*Pakistan J. Zool., vol. 46(6), pp. 1491-1501, 2014.*

# Molecular Cloning and Characterization of *Exochitinase A* Gene of Indigenous *Bacillus thuringiensis* Isolates

**Faiza Saleem[1], Aisha Younas[1], Rasheeda Bashir[1], Shagufta Naz[1], Neelma Munir[1] and Abdul Rauf Shakoori[2]***

[1]*Department of Biotechnology, Lahore College for Women University, Lahore*
[2]*School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore*

**Abstract.-** Chitin is a naturally occurring insoluble linear β1-4 linked polymer of N-acetylglucosamine and a common constituent of the fungal cell wall and exoskeleton of arthropods. Chitinases convert chitin into its economically important derivatives. In the present study, 13 *Bacillus thuringiensis (Bt)* isolates screened on colloidal chitin medium were found to have chitinolytic activity. The maximum chitinase activity (0.23±0.001 U/ml) was shown by CMBL-Bt4 after 4 days of incubation at 37°C (pH 7.0). SBS-Bt1, 3, 5 and 6 were found positive for chitinase (*chi*) gene. The amplified genes were cloned in pTZ57R TA cloning vector, sequenced and submitted to EMBL with accession nos. HG792449 to 52. All these genes, had 99% homology with *Bacillus cereus chiA* (AB041931.1) and *Bacillus cereus* strain 49 *chiA* (GU134904.1), and 97% similarity with *Bacillus thuringiensis* YBT-1518 strain (CP005935). The structure of exochitinase predicted by Phyre2 showed $^{107}$GGQNG$^{111}$, $^{141}$DDLE$^{145}$ and $^{225}$QHYN$^{228}$, regions, respectively for chitin binding, catalysis and substrate hydrolysis. The *exochitinaseA* genes described in this study can be employed in vast applications including the production of transgenic plants and in bringing out commercial formulation for the control of lepidopteran pests.

**Keywords:** Chitinolytic *Bacillus thuringiensis*, chitin, N-acetylglucosamine, chitinase, *exochitinase A* gene

## INTRODUCTION

Chitin is the second most frequent biopolymer in environment, after cellulose. Chitin along with its derivatives is of huge economic interest, as it has a wide range of applications in medical, agricultural and cosmetics industry (Bansode and Bajekal, 2006). The functional properties of chitin such as chemical reactivity, solubility and biological activities (Al Sagheer *et al*., 2009) like biodegradability (Sato *et al.,* 1998; Sorlier *et al*., 2001), are mainly influenced by its physiochemical characteristics. Chitin prepared at lab scale from various crustacean shell wastes has variability in the fat binding capacities (Eijsink *et al.,* 2003). Chitin occurs naturally in three different polymorphic forms, α, β and γ chitins, within the crystal cell these polymorphic forms vary in the array of molecular chains (Dahiya *et al*., 2006; Zhang *et al*., 2005). Among these, naturally α-chitin is the most abundant having parallel chain packing, while β-chitin is found in squid pens and shows antiparallel chains, however γ-chitin is a combination of former chitins (Dahiya *et al*., 2006). Like cellulose, chitin is not soluble in general organic solvents, dilute aqueous solvents and water, owing its strong inter and intra molecular hydrogen bonds (Kurita, 2006; Liu *et al.,* 2002).

The process of chitin catabolism typically occurs in two steps. In the first step chitinase is converted into chitin oligosaccharides and then secondly it is cleaved into N-acetylglucosamine by chitobiases (Suginta *et al*., 2000). Chitinase is an enzyme that hydrolyzes chitin to its monomeric, dimeric and oligomeric components. Chitinases are divided into three main groups: exochitinases, endochitinases and β-N-acetylglucosaminidase. The ultimate aim of chitin degradation is to convert the polymer $(GlcNAc)_n$ into $NH_3$, fructose 6 phosphate and acetate.

Different chitinases are produced by different organisms for different purposes depending on their own use and physiology (Pichyangkura *et al*., 2002). Various microorganisms such as actinomycetes (Akagi *et al.,* 2006), fungi, bacteria and some higher plants produce chitinases. *Bacillus thuringiensis* acts on susceptible insects by producing parasporal crystals and is utilized as a biological pest control agent, worldwide (Höfte and Whiteley, 1989). Under appropriate conditions, it is capable of

* Corresponding author: arshaksbs@yahoo.com
0030-9923/2014/0006-1491 $ 8.00/0

producing many biological active molecules such as bacteriocins, insecticidal proteins and hydrolytic enzymes such as chitinases.

In the present study chitinolytic activity of *Bt* strains was evaluated using chitin derived from shrimp waste as substrate. The *exochitinase A* genes of these isolates were amplified, cloned and characterized using different bioinformatics tools.

## MATERIALS AND METHODS

### Bt *isolates*

Thirteen isolates of *Bacillus thuringiensis* namely SBS-Bt1-6 (Saleem and Shakoori, 2010), CMBL-Bt 3-6 (Khurshid, 2003), 40Bi, 40Cii, 47Cii, 9Cii and 29Ci were obtained from School of Biological Sciences, University of the Punjab, Lahore. All bacterial strains were cultured on nutrient agar medium, overnight incubated at 37°C and further kept at 4°C.

### *Extraction and preparation of colloidal chitin*

Shrimp shells taken from sea food waste were weighed and dipped in 2% NaOH for 30 min with constant stirring. The shrimp shells were filtered and rinsed. Sterile distilled water was used for this purpose. The shells were then treated with 5% conc. HCl for 30 min with constant stirring, filtered, and rinsed with distilled water. For drying, the deproteinated and demineralized shells were placed at 60°C in an oven (Pinelli-Saavedra *et al*., 1998). The dried shrimp shells were then grinded and stored at room temperature.

Chitin powder (5g) was suspended in 60 ml of concentrated HCl. This mixture was constantly stirred for 1 h with a magnetic stirrer. This was followed by centrifugation in Hermle Z 300K at 6000 rpm (3904 rcf) for 15 min at 4°C. Ethanol (50%) 200 ml, was added to the supernatant, shaken on the shaker for few minutes and then centrifuged at 6000 rpm (3904 rcf) for 15 min at 4°C. The pellet of colloidal chitin was washed with 2 liters of sterile distilled water until its pH became neutral. Then colloidal chitin was weighed and kept at 4°C in dark (Roberts and Selitrennikoff, 1988).

### *Screening of* Bt *isolates for chitinolytic activity*

*Bt* isolates were cultured on synthetic medium containing 2% colloidal chitin for screening those with chitinolytic activity (Mejia-Saules *et al*. 2005). The isolates were streaked on the petri plates containing synthetic media and incubated at 30°C for 96 h. After every 24 h, the plates were observed for the presence of a clear zone surrounding the *Bt* colony, as an indicator of chitinolytic activity. The potential of the chitinase producing strains was determined on the basis of zone of clearance (CZ) to colony size (CS) ratio (Cody, 1989; Wirth and Wolf, 1990).

### *Chitinase assay*

Chitinase activity of the sample was determined by estimation of N-acetylglucoseamine (GlcNac), released from the colloidal chitin substrate using dinitrosalicyclic (DNS) method (Miller, 1959). This method tests the presence of free carbonyl group in reducing sugars.

Briefly, the reaction mixture containing 1 ml of substrate (10% colloidal chitin in 0.2 M phosphate buffer, pH 6.0) and 1 ml crude enzyme solution was incubated at 50% for 1 h. Then 1 ml of NaOH was added and boiled for 5 min to stop the enzyme reaction. The mixture was centrifuged for 5 min at 4000 rpm. 1 ml of supernatant was taken and added to 1 ml of DNS reagent and incubated at 100°C for 5 min until red brown colour appeared. The solution was cooled at room temperature and 2.5 ml of distilled water was added. The absorbance was determined at 540 nm using spectrophotometer (Binod *et al.*, 2005).

One unit of chitinase activity was defined as the amount of enzyme required to release 1µmol of NAG per hour at 50°C.

### *Cloning and nucleotide sequencing of* chitinase *gene*

Genomic DNA of all bacterial isolates extracted according to Saleem and Shakoori (2013) was used to amplify full length *exochitinaseA* gene using the following primers (Usharani and Gowda, 2011):

```
CHI-AF 5'CTAGACAACTTTTTGTATAGGAGTGTTGATATG3'
CHI-AR 5'CGATTGATGAGGGCTAATTATAGTTTTACTTTG 3'
```

PCR reaction mixture (50 µl) contained 1X Taq buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 1µM each

primer, 350 µg template DNA, 2.5 units of Taq DNA. The reaction thermal cycle comprised pre-PCR denaturation at 94°C for 3 min followed by 35 cycles, each of 45s at 94°C (denaturation), 45s at 58°C (annealing), 1 min at 72°C (extension) and final extension at 72°C for 5 min. The results of PCR were checked on 1% agarose gel.

The PCR product (1.1 kb) was cloned using InsT/Aclone™ PCR product cloning kit (Fermentas, cat # k1214). The ligation mixture was incubated overnight at 22°C in order to get recombinant vector which was further used for the transformation of *E. coli* DH5α.

Thermoscientific Genejet Miniprep Kit (cat # K0502) was used to purify the recombinant plasmid according to manufacturer's instructions and the insert was sequenced by Macrogen, South Korea. The contigs were aligned for minus and plus strands. The consensus sequence was generated using DNASTAR seqman software. The final sequences were submitted to EMBL database.

*Sequence analysis*

BLASTn search of GenBank was used to select the homologues, while CLUSTALW was used for alignment of the selected sequences. Using different online programs at DDBJ and NCBI, sequence analysis was done for both DNA and predicted protein. The structural prediction of protein was done by Phyre 2 and analysed through pymol. NEBcutter was used to check the restriction sites and variations by comparing sequences. Phylogenetic tree of chitinolytic *Bt* isolates along with other known sequences of *Bacillus* sp. was prepared, using MEGA 5.

*Statistical analysis*

The statistical analysis was done by applying Duncan's multiple range tests for assessment of significant differences. All the statements of significance were based on 0.05 level of probability and shown as $p \leq 0.05$. The experiment was done in triplicates.

## RESULTS

*Chitinase harbouring strains*

The clear halo zones on the chitin containing synthetic media confirmed that all the *Bt* isolates confirmed their chitinolytic activity (Fig. 1).

The production of chitinase by different chitinolytic *Bt* isolates was conducted in batch using shake flask culture technique in colloidal chitin medium. The highest chitinase activity (0.23 U/ml) was shown by CMBL-Bt4 and was detected after the 4th day of incubation, while the lowest activity was observed in the isolate 9C (ii) (Fig. 2).

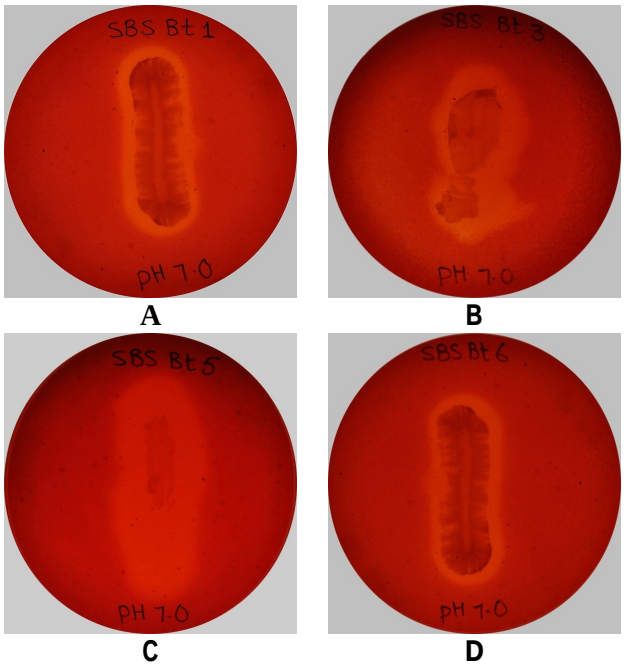


Fig. 1. Chitinolytic activity of *Bacillus thuringiensis* isolates grown on synthetic medium containing chitin (pH 7), stained with Congo red and incubated at 30°C, for 96 h. A, SBS-Bt 1; B, SBS-Bt 3; C, SBS-Bt 5; D, SBS-Bt 6.

*Chitinase gene*

Figure 3A shows the genomic DNA of chitinolytic *Bt* isolates, which was used to amplify the *exochitinase A* genes of SBS-Bt1, 3, 5 and 6. A DNA fragment of about 1.1 kb was amplified by PCR with genomic DNA of four isolates as a template (Fig. 3B). The amplified fragments of *exochitinase A* gene were ligated in pTZ57R/T cloning vector and were used for transformation of *E. coli* DH5α (Fig. 4). The complete gene sequence of SBS-Bt1, 3, 5 and 6 revealed 1083 bp long

coding region that was flanked by 42 bp on N-terminal and by 40 bp on the C-terminal end.

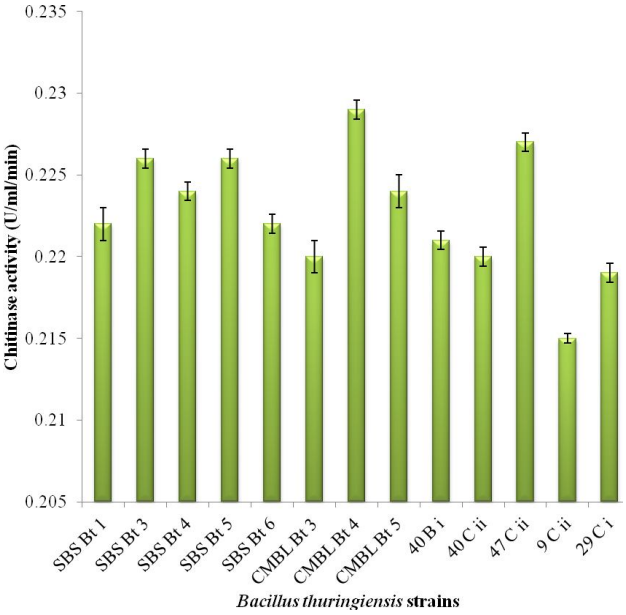


Fig. 2. Extracellular chitinase activity (U/ml/min) of *Bacillus thuringiensis* isolates, incubated at 37ºC for 4 days at 100 rpm. The activity was estimated by DNS method.

The sequences of all the *exochitinase A* genes of SBS-Bt1, 3, 5 and 6 were deposited in EMBL under accession numbers HG792450, HG792451, HG792449, and HG792452, respectively. On the basis of BLASTn search results using nucleotide sequence of *chitinase* gene and using phyre 2 and pymol, it was identified as *exochitinase A* gene.

*Structure of exochitinase A*

The structures of exochitinase type chitinase A produced by SBS-Bt1, 3, 5 and 6 were predicted by using Phyre 2. The black labeled loops (Gly-67—Thr-69 and Ile-106—Val-112) in all the structures are the dynamic saccharide (chitin) binding loops. The purple helix in all the four structures is the endochitinase and exochitinase differentiating α-Helix5 (Figs. 5,6).

Multiple sequence alignment of exochitinase A of *Bt* isolates with those of other *Bacillus* spp taken from NCBI is shown in Figure 7. Out of 360 amino acids 18 are partially conserved and five are non conserved.

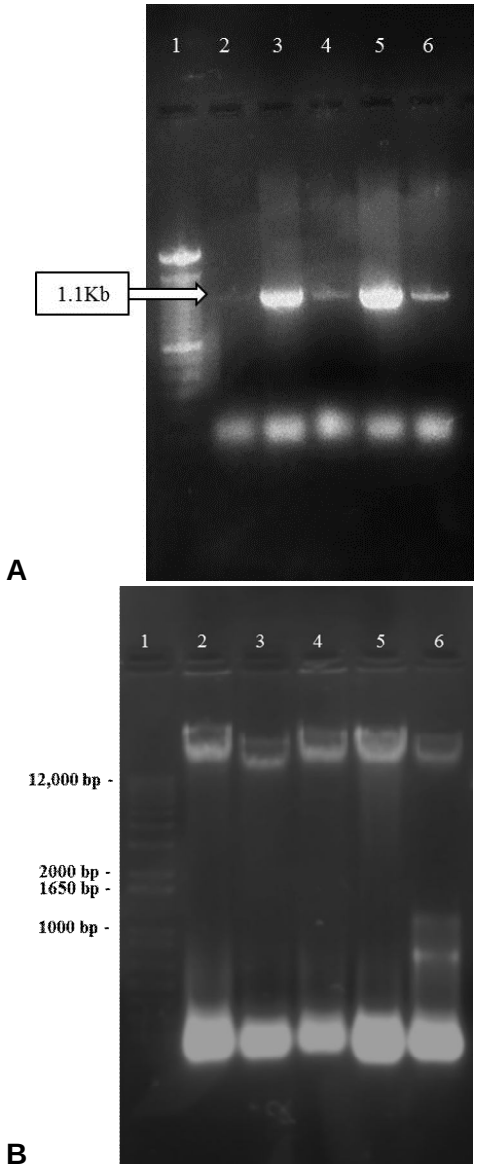


Fig. 3. Agarose gel showing isolated DNA (A) and PCR amplification of *chitinase* gene (B) of chitinolytic strains of *Bacillus thuringiensis*.
**Lanes in A**; Lane 1, DNA ladder; Lane 2, SBS-Bt1; Lane 3, SBS-Bt3; Lane 4, SBS-Bt5; Lane 5, SBS-Bt6; Lane 6, CMBL-Bt4.
**Lanes in B**: Lane 1, DNA ladder; Lane 2, CMBL Bt4; Lane 3, SBS-Bt1; Lane 4, SBS-Bt3; Lane 5, SBS-Bt5, Lane 6, CMBL-Bt6.

Figure 8 shows three amino acid stretches that play vital role in chitin hydrolysis were found conserved in all 4 exochitinase genes described in

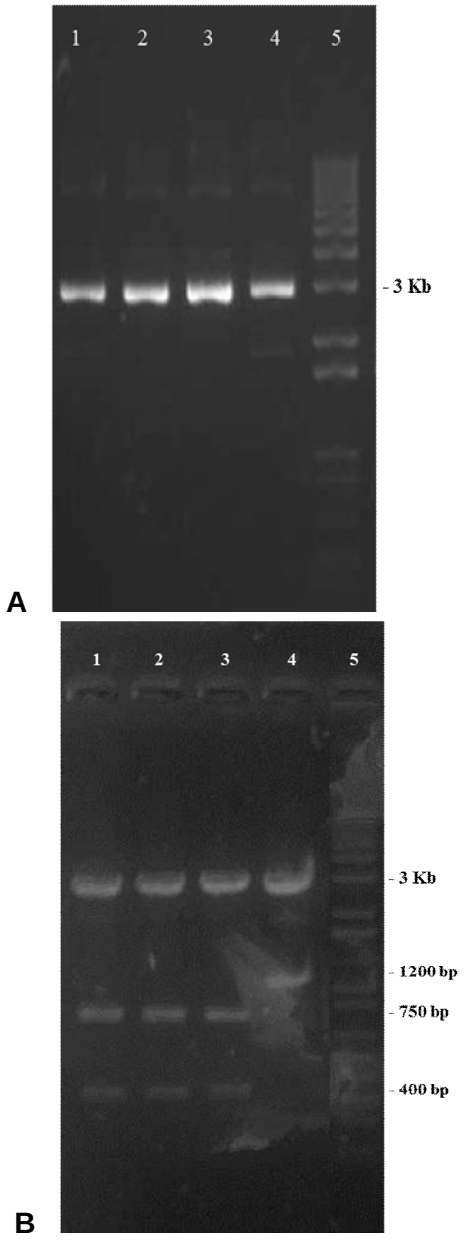


Fig. 4. Agarose gels showing recombinant plasmid with T/A cloned *chitinase* gene A, and its linearized form after digestion with *Eco*RI (B).
Lane: 1, SBS-Bt1; Lane 2, SBS-Bt3; Lane 3, SBS-Bt5; Lane 4, SBS-Bt6; Lane 5, DNA Ladder.

this study including the first fragment $^{107}$GGQNG$^{111}$ which is related to chitin binding, second fragment $^{141}$DIDLE$^{145}$ that serves as catalytic centre, while the third fragment $^{225}$QHYN$^{228}$ which is involved in substrate hydrolysis. The peptides showed two substitutions *i.e.,* S67F and I112V when compared with *B. cereus* exochitinase (accession No. WP_000932444.1|), and one substitution (Y293S) with that of *Bt* (accession No. YP_006601700.1).

*Phylogenetic relationship*

In addition, the phylogenetic tree showed high homology of *exochitinase A* gene from the indigenous strains. The indigenous genes showed 99% similarity with that of *Bt* (accession No. YP_006601700.1|) while 99% homology of *exochitinase A* gene with that of *B. cereus* (Fig. 9). Thus these results reveal close phylogenetic relationship of these two species of genus *Bacillus*.

## DISCUSSION

*Bt* harbours many enzymes including hemolysins, enterotoxins, chitinases and phospholipases apart from the well documented δ-endotoxins and exotoxins that help the bacteria to colonize insects (de Maagd *et al*., 2001). The current study was conducted to screen incidence of chitinase(s) in a collection of indigenous *Bt* isolates toxic to *Helicoverpa armigera* and *Musca domestica* (Saleem and Shakoori, 2010). A greater number of chitin degrading bacteria in agricultural fields have already been revealed (Nawani and Kapadnis, 2003). Bacteria used in the present study, were also capable of cleaving chitin into N-acetyl-glucosamine. Most of the *Bt* isolates, investigated in this study, were found chitinolytic, though chitinases from this bacterial specie have been poorly studied (Barboza-Corona *et al*., 1999; Ramirez *et al*., 2002). The highest chitinase activity (0.23±0.001U/ml) was detected by CMBL-Bt4 after 4 day of incubation at 37ºC. Almost similar results were found in the study conducted by Saadoun *et al*. (2009), where the *Streptomyces* sp. strain $S_{242}$ showed highest activity (0.2 U/ml) after incubation at 30°C with neutral pH for 4 days. In the present study, the *exochitinase A chiA* (gene) could be amplified from 4 *Bt* isolates (SBS-Bt1, 3, 5 and 6) despite higher or similar chitinolytic activities of other *Bt* isolates *i.e.* CMBL BT3, CMBL BT4 and SBS-Bt4. The chitinolytic activities of rest of

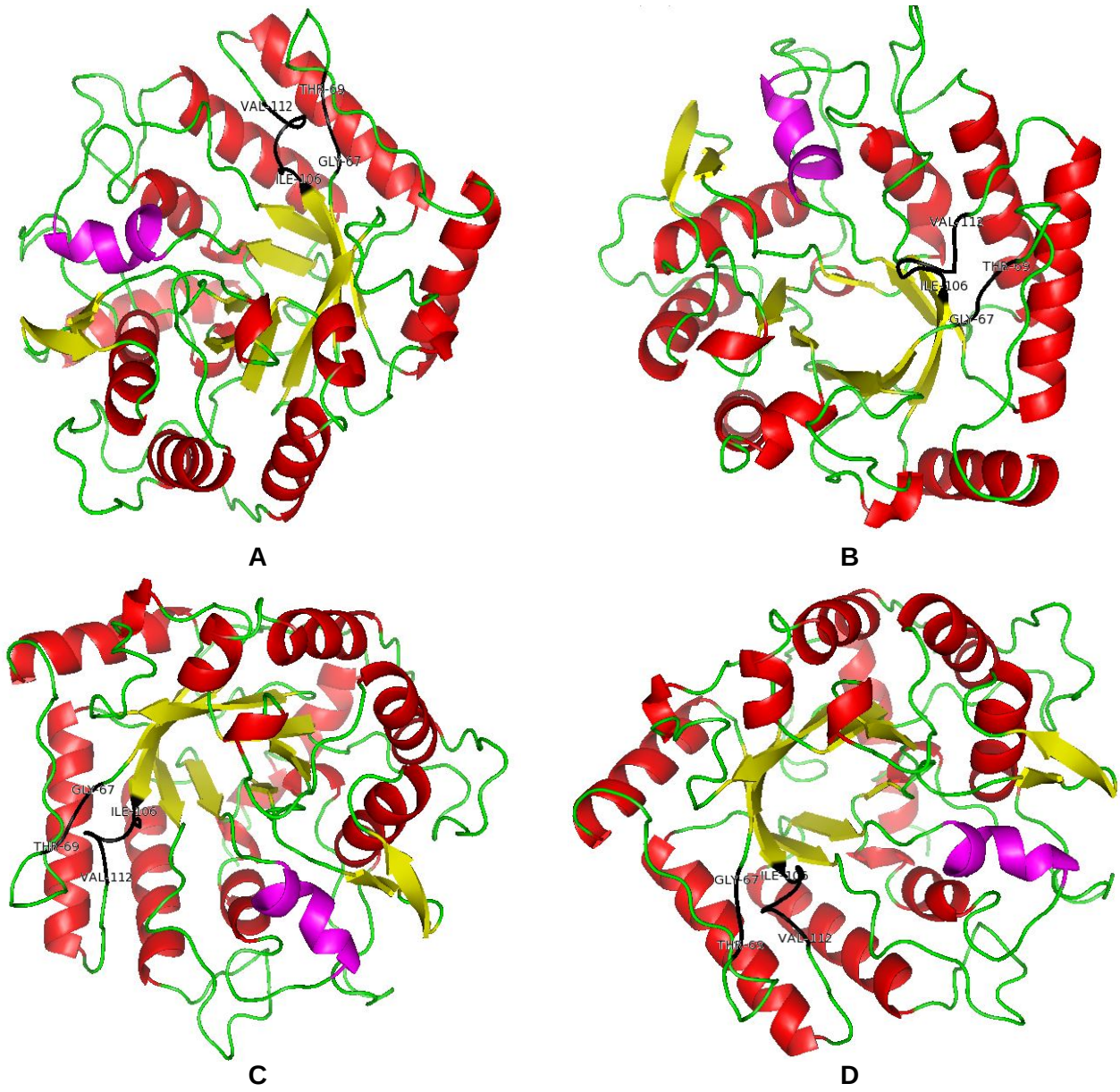


Fig. 5. Structures of exochitinase type chitinase A produced by SBS-Bt1 (A), SBS-Bt3 (B), SBS-Bt5 (C) and SBS-Bt6 (D) (predicted by Phyre 2). The black labeled loops (Gly-67 to Thr-69 and Ile-106 to Val-112) in all the structures are dynamic saccharide (chitin) binding loops, whereas the purple helix in all the four structures is the endochitinase and exochitinase differentiating α-Helix5 (Hsieh *et al.*, 2010).

isolates might be due to occurrence of other types of chitinases *i.e.* chitinase B or chitinase C.

The *exochitinase A* genes of the four isolates consisted of 1083bp, encoding 360 residues (Fig.7). The deduced amino acid sequences of *chiA* gene showed high degree of identity with other exochitinases such as *Bt* BMB171 (100%), *Chi36* of *Bt* HD-771 (100%), and *ChiA* of *B. cereus*. Similar results were reported by Usharani and Gowda (2011), who reported 1129 bp *chi* gene encoding a polypeptide of 360 amino acids. The gene was found to be homologous to that of

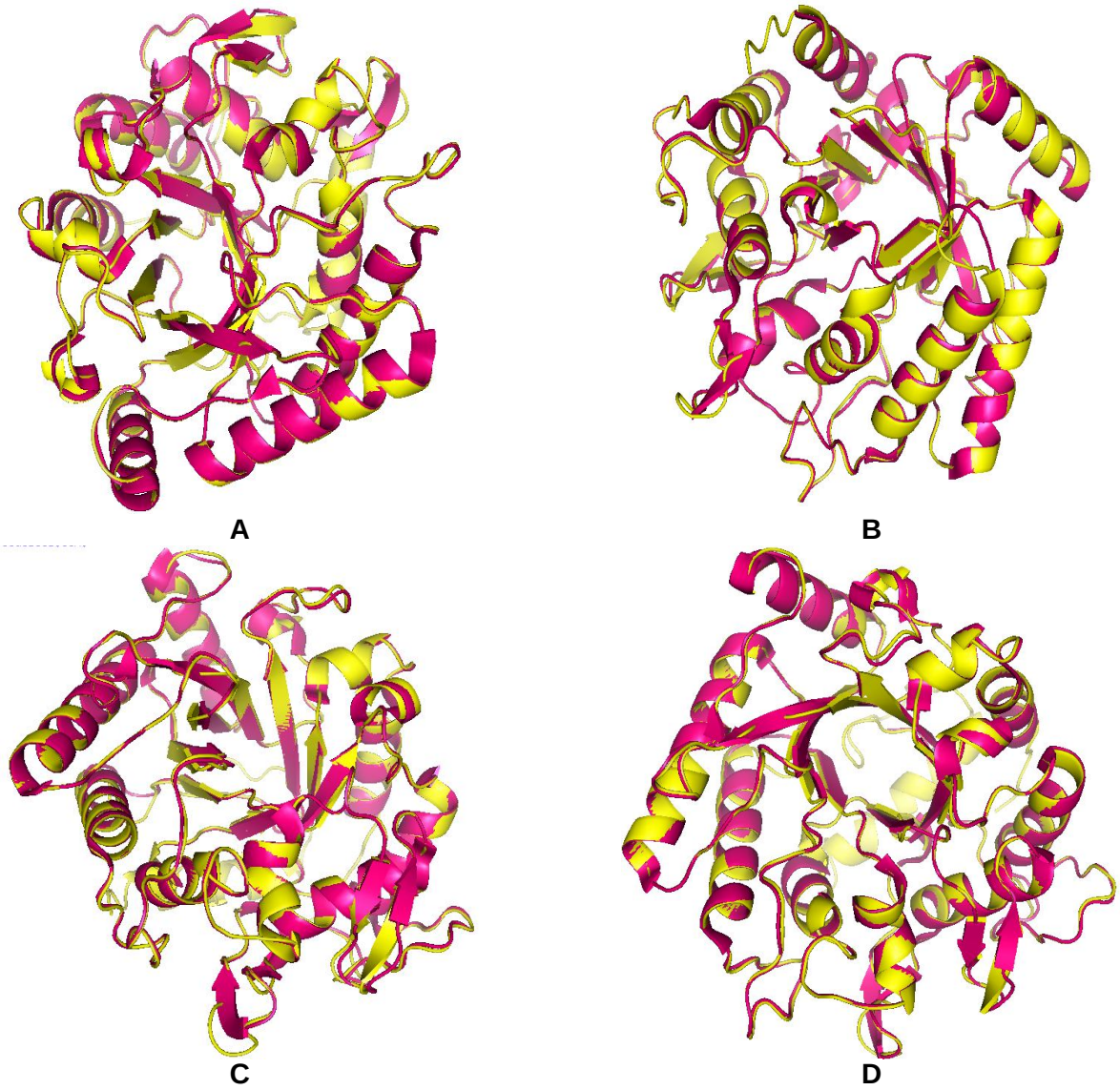


Fig. 6. Chitinase A from four distinct strains *i.e.* SBS-Bt1 (A), SBS-Bt3 (B), SBS-Bt5(C) and SBS-Bt6(D) have been superimposed in order to structurally align them with the most closely related Chitinase A (3N11 EC 3.2.1.1.4) which has been reported through X-ray crystallography techniques (Hsieh *et al.*, 2010). Our structures: Yellow, 3N11: Pink

*B. cereus*. Similarly, Wang *et al.* (2001) reported 1083 bp long ORF encoding a protein of 360 amino acids for *chi36* gene encoding exochitinase from *B. cereus.* The high homology (99%) between the *Bt* chitinase and the *B. cereus* chitinase (Mabuchi and Araki, 2001) supports the view that even though *Bt* and *B. cereus* belong to different species they are closely related evolutionary.

From a comparison with endochitinases, α5 helix (shown in purple colour) exists only in the structure of exochitinases (Hsieh *et al.*, 2010). Amino acid sequences of various chitinases were aligned to analyze their diversity. The sequences share several highly conserved fragments among all chitinases in which three regions ($^{107}$GGQNG$^{111}$, $^{141}$DIDLE$^{145}$ were found conserved in all the 4

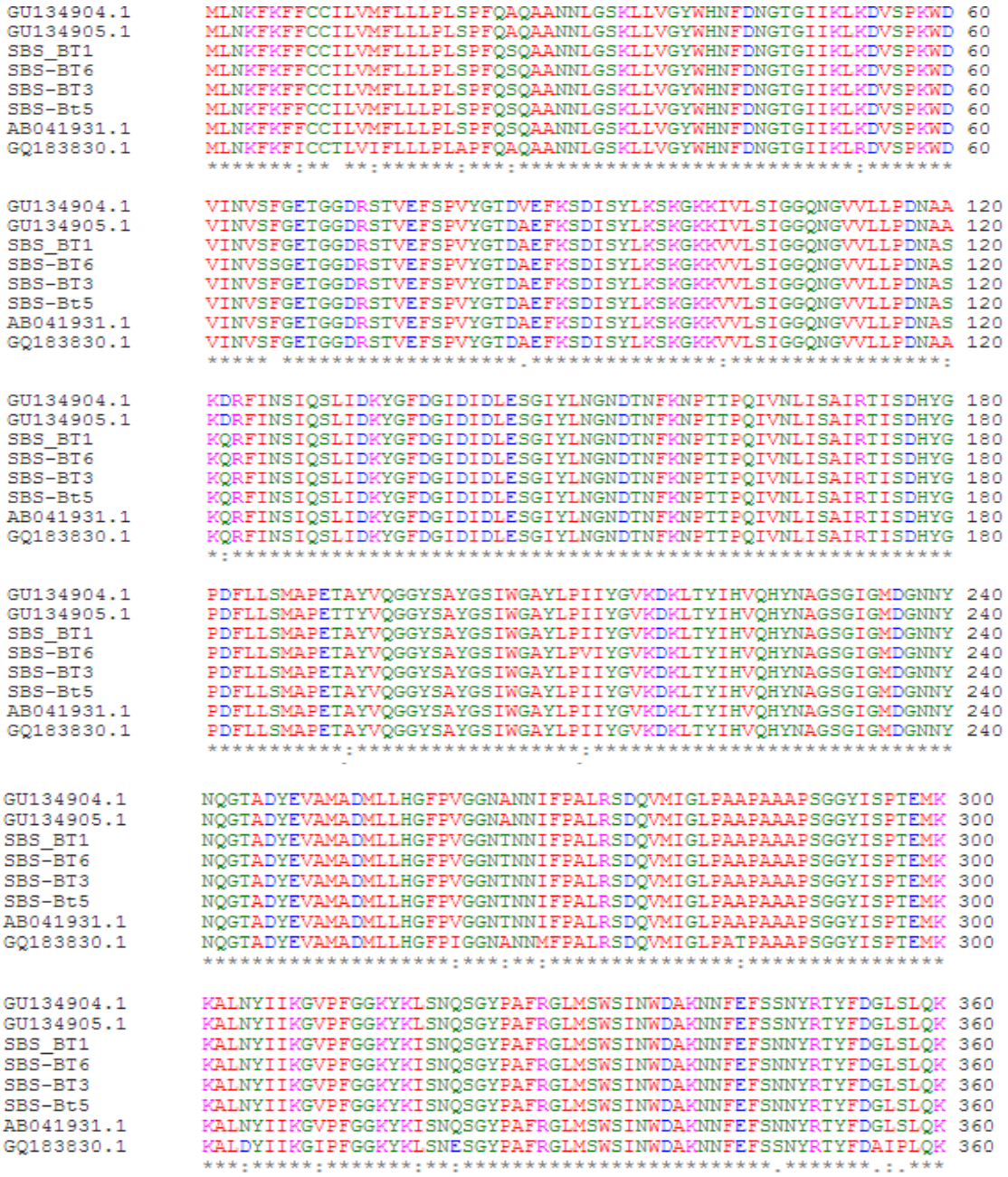


Fig. 7. Multiple sequence alignment of exochitinase A (exochitinase A) of *Bacillus thuringiensis* strains with those of other strains of *Bacillus* taken from NCBI. *Bacillus cereus* strain 49 (Acc. No: GU134904), *Bacillus cereus* strain 79 (Acc. No: GU134905), SBS-Bt1 (Acc. No: HG792450), SBS-Bt3 (Acc. No: HG792451), SBS-Bt5 (Acc. No: HG792449), SBS-Bt6 (Acc. No: HG792452), *Bacillus cereus chiA* (Acc. No: AB041931) and *Bacillus thuringiensis* serovar *konkukian* strain S4 (Acc. No: GQ183830).

Numbers refer to the amino acid residue at the end of the respective lines.

* Fully conserved

: Partially conserved

. Non conserved

```
SBS-BT1 VINVSFGETGGDRSTVEFSPVYGTDAEFKSDISYLKSKGKKVVLSIGGQNGVVLLPDNAS 120
SBS-BT3 VINVSFGETGGDRSTVEFSPVYGTDAEFKSDISYLKSKGKKVVLSIGGQNGVVLLPDNAS 120
SBS-BT5 VINVSFGETGGDRSTVEFSPVYGTDAEFKSDISYLKSKGKKVVLSIGGQNGVVLLPDNAS 120
SBS-BT6 VINVSSGETGGDRSTVEFSPVYGTDAEFKSDISYLKSKGKKVVLSIGGQNGVVLLPDNAS 120

SBS-BT1 KQRFINSIQSLIDKYGFDGIDIDLESGIYLNGNDTNFKNPTTPQIVNLISAIRTISDHYG 180
SBS-BT3 KQRFINSIQSLIDKYGFDGIDIDLESGIYLNGNDTNFKNPTTPQIVNLISAIRTISDHYG 180
SBS-BT5 KQRFINSIQSLIDKYGFDGIDIDLESGIYLNGNDTNFKNPTTPQIVNLISAIRTISDHYG 180
SBS-BT6 KQRFINSIQSLIDKYGFDGIDIDLESGIYLNGNDTNFKNPTTPQIVNLISAIRTISDHYG 180

SBS-BT1 PDFLLSMAPETAYVQGGYSAYGSIWGAYLPIIYGVKDKLTYIHVQHYNAGSGIGMDGNNY 240
SBS-BT3 PDFLLSMAPETAYVQGGYSAYGSIWGAYLPIIYGVKDKLTYIHVQHYNAGSGIGMDGNNY 240
SBS-BT5 PDFLLSMAPETAYVQGGYSAYGSIWGAYLPIIYGVKDKLTYIHVQHYNAGSGIGMDGNNY 240
SBS-BT6 PDFLLSMAPETAYVQGGYSAYGSIWGAYLPVIYGVKDKLTYIHVQHYNAGSGIGMDGNNY 240
```

Fig. 8. Structural-based multiple-sequence alignment showing the sequences (SBS-Bt1, SBS-Bt3, SBS-Bt5 and SBS-Bt6) share several highly conserved fragments among all Chitinases in which three regions are important. The first fragment, with the sequence $^{107}$GGQNG$^{111}$, is related to substrate binding (Perrakis *et al*., 1994); the second fragment, with the sequence $^{141}$DIDLE$^{145}$, serves a catalytic center (Vaaje-Kolstad *et al.*, 2004); the third fragment, with the sequence $^{225}$QHYN$^{228}$, is involved in the substrate hydrolysis (Synstad *et al*., 2004). Numbers refer to the amino acid residue at the end of the respective lines.

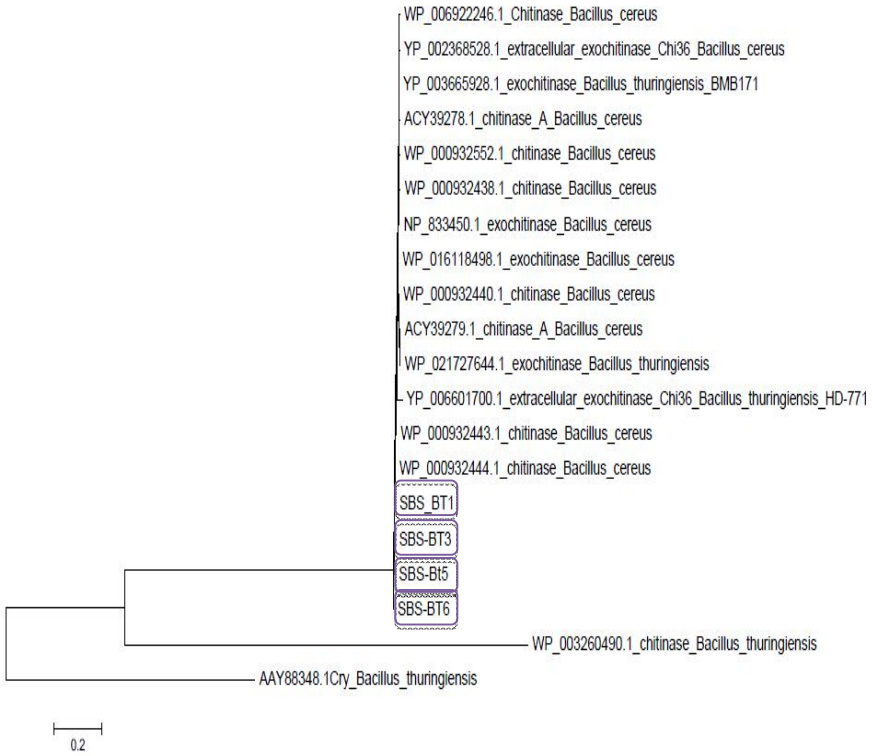


Fig 9: Phylogenetic tree between *chiA* from *Bacillus thuringiensis* strains (SBS-Bt1, SBS-Bt3, SBS-Bt5 and SBS-Bt6) and already known sequences of *chiA* from *Bacillus* spp. by neighbour-joining method using MEGA 5. The tree has been rooted against *Bacillus thuringiensis cry2A* gene.

exochitinases described in this study. The loss of this chitin-binding domain results in a significant decrease in colloidal chitin-hydrolyzing activity (Watanabe *et al.,* 1994). Structures consisting of discrete catalytic and substrate-binding domains similar to that of chitinase A have been described for some other polysaccharide-degrading enzymes such as cellulases, xylanases and amylases.

The *exochitinase A* genes described in this study can be employed in vast applications including the production of transgenic plants and in bringing out commercial formulation for the control of lepidopteran pests.

## REFERENCES


AKAGI, K., WATANABE, J., HARA, M., KEZUKA, Y., CHIKAISHI, E., YAMAGUCHI, T., AKUTSU, H., NONAKA, T., WATANABE, T. AND IKEGAMI, T., 2006. Identification of the substrate interaction region of the chitin-binding domain of *Streptomyces griseus* chitinase C. *J. Biochem.*, **139**: 483-493.

AL SAGHEER, F.A., AL-SUGHAYER, M.A., MUSLIM, S. AND ELSABEE, M., 2009. Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf. *Carbohyd. Polym.*, **77**: 410-419.

BANSODE, V. B. AND BAJEKAL, S.S., 2006. Characterization of chitinases from microorganisms isolated from Lonar Lake. *Ind. J. Biotech*., **5**: 357-363.

BARBOZA-CORONA, J.E., CONTRERAS, J.C., VELAZQUEZ-ROBLEDO, R., BAUTISTA-JUSTO, M., GOMEZ-RAMIREZ, M., CRUZ-CAMARILLO, R. AND IBARRA, J.E., 1999. Selection of chitinolytic strains of *Bacillus thuringiensis. Biotechnol. Lett.,* **21**: 1125-1129.

CODY, R.M., 1989. Distribution of chitinase and chitobiase in *Bacillus. Curr. Microbiol*., **19**: 201-205.

DAHIYA, N., TEWARI, R. AND HOONDAL, G.S., 2006. Biotechnological aspects of chitinolytic enzymes: a review. *Appl. Microbiol. Biotechnol*., **71**: 773-782.

EIJSINK, V.G.H., SYNSTAD, B., KOLSTAD, B., GASEIDNES, G.S., KOMANDER, D., HOUSTON, D., PETER, M.G. AND VANAALTEEN, D.M.F., 2003. Structure and function of chitinolytic enzymes. *Adv. Chitin Sci.,* **2**: 71-78.

HÖFTE, H. AND WHITELEY, H.R., 1989. Insecticidal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.*, **53**: 242-255.

HSIEH, Y.C., WU, Y.J., CHIANG, T.Y., KUO, C.Y., SHRESTHA, K.L., CHAO, C.F., HUANG, Y.C., CHUANKHAYAN, P., WU, W.G., LI, Y.K. AND CHEN, C.J., 2010. Crystal structures of *Bacillus cereus* NCTU2 chitinase complexes with chitooligomers reveal novel substrate binding for catalysis. *J. biol. Chem.*, **285**: 31603-31615.

KHURSHID, N., 2003. *Study on the locally isolates of Bacillus for their possible use as bioinsecticide.* Ph.D. thesis, University of the Punjab, Lahore.

KRONSTAD, J.W., SCHNEPF, H.E. AND WHITELEY, H.R., 1983. Diversity of locations for *Bacillus thuringiensis* crystal protein genes. *J. Bact.*, **154**: 419-428.

KURITA, K., 2006. Chitin and Chitosan: Functional biopolymers from marine crustaceans. *Mar. Biotechnol.*, **8**: 203- 226.

LIU, M., CAI, Q.X., LIU, H.Z., ZHANG, B.H., YAN, J.P. AND YUAN, Z.M., 2002. Chitinolytic activities in *Bacillus thuringiensis* and their synergistic effects on larvicidal activity. *J. appl. Microbiol.*, **93**: 374-379.

MILLER, G.L., 1959. Glucose assay by dinitrosalicylic colorimetric method. *Anal. Chem*. **31**.426

NAWANI, N.N. AND KAPADNIS, B.P., 2003. Chitin degrading potential of bacteria from extreme and moderate environment. *Indian J. exp. Biol.,* **41**: 255-261.

PERRAKIS A., TEWS, I., DAUTER, Z., OPPENHEIM, A.B., CHET, I., WILSON, K.S. AND VORGIAS, C.E., 1994. Crystal structure of a bacterial chitinase at 2.3 A resolution. *Structure*, **2**: 1169-1180.

PICHYANGKURA, R., KUDAN, S., KUTTIYAWONG, K., SUKWATTANASINITT, M. AND AIBAC, S., 2002. Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. *Carbohydr. Res*., **337:**557-9.

PINELLI-SAAVEDRA, A., TOLEDO GUILLÉN, A.R., BRAUER, I.R.E., SILVA, A.R.L. AND CIAPARA, I.H. 1998. Methods for extracting chitin from shrimp shell waste. *Arch. Latinoam. Nutr*., **48:** 58-61.

RAMIREZ, D.M., LEPPLA, S.H., SCHNEERSON, R. AND SHILOACH, J., 2002. Production, recovery and immunogenicity of the protective antigen from a recombinant strain of *Bacillus anthracis. J. indust. Microbiol. Biotechnol*., **28**: 232-238.

ROBERTS, W.K. AND SELITRENNIKOFF, C.P., 1988. Plant and bacterial chitinases differ in antifungal activity. *J. Gen. Microbiol*., **134**: 169-176.

SALEEM, F. AND SHAKOORI, A.R., 2013. A rapid extraction method for *Bacillus thuringiensis*. *Pakistan J. Zool*., **45**: 1470-1472.

SATO, H., MIZUTANI, S.I., TSUGE, S., AOI, K., TAKASU, A., OKADA, M., KOBAYASHI, S., KIYOSADA, T. AND SHODA, S.I., 1998. Determination of the degree of acetylation of chitin/chitosan by pyrolysis-gas chromatography in the presence of oxalic acid. *Anal. Chem.*, **70**: 7-12.

SORLIER, P., DENUZIERE, A., VITON, C. AND DOMARD,

A., 2001. Relation between the degree of acetylation and the electrostatic properties of chitin and chitosan. *Biomacromolecules,* **2**: 765-772

SUGINTA, W., ROBERTSON, P.A., AUSTIN, B., FRY, S.C. AND FOTHERGILL-GILMORE, L.A., 2000. Chitinases from vibrio: activity screening and purification of ChiA from *Vibrio carchariae. J. appl. Microbiol.,* **89**: 76-84.

SYNSTAD, B., GÅSEIDNES, S., VAN AALTEN, D.M.F., VRIEND, G., NIELSEN, J.E. AND EIJSINK, V.G.H., 2004. Mutational and computational analysis of the role of conserved residues in the active site of a family 18 chitinase. *Eur. J. Biochem.,* **271**: 253-262.

USHARANI, T.R. AND GOWDA, T.K.S., 2011. Cloning of chitinase gene from *Bacillus thuringiensis. Indian J Biotechnol.,* **10**: 264-269.

VAAJE-KOLSTAD, G., VASELLA, A., PETER, M.G., NETTER, C., HOUSTON, D.R., WESTERENG, B., SYNSTAD, B., EIJSINK, V.G. AND VAN AALTEN, D.M., 2004. Interactions of a family 18 chitinase with the designed inhibitor HM508 and its degradation product, chitobiono-delta-lactone. *J. biol. Chem.,* **279**: 3612-3619.

WATANABE, T., ITO, Y., YAMADA, T., HASHIMOTO, M., SEKINE, S. AND TANAKA, H., 1994. The roles of the C-terminal domain and type III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. *J. Bact.,* **176:** 4465.

WIRTH, S.J. AND WOLF, G.A., 1990. Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. *J. Microbiol. Methods,* **12**: 197-205.

ZHANG, Y., XUE, C., XUE, Y., GAO, R. AND ZHANG, X., 2005. Determination of the degree of deacetylation of chitin and chitosan by X-ray powder diffraction. *Carbohyd. Res.,* **340**: 1914-1917.

*(Received 24 July 2014, revised 10 October 2014)*