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Cloning, expression, purification and characterisation of a thermostable chitinase from *Bacillus licheniformis* A1

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Abstract - The chitinase B gene (*chi*B65) of *Bacillus licheniformis* A1 (*Blic*A1) isolated from Diyadin hotspring in Turkey was cloned and sequenced. The gene is 1779 bp long and encodes a protein 592 amino acids with a 35-amino acid signal peptide at N-terminal. The gene has 99% percent similarity to *chi*B gene of *Bacillus licheniformis* under the GenBank number AY205293. The gene without signal peptide was overexpressed in *Escherichia coli* and the recombinant protein purified by nickel affinity chromatography. The activity of enzyme was shown on SDS-PAGE with the flourogenic substrate 4-methylumbelliferyl ß-D-*N,N'*-diacetylchitobioside. Kinetic characterisation of the enzyme was performed at 65 °C by using chromogenic substrate *p*-nitrophenyl *N*,*N'*-diacetyl-ß-D-chitobioside, and K_m and V_{max} were found to be 0.02 µM and 1017 U/mg protein, respectively. Enzyme has maximal activity at pH 6.0 and was stable over a broad pH range of 5.0-9.0 for 24 h at room temperature and 4 h at 65 °C. Enzyme was 60% stable at 65 °C for 1.5 h. The inhibition or activation of some substances on the activity of enzyme was determined. High kinetic properties of enzyme open the possibility of an extensive structural and enzyme-substrate interaction studies.

Key words: *Bacillus licheniformis* A1 (*Blic*A1), cloning, *chi*B65, pNP-$(GlcNAc)_2$ and 4-MUF-$(GlcNAc)_2$.

INTRODUCTION

Chitin, an insoluble polysaccharide consisting of ß-1,4-N-acetyl-D-glucosamine (GlcNAc) units is the most abundant renewable natural resource after cellulose (Deshpande, 1986) and degraded by chitinase enzymes. Biodegradation of chitin is performed by chitinases and appears to occur in two steps. An endochitinase degrades the polymer to oligomers, which are subsequently degraded to monomers by exochitinase. Chitinolytic enzymes can be grouped into three types (Shaikh and Deshpande, 1993). Endochitinases (EC 3.2.1.14) are defined as enzymes catalysing the random hydrolysis of 1,4-, linkages of GlcNAc at internal sites over the entire length of the chitin microfibrils. The products are soluble, low-molecular mass oligomers of GlcNAc such as chitotetraose, chitotriose and diacetyl-chitobiose. Exochitinases (chitobiosidases and ß-N-acetyl hexosaminidase, EC 3.2.1.52) catalyse the successive release of diacetylchitobiose units in a stepwise fashion as the sole product from chitin. The third type is N-acetyl-ß-1,4-D-glucosaminidase (EC 3.2.1.30), a chitinolytic enzyme which also acts in exo-splitting mode on diacetylchitobiose and higher analogs (Toharisman *et al*., 2005).

Chitinases exist in many organisms such as crabs, insects, fungi, invertebrates, bacteria, plants, and vertebrates for different roles (Sakai *et al*., 1998). The roles of these chitinases could be divided into several categories and bacteria produce chitinase to digest chitin primarily to utilise it as a carbon and energy source. There are some studies about chitinase enzymes from *Bacillus* species (Watanabe *et al*., 1990; Bhushan, 2000; Huang and Chen, 2004; Toharisman *et al*., 2005; Waldeck *et al*., 2006). During the last decade, chitinases have received remarkable attention due to their wide range of applications. Efforts are going on throughout the world to enhance the production and purity of bacterial chitinases. However, only a few thermostable chitinolytic enzymes from bacteria are

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cloned and characterised especially from *Bacillus* species (Huang and Chen, 2004) that is a big source of chitinases.

We isolated a new chitinase producing *Bacillus* species from Agri-Diyadin hotspring in Turkey. In this paper, we described the cloning, expression, purification and characterisation of a chitinase B gene from this new strain of *Bacillus*.

MATERIALS AND METHODS

Microorganism. *Bacillus* strains were isolated from the Agri-Diyadin hot spring in Turkey. Mud and water samples of hot spring were poured and spread onto nutrient agar plates. These plates were incubated at 60 °C for 2 days. The colonies that were found on the plates were transferred onto M9 agar plates containing 0.5% colloidal chitin and Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g: NH_4Cl, 1 g; $MgSO_4$, 2 ml (1 M stock); and $CaCl_2$ 10 ml, (1 M stock) distilled water 1 l. The plates were incubated at 60 °C for 2 days and a colony showing a clear zone on agar plate was accepted as chitinase producing bacterium. Its morphological, physiological and biochemical characterisation was done according to Bergey's Manual of Systematic Bacteriology (Claus *et al*., 1986) and its molecular identification was done according to 16S rRNA gene sequence (Belduz *et al*., 2003).

Determining the whole gene sequence of *chi*B65. This new *Bacillus* strain showing chitinase activity was grown in Luria-Bertani (LB) medium at 55 °C and genomic DNA was purified using Wizard Genomic DNA Purification Kit (Promega, Madison, USA). The genomic DNA from *Blic*A1 was used as a template to amplify the *chi*65B. Two primers (*Blic*ChiF1: 5'- ATGAAMATCGTGTTGRTCAAC-3' and *Blic*ChiR1:5'-TCAGCCGCCATACGCCCCATTC-3') were designed from the known sequence of *B. licheniformis* chitinase gene (GenBank accession number AY205293). PCR was performed to amplify the whole gene sequence of the chitinase gene in 50 µl reaction volume with 50 ng genomic DNA, forward and reverse primers, 3 mM $MgCl_2$, 0.2 mM dNTP mix and 2 unite *Taq* DNA polymerase (Fermentas). The programme was as follows: 94 °C for 3 min, 32 cycles of 94 °C for 30 s, 50 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 5 min. The PCR product was ligated to pGEM-T easy vector (Promega) and transformed to *Escherichia coli* DH5α strain. Recombinant vector containing *chi*65B gene of *BlicA1* was selected on ampicillin/X-gal plate. Recombinant plasmid was purified using Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced by Macrogen Inc. (Seoul, Korea). Nucleotide sequence was translated to amino acids by using Expasy Proteomics Server (http://www.expasy.ch/tools/dna.html) and amino acid alignments were performed by using the online BLAST search engine at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Gene sequence was submitted to GenBank under the accession number EU314720. Signal peptide of the gene product was determined by using Signal P programme (http://www.cbs.dtu.dk/services/SignalP/).

Cloning and overexpression of chitinase gene in *Escherichia coli*. Chitinase gene without signal peptide corresponding with 567 amino acids was amplified using forward primer (*BlicNde*I-F) 5'-GCACATATGGATTCCGGAAAAAACTATAAAAT-CATCG-3' and reverse primer (*BlicHin*dIII-R) 5'-GTGAAGCTTGCCGCCATACGCCCCATTCACCG-3'. In the forward and reverse primers, the underlined sequences represent the sites of *Nde*I and *Hin*dIII, respectively. The condition for PCR was performed at 94 °C for 3 min; and 94 °C for 30 s; 52 °C for 1 min; 72 °C for 2 min for 30 cycles. The PCR amplified DNA fragments were ligated to pGEM-T Easy vector and positive clones were selected on the ampicillin and X-gal plates. One positive clone was double digested with *Nde*I and *Hin*dIII, ligated into *Nde*I-*Hin*dIII digested pET-28a+, and transformed into *E. coli* BL-21 (DE3) competent cells to construct recombinant plasmid pET-*Blichi*65B. The clone was streaked onto LB agar plate containing kanamycin (50 µg/ml) and the plate was incubated at 37 °C for 16 h. A single colony was used to inoculate 5 ml LB broth containing kanamycin (50 µg/ml) followed by its incubation at 37 °C with vigorous agitation in a shaking incubator. Two millilitres of overnight culture were used to inoculate 250 ml of LB broth containing kanamycin (50 µg/ml) in a 500 ml culture flask and the culture was grown at 37 °C with vigorous agitation. When cells reached an optical density (OD) 0.6 at 600 nm, isopropyl ,-D-thiogalactoside (IPTG, 1 mM final concentration) was added. After 3 h of induction at 37 °C, cells were harvested by centrifugation at 5000 x *g* for 5 min at 4 °C. The recombinant enzyme containing His-tag was purified by using a manual procedure according to the manufacturer by MagneHis Protein Purification System containing paramagnetic precharged nickel particles. Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as the standard.

Detection of chitinolytic activity on SDS-PAGE. Concentrated protein was prepared in sample buffer (Laemmli, 1970) and incubated for 10 min at room temperature prior to loading. Where sample buffer containing 2-mercaptoethanol was used, the samples were boiled for 4 min prior to loading. The proteins were separated by sodium dodecyl sulphate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) according to Sambrook *et al*. (1989).

Enzyme was reactivated in the gel by removing SDS by the casein-EDTA procedure (McGrew and Green, 1990) as modified by Haran *et al*. (1996). Enzyme activity was detected on gel by using 1 mM of fluorescent substrates as described Tronsmo and Harman (1993). 4-Methylumbelliferyl-*N*-acetyl-ß-D-glucosaminide [4-MU-(GlcNAc)] and 4-methylumbelliferyl-ß-D-*N*,*N*'-diacetylchitobioside [4-MU-$(GlcNAc)_2$] were used as flourogenic substrate and prepared in a small amount of dimethylformamide then diluted in 50 mM sodium phosphate buffer (pH 6.5) to a final concentration of 1 mM. The chitinolytic enzyme is appeared as a fluorescent band under UV light because of enzymatic hydrolysis of fluorescent 4-methylumbelliferone from the GlcNAc mono- and oligo-saccharides. The molecular weight of the renaturated chitinase was estimated by using high range prestained standards (Bio-Rad Laboratories). Proteins separated by SDS-PAGE were stained with Coomassie brilliant blue G-250 prepared as described Neuhoff *et al.* (1988).

Enzyme activity assay. Chitinase activity was assayed by using *p*-nitrophenyl *N*,*N*'-diacetyl-α-D-chitobioside [pNP-$(GlcNAc)_2$] (Sigma Biochemicals) as a substrate. This substrate was dissolved initially in a small volume of dimethylformamide and diluted in 50 mM sodium phosphate buffer (pH 6.5) to a final concentration of 1 mM. The chromogenic assay procedure with *p*-nitrophenyl labelled substrate was performed according to the method of Roberts and Selitrennikoff (1988) with minor modifications (Tronsmo and Harman, 1993). The effect of substrate concentration on chitinase activity was determined at different concentrations of *p*-nitrophenyl *N*,*N*'-diacetyl-ß-D-chitobioside [pNP-$(GlcNAc)_2$], varying between 40 µM to 5 mM. Activity assays were carried out in a reaction volume of 50 µl with 50 mM phosphate buffer pH 6.0 and 3.34 µg of the purified enzyme at 65 °C. The reactions were terminated with the addition of 150 µl of 10% Na_2CO_3 (pH 10). Activity was monitored by the release of the *p*-nitrophenyl group from the substrate, resulting in a yellow colour, detected spectrophotometrically at 405 nm. The activity (mmol/min) was calculated using the molar extinction coefficient for the *p*-nitrophenyl group. The K_m and V_{max} values were determined by Lineweaver-Burk's plot.

Optimal pH and stability. The effect of pH on the chitinase activity was determined at 50 µl of the reaction mixture by using 1 mM [pNP$(GlcNAc)_2$] and 3.34 µg enzyme with the following buffers (50 mM) at the indicated pH; acetate buffer (pH 5.0 and 5.5), phosphate buffer (pH 5.5 and 7.5) and Tris-HCl buffer (pH 7.5-9.0). The optimum pH obtained was used for determining thermal properties and other parameters. In order to determine the pH stability of the enzyme, aliquots of enzyme in 1.5 ml plastic tubes were incubated from 30 min to 24 h at room temperature and from 30 min to 4 h at 65 °C in various pHs between 5.0 and 9.0. Then, these enzyme samples were used to determine the pH stability of enzyme. All reactions were performed at 65 °C for 3 min and terminated and measured as above.

Optimal temperature and thermal stability. To determine the optimal temperature for the chitinase activity, enzymatic reactions at various temperatures over the range 30-80 °C were performed using 1 mM pNP-$(GlcNAc)_2$, 3.34 µg enzyme and 50 mM phosphate buffer (pH 6.0). In order to determine the thermal stability of the enzyme, aliquots of enzyme in plastic tubes were incubated from 30 min to 2 h at various temperatures from 30 to 85 °C with 10 °C increments. After incubation, the tubes were rapidly cooled in an ice bath and then brought to room temperature. Then, these heat treated enzymes were used to determine the residual enzyme activity. The percentage residual chitinase activity was calculated by comparison with unincubated enzyme (Yildirim *et al*., 2005).

Effects of some metal ions, denaturants and others on the activity. The enzyme was preincubated for 10 min at room temperature at 10 mM various ion concentrations (NaCl, $MnCl_2$, $CoCl_2$, $MgCl_2$, $CaCl_2$, $CuSO_4$, $ZnSO_4$,), denaturing reagents like urea (2 M), ß-merkaptoetanol (5%), detergents (2%), organic solvents (5%) and other reagents such as EDTA (10 mM). The enzyme activity was measured using 1 mM pNP-$(GlcNAc)_2$ at 65 °C for 5 min.

RESULTS AND DISCUSSION

Morphological, biochemical and molecular identification

According to morphological, biochemical and molecular identification, this new strain is suggested to be representative of the *Bacillus licheniformis* species. However, definite evidence for the affiliation of the isolate with the species *B. licheniformis* was obtained from the sequence comparision based on the 16S rRNA gene sequence of this new strain with the sequences from GenBank. 16S rRNA gene sequence of the new strain has shown 100% identity to the sequence of *Bacillus licheniformis* DSM13/ATCC 14580 and it was designated A1 (*Bacillus licheniformis* A1). This new strain generated a very large zone when grown on chitin-containing M9 minimal agar plates. In the colloidal chitin medium, yeast extract was not essential for the growth of A1.

Molecular cloning and sequence analysis of the chitinase gene

The whole chitinase gene of *B. licheniformis* A1 was obtained by performing PCR with *Blic*chiF1 and *Blic*chiR1 primers designed from the known sequence of the gene from *Bacillus licheniformis*. The *chi*B65 gene from *B. licheniformis* A1 consist of 1779 bp coding for a polypeptide of 592 amino acids (excluding the initiator methionine) with signal peptide at N-terminal. The first 35 amino acids of the gene product belong to signal peptide and most likely cleavage site is between position A[35] and D[36]. The deduced *chi*65B revealed that enzyme is composed of a family 18 catalytic domains responsible for chitinase activity, fibronectin type 3 domains, a chitin-binding domain (CBD) and a domain of unknown function. The nucleotide sequence of chitinase gene was submitted to GeneBank (accession number EU314720). The deduced protein of chitinase of *B. licheniformis* A1 shares 99, 81, 81, 79, 87, 59 and 59% similarity with family 18 chitinase from *Bacillus licheniformis* (GenBank accession no. AAO22144: 592 aa), *Bacillus pumilus* (GenBank accession no. ABI15082: 596 aa), *Bacillus subtilis* (GenBank accession no. ABG57262: 596 aa), *Bacillus amyloliquefaciens* (GenBank accession no. ABO15008: 595 aa), *Bacillus circulans* (GenBank accession no. AAF23368: 496 aa), *Bacillus clausii* KSM-K16 (GenBank accession no. BAD63185: 598 aa), *Bacillus halodurans* C-125 (GenBank accession no. BAB04635: 599 aa), respectively. The deduced protein was compared to the chitinase genes of *Bacillus licheniformis* strains in GenBank accession numbers AAO22144, AAB47847, ABJ74158, YP089989 and AAU39296, the similarity was seen between 99 and 93%. The number of amino acids in chitinase enzymes are generally between 592 and 599 amino acids and conserved among *Bacillus* species.

For expression, the *chi*B65 gene was amplified by two primers designed for the gene sequence without signal part of the gene. The gene was cloned into pET-28a(+) expression vector and transformed into *E. coli* BL21(DE3)pLysS for expression. The purified enzyme has a molecular weight ~71 kDa on 10% SDS-PAGE and a calculated molecular mass of 65185.73 Da and 61202.79 Da with and without signal peptide, respectively. A thermostable chitinase (chi67) was purified from the extracellular supernatant of *Bacillus licheniformis* MB-2 and first 13 amino acids were determined as SGKNYKIIGYYPS (Toharisman *et al*., 2005). These sequences are in agreement with the sequences of chitinase of *B. licheniformis* A1 starting the position S[36].

Enzymatic characterisation and electrophoresis analysis

Recombinant protein was expressed under the control of T7 RNA polymerase promoter with 6xHis-tag in the N-terminal of the protein and purified by MagneHis Protein Purification System (Ertunga *et al*., 2007). SDS-PAGE analysis of the purified chitinase contained 6xHis-tag in the C-terminal of protein showed a single band with molecular weigth of ~71 kDa as shown in Fig. 1, which was seen bigger than the molecular weight calculated from predicated amino acid sequence. This molecular mass lies in around of ~71 kDa is in good agreement with the chitinase enzymes previously observed (Table 1). Bacteria may have several chitinases from 30 kDa to 81 kDa, probably to hydrolyse the different chitin substrates found in nature.

On SDS- PAGE, flourometric detection yielded a brilliant band on UV at the same electrophoretic mobility as developed from Coomassie blue staining. Enzyme produced a fluorescent product from 4-MUF-$(GlcNAc)_2$ (Fig. 1), but it did not hydrolyse MUF from 4-MUF-(GlcNAc) (data not shown). K_m and V_{max} of chitinase from *Bacillus licheniformis* A1 were found to be 0.02 µM and 1017 U/mg protein, respectively. These were comparatively better than reported K_m and V_{max} values of the chitinase I from *Bacillus licheniformis* X-7 using the same substrate pNP-$(GlcNAc)_2$ (Takayanagi *et al*., 1991). These characteristic made this enzyme ideal for many applications in the future.

Effect of pH on chitinase activity.

pH dependent enzyme activity exhibited a sharp peak with an optimum at pH 6.0 (Fig. 2). This optimum is similar with the optimum at pH 6.0 of the chitinase from *B. licheniformis* (Toharisman *et al*., 2005). However, chitinases belong to family 18 from *Bacillus* species have maximum activity between pH

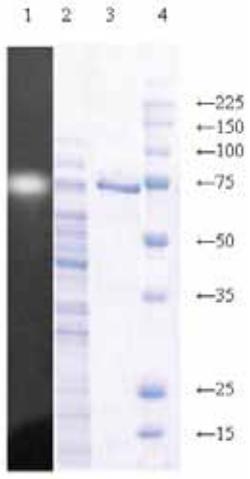

FIG. 1 - SDS-PAGE showing purified recombinant *Bacillus licheniformis* A1 chitinase. Lane 1: zymogram analysis with 4-MU-$(GlcNAc)_2$ of purified enzyme, lane 2: crude extract enzyme, lane 3: the recombinant enzyme contained His-tag and purified by MagneHis Protein Purification System, lane 4: contains the protein molecular weight marker

TABLE 1 - Properties of some bacterial chitinases (Toharisman *et al.*, 2005)

Sources	Molecular mass (kDa)	Optimum pH	Optimum temperature (°C)	Thermal stability (°C, min)	Substrates	References
Bacillus licheniformis A1	65 (calculated) ~71 (on SDS)	6.0	65	65, 90	pNP-$(GlcNAc)_2$	This study
Bacillus licheniformis MB-2	67	6.0	70	60, 80	4-MU-$(GlcNAc)_2$	Toharisman *et al.* (2005)
Streptomyces. thermoviolaceus OPC-520	30	4.0	60	60, 30	Glycol chitin	Tsujibo *et al.* (2000)
Enterobacter sp. G-1	60	7.0	40	< 50, NK	Colloidal chitin	Park *et al.* (1997)
Pseudomonas aeruginosa K-187	30	8.0	50	50, 10	Colloidal chitin	Wang and Chang (1997)
Bacillus MH-1	71	6.5	75	80, 10	pNP-$(GlcNAc)_2$	Sakai *et al.* (1998)
	62	5.5	65			
Streptomyces RC1071	70	8.0	40	60, 60	4-MU-$(GlcNAc)_3$ (4-methylumbelliferyl *N,N,N'*-triacetylchitotrioside)	Gomes *et al.* (2001)
Bacillus sp. NTCU2	36.5	7.0	50-60	60, >30	Colloidal chitin	Wen *et al.* (2002)

NK –Not known.

6.0 and 7.0 (Table 1). Incubation of the enzyme at pH between 5.0 and pH 7.0 at room temperature for 24 h resulted in only 30% reduction of the activity (Fig. 3A). However, the enzyme has shown the same stability only for pH 6.0 when incubated at 65 °C (Fig. 3B). This is well agreement with some other reported chitinases (Table 1).

Effect of temperature on chitinase activity and thermal stability

Chitinase from *Bacillus licheniformis* A1 has a maximum activity at 65 °C (Fig. 4) which coincides with that of thermostable chitinases from *Bacillus* MH-1 (65 °C) (Sakai *et al*., 1998) and *Bacillus licheniformis* Mb-2 (70 °C) (Toharisman *et al*., 2005). These temperature optima of A1, MH-1 and Mb-2 are seen as a common feature of chitinases of *Bacillus* species. Even among fungi or crustaceans found in the Antarctic, chitinase temperature optima of 55 °C were also found (Spindler and Buchholz, 1998). The enzyme had almost no activity at 30 °C and approximately 20% activity below and above 40 °C and 80 °C respectively. Figure 5 shows the loss of activity of the purified enzyme incubated for different lengths of time at various temperatures. The enzyme remained stable more than 50% at temperatures 55 and 65 °C when incubated at pH 6.0 for 90 min. However, the enzyme is unstable at temperatures 75 and 85 °C and it almost fully lost its activity by heating 75 and 85 °C for 90 min.

Effect of some metal ions and chemicals on the enzyme activity

The enzyme had more than 50% stability to denaturation by 2 M urea (54%) and 2% Tween-20 (75%). Enzyme was unstable toward DMSO at a concentration of 5%, indicating that hydrophobic interactions are significantly important for enzyme activity (Toharisman *et al*., 2005). The enzyme was also insensitive toward mercaptoethanol (5%). Besides these, enzyme activity was inhibited more than 70% with EDTA (10 mM). Enzyme has shown

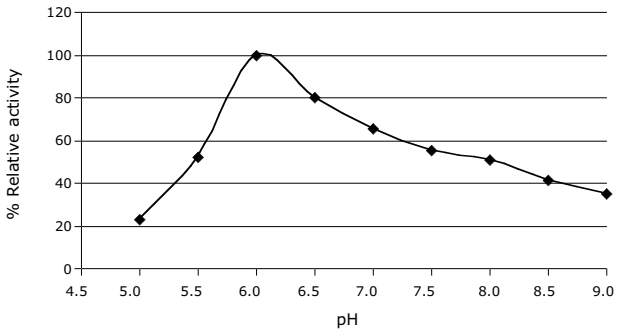

FIG. 2 - Effect of pH on chitinase activity from *Bacillus licheniformis* A1. Assays were performed in 50 mM of different buffers between pH 5.0 and 9.0 at 65 °C.

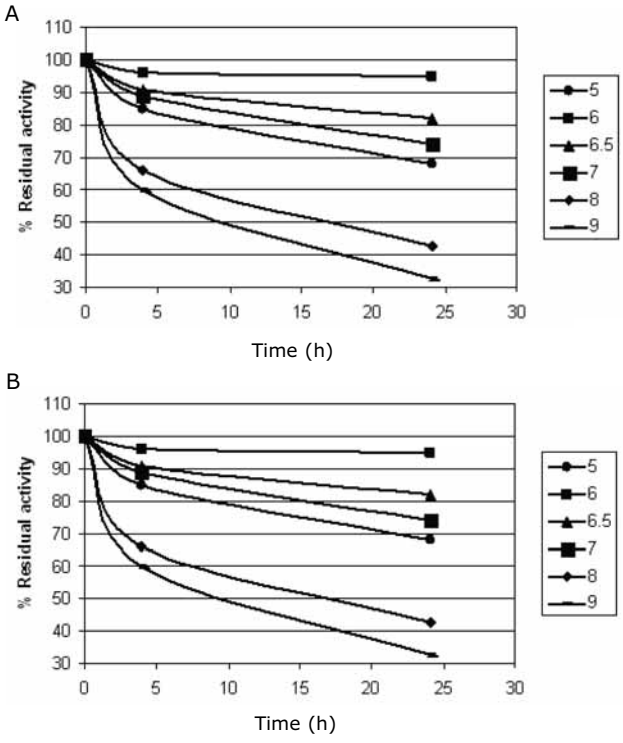

FIG. 3 - pH stability of *Bacillus licheniformis* A1 chitinase at room temperature (A) and at optimum temperature (B).

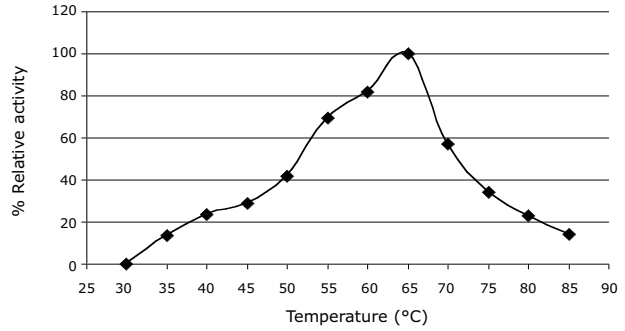

FIG. 4 - Effect of temperature on chitinase activity from *Bacillus licheniformis* A1. The activity reactions were performed at different temperatures from 30 to 85 °C.

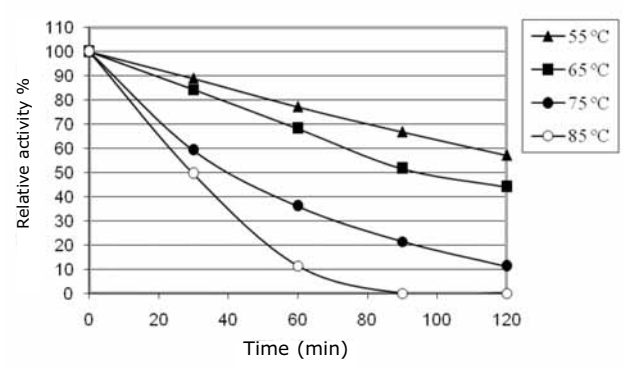

FIG. 5 - Thermal stability of *Bacillus licheniformis* A1 chitinase. Enzyme solutions were incubated from 30 min to 2 h at 55, 65, 75 and 85 °C. Residual enzyme activities were determined by using standard assay procedure at 65 °C.

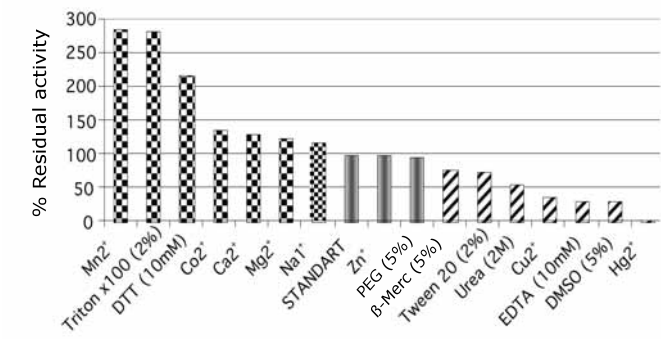

FIG. 6 - Effect of various substances on *Bacillus licheniformis* A1 chitinase activity.

more activity with Mn^{2+} (185%), Co^{2+} (36%), Ca^{2+} (30%), Mg^{2+} (23%), Na^{+} (16%) up to 10 mM concentration and more activity with 2% Triton-X (180%) and 10 mM DTT (118%) and less activity with Cu^{2+} (66%), Hg (100%) up to 10 mM concentration. Zn^{2+} and PEG have no effect on the enzyme activity up to 10 mM and 5% concentration, respectively (Fig. 6).

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