Detection of microbial enzyme: Amylase, lipase, gelatinase, catalase, urease, nitrate reductase, protease and coagulase
To detect amylase enzyme production

• **Introduction:**
  - Amylase is hydrolytic enzyme produced extracellularly and classified in hydrolyases (class III).
  - Starch is composed of two units- Amylose, an unbranched glucose polymer (200-300 unit) and amylpectin, a large branched polymer. In amylose, \( \alpha 1, 4 \) glycosidic bond and \( \alpha 1, 6 \) glycosidic bond are present. Both amylose and amylpectin are rapidly hydrolyzed by certain bacteria using their \( \alpha \)-amylase to yield dextrin, maltose and glucose.
  - The microbes **produces the amylase** are *Bacillus subtilis, Escherichia coli, Proteus vulgaris*. 
Muscles store sugar as glycogen.

The hydrolysis reaction is the reverse of the dehydration reaction
Principle:

- This test is based on starch hydrolyzing ability of microorganism.
- The starch hydrolysis is detected by iodine reaction.
- Starch reacts with iodine and produces blue color.
- If the starch is depleted from medium, the ‘Zone of clearance’ will be observed around the bluish background.
Requirement:

- Suspension of test organism
- Sterile starch agar plates
- Iodine solution
Procedure:

• The given test organism is spot inoculated on starch agar plate.
• Incubate the plate at suitable temperature.
• After incubation, iodine solution is added to medium
Interpretation:

• The blue color indicates the presence of starch while colorless zone around the colony indicates production of amylase and utilization of starch.
To detect catalase enzyme production

- **Introduction:**
- Some bacteria contain flavoprotein that reduces oxygen, resulting in production of super oxides of oxygen. This is extremely toxic compound because they are powerful oxidizing agent and destroy the cellular constituent very rapidly. Bacteria must be able to protect itself against such oxygen product or it will be killed.
- Many bacteria (e.g. *Staphlococcus aureus*, *Strptococcus spp.*.) produce enzyme catalase that protect against toxic oxygen product. Obligate aerobes and facultative anaerobes usually contain catalase or peroxidase which catalyses the destruction of hydrogen peroxide as follows.
- \[ 2H_2O_2 + \text{Catalase} \rightarrow 2H_2O + O_2 \uparrow \]
- Water Free Oxygen
Principle:

- Catalase production activity can be detected by adding the substrate Hydrogen peroxide. If catalase was produced by the bacteria, the above chemical reaction will liberate free oxygen gas. Bubbles of oxygen represent a positive catalase reaction, and the absence of bubble formation is a negative catalase reaction.
Requirements:

• Given bacterial suspension.
• Sterile nutrient agar slant.
• 3%H₂O₂ solution
Procedure:

1) Aseptically streak a loopful of suspension on sterile nutrient agar slant.
2) Incubate the slant at 37°C for 24 hr.
3) One ml of 3% Hydrogen peroxide was added on nutrient agar slant after incubation and observed for evolution of oxygen bubbles.
Result

Adding hydrogen peroxide directly to a pure slant culture
Detection of enzyme activity
Coagulase test

• Introduction
• Coagulase is an enzyme which adds to the invasiveness (the capacity to invade and multiply in healthy tissues) of the pathogenic bacteria. It is thrombin like enzyme which catalyzes the formation of fibrin around the organisms and thus preventing phagocytosis. Phagocytosis. Coagulase is produced by certain strains spp. of Staphylococcus, Yersenia and others. Organisms produce two types of coagulase which is found on the external surface of the wall. Presence of coagulase can be detected by the following tests.
Tube test: Principle:

- Clotting requires interaction of free coagulase with coagulase reacting factors (CRF) in plasma, which is probably a derivative of prothrombin; a coagulase-CRF complex converts fibrinogen to fibrin. Although some fibrinopeptides are released as with thrombin, the process differs from normal clotting in that the multiple accessory factors including Ca$^{++}$, are not required, and the clot is more friable and does not retract. Citrate, oxalates and EDTA are usually added to act as anticoagulants and prevent false positive results.
Requirements

• Test culture
• Sterile Nutrient Broth tube
• Citrated or oxalated human or rabbit plasma (about 2ml)
Procedure

• Inoculate a loopful of test culture into the nutrient broth. Incubate at 37ºC for 18-24 hours.

• Add about 0.5ml of 18-24 hours broth culture to 2ml of plasma in a small test tube.

• Incubate the tube at 37ºC.

• Examine for the clot formation at an hourly intervals up to 4hrs.
Coagulase tubes

Staphylococcus epidermidis
Staphylococcus aureus
Staphylococcus aureus
To detect gelatinase enzyme production

• **Introduction:**
• Gelatin is the protein that derived and from collagen, which is one of the principal components of the connective tissue and is itself insoluble in water but can be rendered soluble by boiling. Gelatin has an unusually high content of proline and glycine.
• Gelatin is hydrolyzed by Gelatinase produced by a variety of bacterial species. In tube test method upon hydrolysis the gelling properties are lost and medium remains liquid even at low refrigerator temperature.
• The ability of the organism to hydrolyse or liquefy gelatin is characteristics of genera e.g. *Enterobacteriaceae*, *Pseudomonaceae*, *Proteus vulgaris* or *Serratia marcescen* Gelatinase of *Clostridium perfrigens* is called collagenase. and anaerobes e.g. *Clostridium* species.
Principle:

• In Frazier’s method gelatin hydrolysis can be demonstrated in nutrient agar with gelatin by adding acidic solution of HgCl2, positive organisms show a zone of clearance.
Requirements:

- Suspension of test organism
- Sterile 1% gelatin agar plate
- Acidic solution of $\text{HgCl}_2$
Procedure:

• The given test organism is spot inoculated on gelatin agar plate.
• Incubate the plate at suitable temperature.
• After incubation, Acidic solution of Hgcl2 is added to medium.
Interpretation:

• The zone of clearance around the colony on gelatin agar indicates production of gelatinase and utilization of gelatin.
Detection of enzyme activity Lipid Hydrolysis Test (Lipase)

- **Introduction**
- Fatty acid esters of the alcohol glycerol are called acylglycerol or glycerides or neutral lipids / fats. Triacylglycerols are the most common of all lipids and are mainly found in plant and animal cells. Triglycerols that are stored at room temperature are often referred to as `fats’ and those which are liquid as `oils’.
- Few organisms can use lipids as alternative source of energy (main being carbohydrates like glucose & sucrose). The breakdown of lipids or fats begin with the cleavage of triglycerides by addition of water to form glycerol and fatty acids by means of enzymes called lipases.
- The glycerol liberated is oxidized to glycerol-3-phosphate and dihydroxy acetone phosphate which then enters EMP pathway. Fatty acids are oxidized by the successive removal of 2-carbon fragments in the form of acetyl-Co A, a process known as β-oxidation. The acetyl-Co A formed then enters the TCA cycle.
- When lipase producing bacteria contaminate food products, the lipoltic bacteria hydrolyze the lipids, causing spoilage termed rancidity.
Triglyceride Hydrolysis by Lipase

**Structure of a Triglyceride**

- Glycerol
- Fatty acid
- Fatty acid

**Chemical Reaction**

Fatty acid

Lipase

Glycerol + Fatty Acids

Glycerol

Fatty Acids
Principle

• Tributyric acid (tributyrene) is commonly used to check the lipase activity by organisms. Teibutyrene is hydrolysed as follows:
  • Tributyrene $+$ $3H_2O$ $\rightarrow$ lipases
  Glycerol $+$ butyric acid
  • The butyric acid so formed reacts with calcium carbonate to form solubilized calcium butyrate:
  • $CH_3COOH$ $+$ $C_aCO_3$ $\rightarrow$ $CH_3COOC_a$ $+$ $CO_2$
  • By adding a $P_H$ indicator to the culture medium, it is also possible to detect
Requirements

• Test culture
• Sterile Tributyrene agar plate
Procedure

• Inoculate the test culture on the plate as spot or line, and incubate at 37 °C for 24-72 hrs.
• Observe for a clear zone due to solubilization of a calcium carbonate is a positive test indicating the production of the enzyme lipase
Results:

- Tributyrin agar plates contain the triglyceride tributyrin and are initially opaque;

- Lipase-positive organisms will exhibit a clear zone around their growth—tributyrin has been hydrolyzed
Detection of enzyme activity Casein Hydrolysis Test (Protease)

• Introduction
  Many organotrophs (heterotrophs) can utilize exogenous proteins as a source of carbon and nitrogen. Since the protein molecules are too large to enter the cell, microorganisms secrete extracellular enzymes called proteases, which hydrolyze peptide bonds to form peptides. Peptides are short chains made up of few amino acids, which can be further broken down to the individual amino acids by peptidases. The sequence of protein breakdown can be summarized as follows.
  • Protein → Proteases → Peptones → Peptides → Amino acids

• Peptides and amino acids being smaller in size can enter the cell where the amino acids undergo oxidation to form the compounds that may enter the TCA cycle.
Principle

• Casein is sparingly soluble in water and hence medium formed due to it’s incorporation is opaque (milkey white). Caseolytic organism produce casease which hydrolyze casein to soluble form paracasein. Hence the clear zone is observed in the medium surrounding the growth.
Requirements

- Test culture
- Nutrient casein agar plate / skim milk agar plate
Procedure

• Inoculate the test culture on the plate as spot or line, and incubate at 37 °C for 24 hrs.
• Observe for a clear zone of casein solubilizing surrounding the growth of organism.
Results:

- Bacteria that secrete the proteolytic exoenzyme casease hydrolyze milk protein thus creating a zone of clearing around the bacterial growth
To detect urease enzyme production

- **Introduction:**
- Urea is waste nitrogenous material and excreted out by animals. Some bacteria degrade the urea into ammonia and CO2. The urase production can be demonstrated by the following reaction.

\[
\text{NH}_2\text{CO}\text{NH}_2 \rightarrow 2\text{NH}_3 + \text{CO}_2
\]
• **Principle:**
  Enzyme urease breaks urea into ammonia and carbon dioxide, due to which pH of medium becomes alkaline and at alkaline pH phenol red indicator turns to pink color while at acidic pH it remains yellow.

• **Example:** Urease producers - *Proteus Vulgaris*
  Urease negative :- *E.Coli, Serratia marcescens*
Requirements:

• Given bacterial suspension.
• Sterile urea agar slant.
Procedure:

• Inoculate the sterile urea agar slant with bacterial culture.
• Incubate the slant at 370C for 48hours.
• After incubation observe the slant and compare with the control.
Interpretation:

- The phenol red indicator will turn to pink due to alkaline nature of the medium because of ammonia production. Otherwise, the indicator will remain yellow at acidic range of pH.
Detection of enzyme activity Nitrate Reductase Test

• **Introduction**
  
  Microorganisms reduce nitrate under two different growth conditions with different purpose.
  
  Certain bacteria carry out nitrate respiration under anaerobic / microaerophilic conditions and use nitrate as the terminal electron acceptor, thereby reducing it.
  
  In contrast to this, assimilatory nitrate reduction generally occurs under aerobic conditions in absence of sources of nitrogen.
  
  Thus in assimilatory nitrate reduction, nitrate is incorporated into organic material and do not participate in energy production. This process is wide spread among bacteria, fungi and algae which can use nitrate as the sole source of nitrogen.
• In the first step nitrate is reduced to nitrite by nitrate reductase:
  \[ \text{NO}_3^- + \text{NADPH} + \text{H}^+ \xrightarrow{\text{nitrreductase}} \text{NO}_2^- + \text{NADP}^+ + \text{H}_2\text{O} \]

• Nitrite is next reduced to ammonia with a series of two electrons addition catalysed by nitrite reductase. Hydroxylamine may be an intermediate.
  \[ \text{NO}_2^- (\text{NH}_2\text{OH}) \xrightarrow{2\text{e}^-, 2\text{H}^+, \text{H}_2\text{O}} \text{NH}_3 \]

• Ammonia is then assimilated by the reaction:
  \[ \alpha\text{-ketoglutarate} + \text{NH}_3 \xrightarrow{\text{glutamate dehydrogen glutamate}} \]

• Test for nitration reduction is performed using nitrate broth.
Principle

• Organism possessing nitrate reductase when grown in a medium containing nitrate as the sole source of nitrogen will reduce nitrate to nitrite. The formation of nitrite can be detected by adding sulphanilic acid, which forms a diazonium salt, which in turn reacts with α-naphthylamine thereby leading to formation of soluble red azo dye (p-sulphobenzene – azo –α – napthymaline).
Requirements

• 1) Peptone nitrate broth (PNB)
• 2) Test culture
• 3) Zinc dust
• 4) \( \alpha \)-napthylamine reagent (reagent A)
• 5) Sulphanilic acid reagent (reagent B).
Procedure

• Inoculate PNB with a loopful of culture and incubate the medium at 37°C.
• Add 0.5 ml of the reagent A & B each to the test medium in this order.
• Observe the development of red colour within 30 seconds after adding test reagent.
• If no colour develops, add a pinch of zinc dust, mix them well and observe the development of red colour.
Nitrate reduction test

Nitrate negative

Nitrate positive