JEB®

Journal of Environmental Biology

p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP

Journal website : www.jeb.co.in ★ **E-mail :** editor@jeb.co.in

Triveni Enterprises (Educational Services Set-up)

Original Research

DOI : http://doi.org/10.22438/jeb/43/4/MRN-2038

Molecular characterization of chitinase producing *Bacillus thuringiensis*

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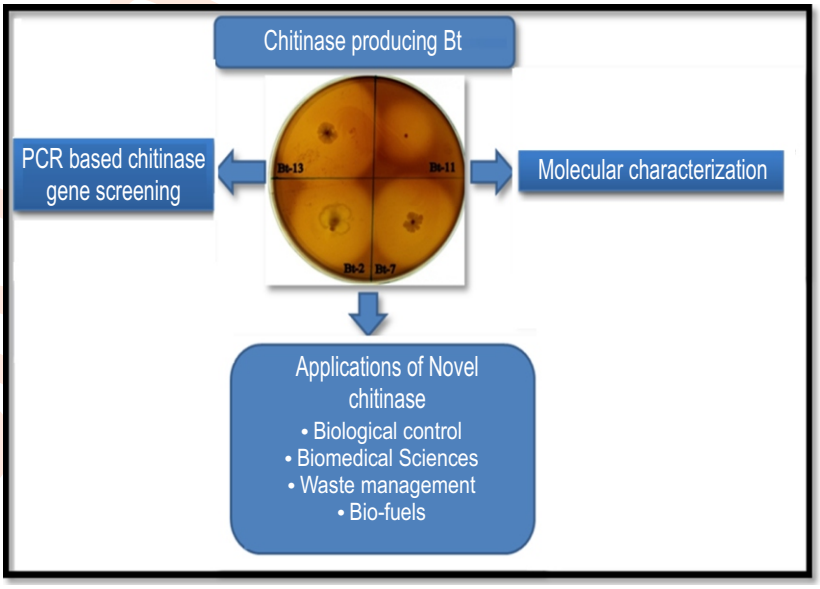
Received: 21.06.2021 *Revised*: 01.10.2021 *Accepted*: 05.12.2021

Abstract

Aim: In the present study, twenty eight *Bacillus thuringiensis* (Bt) strains were studied for their potential to hydrolyze chitin.

Methodology: The chitinolytic potential of Bt strains were determined using modified chitinase screening media (CSM) containing colloidal chitin as substrate. The potential Bt strains were screened for the presence of cry genes using PCR and further molecular characterized using 16S rDNA amplification and sequencing.



Results: Among 28 Bt strains, 10 strains showed positive results by producing clear halo zone around bacterial colonies and the maximum chitinase solubilization index was observed in Bt-13 whereas the minimum solubilization index was observed in Bt-27 strain. Further, the chitinase assay revealed the minimum enzyme activity in Bt-26 (3.78 ± 0.101 U ml^{-1}) whereas the maximum activity was observed in strain Bt-2 (10.19 ± 0.651 U ml^{-1}). PCR based chitinase gene screening also revealed the presence of both endochitinase and exochitinase genes in these Bt strains. The potent Bt strains *viz.,* Bt-7, Bt-10, Bt-11, Bt-13 and Bt-27 on the basis of chitinase production were molecular characterized based on 16S rDNA and sequencing results reveled their greatest sequence identity *Bacillus thuringiensis*.

Interpretation: Results showed that the high chitinase activity of these Bt strains may be due to the presence of chitinase genes, which need to be explored for further biological applications.

Key words: *Bacillus thuringiensis*, Chitinase, Molecular characterization, PCR

How to cite : Singh, P., A. Joshi, D.J. Nath, A. Gayan, S. Sanadhaya, H. Saheewala, P. Jain, L. Gupta, S.R. Mohanty and D. Jain: Molecular characterization of Chitinase producing *Bacillus thuringiensis*. *J. Environ. Biol.*, **43**, 506-513 (2022).

Introduction

Chitin is the second most abundant polysaccharide in nature (Saima *et al.,* 2013). The chitin backbone in crustacean and insect exoskeletons is made up of repeating units of N-acetyl D-glucosamine residues (Franca *et al.,* 2011). Chitin is also present in fungi as well as parasitic nematode micro filarial sheaths. It is found as α & β crystalline chitin forms as well as colloidal chitin and can be degraded by chitin producing microorganisms as one would imagine (Lenardon *et al.,* 2010). As a result, chitin degradation is a critical step in the natural recycling of chitin as nutrients. Chitinases (EC 3.2.1.14) degrade the glycosidic bonds present in chitin. A wide number of microorganisms produces chitinase enzyme which plays an important role and have a broad range of applications (Akeed *et al.,* 2020). Chitinolytic bacteria produces a number of chitinolytic enzymes that convert chitin into carbon and nitrogen sources, which in combination with other metabolites, help the plants to grow. In recent times, the chitinase produced by Bt have been widely studied due to its potential applications not only in insect control but also as antibacterial and fungicidal specialist (Azizoglu, 2019; Casados-Vazquez *et al.,* 2018; Jouzani *et al.,* 2017).

The Gram positive spore forming aerobic bacterium, Bt is an important industrial organism used widely against different insect pests. Bt produces crystalline insecticidal proteins in the sporulating cell and is thus used as a biological pest control agent, all over the world (Jain *et al.,* 2017). The first application of Bt chitinases was demonstrated in 1970s when it was realized that the enzymes secreted by Bt can hydrolyze chitin (Chigalelchik, 1976). Chitinolytic bacteria hydrolyzes chitin as carbon and nitrogen source and chitinase production can be increased further if the medium is supplemented with a chitin source (Gomaa, 2012). The screening of chitinolytic bacteria includes incorporation of colloidal chitin into a growth medium and then observing the breakdown of chitin either in form of halo zones around the bacterial colonies or as colorimetric assays (Veliz *et al.,* 2017). Polymerase Chain Reaction has been used to characterize the exochitinase and endochitinase genes based on the presence of conserved DNA in the genome of producing microorganism. El-Hamshary *et al.* (2008) and Djenane *et al.* (2017) performed PCR based screening of chitinase producing Bt strain using PCR primer specifically designed for chitinase and exochitinase and endochitinase genes.

Chitinase have wide range of use in medicine and agriculture including the manufacture of antifungal substances and the management of plant pathogen (Bhattacharya *et al.,* 2007; Reyes-Ramirez *et al.,* 2004). Presence of chitin in the host indicates bacterial pathogenesis due to chitinase activity (Busby *et al.,* 2012). Chitinase is likewise being recommended to be utilized for identification of intrusive contagious disease in people (Vega and Kalkum, 2012). Chitinase can also be used to produce bio-fertilizers out of chitinous waste (Sakai *et al.,* 1998). Single cell protein can be made in aquacultures using bacteria and yeast that produce chitinases. Chitinase can also be used as a bio pesticide against a variety of fungi and insect (Melchers and Stuiver, 2000). Chitinases are broadly present in Bt strains and plays key role in increasing insecticidal activities. In the present work, an attempt was made to screen and characterize chitinolytic Bt strains having high chitinolytic potential at phenotypic and molecular level.

Materials and Methods

Bacterial strains: Twenty-eight Bt were provided by All India Network Project on Soil Biodiversity and Biofertilizers, Dept. of Molecular Biology and Biotechnology, RCA, MPUAT, Udaipur.

Preparation of colloidal chitin: Colloidal chitin was prepared as per the protocol of Hsu and Lockwood (1975). Chitin powder was broken down in concentrated HCl and blended for 2 hr. During the process of blending, the hydrolyzed chitin was washed several times with distilled water in order to remove acid completely, and to lower its pH to 7. The colloidal chitin was collected by centrifuging the solution and stored in the form of a paste at 4°C until further use.

Screening of chitinase producing *Bacillus thuringiensis* strains: Bacterial isolates were streaked directly onto modified chitinase screening media containing colloidal chitin as substrate followed by incubation at 37°C for 7 days and the plates were then observed for zone of hydrolysis around the inoculated area (Chigalelchik, 1976). The zone was further visualized by flooding the plate with Gram's iodine solution and observed for the formation of clear zone against a brownish background. Chitinolytic index or chitin solubilization index (CSI) was calculated by dividing the diameter of clear zone with the colony diameter (Ravindra *et al.*, 1998).

Chitinase enzyme assay: Chitinase assay was carried out with crude enzyme (Bansode and Bajekal, 2006). Bt isolates that produced chitinase were inoculated to colloidal chitin broth (100 ml) in flasks and incubated for 4 days at 37°C. The cell-free supernatant was obtained after centrifuging the culture broth at 8000 rpm for 20 min. To remove the enzymes, the clear culture filtrates were saturated to 60–70% with ammonium sulphate and held at 4°C overnight. The precipitate was obtained by centrifugation at 10,000 rpm at 4°C, dissolved in 50 mM phosphates buffer (pH 7.0), and used as a crude enzyme.

Qualitative (well diffusion) assay: The chitinase activity of crude enzyme extract was assessed by the method of Agrawal and Kotasthane (2012) using a simple well diffusion assay. Using a 6 mm sterile cork borer, wells were made in 1% colloidal chitin agar, containing BCP dye plates. Each well was filled with a 100 µl culture filtrate from each isolate and incubated at 37°C. Colloidal chitin media containing BCP indicator (pH 4.7) when reacted with crude chitinase enzyme resulted in breakdown of chitin into N- acetyl glucosamine and due to breakdown of chitin, the pH of the medium increased and the colour of BCP dye turned into purple.

Quantitative assay: The chitinase activity was assessed as per protocol of Miller *et al.* (1959). The A mixture of 0.1 % colloidal chitin and 50mm of 0.1 M phosphate buffer pH 7.0 was added to crude enzyme. The reaction mixture was centrifuged at 10,000 rpm for 10 min after being incubated at 55°C (10 minutes). The supernatant was then combined with DNS and Schales reagents and boiled for 10 min. The absorbance of the mixture was read at 530 nm using nanodrop spectrophotometer (Implen, Germany) after cooling. The chitinase activities were calculated based on standard curve of N-Acetyl glucosamine. One unit of chitinase activity is defined as the amount of enzyme that produces 1 l mol^{-1} of reducing sugar as N-acetyl-D-glucosamine equivalent min^{-1}.

PCR based screening of chitinase genes from *Bacillus thuringiensis* isolates: The total genomic DNA from *B. thuringiensis* isolates was extracted as per Kalman *et al.* (1993). Amplification of chitinase genes were performed with primers specific to Bt Chitinase genes (Table 1). PCR reaction was carried out in 25 µl of reaction mix containing 50ng genomic DNA, 2 µl of 10X Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 200 µM each dNTPs, 0.5 µM of primer and 1 unit of Taq DNA polymerase using a Bio-Rad thermocycler with a cycle comprising 94°C for 5 min for initial denaturation, followed by 30 cycles of denaturation at 94°C for 45 sec, 48°C for 45 sec for primer annealing, 72°C for 45 sec for extension, and one cycle of 72°C for 10 min for final extension. The amplified products were analysed using 1 KB and 100bp ladder on 1.2 per cent agarose gel with ethidium bromide staining.

Molecular characterization of chitinase producing native Bt strains: The PCR amplified 16S rDNA region of chitinase producing Bt isolates were amplified and sequenced using automated DNA Sequencer (ABI model 377, Applied Biosystems) as per the method described by Jain *et al.* (2017). Amplification of 16S rDNA region by PCR was performed with universal primers, 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5-'ACGGCTACCTTGTTACGACTT-5') specific to conserved regions. The amplified products were gel purified and sequenced at Biokart India Pvt. Ltd., Bangalore. The 16S rDNA sequences were edited before the BLAST using BioEdit software package. The sequences obtained in the study were compared with previously submitted sequences of nucleotide database GenBank at National Centre for Biotechnology (NCBI) using nucleotide BLAST (blastn). The 16S rDNA consensus sequences were aligned using online tool CLUSTAL-W. This alignment was further used for phylogenetic tree construction through MEGA 6.06 software using Maximum Likelihood method.

Results and Discussion

Chitinolytic potential of twenty-eight *Bt* isolates on chitinase screening media (CSM) and the *Bt* isolates producing clear halo zones on the CSM media were considered chitinase positive. The halo zones were further visualized by the application of Gram's Iodine solution (Fig. 1) and among 28 Bt isolates, 10 *Bt* isolates produced prominent halo zones considered as chitinase producing *Bt* strains. The chitin solubilization indexes (CSI) were calculated using diameter of halo zone of bacterial isolates and is

Table 1: Chitinase gene specific primer used in the present study

Gene name	Primer sequence	Amplicon length	References
Chitinase	F:5' TTCA(T/C)GTTCAACACT ACAA 3' R: 5' CATTAGGCCGCGGA(A/G)TG 3'	350 bp	El-Hamshary *et al.,* 2008
Endochitinase	Chit(F) 5'ATTCACACTGCTATTACTATC3' Chit(R) 5' TGACGGCATTTAAAAGTTCGGC3'	1997 bp	Djenane *et al.,* 2017
Exochitinase	Chi36(F) 5'GATGTTAAACAGGTTCAA 3' Chi36(R) 5'TTATTTTTGCAAGGAAAG 3'	1083 bp	Djenane *et al.,* 2017

Table 2: Chitinase solubilization index of chitinase hydrolyzing bacteria (Bt)

***Bacillus thuringiensis* strains**	**Chitin solubilization index* (cm)**	**Chitinase activity (U ml^{-1})**
Bt2	7.53±0.30	10.19±0.651
Bt7	6.77±0.16	5.24±0.408
Bt10	7.76±0.20	5.20±0.192
Bt11	3.70±0.27	4.83±0.132
Bt13	7.79±0.14	3.82±0.364
Bt19	4.15±0.17	5.18±0.329
Bt20	6.19±0.21	5.36±0.190
Bt26	3.67±0.19	3.78±0.101
Bt27	3.45±0.10	6.53±0.309
Bt28	4.29±0.11	4.41±0.398

*Data are mean of triplicate ± SD

Table 3: Molecular identity of potent Bt isolates

			Closest type strain			
Strains	**Molecular identity**	**Accession number**	**Molecular identity**	**Strain**	**Accession number**	**Percent similarity/ Query coverage**
Bt 7	*Bacillus thuringiensis*	MW406896	*Bacillus thuringiensis*	IAM012 077	NR043403.1	99/99.14
Bt 10	*Bacillus thuringiensis*	MW406897	*Bacillus thuringiensis*	IAM012077	NR043403.1	100/98.92
Bt 11	*Bacillus thuringiensis*	MW406898	*Bacillus thuringiensis*	IAM012077	NR043403.1	100/98.96
Bt 13	*Bacillus thuringiensis*	MW406899	*Bacillus thuringiensis*	IAM012077	NR043403.1	100/98.57
Bt 27	*Bacillus thuringiensis*	MW406900	*Bacillus thuringiensis*	IAM012077	NR043403.1	100/99.05

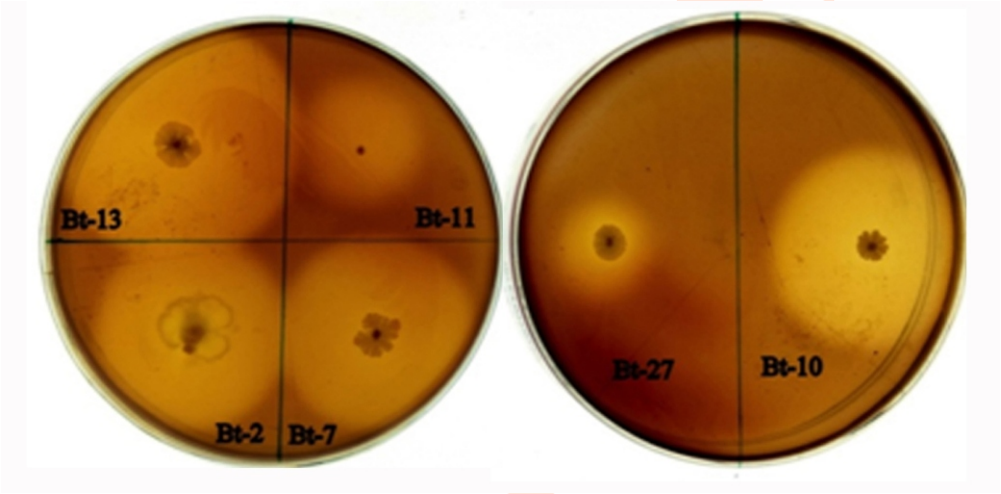


Fig. 1: Chitinase producing Bt strains on chinase screening media (CSM).

presented in Table 2. The maximum solubilization index was observed in Bt-13 with a CSI of 7.79±0.14 whereas the minimum solubilization index was observed in Bt-27 with a CSI of 3.45±0.10. El-Hamshary *et al.* (2008) screened 66 local *Bacillus* isolates recovered from dead *Biomphalaria alexandrina* snails along with reference type strains of Bt for chitinase and reported that all these strains showed chitinase activity. Gupta *et al.* (2018) isolated 28 chitinase producing bacterial isolates from the rhizospheric soil samples and reported that only 12 produced clear halo zone on colloidal chitin containing media plates. The formation of clear zone around the colonies indicates the presence of chitinase activities which helps the bacteria to utilize chitin as a sole carbon and nitrogen source, whereas the strains do not have chitinase activity showed poor growth. Similar results were also observed in the present study.

Colloidal chitin media containing BCP indicator (pH 4.7) when reacted with crude chitinase enzyme resulted in breakdown of chitin into N- acetyl glucosamine, and due to the breakdown of chitin the pH of the medium increased resulted in change the colour of BCP dye into purple (Fig. 2). Petrisor *et al.* (2016) examined *Trichoderma* for production of extracellular enzymes chitinases on 1% colloidal chitin agar plates containing Bromocresol blue dye and two strains *viz.,* Td 49 and Tdal12 exhibited higher chitinase activity by producing a higher diameter of purple colour zone. Narayana and Vijayalakshmi (2009) also studied the enzymatic activities on partially purified chitinase enzyme on chitinase plates by placing the enzymes in the wells and observed that clear chitinolytic zones also support the results observed in the present study. *Bt* isolates were also tested for quantitative chitinase activity in liquid CSM media and the results for quantitative chitinase activity are summarized in Table 2.

The enzyme activity varied from 10.19 to 3.78 U ml^{-1} depending on the bacterial strain. Maximum chitinase activity was shown by Bt-2 (10.19 U ml^{-1}) closely followed by Bt-27 (6.53 Uml^{-1}), Bt-20 (5.36 Uml^{-1}), Bt-7 (5.24 Uml^{-1}) and Bt-10 (5.20 Uml^{-1}), respectively. Similarly, Ong *et al.* (2017) reported highest chitinase production after 24 hr for *Enterobacter* sp. (6.70 µg ml^{-1}) and *Zymomonas* sp (1.68 µg ml^{-1}). Chitinase activity depends on factors like time, pH, incubation time and substrate concentration etc. Chitinase activity in the present study was higher as compared to the results observed by Setia and Suharjono (2015). Gomma (2012) reported that influence of additional carbon sources on chitinase production and reported enhanced chitinase production of 16.02 µg ml^{-1} by *Bt* when colloidal chitin medium

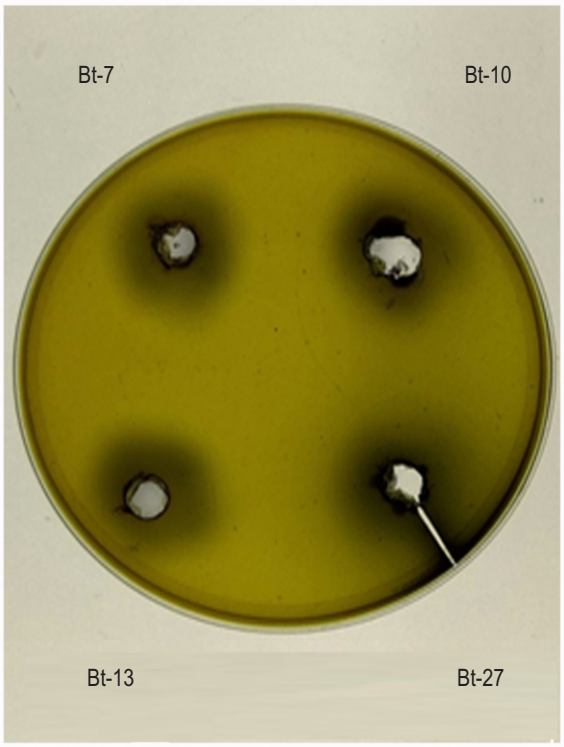


Fig. 2: Activity of crude chitinase on colloidal chitin plate containing BCP indicator (Qualitative assay).

amended with galactose. Saleem *et al.* (2014) reported that the production of chitinase by different chitinolytic *Bt* isolates and reported highest chitinase activity (0.23 µg ml^{-1}) in Bt-4 strain after 4th day of incubation. El-Hamshary *et al.* (2008) reported chitinase production in the supernatant of Bacillus isolate 66 (NCBI accession: DQ524821) isolated from dead snails was 0.66 U ml^{-1}. Subbanna *et al.* (2019) reported the chitinase activities of 80 *Bt* isolates positive on colloidal chitin medium and out of which 23 *Bt* isolates showed a prominent halo zone (4mm size) having chitinase activity ranging from 0.15 to 0.86 U ml^{-1} also support the results observed in the present study.

The genomic DNA from *Bt* isolates were screened by PCR with primer pairs specific to chitinase, exochitinase and endochitinase genes. Amplification of expected size of PCR products *viz.,* 350 bp in chitinase gene, 1083 bp exochitinase and 1997 bp in endochitinase in different primer pairs indicated the presence of above mentioned chitinase genes in *Bt* strains (Fig. 3). All the 10 *Bt* isolates showed PCR amplification with chitinase gene which indicated the presence of any type of chitinase gene. Further, the amplification with specific primers of endochintinase and exochitinase genes also confirmed that the 10 *Bt* stains harbor both endochitinase and exochitinase genes. El-Hamshary *et al.* (2008) designed PCR primers for screening of *Bt* strains for presence of chitinase gene and the sequencing of 350 bp PCR amplicons from local *Bt* isolates from their study revealed 96% similarity with the earlier reported chitinase gene from *B. thuringiensis* A1 Hakam strain. The same primer pair was used in the present study and the expected size of amplification (350bp) was observed in all 10 *Bt* strains confirms that all the *Bt* strains contain any type of chitinase gene. Djenane *et al.* (2017) studied the occurrence of exochitinase and endochitinase genes in *Bacillus* spp. by PCR amplification using gene-specific primers and reported that 88 (64.2%) of 137 *Bt* isolates harbour at least one type of chitinase gene whereas 66 (48.2%) being positive for exochitinase gene and 82 (59.9%) being positive for endochitinase gene. The results observed by Djenane *et al.* (2017) are in agreement with the present study in which 10 isolates (35.7%) out of 28 *Bt* isolates were found to harbour chitinase genes. The production of multiple chitinase in a single microorganism may be attributed to lateral transfer of chitinase genes and these chitinase helps the microorganisms in their antagonistic, nutritional and functional roles (Oyeleye and Normi, 2018).

The expression of *Bt* chitinase is low and hence to take its advantage in different biological processes, the constitutive expression of chitinase at a sufficiently high level is required and can be achieve by cloning and expression of *Bt* chitinase (Honda *et al.,* 2017). Five *Bt* strains *viz.,* Bt-7, Bt-10, Bt-11, Bt-13 and Bt-27 having highest chitinase activities were selected further for molecular characterization based on the amplification and sequencing of 16S rDNA conserved region and the amplification of the specific size~1.4 kb amplicon of 16S rDNA region was sequenced with universal primers at Biokart India Pvt.Ltd., Bangalore, India. The strains were identified and attributed to the closely related taxa based on their homology and matches with earlier reported bacterial rDNA sequences in nucleotide BLAST. The accession numbers of 16S rDNA were also obtained by submitting the DNA sequence at NCBI (Table 3) and the 16S rDNA sequences of these *Bt* strains revealed their greatest sequence identity with *Bacillus thuringiensis* (Fig. 4).

The partial sequence of 16S rDNA of isolate Bt-7, Bt-10, Bt-11, Bt-13 and Bt-27 showed highest homology with 16S rRNA sequence of *Bacillus thuringiensis* (Accession number NR_043403.1) already submitted to NCBI database. The phylogenetic position of species is shown in Fig. 4. Tran *et al.* (2018) isolated thirty one chitinolytic bacteria from Japan and the phylogenetic analysis of these isolates based on 16S rRNA genes sequence revealed that most isolates belonged to families Aeromonadaceae, followed by Paenibacillaceae, Enterobacteriaceae, and Neisseriaceae. Sudha *et al.* (2020) isolated and molecular characterized two chitinolytic bacterial strains a *Bacillus thuringiensis* strain LS1 and *Bacillus cereus* strain. Setia and Suharjono (2015) isolated chitinolytic bacteria from shrimp waste and two isolates *viz.* PBK 2 and SA 1.2 having highest chitinolytic index were molecular characterized and revealed their identity as *Acinetobacter johnsonii* and *Bacillus amyloliquefaciens* respectively. The chitinolytic *Bt* strains showed higher activities due to the presence of chitinase genes compare to previously published reports, hence these stains need to be

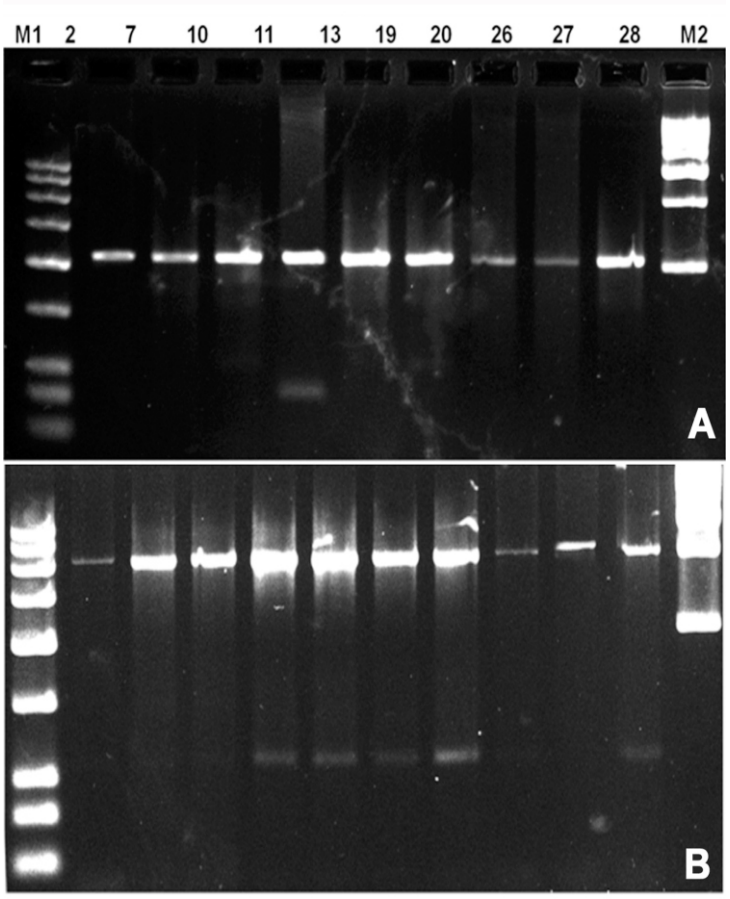


Fig. 3: Amplicons of chitinase genes from Bt with *A. endochitinase* (1997 bp) *B. exochitinase* genes (1083 bp). M1: 100 bp extended ladder M2: 1 Kb ladder.

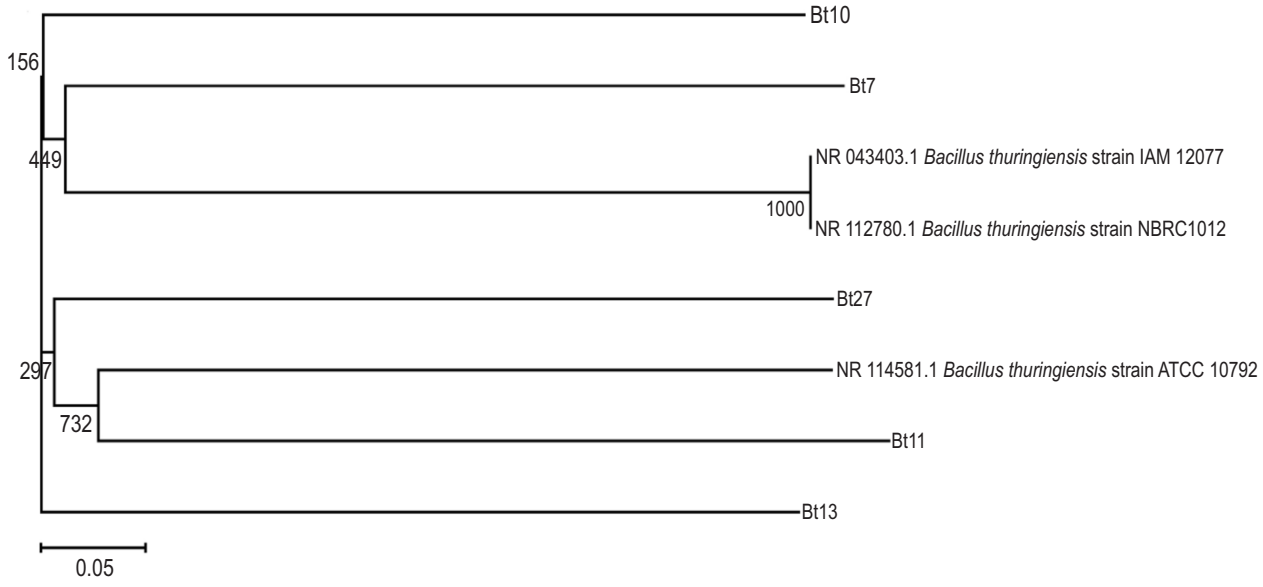


Fig. 4: Phylogenetic tree of chitinase producing *Bacillus thuringiensis* strains based on 16S rDNA.

further evaluated for full length cloning and expression. Indigenous *Bt* were screened for chitinase enzyme activity. Isolate Bt-13 showed significantly high chitinase activity and PCR based screening also revealed the presence of both exochitinase and endochtinase genes in the isolate. The chitinase genes are required to be cloned and express suitable host for its applications under various processes.

Acknowledgments

The financial assistance from All India Network Project on Soil Biodiversity and Biofertilizers, ICAR is highly acknowledged.

Add-on Information

Authors' contribution: P. Singh, S. Sanadhaya, H. Saheewala: Laboratory experiments; **A. Joshi, L. Gupta, S.R. Mohanty:** Manuscript writing; **D.J. Nath:** Bt strain isolation; **A. Gayan:** Bt strain characterization; **P. Jain:** Molecular characterization **and D. Jain:** Conceived and designed the experiments, Manuscript proofreading and communication.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: Not applicable.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology.*

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