Journal of Advanced Laboratory Research in Biology
E-ISSN: 0976-7614
Volume 5, Issue 1, January 2014
PP 1-6
https://e-journal.sospublication.co.in

Research Article

Bacillus thuringiensis var. *aizawai* HD-137 as a Potential Agent for Biological Control

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Abstract: Four strains of *Bacillus thuringiensis* were screened for their chitinolytic activity on colloidal chitin. *B. thuringiensis* var. *aizawai* HD-137 with the GenBank accession number HM173355 showed the highest chitinase activity, which was recorded after 2 days of incubation. The optimum condition for high chitinase production was Nutrient Yeast extract, Salt Medium, NYSM, with 0.2% colloidal chitin, two days of incubation, pH 6 and 30°C. The novel strain *B. thuringiensis* var. *aizawai* HD-137 is also considered as a powerful phytopathogenic control agent in which it showed inhibition of the mycelial growth of some phytopathogenic fungi, *Alternaria solani*, *Rhizopus* B1 and B2, *Fusarium solani* and *Aspergillus flavus*. The clear zones of mycelial inhibition ranged from 12 to 19mm. The partial nucleotides sequence of chitinase gene from *B. thuringiensis* var. *aizawai* HD-137 showed similarities to the chitinase producing bacteria in the GenBank, and it was more related to *B. thuringiensis* (AB699714, GQ921840 and GQ921842) and *B. ehimensis* chi60 (AB110081). It is obvious that the *B. thuringiensis* var. *aizawai* HD-137 is considered as a significant biocontrol agent against phytopathogenic fungi.

Keywords: *Bacillus thuringiensis* HD-137, Chitinase, Antagonism, Phylogenetic.

1.1 Introduction

Chitin $(C_8H_{13}O_5N)_n$ is a long chain polymer of an N-acetylglucosamine, a derivative of glucose. It is the main component of the cell walls of fungi, the exoskeletons of arthropods and insects, the radules of molluscs and beaks of cephalopods. Chitinase (EC 3.2.1.14) is a glycosyl hydrolase that catalyzes the hydrolytic degradation of chitin. Bacteria produce chitinase to digest chitin for use as carbon and energy sources (1). Some chitinolytic bacteria have been shown to be potential agents for the biological control of both phytopathogenic fungi and insect pests (2). It has been reported that chitinases are widely distributed in *B. thuringiensis* strains and these strains can enhance the insecticidal activity of *B. thuringiensis* (3). It is known that *B. thuringiensis* var. *aizawai* HD-137, has strong larvicidal activity against some lepidopteran pest (4). Continuous use of chemical fungicides in agriculture leads to deleterious effects on the environment. Now today, the attention of the world is directed to reduce the application of chemical products and increase the potential for biological control. Some chitinase genes have yet been cloned and their phylogenetic was done (1, 5-7). A phylogenetic tree or evolutionary tree is a branching diagram or "tree" showing the inferred evolutionary relationships among various biological species or other entities, their phylogeny, based upon similarities and differences in their physical and/or genetic characteristics. Joung and Cote (8), reported that most *B. thuringiensis* could be grouped together based on the DNA similarity.

The objectives of this work are to evaluate the efficacy of some strain of *B. thuringiensis* as a biological control agent against plant pathogenic fungi, to optimize conditions for chitinase production by *B. thuringiensis* var. *aizawai* HD-137 and to confirm the relationship with other chitinase bacteria.

2. Material and Methods

2.1 Bacterial strains and culture conditions

Four strains (obtained from the Biocontrol Agricultural Pests Department, NRC, Cairo, Egypt) from previous studies were used in this study, *B. thuringiensis* var. *kurstaki* HD-1-S, *B. thuringiensis* var. *galleriae* HD-129, *B. thuringiensis* var. *aizawai* and *B. thuringiensis* var *aizawai* HD-137. The strains were incubated in L-broth medium (9) at 30°C.

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2.2 Detection of Chitinase

Each strain of *B. thuringiensis* was streaked on nutrient agar supplemented with (0.2% colloidal chitin). Plates were incubated at 30°C until a clear zone was seen around the colonies (10).

2.3 Optimization of culture conditions

The effect of type of media, temperature, pH and the concentration of chitin on bacterial chitinase production were studied. Different media (NYSM, without chitin, NYSM with 0.2% chitin, L.B and N.B), temperatures (25, 30, 35, 40 and 45°C), the pH level (5, 6, 7, 8, 9 and 10) and concentration of chitin on medium (0.2%, 0.5% and 1%) were taken to determine the optimum conditions for chitinase production. After two days of growth, the supernatant was used for chitinase assay.

2.4 Chitinase Assay

Chitinase activity was determined colorimetrically by detecting the amount of N-acetylglucosamine (GlcNAc) released from a colloidal chitin substrate (11). The reaction mixture consisted of equal volumes (0.3ml) of crude protein and 0.5% colloidal chitin in 50mM acetate buffer, pH 6.0. One unit of chitinase activity was defined as the amount of enzyme that released 1µmol GlcNAc or its equivalent from colloidal chitin in 1 min.

2.5 Antagonism test

Antagonistic activity of *B. thuringiensis* var. *aizawai* HD-137 to some phytopathogenic fungi was evaluated *in vitro*. Phytopathogenic fungi used in this study were, *Alternaria solani*, *Rhizopus*, *Fusarium solani* and *Aspergillus flavus*, (obtained from City of Scientific Research and Technological Application, Alexandria, Egypt), which grown on L-agar medium to confirm their sensitivity to chitinase. Antifungal activity of *B. thuringiensis* var. *aizawai* HD-137 was tested according to the bioassay method described (12-13) against test fungi. For test inoculation method, first, test bacterial culture was spot inoculated on nutrient agar medium and allowed to grow at 30°C. After 48 hrs of inoculation, the medium containing the spores of the test fungus was spread on the same plate of bacteria and reincubated at 30°C for 2-3 days. Diameter of inhibition zones was measured after clearly visible pathogen lawns had covered the control dishes after 3 days.

2.6 DNA isolation and PCR Amplification

The genomic DNA was performed according to Ausubel *et al*., (14). Preparation of genomic DNA and separation of fragments by agarose gel electrophoresis were performed as described by Sambrook *et al*., (15). Polymerase Chain Reaction (PCR) technique was performed with *Taq* polymerase (Fermentas) and the amplified fragment was cut and purified from agarose gel using the Sephaglas™ Band Prep Kit. A set of primers designed based on sequencing alignment of chitinase producing Bacilli. The conserved region of aligned nucleotide sequences used to design degenerate 20-mer oligonucleotides forward and reverse PCR primers F: 5 ' TTCA(T/C)GTTCAACACTACAA3', R: 5'CATTAGGCCGCGGA(A/G)TG3', respectively. PCR reaction was performed in 50-µl reaction mixture containing 50ng of template DNA, 1.5µl (10 pmoles) of each primer, 200µM of each dNTP, standard buffer (supplied with the *Taq*) containing 1.5mM $MgCl_2$, and 1 U of *Taq* DNA polymerase, in thermal cycler, as follows: initial denaturation step 94°C for 3 min., then (30 cycles) comprised a denaturing step at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1.5 min. followed by a final extension at 72°C for 7 min.

2.7 Nucleotide Sequence Analysis

Sequencing of PCR product was carried out using the ABI PRISM dye cycle sequencing ready reaction kit - Perkin Elmer and an ABI PRISM 377 sequencer according to the manufacturer's protocol (USA) uses the forward primer. DNA sequencing and chain terminating inhibitors were achieved as described by Sanger *et al*., (16).

2.8 Phylogenetic Analysis

The DNA sequence of the target band was aligned with both the BLAST (http:ncbi.nlm.nih.gov/BLAST) and CLUSTAL W (17) and a phylogenetic tree was constructed using tree Puzzle software, version 5.2.

3. Results and Discussion

3.1 Chitinase productions

The four strains of *B. thuringiensis* were tested for their ability to produce chitinase using colloidal chitin agar. All strains produced clear zones (Fig. 1). The appearance of a clear zone around the colonies indicated the presence of chitinase activity and could be considered as chitinase producers (10).

3.2 Optimization of Culture Conditions

The results of four media tested, indicated that NYSM with 0.2 chitin gave highest chitinase production. Among three different concentrations of colloidal chitin tested in NYSM medium, 0.2% colloidal chitin was considered to enhance chitinase production. Results on the effect of different pH levels in NYSM medium demonstrated that the pH 6 in the media has the highest effect on production of chitinase. A majority of bacteria was reported to produce the maximum level of chitinase at neutral or slightly acidic pH (18-19). For the temperature tested, 30°C was the optimum temperature for enzyme production (Fig. 2). The same result was reported by Gomaa (20) while Shanmugaiah *et al*., (21) reported that the production of chitinase was optimum at a temperature of 30-40°C in *S. aureofaciens* and *B. laterosporus*, respectively.

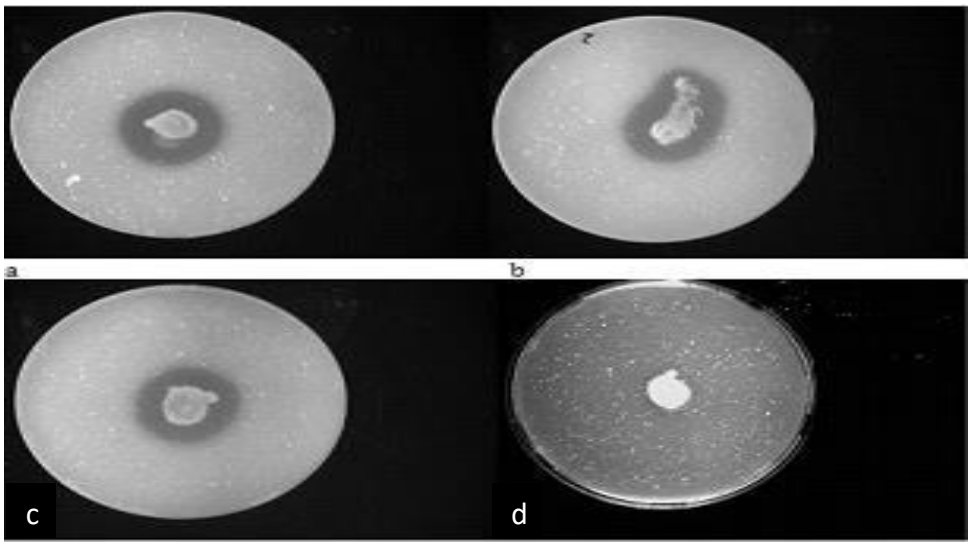

Fig. 1. Clear zone on nutrient agar medium supplemented with (0.2% colloidal chitin) produced by (a) *B. thuringiensis* var. *aizawai* HD-137, (b) *B. thuringiensis* var. *kurstaki* HD-1-S, (c) *B. thuringiensis* var. *galleriae* HD-129, (d) *B. thuringiensis* var. *aizawai*.

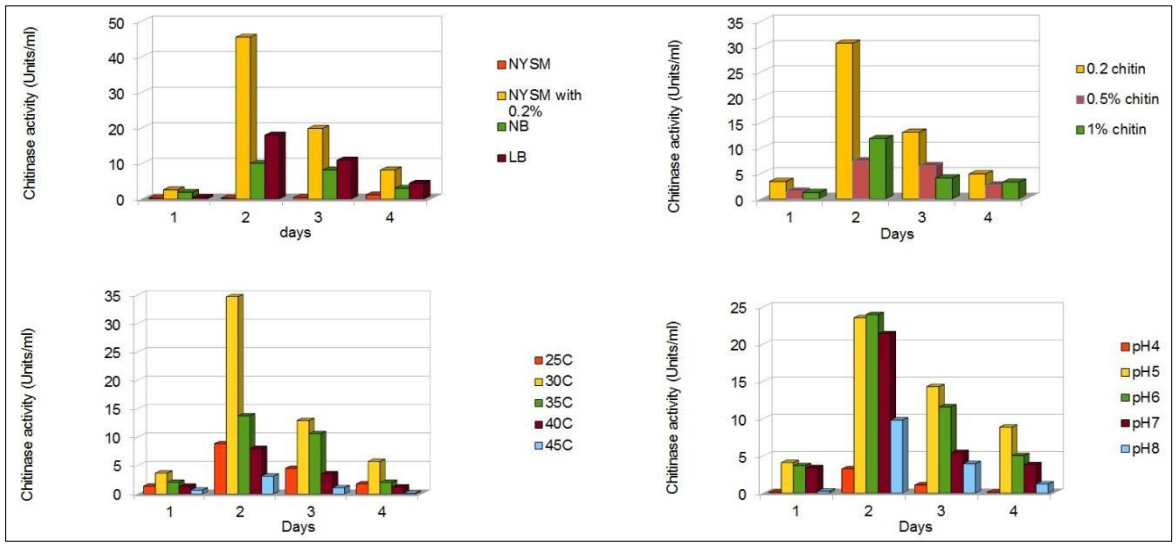

Fig. 2. Production of chitinase by *B. thuringiensis* var. *aizawai* HD-137 on different media, concentrations of colloidal chitin, pH and temperature.

3.3 Chitinase Assay

The activity of chitinase production by *B. thuringiensis* var. *aizawai* HD-137 was measured in NYSM supplemented with 0.2% colloidal chitin and the highest yield was obtained after 2 days with 0.28U/mg proteins, which suggested good production of chitinase during log phase (Fig. 3). A significant decrease in chitinase activity (about 50%) was shown after 6 days of incubation. The reduction of chitinase production was reported Nawania and Kapadnis (22) in *Streptomyces* and Gomaa (20) in *Bacillus thuringiensis* and *Bacillus licheniformis* after 5 days of incubation and the decrease in the production may be affected by increase of pH, the presence of proteolytic enzymes or the accumulation of N-acetylglucosamine resulting from chitin decomposition.

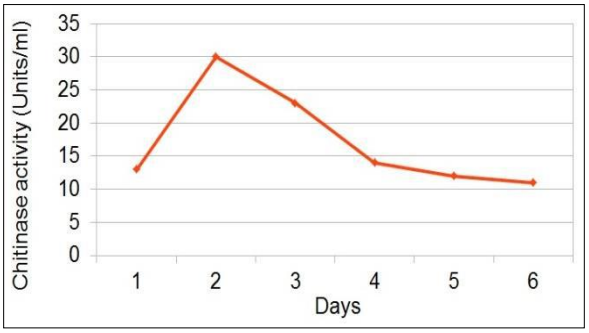

Fig. 3. Chitinase activity of *B. thuringiensis* var *aizawai* HD-137. The bacterium was grown on NYSM medium supplemented with 0.2% colloidal chitin at 1h.

3.4 *In vitro* Antagonism

The antagonistic activity of *B. thuringiensis* var. *aizawai* HD-137 against some phytopathogenic fungi was demonstrated by inhibition of the mycelial formation and the clear zones indicated the mycelial inhibition. A high inhibition of mycelia growth was observed and the clear zones were 12, 15, 16 and 19 for *Alternaria solani*, *Rhizopus*, *Fusarium solani* and *Aspergillus flavus*, respectively (Fig. 4). The results demonstrated the toxicity of *B. thuringiensis* var. *aizawai* HD-137 against the tested fungi and indicated that *B. thuringiensis* may play a potential role in fungus control as a biological agent. *B. thuringiensis* is well known to be a very efficient insect biological control agent. From previous studies, the biological activity of *B. thuringiensis* against insects was enhanced by the addition of chitinase (23-24). A chitinase, belonging to the chitobiose class from *B. thuringiensis* subsp. *kurstaki* inhibited the mycelial growth of *Aspergillus niger* that causes black mold onion and peanut (25). Our results were agreed with Quecine *et al*., (26) and Ganesan *et al.*, (27) that chitinase has an inhibitory role to plant pathogenic fungi and considered effective biological control method of managing plant pathogens.

3.5 PCR Analysis

PCR products amplified from all strains using 20-mer oligonucleotides forward and reverse PCR primers F: 5'TTCA(T/C)GTTCAACACTACAA3', R: 5'CATTAGGCCGCGGA(A/G)TG3', respectively, showed a 259 bp band (Fig. 5). The four strains have the same PCR band and approximately the same clear zone, for this, we have chosen only one strain, *B. thuringiensis* var. *aizawai* HD-137, to determine the sequence.

3.6 Phylogenetic Analysis

Sequenced product of the *B. thuringiensis* var. *aizawai* HD-137 strain (HM173355) showed a similarity to different accessions of GenBank using BLAST (http:ncbi.nlm.nih.gov/BLAST) and showed corresponding to the part of chitinase gene. The partial sequence of the nucleotide of the chitinase gene has been determined and was compared with those of some other bacterial chitinase producers of the GenBank. Results are shown in Fig. 6.

The phylogenetic tree of chitinase genes from various species of Bacillus related to our target sequence was constructed, Fig. 6. The tree divided into 4 subgroups. The most accession related to *B. thuringiensis* var. *aizawai* HD-137 (HM173355) was *B. thuringiensis* (AB699714) with 86%, and subsequently *B. thuringiensis* (GQ921840, GQ921842, EF103273 and EU734811) from 82 to 80%, *B. ehimensis* chitinase chi60 (AB111081) and *Serratia marcescens* Bn10 (DQ165083) with 82% of similarity. The high similarity indicated that maybe these species had been similar evolution way. The results agree with Joung and Cote (8), which reported that most *B. thuringiensis* could be grouped together based on the DNA similarity, but Hansen and Hendriksen (28), provided an argument that can consider *B. cereus* and *B. thuringiensis* to represent one taxonomic unit.

4. Conclusion

This study has demonstrated the potential of *B. thuringiensis* var. *aizawai* HD-137 to produce chitinase and the correlation between chitinase production and biocontrol potential as an antifungal agent, inhibiting mycelial growth of pathogenic fungi (*Rhizopus*, *Aspergillus*, *Alternaria* and *Fusarium)*. Elucidated the relationship between *B. thuringiensis* var. *aizawai* HD-137 and related chitinase producing bacteria in the GenBank, especially *B. thuringiensis, B. ehimensis, Serratia marcescens.* The high similarity between them indicated that possibly related evolutionary. More investigations, additional sequence analysis may be needed.

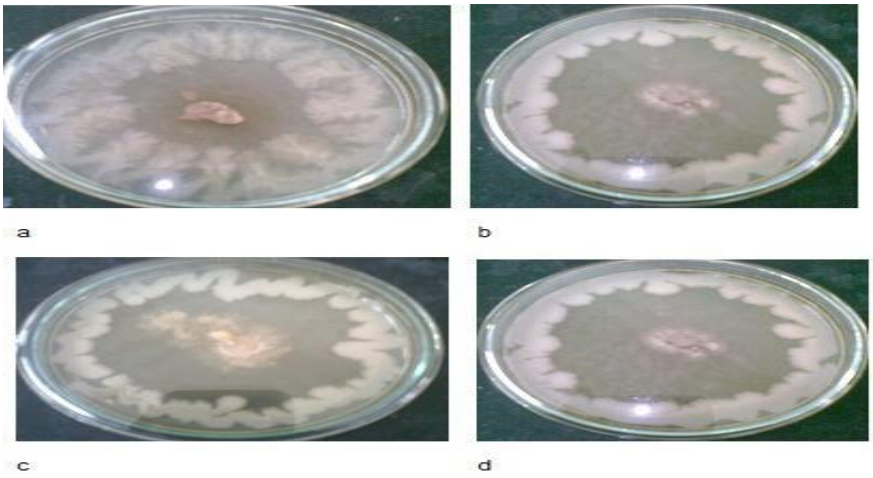

Fig. 4. Antagonism activity of *B. thuringiensis* to some of phytopathogenic fungi was tested *in vitro*. Phytopathogenic fungi (a) *Rhizopus,* (b) *Alternaria solani,* (c) *Fusarium solani* and (d) *Aspergillus flavus* were grown on nutrient agar medium to confirm their sensitivity to chitinase.

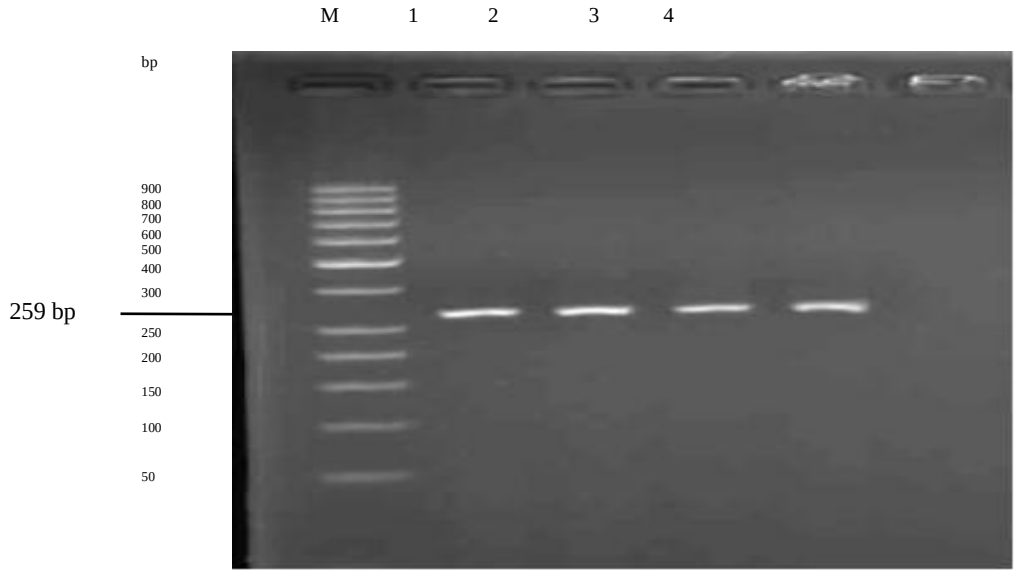

Fig. 5. PCR amplification of *B. thuringiensis* (1 = *B. thuringiensis* var. *kurstaki* HD-1-S, 2 = *B. thuringiensis* var. *galleriae* HD-129, 3 = *B. thuringiensis* var. *aizawai* and 4 = *B. thuringiensis* var. *aizawai* HD-137) in 1% agarose gel, the size of the PCR product was compared with 50 bp DNA ladder (lane M).

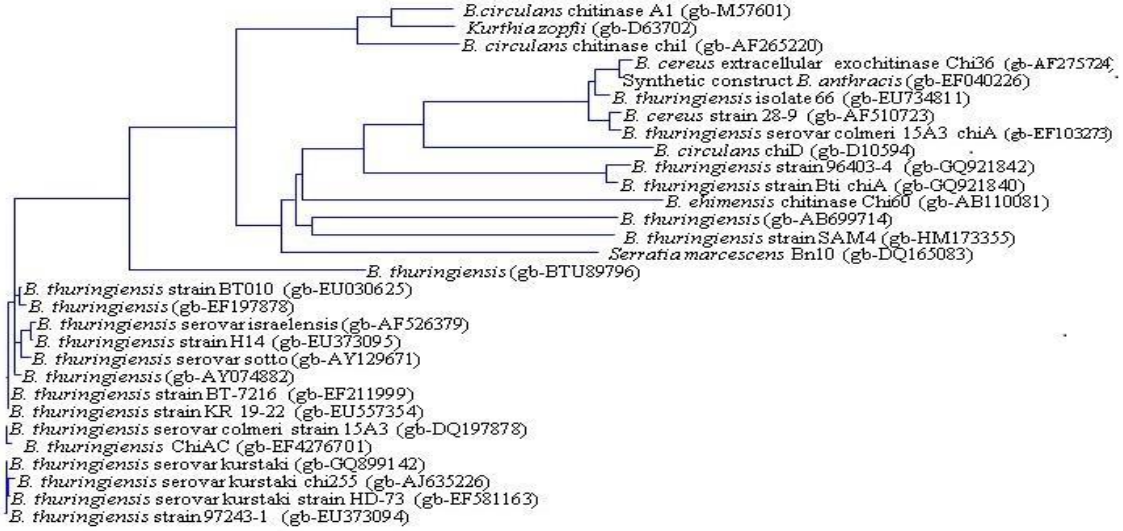

Fig. 6. A rooted neighbour joining tree constructed on the basis of our target sequence showing the relationship between our strain, *B. thuringiensis* var. *aizawai* HD-137, SAM4 (HM173355) and other different related species.

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