CHAPTER II

MATERIALS AND METHODS

2.1 Equipment

-20 °C Freezer

-80 °C Freezer (Revco)

ACTA FPLC (Amersham)

Autoclave Model # LS-2D (Rex all industries Co. Ltd.)

Automatic micropipette P10, P20, P100, P200, and P1000 (Gilson Medical Electrical S.A.)

Balance: Satorius 1702 (Scientific Promotion Co.)

Biochiller 2000 (Fotodyne)

Bio-Dot® microfiltration apparatus (Bio-RAD)

Flexi-dry FTS system (USA)

Fraction collector: FRAC-200 (Pharmacia)

Gene Pulser (Bio-RAD)

GS Gene LinkerTM : UV Chamber (Bio-RAD Laboratories)

Gyrotory water bath shaker Model # G76 (New Brunswick Scientific)

Hitrap chelating 1 ml (Amersham)

Hoefer Mini VE Vertical Electrophoresis System (Amersham Pharmacia)

Hybridization oven (Hybrid)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

Larminar flow: Dwyer Mark II Model # 25 (Dwyer instruments)

Light microscope (Olympus)

Microcentrifuge tube 0.5 and 1.5 ml (Bio-RAD Laboratories)

Minicentrifuge (Costar)

Nipro disposable syringe (Nissho)

Orbital Shaker (Gallenkamp)

PCR thermal cycler: Gene Amp PCR System 2400 (Perkin Elmer)

PCR thin wall microcentrifuge tube 0.2 ml (Perkin Elmer)

Pharmacia LKB-Pump P-l (Pharmacia)

pH meter Model # SA720 (Orion)

Pipette boy ACU (Integra biosciences)

Pipette tips 10, 20, 200, and 1000 µl (Bio-RAD Laboratories)

Power supply: Power PAC 3000 (Bio-RAD Laboratories)

Refrigerated centrifuge Model # J2-21 (Beckman)

Refrigerated micro centrifuge Kubota 1300 (Kubota)

Rocker platform (Belleo)

Sephacryl S-100 HR (Amersham)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Sterile disposable plastic pipettes 1, 5, and 10 ml (Sterilin)

Stirring hot plate (Fisher Scientific)

Tissue culture dish 35 mm (Iwaki)

Tissue culture flask 25, 150, and 225 cm2 (Iwaki)

Touch mixer Model # 232 (Fisher Scientific, USA)

Transilluminator 2011 Macrovue (LKB)

Vacuum blotter Model # 785 (Bio-RAD Laboratories)

Vacuum pump (Bio-RAD Laboratories)

2.2 Chemical reagents

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide, C_3H_5NO (Merck)

Agarose (Sekem)

Ammonium persulfate, $(NH_4)_2S_20_8$ (USB)

Bacto agar (Difco)

Bacto tryptone (Merck)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (Merck)

Bromophenol blue (BDH)

Chloroform, $CHCl_3$ (Merck)

Coomassie brilliant blue R-250, $C_{45}H_{44}N_3O_7S_2Na$ (Sigma)

100 mM dATP, dCTP, dGTP, and dTTP (Promega)

Diaminobenzidine (Sigma)

Dimethyl sulfoxide (DMSO), C_6H_6SO (Amresco)

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid (EDTA), disodium salt dihydrate (Fluka)

Ficoll™ -400 (Amersham)

Fetal bovine serum (Gibco BRL)

Formaldehyde, CH_2O (BDH)

GeneAmp PCR core reagent (Perkin Elmer)

: 10 x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl)

: 25 mM $MgCl_2$

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine NH_2CH_2COOH (Scharlau)

Grace insect medium (Gibco BRL)

Hydrochloric acid, HCl (Merck)

Isoamylalcohol, $C_5H_{12}O$ (Merck)

Isopropanol, C_3H_7OH (Merck)

Kodak tri-X pan400 film

Liquid nitrogen (TIG)

Lysozyme (Sigma)

Methanol, CH_3OH (Merck)

2-mercaptoethanol, C_2H_6OS (Fluka)

0.22 μm millipore membrane filter (Millipore)

N, N'-methylene-bisacrylamide, $C_7H_{10}N_2O_2$ (USB)

N, N, N', N'-tetramethylethylenediamine (TEMED) (BDH)

Neutral red (Fluka)

Nytrans® super charge nylon membrane (Schleicher & Schuell)

Phenol crystals, C_6H_5OH (Carlo Erba)

Potassium chloride, KCl (Merck)

Prestained molecular weight marker (Fermentus)

SeaPlaque GTG agarose (FMC Bioproducts)

Sephadex G-50 (Pharmacia)

Sodium acetate, CH_3COONa (Carlo Erba)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

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จุฬาลงกรณ์มหาวิทยาลัย

Sodium dodecyl sulfate, $C_{12}H_{25}O_4SNa$ (Sigma)

Sodium hydrogen carbonate, $NaHCO_3$ (BDH)

Sodium hydroxide, NaOH (Eka Nobel)

TC-1OO insect medium (Gibco BRL)

Tris-(hydroxy methyl)-aminomethane, $NH_2C(CH2OH)_3$ (USB)

Tryptic soy broth (Difco)

Tween™-20 (Fluka)

Unstained molecular weight marker (Owl separation systems)

Whatman 3 MM™ filter paper (Whatman)

2.3 Enzymes

Eco RI (Biolabs)

Pme I (Biolabs)

RNase A (Sigma)

Taq DNA polymerase (Pharmaeia)

T4 DNA ligase (Gibeo BRL)

Xba I (Biolabs)

Xho I (Biolabs)

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2.4 Bacterial strains

Escherichia coli strain JM 109

E. coli strain XL-I blue

Staphylococcus aureus

Micrococcus luteus

Vibrio harveyi 1526

2.5 Expression of 6XHis Tag anti-LPS factor in the baculovirus expression system

2.5.1 Preparative production of recombinant proteins

The two types of recombinant viruses, full length and NH_2-terminal truncated anti-LPS factor from previous study (Anurakolan Thesis, 2001), were used to produce recombinant proteins. The 70-80 % confluent monolayer of Sf9 cells in 150 cm^2 tissue culture (TC-150) flask were washed with serum free insect medium and infected with 100 µl of each the recombinant baculoviruses. Incubation was allowed to proceed for 1 hour, mixed gently by rocking the flask every 15 minutes, and was followed by addition of 25-30 ml TC-100/10% (v/v) FBS. The Sf9 cell monolayer was incubated at 27 °C for 60-72 hours. During incubation, the cells were checked for signs of infection 2-3 days after inoculation under the light microscope. The infected Sf9 cells should enlarged in size (about 2 fold) and large nucleus should be visible. The infected cells and the supernatant were harvested at 60-72 hours post infection (hpi) by scraping cells with scraper and centrifugation at 3000 rpm for 10 minutes. The cell pellet was stored at -20 °C. The infected cell pellet and protein pellet were divided and resuspended in 1x sample loading buffer (50 mM Tris-HCI, pH 6.8, 2 % (w/v) SOS, 0.1 % (w/v) bromophenol blue, 10 % (v/v) glycerol, and 14.4 mM 2-mercaptoethanol) to analyze by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.5.2 Analysis of recombinant protein by SDS-PAGE

The SDS-PAGE system was performed according to the method of *Bollag et al.* The slab gel (10 x 10 x 0.75 cm) system consisted of 0.1% (w/v) SDS in 15 % (w/v) separating gel and 5 % (w/v) stacking gel. Tris-glycine buffer, pH 8.3 containing 0.1% (w/v) SDS was used as electrode buffer. The gel preparation was described in Appendix A. The cells pellet of recombinant proteins, histidine tag full length and histidine tag NH_2-terminal truncated anti-LPS factor proteins, was treated with sample buffer and boiled for 10 minutes before loading to the gel. The electrophoresis was performed at constant current

of 20 mA per slab at room temperature from cathode towards anode. The gel was stained with Coomassie gel stain solution (0.1 % (w/v) Coomassie brilliant blue R-250, 45 % (v/v) methanol, and 10 % (v/v) glacial acetic acid) at room temperature for at least 4 hours with gently shaking. After staining, the gel was destained by soaking in Coomassie gel destain solution (10 % (v/v) methanol and 10% (v/v) glacial acetic acid) with gently shaking and changed destain solution three or four times for 24 hours.

2.5.3 Detection of recombinant protein by Western blot

The two types of recombinant proteins, full length and NH_2-terminal truncated anti-LPS factor proteins, were separated on 15 % SDS-PAGE gels as describe in section 2.5.2. Remove gel from slab and incubated for 10 min in tank-blotting buffer (25 mM Tris, 150 mM glycine and 20% methanol). Soak filter paper and membrane in tank-blotting buffer. Avoiding air bubbles, place 4 sheets of filter paper on the fiber pad, followed by the gel, the membrane, 4 sheets of filter paper, and finally the other fiber pad (Figure 2.1).

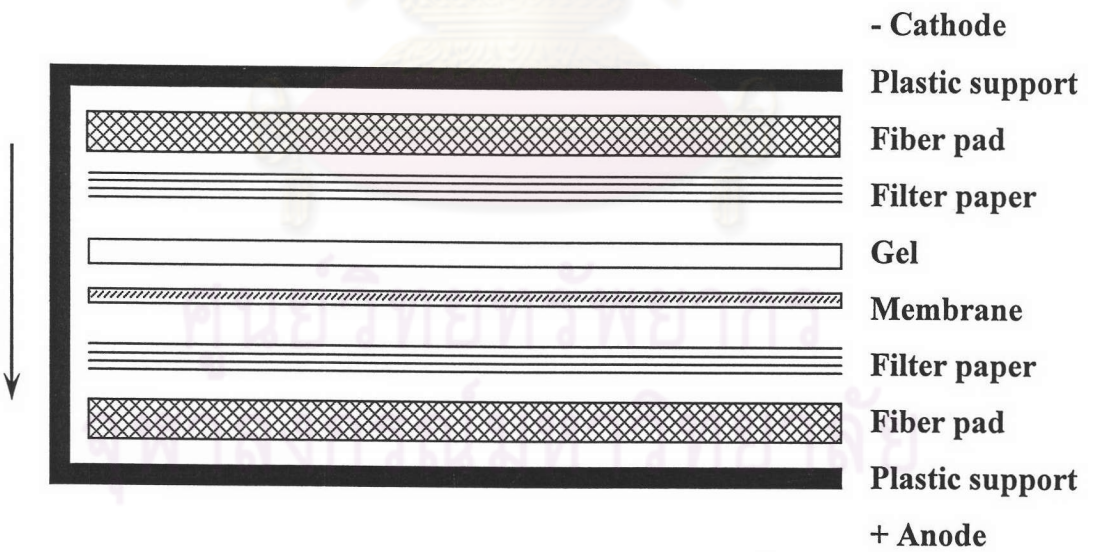

Figure 2.1 Western transfer cassette

Place a cassette in the chamber, protein transfer was performed at constant voltage of 60 V at 4°C from cathode towards anode. After transfer, mark the orientation of the gel on the membrane and washed membrane twice for 10 minutes each time with TBS (10 mM Tris-Cl, 150 mM NaCl, pH 7.5) buffer at

room temperature and then incubate for 1 hour in blocking buffer (3% (w/v) BSA in TBS buffer) at room temperature. Wash membrane twice for 10 min each time in TBS-Tween/triton buffer (20 mM Tris-Cl, 500 mM NaCl, 0.05% (v/v) Tween 20, 0.2% (v/v) Triton X-100, pH 7.5 and incubate in Anti-His antibody 1/1500 dilution diluted in blocking buffer at room temperature for 1 hour and then wash membrane twice for 10 min each time in TBS-Tween/triton buffer at room temperature and wash again for 10 min with TBS buffer at room temperature. After that incubate with secondary antibody solution 1/10000 dilution diluted blocking buffer for 1 hour at room temperature. Wash four times for 10 minutes each time in TBS-Tween/triton buffer at room temperature and stain with HRP staining solution [30 mg diaminobenzidine (DAB) Dissolve in 30 ml Tris-saline (9% (w/v) NaCl in 1 M Tris-Cl, pH 8.0) followed by 60 µl 30% hydrogen peroxide (H_2O_2)] until the signal is clearly visible (approximately 5-15 min). Stop the chromogenic reaction by rinsing the membrane twice with water.

2.5.4 Analysis of Anti-LPS factor gene by dot blot

Assemble of the Bio-dot apparatus and connect the vacuum pump with outlet port. The nylon membrane was soaked in 6x SSC solution (90 mM sodium citrate buffer with 0.9 M NaCl pH 7.0) to pre wet. Place membrane on gasket and remove trapped air bubble. Cover membrane with sample template and tighten the four screws. Apply 100 µl buffers to all 96 sample wells. Gently removed the buffer from the wells by vacuum and adjust flow valve to atmospheric pressure. NH_2-terminal truncated diluted virus stock from 10^{-1} to 10^{-12} was loaded to sample wells and applied a gentle vacuum. The membrane was immersed in 0.1 N NaOH 10 minutes and washed with 3x SSC 10 minutes then with 2x SCC 10 minutes. The membrane was dried on filter paper and baked at 80 °C 30 minutes then cross linked in UV chamber. Membrane was pre-hybridized in hybridization solution (QuikHyb® Hybridization solution) with 1% BSA at 37 °C 30 minutes. The labeled probe, which was anti-LPS gene prepared by ramdom prime labeling system, was denatured by heating for

5 minutes in a boiling water bath and quickly cooled on ice for 5 minutes. The denature labeled probe was spun briefly, then added to the hybridization solution and further incubated at 37 °C with gently shaking 2 hours. After hybridization step, the hybridization solution was removed and the membrane was washed with 1x SSC containing 0.1 % (w/v) SDS and 0.5x SSC containing 0.1 % (w/v) SDS, respectively at 60 °C for 15 minutes. The membrane was then drained off of the excess detection reagent, wrapped in a plastic bag and gentle smooth out air pockets, then it was subjected to autoradiography.

2.6 Expression of 6X His Tag NH_2-terminal truncated anti-LPS factor in the yeast (*Pichia*) expression system

2.6.1 Construct of transfer vector

2.6.1.1 Preparation of 6X His Tag NH_2-terminal truncated anti-LPS factor gene

5'-terminal truncated with 6 histidine gene (HΔNAL) was constructed by polymerase chain reaction (PCR) using p6XHAL7 as a template, which had 6 histidine residue at NH_2 terminal, and oligonucleotide primers incorporating 5' *Eco* RI (5'GCGAATTCATGTCCCCTATAGATC -3') and 3' *Xba* I (5'CGTCTAGAAAAAGGCCTATGAGC -3') cleavage sites. The amplification reaction was prepared in a 25 µl reaction volume containing 50 ng of DNA template, 1.25 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 1x PCR buffer, 1.2 mM $MgCl_2$, 5 pmole of each primers, and 1 unit of *Taq* DNA polymerase (5 units/µl). Sterile distilled water was added to make the final volume to 25 µl. The amplification program of HΔNAL gene was initially denatured at 94 °C for 3 minutes followed by 35 cycles of 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1 minute. The final extension was carried out at 72 °C for 10 minutes. The resulting PCR product was ran on 1.5 % agarose gel to determine whether the DNA fragment was successfully amplified. The size of the DNA product from the amplification was a 442 bp DNA fragment.

2.6.1.2 Restriction enzyme digestion

The amplified HΔNAL fragment gene and plasmid pPICZαA (Appendix D) was digested with restriction enzymes *Eco* RI and *Xba* I. The reaction mixture was plasmid pPICZαA or amplified HΔNAL fragment gene 1 μg, 10x BSA 2 μl, 10x *Eco* RI buffer 2 μl, restriction enzymes *Eco* RI and *Xba* I 1 μl (5 units/μl). Steriled distilled water was added to make the final volume up to 20 μl.

2.6.1.3 Ligation

The mixture of sticky-end ligation must contained a suitable molecular ratio between vector and DNA insert which is usually 1:3. The 15 μl of ligation reaction was composed of 1.5 μl of 10x T4 DNA ligase buffer, 1 μl of 10 mM ATP, 1 μl of T4 DNA ligase (3 units/μl), 150 ng of DNA insert, and 50 ng pPICZαA/*Eco* RI/*Xba* I. Steriled distilled water was added to make the final volume up to 15 μl. The mixture was mixed, quick spun for 30 seconds and incubated at 16 °C overnight.

2.6.1.4Competent cells preparation

A single colony of *E. coli* JM-109 was cultured used as the starter inoculums in 10 ml of LB broth [1 % (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1% (w/v) NaCl] and incubated at 37°C with shaking for overnight. One percent of the microbial starter was inoculated into 1000 ml of LB broth [1 % (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1 % (w/v) NaCl] and the culture was incubated at 37 °C with vigorous shaking for 3-5 hours until the optical density at 600 nm (OD_{600}) of the cells reached 0.5-0.8. Cells were then chilled on ice for 15-30 minutes and harvested by centrifugation at 5000 rpm for 10 minutes at 4°C. The supernatant was removed as much as possible. The cell pellet was washed by resuspending in a total of 1000 ml of cold steriled water, gently mixing and centrifugation. The pellet was washed further with different kinds of solution, first with 500 ml of cold steriled water, followed with 20 ml of ice cold steriled 10% (v/v) glycerol, and resuspended to

a final volume of 2-3 ml ice cold steriled 10 % (v/v) glycerol. This cell suspension was divided into 40 µl aliquots and stored at -80 °C until used. The cells are good for at least 6 months under these conditions.

2.6.1.5 Electrotransformation

The competent cells were gently thawed at room temperature and then immediately placed on ice. Forty microlitres of the cell suspension was mixed with 1 µl of ligation mixture, mixed well and placed on ice for approximately 1 minute. The mixture of cell and DNA was transformed by electroporation in a cold 0.2 cm cuvette with setting the apparatus as follows; 25 µF of the Gene pulser, 200 Ω of the Pulse controller unit, and 2.50 kV of the Gene pulser apparatus (Bio-RAD). After one pulse was applied at the above setting, the cells were immediately resuspended with 1 ml of SOC medium [2 % (w/v) bacto tryptone, 0.5 % (w/v) bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgS0_4$, and 20 mM glucose] and transferred to test tube. The cell suspension was incubated at 37°C with shaking at 250 rpm for 1 hour. The content was spread on the low salt-LB agar plates [1 % (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 0.5 % (w/v) NaCl and 1.5% bacto agar], which contained 50 mg/ml zeocin, and incubated at 37°C overnight. After incubation, colonies were selected randomly for plasmid DNA isolation.

2.6.1.6 Plasmid preparation

The colonies were inoculated into 1.5 ml of low salt-LB broth (one colony per tube) and incubated at 37°C with shaking overnight. The cultures were transferred into 1.5 ml microcentrifuge tube and spun at 8000 rpm for 1 minute. The supernatant was discarded, 100 µl of solution I [25 mM Tris-HC1,pH 8.0, 10mM EDTA, pH 8.0, 50 mM glucose, and 0.5 % (w/v) lysozyme] was added, mixed by vigorous vortexing and placed on ice for 30-60 minutes. Two hundred microlitres of freshly prepared solution II [0.2 N NaOH and 1 % (w/v) SDS] was added for cell lysis and DNA denaturation and mixed gently. After incubating on ice for 10 minutes, the mixture was added, with 150 µl of

solution III (3 M sodium acetate, pH 4.8) for renaturation, mixed gently and placed on ice for 30 minutes. The tube was spun at 10000 rpm for 10 minutes to separate cell debris. The supernatant was transferred into a new microcentrifuge tube, added with an equal volume of Phenol : Chloroform : Isoamyl alcohol (25 : 24 : 1), mixed and spun at 10000 rpm for 10 minutes. The upper aqueous phase was transferred to a new tube. The plasmid DNA was precipitated by adding 2 volumes of absolute ethanol then mixed well and kept at -80°C for at least 1 hour. The mixture was centrifuged at 10000 rpm for 10 minutes. The plasmid DNA was washed with 70 % (v/v) ethanol, air-dried, and then dissolved in 50 µl of TE buffer (10 mM Tris-HC1,pH 8.0 and 1mM EDTA, pH 8.0).

2.6.1.7 Detection of desired recombinant plasmid

Transfer vector pHΔNAL was detected for corrected insertion by digested with restriction enzyme *Eco* RI and *Xba* I and subject to agarose gel electrophoresis. The size of DNA fragment was compared with amplified gene HΔNAL and standard DNA ladder (100 bp marker, Biolabs). The selected Transfer vector pHΔNAL was sequenced for checking gene correction.

2.6.2 Transformation of recombinant plasmid with anti-LPS factor gene into yeast cells

2.6.2.1 Recombinant plasmid preparation

The colonies with pHΔNAL were inoculated into 1.5 ml of LB broth (one colony per tube) and incubated at 37°C with shaking overnight. The cultures were transferred into 1.5 ml microcentrifuge tube and spun at 8000 rpm for 1 minute. The bacterial cell pellet was resuspended in 250 µl of buffer PI and mixed by vortexing. Two hundred and fifty microlitres of buffer P2 was added and mixed gently. Then 350 µl of buffer N3 was added, mixed gently by immediately inverted the tube for 4-6 times, and centrifuged at 12000 rpm for 10 minutes. During centrifugation, QIAprep spin column was placed in a 2 ml collection tube. The supernatant was transferred into QIAprep spin column, centrifuged at 10000 rpm for 1 minute, and discarded the flow-through.

QIAprep spin column was washed with different buffer containing 500 µl of buffer PB and 750 µl of buffer PE, respectively, centrifuged at 10000 rpm for 1 minute and discarded the flow-through. QIAprep spin column was removed residual washed buffer by additional centrifuged at 12000 rpm for 1 minute and then placed the QIAprep spin column into a new 1.5 ml microcentrifuge tube. The plasmid DNA was eluted by adding 50 µl of buffer EB (10 mM Tris-Cl. PH 8.5) or steriled distilled water to the center of QIAprep column, incubated at room temperature for 5 minutes, and centrifuged at 12000 rpm for 1 minute. The extracted pHΔNAL was linearlized by digested with restriction enzyme *Pme* I.

2.6.2.2 Competent cells yeast preparation

A single colony of *Pichia pastoris* KM-71 was cultured and used as the starter 5 ml in YPD [2 % (w/v) peptone, 1 % (w/v) bacto yeast extract and 2% glocose] in a 50 ml conical at 30°C with shaking for overnight. Inoculate 0.1-0.5 ml of the overnight culture in 500 ml of fresh medium used a 2 liter flask. Grow overnight again to an optical density at 600 nm (OD_{600}) reached 1.3-1.5. Cells were then chilled on ice for 15-30 minutes and harvested by centrifugation at 3500 rpm for 5 minutes at 4°C. The supernatant was removed as much as possible. The cell pellet was washed by resuspending in a total of 1000 ml of cold steriled water, gently mixing and centrifugation. The pellet was washed further with 500 ml of cold steriled water, followed with 20 ml of ice cold steriled 1 M sorbitol, and resuspended to a final volume of 2-3 ml ice cold steriled 1 M sorbitol. This cell suspension was divided into 80 µl aliquots and stored at -80 °C until used.

2.6.2.3 Electrotransformation

The competent cells were gently thawed at room temperature and then immediately placed on ice. Eighty microlitres of the cell suspension was mixed with 5-20 µg of linearlized pHΔNAL/*Pme* I, mixed well and placed on ice for approximately 5 minute. The mixture of cell and DNA was transformed by

electroporation in a cold 0.2 cm cuvette with setting the apparatus as follows; 25 μF of the Gene pulser, 200 Ω of the Pulse controller unit, and 1.50 kV of the Gene pulser apparatus (Bio-RAD). After one pulse was applied at the above setting, the cells were immediately resuspended with 1 ml of ice cold steriled 1 M sorbitol and transferred to test tube. The cell suspension was spread 200-600 μl on the YPD agar plates and incubated at 30°C overnight until colonies appear. After incubation, colonies were selected randomly for colonies PCR

2.6.2.4 Detection of desired recombinant plasmid by colony PCR

The PCR reaction using yeast genomic DNA as a template and 5' AOX 1 and 3' AOX 1 primers. The first step was prepare yeast genomic DNA by picked a single colony and resuspend in 10 μl of water. Then add 5 μl of a 5 U/μl solution of lyticase and incubate at 30°C for 10 minutes and freeze the sample at -80°C for 10 minutes or immerse in liquid nitrogen for 1 minute. The amplification reaction was prepared in a 50 μl reaction volume containing 5 μl of cell lysate, 25 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 1x PCR buffer, 2.5 mM $MgCl_2$ and 10 pmole/μl of each primers. Sterile distilled water was added to make the final volume to 45 μl. The amplification program was initially denatured at 95 °C for 5 minutes. Add 5 μl of a 0.16 U/μl solution of *Taq* DNA polymerase (0.8 units) followed by 30 cycles of 95 °C for 1 minute, 54 °C for 1 minute, and 72 °C for 1 minute. The final extension was carried out at 72 °C for 7 minutes. The resulting PCR product was ran on 1.2 % agarose gel to determine whether the DNA fragment was successfully amplified. The size of the DNA product from the amplification was a 1069 bp, which is alcohol oxidase (AOX) gene and α factor signal peptide with HΔNAL gene, DNA fragment.

2.6.3 Expression of recombinant yeasts

2.6.3.1 Screening for more expressed clone

Inoculated each single colony of selected clone in 100 ml of BMGY medium [1% (w/v) bacto yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, 1.34% yeast nitrogen base, $4x10^{-5}$% (w/v) biotin and 1% (v/v) glycerol] in a 1 liter baffled flask. Grow at 30°C in a shaking incubator (300 rpm) until the culture reaches an OD600 about 2-6 (approximately 16-18 hours). Harvest the cells by centrifuged at 1500 g for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend cell pellet in 10 ml BMMY medium [1% (w/v) bacto yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, 1.34% yeast nitrogen base, $4x10^{-5}$% (w/v) biotin and 0.5% (v/v) methanol]. Place suspension in a 100 ml baffled flask and return to incubator to continue to grow. Add 100% methanol to a final concentration of 0.5% every 24 hours to maintain induction. At every 24 hours for 6 days transfer 1ml of the expression culture to a 1.5 ml microcentrifuge tube. Centrifuge at 10000 rpm in a microcentrifuge for 2-3 minutes at .roomtemperature. Transfer the supernatant to a separate tube and store the supernatant at -80°C until ready to assay. Analyze the cell pellets for protein expression by Tricine-SDS- PAGE. These samples are used to analyze expression levels and determine the optimal time post-induction to harvest.

2.6.3.2 Analysis of recombinant anti-LPS factor by Tricine-SDS- PAGE

The Tricine-SDS-PAGE system was performed according to the method of *Schägger et al.* The slab gel (10 x 10 x 0.75 cm) system consisted of 0.1% (w/v) SDS in 12 % (w/v) separating gel and 4 % (w/v) stacking gel. Cathode buffer was 0.1 M Tris-HCl, pH 8.45 containing 0.1% (w/v) SDS and 0.1 M tricine and anode buffer was 0.2 M Tris-HCl, pH 8.9. The gel preparation was described in Appendix B. The supernatant of expressed proteins, histidine tag NH_2-tenmnal truncated anti-LPS factor proteins, was treated with sample buffer and boiled for 10 minutes before loading to the gel. The electrophoresis was

started at constant voltage of 30 V about 1 hour and then raised to 100 V when the sample had completely left the sample pocket. The gel was stained by silver staining method described in Appendix C.

2.6.3.3 Analysis of recombinant anti-LPS factor by antibacterial activity

The crude protein samples were tested for their antibacterial activity against bacteria including the Gram-negative *Vibrio harveyi* 1526 and *Escherichia coli* XL1 blue and the Gram-positive *Staphylococcus aureus* and *Micrococcus luteus.* Inhibitory activity was determined by the assay on bacterial growth rate. *E. coli* XL1 blue, *Micrococcus luteus* and S. *aureus* were grown on LB broth medium at 37 °C overnight and *V. harveyi* 1526 was grown on LM broth medium [1 % (w/v) bacto tryptone, 0.5 % (w/v) bacto yeast extract, and 2 % (w/v) NaCl] at 30 °C overnight. The overnight cultured were diluted with poor broth [1 % (w/v) bacto tryptone and 0.5 % (w/v) NaCl, pH 7.4] while *V. harveyi* in poor broth with 2% NaCl to a final optical density at 600 nm of 0.001. The 100 µl of each suspension were subjected into microtiter plate. Then, the 2, 4, 6, 8 and 10 µl of crude protein samples were added and make a final volume to 110 µl with 20 mM sodium phosphate buffer pH 7.4. The microtiter plate was incubated at 30 °C with shaking. After 18 hours incubation, the bacterial was measured optical density at 600 nm.

2.7 Purification of anti-LPS factor from recombinant yeasts

2.7.1 Preparative production of recombinant anti-LPS factor

Inoculated each single colony of selected clone in 100 ml of BMGY medium in a 1 liter baffled flask. Grew at 30°C in a shaking incubator (300 rpm) until the culture reaches an OD600 about 2-6 (approximately 16-18 hours). The cells were harvested by centrifuged at 1500 g for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspended cell pellet in 10 ml BMMY medium. Place suspension in a 100 ml baffled flask and return to incubator to continue to grow. Add 100% methanol to a final concentration of

0.5% every 24 hours to maintain induction. For 6 days harvest all expression culture and centrifuge at 10000 rpm. Transfer the supernatant to a separate tube and store the supernatant at -80°C.

2.7.3 Affinity column chromatography

The purification was performed by use Hitrap chelating 1 ml column, which bind specifically with histidine amino acid, and ACTA FPLC (amersham). The supernatant from expression culture was dialysed against buffer A (20 mM sodium phosphate buffer with 0.5 M NaCl, pH 7.4) and filtrated through 0.45 μm column filter. The Hitrap chelating 1 ml column was charged with $NiSO_4$ before equilibrated with buffer A at flow rate 1 ml/min. The sample was applied and the unbound proteins were eluded from the column with buffer A (Keep washing until the absorbance at 280 nm decreased to a low and constant value). Next, the bound proteins were eluded from column with imidazole in buffer B (20 mM sodium phosphate buffer, 0.5 M NaCl and 0.5 M imidazloe, pH 7.4). The elution peak were pooled and dialysed, which concentrated by lyophilized to reduce volume for further purification step.

2.7.4 Sephacryl S-100 HR column chromatography

Sephacryl S-100 HR column, was washed with deionized water and then equilibrated with the same buffer as previous buffer A at flow rate 0.5 ml/min. The bound protein from Hitrap column was concentrated and applied to the column. The elution profile was monitored for protein by measuring the absorbance at 280 nm. All elution peaks were pooled then dialyzed and lyophilized respectively.

The protein from each step of purification were analyzed by Tricine-SDS-PAGE as described in section 2.6.3.2 and test for antibacterial activity as described in section 2.6.3.3.