CHAPTER III

RESULTS

1. Screening and isolation of bacteria producing chitinase

Bacteria TU05 was screened from soil in Pathumwan, Bangkok, Thailand. It showed a visible clear zone on 0.02% CCMM plate within 3 days. It indicated that bacteria TU05 can produced extracellular chitinase, which secreted from cell to degrade chitin. Then, TU05 was selected for this study.

2. Identification of selected strain

2.1 Gram-stain, colonial morphology and biochemical test

TU05 was cultivated at 30 °C with aerobic condition. It was grown as round colony with yellow pigments on CCMM plate. Clear zone was found around the colony in the third day (Figure 14). Gram-staining of TU05 showed that it was a gram-positive bacteria, non-motile, and non-spore forming. In rich medium, LB agar plate, the bacteria morphology was shown as small diphtheroid rod with round end in 1 day and became longer and palisade in 3 days. On the other hand, when grown in minimum medium, CCMM agar plate, its morphology appears as small diphtheroid rod with round end in 1 day and changed to coccoid form with age (Figure 15).

From biochemical test, TU05 produced weak acidic products from fermented carbohydrates and catalase positive. Biochemical characteristics were shown in Table 3.

2.2 Identification by 16S rRNA gene

The 16S rRNA gene of bacteria strain TU05 was amplified by PCR and ligated with pGEM-T easy vector (Figure 16). The genomic rRNA gene sequence (1622 bp) was shown in Figure 17. The genomic rRNA gene sequence was aligned and compared to 16S rRNA gene sequence of the other organisms in GenBank. The 16S rRNA gene of bacteria strain TU05 showed the highest sequence similarity to that of *Microbacterium keratanolyticum* (97%).

3. Chitinase production of *Microbacterium* sp. TU05

In 0.2% CCMM and 1% FCMM, as shown in Figure 18, the chitinase activities were measured by colorimetric method. The highest chitinase activity was found in 2 days in CCMM and 10 days in FCMM.

4. Characterization of crude chitinase

4.1 Effects of pH and temperature on the activity

The chitinase activity was measured at various pHs in the range of 2 to 10 with 3 types of buffer that contained overlapping pH. Chitinase activity at different pH was illustrated in Figure 19. We found chitinase activity in range of pH from 4 to 8 (more than 50% relative activity) with the highest chitinase activity, in citrate buffer pH 5.0 and in phosphate buffer pH 7.0, when colloidal chitin was used as substrate. The effect of temperature on crude chitinase activity was determined at pH 5.0 at different temperature from 20 °C to 70 °C for 1 hour. The optimum temperature for crude chitinase activity was 40 °C, as shown in Figure 20.

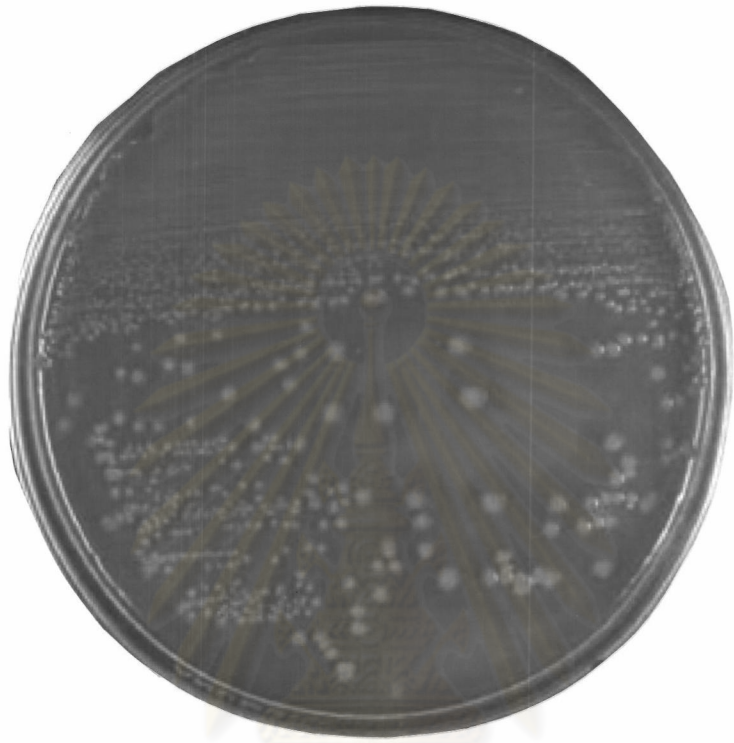


Figure 14 Colonial morphology of *Microbacterium* sp.TU05 on 0.02% CCMM agar plate. *Microbacterium* sp.TU05 was grown on 0.02 % CCMM at 30 °C with aerobic condition for 3 days, clear zone around colony was observed.

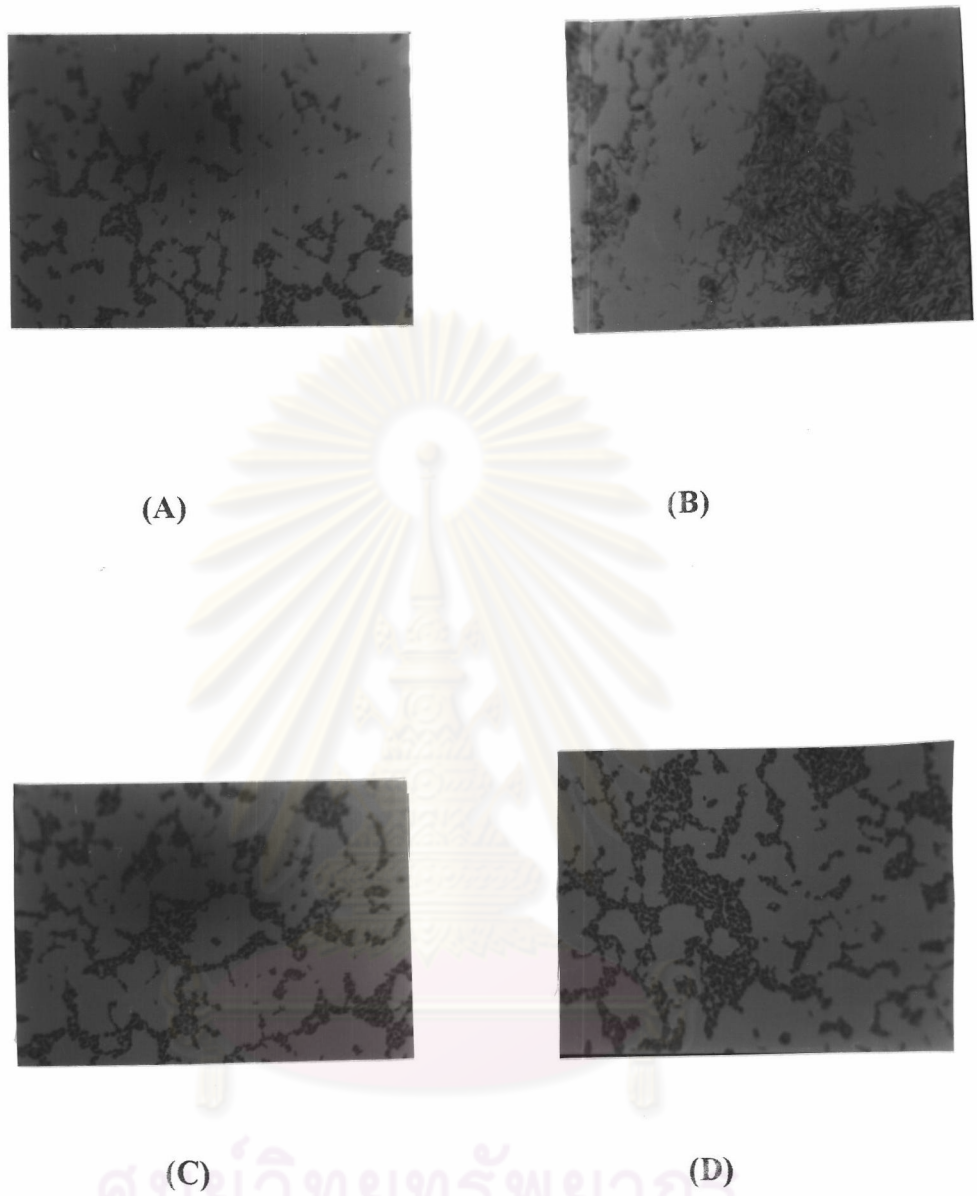


Figure 15 Gram-stain of selected bacteria at different cultivation time and medium. From gram-stain of TU05, it was gram-positive bacteria, non-motile, and non-spore forming. In rich medium, LB agar plate, (A) TU05 was small diphtheroid rod with round end in 1 day and (B) TU05 cell became longer and palisade in 3 days. On the other hand, in minimum medium, CCMM agar plate, (C) it was small diphtheroid rod with round end in 1 day and (D) it changed to coccoid form with age.

Table 3 Biochemical characteristics of selected strain

Biochemical characteristics
Catalase : positive
Acid from Glucose: positive
Sucrose: positive
Ribose: positive
Maltose: positive
Hydrolysis of aryl-substituted glycoside
p-Nitrophenyl-α,D-glucoside: positive
p-Nitrophenyl-β,D-glucoside: positive
p-Nitrophenyl-N-acetyl-β,D-glucosaminide: positive
p-Nitrophenyl-glycoside: positive
o-Nitrophenyl-β,D-galactoside: positive
p-Nitrophenyl phosphate: negative
Hydrolysis of fatty acid ester: negative
Hydrolysis of aryl-amide
Proline-β-naphthylamide: negative
Tryptophane-β-naphthylamide: positive
Pyrrolidine-β-naphthylamide: negative
Leucyl-glycine-β-naphthylamide: positive
Leucine-β-naphthylamide: negative
Hydrolysis of urea: negative
Hydrolysis of nitrate: negative

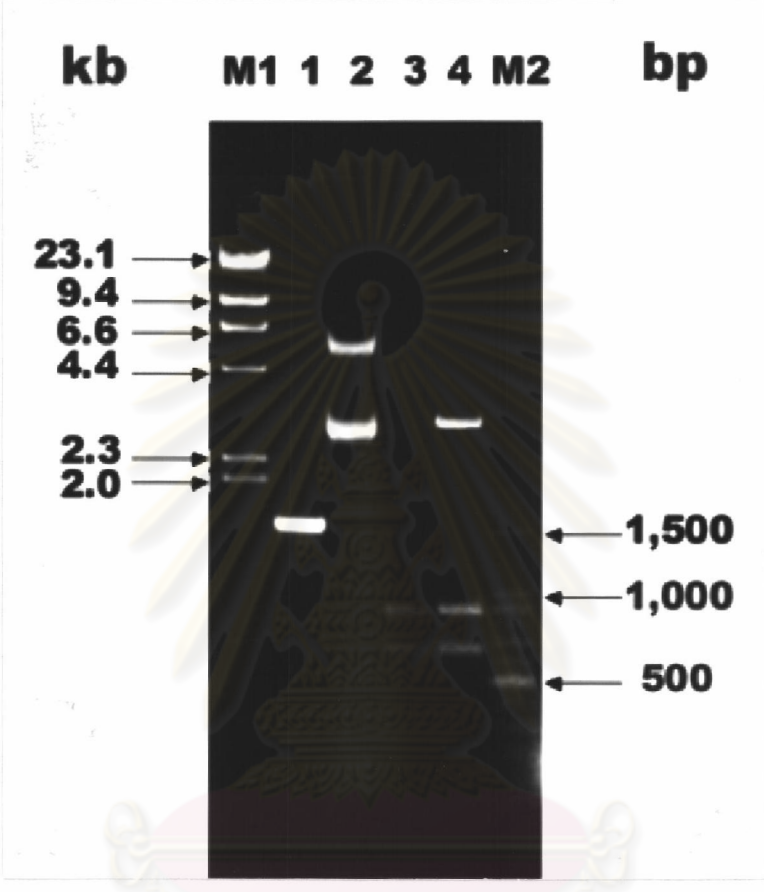


Figure 16 The ligated 16S rRNA gene analyze by 1.5% agarose gel electrophoresis.

Lane M1: λ/*Hind*III marker

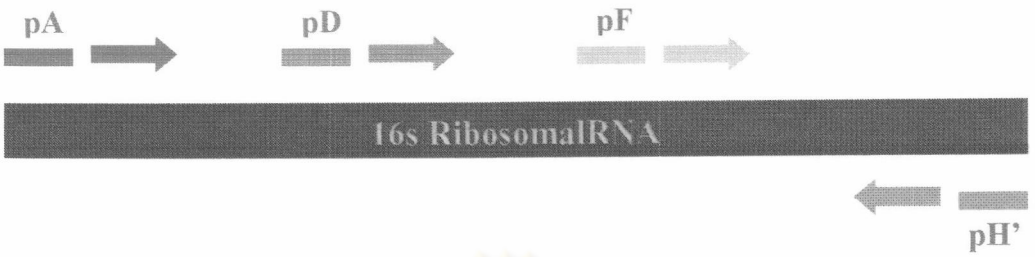
Lane 1: 16S rRNA (PCR product)

Lane 2: 16S rRNA ligated with pGEM-T easy vector

Lane 3: 16S rRNA cut with *Eco*RI

Lane 4: 16S rRNA ligated with pGEM-T easy vector cut with *Eco*RI

Lane M2: 100bp marker

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241  TGTTGGTGAG GTAATGGCTC ACCAAGGCGT CGACGGGTAG CCGGCCTGAG AGGGTGACCG  300
301  GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA GGCAGCAGTG GGGAATATTG  360
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421  AAACCTCTTT TAGTAGGGGA AGAAGCGAAA GTGACGGTAC CTGCAGAAAA AGCGCCGGCT  480
481  AACTACGTGC CAGCAGCCGC GGTAATACGT AGGGCGCAAG CGTTATCCGG AATTATTGGG  540
541  CGTAAAGAGC TCGTAGGCGG TTTGTCGCGT CTGCTGTGAA ATCTGGGGGC TCAACCCCCA  600
601  CCTGCAGTGG GTACCGGCAG ACTAAAGTGC CGTAGGGGAG ATTGGAATTC CTGGTGTAAC  660
661  CGTGGAATGC CCCAAATTTC AGGAGGAACA CCCGATGGCG AAGGCGGATC TTTTGGCCCT  720
721  AACTTGACCC TTACCAAGGC TTGACATATA CGAGACGGGC CCAGAAATGG TCAACTCTTT  780
781  GGACACTCGT AAACAGGTGG TGCATGGCTG TCGTCAGCTG GTGTCGTGAG ATGTTGGGTT  840
841  AAGTCCCGCA ACGAGCGCAA CCCTCGTTTT ATGTTGCCAG CACGTAATGG TGGGAACTCA  900
901  TGGGATACTG CCGGGGTCAA CTCGGAGAAG GTGGGGATGA CGTCAAATCA TCATGCCCCT  960
961  TATGTCTTGG GCTTCACGCA TGCTACAATG GCCGGTACAA AGGGCTGCAA TACCGCGAGG 1020
1021 TGGAGCGAAT CCCAAAAAGC CGGTCCCAGT TCGGATTGAG GTCTGCAACT CGACCTCATG 1080
1081 AAGTCGGAGT CGCTAGTAAT CGCAGATCAG CAACGCTGCG GTGAATACGT TCCCGGGTCT 1140
1141 TGTACACACC GCCCGTCAAG TCATGAAAGT CGGTAACACC TGAAGCCGGT GGCCTAACCC 1200
1201 TTGTGGAGGG AGCCGTCGAA GGTGGGATCG GTAATTAGGA CTAAGTCGTA ACAAGGTAGC 1260
1261 CGTACCGGAA GGTGCGGCTG GATCACCTCC TTTCAAATCA TCATGCCCCT TATGTCTTGG 1320
1321 GCTTCACGCA TGCTACAATG GCCGGTACAA AGGGCTGCAA TACCGCGAGG TGGAGCGAAT 1380
1381 CCCAAAAAGC CGGTCCCAGT TCGGATTGAG GTCTGCAACT CGACCTCATG AAGTCGGAGT 1440
1441 CGCTAGTAAT CGCAGATCAG CAACGCTGCG GTGAATACGT TCCCGGGTCT TGTACACACC 1500
1501 GCCCGTCAAG TCATGAAAGT CGGTAACACC TGAAGCCGGT GGCCTAACCC TTGTGGAGGG 1560
1561 AGCCGTCGAA GGTGGGATCG GTAATTAGGA CTAAGTCGTA ACAAGGTAGC CGTACCGGAA 1620
1621 GGTGCGGCTG GATCACCTCC TT                                          1642
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pA	5'	AGAGTTTGAT CCTGGCTCAG	3'
pD	5'	CAGCAGCCGC GGTAATAC	3'
pF	5'	TGCATGGCTGTCGTCAGCTGGT	3'
pH	5'	**AAGGAGGTGATCCAGCCGCA**	3'
Reverse&complementary	5'	TGCGGCTGGATCACCTCCTT	3'

EcoRI site inside 16sr RNA gene of *Microbacterium* sp. TU05: GAATTC

Figure 17 The 16S rRNA gene sequence of TU05

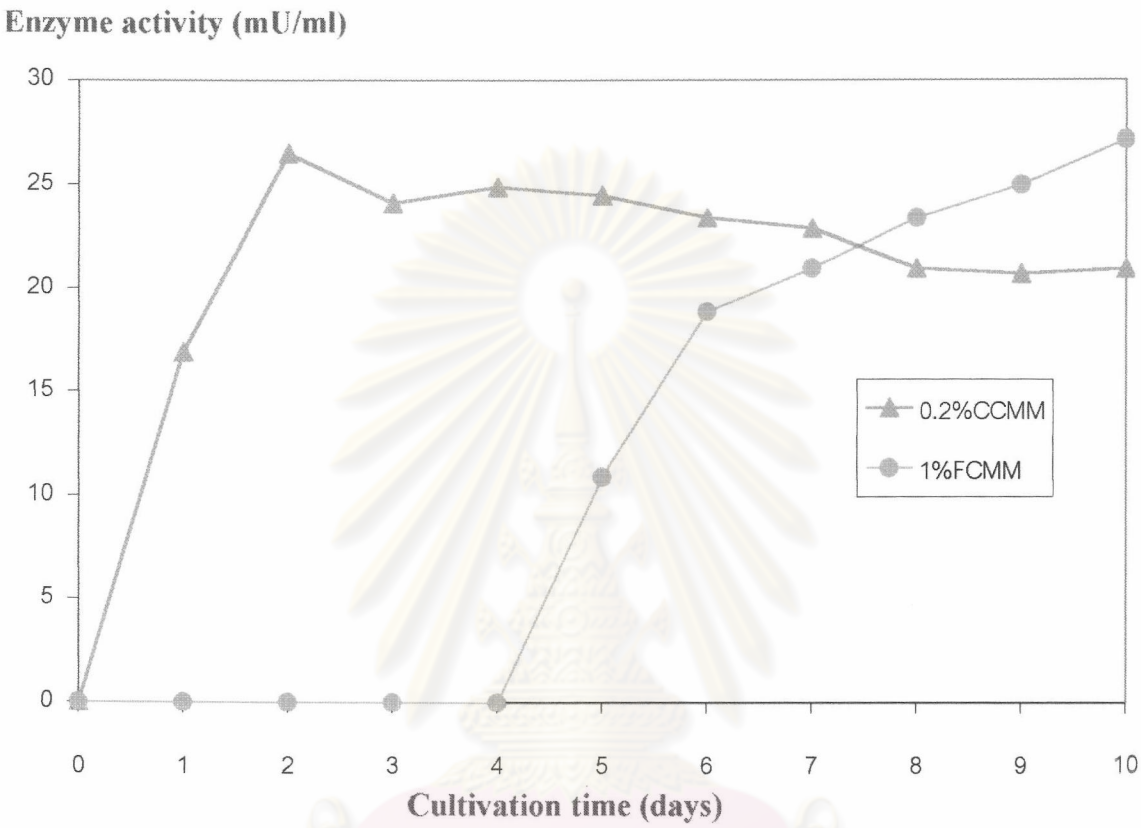

Figure 18 Production of chitinase from *Microbacterium* sp. TU05. Chitinase activity of crude chitinase from strain TU05 in 0.2% CCMM and 1% FCMM was assayed by colorimetric method using colloidal chitin as substrate at pH 7.0. Chitinase activity was checked everyday for 10 days.

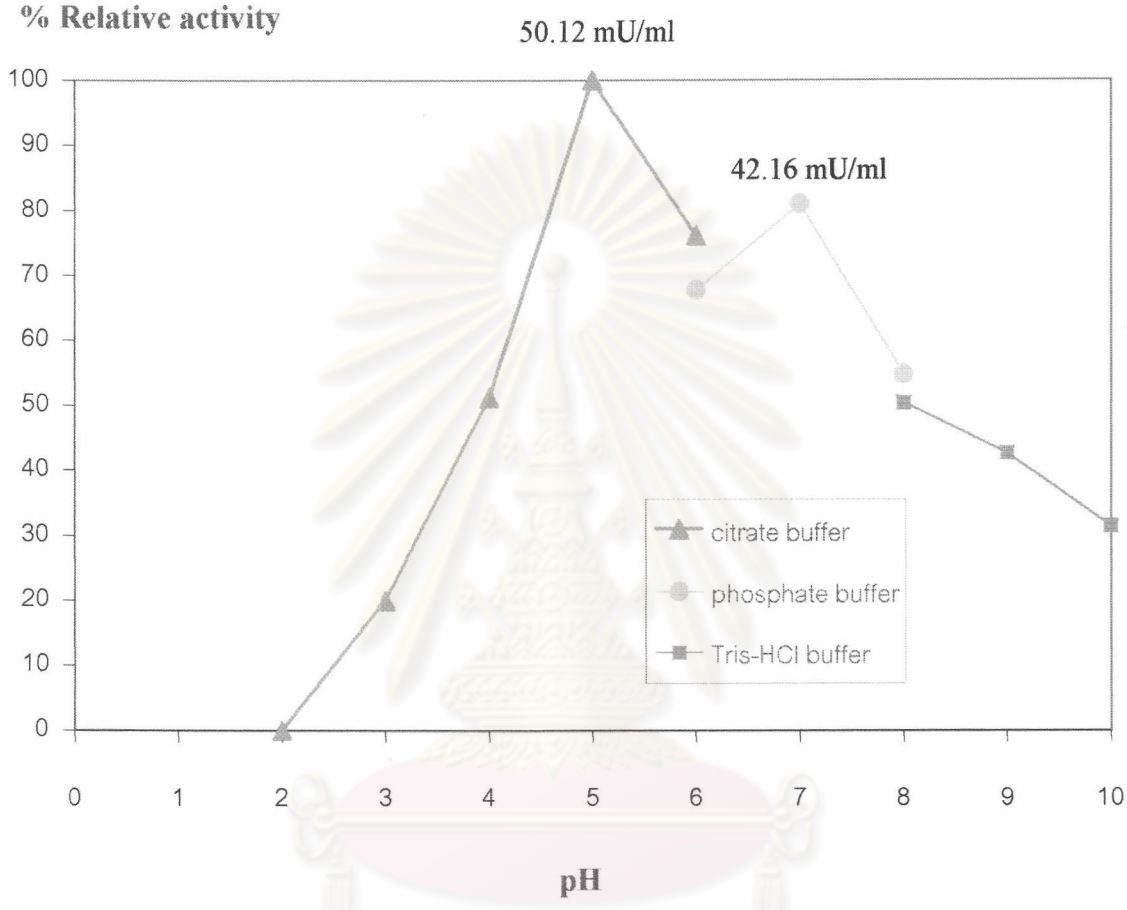

Figure 19 Effect of pH on crude chitinase activity. Chitinase activity was measured by colorimetric method at pH range of 2 to 10 at 37 ° C when colloidal chitin was used as substrate.

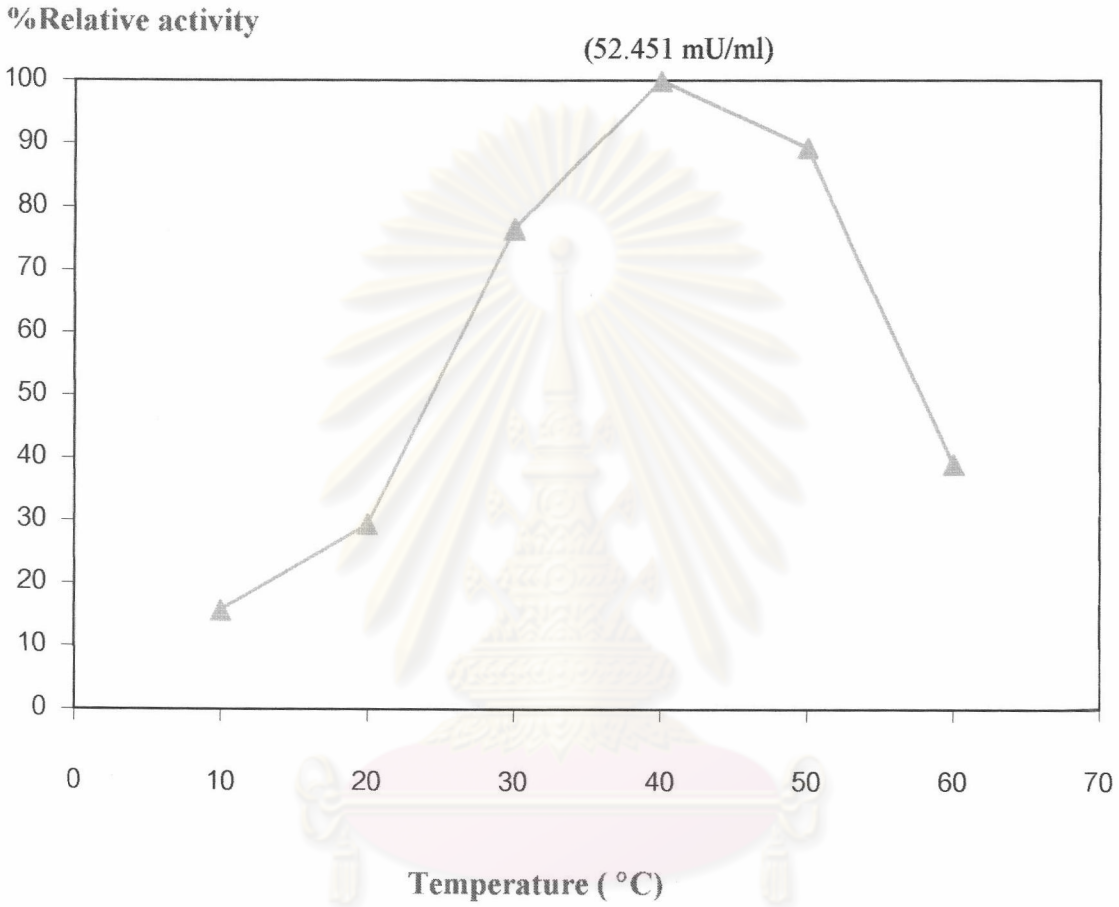

Figure 20 Effect of temperature on crude chitinase activity. Chitinase activity was measured by colorimetric method at 5.0 in temperature range 10 to 60 °C when colloidal chitin was used as substrate.

4.2 Substrate specificity

Chitinase activity on chitin related substrates was assayed by colorimetric method. The reactions were performed in 0.1M citrate buffer, pH5.0 at 40 °C. Crude chitinase, 0.1 unit, was used to hydrolyze10 mg/ml of substrate, including regenerated chitin (RC), colloidal chitin (CC), 45% deacetylated chitosan (45% DD), 80% deacetylated chitosan (80% DD), 90% deacetylated chitosan (90% DD), powder chitin (PC), and flaked chitin (FC). Crude chitinase showed high activity on colloidal chitin, 45% deacetylated chitosan, 80% deacetylated chitosan, 90% deacetylated chitosan, regenerated chitin, powder chitin, and flake chitin, respectively (Figure 21). In the group of amorphous chitin, colloidal chitin and regenerated chitin, the hydrolytic activity on colloidal chitin was higher than regenerated chitin. The crude enzyme has been found hydrolytic activity on chitosan, but the activity was decreased when percent deacetylation was in creased.

4.3 Estimation molecular weight of chitinase

Crude chitinase, containing chitinase and other extracellular proteins, was analyzed by SDS-PAGE, 10%separating gel with 0.01% glycol chitin. After activity staining, chitinase activity was observed at 65 and 30 kDa (Figure 22).

4.4 Hydrolysis products of colloidal chitin

Crude chitinase from *Microbacterium* sp. TU05 was used to digest colloidal chitin. Then the products were separated by HPLC (Figure 23). Two peaks of products was observed. From the retention time compared with the standard, they were identified as monomer and dimer of N-acetylglucosamine. This result suggested that in crude enzyme contained chitinase and chitobiase activity.

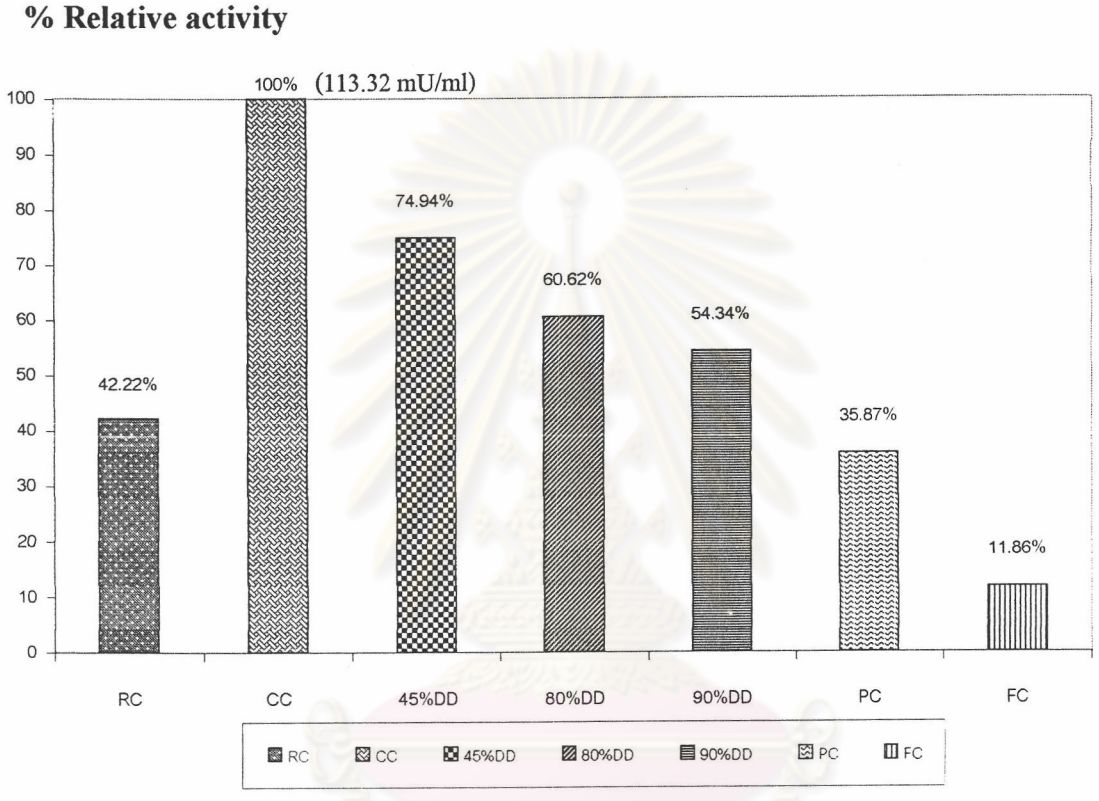

Figure 21 Substrate specificity of crude chitinase of *Microbacterium* sp. TU05. The reactions were done in 0.1M citrate buffer, pH5.0 at 40 °C. Crude chitinase, 0.1 unit, was used for hydrolyzed 10 mg/ml of substrates, including regenerated chitin (RC), colloidal chitin (CC), 45%deacetylated chitosan (45% DD), 80%deacetylated chitosan (80% DD), 90%deacetylated chitosan (90% DD), powder chitin (PC), and flaked chitin (FC).

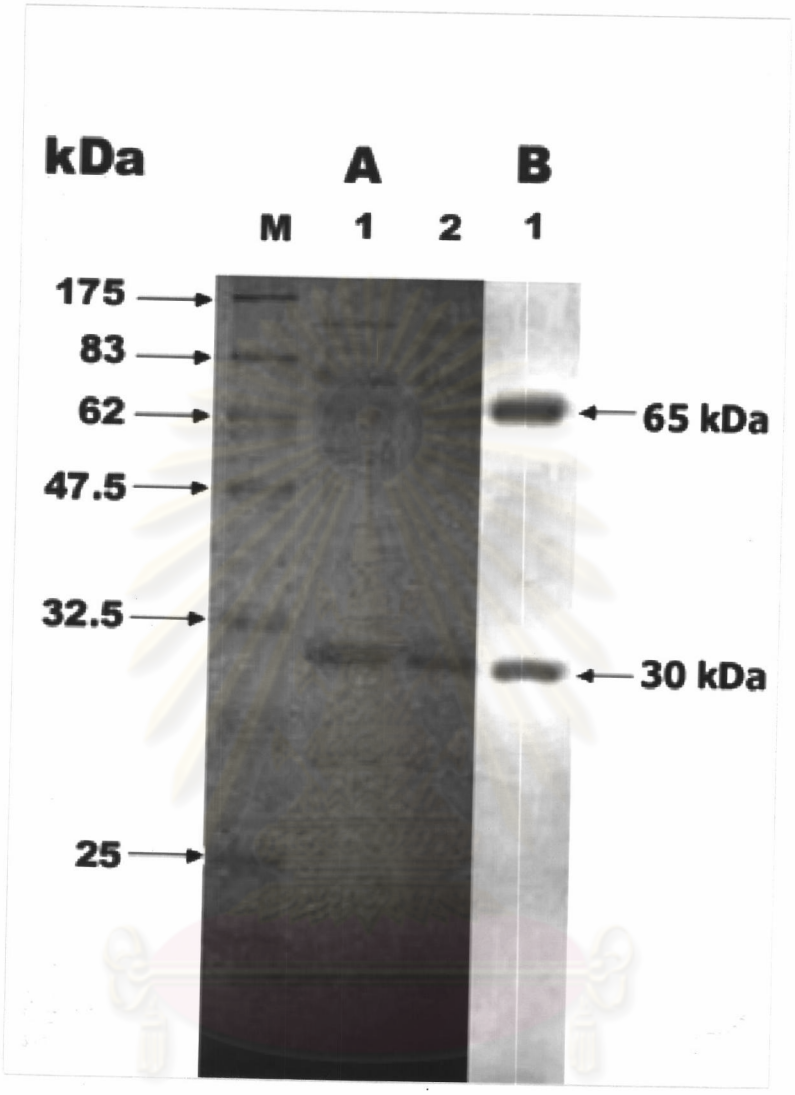

Figure 22 Detection of chitinase activity from crude enzyme of *Microbacterium* sp. TU05 by SDS-PAGE.

Panel A: Protein stain

Lane M: Protein marker

Lane 1: crude enzyme

Lane 2: crude enzyme

Panel B: Activity stain

Lane 1: crude enzyme

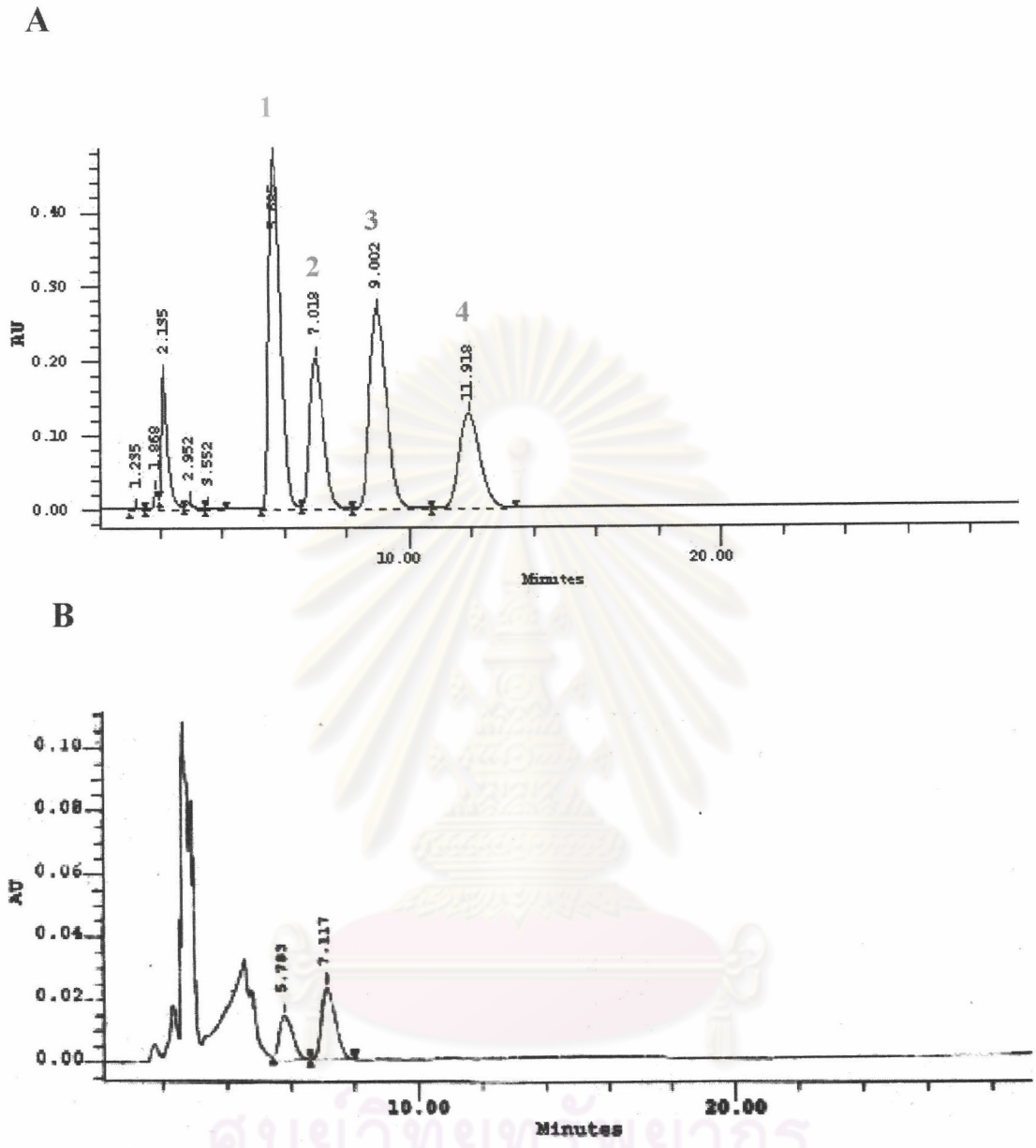

Figure 23 Hydrolysis products of crude chitinase from *Microbacterium* sp. TU05. Colloidal chitin in 0.1M citrate buffer (pH 5.0) was used as substrate and incubated at 40 °C for 24 hours. Hydrolysis products were analyzed by HPLC.

Panel A: Standard oligosaccharides

1: Monomer of N-acetylglucosamine, GlcNAc

2: Dimer of N-acetylglucosamine, $(GlcNAc)_2$

3: Trimer of N-acetylglucosamine, $(GlcNAc)_3$

4: Tetramer of N-acetylglucosamine, $(GlcNAc)_4$

Panel B: Hydrolysis products of crude chitinase.

5. Partial purification of crude chitinase by DEAE-cellulose

At first, concentrated crude chitinase was partially purified by passing through a DEAE-cellulose column. The unbound proteins were washed by 10mM phosphate buffer, pH 7.0. After that, the bound proteins were eluted from the column by gradient elution with 0-1.0 M NaCl in the same buffer. The chromatographic pattern of protein and chitinase activity of the fractions, obtained from the column, were shown in Figure 24. The partially purified chitinase peak1 was pooled from fraction number 81 to 85 and the partially purified chitinase peak2 was pooled from fraction number 92 to 95. Both of them were analyzed for enzyme purity, chitinase activities were measured by colorimetric method in citrate buffer (pH 5.0) and the amount of proteins were measured by dye binding method. The purification data was shown in Table 4.

6. Characterization of partially purified chitinase peak1

6.1 Estimation of molecular weight

There were two peaks containing chitinase activity, then proteins from each peak was analyzed and the molecular weight of partially purified chitinase was estimated by SDS-PAGE followed by activity stain as shown in Figure 25. After activity staining, chitinase activity from peak 1 was observed at 65 kDa and chitinase activity from peak 2 was observed at 55 and 30 kDa.

6.2 Optimum pH of partially purified chitinase peak1

The optimum pH of partially purified chitinase peak1 was determined in the same buffer as crude chitinase at 40 °C. Chitinase activity was assayed by colorimetric method. Like crude chitinase, the activity was found in range of pH from 3 to 8, but the partially purified chitinase peak1 was found only one highest chitinase activity at pH 5.0 (Figure 26).

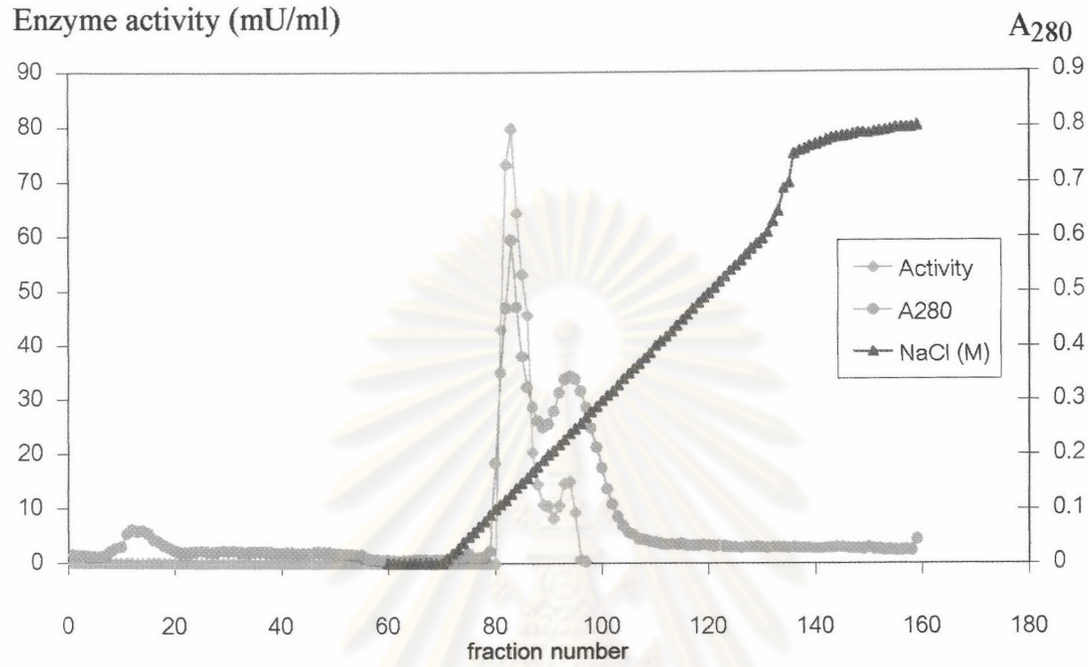

Figure 24 chromatographic pattern of protein and chitinase activity by DEAE-cellulose column. Column size 1.8 x 20 cm, flow rate 1 ml/min. and 5 ml fractions were collected.

Table 4 Partial purification of chitinase from *Microbacterium* sp. TU05

Step	Protein (mg)	Activity (U)	Specific activity (U/mg protein)	Purification fold	%Recovery
Crude chitinase	290	256	0.88	1.0	100
DEAE column					
peak 1	26.15	194.6	7.44	8.5	76
peak2	18.44	34.2	1.85	2.1	13.4

Specific activity = total activity (U) / mg protein
Purification fold = specific activity (crude chitinase)/specific activity
%Recovery = $\frac{\text{total activity of partial purified chitinase}}{\text{total activity of crude enzyme}} \times 100$

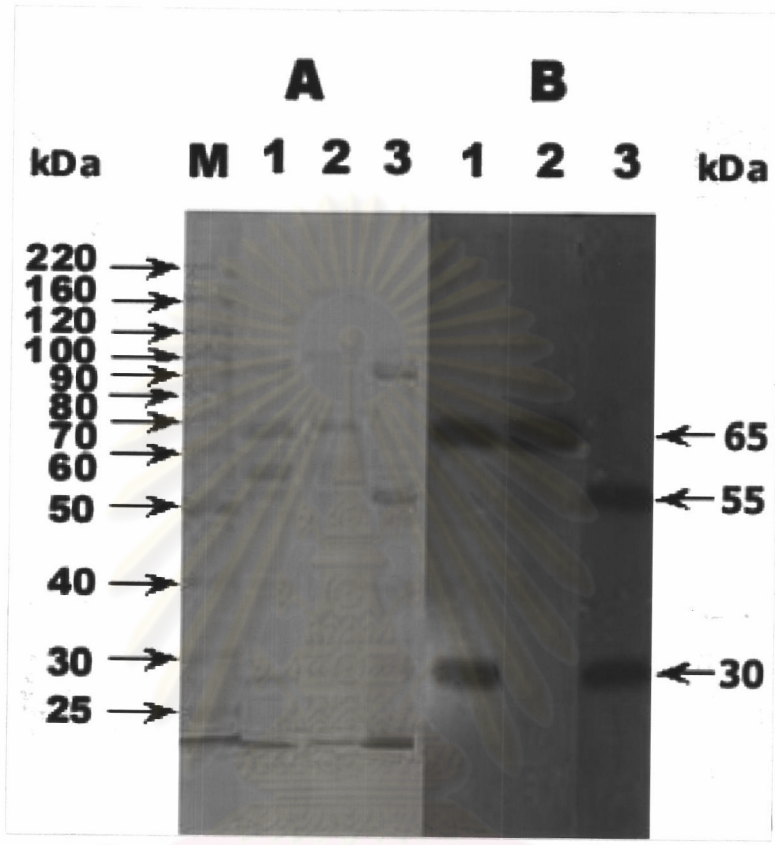

Figure 25 SDS-PAGE of fraction containing maximum chitinase activity.

Panel A: Protein stain

Lane M: Protein marker

Lane 1: Crude enzyme

Lane 2: Partially purified chitinase peak 1

Lane 3: Partially purified chitinase peak 2

Panel B: Activity stain

Lane 1: Crude enzyme

Lane 2: Partially purified chitinase peak 1

Lane 3: Partially purified chitinase peak 2

6.3 Optimum temperature of partially purified chitinase peak1

The optimum temperature of partially purified chitinase peak1 was determined in 0.1M citrate buffer pH 5.0 in various temperatures as crude chitinase. Chitinase activity was assayed by colorimetric method. The chitinase activity was found in range 30 to 40 °C and activity was decreased more than 50% when temperature was 50 °C. For crude chitinase, activity was found from 30 to 50 °C. The highest chitinase activity was found at 40 °C when colloidal chitin was used as substrate (Figure 27).

6.4 Substrate specificity of partially purified chitinase peak1

Partially purified chitinase peak1 was assayed for substrate specificity by colorimetric method. The reactions were done in 0.1M citrate buffer, pH 5.0 at 40 °C. Partially purify chitinase peak1, 0.1 unit, was used for hydrolyzed 10 mg/ml of substrate, including regenerated chitin (RC), colloidal chitin (CC), 45%deacetylated chitosan (45% DD), 80%deacetylated chitosan (80% DD), 90%deacetylated chitosan (90% DD), powder chitin (PC), and flaked chitin (FC). The partially purified chitinase peak1 had the highest hydrolytic activity on colloidal chitin, followed by 45% deacetylated chitosan, powder chitin, regenerated chitin, 80% deacetylated chitosan, flake chitin, and 90% deacetylated chitosan, respectively (Figure 28).

7 DNA Cloning

7.1 Shot gun cloning

Chromosomal DNA of *Microbacterium* sp. TU05 was partially digested with restriction enzyme, *PstI*. Then fragments, 2 to 9 kb, were ligated with dephosphorylated *PstI*-digested pBSSK$^-$. The ligation products were transformed into host cell, *E. coli* JM109, by electrotransformation.

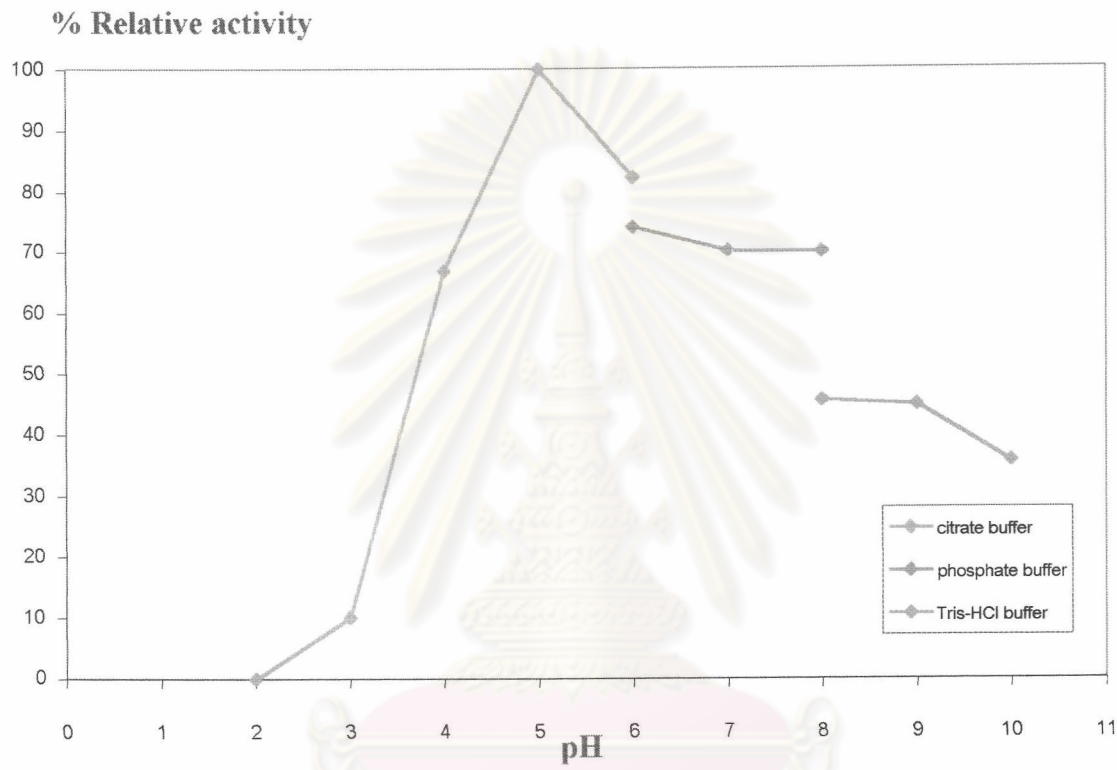

Figure 26 Effect of pH on partially purified chitinase peak1 activity. Chitinase activity was measured by colorimetric method at pH range 2 to 10 at 40 ° C when colloidal chitin was used as substrate.

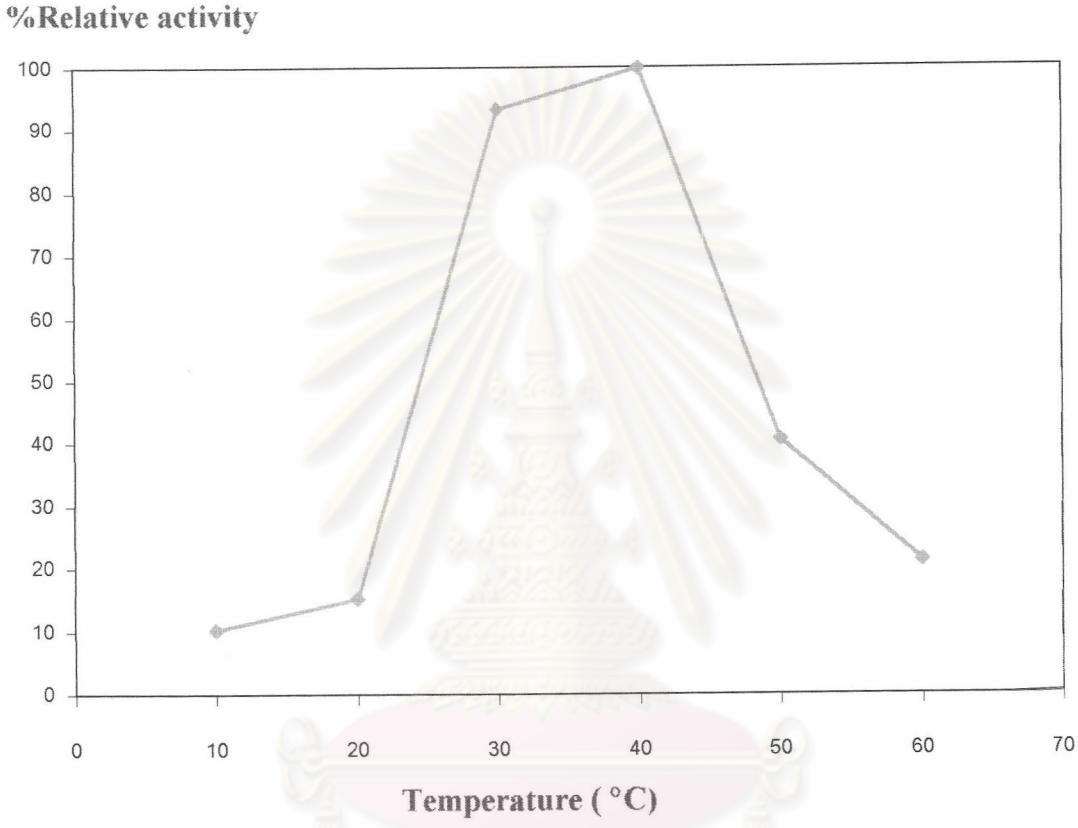

Figure 27 Effect of temperature on partially purified chitinase peak1 activity. Chitinase activity was measured by colorimetric method at pH 5.0 in temperature range 10 to 60 °C when colloidal chitin was used as substrate.

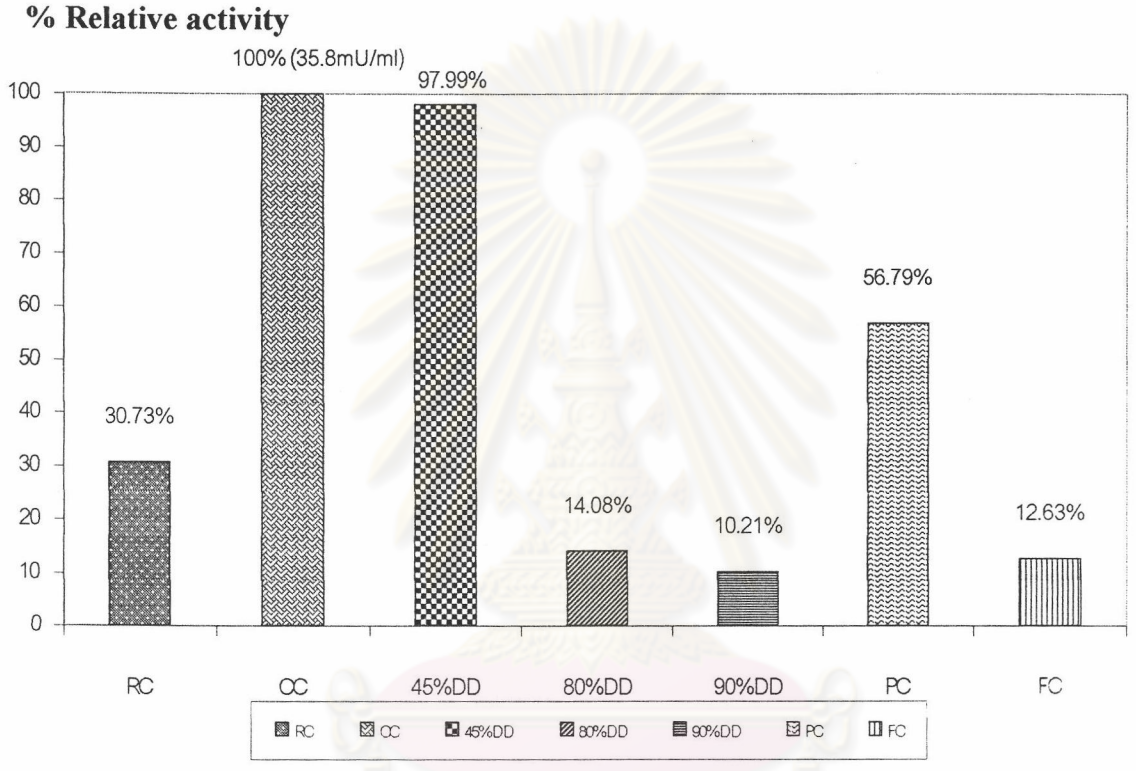

Figure 28 Substrate specificity of partial purified chitinase peak1 from *Microbacterium* sp. TU05. The reactions were done in 0.1M citrate buffer, pH5.0 at 40 °C. Partial purified chitinase, 0.1 unit, was used for hydrolyzed 10 mg/ml of substrates, including regenerated chitin (RC), colloidal chitin (CC), 45% deacetylated chitosan (45% DD), 80% deacetylated chitosan (80% DD), 90% deacetylated chitosan (90% DD), powder chitin (PC), and flaked chitin (FC).

Transformants carrying recombinant DNA were selected by blue-white screening to replicated plates for screening recombinant DNA that contained chitinase gene. About 30,000 transformants were screened, no positive clones were found on selective media.

7.2 Partial sequence of chitinase gene by PCR

The partial sequence of chitinase gene of *Microbacterium* sp. TU05 was amplified by PCR. The PCR products were analyzed by agarose gel electrophoresis (Figure 29). The PCR product from primers BPI and BPVI (636 bp) was selected. Then, PCR products from primers BPI and BP VI were ligated with pGEM-T easy vector before analyzed (Figure 30 and 31). The sequence of PCR product was shown in Figure 32. The DNA sequence of partial chitinase gene was aligned and compared to other proteins in GenBank. The partial sequence of chitinase gene of *Microbacterium* sp. TU05 when translated to protein, it showed the highest similarity to the chitinase of *Arthrobacter* sp. (71%) as shown in Figure 33.

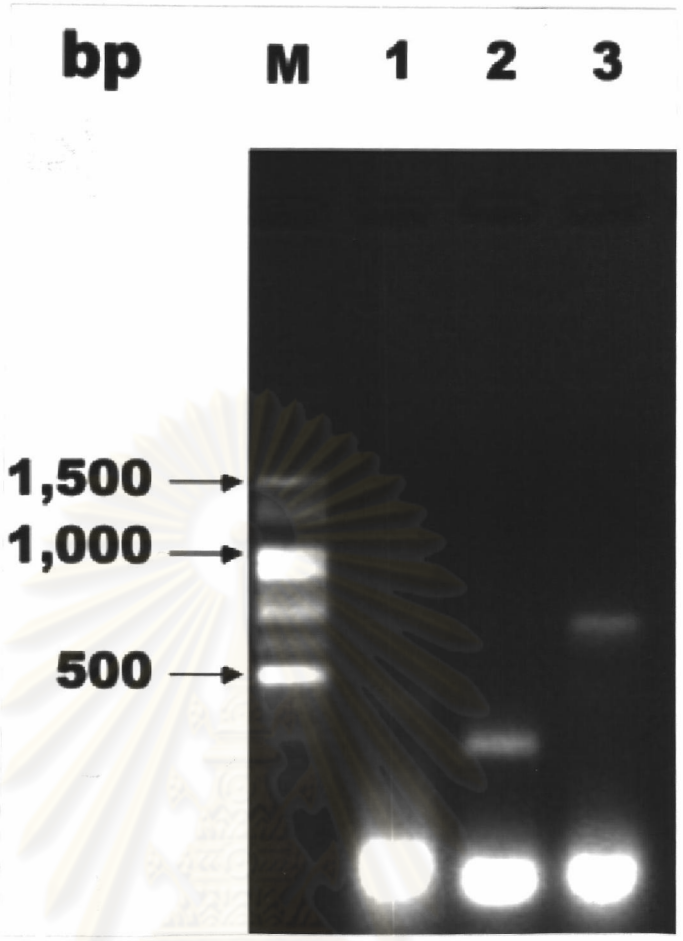

Figure 29 Agarose gel electrophoresis determined PCR products from primer BPI, BPII, BPV, and BPVI.

Lane M: 100bp marker

Lane 1: PCR products from primers BPI + BPV

Lane 2: PCR products from primers BPII + BPVI

Lane 3: PCR products from primers BPI + BPVI

Forward primer BP-I 5'-AAYTAYGCDTTYGCDGAYATHTGYTGGRANGG-3'

BP-II 5'-TTYGAYGGNGTNGAYYTNGAYTGGGARTA-3'

Reverse primer BP-V 5'-RTAYTCCCARTCNARRTCNACNCCRTCRAA-3'

BP-VI 5'-CCANCCNCCRTTRAARTCRTANGTCATGATRTT-3'

Abbreviations: Y = C, T R = A, G D = A, G, T H = A, C, T N = A, C, G, T

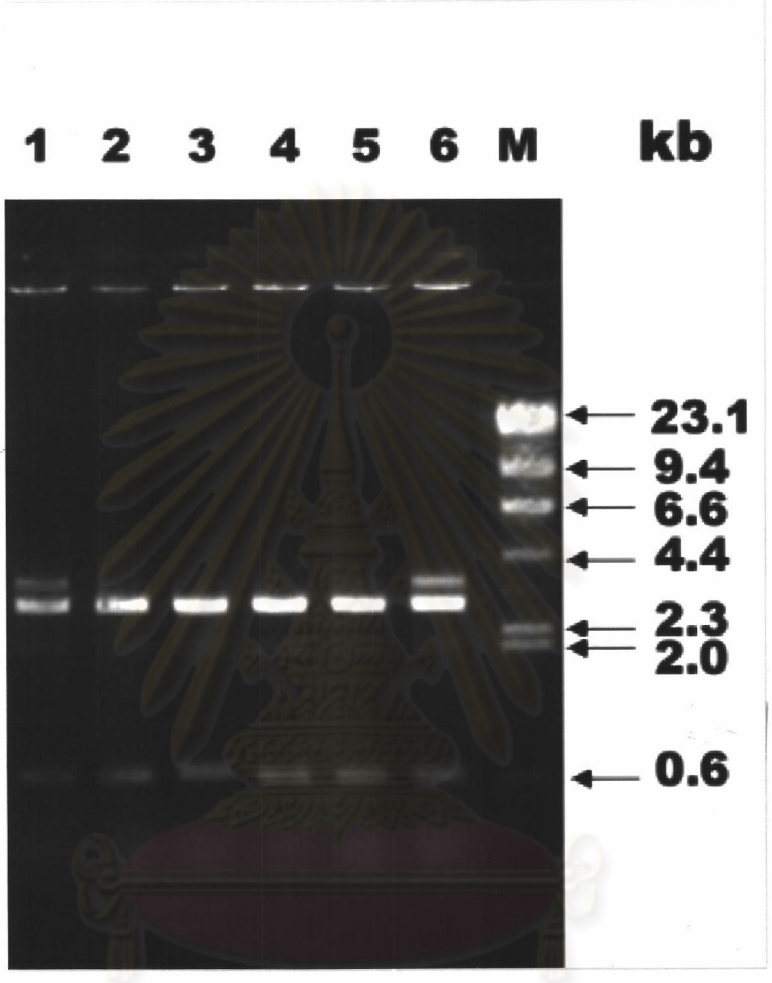

Figure 30 Agarose gel electrophoresis illustrated ligated PCR products from several transformants.

Lane 1 to 6: Ligated PCR products from primers BPI + BPVI cut with *Eco*RI, from several transformants

Lane M: λ/*Hind*III marker

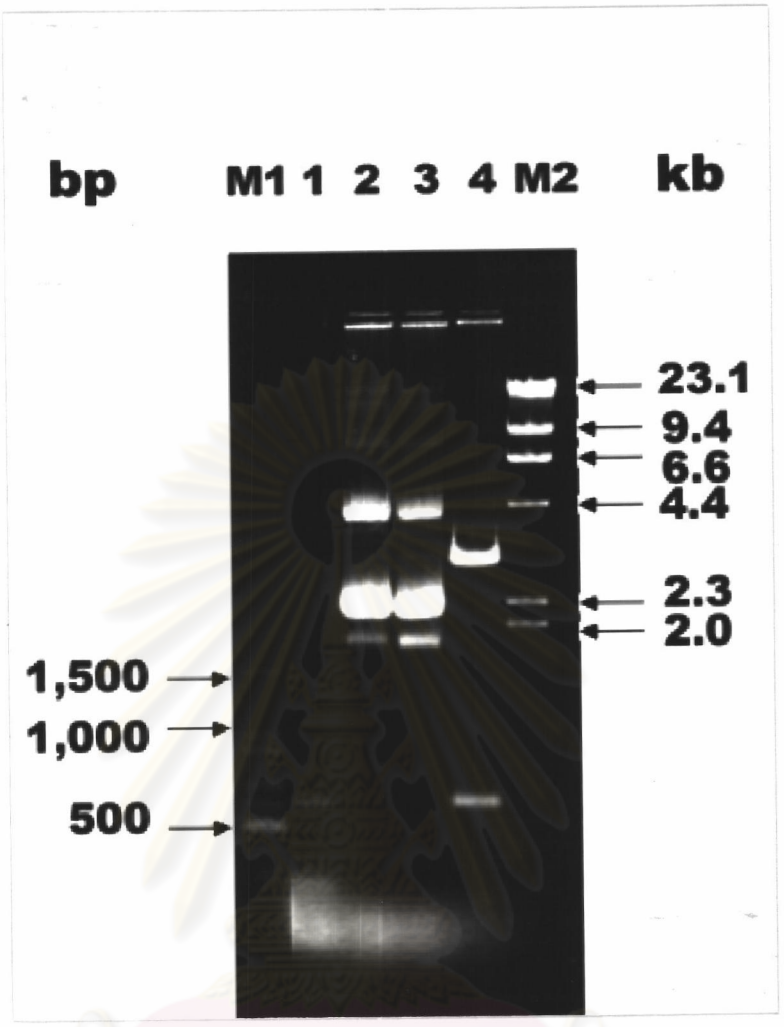

Figure 31 Agarose gel electrophoresis determined partial chitinase gene from PCR amplification.

Lane M1: 100bp marker

Lane 1: PCR products from primers BPI + BPVI

Lane 2: PCR products from primers BPI + BPVI ligated with pGEM-T easy vector

Lane 3: PCR products from primers BPI + BPVI ligated with pGEM-T easy vector

Lane 4: PCR ligation products from primers I + VI and pGEM-T easy vector cut with *Eco*RI

Lane M2: λ/*Hind*III marker

```
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51  GGAGGATCTG GTGGACCTCC GCGCCACGCA GCAGCCAGCC GGAGGCGGGA 100
101 GCTGCGACCG TGAGCATGTA GTGCTTGCCG TCGGCAGCGC CCGCGACGTC 150
151 GAGCTTCTCG CGCAGCGTCT TCATGAGTGC TTCGTAGCCC GACCACAGCT 200
201 TCGCGCGGCG CGGCTCGGAG AACGAGAAGT CATCCGGGCT GCCGGCCTTG 250
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351 GTGTGTCGGT CATCGTGTAG AAACCACCGT CAGCACCGCG GTTGCCGTCC 400
401 GCCCCGAAGT GGCCTCCGGT CTCAGCCCAC CCGCCGACGG AGACGAGCGC 450
451 CTTCACGCCC GGGTTCTGTG CCTTGTAGGT GTTGAGCATG TTGAAGTGCC 500
501 CCGTGTACCG GAAGCGATGC GGTCATCGCA GCGCCGGGCT CTTCGGGCCA 550
551 GGTCATGTCG GTGGGCCGCG TTGCCGGGGA CCTCCGCGTT GACCGACACC 600
601 TTGCCTTTTC AACAAATGTT CCGCGAACGC ATAGTTT              636
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Figure 32 Nucleotide sequences of partially chitinase gene.

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A. sp.      256 YKGHLNQLKKFKKENPGVKTLVSVGGWAETGGHFAADGTRVGDGGFYTLTESQAKIDTFS 315
                Y GH N L  +K +NPGVK LVSVGGWAETGGHF ADG R  DGGFYT+T++QAKIDTF+
M. TU05     507 YTGHFNMLNTYKAQNPGVKALVSVGGWAETGGHFGADGNRGADGGFYTMTDTQAKIDTFA 328

A. sp.      316 DSAVEFIRKYGFSGVDIDYEYATSNSKAGNPDDFAISEPRRAVLFENYMKLMKTLREKLD 375
                 SAVEFIR YGF GVDIDYEYA SN KAG+PDDF+ SEPRRA L+  Y  LMKTLREKLD
M. TU05     327 TSAVEFIRTYGFDGVDIDYEYANSNGKAGSPDDFSFSEPRRAKLWSGYEALMKTLREKLD 148

A. sp.      376 KASVADGTYYQLTVAAPASGWLLRGMEAHQVVKYLDFVNMMSYDLHGAW 424
                A  ADG +Y LTVAAPASGWLLRG E HQ++ YLD+VN+M+YD +G W
M. TU05     147 VAGAADGKHYMLTVAAPASGWLLRGAEVHQILPYLDYVNIMTYDFNGGW 1
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Figure 33 Nucleotide translated to protein alignment of *Microbacterium* TU05 (M. TU05) with *Arthrobacter* sp.(A. sp.). Red alphabet showed the comparison between *Microbacterium* TU05 (M. TU05) with *Arthrobacter* sp.(A. sp.) and plus showed charged amino acid residues.