CHAPTER III

RESULTS

Chitinase production of *Burkholderia cepacia*

Burkholderia cepacia was isolated from soil in Bangkok, Thailand. This organism showed a visible clear zone when grown on colloidal chitin minimum medium plate (Figure 8). This indicates that *Burkholderia cepacia* can produce chitinase which can degrade chitin.

Burkholderia cepacia was cultivated in 0.2% colloidal chitin and 10 g chitin minimum medium. Crude chitinase from both cultured medium was assayed at every 24 hours for a period of 1 week by determination of turbidity reduction. Chitinase production profile in both colloidal chitin and chitin minimum medium was shown in Figure 9. The highest chitinase activity, in both medium, is on the second day of cultivation.

Characterization of crude chitinase

A. Optimum pH

The optimum pH of crude chitinase was determined. Chitinase was assayed in buffer with pH ranging from 2-10. Activities at different pH were shown in Figure 10. Turbidity reduction of a colloidal chitin suspension during chitinolysis was used to determine the activity. The highest chitinase activity was detected when crude enzyme was incubated with substrate, colloidal chitin, in citrate buffer (pH7.0).

B. Optimum Temperature

The optimum temperature of crude chitinase was determined at pH 7.0, in the range of 10-65 °C. The chitinase activities were assayed by the turbidity reduction of a colloidal chitin suspension during chitinolysis.

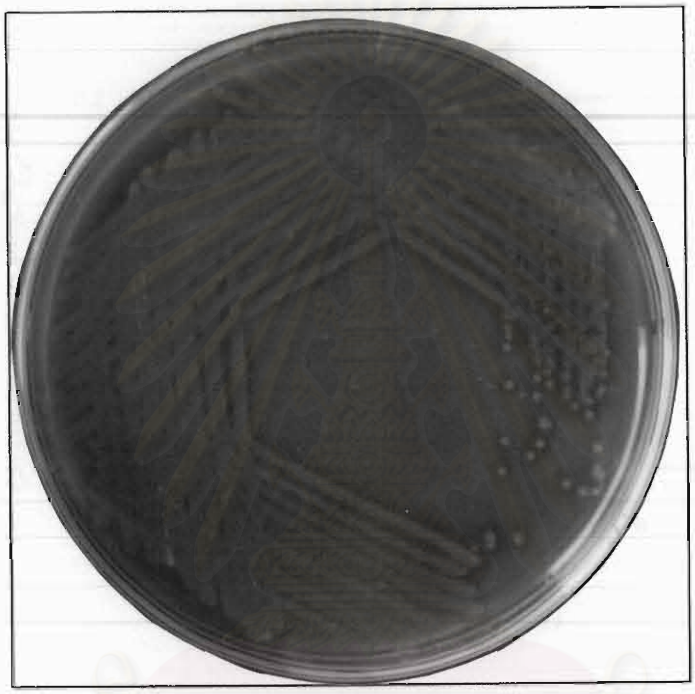

Figure 8 ***Burkholderia cepacia*** **on colloidal chitin minimum plate.** *Burkholderia cepacia* was grown on minimum medium supplimented with 0.2% colloidal chitin. The culture was grown for 3 days at 30 °C.

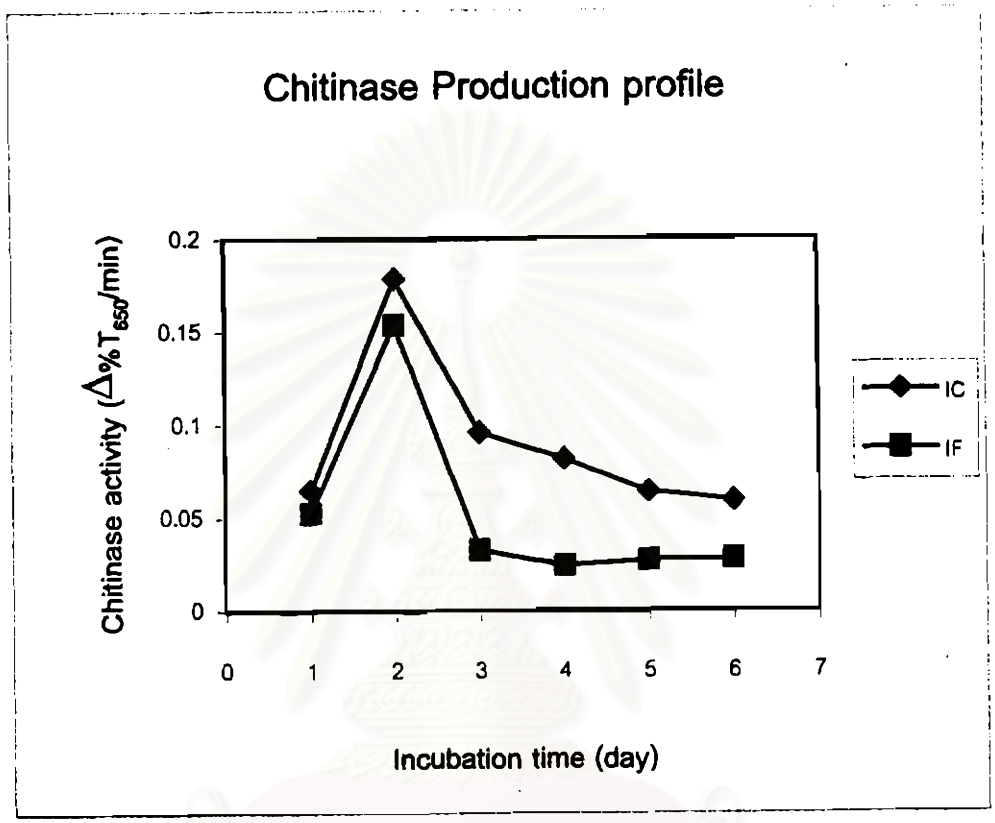

Figure 9 Profile of Chitinase Production, from *Burkholderia cepacia*; IC shows chitinase production in cultured colloidal chitin (0.2%) minimum medium ,and IF shows chitinase production in cultured flake chitin (20% W/V) minimum medium.

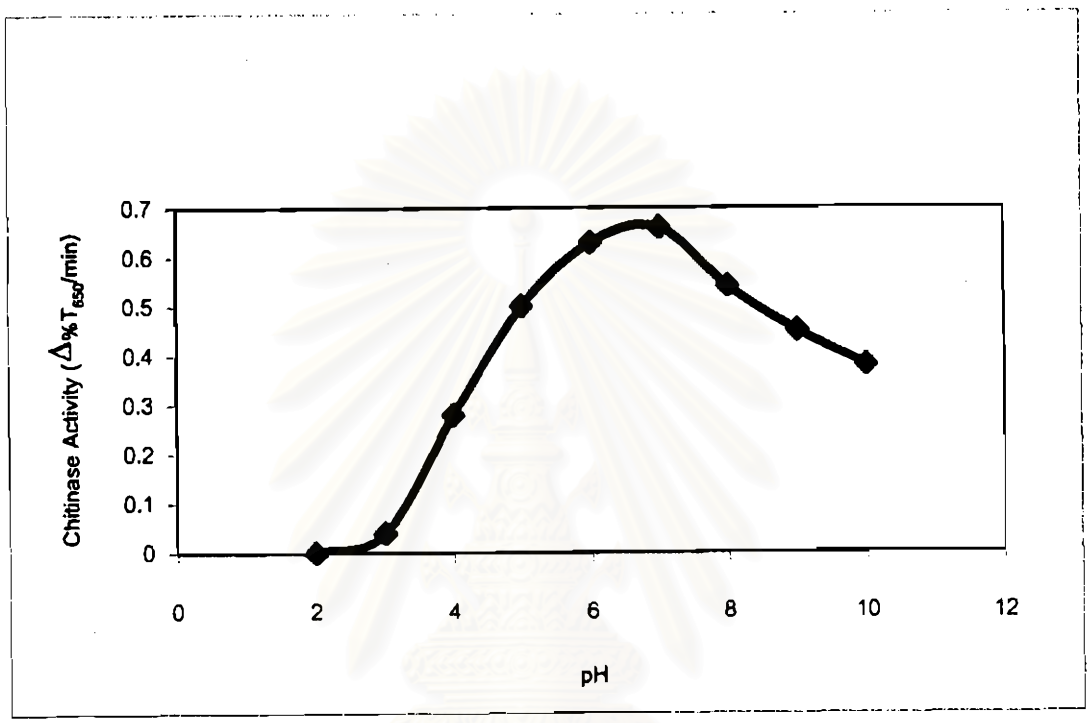

Figure 10 Effect of pH on chitinase activities. Chitinase activity was measured at pH range from 2 – 10, using colloidal chitin as substrate under conditions described in 2.10.1 (materials and methods)
Buffer: pH 2.0-6.0 and 7.0, 0.1 M sodium citrate buffer
pH 6.5-7.5, 0.1 M phosphate buffer
pH 7.0 and 8.0-10.0, 0.1 M Tris-HCl buffer

The results were shown in Figure 11. The highest chitinase activity was detected at 40 °C.

C. Substrate specificity

An action of chitinase on chitin and its related compounds was study in this work. The hydrolysis of chitin and its related compounds with chitinase was examine at pH7.0, 40 °C. The chitinase activities were assayed by determination of reducing sugar, described in 2.7.2 (material and method). The results were shown in Table 4. Chitinase was able to hydrolyze colloidal chitin and glycol chitin well, followed by flake chitin and crab shells. The enzyme can also use 78% and 90% deacetylated chitosan as substrate, 78% deacetylated chitosan was a better substrate than 90% deacetylated chitosan.

D. Estimation of molecular weight

The molecular weight of chitinase was estimated by SDS-polyacrylamide gel electrophoresis followed by activity staining of chitinase. As shown in Figure 13, there were several protein bands in SDS-PAGE, stained with Coomassie Brilliant Blue R-250. After staining chitinase activity, only one protein band, approximately 47.5 kDa for molecular weight was observed on SDS-PAGE.

Detection of Chitinase gene by Southern blot Analysis

DNA was extracted from *Burkholderia cepacia* using procedure as described in 2.11 (material and method). The A_{260}/A_{280} ratio of extracted DNA was 2.18 indicating high purity of DNA. The extracted DNA was completely digested with *Eco*R I, *Bam*H I, *Pst* I or *Hin*d III and subjected to agarose gel electrophoresis. The result was shown in Figure 14A.

To detect the DNA fragment containing chitinase gene by Southern blot analysis, [α-^{35}S]-dATP labeled standard DNA marker (λ/*Hind* III) and chromosomal DNA completely digested with *Eco*R I, *Bam*H I, *Pst* I and *Hin*d III on agarose gel were transferred to nylon membrane by capillary transfer method (Southern, 1975).

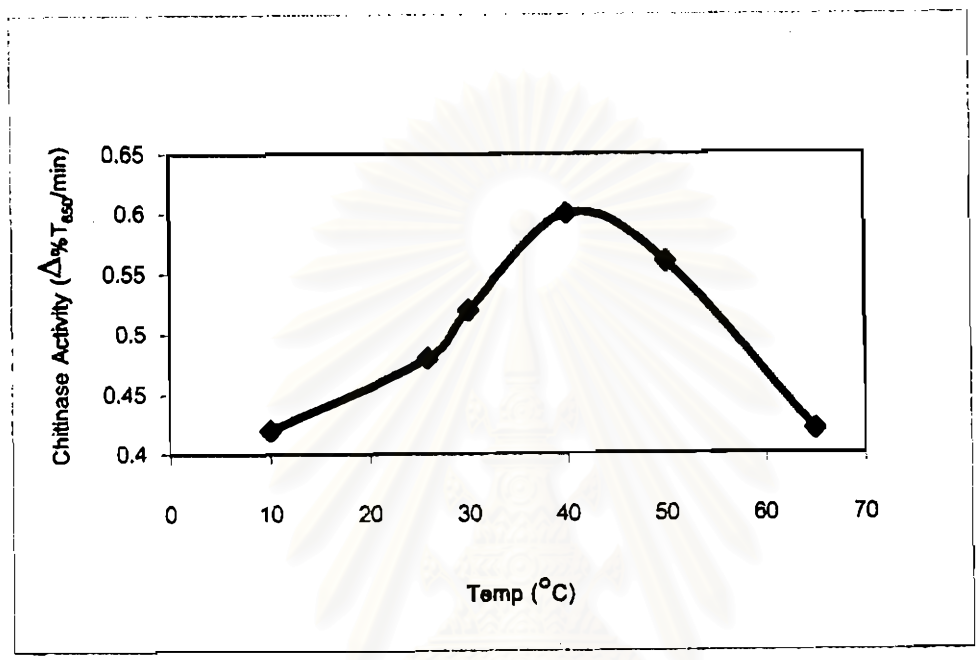

Figure 11 Effect of temperature on chitinase activities. Chitinase activities were measured at 10°, 27°, 30°, 40°, 50°, and 65° C in 0.1 M citrate buffer (pH 7.0) by using colloidal chitin as substrate under conditions that described in 2.10.2 (materials and methods)

Table 4 Hydrolysis of chitin and its related compounds with chitinase.

The reaction mixture (2.0 ml) consisting of 0.2 g/ml of various compounds as substrate and 0.06 units of crude enzyme in 0.2 M citrate buffer, pH 7.0 was incubated with shaking at 37 °C for 1 hour.

Compounds	Reducing sugar produced (μmole/hr)	Relative activity
Colloidal chitin	3.52	100
Glycol chitin	3.06	87
Flake chitin	0.27	8
Crab shells	0.24	7
78% Deacetylated chitosan	0.16	5
90% Deacetylated chitosan	0.07	2

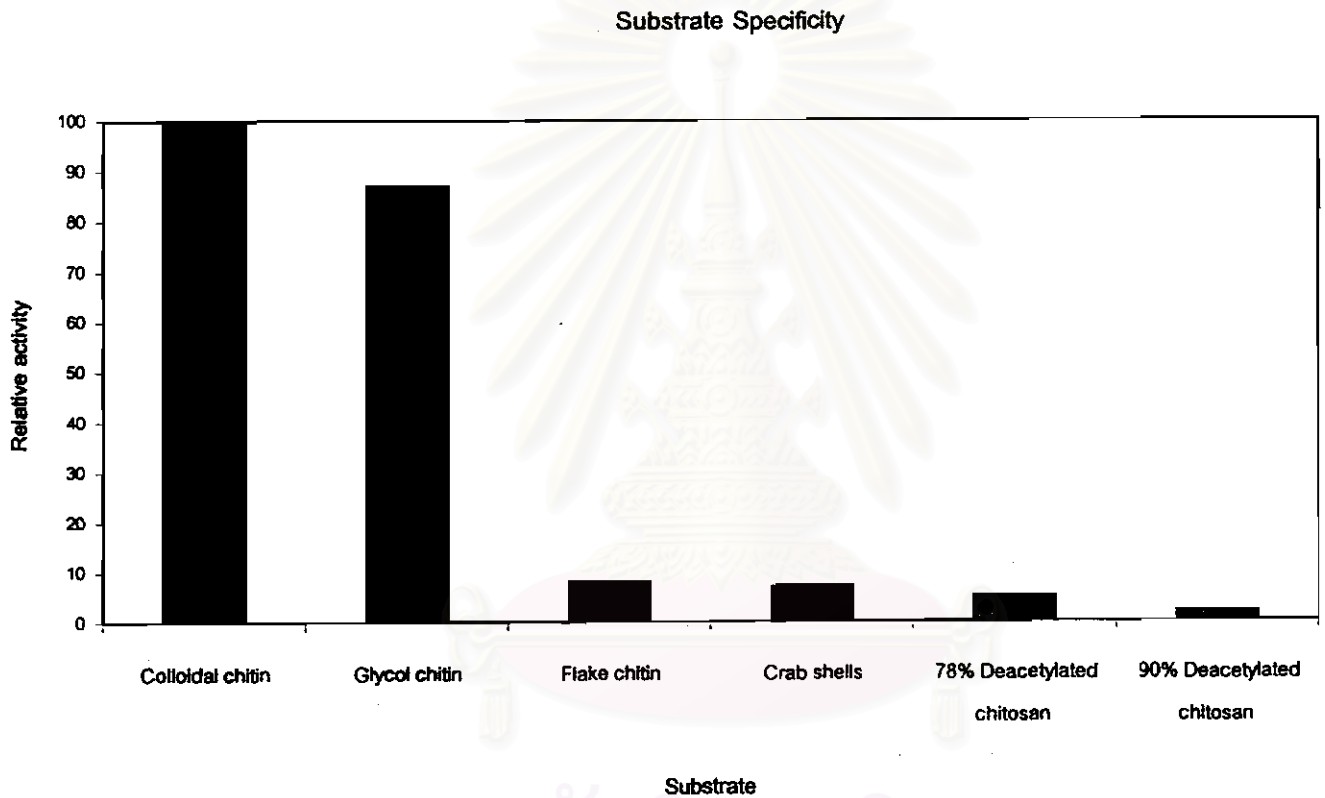

Figure 12 Substrate specificity of crude chitinase from *Burkholderia cepacia*

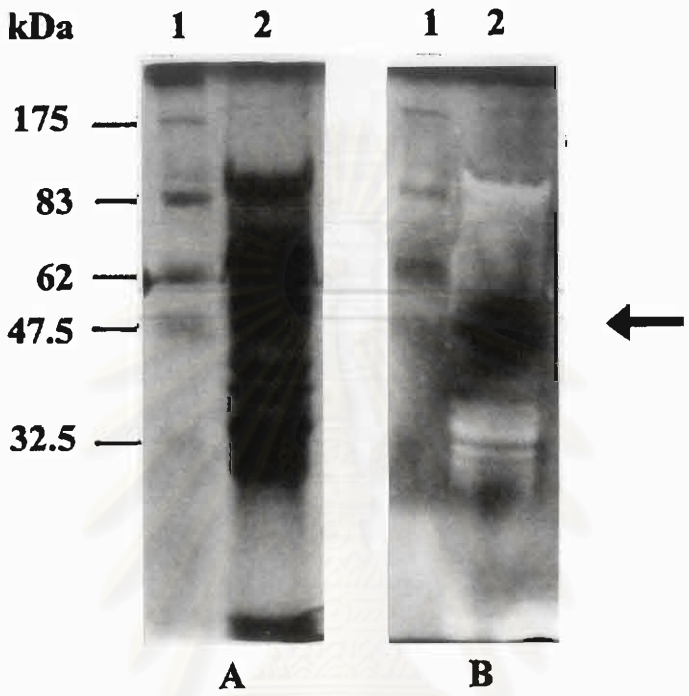

Figure 13 SDS-PAGE of Chitinase from *Burkhloderia cepacia*
Panel A (Coomassie Brilliant Blue R-250 staining), lane 1 shows protein marker and lane 2 shows crude protein in *Burkholderia cepacia*'s cultured medium)
Panel B (Chitinase activity staining), lane 1 shows protein marker and lane 2 shows chitinase in *Burkholderia cepacia*'s cultured medium.
Arrow shows chitinase activity band

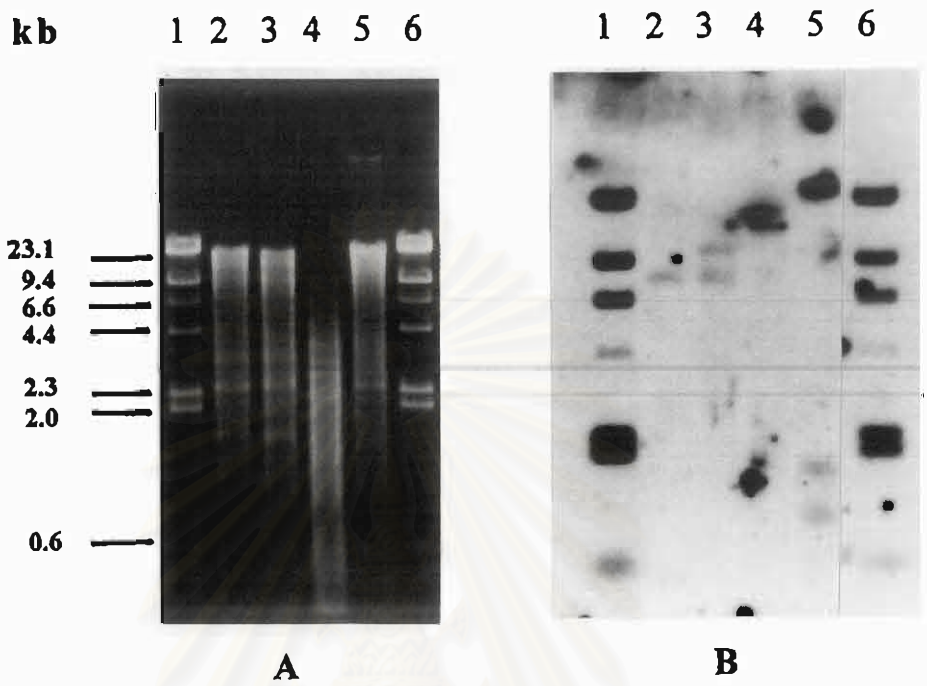

Figure 14 Detection of chitinase gene from *Burkholderia cepacia* by Southern blot analysis

Panel A: Analysis of completely digested chromosomal DNA from *Burkholderia cepacia* with *Eco*R I, *Bam*H I, *Pst* I or *Hin*d III on 1.0 % agarose gel electrophoresis. Standard DNA marker (λ/*Hin*d III) was loaded onto lane 1 and 6. Chromosomal DNA completely digested with *Eco*R I, *Bam*H I, *Pst* I or *Hin*d III was presented on lane 2-5, respectively.

Panel B: Southern blot analysis of *Burkholderia cepacia.* Standard DNA marker (λ/*Hin*d III) labeled with [α-^{35}S]-dATP was shown in lane 1 and 6. Chromosomal DNA completely digested with *Eco*R I, *Bam*H I, *Hin*d III and *Pst* I was in lane 2-5, respectively then probe with CB1.

Nylon membrane containing the target DNA was prehybridized in prehybridization solution at 65 °C and then hybridized with [γ-^{32}P] labeled degenerate probe specific for family 18 chitinase gene, CB1 in 6xSSC at 50 °C overnight. After that, nylon membrane was washed in 2xSSC and 0.2% SDS at 50 °C. The membrane, covered with Saran Wrap, was exposed to Kodak X-Omat film to obtain autoradiographic image.

Shown in Figure 14B, after Southern blot hybridization with the specific probe for chitinase gene, the positive signals were detected on the blot. Southern blot analysis showed 7.0 kb band of *Eco*R I digested chromosomal DNA, 7.8 kb and 10 kb bands of *Bam*H I digested chromosomal DNA and 1.8 kb and 1.2 kb bands of *Pst* I digested chromosomal DNA.

DNA Cloning

Chromosomal DNA from *Burkholderia cepacia* was completely digested with *Eco*R I or *Bam*H I, and then subjected to agarose gel electrophoresis. *Eco*R I digested DNA fragments between 6 to 9 kb and *Bam*H I digested DNA fragments between 6 to 12 kb were harvested by using QIAquick Gel extraction Kit. The pBluescript II KS$^+$, digested with *Eco*R I or *Bam*H I and dephosphorylated by alkaline phosphatase, was used as a cloning vector. Harvested *Eco*R I digested DNA fragments, 6 to 9 kb, and *Bam*H I digested DNA fragments, 6 to 12 kb, were ligated with pBluescript II KS+ digested with *Eco*R I or *Bam*H I, respectively. The ligation products were transformed into host cell *E.coli* strain DH5α F′ by electro-transformation. About 500 white colonies were found on LB agar plate containing ampicillin, IPTG and X-gal. These transformants were picked and screened for chitinase gene.

Detection of transformants containing chitinase gene

A. Southern blot hybridization

Plasmids from approximately 500 colonies of transformants were extracted and blotted onto nylon membrane and then hybridize with DNA probe, CB1. Plasmids which gave positive signal were digested with

*Eco*R I or *Bam*H I and *Pst* I and hybridized with CB1 probe. pKK243B gave a positive result on dot blot analysis (data not shown). Southern blot analysis of pKK243B showed hybridized bands at 7.8 kb and 1.8 kb when pKK243B was digested with *Bam*H I and *Pst* I respectively (Figure 15).

B. Phenotype screening

The colonies containing pKK243B and pKK1.8PP which was generated from ligation of *Pst* I digested pBluescript II KS^+ and 1.8 kb *Pst* I digested pKK243B fragment which gave a positive result on Southern blot analysis (see on plasmid map) were streaked onto LB-glycol chitin agar to detect the chitinase activity. The colonies containing pKK243B gave a positive result by forming of a visible clearing zone around the colony when staining with Congo red (shown in Figure 16).

Determination of restriction sites of recombinant plasmid containing Chitinase Gene (pKK243B)

pKK243B was digested with various restriction enzyme and then subjected to agarose gel electrophoresis to determined the positions of restriction sites on the plasmid. There is no recognition site in the 7.8 kb insert of pKK243B for the following restriction enzymes : *Bgl* II, *Kpn* I, *Spe* I, *Nde* I, *Sma* I and *Xba* I. The restriction map is shown in Figure 17.

pKK1.8PP was created by ligation of 1.8 kb *Pst* I digested pKK243B fragment which gave a positive result on Southern blot analysis and *Pst* I digested pBluescript II KS^+.

Detection of Chitinase activity in recombinant colonies

Colonies containing pKK243B and p1.8PP were cultivated in LB-colloidal chitin at 37°C with 250 rpm rotary shaking overnight. Crude chitinase in cultured medium was assayed by reducing sugar-producing activity assay. *Burkholderia cepacia* and *E.coli* DH5α F′ containing pBluescript II KS^+ was used as a positive and negative control, respectively. The results were shown in Table 5.

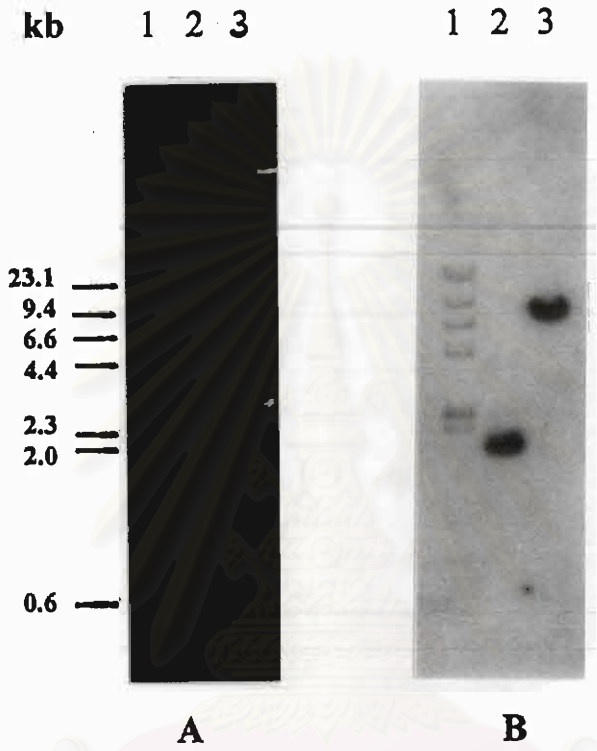

Figure 15 Detection of chitinase gene from transformant containing pKK243B

Panel A: Agarose gel Electrophoresis of pKK243B. Standard DNA marker (λ/*Hin*d III) was loaded onto lane 1, followed by pKK243B digested with *Pst* I and pKK243B digested with *Bam*H I in lane 2 and 3, respectively.

Panel B: Southern blot analysis of pKK243B. Standard DNA marker (λ/*Hin*d III) labeled with α-^{35}S]-dATP was shown in lane 1, followed by pKK243B digested with *Pst* I and pKK243B digested with *Bam*H I in lane 2 and 3, respectively then probe with CB1.

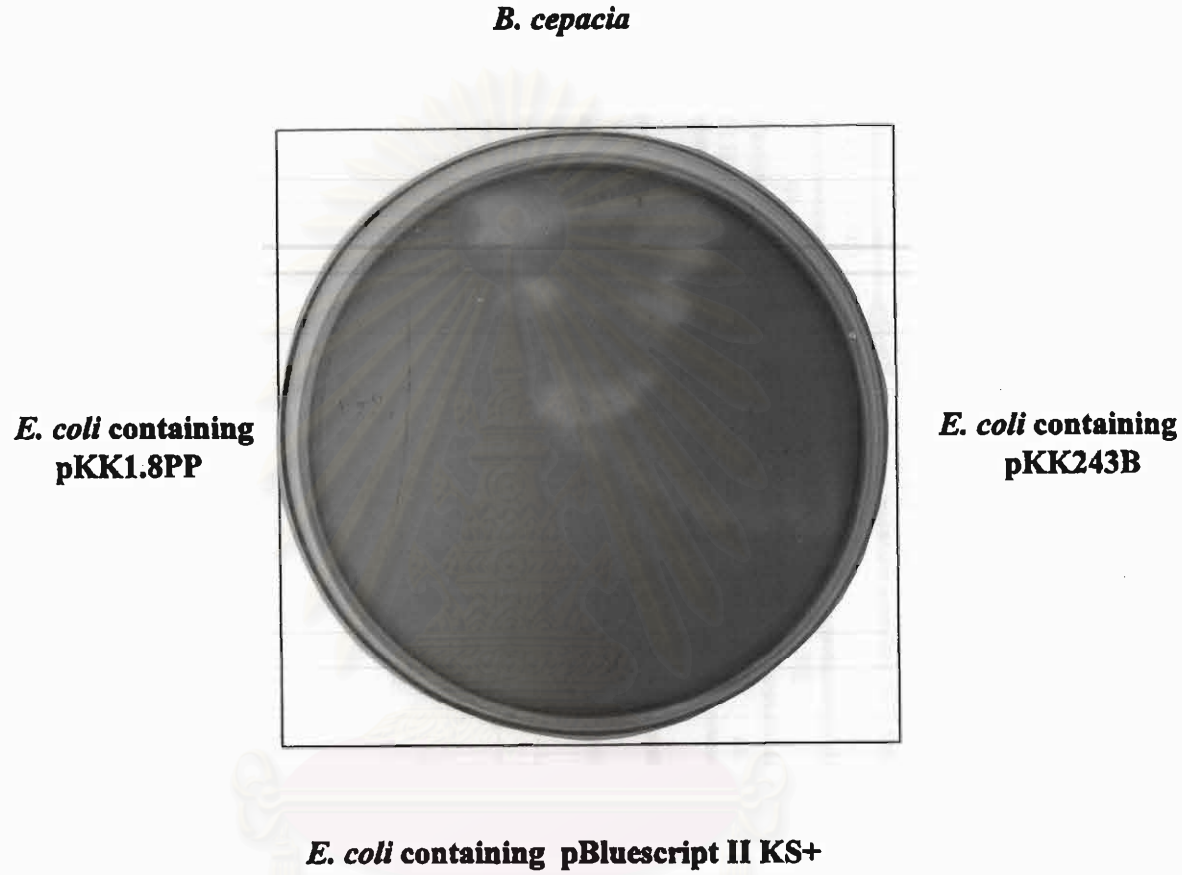

Figure 16 Colonies containing pKK243B and pKK1.8PP on LB-glycol chitin plate stained with Congo red. *Burkholderia cepacia* was used as a positive control, while *E.coli* DH5α F′ containing pBluescript II KS+ was used as a negative control.

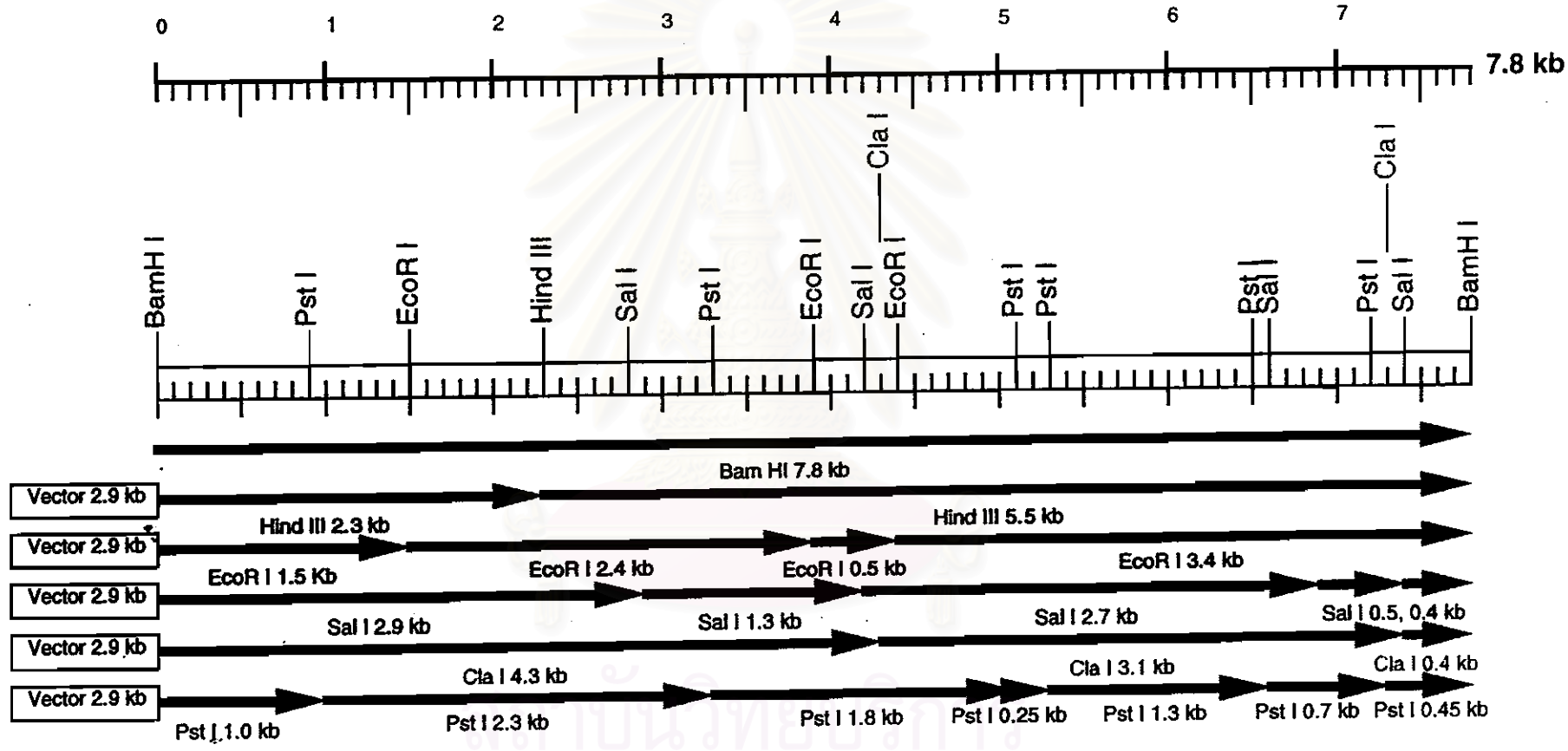

Figure 17 Restriction map of 7.8 kb insert fragment from pKK243B

Table 5 Chitinase activity in cultured LB-colloidal chitin medium.

Cell culture	Reducing sugar produced (μmole/hr)	Relative activity
Burkholderia cepacia	0.052	100
E. coli containing pKK243B	0.017	33
E. coli containing pKK243B (re-transformation)	0.022	42
E. coli containing pKK1.8PP	0.005	10
E. coli containing pBluescript KS+	0	0

Analysis of chitinase gene

pKK1.0PS and pKK0.8PS were generated from pKK1.8PP for sequencing, by method of Sanger *et al.* (1997). pKK1.0PS was generated by digestion of pKK1.8PP with *Sal* I, and then re-ligate. pKK0.8PS was constructed by ligation of 0.8 kb *Sal* I-*Pst* I digested pKK1.8PP fragment with pBluescript II KS^+ digested with the same enzymes. Nucleotide sequence of pKK1.8PP was shown in Figure 18.

Nucleotide sequence of pKK1.8PP ,1706 bp, was translated 6-phase into amino acid sequence. An open reading frame of 206 amino acid translated from 1-618 nucleotide sequence and 244 amino acid translated from 618-1349 nucleotide sequence were found. The deduced amino acid sequences were compared with protein sequences in the Genbank Database. The open reading frame of 206 amino acid is similar to amino acid sequence of putative sensor proteins (Blattner *et al.*, 1997; Fakhr *et al.*, 1999; Goethel *et al.*, 1997; Kunst *et al.*, 1997; Presecan *et al.*, 1997). The reading frame of 244 amino acid sequence shares similarity with amino acid sequence of putative 2-component transcriptional regulator (Blattner *et al.*, 1997; Goethel *et al.*, 1997; Lange *et al.*, 1999; Smith *et al.*,1997). No open reading frame which gives comparable amino acid sequence with chitinase was found in pKK1.8PP.

```
                1 →
1     CTGCAGAGCA  AGATTAACCC  GCACTTCCTG  TTCAACGCGC  TGAACGCCAT    50
51    CTCTTCCTCC  ATCCGCCTGA  ATCCGGACAC  CGCGCGCCAG  CTGATCATCA   100
101   ACCTGTCGCG  CTACCTGCGC  TACAACCTGG  AGCTGAACGA  CGAACTGATC   150
151   GATATCCGTA  AGGAACTGCA  TCAGATTCAG  GACTATATCG  CCATCGAACA   200
201   GGCGCGCTTC  GGCGCCAAGC  TGACGGTGAT  TTACGACATC  GACGACGACG   250
251   TCTCGGTGCG  CATTCCGAGC  CTGCTGATCC  AGCCGCTGGT  GGAGAACGCC   300
301   ATCGTGCACG  GCATCCAGCC  CTGCAAGGGT  AAAGGGGTGG  TGGTGATCGC   350
351   GGTGAAAGAT  CAGGGCGATC  GGGTGAAGAT  TTCGGTGAAG  GATACCGGCC   400
401   ACGGCATCAA  CCAGGAAACC  ATCGACCGGG  TGGCGCGCAA  CGAGATGCCG   450
451   GGCCACAATA  TCGGCCTGCT  CAACGTGCAT  CACCGCGTGT  CGCTGCTGTA   500
501   CGGTGAAGGG  TTGCATATCC  GCCGCCTGGA  GCCGGGCACC  GAAATCGCGT   550
551   TCTATATCAG  CAAAAACGGC  GGCAAGCTGC  ATCAGGAACC  GAGCGCGCCG   600
                2 →
601   CCGGTCGGGG  AGGCCTCATG  AAAGCTATCA  TCGTGGAAGA  CGAATTCCTC   650
651   GCGCAGGAAG  AACTCAGCTA  CCTGATCAAG  AAACACAGCA  ATATCGATAT   700
701   CATCGCTACC  TTCGAGGACG  GCCTCGACGT  TCTGAAATAC  CTGCAAACCC   750
751   ACCAGGTCGA  CGCCATTTTT  CTCGACATCA  ACATCCCGTC  GCTGGACGGC   800
801   GTGCTGCTGG  CGCAAAACAT  CAGCAAGTTC  GCCCATCGGC  CGTCTATCGT   850
851   GTTCATCACC  GCCTATAAAG  AACACGCGGT  GGAAGCCTTC  GAGATCGAGG   900
901   CGTTCGATCA  TATCCTCAAG  CCCTATCACG  AAGCGCGCAT  CGTCACCATG   950
951   TTGCAAAAAC  TGGAGGCGCT  GCATCATCGC  CCCGCCGGCG  CGGCGGAACC  1000
1001  GACCAGCGCG  CCGAGCCGCG  GCAGCCACAG  CATCAACCTG  ATCAAAGACG  1050
1051  AGCGGATCAT  CGTTACCGAC  ATCAACGACA  TCTATTACGC  CGCCGCCGAT  1100
1101  GAAAAGGTGA  CGCGGGTCTA  TACTCGCCGG  GAAGAATTCG  TGATGCCGAT  1150
1151  GAATCTCACC  GAGTTTTACG  GCCGGCTGCC  GGAAGAGCAT  TTCTTCCGCT  1200
1201  GCCACCGCTC  TTACTGCGTT  AACCTGGCCA  AGATCCGCGA  GATCGTGCCC  1250
1251  TGGTTCAACA  ATACCTACAT  TCTGCGGCTG  AGCGATCTTG  AGTTTGAAGT  1300
1301  GCCGGTCAGC  CGCAGCAAGG  TGAAAGAATT  TCGCAAGCTG  ATGCGCCTGT  1350
1351  AAGCGCTTAC  CAGCGCGGGC  CCGGACGGTA  GTAATAGCCG  GCGGCGGCGG  1400
1401  TGGCGGCATG  GCGGTAATGC  GGCCGATCTC  GCCAGTCGCG  CCGCGGCGGC  1450
1451  CCGTAGTAAA  TCACCCGTGG  CGGCGGGCCG  TAATAGTACC  CGCGCGAACG  1500
1501  TTCATACCGG  TGATGATCGG  CCCACCAGCG  CGGATCGCGC  CAGCGATAGC  1550
1551  CGTCCCAGTA  GTGGCCGCGA  TGATCGCGGT  CGCCGATATG  CAACGACAGG  1600
1601  CCCGGCACGT  TCACGCCGAT  GGACACGTCG  GCCTGGCTCG  CCAGCGGCAG  1650
1651  CGCCAGCAGC  GCAGCCAGTA  ACAACAGCGT  TTTTTTCATT  TCATGGACTC  1700
1701  CTGCAG                                                    1750
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Figure 18 Nucleotide sequence of 1.7 kb insert fragment of pKK1.8PP. Arrows indicate the ORF and the direction of transcription of partial sequence of putative sensor protein, 1, and complete sequence of putative 2-component transcription regulator, 2. Underlined nucleotides shows translation start codon, bold type nucleotides show translation stop codon.

```
1/1                                                 31/11
CTG CAG AGC AAG ATT AAC CCG CAC TTC CTG TTC AAC GCG CTG AAC GCC ATC TCT TCC TCC
L   Q   S   K   I   N   P   H   F   L   F   N   A   L   N   A   I   S   S   S
61/21                                               91/31
ATC CGC CTG AAT CCG GAC ACC GCG CGC CAG CTG ATC ATC AAC CTG TCG CGC TAC CTG CGC
I   R   L   N   P   D   T   A   R   Q   L   I   I   N   L   S   R   Y   L   R
121/41                                              151/51
TAC AAC CTG GAG CTG AAC GAC GAA CTG ATC GAT ATC CGT AAG GAA CTG CAT CAG ATT CAG
Y   N   L   E   L   N   D   E   L   I   D   I   R   K   E   L   H   Q   I   Q
181/61                                              211/71
GAC TAT ATC GCC ATC GAA CAG GCG CGC TTC GGC GCC AAG CTG ACG GTG ATT TAC GAC ATC
D   Y   I   A   I   E   Q   A   R   F   G   A   K   L   T   V   I   Y   D   I
241/81                                              271/91
GAC GAC GAC GTC TCG GTG CGC ATT CCG AGC CTG CTG ATC CAG CCG CTG GTG GAG AAC GCC
D   D   D   V   S   V   R   I   P   S   L   L   I   Q   P   L   V   E   N   A
301/101                                             331/111
ATC GTG CAC GGC ATC CAG CCC TGC AAG GGT AAA GGG GTG GTG GTG ATC GCG GTG AAA GAT
I   V   H   G   I   Q   P   C   K   G   K   G   V   V   V   I   A   V   K   D
361/121                                             391/131
CAG GGC GAT CGG GTG AAG ATT TCG GTG AAG GAT ACC GGC CAC GGC ATC AAC CAG GAA ACC
Q   G   D   R   V   K   I   S   V   K   D   T   G   H   G   I   N   Q   E   T
421/141                                             451/151
ATC GAC CGG GTG GCG CGC AAC GAG ATG CCG GGC CAC AAT ATC GGC CTG CTC AAC GTG CAT
I   D   R   V   A   R   N   E   M   P   G   H   N   I   G   L   L   N   V   H
481/161                                             511/171
CAC CGC GTG TCG CTG CTG TAC GGT GAA GGG TTG CAT ATC CGC CGC CTG GAG CCG GGC ACC
H   R   V   S   L   L   Y   G   E   G   L   H   I   R   R   L   E   P   G   T
541/181                                             571/191
GAA ATC GCG TTC TAT ATC AGC AAA AAC GGC GGC AAG CTG CAT CAG GAA CCG AGC GCG CCG
E   I   A   F   Y   I   S   K   N   G   G   K   L   H   Q   E   P   S   A   P
601/201
CCG GTC GGG GAG GCC TCA TGA
P   V   G   E   A   S   *
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Figure 19 Deduced partial amino acid sequence of putative sensor protein from pKK1.8PP

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618/1                                                   648/11
ATG AAA GCT ATC ATC GTG GAA GAC GAA TTC CTC GCG CAG GAA GAA CTC AGC TAC CTG ATC
M   K   A   I   I   V   E   D   E   F   L   A   Q   E   E   L   S   Y   L   I
678/21                                                  708/31
AAG AAA CAC AGC AAT ATC GAT ATC ATC GCT ACC TTC GAG GAC GGC CTC GAC GTT CTG AAA
K   K   H   S   N   I   D   I   I   A   T   F   E   D   G   L   D   V   L   K
738/41                                                  768/51
TAC CTG CAA ACC CAC CAG GTC GAC GCC ATT TTT CTC GAC ATC AAC ATC CCG TCG CTG GAC
Y   L   Q   T   H   Q   V   D   A   I   F   L   D   I   N   I   P   S   L   D
798/61                                                  828/71
GGC GTG CTG CTG GCG CAA AAC ATC AGC AAG TTC GCC CAT CGG CCG TCT ATC GTG TTC ATC
G   V   L   L   A   Q   N   I   S   K   F   A   H   R   P   S   I   V   F   I
858/81                                                  888/91
ACC GCC TAT AAA GAA CAC GCG GTG GAA GCC TTC GAG ATC GAG GCG TTC GAC TAT ATC CTC
T   A   Y   K   E   H   A   V   E   A   F   E   I   E   A   F   D   Y   I   L
918/101                                                 948/111
AAG CCC TAT CAC GAA GCG CGC ATC GTC ACC ATG TTG CAA AAA CTG GAG GCG CTG CAT CAT
K   P   Y   H   E   A   R   I   V   T   M   L   Q   K   L   E   A   L   H   H
978/121                                                 1008/131
CGC CCC GCC GGC GCG GCG GAA CCG ACC AGC GCG CCG AGC CGC GGC AGC CAC AGC ATC AAC
R   P   A   G   A   A   E   P   T   S   A   P   S   R   G   S   H   S   I   N
1038/141                                                1068/151
CTG ATC AAA GAC GAG CGG ATC ATC GTT ACC GAC ATC AAC GAC ATC TAT TAC GCC GCC GCC
L   I   K   D   E   R   I   I   V   T   D   I   N   D   I   Y   Y   A   A   A
1098/161                                                1128/171
GAT GAA AAG GTG ACG CGG GTC TAT ACT CGC CGG GAA GAA TTC GTG ATG CCG ATG AAT CTC
D   E   K   V   T   R   V   Y   T   R   R   E   E   F   V   M   P   M   N   L
1158/181                                                1188/191
ACC GAG TTT TAC GGC CGG CTG CCG GAA GAG CAT TTC TTC CGC TGC CAC CGC TCT TAC TGC
T   E   F   Y   G   R   L   P   E   E   H   F   F   R   C   H   R   S   Y   C
1218/201                                                1248/211
GTT AAC CTG GCC AAG ATC CGC GAG ATC GTG CCC TGG TTC AAC AAT ACC TAC ATT CTG CGG
V   N   L   A   K   I   R   E   I   V   P   W   F   N   N   T   Y   I   L   R
1278/221                                                1308/231
CTG AGC GAT CTT GAG TTT GAA GTG CCG GTC AGC CGC AGC AAG GTG AAA GAA TTT CGC AAG
L   S   D   L   E   F   E   V   P   V   S   R   S   K   V   K   E   F   R   K
1338/241
CTG ATG CGC CTG TAA
L   M   R   L   *
```

Figure 20 Deduced amino acid sequence of putative transcriptional regulator from pKK1.8PP