CHAPTER III

RESULTS

Screening, isolation and identification of a thermotolerant bacterium producing the highest chitinase

Screening and isolation of a thermostable chitinase producing bacteria

Cultivation of bacteria on plates containing colloidal chitin was used as a first screening step for selection of chitinase producers, and an incubation temperature of 50 °C was used as a second screening step for selection of thermotolerant bacteria. At this stage of the screening, 4 bacterial strains were isolated. Bacteria strain SK-1 to SK-4 generated clear zones around colonies indicating produce extracellular chitinase. The chitinase production strains were further grown on 0.02% colloidal chitin minimum medium. Chitinase activity in the culture supernatant was determined as described in the chapter II. Level of chitinase activity among these strain was ranging from 58 to 87 mU/ml (Figure 7). Bacteria strain SK-1 exhibited the highest enzyme activity with 87 mU/ml and produces extracellular chitinase as shown in Figure 8. Thus, the strain SK-1 was selected and used as a source of chitinase enzyme and chitinase gene for further characterization.

Identification of bacterial strain SK-1

Bacteria strain SK-1 was identified by morphological and biochemical characteristics listed in Table 6. This strain was a Gram positive bacilli which formed subterminal ellipsoidal endospores, as shown in Figure 9. SK-1 cells were rod shape, size 0.4x1.3 μM (Figure 10). This bacteria grew both aerobically and anaerobically on LB agar. The limiting growth temperature was 60 °C. The colonies on nutrient agar were reddish-brown in color and had a rough surface with hair-like outgrowths. This bacterium was highly active in producing acids from sugars (see appendix B) and in degrading macromolecule such as starch (Table 6). Thus, it was classified in *Bacillus*

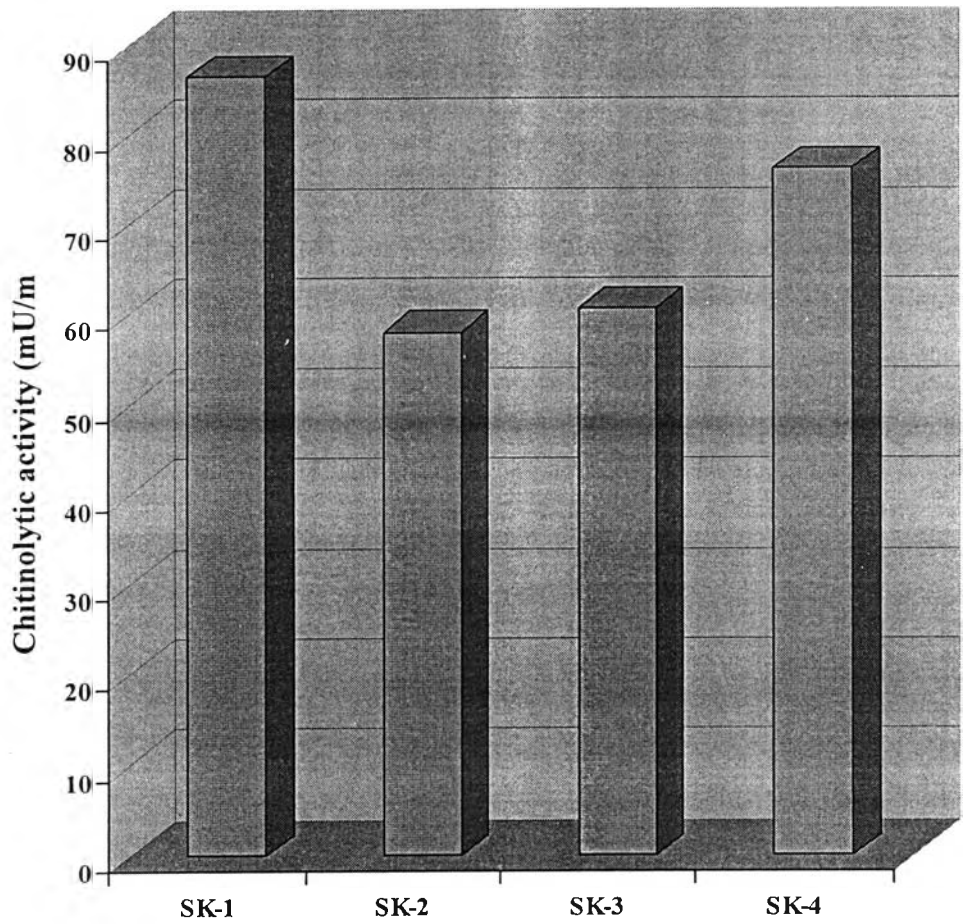

Figure 7. Levels of chitinolytic activity of isolated bacteria.

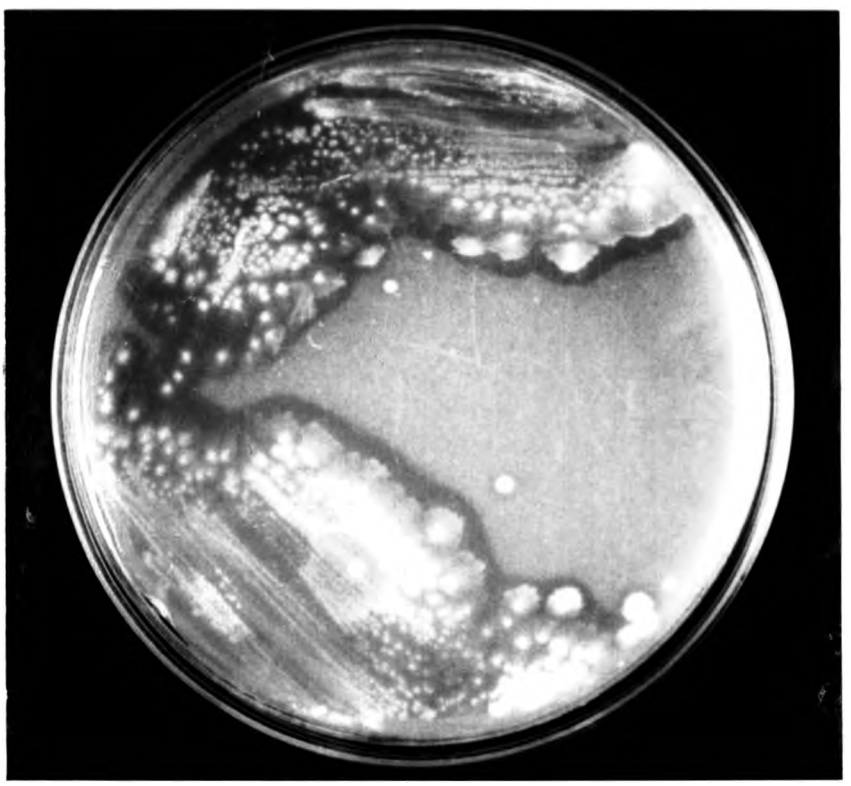

Figure 8. Colony morphology of bacteria strain SK-1 on 0.02% colloidal chitin minimum medium plate. *B. licheniformis* SK-1 was grown on CCMM and produce clear zone around colony. The plate was incubated at 50 °C for 2 days.

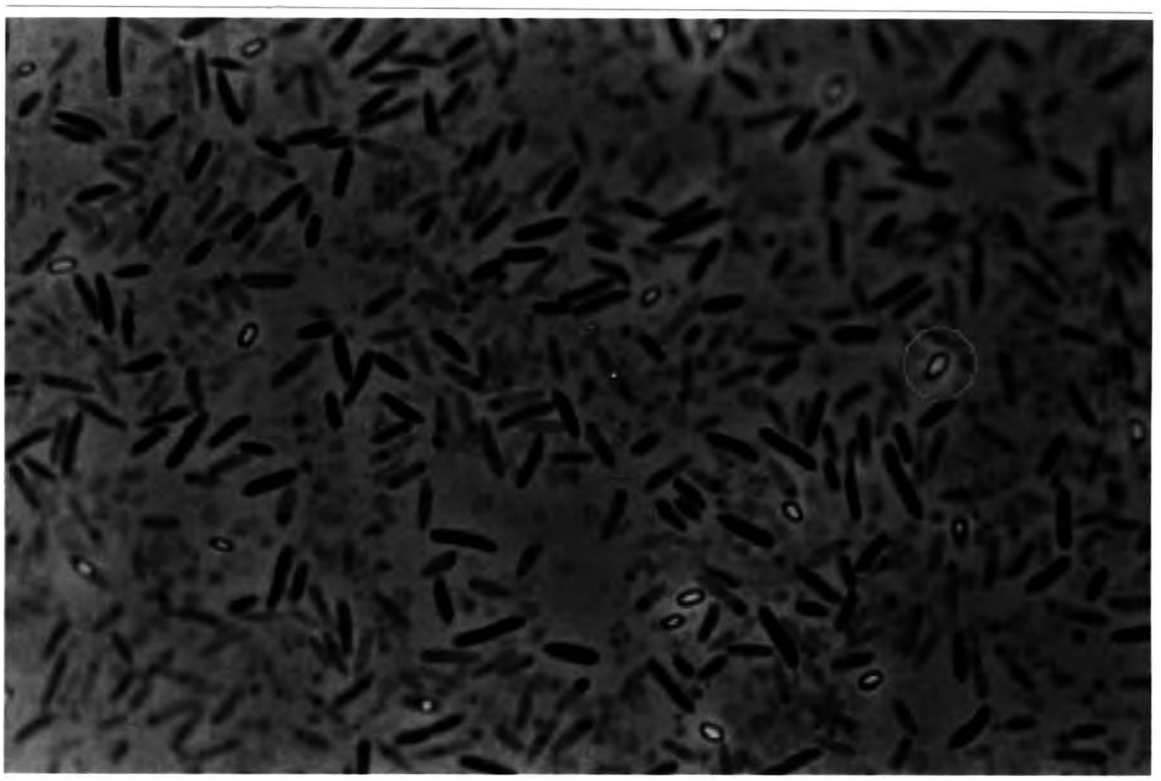

Figure 9. Cell morphology of the bacteria strain (x100 objective, phase contrast microscope). Microscopic morphological of *B. licheniformis* SK-1. Red circle showed shape of bacteria, green circle showed its spore.

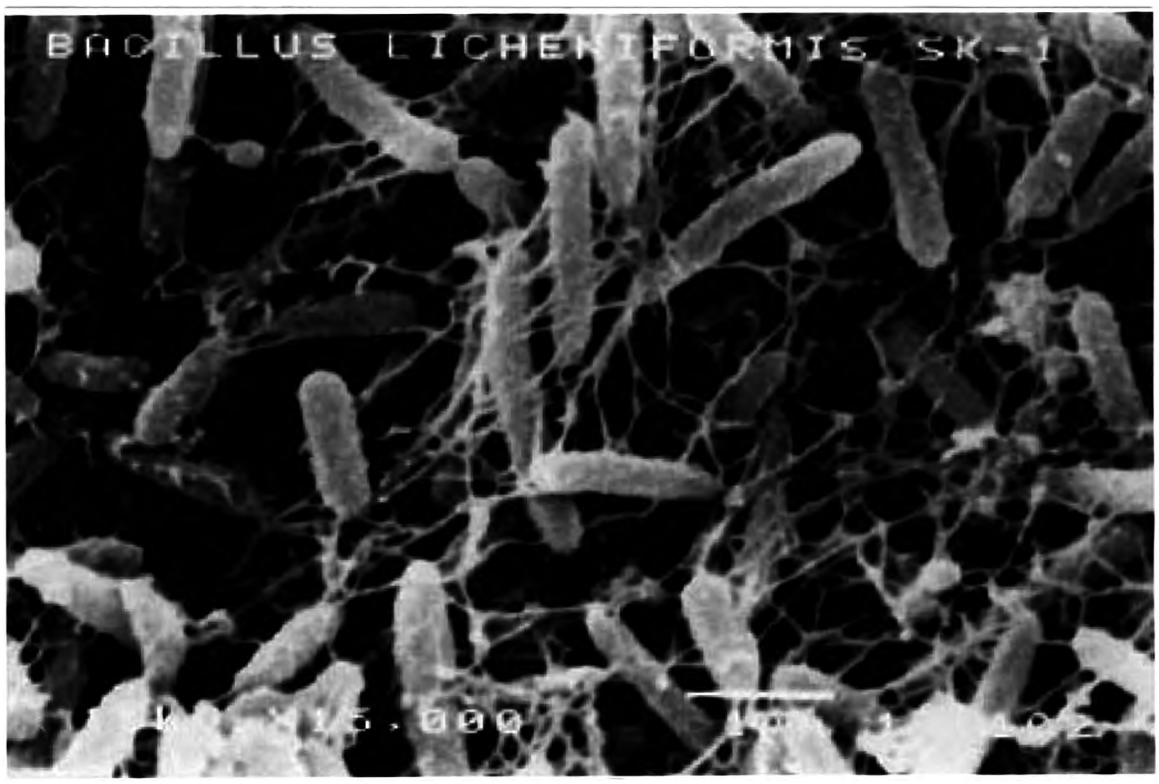

Figure 10. Scanning electron microscopic (SEM) of *B. licheniformis* SK-1.

SK-1 cells were grown on CCMM at 50 °C for 2 days.

Table 6. Morphological and biochemical characteristics of *B. licheniformis* SK-1

Morphological characteristics
Shape: bacilli
Mobility: motile, with peritrichous figella
Spores: subterminal ellipsoidal endospores formed
Gram stain: positive
Culture characteristics
Nutrient agar colony: circular, flat, opaque with rough surface, hair-like outgrowths
Physiological characteristics
Growth at 60 °C: positive
pH for growth 5-9, optimum 7.5
Biochemical characteristics
Growth in anaerobic condition: positive
Growth in 10% NaCl: positive
Phenylamine deaminase: positive
Alkaline phosphatase: positive
Catalase: positive
Acid from Ribose: positive
Manitol: positive
Cellobiose: positive
Lactose: positive
Sucrose: positive
Hydrolysis of Starch: positive
Casein: positive
Tyrosine: negative
Antibiotic resistance
Ampicillin: positive

group BII (Priest, 1988). From these results, strain SK-1 was identified as *Bacillus licheniformis*.

16S rRNA sequence comparison of SK-1

The genomic rRNA gene sequence (1,533 bases) of strain SK-1 was determined as shown in Figure 11. The sequence of SK-1 was aligned and compared to the 16S rRNA gene sequence of various organisms in GenBank. It showed highest sequence similarity to 16S rRNA from *B. licheniformis* (97%) (Figure 12).

Optimization for growth of *B. licheniformis* SK-1.

B. licheniformis SK-1 grew between 30 to 60 °C, the optimum for growth temperature was 50 °C (Figure 13A). The pH range for growth was 6 to 8, and the optimum pH was around 7.5 (Figure 13B). Growth was observed in medium containing 1 to 10 % NaCl, with the optimum at 1% NaCl (Figure 13C).

Chitinolytic enzyme production and growth curve of *B. licheniformis* SK-1.

The relationship between colony forming unit and total chitinolytic activity of all samples were shown in Figure 14. These data, showed that enzyme production of SK-1 in 0.02% CCMM gradually increased with growth rate. The highest chitinase production and growth rate was found in the second day. The maximum chitinase activity was found on the second day.

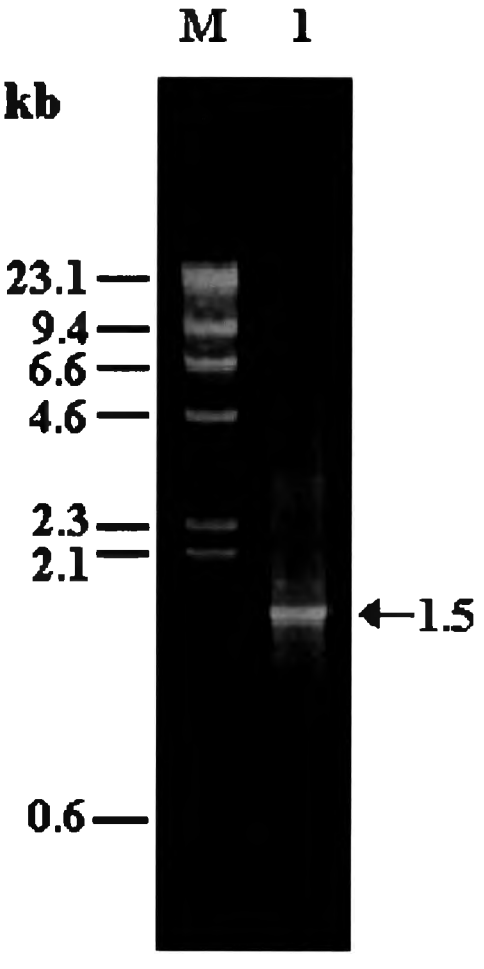

Figure 11. 16S rRNA amplification product from *B. licheniformis* SK-1

Lane M = λ/*Hin*dIII

Lane 1 = 16S rRNA products using genomic DNA of *B. liceheniformis* SK-1 as template

```
SK-1        TTGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGACGGGAGCTTG 60
B           --CGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGACGGGAGCTTG 58
               ************************************************************

SK-1        CTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGG 120
B           CTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGG 118
            ************************************************************

SK-1        GATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATTA 180
B           GATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATCA 178
            ************************************************************ *

SK-1        TAAAAGGGGGGTTTTAACTTCCACTTTCAGATGGACCCCCGGGGCATTAACTTGTTGGGG 240
B           TAAAAGGTGGCTTTCAGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTG 238
            ******* ** *** * ** ****** ********** *** ****** ** ***** *

SK-1        AGGGAACGGGTTACCAAAGGGACAATGCGTAACCCACCTGAGAGGGGGATTGGGCACACT 300
B           AGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT 298
            *** ***** * ***** * *** ******* ** ********** *** ** ******

SK-1        GGGACTGAGAAACGGGCCAAACTTCTACGGGAGGGAGCAGTAGGGAATTTTTCCCAATGG 360
B           GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG 358
            ********** **** *** *** ********** ********** ** * ******

SK-1        ACGAAAGTTTTACCGAGCAACCCCCCGTGAGTGATGAAAGGTTTGGGATCGTAAAACTTT 420
B           ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCT 418
            ******** * ** ******* ** ********** * *** ********** *

SK-1        TTTGTTAGGGAAAAACAAGTACCGTTCGAATAGGGGGGGGCCTTTACAG-GCCTTACCAA 479
B           GTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAG 478
            *********** ********************** **  **** ** *  *** ****

SK-1        AAAGCC-CGGGTTACTTCTTGCCA--AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCC 536
B           AAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCC 538
            ****** *** * *** * *****  ***********************************

SK-1        GGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCG 596
B           GGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCG 598
            ************************************************************

SK-1        GCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAA 656
B           GCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAA 658
            ************************************************************

SK-1        TTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACT 716
B           TTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACT 718
            ************************************************************

SK-1        CTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACC 776
B           CTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACC 778
            ************************************************************

SK-1        CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTG 836
B           CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTG 838
            ************************************************************

SK-1        CTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAA 896
B           CTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAA 898
            ************************************************************

SK-1        GGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA 956
B           GGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA 958
            ************************************************************

SK-1        GAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGGG 1016
B           GAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGGG 1018
            ************************************************************

SK-1        GCAAAGTGACAGGTGGGTGCATGGTTGTCATAAGCTCGTGTGGTAAGATGTTGGGTTAAG 1076
B           GCAGAGTGACAGGTGG-TGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG 1077
            *** ************ ************ * ********* ** ***************

SK-1        TCCCGCAAAGACCAC--CCCTTGATTTTTAGTTTGCCAGGCATTCAGTTGGGCACTCTAA 1134
B           TCCCGCAACGAGCGCAACCCTTGATCTTAG--TTGCCAG-CATTCAGTTGGGCACTCTAA 1134
            ******** ** * *  ******** **    ******* ********************
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SK-1            GG-AACTGCCGGGTACAAACCGAAGGAAGGTGGGAATGACTGTCAGAATCATCATGCCCA 1193
B               GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC-GTCA-AATCATCATGCCC- 1191
                **  ********  ******** ********** ***** **** ************

SK-1            TTTATGACATGGGCTACACAGGGTGATACAATGGGCAGAACAAAGGGCAGCGAAGCCGCG 1253
B               CTTATGACCTGGGCTACACACG-TGCTACAATGGGCAGAACAAAGGGCAGCGAAGCCGCG 1250
                 ******* ********** * ** ***********************************

SK-1            AGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGC 1313
B               AGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGC 1310
                ************************************************************

SK-1            GTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGC 1373
B               GTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGC 1370
                ************************************************************

SK-1            CTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAAC 1433
B               CTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAAC 1430
                ************************************************************

SK-1            CTTTTGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTACCAGGGTAC 1493
B               CTTTTGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAG 1490
                ************************************************** ** ****

SK-1            CTGACGGTAAATGACCATATAAAAAACCGACTCCCGCCCC 1533
B               CCGTATCGGAAGG------------------------- 1503
                * *      ** *
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Figure 12. Alignment of 16S ribosomal RNA sequence of *Bacillus licheniformis* SK-1 (AF411341) with *B. licheniformis* B (AF276309). The asterisks indicated identical sequence. This figure was created using CLUSTAL W multiple sequence alignment version 1.81.

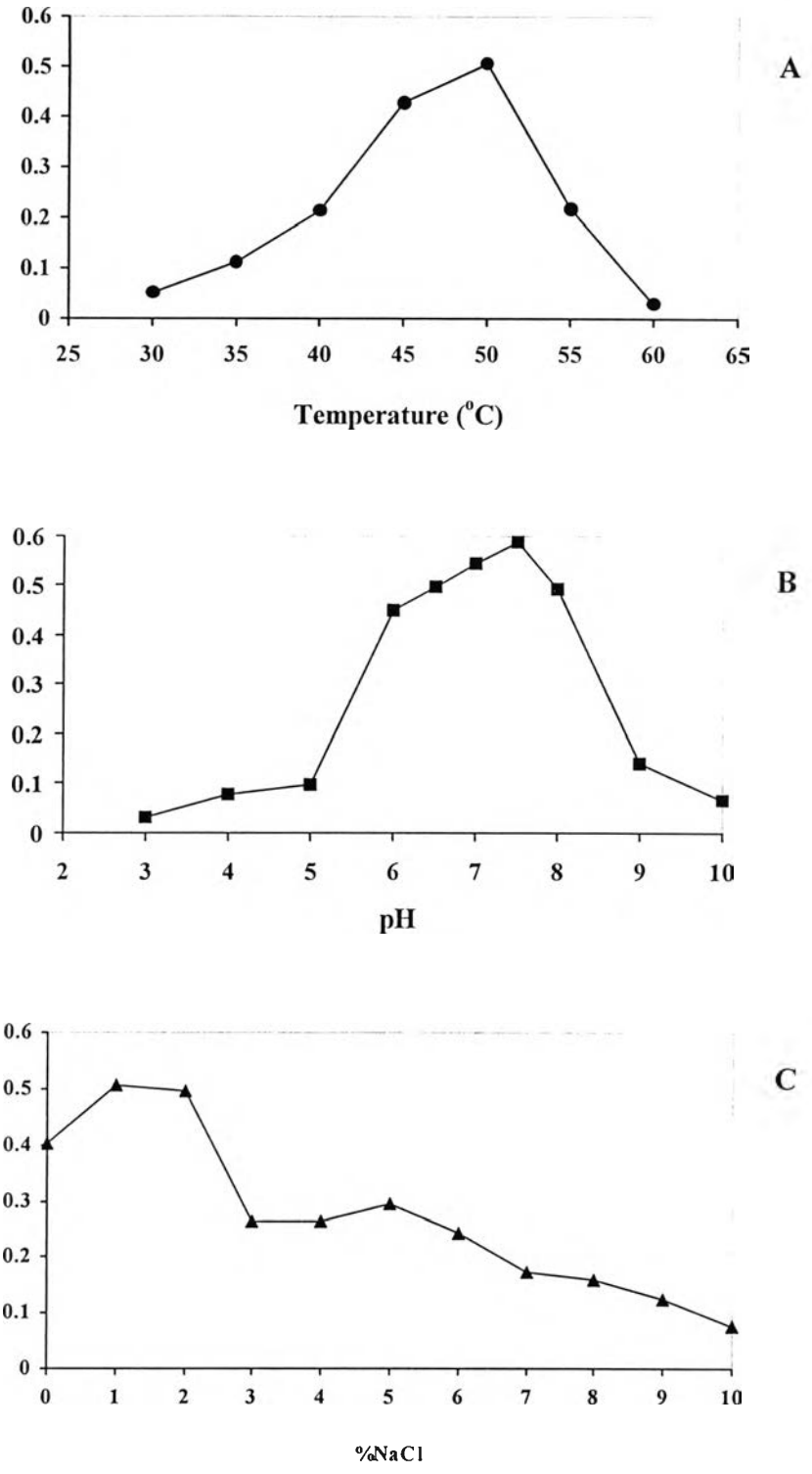


Figure 13. Growth of strain SK-1 in various conditions. SK-1 cultured in LB medium at different pH (A), temperature (B) and NaCl concentration (C) and was measured A_{650} at 2 hrs.

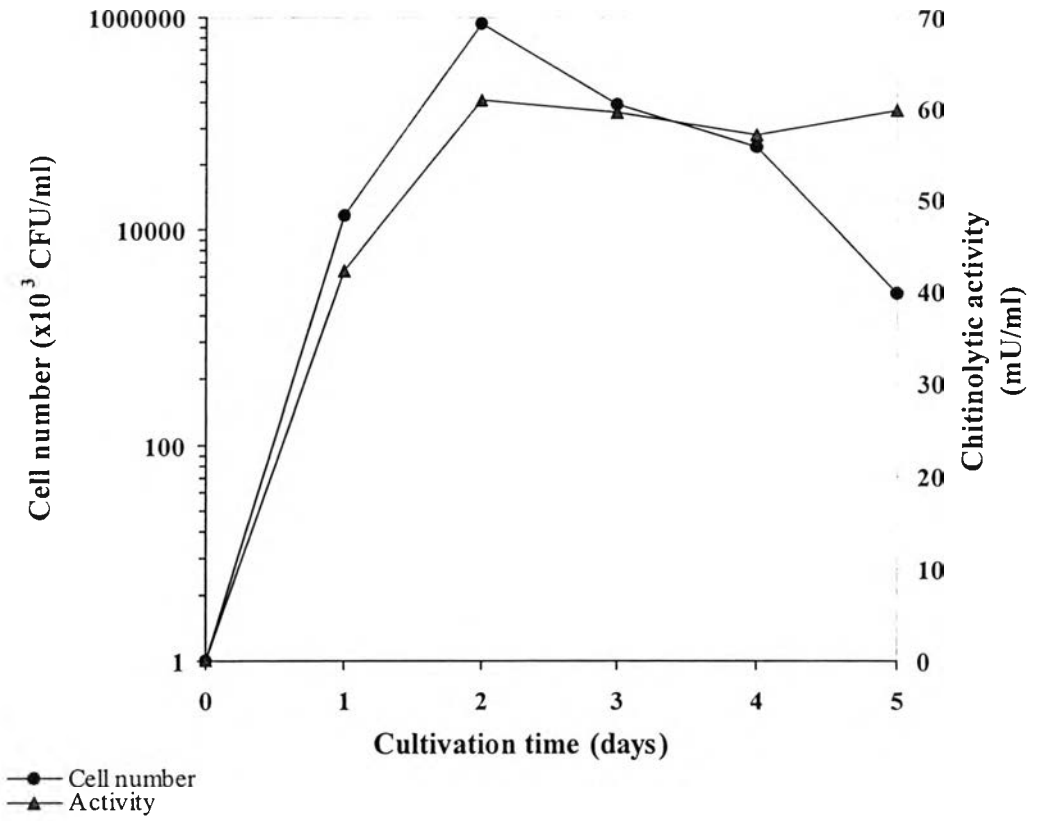


Figure 14. The relationship between cell number (bacterial concentration, CFU/ml) and chitinolytic enzyme production (enzyme concentration, mU/ml) of *B. licheniformis* SK-1.

Optimization for chitinase production

Optimum temperature

The relationship between culturing temperature and total chitinolytic activity in the culture medium was shown in Figure 15. These data, demonstrated that enzyme production of SK-1 in 0.02% CCMM gradually increased with culturing time. The highest chitinase production was at 50 °C. The chitinase activity was maximized at the fourth day.

Effect of various source of chitin on chitinase production

The quantitative activity of chitinase produced in various sources of chitin was further determined by the colorimetric method using colloidal chitin as substrate. The highest activity was obtained at the third day of the cultivation (Figure 16). The maximum activity was detected in CCMM (74 mU/ml). In the culture medium without colloidal chitin, LB medium, chitinase activity increased slowly and remained constant at a low level, 21 mU/ml. The amount of chitinase increased when used colloidal chitin, powdered chitin and regenerated chitin was supplemented as carbon source in minimum medium, and LB medium, respectively. The chitinase activity were found 74, 46, 45 and 21 mU/ml when *B. licheniformis* SK-1 was grown in the medium with colloidal chitin, powdered chitin, and regenerated chitin as carbon source and LB medium, respectively.

Effect of various concentration of colloidal chitin

The quantitative activity of chitinase produced in each concentration of CCMM was further determined by the colorimetric method using colloidal chitin as substrate. The highest activity was obtained at fourth day of the incubation (Figure 17). The maximum activity was detected in 0.08% CCMM (184 mU/ml). The chitinolytic activities were 124, 160, 184, 158, 162 mU/ml when colloidal chitin was added in the minimum medium at final concentrations of 0.02, 0.04, 0.08, 0.16 and 0.2%, respectively.

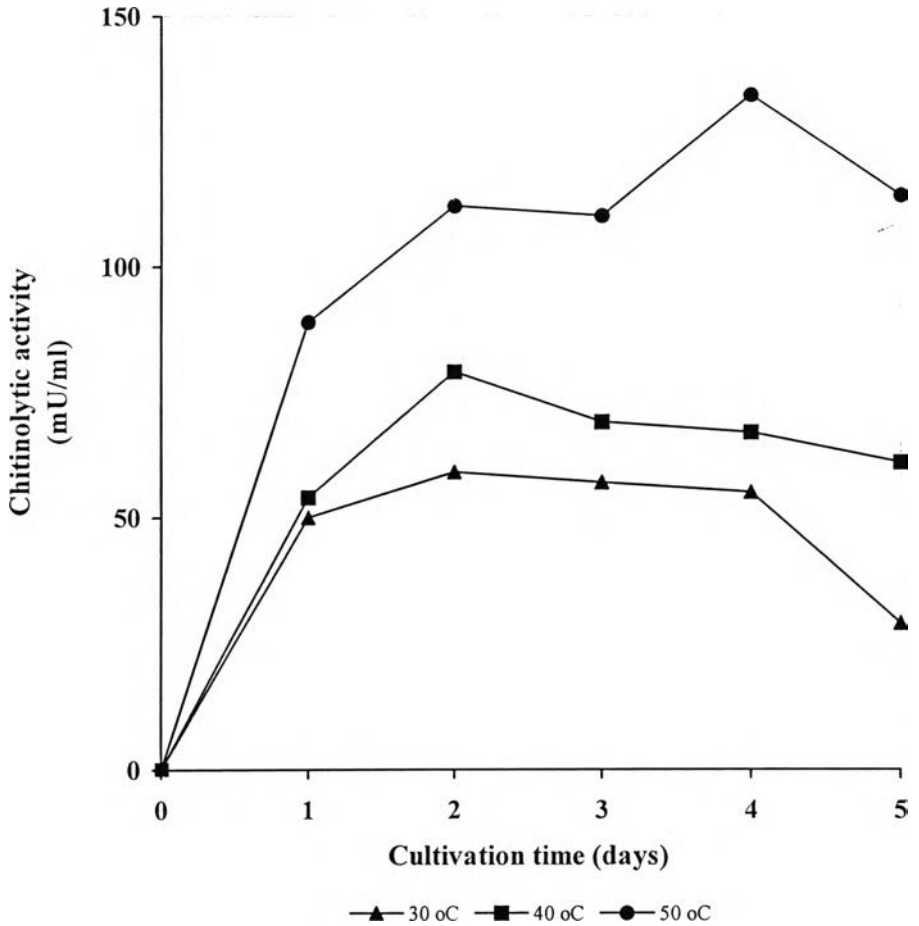


Figure 15. Effect of temperature on the chitinolytic production by *B. licheniformis* SK-1. The enzyme activity was determined from the culture medium, using colloidal chitin as a substrate, chitinolytic activity was expressed as mU/ml under the standard assay condition.

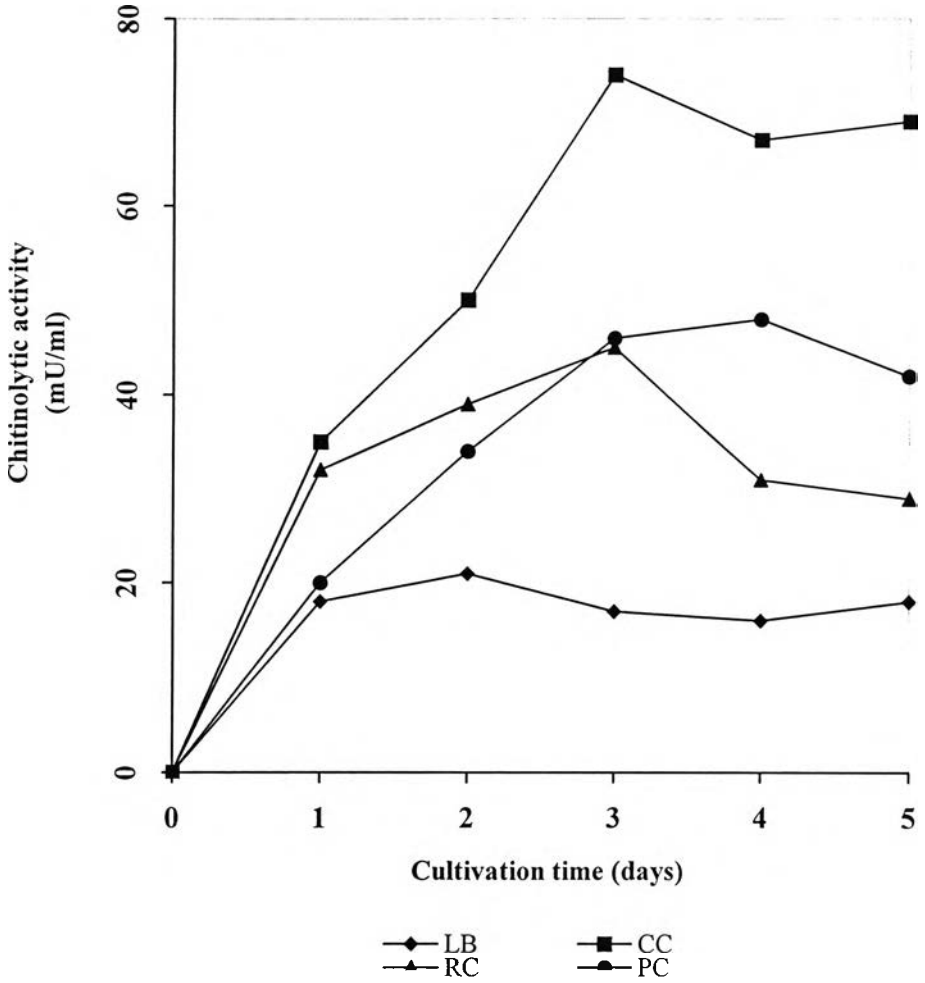


Figure 16. Effect of various source of chitin on the chitinolytic production by *B. licheniformis* SK-1. The enzyme activity was determined from the culture medium, using colloidal chitin as a substrate, chitinolytic activity was expressed as mU/ml under the standard assay condition.

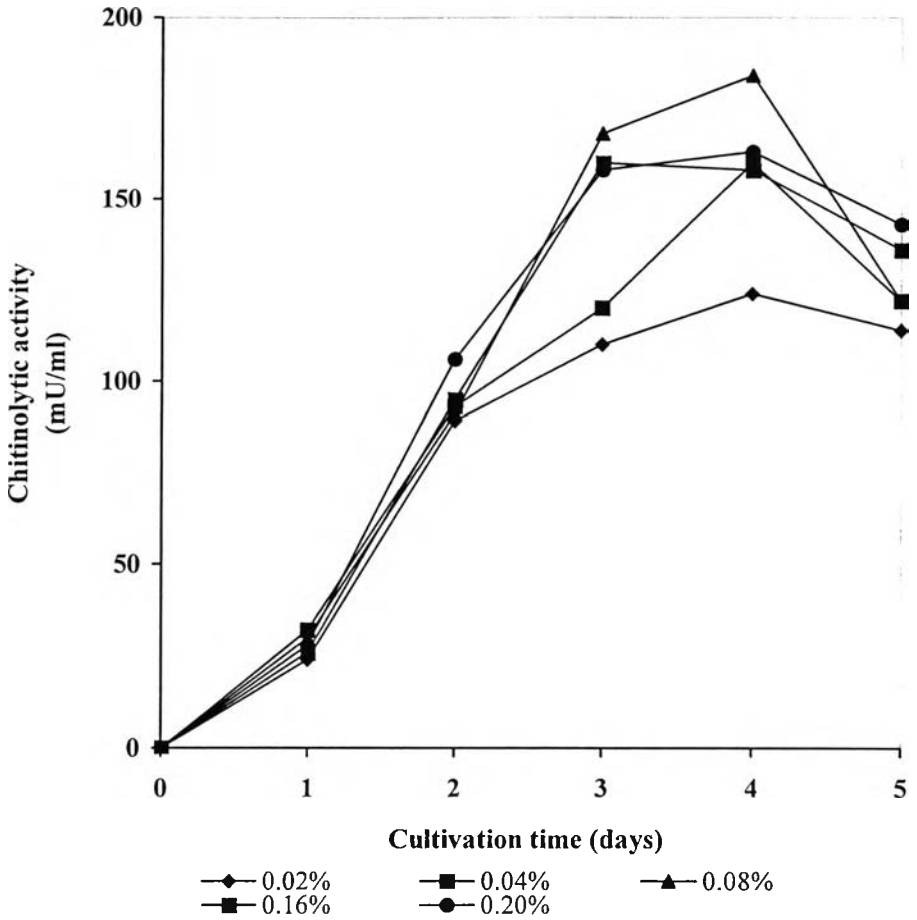


Figure 17. Effect of colloidal chitin concentration on the chitinolytic production by *B. licheniformis* SK-1. The enzyme activity was determined from the culture medium, using colloidal chitin as a substrate, chitinolytic activity was expressed as mU/ml under the standard assay condition.

Partial characterization of crude chitinolytic enzyme from *B. licheniformis* SK-1

The chitinolytic enzyme was studied and assayed by the colorimetric method as previously described for its properties as follows:

Optimum and pH stability for crude chitinolytic enzyme

The optimum pH of crude chitinolytic was determined. Chitinolytic activity was assayed at 50 °C in buffer with pH ranging from 3-10. Activities at different pH was shown in Figure 18. Reducing sugar from a colloidal chitin suspension during chitinolysis was used to determine the activity. Crude enzyme showed a broad pH ranging from 5-10 (80% relative activity), with optimum in phosphate buffer pH 6.0.

The stability of crude enzymes against pH was also determined. Results of the crude enzymes was summarized in Figure 19. The analysis of the chitinolytic enzyme activity of *B. licheniformis* SK-1 indicated that it was more stable at pH 7.0. The activity was decreased about 28, 34 and 15% when the enzyme was kept in pH 5.0, 6.0 and 7.0, respectively at 4 °C for 3 days. The activity of crude enzymes was rapidly lost when it was kept in acid and alkali pH. Whereas, it was found that 60%, of the activity was lost when it was kept at pH 4.0 for 3 days. More than 50% of the activity was lost after incubating at all pHs for 4 days.

Optimum and temperature stability for crude chitinase

The optimum temperature of crude chitinase was determined at pH 6.0, in the range of 30-90 °C. The chitinase activity was assayed by the measuring the increasing reducing sugar from a colloidal chitin suspension during chitinolysis. The crude chitinase showed a broad activity temperature ranging between 55-75 °C, with optimum temperature at 60 °C (Figure 20).

The stability of crude chitinase was analyzed. The activity of chitinolytic enzyme was stable and slightly decreased when incubated at 40 °C and 50 °C for 12 hrs, respectively. About 42% of enzyme activity was detected after it had been incubated at 60 °C for 12 hrs. Thirty nine percents of the enzyme activity was lost when the enzymes was kept at 60 °C for 2 hrs (Figure 21).

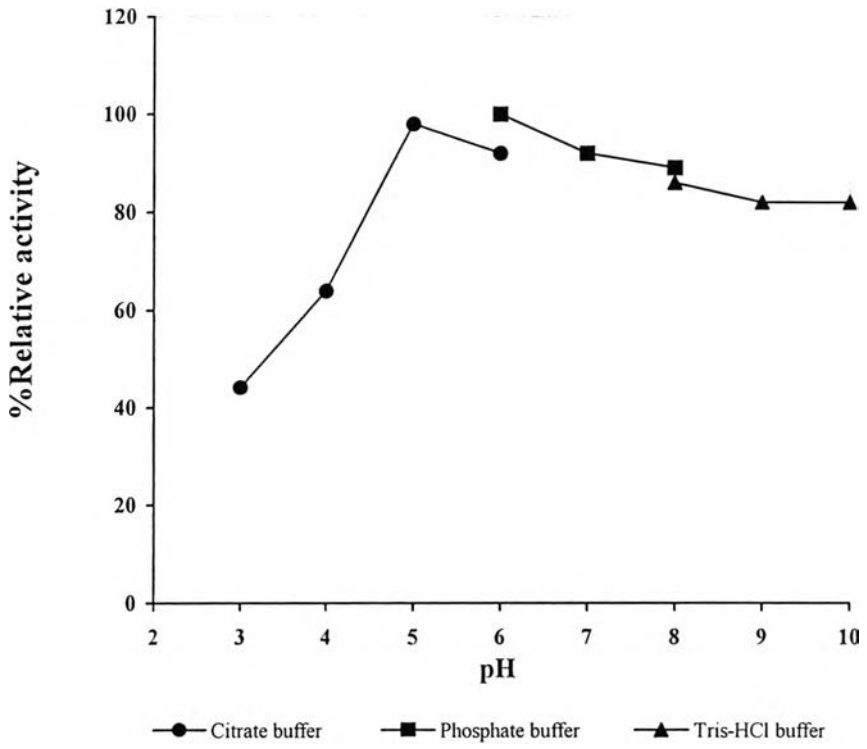


Figure 18. Optimum pH of crude chitinolytic enzyme from *B.licheniformis* SK-1. Chitinolytic activity was measured at pH range from 3-10 by colorimetric method when used colloidal chitin as substrate.

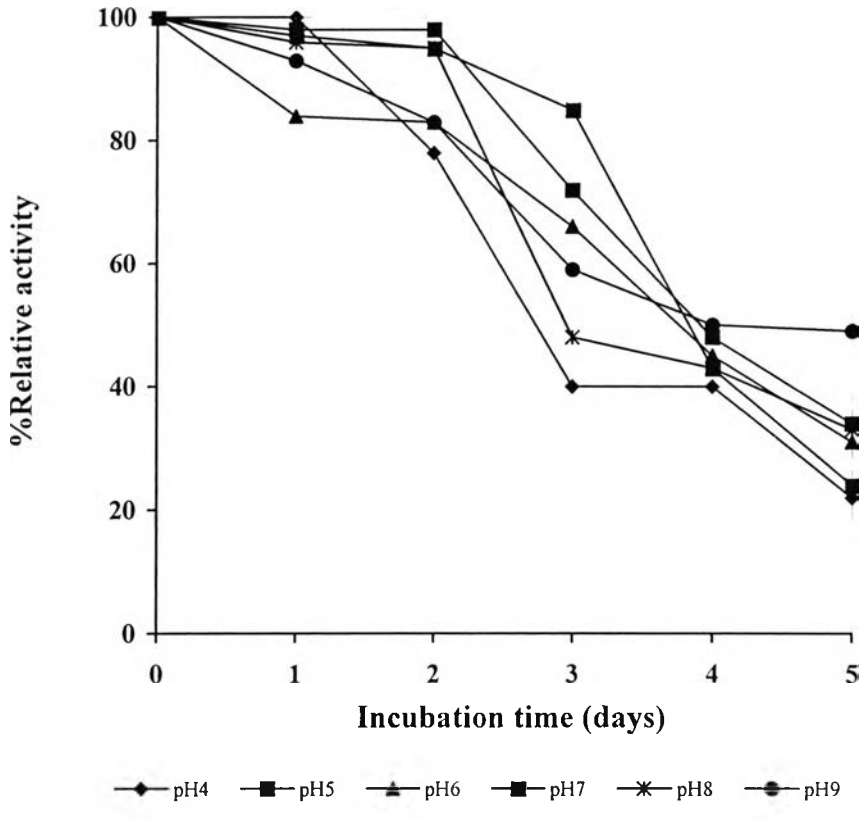


Figure 19. The pH stability of crude chitinolytic enzyme from *B. licheniformis* SK-1. The crude enzyme was incubated in various pH for 5 days, at 4 °C. At each day, it was assayed in 0.1 M phosphate buffer at 60 °C.

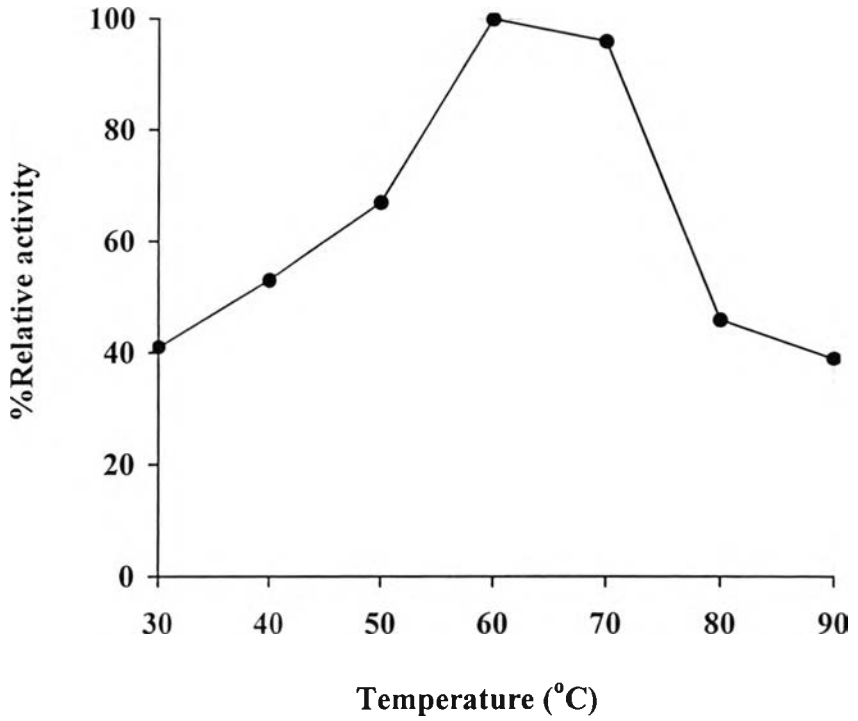


Figure 20. Optimum temperature of crude chitinolytic enzyme from *B. licheniformis* SK-1. Chitinolytic activity was measured at temperature range from 30-90 °C by colorimetric method when used colloidal chitin as substrate.

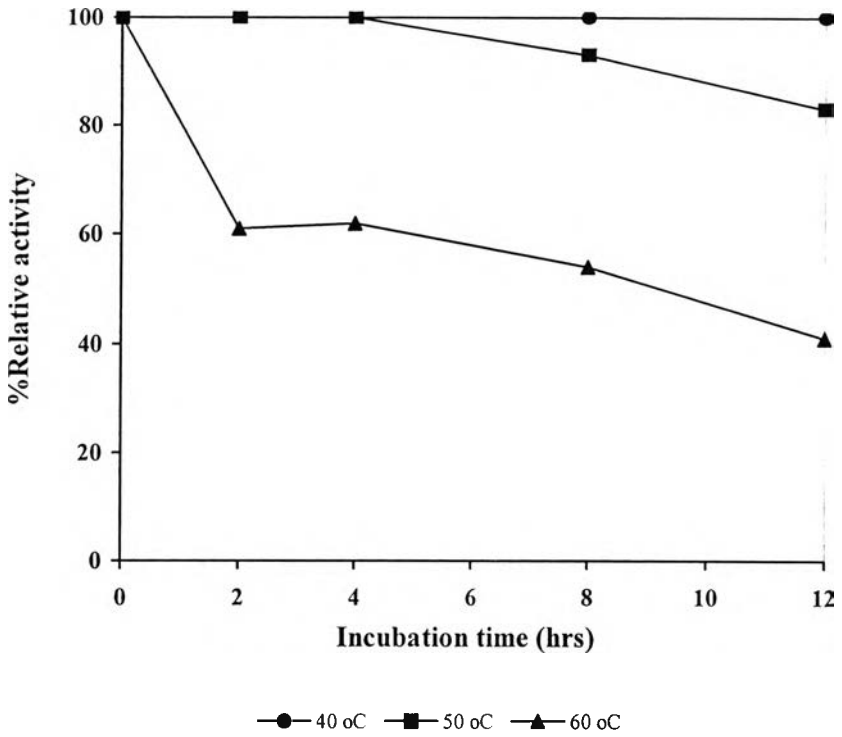


Figure 21. The temperature stability of crude chitinolytic enzyme from *B. licheniformis* SK-1. The crude enzyme was incubated at various temperatures in 0.05 M phosphate buffer pH 7.0 for 12 hrs and then assayed at time intervals in 0.1 M phosphate buffer pH 6.0 at 60 °C.

The chitinolytic activity on different substrates

An action of crude chitinase form *B. licheniformis* SK-1 on chitin and its related compounds was studied in this work. The hydrolysis of chitin and its related compounds with chitinase was examined at pH 6.0, 60 °C. Chitinolytic activity was assayed by determination of reducing sugar produced. Chitinase showed high activity on regenerated chitin, followed by colloidal chitin, powdered chitin, partially N-acetylated chitin, chitosan and flake chitin, respectively (Figure 22).

Products of chitinolytic degradation of colloidal chitin by crude enzyme from *B. licheniformis* SK-1

Digestion of colloidal chitin by chitinase resulted mainly in the production of N,N'-diacetylchitobiose and N-acetylglucosamine (Figure 23).

Detection of crude chitinolytic enzyme and determination of its molecular mass using SDS-PAGE in crude enzyme

The activity of chitinolytic in SDS-PAGE could be renatured (Trudel and Asselin, 1989). Thus, its molecular mass could be determined by comparison the mobility of protein bands with chitinolytic activity (Figure 24) to those of molecular mass markers. A concentrated crude chitinase from 48 hrs culture supernatant gave at least 8 chitinolytic activity bands with molecular mass of 72, 70, 66, 60, 58, 38.5, 30 and 20 kDa, respectively.

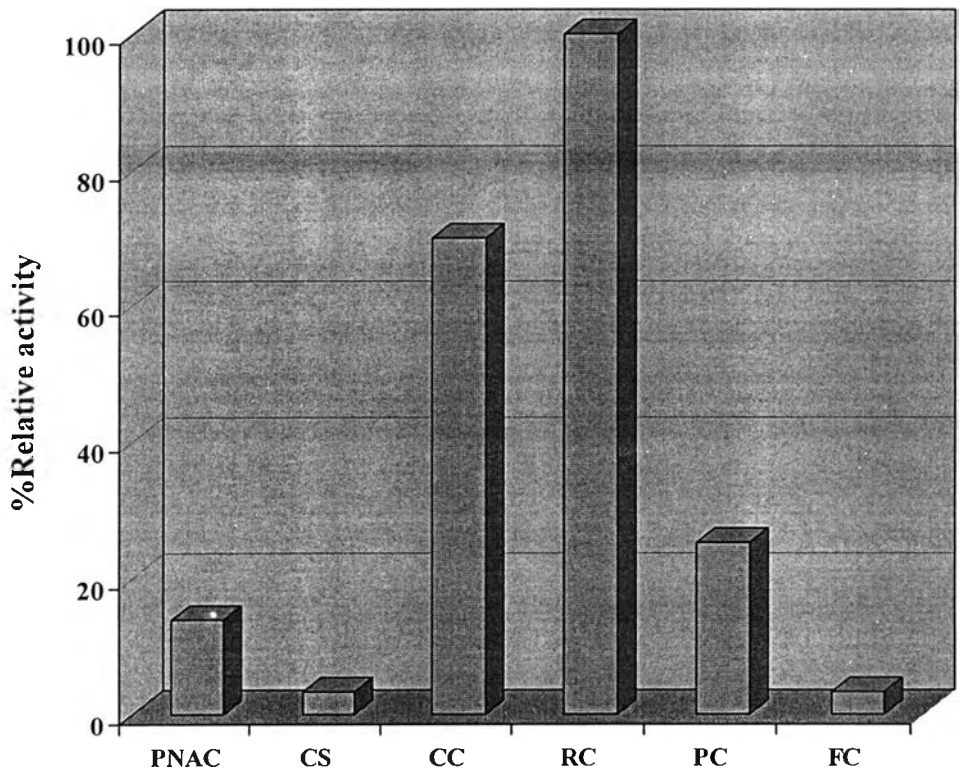


Figure 22. Crude chitinolytic activity from *B. licheniformis* SK-1 on different substrates. Chitinolytic activity was measured in 0.1 M phosphate buffer, pH 6.0 at 60 °C with different substrates.

PNAC = Partially N-acetylated chitin

CS = 80% Chitosan

CC = Colloidal chitin

RC = Regenerated chitin

PC = Powdered chitin

FC = Flaked chitin

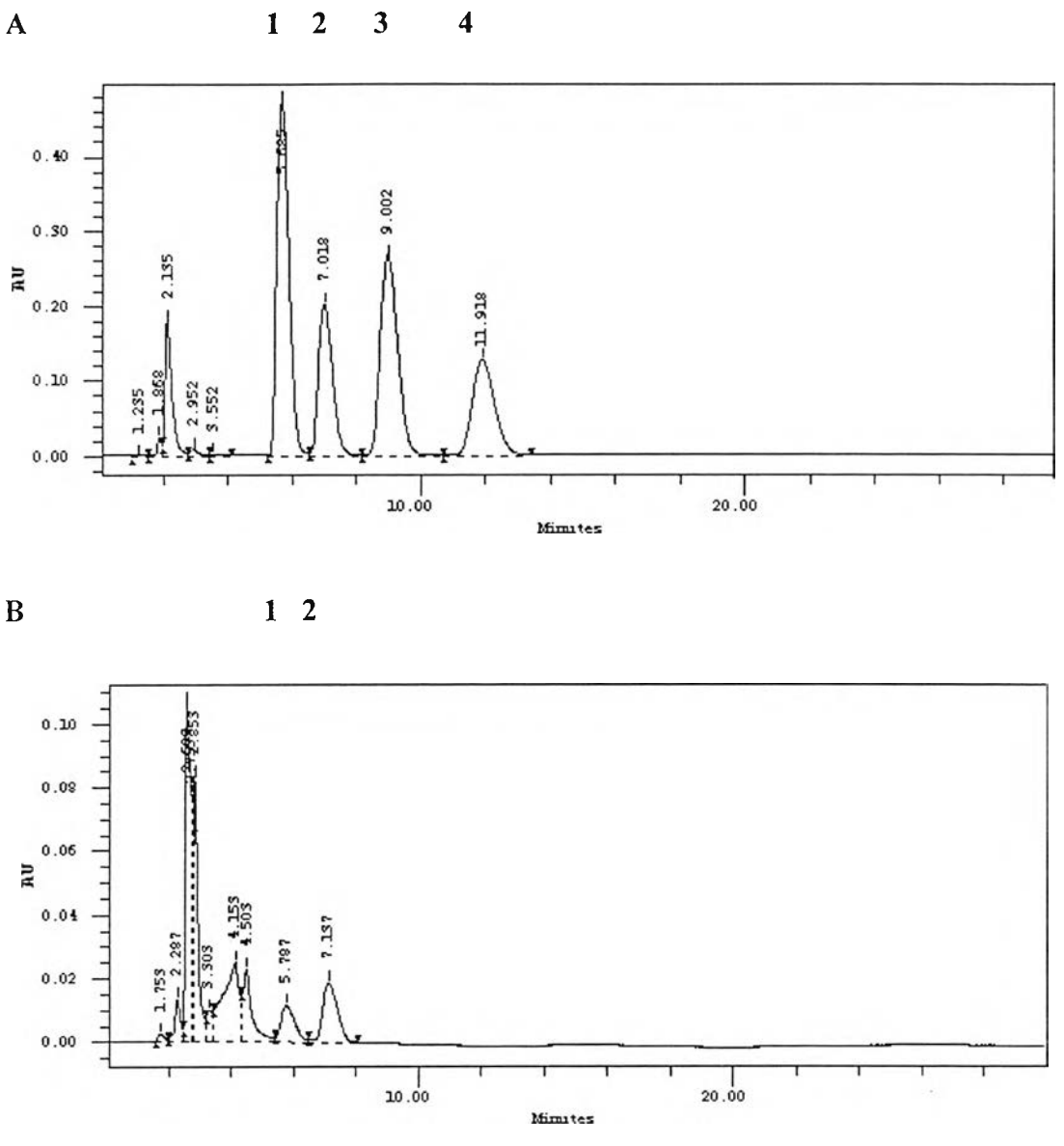


Figure 23. Digestion products of crude chitinolytic enzyme from *B. licheniformis* SK-1. Colloidal chitin was used as substrate for crude enzyme. The reaction was incubated in 0.1 M phosphate buffer pH 6.0 at 60 °C, for 12-24 hrs. Products were analyzed by HPLC.

Panel A; standard chitooligosaccharides

1: Monomer of N-acetylglucosamine

2: Dimer of N-acetylglucosamine

3: Trimer of N-acetylglucosamine

4: Tetramer of N-acetylglucosamine

Panel B; Products from crude enzyme chitinase using colloidal chitin as substrate

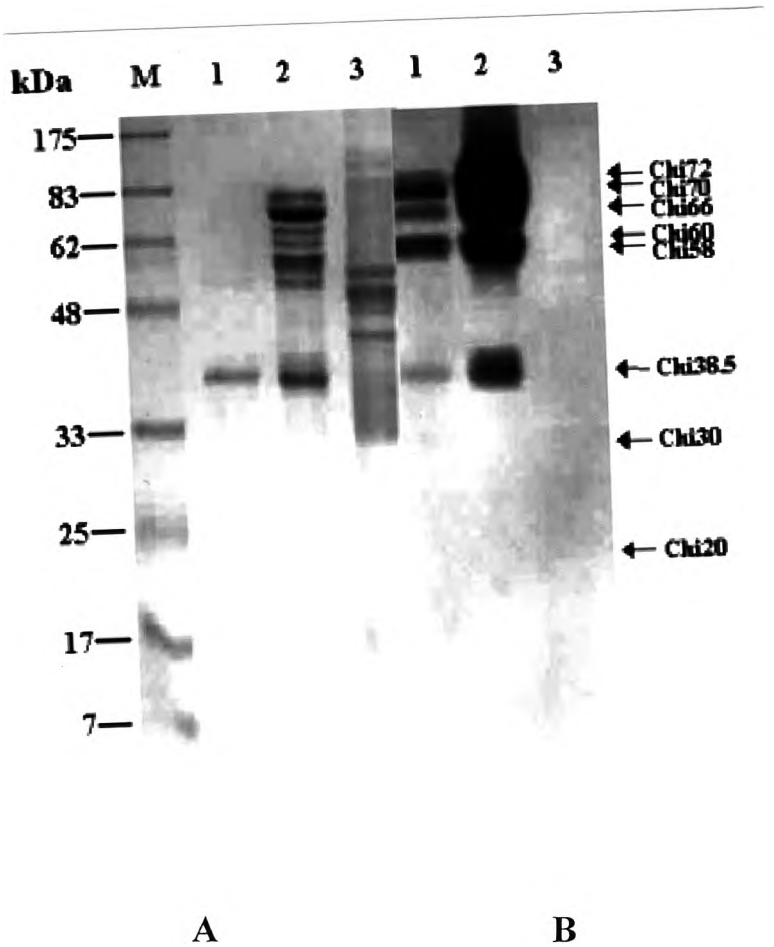


Figure 24. SDS-PAGE determination of protein with chitinolytic activity in the culture medium from *B. licheniformis* SK-1. *Bacillus licheniformis* SK-1 was grown in CCMM. The cells were removed after 2 days and the crude enzyme from culture fluid was used. Panel A: Protein stain; Panel B: Activity stain.

Lane M = Standard Protein

Lane 1 = Crude enzyme

Lane 2 = Affinity adsorped

Lane 3 = Unadsorped

Purification of *Bacillus licheniformis* chitinase

Partial purification of crude chitinase was performed by colloidal chitin affinity adsorption and DEAE cellulose column chromatography. Firstly, 2000 ml of supernatant from 2 days grown culture of *B. licheniformis* SK-1 was adsorbed by colloidal chitin affinity. The adsorbed protein was collected. The chitinases were further purified by passing this fraction through the DEAE-cellulose column. The chromatographic pattern of total protein (absorbance at 280 nm) and chitinase activity of fractions obtained from DEAE-cellulose are shown in Figure 25. Proteins were eluted from column by NaCl gradient concentration of 0-1.0 M in 25 mM Tris-HCl buffer, pH 7.5. There were two dominant peaks as shown in Figure 25. Chitinase peak1 (pooled, fraction 170-210) was eluted between 0.3-0.4 M NaCl and chitinase peak2 was eluted (pooled, fraction 220-260) by 0.45 M NaCl. The protein from peaks (P1 and P2) were analyzed for the enzymatic purity by using chitinolytic activity detection on native PAGE and SDS-PAGE, the proteins were detected by Coomassie blue staining. The table of purification was shown in Table 7.

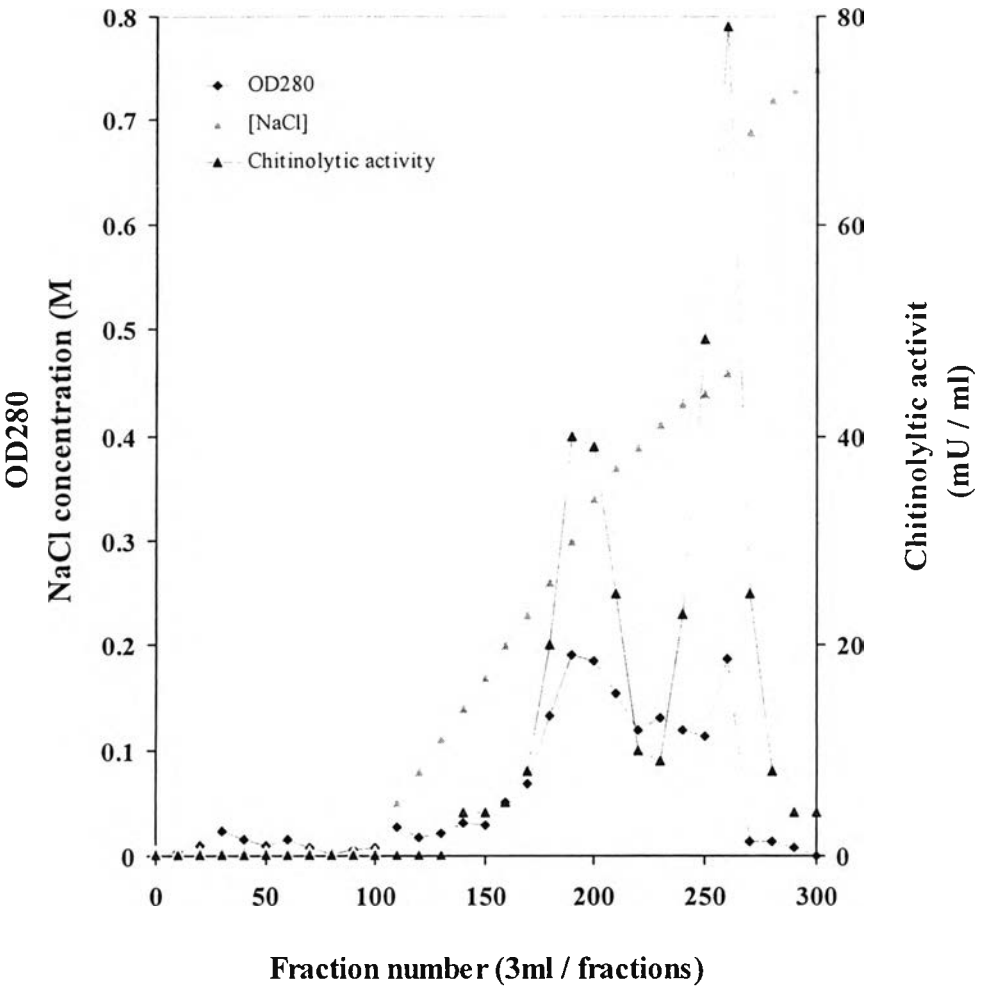


Figure 25. Chromatogram of protein from *B. licheniformis* SK-1 separated by DEAE cellulose column.

Table 7. Purification of chitinase from *B. licheniformis* SK-1.

Step	Protein (mg)	Activity (U)	Specific activity (U/mg protein)	Purification (fold)	Recovery (%)
Crude enzyme	98	190.00	1.95	1.0	100
Affinity fraction	16.75	127.25	5.46	2.8	64
DEAE column					
-P1	3.2	29	9.02	4.0	13
-P2	3.0	67.5	22.5	11.5	35

Specific activity = total activity (U) / mg protein

Purification = specific activity (step n) / specific activity (step 1)

% Recovery = total activity (step n) / total activity (step 1)

Characterization of partial purified chitinase enzyme

The chitinase was studied and assayed by the colorimetric method as previously described for its properties as follows:

Non-denaturing polyacrylamide gel electrophoresis

The partial purified chitinase gave a single protein band on native polyacrylamide gel electrophoresis (Figure 26).

Isoelectric focusing gel electrophoresis

The partial purified enzyme gave a single protein band on isoelectric focusing gel electrophoresis, which corresponded to the isoelectric point of 4.62 (Figure 27).

Glycoprotein staining (PAS staining)

The partial purified chitinase showed a positive with PAS stained in native PAGE (Figure 28), but not in SDS-PAGE (data not shown).

Molecular weight of partial purified chitinase

The molecular weight of partial purified chitinase was estimated by SDS-PAGE from Figure 29. The chitinase showed a single major band with molecular weight 72 kDa and 2 minor bands with molecular weight 70 and 58 kDa on 10% separating gel of SDS-PAGE.

Optimum and pH stability of partial purified chitinase activity

The optimum pH of partial purified chitinase was determined. Chitinase was assayed at 50 °C in buffer with pH ranging from 3-10. Activities at different pH were shown in Figure 30. Reducing sugar of a colloidal chitin suspension during chitinolysis was used to determine the activity. The partial purified chitinase revealed two activity optima at pH 6 and 8.

The stability of partial purified enzyme at different pH was also determined. The analysis of the chitinolytic enzyme activity of *B. licheniformis* SK-1 indicated that it was more stable at pH 7.0. The activity decreased about 28, 37 and 23% when the

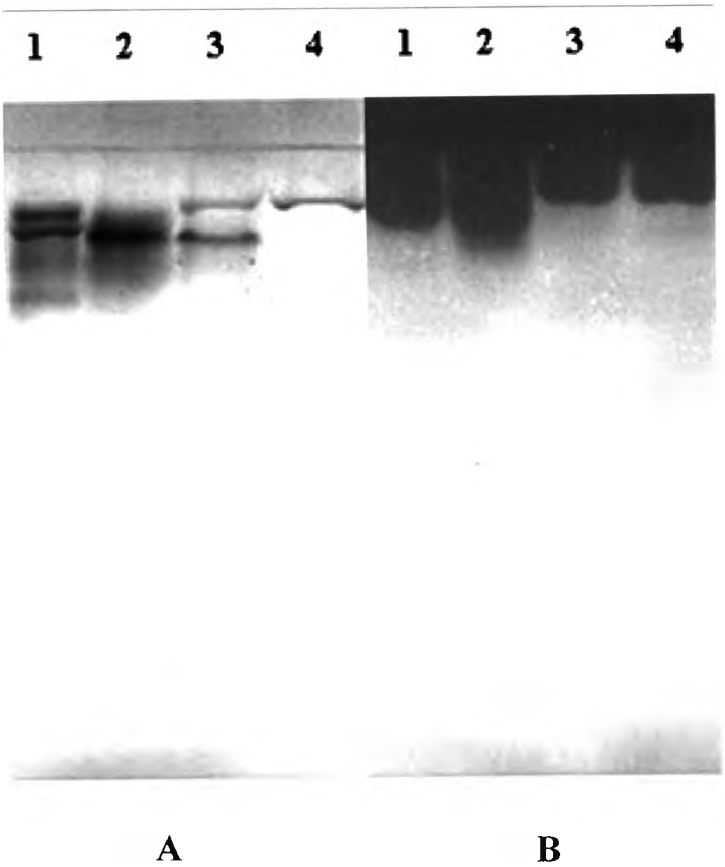


Figure 26. Native gel of each step of chitinase purification from *B. licheniformis* SK-1. Panel A: protein stain, Panel B: activity stain.

Lane 1 = Crude enzyme

Lane 2 = Affinity adsorption

Lane 3 = DEAE peak1

Lane 4 = DEAE peak2

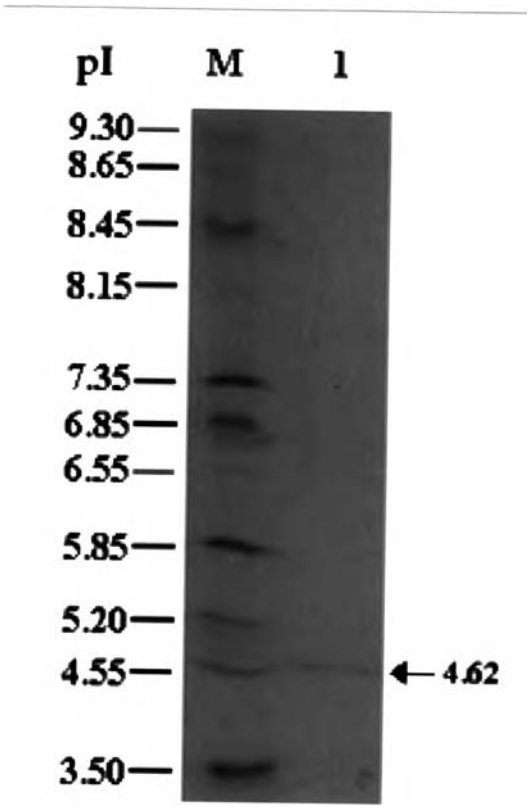


Figure 27. Native IEF pattern of the partial purified chitinase from *B. licheniformis* SK-1.

Lane M = IEF standard marker

Lane 1 = partial purified chitinase

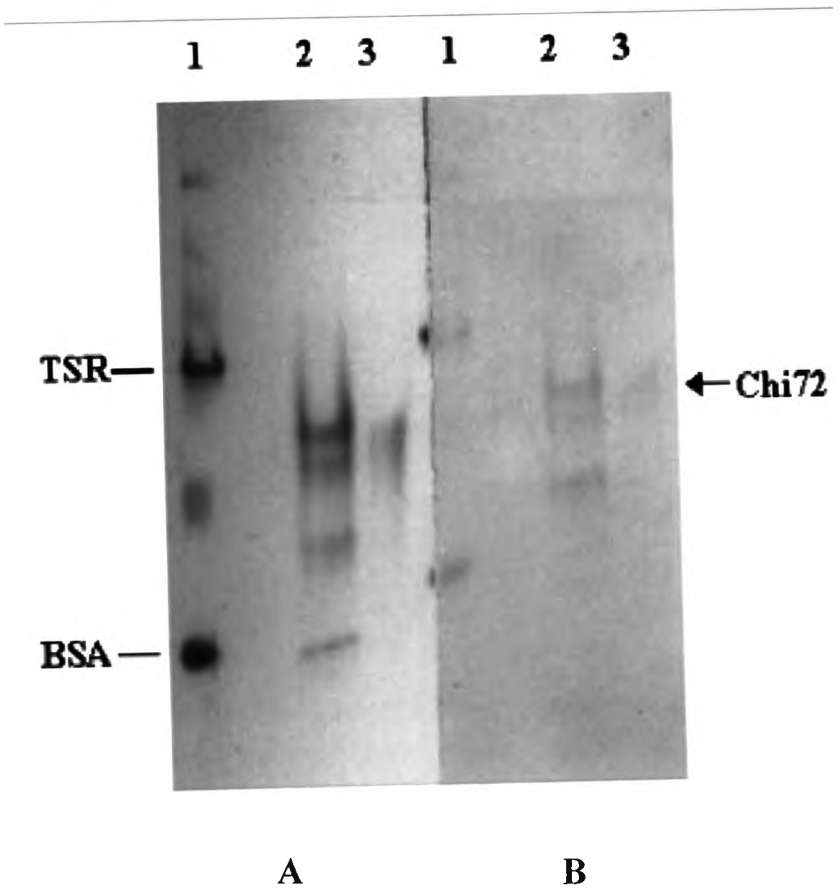


Figure 28. PAS staining of the partial purified chitinase from *B. licheniformis* SK-1 on native PAGE. Panel A: protein stain; Panel B: PAS stain.

Lane 1 = Positive control (transferin, BSA)

Lane 2 = Crude enznyme *B. licheniformis* SK-1.

Lane 3 = Partial purified chitinase from *B. licheniformis* SK-1.

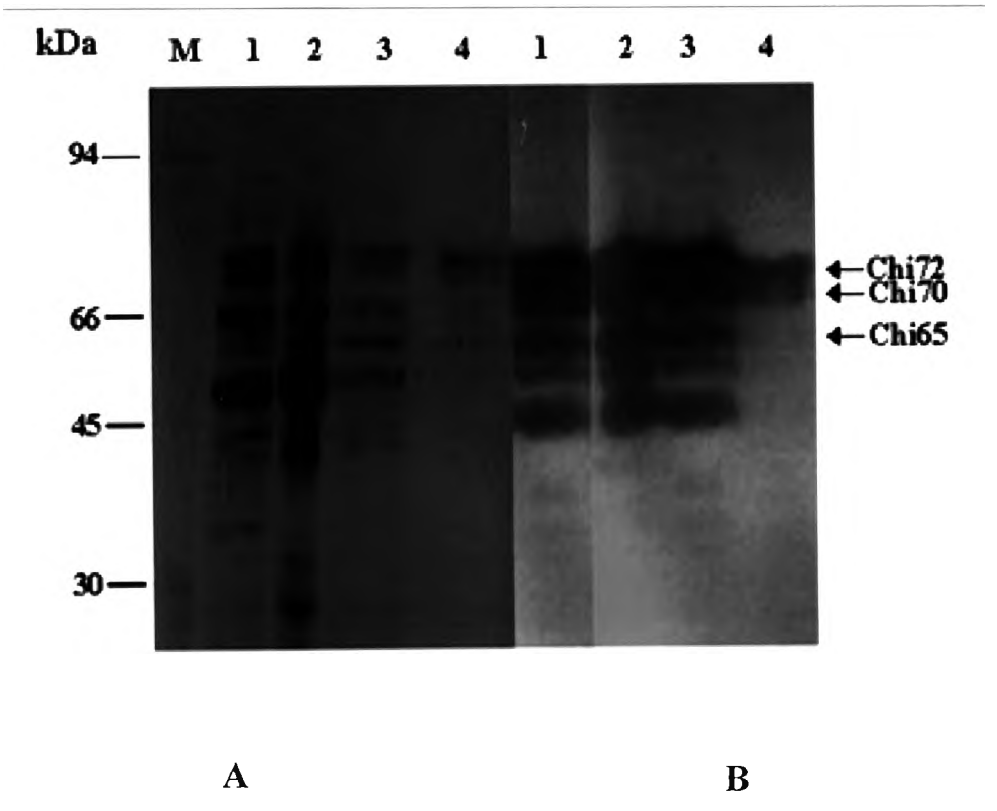


Figure 29. SDS-PAGE of enzyme from *B. licheniformis* SK-1. Panel A: protein stain; Panel B: activity stain.

Lane M = Standard protein

Lane 1 = Crude enzyme from *B. licheniformis* SK-1

Lane 2 = Affinity adsorbed protein

Lane 3 = DEAE peak1

Lane 4 = DEAE peak2

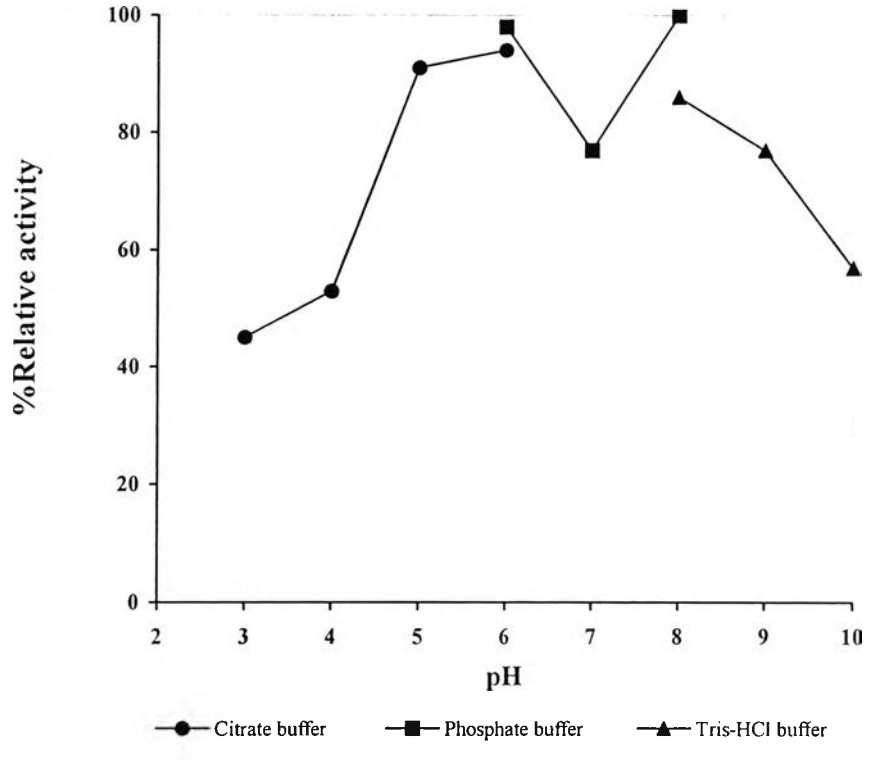


Figure 30. Optimum pH of partial purified chitinase enzyme from *B. licheniformis* SK-1. Chitinolytic activity was measured at pH range of 3-10 by colorimetic method when used colloidal chitin as substrate.

enzyme was kept at 4 °C for 5 days in pH 5.0, 6.0 and 7.0, respectively. The activity of partial purified enzyme rapidly decreased when it kept incubated in acidic and alkali pHs. We found that 47% of the activity was lost when it was kept at pH 4.0 for 2 days (Figure 31).

Optimum and temperature stability for partial purified chitinase activity

The optimum temperature of partial purified chitinase was determined in phosphate buffer, at pH 6.0, in the range of 40-70 °C. The chitinase activity was assayed by colorimetric method. The partial purified chitinase activity exhibited a broad temperature range between 40 to 70 °C, with an optimum at 55 °C (Figure 32).

The stability of partial purified chitinase was analyzed. The activity of chitinolytic enzyme slightly decreased to 94% and 83% when incubated at 40 °C and 50 °C for 12 hrs, respectively. Only 22% of enzyme activity was detected after it had been incubated at 60 °C for 12 hrs. The enzyme activity reduced 62 percent when the enzyme was kept at 60 °C for 2 hrs (Figure 33).

The kinetic of enzyme

The enzyme kinetic parameters such as Michaelis's constant (K_m) and maximum velocity (V_m) of *B. licheniformis* SK-1 chitinolytic enzyme was determined by using various substrate concentrations, i.e., 0.01, 0.05, 0.1, 0.2, 0.4 mg/ml (w/v, dry weight) colloidal chitin substrate in 100 mM phosphate buffer pH 6.0 at 55 °C. Lineweaver- Burk's equation and protein concentration of enzyme that used in the reaction, the Michaelis's constant (K_m) and maximum velocity (V_m) of enzyme was measured. From these results, K_m was obtained from reciprocal absolute value of intersection point of 1/[S] on X-axis in figure and was calculated to be 0.23 mg/ml (0.023%, w/v) while V_m was obtained from reciprocal value of intersection point of 1/ [V] on Y-axis in Figure 34 and was calculated to be 0.45 U/ml. Since the 450 x 10^{-3} μ moles of product was produced by enzyme solutions which contained 6.4 μg of protein as measured by Bradford's method. Results of V_m indicated that the enzyme had activity equal to 7.03 U/mg protein.

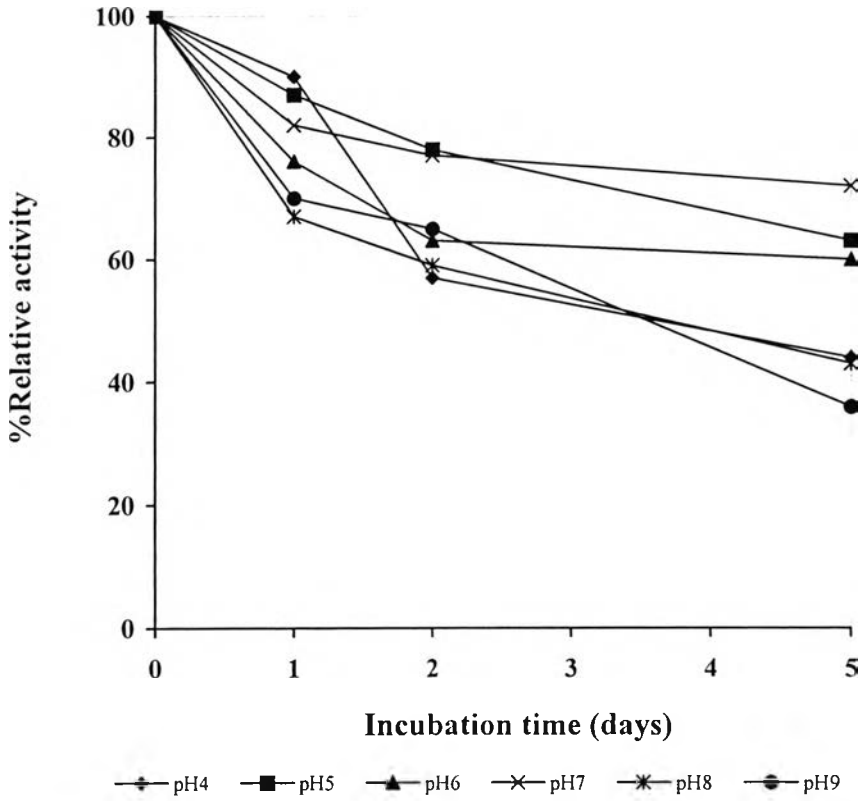


Figure 31. The pH stability of partial purified chitinase enzyme from *Bacillus licheniformis* SK-1. The crude enzyme was incubate at various pHs for 5 days, at 4 ° C and then samples were remained and assayed everyday in 0.1 M phosphate buffer at 55 °C.

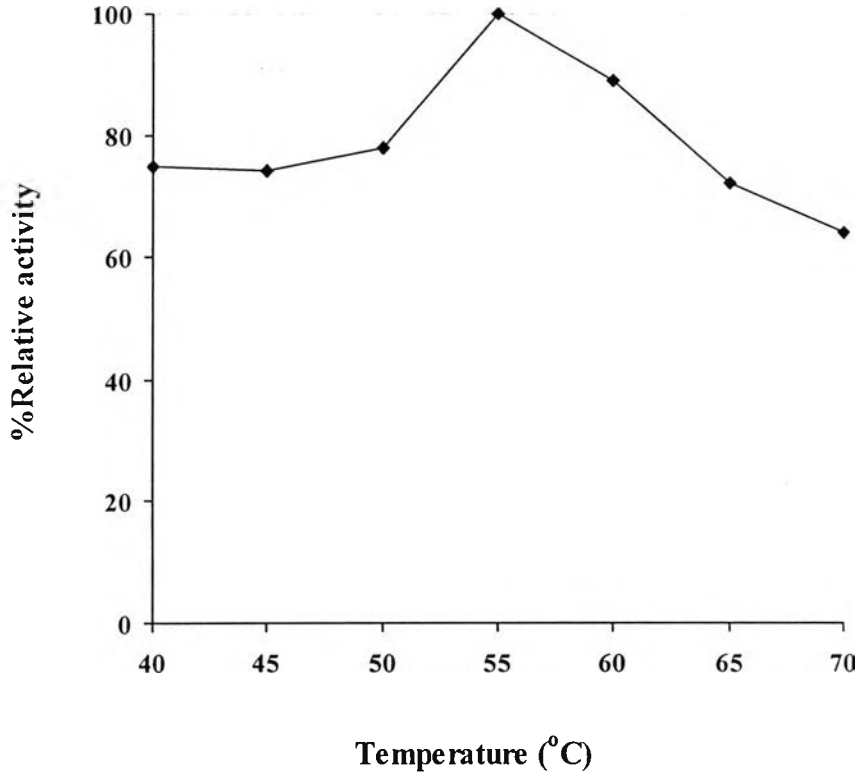


Figure 32. Optimum temperature of partial purified chitinolytic enzyme from *Bacillus licheniformis* SK-1. Chitinolytic activity was measured at temperature range from 30-90 °C by colorimetric method when colloidal chitin was used as substrate.

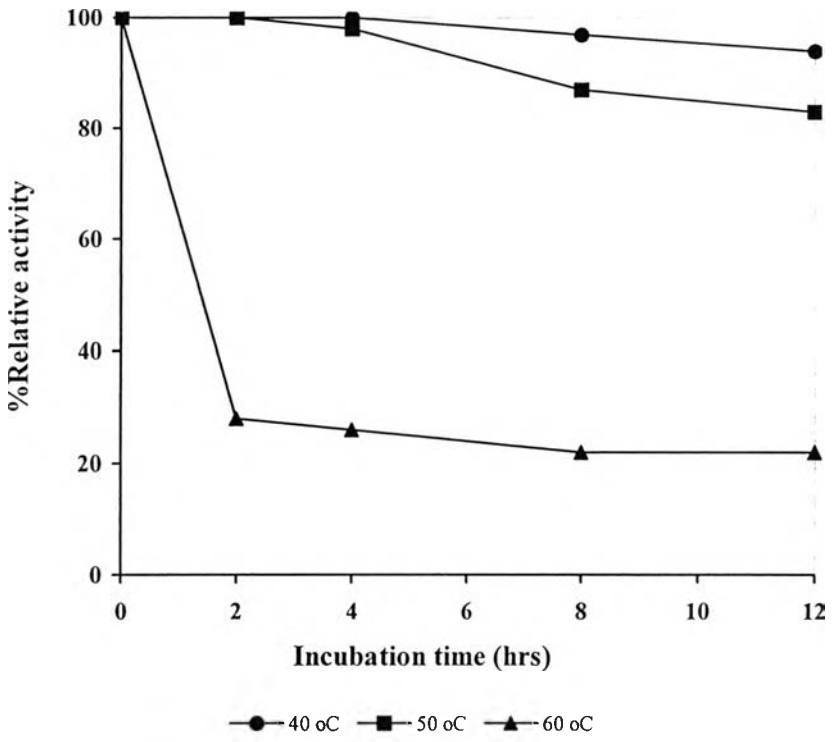
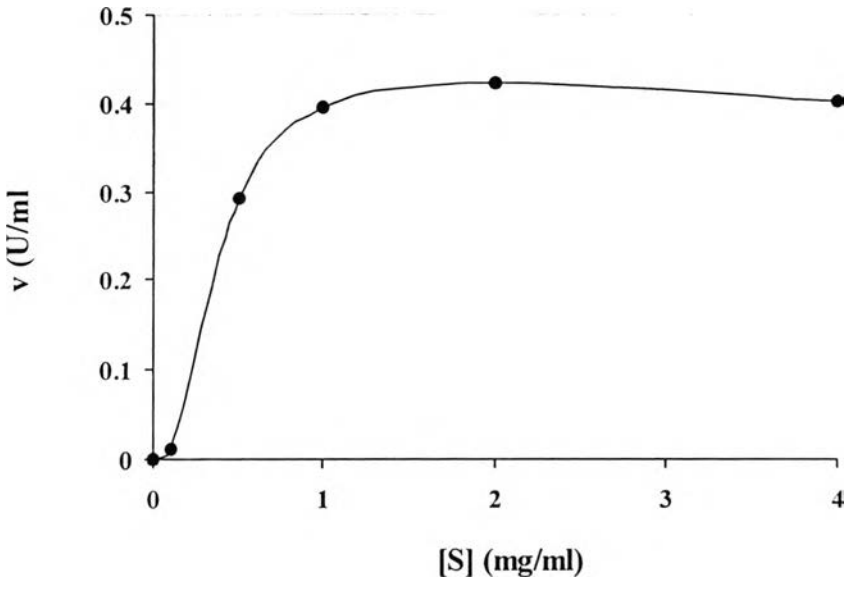


Figure 33. The temperature stability of partial purified chitinolytic enzyme from *B. licheniformis* SK-1. The crude enzyme was kept at various temperature for 12 hrs and then assay at different time every 2 hrs for 12 hrs, in 0.1 M phosphate buffer pH 7.0 at 55 °C.



B

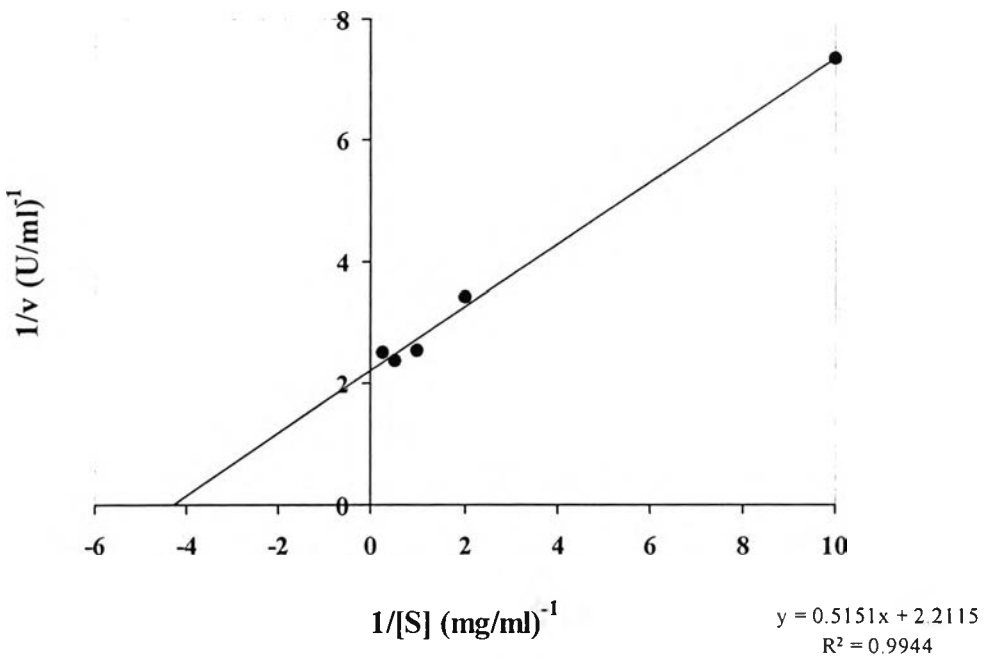


Figure 34. Dependence of the reaction rate of chitinase on the substrate concentration.

A) Saturation curve
B) Lineweaver-Burk plot

Shotgun cloning

Partially *Pst*I-digested fragments of chromosomal DNA of *B. licheniformis* SK-1 were ligated with dephosphorylated *Pst*I-digestd pBSSK$^-$ and transformed into *E. coli* JM109. Transformants carrying chitinase gene was selected by the formation of clear zone around the colonies on CCMM plate. About 5,000 transformant colonies were screened. We did not find any positive clones.

PCR amplification of the whole gene fragment

The whole gene fragment was amplified by using the forward and reverse primers as described in chapter II. About 2 kb fragment was produced as shown in Figure 35.

T-A cloning and transformation

The whole gene fragments were ligated with pGEM-Teasy and transformed into *E. coli* JM109 by electroporation. Transformants carrying chitinase gene was selected by the formation of clear zone around the colonies on CCMM plate. The white colonies which produced clear zone were picked for plasmid extraction. The positive clone contained a plasmid with a 2.0 kb inserted fragment, designated as pSKChi 66. The plasmid was retransformed into other hosts include *E. coli* JM109 *E. coli* XL-1Blue and *E. coli* DH5α (Figure 36).

After retransformation of pSKChi66 into the other strains of *E. coli*, clear zone were detected in all hosts, XL-1Blue and DH5α. The expression of Chi66 was better in XL-1 Blue than the original JM109 and DH5α cells, respectively.

Analysis of *chi66* gene

Homology of *chi66* using BLAST program from GenBank

From sequencing results, using BLAST program to search for homologues of *chi66* gene and protein from other bacteria. The amino acid comparison indicated that Chi66 is 84% similar to chitinase from *B. subtilis* and *B. licheniformis* TP-1.

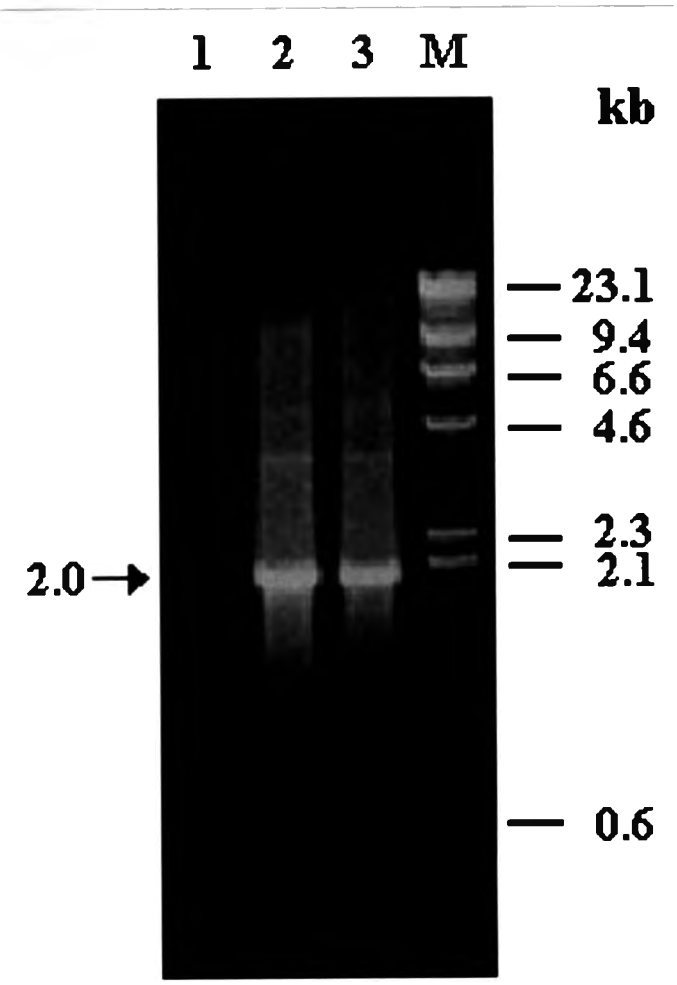


Figure 35. Agarose gel electrophoresis of PCR products from whole chitinase gene amplification

Lane M = Standard DNA marker λ/*Hin*dIII, 100 bp ladder marker

Lane 1 = Negative control (from *Peanibacillus* sp. BT)

Lane 2 = Positive control (from *B. licheniformis* RP-1)

Lane 3 = PCR products from *B. licheniformis* SK-1

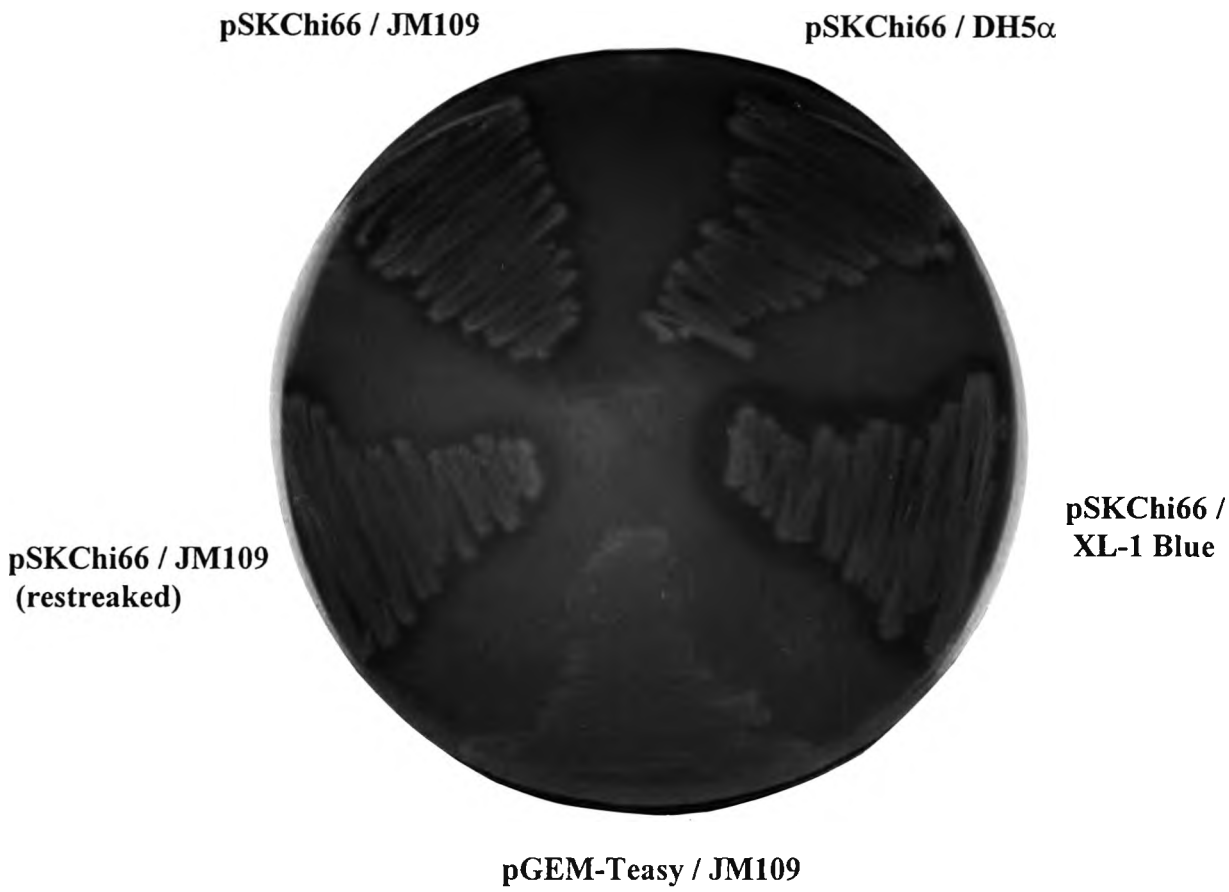


Figure 36. Formation of clear zone in various host strains. JM109 harboring plasmids, pSKChi66 was grown on CCMM for 4 days.

Manipulation of DNA sequence

GENETYX-WIN was used to analysis *chi66* gene in this following: find one open reading frame (ORF), is 1,797 bp (Figure 37) which encoded a polypeptide of 598 amino acid (Figure 37), which correspond to 66 kDa with isoelectric point of 5.02.

Multialignment by CLUSTAL W

The deduced amino acid from Chi66 were align with other chitinase by CLUSTAL W. The amino acid comparison was shown in Figure 38.

Protein prediction by SWISS-Model Protein Modeling

Homology modeling of the structure of Chi66 was accomplished by SWISS Model Protein Modeling (SWISS-MODEL version 36.002, Guex and Peitsch, 1995; Peitsch, 1996; Peitsch, 1997) as shown in Figure 39.

Partial characterization of crude chitinase from Chi66

Optimum pH for crude chitinase activity from Chi66

The optimum pH of chitinase was determined. Chitinase was assayed at 50 °C in buffer with pH ranging from 3-10. Activity at different pHs are shown in Figure 40. Reducing sugar production of a colloidal chitin suspension during chitinolysis was used to determine the activity. The crude chitinase from Chi66 revealed two activity optima at pH 5 and 9.

Optimum and temperature stability for crude chitinase activity from Chi66

The optimum temperature of crude chitinase from Chi66 was determined at pH 5.0, in the rang of 30-90 °C. The chitinase activities were assayed by colorimetric method. The results were shown in Figure 41. Crude chitinase activity from Chi66 exhibited a broad temperature range between 50 to 70 °C, with optimum at 60 °C.

The stability of chitinase was analyzed. The activity of chitinolytic enzyme slightly decreased to 60% and 72% when incubated at 40 °C and 50 °C, respectively for 12 hrs. About 22% of enzyme activity was detected after it had been incubated at

```
        10         20         30         40         50         60         70         80         90
TGTTTTCCCTTGTTGTCTTCAATGTATCTGCTGCTATTAGATGACAAGGAAAAATATAAAACCAGCAAAAAAGGCGGTGAGAAAAAAGAG
       100        110        120        130        140        150        160        170        180
AGTTCTAGTTTCATAGCTTGCCAAAAAATTGCTTGTAAAGGAGAATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTC
                                                  M  K  I  V  L  I  N  K  S  K  K  F  F  V  F  S
      190        200        210        220        230        240        250        260        270
TTTCATTTTTGTTATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAACTATAAAATCATCGGCTA
 F  I  F  V  M  M  L  S  L  S  F  V  N  G  E  V  A  K  A  D  S  G  K  N  Y  K  I  I  G  Y
       280        290        300        310        320        330        340        350        360
CTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGGGATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGA
 Y  P  S  W  G  A  Y  G  R  D  F  Q  V  W  D  M  D  V  S  K  V  S  H  I  N  Y  A  F  A  D
       370        380        390        400        410        420        430        440        450
TATTTGCTGGGAGGGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGATGAAAACGGAGTGATCGA
 I  C  W  E  G  R  H  G  N  P  D  P  T  G  P  N  P  Q  T  W  S  C  Q  D  E  N  G  V  I  D
       460        470        480        490        500        510        520        530        540
CGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGACGCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGG
 A  P  N  G  T  I  V  M  G  D  P  W  I  D  A  Q  K  S  N  P  G  D  V  W  D  E  P  I  R  G
       550        560        570        580        590        600        610        620        630
CAACTTTAAACAATTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGGACTTGGTCTAACCGCTT
 N  F  K  Q  L  L  K  L  K  K  S  H  P  H  L  K  T  F  I  S  V  G  G  W  T  W  S  N  R  F
       640        650        660        670        680        690        700        710        720
TTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCCGCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGA
 S  D  V  A  A  D  P  A  A  R  E  N  F  A  A  S  A  V  E  F  L  R  K  Y  G  F  D  G  V  D
       730        740        750        760        770        780        790        800        810
TCTTGACTGGGAATATCCGGTCAGCGGAGGATTGCCGGGGAACAGCACACGTCCGGAAGATAAAAGAAACTACACGCTGCTCCTGCAAGA
 L  D  W  E  Y  P  V  S  G  G  L  P  G  N  S  T  R  P  E  D  K  R  N  Y  T  L  L  L  Q  E
       820        830        840        850        860        870        880        890        900
GGTGCGCAAAAAACTTGACGCTGCAGAAGCAAAAGACGGCAAGGAATACTTGCTGACGATCGCATCCGGCGCAAGTCCCGATTATGTAAG
 V  R  K  K  L  D  A  A  E  A  K  D  G  K  E  Y  L  L  T  I  A  S  G  A  S  P  D  Y  V  S
       910        920        930        940        950        960        970        980        990
CAACACTGAGCTCGATAAAATCGCTCAAACCGTGGATTGGATTAACATTATGACCTATGACTTTAATGGCGGATGGCAAAGCATAAGCGC
 N  T  E  L  D  K  I  A  Q  T  V  D  W  I  N  I  M  T  Y  D  F  N  G  G  W  Q  S  I  S  A
      1000       1010       1020       1030       1040       1050       1060       1070       1080
CCATAATGCACCGCTGTTCTATGATCCAAAAGCGAAAGAAGCAGGCGTTCCAAACGCTGAGACCTACAATATTGAAAACACTGTGAAACG
 H  N  A  P  L  F  Y  D  P  K  A  K  E  A  G  V  P  N  A  E  T  Y  N  I  E  N  T  V  K  R
      1090       1100       1110       1120       1130       1140       1150       1160       1170
CTACAAGGAAGCCGGTGTCAAGGGTGACAAATTAGTGCTTGGAACACCGTTCTACGGAAGGGGCTGGAGCGGTTGTGAACCAGGGGGGCA
 Y  K  E  A  G  V  K  G  D  K  L  V  L  G  T  P  F  Y  G  R  G  W  S  G  C  E  P  G  G  H
      1180       1190       1200       1210       1220       1230       1240       1250       1260
CGGAGAATATCAGAAATGCGGACCGGCTAAAGAAGGGACATGGGAAAAGGGCGTATTCGATTTTTCAGATCTTGAAAGGAACTATGTGAA
 G  E  Y  Q  K  C  G  P  A  K  E  G  T  W  E  K  G  V  F  D  F  S  D  L  E  R  N  Y  V  N
      1270       1280       1290       1300       1310       1320       1330       1340       1350
TCAAAACGGCTATAAAAGGTATTGGAACGATCAAGCAAAAGTGCCGTTTTTGTATAATGCGGAAAATGGCAATTTCATCACTTATGATGA
 Q  N  G  Y  K  R  Y  W  N  D  Q  A  K  V  P  F  L  Y  N  A  E  N  G  N  F  I  T  Y  D  D
      1360       1370       1380       1390       1400       1410       1420       1430       1440
TGAACAATCATTCGGCCACAAAACGGATTTTATTAAAGCAAACGGATTAAGCGGAGCAATGTTCTGGGATTTCAGCGGCGATTCCAATCG
 E  Q  S  F  G  H  K  T  D  F  I  K  A  N  G  L  S  G  A  M  F  W  D  F  S  G  D  S  N  R
      1450       1460       1470       1480       1490       1500       1510       1520       1530
GACGCTTCTCAATAAATTGGCAGCCGATTTAGATTTTGCACCGGACGGAGGCAATCCGGAGCCGCCTTCATCGGCACCTGTGAATGTGCG
 T  L  L  N  K  L  A  A  D  L  D  F  A  P  D  G  G  N  P  E  P  P  S  S  A  P  V  N  V  R
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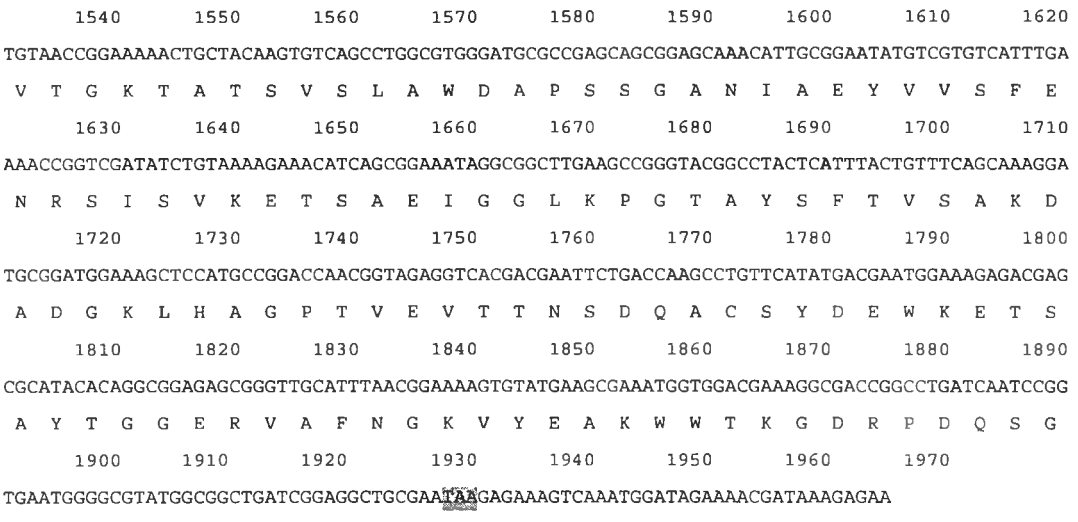

Figure 37. Nucleotide and deduced amino acid sequence of the *chi66* gene. Numbering of amino acids in the sequence started with the beginning of the coding sequence, and the number are shown under the numbering for the nucleotide sequence. The position –10 and –35, putative promoter sequences, are underlined. The putative Shine-Dalgarno (SD) sequence is under doublelined. Active site is shown by dotted line. This figure was created using GENETYX-WIN version 3.1.

```
BL          MNIVLVNKSKKFFVFSFIFVMLLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRNFQVW 60
Chi65       MKIVLINKSKKFFVFSFIFVMMLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDFQVW 60
Chi66       MKIVLINKSKKFFVFSFIFVMMLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDFQVW 60
BC          MNIVLVNKSKKFFVFSFIFVMLLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRNFQVW 60
BS          MKKVFSN--KKFLVFSFIFAMILSLSFFNGESAKASSDKSYKIIGYYPSWGAYGRDFQVW 58
            *: *: *.:***:******.*:*****.*** ***.*.*.***************:****

BL          DMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWID 120
Chi65       DMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWID 120
Chi66       DMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWID 120
BC          DMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWID 120
BS          DMDASKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDVPNGSIVMGDPWID 118
            ***.*****************************************.***:*********

BL          AQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPVARGNFA 180
Chi65       AQKANPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARENSA 180
Chi66       AQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARENFA 180
BC          AQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPVARGNFA 180
BS          VQKSNAGDTWDEPIRGNFKQLLKLKKNHPHLKTFISVGGWSWSNRFSDVAADPAARENFA 178
            .**:*.**.*****************.*************:************.** * *

BL          ASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGK 240
Chi65       ASPVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGK 240
Chi66       ASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGK 240
BC          ASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQECVKNLMLQKQRTAR 240
BS          ASAVNFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQDVREKLDAAEAKDGK 238
            **.*:*****************************************:  ::*   : : .:

BL          EYLLTIASGASDRYVSNTELDKIAQTVDWININIMTYDFNGGWQSISAHNAALFYDPKAKEA 300
Chi65       EYLLTIASGASPDYVSNTELDKIAQTVDWININIMTYDFNGGWQSISAHNAPLFYDPKAKEA 300
Chi66       EYLLTIASGASPDYVSNTELDKIAQTVDWININIMTYDFNGGWQSISAHNAPLFYDPKAKEA 300
BC          EYLLTIASGASPEYVSNTELDKIAQTVDWININIMTYDFNGGWQSISAHNAPLFYDPKAKEA 300
BS          KYLLTTVSGASPEYVSNTELDKIAETVDWININIMTYDFNGGWQSISAHNAPLFYDPKAKEA 298
            :**** .****  ***********:************************.**********

BL          GVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYEGAGAVVNPAATENIRSADRRKKGRG 360
Chi65       GVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRAGAVVNPGGTENIRNADRLKKGHG 360
Chi66       GVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSGCEPGGHGEYQKCGPAKEGTW 360
BC          GVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSGCESGGHGEYQKCGPAKEGTW 360
BS          GVPNAETFNIESTVKRYKEAGVKADKLVLGTPFYGRGWSNCEPADNGEYQKCGPAKEGTW 358
            *******:***.***********.**********  . :  :..  : :...  *:*

BL          KMEYSTFQILKRTYVNQNGYKRYWNDQAKVPFLYNAENGNFITYDDEQSFGHKTDFIKAN 420
Chi65       KRAYSIFQILKGTYVNQNGYKRYWNDQAKVPFLYNAENGNFITYDDEQSFGHKTDFIKAN 420
Chi66       EKGVFDFSDLERNYVNQNGYKRYWNDQAKVPFLYNAENGNFITYDDEQSFGHKTDFIKAN 420
BC          ENGVFDFSDLEKNYVNQNGYKRYWNDQAKVPFLYNAENGNFITYDDEQSFGHKTDFIKAN 420
BS          EKGVFDFSDLEKNYINKNGYKRYWNDRAKVPFLYNAENGNFITYDDEESYGYKTDLIQSN 418
            :     *. *: .*:*:*********:********************:*:*:***:*::*
```

```
BL              GLSGAMFWDFSGDSNRTLLNKLAADLDFAPDGGNPEPPSSAPVNVRVTGKTATSVSLAWD 480
Chi65           GLSGAMFWDFSGDSNRTLLNKLAADLDFAPDGGNPEPPSSAPVNVRVTGKTATSVSLAWD 480
Chi66           GLSGAMFWDFSGDSNRTLLNKLAADLDFAPDGGNPEPPSSAPVNVRVTGKTATSVSLAWD 480
BC              GLSGAMFWDFSGDSNPTLLNKLAAEFKFCTR---------------------------- 451
BS              GLSGAMFWDFSGDSNQTLLNKLAADLGFAPGGGNPEPPSSAPDNLRVTEKTATSISLAWD 478
                *************** ********:: *.. ..........: .  : :::: : : .

BL              AASSGTNITEYVVSFESRSISVKETSAEIGNLNRGTAYSFTVSAKDADGELHTGPTVKVT 540
Chi65           APSSGANIAEYVVSFENRSISVKETSAEIGGLKPGTAYSFTVSAKDADGKLHAGPTVEVT 540
Chi66           APSSGANIAEYVVSFENRSISVKETSAEIGGLKPGTAYSFTVSAKDADGKLHAGPTVEVT 540
BC              ------------------------TEAIRNRLHP-HLWNVLVIRKNCY---KCQPGVGCA 483
BS              APSDGANIAEYVLSYEGGAVSVKDTSATIGQLKPNTTYSFTVSAKDADGKLHTGPTIEAA 538
                :.:...:. :.   : .. : : ..*.*  . *: .  :.. *  *:. .. :  * :  :

BL              TNSDQACSYDEWKETNAYTGGERVAFNGKVYEAKWWTKGDRRINPVNGAYGGWSEAANNR 600
Chi65           TNSDQACSYDEWKETSAYTGGERVAFNGKVYEAKWWTKGDRLINPVNGAYGG-------- 592
Chi66           TNSDQACSYDEWKETSAYTGGERVAFNGKVYEAKWWTKGDR--PDQSGEWGVWRLIGGCE 598
BC              E------------QRNKHYGICRVI---------------------------------- 496
BS              TNSDQTCGYNEWKDTAVYTGGDRVVFNGKVYEAKWWTKGEQ--PDQAGESGVWKLIGDCK 596
                 .:..:.. .. .:   : *  **  ...  .::  :...      .  .     ..

BL              KSNG 604
Chi65           ----
Chi66           ----
BC              ----
BS              ----
```

Figure 38. Amino acids sequences alignment chitinase from *chi66* gene with other chitinase. The asterisks indicated identical residues, colon indicated that very similar residues and dot indicated similar residues. The figure was created using CLUSTAL W multiple sequence alignment version 1.82.

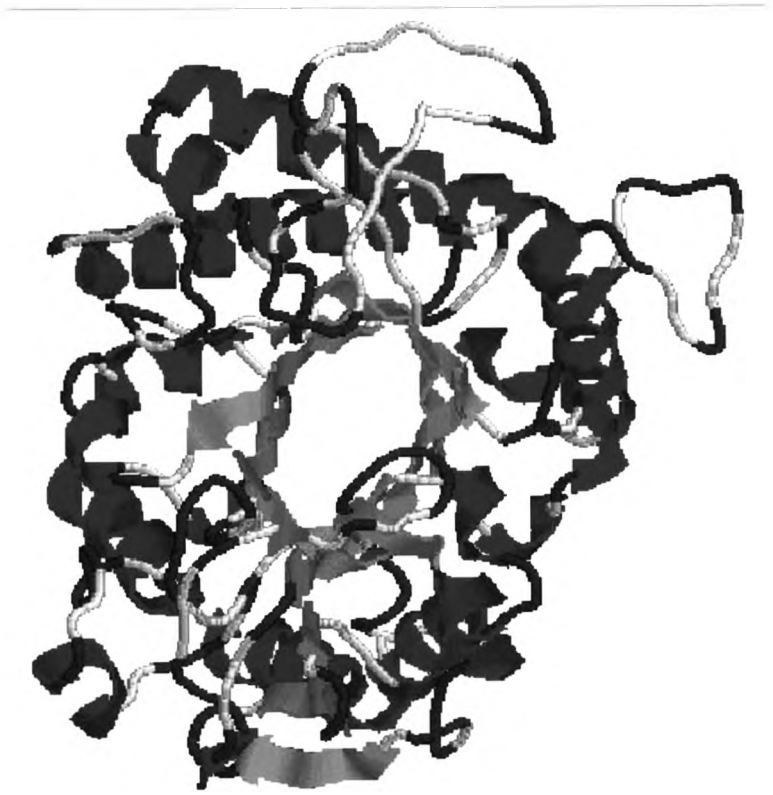

Figure 39. Theoretical model of *B. licheniformis* Chi66. α-helices are showed in pink, β-strands in pink and loops in white and blue. This figure was created using SWISS-MODEL version 36.002.

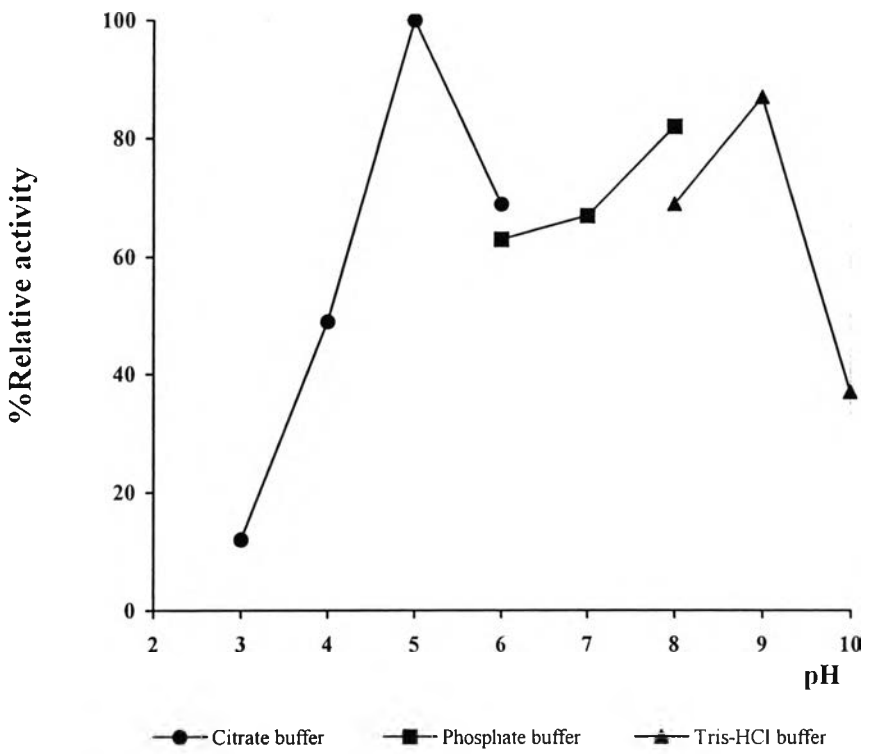

Figure 40. Optimum pH of crude chitinase from Chi66. Chitinolytic activity was measured at pH range of 3-10 by colorimetric method when used colloidal chitin as substrate.

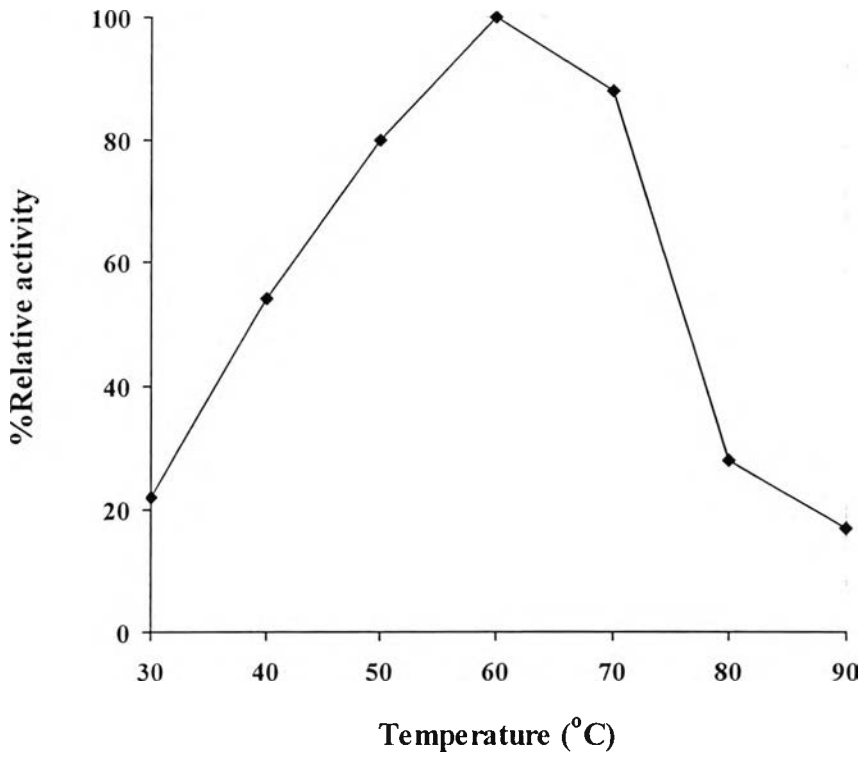

Figure 41. Optimum temperature of crude chitinase enzyme from Chi66. Chitinolytic activity was measured at temperature range from 30-90 °C by colorimetric method when used colloidal chitin as substrate.

60 °C for 12 hrs. The enzyme activity reduced 42 percent when the enzymes was kept at 60 °C for 2 hrs (Figure 42).

The crude chitinase activity from Chi66 on different substrates

The activity of crude chitinase from Chi66 on chitin and its related compounds was studied. The hydrolysis of chitin and its related compounds with chitinase was examined at pH 5.0, 60 °C. Chitinase activity was assayed by determination of reducing sugar. Chitinase was able to hydrolyze colloidal chitin well, followed by powdered chitin, partially N-acetylated chitin, flake chitin and regenerated chitin, respectively (Figure 43).

Products of chitinolytic degradation of colloidal chitin by crude enzyme from Chi66.

Digestion of colloidal chitin by chitinase resulted mainly in the production of N,N'-diacetylchitobiose (Figure 44).

Detection of chitinase and determination of its molecular mass using SDS-PAGE in crude enzyme from Chi66

The activity of chitinase in SDS-PAGE could be renatured (Trudel and Asselin, 1989). Thus, its molecular mass could be determined by comparison the mobility of protein bands with chitinase activity (Figure 45) to those of molecular mass markers. A concentrated crude chitinase from 7 days culture supernatant gave at least 3 chitinolytic activity bands with calculated molecular mass of 70, 65, and 58 kDa, respectively.

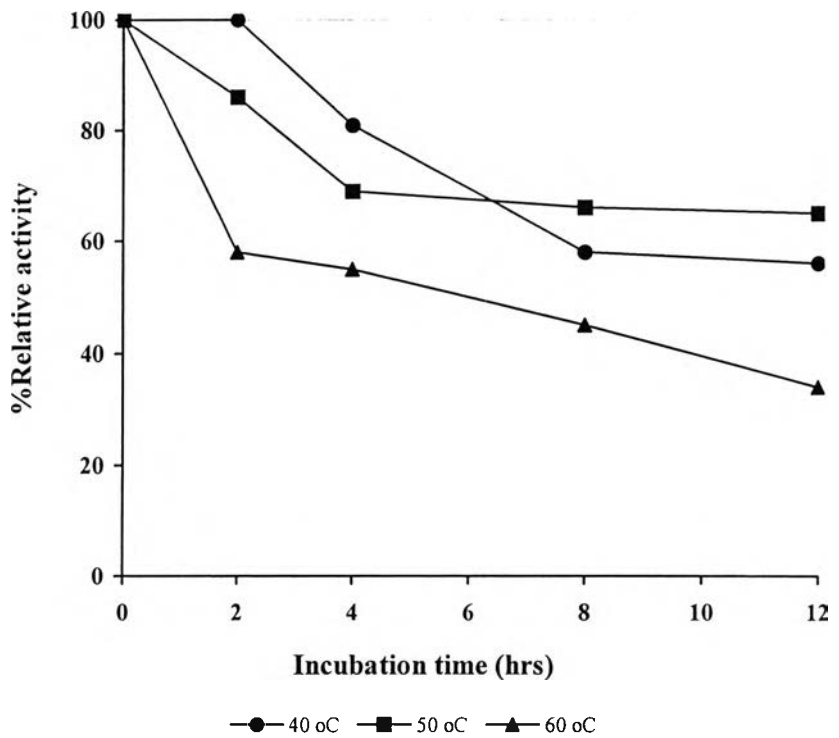

Figure 42. The temperature stability of crude chitinase from Chi66. The crude enzyme was incubated at various temperature for 12 hrs and then assay at different time every 2 hrs for 12 hrs, in 0.1 M citrate buffer pH 5.0 at 60 °C.

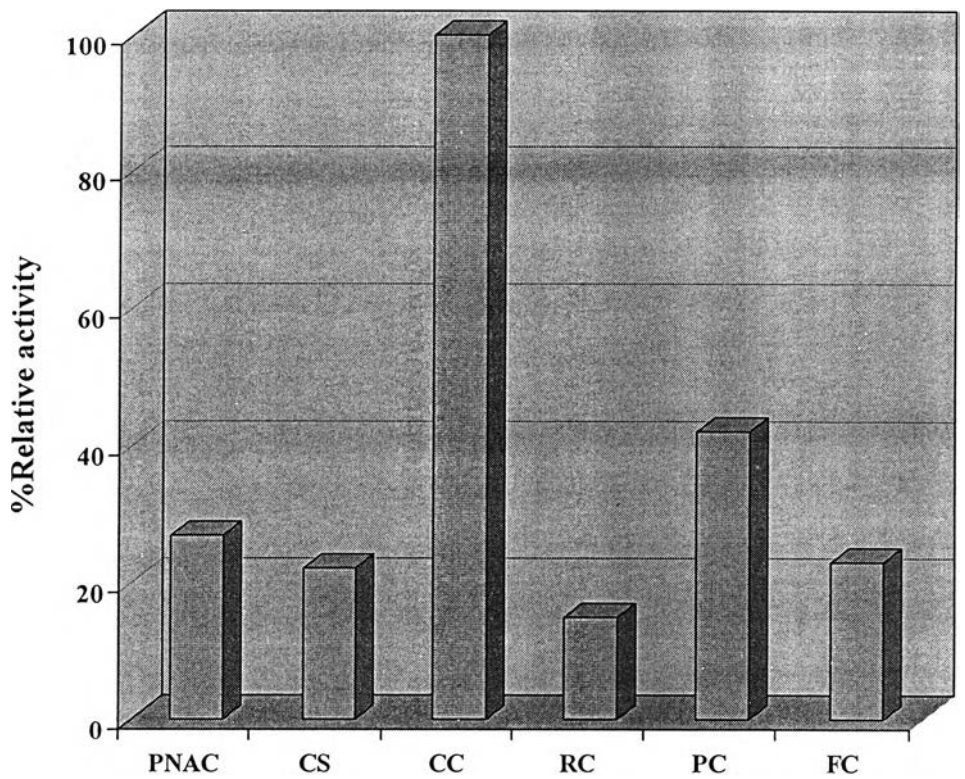

Figure 43. Crude chitinase activity from Chi66 on different substrates. Chitinolytic activity was measured in 0.1 M citrate buffer, pH 5.0 at 60 °C with different substrates.

PNAC = Partially N-acetylated chitin

CS = 80% Chitosan

CC = Colloidal chitin

RC = Regenerated chitin

PC = Powdered chitin

FC = Flaked chitin

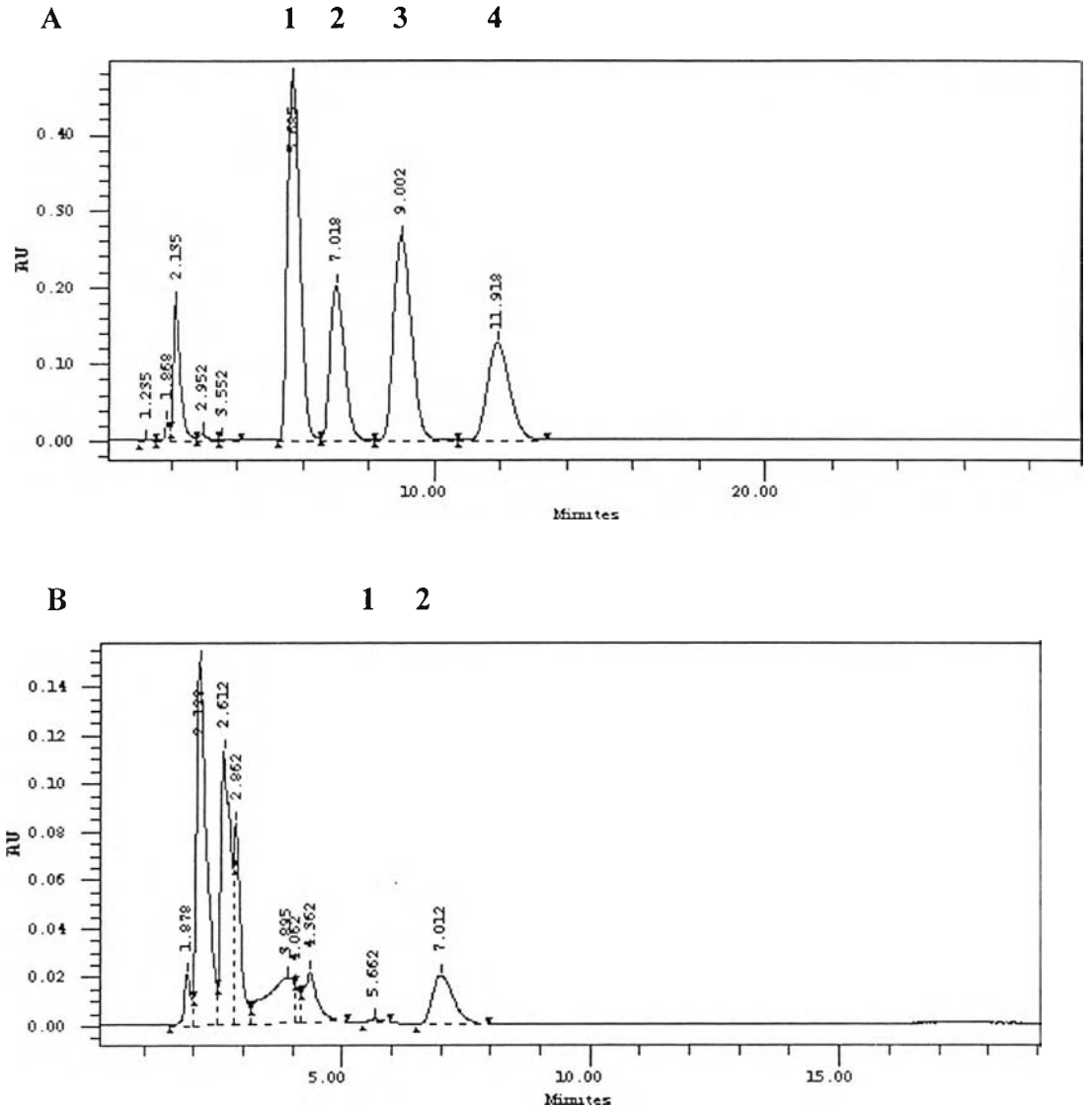

Figure 44. Digestion products of crude chitinase enzyme from pSKChi66. Colloidal chitin was used as substrate for crude enzyme. The reaction was incubated in 0.1 M cicrate buffer, pH 5.0 at 60 °C for 12-24 hrs. Products were analyzed by HPLC.

Panel A; standard chitooligosaccharide

1: Monomer of N-acetylglucosamine

2: Dimer of N-acetylglucosamine

3: Trimer of N-acetylglucosamine

4: Tetramer of N-acetylglucosamine

Panel B; Products from Chi66 using colloidal chitin as substrate

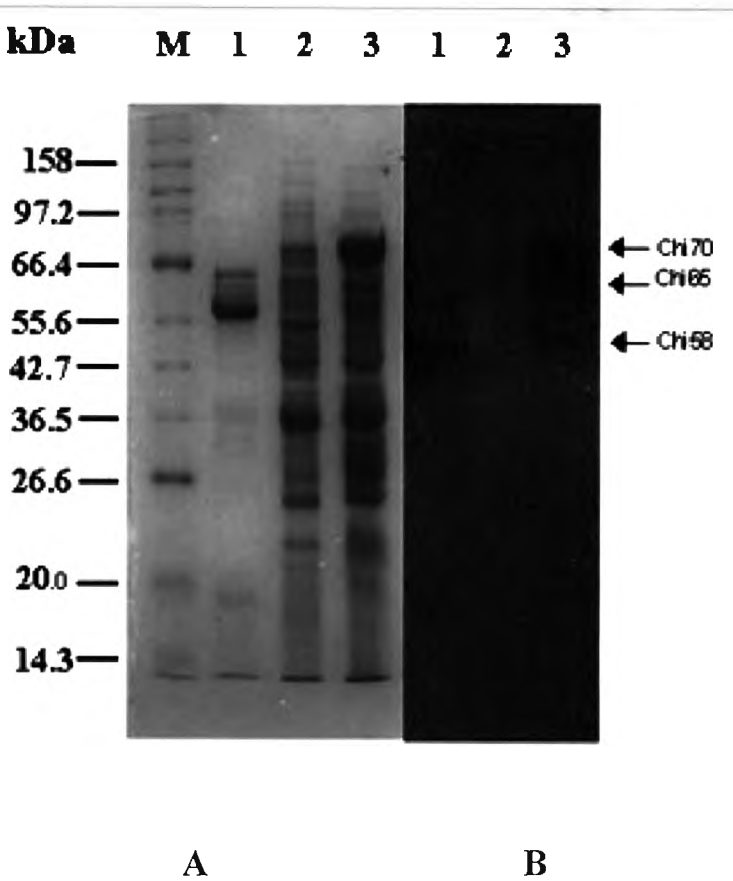

Figure 45. SDS-PAGE of crude chitinase from Chi66. Panel A: protein stain; Panel B: activity stain.

Lane M = Standard protein

Lane 1 = Crude enzyme from *B. licheniformis* SK-1

Lane 2 = Crude protein from JM109/pGEM-Teasy

Lane 3 = Crude enzyme from JM109/pSKChi66