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Isolation and cloning of the *Pseudomonas fluorescens* chitinase gene – An ecofriendly approach for its use as a specific biopesticide

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ABSTRACT

Insect pests are one of the major biotic factors limiting the yield of agricultural food crops. In routine agricultural practice, chemical pesticides are used to control the insect pests; however, repeated spraying leads to numerous environmental and health concerns. The search for ecofriendly and sustainable alternatives to chemical pesticides has led to the exploitation of biological control agents. Among these, chitinase plays a target-specific insecticidal activity by degrading the chitin in the insect exoskeleton and gut regions, making it a desirable alternative to chemical pesticides. Therefore, the objective of the study was to isolate and clone the *Pseudomonas fluorescens* chitinase gene for its use as a specific biopesticide. Full-length chitinase gene coming under family 18 Group D glycosyl hydrolases was isolated from *P. fluorescens* genomic DNA using polymerase chain reaction amplification and cloned into an expression vector pET32C+ and transformed into *Escherichia coli*. The sequencing results showed that the chi gene containing 1800 bp long open reading frame encoding 353 amino acids. Deduced amino acid sequence showed that the protein consisted of chitin-binding domain, a catalytic domain, and a fibronectin Type III domain also an amino terminus signal peptide. This study allows the identification of new, target-specific bacterial metabolite as a biopesticide for safe, environment-friendly pest management strategies.

ARTICLE HIGHLIGHTS

The article highlights the isolation and cloning of a chitinase gene from Pseudomonas fluorescens as a potential eco-friendly biopesticide for precise pest control in agriculture.

1. INTRODUCTION

Agriculture is widely recognized as the foundation of the global economic system. However, insect pests pose a significant threat to the yield and quality of numerous food crops. To combat this issue, farmers resort to excessive spraying of chemical pesticides beyond the recommended dosage, which results in pesticide residues in food crops, the emergence of pesticide-resistant insect pests, and environmental contamination [1]. Furthermore, chemical pesticides are not only toxic to insect pests but also toxic to other organisms, namely, beneficial insects, birds, and animals. In addition, a substantial quantity of chemical pesticides is washed away from the intended plants and accumulates in the soil, causing environmental degradation. These chemical residues have been linked to a range of human health risks, from mild headaches to severe and chronic conditions like cancer [2,3]. Negative impact of pesticide residues on human beings can include allergies, disruption of the immune system, hypersensitivity reactions, and damage to the central and peripheral nervous systems [4]. Hence, there is a need to eliminate pesticide contamination from the environment and its negative influence on food crops.

Farmers nowadays are increasingly turning to biopesticides for pest management due to their low environmental impact and lack of health risks. In addition, consumers are demanding pesticide-free food produced through sustainable agricultural practices. Recently, biological control agents are emerging as an important component of integrated pest management practices in many agricultural food crops. Bacterial biocontrol agents are one of the most exploited microbial agents of insect pest management programs. Targeted use of chitinase, a bacterial metabolite, as a specific biopesticide is a promising alternative to whole bacterial application [5,6]. When compared to plants and animals, microbes show high chitinase activity [7].

Chitin is the principle structural component of the insect outer skeleton (50% of the insect cuticle made up of chitin), foregut, hindgut, and midgut lining of peritrophic membrane and involved in structural integrity [8]. When insects consume plant leaves treated with chitinase, which penetrates the alimentary canal and induces substantial

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impairment to the peritrophic membrane by means of chitinolysis, this leads to a consequential incapacity to feed, culminating in the death of the insect [9]. Two kinds of chitinases are exochitinases and endochitinases. Endochitinase cleaves glycosidic bonds in the chitin randomly at internal points within the polymer, releasing low-molecular weight monomers or oligomers of N-acetyl glucosamine (GlcNAc). Henrissat [10] classified chitinases into three families based on amino acid sequences of glycosyl hydrolases: Family-18, 19, and 20. Bacteria, fungi, viruses, animals, and some plant chitinases are coming under family 18. Plant chitinase is mainly coming under family 19. Amino acid sequence of chitinase commonly composed of three functional domains, namely, chitin-binding domain (ChBD), catalytic domain (CATD), and fibronectin Type III-like domain (Fn3D) [11].

The rhizosphere soil is an excellent reservoir for isolating chitin-degrading bacteria that synthesize chitinase enzymes with insecticidal properties. In this regard, chitinolytic bacterial strains were isolated from tea rhizosphere. Among 113 strains, *Pseudomonas fluorescens* MP-13 reported as a high chitinase producer by our research group [12]. Fluorescent pseudomonads reported to produce chitinase as a lytic enzyme for its antagonistic activity against pest. Chitinase enzyme isolated from *P. fluorescens* MP-13 and its insecticidal activity checked against the major tea pest, tea mosquito bug [13]. In the present study, full-length chitinase gene was isolated *P. fluorescens* MP-13 and cloned in *Escherichia coli* expression vector as a preliminary approach for large-scale production of chitinase. Based on the gene sequence analysis, chitinase grouped into Group D glycosyl hydrolases of family 18. Comparative analysis of chitinase D (chiD) amino acid sequence was done with closely related chitinase sequence. Furthermore, studies about amino acid structure, characteristics, and homology of genes encoding chitinase will enhance our understanding of the mechanism of chitin degradation and the roles and relationships of the different enzymes.

While the isolation and cloning of *P. fluorescens* chi gene is a promising approach for the development of a specific biopesticide, there are still gaps in our understanding of overexpression of chitinase in large-scale, optimum conditions for gene expression, molecular mechanisms involved, and the efficacy, specificity of the biopesticide that need to be addressed in future which is crucial for the development and commercialization of biopesticide effective and safe for the environment.

2. MATERIALS AND METHODS

2.1. Bacterial Strains, Plasmids, and Culture Conditions

The strain utilized in this investigation, namely, *P. fluorescens* MP-13, was initially isolated from a soil sample collected at Meppadi, Tamil Nadu, India. *E. coli* DH5α was used in recombinant plasmid as the recipient strain and it was grown in Luria-Bertani medium (Trypton 10.0 g, Yeast extract 5.0 g, NaCl 10.0 g and double distilled H_2O up to 1 L). pET32C+ vector was used for cloning purpose.

2.2. DNA Template Preparation and Chitinase Gene Amplification

A single colony of *P. fluorescens* MP-13 pure culture was inoculated and grown overnight in 100 mL of Luria Bertani (LB) medium, further genomic DNA was isolated by following the method [14]. Isolated genomic DNA was dissolved in 200 µL Tris-EDTA (TE) buffer, further separated, and analyzed on 0.8% agarose/Ethidium Bromide (EtBr) gel.

Polymerase chain reaction (PCR) amplification reaction was carried out in 25 µL master mix containing 2 µL PCR buffer, 1 µL chitinase forward primer Chi FP (5'-ATCGAATTC ATGTCTAAATTCGACTTTACGTTA-3') and reverse primer Chi RP (5'-ATCGCGGCC AATGTCGCACAATCGCTGAAGCCA-3') with *EcoRI* and *NotI* restriction sites (200 nM of each primer), 1 µL DNA (50–100 ng), 0.3 µL Taq DNA polymerase, and 20.7 µL of distilled water. Chi gene was amplified in the PCR program comprised of pre-denaturation at 95°C for 5 min, followed by 35 cycles of amplification (denaturation at 94°C for 60 s; annealing at 55°C for 50 s; extension at 72°C for 3 min), and final extension at 72°C for 5 min. The PCR product was separated on 1.4% of agarose gel and documented in a UV transilluminator.

2.3. Isolation of pET32C+ Plasmid DNA from *E. coli* by Alkaline Lysis Method

Overnight grown culture of *E. coli* (containing pET32C+ plasmid) was centrifuged at 6000 rpm for 10 min and the supernatant was discarded. Bacterial pellet was resuspended in 2.5 mL of ice-cold solution I containing 20 µL/mL DNase-free RNase and resuspended by vortexing. 5 mL of freshly prepared solution II was added to the above suspension mixture. 3.75 mL of ice-cold solution III was added to the above bacterial lysate and tubes were kept on ice for 3–5 min. Precipitate formed in the mixture was centrifuged at 12,000 rpm for 10 min at 4°C and the supernatant was transferred into a fresh tube with equal volumes of Phenol: Chloroform: Iso amyl alcohol (25:24:1). Contents were mixed by vortexing and followed by centrifugation at 8,000 rpm for 2 min at 4°C. Transferred the supernatant into a fresh tube and added two volumes of 100% of ethanol, tubes were stored at −20°C for 10 min. Again centrifuged at 12,000 rpm for 5 min and the obtained DNA pellet was washed with 70% of ethanol to remove the traces of salt. Subsequently, the plasmid DNA was dried before dissolving it in 50 µL of TE buffer (pH 8.0), vortexed the solution gently for a few seconds and stored it at −20°C.

2.4. Restriction Digestion and Cloning of Chitinase Gene in pET32C+ Vector

NotI and *EcoRI* restriction enzymes were used to digest both pET32C+ plasmid DNA and chitinase gene. QIAquick Gel extraction kit (QIAGEN, Bangalore, India) was used to elute the plasmid DNA and PCR product from the agarose block as described in the user's manual. Ligation reaction was carried out in 20 µL of reaction mixture containing 7 µL digested vector, 7 µL Insert (digested chitinase gene), 4 µL 5X ligation Buffer, 1 µL PEG, and 1 µL T4 DNA ligase. The tubes were incubated at 17°C for overnight.

2.5. Transformation and Selection of Recombinants

The resulting ligated product was transformed into the competent cells of *E. coli*. The axenic 500 µL of overnight grown *E. coli* culture was inoculated into 50 mL of antibiotic-free LB broth. Culture was incubated at 37°C for 2–3 h. The flask was incubated in ice for 30 min. The culture was spinned in falcone tube at 7000 rpm for 10 min at 4°C, the pellet was suspended in 12.5 mL of 0.1 M $CaCl_2$ (ice cold) by hand rotation. The flask was kept in ice for 30 min and spinned at 5000 rpm for 10 min at 4°C. Supernatant was discarded and the pellet was suspended in 25 mL of 0.1 M $CaCl_2$ (ice cold) and incubated in ice for 30 min. The above mixture was centrifuged at 5000 rpm for 10 min at 4°C. Supernatant was discarded and the pellet was suspended in 1 mL of 85 mM $CaCl_2$ with 15 % glycerol. Immediately, 3 µL of ligated product was mixed with 200 µL of competent cells and incubated on ice for 30 min followed by heat treatment at 42°C for 90 s. Transformed cells were incubated for 2 h at 37°C in the shaker. Culture

pellet was dissolved in 100 µL LB broth and plated onto LB agar plates containing amp 100 mg/mL, and incubated overnight at 37°C. The recombinant colonies were identified by blue/white screening method.

Transformed recombinant white colonies containing chitinase insert in pET32C+ plasmid were selected from the LB-amp plate and inoculated in 100 mL of LB broth containing amp (100 mg/mL) and incubated overnight at 37°C at 250 rpm. Plasmid DNA was isolated from recombinant *E. coli* cells and used for PCR confirmation of chitinase gene using chitinase forward and reverse primer.

2.6. Cloning, Sequencing, and Domain Analysis of Chitinase

Chitinase gene was ligated with T/A cloning vector according to the manufacturer's manual and transformed into *E. coli* DH5α strain as per manufacturer's protocol. The plasmids were extracted and sequenced (Macrogen Inc. Seoul, Korea). *In silico* translation of the chi gene was determined using coding sequences feature and pair-wise alignment in Basic Local Alignment Search Tool (BLAST). For homology search, multiple sequence alignment was carried out using Multialin (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl.page=npsa_multialin.html). SignalP (Version 4.1) was used to predict the putative signal peptide sequence. Conserved domains of the amino acid sequence were analyzed by conserved domain architecture retrieval tool.

3. RESULTS

3.1. Isolation and Cloning of Full-Length Chitinase Gene

Gene-specific chitinase primers with specific restriction sites (*NotI* and *EcoRI*) were designed and used in PCR to amplify the chitinase gene. The annealing temperature for chitinase gene amplification was optimized using different annealing temperatures between 48°C and 56°C and finally, the gene was successfully amplified at 55°C. Amplified PCR product 1.8 kb was noticed in Agarose/EtBr gel [Figure 1]. In agarose gel, no extra bands were detected suggesting that designed chi primers were specifically amplified the chitinase gene fragment. PCR product of full-length chitinase gene was sequenced and submitted to NCBI GenBank Ac. No. KM249884. pET32C+ plasmid DNA was isolated successfully from *E. coli* strain [Figure 2].

Isolated pET32C+ plasmid and chitinase gene were digested with *EcoRI* and *NotI* restriction enzymes [Figure 3]. The double-digested PCR product was ligated into the pre-digested plasmid vector (pET32C+) at 5'-*EcoRI* and 3'-*NotI* sites. The ligated product was transformed into *E. coli* and recombinant white colonies were observed in LB-amp plates. Plasmid DNA was isolated from the transformed colonies of *E. coli* and used for PCR confirmation using chitinase gene-specific forward and reverse primer. Analysis of the PCR product (~1.8 kb) on Agarose/EtBr gel revealed the presence of the chitinase gene in the recombinant cells [Figure 4].

3.2. *In Silico* Analysis of Chitinase Gene

Homolog search using the BLAST program revealed that the cloned product was the endochitinase gene. Further analysis of the chitinase gene sequence revealed a 1212 bp open reading frame (ORF) encoding 354 amino acids [Figure 5]. BLAST search revealed a high sequence homology of 97% similarity with putative chitinase from *P. fluorescens* (ADN84073.1), 94% similarity with glycosyl hydrolase family chitinase from *Pseudomonas mandelii* JR-1 (AHZ68227.1), 90% similarity with chitinase from *Pseudomonas* spp. BRG-100 (KFF46732.1), 89% similarity with chitinase from *P. fluorescens* (WP014719207.1), 89% similarity with chitinase from *Pseudomonas synxantha* (WP005789852.1), 88% similarity with chitinase from *Pseudomonas* spp. GM-50 (WP008008538.1), 88% similarity with chitinase from *P. fluorescens* FH5 (ETK38535.1), 86% similarity with chitinase from *Pseudomonas mosselii* (WP028689291.1), chitinase from *P. chlororaphis* (WP025808710.1), 86% similarity with chitinase from *Pseudomonas entomophila* (WP011533766.1), 84% similarity with chitinase from *Pseudomonas protegens* (WP015634913.1), and 78% similarity with chitinase from *Pseudomonas aeruginosa* (WP024918163.1).

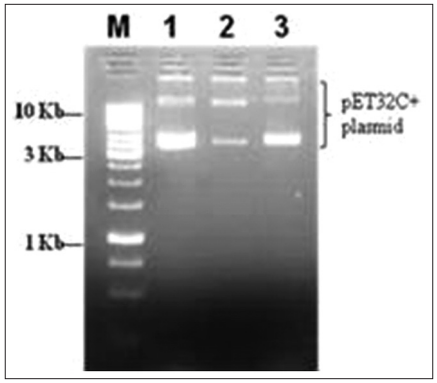

Figure 2: Isolation of plasmid (pET32C+) from *Escherichia coli*. Lane M = 1 kb DNA ladder; Lane 1-3 plasmid DNA.

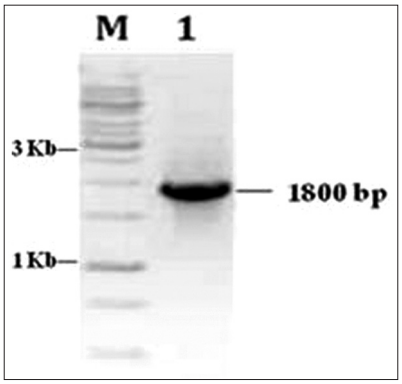

Figure 1: Polymerase chain reaction amplification of full-length chitinase gene. Lane M = 1 kb DNA ladder; Lane 1 - Chitinase gene amplification from *Pseudomonas fluorescens* MP-13.

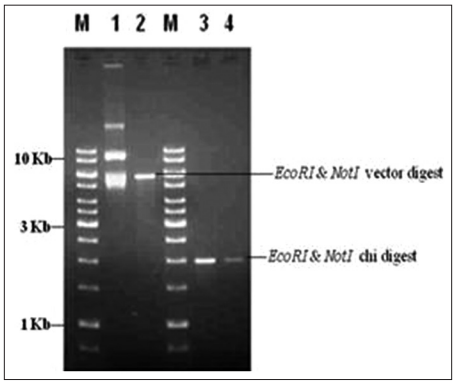

Figure 3: Restriction digestion of pET32C+ vector and chitinase gene. Lane M =1 kb DNA ladder; Lane 1 – Control plasmid, Lane 2 – Digested plasmid, Lane 3 – Control chitinase gene, Lane 4 – Digested chitinase gene.

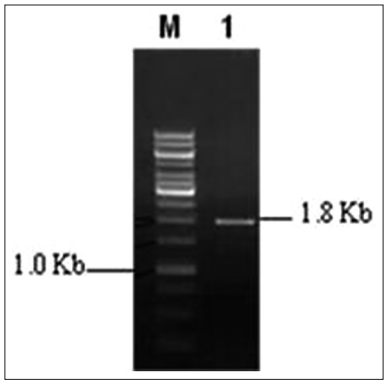

Figure 4: Polymerase chain reaction confirmation of chitinase gene from transformed calories of *Escherichia coli.* Lane M =1 kb DNA ladder; Lane 1 – chi gene from recombinant plasmid.

Figure 5: The amino acid sequence of full-length chitinase gene. The sequences that are underlined are the signal peptide sequence. Baxed residues (solid) indicate the catalytic domain, baxed residues (dotted) indicate the chitin-binding domain and dotted underlined residues indicate the Fn3 modules.

The multiple sequence alignment of the chitinase protein sequence obtained from the present study and its homologus revealed the presence of conserved sequences [Figure 6]. Chitinase amino acid sequence showed a maximum similarity among *Pseudomonas* chitinases and the regions of YGFDGLDIDLEQSAIDFA which was highly conserved among different species of *Pseudomonas* chitinase. The conserved region of glycine [Gly (G): 134,137] and serine [Ser (S) 145] in the box region are known to be hydrophobic and hydrophilic amino acids respectively, during hydrolysis reaction, these Gly and Ser amino acids are taking the role of reacting with the surface of chitin molecules [Figure 6]. The Box was also dominated by other amino acid residues, namely, glutamic acid (E) and aspartic acid (D) which gives the nature of acidic and negatively charged [Figure 5].

3.3. Functional Domains of Chitinase

Prediction results of functional domains of chitinase revealed that the protein belongs to family 18 of glycosyl-hydrolases chiD. The enzyme was composed of three domains, a family 18 chitinase CATD and a ChBD separated by Fn3-like module. Fn3-like module has played a role to maintain the orientation and optimal distance between ChBD and CATD. Protein signal sequence was successfully predicted by SignalP (Version 4.1) [Figure 5].

Using the CLUSTAL W program, amino acid sequence of all three domains was compared with the respective domains of bacterial chitinases. CATD of ChiD showed high sequence similarity to the family 18 catalytic regions of different bacterial chitinases [Figure 7]. CATD and ChBD amino acid sequences of the different chitinases are well conserved, whereas only a considerable variation in sequence length was observed by comparing the Fn3D of chitinase with the same domain of other bacterial chitinases [Figure 8]. The region between the CATD and a ChBD of ChiD was observed to exhibit sequence similarity to the Fn3D. Similarly, Fn3D displayed

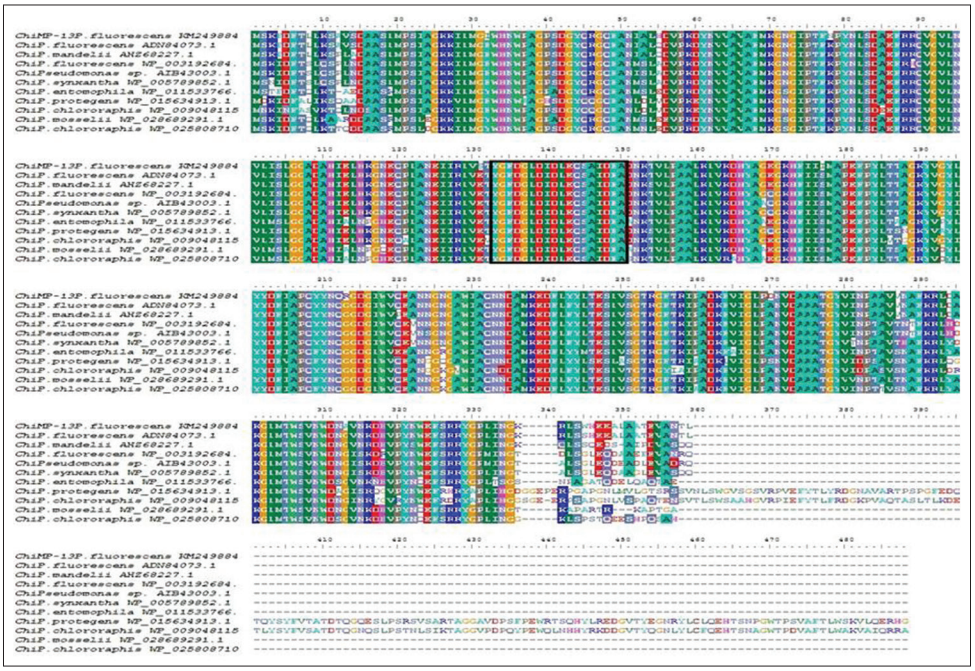

Figure 6: Multiple alignments of chitinase amino acid sequence from *Pseudomonas fluorescens* with chitnase from *Pseudomonas* species. The baxed regions indicate the location of the conserved domain in the protein.

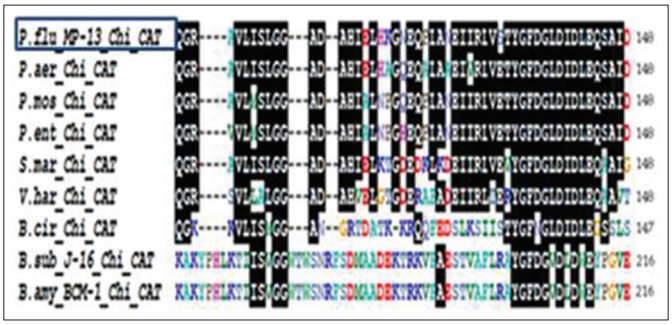

Figure 7: Comparison of chitinase catalytic domain with other bacterial chitnase. The putative catalytic domain of *Pseudomonas fluorescens* MP-13 was compared with other bacterial chitinases. The alignment was obtained using the CLUSTAL W program.

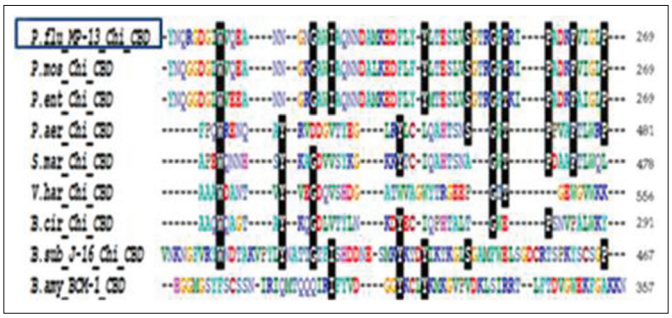

Figure 8: Comparison of chitinase chitin binding domain with other bacterial chitnase. The putative catalytic domain of *Pseudomonas fluorescens* MP-13 was compared with other bacterial chitinases. The alignment was obtained using the CLUSTAL W program.

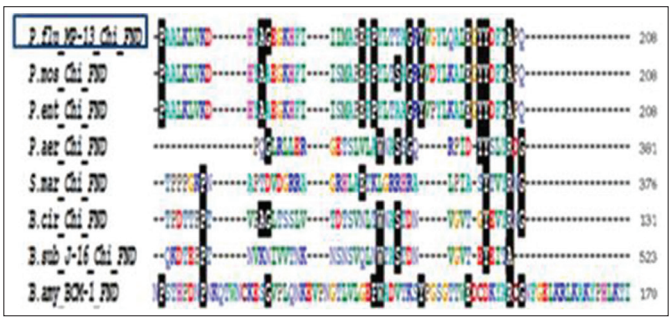

Figure 9: Comparison of chitinase fibronectin Type III domain with other bacterial chitnase. The putative catalytic domain of *Pseudomonas fluorescens* MP-13 was compared with other bacterial chitinases. The alignment was obtained using the CLUSTAL W program.

less sequence similarity to Fn3D of other bacterial chitinases as shown in [Figure 9].

4. DISCUSSION

Public concern over harmful health and environmental impacts of noxious chemical pesticide residues in food crops has been raised. Wanwimolruk *et al*. [15] conducted a study that demonstrated pesticide contamination in common fruits and vegetables, with some samples showing residues above maximum residue limits. Post-harvest, these food products contain one or more pesticide residues, with some, such as grapes and tea, containing up to nine residues, and citrus fruits, such as lemon, orange, peaches, and pears containing up to five to eight residues. Cucumbers, pomegranates, tomatoes, plums, and strawberries contain up to three to five residues [16]. Biocontrol agents are considered as a safe and environment-friendly alternative to chemical pesticides [17]. In recent years, chitinase enzyme from microorganism has attracted substantial attention since it takes part in the defense against chitin-containing pests and pathogens [9]. Isolation and diversity of chitinolytic bacteria, namely, *Bacillus, Pseudomonas* are frequently found in rhizosphere soil [18]. Our research group previously reported the molecular characterization and diversity characterization of different chitinolytic bacteria using 16S rRNA sequencing technology [12]. Among 113 bacterial strains, *P. fluorescens* MP-13 was identified as a high chitinase-producing bacterial strain [19] and chitinase enzyme (~30 kDa) was characterized. Compared to the *Bacillus cereus* chitinase, *P. fluorecsens* chitinase revealed 100% of insecticidal activity against tea mosquito bug under *in vitro* condition [6]. In recent years, instead of using whole micro-organism, microbial metabolites like chitinase act as an efficient biopesticide against agricultural pests and pathogens [20]. Furthermore, researchers are interested in searching the metabolite or enzyme competent of degrading the chitin-containing insect cuticles and peritrophic membranes of insect pests, in which chitinase is the most intensively studied [21].

The present study describes the successful isolation and cloning of the full-length chitinase gene from *P. fluorescens* MP-13. To achieve this, gene-specific chitinase primers were designed that contained specific restriction sites (*NotI* and *EcoRI*) to allow for the subsequent ligation of the PCR product into the pET32C+ plasmid vector. The annealing temperature for chitinase gene amplification was optimized, and the gene was successfully amplified at 55°C. The amplified PCR product was found to be 1.8 kb, and no extra bands were detected in the agarose gel, indicating that the designed chi primers specifically amplified the chitinase gene fragment. Further confirmation of successful cloning was obtained by digesting the isolated pET32C+ plasmid and chitinase gene with *EcoRI* and *NotI* restriction enzymes. The double-digested PCR product was then ligated into the pre-digested plasmid vector at the 5'-*EcoRI* and 3'-*NotI* sites. This ligated product was transformed into *Escherichia coli* and recombinant white colonies were observed in LB-amp plates. Plasmid DNA was isolated from the transformed colonies and used for PCR confirmation using chitinase gene primers. Analysis of the PCR product on Agarose/EtBr gel revealed the presence of the chitinase gene in the recombinant cells. Similarly, in another study, chitinase was successfully cloned and characterized from *Paenibacillus chitinolyticus* strain UMBR 0002. The gene was amplified using PCR with gene-specific primers, and the resulting product was cloned into a pET-28a expression vector. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells for expression [22].

Cloning of the chitinase gene in the model system has been one of the most interesting areas of chitinase applications. In this study, we successfully isolated and cloned the full-length chitinase gene from *P. fluorescens* MP-13, which has been shown to have strong chitinolytic activity. The full-length chitinase gene was identified and characterized by sequencing and analysis, revealing high similarity to previously reported chitinase genes. Our findings suggest that the cloned chitinase gene could be used as a promising candidate for the development of chitinase-based biopesticides, which have been shown to be effective in controlling a variety of insect pests. However, more research is needed to fully understand the molecular mechanisms involved in chitinase expression and to optimize its expression in large-scale bioprocessing. In this study, chitinase gene with 1800 nucleotides of ORF was isolated from the

chromosomal DNA of *P. fluorescens* MP-13. In another report, chitinase gene (ORF 2067 nucleotides) was isolated from *Bacillus thuringiensis* chromosomal DNA [23]. The previous studies have investigated the PCR amplification and cloning of the chitinase gene from *Beauveria bassiana* [24], as well as the isolation and characterization of the gene encoding the extracellular chitinolytic enzyme from the marine psychrophilic bacterium *Moritella marina* [25]. Under laboratory and field conditions, chitinase combination with *B. thuringiensis* subspecies galleriae (Bt) and *Mamestra brassicae* nucleopolyhedrovirus was successful against *M. brassicae* larvae and also provided the concurrent management of all other *Lepidopteran* pests attach in cabbage leaves [5].

In silico analysis was carried out to compare and contrast the amino acid sequence of chitinase with other bacterial chitinase and to find out the different domain structures in it behind the concept to understand the function of chitinase. Signal peptide site in chitinase amino acid sequence predicted that chitinase is secreted as a toxic enzyme from *P. flourescens* MP-13 as a part of its insect pathogenicity [Figure 6]. Like the arrangement of domains in *Bacillus circulans* chiA, in this study, chitinase has its CATD in the N-terminal portion and a ChBD in the C-terminal portion. Watanabe *et al*., [26] reported that chiA1 gene was isolated from *B. cereus*, a ChBD is essential for binding specifically to chitin and hydrolyzation. The glycine [Gly (G): 134,137] and serine [Ser (S) 145] in CATD play an key role for reacting with chitin molecules. In ChBD, conserved tryptophan (W) and tyrosine (Y) play an important role in binding with GlcNAc residues and thereby enhance chitin degradation [11,27]. Apart from chitinase, Fn3D was also found in other bacterial enzymes such as amylases and cellulases [28]. In Fn3D, fibronectin plays an important role in cell adhesion as a multi-functional extracellular matrix protein [29,30].

To enhance the pathogenic and herbicide resistance in wheat cultivars, chitinase gene was transferred into immature embryo-derived callus through biolistics-mediated transformation [31]. Wang *et al*., [32] characterized chitinase from *Pseudomonas* sp. TKU008 and reported the resemblance of the same with chitinase from *B. cereus* (gi 45827175). Chitinolytic extracellular enzyme from *P. fluorescens* was isolated and its pathogenicity against insect larvae of *Culex quinquefasciatus* was studied and found that extracellular insecticidal proteins involved in chitin degradation in insect cuticular and gut regions and leads to insect death [33]. Similarly, in our previous study, chitinase from *P. flourescens* MP-13 was showed 100% insect mortality against *Helopeltis theivora* populations under *in vitro* condition [6].

5. CONCLUSION

The isolation and cloning of the *P. fluorescens* chitinase gene represent a significant step towards developing an ecofriendly and specific biopesticide for controlling insect pests in agriculture. The potent insecticidal activity exhibited by the chitinase enzyme is due to its ability to degrade the chitin present in the insect cuticle, ultimately resulting in insect death. The use of chitinase as a biopesticide has several advantages over conventional chemical insecticides, including its specificity, biodegradability, and lower toxicity to non-target organisms. Significant progress has been made toward the goal of expressing large quantities of chitinase from *E. coli* for large-scale field trials through the successful isolation and cloning of the chitinase gene from *P. fluorescence* MP-13 in the *E. coli* pET32C+ expression vector. This approach has the potential to reduce the environmental impact of conventional chemical insecticides while also improving the sustainability and economic viability of agricultural production.

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. FUNDING

There is no funding to report.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

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