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APPENDICES

APPENDIX A

Ingredients of Sample

Minced pork flavour number 1.

Ingredients	Amounts
Wheat flour	70 %
Palm oil	22 %
Salt	0.68 %
Monosodium glutamate	1.04 %

Minced pork flavour number 2.

Ingredients	Amounts
Wheat flour	62 %
Palm oil	18 %
Monosodium glutamate	1.2 %
Disodium 5′ Inosinate	
Disodium 5′ Guanolate	

Minced pork flavour number 3.

Ingredients	Amounts
Wheat flour	70 %
Palm oil	10 %
Red pepper	0.75 %
Pork extract	0.50 %
Monosodium glutamate	0.7 g

Pa-lo duck flavour number 4.

Ingredients	Amounts
Wheat flour	67 %
Palm oil	15.32 %
Caramel	0.38 %
Duck powder	0.22 %
Star anise powder	0.05 %
Cinnamon powder	0.05 %
Monosodium glutamate	1.27 %
Disodium 5′ Inosinate	
Disodium 5′ Guanolate	

Pa-lo duck flavour number 5.

Ingredients	Amounts
Wheat flour	68.55 %
Palm oil	14.64 %
Duck powder	0.67 %
Monosodium glutamate	1.27 %
Disodium 5′ Inosinate	
Disodium 5′ Guanolate	

Pa-lo duck flavour number 6.

Ingredients	Amounts
Wheat flour	66 %
Palm oil	11 %

Salt	5.50 %
Chili powder	0.84 %
Duck powder	0.50 %
Star anise powder	0.1 %
Monosodium glutamate	0.3 g

Sour shrimp flavour number 7.

Ingredients	Amounts
Wheat flour	70 %
Palm oil	20 %
Salt	1.23 %
Monosodium glutamate	1.08 %
Disodium 5′ Inosinate	
Disodium 5′ Guanolate	

Sour shrimp flavour number 8.

Ingredients	Amounts
Wheat flour	54.2 %
Palm oil	16.5 %
Monosodium glutamate	1.3 %
Disodium 5′ Inosinate	
Disodium 5′ Guanolate	

Sour shrimp flavour number 9.

Ingredients	Amounts
Wheat flour	66 %
Palm oil	13 %
Salt	3.50 %
Dried shrimp	0.45 %
Monosodium glutamate	0.8 g

APPENDIX B

1. Preparation of stock Solution and Media (Maron and Ames, 1983)

1.1 Vogel Bonner media E stock salt solution (VB salt)

Use : Minimal agar

Ingredient	Per liter
Warm distilled water (45 ° C)	670 ml
Magnesium sulfate ($MgSO_4$ $7H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaNH_4$ HPO_4 $4H_2O$)	175 g

Add salts in the order indicated to warm water and allow each salt to dissolve completely before adding the next. Adjust the volume to 1 liter. Filter the solutions and then autoclave at 121 °C for 15 min.

1.2 Minimal glucose agar plate

Use : Mutagenicity assay

Ingredient	Per liter
Bacto agar	15 g
Distilled water	930 ml
VB salts	20 ml
40% glucose	50 ml

Add agar to distilled water in a glass bottle. Autoclave at 121 ° C for 15 min. When the solution has cooled slightly, add sterile VB salts and sterile 40% glucose. Mix and pour 30 ml into each sterile petri plate. Minimal glucose agar plates were

kept in incubator at 37 °C before using. (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 nutrient broth No.2 in 100 ml distilled water. Transfer 12 ml nutrient broth for each 50 ml erlenmeyer flask (covered with sterile gauze). Autoclave at 121 °C for 15 min.

1.4 Top agar

Use : Mutagenic assay

Ingredient	100 ml
Bacto agar	0.6 g
Sodium chloride (NaCl)	0.5 g
Distilled water	100 ml

Dissolve ingredients in distilled water. Store in a glass bottle. Autoclave for 15 min. At 121 °C and then add 10 ml of 0.5 mM L–histidine/biotin solution and mixed thoroughly by swirling.

1.5 0.1 M L-histidine HCl stock

Use : Fortification of minimal agar plate.

Ingredient	10 ml
L-histidine HCl	0.2096 g
Distilled water	10 ml

Dissolve 0.2096 g of L-histidine HCl (MW 209.63) in 10 ml distilled water. Autoclave at 121° C for 15 min. Store in a glass bottle at 4 °C.

1.6 1 mM L-histidine HCl stock

Use : Fortification of minimal agar plate.

Ingredient	100 ml
0.1 M L-histidine HCl	1 ml
Distilled water	99 ml

Dilute 1 ml of 0.1 M L-histidine HCl in 99 ml of distilled water. Autoclave at 121 °C for 15 min.

1.7 1 mM biotin stock

Use : Fortification of minimal agar plate.

Ingredient	100 ml
Biotin	24.43 mg
Distilled water	100 ml

Dissolve biotin (MW 244.3) in distilled H_2O. Warm it until dissolve completely. Autoclave at 121° C for 15 min.

1.8 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.

1.9 1 M potassium chloride

Use : Na_3PO_4 -KCl buffer for mutagenicity assay

Ingredient	100 ml
Potassium chloride	7.456 g
Distilled water	100 ml

Mix and autoclave at 121 °C for 15 min.

1.10 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)(MW 120)

(30 g / 500 ml)

0.5 M Disodium hydrogen phosphate dihydrate ($Na_2HPO_4\ 2H_2O$)

(MW 177.99) (44.5 g / 500 ml)

Dissolve 44.5 g disodium hydrogen phosphate dihydrate 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.

1.11 Na_3PO_4 – KCl buffer

Use : mutagenicity assay

Ingredient	330 ml
0.5 M Sodium Phosphate (Na_3PO_4)pH 7.4	100 ml
1 M Potassium Chloride (KCl)	16.5 ml
Distilled water	213.5 ml

Autocave for 15 min at 121 °C.

2. Recipes for Some Reagents and Test Chemicals

2.1 2M sodium nitrite

Use : Nitrosation

Ingredient	10 ml
Sodium nitrite	1.38 g
Distilled water to	10 ml

Mix and autoclave for 15 min at 121 °C.

2.2 2M ammonium sulfamate

Use : reaction mixture

Ingredient	10 ml
Ammonium sulfamate	2.28 g
Distilled water to	10 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	100 ml
Conc. Hydrochloric acid	1.66 ml
Sterile distilled water	98.34 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. Preparation of this solution 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. These preparation must be used sterile technique.

2.5 8 mg/ml ampicillin solution

Ingredient	10 ml
Ampicillin sodium	80 mg
Distilled water to	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.

3 Procedure for Reisolation and Growing Culture

Tester strains, TA98 and TA100 are grown in Oxoid nutrient broth No.2 and incubated overnight in a 37° C shaking water bath. The growth period should not exceeded 16 h. These cultures are re-isolated by streaking on minimal glucose agar plates which the surface were spread with 0.1 ml of 8 mg/ml ampicillin, 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 4 single colonies per strain TA98 and TA100 are picked up and grown in Oxoid nutrient broth No. 2 overnight at 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 then transfer to a -80 °C freezer.

4. Confirming Genotype of Tester Strains

The broth cultures of TA 98 and TA100 are used to confirm genotypes in the following ways.

4.1 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 are required for each tester strains. Each plate is applied on the surface with 0.1 ml of 1 mM biotin or 0.3 ml of 0.1 M His-HCl, or 0.3 ml of 0.1 M His-HCl plus 0.1 ml of 1 mM Biotin or no application (plate b, c, d, a, respectively). Made a single streak of each strains across these plates. Four strains could be tested on the same plate. Incubated at 37 ° C for 24 h. The growing of bacteria on histidine plus biotin plate is the result of histidine requirement.

4.2 R-factor

The R-factor strains (TA 97, TA 98, TA100 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 0.1 M histidine – HCl followed by adding 20 ml of molten top agar containing 0.5 mM, 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 disc indicate resistance to ampicillin.

4.3 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 transferred the disc to plates, seeded 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.

5. 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 auxotropic 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 advisable to include at least 2-3 spontaneous mutation control plates for each strain in a mutagenicity assay.

Procedure : 0.2 ml of sterile distilled water (solvent in the experiment) is added to capped tube. Add 0.5 ml of $NaPO_4$-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 agar plates, rotate plates and left it to become harden. Incubated at 37 ° C for 48 h.. and the his^- revertant colonies were counted.

6. The Response to Standard Carcinogen

Standard carcinogens or positive carcinogens are used routinely in mutagenicity experiments to confirm the reversion property and specificity of each strain. The standard mutagen which 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, and 0.12 µl per plate for TA 98 and TA 100, respectively) are used instead of sterile distilled water in absence S-9 mix, respectively. The characteristic of 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.

VITA

Miss Oranuch Wongwattanasathien was born on May, 9, 1973 in Khonkaen province, Thailand. She received her Bachelor's degree of Science in Pharmacy in 1996 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.