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APPENDIX 1

STRUCTURAL OF POLYSACCHARIDES

Agar

Agar is a strongly gelling seaweed hydrocolloid composed of polysaccharides. The basic repeating unit of agar consists of alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose units. The disaccharide, agarbiose (figure 1), is the common structural unit in all of the agar polysaccharides. Agar consists of two groups of polysaccharides: agarose, a neutral polysaccharide, and agaropectin, an oversimplified term for the charged polysaccharide. The agaropectin contains sulphuric acid, D-glucuronic and pyruvic acids (Matsuhashi, 1990).

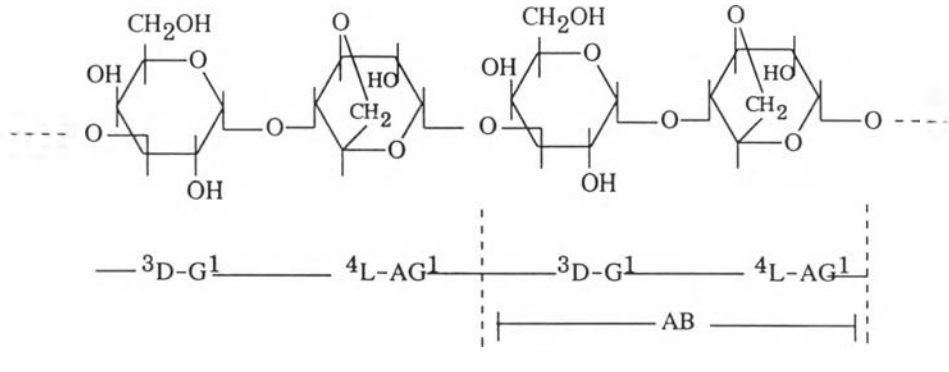


Figure 1 Basic repeating unit of agar. D-G = beta-D-galactopyranose; L-AG = 3, 6-anhydro-α-galactopyranose; AB = agarobiose

Cellulose

Cellulose is the most common component found in the cell walls of higher plants and therefore it is the most abundant organic substance in the world. Cellulose comprises 20 to 50% of the dry matter of many fibrous foods such as vegetables and cereal.

Cellulose is a linear polysaccharide of high molecular weigh, consisting of β-1,4 glucose(glucopyranose) units, having a degree of polymerization ranging from 300 to 15,000 (Figure 2). The degree of polymerization varies depending on the source of the cellulose and the method of isolation. The β-glycosidic bond between the 1,4 linkage of the glucose units can only be broken down by strong mineral acid or a cellulase enzyme. Thus cellulose is not hydrolyzed in the human digestive system (Dreher, 1987b).

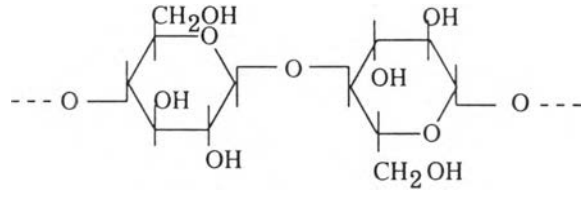


Figure 2 Structure of cellulose

Cellulose is the residue insoluble in strong alkali (17.5%(w/v) NaOH), which is usually called α-cellulose. Glucose polymers of a lower molecular weight

than cellulose are found in the alkali extract, and are presumably formed by degradation of the native cellulose (Southgate, 1991).

Modified Cellulose

Sodium carboxymethylcellulose (CMC) CMC is a water-soluble ether cellulose (figure 3). CMC is produced by treating cellulose sequentially with sodium hydroxide and sodium monochloroacetate. Upon completion of these reactions, food-grade CMC is prepare by washing the material with an alcohol-water mixture to remove excess salt. This process can be regulated for the degree of substitution, the degree of polymerization, and uniformity of substitution to obtain desirable water solubility and other physical properties. Theoretically, a maximum degree of substitution of 3 is possible, since each glucose unit contains three available hydroxyl groups with which the monochloroacetate can react. The most widely used CMC have a degree of substitution of 0.7 (an average of 7 carboxymethyl groups per 10 glucose units); however, the degree of substitution typically ranges from 0.4 to 12. A degree of substitution 0.45 or greater is usually required for water solubility. The degree of polymerization usually ranges from 400 to 3,200 units, which corresponds to a range of molecular weight (MW) from 90,000 to 700,000 at a degree of substitution of 0.75 (the MW is affected by both the degree of substitution and the degree of polymerization).

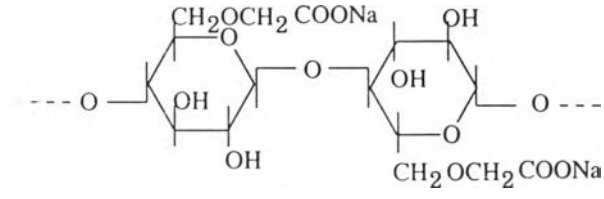


Figure 3 Structure of carboxymethylcellulose

The viscosity of CMC solution is directly related to MW. The degree of polymerization of the CMC greatly affects solution viscosity; the higher the degree of polymerization, the greater the viscosity. CMC has viscosity stability over a wide pH range of between 5 and 11, with the optimal stability at pH 7 (Dreher, 1987b).

Methylcellulose Methylcellulose is the methyl ether of cellulose in the form of a white, fibrous power or granules (figure 4). It is made by reacting cellulose with caustic soda to produce alkali cellulose, which is then mixed with methyl chloride. The maximum degree of substitution is three, and this degree of substitution is very important in determining methylcellulose properties; low degree of substitution products are soluble only in alkali, medium degree of substitution products only in water, and high degree of substitution products only in organic solvents. Medium degree of substitution methylcellulose is more soluble in cold water than hot water. Heated methylcellulose solutions increase in viscosity and can gel at 50-55 ^{o}C. It is nonionic, making it unaffected by ordinary concentrations of electrolytes (Dreher, 1987b).

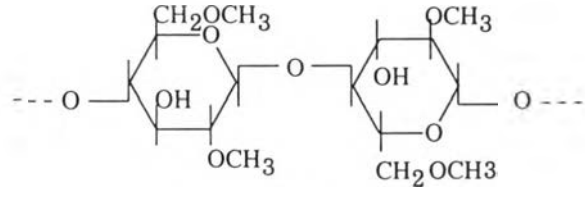


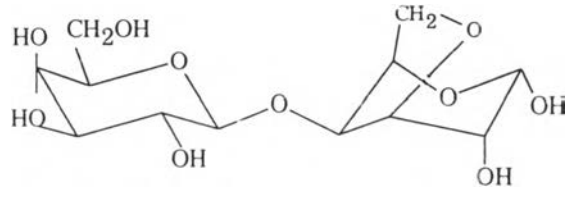
Figure 4 Structure of methylcellulose

Carrageenan

The term "carrageenan" is used to name a class of galactan polysaccharides that occur as intercellular matrix material in numerous species of red seaweeds (marine algae of the class Rhodophyta).

Carrageenan are linear polysaccharides made up of alternating β-1,3- and α-1,4-linked galactose residues. Thus, the repeating units are disaccharides. Carrageenans differ from agars in that the 1,4-linked residue in agars is the L-enantiomer, whereas in carrageenan it is the D-enantiomer; the 1,3-linked residues are D-galactose in both agars and carrageenan (figure 5). Variants on this basic structure result from substitutions on the hydroxyl groups of the sugar residues and from the absence of the 3,6-ether linkage. Substituents may be either anionic (sulphate, pyruvate) or non-ionic (methoxyl). In contrast to agars, carrageenan characteristically are highly sulphated. The 1,3-linked-D-galactose residues occur as the 2-and 4-sulphate, or are occasionally unsulphated, while the 1,4-linked residues occur as the 2-sulphate, the 6-sulphate, the 2,6-disulphate, the 3,6-anhydride and

the 3,6-anhydride 2-sulphate. Sulphate at C-3 apparently never occurs (Stanley, 1990).



Carrabiose (4-β-D-pyranosyl-3, 6-anhydro-α-D-galactopyranose)

Figure 5 Basic repeating unit of carrageenan

Guar Gum

Guar gum is a high MW galactomannan derived from the seed of *Cyamopsis tetragonolobus*, a leguminous plant grown in Pakistan and India. Guar consists of a straight chain β-(1,4)mannan with single unit galactose branches about every other mannose unit (approximately 2:1, manose : galactose ratio). Its MW is about 220,000 as shown in Figure 6 (Dreher,1987b).

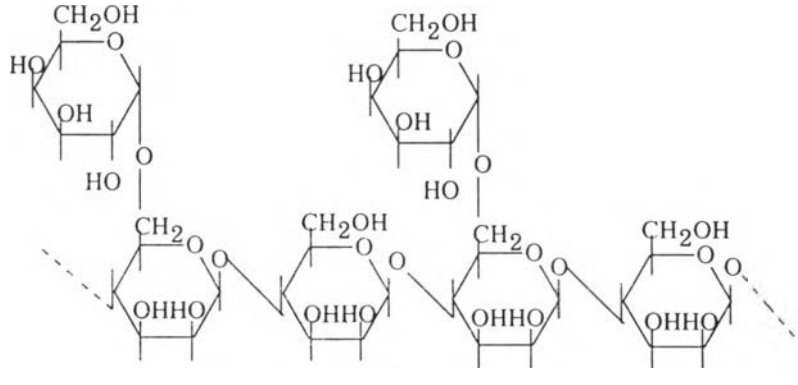


Figure 6 Structure of guar gum (Seaman, 1980)

Gum Arabic

Gum arabic sometimes known as acacia gum or acacia mucilage. It has a comparatively low viscosity, but its function is based primarily on its properties as a protective colloid and emulsifier. The adhesive property of gum arabic is not related to its viscosity. This gum is the amber, amorphous, dried exudate of the acacia tree. Most commercial gum arabic is derived from *Acacia senegal* (Meer, 1980).

Structurally this gum is a heteropolymolecular (e.g. highly variable structure in monomer composition and/or mode of linkage and branching) polysaccharide (Figure 7). Its main chain is composed of D-galactopyranose units joined by β-D-(1,4) and β-D-(1,6) linkages. Its side chains consist of D-galactopyranose attached by β-D-(1,4) linkages and attached to these are L-arabinofuranose or L-rhamnopyranose residues as end units. Additionally, D-glucuronic acid units may be attached by β-D-(1,6) linkages to D-galactose units. The average molecular weight of this gum is approximately 600,000 (Dreher, 1987b).

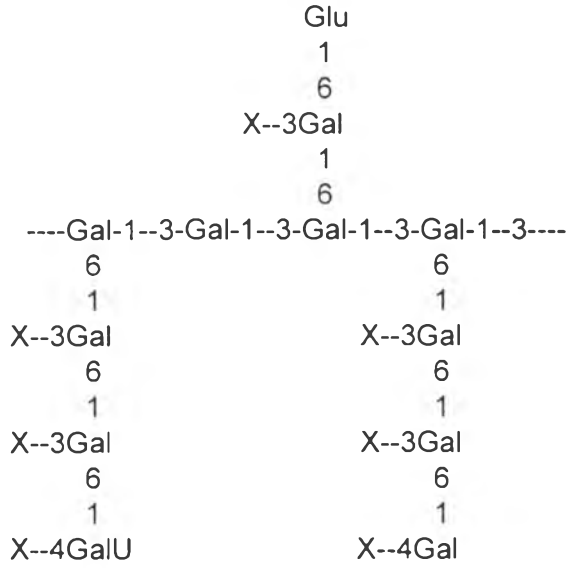


Figure 7 Structure of a gum arabic: X is either arabinose, rhamnose, or galactose; Glu = glucose, Gal = galactose, and GalU = galacturonic acid.

Locust Bean Gum

Locust bean gum is isolated from the endosperm of the seed from the carob tree, *Ceatonic siliqua*, a large leguminous evergreen indigenous to the Mediterranean area. Structurally this gum is a neutral galactomannan polymer consisting of a base chain of D-mannose units with a side chain of D-galactose on every fourth or fifth mannose unit attached through 1,6-glucosidic linkages (Figure 8). Its MW is approximately 310,000 (Dreher, 1987b).

---Man-1--4-Man-1--4-Man-1--4-Man-1--4----
6
1
Gal

Figure 8 Structure of locust bean gum

Pectin

Pectins are a class of polysaccharides found in the primary cell walls and intercellular layers in plants. Pectin consists mainly of the partial methyl esters of polygalacturonic acids and their sodium, potassium, calcium and ammonium salts. The commercial product is normally diluted with sugars for standardization purpose, and mixed with suitable food-grade buffer salts required for pH control and desirable setting characteristics (Annison, Bertocchi, and Khan, 1993).

The polymerized, partly methanol-esterified 1→4 linked α-D-galacturonic acid, accounts for the major part of the material of all commercial pectins (Figure 9). In some of pectins part of the methyl ester groups may be replaced by amide groups (Figure 10). Because ammonia is used for the de-esterification, some of the methyl ester groups are substituted by amide groups. The resulting product is referred to as "amidated pectin". The fraction of the subunits that are esterified may vary from approximately 80% maximum downwards. The sequence in which esterified and free acid groups are arranged along the molecule is not fixed. The substituent that is most abundantly present is the methanol ester of the galacturonate residues. The

distribution of the ester groups depends on the source. The only signification sources of commercial pectins are citrus peel and apple pomace (Rolin and Vries, 1990).

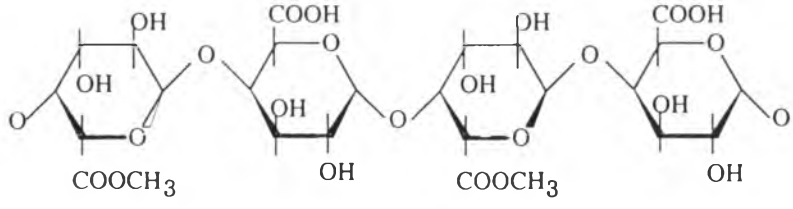


Figure 9 Pectin, main component

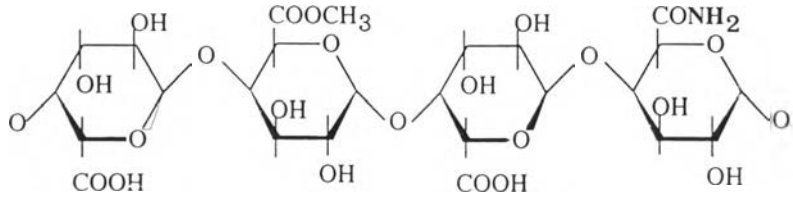


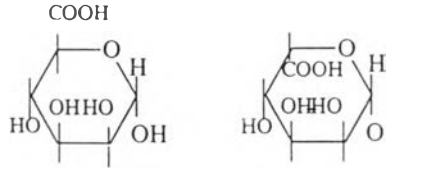
Figure 10 Amidated pectin

Alginates

The term "alginate" (or algin) refers to a group of naturally occurring polysaccharides that are extracted from the brown seaweeds (Phaeophycea). Alginates should be distinguished from the other seaweed extracts agar and carrageenan, which are obtained from red seaweed. Both the chemical composition and properties of alginates differ significantly from those of agar and carrageenan. The most widely used species are *Laminaria hyperborea*, *Macrocystis pyrifera* and *Ascophyllum nodosum*. In its natural environment, alginate exists in the cell wall as

the mixed calcium/ sodium/ potassium salt of alginic acid. It is available commercially principally as the sodium salt (Sime, 1990).

It may described chemically as a linear copolymer made up of β-1,4 linked D-mannuronic and L-guluronic acid units (Figure 11). The ratio of mannuronic/ guluronic acid residues varies from one species of kelp to another (the ratio is 1.6 for *M. pyrifera*). Commercial sodium alginates have MW ranging from 40,000 to 180,000 (approximate degree of polymerization 180 to 930) (Dreher, 1987b).



D-Mannuronic acid L-Guluronic acid

----ManU-1--4-ManU-1--4-ManU-1--4-ManU-1--4----
----GulU -1--4-GulU -1--4-GulU -1--4-GulU -1--4----
----ManU -1--4-GulU-1--4-ManU-1--4-GulU -1--4----

Figuure 11 Structure of alginic acid; ManU = mannuronic acid and GulU = guluronic acid.

Xanthan Gum

Xanthan gum developed by the United States Department of Agriculture and originally designated as B-1459. This gum is produced by the fermentation of

dextrose by the bacterium *Xanthomonas campestris* This gum is a complex polysaccharides with a MW in excess of one million (Dreher, 1987b).

Xanthan gum contains three different monosaccharides: mannose, glucose, and glucuronic acid (as a mixed potassium, sodium, and calcium salt). Each repeating block of the polymer chain has five sugar units (two glucose, two mannose, one glucuronic acid). The polymer's main chain is made up of β-D-glucose units linked through the 1-and 4-position; thus, the chemical structure of the main chain is identical to that of cellulose.

Two mannose units and the glucuronic acid unit make up the side chain. The terminal β-D-mannose units is glycosidically linked to the 4-position of β-D-glucuronic acid, which in turn is glycosidically linked to the 2-position of α-D-mannose. This side chain is linked to the 3-position of every other glucose residue on average in the polymer main chain. Roughly half of the terminal D-mannose residues carry a pyruvic acid residue linked ketalically to the 4-and 6-positions. The nonterminal D-mannose unit on the side chain has an acetyl group at the 6-position as shown in Figure 12 (Cottrel, Kang, and, Kovacs, 1980).

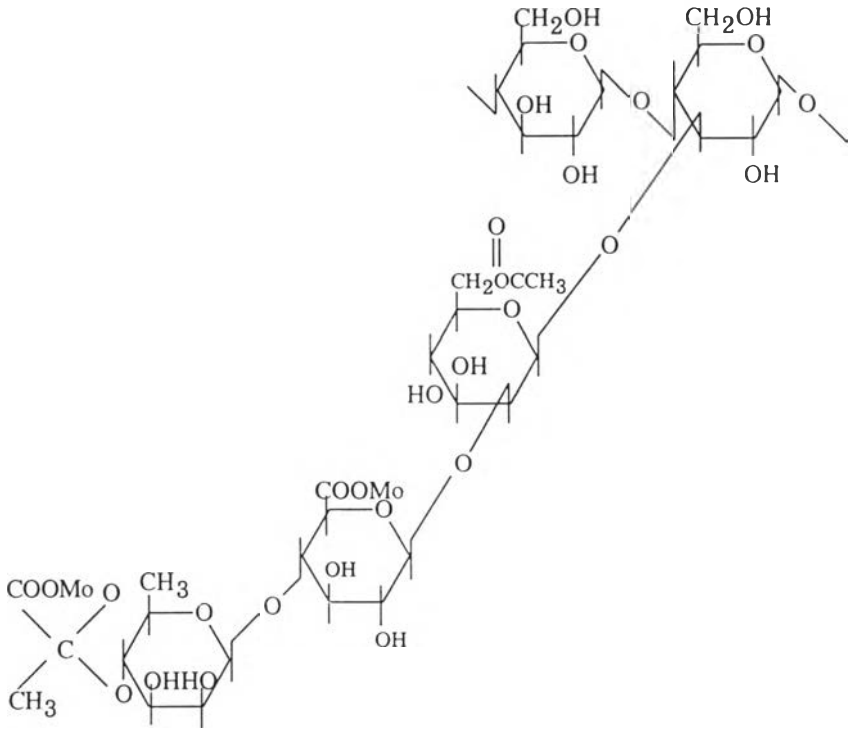


Figure 12 Structure of xanthan gum; Mo = Na, K, 1/2Ca

APPENDIX 2

1. preparation of stock solutions and media (Maron and Ames, 1983)

1.1 Vogel-Bonner medium E stock salt solution (VB salt)

Use : Minimal agar

Ingredient	1000 ml	200 ml
Warm distilled water (45°C)	670 ml	134 ml
Magnesium sulfate ($MgSO_4 . 7H_2O$)	10 g	2 g
Citric acid monohydrate	100 g	20 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	500 g	100 g
Sodium ammonium phosphate ($NaHNH_4PO_4 . 4H_2O$)	175 g	35 g

Add salts in the order indicated to warm water in beaker placed on a magnetic stirring hot plate. Allow each salt to dissolve completely before adding the next. Adjust the volume and filter the solutions into the glass bottles with screw caps and then autoclave at 121°C for 15 min.

1.2 Minimal glucose agar plate

Use : Mutagenicity assay

Ingredient	1000 ml	350 ml
Bacto agar	15 g	5.25 g
Distilled water	930 ml	330 ml

Ingredient	1000 ml	350 ml
VB salts	20 ml	7 ml
40% glucose	50 ml	17.5 ml

Add agar to distilled water in a glass bottle. Autoclave at 121°C for 15 min using slow exhaust. When the solution has cooled slightly, add sterile VB salt and sterile 40% glucose. After all the ingredients have been added, the solution should be swirled thoroughly. Pour 30 ml into each sterile petri plate. Minimal glucose agar plate were kept in incubator at 37°C before using.

note : The VB salts and 40% glucose should be autoclaved separately.

1.3 Oxoid nutrient broth No.2

Use : Growing culture

Dissolve 2.5 g of Oxoid nutrient broth No. 2 in 100 ml distilled water. Transfer 12 ml of nutrient broth for each 50 ml erlenmeyer flask. Autoclave at 121°C for 15 min.

1.4 0.1 M L-histidine HCl stock

Use : Fortification of minimal agar plate

Ingredient	1000 ml	100 ml
L-Histidine Hcl (MW 191.7)	19.17 g	1.92 g
Distilled water	1000 ml	100 ml

Dissolve L-histidine HCl in distilled water. Dilute 1 ml of 0.1 M L-histidine HCl in 99 ml of distilled water for prepared 1 mM L-histidine HCl.

1.5 1mM biotin stock

Use : Fortification of minimal agar plate

Ingredient	**100 ml**
Biotin (MW 244.3)	24.43 mg
Distilled water	100 ml

Dissolve biotin in distilled water. Warm it until dissolve completely. Autoclave at 121°C for 15 min.

1.6 0.5 mM L-histidine/biotin solution

Use : Mutagenicity assay (add 10 ml to 100 ml of Top agar)

Ingredient	**200 ml**
1 mM L-histidine HCl	100 ml
1 mM biotin	100 ml

Mix and autoclave at 121°C for 15 min.

1.7 Top agar

Use : Mutagenicity assay

Ingredient	**1000 ml**	**100 ml**
bacto agar	6 g	0.6 g
sodium chloride	5 g	0.5 g
distilled water	1000 ml	100 ml

Dissolve ingredients in distilled water. Store in a glass bottle. Autoclave for 15 min at 121°C, and then add 0.5 mM L-histidine/ biotin solution (10 ml for 100 ml of Top agar).

1.8 1M potassium chloride

Use : Na_3PO_4 - KCl buffer

Ingredient	**1000 ml**	**100 ml**
Potassium chloride	74.56 g	7.456 g
Distilled water	1000 ml	100 ml

Mix and autoclave at 121°C for 15 min.

1.9 0.5 M sodium phosphate pH 7.4

Use : Na_3PO_4 - KCl buffer for mutagenicity assay

Ingredient

0.5 M Sodium dihydrogen phosphate (NaH_2PO_4)

(30 g / 500 ml)

0.5 M Disodium hydrogen phosphate ($Na_2HPO_4 . 2H_2O$)

(35.5 g / 500 ml)

Dissolve 35.5 g disodium hydrogen phosphate in 300 ml of distilled water. Add 0.5 M sodium dihydrogen phosphate until to pH 7.4, then adjust volume to 500 ml. Sterilize by autoclaving for 15 min at 121°C.

1.10 Na_3PO_4 - KCl buffer

Use : mutagenicity assay

Ingredient	330 ml
0.5 M Na_3PO_4 pH 7.4	100 ml
1 M KCl	16.5 ml
Distilled H_2O	213.5 ml

Autocave for 15 min at 121°C.

2. Recipes for some reagents and test chemicals

2.1 2 M sodium nitrite

Use : Nitrosation

Ingredient	1000 ml	10 ml
Soduim nitrite	138 g	1.38 g
Distilled water to	1000 ml	10 ml

Autoclave for 15 min at 121°C.

2.2 2 M ammonium sulfamate

Use : reaction mixture

Ingredient	1000 ml	50 ml
Ammonium sulfamate	228.24 g	11.41 g
Distilled water to	1000 ml	50 ml

Dissolve ammonium sulfamate in distilled water and adjust volume. Autoclave for 15 min at 121°C.

2.3 0.2 N hydrochloric acid

Use : reaction mixture

Ingredient	**1000 ml**	**100 ml**
Conc. Hydrochloric acid	15.36 ml	1.54 ml
Sterile distilled water	984.64 ml	98.46 ml

Dissolve conc hydrochloric acid in sterile water. Store in sterile glass tubes or bottles with screw caps.

Note : Preparation of 0.2 N HCl must be used sterile technique because hydrochloric acid cannot be autoclaved.

2.4 0.3 mg/ml aminopyrene

Use : standard solution for mutagenicity assay

Ingredient	**1 ml**
Aminopyrene	3 mg
Acetonitrile	1 ml

Dissolve aminopyrene in acetonitrile. Store in sterile vial with screw caps in the freezer. This preparation must be used sterile technique.

Ingredient	1 ml
3 mg/ml aminopyrene	0.1 ml
Acetonitrile	0.9 ml

Dissolve 3 mg/ml aminopyrene in acetonitrile. Store in sterile vial with screw cap in the freezer. This preparation must be used sterile technique.

2.5 8 mg/ml ampicillin solution

Ingredient	10 ml
Ampicillin (sodium)	800 mg
0.02 N sodium hydroxide	10 ml

2.6 0.1% crystal violet

Ingredient	10 ml
Crystal violet	10 mg
Distilled water to	10 ml

2.5 and 2.6 : Store at 4°C in glass bottle with screw cap.

2.7 Gastric condition mixture

Ingredient	100 ml
Sodium chloride	0.2 g
Bovine serum albumin	30 mg
Sodium thiocyanate	2.43 mg
Distilled water to	100 ml

3. Procedure for Reisolation and Growing Culture

Tester strains, TA 98 and TA 100 are grown in Oxoid nutrient broth No.2 and incubated overnight in a 37°C shaking water bath. The growth period should not exceed 16 h (Ames *et al.*, 1973a). These cultures are reisolated by streaking on minimal glucose agar plates which the surface were spread with 0.1 ml of 8 mg/ml amipicllin. 0.3 ml of 0.1 M histidine HCl and 0.1 ml of 1 mM biotin. These plates are incubated at 37°C for 48 h. After incubation, the 5 single colonies per strain TA 98 and TA 100 are picked up and grown in Oxoid nutrient broth No. 2 overnight 37°C in shaking water bath. Each culture is confirmed genotypes of the strains and kept the cultures as the source of bacteria for mutagenicity testing. For each 1.0 ml of culture, add 0.09 ml of spectrophotometric grade DMSO. Combine the culture and DMSO in a sterile tube and distribute 400 µl of the culture aseptically into sterile cryotubes (Nunc). The tubes should be filled nearly full and then transfer to a -80°C freezer.

Confirming Genotype of Tester Strains The broth cultures of TA 98 and TA 100 are used to confirm genotypes in the following ways.

Histidine requirement The His+ character of the strains is confirmed by demonstrating the histidine requirement for growth on the minimal glucose agar plates enriched with histidine and biotin.

Procedure :plate a no histidine and biotin

plate b 0.1 ml of 1 mM biotin

plate c 0.3 ml of 0.1 m His-HCl

plate d 0.3 ml of 0.1 M His-HCl + 0.1 ml of 1 mM biotin

Four minimal glucose agar plates is required for each tester strains. Each of the plates is applied on the surface with 0.1 ml of 1 mM biotin, 0.3 ml of 0.1 M His-HCl, 0.3 ml of 0.2 M His-HCl plus 0.1 ml of 1 mM biotin and no application (plate b,c,d,a respectively). Made a single streak of each strains across these plates. Five strains could be tested on the same plate. Incubated at 37°C for 48 h. The growth of bacteria in histidine plus biotin plate is the result of histidine requirement.

R Factor The R-factor strains (TA 97, TA 98, TA 100 and TA 102) should be tested routinely for the presence of the ampicillin resistance factor because the plasmid is somewhat unstable and can be lost from the bacteria.

Procedure : For each tester strain, add 0.3 ml of fresh overnight culture to a tube containing 0.1 ml of 0.2 M histidine-HCl followed by adding 2.0 ml of molten top agar containing 0.5 mM histidine and 0.5 mM biotin. Mixed and poured on a minimal glucose agar plate. Rotated the plate to distribute the mixtures and allowed several minutes for agar to become firm. R factor and rfa mutation (see the next

section) are performed in the same plate by dividing the plate into 2 areas, one for R factor and the other for rfa mutation. For R factor, commercial ampicillin disc or filter paper disc containing 8 mg/ml ampicillin is applied on the surface of the agar by using sterile forceps. The disc is pressed lightly to embed in the overlay. The plates are incubated at 37°C for 24 h. The absence of the clear zones of inhibition around the discs indicate resistance to ampicillin.

rfa Mutation Strains having the deep rough (rfa) character should be tested for crystal violet sensitivity .

Procedure : Pipetted 0.1% solution of crystal violet to the sterile filter paper disc (1.4 inch) and transfered the disc to plates, seed with bacteria (the procedure is similar to R factor). Incubated at 37°C for 48 h. The clear zone appeared around the disc indicated the presence of the rfa mutation that permitted crystal violet to enter and kill bacteria.

Spontaneous Reversion Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous revertants per plate. The revertant colonies are clearly visible in a uniform background lawn of auxotrophic bacteria. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. Nevertheless, there is variability in the number of spontaneous revertants from one experiment to another and from one plate to another, and it is advisible to include at least 2-3 spontaneous mutation control plates for each strain in a mutagenicity assay.

Procedure : 0.1 ml of DMSO (Solvent in the experiment) is added to capped culture tube. Add 0.5 ml of Na_3PO_4-KCl buffer pH 7.4 in the absence of metabolic activation, 0.1 ml of fresh overnight culture of TA 98 or TA 100, followed by 2.0 ml of molten top agar. Mixed and then poured on minimal glucose agar plate. Rotated plates and left it to become harden. Incubated at 37°C for 48 hr and the His+ revertant colonies are counted.

The Response to Standard Mutagen

Standard mutagens or positive mutagens are used routinely in mutagenicity experiments to confirm the reversion property and specificity of each strain. The standard mutagen used in these experiments is aminopyrene in the absence of metabolic activation. Tester strain which highly response to positive mutagens must be collected.

Procedure : The procedure is as described in spontaneous reversion except aminopyrene (0.06, 0.12 and 0.24 μg/plate for TA 100 and TA 98, respectively) are used instead of DMSO in the absence of S9 mix, respectively. The characteristic of the stock culture for TA 98 and TA 100 as the source of bacteria for mutagenicity is

a. contained R factor (pKM 101) and rfa mutation
b. His^+ requirement
c. low spontaneous reversion
d. highly response to standard carcinogen

After the characteristic of the culture was tested, the mutagenicity test was started

4. The mutagenicity test using *Salmonella typhimurium*.

Plate incorporation test The test is the standard method that has been used for test the mutagenicity of chemicals. This test consists of combining the test compound and the bacterial tester strain in soft agar which is poured onto a minimal agar plate. Positive and negative controls are also included in each assay. After incubation at 37°C for 48 h revertant colonies are counted (Ames et al., 1973b). For initial screening chemicals are tested in concentrations over a three-log dose. A positive or questionable result should be confirmed by demonstrating a dose-response relationship using a narrower range of concentrations. For most mutagens, there is a concentration range that produces a linear dose-response curve and the number of revertants per plate reported for a mutagen should be taken from the region of the curve. However, a few mutagens such as 9-aminoacridline, MNNG, diethylsulfate and ethylmethanesulfonate produce non liner dose-response curve (McCann et al., 1975). The compounds that are negative can be retested using the preincubation method.

Preincubation method Some mutagens, such as dimethyl- and diethyl-nitrosamine are poorly detected in the standard plate incorporation assay and should be tested using a modification of the standard procedure. The most widely used test modification is the preincubation assay first described by Yahagi et al, (1975), in which carcinogenic azo dyes were found to be mutagenic. They incubated the mutagen and bacteria for 20-30 min at 37°C and then added the top agar. The assay has been also used to detect the mutagenicity of 10 carcinogenic nitrosamines and several carcinogenic alkaloids (Yamanaka et al., 1979). The mutagenic activity of aflatoxin B1, benzidine, benzo(a)pyrene and methylmethanesulfonate has been determined using both plate incorporation and preincubation procedures and in all

cases the preincubation assay is of equal or greater sensitivity than the plate incorporation assay (Matsushima et al., 1980). The increased activity is attributed to the fact that the test compound and bacteria are incubated at higher concentration in the preincubation assay than in the standard plate incorporation test (Prival, King, and Sheldon, 1979). The procedure described below is based on recommendation of Matsushima et al., (1980).

The preincubation modification can be used routinely or when inconclusive results are obtained in the standard plate incorporation assay. This assay requires an extra step and therefore involves more work than the standard test but many laboratories use it routinely because of the increased sensitivity of some compounds. Its use in screening assays has been recommended by De Serres and Shelby (1979).

Spot test The spot test is the simplest way to test compounds for mutagenicity and is useful for the initial rapid screening of large numbers of compounds. Ames, McCan, and Yamasaki.(1975) have tested 169 different hair dyes for mutagenicity using this method. This test has several advantages. A few crystals of a solid mutagen or μl of a liquid mutagen can be put directly on the agar surface, thus eliminating the time-consuming preparation of solutions of the chemicals to be tested. As the test compound diffuse out from the central spot, a range of concentrations is tested simultaneously.

This test is primarily a qualitative test and has distinct limitations. It can be used only for testing chemicals that are diffusable in the agar. It is much less sensitive than the standard plate incorporation test. Mutagenicity should be

confirmed by demonstrating a dose-response relationship using the standard plate incorporation test.

Positive control (diagnostic mutagens) In each experiment positive mutagenesis controls using diagnostic mutagens to confirm the reversion properties and specificity of each strain. The characteristic reversion patterns of the standard strains to some diagnostic mutagens are described by Maron and Ames (1983).

Evaluation criteria for Ames assay Because the procedures to be used to evaluate the mutagenicity of the test article are semiquantitative. Each tester strian is specific to each type of mutation such as frameshift mutation, base pair substitute or oxidative mutation etc. The criteria used to determine positive effects are, therefore, inherently subjective and based primarily on the information shown in Table 2.3. Most data sets should be evaluated using the following criteria

a. Strains TA 1535, TA 1537, and TA 1538. If the solvent control value is within the typical range for the laboratory, a test article that produces a positive dose response over three concentrations, with the highest increase equal to three times the solvent control value, is considered mutagenic.

b. Strains TA 98 and TA 100. If the solvent control value is within the normal range for the laboratory, a test article that produces a positive dose response over three concentration, with the highest increase equal to twice the solvent control value, is considered mutagenic. Occasionally a doubling is not necessary for TA 100 if a clear dose-related pattern is observed over several concentrations.

c. Pattern. Because TA 1535 and TA 100 are derived from the same parental strain (G46), and TA 1538 and TA 98 are derived from the same parental strain (D3052), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen, and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it should do so in activation tests.

d. Reproducibility. If a test article produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute and other extenuation factors may enter into a final evaluation decision. However, these criteria can be applied to the majority of situations and are presented to aid those individuals not familiar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established. It must be emphasized that modifications of the procedure involving preincubation conditions is necessary for evaluation of specific chemicals or classes of chemicals.

VITA

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