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APPENDICES

APPENDICES I

ANTIOXIDANT TEST

APPENDICES I

ANTIOXIDANT ACTIVITY

Preparation of the solution (DPPH assay, Blois *et. al.*, 1983)

1,1-diphenyl-2-picrylhydrazyl (DPPH) solution at 200 μM

DPPH	0.00789 g
Absolute ethanol	100 ml

DPPH solution was mixed well before, kept in the dark by covering with aluminum foil until use.

APPENDICES II

MUTAGENICITY AND ANTIMUTAGENICTY TEST BY AMES' TEST

APPENDICES II

REVERSION MUTATION ASSAY (Ames' test)

Tester strains

1. Reisolation of tester strains

1.1 Take one frozen permanent of the standard tester strains, S. Typhimurium TA98 and TA100 from the freezer, thaw at room temperature.

1.2 Apply small aliquot (0.05 ml) of thawed cultures into 12 ml of Oxoid nutrient broth No. 2

1.3 Incubate the plates at 37 ^{0}C for 14 hours.

1.4 The cultures are reisolated by streaking on Ampicillin plates.

1.5 Incubate the plates at 37 ^{0}C for 48 hours, 5 single colonies of each strain are picked up and grown in Oxoid nutrient broth No. 2 on a shaker water bath at 37 ^{0}C for 14 hours.

1.6 The cultures are used in preparation of master plate and confirming of the genotypes of the tester strains.

2. Preparation of master plates

Master plates are used as the source of starting bacteria for inoculating the overnight culture of the tester strains. Using of these plates should avoid the problems that arise when the frozen permanents are opened frequently.

2.1 Each of the overnight cultures (0.3 ml) of the single colony isolation is spreaded on Ampicillin plates.

2.2 Incubate the plate at 37 ^{0}C for 24 hours.

2.3 The master plates are then stored at 4 ^{0}C and are discarded after 2 mouths or sooner if the number of spontaneous revertant colonies per plate falls out of the range specified for a strain.

3. Confirming genotypes of tester strains

The genotypes of bacterial strains used in mutation assay should be checked from time to time. Checks are necessary when strains are first received or the results of positive control assays fail to meet the standards accepted by the particular laboratory. A good compromise would be to check genotypes at regular intervals such as two month. Confirming genotypes were used with five recommendation; histidine requirement, R-factor, rfa mutation, uvr B mutation, procedure for growing cultures, spontaneous reversion and the response to standard carcinogen.

3.1 Histidine requirement

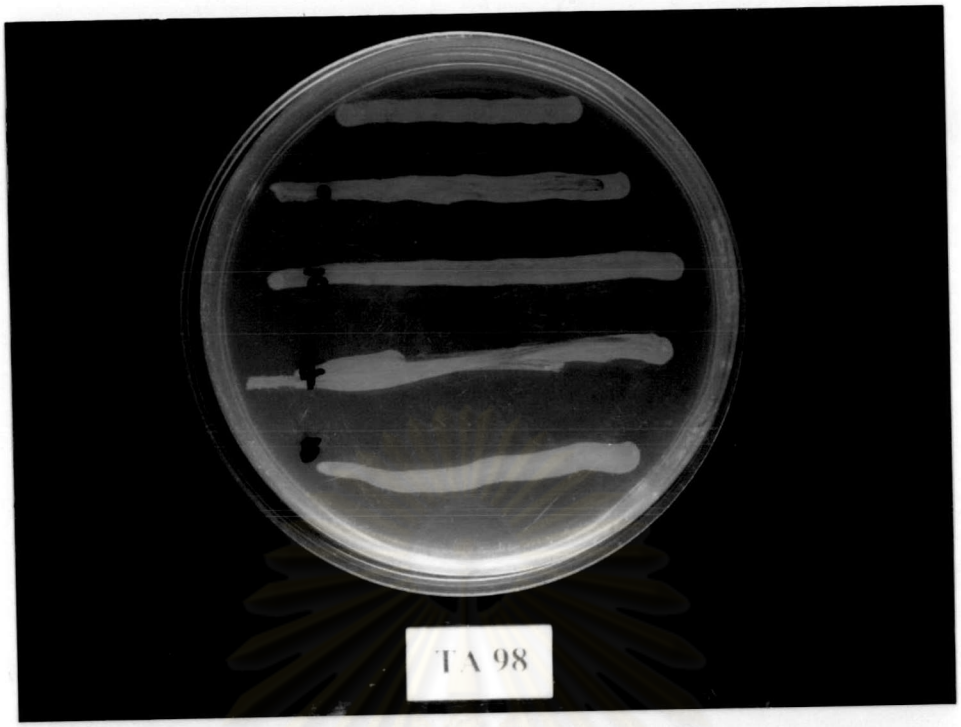
The several single colonies of tester strains can be tested on the same plate, which can be checked as follow;

3.1.1 Take a single streak of each single colony with a sterile loop on the histidine-plus plate (histidine/biotin plate) and flame the loop before streaking the next single colonies. Mark the position and identify of each strain and single colonies on the bottom of the plate.

3.1.2 Repeat steps 3.1.1 using hisitdine-minus plated (Minimal glucose agar plate which the surface are spread with 0.1 ml of 1 mM biotin)

3.1.3 Incubate at 37 °C for 24 hours

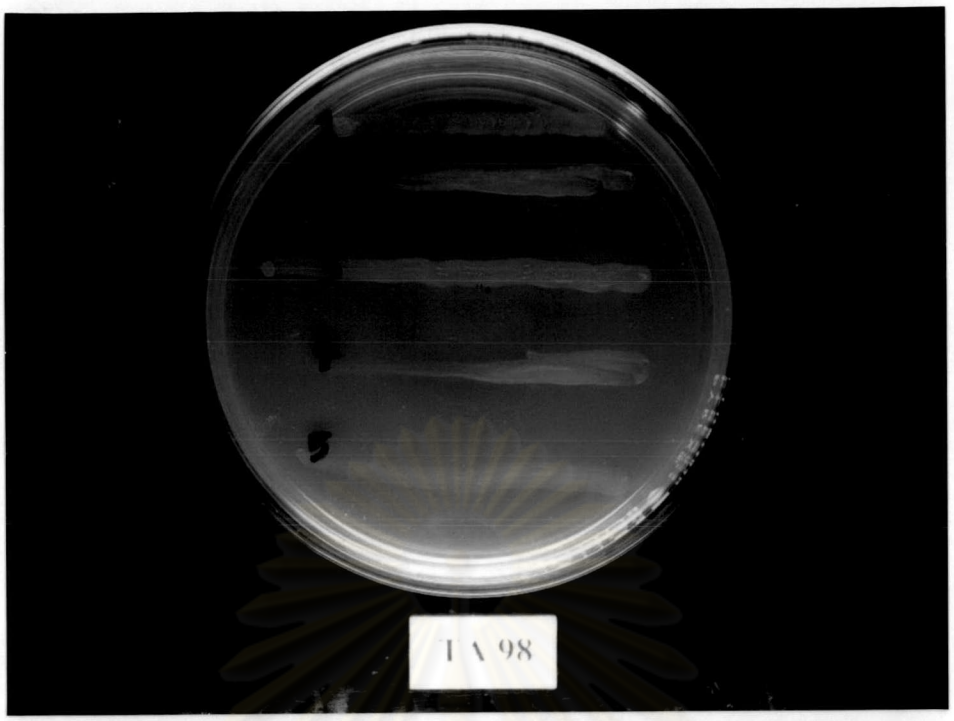
3.1.4 Check the plates: the bacteria should be good growth on the histidine-plus plate and no growth on histidine-minus plate. (Figure II-1, II-2)



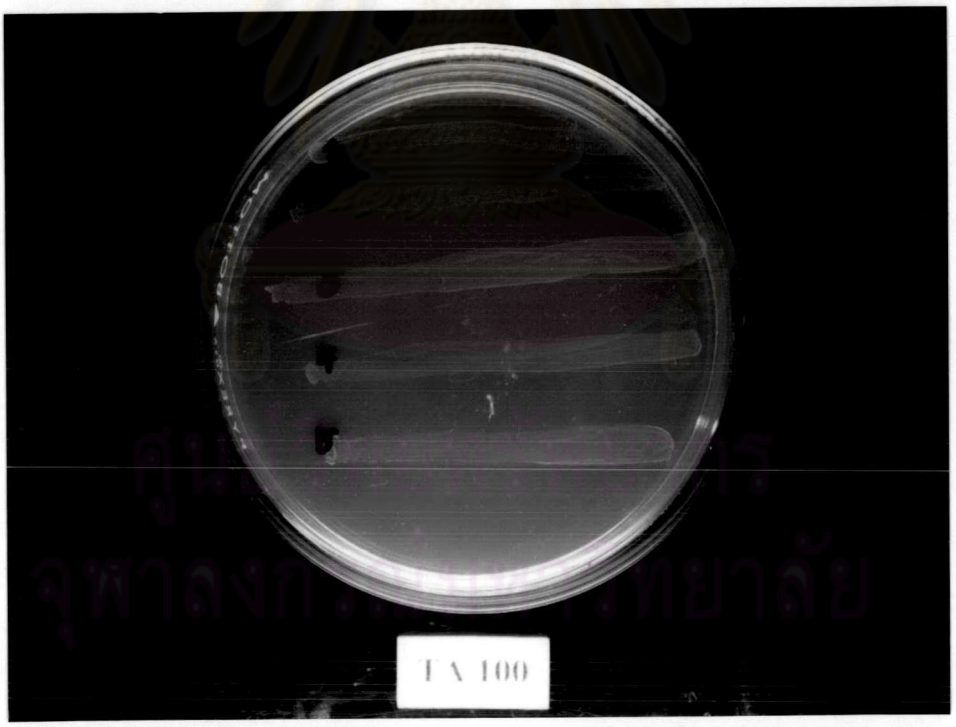
(a)

(b)

Figure II-1 Each of single revertant colonies of S. Typhimurium strains TA98 (a) and TA100 (b) on histidine-plus plate for confirming genotype: histidine requirement



(a)



(b)

Figure II-2 Each of single revertant colonies of S. Typhimurium strains TA98 (a) and TA100 (b) on ampicillin plate for confirming genotype: biotin plus

3.2 R-factor

Tester strains TA98 and TA100 contain R-factor plasmid, pKM 101, should be tested for the presence of the ampicilin resistance factor because the plasmid is some what unstable and can be lost from the bacteria which can be checked as follow;

3.2.1 Each of single colonies of required strains was streak on the Ampicilin plates.

3.2.2 Incubate the plates at 37 ^{0}C for 24 hours

3.2.3 Checks the plate: the bacteria should be growth on the Ampicillin plates as indicate resistance to ampicillin so the presence of R-factor (Figure II-3)

3.3 rfa mutation

Tester strains TA98 and TA100 as carrying the rfa mutation are more permeable to large molecules. Thus, this characteristic is confirmed by testing their sensitivity to the lethal effect of a high molecular weight which the crystal violet dye was used which can be checked as follow;

3.3.1 Add 0.1 ml of fresh overnight culture of each tester strains TA98 and TA100 to a tube containing 2 ml of molten top agar held at 45 ^{0}C and vortex for 3 sec at low speed and pour on a nutrient agar plate. Rotate the plate to distribute the top agar evenly, place it on a level surface.

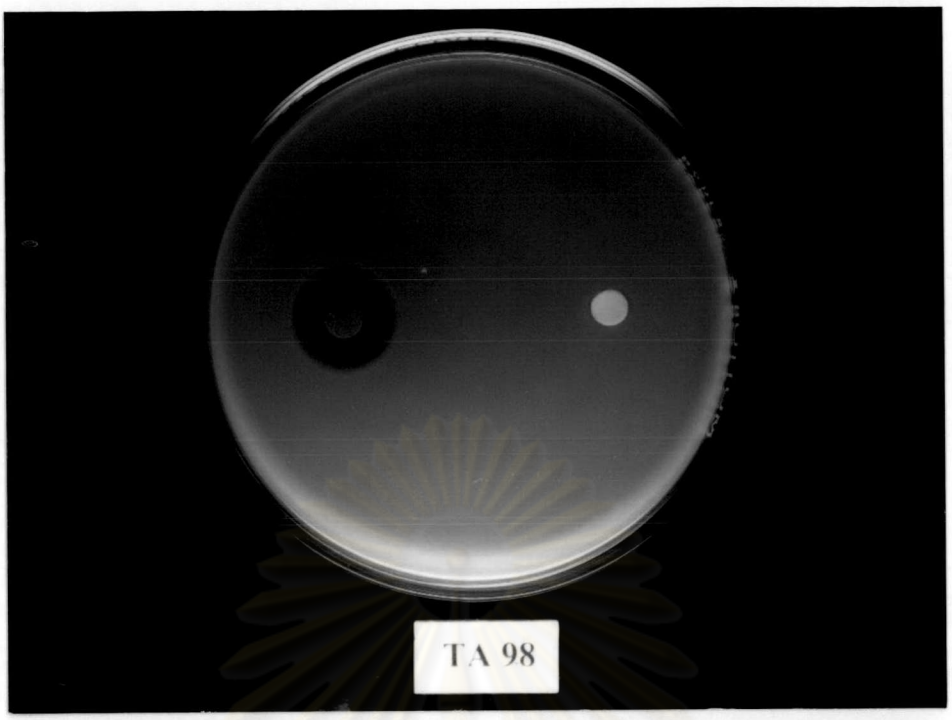
3.3.2 Allow several minutes for the agar to become firm

3.3.3 The sterile filter paper disc was drop with 10 µl of 1 mg/ml solution of crystal violet and using sterile forceps for transfer one disc to each of the seeded plates. Press the disc lightly with the forceps to embed it slightly in the overlay.

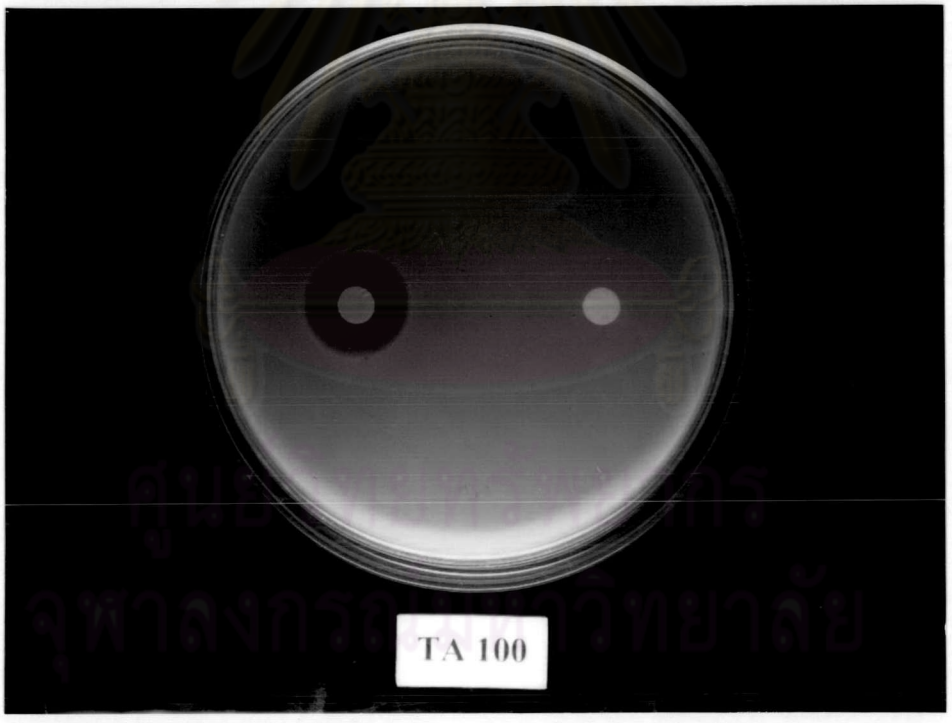
3.3.4 Incubate the plate at 37 ^{0}C for 12 hours

3.3.5 Check the plates: The bacteria could be growth on overlay, except around the disc, which showed a clear zone of inhibition (12 mm).

Therefore, indicating the presence of rfa mutation as permits large molecules and kills these bacteria (Figure II-3).



(a)



(b)

Figure II-3 Revertant colonies of S. Typhimurium strains TA98 (a) and TA100 (b) on nutrient agar plate for confirming genotype: rfa mutation (clear zone) and R-factor mutation (non-clear zone)

3.4 uvr B mutation

Tester strains TA98 and TA100 contain the uvr B mutation that make them more sensitive to the ultraviolet light which can be checked as follow;

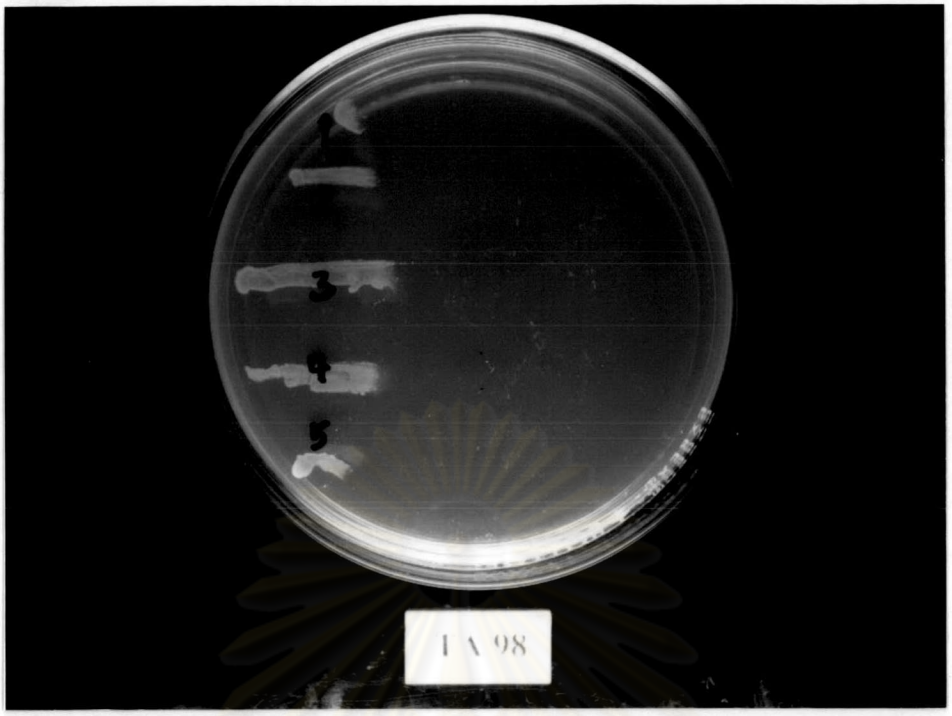
3.4.1 The cultures were streaked across the nutrient agar plate in a parallel stripe.

3.4.2 Place a piece of card board over the uncovered plate so that the half of each bacterial streak is covered

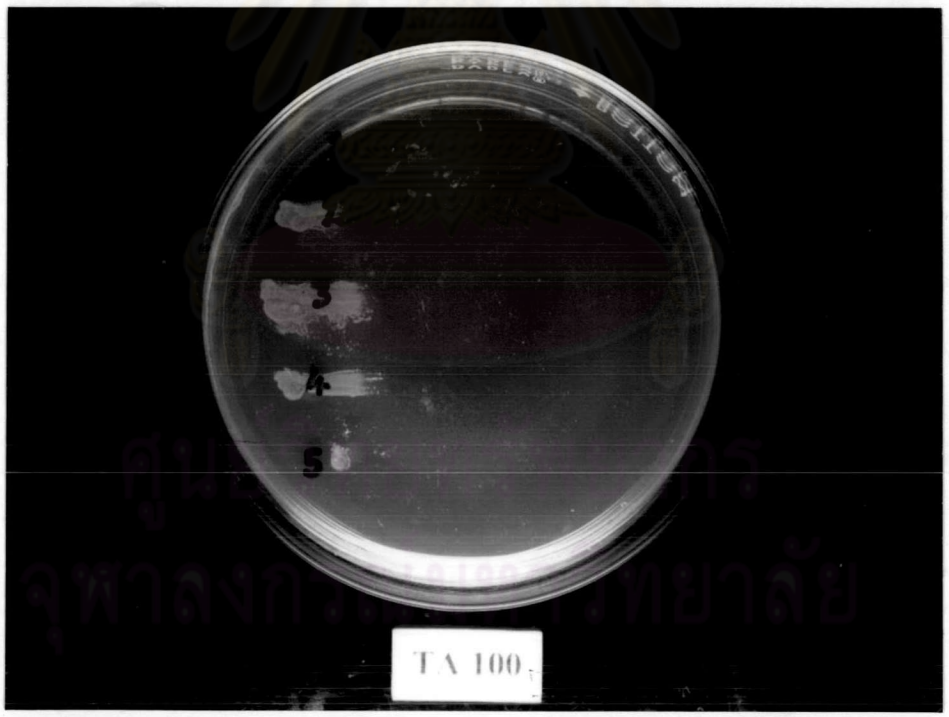
3.4.3 Plates were irradiated with a 15-W germicidal lamp at a distance of 33 cm for 8 sec.

3.4.4 Incubate the plates at 37 ^{0}C for 24 hours

3.4.5 Check the plates: the bacteria should be no growth on the uncovered slide of the plate (Figure II-4)



(a)



(b)

Figure II-4 Each of single revertant colonies of S. Typhimurium strains TA98 (a) and TA100 (b) on nutrient agar plate for confirming genotype: uvr B mutation

3.5 Procedure for growing cultures

3.5.1 Taking a single sweep from the master plates with a sterile wire loop, and grow in a L-tube containing 12 Oxoid nutrient broth No.2

3.5.2 Incubate the L-tube at 37 ^{0}C in shaking water bath for 14-16 hours.

3.5.3 The culture will grow to a density of 1-2x10^{9} cells per ml.

3.5.4 Remove the culture to an ice bath until it is required for the assay.

3.6 Spontaneous reversion

The condition of spontaneous reversion of the tester strains to histidine independence as well as in mutagenicity test by using solvent (DMSO) instead of the test chemicals. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. With out S9 mix, S. Typhimurium TA98 express to 30-50 revertants/plate, whereas strain TA10 express to 120-200 revertants/plate and with S9 mix condition, the numbers may be slightly different on plates which can be checked as follow;

3.6.1 Taking the S9 mix 0.5 ml (or $NaPO_4$-KCl buffer pH 7.4; in case of without S9 activation) to sterile capped culture tubes as placed in an ice bath

3.6.2 0.1 ml of fresh overnight culture 14-16 hours was added.

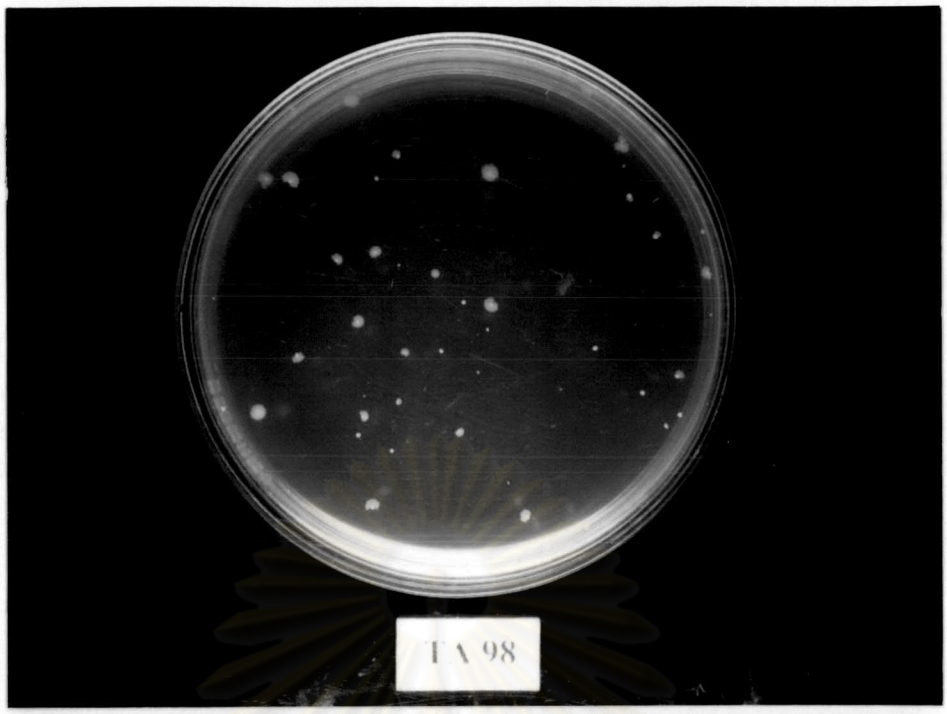
3.6.3 0.1 ml of DMSO was added.

3.6.4 Vortex the tube gently and pour on the minimal glucose agar plates.

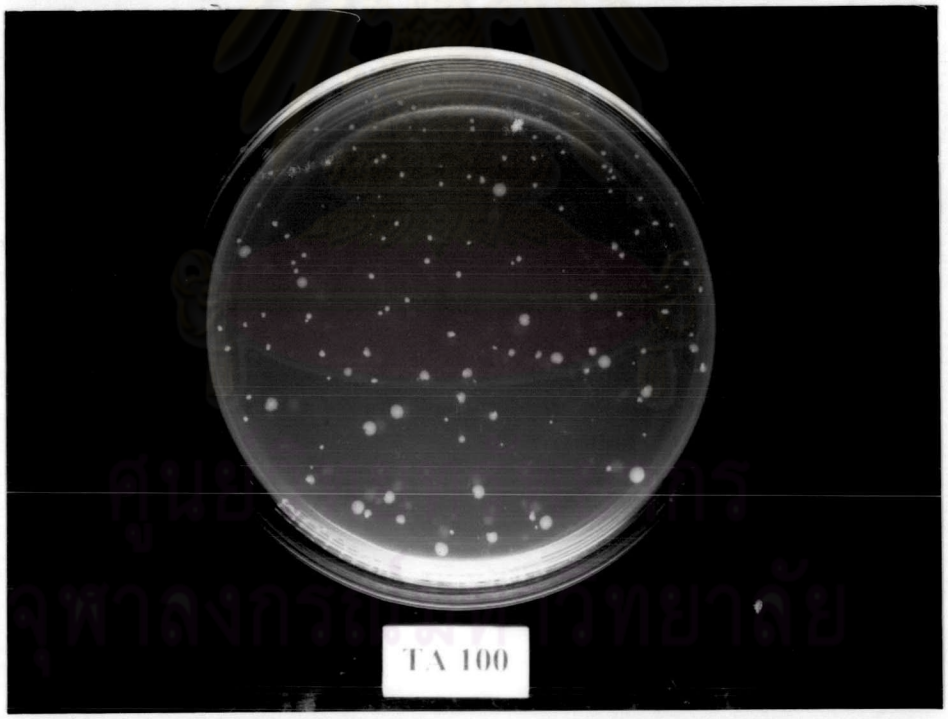
3.6.5 Rotate the plates then left it to become harden

3.6.6 Incubate the plates at 37 ^{0}C for48 hours

3.6.7 The histidine revertant colonies of each strain were counted as shown in Figure II-5



(a)



(b)

Figure II-5 Revertant colonies of S. Typhimurium strains TA98 (a) and TA100 (b) on minimal glucose agar plate for confirming genotype: Spontaneous reversion

3.7 The response to standard carcinogens

The standard carcinogens were used to confirm the sensitivity and specificity of each strain and the efficacy of the S9 mix. These experiment are B(a)P for TA98 and TA100 strains in the presence of S9 mix. AF_2 for TA98 and TA100 strains in the absence of S9 mix. Testing as well as the method in the spontaneous reversion but 0.1 ml of B(a)P for TA98 (10 μg/plate) and TA100 (5 μg/plate) in the presence of S9 mix and AF_2 for TA98 (0.1 μg/plate) and TA100 (0.01 μg/plate) in absence of S9 mix are used instead of 0.1 ml of DMSO.

Preparation of stock solution for Ames' test

1. Vogel-Bonner medium E (50X VB salts) **1000 ml**

(For Minimal glucose agar plate)

Distill water	670 ml
Magnesium sulfate ($MgSo_4.7H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate dibasic (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaHNH_4 .4H_2O$)	175 g

Add these ingredients in the order and allow each chemical to dissolve completely before adding the next. Adjust the volume to 1000 ml. Autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min.

2. Minimal glucose agar plates **1000 ml**

(For the mutagenicity test)

Bacto agar	15 g
Distilled water	930 ml
50 X VB salts	20 ml
40% glucose	50 ml

Add 15 g of agar to 930 ml of distilled water. Autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min. Add sterile 50 X VB salts and 40% glucose, after that pour 30 ml of the mixed into each Petri plate.

3. Oxiod nutrient broth No. 2 **100 ml**

(For growing the strains (overnight) for mutagenicity test)

Nutrient broth No. 2	2.5 g
Distilled water	100 ml

The media has to be dissolved completely. Then dispense 12 portions to flask or L-shape test tube with loose fitting cotton or cap plug. Autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min.

4. Top agar **200 ml**

(For the mutagenicity test)

Bacto agar	1.2 g
Sodium chloride	1 g
Distilled water	200 ml

Top agar, autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min. Add 20 ml of 0.5 mM histidine/biotin solution before use for assay

5. 0.5 mM histidine-biotin solution **400 ml**

(For the mutagenicity test)

L-histidine.HCl	41.93 mg
D-biotin	48.86 mg
Distilled water	400 ml

After autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min, store at 4 °C

6. Crystal violet solution (0.1%) **100 ml**

(To confirm rfa mutation)

Crystal violet powder	0.1 g
Sterile distilled water	100 ml

After the powder of crystal violet was completely dissolve, store at 4 °C in glass bottle with cap and wrap the bottle with metal foil to prevent it from light.

7. Histidine-biotin plates **1000 ml**

(To confirm histidine requirement)

Bacto agar	15 g
Distilled water	914 ml
50X VB salts	20 ml
40% glucose	50 ml
Sterile 0.5 mM histidine.HCl.H_2O	10 ml
Sterile 0.5 mM biotin	6 ml

15 g of agar was added to 914 ml distilled water. Autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min. Add sterile 50X VB salts, 40% glucose, sterile 0.5 mM histidine-HCl.H_2O and sterile 0.5 mM biotin. The mixtures were mixed and pour approximate 30 ml into each petri plate.

8. 0.5 mM D-biotin **25 ml**

(To histidine/biotin plate and Ampicillin plate)

D-biotin (MW.244.3)	3.054 mg
Distilled water	25 ml

Autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min and store at 4 °C

9. 0.15 M KCl **1000 ml**

(To prepare the liver homogenate S9 fraction)

Potassium chloride	11.18 g
Distilled water	1000 ml

Autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min and store at 4 °C

10. Ampicillin plates **1000 ml**

(To confirm ampicillin resistance phenotype, master plates)

Bacto agar	15 g
Distilled water	910 ml
50X VB salts	20 ml
40% glucose	50 ml
Sterile 0.5 mM histidine.Hcl.H_2O	10 ml
Sterile 0.5 mM biotin	6 ml
Sterile ampicillin solution (8 mg/ml)	3.15 ml

Procedure of Ampicillin plates; add Bacto agar to distilled water and autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min. Add sterile 40% glucose, 50X VB salts and sterile 0.5 mM histidine.Hcl.H_2O on hot plate. Mix and cool to approximately 50 °C. Add sterile 0.5 mM biotin and ampicillin solution, mix and pour approximate 30 ml into each petri plate.

11. Ampicillin solution (8mg/ml) **100 ml**

(To ampiclling plates)

Ampicillin	800 mg
Sodium hydroxide	100 ml

The solution was filtered through a 0.22 μM membrane filter and store in a glass bottle at 4 °C (Unnecessary to sterilize).

12. Nutrient agar plates **1000 ml**

(To test the genotypes: Crystal violet sensitivity (rfa mutation) and UV sensitivity (uvr B mutation)

Oxoid nutrient broth No. 2	25 g
Bacto agar	15 g
Distilled water	1,000 ml

Autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min and mix and pour approximately 30 ml into each Petri plate.

13. 0.16 M $MgCl_2$ **100 ml**

(To mix with S9 mix)

Magnesium chloride ($MgCl_2.6H_2O$)	3.25 g
Distilled water	100 ml

Autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min and store at 4 °C

14. 0.1 M NADP solution **1 ml**

(For S9 mix)

B-NADP	63 mg
Sterile distilled water	1 ml

Aseptic for these procedure and not autoclave.

15. 0.1 M G6P **10 ml**

(To mix with S9 mix)

Glucose-6-phosphate (MW 282.1)	282.1 mg
Sterile distilled water	10 ml

Aseptic for these procedure and not autoclave.

16. 0.5 M Sodium phosphate buffer, pH 7.4 **100 ml**

(For the mutagenicity assay)

0.5 M Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$, 6.9 g/100 ml)	60 ml
0.5 M disodium hydrogen phosphate (Na_2HPO_4, 35.5 g/500 ml)	440 ml

Mix well and adjust the pH to 7.4 and Autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min

17. Sodium phosphate -potassium chloride buffer **350 ml**

(To mix with S9 mix)

0.5 M M Sodium phosphate buffer, pH 7.4	100 ml
1 M Potassium chloride	16.5 ml
Distilled water	213.5 ml

Autoclave at 15 lbs/in^2, 121 ^{0}C for 15 min

18. Liver S9 mix **10 ml**

(For the mutagenicity assay)

Sodium phosphate -potassium chloride buffer	6.6 ml
0.16 M $MgCl_2$	0.5 ml
0.1 M NADP	0.4 ml
0.1 M G6P	0.5 ml
S9 fraction	2 ml

Aseptic for this procedure, not to autoclave. The mixture must not reuse.

APPENDICES III

MUTAGENICITY TEST BY MICRONUCLEUS TEST

APPENDICES III

MICRONUCLEUS TEST

1) Giemsa's stain

Giemsa powder	1 g
Absolute methanol	100 ml

The mixtures are warm to 50 °C and keep at this temperature for overnight with incubator, and then filter the solution.

2) May-Grunwald's stain

May-Grunwald power	0.3 g
Absolute methanol	100 ml

3) Cyclophosphamide solution

Cyclophophamide	20 mg
Distilled water	1 ml

Cyclophophamide was completely dissolved and freshly preparation.

BIOGRAPHPY

Miss Wandee Sutjit was born on March 18, 1976 in Phang nga Province, Thailand. She received her Bachelor of Science in Food Science, Faculty of Science, The University of the Thai Chamber of Commerce in 1999. She had been work as Supervisor Production position, Serm Suk Y. H. S. Beverage Co. Ltd. During 1999. She has studied for Master's Degree in Biotechnology program at Faculty of Science and establishes a lab work in the Department of Biology, Chulalongkorn University since 2001.