CHAPTER FOUR

DISCUSSIONS

The recombinant plasmid, pWHM3-*picK* was constructed from 7.2 kbp of pWHM3 (Vara *et al.* 1989) and 1.4 kpb of *picK* gene fragment from (personal communication, Chanama, M). The sized of constructed plasmid was approximately 8.6 kbp. The nucleotide 1410 base pairs and putative amino acid 436 residues were resulted. The putative start codon of *picK* ORF in the recombinant plasmid was selected on the basis of codon composition (Bibb *et al.* 1984) and is located 8 nucleotides downstream of a putative ribosome binding site (GAAGGA). The one translated amino acid sequence was changed from Glu146 to Gly146.

The pWHM3-*picK* is consisted of the origin of replication (*ori*) of *E. coli* and *streptomyces*, Thiostrepton resistant (*tsr*), *lacZ* containing 54 base pairs of multiple cloning sites (MCS) and ampicillin resistant gene (*bla* gene or Amp^r). Thus, pWHM3 are ably replicated both in *Streptomyces* and *E. coli* hosts. Thus, *picK* gene fragment was inserted to plasmid at MCS underneath *lac* operon containing promoter P_{lac} and the 5' terminal part of the *lacZ* gene encoding the N-terminal fragment of β-galactosidase (Yanisch-Perron *et al.* 1985). The recombinant clone number 8 (from 24 colonies of *E. coli* XL1-blue) was chosen for study of *picK* expression. The potential PicK was estimated to nearly 45 kDa by SDS-PAGE and compared to molecular weight of other reports. Betlach and coworker reported the 46 kDa of purified PicK (Betlach *et al.* 1998). The PicK is a cytochrome P450. P450 was observed by a carbon monoxide difference spectrum with a maximum wave length at 450 nm when carbon monoxide was added to sample that had been treated with dithionite (Omura and Sato 1962). In confirmation, PicK activity was measured by

this analysis. The predicted protein was appeared at the peak of 420 and 450 nm. Omura and Sato described that the P420 is a denaturized inactive form of P450 (Omura and Sato 1964b). The concentration of P450 was determined by calculation using molar extinction difference of 91 $cm^{-1}nm^{-1}$ (Omura and Sato 1964a). The P450 content of PicK was calculated to be 16.5 nM. In parallel, *E. coli* BL21 (DE3) pLysS carrying pMC-*picK* was induced by 0.4 mM IPTG and protein expression profile was observed. The molecular weight of PicK was approximately 45 kDa and the CO-difference spectrum analysis shown peaks at 420 and 450 nm which are similar to the results obtained from picK expression under controlled of P_{lac} in *E. coli* XL1-Blue. Similarly, the P450 content was calculated to be 57.1 nM The CO difference spectra peak 420 nm should be the denature form of P450. Therefore, Cheesman and coworker reported the stabilization of P450 by adding α-naphathoflavone to stabilizing ligand (Cheesman *et al.* 2003).

The pMC-*picK,* a derivative of pET-17b, is an overexpression vector because it contains the T7 promoter. The T7-based systems utilize the *lac* repressor for regulation. Woyski and Cupp-Vickery could express of P450 *picK* in *E. coli* using pTrc99A and pET-22b vector. They examined the expression of the P450 under the T7 system in pET-22b vector (Woyski and Cupp-Vickery 2001) and shows that PicK is unstable in *E. coli* using pTrc expression system. For this reason, the use of the strong T7 system was the only expression system that yields measurable amounts of active P450. The expression of *picK* from pET-22b vector using IPTG induction yields 0.26 μM of P450. Thus, the *picK* gene is able to express in *E. coli* host using pMC-*picK* and pWHM3-*picK*.

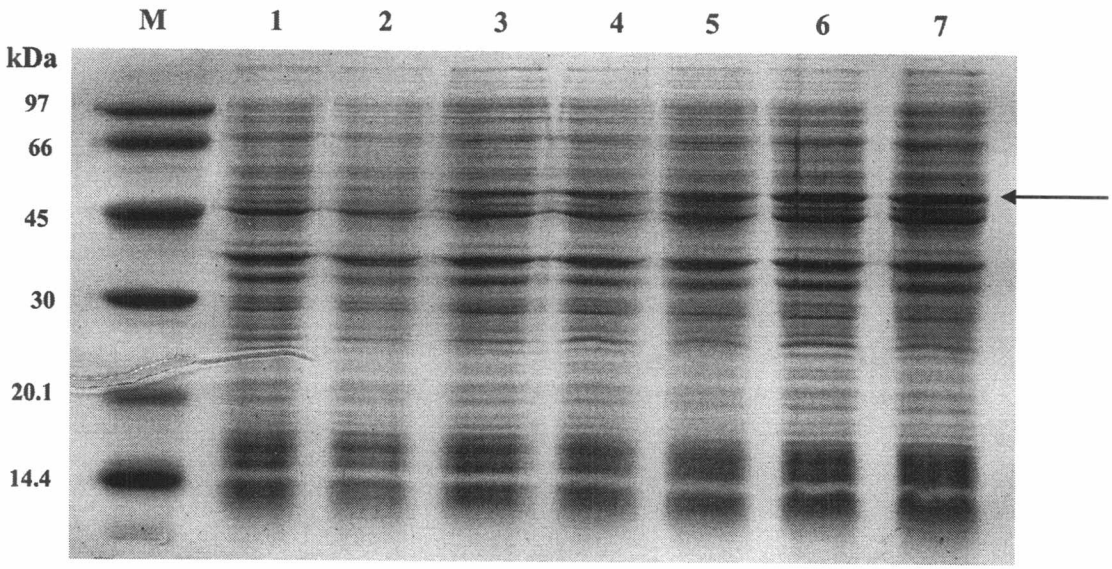

Figure 4.1 SDS-PAGE analysis of protein pattern of *E. coli* BL21 (DE3)pLysS carrying pMC-picK

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

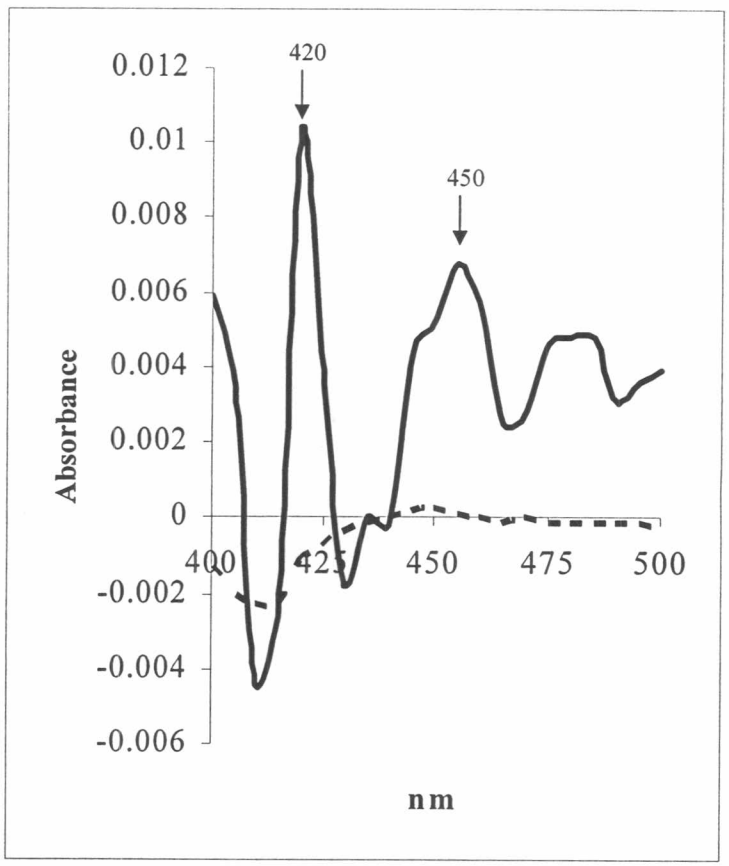

Figure 4.2 The carbon monoxide difference spectra of the *E. coli* BL21 (DE3)pLysS carrying pMC-*picK* at 6 hours induction

(····· uninduced, —— induced)