ภาคผนวก ก
การเตรียมอาหารเลี้ยงเชื้อ สารละลาย และสับสเตรท

1.1. LB Medium (Luria-Bacterial Medium)

Per litre:

To 950 ml of deionised H_2O add:

bacto-tryptone	10 g
bacto-yeast extract	5 g
NaCl	10 g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (0.2 ml). Adjust the volume of the solution to 1 litre with deionised H_2O. Sterilise by autoclaving for 20 min at 15lb/sq. in. on liquid cycle.

1.2. Preparation of colloidal chitin

Chitin powder from crab shells (5 g) was added slowly into 60 ml of concentrated HCl and left at 4 °C overnight with vigorous stirring. The mixture was added to 2 litres of ice-cold 95% ethanol with rapid stirring and kept overnight at 25 °C. The precipitant was collected by centrifugation at 5000 ***g*** for 20 min at 4 °C and was washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). Colloidal chitin was stored at 4 °C until further applications.

1.3. Preparation of Bradford's solution

The dye reagent is prepared by dissolving 0.01% Coommassie blue G-250 in a mixture of 85% (v/v) phosphoric acid, 95% (v/v) ethanol, and water in a ratio of 10:5:85 by vol.

The protein concentration was determined by Micro-assay method

1. Pipet 20 µl samples containing between 1 and 10 µg into 1.5-mL polyethylene microfuge tubes. For the calibration curve, pipet triplicate volumes of 0-20 µg/mL BSA standard solution into microfuge tubes.

2. Add 1 mL of protein reagent to each tube and mix gently, but thoroughly.

3. Measure the absorbance of each sample between 2 and 60 min after addition of the protein reagent.

1.4. SDS-PAGE

- Solutions for preparing 12% resolving SDS-polyacrylamide gel

Solution component	Component volume (ml)		
	5 ml	10 ml	20 ml
H_2O	1.6	3.3	6.6
30% (w/v) acrylamide mix	2.0	4.0	8.0
1.5 M Tris (pH 8.8)	1.25	2.5	5.0
10% SDS	0.05	0.1	0.2
10% ammonium persulfate (freshly prepared)	0.05	0.1	0.2
TEMED	2 µl	4 µl	6 µl

- Solutions for preparing 5% stacking SDS-polyacrylamide gel

Solution component	Component volume (ml)	
	2 ml	5 ml
H_2O	1.4	3.4
30% (w/v) acrylamide mix	0.33	0.83
1.0 M Tris (pH 6.8)	0.25	0.63
10% SDS	0.02	0.05
10% ammonium persulfate (freshly prepared)	0.02	0.05
TEMED	2 µl	5 µl

- Buffers for SDS-PAGE

SDS-gel loading buffer (3 x stock)

150 mM Tris.Cl (pH6.8)

300 mM dithiothreitol

6% SDS (electrophoresis grade)

0.3 % bromophenol blue

30% glycerol

- Tris-Glycine electrophoresis buffer (5 x stock)

250 mM Tris.Cl (pH 8.3)

1.25 M glycine (electrophoresis grade) (pH 8.3)

0.5 % SDS

- Staining solution with Coomassie Brilliant Blue for Protein

Dissolve 0.25 g of Coomassie Brilliant Blue R250 in 90 ml of methnol:H_2O (1:1v/v) and 10 ml of glacial acetic acid. Filter the solution through a Whatman No. 1 filter to remove any particulate matter.

- Destaining Solution for Coommassie Stain

30% methanol

10% acetic acid

dH_2O is added to bring volume to 100 ml.

1.5. Preparation of competent cells

1. Streak *E. coli* host cells on an LB plate+100 µg/ml Amp)
2. Allow cells to grow at 37°C overnight
3. Place one colony in 10 mL LB media (+antibiotic selection if necessary), grow overnight at 37°C
4. Transfer 5 mL overnight DH5a culture into 500 mL LB media in 2-L flask
5. Allow cell to grow at 37°C (250 rpm), until OD_{600}= 0.6 (~2-3 hours)
6. Transfer cells to 2 centrifuge bottles (250 mL), and place cells on ice for 20 mins
7. Centrifuge cells in Sorval GSA rotor at 4°C for 10 mins at 3,000 g (2500 rpm). **Cells must remain cold for the rest of the procedure**
8. Pour off media and resuspend cells in 30 mL of **cold** 0.1 M $CaCl_2$. Transfer the suspended cells into 50 mL polypropylene falcon tubes, and incubate on ice for 30 mins
9. Centrifuge cells using rotor at 4 °C for 10 mins at 3,000 g
10. Pour supernatant and re-suspend cells (by pipetting) in 8 mL cold 0.1M $CaCl_2$ containing 15% glycerol. Transfer 100 µL into (1.5 mL) Eppendorff tubes placed on ice. Freeze the cells in liquid nitrogen. Cells stored at -80°C can be used for transformation for up to ~6 months.

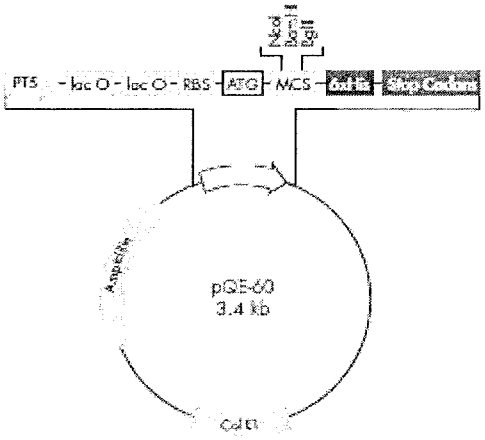
ภาคผนวก ข

แผนที่พลาสมิด pQE60 expression vector

pQE-60 Vector

Positions of elements in bases

Vector size (bp)	3431
Start of numbering at XhoI (CTCGAG)	1–6
T5 promoter/lac operator element	7–87
T5 transcription start	61
6xHis-tag coding sequence	133–150
Multiple cloning site	113–132
Lambda t_0 transcriptional termination region	173–267
rrnB T1 transcriptional termination region	1033–1131
ColE1 origin of replication	1608
β-lactamase coding sequence	3226–2366



pQE-60

Eco RI/BSS Nco I Bam HI Bgl II 6xHis Hind III t_0

CCATGGGCAGGATCCATGATCT TAAGCTTAATTAGCTGAG