CHAPTER 3

RESULTS

3.1 Construction of plasmid carrying *pcpD* open reading frame (ORF)

PCR product (*pcpD*) was purified by phenol/chloroform extraction and precipitated with 2.5 volumes of ice-cold absolute ethanol in the present of 0.1 volume of 3M NaOAc pH 7.0 and dissolved in TE buffer.

pcpD PCR product was digested with *Bam*H I and *Pst* I, resulting in DNA fragments of 178 and 822 bp in length. pUC 19 was digested with *Bam*H I and *Pst* I resulting in 18 and 2668 bp fragments. The 822 bp fragment of *pcpD* PCR product and was ligated into 2668 bp linearized pUC 19. The ligation products were transformed into *E. coli* strain XL 1-BLUE by electroporation and the cells were grown on LB medium plate with X-gal, IPTG and ampicillin (Amp). White colonies were selected for extraction of recombinant plasmids by alkaline lysis method. These recombinant plasmids were digested with restriction endonucleases in order to determine the correct insertion of *pcpD*. Several steps for cloning of *pcpD* gene into pUC 19 and restricted fragment size generated from digestion of plasmid containing *pcpcD* ORF are despicted in Fig 3.1 and Table 3.1, respectively. The restriction endonucleases mapping of selected plasmid containing *pcpD* ORF were shown in Fig 3.2 and called pPA 02.

3.2 Construction of targeting vector for disruption of genomic *pcpD*

pVR 46 was digested with *Bam*H I, to release 1 kb fragment of kanamycin resistance gene (kan^r gene). The 1kb fragment was inserted into pPA 02 at the middle of *pcpD* ORF (at *Apa* I site) by blunt-end ligation. The ligation mixture was transformed into *E. coli* strain XL 1-BLUE by electroporation and grown on LB plate containing ampicillin (Amp) and kanamycin (Kan). Amp^r and Kan^r colonies were extracted for recombinant plasmids by alkaline lysis method. The correct clones were determined by restriction endonuclease digestion. Several steps for interruption of *pcpD* ORF and restricted fragment size generated from digestion of recombinant plasmid, carrying kan^r gene interrupted the *pcpD* ORF, with some restriction endonuclease are despicted in Fig 3.3 and Table 3.2, respectively. The recombinant plasmid carrying kan^r gene interrupted the *pcpD* ORF is a targeting vector used for disrupting the genomic *pcpD* in *S. chlorophenolica* in the next experiment. Restriction endonucleases mapping of selected targeting vector were shown in Fig.3.4.

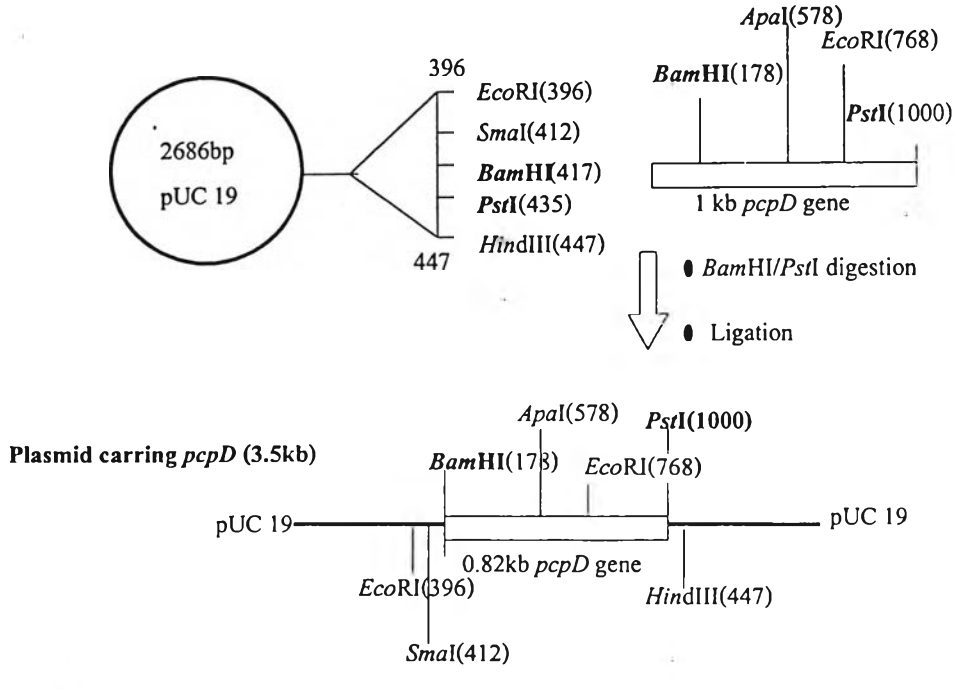

Figure 3.1 Several steps for cloning of *pcpD* gene fragment into pUC 19.

Table 3.1 Restricted fragment size generated from digestion of plasmid containing *pcpD* ORF with some selected restriction endonucleases.

Restriction endonuclease	Restircted fragment size (kb)
*Bam*H I and *Pst* I	0.82, 2.67
*Eco*R I	0.61, 2.88
Apa I	3.49

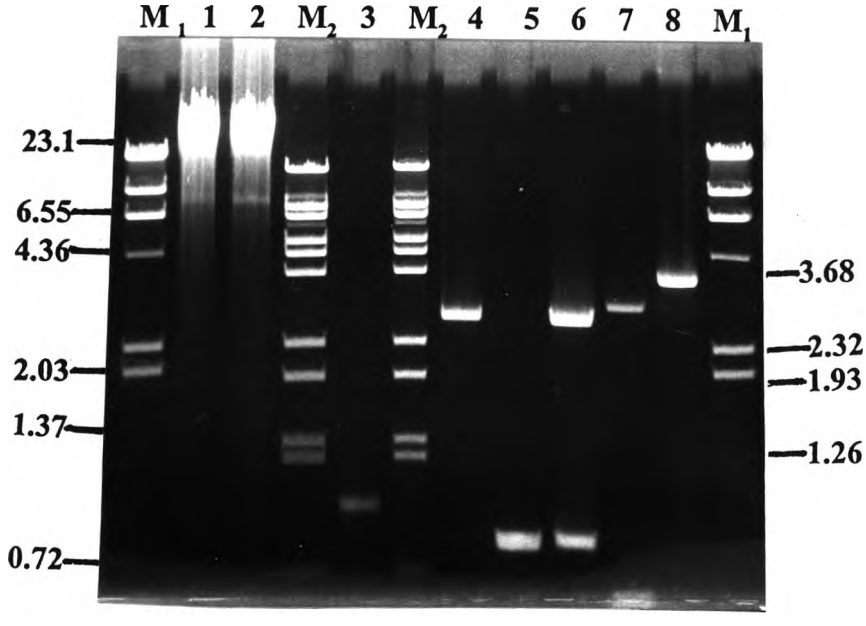

Figure 3.2 Ethidium bromide staining of DNA from various original sources:

Chromosomal DNA of *S. Chlorophenolica*, PCR product (*pcpD*) and restriction endonuclease digested plasmid containing *pcpD*.

Lane M_1 = lamda DNA/*Hin*d III digested marker

Lane M_2 = lamda DNA/*Bst*E II digested marker

Lane 1-2 = genomic DNA from *S. chlorophenolica*

Lane 3 = PCR product (1 kb of *pcpD*)

Lane 4 = *Bam*H I and *Pst* I digested pUC 19 (2.68 kb)

Lane 5 = *Bam*H I and *Pst* I digested *pcpD* fragment (0.82 kb)

Lane 6 = *Bam*H I and *Pst* I digested pPA 02 (2.68, 0.82 kb)

Lane 7 = *Eco*R I digested pPA 02 (2.88, 0.61 kb)

Lane 8 = *Apa* I digested pPA 02 (3.5 kb)

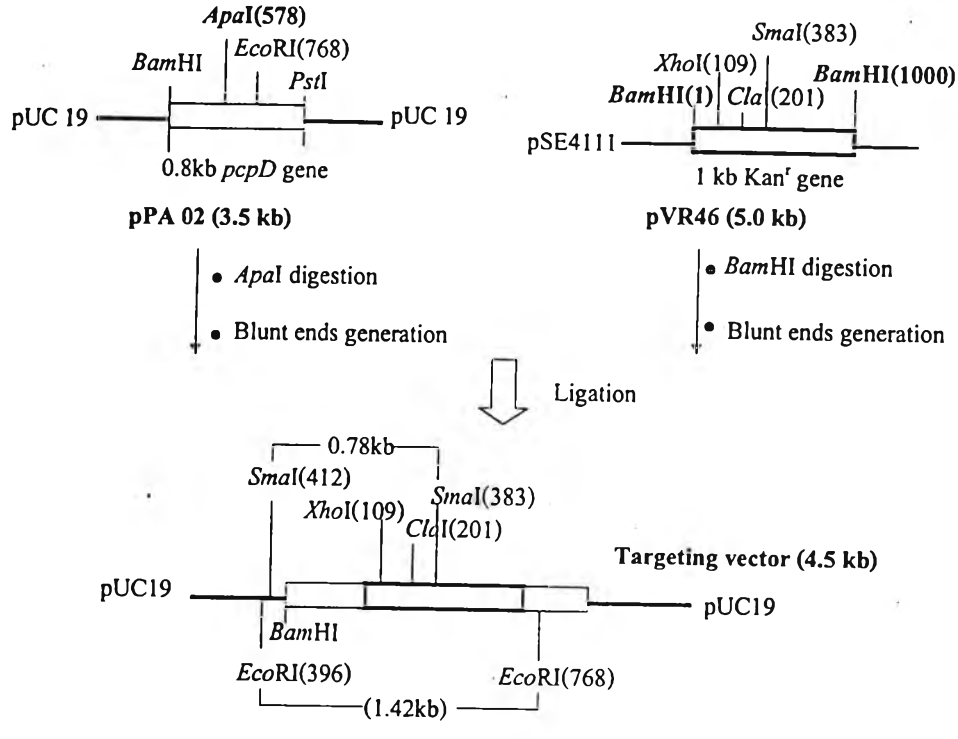

Figure 3.3 Several steps for insertion of kanamycin resistance gene into plasmid carrying *pcpD* ORF.

Table 3.2 Restricted fragment size generated from digestion of the targeting vector with some selected restriction endonucleases.

Restriction endonuclease	Restricted fragment size (kb)
*Eco*R I	1.42, 3.07
Sma I	0.78, 3.71
Cla I	4.49
Xho I	4.49

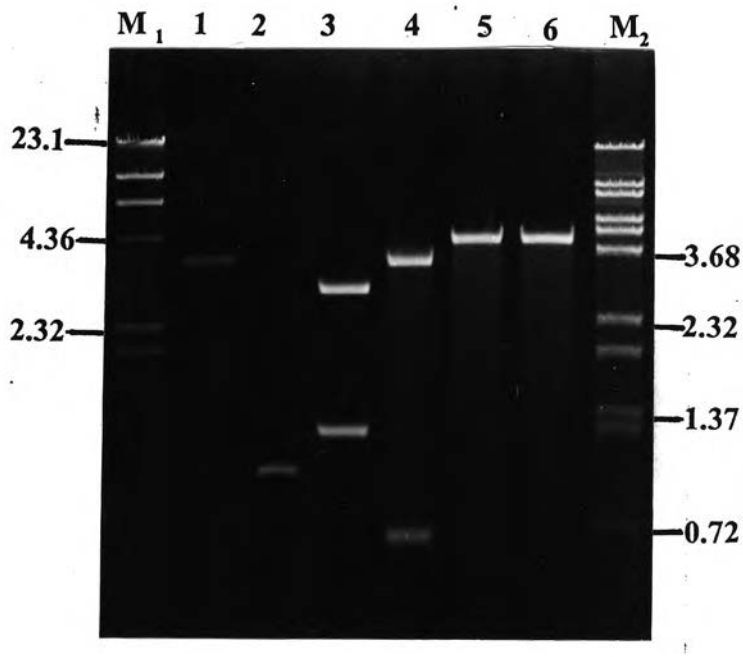

Figure 3.4 Ethidium bromide staining of targeting vector digested with various restriction endonuclease.

Lane M_1 = lamda DNA/*Hin*d III digested marker

Lane M_2 = lamda DNA/*Bst*E II digested marker

Lane 1 = *Apa* I digested pPA 02 (3.5 kb)

Lane 2 = kan^r gene cassette (1 kb *Bam*H I fragment from pVR 46)

Lane 3 = *Eco*R I digested targeting vector (2.9,1.28 kb)

Lane 4 = *Sma* I digested targeting vector (3.52, 0.67 kb)

Lane 5 = *Cla* I digested targeting vector (4.2 kb)

Lane 6 = *Xho* I digested targeting vector (4.2 kb)

3.3 Gene targeting disruption of *pcpD* in genomic DNA of *S. chlorophenolica*

The targeting vector was transformed into *S. chlorophenolica* by electroporation and transformants were selected in mineral salt medium plates containing kanamycin (Kan). Electrotransformation of *S. chlorophenolica* with targeting vector was performed twenty-five times which gave no transformant, except the last experiment that gave apparent kanamycin resistance (Kan^r) colonies within 3-5 days after incubation. Morphological characterization of the colonies was apparently similar to those of wild type. The Kan^r colonies were tested for the event of crossover of the targeting vector and genomic *pcpD* in *S. chlorophenolica*, and ability to utilize PCP. The Kan^r colonies were transferred to agar medium containing ampicillin (Amp) and Kan and agar medium containing Kan and PCP. All of the Kan^r colonies could grow in Amp-Kan plate and PCP-Kan plate. In Amp-Kan plate, colonies were visible in third day whereas in PCP-Kan plate colonies could be seen after incubate for seven days. Wild type strain was also tested for the resistance to Amp and Kan. The wild type was resistant to Amp but sensitive to Kan. Because of its resistance to Amp, Amp-Kan agar medium should not be used to test the event of crossover. Other method, Southern blot analysis should be used to examine the clones.

Twenty-seven Kan^r colonies were selected to be potential *pcpD* mutants. Their chromosomal DNAs were isolated and completely digested with *Eco*R I, then resolved by 0.8% agarose gel electrophoresis. Their digested DNAs were subjected to Southern blot hybridization with *pcpD* and kan^r gene probes by nonradioactive method. All of 27 potent clones gave hybridization signal with *pcpD* probe but did not gave any signal with kan^r gene probe. Therefore, this showed that all clones were mutant strain of *S. chlorophenolica* with no kan^r gene inserted in genomic *pcpD* (Fig 3.5-3.7). The mutants were extracted for targeting vector by alkaline lysis method, and were not found the targeting vector in all mutants.

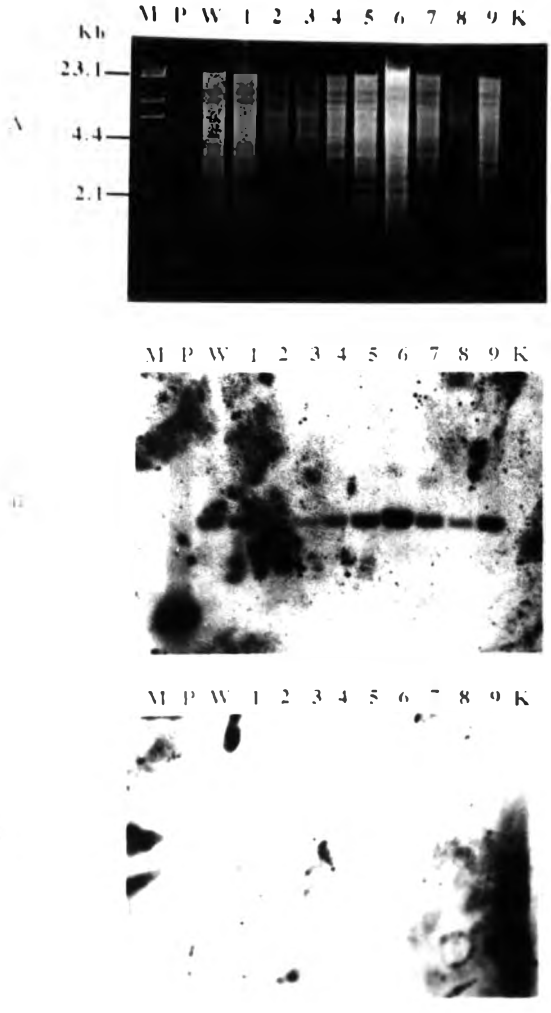

Figure 3.5 Southern-blot analysis in mutant strain of *S. chlorophenolica* no. 1-9.

A. DNA digested with *Eco*R I

B. Hybridization analysis with *pcpD* gene probe

C. Hybridization analysis with kan^r gene probe

Lane M = lamda DNA/*Hin*d III digested marker

Lane P = *pcpD* fragment (used as probe)

Lane W = DNA of wild type strain digested with *Eco*R I

Lane 1-9 = DNA of mutant strain no. 1-9 digested with *Eco*R I, respectively

Lane K = kan^r gene (used as probe)

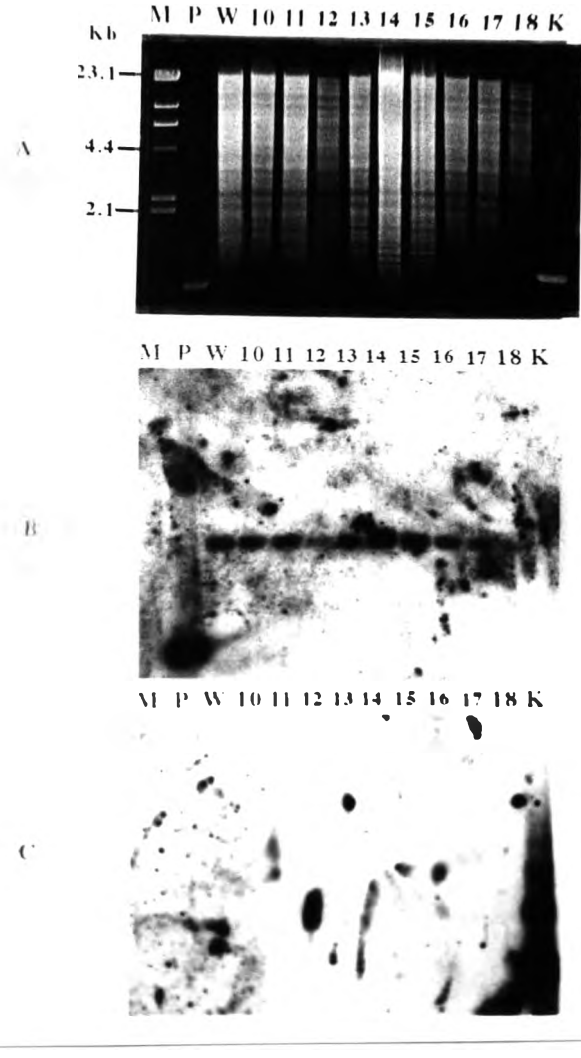

Figure 3.6 Southern-blot analysis in mutant of *S. chlorophenolica* no. 10-18.

A. DNA digested with *Eco*R I

B. Hybridization analysis with *pcpD* gene probe

C. Hybridization analysis with kan^r gene probe

Lane M = lamda DNA/*Hin*d III digested marker

Lane P = *pcpD* fragment (used as probe)

Lane W = DNA of wild type strain digested with *Eco*R I

Lane 10-18 = DNA of mutant strain no. 10-18 digested with *Eco*R I, respectively

Lane K = kan^r gene (used as probe)

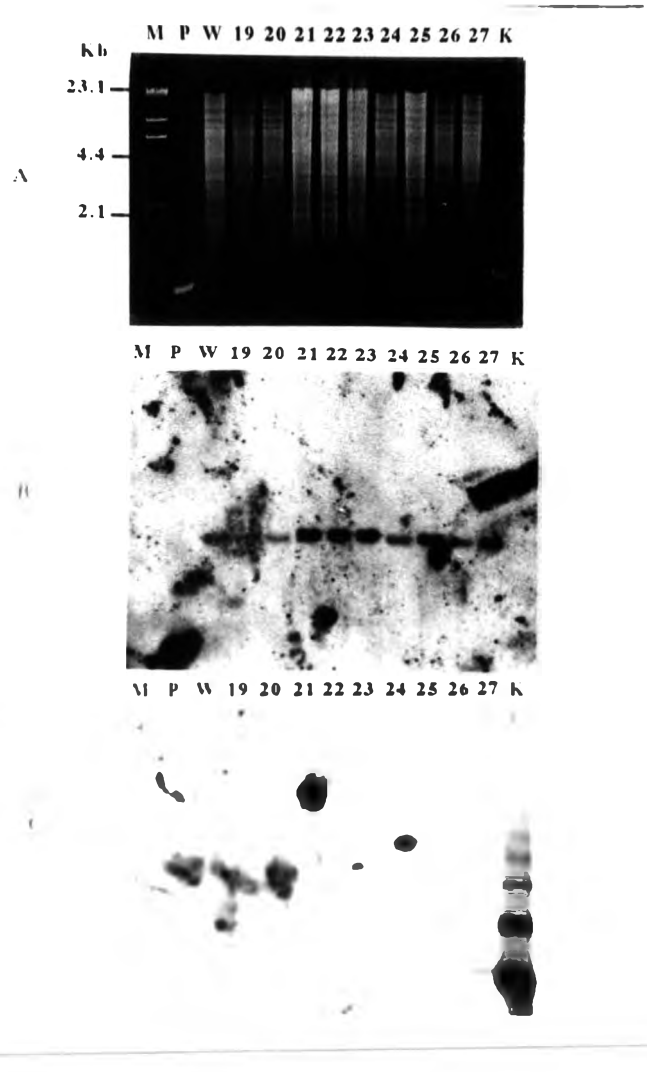

Figure 3.7 Southern-blot analysis in mutant of *S. chlorophenolica* no. 19-27.

A. DNA digested with *Eco*R I

B. Hybridization analysis with *pcpD* gene probe

C. Hybridization analysis with Km^r gene probe

Lane M = lamda DNA/*Hin*d III digested marker

Lane P = *pcpD* fragment (used as probe)

Lane W = DNA of wild type strain digested with *EcoR* I

Lane 19-27 = DNA of mutant strain no.19-27 digested with *Eco*R I, respectively

Lane K =kan^r gene (used as probe)

3.4 Pentachlorophenol degradation by mutant strains of *S. chlorophenolica*

All mutant strains were tested for the PCP degradation activity in liquid media containing 50 mg/l PCP and PCP degradation was monitored by spectrophotometric method at wavelength 318 nm.

Time-course study of the PCP degradation by twenty-seven mutants revealed that most of the mutants still had the ability to degrade PCP completely within 2 hours. Only two mutant strains, no. 2 and 14, showed dramatically slow degradation of PCP, they could degrade 25-45% of PCP in 2 hours (Table. 3.1). Their PCP degradation activity were retarded in some ways which we intent to investigate in future.

Table 3.3 Pentachlorophenol degradation activity of mutant strains of *S. chlorophenolica* and their wild type

S. chlorophenolica Strain	% PCP degraded by 2 hours.	*S. chlorophenolica* strain	% PCP degraded by 2 hours.
Mutant no. 1	97.84	mutant no. 15	92.24
Mutant no. 2	45.91	mutant no. 16	91.01
Mutant no. 3	96.92	mutant no. 17	97.03
Mutant no. 4	85.45	mutant no. 18	93.44
Mutant no. 5	86.86	mutant no. 19	93.78
Mutant no. 6	88.81	mutant no. 20	98.94
Mutant no. 7	97.21	mutant no. 21	78.56
Mutant no. 8	72.65	mutant no. 22	90.92
Mutant no. 9	98.59	mutant no. 23	88.80
Mutant no. 10	85.87	mutant no. 24	90.22
Mutant no. 11	97.16	mutant no. 25	90.40
Mutant no. 12	79.72	mutant no. 26	88.50
Mutant no. 13	98.56	mutant no. 27	93.71
Mutant no. 14	25.87	wild type	99.16

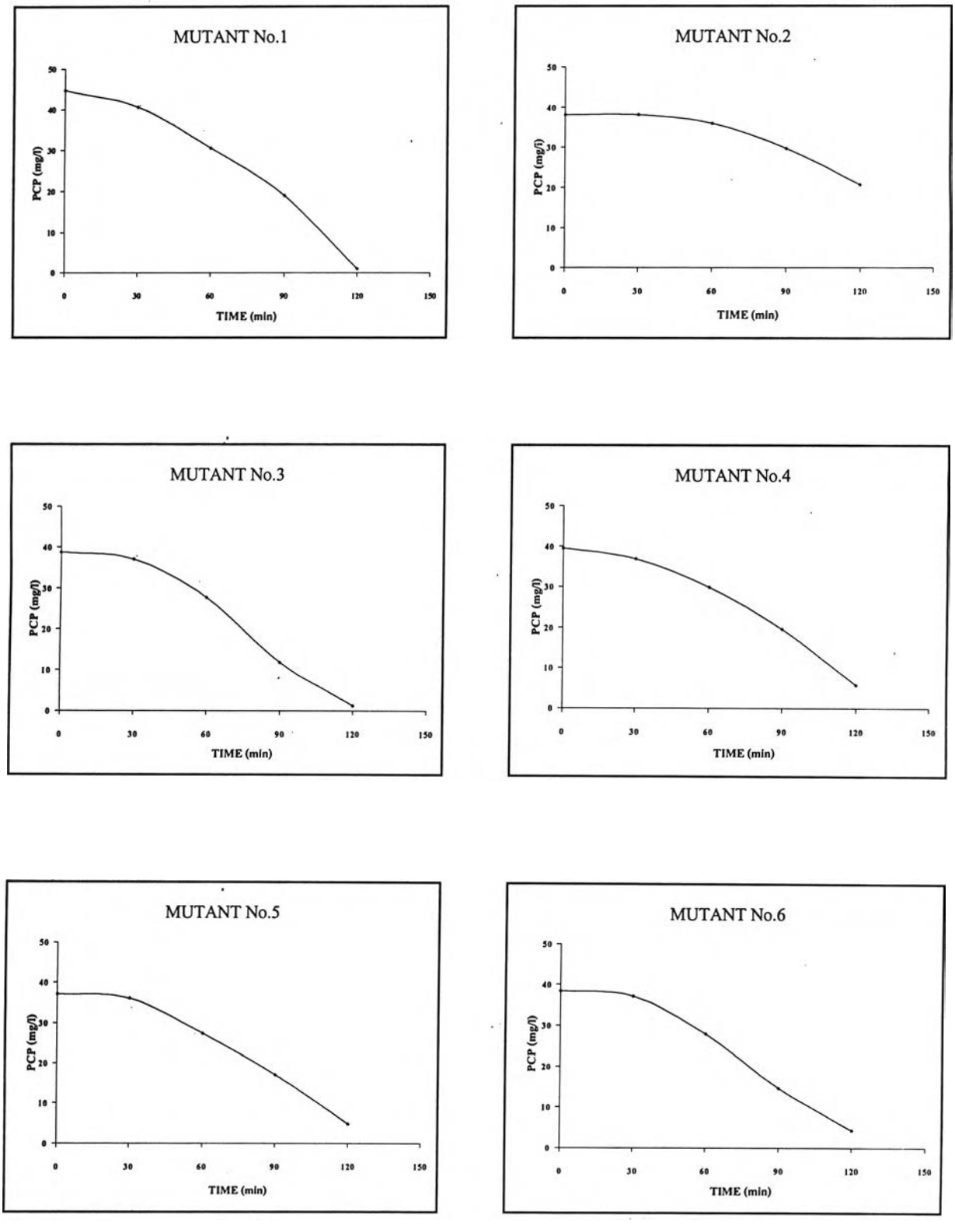

Figure 3.8 Degradation of pentachlorophenol by mutant strains of *S. chlorophenolica* no. 1-6 as monitored by UV-spectrophotometric method at wavelength of 318 nm.

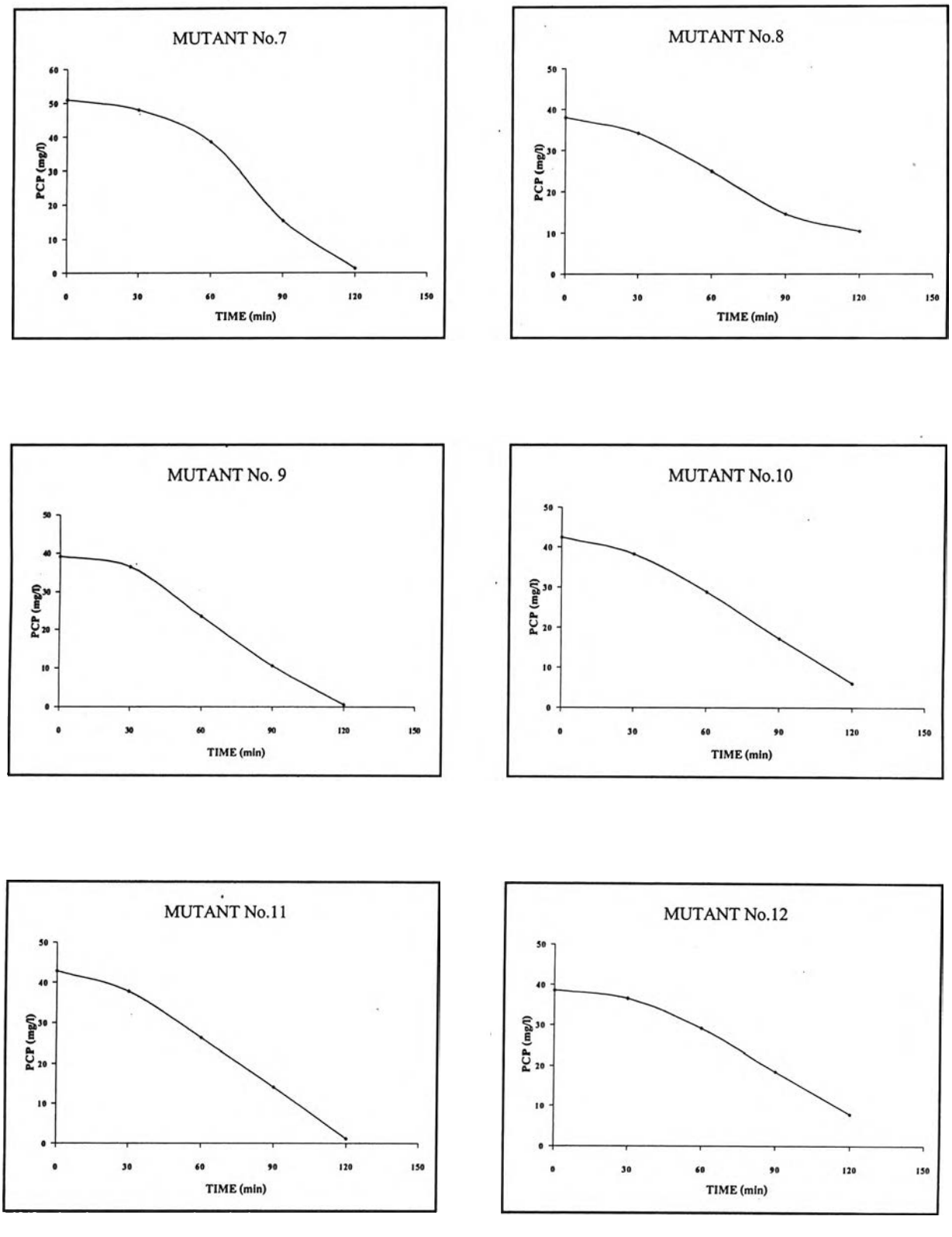

Figure 3.9 Degradation of pentachlorophenol by mutant strains of *S. chlorophenolica* no.7-12 as monitored by UV-spectrophotometric method at wavelength of 318 nm.

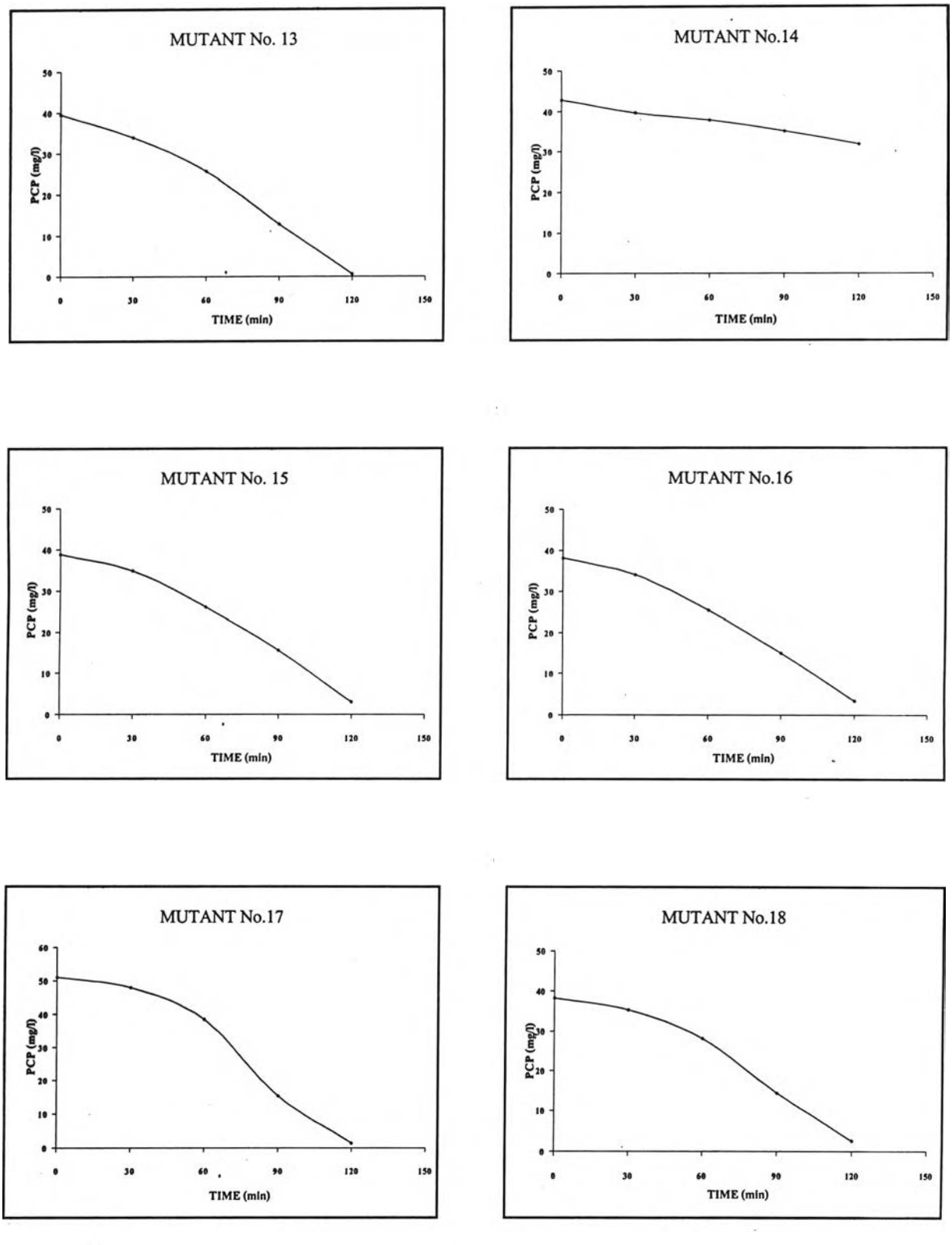

Figure 3.10 Degradation of pentachlorophenol by mutant strains of *S. chlorophenolica* no. 13-18 as monitored by UV-spectrophotometric method at wavelength of 318 nm.

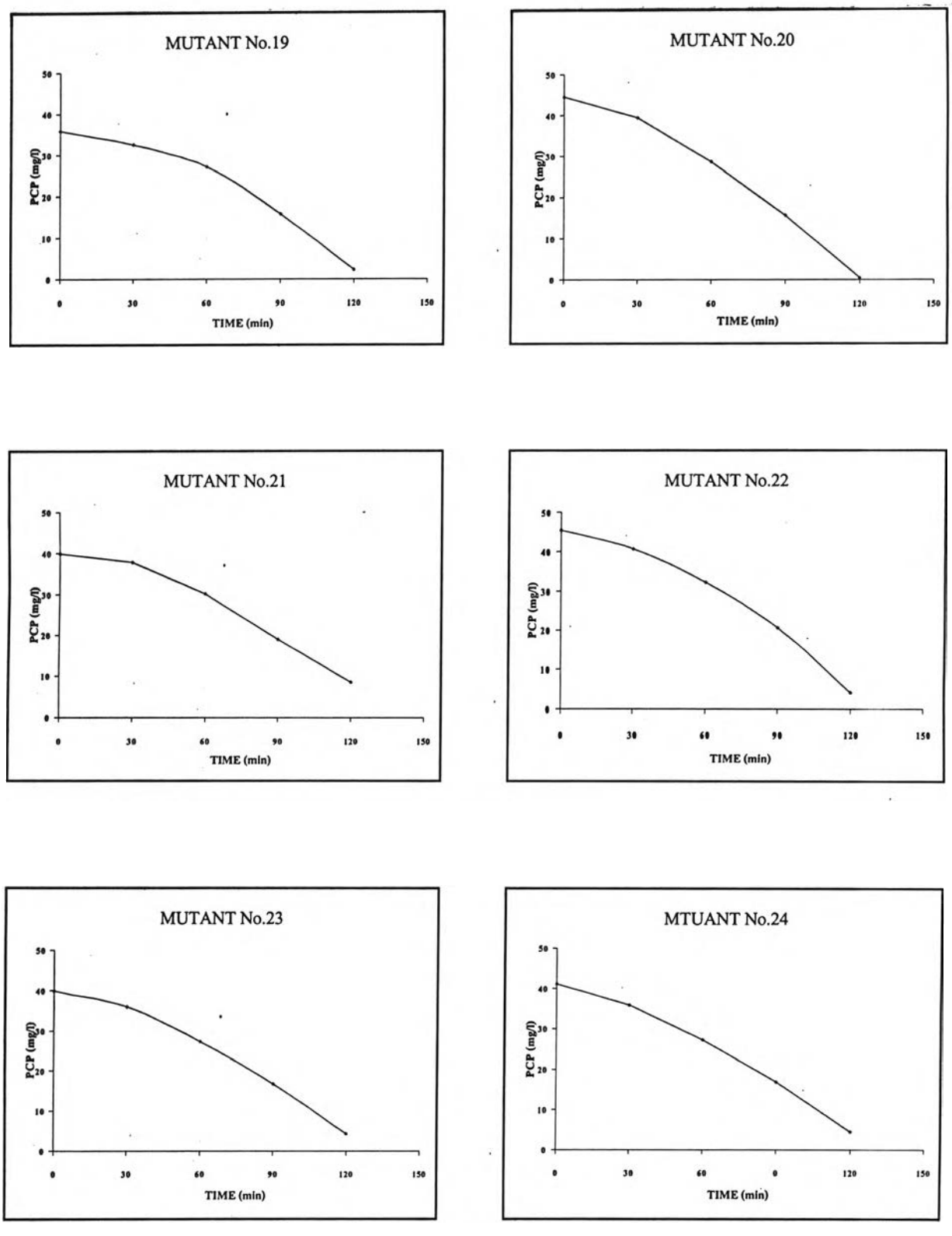

Figure 3.11 Degradation of pentachlorophenol by mutant strains of *S. chlorophenolica* no.19-24 and their wild type as monitored by UV-spectrophotometric method at wavelength of 318 nm.

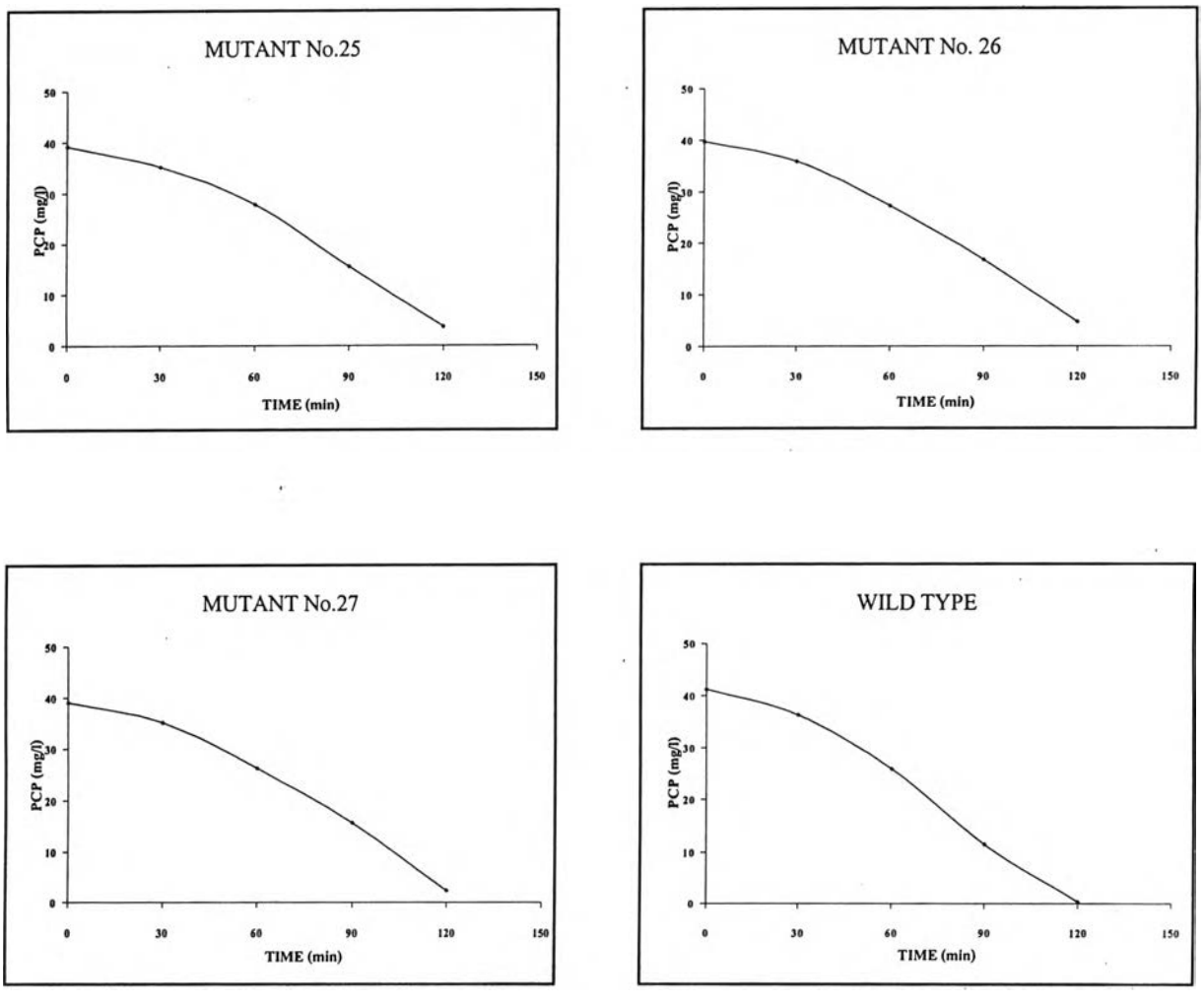

Figure 3.12 Degradation of pentachlorophenol by mutant strains of *S. chlorophenolica* no.25-27 and their wild type as monitored by UV-spectrophotometric method at wavelength of 318 nm.