CHAPTER II

MATERIALS AND METHOD

1. Materials for maintenance the Lactobacillus casei and assay of folic acid.

1.1 Stock Culture of L. casei.

L. casei, ATCC (American Type Culture Collection) No. 7469, was obtained from American Type Culture Collection, 2112 M Street, Washington, D.C., U.S.A.

1.2 Microbiological Assay Inoculum Broth Dehydrated or Maintenance Medium. (Fisher Scientific, Fair Lawn, N.J., U.S.A.)

1.3 Folic Acid Assay PGA Broth.

Microbiological Culture Media (Baltimore Biological Laboratory (BBL), Cockeysville, Maryland 21030, U.S.A.

Folic Acid Casei Medium (Difco Laboratories, Detroit, Michigan, U.S.A.).

1.4 Chemicals.

Folic acid, chemically pure (Koch-Light Laboratories Ltd., Colnbrook Bucks, England).

Chicken Pancreas (conjugase) (Difco Laboratories, Detroit, Michigan, U.S.A.).

Tween 80.

Sodium hydroxide A.R.

Disodium hydrogen phosphate (Na_2HPO_4) A.R.

Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) A.R.

Ascorbic acid A.R.

Potassium dichromate, commercial grade.

Concentrated sulphuric acid, commercial grade.

1.5 Glasswares.

Screw caps bottles (2 x 7 and 2.5 x 9 cm).

Screw caps test tubes (2 x 10 cm).

Rimless tubes.

Centrifuge tubes (15 ml)

Pasteur pipettes

Ground glass blender (glass tissue grinder).

1.6 Instruments.

Refrigerator (4° C).

Deep freezer (-20° C).

Incubator (37° C).

Autoclave.

Hot air oven.

Nephelometer (Nepho-Colorimeter, Model 9, Coleman Instruments Corporation).

Centrifuge (International Portable Refrigerated Centrifuge, Model PR-2).

Micropipettes : 50, 100, 500 microlitres ("Sampler" Oxford Laboratories, 1149 chess Drive, Foster City, C.A.

94404 U.S.A.).

Mixer (Vortex-Genie) Scientific Industries Supplied, Massachusetts).

Mixed ion-exchange resin bed (Barnstead, D 5040 Standard cartridge, Sybron corporation, Boston, Massachusetts 02132 U.S.A.).

2. Method of Preparation.

All glasswares used in the experiment must be free from folate by boiling in the teepol solution for at least 30 minutes. They were then soaked overnight in chromic-sulphuric acid and rinsed well with tap water and distilled water.

Preparation of Micro Inoculum Broth (Maintenance Medium).

The maintenance medium is available ready-made in dry form with pH 7, and must be kept in the refrigerator. Thirty-seven grams of the dehydrated medium was dissolved in 2 litres of deionized distilled water. Ten millilitres of dissolved media was dispensed into screw cap tubes. The tubes were pluged with cotton wool and capped, then sterilized in the autoclave at 121° C for 15 minutes.

The sterility was checked by incubation all tubes in the incubator at 37° C for overnight. There should not be any cloudiness or growth of bacteria. The tubes were then stored at 5° C in the refrigerator until required for using.

Maintenance of Stock Culture of Lactobacillus casei.

L. casei is maintained in the Micro Inoculum Broth at 4^{o} C.

One ampoule of the lyophilized culture is transferred to a 10 ml volume of sterile Micro Inoculum Broth and incubated at 37^{o} C for 18 hours.

It is checked for purity by plating onto a Blood Agar Medium, and meanwhile is stored at 5^{o} C. If pure, a drop is inoculated into another sterile 10 ml volume of Micro Inoculum Broth and incubated at 37^{o} C for 18 hours, checked for purity and stored at 5^{o} C. It is maintained at the refrigerated temperature for assays and sub-culture into another fresh Micro Inoculum Broth within two weeks.

These two-weekly sub-cultures are done to maintain the L. casei and also to avoid possible mutation. Strict aseptic technique were observed in sub-culturing and inoculating and the old culture is not discarded until the new culture is proved to be a pure one.

Stock Solutions for extraction of food samples.

1. 0.1 N Sodium Hydroxide Solution.

Two grams of sodium hydroxide is dissolved in 500 ml of deionized water.

2. Phosphate Buffer pH 6.1.

Solution (a) : Dissolve 31.2 g of sodium

dihydrogen phosphate in deionized water and make up to 1 litre.

Solution (b) : Dissolve 28.4 g of disodium hydrogen phosphate in deionized water and make up to 1 litre.

Add together 212.5 ml of (a) to 37.5 ml of (b) and make up to 1 litre with deionized water, the pH is 6.1.

Ascorbic Acid Phosphate Buffer.

Dissolve 150 mg of ascorbic acid in every 100 ml of the phosphate buffer solution of pH 6.1 (150 mg %). This buffer is freshly prepared each time before use, because it is rapidly deteriorated in the solution.

Preparation of Food Samples.

1. Solid samples are prepared for assay as follows :

Accurately weigh 1 g of sample and grinded in a glass tissue grinder with 20 ml ascorbic acid phosphate buffer. The homogenized sample is centrifuged for five minutes at 3,000 r.p.m. and the supernatant is kept at -20° C until assay.

2. Liquid samples.

(a) Acid sample (vinegar) is neutralized with 0.1 N sodium hydroxide solution before assay.

(b) Milk is added with 150 mg % of ascorbic acid and autoclaved at 15 lb psi for 15 minutes,

then centrifuged at low temperature. The supernatant is kept at -20° C until assay.

(c) Other neutral liquid samples are assayed directly without any preparation.

Preparation and Use of Conjugase Solution.

Desiccated chicken pancreas is dissolved in the ascorbic acid phosphate buffer solution (3 mg/ml). The solution is centrifuged for five minutes at 3,000 r.p.m. and the precipitate is discarded. The clear solution is kept at -20° C.

Preparation of food sample with conjugase treatment.

The freezed sample and conjugase solutions are thawed in running tap water. Food sample is divided into two parts. One part is incubated with the conjugase solution and the other for assays directly without adding the conjugase solution. One millilitre of conjugase solution is added to 9 ml of the food sample. After shaking, the mixture is incubated at 37° C under toluene solution for 18 hours or overnight. The samples then are boiled for stopping the conjugase activity and aliquots are taken for assay.

Both of samples with and without conjugase treatment are diluted to the appropriate concentration (0.6 - 9.0 ng/ml) with ascorbic acid phosphate buffer before adding

to folic acid assay medium.

Preparation of Inoculum (L. casei suspension).

A ten millilitres of sterile Micro Inoculum Broth is inoculated with one drop of the 18 hour maintenance culture and incubated at 37° C for 18 hours. The solution is mixed well and 0.5 ml of this 18-hour culture is inoculated into another 10 ml sterile Micro Inoculum Broth. This is the 6 - 8 hour cultures. And then 0.05 ml of this 6 - 8 hour growth is added into a 18 ml sterile single strength assay medium. Mix well to get an even suspension of bacteria. One drop of this suspension is used as the inoculum for each of the assay bottles.

Preparation of Folic Acid Assay PGA Broth (Double Strength).

This media is available ready-made in the form of dry mixture. It is stored at 5° C and once the seal is broken, it must be stored with the desiccant in a sealed container.

Method of Preparation.

Dissolve 9.4 g of the dry material in 100 ml of deionized water, to make double strength medium. Add 0.01 ml (one drop) of Tween 80, mix and heat with slight stirring. Add 50 mg of ascorbic acid and filter through the filter paper. Let it cool, and dispense 3 ml into assay

bottles.

Folic Acid Standards.

Two working solutions of standard folic acid are freshly prepared from the stock bottle each time before use.

Stock 10^{-5} g/ml.

Accurately weigh 10 mg of dry folic acid and dissolve in 100 ml of 20 % ethanol in deionized water (V/V). One millilitre of 0.1 N sodium hydroxide solution is added. The solution is diluted to 1,000 ml with the deionized water. Store in aliquots of 5 ml in brown plastic bottles at -20° C. Prepare fresh standard solution every 12 months.

To prepare 10^{-7} g/ml Folic Acid Solution.

Thaw one of the stock folic acid solution with a concentration of 10^{-5} g/ml. Dilute one millilitre of this solution with the deionized water to 100 ml in a volumetric flask. The stock solution which is once defrosted should be discarded to avoid the deteriorated effect.

3. Techniques of assaying folic acid.

3.1 The evening before the plan of assaying day, prepare L. casei suspension as described on page 21. Place prepared inoculum tube in the incubator at approximately

3 - 5 p.m. and let it remain overnight at 37° C.

3.2 The morning of assaying day, prepare 6 - 8 hour growth culture as follow : take 0.5 ml of the 16 - 18 hour growth culture which has been prepared the day before and inoculate into a fresh tube of 10 ml of maintenance medium. Shake, and incubate in the incubator till approximately 3 p.m. It is from this 6 - 8 hour growth culture that the assay bottles will be inoculated. Store the 16 - 18 hour growth culture in a refrigerator.

3.3 Number the assay bottles (2 x 7 cm glass bottle with plastic screw cap), for standard curve in triplicate (0a, 0b, 0c, 1a, 1b, 1c, 2a, 2b, 2c, ... to 9a, 9b, 9c), for unknown samples in double dilution and each dilution in duplicate (U1a, U1$\bar{a}$, U1b, U1$\bar{b}$, U2a, U2$\bar{a}$, U2b, U2$\bar{b}$, ...etc. and samples with conjugase as : C1a, C1$\bar{a}$, C1b, C1$\bar{b}$, C2a, C2$\bar{a}$, C2b, C2$\bar{b}$, ...etc.).

3.4 Prepare the Folic Acid Assay PGA Broth (double strength) as described above. Add 3 ml double strength assay medium to each assay bottle.

3.5 Add the standard solution from working solutions (10^{-9}, 10^{-10} g/ml) into each of assay medium bottle for preparing the standard curve in known amounts of folic acid except bottle No. 0 and the blank control (No. 1a, 1b, 1c = 60 pg, No. 2a, 2b, 2c = 120 pg, ...etc.).

3.6 Add unknown samples which were diluted to the

appropriate concentration by using micropipettes "Sampler", 0.1 ml for No. 1a and 1$\bar{a}$, 0.2 ml for No. 1b, and 1$\bar{b}$, ...etc.

3.7 Add deionized water into every assay bottles to make the final volume 6 ml for each bottle.

3.8 Fill into 'LC' bottle 18 ml of single strength of assay medium for using as bacterial dilution.

Table 1

Volume adjustment of assay bottles.

Reference	Tube No.	Double Strength F.A.A. medium (ml)	Folic Acid Std. (ml)		Food without conjugase (ml)	Food with conjugase (ml)	Deionized water (ml)
			10^{-10} g/ml	10^{-9} g/ml			
Blank	Blank	3	Nil.	Nil.	-	-	3.0
Control	0a,0b,0c	3	Nil.	Nil.	-	-	3.0
Std. 10 pg/ml	1a,1b,1c	3	0.6	-	-	-	2.4
Std. 20 pg/ml	2a,2b,2c	3	1.2	-	-	-	1.8
Std. 40 pg/ml	3a,3b,3c	3	2.4	-	-	-	0.6
Std. 60 pg/ml	4a,4b,4c	3	0.6	0.3	-	-	2.1
Std. 80 pg/ml	5a,5b,5c	3	0.8	0.4	-	-	1.8
Std.100 pg/ml	6a,6b,6c	3		0.6	-	-	2.4
Std.150 pg/ml	7a,7b,7c	3		0.9	-	-	2.1
Std.200 pg/ml	8a,8b,8c	3		1.2	-	-	1.8
Std.300 pg/ml	9a,9b,9c	3		1.8	-	-	1.2

Table 1 (continued)

Reference	Tube No.	Double Strength F.A.A. medium (ml)	Folic Acid Std. (ml)		Food without conjugase (ml)	Food with conjugase (ml)	Deionized water (ml)
			10^{-10} g/ml	10^{-9} g/ml			
Unknown 1	U1a U1ā	3	-	-	0.1	-	2.9
Sample	U1b U1b̄	3	-	-	0.2	-	2.8
	C1a C1ā	3	-	-	-	0.1	2.9
	C1b C1b̄	3	-	-	-	0.2	2.8
2	U2a U2ā	3	-	-	0.1	-	2.9
	U2b U2b̄	3	-	-	0.2	-	2.8
	C2a C2ā	3	-	-	-	0.1	2.9
	C2b C2b̄	3	-	-	-	0.2	2.8
etc.							
LC Tube or Single Strength F.A.A. medium	"LC"	9	-	-	-	-	9

3.9 Cap all bottles, and autoclave at 10 lb psi for 10 minutes and let them cool for approximately half an hour.

3.10 Method of inoculation of 6 - 8 hour growth bacteria in the assay media.

Add aseptically 0.05 ml of 6 - 8 hour growth

culture into the "LC" bottle at 3 - 5 p.m. and mix well. Inoculate one drop of this diluted culture into each assay bottle, except for the blank using sterile pasteur pipette. This must be done with aseptic technique.

3.11 Mix and incubate at 37° C for approximately 40 hours or until growth is satisfactory.

3.12 The assay is read in a Nepho-colorimeter which has been turned on for at least 20 minutes. Record the absorbance value of each bottle on the data book.

3.13 Plot a graph of the standard curve.

3.14 Calculate the unknown in terms of the standard curve by the following formula :-

$$\text{F.A. Concentration (pg/ml)} = \frac{\text{F.A. from Std. Curve x dilution x total volume of assay media}}{\text{Vol. of sample added to the assay media}}$$

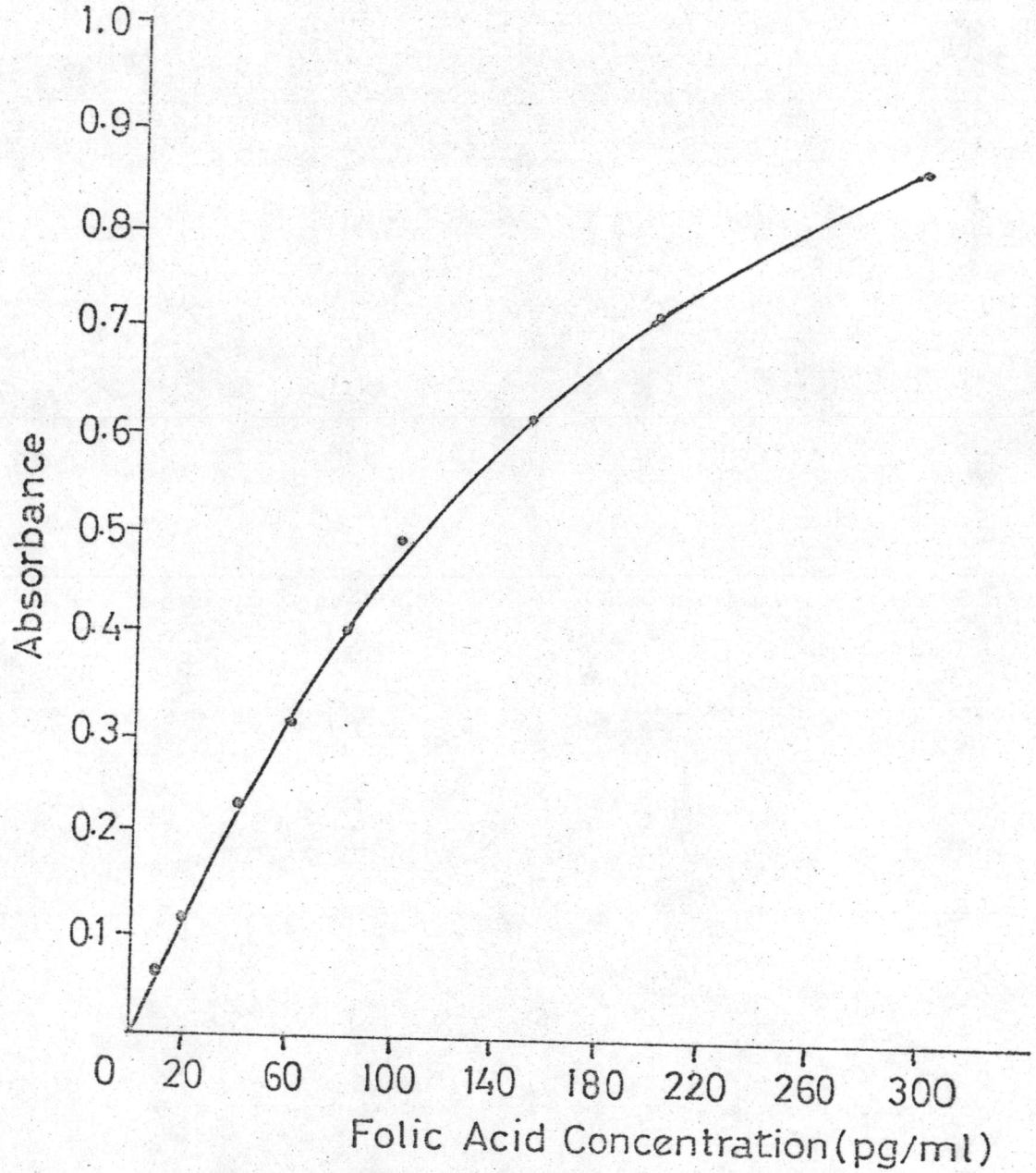

Fig. 2 Standard curve