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APPENDICES

APPENDIX A

A.1 Gram stain

A.1.1 Dye and chemical solution for bacterial staining

The widely used staining technique in bacteriology is the Gram stain developed in 1884 by Hans Christain Gram. This procedure is a differential stain method meaning that bacteria will give different results depending on their cell wall chemistry. The Gram stain divides bacteria will be purple after the last step; gram negative bacteria will be red. The size, shape and arrangement of the organisms can also be determined from a stained specimen using microscope.

1. Gram's crystal violet

Solution A	Crystal violet	2	grams
	Ethyl alcohol	20	grams
	Distilled water	100	milliliters
Solution B	Smmonium oxalate	0.8	milliliters
	Distilled water	100	milliliters

Solution A and B were mixed and filtrated in order to remove dregs before using.

2. Gram's iodine

Iodine	1	grams
KI	2	grams
Distilled water	300	milliliters

KI was dissolved in distilled water and added iodine before using.

3. Gram's alcohol

Ethyl alcohol	98	milliliters

Acetone	2	milliliters

All of solutions were mixed before using.

4. Gram's safranin

Safranin o (2.5% solution in 95% ethyl alcohol)	10	milliliters
Distilled water	100	milliliters

All of solutions were mixed before using.

A.1.2 Gram-staining Procedure

The four steps of the Gram stain can be summarized as follows:

1. Primary stain-cover the smear with crystal violet for one minute. All bacteria will take up this dye and appear purple. Rinse off the excess dye with water.

2. Mordant-Gram's iodine is added to interact for one minute with the crystal violet. This complex will be difficult to remove from certain bacteria during the next (decolorization) step. Excess iodine is rinsed is off with water.

3. decolorization-95% ethanol is briefly (10-20 seconds) applied to the smear followed by a water rinse.

4. Counterstain-Safranin is added for 20 seconds to dye any decolorized cells. It will not change the color of the cells that retain the crystal violet.

A.2 Biochemical test

Oxidase test

This test determines the presence of oxidase enzymes. Use only plastic or platinum loops for this test (A platinum loop may be used to transfer organisms but iron in a nichrome loop may interfere with the reaction.). Using a sterile swab, transfer a heavy inoculum of the bacteria to a slide. Place the slide on a white paper and add the

oxidase reagent. Observe for a color change. A positive reaction appears pink, then maroon and finally black. Take care to avoid contact with the oxidase reagent.

Catalase test

This test determines bacterial production of catalase enzymes. Place a drop of hydrogen peroxide (3% H_2O_2-reagent grade) on a microscope slide or in the concave surface of a hanging drop slide. With a sterile loop, collect a sample of 18-24 hour old pure bacterial culture. Place the loop in the hydrogen peroxide. If the test is positive, there will be immediate bubbing or foaming and liberation of o_2 gas. Records results

TSI/H_2S production

This medium can determine the ability of an organism to utilize specific carbohydrates incorporated in basal growth medium, with or without the production of gas, along with the determination of hydrogen sulfide (H_2S) production. TSI agar contains the three sugars in varying concentration: glucose (1X), lactose (10X) and sucrose (10X). It also contains the pH indicator phenol red. If sugar fermentation occurs, glucose will be initially used and the butt of the tube will be acidic (yellow). After glucose utilization the organism may continue to ferment the remaining sugars. If this occurs the entire tube will become acidic. Certain bacteria are unable to utilize any sugars and will breakdown the peptone present. Peptone utilization causes an alkaline (red) shift in the medium that causes a color changes from orange to red. Blackened medium is caused by hydrogen sulfide production, splitting of the medium or presence of bubble in the butt of the tube can determine gas production. With a sterile needle inoculate the TSI slant by stabbing to the bottom of the tube and then streaking the surface of the slant as

the needle is drawn out of the tube. Screw the cap on loosely. Incubate at 20-24°C and read after 18-24 hours.

RESULS: A = Acid; K = Alkaline; H_2S = Hydrogen sulfide produced; N = No change

Slant/Butt	Color/Reaction	Interpretation
K/N or K/A	red/orange (oxidative) or red/yellow (fermentative)	only peptone utilized or only glucose-fermented
A/A	Yellow/yellow and/or sucrose-fermented	glucose, plus lactose
Gas	Splitting or bubbles	Gas production
H_2S	Black butt	Hydrogen sulfide produced

Motility

This test determines I a bacterial isolate is motile by means of flagella. Place a drop of distilled water or sterile PBS onto the center of a clean microscope cover glass. Place an additional tiny drop in one corner of the cover glass (to adhere the cover glass to the depression slide when it is inverted). Incubate the center drop from a pure stain culture that is 24-48 hours old using a sterile loop. Carefully invert the cover glass and place over the concave portion of a hanging drop slide. Observe or motility using phase contrast at 400x magnification on a compound microscope. Care shoud be taken to not interpret "drift" or "Brownian motion" as motility. Record results as motile or non-motile.

If this method fails to show motility then inoculate a nutrient broth with the isolate and incubate at room temperature until growth is obtained usually 24 hours. After incubation use a sterile loop or sterile dropper and place a drop on a clean cover glass. Place a tiny drop of distilled water in one corner of the same cover glass. Continue as above. Semi-solid motility test medium can be used. Stab the medium with a small amount of inoculum. Incubte overnight at room temperature. If the bacterial species is motile, the medium will become turbid with growth that radiates from the line of inoculum. If the bacterial species is non-motile, only the stab line will have visible bacterial growth.

Citrate Utilization

Organisms that are able to use citrate as the sole source for metabolism and growth are able to grow on Simmons citrate agar. By metabolizing citrate by the bacteria alkaline conditions are formed in the medium. The pH indicator in on Simmons citrate agar, bromothymol blue, will turn from green from acidic conditions to a royal blue when the medium becomes alkaline.

Inoculate the Simmons Citrate agar by making a streak onto the surface of the slant with an 18-24 hour old pure culture. Incubate for up to 4 days at 20-24°C.

RESULTS: Positive-growth and medium color change to a blue-green or royal blue.

Negative-little or no growth and no color change in the medium,remaining dark green.

Urease test

Inoculate the surface of a Christensen urea agar slant and incubate. A position test is indicated by a red-violet color

Nitrate reduction

To determine the ability o an organism to reduce nitrate (NO_3) to nitrite (NO_2) or further reduced products. Inoculate the Nitrate Broth with 18-24 hour old pure culture. Incubate 24-48 hour at 20-24°C aerobically. After incubation add about 5 drops of α-naphthylamine and sulfanilic acid, add a small amount of zinc dust.

RESULTS: Positive-Formation of a pink or red color in the medium within 1-2 minutes following the addition of naphthylamine and sulfanilic acid or mo color development within 5-10 minutes after adding zinc dust.

Negative-No pink or red color development within 1-2 minutes following the addition of naphthylamine and sulfanilic acid or red color development within 5-10 minutes after adding zinc dust.

Esculin hydrolysis

To determine the ability of an organism to hydrolyze the glycoside esculin (aesculetin) to esculetin (aesculetin) and glucose in the presence of bile (10 to 40%). Inoculate the surface of the bile esculin slant with inoculum from an 18 to 24 hour old pure culture. Incubate 20-24°C for 24 to 48 hour.

RESULTS: Positive-Presence of black to dark brown color on the slant.

Negative-No blackening of the medium.

Vogas-Proskauer reaction

To detect the production of acetylmethylcarbinol (atoin), a natural product formed from pyruvic acid in the course of glucose fermentation. Inoculate buffered glucose broth with the organism and incubate at 37°C for 3 days. Add approximately 3

ml of alpha napthol, followed by 1 ml of 40% KOH. Mix well and allow to stand for 30 minutes.

RESULTS:

Pink	VP(+)
No change	VP(-)

Malonate Utilization

A method to establish if a bacterial isolate is able to utilize sodium malonate as its only source of carbon. Inoculate malonate broth with a light inoculum from an 18 to 24 hour pure culture. Incubate 24-48hours at 20-24°C.

RESULTS: Positive-Light blue to deep blue color throughout the media

Negative-Color re mains the same as un-inculated tube-green

Gelatinase Liquefaction

A test to determine bacterial production of gelatinase enzymes that liquefy gelatin. Inoculate by stabbing ½ to 1 inch deep into the nutrient gelatin media with a heavy inoculum from an 18 to 24 hour pure culture. Incubate 18 to 24 hours at 20-24°C.

RESULTS: Positive-Media is liquefied. Weak results can be visualized by rapping the tube against the palm of the hand to dislodge droplets of liquid from the media. Any drops seen are considered positive.

Negative-No liquefaction occurs in media.

Carbohydrate fermentation test

Bacteria metabolize carbohydrates by oxidative and / or fermentative pathways. Oxidation occurs in the presence of atmospheric oxygen (aerobic), whereas fermentation tales place in an anaerobic environment. Metabolism of the carbohydrate dextrose by either an aerobic or anaerobic pathway results in acid production. The resulting acidic environment causes the Brom Thymol blue pH indicator in the medium to turn from green to yellow. The presence of bubbles in the tube indicates gas produmtion(aerogenuc). If no reaction occurs, the medium can remain unchanged or become alkaline (blue at the surface). A deep butt tube (~7 ml in 16×125mm) is used for this test. With a sterile needle to a small inoculums from an isolated colony and stab to the bottom of the tube. Incubate at 20-24°C for 24-48 hour. Check rubes at 24 hour for acid and /or gas production.

RESULTS: A = acid (yellow); AG = acid + gas, N = no change or alkaline

	Top of Tube	Bottom of Tube
Oxidative	A	N
Fermentative	AG or A	AG or A
Non-reactive	N	N

Decarboxylase test (Lysine or Ornithine)

A determination of bacterial enzymatic capability to decarbozylate an amino acid to form an amine with resultant alkalinity. For each isolate to be tested, it is necessary to inoculate a decarboxylase control tube and lysine or ornithine test tube. Use light inoculum from 18 to 24 hour pure culture. Add 1 to 2 ml oil overlay to each tube.

Incubate 24 hours at 20-24 °C. A prolonged incubation or up to four days may be necessary.

RESULTS:

Test Result	Lysine or ornithine Tube	Control Tube
Positive	Turbidto faded purple (glucose fermented, Decarboxylase produced)	Yellow (glucose fermented)
Negative	Yellow (glucose fermented, Decarboxylase not produced)	Yellow (glucose fermented)
Negative	Purple (glucose not fermented, Decarboxylase nor produced)	Purple (glucose not fermented)

Arginine Dihydrolase

This method is based on the direct measurement of the disappearance of arginine. Make a dense suspension of the bacteria in 0.033 mM phosphate buffer (pH 6.8). Purge 4 ml pf the suspension by bubbling nitrogen through the suspension for several minutes, and add 1 ml of 0.001 M L-arginine monohydrochloride. After purging again, stopper the tubes, incubate them for 2 hours, and heat them at 100 °C for 15 min. After removing the cells by centrifugation, determine the concentration of arginine in the supernatant by the method of Rosenberg et al. as follows. Mix 1 ml of sample with 1 ml of 3 N NaOH, 2 ml of developing solution, and 6 ml of water; read the tubes at 30 min against a blank prepared without arginine by using a colorimeter equipped with a green filter (540nm); and compare the reading with those obtained with an uninoculated control containing arginine.

RESULTS: A positive test is indicated by the disappearance of some or all of the arginine.

A.3 Preparation of *E. coli* competent cells for heat shock method

Inoculate 100 ml sterile LB medium with 200 µl overnight culture of *E. coli* DH5α then grown at 37°C, 250 rpm until OD600 reach 0.3-0.4. The culture was incubated on ice for 30 minutes. The cells were collected by centrifugation at 5,000 rpm, 4°C for 10 minutes. A supernatant was discarded. The pellet was resuspended with equal volume of cold 0.1 M $MgCl_2$ and centrifuge at 5,000 rpm, 4°C for 10 minutes. A supernatant was discarded then the pellet was resuspended in 10 ml cold 0.1 M $CaCl_2$ and incubated on ice for 30-60 minutes. After incubation, the cell suspension was centrifuge at 5,000 rpm, 4°C, for 10 minutes. A supernatant was discarded then the pellet was resuspended in 1.8 ml cold 0.1 M $CaCl_2$ and 0.4 ml sterile glycerol. The cell suspension was aliquot 100 µl / tube on ice prior to immediate drop into liquid nitrogen. The competent cells were stored at -80°C until use.

The plasmid was mixed with cold cell suspension in microtube and place on ice for 1 hour. The mixture was incubated at 42°C for 90 seconds and placed on ice for 5 minutes. The cell suspension was transferred into a new sterile tube containing 1 ml of LB broth. The transformed cells were incubated at 37°C, 200 rpm for 1hour. The transformed cells were then spreaded onto the LB agar plate containing appropriate antibiotic. The plate was incubated at t 37°C for overnight.

Table A.1 The biochemical test result of four bacterial isolates were chosen

Biochemical Tests	**Bacterial isolates**			
	No.45	**No.4/1**	**No.13**	**No.27**
Gram stain	+ bacilli	+ bacilli	+ bacilli	+ cocci
Hemolysis	β	β	β	
Anaerobic growth		+		
TSI	K/A	K/A	K/K	N/N
H_2S				-
Catalase	+	+	+	+
Oxidase	+	+	+	+
Motility	+	+	+	-
Indole				
Citrate	+	+	-	-
Urease	+	+	-	-
Nitrate	+	+	-	-
N_2 Gas				-
Esculin				-
42°C(Growth)				
Acetate				
VP	+	+	-	+
Gelatinase	+	+	+	+w
Egg Yolk	-	+	-	
Glucose/Gas	+	+	+	
Lactose				-
Maltose				
Mannitol	+	-	+	-
D-Xylose	+	-	-	-
Sucrose				
L-Arabinose	+	-	-	
Fructose				-
Trehalose	+	+	+	
Starch hydrolysis	+	+	+	+
LDA		-		
Lysine				
Arginine	-		-	-
Ornithine		-		
Alkaline phos.				
Camp test				
Flagella				
Bacterial identification	*Bacillus subtilis*	*Bacillus cereus*	*Bacillus circulans*	*Dienococcus sp.*

APPENDIX B

Esterase activity assay

Principle:

p-Nitrophenyl acetate + H_2O $\xrightarrow{\text{Esterase}}$ *p* -Nitrophenol + Acitic acid

Condition:

Temperature = 25°C, pH = 8.0, $A_{420\ nm}$, Light path = 1 cm

Method: Continuous spectrophotometric rate determination

Reagents:

A. 50 mM potassium phosphate buffer, pH 8.0 at 25°C

(Prepare 100 ml in deionized water using potassium phosphate, monobasic, anhydrous and adjust pH 8.0 with 1 M of KOH.)

B. 100 mM *p*-nitrophenyl acetate (PNPB)

(Prepare 10 ml in dimethylsulfoxide using *p*-nitrophenyl acetate)

C. Esterase enzyme solution

(Immediately before use, prepare a solution containing 0.5-1.0 unit/ml of esterase in cold reagent A.)

Procedure:

Pipette (in milliliter) the following reagents into suitable cuvettes:

	Test	Bank
Reagent A (buffer)	0.99	0.99
Reagent B (PNPB)	0.01	0.01

Mix by inversion and equilibrate to 25°C. Monitor the $A_{420\ nm}$ until constant, using a suitably thermostatted spectrophotometer. Then add:

	Test	Bank
Reagent A (buffer)	0.10	-
Reagent B (PNPB)	-	0.01

Immediately mix by inversion and record the increase in $A_{420\ nm}$ for approximately 5 minutes. Obtain the $\Delta A_{420\ nm}$ /minute using the miximum linear rate for both the test and blank.

Calculations:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{420\ nm}\text{/min test} - \Delta A_{420\ nm}\text{/min bank})\ (1)\ (df)}{(12.95)\ (0.1)}$$

1 = Total volume (in milliliters) of assay

df = Dilution factor

12.95 = Millimolar extinction coefficient of *p* -nitrophenol at 420 mm at pH 8.0

0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

Unit definition:

One unit will hydrolyze 1.0 μmole of *p*-nitrophenyl acetate and *p*-nitophenol per minute at pH 8.0 at 25°C.

Final assay concentration:

In a 1 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 1 mM *p*-nitrophenyl acetate and 0.05-0.10 unit of esterase.

APPENDIX C

Bacterial volume and surface of gram-positive bacilli (Neumann *et al.*,2005)

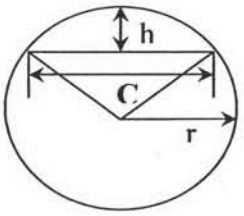
Cells dimensions of cylindrical bodies were directly measured from the TEM photographs to calculate cell volume and cell surface area by the following equations:

$$V\ (\mu m^3) = r^2\pi h,$$

$$A\ (\mu m^2) = 2r^2\pi + 2\pi rh,$$

where r is the radius and h the length of the cylindrical cells. Average cellular volumes and surface areas were calculated by using 30 individual bacteria per population. Cells showing deformations depressions were not considered.

Bacterial volume and surface of gram-negative cocci (Tumber *et al.*,1993)



$V = 2\,\pi\,r^3\,h \quad = \quad 2.0944\,r^2\,h$

$A = 3.1416\,r\,(2\,h + 1c)$

$c = 2\sqrt{h\,(2\,r - h)}$

BIOGRAPHY

Miss Ajiraporn Kongpol was born on June 1982, in Krabi province, Thailand. She received Bachelor's Degree in Applied biology and Microbiology, Faculty of science, Suan Sunandha Rajaphat University in 2005. She Master degree study in the Biotechnology Program, Faculty of science, Chulalongkorn University, Bangkok, Thailand in June 2005. She finished Master Degree of science in Biotechnology Program in May 2008. During her study, the output of her research work can be summarized as follows:

1. **Ajiraporn Kongpol,** Junichi Kato, and Alisa S. Vangnai. (2008) Isolation and characterization of *Deinococcus geothermalis* T27, a thermophilic and organic solvent-tolerant bacterium able to grow in the presence of high concentrations of ethyl acetate. *Submitted to FEMS Microbiology Letters*
2. Alisa S. Vangnai, **Ajiraporn Kongpol,** Thitikamon Sitthisorn, and Junichi Kato. (2007) Biotransformation of indole using thermo-tolerant, organic solvent-tolerant bacteria. Asia Pacific Biochemical Engineering Conference, Taiwan, November 4-7.
3. **Ajiraporn Kongpol,** Junichi Kato, and Alisa S. Vangnai. (2007) Characterization of thermo-tolerant, organic solvent-tolerant bacteria for industrial biotechnology application JSPS-NRCT Concluding Joint Seminar on Development of Thermotolerant Microbial Resources and their Application. The33 rd Congress on Science & Technology, Thailand (STT33) Science and Technology for Global Sustainability. Walailak University, Nakhon Si Thammarat, Thailand. October, 18-20.