PRACTICAL 9 BIOCHEMICAL TESTS BACTERIAL TESTING

Structure

9.1 INTRODUCTION

Practical 9 deals with the various biochemical tests used to differentiate microorganisms. Identification of an unknown organism isolated from natural environment or any other source to a genus and species level is necessary to determine its characteristics, to exploit it industrially, to determine the cause of the disease or for its categorization into proper group or taxa. This can be accomplished by a combination of microscopic observations like morphology, cell size, shape and arrangement, gram staining and other staining reactions, motility etc. with cultural characteristics, nutritional requirements and biochemical tests. We will learn about the different microbial enzymatic activities and the importance of various biochemical characteristics in identification process in this practical.

Objectives

After undertaking this practical, you will be able to:

- recognize the different microbial enzymatic activities,
- explain the importance of various biochemical characteristics in microbial identification process,
- perform biochemical tests on a given bacterial culture, and
- differentiate bacteria based on their biochemical characteristics.

9.2 IMPORTANCE OF BIOCHEMICAL TESTS

Specific series of biochemical tests have been developed for fast identification of microorganisms in laboratories. These biochemical tests are based on the properties governed by the cell's enzymatic system. Both extracellular (exoenzymes) and intracellular

(endoenzymes) enzymes are synthesized by the cell to carry out various biochemical activities. Exoenzymes are released into the environment where these degrade high molecular weight polymers like proteins, carbohydrates etc. into low molecular weight materials. These low molecular weight nutrients are transported into the cell where endoenzymes (functions inside the cell) metabolize them to provide energy and metabolic products. These metabolic products are useful in characterizing the genera.

As different microorganisms have different enzymatic profile, these biochemical activities can be used to separate even very closely related microorganisms, e.g. members of enteric bacteria, which you may be aware are a large heterogenous group of microbes present in intestinal tract of humans and other animals. Phenotypically, these are characterized as gram negatives, non-sporulating, non-motile/motile rods which on fermenting sugar produces a variety of end products and usually reduces nitrate to nitrite. Several members of the enteric group are pathogens and medically important, e.g., *Salmonella, Shigella* etc. which causes diarrhoeal illnesses. To combat these infections, quick identification of the pathogen is needed, sometimes even to a species or strain level. Though marked morphologic and genetic relatedness exist among many of the enteric bacteria there is a difference in type and proportion of the fermentation products, produced by them on sugar fermentation and in other biochemical properties. Variety of diagnostic tests and differential media can be used to differentiate among different bacteria. Some more commonly used tests are those for the type of formic acid fermentation, lactose and citrate utilization, indole production, urea hydrolysis etc. Identification of the bacteria is based on the analysis of a large number of such diagnostic tests, which are specific to the biochemical characteristics of microorganisms. What are these biochemical characteristics? Let us find out.

9.3 BIOCHEMICAL CHARACTERISTICS

In the last section we have learnt about the importance of biochemical tests. It must be clear now that biochemical tests are used to –

(1) identify the bacteria, and

(2) to provide insight into species's niche in the ecosystem.

Next, what are the biochemical characteristics specific to the microbial identification process? The characteristics considered for this purpose include:

1. Fermentation of Dextrose, sucrose, mannitol and lactose.
2. Gelatin liquefaction
3. Starch hydrolysis
4. Lipid hydrolysis
5. Casein hydrolysis
6. Litmus milk reaction
7. Nitrate reduction
8. Indol production
9. H_2S Production
10. Methyl red test
11. Voges – Proskauer test
12. Citrate Utilization
13. Catalase activity
14. Urease activity
15. Oxidase activity, etc.

These biochemical tests can be used for differentiation among enteric bacteria and also bacteria other than enteric ones.

Many of these biochemical tests are discussed in the practical. The genus of bacteria can be identified by referring to the Table 9.1. It may be noted, however, that biochemical results may not be identical as given in table because the results may vary depending on the strains of each species used and the length of time the organism has been maintained in the stock culture. The following sub-section(s) discusses the various tests.

9.3.1 Tests for the Presence of Exoenzymatic Activity

Microorganisms require various micro- and macro-nutrients for energy production and growth. These are obtained from the environment. However, many of these are present in polymeric form, like starch, lipid, casein etc. which because of their high molecular weight and size are not able to enter in the cell. Microorganisms secrete exoenzymes to hydrolyze these macromolecules into respective building blocks to transport into the cell. Different microorganisms show different enzymatic activities.

Starch is a polymer of glucose linked together by glycosidic bonds. Microorganisms produce hydrolytic enzyme *amylase* which degrade starch into short polysaccharide units – dextrins and ultimately into disaccharide maltose. Maltose is finally hydrolyzed to soluble glucose molecules by enzyme *maltase*. Glucose is transported into the cell and is involved in cellular processes. For observing hydrolytic activity of these exoenzymes, starch agar is used. Detection of starch hydrolysis after growth is made by observing for the presence or absence of starch using *iodine*. Iodine imparts blue black colour to the medium, if starch is present. Hydrolysis of starch is *demonstrated by a clear zone surrounding the growth of the organism*. This is a *positive reaction*.

Lipids are also high molecular weight compounds. Enzyme *lipases* (*esterases*) cleaves the ester bond to form glycerol and fatty acids. Once assimilated inside the cell, these are further metabolized to produce cellular energy ATP and other cellular protoplasmic macromolecules. Presence of exoenzymatic activity for lipid hydrolysis can be demonstrated by using *tributyrin agar*, an opaque medium formed due to emulsion formation by tributyrin in agar medium. Lipolysis is shown by *a clear zone of hydrolysis surrounding the growth*. In the absence of lipolytic enzymes, the medium retains its opacity. It represents a *negative reaction*.

Casein is a major milk protein composed of various amino acids linked through peptide bonds. Extra-cellular enzyme *protease* degrades proteins to produce peptones, polypeptides, dipeptides and finally to amino acids (proteolysis). Amino acids are transported to the cell and used for synthesis of structural and functional cellular proteins. Release of proteases by microorganisms can be detected by use of milk agar (nutrient agar supplemented with milk). The medium is opaque. Following inoculation and incubation, loss of opacity *around the growing organism indicate positive result, i.e., synthesis of proteases*.

Gelatin is an incomplete protein as it lacks amino acid tryptophan. It is a major component of connective tissue and tendons in humans and other animals. Many microorganisms produce an enzyme *gelatinase*, which result in its hydrolysis. Gelatin deep tubes (Nutrient broth supplemented with 12% gelatin) are used to demonstrate the hydrolytic activity of gelatinase.

Gelatin is solid at temperature less than 25^{o}C and liquid at temperature above 25^{o}C. Gelatinase degrades gelatin to produce amino acids and liquefies gelatin. Once this degradation occurs, gel characteristic is not restored even at 4^{o}C. Following inoculation and incubation for 48 hours, *if the culture remains liquid*, it indicates fast gelatin *hydrolysis*. If it gets solidified, further incubate for 5 days. If it remain liquefy on refrigeration for 30 minutes, it indicates slow gelatin hydrolysis.

Table 9.1: Cultural and Biochemical Characteristics of Unknown Organisms

Organism	Gram Stain	Agar Slant Cultural Characteristics	Litmus Milk Reaction	Fermentation			H_2S production	NO_3 Reduction	Indole Production	MR Reaction	VP Reaction	Citrate Use	Urease Activity	Catalase Activity	Oxidase Activity	Gelatin Liquefaction	Starch Hydrolysis	Lipid Hydrolysis
				Lactose	Dextrose	Sucrose												
Escherichia coli	Rod -	White, moist, glistening growth	Acid, curd ±, gas ± and reduction ±	AG	AG	A±	–	+	+	+	–	–	–	+	–	–	–	–
Enterobacter aerogenes	Rod -	Abundant, thick, white, glistening growth	Acid	AG	AG	AG±	–	+	–	–	+	+	–	+	–	–	–	–
Klebsiella pneumoniae	Rod -	Slimy, white, somewhat translucent, raised growth	Acid, gas, curd ±	AG	AG	AG	–	+	–	±	±	+	+	+	–	–	–	–
Shigella dysenteriae	Rod -	Thin, even, grayish growth	Alkaline	-	A	A±	–	+	±	+	–	–	-	+	–	–	–	–
Salmonella typhimurium	Rod -	Thin, even, grayish growth	Alkaline	-	AG±	A±	+	+	–	+	–	+	–	+	–	–	–	–

A → Acid; AG → Acid and gas; ± Variable reaction; Rod –Coccobacillus; - negative; + positive

Organism	Gram Stain	Agar Slant Cultural Characteristics	Litmus Milk Reaction	Fermentation			H_2 production	NO_3 Reduction	Indole Production	MR Reaction	VP Reaction	Citrate Use	Urease Activity	Catalase Activity	Oxidase Activity	Gelatin Liquefaction	Starch Hydrolysis	Lipid Hydrolysis
				Lactose	Dextrose	Sucrose												
Proteus vulgaris	Rod -	Thin, blue-gray, spreading growth	Alkaline	–	AG	AG ±	+	+	+	+	–	+	+	+	–	+	–	–
Pseudomonas aeruginosa	Rod -	Abundant, thin, white growth, with medium turning green	Rapid peptonization	–	–	–	–	+	–	–	–	+	–	+	+	+ Rapid	–	+
Alcaligenes faecalis	Rod* -	Thin, white, spreading, viscous growth	Alkaline	–	–	–	–	–	–	–	–	±	–	+	+	–	–	–
Staphylococcus aureus	Cocci +	Abundant, opaque, golden growth	Acid, reduction ±	A	A	A	–	+	–	+	±	–	–	+	–	+	–	–
Streptococcus lactis	Cocci +	Thin, even growth	Acid, rapid reduction with curd	A	A	A	–	–	–	+	–	–	–	–	–	–	–	–
Micrococcus luteus	Cocci +	Soft, smooth, yellow growth	Alkaline	–	–	–	–	±	–	–	–	–	+	+	–	+ Slow	–	–
Corynebacterium xerosis	Rod +	Grayish, granular, limited growth	Alkaline	–	A±	A±	–	+	–	–	–	–	–	+	–	–	–	–
Bacillus cereus	Rod +	Abundant, opaque, white waxy growth	Peptonization	–	A	A	–	+	–	–	±	–	–	+	–	+ Rapid	+	±

9.3.2 Carbohydrate Utilization Pattern Test

Carbohydrates are composed of carbon, hydrogen and oxygen. These organic molecules are catabolized by large number of microorganisms to obtain energy. However, organisms use carbohydrates differently depending upon their enzyme complement. The pattern of carbohydrate fermentation is a characteristic of species and can be used to differentiate among different genera of enteriobacteriaceae and from other gram-negative intestinal bacilli.

Triple sugar iron (TSI) agar is used to observe carbohydrate utilization pattern. The medium contains 1% concentration of each of lactose and sucrose and 0.1% concentration of glucose. Acid base indicator phenol red is also incorporated in the medium to detect the production of acid during carbohydrate fermentation. Presence of acid results in the change of medium colour from *orange red* to *yellow* and indicate the carbohydrate fermentation. TSI medium also contains sodium thiosulphate as substrate and ferrous sulphate for detection of H_2S production by certain organisms through reduction of sulphur containing amino acids (cysteine, cystine and methionine) or inorganic sulphur compounds (thiosulphates, sulphates, sulphites). H_2S is a colourless gas commonly called "rotten egg gas" which when react with metal salts (e.g. ferrous sulphate) form visible insoluble black ferrous sulfide precipitates.

$$3Na_2S_2O_3 \longrightarrow 2Na_2SO_3 + 2H_2S\uparrow$$

(Sodium thiosulphate) (Sodium sulphite) (Hydrogen Sulphite gas)

$$\downarrow +FeSO_4$$

FeS
Ferrous sulphide
(Black precipitate)

TSI medium is inoculated by means of a stab and streak procedure. From the base of the slant, culture is inoculated into the butt by using straight, sterile needle. The slanted surface is streaked. The results are observed within 18-24 hours after incubation otherwise peptone will start degrading after depletion of carbohydrates and produces alkaline reaction. Slants and butts are seen separately for acid production along with gas production (breaks in agar butt). Following observations can be made:

(1) Alkaline slant (red) and acid butt (yellow) with or without gas production – This indicates fermentation of glucose only. As glucose concentration is low, only small amount of acid is produced which gets oxidized rapidly on a slant surface. The peptones in the medium are also used in the production of alkali so slant is red. The acid reaction is maintained in the butt due to slow growth of organism and low oxygen tension so it become yellow.

(2) Acid butt (yellow) and acid slant (yellow) with or without gas production (refer to margin illustration tube 1,2,3 and 5) – Glucose along with lactose and/or sucrose, is degraded by microorganisms. Large amount of acid is produced due to higher concentration of substrates which is maintained both in the slant and butt due to continued fermentation.

(3) Alkaline slant (red) and alkaline butt (red) or no change (orange-red) butt (refer to margin illustration tube no. 4) – Instead of carbohydrates, peptone in the medium produces ammonia on fermentation, resulting in alkaline pH. Fermentation occurs either aerobically (alkaline reaction is produced only in slant) or anaerobically (alkaline reaction is present on the slant and the butt).

Along with carbohydrate fermentation, tubes should also be examined for the presence or absence of black colouration along with the line of stab inoculation. Growth of some common bacteria on TSI medium is given in the Table 9.2. We shall use this information for identification of bacteria.

Table 9.2: Growth of Bacteria on TSI medium

S.No.	Colour of		Presence/Absence of black precipitate	Organisms
	Slant	Butt		
1.	Yellow (Acid production)	Yellow (Acid production)	Absent (No H_2S production)	*Escherichia Klebsiella Enterobacter*
2.	Yellow	Yellow	Present	*Citrobacter Ariozona Some proteus* sp.
3.	Red	Yellow	Absent	*Shigella* Some *proteus* sp.
4.	Red	Yellow	Present	Most *Salmonella Arizona Citrobacter*
5.	Red/ No Change	Red/ No Change		*Alcaligenes Pseudomonas Acinetobacter*

9.3.3 IMViC Test

IMViC test is a combination of four tests:

(1) Indole production

(2) Methyl Red test

(3) Voges Proskauer, and

(4) Citrate Utilization test

The name IMViC includes the first letter of each test and the lower case 'i' is included to facilitate pronunciation. The test is used to differentiate among different genera of the family Enterobacteriaceae particularly *E. coli* and *Enterobacter*. Enterobacteriaceae family include pathogens like *Salmonella, Shigella*; occasional pathogens like *Proteus*, *Klebsiella* and normal saprophytes, e.g., *E. coli* and *Enterobacter*. Identification of these organisms is essential to control the intestinal infections by preventing contamination of food and water by these organisms. Checking for each and every organism in food is, however, a tedious job. So detection of indicator organisms in food and water may be used as indication of faecal contamination and possibility of the presence of pathogens in food and water. Coliforms are used as indicator organisms. We shall study more on this aspect later in Practical 10. *E. coli* is a normal inhabitant of intestine while *Enterobacter* is present on plant surfaces. For better indication of faecal contamination, the two, i.e., *E. coli* and *Enterobacter* can be separated by performing IMViC test. The test can also be used to differentiate other members of Enterobacteriaceae and also other intestinal organisms on the basis of their enzymatic reactions in presence of specific substrates.

Let us learn about the four tests which constitute the IMViC test.

(1) *Indole Production test:* Many microorganisms produce an enzyme *tryptophanase* which oxidizes amino acid tryptophan to produce pyruvic acid, indole and ammonia. We can analyze the production of tryptophanase by using SIM agar containing tryptophan. The presence of indole is then detected by adding Kovac's reagent which contains p-dimethyl amino benzaldehyde, butanol and hydrochloric acid. It produces a cherry-red reagent layer with indole (positive test), as illustrated in the Figure 9.1.

Figure 9.1: Indole test

The role of acidified butyl alcohol is to extract the indole into the reagent layer which then react with dimethyl amino benzaldehyde.

Appearance of *cherry red colour reagent layer is a positive test. E. coli is an indole positive bacterium* (as seen in tube 2 in margin illustration).

(2) *Methyl red test:* As you have read in previous section, all enteric bacteria utilize glucose to obtain energy. Depending upon the end products formed, all enteric bacteria can be categorized into two major groups. One group *produces large amount of acid*, i.e., formic, acetic, lactic and succinic acid from glucose, which is stabilized and maintained in the culture while other group produces *low amount of acid* which soon get converted to neutral product, acetoin. The end products formed depend upon the enzymatic pathways present in the organism. These two groups can be differentiated by MR-VP test, the two tests performed simultaneously on same MR-VP medium. Generally the results are opposite, i.e., if organism is giving MR test positive, VP test is negative and vice-versa. *MR-VP test is of value in separating E. coli and Enterobacter aerogenes* in particular. Both are coliforms and are identical except in certain physiological characteristics.

In MR test, methyl red is used as pH indicator. If the methyl red remains red on addition into the culture medium, it indicates the production of large amount of acid (pH 4.0) and MR test is positive for the organism, as shown in Figure 9.2. However, if methyl red turns yellow, it indicates that high pH (pH 6.0) is produced because of conversion of organic acids to non-acidic end components, i.e., ethanol and acetoin (acetyl methyl carbinol). It indicates negative MR (refer to margin illustration).

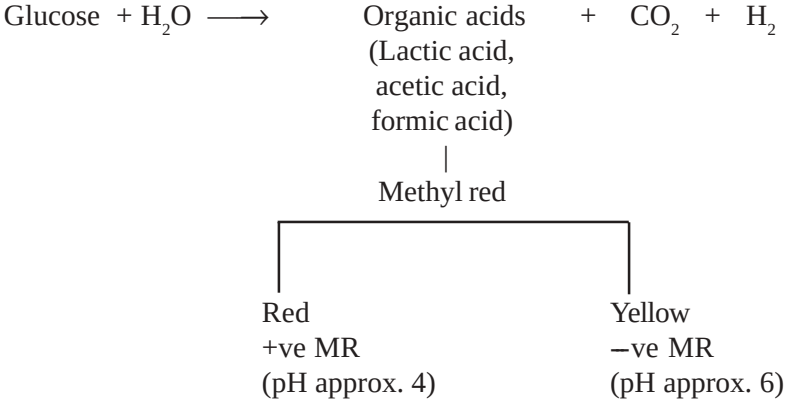

Figure 9.2: Methyl Red test

(3) *Voges Proskauer (VP) test:* As it has been discussed in previous exercise, second group of enteric bacteria produces acid during early incubation which rapidly converted to non-acidic components, i.e., 2, 3 butanediol and acetoin (acetylmethylcarbinol). These components can be detected by using Barritt's reagent, which is a mixture of alcoholic alpha-naphthol and 40% potassium hydroxide solution. Acetylmethylcarbinol is oxidized to a diacetyl compound in presence of the alpha-naphthol catalyst and a guanidine group present in the peptone of MR-VP medium. This results in formation of a *pink complex imparting rose red colouration* to a medium after 15 minutes. Development of rose red colour indicates positive VP reaction while absence of it is a negative result. Figure 9.3 highlights the VP test.

Figure 9.3: Voges-Proskauer test

(4) *Citrate Utilization test:* Many microorganisms have the ability to use citrate as the sole source of carbon and energy. This ability depends on the presence of *citrate permease* and *citrase* in the organism. *Citrate permease* facilitates the transport of citrate in the cell while *citrase* acts upon citrate to produce oxaloacetic acid and acetate as indicated in the reaction given in Figure 9.4.

Citrate utilization by microorganisms can be detected by using Simmons citrate medium which contains sodium citrate as the sole carbon source and bromothymol blue as pH indicator. When citrate in the medium is used by microorganisms, resulting CO_2 reacts with sodium and water to form sodium carbonate. This changes the pH of the medium to alkaline and its colour from green to deep prussian blue. Bromothymol blue is green when acidic (pH 6.8 and below) and blue when alkaline (pH 7.6 and higher). Figure 9.4 shows the reaction.

Figure 9.4: Citrate utilization test

Following incubation, presence of growth in slants and blue colour of medium indicates positive test, i.e., citrate is used as carbon source. Absence of growth and green colour shows absence of citrate utilization, i.e., negative test.

9.3.4 Nitrate Reduction Test

Many microorganisms reduce nitrates and sulphates under anaerobic conditions to supply oxygen which in turn utilized as a final hydrogen acceptor during energy formation. Nitrates reduces to form nitrites which may be degraded further enzymatically to ammonia or molecular nitrogen as shown herewith:

$$\underset{\text{Nitrate}}{NO_3^-} + \underset{\text{Hydrogen}}{2H^+} + \underset{\text{Electrons}}{2e^-} \xrightarrow{\text{Nitrate reductase}} \underset{\text{Nitrite}}{NO_2^-} + H_2O$$

$$\downarrow$$

$$NH_3 \text{ (ammonia)}$$

OR

$$2NO_3^- + 12H^+ + 10e^- \longrightarrow \underset{\text{molecular nitrogen}}{N_2} + 6H_2O \downarrow$$

For determining the nitrate reduction ability of microorganisms, nitrate broth is used for microbial growth. The medium is basically a nutrient broth supplemented with 0.1% potassium nitrate and 0.1% agar. Potassium nitrate is used as the nitrate substrate. Agar is used to make the medium semisolid to impede the diffusion of oxygen into the medium and creating anaerobic conditions to favour nitrate reduction.

Reduction of nitrate in the medium is assessed by using sulfanilic acid solution (Solution A) and alphanaphthylamine solution (solution B). Development of cherry red colour indicates positive test, i.e., reduction of nitrate to nitrite (see margin illustration). Figure 9.5 illustrates the reaction.

Figure 9.5: Nitrate reduction test

If there is no change in the colour, it suggests that either there is no reduction of nitrate or nitrate is converted to ammonia or molecular nitrogen. As mentioned earlier, many microorganisms have the ability to rapidly convert nitrite to molecular nitrogen or ammonia. This can be judged by adding a pinch of Zinc powder to the basically colourless culture already containing solutions A and B. Zinc reduces nitrate to nitrite and this results in the development of cherry red colour if no reduction of nitrate has occurred earlier. This verifies that nitrate is not reduced by the organism. If no colour change occurs even after adding the zinc, it means nitrate is reduced beyond nitrites to ammonia and molecular nitrogen. This is a positive reaction, i.e., the organism is able to reduce nitrate.

9.3.5 Urease Activity Test

The test is used for identifications of bacteria *Proteus vulgaris* which is a fast producer of enzyme urease. Urease attacks on urea, a waste product in urine and produces ammonia.

Urease production by microorganism can be detected by growing it on urea broth or urea agar medium containing phenol red as an indicator (pH 6.8). If enzyme urease is possessed by the microorganism, it would result in the production of ammonia which raises the pH of the medium. At higher pH, phenol red changes its colour from yellow to red or deep pink. The development of a red colour is a positive test for urease production. *Proteus* gives positive test for urease.

9.3.6 Catalase Activity Test

As you know, microorganisms employ different metabolic pathways to obtain energy in presence or absence of oxygen. Oxygen requirement for microbes vary with the type. Depending upon the effect of oxygen, microbes can be divided into different groups. These include:

(1) *Obligate aerobes* – Obligate aerobes use molecular oxygen as terminal electron acceptor for electron transport chain in aerobic respiration. In addition, aerobic eukaryotes employ oxygen for synthesis of sterols and unsaturated fatty acids. These organisms require atmospheric level of oxygen for their survival and growth, example, *Micrococcus luteus*. All multi-cellular organisms are obligate aerobes.

(2) *Facultative anaerobes* – These organisms can grow both in presence or absence of oxygen, i.e., use oxygen when it is present but are able to continue growth by using fermentation or anaerobic respiration when oxygen is not available. Growth in presence of oxygen is better. Also energy production is more during aerobic growth as they use aerobic respiration in presence of oxygen e.g., *E. coli*, yeasts.

(3) *Obligate anaerobes* – These organisms do not need molecular oxygen for their growth rather these are harmed by it and die in its presence. These organisms use fermentation or anaerobic respiration for energy production, e.g., *Clostridium, Bacteroides, Fusobacterium, Methanococcus, Neocallimastix, Methanobacterium formicicum.*

(4) *Aerotolerant anaerobes* – These organisms do not use oxygen for their growth but tolerate it if it is present in the surrounding. These grow equally well whether O_2 is present or not e.g., *Lactobacillus, Enterococcus faecalis, Streptococcus pyogenes*. These use fermentation or anaerobic respiration instead of aerobic respiration.

(5) Microaerophiles – These organisms do need molecular oxygen for survival and growth but the level required is less than atmospheric level (i.e. 2-10%) e.g., *Campylobacter, Spirillum volutans*. These are damaged by normal atmospheric level of oxygen (21%).

All 5 types of microbial groups discussed are found among prokaryotes. *Fungi* are normally *aerobic*, but *yeasts* are *facultative anaerobes*. *Algae* are almost always *obligate*

aerobes. Effect of oxygen on bacterial growth pattern can be readily determined by growth in tubes containing solid growth medium. Obligate aerobes grow near surface where high concentrations of oxygen have diffused into the medium, as illustrated in Figure 9.6.

Facultative anaerobes grow throughout the tube though growth is best where high oxygen concentration is present.

Obligate anaerobes grow at the bottom where there is no oxygen.

Aerotolerants grow evenly in the tube because of no effect of oxygen. Microaerophiles growth can be seen slightly below the surface where a low concentration of oxygen has diffused into the medium.

Figure 9.6: Catalase activity test

Oxygen is harmful also as it accept electrons and is readily reduced because of its two outer unpaired orbital electrons. This results in formation of many toxic forms of oxygen which may inactivate many proteins and enzymes. These toxic products are:

(a) Singlet oxygen - Here molecular oxygen is boosted into a high energy state. It is highly reactive and may kill microorganisms. These are powerful oxidizing agents which rapidly destroy cellular constituents.

(b) Superoxide free radicals (O_2^-) – These are very toxic to cellular components. These are very unstable and can take electrons from a neighbouring molecule, which in turn becomes a radical and steals an electron and so on.

(c) Hydrogen peroxide (H_2O_2) – It is very toxic and contains the peroxide anion O_2^{2-}.

(d) Hydroxyl radical (OH^-) – It is another form of oxygen which is most reactive and formed in cellular cytoplasm by ionizing radiations.

Biochemically

$O_2 + e^- \rightarrow O_2^-$ (Superoxide radical)

$O_2^- + e^- + 2H^+ \rightarrow H_2O_2$ (Hydrogen peroxide)

$H_2O_2 + e^- + H^+ \rightarrow H_2O_2 + OH^-$ (Hydroxyl radical)

Many microorganisms possess enzymes like superoxide dismutase (SOD) and catalase to neutralize these toxic compounds. Aerobes, facultative anaerobes and aerotolerant anaerobes produce SOD, as shown in Figure 9.6, which converts superoxide free radical into molecular oxygen and hydrogen peroxide, i.e.

$$2\,O_2^- + 2H^+ \xrightarrow{\textit{Superoxide dismutase}} O_2 + H_2O_2$$

Catalase enzyme is produced by aerobes and facultative anaerobes (refer to Figure 9.6) to neutralize hydrogen peroxide to form water and oxygen

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

Another enzyme peroxidase is also used by many microorganisms to breakdown hydrogen peroxide to water. Here no release of oxygen occurs.

$$H_2O_2 + 2H^+ \longrightarrow 2H_2O$$

Aerotolerant microorganisms may lack catalase but almost always have superoxide dismutase or equivalent system which partially neutralized toxic oxygen forms and lead to O_2 tolerance by them. Aerotolerant *Lactobacillus plantarum* uses manganous ions instead of superoxide dismutase to destroy superoxide radical. Obligate anaerobes lack these enzymes or have these in very low concentration so cannot tolerate oxygen.

Production of catalase by microorganisms can be determined by adding a few drops of substrate, i.e., H_2O_2 on the bacterial culture in trypticase soy agar slants. *Release of oxygen gas (indicated by bubbles) is the positive test and confirms the production of catalase*. Absence of bubble formation indicates negative catalase test.

With this, we end our study about catalase activity test. Finally we shall get to know about the Cytochrome oxidase activity.

9.3.7 Cytochrome Oxidase Activity

Microorganisms vary not only in their energy source but also in the kinds of electron acceptors used by them. In fermentation substrate is oxidized without the participation of exogenous electron acceptors. Rather intermediates produced during catabolic pathway like pyruvate may act as electron accepter. In other energy yielding metabolic processes exogenous electron acceptors are used. These metabolic processes are called respirations where either oxygen (aerobic respiration) or inorganic and sometimes organic compounds like NO_3^-, SO_4^{2-}, CO_2, Fe^{3+}, fumarate (anaerobic respiration) are used as final electron acceptors. Most respiration involves the activity of an electron transport chain.

Electron transport chain is composed of a series of electron carriers, which together transfer the electrons from donor, i.e., $FADH_2$ and NADH to acceptor, i.e. O_2 and releases energy in form of ATP. Electron transport chain carriers reside within the inner mitochondrial membrane whereas in bacteria these are present in the bacterial plasma membrane. Oxidase enzyme plays an important role in the operation of electron transport system during aerobic respiration. Cytochrome oxidase catalyzes the oxidation of reduced cytochrome by molecular oxygen resulting in formation of water or hydrogen peroxide. Aerobic microorganisms and some facultative anaerobe and microaerophiles shows oxidase activity. Oxidase positive bacteria belong to the genera *Pseudomonas, Vibrio, Aeromonas, Alcaligenes, Achromobacter, Flavobacterium, Neisseria, Moraxella* etc. All enteries are, however, oxidase negative.

Oxidase test can be used to differentiate *Neisseria* and *Moraxella* (positive) from *Acinetobacter* (negative) and enteric bacteria (all negative) from *Pseudomonas*. It also helps in identification of *Aeromonas* (positive). To perform for the oxidase test, light pink coloured reagent p-aminodimethylaniline oxalate is added to the culture grown on trypticase soy agar. The test reagent acts as a substrate for cytochrome and donate electrons to become oxidized which is a blackish compound in presence of oxidase and free oxygen. So development of pink, then maroon and finally black colouration on surface colony is indicative of positive test, i.e, oxidase activity is present in the given microorganism. No colour change is a negative oxidase test.

So we have learnt about the different biochemical tests, which we can carry out in the laboratory to differentiate between microorganisms. Now try to answer the questions given in Review Questions section here and check your understanding of the topic so far.

9.4 REVIEW QUESTIONS

1. Name the characteristics used to identify the microorganism.

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2. What is the significance of performing biochemical tests?

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3. What is the difference between exoenzyme and endoenzyme?

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   .....

4. List various biochemical characteristics, which can be used for microbial identification purpose.

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   .....

Now move on to the exercises. There are 7 exercises in this practical. Carry out the exercises and learn about the biochemical test.

EXERCISE 1

PERFORMING BIOCHEMICAL TESTS ON A GIVEN BACTERIAL CULTURE

Date : .....

Aim : To demonstrate the exoenzymatic activities of different microorganisms by starch, casein, lipid and gelatin hydrolysis.

Requirements

Culture : *E. coli, Bacillus* sp., *Pseudomonas* sp., *Staphylococcus aureus.*

Medium : Starch agar, tributyrin agar, milk agar, gelatin deep tubes.

Reagent : Gram's Iodine solution

Equipments : Bunsen burner, inoculating loop, glass marker, incubator, refrigerator.

Theory/Principle:

(Refer to sub-section 9.3.1 above. Write the principle for the biochemical test in the space provided.)

Procedure:

Conduct the exercise following the steps enumerated herewith:

(1) Divide the plate of each medium into 4 sections at the bottom and label them with the name of the organism to be inoculated.

(2) Inoculate each section of a plate by different organism by making a single streak inoculation. For gelatin deep agar tubes, stab inoculation is done under aseptic conditions.

(3) Incubate all the plates for 24-48 hours at 37 ^{o}C and examine for the hydrolysis. For starch agar plates, flood the plate with Gram's iodine and observe for the presence or absence of blue-black colour after 30 seconds to one minute. Examine tributyrine agar plate and milk agar plate for the presence or absence of a clear area around the growth of each organism. Incubate gelatin deep tubes for 48 hours and observe for liquefaction. If liquefied, observe after 30 minutes of refrigeration. For slow gelatin hydrolysis, re-incubate the tubes for additional 5 days and then observe after 30 minutes of refrigeration.

Precautions:

1. Follow aseptic conditions.
2. Label the plates or tubes with the organism's name correctly.

Observations and Results:

Record your observations in the format presented herewith:

Observations			Results
Organism	**Characteristics**	**Appearance of Medium**	
E. coli	Starch hydrolysis		
	Lipid hydrolysis		
	Casein hydrolysis		
	Gelatin hydrolysis		
Pseudomonas	Starch hydrolysis		
	Lipid hydrolysis		
	Casein hydrolysis		
	Gelatin hydrolysis		
Bacillus sp.	Starch hydrolysis		
	Lipid hydrolysis		
	Casein hydrolysis		
	Gelatin hydrolysis		
Staphylococcus sp.	Starch hydrolysis		
	Lipid hydrolysis		
	Casein hydrolysis		
	Gelatin hydrolysis		

Inference/Conclusion:

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Now answer the following review questions. This will help you consolidate your knowledge about exoenzymatic activity of different microorganisms.

Review Questions

1. Why gelatin is an incomplete protein?

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2. What are exoenzymes?

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3. Why exoenzymes are released by microorganisms?

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4. Why iodine is used to detect starch hydrolysis?

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5. How is the presence of proteases and lipases in microbial culture detected?

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6. Why refrigeration is needed to detect gelatin hydrolysis?

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Now submit the exercise for evaluation.

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Counsellor Signature

EXERCISE 2

EVALUATION OF CARBOHYDRATE FERMENTATION ABILITY OF MICROORGANISMS

Aim : To evaluate carbohydrate fermentation ability of microorganisms. Date : .....

Requirements

Medium : TSI (Triple Sugar Iron) agar medium

Culture : *Proteus* species, *E. coli*, *Bacillus* species, *Pseudomonas* species, *Klebsiella* species.

Equipments : Inoculating loop, inoculating needle, incubator, bunsen burner, test tubes, test tube rack.

Theory/Principle:

Write the principle in the space provided. Refer to sub-section 9.3.2 earlier for information.

Procedure:

(1) Label the TSI medium tubes and inoculate with different organisms by stab and streak method.

(2) Incubate for 18-24 hours at 37^{o}C.

(3) Record the observations.

Precautions:

1. Incubate the TSI culture for 18-24 hours only.
2. Maintain aseptic conditions.

Observations and Results:

Record your observations in the format provided.

Organism	Observations			Result
	Colour of			
	Slant	Butt	H_2S Production	
E. coli				
Bacillus				
Pseudomonas				
Proteus				
Klebsiella				

Inference/Conclusion:

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Now answer the following review questions:

Review Questions

1. Name the carbohydrates and their concentration in TSI medium.

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2. Why the concentration of glucose is low in TSI medium as compared to lactose and sucrose?

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3. Name the substrate and indicator used for production and detection of H_2S in TSI medium.

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4. Why the results should be recorded within 18-24 hours of incubatim test in this test?

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5. Explain the purpose of phenol red in the medium.

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Submit your exercise for evaluation.

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Counsellor Signature

EXERCISE 3

DIFFERENTIATION BETWEEN BACTERIA USING IMViC TEST

Date : .....

Aim : To perform IMViC test (including the four tests which are a part of IMViC test) with the given bacterial culture and to differentiate between *E.coli* and *Enterobacter*.

Theory/Principle:

Look up sub-section 9.3.3. the theory behind the IMViC test is elaborated therein. Based on your understanding of the test, write the theory and the principle behind the IMViC test here very briefly in the space provided.

IMViC Test Basic Concept:

IMViC test: The four tests (Write the name and the theory of four test)

A	
B	
C	
D	

Now that you are familiar with the four tests which constitute the IMViC test, move on to conducting these four tests. Start with the Indole Production test.

A: Indole Production Test

Aim : To determine the ability of microorganisms to degrade tryptophan.

Requirements

Culture : *E.coli, Pseudomonas, Klebsiella, Proteus, Bacillus, Enterobacter*

Media and Reagents : SIM agar tubes, Kovac's reagent.

Equipments and Glassware : Bunsen Burner, inoculating needle, glass marker, incubator.

Composition of Medium and Reagent

SIM Agar (pH 7.3)

Peptone	30 gm
Beef Extract	3.0 gm
Ferrous Ammonium Sulphate	0.2 gm
Sodium thiosulphate	0.025 gm
Agar	3 gm

Dissolve above said ingredients in 1000 ml distilled water and autoclaved. Dispense in sterilized test tubes and make deep agar tubes.

Kovac's reagent

p-diemthylamino benzaldehyde	5.0 gm
Amyl alcohol	75.00 m
Hydrochloric acid (concentrated)	25.0 ml

Dissolve p–dimethylamino benzaldehyde in the amyl alcohol. Add the hydrochloric acid.

Theory/Principle:

Look up sub-section 9.3.3. the theory behind the Indole test is elaborated therein. Based on your understanding of the test, write the theory and the principle behind the test here very briefly in the space provided.

Procedure:

Carry out the exercise following the steps enumerated herewith:

1. Label SIM agar deep tubes with the name of the organisms.
2. Inoculate the agar tubes each with one of the given culture aseptically and incubate at 37^{O}C for 24 hours to 48 hours.
3. Add about 1 ml of Kovac's reagent to each of the cultured tube and agitate gently.
4. Examine the colour of the reagent layer and record the observations.

Precautions:

1. After adding Kovac's reagent, gentle shaking of tubes is necessary for 10-15 minutes to develop a colour.
2. Results should be read within 48 hours otherwise indole would be degraded.

Observations and Results:

Record your observations in the format given herewith.

Observations

Organism	Colour of the reagent layer	Result
E. coli		
Enterobacter		
Pseudomonas		
Klebsiella		
Proteus		
Bacillus		

Inference/Conclusion:

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B. Methyl Red Test

Aim : To perform methyl red test to know the ability of microorganisms to degrade glucose.

Requirements

Culture : 24 hours culture of *E. coli, Pseudomonas, Klebsiella, Bacillus, Proteus, Enterobacter.*

Media and Reagent : MR-VP broth, methyl red indicator

Equipments and Glassware : Inoculating loop, Bunsen burner, test tubes, permanent marker, incubator.

Composition of medium and reagent

- *MR-VP broth (pH 6.9)*

Peptone	7.0 gm
Dextrose	5.0 gm
Potassium Phosphate	5.0 gm
Distilled water	1000 ml

Methyl Red Solution

Methyl red	0.1 gm
Ethyl alcohol	300 ml
Distilled Water	200 ml

Dissolve the methyl red in 95% ethyl alcohol. Dilute to 500 ml with distilled water.

Theory/Principle:

(Write the principle in the space provided)

Procedure:

Now carry out the exercise following the steps enumerated herewith:

(1) Prepare MR-VP broth and pour 5 ml of it in seven tubes. Sterilize these tubes by autoclaving at 15 lbs for 15-20 minutes.

(2) Inoculate MR-VP broth tubes each with separate culture under aseptic conditions by means of inoculating loop. Label each tube with the name of the organism. Keep one uninoculated MR-VP broth tube as a control.

(3) Incubate tubes for 24 hours at 37 ^{0}C.

(4) Divide each culture into 2 parts. One part is kept aside for VP assay while in second part of each culture add 4-5 drops of methyl red as indicator.

(5) Observe the change in colour of the media and record the observations.

Precautions:

1. Inoculation should be done aseptically.
2. MR-VP tests should be performed on the same culture.

Observations and Results:

Record your observations in the format provided herewith:

Observations		**Result**
Colour change on MR addition		
Organism	**Colour change**	**Positive/Negative**
E. coli		
Klebsiella		
Bacillus		
Pseudomonas		
Proteus		
Enterobacter		

Inference/Conclusion:

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C. Voges Proskauer test

Aim : To perform the Voges Proskauer test with the given bacterial culture(s).

Requirements

Culture : E. coli, *Klebsiella, Enterobacter aerogenes, Pseudomonas, Bacillus, Proteus.*

Media and Reagent : MR-VP broth, Barritt's reagent

Equipments and Glassware : Inoculating loop, bunsen burner, incubator

Composition of Medium and Reagent:

Barritt's reagent:

Solution A

Alpha – naphthol : 5.0 gm

Ethanol absolute : 95.0 ml

Dissolve alpha-naphthol in the ethanol with constant stirring.

Solution B

Potassium hydroxide : 40.0 gm

Creatine : 0.3 gm

Distilled water : 100.0 ml.

Dissolve the potassium hydroxide in 75.0 ml of distilled water. The solution will become warm. Allow to cool and then dissolve creatine with continuous stirring. Add remaining water.

Theory/Principle:

Look up the Voges-Proskauer Test in the sub-section 9.3.3. Write down the theory here in the space provided.

Procedure:

Carry out the test following the steps enumerated herewith:

(1) Add 10 drops of Barritt's reagent A solution to MR-VP aliquot kept during MR test. Shake well. Then add 10 drops of Barritt's B solution and shake.

(2) Reshake the culture every 4-5 minutes and record observations.

(3) Note the colour change in the culture.

Precautions:

1. Shake well after adding Barritt's reagent A and B.
2. Observe the results after 15 minutes.

Observations and Results:

Record your observations in the format given herewith.

Observations		Result
Organisms	**Colour change**	**VP Positive / Negative**
E. coli		
Klebsiella		
Proteus		
Pseudomonas		
Bacillus		
Enterobacter		

Inference/Conclusion:

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D. Citrate Utilization

Aim : To detect the ability of citrate utilization in given bacterial culture(s).

Requirements

Culture : E. coli, B*acillus, Klebsiella, Pseudomonas, Proteus, Enterobacter*.

Medium : Simmons citrate agar slants.

Equipments and Glassware : Inoculating needle, bunsen burner, incubators, test tubes, test tube stand.

Composition of Simmons citrate agar (pH 6.9) :

Ammonium dihydrogen phosphate	:	1.0 gm
Dipotassium phosphate	:	1.0 gm
Sodium chloride	:	5.0 gm
Sodium citrate	:	2.0 gm
Magncoesium sulphate	:	0.2 gm
Bromothymol blue	:	0.8 gm
Agar	:	15.0 gm
Distilled water	:	1000 ml

Theory / Principle:

Look at the Citrate utilization test given in the sub-section 9.3.3 earlier. Write down the theory here in the space provided.

Procedure:

Now carry out the exercise following the steps given herewith.

1) Prepare Simmon's citrate medium as per the given composition. Dissolve all the ingredients except phosphate in distilled water which are to be dissolved separately in 100 ml of water. Then set the pH to 6.9 after making volume to 1 litre and dispense the medium to culture tubes. Sterilize it by autoclaving at 15 psi for 15 minutes. Make the slants after autoclaving.

2) Inoculate Simmon's citrate agar slants with given cultures by means of stab and streak inoculation. keep one tube as a control.

3) Incubate all the tubes at 37°C for 24-48 hours.

(4) Observe the slants for growth and colour change. Record your observations.

Precautions:

(1) Adopt aseptic conditions during inoculation.

(2) Use light inoculation to minimize carry over of nutrients from the medium to grow the inoculation on Simmons citrate agar slants.

Observations and Results:

Now record your observations in the format given herewith.

Observations			Result
Organism	**Presence/Absence of Growth**	**Colour of medium**	**Citrate utilization Positive/Negative**
E.coli *Bacillus* *Pseudomonas* *Proteus* *Klebsiella* *Enterobacter*			

Inference/Conclusion:

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Now that we have carried out the four tests, which constitute the IMViC test, let us test our understanding about this test by answering the review questions given herewith. This in a way will sum up our understanding about the topic.

Review Questions

1. What is the significance of IMViC test?

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2. Name the tests included in IMViC tests.

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3. Which of the four tests in IMViC series are positive for *E. coli*?

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4. Write down the equation for indole production and development of colour.

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5. What are the components of Kovac's reagent? Write their role in colour development.

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6. Explain the chemical mechanism for detecting acetoin in the culture media.

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7. Write down the equation of oxidation of glucose to acetoin.

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8. Explain the chemical mechanism of citrate utilization.

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9. What is the pH indicator used in Simmons citrate?

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10. Why the colour of Simmons citrate changes on citrate utilization?

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11. Write the IMViC test results for the following microorganisms:

Bacillus .....

Enterobacter .....

E. coli .....

Proteus .....

Pseudomonas .....

12. Why is methyl red used as pH indicator?

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    .....

13. What is the significance of MR-VP test?

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    .....

14. Write down the equation of glucose oxidation.

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    .....

15. Both *E. coli* and *Enterobacter aerogenes* ferment carbohydrate to produce acid and gas. Explain why *E. coli* is MR positive and *Enterobacter aerogenes* MR negative.

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Now submit the exercise for evaluation.

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Counsellor Signature

EXERCISE 4

DETERMINE THE ABILITY OF BACTERIA TO REDUCE NITRATES

Date : .....

Aim : To determine the ability of given bacterial culture(s) to reduce nitrates.

Requirement

Culture : *E. coli, Proteus, Pseudomonas, Klebsiella, Bacillus, Enterobacter*

Medium and Reagent : Nitrate broth

Solution A — Sulfanilic acid

Solution B — Alpha-naphthylamine

Zinc powder

Equipments and Glassware : Inoculating needle, bunsen burner, incubator, test tubes and test tube rack, permanent marker.

Composition of Medium and Reagents

Nitrate Broth (pH 7.2)

Peptone	:	5.0 gm
Beef extract	:	3.0 gm
Potassium nitrate	:	5.0 gm
Distilled water	:	1000 ml

Solution A – Sullfanilic acid

Sulfanidic acid	:	8.0 gm
Acetic acid 5N: 1parts	:	1 part
Glacial Acetic acid to 2.5 parts	:	1000 ml

Distilled water

Solution B — Alpha—naphthylamine

Alpha-naphthylamine	:	5.0 gm
Acetic Acid 5N	:	1000 ml

Theory/Principle:

Look up the theory of the nitrate reduction test in sub-section 9.3.4 earlier. Accordingly summarize the theory here in the space provided.

Procedure:

Now carry out the exercise following the steps given herewith.

1) Prepare nitrate broth as per the composition. Sterilize it by autoclaving at 15 psi for 15 minutes. Dispense the medium into sterile test tubes.

2) Label the tubes with the organism's name. Inoculate each organism into appropriately labelled tube aseptically. One tube was kept as a control.

3) Incubate the tubes at 37°C for 24-48 hours.

4) Following incubation, determine the nitrate reduction by adding 5 drops each of solution A and solution B.

5) Note the colour change. If no red colour developed, add zinc powder to the tube and observe for the colour change.

6) Record the observations.

Precautions:

1. Follow aseptic conditions during inoculation.

2. Add equal amount of solution A and B.

Observations and Results:

Record your observations in the format given herewith.

Organisms	Observations		Result
	Colour change with solution A & B	Colour change after adding Zn	Nitrate Reduction Positive/Negative
E. coli			
Bacillus			
Pseudomonas			
Proteus			
Enterobacter			
Klebsiella			

Inference/Conclusion:

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Answer the review questions given herewith.

Review Questions

1. Why 0.1% agar is used in nitrate broth?

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2. What is the function and concentration of potassium nitrate in the medium?

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3. Explain the biochemical reaction of nitrate reduction.

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4. Why Zinc was used during detection?

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Now submit for evaluation.

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Counsellor Signature

EXERCISE 5

DETERMINE THE ABILITY OF MICROORGANISMS TO PRODUCE UREASE

Date : .....

Aim : To determine the ability of microorganisms such as *Proteus vulgaris* to produce urease.

Requirements

Culture : *E. coli, Proteus, Pseudomonas, Staphylococcus, Bacillus, Klebsiella*.

Media : Urea agar/Urea broth medium.

Equipments and Glassware : Inoculating loop, bunsen burner, test tube rack, permanent glass marker.

Composition of Urea agar medium

Peptone	1.0 gm
Sodium chloride	5.0 gm
Potassium mono hydrogen (or dihydrogen) phosphate	2.0 gm
Agar	20.0 gm
Distilled Water	1000 ml

Dissolve the ingredients by heating, adjust the pH to 6.8 and autoclave at 15 psi for 15 minutes. Cool to 50°C and add glucose 1.0 gm and phenol red (0.2% solution) 6.0 ml and steam for 1 hour. Cool to 50°C. Urea 20% aqueous solution (100 ml) is sterilized by filtration and add to medium. Mix well and dispense in tubes and plates.

Theory/Principle:

Look at the sub-section 9.3.5. Read the theory of urease production. Now write the theory here in the space provided briefly.

Procedure:

Now carry out the exercise following the steps given herewith.

1. Label and inoculate urea medium tubes with the given culture. One tube is kept as a control.
2. Incubate the tubes at 37°C for 24-48 hours.
3. Observe the tubes for colour change.

Precautions:

1. As urea is unstable and breaks down at 15 psi, it should not be sterilized by autoclaving.

Observations and Results:

Write your observations in the format given herewith.

Organisms	Observations		Result
	Colour of the medium	Urea hydrolysis + / —	
E. coli			
Proteus			
Pseudomonas			
Bacillus			
Klebsiella			

Inference/Conclusion:

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Check your understanding on this topic by answering the review questions given herewith.

Review Questions

1. Which indicator is used for urease test?

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2. Write down the mechanism of urease activity

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3. Which colour is produced in the medium when urease is produced by the microorganisms?

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   .....

4. Explain how the urease test is useful for identifying *Proteus* species.

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Now submit the exercise for evaluation.

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Counsellor Signature

EXERCISE
6

CATALASE ACTIVITY TEST

Aim : To assess the ability of given microorganisms to produce the enzyme catalase.

Date : .....

Requirements

Culture : 24 hours cultures of *E. coli, Bacillus, Staphylococcus, Pseudomonas, Proteus* and *Klebsiella, Enterobacter*

Medium and Reagents : Trypticase soy agar slants, 3% hydrogen peroxide (refrigerate when not in use)

Equipments and Glassware : Inoculating loop, bunsen burner, test tubes, test tube rack, permanent glass marker.

Composition of Trypticase soy agar (pH 7.3) :

Trypticase	:	15.0 gm
Phytane	:	5.0 gm
Sodium chloride	:	5.0 gm
Agar	:	15.0 gm
Distilled water	:	1000.0 ml

Theory/Principle:

(Write the principle in the space provided herewith).

Procedure:

Carry out the test based on the steps enumerated herewith.

1) Prepare trypticase soy agar medium and dispense it in the culture tubes. Sterilize the medium tubes by autoclaving at 15 psi for 15 minutes.
2) Inoculate each appropriately labeled tube with the test organisms. One tube was kept as a control.
3) Incubate the cultures at 37^{o}C for 24-48 hours.
4) Add 3-4 drops of hydrogen peroxide over the culture and examine each culture for the presence or absence of bubbling or foaming.
5) Record your observations and discuss the results.

Precautions:

1. Add hydrogen peroxide (H_2O_2) carefully.
2. Record observations immediately.

Observations and Results:

Record your observations in the format provided herewith:

Organism	Observations	Result
	Bubbling (+ ve/—ve)	Catalase Production Positive / Negative
E. coli		
Proteus		
Staphylococcus		
Bacillus		
Pseudomonas		
Enterobacter		
Klebsiella		

Inference/Conclusion:

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Answer the following review questions.

Review Questions

1. How oxygen is harmful to organisms?

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2. Would catalase be classified as an endoenzyme or exoenzyme?

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3. Describe the microorganisms groups on the basis of oxygen requirement.

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4. Name the enzymes produced by microorganisms to detoxify oxygen products.

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5. Give examples of

   Microaerophiles .....

   Aerotolerants .....

   Obligate anaerobes .....

   Aerobes .....

   Facultative anaerobes .....

6. Why strict anaerobes are not able to tolerate presence of oxygen?

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Submit the exercise for evaluation

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Counsellor Signature

EXERCISE 7

CYTOCHROME OXIDASE ACTIVITY

Date : .....

Aim : To distinguish given bacterial culture on the basis of cytochrome oxidase activity.

Requirements

Culture : Cultures of *E. coli, Bacillus, Staphylococcus, Klebsiella, Pseudomonas, Proteus, Enterobacter*

Media and Reagent : Trypticase soy agar plates (composition given in previous exercises) and p-aminodimethylaniline oxalate

Composition of p– aminodimethylaniline oxalate reagent

p-aminodimethylaniline oxalate - 0.5 gm

Distilled water - 50 ml

Gently warm to fully dissolve.

Equipments and Glassware : Inoculating loop, Bunsen burner, permanent glass marker, test tubes.

Theory/Principle:

Write the principle in the space provided. (Look up sub-section 9.3.7 earlier for details).

Procedure:

Now carry out the test following the steps enumerated herewith.

1. Prepare trypticase soy agar culture plates. For the same, dissolve all the ingredients in 1000 ml distilled water. Adjust the pH and add agar. Heat it to dissolve the agar. Sterilize the medium by autoclaving at 15 psi for 15 minutes and after cooling, pour it in sterile culture plates.

2. Label each plate appropriately and inoculate with the given cultures by single line streak method under aseptic conditions.

3. Incubate the plates at 37°C for 24-48 hours.
4. Add 2-3 drops of p-aminodimethylaniline oxalate to the surface of bacterial growth and observe for the colour change within 10-30 seconds.
5. Examine the plates and record your observations.

Precautions:

1. Incubate the plates in inverted position.
2. Maintain aseptic conditions.

Observations and Results:

Record your observations in the format given herewith:

Organism	**Observations**	**Result**
	Colour change +ve / —ve	**Positive/Negative**
E. coli		
Bacillus		
Klebsiella		
Proteus		
Pseudomonas		
Enterobacter		
Staphylococcus		

Inference/Conclusion:

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Now submit the exercise for evaluation.

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Counsellor Signature