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Microbial Biotechnology Review in Laboratory Safety, Media Formulation, Microbial Isolation and Methods for Identification of Microorganisms

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ABSTRACT

There is growing interest in research and development to develop novel tools to study, detect, and characterize microbes and their communities in various areas. However, knowledge about their validity in practical is still scarce. This review describes the advantages of safety security in the laboratory and microbial screening and molecular methods used for identifications. In addition, the review addresses the importance of isolating the microorganisms from different agro ecology and the possibilities and future prospects for exploiting the described methods in different biotechnology areas.

Introduction

Microorganisms (microbes) are such life forms which cannot be seen by nacked eye. These microbes are omnipotent with nature that their existence is proved everywhere. They are present in soil, animal intestine, water, air, food and even with extreme condition like within rocks, in glaciers, in hot springs and so on.

Microbial biotechnology (MB) explores and exploits the beneficial microbial wealth for various human requirements. Human made lot many modifications toward microbial genomes with the help of biotechnological tools that improved the potency of microbes in terms of desired criteria accordingly the need.

There is different application of microbes in biotechnology. Thus microbes (microorganisms) are very important in agriculture, in industry, in medicine, in environment and in other molecular biology. This book contains three parts .The first part is safety techniques, the second one is media formulation and solution preparation and the third is microbiological techniques. Here with the specific area the book deals with microbial safety techniques, standard based media formulation and improvement according to the product to be isolated and methods to isolate and purify the required microbial strain out of mixed population and its various strain improvement techniques up to genetic level.

1.1. Safety rule of microbial biotechnology laboratory

Documentation: The lab note book.

Documentation in lab note book is very essential skill for any biotecnicial. An important parts of this documentation process is to record what equipment and materials where used and to show that the equipment and materials were validated and used in correct manner.

The objectives of these are:-

- To record what an individual has done and observed
- To prove that a procedure was done correctly
- To adhere, evaluate, and develop standard operating procedures (SOP)

1.2. Laboratory safety and security

Number of chemicals used in the laboratory is hazardous and can Couse harm to human and environment. It is important that laboratory personnel take specific actions to prevent unauthorized entry to labs, secure highly hazardous materials against theft, and ensure compliance new security regulation. Hence, the following objectives are helps to attain and benefit from safety and security to intervene the negative impacts associated with hazardous materials.

Objective of Safety and Security

- To recognize the correct procedure for storing and handling hazardous materials
- To find information on the classification of chemical hazards
- To know what types of health hazards a chemical may pose
- To know what personal protective equipment is required for handling a hazardous chemical
- To locate the lab safety equipment

This is often the responsibility of any bio technician to make sure that safety rules are followed, and any one working in the laboratory must pay attention to what they are doing and use common sense to avoid hazardous chemical situations.

It is important that laboratory personnel take specific actions to prevent unauthorized entry to labs, secure highly hazardous materials against theft, and ensure compliance new security regulation. Due these the following laboratory safety instructions are very important.

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- Do not eat or drink in the laboratory.
- Wear laboratory coats and gloves while working in the laboratory
- Wipe the working area with a disinfectant at the beginning and end of the laboratory session.
- Avoid any activity that introduces objects into the mouth, e.g. mouth pipetting. Cover any open cuts on hands and other exposed skin surfaces with a water resistant dressing.
- Carry out all procedures in a way so as to minimize the risks of spills, splashes and the production of aerosols.
- Always wash your hands before leaving the laboratory. Benches should be clear of all non-essential materials including books and notes

1.3. Lab equipment and reagent orientation

Before beginning to work in laboratory, is best to get an idea of where things are stored. This is to identify common lab equipment and describe their functions as well as to understand the role of the reagents you use in the laboratory

Lab courtesy

❖ If your lab session has been messy or you have worked with organism or hazardous chemicals, you have to clean the bench tops, laminar flow wood with bleach, ethanol or soapy water**.**

❖ Never place disposal box, white bio hazard envelopes on the bench and any sharp materials and broken glass in domestic bin

❖ Make sure to turn off equipment when you leave the lab.

Solution/Sample Storage

- Please thoroughly label everything you store in a refrigerator or freezer.

2. Media formulation and solution preparation

Bacteria and fungi are grown on or in microbiological media of various types. The medium that is used to culture the microorganism depends on the microorganism that one is trying to isolate or identify. Different nutrients may be added to the medium, making it higher in protein or in sugar. Various pH indicators are often added for differentiation of microbes based on their biochemical reactions: the indicators may turn one color when slightly acidic, another color when slightly basic. Other added ingredients may be growth factors, NaCl, and pH buffers which keep the medium from straying too far from neutral as the microbes metabolize.

The only time that is done today is when making a special medium to grow a certain finicky organism, where particular growth factors, nutrients, vitamins, and so on, have to be added in certain amounts. This medium is called a chemically defined medium (synthetic). Fortunately, the most common bacteria that we want to grow will do nicely with media that we commonly use in lab. Some of our media is bought, but most is produced in the prep area behind the lab. Since this type of medium has some unknown ingredients, or sometimes unknown quantities it is called complex media.

2.1. Culture media for fungi

A wide range of media are used for growing fungi. But most preferences for certain types of media based on experience and peculiarities of the type of fungi that are routinely grown. Media will affect colony morphology and color, whether particular structures are formed or not, and may affect whether the fungus will even grow in culture. For example, some fungi lack the necessary enzymes to utilize different carbon sources. All fungi require several specific elements for growth and reproduction. The requirements for growth are generally less stringent than for sporulation, so it is often necessary to try several types of media when attempting to identify a fungus in culture.

Most fungi thrive on Potato Dextrose Agar (PDA), but this can be too rich for many fungi, so that excessive mycelial growth is obtained at the expense of sporulation. I have found that most of the fungi isolated from soil, or from substrates in the soil, i.e., plant debris, grow well on Corn Meal Agar (CMA), a relatively weak medium compared to PDA.

Cellulose-destroying fungi and spoilage fungi retain their ability to produce cellulose when grown on a weak medium such as Water Agar (WA) or Potato Carrot Agar (PCA) with a piece of sterile filter paper, wheat straw or lupin stem placed on the agar surface. The introduction of pieces of tissue, such as filter paper, wheat straw, rice, grains, leaves or dung, often produces good sporulation dependent on the organism grown

2.1.1. Constituents of Media

Media generally contain a source of carbon, nitrogen and vitamins. Glucose (dextrose) is the most widely utilizable carbon source, and hence is the most commonly used in growth media. Fructose and mannose are the next most commonly utilized sugars by fungi and are found in media from natural sources. Sucrose (table sugar) may be used in some media. Nitrogen sources include peptone, yeast extract, malt extract, amino acids, ammonium and nitrate compounds.

Casamino Acids, a Difco product, is acid-hydrolized casein, a mixture of amino acids. It is a good general source of nitrogen but is vitamin free. Bacto-Peptone, another Difco product, contains nitrogen and a high peptone and amino acid content. Salts, including Fe, Zn and Mn, are often added to 'defined' media, but are usually not added to the common media used for routine culture. Fungi have natural deficiencies for vitamins that are satisfied at µM to nM concentrations. The most common naturally occurring vitamin deficiencies are thiamin and biotin. Deficiency of both is quite common among the Ascomycota. Other organic nutrients such as glucose are often contaminated with vitamins sufficient to supply the growth requirements of fungi.

2.1.2. Growth Media for fungi

Ascomycota isolated from fruiting bodies forming on dung, wood or soil can be grown on Malt Agar, Potato Carrot Agar and Potato Dextrose Agar. The first step is to attempt to get the ascospores to germinate by streaking them out or getting forcible discharge onto a selective or isolation medium. Look for spore germination and transfer a small piece of agar with the germinating spore(s) to one of these richer media. Basidiomycota growing on wood can be grown on Malt Extract Agar. Cultures derived from mushrooms can be grown on Potato Dextrose Agar, Potato Sucrose Agar and Malt Extract Agar. Potato Dextrose Yeast Extract Agar (YPDA) also is a recommended.

2.2. Culture media for bacteria

Our ability to study different types of bacteria ultimately relies upon knowing their nutritional requirements. The bacteria with which we are most familiar are generalists (which are able to use a wide range of nutrients) and/or nutrients that are commonly available. Some bacteria can synthesize all of their growth requirements from common mineral nutrients and simple carbohydrates. However, some bacteria are classified as auxotrophs because, even given a Carbohydrate carbon source, they cannot synthesis one or more organic molecules required for their growth – these molecules must be also provided in growth media.

However, if a sample swabbed from your mouth were inoculated on a plate of common culture medium, only a small percentage of the hundreds of different bacteria will

grow and form colonies. This is because most bacteria are fastidious, meaning that they have very specific and/or complex nutritional requirements. These species do not grow because they cannot use one or more nutrients in the form provided in the medium (e.g, they might require H2S rather than SO4 as a sulfur source), have requirements for very specific types of nutrients (such as certain complex organic molecules), and/or require unusual growth conditions (such as growth in living cells or at high temperature or pressure). We presently know very little about many of these bacteria because we do not know how to grow them under artificial laboratory conditions.

2.3. Types of culture media

Media are classified on the basis of composition or application.

1. Complex Media.

Today, many modern complex media (such as Tryptic SoyAgar) contain extracts of beef (peptone), milk (tryptone), soybean meal (soy tone), or yeast are present. The protein in these extracts is broken down into small peptides and amino acids. Although the specific amount of these molecules is not precisely determined, their wide assortment allows complex media to support a wide range of bacterial types.

2. Defined Media.

Defined media are formulated from pure substances at predetermined concentrations. Thus, unlike complex media, the exact chemical composition of defined media is known precisely. Because the composition is precisely established, defined media are often used to determine the nutritional requirements of bacterial species.

3. Selective Media.

Complex or defined media will also be classified as 'selective' if they support the growth of only certain types of bacteria. Media can be made selective through the addition of substances that enhance or inhibit the growth of particular types of bacteria. For example MacConkey Agar.

4. Differential Media

Any of the above types of media might also be formulated as a differential medium. A differential medium reveals specific metabolic or metabolic characteristics of bacteria grown on it. For example, the medium called MacConkey Agar is selective for gram-negative bacteria and will indicate whether bacteria can ferment lactose.

The procedure

The medium that we will prepare is called Tryptic Soy Broth (TSB) which is designed to support the growth of a wide range of bacterial types.

Supplies:

Culture Media, 1 empty 1L flask, autoclave tape, 1 empty test tubes (red cap), TSB (clear cap) and 1 5 ml pipet.

TRYPTIC SOY BROTH (TSB) **Component g/L medium**

Tryptone 17.0
Soytone 3.0
NaCl 5.0
K2HPO4 . . . . . 2.5
Glucose 2.5

1. Transfer the dehydrated TSB medium to a 1L flask
2. Add 1000ml of deionized water and swirl until all of the powder is completely dissolved.
3. To emphasize the importance of media sterilization, pipet 9ml of TSB from the bottle into a 16mm test tube. Label and place it in your drawer.
4. Examine the appearance of the medium next time you come to lab, and then dispose of this medium.

Preparing solid medium (TSA):

Supplies. Culture Media, 1 empty 1L flask, autoclave tape, agar (white cap), measuring boot, balance.

1. Measure and Transfer the agar to the TSB solution.
2. Cover the mouth of the flask with foil, and label it on the autoclave tape as "TSA" with your name and date.
3. Place the flask in the tray to be autoclaved.
4. After autoclaving, the flasks will be placed in a $_{50O}{}^{o}{}_{c}$ water bath to cool.
5. Label the base of 4 petri plates with your name and date.
6. Remove your flask from the water bath, dry the outside with a paper towel, and swirl the medium to mix the dissolved agar completely.
7. Return to your work station. You will need to work quickly but carefully now because the medium will begin to solidify when the temperature drops to 50 ^{0}C.
8. Remove the foil from the flask and hold the flask at an angle at all times to minimize the chance for contamination.
9. Sequentially, lift the lid of each plate just enough to allow the medium to be poured in.
10. Add enough to each plate to cover the base.
11. Let the medium solidify for about 15 minutes before moving the plates.
12. invert the solidified plate and Save the solidified plates in your drawer

2.4. Sterilization and the autoclave

When microbiological media has been made, it still has to be sterilized because of microbial contamination from air, glassware, hands, etc. Within a few hours there will be thousands of bacteria reproducing in the media so it has to be sterilized quickly before the microbes start using the nutrients up. Media sterilization is carried out with the autoclave, basically a huge steam cooker.

The prepared media is distributed in different ways, depending on the form one is making. Agar medium to be be poured into plates is sterilized in a flask, and then poured afterward. Broths and agar deeps are dispensed into tubes and then sterilized. Not all media or solutions can be sterilized via an autoclave. Certain high-protein solutions such as urea, vaccines, and serum will denature in the extreme heat, and so they may have to be filter-sterilized without heat. You will be making slant and broth media, but not plate media.

2.5. Solution Preparation

Whether it is an organism or an enzyme, most biological activities function at their optimum only within a narrow range of environmental conditions. From growing cells in culture to sequencing of a cloned DNA fragment or assaying an enzyme's activity, the success or failure of an experiment can hinge on paying careful attention to a reaction's components. This section outlines the mathematics involved in making solutions. There are several methods that can be used to calculate the concentration of a diluted reagent. Typically, the method chosen by an individual has more to do with how his or her brain approaches mathematical problems than with the legitimacy of the procedure. One approach is to use the equation **C1 V1 = C2V2** where:-

C1 is the initial concentration of the stock solution,
V1 is the amount of stock solution taken to perform the dilution
C2 is the concentration of the diluted sample and
V2 is the final, total volume of the diluted sample

For example, if you were asked how many µL of 10% sugar should be used to make 2 mL of 5% sucrose, the C_1V_1-C_2V_2 equation could be used. However, to use this approach, all units must be the same.

Therefore, you first need to convert 2 mL into a microliter amount. This can be done as follows:

C1=10%, v_2=2

C2=5% v_1=?

Answer $c_1v_1=c_2v_2$, (10%) V1 = (5%) (2000 µL)

Solving for V1 gives the following result

$$V1 = \frac{(5\%)(2000\ \mu L)}{10\%} = 1000\ \mu l$$

Therefore, you would need 1000 µL of 10% sucrose plus 1000 µL (2000 µL- 1000 µL = 1000 µL) of water to make a 5% sucrose solution from a 10% sucrose solution.

Concentrations by a Factor of X

The concentration of a solution can be expressed as a multiple of its standard working concentration. For example, many buffers used for agarose or acrylamide gel electrophoresis are prepared as solutions 10-fold (10X) more concentrated than their standard running concentration (IX). To prepare a 1X working buffer, a dilution of the more concentrated 10X stock is performed in water to achieve the desired volume.

Example: prepare 1000 mL (1 L) of 1X Tris-borate-EDTA (TBE) gel running buffer from a 10X TBE concentrate

C_1=10X TBE V_2=1000ML

C_2=1X TBE V_1= X

$C_1V_1=C_2V_2$,

$V_1 = \frac{C_2V_2}{C_1} = \frac{1X\ TBE \times 1000ml}{10X\ TBE} = 100ml$

Therefore, to make1000 mL of 1X buffer, add 100 mL of 10X buffer stock to 900 mL of distilled water (1000 mL-100 mL contributed by the 10X buffer stock = 900 mL).

2.5.1. Preparing Percent Solutions

Many reagents are prepared as a percent of solute (such as salt, cesium chloride, or sodium hydroxide) dissolved in solution. Percent, by definition, means "per 100." 13%, therefore, means 13per 100, or 13 out of every 100. 13% may also be written as the decimal 0.13 (derived from the fraction 13/100 = 0.13).

Depending on the solute's initial physical state, its concentration can be expressed as a weight per volume percent (% W/v) or a volume per volume percent (% v/v).

Percentage in weight per volume refers to the weight of solute (in grams) in a total of 100 mL of solution. A percentage in volume per volume refers to the amount of liquid solute (in mL) in a final volume of 100 mL of solution.

Most microbiology laboratories will stock a solution of 20% (w/v) glucose for use as a carbon source in bacterial growth media. To prepare 50 mL of 20% (w/v) glucose, 20 grams of glucose are dissolved in enough distilled water so that the final volume of the solution, with the glucose completely dissolved, is 50 mL.

For example: To prepare

A, 47 mL of a 7% (w/v) solution of sodium chloride

B, 200 mL of a 95% (v/v) solution of ethanol

Solution:

A, 7%x47=0.07x47=3.29

Therefore, to prepare 47 mL of 7% sodium chloride, weigh out 3.29 grams of sodium chloride and dissolve the crystals in some volume of distilled water less than 47 mL, a volume measured so that, when the 3.29 grams of NaC1 are added, it does not exceed 47 mL.

B, 95%x200ml=0.95x200ml = 190ml

Therefore, to prepare 200 mL of 95% ethanol, measure 190 mL of 100% (200 proof) ethanol and add 10 mL of distilled water to bring the final volume to 200 mL.

2.5.2. Molarity

A 1 molar (1 M) solution contains the molecular weight of a substance (in grams) in 1 liter of solution. For example, the molecular weight of NaOH is 40. A 1 M solution of sodium hydroxide (NaOH), therefore, contains 40 grams of NaOH dissolved in a final volume of 1000 mL (1 L) water. A 2 M solution of NaOH contains twice that amount (80 grams) of NaOH dissolved in a final volume of 1000 mL water.

Example 1. How are 200 mL of 0.3 M NaOH prepared?

Solution: The molecular weight of NaOH is 40. The first step in solving this problem is to calculate how many grams are needed for 1 L of a 0.3 M solution. This can be done by setting up a ratio stating, "40 grams is to 1 M as x grams is to 0.3 M." This relationship, expressed mathematically, can be written as follows. We then solve for x.

1M NaOH=40grams

0.3m NaOH=x

40x0.3m=1M*x

X=12g of NaOH needed

Therefore, to prepare 1 L of 0.3 M NaC1, 17.53 grams of NaC1 are required

Another ratio can now be written to calculate how many grams of NaOH are needed if 200 mL of a 0.3 M NaOH solution are being prepared. It can be expressed verbally as "12 grams is to l000 mL as x grams is to 200 mL," or written in mathematical terms"

12g NaOH =1000ml

X =200ml

12x200ml=x*1000ml

X=2.4g

Therefore, to prepare 200 mL of 0.3 M sodium hydroxide solution, 2.4 grams of NaOH are dissolved in distilled water to a final volume of 200 mL.

Example 2. How are 50 mL of 20 millimolar (mM) sodium hydroxide (NaOH) prepared?

First, convert the 20 mM value to an M value

20 mM x 1M/1000mM =0.02M

Next, set up a ratio to calculate the amount of NaOH (40.0 gram molecular weight) needed to prepare 1 L of 0.02 M NaOH. Use the expression "40.0 grams is to 1 M as x grams is to 0.02 M." Solve for x.

40g x0.02M=1MXg

X=0.8g

Therefore, if 1 L of 0.02 M NaOH is to be prepared, add 0.8 grams of NaOH to water to a final volume of 1000 mL.

Now, set up a ratio to determine how much is required to prepare 50 mL and solve for x. (The relationship of ratios should read as follows: 0.8 grams is to 1000 mL as x grams is to 50 mL.)

0.8g= 1000ml

Xg =50ml

0.8g x50ml=xg x1000ml

X= 0.04

Therefore, to prepare 50 mL of 20 mM NaOH, 0.04 grams of NaOH is dissolved in a final volume of 50 mL of distilled water.

A. Converting Molarity to Percent

Since molarity is a concentration of grams per 1000 mL, it is a simple matter to convert it to a percent value, an expression of a gram amount in 100 mL. The method is demonstrated in the following problem.

Example 1. Express 2.5 M NaC1 as a percent solution.

Solution:- The gram molecular weight of NaC1 is 58.44. The first step in solving this problem is to determine how many grams of NaC1 are in a 2.5 M NaC1 solution.

This can be accomplished by using an equation of ratios: "58.44 grams is to 1 M as x grams is to 2.5 M." This relationship is expressed mathematically as follows.

58.44=1M 58.44x2.5 =x*1M

x =2.5M X=146.1

Therefore, to prepare a 2.5 M solution of NaC1, 146.1 grams of NaC1 are dissolved in a total volume of 1 liter.

Percent is an expression of concentration in parts per 100. To determine the relationship between the number of grams of NaC1 present in a 2.5 M NaC1 solution and the equivalent percent concentration, ratios can be set up that state, "146.1 g is to 1000 mL as x g is to 100 mL

146.1=1000ml

X =100ml, $\frac{146.1 \times 100}{1000}$

=14.61

Therefore, a 2.5 M NaC1 solution contains 14.6 grams of NaC1 in 100 mL, which is equivalent to a 14.6% NaC1 solution.

Converting a solution expressed as percent to one expressed as a molar concentration is a matter of changing an amount per 100 mL to an equivalent amount per liter (1000. mL) as demonstrated in the following problem.

Example:- What is the molar concentration of a 10% NaC1 solution?

Solution: A 10% solution of NaC1, by definition, contains 10 grams of NaC1 in 100 mL of solution.

So, to solve this problem:

10g=100ml

X =1000ml, $\frac{10g \times 1000}{100}$

=100g

Therefore, a 1000-mL solution of 10% NaC1 contains 100 grams of NaC1.

Using the gram molecular weight of NaC1 (58.44), an equation of ratios can be written to determine molarity.

1M Nacl=58.44

X =100g, $\frac{1M\ Nacl \times 100}{58.44}$

=1.71

Therefore, a 10% NaC1 solution is equivalent to 1.71 M NaC1.

2.5.3. Normality

A 1 normal (1 N) solution is equivalent to the gram molecular weight of a compound divided by the number of hydrogen ions present in solution (i.e., dissolved in one liter of water). For example, the gram molecular weight of hydrochloric acid (HC1) is 36.46. Since, in a solution of HC1, one H § ion can combine with C1- to form HC1, a 1 N HC1 solution contains 36.46/1 = 36.46 grams HC1 in 1 liter. A 1 N HC1 solution, therefore, is equivalent to a 1 M HC1 solution.

As another example, the gram molecular weight of sulfuric acid (H2SO4) is 98.0. Since, in a H2SO 4 solution, two H § ions can combine with SO42- to form H2SO 4, a 1 N H2SO4 solution contains 98.0/2 = 49.0 grams of H2SO 4 in 1 liter. Since half the gram molecular weight of H2SO4 is used to prepare a 1 N H2SO4 solution, a 1 N H2SO 4 solution is equivalent to a 0.5 M H2SO4 solution.

Normality and molarity are related by the equation N=nM

Where "n" is equal to the number of replaceable H (or Na+ or OH- ions per molecule.

What is the molarity of a 1 N sodium carbonate (Na2CO3) solution?

Solution:- Sodium carbonate has two replaceable Na + ions.

The relationship between normality and molarity is N=nM

Solving for M gives the following result.

N/n = M 1/2=M

0.5=M

Therefore, a 1 N sodium carbonate solution is equivalent to a 0.5 M sodium carbonate solution.

3. General microbiological techniques

3.1. Microbial isolation

The utilization of microbes in biotechnology depends on pure cultures, which consist of only a single species, and the maintenance of the purity of the isolates through subsequent manipulations.

Most methods for obtaining pure cultures rely on some form of dilution technique. The most useful and pragmatic method is the streak plate, in which a mixed culture is spread or streaked over the medium surface in such a way that individual cells become separated from one another. Each isolated cell grows into a colony and, therefore, a pure culture (or clone) because cells are the progeny of the original single cell.

The initial step in microbial isolation is:-

- Selecting appropriate artificial media(example: Nutrient broth, Nutrient agar,MRS broth,M17 broth,MRS agar, PDA,YPGA etc)
- Prepare the media based on standard and autoclave it at 121oc 15min
- Measure the collected sample and make it serial dilution (example if the sample is solid like soil, enset and other agro industrial waste measure it by using sensitive balance. If the sample is liquid sample like milk, water etc we can measure it by graduate cylinder or pipette
- Spread on agar plate and incubate at appropriate temp and time.

3.1.1. Purification of the culture

To establish the purity of a culture an isolated colony from an initial spread plate should be streaked on agar media and only a single type of isolated colony whose colonial morphology is consistent with the initial isolate will be harvested.

3.1.2. Maintenance of pure culture

Aseptic technique is required to transfer pure cultures and to maintain sterility of media and solutions (Safe Handling of Microorganisms). Proper aseptic transfer technique also protects the biotechnologist from contamination with the culture, which should always be treated as a potential pathogen. Theses aseptic techniques are:-

- Avoiding any contact between the pure culture
- Avoiding any contact between sterile medium, and sterile surfaces of the growth vessel with contaminating microorganisms.
- Sterilize the transfer instruments; for example, the transfer loop is sterilized by heating with a Bunsen burner before and after transferring.

3.2. Microbial characterization

There are different microbial characterization techniques.

A. Morphological (macroscopic) characterization :-(example: size, shape, texture, color etc.).

B. Microscopic characterization

- **Gram Stain:**

Gram staining detects a fundamental difference in the cell wall composition of bacteria. Pure stain culture to determine gram positive or gram negative bacteria. In gram staining the first step is to prepare bacterial smear from pure culture.

- Put a drop of saline, distilled water or PBS on clean glass slide
- Using sterile loop or needle touch an isolated colony and mix in water drop
- Mix until just slightly turbid (light inoculum is best, excess bacteria will not stain properly.
- Let air dry and heat fix
- Allow to cool.
- Flood the slide with crystal violet and allow to remain on the slide for 60s
- Wash off the crystal violet with the running tap water
- Flood the slide with grams iodine and allow to remain on the slide for 60s
- Wash off with the running tap water
- Decolorize with 50%alcohol
- Rinse immediately with running tap water
- Counter stain with safaranin with 30-60s
- Rinse with running tap water and allow to dry

C. Biochemical characterization

• **Catalase test:-** One ml of a 3% solution of hydrogen peroxide will added to a Petri dish and a loop of fresh culture will rubbed into the solution. Release of bubbles from the culture should be recorded as catalase positive.

• **KOH solubility test:- :** Two drops of 3% KOH will put onto glass slide and colony will stirred into the solution with clean loop for 10 s. a thin strand of slime like is +ve result

• **Carbohydrate utilization test:** The following carbohydrate utilization can aid in bacterial species identification. Aibinose, rhamanose, mannitol, sorbitol and sucrose. The procedure to be followed for each of these media is identical.

1, Inoculate carbohydrate media with pure culture 18-24hr

2, Incubate at appropriate temp and hr.

Positive result is produced from fermentation which turns the media to yellow.

• **Citrate utilization test**

Citrate test should be used to differentiate bacteria on the basis of their ability to utilize citrate as the sole carbon source. The utilization of citrate depends on the presence of an enzyme citrase produced by the organism that breaks down the citrate to oxaloacetic acid and acetic acid. Simmon's Citrate agar slants were inoculated with the selected isolate.

• **Nitrate Reduction**

Nitrate broth should be inoculated with a loopful of selected isolate and incubated at 28ºC for 7 days. Uninoculated nitrate broth should be kept as control. Control should be also run without inoculation. After incubation two drops of sulphanilic acid followed by two drops of α – napthylamine solution should be added. The presence of nitrate is indicated by a pink, red or orange colour and absence of colour change should be considered as nitrite negative.

• **Starch hydrolysis**

The ability to degrade starch should be used as a criterion for the determination of amylase production by a microbe. Starch hydrolysis test should be determined the absence or presence of starch in the medium by using iodine solution as an indicator. Starch in the presence of iodine produces a dark blue colouration of the medium and a clear zone around a colony indicates amylolytic activity. The isolate should be streaked on sterile starch agar plates and incubated at 37ºC for 48 hrs. The plates should be flooded with 1 % of iodine. Amylase production should be indicated by colourless clear zone surrounded by the microbial isolate and rest of the plate appeared dark blue colouration.

4. Methods for Identification of Microorganisms

4.1. Classical Methods

This method could identify microorganisms using in-house biochemical, manual phenotypic methods (API Strips, BBL etc.), automated phenotypic methods (Vitek), Cellular Fatty Acids (MIDI Sherlock System), Carbon Source Utilization (Biolog) and Genetic (MicroSeq, Riboprinter).

4.1.1. Limitations of the Methods

- Phenotypic properties (biochemical / carbon source utilization) can be variable, subjective, and dependent on growth parameters and health of organism.
- Cellular fatty acid profiles change with temperature, age of culture and growth medium.
- Some systems require subjective off-line testing such as gram stain, oxidase, coagulase, etc., before determining the appropriate test card.
- DNA Sequencing system is not automated, numerous, somewhat complicated lab procedures, and manual interpretation of results requires microbial phylogenetic experience.

4.2. A Revolution in Bacterial Taxonomy

This is towards a natural system of classification based on phylogenetics rather than taxonomic characters. In many cases taxonomic characters are not phylogenetically valid- i.e. morphological characters such as cell shape, mode of cell division, lack of cell wall can be misleading.

4.2. Road Map to Bergey's

Bergey's Manual of systematic bacteriology, one of the most comprehensive and authoritative works in the field of bacterial taxonomy, has been extensively revised in the form of a five volume second edition. Numerous taxonomic rearrangements and changes in nomenclature have resulted from more than 850 published new combinations.

These developments, which are attributable to rapid advances in molecular sequencing of highly conserved regions of the prokaryotic genome, most notably genes coding for the RNA of the small ribosomal subunit, have led to a natural classification that reflects the evolutionary history of Bacteria and Archaea, and to the development of new, universally applicable methods of identifying these organisms."

4.3. Phylogenetic Taxonomy

This method is universal system and reproducible from laboratory to laboratory and over time. Sequence characters are not subjective and more informative than phenotypic ID with no specific growth requirements and accepted gold standard for taxonomy.

4.4. Methods of Microbial genomic DNA Isolation

4.5. CTAB

In this method total genomic DNA from the bacteria is isolated by N- Cetyl- N, N, Ntrimethyl-ammonium bromide (CTAB). In brief, the culture should centrifuged at 10000 rpm at 4°C and should lysed with 675µl extraction buffer (100mM Tris HCl, 100mM EDTA, 1.4M NaCl, 1% CTAB and Proteinase K -0.03µg/µl). The suspension should be incubated at about 37^0C for 30 minutes. To the mixture 75µl of 20% SDS should be added and incubated at 65°C for 2 hours. The suspension should then centrifuge and the supernatant is extracted with equal volumes of Chloroform and Isoamyl alcohol (24:1). The aqueous phase obtained after centrifugation should be then extracted with 0.6 volumes (600 µl) of isopropyl alcohol. The mixture should be allowed to stand undisturbed at RT for 1hour. The suspension is then centrifuged again and the DNA should be pelleted with 500µl of 70% ethanol.

The DNA collected should be then quantified using UV spectrophotomer (Vivaspec Biophotometer, Germany).

4.6. QIGAN Kit

DNeasy Blood & Tissue Kits are designed for rapid purification of total DNA (e.g., genomic, mitochondrial, and pathogen) from a variety of sample sources including fresh or frozen animal tissues and cells, blood, or bacteria. DNeasy purified DNA is free of contaminants and enzyme inhibitors and is highly suited for PCR, Southern blotting, RAPD, AFLP, and RFLP applications. Purification requires no phenol or chloroform extraction or alcohol precipitation, and involves minimal handling. This makes DNeasy Blood & Tissue Kits highly suited for simultaneous processing of multiple samples. For higher-throughput applications, the DNeasy 96 Blood &

Tissue Kit enables simultaneous processing of 96 or 192 samples.

The buffer system is optimized to allow direct cell lysis followed by selective binding of DNA to the DNeasy membrane. After lysis, the DNeasy Blood & Tissue spin-column procedure can be completed in as little as 20 minutes. Using the DNeasy 96 Blood and Tissue Kit, 96 or 192 samples can be processed in just 1 hour after lysis

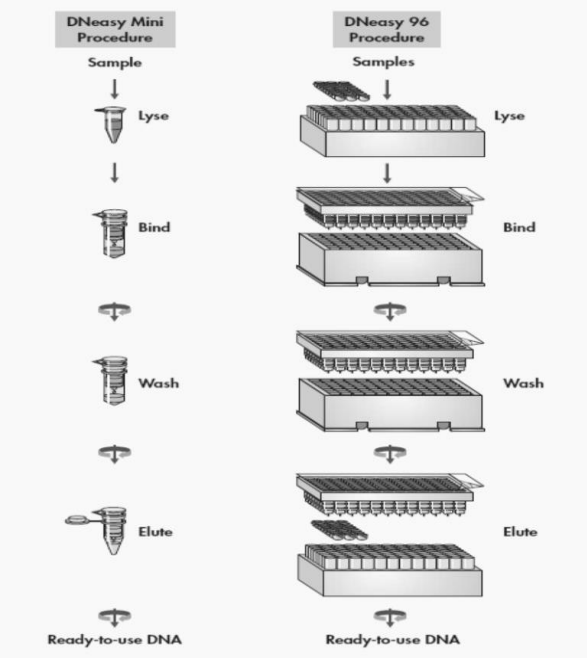

Fig 1. GIAGEN Procedure.

4.7. Boiling

There are two methods in these techniques and two colonies of overnight growth bacteria should be used. The colonies should put in a test tube containing one ml of distilled water and boiled for 10 minutes in a water bath, and then should centrifuged for 5 minutes at1000rpm. Five microliters of the supernatant should be used for the PCR. The second method should be based on using a National microwave oven (Matsushita Electric Industrial Company, Japan) to heat the bacterial colonies (two colonies dissolved in 500 µl distilled water) for 10 seconds, followed by centrifugation for two minutes at 1000 rpm. Similarly, 5 µl of the supernatant should used for the PCR.

5. PCR amplification of microbial genome

The extracted gene should be amplified by PCR using purified genomic DNA as a template. Oligonucleotide primers should be synthesized to amplify the intact region of gene. The forward primer and the reverse primer should be used. These primers correspond to the gene extracted and thus the final PCR product should determine. The PCR mixture consisted of;

- 10x reaction buffer with $MgCl_2$ (1.5mM)
- 2µL of dNTP mix (2.5mM)
- 2µL each of forward and reverse primers (10picomoles/µl each primer)
- 0.3µL of Taq DNA polymerase (5 U/µL)
- 50ng/ µL of template DNA in a total volume of 20µL.

The PCR should be performed with the following cycling profile:

- Initial denaturation at 94°C for 2-5min
- denaturation at 94°C for 45s
- Annealing at 50-61°C for 45s
- Extension at 72°C for 1min
- Final extension at 72°C for 4-10 min
- followed by 26-38 cycle

The PCR products amplified should then qualitatively analysed on 2-3% agarose gel. The PCR product is recovered using the QIA quick gel extraction kit, and the amplified product should be then purified and used for cloning purpose.

6. Sequencing

The DNA eluted from agarose gel should be sequenced using ABI PRISM Big Dye Terminators v1.1 cycle sequencing kit (Applied Biosystems Foster city, CA, USA) according to the manufacturer's instruction employing T7 or M13 primers. The comparison of the nucleotide sequences of the unique fragment with the sequences available in the GenBank database should be carried out using the NCBI BLAST program (http//www.ncbi.nlm.nih.gov/blast).

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