CHAPTER THREE

RESULTS

Construction of pWHM3-*picK*

The recombinant plasmid, pWHM3-*picK* was constructed from 7.2 kbp of pWHM3 and 1.4 kbp *picK* gene fragment of pMC-*picK*. Thus, pWHM3-*picK* was approximately sized 8.6 kilobase pairs (kpb) *E. coli* strain XL1-Blue was used to be electrotransformation host. The twenty- four of potential clone was chosen and pWHM3-*picK* was screened by plasmid isolation as shown in **Figure 3.1.** The picture show mobility shift of recombinant plasmid that was compared by plasmid pWHM. The recombinant clone number (lane 9) was chosen for restriction enzymes digestion.

The clone was further confirmed by restriction enzyme digestion and DNA sequencing. The **Figure 3.2** shows restriction map of clone number 8. The unique site of recombinant plasmid was digested by *Eco*RI, *Eco*RV, *Pst*I, *Xba*I and *Xho*I (lane 5, 6, 10, 14 and 15, respectively). Digestion of *Hin*dIII yielded 2 fragments of 1.4 kbp and 7.0 kbp (lane 7). The six kilobase pairs and 1.6 kbp were observed by *Nde*I digestion (lane 9). The linear form of plasmid expected sized of recombinant clone, pWHM3-*picK* (8.6 kbp). The restriction map of pWHM3-*picK* was drawn and shown in **Figure 3.3.**

The picK gene in recombinant plasmid was confirmed by sequencing. The sequence of 1.4-kbp of *picK* gene fragment shown in **Figure 3.4** and two nucleotides was changed (indicated by arrow) by comparing with picK gene from *Streptomyces*

venezuelae. Thus, the deduced amino acid was resultant changing Glu 146 to Gly146 that only first position of nucleotide was changed. **Figure 3.5** represented the deduce amino acid of PicK and the changed amino acid was indicated by arrow. The changing of amino acid residue was not interfered to important sites. The important sites of P450 are oxygen binding site and heme binding regions (underline).

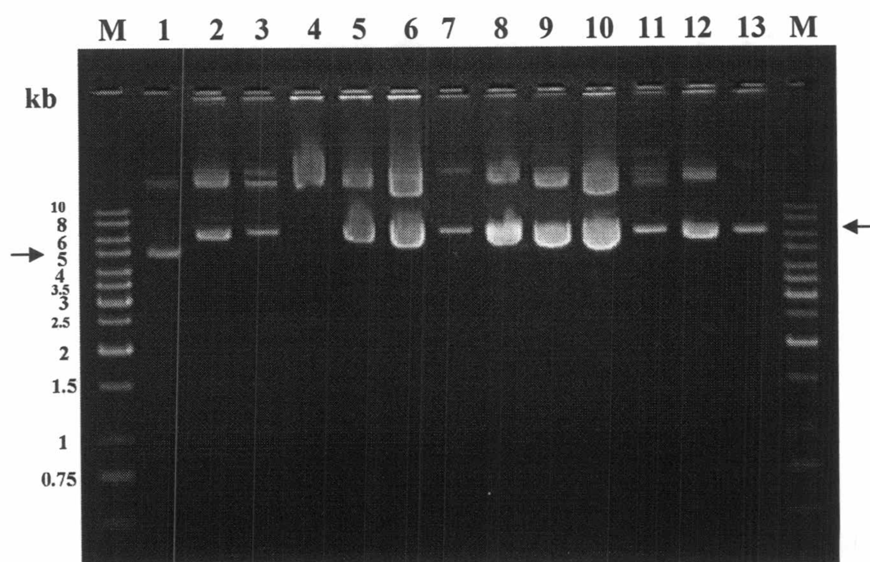

Figure 3.1a Agarose gel electrophoresis of recombinant plasmid extraction. The arrow indicated mobility of plasmid, pWHM3 and arrow was recombinant plasmid.

Lane M	GeneRuler™ 1 kb DNA Ladder
Lane 1	pWHM3
Lane 2-13	Extracted of recombinant clone no. 1 - 12

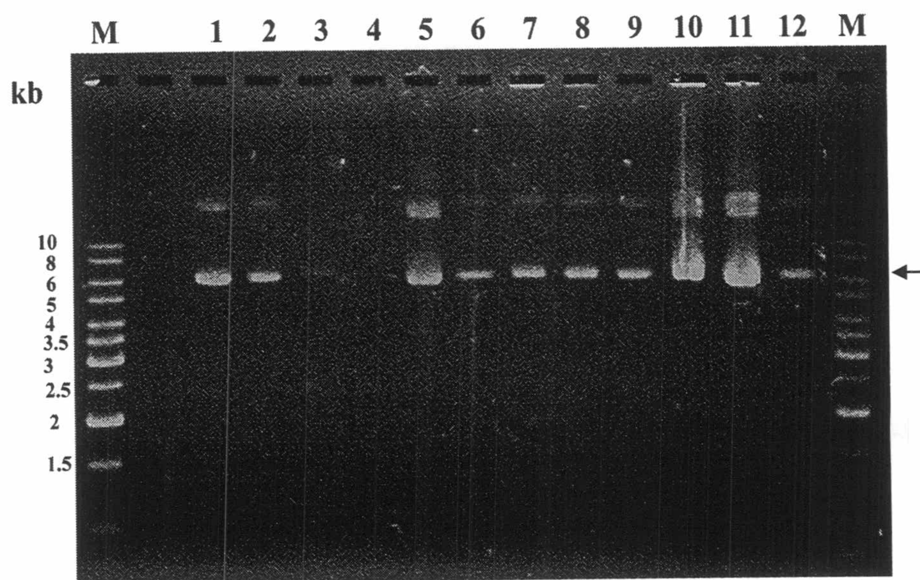

Figure 3.1b Agarose gel electrophoresis of recombinant plasmid extraction. The arrow indicated mobility of plasmid, pWHM3 and arrow was recombinant plasmid.

Lane M GeneRuler™ 1 kb DNA Ladder

Lane 1-12 Extracted of recombinant clone no. 13 - 24

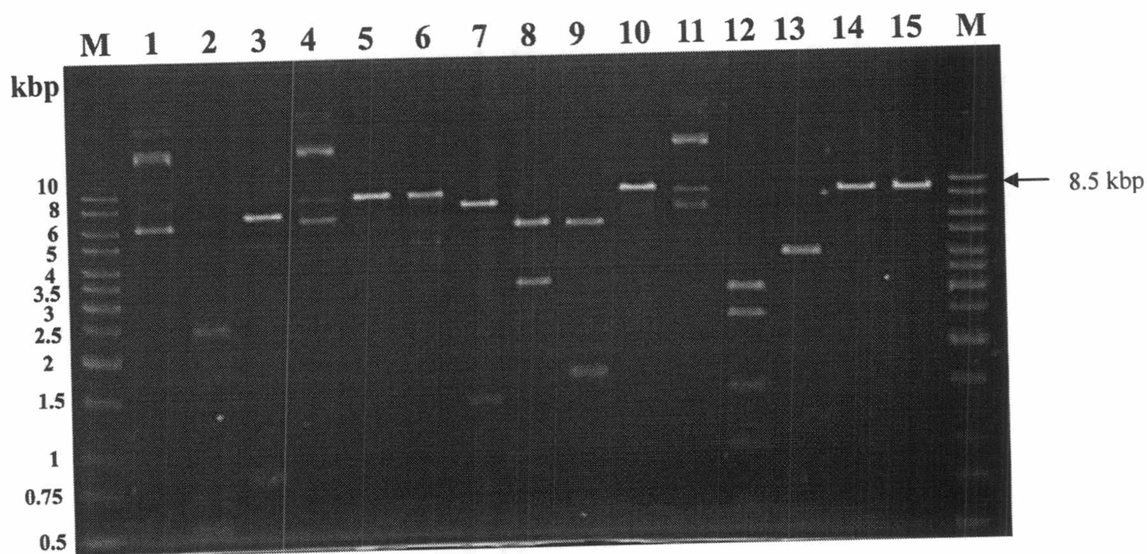

Figure 3.2 Restriction enzymes digestion of constructed plasmids, pWHM3-*picK*. The arrow pointed 8.5 kbp of unique site was digested by *Eco*RI, *Eco*RV, *Pst*I, *Xba*I and *Xho*I, respectively.

Lane M GeneRuler™ 1 kb DNA Ladder

Lane 1 Clone no. 8 undigested

Lane 2 Clone no. 8/ *Bam*HI

Lane 3 Clone no. 8/ *Bst*EII

Lane 4 Clone no. 8/ without enzyme

Lane 5 Clone no. 8/ *Eco*RI

Lane 6 Clone no. 8/ *Eco*RV

Lane 7 Clone no. 8/ *Hin*dIII

Lane 8 Clone no. 8/*Kpn*I

Lane 9 Clone no. 8/*Nde*I

Lane 10 Clone no. 8/*Pst*I

Lane 11 Clone no. 8/*Pvu*I

Lane 12 Clone no. 8/*Sal*I

Lane 13 Clone no. 8/*Sma*I

Lane 14 Clone no. 8/*Xba*I

Lane 15 Clone no. 8/*Xho*I

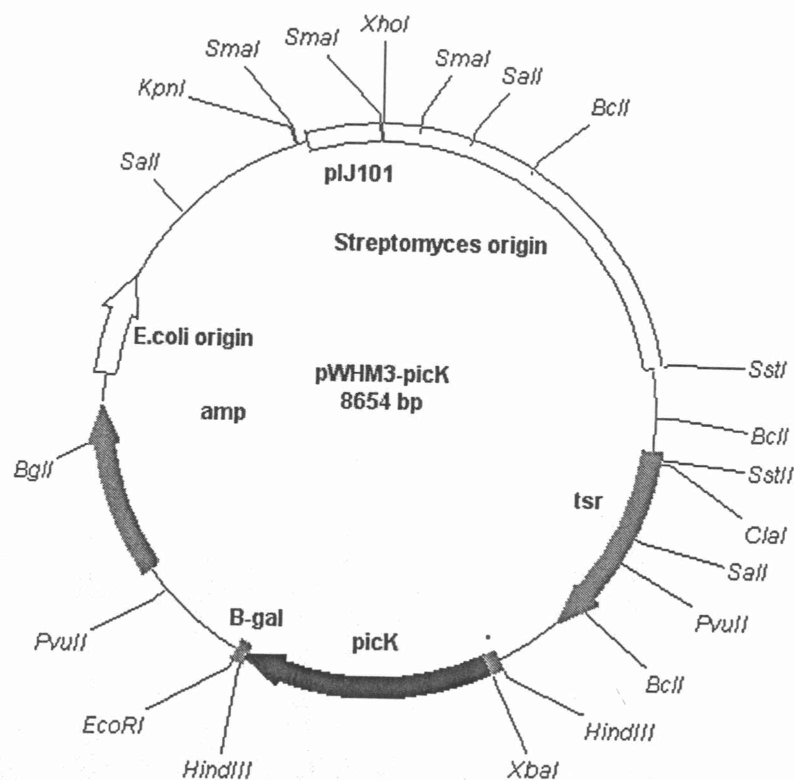

Figure 3.3 The restriction map of plasmid, pWHM3-*picK*. The *Streptomyces* origin, *E. coli* origin, thiostrepton resistant gene (*tsr*), ampicillin resistant gene (amp) and β-galactosidase gene (β-gal) inserted *picK* are composed to pWHM3-*picK*.

*Hind*III
AACAGCTATGACCATGATTACGCCAAGCTTG
*Sph*I *Pst*I *Sal*I *Xba*I **rbs** *Nde*I
CATGCCTGCAGGTCGACTCTAGAAATAATTTTGTTTAACTTTAAGA**AGGAGA**TATACAT

ATGCGCCGTACCCAGCAGGGAACGACCGCTTCTCCCCCGGTACTCGACCTCGGGGCCCTG
GGGCAGGATTTCGCGGCCGATCCGTATCCGACGTACGCGAGACTGCGTGCCGAGGGTCCG
GCCCACCGGGTGCGCACCCCCGAGGGGACGAGGTGTGGCTGGTCGTCGGCTACGACCGG
GCGCGGGCGGTCCTCGCCGATCCCCGGTTCAGCAAGGACTGGCGCAACTCCACGACTCCC
CTGACCGAGGCCGAGGCCGCGCTCAACCACAACATGCTGGAGTCCGACCCGCCGCGGCAC
ACCCGGCTGCGCAAGCTGGTGGCCCGTGAGTTCACCATGCGCCGGGTCGAGTTGCTGCGG
CCCCGGGTCCAGGAGATCGTCGACGGGCTCGTGGACGCCATGCTGGCGGCGCCCGACGGC

↓
CGCGCCGATCTGATGGGGTCCCTGGCCTGGCCGCTGCCGATCACCGTGATCTCCGAACTC
CTCGGCGTGCCCGAGCCGGACCGCGCCGCCTTCCGCGTCTGGACCGACGCCTTCGTCTTC
CCGGACGATCCCGCCCAGGCCCAGACCGCCATGGCCGAGATGAGCGGCTATCTCTCCCGG

↓
CTCATCGACTCCAAGCGCGGGCAGGATGGCGAGGACCTGCTCAGCGCGCTCGTGCGGACC
AGCGACGAGGACGGCTCCCGGCTGACCTCCGAGGAGCTGCTCGGTATGGCCCACATCCTG
CTCGTCGCGGGGCACGAGACCACGGTCAATCTGATCGCCAACGGCATGTACGCGCTGCTC
TCGCACCCCGACCAGCTGGCCGCCCTGCGGGCCGACATGACGCTCTTGGACGGCGCGGTG
GAGGAGATGTTGCGCTACGAGGGCCCGGTGGAATCCGCGACCTACCGCTTCCCGGTCGAG
CCCGTCGACCTGGACGGCACGGTCATCCCGGCCGGTGACACGGTCCTCGTCGTCCTGGCC
GACGCCCACCGCACCCCCGAGCGCTTCCCGGACCCGCACCGCTTCGACATCCGCCGGGAC
ACCGCCGGCCATCTCGCCTTCGGCCACGGCATCCACTTCTGCATCGGCGCCCCCTTGGCC
CGGTTGGAGGCCCGGATCGCCGTCCGCGCCCTTCTCGAACGCTGCCCGGACCTCGCCCTG
GACGTCTCCCCCGGCGAACTCGTGTGGTATCCGAACCCGATGATCCGCGGGCTCAAGGCC
*Hind*III
CTGCCGATCCGCTGGCGGCGAGGACGGGAGGCGGGCCGCCGTACCGGTTGAAAGCTTGGT
*Kpn*I *SacI* *Bam*HI *Spe*I *BstX*I *Eco*RI
ACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCGTGGAATTCACTGGCCGTCG

Figure 3.4 Nucleotide sequence of *picK* gene in pWHM3-*picK*. The arrows were changing residues and rbs are ribosome binding site.

MRRTQQGTTASPPVLDLGALGQDFAADPYPTYARLRAEGPAHRVRTPEGDEVWLVVGYDR

ARAVLADPRFSKDWRNSTTPLTEAEAALNHNMLESDPPRHTRLRKLVAREFTMRRVELLR

↓

PRVQEIVDGLVDAMLAAPDGRADLM**G**SLAWPLPITVISELLGVPEPDRAAFRVWTDAFVF

PDDPAQAQTAMAEMSGYLSRLIDSKRGQDGEDLLSALVRTSDEDGSRLTSEELLGMAHI***L***

oxygen binding region

LVAGHETTVNLIANGMYALLSHPDQLAALRADMTLLDGAVEEMLRYEGPVESATYRFPVE

heme binding pocket

PVDLDGTVIPAGDTVLVVLADAHRTPERFPDPHRFDIRRDTAGHLA**FGHGIHFCIGAPLA**

RLEARIAVRALLERCPDLALDVSPGELVWYPNPMIRGLKALPIRWRRGREAGRRTG

Figure 3.5 Deduced amino acid sequence with PicK. The arrow indicates amino acid changed from Glu146 to Gly146 and indicated oxygen binding region and the heme binding pocket, respectively.

Expression of *picK* gene in *E. coli* strain XL1-Blue

E. coli XL1-Blue carrying pWHM3-*picK* was expressed by 1.0 mM IPTG induction. The samples of the cell lysate were analyzed using SDS-PAGE as shown in **Figure 3.6** and potential PicK protein band was observed. The molecular weight of the potential PicK was estimated to be nearly 45,000 daltons. The P450 was detected by carbon monoxide difference spectra measurement of whole cell. The induced cell culture was measured difference spectra by scanning 400 to 500 nm and represented in **Figure 3.7**. The uninduced cell was indicated by dash line and strong line was CO difference spectrum of 24 hours post-induction cell which was observed the peaks at 420 and 450 nm, approximately. The difference spectrum of P450 was used to calculation of P450 content. The content of P450 was estimated to be 16.5 nM.

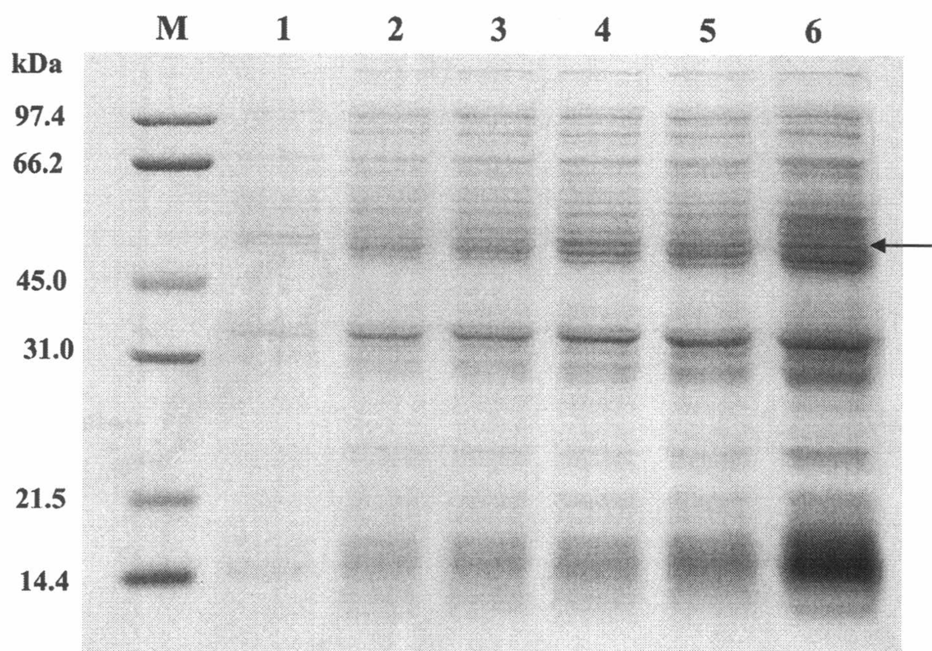

Figure 3.6 SDS-PAGE analysis of protein pattern of *E. coli* XL1-Blue carrying pWHM3-*picK*. The arrow indicates the potential PicK.

Lane M	Molecular weight standard marker
Lane 1	cell lysate of uninduced
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

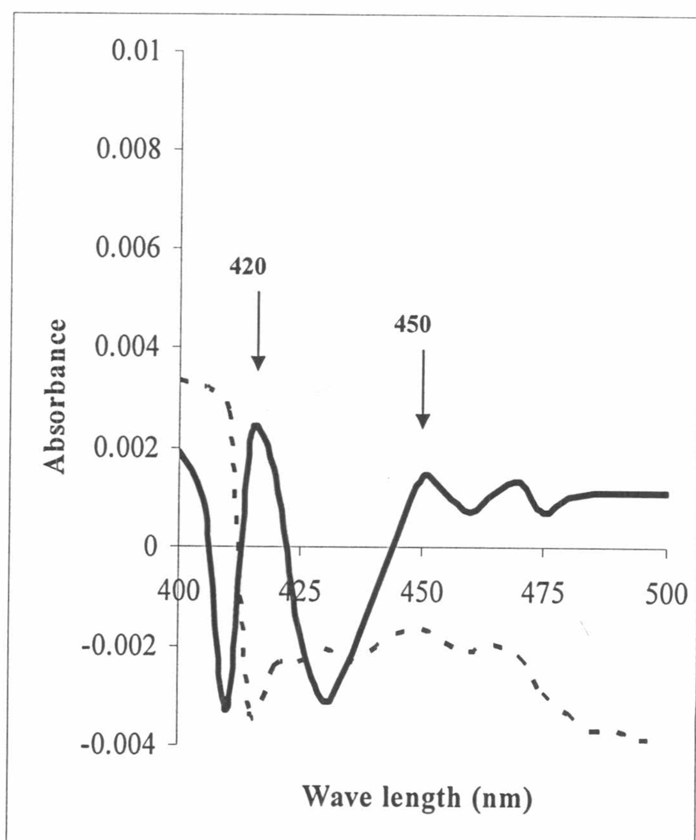

Figure 3.7 The carbon monoxide difference spectra of whole cell of the *E. coli* XL1-Blue transformants carrying pWHM3-*picK* of 24 hours induction.

(......uninduced, ——— induced)

Expression of pWHM3-*picK* in *Streptomyces* species

Expression of pWHM3-*picK* in *Streptomyces lividans* TK24

The *Streptomyces lividans* TK24 protoplasts suspension were assessed to protoplasts and non-protoplasts and estimated to 5.13×10^{13} protoplasts per milliliter. Percentage of protoplast forming was 99.80 %. The transformation of *S. lividans* TK24 was optimized by various concentration of plasmid, pWHM3. The concentration of plasmid was used by 0, 0.5, 1.0, 2.0 and 3.0 μg per one transformation. The transformant colony was monitored by thiostrepton resistant forming colony. The transformant colony was absented in plates of 0 and 0.5 μg of DNA. When protoplasts suspension was transformed by 1.0 μg of DNA, resistant colony yielded 36 colonies and estimated to be 7.2×10^{4} per μg DNA and 2.0 μg of DNA was scored 394 colonies and 7.88×10^{5} was transformation efficiency. Both transformation of 1.0 and 2.0 μg DNA was observed from colonies of 50 μl undiluted transformation mixture plates. Late, the tree microgram of DNA was transformed and scored resistant colony; 50 μl of undiluted transformation mixture was large number of colony (uncountable), 100-fold dilution was observed 250 resistant colonies, and 10,000-fold dilution was score the resistant colonies, yielded 18 colonies. The transformant efficiency of 3.0 μg DNA transformation was calculated to be 7.17×10^{4} and this concentration was chosen for recombinant plasmid, pWHM3, transformation. The transformation of pWHM3-picK was yield 400 resistant colonies on 50 μl of undiluted transformation mixture plates and 68 colonies on 100-fold dilution plates. Thus, the transformant efficiency was estimated to be 2.77×10^{5} per μg DNA.

The thiostrepton resistant colonies were screened pWHM3-*picK* by plasmid extraction. Eleven colonies which were single colony were selected. The plasmid was

isolated and agarose gel electrophoresis of eleven colonies was shown in **Figure 3.8**. Lane one was pWHM3-*picK* from *E.coli* XL1-blue. The transformant number 1 and 2 represented low quantity of plasmid (Lane 2 and 3). The plasmid showed 2 bands in transformant number 6, 8, 9, and 10 have 3 bands of plasmid.

The size of transformant plasmid was confirmed by restriction enzymes digestion as shown **Figure 3.9.** The transformant number 10 was digested by *Eco*RI, *Xba*I, *Hin*dIII, *Eco*RV and *Nde*I The unique site of pWHM3-*picK* was cut by *Eco*RI, *Xba*I and *Eco*RV (shown in lane 2, 3 and 5) and the yield fragment was 8.5 kbp. The double site was digested by *Hin*dIII and *Nde*I (shown in lane 4 and 6). These results of digestion were related to pWHM3-*picK* of *E. coli* XL1-Blue.

However, the growth periods of *Streptomyces lividans* TK24 was differed from *E. coli.* The log phase was begun at 30 hours and reached to mid-log phage at 36 hours until the stationary phase at 40 hours. The SDS-PAGE of protein expression was represented at **Figure 3.10**. Similarly, the transformant of *Streptomyces lividans* TK24 was measured difference spectra by scanning between 400 to 500 nm and represented in **Figure 3.11**.

Table 3.1 The transformation efficiency of *Streptomyces lividans* TK24 protoplasts

Concentration of DNA (μg)	Viable count (CFU)	Transformant colony (CFU)	Transformantion efficiency (per μg DNA)
pWHM3			
0	5.20×10^9	0	0
0.5	1.40×10^9	0	0
1.0	1.50×10^9	7.20×10^4	7.20×10^4
2.0	2.70×10^9	7.88×10^5	3.99×10^5
3.0	4.30×10^9	2.15×10^5	7.17×10^4
pWHM3-*picK*			
3.0	1.10×10^8	6.8×10^5	2.27×10^5

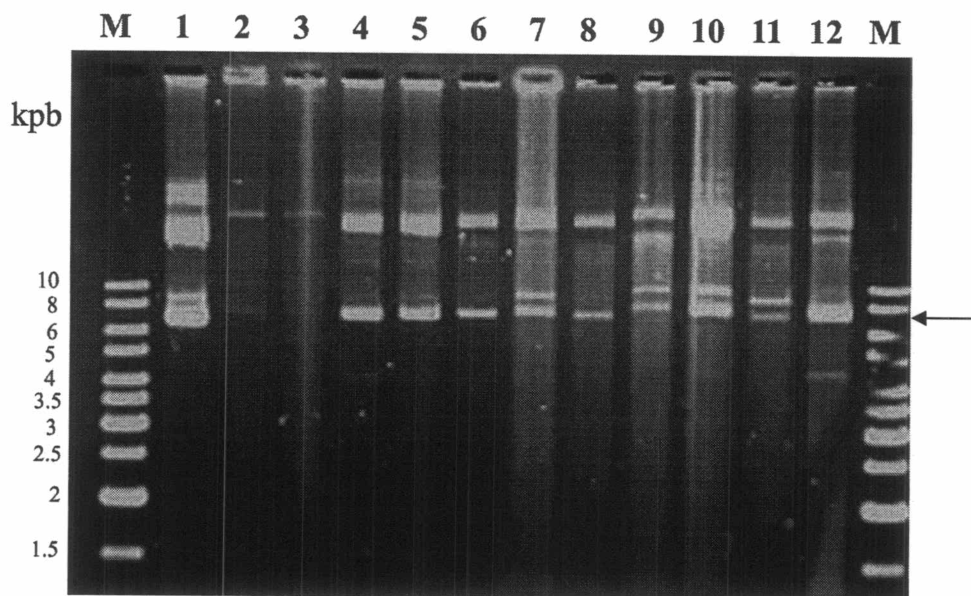

Figure 3.8 Agarose gel electrophoresis of isolated plasmid from *S. lividans* TK24 transformant

Lane M	GeneRuler™ 1 kb DNA Ladder
Lane 1	pWHM3-*picK* isolated from *Escherichia coli* XL1-Blue
Lane 2-12	Isolated plasmids from *S. lividans* TK24 transformant no. 1 - 11

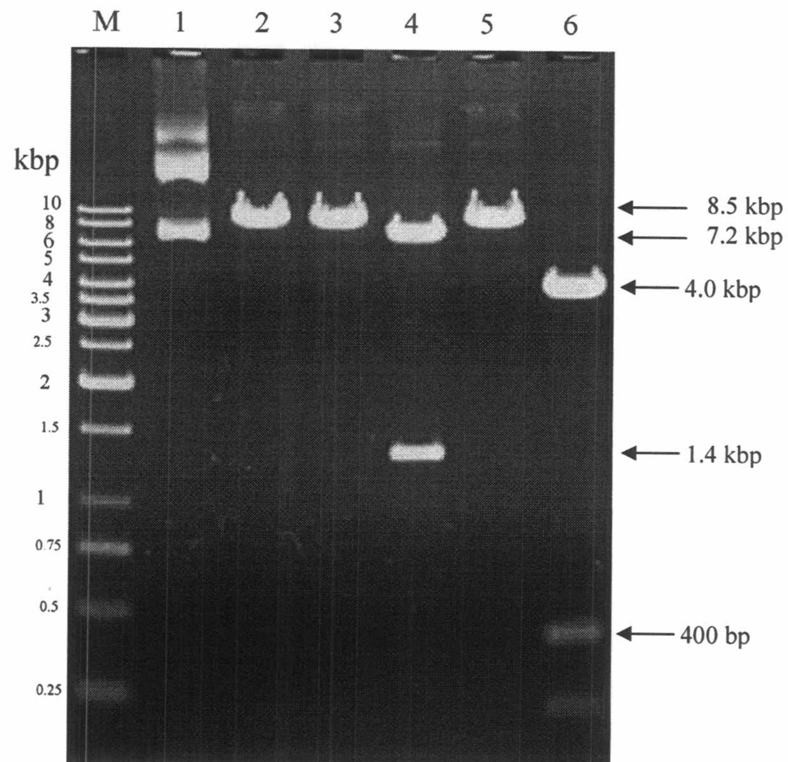

Figure 3.9 Restriction enzymes digestion of *S. lividans* TK24 transformants no. 10.

Lane M	GeneRuler™ 1 kb DNA Ladder
Lane 1	pWHM3-*picK* undigested
Lane 2	pWHM3-*picK* digested *Eco*RI
Lane 3	pWHM3-*picK* digested *Xba*I
Lane 4	pWHM3-*picK* digested *Hin*dIII
Lane 5	pWHM3-*picK* digested *Eco*RV
Lane 6	pWHM3-*picK* digested *Nde*I

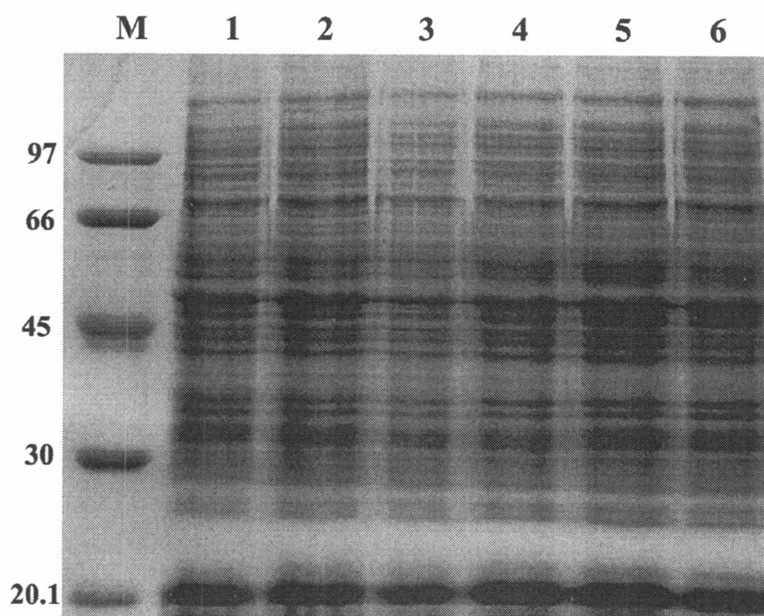

Figure 3.10 Protein pattern of *Streptomyces lividans* TK24 transformant on SDS-PAGE

Lane M	Molecular weight standard marker
Lane 1	cell extracts uninduced
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

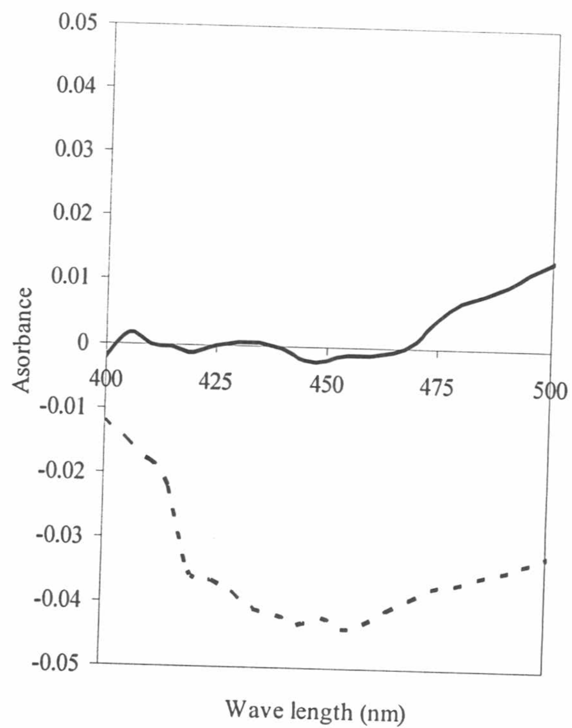

Figure 3.11 The carbon monoxide difference spectra of whole cell of the *Streptomyces lividans* TK24 transformant at 6 hours of post-induction. (······ uninduced, —— induced)

Expression of pWHM3-picK in *Streptomyces narbonensis*

The *Streptomyces narbonensis* protoplasts were estimated to 10^7 protoplasts per milliliter and transformation was carried out by PEG-mediated protoplast transformation. The transformation efficiency was shown in **Table 3.2**. The transformation of *Streptomyces narbonensis* was unsuccessful.

Table 3.2 The transformation efficiency of *Streptomyces narbonensis* protoplasts.

Concentration of DNA (µg)	Viable count (CFU)	Transformant colony (CFU)	Transformation efficiency (per µg DNA)
0	4×10^6	0	0
3.0	3×10^6	0	0