CHAPTER III
RESULTS AND DISCUSSIONS

In our previous experiment, to improve L-Phe production from phenylpyruvate substrate by resting cell of *E. coli* BL(DE3) expressing PheDH. The *kgtP* gene encoding α-ketoglutarate permease that might be responsible for an increase in phenylpyruvate uptake into *E. coli* cell was selected to co-express with *phedh* in *E. coli* BL(DE3). The *kgtP* gene was cloned into pET-17b and the recombinant plasmid obtained was used as a source for amplification of the *kgtP* gene preceded by T7 promoter. After transformation into *E. coli* BL21(DE3), colony that could grow on selective plate containing ampicillin was hardly found. It was found that certain morphology of recombinant *E. coli* colony on agar plate was changed, and it appeared irregular in shape and the elevation of colony was seen to be flat with undulate margin. The altered morphology led to terrible retardation of cell growth in LB broth. This incident might be caused by the basal level expression in the absence of induction due to some expression of T7 RNA polymerase from the *lac*UV5 promoter in *E. coli* BL21(DE3) host. If that gene product is sufficiently toxic to host cell, this basal level expression can be enough to interfere with normal function of cell and restrain cell growth (Novagen, 2003: online). In 1996, Miroux and Walker investigated the over-production of seven membrane proteins in an *E. coli*-bacteriophage T7 RNA polymerase expression system. It was reported that most of the BL21(DE3) host cells died when genes encoding membrane proteins cloned into pET were over-expressed by induction with IPTG suggesting that over-production of proteins in this expression system is either limited or prevented by bacterial cell death. Moreover, the toxicity of the expression plasmids prevented transformation into this host. Therefore, pET-17b was not suitable for membrane protein expression.

The transmembrane proteins YddG and GlpF are relatively hydrophobic and are believed to be toxic to cells when expressed at a high level. Therefore, both genes typically need to be placed under stringent regulation. The control by T7*lac* promoter is stricter than that of the T7 promoter. Most toxic proteins are expressed well under control of the T7*lac* promoter in BL21(DE3). In addition, the strict gene expression is

necessary to evaluate protein folding kinetics and metabolic rates, as well as to propagate proteins that are toxic to *E. coli* (Gruber et al., 2008). Thus, to ensure the sufficient expression of *lac* repressor, pRSFDuet-1 vector carrying T7*lac* promoter and the *lacI* gene is useful in the BL21(DE3) host to reduce the basal transcription level (leakiness) of the proteins in the absence of induction with IPTG and help in keeping stable a plasmid containing toxic genes (Mierendorf et al., 1994: online) Therefore, *phedh* gene from *B. lentus* was subcloned into pRSFDuet-1 vector.

3.1 Subcloning of phenylalanine dehydrogenase gene

3.1.1 Plasmid extraction

pBLPheDH (4,388 bp), pET-17b inserted with *B. lentus phedh* under T7 promoter, was extracted from *E. coli* BL21(DE3) host as described in section 2.9.1. The concentration of the obtained plasmid was examined on agrose gel electrophoresis. Generally, a yield of approximately 50 ng/μL of the pBLPheDH concentration was obtained from 5 mL of cultured bacterial cells together with the elution step by 30 μL of ultrapure water. However, this yield depended on the amount of copy number of plasmid. The plasmid solution was used as a DNA template for *phedh* gene PCR amplification.

3.1.2 PCR amplification of *phedh* gene

The *phedh* gene (1,143 bp) was cloned into recognition sites *Nde*I and *Eco*RV of pRSFDuet-1 vector. After *phedh* amplification, the 3'-end of PCR fragment was spontaneously compatible with blunt end of *Eco*RV-digested pRSFDuet-1. When theses sites had been ligated as GAT$^{\downarrow}$ATC, it was able to be digested by *Eco*RV. The PCR product of approximately 1.1 kb which correlated to the expected length of *phedh* was amplified from pBLPheDH at different annealing temperatures as shown in Figure 3.1A. All annealing temperatures showed high intensity of specific band of *phedh* with non-specific DNA fragments of higher size. These PCR products were pooled and run on the agarose gel to separate the desired fragment through the instruction of gel/PCR DNA fragment extraction kit.

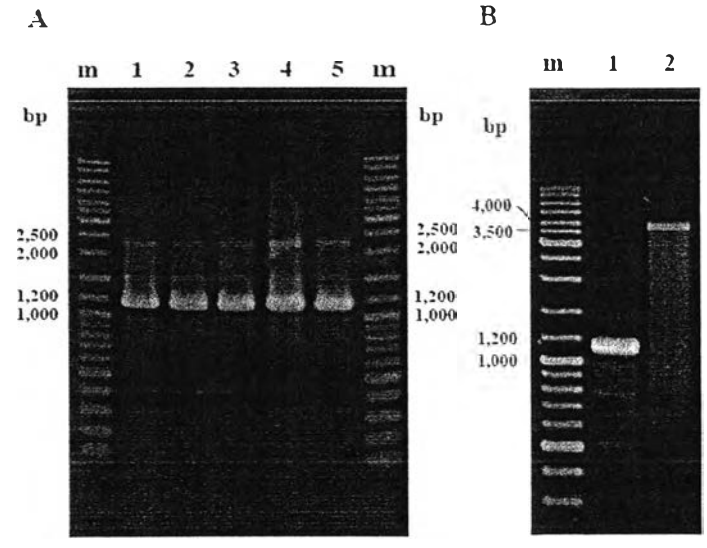


Figure 3.1 Electrophoretic patterns of recombinant plasmid pPheDH

A: PCR products of *phedh* amplification using various annealing temperatures

Lane m = 100 bp DNA ladder

Lane 1 = annealing temperature of 58.5 °C

Lane 2 = annealing temperature of 60.7 °C

Lane 3 = annealing temperature of 62.9 °C

Lane 4 = annealing temperature of 64.9 °C

Lane 5 = annealing temperature of 66.6 °C

B: Pattern of digested fragments

Lane m = 100 bp DNA ladder

Lane 1 = *Nde*I-digested PCR product of *phedh* gene

Lane 2 = *Nde*I/*Eco*RV-digested pRSFDuet-1

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

The purified PCR product of *phedh* gene was only digested with *Nde*I. In the meantime, pRSFDuet-1 was digested with *Nde*I and *Eco*RV (Figure 3.1B). Both of the digested fragments were joined by ligase resulting in a recombinant plasmid pPheDH (Figure 2.1A). After transformation into *E. coli* BL21(DE3) by electroporation, the recombinant clones were screened on LB agar plates containing 30 μg/mL of kanamycin and incubated at 37 °C for 18 h as described in 2.9.4.1 to 2.9.4.4, respectively.

For selection of recombinant clone as described in section 2.9.4.5.1, the colonies containing recombinant plasmid with slower mobility on the agarose gel than that of parental plasmid (pRSFDuet-1) were predicted to be a recombinant plasmid inserted with *phedh* (Figure 3.2). The mobilities of recombinant plasmids from various clones (lane 1-16) were compared with that of pRSFDuet-1 vector (lane C). For colony in lane 5, its recombinant plasmid seemed to be generated from self ligation of vector. Thus, all of these colonies except colony of lane 5 were cultivated in LB broth containing kanamycin. The plasmid of each colony was extracted and digested *Nde*I and *Eco*RV. The digestion products were characterized by agarose gel electrophoresis. In case of successful cloning, pPheDH (4,955 bp) digested with *Nde*I and *Eco*RV had to comprise of two DNA fragments: 3,808 bp (pRSFDuet-1 part) and 1,147 bp (*phedh* gene). Later on, eighteen recombinant clones harbouring correct size of recombinant plasmid were chosen to assay for their PheDH activity.

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

For expression of the *E. coli* BL21(DE3) transformants harbouring *phedh*, 1 mM IPTG was added to induce *phedh* expression. Generally, for pET constructions carrying the "plain" T7 promoter, a final concentration of 0.4 mM IPTG is recommended for full induction, while 1 mM IPTG is recommended for full induction with vectors having the T7*lac* promoter (Novagen, 2003: online).

After crude extracts of each of eighteen recombinant clones had been prepared, their PheDH activity in the direction of an oxidative deamination was assayed. *E. coli* BL21(DE3) with and without pRSFDuet-1 were used as references as specified in 2.9.5. The result was shown in Table 3.1. The clones showed different

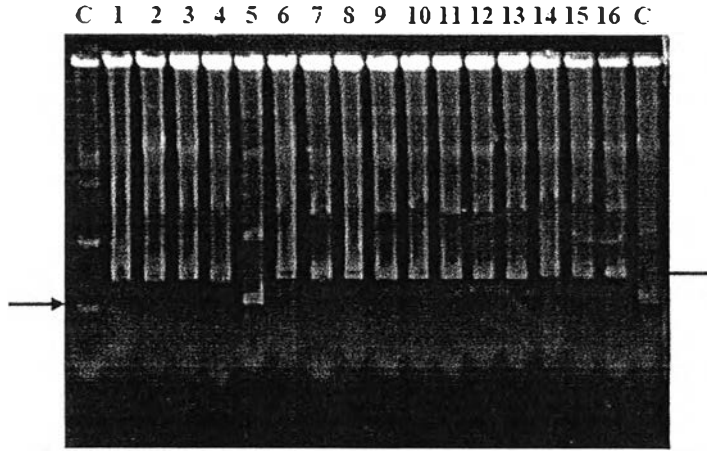


Figure 3.2 Electrophoretic patterns of rapid selection of pPheDH clone.
The arrows showed the positions of supercoiled form of plasmids.

Lane C = *E. coli* BL21(DE3)/pRSFDuet-1
Lane 1-4, 6-16 = putative *E. coli* BL21(DE3)/pPheDH
Lane 5 = nonputative *E. coli* BL21(DE3)/pPheDH

Table 3.1 Phenylalanine dehydrogenase activity from each crude extract of *E. coli* BL21(DE3) transformants*

Source	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
Bacillus lentus	2,230	2,170	1.0**
E. coli BL21(DE3)/pBLPheDH	6,937	89.0	77.9***
E. coli BL21(DE3)	0	78.3	0.0
E. coli BL21(DE3)/pRSFDuet-1	0	41.6	0.0
Transformant No.1	4,992	81.2	61.5
Transformant No.2	**5,491**	**76.5**	**71.7**
Transformant No.3	4,299	77.1	55.7
Transformant No.4	4,992	83.7	59.7
Transformant No.5	5,325	90.4	58.9
Transformant No.6	5,158	91.7	56.3
Transformant No.7	2,413	59.6	40.5
Transformant No.8	5,276	96.0	54.9
Transformant No.9	4,659	94.1	49.5
Transformant No.10	5,325	95.3	55.9
Transformant No.11	5,657	108.7	52.1
Transformant No.12	2,219	47.8	46.4
Transformant No.13	5,949	104.3	57.0
Transformant No14	3,883	94.0	41.3
Transformant No.15	3,550	101.1	35.1
Transformant No.16	4,825	100.4	48.1
Transformant No.17	5,713	102.8	55.6
Transformant No.18	5,325	98.1	54.3

*Crude extracts were prepared by culturing each *E. coli* cells in 200 mL of LB medium in the presence of 1 mM IPTG for 3 h.

**This was obtained by culturing *B. lentus* with the optimum condition for enzyme production; peptone medium (2 L), pH 7.0 supplemented with 0.4% L-Phe at 37 °C for 18 h. The peptone medium consisted of 1% peptone, 0.2% sodium chloride, 0.01% magnesium sulfate and 0.01% yeast extract (Inkure, 2005).

***The soluble crude extract was prepared by cultivation of recombinant *E. coli* BL21(DE3) containing pBLPheDH in 200 mL of LB medium induced by 0.4 mM IPTG for 4 h (Thongchuang, 2006).

levels of the specific activity in the range of 35.1 to 71.7 units/mg protein. The highest specific activity was reached by *E. coli* BL21(DE3)/pPheDH transformant No. 2 which was 71.7-fold higher than that of *Bacillus lentus* (Inkure, 2005). The specific PheDH activity of pBLPheDH recombinant clone was 77.9 units/mg protein (Thongchuang, 2006). It was found that the PheDH activity obtained from pET-17b (pBLPheDH) did not significantly differ from that of using pRSFDuet-1 (pPheDH). The copy number of pET-17b with T7 promoter is 40 per cell while that of pRSFDuet-1 with T7*lac* promoter is more than 100 per cell.

The nucleotide sequence of inserted *phedh* in pPheDH from *E. coli* BL21(DE3) transformant No. 2 was verified by DNA sequencing. The obtained sequence consisted of a 1,143 bp open reading frame of *phedh* specifying a protein of 380 amino acid residues. It was 100% identical to *phedh* gene from *B. lentus*. Figure 3.3 showed sequence of *phedh* gene inserted between *Nde*I and *Eco*RV sites of pRSFDuet-1. This recombinant clone (pPheDH) was used as parental plasmid as well as a source for a basal level of L-Phe production.

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

3.2.1 Chromosomal DNA extraction of *E. coli* TOP10

From agarose gel electrophoresis, the extracted chromosomal DNA from *E. coli* TOP10 showed high purity of molecular weight over 23.1 kb without RNA and protein contamination. This significantly corresponded to the purity assessed by A_{260}/A_{280} ratio which was around 1.8 to 2.0. It was also indicated that the DNA solution had less phenol and protein contaminant.

3.2.2 Template preparation for PCR amplification

At the beginning of this work, the complete genome sequence of *E. coli* BL21(DE3) (Genbank accession no. CP001509) used as host strain was still not published. Therefore, the complete genome sequence of *E. coli* DH10B (Genbank accession no. CP000948.1) was used for primers design in amplification of genes of

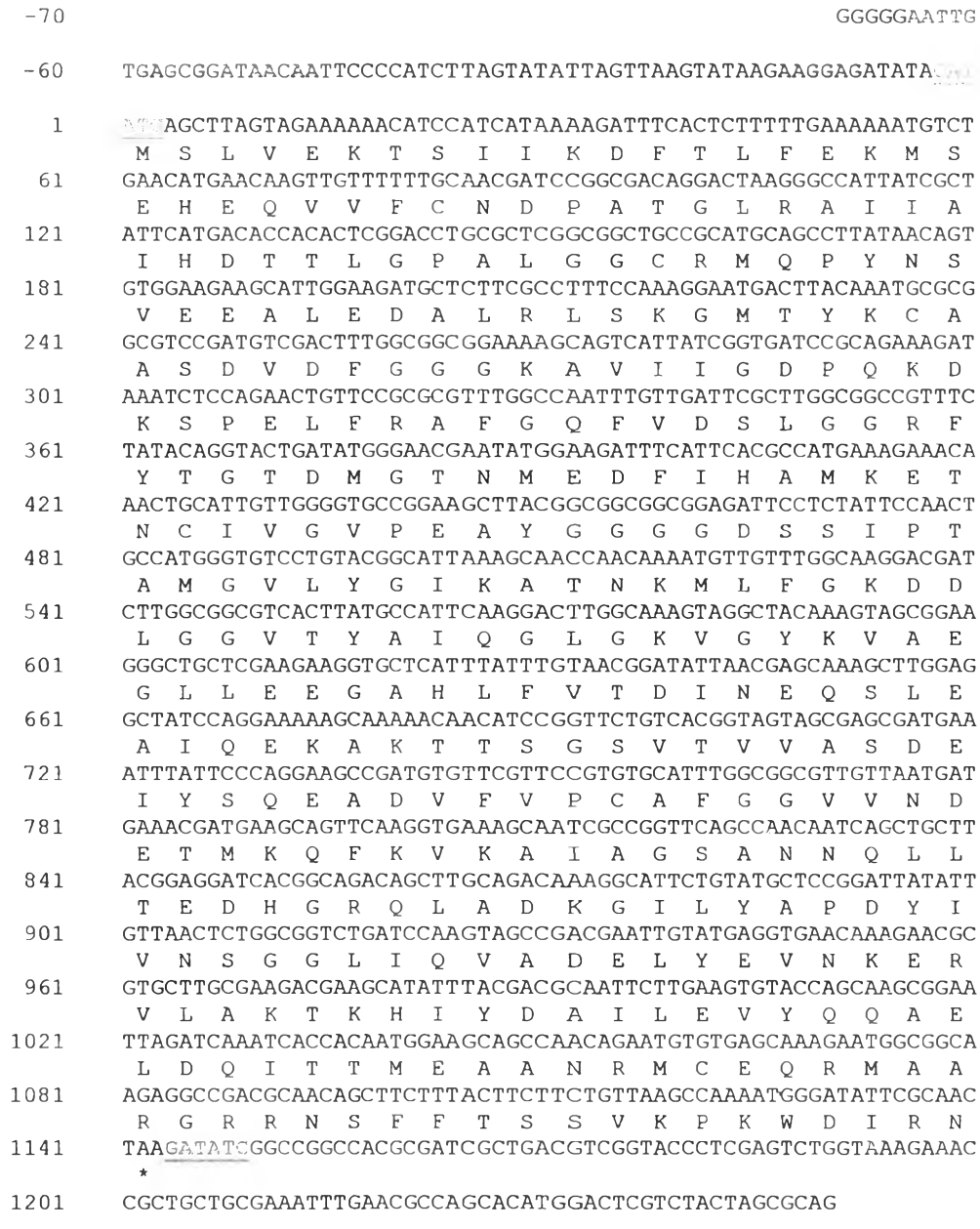


Figure 3.3 The nucleotide sequence and the deduced amino acid sequence of *phedh* gene of recombinant plasmid pPheDH. The *lac* operator and ribosome binding site are indicated in blue and green, respectively. The restriction sites *Nde*I and *Eco*RV are shown by the underlined pink and red, respectively.

interest and *E. coli* TOP10, a strain with a close similarity to the strain DH10B, was used as a DNA template for amplification.

Comparison of nucleotide sequences and deduced amino acid sequences of *aroB* encoding 3-dehydroquinate synthase, *aroL* encoding shikimate kinase II, *glpF* encoding glycerol facilitator, *glpK* encoding glycerol kinase, *tktA* encoding transketolase, *pheA* encoding chorismate mutase/prephenate dehydratase and *yddG* encoding aromatic amino acid exporter reported in *E. coli* DH10B with those of *E. coli* BL21(DE3) revealed high levels of homology (Table 3.2). The nucleotide sequence identities of all 7 genes of strain DH10B and strain BL21(DE3) were ranged from 97% to 100%. The deduced amino acid sequences were also highly similar (99 or 100% homology).

Prior to PCR amplification of each gene, the DNA template was prepared by cleaving chromosomal DNA of *E. coli* TOP10 with restriction enzyme because the small fragment of chromosomal DNA was easily accessed for annealing with primers in PCR reaction. For these six genes (i.e. *aroB, aroL, glpF, pheA, tktA* and *yddG*), no recognition site of *Bam*HI was found. Thus, *Bam*HI-digested chromosomal DNA was used as PCR template for amplification of six genes. In contrast to *glpK* gene amplification, the template was prepared by digestion with *Xho*I because *glpK* gene could be cut by *Bam*HI. After digestion and separation on agarose gel, it was found that the digestion gave the DNA fragments in the range of 1 kb to 23.1 kb. The digestion reaction was purified and then used as template for gene amplifications.

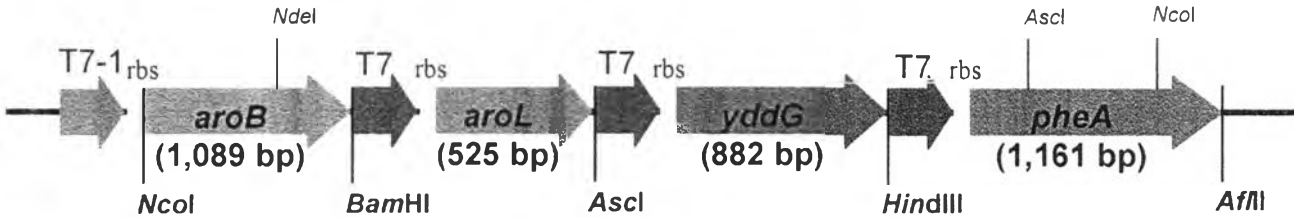
3.2.3 PCR amplification of *aroL, pheA* and *yddG* genes

The rate of expression of a gene in a vector is known to depend on the distance between the gene and its promoter. A gene located close to the promoter is expressed with a much higher rate compared to a gene that is located far from the promoter (Weckbecker and Hummel, 2004). Therefore, to control the expression of each gene at similar level, each gene was required to be preceded by its own T7*lac* promoter. Figure 3.4 showed the position of each of these seven genes located on the single pRSFduet-1 vector by rational design.

Table 3.2 The identity of interested genes from *E. coli* strains BL21(DE3) and DH10B

Gene	% Identity	
	Nucleotide sequence	Deduced amino acid sequence
aroB	99	99
aroL	99	100
glpF	99	100
glpK	100	100
pheA	98	100
tktA	99	100
yddG	97	99

A



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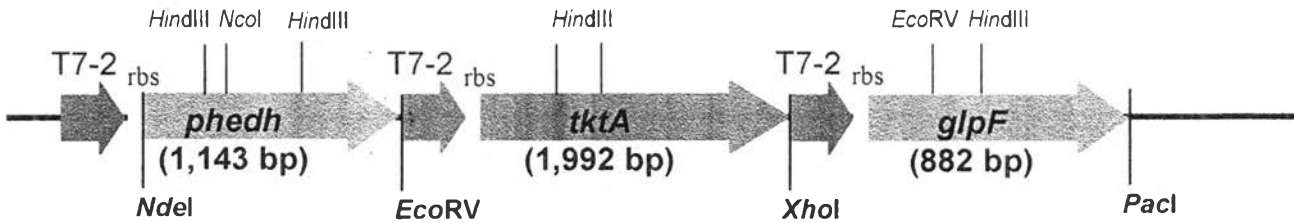

Figure 3.4 The positions of all seven genes (*aroB*, *aroL*, *glpF*, *pheA*, *phedh*, *tktA* and *yddG*) inserted into multiple cloning site-1 (A) and multiple cloning site-2 (B) of single pRSFDuet-1 vector to produce a recombinant plasmid pPTFBLYA

To facilitate the construction of all seven genes in single vector that each gene preceded by its own promoter, firstly, each gene was cloned into individual pRSFDuet-1 vector to yield pAroB, pAroL, pGlpF, pPheDH, pPheA, pTktA and pYddG. Each of the three genes (*aroL, pheA* and *yddG*) that was planned to be placed into multiple cloning site-1 of pRSFDuet-1 vector was initially cloned into an expression vector pET-22b(+) since pRSFDuet-1 was not available in the lab and pET-22b(+) has a same T7*lac* promoter as pRSFDuet-1 vector.

For *aroL* (525 bp) and *yddG* (882 bp) amplifications, agarose gel electrophoresis revealed that the amplified PCR fragment lengths of approximately 0.55 and 0.90 kb were in good agreement with the actual lengths of *aroL* and *yddG* genes of DH10B strain, respectively. The PCR products of both genes showed strong band with specific size at all annealing temperatures. However, non specific amplified DNA fragments of bigger size were detected (Figure 3.5A). For *pheA* (1,161 bp) amplification, the amplified 1.2 kb fragment was detected with high intensity together with trace of non-specific band of approximately 2.3 kb at all annealing temperatures (Figure 3.5B). All PCR products were pooled and then purified.

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

The PCR products of these three genes were digested with restriction enzymes, and ligated into pET-22b(+) digested with the same restriction enzymes. As a result of this, the recombinant plasmids pETAroL (5,890 bp), pETYddG (6,247 bp) and pETPheA (6,560 bp) were obtained (Figure 2.1A, 2.2A and 2.2B). Each plasmids was introduced into *E. coli* BL21(DE3) and ampicillin-resistant colonies harbouring expected genes-incorporated pET-22b(+) were examined on agarose gel. The plasmids pETAroL and pETYddG, after digested with *Nde*I and *Xho*I, showed the vector fragment of 5,363 bp, and the fragments of 527 bp of *aroL* gene and 884 bp of *yddG* gene (Figure 3.6A and 3.6B). The 5,397 bp fragment of pET-22b(+) part and the 1,163 bp fragment containing *pheA* gene were derived from cutting pETPheA with *Nde*I and *Eco*RI (Figure 3.6C). It was concluded that the specified PCR product was indeed ligated to the target plasmid.

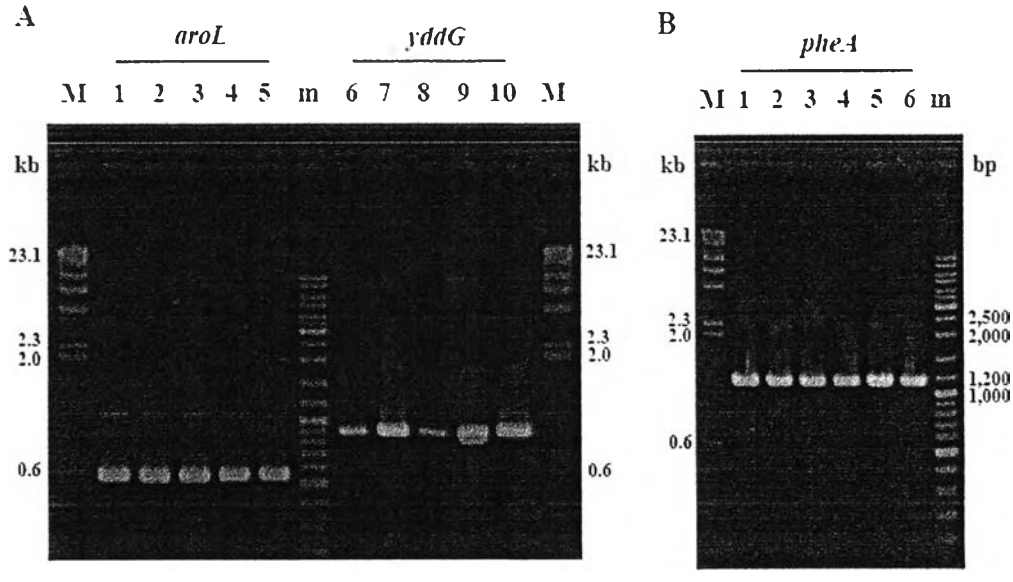

Figure 3.5 PCR products of *aroL*, *yddG* and *PheA* genes using various annealing temperatures

A: PCR products of *aroL* and *yddG* genes

Lane M = λ/*Hin*dIII standard DNA marker
Lane 1-6 = annealing temperature of 50.0 °C
Lane 2-7 = annealing temperature of 52.7 °C
Lane 3-8 = annealing temperature of 55.4 °C
Lane 4-9 = annealing temperature of 58.1 °C
Lane 5-10 = annealing temperature of 60.0 °C
Lane m = 100 bp DNA ladder

B: PCR products of *pheA* gene

Lane M = λ/*Hin*dIII standard DNA marker
Lane 1 = annealing temperature of 50.0 °C
Lane 2 = annealing temperature of 52.7 °C
Lane 3 = annealing temperature of 55.4 °C
Lane 4 = annealing temperature of 58.1 °C
Lane 5 = annealing temperature of 60.0 °C
Lane m = 100 bp DNA ladder

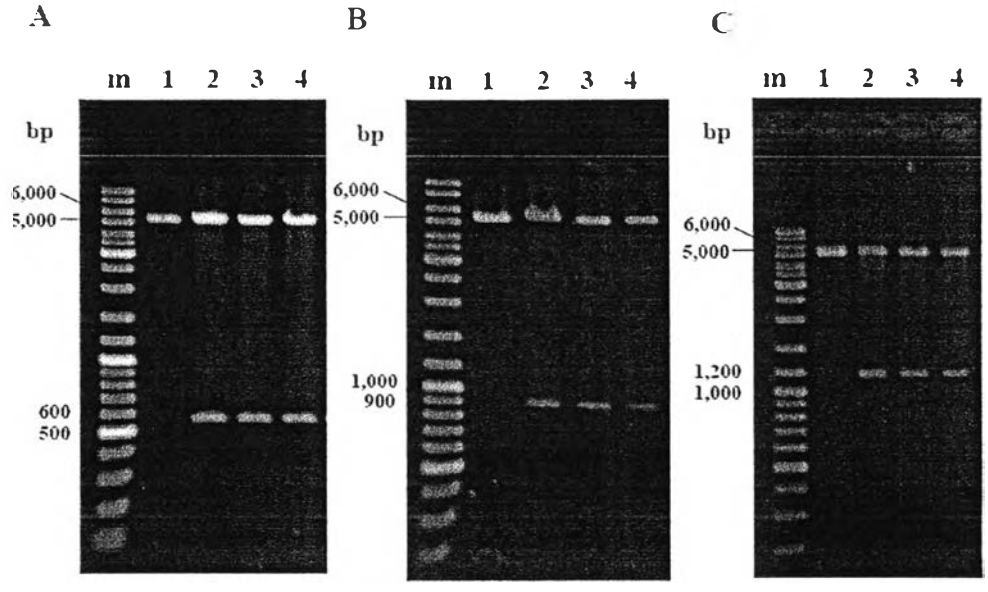

Figure 3.6 Restriction patterns of recombinant plasmids pETAroL, pETYddG and pETPheA

A: Restriction pattern of pETAroL

Lane m = 100 bp DNA ladder

Lane 1 = *Nde*I/*Xho*I-digested pET-22b(+)

Lane 2-4 = *Nde*I/*Xho*I-digested pETAroL

B: Restriction pattern of pETYddG

Lane m = 100 bp DNA ladder

Lane 1 = *Nde*I/*Xho*I-digested pET-22b(+)

Lane 2-4 = *Nde*I/*Xho*I-digested pETYddG

C: Restriction pattern of pETYddG

Lane m = 100 bp DNA ladder

Lane 1 = *Nde*I/*Eco*RI-digested pET-22b(+)

Lane 2-4 = *Nde*I/*Eco*RI-digested pETPheA

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

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

To integrate the *aroL* gene fragment with T7*lac* promoter and the ribosome binding site into *Bam*HI and *Asc*I sites of pRSFDuet-1 vector, constructed pETAroL was used as DNA template for PCR amplification. It was found that all annealing temperatures gave strong product of approximately 0.7 kb that was 100 bp bigger than the *aroL* gene. This 100 bp bigger included T7*lac* promoter, *lac* operator and ribosome binding site. From analysis of the separation on agarose gel in Figure 3.7A, no interference of non-specific PCR product with the main product was found. Thus, the step of separation of PCR product on agarose gel for eluting the desired fragment was not needed. The PCR product was simply harvested, and then cloned into pRSFDuet-1 to get pAroL (4,447 bp) (Figure 2.3A). The obtained plasmid was introduced into *E. coli* BL21(DE3) and colonies harbouring expecting gene were examined on agarose gel. The plasmid that was digested with *Bam*HI and *Asc*I resulting in the fragments of 3,810 bp and 637 bp (Figure 3.7B) was selected to be investigated by DNA sequencing to make sure the correct nucleotide sequence of *aroL* gene and T7*lac* promoter. The nucleotide sequence of *aroL* gene of pAroL is shown in Figure 3.8. The obtained sequence consisted of a 525 bp open reading frame of *aroL* encoding a protein of 174 amino acid residues. It showed 100% identity to *aroL* gene from *E. coli* DH10B.

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

To subclone the DNA fragment containing the *yddG* gene including the T7*lac* promoter and the ribosome binding site from pETYddG into *Asc*I and *Hin*dIII sites of pRSFDuet-1 vector, PCR amplification of this fragment was performed. All annealing temperatures showed strong main product of 1.0 kb which was 100 bp bigger than the size of *yddG* gene after separation on agarose gel (Figure 3.9A). The main product of gene fragment was obtained without any non-specific product. The PCR product was simply harvested, and then cloned into pRSFDuet-1 leading to pYddG (4,805 bp) (Figure 2.3B). The obtained plasmid was introduced into

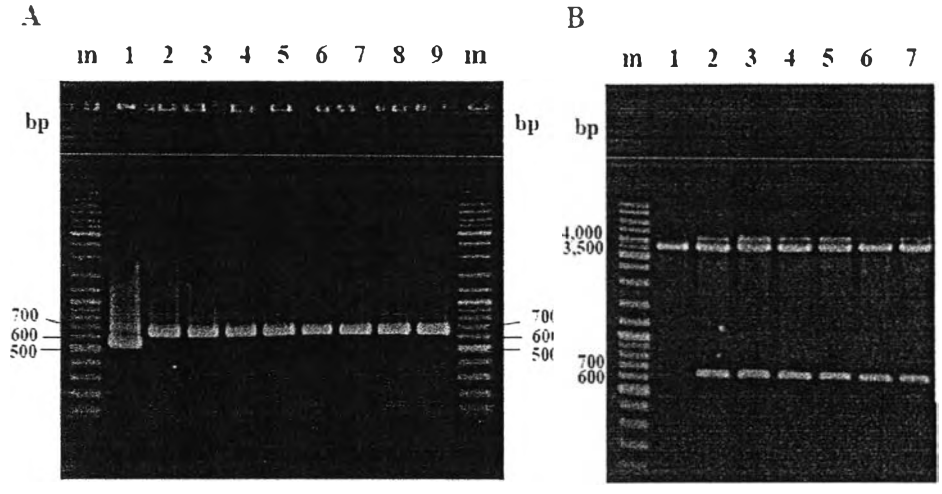

Figure 3.7 Electrophoretic patterns of recombinant plasmid pAroL

A: PCR products of *aroL* gene preceded by T7*lac* promoter using various annealing temperatures

Lane m = 100 bp DNA ladder
Lane 1 = PCR product of *aroL* gene
Lane 2 = annealing temperature of 50.0 °C
Lane 3 = annealing temperature of 52.5 °C
Lane 4 = annealing temperature of 54.4 °C
Lane 5 = annealing temperature of 56.5 °C
Lane 6 = annealing temperature of 58.7 °C
Lane 7 = annealing temperature of 60.8 °C
Lane 8 = annealing temperature of 62.9 °C
Lane 9 = annealing temperature of 64.6 °C

B: Restriction pattern of pAroL

Lane m = 100 bp DNA ladder
Lane 1 = *Bam*HI/*Asc*I-digested pRSFDuet-1
Lane 2-7 = *Bam*HI/*Asc*I-digested pAroL

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-232          ATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTGTA

-180    GAAATAATTTTGTTTAACTTTAATAAGGAGATATACCATGGGCAGCAGCCATCACCATCA

-120    TCACCACAGCCA[illegible]CGATCCCGCGAAAT[illegible]AGGGGAATTGTG

 -60    AGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT

   1    ATGACACAACCTCTTTTTCTGATCGGGCCTCGGGGCTGTGGTAAAACAACGGTCGGAATG
         M  T  Q  P  L  F  L  I  G  P  R  G  C  G  K  T  T  V  G  M
  61    GCCCTTGCCGATTCGCTTAACCGTCGGTTTGTCGATACCGATCAGTGGTTGCAATCACAG
         A  L  A  D  S  L  N  R  R  F  V  D  T  D  Q  W  L  Q  S  Q
 121    CTCAATATGACGGTCGCGGAGATCGTCGAAAGGGAAGAGTGGGCGGGATTTCGCGCCAGA
         L  N  M  T  V  A  E  I  V  E  R  E  E  W  A  G  F  R  A  R
 181    GAAACGGCGGCGCTGGAAGCGGTAACTGCGCCATCCACCGTTATCGCTACAGGCGGCGGC
         E  T  A  A  L  E  A  V  T  A  P  S  T  V  I  A  T  G  G  G
 241    ATTATTCTGACGGAATTTAATCGTCACTTCATGCAAAATAACGGGATCGTGGTTTATTTG
         I  I  L  T  E  F  N  R  H  F  M  Q  N  N  G  I  V  V  Y  L
 301    TGTGCGCCAGTATCAGTCCTGGTTAACCGACTGCAAGCTGCACCGGAAGAAGATTTACGG
         C  A  P  V  S  V  L  V  N  R  L  Q  A  A  P  E  E  D  L  R
 361    CCAACCTTAACGGGAAAACCGCTGAGCGAAGAAGTTCAGGAAGTGCTGGAAGAACGCGAT
         P  T  L  T  G  K  P  L  S  E  E  V  Q  E  V  L  E  E  R  D
 421    GCGCTATATCGCGAAGTTGCGCATATTATCATCGACGCAACAAACGAACCCAGCCAGGTG
         A  L  Y  R  E  V  A  H  I  I  I  D  A  T  N  E  P  S  Q  V
 481    ATTTCTGAAATTCGCAGCGCCCTGGCACAGACGATCAATTGTTGAGGCGCGCCTGCAGGT
         I  S  E  I  R  S  A  L  A  Q  T  I  N  C  *
 601    CGACAAGCTTGCGGCCGCATAATGCTTAAGTCGAACAGAAAGTAATCGTATTGTACACGG

 661    CCGCATAATCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACATTCCCC

 721    ATCTTAGTATATTAGTTAAGTATATGAGGAGATATACATATGGCAGAT
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Figure 3.8 The nucleotide sequence and the deduced amino acid sequence of *aroL* gene of recombinant plasmid pAroL. The T7 promoter, *lac* operator and ribosome binding site are indicated in orange, blue and bold green, respectively. The restriction sites *Bam*HI and *Asc*I are shown by the underlined pink and red, respectively.

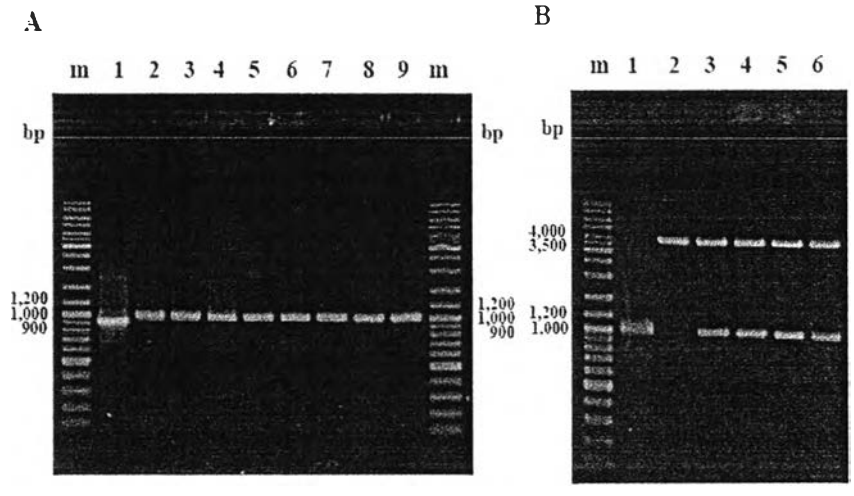


Figure 3.9 Electrophoretic patterns of recombinant plasmid pYddG

A: PCR products of *yddG* gene preceded by T7*lac* promoter using various annealing temperatures

Lane m = 100 bp DNA ladder

Lane 1 = PCR product of *yddG* gene

Lane 2 = annealing temperature of 50.0 °C

Lane 3 = annealing temperature of 52.5 °C

Lane 4 = annealing temperature of 54.4 °C

Lane 5 = annealing temperature of 56.5 °C

Lane 6 = annealing temperature of 58.7 °C

Lane 7 = annealing temperature of 60.8 °C

Lane 8 = annealing temperature of 62.9 °C

Lane 9 = annealing temperature of 64.6 °C

B: Restriction pattern of pYddG

Lane m = 100 bp DNA ladder

Lane 1 = PCR product of *yddG* gene preceded by T7*lac* promoter

Lane 2 = *Asc*I/*Hin*dIII-digested pRSFDuet-1

Lane 3-6 = *Asc*I/*Hin*dIII-digested pYddG

E. coli BL21(DE3) and colonies harbouring expecting gene were examined on agarose gel. The selected clone containing recombinant plasmid needed to give the fragments of 3,811 bp and 994 bp after digestion with *Asc*I and *Hin*dIII (Figure 3.9B). This plasmid was confirmed again by sequencing using only one primer because the fragment length was not long. The nucleotide sequence of *yddG* gene of pYddG is shown in Figure 3.10. The obtained sequence consisted of an 882 bp open reading frame of *yddG* encoding a protein of 293 amino acid residues. It was found that the *yddG* sequence was entirely homologous (100%) to that from *E. coli* DH10B.

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

To switch an expression vector from pET-22b(+) to pRSFDuet-1 vector, the DNA fragment containing the *pheA* gene linking to the T7*lac* promoter and the ribosome binding site was amplified from previous constructed pETPheA. The PCR products were separated on the agrose gel (Figure 3.11A). The thick band of DNA fragment of 1.3 kb which was compatible with the sum of the length of *pheA* gene (1.2 kb) and T7*lac* promoter-ribosome binging site (100 bp) was obtained with an occurrence of smear non-specific product of 2.5-3.0 kb. After collection of the relevant fragment from PCR reaction, it was cloned into pRSFDuet-1 to produce pPheA (5,078 bp) (Figure 2.4A). Then, the pPheA was transformed into *E. coli* BL21(DE3) and screened on agarose gel. After digestion with *Hin*dIII and *Afl*II, the recombinant plasmid giving the fragments of 3,809 bp and 1,269 bp (Figure 3.11B) was sequenced. The nucleotide sequence of *pheA* gene of pPheA is shown in Figure 3.12. The obtained sequence consisted of a 1,161 bp open reading frame of *pheA* encoding a protein of 386 amino acid residues. It was 100% homology to *pheA* gene from *E. coli* DH10B.

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

3.4.1 Cloning of *aroB* gene into pRSFDuet-1

Based on the plan of arrangement of all seven gene positions on only one of pRSFDuet-1 vector as shown in Figure 3.4, it was found that just one gene (*aroB*

```
-26                                                    TTTAACTTTAAGAAGGAGATATACAT

  1     ATGACACGACAAAAAGCAACGCTCATAGGGCTGATAGCGATCGTCCTGTGGAGCACGATG
         M  T  R  Q  K  A  T  L  I  G  L  I  A  I  V  L  W  S  T  M
 61     GTAGGATTGATTCGCGGTGTCAGTGAGGGGCTCGGCCCGGTCGGCGGCGCAGCTGCTATC
         V  G  L  I  R  G  V  S  E  G  L  G  P  V  G  G  A  A  A  I
121     TATTCATTAAGCGGGCTGCTGTTAATCTTCACGGTTGGATTTCCGCGTATTCGGCAAATC
         Y  S  L  S  G  L  L  L  I  F  T  V  G  F  P  R  I  R  Q  I
181     CCGAAAGGCTATTTACTCGCCGGGAGTCTGTTATTCGTCAGCTATGAAATCTGTCTGGCG
         P  K  G  Y  L  L  A  G  S  L  L  F  V  S  Y  E  I  C  L  A
241     CTTTCCTTAGGGTATGCGGCGACCCATCATCAGGCGATTGAAGTGGGTATGGTGAACTAT
         L  S  L  G  Y  A  A  T  H  H  Q  A  I  E  V  G  M  V  N  Y
301     CTGTGGCCCAGCCTGACAATTCTCTTTGCCATTCTGTTTAATGGTCAGAAAACCAACTGG
         L  W  P  S  L  T  I  L  F  A  I  L  F  N  G  Q  K  T  N  W
361     TTGATTGTACCTGGATTATTATTAGCCCTCGTCGGCGTCTGTTGGGTGTTAGGCGGTGAC
         L  I  V  P  G  L  L  L  A  L  V  G  V  C  W  V  L  G  G  D
421     AATGGGTTACATTATGATGAAATCATCAATAATATCACCACCAGCCCATTGAGTTATTTC
         N  G  L  H  Y  D  E  I  I  N  N  I  T  T  S  P  L  S  Y  F
481     CTGGCGTTCATTGGTGCGTTTATCTGGGCAGCCTATTGCACAGTAACGAATAAATACGCA
         L  A  F  I  G  A  F  I  W  A  A  Y  C  T  V  T  N  K  Y  A
541     CGCGGATTTAATGGAATTACCGTTTTTGTCCTGCTAACGGGAGCAAGTCTGTGGGTTTAC
         R  G  F  N  G  I  T  V  F  V  L  L  T  G  A  S  L  W  V  Y
601     TATTTTCTTACGCCACAACCAGAAATGATATTTAGCACGCCCGTCATGATTAAACTCATC
         Y  F  L  T  P  Q  P  E  M  I  F  S  T  P  V  M  I  K  L  I
661     TCTGCGGCATTTACCTTAGGATTTGCTTATGCTGCATGGAATGTCGGTATATTGCATGGC
         S  A  A  F  T  L  G  F  A  Y  A  A  W  N  V  G  I  L  H  G
721     AATGTCACCATTATGGCGGTAGGTTCGTATTTTACGCCTGTACTTTCCTCAGCGCTTGCA
         N  V  T  I  M  A  V  G  S  Y  F  T  P  V  L  S  S  A  L  A
781     GCCGTGCTGCTCAGCGCCCCGCTGTCGTTCTCGTTCTGGCAAGGCGCGCTGATGGTCTGC
         A  V  L  L  S  A  P  L  S  F  S  F  W  Q  G  A  L  M  V  C
841     GGCGGTTCCCTGCTCTGCTGGCTGGCGACACGTCGTGGTTAAAAGCTTGCGGCCGCATAA
         G  G  S  L  L  C  W  L  A  T  R  R  G  *
901     TGCTTAAGTCGAAC
```

Figure 3.10 The nucleotide sequence and the deduced amino acid sequence of *yddG* gene of recombinant plasmid pYddG. The ribosome binding site is indicated in green. The restriction site *Hin*dIII is shown by the underlined red.

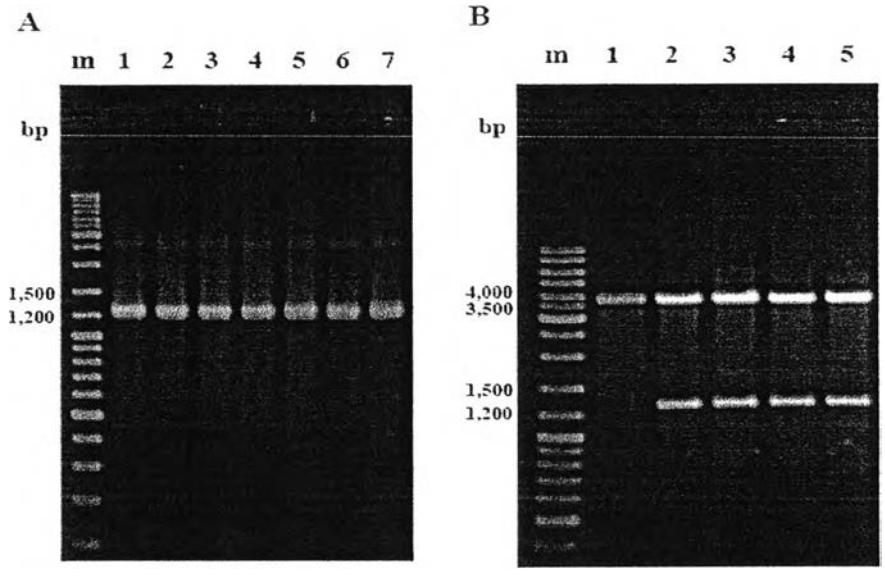


Figure 3.11 Electrophoretic patterns of recombinant plasmid pPheA

A: PCR products of *pheA* gene preceded by T7*lac* promoter using various annealing temperatures

Lane m = 100 bp DNA ladder

Lane 1 = annealing temperature of 50.0 °C

Lane 2 = annealing temperature of 52.5 °C

Lane 3 = annealing temperature of 54.4 °C

Lane 4 = annealing temperature of 58.7 °C

Lane 5 = annealing temperature of 60.8 °C

Lane 6 = annealing temperature of 62.9 °C

Lane 7 = annealing temperature of 64.6 °C

B: Restriction pattern of pPheA

Lane m = 100 bp DNA ladder

Lane 1 = *Hin*dIII/*Afl*II-digested pRSFDuet-1

Lane 2-5 = *Hin*dIII/*Afl*II-digested pPheA

```
-273                                        TAGGAATTATACGACTCACTATAGGGGAATTGT

-240      GAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAACTTTAATAAGGAGATATACC

-180      ATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCGAATTCGAGCTCGGCGCGC

-120      CTGCAGGTCGACAAGCTTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTG

 -60      AGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT

   1      ATGACATCGGAAAACCCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGGATGAAAAA
           M  T  S  E  N  P  L  L  A  L  R  E  K  I  S  A  L  D  E  K
  61      TTATTAGCGTTACTGGCAGAACGGCGCGAACTGGCCGTCGAGGTGGGAAAAGCCAAACTG
           L  L  A  L  L  A  E  R  R  E  L  A  V  E  V  G  K  A  K  L
 121      CTCTCGCATCGCCCGGTACGTGATATTGATCGTGAACGCGATTTGCTGGAAAGATTAATT
           L  S  H  R  P  V  R  D  I  D  R  E  R  D  L  L  E  R  L  I
 181      ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCTGTTCCAGCTCATC
           T  L  G  K  A  H  H  L  D  A  H  Y  I  T  R  L  F  Q  L  I
 241      ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT
           I  E  D  S  V  L  T  Q  Q  A  L  L  Q  Q  H  L  N  K  I  N
 301      CCGCACTCAGCACGCATCGCTTTTCTCGGCCCCAAAGGTTCTTATTCCCATCTTGCGGCG
           P  H  S  A  R  I  A  F  L  G  P  K  G  S  Y  S  H  L  A  A
 361      CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCAAATTTGCC
           R  Q  Y  A  A  R  H  F  E  Q  F  I  E  S  G  C  A  K  F  A
 421      GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGTACCGATTGAAAAT
           D  I  F  N  Q  V  E  T  G  Q  A  D  Y  A  V  V  P  I  E  N
 481      ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT
           T  S  S  G  A  I  N  D  V  Y  D  L  L  Q  H  T  S  L  S  I
 541      GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA
           V  G  E  M  T  L  T  I  D  H  C  L  L  V  S  G  T  T  D  L
 601      TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT
           S  T  I  N  T  V  Y  S  H  P  Q  P  F  Q  Q  C  S  K  F  L
 661      AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG
           N  R  Y  P  H  W  K  I  E  Y  T  E  S  T  S  A  A  M  E  K
 721      GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG
           V  A  Q  A  K  S  P  H  V  A  A  L  G  S  E  A  G  G  T  L
 781      TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT
           Y  G  L  Q  V  L  E  R  I  E  A  N  Q  R  Q  N  F  T  R  F
 841      GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG
           V  V  L  A  R  K  A  I  N  V  S  D  Q  V  P  A  K  T  T  L
 901      TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC
           L  M  A  T  G  Q  Q  A  G  A  L  V  E  A  L  L  V  L  R  N
 961      CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG
           H  N  L  I  M  T  R  L  E  S  R  P  I  H  G  N  P  W  E  E
1021      ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCATTGAAA
           M  F  Y  L  D  I  Q  A  N  L  E  S  A  E  M  Q  K  A  L  K
1081      GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA
           E  L  G  E  I  T  R  S  M  K  V  L  G  C  Y  P  S  E  N  V
1141      GTGCCTGTTGATCCAACCTGACTTAAGTCGAACAGAAAGTAAT
           V  P  V  D  P  T  *
```

Figure 3.12 The nucleotide sequence and the deduced amino acid sequence of *pheA* gene of recombinant plasmid pPheA. The T7 promoter, *lac* operator and ribosome binding site are indicated in orange, blue and green, respectively. The restriction sites *Hin*dIII and *Afl*II are shown by the underlined pink and red, respectively.

gene) could be inserted at *Nco*I site in multiple cloning site-1 of pRSFDuet-1. The recognition site of restriction endonuclease *Nco*I is C'**CATGG**. The 5'-end of *aroB* gene is **ATGG**AGAGGATTGTC.... Therefore, the forward primer (aroB-*Nco*I) for amplifying this gene was 5'-CATGC'C**ATGG**AGAGGATTGTCGTTACTCTCG-3'. In case of inserting the other genes (i.e. *aroL, glpF, pheA, phedh, tktA* or *yddG*) into *Nco*I site, it was needed to put nitrogenous base G after start codon in forward primer to get the amplified fragment enabling to be digested with *Nco*I. Subsequently, it would result in the frameshift mutation of that gene.

To clone *aroB* gene (1,089 bp) under T7*lac* promoter of multiple cloning site-1 of pRSFDuet-1 vector, the *E. coli* TOP10 chromosomal DNA digested with *Bam*HI was used as DNA template for *aroB* gene amplification. It was found that the alteration of annealing temperatures in range of 58.5-66.6 ^{o}C did not change the pattern of amplified PCR product. The dense specific band of 1.1 kb of *aroB* gene was obtained. The PCR products were pooled and then purified. The 1.1 kb PCR fragment was digested with *Nco*I and *Bam*HI (Figure 3.13A; lane 1) as same as pRSFDuet-1 (Figure 3.13A; lane 2) and then ligated together leading to pAroB (4,883 bp) (Figure 2.4B). The obtained plasmid was introduced into *E. coli* BL21(DE3) and kanamycin-resistant colonies harbouring expected genes-incorporated pRSFDuet-1 were examined on agarose gel. Figure 3.13B showed the restriction pattern of pAroB after cleaving with *Nco*I and *Bam*HI. It corresponded to the calculated lengths of 3,792 bp and 1,091 bp from its restriction map. The nucleotide sequence of *aroB* gene of pAroB clone verified by sequencing is shown in Figure 3.14. The obtained sequence consisted of a 1,089 bp open reading frame of *aroB* encoding a protein of 362 amino acid residues. The *aroB* nucleotide sequence was absolutely similar (100% homology) to that of strain DH10B.

3.4.2 Cloning of *tktA* gene into pRSFDuet-1

To construct recombinant plasmid pTktA containing *tktA* gene, the gene was amplified from *Bam*HI-digested *E. coli* TOP10 chromosomal DNA with different annealing temperatures. Each PCR condition gave the same approximately 2.0 kb fragment of *tktA* gene (Figure 3.15A). The gene fragment was cut with restriction endonucleases *Nde*I and *Xho*I and inserted under T7*lac* promoter-2 of

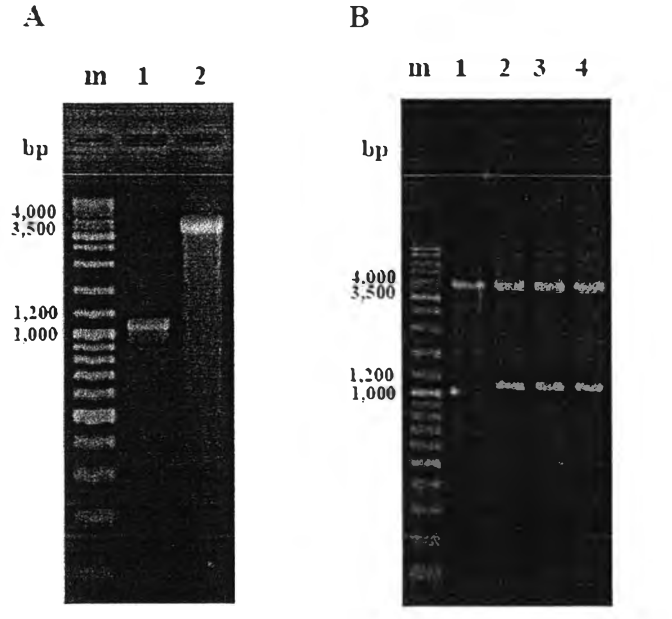


Figure 3.13 Electrophoretic patterns of recombinant plasmid pAroB

A: Pettern of digested fragments

Lane m = 100 bp DNA ladder

Lane 1 = *Nco*I/*Bam*HI-digested PCR product of *aroB* gene

Lane 2 = *Nco*I/*Bam*HI-digested pRSFDuet-1

B: Restriction pattern of pAroB

Lane m = 100 bp DNA ladder

Lane 1 = *Nco*I/*Bam*HI-digested pRSFDuet-1

Lane 2-4 = *Nco*I/*Bam*HI-digested pAroB

```
-89                                          ATTAATACGACTCACTATAGGGGAATTGT

-60      GAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAACTTTAATAAGGAGATATACC

  1      ATGGAGAGGATTGTCGTTACTCTCGGGGAACGTAGTTACCCAATTACCATCGCATCTGGT
          M  E  R  I  V  V  T  L  G  E  R  S  Y  P  I  T  I  A  S  G
 61      TTGTTTAATGAACCAGCTTCATTCTTACCGCTGAAATCGGGCGAGCAGGTCATGTTGGTC
          L  F  N  E  P  A  S  F  L  P  L  K  S  G  E  Q  V  M  L  V
121      ACCAACGAAACCCTGGCTCCTCTGTATCTCGATAAGGTCCGCGGCGTACTTGAACAGGCG
          T  N  E  T  L  A  P  L  Y  L  D  K  V  R  G  V  L  E  Q  A
181      GGTGTTAACGTCGATAGCGTTATCCTCCCTGACGGCGAGCAGTATAAAAGCCTGGCTGTA
          G  V  N  V  D  S  V  I  L  P  D  G  E  Q  Y  K  S  L  A  V
241      CTCGATACCGTCTTTACGGCGTTGTTACAAAAACCGCATGGTCGCGATACTACGCTGGTG
          L  D  T  V  F  T  A  L  L  Q  K  P  H  G  R  D  T  T  L  V
301      GCGCTTGGCGGCGGCGTAGTGGGCGATCTGACCGGCTTCGCGGCGGCGAGTTATCAGCGC
          A  L  G  G  G  V  V  G  D  L  T  G  F  A  A  A  S  Y  Q  R
361      GGTGTCCGTTTCATTCAAGTCCCGACGACGTTACTGTCGCAGGTCGATTCCTCCGTTGGC
          G  V  R  F  I  Q  V  P  T  T  L  L  S  Q  V  D  S  S  V  G
421      GGCAAAACTGCGGTCAACCATCCCCTCGGTAAAAACATGATTGGCGCGTTCTACCAACCT
          G  K  T  A  V  N  H  P  L  G  K  N  M  I  G  A  F  Y  Q  P
481      GCTTCAGTGGTGGTGGATCTCGACTGTCTGAAAACGCTTCCCCCGCGTGAGTTAGCGTCG
          A  S  V  V  V  D  L  D  C  L  K  T  L  P  P  R  E  L  A  S
541      GGGCTGGCAGAAGTCATCAAATACGGCATTATTCTTGACGGTGCGTTTTTTAACTGGCTG
          G  L  A  E  V  I  K  Y  G  I  I  L  D  G  A  F  F  N  W  L
601      GAAGAGAATCTGGATGCGTTGTTGCGTCTGGACGGTCCGGCAATGGCGTACTGTATTCGC
          E  E  N  L  D  A  L  L  R  L  D  G  P  A  M  A  Y  C  I  R
661      CGTTGTTGTGAACTGAAGGCAGAAGTTGTCGCCGCCGACGAGCGCGAAACCGGGTTACGT
          R  C  C  E  L  K  A  E  V  V  A  A  D  E  R  E  T  G  L  R
721      GCTTTACTGAATCTGGGACACACCTTTGGTCATGCCATTGAAGCTGAAATGGGGTATGGC
          A  L  L  N  L  G  H  T  F  G  H  A  I  E  A  E  M  G  Y  G
781      AATTGGTTACATGGTGAAGCGGTCGCTGCGGGTATGGTGATGGCGGCGCGGACGTCGGAA
          N  W  L  H  G  E  A  V  A  A  G  M  V  M  A  A  R  T  S  E
841      CGTCTCGGGCAGTTTAGTTCTGCCGAAACGCAGCGTATTATAACCCTGCTCAAGCGGGCT
          R  L  G  Q  F  S  S  A  E  T  Q  R  I  I  T  L  L  K  R  A
901      GGGTTACCGGTCAATGGGCCGCGCGAAATGTCCGCGCAGGCGTATTTACCGCATATGCTG
          G  L  P  V  N  G  P  R  E  M  S  A  Q  A  Y  L  P  H  M  L
961      CGTGACAAGAAAGTCCTTGCGGGAGAGATGCGCTTAATTCTTCCGTTGGCAATTGGTAAG
          R  D  K  K  V  L  A  G  E  M  R  L  I  L  P  L  A  I  G  K
1021     AGTGAAGTTCGCAGCGGCGTTTCGCACGAGCTTGTTCTTAACGCCATTGCCGATTGTCAA
          S  E  V  R  S  G  V  S  H  E  L  V  L  N  A  I  A  D  C  Q
1081     TCAGCGTAAGGATCCGAATTCGAGCTCGGCGCGCCTGCAGGTCGACAAGCTTGCGGCCGC
          S  A  *
1141     ATAATGCTTAAGTCGAAC
```

Figure 3.14 The nucleotide sequence and the deduced amino acid sequence of *aroB* gene of recombinant plasmid pAroB. The T7 promoter, *lac* operator and ribosome binding site are indicated in orange, blue and green, respectively. The restriction sites *Nco*I and *Hin*dIII are shown by the underlined pink and red, respectively.

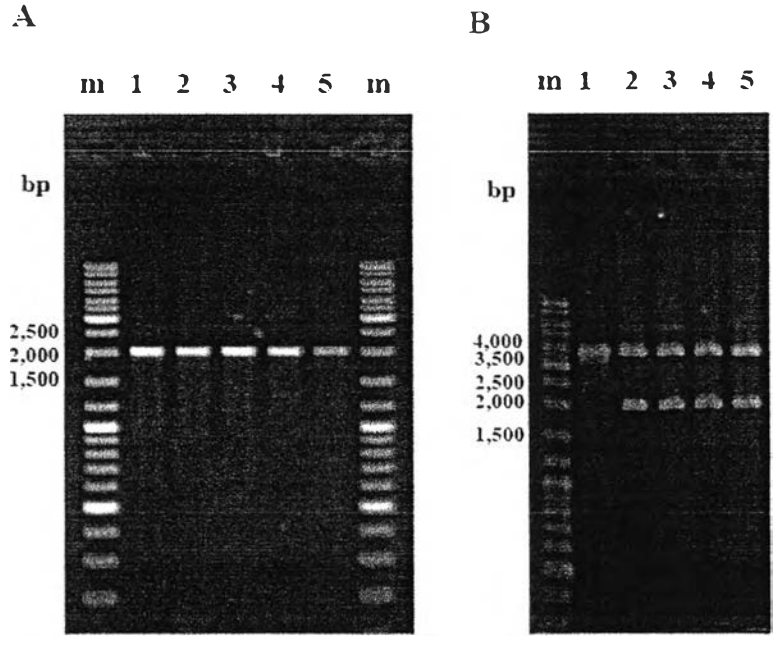


Figure 3.15 Electrophoretic patterns of recombinant plasmid pTktA

A: PCR products of *tktA* gene using various annealing temperatures

Lane m = 100 bp DNA ladder

Lane 1 = annealing temperature of 58.5 °C

Lane 2 = annealing temperature of 60.7 °C

Lane 3 = annealing temperature of 62.9 °C

Lane 4 = annealing temperature of 64.9 °C

Lane 5 = annealing temperature of 66.6 °C

B: Restriction pattern of pTktA

Lane m = 100 bp DNA ladder

Lane 1 = *Nde*I/*Xho*I-digested pRSFDuet-1

Lane 2-5 = *Nde*I/*Xho*I-digested pTktA

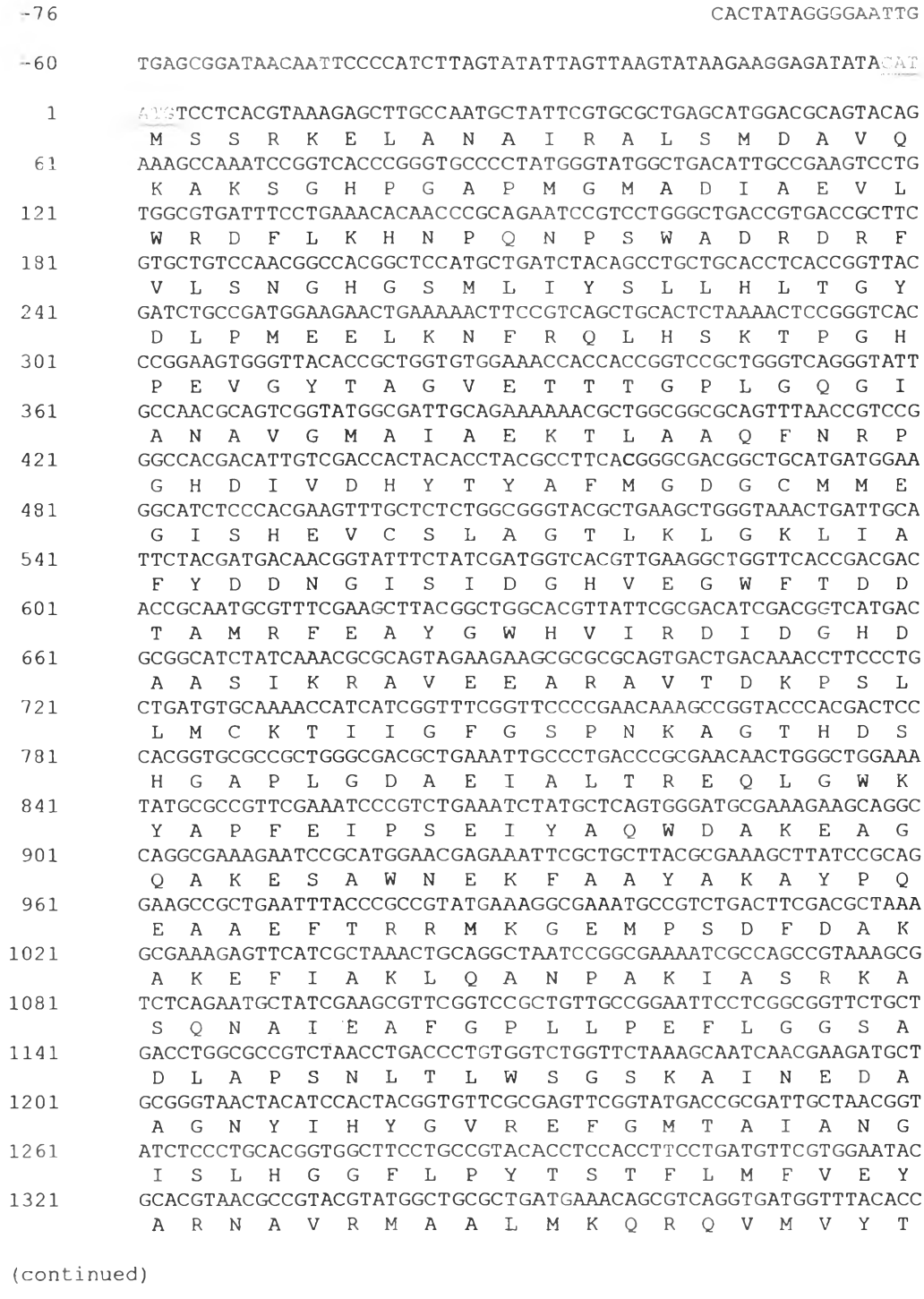
pRSFDuet-1 to create pTktA (5,767 bp) (Figure 2.5A). This recombinant was transformed into *E. coli* BL21(DE3) and screened on agarose gel. After excision of pTktA with *Nde*I and *Xho*I, the restriction fragments of 3,773 bp and 1,994 bp were generated (Figure 3.15B). The sequence of *tktA* gene was investigated by sequencing. The nucleotide sequence of *tktA* gene is shown in Figure 3.16. The obtained sequence consisted of a 1,992 bp open reading frame of *tktA* encoding a protein of 663 amino acid residues. The *tktA* nucleotide sequence showed 100% homology to that of strain DH10B.

3.4.3 Cloning of *glpF* gene into pRSFDuet-1

The recombinant plasmid pGlpF was produced by the insertion of *glpF* gene (846 bp) under T7*lac* promoter-2 of pRSFDuet-1. The *glpF* gene fragment was amplified from *Bam*HI-digested chromosomal DNA of *E. coli* TOP10. After varying annealing temperatures of PCR reaction ranging from 49.5 °C onwards, it was found that an increase in annealing temperature effected on the intensity of 0.85 kb target product (Figure 3.17A). The higher annealing temperature caused the less dense specific band of *glpF* gene. The annealing temperature was greater than 60 °C resulting in no desired band product. The target fragment was harvested and cut with *Nde*I and *Pac*I. The *glpF* fragment was incorporated into pRSFDuet-1 vector by ligase activity to give pGlpF (4,550 bp) (Figure 2.5B). This recombinant plasmid was introduced into *E. coli* BL21(DE3). After selection of recombinant clone, the plasmid was digested with *Nde*I and *Pac*I and the inserted fragment of 852 bp was removed from that of the original plasmid pRSFDuet-1 (3,698 bp) (Figure 3.17B). The nucleotide sequence of *glpF* gene of pGlpF verified by sequencing is shown in Figure 3.18. The obtained sequence consisted of an 846 bp open reading frame of *glpF* encoding a protein of 281 amino acid residues. The nucleotide sequence of *glpF* gene from TOP10 was absolutely consistent with that from DH10B.

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

Primarily, the *glpK* gene was not considered to be combined with other seven genes (*aroB, aroL, glpF, pheA, phedh, tktA* and *yddG*) in single pRSFDuet-1 vector. From the reasonable design of pPTFBLYA in Figure 3.4, there was no available



(continued)

Figure 3.16 The nucleotide sequence and the deduced amino acid sequence of *tktA* gene of recombinant plasmid pTktA. The *lac* operator and ribosome binding site are indicated in blue and green, respectively. The restriction sites *Nde*I and *Xho*I are shown by the underlined pink and red, respectively.

(continued)

```
1381      CACGACTCCATCGGTCTGGGCGAAGACGGCCCGACTCACCAGCCGGTTGAGCAGGTCGCT
           H  D  S  I  G  L  G  E  D  G  P  T  H  Q  P  V  E  Q  V  A
1441      TCTCTGCGCGTAACCCCGAACATGTCTACATGGCGTCCGTGTGACCAGGTTGAATCCGCG
           S  L  R  V  T  P  N  M  S  T  W  R  P  C  D  Q  V  E  S  A
1501      GTCGCGTGGAAATACGGTGTTGAGCGTCAGGACGGCCCGACCGCACTGATCCTCTCCCGT
           V  A  W  K  Y  G  V  E  R  Q  D  G  P  T  A  L  I  L  S  R
1561      CAGAACCTGGCGCAGCAGGAACGAACTGAAGAGCAACTGGCAAACATCGCGCGCGGTGGT
           Q  N  L  A  Q  Q  E  R  T  E  E  Q  L  A  N  I  A  R  G  G
1621      TATGTGCTGAAAGACTGCGCCGGTCAGCCGGAACTGATTTTCATCGCTACCGGTTCAGAA
           Y  V  L  K  D  C  A  G  Q  P  E  L  I  F  I  A  T  G  S  E
1681      GTTGAACTGGCTGTTGCTGCCTACGAAAAACTGACTGCCGAAGGCGTGAAAGCGCGCGTG
           V  E  L  A  V  A  A  Y  E  K  L  T  A  E  G  V  K  A  R  V
1741      GTGTCCATGCCGTCTACCGACGCATTTGACAAGCAGGATGCTGCTTACCGTGAATCCGTA
           V  S  M  P  S  T  D  A  F  D  K  Q  D  A  A  Y  R  E  S  V
1801      CTGCCGAAAGCGGTTACTGCACGCGTTGCTGTAGAAGCGGGTATTGCTGACTACTGGTAC
           L  P  K  A  V  T  A  R  V  A  V  E  A  G  I  A  D  Y  W  Y
1861      AAGTATGTTGGCCTGAACGGTGCTATCGTCGGTATGACCACCTTCGGTGAATCTGCTCCG
           K  Y  V  G  L  N  G  A  I  V  G  M  T  T  F  G  E  S  A  P
1921      GCAGAGCTGCTGTTTGAAGAGTTCGGCTTCACTGTTGATAACGTTGTTGCGAAAGCAAAA
           A  E  L  L  F  E  E  F  G  F  T  V  D  N  V  V  A  K  A  K
1981      GAACTGCTGTAACTCGAGTCTGGTAAAGAAACCGCTGCTGCGAAATTTGAACGCCAGCAC
           E  L  L  *
2041      ATGGACTCGTCTACTAGCGCAGCTTAATAGCT
```

Figure 3.16 The nucleotide sequence and the deduced amino acid sequence of *tktA* gene of recombinant plasmid pTktA. The *lac* operator and ribosome binding site are indicated in blue and green, respectively. The restriction sites *Nde*I and *Xho*I are shown by the underlined pink and red, respectively.

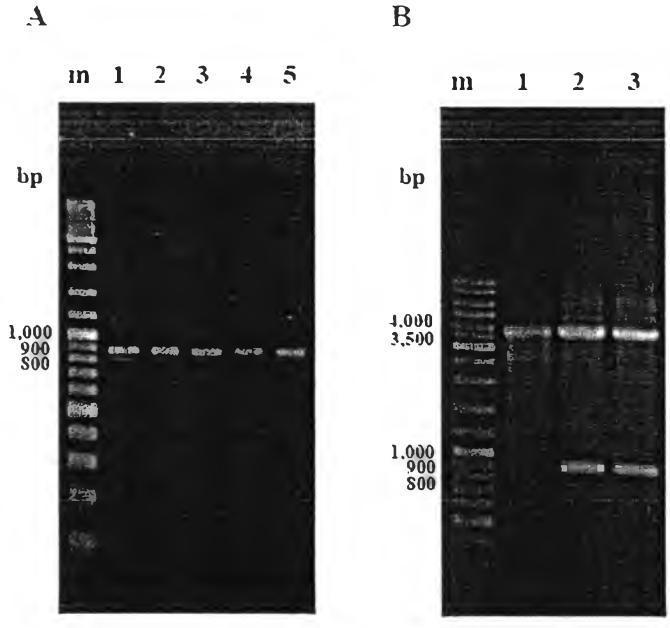


Figure 3.17 Electrophoretic patterns of recombinant plasmid pGlpF

A: PCR products of *glpF* gene using various annealing temperatures

Lane m = 100 bp DNA ladder

Lane 1 = annealing temperature of 49.5 °C

Lane 2 = annealing temperature of 51.3 °C

Lane 3 = annealing temperature of 53.4 °C

Lane 4 = annealing temperature of 55.6 °C

Lane 5 = annealing temperature of 57.8 °C

B: Restriction pattern of pGlpF

Lane m = 100 bp DNA ladder

Lane 1 = *Nde*I/*Pac*I-digested pRSFDuet-1

Lane 2-3 = *Nde*I/*Pac*I-digested pGlpF

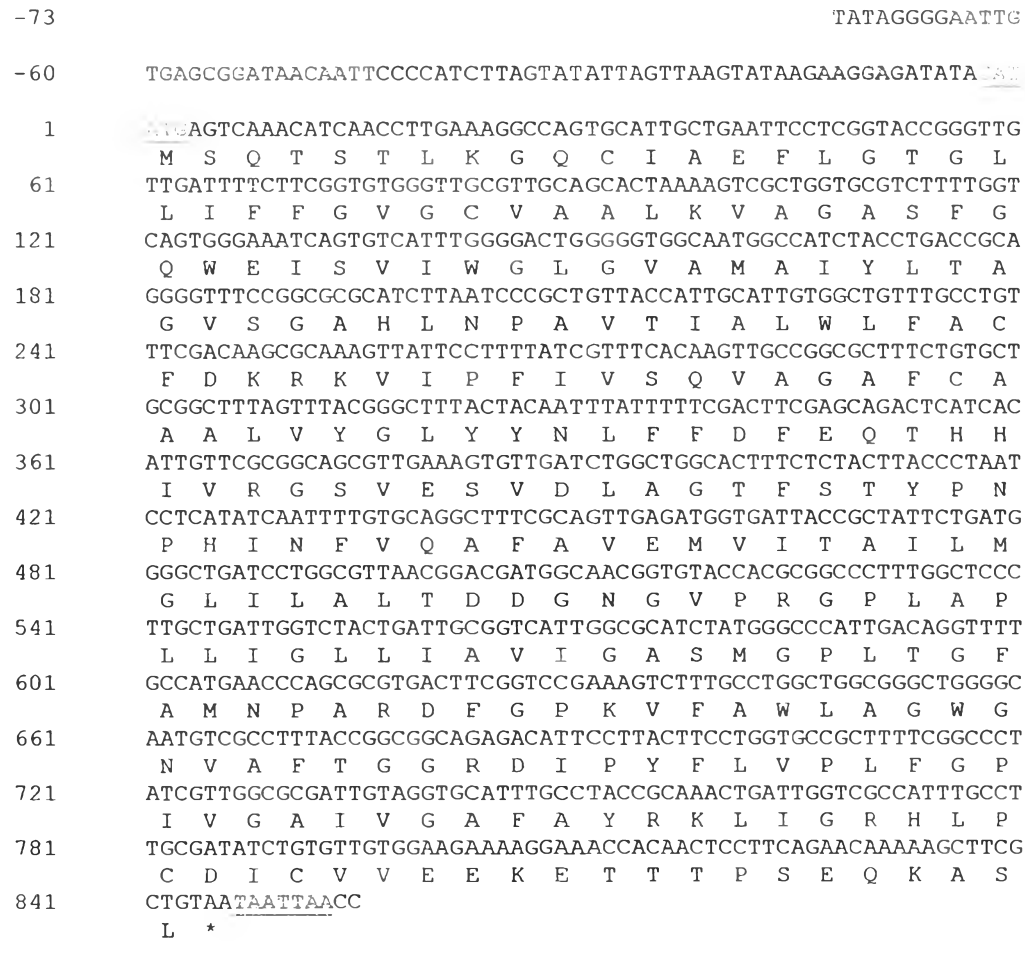


Figure 3.18 The nucleotide sequence and the deduced amino acid sequence of *glpF* gene of recombinant plasmid pGlpF. The *lac* operator and ribosome binding site are indicated in blue and green, respectively. The restriction sites *Nde*I and *Pac*I are shown by the underlined pink and red, respectively.

recognition site for addition of another gene in both multiple cloning sites of pRSFDuet-1. Nevertheless, it was found that the *glpFKX* operon of *E. coli* genome comprising of the organization of three genes (*glpF* encoding glycerol facilitator, *glpK* encoding glycerol kinase and *glpX* gene encoding fructose 1, 6-bisphosphatase II) as shown in section 2.13. The simple manner to add *glpK* gene in pRSFDuet-1 vector was to clone both *glpF* and *glpK* in the same time (designated as *glpFK* gene). The *glpFK* gene was the 2,377 bp DNA fragment containing *glpF* gene (846 bp) upstream of *glpK* gene (1,509 bp). Like the construction of pGlpF, the *glpFK* gene was inserted between *Nde*I and *Pac*I sites of pRSFDuet-1 to produce recombinant plasmid pGlpFK. Chromosomal DNA of *E. coli* TOP10 digested with *Bam*HI that was used for amplifying previous genes (i.e. *aroB, aroL, glpF, pheA, tktA* and *yddG*) was not capable for amplifying this region because *Bam*HI could cleave the *glpK* gene at location of 431 bp. Alternatively, the template was prepared by cutting the chromosomal DNA with *Xho*I. The amplification of *glpFK* gene was accessed using primers glpF-*Nde*I and glpK-*Pac*I. The forward primer (glpF-*Nde*I) bound to 5′-terminus of *glpF* and the reverse primer (glpK-*Pac*I) bound to 3′-terminus of *glpK* gene. The PCR amplification was done with one annealing temperature of 50 ^{o}C. The result from separation of PCR product on agarose gel electrophoresis is shown in Figure 3.19A. The sharp band fragment of approximately 2.4 kb containing both *glpF* and *glpK* genes was appeared when illuminated with UV light. This product was collected, cloned into pRSFDuet-1 vector at *Nde*I and *Pac*I sites resulting in pGlpFK (6,081 bp) (Figure 2.6A) and transformed into *E. coli* BL21(DE3). After selection on agarose gel, the pGlpFK was digested with appropriate restriction enzyme *Eco*RV. As shown in Figure 3.19B, the two DNA fragments of 5,792 bp and of 289 bp were found because *glpFK* gene contained two cleavage sites of *Eco*RV. The nucleotide sequence of *glpFK* gene of pGlpFK was evaluated by DNA sequencing. Because *glpFK* gene was rather long, after getting the gene sequence at 5′-end, this sequence was used for generating glpF-[674-700] primer to continue sequencing the internal fragment which could not be sequenced by DuetUP2 and T7 Terminator primers. The whole nucleotide sequence of *glpFK* gene is shown in Figure 3.20. The obtained sequence consisted of two open reading frames of *glpF* (846 bp) and *glpK* (1,509 bp) which encode proteins of 281 and 502 amino acid residues, respectively.

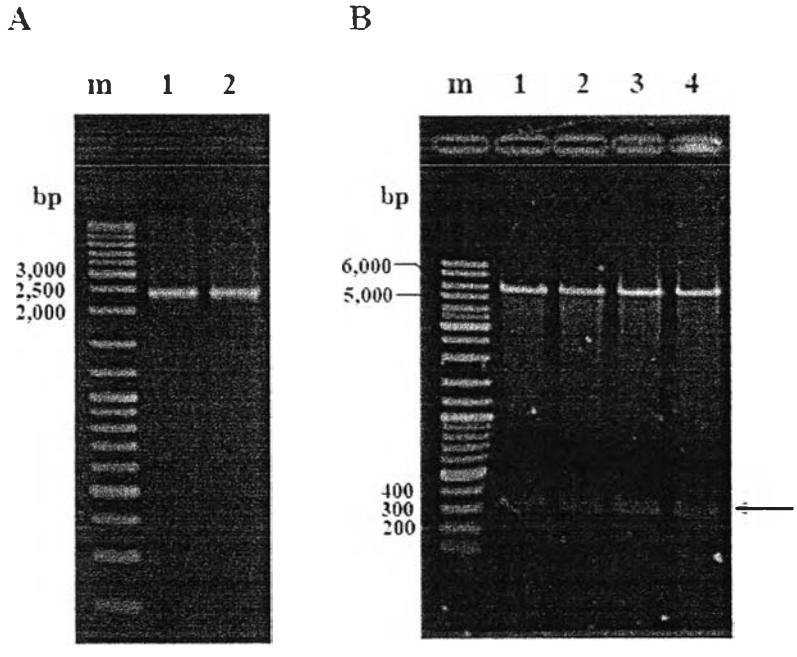


Figure 3.19 Electrophoretic patterns of recombinant plasmid pGlpFK. The arrow indicated the digested fragment of 289 bp.

A: PCR products of *glpFK* gene

Lane m = 100 bp DNA ladder

Lane 1-2 = annealing temperature of 50 °C

B: Restriction pattern of pGlpFK

Lane m = 100 bp DNA ladder

Lane 1-4 = *Eco*RV-digested pGlpFK

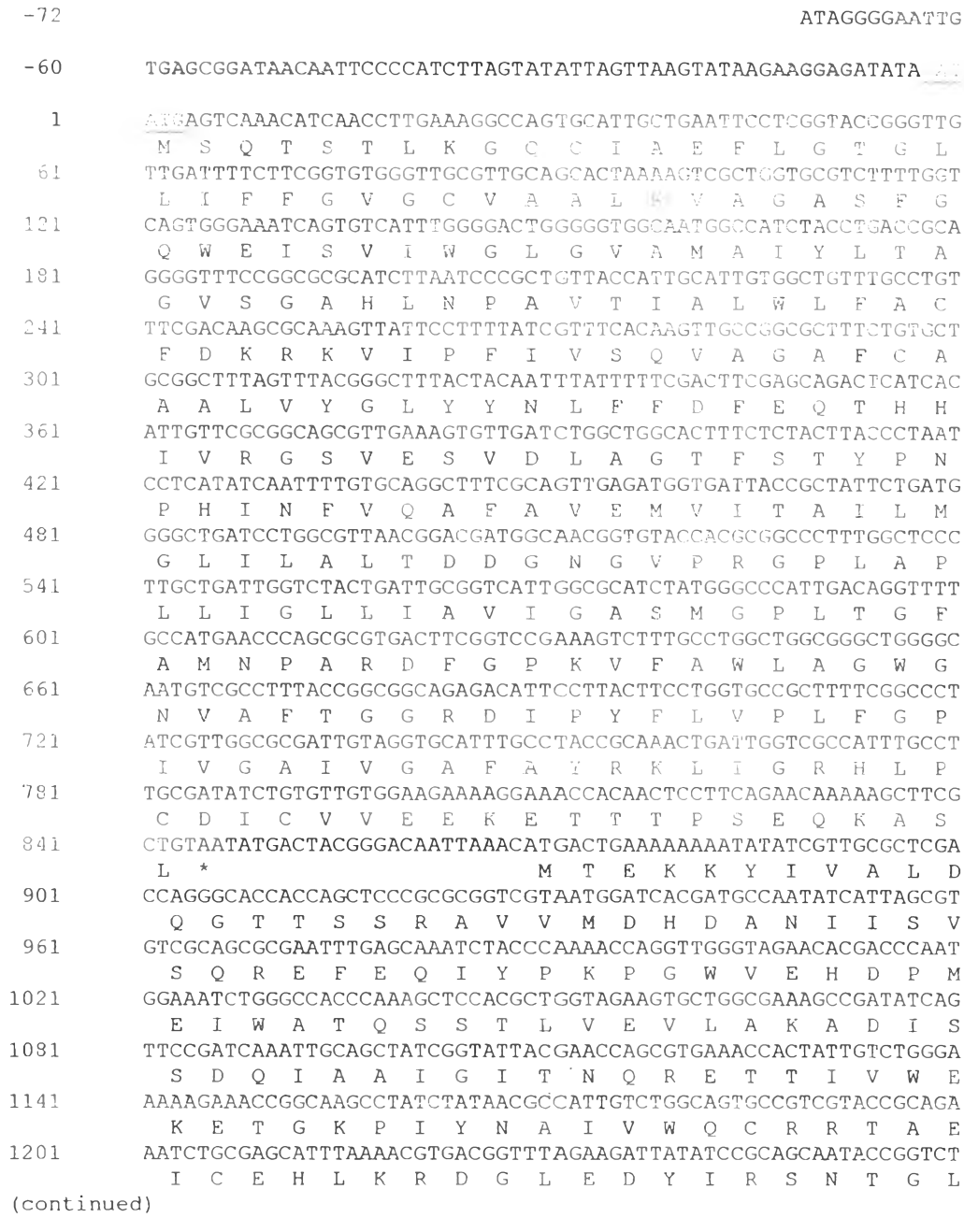


Figure 3.20 The nucleotide sequence and the deduced amino acid sequence of *glpFK* gene of recombinant plasmid pGlpFK. The *lac* operator and ribosome binding site are indicated in blue and green, respectively. The restriction sites *Nde*I and *Pac*I are shown by the underlined pink and red, respectively. The nucleotide sequences and the deduced amino acid sequences of *glpF* and *glpK* genes are indicated in brown and purple, respectively.

(continued)

```
1261      GGTGATTGACCCGTACTTTTCTGGCACCAAAGTGAAGTGGATCCTCGACCATGTGGAAGG
            V  I  D  P  Y  F  S  G  T  K  V  K  W  I  L  D  H  V  E  G
1321      CTCTCGCGAGCGTGCACGTCGTGGTGAATTGCTGTTTGGTACGGTTGATACGTGGCTTAT
            S  R  E  R  A  R  R  G  E  L  L  F  G  T  V  D  T  W  L  I
1381      CTGGAAAATGACTCAGGGCCGTGTCCATGTGACCGATTACACCAACGCCTCTCGTACCAT
            W  K  M  T  Q  G  R  V  H  V  T  D  Y  T  N  A  S  R  T  M
1441      GTTGTTCAACATCCATACCCTGGACTGGGACGACAAAATGCTGGAAGTGCTGGATATTCC
            L  F  N  I  H  T  L  D  W  D  D  K  M  L  E  V  L  D  I  P
1501      GCGCGAGATGCTGCCAGAAGTGCGTCGTTCTTCCGAAGTATACGGTCAGACTAACATTGG
            R  E  M  L  P  E  V  R  R  S  S  E  V  Y  G  Q  T  N  I  G
1561      CGGCAAAGGCGGCACGCGTATTCCAATCTCCGGGATCGCCGGTGACCAGCAGGCCGCGCT
            G  K  G  G  T  R  I  P  I  S  G  I  A  G  D  Q  Q  A  A  L
1621      GTTTGGTCAGTTGTGCGTGAAAGAAGGGATGGCGAAGAACACCTATGGCACTGGCTGCTT
            F  G  Q  L  C  V  K  E  G  M  A  K  N  T  Y  G  T  G  C  F
1681      TATGCTGATGAACACTGGCGAGAAAGCGGTGAAATCAGAAAACGGCCTGCTGACCACCAT
            M  L  M  N  T  G  E  K  A  V  K  S  E  N  G  L  L  T  T  I
1741      CGCCTGCGGCCCGACTGGCGAAGTGAACTATGCGTTGGAAGGTGCGGTGTTTATGGCAGG
            A  C  G  P  T  G  E  V  N  Y  A  L  E  G  A  V  F  M  A  G
1801      CGCATCCATTCAGTGGCTGCGCGATGAAATGAAGTTGATTAACGACGCCTACGATTCCGA
            A  S  I  Q  W  L  R  D  E  M  K  L  I  N  D  A  Y  D  S  E
1861      ATATTTCGCCACCAAAGTGCAAAACACCAATGGTGTGTATGTGGTTCCGGCATTTACCGG
            Y  F  A  T  K  V  Q  N  T  N  G  V  Y  V  V  P  A  F  T  G
1921      GCTGGGTGCGCCGTACTGGGACCCGTATGCGCGCGGGGCGATTTTCGGTCTGACTCGTGG
            L  G  A  P  Y  W  D  P  Y  A  R  G  A  I  F  G  L  T  R  G
1981      GGTGAACGCTAACCACATTATACGCGCGACGCTGGAGTCTATTGCTTATCAGACGCGTGA
            V  N  A  N  H  I  I  R  A  T  L  E  S  I  A  Y  Q  T  R  D
2041      CGTGCTGGAAGCGATGCAGGCCGACTCTGGTATCCGTCTGCACGCCCTGCGCGTGGATGG
            V  L  E  A  M  Q  A  D  S  G  I  R  L  H  A  L  R  V  D  G
2101      TGGCGCAGTAGCAAACAATTTCCTGATGCAGTTCCAGTCCGATATTCTCGGCACCCGCGT
            G  A  V  A  N  N  F  L  M  Q  F  Q  S  D  I  L  G  T  R  V
1261      TGAGCGCCCGGAAGTGCGCGAAGTCACCGCATTGGGTGCGGCCTATCTCGCAGGCCTGGC
            E  R  P  E  V  R  E  V  T  A  L  G  A  A  Y  L  A  G  L  A
2221      GGTTGGCTTCTGGCAGAACCTCGACGAGCTGCAAGAGAAAGCGGTGATTGAGCGCGAGTT
            V  G  F  W  Q  N  L  D  E  L  Q  E  K  A  V  I  E  R  E  F
2281      CCGTCCAGGCATCGAAACCACTGAGCGTAATTACCGTTACGCAGGCTGGAAAAAAGCGGT
            R  P  G  I  E  T  T  E  R  N  Y  R  Y  A  G  W  K  K  A  V
2341      TAAACGCGCGATGGCGTGGGAAGAACACGACGAATAATAATTAA
            K  R  A  M  A  W  E  E  H  D  E  *
```

Figure 3.20 The nucleotide sequence and the deduced amino acid sequence of *glpFK* gene of recombinant plasmid pGlpFK. The *lac* operator and ribosome binding site are indicated in blue and green, respectively. The restriction sites *Nde*I and *Pac*I are shown by the underlined pink and red, respectively. The nucleotide sequences and the deduced amino acid sequences of *glpF* and *glpK* genes are indicated in brown and purple, respectively.

The nucleotide sequences of *glpFK* gene from TOP10 and DH10B were in good agreement with 100% identity.

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

The expression of individual AroB, AroL, GlpF, PheA, PheDH, TktA, YddG and GlpK proteins under the control of the T7*lac* promoter in each recombinant *E. coli* was evaluated by SDS-PAGE analysis (Figure 3.21). *E. coli* BL21(DE3) harbouring parental plasmid, pRSFDuet-1, was used as a control (lane 1). It was disclosed that AroB, AroL, PheA, PheDH, TktA, and GlpK proteins (lane 2-3, 5-7 and 9) could be clearly individually expressed in each genetically modified *E. coli* BL21(DE3) by IPTG induction. SDS-PAGE analysis remarkably showed the relevant intense protein bands of AroB, AroL, PheA, PheDH, TktA, and GlpK after 3 h induction at the apparent molecular weights of approximately 39, 19, 43, 42, 73 and 57 kDa, respectively. These values were consistent with the theoretical molecular weights of 38,881, 19,151, 43,111, 41,330, 72,212 and 56,231 Da estimated from deduced amino acid sequences of each protein deposited in GenBank databases by using the ProtParam tool (http://web.expasy.org/protparam/). The calibration curve for determination of protein molecular weight is shown in Appendix J. These six proteins were synthesized in cytoplasm and their amounts were sufficient to be detected on Coomassie Brilliant Blue stained SDS-PAGE gel. In contrast, the membrane proteins YddG and GlpF synthesized were embedded in cell membrane and their amounts in membrane fractions were insufficient to be detected on gel stained with Coomassie Blue (lane 4, 8 and 9). Their expressions could be determined indirectly by evaluation of an increase in L-Phe production of engineered *E. coli* containing relevant gene combination.

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

To successfully attain pPTFBLYA as shown in Figure 3.4, each gene had to be sequencely placed on pRSFDuet-1 to avoid the cleavage of restriction enzymes at improper locations on backbone recombinant plasmid. The insertion of genes

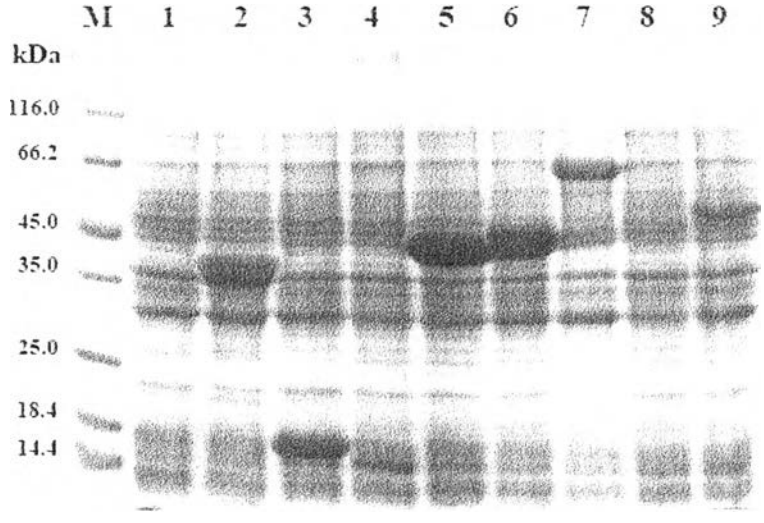


Figure 3.21 SDS-PAGE of whole cell extracts of recombinant *E. coli* BL21(DE3) carrying each gene after induction with 1 mM IPTG for 3 h

Lane M = protein molecular weight marker
Lane 1 = *E. coli* BL21(DE3)/pRSFDuet-1
Lane 2 = *E. coli* BL21(DE3)/pAroB
Lane 3 = *E. coli* BL21(DE3)/pAroL
Lane 4 = *E. coli* BL21(DE3)/pGlpF
Lane 5 = *E. coli* BL21(DE3)/pPheA
Lane 6 = *E. coli* BL21(DE3)/pPheDH
Lane 7 = *E. coli* BL21(DE3)/pTktA
Lane 8 = *E. coli* BL21(DE3)/pYddG
Lane 9 = *E. coli* BL21(DE3)/pGlpFK

in multiple cloning site-2 of pRSFDuet-1 was carried out first followed by the insertion of genes in multiple cloning site-1. Every single gene (i.e. *tktA, glpF, aroB, aroL, yddG* and *pheA* genes) was added to previous constructed plasmid pPheDH one by one to produce recombinant plasmids pPT, pPTF, pPTFB, pPTFBL, pPTFBLY and pPTFBLYA.

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

To generate recombinant plasmid pPT containing *phedh* and *tktA*, the DNA fragment containing the *tktA* gene with its own T7*lac* promoter was amplified using the plasmid pTktA as template and a pair of specific primers T7-*Eco*RV and tktA-*Xho*I was used. As shown in Figure 3.22A, all annealing temperatures gave strong band of 2.1 kb together with smear tail at lower size. After recovering the desired fragment from the gel, it was cleaved with *Xho*I and ligated into the vector pPheDH (4,955 bp) digested with *Eco*RV and *Xho*I to construct pPT (7,021 bp) in which the *tktA* gene fragment was placed downstream of *phedh* gene as shown in Figure 2.6B. The digestion fragments of 2,101 bp (insert DNA) and 4,920 bp (vector DNA) for ligation were run on agarose gel to determine purity as shown in Figure 3.22B. Plasmid pPT was then transformed into *E. coli* BL21(DE3), confirmed the correct insertion by digestion and sequencing. The nucleotide sequence of *tktA* gene including its T7*lac* promoter and ribosome binding site is shown in Figure 3.23. This obtained sequence has 100% match against the nucleotide sequence of *tktA* gene from *E. coli* DH10B, the T7*lac* promoter and ribosome binding site from pRSFDuet-1. This pPT was used as a cloning vector for further insertion of *glpF* gene.

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

Using pGlpF as template, PCR amplification of the *glpF* gene preceded by the T7*lac* promoter fragment of 961 bp was conducted using sense T7-*Xho*I and antisense glpF-*Pac*I primers. It was found that all annealing temperatures gave the intense band of roughly 0.97 kb (Figure 3.24A). Subsequently, this PCR product was harvested and inserted between *Xho*I and *Pac*I sites linking to stop codon of *tktA* gene of plasmid pPT yielding plasmid pPTF (7,907 bp) containing *phedh*, *tktA* and

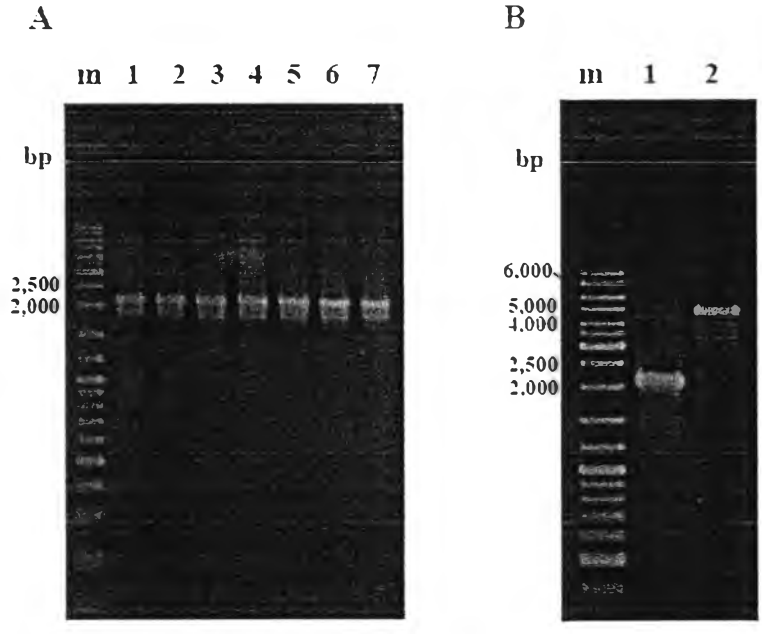


Figure 3.22 Electrophoretic patterns of recombinant plasmid pPT

A: PCR products of *tktA* preceded by T7*lac* promoter (*tktA* gene fragment) using various annealing temperatures

Lane m = 100 bp DNA ladder

Lane 1 = annealing temperature of 50.0°C

Lane 2 = annealing temperature of 54.4 °C

Lane 3 = annealing temperature of 56.5 °C

Lane 4 = annealing temperature of 58.7 °C

Lane 5 = annealing temperature of 60.8 °C

Lane 6 = annealing temperature of 62.9 °C

Lane 7 = annealing temperature of 64.6 °C

B: Pattern of digested fragments

Lane m = 100 bp DNA ladder

Lane 1 = *Xho*I-digested PCR product of *tktA* gene fragment

Lane 2 = *Eco*RV/*Xho*I-digested pPheDH

```
-402                          GGCAGACAGCTTGCAGACAAAGGCATTCTGTATGCTCCGGAT

-360      TATATTGTTAACTCTGGCGGTCTGATCCAAGTAGCCGACGAATTGTATGAGGTGAACAAA

-300      GAACGCGTGCTTGCGAAGACGAAGCATATTTACGACGCAATTCTTGAAGTGTACCAGCAA

-240      GCGGAATTAGATCAAATCACCACAATGGAAGCAGCCAACAGAATGTGTGAGCAAAGAATG

-180      GCGGCAAGAGGCCGACGCAACAGCTTCTTTACTTCTTCTGTTAAGCCAAAATGGGATATT

-120      CGCAACTAAGAATATCACGGCCGCATAATCGAAATTAATACGACTCACTATAGGGGAATTG

 -60      TGAGCGGATAACAATTCCCCATCTTAGTATATTAGTTAAGTATAAGAAGGAGATATACAT

   1      ATGTCCTCACGTAAAGAGCTTGCCAATGCTATTCGTGCGCTGAGCATGGACGCAGTACAG
           M  S  S  R  K  E  L  A  N  A  I  R  A  L  S  M  D  A  V  Q
  61      AAAGCCAAATCCGGTCACCCGGGTGCCCCTATGGGTATGGCTGACATTGCCGAAGTCCTG
           K  A  K  S  G  H  P  G  A  P  M  G  M  A  D  I  A  E  V  L
 121      TGGCGTGATTTCCTGAAACACAACCCGCAGAATCCGTCCTGGGCTGACCGTGACCGCTTC
           W  R  D  F  L  K  H  N  P  Q  N  P  S  W  A  D  R  D  R  F
 181      GTGCTGTCCAACGGCCACGGCTCCATGCTGATCTACAGCCTGCTGCACCTCACCGGTTAC
           V  L  S  N  G  H  G  S  M  L  I  Y  S  L  L  H  L  T  G  Y
 241      GATCTGCCGATGGAAGAACTGAAAAACTTCCGTCAGCTGCACTCTAAAACTCCGGGTCAC
           D  L  P  M  E  E  L  K  N  F  R  Q  L  H  S  K  T  P  G  H
 301      CCGGAAGTGGGTTACACCGCTGGTGTGGAAACCACCACCGGTCCGCTGGGTCAGGGTATT
           P  E  V  G  Y  T  A  G  V  E  T  T  T  G  P  L  G  Q  G  I
 361      GCCAACGCAGTCGGTATGGCGATTGCAGAAAAAACGCTGGCGGCGCAGTTTAACCGTCCG
           A  N  A  V  G  M  A  I  A  E  K  T  L  A  A  Q  F  N  R  P
 421      GGCCACGACATTGTCGACCACTACACCTACGCCTTCACGGGCGACGGCTGCATGATGGAA
           G  H  D  I  V  D  H  Y  T  Y  A  F  M  G  D  G  C  M  M  E
 481      GGCATCTCCCACGAAGTTTGCTCTCTGGCGGGTACGCTGAAGCTGGGTAAACTGATTGCA
           G  I  S  H  E  V  C  S  L  A  G  T  L  K  L  G  K  L  I  A
 541      TTCTACGATGACAACGGTATTTCTATCGATGGTCACGTTGAAGGCTGGTTCACCGACGAC
           F  Y  D  D  N  G  I  S  I  D  G  H  V  E  G  W  F  T  D  D
 601      ACCGCAATGCGTTTCGAAGCTTACGGCTGGCACGTTATTCGCGACATCGACGGTCATGAC
           T  A  M  R  F  E  A  Y  G  W  H  V  I  R  D  I  D  G  H  D
 661      GCGGCATCTATCAAACGCGCAGTAGAAGAAGCGCGCGCAGTGACTGACAAACCTTCCCTG
           A  A  S  I  K  R  A  V  E  E  A  R  A  V  T  D  K  P  S  L
 721      CTGATGTGCAAAACCATCATCGGTTTCGGTTCCCCGAACAAAGCCGGTACCCACGACTCC
           L  M  C  K  T  I  I  G  F  G  S  P  N  K  A  G  T  H  D  S
 781      CACGGTGCGCCGCTGGGCGACGCTGAAATTGCCCTGACCCGCGAACAACTGGGCTGGAAA
           H  G  A  P  L  G  D  A  E  I  A  L  T  R  E  Q  L  G  W  K
 841      TATGCGCCGTTCGAAATCCCGTCTGAAATCTATGCTCAGTGGGATGCGAAAGAAGCAGGC
           Y  A  P  F  E  I  P  S  E  I  Y  A  Q  W  D  A  K  E  A  G
 901      CAGGCGAAAGAATCCGCATGGAACGAGAAATTCGCTGCTTACGCGAAAGCTTATCCGCAG
           Q  A  K  E  S  A  W  N  E  K  F  A  A  Y  A  K  A  Y  P  Q

(continued)
```

Figure 3.23 The nucleotide sequence and the deduced amino acid sequence of *tktA* gene of recombinant plasmid pPT. The nucleotide sequence of *phedh* gene at upstream of *tktA* gene is indicated in purple. The T7 promoter, *lac* operator, ribosome binding site are indicated in orange, blue and green, respectively. The restriction sites *Eco*RV and *Xho*I are shown by the underlined pink and red, respectively.

(continued)

```
 961      GAAGCCGCTGAATTTACCCGCCGTATGAAAGGCGAAATGCCGTCTGACTTCGACGCTAAA
           E  A  A  E  F  T  R  R  M  K  G  E  M  P  S  D  F  D  A  K
1021      GCGAAAGAGTTCATCGCTAAACTGCAGGCTAATCCGGCGAAAATCGCCAGCCGTAAAGCG
           A  K  E  F  I  A  K  L  Q  A  N  P  A  K  I  A  S  R  K  A
1081      TCTCAGAATGCTATCGAAGCGTTCGGTCCGCTGTTGCCGGAATTCCTCGGCGGTTCTGCT
           S  Q  N  A  I  E  A  F  G  P  L  L  P  E  F  L  G  G  S  A
1141      GACCTGGCGCCGTCTAACCTGACCCTGTGGTCTGGTTCTAAAGCAATCAACGAAGATGCT
           D  L  A  P  S  N  L  T  L  W  S  G  S  K  A  I  N  E  D  A
1201      GCGGGTAACTACATCCACTACGGTGTTCGCGAGTTCGGTATGACCGCGATTGCTAACGGT
           A  G  N  Y  I  H  Y  G  V  R  E  F  G  M  T  A  I  A  N  G
1261      ATCTCCCTGCACGGTGGCTTCCTGCCGTACACCTCCACCTTCCTGATGTTCGTGGAATAC
           I  S  L  H  G  G  F  L  P  Y  T  S  T  F  L  M  F  V  E  Y
1321      GCACGTAACGCCGTACGTATGGCTGCGCTGATGAAACAGCGTCAGGTGATGGTTTACACC
           A  R  N  A  V  R  M  A  A  L  M  K  Q  R  Q  V  M  V  Y  T
1381      CACGACTCCATCGGTCTGGGCGAAGACGGCCCGACTCACCAGCCGGTTGAGCAGGTCGCT
           H  D  S  I  G  L  G  E  D  G  P  T  H  Q  P  V  E  Q  V  A
1441      TCTCTGCGCGTAACCCCGAACATGTCTACATGGCGTCCGTGTGACCAGGTTGAATCCGCG
           S  L  R  V  T  P  N  M  S  T  W  R  P  C  D  Q  V  E  S  A
1501      GTCGCGTGGAAATACGGTGTTGAGCGTCAGGACGGCCCGACCGCACTGATCCTCTCCCGT
           V  A  W  K  Y  G  V  E  R  Q  D  G  P  T  A  L  I  L  S  R
1561      CAGAACCTGGCGCAGCAGGAACGAACTGAAGAGCAACTGGCAAACATCGCGCGCGGTGGT
           Q  N  L  A  Q  Q  E  R  T  E  E  Q  L  A  N  I  A  R  G  G
1621      TATGTGCTGAAAGACTGCGCCGGTCAGCCGGAACTGATTTTCATCGCTACCGGTTCAGAA
           Y  V  L  K  D  C  A  G  Q  P  E  L  I  F  I  A  T  G  S  E
1681      GTTGAACTGGCTGTTGCTGCCTACGAAAAACTGACTGCCGAAGGCGTGAAAGCGCGCGTG
           V  E  L  A  V  A  A  Y  E  K  L  T  A  E  G  V  K  A  R  V
1741      GTGTCCATGCCGTCTACCGACGCATTTGACAAGCAGGATGCTGCTTACCGTGAATCCGTA
           V  S  M  P  S  T  D  A  F  D  K  Q  D  A  A  Y  R  E  S  V
1801      CTGCCGAAAGCGGTTACTGCACGCGTTGCTGTAGAAGCGGGTATTGCTGACTACTGGTAC
           L  P  K  A  V  T  A  R  V  A  V  E  A  G  I  A  D  Y  W  Y
1861      AAGTATGTTGGCCTGAACGGTGCTATCGTCGGTATGACCACCTTCGGTGAATCTGCTCCG
           K  Y  V  G  L  N  G  A  I  V  G  M  T  T  F  G  E  S  A  P
1921      GCAGAGCTGCTGTTTGAAGAGTTCGGCTTCACTGTTGATAACGTTGTTGCGAAAGCAAAA
           A  E  L  L  F  E  E  F  G  F  T  V  D  N  V  V  A  K  A  K
1981      GAACTGCTGTAACTCGAGTCTGGTAAAGAAACCGCTGCTGCGAAATTTGAACGCCAGCAC
           E  L  L  *
2041      ATGGACTCGTCTACTAGCGCAGCTTAAT
```

Figure 3.23 The nucleotide sequence and the deduced amino acid sequence of *tktA* gene of recombinant plasmid pPT. The nucleotide sequence of *phedh* gene at upstream of *tktA* gene is indicated in purple. The T7 promoter, *lac* operator, ribosome binding site are indicated in orange, blue and green, respectively. The restriction sites *Eco*RV and *Xho*I are shown by the underlined pink and red, respectively.

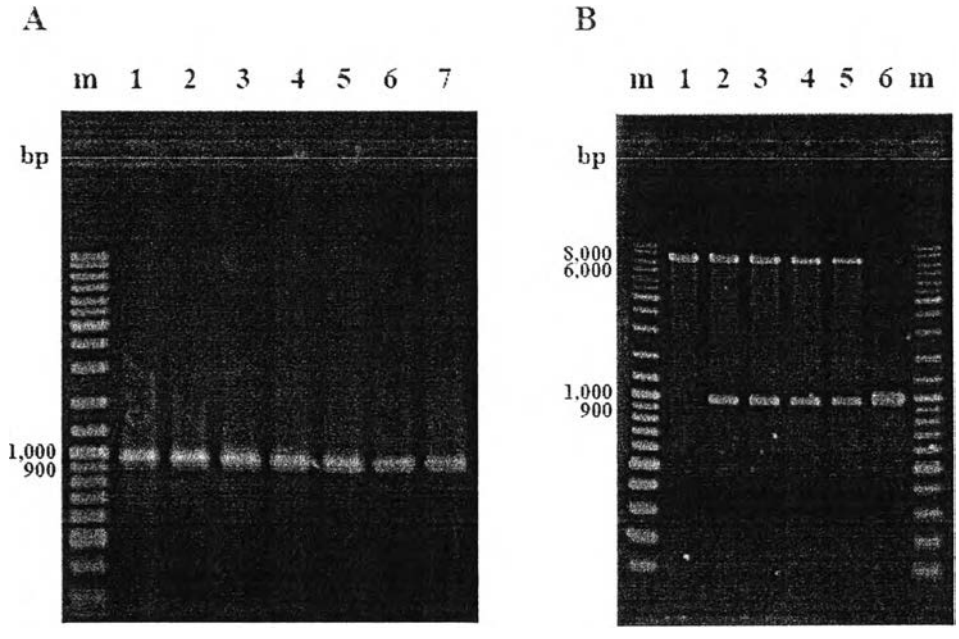


Figure 3.24 Electrophoretic patterns of recombinant plasmid pPTF

A: PCR products of *glpF* preceded by T7*lac* promoter (*glpF* gene fragment) using various annealing temperatures

Lane m = 100 bp DNA ladder

Lane 1 = annealing temperature of 36.4°C

Lane 2 = annealing temperature of 38.2 °C

Lane 3 = annealing temperature of 40.3 °C

Lane 4 = annealing temperature of 42.5 °C

Lane 5 = annealing temperature of 44.7 °C

Lane 6 = annealing temperature of 46.8 °C

Lane 7 = annealing temperature of 48.5 °C

B: Restriction pattern of pPTF

Lane m = 100 bp DNA ladder

Lane 1 = *Xho*I/*Pac*I-digested pPT

Lane 2-5 = *Xho*I/*Pac*I-digested pPTF

Lane 6 = PCR product of *glpF* gene fragment

glpF as shown in Figure 2.7A. The pPTF was transformed into *E. coli* BL21(DE3) competent cell and kanamycin-resistant colonies were selected on agarose gel. To analyze restriction pattern of pPTF, it was cut by *Xho*I and *Pac*I leading to two separated fragments: the inserted fragment containing *glpF* gene (961 bp) and the part of pPT (6,946 bp) (Figure 3.24B). The pPTF was confirmed the correct insertion by restriction enzyme digestion and DNA sequencing. The nucleotide sequence of *glpF* gene of pPTF is shown in Figure 3.25. It showed 100% sequence identity to *glpF* gene of *E. coli* DH10B.

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

The recombinant plasmid pPTFK containing *phedh*, *tktA, glpF* and *glpK* was constructed to be used as a source of *glpFK* gene with its promoter for ease of further cloning.

The constructed plasmid pGlpFK was used as a DNA template for amplification of the *glpFK* flanking with promoter using T7-*Xho*I and glpK-*Pac*I primers. The result from agarose gel electrophoresis is shown in Figure 3.26A. It was clear that all annealing temperatures yielded the expected DNA fragment approximately 2.5 kb corresponding to the sum of the *glpFK* gene and the promoter lengths. However, a few non-specific bands were appeared. The expected fragment was recovered and ligated into pPT at *Xho*I and *Pac*I sites to create the 9,438 bp pPTFK vector as shown in Figure 2.7B. After transformation into competent *E. coli* BL21(DE3) host, the recombinant plasmid was confirmed by double digestion with *Bam*HI and *Xho*I. The restriction pattern contained three bands of 4,659 bp, 3,480 bp and 1,300 bp (Figure 3.26B) because each enzyme cloud digest at one site of pPT and *Bam*HI could cut *glpK* gene. As shown in Figure 3.27, the nucleotide sequence of long fragment of *glpFK* gene and its T7*lac* promoter (2,492 bp) inserted into pPT was verified by sequencing. The sequence showed 100% homology to *glpFK* gene of *E. coli* DH10B reported in sequence database.

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

To achieve the recombinant plasmid pPTFB carrying *phedh*, *tktA*, *glpF* and *aroB*, the digestions of pAroB (4,883 bp) and pPTF (7,907 bp) with *Bam*HI and

```
-96                                  TAATCGAAATTAATACGACTCACTATAGGGGAATTG

-60      TGAGCGGATAACAATTCCCCATCTTAGTATATTAGTTAAGTATAAGAAGGAGATATACAT

  1      ATGAGTCAAACATCAACCTTGAAAGGCCAGTGCATTGCTGAATTCCTCGGTACCGGGTTG
          M  S  Q  T  S  T  L  K  G  Q  C  I  A  E  F  L  G  T  G  L
 61      TTGATTTTCTTCGGTGTGGGTTGCGTTGCAGCACTAAAAGTCGCTGGTGCGTCTTTTGGT
          L  I  F  F  G  V  G  C  V  A  A  L  K  V  A  G  A  S  F  G
121      CAGTGGGAAATCAGTGTCATTTGGGGACTGGGGGTGGCAATGGCCATCTACCTGACCGCA
          Q  W  E  I  S  V  I  W  G  L  G  V  A  M  A  I  Y  L  T  A
181      GGGGTTTCCGGCGCGCATCTTAATCCCGCTGTTACCATTGCATTGTGGCTGTTTGCCTGT
          G  V  S  G  A  H  L  N  P  A  V  T  I  A  L  W  L  F  A  C
241      TTCGACAAGCGCAAAGTTATTCCTTTTATCGTTTCACAAGTTGCCGGCGCTTTCTGTGCT
          F  D  K  R  K  V  I  P  F  I  V  S  Q  V  A  G  A  F  C  A
301      GCGGCTTTAGTTTACGGGCTTTACTACAATTTATTTTTCGACTTCGAGCAGACTCATCAC
          A  A  L  V  Y  G  L  Y  Y  N  L  F  F  D  F  E  Q  T  H  H
361      ATTGTTCGCGGCAGCGTTGAAAGTGTTGATCTGGCTGGCACTTTCTCTACTTACCCTAAT
          I  V  R  G  S  V  E  S  V  D  L  A  G  T  F  S  T  Y  P  N
421      CCTCATATCAATTTTGTGCAGGCTTTCGCAGTTGAGATGGTGATTACCGCTATTCTGATG
          P  H  I  N  F  V  Q  A  F  A  V  E  M  V  I  T  A  I  L  M
481      GGGCTGATCCTGGCGTTAACGGACGATGGCAACGGTGTACCACGCGGCCCTTTGGCTCCC
          G  L  I  L  A  L  T  D  D  G  N  G  V  P  R  G  P  L  A  P
541      TTGCTGATTGGTCTACTGATTGCGGTCATTGGCGCATCTATGGGCCCATTGACAGGTTTT
          L  L  I  G  L  L  I  A  V  I  G  A  S  M  G  P  L  T  G  F
601      GCCATGAACCCAGCGCGTGACTTCGGTCCGAAAGTCTTTGCCTGGCTGGCGGGCTGGGGC
          A  M  N  P  A  R  D  F  G  P  K  V  F  A  W  L  A  G  W  G
661      AATGTCGCCTTTACCGGCGGCAGAGACATTCCTTACTTCCTGGTGCCGCTTTTCGGCCCT
          N  V  A  F  T  G  G  R  D  I  P  Y  F  L  V  P  L  F  G  P
721      ATCGTTGGCGCGATTGTAGGTGCATTTGCCTACCGCAAACTGATTGGTCGCCATTTGCCT
          I  V  G  A  I  V  G  A  F  A  Y  R  K  L  I  G  R  H  L  P
781      TGCGATATCTGTGTTGTGGAAGAAAAGGAAACCACAACTCCTTCAGAACAAAAAGCTTCG
          C  D  I  C  V  V  E  E  K  E  T  T  T  P  S  E  Q  K  A  S
841      CTGTAATAATTAA
          L  *
```

Figure 3.25 The nucleotide sequence and the deduced amino acid sequence of *glpF* gene of recombinant plasmid pPTF. The T7 promoter, *lac* operator, ribosome binding site are indicated in orange, blue and green, respectively. The *Pac*I restriction site is shown by the underlined red, respectively.

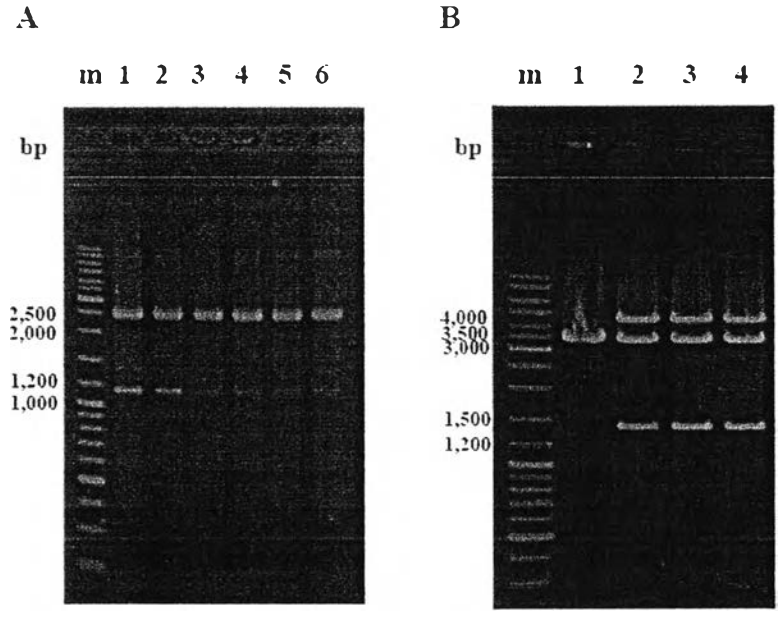


Figure 3.26 Electrophoretic patterns of recombinant plasmid pPTFK

A: PCR products of *glpFK* preceded by T7*lac* promoter (*glpFK* gene fragment) using various annealing temperatures

Lane m = 100 bp DNA ladder

Lane 1 = annealing temperature of 46.4°C

Lane 2 = annealing temperature of 48.9 °C

Lane 3 = annealing temperature of 51.0 °C

Lane 4 = annealing temperature of 52.7 °C

Lane 5 = annealing temperature of 53.8 °C

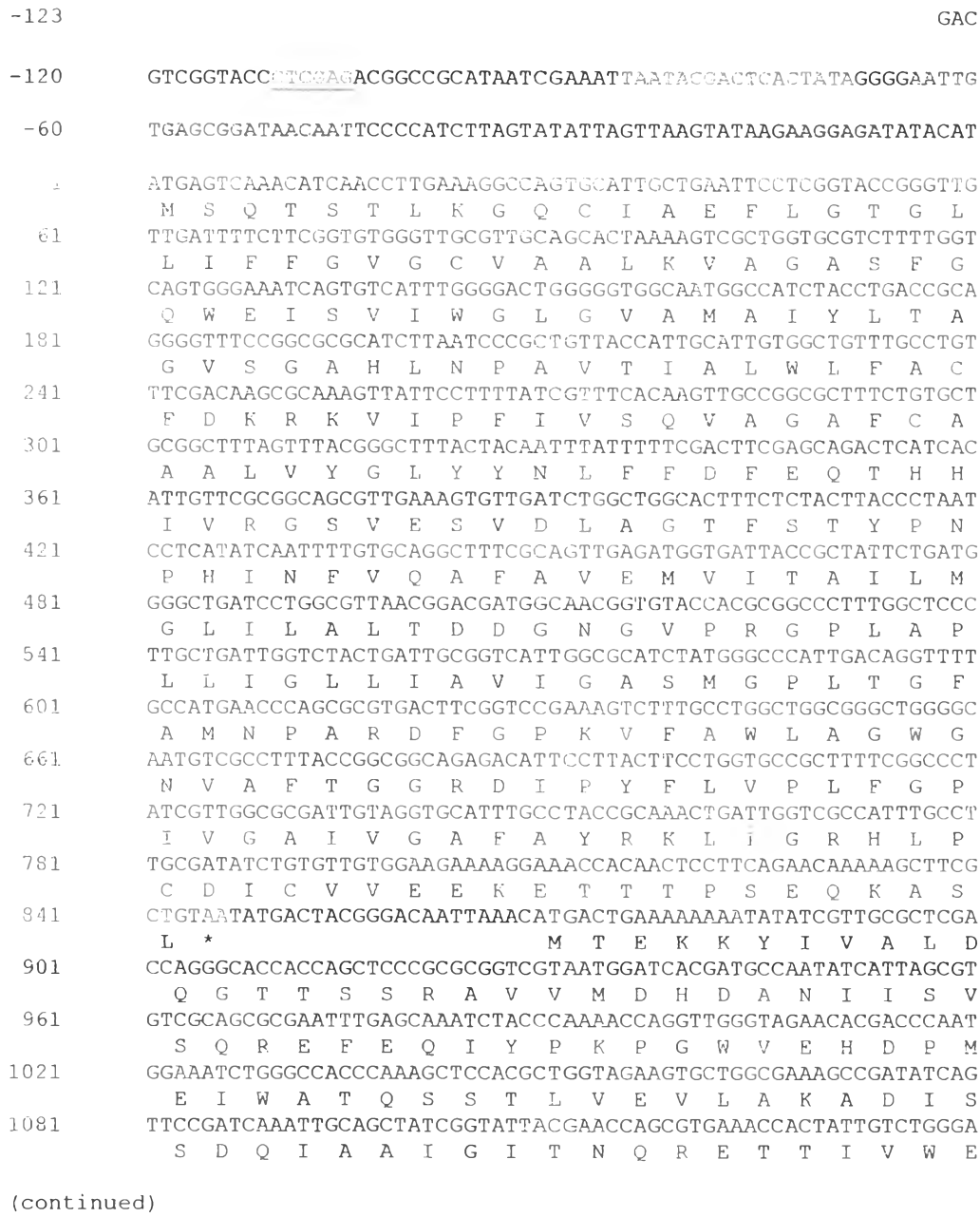
Lane 6 = annealing temperature of 55.0 °C

B: Restriction pattern of pPTFK

Lane m = 100 bp DNA ladder

Lane 1 = *Bam*HI/*Xho*I-digested pPT

Lane 2-4 = *Bam*HI/*Xho*I-digested pPTFK



(continued)

Figure 3.27 The nucleotide sequence and the deduced amino acid sequence of *glpFK* gene of recombinant plasmid pPTFK. The T7 promoter, *lac* operator and ribosome binding site are indicated in orange, blue and green, respectively. The restriction sites *Xho*I and *Pac*I are shown by the underlined pink and red, respectively. The nucleotide sequences and the deduced amino acid sequences of *glpF* and *glpK* genes are indicated in brown and purple, respectively.

(continued)

```
1141    AAAAGAAACCGGCAAGCCTATCTATAACGCCATTGTCTGGCAGTGCCGTCGTACCGCAGA
          K  E  T  G  K  P  I  Y  N  A  I  V  W  Q  C  R  R  T  A  E
1201    AATCTGCGAGCATTTAAAACGTGACGGTTTAGAAGATTATATCCGCAGCAATACCGGTCT
          I  C  E  H  L  K  R  D  G  L  E  D  Y  I  R  S  N  T  G  L
1261    GGTGATTGACCCGTACTTTTCTGGCACCAAAGTGAAGTGGATCCTCGACCATGTGGAAGG
          V  I  D  P  Y  F  S  G  T  K  V  K  W  I  L  D  H  V  E  G
1321    CTCTCGCGAGCGTGCACGTCGTGGTGAATTGCTGTTTGGTACGGTTGATACGTGGCTTAT
          S  R  E  R  A  R  R  G  E  L  L  F  G  T  V  D  T  W  L  I
1381    CTGGAAAATGACTCAGGGCCGTGTCCATGTGACCGATTACACCAACGCCTCTCGTACCAT
          W  K  M  T  Q  G  R  V  H  V  T  D  Y  T  N  A  S  R  T  M
1441    GTTGTTCAACATCCATACCCTGGACTGGGACGACAAAATGCTGGAAGTGCTGGATATTCC
          L  F  N  I  H  T  L  D  W  D  D  K  M  L  E  V  L  D  I  P
1501    GCGCGAGATGCTGCCAGAAGTGCGTCGTTCTTCCGAAGTATACGGTCAGACTAACATTGG
          R  E  M  L  P  E  V  R  R  S  S  E  V  Y  G  Q  T  N  I  G
1561    CGGCAAAGGCGGCACGCGTATTCCAATCTCCGGGATCGCCGGTGACCAGCAGGCCGCGCT
          G  K  G  G  T  R  I  P  I  S  G  I  A  G  D  Q  Q  A  A  L
1621    GTTTGGTCAGTTGTGCGTGAAAGAAGGGATGGCGAAGAACACCTATGGCACTGGCTGCTT
          F  G  Q  L  C  V  K  E  G  M  A  K  N  T  Y  G  T  G  C  F
1681    TATGCTGATGAACACTGGCGAGAAAGCGGTGAAATCAGAAAACGGCCTGCTGACCACCAT
          M  L  M  N  T  G  E  K  A  V  K  S  E  N  G  L  L  T  T  I
1741    CGCCTGCGGCCCGACTGGCGAAGTGAACTATGCGTTGGAAGGTGCGGTGTTTATGGCAGG
          A  C  G  P  T  G  E  V  N  Y  A  L  E  G  A  V  F  M  A  G
1801    CGCATCCATTCAGTGGCTGCGCGATGAAATGAAGTTGATTAACGACGCCTACGATTCCGA
          A  S  I  Q  W  L  R  D  E  M  K  L  I  N  D  A  Y  D  S  E
1861    ATATTTCGCCACCAAAGTGCAAAACACCAATGGTGTGTATGTGGTTCCGGCATTTACCGG
          Y  F  A  T  K  V  Q  N  T  N  G  V  Y  V  V  P  A  F  T  G
1921    GCTGGGTGCGCCGTACTGGGACCCGTATGCGCGCGGGGCGATTTTCGGTCTGACTCGTGG
          L  G  A  P  Y  W  D  P  Y  A  R  G  A  I  F  G  L  T  R  G
1981    GGTGAACGCTAACCACATTATACGCGCGACGCTGGAGTCTATTGCTTATCAGACGCGTGA
          V  N  A  N  H  I  I  R  A  T  L  E  S  I  A  Y  Q  T  R  D
2041    CGTGCTGGAAGCGATGCAGGCCGACTCTGGTATCCGTCTGCACGCCCTGCGCGTGGATGG
          V  L  E  A  M  Q  A  D  S  G  I  R  L  H  A  L  R  V  D  G
2101    TGGCGCAGTAGCAAACAATTTCCTGATGCAGTTCCAGTCCGATATTCTCGGCACCCGCGT
          G  A  V  A  N  N  F  L  M  Q  F  Q  S  D  I  L  G  T  R  V
1261    TGAGCGCCCGGAAGTGCGCGAAGTCACCGCATTGGGTGCGGCCTATCTCGCAGGCCTGGC
          E  R  P  E  V  R  E  V  T  A  L  G  A  A  Y  L  A  G  L  A
2221    GGTTGGCTTCTGGCAGAACCTCGACGAGCTGCAAGAGAAAGCGGTGATTGAGCGCGAGTT
          V  G  F  W  Q  N  L  D  E  L  Q  E  K  A  V  I  E  R  E  F
2281    CCGTCCAGGCATCGAAACCACTGAGCGTAATTACCGTTACGCAGGCTGGAAAAAAGCGGT
          R  P  G  I  E  T  T  E  R  N  Y  R  Y  A  G  W  K  K  A  V
2341    TAAACGCGCGATGGCGTGGGAAGAACACGACGAATAATAATTAA
          K  R  A  M  A  W  E  E  H  D  E  *
```

Figure 3.27 The nucleotide sequence and the deduced amino acid sequence of *glpFK* gene of recombinant plasmid pPTFK. The T7 promoter, *lac* operator and ribosome binding site are indicated in orange, blue and green, respectively. The restriction sites *Xho*I and *Pac*I are shown by the underlined pink and red, respectively. The nucleotide sequences and the deduced amino acid sequences of *glpF* and *glpK* genes are indicated in brown and purple, respectively.

*Xba*I were performed to get the desirable fragments for cloning (Figure 2.8A). For pPTF digestion, the shorter *Bam*HI-*Xba*I fragment of 1,521 bp was removed whereas the longer fragment of 6,386 bp containing *phedh*, *tktA*, and *glpF* was eluted from agarose gel. For pAroB digestion, the *Bam*HI-*Xba*I fragment of 2,575 bp containing the *aroB* gene was purified from gel and ligated with the prepared fragment of 6,386 bp resulting in recombinant plasmid pPTFB (8,961 bp). Each fragment for ligation is shown in agrose gel (Figure 3.28A). Though the two similar sizes of resulting restriction fragments of pAroB were separated on 1.4% agrose gel before gel extraction, it was still difficult to purify the desired fragment (2,575 bp) from the contaminated 2,308 bp fragment of. However, the undesirable recombinant plasmid could be excluded by mobility of recombinant plasmid during rapid selection on agarose gel electrophoresis as section 2.9.4.5.1. This pPTFB plasmid was transformed into competent *E. coli* BL21(DE3) and transformant was selected on agarose gel. After digestion of pPTFB with *Bam*HI and *Xba*I, the two fragments of 6,386 bp and 2,575 bp were separated (Figure 3.28B).

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

The engineered plasmid pPTFBL harbouring *phedh*, *glpF*, *aroB* and *aroL* was created by the fragment combination between the restriction fragments from pPTFB and pAroL digestions. The pPTFB plasmid (8,961 bp) was digested with *Bam*HI and *Bsr*GI to remove the 84 bp *Bam*HI-*Bsr*GI fragment. The retaining fragment (8,877 bp) was inserted with the target fragment. The target fragment of 702 bp containing the *aroL* gene was prepared by digestion of pAroL (4,447 bp) with the same restriction enzymes. After purification of the specified fragments, both were treated with ligase to give pPTFBL (9,579 bp) as in Figure 2.8B and then introduced into *E. coli* BL21(DE3) host cell. The recombinant cells containing pPTFBL were selected and their recombinant plasmids were cut with *Bam*HI and *Bsr*GI. The digestion of recombinant plasmid with right cloning needed to be composed of two fragments that were 8,877 bp and 702 bp as in Figure 3.29.

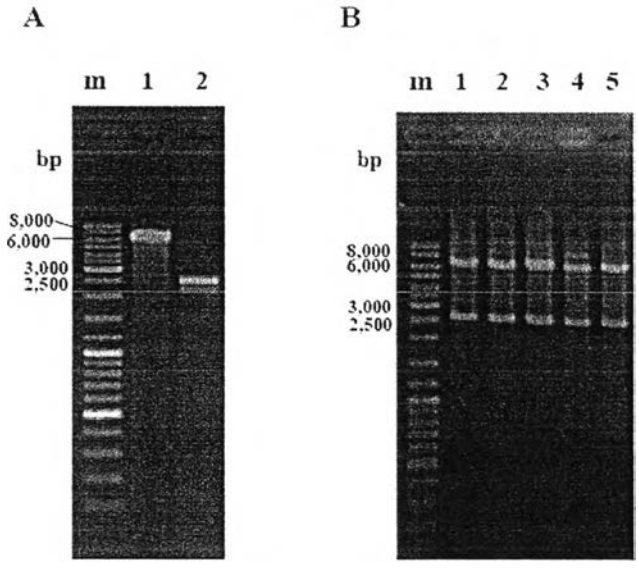


Figure 3.28 Electrophoretic patterns of recombinant plasmid pPTFB

A: Pattern of digested fragments

Lane m = 100 bp DNA ladder

Lane 1 = *Bam*HI/*Xba*I-digested fragment of pPTF

Lane 2 = *Eco*RV/*Xho*I-digested fragment of pAroB

B: Restriction pattern of pPTFB

Lane m = 100 bp DNA ladder

Lane 1-5 = *Bam*HI/*Xho*I-digested pPTFB

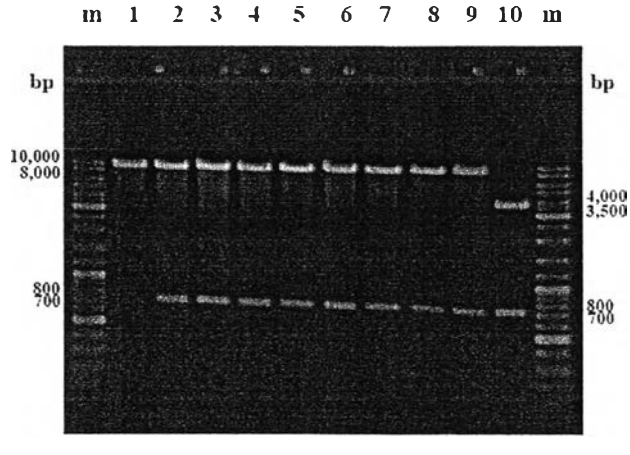


Figure 3.29 Restriction pattern of recombinant plasmid pPTFBL

Lane m = 100 bp DNA ladder

Lane 1 = *Bam*HI/*Bsr*GI-digested pPTFB

Lane 2-9 = *Bam*HI/*Bsr*GI-digested pPTFBL

Lane 10 = *Bam*HI/*Bsr*GI-digested pAroL

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

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

To construct the pPTFBLY carrying *phedh*, *tktA*, *glpF*, *aroB*, *aroL* and *yddG*, the recombinant plasmid pLY containing *aroL* and *yddG* genes was first generated so that it was used as source of both *aroL* and *yddG* genes for insertion to pPTFB. The constructed pAroL was used as backbone plasmid for insertion of the *yddG* gene fragment from digested pYddG. The pYddG (4,805 bp) was digested with *Asc*I and *Hin*dIII to recover the target fragment (994 bp) containing the *yddG* gene with its promoter, while the 3,811 bp fragment was eliminated. Similarly, the pAroL was digested with the same restriction endonucleases. The target fragment was cloned into the *Asc*I-*Hin*dIII pAroL vector (4,429 bp) leading to pLY (5,423 bp) as in Figure 2.9A. After transformed to host cell, pLY was extracted and verified by digestion with *Asc*I and *Hin*dIII. The two obtained fragments of 4,429 bp and 994 bp are shown in Figure 3.30.

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

To obtain recombinant plasmid pPTFBLY, the constructed plasmids pPTFB and pLY were used. The backbone plasmid pPTFB was prepared by cleaving with *Bam*HI and *Afl*II to give the 8,904 bp fragment. The pLY was digested with the same restriction enzymes and the 1,651 bp fragment containing both *aroL* and *yddG* genes was purified from the gel. The desired fragment of 1,651 bp was cloned into the *Bam*HI-*Afl*II pPTFB vector to get pPTFBLY (10,555 bp) as in Figure 2.9B. The prepared plasmid was transformed to competent cell. To confirm whether cloning was correct, the plasmid pPTFBLY was extracted and digested with *Bam*HI and *Afl*II as shown in Figure 3.31. The restriction fragments of 8,904 bp and 1,651 bp were obtained.

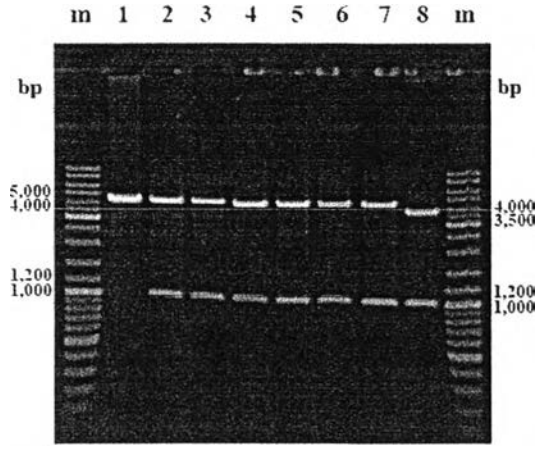


Figure 3.30 Restriction pattern of recombinant plasmid pLY

Lane m = 100 bp DNA ladder

Lane 1 = *Asc*I/*Hin*dIII-digested pAroL

Lane 2-7 = *Asc*I/*Hin*dIII-digested pLY

Lane 8 = *Asc*I/*Hin*dIII-digested pYddG

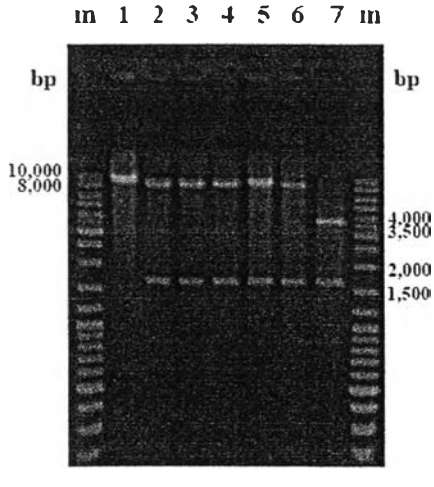


Figure 3.31 Restriction pattern of recombinant plasmid pPTFBLY

Lane m = 100 bp DNA ladder

Lane 1 = *Bam*HI/*Afl*II-digested pPTFB

Lane 2-6 = *Bam*HI/*Afl*II-digested pPTFBLY

Lane 7 = *Bam*HI/*Afl*II-digested pLY

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

The recombinant plasmid pPTFBLYA carrying *phedh*, *tktA*, *glpF*, *aroB*, *aroL*, *yddG* and *pheA* was created using similar strategy of previous constructed pPTFBLY.

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

Firstly, the three genes, *aroL*, *yddG* and *pheA*, were deposited in the same pRSFDuet-1 vector resulting in pLYA. This plasmid was used as source of genes to facilitate construction of pPTFBLYA from initial vector pPTFB. The pLYA was constructed by the insertion of the *pheA* gene fragment from pPheA into backbone vector pLY at the recognition sites of *Hin*dIII and *Bsr*GI. The pPheA was digested with *Hin*dIII and *Bsr*GI to delete the unwanted fragment of 3,782 bp and to recover the desired fragment of 1,296 bp containing T7*lac* promoter followed by the *pheA* gene. At the same time, pLY was digested with the same restriction enzymes leading to the 5,376 bp potential vector containing *aroL* and *yddG* genes. The *pheA* fragment was cloned into the vector to generate pLYA (6,672 bp) as shown in Figure 2.10A. After transformation, *E. coli* BL21(DE3) containing pLYA was selected and its plasmid was confirmed by cutting with *Hin*dIII and *Bsr*GI as shown in Figure 3.32.

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

The pLYA was cut with *Bam*HI and *Afl*II to give the 2,900 bp DNA cassette containing *aroL*, *yddG* and *pheA* genes and the undesirable fragment of 3,772 bp. The desired fragment 2,900 bp was purified from the gel and ligated to the *Bam*HI-*Afl*II pPTFB fragment of 8,904 bp resulting in pPTFBLYA (11,804 bp) as shown in Figure 2.10B. Both specified fragments for cloning were run on agarose gel (Figure 3.33A). Then, the pPTFBLYA vector was introduced into host cell to produce *E. coli* BL21(DE3)/pPTFBLYA clone. An expected *E. coli* clone was selected and the pPTFBLYA was verified by digestion with appropriated restriction enzyme *Nde*I. The restriction pattern analysis of pPTFBLYA with *Nde*I is shown in Figure 3.33B. It contained the seven fragments of 5275, 2103, 1298, 1254, 990, 638 and 246 bp

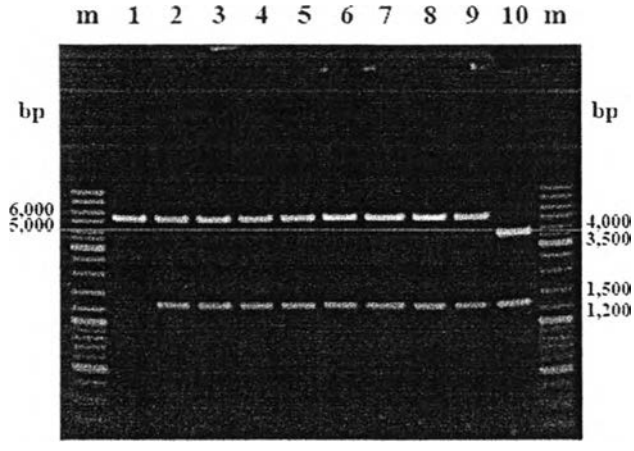


Figure 3.32 Restriction pattern of recombinant plasmid pLYA

Lane m = 100 bp DNA ladder

Lane 1 = *Hin*dIII/*Bsr*GI-digested pLY

Lane 2-9 = *Hin*dIII/*Bsr*GI-digested pLYA

Lane 10 = *Hin*dIII/*Bsr*GI-digested pPheA

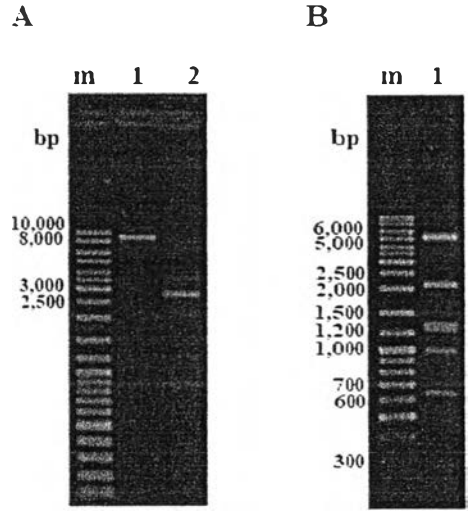


Figure 3.33 Electrophoretic patterns of recombinant plasmid pPTFBLYA

A: Pattern of digested fragments

Lane m = 100 bp DNA ladder

Lane 1 = *Bam*HI/*Afl*II-digested fragment of pPTFB

Lane 2 = *Bam*HI/*Afl*II-digested fragment of pLYA

B: Restriction pattern of pPTFBLYA

Lane m = 100 bp DNA ladder

Lane 1 = *Nde*I-digested pPTFBLYA

because the recognition sites of *Nde*I located at 954, 1200, 1838, 2828, 4126, 5380 and 7483 bp of pPTFBLYA.

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

To prove whether overexpression of *yddG* gene in *E. coli* BL21(DE3) containing *phedh* gene could improve the production of L-Phe, both *phedh* and *yddG* genes were cloned into the single pRSFDuet-1 to get pPY. The pYddG was digested with *Bam*HI and *Afl*II to generate the 1,033 bp desirable fragment containing the *yddG* gene together its promoter and the unwanted fragment of 3,772 bp which was the *Bam*HI-*Afl*II pRSFDuet-1 vector part. Then, the eligible fragment was purified from gel and cloned to the backbone plasmid pPheDH (4,898 bp) leading to pPY (5,931 bp) (Figure 2.11A). After transformation into *E. coli* host cell, potential cells containing pPY were selected and their plasmids were analyzed by digestion with *Bam*HI and *Afl*II. The restriction pattern of pPY composed of two fragments of 4,898 bp and 1,033 bp (Figure 3.34).

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

In order to study whether the *glpF* overexpression along with *phedh* and *yddG* genes in *E. coli* would affect on increasing of L-Phe production, *E. coli* BL21(DE3) containing pPYF (*phedh*, *yddG* and *glpF* genes) was designed. The recombinant plasmid pPYF was produced by the insertion of DNA fragment containing *phedh* and *yddG* genes into the pPTF in which the fragment containing *phedh* and *tktA* gene was removed. After digestion of both pPY and pPTF with *Bam*HI and *Xho*I, the 2,350 bp defined fragment containing *phedh* and *yddG* genes of pPY digestion was purified from gel. The 4,467 bp fragment of pPTF digestion acted as vector inserted with *glpF* gene was also recovered. After both of these fragments had been combined by ligase producing pPYF (6,817 bp) (Figure 2.11B), the ligation product was transformed to *E. coli* competent cell and the defined engineered clone/pPYF was examined. After digestion of pPYF with *Bam*HI and *Xho*I, the restriction fragments of 4,467 and 2,350 bp were obtained (Figure 3.35).

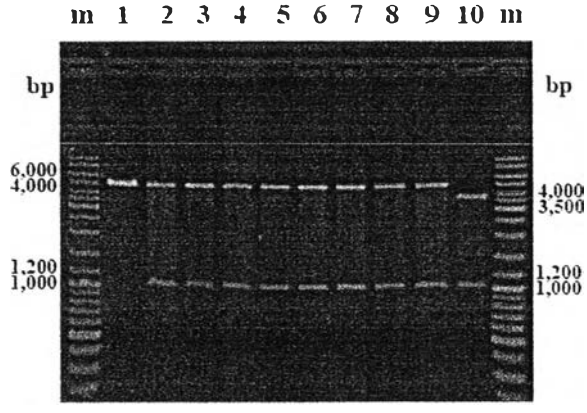


Figure 3.34 Restriction pattern of recombinant plasmid pPY

Lane m = 100 bp DNA ladder

Lane 1 = *Bam*HI/*Afl*II-digested pPheDH

Lane 2-9 = *Bam*HI/*Afl*II-digested pPY

Lane 10 = *Bam*HI/*Afl*II-digested pYddG

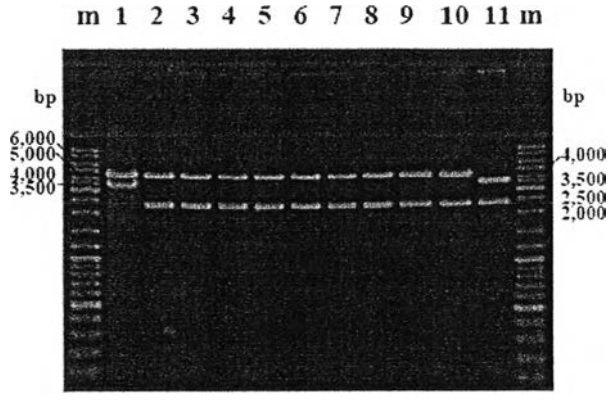


Figure 3.35 Restriction pattern of recombinant plasmid pPYF

Lane m = 100 bp DNA ladder

Lane 1 = *Bam*HI/*Xho*I-digested pPTF

Lane 2-10 = *Bam*HI/*Xho*I-digested pPYF

Lane 11 = *Bam*HI/*Xho*I-digested pPY

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

To elucidate whether the co-existence of the *glpK* gene prevented the reverse of cytoplasmic glycerol to exterior with *phedh, yddG* and *glpF* genes in pRSFDuet-1 vector was responsible for an increase in L-Phe production, the recombinant plasmid pPYFK containing four genes (*phedh*, *yddG*, *glpF* and *glpK* genes) was constructed. The 5,856 bp fragment containing *phedh* and *yddG* was recovered from the *Xho*I and *Pac*I-digested pPY reaction and acted as backbone plasmid. After the digestion of pPTFK, the target fragment of 2,493 bp containing the *glpF* and *glpK* genes was separated apart from the undesirable fragment of 6,945 bp. The two specified fragments was ligated to yield pPYFK (8,348 bp) (Figure 2.12A) and transformed into *E. coli*. The engineered *E. coli*/pPYF was selected and the successful of cloning was confirmed by cleaving the pPYF with *Xho*I and *Pac*I resulting in two fragments of 5,856 and 2,493 bp (Figure not shown).

The recombinant plasmids pPheDH (4,955 bp), pPY (5,931 bp), pPYF (6,817 bp) and pPYFK (8,348 bp) digested with *Xho*I were analyzed on agarose gel as shown in Figure 3.36.

3.8 Expression of recombinant plasmids pPY, pPYF, pPYFK in *E. coli* BL21(DE3)

To select one transformant that was a representative of each recombinant *E. coli* for evaluation of L-Phe production, five transformants of each were compared for the expression level (section 2.14). All cells of each recombinant clone showed similar intensity of protein pattern in SDS-PAGE gel stained by Coomassie blue (data not shown). Therefore, one was picked for shake flask cultivation. As seen in Figure 3.37A, the protein patterns of cell extracts by all *E. coli* containing *phedh* (pPheDH, pPY, pPYF and pPYF clones) showed the additional protein band with the expected size of PheDH (42 kDa) (lane 2-5). From the comparison of the intensity of PheDH bands in different clones containing the same *phedh* gene (lane 2-5), it was revealed that PheDH protein band by pPheDH clone showed the highest level. In the pPY, pPYF and pPYFK clones, the production level of PheDH was similar (lane 3-5) and at the lower level. Possibly, the pPheDH clone contained only one inserted gene.

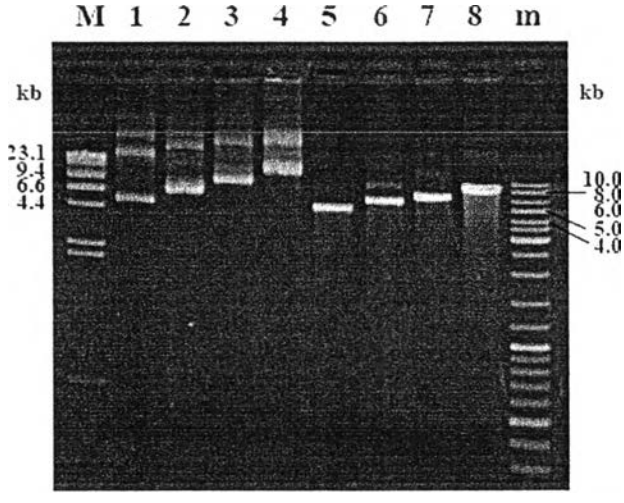


Figure 3.36 Restriction patterns of recombinant plasmids pPheDH, pPY, pPYF and pPYFK

Lane M = λ/*Hin*dIII standard DNA marker
Lane 1 = undigested pPheDH
Lane 2 = undigested pPY
Lane 3 = undigested pPYF
Lane 4 = undigested pPYFK
Lane 5 = *Xho*I-digested pPheDH
Lane 6 = *Xho*I-digested pPY
Lane 7 = *Xho*I-digested pPYF
Lane 8 = *Xho*I-digested pPYFK
Lane m = 100 bp DNA ladder

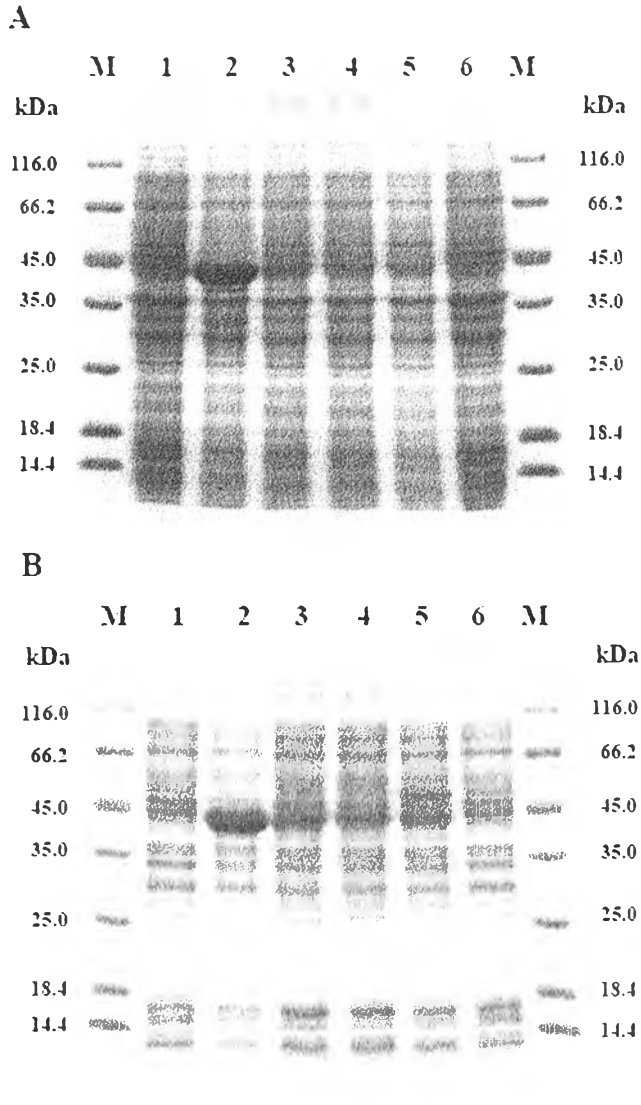


Figure 3.37 SDS-PAGE of recombinant *E. coli* BL21(DE3) carrying different gene combination after induction with 1 mM IPTG for 3 h

A: whole cell

B: crude extract

Lane M = protein molecular weight marker

Lane 1,6 = *E. coli* BL21(DE3)/pRSFDuet-1

Lane 2 = *E. coli* BL21(DE3)/pPheDH

Lane 3 = *E. coli* BL21(DE3)/pPY

Lane 4 = *E. coli* BL21(DE3)/pPYF

Lane 5 = *E. coli* BL21(DE3)/pPYFK

The building blocks for approaching central dogma were not shared as in case of more genes inserted. Thus, the pPheDH clone could produce higher PheDH in the same inducible period. Alternatively, it might be caused by the disturbance of the toxicity tendency of overexpressed YddG and GlpF proteins to host cell that led to the reduction or retardation of cell growth and production of desired compounds. Furthermore, the appearance of the slightly higher intensity of the protein band of GlpK of pPYFK clone was elucidated at approximately 56 kDa (lane 5). The *glpK* gene did not have its own T7*lac* promoter like the others. It was controlled under the same T7*lac* promoter of *glpF* gene at the downstream of this gene. This might be the reason why the protein band of GlpK was relatively low as also mentioned by Weckbecker and Hummel, 2004. Unsuspectedly, the expression of YddG and GlpF proteins could not be detectable by this procedure as previously mentioned. This result illustrated that overexpression of *phedh* or *glpK* genes was achieved individually or simultaneously in recombinant *E. coli* (pPheDH vs pPY, pPYF, pPYFK and pGlpFK vs pPYFK).

Although the *phedh* expression in whole cell extracts of pPheDH, pPY, pPYF and pPYFK clones were examined by SDS-PAGE analysis, in parallel, its PheDH activity in each of those crude extracts was also determined by activity assay (section 2.9.5.2) as summarized in Table 3.3. The pPheDH clone exhibited the highest specific PheDH activity of 71.8 unit/mg protein whereas specific acitivities of pPY, pPYF and pPYFK were 5.5, 3.0 and 6.1 unit/mg protein, respectively. The levels of activities were consistent with the intensities of PheDH band as shown in Figure 3.37A. Also, the intensity of individual target proteins (PheDH and GlpK) of each different clone seemed to correspond in both crude extract and whole cell extract (Figure 3.37A and 3.37B). It was confirmed that PheDH could be produced in soluble form and function when induced by 1 mM IPTG. Although glycerol kinase (GlpK) activity was not monitored, it was believed that it could function because it was produced in soluble form and its nucleotide sequence was 100% correct. However, an amount of IPTG (1 mM) might not be an optimum for *phedh* gene or all co-expressed genes. In general, the optimum condition to attain preferable yield and solubility of protein was systematically varied depending on the induction temperature, concentration of IPTG, location of the enzyme in different compartment (cytosol, periplasm or culture broth)

Table 3.3 Phenylalanine dehydrogenase activity from crude extract of each engineered *E. coli* BL21(DE3)*

Source	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
E. coli BL21(DE3)/pRSFDuet-1	0	63.3	0.0
E. coli BL21(DE3)/pPheDH	5,691.6	79.3	71.8
E. coli BL21(DE3)/pPY	280.5	50.8	5.5
E. coli BL21(DE3)/pPYF	140.6	47.6	3.0
E. coli BL21(DE3)/pPYFK	326.7	53.8	6.1
E. coli BL21(DE3)/pPTY	699.6	61.2	11.4
E. coli BL21(DE3)/pPTFY	244.8	57.6	4.2
E. coli BL21(DE3)/pPTFKY	672.3	42.5	15.8
E. coli BL21(DE3)/pPTFBY	330.0	50.7	6.5
E. coli BL21(DE3)/pPTFBLY	349.9	55.2	6.3
E. coli BL21(DE3)/pPTFBLYA	281.9	61.1	4.6
E. coli BL21(DE3)/pPT	2,353.7	86.5	27.2
E. coli BL21(DE3)/pPTF	1,827.0	76.1	24.0
E. coli BL21(DE3)/pPTFK	2,273.8	104.3	21.8
E. coli BL21(DE3)/pPTFB	2,106.5	99.0	21.3
E. coli BL21(DE3)/pPTFBL	2,441.0	101.4	24.1

*Crude extracts were obtained by culturing each *E. coli* cell in 200 mL of LB medium supplemented with IPTG at final concentration of 1 mM for 3 h.

and most importantly, the nature of each enzyme (Tolia and Joshua-Tor, 2006; Yamabhai et al., 2011).

3.9 Shake flask cultures

3.9.1 Optimization of production medium composition for L-Phe production

In 2009, Khamduang and colleagues carried out the fermentation for L-Phe production from 10 g/L of glycerol to attain the highest yield of L-Phe using recombinant *E. coli* BL21(DE3) containing *phedh* from *Acinetobacter lwoffii*. This indicates that glycerol tends to be effective and alternative carbon source for microbial fermentation of desirable product. Similar approach to Khamduang's work, *E. coli* BL21(DE3) bearing pPheDH was preliminary cultured in the Khamduang's medium containing 10 g/L of glycerol. It was found that this amount of glycerol was not enough for cell culture to reach the stationary phase or the highest biomass because the depletion of glycerol was completely attained within 72 h of induction with a maximum yield of biomass ~2 g/L. In this stage, bacterial growth was still in mid-log phase. In contrast, Khamduang et al. reported that even 10 g/L of glycerol was nearly completely consumed within 72 h of cultivation, cells reached the stationary phase with a maximum yield of biomass ~5 g/L. L-Phe was reported to be highly accumulated in stationary phase of cell growth (Khamduang et al., 2009b; Doroshenko et al., 2010; Wang et al., 2011). Thus, to accomplish the maximum cell biomass and L-Phe concentration, the concentration of carbon and nitrogen sources of Khamduang's medium was needed to partially optimize. Markedly, it was reported that the L-Phe production was growth-associated (Khamduang et al., 2009b, Wang et al., 2011; Srinophakun et al., 2012). Hence, the concentration of glycerol in medium was optimized first, and followed by the concentration of nitrogen source, $(NH_4)_2SO_4$.

The pPheDH clone was chosen as the representative of other clones for optimization of carbon and nitrogen concentration (glycerol and $(NH_4)_2SO_4$) in production medium because pPheDH clone was used as basal titer of L-Phe production for comparison with other clones.

3.9.1.1 Optimization of glycerol concentration in production medium

Glycerol at different concentrations of 10 to 50 g/L were used as a sole carbon source in shake flask cultivation of recombinant *E. coli* BL21(DE3) containing pPheDH (section 2.18.1.1) at fixing concentration of $(NH_4)_2SO_4$ (50 g/L). After OD_{600} reached 0.6, each flask was induced with 1 mM IPTG.

During cultivation, the cell density, glycerol assimilation and L-Phe production were monitored every 24 h after induction as shown in Figure 3.38. The HPLC and TLC chromatograms of L-Phe are illustrated in Appendix K and L, respectively. The cell growth profile of pPheDH clone in each different glycerol concentration medium had a similar trend as that of L-Phe production profile (Figure 3.38A and 3.38C). The growth profile relating to the biomass concentration, which was calculated from the equation: dry biomass concentration (g/L) = 0.99 × OD_{600} (section 2.19.1), and L-Phe production profile at 10 g/L glycerol were clearly different from higher glycerol concentrations (20, 30, 40 and 50 g/L). At 240 h after induction, the lowest L-Phe production (85.8 mg/L) was observed at 10 g/L of glycerol corresponding to the obtained lowest biomass concentration of 1.27 g/L. However, the highest biomass concentration of 2.04 g/L was attained at 96 h after induction but after this time, cell density was slightly gradually decreased. After 72 h, the exhaustion of glycerol was corresponded to the plateau of L-Phe and biomass concentration. Thus, it was confirmed that 10 g/L of glycerol was not enough to yield high biomass and L-Phe production.

The concentrations of glycerol in the range of 20-50 g/L yielded a relatively similar growth profile with final biomass concentrations of 4.96-5.33 g/L. It was implied that an increase in glycerol content exceeding 10 g/L could increase cell biomass concentration by approximately 4-fold at final cultivation. Considering the residual glycerol in medium (Figure 3.38B), it was obviously found that in all four different conditions, glycerol was utilized at the same rate during the initial period of cultivation (0-120 h). Glycerol of ~10 g/L and ~20 g/L were depleted at 72 h and at 144 h of induction, respectively. Particularly, glycerol of ~24 g/L was consumed at 240 h so that the final biomass concentration reached the highest level with a yield of ~5 g/L.

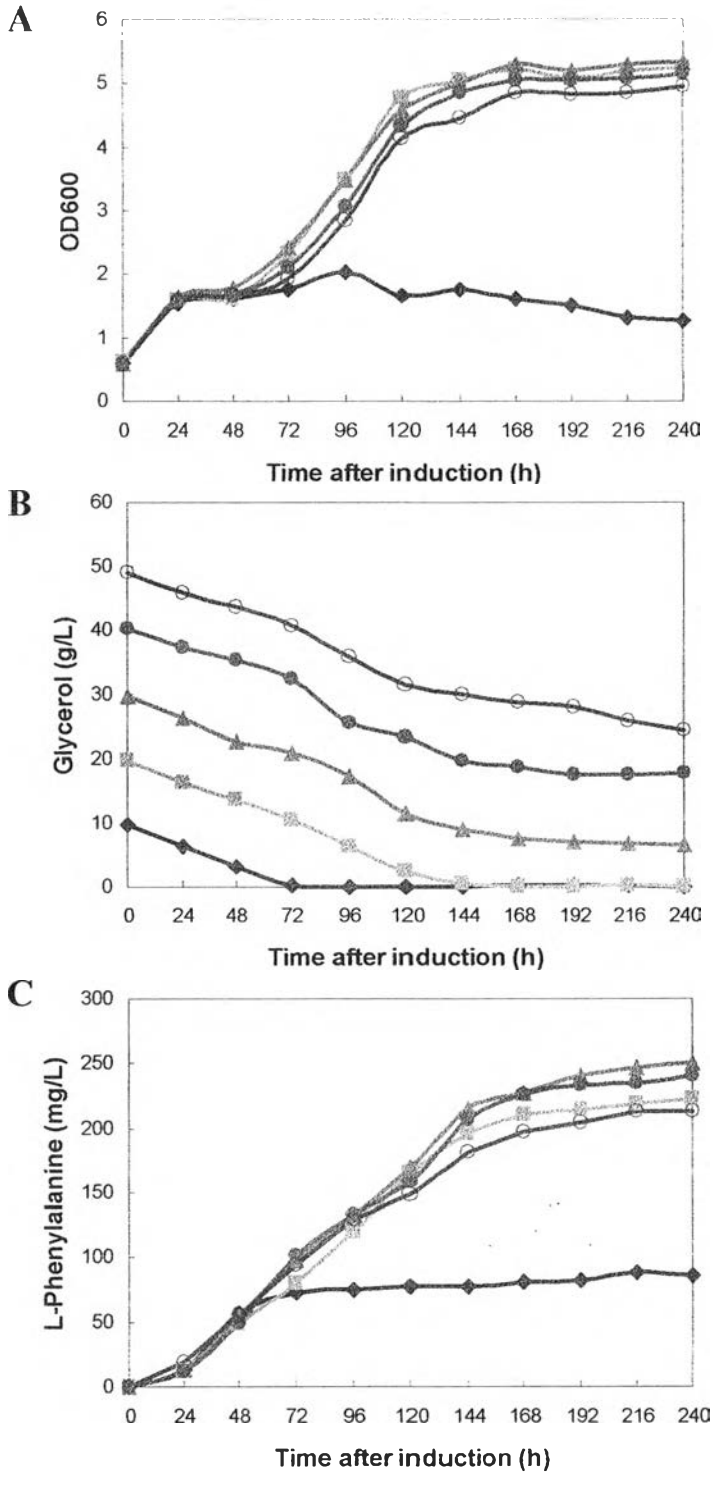


Figure 3.38 Time courses of cell growth (A), residual glycerol (B) and concentration of L-Phe (C) by *E. coli* BL21(DE3) harbouring pPheDH in production medium containing different glycerol concentrations of 10 g/L (◆), 20 g/L (▩), 30 g/L (▲), 40 g/L (●) and 50 g/L (○) supplemented with 1 mM IPTG at fixing concentration of $(NH_4)_2SO_4$ (50 g/L) in shake flask culture. Data shown were the means of two independent cultivations.

The glycerol concentrations of 30 and 40 g/L led to high L-Phe production of ~250 mg/L at final fermentation. These glycerol concentrations resulted in a ~3-fold increase in the L-Phe production compared to 10 g/L glycerol. Although both concentrations gave a similar yield of L-Phe, a lower remained glycerol content in medium was observed in the former. Thus, glycerol of 30 g/L was used for further experiments. The cell dry weight at 50 g/L of glycerol was lower than those at 20, 30 and 40 g/L in conformity with a lower L-Phe production. The biomass concentration slightly decreased when the concentration of glycerol was increased from 30 to 40 and 50 g/L. It may be presumed that an excess of glycerol substrate could inhibit cell growth (Khamduang et al., 2009a; Wendisch et al., 2011).

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

Batch fermentation of *E. coli* BL21(DE3)/pPheDH in the medium containing 30 g/L of glycerol with varying concentrations of $(NH_4)_2SO_4$ from 10 to 50 g/L was then performed.

As displayed in Figure 3.39A, the cell growth profiles at 10 and 20 g/L of $(NH_4)_2SO_4$ showed the closed similar trend with the same biomass concentration of ~2.8 g/L. However, L-Phe production could not be detected at these two concentrations (Figure 3.39C). When increased $(NH_4)_2SO_4$ to 30 g/L or more, the L-Phe concentration could be detected suggesting that less than 20 g/L $(NH_4)_2SO_4$ was insufficient for L-Phe production. When the concentration of $(NH_4)_2SO_4$ was increased from 30 g/L to 40 and 50 g/L, the obtained cell dry weight and L-Phe titer increased in order. The highest biomass concentration of ~5 g/L and the maximum yield of L-Phe (~250 mg/L) were obtained at 50 g/L of $(NH_4)_2SO_4$. This result was about the same when 30 g/L of glycerol was used in section 3.9.1.1. It was observed that glycerol was consumed more at increasing concentration of $(NH_4)_2SO_4$ (Figure 3.39B). Thus, the best concentration of $(NH_4)_2SO_4$ for maximizing L-Phe production was 50 g/L which was consistent with the previous report (Khamduang et al., 2009a).

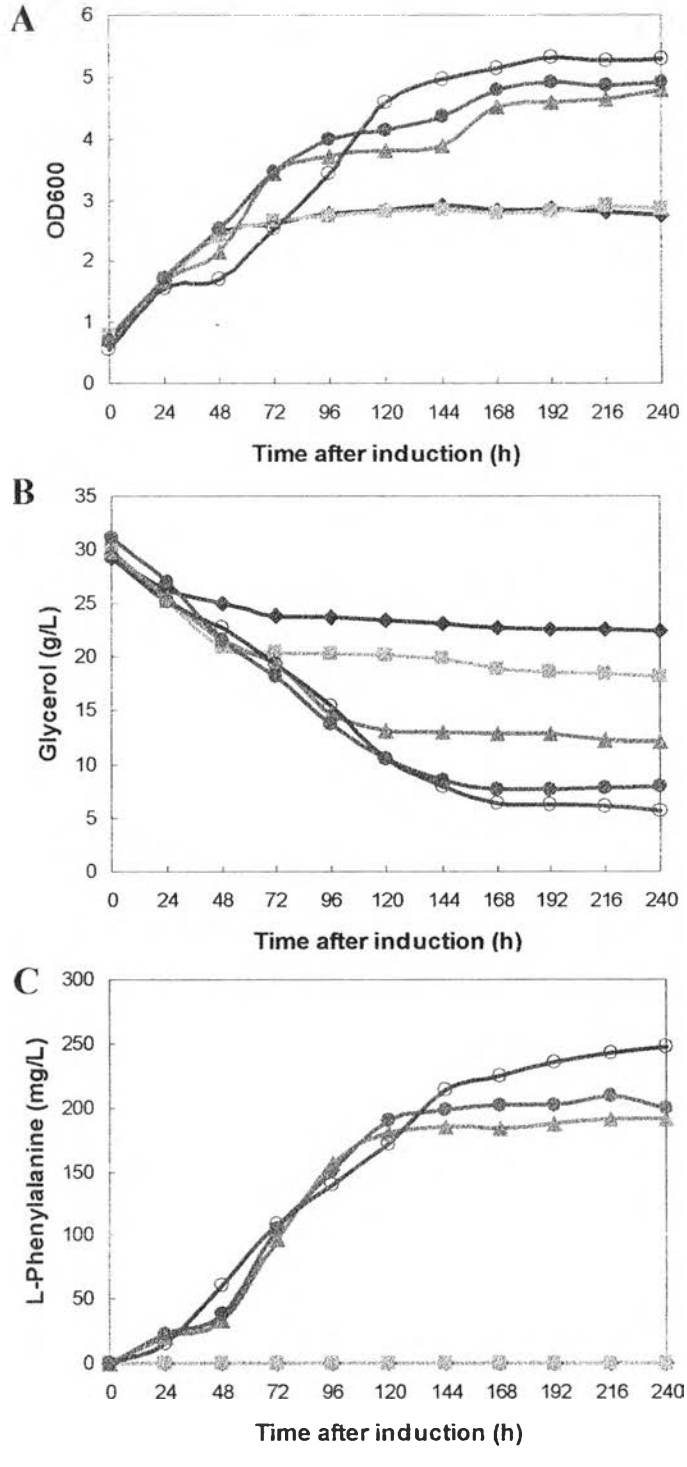


Figure 3.39 Time courses of cell growth (A), residual glycerol (B) and concentration of L-Phe (C) by *E. coli* BL21(DE3) harbouring pPheDH in production medium containing different $(NH_4)_2SO_4$ concentrations of 10 g/L (◆), 20 g/L (▩), 30 g/L (▲), 40 g/L (●) and 50 g/L (○) supplemented with 1 mM IPTG at fixing concentration of glycerol (30 g/L) in shake flask culture. Data shown were the means of two independent cultivations.

3.9.2 Optimization of IPTG concentration for L-Phe production in cultivation of pPheDH, pPY, pPYF and pPYFK clones

In this expression system, the target plasmids were established in *E. coli* BL21(DE3), an expression of desirable genes was induced by the addition of IPTG to a growing culture. As specified above, IPTG at final concentration of 1 mM was purposed for full induction with vector having the T7*lac* promoter. IPTG concentration and induction duration can both influence recombinant protein solubility. Lower concentrations of IPTG may induce protein expression at a slower rate resulting in better folding. Also, it may reduce yield. Higher IPTG concentrations may cause an excessive overexpression level allowing an accumulation of inactive aggregated proteins called inclusion bodies (Nilsson and Anderson, 1991) or inhibition of cell viability. In addition, longer induction time, which increases expression, may also induce proteolysis of the target, leading to heterogeneity in the sample and reducing yield. Different concentration of IPTG and a time course of induction should be tested to determine the optimal combination (Tolia and Joshua-Tor, 2006).

The effects of the added *yddG, glpF* and *glpK* genes on L-Phe production from glycerol were further characterized by comparison of the production of the different clones and the parent strain that did not contain the genes. Thus, the clones pPheDH, pPY, pPYF and pPYFK were cultured in a minimal medium containing 30 g/L of glycerol and 50 g/L of $(NH_4)_2SO_4$. When OD_{600} nm reached 0.6, 0-1 mM IPTG was added. In this work, an induction did not perform at inoculation as few previous reports because if the gene product is toxic to *E. coli* cell, the expression hinders cell replication and decreases protein production. Thus, transcription is typically suppressed until cells have reached a high cell density before activating gene expression (Gruber et al., 2008). It was found that the time elapsed prior to induction was varied in different clones. The growth of the cells was progressively reduced as the number of inserted genes increased.

It was reported that the optimum condition for *phedh* gene expression in *E. coli* BL21(DE3)/pBLPheDH was induction with 0.2 mM IPTG for 8 h (Thongchuang, 2006). The optimal condition for L-Phe production by resting cell was

obtained when the recombinant cells were cultured in LB medium for 6 h after OD_{600} reached 0.6 without IPTG induction (Ratanasuwanasri, 2008). The result indicated that the condition giving the best level of PheDH activity did not bring about the highest yield of L-Phe production. It might be due to different environmental conditions such as PheDH conformation or sufficient substrate concentration between intact cell (*In vivo*) and crude extract (*In vitro*). Considering this, only the L-Phe production level was used for a comparative evaluation of different clones. Similarly, because of the difficulty in directly detecting the membrane-associated protein products of *yddG* and *glpF* genes, only the level of L-Phe production of the various clones was used to infer about overall activities of these proteins in a given clone.

The growth profiles of *E. coli* clones harbouring pPheDH, pPY and pPYF and pPFK with and without induction are shown in Figure 3.40. In the glycerol medium, all the IPTG induced clones generally grew better and attained a higher biomass concentration than the same clones without induction. The concentration of the IPTG inducer (0.25, 0.5 and 1 mM) had no effect on a clone's ability to grow so long as the concentration was at least 0.25 mM. For induced pPheDH clones (Figure 3.40A), the induction reduced growth up to 96 h relative to noninduced cells after induction. After that, the induced cells ultimately grew faster and attained a higher biomass concentration (~5 g/L) than the noninduced cells (~4 g/L). For the noninduced pPY, pPYF and pPYFK clones, the final biomass concentrations were in the range of 1.2 to 2.0 g/L (Figure 3.40B, C and D), but with induction it increased by 2.2- to 3.5-fold depending on the clone. Although IPTG is known to negatively affect the growth of *E. coli* (Law et al., 2002), the IPTG induced pPY, pPYF and pPYFK greatly outperformed the corresponding noninduced clones.

The glycerol consumption profiles of the various clones (Figure 3.41) were quite consistent with their growth patterns (Figure 3.40). Thus, the clones that grew poorly consumed glycerol slower than the faster growing clones. Poor growth always led to more glycerol remained in the culture broth. The induced pPheDH, pPY, pPYF and pPYFK clones had similar rates of glycerol consumption, but the noninduced pPY, pPYF and pPYFK barely consumed glycerol (Figure 3.41B, C and D). Clearly, the glycerol utilization ability of the noninduced pPY, pPYF and pPYF clones was poor, but was restored by induction. In cultures of clones that effectively consumed

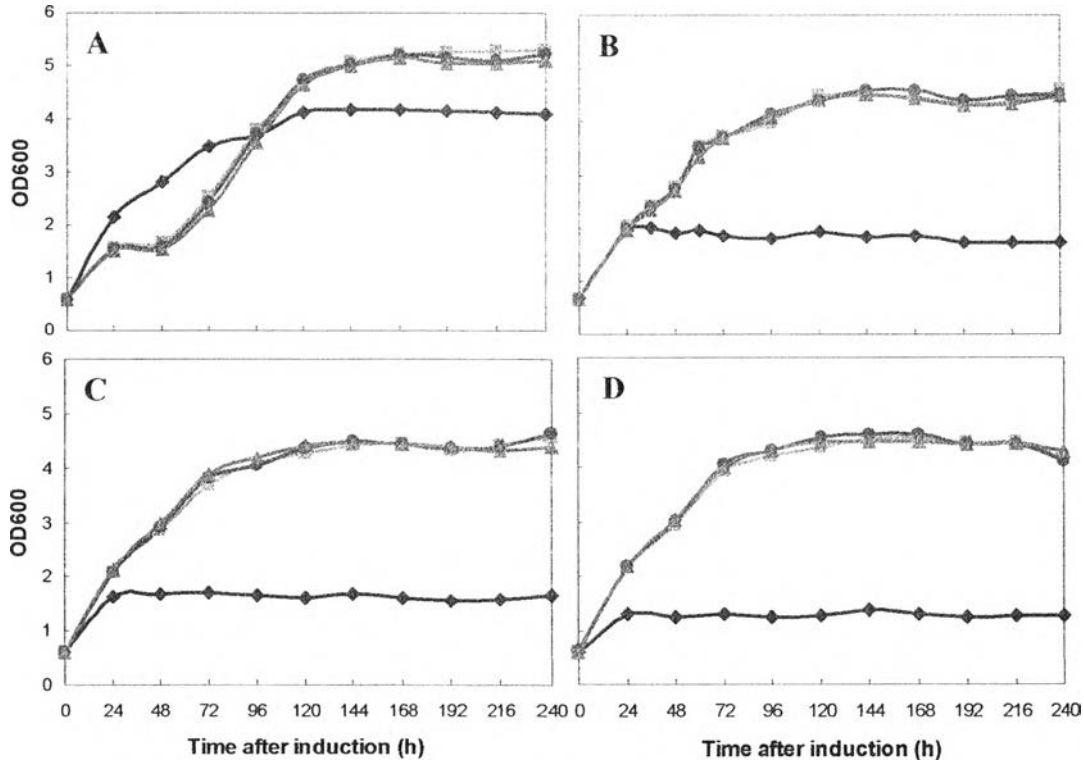


Figure 3.40 Growth profiles of *E. coli* BL21(DE3) harbouring pPheDH (A), pPY (B), pPYF (C) and pPYFK (D) in glycerol medium without IPTG induction (◆) and with IPTG induction to final concentrations of 0.25 mM (▒), 0.5 mM (●) and 1 mM (▲) in shake flask culture. Data shown are averaged values from three independent experiments. The standard deviation of the measurements was within ±10% of the average values shown.

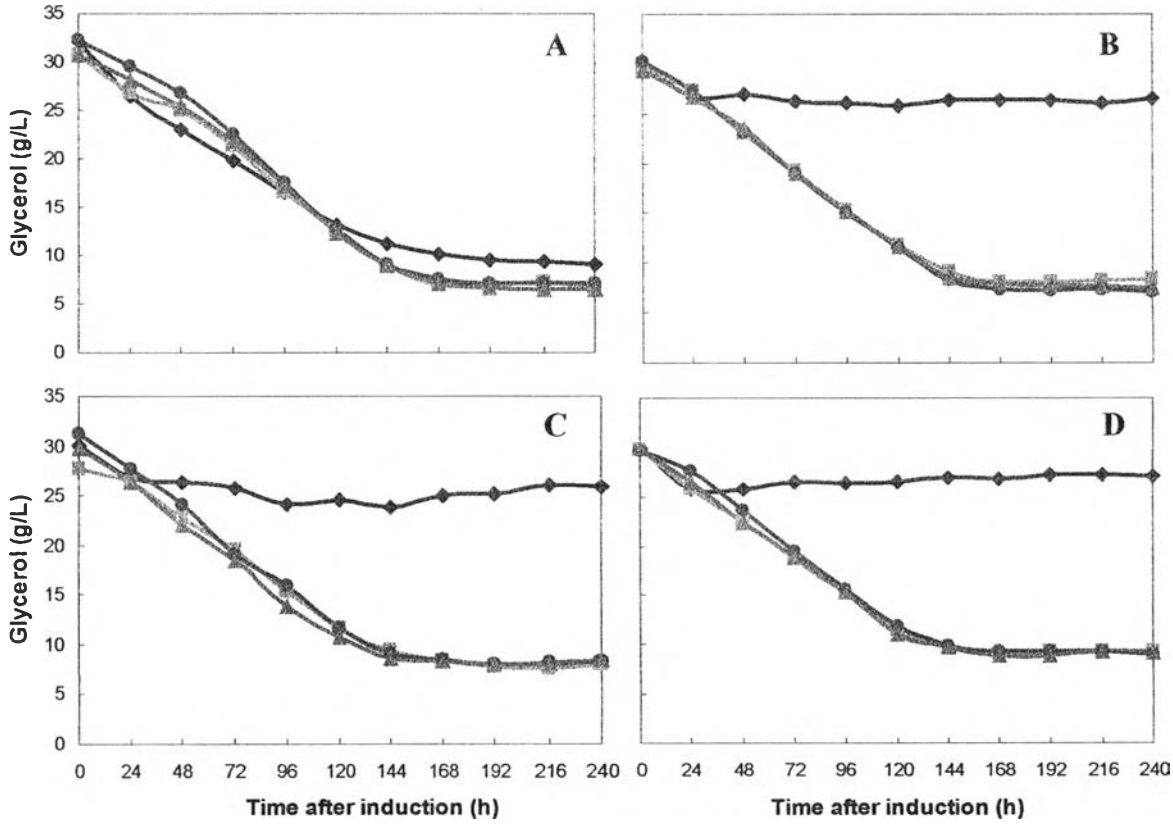


Figure 3.41 Glycerol consumption profiles by *E. coli* BL21(DE3) harbouring pPheDH (A), pPY (B), pPYF (C) and pPYFK (D) in glycerol medium without IPTG induction (◆) and with IPTG induction to final concentrations of 0.25 mM (▩), 0.5 mM (●) and 1 mM (▲) in shake flask culture. Data shown are averaged values from three independent experiments. The standard deviation of the measurements was within ±10% of the average values shown.

glycerol, a significant amount of glycerol was remained after the stationary phase had been reached. This suggested a sufficiency of glycerol in the medium and implicated an insufficiency of some other factors (e.g. the concentration of dissolved oxygen, limited supply of growth factor) in the medium as the cause of the stationary phase (Wang et al., 2011).

From an initial pH of medium of 7.4, it was found that at the end of shake flask cultivation, the pH value of induced clones was approximately 4-4.2 (Figure 3.42). For noninduced clones, the pH value (4.2-4.8) was slightly higher than that of induced clones. pH is another limiting factor for cell growth in addition to nutrition exhaustion and accumulation of toxic metabolites. The medium's pH is governed by medium compositions, buffers, cellular metabolites, and aeration conditions. The optimal pH for *E. coli* is near neutral. *E. coli* cells can grow reasonably well over a range of three pH units (from pH 5.5 to 8.5). Extreme pH beyond this range will significantly decrease the cell growth rate and may sometimes even cause cell death. The minimum and maximum growth pHs for *E. coli* are pH 4.4 and 9.0 respectively. At the stationary phase, the pH of the *E. coli* culture in commonly used media is near its pH limits. *E. coli* cells produce large quantities of acetic acid if the growth medium contains little or no oxygen causing the growth medium to reach pH 4 or lower. Acetic acid is an extracellular co-product of aerobic fermentation by *E. coli* cells, and this exists as acetate ion at the neutral pH used in *E. coli* fermentations. The acetate formation rate is directly related to the cell growth rate or the substrate consumption rate (Eiteman and Altman, 2006). Moreover, acetic acid is the major metabolic inhibitor under anaerobic growth condition.

Aeration is another critical parameter to optimize individual protein expression and cell growth in small-scale culture because the expression of more than 200 genes by *E. coli* is dependent on the availability of oxygen (Losen et al., 2004). Aeration is directly related to the medium/container volume ratio. For good aeration, medium volume should be added up only 20-25% of the total flask volume. In this experiment, because of the limitation of various factors such as flask size, sampling volume and holder size, minimal medium must be filled in a 500 mL flask with volume of 200 mL (40% of the total flask volume) that might lead to bad aeration.

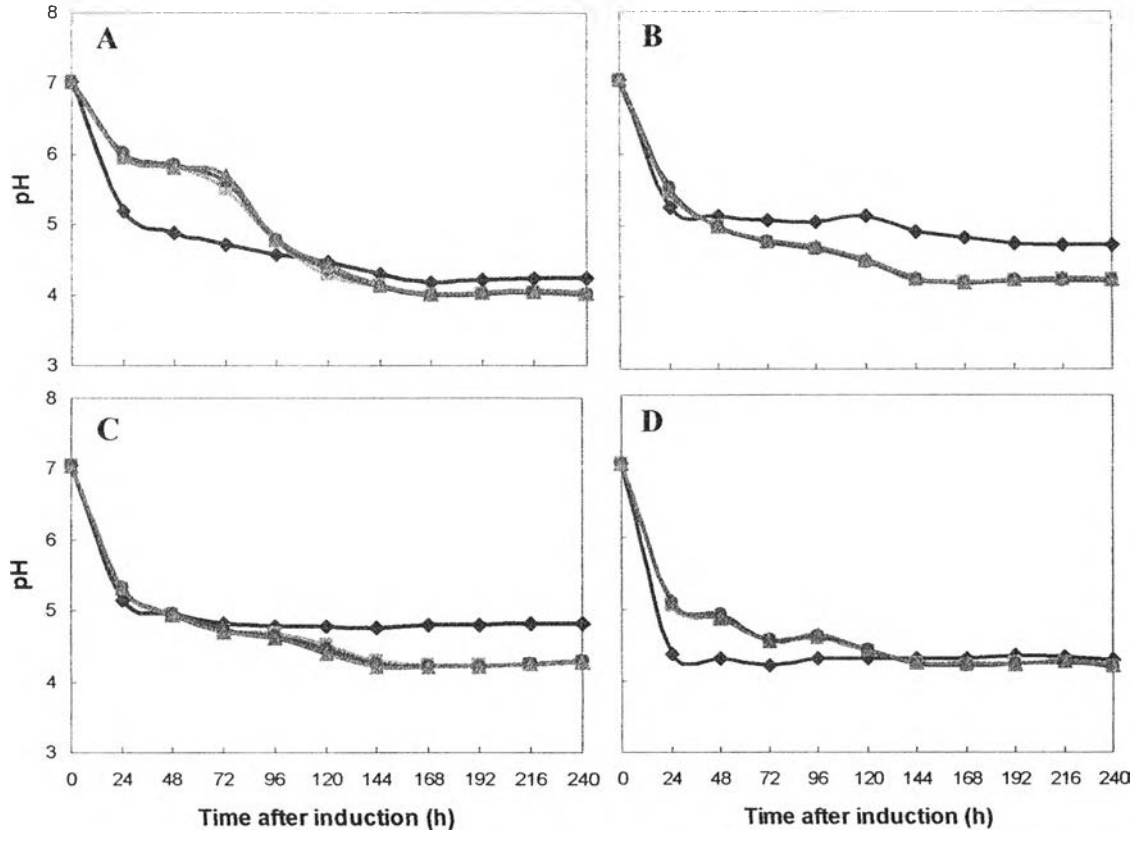


Figure 3.42 Profiles of the medium pH values when *E. coli* BL21(DE3) harbouring pPheDH (A), pPY (B), pPYF (C) and pPYFK (D) were grown in glycerol medium without IPTG induction (◆) and with IPTG induction to final concentrations of 0.25 mM (▩), 0.5 mM (●) and 1 mM (▲) in shake flask culture. Data shown are averaged values from three independent experiments. The standard deviation of the measurements was within ±10% of the average values shown.

An increase in aeration without changing culture volume was proportionally fulfilled by increasing the shaking speed (Maier, Losen, and Büchs, 2004)

The lower pH of medium (4.2) at the end of fermentation (240 h) might be caused by an inadequate aeration during long period of cultivation which led to a faster rate to reach an acidic pH of medium due to the formation of formate, succinate, acetate and lactate from glycerol metabolism under anaerobic condition (Zhang, Shanmugam, and Ingram, 2010). Furthermore, in *E. coli* fermentation using mineral medium with glucose or glycerol as sole carbon source, the pH value is highly dependent on ammonium consumption. One molecule of ammonium taken into cells generates one proton resulting in declining of pH value in the medium (Scheidle, Klinger, and Büchs, 2007).

The L-Phe production profiles for the four clones are shown in Figure 3.43. Suitably induced recombinant clones pPheDH, pPY, pPYF and pPYFK produced a final L-Phe concentration of about 250 mg/L by 240 h, but the clones varied in the inducer concentrations required to achieve this result and the rate at which the peak L-Phe concentration was attained. This was shown clearly for the four clones in Figure 3.44.

In general, an induction with IPTG leads to more protein expression level than that without induction. Consequently, an increase in *phedh* gene expression with induction should result in driving to produce more L-Phe titer than that absence of IPTG but the observed result did not correspond to an expected one. For pPheDH clone, although an increasing amount of IPTG caused a better *phedh* gene expression (Figure 3.45B, C and D) than that of noninduced pPheDH clone (Figure 3.45A), their L-Phe productions were relatively similar (Figure 3.43A). From our experiences about IPTG induction in various clones, the results on cell growth, carbon source utilization and production of target product were varied in both pET and pRSFDuet-1 expression systems. It could not certainly explain why pPheDH generates the same level of L-Phe in the presence or absence of IPTG. Possibly, it depends on overall characteristics of each clone affected by several factors including expression vector, host cell, cultivation condition, IPTG concentration, induction duration, induction temperature and protein solubility. In general, increasing of IPTG concentration is expected for higher protein expression level than that without induction. However, in

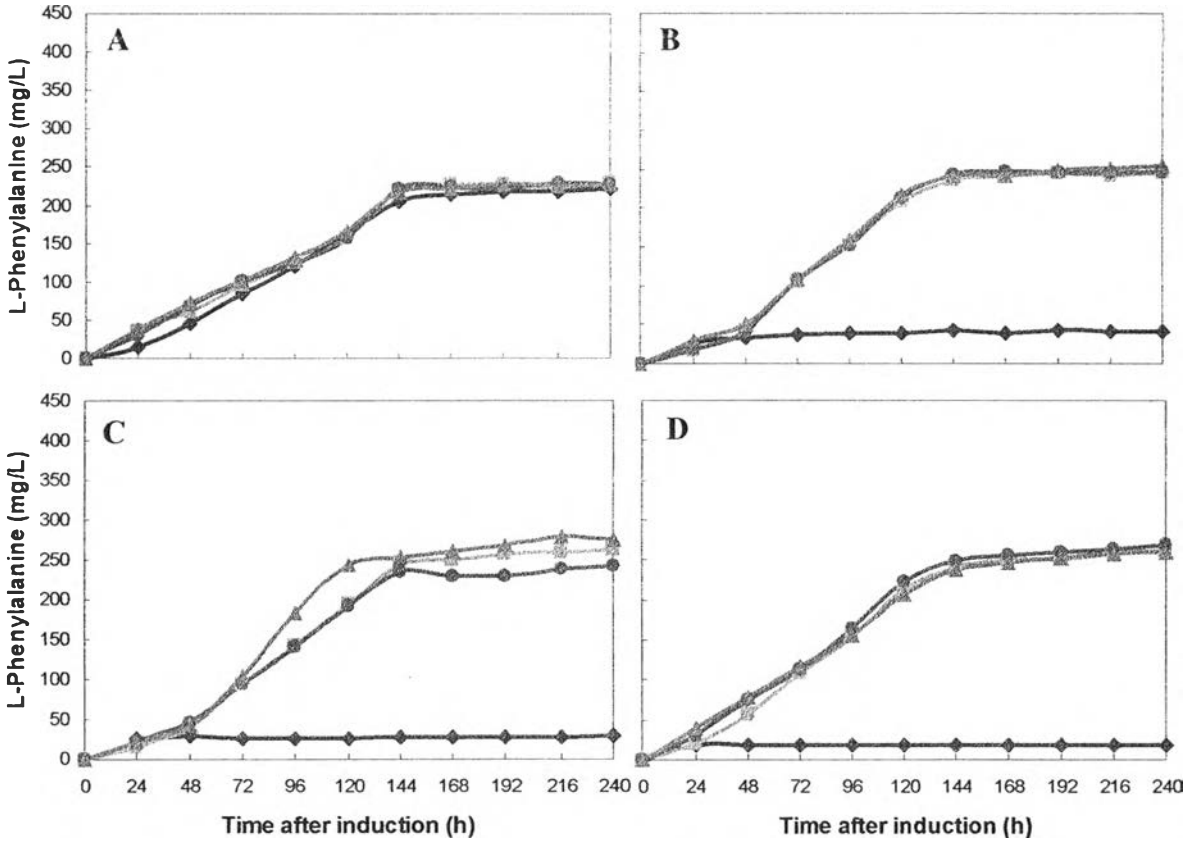


Figure 3.43 L-Phe production profiles by *E. coli* BL21(DE3) harboring pPheDH (A), pPY (B), pPYF (C) and pPYFK (D) in glycerol medium without IPTG induction (◆) and with IPTG induction to final concentrations of 0.25 mM (▩), 0.5 mM (●) and 1 mM (▲) in shake flask culture. Data shown are averaged values from three independent experiments. The standard deviation of the measurements was within ±10% of the average values shown.

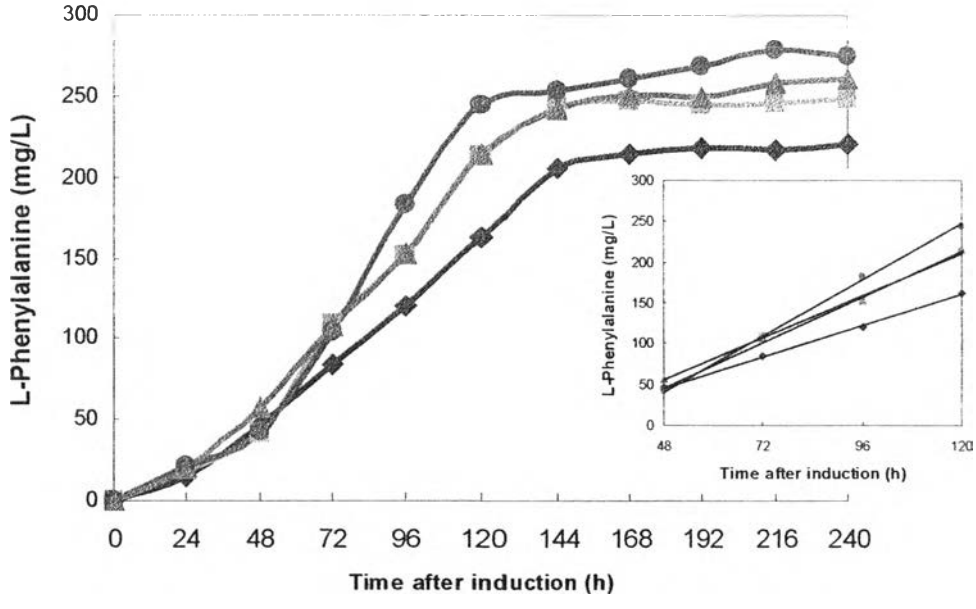


Figure 3.44 Comparison of L-Phe production profiles and maximum production rates (inset) of the recombinant clone pPheDH without IPTG addition (◆); clone pPY induced with 0.5 mM IPTG (▩); clone pPYF induced with 1 mM IPTG (●); and clone pPYFK induced with 0.25 mM IPTG (▲). Data shown are averaged values from three independent experiments. The standard deviation of the measurements was within ±10% of the average values shown.

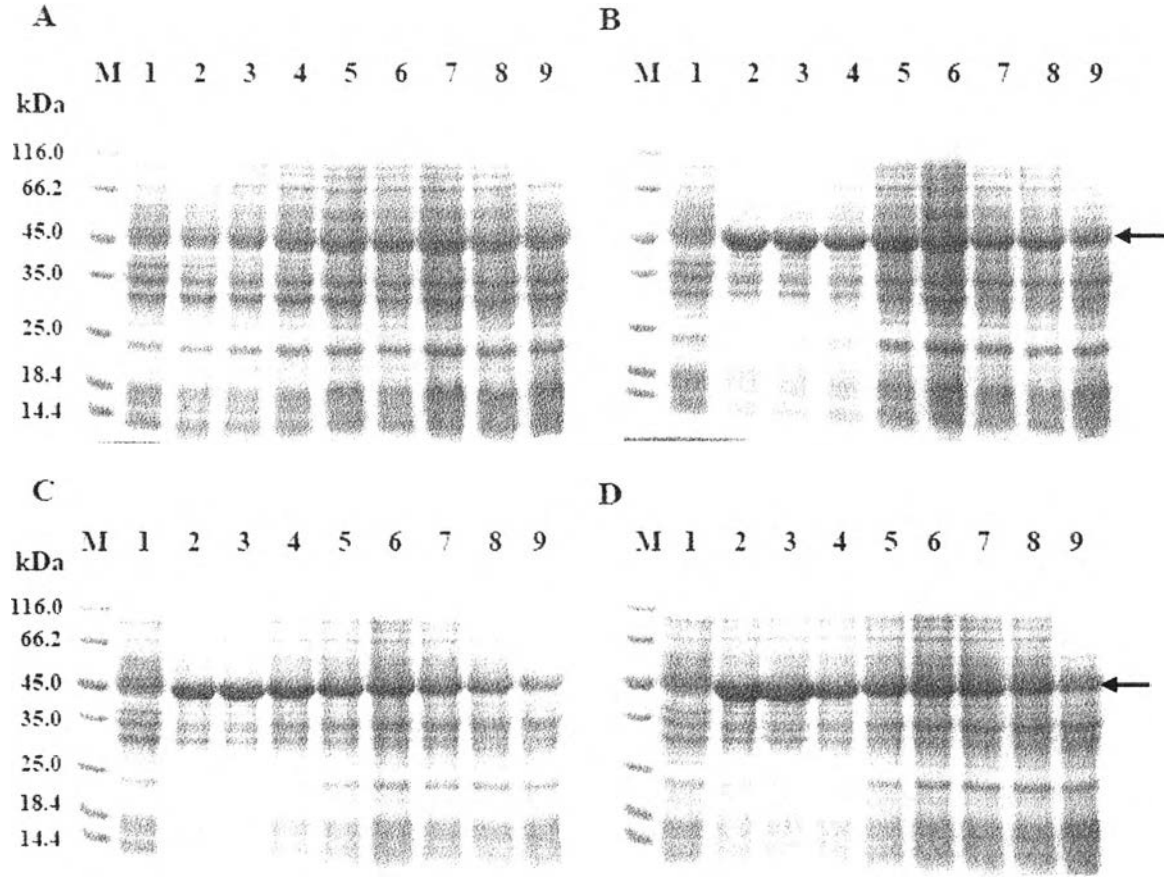


Figure 3.45 SDS-PAGE of whole cell extracts of *E. coli* harbouring pPheDH induced by 0 mM (A), 0.25 mM (B), 0.5 mM (C) and 1 mM (D) IPTG at various times of shake flask cultivation. The arrows indicated PheDH band.

Lane M = protein molecular weight marker

Lane 1 = *E. coli* BL21(DE3)/pRSFDuet-1

Lane 2-9 = *E. coli* BL21(DE3)/pPheDH at 24, 48, 72, 96, 120, 144, 168, 192 and 192 h after induction, respectively

our experiments, the induction condition possessing intense target protein band in SDS-PAGE gel may not confer the best level of enzyme activity. Also, the peak of production of target product did not correspond to the best level of enzyme activity. Thus, the induction condition should be varied to reach the peak of target production (Liu et al., 1999; Moon et al., 2009; Tsao et al., 2011).

When comparing the intensity of PheDH band of pPheDH clone in cases of with and without induction in SDS-PAGE, it was found that induced cells showed more greatly intense band than that of uninduced cells (Figure 3.45). If this more intense band can refer to more PheDH activity, induced cells should contribute higher L-Phe production and did not generate the same level of L-Phe as that of uninduced cells. It indicated that the pPheDH clone might have a limited ability to produce L-Phe, possibly due to poor glycerol uptake, low ability to excrete L-Phe, or tightly regulated steps in L-Phe biosynthesis. Thus, this work aimed to improve L-Phe production from glycerol by metabolic engineering process.

For the pPheDH clone, L-Phe was produced without induction at a peak production rate of 1.61 mg/L h. The pPY clone required induction with 0.5 mM IPTG to attain a maximum L-Phe production rate of 2.33 mg/L h. The pPYF clone achieved a maximum L-Phe production rate of 2.86 mg/L h on induction with 1 mM IPTG. The pPYFK clone induced with 0.25 mM IPTG reached a maximum L-Phe production rate of 2.15 mg/L h (Figure 3.44). The maximum L-Phe production rates of the induced pPY, pPYF and pPYFK clones were 1.4-, 1.8- and 1.3-fold higher than the maximum production rate of the pPheDH clone.

This experimental part demonstrated the feasibility of enhancing the production of L-Phe from glycerol in an engineered *E. coli* expressing PheDH together with an aromatic amino acid exporter, a glycerol transport facilitator and glycerol kinase. Co-expression of the aromatic amino acid exporter in *E. coli* containing *phedh* promoted the production of L-Phe and production could be improved a little more by also co-expressing a glycerol facilitator. In contrast, the expression tandem of glycerol facilitator and glycerol kinase could not increase the production rate of L-Phe from *E. coli* containing *phedh* and *yddG* genes but it showed insignificantly different from pPY clone's productivity. Also, its production rate was decreased when comparing to that of pPYF clone.

In this work, the function of all expressed proteins could not be determined directly because some proteins were difficult to measure their activity (YddG and GlpF proteins). As firstly mentioned, to avoid the trouble usage of radioisotope and antibody conjugated probe, the western blot analysis was not approached. Moreover, the detection of expressed YddG and GlpF proteins by purification of the His-tagged protein was impossible due to the fact that the construction of recombinant plasmids was not planned to express protein fused with His-Tag. Many previous works on identification and characterization of specific functions of YddG and GlpF proteins (Sweet et al., 1990; Lu et al., 2003; Doroshenko et al., 2007; Airich et al., 2010) were reported. Therefore, if the nucleotide sequences of inserted *yddG* and *glpF* genes in the recombinant plasmid were actually correct and did not show reading frame shift, as well as the addition of these genes in *E. coli* contributed positive result to L-Phe production, it is likely that both genes were able to express and function as specified membrane proteins. In addition, we observed that colony of recombinant cells harbouring gene encoding membrane protein appeared to show different morphology from that of their parental strain. The small flat colonies were formed on agar plate with undulate margin. The altered morphology of colony was shown to occur from basal level expression of membrane-associated protein without induction of *E. coli*-bacteriophage T7 RNA polymerase expression system (Miroux and Walker, 1996). This supported that genes encoding membrane proteins in this work were rather expressed. Moreover, when compared PheDH band intensity in whole cells of clones, an expression of *phedh* gene by pPY, pPYF and pPYFK clones had drastically lower expression levels than that of pPheDH clone both with and without IPTG inductions. However, pPY, pPYF and pPYFK clones gave the higher L-Phe titer than that of pPheDH clone. This result confirmed that genes encoding membrane proteins were expressed. Protein patterns of whole cell of these three clones at specified times after induction were closely similar, only those of pPY clone are showed in Figure 3.46.

In *E. coli*, glycerol molecules enter the cell by passive diffusion via channel proteins called porins that are located in the outer membrane and then glycerol molecules cross the inner membrane by facilitated diffusion achieved through an integral membrane protein, glycerol facilitator GlpF. The cytoplasmic

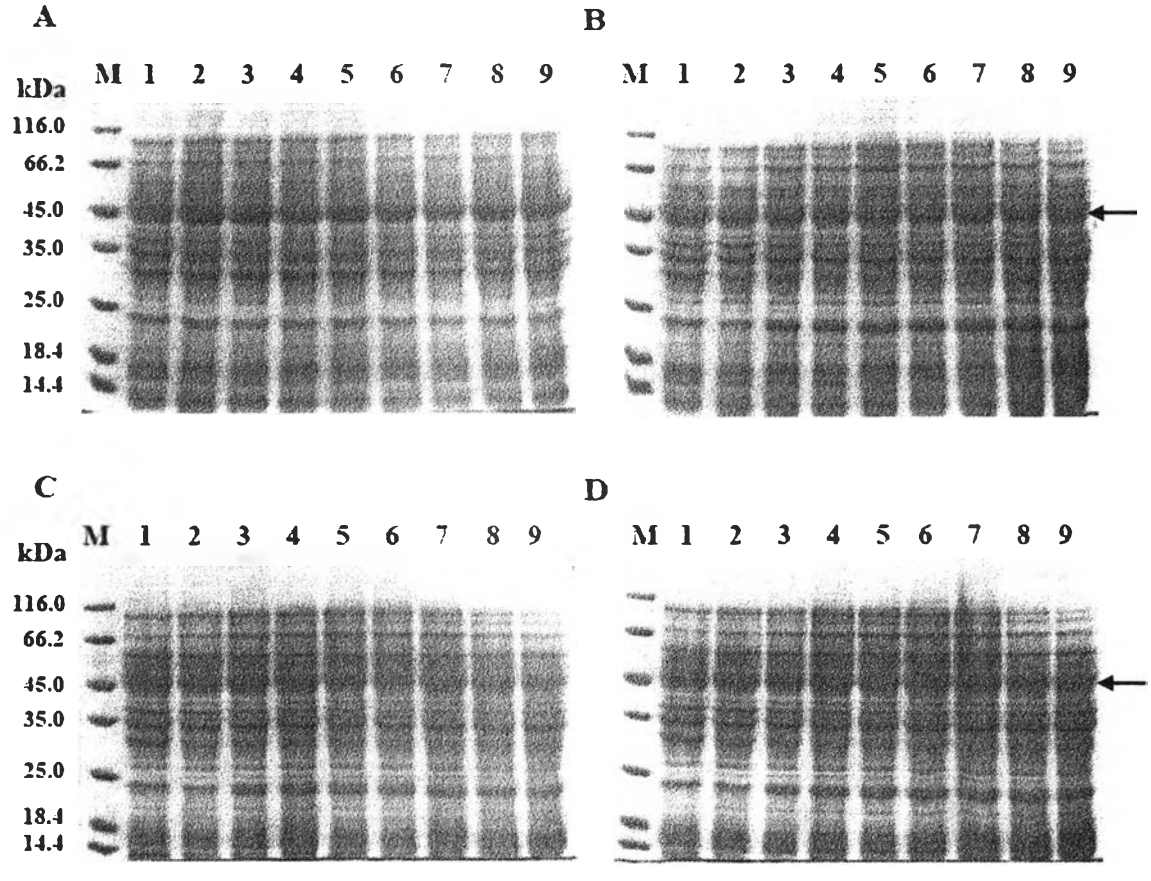


Figure 3.46 SDS-PAGE of whole cell extracts of *E. coli* harbouring pPY induced by 0 mM (A), 0.25 mM (B), 0.5 mM (C) and 1 mM (D) IPTG at various times of shake flask cultivation (For (A), sample volume for loading was two times higher than that of the others). The arrow indicated PheDH band.

Lane M = protein molecular weight marker

Lane 1 = *E. coli* BL21(DE3)/pRSFDuet-1

Lane 2-9 = *E. coli* BL21(DE3)/pPY at 24, 48, 72, 96, 120, 144, 168, 192 and 192 h after induction, respectively

glycerols are phosphorylated to glycerol 3-phosphate (G3P) by glycerol kinase to prevent glycerol from passively diffusing out of the cell. The resulting G3P is trapped in the cell until it is further metabolized since it is not a substrate for the glycerol facilitator (Voegele et al., 1993). G3P is then oxidized through the action of G3P dehydrogenase encoded by *glpD* gene to form dihydroxyacetone phosphate (DHAP), which is in turn isomerized to glyceraldehyde 3-phosphate (GA3P) (da Silva et al., 2009). Both DHAP and GA3P are further metabolized in both the glycolysis and the pentose phosphate pathways. As a consequence of this, the overexpressed glycerol kinase should increase the trapped glycerol inside the cells leading to enhancement of carbon flux into further metabolisms. From the result of L-Phe production by pPYFK clone, overexpression of glycerol kinase together with glycerol facilitator was unable to support L-Phe productivity in *E. coli* containing *phedh* and *yddG* genes. Possibly, this approach might not be effective enough to increase glycerol utilization. In 2008, *Corynebacterium glutamicum* producing amino acid that cannot utilize glycerol was successfully engineered to grow on glycerol by heterologous expression of *E. coli* glycerol utilization genes. Simultaneous expression of *glpK* encoding glycerol kinase and *glpD* encoding glycerol 3-phosphate dehydrogenase was sufficient for growth on glycerol and an additional expression of the *glpF* gene increased growth rate and biomass formation. This *C. glutamicum* strain expressing *E. coli glpF*, *glpK*, and *glpD* genes was able to produce glutamate and lysine from glycerol as the sole carbon and energy sources. Moreover, the increased glycerol kinase level caused the intracellular accumulation of G3P that is a growth inhibitor. Co-expression of *glpF* and *glpK* genes in *C. glutamicum* led to a maximum accumulated intracellular G3P compared to clone expressing *glpF* gene and clone expressing *glpF*, *glpK* and *glpD* genes. To prevent intracellular accumulation of G3P, *glpD* gene is required to co-express with *glpK* gene to balance both expression levels (Rittmann, Lindner, and Wendisch, 2008). Additionally, the simultaneous expression of *gldA* encoding glycerol dehydrogenase and *dha*KLM encoding dihydroxyacetone kinase was found to accelerate the formation of DHAP from glycerol to promote the rates of glycerol utilization and product synthesis in *E. coli* either through the fermentative or the respiratory glycerol dissimilation pathway (Yazdani and Gonzalez, 2008).

As the production rates of L-Phe were improved (1.3-, 1.4- and 1.8-fold) whereas the L-Phe titer was not changed (~250 mg/L), some other bottlenecks in the common L-Phe synthesis pathway should exist. This section was likely focused only on improving transport of the substrate and the excretion of the product, but not on the steps of the tightly regulated biosynthesis pathway of aromatic amino acids. Consequently, metabolic engineering process of an aromatic amino acid biosynthesis pathway from starting carbon source, glycerol was further focused on.

To determine the utilization of glucose as carbon source for L-Phe production, glycerol, glucose and glucose-glycerol mixture were used as single nutrient or co-nutrient. Each of these recombinant cells pPheDH, pPY, pPYF and pPYFK was cultivated in production medium in shake flask. Normally, glycerol concentration of 30 g/L was always fixed throughout experiments. Instead of glycerol, glucose was used either in the same gram amount of glycerol (30 g/L) or in the same mole amount of glycerol (326 mmole/L). Additionally, to investigate co-utilization of two carbon sources (glucose and glycerol), the mixture of the same amount of each (15 g/L) was used. Each clone was induced by IPTG to the same final concentration to attain the maximum yield of L-Phe as previously identified. As shown in Figure 3.47-3.50, it was found that all four clones had closely similar profiles of growth, substrate utilization, L-Phe production and the pH values of medium, except for the growth curve of pPYFK clone in medium containing 30 g/L of glucose (Figure 3.47D) when they were cultured in different media (i.e. glycerol (30 g/L), glucose (30 g/L), glucose (326 mmole/L) and glucose-glycerol mixture (15 g/L of each). The medium containing glucose gave the values of biomass concentration (1.9- to 2.6-fold) (Figure 3.47) and L-Phe production (4.5- to 9.6-fold) (Figure 3.49) lower than those of medium containing sole glycerol.

It was found that in glucose-glycerol mixture medium, glucose was gradually utilized while glycerol seemed hardly utilized (Figure 3.48). This result could be explained well with previous observation in *E. coli* JM101 that was cultured in a mixture of glucose and glycerol as carbon sources. Clearly, after glucose had been completely exhausted, glycerol was initially consumed. Moreover, for *E. coli*, the presence of glucose inhibits the utilization of secondary carbon sources known as carbon catabolite repression (Martínez et al., 2008: online). Furthermore, this event

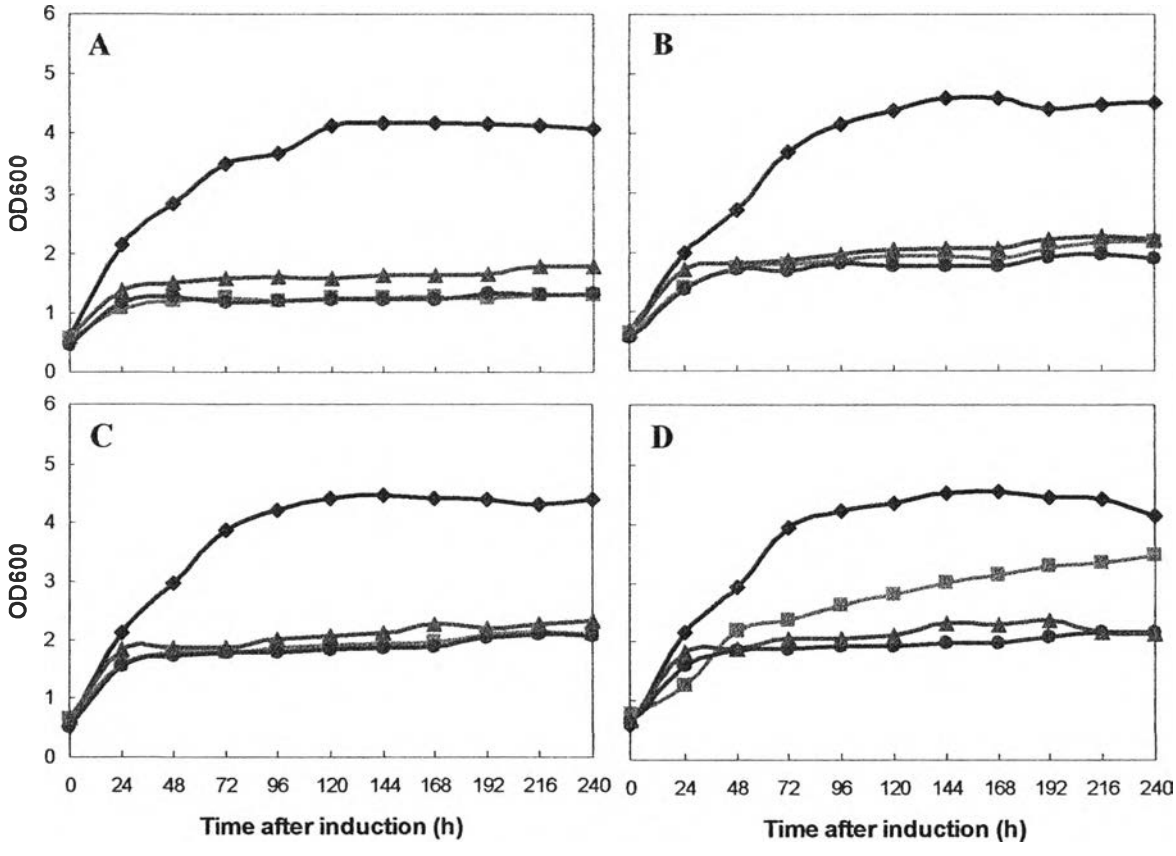


Figure 3.47 Growth profiles of *E. coli* BL21(DE3) harbouring pPheDH (A), pPY (B), pPYF (C) and pPYFK (D) in production medium containing 30 g/L of glycerol (◆), 30 g/L of glucose (■), 326 mmole/L (or 58.73 g/L) of glucose (●) and the mixture of 15 g/L of glucose and 15 g/L of glycerol (▲) in shake flask culture. Data shown were the means of two independent cultivations.

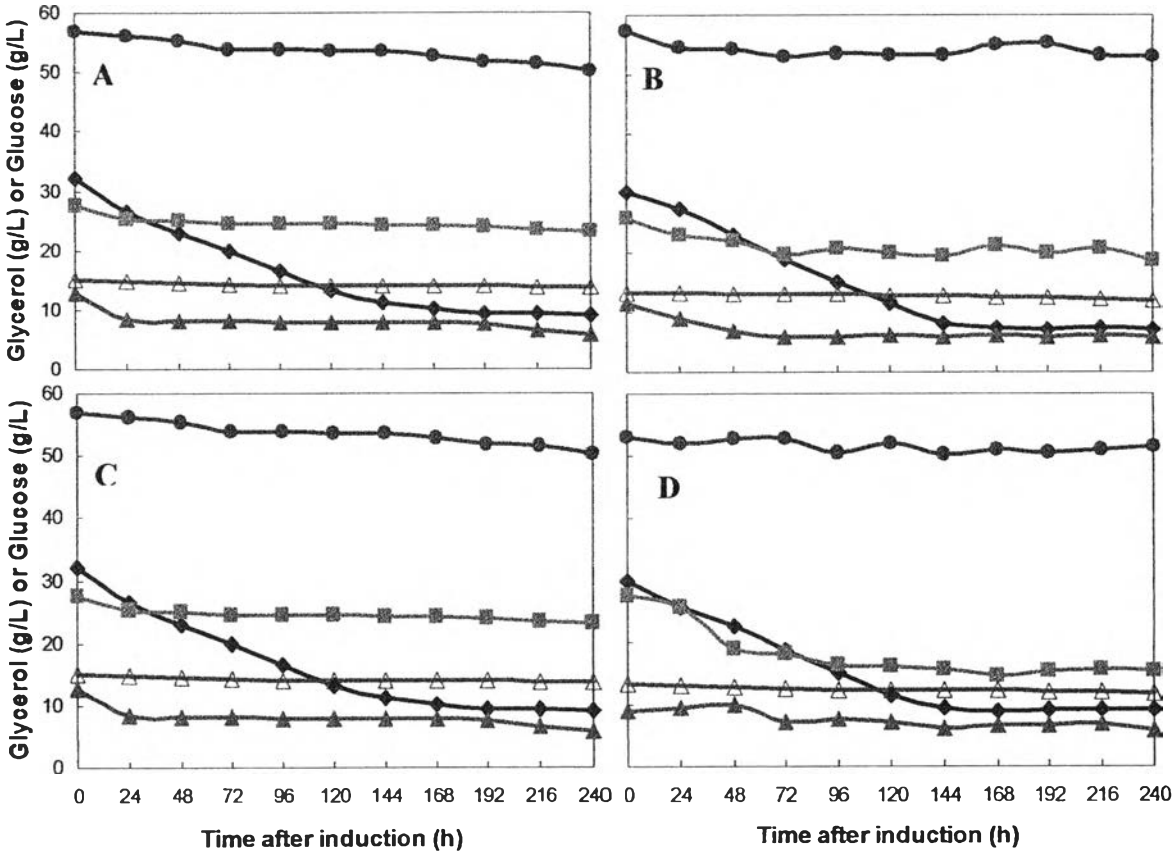


Figure 3.48 Carbon source consumption profiles of *E. coli* BL21(DE3) harbouring pPheDH (A), pPY (B), pPYF (C) and pPYFK (D) in production medium containing 30 g/L of glycerol (◆), 30 g/L of glucose (▩), 326 mmole/L (or 58.73 g/L) of glucose (●) and the mixture of 15 g/L of glucose (▲) and 15 g/L of glycerol (△) in shake flask culture. Data shown were the means of two independent cultivations.

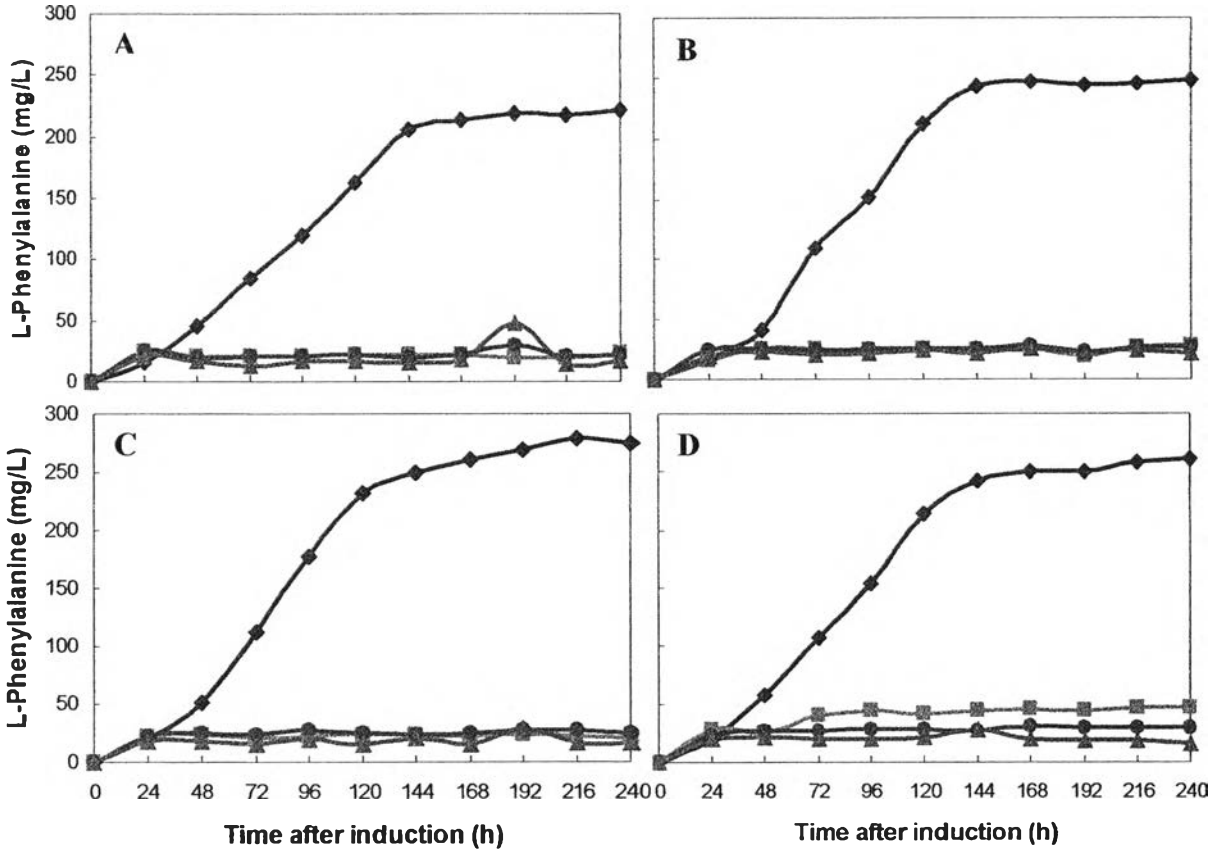


Figure 3.49 L-Phe production profiles of *E. coli* BL21(DE3) harbouring pPheDH (A), pPY (B), pPYF (C) and pPYFK (D) in production medium containing 30 g/L of glycerol (◆), 30 g/L of glucose (■), 326 mmole/L (or 58.73 g/L) of glucose (●) and the mixture of 15 g/L of glucose and 15 g/L of glycerol (▲) in shake flask culture. Data shown were the means of two independent cultivations.

may occur from glucose inhibition of glycerol utilization. In glycerol utilization, *E. coli* glycerol kinase catalyzes the rate-limiting step. Its catalytic activity is allosterically regulated at the protein level by two effectors, the fructose 1,6-bisphosphate and the phosphocarrier protein of phosphotransferase system (Pettigrew et al., 1996). Also, it was reported that if possible, media containing glucose should usually be avoided for culturing *E. coli* in shake flasks because this induces overflow metabolism despite of under unlimited-oxygen conditions (Losen et al., 2004) leading to acetate undesirable by-product formation that is known to cause several negative effects on growth and protein production (Kleman and Strohl, 1994; Eiteman and Altman, 2006). High acetate titers not only caused a reduction of glucose uptake but also a reduction of L-Phe formation (Gerigk et al., 2002).

When observing the pH value of glycerol and glucose medium, it was found that all medium containing glucose had a faster rate of reducing pH value compared to that in medium containing glycerol (Figure 3.50). This was consistent with previous report in which the formation of acetic acid was taken place during the earlier phase of cultivation in glucose medium. In contrast to in glycerol medium, acetic acid was initially formed later and was particularly rapid at the end of the cultivation (Nakano et al., 1997).

From these results, it was suggested that glycerol was absolutely a better carbon source than glucose. This was mainly due to higher biomass and L-Phe concentrations of recombinant clones in glycerol medium than those in medium containing glucose.

From section 3.9.2, the result of L-Phe production in different engineered *E. coli* (pPheDH, pPY, pPYF and pPYFK clones) led to an important information. It was clear that the overexpression of *yddG* gene encoding aromatic amino acid exporter could significantly improve the L-Phe production in *E. coli* containing *phedh* gene. Consequently, each of these previous constructed plasmids pPT, pPTF, pPTFK, pPTFB should be added with the *yddG* genes and its T7*lac* promoter to generate pPTY, pPTFY, pPTFKY and pPTFBY, respectively to ensure that the L-Phe production of all clones was significantly increased.

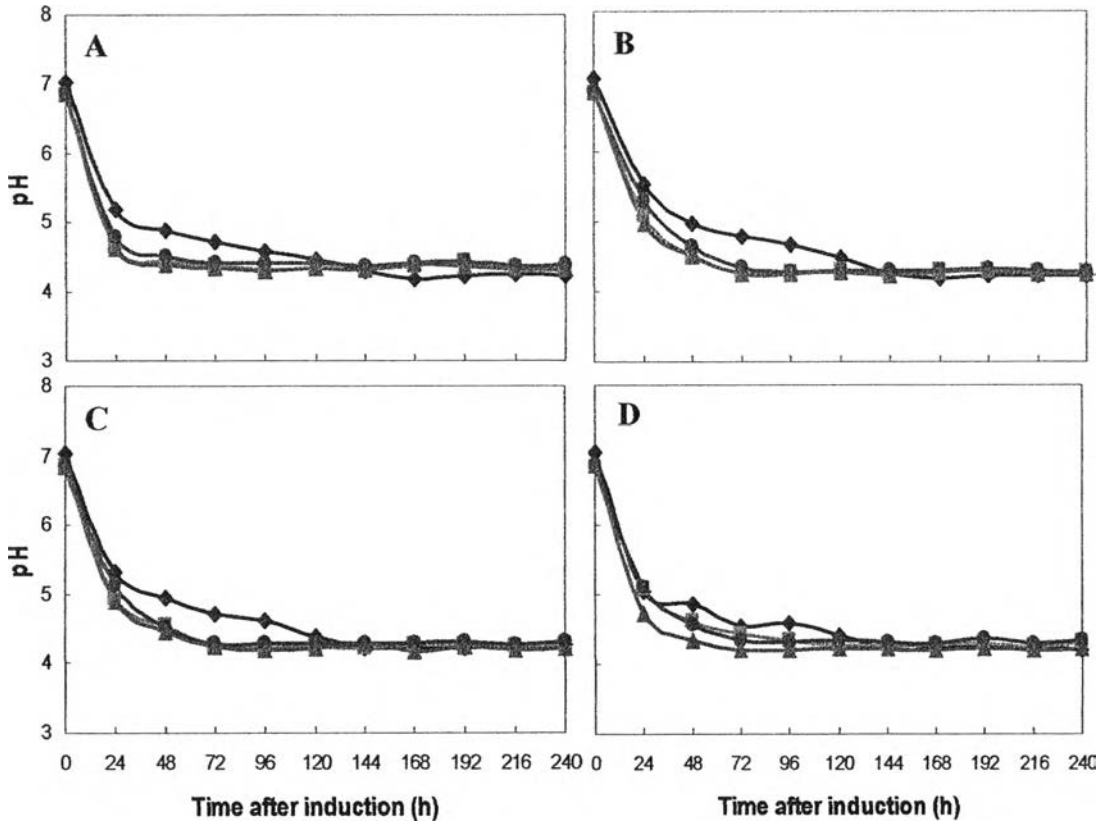


Figure 3.50 The medium pH profiles when *E. coli* BL21(DE3) harbouring pPheDH (A), pPY (B), pPYF (C) and pPYFK (D) were grown in production medium containing 30 g/L of glycerol (◆), 30 g/L of glucose (■), 326 mmole/L (or 58.73 g/L) of glucose (●) and the mixture of 15 g/L of glucose and 15 g/L of glycerol (▲) in shake flask culture. Data shown were the means of two independent cultivations.

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

3.10.1 Cloning of *yddG* gene into recombinant plasmid pPT

To prepare pPTY containing *phedh, tktA* and *yddG* genes, the pPT (7,201 bp) was digested with *Bam*HI and *Eco*RV to retain the 5,682 bp fragment containing *tktA* gene and to delete the DNA fragment of 1,339 bp containing *phedh* gene. The specified fragment of 5,682 bp performed as vector was purified from the gel. The insert fragment 2,315 bp consisting of *phedh* and *yddG* genes was obtained by cutting the pPY (5,931 bp) with the same restriction enzymes. The insert fragment and the vector fragment were ligated resulting in pPTY (7,997 bp) (Figure 2.12B). After the resulting ligation had been introduced into *E. coli* BL21(DE3), the desired clone was selected and its plasmid was identified by cleaving with *Bam*HI and *Eco*RV. The fragments of 5,682 bp and 2,315 bp were obtained (Figure 3.51).

3.10.2 Cloning of *yddG* gene into recombinant plasmid pPTF

The recombinant plasmid pPTFY containing *phedh, tktA, glpF* and *yddG* genes was created by the combination of the DNA fragments obtained from pPTFY (6,817 bp) and pPTY (7,997 bp) digestions. The pRSFDuet-1 vector fragment of 4,467 bp containing *glpF* gene was prepared by digestion of pPTFY with restriction enzymes *Bam*HI and *Xho*I. Also, the insert fragment (4,416 bp) containing three genes, *phedh, tktA* and *yddG* genes, was prepared by digestion of pPTY with *Bam*HI and *Xho*I. After both of these eligible fragments had been purified from the gel, these were ligated to form pPTFY (8,883 bp) (Figure 2.13A) and transformed into *E. coli* host cell. The engineered *E. coli* BL21(DE3) bearing pPTFY was selected. The plasmid pPTFY was digested with *Bam*HI and *Xho*I. It was likely that only single intense DNA band of approximately 4.5 kb was appeared as shown in Figure 3.52. However, this band virtually contained the two bands of 4,416 and 4,467 bp. These fragment sizes were too close to be separated apart on 0.8% agarose gel.

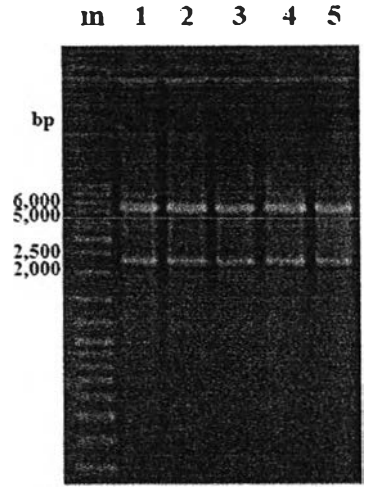


Figure 3.51 Restriction pattern of recombinant plasmid pPTY

Lane m = 100 bp DNA ladder

Lane 1-5 = *Bam*HI/*Eco*RV-digested pPTY

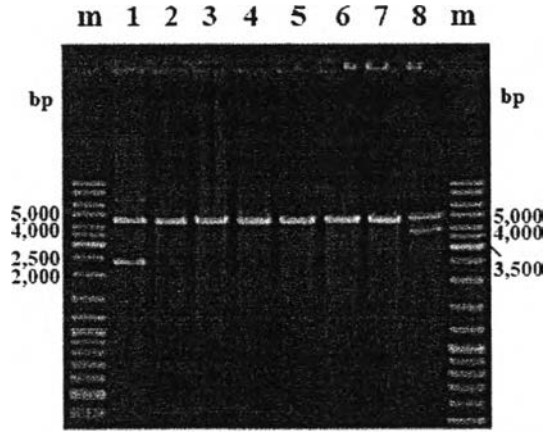


Figure 3.52 Restriction pattern of recombinant plasmid pPTFY

Lane m = 100 bp DNA ladder

Lane 1 = *Bam*HI/*Xho*I-digested pPYF

Lane 2-7 = *Bam*HI/*Xho*I-digested pPTFY

Lane 8 = *Bam*HI/*Xho*I-digested pPTF

3.10.3 Cloning of *yddG* gene into recombinant plasmid pPTFK

To produce pPTFKY containing *phedh, tktA, glpF, glpK* and *yddG* genes, the pPTFK (9,438 bp) was cut with *Asc*I and *Afl*II to give the pRSFDuet-1 vector fragment inserted with four genes of 9,400 bp (i.e. *phedh, tktA, glpF* and *glpK* genes). The 1,014 bp fragment containing *yddG* gene was prepared by digesting pYddG with *Asc*I and *Afl*II and played as insert fragment. The ligation between the vector and the insert fragments was performed leading to pPTFKY (10,414 bp) as shown in Figure 2.13B. The pPTFKY was transformed into host cell and the plasmid was verified by double digestion with *Asc*I and *Afl*II. The obtained fragments in lengths of 9,400 bp and 1,014 bp are showed in Figure 3.53.

3.10.4 Cloning of *yddG* gene into recombinant plasmid pPTFB

Eventually, the plasmid pPTFBY containing *phedh, tktA, glpF, aroB* and *yddG* genes was constructed. The pPTFB was digested by *Bam*HI and *Xho*I to give the potential vector containing *glpF* and *aroB* genes of 5,186 bp. Similarly, the pPTY was digested with the same restriction enzymes to produce the DNA fragment of 4,416 bp containing *phedh, tktA* and *yddG* genes. After recovering each of these two wanted fragments from the gel, they were ligated together to carry out pPTFBY (9,602 bp) (Figure 2.14). This ligation was transformed into *E. coli* BL21(DE3) host cell. The *E. coli* BL21(DE3) harbouring pPTFBY was selected and the plasmid pPTFBY was analyzed by digestion of *Bam*HI and *Xho*I. The restriction fragments of approximately 5.2 and 4.5 kb were showed in Figure 3.54.

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

When observing the gene expression levels of five transformants of individual recombinant clone as previously mentioned in section 3.8, it was found that they had a similar level of specified gene expression. Therefore, one transformant was randomly chosen for exploring ability for L-Phe production in shake flask. Primarily, these six clones (i.e. pPTY, pPTFY, pPTFKY, pPTFBY, pPTFBLY and pPTFBLYA) were investigated whether each gene appeared on recombinant plasmid was simultaneous

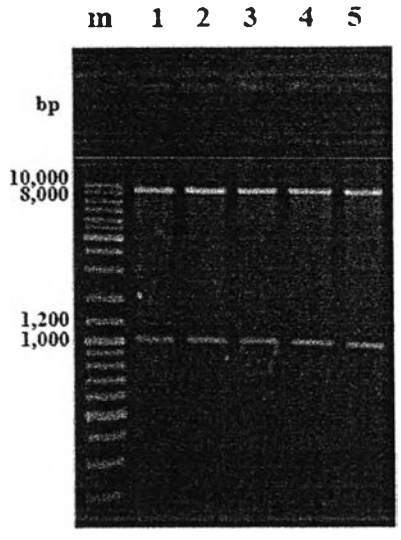


Figure 3.53 Restriction pattern of recombinant plasmid pPTFKY

Lane m = 100 bp DNA ladder

Lane 1-5 = *Asc*I/*Afl*II-digested fragment of pPTFKY

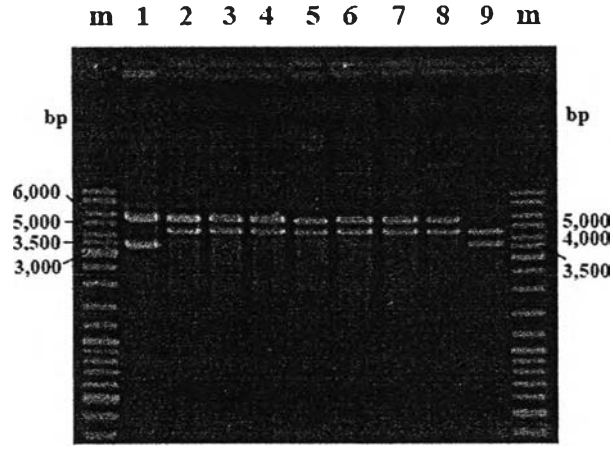


Figure 3.54 Restriction pattern of recombinant plasmid pPTFBY

Lane m = 100 bp DNA ladder

Lane 1 = *Bam*HI/*Xho*I-digested fragment of pPTFB

Lane 2-7 = *Bam*HI/*Xho*I-digested fragment of pPTFBY

Lane 8 = *Bam*HI/*Xho*I-digested fragment of pPTY

expressed in the same *E. coli* host cell as judged by distinct band on Coomassie Blue stained SDS-PAGE gel. The protein patterns obtained from both crude extract and whole cell extract of these clones were compared to that of the control *E. coli* BL21(DE3) harbouring pRSFDuet-1 vector, pPheDH and pPY as shown in Figure 3.55. It was shown that each clone could produce proteins in soluble form because band density of proteins in crude extracts and whole cell extracts after induction with 1 mM IPTG for 3 h were in good agreement (Figure 3.55A and B).

When considering each desired protein band (PheDH, TktA, GlpK, AroB, AroL and PheA) that could be detected upon SDS-PAGE stained with Coomassie Blue, it was found that *phedh* gene in all recombinant clones were expressed with apparent molecular weight of 42 kDa (lane 2-9) that was matched to its theoretical one. The levels of visualized PheDH protein band in each of these different clones were varied as similar to PheDH activity. These six clones showed their PheDH activity ranging from 4.2-15.8 unit/mg protein (Table 3.3). Distinctly, all clones had lower PheDH activities compared to clone expressing *phedh* gene only (pPheDH clone).

All clones containing *tktA* gene encoding transketolase A (i.e. pPTY, pPTFY, pPTFKY, pPTFBY, pPTFBLY and pPTFBLYA) showed similar visible levels of *tktA* expression with sharp TktA protein band of 72 kDa (lane 4-9) in both crude extract and whole cell. For pPTFKY clone, *glpK* gene encoding glycerol kinase showed the higher intensity of protein band of 56 kDa (lane 6). For recombinant cells containing *aroB* gene encoding 3-dehydroquinate synthase (pPTFBY, pPTFBLY and pPTFBLYA clones), it was found that no extra intensity of AroB protein band at predicted size of 39 kDa could be detected (lane 7-9). Possibly, this concentration of IPTG (1 mM) and induction duration (3 h) was not suitable for *aroB* gene expression within these three clones. The useful method for optimization of the expression of multiple genes is the arrangement of several genes as an operon to facilitate coordinated expression of multiple genes (Pfleger et al., 2006). Moreover, an expression level for producing AroB protein was too low to be visualized by Coomassie Blue stained SDS-PAGE gel. The low expression titer of *aroL* gene encoding shikimate kinase II in (pPTFBLY and pPTFBLYA clones) was observed as faint band at 19 kDa (lane 8-9). For the latter, expression of *pheA* gene encoding

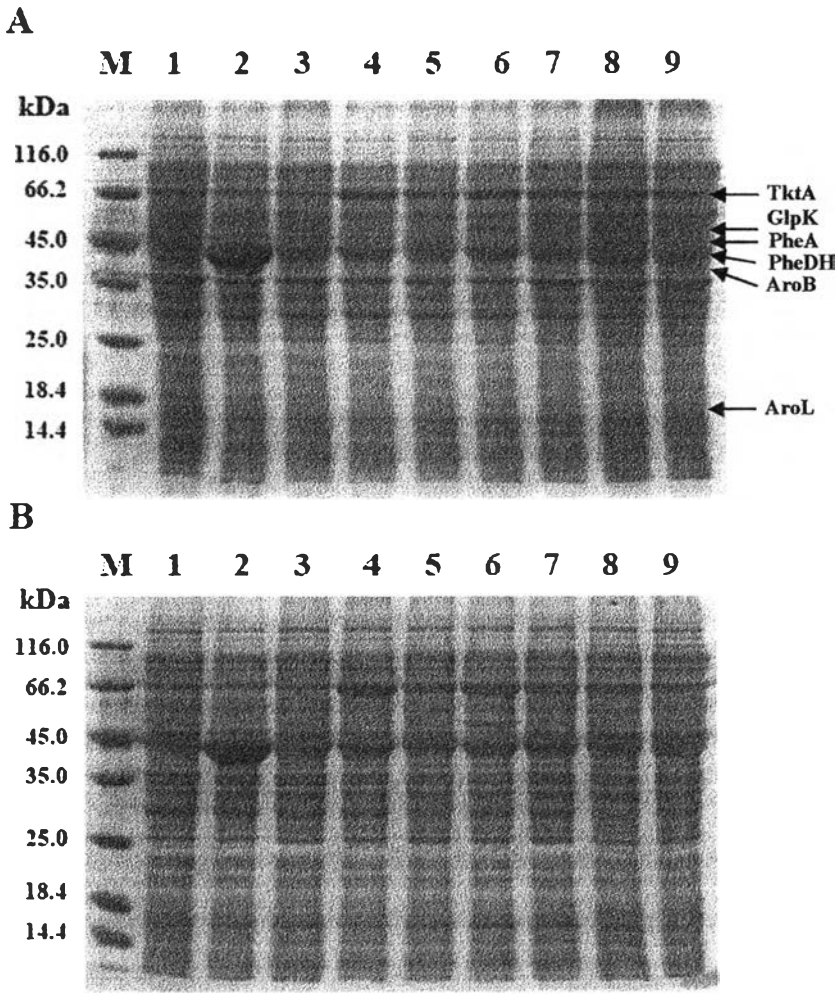


Figure 3.55 SDS-PAGE of recombinant *E. coli* BL21(DE3) carrying different gene combination after induction with 1 mM IPTG for 3 h. The arrows showed the specified protein bands.

A: whole cell

B: crude extract

Lane M = protein molecular weight marker

Lane 1 = *E. coli* BL21(DE3)/pRSFDuet-1

Lane 2 = *E. coli* BL21(DE3)/pPheDH

Lane 3 = *E. coli* BL21(DE3)/pPY

Lane 4 = *E. coli* BL21(DE3)/pPTY

Lane 5 = *E. coli* BL21(DE3)/pPTFY

Lane 6 = *E. coli* BL21(DE3)/pPTFKY

Lane 7 = *E. coli* BL21(DE3)/pPTFBY

Lane 8 = *E. coli* BL21(DE3)/pPTFBLY

Lane 9 = *E. coli* BL21(DE3)/pRTFBLYA

chorismate mutase/ prephenate dehydratase in pPTFBLYA clone, the PheA protein produced was observed as faint visible band of 43 kDa (lane 9).

By comparison of protein pattern of cells with (Figure 3.55A; lane 4-8) and without (Figure 3.56A; lane 3-7) additional expression of *yddG* gene such as pPT/pPTY; pPTF/pPTFY; pPTFK/pPTFKY; pPTFB/pPTFBY; and pPTFBL/pPTFBLY), it was found that the presence of additional *yddG* gene rendered that clone to have a low expression level of other co-expressed genes. It was possible that the toxicity of YddG disturbed the expression of other genes. The PheDH activities of pPT, pPTF, pPTFK, pPTFB and pPTFBL clones were varied from 21.3 to 27.2 unit/mg protein (Table 3.3). These activities were higher than those of clones existing additional *yddG* gene (pPTY, pPTFY, pPTFKY, pPTFBY and pPTFBLY clones).

3.12 Optimization of IPTG concentration for L-Phe production in cultivation of pPTY, pPTFY, pPTFKY, pPTFBY, pPTFBLY and pPTFBLYA clones

In order to investigate whether heterologous expression of the *E. coli* genes *yddG*, *tktA*, *glpF*, *glpK*, *aroB*, *aroL* and *pheA* together with *phedh* gene was sufficient to increase capability of L-Phe production in *E. coli* from glycerol comparing to that of *E. coli* containing *phedh* gene alone. In shake flask, individual engineered *E. coli* that harbouring different combinations of genes arranged on single plasmid pRSFDuet-1 with fixing existence of *phedh* and *yddG* genes (pPTY, pPTFY, pPTFKY, pPTFBY, pPTFBLY and pPTFBLYA) was inoculated in a minimal medium containing 30 g/L of glycerol and 50 g/L of $(NH_4)_2SO_4$ as sole carbon and nitrogen sources, respectively. The culturing flasks of each clone were added by 0-1 mM IPTG.

The cell growth, glycerol utilization, medium pH and L-Phe production profiles of all six clones were monitored. The growth curves of these six clones showed variable types of growth (Figure 3.57). A few clones (pPTFKY and pPTFBLY) showed similar biomass level both with and without induction (Figure 3.57C and E). The highest yield of biomass of all clones was observed in range of 4-5 g/L and

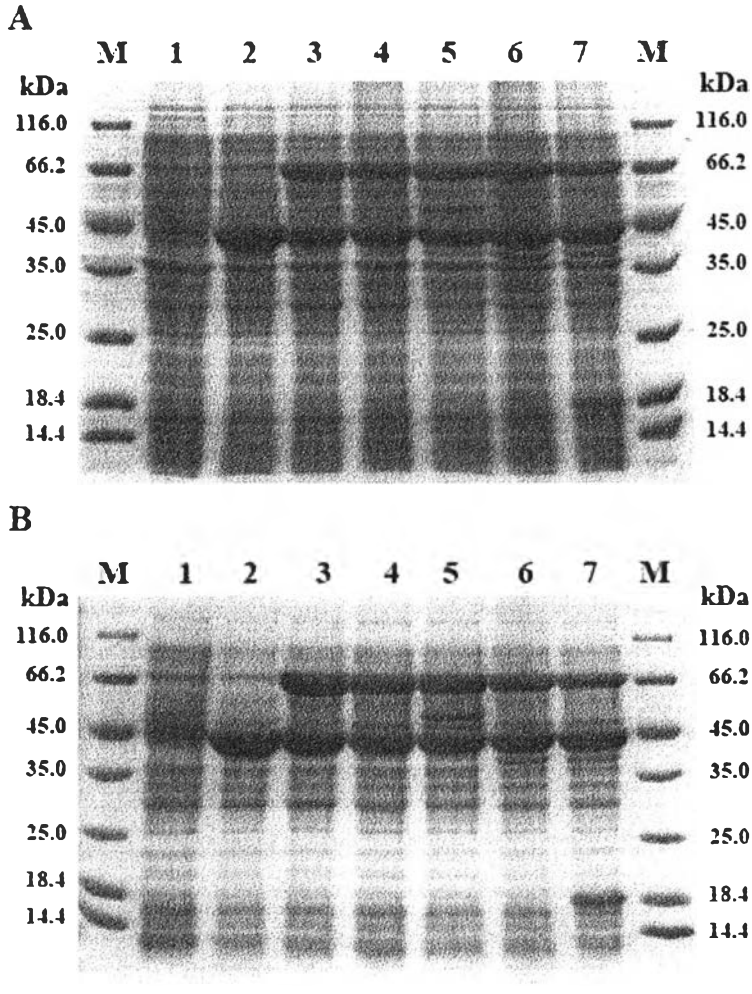


Figure 3.56 SDS-PAGE of whole cell extracts derived from recombinant *E. coli* BL21(DE3) carrying different gene combination after induction with 1 mM IPTG for 3 h

A: whole cell

B: crude extract

Lane M = protein molecular weight marker

Lane 1 = *E. coli* BL21(DE3)/pRSFDuet-1

Lane 2 = *E. coli* BL21(DE3)/pPheDH

Lane 3 = *E. coli* BL21(DE3)/pPT

Lane 4 = *E. coli* BL21(DE3)/pPTF

Lane 5 = *E. coli* BL21(DE3)/pPTFK

Lane 6 = *E. coli* BL21(DE3)/pPTFB

Lane 7 = *E. coli* BL21(DE3)/pPTFBL

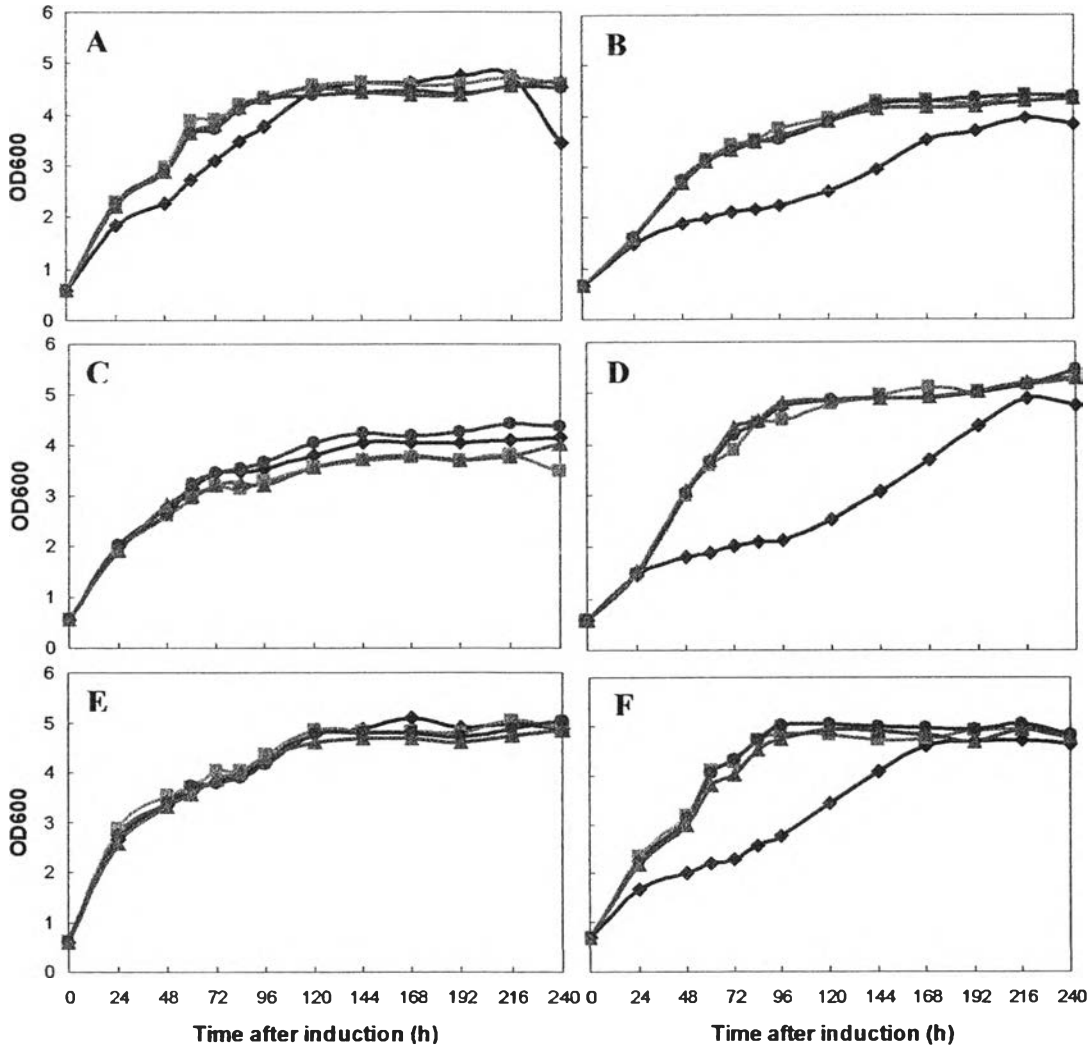


Figure 3.57 Growth profiles of *E. coli* BL21(DE3) harbouring pPTY (A), pPTFY (B), pPTFKY (C), pPTFBY (D), pPTFBLY (E) and pPTFBLYA (F) in glycerol medium without IPTG induction (◆) and with IPTG induction to final concentrations of 0.25 mM (▩), 0.5 mM (●) and 1 mM (▲) in shake flask culture. Data shown were the means of two independent cultivations.

mostly, biomass concentration of uninduced clone was lower than those of induced clones (pPTY, pPTFY, pPTFBY and pPTFBLYA; Figure 3.57A, B, D and F). In the absence of IPTG, pPTFY, pPTFBY and pPTFBLYA clones could consume more glycerol than those of induced clones with remaining less amount of glycerol (~1 g/L) at the end of cultivation (Figure 3.58B, D and F). The pH value of medium of induced clones was gradually decreased to the same value of 4.2 (data not shown).

As mentioned in section 3.9.2, to select which clone was the best for L-Phe production (pPheDH, pPY, pPYF and pPYFK clones), the L-Phe production rate was considered due to the fact that their highest values of L-Phe concentration were approximately 250 mg/L (230-280 mg/L) which were not clearly different among the four clones. The production rate should be considered within 120 h after induction in which it was still linear. However, in this section, the critical parameters used for monitoring these clones were the L-Phe production rate and the highest value of L-Phe. The production rates of these remaining six clones are shown in Figure 3.59 and summarized in Table 3.4. It was found that all clones showed higher in both L-Phe production rate and the maximum value of L-Phe than those of pPheDH clone indicating all combinations of gene expression actually caused an improvement of L-Phe production from glycerol. The pPTFBLY clone gave the highest production rate of 3.36 mg/L h which was 2.1-fold higher when compared to rate of pPheDH clone. The highest L-Phe production of pPTFBLY clone was observed at 429 mg/L. Surprisingly, this value was obtained from the condition without induction. This was consistent with Moon and coworkers' work. It was reported that increasing the inducer (IPTG) concentration to increase expression resulted in lower product concentration. They investigated the production of glucaric acid from a synthetic pathway in recombinant *E. coli* by co-expression of pTrc99A and pRSFD-IN-MI (a derivative of pRSFDuet-1) (Moon et al., 2009). The other five clones (pPTY, pPTFY, pPTFKY, pPTFBY and pPTFBLYA) had the production rates of 2.21-2.94 mg/L h with final values of L-Phe concentrations of 340-390 mg/L. As indicated in Table 3.4, it was suggested that some clones used different concentrations of IPTG to reach the highest L-Phe production rate and L-Phe concentration. As shown in Figure 3.59, some clones had a lower rate of production during initial cultivation but had the highest L-Phe concentration at 240 h of cultivation. Thus, to attain an appreciable

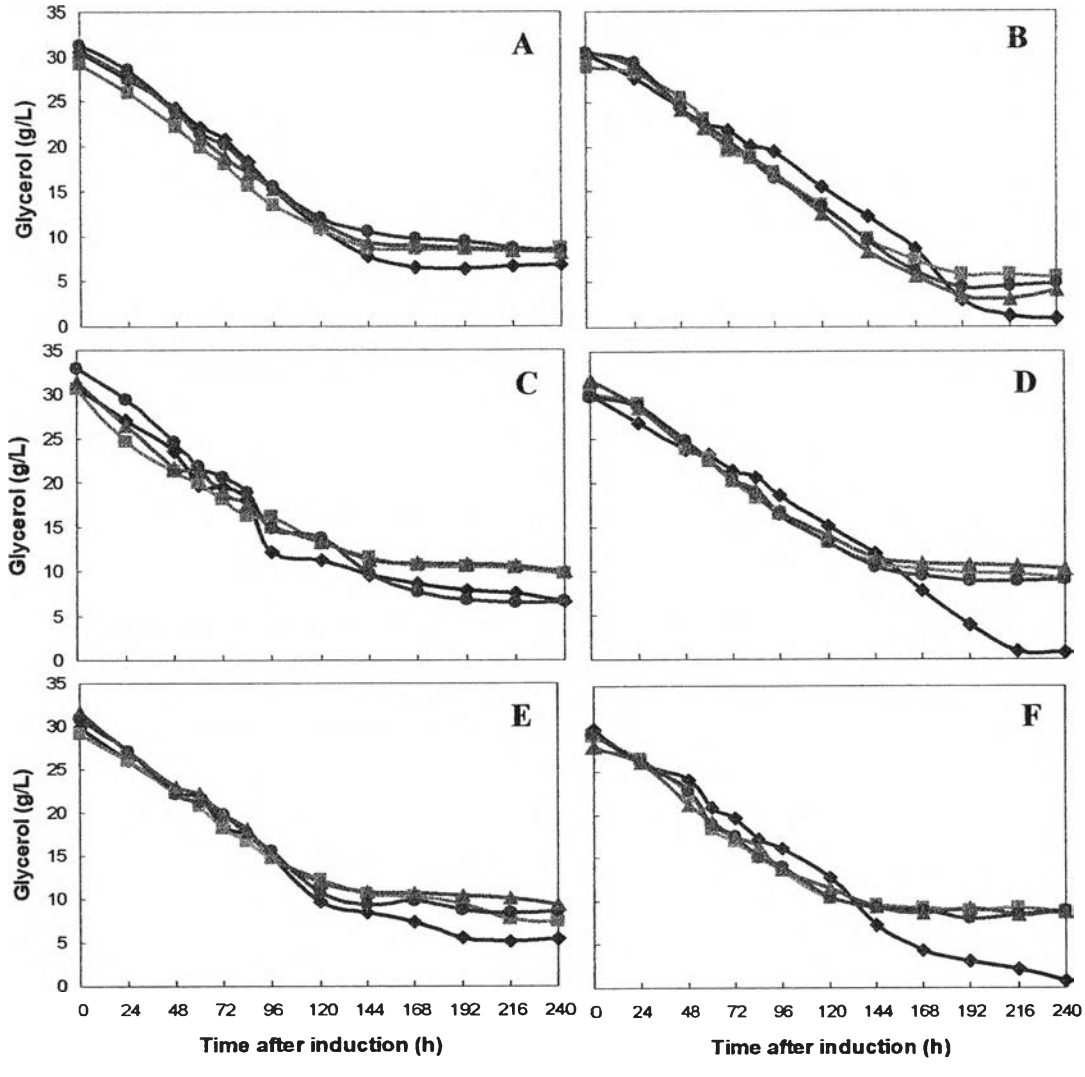


Figure 3.58 Glycerol consumption profiles of *E. coli* BL21(DE3) harbouring pPTY (A), pPTFY (B), pPTFKY (C), pPTFBY (D), pPTFBLY (E) and pPTFBLYA (F) in glycerol medium without IPTG induction (◆) and with IPTG induction to final concentrations of 0.25 mM (▩), 0.5 mM (●) and 1 mM (▲) in shake flask culture. Data shown were the means of two independent cultivations.

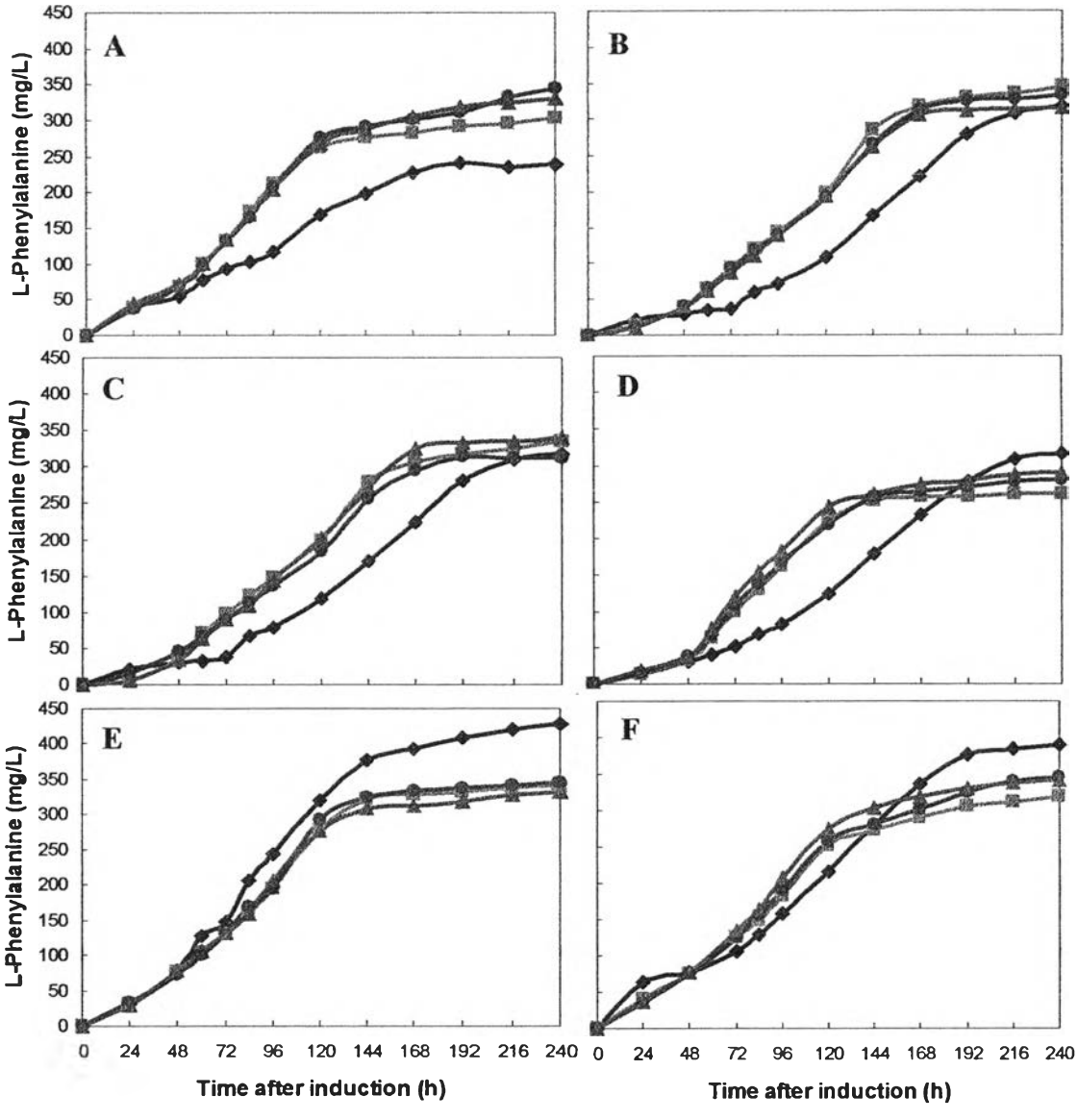


Figure 3.59 L-Phe produciton profiles of *E. coli* BL21(DE3) harbouring pPTY (A), pPTFY (B), pPTFKY (C), pPTFBY (D), pPTFBLY (E) and pPTFBLYA (F) in glycerol medium without IPTG induction (◆) and with IPTG induction to final concentrations of 0.25 mM (■), 0.5 mM (●) and 1 mM (▲) in shake flask culture. Data shown were the means of two independent cultivations.

Table 3.4 Comparison of parameters in shake flask cultivation of different recombinant *E. coli* BL21(DE3)

No.	Recombinant clone	L-Phe production		L-Phe production (mg/L) at 240 h of induction (IPTG (mM))
		Rate* (mg/L h) (IPTG (mM))	Fold**	
1	pPheDH	1.61 (0)	1.0	230 (0.5)
2	pPY	2.33 (0.5)	1.4	255 (1)
3	pPYF	2.86 (1)	1.8	280 (1)
4	pPYFK	2.15 (0.25)	1.3	268 (0.5)
5	pPTY	2.94 (0.5)	1.8	345 (0.5)
6	pPTFY	2.21 (0.25)	1.4	345 (0.25)
7	pPTFKY	2.32 (1)	1.4	340 (1)
8	pPTFBY	2.90 (1)	1.8	316 (0)
9	**pPTFBLY**	**3.36 (0)**	**2.1**	**429 (0)**
10	pPTFBLYA	2.76 (1)	1.7	389 (0)

*The L-Phe production rate was considered within 120 h after induction.

**Calculated by comparison to L-Phe production rate of pPheDH clone.

value of L-Phe, the inducer concentration and time profile of L-production of each clone needed to be selected. It was difficult to indicate whether addition of one gene led to the improvement of L-Phe production when compared to clone that did not contain the gene because the yield of production was caused by overall collaboration of over-produced proteins.

Also, SDS-PAGE analysis was used to determine heterologous expression of each clone in glycerol medium in the absence and presence of 0.25 to 1 mM IPTG, it was found that if genes were co-expressed with *yddG* gene (pPTY, pPTFY, pPTFKY, pPTFBY, pPTFBLY and pPTFBLYA), an expression of individual gene in protein pattern of whole cell extract was not clearly observed when staining gel by Coomassie Blue as shown in Figure 3.46. Possibly, all inserted genes did not simultaneously express in a given time. It is not easy to co-express genes since they must be expressed at approximately balanced levels to avoid the accumulation of toxic intermediates or bottlenecks that result in growth inhibition or suboptimal yields (Pfleger et al., 2006).

To improve L-Phe titer produced, the recombinant clone should be cultured in a bioreactor with additional controlled parameters (i.e. pH, aeration). However, the bioreactor experiments were operated when the optimization of L-Phe production of recombinant clones pPTY, pPTFY, pPTFKY, pPTFBY, pPTFBLY and pPTFBLYA has not completed. Thus, pPYF clone, that was proven to be the best for producing L-Phe among the four clones (pPheDH, pPY, pPYF and pPYFK) as reported in section 3.9.2, was used as a model to examine L-Phe production under the control of a bioreactor system.

3.13 Fermenter cultures

3.13.1 Batch fermentation

L-Phe production by the pPYF clone was examined in a 5 L stirred bioreactor (3.5 L working volume). The cultivation (37 °C, pH 7.4) was carried out for 84 h at an agitation rate of 400 rpm and an aeration rate 1 vvm. The time courses of L-Phe production, biomass and residual glycerol were shown in Figure 3.60.

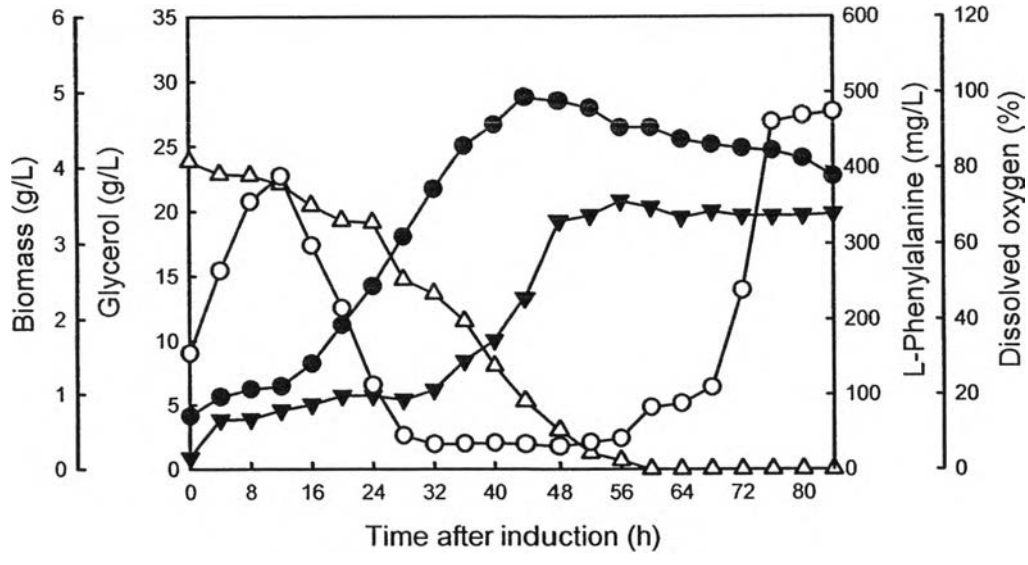


Figure 3.60 Fermentation profile of cell growth (●), residual glycerol (△), concentration of L-Phe (▼) and dissolved oxygen (○) of *E. coli* BL21(DE3) harbouring pPYF. Glycerol medium at an aeration rate of 3.5 L/min and an impeller speed of 400 rpm was used. Data shown are averaged values of two independent experiments.

The L-Phe production was accumulated in the medium before starting of induction with 1 mM IPTG (0 h) and reached a maximum value or 366 mg/L at 56 h after induction. This profile coincided with exhaustion of glycerol in the medium and consequently there was no further increase either in the concentration of L-Phe or of the biomass.

In comparison with shake-flasks, the stationary phase of growth occurred earlier (at 64 h from start of induction) in the bioreactor because of highly aerobic condition. Consequently, all glycerol was consumed. In shake flasks, L-Phe production at the beginning of induction (0 h) cloud not be detected and the first time that L-Phe could be detected by HPLC was at 24 h after induction while the production in the bioreactor could be detected at T_0 of induction. It means that L-Phe was produced and accumulated in medium prior to induction suggesting that a pRSFDuet-1 vector system under the control of T7*lac* promoter had a leaky production of protein similar to the pET vector system under the control of plain T7 promoter although T7*lac* promoter-controlled system had a strictly greater regulation than that of T7 promoter-driven system. Possibly, in bioreactor condition, the leakiness of gene expression without induction could take place faster than that in shake flask.

An expression vector, pRSFDuet-1 has a relatively high-copy number containing the RSF origin of replication allowing copy numbers of greater than 100 per cell. High-copy plasmids are widely useful for generating numerous quantities of plasmid DNA, but are generally inadequate for tightly regulation of gene expression. Although pRSFDuet-1 vector was reasonably designed to carry the *lacI* gene to ensure the expression of sufficient LacI repressor to control basal expression, gene expression using the *lac* promoter is well known "leaky" due to insufficient repressor molecules available to suppress gene expression from a high-copy number plasmid. It is especially difficult to avoid basal gene expression when using the popular *lac* repressor system, because four repressor molecules (forming a tetramer) are needed to bind to a single operator sequence in each cell in order to suppress gene expression (Gruber et al., 2008). However, to convert a high-copy plasmid to a regulatable expression plasmid by avoiding the process of subcloning DNA fragments into low-copy plasmids and to maintain a low residual gene expression,

Gruber and coworkers (2008) successfully engineered a series of compatible plasmids that permit titration of the LacI repressor protein in *E. coli* by co-expression with a high-copy plasmid. One advantage of this method is that it can be applied to an expression of membrane proteins and toxic or poorly tolerated proteins to *E. coli*.

The peak biomass concentration of 4.9 g/L in the bioreactor was not significantly different compared to in the shake flask culture (4.4 g/L). In bioreactor, the peak biomass concentration of 4.9 g/L was observed at 44 h of induction. After that, it was gradually decreased and attained 3.9 g/L at the end of fermentation (84 h). In shake flasks, the biomass concentration of 4.5 g/L was obtained at 120 h of induction and this level still remained until the end of cultivation (240 h). Probably, in bioreactor glycerol was fully consumed and insufficient. But in shake flasks, glycerol concentration was sufficient and still retained ~8 g/L at 240 h.

The peak L-Phe concentration in the bioreactor (366 mg/L) was nearly 1.3-fold the peak value obtained in the shake flasks (280 mg/L). In the bioreactor, the peak L-Phe concentration was attained at 56 h, but shake flask cultures required 216 h to reach the peak concentration. Thus, L-Phe productivity of the bioreactor was nearly 5-fold greater than the shake flask productivity. A better supply of oxygen in the bioreactor contributed its higher productivity compared to the shake flasks.

To mimic the pH value adjustment of medium to 7.4 as in the bioreactor, the preliminary experiment concerning with/without pH control of medium to 7.4 of pPheDH and pPY clones in production medium containing 40 g/L of glycerol and 50 g/L of $(NH_4)_2SO_4$ supplemented with 0.25 mM IPTG was investigated. In shake flasks without pH control, the pH gradually declined from an initial value of 7.4 to 4.2 at 168 h; however, daily adjustments of pH to 7.4 during the cultivation did not show any improvement in L-Phe titer compared to control flasks (no pH adjustment), although the final biomass concentration in the pH-adjusted flasks was nearly twice as high as in control cultures. It was confirmed that cells grew well in neutral pH. Beyond pH, there are other factors causing limitation of L-Phe production in shake flasks. Possibly, an insufficient oxygen supply in shake flask as aforementioned in section 3.9.2 might be one reason leading to limited L-Phe production. This supported why L-Phe production in bioreactor was better than that in shake flask.

3.13.2 Fed-batch fermentation

As reported in section 3.13.1, L-Phe concentration of around 366 mg/L was achieved. This was of course low compared to the best published L-Phe production processes (50 g/L, Backman et al., 1990; 46 g/L, Konstantinov et al., 1991; 45 g/L, Wang et al., 2011). The final concentration of L-Phe approached 50 g/L in the fed-batch fermentation. Batch fermentations invariably attain a low final product concentration because only a small concentration of substrate can be added to the medium at the start to prevent substrate inhibition of fermentation (Khamduang et al., 2009b). Thus, fed-batch fermentation in attempts to increase the L-Phe production was performed.

In batch fermentation, it was found that glycerol was completely depleted at 60 h while at the same time the obtained maximum biomass concentration was gradually decreased. An optimum glycerol concentration of 30 g/L in shake flasks could be identified that allowed an insufficient carbon supply for L-Phe production in bioreactor. Consequently, the fermentation was started with a relatively low initial volume of 3 L using exactly the same condition as used in the batch operation (an aeration rate of 3 L/min (1 vvm) and an impeller speed of 400 rpm). One mM IPTG was added into culture with an OD_{600} value of 0.6 for induction.

For fed batch operation, to maintain glycerol concentration nearly to 30 g/L, the feeding of a concentrated medium, containing 200 g/L of glycerol was started at 40 h after induction in which the high cell density was reached as well as residual glycerol was approximately of 10 g/L. For the next 20 h, the feeding was applied again. An air flow rate of 1 vvm and an agitation speed of 400 rpm were operated throughout the experiment. The medium from the bioreactor was continuously withdrawn every 4-h interval until 120 h. Time course of L-Phe production, biomass growth and residual glycerol were shown in Figure 3.61. The highest yield of biomass concentration was 3.8 g/L, that was 1.3 times lower than that in the former batch (4.9 g/L). The maximum value of L-Phe titer in fed-batch was 445 g/L leading to 1.3-fold above the level of maximal titer in batch culture.

For the first and second times of feeding new medium (at 40 h and 60 h), residual glycerol were observed to be 26 and 30 g/L, respectively. After first feeding, glycerol was progressively consumed but after second feeding, glycerol still remained

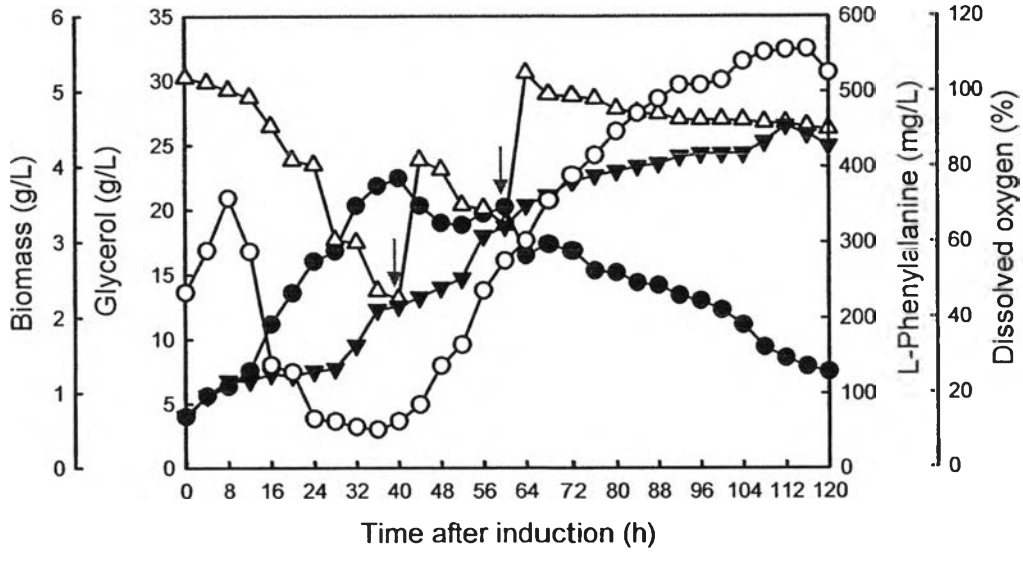


Figure 3.61 Fermentation profile of cell growth (●), residual glycerol (△), concentration of L-Phe (▼) and dissolved oxygen (○) of *E. coli* BL21(DE3) harbouring pPYF with two times feeding of glycerol. Glycerol medium at an aeration rate of 3.5 L/min and an impeller speed of 400 rpm was used. The feeding of new concentrated medium is indicated by arrow.

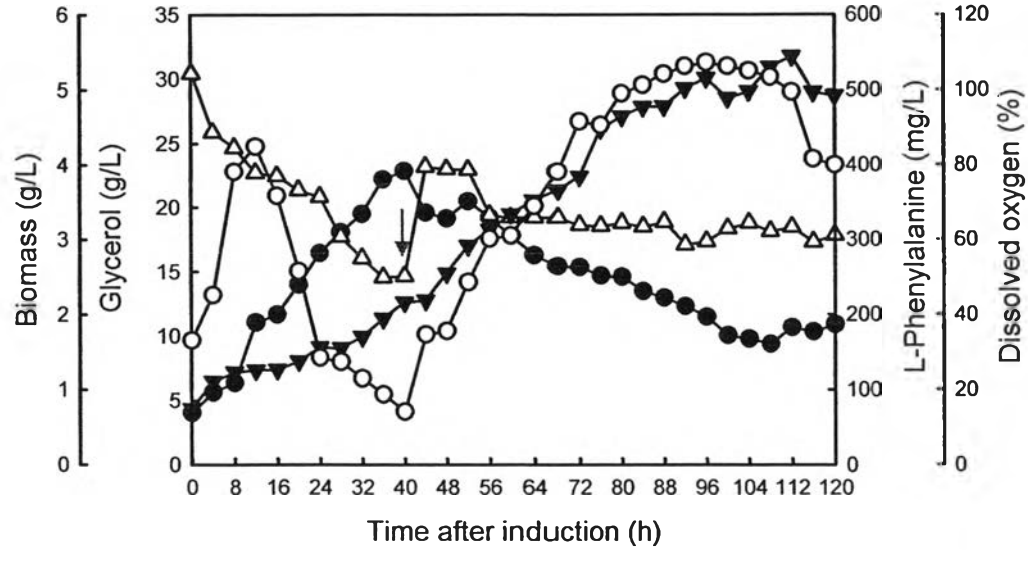


Figure 3.62 Fermentation profile of cell growth (●), residual glycerol (△), concentration of L-Phe (▼) and dissolved oxygen (○) of *E. coli* BL21(DE3) harbouring pPYF with one time feeding of glycerol. Glycerol medium at an aeration rate of 3.5 L/min and an impeller speed of 400 rpm was used. The feeding of new concentrated medium is indicated by arrow.

nearly 30 g/L. It suggested that one feeding of glycerol was clearly sufficient. The profiles resulting from one feeding at 40 h was shown in Figure 3.62. This result was closely similar to the first fed batch. The maximal L-Phe production of 545 mg/L was attained at 112 h with residual glycerol of 18 g/L. This was 1.4- and 1.2-fold greater than those in batch and first fed batch cultures, respectively.

Owing to the supply of dissolved oxygen (DO) to bioreactor for L-Phe production by *E. coli*, a number of previous works reported the variation of dissolved oxygen concentration that each fermentation system intensively required. To prevent oxygen limitation, some experimental works kept the dissolved oxygen concentration above 20% (Gerigk et al., 2002), or at 40% (Rüffer et al., 2004; Wang et al., 2011), or above 70% (Förberg and Häggström, 1988) by cascading the agitation speed or aeration rate. In our work, the bioreactor system did not control DO concentration. DO levels were always above 10% saturation.

To enable sufficient oxygen supply to this fed-batch, an aeration rate of 3 L/min (1 vvm) and an agitation speed of impeller of 400 rpm were modified. Firstly, the agitation speed was increased from 400 rpm to 600 rpm with the same aeration rate of 3 L/min. It was found that the maximum attainable biomass concentration was 2 g/L, a 2-fold lower titer compared with that of the best fed-batch fermentation. Later on, the aeration rate of 4.5 L/min (1.5 vvm) was used with fixing the agitation speed of 400 rpm. The peak biomass concentration of 1.7 g/L was observed leading to 2.2-fold decrease from that of the best fed-batch fermentation. The highest yield of L-Phe did not exceed 1.96 g/L. The dissolved oxygen concentration was maintained nearly 100% in these two altered fermentation conditions. Thus, an aeration rate of 3 L/min (1 vvm) and agitation speed of 400 rpm were optimum values for supply oxygen to this fed-batch fermentation.

In this work, although the fed-batch fermentations were operated, the L-Phe production was still lower than previously published by *E. coli* producer, especially L-Tyr auxotrophic strain that can produce substantially higher level of L-Phe from glucose. In order to enhance L-Phe fermentation process using L-Tyr auxotrophic *E. coli*, the effects of several factors (glucose feeding, L-Tyr feeding and oxygen supply) were investigated (Konstantinov et al., 1991; Gerigk et al, 2002; Wang et al., 2011) normally, in higher scale of bioreactor (20 L).

3.14 Further works

In the present study, the combination of six genes encoding PheDH, transketolase, glycerol facilitator, 3-dehydroquinate synthase, shikimate kinase II, and an aromatic amino acid exporter of pPTFBLY clone conferred the best L-Phe production from glycerol but the maximum final concentration of L-Phe was less than previously achieved in production by *E. coli* from glucose (50 g/L) (Backman et al., 1990) or glycerol (5.6 g/L) (Khamduang et al., 2009b). Probably, several compositions of production medium might not be suitable for this constructed *E. coli* because this work used an optimized medium as previously published (Khamduang et al., 2009b). Furthermore, this work used the wild type *E. coli* BL21(DE3) as producer microorganism whereas other studies used *E. coli* with extensively engineered pathway of aromatic amino acid biosynthesis.

To improve L-Phe production of pPTFBLY clone, further works suggested are:

1. To optimize the compositions of production medium in shake flask cultivation
2. To improve the L-Phe production in bioreactor