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**18/SCI05/010.**

**MEDICAL LABORATORY SCIENCES.**

**GENERAL MICROBIOLOGY (MCB 202).**

**1.**Explain (step-by-step) at least ten (10) biochemical reactions of bacteria.

 1. Heterotrophic Metabolism

 Heterotrophic metabolism is the biologic oxidation of organic compounds, such as glucose, to yield ATP and simpler organic (or inorganic) compounds, which are needed by the bacterial cell for biosynthetic or assimilatory reactions.

2. Respiration

 Respiration is a type of heterotrophic metabolism that uses oxygen and in which 38 moles of ATP are derived from the oxidation of 1 mole of glucose, yielding 380,000 cal. (An additional 308,000 cal is lost as heat.)

3.Sugar Fermentation.

 Sugar media are composed of;

* Peptone water’’’+1% test sugar, +Andrade’s indicator, +A small inverted tube (Durham’s tube).

In fermentation, another type of heterotrophic metabolism, an organic compound rather than oxygen is the terminal electron (or hydrogen) acceptor. Less energy is generated from this incomplete form of glucose oxidation, but the process supports anaerobic growth.

 Sugar fermentation can be indicated by change in the colour of the medium from yellow to red.

* Glucose +ve
* Lactose +ve
* Maltose +ve
* Mannite +ve

In the late 1850s, Pasteur demonstrated that fermentation is a vital process associated with the growth of specific microorganisms, and that each type of fermentation can be defined by the principal organic end product formed (lactic acid, ethanol, acetic acid, or butyric acid). His studies on butyric acid fermentation led directly to the discovery of anaerobic microorganisms. Pasteur concluded that oxygen inhibited the microorganisms responsible for butyric acid fermentation because both bacterial mobility and butyric acid formation ceased when air was bubbled into the fermentation mixture. Pasteur also introduced the terms aerobic and anaerobic. His views on fermentation are made clear from his microbiologic studies on the production of beer (from Etudes sur la Biere, 1876): In the experiments which we have described, fermentation by yeast is seen to be the direct consequence of the processes of nutrition, assimilation and life, when these are carried on without the agency of free oxygen. The heat required in the accomplishment of that work must necessarily have been borrowed from the decomposition of the fermentation matter…. Fermentation by yeast appears, therefore, to be essentially connected with the property possessed by this minute cellular plant of performing its respiratory functions, somehow or other, with the oxygen existing combined in sugar.

For most microbial fermentations, glucose dissimilation occurs through the glycolytic pathway (Fig. 4-1). The simple organic compound most commonly generated is pyruvate, or a compound derived enzymatically from pyruvate, such as acetaldehyde, α-acetolactate, acetyl ~ SCoA, or lactyl ~ SCoA

3. Krebs cycle (also tricarboxylic acid or citric acid cycle).

 The Krebs cycle is therefore another preparatory stage in the respiratory process. If 1 molecule of pyruvate is oxidized completely to 3 molecules of CO2, generating 15 ATP molecules, the oxidation of 1 molecule of glucose will yield as many as 38 ATP molecules, provided glucose is dissimilated by glycolysis and the Krebs cycle (further assuming that the electron transport/oxidative phosphorylation reactions are bio-energetically identical to those of eukaryotic mitochondria).

 If 2 pyruvate molecules are obtained from the dissimilation of 1 glucose molecule, then 30 ATP molecules are generated in total. The decarboxylation of pyruvate, isocitrate, and α-ketoglutarate accounts for all CO2 molecules generated during the respiratory process. It shows the enzymatic reactions in the Krebs cycle. The chemical energy conserved by the Krebs cycle is contained in the reduced compounds generated (NADH + H+, NADPH + H+, and succinate). The potential energy inherent in these reduced compounds is not available as ATP until the final step of respiration (electron transport and oxidative phosphorylation) occurs.

4. Glyoxylate Cycle

In general, the Krebs cycle functions similarly in bacteria and eukaryotic systems, but major differences are found among bacteria. One difference is that in obligate aerobes, L-malate may be oxidized directly by molecular O2 via an electron transport chain. In other bacteria, only some Krebs cycle intermediate reactions occur because α-ketoglutarate dehydrogenase is missing.

 A modification of the Krebs cycle, commonly called the glyoxylate cycle, or shunt, which exists in some bacteria. This shunt functions similarly to the Krebs cycle but lacks many of the Krebs cycle enzyme reactions. The glyoxylate cycle is primarily an oxidative pathway in which acetyl~SCoA is generated from the oxidation, of acetate, which usually is derived from the oxidation of fatty acids. The oxidation of fatty acids to acetyl~SCoA is carried out by the β-oxidation pathway. Pyruvate oxidation is not directly involved in the glyoxylate shunt, yet this shunt yields sufficient succinate and malate, which are required for energy production. The glyoxylate cycle also generates other precursor compounds needed for biosynthesis. The glyoxylate cycle was discovered as an unusual metabolic pathway during an attempt to learn how lipid (or acetate) oxidation in bacteria and plant seeds could lead to the direct biosynthesis of carbohydrates. The glyoxylate cycle converts oxaloacetate either to pyruvate and CO2 (catalyzed by pyruvate carboxylase) or to phosphoenolpyruvate and CO2 (catalyzed by the inosine triphosphate [ITP]-dependent phosphoenolpyruvate carboxylase kinase). Either triose compound can then be converted to glucose by reversal of the glycolytic pathway. The glyoxylate cycle is found in many bacteria, including Azotobacter vinelandii and particularly in organisms that grow well in media in which acetate and other Krebs cycle dicarboxylic acid intermediates are the sole carbon growth source. One primary function of the glyoxylate cycle is to replenish the tricarboxylic and dicarboxylic acid intermediates that are normally provided by the Krebs cycle. A pathway whose primary purpose is to replenish such intermediate compounds is called anaplerotic.

5. Electron Transport and Oxidative Phosphorylation

The final stage of respiration occurs through a series of oxidation-reduction electron transfer reactions that yield the energy to drive oxidative phosphorylation; this in turn produces ATP. The enzymes involved in electron transport and oxidative phosphorylation reside on the bacterial inner (cytoplasmic) membrane. This membrane is invaginated to form structures called respiratory vesicles, lamellar vesicles, or mesosomes, which function as the bacterial equivalent of the eukaryotic mitochondrial membrane.

 Respiratory electron transport chains vary greatly among bacteria, and in some organisms are absent. The respiratory electron transport chain of eukaryotic mitochondria oxidizes NADH + H+, NADPH + H+, and succinate (as well as the coacylated fatty acids such as acetyl~SCoA).

6. Respiratory electron transport chains.

In bacteria cytochrome oxidases usually occur as combinations of a1: d: o and a + a3:o. Bacteria also possess mixed-function oxidases such as cytochromes P-450 and P-420 and cytochromes c' and c'c', which also react with carbon monoxide. These diverse types of oxygen-reactive cytochromes undoubtedly have evolutionary significance. Bacteria were present before O2 was formed; when O2 became available as a metabolite, bacteria evolved to use it in different ways; this probably accounts for the diversity in bacterial oxygen-reactive hemoproteins.

Cytochrome oxidases in many pathogenic bacteria are studied by the bacterial oxidase reaction, which subdivides Gram-negative organisms into two major groups, oxidase positive and oxidase negative. This oxidase reaction is assayed for by using N,N,N', N'-tetramethyl-p-phenylenediamine oxidation (to Wurster's blue) or by using indophenol blue synthesis (with dimethyl-p-phenylenediamine and α-naphthol).

7. Mitchell or Proton Extrusion Hypothesis

 A highly complex but attractive theory to explain energy conservation in biologic systems is the chemiosmotic coupling of oxidative and photosynthetic phosphorylations, commonly called the Mitchell hypothesis. This theory attempts to explain the conservation of free energy in this process on the basis of an osmotic potential caused by a proton concentration differential (or proton gradient) across a proton-impermeable membrane. Energy is generated by a proton extrusion reaction during membrane-bound electron transport, which in essence serve as a proton pump; energy conservation and coupling follow. This represents an obligatory “intact” membrane phenomenon. The energy thus conserved (again within the confines of the membrane and is coupled to ATP synthesis. This would occur in all biologic cells, even in the lactic acid bacteria that lack a cytochrome-dependent electron transport chain but still possesses a cytoplasmic membrane. In this hypothesis, the membrane allows for charge separation, thus forming a proton gradient that drives all bio-energization reactions.

8. Bacterial Photosynthesis

 Many prokaryotes (bacteria and cyanobacteria) possess phototrophic modes of metabolism (Table 4-1) . The types of photosynthesis in the two groups of prokaryotes differ mainly in the type of compound that serves as the hydrogen donor in the reduction of CO2 to glucose (Table 4-1). Phototrophic organisms differ from heterotrophic organisms in that they utilize the glucose synthesized intracellularly for biosynthetic purposes (as in starch synthesis) or for energy production, which usually occurs through cellular respiration.

 Unlike phototrophs, heterotrophs require glucose (or some other preformed organic compound) that is directly supplied as a substrate from an exogenous source. Heterotrophs cannot synthesize large concentrations of glucose from CO2by specifically using H2O or (H2S) as a hydrogen source and sunlight as energy. Plant metabolism is a classic example of photolithotrophic metabolism: plants need CO2 and sunlight; H2O must be provided as a hydrogen source and usually NO3– is the nitrogen source for protein synthesis. Organic nitrogen, supplied as fertilizer, is converted to NO3– in all soils by bacteria via the process of ammonification and nitrification.

9. The nitrogen cycle.

 When the specific breakdown of organic nitrogenous compounds occurs, that is, when proteins are degraded to amino acids (proteolysis) and then to inorganic NH3, by heterotrophic bacteria, the process is called ammonification. This is an essential step in the nitrogen cycle. At death, the organic constituents of the tissues and cells decompose biologically to inorganic constituents by a process called mineralization; these inorganic end products can then serve as nutrients for other life forms. The NH3 liberated in turn serves as a utilizable nitrogen source for many other bacteria. The breakdown of feces and urine also occurs by ammonification.

 The other important biologic processes in the nitrogen cycle include nitrification (the conversion of NH3 to NO3by autotrophes in the soil; denitrification (the anaerobic conversion of NO3 to N2 gas) carried out by many heterotrophs); and nitrogen fixation (N2to NH3, and cell protein). The latter is a very specialized prokaryotic process called diazotrophy, carried out by both free-living bacteria (such as Azotobacter, Derxia, Beijeringeia, and Azomona species) and symbionts (such as Rhizobium species) in conjunction with legume plants (such as soybeans, peas, clover, and bluebonnets). All plant life relies heavily on NO3– as a nitrogen source, and most animal life relies on plant life for nutrient.

2.Explain the identification/ staining techniques of fungi.

 a. Potassium Hydroxide Wet Mount P otassium hydroxide (KOH) is used to dissolve proteinaceous material and facilitate detection of fungal elements that are not affected by strong alkali solution. It is a strong alkali used as a clearing agent to observe fungi in a wet mount preparation. The concentration of KOH is usually based on the specimen that is being used. Normally, 10–20% KOH is used; occasionally, 40% is used when the specimen is not cleared by 10–20% of KOH. In this method, the fungal structures, such as hyphae, large yeasts ( Blastomyces ), spherules, and sporangia, are well distinguished. In unstained preparations (KOH without stain), the fungal structures may be enhanced by using a phase contrast microscope (18, 24). The clearing effect throughout the specimen can be accelerated by gently heating the KOH preparation. V isualization of fungi can be further enhanced by the addition of dyes to the preparation. This method is quick, simple, and inexpensive [ 25, 26 ] .

1.Place a large drop of KOH solution with a Pasteur pipette.

2.Transfer small quantity of the culture with a loop or the tip of a scalpel into the KOH drop.

3.Put a clean coverslip over the drop gently so that no air bubble is trapped. 4.Clearing can be hastened by gentle heating of the slide, but it is best avoided.

 5.Observe under 20× and 40× objective of light or phase contrast microscope.

 6.Look for budding yeast cells; branching hyphae; type of branching; and the color, separation, and thickness of hyphae.

**b**. 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 modi ﬁ cations. Giemsa stain is a member of the Romanowsky group of stains, which are de ﬁ ned as being the black precipitate formed from the addition of methanol [ 37 ] . 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 ﬁ x ation as a single step for smears and thin ﬁlms. This stain has widely been used to examinePneumocystis jiroveci, Rhinosporidium seeberi, and Histoplasma capsulatum [38– 40] .

 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.

**c.** 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 s afranin [24, 30, 33–36 ] .

1.Apply two drops of crystal violet on smear for 30s.

 2.Wash with tap water.

3.Add two drops of Gram’s iodine for 30s.

4.Repeat step 2.

5.Add 95% ethanol.

 6.Repeat step 2.

7.Add two drops of safranin.

 8.Repeat step 2.

**D.**

Weigert’s Iron Hematoxylin Staining. This stain can be used with ﬁxatives that include polyvinyl alcohol, sodium acetate, and formalin. The staining method involves application of haemalum, which is a complex formed from aluminium ions and oxidized hematoxylin. This stains nuclei of cells blue. Counterstain eosin Y may also be used to colour other structures in various shades of red, pink, and orange [ 28, 43– 47 ] .

1.Add staining solution on a smear and leave for 1–2 h.

 2.Rinse with tap water.

 3. Add 1% HCl.

 4.Add 70% ethanol.

 5.Repeat step 2.

 6.Counterstain with eosin Y, if necessary.

7.Dehydrate with ethanol.

 8.Clear with xylene and observe under microscope.

9.Yeast cells stain blue–grey to black.

**E.** 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 [ 28, 47, 51, 54, 55 ] .

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 ﬂ uorescence microscope.

15.Fungi show purple color with yellow background (see Note 13).

F. Periodic Acid–Schiff Staining Periodic acid–Schiff (PAS) reactions are effective stains for demonstrating fungal elements of essentially all fungi. Periodic acid attacks some carbohydrates 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 color [ 25, 32 ] . Identiﬁcation of fungal elements can be enhanced if a counterstain such as light green is used. Species of Coccidioides, Cryptococcus, Histoplasma , Candida, Malassezia , and Aspergillus can be stained with this stain [ 65 ] .

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.Observe under microscope.

11.Fungi stain bright pink-magenta or purple against green background when light green is used as a counterstain.

**G.**Toluidine Blue O Staining ;

This stain is primarily used for the detection of Candida albicans, Rhinosporidium seeberi, and Pneumocystis carinii. Background staining is removed by sulfation reagent. Yeast cells get stained differentially and are difﬁ c ult to distinguish from Pneumocystis cells. The stain can be replaced with speciﬁc ﬂuorescent stains. Toluidine blue O gives polychromatic staining for all the fungal structures (such as conidia, germ tubes, haustoria, and hyphae) as well as cells [ 55,64, 70– 73 ] .

 1.Add sulfation reagent for 10 min.

 2.Wash with tap water.

 3.Add toluidine blue O for 3 min.

 4.Add 95% ethyl alcohol, absolute ethyl alcohol, and xylene, each for 10 s for decolorizing.

 5. Place a coverslip on the slide.

 6. Observe with 20× and 40× objectives.

 7. Fungi stain reddish blue to dark purple on light-blue background.