4. Techniques for Microbial Study and Identification:
(Phenotypic (Phenetic) Identification of Bacteria)

4. Phenotypic (Phenetic) Identification of Bacteria

Many characteristics (cultural, morphological, biochemical, physiological, immunological, and pathogenic) are used to identify a bacterium that has already been isolated in pure form.

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4.1 Cultural characteristics (Macroscopic study)

Size, shape, colour, opacity, surface, texture, odour, and, of course, any changes occurring on the surface of the solid medium, are the characteristics frequently used to macroscopically characterise a bacterial colony (cluster of cells).

With the naked eye or using a stereomicroscope, colonies are observed in natural and/or artificial light.

- **Size or diameter:** can be measured using a graduated millimetre ruler;
- **Surface texture:** may be smooth or rough;
- **Shape:** (flat, raised, edge) sometimes with a raised centre, or a hollowed-out centre;
- **Opacity:** colonies are described as: opaque (do not allow light to pass through); translucent (allow light to pass through but shapes cannot be seen through them, like frosted glass); transparent (allow light to pass through and shapes to be seen through them, like glass);
- **Consistency:** at the time of sampling, it is possible to assess whether the colonies are: greasy/creamy (homogeneous suspensions are easily obtained); dry/mucous (homogeneous suspensions are difficult to obtain).
- **Color (pigmentation):** on standard agar plates, colonies are usually cream-coloured, whereas on selective media, colonies are a different colour; this is due to various pigments such as yellow, red, orange, purple, etc.;
- **Odor:** a characteristic odor may be present (*Pseudomonas aeruginosa*).

By combining the characteristics listed above, three types of colonies can be distinguished:

1. **S colony (Smooth):** with a smooth surface and regular, rounded edges; creamy in consistency and producing homogeneous suspensions;
2. **R colony (Rough):** with a rough surface and jagged, flat edges, dry in consistency and yielding heterogeneous suspensions;
3. **M colony (Mucous):** with a smooth surface and regular, domed edges, stringy when pulled with a loop, and yielding heterogeneous suspensions.

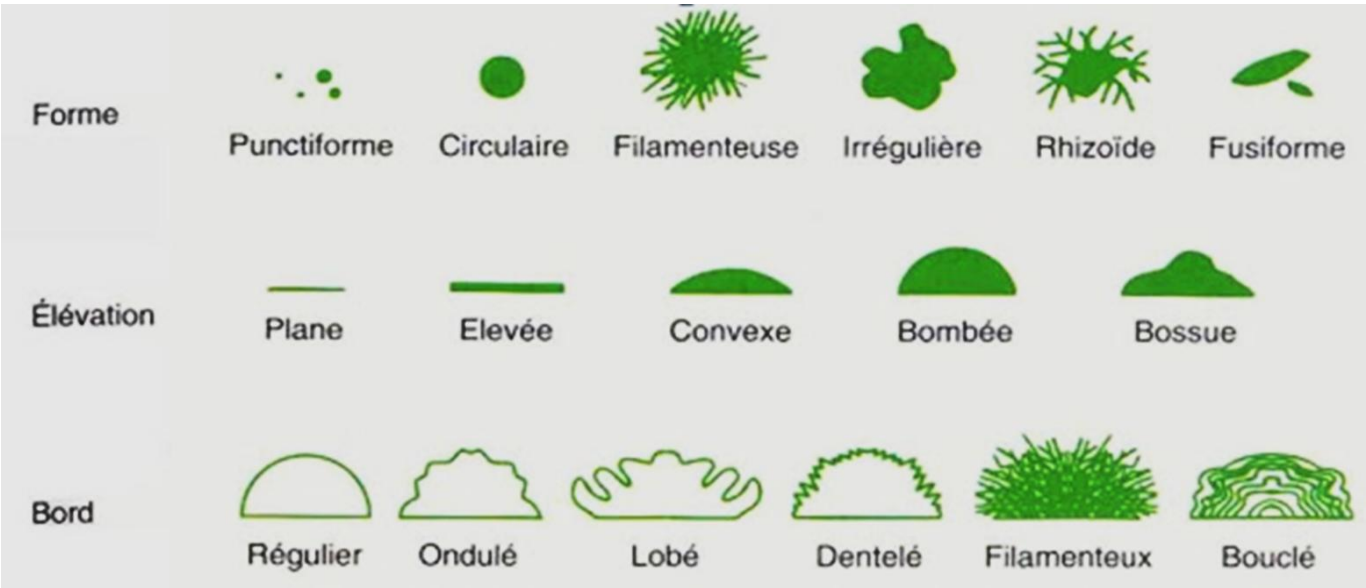


Figure : représentation schématique des caractères fréquemment utilisés pour caractériser macroscopiquement une colonie bactérienne.

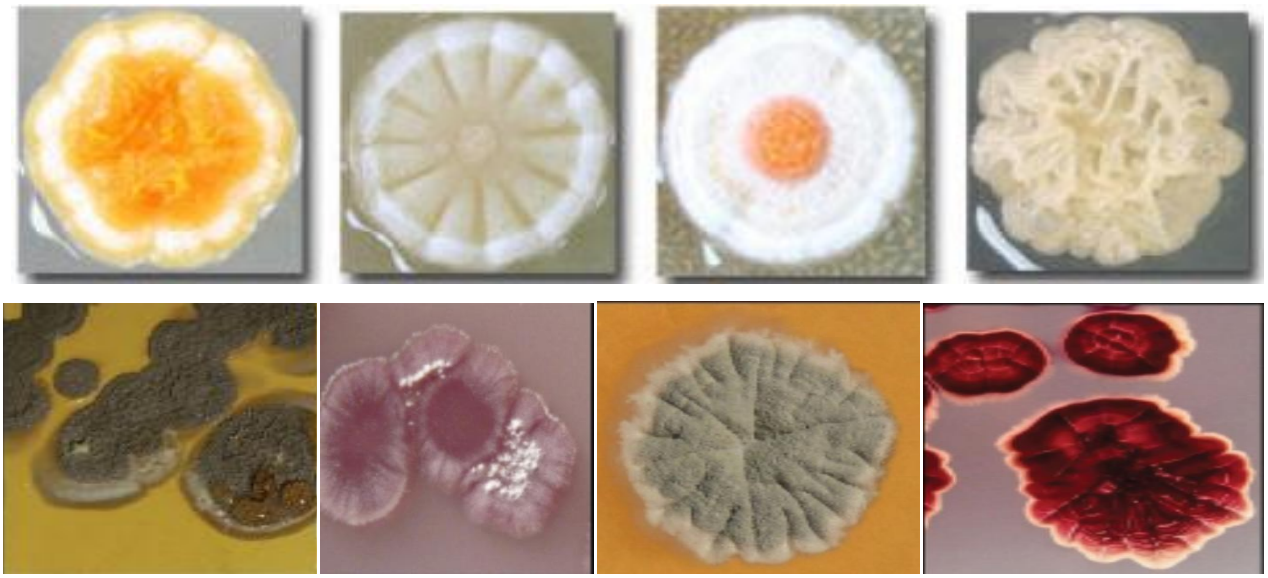
Bacterial colonies

Actinomycete colonies

The colonies formed on solid medium result from the accumulation of branched hyphae and not from individual cells as is the case with non-filamentous bacteria. The colony diameter is variable, ranging from 1 to 10 mm.

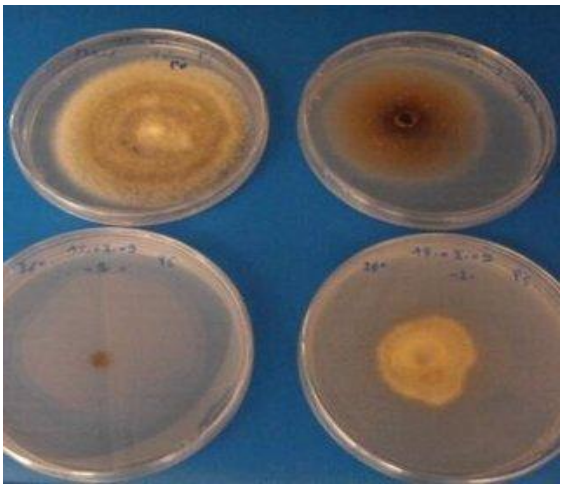
The appearance of the colonies can be compact, dry, smooth, rough with smooth or indented margins.

The colonies are often pigmented (white, cream, yellow, purple, pink, gray, etc.).



Fungi colonies

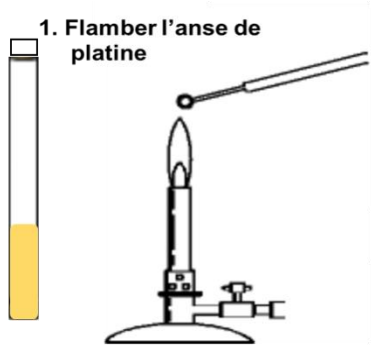
We will observe fluffy, large colonies that take up almost the entire plate, unlike bacterial colonies.

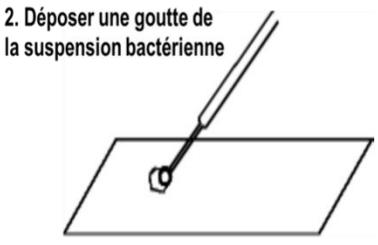


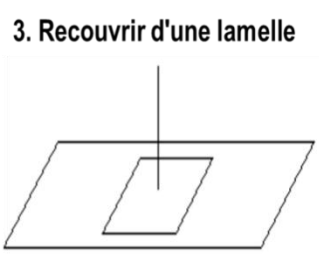
4.2 Microscopic examination (morphological and structural characteristics)

4.2.1. Wet mount examination :

This is the microscopic examination of live bacteria in a liquid medium. It allows for the assessment of their motility (or immotility).





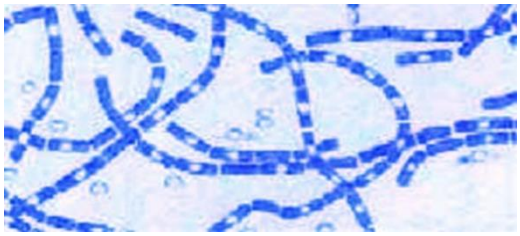


4.2.2. Staining Examination:

Allows for the observation of killed (fixed) bacteria on a slide that have been treated with one or more stains.

a. Simple staining (a single dye) (Methylene Blue staining)

Methylene blue staining can provide information regarding the morphology and grouping pattern of microbes. It also allows for the differentiation between dead and living cells in the case of a control (unfixed) analysis.

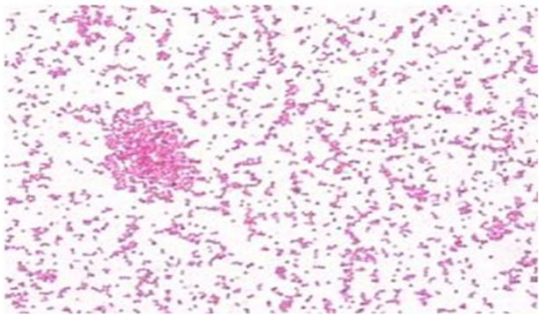


b. Differential Staining (Gram stain type)

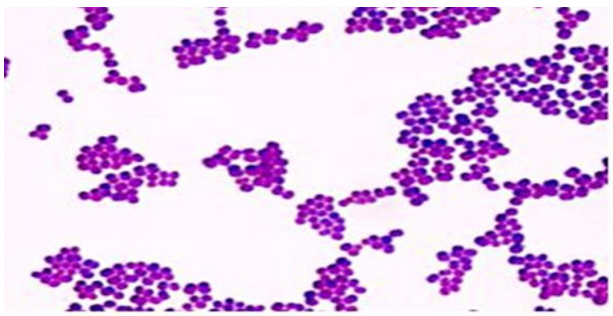
The Gram stain (the first test to be carried out) allows bacteria to be divided into two main groups: those with a Gram-positive cell wall (which retain gentian violet) and those with a Gram-negative cell wall (which take on the colour of safranin after washing with alcohol).

This method is based on the structure and composition of the cell wall. The lipids, which are more abundant in Gram- bacteria than in Gram+ bacteria, are extracted by the action of alcohol, leading to increased permeability and the extraction of the gentian violet-iodine complex, allowing the cell to take on the colour of safranin (fuchsin).

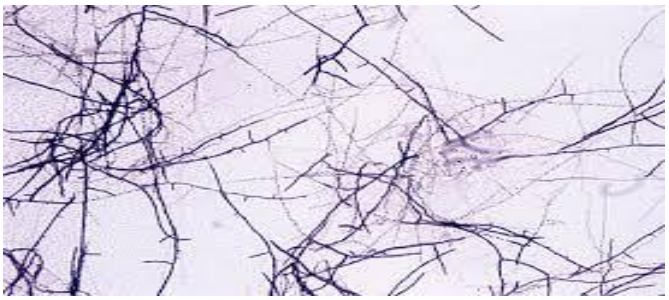
Furthermore, this test allows bacteria to be classified into two main groups based on their shape: cocci and bacilli, and it provides valuable information regarding their arrangement, which may take the form of diplococci, chains, tetrads, clusters, and palisades. It should be noted that even without this type of staining (complex and/or double staining), the shape, grouping and motility can be observed when the sample is fresh..



Gram-negative



Gram-positive

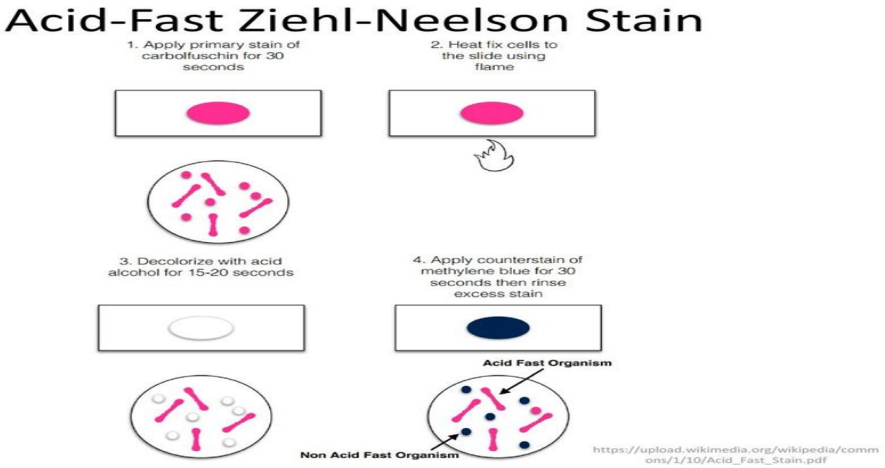


Actinomycete

c. Ziehl-Neelsen staining (acid-fast stain)

The Ziehl-Neelsen stain is specifically designed for mycobacteria, which are characterised by their ability to resist decolourisation by dilute acids and alcohol after being stained with safranin or fuchsin.

These are known as acid-fast bacilli (AFB), for example: Mycobacterium tuberculosis, which appears pinkish-red, often rod-shaped and slightly curved.

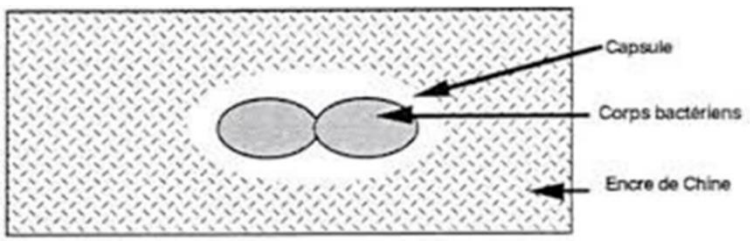


- **Special stains for bacterial structures.**

d. Coloration de la capsule

The presence of a capsule is revealed by India ink staining; its detection is a key indicator for classifying microorganisms into two groups: those with capsules and those without.

For example, Gram-positive diplococci surrounded by a prominent capsule are characteristic of *Streptococcus pneumoniae*.



d. Endospore staining (sporulation)

Sporulation allows bacteria to be divided into two groups: *spore-forming* and *non-spore-forming*. This test is performed either by malachite green staining or by a culture assay following pasteurization.

The position of the spore enables bacteria to be classified into groups within the same family.

4.3. Biochemical and Physiological Study.

a. Oxygen requirement

This characteristic allows for the classification of bacteria into 5 groups based on their growth responses in the presence and absence of oxygen :

1. Strict Aerobe: The final hydrogen acceptor is necessarily atmospheric O_2., example : *Pseudomonas aeruginosa*.

2. Strict Anaerobe: The acceptor is of a different nature, and O_2 is toxic.

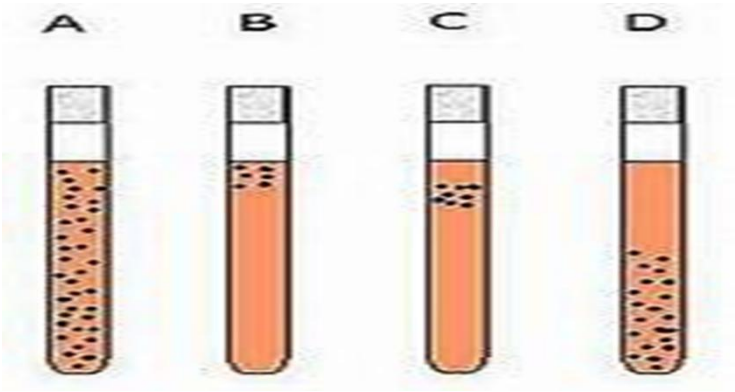
3. Aerotolerant anaerobes: Capable of growing in the presence or absence of O_2, the acceptor is of a different nature, and O_2 is not toxic, example: *Campylobacter jejuni* ;

4. Facultative Anaerobe: The bacterium grows indifferently under aerobic or anaerobic conditions but grows better in the presence of oxygen. exemple : Entérobactéries ;

5. Microaerophile: Require O_2, but at a low (tolerable) concentration.

Test for studying oxygen requirement.

Culture in a tube containing Meat Liver Agar (MLA) medium.



b. Respiratory enzymes

1. Cytochrome c oxidase test.

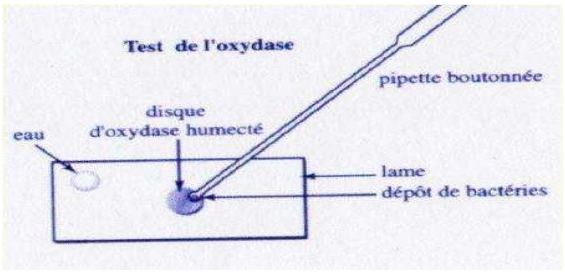
Oxidase or cytochrome oxidase: It is an enzyme that catalyzes an oxidation-reduction reaction, using an oxygen molecule (O_2) as an electron acceptor.

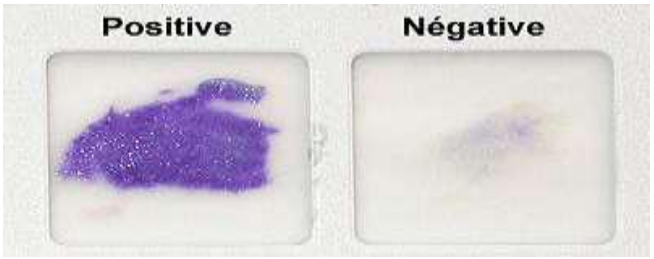
Principle

This test allows for the detection of phenylenediamine oxidase or cytochrome oxidase; an enzyme involved in various oxidation-reduction couples.

Method

This involves bringing a bacterial colony into contact with an oxidase disk. A positive reaction is indicated by the appearance of a violet color, either immediately or after a few seconds.





2. Catalase test.

Some oxidation reactions lead to the production of hydrogen peroxide (H_2O_2). Hydrogen peroxide is toxic to the cell unless the cell possesses the enzyme catalase.

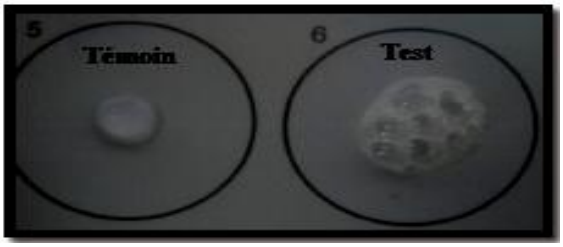
Principle:

Catalase is an enzyme with the property of decomposing hydrogen peroxide (H_2O_2) with the release of oxygen according to the following reaction:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Method :

This involves bringing a bacterial colony into contact with a drop of hydrogen peroxide. A positive reaction is indicated by the appearance of gas bubbles.



c. Hydrogen Acceptors.

Anaerobic Respiration: This is a process where the final hydrogen acceptor is an oxidized inorganic substance.

Many microorganisms are capable of completely oxidizing glucose in the absence of air, provided that nitrate, nitrite, sulfates (SO_4), or CO_2 are present.

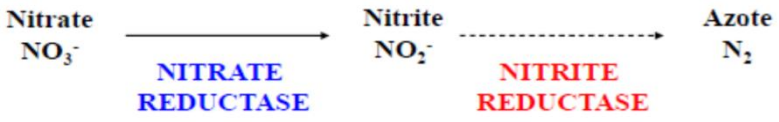
Sulfite reduction :

Sulfite-reducing anaerobes are capable of using sulfates (SO_4^{2-}) as hydrogen acceptors, reducing them to sulfides (S^{2-}). This test is performed on solid (MLA) or semi-solid media containing sodium sulfate and iron alum. After incubation, reduction is indicated by blackening (the production of H_2S).

Nitrate reduction :

Anaerobes are also capable of using nitrates as hydrogen acceptors, reducing them to nitrites.

This test is performed on solid media or broths containing 1‰ potassium nitrate. After incubation, the reduction of nitrates to nitrites is detected by adding 0.1 ml each of reagents NiT1 and NiT2.



Detection of nitrate reductase.

Nitrate broth + bacteria
Incubated for 24 hours at 37 °C.

GRIESS 1 (Nit 1)
+
GRIESS 2 (Nit 2)

Presence of a red color: The Griess reagents indicate the presence of nitrites. Therefore, the bacterium is NR+ (Nitrate Reductase positive).

Absence of color: Absence of nitrites:
- Either because they were not formed
- Or because they were reduced to nitrogen gas.

Zn

Absence of red color: Disappearance of nitrates from the medium and their conversion into nitrites and then into nitrogen gas. The bacterium is NR+ (Nitrate Reductase positive).

Presence of a red color: Zinc reduced the nitrates to nitrites, which produce a red color with Griess reagents. Therefore, the bacterium is NR- (Nitrate Reductase negative).

b. Oxidation-fermentation and sugar assimilation.

The determination of oxidation-fermentation and the possibility that other sugars are assimilated as the sole carbon source are important for taxonomic purposes.

Bacteria assimilate sugar by producing acid and frequently CO_2, in the presence or absence of O_2.

Acidification of the medium is detected by a color change due to the presence of a colored indicator (BCPL, phenol red, bromothymol blue, etc.). CO_2 is indicated by the lifting of the Durham bell, as it is released at the bottom of the tube (in the case of the oxidative pathway, it is produced at the surface, so the bell does not lift). A number of techniques using different media (MEVAG, TSI, various broths, etc.) are employed.

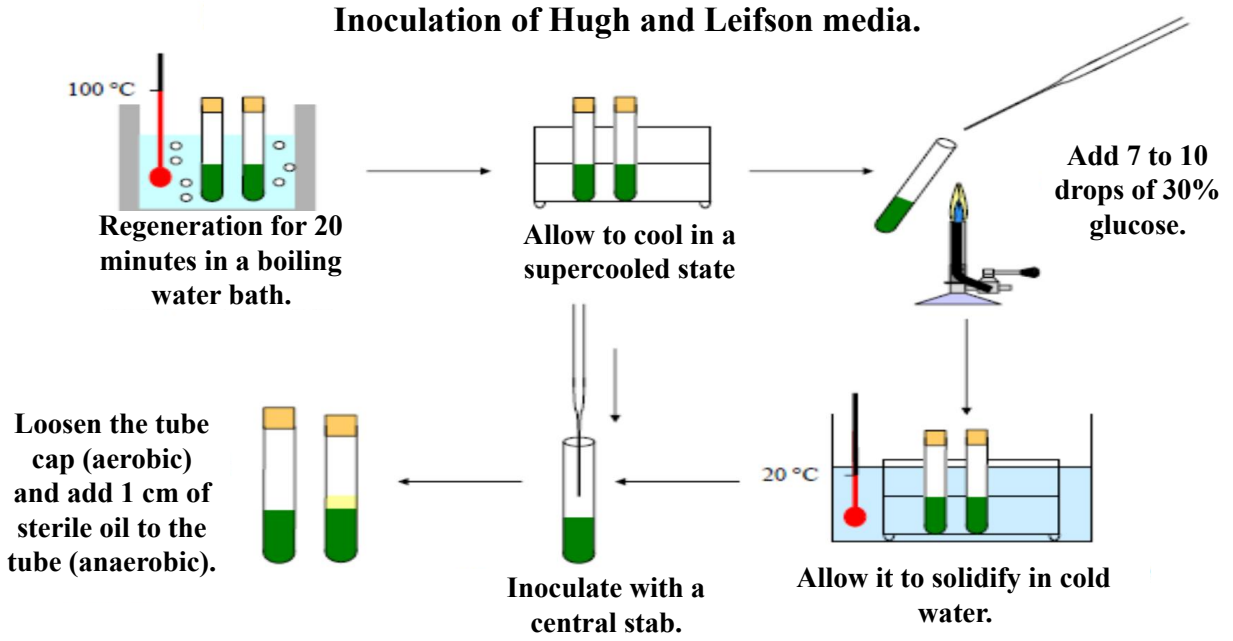
Carbohydrate metabolism.

The medium used is Hugh and Leifson's medium, which is green in color. This medium is used to determine the pathway of carbohydrate metabolism (glucose, arabinose, and xylose).

Principle

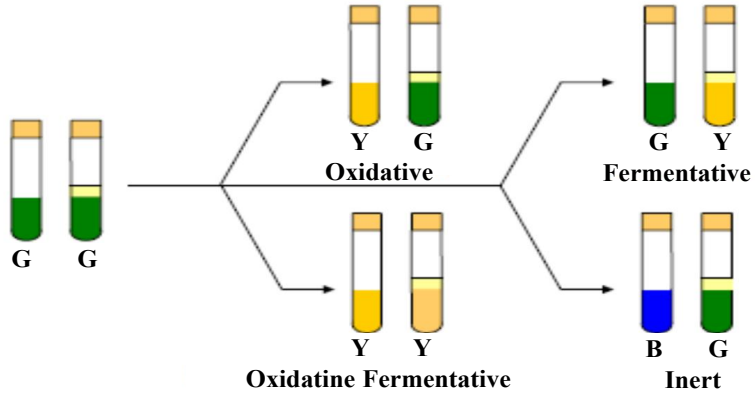
The study of energy metabolism reveals that a carbohydrate can be catabolized via respiratory or fermentative pathways. Hugh and Leifson's medium allows differentiation between these two processes. The degradation of a carbohydrate is generally accompanied by acidification of the medium. Hugh and Leifson's medium contains a pH indicator, bromothymol blue, which turns yellow in an acidic environment.

Method



Reading/Interpretation of Hugh and Leifson medium.:

Sealed tube (anaerobic conditions)	Open tube (aerobic conditions)	Metabolism
-	+	Oxidative.
+	+	Fermentative
+	-	Fermentative
-	-	Inert



c. Study of different intermediate fermentative pathways : Methyl Red test, Voges-Proskauer reaction (Acetoin production).

- Principle

  This study allows for differentiation between mixed acid fermentation (Methyl Red test: MR) and butanediol fermentation (Voges-Proskauer test, VP). These two reactions serve different purposes but can be performed on the same medium and often verify each other—an MR-positive strain is usually VP-negative, and vice versa.

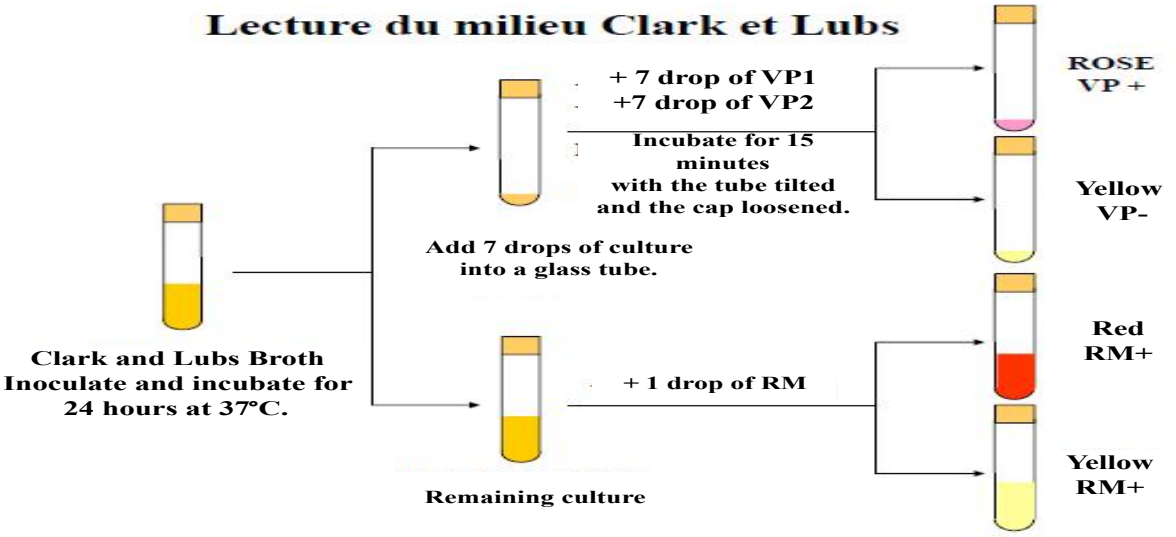
- **Methyl red reaction**

  This reaction demonstrates the final acidification of a glucose-containing medium resulting from glucose fermentation; this acidification causes methyl red to change colour (red at pH $\leqslant$ 5; yellow at pH $\geqslant$ 5.8).

- **Voges-Proskauer reaction (Production of acetoin)**

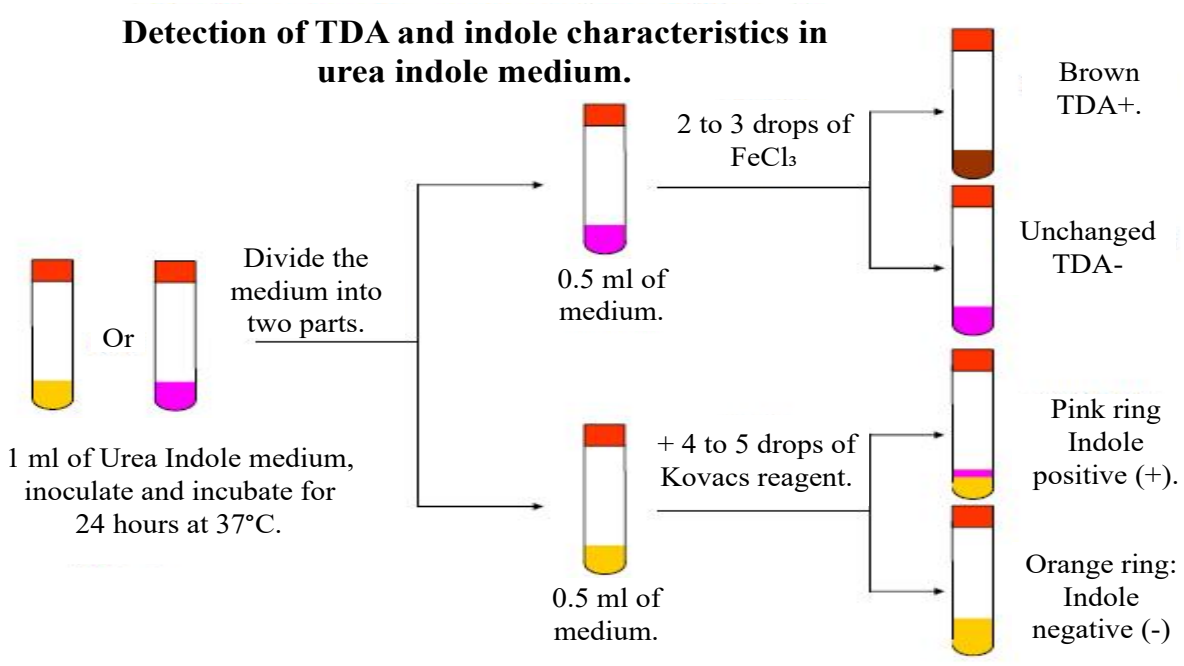
  Certain bacteria are capable of producing acetyl-methyl carbinol, either from two molecules of pyruvic acid or, more commonly, during the fermentation of 2,3-butylene glycol. In the presence of a strong base, acetoin produces a red colour in an oxygenated medium (oxidation to diacetyl). The diacetyl formed reacts with the guanidine group of α-naphthol to yield a red compound.

Method



f. Proteolytic metabolism

- **TDA, Tryptophanase et PDA :** enzymes used in bacterial identification. Tryptophan deaminase (TDA) catalyses the deamination of tryptophan to form indole pyruvic acid and ammonia. Tryptophanase, on the other hand, catalyses the breakdown of tryptophan into indole, pyruvic acid and ammonia. The addition of ferric chloride ($FeCl_3$) reacts with indolepyruvic acid to form a brown precipitate, and the addition of Kovacs' reagent reacts with indole to form a red ring on the surface. Example: *E. coli* is Indole+. Whereas, phenylalanine deaminase (PDA) catalyses the deamination of phenylalanine to phenylpyruvic acid and ammonia. The activity of phenylalanine deaminase (PDA) is tested on a phenylalanine-slanted agar plate. Phenylpyruvic acid (PPA) produces a green colour in the presence of ferric chloride.



- **Decarboxylase tests:**

➢ **L'ornithine-décarboxylase (ODC), la lysine-décarboxylase (LDC) et l'arginine-déhydrolase (ADH) :**

The presence of these enzymes is a characteristic frequently studied in the identification of Gram-negative bacilli, particularly Enterobacteria and Pseudomonas. The most commonly used media, under anaerobic conditions, are Falkow's media (the technique for which was extended by Möller); these media contain glucose, a colour indicator (usually BCPL) and contain only a single amino acid, namely the one whose utilisation is to be studied.

Principle:

Decarboxylases cleave amino acids, leading to the formation of the corresponding amine with the release of CO_2 according to the reaction. These enzymes are favored by an acidic pH (pH = 3.5–5.5) and anaerobic conditions.

*La lysine décarboxylase (LDC): Lysine $\xrightarrow{LDC}$ Cadavérine + CO_2

*L'ornithine décarboxylase ou ODC: Ornithine $\xrightarrow{ODC}$ Putricine + CO_2

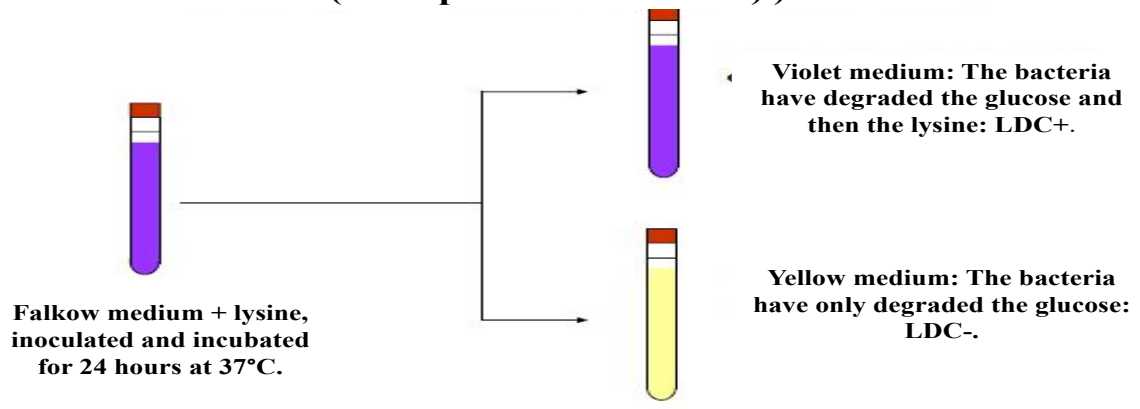
*L'argenine dihydrolase ou ADH : Argénine $\xrightarrow{ADH}$ Agmatine + CO_2.

Method:

The test is performed using Falkow medium distributed into four different hemolysis tubes.

1. The first tube serves as the control. It primarily contains a small amount of glucose and bromocresol purple as a pH indicator.
2. The three other tubes contain, in addition to the control medium, one of the following three amino acids: Arginine, Lysine, or Ornithine.
3. After inoculation, 1 ml of sterile vaseline is added to each tube, and the entire setup is incubated at 30°C for 24 to 48 hours..

Reading of Falkow medium (Example of the LDC test).)



g. Lactose breakdown

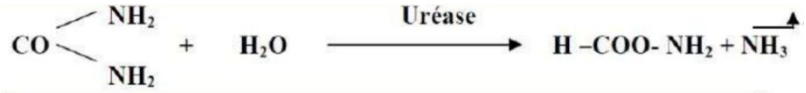
ONPG: The presence of β-galactosidase is a commonly used characteristic for identifying bacteria; it is particularly applied to Enterobacteria, specifically those that are lactose-negative. This enzyme hydrolyses a lactose analogue—ortho-nitro-phenyl-galactopyranoside (ONPG)—into galactose and ortho-nitro-phenol, which is yellow in colour.

h. Dégradation de l'urée

Urease : hydrolyses urea into ammonia and ammonium carbonate, leading to alkalisation of the medium, which is detectable by a colour change in the medium due to the presence of a colour indicator (usually phenol red). This test enables the identification of certain species of Enterobacteriaceae, Corynebacterium urealyticum, and Helicobacter pylori.

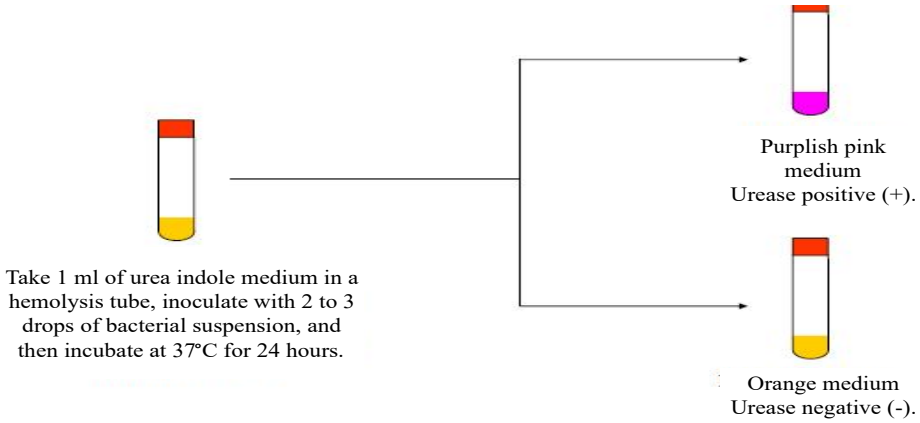
Principle:

All bacteria hydrolyze urea through the following reaction:



The CO_2 and NH_3 combine to form ammonium carbonate:

Detection of urease activity in urea indole medium



i. Various biochemical and physiological characteristics

- **Motility:** is tested on a semi-solid medium (in a dish). The inoculation is carried out by a central puncture; after incubation, motility is indicated by the cells spreading across the medium.
- **Growth temperatures, halophilia-osmophilia and pH:** assessing the ability to grow under adverse conditions is sometimes of great value for identification purposes. The ability to grow at different temperatures, at different concentrations of NaCl or sucrose, and at different pH levels is tested on liquid or solid media.

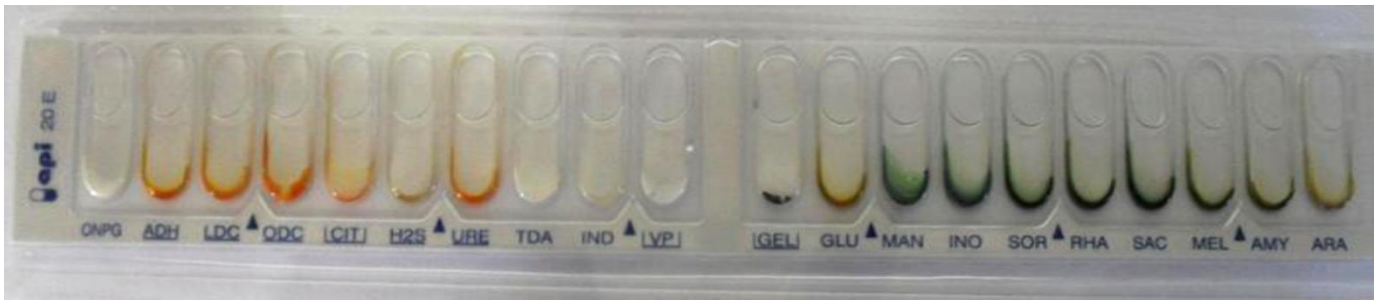
- **Resistance to antibiotics and inhibitors:** the ability to grow in the presence of certain antibiotics or inhibitory agents is widely used in taxonomy. The presence or absence of growth indicates the bacterium's susceptibility or resistance. This test is carried out on liquid or solid media. It is important to note that, unlike Gram-negative bacteria, Gram-positive bacteria are generally sensitive to vancomycin, with a few exceptions (Enterococci, Lactobacilli, Leuconostoc and Pediococcus spp.). Conversely, Gram-negative bacteria are sensitive to colistin and polymyxin, whereas Gram-positive bacteria are not.

Commercial microbial identification systems: The API 20E gallery

The API 20E biochemical gallery is a miniaturized, ready-to-use, and standardized system. It consists of 20 microtubes containing dehydrated substrates.

The tubes are inoculated with a bacterial suspension prepared in physiological saline.

The reactions occurring during the incubation period result in spontaneous color changes or are revealed by the addition of reagents (TDA, IND, and VP).

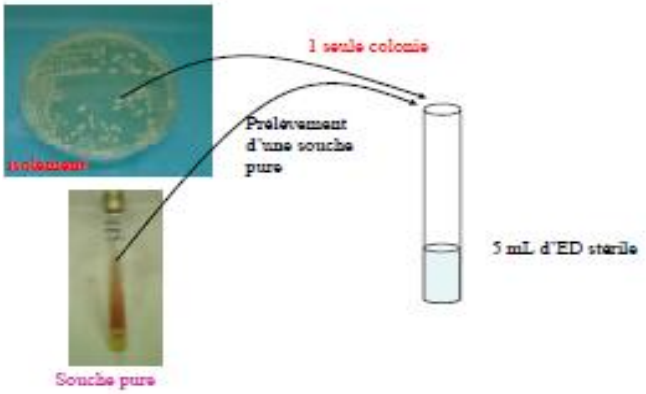


Preparation of the gallery

- Assemble the base and lid of an incubation box and distribute approximately 5 ml of distilled or demineralized water into the wells to create a humid atmosphere.
- Record the strain reference on the lateral tab of the box. (Do not write the reference on the lid, as it may be moved during handling).
- Remove the gallery from its individual packaging.
- Place the gallery in the incubation box.

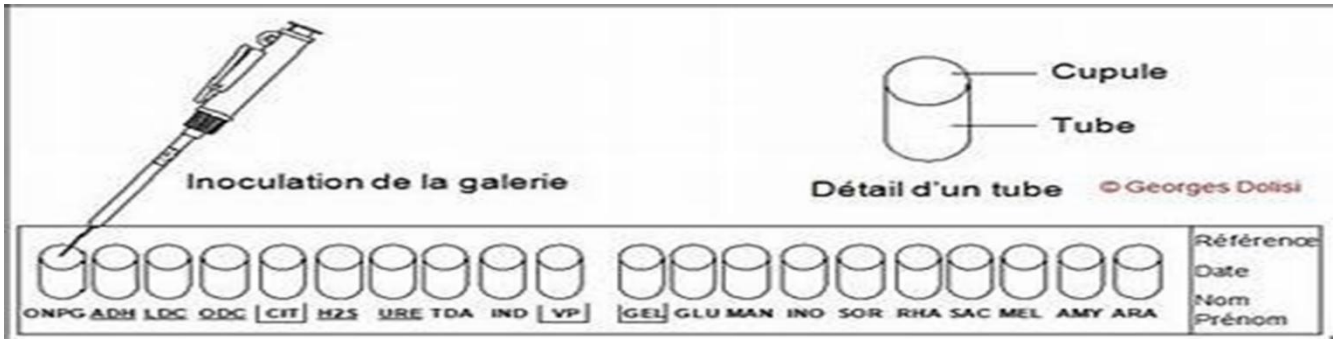
Preparation of the inoculum.

- Open an ampoule of API NaCl 0.85% Medium (2 ml) or use a tube containing 2 ml of 0.85% saline solution, without additives.
- Using a pipette, pick 1 to 4 colonies of identical morphology by successive touches. Preferably use young cultures (18-24 hours).
- Prepare a suspension with an opacity equal to 0.5 McFarland. This suspension must be used immediately.



Inoculation of the gallery

- Fill the tubes (not the cups) of the tests with the previously prepared suspension using the pipette.
- Fill the tubes and cups of the CIT, VP, and GEL tests.
- Fill the cups of the underlined tests (ADH, LDC, ODC, H_2S, and URE) with paraffin oil.
- Close the incubation box and incubate at 29°C ± 2°C for 24 hours.



After 24 and 48 hours of incubation at 30°C, the results are read by referring to the results table, where the reactions that have taken place during the incubation period are indicated by spontaneous colour changes or changes brought about by the addition of reagents (TDA, IND and VP).

Determination of the numerical profile :

On the result sheet, the tests are separated into groups of 3 and a value 1, 2 or 4 is indicated for each. By adding together the values corresponding to positive reactions within each group, a 7-digit profile number is obtained for the 20 tests of the API 20 E strip. The oxidase reaction constitutes the 21st test and has a value of 4 if it is positive.

This yields a seven-digit number that constitutes the digital profile of the strain under study

Identification is carried out using the digital profile via the apiwebTM identification software.

Reading Table

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS/ENZYMES	RESULTS	
				NEGATIVE	POSITIVE
ONPG	2-nitrophenyl-ßD-galactopyranoside	0.223	ß-galactosidase (Ortho NitroPhenyl-ßD-Galactopyranosidase)	colorless	yellow (1)
ADH	L-arginine	1.9	Arginine DiHydrolase	yellow	red / orange (2)
LDC	L-lysine	1.9	Lysine DeCarboxylase	yellow	red / orange (2)
ODC	L-ornithine	1.9	Ornithine DeCarboxylase	yellow	red / orange (2)
CIT	trisodium citrate	0.756	CITrate utilization	pale green / yellow	blue-green / blue (3)
H2S	sodium thiosulfate	0.075	H2S production	colorless / greyish	black deposit / thin line
URE	urea	0.76	UREase	yellow	red / orange (2)
TDA	L-tryptophane	0.38	Tryptophane DeAminase	TDA / immediate: yellow	TDA / immediate: reddish brown
IND	L-tryptophane	0.19	INDole production	JAMES / immediate: colorless pale green / yellow	JAMES / immediate: pink
VP	sodium pyruvate	1.9	acetoin production (Voges Proskauer)	VP 1 + VP 2 / 10 min: colorless	VP 1 + VP 2 / 10 min: pink / red (5)
GEL	Gelatin (bovine origin)	0.6	GELatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	1.9	fermentation / oxidation (GLUcose) (4)	blue / blue-green	yellow / greyish yellow
MAN	D-mannitol	1.9	fermentation / oxidation (MANnitol) (4)	blue / blue-green	yellow
INO	inositol	1.9	fermentation / oxidation (INOsitol) (4)	blue / blue-green	yellow
SOR	D-sorbitol	1.9	fermentation / oxidation (SORbitol) (4)	blue / blue-green	yellow
RHA	L-rhamnose	1.9	fermentation / oxidation (RHAmnose) (4)	blue / blue-green	yellow
SAC	D-sucrose	1.9	fermentation / oxidation (SACcharose) (4)	blue / blue-green	yellow
MEL	D-melibiose	1.9	fermentation / oxidation (MELibiose) (4)	blue / blue-green	yellow
AMY	amygdalin	0.57	fermentation / oxidation (AMYgdalin) (4)	blue / blue-green	yellow
ARA	L-arabinose	1.9	fermentation / oxidation (ARAbinose) (4)	blue / blue-green	yellow
OX	(see oxidase test package insert)		cytochrome-OXidase	(see oxidase test package insert)	

4.4 Immunological characteristics (serological)

Serological tests : of the bacterium-antibody type (agglutination reaction) or the antigen-antibody type (precipitation reaction) – are used in taxonomy primarily for Enterobacteria, which contain three types of antigens: H, O, and K; and for streptococci, the most significant of which is type C: A, B, C, D, N. These serological tests are mainly carried out using the Lancefield technique, which relies on the use of cell wall polysaccharides (notably polysaccharide C) as antigens.

4.5 Pathogenicity

- **Coagulase:** this characteristic alone is sufficient to confirm the presence of S. aureus, which is coagulase-positive, as opposed to other Staphylococcus species, which are coagulase-negative (S. epidermidis, S. saprophyticus). Staphylococcus aureus produces two types of coagulase: (1) free coagulase (extracellular enzyme) and (2) bound coagulase (wall-associated protein). Both enzymes are capable, in vitro, of coagulating rabbit plasma (formation of an insoluble fibrin clot).
- **α, ß and γ haemolysins:** enzymes responsible for the lysis of red blood cells; these are detected by culture on blood agar. α-haemolysins (greenish zone due to methaemoglobin, e.g. S. pneumoniae); β-haemolysins (pale halo due to the release of haemoglobin, e.g. S. aureus); γ-haemolysins (no change, no haemolysis around the colonies, e.g. E. faecalis).
- **DNase:** an enzyme that breaks down the cell nucleus; it is detected on media containing DNA. The hydrolysis of DNA is indicated by a clear zone; for example: *S. aureus*+ and *S. epidermidis.*