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APPENDICES

APPENDIX A

QIAquick gel extraction kit protocol

1. The DNA fragment from the agarose gel was excised with a clean and sharp scalpel.
2. The gel slice was weighed in a colorless tube. Then, 3 volumes of buffer QG was added to 1 volume of gel (100 mg ~ 100 µl).
3. The tube was incubated at 50 °C and mixed by vortexing the tube every 2-3 minutes until the gel slice had completely dissolved.
4. After the gel slice had dissolved completely, 1 gel volume of isopropanol was added to the sample and mixed.
5. QIAquick spin column was placed in a provided 2-ml collection tube.
6. To trap DNA, the sample was applied to the QIAquick column and centrifuged at 10,000 rpm for 1 minute.
7. The flow-through was discarded and QIAquick column was placed back in the same collection tube.
8. Then, 0.5 ml of buffer QG was added to QIAquick column and centrifuged at 10,000 rpm forl minute.
9. Buffer PE 0.75 ml was added to QIAquick column to wash and further centrifuged at 10,000 rpm for 1 minute.
10. The flow-through was discarded and QIAquick column was centrifuged at 10,000 rpm for an additional 1 minute.
11. Finally, 50 µl of buffer EB (10 mM Tris-Cl, pH 8.5) was added to elute DNA and centrifuged at 10,000 rpm for 1 minute.

APPENDIX B

Preparation of *E. coli* competent cells for electroporation (Dower, 1988)

1. A fresh overnight culture of *E. coli* XL1-Blue was inoculated into 1 liter of LB broth, which 1 volume of overnight culture to 100 volume of LB broth.
2. Cells were grown to log phase at 37 °C with vigorous shaking. The OD_{600} was about 0.5 to 0.8.
3. To harvest, the culture was chilled on ice for 15 to 30 minutes, and then centrifuged at 8,000 x g for 15 minutes at 4 °C.
4. The cells were washed with 1 liter of cold water, were spun down and washed again with 0.5 liter of cold water.
5. After the centrifugation, cells were resuspended in approximately 20 ml of 10% glycerol in distilled water and centrifuged at 8,000 x g for 15 minutes at 4 °C.
6. The cell pellets were resuspended to a final volume of 2 to 3 ml in 10 % glycerol. This suspension was stored at –70 °C until used.

APPENDIX C

Media, Buffers and suppliers

1. Yeast extract – malt extract medium (YEME), 1 liter

Difco yeast extract		3	g
Difco Bacto – peptone		5	g
Difco malt extract		3	g
Glucose		10	g
Sucrose		340	g (34% final)
Distilled water	up to	1000	ml

After autoclaving 5 mM magnesium chloride hexahydrate was added.

2. R2YE Medium, 1 liter

Sucrose		103	g
K_2SO_4		0.25	g
$MgCl_2.6H_2O$		10.12	g
Glucose		10	g
Difco Casaminoacids		0.1	g
Difco yeast extract		3	g
Distilled water	up to	800	ml

The 80 ml of the solution was poured into Erlenmeyer flasks each containing 2.2 g Difco Bacto agar. Then autoclave R2YE was added sterile solution in the order listed.

0.5% KH_2PO_4	1	ml
3.68% $CaCl_2.2H_2O$	8	ml
20% L – proline	1.5	ml
5.73% TES, pH 7.2	10	ml
*Trace element solution	0.2	ml
1 N NaOH	0.5	ml

3. *Trace element solution, 1 liter

$ZnCl_2$	40	mg
$FeCl_3.6H_2O$	200	mg
$CuCl_2.2H_2O$	10	mg
$MnCl_2.4H_2O$	10	mg
$Na_2B_4O_7.10H_2O$	10	mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	10	mg

4. P (protoplast) Buffer, 1 liter

Sucrose	103	g
K_2SO_4	0.25	g
$MgCl_2.6H_2O$	2.02	g
*Trace element solution	0.2	ml
Distilled water	up to 800	ml

Each 80 ml aliquots buffer was autoclaved and added to each flask in order before used.

0.5% KH_2PO_4	1	ml
3.68% $CaCl_2.2H_2O$	10	ml
5.73% TES, pH 7.2	10	ml

5. T (transformation) Buffer

10.3% sucrose	25	ml
Distilled water	75	ml
*Trace element solution	0.2	ml
2.5% K_2SO_4	1	ml

Each 9.3 ml of solution was added:

5 M $CaCl_2$	0.2	ml
Tris – maleic acid buffer[1]	0.5	ml

[1] 1 M Tris was adjusted with maleic acid buffer to pH 8.0.

T buffer was used 3 parts by volume of the above solution to 1 part by weight of PEG 1000, previously sterilised by autoclaving.

6. T (transformation) Buffer

Mixtures of sterile solutions were followed:

10.3% sucrose	100	ml
5.73% TES, pH 7.2	10	ml
2.5% K_2SO_4	1	ml
*Trace element solution	0.2	ml
0.5% KH_2PO_4	1	ml
2.5 M $MgCl_2.6H_2O$	0.1	ml
0.25 M $CaCl_2$	1	ml

This stock solution was kept indefinitely. Lysozyme was dissolved at concentration 1 mg/ml and sterilized by filtration.

APPENDIX D

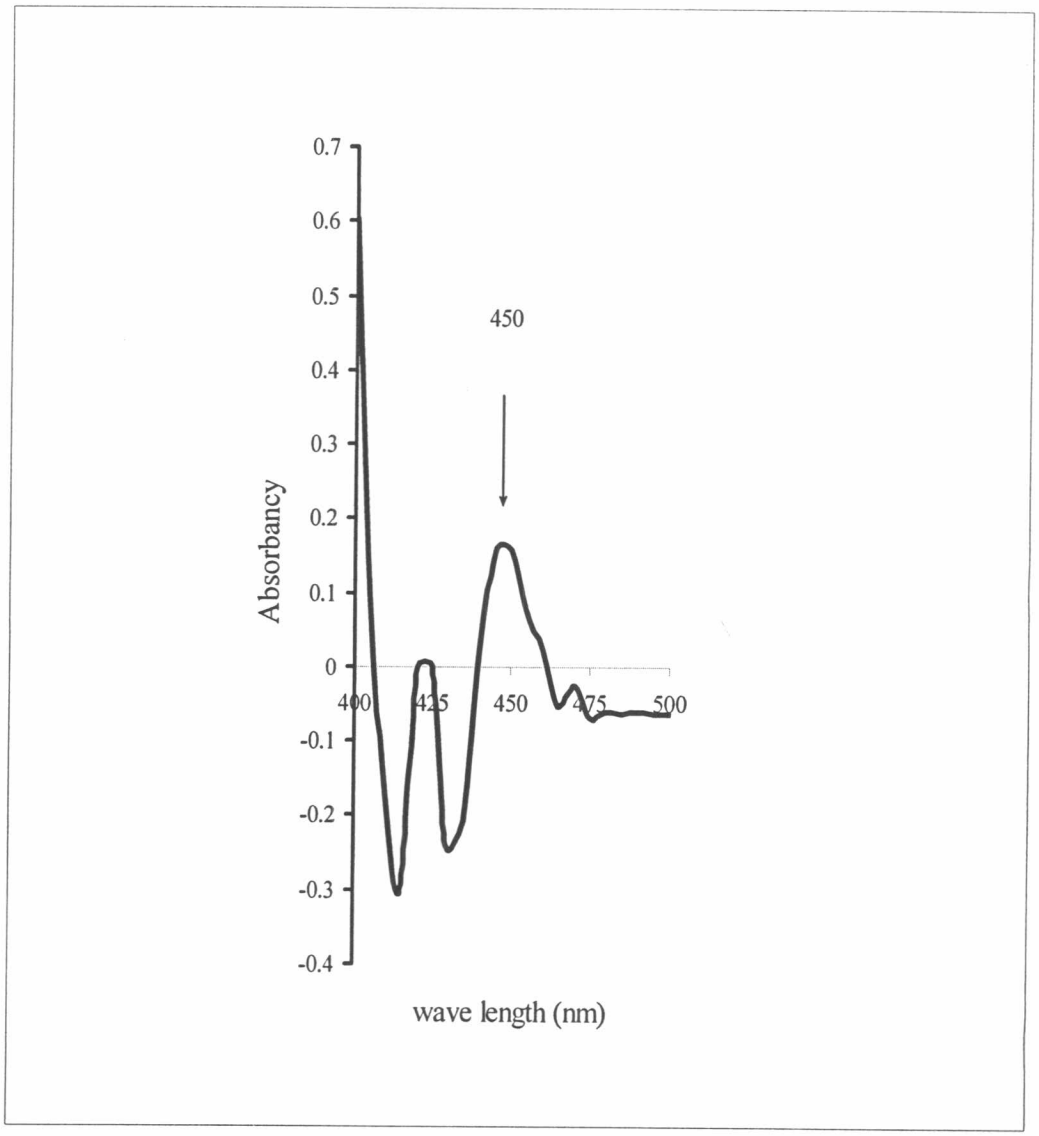
Microsomal preparations (Omura and Sato 1964)

The microsomal used prepared from liver by modified method of Omura and Sato, 1964. The weighted liver was finely chopped with a razor, and homoginized with 4 volumes of isotonic (1.15%) KCl solution in a potter glass homoginizer. The homogenate liver was centrifuged at 12,000 *x g* for 25 min in a refrigerated centrifuge, and the precipitate was discarded. The microsomes were sedimented by centrifugation at 78,000 x g for 90 min in ultracentrifuge. The firmly packed pellet of microsomes was resuspended in isotonic KCl solution with the homoginizer and again centrifuged as above. The washed microsomes were finally suspended in isotonic KCl, usually at a concentration of 10 mg of protein per ml. The protein concentration was determined by Lowry assay and shown in appendix G. The resultant microsomal suspensions were stored at 4 oC and used within 2 to 3 days.

In preparations isotonic KCl (ionic strengths, about 0.15) was employed, instead of the more usual 0.25 M sucrose, so as to minimize the adsorption of hemoglobin on microsomes. The microsomal preparations thus obtained were found to be practically free of adsorbed hemoglobin, when examined by zone electrophoresis as described by Paigen. As shown in section 2.19, the absence of hemoglobin could be confirmed further spectrophotometrically.

APPENDIX E

The carbon monoxide difference spectra of microsome



The carbon monoxide difference spectra of microsome 4 mg/ml protein was used to control.

APPENDIX F

Calculation of cytochrome P450 content

The content of P450 is determined from carbon monoxide difference spectrum of dithionite reduction, with a molar extinction difference of 91 $cm^{-1}mM^{-1}$.

From Beer-Lambert Law (Silverstein *et al.*, 1981)

$$A = \varepsilon bc$$

A: Absorbance

ε: Molar absorptivity ($cm^{-1}mM^{-1}$)

b: path length of cuvette (cm)

c: concentration of solution

The P450 content is calculated by

ε is 91 $cm^{-1}mM^{-1}$

A is difference spectra between 450 and 490 nm (ΔA)

b is 1 cm of cuvette path length

$$A = (91\ cm^{-1}mM^{-1})(1\ cm)(x)$$

Example;

$$\Delta A = 0.2$$

$$0.2 = (91\ cm^{-1}mM^{-1})(1\ cm)(x)$$

$$x = 0.2/91\ mM$$

$$= 0.0022\ mM$$

$$= 2.2\ \mu M$$

APPENDIX G

Preparation for protein determination

Reagent for determination of protein concentration (modified from Lowry *et al.*, 1951)

Solution A (0.5 % copper sulfate, 1% potassium tertate, pH 7.0)

Potassiun tartate	1	g
Copper sulfate	0.5	g

Adjusted pH to 7.0 and adjusted the solution volume to 100 ml.

Solution B (2 % sodium carbonate, 1 N sodium hydroxide)

Sodium carbonate	20	g
Sodium hydroxide	4	g

Dissolved in distilled water to 1 liter.

Solution C (phenol reagent)

Sodium tungstate	50	g
Sodium molybdate	12.5	g
85 % phosphoric acid	25	ml
Distilled water	350	ml
Concentrated hydrohloric acid	50	ml

Reflux for 10 hours

Lithium sulphate	75	ml
Distilled water	25	ml
Bromine solution	75	g

Boiled the solution to reduced excess bromine for 15 minutes, then adjusted volume to 500 ml with distilled water and stored at 4 °C. The stock solution was diluted with distilled water in ratio 1:1 (V/V) before using.

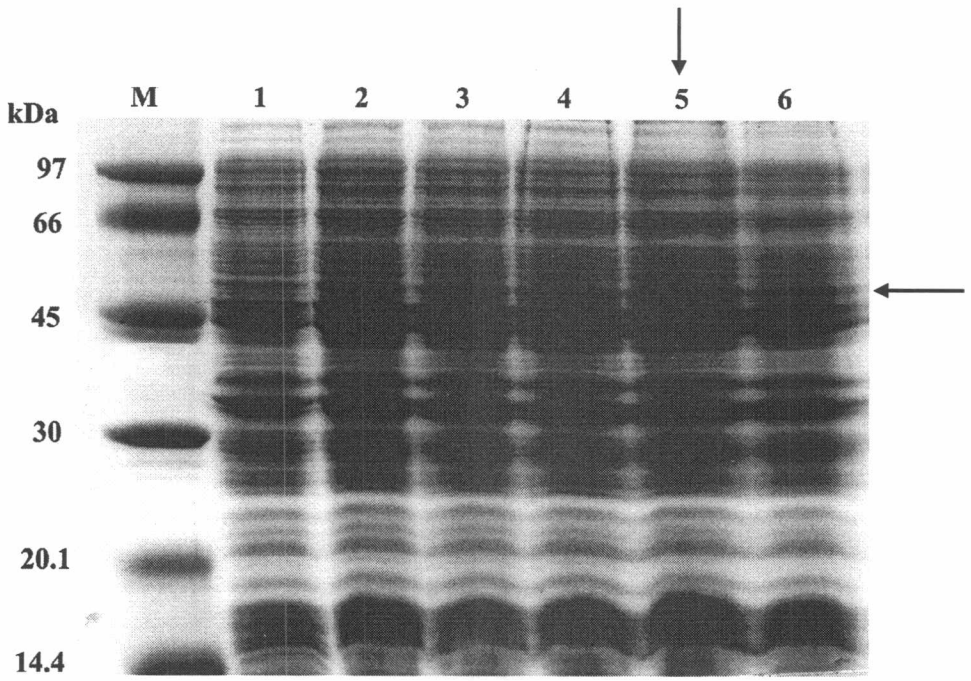
The reaction of Lowry assay

1. The appropriated protein was made up to standard by using Bovine Serum Albumin. Their concentrations were varied 20, 50, 100, 200 and 300 mg/ml per reaction.
2. Standard protein and sample was mixed in distilled water up to 0.5 ml and added solution A and B. The reaction mixtures were incubated 10 minutes at 30 °C.
3. Then solution C was added. After thoroughly mixed reactions were left 30 minutes at room temperature.
4. The absorption was measured at 610 manometers and plotted to standard for determination of protein concentration.

APPENDIX H

SDS-PAGE

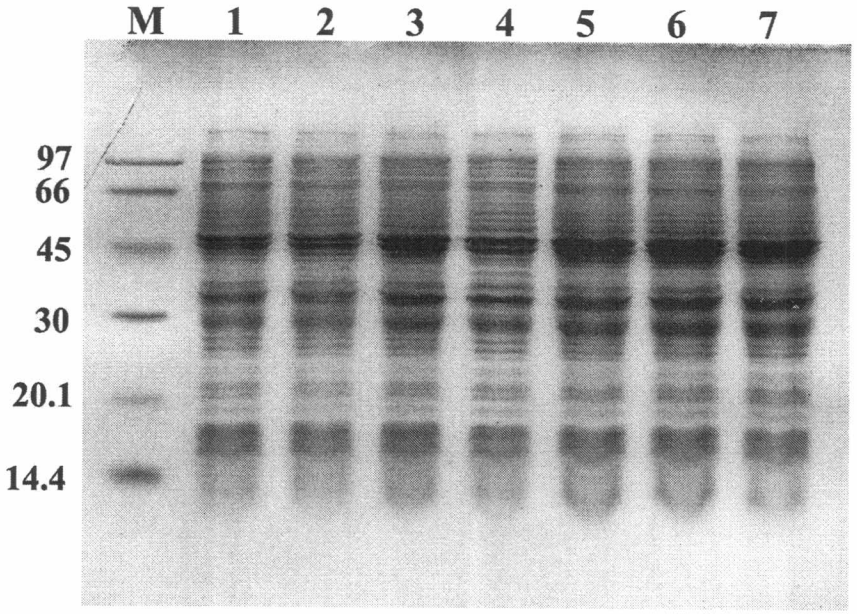
1. The induction of *picK* gene in *E. coli* XL1-Blue



The SDS-PAGE of protein pattern of *E. coli* XL1-Blue carrying pWHM3-*picK*

Lane M	Molecular weight standard marker
Lane 1	cell extracts uninduced
Lane 2	cell lysate of 6 hour post-induction
Lane 3	cell lysate of 12hours post-induction
Lane 4	cell lysate of 18 hours post-induction
Lane 5	cell lysate of 24 hours post-induction
Lane 6	cell lysate of 36 hours post-induction

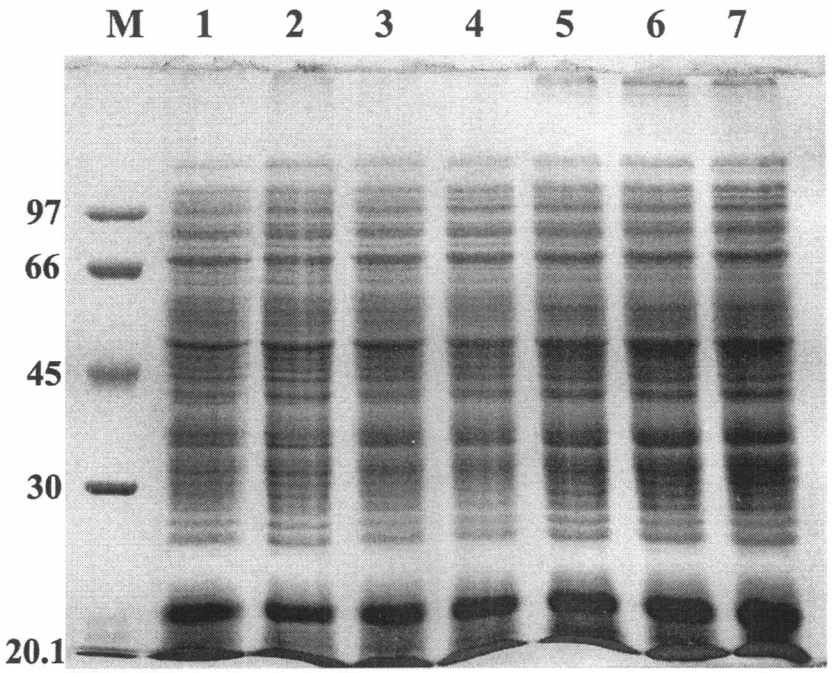
2. The induction of *E. coli* XL1-Blue carrying pWHM3 (not insert *picK* gene)



The SDS-PAGE of protein pattern of *E. coli* XL1-Blue carrying pWHM3

Lane M	Molecular weight standard marker
Lane 1	cell extracts uninduced
Lane 2	cell lysate of 0 hour post-induction
Lane 3	cell lysate of 1hours post-induction
Lane 4	cell lysate of 2 hours post-induction
Lane 5	cell lysate of 3 hours post-induction
Lane 6	cell lysate of 4 hours post-induction
Lane 7	cell lysate of 5 hours post-induction

3. The induction of *S. lividans* TK24 carrying pWHM3 (not insert *picK* gene)



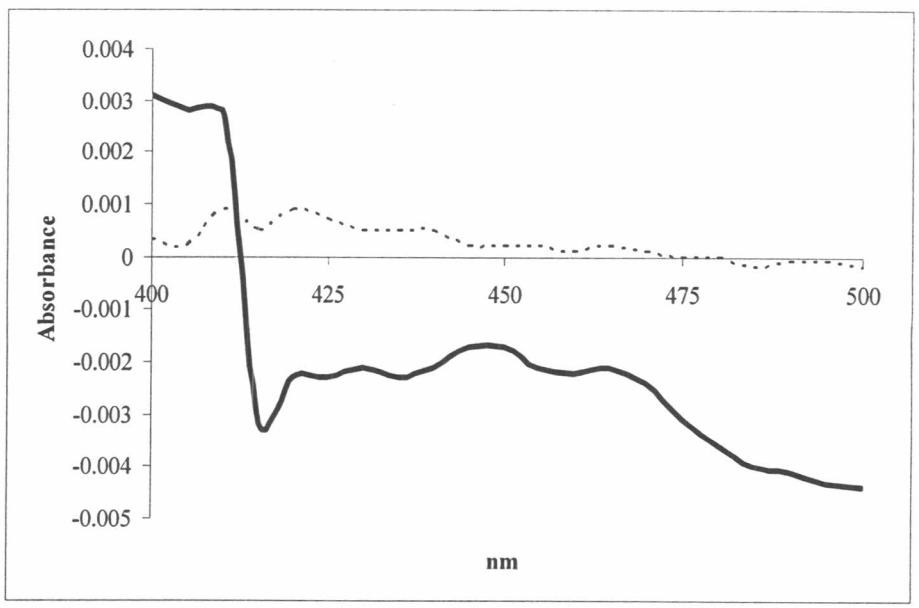
The SDS-PAGE of protein pattern of *S. lividans* TK24 carrying pWHM3

Lane M	Molecular weight standard marker
Lane 1	cell extracts uninduced
Lane 2	cell lysate of 0 hour post-induction
Lane 3	cell lysate of 1hours post-induction
Lane 4	cell lysate of 2 hours post-induction
Lane 5	cell lysate of 3 hours post-induction
Lane 6	cell lysate of 4 hours post-induction
Lane 7	cell lysate of 5 hours post-induction

APPENDIX I

The carbon monoxide difference spectra

1) The *E. coli* XL1-Blue host (no plasmid).



APPENDIX J

Protoplast density and transformation efficiency

1. Protoplast density

1.1. The CFU was calculated by counting colony on agar or selective plate and calculate by

$$\text{CFU} = \frac{\text{Colony x dilution factor x 1000}}{\text{plate out volume (µl)}}$$

1.2. Protoplast density was determined by

Protoplast unit = viable count – non-protoplast unit

and calculated to CFU. The **viable count** was scored from protoplast suspension diluted with P-buffer. The **non-protoplast unit** was scored from protoplast suspension diluted with detergent.

2. transformation efficiency

The transformation efficiency was calculated by

$$\textbf{Transformation efficiency} = \frac{(\text{viable colony} - \text{resistant colony}) \times \text{dilution factor}}{\text{volume of plate out (ml)}}$$

BIOGRAPHY

Miss Nongnuch Boonderm was born on January 11, 1977. She finished High school at Benjamarajrangsarit Chachaengchao School and enrolled in the Faculty of Science, King Mongkut's Institute of Technology Ladkrabang. She graduated with the B. Sc. In Biotechnology in 1999 and continue studying for M.Sc. in Biochemistry program, Faculty of science, Chulalongkorn University in 2000.