

Practical-4: Bioassay of penicillin using *Bacillus subtilis*

Introduction

Many analytical methods are available to quantify the concentration of Antibiotics, such as microbiological assays (bioassay), physicochemical assays (i.e. HPLC) and immunological assays. But other assays are replaced by the microbiological assays to determine the antibiotic concentrations as they are more attractive in terms of speed, accuracy and precision.

Microbiological assays involve technique of analytical microbiology in which microorganisms are used as reagents in performing the analytical tests. Microbiological assays are useful in qualitative as well as quantitative estimation of antibiotics, vitamins, and amino acids. For assay of growth promoting substance auxotrophic mutants are used, while for assay of growth inhibitory substance sensitive strains are used.

Some of the examples are as follows

Organisms	Antibiotics
<i>Lactobacillus planetarium</i>	Niacin
<i>Neurospora</i>	Pyrindocin (Vitamin B6)
<i>Saccharomyces (yeast)</i>	Thiamine (Vitamin-B)
<i>Streptomyces lactis</i>	Follic acid, Lincomycin
<i>Staphylococcus aureous</i>	Penicillin, Erythromycin
<i>Micrococcus luturs</i>	Penicillin, Erythromycin

Microbiological assays are performed by two reasons:

1. They are more specific than chemical assays.
2. The antimicrobial activity of antibiotic is the property which can only be measured qualitatively by the use of microorganisms

Principle

In agar diffusion assay, the potency of growth-inhibiting (i.e. antibiotics) and growth-promoting substances (i.e. vitamins) are determined. Antibiotic diffuses readily in agar and usually gives clear zone of inhibition. Against many sensitive organisms, the diameter of this zone gives a good graded response to graded doses of compound.

Requirements

1. Sensitive test culture of *B. subtilis*
2. Standard antibiotic sample.
3. Unknown antibiotic sample.
4. Phosphate buffer (pH 6.8-7)
5. Top agar
6. Glass wares and other lab requirements.

Procedure

- Take a sterile Petri dish and pour approx 15 ml of the base agar into it. Allow it to solidify. Incubate 0.1 ml of test organisms to seed agar which is previously cooled to around 46°C to 47°C. mix it well.
- Pour seed agar immediately on base agar and allow it to solidify to form uniform layer.
- With the help of cup borer have 4 cups of 8mm diameter in agar; each should be at equal distance and as far as possible from each other.
- Mark the 4 wells as SH (standard high), SL (standard low), UH (unknown high), UL (unknown low). SH & UH should be diagonally opposite to each other to prevent interference of zone of inhibition.
- Add Penicillin solution into the respective cells and fill it up in such a way that it should not spread on the agar surface (add 0.1 ml of given sample).
- Allow the penicillin solution to diffuse for half an hour without disturbing plate.
- Incubate the plate at 37°C for 24 hrs.
- After incubation observe the plate for zone of inhibition around the cell.
- Measure the diameter of each zone in mm.
- Calculate potency of unknown sample of penicillin using the following formula:

$$P = \text{antilog} [D/B \times I] \times M \times F$$

Where

- ✓ P = potency
- ✓ $D = [(UH + UL) - (SH + SL)]$
- ✓ $B = [(UH - UL) + (SH - SL)]$
- ✓ $I = \log SH/SL = \text{LOG } 4/1$
- ✓ $\text{LOG } 4/1 = 0.6021$
- ✓ M = Conc. of SH = 4 units/mm
- ✓ D = dilution factor to get UH = 1 units/mm

This formula will give potency of penicillin in International Units.

1 international unit of penicillin = 0.6 micro gram of penicillin

- *Microbiology- A laboratory Manual-International student Edition: 4th edition. By James G. Cappuccino & Natalie Sherman*
- *Bacteriological Techniques By F.J. Baker*
- *Introduction to Microbial Techniques By Gunasekaran*
- *Manual of Industrial Microbiology and Biotechnology, 2nd Edition by Arnold ; . Demain and Julian, E. Davie Ronald M. Atlas, Gerald Cohen, Charles L. Hershberger, Wei-Shou Hu, David H. Sherman, Richard C. Wilson and J. H. David Wu*