PCR based Characterization of *Chitinase* gene from *Bacillus thuringiensis*

काइटिनेस बनाने वाले बैसिलस थुरिंजेंसिस में काइटिनेस जीन का पीसीआर आधारित लक्षण वर्णन

PAVITRA SINGH

Thesis

Master of Science in Agriculture

(Molecular Biology and Biotechnology)

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RAJASTHAN COLLEGE OF AGRICULTURE

MAHARANA PRATAP UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

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Thesis

Submitted to the

Maharana Pratap University of Agriculture and Technology, Udaipur

In partial fulfillment of the requirement for the degree of

Master of Science in Agriculture

(Molecular Biology and Biotechnology)

By

PAVITRA SINGH

2021

CERTIFICATE-I

CERTIFICATE OF ORIGINALITY

The research work embodied in the thesis titled **"PCR based characterization of *Chitinase* gene from *Bacillus thuringiensis*"** submitted for the award of degree of **M.Sc. (Ag.) Molecular Biology and Biotechnology** to Maharana Pratap University of Agriculture and Technology, Udaipur (Rajasthan), is original and bonafied record of research work carried out by me under the supervision of (Dr. Arunabh Joshi, Head, Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Udaipur). The contents of the thesis, either partially or fully, have not been submitted or will not be submitted to any other institute or university for the award of any degree or diploma.

The work embodied in the thesis represents my ideas in my own words and where others ideas or words have been included. I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be cause for disciplinary action by the university and can also evoke panel action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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MAHARANA PRATAP UNIVERSITY OF AGRICULTURE AND TECHNOLOGY, UDAIPUR

CERTIFICATE - II

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(Dr. Arunabh Joshi)
Head
Department of Molecular Biology and Biotechnology
RCA, Udaipur (Rajasthan)

(Dr. Arunabh Joshi)
Major Advisor
Head
Department of Molecular Biology and Biotechnology
RCA, Udaipur (Rajasthan)

(Dr. Dilip Singh)
Dean
Rajasthan College of Agriculture
Udaipur (Raj.)

RAJASTHAN COLLEGE OF AGRICULTURE

MAHARANA PRATAP UNIVERSITY OF AGRICULTURE AND TECHNOLOGY, UDAIPUR

CERTIFICATE - III

Dated: 18/06 /2021

This is to certify that this thesis entitled **"PCR based characterization of *Chitinase* gene from *Bacillus thuringiensis*"** submitted by **Mr. Pavitra Singh** to Maharana Pratap University of Agriculture and Technology, Udaipur in partial fulfillment of the requirements for the degree of **Master of Science in Agriculture in the subject of Molecular Biology and Biotechnology** after recommendation by the external examiner was defended by the candidate before the following members of the examination committee. The performance of the candidate in the oral examination held on 16/06/2021 was found satisfactory; we therefore, recommend that the thesis be approved.

(Dr. Arunabh Joshi)
Major Advisor

(Dr. Devendra Jain)
Advisor

(Dr. Amit Trivedi)
Advisor

(Dr. S.K. Intodia)
DRI, Nominee

(Dr. Arunabh Joshi)
Head
Department of Molecular
Biology and Biotechnology
RCA, Udaipur (Raj.)

(Dr. Dilip Singh)
Dean
Rajasthan College of Agriculture
Udaipur (Raj.)

Approved

(Dr. S. R. Bhakar)
Director Resident Instructions
MPUAT, Udaipur

RAJASTHAN COLLEGE OF AGRICULTURE
MAHARANA PRATAP UNIVERSITY OF AGRICULTURE AND TECHNOLOGY, UDAIPUR

CERTIFICATE – IV

Dated: 18 /06 /2021

This is to certify that **Mr. Pavitra Singh** Student of **Master of Science in Agriculture, Department of Molecular Biology and Biotechnology**, Rajasthan college of Agriculture, Udaipur has made all correction/modification in the thesis entitled **"PCR based characterization of *Chitinase* gene from *Bacillus thuringiensis*"** which were suggested by the external examiner and the advisory committee in the oral examination held on 16/06/2021. The final copies of the thesis duly bound and corrected were submitted on 18/06/2021.

(Dr. Arunabh Joshi)
Head
Department of Molecular Biology and Biotechnology
Rajasthan College of Agriculture
Udaipur (Rajasthan)

(Dr. Arunabh Joshi)
Major Advisor
Department of Molecular Biology and Biotechnology
Rajasthan College of Agriculture
Udaipur (Rajasthan)

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Palace: Udaipur

Dated: 18 June, 2021 ***(Pavitra Singh)***

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ABBREVIATIONS

- % Percent
- > More than
- µl Microlitre
- µM Micromole(s)
- ARDA Adenine
- bp Base pair
- Chi Chitinases
- e.g Exempli gratia
- *et al.* Coworkers
- Fe Iron
- Pb Lead
- Fe^{+2} Ferrous ion
- Fig. Figure
- G Gram
- h Hour
- HCl Hydrochloride
- Hr Hour(s)
- i.e. That is
- Kb Kilo base
- kDa Kilodaltons
- Min Minute(s)
- ml Millilitre
- mM Milli molar
- Mn^{+2} Manganese ions
- NH_4 NO_3 Ammonium Nitrate
- N Normal
- Na Sodium
- NaCl Sodium Chloride
- NB Nutrient Broth
- kg Kilo gram
- LB Luria Broth
- MR Methyl-Red
- mg Milligram
- Ni Nickel
- nm Nano meter
- No. Number
- U Unit(s)
- TE Tris EDTA
- U/ml Units per millilitre
- ng Nano gram
- ºC Degree centigrade
- OD Optical Density

- P Phosphorus
- ppm Part per million
- rDNA Ribosomal DNA
- Sec Second(s)
- *Viz* as follows
- VP Voges-Proksauer
- NaCl Sodium chloride
- SI Solubility index
- SCP Single Cell Protein
- SD Standard Deviation
- NH_4NO_3 Ammonium nitrate
- Mg^{+2} Magnesium ions
- KCl Potassium chloride
- 3D Three-dimensional
- BCP Bromocresol Blue
- Bt Bacillus thuringiensis
- DNA Deoxyribo nucleic acid
- CSM Chitin solubilizing medium
- rpm Revolutions per minute
- rRNA Ribosomal ribo-nucleic acid
- SDS Sodium do-dicyle sulphate
- Tris Tris-hydroxy methyl aminoethane
- blastn Basic local alignment search tool
- dNTPs Deoxy-nucleotide triphosphates
- CSI Chitin solubilizing Index
- DEAE Diethylaminoethyl cellulose
- µg/ml Micro gram per millilitre
- EDTA Ethylene Diamine Tetraacetic acid
- ERIC Enterobacterial Repetitive Intergenic Consensus
- IUPAC–IUB International Union of Pure and Applied Chemistry-International Union of Biochemistry
- KH_2PO_4 Potassium di hydrogen phosphate
- $MgSO_47H_2O$ Magnesium sulfate heptahydrate
- NCBI National Center for Biotechnology Information.
- NTSYS- pc Numerical Taxonomy SYStem for personal computer
- PsChi Pseudomonas Chitinase
- PCR Polymerase Chain Reaction
- pH Negative Logarithm hydrogen ion concentration
- REP Repetitive Element Sequence-based PCR

1. INTRODUCTION

Next to cellulose, chitin is the most abundant biopolymer on the planet (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 and yeast cell walls, as well as parasitic nematode micro filarial sheaths. Chitin is found as α & β crystalline chitin forms as well as colloidal chitin. Chitin 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) are enzymes that degrade chitin's glycosidic bonds. 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 *(chi)* enzyme produced by *Bacillus thuringiensis* received a great deal of attention 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).

Bacillus thuringiensis (Bt) is a gram positive rod shaped aerobic spore forming soil bacterium that produces crystalline insecticidal proteins in the sporulating cell cytoplasm. Inside the cytoplasm of the sporulating cell, they ordinarily contain insecticidal proteins (Jain *et al.,* 2017). *Bacillus thuringiensis* causes parasporal crystals in susceptible insects and is used as a biological pest control agent, all over the world. (Hofte and Whiteley, 1989). The first application of *Bacilus thuringiensis* Chitinases (Bt Chi) was demonstrated in 1970s when it was realized that the enzymes secreted by Bt can hydrolyzed chitin (Chigalelchik *et al*., 1976).

Chitinase have wide range of use in medicine and agriculture including the manufacture of antifungal substances and the management of plant pathogens. (Bhattacharya *et al*., 2007; Reyes-Ramirez *et al*., 2004). Wherever chitin is present in the host, Chitinase play an important role in bacterial pathogenesis (Busby *et al.,* 2012). Chitinase is also utilized for the identification of intrusive contagious diseases (Vega *et al.,* 2012).

Chitinase can also be used to produce biofertilizers out of chitinous waste (Sakai *et al.,* 1998). Single cell protein (SCP) 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 *et al*., 2000). The Chitinases are broadly present in *Bacillus thuringiensis* strains and plays a key role in increasing the insecticidal activities. Hence, the present investigation was made to screen chitinolytic strains and isolate chitinase gene from among the Bt isolate collections.

The present investigation was carried out with the following objectives.

1. Screening of *chitinase* producing *Bacillus thuringiensis* strains.
2. PCR based screening of *chitinase* gene from *Bacillus thuringiensis* strains.
3. Molecular characterization of *chitinase* producing *Bacillus thuringiensis* strains.

2. REVIEW OF LITERATURE

The review of literature related to the present investigation is presented below under the following headings and subheadings:

2.1 Chitin:

After cellulose, chitin is the second most abundant biopolymer on the earth. Chitin along with its derivatives has vast economic significance in medical sciences, agricultural sciences, and foundation industries *i.e.* chemical reactivity and biological activities (Al-Sagheer *et al.,* 2009). Chitin extracted from the crustacean shell wastes has variability in the fat binding capacities (Eijsink *et al*., 2003). It is naturally present in three different polymorphic forms *i.e.,* α (parallel chain structure), β (antiparallel structure) & γ chitins (combination of parallel and antiparallel structure) (Rudall *et al.,* 1963).

The process of chitin catabolism occurs with the help of Chitinase enzyme and process takes 2 steps for catabolism. Firstly, Chitin is converted into chitin oligosaccharides by Chitinase and secondly, it is further cleaved into N-acetylglucosamine by Chitobiases (Suginta *et al.,* 2000). The occurrence of exo and endochitinase genes was assessed by PCR amplification *via* gene-specific primers. Different types of Chitinases enzymes are produced by different microorganisms such as actinomycetes, fungi, bacteria and some higher plants for various purposes depending on their own usage and physiology (Pichyangkura *et al*., 2002).

Bacillus thuringiensis producing Cry and Vip proteins acts on the larvae of the insects and is exploited worldwide for the biological pest management (Hofte and Whiteley, 1989). Under optimum conditions, Bt also produces many biologically active molecules such as bacteriocins and hydrolytic enzymes i.e. Chitinase. Bt produces small quantities of chitinases, sometimes difficult to detect with traditional methods using chitin as carbon and nitrogen sources (i.e. halo formation) (Jain *et al.,* 2017).

2.2 Chitinases:

Chitinases are produced by different microorganisms which generally present a wide multiplicity of enzymes that are mainly extracellular. Chitinases are made up of a catalytic domain, carbohydrate binding modules (one or more), and other modules *viz.*

fibronectin type III domain (This domain hydrolyze the β-(14)-glycosidic linkages in chitin) (Mehmood *et al.*, 2010). However, some chitinases only have one catalytic domain. On the basis of amino acid sequence similarity, Chitinases are divided into GH18 and GH19 families (CAZy database: http://www.cazy.org) (Lombard *et al.*, 2014). Some chitinases, especially in combination with [3-1,3 glucanases, inhibit fungal growth *in-vitro* substantiating the idea that these enzymes play an important role in plant defense against pathogens, Chitinases also releases elicitors from fungal cell walls (Fritig *et al.*,1998; Boller,1988).

Plant chitinases have been divided into several classes based on amino acid sequence. In the classification system of glycosyl hydrolases, all chitinases are grouped into two families. Class I, II, IV and V chitinases form the family 19 that is only known in plants. Family 18 includes all fungal, animal and bacterial chitinases as well as plant chitinases of classes III and VI. The hydrolytic action of glycosyl hydrolases can take place with either retention or inversion of the anomeric configuration (Ohno *et al.,* 1996).

Microbial chitinases fascinated the consideration as one of the potential enzymes for applications in agriculture, pharmaceutical, waste management, biotechnology, and industry (Neeraja *et al*., 2010). Their high demand and wide potential use have led to the discovery of new strains of microorganisms that are capable to produce enzymes with novel properties and the development of low-cost industrial media formulations (Jaeger *et al.,* 2002).

2.3 *Bacillus thuringiensis* Chitinases:

Bt Chitinases plays an important role in the survival of itself under different conditions. For example, it seize and assimilate chitin in order to use it as a source of carbon and act as a virulence factor that promotes the establishment of successful infection since Bt propagate in moribund and deceased larvae. Chitinases can participate in the destruction of the cuticle thereby facilitating the release and dissemination of toxins and establishment of the bacterium in other ecological niches (Martinez-Zavala *et al.,* 2020).

Fifty chitinase genes in different Bt subspecies isolated from China (41%), Mexico (17%), Pakistan (15%), Thailand, Tunisia, and India have been deposited in NCBI GenBank (Barboza Corona *et al*., 2008). To date, at least 38 chitinase genes from

different subspecies of Bt such as kenyea, pakistani, colmeneri, canadiensis, entomocidus, kurstaki, israelensis and konkukian have been reported in the GenBank database (http://www.ncbi.nlm.nih.gov). Most of Bt Chitinases showed 93.8–99.6% similarities with each other (Barboza-Corona *et al*., 2012).

Chitinases of Bt play important roles in nature and can be used for different environment purposes, for example: (i) in the degradation of shrimp wastes, (ii) improving the insecticidal activity of Cry or (iii) in the control of phytopathogenic fungi. The last two options, implicate alternatives to reduce the use of chemicals. Bt secretes a chitinases and proteases which can act synergistically to hydrolyzes chitin containing wastes from shrimps and other non-edible food materials that can be used in pharmacy, medicine, food, water treatment, as ingredient in culture media, in pigment extraction, in the formulation of animal food, for reducing nematode population in soils and to produce single-cell protein (Barboza-Corona *et al*., 2012).

2.3 Mechanism of Chitin production:

Although chitin is one of the most important biopolymers in nature, knowledge of its biosynthesis is still fragmentary. Formation of the different chitin forms is catalyzed by chitin synthase (UDP-N-acetyl-D glucosamine: chitin 4-β-Nacetylglucosaminyltransferase, a highly conserved enzyme found in every chitin synthesizing organism. It utilizes UDP-N-acetyl-glucosamine (UDPGlcNAc) as the activated sugar donor to form the chitin polymer (Glaser and Brown, 1957).

Candy and Kilby (1962) were the first to propose a biosynthetic pathway for insect chitin synthesis starting with glucose and ending with UDP-GlcNAc. The pathway from UDP-GlcNAc to chitin was finally established in insects by Jaworski *et al*. (1963). One intermediate step might be the transfer of GlcNAc onto a lipid to form dolichyldiphospho-N-acetylglucosamine, as was deduced from studies with microsomal fractions of bugs and brine shrimps (Horst, 1983; Quesada-Allue *et al*., 1976). The individual sugar units in its structure are rotated 180° with respect to each other, and each pair forms the disaccharide N, N′-diacetylchitobiose [(GlcNAc)2. The individual polymer chains can be described as helices. Such a structure leads to high stability as the rigid ribbons are connected by 03-H→05 and 06-H→07 hydrogen bonds. Chitin also has three different crystalline allomorphs: α-, β- and γ-forms. These differ in the orientation of the micro-fibrils.

2.4 Applications of Chitinase:

The role of Chitinases resulting from chitin and chitosan degradation have shown immense potential in several fields. They have dissolves cell walls of various fungi, cell wall synthesis, secretion, strain improvement as well as other biotechnological applications. *Bt* has become an important tool in the control of pests that affect humans, whether in agriculture or in combating vectors of disease. Since the first commercial use of Bt formulations for the control of caterpillars, the spectrum of applications has increased and is no longer restricted to the initial function. It has become apparent that the potential of *Bt* transcends just biological control, and it can be used in other branches of science to assist humans. The applicability of bacterial chitinases is discussed as follows:

1. **Defense:** Plant Chitinases are involved in defense mechanism against pathogens either directly through their antifungal properties or indirectly through release of chitin oligomers. A model of the roles of chitinases in plant defence response, proposed by (Mauch and Staehelin1989) suggests that these enzymes are involved at different stages of pathogenesis. By releasing elicitor molecules chitinase play an important role in early stage of infection and involved in transfer of information (de AGerhardt *et al*., 1997). Root nodule and nodule development, germination of seed and somatic embryogenesis also plays an important role in development process of some chitinase genes. Chitinases are known biocontrol agents with significant potential to protect plants against fungal pathogens. Chemically, chitinases act by hydrolyzing N-acetyl D-glucosamine, and this capability has great industrial potential. By having the affinity to the polymer and the ability to degrade it, chitinases have many applications in the field of health care for their antibacterial and immune modulating effects and in agriculture for the production of antifungal substances and the control of plant pathogens (Bhattacharya *et al*., 2007; Reyes-Ramirez *et al*., 2004).

2. **Production of single cell protein:** Revah-Moiseevand Carrod (1981) worked on shellfish waste for the conversion of chitin to yeast single-cell protein (SCP). They used the *Serratia marcescens* chitinase system to hydrolyze the chitin and *Pichia kudriavazevii*. Growth yield, total protein and nucleic acid contents were the criteria to assess single cell protein production.

3. **Chitinases as biopesticides:** It has been implicated in different forms of morphological event in fungi (Villagomej-Castro and Lopez-Romero, 1996). Although chitinases stimulates increased toxicity of *Bt* products, its use in bio pesticides is just one of the potential uses of these enzymes. In a study of soybeans, Reyes-Ramirez *et al*. (2004) investigated the protective, antifungal activity of chitinase from *Bacillus thuringiensis.*

4. **Miscellaneous applications:** Chitinase are increasingly used in the food processing industry to degrade waste and make it useful for humans. Food processing produces large quantities of by-products. Disposal of waste can lead to environmental and human health problems, yet often they can be turned into high value useful products. Crustacean shells waste from shrimp, lobster and crab contain large amount of chitin, a polysaccharide that may be extracted after deproteinization and demineralization of the exoskeletons (Hamed *et al*., 2016)

2.5 Biochemical Characterization:

Biochemical characterization is used for microbial identification based on differences lies in their biochemical activities exhibited by different types of chitinase producing bacteria. Wafula *et al*. (2014) performed biochemical studies included, gelatin liquefaction, methyl red-Voges Proskauer, catalase test, and oxidase test in which isolates D16, D19 and D51 showed positive results for oxidase and MR-VP (Methyl Red Voges-Proskauer) test and also suggested their close relatedness with *Bacillus cereus*. Isolates D5, D16, S31, D70 and D2 were biochemically and morphologically closely related to *Bacillus thuringiensis* with the same characteristics as *Bacillus cereus* only that they had large rods upon gram staining.

Jyoti *et al*. (2018) performed various biochemical test gelatine liquefaction, methyl red-Voges Proskauer, catalase test, and oxidase test for confirming the morphology of the *Bacillus thuringiensis*. This genus was found positive for all microscopic tests.

2.6 PCR based screening of Chitinase genes:

The Polymerase Chain Reaction (PCR) has been used to characterize the chitosan producing bacterium *Bt* strain collections. This technique is a highly sensitive and effective in rapidly detecting and identifying target DNA sequences requires small amounts of DNA and allows screening of many *Bt* samples to classify them and predict

their chitinolytic activities. The efficacy of PCR in identifying the large family of exochitinase and endochitinase genes is based on the presence of conserved regions.

Zhong *et al*. (2015) isolated Pseudomonas *chitinase C* gene (PsChiC) from the chromosomal DNA of this bacterium using a pair of specific primers. The *PsChiC* gene having 1443 bp sequence resulted in 480 amino acid protein with a molecular mass of 51.66 kDa. Chitinase assays revealed that the chitobiosidase and endochitinase activities of PsChiC were 51.6-fold and 84.1-fold higher than those of pET30a, respectively. These results suggest that PsChiC from *Pseudomonas* sp. could be useful in improving the pathogenicity of baculoviruses.

El-Hamshary *et al.* (2008) performed PCR based screening of chitinase producing strain using the PCR primer was designed from chitinase nucleotide sequences of different species of bacilli with the amplicons size of 310 bp from the conserved region of the *chitinase* gene (310bp). On sequencing the PCR fragment from the local Bt isolates, around 96% similarity to chitinase gene from *B.thuringiensis* A1 Hakam was reported.

Shahbaz and Yu (2020) screened chitinase-coding genes by PCR using the degenerative primer. Chitinase18 (glycoside hydrolase family 18) produced 1024 bp long amplicons and sequence comparison revealed that the gene is derived from *Serratia Marcescens* strain WVU-005 with the sequence identity of 94.93% and the E value = 0.

Saleem *et al*. (2015) screened chitinase gene in bacterial strains and eight DNA samples were found positive when subjected to PCR amplification using the designed primers of the chitinase gene with the chitinase gene fragment size of approximately 2300 bp in length by PCR amplification.

2.6 Molecular characterization of chitinase producing bacteria:

Molecular techniques have helped to develop easy, fast and reliable method to achieve microbial characterization at genus, species and even at strain level. Molecular marker technique has been found strain specific and have proved to be valuable and effective tools in the characterization and evaluation of genetic diversity within and between species and populations. Identification of *Bacillus* species using conventional sequencing methods can divulge their taxonomic affiliation but there are certain groups

of *Bacillus* where alternate methods like 16S rDNA and PCR fingerprinting can expose their exact lineage of the species quickly.

Krithika *et al*. (2016) isolated and characterized chitinase producing strains at molecular level by 16s rRNA amplification and sequencing and the indentified species are *Chitiniphilus shianonensis*, *Acinetobacter sp*., *Bacillus badius*, *Acinetobacter venetianus*, and *Brevibacillus borstelensis*.

Setia and Suharjono (2015) amplified 16S rDNA with universal primers 27F (5'GAGAGTTTGATCCTGGCTCAG3') and 1492R (5'CTACGGCTACCTTGTTACGA3') and 16S rDNA was separated by agarose gels electrophoresis. The ARDRA pattern obtained is said to be representative of the species analyzed and important for their molecular characterization. In the present study ARDRA produced a fingerprint based on length polymorphism for molecular characterization of native *Bacillus thuringiensis* strains.

3. MATERIALS AND METHODS

The present investigation entitled "**Molecular characterization of chitinase producing *Bacillus thuringiensis* strains**" was undertaken in the Department of Molecular Biology and Biotechnology (MBBT), Rajasthan College of Agriculture (RCA), MPUAT, Udaipur (Rajasthan) during 2019-2020.

3.1 Chemicals, Glasswares, Plasticwares and Equipments:

All chemicals used in the study were of molecular and analytical grade purchased from Hi-Media, SRL and Bangalore Genei Pvt. Ltd. etc.

The glasswares and plasticwares were procured from Borosil, Tarson (India), Thermo (India) etc.

The following instruments were used in the study *i.e.,* Centrifuge (Eppendorf), Vortex (REMI), Nano-Spectrophotometer (IMPLEN), Hot air oven (Yorco), Deionised water (Milli Q, Millipore), Incubator-shaker (MAC), Laminar airflow (Rescholar), Electrophoresis Unit (Bio-Rad), Gel documentation system (Eppendrof), PCR machine (Bio-Rad), Magnetic stirrer machine, Double distilled water etc.

3.2 Bacterial Strains:

Twenty eight *Bacillus thuringiensis* strains were provided by All India Network Project on Soil Biodiversity and Biofertilizers, Dept. of Molecular Biology and Biotechnology, RCA, MPUAT, Udaipur.

3.3 Preparation of colloidal chitin:

Colloidal chitin was prepared using previously published protocol of Krithika *et al*. (2016). The practical grade chitin powder purchased from (Hi Media, India) was utilized to set up the colloidal chitin. Chitin powder was broken down in concentrated HCl and blended at for 2 h. In the process of blending's, the hydrolyzed chitin was washed for a number of times with distilled water in order to remove the acid completely to bring its pH to 7. The colloidal chitin was collected by centrifuging the solution and stored in the form of a paste at 4°C till its further use.

3.4 Screening of Chitinase producing *Bacillus thuringiensis* strains:

These bacterial strains were plated directly onto modified chitinase screening media (CSM) containing chitinase as substrate (Saima *et al*., 2013). The CSM medium composition was adapted as follows (6 g/l Disodium hydrogen phosphate; 3 g/l Potassium dihydrogen phosphate; 1gram/l NH4NO3; 0.5 gram/litre Sodium chloridel; 0.05 g/l Yeast extract; 0.5%(w/v) Colloidal chitin; 20 g/l agar with 5.5 pH. All the strains were streaked on media. Freshly grown bacterial culture was spot inoculated at five equidistant points followed by incubation at 37 °C for 7 days and the plates were then observed for zone of hydrolysis around the inoculated area. The zone was further visualize by floodings the plate with Gram′s iodine solutions observe the formation of clear zone against a brownish background (Kasana *et al.* 2008) All the experiments were carried out in triplicate and averages were reproduced.

3.5 Qualitative assay and Quantitative chitinase enzyme assay:

The assay for chitinase enzyme was carried out with crude enzyme (Sudha *et al*., 2020). The Bt strains that produce chitinase were inoculated in colloidal chitin broth (100 ml) in 250 ml Erlenmeyer flasks and incubated for 4 days at 37°C. The cell-free supernatant was obtained after centrifuging the culture broths at 8000 rpm for 20 minutes. To remove the enzymes, the clear culture filtrates were saturated to 60–70% with ammonium sulphate and held at 4°C overnight. The precipitate could be obtained by centrifugation at 10,000 rpm at 4°C, dissolved in 50 mM phosphates buffer (pH 7.0), and used as a crude enzymes supply.

3.5.1 Qualitative (well diffusion) assay:

The Chitinase enzyme activity of the crude enzyme extract was assessed using the method of Lunge and Patil (2012) techniques by using a simple well diffusion assay. Using a 6 mm sterile cork borer, wells were drilled in 1 percent colloidal chitin agar containing BCP dye plates. Every well will be filled with a 100 litre culture filtrate from each isolate and incubated at 37°C. The formation of clear zone around the well will be monitored for 24 hours.

3.5.2 Quantitative assay:

The Chitinase enzyme was assessed using the previously published protocol of Miller *et al.* (1959). A mixture of 0.1 percent colloidal chitin and 50mm of 0.1 M phosphate buffer pH 7.0 was added to the crude enzyme. The reaction mixture was

centrifuged at 10,000 rpm for 10 minutes after being incubated at 55° C for 10 minutes. The supernatant was then combined with DNS and Schales reagents and boiled for 10 minutes. The absorbance of the mixture was measured at 530 nm after cooling. One unit of chitinase activity is defined as the amount of enzyme that produces 1 L/mol of reducing sugar as N-acetyl-D-glucosamine equivalent/min.

3.6 Biochemical characterization of chitinase producing *Bacillus thuringiensis* strains:

The following biochemical test was performed in order to characterize chitinase producing *Bacillus thuringiensis* strains. The standard methodologies were preformed.

1. Oxidase test (Al.Tariq *et al*., 2016)
2. Gelatin liquefaction test (Krithika *et al*., 2016)
3. Catalase test (Krithika *et al*., 2016)
4. Methyl red-Voges proskauer test (Wafula *et al*., 2014).

3.7 PCR based screening of chitinase gene from *Bacillus thuringiensis* strains:

3.7.1 Isolation of genomic DNA from *B. thuringiensis* strain (Kalman *et al.,* 1993):

A loopful of Bt strains was streaked on LB agar plate and brooded at 30°C short-term. Single state from the short-term developed culture was vaccinated into 30 ml LB stock and kept at 30°C for shaking. Bt cells were collected for DNA seclusion when development arrived at 1.0 OD at 600 nm. The cells were gathered from 30 ml culture by centrifugation at 5000 X g for 5 min. The supernatant was disposed of and the cells were washed with washing arrangement (TES: Tris-HCl (pH 8.0) 10 mM; EDTA 1 mM; Sodium chloride 100 mM), and resuspended in resuspension arrangement (Tris – HCl 25 mM; Sucrose 25%; EDTA 25 mM; Lysozyme 1mg/ml) and hatched at 37 °C for 1h. SDS was added to a last convergence of 2% and hatched at 50 °C for 15 min. NaCl was added to a last centralization of 1M and hatched at 50 °C for 5 min followed by for the time being brooding at 4 °C. Arrangement was centrifuged at 10, 000 X g for 5 min at 4°C and DNA in the supernatant was encouraged with equivalent volume of isopropanol.

The DNA was resuspended in 30 µl of TE cradle containing 1mM NaCl and 10 µg of RNase 10mg ml-1. After 30 min of hatching at 37°C, Proteinase K (0.6 mg ml-1) was added and brooded at 37 °C for 30 min. The blend was separated with equivalent

volume of phenol, phenol: chloroform (1:1) and chloroform. The DNA was encouraged with 2.5 ml of total ethanol and 1/tenth volume of 3M sodium acetic acid derivation (pH 5.2). The DNA pellet was washed with 70% ethanol and air dried. The dried DNA pellet was broken down in suitable amount of TE support and put away at – 20 °C for additional utilization. The complete DNA was broke down utilizing 1Kb stepping stool on 0.8 percent agarose gel with ethidium bromide. The disconnected DNA was likewise dissected utilizing spectrophotometer at 260 and 280 nm for subjective and quantitative analysis.

3.8 Amplification of Chitinase gene:

Amplification of the following chitinase genes were performed with the following primers specific.

Table 3.1: Chitinase gene specific primer used in the present study

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

The PCR reaction was done using an Eppendorf thermal cycler and the reaction mixture was 25 The PCR reaction was done using an Eppendorf thermal cycler and the reaction mixture was 25 µl containing 50 ng of genomic DNA, 75 ng each of forward and reverse primers and 12.5 µl 2X PCR master mix (Bangalore Genie, India). The PCR reaction system was optimized based on the previously published protocol from Jain et al. (2021) and briefly, the annealing temperature for all these primers were used as 48 ^{o}C for 45 sec in each cycles of the PCR. The PCR mixture without genomic DNA was used as negative control. Amplification accounted for 30 cycles. Seven microliters of the amplified products were analysed using 1 KB and 100bp ladder on 1.2 per cent agarose gel with ethidium bromide staining.

3.9 Molecular Characterization of Chitinase producing native *Bacillus thuringiensis* strains:

The PCR amplified 16S rDNA region of chitinase producing *Bacillus thuringenisis* stains 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'AGAGTTTGATCCTGGCTCAG 3') and 1492R (5-'ACGGCTACCTTGTTACGACTT -5') specific to conserved regions. The amplified product were gel purified and sequenced at Biokart India Pvt Ltd, Bangalore. The 16S rDNA sequences were edited before the BLAST using the 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 the 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 the Maximum Likelihood method.

4. RESULTS AND DISCUSSION

The research entitled "**PCR based characterization of chitinase gene from *Bacillus thuringiensis***" was undertaken in the laboratory of All India Network Project on Soil Biodiversity & Biofertilizers, Department of Molecular Biology and Biotechnology (MBBT), Rajasthan College of Agriculture (RCA), MPUAT, Udaipur (Rajasthan) during 2019-2020. The results obtained in the research were discussed below under separate subheadings.

4.1 Screening of chitinase producing *Bacillus thuringiensis* strains:

4.1.1 Qualitative assay:

The chtinolytic potential of twenty-eight *Bacillus thuringiensis* strains was assessed on chitinase screening media (CSM) supplemented with colloidal chitin as substrate for the determined of their chitinase solubilization activity. The Bt strains developed transparent holo zones of the CSM medium were considered positive for producing chitinase enzyme. The holo zones waere further visualized by the application of Gram's Iodine solution which producing a transparent holo zone around the bacterial colonies (Figure 4.1) and among 28 Bt strains, 10 Bt strains produced prominent holo zone and having Chitinase producing activities.

Further from the diameter of the holo zone and bacterial colony, the chitinase solubilization indexes (CSI) were calculated and summarized in Table 4.1. The maximum solubiliztion index was observed in Bt-13 with the CSI of 7.79±0.14 closely followed by Bt-2 (7.53±0.30), Bt-7 (6.77±0.16), Bt-10 (7.76±0.20), Bt-11 (3.70±0.27), Bt-19 (4.15±0.17), Bt-20 (6.19±0.21) and Bt-26 (3.67±0.19), Bt-28 (4.29±0.11) respectively. The minimum solubilization index was observed in Bt-27 with the CSI of 3.45±0.10.

The results obtained in the present study were well supported by the observations of Gupta *et al.* (2018) isolated 28 chitinase producing bacterial strains from the soil samples and reported that out of 28 strains only 12 showed clear holo zone when incubated in different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%) of 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 the chitin as a sole carbon and nitrogen source.

Ong *et al*. (2017) reported the Chitinase activities using chitinase selective medium supplemented with colloidal chitin and bromocresol purple as an indicator in 23 chitinase producing bacterial strains isolated from Malaysian soil samples, and the positive colonies produced a purple zone on the plate. Further, the two most potent isolates were chosen for identification and tentatively identified as *Enterobacter* sp. and *Zymomonas* sp., respectively, based on their morphological and biochemical test.

Krithika *et al*. (2016) isolated 35 different bacterial strains from marine waste and out of which 14 isolates were showed chitinolytic activities by producing clear zone on chitin-containing media and highest activates were showed by the strains ASK5, ASK18, ASK19, ASK22, and ASK31 when the plates were further stained with Lugmann's Iodine.

Setia *et al*. (2015) isolated 18 chitinolytic bacterial isolates with different morphology from shrimp waste and based on primary screening and two isolates *viz.,* PBK 2 and SA showed the highest chitinolytic index of 2.069 and 2.084 respectively. Similar results were also observed in the present study.

Table 4.1: Chitinase solubilization index of chitinase hydrolyzing Bacteria (Bt)

S.N.	***Bacillus thuringiensis* Strains**	**Chitinase solubilization index***
1	Bt2	7.53±0.30 cm
2	Bt7	6.77±0.16 cm
3	Bt10	7.76±0.20 cm
4	Bt11	3.70±0.27 cm
5	**Bt13**	**7.79±0.14 cm**
6	Bt19	4.15±0.17 cm
7	Bt20	6.19±0.21 cm
8	Bt26	3.67±0.19 cm
9	Bt27	**3.45±0.10 cm**
10	Bt28	4.29±0.11 cm

***Data are mean of triplicate values; Data ± SD**

4.1.2 Qualitative assay for Chitinase enzyme production

The crude Chitinase enzyme from the *Bacillus thuringiensis* strains produced clear zones on colloidal chitin plate were further checked for qualitative examination by well dissemination strategy (Lunge and Patil, 2012). The crude chitinase enzyme from 10 Bt strains were produced according to Sudha *et al*. (2020) and further the 100 µL crude Chitinase enzyme was added in the wells made on 1% colloidal chitin agar plates containing Bromocresol blue (BCP) color and incubated at 37°C (Sudha *et al*., 2020). 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 increase of the medium was increased and the color of BCP dye turned into purple (Figure 4.2).

Petrisor *et al*. (2015) isolated the cultures of *Trichoderma* were examined qualitatively for production of extracellular enzymes chitinases on 1% colloidal chitin agar plates containing Bromocresol blue (BCP) dye and two strains *viz.,* Td49 and Tdal12 strains exhibited higher chitinase activity by producing a higher diameter of the purple color zone on medium. 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 clear chitinolytic zones also supports the results observed in the present study.

4.1.3 Quantitative Chitinase enzyme production assay based on standard curve of N-acetyl glucosamine

Based on the chitinase solubilization index (Table 4.1), the ten isolates *viz*., Bt-2, Bt-7, Bt-10, Bt-11, Bt-13, Bt-19, Bt-20, Bt-26, Bt-27 and Bt-28 were selected further for quantitative chitinase enzyme activity (U/ ml) assay in liquid CSM media as per Sudha *et al*. (2020). The Chitinase activities were calculated based on standard curve of N-Acetyl glucosamine. The results for quantitative chitinase enzyme activity were summarized in (Table 4.2). The results revealed the enzyme activity varied from 0.130 to 0.651 U/ml depending on the bacterial strain. Maximum chitinase activity was shown by Bt-2 (10.19±0.651 U/ml) closely followed by Bt-27 (6.53±0.309 U/ml), Bt-20 (5.36±0.190 U/ml), Bt-7 (5.24±0.408 U/ml) and Bt-10 (5.20±0.192 U/ml) respectively.

Similar results were observed by Ong *et al*. (2017) reported the highest chiltinase production after 24 h for *Enterobacter* sp. (6.70±0.15 U/mL) and *Zymomonas*

sp (1.68±0.14 U/mL). Setia *et al*. (2015) reported the chitinase acitivity of isolates PBK 2 and SA1.2 to 0.213 and 0.219 U/ml respectively. Chitinase activity depends on various factors such as time, pH, incubation time and substrate concentration etc. The chitinase enzyme activites observed in the present study was higher compare to the results observed by Setia *et al.* (2015).

Gomma *et al*., (2012) reported that influence of additional carbon sources on chitinase production and reported enhanced chitinase production of 16.02 U/ml by *B. thuringiensis* when colloidal chitin medium amended with galactose. Saleem *et al*., (2014) reported that the production of chitinase by different chitinolytic Bt isolates and reported the highest Chitinase activity (0.23 U/ml) was reported in Bt-4 strain after the 4th day of incubation.

Table 4.2: Estimation of Chitinase activity

S.N.	BT STRAINS	Chitinase activity (U/ ml)
1	Bt-2	**10.19±0.651**
2	Bt-7	5.24±0.408
3	Bt-10	5.20±0.192
4	Bt-11	4.83±0.132
5	Bt-13	3.82±0.364
6	Bt-19	5.18±0.329
7	Bt-20	5.36±0.190
8	Bt-26	**3.78±0.101**
9	Bt-27	6.53±0.309
10	Bt-28	4.41±0.398

***Data are mean of triplicate values; Data ± SD**

4.2 Biochemical characterization of Chitinase producing *Bacillus thuringiensis* strain:

Biochemical characterization offers an initial evaluation of the microorganism. Each bacterial species has its own metabolic activities, which are regulated by enzyme/biochemical activity. As a result, bacterial biochemical characterization can be

used to distinguish distinct bacterial strains based on their precise biochemical profiles, as well as provide information about the ecology and physiology of the microorganisms being examined. The biochemical test was used in this analysis to look at the different enzymes of these Chitinase producing bacterial strains.

All the 10 isolates were subjected to various biochemical studies and the results were summarized. Out of ten isolates, seven isolates were founded positive for oxidase activity, nine isolates were founded positive for gelatine liquification, eight isolates were founded positive for catalase activity, ten isolate were founded positive for Methyl-Red test and seven isolates were founded positive for Voges-Proksauer (Figure 4.3).

Das *et al*. (2015) studied four new Bt strains for various biochemical tests and all strains were positive for catalase activity whereas all isolates were negative for Methyl-Red and Voges-Proksauer test. Krithika *et al.* (2016) studied 5 bacterial isolates for biochemical characterization and reported that out of 5 isolates 3 were positive for oxidase activity, 1 was positive for catalase test, 1 showed positive results for gelatine liquification, all 5 isolates were positive for Methyl red test and all 5 isolates were negative for Voges-Proskauer test.

Wafula *et al*. (2014) identified 10 isolates of gram-positive rod shaped bacteria and out of 10 isolates all were positive for oxidase test, all were also positive for catalase test, whereas 6 were positive for gelatine liquification and all were negative for methyl red test while positive for Voges-Proskauer test. Results obtained in the present study were in close agreement with the above mentioned reports.

Table 4.3: Biochemical characterization of chitinase producing Bt strains

Bt Strains	**Bt-2**	**Bt-7**	**Bt-10**	**Bt-11**	**Bt-13**	**Bt-19**	**Bt-20**	**Bt-26**	**Bt-27**	**Bt-28**
Oxidase	+	+	-	+	+	-	+	-	+	+
MR	-	-	-	-	-	-	-	-	-	-
VP	+	+	+	+	+	+	+	+	+	+
Gelatin Liquefication	+	+	+	+	+	+	-	+	+	+
Catalase	-	+	+	+	+	+	-	+	+	+

MR: Methyl Red ; VP: Voges Proskauer + Positive result; - Negative result

4.3 PCR based screening of *Chitinase* genes:

4.3.1 Isolation of genomic DNA of *Bacillus thuringiensis* isolates:

The genomic DNA from ten different chitinase producing Bt strains were isolated and run on agarose gel to check its purity and quantity by standard procedures. Agarose gel electrophoresis of the Bt genomic DNA showed intact band (Figure 4.4). Previously, Jain *et al*. (2017) isolated the total genomic DNA from the native Bt strains and further use the isolated DNA for PCR based screening of different *cry* and *vip* genes and also used for molecular characterization of these Bt stains.

Isolation of good quality DNA is the most important step. The DNA quantification was carried out by nano-spectrophometer and further based on the A_{260}/A_{280} ratio, the quality of DNA was found to be in the range from 1.7-2.1 which is an indicator of moderate good quality DNA for the 10 chitinase producing Bt strains. This was also supported by appearance of single, compact and sharp band when resolved on agarose gel electrophoresis. The DNA concentration was diluted to 50ng/ul for the PCR based screening of chitinase genes.

4.3.2 Screening of chitinase, exochitinase and endochtinase genes by PCR

The genomic DNA from Bt isolates were further 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 (Figure 4.5) indicated the presence of the above mentioned *chitinase* genes in Bt strains. All the 10 Bt strains showed the PCR amplification with *chitiniase* gene which indicate the presence of any type of chitinase gene. Further, the amplification with specific primers of *endochintinase* and *exochitinase* genes also confirmed that these Bt strains are harbouring both endochitinase and exochitinase genes.

El-Hamshary *et al*. (2008) designed PCR primers for PCR amplification of conserved region of the *chitinase* gene and screened local and reference type strains of *Bacillus thuringiensis* by PCR for the presence of *chitinase* gene. The primers designed by El-Hamshary *et al*. (2008) showed expected molecular size amplification in both type strains as well the local isolate. Further sequencing of the PCR amplicons from local Bt isolates revealed 96% similarity with the reported *chitinase* gene from *B.*

thuringiensis A1 Hakam. The same primer pair was used in present study and expected size of amplification was observed.

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 the 137 *Bacillus thuringiensis* isolates harbored at least one type of the *chitinase* gene whereas 66 (48.2%) being positive for the *exochitinase* gene and 82 (59.9%) being positive for the *endochitinase* gene. The results observed by Djenane *et al*. (2017) are in close agreement with the present study in which 10 strains (35.7%) Bt strains were hourboring *chitinase* genes.

4.4 Molecular Identification of chitinase producing bacteria:

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. The total genomic DNA from these potent five Bt isolates was used for the amplifcation of 16S rDNA region with the universal primers, 27F and 1492R as described by Jain *et al*. (2021) and the amplification of the specific size ~ 1.4 kb amplicon of 16S rDNA region was observed in all the five Bt strains (Figure 4.6).

The 16S rDNA amplicon was further gel purified and the purified DNA was sequenced with universal primers at Biokart India Pvt Ltd., Bangalore, India and analyzed using the nucleotide-nucleotide BLAST for the molecular identification. 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 the 16S rDNA were also obtained by submitting the DNA sequence at NCBI (Table 4.4) and the 16S rDNA sequences of these Bt strains revealed their greatest sequence identity with *Bacillus thuringiensis*.

Table 4.4: Molecular Identity of Potent Bt strains

Strains	Molecular identity	Accession number	Closest type strain			
			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

The partial sequence of 16S rRNA gene of isolate Bt-7 showed 100% homology with 16S rRNA sequence of *Bacillus thuringiensis* (Accession number NR_043403.1) already submitted to NCBI database. The partial sequence of 16S rRNA gene 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 the species is shown in (Fig. 4.8-4.12).

Tran *et al*., (2018) isolated thirty one chitinolytic bacteria from sediments and chitin flakes immersed in the water of a sand dune lake, Japan and the phylogenetic analysis of these isolates based on the 16S rRNA genes sequence reveal that most isolates belonged to the families Aeromonadaceae, followed by Paenibacillaceae, Enterobacteriaceae, and Neisseriaceae. Ten strains were estimated to be *Aeromonas hydrophila*; two strains to be *Aeromonas salmonicida;* and one strain to be *Serratia plymuthica*.

Sudha *et al.,* (2020) isolated and characterized two bacterial at molecular level and identified these strains as *Bacillus thuringiensis* strain LS1 and *Bacillus cereus* strain LS2 by 16S rDNA analysis. The BLAST analysis revealed that the bacterial species LS1 and LS2 showed a very high percentage of similarity (98.87%) with the

sequences of *B. thuringiensis* and *B. cereus*, respectively, with a reasonably high score and E-value being 0.

Hardoko *et al*., (2020) isolated and characterized the strong chitinolytic bacterial isolate A12 from rotten tiger shrimps (*P. monodon*) shells. Further the molecular identification based on 16S rDNA gene sequence revealed the identity of the isolate A12 was *Providencia stuartii*, with similarity of 99.33%.

Setia and Suharjono (2015) isolated chitinolytic bacteria from shrimp waste and two isolates *viz.* PBK 2 and SA 1.2 having the highest chitinolytic index were molecular characterized based on 16S rDNA sequences and revealed their identity as *Acinetobacter johnsonii* and *Bacillus amyloliquefaciens* respectively.

The present study addressed that screening and ability of local isolate of *Bacillus thuringiensis* strains to produce the chitinase enzyme. The Chitinase activity of these strains i.e. Bt-13 were significantly high and PCR based screening also revealed the presence of both exochitinase and endochtinase genes. The Chitinase activity of these strains might be the presence of novel *chitinase* genes, hence, these genes need to be cloned and expressed in the suitable host for its applications under various processes.

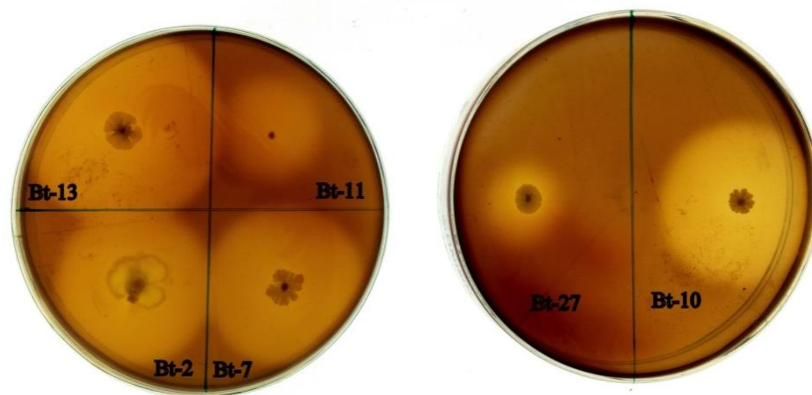

Figure 4.1: Screening of Chitinase producing Bt strains on chtinase screening media (CSM) containing colloidal chitin as substrate.

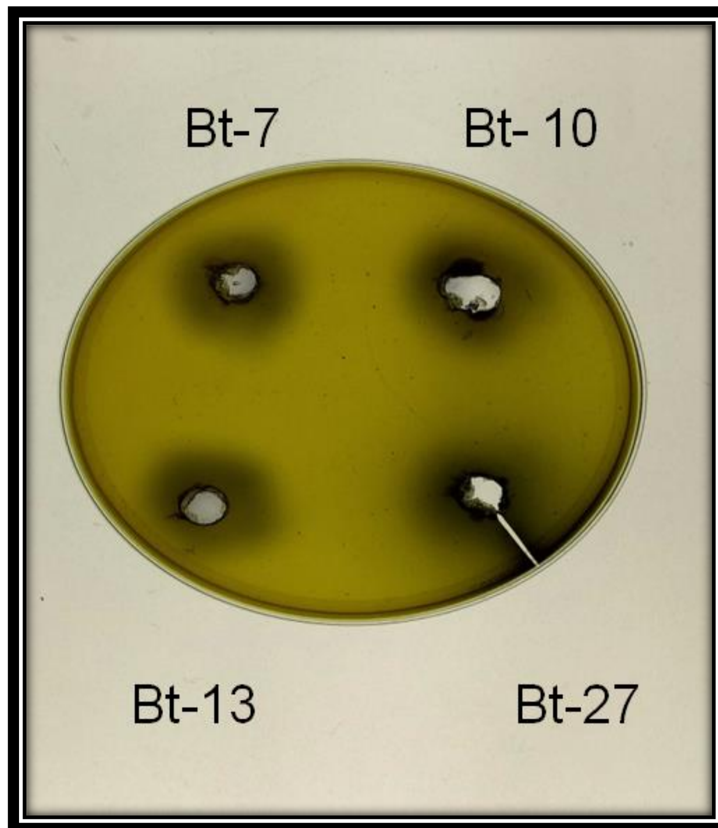

Figure 4.2: Qualitative assay of Chitinase crude enzyme activities on colloidal chitin plate containing BCP dye as color indicator

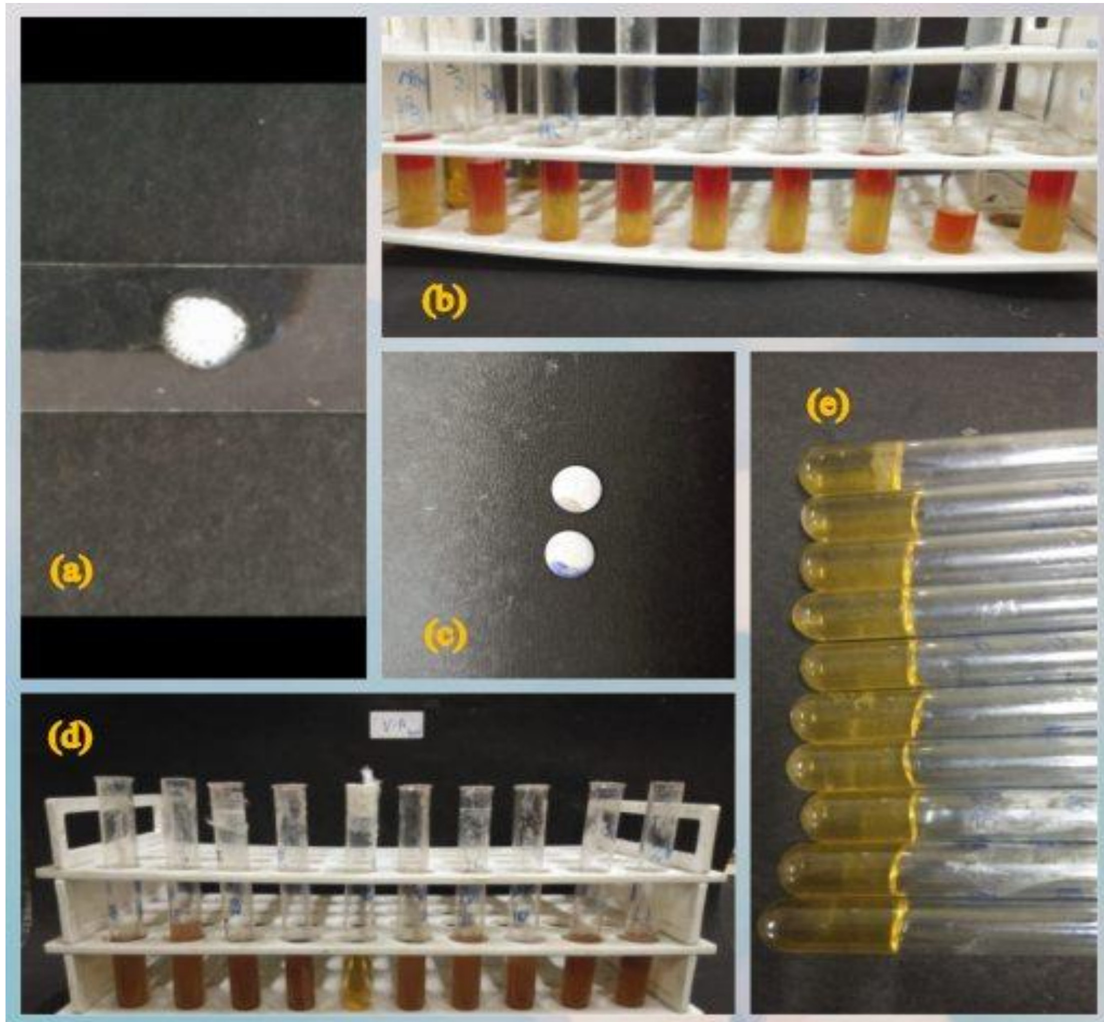

Figure 4.3: Biochemical studies of Chitinase producing Bt strains; (a) Oxidase test ;(b) Gelatin Liquification Test ;(c) Catalase test ;(d) Methyl – Red ;(e) Voges-Proksauer

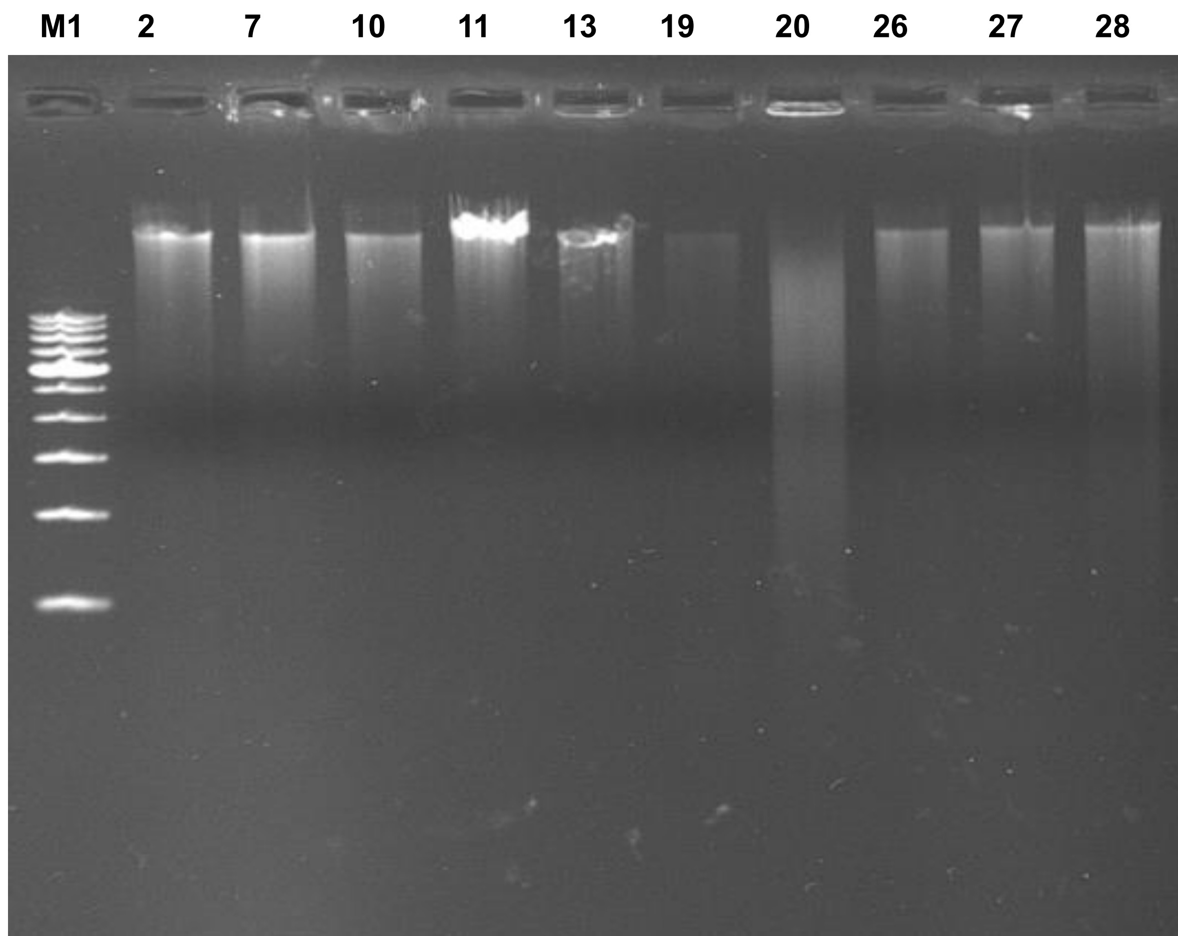

Figure 4.4: Agarose gel electrophoresis of the genomic DNA isolated from Chitinase producing Bt strains; M1: 1 Kb ladder DNA

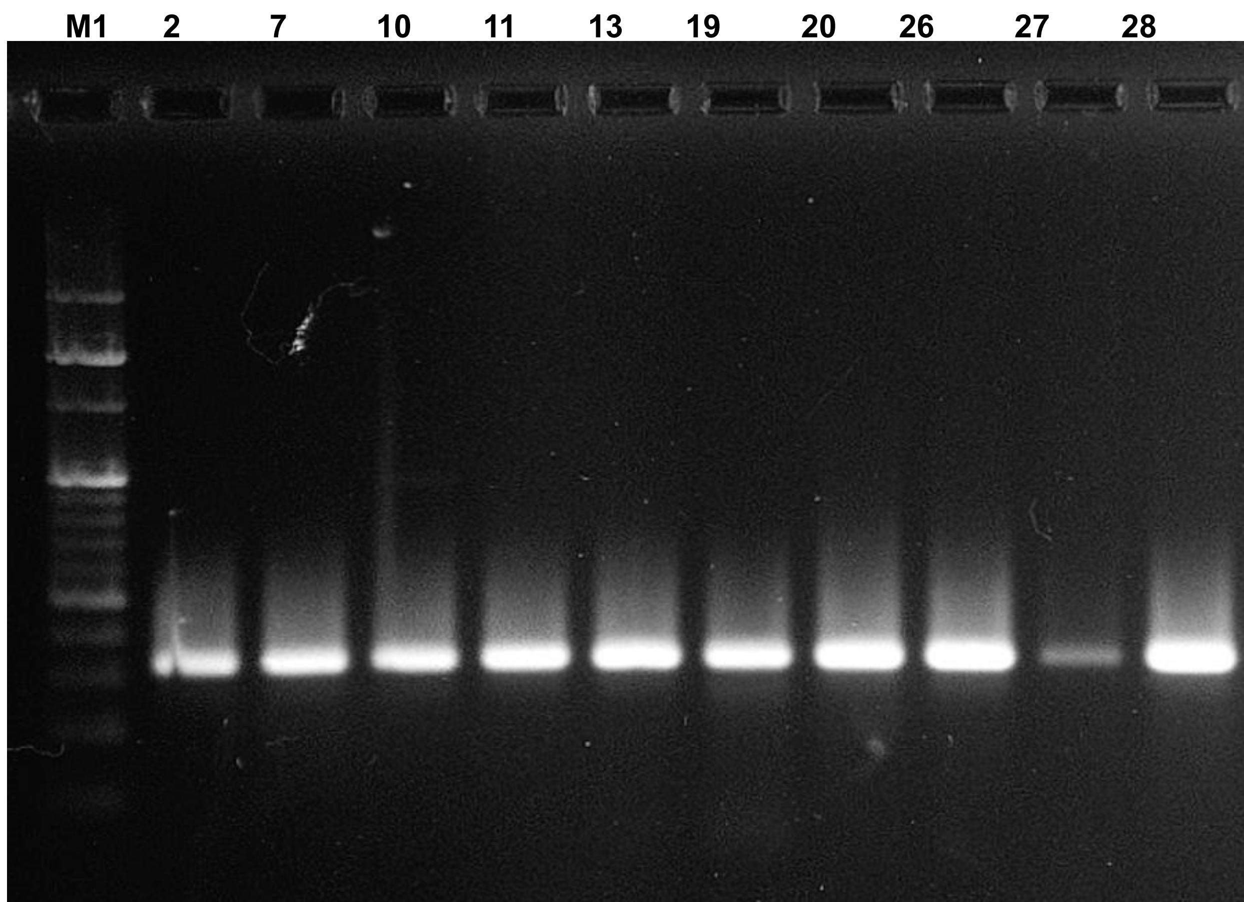

Figure 4.5: Agarose gel electrophoresis of the PCR amplification screened with chitinase gene specific primers. M1: 100 bp extended ladder.

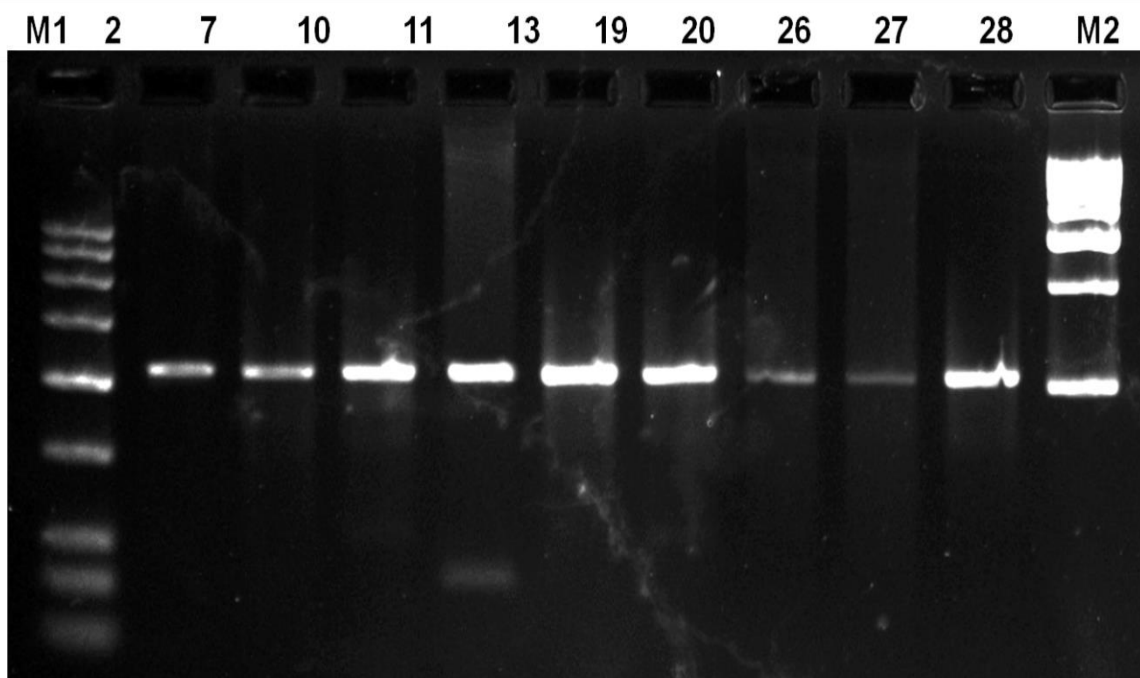

Figure 4.6: Agarose gel electrophoresis of the PCR amplification screened with exochitinase gene specific primers. M1: 100 bp extended ladder M2: 1 Kb ladder.

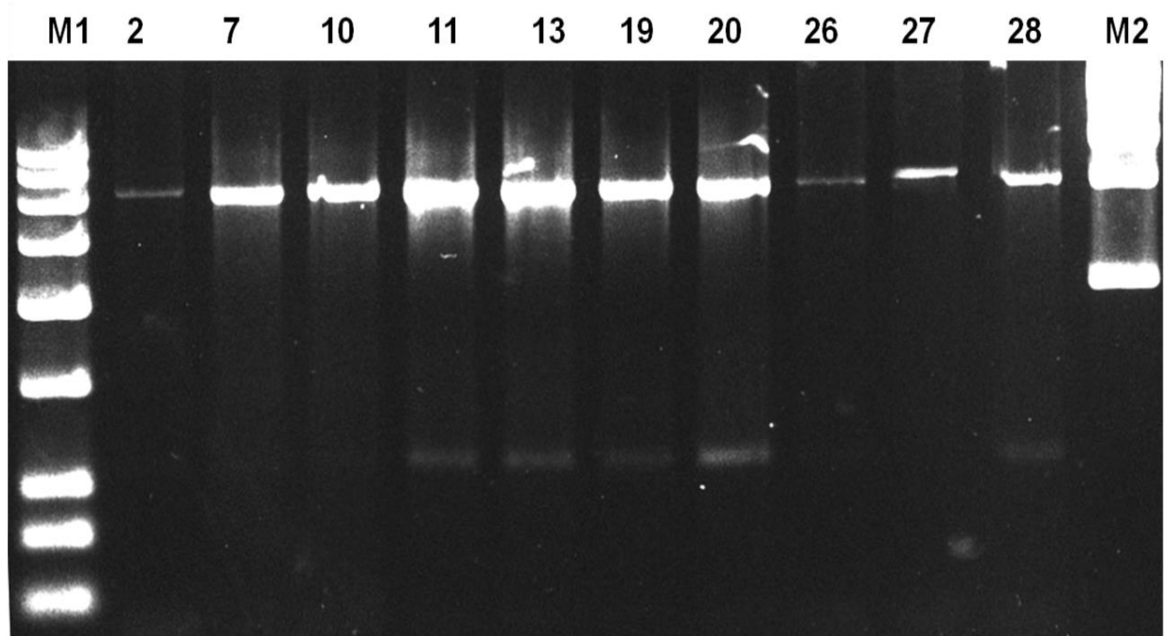

Figure 4.7: Agarose gel electrophoresis of the PCR amplification screened with endochitinase gene specific primers. M1: 100 bp extended ladder M2: I Kb Ladder.

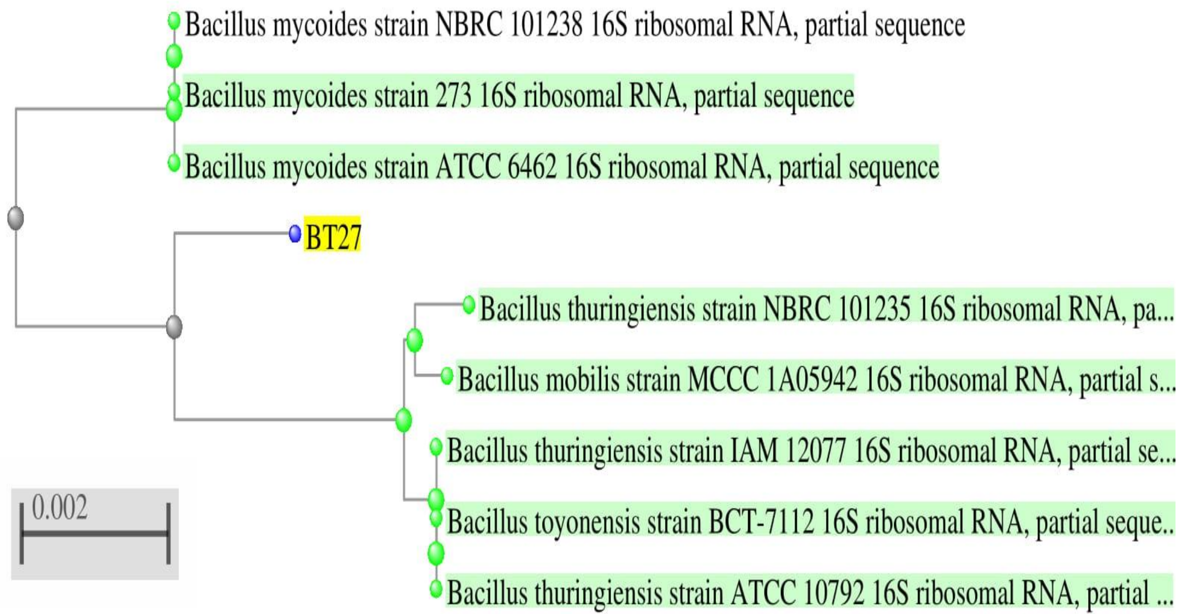

Figure 4.12: Sequencing and Phylogenetic Analysis of 16S rDNA of Bt-27 strain.

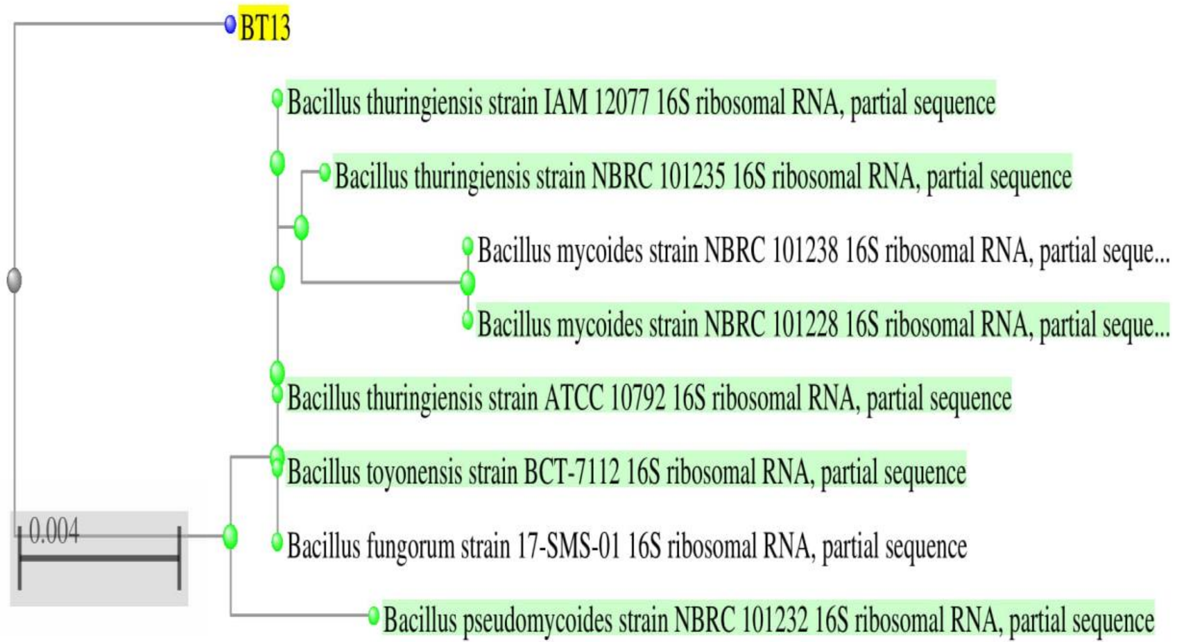

Figure 4.11: Sequencing and Phylogenetic Analysis of 16S rDNA of Bt-13 strain.

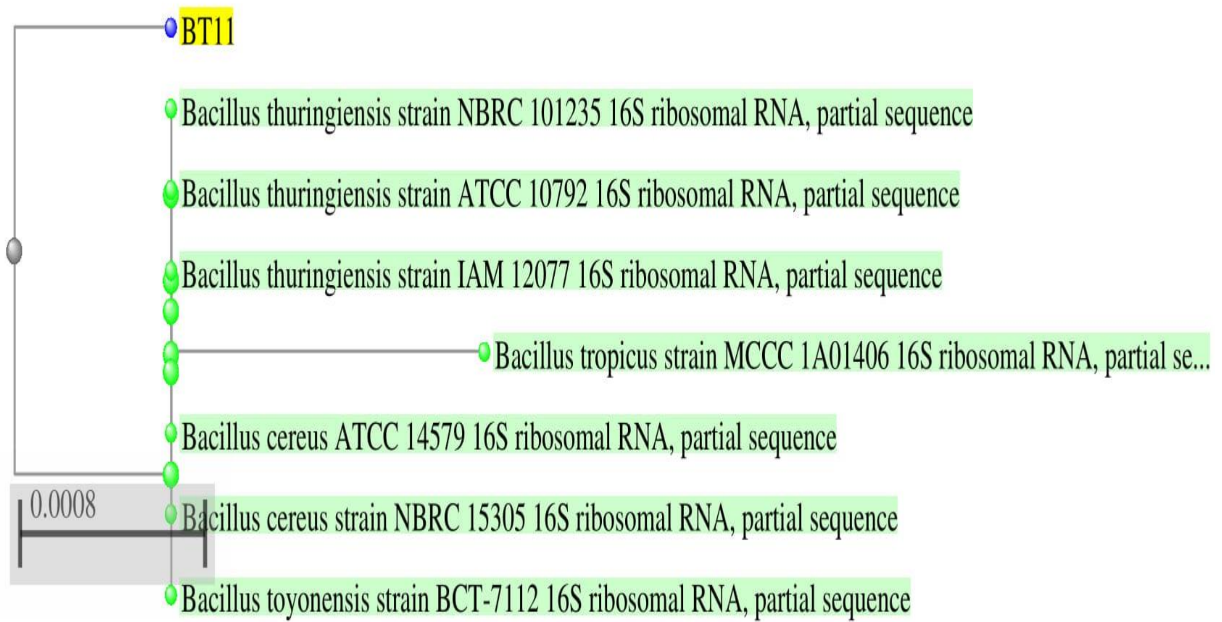

Figure 4.10: Sequencing and Phylogenetic Analysis of 16S rDNA of Bt-11 strain.

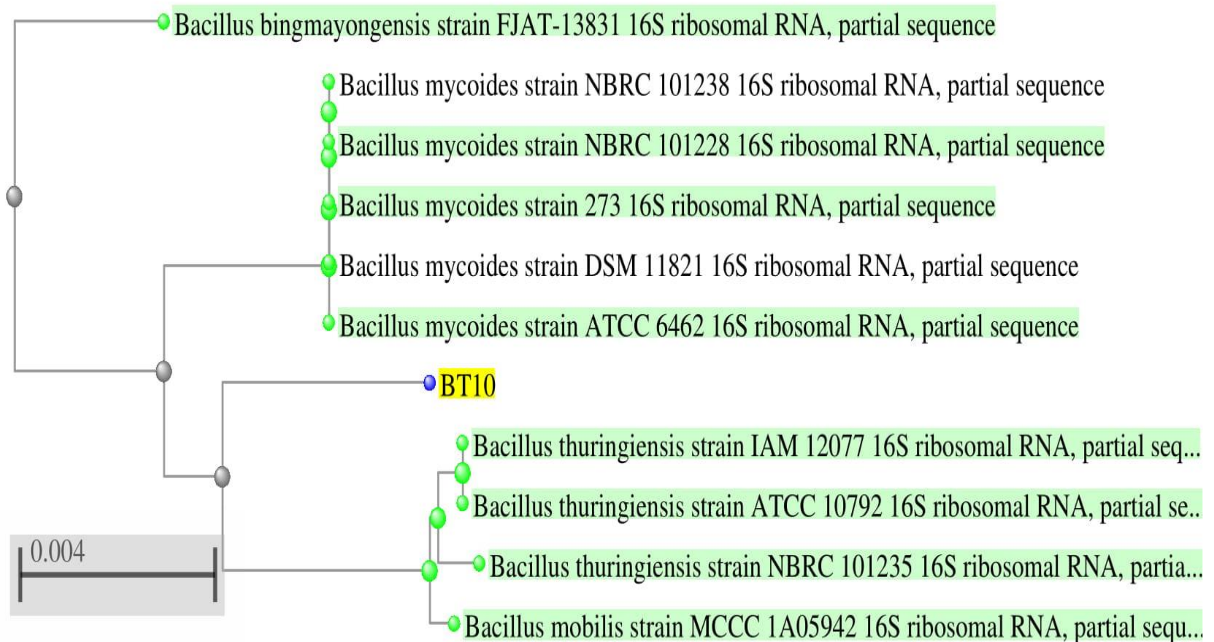

Figure 4.9: Sequencing and phylogenetic analysis of 16S rDNA of Bt-10 strain.

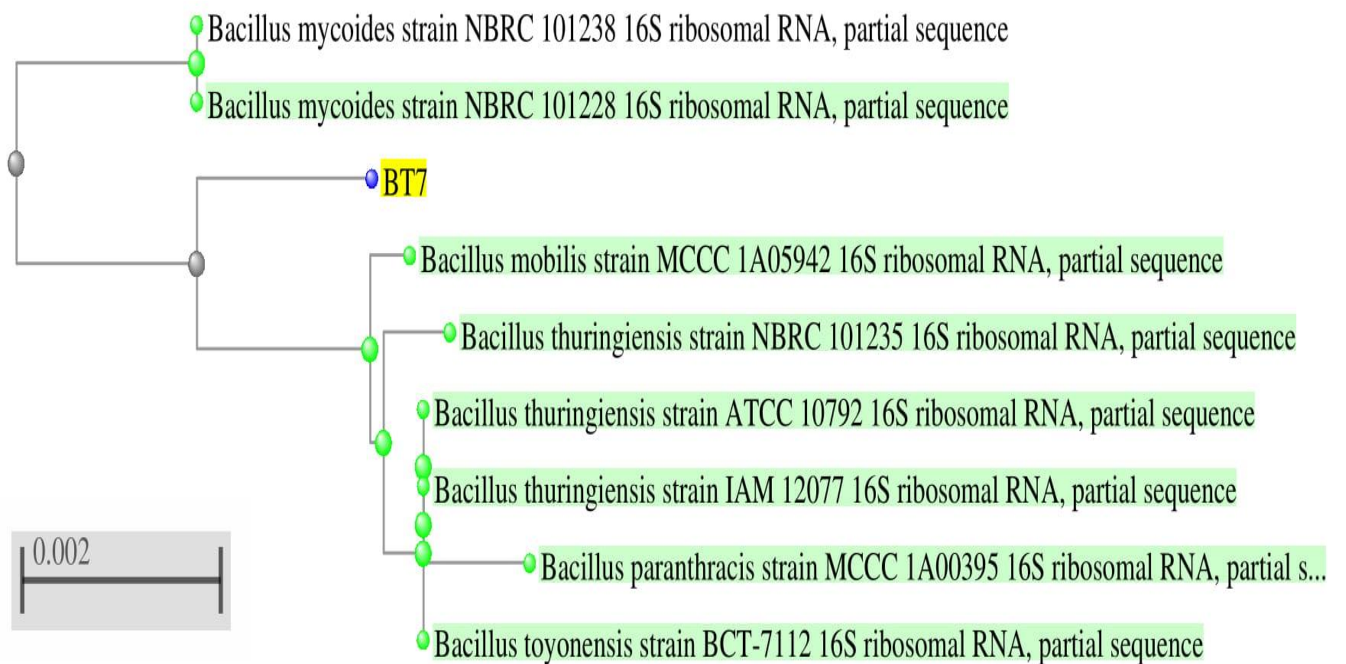

Figure 4.8: Sequencing and phylogenetic analysis of 16S rDNA of Bt-7 strain.

5. SUMMARY AND CONCLUSION

This chapter summarizes the research findings with concluding remarks on "PCR based Characterization of Chitinase gene from *Bacillus thuringiensis*" carried out at the AINP-SBB, MBBT Department, Rajasthan College of Agriculture, MPUAT, Udaipur (Rajasthan).

In the present study, twenty eight strains having chitinase solubilizing activity were used for determination of their potential to hydrolyze chitinase solubilization on CSM plate's supplemented colloidal chitin as substrate and the ten strains showed positive results by producing clear holo zone around the bacterial colonies. The maximum solubilization index was observed in Bt-13 with the SI of (7.79±0.14) closely followed by Bt-2 (7.53±0.30), Bt-7 (6.77±0.16), Bt-10 (7.76±0.20), Bt-11 (3.70±0.27), Bt-19 (4.15±0.17), Bt-20 (6.19±0.21) and Bt-26 (3.67±0.19), Bt-28 (4.29±0.11), whereas the minimum solubilization index was observed in Bt-27 with the SI of (3.45±0.10). Further, these ten isolates were selected further were selected for quantitative and qualitative chitinase enzyme production assay and the maximum Chitinase enzyme activity was observed in the strain Bt-2 (10.19±0.651 U/ml) followed by Bt-27 (6.53±0.309 U/ml), Bt-20 (5.36±0.190 U/ml), Bt-7 (5.24±0.408 U/ml) and Bt-10 (5.20±0.192 U/ml) respectively.

In the present study the biochemical tests were performed to understand the various enzymes produced by these Chtinase producing Bt strains. Out of ten isolates, seven isolates were found positive for oxidase activity, nine isolates were found positive for gelatine liquification, eight isolates were found positive for catalase activity, ten isolate were found positive for Methyl-Red test and seven isolates were found positive for Voges-Proksauer.

PCR based screening of chitinase gene was conducted along with *chitinase*, *endochitinase* and *exochitinase* genes. All these 10 Bt strains showed the presence of *chitinase*, *endochitinase* and *exochitinase* genes by PCR confirmed that these strains are harbouring both endochitinase and exochitinase genes.

Based on zone of hydrolysis and chitinase activities five Bt strains *viz*., Bt-7, Bt-10, Bt-11, Bt-13 and Bt-27 were selected further for molecular characterization based on the amplification and sequencing of 16S rDNA conserved region and the 16S rDNA sequences of these Bt strains revealed their greatest sequence identity with genus

Bacillus. The partial sequence of 16S rRNA gene of isolate Bt-7 showed 100% homology with 16S rRNA sequence of *Bacillus thuringiensis* (Accession number NR_043403.1) already submitted to NCBI database. The partial sequence of 16S rRNA gene 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.

In conclusion, the Bt strains isolated in the present studies addressed that screening & abilities of the local isolates of *Bacillus thuringeisis* strain to produce the chitinase enzymes. The Chitinase activity of these strains i.e. Bt-13 were significantly high and PCR based screening also revealed the presence of both exochitinase and endochtinase genes. The Chitinase activity of these strains might be the presence of novel *chitinase* genes, hence, these genes need to be cloned and expressed in the suitable host for its applications under various processes.

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PCR based characterization of *Chitinase* gene from *Bacillus thuringiensis*

Pavitra Singh* — Research Scholar

Dr. Arunabh Joshi** — Major Advisor

ABSTRACT

Chitinase are prevalent in life and are found in species including archaea, bacteria, fungi, plants, and animals. Chitinase break down chitin, which is the second most abundant carbohydrate in nature after cellulose. Chitinases produced by different microbes have received great attention in recent times due to their wide range of biotechnological applications *i.e.* biocontrol of pathogen and pests etc. In the present study, twenty eight *Bacillus thuringiensis* (Bt) strains were studied for their potential to hydrolyze chitinase on chitin solubilizing medium (CSM) supplemented colloidal chitin as substrate and ten Bt strains showed positive results by producing clear holo zone around the bacterial colonies. The maximum chitinase solubilization index was observed in Bt-13 whereas the minimum solubilization index was observed in Bt-27 strain. The Chitinase enzyme production assay revealed the minimum enzyme activity in Bt-26 (3.78 ± 0.101 U/ml) whereas the maximum activity was observed in the strain Bt-2 (10.19 ± 0.651 U/ml). Biochemical studies were also conducted to characterize the Bt strains on basis of different biochemical test. Further, the PCR based chitinase gene screening also revealed that all these 10 strains were harboring both endochitinase and exochitinase genes. The potent Bt strains *viz.*, Bt-7, Bt-10, Bt-11, Bt-13 and Bt-27 on the basis of Chitinase enzyme production were selected further for molecular characterization and the 16S rDNA sequences of these Bt strains revealed their greatest sequence identity with 16S rRNA sequence of *Bacillus thuringiensis* (Accession number NR_043403.1) already submitted to NCBI database. The Chitinase activity of these strains may be due to the presence of novel *chitinase* genes, hence, these genes need to be cloned and expressed in the suitable host for its biological applications.

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* M.Sc. Scholar, Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Udaipur.

** Assistant Professor, Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Udaipur

काइटिनेस बनाने वाले *बैसिलस थुरिंजेंसिस* में काइटिनेस जीन का पीसीआर आधारित लक्षण वर्णन

पवित्र सिंह *
शोधकर्ता

डॉ अरुणाभ जोशी **
प्रमुख सलाहकार

अनुक्षेपण

काइटिनेस जीवन में प्रचलित हैं और ये आर्किया, बैक्टीरिया, कवक, पौधों और जानवरों सहित प्रजातियों में पाए जाते हैं। काइटिनेस काइटिन को तोड़ता है जो की सेलुलोज के बाद प्रकृति में दूसरा सबसे प्रचुर कार्बोहाइड्रेट है। विभिन्न सूक्ष्मजीवो द्वारा उत्पादित काइटिनेस ने हाल के दिनों में जैव–प्रौद्योगिकीय अनुप्रयोग जेसे की् रोगजनकों और कीटों के बायोकेन्ट्रोल आदि से ध्यान अर्जित किया है। वर्तमान अध्ययन में, अट्ठाईस *बैसिलस थुरिंजेंसिस* (बीटी) उपभेदों का अध्ययन किया गया और अट्ठाईस उपभेदों में से दस बीटी उपभेदों ने काइटिन सोलुबिलाइजिंग माध्यम (सीएसएम) पर स्पष्ट होलो जोन का निर्माण करके सकारात्मक परिणाम दिखाए। बीटी–13 स्ट्रेन में अधिकतम काइटिनेस घुलनशीलता सूचकांक देखा गया जबकि बीटी –27 स्ट्रेन में न्यूनतम घुलनशीलता सूचकांक देखा गया था। काइटिनेस एंजाइम उत्पादन क्षमता ने बीटी –26 स्ट्रेन में न्यूनतम एंजाइम गतिविधि (3.78 ± 0.10) यू/मिलीलीटर को प्रकट किया जबकि अधिकतम गतिविधि बीटी –2 स्ट्रेन (10.19 ± 0.651) यू/मिलीलीटर में देखी गई थी। जैव रासायनिक परीक्षण के आधार पर बीटी उपभेदों को चिह्नित करने के लिए जैव रासायनिक अध्ययन भी किए गए थे। इसके अलावा, पीसीआर आधारित काइटिनेस जीन स्क्रीनिंग से यह भी पता चला है कि ये सभी 10 उपभेद एंडोकाइटिनेस और एक्सोकाइटिनेस जीन दोनों में अपनी प्रतिक्रिया दिखा रहे थे। प्रबल बीटी उपभेदों, बीटी –7, बीटी –10, बीटी –11, बीटी –13 और बीटी –27 काइटिनेस एंजाइम उत्पादन के आधार पर आणविक लक्षण वर्णन के लिए आगे चुने गए और इन बीटी उपभेदों के 16 एस आरडीएनए दृश्यों ने बेसिलस थुरिंजेंसिस के 16 एस आरआरएनए अनुक्रम के साथ अपनी सबसे बड़ी अनुक्रम पहचान का पता लगाया (परिग्रहण संख्या एन.आर.–043403.1) जो की एन सी बी आई डेटाबेस में जमा किया गया है। इन उपभेदों की काइटिनेस गतिविधि नई काइटिनेस जीन की उपस्थिति के कारण हो सकती है, इसलिए, इन जीनों को क्लोन किया जाना चाहिए और इसके जैविक अनुप्रयोगों के लिए उपयुक्त मेजबान में व्यक्त किया जाना चाहिए।

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* स्नातकोतर शोध छात्र, आण्विक जीव विज्ञान एवं जैव प्रौद्योगिकी विभाग, राजस्थान कृषि महाविद्यालय, उदयपुर (राजस्थान)

** सहायक प्राध्यापक, आण्विक जीव विज्ञान एव ंजैव प्रौद्योगिकी विभाग, राजस्थान कृषि महाविद्यालय, उदयपुर (राजस्थान)

ANNEXURE-I

ANNUXURE - I

1. Medium composition of Colloidal Chitin Agar

Ingredients	Grams/Litre
Disodium hydrogen phosphate	6 g
Potassium dihydrogen phosphate	3 g
NH_4Cl(Ammonium Chloride)	1 g
NaCl (Sodium chloride)	0.5 g
Yeast Extract	0.05 g
Colloidal Chitin	5 g
Agar agar	20g
pH	7
Distilled Water	1 liter

**Formula adjusted, standardized to suit performance parameters

All ingredients are mixed, strained and the volume is made 1000 ml and boiled till all ingredients get dissolved.

2. Medium composition of Chitinase detection medium

Ingredients	Grams/Litres
Magnesium sulphate heptahydrate	0.3 g
Ammonium Sulphate	3 g
Potassium Dihydrogen phosphate	2 g
Citric acid monohydrate	1 g
Agar	15 g
Tween-80	0.2ml
Colloidal chitin	4.5g
Bromocresol Purple	0.15g
pH	4.5-4.7
Distilled water	1 litre

All ingredients are mixed in boiled water.

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Enrollment No.	:	2018/RCA/140
Department	:	Molecular Biology and Biotechnology
College	:	Rajasthan College of Agriculture, Udaipur
E-mail	:	singhpavitra832@gmail.com
Name and E-mail ID of the Major Advisor	:	Dr. Arunabh Joshi (arunabhjoshi@gmail.com)
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