Chapter ... 13

MICROBIOLOGICAL ASSAYS

♦ LEARNING OBJECTIVES ♦

After completing this chapter, reader should be able to understand:

- *Techniques for Standardization of Pharmaceuticals by Microbiological Assays*
- *The Importance of Assessment of new Antibiotic by MIC*

13.1 INTRODUCTION

A microbiological assay may be defined as qualitative or quantitative determination of any chemical compound from a simple or even complex material with the use of microorganisms. It is necessary to assay antimicrobial agents for determination of potency, for determining the pharmacokinetics of a drug in animals or man and for monitoring and controlling antimicrobial chemotherapy. Quantitative chemical or physical methods can assay most of the currently employed therapeutic agents. Many therapeutic agents, which either inhibit the growth of microorganisms (antibiotics) or are essential for their growth (vitamins and amino acids) can be standardized by microbiological assays.

Microbiological assays are relatively as accurate as chemical methods. It is a simple, specific, inexpensive and convenient method. Compared with biological assay methods using animals, microbiological techniques possess the advantages of minimal requirements of space, labour, materials and time. Microbiological assays are very useful for detecting changes in potency of antibiotics and their preparations. Microbial assays are more difficult to perform as compared to physical and chemical assays and also require proper calibration. They are less reproducible and have greater error as compared to other assays. Microbiological assay are not used if a good alternative physical or chemical assay is available.

13.2 MICROBIOLOGICAL ASSAY OF ANTIBIOTICS

The inhibition of microbial growth under standardized conditions may be utilised for demonstrating the therapeutic efficacy of antibiotics. The microbiological assay is based upon a comparison of the inhibition of growth of microorganisms by measured concentrations of the antibiotics to be examined with that produced by known concentrations of a standard preparation of the antibiotic having a known activity.

13.2.1 Media used for Antibiotic Assay

The media required for the preparation of test microorganisms, inoculums are made from the ingredients listed in Table 13.1. Dissolve the ingredients (as per Table 13.1) in sufficient water to produce 1000 ml and add sufficient 1M sodium hydroxide or 1M hydrochloric acid, as required, so that after sterilization the pH is adjusted as given in Table 13.1.

Table 13.1: Composition of media: Quantities in gm per 1000 ml medium

Ingredient	A	B	C	D	E	F	G	H	I	J
Peptone	6.0	6.0	5.0	6.0	6.0	6.0	9.4	–	10.0	–
Yeast extract	3.0	3.0	1.5	3.0	3.0	3.0	4.7	–	–	–
Beef extract	1.5	1.5	1.5	1.5	1.5	1.5	2.4	–	10.0	–
Pancreatic digest of casein	4.0	–	–	4.0	–	–	–	17.0	–	15.0
Dextrose	1.0	–	1.0	1.0	–	–	10.0	2.5	–	–
Papaic digest of soyabean	–	–	–	–	–	–	–	3.0	–	5.0
Agar	15.0	15.0	–	15.0	15.0	15.0	23.5	12.0	17.0	15.0
Glycerin	–	–	–	–	–	–	–	–	10.0	–
Polysorbate 80	–	–	–	–	–	–	–	10.0	–	–
Sodium chloride	–	–	3.5	–	–	–	10.0	5.0	3.0	5.0
Dipotassium hydrogen phosphate	–	–	3.68	–	–	–	–	2.5	–	–
Potassium dihydrogen phosphate	–	–	1.32	–	–	–	–	–	–	–
Final pH	6.5 – 6.6	6.5 – 6.6	6.95 – 7.05	7.8 – 8.0	7.8 – 8.0	5.8 – 6.0	6.0 – 6.2	7.1 – 7.3	6.9 – 7.1	7.2 – 7.4

13.2.2 Preparation of Standard, Test Solution and Innoculums

Preparation of standard solution: A standard preparation is an authentic sample of the appropriate antibiotic for which the potency has been precisely determined by reference to the appropriate international standard. The potency of the standard preparation may be expressed in International Units or in ug per mg of the pure antibiotic.

Dissolve a quantity of the standard preparation of a given antibiotic in the solvent specified in Table 13.2. Dilute the preparation to get the required concentration as stated and store in a refrigerator. On the day of assay, prepare from stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio 1:1.25 for Method A or for Method B.

Preparation of sample solution: From the information available for the substance under examination (test sample), assign to it an assumed potency per unit weight or volume, and on this assumption prepare on the day of assay a stock solution and test dilution as specified for each antibiotic in Table 13.2.

Preparation of buffer solution: Prepare the buffer solutions of given quantities in Table 13.3 of dipotassium hydrogen phosphate and potassium dihydrogen phosphate in sufficient water to produce 1000 ml after sterilization, adjusting the pH with 8 M phosphoric acid or 10 M potassium hydroxide.

Table 13.2: Stock solutions and test dilutions of standard preparation

Antibiotic	Assay method	Initial solvent for std. stock solution	Final std. stock conc^{n}/ml	Final diluent for test dilution	Median dose ug or Units/ml of test sol^{n}.
Amikacin	B	Water	1 mg	Water	10 µg
Amphotericin B	A	Dimethyl sulphoxide	1 mg	B5	1 µg
Bacitracin	A	0.01 M HCl	100 units	B1	1 unit
Bleomycin	A	B6	2 units	B6	0.04 unit
Capreomycin	B	Water	1 mg	Water	100 µg
Carbenicillin	A	B1	1 mg	B6	20 µg
Chlortetracycline	A[1]	0.1 M HCl	1 mg	Water	2.5 µg
	B[2]	0.1 M HCl	1 mg	Water	0.24 µg
Colistimethate sodium	A	Water	1 mg	B4	1 unit
	B	Water	1 mg	B6	1 unit
Colistin sulphate	A	Water	1 mg	B6	1 µg
Erythromycin	A	Methanol	1 mg	B2	1 µg
Framycetin	A	B2	1 mg	B2	1 µg
Gentamicin	A	B2	1 mg	B2	0.1 µg
Kanamycin sulphate	A[1]	B2	800 units	B2	0.8 unit
	B[3]	Water	1000 units	Water	10 unit
Neomycin	A	B2	1 mg	B2	1 µg
Novobiocin	A	Ethanol	1 mg	B4	0.5 µg
Nystatin	A	Dimethyl formamide	1000 units	B4	20 unit
Oxytetracycline	A[4]	0.1 M HCl	1 mg	B3	2.5 µg
	B[3]	0.1 M HCl	1 mg	Water	0.24 µg
Polymyxin B	A	Water	10,000 units	B4	10 unit
Spiramycin	A[5]	Methanol	1 mg	B2	12-50 units
Streptomycin	A[5]	Water	1 mg	Water	1 µg
	B[6]	Water	1 mg	Water	30 µg
Tetracycline	A[4]	0.1 M HCl	1 mg	Water	2.5 µg
	B[3]	0.1 M HCl	1 mg	Water	0.24 µg
Tobramycin	B	Water	1 mg	Water	2.5 µg
Vancomycin	A	Water	1 mg	B3	10 µg

P.T.O. ...

1 → *Bacillus pumilus* ATCC 14884 as test organism; 2 → *Staphylococcus auveus* ATCC 9144 as test organism; 3 → *Staphyococcus aureus* ATCC 29737 at test organism; 4 → *Bacillus cereus var mycoides* ATCC 11778 as test organism; 5 → *Bacillus subtilis* ATCC 6635 as test organism. 6 → *Klebsiella pneumoniae* ATCC 10031 as test organism; B denotes buffer solution and number following refers to the buffer number.

Table 13.3: Buffer solutions

Buffer No.	Dipotassium hydrogen phosphate, K_2HPO_4 (g)	Potassium dihydrogen phosphate, KH_2PO_4 (g)	pH adjusted after sterilization to
1.	2.0	8.0	6.0 ± 0.1
2.	16.73	0.523	8.0 ± 0.1
3.	–	13.61	4.5 ± 0.1
4.	20.0	80.0	6.0 ± 0.1
5.	35.0	–	10.5 ± 0.1
6.	13.6	4.0	7.0 ± 0.2

Test microorganisms: The test microorganism for each antibiotic is listed in Table 23.4 with its identification number in the American Type Culture Collection (ATCC). Maintain a culture on slants of the medium under the incubation conditions (Table 13.5) and transfer weekly to fresh slants.

Preparation of inoculum: Prepare the microbial suspensions for the inoculum for the assay as given in Table 13.5. Test microorganism suspensions are prepared by any one of the following methods:

(i) Maintain the test microorganism on slants of medium A and transfer to a fresh slant once a week. Incubate the slants at the specified temperature for 24 hours. Using 3 ml of saline solution, wash the microorganisms from the agar slant onto a large agar surface of medium A such as a Roux bottle containing 250 ml of agar. Incubate for 24 hours at the appropriate temperature. Wash the growth from the nutrient surface using 50 ml of saline solution. Store the test microorganisms under refrigeration. Determine the dilution factor which will give 25% light transmission at about 530 nm. Determine the amount of suspensions to be added to each 100 ml of agar of nutrient broth by use of test plates or test broth. Store the suspension under refrigeration.

(ii) Proceed as described in method 1 but incubate the Roux bottle for 5 days. Centrifuge and decant the supernatant liquid. Resuspend the sediment with 50 to 70 ml of saline solution and heat the suspension for 30 minutes at 70°C. Wash the spore suspension three times with 50 to 70 ml of saline solution and heat shock again for 30 minutes. Use test plates to determine the amount of the suspension required for 100 ml agar. Store the suspension under refrigeration.

(iii) Maintain the test microorganisms on 10 ml agar slant of medium G (Table 13.1). Incubate at 32 to 35°C for 24 hours. Inoculate 100 ml of nutrient broth. Incubate for 18 to 24 hours at 37°C and proceed as described in method 1.

(iv) Proceed as described in method 1 but wash the growth from the nutrient surface using 50 ml of medium 1 (prepared without agar) in place of saline solution.

Table 13.4: Test organisms for microbiological assay of antibiotics

Antibiotic	Test organism	ATCC No.
Amikacin	*Staphylococcus aureus*	29737
Amphotericin B	*Saccharomyces cerevisiae*	9763
Bacitracin	*Micrococcus luteus*	10240
Bleomycin	*Mycobacterium smegmatis*	607
Capreomycin	*Klebsiella pneumoniae*	10031
Carbenicillin	*Pseudomonas aeruginosa*	25619
Chlortetracycline	*Bacillus pumilus*	14884
Colistimethate sodium	*Bordetella bronchiseptica*	4617
	Escherichia coli	10536
	Escherichia coli	9637
Colistin sulphate	*Bordetella bronchiseptica*	4617
	Escherichia coli	10536
Erythromycin	*Kocuria rhizophila*	9341
Framycetin	*Bacillus pumilus*	14884
	Bacillus subtilis	6633
Gentamicin	*Staphylococcus epidermidis*	12228
Gramicidin	*Entrococcus hirae*	10541
	Staphylococcus aureus	6538
Kanamycin sulphate	*Bacillus pumilus*	14884
	Staphylococcus aureus	29737
Neomycin	*Staphylococcus epidermidis*	12228
Netilmicin sulphate	*Staphylococcus aureus*	6538
Novobiocin	*Staphylococcus epidermidis*	12228
Nystatin	*Saccharomyces cerevisiae*	2601
Oxytetracycline	*Bacillus cereus var, mycoides*	11778
	Staphylococcus aureus	29737
Polymyxin B	*Bordetella bronchiseptica*	4617
Sisomicin	*Staphylococcus epidermidis*	12228
Spiramycin	*Bacillus subtilis*	6633
Streptomycin	*Bacillus subtilis*	6633
	Klebsiella pneumoniae	10031
Teicoplanin	*Bacillus subtilis*	6633
Tetracycline	*Bacillus cereus*	11778
	Staphylococcus aureus	29737
Tobramycin	*Staphylococcus aureus*	29737
Tylosin	*Staphylococcus aureus*	9144
Tyrothricin	*Entrococcus hirae*	10541
Vancomycin	*Bacillus subtilis*	6633

Table 13.5: Preparation of inoculum

Test organism	Incubation conditions			Suggested inoculums composition		
	Medium/ Method of Preparation	Temp. (°C)	Time (hr)	Medium	Amount (ml/100 ml)	Antibiotic assayed
Bacillus cereus var mycoides	A/2	32-35	120	F	As required	Oxytetracycline/ Tetracycline
Bacillus pumilus	A/2	32-35	120	D	As required	Chlortetracycline/ Framycetin/ Kanamycin Sulphate
Bacillus subtilis	A/2	32-35	120	E	As required	Teicoplanin/ Framyetin/ Kanamycin B.
				A	As required	Streptomycin/ Vancomycin
				B	As required	Spiramycin
Bordetella bronchiseptica	A/1	32-35	24	H	0.1	Colistimethate Sodium/ Polymyxin B, Colistin sulphate
Escherichia coli (10536)	A/1	35-39	24	H	0.1	Colistimethate Sodium/Colistin Sulphate
Escherichia coli (9637)	A/1	35-37	24	C	0.1	Colistimethate Sodium
Entrococcus hirae	A/1	36-38	16-18	C	1.0	Gramicidin/ Tyrothricin
Klebsiella pneumoiniae	A/1	36-37	24	C	0.1	Capreomycin/ Streptomycin
Kocuria rhizophila	A/1	32-35	24	D	1.5	Erythromycin
Micrococcus luteus	A/1	32-35	24	A	0.3	Bacitracin
Mycobacterium smegantis	J/4	36-37.5	48	I	1.0	Bleomycin

contd. ...

Pseudomonas aeruginosa	A/1	36-37.5	24	H	0.5	Carbenicillin
Saccharomyces cerevisiae (9763)	G/3	29-31	48	G	1.0	Amphotericin B
Saccharomyces cerevisiae (2601)	G/3	29-31	48	G	1.0	Nystatin
Staphylococcus aureus (6538)	A/1	32-35	16-18	C	1.0	Gramicidin
			24	A	1.0	Netilmicin sulphate
Staphylococcus aureus (29737)	A/1	32-35	24	C	0.1	Amikacin/Tylosin/ Doxycycline/ oxytetrncycline/ Tetracycline/ Tobramycin
Staphylococcus epidermidis	A/1	32-35	24	D	0.03/0.4	Gentamicin/ Neomycin
				A/D	4.0/0.03	Norobiocin/ Sisomicin

13.2.3 Assay Methods

The microbiological assay of antibiotics may be carried out by the following two methods:

(i) Method A: Cup-plate or cylinder-plate method.

(ii) Method B: Turbidimetric or tube assay method.

(i) Method A: Cup-plate or cylinder-plate method: This method depends on the diffusion of an antibiotic from a vertical cavity or a cylinder, through the solidified agar layer in a Petri plate. The growth of test microorganisms is inhibited entirely in a circular area or zone around the cavity or cylinder containing a solution of the antibiotic.

A liquified assay medium (43 to 45°C) is inoculated by suspension of test microorganisms and the inoculated medium is poured immediately into sterile Petri plate or pre-prepared agar plates by using an assay medium and then spread the test culture or microorganisms on the surface of plates (spread plate technique).

Solutions of known concentrations of the standard preparation and the test antibiotic are prepared in appropriate solutions as given in Table 13.2. Preparation of the standard solutions and potency of antibiotics for assay of penicillin and assay of streptomycin is given in Table 13.6. These solutions are added in sterile cavities or cylinders prepared in a solid medium (Fig. 13.1.)

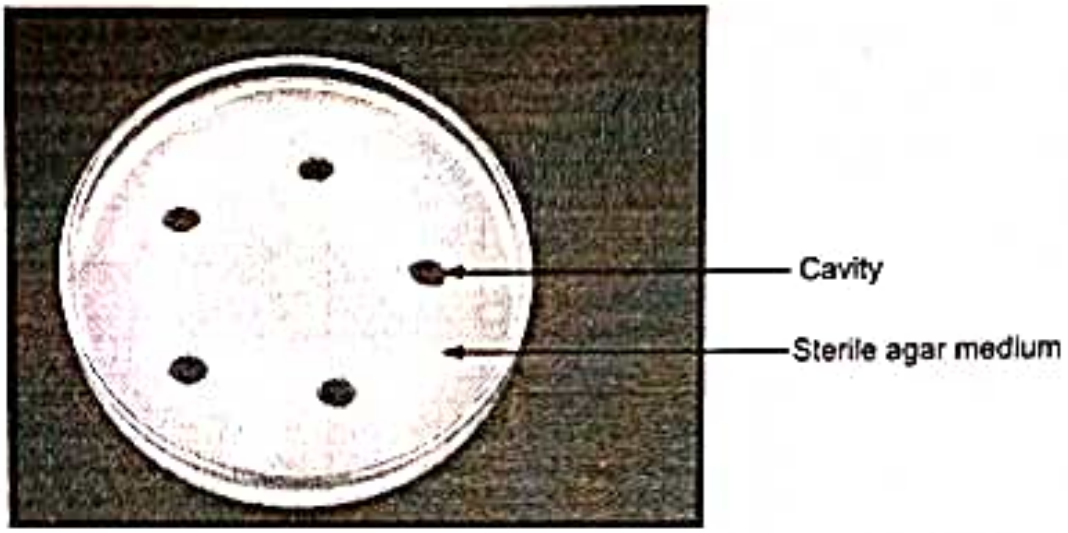

Fig. 13.1: Cavities prepared in sterile agar medium for assay of antibiotics

Table 13.6: Comparison of assay of penicillin and streptomycin

Properties	Penicillin	Streptomycin
Form	Crystalline sodium salt of benzyl penicillin	Streptomycin sulfate
Units/mg	1670	780
Potency in units/ml	0.5 – 8	5 – 20
mg containing 1 unit	0.0005988	0.001282
Units for standards	1, 2, 3, 4, 5 and 6	10, 12, 14, 16, 18 and 20
Preparation of antibiotic solution	Dissolve 35 mg in 100 ml. Take one ml from above solution and dilute to 100 ml. Each ml of this contains 6 unit activity.	Dissolve 256 mg in 100 ml. Take 1 ml from the above solution and dilute to 100 ml. Each ml of this contains 20 units of activity.
Amount to be added in cavity	1 ml or as per size of the cavity.	1 ml or as per size of the cavity.

The volume of solution added to each cavity or cylinder must be uniform and sufficient to fill the holes. When paper discs are used, these discs should be sterilized first and then dipped in the standard solutions or the test solutions and placed on the surface of the medium.

The plates are left standing for 1 to 2 hours at room temperature or at 4°C, as a period of pre-incubation diffusion to minimize the effects of variation in time between the applications of the different solutions. All plates are then incubated for about 18 to 24 hours at the temperature indicated in Table 13.5. The diameters or areas of the circular inhibition zones (Fig. 13.2) produced by standard and test antibiotic solutions are accurately measured. The graph which relates zone diameter to the logarithm of the concentration of antibiotics is plotted (Fig. 13.3) and the unknown concentration of test antibiotics is calculated.

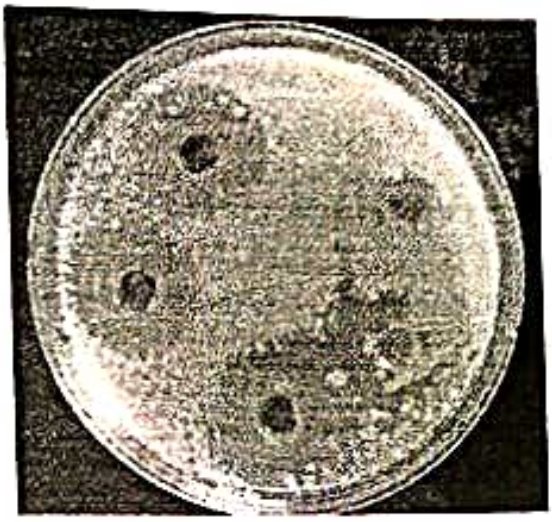

Fig. 13.2: Zone of inhibition observed in plate (Assay of streptomycin)

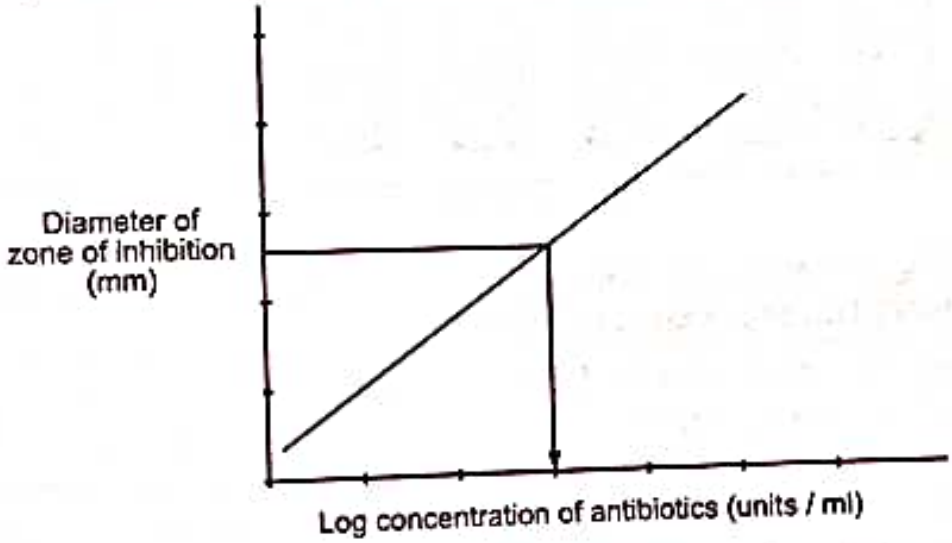

Fig. 13.3: Standard curve of microbiological assay of antibiotics

(ii) Method B: Turbidimetric or tube assay method: This method depends upon the growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favourable to its rapid growth in the absence of the antibiotic. This method has the advantage of a shorter incubation period for the growth of the test microorganisms (4 to 5 hours). However, the presence of solvent residues or other inhibitory substances affects this assay more than the cup-plate assay. This method is not recommended for cloudy or turbid preparations.

Five different concentrations of the standard solution are prepared by diluting the stock solution (Table 13.2) for making the standard curve. A median concentration is selected and the test sample of the antibiotic solution is adjusted by dilution to obtain approximately this concentration. One ml of each concentration of the standard solution and of the sample solution are placed in each of the tubes in duplicate. To each tube, 9 ml of nutrient medium (Table 13.1) previously seeded with the appropriate test microorganism is added.

At the same time, three control tubes, one containing the inoculated culture medium (culture control), another identical with it but treated immediately with 0.5 ml of dilute formaldehyde solution (blank) and a third containing uninoculated culture medium are prepared.

All the tubes are placed in an incubator at the specified temperature (Table 13.5) for 4 to 5 hours. After incubation add 0.5 ml of dilute formaldehyde solution to each tube. The growth of the test microorganisms (Fig. 13.4) is measured by determining the absorbance at about 530 nm of each of the solutions in the tubes against the blank.

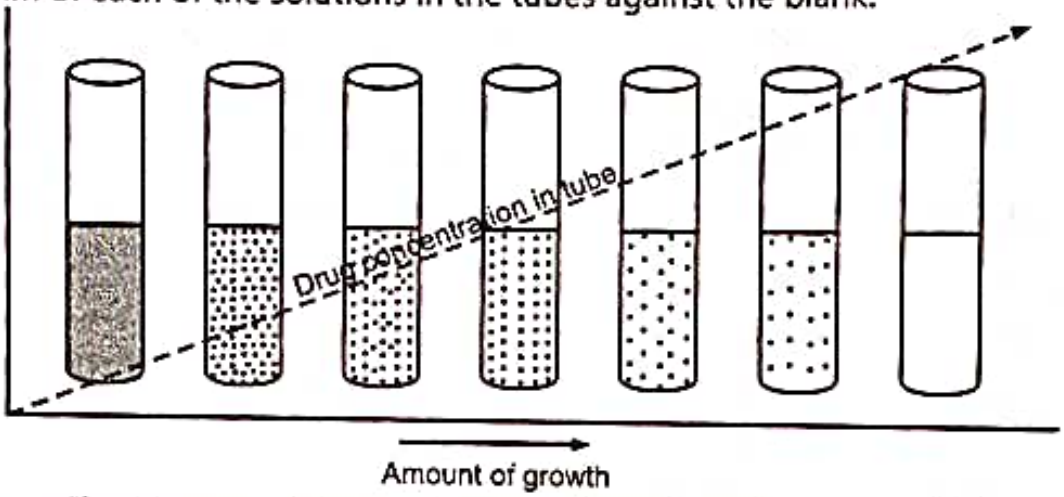

Fig. 13.4: Microbiological assay of antibiotics by tube assay method

13.3 STANDARDIZATION OF VITAMINS

Vitamins are important growth factors needed for growth and multiplication of microorganisms. They are very sensitive to small amounts of growth factors. It is the ability of these test microorganisms to synthesize the factor being assayed that forms the basis of the microbiological assay of vitamins and amino acids. Test microorganisms used for assaying the water soluble vitamins are listed in Table 13.7.

Table 13.7: Test microorganisms and conditions for the microbial assay of vitamins

Vitamin	Test Microorganism	Incubation Temp. (°C)	Assay pH
Vitamin B_{12}	*Lactobacillus leichamannii* ATCC 7830	37	6.1
	Poteriochromonas stipitata ATCC 11532	30	5.5
Vitamin B_6	*Saccharomyces uvarum* ATCC 9080	30	4.5
	Tetrahymena thermophila ATCC 30008	30	6.1
Riboflavin	*Lactobacillus casei* ATCC 7469	37	6.8
	Tetrahymena thermophila ATCC 30008	30	6.1
Thiamine	*Lactobacillus viridescens* ATCC 12706	30	6.0
	Ochromonas danica ATCC 30004	30	5.5
Biotin	*Lactobacillus plantarum* ATCC 8014	37	6.8
	Ochromonas danica ATCC 30004	30	5.5
Niacin	*Lactobacillus plantarum* ATCC 8014	37	6.8
	Tetrahymena thermophila ATCC 30008	30	6.1
Pantothenate	*Lactobacillus plantarum* ATCC 8014	37	6.7
	Tetrahymena thermophila ATCC 30008	30	6.1

Microbiological assay of cyanocobalamin and other vitamins can be performed by the following methods:

(i) Titrimetric method.

(ii) Turbidimetric method.

13.3.1 Titrimetric Method

Procedure: Clean ten test tubes and add 0.0 ml, 0.5 ml, 1.0 ml, 1.5 ml, 2 ml, 2.5 ml, 3 ml, 4 ml, 4.5 ml and 5 ml respectively of standard cyanocobalamin solution (0.01 – 0.04 ug/ml). To each test tube add 5 ml Basal medium stock solution. Adjust the final volume (10 ml) by using water. In the other four test tubes add 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml respectively of the test solution to be assayed. To each test tube add 5 ml Basal medium stock solution and adjust the final volume (10 ml) by using water.

Sterilize all test tubes in autoclave at 121°C for 5 minutes. After sterilization, cool all test tubes upto room temperature and inoculate with one drop of inoculum (*Lactobacillus leichmanii* ATCC 7830). Incubate the tubes for 64 to 72 hours at any chosen temperature within the range of 30 to 37°C (Table 13.8).

Table 13.8: Procedure for microbiological assay of Vitamin B_{12} (Titrimetric method)

Tube number	Std. Cyanocobalamin solution (0.01 to 0.04 µg/ml) (ml)	Basal medium stock solution (ml)	Volume of sterile water (ml)
1	0.0	5	5.0
2	0.5	5	4.5
3	1.0	5	4.0
4	1.5	5	3.5
5	2.0	5	3.0
6	2.5	5	2.5
7	3.0	5	2.0
8	4.0	5	1.0
9	4.5	5	0.5
10	5.0	5	0.0
1'	1.0 (Test solution)	5	4.0
2'	2.0 (Test solution)	5	3.0
3'	3.0 (Test solution)	5	2.0
4'	4.0 (Test solution)	5	1.0

Titrate the contents of each tube with 0.05 N NaOH, electrometrically or using 0.1% w/v bromothymol blue as an indicator (converts to green colour). Determine the average of the titration values for each level of standard and test samples used. Plot the graph by considering the average titration values expressed in ml of 0.05 N NaOH against the corresponding levels of standard cyanocobalamin solution added (Fig. 13.5). Draw a smooth curve and determine the concentration as activity per ml of test solution by interpolation of vitamin B_{12} activity.

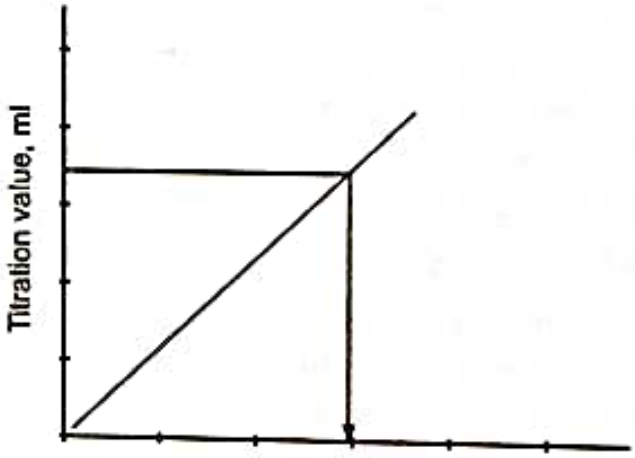

Fig. 13.5: Assay of Vitamin B_{12}

Standard cyanocobalmin stock solution: 0.1 µg/ml in 25% alcohol. Store in a refrigerator for not more than two months.

Basal medium stock solution: Composition of Basal medium stock solution is as follows:

L – Cystine	0.1 gm
DL – Tryptophan	0.1 gm
1N HCl	10 ml
Adenine - Guanine - Uracil solution	5 ml
Xanthine Solution	5 ml
Riboflavin - Thiamine - Biotin - Nicotinic acid solution	10 ml
P-Aminobenzoic acid – Pyridoxine - Pyridoxal - Pyridoxamine solution	10 ml
Calcium pantothenate - Folic acid solution	5 ml
Salt solution A	5 ml
Salt solution B	5 ml
Asparagine solution	5 ml
Acid - hydrolysed casein solution	25 ml
Dextrose	10 gm
Sodium acetate	5 gm
Ascorbic acid	1 gm
Sorbitan mono-oleate derivative solution	5 ml

First dissolve L-cystine and DL-tryptophan in hydrochloric acid and add the other ingredients/reagents in the order listed in the formula. Dissolve dextrose, sodium acetate and ascorbic acid separately in 100 ml of water and add to the mixture. Adjust the reaction of the medium to pH 6.0 with 1N sodium hydroxide. Add the sorbitan mono-oleate derivative solution and make-up the volume to 250 ml with distilled water.

Test solution of the material to be assayed: Take accurate amount of the material to be assayed and dissolve it in water or dilute if necessary. Add dil. HCl or solution of sodium hydroxide to adjust the pH (6.0) and add water to make a final volume.

Preparation of innoculum: Transfer a few cells of *Lactobacillus leichmannii* from a recent sub-culture into two sterile tubes each containing 10 ml of culture medium (yeast extract – 0.75 gm, peptone – 0.75 gm, dextrose – 1 gm, potassium dihydrogen phosphate – 0.2 gm, tomato juice filtrate – 10 ml, sorbitan mono-oleate derivate solution – 1 ml, pH 6.8 and water to make 100 ml). Incubate the tubes for 18 to 24 hours at 37°C. After incubation, centrifuge the culture till the cells settle to the bottom of the tube (as mat). Decant off the supernatant fluid, under aseptic conditions and suspend the cells in 10 ml of sterile suspension medium (Basal medium stock solution – 25 ml + water – 25 ml). Centrifuge again and decant off the supernatant fluid. Repeat this procedure a third time if necessary. Finally suspend the cells uniformly in 10 ml of sterile suspension medium. Aspetically transfer 1 ml of the suspension of the cells to 10 ml of sterile suspension medium and mix. The resulting cell suspension is the inoculum.

13.3.2 Turbidimetric Method

Apparatus, reagents and procedures are same as titrimetric method but this test includes two more test tubes to which neither standard cyanocobalmin solution nor test solution, nor inoculum is added. Incubate all test tubes at 30 to 37°C for 16 to 24 hours.

By using an 'uninoculated blank tube' adjust the transmittance at 640 mμ to 100% in the photoelectric colorimeter. Thoroughly mix the contents of each tube and record the transmittance reading. Plot the graph by considering transmittance value against the corresponding levels of standard cyanocobalmin solution. Draw a smooth curve and calculate the concentration of the test solution of cyanocobalmin.

13.4 STANDARDISATION OF AMINO ACIDS

The principle and procedure of assay of amino acids is same as that of assay of vitamins (Refer Section 13.3). The only difference is the factor is an amino acid instead of a vitamin.

Microbiological methods mainly used for determination of amino acids because of their specificity, accuracy, sensitivity and ability to yield many replicate results within a short time. Amino acids are essential components for the growth and multiplication of different microorganisms. Growth of many strains of microbes depends on a specific amino acid. The

amino acids such as arginine, leucine, histidine, isoleucine, methionine, threonine, phenylalanine, tryptophan, valine, glutamic acid, serine, cystine, tyrosine, etc. are essential for the growth of cells. The concentration of this substances present in the sample are determined by comparing the effect of sample and standard on growth of microorganisms. The reaction is the response of formation of lactic acid or growth of microorganisms. Lactic acid formation is determined by titration method or pH change and growth is determined by turbidimetry or gravimetry method. *Leuconostoc mesenteroides* ATCC 8042, *Streptococcus faecalis ATCC* 8043, *Lactobacillus arabinosus* 1715, etc. are commonly used as test organisms for assay of amino acids.

13.5 ASSESSMENT OF NEW ANTIBIOTIC

Assessment of new antibiotic or antimicrobial agent is the process of discovery and evaluation of efficacy of isolated synthetic or natural chemicals against different types of microorganisms. Antibiotic is a antibacterial substance active against bacteria and is the most important type of antibacterial agent. Antibiotic medications are widely used in treatment and prevention of bacterial, fungal and protozoal infections. Effective concentration of new compounds are evaluated by method of Minimum Effective Concentration (MEC), means determination of concentration at which the chemical or antibiotic effectively kills the microbial strains.

The different techniques are used for assessment of new antimicrobial agents such as agar diffusion method, antimicrobial gradient method (E-test), Minimum Effective Concentration (MEC), dilution test etc.

Agar diffusion method:

This method is most applied method for assessment of antibiotics and it is also called as disc method. In this test, standard suspensions of log phase growth microbial cells are prepared and inoculated onto the surface of agar plates to form lawn. Then a filter paper discs are taken and wetted with the fixed known concentration of chemical to be tested. These wet paper discs are placed over the surface of culture media or dried lawn and plates are incubated aerobically at 35 to 37°C for 18 to 24 hours. The test chemical is diffused from paper disc and also diffuse into the agar media. The clear area formed around the paper discs is called zone of inhibition (Fig. 13.6). Zone of inhibition formation indicates the microbial cells are killed by the test antibiotic. This shows that the new test antibiotic or antimicrobial substance is effective against specific microbial cells. The diameter of the zone of inhibition is more, that indicates test chemical is more effective against selected microbial cells.

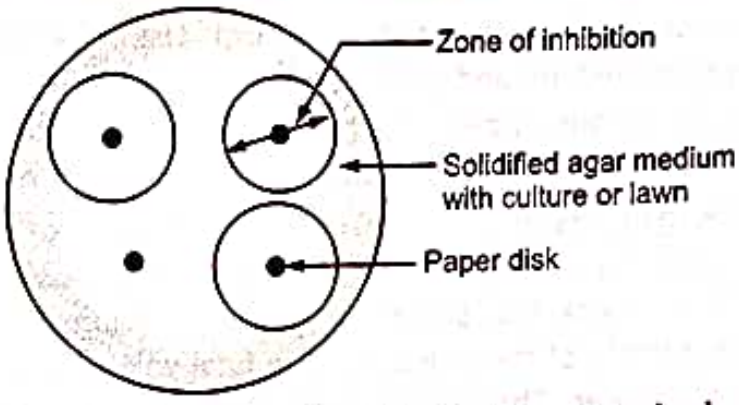

Fig. 13.6: Paper disc or agar diffusion method

Antimicrobial gradient test:

This test is called as Epsilometer (E) test. This method shows sensitivity of selected microbial cells towards any test chemical as well as information about MIC. Bacterial cell lawn culture is prepared in a similar manner of agar diffusion method. The test chemical is applied on paper strip by increasing concentration at fixed interval or gap (Fig. 13.7). The paper strip is placed on the freshly prepared bacterial lawn and incubated at 35°C for 18 to 20 hours. The test chemical is diffused from the strip to the culture medium and kills bacterial cells as per its efficacy to form zone of inhibition (Fig. 13.7).

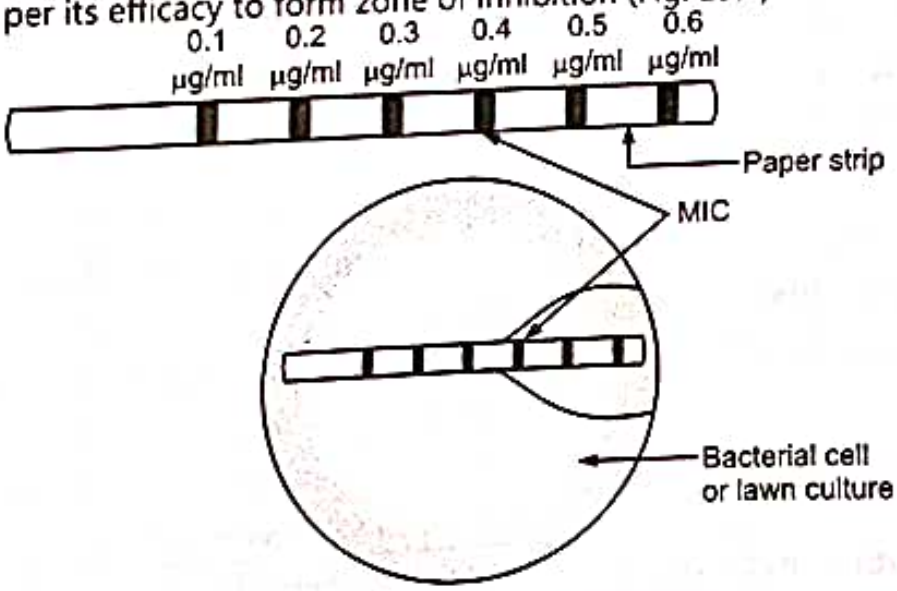

Fig. 13.7: Determination of MIC by E-test

Dilution method or MIC method:

Minimum Inhibitory Concentration (MIC) is the lowest concentration of antimicrobial compound found to inhibit the growth of a particular test microorganism. It may be applied to assess new disinfectants, antiseptics, preservatives and antibiotics. MIC values are usually expressed in terms of µg/ml or units/ml. Minimum inhibitory concentration of different anti-microbial compounds may be determined by the liquid dilution method or the solid dilution method.

1. Liquid dilution method or test tube method: Use a series of test tubes which contain a double - strength medium and are labelled as shown in Table 13.9. In the first tube (un-inoculated), inoculum is not added which is used for checking the sterility of the medium. All other in a eleven test tubes, inoculum (3 to 4 drops) is added to reach the final concentration of microorganisms is 10^5 to 10^6 cells/ml. In all test tubes, test chemical is added ranging from 0.5 to 5 ml except in the uninoculated and control tube. The second tube (control) is used to check the suitability of the medium for growth of the test microorganism and the viability of the inoculum. The final volume (10 ml) in all test tubes is adjusted by using sterile water. The contents of all test tubes are properly mixed and incubated at 37°C for 2 to 3 days. After incubation, all test tubes are examined for the growth in the form of turbidity and the results are recorded and minimum inhibitory concentration is calculated. It is also necessary to conduct a preliminary experiment to determine the approximate range (test solution) which would be suitable for the test.

Table 13.9: Determination of MIC by liquid dilution method

Tube number	Volume of double strength medium (ml)	Volume of test chemical (ml)	Volume of sterile water (ml)
0 (uninoculated)	5	0.0	5
0' (control)	5	0.0	5
1	5	0.5	4.5
2	5	1.0	4.0
3	5	1.5	3.5
4	5	2.0	3.0
5	5	2.5	2.5
6	5	3.0	2.0
7	5	3.5	1.5
8	5	4.0	1.0
9	5	4.5	0.5
10	5	5.0	0.0

2. Solid dilution method: In this method, test chemical is first mixed into molten agar and then poured into Petri plates. After solidification, the innoculum is spread on the surface of agar medium. All plates are incubated at 37°C for 2 to 3 days. After incubation, all plates are observed for growth of inoculum and the minimum inhibitory concentration of the test chemical is calculated.

The advantages of this method are:

(i) Several microorganisms can be tested at the same time by use of multipoint inoculator.

(ii) Contaminations are easily detected, because colony features on solid media are more distinctive than turbidity differences in fluid media.

QUESTIONS

(A) Objective type questions:

1. What are microbiological assays? Write advantages and disadvantages.
2. How will you prepare an inoculum for assay of antibiotics?

(B) Short answer questions:

1. How will you assess new antibiotic by MIC?
2. Write a note on:
   (i) Assay of Vitamin B_{12}
   (ii) Assay of Streptomycin.

(C) Long answer questions:

1. Explain in detail about the importance of microbiological assays with special reference to antibiotics.
2. How will you perform assay of antibiotics by turbidimetric method?

(D) Multiple choice questions:

1. Test microorganism used for microbiological assay of Neomycin is _____.
   (a) *Bacillus pumilus* (b) *Staphylococcus aureus*
   (c) *Staphylococcus epidermidis* (d) *Saccharomyces cerevisiae*
2. Test microorganism used for microbiological assay of Vitamin B_{12} is _____.
   (a) *Lactobacillus leichamannii* (b) *Lactobacillus casei*
   (c) *Lactobacillus viridescens* (d) *Lactobacillus plantarum*
3. In broth dilution test, the final inoculum used for determination of minimum inhibitory concentration (MIC) of antimicrobial compound is _____.
   (a) 10^3 CFU/ml (b) 10^5 CFU/ml
   (c) 10^7 CFU/ml (d) 10^{10} CFU/ml
4. *Staphylococcus aureus* is used for IP assay of _____.
   (a) Bleomycin (b) Carbenicillin
   (c) Doxycycline (d) Kanamycin
5. In the microbiological assay of bacitracin IP, the test organism used is _____.
   (a) *Micrococcus luteus* (b) *Pasteurella pestis*
   (c) *Bacillus subtilis* (d) *Staphylococcus aureus*
6. Microbiological assay of Nystatin, test organism used is _____.
   (a) *Saccharomyces cerevisiae* (b) *Bacillus subtilis*
   (c) *Escherichia coli* (d) *Bacillus pumilus*

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