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Gene cloning and biochemical characterization of chitinase CH from *Bacillus cereus* 28-9

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Abstract - *Bacillus cereus* 28-9 is a chitinolytic bacterium showing antagonistic activity against several fungi. One chitinase of 37 kDa, named chitinase CH (ChiCH), was purified by ammonium sulphate fractionation and anion exchange chromatography. The N-terminal sequence of purified ChiCH was determined as ANNLGSKLLVGYWHNFD. The *chiCH* (1,083 bp), cloned from the genomic DNA of *B. cereus* 28-9, encodes a polypeptide of 360 amino acids containing the N-terminal signal peptide and a catalytic domain. ChiCH, partially purified from an *Escherichia coli* transformant harbouring *chiCH*, exhibited chitinase activity with an optimal pH of 6.0 and an optimal temperature of 40 °C. This ChiCH was slightly inhibitory to conidial germination of *Botrytis elliptica*. It was suggested that ChiCH is one of the factor involved in the antagonism of *B. cereus* 28-9 toward fungi.

Key words: chitinase, ChiCH, glycosyl hydrolase family 18, gene cloning.

INTRODUCTION

Bacillus cereus is a large, Gram-positive, endospore-forming bacterium that is very common in soils and plants (Brunel *et al*., 1994; Martinez *et al*., 2002). For plant disease control, *B. cereus* UW85 has been proven as a reliable biocontrol agent of Phytophthora damping off and root rot of soybean (Emmert and Handelsman, 1999), and capable of producing two antibiotics responsible for disease suppression (Silo-Suh *et al*., 1994). In addition, an endophytic *B. cereus* strain 65 producing a chitobiosidase is effective against *Rhizoctonia solani* in cotton (Pleban *et al*., 1997). However, the role of chitobiosidase in the antagonism of *B. cereus* strain 65 toward fungal plant pathogens is not clearly understood.

In this study, we analysed the chitinases produced by a chitinolytic strain of *B. cereus* and found that this *B. cereus* strain excreted two chitinases. One of them was partially purified and its encoding gene was cloned. In addition, this chitinase was characterized and investigated on its antifungal activity toward *Botrytis elliptica*, a fungal pathogen of lily leaf and blossom blight.

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MATERIALS AND METHODS

A chitinolytic strain 28-9 was classified as *Bacillus cereus* / *Bacillus thuringiensis* according to carbon source utilization ability by using BIOLOG plate (Bacteria & Yeast Identification System, Biolog, Inc., Hayward, CA) and identified as a strain of *B. cereus* by PCR analysis of a gyrase gene (Yamada *et al*., 1999).

Chitinase activity was determined using a fluorometric substrate, 4-methylumbelliferyl β-D-N, N'-diacetylchitobioside (Sigma, St. Louis, MO, USA), following the method of Morimoto *et al*. (1997). One unit of chitinase activity was defined as the amount of enzyme required to release 1 μmol of 4-methylumbelliferone per min. In addition, protein concentration was measured using Bradford's method (1976) and bovine serum albumin was used as a standard.

For the purification of ChiCH, all steps were carried out at 4 °C. *Bacillus cereus* 28-9 was cultured in 500 mL of M9 broth that contained 0.4% GlcNAc at 37 °C on a rotary shaker at 175 rpm for three days. The culture supernatant was collected by centrifugation at 10,000 × *g* for 15 min, and proteins in the supernatant were precipitated with ammonium sulphate at 40-70% saturation. The precipitate was dissolved in 0.1 M of Tris-HCl buffer (pH 8.0) and dialyzed overnight in the same buffer. The dialysate was loaded onto a Hyper-D anion exchange column (Sigma) and proteins were eluted with 0.1-0.5 M NaCl gradient in 0.1 M of Tris-HCl buffer (pH 8.0). ChiCH was eluted with 0.1 M NaCl and fractions that exhibited chitinase activity were pooled, concentrated by ultrafiltration through a Centriplus YM-10 membrane (10 kDa MW cut-off, Millipore, Bedford, MA, USA), and finally stored at –20 °C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970) using Mini-Protein II apparatus (Bio-Rad, Herculus, CA, USA). A separating gel (10%) containing 0.01% of glycol chitin was used for detection of chitinase activity. After electrophoresis, separated proteins were renatured by soaking the gel in 0.1 M acetate buffer (pH 5.0) containing 1% Triton X-100 at 37 °C with gentle shaking for 2 h. The gel was stained with 0.01% Calcofluor White M2R (Sigma) in 0.5 M Tris-HCl (pH 8.9). Protein bands exhibiting chitinolytic activities were visualized under a UV transilluminator (Trudel and Asselin, 1989). Proteins in the polyacrylamide gel were stained with Coomassie Brilliant Blue G-250.

The protein was electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore), using a Mini-Electroblot apparatus (Bio-Rad). Proteins on the membrane were stained with 0.1% amido black. The protein band corresponding to that exhibiting chitinase activity was cut out from the membrane and subjected to N-terminal amino acid sequencing by automated Edman degradation using the Applied Biosystems model 477A protein sequencer (Applied Biosystems, Perkin Elmer, Foster City, Calif., USA).

The N-terminal amino acid sequence of ChiCH and the conserved sequence of family 18 chitinases were used to design degenerated primers. Primer dchf (5'-TAITGGCAIAACTTTG-3') corresponding to the amino acid sequence YWHNF and primer dchr (5'-TTCITCITCIATITCTATTCC-3') corresponding to the amino acid sequence G(L/I)D(L/I)DXE were used in polymerase chain reaction (PCR). "I" refers to inosine. For DNA amplification, PCR was done with melting at 94 °C for 10 min, followed by 30 cycles of 94 °C 1 min, 54 °C 1.5 min, and 72 °C 1 min, with final extension at 72 °C for 10 min after the

last cycle. Amplified DNA fragments were cloned into pGEMT-easy vector and sequenced. The insert of recombinant plasmid, encoding amino acid sequence of chitinase was used as a probe in Southern blot analysis and subsequent colony hybridisation. Probe was prepared using a PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals, Mannheim, Germany) following the method described by the manufacturer. A subgenomic library of *B. cereus* 28-9 was constructed in pBluescript II KS(-) and transformed into *E. coli* TOP10F'. After colony hybridisation, the insert DNA from a selected clone was sequenced using the ABI-310 autosequencer (Applied Biosystems).

The DNA fragment carrying the *chiCH* gene and 17-bp upstream region was amplified by PCR with primer chf, 5'-GTATAGGAGTGTTGATAATGTTAAA CAAG-3', and primer chr, 5'-GTTATTTTTCGAAGGAAAGACCATC-3'. The amplified *chiCH*-containing fragment was cloned into pGEMT-easy vector to create recombinant plasmid, pGH51, and transformed into *E. coli* DH5α. The resulting *E. coli* DH5α(pGH51) was cultured in LB broth containing 50 μg/ml ampicillin under constant shaking at 37 °C for 20 h. The periplasmic protein of *E. coli* DH5α(pGH51) was extracted following the method of Manoil and Beckwith (1986). Purification of ChiCH from the periplasmic fraction of *B. cereus* 28-9 was performed by the same procedures used for purification of ChiCH from culture supernatant of *B. cereus* 28-9.

ChiCH purified from the periplasmic fraction of *E. coli* DH5α(pGH51) was used to determine the effects of pH and temperature on chitinase activity of ChiCH. Glycol chitin was used as a substrate. Chitinase activity was analysed by a procedure described by Imoto and Yogishita (1971). Hydrolysis reaction of ChiCH was performed at 37 °C for 25 min in the following buffers of 0.1 M: sodium citrate (pH 3-5), potassium phosphate (pH 6-7), Tris-HCl (pH 8), and glycine-NaOH (pH 9-11) buffers. Temperature effect on chitinase activity of ChiCH was measured in 0.1 M potassium phosphate buffer (pH 6.0) from 20 °C to 80 °C. In addition, substrate specificity of ChiCH was examined on soluble substrates, namely glycol chitin (Sigma), glycol chitosan (Sigma), carboxymethylcellulose (Hayashi, Osaka, Japan), laminarin (Sigma), and soluble starch (Hayashi).

For antifungal assay, conidial suspension of *Botrytis elliptica*, at a final concentration of 4×10^5 conidia/ml, was mixed with purified ChiCH and incubated at room temperature for 12 h. After incubation, the percentage of germinated spores of *Botrytis elliptica* was calculated and the inhibition rate was used as an indication of antifungal activity. The experiment was repeated for three times and analysed statistically by Duncan's Multiple Range Test.

The nucleotide sequence data of *chiCH* have been submitted to GenBank/EMBL DNA Databases under accession numbers AF510723.

RESULTS AND DISCUSSION

Three chitinolytic *B. cereus* strains isolated from Israel (Pleban *et al*., 1997), United States (Wang *et al*., 2001), and Japan (Mabuchi *et al*., 2000), have been reported. These *B. cereus* strains produce chitinases, such as chitobiosidase of strain 65 (Pleban *et al*., 1997), Chi36 of strain 6E1 (Wang *et al*., 2001), and ChiA of strain CH (Mabuchi and Araki, 2001). In this report, we found that

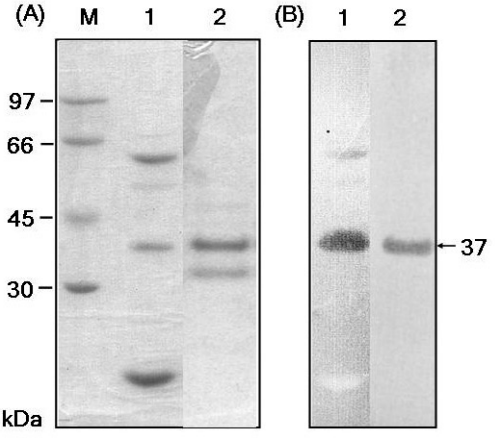

FIG. 1 – SDS-PAGE and zymogram analysis. (A) Gel was stained with Coomassie Brilliant Blue G-250. (B) Chitinase activity was detected by staining the gel containing 0.01% glycol chitin with 0.01% Calcofluor. Lanes: 1, proteins partially purified from the culture supernatant of *B. cereus* 28-9; 2, ChiCH partially purified from *E. coli* DH5α(pGH51). ChiCH with estimated molecular mass of 37 kDa is indicated by an arrow.

ChiCH produced by the chitinolytic *B. cereus* 28-9 from Taiwan was similar to these chitinases. However, the biological function of this kind of chitinases from different *B. cereus* strains has not yet been studied. Therefore, we cloned the ChiCH-encoding gene and investigated the biological function of ChiCH of *B. cereus* 28-9 herein.

B. cereus 28-9 produced at least two chitinases and secreted both enzymes into culture medium. Fig. 1 shows the zymogram of partially purified chitinases produced by *B. cereus* 28-9. One chitinase with estimated molecular mass of 37 kDa was named ChiCH and its N-terminal amino acid sequence was determined as ANNLGSKLLVGYWHNFD.

The N-terminal amino acid sequence of ChiCH and the conserved amino acid sequence of catalytic domains of family 18 chitinases were used to design degenerate primers, dchf and dchr. A DNA fragment of about 300 bp was amplified and determined as the partial sequence of a chitinase gene based on sequence analysis data. This fragment was subsequently used as a probe in Southern blot analysis and colony hybridisation.

Figure 2 shows the result of Southern blot analysis using the 300-bp DNA probe. *Eco*RI-digested genomic DNA of *B. cereus* 28-9 yielded a single band at the 1.7 kb-position. Single bands were also detected in the *Eco*RV- and *Pvu*II-digested *B. cereus* 28-9 genomic DNA at the positions of about 4.0 kb and 8.0 kb, respectively. Therefore, *Eco*RI fragments (1.5-3 kb) of *B. cereus* 28-9 were used to construct a subgenomic library. One clone, screened from this subgenomic library, harboured a recombinant plasmid carrying a 1.7-kb *Eco*RI insert. Sequence analysis revealed an open reading frame (ORF) in this region. The N-terminal amino acid sequence, ANNLGSKLLVGYWHNFD, of excreted ChiCH coincided with that deduced from the nucleotide sequence of the predicted ORF as shown in Fig. 3.

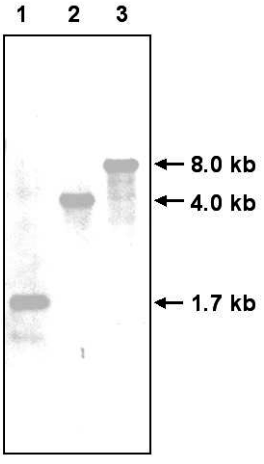

FIG. 2 – Southern blot analysis of the genomic DNA of *B. cereus* 28-9. The genomic DNA was digested with *Eco*RI (lane 1), *Eco*RV (lane 2) and *Pvu*II (lane 3). The Southern blot showed signals of hybridization with the 300-bp DNA probe corresponding to *chiCH* sequence. The estimated DNA fragment size of each signal is indicated beside the arrow.

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                                                        GT ATA GGA GTG TTG ATA  -1
    ATG TTA AAC AAG TTC AAA TTT TTT TGT TGT ATT TTA GTA ATG TTC TTA CTT CTA CCG CTA  60
1   M  L  N  K  F  K  F  F  C  C  I  L  V  M  F  L  L  L  P  L
    TCC CCT TTC CAA GCA CAA GCA GCA AAC AAT TTA GGT TCA AAA TTA CTC GTT GGA TAT TGG 120
21  S  P  F  Q  A  Q  A  A  N  N  L  G  S  K  L  L  V  G  Y  W
    CAT AAC TTT GAT AAC GGT ACT GGC ATT ATT AAA TTA AAA GAC GTT TCA CCA AAA TGG GAT 180
41  H  N  F  D  N  G  T  G  I  I  K  L  K  D  V  S  P  K  W  D
    GTA ATC AAT GTA TCT TTT GGT GAA ACT GGT GGT GAT CGT TCC ACT GTT GAA TTT TCT CCT 240
61  V  I  N  V  S  F  G  E  T  G  G  D  R  S  T  V  E  F  S  P
    GTG TAT GGT ACA GAT GCA GAC TTC AAA TCA GAT ATT TCT TAT TTA AAA AGT AAA GGA AAG 300
81  V  Y  G  T  D  A  D  F  K  S  D  I  S  Y  L  K  S  K  G  K
    AAA GTA GTT CTT TCA ATA GGT GGA CAA AAT GGA GTC GTT TTA CTT CCT GAC AAT GCC GCT 360
101 K  V  V  L  S  I  G  G  Q  N  G  V  V  L  L  P  D  N  A  A
    AAG GAT CGT TTT ATT AAT TCC ATA CAG TCT CTA ATC GAT AAA TAC GGT TTT GAT GGA ATA 420
121 K  D  R  F  I  N  S  I  Q  S  L  I  D  K  Y  G  F  D  G  I
    GAT ATT GAC CTT GAA TCA GGT ATT TAC TTA AAC GGA AAT GAT ACT AAT TTC AAA AAT CCA 480
141 D  I  D  L  E  S  G  I  Y  L  N  G  N  D  T  N  F  K  W  P
    ACT ACT CCC CAA ATC GTA AAT CTT ATA TCA GCT ATT CGA ACA ATC TCA GAT CAT TAT GGT 540
161 T  T  P  Q  I  V  N  L  I  S  A  I  R  T  I  S  D  H  Y  G
    CCA GAT TTT CTA TTA AGC ATG GCT CCT GAA ACA GCT TAT GTT CAA GGC GGT TAT AGC GCA 600
181 P  D  F  L  L  S  M  A  P  E  T  A  Y  V  Q  G  G  Y  S  A
    TAT GGA AGC ATA TGG GGT GCA TAT TTA CCA ATT ATT TAC GGA GTG AAA GAT AAA CTA ACA 660
201 Y  G  S  I  W  G  A  Y  L  P  I  I  Y  G  V  K  D  K  L  T
    TAC ATT CAT GTT CAA CAC TAC AAC GCT GGT AGC GGG ATT GGA ATG GAC GGT AAT AAC TAC 720
221 Y  I  H  V  Q  H  Y  N  A  G  S  G  I  G  M  D  G  N  N  Y
    AAT CAA GGT ACT GCA GAC TAC GAG GTC GCT ATG GCA GAT ATG CTC TTA CAT GGT TTT CCT 780
241 N  Q  G  T  A  D  Y  E  V  A  M  A  D  M  L  L  H  G  F  P
    GTA GGT GGT AAT GCA AAT AAC ATT TTC CCA GCT CTT CGT TCA GAT CAA GTC ATG ATT GGG 840
261 V  G  G  N  A  N  N  I  F  P  A  L  R  S  D  Q  V  M  I  G
    CTT CCA GCA GCA CCA GCG GCA GCT CCA AGT GGT GGA TAC ATT TCG CCA ACT GAA ATG AAA 900
281 L  P  A  A  P  A  A  A  P  S  G  G  Y  I  S  P  T  E  M  K
    AAA GCT TTA AAT TAT ATC ATT AAA GGA GTT CCG TTC GGA GGA AAG TAT AAA CTT TCT AAC 960
301 K  A  L  N  Y  I  I  K  G  V  P  F  G  G  K  Y  K  L  S  N
    CAG AGT GGC TAT CCT GCA TTC CGC GGC CTA ATG TCT TGG TCT ATT AAT TGG GAT GCA AAA 1020
321 Q  S  G  Y  P  A  F  R  G  L  M  S  W  S  I  N  W  D  A  K
    AAC AAC TTC GAA TGC TCA AAT AAC TAT AGA ACA TAT TTT GAT GGT CTT TCC TTC GAA AAA 1080
341 N  N  F  E  C  S  N  N  Y  R  T  Y  F  D  G  L  S  F  E  K
    TAA  C                                                                    1084
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FIG. 3 – Nucleotide and the deduced amino acid sequences of *chiCH*. Amino acid residues corresponding to those determined by N-terminal sequencing are in bold type. The putative active site of ChiCH is underlined. The stop codon of *chiCH* is indicated by an asterisk. The putative Shine-Dalgarno sequence, AGGAG, is italicized.

Sequence analysis indicated that *chiCH* gene is 1,083 bp in length with an ATG start codon and a TAA stop codon (Fig. 3). The putative Shine-Dalgarno sequence, AGGAG, was located 8 nucleotides upstream of the start codon. The deduced protein (ChiCH precursor) consisted of 360 amino acid residues with a calculated molecular weight of 39,372 and isoelectric point of 6.21.

Alignment of the deduced amino acid sequence of ChiCH precursor with the N-terminal amino acid sequence of excreted ChiCH of *B. cereus* 28-9 showed that the deduced amino acid sequence of ChiCH precursor contained a signal peptide which was cleaved off between Ala-27 and Ala-28 by the signal peptidase. The signal peptide of ChiCH produced by *B. cereus* 28-9 had 27 amino acid residues with the common characteristics of a signal peptide, including a positive-charged region, a hydrophobic central core and a signal peptidase recognition site, Ala-X-Ala (Perlman and Halvorson, 1983).

In addition to the N-terminal signal peptide, the deduced ChiCH contained a catalytic domain as that shown in the conserved domain database of National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The conserved amino acid sequence of the catalytic domain, from Gly-139 to Leu-150, was homologous to a number of family 18 chitinases (Henrissat and Bairoch, 1993) (Fig. 3). This region included two aspartate residues, Asp-141 and Asp-143, and one glutamate residue, Glu-145. These residues have also been found in ChiA1 of *Bacillus circulans* WL-12 (Watanabe *et al*., 1993) and ChiA of *Serratia marcescens* (Perrakis *et al*., 1994). Furthermore, Glu-145 in the deduced ChiCH seemed to correspond to Glu-315 of *S. marcescens* ChiA, which has been reported to be involved in the catalysis of chitinase (Perrakis *et al*., 1994).

The amino acid sequence of the deduced ChiCH showed 97.5% homology to that of the ChiA of *B. cereus* CH (Mabuchi and Araki, 2001) and 94.7% homology to that of the Chi36 of *B. cereus* 6E1 (Wang *et al*., 2001) as analysed by the comparison program in the GCG package (Fig. 4). In addition, Wang *et al.* (2001) have speculated that the chitobiosidase of *B. cereus* strain 65 is similar to Chi36 of strain 6E1 (Pleban *et al*., 1997; Wang *et al*., 2001). Although homologous chitinases are distributed in different *B. cereus* strains from different countries, it is unclear whether this kind of family 18 chitinase gene is species-specific in *B. cereus*. However, a species-specific family 19 chitinase gene has been found in *Burkholderia gladioli* (Kong *et al*., 2001). Therefore, the distribution of *chiCH*-like genes in *B. cereus* strains and other *Bacillus* species becomes a subject to study.

ChiCH was expressed and purified from the periplasmic fraction of *E. coli* DH5α(pGH51) as shown by SDS-PAGE and in-gel activity assay (Fig. 1, lane 2). A 33-kDa protein without chitinase activity as shown by in-gel activity assay was consistently co-purified with ChiCH in our procedure. Therefore, the partially purified ChiCH was used for further characterization. The results indicated that ChiCH had an optimal pH of 6 (Fig. 5) and an optimal temperature of 40 °C (Fig. 6). It retained over 75% of optimal activity between pH 5.0–7.0. Furthermore, pH and temperature stabilities of ChiCH could be maintained in the ranges of pH 3-8 (Fig. 5) and 40-50 °C (Fig. 6), respectively.

The ability of ChiCH to hydrolyse various carbohydrates was examined. The result showed that glycol chitin was efficiently hydrolysed among five soluble substrates. In addition, when glycol chitosan was used as a substrate, chitinase

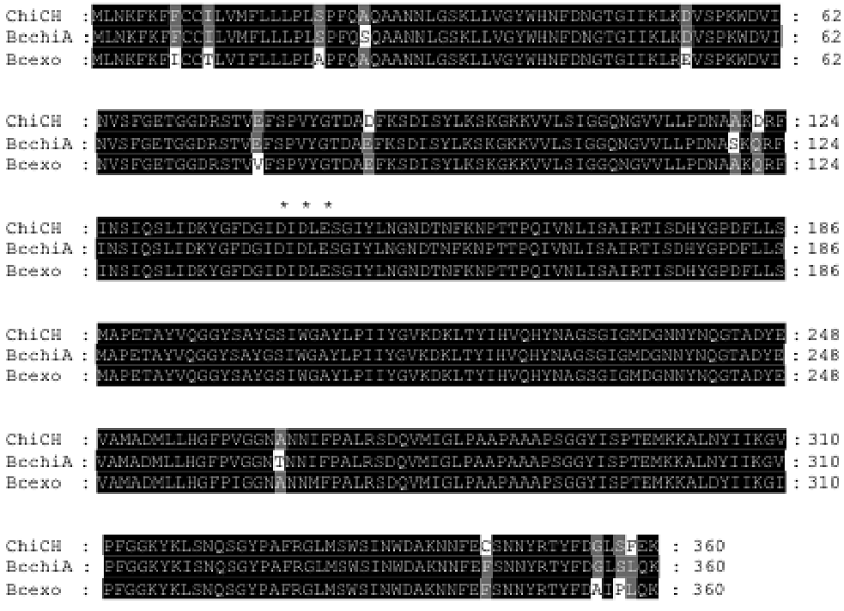

FIG. 4 – Sequence comparison of three chitinases from *B. cereus* strains. ChiCH (this study); BcchiA, chitinase A of *B. cereus* CH (accession number AB041931); Bcexo, exochitinase (Chi36) of *B. cereus* 6E1 (accession number AF275724). The asterisks indicate conserved amino acid residues, two Asp and one Glu, which have been identified as essential amino acid residues.

ChiCH exhibited 10% of relative enzyme activity. However, null effect on other substrates, including laminarin (β-1,3-glucan), carboxymethyl cellulose (β-1,4-glucan), and soluble starch (β-1,4/1,6-glucan), was observed.

ChiCH of 17 μunit inhibited about 10% of the conidial germination of *Botrytis elliptica* and the inhibition level was not augmented by increasing chitinase activity from 17 to 66 μunit. On the other hand, inhibition of the conidial germination of *Botrytis elliptica* was much stronger (55.2% of inhibition) by the culture supernatant of *B. cereus* 28-9 at 20 μunits than by the partially purified ChiCH from *E. coli* DH5α(pGH51).

According to the study of Pleban *et al*. (1997), a chitobiosidase is present in the endophytic *B. cereus* strain 65. They have suggested that chitobiosidase activity is important for antifungal activity of strain 65. Our present work indicated that ChiCH possibly has antifungal activity of mild strength. Since this antifungal level was much lower than that exhibited by the culture supernatant of *B. cereus* 28-9, we presume that not only ChiCH but also other antifungal factors were produced by *B. cereus* 28-9 and exhibited synergistic or combined effect against target fungi.

Acknowledgements
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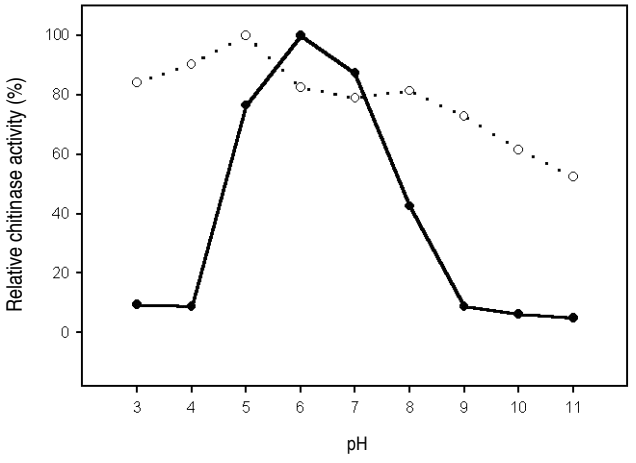

FIG. 5 - Effect of pH on the chitinase activity and stability of ChiCH. ChiCH partially purified from *E. coli* DH5α(pGH51) was assayed for the effect of pH on its enzyme activity (solid circle) and stability (open circle).

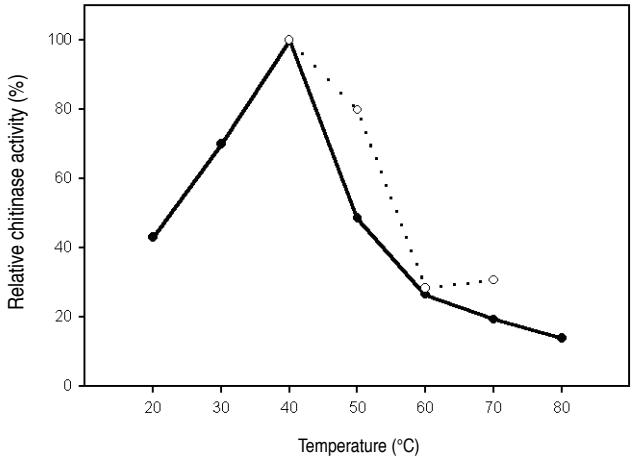

FIG. 6 - Effect of temperature on the chitinase activity and stability of ChiCH. ChiCH partially purified from *E. coli* DH5α(pGH51) was assayed for the effect of temperature on its enzyme activity (solid circle) and stability (open circle).

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