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The Effectiveness of Native *Bacillus thuringiensis israelensis* Culture Supernatant and one of its Recombinant Components on *Culex pipiens* Larvae.

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ABSTRACT

It infers that the chitinase produced by *B. thuringiensis* might play a role in the activity of the biopesticide. The **toxicity** of suspended culture pellet and supernatant of *B. thuringiensis israelensis* (NRRL HD-522) **was** assayed toward laboratory reared second and third-instar larvae of *Culex*. *pipiens*. Results showed 100% mortality after 30 min with $2.4x10^2$ spores/ml, 50% and 100% mortality by 6h and 12h, respectively with $0.024x10^2$ spores/ml. Each of portions of 1000, 500, 250 and 100µ1 of culture supernatant persisted to eliminate all larvae during the day experiment period. No larvicidal activity occurred in containers treated with portions of 50µ1 (or less) of culture supernatant. The activity of the chitinase enzyme of the highest culture supernatant concentration was 3.5U/ml. Chitinase gene from *B. thuringiensis israelensis* was cloned and expressed in *Escherichia coli*. The gene sequence alignment showed 99% similarity to Chi36 of *B. thuringiensis* HD-789 (accession number: CP003763). The sequence result was submitted into GenBank under accession number JX474751. The recombinant chitinase reached maximal activity after 2h of incubation (about 133.4U/ml) in liquid medium. It did not exhibit any insecticidal activity at maximum concentration obtained in the bioassay experiment (about 57.5 U/ml) toward second and third-instar *Culex*. *pipiens* larvae).

Keywords: larvicidal, chitinase, *Bacillus* sp., *Culex*. *pipiens*

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INTRODUCTION

Bacillus thuringiensis subsp. *israelensis* (*Bti*), serotype H14, is a subspecies of the diversified *Bt* species, an entomopathogenic bacterium able to survive in the environment as a spore and producing insecticidal toxins within an inclusion body during the process of sporulation. *Bti* was first isolated from a water pond in the Negev desert and was the first strain described for having insecticidal activity outside Lepidoptera [1]. *Bti* is highly toxic to different *Aedes, Culex* and *Anopheles* mosquito species that are vectors of human diseases [2]**.** Its larvicidal activity resides in four major (of 134, 128, 72 and 27 kDa) and at least two minor (of 78 and 29 kDa) polypeptides encoded respectively by *cry4Aa*, *cry4Ba*, *cry11Aa*, *cyt1Aa*, *cry10Aa* and *cyt2Ba*, all mapped on the 128 kb plasmid known as pBtoxins [3]. The primary action of *Cry* toxins is to lyse midgut epithelial cells in the target insect by forming pores in the apical microvilli membrane of the cells [4- 6]. Recent evidence suggested that *Cyt* synergize or overcome resistance to mosquitocidal *Cry* proteins by functioning as a *Cry* membrane bound receptor [7].

Nevertheless, it has been recently suggested that toxicity could be related to G-protein mediated apoptosis following receptor binding [8].

Even if the detection of the *cry* and *cyt* content of a *Bacillus thuringiensis* strain may allow, to certain extent, the prediction of the insecticidal activity of its purified parasporal crystals, the complete pathogenic effect of a strain may involve other factors. Crickmore *et al* [9] reported that other insecticidal proteins that are not related phylogenetically to the three-domain *Cry* family have been identified. Among these, are binary-like toxins and Mtx-like toxins related to *Bacillus sphaericus* toxins, and parasporins produced by *Bt***.** Additionally, there are series of extracellular compounds synthesized by *Bt*, such as L-exotoxins, phospholipases, proteases, chitinases and the secreted VIPs (vegetative insecticidal proteins) contribute to virulence [10].

Although these other factors are important to ***Bt*** insecticidal activity, regardless of the target insect, *Cry* proteins are the most important of the insecticidal components found in commercial ***Bt*** formulations. Without these, for example, when endotoxin plasmids are eliminated from ***Bt*** strains by curing, the resulting spores, which lack a parasporal body containing endotoxins, are in essence not toxic or pathogenic to insects that eat those [11].

Chitin is a major component of the cuticle, the peritrophic membrane, and also functions as sleeve lining the gut of many insects. Hydrolysis of chitin to disaccharides and larger oligomeric saccharides usually takes place extra-cellular by the action of chitinases [12]. It is well documented that pathogens play role in infection through the gut penetration for chitin-rich barrier [13].

Chitinase (EC 3.2.1.14) is a glycosyl hydrolase that catalyzes the hydrolytic degradation of chitin, and is found in a wide variety of organisms including bacteria, fungi, invertebrates, plants and animals, but the roles of chitinases in different organisms are diverse [14]. Chitin hydrolysis is performed by two types of enzymes: The first, chitinases [poly-b-1, 4-(2-acetamido-2-deoxy) glucoside glycanohydrolases, EC 3.2.1.14] are the major enzymes; endochitinase which produces multimers of N-acetylglucosamine and exochitinase which catalyzes the progressive release of soluble low molecular mass dimers starting at the non-reducing end of the polymer. The second, chitobiases (N acetyl glucosaminidase, EC 3.2.1.30) which hydrolyze chitobiose to monomers of N acetylglucosamine [15, 16].

In the same concern, Sampson and Gooday [17] reported that several strains of *Bt* were found to produce chitinolytic enzymes, e.g., *Bt aizawai* HD133 and *Bt israelensis* IPS78. In addition, several studies have demonstrated that chitinase enzyme hydrolyzes chitin, can enhance the insecticidal activity of *Bt* [17-26].

Chitinase could increase toxicity by perforating the peritrophic membrane barrier in the larval midgut and thus increase the accessibility of the *Bt* δ- endotoxin molecule to its receptor on epithelial cell membranes and increase in the amount and rate of *Bt* insecticidal compounds reaching the insect midgut [19, 27, 28].

Chitinase genes have been cloned and characterized from many microorganisms such as; *B. thuringiensis* [22] and *S.* marcescens [29- 31].

The involvement of endogenous chitinases during *B. thuringiensis* infection of insect larvae was demonstrated by different methods such as; the addition of a chitinase inhibitors [17], with Chi^- mutants [22] or cloning of the corresponding gene, and its heterologous expression in *E. coli* [32, 33] or its expression in wild-type *B. thuringiensis* strain HD73 and the acrystalliferous strain Cry-B [34].

So far, genes coding for endogenous chitinases have been cloned from *Bacillus thuringiensis subsp israelensis*, *kurstaki*, *Pakistani*, *kenyae* and *sotto* [24]. Downing *et al* [29] co-introduced *cry*1Ac7 gene of *Bt* strain 234 and *chiA* gene of *S. marcescens* into strains of *P. fluorescens*, and achieved an increased efficacy in biocontrol of sugarcane borer *E. saccharina* at lower levels of Cry1Ac7 protein expression. This is advantageous, since lower expression may enable the bacteria to compete better in the environment with a diminished risk of generation of resistant larval populations resulting from exposure to high levels of Cry protein.

A number of reports are available on the cloning of chitinases either to increase biocontrol efficiency of *Bacillus thuringiensis* to prepare highly active chitinase preparation or to produce transgenic plants for increased resistance against insects [35].

Henrissat and Bairoch [36] cloned the *chiA*71 gene from *Bacillus thuringiensis* subsp *Pakistani* and its deduced amino acid sequence was analyzed. The N-terminus of mature ChiA71 showed sequence homology to the catalytic domain of chitinases which belong to family 18 of the glycosyl hydrolases.

Expression of a chitinase gene, *chiA*C, from *Bt* in *Bacillus sphaericus* 2297 using the binary toxin promoter yielded a recombinant strain that was 4,297-fold more toxic than strain 2297 against resistant *Culex quinquefasciatus*. These results show that this chitinase can synergize the toxicity of the binary toxin against mosquitoes and thus may be useful in managing mosquito resistance to *Bacillus sphaericus* [37].

Zhong *et al* [38] cloned the chitinase (*Schi*) gene from *Bacillus thuringiensis* serovar *sotto* chromosomal DNA. DNA sequencing analysis revealed that the *Bacillus thuringiensis sotto Schi* gene consists of an open reading frame (ORF) of 2067 nucleotides with codes for the chitinase precursor. The putative promoter consensus sequences (the -35 and -10 regions) of the *Bacillus thuringiensis sotto Schi* gene are identical to those of the *chiA*71 gene from *Bacillus thuringiensis subsp Pakistani*, the *chiA*74 gene from *Bacillus thuringiensis kenyae* and the *ich*i gene from *Bti*.

Sirichotpakorn [20] cloned and co-expressed chitinase gene from a high chitinase producer, *Bacillus licheniformis* TP-1, with the regulatory gene p19 and the toxin gene *cry1*1Aa1 in the host *Bti* strains 4Q2-72 and c4Q2-72 to improve the insecticidal activity by chitinases.

A transcriptionally fused gene comprising the P19 gene from *Bti* fused with a chitinase gene (chiBlA) from *Bacillus licheniformis* was integrated into the *Bacillus thuringiensis* subsp. *aizawai* BTA1 genome by homologous recombination. The resulting *Bacillus thuringiensis* subsp. *aizawa*i strain (INT1) showed growth and sporulation comparable with that of the wild-type strain. These results show that chitinase can increase the activity of *Bacillus thuringiensis* subsp. *aizawai* against *S. exigua* [39].

The objective of this study is to determine if a chitinase, obtained from *B. thuringiensis subsp*. *israelensis*, had a synergistic effect on *B. thuringiensis subsp*. *israelensis* toxin directed against second and third-instar *Culex pipiens* larvae. The synergistic action of this chitinase was demonstrated by cloning of the corresponding gene, and its heterologous expression in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacillus thuringiensis israelensis (NRRL HD-522) was kindly supplied by Prof. Nakamura, LK, United State Department of Agriculture and *Esherichia coli* DH5α was kindly provided from Stuttgart University, Germany. The vector pTZ57R/T (Viogen Biotek Corporation and Fermentas) and the vector pCYTEXP1Vieira & Messing [40] (Stuttgart University, Germany) were used for cloning and expression studies, respectively.

Mosquitoes

Culex pipiens eggs and larvae were kindly provided from laboratory of Medical Entomology, Department of Applied Entomology, Faculty of Agriculture, Alexandria University, Egypt. The larvae were hatched and raised in distilled water at room temperature for 10 to 12 days and fed mosquito diet for acclimatization with laboratory conditions.

Mosquito bioassay

Preparation of bacterial toxin

An appropriate amount of freshly prepared *Bacillus thuringiensis israelensis* culture was used to inoculate 50ml of NB broth supplemented with 0.2% colloidal chitin for chitinase production. The culture was grown at 30°C with shaking for 8 -11 days until most of the cells was lysed, crystals had been released and chitinase had produced. The spores and crystals (biomass yield) were harvested by centrifugation at 12,000 rpm for 10 min. Both the resultant supernatant and pellet were stored at -20 °C until be used for bioassay purposes. During the culturing period, samples were collected and used for determining viable count, spore count and chitinase activity.

The concentration of *Bt* was evaluated by the mean of bacteriological counts. *Bacillus thuringiensis* spore count was determined as described by Thomas *et al* [41].

Toxicity test

Toxicity of both culture pellet and supernatant was determined by bioassay with laboratory reared second and third-instar larvae of *C. pipiens*. Groups of 10 mosquito larvae were placed into sterile cups containing 50ml distilled water and different dilutions of either culture pellet or supernatant. The assay cups were kept at 26±2 °C. Distilled water was used as negative controls. Each experiment was performed in duplicate and two independent experiments were performed. Mortalities % were recorded after different periods. The mortality percentage was recorded by counting the number of living larvae and corrected by using appropriate control and adopting Abbot's formula [42].

Abbot's formula:

$$\text{Corrected mortality \%} = \frac{\text{Observed mortality \% - control mortality \%}}{\text{100 - Control mortality \%}} \times 100$$

Chitinase detection

A preliminary test for production of chitinase enzyme by *B. thuringiensis israelensis* strain was carried out as previously described [43].

Enzyme Assay

Quantitative estimation of chitinase activity was measured using colloidal chitin as the assay substrate. Crude enzyme was prepared as previously described [43]. The reaction mixture consisted of equal volumes of crude protein and 0.5% colloidal chitin in 50 mM acetate buffer, pH 6.0. The reaction was performed at 37 °C for 60 min and terminated by adding one volume of 1% dinitrosalicylic acid solution. One unit of chitinase activity was defined as the amount of enzyme which produces 1µmol of reducing sugar (*N*-acetylglucosamine (GlcNAc) or its equivalent) from colloidal chitin per 1hr at 37ºC. The reducing sugar released during the reaction, using colloidal chitin as substrate was measured, according to Somogyi-Nelson method [44].

DNA extraction and manipulation

The genomic DNA was isolated from *Bacillus thuringiensis israelensis* strain by the method of Sambrook *et al* [45]. Plasmid DNA was isolated using easy nucleic acid isolation kit [E.Z.N.A. ® Plasmid Miniprep

Kit (OMEGA BIO-TEK) (USA)] according to manufacturer's recommendations. Extraction of PCR products and plasmid DNA from agarose gel was done with the peqGOLD gel extraction kit (Viogen Biotek Corporation and Fermentas). Restriction analysis and DNA modifications were performed using enzymes purchased from Viogen Biotek Corporation and Fermentas according to the recommendations of the manufacturer.

TA-cloning of chitinase gene from *Bacillus thuringiensis israelensis*:

A conserved region of chitinase gene (about 310- bp) was amplified from genomic DNA of *Bacillus thuringiensis israelensis* strain as described by El-Hamshary *et al* [43]**.** Full length chitinase gene was amplified using the primers; chit F and chit R 5'ATATGTTAAACAAGTTCAAATTTTTTT3' and 5' TTATTTTTGCAAGGAAAG ATTATC 3'respectively, which were designed based on the sequence data of obtained partial chintinase gene. PCR product of expected full length chitinase gene (~1200bp) using these primers was performed then checked on agarose gel. Afterwards, the PCR product was purified from agarose gels using (peqGOLD Gel Extraction Kit). TA-cloning of purified fragment was carried out to clon into the vector pTZ57R/T according to the protocol of InsTAclone ™ PCR Cloning Kit (Sigma). The ligation mixture was used directly to transform *E. coli* DH5 α cells. Transformants were selected in LB agar supplemented with ampicillin (100mg/ml), 5-bromo-4-chloro-3-indolyl-D-galactoside, (20mg/ml) X-Gal and (0.2mM) isopropylthio-D-galactoside (IPTG). Plasmids of selected colonies were analyzed by PCR using universal M13 primers then subjected to DNA sequencing.

DNA sequencing

Automated DNA sequencing based on enzymatic chain terminator technique, developed by Sanger *et al* [46], was done using 3130 X DNA Sequencer (Genetic Analyzer, Applied Biosystems, Hitachi, Japan). Chitinase gene was sequenced partially using the specific primers previously mentioned [43]**.** Full length chitinase gene cloned into pTZ57R/T vector was sequenced using standard M13/pUC universal primer. The full length gene sequence has been submitted, deposited into GenBank under accession number (ac: JX474751.1). Deduced amino acid sequences were analyzed using the BLASTX family program.

Phylogenetic analysis:

Multi alignment between the target sequence and the closely related (ac: CP003763; CP001186; CP003752; CP001903; CP001176; AF510723; GU134905; EF103273; CP003889; CP001907; CP001746; JQ739168; CP001598; GQ183830; CP000485; AE017194 and CP000903) was performed with CLUSTAL W. On the basis of the resulting multiple sequence alignments a phylogenetic tree was calculated for each protein by applying the maximum-likelihood method implemented in the Tree-Puzzle software, version 5.2. Multiple sequence alignment and molecular phylogeny were performed using BioEdit [47]. Subsequently, the phylogenetic tree was displayed using the TREEVIEW program [48].

Construction of expression plasmids carrying chitinase gene from *Bacillus thuringiensis israelensis* strain:

For expression, the target gene was amplified from the pTZ57R/T plasmid carried insert using modified specific primers introduced with recognition sites (*Nde*I and *Bam*HI) for forward and reverse direction, respectively: chit F (*Nde*1) 5'AAT TCC ATA TGT TAA ACA AGT TCA AAT TTT TTT3' and chit R (*Bam* H1) 5' AAA GGATCC TTA TTT TTG CAAGGA AAG ATTATC3'. The purified PCR product was digested by *Nde*I/*Bam*HI according to manufacturers'ˢ instructions and ligated into *Nde*I/*Bam*HI digested expression vector pCYTEX-P1. The construct (pCYTEX-Chitinase) was developed after ligation and transformation in *E. coli* DH5α. Plasmids were prepared from some picked colonies, inserts' verification were confirmed by PCR using universal primers SDM1&3 [49] and restriction digestion.

Heterologous expression of chitinase gene under lambda promoter

After sub-cloning of the chitinase gene in *E. coli* DH5α as mentioned above, its expression was achieved under control of the temperature-inducible λ promoter [49]. A portion of 50ml LB ampicillin medium in 250ml Erlenmeyer conical flask was inoculated with 500µl overnight culture of *E. coli* DH5α carrying the target plasmid at 37ºC with shaking (200 rpm) till OD_{600} 0.7-1.0. For induction, the temperature was shifted to 42ºC [49] and sample was taken every hour for four hours. The cell pellet was separated by centrifugation for 10min at 5000rpm then suspended in phosphate buffer pH (7.0), disrupted by sonication for 1min, then the

soluble recombinant protein was separated by centrifugation for 10min at 10,000 rpm. Mosquito bioassay and chitinase activity was measured in each sample.

Chitinase activity:

The chitinase activity of the crude extracts (recombinant protein) was determined spectrophotometrically using colloidal chitin as the assay substrate as mentioned above.

Mosquito Bioassay Using Crude Recombinant Protein:

Groups of 10 mosquito larvae were placed into sterile cups containing 10ml distilled water and 500µl of separated soluble recombinant protein. The assay cups were kept at 26±2 °C for 3 days. Distilled water was used as negative controls. Each experiment was performed in duplicate and two independent experiments were performed. The mortality percentage was calculated as previously, mentioned.

RESULTS

Larvicidal activity of the spore/ crystal complex of *B. thuringiensis var. israelensis* toward *Culex pipiens* larvae:

The toxicity of various concentrations from mixture of spores and crystals of *B. thuringiensis var. israelensis* was assayed toward *Culex pipiens* larvae. In all tests carried out, a100% mortality rate was observed during the experiment period. It was found that $2.4x10^2$ spores/ml causes100% mortality after 30min. while, $0.024x10^2$ spores/ml causes 50% and100% mortality by 6h and 12h, respectively **(Table 1)**.

Table. 1: Mortality % after exposure periods at different viable count, spore count dilutions.

Viable Count	Spore Count	Mortality % after different exposure periods												
		½	1h	2	3	4	5	6	7	8	9	10	11	12
$98x10^2$	$2.4x10^2$	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
$49x10^2$	$1.2x10^2$	80%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
$24.5x10^2$	$0.6x10^2$	50%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
$9.8x10^2$	$0.24x10^2$	40%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
$4.9x10^2$	$0.12x10^2$	Nil	Nil	10%	40%	60%	70%	80%	90%	100%	100%	100%	100%	100%
$2.45x10^2$	$0.6x10^2$	Nil	Nil	Nil	Nil	10%	60%	80%	90%	90%	90%	100%	100%	100%
$0.98x10^2$	$0.024x10^2$	Nil	Nil	Nil	Nil	10%	30%	50%	60%	70%	80%	80%	90%	100%

Larvicidal activity the culture supernatant of *B. thuringiensis var. israelensis*

Also, the toxicity of various concentrations from culture supernatant of *B. thuringiensis var. israelensis* containing chitinase was assayed toward *Culex pipiens* larvae. It was found that each of portions of 1000, 500, 250 and 100µ1 of culture supernatant persisted to eliminate all larvae during the day experiment period. No larvicidal activity occurred in containers treated with portions of 50µ1 (or less) of culture supernatant **(Table 2)**. The activity of the enzyme of the highest culture supernatant concentration was 3.5U/ml.

Table. 2: Mortality % after exposure periods at different supernatant dilutions.

culture supernatant	Mortality % after different exposure periods												
	½	1h	2	3	4	5	6	7	8	9	10	11	12
1000 µl (3.5U/ml)	Nil	Nil	80%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
500µl	Nil	Nil	40%	80%	90%	90%	100%	100%	100%	100%	100%	100%	100%
250µl	Nil	Nil	30%	60%	70%	80%	90%	100%	100%	100%	100%	100%	100%
100 µl	Nil	Nil	10%	30%	40%	50%	70%	90%	100%	100%	100%	100%	100%
50µl	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
25 µl	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
10µl	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

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Chitinase activity of *B. thuringiensis israelensis* strain

A preliminary test was performed by growing *B. thuringiensis var. israelensis* strain in solid chitinase-inducing medium plate containing chitin. The strain exhibited zone of degradation of colloidal chitin on solid chitinase-inducing medium after 8 days of incubation evidencing its chitinolytic activity **(Figure 1)**. The activity was detected through floating the plate with congo red solution.

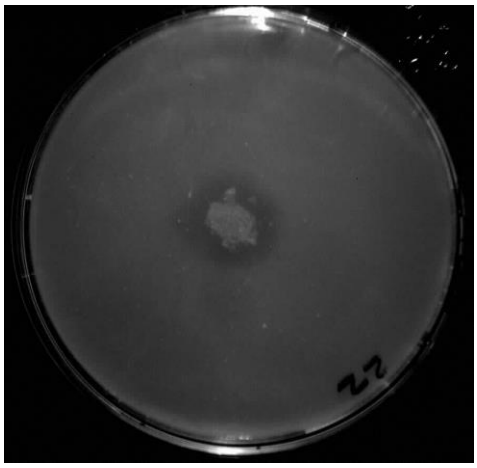

Fig. 1. Chitinolytic activity of *B. thuringiensis israelensis* strain on colloidal chitin containing agar plate.

When the tested strain was grown in nutrient broth supplemented with 0.3% colloidal chitin, the chitinase activity was found to increase during the exponential growth and reach maximal level after 3 days of incubation (174U/ml). The activity decreased slightly after further incubation **(Figure 2)**.

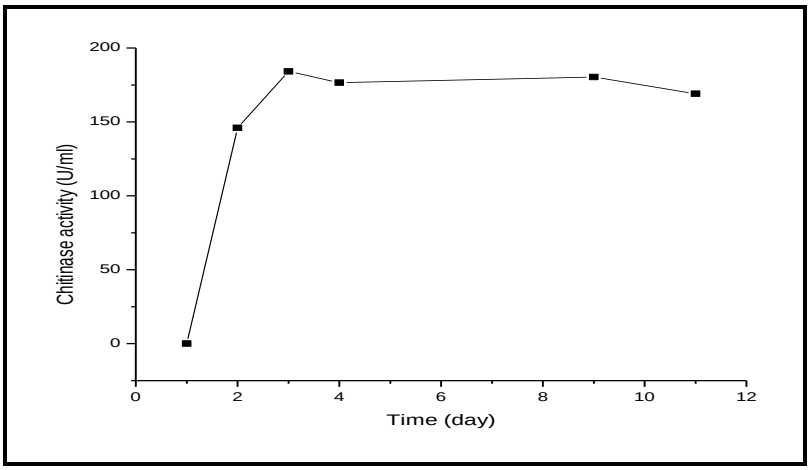

Fig. 2. Chitinase activity of *B. thuringiensis var. israelensis* strain in respect to incubation time.

Cloning of chitinase gene from *Bacillus thuringiensis israelensis*:

The primers designed according to the sequence of *B. cereus* G9842 chitinase gene (GeneBank Accession No. CP001186), were used in order to amplify the full length chitiase gene from *B. thuringiensis israelensis*. The single sharp band (≈1100-bp) corresponding to chitinase gene insert was cut out from agarose gel, purified and ligated into pTZ57R/T plasmid. The recombinant plasmid was transferred into *E. coli* DH5α. Super coiled plasmid DNA of pTZ57R/T was used as positive control. The transformation efficiency was found to be 0.55 x 10^3 CFU/µg.

The transformed cells were picked up and streaked on Luria agar containing amplicillin (100mg/ml), X-gal and isopropyl β-D-thiogalactosidase (IPTG). The clones containing recombinant molecules were selected based on blue-white assay.

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Plasmids were isolated from clones containing of chitinase gene and the clones were confirmed through PCR amplification using both specific primers and M13 primers as shown in **Figure 3**. The plasmids carried insert were subjected to sequencing using universal primers (M13 F& R).

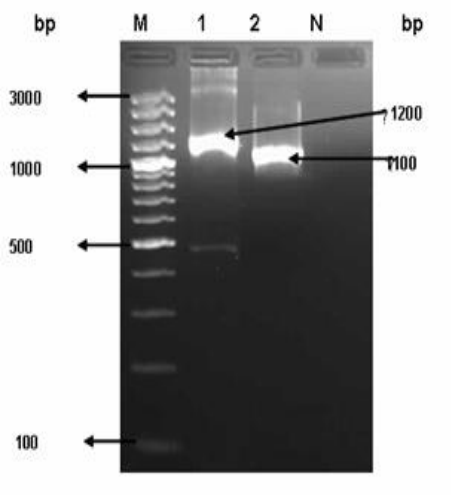

Fig. 3. Agarose gel electrophoresis of PCR products Lane M: Marker [Fermentas gene ruler™ 100 bp plus DNA ladder], Lane 1: PCR fragment resulting from usage of (M13 F & R) primers and recombinant pTZ57R/T plasmid as a template, Lane 2: PCR fragment resulting from usage of specific chitinase gene primers and recombinant pTZ57R/T plasmid as a template and Lane N: Negative control (no template).

Expression of chitinase gene from *Bacillus thuringiensis israelensis* into *E. coli*

The chitinase gene cloned into pTZ57R/T was amplified using modified primers started with restriction sites *Nde*1*/Bam*H*1* to expresses the gene under lambda-promoter using pCYTEXP1. Both of expression vector and chitinase gene cut with *Nde*1/*BamH*1 then ligated. The ligate mixture was transferred into *E. coli* DH5α competent cells. The transformation efficiency was found to be 0.7 x 10^3 CFU/µg.

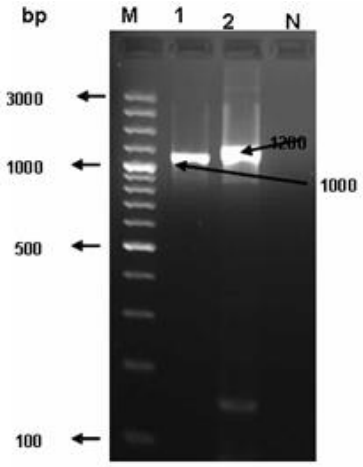

Fig. 4. Agarose gel electrophoresis of PCR products. Lane M: Marker [Fermentas gene ruler™ 100 bp plus DNA ladder], Lane 1: PCR fragment resulting from usage of specific chitinase gene primers and (pCYTEX-Chitinase) constructs as a template, Lane 2: PCR fragment resulting from usage of SDM1&3 primers and (pCYTEX-Chitinase) constructs as a template and Lane N: Negative control (no template).

The positive clones containing chitinase gene were confirmed by PCR. PCYTEX-chitinase gave amplicons of about 1100bp and about 1200bp from usage of specific chitinase gene and universal SDM1&3 primers, respectively **(Figure 4)**. The positive clones were also confirmed by restriction digestion with *Nde*I and *BamH1*.

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Nucleotide sequence of the chitinase gene

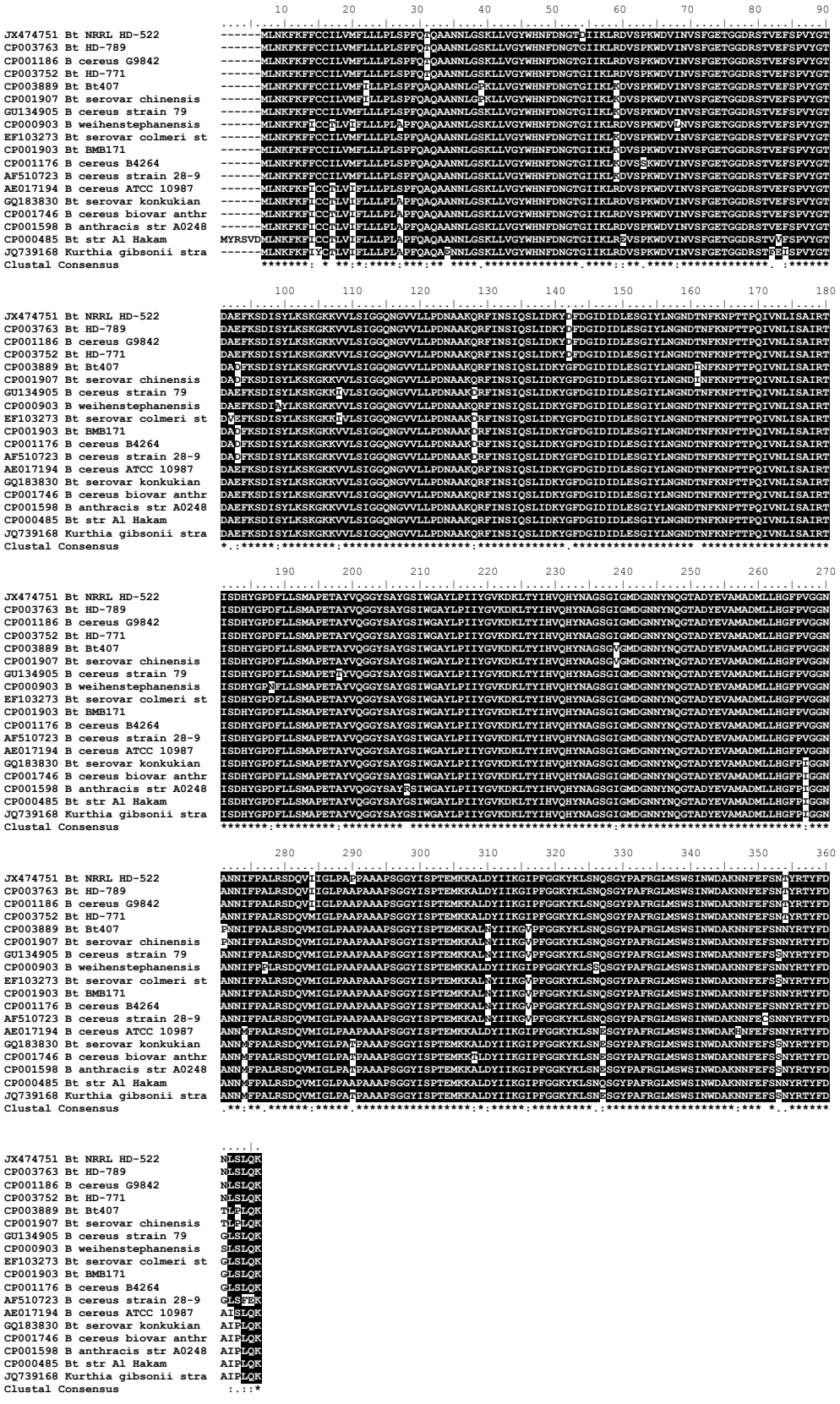

Fig. 5. Multiple-alignment of amino acid sequences of chitinase gene from various species under ac: CP003763; CP001186; CP003752; CP001903; CP001176; AF510723; GU134905; EF103273; CP003889; CP001907; CP001746; JQ739168; CP001598 and GQ183830 in a graphic view.

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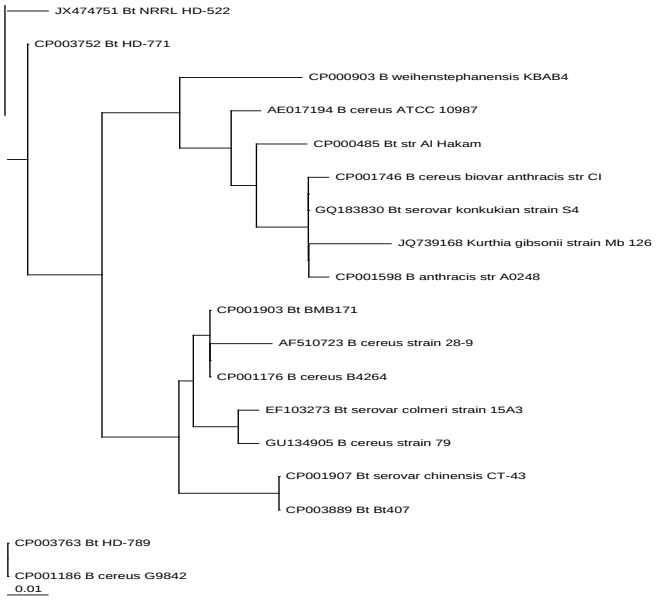

Fig. 6. Phylogenetic tree of *B. thuringiensis israelensis* chitinase gene amino acid sequences. (Multiple sequence alignment and molecular phylogeny were performed using BioEdit.The phylogenetic tree was displayed using the TREEVIEW program subsequently).

The chitinase gene was sequenced completely in both recombinant pTZ57R/T plasmids and (pCYTEX-Chitinase) constructs using M13 and SDM 1& 3 primers respectively. The sequence was deposited into GenBank under accession no. (JX474751.1). Chitinase gene of the tested *B. thuringiensis israelensis* strain had 99% percent homology with each of reported extracellular exochitinase Chi36 of *B. thuringiensis* HD-789, extracellular exochitinase Chi36 of *B. cereus* G9842 and extracellular exochitinase Chi36 of *B. thuringiensis* HD-771. Multiple-alignment between amino acid sequence of *B. thuringiensis israelensis* (ac: JX474751) and the closely related sequences (ac: CP003763; CP001186; CP003752; CP001903; CP001176; AF510723; GU134905; EF103273; CP003889; CP001907; CP001746; JQ739168; CP001598; GQ183830; CP000485; AE017194 and CP000903) showed minor differences in the deduced amino acids **(Figure 5)**. Also, a phylogenetic tree was displayed and presented in **Figure 6**, where chitinase genes under ac: CP000485; AE017194 and CP000903 compared to chitinase sequence of *Bacillus thuringiensis israelensis* (ac: JX474751). The alignment was performed with CLUSTAL W as described under material and methods.

Activity of recombinant chitinase gene:

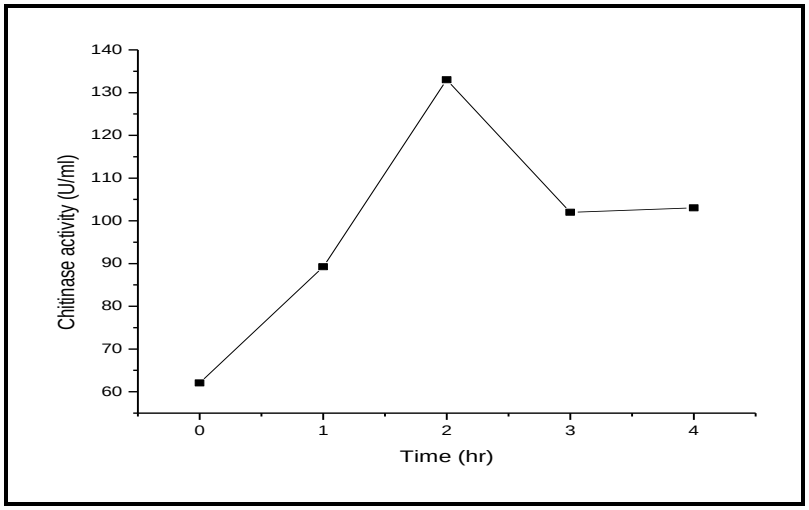

Fig. 7. Chitinase activity of recombinant chitinase gene.

The expression of the cloned chitinase gene, was checked by culturing *E. coli* harbouring (pCYTEX-Chitinase) in LB medium and the enzyme activity of the crude extracts (recombinant protein) was determined after heat induction (shift of temperature to 42◦C) of the culture. It was noticed that the chitinase activity started increasing at 1 h during the exponential growth and reach maximal level after 2 h of incubation (about 133.4U/ml), followed by a decrease until the end of exponential growth, respectively **(Figure 7)**.

Insecticidal activity of chitinase gene expressed in *E. coli*.:

To investigate if the insecticidal activity of *Bacillus thuringiensis israelensis* culture supernatant was potentates by the addition of chitinase, crude recombinant chitinase was assayed toward *Culex pipiens* larvae as described in material and methods section**.** The results revealed that the expressed chitinase alone has no insecticidal activity at maximum concentration obtained in this experiment (about 57.5 U/ml).

DISCUSSION

B. thuringiensis was initially characterized as an insect pathogen, and its insecticidal activity was attributed largely or completely (depending on the insect) to the parasporal crystals. Moreover, some strains of *B. thuringiensis* have been reported to produce pesticidal proteins unrelated to the Cry proteins during vegetative growth [50]. Additionally, *B. thuringiensis* strains are known to produce chitinase enzymes if chitin is used as a sole carbon source [51], and chitinases are known to synergize the activity of *B. thuringiensis* spore-toxin complexes against various *Lepidoptera* [18, 19, 52].

Our objective was to determine if a chitinase, obtained from *B. thuringiensis israelensis* strain, had a synergistic effect on *B. thuringiensis* israelensis toxin directed against *Culex pipiens* larvae.

In this study, the toxicity of various concentrations from mixture of spores and crystals of *B. thuringiensis var. israelensis* (Pellet) was assayed toward *Culex pipiens* larvae. It was found that $2.4x10^2$ spores/ml causes100% mortality after 30 minute while, $0.024x10^2$spores/ml causes 50% and100% mortality by 6h and 12h, respectively. The toxicity of *B. thuringiensis israelensis* against mosquito vectors of human diseases is because of the production of multiple toxins with different modes of action including the four major (of 134, 128, 72 and 27 kDa) and at least two minor (of 78 and 29 kDa) polypeptides which encoded respectively by *cry4Aa*, *cry4Ba*, *cry11Aa*, *cyt1Aa*, *cry10Aa* and *cyt2Ba* [3]**.** The results obtained showed that the larvae of *Culex* are sensitive to the used spores and crystals of *B. thuringiensis var. israelensis*. These results similar to those obtained by Gbehou *et al* [53]**,** who used the granular formulation of *Bacillus thuringiensis israelensis*, serotype H-14 VectoBac G–200 International toxic units per mg [(ITU/mg]) at concentration of 10 mg/L against two larval stages of *Culex* spicies (L2 and L4)] in the laboratory, their results showed the death rate of 100% after 3 h and half of exposure time and 5 h for L2 and L4 respectively. They explained that their results were due to the sensitivity of *Culex larvae* to the crystals of *B. thuringiensis var. israelensis* which would be caused by behavioral and physiological properties for species and also related to the dispersion of the crystals of *B. thuringiensis var. israelensis*. These results were also connected with those provided by Lacoursière *et al* [54].

On the other hand, the toxicity of various concentrations from culture supernatant of the studied strain was assayed toward *Culex pipiens* larvae. It was found that 1000µlof culture supernatant cause 100% mortality after 3 hours while 50µl did not cause mortality, where activity of the chitinase enzyme of the highest culture supernatant concentration was 3.5U/ml. Studies by Wiwat *et al* [55]**,** had indicated that the toxicity of *B.thuringiensis* ssp. *kurstaki* HD-1(G) for diamondback moth larvae was increased when in combination with its supernatant and they suggested that the chitinase present in the supernatant of *B. thuringiensis* ssp. *kurstaki* HD-1(G) culture and/or some factor(s) could enhance the toxicity for diamondback moth larvae. It has been well established that entomotoxicity of *B. thuringiensis* is due to the toxin crystal, *B. thuringiensis* cell-spore complex, and vegetative insecticidal protein (VIPs) [56]**.** Recently, they also demonstrated that the residual toxin, spores, cells, and VIPs could be recovered from the supernatant employing ultrafiltration technique, which in turn could be mixed with the centrifuged or the concentrated toxin, spores and cells to prepare a high entomotoxicity value product. Vu *et al* [57] reported that the chitinase produced during *B. thuringiensis kurstaki* fermentation, which would normally be discarded in the supernatant, could be recovered during ultrafiltration along with residual toxin, spores, cells and soluble VIPs, could further enhance the entomotoxicity value of the final product.

This study also revealed that the tested *B. thuringiensis israelensis* strain exhibited zone of degradation of colloidal chitin on solid chitinase-inducing medium after 8 days of incubation. On the other hand, the chitinase activity from the tested strain was found to increase during the exponential growth in induced broth and reach maximal level after 3 days of incubation (174U/ml). The activity decreased slightly and still detected after further incubation. It is possible that the chitinase of the tested strain may not be produced in sufficient quantity, for detection by solid chitinase-inducing plates at the third day of incubation as in case of induced broth. Long incubation time was reported for *Stenotrophomonas maltophilia* C3, where cultures in crude chitin had the highest chitinase activity, reaching peak level after 9 days **[**58]. These results were also in agreement with those obtained by Liu *et al* [18] who found that thirty-eight out of 70 *B. thuringiensis* strains tested exhibited endochitinase activity on solid chitinase-inducing plates at pH 7.0, and most of them produced chitinase activities of 150 ~ 280 U/ ml in inducing broth. In addition, it was reported that the expression of *B. thuringiensis* chitinase is rather low and needs induction by chitin [22, 32, 33]**.** Most of the researches on endogenous chitinase production by *B. thuringiensis* demonstrated that there were differences in the activities of the chitinase enzymes from tested strains [17, 18, 26]. The range in activity levels may be due to the biochemical and physicochemical characteristics of the *B. thuringiensis* chitinases **[**59]**.**

Whenever information on the entire or conserved domains of the target gene is known, PCR based techniques are used to detect and also pull out the desired sequences from genomic DNA or from clones. The database at http://www.ncbi.nlm.nih.gov also houses sequences of chitinase encoding genes from different sources. PCR primers were designed based on conserved nucleotide sequences of chitinase genes in different bacilli [43] and used to amplify a conserved region of chitinase gene from *B. thuringiensis subsp israelensis*, and amplicone with molecular size 310 pb was obtained. The purified amplicon was sequenced and based on alignment of the obtained sequences with those available in database at http://www.ncbi.nlm.nih.gov, specific primers were designed for full-length chitinase gene. About 1100bp amplicon picked up and cloned into pTZ57R/T and transformants having recombinants were isolated through blue-white assay. The white colonies were confirmed as recombinants by gene specific PCR, restriction analysis and through sequencing. Generally, several chitinase genes of *B. thuringiensis* have been cloned and intensively characterized [24, 32, 33, 38].

In the current study, the gene sequence alignment indicates that chitinase gene belongs to glycoside hydrolase (GH) family 18, has an open reading frame comprising of 1083 bp nucleotide sequences and encodes 361 amino acids. The deduced 361 amino acid sequence showed a high degree of similarity with other *Bacillus* chitinases. It was reported that chitinases are grouped, based on their sequence similarities, into two families of glycoside hydrolases (GH), family 18 and 19 [13, 60, 61**.** GH Family 18 chitinases are widely distributed in bacteria, fungi, viruses and some plants [62]. To demonstrate the synergistic action of this chitinase along with the *B. thuringiensis* israelensis toxins toward *Culex pipiens* larvae, chitinase gene of *B. thuringiensis subsp israelensis* was expressed in *E. coli* DH5α using pCYTEXP1 vector. The recombinant clones were analyzed by PCR amplification and restriction mapping. The individual role of chitinase from *B. thuringiensis* israelensis was produced in *E.coli* and its enzymatic effect on chitin degradation was studied by culturing *E. coli* harbouring (pCYTEX-Chitinase) in LB medium and the enzyme activity of the crude extracts (recombinant protein) was determined after heat induction of the culture. It was noticed that the chitinase activity reached maximal level after 2h of induction (about 133.4U/ml), indicating expression of the cloned gene. This result also indicated that the enzyme activity of chitinase produced from transformant strain is higher than that produced by parental *B. thuringiensis israelensis* strain because the transformant strain produced approximately similar quantities of enzyme activity in shorter time. The same vector was successfully used by Mc Carthy [63] and Rua *et al* [64] for expression and producing large amounts of an active enzyme.

The chitinase gene from *Bacillus circulans* number 4.1 (pCHIB1) was subcloned into the *HindIII* sites of a 6.96-kbp plasmid vector pBCX and designated as pBX43 (9.56 kbp). This plasmid produced three times as much chitinase in *Bacillus thuringiensis subsp. israelensis* strain c4Q272 as pHYB43, which comprises the commercial shuttle vector pHY300PLK plus the chitinase gene [65]**.** Additionally, Okay and Özcengiz [66] used the *E. coli-Bt* shuttle vector pHT315 for homologous expression of endochitinase gene (*chi3023)* from *Bt* serovar *morrisoni* 3023 and they reported that, introduction of recombinant pHT315BTC, carrying *chi3023* into *Bt* serovar *morrisoni* 3023, resulted in a 23-fold increase in endochitinase activity (0.185 U/mg versus 4.256 U/mg).

As the insect peritrophic membrane consists of a chitin fibril network embedded in a protein-carbohydrate matrix [67], pathogens have to cross the chitin-rich barrier to exert their virulence [33]. Several

studies have demonstrated that chitinase, the enzyme that hydrolyzes chitin, can enhance the insecticidal activity of *B. thuringiensis* [17-22]. It has been suggested that chitinase might hydrolyze chitin in the peritrophic membrane and cause pore formation, thus allowing for an increase in the amount and rate of *B. thuringiensis* insecticidal compounds reaching the insect midgut [27, 28]. Additionally, Granados *et al* [68] showed that the enhancin from the *Trichoplusia ni granulovirus* could also degrade the peritrophic membrane of various lepidopteran larvae and could increase the activity of *B. thuringiensis* against lepidopteran larvae, including *Spodoptera exigua*.

The positive role of endogenous chitinase in *B. thuringiensis* pathogenicity has been demonstrated [17, 18, 19, 22, 23, 55]**.** In addition, several studies have demonstrated that co-expression of heterologous chitinase genes in *B. thuringiensis* can enhance the insecticidal activity of this bacterium [18,20, 25, 37,39]**.** In this study, crude recombinant chitinase was assayed toward *Culex pipiens* larvae. The results revealed that the expressed chitinase alone has no insecticidal activity at maximum concentration obtained in this experiment (about 57.5 U/ml). These results may be due to the tested native strain may express more than one chitinases but in this study, only one chitinase gene has been cloned from tested *B. thuringiensis* strain. This enzyme may differs biochemically and physicochemically from those previously described. Many chitinases that vary widely in their biochemical and physicochemical characteristics have been reported. For example, six distinct chitinases were detected in the cultural supernatant of *B. circulans* WL-12 when it was grown in the medium containing chitin as an inducer substrate, and one of these six chitinases, chitinase Al, showed strong affinity to chitin and was suggested to play a major role in the degradation of chitin in the chitinase system of *B. circulans* WL-12 [69]. Also, Liu *et al* [70] have reported that *B. thuringiensis*. subsp. colmeri 15A3 (Bt. 15A3) constitutively expresses two chitinases, namely, ChiB and ChiA, with molecular masses of 70 and 36 kDa, respectively and the biocontrol potentials of ChiA and ChiB would appear to be different. Cai *et al* [37], expressed a chitinase gene, chiAC, from *B.thuringiensis*, in *B.sphaericus* 2297 using the binary toxin promoter, yielded a recombinant strain that was 4,297-fold more toxic than strain 2297 against resistant *Culex quinquefasciatus* and concluded that this chitinase can synergize the toxicity of the binary toxin against mosquitoes and thus may be useful in managing mosquito resistance to *Bacillus sphaericus***.**

On the other hand, *chi*A74 under the control of its native promoter has been used to transform *B.thuringiensis*, and although the recombinant strain showed threefold increase in chitinase production, the insecticidal activity was not improved [71]. Similarly, Barboza-Corona *et al* [72] noticed that despite the chitinolytic activity of recombinant HD-73 strain, expressing chiA74 under the control of its native promoter (HD-73-pEHchi-A74), was markedly elevated, being *58- and 362-fold higher than, respectively, HD-73-pEHchiA74 and parental HD-73, no change in cellular protease activity was observed. Edwards and Jacobs-Lorena [73], noticed that the larval peritrophic matrix (PM) differ considerably in their susceptibility to chitinase. Many chitinases have been reported to be alkali-tolerant [74, 75]. INT1 and similar strains should find applications as more effective bio-insecticides, due to their combined action of Cry toxin(s) and chitinase [39]. However, it is possible that the activity of the bacterial chitinase, which has a pH optimum of around 5.0–6.0 [76], may have been impaired in the mosquito gut lumen, which is alkaline [77, 78].

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