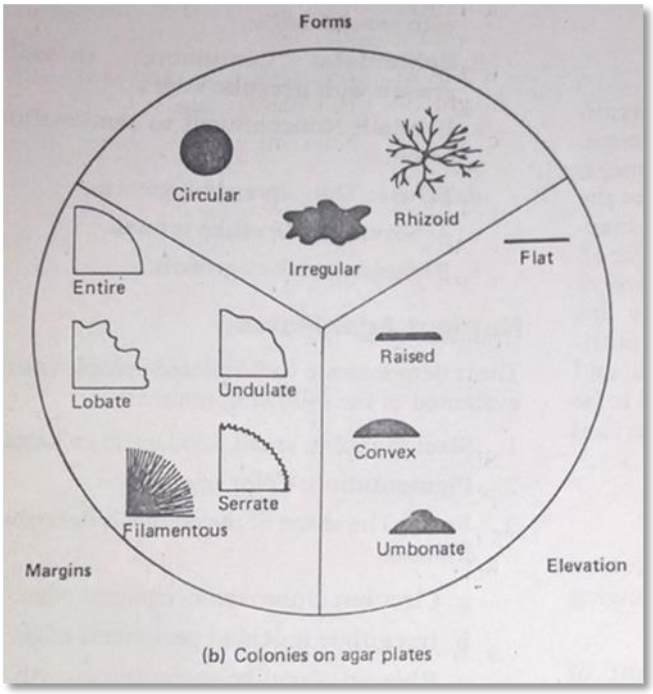
Among these methods' identification based on colony morphology and culture characteristics; (cell arrangement, Gram's reaction, characteristics of other structures) based on staining and microscopy; and biochemical tests are considered as conventional methods of identification of bacteria. These difference in culture characteristics, macromolecule structures, etc plays vital role in the detection or differentiation of the bacteria to the genus or species level. Observation for the abundance of growth, pigmentation, optical characteristic, form of the culture etc in the line of streaking on the nutrient agar or any other non-selective enrichment slants. Colony characteristics on the nutrient agar plates or any other non-selective enrichment plates. Observe for the size, pigmentation, form, margin, elevation in the colony. Observe for the distribution and appearance of the growth in the nutrient broth such as uniform turbidity, flocculants, pellicle, sediment characteristics. Observe in the nutrient gelatine stab culture for the liquefaction pattern. All these characteristics are explained in the diagrammatic representation below and the individual conventional method of identification are addressed in each chapter.



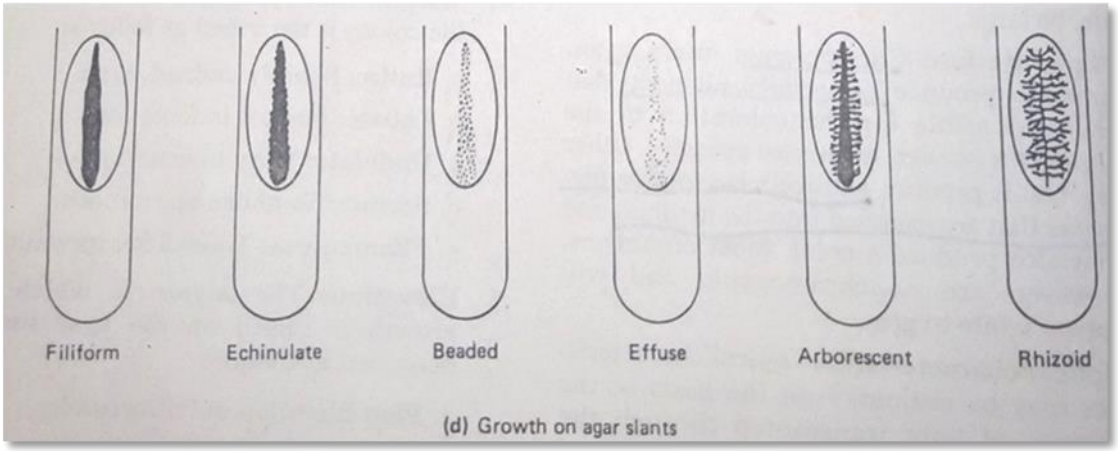
(a) Gelatin liquefaction



(c) Growth in broth media



(b) Colonies on agar plates



(d) Growth on agar slants

Motility test

Organ of locomotion in bacteria is flagella. Flagella mostly identified in Rods with exception of few cocci. Hence bacteria possessing flagella are motile. Bacteria may be flagellated either single or two or in clusters and they located either at ends or throughout the body. In rare case non-motile variants of motile bacteria do exist. Non-motile bacteria generally lack flagella.

Principle

To determine the bacteria is motile or non-motile. Motility of the bacteria can be determined in liquid medium by their movement. Motility can be observed at high power objective (40X)

Materials required

1. Cavity slide
2. Cover slip
3. Motility test medium

Method

Hang's motility test (Direct Method)

- Grow cultures to be tested for motility in broth or on agar slant
- Add a small drop of overgrown cultures or young culture from slants and emulsify with a drop of distilled water on middle of the cover slip.
- Place paraffin jelly on the sides of the cavity slides.
- Invert the slide on the cover slip and make it attached to it.
- Invert it back and observe the drop of culture in 40X objective of microscope at the periphery for the motility of bacteria.

Motility test medium (Semisolid medium)

- Grown the culture to be tested for motility in the plate or slant.
- Stab the centre of the medium with an inoculating needle to a depth of 1/2 inch and incubate it at their desired temperature.

Observation

Positive test means motile ie organism migrate from the stab line and diffuse into the medium as turbidity or as fuzzy streaks of growth. Negative test means no growth or turbidity and clear surrounding the inoculated line. In general, incubate the motility medium at 22-25^{o} C rather than 37^{o} C even if the optimum growth temperature is 35^{o} C. Incubate the tube at 35 for first two days and at 22-25^{o} C for next 5 days.

Biochemical tests

Bacteria do have the biochemical fingerprints that are properties controlled by the cellular enzymatic activity. Biochemical test always has to be related to the bacterial metabolism. Biochemical characterization of bacteria is based on the extracellular enzyme activity and intracellular enzyme activity. Extracellular enzymes are elaborated out of the bacterium

and usually performs the action of hydrolysis to break down complex molecules to simpler building block units which can be further utilized by the bacteria after transporting into the cell. Whereas on the other hand the intracellular enzyme functions inside the cell for the metabolism and the metabolic products are excreted out of the bacterium. This metabolic product accumulated outside of the bacterium is detected in the biochemical test. Biochemical methods involve the identification of activity of both the types of enzymes.

Tests used to identify the extracellular enzymes activity are starch hydrolysis, lipid hydrolysis, casein hydrolysis, chitin hydrolysis etc.,

Tests used to identify the intracellular enzyme activity basically identifying the end product of the reaction are carbohydrate fermentation, litmus reaction, H_2S production, nitrate reduction, catalase, oxidase, IMVC, TSI etc.,

For the starch, lipid and protein hydrolysis test starch, tributyrin, skim milk powder is added in the nutrient agar or composition mentioned in appendix section and checked for their respective activity.

Starch hydrolysis

The degradation of starch molecule by amylase to shorter polysaccharides maltose and dextrin. Overnight grown cultures were streaked onto the starch agar and incubated at different temperature according to the optimum growth of the different bacteria for 24 or 48h. Pour potassium iodide solution or gram's iodine solution over the colony and observe it under the light.

Observing a zone of clearance against the dark blue background is the positive and no clearance zone around the colony is negative for the starch hydrolysis test.

Lipid hydrolysis

The degradation or hydrolysis of lipid molecule by lipase to shorter fatty acid molecule and glycerol or alcohol. Overnight grown cultures were streaked onto the tributyrin agar and incubated at different temperature according to the optimum growth of the different bacteria for 24 to 48h. observe it under the light.

Observing a zone of clearance around the colony is considered as positive and no clearance zone around the colony is negative for the lipid hydrolysis test.

Protein hydrolysis

The degradation or hydrolysis of high molecular weight protein molecule by protease to shorter peptides. Overnight grown cultures were streaked onto the skim milk or casein agar and incubated at different temperature according to the optimum growth of the different bacteria for 24 to 48h. observe it under the light.

Observing a zone of clearance around the colony is considered as positive and no clearance zone around the colony is negative for the lipid hydrolysis test.

Carbohydrate fermentation test

Bacteria obtain their energy through series of enzymatic reactions by majority of cases oxidation of carbohydrate substrates. Some bacteria utilize sugars either in aerobic respiration or through fermentation pathway. Whereas the facultative anaerobes use both pathways. Some of the bacteria do not use sugar also.

Bacteria can be differentiated based on the carbohydrate fermentation for many types of sugars. The media required for the carbohydrate fermentation are described in appendix.

Inoculate overnight grown fresh cultures into the carbohydrate fermentation broth incorporated individually with various sugar. Incubate at various temperature according to the requirement of bacteria and incubate for 24h to 48h. Observe it for the characteristic colour change.

Oxidase test

During aerobic respiration, oxidase enzymes (intracellular cytochrome) catalyzes the oxidation of reduced cytochrome by molecular oxygen which results in the formation of H20 or H2O2 depending on the type of enzyme system they possess.

Oxidase activity was found in the aerobic, facultative anaerobes and microaerophiles. Obligate anaerobes were negative for the oxidase activity. In general, Gram positive organism was oxidase negative with exception of

Bacillaceae and Gram negative in exception to the Enterobacteriaceae were found in majority of the cases.

Principle

Determination of ability of bacteria to produce cytochrome oxidases. This is confirmed by the oxidization of light pink substrate (p-aminodimethyl alaniline oxalate) as electron donors and the substrate is oxidized to the blackish compound in the presence of free oxygen and oxidase enzyme.

Method

- Prepare for the young culture in TSA slant or plate
- Add directly the substrate containing solution as 1% or 0.5% on the colony or pour the solution on to the Whatman filter paper No.1 and pick a colony of the young culture and streak onto the filter paper loaded with substrate.

Observation

Dark pink, maroon, finally black or purple colour development denotes positive for oxidase test. No colour change or light pink indicates negative for oxidase test. The result should be read within 10 to 30 seconds.

Catalase test

In aerobic respiration the bacteria produce hydrogen peroxide and toxic superoxide. Accumulation of these toxic compound result in death of cell. In order to avoid this the bacteria, produce catalase to rapidly degrade hydrogen peroxide. Superoxide dismutase is the enzyme used for the degradation of the toxic superoxide. So, catalase production can be determined by the addition of 3% H_2O_2 and observe for the bubbles of free oxygen as gas in the slide.

Keep three drops of 3% H_2O_2 and add a minute quantum of culture picked out from individual isolated colony or drop H2O2 on to the colony and observe for bubbling or foaming.

Other biochemical test is Indole production, H_2S production, Methyl red test, Voges Proskauer test, Citrate utilization, Urease test, Gelatin liquefaction test.

The list of test performed such as grams reaction or any other staining methods to detect or identify the macromolecular structure such as capsule,

spore, or acid fastness or shape and arrangements, cultural characteristics, and other biochemical tests such as oxidase, catalase, H2S production, IMVC, urease, gelatin liquefaction, nitrate reduction, carbohydrate fermentation, O-F test, starch hydrolysis, lipid hydrolysis, and protein hydrolysis should be tabulated and should be checked with the standard reference books such as Bergey's manual of Systematic Bacteriology for arriving at genus or sometimes species level identification of bacteria.

Miniaturized biochemical identification systems

In order to reduce the consumption of media, reagents in the biochemical test where larger volume are consumed for identifying different group of bacteria several modifications were done in the biochemical identification scheme. In economic way multitest kits were developed with identification schemes. The bacteria were first grouped based on Gram's reaction, oxidase, catalase, motility test and then identified with the help of multitest kits where in bacteria can be identified even up to species level.

These are comprehensive biochemical identification kit proposed to identify gram positive bacteria, gram negative bacteria, *Enterobacteriaceae*, non-*Enterobacteriaceae* with twenty biochemical tests in majority of the cases.

Numerous commercial kits are available in the market and our laboratory concentrate on the API kit sold by Biomerieux. In case the definitive identification could not be done with this method few more supplementary tests should be performed. The results of these kits should be correlated with the type and source of sample, colony morphology etc.

It is absolutely essential to use the manufacturers instruction and guidelines to perform the test and read the result and interpretation of results. Some of the kits used by our laboratory for the identification of bacteria based on the conventional identification scheme with the help of kit are API 20E for *Enterobacteriaceae*, API 20NE for non-*Enterobacteriaceae*, API 50CHB for *Bacillus*, and API STAPH for species differentiation of *Staphylococcus*.

Recently completely automated systems have been developed such as vitek, bactec, remel, biolog systems for the rapid identification of bacteria based on biochemical profiles are now in the market.