Collection, isolation and identification of bacillus subtilis from soil report ex...

Technology, Development

Introduction

Bacillus Subtilis are the organisms that are aerobic, Gram Positive, spore-forming belonging to the Bacillus genus. The organisms are together with other species that are closely related play a crucial role in disease, food poisoning as well as food spoilage. Lack of methods that are standardized for the identification of the organisms whether coming from the food or environment has made the tests to be difficult. Use of morphological as well as physiological tests have offered the best ways to identify organisms in the laboratory although there are inconsistent and lack of reliance in these methods.

Identification of an organism refers to the determination of whether an isolate or an organism should be put within a group of organisms that are known to conform to within some scheme of classification. Microorganisms that are found in the environments need to be identified in order to identify species to which they belong. This helps in the assessment of the source of contamination as well as the organisms that may cause diseases to the population. Identification is also meant to help in the diagnosis of disease (Fox, 2011). The identification of organisms is usually done using standardized techniques.

The first step in the identification schemes involves describing the colony as well as the cellular structure of the microorganism. The morphology of the colony is usually described by observing the growth of the organism directly on the agar. This describes the shape such as spherical, raised or crenated and the pigment of the colony. Although some microbiologists have attempted to identify the microorganism using only the visual identification,

this is usually discouraged since only experienced people can positively identify an organism in this manner (Sandle, 2011).

The techniques that are available for the identification of organisms include morphological identification, serological methods, protein analysis, and differential staining among others (Sandle, 2011). Bacillus subtilis is a one of the remarkably diverse species and can grow within a variety of environments. Using microarray-based comparative analysis of the genome, there has been a revelation that this species has a considerable genomic diversity. Some of the environments from which Bacillus subtilis has been isolated include the terrestrial environment and the aquatic environment. These species is thus adapted to living and growing in the biosphere that is most diverse (Lovett & Young, 1969).

Through the identification of the physiological, as well as, biochemical properties of a species, it is possible to classify an organism into either a genus, species or even as a strain of a known species. Currently, these techniques are not the only ones used in the identification of an organism. The sequences of the 16S rRNA gene have also been employed in the study of taxonomy as well as phylogeny in bacteria. Some of the reasons for applying this technique include the fact that the 16S rRNA is available in all bacteria, and function of the gene has not changed for a long time. The other reason is that the gene is long enough to be used for informatics purposes (Janda & Abbott, 2007).

A significant number of bacteria have been identified although this number is still low compared with the proportion of plants or animals that have been identified. This is mainly because microbes are more diverse than the higher

organisms and their identification is more complex than that of higher organisms. The make-up of bacterial flora differs from location to location. This is because of the different nutrients that are available in the different locations making the different bacteria to evolve accordingly. Bacillus subtilis has been isolated, identified and characterized as a versatile bacterium capable of converting nitrile in the soil.

Since the collection was done in the terrestrial where Bacillus species are known to be abundant, the isolate was predicted to be a Bacillus species. The experiment aimed at taking swabs from trees in Flagstaff, Arizona and performing different stain and biochemical test to identify the organism collected. It was hypothesized that proper use of physical, as well as, biochemical tests would result in the identification of the organism collected.

Materials and Methods

The procedure of identifying the unknown organism from the environment was done following the illustrations that are given in the Bergey's Manual of Determinative Bacteriology.

Cotton swab was used to swab the snow and streaked into trypticase soy agar (TSA) plates. These plates were placed for incubated at room temperature for 72 hours after which they were refrigerated. The plates were checked for any growth and number as well as the features such as the size, color and the shape recorded. Two plates were selected and purified by streaking isolated colonies on TSA plates and then incubated in room temperature for 48 hours.

The colony was purified for the second time, and the first attempt of

inspection was done. A colony was selected from the first purification plate and re-streaked onto a new TSA plate to confirm purification. Incubation of the plate was done for 48 hours at room temperature. The environmental unknown was inspected for purity, and when this was confirmed, a large colony was selected, and about two third of the colony used to inoculate 2 TSA (TSA in a screw –capped tube) for storage. The TSA slants were incubated at room temperature with the caps unscrewed ½ turned from its fully tightened position. The growth was monitored with an aim of determining the type of bacterial. The screw cap was tightened and one of the tubes stored at room temperature while the other one was stored at 4OC refrigerator for 2 weeks.

The storage slant was sub-cultured onto 2 fresh TSA slants and allowed to grow at room temperature with the cap ½ turn screwed. The slants were observed frequently to determine whether one of them regenerated better than the other. The best storage conditions were determined and were used all through the experiment and sub-cultured after every two weeks.

Simple stain was used in the determination of the morphology of the microorganism while Gram stain was used to determine if the organism was a Gram positive or negative. A Gram positive result is indicated by a purple/blue color while the results for Gram negative are indicated by a pink/red color. Other stain that was used was the endospore stain whose positive indicator is green color and negative indicator is the lack of the green color.

Biochemical Tests

Catalase Test

The catalase test was done by removing a small amount of the environmental unknown from the agar slant, or a loopful of control test organisms form a broth culture and placed on a glass slide. The organism was mixed with a drop of 3% H_2O_2, and the appearance of gas bubbles checked as the positive indicator of the test. Staphylococcus epidermidis was used as the positive control while Streptococcus lactis was used as the negative control.

Oxidase

A commercially prepared test known as Dry Slide oxidase test was used. One window was used for the unknown while the second window was used for the positive control. The cells were rubbed using plastic steri-loop on the filter paper in one the dry slide, and the where change in color was recorded within 20 seconds. If the organism is oxidase positive, the reaction turned dark purple, and if oxidase negative, there was no color change or change of color form colorless to gray. Pseudomonas aeruginosa was used as a positive control.

Carbohydrates Fermentation

The ability to ferment carbohydrates and the type of fermentation end products that are formed are useful in bacterial identification. The environmental isolate for the ability to ferment glucose sucrose, lactose and mannose. A tube containing the sugar to be tested was inoculated and kept at room temperature. The tubes were scored at 24 and 48 hours, and the

results recorded. A yellow color is a gives a positive indication for the fermentation of carbohydrate while orange is the negative indication that the organism does not ferment carbohydrate. Gas in the Durham tube acts as an indication of gas production through aerobic fermentation. Absence of gas in the Durham tube indicates that no gas was produced, or the organism is anaerogenic organism

Oxygen Requirement

Bacteria are divided into either obligate aerobes, cannot survive without oxygen, strict anaerobes, those that cannot survive in the presence of oxygen facultative anaerobes, those can do well using free oxygen, or through alternative sources, aerotolerant, those that have no preference for either aerobic or anaerobic condition, and microaerophiles refer to those organisms that require free oxygen, but not in a limited amount.

Figure 1: Growth results of organisms at various oxygen conditions

Motility Test

A tube of motility medium using the inoculating needle rather than the loop was inoculated. The needle was flame sterilized, and when cool, the cells were transferred onto the very tip. The motility medium was stabbed to about two third of its depth and the needle withdrawn straight out using the same path that was used to go in. The needle was sterilized and incubation of the tube done for 48 hours. The test was positive when there was red cloudiness around the stab pathway. Lack of cloudiness outside the area was an indication of negative results.

Facultative Anaerobes

Simmons Test

Simmons citrate test is used to see whether the microorganisms can use citrate as the only carbon source. The agar contains sodium citrate making it the sole source of carbon, the sole source of nitrogen as ammonium dihydrogen phosphate. The inoculating needle was flame sterilized and allowed to cool. Some of the rest microorganism were transferred onto the tip of the inoculating needle, and the agar was stabbed. The needle was sterilized, and the sample incubated at room temperature for 48 hours. A positive test was indicated by a color change from green to blue while no change in color indicated negative test.

Urea Hydrolysis

Using urea's broth that was composed of yeast extract, urea as well as pH indicator phenol red, incubation of the tube was done in the broth and incubated for 48 hours at room temperature. If the urease was present, ammonia was released, and there was a rise in pH. A positive urease test was thus indicated by color change from yellow to cerise, and no color change was an indication of a negative test.

Kligler Iron Agar

The test was done by inoculating a tube of Kleigler's iron agar with some of the test organism using the inoculating needle. The stab was made to two third of the way into the agar and incubated for room temperature for 48 hours. A positive test was shown by a dark precipitate that formed in the tube while the absence of the precipitate was an indication of negative test.

Since the medium also contained glucose, lactose and phenol red which may result to yellow color after fermentation, the dark precipitate still remained the indicator for a positive test.

Starch Hydrolysis

The test was done by inoculating a single starch-gelatin agar plate with a small amount of the environmental unknown and used B. megaterium for the positive control and E. coli for the negative control. Incubation of the plate was done for 48 hours at room temperature and then refrigerated. A few drops of Gram’s iodide were added. Areas on the plate that contained starch formed a dark blue or purple complex while areas around the colonies where the starch had been hydrolyzed appeared as clear zones. A clear zone around the test organism was thus an indication of a positive test.

Results

The unknown organism was studied in detail for color, colony, growth temperature, cell morphology, spore formation. The results from the physical tests of the unknown organism revealed that the color of the colony had a pigmentation that was yellow in color while its configuration was irregular and spreading. The margin of the colony was irregular with raised elevation and a filliform pattern of growth. The organism was rod-shaped in terms of the cell morphology and tetrads cellular arrangement. Gram staining technique, which was used to determine whether the organism was Gram positive as a result of the presence of peptidoglycan, gave a positive test. Similarly, the test to determine production of spores, endospore test, was positive indicating production of spores by the organism.

Catalase enzyme is the enzyme necessary in the conversion the H2O2 molecules into oxygen and water products that are not harmful to the cells. The produced oxygen is the basis behind the catalase test where H2O2 is introduced to the organism and appearance of bubbles confirms degradation of H2O2 by the catalase enzyme. In this experiment, introduction of H2O2 resulted into bubble production.

In the oxidation test, the reaction area did not turn to purple meaning that the artificial substrate that was introduced was not oxidized. The test was thus declared to be negative. Carbohydrate fermentation test was used to test for the ability of the organism to digest carbohydrates. The test was done by using a pH indicator to monitor changes in pH through color change. Change in color indicated a positive result while an incident where there was no color change indicated results that were negative. In the test, all the sugars that were tested resulted into an orange color and therefore, negative for the test.

The other test that was done involved determination of the oxygen requirements by the environmental unknown organism. This was done to help classify the organism either as obligate aerobe, facultative anaerobe, microaerophile, aerotolerants or even strict anaerobes. The unknown organism was an obligate aerobe since all of them were found near the top of the tube. Similarly, there was also no growth in the facultative anaerobe test.

The motility test that was done in the identification of organisms involved testing whether the organism has structures such as flagella that can be used for movement. True motility is observed in a wet mount or hanging

drop preparation of the organism or even by using semi-solid medium and using TTC (2, 3, 5-triphenyltetrazolium chloride as an indicator. When the organism that is motile moves to the TTC and transport it into the cytoplasm where it is reduced forming an insoluble red formazan pigment. The appearance of red color is the positive indicator for the test. The motility test was negative since there was no red cloudiness outside of the initial stab pathway.

In the Simmons test, results were negative as there was no change of the green color to blue. The urea hydrolysis test was negative since there was no change of the yellow color to cerise.

Kligler Iron Agar involved the test of the production of hydrogen sulfide gas that results from the deamination of sulfur containing amino acid such as cysteine. The medium contained ferrous sulfate, which reacts with H2S forming iron sulfide precipitate, which is dark in color as a positive result for the test. The Kligler iron agar test was negative since there was no dark precipitate that formed in the tube.

The starch is hydrolyzed outside the cell since the molecules cannot pass through the cell membrane. The organism is incubated with starch and left overnight. The availability of the α-amylases results in the hydrolysis of the starch and a clear zone is observed around the organism. The test indicated a clear zone in the medium and thus the starch hydrolysis test was positive.

The Table 1 below summarizes the results for the identification of the unknown organism.

Discussion

The experiment aimed at taking swabs from trees in Flagstaff, Arizona and performing different stain and biochemical test to identify the organism collected. The environmental organism was collected and through different stains as well as biochemical tests identified. The physical and stain tests that were performed indicated that the organism color of the colony was yellow while its configuration was irregular and spreading. The margin of the colony was irregular with raised elevation and a filliform pattern of growth. The organism had the rods form of cell morphology and tetrads cellular arrangement. Both the Gram stain and endospore stain gave positive results.

Out of the four genera, both the Bacillus and Streptomyces are strictly aerobes while the Clostridium and Corynebacterium are not. The other tests that were used to distinguish the four genera were the motility of the organism as well as the presence of a capsule. All the genera other than Clostridium had a capsule while and were immotile.

Fermentation of carbohydrate test is a test aimed at identifying bacteria that use different carbohydrates such as glucose, sucrose, lactose, as well as mannose. The test did not give a positive test for all the carbohydrates used. Out of the four genera Bacillus genus did not show carbohydrates fermentation. The genus that has the strongest correlation with the isolate was the Bacillus, and this suggested that the isolate would be that of Bacillus. The correlation results for the different genera were as in Table 2 below.

The successful prediction of the genus necessitated for the identification of the species that the organism belonged to using the various biochemical tests. The catalase test gave positive results as there was no air bubbles after the experiment. The Bacillus species are positive for catalase test (Engelkirk & Duben-Engelkirk, 2008) since most of them are obligate aerobes while others are facultative aerobes. Catalase and peroxidase are enzymes which convert H2O2 to water and free oxygen gas (O2). The liberation of oxygen gas is the basis for the catalase test.

True motility is normally different from the Brownian movement that is observed under a microscope. Bacteria move through flagella and follow either the polar or peritrichous patterns. Using the motility medium the motility of the organism was confirmed by the appearance of red cloudiness in the tube. This test was negative meaning that the organism was non-motile. From the various Bacillus species that are commonly occurring some of the species that closely match the results obtained were Bacillus anthracis and Bacillus subtilis. Some of the characteristics include being obligate, and the ability to hydrolyze starch. While the Bacillus anthracis is non-motile the Bacillus subtilis is motile using the flagella (CDC, 2001; Masahiro, Naoya, Shun, & Terry, 2005)

The Simmon test on the environmental unknown was negative and was similar to the Bacillus subtilis. The results for the urea hydrolysis test were negative for the environmental unknown. This means that the organism does not possess the urease enzyme which is responsible for the hydrolysis.

Kligler Iron Agar involves the test of the production of hydrogen sulfide gas that results from the deamination of sulfur containing amino acid, cysteine.

The medium contains ferrous sulfate, which reacts with H2S forming iron sulfide precipitate, which is dark in color. For the unknown organism, there was no dark precipitate formed. These results were identical to those obtained in the B. subtilis. However other Bacillus species such as B. anthracis give positive results for the test (Shatalin, Shatalina, Mironov, & Nudler, 2011).

The starch hydrolysis test determines the ability of the organism to hydrolyze the starch using the extracellular enzymes called α-amylases. Most of the Bacillus species are known to have the enzyme and thus positive for the test. The organism that was highly correlated with the environmental unknown organism was B. subtilis and thus the unknown organism was most probably the B. subtilis. The correlation results were as shown in the Table 3 below.

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