CHAPTER II
MATERIALS AND METHODS

2.1 Equipments

Autoclave (MLS-3020, SANYO electric Co., Ltd., Japan)

Autopipette (Pipetman, Gilson, France)

Benchtop centrifuge (Sorvall Biofuge Primo, Kendro Laboratory Products L.P., USA)

Bioreactor (BioFlo IIc, New Brunswick Scientific, USA)

Electrophoresis unit (Gelmate 2000, TOYOBO Co., Ltd., Japan)

Electroporator (MicroPulser™ electroporator, Bio-Rad Laboratories, Inc., USA)

Gel Doc (BioDoc-It® Imaging System with M-20 UV Transilluminator, UVP®, Inc., USA)

High Performance Liquid Chromatography (CLASS-VP 10A*vp,* SHIMADZU, Japan)

Magnetic hotplate stirrer (CH-1E, Nickel Electro-Clifton, UK)

Microwave oven (TRX1500, Turbora International Co., Ltd., Korea)

pH electrode (model no. 405-DPAS-SC-K8S/225, Mettler-Toledo, Switzerland)

pH meter (S20-K SevenEasy™, Mettler-Toledo, Switzerland)

Polarographic electrode (InPro® 6800 Series O_2 Sensors, Mettler-Toledo, Switzerland)

Power supply (POWER PAC 300, Bio-Rad, USA)

Scanner (CanoSan LiDE 100 Color Image Scanner, Canon, Thailand)

Refrigerated centrifuge (Avanti J-30I High-Performance Centrifuge, Beckman Coulter, Inc., USA)

Reverse phase column chromatography (CROWNPAK CR(+) column, 150 mm x 4 mm ID, Daicel Chemical Industries, Ltd., Japan)

Shaking incubator (Excella E24R, New Brunswick Scientific, USA)

Solvent filtration apparatus (Part no. 0210-0005, 1 L flask 40/35, 300 mL glass funnel, fritted glass support base 40/35, 47 mm aluminum clamp, Vertical Chromatography Co., Ltd., Thailand)

Sonicator (Vibra cell^{Tm}, SONICS & MATERIALS, Inc., USA)

Spectrophotometer (Beckman DU 530, Beckman Coulter, Inc., USA)

Spectrophotometer (DU Series 650, Beckman Instrument Inc., USA)

Thermoblock Reactor (MD-01-220, Major Science, USA)

Thermo cycler (Mastercycler® Family, Eppendorf, Germany)

UV Transilluminator (MacroVue™ UV-25, Hoefer Inc., USA)

Vacuum/pressure pump (Model number. WP6111560, Millipore Inc., USA)

Vortex shaker (Topmix FB15024, Thermo Fisher Scientific Inc., USA)

Waterbath (WB14, Memmert, Germany)

2.2 Disposable materials

Membrane filter (GSWG047S6, 0.22 μm, 47 mm, white gridded, sterile, mixed cellulose esters, Millipore Inc., USA)

Membrane filter (Midisart 2000, 0.22 μm, 64 mm, PTFE, sterile, Sartorius Stedim Biotech S.A., Germany)

Membrane filter (NYLON Membrane filters, 0.45 μm, 47 mm, Vertical Chromatography Co., Ltd., Thailand)

Microcentrifuge tube (1.5 mL microcentrifuge tube, MCT-150, Axygen Inc., USA)

PCR tube (0.2 mL thin-wall domed-cap PCR tube, PCR-02D-C, Axygen Inc., USA)

Pipette tip (10 μL, 200 μL and 1000 μL pipette tip, Axygen Inc., USA)

Syringe (3 mL, 5 mL and 20 mL disposable syringe, Nissho Nipro Co., Ltd., Japan)

Syringe filter (0.2 μm, 13 mm, VertiPure^{TM} PTFE, Vertical Chromatography Co., Ltd., Thailand)

2.3 Markers

Lambda DNA/*Hin*dIII Marker (#SM0103, Fermentas Inc., USA)

Protein molecular weight marker (#SM0431, Fermentas Inc., USA)

100 bp DNA marker (#SM0334, Fermentas Inc., USA)

2.4 Kits

Gel/PCR DNA fragment extraction kit (DF300, Geneaid, Biotech Ltd, Taiwan)

High-speed plasmid mini kit (PD300, Geneaid, Biotech Ltd, Taiwan)

2.5 Chemicals

Acetylacetone (Carlo Erba, Italy)

Acrylamide (Sigma, USA)

Agar, Bacteriological grade (Criterion, USA)

Agarose (FMC Bioproducts, USA)

Ammonium persulphate (Sigma, USA)

Ammonium sulphate (Carlo Erba, Italy)

Ampicillin sodium salt (US Biological, UK)

Bovine serum albumin (Sigma, USA)

Bromphenol blue (Merck, Germany)

Chloroform (Lab Scan, Thailand)

Coomassie brilliant blue R-250 (Sigma, USA)

Folin-Ciocalteu phenol reagent (Carlo Erba, Italy)

Ethidium bromide (Sigma, USA)

Ethylenediaminetetraacetic acid disodium salt, EDTA (Merck, Germany)

Glycerol (Ajax Finechem, Australia)

Isopropyl-β-D-thiogalactopyranoside (IPTG), Dioxane Free (US Biological, UK)

β-mercaptoethanol (Fluka, Switzerland)

Methanol, HPLC grade (Merck, Germany and LAB SCAN, Thailand)

N, N'-methylene-bis-acrylamide (Sigma, USA)

β-Nicotinamide adenine dinucleotide, NAD^+ (Sigma, USA)

Pancreatic digest of casein (Criterion, USA)

Phenol (BDH, UK)

L-phenylalanine (Sigma, USA)

Phenylmethylsulfonyl fluoride, PMSF (Sigma, USA)

Potassium hydroxide (Ajax Finechem, Australia)

Silicon antifoam agent (Ajax Finechem, Australia)

Sodium dodecyl sulfate (Sigma, USA)

Sodium metaperiodate (BDH, UK)

N,N,N',N'-tetramethyl-1, 2-diaminoethane, TEMED (Carlo Erba, Italy)

Yeast extract (Scharlau, Spain)

Other common chemicals were products obtained from Sigma, USA; BDH, UK; Fluka, Switzerland; Merck, Germany; Ajax Finechem, Australia; Carlo Erba, Italy; and Lab Scan, Thailand.

2.6 Enzymes and restriction enzymes

Lysozyme (Sigma, USA)

Pfu DNA polymerase (Promega, USA)

Proteinase K (Sigma, USA)

Restriction enzymes (New England BioLabs, Inc., USA)

RNase A (Sigma, USA)

T4 DNA ligase (New England BioLabs, Inc., USA)

2.7 Bacterial strains and plasmids

The bacterial strains and plasmids used are shown in Table 2.1.

E. coli BL21(DE3) was the host strain used to overexpress all genes throughout this work. *E. coli* TOP10 was used as the source of genomic DNA for amplification of *aroB, aroL, glpF, glpK, pheA, tktA* and *yddG* genes.

pET-22b(+) (5,493 bp), containing an ampicillin resistance gene as the selection marker gene, was used as cloning vector for *aroL*, *pheA*, and *yddG* genes. pRSFDuet-1 (3,829 bp), containing a kanamycin resistance gene, was used as an expression vector. The restriction maps of these plasmids are shown in Appendix A and B.

For short-term storage, all constructed recombinant strains were maintained on Luria-Bertani (LB) agar (1% peptone from casein, 0.5% yeast extract 0.5% NaCl and 1.5% agar, pH 7.4) containing either 100 μg/mL of ampicillin or 30 μg/mL of kanamycin depending on the selective marker on plasmid. Agar plates were incubated at 37 °C for 18 h and then stored at 4 °C. The strains were subcultured once a month.

Table 2.1 *E. coli* strains and plasmids used throughout this work

	Description	**Source**
Strains		
BL21(DE3)	F⁻ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen, Merck
TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen, CA
Plasmids		
pET-17b	Expression vector with T7 promoter	Novagen, Merck
pET-22b (+)	Expression vector with T7*lac* promoter	Novagen, Merck
pRSFDuet-1	Expression vector with T7*lac* promoter containing two multiple cloning sites	Novagen, Merck
pBLPheDH	*B. lentus phedh* gene inserted under T7 promoter of pET-17b	Thongchuang, 2006
pPheDH	*B. lentus phedh* gene inserted under T7*lac* promoter of pRSFDuet-1	This study
pETAroL	*E. coli* TOP10 *aroL* gene inserted under T7*lac* promoter of pET-22b(+)	This study

(continued)

Table 2.1 *E. coli* strains and plasmids used throughout this work

	Description	**Source**
pETYddG	*E. coli* TOP10 *yddG* gene inserted under T7*lac* promoter of pET-22b(+)	This study
pETPheA	*E. coli* TOP10 *pheA* gene inserted under T7*lac* promoter of pET-22b(+)	This study
pAroL	*E. coli* TOP10 *aroL* gene inserted under T7*lac* promoter of pRSFDuet-1	This study
pYddG	*E. coli* TOP10 *yddG* gene inserted under T7*lac* promoter of pRSFDuet-1	This study
pPheA	*E. coli* TOP10 *pheA* gene inserted under T7*lac* promoter of pRSFDuet-1	This study
pAroB	*E. coli* TOP10 *aroB* gene inserted under T7*lac* promoter of pRSFDuet-1	This study
pGlpF	*E. coli* TOP10 *glpF* gene inserted under T7*lac* promoter of pRSFDuet-1	This study
pTktA	*E. coli* TOP10 *tktA* gene inserted under T7*lac* promoter of pRSFDuet-1	This study
pGlpFK	*E. coli* TOP10 *glpF* and *glpK* genes inserted under T7*lac* promoter of pRSFDuet-1	This study
pPT	Each *phedh* and *tktA* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study

(continued)

Table 2.1 *E. coli* strains and plasmids used throughout this work

	Description	**Source**
pPTF	Each *phedh, tktA* and *glpF* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pPTFK	Each *phedh, tktA*, *glpF* and *glpK* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pPTFB	Each *phedh, tktA*, *glpF* and *aroB* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pPTFBL	Each *phedh, tktA, glpF, aroB* and *aroL* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pLY	Each *aroL* and *yddG* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pLYA	Each *aroL, yddG* and *pheA* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pPTFBLY	Each *phedh, tktA*, *glpF, aroB, aroL* and *yddG* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pPTFBLYA	Each *phedh, tktA*, *glpF, aroB, aroL, yddG* and *pheA* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study

(continued)

Table 2.1 *E. coli* strains and plasmids used throughout this work

	Description	**Source**
pPY	Each *phedh* and *yddG* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pPYF	Each *phedh*, *yddG* and *glpF* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pPYFK	Each *phedh*, *yddG*, *glpF* and *glpK* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pPTY	Each *phedh*, *tktA* and *yddG* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pPTFY	Each *phedh, tktA*, *glpF* and *yddG* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pPTFKY	Each *phedh, tktA*, *glpF, glpK* and *yddG* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study
pPTFBY	Each *phedh, tktA*, *glpF, aroB* and *yddG* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	This study

For long-term storage, the glycerol stock of each strain was prepared and stored at -80 °C.

2.8 Oligonucleotides

Oligonucleotide synthesis was performed by 1st BASE, Singapore. The oligonucleotide primers used for polymerase chain reaction (PCR) amplification and DNA sequencing are shown in Table 2.2 and Table 2.3, respectively.

2.9 Subcloning of *phedh* gene to pRSFDuet-1 vector

2.9.1 Plasmid extraction

The *E. coli* BL21(DE3) harboring previously constructed pBLPheDH (4,388 bp), pET-17b (Appendix C) inserted with *B. lentus phedh* under T7 promoter, was grown in 5 mL of LB medium (1% peptone from casein, 0.5% yeast extract and 0.5% NaCl, pH 7.4) supplemented with 100 μg/mL ampicillin at 37 °C for 18 h with shaking at 250 rpm. The cell culture was collected by centrifugation at 12,000xg for 1 min. Then, the plasmid was extracted using the high-speed plasmid mini kit. The obtained plasmid solution was used as DNA template for *phedh* PCR amplification. Ultimately, DNA concentration was estimated using submarine agarose gel electrophoresis by comparing the intensity of the fluorescence of ethidium bromide-DNA complex with known amount of λ/*Hin*dIII marker.

2.9.2 Agarose gel electrophoresis

The appropriate amount (g) of agarose (normally, 0.7 or 1.0 g used) relying on the size of the DNA fragments to be separated, was added to 100 mL of electrophoresis buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) and melted in a microwave oven. The solution was cooled to about 55-50 °C before pouring into the casting tray inserted with proper comb and allowed to solidity. The DNA samples were mixed with 6x loading buffer (30% glycerol and 0.25% bromphenol blue), then loaded into the gel. Electrophoresis was run at constant voltage of 100 volts until the bromophenol blue loading dye front approached the end

Table 2.2 The oligonucleotide primers used for PCR amplification in this work

Primer	Sequence	T_m (°C)	Restriction site	Direction
phedh-*Nde*I	5′-GGA ATT CCA TAT GAG CTT AGT AGA AAA AAC ATC CAT CAT A-3′		*Nde*I	Forward
phedh-*Eco*RV	5′-ATC TTA GTT GCG AAT ATC CCA TTT TGG CTT AA-3′	58.5	Three bases of *Eco*RV	Reverse
aroL-*Nde*I	5′-GGA ATT CCA TAT GAC ACA ACC TCT TTT TCT GAT CGG-3′		*Nde*I	Forward
aroL-*Xho*I	5′-CCG CTC GAG TCA ACA ATT GAT CGT CTG TGC CAG G-3′	60.0	*Xho*I	Reverse
yddG-*Nde*I	5′-GGA ATT CCA TAT GAC ACG ACA AAA AGC AAC GCT CAT A-3′		*Nde*I	Forward
yddG-*Xho*I	5′-CCG CTC GAG TTA ACC ACG ACG TGT CGC CAG CC-3′	60.0	*Xho*I	Reverse
pheA-*Nde*I	5′-GGA ATT CCA TAT GAC ATC GGA AAA CCC GTT ACT GG-3′		*Nde*I	Forward
pheA-*Eco*RI	5′-CGG AAT TCT CAG GTT GGA TCA ACA GGC ACT ACG-3′	60.0	*Eco*RI	Reverse
T7-*Bam*HI	5′-CGC GGA TCC ACG GCC GCA TAA TCG AAA TTA ATA CG-3′		*Bam*HI	Forward
aroL-*Asc*I	5′-TTG GCG CGC CTC AAC AAT TGA TCG TCT GTG CCA GG-3′	50.0	*Asc*I	Reverse

(continued)

Table 2.2 The oligonucleotide primers used for PCR amplification in this work

Primer	Sequence	T_m (°C)	Restriction site	Direction
T7-*Asc*I	5′-TTG GCG CGC CAC GGC CGC ATA ATC GAA ATT AAT ACG-3′	50.0	*Asc*I	Forward
yddG-*Hin*dIII	5′-CCC AAG CTT TTA ACC ACG ACG TGT CGC CAG CC-3′		*Hin*dIII	Reverse
T7-*Hin*dIII	5′-CCC AAG CTT CGA TCC CGC GAA ATT AAT ACG ACT C-3′	50.0	*Hin*dIII	Forward
pheA-*Afl*II	5′-CCC CTT AAG TCA GGT TGG ATC AAC AGG CAC TAC G-3′		*Afl*II	Reverse
aroB-*Nco*I	5′-CAT GCC ATG GAG AGG ATT GTC GTT ACT CTC G-3′	58.5	*Nco*I	Forward
aroB-*Bam*HI	5′-CGG GAT CCT TAC GCT GAT TGA CAA TCG GCA ATG G-3′		*Bam*HI	Reverse
tktA-*Nde*I	5′-GGA ATT CCA TAT GTC CTC ACG TAA AGA GCT TGC C-3′	58.5	*Nde*I	Forward
tktA-*Xho*I	5′-CCG CTC GAG TTA CAG CAG TTC TTT TGC TTT CGC AA-3′		*Xho*I	Reverse
glpF-*Nde*I	5′-GGA ATT CCA TAT GAG TCA AAC ATC AAC CTT GAA AGG-3′	49.5	*Nde*I	Forward
glpF-*Pac*I	5′-CCT TAA TTA ATT ACA GCG AAG CTT TTT GTT CTG AAG G-3′		*Pac*I	Reverse
glpF-*Nde*I	5′-GGA ATT CCA TAT GAG TCA AAC ATC AAC CTT GAA AGG-3′	50.0	*Nde*I	Forward
glpK-*Pac*I	5′-CCT TAA TTA ATT ATT CGT CGT GTT CTT CCC ACG CC-3′		*Pac*I	Reverse

(continued)

Table 2.2 The oligonucleotide primers used for PCR amplification in this work

Primer	Sequence	T_m (°C)	Restriction site	Direction
T7-*Eco*RV	5′-ATC ACG GCC GCA TAA TCG AAA TTA ATA CG-3′	64.6	Three bases of *Eco*RV	Forward
tktA-*Xho*I	5′-CCG CTC GAG TTA CAG CAG TTC TTT TGC TTT CGC AA-3′		*Xho*I	Reverse
T7-*Xho*I	5′-CCG CTC GAG ACG GCC GCA TAA TCG AAA TTA ATA CG-3′	48.5	*Xho*I	Forward
glpF-*Pac*I	5′-CCT TAA TTA ATT ACA GCG AAG CTT TTT GTT CTG AAG G-3′		*Pac*I	Reverse
T7-*Xho*I	5′-CCG CTC GAG ACG GCC GCA TAA TCG AAA TTA ATA CG-3′	55.0	*Xho*I	Forward
glpK-*Pac*I	5′-CCT TAA TTA ATT ATT CGT CGT GTT CTT CCC ACG CC-3′		*Pac*I	Reverse

Table 2.3 The oligonucleotide primers used for DNA sequencing in this work

Primer	Sequence	T_m (°C)	Target gene	Direction
DuetUP1	5′-GGA TCT CGA CGC TCT CCC T-3′	64.5	Gene inserted into multiple cloning sites-1 of pRSFDuet-1	Forward
DuetDOWN1	5′-GAT TAT GCG GCC GTG TAC AA-3′	60.4	Gene inserted into multiple cloning sites-1 of pRSFDuet-1	Reverse
DuetUP2	5′-TTG TAC ACG GCC GCA TAA TC-3′	60.4	Gene inserted into multiple cloning sites-2 of pRSFDuet-1	Forward
T7 terminator	5′-GCT AGT TAT TGC TCA GCG G-3′	51.0	Gene inserted into multiple cloning sites-2 of pRSFDuet-1	Reverse
phedh-[813-832]	5′-CGC CGG TTC AGC CAA CAA TC-3′	72.4	*tktA* gene in pPT	Forward
tktA-[1939-1971]	5′-GAG TTC GGC TTC ACT GTT GAT AAC GTT GTT GCG-3′	64.4	*glpF* gene in pPTFK	Forward
glpF-[674-700]	5′-CCG GCG GCA GAG ACA TTC CTT ACT TCC-3′	70.7	*glpK* gene in pGlpFK and pPTFK	Forward

of the gel. The gel was stained with 2.5 μg/mL of ethidium bromide solution for 5 min and destained in tap water for 10 min. DNA fragments were visualized under a long wavelength UV light. Photograph of the gel was obtained by gel documentation instrument. The concentration and molecular weight of DNA sample was compared with those of the standard DNA markers (λ/*Hin*dIII and 100 bp ladder).

2.9.3 PCR amplification of *phedh* gene

The *phedh* gene (1,143 bp) from *B. lentus* was amplified with a pair of primers designed based on the nucleotide sequence of *B. lentus phedh* (GenBank accession no. EU880599) using pBLPheDH as the template. The 5′-terminal of forward primer (phedh-*Nde*I) possessed restriction endonuclease site for *Nde*I. As PCR amplification by *Pfu* DNA polymerase generated blunt-end PCR fragment, the 3′-terminal of reverse primer (phedh-*Eco*RV) was added by three bases of *Eco*RV site (ATC). For PCR amplification, a total reaction volume of 50 μL contained 1 U of *Pfu* DNA polymerase, 1x *Pfu* DNA polymerase buffer with $MgSO_4$, 0.2 mM of each dNTP, 50 ng of pBLPheDH obtained from section 2.9.1 and 10 pmoles of each of primers phedh-*Nde*I and phedh-*Eco*RV. The *Pfu* DNA polymerase allowed approximately 2 min for every 1 kb to be amplified.

The *phedh* gene was amplified using gradient PCR method. The PCR step was initiated by pre-denaturation at 95 °C for 10 min followed by the cycling profile of 35 cycles containing denaturation at 95 °C for 1 min, annealing at 58.5, 60.7, 62.9 64.9 and 66.6 °C for 30 sec and extension at 72 °C for 3 min. The last step was final extension at 72 °C for 7 min. The PCR products were subjected to an agarose gel. The *phedh* gene fragment was harvested from the gel by gel/PCR DNA fragment extraction kit.

2.9.4 Cloning of *phedh* gene to pRSFDuet-1 vector

2.9.4.1 Vector DNA preparation

After *E. coli* BL21(DE3) containing pRSFDuet-1 was cultured in LB medium supplemented with 30 μg/mL of kanamycin, pRSFDuet-1 was extracted as described in section 2.9.1. It was linearized with *Nde*I and *Eco*RV. The reaction mixture for double digestion containing 1 μg of pRSFDuet-1, 1x NEBuffer 2, 100 μg/mL of BSA, 10 U of *Nde*I, and 10 U of *Eco*RV in a total volume of 20 μL was incubated at 37 °C for 3 h. Electrophoresis step may be omitted in case the plasmid was completely digested and the eliminated fragment must not exceed 50 bp. Thus, the linear form of pRSFDuet-1 (3,808 bp) was easily recovered and concentrated using PCR clean up protocol of gel/PCR DNA fragment extraction kit.

2.9.4.2 Insert DNA preparation

The purified *phedh* gene fragment from 2.9.3 was digested only with *Nde*I. The reaction mixture for digestion consisting of 1 μg of gene fragment, 1x NEBuffer 4, and 10 U of *Nde*I in a total volume of 20 μL was incubated at 37 °C for 18 h. The DNA fragment (1,147 bp) was collected using PCR clean up protocol.

2.9.4.3 Ligation of vector DNA and insert DNA

The gene fragment (2.9.4.2) was inserted into the pRSFDuet-1 vector (2.9.4.1) at vector: insert molar ratio of 1: 5. The ligation mixture of 20 μL consisting of 100 ng of vector DNA, 500 ng of the gene fragment, 1x T4 DNA ligase reaction buffer and 10 U of T4 DNA ligase was incubated overnight at 22 °C. This obtained ligation reaction was further used for transformation.

The construction of recombinant plasmid pPheDH (4,955 bp), pRSFDuet-1 inserted with *B. lentus phedh* under T7*lac* promoter, is shown in Figure 2.1A.

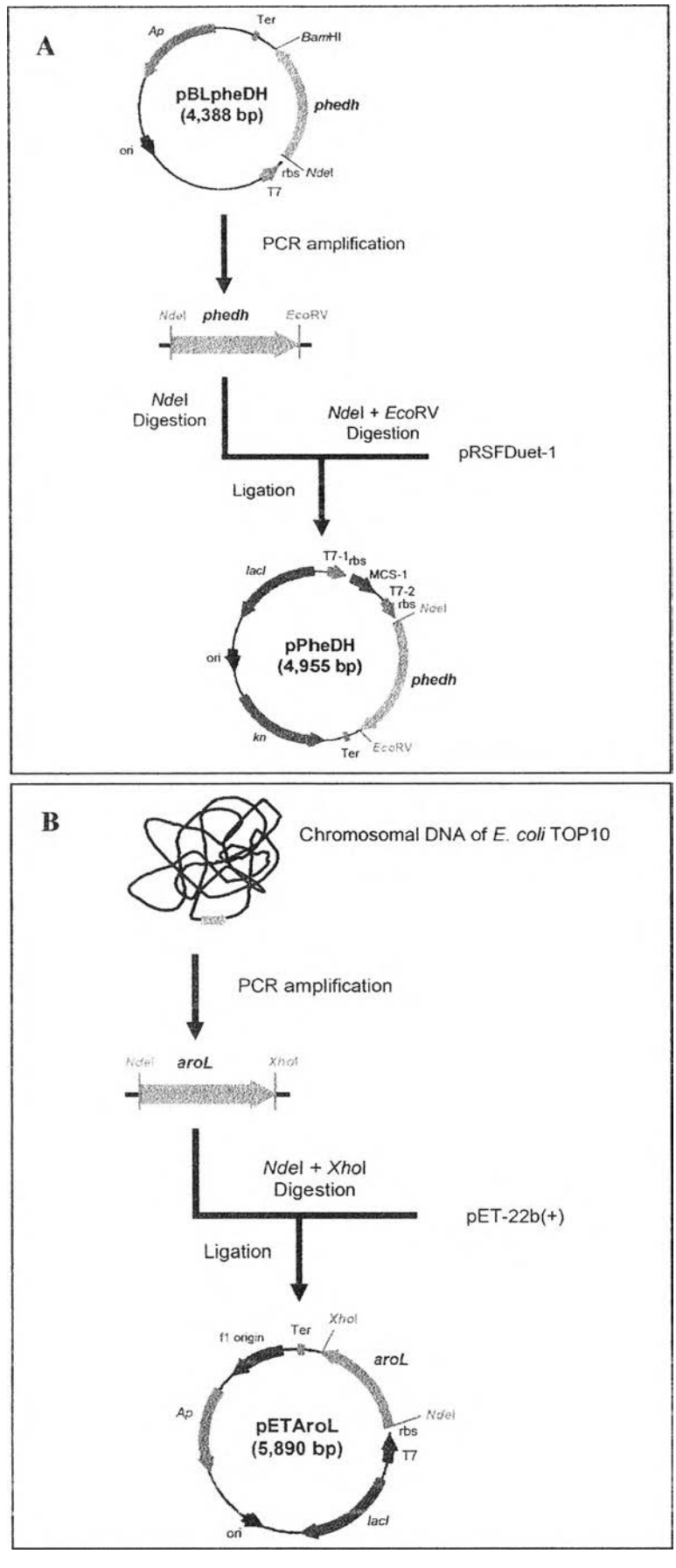


Figure 2.1 The constructions of recombinant plasmids pPheDH (A) and pETAroL (B)

2.9.4.4 Transformation

2.9.4.4.1 Competent cell preparation

One colony of the freshly streaked *E. coli* BL21(DE3) from LB agar plate was resuspended in 5 mL of LB medium, pH 7.4 and grown for 18 h (37 °C, 250 rpm shaker speed). Overnight seed culture (5 mL) was used to inoculate (1% v/v) to 500 mL of the same medium in a 1 L shake flask. Cell culture was shaken until the optical density at 600 was 0.6-0.8. The culture was chilled on ice for 30 min and then centrifuged at 8,000xg for 10 min at 4 °C. The cell pellets were washed with cold autoclaved distilled water 1.5-2 volumes of medium used. After that, the cells were washed with approximately 20 mL of cold 10% glycerol, centrifuged at 8,000xg for 10 min at 4 °C, and resuspended with cold 10% glycerol to a final volume of 3 mL. Then, a 40 μL aliquot was transferred to each 1.5 mL microcentrifuge tube and placed on ice. The remainders were maintained at -80 °C.

2.9.4.4.2 Electroporation

The ligation reaction from 2.9.4.3 was introduced into competent cells of *E. coli* BL21(DE3) by electroporation. The 0.1-cm gap width cuvette was chilled on ice and the competent cells were gently thawed on ice. Two μL of ligation reaction was mixed well with 40 μL of the competent cells and then placed on ice for 1 min. This mixture was transferred to the cuvette. After electroporation, one mL of LB medium was immediately added to the cuvette. The entire volume was transferred to a new microcentrifuge tube, mixed by inverting the tube and incubated at 37 °C for 1 h with shaking. After incubation, if desired, the transformed cells may be concentrated by centrifugation at 8,000xg for 1 min. The cell pellet was resuspended in remaining supernatant, plated onto the LB agar plates containing 30 μg/mL of kanamycin and incubated at 37 °C for 18 h. The transformed cells containing plasmid growing on these selective plates were picked and marked onto the new LB agar plates containing 30 μg/mL of kanamycin called as the master plates.

2.9.4.5 Selection of recombinant clone

2.9.4.5.1 Rapid selection by agarose gel electrophoresis

The transformed cells in master plates from above section were identified if each clone contained recombinant plasmid or vector by agarose gel electrophoresis. After the 0.5% agarose gel preparation, fifteen μL of the resuspension buffer was added to each microcentrifuge tube. The cells from the master plates (about 2 fold of end of a 200 μL pipette tip-sized amount) were subjected into these microcentrifuge tubes by a pipette tip, resuspended and placed for 20 min at room temperature. Five μL of the lysis buffer was loaded into each well of the gel and followed by the above incubated resuspension from each tube. All solutions for this selection could be prepared in accordance with Appendix D. Clones containing desired recombinant plasmid were picked and cultured in LB medium. After that, their plasmids were extracted and confirmed again by restriction enzyme digestion as described below.

2.9.4.5.2 Plasmid extraction and restriction enzyme digestion

E. coli BL21(DE3) recombinant clones were grown in 5 mL of LB medium containing 30 μg/mL of kanamycin at 37 °C for 18 h at 250 rpm. The plasmid from individual clone was isolated as described in 2.9.1. After that, the plasmids were completely digested with *Nde*I and *Eco*RV (section 2.9.4.1). The sizes of digested recombinant plasmids were estimated by agarose gel electrophoresis compared with λ/*Hin*dIII marker. The crude extract of clones harboring recombinant plasmid containing *phedh* inserted at *Nde*I and *Eco*RV sites of pRSFDuet-1 was prepared and assayed for the PheDH activity and protein concentration as described in the following section.

2.9.5 Expression of *phedh* in *E. coli* BL21(DE3)

2.9.5.1 Crude extract preparation

The *E. coli* BL21(DE3) transformants harboring *phedh* was cultured in 5 mL of LB medium containing 30 μg/mL of kanamycin at 37 °C for 18 h with shaking. After that, 2.5% v/v of the cell culture was inoculated into 200 mL of the same medium and kept on cultivation at 37 °C. When the turbidity of the culture at 600 nm reached 0.6-0.8, IPTG was added to final concentration of 1 mM to induce *phedh* expression, and cultivation was continued at 37 °C for 3 h as recommended by pET system manual (Novagen, 2003: online). The cells were harvested by centrifugation at 8,000xg for 10 min and washed with cold 0.85% NaCl. Later on, the cell pellet was washed once in cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01% β-mercaptoethanol and 1.0 mM EDTA) and centrifuged again. The cell pellets were stored at -80 °C until used. For crude extract preparation, the cell pellets were resuspended in 5 mL of cold extraction buffer and sonicated on ice. Unbroken cells and cell debris were excluded by centrifugation at 17,500xg for 30 min. The supernatants were kept at 4 °C for determination of PheDH activity and protein concentration.

2.9.5.2 PheDH activity assay

The PheDH activity for oxidative deamination of L-Phe was spectrophotometrically assayed. One mL of standard reaction mixture contained 200 μmols of glycine-KCl-KOH buffer, pH 11.5, 20 μmols of L-Phe, 1 μmol of NAD^+, and the crude extract. Each of above components, except NAD^+ was added into a cuvette of 1-cm light path. The cuvette was incubated by putting in a water bath set at 30 °C for 5 min. After incubation, the oxidation reaction was initiated by the addition of NAD^+ and monitored by measuring the increase in absorbance at 340 nm using the extinction coefficient 6.22 $mM^{-1}cm^{-1}$ for NADH.

One unit of the enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH in 1 min. Specific activity is expressed as units per milligram of protein.

2.9.5.3 Protein determination

Protein concentration was quantified by the modified method based on Lowry et al., (1951). For preparation of calibration curve, the reaction mixture (3.05 mL) containing the standard protein BSA in the range from 20 to 100 μg, 50 μL of solution A (0.5% copper sulfate and 1% potassium tartate, pH 7.0), and 2.5 mL of solution B (2% sodium carbonate and 0.1 N sodium hydroxide) was eventually mixed by vortex and then incubated in a water bath thermostated at 30 °C for 10 min. After that, 250 μL of the freshly prepared solution C (1:1 (v/v) phenol reagent: distilled water) was added to the mixture, let rest for 20 min at room temperature, and then measured the absorbance at 610 nm. The standard curve for protein determination is shown in Appendix E.

2.9.6 Nucleotide sequencing of *phedh*

The plasmid of *E. coli* BL21(DE3) transformant showing the highest PheDH specific activity was prepared and the nucleotide sequence of inserted *phedh* was verified by DNA sequencing at Macrogen Inc., Republic of Korea using primers DuetUP2 and T7 terminator due to the insertion of *phedh* at multiple cloning sites-2 of pRSFDuet-1 vector. The plasmid consisting of correct nucleotide sequence of *phedh* or pPheDH was used as a parental plasmid for gene combination construction in further experiments.

2.10 Cloning of *aroL*, *pheA* and *yddG* genes into pET-22b(+) vector

2.10.1 Chromosomal DNA extraction

Chromosomal DNA of *E. coli* TOP10 was isolated by the following method of Frederick et al., (1995). A single colony of *E. coli* TOP10 was inoculated into 100 mL of LB medium and incubated at 37 °C for 18 h with shaking at 250 rpm. Each 1.5 mL of cell culture was centrifuged at 12,000xg for 2 min. The pellet was resuspended in 550 μL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The cell suspension was then treated with 3 μL of 5 mg/mL lysozyme, 2 μL of 10 mg/mL RNase A, 30 μL of 10% SDS and 3 μL of 20 mg/mL proteinase K and

incubated at 37 °C for 1 h. After incubation, the DNA was extracted with an equal volume of phenol-chloroform (1:1 v/v) and centrifuged at 12,000xg for 10 min. The upper phase was carefully transferred to a new microcentrifuge tube. DNA was precipitated by the addition of 5 M NaCl to the final concentration of 1 M and 2 volumes of cold absolute ethanol and kept at -20 °C for at least 30 min. Subsequently, the DNA pellet was collected by centrifugation at 12,000xg for 10 min at 4 °C and washed well with cold 70% ethanol. After drying, the pellet was dissolved in an appropriate volume of sterile ultrapure water. DNA concentration was estimated by agarose gel electrophoresis.

2.10.2 PCR amplification of *aroL*, *pheA* and *yddG* genes

2.10.2.1 Template preparation for PCR amplification

The isolated *E. coli* TOP10 genomic DNA was digested with *Bam*HI. The reaction mixture containing 5 µg of chromosomal DNA, 1x NEBuffer 3, 100 µg/mL of BSA and 20 U of *Bam*HI in a total volume of 50 µL was incubated at 37 °C for 18 h. After incubation, the obtained DNA fragments were concentrated according to the PCR clean up protocol. These purified DNA fragments were finally eluted with 50 µL of sterile distilled water. Two µL of this solution (about 200 ng) was used as template in each PCR reaction for amplification of all genes throughout this work, except *phedh*.

2.10.2.2 PCR condition for amplification of *aroL*, *pheA* and *yddG*

The primers used in PCR amplification of *aroL* (525 bp), *pheA* (1,161 bp) and *yddG* (882 bp) genes were designed based on the nucleotide sequence of each gene from *E. coli* DH10B (Genbank accession no. CP000948.1), a strain with a close similarity to the strain TOP10. The primers used in *aroL* amplification were aroL-*Nde*I and aroL-*Xho*I. Primers pheA-*Nde*I and pheA-*Eco*RI were used in *pheA* amplification. Also, the primers used in *yddG* amplification were yddG-*Nde*I and yddG-*Xho*I. The reaction mixture for each PCR amplification in a final volume of 50 µL contained 1 U of *Pfu* DNA polymerase, 1x *Pfu* DNA polymerase buffer with

$MgSO_4$, 0.2 mM of each dNTP, 200 ng of *Bam*HI-digested *E. coli* TOP10 chromosomal DNA as template and 10 pmoles of each of forward and reverse primers.

The *aroL* and *yddG* genes were amplified using gradient PCR method. The PCR steps were composed of an initial denaturation at 95 °C for 10 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 50, 54.4, 58.7, 60.8, 62.9, and 64.6 °C for 30 sec, extension at 72 °C for 2 min and a final elongation at 72 °C for 7 min. For *pheA* gene amplification, the annealing temperatures were varied at 50, 52.7, 55.4, 56.8, 58.1 and 60 °C for 30 sec and extension was done at 72 °C for 3 min. The other steps were conducted like above.

The PCR products obtained (i.e. *aroL, pheA* and *yddG* fragments) were separated by agarose gel electrophoresis. The specific band of each product was harvested from the gel.

2.10.3 Cloning of *aroL, pheA* and *yddG* genes

The *aroL* and *yddG* fragments and pET-22b(+) were digested with *Nde*I and *Xho*I. The reaction mixture for double digestion consisting of 1 μg of DNA, 1x NEBuffer 4, 100 μg/mL of BSA, 10 U of *Nde*I and 10 U of *Xho*I in a total volume of 20 μL was incubated at 37 °C for 18 h, except for 3 h in plasmid digestion.

The *pheA* gene fragment and pET-22b(+) were digested with *Nde*I and *Eco*RI. The reaction mixture for double digestion contained 1 μg of DNA, 1x NEBuffer *Eco*RI, 10 U of *Nde*I and 10 U of *Eco*RI in a total volume of 20 μL and then incubated at 37 °C for 18 h, except for 3 h in plasmid digestion.

Each gene was ligated into pET-22b(+) plasmid digested with the same restriction enzymes (*Nde*I-*Xho*I or *Nde*I-*Eco*RI). This produced the recombinant plasmids pETAroL (5,890 bp), pETYddG (6,247 bp) and pETPheA (6,560 bp). Subsequently, each of the resulting plasmids was introduced into *E. coli* BL21(DE3) and screened on the LB agar plates containing 100 μg/mL of ampicillin. The constructions of recombinant plasmids pETAroL, pETYddG and pETPheA are shown in Figure 2.1B, 2.2A and 2.2B, respectively. The *E. coli* BL21(DE3) containing each type of plasmid was selected as section 2.9.4.5. The plasmid of each

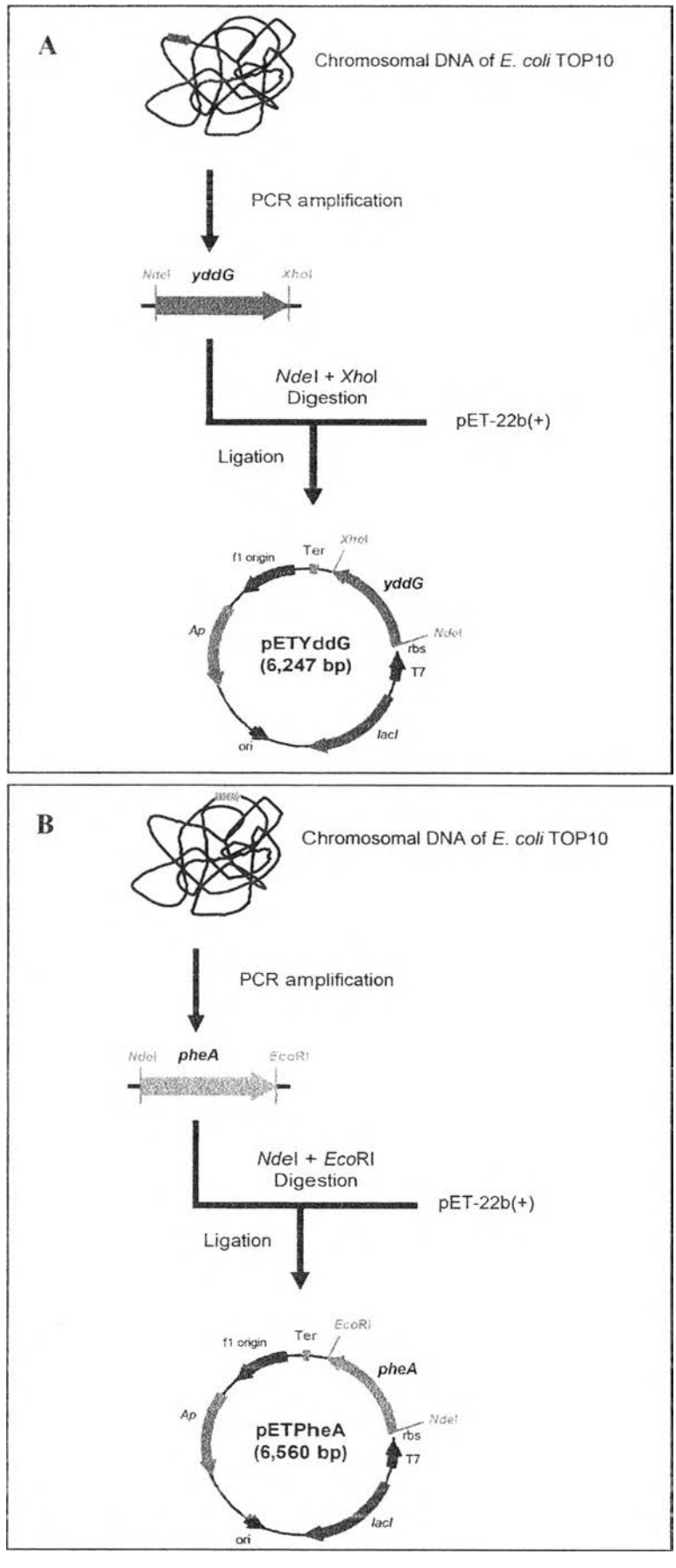


Figure 2.2 The constructions of recombinant plasmid pETYddG (A) and pETPheA (B)

E. coli BL21(DE3) clone was extracted and the existence of the gene was confirmed by digestion with the appropriate restriction enzyme. The *E. coli* BL21(DE3) clone containing each gene-inserted pET-22b(+) was used as source of the relevant gene fragment preceded by the T7*lac* promoter and the ribosome binding site. Each fragment was further cloned into pRSFDuet-1 vector.

2.11 Subcloning of *aroL*, *pheA* and *yddG* into pRSFDuet-1 vector

Each of *aroL*, *pheA* and *yddG* genes under T7*lac* promoter in a backbone plasmid pET-22b(+) (pETAroL, pETYddG and pETPheA) was subcloned into suitable restriction sites of pRSFDuet-1 so that construction of gene combination in future was conveniently attained.

2.11.1 Cloning of *aroL* gene into pRSFDuet-1 vector

To clone the DNA fragment (637 bp) containing the T7*lac* promoter, the ribosome binding site and the *aroL* gene from pETAroL into *Bam*HI and *Asc*I sites of pRSFDuet-1, the primers used for this fragment amplification were designed from nucleotide sequence of T7 promoter and 3'-sequence of *aroL* gene flanking with respective *Bam*HI and *Asc*I sites as T7-*Bam*HI and aroL-*Asc*I primers. After the pETAroL extraction, PCR amplification was conducted using pETAroL as template at annealing temperature of 50, 52.5, 54.4, 56.5, 58.7, 60.8, 62.9, and 64.6 °C for 30 sec and extension at 72 °C for 2 min.

The amplified PCR product was harvested from the agarose gel and digested with *Bam*HI and *Asc*I. A total volume of 20 µL containing 1 µg of DNA, 1x NEBuffer 4, 100 µg/mL of BSA, 10 U of *Bam*HI and 10 U of *Asc*I was incubated at 37 °C for 18 h. Similarly as PCR product, pRSFDuet-1 was digested for 3 h. After purification, this gene fragment was inserted between *Bam*HI and *Asc*I sites of pRSFDuet-1 by ligase leading to a recombinant plasmid pAroL (4,447 bp). The construction of recombinant plasmid pAroL is shown in Figure 2.3A.

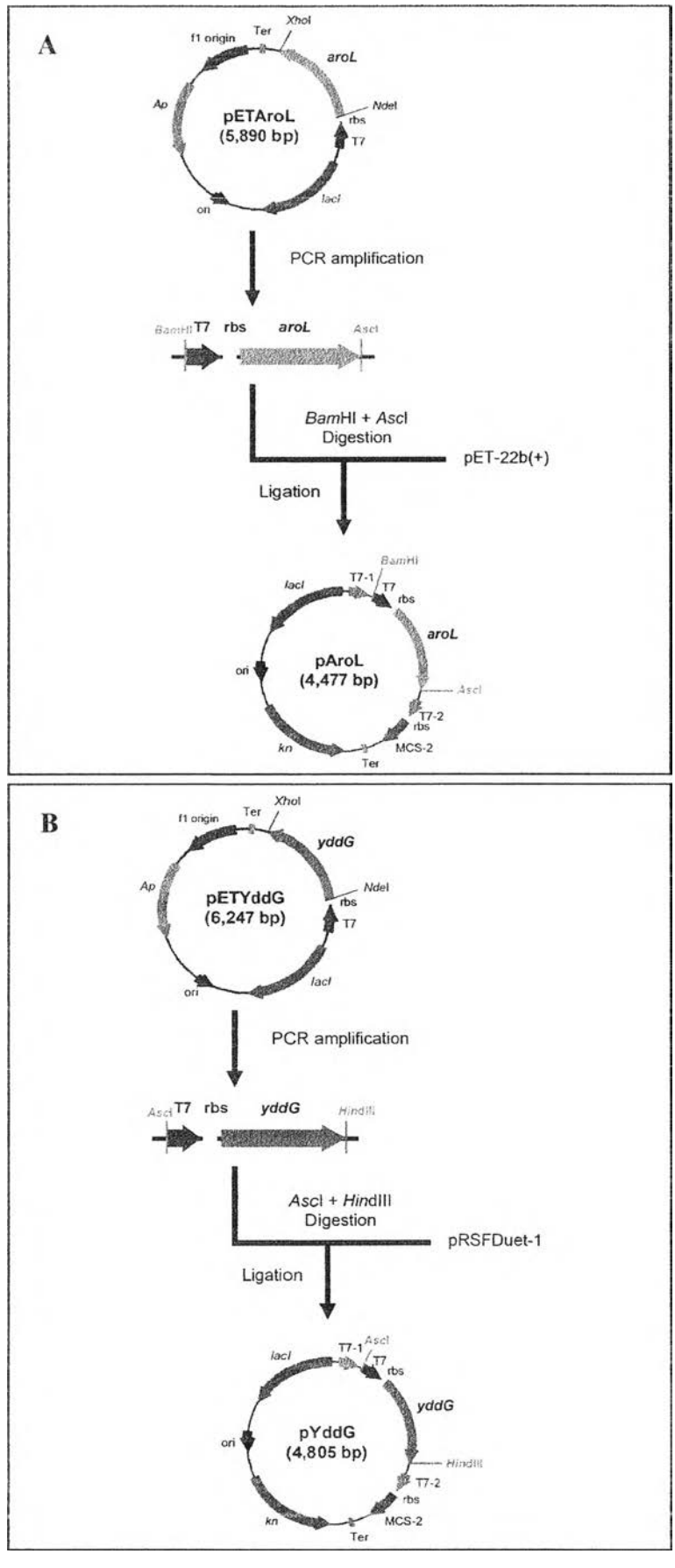


Figure 2.3 The constructions of recombinant plasmids pAroL (A) and pYddG (B)

2.11.2 Cloning of *yddG* gene into pRSFDuet-1 vector

The primers used for amplification of the DNA fragment (994 bp) containing the T7*lac* promoter, the ribosome binding site and the *yddG* gene were designed from nucleotide sequence of T7 promoter and 3'-sequence of *yddG* gene linked to site of *Asc*I and *Hin*dIII as respective T7-*Asc*I and yddG-*Hin*dIII primers to clone this fragment from pETYddG into *Asc*I and *Hin*dIII sites of pRSFDuet-1. PCR amplification was then performed using pETYddG as template by varying annealing temperature.

The amplified PCR product was purified from the agarose gel and double digested with *Hin*dIII and *Asc*I in a total volume of 20 µL containing 1 µg of DNA, 1x NEBuffer 4, 10 U of *Asc*I and 20 U of *Hin*dIII. In the meanwhile, pRSFDuet-1 was digested with *Hin*dIII and *Asc*I. After purification, gene fragment and pRSFDuet-1 digested with *Asc*I and *Hin*dIII were ligated together resulting in a recombinant plasmid pYddG (4,805 bp). The construction of recombinant plasmid pYddG is schemed in Figure 2.3B.

2.11.3 Cloning of *pheA* gene into pRSFDuet-1 vector

Recombinant plasmid pPETPheA was used as template to amplify the DNA fragment (1,269 bp) consisting of the T7*lac* promoter, the ribosome binding site and the *pheA* gene. The forward and reverse primers containing *Hin*dIII and *Afl*II (T7-*Hin*dIII and pheA-*Afl*II) were designed from T7 promoter and 3'-sequence of *pheA* gene. This fragment was inserted into *Hin*dIII and *Afl*II sites of pRSFDuet-1. After pETYddG was isolated, PCR reaction was done by varying annealing temperature.

The specific fragment was recovered from the agarose gel. The *Hin*dIII and *Afl*II excision of the DNA fragment was occurred in a total volume of 20 µL containing 1 µg of DNA, 1x NEBuffer 2, 100 µg/mL of BSA, 10 U of *Hin*dIII and 10 U of *Afl*II. Similarly, pRSFDuet-1 was digested with *Hin*dIII and *Afl*II. Later on, the digested gene fragment and pRSFDuet-1 were ligated resulting in a recombinant plasmid pPheA (5,078 bp). The construction of recombinant plasmid pYddG is shown in Figure 2.4A.

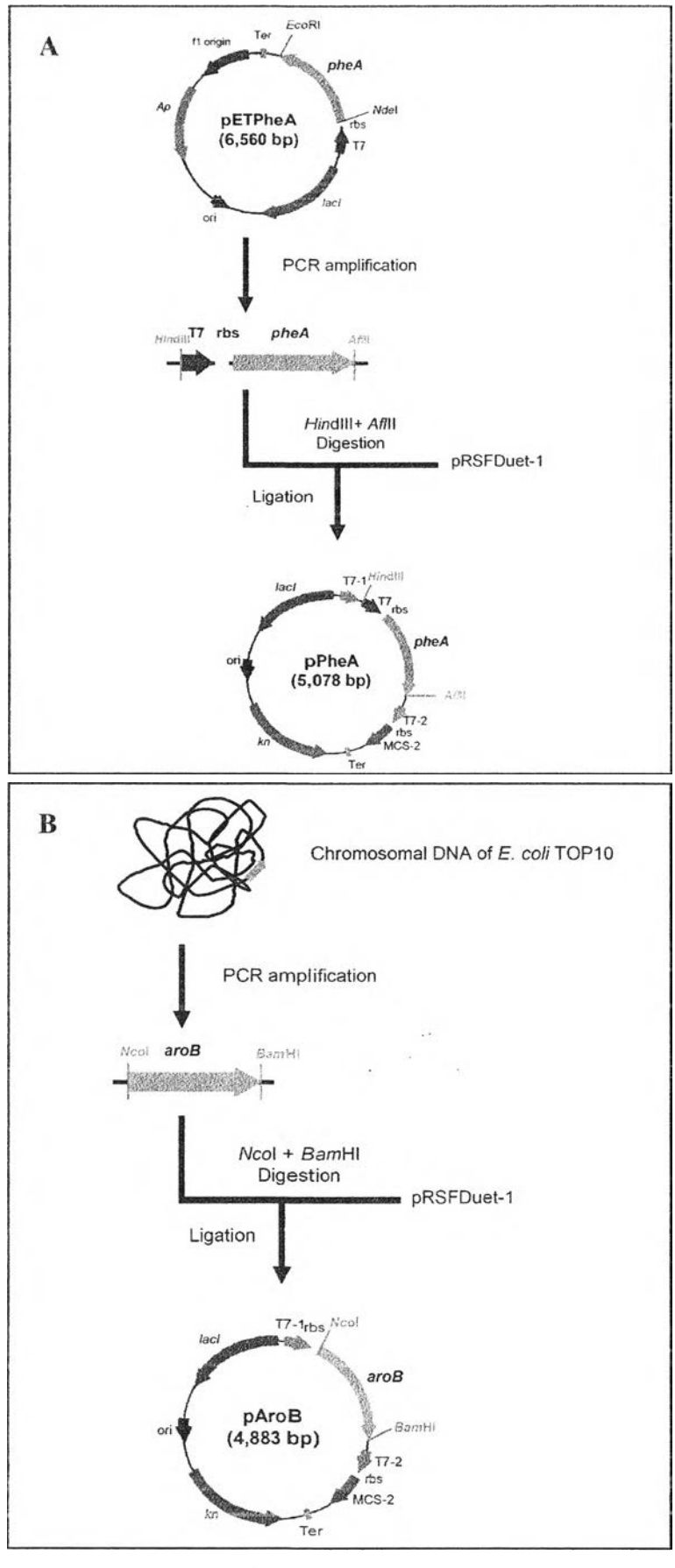


Figure 2.4 The constructions of recombinant plasmids pPheA (A) and pAroB (B)

The plasmids pAroL, pPheA and pYddG were separately transformed into *E. coli* BL21(DE3). The transformed cells were grown on LB agar plates containing 30 μg/mL of kanamycin and incubated at 37 °C for 18 h. Each of transformed cells was briefly screened on agarose gel as section 2.9.4.5.1 to find out cells containing the desired fragment inserted into pRSFDuet-1. The plasmid from each *E. coli* BL21(DE3) transformant was extracted and confirmed the gene existence by appropriate restriction enzyme digestion and sequencing. The primers DuetUP1 and DuetDOWN1 were used to sequence gene inserted into multiple cloning sites-1 of pRSFDuet-1 vector (i.e. pAroL, pPheA and pYddG). Then, the expression of each gene of the *E. coli* BL21(DE3) clone containing recombinant plasmid with correct nucleotide sequence was further identified.

2.12 Cloning of *aroB*, *glpF* and *tktA* into pRSFDuet-1

The primers used for PCR amplification of *aroB* (1,089 bp), *glpF* (846 bp) and *tktA* (1,992 bp) genes were designed based on the nucleotide sequence of each gene from *E. coli* DH10B (Genbank accession no. CP000948.1). The primers comprised of appropriate recognition sites to insert each of these genes into the desired sites of pRSFDuet-1 vector. The *E. coli* TOP10 genomic DNA digested with *Bam*HI was used as template for these gene amplifications.

2.12.1 Cloning of *aroB* gene into pRSFDuet-1 vector

The *aroB* gene amplification was performed using primers aroB-*Nco*I and aroB-*Bam*HI to introduce *aroB* gene into *NcoI* and *Bam*HI sites at multiple cloning sites-1 of pRSFDuet-1. PCR reaction was done by varying annealing temperature.

The specific band of PCR product was recovered from the agarose gel. It was simultaneously digested with *Nco*I and *Bam*HI in a total volume of 20 μL containing 1 μg of DNA, 1x NEBuffer 3, 100 μg/mL of BSA, 10 U of *Nco*I and 10 U of *Bam*HI, the same condition as that of pRSFDuet-1 vector. Accordingly, the digested gene fragment and pRSFDuet-1 were linked together as circular DNA by

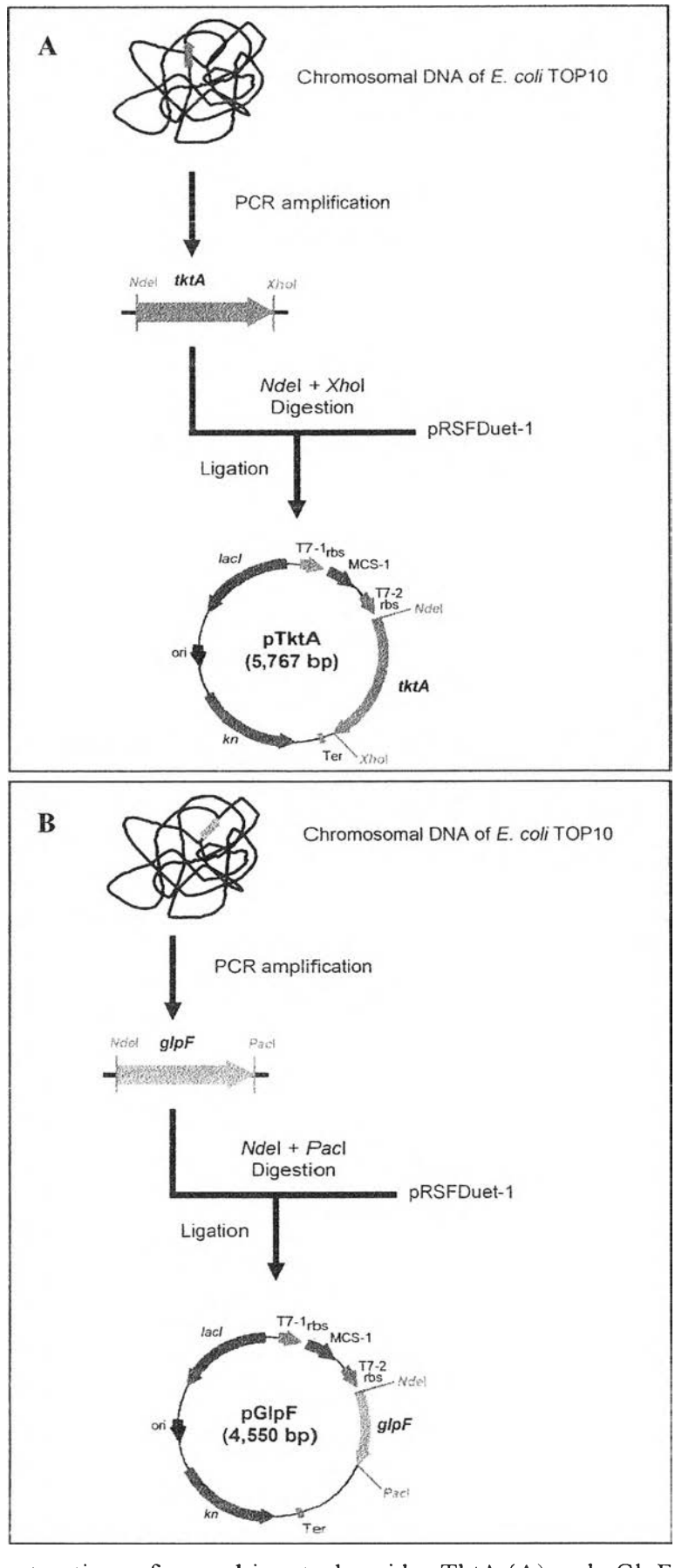


Figure 2.5 The constructions of recombinant plasmids pTktA (A) and pGlpF (B)

ligase resulting in a recombinant plasmid pAroB (4,883 bp). The construction outline of recombinant plasmid pAroB is shown in Figure 2.4B.

2.12.2 Cloning of *tktA* gene into pRSFDuet-1 vector

The *tktA* gene was introduced into *NdeI* and *Xho*I sites at multiple cloning sites-2 of pRSFDuet-1. The designed primers were tktA-*Nde*I and tktA-*Xho*I. The PCR reaction was accomplished at various annealing temperatures.

The specific fragment was concentrated from the agarose gel. The gene fragment and pRSFDuet-1 were simultaneously digested with *Nde*I and *Xho*I in a total volume of 20 μL containing 1 μg of DNA or pRSFDuet-1, 1x NEBuffer 4, 100 μg/mL of BSA, 10 U of *Nde*I and 10 U of *Xho*I. After purification, they were ligated resulting in a recombinant plasmid pTktA (5,767 bp). The construction of recombinant plasmid pTktA is shown in Figure 2.5A.

2.12.3 Cloning of *glpF* gene into pRSFDuet-1 vector

The *Bam*HI-digested chromosomal DNA was used as source for *glpF* amplification using primers glpF-*Nde*I and glpF-*Pac*I to clone *glpF* gene into *Nde*I and *Pac*I sites at multiple cloning sites-2 of pRSFDuet-1. PCR was performed in a total reaction volume of 50 μL as specified in section 2.6.3. The PCR reaction was accomplished at various annealing temperatures of 46.4, 48.9, 51.0, 52.7, 53.8 and 55.0 °C.

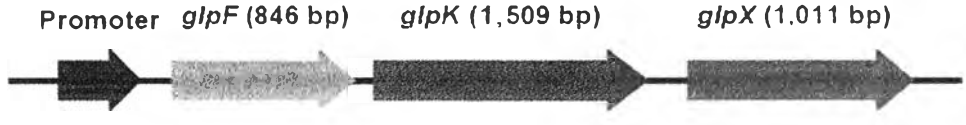
The specific fragment was concentrated from the agarose matrix. The double digestion of the gene fragment and pRSFDuet-1 were separately taken place by *Nde*I and *Pac*I in a reaction of 20 μL containing 1 μg of DNA, 1x NEBuffer 4, 100 μg/mL of BSA, 10 U of *Nde*I and 10 U of *Pac*I. After cleaning up by kit, both of the digested DNA fragments were joined together by ligase resulting in a recombinant plasmid pGlpF (4,550 bp). The construction of recombinant plasmid pGlpF is shown in Figure 2.5B.

The plasmids pAroB, pTktA and pGlpF were separately introduced into *E. coli* BL21(DE3). The transformed cells were grown on LB agar plates containing 30 μg/mL of kanamycin and incubated at 37 °C for 18 h. Each of

transformed cells was rapidly screened on agarose gel (section 2.9.4.5.1) for cells containing expected gene inserted into pRSFDuet-1. The plasmid of each *E. coli* BL21(DE3) transformant was extracted and the existence of the gene was confirmed by digestion with the appropriate restriction enzymes and sequencing. The primers DuetUP1 and DuetDOWN1 were used to sequence gene inserted into multiple cloning sites-1 of pRSFDuet-1 vector (i.e. pAroB), while the primers DuetUP2 and T7 terminator were used to sequence gene inserted into multiple cloning sites-2 (i.e. pTktA and pGlpF). Then, the gene expression of each *E. coli* BL21(DE3) clone containing recombinant plasmid with correct nucleotide sequence was further examined.

2.13 Cloning of *glpF* and *glpK* genes into pRSFDuet-1 vector

In *E. coli* genome, *glpF* encoding glycerol facilitator, *glpK* encoding glycerol kinase and *glpX* gene encoding fructose 1, 6-bisphosphatase II were organized in the *glpFKX* operon belonging to the *glp* regulon as shown below.



To simultaneously clone both *glpF* (846 bp) and *glpK* (1,509 bp) genes or *glpFK* gene (2,377 bp) from *E. coli* into *Nde*I and *Pac*I sites of pRSFDuet-1 vector, the primers for the *glpFK* amplification were created based on the orientation of *glpF* and *glpK* gene on the operon. The forward primer was designed from 5'-sequence of *glpF* gene containing *Nde*I site (glpF-*Nde*I) and the reverse primer was designed from 3'-sequence of *glpK* gene containing *Pac*I site (glpK-*Pac*I). PCR amplification was achieved using *Bam*HI-digested chromosomal DNA as template at various annealing temperatures. After that, the construction of recombinant plasmid was done as section 2.12.3 resulting in a recombinant plasmid pGlpFK (6,081 bp). The construction of recombinant plasmid pGlpFK is shown in Figure 2.6A. The plasmid was introduced into *E. coli* BL21(DE3). The pGlpFK clone containing recombinant plasmid with correct nucleotide sequence of *glpFK* gene was further analyzed for gene expression.

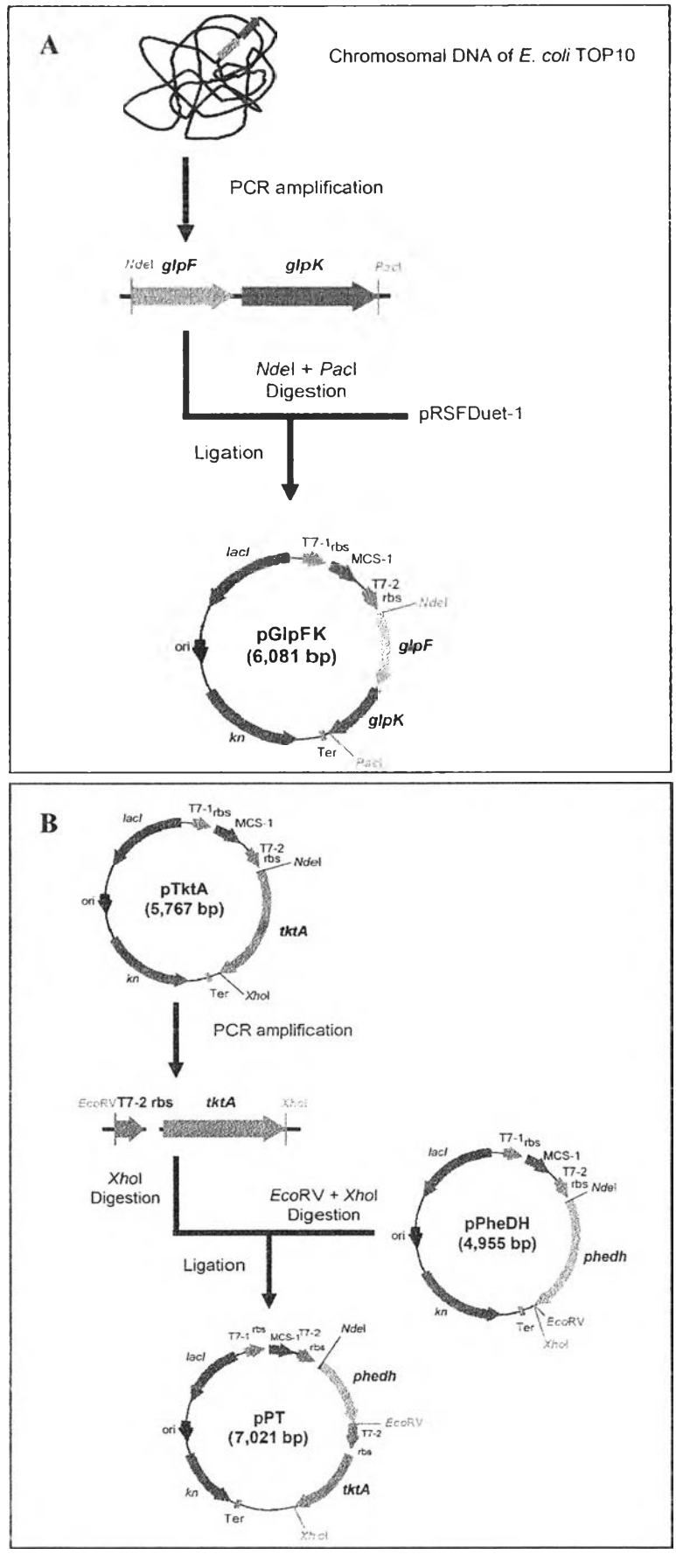


Figure 2.6 The constructions of recombinant plasmids pGlpFK (A) and pPT (B)

2.14 Expression of recombinant plasmids pAroB, pAroL, pGlpF, pPheDH, pPheA, pTktA, pYddG and pGlpFK in *E. coli* BL21(DE3)

2.14.1 Cultivation condition for gene expression

E. coli BL21(DE3) cells containing the recombinant plasmids were cultured at 37 °C for 18 h in 5 mL of LB medium supplemented with 30 μg/mL of kanamycin. The cell culture was inoculated (5% v/v) to 100 mL of the same medium and incubation was continued until OD_{600} was about 0.6. IPTG was then added to a final concentration of 1 mM and the cultivation was continued for 3 h. The cells were harvested by centrifugation at 8,000xg for 5 min and stored at -20 °C for further analysis.

2.14.2 SDS-polyacrylamide gel electrophoresis

The analysis of gene expression was carried out by SDS-PAGE according to Bollag et al., 1996. The slab gel system consisted of 0.1% SDS (w/v) in 12% separating gel and 5% stacking gel. The cell pellet from section 2.14.1 was resuspended in 100 μL of the 5x sample buffer (312.5 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 1% (w/v) bromophenol blue) and boiled for 15 min. After centrifugation at 12,000xg for 5 min, eight μL of each sample was loaded to the gel. The cell extract of *E. coli* BL21(DE3) containing pRSFDuet-1 under induction with 1 mM IPTG was loaded as reference of protein pattern. The protein molecular weight markers were β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease Bsp98I (25 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa). After electrophoresis, the gel was stained with Coomassie blue solution and then destained by destaining solution.

2.15 Subcloning of genes combined with *phedh* in a single vector

2.15.1 Cloning of *tktA* gene into a recombinant plasmid pPheDH

To clone the DNA fragment (2,101 bp) containing the *tktA* gene with its own T7*lac* promoter and ribosome binding site into *Eco*RV and *Xho*I sites of pPheDH at the 3′ terminus of *phedh* gene, the primers used were designed from T7 promoter-2 sequence of pRSFDuet-1 containing three bases (ATC) of *Eco*RV site (T7-*Eco*RV) and 3′-sequence of *tktA* gene containing *Xho*I site (tktA-*Xho*I). The PCR reaction was accomplished using pTktA as template at various annealing temperatures.

The specific band of PCR product was purified from the agarose gel. The pPheDH was digested with *Eco*RV and *Xho*I endonucleases in a total volume of 20 µL containing 1 µg of plasmid, 1x NEBuffer 3, 100 µg/mL of BSA, 10 U of *Eco*RV and 10 U of *Xho*I, and incubated at 37 °C for 3 h. The gene fragment was digested only with *Xho*I in a total volume of 20 µL containing 1 µg of DNA, 1x NEBuffer 4, 100 µg/mL of BSA, and 10 U of *Xho*I and incubated at 37 °C for 18 h. Both were purified by kit and ligated to produce recombinant plasmid pPT (7,021 bp). The construction of recombinant plasmid pPT is elucidated in Figure 2.6B. The recombinant plasmid was introduced into *E. coli* BL21(DE3) and screened on LB agar plates containing 30 µg/mL of kanamycin. The nucleotide sequence of *tktA* and its promoter in the clone was verified by sequencing using primers phedh-[813-832] and T7 terminator. The pPT containing correct sequence of *tktA* was used as backbone plasmid for next cloning.

2.15.2 Cloning of *glpF* gene into a recombinant plasmid pPT

To amplify the DNA fragment (961 bp) containing the *glpF* gene preceded by the T7*lac* promoter and ribosome binding site, pGlpF was used as DNA template. The primers were designed from T7 promoter-2 sequence of pRSFDuet-1 containing *Xho*I site (T7-*Xho*I) and 3′-sequence of *glpF* gene containing *Pac*I site (glpF-*Pac*I). This fragment was placed between *Xho*I and *Pac*I sites of pPT at

the 3'-end of *tktA* gene. The PCR reaction was carried out at various annealing temperatures.

The expected band of PCR product was purified from the agarose gel. The pPT and the PCR product fragment were separately digested with *Xho*I and *Pac*I in a total volume of 20 µL containing 1 µg of DNA, 1x NEBuffer 4, 100 µg/mL of BSA, 10 U of *Xho*I and 10 U of *Pac*I and incubated at 37 °C for 3 h and 18 h, respectively. After cleaning up, both were purified by kit and ligated to produce recombinant plasmid pPTF (7,907 bp). The construction of recombinant plasmid pPTF is outlined in Figure 2.7A. The introduction into host cells, screening for clones, and sequence verified using primer T7 terminator was performed. The pPTF containing correct sequence of *glpF* was used as backbone plasmid for further cloning.

2.15.3 Cloning of *glpFK* gene into a recombinant plasmid pPT

The DNA fragment (2,493 bp) containing the *glpFK* gene preceded by the T7*lac* promoter and ribosome binding site, which was amplified from pGlpFK using primers T7-*Xho*I and glpK-*Pac*I was cloned into *Xho*I and *Pac*I sites of pPT at the downstream of *tktA* gene as same as *glpF* gene mentioned in section 2.15.2. The obtained recombinant plasmid pPTFK (9,439 bp) was transformed into *E. coli* BL21(DE3). The construction of recombinant plasmid pPTFK is outlined in Figure 2.7B. The nucleotide sequence of *glpFK* and its promoter in the clone was verified using primers tktA-[1939-1971], glpF-[674-700] and T7 terminator. The pPTFK containing correct sequence of *glpFK* was used as backbone plasmid for further cloning.

In general, prior to expression, selected clones were confirmed for correct amplification and absence of PCR/cloning mediated mutations by sequencing. For the following experiments, there was cloning by digestion of recombinant plasmid with restriction enzymes and ligation but not by PCR amplification. Thus, DNA sequencing of each of constructed plasmids was not determined.

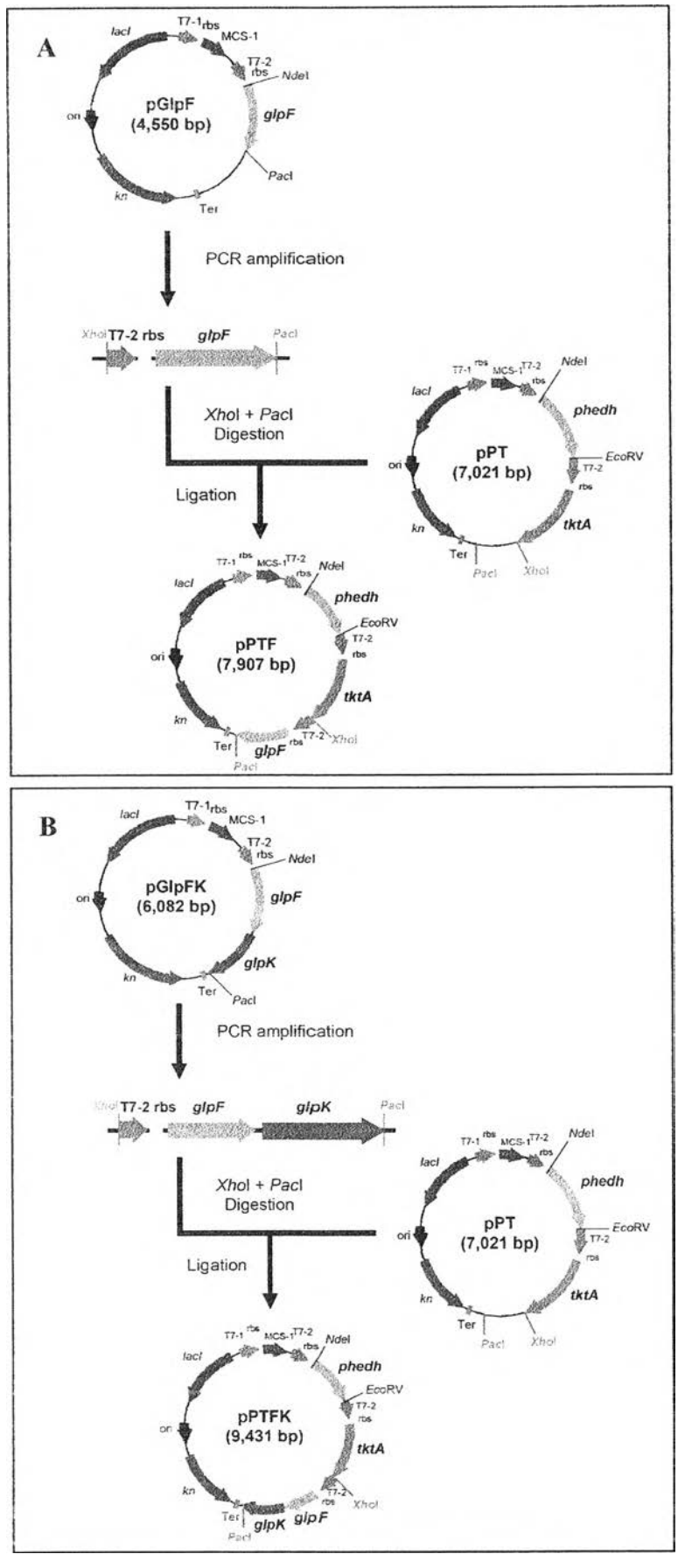


Figure 2.7 The constructions of recombinant plasmids pPTF (A) pPTFK (B)

2.15.4 Cloning of *aroB* gene into a recombinant plasmid pPTF

As simple construction, pAroB should be cleaved by *Nco*I and *Bam*HI to insert the *aroB* gene into the same sites of pPTF. However, *phedh* gene inserted into pPTF is able to be digested by *Nco*I. To avoid the incident, the digestion of pPTF and pAroB with *Xba*I and *Bam*HI is a reasonable alternative for cloning *aroB* gene into pPTF.

The long length DNA fragment (6,386 bp) containing the respective *phedh*, *tktA* and *glpF* genes closing to each own T7*lac* promoter and ribosome binding site was obtained by digestion of pPTF with *Xba*I and *Bam*HI. The digestion reaction contained 1 μg of pPTF, 1x NEBuffer 3, 100 μg/mL of BSA, 10 U of *Xba*I and 10 U of *Bam*HI in a total volume of 20 μL and incubated at 37 °C for 3 h. The digestion products were separated by agarose gel electrophoresis. The 6,386 bp DNA fragment was harvested from the gel and used as a vector DNA for ligation.

The 2,575 bp DNA fragment containing the *aroB* gene preceded by the T7*lac* promoter and ribosome binding site was obtained by digestion of pAroB with the same restriction endonucleases (*Xba*I and *Bam*HI). The digestion was done as mentioned above. The 2,575 bp DNA fragment was collected from the gel and used as an insert DNA for ligation.

Both of the DNA fragments were linked together by ligase to produce a recombinant plasmid pPTFB (8,961 bp) (Figure 2.8A). After transformation into *E. coli* BL21 (DE3), pPTFB was isolated and used as source of gene combination for further cloning.

2.15.5 Cloning of *aroL* gene into a recombinant plasmid pPTFB

The construction of recombinant plasmid pPTFBL is shown in Figure 2.8B. pPTFBL containing *phedh, tktA, glpF, aroB* and *aroL* genes with each own T7*lac* promoter and ribosome binding site was derived from the ligation of the DNA fragments obtained from the digestion of pPTFB and pAroL with *Bam*HI and *Bsr*GI.

The long length DNA fragment (8,877 bp) containing the *phedh*, *tktA, glpF* and *aroB* genes with each own T7*lac* promoter and ribosome binding site was obtained by the digestion of pPTFB with *Bam*HI and *Bsr*GI. The digestion reaction

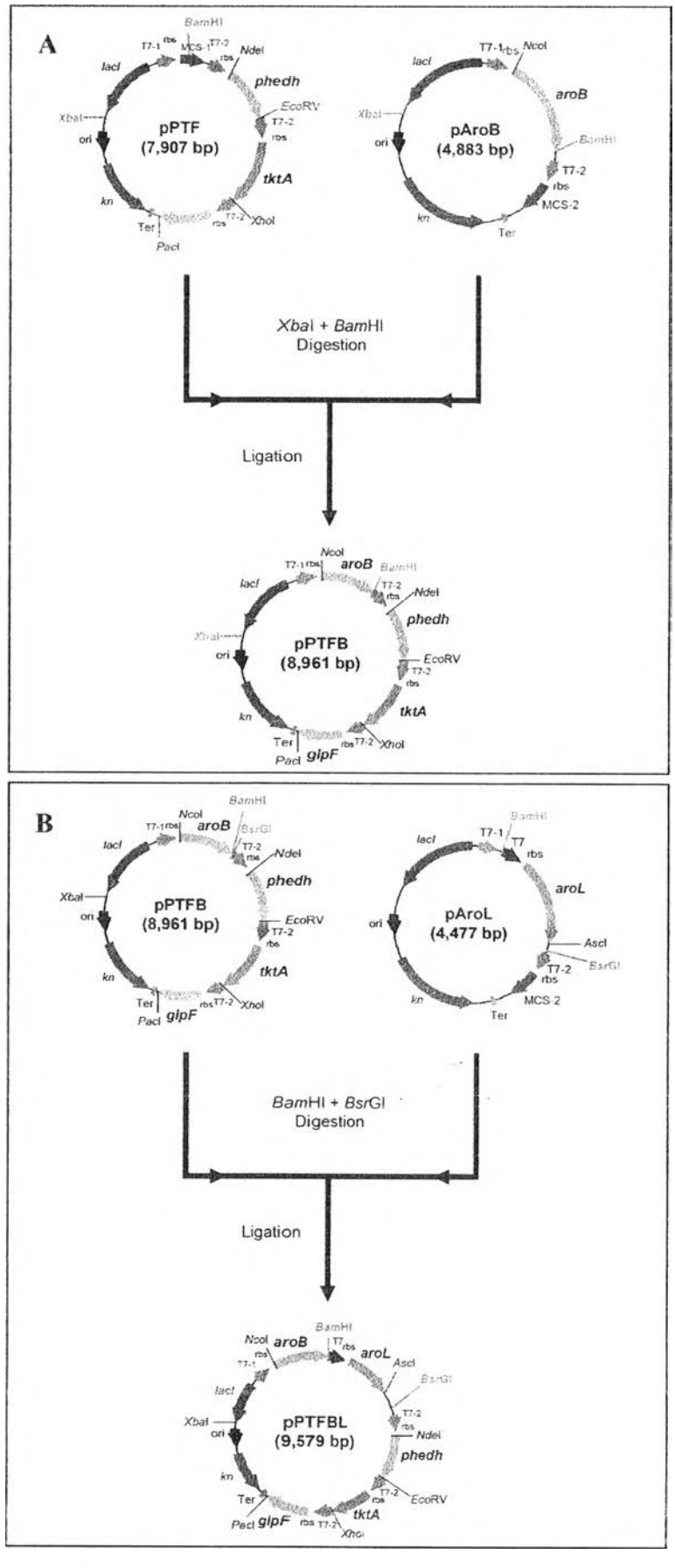


Figure 2.8 The constructions of recombinant plasmids pPTFB (A) and pPTFBL (B)

contained 1 μg of pPTFB, 1x NEBuffer 2, 100 μg/mL of BSA, 10 U of *Bam*HI and 10 U of *Bsr*GI in a total volume of 20 μL and incubated at 37 °C for 3 h. The digestion products were separated by agarose gel electrophoresis. The 8,877 bp DNA fragment was harvested from the gel and used as a vector DNA for ligation.

The short length DNA fragment (702 bp) containing the *aroL* gene linking with the T7*lac* promoter and ribosome binding site was obtained by digestion of pAroL with the same restriction enzymes as mentioned above. The 702 bp DNA fragment was collected from the gel and used as an insert DNA for ligation.

Both of the DNA fragments were linked together by ligase leading to a recombinant plasmid named as pPTFBL (9,579 bp). After that, pPTFBL was transformed into *E. coli* BL21(DE3).

2.15.6 Cloning of *yddG* gene into a recombinant plasmid pPTFBL

Since *phedh, tktA* and *glpF* genes inserted into pPTFB could be cleaved by *Hin*dIII, a recombinant plasmid pLY containing the DNA fragment of the *yddG* gene placed into downstream of *aroL* gene in pAroL was firstly prepared. This recombinant plasmid pLY was used as source of both *aroL* and *yddG* for construction of pPTFBLY from an original plasmid pPTFB.

2.15.6.1 Cloning of *yddG* gene into a recombinant plasmid pAroL

To obtain a recombinant plasmid pLY, the recombinant plasmid pAroL was used as parental plasmid for insertion of the DNA fragment (994 bp) containing the *yddG* gene and its promoter derived from pYddG digestion.

The 994 bp DNA fragment generated from the excision of pYddG with *Asc*I and *Hin*dIII was separated and purified from the agarose gel. The *Asc*I/*Hin*dIII-digested pAroL (4,429 bp) was cleaned up by kit. Both digested DNA fragments were ligated to produce recombinant plasmid pLY (5,423 bp) as shown in Figure 2.9A. After ligation and transformation, *E. coli* BL21(DE3) transformed cells were screened on LB agar plates containing 30 μg/mL of kanamycin.

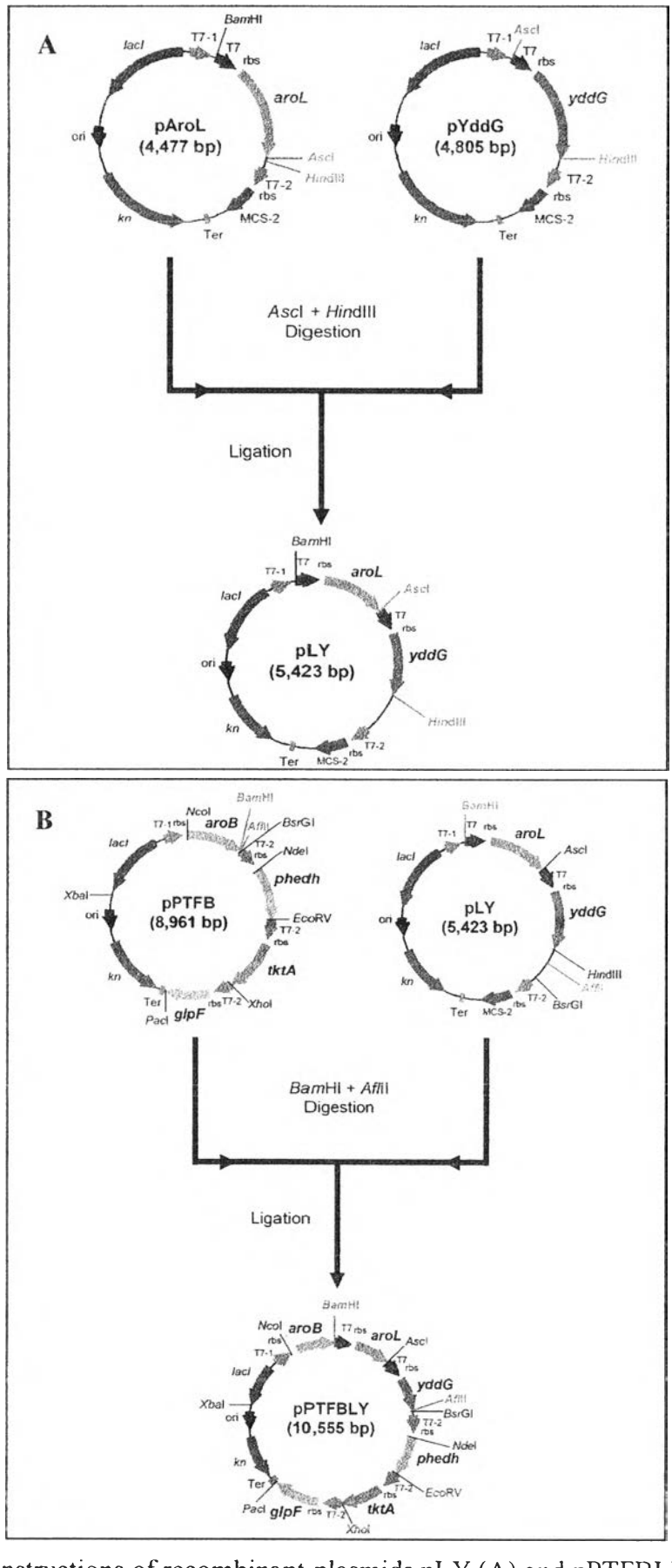


Figure 2.9 The constructions of recombinant plasmids pLY (A) and pPTFBLY (B)

2.15.6.2 Cloning of *AroL* and *yddG* genes into a recombinant plasmid pPTFB

The construction of a recombinant plasmid pPTFBLY containing p*hedh, tktA, glpF, aroB, aroL* and *yddG* genes with each own T7*lac* promoter and ribosome binding site was carried out by the ligation of the DNA fragments obtained from the digestions of pPTFB and pLY with *Bam*HI and *Afl*II. Each DNA fragment was prepared in the following manner.

The long length DNA fragment (8,904 bp) containing *phedh*, *tktA*, *glpF* and *aroB* genes attaching to each own T7*lac* promoter and ribosome binding site was received from the digestion of pPTFB with *Bam*HI and *Afl*II. The obtained fragments were separated on the agarose gel. The 8,904 bp DNA fragment was harvested from the gel and used as a vector DNA for ligation.

The short length DNA fragment (1,651 bp) containing *aroL* and *yddG* genes closed to each T7*lac* promoter and ribosome binding site was obtained by the digestion of pLY with the same restriction enzymes as mentioned above. The 1,651 bp DNA fragment was collected from the gel and used as an insert DNA for ligation.

Both vector DNA and insert DNA were linked together by ligase leading to a recombinant plasmid named pPTFBLY (10,555 bp) (Figure 2.9B). After that, pPTFBLY was transformed into *E. coli* BL21 (DE3).

2.15.7 Cloning of *pheA* gene into a recombinant plasmid pPTFBLY

As the same reason for the construction of pPTFBLY, a recombinant plasmid pPTFBLYA (pPTFBLY containing *pheA* gene) was constructed like in the section 2.15.6. The DNA fragment containing the *pheA* gene and its T7*lac* promoter could not be directly obtained by digestion of pPheA with *Hin*dIII and *Afl*II to insert into the same sites of pPTFBLY due to the fact that *phedh, tktA* and *glpF* genes could be cleaved by *Hin*dIII. Hence, a recombinant plasmid pLYA, the *pheA* gene with its promoter placed into the 3' terminus of *yddG* gene in pLY, was prepared first. This recombinant plasmid pLYA was used as source of *aroL*, *yddG* and *pheA* genes for the construction of pPTFBLYA by addition the DNA fragment containing these three genes into pPTFB.

2.15.7.1 Cloning of *pheA* gene into a recombinant plasmid pLY

To construct a recombinant plasmid pLYA, the pLY was used as parental plasmid for incorporation of the DNA fragment (1,296 bp) containing the *pheA* gene and its promoter derived from pPheA digestion. The excision of pPheA and pLY with *Hin*dIII and *Bsr*GI were separately occurred in the reaction.

Each of the obtained products was separated on the agarose gel. The 1,296 bp DNA fragment of pPheA digestion and the 5,376 bp DNA fragment of pLY digestion were harvested by kit. Both of them were ligated to produce recombinant plasmid pLYA (6,672 bp) as shown in Figure 2.10A. After ligation, pLYA was transformed into *E. coli* BL21(DE3).

2.15.7.2 Cloning of *AroL, yddG* and *pheA* genes into a recombinant plasmid pPTFB

The construction of a recombinant plasmid pPTFBLYA containing p*hedh, tktA, glpF, aroB, aroL, yddG* and *pheA* genes preceded by each own T7*lac* promoter and ribosome binding site was carried out by the ligation of the DNA fragments obtained from the digestion of pPTFB and pLYA with *Bam*HI and *Afl*II.

The long length DNA fragment (8,904 bp) containing *phedh*, *tktA*, *glpF* and *aroB* genes attaching to each own T7*lac* promoter and ribosome binding site was received from the digestion of pPTFB with *Bam*HI and *Afl*II and used as vector DNA for ligation.

The short length DNA fragment (2,900 bp) containing *aroL, yddG* and *pheA* genes closed to each T7*lac* promoter and ribosome binding site was also obtained by the digestion of pLY with *Bam*HI and *Afl*II and used as insert DNA for ligation.

Both of the DNA fragments were linked together by ligase leading to the recombinant plasmid pPTFBLYA (11,804 bp) as shown in Figure 2.10B and then pPTFBLYA was transformated into *E. coli* BL21 (DE3).

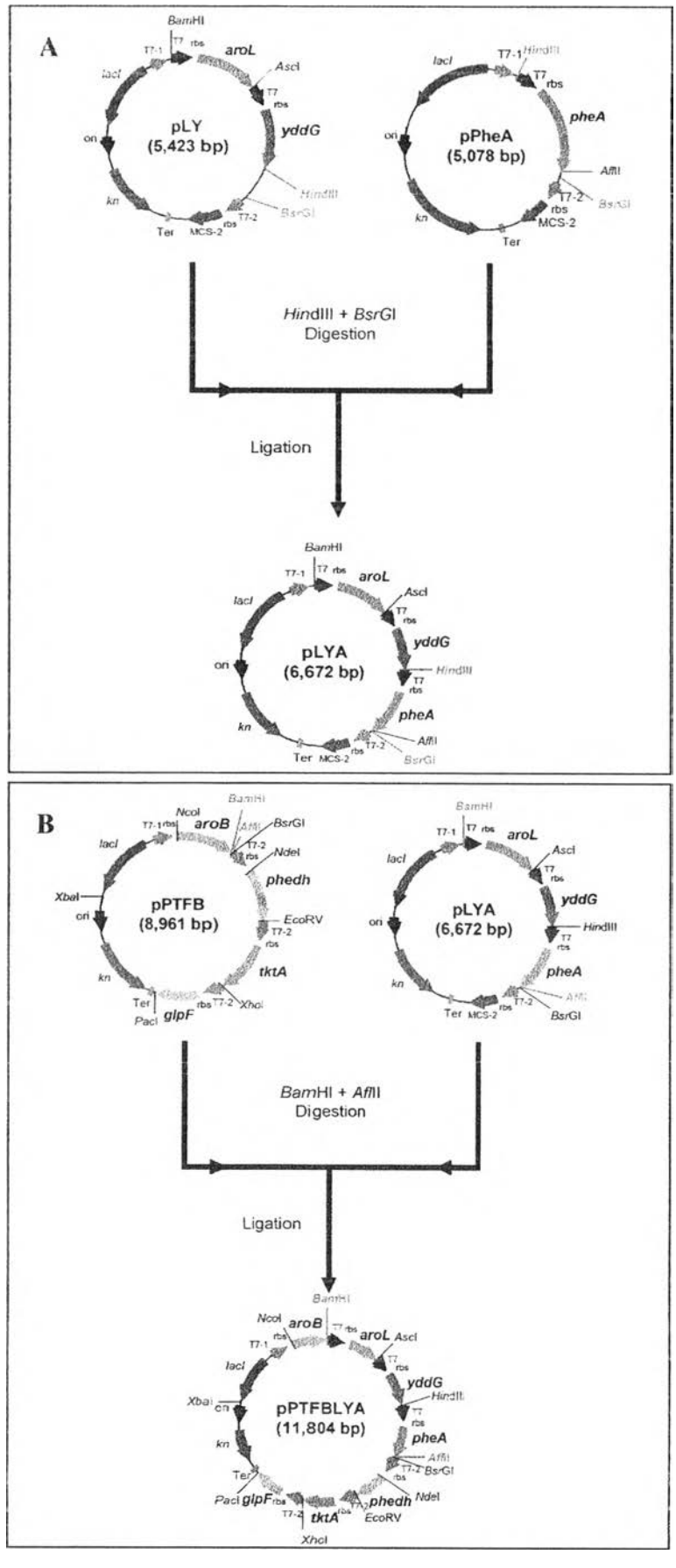


Figure 2.10 The constructions of recombinant plasmids pLYA (A) and pPTFBLYA (B)

2.15.8 Cloning of *yddG* gene into a recombinant plasmid pPheDH

To study whether only the overexpression of *yddG* gene encoding aromatic amino acid transporter could increase L-Phe production in *E. coli* containing *phedh*, a recombinant pPY containing *phedh* and *yddG* in a single pRSFDuet-1 was constructed.

To construct the recombinant plasmid pPY, the previous constructed pPheDH was used as parental plasmid for incorporation of the DNA fragment (1,033 bp) containing the *yddG* gene with its promoter derived from pYddG digestion. The pPheDH and pYddG were separately digested with *Bam*HI and *Afl*II.

Each of the reaction products was separated on the agarose gel. The 1,033 bp DNA fragment of pYddG reaction and the 4,898 bp DNA fragment of pPheDH reaction were harvested by kit. Both of them were ligated to produce the recombinant plasmid pPY (5,931 bp). The construction of pPY is schemed in Figure 2.11A. After ligation and transformation, *E. coli* BL21(DE3) transformed cells were screened on LB agar plates containing 30 μg/mL of kanamycin.

2.15.9 Cloning of *glpF* gene into a recombinant plasmid pPY

To justify if the addition of an overexpressed *glpF* gene into *E. coli* containing *phedh* and *yddG* genes could provide increase in L-Phe production, a recombinant plasmid pPYF containing three genes (*phedh*, *yddG* and *glpF* genes) was created by the ligation of the DNA fragments obtained by cleaving pPTF and pPY with *Bam*HI and *Xho*I.

After agarose gel electrophoresis, the 4,467 bp fragment obtained from pPYF digestion, containing *glpF* gene with its promoter was harvested and used as a vector DNA for ligation. The 2,350 bp DNA fragment generated from pPY digestion, containing *phedh* and *yddG* genes with its promoter was collected and used as an insert DNA for ligation. Both were ligated to produce the recombinant plasmid pPYF (6,817 bp) as shown in Figure 2.11B. After transformation into *E. coli* BL21(DE3), the transformed cells were screened on LB agar plates containing 30 μg/mL of kanamycin.

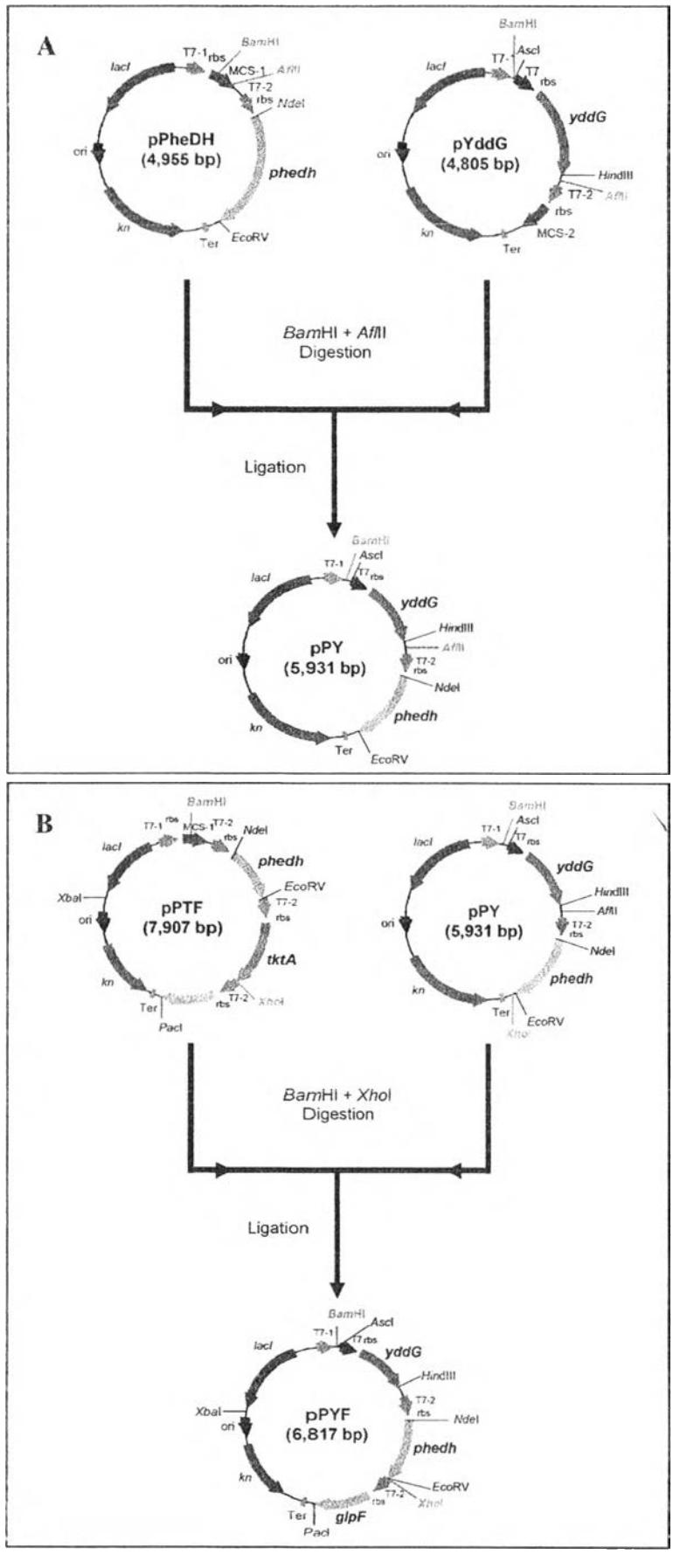


Figure 2.11 The constructions of recombinant plasmids pPY (A) and pPYF (B)

2.15.10 Cloning of *glpFK* gene into a recombinant plasmid pPY

The recombinant plasmid pPYFK containing four genes (*phedh*, *yddG*, *glpF* and *glpK* genes) was constructed by the combination of DNA fragments derived from pPTFK and pPY digested by restriction enzymes *Xho*I and *Pac*I. The digestion reaction of individual pPTFK and pPY contained the ingredients as shown in section 2.15.2.

The digested products were separately run on the agarose gel. The 2,493 bp DNA fragment containing *glpFK* gene with its own promoter from the pPYF digestion was harvested and used as an insert DNA for ligation. The 5,856 bp DNA fragment containing *phedh* and *yddG* genes with each own promoter resulted from the pPY digestion was collected and used as a vector DNA for ligation. Both were ligated as the recombinant plasmid pPYFK (8,349 bp) and then transformed into *E. coli* BL21(DE3). The construction of pPYFK is shown in Figure 2.12A. The transformants were screened on LB agar plates containing 30 μg/mL of kanamycin.

2.16 The addition of *yddG* gene into pPT, pPTF, pPTFK, pPTFB to produce pPTY, pPTFY, pPTFKY and pPTFBY

2.16.1 The construction of recombinant plasmid pPTY

To prepare a recombinant plasmid pPTY containing *phedh*, *tktA* and *yddG* genes with each own T7*lac* promoter, the pPT and pPY were used as source of these genes.

Each plasmid (i.e. pPT and pPY) was digested with *Bam*HI and *Eco*RV. After the digested products were separated on agarose gel electrtophoresis, the 5,682 bp DNA fragment resulted from the pPY digestion containing the *glpF* gene and its promoter was recovered from the gel and used as a vector DNA for ligation. An insert DNA was the 2,315 bp DNA fragment derived from *Bam*HI/*Eco*RV-digested pPY containing the *phedh* and *yddG* genes preceded by each own promoter. These two DNA fragments were ligated as circular DNA designated as pPTY (7,997 bp) as shown in Figure 2.12B.

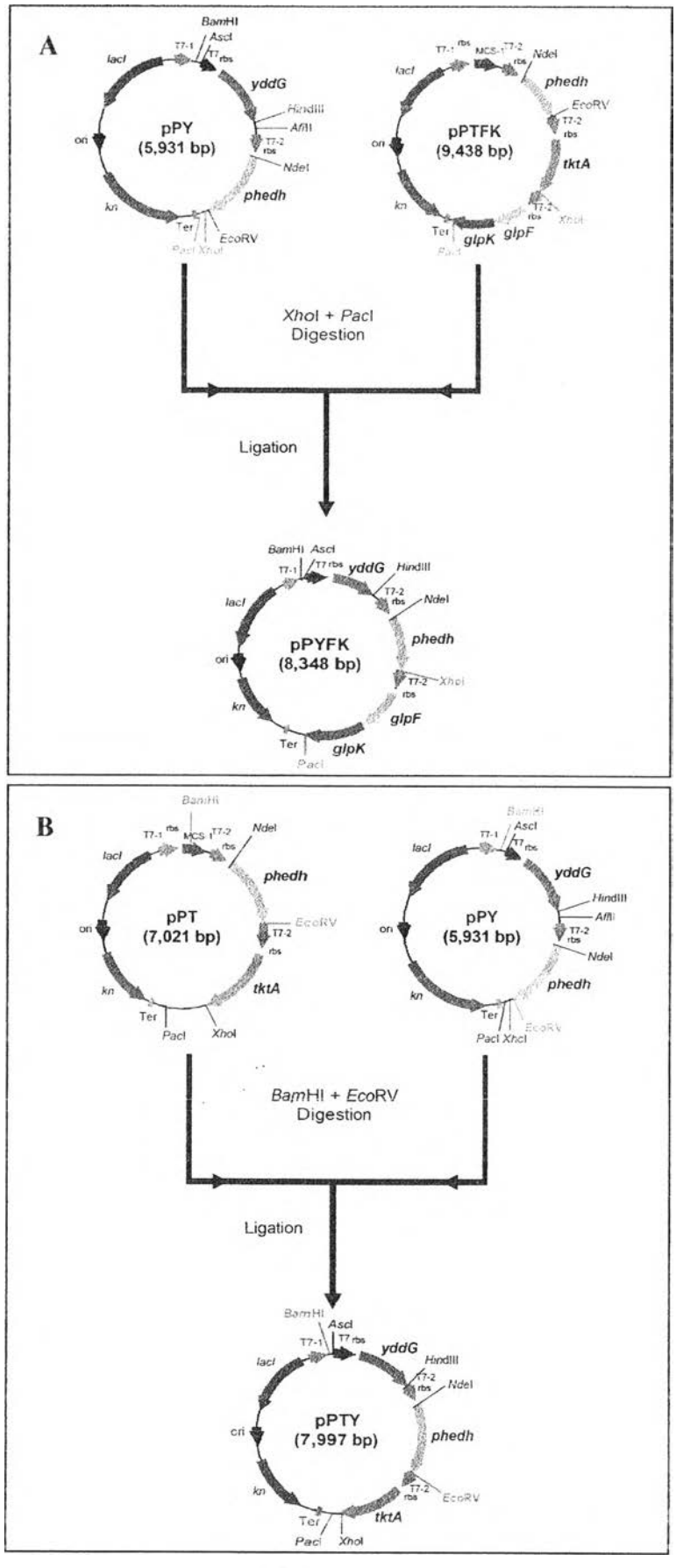


Figure 2.12 The constructions of recombinant plasmids pPYFK (A) and pPTY (B)

2.16.2 The construction of recombinant plasmid pPTFY

The recombinant plasmid pPTFY was generated by the combination of genes from pPTY and pPYF.

For pPTFY construction, pRSFDuet-1 vector inserted with the *glpF* gene in which each terminus was digested with restriction enzymes *Bam*HI and *Xho*I was used as a vector DNA (4,467 bp). pPYF was excised by *Bam*HI and *Xho*I. DNA fragment (3,440 bp) containing *phedh* and *yddG* genes was excluded from the vector DNA by agarose gel electrophoreis. Similarly, an insert DNA (4,416 bp) containing the *phedh, tktA* and *yddG* genes including individual own promoter was prepared by digestion of pPTY with the same restriction enzymes. After both fragments were harvested from the gel, they were ligated leading to the recombinant plasmid pPTFY (8,883 bp) as shown in Figure 2.13A.

2.16.3 The construction of recombinant plasmid pPTFKY

The recombinant plasmid pPTFKY containing *phedh, tktA, glpF, glpK* and *yddG* genes was generated by the combination of genes from pPTFK and pYddG.

For pPTFKY construction, pPTFK was digested with *Asc*I and *Afl*II to create a vector DNA (9,400 bp), pRSFDuet-1 vector inserted with the *phedh, tktA, glpFK* closed to each own T7*lac* promoter between *Nde*I and *Pac*I sites in consecutive order. An insert DNA (1,014 bp) containing the *yddG* genes with its own promoter was prepared by digestion of pYddG with the same above restriction enzymes. After both of these desired fragments had been harvested from the gel, they were ligated leading to the recombinant plasmid pPTFKY (10,414 bp) as shown in Figure 2.13B.

2.16.4 The construction of recombinant plasmid pPTFBY

The recombinant plasmid pPTFBY containing *phedh, tktA glpF, aroB* and *yddG* genes with each own T7*lac* promoter was constructed. The previous constructed pPTFB and pPTY were used as source of these genes.

An insert DNA (4,416 bp) containing the *phedh, tktA* and *yddG* genes including individual own promoter was prepared. A vector DNA (5,186 bp), pRSFDuet-1 vector inserted with the *glpF* gene and its T7*lac* promoter at *Xho*I-*Pac*I sites and the *aroB* gene at *Nco*I-*Bam*HI sites, was prepared by cleaving pPTFB with

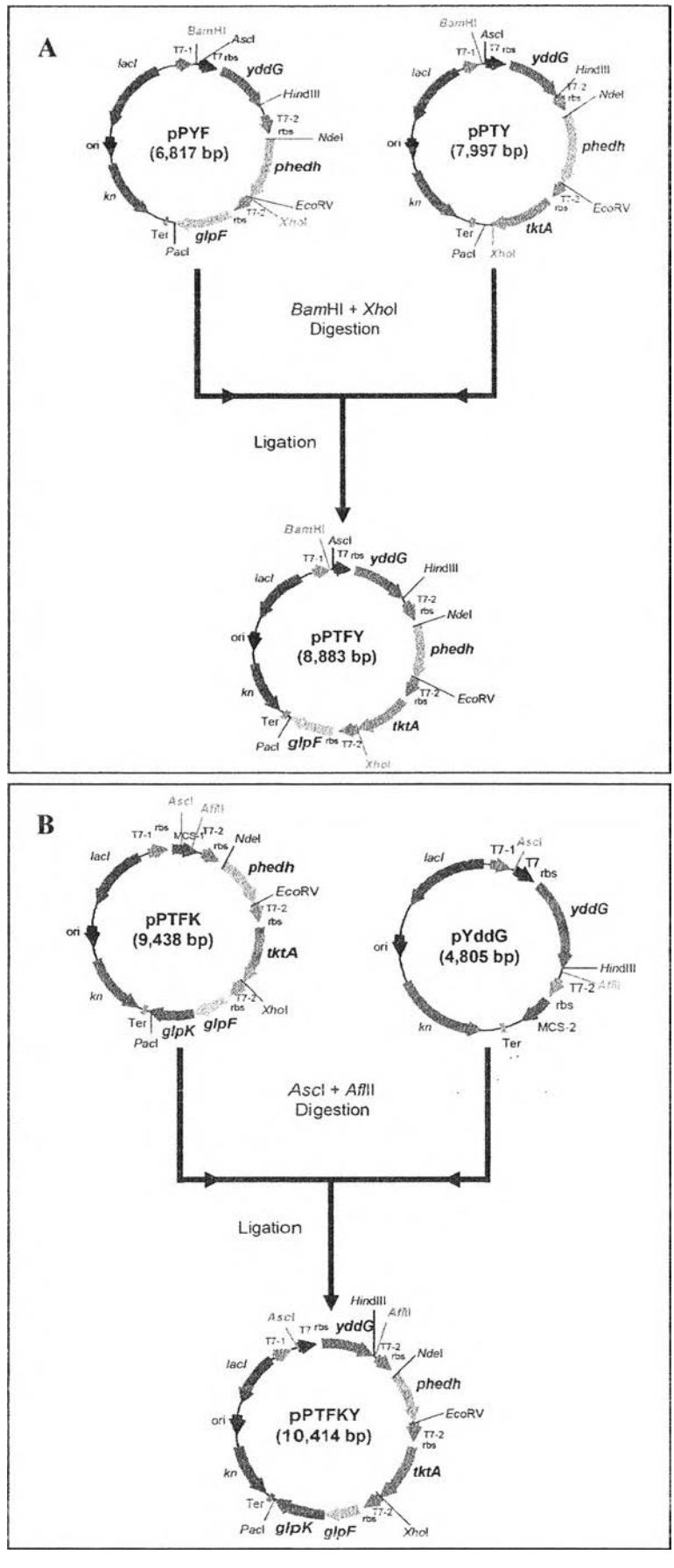


Figure 2.13 The constructions of recombinant plasmids pPTFY (A) and pPTFKY (B)

*Bam*HI and *Xho*I. Both fragments were harvested from the gel and ligated to produce the recombinant plasmid pPTFBY (9,602 bp) as illustrated in Figure 2.14.

These four recombinant plasmids (i.e. pPTY, pPTFY, pPTFKY and pPTFBY) were separately transformed into *E. coli* BL21(DE3) prior to plating on LB agar containing 30 μg/mL of kanamycin.

2.17 Expression of recombinant plasmids pPY, pPYF, pPYFK, pPTY, pPTFY, pPTFKY, pPTFBY, pPTFBLY and pPTFBLYA in *E. coli* BL21(DE3)

Prior to optimization of L-Phe production by culturing in production medium, five transformants of each of nine recombinant clones (pPY, pPYF, pPYFK, pPTY, pPTFY, pPTFKY, pPTFBY, pPTFBLY and pPTFBLYA) were examined for their expected gene expression in LB medium by SDS-PAGE, and the highest over-expression of each genes was selected.

2.18 Shake flask cultures

Ten recombinant clones (i.e. pPheDH, pPY, pPYF, pPYFK, pPTY, pPTFY, pPTFKY, pPTFBY, pPTFBLY and pPTFBLYA) showing intense desirable protein bands that were overexpressed from genes inserted in its recombinant plasmid in SDS-PAGE gel derived from above sections (exception for pPheDH clone that was selected from its highest PheDH activity) were examined for their ability to produce L-Phe when they were grown in the minimum medium containing glycerol as the carbon source.

2.18.1 Optimization of production medium composition for L-Phe production

The basic culture medium for L-Phe production contained carbon source, nitrogen source, salts, vitamins, and trace elements. In this work, glycerol and $(NH_4)_2SO_4$ were used as carbon and nitrogen sources, respectively. This medium, pH 7.4 contained (g/L): glycerol 10; $(NH_4)_2SO_4$ 50; $MgCl_2 \cdot 6H_2O$ 0.81; KH_2PO_4 2.43;

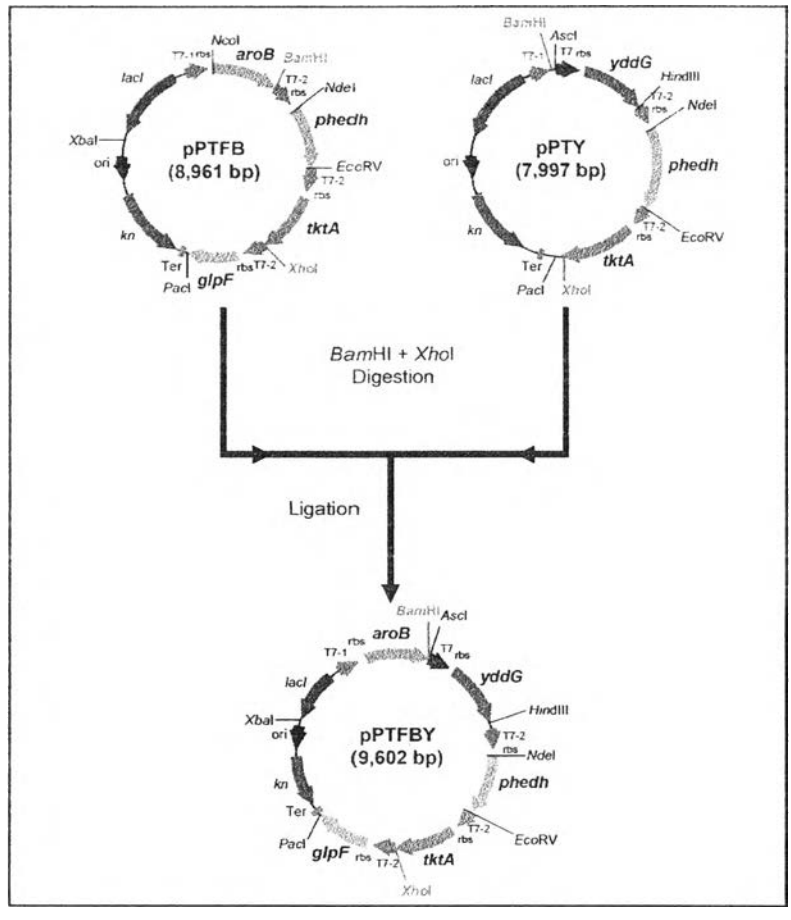


Figure 2.14 The construction of recombinant plasmid pPTFBY

K_2HPO_4 2.43; yeast extract 0.085; thiamine-HCl 0.0085; $FeSO_4 \cdot 7H_2O$ 0.002; $MnSO_4 \cdot H_2O$ 0.002; $CaCl_2 \cdot 2H_2O$ 0.05; $ZnSO_4 \cdot 7H_2O$ 0.01 (Khamduang et al., 2009b). The $MgCl_2 \cdot 6H_2O$ solution and the thiamine-HCl solution were sterilized separately. The first one was autoclaved at 121 °C for 15 min and the latter was filtered through a 0.22 μm membrane filter. These were added into cooled medium prior to inoculation.

To obtain the optimum composition of production medium, this work was focused only on the concentration of carbon and nitrogen sources (glycerol and $(NH_4)_2SO_4$. The *E. coli* recombinant clone containing pPheDH was used as a model.

2.18.1.1 Optimization of glycerol concentration in production medium

Shake flask cultures were used to examine the L-Phe production of the *E. coli* BL21 (DE3) containing pPheDH. A fresh single colony from LB agar plate was resuspended in 100 mL of LB medium (pH 7.4, supplemented with 30 μg/mL of kanamycin) and grown for 18 h (37 °C, 250 rpm). Overnight seed culture (10 mL) was inoculated to 200 mL of the production medium, pH 7.4 containing (g/L): glycerol 10-50; $(NH_4)_2SO_4$ 50; $MgCl_2 \cdot 6H_2O$ 0.81; KH_2PO_4 2.43; K_2HPO_4 2.43; yeast extract 0.085; thiamine-HCl 0.0085; $FeSO_4 \cdot 7H_2O$ 0.002; $MnSO_4 \cdot H_2O$ 0.002; $CaCl_2 \cdot 2H_2O$ 0.05; $ZnSO_4 \cdot 7H_2O$ 0.01; and 30 μg/mL of kanamycin. Incubated was at 37 °C, 250 rpm until OD_{600} reached 0.6 measured. IPTG was then added to the final concentration of 1 mM and incubation was continued. Samples were withdrawn at every 24 h until 240 h, and OD_{600} was measured. For measurements of glycerol and L-Phe concentrations, a 5 mL aliquot of each culture broth was centrifuged at 8,000xg for 15 min to remove the cells and each supernatant was monitored for pH of the broth. The remaining supernatant was maintained at -20 °C for further analysis. The experiments were carried out in duplicate.

2.18.1.2 Optimization of $(NH_4)_2SO_4$ concentration in production medium

The pPheDH recombinant clone was cultured as described in section 2.18.1.1. The production medium (pH 7.4) contained (g/L): glycerol 30; $(NH_4)_2SO_4$ 10-50; $MgCl_2 \cdot 6H_2O$ 0.81; KH_2PO_4 2.43; K_2HPO_4 2.43; yeast extract 0.085; thiamine-

HCl 0.0085; $FeSO_4 \cdot 7H_2O$ 0.002; $MnSO_4 \cdot H_2O$ 0.002; $CaCl_2 \cdot 2H_2O$ 0.05; $ZnSO_4 \cdot 7H_2O$ 0.01; and 30 μg/mL of kanamycin.

The optimum concentrations of glycerol and $(NH_4)_2SO_4$ from section 2.18.1.1 and 2.18.1.2 were used as components of production medium for further shake-flask cultivation.

2.18.1.3 Optimization of IPTG concentration for L-Phe production

The L-Phe productions of all nine *E. coli* BL21(DE3) recombinant clones (pPY, pPYF, pPYFK, pPTY, pPTFY, pPTFKY, pPTFBY, pPTFBLY and pPTFBLYA) in comparison with production by the basal clone, *E. coli* BL21 (DE3) containing pPheDH, were examined in the production medium by varying concentration of inducer or IPTG.

One colony of freshly grown clones from LB agar plates was cultivated according to section 2.18.1.1 The production medium contained 30 g/L of glycerol and 50 g/L of $(NH_4)_2SO_4$ and other components as given above. After OD_{600} reached 0.6, IPTG was added to different final concentrations (0, 0.25, 0.5, 1 mM) and incubation was continued. The samples were collected as section 2.18.1.1. In addition, a 1 mL sample of each broth was centrifuged at 12,000xg for 5 min to get cell pellet to determine the expressed proteins as mentioned in section 2.14. All experiments were carried out in duplicate or triplicate.

2.19 Analytical methods

2.19.1 Biomass concentration

The following method was used to measure the dry cell weight. Firstly, a 0.22 μm membrane filter, 47 mm in diameter was placed on Petri dish and dried in an oven at 105 °C for 24 h. After cooling down in a desiccator, the dried filter was weighed and stored in the desiccator until used. After the cells had reached the stationary phase, they were separated from the broth by filtration. For filtration, a 5 mL aliquot of the culture was poured into the holding reservoir fitted on the preweighed membrane filter and vacuum was applied. A few mL of water was

added to rinse and the cell paste trapped on the membrane filter was completely dried in an oven for 24 h. After cooling down, the filter plus the cell paste was weighed. The cell dry weight was calculated in g/L.

The biomass concentration in the culture broth was monitored by measuring OD_{600}. A calibration curve (Appendix F) is used to convert the OD to the dry biomass concentration. The equation of the calibration curve was Dry biomass concentration (g/L) = $0.99 \times OD_{600}$.

2.19.2 Glycerol concentration

The residual glycerol in the supernatant was determined by A_{410} of the periodate oxidation method (Bondioli and Bella, 2005). The working reagents for glycerol determination are described in Appendix G. The supernatant sample containing glycerol was appropriately diluted with working solution so that its concentration is in the range of the standards. Sodium periodate solution (1.2 mL) was added to the diluted sample (2 mL) and thoroughly shaken for 30 sec with vortex. After that, acetylacetone solution (1.2 mL) was added, then incubated at 70 °C for 1 min. The sample was immediately cooled by immersing the tube in a plastic basin containing tap water which was maintained at 20-25 °C for 30 min. The A_{410} is measured to glycerol concentration using a standard curve (Appendix H). The concentration of glycerol was calculated using equation: Glycerol concentration (g/L) = $0.0486 \times A_{410}$.

2.19.3 L-phenylalanine concentration

For measuring L-phenylalanine concentration, the culture supernatant was filtered through a 0.2 μm syringe filter. The concentration of L-Phe was then quantitatively measured by HPLC using a CROWNPAK CR(+) column equipped with a UV absorbance detector at 200 nm. The column temperature was maintained at room temperature. The mobile phase was 85: 15 v/v mixture of perchloric acid (pH 1.0) and methanol, with the flow rate of 0.8 mL/min. The injected sample volume was 20 μL. L-Phenylalanine is quantified by comparison with the calibration curve (Appendix I).

2.20 Fermenter cultures

The appropriate IPTG concentration used to yield the highest production of L-Phe of each *E. coli* recombinant clone in previous shake flask cultures was used in fermenter cultures.

2.20.1 Batch cultivation

2.20.1.1 Inoculum preparation

Luria-Bertani medium is used as the seed medium. One fresh colony of *E. coli* BL21(DE3) recombinant clone grown on LB agar plate was resuspended into 100 mL of LB medium, pH 7.4, containing 30 μg/mL of kanamycin. Incubation was at 37 °C, 250 rpm for 18 h. An overnight culture was diluted with fresh LB medium to bring OD_{600} to 0.6-0.8. This served as the inoculum for the fermenter cultures.

2.20.1.2 Batch cultivation condition

Batch fermentations were conducted in a 5 L glass stirred tank bioreactor (Figure 2.15). The above prepared inoculum (10% of working volume) was transferred to the bioreactor that had been prefilled with 3.5 L of an optimized production medium as specified in the section of shake flask cultures. The cells were grown aerobically and the aeration rate was 1 vvm (3.5 L/min). The incubation temperature was 37 °C and mixing speed of 400 rpm. The dissolved oxygen concentration was monitored using a polarographic electrode. The pH of the broth was controlled at 7.4 by automated addition of 5 M NaOH in response signals from the pH electrode. Foaming was controlled by automated addition of a sterile silicon antifoam agent. Once the OD_{600} reached 0.6, the appropriate concentration of IPTG was added. Samples were taken at 4-h intervals for 84 h after induction to measure OD_{600}. For the other analyses, the samples were centrifuged at 12,000xg for 5 min and the supernatants were kept at -20 °C.

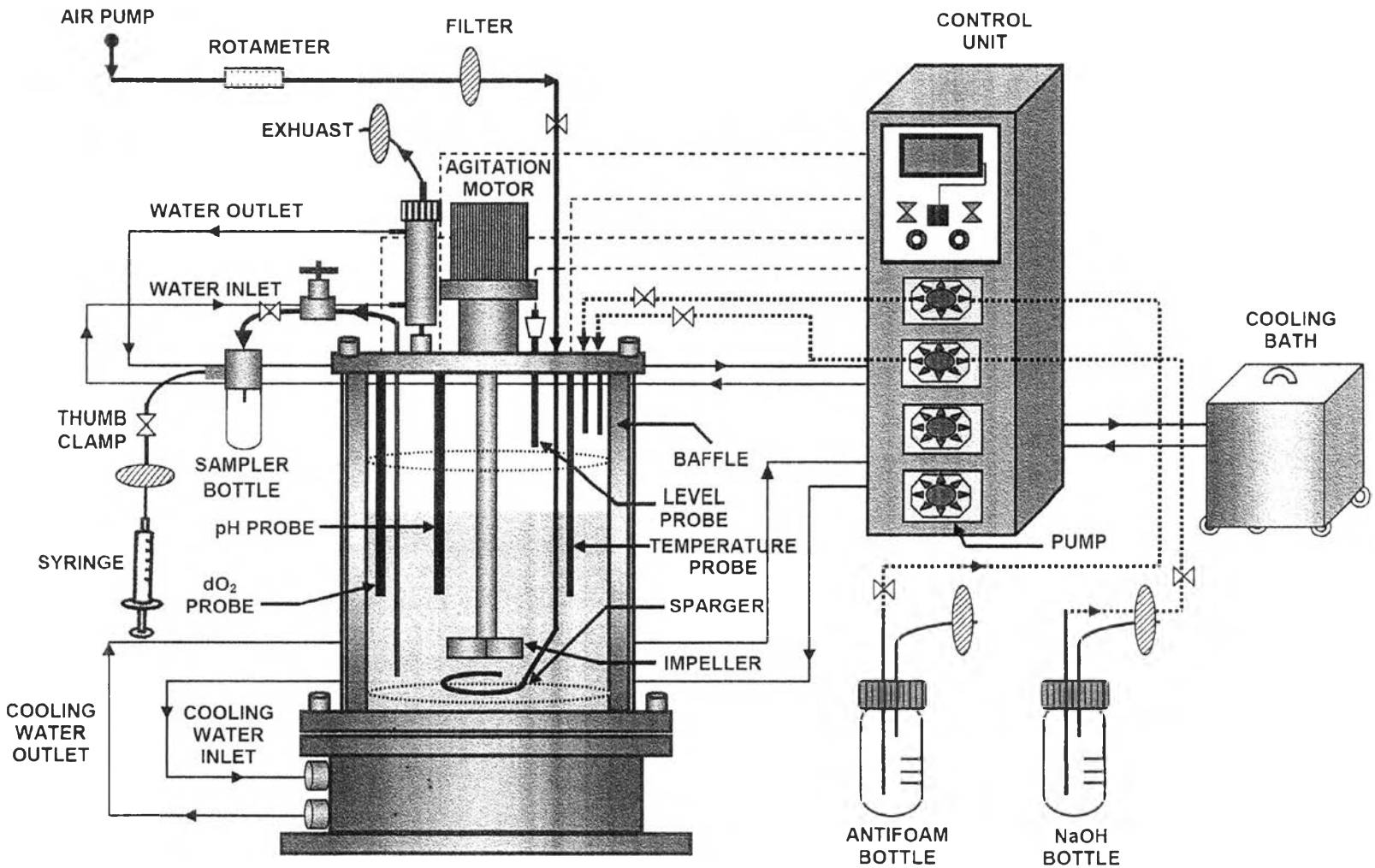


Figure 2.15 Fermentation system

2.20.2 Fed-batch cultivation

The starter culture was prepared according to section 2.20.1.1. The fermentation was started with a relatively low initial volume of 3 L using exactly the same condition as used in the batch culture (section 2.20.1.2).

Before glycerol in the medium was mostly consumed, feeding of new concentrated medium had started. The feeding medium contained 200 g/L of glycerol and other media components in the same proportion of batch medium or shake flask medium (Table 2.4), except for the concentrations of IPTG and kanamycin (previously determined optimal concentration and 30 μg/mL, respectively, were used) After 40 h, the fed-batch cultivation was started by addition of the new concentrated medium (200 mL) to the fermenter, then at 60 h another lot (200 mL) was added. A air flow rate of 1 vvm and agitation speed of 400 rpm were used throughout the experiment. The medium was continuously withdrawn from the bioreactor every 4 h until 120 h. The samples were analyzed for biomass concentration, glycerol depletion and L-Phe production as previously explained.

Table 2.4 The compositions of batch culture medium and feeding medium

Component	Batch medium (g/L)	Feeding medium (g/L)
glycerol	30	200
$(NH_4)_2SO_4$	50	333.33
$MgCl_2 \cdot 6H_2O$	0.81	5.4
KH_2PO_4	2.43	16.2
K_2HPO_4	2.43	16.2
yeast extract	0.085	0.57
thiamine-HCl	0.0085	0.057
$FeSO_4 \cdot 7H_2O$	0.002	0.013
$MnSO_4 \cdot H_2O$	0.002	0.013
$CaCl_2 \cdot 2H_2O$	0.05	0.33
$ZnSO_4 \cdot 7H_2O$	0.01	0.067