Kingdom of Saudi Arabia
Ministry of Education
Northern Border University
Faculty of Science and Arts
Department of Biology
(Microbiology)

# PRACTICAL INDUSTRIAL MICROBIOLOGY COURSE (3303-411)

Prepared by

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# Lab (1): Introduction to Industrial Microbiology

## Equipment's of Industrial Microbiology Lab

### (1)Shaker Incubator

**Function:** In the microbiology laboratories it is among the essential devices which are based on the principle of shaking at different temperatures according to the purpose and the work load of the laboratory. It is used in cultivating, multiplying and in the characterization tests of microorganisms. This device provides the heat necessary for the growth of microorganisms.

## (2) Rotary Evaporator


**Function:** is a device used in laboratories for the efficient and gentle removal of solvents from samples (in extraction of bio-product) by evaporation.

## (3)Seed Fermentor

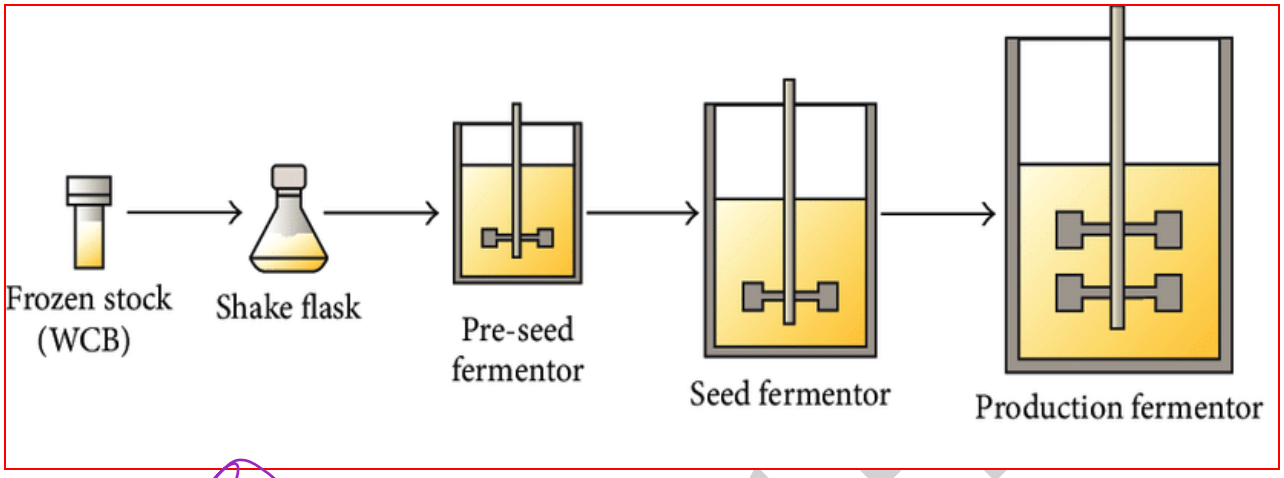

**Function:** Seed fermenter is usually required to produce the inoculum volume of the producer microorganism. Seed vessel is essential for the growth of organism and nor the production of the target product.

What capacity you are planning?

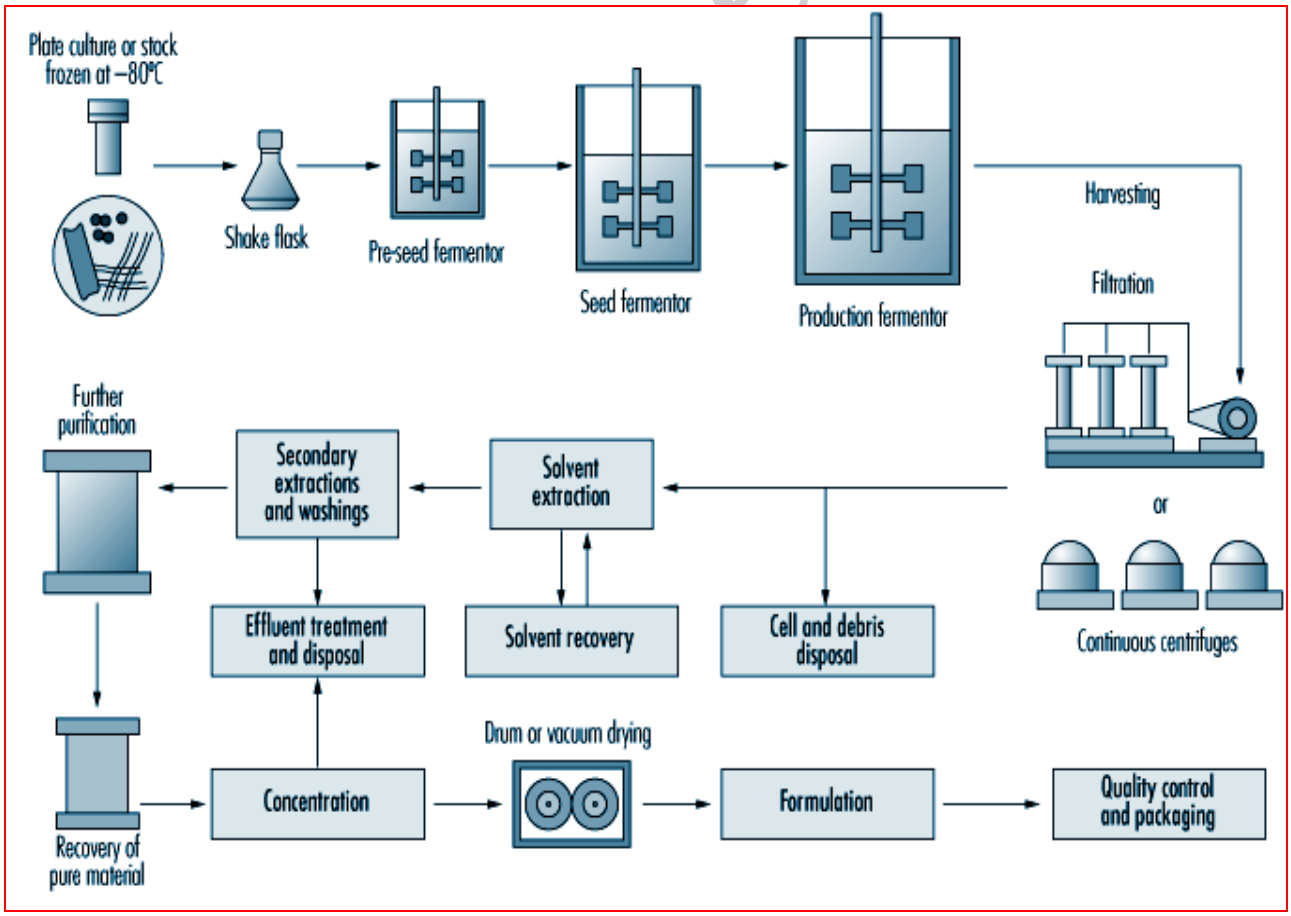
In case you are planning for 100 liters of production fermenter you may require a 20-25 liters of seed fermenter this mainly depends on the inoculum percentage you are adding to the production media.

## Production Fermentor

**Function:** Production fermenter is used for the production of the target product in the desired quantity.




**Flow diagram for the classical fermentation process**




**Flow diagram for Production Process in a typical Industrial Microbiology**

# Lab (2): Microbial Production of Foods

## (1)Baker's Yeast Production

## Background:

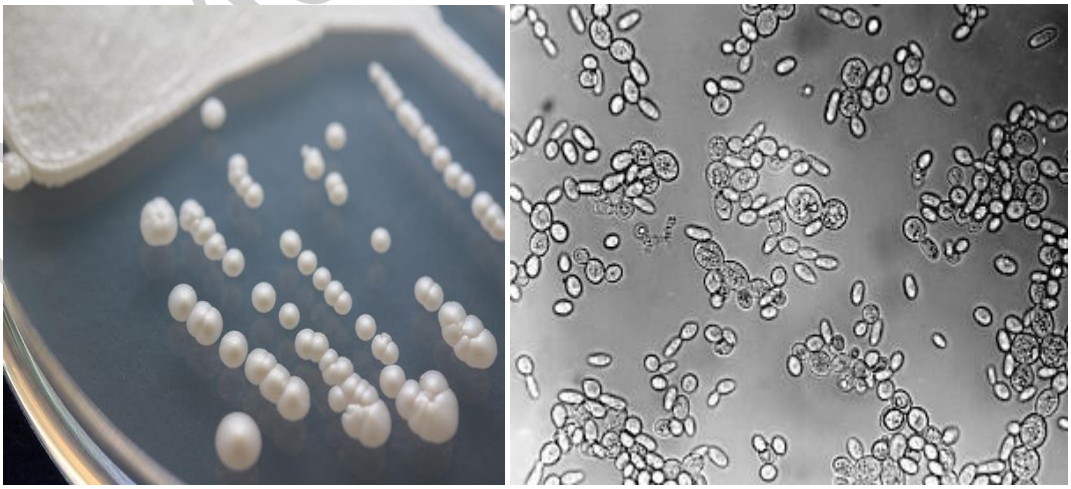
Baker's yeast is the common name for the strains of yeast commonly used as a leavening agent in baking bread and bakery products, where it converts the fermentable sugars present in the dough into carbon dioxide and ethanol.

Baker's yeast is of the species *Saccharomyces cerevisiae*, which is the same species (but a different strain) commonly used in alcoholic fermentation, which is called brewer's yeast. Baker's yeast is also a single-cell microorganism found on and around the human body.

The produced Baker's yeast is marketed in the form of cake, powder or cream.

## Microorganism used:

*Saccharomyces cerevisiae*



### (1) Characteristics of the microorganism

- Most commonly used organism
- Unicellular

- Rich in protein & vitamin B
- Budding

**(2) Enzymes produced by the microorganism:**

- Maltase; converts maltose to glucose
- Invertase; sucrose to glucose & fructose
- Zymase complex; sugars to $CO_2$ & ethanol

**(3) Process Biochemistry**

- Grow either in the absence or presence of $O_2$.
- Grows efficiently… $O_2$ present.
- Grows inefficiently… $O_2$ not present.
- Produces ethanol in large quantity.
- Fed-batch is best method.
- Incremental feeding & high aeration.

## Isolating *Saccharomyces cerevisiae* yeast from natural sources:

1) Samples collected from different natural sources such as soil and fruit.
2) Make a series of dilution of the sample, then moved to the culture media to isolate yeasts such as yeast extract agar or malt extract agar.
3) Incubated for 2-5 day at 25-30°C.
4) Get a pure culture for yeasts through a two or more on the same media.

## Types of Baker's Yeast

### (1) Cream Yeast

- Suspension of yeast cells.
- Cream yeast is not termed as baker's yeast but is a marketable product.
- Solid contents about 18-20%.

### (2) Compressed Yeast


- Solid contents range between 27-33%.
- Compressed yeast can be granular or in the form of cake.

**(A) Granular Yeasts**


- Small granules
- Can be added to driest doughs

**(B) Cake Yeast**

- Also known as active dry yeast
- Long shelf life
- Cells encapsulated in a thick jacket of dead cells

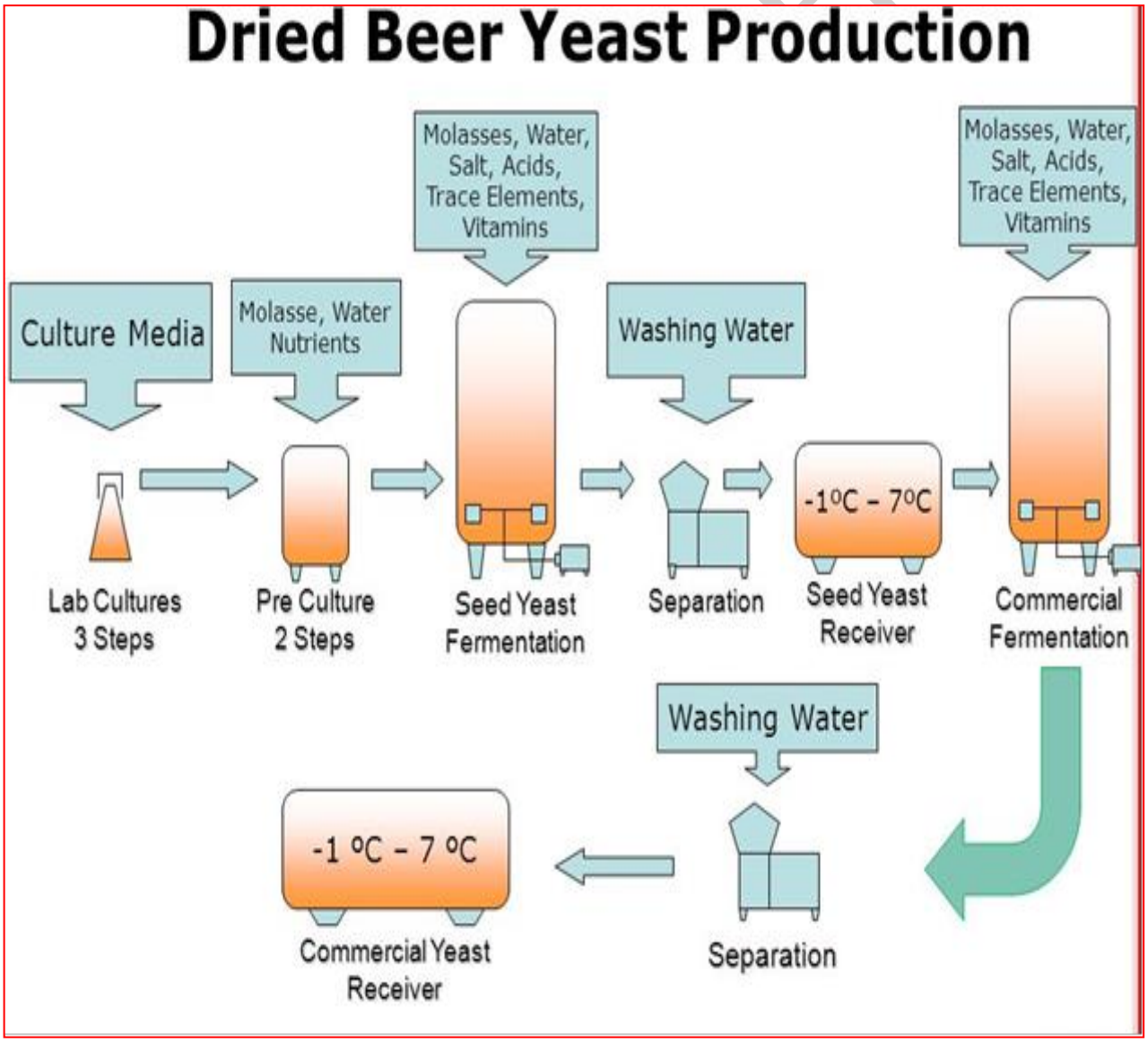
Shelf life of compressed yeast is about 1-2 years.

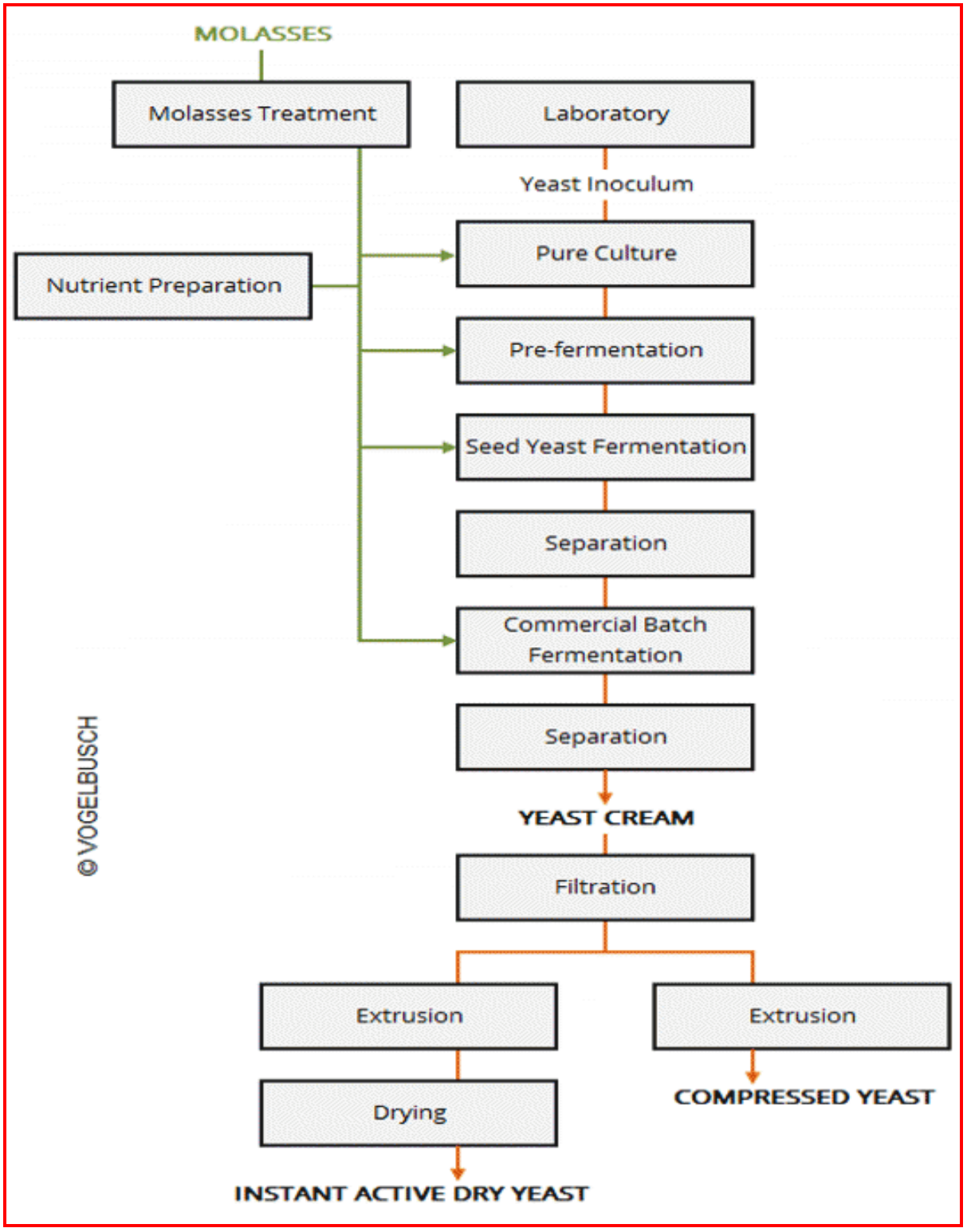
## Bread and Bread-Making:

Made by mixing water, flour, salt and yeast

## Importance of Yeast in the manufacturing of Bread

- It provides the $CO_2$ needed in order for the dough to expand.
- It strengthens bread dough.
- During fermentation, yeast provides the metabolites necessary for the characteristic flavour of bread.






**Flow diagram for Baker's Yeast Production**

# (2) Cheese Production

## Background:

Cheese is a generic term for a diverse group of milk-based food products. Cheese is produced throughout the world in wide-ranging flavours, textures, and forms.

- Cheese consists of proteins and fat from milk, usually the milk of cows, buffalo, goats, or sheep.
- It is produced by coagulation of the milk protein casein.

What kind of cheese are you making?

- ✓ Select the milk first!

The origin of the milk is the first step in determining the flavor and consistency of the cheese. Although most cheese is made from cow's milk, sheep and goat's milk are also used.

## The 2 parts of milk...

- ✓ We already learned that milk has two main parts…the water and the solids. There are other names for these two parts:

As milk separates into the two parts, we call it "**curdling**"or "**clabbering**".

- ✓ **Whey** is the correct name for the liquid.
- ✓ **Curds** is the correct name for the solids.

Curdling happens naturally as the milk sours, but it is done intentionally as the first step in making cheese.

The art of making natural cheeses...

The enzyme rennin is obtained from the stomach of young calves. Added to raw, whole milk in liquid or tablet form, it causes the milk protein casein to clabber.

In the microbial production of cheese the coagulation of milk is carried out by action of caseinase enzyme which produced by some bacterial species such as *Lactobacillus.*

In the dairy industry, some enzymes are required for the production of cheeses, yogurt, and other dairy products, while others are used in a more specialized fashion to improve texture or flavor. Five of the more common types of enzymes and their role in the dairy industry are described below.

### (1) Rennet

Milk contains proteins, specifically caseins, that maintain its liquid form. Proteases are enzymes that are added to milk during cheese production, to hydrolyze caseins. Rennet and rennin are general terms for any enzyme used to coagulate milk. Technically rennet is also the term for the lining of a calf's fourth stomach.

The most common enzyme isolated from rennet is chymosin. Chymosin can also be obtained from several other animals, microbial or vegetable sources, but indigenous microbial chymosin (from fungi or bacteria) is ineffective for making cheddar and other hard cheeses.

### (2) Lactalbumin and Lactoglobulin

Milk contains a number of different types of proteins, in addition to the caseins. Cow milk also contains whey proteins such as lactalbumin and lactoglobulin. The denaturing of these whey proteins, using proteases, results in a creamier yogurt product. Destruction of whey proteins is also essential for cheese production.

During the production of soft cheeses, whey is separated from the milk after curdling and may be sold as a nutrient supplement for bodybuilding, weight loss, and lowing blood pressure, among other things.

Proteases are used to produce hydrolyzed whey protein, which is whey protein broken down into shorter polypeptide sequences.

Hydrolyzed whey is less likely to cause allergic reactions and is used to prepare supplements for infant formulas and medical uses.

### (3) Lactase

Lactase is a glycoside hydrolase enzyme that cuts lactose into its constituent sugars, galactose, and glucose. Without sufficient production of lactase enzyme in the small intestine, humans become lactose intolerant, resulting in discomfort (cramps, gas, and diarrhea) in the digestive tract upon ingestion of milk products.

Lactase is used commercially to prepare lactose-free products, particularly milk, for such individuals. It is also used in the preparation of ice cream, to make a creamier and sweeter tasting product. Lactase is usually prepared from *Kluyveromyces* sp. of yeast and *Aspergillus* sp. of fungi.

### (4) Catalase

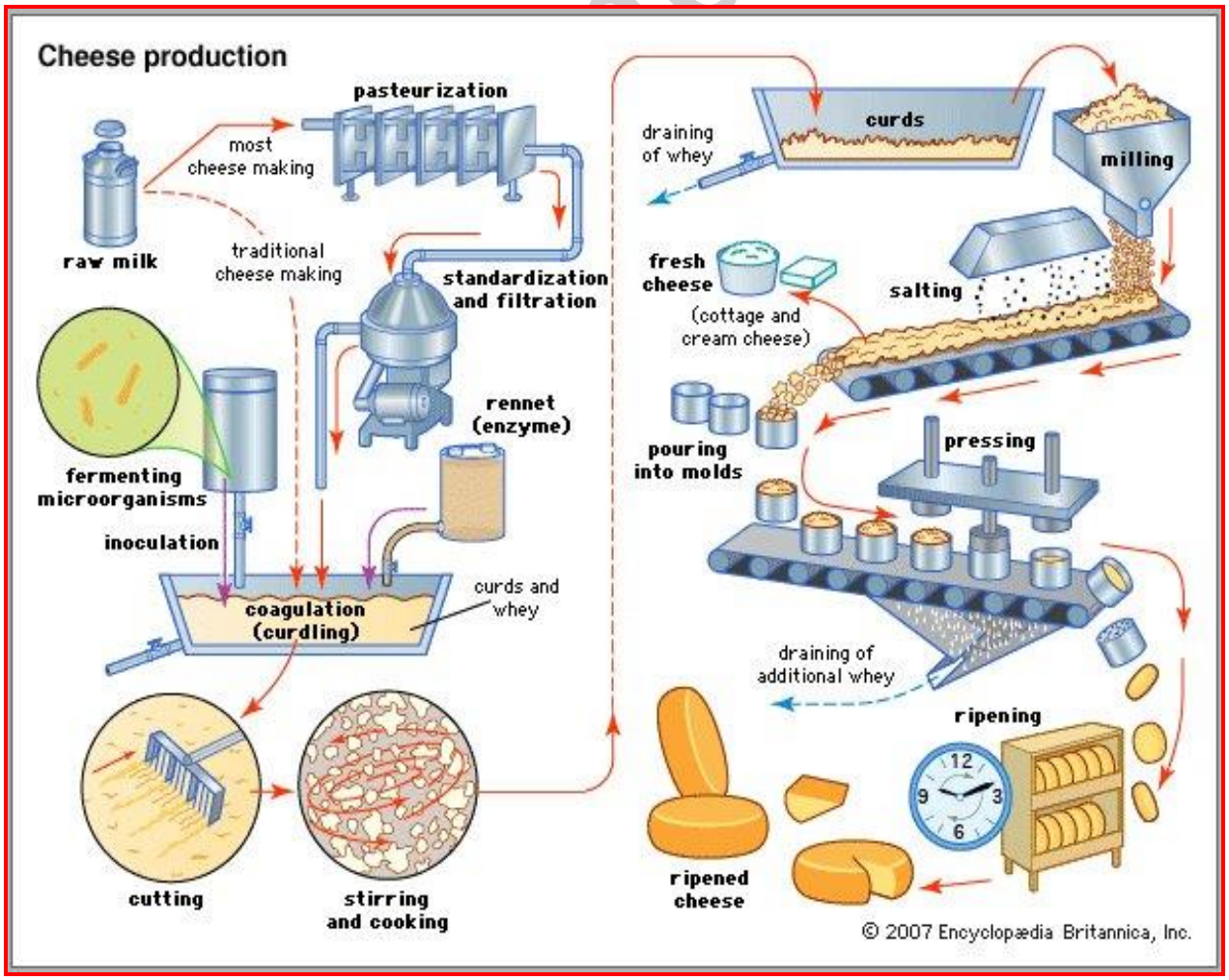
The enzyme Catalase has found limited use in one particular area of cheese production. Hydrogen peroxide is a potent oxidizer and toxic to cells. It is used instead of pasteurization, when making certain cheeses such as Swiss, in order to preserve natural milk enzymes that are beneficial to the end product and flavor development of the cheese.

These enzymes would be destroyed by the high heat of pasteurization. However, residues of hydrogen peroxide in the milk will inhibit the bacterial cultures that are required for the actual cheese

production, so all traces of it must be removed. Catalase enzymes are typically obtained from bovine livers or microbial sources and are added to convert the hydrogen peroxide to water and molecular oxygen.

### (5) Lipases

Lipases are used to break down milk fats and give characteristic flavors to cheeses. Stronger flavored cheeses, for example, the Italian cheese, Romano, are prepared using lipases. Animal lipases are obtained from kid, calf, and lamb, while microbial lipase is derived by fermentation with the fungal species *Mucor meihei*.




**Flow diagram for Cheese Production**

# Lab (3): Microbial Production of Enzymes

## (1) Microbial Amylase

### Background:

Amylases are enzymes that break down starch or glycogen. Amylases are produced by a variety of living organisms, ranging from bacteria to plants and humans. Bacteria and fungi secrete amylases to the outside of their cells to carry out extra-cellular digestion. When they have broken down the insoluble starch, the soluble end products such as (glucose or maltose) are absorbed into their cells.

Amylases are classified based on how they break down starch molecules:

1. α-amylase (alpha-amylase) - Reduces the viscosity of starch by breaking down the bonds at random therefore, producing varied sized chains of glucose.

2. β -amylase (Beta-amylase) - Breaks the glucose-glucose bonds down by removing two glucose units at a time, thereby producing maltose.
3. Amyloglucosidase (AMG) - Breaks successive bonds from the non-reducing end of the straight chain, producing glucose. Many microbial amylases usually contain a mixture of these amylases.

- Humans exploit microbial amylases for the following purposes:

  (1) High Fructose Corn syrup preparation.

  (2) Additives to detergents for removing stains

  (3) Saccharification of starch for alcohol production.

  (4) Brewing.

The soil contains a rich deposit of both bacteria and fungi, which produce amylases. Starch hydrolyzing fungi or bacteria could be isolated from the soil, foods or could be purchased. Buying saves time and ensures a high yielding strain. However, isolating could be fun, and constitutes an additional lab.

Although many microorganisms produce this enzyme, the ones most commonly used for their industrial production are *Bacillus subtilis* and *Aspergillus niger*

## Materials Required:

Nutrient agar, potato dextrose agar, soluble starch (1%) and Gram's iodine.

## Procedures:

1) Suspend about 10 grams of either soil or rotten potato in 90 mL sterile distilled water and mix properly.
2) Pipette 10 mL of the above and transfer to another 90 mL of water.
3) Dilute further in two more 90 mL sterile water blanks.
4) For Fungi: Spread 0.1 mL from the dilutions on Potato Dextrose Agar plates (fortified with 0. 1 mg/mL streptomycin sulfate) with a glass spreader. (The glass spreader is quickly sterilized by dipping in 95%) ethanol and putting in the flame, so that the alcohol burns off)

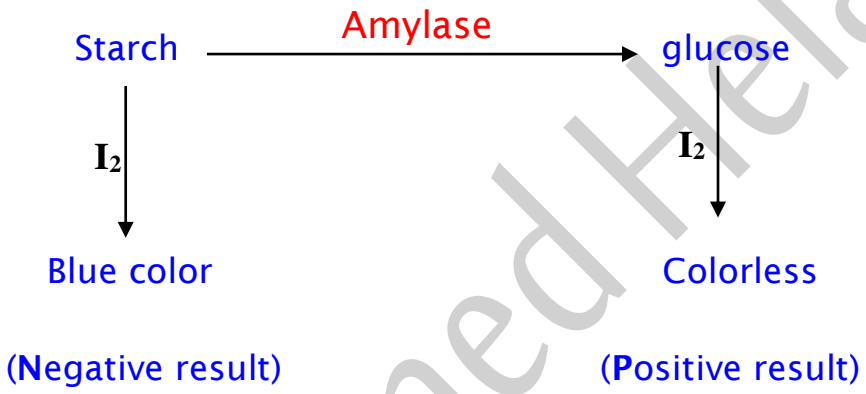
- Incubate at room temperature for about 3 days.

5) For Bacteria: Spread 0.1 mL of the diluted samples on Nutrient Agar plates containing I % w/v soluble starch and incubate at 30"C for 24 hours.

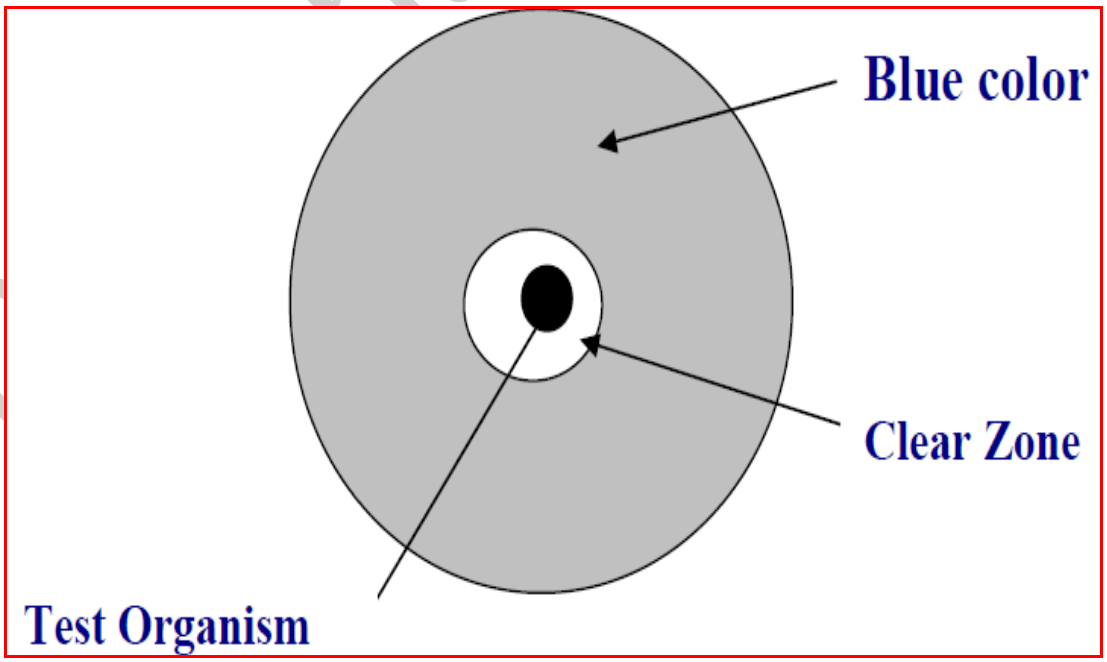
Observations:

- Starch-producing colonies will have an area of clearing around them.
- Confirm by flooding plates with Gram's iodine.
- Transfer distinguishable, amylase-producing fungi to fresh plates of Potato Dextrose agar.
- Containing I % starch, using a sterilized dissecting needle. For bacteria, streak on a fresh.
- Plate of nutrient agar containing $I_2$ % starch.

- Transfer your isolated amylase-producing fungi to potato dextrose agar slants, and the bacteria to nutrient agar. Allow bacteria to grow for 24 hours and fungi to grow for 72 hours, then store in the refrigerator until needed.




## Results and Interpretations:




**Result of Amylase production**

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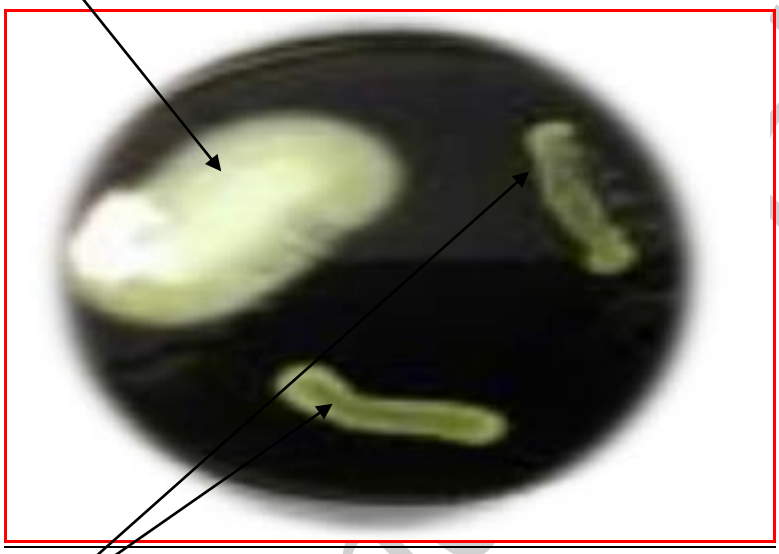
## Control:

**Positive control:** *Bacillus subtilits*

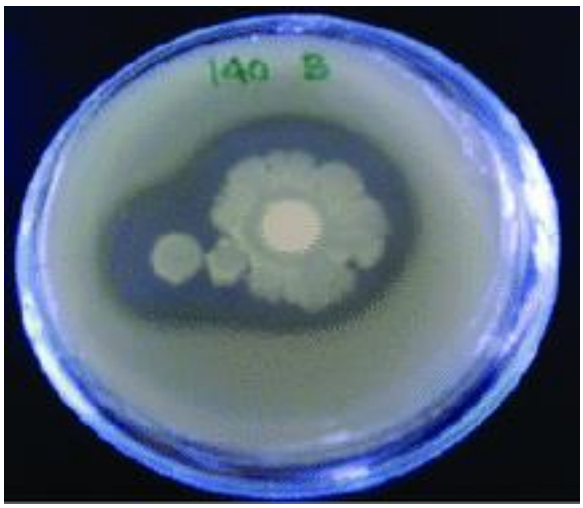


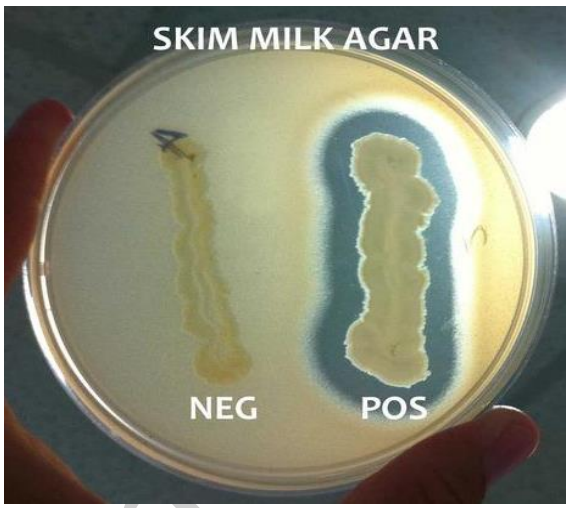
**Negative control:** *E.coli*

*Note that:*

- *Name of Enzyme: Amylase.*
- *Name of Substrate: Starch.*
- *Name of Reagent: Gram's Iodine ($I_2$).*
- *Result of Amylase Positive Reaction: Clear zone.*
- *Result of Amylase Negative Reaction: Blue color.*

# (2) Microbial Protease






## Background:

Proteases are the key enzyme in the industrial application. Microbial proteases play important role in biotechnological process with worldwide sale representing about 60% of the total enzyme marked.

A protease (also termed peptidase or proteinase) breaks down proteins. A protease is any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino ac ids together in the polypeptide chain forming the protein.

## Required Materials:

*Aspergillus niger* or *Bacillus subtilis*, potato dextrose agar medium. nutrient agar, enzyme production medium such as skim milk agar media or tributyrin agar media.

## Procedure:

Preparation of enzyme production medium:

## (A) Production medium for *Bacillus subtilis*:

| Components: | (g/L) |
|---|---|
| Peptone | 1 |
| NaCl | 5 |
| Skim milk | 10 |
| Agar | 20 |
| $H_2O$ | 1 |
| pH 7.0-7.2 at 25 ˚C | |

1) Sterilize peptone separately and add aseptically to the flask containing the liquid medium, after cooling.
2) Inoculate the medium (50ml in 250mI conical flask) with 1ml of an overnight culture of Bacillus Subtilis.
3) Incubate at 50□C in a rotary shaker at 150 rpm for 12hr.
4) At time intervals determine the turbidity of the culture measuring the increase in optical density at 450 nm with a spectrophotometer.

## (B) Production medium for *Aspergillus niger*

| Components: | (g/L) |
|---|---|
| Ammonium sulphate | 1.0 |
| Magnesium sulphate heptahydrate | 5.0 |
| Potassium di-hydrogen phosphate | 5.0 |
| Ferrous sulphate heptahydrate | 0.005 |
| Glucose | 5.0 |
| Jowar seeds | 3.0 |
| $H_2O$ | 1 |
| pH 5.0 at 25 ˚C | |

5) Inoculate the liquid medium with overnight grown *Aspergillus* culture.
6) Examine fermentation duration for 24 to 120 hrs.
7) Kept the culture flask on rotary shaker at 300 rpm at 28˚C.

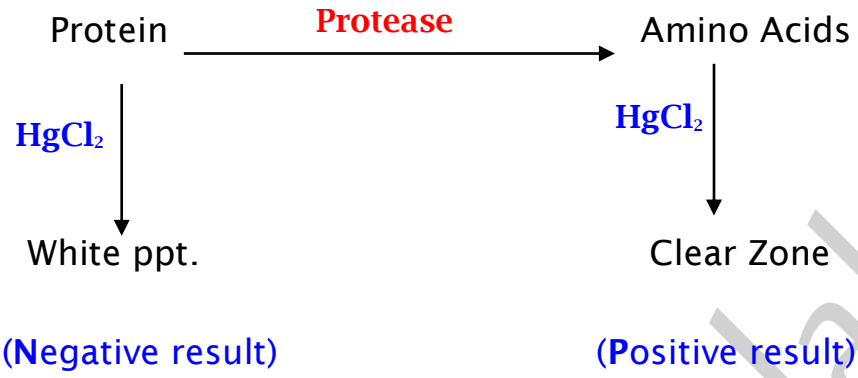
## Enzyme assay:

Casein solution of 2% (1ml) was incubated with 0.1 ml of enzyme solution and 0.9 ml of sodium phosphate buffer (pH 7) for 10 minutes at 40˚C. The reaction was stopped using 10% TCA solution. After 20 minutes the mixture was centrifuged 10,000 rpm for 5 minutes. The color intensity of supernatant was read at 280 nm.

The enzyme activity was calculated from standard curve of L-tyrosine. The serial increase in concentration of tyrosine (10-100tg/ml) was read at 280 nm for the standard graph. Enzyme activity was depends on the temperature, particular pH, substrate concentration and the active site of enzyme.

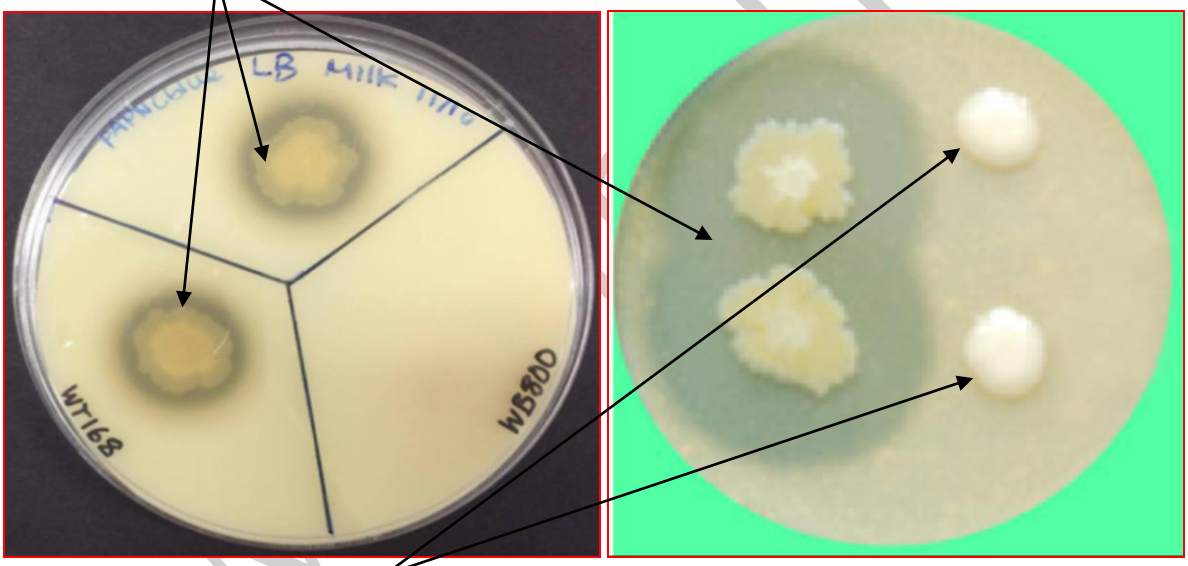
## Product recovery and purification:

Protease is an extracellular enzyme so its recovery is quite easy. After incubation centrifuge the production medium at 12.200 rpm for 15 min to separate the cells. Collect the supernatant as it will contain the crude enzyme and store at 14˚C till further use.




## Results and Interpretations:

**Positive control:** *Bacillus subtilits*



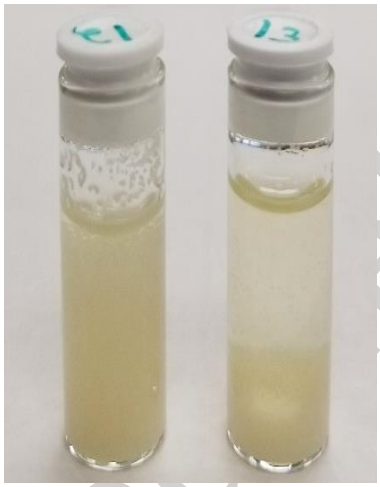

**Negative control:** *Candida* sp

*Note that:*

- *Name of Enzyme: Protease.*
- *Name of Substrate: Skim milk.*
- *Name of Reagent: Mercuric chloride ($HgCl_2$)*
- *Result of Protease Positive Reaction: Clear zone.*
- *Result of Protease Negative Reaction: White ppt.*

# Lab (4): Microbial Production of Organic Acids

## Citric Acid Production

### Background:

Citric acid, a carboxylic organic acid, soluble in water with a pleasant taste, is the most important acid used in the food industries. citric acid can be produced by fermentation process using species of microorganisms namely *Aspergillus niger*, a fungus which was used commercially for the first time in 1923.

Factors affecting the production of citric acid by fermentation include:

(1) Nutritional composition of the media.

(2) Environmental conditions.

(3) Deficiency of manganese and other metals.

(4) pH

(5) Dissolved oxygen tension.

At present time citric acid is produced commercially using mutant strains of *Aspergillus niger*, and with a significant amount by *Saccharomycopsis lipolytica*, *Pencillium simplicissimum* and *Aspergillus foeitidus*.

Other carbohydrates and wastes that have been considered, experimentally, to produce citric acid by *Aspergillus niger* includes inulin, date fruit syrup, sugar cane molasses, soya whey, Carob pod and cheese whey.

Large amounts of whey are produced worldwide as a by-product of cheese and other dairy products manufacturing. Whey in the Middle Eastern region is generally considered a waste and disposed in the sewage system leaving a small amount for drinking for domestic animals. The aim of this study was to produce citric acid by *Aspergillus niger* from cheese whey fortified with different sucrose, tricalcium phosphates and riboflavin in a liquid surface culture process.

## Materials

Pasteurized cheese whey, sucrose, tricalcium phosphate, riboflavin, Fresh *Aspergillus niger* culture (approx $10^3$ spore suspension).

## Procedures:

1) Take 100 mL cheese whey in 500 mL Erlemyer flask and add sucrose (15g) and tricalcium phosphate (1g).

2) Pateurized cheese whey at 60˚C for 30 minutes and add filter sterilized riboflavin (10 mg/L) to fortify the media.
3) Adjust the initial pH of the fermentation media to 3.0 using I N of HCl and/or NaOH.
4) Carry out surface liquid culture fermentation process by inoculating the media with the fungal culture (approx $10^3$ spore suspension) and incubate at 30˚C for up to 20 days.
5) Determine citric acid concentration via titrating with 0.1 N NaOH and phenolphthalein as indicator and calculated as % according to the following formula:

$$\% \text{ of Citric acid} = \frac{\text{Normality x Volume of NaOH x Equiv. wt. of CA}}{\text{Weight of sample (g)}} \text{ x } 10$$

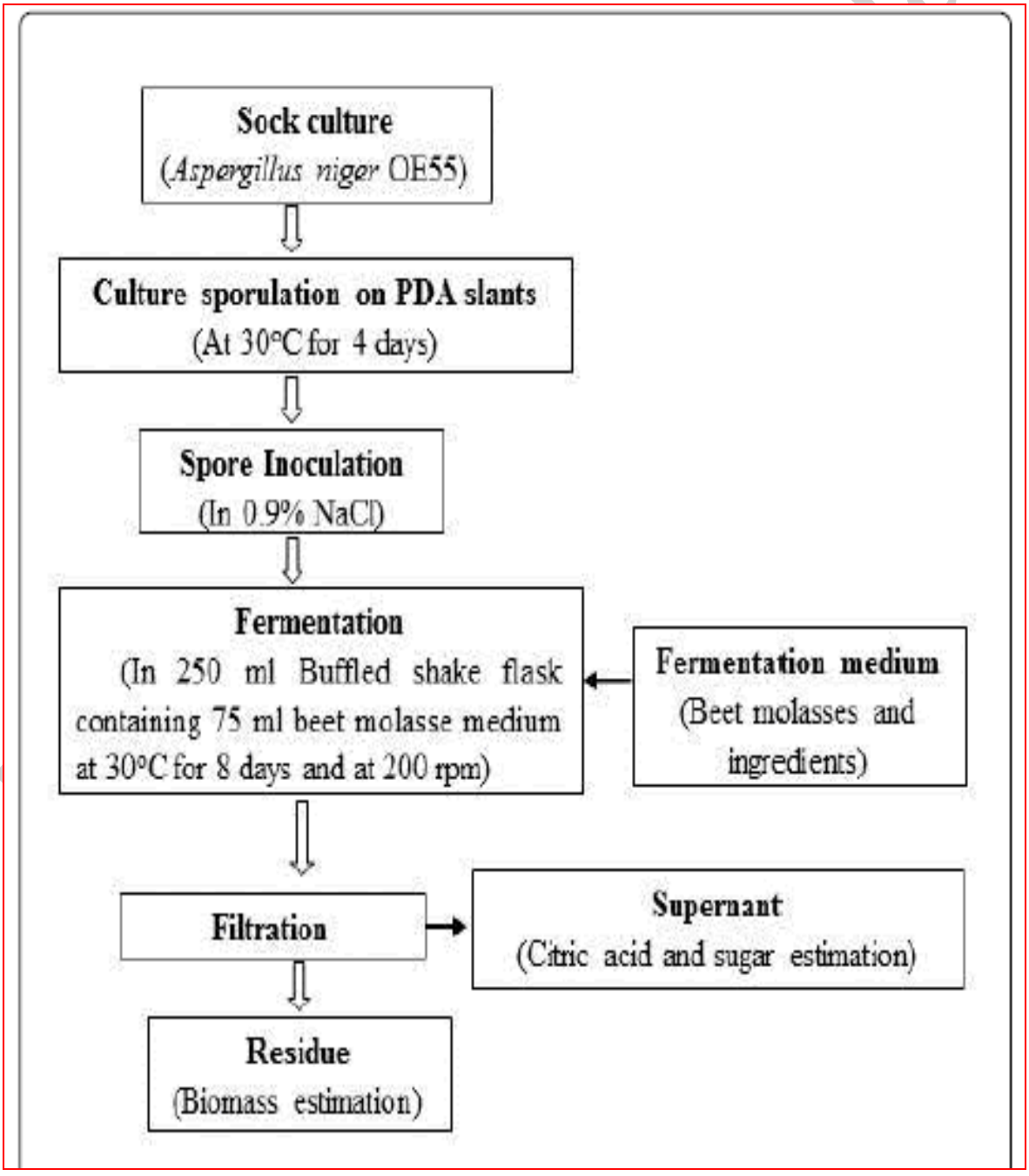
6) For determining biomass, take the whole fungal culture growth and filter with Whatman filter paper No.4, Wash with distilled water (250 ml) and dry at 105˚C to constant weight.
7) Measure culture pH by pH meter.

## Table of Results:

| Parameters | Citric acid |
|---|---|
| Citric acid (%) | |
| Biomass (g/L) | |
| pH | |

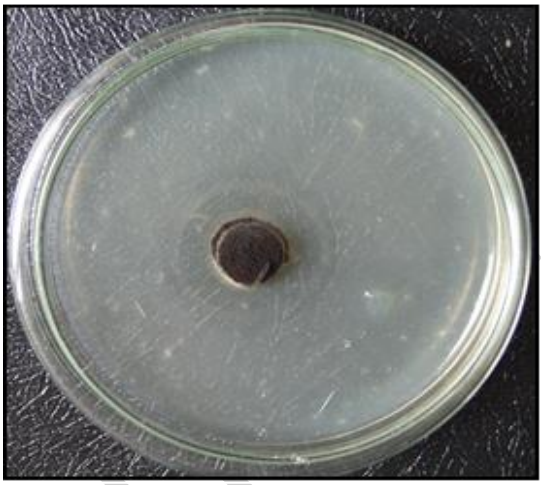
*Note that:*

- *Name of organic Acid: Citric Acid.*
- *Name of Producer Organism: Aspergillus niger.*
- *Name of Raw Material (Substarte): Cheese Whey*
- *Name of Indicator:* 0.1 N NaOH and phenolphthalein.




**Flow sheet of citric acid fermentation process by *Aspergillus niger***

# Lab (5): Microbial Production of Antibiotics



## (A) Required Materials:

(1) Soil or water sample for isolation antibiotic-producing microorganism.

(2) Selective media for isolation of antibiotic-producing microorganism such starch-nitrate agar media for isolation of actinobacteria.

(3) Microbial standard test strains such as:

(1) Bacterial test strains:

- *Bacillus subtilis* ATCC 6633
- *Staphylococcus aureus* ATCC 6538
- *Escherichia coli* ATCC 7839
- *Pseudomonas aeruginosa* ATCC 9027

(2) Unicellular fungi:

- *Candida albicans* ATCC 10231

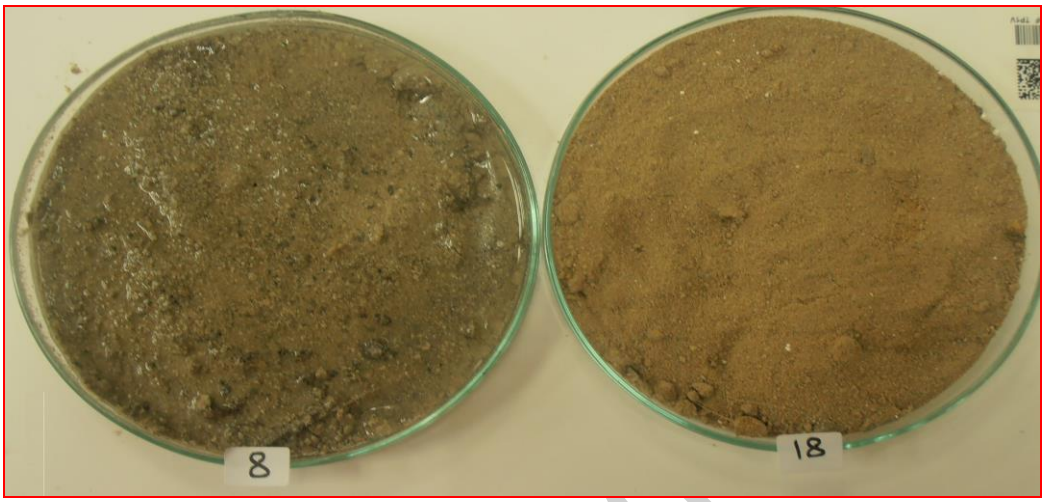
(3) Multicellular fungi:

- *Aspergillus niger* ATCC 16404
- *Aspergillus flavus* ATCC 16883

(4) Antibiotic assay-media such as Muller-Hinton agar media and nutrient agar media.

## (B) Procedures of Antibiotic Assay:

### 1) Collection of Soil Samples:

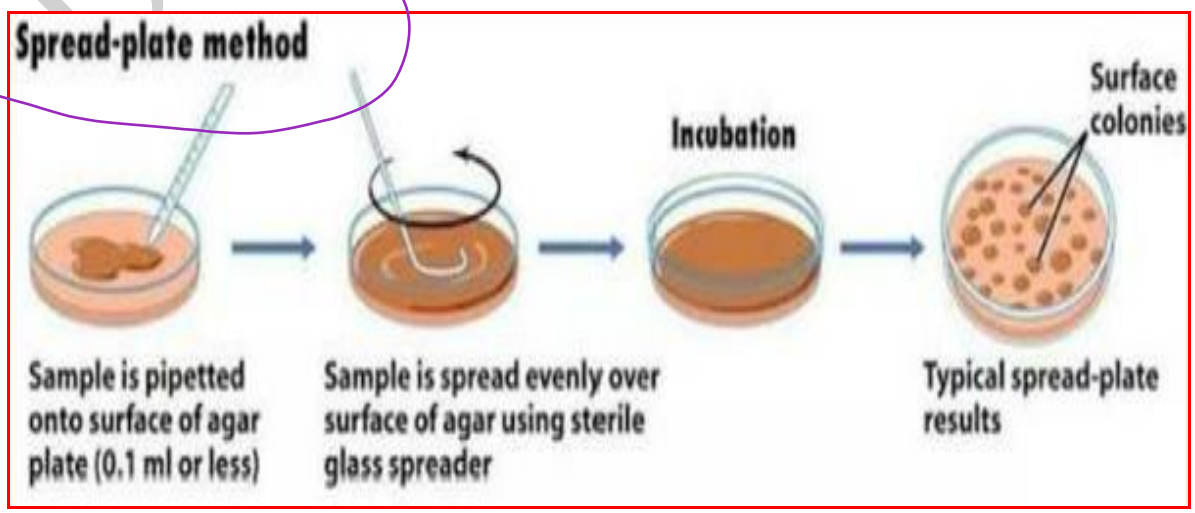



### 2) Isolation of antibiotic-producing microorganism from soil

There are two methods were used for the isolation of the target organism from the collected samples:

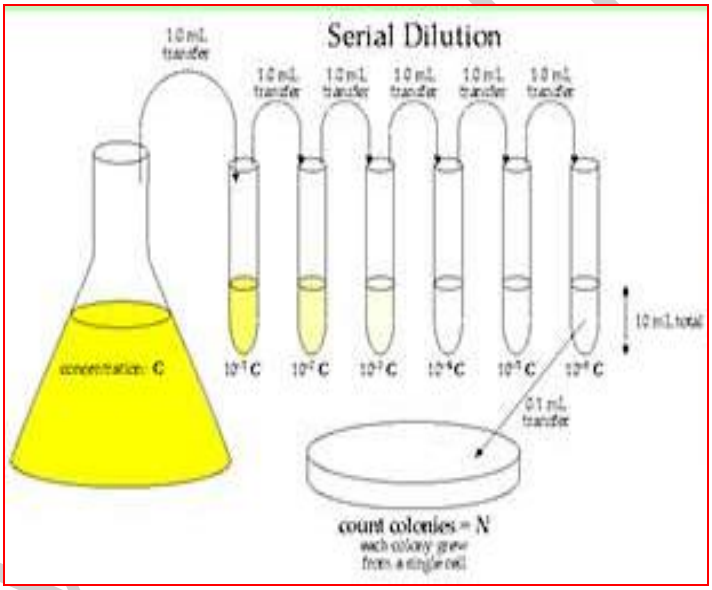
#### (A) Spread method:

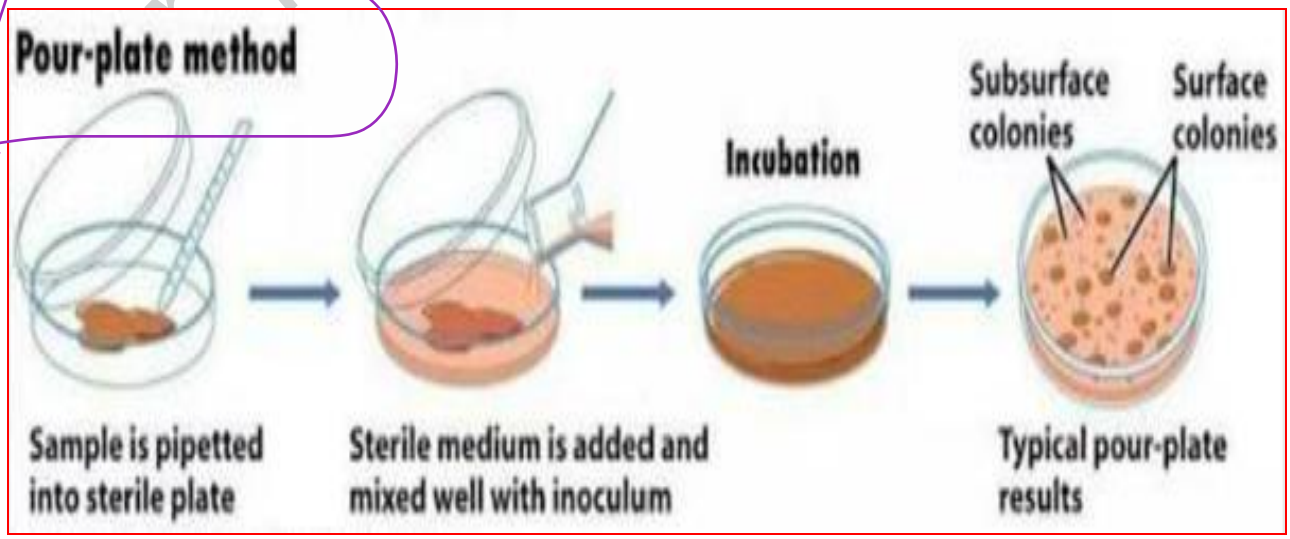
This was performed by the spatula spread technique. In which one gram of the collected soil sample is spread over the surface of the isolation media by sterile glass spreader.



## (B) Dilution Method:

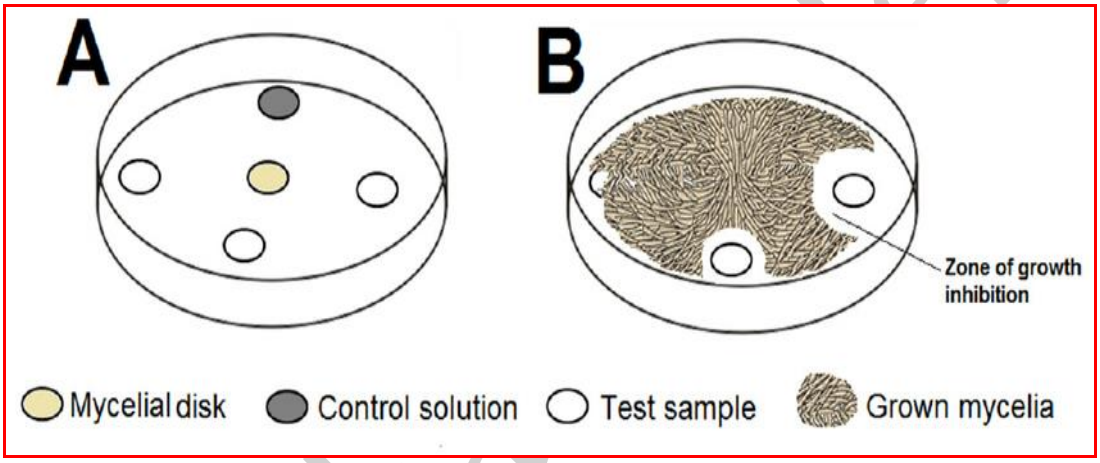
A suspension of the soil sample was prepared by shaking 10 g of the air dried soil in 100 ml sterile distilled water for about 20 min. Serial dilutions were made to cover the range of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$ and $10^{-7}$. Tenth ml of each dilution was transferred to plate medium and spread evenly on the surface of the medium using the sterile glass spreader under aseptic conditions.



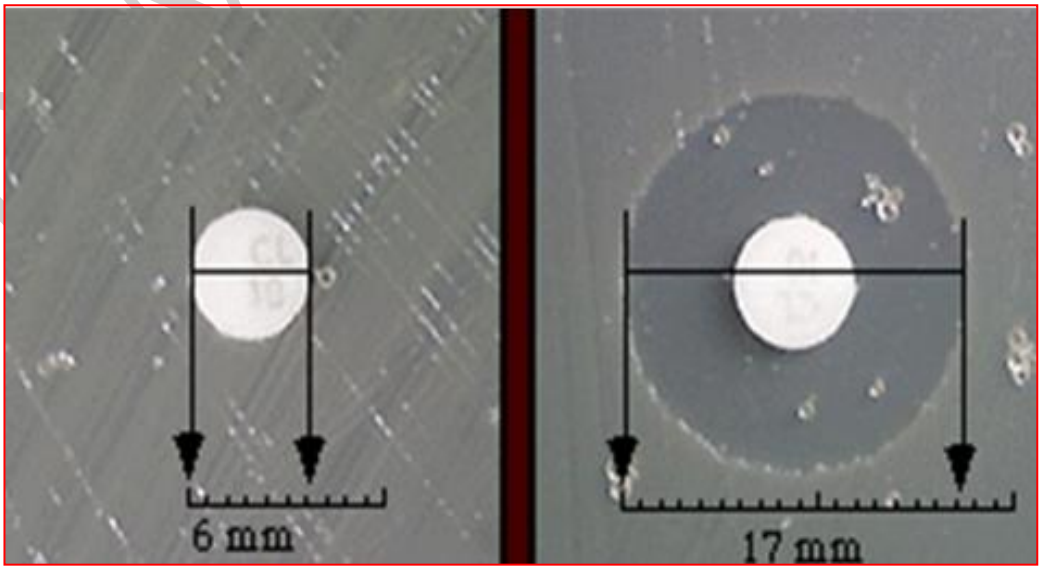



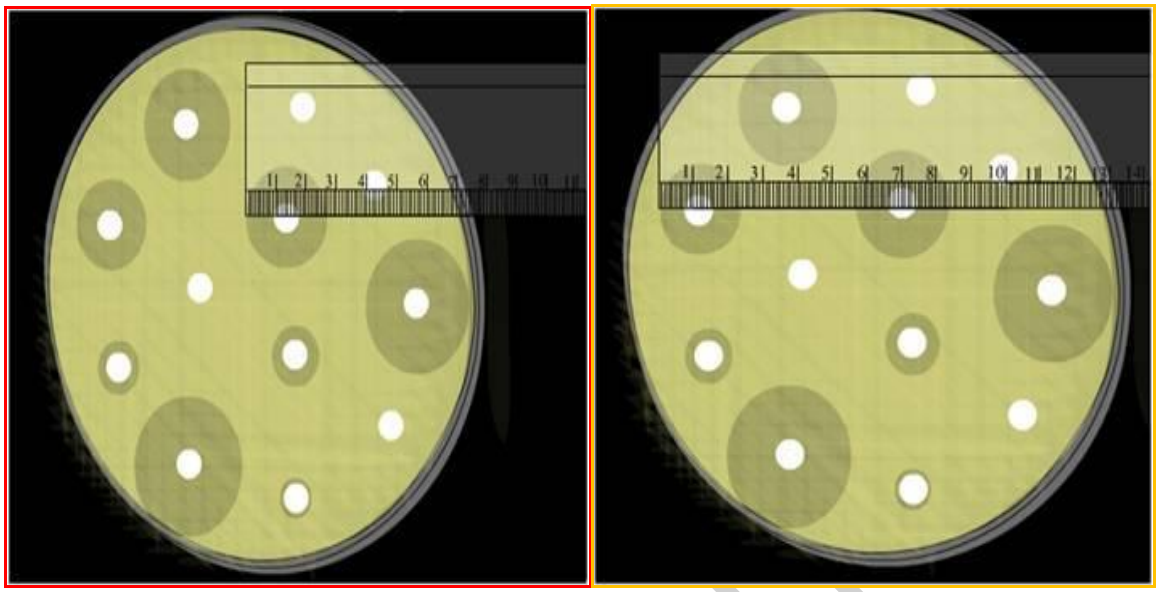
## 3) Methods of Antibiotic Assay

- To test for antibiotic production, inoculate the plates with different bacterial and fungal standard test strains.
- Incubate the plated at 30˚C for 24 hours.
- Check the plates for zone of inhibition surrounding potential antibiotic-producing organisms.




- Record your observations of each plates. Measure the diameters of the clear zones (in mm) around the antibiotic and antiseptic sensitivity disks (Figure below).



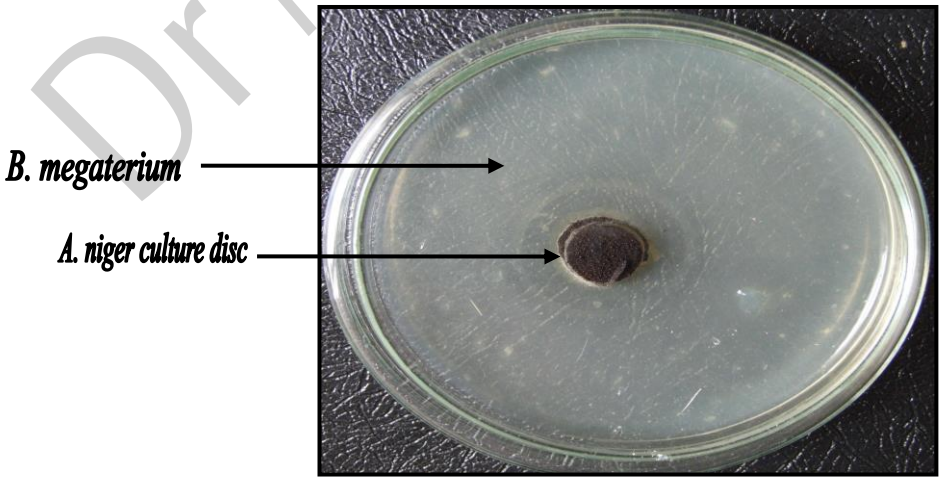


Measurement of inhibition zone diameter

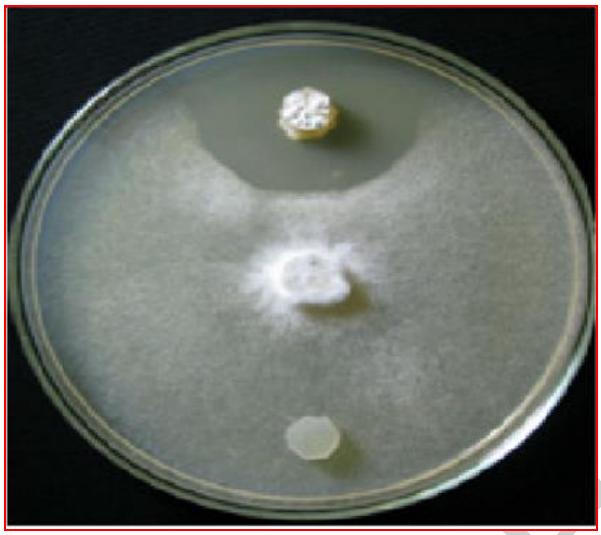
## Techniques of Antibiotic Assay:

There are 3 techniques used for assay of any antibiotic activity that are:

### (A) Agar plug/Culture disc technique

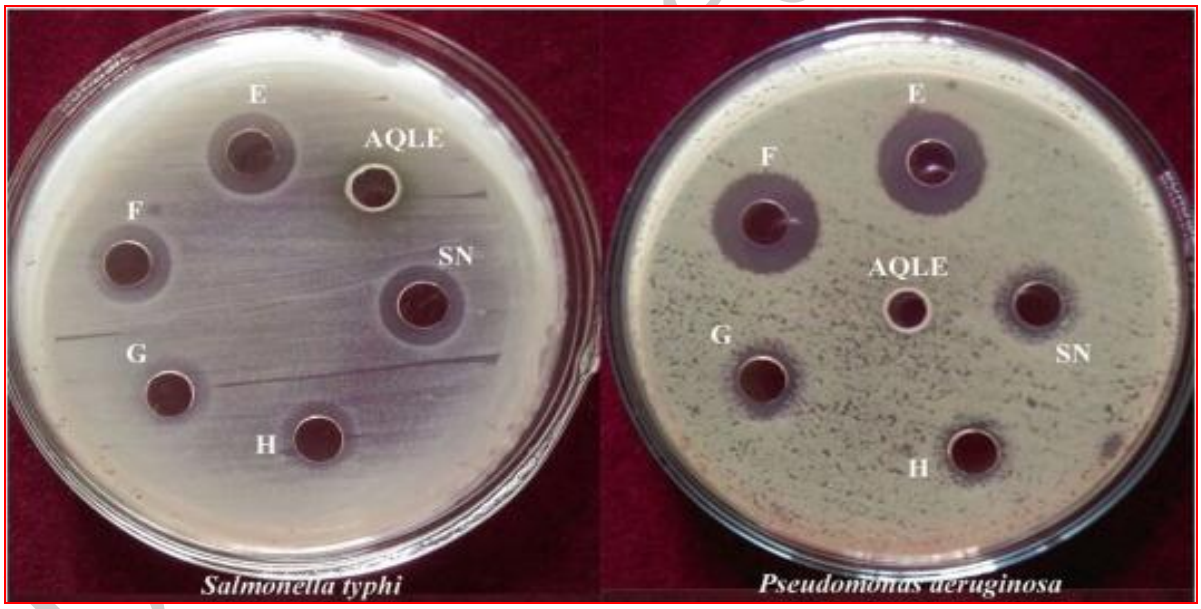



Assay of Antibacterial activity of *A. niger* using agar plug/Culture disc technique



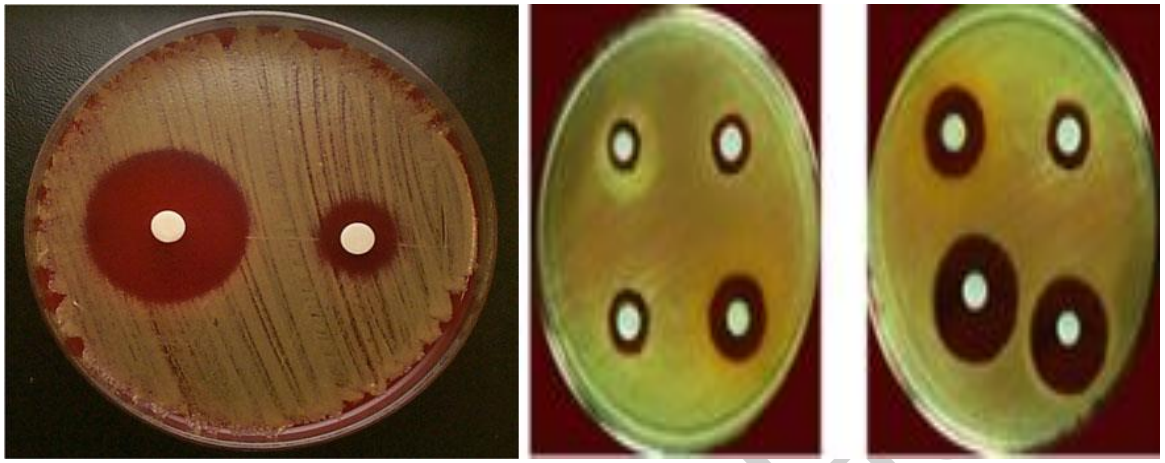
Assay of Antifungal activity of *Bacillus* using agar plug/Culture disc technique

**(B) Agar well/hole technique**




Assay of Antibacterial activity using Agar well/hole technique

## (C) Paper disc technique

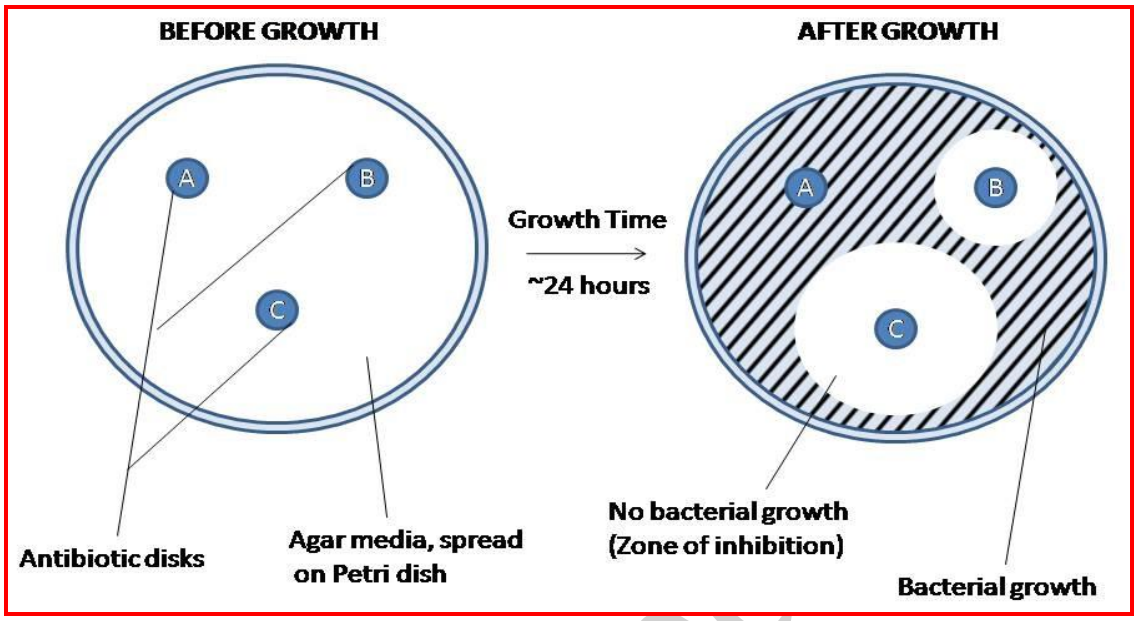


Assay of Antibacterial activity using paper disc technique

- **Test to check the antibiotic spectrum of a suspected antibiotic producer**

  - Streak the suspected antibiotic producer across a fresh Muller-Hinton agar plate.
  - Incubate the plate to permit bacterial growth and antibiotic production.
  - Cross-streak test organisms along the plate.
  - Incubate the plates at 37˚C for overnight. And check for growth inhibition.
  - Observe the growth of the test organisms.

## Results:



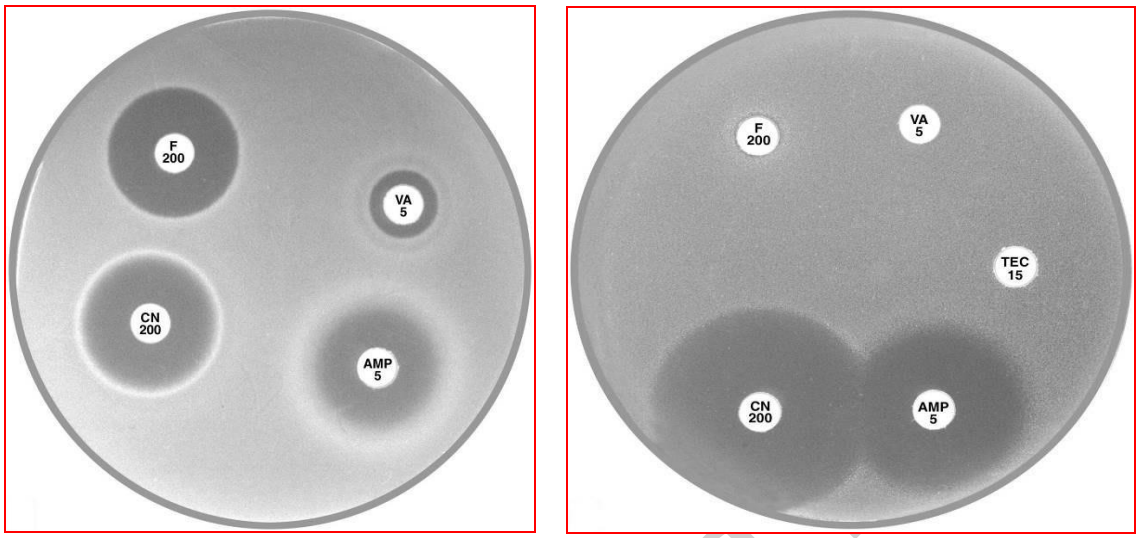

### Results of Antibiotic Assay

The obtained results of antibiotic assay are recorded as in table below:

Table: Antimicrobial activities of the isolated microorganism against different microbial strains.

| Isolate No. | Mean diameter of inhibition zone (mm) of the tested microbial strains | | | | | | |
|---|---|---|---|---|---|---|---|
| | *B. subtilus* ATCC 6633 | *S. aureus* ATCC 6538 | *P. aeruginosa* ATCC 9027 | *E. coli* ATCC 7839 | *C. albicans* ATCC 10231 | *A. niger* ATCC 16404 | *A. flavus* ATCC 16883 |
| 1 | 0.0 | 15.0 | 0.0 | 0.0 | 19.0 | 17.0 | 14.0 |
| 2 | 13.0 | 16.0 | 15.0 | 15.0 | 15.0 | 14.0 | 15.0 |

## Types of antibiotic activity:




**Gram positive bacteria** **Gram negative bacteria**

**Broad and narrow spectrum antibiotics**

As shown in the above figure:

- Broad-spectrum: Drugs that are effective against a variety of both gram-positive and gram negative bacteria.
- Narrow-spectrum: Drugs that are effective against either gram-positive or gram negative bacteria only.

As shown in figure below: antibiotics (ampicillin (Amp) & gentamycin (CN)) are broad spectrum antibiotics where it has inhibitory effect on both gram-positive and gram negative bacteria.

While the antibiotics (vancomycin (VA) & nitrofurantoin (F)) are narrow spectrum antibiotics.