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Medical laboratory science
General microbiology
MCB 202

Question
1. Explain (step-by-step) at least ten (10) biochemical reactions of bacteria.
2. Explain the identification/staining techniques of fungi.

1. BIOCHEMICAL REACTIONS OF BACTERIA

To identify bacteria, we must rely heavily on biochemical testing. The types of biochemical reactions each organism undergoes act as a thumbprint for its identification. This is based on the following chain of logic:

- Each different species of bacterium has a different molecule of DNA (i.e., DNA with a unique series of nucleotide bases).
- Since DNA codes for protein synthesis, then different species of bacteria must, by way of their unique DNA, be able to synthesize different protein enzymes.
- Enzymes catalyze all the various chemical reactions of which the organism is capable. This in turn means that different species of bacteria must carry out different and unique sets of biochemical reactions.

When identifying a suspected organism, you inoculate a series of differential media. After incubation, you then observe each medium to see if specific end products of metabolism are present. This can be done by adding indicators to the medium that react specifically with the end product being tested, giving some form of visible reaction such as a color change. The results of these tests on the suspected microorganism are then compared to known results for that organism to confirm its identification.

a. Catalase test
b. Citrate test
c. Coagulase test
d. DNase test
e. Hippurate test
f. Indole test
g. Voges-Proskauer (VP) test
h. Potassium hydroxide test
i. Mixed acid fermentation
j. Oxidase reaction

CATALASE TEST

Catalase is the name of an enzyme found in most bacteria which initiates the breakdown of hydrogen peroxide (H_2O_2) into water (H_2O) and free oxygen (O_2).

Many aerobic bacteria and most of those which are facultatively anaerobic produce the enzyme catalase. The function of this enzyme is to detoxify hydrogenperoxide (H_2O_2), which is formed from the superoxide radical by superoxide dismutase. Many aerotolerant anaerobic bacteria have peroxidase (which is not the same enzyme as cytochrome c oxidase) instead of catalase. Obligate anaerobic bacteria lack superoxide dismutase and catalase.

Catalase contains a heme group at the active site and it is catalyzing the following reaction with a very high turnover number:

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

Catalase test

A. Apply one drop of 3% hydrogene peroxide on a microcscopic slide.
B. Transfer bacteria with a plastic loop to the H_2O_2 solution.
C. Catalase positive bacteria produce gas (O_2) in form av bubbles which shows that the bacterium has a catalase.
D. Catalase negative bakteria does not have a catalase and therefore oxygene is not formed. Note the fragments of bacterial colonies, but there are no bubbles in the solutions.

Method

1. Spread the bacteria on an agar plate and incubate the plate over night (18-24 h) under appropriate conditions.

2 Collect bacteria from one colony with a sterile inoculating loop (of plastic or platinum) and apply the bacteria on a microscope slide.

3 If the bacteria are collected from a blood agar plate, one has to avoid contamination of agar, because hemoglobin also contains heme groups which can cause a false positive reaction.

4 Add one drop of 3% H_2O_2 to the bacteria and observe the suspension. Be careful with the handling of H_2O_2 which is corrosive!!!

- Positive test result: Gas formation (O_2) in the form av bubbles shows that the bacterium has a catalase.
- Negative test result: No gas formation.

Applications

The catalse test is primarily used for gram positive bacteria and can for instance be utilized to distinguish Staphylococcus spp. and Micrococcus spp., which are catalase positive from Streptococcus spp. and Enterococcus spp., respectively, which are catalase negative.

CITRATE TEST

Some bacteria can utilize citrate as the only carbon source and the citrate test shows if the actual bacterium has this capability.

Method

1 Inoculate a tube containing citrate medium with a small amount of bacteria. It is also possible to streak or perform a deep inoculation into "Simmons citrate tube".

2 Incubate at 30-37ºC during 24-48 h.

- Positive test result: growth in citrate medium or growth with colour change to blue in Simmon's citrate tube.
- Negative test result: no growth in citrate medium eller growth but no colour change (still green colour) in Simmon's citrate tube.

Use

The citrate test is used to distinguish between, among others Citrobacter freundii and Escherichia coli.

COAGULASE TEST

Some bacteria produce coagulase, which is an enzyme that converts fibrinogen to fibrin, which means that it can coagulate plasma. The ability to produce coagulase is assumed to be associated to the virulence of staphylococci. The test is used to distinguish between coagulase positive and coagulase negative staphylococci.

Method

1 Suspend one colony from the suspected pure culture in 0.5 ml of plasma from horse, rabbit or man.

2 Incubate at 37ºC.

3 Read the test after 4 h. If the result is negative (see below), continue with the incubation.

4 Perform the final read after 24 h.

- Positive reaction if the plasma coagulates and the coagulate is stable. It must not be dissolved upon stirring.
- Negative reaction if the plasma does not coagulate or if the coagulate is dissolved again upon stirring.

Coagulase test of Staphylococcus spp. The upper tube shows positive result (the plasma has coagulated) and the lower tube shows a negative result.

Use

The coagulase test is used to distinguish between Staphylococcus aureus from coagulase negative Staphylococcus spp. Note, however, that some strains of S. aureus can be coagulase negative, but it is unusual. Some strains of S. hyicus and S. intermedius can br coagulase positve. S. pseudintermedius is coagulase positive, but not until after 24 h.

DNase TEST

Many bacteria have enzymes that break down nucleic acids. The bacteria can then use the resulting nucleotides to build up their own nucleic acids. DNase is such an enzyme, which thus hydrolyzes DNA. Existence of DNase is characteristic for certain species or strains of bacteria and can be used for typing.

METHOD

Presence of DNase can be determined by cultivation on an agar plate, which contains DNA. If the bacterium has DNase and if the bacteria are allowed to grow over night, the DNA will be hydrolyzed into the constituting nucleotides. Diluted hydrochloric acid (HCl) is then poured onto the plate and there will be a clear zone close to the colonies or the streak, because individual nucleotides are soluble in diluted HCl, but not DNA, which precipitates in the rest of the plate.

Use

The test is useful to distinguish between:

- Serratia spp. and Enterobacter spp.
- Staphylococcus aureus (most strains are coagulase positive) and coagulase negative Staphylococcus spp.
- Moraxella catarrhalis and Neisseria spp.

HIPPURATE TEST

Some bacteria can hydrolyze hippurate to the amino acid glycine and benzoate by means of the enzyme hippuricase. Glycine can be detected with ninhydrin (2,2-Dihydroxyindane-1,3-dione), which reacts with free amino groups (-NH2) and a blue product is formed.

Method

1 Suspend a loop-full of bacteria (for instance Campylobacter sp.) in 0.5 ml of sodium hippurate siolution.

2 Incubate the suspensionen at 37ºC during 2 h in a water bath.

3 Then carefully add 0.2 ml of ninhydrin solution without mixing.

4 Incubete the dube during another 10 min. at 37ºC before reading the result.

- Positive test resultat: Deep blue colour.
- Negative test result: Pale blue colour.

Use

The hippurate test is primarely used to distinguish between Campylobacter jejuni (hip+) and Campylobacter coli (hip-) and to distinguish between different streptococci (see figure).The test is also used, in combination with other methods, to type Brachyspira spp.

INDOLE TEST

Bacteria, which express the enzyme tryptophanase can hydrolyze the amino acid tryptophan to indole, pyruvic acid and ammonia. Presence of indole can be shown by means of Kovác's reagent or by spot indole test. In the spot test indole reacts with p-Dimethylaminocinnamaldehyde to produce a blue to blue-green product. Kovác's reagent contains p-dimethylaminobenzaldehyde, which forms a red complex with indole.

Spot indole test.

A. Application of spot indole reagent.
B. Application of indole positive bacterium.
C. Application of indole negative bacterium.

Method, spot indole test

1 Place several drops of Spot Indole Reagent on a piece of filter paper.

2 Fill a plastic loop with bacteria from a colony cultivated for 24-48 h.

3 Rub it onto the reagent saturated area of the filter paper.

- Positive reaction: Appearance of a blue to blue-green color change within 10 seconds.
- Negative reaction: Remain colorless or light pink.

Method, Kovac´s reagent

1 Suspend one colony from a pure culture of the bacterium to be investigated, in a suitable medium (for instance LTLSB or tryptophan medium).

2 Incubate the medium at 37°C during 20-28 h.

3 Add a few drops of Kovác's reagent.

- Positive test result: The indole reagent change colour to cerise red.
- Negative test result: The indole reagent remains pale yellow.

Use

Confirmation of suspected E. coli-strains. Typing (species determination) of Brachyspira spp. in combination with other tests. Kovac's indole reagent is more sensitive than the indole spot reagent,

but it is not recommended for use with anaerobic bacteria. The indole spot reagen is suitable for both aerobic and anaerobe use.

VOGES-PROSKUAER TEST

The VP test shows if the bacterium has butanediol fermentation and can split glucose to acetoin via pyruvat and further to 2,3-butanediol according to:

2 pyruvate + NADH --> 2CO2 + 2,3-butanediol.

If KOH (potassium hydroxide) is added, acetoin will be converted to diacetyl (= 2,3-butanedione), which reacts with alpha-naphtol and forms a pink complex.

Method

1 Suspend one colony from the pure culture, which is to be investigated, in VP/MR medium.

2 Incubate at 30-37ºC during 24-48 h.

3 Add 0.2 ml of 40% KOH and then 0.6 ml of alpha-naphtol solution.

- Positive test result: colour change to pink.
- Negative test result: no colour change.

Use

Klebsiella spp. and Enterobacter spp. has the capacity to perform butanediole fermentation in contrast to Escherichia coli, Salmonella spp. and Shigella spp.

POTASSIUM HYDROXIDE TEST

The purpose of the potassium hydroxide test (KOH test) is to identify gram negative bacteria. KOH dissolves the thin layer of peptidoglycan of the cell walls of gram negative bacteria, but does not affect gram positive cell walls. Disintergration of gram negative cell walls lyses the cell and release its contents, including the DNA. The DNA will make the solution very viscous and the solution will stick to the plastic loop when touched. Gram positive bacteria will not be affected by KOH, because they have thicker peptidoglycan layer in the cell wall. Thus, the cells will not be lysed, the DNA not released and no viscosity will be observed.

Potassium hydroxide test.
A. Application of potassium hydroxide solution onto a microscopic slide.
B. Bacteria are transferred and mixed with the KOH solution.
C. Result with gramnegative bacteria where the solution will be viscous and form a mucoid string.
D. Result with grampositive bacteria where the solution will not be viscous.

Material

- Microscopic slide
- Plastic loop
- 3% KOH

Method

- Apply one drop of 3% KOH on a microscopic slide.
- Use o loop to transfer a generous amount of bacteria (cultivated for 24-48 h) to the drop of KOH
- Stir carefully
- The solution of gram negative bacteria will be viscous and form a mucoid string within 30 sec
- Use positive and negative controls

Positive results: The solution with the bacteria (gram negative) will be viscous
Negative results: The solution with the bacteria (gram positive) will not be viscous

Use

The purpose of the KOH test is to quickly distinguish between gram negative and gram positive bacteria as a complement to Gram staining. The test is not useful for anaerobic bacteria.

MIXED ACID FERMENTATION

Some bacteria can ferment glucose to a mixture of the following organic acids: formic acid, acetic acid and lactic acid. This is called mixed acid fermentation and it causes highly decreased pH in the medium. Mixed acid fermentation can, therefore, be detected by

addition of the pH indicator methyl red (MR). The test method is sometimes called the MR test.

Method

1 Suspend a bacterial colony from a pure culture in the MR/VP medium.

2 Incubate at 30°C during 3 days or at 37°C during 48 h.

3 Add 2-3 drops of a solution of methyl red.

- Positive test result: red colour change
- Negative test result: no colour change.

Use

Some members of the family Enterobacteriaceae have mixed acid fermentation (see the respective bacterial page), which can be used to differentiate these bacteria.

OXIDASE REACTION

Bacteria, which have aerobic respiration, often have cytochrome c and a cytochrome c oxidase. The presence of these components can in combination with other methods be used for typing. A commersial test, which contains an artificial electron acceptor (N, N, N', N'-tetramethyl-p-phenylenediamine, see Fig. 1), is often used. This artificial electron acceptor change colour depending upon redox state. The substance is also referred to as a redox indicator and it can be oxidized by the oxidized form of cytochrome c. Cytochrome c oxidase is the last enzyme of the electron transport chain, where it normally reduces oxygen to water and pump protons to the outside according to the following net reaction:

$4\ Fe^{2+}\text{-cytochrome c}_{red} + 8H^{+}_{in} + O_2 \rightarrow 4\ Fe^{3+}\text{-cytochrome c}_{ox} + 2H_2O + 4H^{+}_{out}$

Cytochrome c oxidase is a transmembrane protein complex (Complex IV), which is also present in the cytoplasmic (inner) membrane of mitochondria.

Oxidase test

A. Two drops of oxidase reagent is applied onto a piece of filter paper.
B and C. Colony material is transferred by a plastic loop to the spot of oxidase on the filter paper.

C. The left spot includes oxidase positive bacteria and the spot to the right oxidase negative bacteria.
D. The final results can be observed after 30s.

Method

1 Keep the plastic ampoule (which contains a glass ampoule) between your fingers and with the opening upwards and away from your face. Press the ampouls with your fingers until the glass ampoule breaks.

2 Apply two drops of the oxidase reagent onto a piece of filter paper.

3 Transfer bacteria from one colony with a plastic or platinum loop onto the spot with the oxidase reagent. The colonies should have been incubated at the appropriate temperature for 18-24 h .

- Positive test resultat: Dark blue-purple colour change within 10-30 sec.
- Negative test resultat: No colour change or colour change after more than 30 sec.

Note that the oxidase reagent is not stable after that the ampoule has been opened. It may be used for a couple of hours, but eventually it will be oxidized by the oxygen in the air.

Applications

The oxidase test is used for identification of gram negative bacteria. For instance to identify members of the family Enterobacteriaceae, which are oxidase negative, except members of the genus Plesiomonas (oxidase positive). Members of the family Pseudomonadaceae, and the genera Aeromonas and Campylobacter are oxidase positive.

2. IDENTIFICATION OR STAINING TECHNIQUES OF FUNGI

In general, fungal identification requires greater visual acuity than bacteria. Unlike other important microorganisms such as bacteria and viruses, the identification of fungi heavily relies on morphological criteria. The characteristics of fungal structure are identified by

observing colonial growth both macroscopically and microscopically. These morphological features and other classical methods that are routinely used in
classification are also useful in fungal identification. A recent review illustrates the inability to fungi at the species, or even at the genus, level in many cases. Many species of fungi, ascomycetes, basidiomycetes, and zygomycetes in particular, have different microscopic and macroscopic characteristics in each stage of their life cycle. Moreover, they are synonymous to each other with many names used to describe the same organism

STAINING TECHNIQUES

Direct microscopic examination without stain lacks sensitivity, especially when hyphae are
sparse in the specimen. A variety of differential stains are commonly used like Gram, Giemsa, Wright stain, toluidine blue O, and Weigert's iron hematoxylin to stain fungi. The sensitivity of microscopic examination is improved when fungus-enhancing stains like Mayer's mucicarmine, periodic acid Schiff, Gomori's methenamine silver, acridine orange fluorescent, calcoflour white, thiosemicarbazide, Fontana- Masson, and Gridley's stains are used. Since the stain is immediately taken up by the fungal cell wallinthescraping,thestainingusuallybecomes much brighter after 5–10 min. Some specimens need alkali pretreatment. In that case, it is important to make sure that they do not react for a long period of time; otherwise, a gelatinous consistency will form, and the specimen should be neutralized with 10% lactic acid before staining and adjusted to pH 3.0–5.0. The method of preparation of smear for staining is as follows:

1. Take a clean grease-free glass slide.
2. Place a large drop of saline solution.
3. Transfer a small quantity of the culture with a loop or the tip of a scalpel into the saline drop.
4. Make a smear over the surface of the slide.
5. Fix by heat, if necessary.

1. GRAM STAINING

Gram stain is a key starting point to identify microbial species. The stain differentiates membrane structures between gram-positive and gram-negative microorganisms. Gram-positive microbes have a thick cell wall made up of peptidoglycan (50–90%), which are stained purple by crystal violet, whereas gram-negative microbes have a thinner layer (10% of cell wall), which are stained pink by the counter-stain safranin [24, 30, 33–36].

1. Apply two drops of crystal violet on smear for 30 s.
2. Wash with tap water.
3. Add two drops of Gram's iodine for 30 s.
4. Repeat step 2.
5. Add 95% ethanol.
6. Repeat step 2.
7. Add two drops of safranin.
8. Repeat step 2.
9. Observe in microscope under oil immersion. 10. Yeasts are gram-positive, but poorly stained; Cryptococcus neoformans is a notable exception (gram-negative).

2. PERIODIC ACID-SCHIFF STAINING

Periodic acid–Schiff (PAS) reactions are effective stains for demonstrating fungal elements of essentially all fungi. Periodic acid attacks some carbo- hydrates containing 1,2-glycol or OH group with the conversion of this group to 1,2-aldehydes, which then react with the fuchsin-sulfurous acid to form the magenta colour. Identification of fungal elements can be enhanced if a counter- stain such as light green is used. Species of Coccidioides, Cryptococcus, Histoplasma, Candida, Malassezia, and Aspergillus can be stained with this stain.

1. Immerse the smear in ethanol for 1 min.
2. Place 5% periodic acid for 5 min.
3. Wash gently in running tap water.
4. Place basic fuchsin for 2 min.
5. Repeat step 3.
6. Add sodium metabisulphite (0.5%) for 3–5 min.

7. Repeat step 3.
8. Counterstain with dilute aqueous light green
(0.2%) for 2 min.
9. Dehydrate with 70%, 80%, 95%, 100% ethanol and xylene, each for 2 min. 10.Observeundermicroscope.
11. Fungi stain bright pink-magenta or purple
against green background when light green is used as a counterstain.

3. GRIDLEY STAINING

Gridley staining method is used to identify fungi, based on Bauer chromic acid leucofuchsin stain with the addition of Gomori's aldehyde fuchsin stain and metanil yellow as counterstains. Against a yellow background, hyphae, conidia, yeast capsules, elastin, and mucin appear in different shades of blue to purple. It can be used to identify Rhiosporidium seeberi and Histoplasma capsulatum.
1. Place chromic acid on smear for 1 h.
2. Wash well with tap water.
3. Treat with sodium metabisulphite bleach for
1 min.
4. Repeat step 2.
5. Rinse with distilled water.
6. Place in Schiff's reagent for 20 min. 7. Repeat step 2.
8. Rinse with 70% ethanol.
9. Place in aldehyde fuchsin for 30 min.
10. Rinse off excess with 95% ethanol.
11. Repeat step 2.
12. Counterstain with metanil yellow for 1 min. 13. Rinse well with distilled water.
14. Dehydrate and observe under fluorescence
microscope.
15. Fungi show purple color with yellow back-
ground.

4. GIEMSA STAINING

A variety of "Romanowsky-type" stains with mixtures of methylene blue and azure eosin compounds have been used successfully for

many years on diverse fungi with various procedures and modifications. Giemsa stain is a member of the Romanowsky group of stains, which are defined as being the black precipitate formed from the addition of methanol. In this stain, eosin ions are negatively charged and stain basic components of cells orange to pink. It was also originally designed to incorporate cytoplasmic (pink) staining with nuclear (blue) staining and fixation as a single step for smears and thin films. This stain has widely been used to examine Pneumocystis jiroveci, Rhinosporidium seeberi, and Histoplasma capsulatum.

1. Flood the smear with methyl alcohol and leave for 3–5 min for fixation.
2. Add prepared Giemsa stain and leave for 45 min.
3. Wash slide thoroughly with running tap water.
4. Blot dry with absorbent paper.
5. Observe under oil immersion.
6. Look for intracellular budding yeasts; fungi stain with purplish-blue.

4. WRIGHT STAINING

The Wright stain is an alcoholic solution of methylene blue, azure A, thionin, and eosin Y. Methyl groups are activated and react with charged components of the cell to produce coloration. It is used to detect blood parasites, viral and chlamydial inclusion bodies, yeast cells, and species of Pneumocystis. Eosin ions are negatively charged and stain basic components of cells orange to pink, whereas other dyes stain acidic cell structures to various shades of blue to purple.

1. Cover the smear with freshly filtered Wright stain and leave for 1–3 min.
2. Without removing the stain, pour on buffer solution (pH 6.4)
3. Gently mix buffer and stain; upon proper mixing, metallic green sheen (green scum) rises to the surface of the fluid.
4. Leave for 3 min or longer.
5. Wash the slide gently with flowing tap water and wipe the bottom of the slide with a clean filter paper.
6. Air-dry the slide and observe under the

microscope.
7. Intracellular yeast cells are typically stain blue and species of Pneumocystis stain purple.

5. FONTANA-MASSON STAINING

The Fontana-Masson (FM) stain can be used to detect the presence of melanin in cell walls of dematiaceous fungi such as species of Bipolaris, Curvularia, Exophiala, and Phialophora. FM stain is often believed to be a diagnostic tool to differentiate dematiaceous fungi from Aspergillus sp. and some Zygomycetes. Also, it is particularly useful for distinguishing capsule-deficient Cryptococcus neoforamans from Histoplasma capsulatum and Blastomyces dermatitis. Melanin has the ability to reduce solutions of ammonical silver nitrate to metallic silver without the use of an external reducing agent. The intensity and amount of staining may reflect differences in melanin deposition owing to growth rate, age, availability of precursors, or loss of pigment staining associated with hyphal death and destruction. Extent of stain intensity and its distribution in fungal elements in tissue were evaluated by means of intensity; for exam- ple, dark brown (strong intensity), medium brown (moderate intensity), and pale brown (weak intensity).

1. Treat smear with ammonical silver nitrate solution for 20 min at 60 °C.
2. Check microscopically after 15 min and repeat step 1 if necessary.
3. Wash well in distilled water.
4. Tone with 0.1% gold chloride for 2 min.
5. Repeat step 3.
6. Fix in 2% aqueous sodium thiosulphate for 2 min.
7. Repeat step 3.
8. Counterstain with neutral red stain for 1 min.
9. Repeat step 3.
10. Rapidly dehydrate well in absolute alcohol, clear, and mount.
11. Observe under microscope.
12. All dematiaceous fungi show strong inten- sity (black); species such as Bipolaris, Exophiala, Fonsecaea, and Phialophora are darkly pigmented because of melanin.

6. GROCOTT-GOMORI METHENAMINE SILVER STAINING

Grocott-Gomori methenamine silver (GMS) staining is preferred for screening degenerated and nonviable fungi because it provides better contrast. The fungal cell wall contains mucopolysaccharides that are oxidized by GMS to release aldehyde groups, which later react with silver nitrate. Silver nitrate is converted to metallic silver, which becomes visible in the silver stains; this is useful in detecting fungal elements. Fungi stain in black against a pale-green background. Pneumocystis jiroveci, Cryptococcus neoformans, Coccidiodes immitis, Histoplasma capsulatum, Aspergillus fumigatus, and Candida albicans can be detected by this staining technique

1. Add two drops of absolute ethanol for 5min.
2. Wash in distilled water.
3. Flood the smear with 4% chromic acid for 45 min.
4. Repeat step 2.
5. Add 1% sodium metabisulphite for 1–2 min.
6. Repeat step 2.
7. Add working solution of hexamine (smear becomes dark brown).
8. Wash with distilled water or if smear turns black, wash with 0.1% ferric chloride.
9. Add 5% sodium thiosulphate for 2 min.
10. Repeat step 2.
11. Wash with 1% light green solution for 1 min.
12. Dry and view under oil immersion.
13. The slide with fungal elements stains black; inner part of micelle or hyphae stains pink with background in pale green.

OTHER STAINING TECHNIQUES INCLUDE:

7. Weigert's Iron Hematoxylin Staining
8. Acridine orange staining
9. Calcoflour white staining
10. Mayer's Mucicarmine staining.