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APPENDICES

APPENDIX I

Media and Identification Procedures.

Media

1. Mueller Hinton II agar

Beef extract	2.0	g
Acid hydrolysate of casein	17.5	g
Starch	1.5	g
Agar	17.0	g
Distilled water	1,000.0	ml

Preparation:

Dissolve the dehydrated medium in water by heating by heating if necessary. Adjust pH to 7.2 - 7.4, transfer into bottles and autoclave at 110 °C for 20 min.

2. Nutrient agar

Meat extract	3.0	g
Peptone	5.0	g
Agar	12.0 – 18.0	g [(1)]
Water	1,000.0	ml

Preparation:

Dissolve the dehydrated medium in the water, may be by heating. Adjust pH ~ 7.0 after sterilization, transfer into bottles and autoclave at 121°C for 20 min. Pour plates with about 15 ml melted medium each.

3. ONPG medium

Sodium dihydrogen phosphate (NaH_2PO_4)	6.9	g
o-Nitrophenyl β-D-galactopyranoside (ONPG)	2.0	g
Water	1,000.0	ml

Adjust pH to 7.0. Dispense 0,5 ml into tubes.

4. Schwärm agar

Bacto beef extract	3.0	g
Bacto peptone	5.0	g
Bacto agar		
Natriumdexoxycholat ($C_{24}H_{39}NaO_4$)	0.3	g
Water	1,000.0	ml

Preparation:

Dissolve medium and chemicals in water by heating if necessary. pH is adjusted to 7.4 , the medium is filled into 250 ml flasks and autoclaved at 121 C for 15 min.

5. Triple sugar/ Iron agar (TSI agar) (ref. 1)

Meat extract	3.0	g
Yeast extract	3.0	g
Peptone	20.0	g
Sodium chloride	5.0	g
Lactose	10.0	g
Sucrose	10.0	g
Glucose	1.0	g
Iron(III) citrate	0.3	g
Sodium thiosulfate	0.3	g
Phenol red	0.024	g
Agar	12.0 – 18.0	g [(1)]
Water	1,000.0	ml

[(1)] Depending on the gel strength of the agar.

Preparation

Dissolve the dehydrated medium in the water by heating if necessary. Adjust pH ~ 7.4 after sterilization, dispense into 10 ml tubes and autoclave at 121 °C for 10 min. Place tubes in a sloping position to obtain a butt depth of 2.5 cm.

6. Trypticase soy agar (Mearck, Germany)

Peptone from casein	15.0	g

Peptone from soymeal	5.0	g
Sodium chloride	5.0	g
Agar	15.0	g
Distilled water	1,000.0	ml

pH: 7.3 +/- 0.2 at 25°C

preparation:

All of ingradients were dissolved in distilled water, heat to boiling and then steriled by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The sterile medium was cooled to 45°C to 50°C, and dispensed into sterile plates.

Identification procedures

1. Biochemical tests

1.1 Triple sugar iron agar

Purpose: Triple sugar iron (TSI) agar is a screening medium used to identify gram- negative bacilli based on ability to ferment the carbohydrates glucose, sucrose, and lactose to produce H_2S gas.

Principle and interpretation: TSI agar contain protein, NaCl, Lactose, sucrose, dextrose, a sulfur source, an H_2S indicator, a pH indicator, and agar. The medium includes ten times as much lactose and sucrose as glucose. Bacteria that ferment glucose produce a variety of acids, turning the colour of the medium from red to yellow. Larger amounts of acid are produced in the butt of the tube (fermentation) than in the slant of the tube (respiration). Organisms growing on TSI also from alkaline products from the oxidative decarboxylation of peptone. These alkaline products neutralize the small amounts of acids present in the slant but are unable to neutralize the large amounts of acid present in the butt. Thus, the appearance of an alkaline (red) slant and an acid (yellow) butt after 24 hours incubation indicates that the organisms is a glucose fermenter but is unable to ferment lactose and sucrose.

Bacteria that ferment lactose or sucrose (or broth), in addition to glucose, reduce such large amonts of acid that the oxidative deamination of protein that may occur in the slant does not yield enough alkaline products to cause a reversion of pH in that region. Thus, these bacteria produce an acid slant and acid butt. It is impossible to determine from the TSI reaction whether both lactose and sucrose are being fermented or only one of these carbohydrates is being fermented; individual carbohydrate fermentation tests are required to make this assessment.

Gas production (CO_2 and hydrogen) is detected by the presence of cracks or bubbles in the medium. These are formed when the accumulated gas escapes.

H_2S gas is produced as a results of the reduction of thiosulfate. H_2S is a colourless gas and can be detected only in the presence of an indicator, in this case ferric ammonium sulfate. H_2S combines with the ferric ions of ferric ammonium sulfate to produce the insoluble black precipitate ferrous sulfide. Reduction of thiosulfate proceeds only in an acid environment, and blackening usually occurs in the butt of the tube. Although the black precipitate may frequently obscure the colour of the butt, it can be assumed that the organism is a glucose fermenter because of the requirement for an acid environment. The reactions can be summarized as follow:

Alkaline slant/acid butt: glucose only fermented

Acid slant/acid butt: glucose and sucrose fermented or glucose and lactose fermented or glucose, lactose, and sucrose fermented

Bubbles or cracks present: gas produced

Black precipitate present: H_2S gas produced

Ingredients and preparation: Mix the ingredients, heat to boiling, dispense into tubes, and sterilize at 121°C for 15 minutes, and allow tubes of medium to cool in a slanted position.

Pancreatic digest of casein, USP	10 g
Peptic digest of animal tissue, USP	10 g
NaCl	1 g
Lactose	10 g
Sucrose	10 g
D-Glucose	1 g

Ferric ammonium sulfate	0.2 g
Sodium thiosulfate	0.2 g
Phenol red	25 g
Agar	13 g
Distilled water	1 L

Final pH 7.3-7.4

Procedure: Inoculate test cultures to TSI agar by first touching a sterile bacteriologic needle to a colony and then stabbing the needle into the deep agar region of the mediun. Hence withdrawing the needle, move it from side to side over the surface of the medium. Incubate cultures at 37°C for 18 to 24 hours. Examine cultures for colour of the slant, butt, gas cracks, and blackening caused by H_2S.

1.2 Indole test

Purpose: Indole broth is used for distinguishing between bacteria based on ability to produce indole from tryptophan.

Principle and interpretation: Indole broth contains tryptophan-rich peptone and NaCl. The tryptophan present in peptone is oxidized by certain bacteria to indole, skatole, and indoleacetic acid. The intracellular enzymes that are responsible for metabolizing tryptophan to these compounds are collectively termed tryptophanase. Indole is detected in broth cultures of bacteria with an alcoholic *p*-dimethylaminobenzaldehyde reagent. Indole reacts with the aldehyde to give a red product in the alcoholic layer of the broth-reagent mixture.

Two reagents were used to detect indole: Kovac's and Ehrlich. Ehrlich reagent is believed to be more sensitive than Kovac's and is recommended for detection of indole production by anaerobic bacteria and nonfermentative gram negative organisms. Kovac's reagent was used initially to classify members of the family *Enterobacteriaceae* and should be used with these organisms.

Ingredients and preparation: Mix the ingredients, heat to boiling, dispense into tubes, and sterilize at 121°C for 15 minutes.

Indole broth:

Pancreatic digest of casein, USP	20 g

NaCl	5 g
Distilled water	1 L
Final pH 7.2	

Reagents:

Kovac's indole reagent. Dissolve the aldehyde in the alcohol and slowly add acid to the mixture.

Alcohol, amyl or isoamyl	150 ml
p-Dimethylaminobenzaldehyde	10 g
Hydrocholic acid, concentrated	50 ml

Procedure: Inoculate the test organism into indole broth, incubate at 35°C for 18 to 24 hours, and test as follows.

Indole test: Add 2 to 3 drops of Kovac's reagent directly to the broth culture, shake gently, and observe for development of a red colour in the upper alcohol layer.

1.3 Urea agar

Purpose: Urease agar are used for distinguishing between species of aerobic bacteria based on ability to hydrolyze urea.

Principle and interpretation: A variety of media are used to test for ability to hydrolyze urea. The hydrolysis of urea by urease to ammonia is accompanied by a rise in pH of the medium and a concomitant change in the color of the indicator from yellow to pink-red.

Ingredients and preparation: Mix urea basal ingredients, sterilize by filtration, and add sterile agar solution (50°C). Mix and dispense into tubes, and allow tubes of medium to cool in a slanted position.

Urea base:

Pancreatic digest of gelatin, USP	1 g
NaCl	5 g
Monopotassium phosphate	2 g
D-Glucose	1 g
Urea	20 g
Phenol red	12 mg

Distilled water	100 ml

Final pH 6.8

Agar solution:

Agar	15 g
Distilled water	900 ml

Urea agar:

Urea base	100 ml
Agar solution	900 ml

Procedure: Inoculate the organism to the urea agar, incubate for 24 to 48 hours at 35°C, and observe for a red color change in the medium.

1.4 Citrate agar, Simmons

Purpose: Simmons citrate agar is used to distinguish gram-negative bacteria based on their ability to utilize as a sole source of carbon.

Principle and interpretation: Several theories have been proposed to explain the mechanism of citrate agar. Only one is presented here. Organisms that metabolize citrate as a sole source of carbon cleave citrate to oxaloacetate and acetate via the citrate enzyme. Another enzyme, oxaloacetate decarboxylase, then converts oxaloacetate to pyruvate and CO_2. CO_2 combines with sodium and water to form Na_2CO_3, an alkaline compound. As a result, the pH of the medium rises and the indicator (bromthymol blue) changes from green to Prussian blue. Presence of the blue color constitutes a positive finding for citrate utilization.

Ingredients and Preparation: Mix the following ingredients, heat to boiling, dispense into test tubes, and sterilize at 121°C for 15 minutes. Cool each tube of medium in a slanted position.

Sodium citrate	2 g
NaCl	5 g
$MgSO_4$	0.2 g
Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Bromthymol blue	80 mg

Agar	15 g
Distilled water	1 L

Final pH 6.9

Procedure: Lightly inoculate the test organism to the surface of citrate medium, incubate at 35°C for 24 to 48 hours, and observe for a Prussian blue color change.

1.5 Malonate broth

Purpose: Malonate broth is used for differentiation of members of the family *Enterobacteriaceae*, especially *Salmonella* species.

Principle and interpretation: Malonate broth tests for utilization of sodium malonate as a sole source of carbon. The medium contains buffer, pH indicator, sodium malonate, required salts, and a small amount of yeast extract and glucose. The pH indicator, bromthymol blue, is a deep Prussian blue at its alkaline ent point (pH 7.6), yellow at its acidic end point (pH 6.0), and green when uninoculated (pH 6.7). Bacteria that are capable of using malonate as a source of energy and carbon produce alkaline by products that change the color of the medium to blue. Bacteria that are unable to use malonate as a carbon source usually do not grow and the pH of the medium does not change; the indicator remains green. Some malonate-negative strains may produce a yellow color owning to fermentation of glucose.

Ingredients and preparation: Mix the ingredients, heat to boiling, dispense into tubes, and sterilize at 121°C for 15 minutes.

Yeast extract	1 g
Ammonium sulfate	2 g
Dipotassium phosphate	0.6 g
Monopotassium phosphate	0.4 g
NaCl	2 g
Sodium malonate	3 g
D-Glucose	0.25 g
Bromthymol blue	0.025 g
Distilled water	1 L

Final pH 6.7

Procedure: Inoculate the test organism into molonate broth and incubate at 35°C for 18 to 24 hours.

APPENDIX II

Formulas for reagents used for PEGE

Stock solution

1. 5 M NaCl (pH 8.0)

NaCl	292.2 g
Deionized water	1,000 ml

This stock reagent was prepared by dissolved by 292.2 g of NaCl in 750 ml of deionized water, then the pH was adjusted to 8.0 with conc. HCl. The final volume was bought up to 1,000 ml with deionized water. The stock reagent steriled by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The stock reagent was stored at room temperature.

2. 0.5 M EDTA (pH 8.0)

Ethylene diaminetetraacetic acid	186.12 g
Deionized water	1,000 ml

This stock reagent was prepared by dissolved by 186.12 g of ethylene diaminetetraacetic acid in 750 ml of deionized water, then the pH was adjusted to 8.0 with conc. HCl. The final volume was bought up to 1,000 ml with deionized water. The stock reagent steriled by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The stock reagent was stored at room temperature.

3. 1 M Tris (pH 8.0)

Tris base	121.14 g
Deionized water	1,000 ml

This stock reagent was prepared by dissolved by 121.14 g of Tris base in 750 ml of deionized water, then the pH was adjusted to 8.0 with conc. HCl. The final volume was bought up to 1,000 ml with deionized water. The stock reagent steriled by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The stock reagent was stored at room temperature.

4. 10 X TBE

Tris base	108	g
Boric acid	55	g
EDTA	7.44	g
Deionized water	1,000	ml

This stock reagent was prepared by dissolved all of ingredients in

1,000 ml of deionized water. The stock reagent steriled by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The stock reagent was stored at room temperature.

Buffer solution

1. Cell Suspension Buffer (CSB; 100 mM Tris: 100 mM EDTA, pH 8.0)

10 ml of 1 M Tris, pH 8.0

20 ml of 0.5 M EDTA, pH 8.0

Dilute to 100 ml with sterile Type 1 water

2. Tris: EDTA Buffer (TE; 10 mM Tris: 1 mM EDTA, pH 8.0)

10 ml of 500 M Tris, pH 8.0

20 ml of 50 M EDTA, pH 8.0

Dilute to 1000 ml with sterile Type 1 water

3. 1% SeaKem Glod: 1% Sodium Dodecyl Sulfate Agarose (1% SKG: 1% SDS) in TE Buffer

a. Weigh 0.05 (or 0.25) gram SKG into 250 ml screw-cap flask.
b. Add 47.0 (or 23.5) ml TE Buffer; swirl gently to disperse agarose.
c. Remove cap, cover loosely with clear film, and microwave for 30-sec; mix gently and repeat for 10-sec intervals until agarose is completely dissolved.
d. Place flask in 65EC water bath for at least 5 minutes.
e. Add 2.5 (or 1.25) ml of 20% SDS that has equilibrated to 56°C and mix well.
f. Recap flack and return to 56°C water bath until ready to use.

4. Cell Lysis Buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosine)

25 ml of 1 M Tris, pH 8.0

50 ml of 0.5 M EDTA, pH 8.0

5 g of Sarcosyl (N-Lauryl-Sarcosine, Sodium salt)

Or

50 ml of 10% Sarcosyl (N-Lauryl-Sarcosine, Sodium salt)

Dilute to 500 ml with sterile type 1 water

Add 25 µl Proteinase K stock solution (20 mg/ml) per 5 ml of cell lysis buffer just before use.

The final concentration of Proteinase K is 0.1 mg/ml in the buffer.

5. 1% SeaKem Gold Agarose (1% SKG) in 0.5X TBE Buffer

a. Weigh 1.0 gram SKG into 500 ml screw-cap bottle.
b. Add 100.0 ml 0.5 X TBE Buffer; swirl gently to disperse agarose.
c. Remove cap, cover loosely with clear film, and microwave for 60-sec; mix gently. And repeat for 15-sec intervals until agarose is completely dissolved.
d. Place bottle in 54-58°C water bath for at least 15 minutes before pouring gel.

Use of trade names and commercial sources is for identification only and does not imply endorsement by the CDC or the U.S. Department of Health and Human Services.

Enzymes

1. Proteinase K (10 mg/ml)

Proteinase K	100	mg
Sterile deionized water	10	ml

To prepared this stock reagent, 100 mg of Proteinase K was dissolved in 10 ml of sterile deionized water. The stock reagent was stored at -20°C

2. Restriction endonuclease enzyme *Xba*I

2.1 Pre-incubation (per reaction)

10X restriction enzyme *Xba*I buffer	10	µl

Sterile deionized water	90	µl

This buffer was freshly prepred by mixing before use.

2.2 Restriction enzyme digestion (per reaction)

Restriction enzyme *X*baI (15 units/µl)	1.33	µl
10X restriction enzyme *X*baI buffer	5	µl
0.1% bovine serum albumin	5	µl
Sterile deionized water	38.67	µl

This restriction enzyme *X*baI solution was freshly prepred by mixing before use.

Agarose gel

1. 1% Low melting point agarose gel (per sample)

Low melting point agarose	0.1	g
TE buffer	10	ml

This 1% low melting point agarose gel was prepared by suspending 0.1 g of low melting point agarose in 10 ml of TE buffer. The agarose was melted by microwave oven.

2. 1% Ultrapure high melting temperature agarose gel

Ultrapure high melting temperature agarose	0.9	g
0.5X TBE	90	ml

This 1% ultrapure high melting temperature agarose gel was prepared by suspending 0.9 g of ultrapure high melting temperature agarose in 90 ml of 0.5X TBE buffer. The agarose was melted by microwave oven.

Reagent

Ethidium bromide solution

One pellet (11 mg) of ethidium bromide was dissolved in 11 ml of ultrapure water. Working solution: The 40 µl ethidium bromide stock solution was mixed with 300 ml ultrapure water before use.

APPENDIX III

Chemical agents, Enzyme, Molecular marker, Materials, and Instruments

1. Chemical agents & Media

Low melting point agarose (Bio-Rad, USA)

Ultrapure high melting temperature agarose (USB, USA)

Tris base (USB, USA)

EDTA (USB, USA)

Sodium chloride (Merch, USA)

Sodium lauroyl sarcosine (USB, USA)

Boric acid (Bio-Rad, USA)

Ethidium bromide solution (USB, USA)

Anti-serum for *Salmonella* species (SAP, Thailand)

Mueller-Hinton II agar plates without blood (Bio-Rad, USA)

Disks with antimicrobial agents (Oxoid)

2. Enzyme and Molecular Marker

Proteinase K (USB, USA)

Restriction endonuclease enzyme *X*baI (USB, USA)

Restriction endonuclease enzyme *X*baI buffer (USB, USA)

Bovine Serum Albumin (USB, USA)

Lambda ladder marker (USB ,USA)

3. Materials

Eppendorf tube (Falcon, USA)

Test tube (Pyrex, USA)

Plastic tube bottom size 50 ml (Falcon, USA)

Tip (Falcon, USA)

Glass bottle (Duran, Germany)

4. Instruments

Incubator 37°C (Memmert, Germany)

Shaking waterbath (Memmert, Germany)

Vortex mixer (Scientific, USA)

Eppendorf microcentrifuge (Tomy Seiko, Japan)

Freezer (-20°C) (Sanyo, Japan)

Refrigerator (Sanyo, Japan)

Automatic pipette (Gilson Medical Electronic, France)

pH meter (Beckman, USA)

Pulsed-Field Gel Box (Bio-Rad, USA)

Pump, Gel Molds (Bio-Rad, USA)

Cooling system (Bio-Rad, USA)

Contour-clamped homogenous electric field apparatus (Bio-Rad, USA)

UV transilluminator (Bio-Rad, USA)

Polaroid camera (Bio-Rad, USA)

Biological safety cabinet (Yamato, Japan)

BIOGRAPHY

NAME:	Miss Jharuwan Pansung
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INSTITUTION ATTENDED:	I graduated with the Bachelor degree of Science in Microbiology from the Faculty of Since, Chulalongkorn University in 1996.
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