Biochemical tests for identification of microorganisms

To aid in the more definitive identification of bacteria, microbiologists have developed a series of biochemical tests that can be used to differentiate even closely related organisms. There are various tests for identification of metabolic products of 148 different bacterial species. Following biochemical tests were used for identification of isolated bacteria.

Indole test:

Principle:

The amino acid tryptophan is found in nearly all proteins. Bacteria that contain the enzyme tryptophanase can hydrolyze tryptophan to its metabolic products such as indole, pyruvic acid, and ammonia. The bacteria use the pyruvic acid and ammonia for their metabolism. Indole is not used and accumulates in the medium. Some bacteria can produce indole from amino acid tryptophan using the enzyme typtophanase. Production of indole is detected using Ehrlich reagent or Kovacs reagent. The formation of a deep red colour in the reagent layer after gentle agitation indicated positive indole test.

Procedure:

Bacterium to be tested was inoculated in peptone water, containing amino acid tryptophan and incubated overnight at 37°C. After incubation few drops of Kovacs reagent were added. Kovacs reagent consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol and conc. HCl. Formation of a red or pink colour indicated the positive reaction.

Methyl red (MR) test:

Principle:

This test is carried out to detect the ability of an organism to produce and maintain stable acid end products from glucose fermentation. Some bacteria produce large amounts of acids from glucose fermentation because of which they overcome the buffering action of the system. Methyl Red is a pH indicator, which remains red in colour.

Procedure:

The bacterium to be tested was inoculated into glucose phosphate broth containing glucose and phosphate buffer, and incubated at 37°C for 48 hours. Over the 48 hrs the mixed-acid producing organisms produce sufficient acid to overcome the phosphate buffer and remain acidic. The pH of the medium was tested by addition of 5 drops of MR reagent. Development of red colour was taken as positive. MR negative organisms produced yellow colour.

VogesProskaeur (VP) test:

Principle:

VP test is used to identify bacteria that ferment glucose, leading to 2, 3- butanediol accumulation in the medium. The addition of 40% KOH and a 5% solution of alpha-naphthol in absolute ethanol (Barritt‟s reagent) detects the presence of acetoin, a precursor in the synthesis of 2, 3-butanediol. In the presence of the reagents and acetoin, a cherry-red colour develops. Development of a red colour in the culture medium 15 minutes following the addition of Barritt‟s reagent represents a positive VP test; absence of a red colour is a negative VP test.

Procedure:

Bacterium to be tested was inoculated into glucose phosphate broth and incubated for 48 hours. 0.6 ml of alpha-naphthol was added to the test broth and shaked. 0.2 ml of 40% KOH was added to the broth with shaking. The tube was allowed to stand for 15 minutes. Appearance of red colour was taken as a positive test. The tubes showing negative result were held for one hour, since maximum colour development occurs within one hour after addition of reagents.

Citrate utilization test:

Principle:

This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Ammonium dihydrogen phosphate and sodium citrate serve as the source of nitrogen and carbon, respectively. Microorganisms also use inorganic ammonium salts as their sole nitrogen source. Utilization of citrate involves the enzyme citrase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO2. Production of Na2CO3 as well as NH3 from utilization of sodium citrate and ammonium salt respectively results in alkaline pH. This results in the change of medium‟s colour from green to blue.

Procedure:

Bacterial colonies were picked up by a nichrome loop and inoculated into slope of Simmon‟s citrate agar and incubated overnight at 37°C. If the organism has the ability to utilize citrate, the medium changes its colour from green to blue (Pommerville 2010).

Starch hydrolysis:

Principle:

The starch molecule consists of two constituents: amylose, an unbranched glucose polymer (200 to 300 units) and amylopectin, a large branched polymer. Both amylopectin and amylose are rapidly hydrolyzed by certain bacteria, using their αamylases, to yield dextrins, maltose, and glucose. Gram‟s iodine can be used to indicate the presence of starch. When it comes into

contact with starch, it forms a blue to brown complex. Hydrolyzed starch does not produce a colour change. If a clear area appears after adding Gram‟s iodine to a medium containing starch and bacterial growth, α-amylase has been produced by the bacteria. If there is no clearing, starch has not been hydrolyzed.

Procedure:

Starch agar plates were inoculated with the bacterium by making a single streak line on the half of the plate and incubated the plate for 24 to 48 hrs at 35°C. After 48 hrs, iodine was added to see whether the starch remained in the agar or had been hydrolyzed by the α-amylase. Iodine reacts with starch to produce a dark brown or blue/black colour. When starch had been hydrolyzed there was clear zone around the bacterial growth because the starch was no longer in the agar to react with the iodine. When starch had not been hydrolyzed, the agar remained as a dark brown or blue/black colour.

Urea hydrolysis test:

Principle:

The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. Urease activity (urease test) is detected by growing bacteria in a medium containing urea and using a pH indicator such as phenol red. When urea is hydrolyzed, ammonia accumulates in the medium and makes it alkaline. This increase in pH causes the indicator to change from orange-red to deep pink or purplish red (cerise) and is a positive test for urea hydrolysis. Failure to develop a deep pink colour is an indication of negative test. Christensen‟s urea agar is used to determine urease activity.

Procedure:

Slant of urea agar medium was prepared and inoculated with isolated bacteria on the entire surface of the slant. The tubes were inoculated at 37°C. The slant was observed for a colour change at 6 hrs, 24 hrs and every day up to 6 days. Urease production was indicated by a bright pink (fuchsia) colour on the slant. Any degree of pink colour development was considered as a positive reaction. Prolonged incubation was avoided as it might result in a false-positive test due to hydrolysis of proteins in the medium. To eliminate protein hydrolysis as the cause of a positive test, a control medium lacking urea was used.

Nitrate reduction test:

Principle:

This test determines the production of an enzyme called nitrate reductase, resulting in the reduction of nitrate (NO3); the test is also called the nitrate reduction test. Nitrate is reduced to nitrite (NO2) by this enzyme. The nitrite ions are detected by the addition of sulfanilic acid and

N, N-dimethyl-1-naphthylamine to the culture. Any nitrite in the medium reacts with these reagents to produce a pink or red colour.

There are various ways that a bacterium can utilize nitrate as the final electron acceptor in anaerobic respiration. The first product of reduction within the Durham tube due to denitrification is N2 gas. This is examined first before any reagents are added. If there is no nitrogen gas, there are still a couple of possible interpretations: nitrate reduction to nitrite (NO2), reduction to ammonia, or no reduction of nitrate at all.

A red colour is produced in the medium only when nitrite is present in the medium. There may be two explanations for the lack of nitrite: the nitrate may not have been reduced; the bacterium cannot use nitrate (- test) or the nitrate may have been reduced to nitrite which has then been completely reduced to ammonia. To differentiate between the above two possibilities, powdered zinc is added. If the bacterium has not used the nitrate, it is still present in the tube. Zn reduces the nitrate forming nitrite, which then reacts with the two reagents already added to the tube. A pink-red colour development is taken as confirmation of a negative nitrate reduction. The last possibility, production of ammonia, is indicated when no pink colour forms.

Procedure:

Bacterium to be tested was inoculated in the tubes containing nitrate broth and the Durham tubes were incubated at 37°C for 24 to 48 hrs. After incubation, tubes were first examined for N2 gas and added 6-8 drops of sulfanilic acid followed by the addition of drops of napthylamine. A reaction occurred within a minute or less or added a bit of powdered zinc. The reduction of unused nitrate by zinc takes a couple of minutes.

H2S Production:

Principle:

TSI agar (triple sugar iron agar) is a differential media that can detect fermentation and hydrogen sulfide production. It is a rich medium containing a pH indicator, four protein sources or extracts, three sugars (testing for fermentation), iron and sulfur compounds (testing for the production of hydrogen sulfide gas). Aerobic growth takes place on the slant and the butt favours the anaerobic growth. If the sulfur compound is reduced, hydrogen sulfide will form and interact with the iron compound to form a black precipitate, which especially is visible in the butt. If there is no H2S formation, no change in the colour of the medium occurs and it remains orange.

Procedure:

Tubes was poured with TSI agar such that each tube contained both a slant (on the top) and a butt (on the bottom) and inoculated with bacteria. The tubes were then observed to examine the development of black colour.

Cytochrome oxidase test:

Principle:

The final stage of bacterial respiration involves a series of membraneembedded components, collectively known as the electron transport chain. The final step in the chain may involve the use of the enzyme cytochrome oxidase, which catalyzes the oxidation of cytochrome c while reducing oxygen to form water. The oxidase test often uses the reagent, tetra-methyl-p-phenylenediaminedihydrochloride, as an artificial electron donor for cytochrome c. When the reagent is oxidized by cytochrome c, it changes from colourless to a dark blue or purple compound, indophenol blue.

Microorganisms are oxidase positive when the colour changes to blue within 15 to 30 seconds. Microorganisms are delayed oxidase positive when the colour changes to purple within 2 to 3 minutes. Microorganisms are oxidase negative if the colour does not change at all.

Procedure:

The isolated bacteria was grown in nutrient broth for 24 hrs at 37°C.After 24 hrs 0.2 ml of 1% α-naphthol followed by 0.3 ml of 1% p-aminodimethylaniline oxalate was added and observed for colour change..

Catalase test:

Principle:

In order to survive, organisms must rely on defence mechanisms that allow them to repair or escape the oxidative damage of hydrogen peroxide (H_2O_2). Some bacteria produce the enzyme catalase which facilitates cellular detoxification. Catalase neutralizes the bactericidal effects of hydrogen peroxide and therefore, the catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Its concentration in bacteria is correlated with pathogenicity. Catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen, ($2H_2O_2$ + Catalase → $2H_2O + O_2$). This reaction is evident by the rapid formation of bubbles.

Procedure:

A small amount of culture from isolated bacteria colony was placed on the microscopic slide. Using a dropper or Pasteur pipette, 1 drop of 3% H_2O_2 was placed onto the organism on the microscope slide. Immediately the petri dish was covered with a lid and observed for immediate bubble formation (O_2 + water = bubbles). Positive reactions were evident by immediate effervescence (bubble formation).

Gelatine liquefaction / gelatine hydrolysis test:

Principle:

Gelatin is a protein derived from the connective tissues of vertebrates (collagen). It is produced when collagen is boiled in water. Gelatin hydrolysis detects the presence of gelatinases. Gelatinases are proteases secreted extracellularly by some bacteria which hydrolyze or digest gelatin. This process takes place in two sequential reactions: In the first reaction, gelatinases degrade gelatin to polypeptides. Then in the second reaction the polypeptides are further converted into amino acids. The bacterial cells can then take up these amino acids and use them in their metabolic processes.

Procedure:

Gelatin hydrolysis was detected using a nutrient gelatin medium. This medium contained peptic digest of animal tissue (peptone), beef extract, and gelatin. Gelatin served as both solidifying agent and substrate for gelatinase activity. Bacterium to be tested was stab-inoculated in the tubes containing nutrient gelatin with a gelatinasepositive bacterium, the secreted gelatinases hydrolyzed the gelatin resulting in the liquefaction of the medium. Since gelatin was digested and unable to form gel, the medium remained liquid when placed inside a refrigerator or in an ice bath. A nutrient gelatin medium inoculated with a gelatinase-negative bacterium remains as solid after the cold treatment. The medium was inoculated with both aerobic and anaerobic bacteria and incubated at appropriate condition.

DNAase agar test:

Principle:

The purpose of this experiment is to see whether the microbe can use DNA as a source of carbon and energy for growth. Use of DNA is accomplished by an enzyme called DNase. An indicator used here complexes with intact DNA, making the medium appear mint green. When DNA is digested, the indicator changes from mint green to clear. Appearance of Halos surrounding colonies is indicative of their ability to digest the DNA in the medium due to the presence of DNase

Procedure:

All bacteria were inoculated in DNase agar and examine for the development of colour.

Carbohydrate fermentation tests:

Principle:

Carbohydrate fermentation tests detect the ability of microorganisms to ferment a specific carbohydrate. Fermentation patterns can be used to differentiate the bacterial groups or species.

During the fermentation process, an organic substrate serves as the final electron acceptor. The end-product of carbohydrate fermentation is an acid or acid with gas production. Carbohydrate fermentation leads to the production of various end-products. The end-product formed depends on the organisms involved in the fermentation reaction, the substrate being fermented, the enzymes involved, and environmental factors such as pH and temperature. Common end-products of bacterial fermentation include lactic acid, formic acid, acetic acid, butyric acid, butyl alcohol, acetone, ethyl alcohol, carbon dioxide, and hydrogen.

Fermentation reactions are detected by the colour change of a pH indicator when acid products are formed. This is accomplished by adding a single carbohydrate to a basal medium containing a pH indicator. Because bacteria can also utilize peptones in the medium resulting in alkaline by-products, the pH change takes place only when excess acid is produced as a result of carbohydrate fermentation. Phenol red is commonly used as a pH indicator in carbohydrate fermentation tests, because most of the end-products of carbohydrate utilization are organic acids. However, other pH indicators such as bromocresol/bromcresol purple, bromothymol/ bromthymol blue, and Andrade‟s can be used.

Fermentation tubes or Durham tubes are used to detect gas production. These small, slender test tubes (6 by 50 mm) are inserted upside down inside larger (13 by 100 mm) test tubes. After sterilization, Durham tubes become filled with the media. If gas is produced, it is trapped inside the Durham tube and is evident by the presence of a visible air bubble.

Three characteristic reactions can be observed from the fermentation of a specific carbohydrate. Based on these reactions, bacteria are classified as:

- Fermenter with acid production only
- Fermenter with acid and gas production
- Nonfermenter

Procedure:

Broth medium was prepared by mixing all ingredients (Table 6.3) in distilled water. A single carbohydrate was used for each batch of medium prepared. The larger test tube was filled with 4 to 5 ml of phenol red carbohydrate broth. An inverted fermentation tube or durham tube was inserted to detect gas production. Media was sterilized by autoclaving for 15 minutes at 116° to 118°C. The sterilization process also drived the broth into the inverted fermentation tube or durham tube. Sterilized base broth was cooled in a 42° to 50°C water bath before adding carbohydrates. Carbohydrate disc was used as a source of carbohydrate and aseptically transferred into the tube.

Each test tube was inoculated with the test microorganism using an inoculating needle or loop. The tube was swirled gently to mix the contents. Tubes were incubated at 35° to 37°C for 18 to 24 hrs. Longer incubation periods were required to confirm a negative result. Fermentation

results were recorded on the basis of colour development. When using phenol red as the pH indicator, a yellow colour indicated that enough acid products had been produced by fermentation of the sugar to lower the pH to 6.8 or less. A delayed fermentation reaction produced an orange colour. In such cases tubes were reincubated.

Bubbles trapped within the Durham tube indicated the production of gas. Even a single bubble was significant and denoted evidence of gas production. No bubble within the Durham tube indicated a non-gas-producing or anaerogenic organism. A reddish or pink colour indicated a negative reaction. In negative tubes, the presence of turbidityserved as control for growth. However appearance of a reddish or pink colour in a clear tube may indicate a false negative.

Amino acid decarboxylation test:

Principle:

The amino acid decarboxylase tests are useful to examine the production of the enzyme decarboxylase, which can remove the carboxyl group from an amino acid. Decarboxylase broth base is used to perform the tests. It contains nutrients, dextrose (a fermentable carbohydrate), pyridoxal (an enzyme cofactor for decarboxylase), and the pH indicators bromcresol purple and cresol red. Decarboxylation produces alkalinity and raises pH,Bromcresol purple turns purple at an alkaline pH and turns yellow at an acidic pH. It also requires the addition of a single amino acid to each batch of decarboxylase broth. Amino acids tested in decarboxylase broth are arginine, lysine, and ornithine. The decarboxylase test is useful for differentiating the Enterobacteriaceae. Each decarboxylase enzyme produced by an organism is specific to the amino acid on which it acts. Therefore, the ability of organisms to produce arginine decarboxylase, lysine decarboxylase, and ornithine decarboxylase is tested using three different but very similar media.

If the organism is unable to ferment dextrose, there is no colour change in the medium. If an organism is able to ferment the dextrose, acidic byproducts are formed, and the media turns yellow. As the organisms ferment the dextrose, the media initially turns yellow, even when it has been inoculated with a decarboxylase-positive organism. The low pH and the presence of the amino acid causes the organism to begin decarboxylation.

Fermentation result:

If an organism is able to decarboxylate the amino acid present in the medium, alkaline byproducts are then produced. Arginine is hydrolyzed to ornithine and is then decarboxylated. Ornithine decarboxylation yields putrescine. Lysine decarboxylation results in cadaverine. These byproducts are sufficient to raise the pH of the media so that the broth turns purple.

Negative result:

If the inoculated medium is yellow, or if there is no colour change, the organism is decarboxylase-negative for that amino acid.

Procedure:

The experiment was conducted as described above and examined to record positive or negative result.

Results & comment: