18BTC103J-MICROBIOLOGY LABORATORY MANUAL

B.TECH. BIOTECHNOLOGY

THIRD SEMESTER

DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIOENGINEERING
SRM INSTITUTE OF SCIENCE AND TECHNOLOGY
KATTANKULATHUR-603 203

List of Experiments

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Experiment No.1: Aseptic techniques and Media preparation

Aim:

To acquire the knowledge on aseptic techniques and develop the skill of media preparation for culturing the microbes

Principle:

Aseptic techniques are a fundamental and important laboratory skill in the field of microbiology. Microbiologists use aseptic techniques for a variety of procedures such as transferring cultures, inoculating media, isolation of pure cultures, and for performing microbiological tests. Proper aseptic technique prevents contamination of cultures from foreign microbes inherent in the environment. For example, airborne microorganisms (including fungal spores), microbes picked up from the researcher's body, the lab bench-top or other surfaces, microbes found in dust, as well as microbes found on unsterilized glassware and equipment, etc. may potentially contaminate cultures, thus interfering with the lab results. Using proper aseptic technique can greatly minimize or even eliminate the risk of microbial contamination. In addition, aseptic technique is of utmost importance to maintain pure stock cultures while transferring cultures to new media. Aseptic technique is also essential for isolation of a single species of microorganism from a mixed culture to obtain a pure culture. Furthermore, proper aseptic technique prevents microbes used in the laboratory from accidentally being released into the environment and/ or infecting people working in the laboratory. This is especially relevant when pathogens are being handled.

As microorganisms are ubiquitous, it is always safe to assume that any environment we are in is filled with them. Before starting today's exercise let us acquaint ourselves with certain important aspects of asepsis.

a) **Work Area Disinfection**: Before starting the experiment, the work bench surface must be sprayed with 70% ethanol to disinfect the place. It must be rubbed down into all regions. The mistake of wiping the work bench with towels or tissues to spread the ethanol will only introduce new microbes. Therefore, the best thing to do is to let the volatile ethanol evaporate on its own.
b) **Transfer Instruments - Loops and Needles**: Wire loops and needles are made out of inert materials such as platinum. They are extremely durable and can be sterilized by incineration. To do this, place the loop in the blue part of the Bunsen burner flame as it is the hottest and allow the loop to get red hot and then remove. Cool the transfer instrument down before inserting into culture. This procedure has to be carried out prior to any inoculation and after any inoculation.
c) **Culture Tube Flaming**: Prior to inserting the loop into the culture tube, remove the lid of the tube and flame the tip of the tube so as to avoid entry of any air contaminants. This has to be carried out before and after insertion of loop into tube culture.
d) **General Sterilization:**

Sterilization is the process of destroying all forms of microbial life-vegetative and sporulation. It is important that all equipment used in a microbiological experiment is

sterilized in order that a particular organism of interest is grown, without contamination from the surrounding environment.

Several methods are employed in the process of sterilization to sterilize the various equipments used in an experiment. These methods are broadly classified as PHYSICAL and CHEMICAL methods:

I) PHYSICAL AGENTS:

The major physical agents used for the control of microorganisms are TEMPERATURE, RADIATION, and FILTRATION.

1. TEMPERATURE: Microorganisms can grow over a range of temperatures, from very low temperatures characteristics of psychrophiles, to very high temperatures characteristics of thermophiles. Temperatures above a maximum generally kill microbes; sterilization can be achieved by high temperatures as DRY HEAT or MOIST HEAT.

- **DRY HEAT STERILIZATION**: Dry heat sterilization is achieved in hot air oven. Laboratory items like petriplates, Erlyn Meyer flasks, pipettes, test tubes, dry powders, glycerol and other oils etc. are sterilized in hot air oven. Such material can be sterilized by placing in hot air, at a temperature of 160°C for 120 mins.

  Alternatively, equipment like forceps, inoculation needle etc. can be sterilized by direct heating on a flame till red hot. Thus it brings about destruction of unwanted organisms without changing the nature (flavor) of the material. This process involves heating at that temperature for 15 mins and then cooling it quickly to 0-5°C.

  PRINCIPLE INVOLVED IN DRY HEAT STERILIZATION: Dry heat oxidizes chemical components of organisms and thus destroying them.

- **MOIST HEAT STERILIZATION**: High temperatures combined with high moisture are one of the most effective ways of sterilization.

  PRINCIPLES INVOLVED IN MOIST HEAT STERILIZATION: Moist heat coagulates the microbial proteins, and is hence more rapid in killing microbes.

  Moist heat can be applied in the following ways in order to bring about sterility:

  STEAM UNDER PRESSURE: Provides temperatures higher than those obtainable by any coagulation of proteins. Autoclave is a device used in the laboratory to sterilize media solution and to kill discarded cultures.

  It is operated at 151lbs/sq. inch pressure, which yields a temperature of 121°C effective in bringing about sterility in 15 min.

- **FRACTIONAL STERILIZATION OR TYNDALLISATION**: Some microbial solutions cannot be heated over 100°C without being damaged. Such materials are sterilized by tyndallisation, which involves heating at 100°C on three successive days with incubation periods in between. Resistant spores germinate during the period of incubation that is killed on heating the subsequent day.
- **PASTEURISATION**: Milk, cream and other alcohol beverages are subjected to controlled heat treatment which kills microbes of a certain type alone.

2. RADIATION: When ionizing radiation pass through cells, they create free hydrogen radicals,, hydroxyl radicals and peroxides that cause intracellular damage, resulting in destruction of microbes. This method of sterilization is effective for sterilizing heat liable materials. Thus, it is also called COLD STERILIZATION.

UV light is the most effective region of the electromagnetic spectrum, and is employed in disinfecting of inoculation chamber and hospital operating rooms. UV light alters nucleic acids and results in a pyrimidine dimer, thus inhibiting DNA replication.

3. FILTRATION: This technique is used when the material to be used is heat liable and cannot be sterilized by heating for e.g. Solutions of proteins, vitamins etc. Filters of pore size 0.03µ-0.08µ are used to filter off microbes, thus rendering the filtrate sterile. Pore size, electric charge of filter, charge carried by the organisms and nature of fluid being filtered affect efficiency of filtration. E.g. Acity filters, Berkefeld filter, membrane filter are all microbial filters.

II.CHEMICAL AGENTS:

Ethyl Alcohol (70%) finds indispensable use for disinfecting hands before and after a microbiological experiment, and also to disinfect the inoculation chamber or area where the experiment is conducted.

Several groups of chemicals can be used as antimicrobial agents:

S.No.	Group	Action	Example
1.	Alcohol	Denature proteins and solubilize lipids	Ethanol, Glutaraldehyde
2.	Aldehyde	Alkylate, reacts with-NH_2,SH-	I_2
3.	Halogens	COOH	Cl_2
4	Heavy Metals	I_2 inactivates proteins oxidize cells.	$HgCl_2$
5	Gases	Precipitates and inactivates proteins (used for surface sterilization)	Ethylene Dioxide
6	Detergents	Alkylates organic compounds; Disrupt cell membrane	
7	Phenols	Denature protein & disrupt cell membrane	

Media Preparation

To prepare the different culture media for growing microorganisms and to learn method for maintaining bacteria in laboratory for long time

GENERAL PURPOSE OF MEDIA PREPARATION

1. Compare the different types of media.
2. Describe the different formats of media, plate, tube, etc.
3. Explain how to sterilize it, and how to distribute it in different formats.
4. Produce Nutrient agar (NA) plates, NA slants, and Nutrient broths (NB) which will be used in subsequent lab periods.
5. Know the laboratory equipment and culture media needed to develop and maintain pure cultures.

6. Carry out aseptic technique for the removal and transfer of microorganisms for culturing.
7. Correctly sterilize and flame transfer instruments and tubes.
8. Properly incubate culture media at the appropriate temperature for the organism and the length of incubation.

PROCEDURE

A clean Erlenmeyer flask was taken with 200 ml of distilled water. Appropriate quantities of chemicals are weighed accurately and dissolved one by one taking care to add the next chemical only after the dissolution of the first one, except agar. The total volume of the medium was made up to 1 litre. The medium is then distributed into 250 ml. Erlenmeyer flasks of 100 ml each and were plugged tightly with non-absorbent cotton and wrapped with a paper and tied with thread. Then the flasks were sterilized at 121°C for 15 minutes with 15 lbs/in in an autoclave.

ENRICHED MEDIA

Enriched media are media which allow fastidious organism to grow because of the presence of specific nutrient additives such as blood, serum, hemin, etc. An example of this category is the blood agar and chocolate agar.

Blood agar was prepared by adding 5% of sheep blood or blood from other sources to precooled nutrient agar. It is also a differential medium to differentiate hemolytic and non-hemolytic colonies. It is widely used to isolate *Streptococcus* spp. from clinical specimens.

Chocolate agar is prepared by slightly heating the media in a water bath to about 65-70°C.

Bright red color of blood agar will change to chocolate color due to the oxidation of haem to hemin. It is generally used to isolate fastidious pathogens like *Neisseria* and *Haemophilus*.

DIFFERENTIAL MEDIA

Differential media are media that aid in the presumptive identification of bacteria based on the organisms appearance in the medium. Common example of differential media is MacConkey agar. MacConkey agar is used to differentiate lactose fermenting gram negative bacteria from lactose non-fermenting bacteria.

MacConkey is a differential and selective medium. The nutritive base included the peptone. The medium is made differential by incorporating lactose and a pH indicator neutral red to detect the acid formation by fermentation of lactose. It is made selective for Gram negative bacteria by incorporating sodium taurocholate. Lactose fermenting colonies appear pink color with a pink halo, against the light pinkish medium. Non-lactose fermenting colonies are colorless or with the color of the medium.

SELECTIVE MEDIA

Selective media are media that contain additives that enhance the presence of the desired bacteria by inhibiting the other bacteria. Example is again MacConkey agar whose preparation is explained in previous section. MacConkey is selective for Gram positive as it contains selective inhibitor of Gram positive bacteria, Sodium taurocholate.

Eosin methylene blue (EMB) agar is another selective medium for isolating *E.coli*. Pancreatic digest is the nutrient component and Eosin and methylene blue are the selective agents inhibiting the Gram positive bacteria. Bacteria that ferment lactose bind to the dye under acidic condition and appear as blue-black colonies with metallic sheen. Under less acidic conditions, other colonies appear pinkish without metallic sheen.

MYCOLOGICAL MEDIA

Sabouraud's Dextrose agar (SDA) is a general purpose media to isolate and cultivate fungi. It has 4% dextrose and a pH of 5.6. This low pH reduces the bacterial contamination. The original composition is now modified by adding antibiotics to prevent bacterial contamination.

PLATES:

The sterilized medium is cooled to 45-50°C and poured into sterile petriplates. About 20 ml of the medium poured into each of the sterile petriplates and allowed to solidify. Incubate in an incubator overnight before use.

SLANTS:

The molten medium after adjusting the pH is poured into test tubes, 5 ml each and the test tubes are placed in a slanted position so that they with solidify with maximum surface area on the slant region and butt region.

DEEPS:

Deeps are prepared by pouring the molten medium in sterile glass test tubes and then placed in a vertical position. The tubes are then cooled in cold water and are used to maintain cultures for long periods of incubation.

1.Composition of Nutrient of nutrient agar (g/L):

pH (7.2)

Beef extract	3.0g
Peptone	5.0g
NaCl	5.0g
Distilled water	1 litre
Agar	20g

2. Composition of MacConkey agar (g/100 ml)

Peptone	2.0g
Lactose	1.0g
Sodium taurocholate	0.2g

Sodium chloride	0.5g
Agar	1.5g
Neutral red	0.03g
Distilled water	100 ml
pH 7.2 ±0.2	

Weighed the ingredients, dissolved by boiling in a water bath and then autoclaved at 15lb pressure for 15 minutes.

3. Composition of EMB agar (g/L)

Peptone	10.0g
Lactose	10.0g
Di-pottassium hydrogen phosphate	2g
Eosin-Y	0.4g
Methylene blue	0.065g
Agar	15g
Distilled water	1 litre
pH 7.1	

Weighed these chemicals and dissolved in one litre of distilled water and autoclaved.

4. Composition of PDA (g/L):

Potato	200 g
Dextrose	20 g
Agar	18 g
Distilled water	1000 ml

pH 5.6. Mixed the ingredients and autoclaved.

5. Composition of Martins Rose Bengal agar (g/L)

KH_2PO_4	20.0g
$MgSO_4.7H_2O$	1.0g
Peptone	5.0g
Dextrose	10.0g
Distilled water	1 litre
Agar	20.0g
Rose Bengal	3.3 ml
Streptomycin	30.3mg (added after autoclaving)

RESULT AND INTERPRETATION:

Expt. No: 2

Isolation and enumeration of bacteria from soil sample

AIM:

To isolate and enumerate bacteria from the given soil sample by the serial dilution technique using aseptic techniques.

PRINCIPLE:

Microorganisms are persisting everywhere including soil, water, and air. The isolation of microorganisms is very much required for various purposes such as the production of industrially important products like enzymes, antibiotics, vitamins, proteins, etc.

Addition of samples directly to the medium will lead to dense colonies, from which we could not able to differentiate the colonies and their morphology and also the purification of colonies become very difficult. Serial dilution of the samples is a viable technique to isolate the microorganisms as individual colonies and differentiate the morphology of isolated bacterial strains. Two types of plating methods are used for the isolation and cultivation of microorganisms: 1) Pour plate and 2) Spread plate.

REQUIREMENTS:

Sterile blanks (9 ml), 1 sterile blank (10ml), soil samples, 18-20 sterile petriplate, nutrient agar plates, 10 sterile pipettes (1ml), melted nutrient agar, inoculation loop, bunsen burners, marker pen and sterile chamber.

I. Isolation

Procedure:

1. SERIAL DILUTION:

Exactly 1 ml of the given soil sample is dissolved well in the 10 ml blank and large particles are dissolved to settle. This solution is labelled as 10^{-1}. From this test tube, 1 ml of solution is taken and added to a 9 ml blank, mixed evenly and labelled as dilution 10^{-2}. In the same manner, the dilutions are prepared up to 10^{-8} using sterile pipettes for each transfer. For the given soil sample, aliquots from the 10^{-5}, 10^{-6} and 10^{-7} were taken for the plating.

2. ISOLATION OF MICROORGANISM BY POUR PLATE METHOD

Pour plating is a technique useful for isolation and enumeration of microbes. 1 ml of the above selected dilutions was pipetted into sterile petriplates in the close proximity of a Bunsen burner flame. Melted nutrient agar in appropriate warmth (about 50ºC) was poured over the inoculum and the plates were swirled to evenly distribute the inoculum. Plates of a particular

dilution were prepared in duplicates. The agar was allowed to solidify and the plates were inoculated at a temperature of 35ºC in an inverted manner for a period of 24-72 hours. The colonies formed were counted using a colony counter and plates having colonies between 30 and 300 are selected for determining colony forming units.

3. ISOLATION OF MICROORGANISM BY SPREAD PLATE METHOD

From the above selected dilution, 0.5 ml is transferred to the surface of a solid agar medium. The inoculum is spreaded over the agar evenly by using an L- shaped bent glass rod by rotating the petri plates. The bent glass rod is sterilized by showing to the flame after dipping in alcohol. Plates can also be spin for spreading on a turn table. While the turn table is spinning, lightly touch the sterile bent rod to the surface of the agar and move it back and forth. This will spread the culture over the agar surface.

II. Enumeration

The number of colony forming units (CFU) was calculated using the formula:

No. of colonies x Dilutions factor = CFU/gram of sample

(Dilution factor is the reciprocal of the dilution taken for the plating)

A. Pour plate method

DILUTION	No. of colonies

Total No. of bacteria present in the soil sample = CFU/g of soil

B. Spread plate method

DILUTION	No. of colonies

Total No. of bacteria present in the soil sample = CFU/g of soil

Growing microorganisms directly from any source is not viable method. Serial dilution is the best method.

In order to execute a reliable colony count, the suspension containing the desired microorganism must be diluted first to an appropriate concentration.

III. Purification:

The selected bacterial colonies were further sub cultured several times (3-5 days) on the same medium until the purity.

RESULT:

Expt. No: 3

Date:

CULTURING AND PRESERVATION TECHNIQUES OF MICROORGANISMS (BACTERIA)

AIM:

To culture a bacterium from soil samples using sub-culture methods and to demonstrate methods for purifying and maintaining bacterial cultures in laboratories for long term.

PRINCIPLE:

Purification of microorganisms is carried out to get a pure colony of organism from a mixture of microbial consortium after the plating of samples through serial dilution. Various streaking methods such as simple streak, T-streak and quadrant streak are used to obtain pure colony. Understanding the storage/preservation methods to store the microorganisms without contamination is very important as the proper storage will help to maintain the cell viability for longer period of time and for the further application studies.

PROCEDURE:

Bacterial colonies that have been isolated in the nutrient agar plates in previous experiment could be purified by the following purification techniques.

Simple streak:

The inoculation loop was sterilized by heating it in the flame till it becomes red hot. The bacterial strain (single colony) was picked up from the plate using the inoculation loop and smeared in one corner of the agar media containing plate. This is called as the mother smear. From this smear, a simple streak is done.

T- streak:

Mother smear was made at one corner of the plate and made a few streaks from this smear. Streaking was continued from one of the made streaks to obtain a T-streak after sterilizing the loop.

Quadrant streak:

Mother smear was made at one corner of the plate and made a few streaks from it. Rotated the plate and made the second set of streaks from the first set. Repeated the procedure till the quadrant streak is achieved. Before streaking each quadrant, loop was heat sterilized.

The purified microbial strains were stored/preserved by using following techniques.

1. **Sub-culturing:** Transferring organisms from one plate (solid medium) to another medium (liquid or solid medium).

a. Slant:

The medium was taken in a test tube and was allowed to solidify by placing the test tube in a slanting position. After solidification, the microorganisms were streaked on the surface of the slant. This storage technique provides short term storage.

b. Deep:

The medium was taken in a test tube and was allowed to solidify by placing the test tube in a vertical position. Microorganisms were inoculated in the medium by inserting a loop containing the colony into the solidified medium. This storage technique provides short term storage.

c. Glycerol broth:

Glycerol broth can be prepared by adding 20% glycerol solution to the culture medium. Microorganisms can be stored in this liquid medium without loss of viability.

d. Mineral oil:

The mineral oil can be added on the surface of the agar medium after the cell growth. This will prevent the loss of moisture from the agar medium, so that the medium will not get dry easily.

2. Refrigeration:

Storage of colonies at 4°C: In the refrigeration temperature (4°C), the fungal samples can be stored for 3-4 months, while the bacteria can be stored for 2-3 weeks without the loss of viability.

3. Cryopreservation:

In this method, liquid nitrogen (-196°C) is used. The cultures are freeze dried using liquid nitrogen and then stored in the deep freezer (at -20°C or -80°C). Bacteria can be stored for long duration using this method.

4. Lyophilization:

The lyophilizer is used to freeze dry the sample, where the adequate moisture is retained and the rest is removed by vacuum under low temperature. The resulted dry powder (no loss of viability) containing the microbial strains are stored in the deep freezer (below -20°C) for long term. The microbial strains can be revived even after many years without losing their viability.

RESULT:

Expt. No: 4

DATE:

SIMPLE STAINING

AIM:

To perform the simple staining procedure to compare morphological features and arrangement of bacterial cells.

PRINCIPLE:

In simple staining, the bacterial smear is stained with a single reagent. Basic stains with positively charged chromogen are preferred, because nucleic acids and certain cell wall components carry a negative charge that strongly attracts and binds to the cationic chromogen. The purpose of simple staining is to elucidate the morphology and arrangement of bacterial cells. The most commonly used basic stains are methylene blue, crystal violet, and carbol fuschin.

MATERIALS:

Cultures: 18-24 hours old bacterial
cultures. Reagents: Crystal violet/safranine.
Equipment: Inoculating loop, staining tray, microscope, blotting paper, and glass slides.

PROCEDURE:

1. Prepared separate bacterial smears of the organisms following the procedure described.
   Note: All smears must be heat fixed prior to staining.
2. Slide is placed on the staining tray and flooded the smear with the stain, using the appropriate exposure time for each: Carbol fuchsin, 30 seconds; crystal violet, 1 minute.
3. Washed the smear with tap water to remove excess stain. During this step, the slide is held parallel to the stream of water, as in this way the loss of oraganisms from the preparation can be reduced.
4. Air dried the smear, do not wipe the slide.
5. Examined the slide using high power and oil immersion objectives.

OBSERVATION:

RESULT:

Expt. No: 5
Date :

GRAM STAINING

AIM

To become familiar with :

1. The chemical and theoretical bases for differential staining procedures.
2. The chemical basis of the Gram stain.
3. Performance of the procedure for differentiating between the two principal groups of bacteria : gram-positive and gram-negative.

PRINCIPLE

Gram staining was introduced by Danish bacteriologist Christian Gram in 1880. It is the most widely used differential staining in bacteriology which permits to classify bacteria as either gram-positive or gram-negative. Gram-positive bacteria have cell walls composed of peptidoglycan with peptide interbridges. Teichoic acids are covalently linked to the peptidoglycan layer or to the plasma lipids (lipoteichoic acids).Teichoic acids appear to extend to the surface of the peptidoglycan, and, because they are negatively charged, help give the gram-positive cellwall a negative charge. Gram-negative bacteria have a thin peptidoglycan layer and an outer membrane composed of lipopolysaccharides. The outer membrane consists of three parts – Lipid A, core polysaccharide and O side chain. The lipopolysaccharides give negative charge to the gram-negative bacteria cell walls. Basic dyes (positively charged) are used to bind to the negatively charged cell wall.

Differential staining requires the use of at least four chemical reagents that are applied sequentially to a heat-fixed smear – the primary stain, the mordant, the decolourising agent and the counter-stain.

The procedure involves four steps :

- Heat-fixed smear on the slide is first treated with a basic dye, crystal violet (CV). At this stage all the bacteria take up the primary stain and appear violet.
- In the second step, after washing, the smear is treated with iodine solution which acts as a mordant. Iodine complexes with crystal violet to form a CV-I complex inside the cell which intensifies the colour of the stain and all the cells will appear purple-black at this point.
- In the third step, smear is treated with alcohol to decolourise the stained cells. During this process, some bacteria will lose the CV-I complex whereas others will retain it.
- In the fourth step, after washing, smear is treated with a counter-stain, safranin. At this stage, all the bacteria which had lost the primary stain will take up the counter-stain and appear red. The rest, which retained the CV-I complex will remain violet.

Thus, after the completion of the above four steps, bacteria may appear either violet or red. The violet-coloured ones are called gram-positive and the red-coloured ones are called gram-negative.

Alcohol is a lipid solvent and it extracts some of the lipid content of the outer liposaccharide layer of gram-negative bacteria leaving minute pores through which the CV-I complex oozes out. In gram-positive bacteria this CV-I complex binds to the cell wall more firmly as magnesium-ribonucleic acid-crystal violet-iodine (Mg-RNA-CV-I) complex, which is difficult to remove.

Factors such as age of the culture, density of the smear, the length of the time involved in different steps etc. will affect the staining result.

REQUIREMENTS

- *Cultures* : 18-24 hours old nutrient agar slant cultures
- *Reagents* : Crystal violet, Gram's iodine, 95% ethyl alcohol, safranin
- *Equipment* : Inoculating loop, staining tray, glass slides, blotting paper, microscope

Preparation of reagents

1. **Gram's Crystal Violet (Huckel's modification)**

   Solution A

   Crystal violet – 2.0g
   Ethyl alcohol – 120ml

   Dissolve the stain completely in solution A.

   Solution B

   Ammonium Oxalate – 0.8g
   Distilled water – 80ml

   Solutions A and B are mixed, filtered and stored in bottles.

2. **Gram's Iodine**

   Potassium iodide – 2.0g
   Distilled water – 10ml

   Dissolve the potassium iodide in the distilled water. Add 1.0g iodine and dissolve completely by grinding. Add 290ml of distilled water to make up to 300ml.

3. **Safranin solution**

   Safranin – 1.0g
   Distilled water – 100ml

   Dissolve the safranin in the distilled water and filter.

PROCEDURE

- Microscopic slides are cleaned thoroughly with detergents and water, followed by wiping with alcohol.

- Using sterile technique prepare a smear of the test bacteria. This is done by placing a drop of water on the slide and then transferring a small portion of the colony to the drop of water on the slide with a sterile, cooled loop. Mix and spread the bacteria by means of a circular motion of the inoculating loop.
- Allow the smear to air-dry and then heat-fix it.
- Apply crystal violet to the smear and leave it undisturbed for one minute.
- Wash the slide with tap water.
- Apply Gram's iodine mordant to the smear and leave it undisturbed for one minute.
- Wash the slide with tap water.
- Decolourise the smear with 95% ethyl alcohol (DO NOT OVER-DECOLOURISE). Add the alcohol drop-wise until the crystal violet fails to get washed away from the smear.
- Wash the slide with tap water.
- Counter-stain the smear with safranin and leave the slide undisturbed for one minute.
- Wash the slide with tap water.
- Air-dry the slide and observes it under the microscope.

PRECAUTIONS

1. The most critical phase of the procedure is the decolourisation step, which is based on the ease with which the CV-I complex is released from the cell. Over-decolourisation will remove the primary stain in gram-positive bacteria also and cause them to appear gram-negative. Under-decolourisation will not completely remove the CV-I complex and cause gram-negative bacteria to appear gram-positive. Strict adherence to all instructions will help remedy part of the difficulty, but individual practice and experience are the keys to obtain correct decolourisation.
2. It is imperative that the slide be thoroughly washed under running tap water between applications of the reagents. This removes excess reagent and prepares the slide for application of the subsequent reagent.
3. The best Gram stained preparations are made with fresh cultures, i.e., those not older than 24 hours. As cultures age, especially in the case of gram-positive cells, the bacteria lose their ability to retain the primary stain and may appear gram-variable; i.e., some cells will appear purple while others will appear red.

OBSERVATIONS:

INFERENCE:

RESULT:

Expt. No: 6
Date :

SPORE STAINING

AIM: To identify endospore producing bacteria.

PRINCIPLE:

The function of endospores is to enable the bacteria to withstand unfavourable environmental conditions. It protects them from heat, UV rays and chemicals. *Bacillus* (aerobic bacteria) and *Clostridium* (anaerobic bacteria) show endospore formation.

Only some types of gram positive bacteria show endospore formation. Sporogenesis allows bacteria to survive unfavourable conditions. Endospore is a dormant, tough , and non-reproductive structure produced by certain bacteria. They form through the production of an encapsulating spore coat within the spore-forming cell. Germination of endospores will lead to the formation of vegetative cells. The tough outer layer is called as Exosporium and is composed of Keratin which provides resistance against unfavourable conditions . It is followed by Spore coat, Spore cortex and Core wall. The core contains the spore chromosomal DNA. The presence of DNA in endospores allows it to germinate once again and enter into vegetative state. Examples of bacteria having terminal endospores include *Clostridium tetani*, the pathogen that causes the disease tetanus and *Bacillus anthrasis* that causes Anthrax.

Spore formation is not good for other organisms as the spores remain dormant for millions of years and cause diseases. Inhalation, ingestion or skin contaminations of these endospores lead to a number of deaths.

REQUIREMENTS:

Cultures: 48-to 72 hour old nutrient agar slant cultures.

Reagents: Malachite green and safranine.

Equipment: Hot plate, staining tray, inoculating loop, glass slides, blotting paper and microscope.

PRIMARY STAIN:

Malachite Green can be used as a blue-green stain. It is used to directly stain spores.

COUNTER STAIN:

Safranine is a nuclear stain.It is used as the second reagent to colour the decolorized vegetative cells, which will absorb the counter stain and appear red. The spores retain the green of the primary stain.

PROCEDURE:

1. Take a clean slide and make individual smears in the usual manner using sterile technique.
2. Allow the smear to air dry, and heat fix in the usual manner.
3. Flood the smears with malachite green and place it on a hot plate allowing the preparation to steam for 2 to 3 minutes. **Caution: Do not allow stain to evaporate; replenish stain as needed.** The stain is prevented from boiling by adjusting the hot plate temperature.(The dye binds when it is heated).
4. Remove the slide from hot plate. Wash with tap water to remove excess stain.
5. Counterstain with safranine for 30 seconds.
6. Wash again with tap water.
7. Blot dry with blotting paper and examine under oil immersion.

OBSERVATION:

RESULT:

Expt. No: 7
DATE:

HANGING DROP TECHNIQUE

AIM:

To demonstrate motility of the given bacterial culture.

PRINCIPLE:

Microorganisms are two types: motile and non motile. Non motile microorganisms are called as atrichous. The motile microorganisms use their flagella to the movement. Many types of flagellar arrangement are present in the bacteria. The flagella cannot be seen under normal compound microscope; only the movement of bacteria can be seen using compound microscope. The flagella can be seen under the phase contrast, scanning electron microscope and transmission electron microscope. There are 2 methods to identify the bacterial motility

1) Microscopical- most widely used method. Because this method is simple and the motility can be seen within few minutes.

2) Agar deep method using TTC (Triphenyl Tetrazolium Chloride) medium

   In the TTC medium the Tetrazolium is a dye compound. The motile bacteria will diffuse around the agar medium from the inoculated line. This can be identified by the dye diffusion (red colour). The non motile bacteria will grow only on the line.

MATERIALS:

12 hour –old broth culture
Cavity slide
Cover slip
Vaseline/ Petroleum jelly
Tooth pick

PROCEDURE:

1. A hanging-drop slide is cleaned and placed on the table with the depression uppermost.
2. Spread a little Vaseline or petroleum jelly around the cavity of the slide.
3. A clean cover slip was taken and applied petroleum jelly on the four corners using a toothpick.
4. Placed the cover slip on a clean paper with petroleum jelly side up.
5. One loopful of culture was transferred to the centre of the cover slip.
6. Cavity slide is carefully kept over the drop on the cover slip, with the cavity facing down so that the depression covers the suspension.

7. Press the slide gently to form a seal between the cover slip and the slide.
8. The preparation is quickly lifted upside so that culture drop is suspended.
9. The preparation is examined under low power objective with reduced light.
10. Switched to high-power objective and examined the preparations again.
11. Placed a drop of oil on cover slip and examined the preparation under oil-immersion objective.

PRECAUTIONS:

1. If a bacterial growing on a solid medium is to be examined, a loopful of culture should be mixed with a drop of 2% CMC, in the centre of the cover slip.
2. The depression slide is inverted over the cover slip in such a way that the suspension does not touch the surface of concavity at any point.
3. The slide and cover slip should be sterilized after examination is finished.
4. Petroleum jelly from depression slide and cover slip should be removed at the end of the experiment, with xylene.

RESULT:

Expt. No: 8
DATE:

BIOCHEMICAL CHARACTERIZATION OF BACTERIAL ISOLATES

by

IMViC Test

The IMViC test is used to differentiate the Gram-negative enteric bacteria of the family *Enterobacter*iacae. The basis of differentiation is the biochemical diversity of different genera. The **IMViC** test includes four different tests such as **indole** production, **methyl red** test, **Voges-Proskauer** test and **citrate** utilization test.

INDOLE PRODUCTION TEST

There are some enteric bacteria which produce indole by hydrolysis of tryptophan with the enzyme tryptophanase. However there are few bacteria that do not produce indole. Indole production can be detected using Kovacs reagent that gives cherry red layer of reagent

$$\text{Tryptophane} \xrightarrow{\text{Tryptophanase}} \text{Indole} + \text{pyruvic acid} + H_2O$$

REQUIREMENTS

- Nutrient broth cultures of *Escherichia coli* and *Enterobacter aerogenes*
- Tubes containing 5ml of 1% tryptone broth
- Kovac's reagent
- Dropper bottle/1ml pipette
- Bunen burner
- Inoculating needle.

PROCEDURE

- Inoculated tryptone broth with *E.coli* and *E.aerogenes* . one uninoculated tube served as the control.
- Incubated the tubes at 35^{o}C for 48 hrs.
- After 48 hrs of incubation, added 1ml of Kovac's reagent to each tube including control.
- The tubes were gently mixed after intervals for 10-15 mins. Allow the tubes to stand to permit the reagent to come to the top. Examine the tubes for the tubes for the colour of the top layer.

OBSERVATION & RESULT

METHYL RED TEST AND VOGES-PROSKAUER TEST

AIM

To determine the ability of the bacteria to oxidize glucose with the production and stabilization of high concentrations of acid end products. To differentiate between all glucose-oxidizing enteric organisms, particularly *Escherichia coli* and *Enterobacter aerogenes.*

PRINCIPLE

The hexose monosaccharide, glucose, is the major substrate oxidized by all entericorganisms for energy production. The end products of this process will vary depending on the specific enzymatic pathways present in the bacteria. The enteric bacteria fallunder two groups-the mixed fermenters and 2,3-butanediol producers- as far as fermentation of glucose is concerned. The mixed fermenters produce large amount of acids and the butanediol fermenters produce neutral end products called acetoin (acetyl methyl carbinol). Since both the tests are performed simultaneously and on same medium, it is called MR-VP test. In this test the pH indicator methyl red detects the presence of large concentrations of acid end products. Up on the addition of methyl red if the medium turns yellow it shows negative test because the pH of the medium is increased to 6 or above due to production of ethanol and acetoin. To detect the presence of acetoin, Barrits reagents are used.

Although all enteric microorganisms ferment glucose with the production of organic acids, this test is of value in separation of *E.coli* and *E.aerogenes.* Both of these organisms initially produced organic acid end products during the early incubation period. The low acidic pH (4.6) is stabilized and maintained by*E.coli*at the end of incubation. During the later incubation period, *E.aerogenes* enzymatically converts these products to nonacidic end products such as 2, 3 butanediol and acetoin, resulting in an elevated pH of approximately 6. The methyl red indicator, in the pH range of 4 will turn red, which is indicating a positive test. At a pH of 6 still indicating an acid but with a lower hydrogen ion concentration, the indicator turns yellow and is negative test.

REQUIREMENTS

- 24 – 48 hrs old cultures of *E.coli*, *Enterobacter aerogenes,* and *Klebsiella pneumoniae*
- MR-VP broth
- Reagent: Methyl red indicator
- Barrits reagent I
- Barrits reagent II
- Bunsen burner
- Inoculating loop

Preparation of MR-VP broth

Peptone	- 7.0g
Glucose	- 5.0g
Potassium phosphate	- 5.0g
Distilled water	- 1000 ml

pH 6.1. 0.5ml broth was taken in test tubes and sterilized at 15lb/inch^2 for 15 min.

Voges-Proskauer test
reagents <u>Barrits reagent- I</u>
5% a-naphthol dissolved in absolute alcohol.
<u>Barrits reagent- II</u>
40% KOH

PROCEDURE

1. Using a aseptic technique, inoculate each bacterium appropriately labelled tube of medium by means of loop inoculation. The last tube served as a control
2. Incubate the cultures for 24 – 48 hrs at 37^{o}C.
3. After incubation tubes are tested by adding a few drops of methyl red indicator and observed for colour change of the broth
4. For VP test, add 10 drops of Barrit I reagent and 2 – 3 drops of Barrits II reagent. Mix well and kept aside under observation for up to 30 min.

OBSERVATION & RESULT:

CITRATE UTILIZATION TEST

AIM

This test is used to differentiate *E.coli* from *Enterobacter* because *E.coli* is not able to utilize citrate as its sole carbon source, whereas *Enterobacter* can use it . this test is based on the ability of the bacteria to utilize citrate as its only source of carbon and ammonia as its nitrogen source.

REQUIREMENTS

- Nutrients broth cultures of *E.coli* and *Enterobacteraerogenes*
- Simmons's citrate agar slants
- Bunsen burner
- Inoculating loop

Preparation of Simmon's citrate agar (pH6.9) slants

Ammonium dihydrogen phosphate	-	1.0g
Dipotassium phosphate	-	1.0g
Sodium chloride	-	5.0g
Sodium citrate	-	2.0g
Magnesium sulphate	-	0.2g
Agar	-	15g
Bromothymol blue	-	0.8g
Distilled water	-	1000ml

PROCEDURE

Inoculated simmon's citrate agar slants by means of a stab and streak inoculation. One uninoculated control is also inoculated. All the slants are incubated at 37^{o}C for 48 hrs. observe the slant cultures for the growth and colouration of the medium.

OBSERVATION & RESULT:

Expt. No: 9

Date:

DETERMINATION OF ANTIBIOTIC SENSTIVITY BY KIRBY-BAUER ASSAY

AIM

To determine the sensitivity of bacterial isolates to antibiotics using Kirby-Bauer method of agar disc diffusion.

PRINCIPLE

Rapidly growing test bacterium is lawn plated on the surface of Muller-Hinton agar plates. Antibiotic disc containing specific concentration of particular antibiotic is pressed on to the surface and incubated at 35°C overnight. Zone of inhibition of growth around each disc is measured and the susceptibility determined by comparing with a standard chart.

Microorganisms may also show resistance towards antibiotics which can be of two types-

Intrinsic resistance: Inborn resistance of a microorganism to an antibiotic.

Acquired resistance: Resistance gained due to previous exposure to an antibiotic.

In bacteria, antibiotic resistance is conferred by plasmid DNA which carries antibiotic resistance gene sequences.

Commonly used antibiotics are Ampicillin, Tetracyclin, Streptomycin and Penicillin.

There are various methods to detect antibiotic sensitivity:

1. **Disc diffusion method**

Antibiotic discs are place in Muller Hinton agar plates and diameter of zone of inhibition is measured to determine the concentration of the antibiotic for which the bacteria is susceptible for growth inhibition

Minimum inhibitory concentration is the minimum antibiotic concentration which causes maximum growth inhibition.

2. **Broth dilution method**

Microorganisms are inoculated in broth at 37^0 C and incubated. Check for turbidity and measure the optical density using colorimeter of UV-Vis spectrophotometer. This method is based on

Beer Lambert's law, according to which absorbance is directly proportional to the concentration of the solution.

3. Molecular biology techniques

These techniques involve PCR amplification and DNA hybridization of genetic material of the desired microorganism to detect the presence of antibiotic resistance genes.

REQUIREMENT

Muller Hinton agar
Bacterial strains
Antibiotics Discs
Sterile swabs for lawn cultures

PROCEDURE

Inoculated the surface of Muller-Hinton agar plates with actively growing culture broth using a sterile cotton swab. To ensure that the growth is uniform and confluent, pass the swab at least three times over the entire surface of the plate. Antibiotic discs are applied in such a way that each disc is made to adhere perfectly to the surface of the agar by gentle pressing. Disc should be at least 2cm apart to prevent the merging of zones of inhibitions. The plates are incubated at 37^0C for 24 hours. The zone of inhibition of individual discs are observed and the diameters are noted down in mm.

OBSERVATION

Plates are examined for the presence and size of inhibitory zones. The diameter is measured with a scale and including the diameter of the disc. The diameter is compared with the standard chart for antibiotic sensitivity and interpreted the result as sensitive, intermediate and resistant.

Also, the extent of inhibition of growth of microorganism is proportional to the volume of antibiotic used.

RESULT

Antibiotic sensitivity test was performed on *Bacillus* spp. and *E.coli* and the following results were obtained:

1. ***Bacillus*** spp. is **resistant** to the antibiotic used
2. ***E. coli*** is **sensitive** to the antibiotic used

Expt. No: 10

Date:

HYDROLYSIS TEST

I. CASEIN HYDROLYSIS

AIM :

To demonstrate the ability of hydrolysis of casein by the given bacterial culture.

PRINCIPLE:

The enzyme protease is secreted out of the cells (an **exoenzyme**) into the surrounding media, catalyzing the breakdown of milk protein, called casein, into small peptides and individual amino acids which are then taken up by the organism for energy use or as building material. The hydrolysis reaction causes the milk agar, normally the opacity of real milk, to clear around the growth area as the casein protein is converted into soluble and transparent end products—small chains of amino acids, dipeptides, and polypeptides.

REQUIREMENTS:

- 24-hour to 48-hour nutrient agar slant cultures of *Bacillus subtilis*, *E. coli* and *Proteus vulgaris*
- Skim milk agar plates
- Inoculating loop
- Bunsen burner
- Wax marking pencil

PROCEDURE :

Labelled skim milk agar plates with the name of the bacterial organisms to be inoculated and one control. Single line streaks are made with all the test cultures into its appropriately labelled petriplate. Incubated the petriplates for 24-48 hours at 37°C in an inverted position and observe for clear zones.

OBSERVATION & RESULT:

II. STARCH HYDROLYSIS

AIM:

Starch is broken into monomer units by the enzyme amylase produced by some bacteria. Starch reacts with iodine and develops a blue coloured complex. Moreover, intensity of the colour developed is directly proportional to the concentration of starch present in the sample.

PRINCIPLE:

Starch is a polysaccharide made up of α -D-glucose subunits. It exists as a mixture of two forms, linear (amylose) and branched (amylopectin), with the branched configuration being the predominant form. The a-D-glucose molecules in both amylose and amylopectin are bonded by 1,4-α-glycosidic (acetal) linkages. The two forms differ in that the amylopectin contains polysaccharide side chains connected to approximately every 30th glucose in the main chain. These side chains are identical to the main chain except that the number 1 carbon of the first glucose in the side chain is bonded to carbon number 6 of the main chain glucose. The bond is, therefore, a 1,6-α-glycosidic linkage.

Starch is too large to pass through the bacterial cell membrane. Therefore, to be of metabolic value to the bacteria it must first be split into smaller fragments or individual glucose molecules. Microorganisms that produce and secrete the extracellular enzymes a-amylase and oligo-l,6-glucosidase are able to hydrolyze starch by breaking the glycosidic linkages between the sugar subunits. Although there usually are intermediate steps and additional enzymes utilized, the overall reaction is the complete hydrolysis of the polysaccharide to its individual a-glucose subunits.

$$\text{Starch} \xrightarrow{\text{Amylase}} \text{Monosaccharides + Disaccharides}$$

REQUIREMENTS :

- Nutrient agar slant cultures of *Bacillus subtilis* and *Escherichia coli*.
- Gram's Iodine solution
- Starch agar
- Sterile petri dishes
- Dropper
- Inoculating loop
- Bunsen burner

PREPARATION OF STARCH AGAR:

Yeast extract	-	0.3g
Malt extract	-	0.3g
Peptone	-	0.5g
Soluble starch	-	1.0g
Distilled water	-	1 litre

pH : 6.1

sterilised at 10lb pressure for 20min and poured into sterile plates.

PROCEDURE:

1. Labelled each of the starch agar plate with the name of the organisms to be inoculated.
2. Using sterile technique, made a single streak inoculation of the test bacteria into the centre of the plates.
3. Incubated the bacterial inoculated plates for 48 hours at 37 °C in an inverted position.
4. The surface of the plates is flooded with iodine solution with a dropper for 30 seconds.
5. Poured off the excess iodine solution and observed for the development of clear zone of hydrolysis against dark purple colour.

OBSERVATION & RESULT:

Expt. No.:11

Date:

DETERMINATION OF ANTIBIOTIC SENSITIVITY BY KIRBY-BAUER ASSAY

AIM:

To determine the sensitivity of bacterial isolates to antibiotics using Kirby-Bauer method by agar disc diffusion technique.

PRINCIPLE:

An antibiotic is an antimicrobial chemical compound produced by microorganisms against other microorganisms. These antimicrobial compounds can be used for the treatment of microbial infections in human beings. The purpose of the Kirby-Bauer disk diffusion susceptibility test is to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds. The pathogenic organism is grown on Mueller-Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism. During incubation, the antibiotic diffuses from the disc into the agar in decreasing amounts the further it is away from the disc. If the organism is killed or inhibited by the concentration of the antibiotic, there will be no growth in the immediate area around the disc which is called as the zone of inhibition. In this test, results are usually reported as sensitive, intermediate, or resistant, based on the size of the zone of inhibition. If the observed zone of inhibition is greater than or equal to the size of the standard zone, the microorganism is considered to be sensitive to the antibiotic. Conversely, if the observed zone of inhibition is smaller than the standard size, the microorganism is considered to be resistant. The antibiotic sensitivity test is also used to identify the organisms based on its sensitivity to various antibiotics.

MATERIALS REQUIRED:

Muller Hinton agar plates, Bacterial strains, Antibiotics Discs, Sterile swabs for lawn cultures

PROCEDURE:

1. The surface of Muller-Hinton agar plates were inoculated with the well grown culture broth using a sterile cotton swab.
2. To ensure the uniform and confluent growth, the swab was passed over the entire surface of the plate for at least three times.
3. Each antibiotic disc was made adhere perfectly to the surface of the agar by gentle pressing. Disc should be at least 2cm apart to prevent the merging of zones of inhibitions.
4. The plates were incubated at 37℃ for 24 hours. The zone of inhibition around individual disc was observed and the diameters were noted down in mm.

RESULTS & INTERPRETATION:

Expt. No.: 12
Date:

DIFFERENTIATION OF LIVE AND DEAD CELLS BY FLUORESCENCE MICROSCOPY-DEMONSTRATION

AIM:

To differentiate the live and dead bacterial cells upon exposure to antibiotics using fluorescence microscopy.

PRINCIPLE:

Fluorescence microscope has enormous applications in various research fields. In bacteriology, the fluourescence microscope is used to differentiate the live and dead bacterial cells from the bacterial cultures of before and after antibiotic treatment by subjecting the bacterial cells to dual staining method using 4,6-diamidine-2-phenylindole dihydrochloride (for live cells) and Propidium Iodide (PI) (for dead cells). DAPI (4', 6-diamidino-2-phenylindole) is a fluorescent dye which can bind DNA strands robustly and it can dye both live and fixed cells as it can cross intact membrane, with higher efficiency in fixed cells. DAPI could pass through the cell and nucleic membranes and bind the double-strand DNA in the nucleus, producing 20 times stronger fluorescence than itself. The efficiency detected by fluorescence microscope is very high (almost 100%), having no side effects for the live cells. Propidium iodide (PI) is a membrane impermeant dye that is generally excluded from viable cells. It can only pass through disordered areas of membrane of dead cells and intercalates with the DNA of the nuclei, emitting red fluorescence light.

MATERIALS REQUIRED:

Preparation of DAPI (4, 6-diamidine-2-phenylindole dihydrochloride) stock solution:

Dissolved 1mg /ml of powder in PBS (pH 7.2) and make appropriate aliquots that can be stored at -20°C.

Phosphate buffered saline (PBS) solution (1L):

1. Dissolved 0.69 g $NaH_2PO_4.H_2O$, 1.34 g $NaH_2PO_4.7H_2O$ and 7.6 g NaCl in 800 ml of distilled water.
2. Adjust pH to 7.5 with 1N HCl or 1N NaOH
3. Made to 1L with distilled water.
4. Autoclaved at 121°C for 15 minutes.

CAUTION: Use caution when using DAPI; it can potentially bind with your own DNA if you spill it on yourself. Wear a pair of latex gloves for the adequate protection while using DAPI.

Preparation of Propidium Iodide (PI) stock solution:
Propidium Iodide Stock solution: 1 mg/ml in PBS (pH 7.5).
PROCEDURE:

1. The mid log culture was inoculated in the Muller Hinton broth and the optical density of the initial cell concentrations were noted.
2. One mg/ml of antibiotic was added to the above culture tube and incubated at 37°C for 24 hours.
3. The inhibition of growth of the microorganisms by the antibiotics was determined by measuring the optical density (turbidity) of the microorganisms.
4. One ml of bacterial culture before or after the treatment with antibiotics is collected in clean, sterile test tube.
5. Added 25µl DAPI and PI stains from stock solution and allowed standing incubation for 20 minutes.
6. After 20 minutes, 1 drop of sample was placed on top of the glass slide and then the cover slip was kept above the sample without any air bubbles.
7. Removed the excess of liquid and observed under fluorescence microscope.

** [DAPI (excitation 358 nm, emission 461 nm).Propidium iodide (excitation 536 nm, emission 617 nm)].

RESULTS & INTERPRETATION:

Exp.No.13 Identification of bacterial strains by using 16S rRNA gene sequencing Demonstration

DATE:

Objectives:

- To study the technique involved in the sequencing of a gene.
- To learn the importance of 16s ribosomal RNA in the identification of bacteria

16S Ribosomal RNA (rRNA) sequencing

Ribosomes are complex structures found in all living cells which functions in protein synthesis machinery. Basically ribosome's consists of two subunits, each of which is composed of protein and a type of RNA, known as ribosomal RNA (rRNA). Prokaryotic ribosomes consist of 30S subunit (small sub unit) and 50S subunit (large sub unit) which together make up the complete 70S ribosome, where S stands for Svedberg unit non-SI unit for sedimentation rate. 30S subunit is composed of 16S ribosomal RNA and 21 polynucleotide chains while 50S subunit is composed of two rRNA species, the 5S and 23S rRNAs. The presence of hyper variable regions in the 16S rRNA gene provides a species specific signature sequence which is useful for bacterial identification process. 16S Ribosomal RNA sequencing is widely used in microbiology studies to identify the diversities in prokaryotic organisms as well as other organisms and thereby studying the phylogenetic relationships between them.

In the past years, conventional microbiology techniques such as culturing of microorganisms, biochemical tests and other related methods are used worldwide to identify most of the bacteria, fungi and other pathogens, still it takes about 8 to 20 hours for an accurate result. New diagnostic techniques have been developed to overcome the limitations of conventional microbiological methods for identifying etiological agents of infections and other microorganisms. Nucleic acid based detection methods help in the detection of genomic materials and thus many genetic or infectious diseases can now be diagnosed by performing a study of relevant DNA sequence by nucleic acid–based techniques.

16S ribosomal RNA (rRNA) sequencing is a common amplicon sequencing method used to identify and compare bacteria present within a given sample. It is a well–established method for studying phylogeny and taxonomy of samples from complex microbiomes or environments that are difficult or impossible to study.

Applications of 16S Ribosomal RNA in microbiology

1. 16S rRNA gene sequencing has been established as the "gold standard" for identification and taxonomic classification of bacterial species.
2. Comparison of the bacterial 16S rRNA sequence has been emerged has a viable genetic technique and can lead to the recognition of novel pathogens such as Mycobacterium species.
3. The hyper variable regions of 16S rRNA gene sequence provide species-specific signature sequences useful for bacterial identification.
4. In medical microbiology,16S rRNA sequencing serves as a rapid and cheap alternative to phenotypic methods of bacterial identification.

5. It is also capable of reclassifying bacteria in to completely new species, or even genera.
6. The sequencing techniques can be used to describe new species that have never been successfully cultured in laboratories.

Steps involved:

Isolation of bacteria

Bacterial DNA extraction

Amplification of 16S rRNA genome

Sequence a portion of the 16S rRNA gene

Compare the sequenced gene with NCBI to obtain a match

Construction of phlogenetic tree

Steps Involved in the Phylogentic tree construction:

Obtained sequence of unidentified bacterial strain

>MPA4-16s-Forward

AACACATGCAAGTCGAGCGGGGAGATGTAGCTTGCTACATTTCCTAGCGGCGGACGGGTGAGTAA
TGCTTAGGAATCTGCCTATTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGCC
CTACGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCAGATTAGCTA
GTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACA
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GATCTGGAGGAATACCGATGGCgAAgGCAGCCATCTGGCCTAgTACTGACgCTGAgGTACgAAAGCA
TGGGGAGCAAACAGGATTAGATACCCTGgtagtCCATGCCgtAAACgATGtc

>MPA4-16s-Reverse
TCCTCCTTGCGGTTAGACTACCTACTTCTGGTGCAACAAATTCCCATGGTGTGACGGGCGGTGTGT
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GGAGTCGAGTTGCAGACTCCAATCCGGACTACGATCGGCTTTTTGAGATTAGCATCCTATCGCTAG

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TTGACGTCGTCCCCGCCTTCCTCCAGTTTGTCACTGGCAGTATCCTTAAAGTTCCCGGCTTAACCC
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CGCGTTAGCTGCGCCACTAAAGCCTCAAAGGCCCCAACGGCTAGTAGACATCGTTTAcGGCATGG
ACTACCAGGGTATCTAATCCTGtTTgCTCCCCaTGCTTTCgtACCTCAGCGtCAGTATTAGGCCAgATG
GcTGcCTTCgCCatCGGTATTCCTCCC

>MPA4-16s-Full length-1404 bp

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TGCTTAGGAATCTGCCTATTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGCC
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AGGAGGAGGCTACTAGTACTAATACTACTGGATAGTGGACGTTACTCGCAGAATAAGCACCGGCT
AACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAA
AGCGTACGTAGGCGGCTTTTTAAGTCGGATGTGAAATCCCTGAGCTTAACTTAGGAATTGCATTCG
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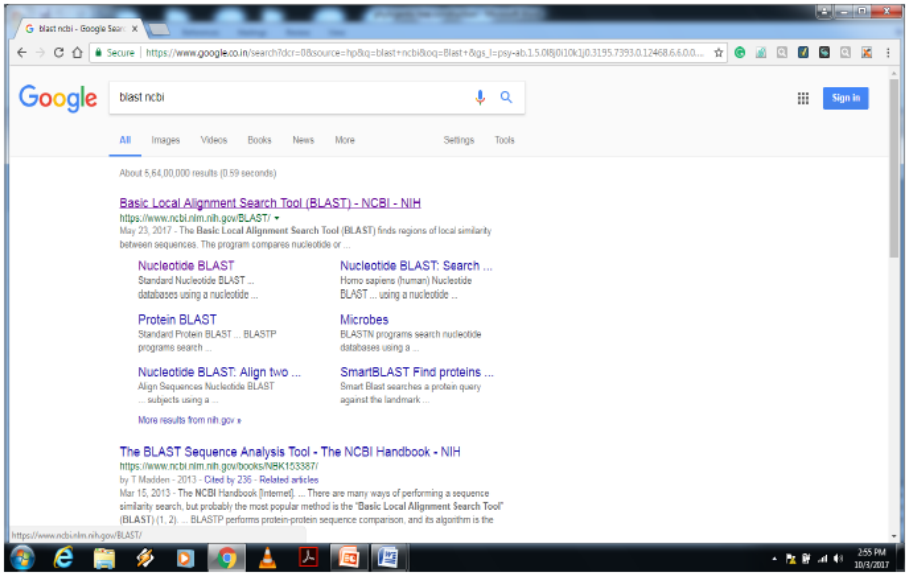

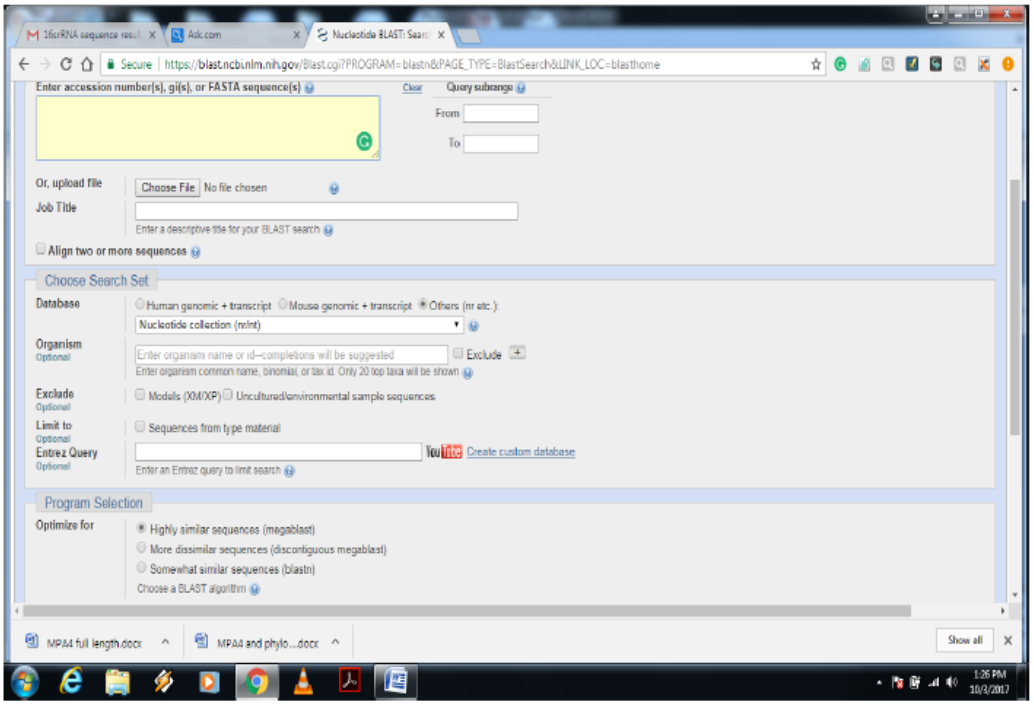
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Enter accession number(s), gi(s), or FASTA sequence(s)
Clear
Query subrange
From
To
Or, upload file
Choose File
No file chosen
Job Title
Enter a descriptive title for your BLAST search
Align two or more sequences
Choose Search Set
Database
Human genomic + transcript
Mouse genomic + transcript
Others (nr etc.)
Nucleotide collection (nr/nt)
Organism
Optional
Enter organism name or id--completions will be suggested
Exclude
Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown
Exclude
Optional
Models (XM/XP)
Uncultured/environmental sample sequences
Limit to
Optional
Sequences from type material
Entrez Query
Optional
Create custom database
Enter an Entrez query to limit search
Program Selection
Optimize for
Highly similar sequences (megablast)
More dissimilar sequences (discontiguous megablast)
Somewhat similar sequences (blastn)
Choose a BLAST algorithm
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Show all
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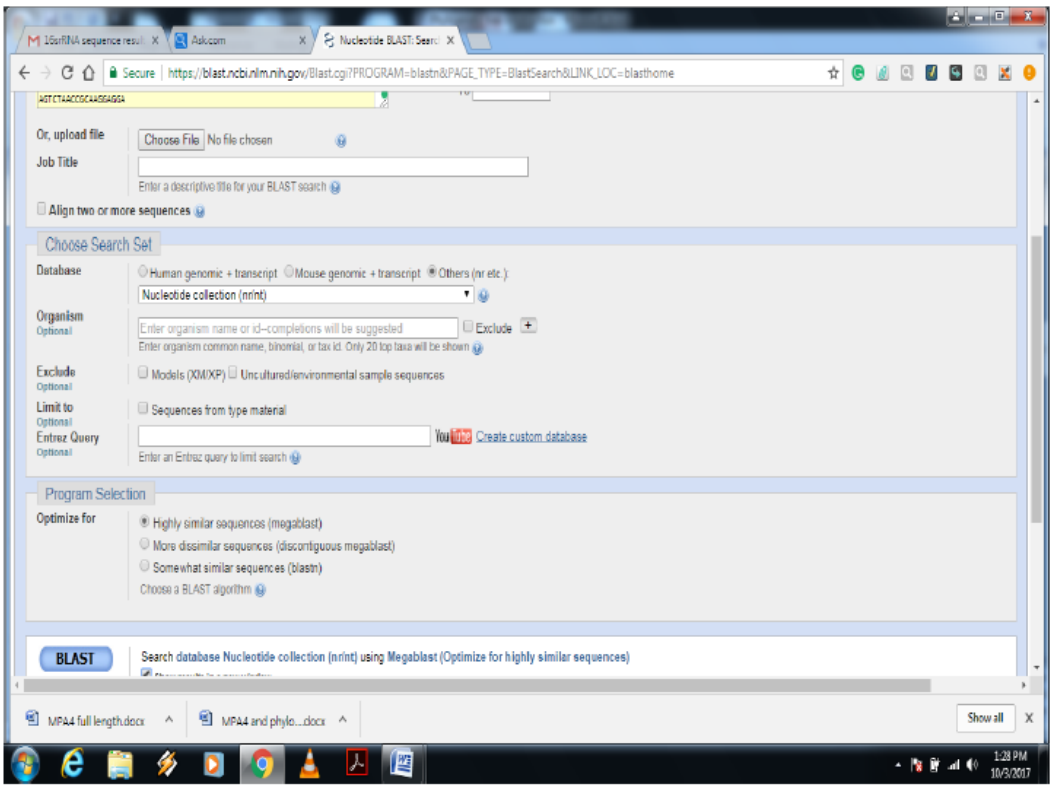
Secure | https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome
Or, upload file
Choose File
No file chosen
Job Title
Enter a descriptive title for your BLAST search
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Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown
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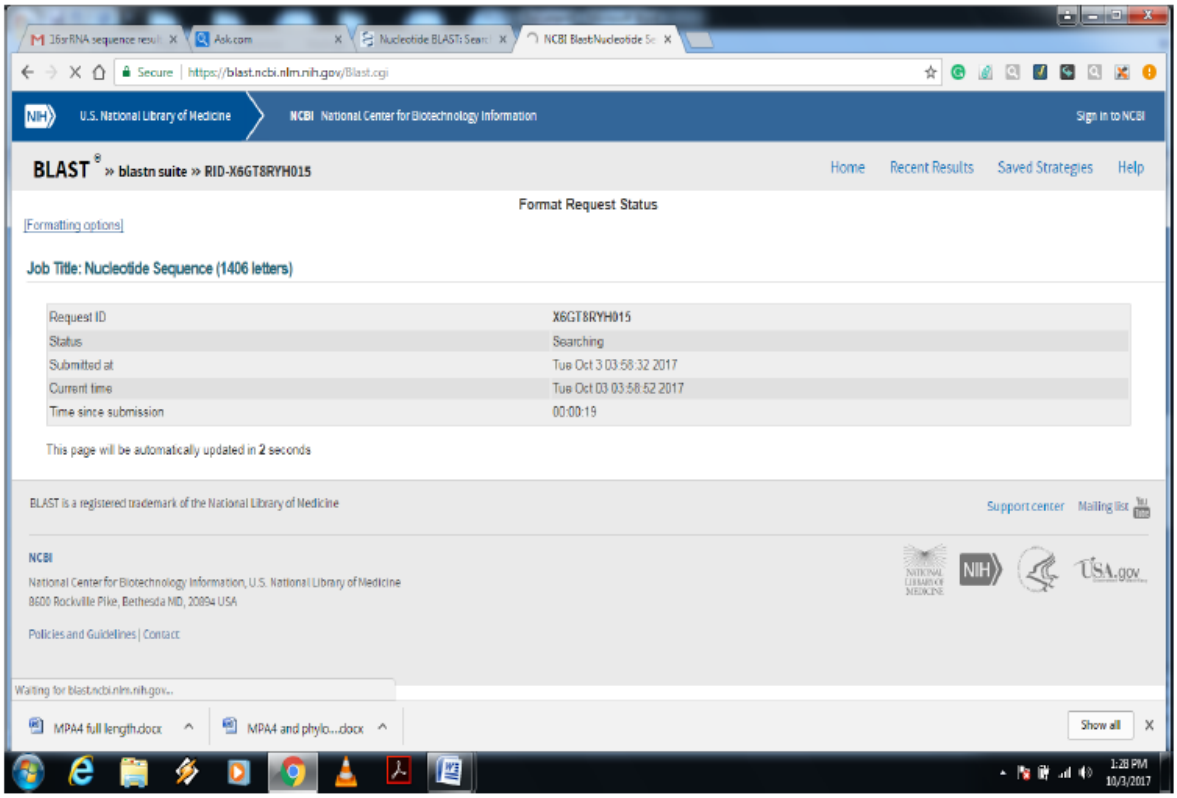
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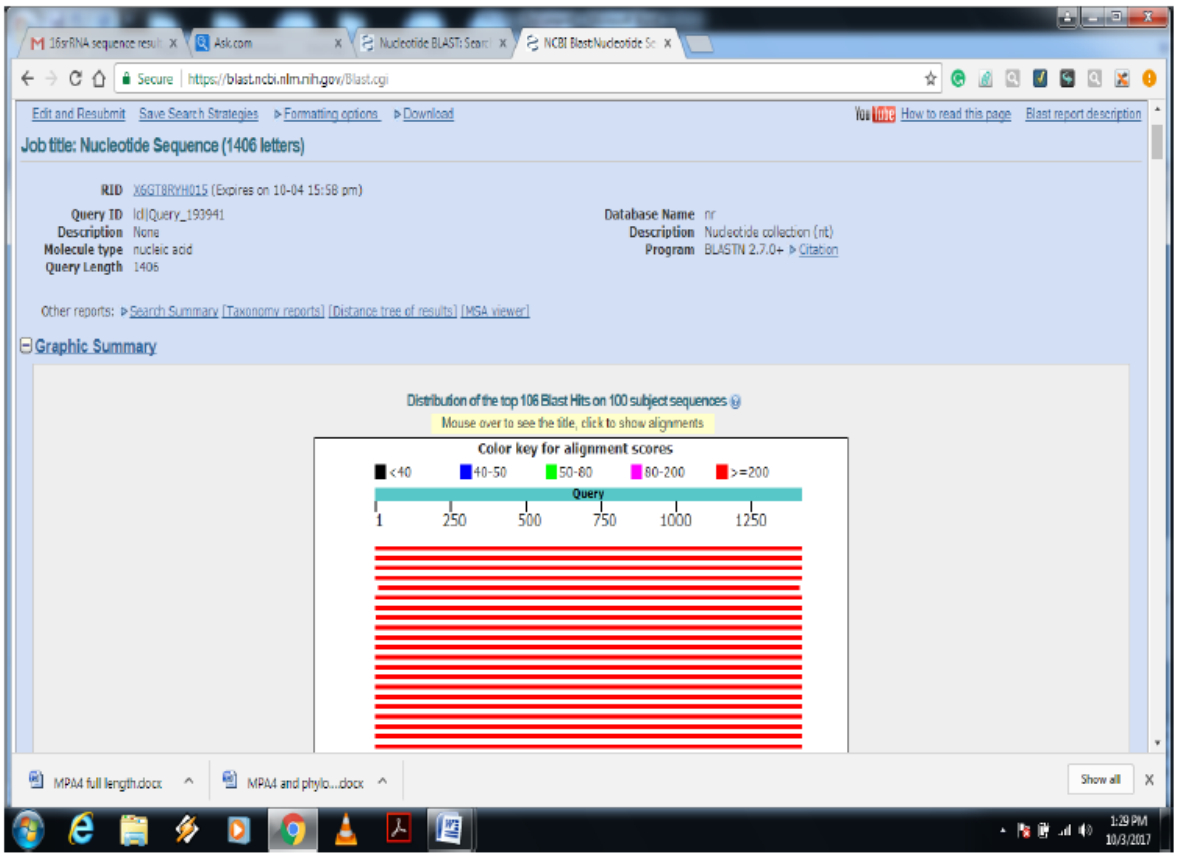
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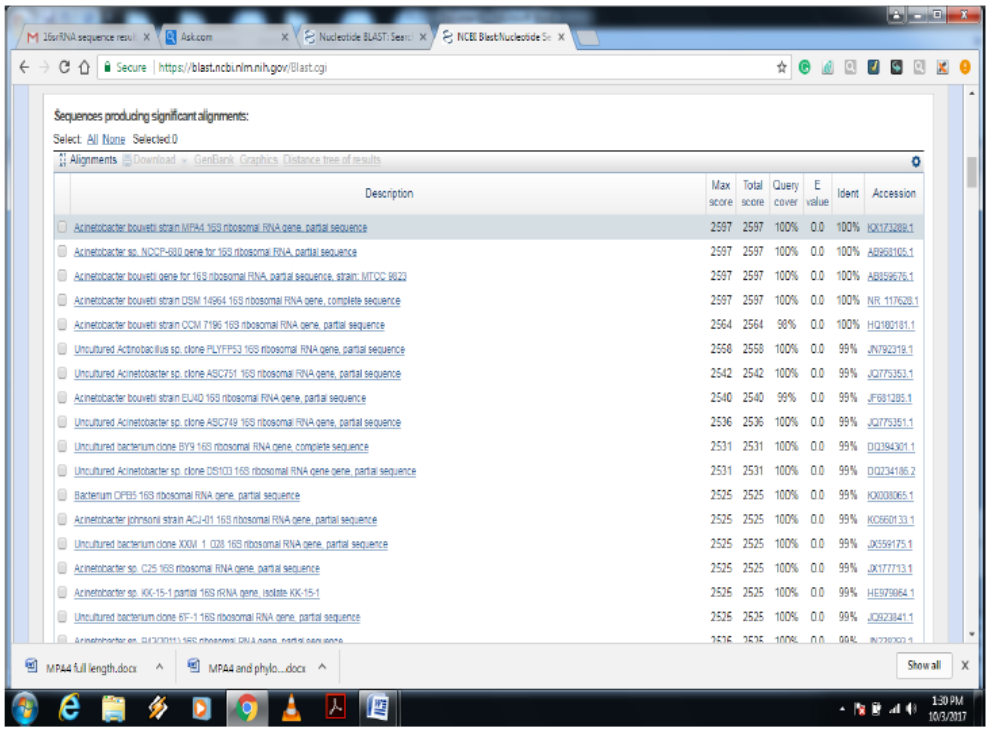

Phylogenetic Tree Construction

>MPA4 (Present Study-unidentified strain)

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> Acinetobacter johnsonii (NR_044975.1)

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>Acinetobacter bouvetii (NR_117628.1)

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>Acinetobacter schindleri (NR_025412.1)

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>Acinetobacter lwoffii (NR_113346.1)

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Acinetobacter kyonggiensis(NR_116714.1)

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>Acinetobacter gandensis (NR_133953.1)

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>Acinetobacter pittii(NR_117621.1)

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>Acinetobacter parvus(NR_025425.1)

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>Acinetobacter nosocomialis(NR_117931.1)

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>Acinetobacter tandoii(NR_028853.1)

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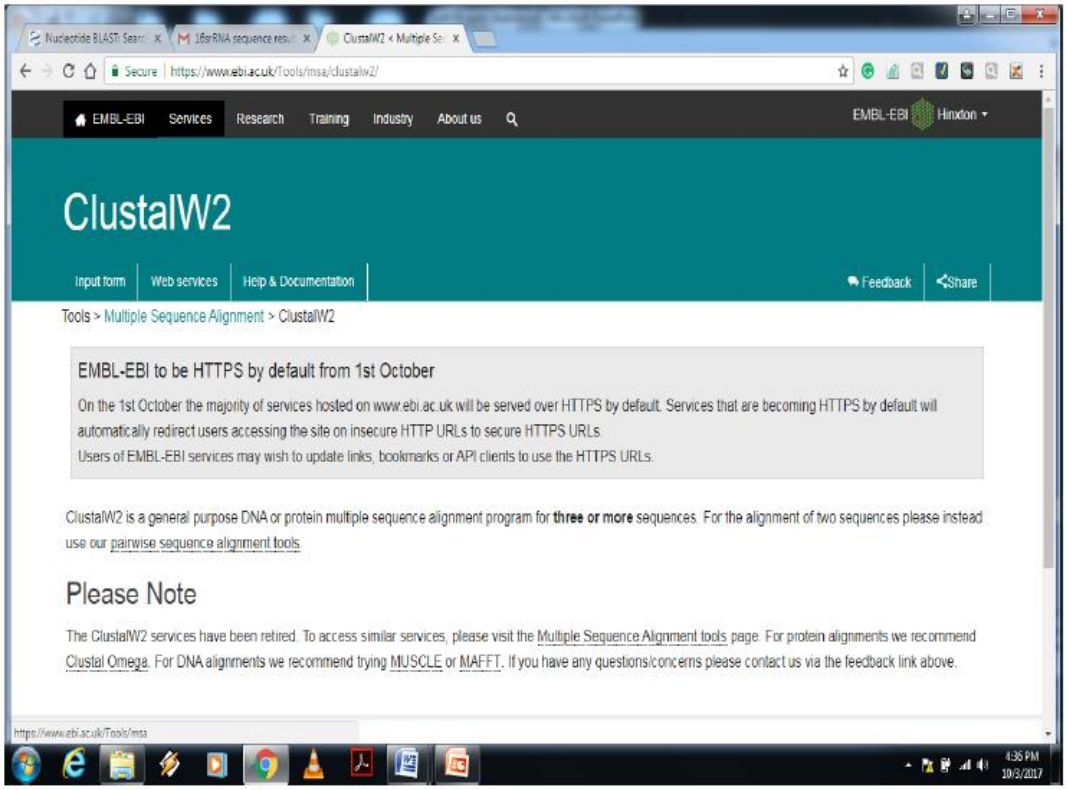
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EMBL-EBI to be HTTPS by default from 1st October
On the 1st October the majority of services hosted on www.ebi.ac.uk will be served over HTTPS by default. Services that are becoming HTTPS by default will automatically redirect users accessing the site on insecure HTTP URLs to secure HTTPS URLs.
Users of EMBL-EBI services may wish to update links, bookmarks or API clients to use the HTTPS URLs.
ClustalW2 is a general purpose DNA or protein multiple sequence alignment program for three or more sequences. For the alignment of two sequences please instead use our pairwise sequence alignment tools.
Please Note
The ClustalW2 services have been retired. To access similar services, please visit the Multiple Sequence Alignment tools page. For protein alignments we recommend Clustal Omega. For DNA alignments we recommend trying MUSCLE or MAFFT. If you have any questions/concerns please contact us via the feedback link above.
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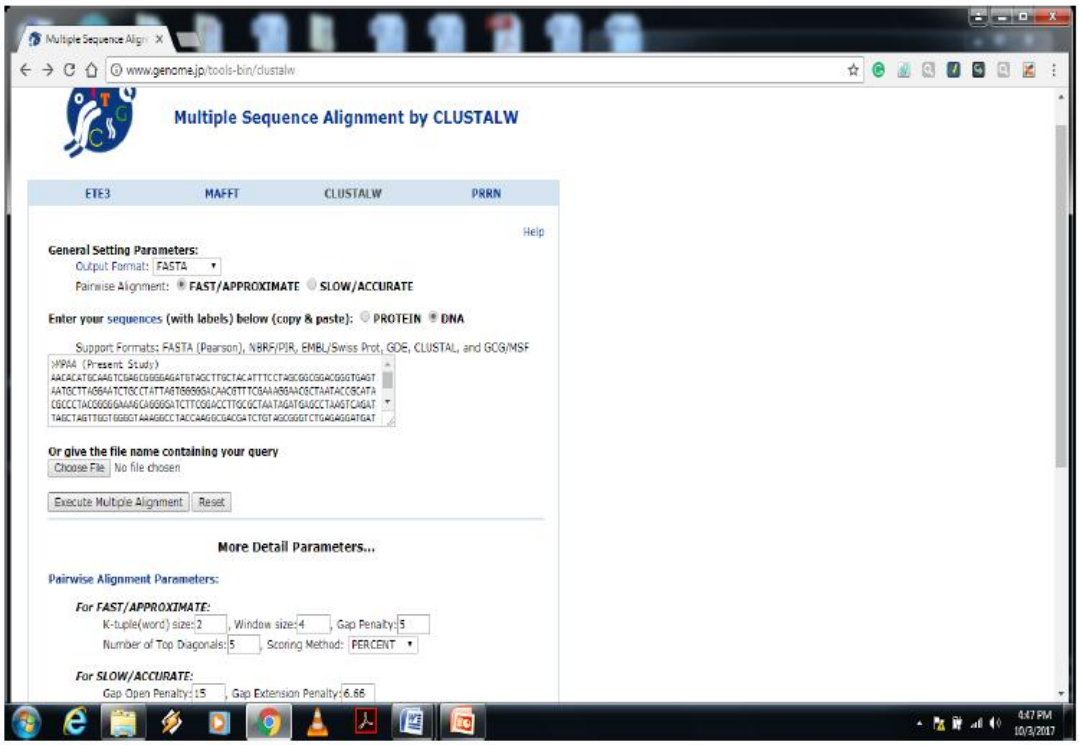
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Or give the file name containing your query
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More Detail Parameters...
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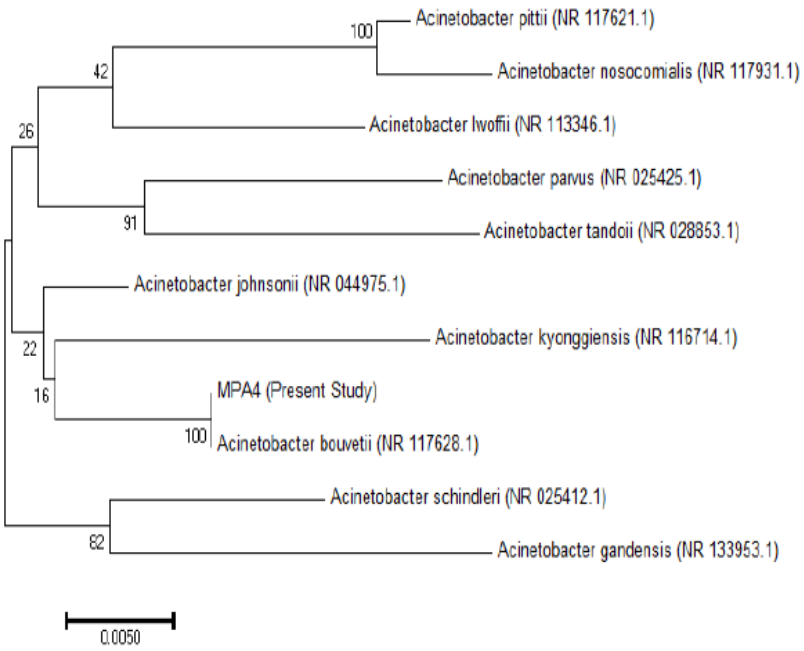

The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA7.

Results:

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