Cloning and expression of a chimeric protease and chitinase fusion protein from *Metarhizium anisopliae* into *Escherichia coli* for enhanced enzyme activity

A thesis submitted for the award of the degree of

Doctor of Philosophy

Under the Supervision of

Prof. Gurvinder Kaur Saini

By

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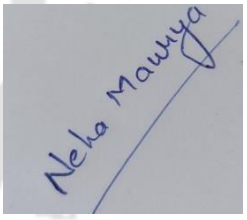
DECLARATION

I, Neha Maurya hereby declare that this thesis, entitled **"Cloning and expression of a chimeric protease and chitinase fusion protein from *Metarhizium anisopliae* into *Escherichia coli* for enhanced enzyme activity"**, submitted for the degree of philosophy is my original work conducted under the supervision of Dr. Gurvinder Kaur Saini at the Fungal Biotechnology research laboratory in the Department of Biosciences and Bioengineering, IIT Guwahati. I would also like to state that no part of the research has been submitted for a degree or examination at any university. References, assistance, and material obtained from other sources have all been properly acknowledged.



Place: IIT Guwahati
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CERTIFICATE

This is to certify that the work incorporated in this thesis "Cloning and expression of a chimeric protease and chitinase fusion protein from *Metarhizium anisopliae* into *Escherichia coli* for enhanced enzyme activity" submitted by Neha Maurya was carried out under my supervision. No part of the thesis has been submitted for a degree or examination at any university. References, help, and material obtained from other sources have been duly acknowledged.

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Neha Maurya
October 2023

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LIST OF ABBREVIATIONS

EMP	Entomopathogenic
Mcl1	Collagen-like protein of *Metarhizium anisopliae*
Pr1a	Protease enzyme of *Metarhizium anisopliae*
Chit1	Chitinase-1 gene of *Metarhizium anisopliae*
Chi2	Chitinase-2 gene of *Metarhizium anisopliae*
Chit3	Chitinase-3 gene of *Metarhizium anisopliae*
Chi4	Chitinase-4 gene of *Metarhizium anisopliae*
EST	Expressed sequence tag
GH-18	Glycosyl hydrolase-18 family
RDT	Recombinant DNA technology
CBM	Carbohydrate binding module
GlcNAc	N-acetyl-D-glucosamine
ChBD	Chitin-binding domain
SpChiD	*Serratia proteamaculans* chitinase
CDEP	*Beauveria bassiana* protease
PCR	Polymerase chain reaction
CBB	Coomassie brilliant blue
EDTA	Ethylenediamine tetra acetate
SDS	Sodium dodecyl sulfate
DNSA	3, 5 – Dinitrosalicyclic acid
SDB	Sabouraud dextrose broth
PIM	Protease induction media
HCl	Hydrochloric acid
TAE buffer	Tris-acetate-EDTA buffer

EtBr	Ethidium bromide
Bmcbd	*Bombyx mori* chitin-binding domain
Pr1abm	Pr1a protease gene of *Metarhizium anisopliae* with Bmcbd overhang at 3’ end
Bmpr1a	Bmcbd with *Metarhizium anisopliae* Pr1a overhang at 5’ end
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
NTA	Nitriloaceticacid, chelating agent
PAGE	Polyacrylamide gel electrophoresis
cPr1a	Chimeric protease gene of *Metarhizium anisopliae* with chitin-binding domain of *Bombyx mori*
cChi2	Chimeric chitinase gene of *Metarhizium anisopliae* with chitin-bindingdomain of *Bombyx mori*
TEMED	Tetramethylenediamine
O.D.	Optical density
BSA	Bovine serum albumin
Chi2bm	Chitinase -2 gene of *Metarhizium anisopliae* with Bmcbd overhang at 3’ end
Bmchi2	*Bombyx mori* chitin-binding domain with Pr1a overhang at 5’ end
f-PC (f-cPr1a-cChi2)	Fused cPr1a- cChi2 chimeric gene


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SYNOPSIS

Synopsis

The world's population has quadrupled past century. By 2050 we may have reached a population of 9.7 billion people and by then the global food demand is anticipated to rise anywhere between 59% to 98%. This much food demand can be met only if we either increase the crop yield by various means of advanced agriculture technologies or by reducing the crop loss due to pests. However various kinds of chemical pesticides are there to counteract the insect pests but they have innumerable disadvantages in comparison to benefits, like DNA mutagenesis, human mortalities due to acute poisoning, degradation of water quality, and serious environmental issues (1). The evolution of resistance to pesticides in various pest organisms is another serious concern (2). Alternatively, microbial pesticides such as viruses, bacteria, and fungi are being used as a lucrative substitute for chemical pesticides to control insect pests. EPF is highly biodegradable and comes with low chances of resistance development and with no possibility of health risks (3).

Metarhizium anisopliae kills the target host by mycosis and provides a feasible system for pest control, as they are an environment-friendly substitute for chemical pesticides, exist ubiquitously, are active against a wide range of insect pests, are harmless and safe to non-target organisms, and the most importantly, they need not be ingested by the host to cause the disease, unlike entomopathogenic bacteria and virus and nematodes. The United States Environmental Protection Agency (US-EPA) has certified *Metarhizium anisopliae* for the management of insect pests. Despite having many advantages, the slow killing of insect pests is a huge downside of it, thence it is not able to stop the crop loss entirely even after the treatment. The incompetency of *Metarhizium anisopliae* due to its slow killing speed makes it futile compared to chemical pesticides (4,5,6 &7). The efficacy of the fungus can be improved by targeting the various kinds of hydrolytic enzymes which plays a pivotal role in the penetration of the insect's cuticle.

The hydrolytic enzymes are those crucial factors that decide the feasibility of fungal infection and are crucial for the penetration of an insect's cuticle. The cuticle of an insect is made

up of an upper layer epicuticle which is composed of lipids and protein and another layer procuticle, which subsides below the epicuticle and consists of chitin. The most crucial step of the fungal infection commences by adhesion of spore with epicuticle of insect integument. Germination of spore after attachment to the cuticle forms germ tube, which leads to the formation of appressoria. The appressoria produce a penetration peg across the epicuticle with the help of turgor pressure and various hydrolytic enzymes.

Metarhizium secrets numerous hydrolytic enzymes viz. lipases, proteases, and chitinase in a sequential manner along with many secondary metabolites (destruxins a, b, c, d, e, and ferricrocin) and proteins during the infection process.

Proteases (Pr1a, Pr1b, Pr1c, Pr1d to Pr1k) are secreted by the fungus during the initial stage of infection after lipases, and out of all the known proteases, Pr1a is one of the most highly expressed subtilisin protease during the breaching of insect cuticles and involved in fungal virulence (4). Studies by St. leger et al have revealed that an engineered strain that constitutively expressed a protease gene (Pr1a) killed caterpillars (*Manduca sexta*) 25% faster than the wild-type strain (8).

Chitinase (Chit1, Chi2, Chit3) is secreted after proteases. The Chi2 gene is a virulence determinant gene of the entomopathogenic fungus *Metarhizium anisopliae*. Chitinase of *Beauveria bassiana* (Bbchit1) when overexpressed under the constitutive promoter of *Aspergillus nidulans*, resulted in enhanced virulence of *Beauveria bassiana* against aphids, transformants show 50% reduction in lethal concentration and 50% lethal time compared to wild type (9). In vitro studies showed that proteases and chitinases secreted by insect pathogenic fungus act synergistically to degrade the cuticle (10). The synergistic action of chitinase and protease were reconfirmed by the studies of which proved that the expression of the fusion protein with chitinase and protease activities results in significantly faster penetration of cuticle than wild-type or transformants overexpressing either chitinase or protease and increases the

virulence of the insect pathogen *Beauveria bassiana* (11).

Rational design approach was employed by the scientists successfully to improve the virulence of EPF. Engineered hybrid chitinase (Bbchit1-BmChBD) and hybrid protease (CDEP: BmChBD) of *Beauveria bassiana* are set of examples of rational design which have increased the pathogenicity of the fungus markedly (12 &13).

It is hypothesized in this study that the fusion of *Bombyx mori* chitin-binding domain (Bmcbd) with protease (Pr1a) and its swapping with the chitin-binding domain of chitinase (Chi2) of *Metarhizium anisopliae* would result in an increase in the enzyme activities of cChi2 and cPr1a chimeric enzymes and this enhanced enzyme activity would result in the augmentation of the insect pest's cuticle digesting potential. An amplified activity of fusion protein (f-cPr1a-cChi2) would take place as a consequence of the synergistic action of cChi2 and cPr1a.

Brief Discussion of the Research Work

Chapter-1: Introduction, Review of Literature, and Objectives

This chapter explains the significance of enzyme secreted by *Metarhizium anisopliae*. Based on the established literature, the enzymes secreted by the fungus are explored in detail. The role of various cuticle-degrading enzymes produced by the *Metarhizium* was discussed. Lipases, proteases, and chitinases enzymes are secreted by the fungus in a sequential manner to degraded lipids and proteins present in the epicuticle and chitin in the procuticle. Analysis of virulence-determining genes was also explored; for instance, three variants (Chit1, Chi2, and Chit3) of chitinase are secreted during the infection phase, but only Chi2 is known to contribute to the virulence of emp fungi, *Metarhizium anisopliae*. Similarly, out of 11 protease variants (Pr1a to Pr1k), Pr1a protease is secreted in larger quantities during infection phase and majorly involved in breaching of insect cuticle. The application of chitinase and protease enzymes in the biopesticide industry is explored thoroughly. Eventually, the approaches of fusion of the chitin-binding domain of *Bombyx mori* to the protease (Cdep-1) and chitinase (Bbchit-1) gene of *Beauveria bassiana* for increasing cuticle digesting capability and associated increased virulence of the fungi were discussed. The concept of construction of chimeric chitinase and chimeric protease through the fusion of the *Bombyx mori* chitin-binding domain to the (Pr1a) protease of entomopathogenic fungi *Metarhizium ansiopliae* and the swapping of a chitin-binding domain of Chi2 chitinase of *M. anisopliae* has been analyzed, which confirms the increased catalytic activity of theproteins, all of which are pertinent to the proposed work's stated objectives.

The advantages of chimeric protease and chimeric chitinase enzymes go beyond pest control, extending into waste management applications. The creation of these enzymes with improved binding affinity and increased catalytic efficiency shows significant potential in various sectors, emphasizing their versatile capabilities beyond just agricultural pest control. Chimeric chitinases and proteases can be valuable in the seafood industry for managing shrimp

shell waste. Traditional chemical hydrolysis is costly and environmentally harmful, whereas these engineered enzymes offer an eco-friendly alternative. With enhanced binding affinity and catalytic efficiency, chimeric enzymes can more rapidly and effectively degrade shrimp shell waste compared to wild-type enzymes.

Objectives

The objective of this study was to clone and express the cPr1a, cChi2, and f-cPr1a-cChi2 proteins in an *E. coli* expression system and to evaluate the enzyme activities or cuticle digesting effect of wild-type and chimeric, purified recombinant proteins using the *Philosamia ricini's* cuticle as the substrate.

1. Cloning and expression of wild-type and chimeric protease from *Metarhizium anisopliae* into *E. coli* and to evaluate the binding capacities and enzyme activities of wild-type and chimeric protease by binding assays and protease enzyme assays.
2. Cloning and expression of wild-type and chimeric chitinase from *Metarhizium anisopliae* into *E. coli* and to check the binding efficiency and enzyme activities of wild-type and chimeric chitinase by binding assays and chitinase enzyme assays.
3. Construction of fusion protein by merging the chimeric protease and chimeric chitinase and checking the enzyme activity of fusion protein by enzyme assays.

Chapter-2: Cloning and expression of wild-type protease Pr1a (M73795.1) and chimeric protease cPr1a.

The chapter aims to analyze the enzyme activity of recombinant purified wild-type and chimeric protease against (Eri silkworm) *Philosamia ricini* insect cuticle. Cloning, expression, and purificationof the wild-type Pr1a and cPr1a chimeric proteins were done, and subsequently, binding and enzyme assays were done to assess any improvement in the binding efficiency and enzyme activity of the chimeric cPr1a protease with respect to wild-type Pr1a protease.

Results:

2.1 Cloning, expression, and purification of wild-type protease Pr1a and chimeric protease cPr1a.

A cDNA was prepared from the RNA isolated from the mycelia harvested from PIM (protease induction media), and cDNA was used for the amplification of Pr1a using gene-specific primers **(Fig. 1)**. The Pr1a gene and Bmcbd (*Bombyx mori* chitin-binding domain) were re-amplified using a new set of primers in order to introduce the compatible overhangs to give Pr1abm (Pr1a gene with Bmcbd overhang at 3' end) and Bmpr1a (Bmcbd with Pr1a overhang at 5' end) and fused using the Gibson assembly method **(Fig. 2)**. Pr1a gene and cPr1a were successfully cloned into the pET-28a vector using the Gibson assembly method, resulting in the pET-28a-Pr1a and pET-28a-cPr1a constructs.

A) The pET-28a-Pr1a and pET-28a-cPr1a constructs were transformed into *E. coli* (BL21 DE3) competent cells. The expression of the Pr1a and cPr1a proteins was done in LB media supplemented with kanamycin (50 μg/ml) with 1.0 mM IPTG.

B) Protein purification was done using Ni-NTA affinity chromatography **(Fig. 3 & Fig. 4)**.

C) Binding assays were done in 50 mM potassium phosphate buffer for 4 mins with 100 μg of purified recombinant Pr1a and cPr1a enzymes, and the results revealed a significant

increase in the binding efficiency of chimeric protease (cPr1a) compared to wild-type protease (Pr1a) **(Fig. 5)**.

D) Enzyme assays were done in 50 mM potassium phosphate buffer (pH-6.0) with purified Pr1a and cPr1a enzymes. The chimeric protease cPr1a enzyme showed a significant increase in enzyme activity in comparison to the wild-type protease Pr1a enzyme **(Fig. 6)**.

E) The 3-D structure of Pr1a and cPr1a proteins were modeled using Phyre2 software**(Fig. 7 and Fig. 8)**.

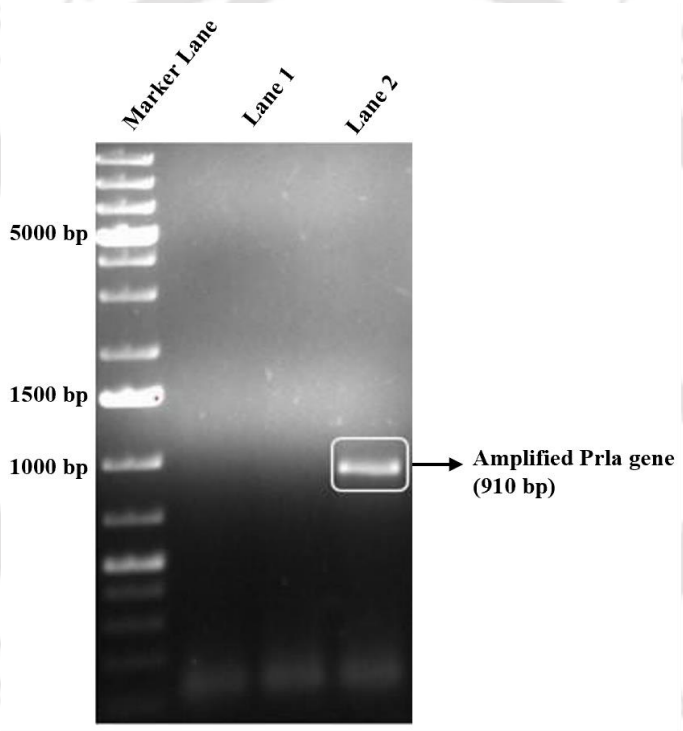

Fig. 1– Agarose gel electrophoresis analysis of Pr1a gene (910 bp) amplified from the cDNA of *Metarhizium anisopliae* using polymerase chain reaction with gene-specific primers at annealing temperatures, 64°C (Lane 1), 66°C (Lane 2), 68°C (Lane 3).
Total RNA was isolated from the mycelia harvested from PIM (Protease induction media) & reverse transcribed into cDNA.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific, 75 bp to 20 kbp).

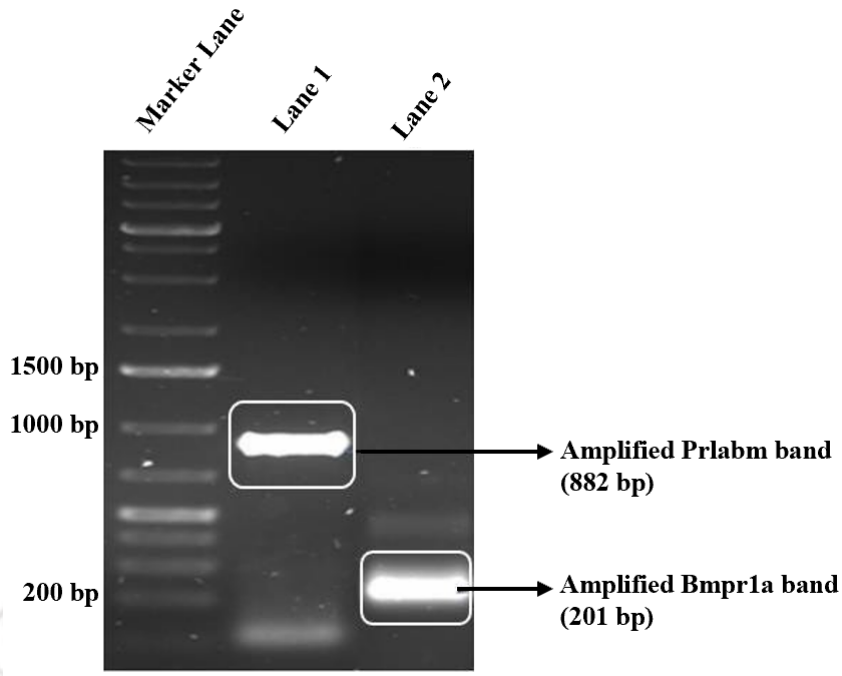

Fig. 2 - - Agarose gel electrophoresis analysis showing PCR amplified fragments of Pr1abm (Pr1a gene with *Bombyx mori* chitin-binding domain overhang at 3' end and Bmpr1a (*Bombyx mori* chitin-binding domain with Pr1a overhang at 5' end.
1% agarose gel was used to resolve the PCR products
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).

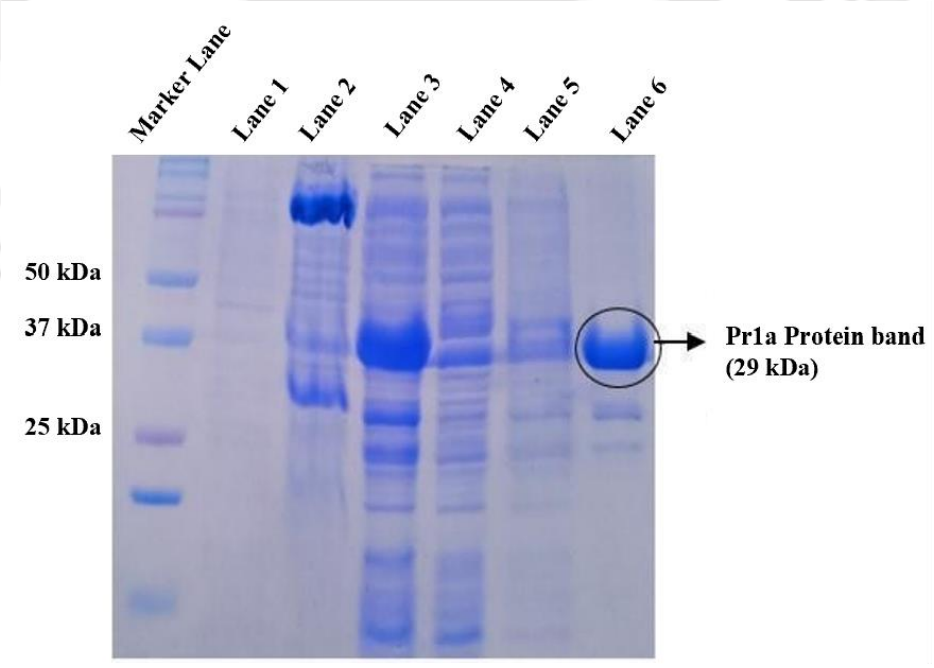

Fig. 3 - Purification profile of recombinant Pr1a protein on 12% (w/v) polyacrylamide gel. SDS-PAGE was used to resolve the proteins and Coomassie Brilliant Blue was used to stain them. Marker Lane: Precision Plus protein ladder, (Biorad 1610374), lane 1: cell pellet after sonication, lane 2: cell-freemedium supernatant after sonication, lane 3: flow-through, lane 4: last column wash, lane 5: purified Pr1a protein.

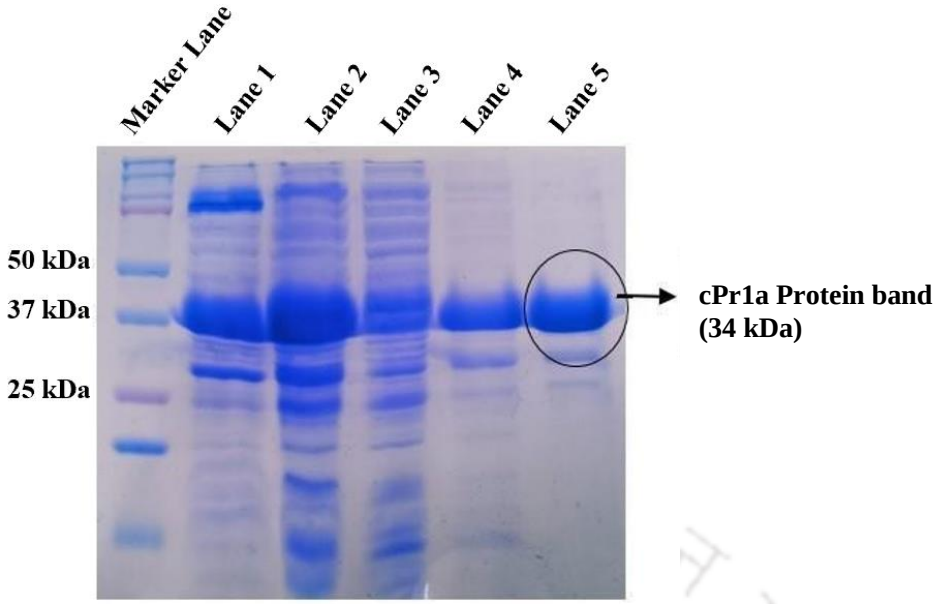

Fig. 4 - Purification profile of recombinant cPr1a protein on 12% (w/v) polyacrylamide gel. SDS-PAGE was used to resolve the proteins and Coomassie Brilliant Blue was used to stain them. Marker Lane:Precision Plus protein ladder, (Biorad 1610374), lane 1: cell pellet after sonication, lane 2: cell free medium supernatant after sonication, lane 3: flow-through, lane 4: last column wash, lane 5: purified cPr1a protein.

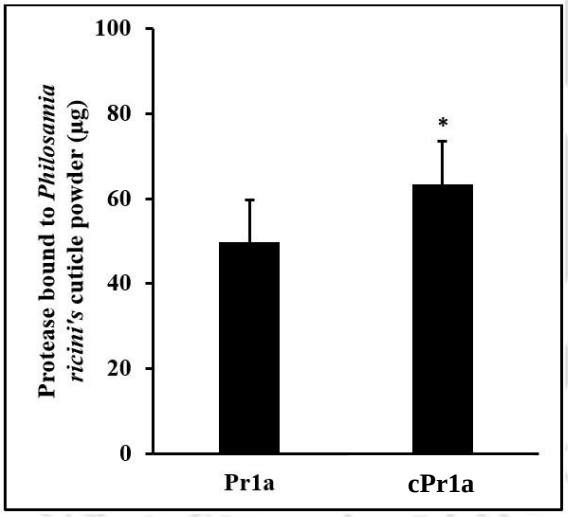

Fig. 5 - Cuticle binding analysis of wild-type Pr1a and chimeric cPr1a protease
The percent protein bound was determined from assay mixtures containing 5 mg *Philosamia ricini*'s cuticle powder in 50 mM potassium phosphate buffer (pH 6.0) plus 100 µg protease.
Pr1a - the amount of Pr1a protein bound (49.73 µg) to the powdered cuticle.
cPr1a- the amount of cPr1a chimeric protein bound (63.42 µg) to the powdered cuticle.
The chimeric protease cPr1a demonstrated significantly higher binding affinity compared to the wild-type Pr1a protease.
* - The results of the student t-test are statistically significant ($P < 0.03$) and represent the mean values from three independent experiments.

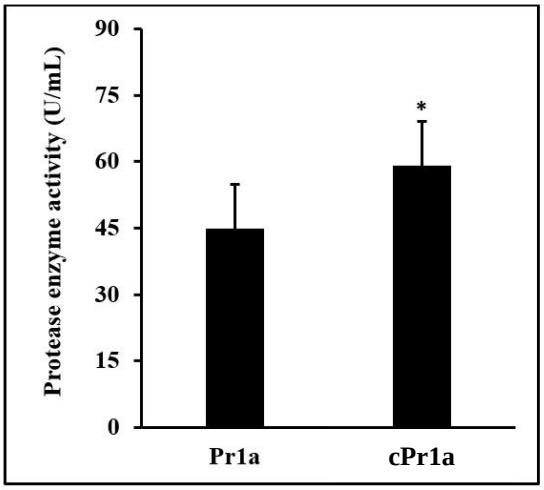

Fig. 6 - Comparative enzymatic analysis with purified wild-type Pr1a protease and chimeric cPr1a protease using *Philosamia ricini's* cuticle powder as substrate.
Pr1a – Enzyme activity of wild-type Pr1a protein (44.94 U/mL).
cPr1a - Enzyme activity of chimeric cPr1a protein (59.06 U/mL).
The chimeric protease cPr1a exhibited significantly higher enzyme activity compared to the wild-type Pr1a protease.
*-The results of the student t-test are statistically significant ($P < 0.02$) and represent the mean values from three independent experiments.

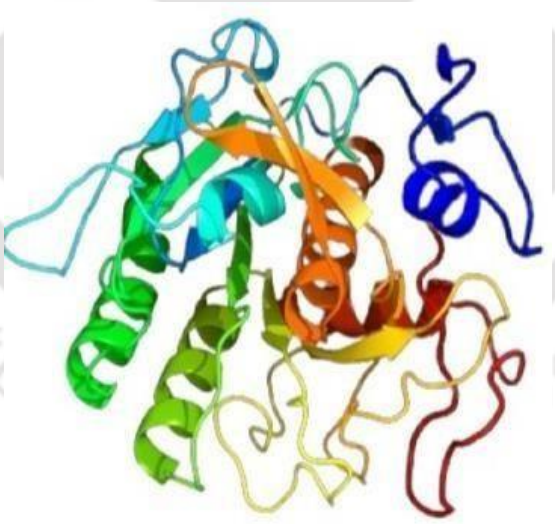

Fig. 7 - The 3D protein structure of wild-type Pr1a protease was modeled by the PHYRE2 web server. The predicted model attains a 100.00% confidence with 100% coverage and 76% identity to the template c3f7oB (PDBe-3f7o) with a PDBe title of Crystal structure of Cuticle-Degrading Protease from *Paecilomyces lilacinus* (PL646).

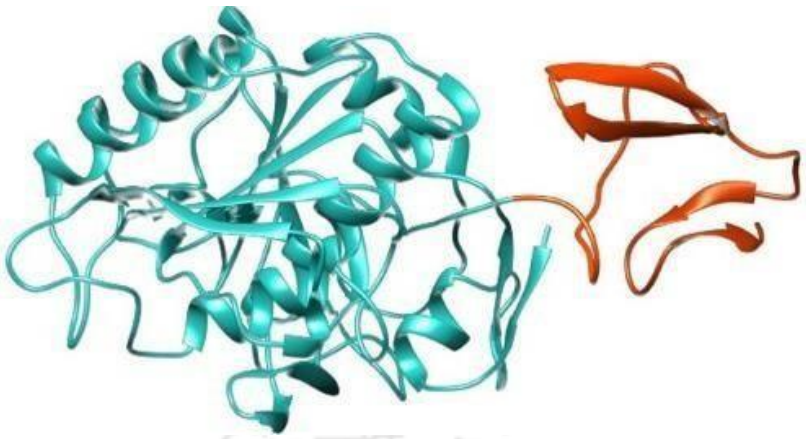

Fig. 8 - The structure of Bmcbd (*Bombyx mori* chitin-binding domain) was generated by the PHYRE2 web server. The predicted model attains a 99.4 % confidence with 96 % coverage and 35 % identity to the template c6bn0C (PDBe- 6bn0) with a PDBe title of Avirulence protein 4 (Avr4) from *Cladosporium fulvum* bound to the hexasaccharide of chitin. The 3D protein structure of chimeric cPr1a protease was generated by merging the modeled structure of Pr1a and Bmcbd using UCSF-Chimera software. Green part - Pr1a, Red part – Bmcbd.

Chapter-3: Cloning and expression of wild-type Chi2 (DQ011663) and chimeric cChi2.

The purpose of this chapter was to test the enzyme activity of bacterially purified wild-type and chimeric chitinase against the (Eri silkworm) *Philosamia ricini* insect cuticle. Cloning, expression analysis, and purification of the Chi2 and cChi2 proteins were done, and eventually, enzyme assays were done for the evaluation of the enzyme activities of Chi2 and cChi2 enzymes. Binding assays were done to check any upgrade in the binding capacity of chimeric cChi2 chitinase.

Results:

3.1 Cloning, expression, and purification of wild-type chitinase Chi2 and chimeric chitinase cChi2.

A) The three exons of the Chi2 gene were amplified using three sets of exon-specific primers. The three exons with overlapping sequences were fused using the overlap extension PCR method **(Fig. 9)**. The Chi2 gene (excluding the chitin-binding domain) and, Bmcbd (*Bombyx mori* chitin-binding domain) were re-amplified using a new set of primers in order to introduce the compatible overhangs to give Chi2bm (Chi2 gene with Bmcbd overhang at 3' end) and Bmchi2 (Bmcbd with Chi2 overhang at 5'end) and fused using the Gibson assembly method **(Fig.10)**. Chi2 gene and cChi2 were successfully cloned into pET-28a vector using the Gibson assembly method, resulting in the pET-28a-Chi2 and pET-28a- cChi2 constructs.

B) The pET-28a-Chi2 and pET-28a- cChi2 constructs were transformed into *E. coli* (BL21 DE3) competent cells. The expression of the Chi2 and cChi2 proteins was done in LB media supplemented with kanamycin (50 μg/ml) with 0.5 mM IPTG.

C) Protein purification was done using Ni-NTA affinity chromatography **(Fig. 11)**.

D) Binding assays were done in 50 mM potassium phosphate buffer for 4 mins with 100 μg of purified recombinant Chi2 and cChi2 enzymes, and the results revealed a significant

increase in the binding efficiency of chimeric chitinase cChi2 compared to wild-type Chi2 chitinase (**Fig. 12)**.

E) Enzyme assays were done in 50 mM potassium phosphate buffer for 4 mins and 1 min with purified Chi2 and cChi2 enzymes **(Fig. 13 and Fig. 14)**. The chimeric cChi2 enzyme showed a marked increase in its enzyme activity relative to the wild-type Chi2 enzyme.

F) The 3-D structure of Chi2 and cChi2 proteins were modeled using Phyre2 software**(Fig. 15 and Fig. 16)**.

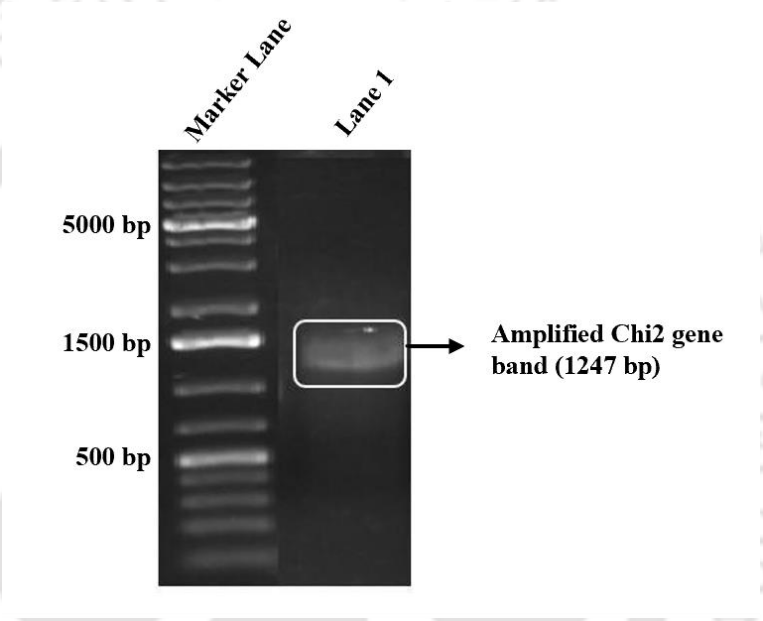

Fig. 9 - Agarose gel image showing PCR amplified Full Chi2 gene of *Metarhizium anisopliae* using Exon-1 Forward primer and Exon-3 Reverse primer.
The three exons of the Chi2 gene were fused using the Gibson assembly method, and the resulting Chi2 gene DNA-Gibson mixture was used to amplify the entire Chi2 gene.
Phusion DNA polymerase was used to amplify the Chi2 gene
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 – Chi2 gene (1247 bp).

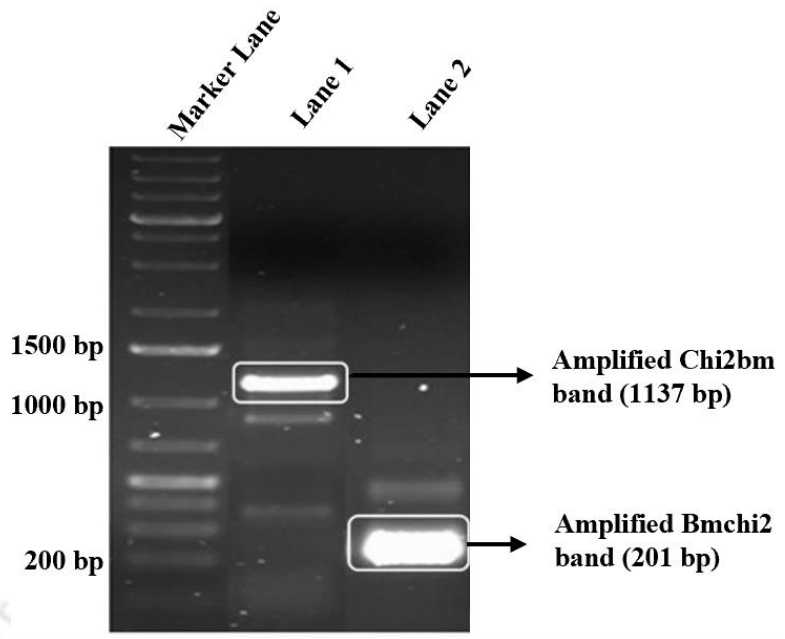

Fig. 10 - Agarose gel image showing PCR amplified Chi2bm (Chi2 gene with Bmcbd overhang at 3'end) & Bmchi2 (Bmcbd with Chi2 gene overhang at 5' end).
Phusion DNA polymerase was employed to amplify the Chi2bm and Bmchi2 fragments.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1– PCR amplified Chi2bm fragment (1137 bp).
Lane 2 – PCR amplified Bmchi2 fragment (201 bp).

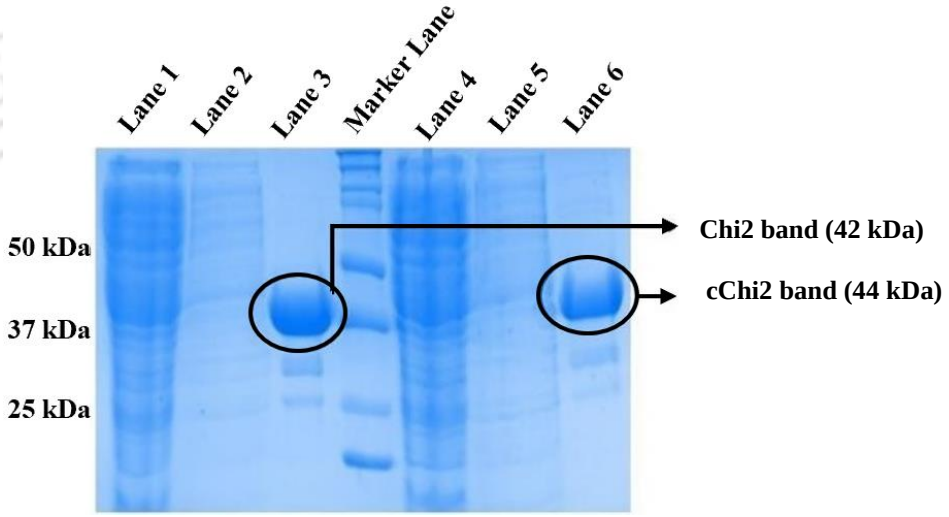

Fig. 11 - Purification profile of recombinant Chi2 and cChi2 on 12% (w/v) polyacrylamide gel. SDS-PAGE was used to resolve the proteins and Coomassie Brilliant Blue was used to stain them. Marker Lane: Precision Plus protein ladder, (Biorad 1610374), lane 1: cell pellet after sonication, lane 1: flow-through (Chi2), lane 2: last column wash (Chi2), lane 3: purified Chi2 protein (42 kDa)cell free medium supernatant after sonication, lane 4: flow-through (cChi2) lane 5: last columnwash (cChi2), lane 6: purified cChi2 (44 kDa).

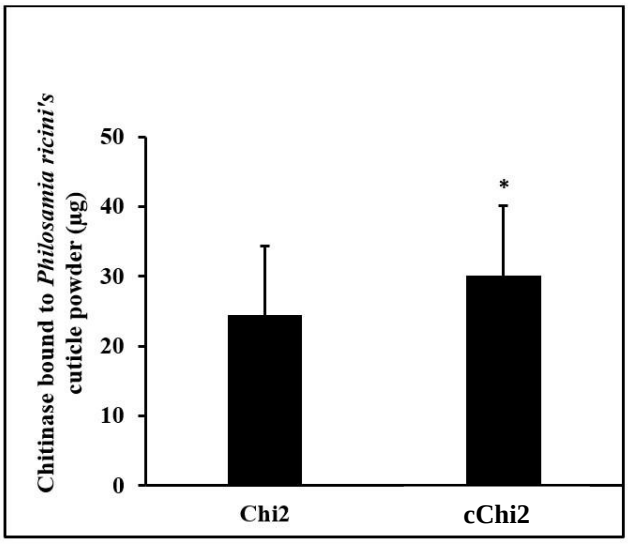

Fig. 12 – Cuticle binding analysis of wild-type Chi2 and chimeric cChi2 chitinase
The percent protein bound was determined from assay mixtures containing 5 mg *Philosamia ricini's* cuticle powder in 50 mM potassium phosphate buffer (pH 6.0) plus 100 µg chitinase.
Chi2 - the amount of Chi2 protein bound (24.41 µg), with the powdered cuticle.
cChi2- the amount of cChi2 chimeric protein bound (30.18 µg) with the powdered cuticle.
*- The results of the student t-test are statistically significant ($P < 0.02$) and represent the mean values from three independent experiments.

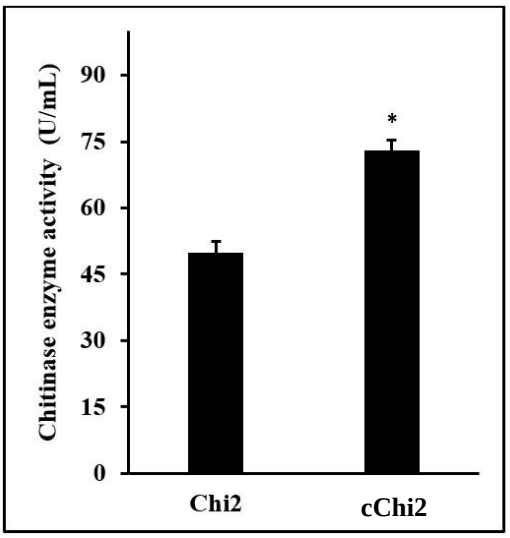

Fig. 13 - Comparative enzymatic analysis (1 min) with wild-type Chi2 chitinase and chimeric cChi2 chitinase using *Philosamia ricini's* cuticle powder as substrate.
Chi2 – Enzyme activity of wild-type Chi2 enzyme (49.97 U/mL).
cChi2 - Enzyme activity of chimeric cChi2 enzyme (72.93 U/mL).
The chimeric chitinase cChi2 exhibited significantly higher enzyme activity compared to the wild-type Chi2 chitinase.
*- The results of the student t-test are statistically significant ($P < 0.05$) and represent the mean values from three independent experiments.

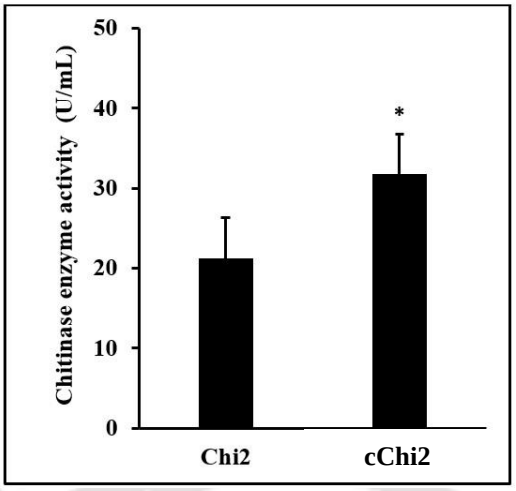

Fig. 14 - Comparative enzymatic analysis (4 min) with wild-type Chi2 chitinase and chimeric cChi2 chitinase using *Philosamia ricini's* cuticle powder as substrate.
Chi2 – Enzyme activity of wild-type Chi2 enzyme (21.28 U/mL).
cChi2 - Enzyme activity of chimeric cChi2 enzyme (31.76 U/mL).
The chimeric chitinase cChi2 exhibited significantly higher enzyme activity compared to the wild-type chitinase.
*- The results of the student t-test are statistically significant ($P < 0.02$) and represent the mean values from three independent experiments.

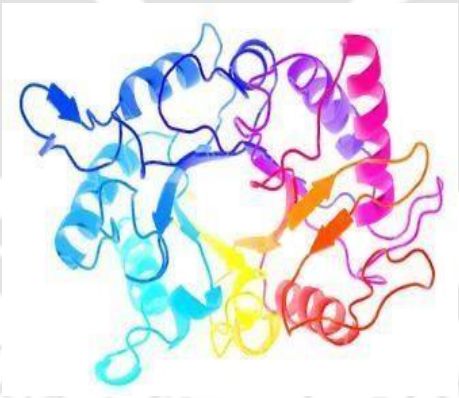

Fig. 15 - The 3D protein structure of wild-type Chi2 chitinase was modeled by the PHYRE2 web server. The predicted model attains a 100.00% confidence with 77% coverage and 36% identity to the template d2hvma (PDBe-2hvm) with a PDBe title of Hevamine a at 1.8-angstrom resolution.

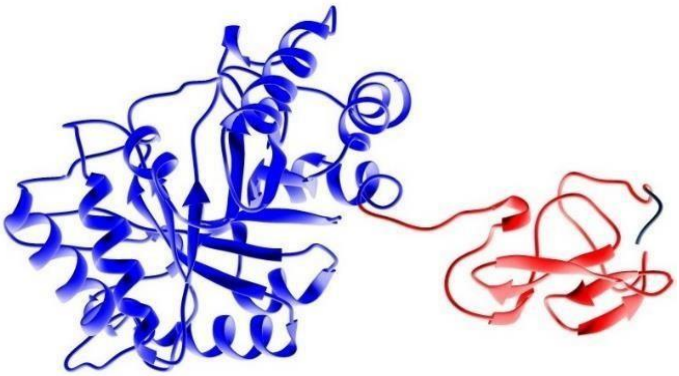

Fig. 16 - The structure of Bmcbd (*Bombyx mori* chitin-binding domain) was generated by the PHYRE2 web server. The predicted model attains a 99.4 % confidence with 96 % coverage and 35 % identity to the template c6bn0C (PDBe- 6bn0) with a PDBe title of Avirulence protein 4 (Avr4) from *Cladosporium fulvum* bound to the hexasaccharide of chitin. The 3D protein structure of chimeric cChi2 was generated by merging the modeled structure of Chi2 (excluding chitin-binding domain) and Bmcbd using UCSF-Chimera software. Blue part - Chi2 (without chitin-binding domain), Red part – *Bombyx mori* chitin-binding domain.

Chapter-4: Cloning and expression of fusion protein f-cPr1a–cChi2 into *Escherichia coli*.

The purpose of this chapter is to evaluate any increase in the enzyme activity of recombinant fusion protein f-cPr1a- cChi2 against *Philosamia ricini's* cuticle. The f-cPr1a-cChi2 protein was cloned, expressed, and purified, and then enzyme assays were performed to determine the difference in enzyme activity of fusion protein compared to wild-type Pr1a, Chi2 and chimeric (cPr1a & cChi2) enzymes.

Results:

4.1 Cloning, expression, and purification of fusion f-cPr1a- cChi2 protein.

A) cPr1a and cChi2 were amplified using cPr1a F.P (old) and R.P (new) and cChi2 F.P (new), R.P (old) respectively **(Fig. 17 & Fig. 18).** The cPr1a & cChi2 were fused successfully and cloned into a pET-28a vector using the Gibson assembly method, resulting into a pET-28a-f-cPr1a- cChi2 construct.

B) The pET-28a-f-cPr1a- cChi2 construct was transformed into *E. coli* (BL21 DE3) competent cells, and the expression was carried out in LB media supplemented with kanamycin (50 μg/ml) with 1.0 mM IPTG.

C) Protein purification was done using Ni-NTA affinity chromatography **(Fig. 19)**.

D) Protease and enzyme assay were done in 50 mM potassium phosphate buffer for 4 mins and 1 min with the purified f-cPr1a- cChi2 enzyme. The truncated fusion f-cPr1a- cChi2 protein showed a significant increase in enzyme activity relative to wild-type Pr1a enzyme **(Fig 20)**.

E) Comparative analysis of Protease enzyme activities of wild-type Pr1a, chimeric cPr1a, and f-cPr1a- cChi2 was done and a marked augmentation has been observed in the enzyme activity of fusion protein with respect to wild-type Pr1a and chimeric cPr1a enzymes **(Fig. 21)**.

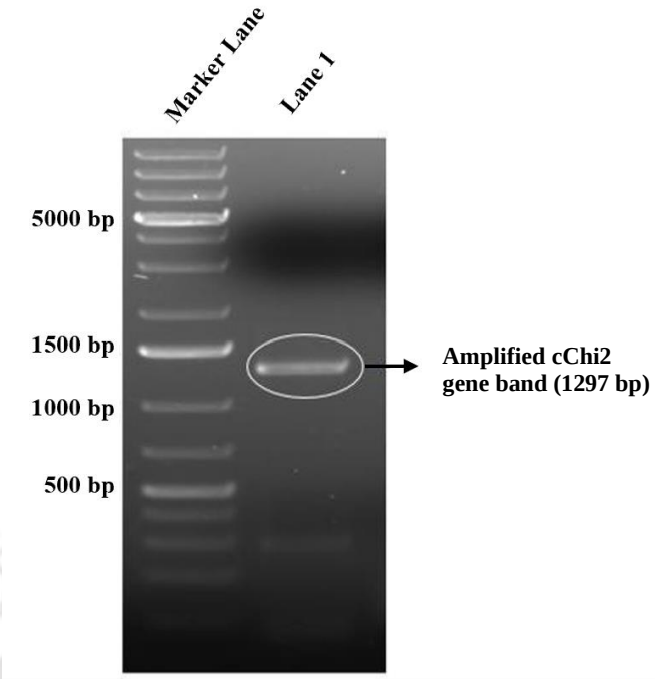

Fig 17 - Agarose gel electrophoresis analysis showing PCR amplified cChi2 (1297 bp) gene amplified using Chi2 gene as template of *Metarhizium anisopliae* employing polymerase chain reaction with gene-specific primers at annealing temperature 64°C, using Chi2 forward primer and Bmcbd reverse primer with cPr1a overhang at 5'end.

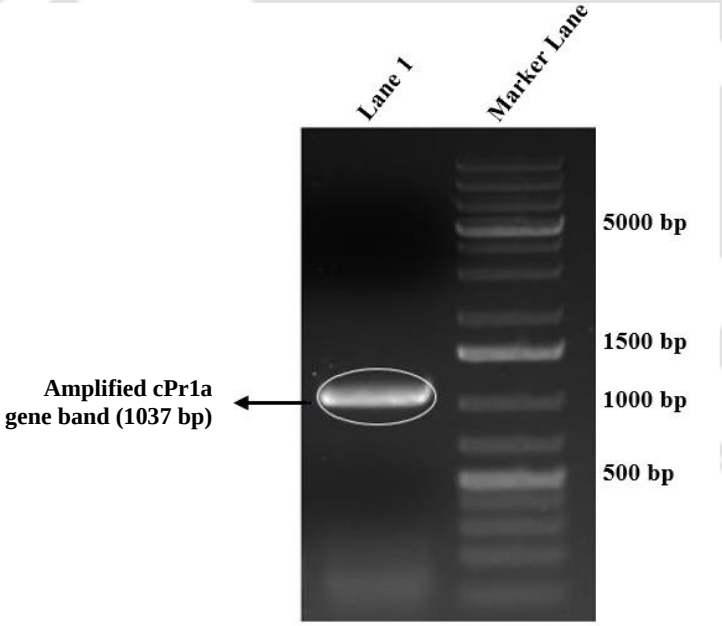

Fig. 18 - Agarose gel electrophoresis analysis showing cPr1a (1037 bp) gene amplified from the cDNA of Metarhizium anisopliae using polymerase chain reaction at annealing temperature 66°C with Pr1a forward primer and Bmcbd reverse primer with cChi2 gene overhang at 3' end.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 – PCR amplified cPr1a gene (1037 bp).

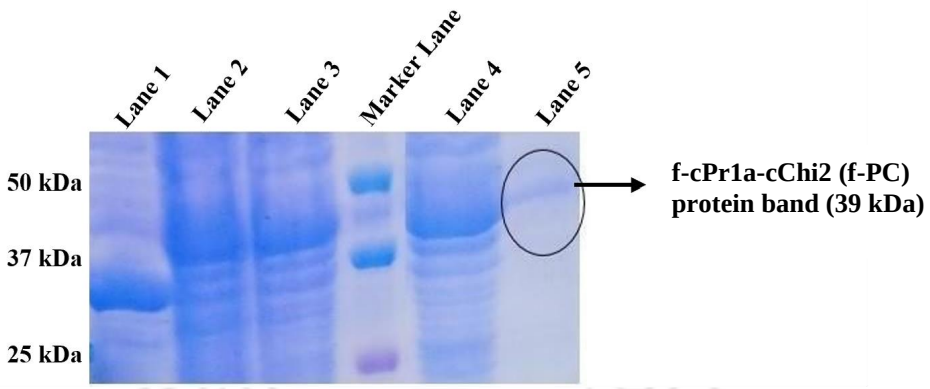

Fig. 19 - Purification profile of recombinant f-cPr1a- cChi2 protein on 12% (w/v)polyacrylamide gel. SDS-PAGE was used to resolve the proteins and Coomassie Brilliant Blue was used to stain them. Marker Lane: Precision Plus protein ladder, (Biorad 1610374), lane 1: cell pellet after sonication, lane 2: cell-free medium supernatant after sonication, lane 3: flow-through, lane 4: column wash, lane 5: purified f-cPr1a- cChi2 protein.

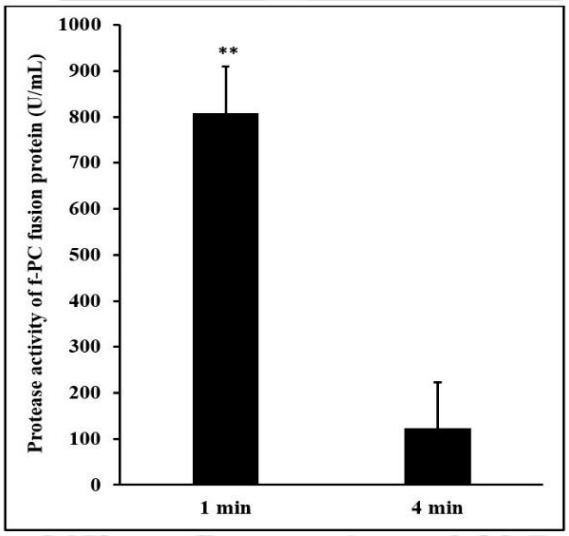

Fig. 20 - Comparative enzymatic analysis protease of f-PC truncated fusion protein using *Philosamia ricini* cuticle as substrate.
1 min – Protease enzyme activity of the fused truncated protein in the 1-minute assay (873.09 U/ mL).
4 mins – Protease enzyme activity of the fused truncated protein in the 4-minute assay (117.98 U/mL).
The protease enzyme activity of the fused truncated protein in the 1-minute assay exhibited significantly higher enzyme activity than in the 4-minute assay.
** - The results of the student t-test are statistically significant ($P < 0.001$) and represent the mean values from three independent experiments.

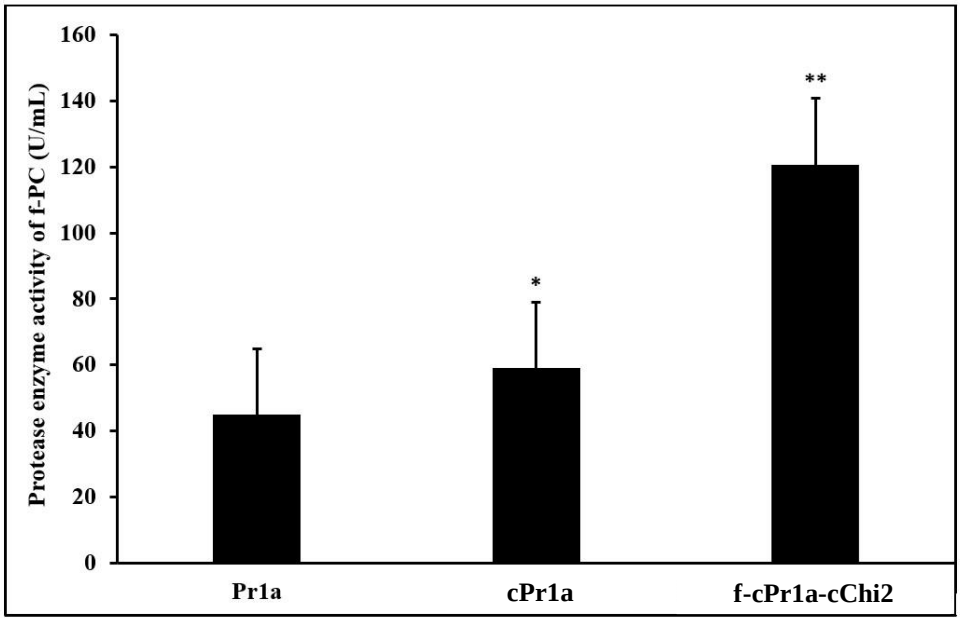

Fig. 21 - Comparative enzymatic analysis of protease activity of wild-type (Pr1a), chimeric protease (cPr1a), and truncated f- f-cPr1a- cChi2 (f-PC) fusion protein using *Philosamia ricini* cuticle as substrate in the 4-minute assay.
Pr1a – Protease enzyme activity of wild-type Pr1a enzyme (44.94 U/mL).
cPr1a - Protease enzyme activity of chimeric cPr1a enzyme 59.06 U/mL).
f-cPr1a- cChi2– Protease enzyme activity of truncated protease-chitinase fusion protein (120.69 U/mL).
* - The results of the student t-test are statistically significant ($P < 0.02$) and represent the mean values from three independent experiments.
**- The results of the student t-test are statistically significant ($P < 0.001$) and represent the mean values from three independent experiments.

Thesis Abstract

This thesis demonstrates the research focused on the development of chimeric protease, chimeric chitinase, and protease-chitinase fusion proteins. These proteins have been engineered to possess increased binding affinity to insect cuticles and improved catalytic activity, all in pursuit of enhancing their capacity to degrade cuticles effectively. In summary, this research delves into the insecticidal capabilities of chitinase and protease enzymes that target the insect cuticle, aiming to combat insect pests effectively. Before delving into the scope of the research and outlining the specific objectives, we provide an introductory exploration of biological control agents and their potential applications. The existing control methods are elucidated, along with their inherent limitations and constraints. In addition to this, the substantial significance of bio-control agents, specifically insect-pathogenic fungi *Metarhizium anisopliae* that overexpress insecticidal enzymes for cuticle degradation, has been emphasized.

This thesis presents a series of studies focused on the development of chimeric protease, chimeric chitinase, and chimeric protease-chitinase fusion proteins by harnessing the properties of the wild-type Pr1a protease and wild-type Chi2 chitinase from *Metarhizium anisopliae*, in combination with the chitin-binding domain from *Bombyx mori*. The primary objective is to engineer these proteins for potential application in degrading the insect cuticle in a faster manner, which serves as the initial barrier of defense for the insect host. The cuticle serves as the insect's primary defense mechanism, and it can be breached by a range of cuticle-degrading enzymes, including proteases, lipases, and chitinases. The efficiency of these enzymes is directly linked to the virulence of fungal entomopathogens. Therefore, enhancing the efficiency of these enzymes can significantly augment the virulence of the fungus, ultimately making it a more potent and effective pathogen.

CHAPTER-1
Introduction & Review of Literature

Chapter-1

1. General introduction

1.1 Crop loss and chemical pesticides

The agricultural system faces the challenge of producing enough food for a growing population, with food scarcity causing the deaths of 11 million children under five annually and over 400 million people chronically malnourished. By 2050, the world population will reach 9.1 billion, requiring a 70% increase in food production, including 3 billion tonnes of cereals annually. However, the growth rate of cereal yields has declined from 3.2% in 1960 to 1.5% in 2000. Reversing this trend is crucial to meet future food demands (14). Crop production is threatened by insect pests, weeds, and pathogens, causing potential global losses of about 50% in wheat and over 80% in cotton (15). Crop losses are significant and can be reduced by implementing various crop protection measures. To cope with this situation, we can focus on either increasing crop yields or reducing crop losses.

Crop loss is depletion in the yields of crops qualitatively as well as quantitatively. Crop loss can be classified into pre-harvest crop loss which occurs in fields & post-harvest crop loss eventuates during storage. Both biotic and abiotic factors contribute to crop losses (16). Reducing crop losses from insects can increase food supply, but using chemical pesticides poses serious health risks. These include acute effects like headaches, nausea, and poisoning, and chronic effects such as cancer and reproductive harm. Pesticides, which can cause various cancers, are classified into four groups: organochlorines, organophosphates, carbamates, and pyrethrins. (17).

Fig. 1.1 - Maize damaged by fall armyworm, (18).

Fig. 1.2 - Rice suffering from bacterial rice blight caused by *Xanthomonas oryzae*, (19).

1.2 Limitations of chemical pesticides

Pesticides, formulated to kill agricultural pests and control disease vectors, also harm non-target organisms, including humans. While effective against insects, they pose environmental hazards and risks to human health and other living organisms.

1.3 Pesticides consequences on health

According to the Environment Forum 1999, pesticide poisoning causes about 1 million deaths and chronic diseases annually. People who are involved in producing, formulating, and spraying pesticides, as well as agricultural and industry workers handling toxic chemicals are at

high risk (20). People or workers who were exposed to HCH hexachlorocyclohexane through the skin were found to have higher residues of neurological symptoms of headache, giddiness, sleeplessness, skin problems, nausea, dyspnea. HCH intoxication also showed ECG alterations like left ventricular hypertrophy, right ventricular hypertrophy sinus bradycardia etc. (21). According to the US National Academy of Sciences, DDE, a metabolite of DDT, is responsible for the thinning of eggshells in raptorial birds like the bald eagle (22). Avian fauna suffered severe effects from DDT pesticide, leading to thinning of eggshells and a decline in bird populations. Exposure to DDT and EE2 resulted in eggshells being 5-11% thinner. Research suggests disruption in the expression of the carbonic anhydrase enzyme may be responsible for this thinning (23).

Pesticides disrupt the growth, development, physiology, and reproduction of diverse animals. They induce atypical sexual development, alter sex ratios, and trigger abnormal mating behavior by blocking sex hormone receptors. Agrochemicals also prompt biochemical changes, disrupting metabolism, inhibiting enzymes, and causing growth retardation (24). Pesticides can disrupt hormones by mimicking them (agonist action) or blocking hormone receptors (antagonistic action), interfering with natural hormone functions (25).

Pesticides have a negative impact on the behavior of honey bees, which are vital pollinators of our ecosystem and responsible directly or indirectly for pollinating about one third of the food consumed by humans (26) Insecticides Endosulfan, baytroid & sevin were liable for the interference in the learning behaviour of honey bees (27). **Colony collapse disorder**- This phenomenon involves the disappearance of most of the worker bees from the colony leaving behind sufficient amount of food, queen & few immature bees which would be well taken care of by nurse bees (28). Imidacloprid, a neonicotinoid insecticide applied to sunflower seeds, is associated with abnormal bee behavior. Residues of imidacloprid (about 13 ng/g) were found in sunflower pollen due to its translocation within the plant (29).

Michael C. R. Alavanja et al. inferred that pesticides from various classes and with different functional groups pose a risk for non-Hodgkin lymphoma (30). Liqin Hu et al. studied the interconnection between three organophosphorus pesticides - Terbufos, Malathion, and Diazinon - and the risk of non-Hodgkin lymphoma. Their research concluded that Diazinon, among the three, disrupts the neuroimmune system, indirectly damaging the immune system (31).

M. Lacasana et al. discovered that pesticide exposure by both parents before or during the periconceptional period raises the risk of anencephalic children. Mothers exposed to pesticides during agricultural work in this time face a four-fold higher risk. The organogenesis phase is particularly vulnerable to exposure, potentially causing teratogenic effects (32).

1.4 Pesticide resistance

Pesticide resistance is defined as the reduced sensitivity of a pest population towards a pesticide. The evolution of pesticide resistance occurs through the process of natural selection, some pests in the population who have resistance to the pesticide survive and transmit these pesticide resistance inheritable characteristics to their offspring. All kinds of pesticides used for crop disease, rodents, and weeds were reported to show resistance. The evolution of resistance towards pesticides in insect pests is significant and due to this pesticide resistance agriculture is in peril. According to Fengying Guo et al pesticide resistance was developed in *Tetranychus cinnabarinus* towards 25 pesticides in China, and resistance against parathion and demeton resulted into the failure of managing the growth of this mite on cotton in China (33).

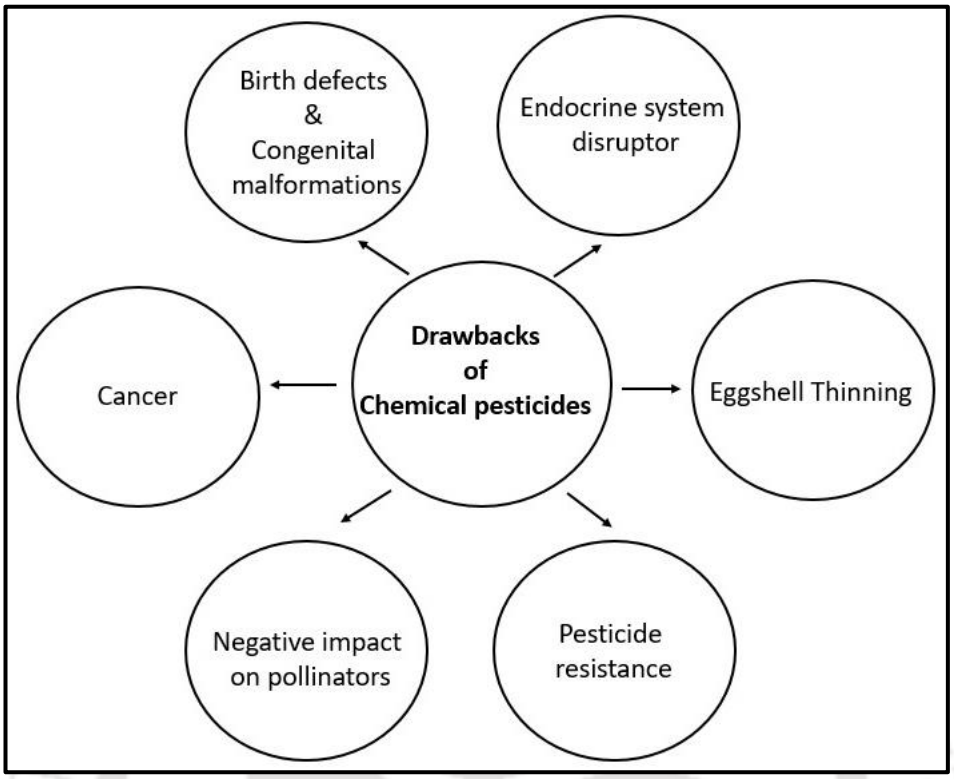

Fig. 1.3 - Drawbacks of chemical pesticides, (22 to 33).

1.5 Integrated pest management

IPM, Integrated Pest Management refers to the meticulous evaluation of all available pest control techniques and the subsequent integration of appropriate strategies that impede the growth of pest populations. It integrates chemical, physical, and biological pest management approaches to growing healthy crops while minimizing pesticide use and reducing pesticide hazards to human beings and the environment for sustainable pest management (34). IPM amalgamates various types of management approaches and practices for the protection of growing crops from insect pests by curtailing the use of chemical pesticides in order to minimize the risk to the environment and agrosystems. The integrated pest management system of America is devised on six basic elements viz. Acceptable pest levels, monitoring, Preventing culture practices, mechanical control, biological control and responsible use (35).

1.6 Biopesticides

Biopesticides are naturally sourced compounds originating from animals, plants, bacteria, and certain minerals, utilized for the purpose of controlling pests (36). Bio-pesticides include several types of pest management intervention through predatory, parasitic or chemical relationships. They are typically created by growing and concentrating naturally occurring organisms or their metabolites including bacteria and other microbes, fungus, nematodes etc. According to a precise definition outlined by the United States Environmental Protection Agency (EPA), these biopesticides can be categorized into three primary classes. (i) Biopesticides encompass naturally occurring biochemicals that operate through non-toxic mechanisms. (ii) Another category comprises microbial entomopathogens. (iii) The third class consists of plant-incorporated protectants originating from genetically modified plants (37). In 2019, the global biopesticides market achieved a valuation of approximately US$ 3.5 billion. Anticipations indicate that the market is poised to attain a valuation of around US$ 7.8 billion by the year 2025 (38). In the year 2019 biopesticides constitute approximately 5% of the pesticide market in India, with a minimum of 15 microbial species and 970 microbial formulations. By the year 2017, more than 200 products formulated from entomopathogenic fungi like *Beauveria bassiana, B. brongniartii, Metarhizium anisopliae., Lecanicillium lecanii,* and *Hirsutella thompsonii*, as well as nematicidal fungi including *Purpureocillium lilacinum* and *Pochonia chlamydosporia*, had obtained registrations for their usage in controlling various arthropods and plant parasitic nematodes (39).

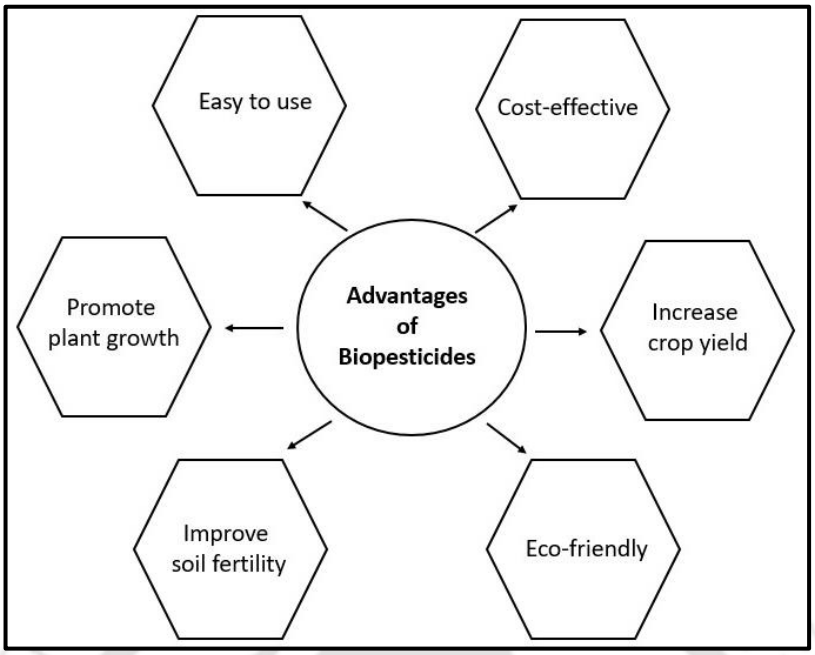

Fig. 1.4 - Advantages of Biopesticides, (40).

1.6.1 Different Classes of Biopesticides

There are three primary categories into which biopesticides can be classified.

1. **Biochemical pesticides** encompass natural substances that regulate pests through non-toxic mechanisms. Biochemical pesticides comprise compounds that disrupt mating behaviors, like insect sex pheromones, and scented plant extracts that lure insect pests into traps.
2. **Microbial pesticides** are composed of microorganisms, such as a bacterium, fungus, virus, or protozoan, serving as the primary active ingredient. Microbial pesticides have the potential to manage a wide range of pest types; however, each individual active ingredient tends to be relatively specific to its intended target pest or pests.
3. **Plant-Incorporated-Protectants (PIPs)** are pesticidal compounds that plants generate using genetic material introduced into the plant. As an illustration, researchers can extract the gene responsible for producing the Bt pesticidal protein and insert it into the plant's

native genetic material. Consequently, the plant itself, rather than relying on the Bt bacterium, produces the substance that eradicates the pest (41).

Microbial pesticides can be subdivided into distinct categories, including bacterial, fungal, viral, and protozoan biopesticides.

1. **Bacterial bio-pesticides**- *Bacillus thuringiensis* (Bt) is a gram-positive, soil-dwelling protein or cry protein, which is encoded by cry genes. Cry toxins have specific activities against insect species of order Lepidoptera (Moth and butterflies), bacterium, commonly used as biological pesticide. During sporulation *Bacillus thuringiensis* forms crystals of proteinaceous insecticidal endotoxins called crystal Diptera (flies and mosquitoes), Coleoptera (beetles), Hymenoptera (wasps, bees, ants, and sawflies), and nematodes. *Bacillus Thuringiensis* serves as an important reservoir of cry toxins for the production of biological insecticides and insect-resistant GMO crops.
2. **Viral bio-pesticides**- Baculovirus are used as viral biopesticides. Alkaline conditions of insect's midgut dissolve the protein coat of virus and expose the viral particles which fuse with midgut epithelial cells, multiply rapidly, and kill the host. Example- Nucleopolyhedrosis (42 & 43).
3. **Protozoa biopesticides**–Protozoa pathogens naturally infect a wide range of insect's hosts. Example- *Nosema Locustae* is known to be a natural biocontrol agent of many grasshopper species. *Nosema* infects at least 90 species of grasshoppers.
4. **Fungal biopesticides** - Fungal biopesticide - Entomopathogenic fungus – are a group of fungus that can act as a natural parasite of insects and kills or seriously disables them (*Beauveria bassiana, Metarhizium anisopliae, Lecanicillium lecanii*). These entomopathogenic fungi are used to control crop pests and vectors of disease-causing insects (44 & 45). *Metarhizium anisopliae* is a deuteromycete i.e. only an asexual conidial form is known which produces green cylindrical conidia and occurs worldwide

(46). Three genera of entomopathogenic fungi, *Beauveria bassiana, Metarhizium anisopliae, and Verticillium lecanii* are well studied with respect to molecular genetics and genomics (47). *Metarhizium* is one of the most studied fungi, it is approved as safe by EPA (48). This does not need to be ingested by the host, unlike other bacterial and viral biopesticides.

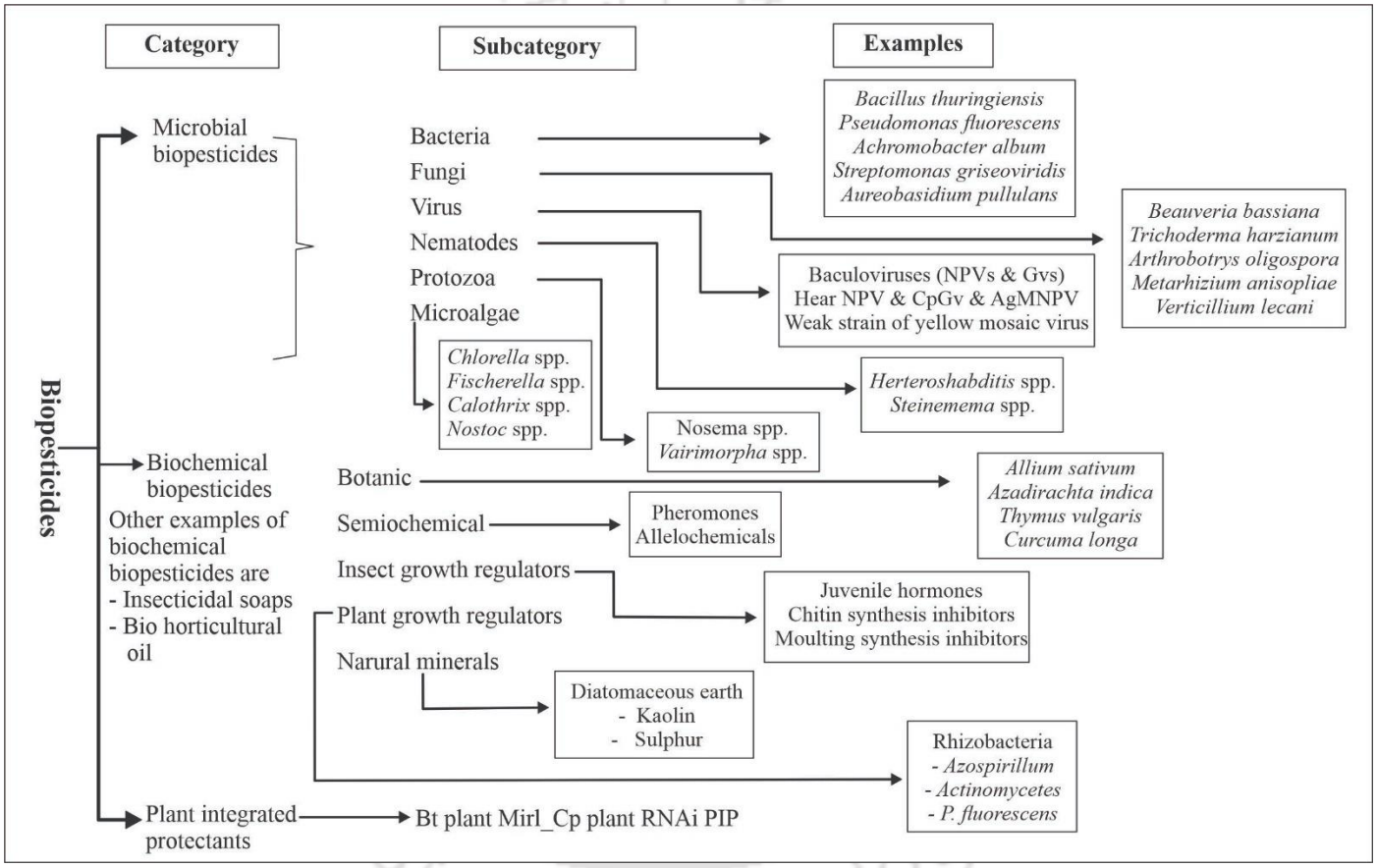

Fig. 1.5 -Classification of Biopesticides, (49).

1.7 Entomopathogenic Fungi

The species that have received the most extensive research attention for pest control encompass *Beauveria bassiana, Metarhizium anisopliae, Lecanicillium lecanii, Nomuraea rileyi,* and various *Hirsutella* species (44). Entomopathogenic fungi such as *Metarhizium spp, Beauveria bassiana,* and *Lecanicillium spp* have the potential to act as biopesticides. All these

fungi are called entomopathogenic fungi because all these fungi can naturally grow and parasitize on their respective insect hosts. One of the main components of integrated pest management is insect pathogenic fungus, which have great capacity as biocontrol agents against insects. They can be produced on a large scale inexpensively and are eco-friendly too.

1.7.1 *Beauveria bassiana*

Beauveria bassiana (Bals.) Vuill. is a ubiquitous pathogen that infects a diverse array of insect host. Hall and Papierok (1982) documented its presence on over 500 different host species (50). During the early 1770s, Italian scientist Agostino Bassi made a significant discovery regarding the appearance of white powdery structures that were wreaking havoc in the silkworm industry. It was later identified that these structures were caused by *Beauveria bassiana*, a pathogenic agent responsible for white muscardine fungal disease. Subsequently, in 1912, the fungus was officially named *Beauveria bassiana*, in tribute to the French scientist Jean Beauverie, by Jean-Paul Vuillemin (1861-1932) (Paul Vuillemin 1912). In its natural habitat, this fungus can be found in the soil, where it presents itself as a mycelium covered in white and forms clusters of conidiophores that exhibit a distinctive dentriculate (branching) pattern. The conidia or spores of these fungi initiate their growth on the surface of the insect host. They then penetrate through the insect's outer protective layer (cuticle), disseminate throughout the insect's circulatory system (hemolymph), and eventually lead to the death of the host. During colonization, a range of toxic metabolites, including antimicrobial peptides, are generated. These compounds play a role in suppressing the host's immune system, causing damage to the host's internal tissues, and depleting nutrients, ultimately resulting in the death of the host (51 & 52). *B. bassiana* produces secondary metabolites like beauvetetraones, bassiatin, beauverolides, beauvericin, dipicolinic acid, bassianolide, bassianin, oxalic acid, tenellin, basiacridin, and oosporin. Of these, beauvericin, bassianolide, oxalic acid, and dipicolinic acid are the ones that

demonstrate insecticidal properties (53). This fungus is recognized as a potent pathogen with a wide range of hosts, having the ability to infect over 700 species, including various species within Acari and Insecta (54 & 55). *B. bassiana* has been documented to parasitize numerous insect species spanning various insect orders, including those within Lepidoptera (*P. xylostella, S. litura*), Coleoptera (*Dendroctonus rufipennis*), Hemiptera (*B. tabaci*), Diptera (*Drosophila suzukii*) (56, 57, 58, 59 & 60). The taxonomical categorization of *B. bassiana* can be outlined as follows:

Scientific classification of *Beauveria bassiana*	
Kingdom	Fungi
Division	Ascomycota
Class	Sordariomycetes
Order	Hypocreales
Family	Cordycipitaceae
Genus	*Beauveria*
Species	*B. bassiana*

a) b) c)

Fig.1.6 - Shows three *Bombyx mori* silkworms dead larvae infected by SZY1 *Beauveria bassiana* strain for 6 days, 7days, and 10 days respectively (61).

1.7.2 *Metarhizium anisopliae*

Metarhizium anisopliae, formerly known as *Entomophthora anisopliae*, is a widely distributed soil-inhabiting fungus. The first use of it as a microbial agent against insects was in 1879, when Elie Metchnikoff used it in experimental tests to control the wheat grain beetle, *Anisoplia austriaca*. A member of the Hyphomycetes class of fungi, *M. anisopliae* is categorized as a green muscardine fungus due to the green color of the sporulating colonies. It has been reported to infect approximately 200 species of insects and other arthropods. The host range of *Metarhizium anisopliae* includes Coleoptera, Diptera, Dermaptera, Heteroptera, Homoptera, Hymenoptera, Isoptera, Lepidoptera, Orthoptera, Siphonaptera, Symphyla (62). The taxonomic classification of *Metarhizium* is as follows:

Scientific classification of *Metarhizium anisopliae*	
Kingdom	Fungi
Division	Ascomycota
Class	Sordariomycetes
Order	Hypocreales
Family	Clavicipitaceae
Genus	*Metarhizium*
Species	*M. anisopliae*

Beauveria bassiana and *Metarhizium anisopliae* have shown potential for biocontrol of *Haemaphysalis ginghaiensis* (63). *Metarhizium anisopliae* target insect pests and unlike bacteria and viruses they do not require ingestion by the host. During the infection cycle, infection is initiated by a conidium that adheres to an insect cuticle using an adhesion protein (64). The fungus infects by direct penetration of the cuticle and utilizes a number of enzymes along with

mechanical pressure (4). Fungus grows in hemolymph and evades recognition by hemocytes by masking itself with a collagen-like protein Mcl1 (65). Conidia are produced massively on the mycosed insect cadaver and infects new host. This fungus uses a variety of enzymes to breach the cuticle, like chitinases, proteases, lipases, etc. and uses hyphae to penetrate inside the host. This fungus is known to cause infections in a wide array of agricultural pests and vectors of disease-causing agents, encompassing a diverse range of hosts including Lepidoptera (*P. xlyostella,* (66), *S. frugiperda,* (67), *S. litura, (*68)), Homopteran (*A. gossypii,* (69)), Hemipteran (*Aphis craccivora,* (70)). *Metarhizium* produces diverse secondary metabolites in response to varying ecological growth conditions (71). Destruxin is a cyclic hexadepsipeptide compound synthesized by polyketide synthase clusters and produced by *Metarhizium* as one of its secondary metabolites (7). This peptide exhibits the remarkable ability to effectively inhibit the growth and proliferation of drug-resistant cancer cells (72).

Fig. 1.7 - A Cockroach infected by *Metarhizium anisopliae*, (73).

1.7.2.1 Life cycle of Entomopathogenic fungi

Entomopathogenic fungi target insect pests and unlike bacteria and viruses, they do not require ingestion by the host. *Metarhizium* and *Beauveria*, both well-studied entomopathogenic

fungus, belongs to the same class and order Sordariomycetes and Hypocreales, respectively but to different families, Cordycipitaceae (*Beauveria*) and Clavicipitaceae (*Metarhizium*). Both the fungus has almost the same mechanism of host infection. Fungal infections typically commence when a conidium comes into physical contact with an insect host and adheres to its cuticle using an adhesion protein triggering the recognition of the host (64). In *B. bassiana*, adhesins (MAD1 and MAD2) and hydrophobins (HYD1 and HYD2) each have a specific purpose. Hydrophobins improve the hydrophobicity of the cell surface, whilst adhesins facilitate attachment to insect hosts (64 & 74). This recognition event initiates spore germination and prompts the formation of a specialized cell called an appressorium, which serves as a highly adapted structure for penetrating the host's protective cuticle. The epicuticle, which protects the insects from desiccation and microbes, consists of a range of lipids, fatty acids, and wax ester layers. *B. bassiana's* Cytochrome P450 monooxygenase (CYP52X1) assists in the degradation of specific varieties of waxy compounds found in the insect's epicuticle (75). The fungi infect by direct penetration of the cuticle and utilize a number of enzymes along with mechanical pressure (4). *Metarhizium* secrets numerous hydrolytic enzymes viz. lipases, proteases (Pr1a, Pr1b, Pr1c, Pr1d to Pr1k) and chitinase (Chit1, Chi2, Chit3) in a sequential manner and many secondary metabolites (destruxins a, b, c, d, e and ferricrocin) and proteins during the infection process which plays a key role in the pathogenicity of the fungus (4, 7, 5, 76). After entering inside the insect body, the fungi transform into blastospores, which then bud and disseminate throughout the insect's body, invading its hemolymph and evading recognition by hemocytes by masking itself with a collagen-like protein Mcl1 (65). Following the death of the insect host, hyphae resurface, enveloping the cadaver and generating a substantial quantity of conidia to initiate infection in a fresh host.

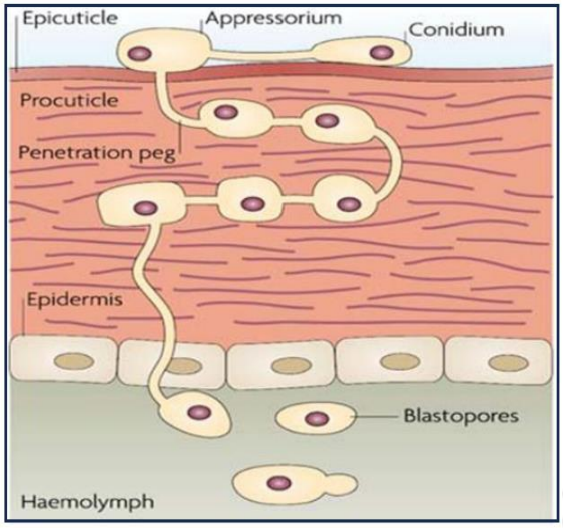

Fig. 1.8 - Invasion of insect cuticle and penetration inside the haemolymph by *Metarhizium anisopliae*, (77).

1.7.2.2 Advantages of entomopathogenic fungus *Metarhizium anisopliae*

Metarhizium anisopliae is a promising option for pest control because it is simple and inexpensive to produce asexual spores. This ease of use can result in lower production costs and improved accessibility for farmers and agricultural businesses looking for environmentally friendly pest management methods (46). These biopesticides can be effective against a wide range of insect pests, making them versatile tools for integrated pest management. Their self-perpetuating nature implies that once applied, they can continue to reproduce and persist in the environment, providing long-term pest control. Insect pests do not acquire resistance to these biopesticides, which further distinguishes them from conventional pesticides. These biopesticides maintain their efficacy over time, in contrast to chemical pesticides, which frequently lose effectiveness over time as pests develop resistance mechanisms. They do not contribute to environmental pollution, water contamination, or harm to non-target species, helping to preserve biodiversity. They are generally safe for the environment and pose little to no risk to humans, animals, or beneficial insects. This makes them an eco-friendly alternative to chemical pesticides.

1.7.2.3 Limitations of *Metarhizium anisopliae* based biopesticide

A significant challenge associated with biopesticides derived from entomopathogenic fungi is their relatively slow speed compared to chemical pesticides, in causing host mortality. Even the most aggressive strains of these fungi may require 2 to 5 days to effectively eliminate the target insect. Consequently, there's a notable risk that insect pests can continue to inflict substantial damage to crops even after the application of these biopesticides. This delay in host mortality can limit the immediate control of pest populations and potentially result in significant agricultural losses during the intervening period. Hence, there is a pressing need for strategies aimed at reducing the time it takes for the biopesticide to achieve lethal effects (reducing the median lethal time (LT_{50}) and enhancing its potency by lowering the dosage required for lethal effects (reducing the median lethal dose (LD_{50}). Notable influences on the pathogen's virulence can arise from abiotic factors such as UV radiation, temperature, and humidity. Hence, there is a need for effective strategies aimed at decreasing the median lethal time (LT_{50}) and the median lethal dose (LD_{50}) while also developing new fungal variants capable of withstanding abiotic stress. This would enable the fungi to act more swiftly upon contact with the target insects, ultimately achieving the desired level of pest mortality. Achieving this objective could involve genetic manipulation of the fungi using transgenic methodologies (78 & 79).

1.7.2.4 Genes involved in virulence of *Metarhizium anisopliae*

Pr1a is one of the most highly expressed subtilisin protease enzymes during the breaching of insect cuticles (4). Pr1a plays an important role in the parasitic phase and it suggests that this gene is a prospective target for the development of advanced engineered bio-pesticides (80). Subtilisin like serine protease can greatly degrade the protein of hot insect cuticle, has the ability to breach the cuticle, colonize and destroy the insect host tissues (81). Subtilisin proteins secreted by entomopathogenic fungus *Metarhizium anisopliae* are classified on the basis of sequence

similarities and exon – intron structure, class 1- subtilisin (Pr1c), class 2-proteinase k like subtilisin. Class 2 subtilisin are further classified into three clusters, extracellular subfamily 1- Pr1a, Pr1b, Pr1g, Pr1i, Pr1k, extracellular subfamily 2- Pr1d, Pr1e, Pr1f, Pr1j and endocellular subtilisin- Pr1h. EST's for Pr1a are found ten times more abundant than second most highly expressed Pr1j (4). Subtilisin has been classified into SB clan which have 3-layer sandwich fold and asparagine, histidine and serine as catalytic residues (82). Serine protease are enzymes that cleave peptide bond in protein in which serine serve as nucleophile amino acid at enzymes active site. Serine proteases fall into two broad categories, Chymotrypsin like (trypsin like) and Subtilisin like. (83). Pr1a gene synthesizes as large precursor (40.3 KDa) containing an 18 amino acid signal peptide and 89 amino acid propeptide (84). Chitinolytic enzymes are classified into two groups, N-acetylglucosaminidases and chitinases (85). Fungal chitinases comes under GH-18 family. Fungal chitinases can be divided into three subgroups, subgroup A, subgroup B and subgroup C (86). Basic structure of family 18 fungal chitinases consists of five domains or regions, catalytic domain, N-terminal signal peptide region, chitin binding domain, serine/threonine rich region and C-terminal extension (87). Genome sequencing and in silico analysis has shown that there are 30 putative chitinases in *Metarhizium anisopliae* (88) and 20 in *Beauveria bassiana* (89). Chi2 is a 419 amino acid protein with 19 amino acid signal peptide and it is upregulated by chitin source and downregulated by glucose (90). Chi2 gene is a virulence determinant gene of entomopathogenic fungus *Metarhizium anisopliae* and is directly involved in host cuticle infection (91).

1.7.2.5 Strategies used to improve the virulence of *Metarhizium anisopliae*

17.2.5.1 Heterologous gene expression

While the predominant approach in research communities involves utilizing endogenous genes, there is a perspective that holds significant promise: genetically modifying entomopathogenic fungi by incorporating genes from a heterologous source. In the initial stages,

baculovirus was genetically modified to facilitate the expression of AaIT1, a neurotoxin sourced from *Andructonus australis*, known for its specific action on insect Na+ channels (92). The same gene was employed by Wang and Leger (2007), they expressed it in *M. anisopliae*, targeting the insect hemolymph by employing the *Metarhizium* collagen-like (Mcl1) promoter. This strategic approach led to a remarkable 22-fold enhancement in fungal toxicity against tobacco hornworm caterpillars and adult yellow fever mosquitoes (93). Co-expression of AaIT, a single chain neurotoxic polypeptide derived from the venom of the scorpion *Androctonus australis* and the Pr1a protease from *M. anisopliae* within *B. bassiana* resulted in a significant reduction of the LT_{50} value by 40% when targeting the larvae of pine caterpillar *Dendrolimus punctatus* (94). Genetic modification of *M. acridum* to introduce BjαIT, an insect-selective neurotoxin sourced from *Buthotus judaicus*, led to augmented virulence when targeting locusts of the species *Locusta migratoria manilensis* (95). *M. anisopliae* was genetically engineered to enable the expression of AaIT. This genetic modification significantly enhanced the toxicity of AaIT in the resulting recombinant fungal pathogen, particularly against mosquitoes, tobacco hornworms, and coffee berry borer beetles (96).

1.7.2.5.2 Targeting genes involved in breaching of insect cuticle.

Although entomopathogenic fungi naturally attack insects as pathogens, their ability to cause mortality is very slow with respect to chemical pesticides, making them ineffective. Scientists have conducted extensive research to accelerate the killing process and boost its virulence. The primary focus of research aimed at enhancing the virulence of *Metarhizium anisopliae* has been on genes responsible for cuticle penetration. These genes, including proteases (Cdep1), chitinases (Bbchit1), lipases, and others, are endogenous to the fungi and actively expressed when they infect their host. Scientists have concentrated their efforts on understanding and optimizing these genes to improve the fungus's ability to effectively breach

the host's protective cuticle, thereby increasing its virulence as a biocontrol agent against insect pests.

1.7.2.6 Wild-type cuticle degrading enzymes of *Metarhizium anisopliae*

Insect cuticles primarily consist of interconnected protein networks and the highly insoluble polysaccharide known as chitin. Entomopathogenic fungi, such as *Beauveria bassiana*, invade insects by directly penetrating the host cuticles. This penetration is facilitated by a range of hydrolases, including proteases and chitinases, working in conjunction with mechanical pressure. Numerous proteases and chitinases are induced and expressed during the infection process but only a few are found to be associated with the virulence of the *M. anisopliae* fungi. The Pr1a gene of *Metarhizium anisopliae* expresses differentially during pathogenesis and different growth conditions and is one of the main virulence-determining factors against *Diatraea saccharalis* (80). When the inherent wild-type genes encoding cuticle-degrading enzymes were constitutively overexpressed, they resulted in increased virulence of the fungus. St. Leger and his co-workers in 1996, engineered a strain that constitutively expressed a protease gene (Pr1a) and killed caterpillars (*Manduca sexta*) 25% faster than wild type strain (8). Overproduction of Bbchit1 genes under the constitutive promoter of *Aspergillus nidulans* enhanced the virulence of *Beauveria bassiana* for aphids, transformants showed transformants show 50% reduction in lethal concentration and 50% lethal time compared to wild-type (9).

1.8 Chimeric Enzyme

A chimeric enzyme is formed by merging two or more enzymes, capable of exhibiting characteristics of each of its parent enzymes (97). The development of chimeric enzymes through genetic engineering is a means of generating entirely new enzymes with customized properties and functionalities. This approach involves combining segments or domains from different enzymes, allowing researchers to design enzymes tailored for specific industrial, medical, or research applications. Chimeric enzymes can harness the beneficial traits of their parent enzymes

and exhibit novel characteristics, expanding the toolbox of biotechnology and enzyme-based processes. In the past, the creation of chimeric enzymes through the fusion or swapping of carbohydrate-binding modules to the polysaccharide degrading enzymes has yielded more proficient enzymatic variants compared to the original or native enzyme. For instance, the swapping of ChBDs (Chitin-Binding Domains) between *B. licheniformis* and *B. thuringiensis* chitinases resulted in increased affinity for colloidal chitin and insoluble chitinous substrate, as well as enhanced conformational stability. Furthermore, domain swapping substantially improved the ability of both chimeric enzymes to bind to β-chitin (98).

1.8.1 Cuticle-degrading chimeric protease and chimeric chitinase enzymes

Polysaccharide-degrading enzymes are typically categorized into distinct glycoside hydrolase families, and they commonly exhibit a modular structure consisting of a catalytic domain linked to one or multiple carbohydrate-binding modules. Carbohydrate-binding modules (CBMs) have been demonstrated to facilitate the enzyme's proximity to its substrate, particularly when dealing with insoluble substrates. As a result, these modules are regarded as enhancers of enzymatic hydrolysis. These characteristics have proven crucial in numerous biotechnological applications focused on enhancing the efficiency of polysaccharide degradation (99). The fusion and interchange of Carbohydrate Binding Modules had a significant impact not only on polysaccharide-degrading enzymes like chitinase and cellulase but also on other enzymes such as proteases, by increasing their binding affinities and catalytic efficiencies.

The chitinase and protease genes from *Beauveria bassiana* were genetically engineered for the development of chimeric protease and chitinase. The modification aimed to enhance their binding affinity with the insect cuticle with simultaneous increase in cuticle digesting capability. The increased binding affinity was expected to elevate the cuticle-digesting capability of the chimeric enzymes with respect to wild-type protease and chitinase enzymes. To construct the

chimeric chitinase, scientists fused *Beauveria bassiana's* chitinase (Bbchit1) with chitin-binding domains sourced from bacteria, plants, and insects. Among these hybrid chitinases, the one utilizing the chitin-binding domain (BmChBD) derived from the silkworm *Bombyx mori* chitinase displayed the highest affinity for binding to chitin when compared to the other hybrid chitinases. The transformants overexpressing hybrid chitinase showed a 23% reduction in time to death in the transformant compared to the wild-type fungus (12). To enhance the targeting of cuticle protein-chitin structures and accelerate penetration, a hybrid protease (CDEP-BmChBD) was developed. This hybrid protease was created by fusing a chitin-binding domain (BmChBD) from *Bombyx mori* chitinase to the C-terminal of CDEP-1, a subtilisin-like protease from *B. bassiana*. When compared to the wild-type protease, the hybrid protease exhibited an increased ability to bind to chitin and release larger quantities of peptides/proteins from insect cuticles. The expression of the hybrid protease in *B. bassiana* resulted in a notable enhancement of fungal virulence when compared to the wild-type strain and strains that were overexpressing the native protease (13). Based on the research carried out by Weiguo Fang et al., it was found that a fused construct of *B. bassiana's* Pr1a homolog (CDEP1) and Bbchit1 demonstrated a higher efficiency in degrading insect cuticle in vitro compared to the individual actions of CDEP1 or Bbchit1 alone. The transformant expressing CDEP1:Bbchit1 exhibited a 24.9% decrease in LT_{50}. Strikingly, the expression of CDEP1:Bbchit1 led to a remarkable 60.5% reduction in LC_{50}, which is more than twice the reduction achieved through the overexpression of Bbchit1 (28.5%). The overexpression of protease-chitinase fusion protein in the transformants leads to notably swifter cuticle penetration compared to both the wild-type strain than the transformants that overexpress either chitinase or protease alone (11). In vitro, studies demonstrated the synergistic degradation of cuticles by proteases and chitinases secreted by insect pathogenic fungi, and the expression of fusion proteins with both chitinase and protease activities enhances the virulence of the insect pathogen *Beauveria bassiana*.

1.9 Versatile roles of chimeric protease and chimeric chitinase enzymes: extending beyond pest control"

The benefits of chimeric protease and chimeric chitinase enzymes extend beyond pest control into waste management applications. The development of chimeric chitinase and chimeric protease enzymes with enhanced binding affinity and augmented catalytic efficiency holds significant promise across multiple sectors, highlighting their versatile potential beyond agricultural pest control.

1.9.1. Chimeric chitinase and chimeric protease for shrimp shell waste management and valorization into valuable chitooligosaccharides

Chimeric chitinases and chimeric protease can be utilized in the seafood industry. Increasing seafood consumption generates tons of shrimp shell waste, which is costly and environmentally harmful to process traditionally. Chemical hydrolysis can effectively breakdown the shrimp cuticle, but it significantly pollutes the environment. Therefore, chimeric protease and chimeric chitinase could serve as an eco-friendly alternative for degrading shrimp shell waste. Enzymes with enhanced binding affinity for the shrimp cuticle and increased catalytic efficiency can digest shrimp shell waste more quickly and effectively than wild-type enzymes.

In this context, the innovative approach demonstrated by Jun-Jin Deng et al. provides a significant breakthrough. Their study showed that attaching three carbohydrate-binding modules (CBMs) to the C-terminal of chitinase significantly enhances its activity and substrate-binding capacity. The modified chitinase, ‘Chit46-CBM3’, enabled a one-step process that converted 46.5% of chitin into chitin oligomers within 12 hours, along with partial protein release (100). Chitooligosaccharides (COS) exhibit a wide range of biological activities and find numerous applications across diverse fields including medicine, cosmetics, food, and agriculture. With

antioxidant, antimicrobial, anti-inflammatory, anti-obesity, and antidiabetic properties, COS offer promising therapeutic potential. They are beneficial in conditions such as osteoporosis, effectively controlling hypertension by inhibiting renin or angiotensin-converting enzyme activity. Moreover, COS have shown to reduce neuronal damage caused by oxidative stress, suggesting their potential as neuroprotective agents against neurodegenerative diseases like Alzheimer's. Additionally, COS and their derivatives demonstrate potent antitumor activity against human cancer cells, making them promising candidates in cancer therapy. Furthermore, COS contribute to tissue repair and wound healing, comparable to glycosaminoglycans, crucial components of the extracellular matrix (ECM). Their ability to mimic the ECM environment makes them suitable for tissue engineering applications, facilitating cell attachment and growth factor preservation. Traditionally, chitin is extracted from crustacean shells using inorganic acids and strong alkali (Potential Medical Applications of Chitooligosaccharides). Over the past few decades, enzymatic hydrolysis of chitin and chitosan has been proposed as an alternative. Enzymes that can hydrolyze chitin and chitosan include chitinase, chitosanase, lysozyme, cellulase, pectinase, protease, lipase, and pepsin. Enzymatic hydrolysis is generally preferable to chemical methods because it occurs under milder conditions and allows for better control of the molecular weight distribution of the products (101). Therefore, chimeric chitinase and chimeric protease enzymes are poised to play a pivotal role in degrading shrimp shell waste. Through deproteinization of proteins and degradation of chitin within the shell, these enzymes can facilitate the conversion of shrimp shell waste into valuable products such as chitin, chitosan, and chitooligosaccharides. The inclusion of a chitin-binding domain in chimeric enzymes could enhance their proximity to the insoluble substrate, resulting in more efficient and rapid conversion of shell waste into useful and valuable products compared to wild-type enzymes.

1.10 Hypotheses

1. Integrating the chitin-binding domain from *Bombyx* mori chitinase at the C-terminal of *Metarhizium anisopliae's* Pr1a protease is anticipated to enhance the binding capacity of cPr1a. This modification is expected to enable more efficient interaction with the insect host's insoluble cuticle, consequently leading to an increase in the enzymatic activity of cPr1a chimeric protease.
2. Swapping the chitin-binding domain of Chi2 chitinase of *Metarhizium anisopliae* with that of *Bombyx mori* chitin-binding domain could result in more efficient enzyme activity of cChi2 chitinase with a simultaneous increase in its binding affinity with the insect cuticle.
3. Construction of chimeric protease (cPr1a) and chimeric chitinase (cChi2) will result in a bifunctional fusion protein (f-cPr1a- cChi2 (f-PC)) with more pronounced protease and chitinase activities than either of chimeric protease or chimeric chitinase.

1.11 Objectives

The objective of this study was to clone and express the Pr1a, cPr1a, Chi2, cChi2, and f-cPr1a: cChi2 proteins in an *E. coli* expression system and to evaluate the enzyme activities or cuticle digesting effect of wild-type and chimeric, purified recombinant proteins using the *Philosamia ricini's* cuticle as the substrate.

1. Cloning and expression of wild-type and chimeric protease from *Metarhizium anisopliae* into *E. coli* and to evaluate the binding capacities and enzyme activities of wild-type and chimeric protease by binding assays and protease enzyme assays.
2. Cloning and expression of wild-type and chimeric chitinase from *Metarhizium anisopliae* into *E. coli* and to check the binding efficiency and enzyme activities of both wild-type and chimeric chitinase by binding assays and chitinase enzyme assays.
3. Construction of fusion protein by merging the chimeric protease and chimeric chitinase and checking the enzyme activity of fusion protein by chitinase and protease enzyme assays.

1.12 The translational potential of current studies

Metarhizium anisopliae, a naturally occurring entomopathogenic fungus, has the ability to infect and kill a wide range of insect pests. The chitinase and protease enzymes secreted by this fungus, contribute to its pathogenicity by breaking down the chitin and protein barrier, allowing it to penetrate the insect cuticle and cause lethal infections. The chitinase and proteases enzymes have been targeted and improved over time utilizing various techniques with respect to their substrate binding affinity, and catalytic efficiency. The improved enzymes with superior properties have shown promising results as a potent component of fungal biopesticides. The chimeric chitinase and protease enzymes could be of great application as biopesticides when overexpressed in the *Metarhizium anisopliae* fungi constitutively. The enhanced ability of

chimeric chitinase and chimeric protease enzymes to degrade chitin and protein respectively, provides opportunities for sustainable pest management in agriculture. By fusing the chitinase with different chitin-binding domains through genetic engineering techniques, chimeric chitinase enzymes can be created with enhanced catalytic activity, stability, and substrate specificity. These engineered enzymes have the potential to be used as components of fungal biopesticides. In agriculture, chimeric chitinases can be employed to control pests by targeting and degrading their chitinous exoskeletons. The chimeric chitinase with increased binding affinity and catalytic activity has been shown to improve the virulence of the fungi compared to wild-type chitinase. The protease enzyme of *Metarhizium anisopliae* shows potential in various medical and biotechnological applications. The chimeric protease of *Metarhizium anisopliae* is a remarkable enzyme with hybrid characteristics derived from the fusion of the protease domain with the chitin-binding domain. This fungal chimeric protease displays an exceptional ability to degrade the cuticle of insect, making it a potent tool in various biopesticide industry. The chimeric nature of this protease allows it to exhibit enhanced substrate specificity, and catalytic efficiency compared to its individual component proteases.

CHAPTER-2
Cloning & expression of wild-type Pr1a and chimeric protease cPr1a into *E. coli*

Chapter-2

2.1 Introduction

Insect pathogenic fungi, *Metarhizium anisopliae*, offer significant promise as biological control agents for pests, especially as viable substitutes for chemical pesticides. As an invertebrate fungal pathogen, *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae, *M. anisopliae*) has been crucial in the management of numerous agricultural pests and human disease vectors (102). *M. anisopliae* is found globally, spanning from arctic regions to tropical areas, where it inhabits insects and soil. (47). *Metarhizium* species are generally considered soil saprophytes and are commonly observed in disturbed environments such as agricultural fields, rather than in forest ecosystems (103). Specific strains of *M. anisopliae* have been utilized for their ability to effectively target and control crop pest insects and disease vectors, such as mosquitoes and ticks, termites (104, 105, 106 & 107). However, the effective utilization of mycoinsecticides in pest control methods has faced challenges mainly because of their slow killing speed, they are not very efficient in terms of the average time it takes to attain considerable mortality. This time frame can range from several days to weeks, which makes them less competitive compared to chemical insecticides that act more quickly and are less expensive (8).

The fungus utilizes mechanical pressure developed by the appressorium as well as a variety of hydrolytic enzymes such as chitinase, proteases, and lipases to breach the insect cuticle (4). *Metarhizium anisopliae* secretes a variety of proteases such as subtilisin (Pr1), trypsin (Pr2), metalloproteases, and cysteine proteases (80). Subtilisin like serine protease can greatly degrade the protein of host insect cuticle, has the ability to breach the cuticle, colonize and destroy the insect host tissues (8). Pr1a Proteases are the hallmark of the infection process and these hydrolytic enzymes breach the cuticle of insect pests during the infection process by degradation of the protein matrix of the insect cuticle in order to utilize the cuticle as a nutrient source (108). The genome of *Metarhizium anisopliae* contains 11 Pr1 subtilisin genes (Pr1a to Pr1k) and out of these. Pr1a is one of the most highly expressed subtilisin proteases during the breaching of insect

cuticles (4). Pr1a protease plays an important role in the parasitic phase and it suggests that this gene is a prospective target for the development of advanced engineered biopesticides (80). An increase in the binding efficiency of protease enzymes had resulted in an enhancement in its enzyme activity, which can be related to increased cuticle digesting capacity. For instance, the construction of hybrid protease (CDEP-BmChBD) by incorporation of chitin-binding domain from *Bombyx mori's* chitinase (BmChBD) to a subtilisin-like serine protease (CDEP) from *Beauveria bassiana* had resulted in the enhanced insecticidal activity of protease against the cuticles of the larva of *Myzus persicae*. The strains of *Beauveria bassiana* overexpressing the hybrid protease construct also showed augmented fungal virulence (13). The Pr1a protease of *Metarhizium anisopliae* contributes majorly to fungal virulence; hence, an increase in its binding capacity can greatly enhance its hydrolytic activity and hence the virulence of the fungus.

Our hypothesis revolves around the fusion of a *Bombyx mori* Chitin-Binding Domain (CBD) with the Pr1a protease genes. This fusion aims to improve the binding affinity of protease with insect cuticles, which primarily consists of chitin embedded in a matrix of cross-linked proteins. By doing so, we anticipate that the chimeric protease cPr1a's potential to digest cuticles may be enhanced by improving its binding affinity. In this research study, we aimed to enhance the binding ability of Pr1a, a serine protease gene from *M. anisopliae* to chitin in the insect cuticle. To achieve this, we introduced a *Bombyx mori* chitin-binding domain (CBD) at the C-terminal end of the Pr1a gene. The rationale behind this modification was to increase the interaction between the chimeric protease and the chitin present in the insect cuticle, ultimately improving its capability to degrade cuticle proteins.

2.2 Material and Methods

2.2.1 Bacterial strains, reagents, and kits

The *E. coli* Top-10 strain was used for the maintenance of cloning and expression vectors. *E. coli* (BL21 DE3) competent cell (New England Biolabs, USA) was used for the expression of Chi2 (chitinase) and Pr1a (protease) genes. Phusion DNA polymerase (Thermo Scientific) for PCR amplification and restriction enzymes (NcoI and XhoI) and ligation enzymes (Taq DNA ligase were purchased from New England Biolabs, USA. GCC Biotech (I) Pvt. Ltd., India, synthesized all the oligonucleotide primers for the amplification of Pr1a and cPr1a genes. The gene encoding Pr1a and cPr1a were cloned and expressed using the expression vector pET-28a (+). Nucleospin gel extraction kits were purchased from Macherey Nagel, GmBH. The tris-free base, Bradford reagent, kanamycin antibiotic, Coomassie Brilliant Blue R250 (CBB) for protein staining, Luria Bertani media, disodium ethylenediamine tetra acetate salts (EDTA), sodium hydroxide were procured from Himedia, whereas sodium dodecyl sulfate.

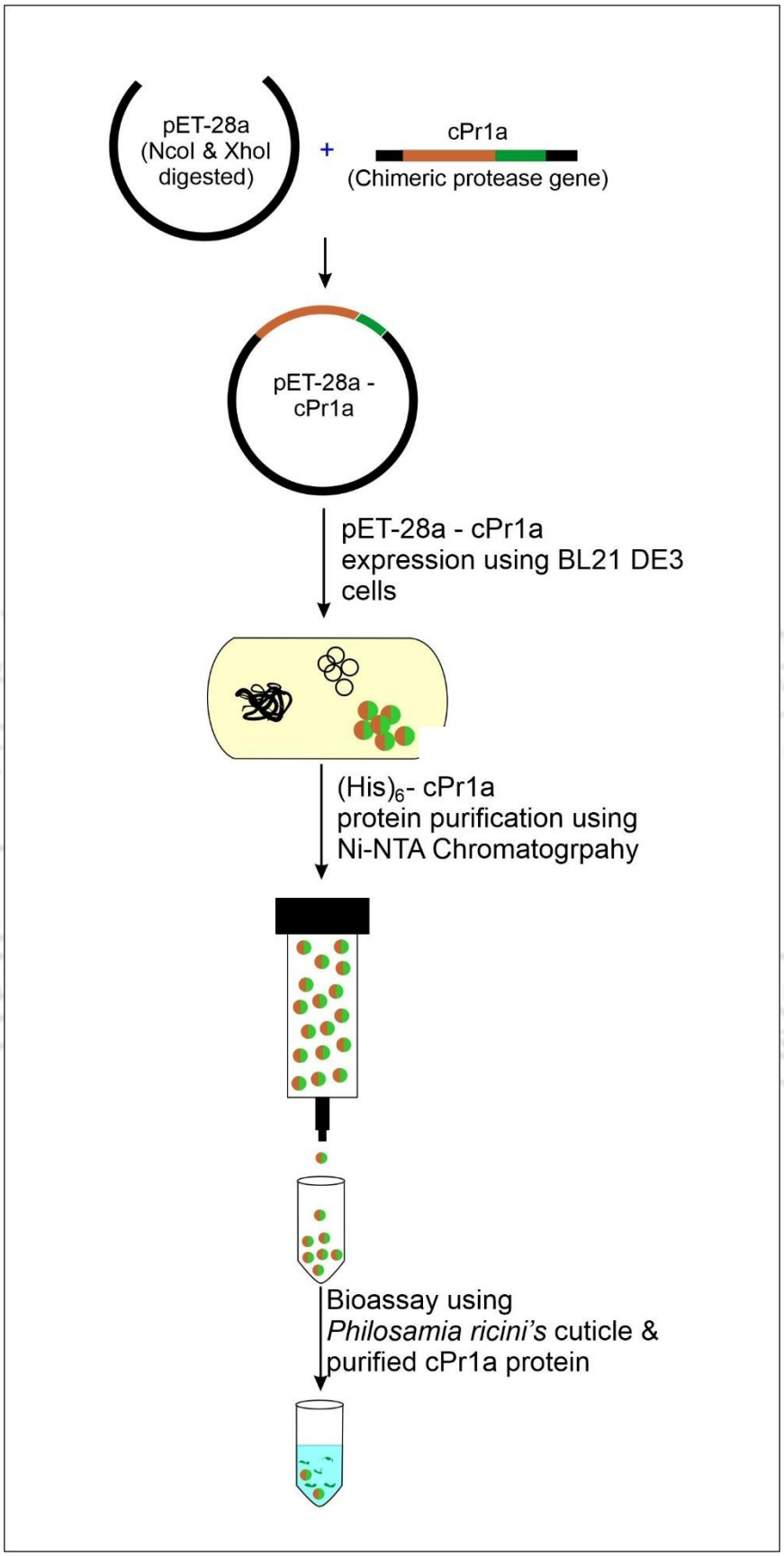

Fig. 2.2.1 - Schematic representation of cPr1a-$(His)_6$ protein expression and purification.

(SDS) and DNSA reagents were obtained from SRL. The DNA ladder was purchased from Thermo Scientific, India, while the protein ladder was purchased from Bio-Rad, India.

2.2.2 Pr1a gene primer designing

The DNA sequence of Pr1a was obtained from National Center for Biotechnology Information (NCBI) database with the accession number: M73795.1 (Pr1a). Forward and reverse primers were designed based on the sequence of the Pr1a gene.

2.2.3 Fungal spores harvesting

The spores (1×10^6 mL^{-1}) of *Metarhizium anisopliae* were subcultured and maintained on potato dextrose agar (PDA) plates (pH-5.6) for 7-10 days at 28°C. The conidial suspension was prepared by harvesting the spores from Petri plates in 0.05% Triton X- 100 using an autopipette.

2.2.4 Mycelia preparation

Conidia ($1x10^6$ conidia/mL) were inoculated into Sabouraud dextrose broth (SDB - (dextrose (2%), peptone (1%)). The pH of the culture media was adjusted to 5.6, and incubation of cultures was done in an orbital incubator shaker at 28°C and 180 rpm for 48 h. After 48 h, mycelia were harvested by filtration using Whatman filter paper no.1. and washed with sterile distilled water. Harvested mycelia were inoculated into (PIM) protease induction media (Potassium dihydrogen phosphate (KH_2PO_4 - 0.1%), Magnesium sulfate ($MgSO_4$.$7H_2O$ – 0.05%), Colloidal chitin – 1%). Mycelia were harvested from PIM (Protease induction media) by filtration and washed with sterile distilled water, and collected for RNA isolation.

2.2.5 Colloidal chitin preparation

The preparation of colloidal chitin was done with a slight modification in the method of Simhara and Takiguchi., (1988). Crab shell chitin was dissolved using 12 N HCl in a 1-liter glass beaker with stirring every couple of minutes for 2 h at 4°C. The suspension was mixed with a liter of distilled water, and the precipitate was collected by centrifugation at 8000 rpm for 10 minutes (Thermo scientific ST16R Refrigerated centrifuge) in 250 mL bottles. The precipitate was washed many times with distilled water until the pH of the wash came to 7.0. and collected and stored in a refrigerator at 4°C further for use.

2.2.6 RNA isolation, cDNA preparation, and Pr1a gene amplification

The RNA was isolated from mycelia harvested from PIM (Protease induction media) following the protocol provided with the RNAiso Plus kit (Takara, Japan), and any traces of DNA contamination were removed by using DNAse I treatment (Sigma Aldrich). Quantitative analysis of RNA was done using a Nanodrop photometer, and agarose gel electrophoresis was used for its qualitative analysis. One microgram of total RNA was converted into cDNA using (SuperScript™ III first-strand synthesis system, Invitrogen, USA). Synthesized cDNA was used as a template for the amplification of the Pr1a gene using gene-specific primers.

2.2.7 Amplification and cloning of wild-type Protease (Pr1a) gene

Primer designing was done using the DNA sequence of Pr1a retrieved from the National Centre for Biotechnology Information (NCBI) database with Accession no.- M73795.1 to amplify and clone the Pr1a gene with a His-tag into pET-28a bacterial expression vector. Amplification of the Pr1a gene sequence was done using a set of forward and reverse primers, and cDNA was used as a template for amplification of the pr1a gene. The reaction conditions followed for amplification were - step 1: Initial denaturation 98ºC for 2 mins, step 2:

Denaturation 98ºC for 10 sec, step 3: Annealing 68ºC for 30 sec, step 4: Extension 72ºC for 30 sec, and step 5: Final extension 72ºC for 10 min. Amplified products were segregated on 1% agarose gel and purified using a Nucleospin gel extraction kit (Macherey Nagel), GmBH. The pET-28a plasmid was digested with NcoI and XhoI restriction enzymes (New England Biolabs, U.S). Digested plasmid products were separated on 1% agarose gel electrophoresis and purified using a Nucleospin gel extraction kit. The Pr1a gene was cloned into NcoI, and XhoI digested pET-28a plasmid using the Gibson assembly method, and a reaction was performed at 50ºC for 60 min.

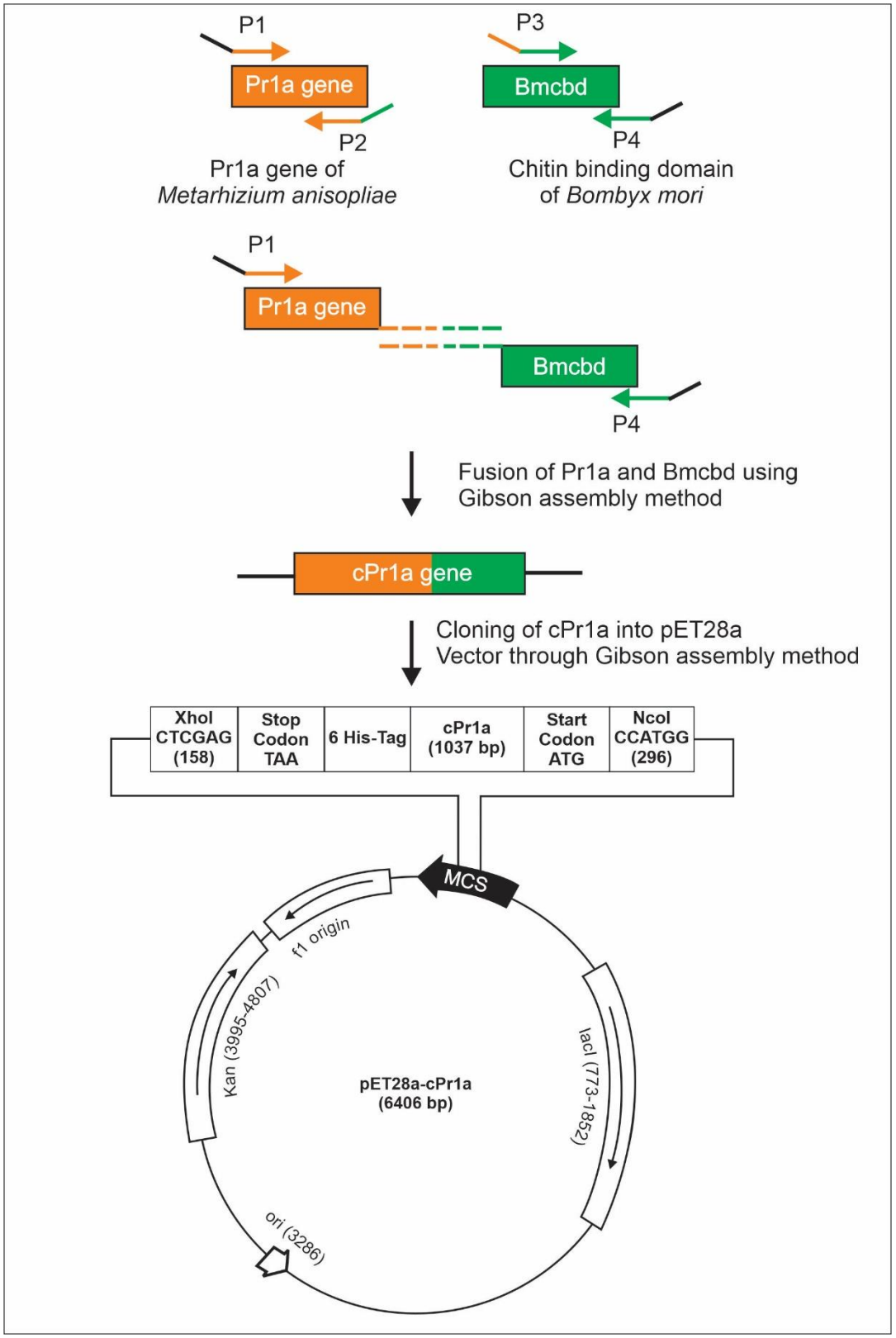

Fig. 2.2.2 - Pictorial illustration of construction and cloning of chimeric cPr1a gene into pET-28a vector.

2.2.8 Amplification of *Bombyx mori* chitin-binding domain with Pr1a overhang and Pr1a gene with *Bombyx mori* chitin-binding domain overhang

The DNA sequence of the *Bombyx Mori* Chitin binding domain was obtained from National Center for Biotechnology Information (NCBI) database with the accession number: AF273695.1. Bmpr1a was amplified using cDNA clones of *Bombyx mori* chitinase (fepM03A03) as a template and gene-specific primers. The reaction conditions followed for amplification were – step 1: Initial denaturation 98ºC for 2 mins, step 2: Denaturation 98ºC for10 sec, step 3: Annealing 70ºC for 30 sec, step 4: Extension 72ºC for 15 sec and step 5: Final extension 72ºC for 10 min. The Pr1a gene was reamplified using the old forward primer and new reverse primer in order to introduce a compatible end with bmcbd (cPr1a).

2.2.9 Analysis of amplified fragments by agarose gel electrophoresis

The amplified Pr1a DNA bands were analyzed using agarose gel electrophoresis. 1% gel was prepared by dissolving 500 mg of agarose powder in 50 mL of 1x TAE buffer, and the mixture was heated in a microwave until it became a clear solution. Next, 2.0 µl ethidium bromide (EtBr, 10 mg/mL) was added to the solution when the temperature reached approximately 40°C, and the solution was mixed by swirling and then filled into the casting tray containing appropriate combs. Amplified DNA fragments were mixed with loading dye, and this DNA-dye mixture was then loaded into wells of agarose gel, and the gel was run in 1x TAE buffer at 80 v for 1 h. The bands were then seen in a gel documentation system under UVlight.

2.2.10 DNA loading dye recipe

The ingredients listed in Table 2.2.4 were mixed to prepare the DNA-loading dye. The final pH of the loading buffer was set to 7.5.

2.2.11 Construction and cloning of chimeric cPr1a gene

The Pr1a gene was fused to *Bombyx mori* chitin-binding domain (Bmpr1a) using the Gibson assembly method. Amplified products were segregated on 1% agarose gel and purified using a nucleospin gel extraction kit (Macherey Nagel), GmBH. The pET-28a plasmid was digested with NcoI and XhoI restriction enzymes (New England Biolabs, U.S.). Digested plasmid products were separated on 1% agarose gel electrophoresis and purified using a Nucleospin gel extraction kit. cPr1a construct was cloned into NcoI and XhoI digested pET- 28a plasmid using the Gibson assembly method, and the reaction was performed at 50ºC for 60 min.

$$\frac{\text{Vector (ng)} \times \text{size of insert (kb)}}{\text{size of vector (kb)}} \times \text{insert: vector molar ratio} = \text{Mass of insert (ng)}$$

$$\frac{50 \text{ ng} \times 0.91 \text{ kb}}{5.369 \text{ kb}} \times 3{:}\ 1 = 25.4 \text{ ng of insert (Pr1a)}$$

$$\frac{50 \text{ ng} \times 1.035 \text{ kb}}{5.369 \text{ kb}} \times 3{:}\ 1 = 28.9 \text{ ng of insert(cPr1a)}$$

2.2.12 *E. coli* (Top-10) Competent cells Preparation

E. coli cells were made chemically competent by using two different concentrations of calcium chloride solutions following the protocol by Cohen et al. (1972) with diminutive modifications.

1. A single Top-10 colony was inoculated into 5mL LB broth and incubated for 12 h at 37ºC, 200 rpm, in a rotatory incubator shaker.
2. 0.1% of overnight grown culture was inoculated into 200 mL of LB broth and incubated at 37ºC in an incubator shaker until the optical density of the culture reached up to OD_{600} 0.3-0.5.
3. Post 2-3 h of incubation, cells were kept on ice for half an hour and subsequently collected by centrifugation at 8000 rpm for 15 min at 4ºC.
4. The cell pellet collected was washed with 100 mM $CaCl_2$ and incubated in ice-cold 100 mM $CaCl_2$ for 30 mins. Post incubation, the cell pellet was collected by centrifugation and resuspended in 1.0 ml of ice-cold 200 mM $CaCl_2$ prepared with 50% glycerol.
5. A 50 µl cell aliquots were made in 1.5mL vials and stored in a -80ºC freezer till further use.

2.2.13 *E. coli* Top-10 Transformation

The recombinant plasmid constructs (pET-28a: Pr1a & pET-28a: cPr1a) were made using the Gibson assembly method and immediately transformed into Top-10 competent cells. The transformation protocol involves the following steps:

1. The microcentrifuge tube containing the competent cell (50 µl) was removed from the -80°C refrigerator, thawed on ice for 10 minutes, and then given 5 µl of the Gibson mixture containing recombinant plasmid construct was added to the competent cells. The mixture of cells and plasmids was mixed gently and kept on ice for 30 minutes.
2. Heat shock was given to the cells by keeping the tubes in a water bath at 42°C for 1

minute.

3. The cells were immediately transferred to ice and kept there for 3 minutes.

4. One mL of sterile LB media was poured into the tubes containing transformed cells and incubated at 37°C, 180 rpm for an hour.

5. The cell pellet was harvested by centrifugation at 8000 rpm for 2 minutes.

6. The cell pellet was resuspended in 100 µl of media after discarding 900 µl of media and spread on LB agar plates supplemented with kanamycin (50 µg/mL).

7. The plates were incubated at 37°C for 12 – 14 h.

8. Transformation efficiency was calculated by using the following formula.

$$\text{Transformation efficiency} = \frac{\text{Total no. of transformants} \times 1000}{\text{ng of plasmid DNA insert}} = \text{CFU/µg}$$

2.2.14 Plasmid DNA isolation

Isolation of plasmid DNA from transformants was done by a modified alkaline lysis method with some modifications in Bimboim and Doly's (1979) protocol.

1. Colonies were picked and Inoculated in 5 mL of LB broth and incubated for 12 – 15 h at 37ºC at 180 rpm in an orbital shaker incubator. The cell pellet was collected by centrifugation of overnight grown cultures at 10,000 rpm for 30 sec, at 4ºC. The cell pellets were resuspended in 200 µl Resuspension buffer (50 mM glucose, 25 mM Tris- Cl (pH 8.0), 10 mM EDTA (pH 8.0), and kept for 5 mins at room temperature.

2. 200 µl of freshly prepared Lysis buffer (0.2 N NaOH, 1% (w/v) SDS) was added to the culture and incubated for 2 mins to 3 mins.

3. Renaturation of plasmid DNA was done by the addition of 300 µl of Neutralization buffer (5 M potassium acetate, glacial acetic acid) to the suspension, followed by incubation at room temperature for 5 min

4. The lysate was centrifuged at 12,000 rpm for 10 min at 4ºC, and the upper aqueous layer

was transferred to fresh microcentrifuge tubes, followed by the treatment with RNase-A at 37ºC for 30-60 min.

5. Post-RNase treatment, 200 µl of ice-cold Chloroform was added into the aqueous solution and vortexed briefly. After centrifugation, the upper aqueous layer was extracted and added with 0.8 volume of ice-cold isopropanol and kept for 10 mins at room temperature, centrifuged at 12,000 rpm, for 10 min at 4ºC.

6. DNA pellet obtained from centrifugation was washed with 70% ice-cold ethanol. The plasmid DNA pellet was dissolved in 50 µl nuclease-free water and analyzed on 1% agarose gel electrophoresis.

2.2.15 Screening of Pr1a & cPr1a positive clones using PCR method

The confirmation of positive clones was done by PCR method using gene-specific primers. Plasmids isolated from the Pr1a & cPr1a transformants were used as templates for PCR reactions. PCR was performed using Taq DNA polymerase (New England Biolabs). Analysis of PCR amplified products was done on 1% agarose gel.

2.2.16 Confirmation of Pr1a & cPr1a positive clones using restriction digestion method

Restriction digestion analysis was performed in a microcentrifuge tube containing plasmid DNA. The double digestion of recombinant plasmid DNA was done in a cut smart reaction buffer with NcoI and XhoI restriction enzymes NEB) to check the desirable positive clones. The reaction component's details are given in **Table 2.2.12**. The reaction mixtures were incubated at 37 ºC for 2 h. Analysis of digested products (pET-28a, Pr1a & cPr1a inserts) was done on 1% agarose gel. Gel imaging was done using the ChemiDoc XRS™ gel. documentation system (Bio-Rad, USA). To confirm the size of the DNA insert and the vector pET-28a, the

digested fragments (insert and vector) were analyzed, and the positive clones wereidentified.

2.2.17 *E. coli* (BL21 DE3) competent cells preparation

E. coli (BL21 DE3) competent cells were prepared using the same protocol as described in section 2.2.12.

2.2.18 Transformation of pET-28a-Pr1a & pET-28a-cPr1a constructs into the expression host

The plasmid pET-28a-Pr1a and pET-28a-cPr1a were transformed into bacterial expression host *E. coli* (BL21 DE3) using the chemical transformation method. The selection was carried out on LB agar plates supplemented with kanamycin (50 µg mL^{-1}) and incubated at 37ºC for 12-14 h. After 12 h, the colonies were chosen at random from LB agar plates and inoculated into 5.0 ml of LB medium supplemented with 50 µg/mL kanamycin.

2.2.19 Pr1a and cPr1a Protein Expression

A single colony of *E. coli* (BL21 DE3) carrying the pET-28a-Pr1a and pET-28a-cPr1a was used to inoculate 5 mL of LB broth supplemented with kanamycin (50 µg mL^{-1}) and incubated overnight at 37ºC at 200 rpm. One percent of overnight grown culture was used to inoculate the expression medium. Bacterial culture was grown until OD_{600} reached 0.6 at 37ºC. The Pr1a protein expression was carried out using LB Broth (Tryptone 12 g l^{-1}, yeast extract 5g l^{-1}, NaCl10 g l^{-1}). To overexpress the protein, protein induction was done with 1.0 mM isopropyl-1-thio-β-D- galactopyranoside (IPTG), and followed by induction, the cultures were incubated for 16h at 16°C in a refrigerated incubator shaker at 200 rpm. One mL of cultures was collected from uninduced and induced samples and was centrifuged at 13000 rpm for 5 mins to harvest the cell pellet. Subsequently, the cell pellet was resuspended in 50 µl buffer, and the protein expression

analysis was done by SDS-PAGE. The purification of proteins was done using Ni-NTA affinity chromatography.

2.2.20 Pr1a and cPr1a protein affinity purification

Post-incubation, the cell pellets of Pr1a and cPr1a were harvested by centrifugation of culture at 10,000 rpm for 30 min at 4ºC, and the cell pellet was resuspended in binding buffer A (40 mM Tris-Cl, 400 mM NaCl, 10 mM Imidazole, 10% Glycerol). The Vibra Cell Sonicator was used for the lysis of cells with 4-sec pulse ON and 20-sec pulse OFF conditions. The lysate was centrifuged at 13,000 rpm for 1 h at 4ºC, and the clarified supernatantsolution was loaded into Nickel- Nitrillo Triacetic Acid (Ni-NTA) affinity chromatography column pre-equilibrated with binding buffer A. Different fractions were eluted with Elution buffer containing 400 mM imidazole and analyzed using 15% Sodium Dodecyl Sulfate- Poly Acrylamide Gel electrophoresis (SDS-PAGE). Gel imaging was done using a Biorad Gel Doc EZ gel documentation system and analyzed with Image Lab software 4.0 from Biorad.

2.2.21 Protein expression analysis of recombinant proteins Pr1a and cPr1a using Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

2.2.21.1 Polymerization of acrylamide gel for SDS-PAGE.

The SDS-PAGE gel was prepared using the following components: milli-q water, acrylamide (30% w/v), Tris-HCl, pH 8.8 (resolving gel), Tris-HCl, pH 6.8 (stacking gel), SDS (10% w/v), APS (10% w/v), TEMED. Using the composition listed in **Tables 2.2.9** and **2.2.10**, the resolving gel and stacking gels were prepared in accordance with the methods from Sambrook et al. (1989). In a 50 mL falcon, the ingredients were added in the order listed in table 2.2.9 to form the resolving gel while maintaining an acrylamide concentration of 12 percent

(w/v). In a similar manner, the stacking gel (4%, w/v) was created by dissolving all the elements listed in Table 2.2.10.

2.2.21.2 10x SDS-PAGE running buffer preparation

A 10x stock solution of SDS running buffer was prepared by mixing the Tris base, glycine, and SDS. The buffer was filtered using Whatman filter paper (No. 1), and the buffer was stored at room temperature.

2.2.21.3 Preparation of SDS loading dye

The 4x SDS loading dye is prepared by mixing all the components listed in the following table. The final pH was adjusted to 6.8. The final concentration while loading to the SDS-PAGE gel was always kept to 1x by mixing three volumes of protein sample with one volume of 4x loading dye.

2.2.21.4 Staining and De-staining solution preparation

To prepare 100 mL of the staining solution, 0.25% of Coomassie brilliant blue R-250 (250 mg) was dissolved in a mixture of a solution containing 10 mL glacial acetic acid, 40 mL methanol, and 50 mL water (1:4:5). The staining solution was stored at room temperature in an amber colored bottle. The de-staining solution was made by dissolving glacial acetic, methanol, and deionized water in a 1:3:6 ratio; the gels were de-stained by submerging them in a de-staining solution while gently shaking them until the protein bands were clear.

2.2.22 Quantitative analysis of protein

Bradford's method of estimating proteins at a wavelength of 595 nm was used to determine the concentrations of the purified recombinant proteins (Bradford, 1976). The standard protein was bovine serum albumin (BSA), which was purchased from Sigma-Aldrich Co. LLC in

the United States. A standard plot was created by measuring the O.D. of different concentrations (10 – 100 µg/mL) of BSA protein at 595 nm.

2.2.23 Cuticle preparation

The Eri silkworm *(Philosamia ricini)* fourth instar larvae were obtained from the local market of Guwahati. The larvae were kept in a refrigerator at -20°C overnight. The next day larvae were taken out from the refrigerator. Larvae were pinched by the fingers in order to expelall the visceral organs from their body. Subsequently, the cuticles obtained were washed thoroughly under tap water, and finally, the second washing of cuticles was done with distilled water. Washed cuticles were kept in the oven for 5 h for drying. The dried cuticles were grounded to get a coarse powder.

2.2.24 Binding assays

Binding assays were performed in 1.5 mL Eppendorf tubes containing 5mg of cuticle powder in 1 mL of 50 mM potassium phosphate buffer (pH-6.0) and 2µl (100µg) of Pr1a and cPr1a enzymes. The mixture was incubated on ice for 4 mins. Subsequently, the mixture was centrifuged at 10000 rpm for 5 mins to separate the solid cuticle and supernatant. The amount of unbound enzyme was determined from the supernatant using Lowry's method of protein estimation. The amount of cuticle-bound enzyme was calculated from the difference between the initial amount of enzyme taken for the assay and the amount of unbound enzyme present in the supernatant.

2.2.25 Protease enzyme assays

The activity of the protease enzyme was assayed by UV absorbance protocol using *Philosamia ricini's* cuticle as a substrate. The enzyme assays were performed in a 1.5 mL

microcentrifuge tube containing 1 mL potassium phosphate buffer (pH - 6.0) and 5mg of cuticle powder, and 5µl (250µg) of Pr1a and cPr1a enzymes. The mixtures were incubated at 37°C for 4 mins, and after incubation, mixtures were centrifuged at 10000 rpm for 1 min to collect the supernatant. The absorbance was measured by a UV-Visible spectrophotometer at 280 nm A_{280}. To estimate the amount of protein in the reaction mixture, a standard plot of L-tyrosine was created.

2.2.26 L-tyrosine standard curve preparation

The standard solutions of L-Tyrosine were prepared by dissolving the varying concentrations (10 – 100 µg/mL) of tyrosine in 50 mM phosphate buffer pH- 6.0. One mL of solution was added to the cuvette, and the absorbance was measured in a UV-visible spectrophotometer at 280 nm wavelength. A standard plot of L-tyrosine was generated using the O.D.'s of different concentrations of tyrosine. One equivalent of tyrosine was determined, and to calculate the enzyme activity, 1 A_{280} equivalent of L-Tyrosine (µg/mL) was converted to mg/mL.

2.2.27 Enzyme activity calculation

The enzyme activities were expressed as U/mL (µmol/min/mL). One unit (U) of enzyme activity is defined asthe amount of an enzyme that liberates 1 µmole of tyrosine per min.

2.2.28 Sequence analysis

Multiple sequence alignment of sequences of various proteases was done using Clustal omega software, and a cladogram was generated using the Ebi website.

2.2.29 Homology modeling

Homology modeling of Pr1a and Bmcbd proteins was done using PHYRE2 software and UCSF chimera software was used to fuse the 3D structures of Pr1a and Bmcbd proteins in order

to get a whole 3D structure of the chimeric cPr1a protein.

2.2.30 Statistical analysis

` All the experiments were done three times independently in triplicates. Student's t-test was used to statistically analyze the mean of three replicates of all enzyme and binding assays.

2.3 Results

2.3.1 Amplification of Pr1a & cPr1a using polymerase chain reaction(PCR)

2.3.1.1 Amplification of wild-type Pr1a protease

In this study, the fungal spores were harvested from the PDA (Potato dextrose agar) petri plate and inoculated into the SDB (Saboraud dextrose broth) for 48 hours. The fungal mycelia were harvested from the SDB, washed twice with sterile distilled water, and transferred into PIM (Protease induction media). The total RNA was isolated using the Trizol method from the *Metarhizium anisopliae* fungal mycelia harvested from protease induction media after 24 h from inoculation. The three distinct bands of 28s rRNA, 18s rRNA, and 5srRNA were confirmed by agarose gel electrophoresis **(Fig 2.3.1)**. The RNA was quantified before its conversion into cDNA **(Table 2.3.1)**. The Pr1a gene was amplified employing the PCR method with forward and reverse primers containing NcoI and XhoI restriction enzyme overhangs at the 5' and 3' end respectively using the cDNA as a template DNA. The cycling conditions used for the amplification of the Pr1a gene are described in **(Table 2.2.2)**. The confirmation of successful amplification of the Pr1a gene from *Metarhizium anisopliae* was obtained by observing the presence of an expected DNA band with a size of 910 base pairs (bp) **(Fig. 2.3.2)**. The PCR amplicons were extracted from the gel using the Nucleospin gel extraction kit, and a sharp, intense, and purified band of Pr1a gene product was analyzed using agarose gel electrophoresis **(Fig 2.3.3)**. The purified Pr1a amplicons were stored at -20°C for subsequent molecular cloning.

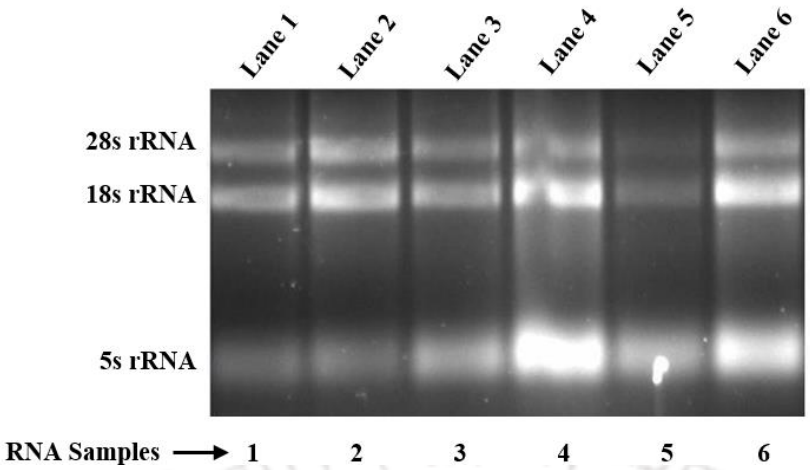

Fig 2.3.1 - Agarose gel analysis showing total RNA isolated following the Trizol method from *Metarhizium anisopliae* mycelia harvested from protease induction medium post 24 h of inoculation.
Three distinct bands corresponding to 28S and 18S & 5S ribosomal RNA

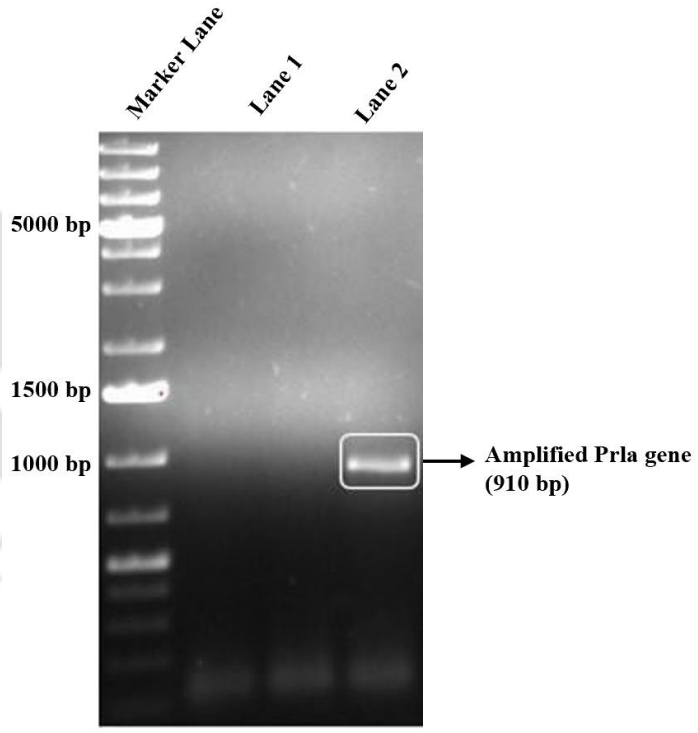

Fig 2.3.2 – Agarose gel electrophoresis analysis of Pr1a gene (910 bp) amplified from the cDNA of *Metarhizium anisopliae* using polymerase chain reaction with gene-specific primers at annealing temperatures, 64°C (Lane 1), 66°C (Lane 2), 68°C (Lane 3).
Total RNA was isolated from the mycelia harvested from PIM (Protease induction media) & reverse transcribed into cDNA
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific, 75 bp to 20 kbp).

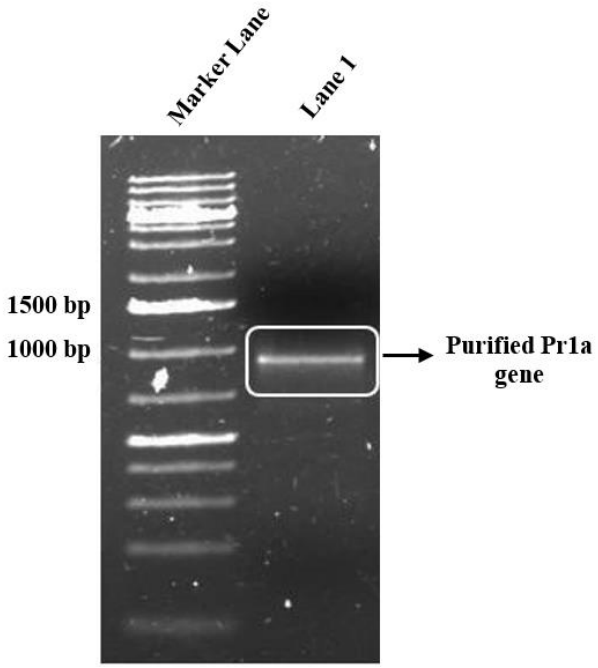

Fig 2.3.3 - Agarose gel electrophoresis analysis of gel-purified DNA fragments of Pr1a gene.
1% agarose gel was used for electrophoresis to resolve the gel-purified DNA fragments of Pr1a gene.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).

2.3.2 Restriction enzyme digestion of pET-28a plasmid

The pET-28a is a widely used expression plasmid that is highly popular in molecular biology research. This plasmid is designed for expression in prokaryotic systems and offers high efficiency. It features the T7 promoter, which enables strong transcription initiation, along with an adjacent lac operator sequence. The lac operator sequence plays a crucial role in suppressing expression when the system is not induced. Furthermore, pET-28a includes a kanamycin resistance gene, making it suitable for selection and maintenance in host cells. The pET-28a plasmids were isolated from Dh5α bacterial cells following the modified alkaline lysis protocol described in section **2.2.14**. The isolated plasmids (5369 bp) were analyzed on 1% agarose gel, and the three separate bands of plasmid DNA in lanes 1, 2, and 3 of the gel indicated the three different forms of pET-28a plasmids (**Fig 2.3.4**). To prepare the pET-28a plasmids for cloning, they were digested with the NcoI and XhoI restriction enzymes. The digested fragment of pET-28a was further purified from the gel using the Nucleospin gel extraction and visualized using

the Bio-Rad Gel Doc™ XR gel documentation system. A single linearized band of the digested pET-28a plasmid (5.3 kbp) is shown in lane 2 (**Fig 2.3.5).**

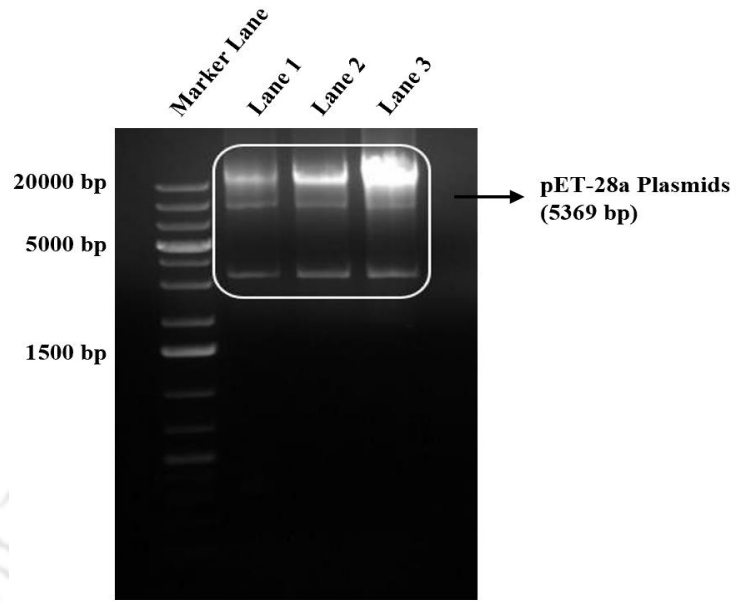

Fig 2.3.4 - Agarose gel electrophoresis analysis of pET-28a plasmid isolated from DH5α cells. Three forms of the plasmid DNA can be observed in the gel picture, Supercoiled form, nicked circular form & Linear form.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).

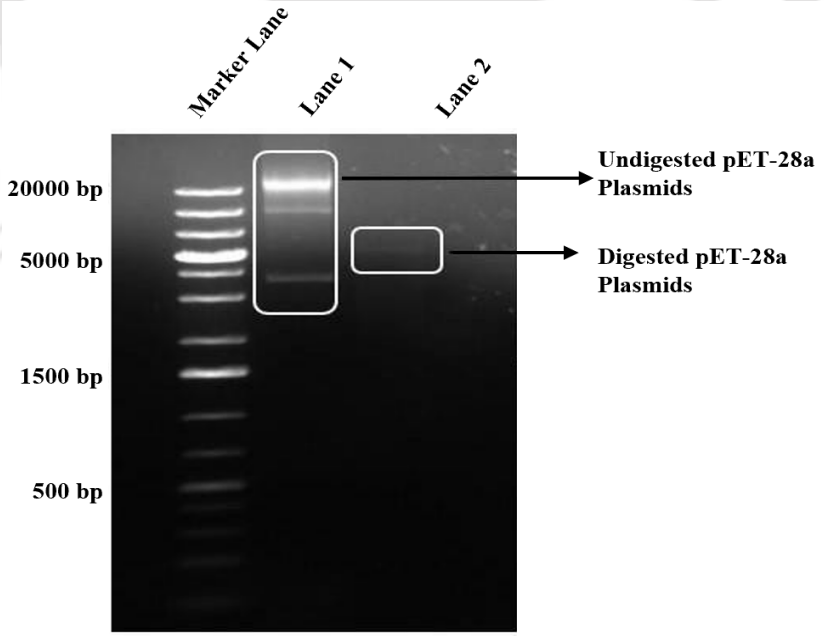

Fig 2.3.5 – Agarose gel electrophoresis image of pET-28a plasmid following restriction enzyme digestion reaction using NcoI and XhoI restriction enzymes.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 – Three bands (Nicked, linear & supercoiled) of Undigested pET-28a plasmid.
Lane 2 – NcoI & XhoI digested single band of pET-28a plasmid.

2.3.3 Amplification of cPr1a chimeric protease

The construction of the chimeric cPr1a gene requires the fusion of the Pr1a gene from *Metarhizium anisopliae* with the chitin-binding domain of *Bombyx mori.* In order to construct a chimeric protease, the 3' end of the Pr1a gene and the 5' end of Bmcbd must have compatible ends. The Pr1a gene was amplified using a set of gene-specific primers so as to introduce a compatible chitin-binding domain overhang at the 3' end of the Pr1a gene (882 bp). The chitin-binding domain of *Bombyx mori* (Bmcbd) was amplified from *Bombyx mori* chitinase cDNA clone construct (fepM03A03) using gene-specific primers with a Pr1a overhang (Pr1a overhang - Bmcbd) at 5' end of Bmcbd. The confirmation of the successful amplification of the Pr1abm and Bmpr1a fragments was achieved by detecting the presence of DNA bands of the anticipated sizes 882 bp and 201 bp, respectively, based on the results of the agarose gel electrophoresis analysis **(Fig. 2.3.6)**. The amplified products (Pr1abm and Bmpr1a) were purified from the gel using Nucelospin gel and PCR clean up kit. Purified bands of Pr1abm (882 bp) and Bmpr1a (210 bp) were observed on 1% agarose gel using the gel electrophoresis technique **(Fig. 2.3.7)**. The purified Pr1abm and Bmpr1a amplicons were stored at -20°C for construction and cloning of cPr1a.

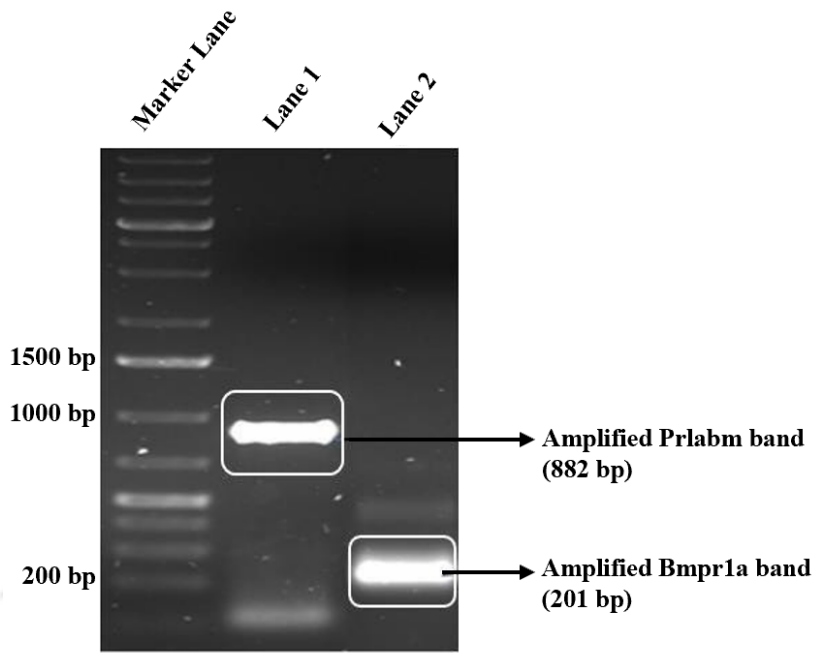

Fig 2.3.6 - Agarose gel electrophoresis analysis showing PCR amplified fragments of Pr1abm (Pr1a gene with *Bombyx mori* chitin-binding domain overhang at 3' end and Bmpr1a (*Bombyx mori* chitin-binding domain with Pr1a overhang at 5' end.
1% agarose gel was used to resolve the PCR products
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 – Pr1abm fragment (882 bp).

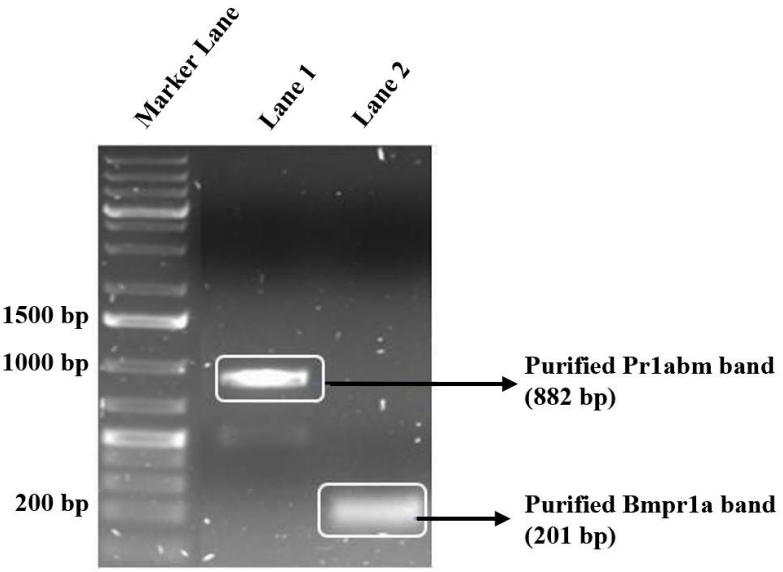

Fig 2.3.7 - Agarose gel showing gel-purified fragments of Pr1abm and Bmpr1a.
Pr1abm and Bmpr1a fragments were purified using nucleospin gel purification kit.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 – Purified Pr1abm fragment (882 bp).
Lane 2 – Purified Bmpr1a fragment (201 bp).

2.3.3.1 Transformation of recombinant plasmid DNA (pET-28a-Pr1a) and (pET-28a-cPr1a) into *E. coli* (Top-10) competent cells

The cloning of the wild-type Pr1a gene (910 bp) and chimeric cPr1a gene (1037 bp) into the NcoI & XhoI restriction enzymes digested pET-28a (5.3 kbp) vector was done using the Gibson assembly method resulting in pET-28a-Pr1a (6.2 kbp) and pET-28a-cPr1a (6.4 kbp) vector-insert system. The recombinant vector-insert constructs were transformed into the *E. coli* (Top-10) competent cells. The transformants were grown on LB agar plates supplemented with kanamycin at 37°C for 12 -14 hours under static conditions **(Fig. 2.3.8 & Fig 2.3.9).** Colonies were picked up and inoculated into a 5 mL LB medium for isolation of plasmids from them. The screening and confirmation of recombinant plasmids (positive clones) was done using restriction digestion and PCR method.

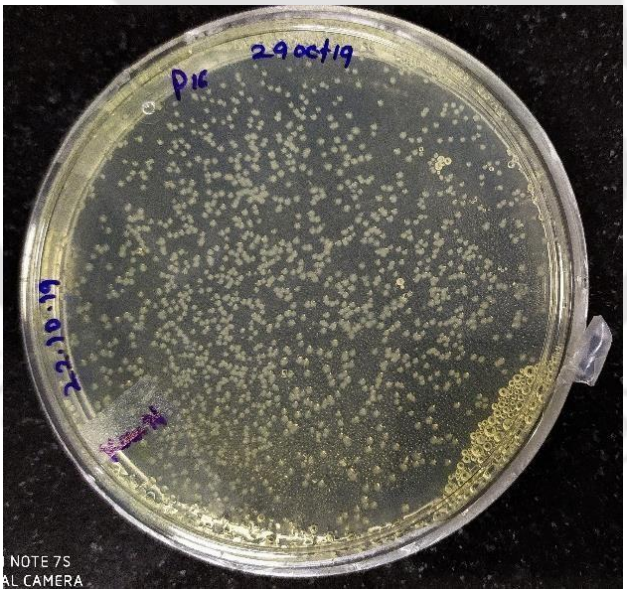

Fig 2.3.8 - The Top -10 cells were transformed with pET-28a-Pr1a construct. The figure shows the Top 10 colonies carrying the pET-28a-Pr1a construct, appeared on the LB agar plate (supplemented with kanamycin (50 µg /mL) after transformation followed by incubation at 37°C for 14 h.

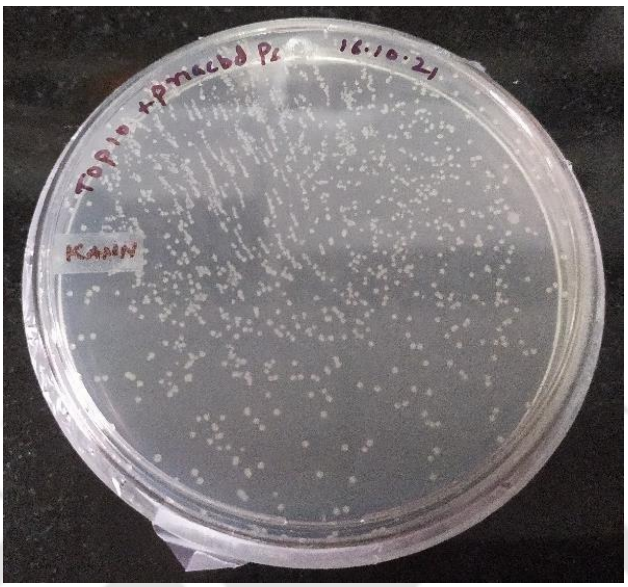

Fig 2.3.9 - The Top -10 cells were transformed with pET-28a-cPr1a construct. The figure shows the Top 10 colonies carrying the pET-28a-cPr1a construct, appeared on the LB agar plate (supplemented with kanamycin (50 µg /mL) after transformation followed by incubation at 37°C for 14 h.

2.3.3.2 Plasmid DNA isolation from Pr1a & cPr1a transformants and verification of positive clones

The plasmid DNA was isolated from the Pr1a and cPr1a transformant colonies following the modified alkaline lysis method described in section **2.2.14**. The recombinant plasmids DNA isolated from the transformants were analyzed on 1% agarose gel **(Fig 2.3.10, Fig 2.3.13, and Fig. 2.3.14).** The recombinant plasmids were subjected to screening using both PCR and restriction digestion methods to identify the positive clones. To confirm the presence of positive clones, the isolated recombinant plasmids were subjected to digestion using NcoI and XhoI restriction enzymes. The plasmid, following the restriction digestion, was subjected to electrophoresis on a 1% agarose gel to visualize the DNA fragments based on their size. The linearized pET-28a plasmid was observed to have a size of approximately 5.3 kilobases (kb)

when analyzed on an agarose gel. The release of 3 parts of Pr1a and cPr1a inserts from the vector's backbone of 6.2 kb (217 bp, 467 bp & 184 bp) and 6.4 kb (217 bp, 467 bp & 332 bp) confirmed the cloning of Pr1a and cPr1a in the pET-28a vector. The agarose gel results showed that the recombinant plasmids obtained from 4th, 9th, and 11th Pr1a colonies showed four DNA bands equivalent to the sizes of the pET-28a vector (5369 bp) and Pr1a (217 bp, 467 bp & 184 bp). There are two NcoI restriction sites inside the Pr1a gene, one at 236 bp and another at 703 bp; therefore, instead of one band of 910 bp, three bands of 217 bp, 467 bp & 184 bp were observed, verifying that Pr1a was successfully cloned into the pET-28a vector **(Fig. 2.3.11)**. The agarose gel analysis of digested cPr1a recombinant plasmids showed that the 1st to 9th, 15th to 17th, and 20th to 23rd cPr1a transformant colonies carrying the positive clones. Four DNA bands obtained after the restriction digestion of cPr1a plasmids, and those are corresponding in size to the pET28a vector (5369 bp) and cPr1a (217 bp, 467 bp, and 332 bp) **(Fig. 2.3.17 and Fig 2.3.18)**. The second confirmation of positive clones was done using the polymerase chain reaction (PCR) method. The plasmids extracted from the colonies of Pr1a and cPr1a transformants were individually subjected to PCR amplification using specific primers designed for the Pr1a and cPr1a genes, respectively. The resulting PCR amplicons were then analyzed by running them on a 1.0 % agarose gel. The electrophoresed amplified products showed that 1st to 12th Pr1a plasmids, and 1st to 16th cPr1a plasmids, carry the Pr1a gene and cPr1a genes, respectively **(Fig 2.3.12, Fig 2.3.15, and Fig 2.3.16)**. The Pr1a and cPr1a colonies carrying the positive clones were grown in 10 mL LB media, and plasmids were isolated from them and stored at -20°C for further protein expression experiments.

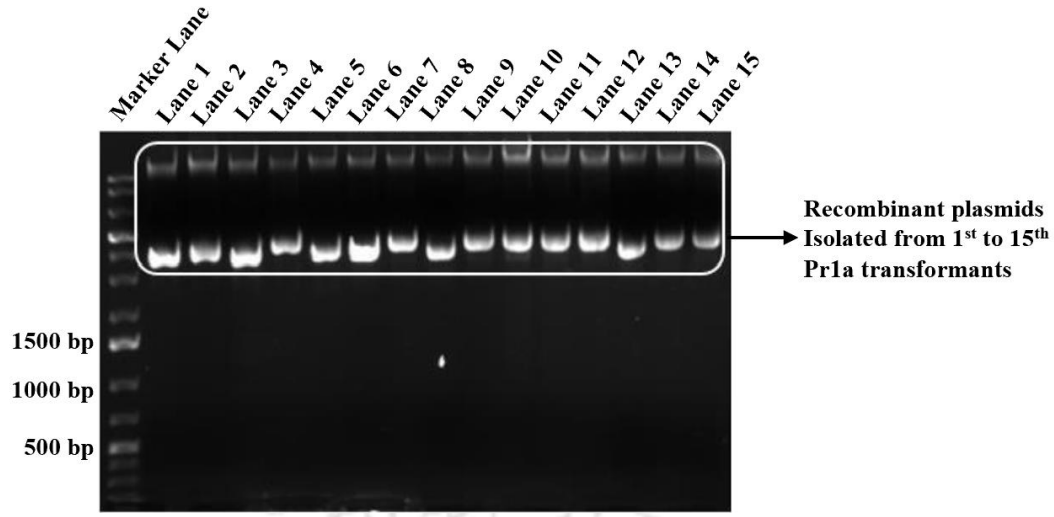

Fig 2.3.10 - Agarose gel electrophoresis analysis showing recombinant plasmids isolated from Pr1a transformants.
E. coli Top 10 cells were transformed with the pET-28a-Pr1a plasmid and selected on LB agar plates containing kanamycin.
Plasmids were isolated from selected transformants and resolved using 1 % agarose gel electrophoresis.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 15 – Recombinant plasmids isolated from Pr1a transformants 1st to 15th.

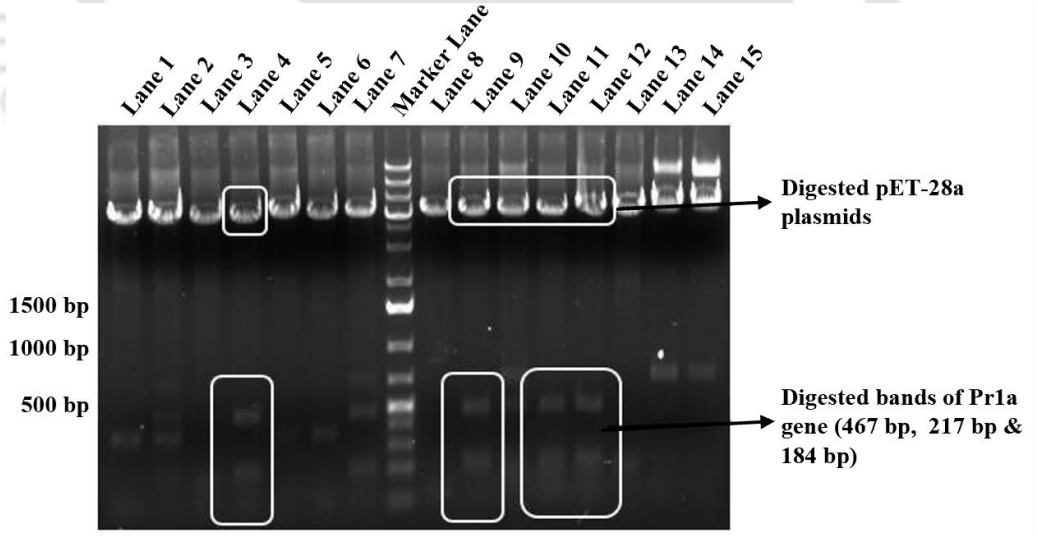

Fig 2.3.11 – Agarose gel electrophoresis image of clone confirmation analysis by restriction enzyme digestion.
Clones (pET-28a - Pr1a) digested with NcoI and XhoI enzymes.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 4, 9, 11 & 12 - Clone's 4th, 9th, 11th & 12th are showing the Pr1a gene insert.
The Pr1a gene has two NcoI restriction sites at 213 bp and 680 bp, giving three bands 467 bp, 217 bp, and 184 bp instead of a single 910 bp band.

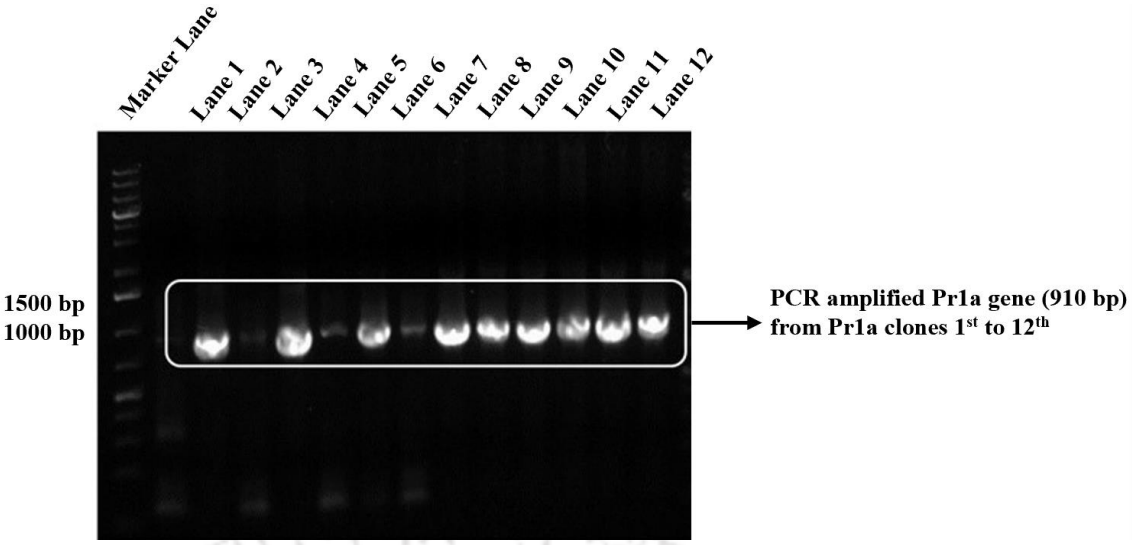

Fig 2.3.12 - Agarose gel analysis showing PCR amplified Pr1a gene (910 bp) fragments from Pr1a clones.
PCR confirmation for positive clones was done using Taq DNA polymerase and Pr1a gene-specific primers, utilizing plasmid DNA isolated from Pr1a clones 1st to 12th as templates.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 2 to 13 – Pr1a clone's 1st to 12th are showing Pr1a gene insert.

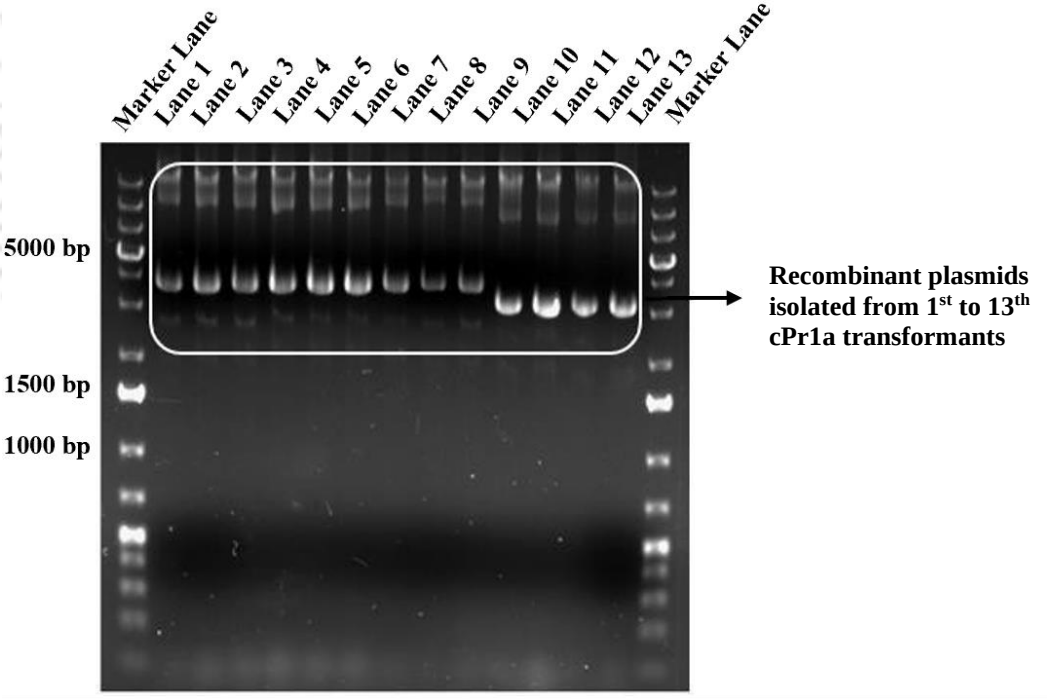

Fig 2.3.13 - Agarose gel analysis showing PCR amplified cPr1a gene (1037 bp) fragments from cPr1a clones.
PCR confirmation for positive clones was done using Taq DNA polymerase and cPr1a gene-specific primers, utilizing plasmid DNA isolated from cPr1a clones 1st to 13th as templates.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 13 – Recombinant plasmids isolated from 1st to 13th cPr1a clones.

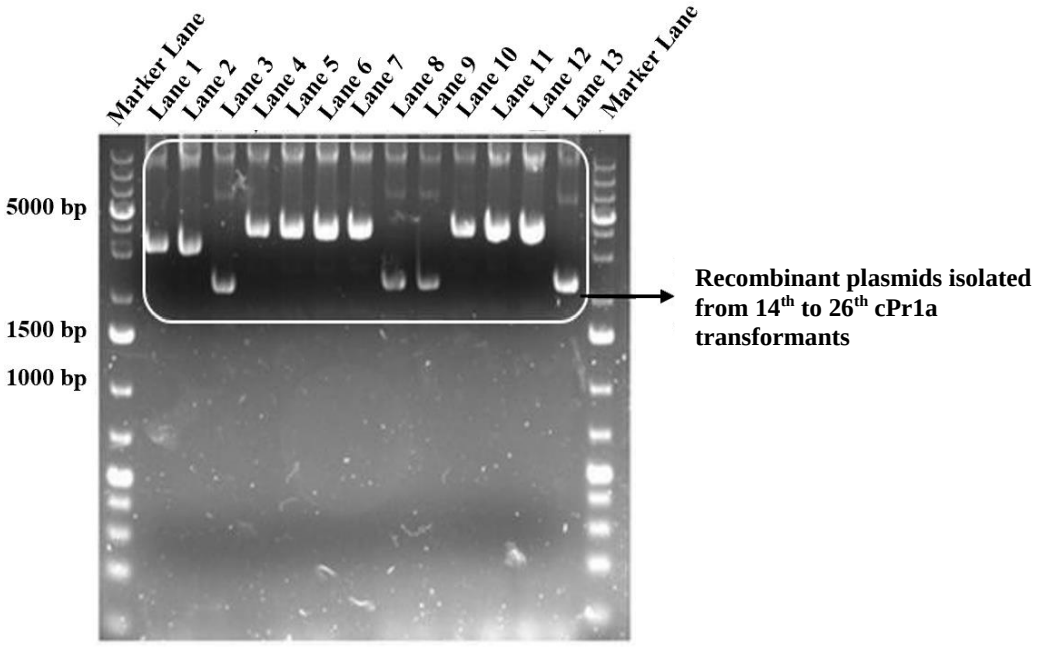

Fig 2.3.14 - Agarose gel analysis showing PCR amplified cPr1a gene (1037 bp) fragments from cPr1a clones.
PCR confirmation for positive clones was done using Taq DNA polymerase and cPr1a gene-specific primers, utilizing plasmid DNA isolated from cPr1a clones 14th to 26th as templates.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 13 – Recombinant plasmids isolated from 14th to 26th cPr1a clones.

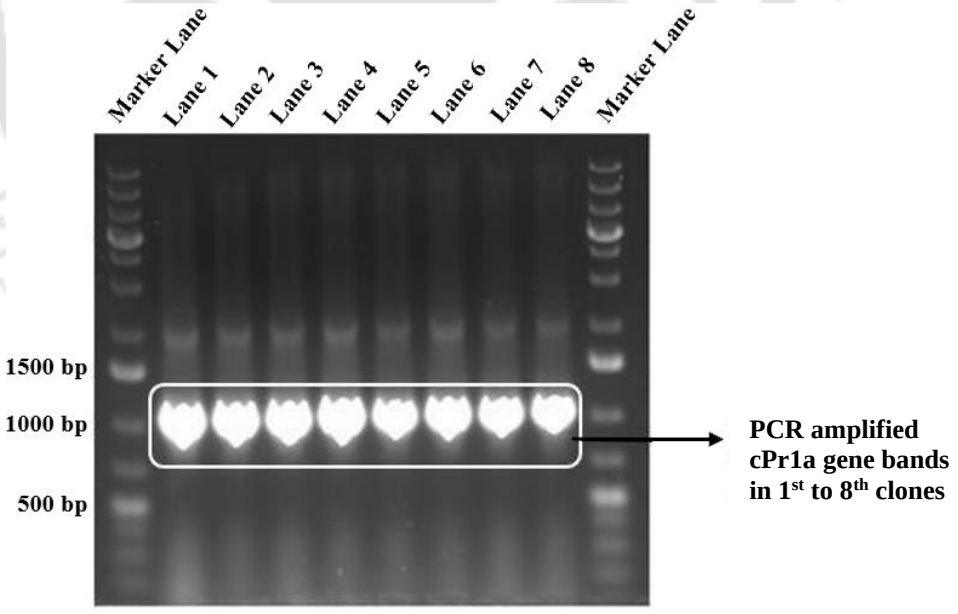

Fig 2.3.15 – Agarose gel analysis showing PCR amplified cPr1a gene (1037 bp) fragments from cPr1a clones.
PCR confirmation of positive clones was done using Taq DNA polymerase and cPr1a gene-specific primers, utilizing plasmid DNA isolated from cPr1a clones 1st to 8th as templates
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 8 – PCR amplified cPr1a gene band in clone's 1st to 8th.

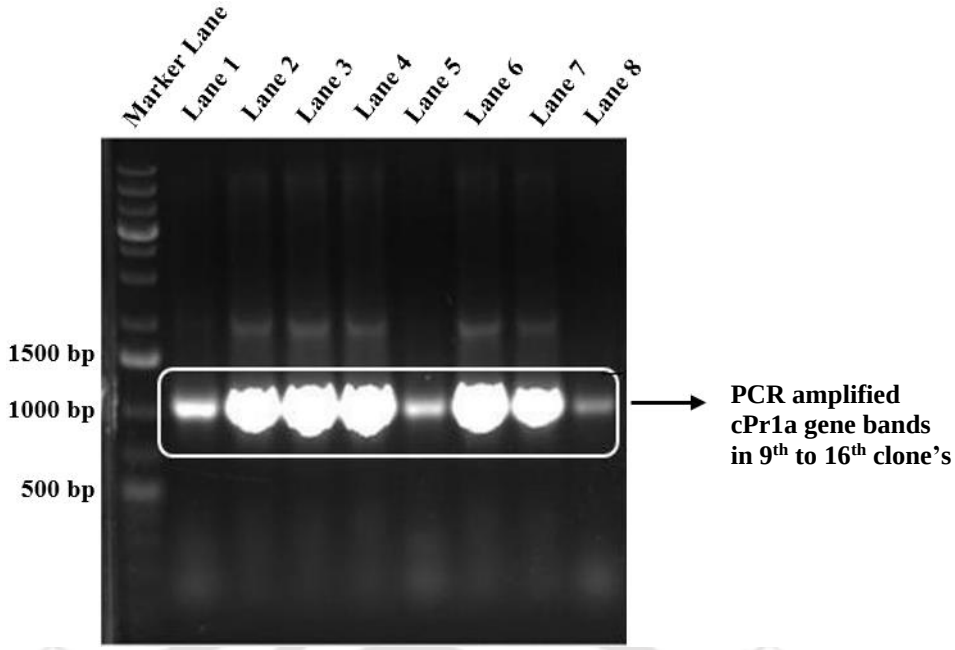

Fig 2.3.16 – Agarose gel analysis showing PCR amplified cPr1a gene (1037 bp) fragments from cPr1a clones.
PCR confirmation of positive clones was done using Taq DNA polymerase and cPr1a gene-specific primers, utilizing plasmid DNA isolated from cPr1a clones 9th to 16th as templates.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1st to 8th – PCR amplified cPr1a gene band in clone's 9th to 16th.

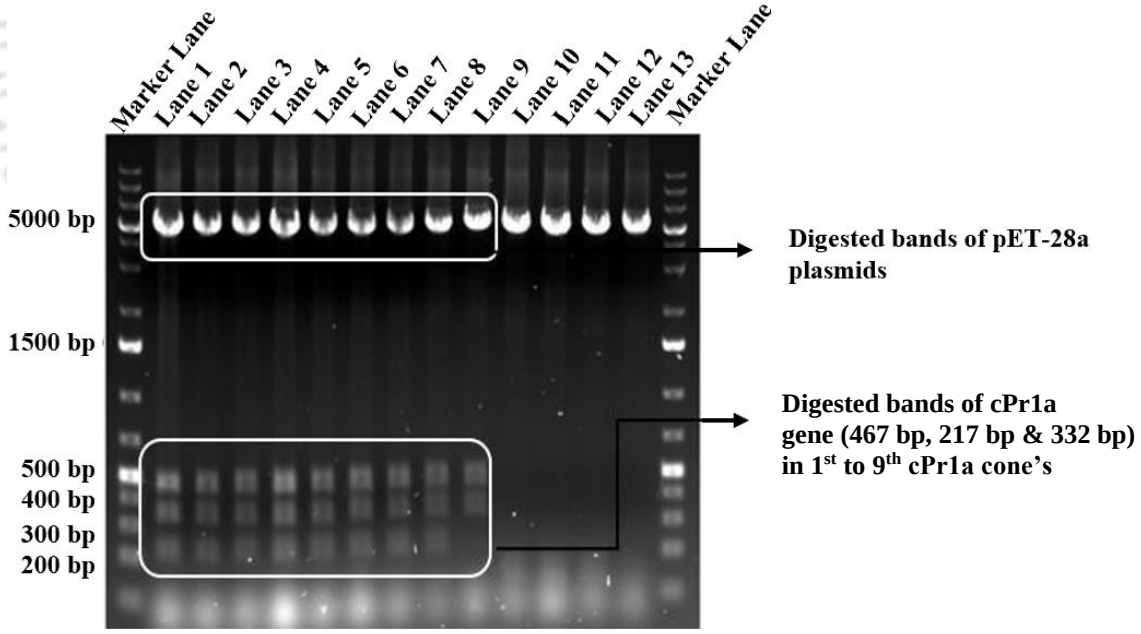

Fig 2.3.17 – Agarose gel electrophoresis image of clone's confirmation analysis by restriction enzyme digestion.
Plasmids (pET-28a - cPr1a) digested with NcoI and XhoI enzymes.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific) enzymes.
The cPr1a gene has two NcoI restriction sites at 213 bp and 680 bp, giving three bands 467 bp, 217 bp, and 332 bp instead of a single 1037 bp band.
Clone's 1st to 9th are showing the cPr1a gene inserts.

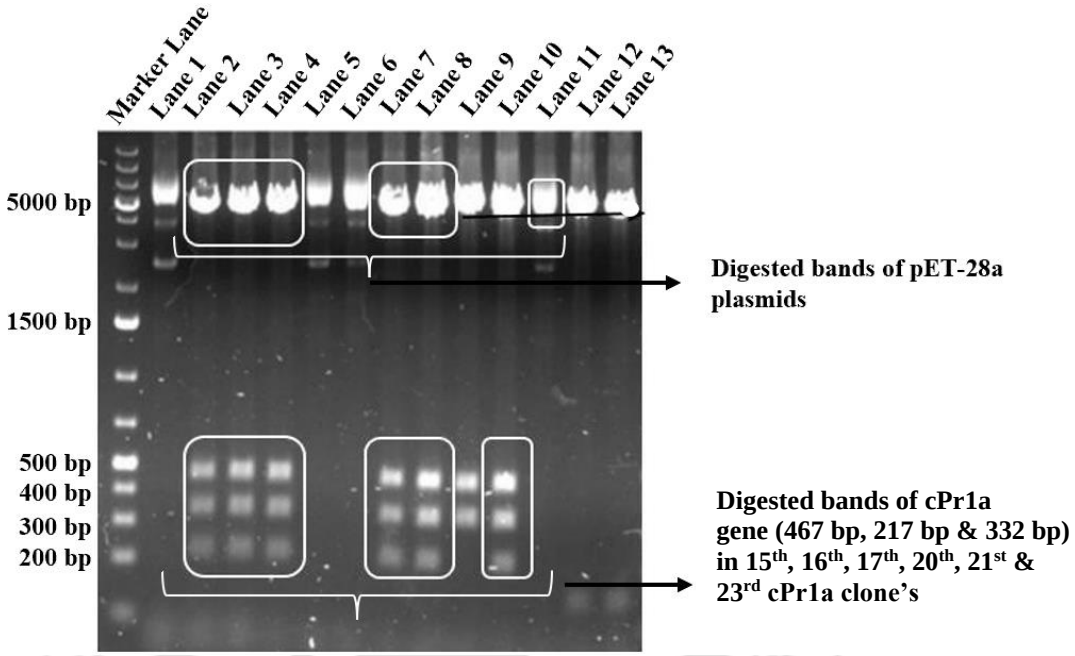

Fig 2.3.18 –Agarose gel electrophoresis image of clone's confirmation analysis by restriction digestion.
Plasmids (pET-28a - cPr1a) digested with NcoI and XhoI enzymes.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Clone's 15th, 16th, 17th, 20th, 21st and 23rd are showing the Pr1a gene insert.
The Pr1a gene has two NcoI restriction sites at 213 bp and 680 bp, giving three bands 467 bp, 217 bp, and 184 bp instead of a single 910 bp band.

2.3.4 Expression, affinity purification of wild-type (Pr1a) and chimeric (cPr1a) proteins

The Purified recombinant plasmids were introduced into *E. coli* BL-21(DE3)- competent cells (**Fig 2.3.19**) and **(Fig. 2.3.20)**, where Pr1a-$(His)_6$ and cPr1a-$(His)_6$ were hyper-expressed and purified using immobilized metal ion affinity chromatography in accordance with Section **2.2.20**. The Pr1a-$(His)_6$ and cPr1a-$(His)_6$ protein expression was optimized in LB Broth at 16ºC. Final purification was performed using Ni-NTA affinity chromatography. The Pr1a and cPr1a were expressed as soluble proteins. The purified Pr1a and cPr1a had a molecular size of about 29 kDa and 34 kDa, which are consistent with the theoretically expected size. Bradford assay was done to quantify the purified Pr1a and cPr1a proteins. The purified protein and other fractions eluted from the column were analyzed on 12% (SDS-PAGE) Sodium Dodecyl Sulfate - Poly

Acrylamide Gel electrophoresis **(Fig. 2.3.21 & Fig. 2.3.22)**. Gel imaging was done by using a Bio-Rad Gel Doc EZ gel documentation system and analyzed with Image Lab software 4.0 from Bio-Rad. The amount of purified Pr1a and cPr1a proteins achieved from the 2-liter culture was 26 mg and 28 mg, respectively.

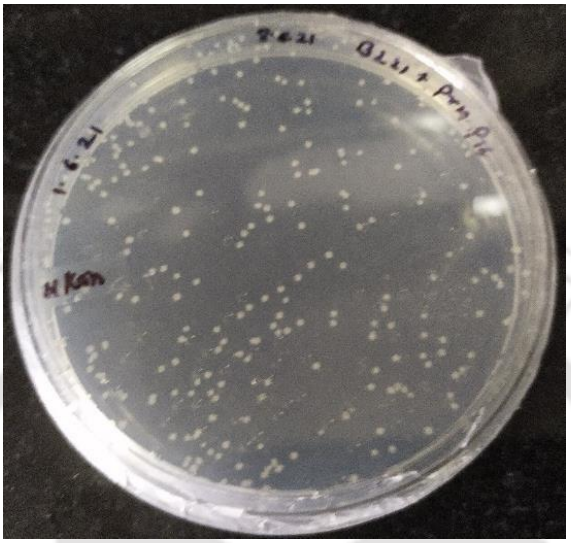

Fig 2.3.19 - The *E.coli* BL21 DE3 cells were transformed with pET-28a-Pr1a plasmids isolated from confirmed positive clones. The figure shows the BL21 DE3 colonies carrying the pET-28a-Pr1a plasmid, appeared on the LB agar plate (supplemented with kanamycin (50 µg /mL) after transformation followed by incubation at 37°C for 14 h.

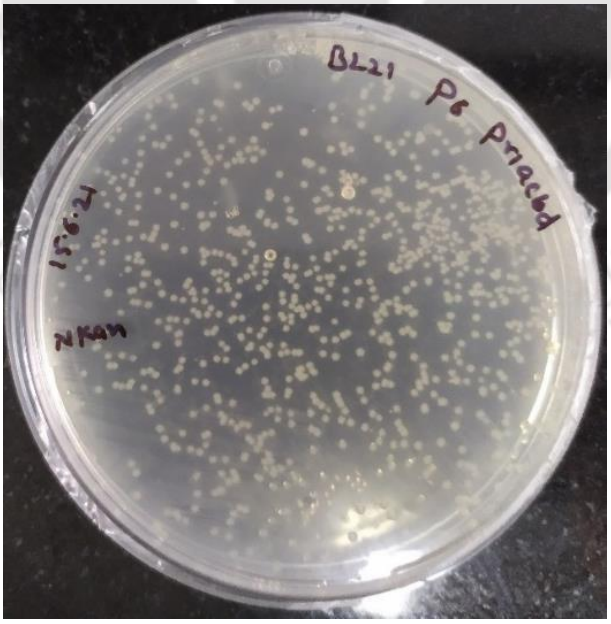

Fig 2.3.20 - The *E.coli* BL21 DE3 cells were transformed with pET-28a-cPr1a plasmids isolated from confirmed positive clones. The figure shows the BL21 DE3 colonies carrying the pET-28a-cPr1a plasmid, appeared on the LB agar plate (supplemented with kanamycin (50 µg /mL) after transformation followed by incubation at 37°C for 14 h.

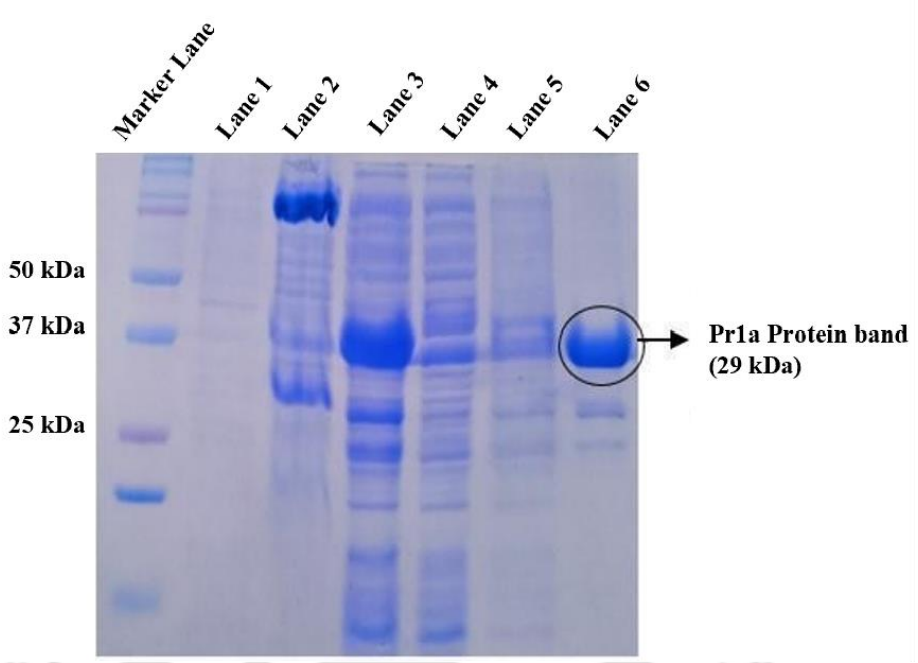

Fig 2.3.21 - Purification profile of recombinant Pr1a protein on 12% (w/v) polyacrylamide gel. SDS-PAGE was used to resolve the proteins and Coomassie Brilliant Blue was used to stain them. Marker Lane: Precision Plus protein ladder, (Biorad 1610374), lane 1: cell pellet after sonication, lane 2: cell-free medium supernatant after sonication, lane 3: flow-through, lane 4: last column wash, lane 5: purified Pr1a protein.

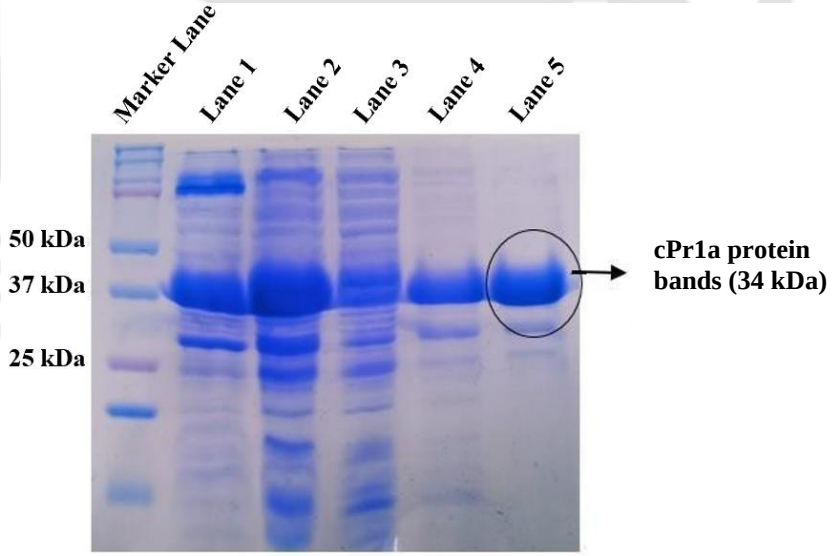

Fig 2.3.22 - Purification profile of recombinant cPr1a protein on 12% (w/v) polyacrylamide gel. SDS-PAGE was used to resolve the proteins and Coomassie Brilliant Blue was used to stain them. Marker Lane: Precision Plus protein ladder, (Biorad 1610374), lane 1: cell pellet after sonication, lane 2: cell free medium supernatant after sonication, lane 3: flow-through, lane 4: last column wash, lane 5: purified cPr1a protein.

2.3.5 Binding assays

Chitin-binding domains from insect (*Bombyx mori*) were fused to the Pr1a protease of fungi (*Metarhizium anisopliae*), resulting in chimeric protease cPr1a. The chimeric protease was successfully expressed in bacteria (*E. coli*), which was confirmed by SDS-PAGE. The Pr1a and cPr1a protease were subsequently purified by affinity chromatography through a Ni-NTA column and used for chitin-binding assays. The chitin-binding domain merged with the protease influenced the binding efficiency of chimeric protease to the insect cuticle in a positive manner.

The fusion of *Bombyx mori* chitin-binding domain to the Pr1a protease of *Metarhizium ansiopliae* increased the affinity of chimeric protease (cPr1a) towards the *Philosamia ricini* silkworm's cuticle. The chimeric protease cPr1a showed markedly more binding affinity than wild-type protease with the silkworm cuticle. A 21.5% increase in the binding efficiency (63.42 µg) of chimeric protease cPr1a was recorded with the powdered cuticle with respect to the wild-type Pr1a protease (49.73 µg) **(Fig 2.3.23).**

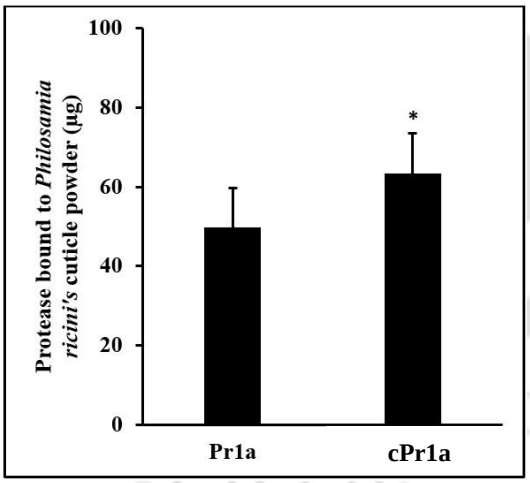

Fig 2.3.23 – Cuticle binding analysis of wild-type Pr1a and chimeric cPr1a protease
The percent protein bound was determined from assay mixtures containing 5 mg *Philosamia ricini*'s cuticle powder in 50 mM potassium phosphate buffer (pH 6.0) plus 100 µg protease.
Pr1a - the amount of Pr1a protein bound (49.73 µg) to the powdered cuticle.
cPr1a- the amount of cPr1a chimeric protein bound (63.42 µg) to the powdered cuticle.
The chimeric protease cPr1a demonstrated significantly higher binding affinity compared to the wild-type Pr1a protease.
* - The results of the student t-test are statistically significant ($P < 0.03$) and represent the mean values from three independent experiments.

2.3.6 Protease enzyme assay

To assess whether fusing the *Bombyx mori* chitin-binding domain Bmcbd to the Pr1a could improve the degradation of protein constituents within the insect cuticle, we conducted protease enzyme assay experiments where purified Pr1a and cPr1a proteins were incubated with Eri silkworm cuticles. Subsequently, we measured the quantity of protein peptides released into the assay supernatant. As depicted in Figure 2.3.24, there was a notable increase in the enzyme activity of cPr1a (59.06 U/mL) when compared to Pr1a (44.95 U/mL) This suggests that the protease's ability to target the insect cuticle is enhanced by the presence of a *Bombyx mori* chitin-binding domain, thus facilitating the degradation of the cuticle. Significant levels of cuticle degradation were observed at $P < 0.05$ with the enzymes **(Fig 2.3.24).**

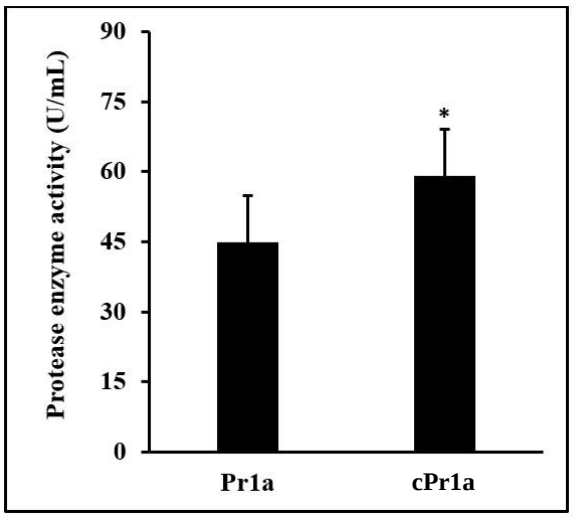

Fig 2.3.24 - Comparative enzymatic analysis with purified wild-type Pr1a protease and chimeric cPr1a protease using *Philosamia ricini's* cuticle powder as substrate.
Pr1a – Enzyme activity of wild-type Pr1a protein (44.94 U/mL).
cPr1a - Enzyme activity of chimeric cPr1a protein (59.06 U/mL).
The chimeric protease cPr1a exhibited significantly higher enzyme activity compared to the wild-type Pr1a protease.
*-The results of the student t-test are statistically significant ($P < 0.02$) and represent the mean values from three independent experiments.

2.3.7 Homology Modeling

The web server PHYRE2 was utilized for the homology modeling of Pr1a and Bmcbd proteins. For structure prediction of Pr1a, PHYRE 2 used the most appropriate model structure of cuticle-degrading protease from *Paecilomyces lilacinus* (PDBe- 3F7O) with 100% confidence and 100% coverage **(Fig. 2.3.25)** and protein structure of Bmcbd was modeled against an avirulence protein4 (avr4) from *Cladosporium fulvum* with 99.4% confidence and 96%coverage. The percentage of different secondary structures was predicted through i-tasser anddescribed in **Table 2.3.2**. The secondary structure analysis showed 56.6 % random coils, 17.99 % β-strands, 25.4 % helix in wild-type Pr1a protease and 59.9 % random coils, 18.34 % β- strands, and 21.7 % helix in chimeric cPr1a protease. The 3D protein structures of Pr1a and Bmcbd were fused using UCSF chimera software in order to generate a 3D protein structureof the cPr1a enzyme **(Fig. 2.3.26)**. The conserved domains in the protease of *M. anisopliae* were found using the NCBI conserved domain search-NIH tool. Peptidases S8 PCSK9 Proteinase K and Peptidase S8 S53 superfamily were found to be two highly conserve domains. The peptidase S8 domain of protease is usually comprised of Asp/His/Ser catalytic triad. The peptidase domain present in the S8 and S53 families comprises both endopeptidases and exopeptidases (NCBI conserved domain). The S53 family has a catalytic triad consists of Glu/Asp/Ser. The S53 family shows a serine-type peptidase activity, which involves the hydrolysis of peptide bonds in a polypeptide chain, which is catalyzed by a catalytic triad that includes a serine nucleophile that is triggered by a proton relay that includes an acidic residue such as aspartate or glutamate and a basic residue like histidine (https://www.ebi.ac.uk/QuickGO/GTerm?id=GO:0008236).

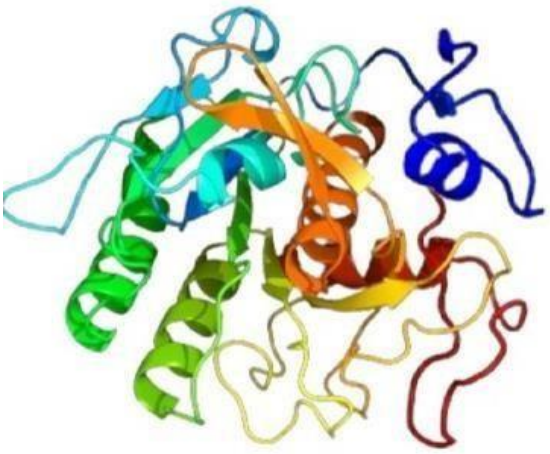

Fig. 2.3.25 - The 3D protein structure of wild-type Pr1a protease was modeled by the PHYRE2 web server. The predicted model attains a 100.00% confidence indicating a highly reliable prediction and 100% coverage, indicating that the entire protein sequence was fully modeled without any missing regions and 76% identity to the template c3f7oB (PDBe-3f7o) with a PDBe title of Crystal structure of Cuticle-Degrading Protease from *Paecilomyces lilacinus* (PL646).

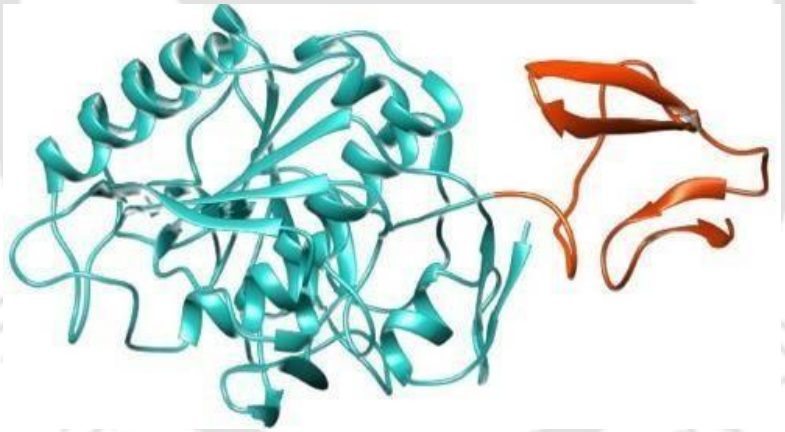

Fig. 2.3.26 - The structure of Bmcbd (*Bombyx mori* chitin-binding domain) was generated by thePHYRE2 web server. The predicted model attains a 99.4 % confidence with 96 % coverage and 35 % identity to the template c6bn0C (PDBe- 6bn0) with a PDBe title of Avirulence protein 4 (Avr4) from *Cladosporium fulvum* bound to the hexasaccharide of chitin. The 3D protein structure of chimeric cPr1a protease was generated by merging the modeled structure of Pr1a and Bmcbd using UCSF-Chimera software. Green part - Pr1a, Red part – Bmcbd.

2.3.8 Sequence analysis

Multiple sequence alignment was done using clustal omega, and a phylogenetic tree was generated from MSA values using Mega 11 software **(Fig. 2.3.27)**. Using the Blastp and Blastn programs, the homology of the *M. anisopliae* protease gene with protease genes reported from other organisms was investigated. At the protein level, the protease gene of *M. anisopliae* revealed 76% homology with the sequence of a subtilisin protease Pr1a from *Paecilomyces lilacinus*. It was 68% identical to the alkaline protease of *Parengydontium album* sp. at the nucleotide level. It also shared 82% nucleotide identity with the *Metapochonia rubescens* strain CBS P32 gene and 48% protein identity with *Thermus aquaticus*. The identities of other protease genes from different organisms with Pr1a gene were found as *Lecanicillium psalliotae* (70%), *Vibrio* sp. PA-44 (45%), *Lederbergia lenta* (35%), *Thermococcus kodakarensis* (32%), *Thermoactinomyces vulgaris* (39.02%), and *Homo sapiens* (34.53 %), respectively.

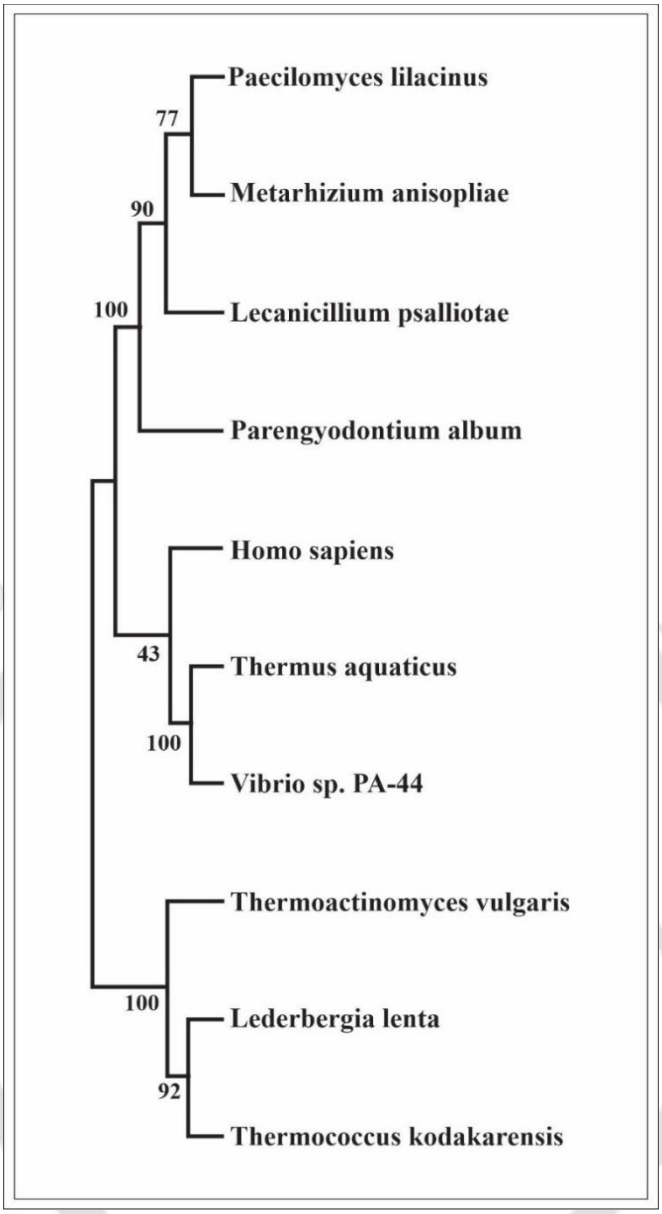

Fig. 2.3.27 - The figure illustrates a phylogenetic tree depicting the evolutionary relationship of *M. anisopliae* Pr1a protease with proteases of different organisms. The construction of this phylogenetic tree was carried out using the MEGA version 11 software, a robust and widely used tool for conducting evolutionary analysis and constructing phylogenetic trees. Neighbor-joining approach with the Poisson model for multiple amino acid substitution and 1000 random bootstrap repeats was used.
High bootstrap values (close to 100%) indicate strong support for the branches. Alignment of all sequences was done using clustal omega. Binomen genus-species names are used. The evolutionary relationship revealed that *Metarhizium anisopliae's* protease was most closely related Pr1a protease of *Paecilomyces lilacinus*.

2.4 Discussion

The application of *Metarhizium anisopliae* protease in the agricultural industry holds significant promise. The pathogenicity of entomopathogenic fungus, *M. anisopliae* is believed to be facilitated by extracellular chitinases and proteases. These enzymes have been identified as potential virulence factors. Extracellular chitinases and proteases are considered significant virulence factors in the pathogenicity of *M. anisopliae*. Expressed sequence tagged (EST) analyses of *M. anisopliae* have identified a range of enzymes produced in vivo. These include various proteases such as Pr1a, chymotrypsin, trypsins, carboxypeptidase, as well as chitinases. Additionally, a cAMP-dependent protein kinase catalytic subunit (AJ273794) has also been detected in these analyses (109,110, 81, 111).

The protease, derived from the fungus *Metarhizium anisopliae*, has demonstrated remarkable potential in the development of potent biopesticides. Protease (Pr1a), plays a crucial role by exhibiting the ability to degrade the cuticles of insect pests, effectively compromising their outer protective layers during the infection phase of insect hosts (112).

A chitin-binding domain has been identified in certain chitinases, including those found in plants and insects. The main role of this domain is thought to be facilitating the binding of the chitinase enzyme to the insoluble chitin substrate. This interaction helps enhance the enzyme's activity by allowing it to efficiently degrade the chitin present in the substrate. The presence of a chitin-binding domain is considered crucial for the biochemical and functional characteristics of the chitinase protein (113, 114, 115, 116, 117 & 118).

Multiple proteases with chitin-binding-like domains have been documented in different organisms. Examples of such proteases include Sp22D from the mosquito *Anopheles gambiae* [119, 120], AprIV from *Alteromonas sp.* Strain O-7 [121], and protease C from *Streptomyces griseus* [122], as well as two putative enzymes from *Streptomyces coelicolor*. These proteases exhibit structural features resembling chitin-binding domains, suggesting their potential involvement in chitin-related processes. The identification of these proteases highlights the diversity of organisms that possess chitin-binding-like proteases and indicates their potential roles in various biological functions.

Sp22D is a serine protease with multiple domains, and among them, it possesses a type 2

chitin-binding domain (ChBD). This protease is believed to have a role in insect immunity and/or in the metamorphic transition from the pupal stage to the adult stage. On the other hand, the bacterial protease AprIV is part of a group of proteases produced by *Alteromonas*. However, it is unique in that it contains ChBD, and it is thought to be involved in the degradation of crustacean cuticles by this chitinolytic marine bacterium (121).

According to the findings reported by Fan Y et al., the introduction of a chitin-binding domain (ChBD) to fungal protease CDEP-1 led to increased binding of the enzyme to insect cuticles. This enhanced binding resulted in an accelerated degradation rate of cuticular proteins. Based on these results, it is highly likely that the observed enhanced virulence in strains expressing CDEP: BmChBD is attributed to the improved efficiency in targeting the enzyme to insect cuticle protein-chitin structures. This, in turn, facilitates the penetration of the cuticle and potentially provides greater nutrient availability for fungal hyphal growth, surpassing the capabilities of the wild-type strain that produces the native protein CDEP-1 (13).

Protease assays with purified wild-type Pr1a and chimeric cPr1a proteases and *Philosamia ricini's* cuticle as the substrate revealed that the fusion of *Metarhizium anisopliae's* chitin-binding domain with that of *Bombyx mori* resulted in increased catalytic efficiency of chimeric protease compared to wild-type protease. About 23.98 % increase in enzyme activityof chimeric cPr1a protease (59.07 U/mL) with respect to wild-type Pr1a protease (44.9 U/mL) 1has been evaluated. The binding assays revealed that the chimeric protease, cPr1a, showed significant enhancement (about 21.59 %) in binding efficiency (63.42 μg) with *Philosamia ricini's* cuticle compared to wild-type Pr1a protease (42.54 μg). Apart from an increase in binding efficiency,the chimeric protease revealed an increase in the enzyme's activity, which can be very useful in biopesticide industries because protease is one of the major components of *Metarhizium*-based fungal biopesticides that has been modulated in the past to improve the virulence of fungal biopesticides.

2.5 Conclusion

The present study provided an overview of the creation of chimeric protease, cloning of wild-type and chimeric protease into the pET-28a vector, optimized expression of soluble Pr1a and cPr1a proteins in a bacterial system, and affinity purification of both proteins using Ni-NTA chromatography preceded by enzyme and binding assays. The results obtained from our study, where we created a chimeric protease with the ability of chitin binding, highlight the inherent structural and functional flexibility of many enzymes. By engineering chimeric genes to possess new properties, we have gained valuable insights into the various parameters that can be manipulated to develop more efficient enzymes utilized by entomopathogenic fungi. This demonstrates the potential for constructing enhanced versions of these fungi with improved capabilities. The chimeric proteases could be exploited in the biopesticide industry to enhance the virulence of *Metarhizium anisopliae*.

Table 2.2.1 Components of PCR mixture for amplification of gene encoding Pr1a from *Metarhizium anisopliae.*

PCR components	Volume (µl)	Final concentration
Nuclease-free water	30.0	--
5x reaction buffer	10.0	1x
dNTP mix (10 mM)	1.0	0.2 mM
Forward primer (10 µM)	2.5	0.5 µM
Reverse primer (10 µM)	2.5	0.5 µM
DMSO	1.5	3%
cDNA (30.0 µg/mL)	2.0	60.0 ng
Phusion DNA polymerase (2 U/µl)	0.5	0.02 U/µl
Total	50.0	--

Table 2.2.2 PCR cycles conditions for amplification of Pr1a from *Metarhizium anisopliae*

Steps		Time
		2 min
I. Denaturation at 98°C		
II. 30 cycles of	i) Denaturation at 98°C	10 s
	ii) Annealing at 68°C	30 s
	iii) Extension at 72°C	30 s
III. Final extension at 72°C		10 min

Table 2.2.3 Primer sequences used for cloning of Pr1a gene from *Metarhizium anisopliae*

Gene construct	Primer sequence
Pr1a_FP_NcoI_pET28a_Over Hang	CTTTAAGAAGGAGATATACCATG GGCATCACTGAGCAGAGCGG
Bmpr1a_RP_His Tag_XhoI_pET28a_Overhang	GGTGGTGGTGGTGCTCGAGTTAATGATGATGATGATGATGGGCACC GTTG TAGGCAA

Table 2.2.4 Components of PCR mixture for amplification of gene encoding Pr1abm/Bmpr1a from *Metarhizium anisopliae.*

PCR components	Volume (µl)	Final concentration
Nuclease-free water	60.0	--
5x reaction buffer	20.0	1x
dNTP mix (10 mM)	2.0	0.2 mM
Forward primer (10 µM)	5.0	0.5 µM
Reverse primer (10 µM)	5.0	0.5 µM
DMSO	3.0	3%
Pr1a gene (50.0 µg/mL)	2.0	50.0 ng
Phusion DNA polymerase (2 U/µl)	1.0	0.02 U/µl
Total	100.0	--

Table 2.2.5 PCR cycles conditions for amplification of Pr1abm from *Metarhizium anisopliae*

Steps		Time
I. Denaturation at 98°C		2 min
II. 30 cycles of	i) Denaturation at 98°C	10 s
	ii) Annealing at 68°C	30 s
	iii) Extension at 72°C	45 s
III. Final extension at 72°C		10 min

Table 2.2.6 PCR cycles conditions for amplification of Bmpr1a from *Bombyx mori*

Steps		Time
I. Denaturation at 98°C		2 min
II. 30 cycles of	i) Denaturation at 98°C	10 s
	ii) Annealing at 70°C	30 s
	iii) Extension at 72°C	15 s
III. Final extension at 72°C		10 min

Table 2.2.7 Composition of 6x DNA loading buffer.

Constituents	Final concentration (6x)
Tris-HCl	10 mM
Glycerol	60% (w/v)
EDTA	60.0 mM
Bromophenol blue	0.03% (w/v)
Xylene cyanol	0.03% (w/v)

Table 2.2.8 Primer sequences used for the amplification of Pr1abm and Bmpr1a of Pr1a gene from *Metarhizium anisopliae* and *Bombyx mori*.

Gene construct	Primer sequence
Pr1abm_FP_NcoI_pET28a_Overhang	CTTTAAGAAGGAGATATACCATG GGCATCACTGAGCAGAGCGG
Pr1abm _RP_Bmchi2 _Overhang	GTTTCACGGTGGTGGTGGT GGCACCGTTGTAGGCAAGGTAG
Bmpr1a_FP_Bmcbd_Overhang	TTGCCTACAACGGTGCC ACCACCACCACCGTGAAAC
Bmpr1a_RP_His Tag_XhoI_pET28a_Overhang	GGTGGTGGTGGTGCTCGAGTTAATGATGATGATGATGATG AGGCCAATCGCAAACGTTAAG GGTGGTGGTGGTGCTCGAGTTAATGATGATGATGATGATGTGAACAT TCCGGTCTGT

Table 2.2.9 Components of PCR mixture for amplification of gene encoding Pr1a from plasmid DNA.

PCR components	Volume (µl)	Final concentration
Nuclease-free water	18.4	--
10x reaction buffer	2.5	1x
dNTP mix (10 mM)	0.5	0.2 mM
Forward primer (10 µM)	0.5	0.2 µM
Reverse primer (10 µM)	0.5	0.2 µM
25 mM $MgCl_2$	1.5	1.5 mM
Plasmid DNA (50.0 µg/mL)	1.0	50.0 ng
Neb Taq DNA polymerase (5 U/µl)	0.1	0.02 U/µl
Total	25.0	--

Table 2.2.10 PCR cycles conditions for amplification of Pr1a gene from plasmid DNA

Steps		Time
		2 min
I. Denaturation at 95°C		
II. 30 cycles of	i) Denaturation at 95°C	10 s
	ii) Annealing at 61°C	30 s
	iii) Extension at 68°C	1min
III. Final extension at 68°C		10 min

Table 2.2.11 PCR cycles conditions for amplification of cPr1a gene from plasmid DNA

Steps		Time
		2 min
I. Denaturation at 95°C		
II. 30 cycles of	i) Denaturation at 95°C	20 s
	ii) Annealing at 53°C	30 s
	iii) Extension at 68°C	1 min 30 s
III. Final extension at 68°C		10 min

Table 2.2.12 Composition of restriction enzymes digestion reaction

Components	Volume (µl)
10x cut smart buffer	2.0
Nuclease free water	7.0
Plasmid DNA (50 ng/µl)	10.0
*Nco*I (20 U/µl)	0.5
*Xho*I (20 U/µl)	0.5
Total	20.0

Table 2.2.13 Constitution of 10x SDS running buffer.

Ingredients	Quantity (For 1000 mL)	Final Concentration of components in 1x buffer
Tris Base	30 g	0.25 M
Glycine	144 g	1.92 M
SDS	10 g	1 % (w/v)

Table 2.2.14 Composition of 4x SDS loading dye

Ingredients	Final concentration (4x dye)
1M Tris-HCl (pH 6.8)	240 mM
Glycerol	40.0 (%)
SDS	8.0 (%)
Bromophenol Blue	0.04 (%)
β-mercaptoethanol	5.0 (%)

Table 2.3.1: **Quantification of RNA**

RNA Sample	Concentration (ng/µl)	A260/280
1	232.1	1.49
2	237.5	1.51
3	382.4	1.46
4	420.0	1.62
5	257.5	1.39
6	432.7	1.59

Table 2.3.2: Secondary structure analysis of Pr1a and cPr1a proteins

Protein	α helix	β strands	Random coils
Pr1a	25.4 %	17.99 %	56.6 %
cPr1a	21.7 %	18.34 %	59.9 %

CHAPTER-3

Cloning and expression of wild-type Chi2 and chimeric chitinase cChi2 into *E. coli*

Chapter-3

3.1 Introduction

Entomopathogenic fungi are potential alternatives to chemical pesticides, but their use as mycoinsecticides is currently limited in the overall insecticide market. A major challenge is the longer time required for mycoinsecticides to control insects after application, which can result in crop damage by infected insects (8). Improving the virulence of *Metarhizium anisopliae* involves understanding pathogenesis mechanisms and genetically modifying targeted virulence factors, thereby enhancing their effectiveness in insect control.

Entomopathogenic fungi, like *Metarhizium anisopliae*, offer the advantage of infecting host insects without requiring ingestion. They penetrate the insect's cuticle through conidial attachment, germination, and enzymatic/mechanical penetration (77 & 123). The insect cuticle consists of chitin fibrils that are embedded within a matrix of proteins (124 & 125). Chitin, as a crucial component of the insect cuticle, plays a significant role in serving as the primary protective barrier against pathogens.

The entomopathogen, *Metarhizium anisopliae*, uses a plethora of hydrolytic enzymes to breach the cuticle of insect pests during the infection process and these are the hallmark of the infection process and include various types of chitinases viz. (Chit1, Chi2 Chit3, and Chi4), proteases (Pr1a, Pr1b Pr1c to Pr1k), and lipases. Among all chitinase enzymes secreted by *Metarhizium ansiopliae*, Chi2 is a pathogenicity-determining enzyme and plays a key role during the infection process (90).

Chitinases are extensively distributed across various organisms, including viruses, bacteria, fungi, plants, and animals. Certain chitinases feature a chitin-binding domain connected to the catalytic site through an intermediate region (126).

Chitinase (E.C.3.2.1.14) has been classified into two families - Glycosyl Hydrolase-18 (GH-18) Family and Glycosyl Hydrolase-19 (GH-19) Family. Fungal chitinases, which are classified as class III chitinases, are categorized within family 18 of glycosyl hydrolases (85 &

127). Fungal chitinases from Subgroup B primarily play a role in nutritional functions, particularly in more aggressive activities exhibited by mycoparasitic and entomopathogenic fungi. The majority of tested Subgroup B chitinase genes from various fungal species are regulated by nutritional stimuli. Typically, they are induced by chitin or specific carbon sources found in the hosts, particularly in mycoparasitic and entomopathogenic fungi. Additionally, many of these chitinase genes, such as *T. atroviride* chit33 and ech30, as well as *M. anisopliae* Chi2, are upregulated in response to starvation conditions. Conversely, they are repressed when exposed to glucose or other readily metabolizable carbon sources (128, 129, 130, 90, 131).

The chitin-binding domains present in GH-18 chitinases are considered crucial for their interaction with chitin polymers (132, 133, 134). The presence of the chitin-binding domain enables chitinase to bind effectively to chitin, facilitating its efficient degradation, while not affecting the degradation of soluble substrates (135).

The chimeric chitinase constructed by the swapping of the chitin-binding domain of *Bacillus thuringiensis* chitinase with that of *Bacillus licheniformis* resulted in the enhanced affinity towards colloidal chitin. The affinity of enzymes to bind their respective substrates relies on their substrate binding domain. Engineering of these domains for the development of hybrid domains with an increased affinity towards the substrate is the trend. Hybrid chitinase created by the chitin-binding domain-swapping of *Bacillus thuringiensis* and *Bacillus licheniformis* had a significantly higher affinity for the strongly crystalline substrate (90).

Our main aim is the construction of a chimeric cChi2 chitinase through the swapping of the chitin-binding domain of *Metarhizium anisopliae* Chi2 chitinase with the chitin-binding domain of *Bombyx mori* chitinase. This domain-swapping approach is aimed at improving the binding affinity and increasing the chitinolytic activity of the chimeric cChi2 chitinase.

3.2 Material and Methods

3.2.1 Bacterial strains and reagents

The *E. coli* Top-10 strain was used for the maintenance of cloning and expression vectors. *E. coli* (BL21 DE3) competent cell (New England Biolabs, USA) was used for the expression of wild-type (Chi2) and chimeric chitinase (cChi2) genes. All restriction enzymes, Phusion DNA polymerase, and ligation enzymes were purchased from New England Biolabs, USA. GCC Biotech (I) Pvt. Ltd., India, synthesized all the primers. Plasmid DNA and Nucleospin gel extraction kits were purchased from Macherey Nagel, GmBH.

3.2.2 Amplification and cloning of wild-type Chi2 gene

The DNA sequence of Chi2 was obtained from the National Center for Biotechnology Information (NCBI) database with the accession number DQ011663. Three sets of primers were designed using the DNA sequence of three exons of the Chi2 gene. The sequences of the six DNA primers used for the amplification of three exons are described in **Table 3.2.1**. The PCR mixture composition and the reaction conditions followed for the amplification of the three exons are described in **Tables 3.2.2, 3.2.3, 3.2.4,** and **3.2.5**. The amplified products were segregated on 1% agarose gel and purified using a Nucleospin gel extraction kit (Macherey Nagel), GmBH. The three exons were fused using the overlap-extension PCR method to give the Chi2 gene. The PCR components and cycling conditions for the amplification of the Chi2 gene are given in **Table 3.2.6** & **Table 3.2.7**. The amplified products were segregated on 1% agarose gel and purified using a Nucleospin gel extraction kit (Macherey Nagel), GmBH. The pET-28a plasmid was digested with NcoI and XhoI restriction enzymes (New England Biolabs, U.S.). Digested plasmid products were separated on 1% agarose gel electrophoresis and purifiedusing a Nucleospin gel extraction kit. The Chi2 gene was cloned into NcoI and XhoI digested pET-28a plasmid using the Gibson assembly method, and the reaction was performed at 50ºC for 60 min.

3.2.3 Amplification of *Bombyx mori* chitin-binding domain with Chi2 overhang

The DNA sequence of the *Bombyx Mori* Chitin binding domain was obtained from National Center for Biotechnology Information (NCBI) database with the accession number: AF273695.1. Bmchi2 was amplified using cDNA clones of *Bombyx mori* chitinase (fepM03A03) as a template and gene-specific primers. The sequence of the DNA primers used for the amplification of the Bmchi2 fragment is given in **Table 3.2.1**.

The PCR mixture composition and reaction conditions followed for the amplification of Bmchi2 are given in **Tables 3.3.10** and **3.3.11**.

3.2.4 Construction and cloning of chimeric cChi2 gene

The Chi2 gene was reamplified using an old forward primer and a new reverse primer in order to introduce a Bmcbd overhang at the 3' end to obtain the Chi2bm fragment. The PCR mixture components and cycling conditions for the amplification of Chi2bm are given in **Table 3.3.8** and **Table 3.3.9**. The Bmcbd *Bombyx mori* chitin-binding domain was reamplified using a new forward primer and an old reverse primer to introduce a Chi2 overhang at 5'end to give the Bmchi2 fragment. The Chi2bm and Bmchi2 fragments were fused using the Gibson assembly method to give a chimeric cChi2 gene. The amplified products were segregated on 1% agarose gel and purified using a Nucleospin gel extraction kit (Macherey Nagel), GmBH. The pET-28a plasmid was digested with NcoI and XhoI restriction enzymes (New England Biolabs, U.S.). Digested plasmid products were separated on 1% agarose gel electrophoresis and purified using a Nucleospin gel extraction kit. The cChi2 construct was cloned into NcoI and XhoI digested pET-28a plasmid using the Gibson assembly method, and the reaction was performed at 50ºC for 60 min.

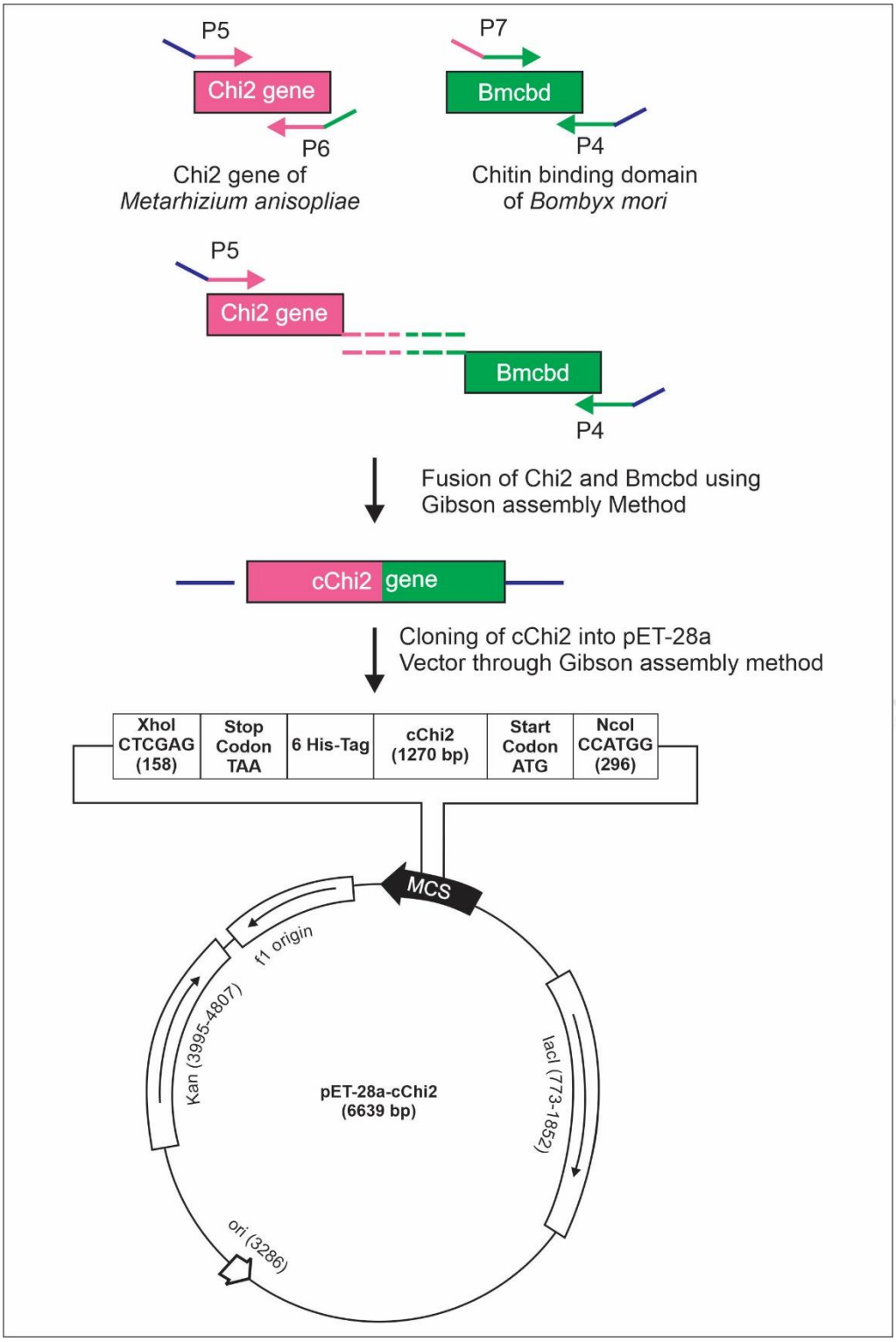

Fig. 3.2.1 - Schematic representation of cChi2-$(His)_6$ protein expression and purification.

3.2.5 *E. coli* (Top 10) Competent Cells Preparation

E. coli competent cell preparation was done following the protocol already describedin section **2.2.12** of Chapter 2.

3.2.6 *E. coli* Top 10 Transformation

The construct of Chi2 and cChi2 was made using the Gibson assembly method and immediately transformed into the Top-10 competent cells after incubation. Screening of positive clones was done on LB agar plates supplemented with kanamycin (50 µg mL^{-1}).

3.2.7 Plasmid DNA isolation

The plasmid DNA isolation was done following the protocol already described in section **2.2.14** of Chapter 2.

3.2.8 Screening of Chi2 & cChi2 positive clones using the PCR method

Confirmation of positive clones harboring Chi2 and cChi2 constructs was done by the PCR method using gene-specific primers. The plasmids were isolated from the transformant colonies randomly picked from the Petri plates. PCR was performed using Taq DNA polymerase (New England Biolabs). Analysis of PCR amplified products was done on 1% agarose gel.

3.2.9 Confirmation of Chi2 & cChi2 clones using restriction digestion method

Restriction digestion analysis was performed in a microcentrifuge tube. Containing plasmid DNA. The double digestion of recombinant plasmid DNA was done in a cut smart reaction buffer with NcoI and XhoI restriction enzymes NEB) to check the desirable positive clones. The reaction component's details are given in the **Table 3.2.12**. The reaction mixtures

were incubated at 37 ºC for 2 h. Analysis of digested products (pET-28a, Chi2 & cChi2 inserts) was done on 1% agarose gel. Gel imaging was done using the ChemiDoc XRS™ gel documentation system (Bio-Rad, USA). To confirm the size of the DNA insert and the vector pET-28a, the digested fragments (insert and vector) were analyzed, and the positive clones were identified.

3.2.10 *E. coli* (BL21 DE3) competent cells preparation

E. coli (BL21 DE3) competent cells were prepared using the same protocol as described in section **2.2.6**.

3.2.11 Transformation of pET28a-Chi2 & pET28a- cChi2 constructs into the expression host

The plasmid constructs pET-28a-Chi2 & pET-28a- cChi2 were transformed into bacterial protein expression host *E. coli* (BL21 DE3) using the chemical transformation method. The selection was carried out on LB agar plates supplemented with kanamycin (50 µg m^{-l}) and incubated at 37ºC for 12-14 h.

3.2.12 Chi2 and cChi2 protein expression

A single colony of *E. coli* (BL21 DE3) carrying the pET-28a-Chi2 & pET-28a- cChi2 was used to inoculate 5 mL of LB broth supplemented with kanamycin (50 µg mL^{-l}) and incubated overnight at 37ºC at 200 rpm. One percent of overnight grown culture was used to inoculate the expression medium. A bacterial culture was grown until OD_{600} reached 0.6 at 37ºC. The Chi2 and cChi2 protein expression was carried out using LB Broth (Tryptone 12 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 10 g l^{-1}). Protein induction was done with 0.5 mM isopropyl thiogalactopyranoside (IPTG) and incubated for 16 h at 16°C in a refrigerated incubator shaker at 200 rpm. Purification of protein was done using Ni-NTA affinity chromatography.

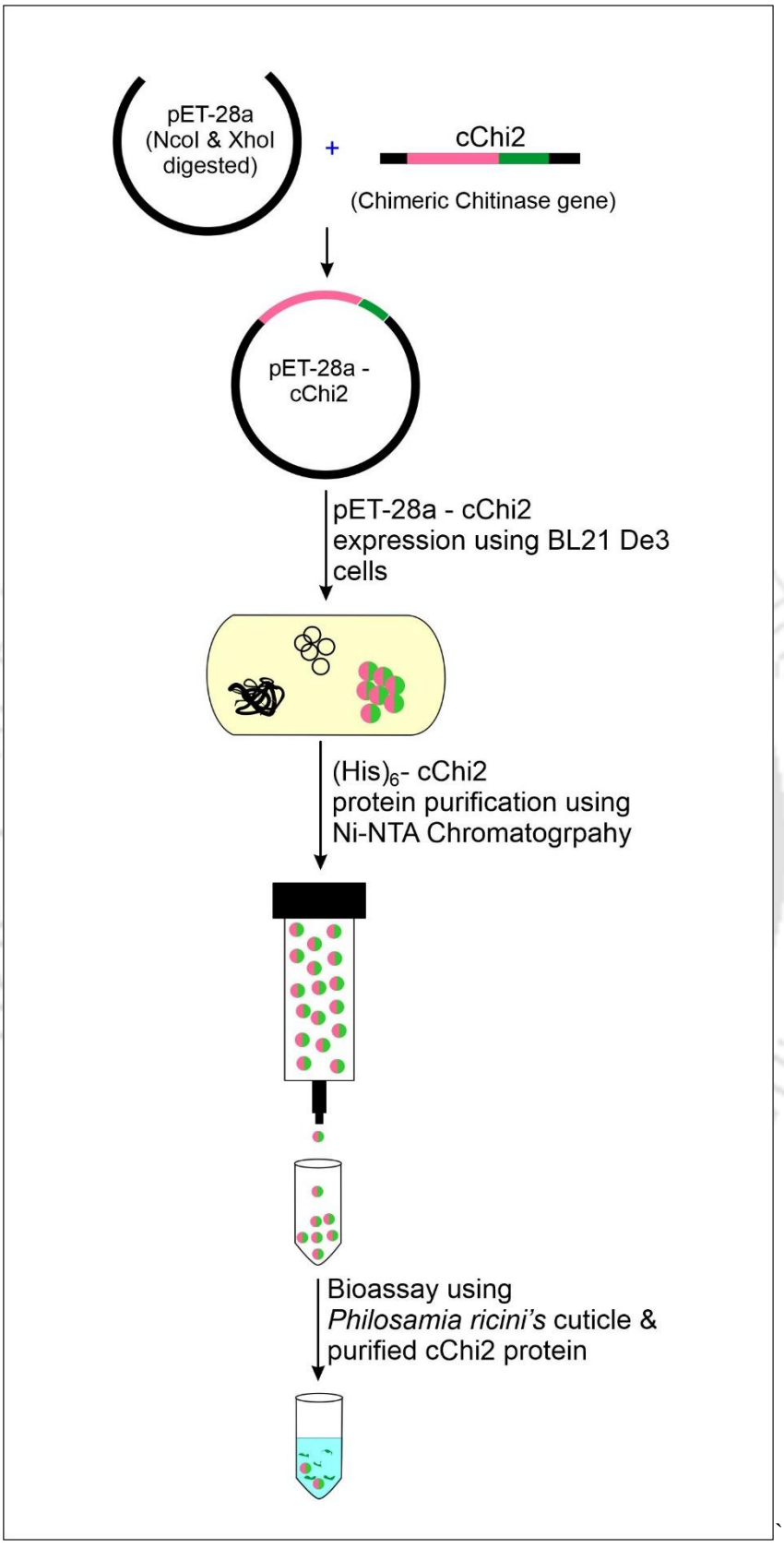

Fig. 3.2.2 – Schematic representation of cChi2-$(His)_6$ protein expression and purification.

3.2.13 Chi2 & cChi2 protein affinity purification

Post-incubation, the cell pellet was harvested by centrifugation of culture at 10,000 rpm for 30 min at 4ºC, and the cell pellet was resuspended in binding buffer A (40 mM Tris-Cl, 400 mM NaCl, 10 mM Imidazole, 10% Glycerol). Vibra Cell Sonicator was used for lysis of cells with 4 sec pulse ON and 20 sec pulse OFF conditions. The lysate was centrifuged at 13,000 rpm for 1 h at 4ºC, and the clarified supernatant solution was loaded into Nickel- Nitrillo Triacetic Acid (Ni-NTA) affinity chromatography column pre-equilibrated with binding buffer A. Different fractions were eluted with Elution buffer containing 400 mM Imidazole and analyzed using 15% Sodium Dodecyl Sulfate- Poly Acrylamide Gel electrophoresis (SDS-PAGE). Gel imaging was done using a Biorad Gel Doc EZ gel documentation system and analyzed with Image Lab software 4.0 from Biorad.

3.2.14 Quantitative analysis of protein

The protein was quantified following Bradford's method of protein estimation described in section **2.2.22** of Chapter 2.

3.2.15 Cuticle preparation

The cuticle preparation was done according to the protocol described in the section **2.2.23** of Chapter 2.

3.2.16 Binding assays

Binding assays were performed in 1.5 mL Eppendorf tubes containing 5mg of cuticle powder in 1 mL of 50 mM potassium phosphate buffer (pH-7.0) and 2 µl (100 µg) of Chi2 and cChi2 enzymes. The mixture was incubated on ice for 4 mins. Subsequently, the mixture was centrifuged at 10000 rpm for 5 mins to separate the solid cuticle and supernatant. The amount of unbound enzyme was determined from the supernatant using Lowry's method of protein

estimation. The amount of cuticle-bound enzyme was calculated from the difference between the initial amount of enzyme taken for the assay and the amount of unbound enzyme present in the supernatant.

3.2.17 N-Acetylglucosamine standard curve preparation

Standard solutions of N-Acetylglucosamine (NAGA) were prepared by dissolving the varying concentrations (10 – 100 µg/mL) of tyrosine in 50 mM phosphate buffer pH- 7.0. one mL of solution was added to the cuvette, and the absorbance was measured in a UV-Visible spectrophotometer at 540 nm wavelength. A standard plot of NAGA was generated using the O.D.s of different concentrations of tyrosine. One equivalent of NAGA was determined, and to calculate the enzyme activity, 1 A_{540} equivalent of N-Acetylglucosamine (µg/mL) was converted to mg/mL.

3.2.18 Chitinase enzyme assay

Chitinase assay was done using 1 % substrate (powdered insect cuticle). 495 µl of the substrate was mixed with 5 µl (250 µg) of wild-type (Chi2) and chimeric (cChi2) chitinase enzymes, and the reaction was incubated at 28°C for 4 mins. After adding 500 uL of DNS reagent, the reaction system was boiled for 10 minutes at 100°C. After centrifugation, the OD_{540} value of the supernatant was determined using the spectrophotometer. Under standard conditions, one unit of chitinase was defined as the amount of enzyme that liberated 1 µg of D-glucosamine per minute.

3.2.19 Enzyme activity calculation

The enzyme activities were expressed as U/mL (µmol/min/mL. One unit (U) of enzyme activity is defined as the amount of an enzyme that liberates 1 µmole of N-Acetylglucosamine per min.

3.2.20 Sequence analysis

Multiple sequence alignment of sequences of various chitinase was done using Clustal omega software, and a cladogram was generated using the Ebi website.

3.2.21 Homology modeling

Homology modeling of Chi2 and Bmcbd proteins was done using Phyre2 software and UCSF chimera software was used to fuse the 3D structures of Chi2 (Without chitin-binding domain) and Bmcbd proteins in order to get a full 3D structure of chimeric cChi2 protein.

3.2.22 Statistical analysis

All the experiments were done three times independently in triplicates. Student's t-test was used to statistically analyze the mean of three replicates of all enzyme and binding assays.

3.3 Results

3.3.1 Amplification of Chi2 gene

The Chi2 DNA sequence (1247 bp) was retrieved from NCBI (National Centre for Biotechnology Information) database (DQ011663). Three sets of forward and reverse primers were designed based on the three exons of the Chi2 gene. The successful amplification of three exons of the Chi2 gene using exon-specific primers from Metarhizium anisopliae was confirmed by detecting the presence of three expected distinct bands in the agarose gel electrophoresis analysis. The observed band sizes were 251 base pairs, 961 base pairs, and 155 base pairs, for exon-1, exon-2 and, exon-3 respectively **(Fig.3.3.1, Fig 3.3.2 and Fig 3.3.3)**. During the PCR amplification, compatible ends for fusion were introduced using exon-specific primers at the 3' end of exon-1, the 5' and 3' ends of exon-2, and the 5' end of exon-3. The exon-1 exon-2 and exon-3 with compatible overhangs were fused using the overlap extension PCR method to give the full Chi2 gene (1247 bp) **(Fig 3.3.4).** The full Chi2 gene was amplified using the PCR method with the forward primer of exon -1 and reverse primer of exon-3, introducing NcoI and XhoI restriction enzyme overhangs at the 5’ and 3’ end of the Chi2 gene. The DNA primers used for the amplification of three exons of the Chi2 gene are shown in **(Table 3.3.1).** The PCR-amplicon Chi2 gene and NcoI and XhoI digested pET-28a plasmid were extracted from the gel using the Nucleospin gel extraction kit, and the purified bands of Chi2 gene (1247 bp) and digested pET-28a were analyzed using 1% agarose gel using electrophoresis method **(Fig 3.3.5)**. The purified Chi2 amplicons were stored at -20°C for subsequent molecular cloning.

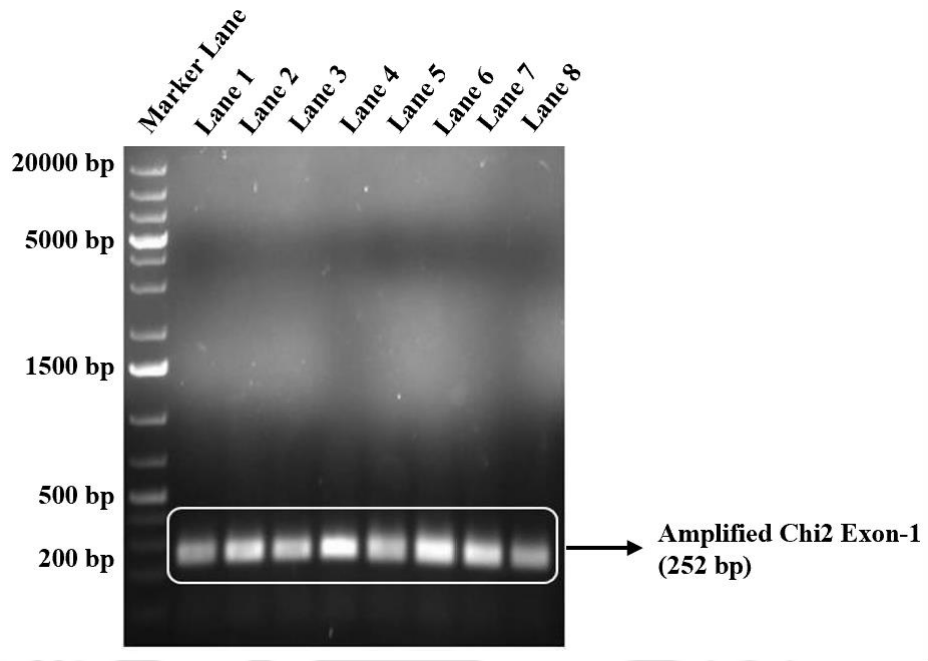

Fig 3.3.1 - Agarose gel electrophoresis analysis showing PCR amplified Exon-1 of Chi2 gene of *Metarhizium anisopliae* using Exon-1 specific primers. The most intense Exon-1 band appeared at 65.2°C. Phusion DNA polymerase was used to amplify the Chi2 Exon-1
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 8 – Exon-I of Chi2 gene (252 bp) (Annealing temperature range - 62°C to 68.9 °C).

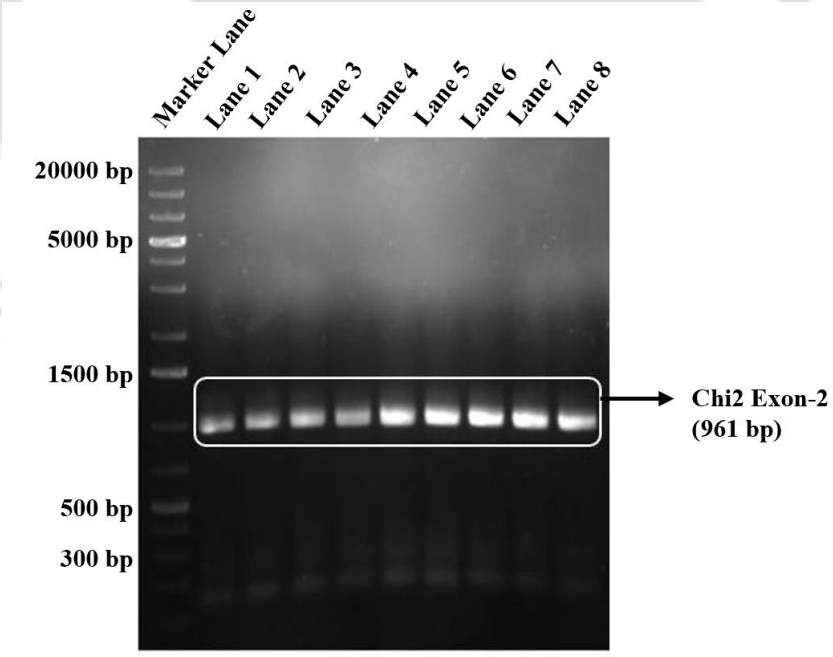

Fig 3.3.2 - Agarose gel electrophoresis analysis showing PCR amplified Exon-2 of Chi2 gene of *Metarhizium anisopliae* using Exon-2 specific primers. The most intense band appeared at 67.5°C. Phusion DNA polymerase was used to amplify the Chi2 Exon-2
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 9 – Exon-2 of Chi2 gene (961 bp) (Annealing temperature range - 62°C to 68.9 °C).

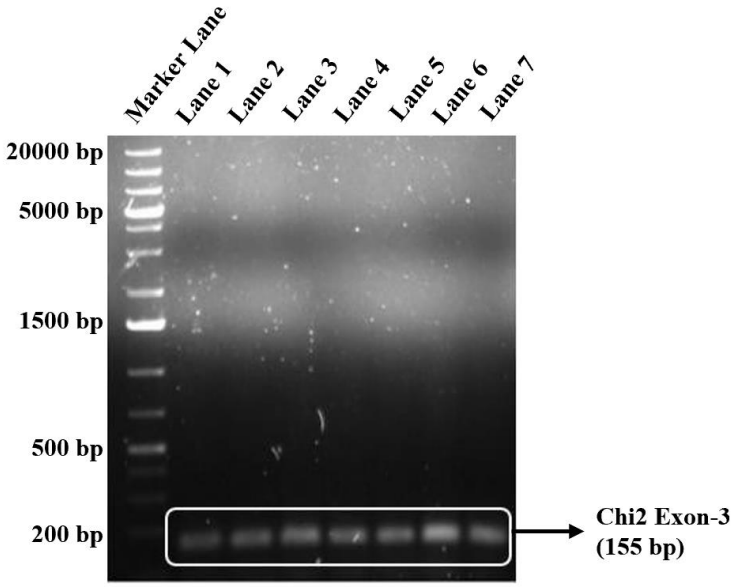

Fig 3.3.3 - Agarose gel electrophoresis analysis showing PCR amplified Exon-3 of Chi2 gene of *Metarhizium anisopliae* using Exon-specific primers. The most intense Exon-3 band appeared at 70.3°C. Phusion DNA polymerase was used to amplify the Chi2 Exon-3

Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 8 – Exon-3 of Chi2 gene (155 bp) (Annealing temperature range - 65°C to 70.9 °C).

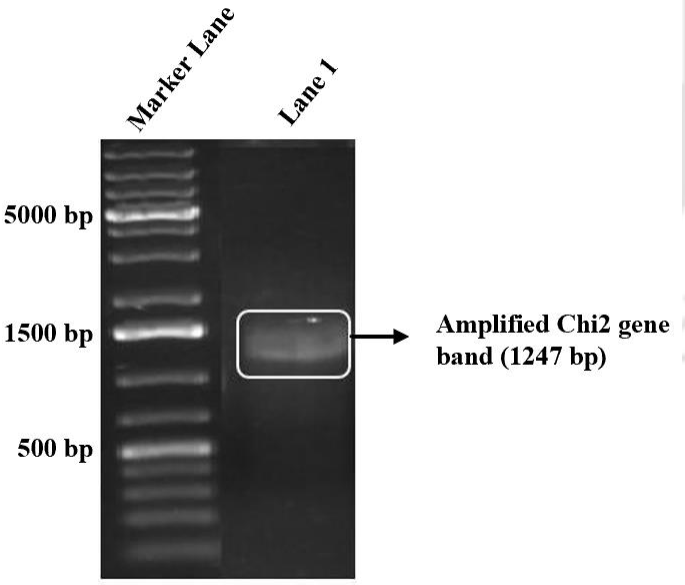

Fig 3.3.4 - Agarose gel image showing PCR amplified Full Chi2 gene of *Metarhizium anisopliae* using Exon-1 Forward primer and Exon-3 Reverse primer. The three exons of the Chi2 gene were fused using the Gibson assembly method, and the resulting Chi2 gene DNA-Gibson mixture was used to amplify the entire Chi2 gene. Phusion DNA polymerase was used to amplify the Chi2 gene
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 – Chi2 gene (1247 bp).

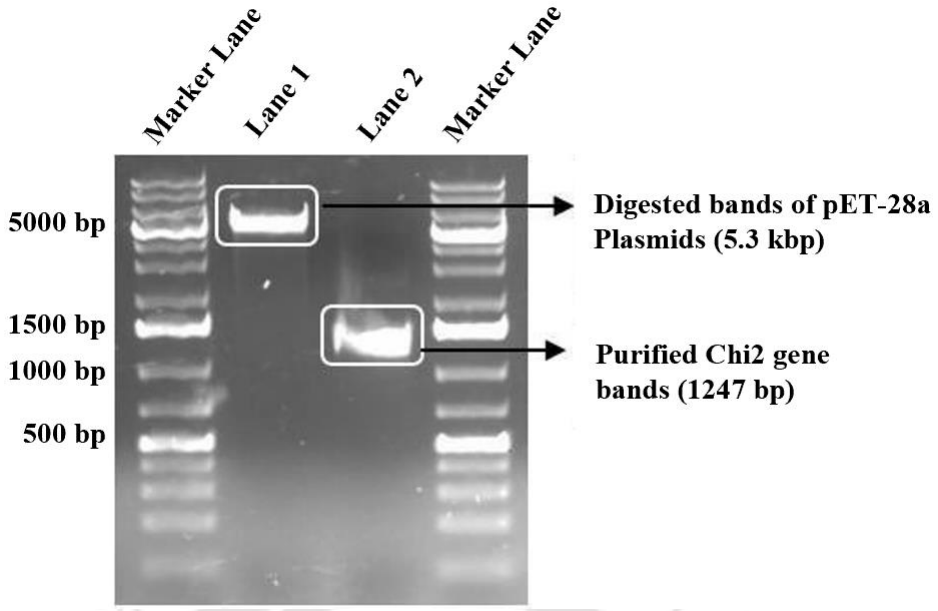

Fig 3.3.5 - Agarose gel image showing bands of gel purified Chi2 gene and digested pET-28a plasmid.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 – Purified Digested pET-28a plasmid (5.3 kbp).
Lane 2 –Purified Chi2 gene (1247 bp).

3.3.2 Amplification of cChi2 chimeric chitinase

To develop the chimeric cChi2 gene, the process involves merging two genetic components: Chi2 gene from *Metarhizium anisopliae* and the chitin-binding domain of *Bombyx mori.* To construct a chimeric protease, it is essential for the 3' end of the Chi2 gene and the 5' end of Bmcbd to have compatible ends. The reamplification of the Chi2 gene was carried out using gene-specific primers, with the specific aim of introducing a compatible chitin-binding domain overhang at its 3' end. The chitin-binding domain of Bombyx mori (Bmcbd) was amplified from the *Bombyx mori* chitinase cDNA clone construct (fepM03A03) using gene-specific primers. During this amplification process, a Chi2 overhang was introduced at the 5' end of the Bmcbd sequence. The Chi2 gene obtained from *Metarhizium anisopliae* and the chitin-binding domain derived from *Bombyx mori*. The Chi2 gene was re-amplified using a set of a new set of primers so as to introduce a compatible chitin-binding domain overhang at the 3’ end of the Chi2bm (Chi2 gene without its chitin-binding domain) (1137 bp). The chitin-binding domain

(Bmcbd) of *Bombyx mori* chitinase was amplified from a chitinase cDNA clone (fepM03A03) using gene-specific primers with a Chi2 overhang (Chi2 overhang -Bmcbd) at the 5' end of Bmcbd. The successful amplification of the Chi2bm and Bmchi2 fragments was verified by observing the presence of DNA bands on the agarose gel. The bands appeared at the anticipated sizes of 1137 bp for the Chi2bm fragment and 201 bp for the Bmchi2 fragment **(Fig. 3.3.6)**. The Nucelospin gel extraction kit was used to extract the amplified Chi2bm and Bmchi2 fragments from the gel, the purified fragments were observed on 1 % agarose gel **(Fig. 3.3.7).** The Chi2bm and Bmchi2 were fused and cloned into the pET-28a vector using the Gibson assembly method. The purified Chi2bm and Bmchi2 amplicons were stored at -20°C for cloning experiments.

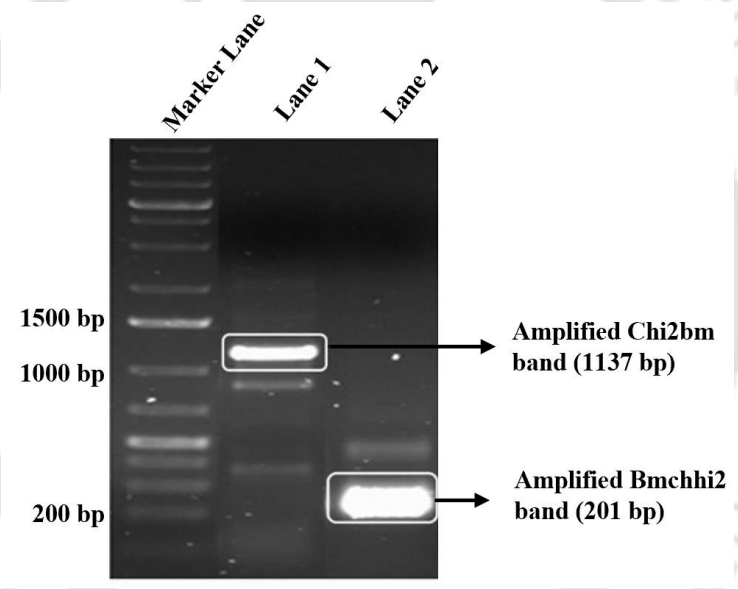

Fig 3.3.6 - Agarose gel image showing PCR amplified Chi2bm (Chi2 gene with Bmcbd overhang at 3'end) & Bmchi2 (Bmcbd with Chi2 gene overhang at 5' end).
Phusion DNA polymerase was employed to amplify the Chi2bm and Bmchi2 fragments.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1– PCR amplified Chi2bm fragment (1137 bp).
Lane 2 – PCR amplified Bmchi2 fragment (201 bp).

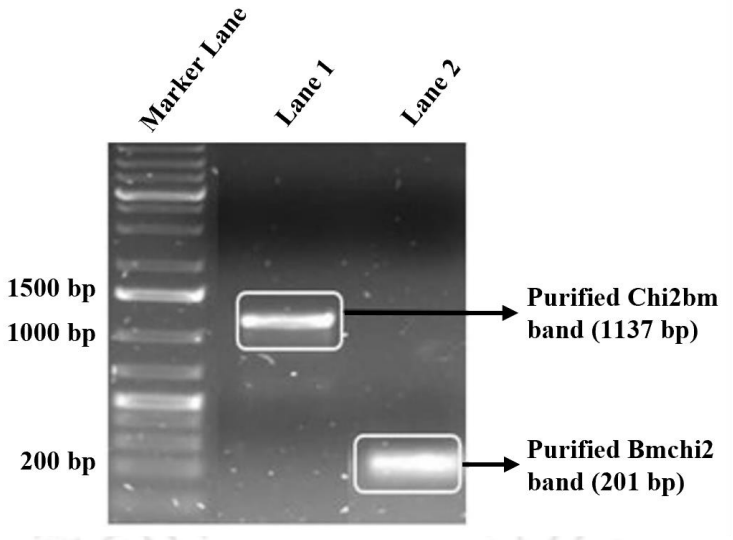

Fig 3.3.7 - Agarose gel electrophoresis analysis showing gel-purified fragments of Chi2bm and Bmchi2.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 – Purified Chi2bm fragment (1137 bp).
Lane 2 – Purified Bmchi2 fragment (201 bp).

3.3.3 Molecular cloning and transformation of recombinant plasmid DNA (pET-28a-Chi2) and (pET-28a- cChi2) into *E. coli* (Top-10) competent cells

The cloning of wild-type Chi2 gene (1247 bp) and the chimeric cChi2 gene (1270 bp) into the NcoI and XhoI digested pET-28a vector was carried out using the Gibson assembly method, yielding two new vector-insert systems, pET-28a-Chi2 (6616 bp) and pET-28a- cChi2 (6666 bp). Subsequently, the recombinant vector-insert constructs were transformed into the *E. coli* Top-10 competent cells. The transformants were grown on LB agar plates supplemented with kanamycin at 37°C for 12 -14 hours under static conditions **(Fig. 3.3.8)** and **(Fig. 3.3.9)**. To isolate the plasmids colonies were selected randomly and inoculated into a 5 mL LB medium. PCR and restriction digestion techniques were used for the screening and verification of recombinant plasmids (positive clones). The restriction digestion technique was employed using the enzymes NcoI and XhoI to verify the positive clones.

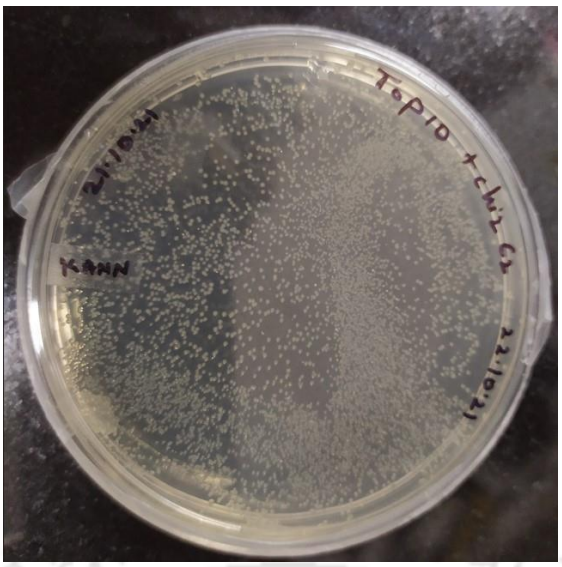

Fig 3.3.8 - The Top -10 cells were transformed with pET-28a-Chi2 construct. The figure shows the Top 10 colonies carrying the pET-28a-Chi2 plasmid, appeared on the LB agar plate (supplemented with kanamycin (50 µg /mL) after transformation followed by incubation at 37°C for 14 h.

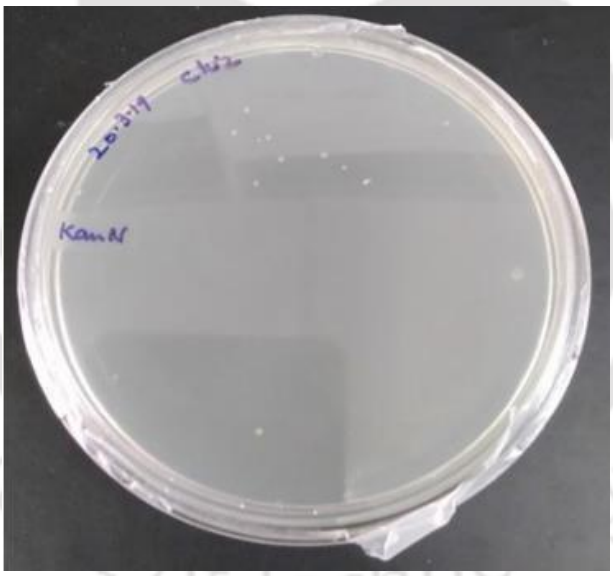

Fig 3.3.9 - The Top -10 cells were transformed with pET-28a- cChi2 construct. The figure shows the Top 10 colonies carrying the pET-28a- cChi2 plasmid, appeared on the LB agar plate (supplemented with kanamycin (50 µg /mL) after transformation followed by incubation at 37°C for 14 h.

3.3.4 Plasmid DNA isolation from transformants and confirmation of Chi2 and cChi2 positive clones

The modified alkaline lysis procedure described in section **2.2.14** was used to isolate the plasmid DNA from the Chi2 and cChi2 transformant colonies. The plasmid DNA isolated from the Chi2 and cChi2 transformant colonies were analyzed on 1% agarose gel. **(Fig 3.3.10** and **Fig. 3.3.11).** The recombinant plasmids were screened for positive clones using both PCR and restriction digestion techniques**.** To confirm the positive clones, the restriction enzyme digestion method was employed, and NcoI & XhoI restriction enzymes were utilized for the digestion of plasmids. Following restriction digestion, the digested plasmids were electrophoresed on a 1% agarose gel in order to see the DNA fragments according to their size. The analysis of the agarose gel revealed that the plasmids obtained from the 2^{nd}, 4^{th}, and 9^{th} Chi2 colonies showed two DNA bands that are equivalent to the sizes of the linearized pET28a vector (5369 bp) and Chi2 (1247 bp), confirming the successful cloning of Chi2 into the pET28a vector **(Fig. 3.3.13)**. The agarose gel evaluation of digested cChi2 recombinant plasmids revealed that the 7th and 8th cChi2 transformant colonies carried positive clones. DNA bands obtained after restriction digestion of plasmids isolated from cChi2 2^{nd} to 12^{th} colonies (except 6^{th}) correspond in size to the pET28a vector (5369 bp) and cChi2 gene (1270 bp) and therefore, confirm the positive cChi2 clones **(Fig.3.3.15)**. The polymerase chain reaction (PCR) was used for the second confirmation of positive clones. The plasmids isolated from the Chi2 and cChi2 transformants' colonies were subjected to PCR amplification using specific primers designed for the Chi2 and cChi2 genes, respectively. A 1.0% agarose gel was used to analyze the PCR amplicons. The agarose gel analysis of the amplified products revealed that the 2^{nd} and 9^{th} Chi2 plasmids and 1^{st} to 12^{th} cChi2 plasmids except the 7th plasmid, respectively, carry the Chi2 gene and the cChi2 gene inserts. **(Fig 3.3.12 and Fig 3.3.14)**. The Chi2 and cChi2 colonies harboring the positive clones were grown in 5 mL LB media, and their plasmids were isolated and stored at -20 °C for protein expression experiments.

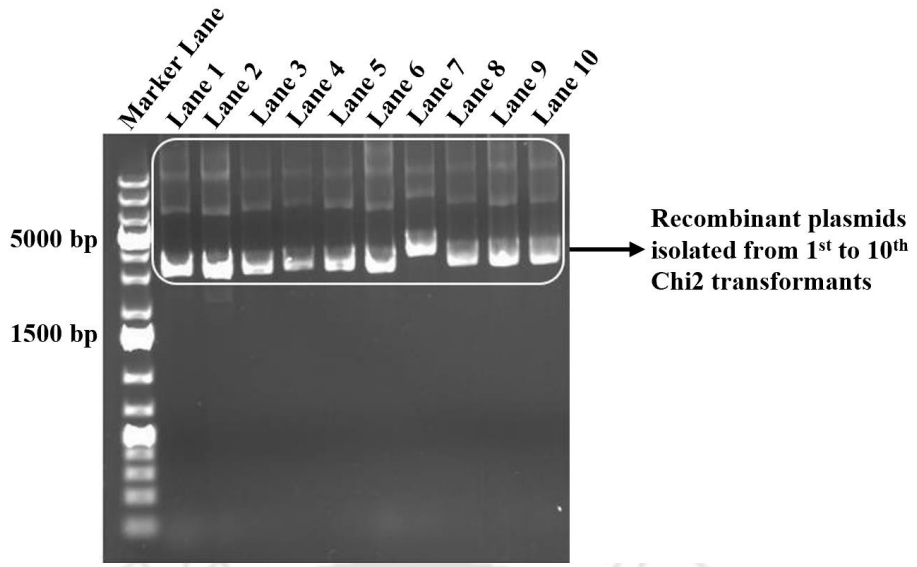

Fig 3.3.10 - Agarose gel electrophoresis analysis showing recombinant plasmids isolated from Chi2 transformants.
E. coli Top 10 cells were transformed with the pET-28a-Chi2 plasmid and selected on LB agar plates containing kanamycin.
Plasmids were isolated from selected transformants and resolved using 1 % agarose gel electrophoresis.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 10 – Recombinant plasmid isolated from Chi2 transformants 1st to 10th.

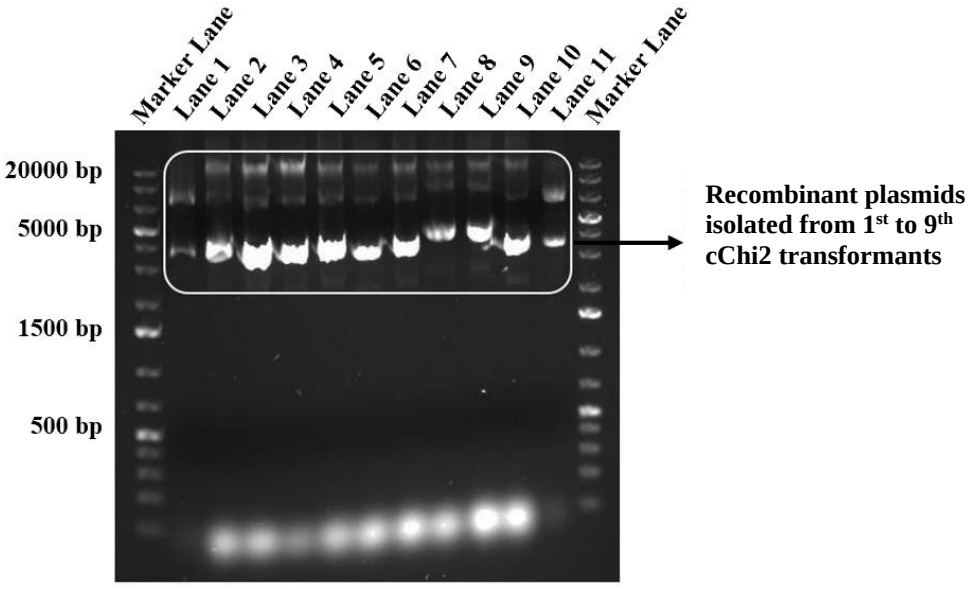

Fig 3.3.11 -Agarose gel electrophoresis analysis showing recombinant plasmids isolated from cChi2 transformants.
E. coli Top 10 cells were transformed with the pET-28a- cChi2 plasmid and selected on LB agar plates containing kanamycin.
Plasmids were isolated from selected transformants and resolved using 1 % agarose gel electrophoresis
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 & 10 – pET-28a (Negative control).

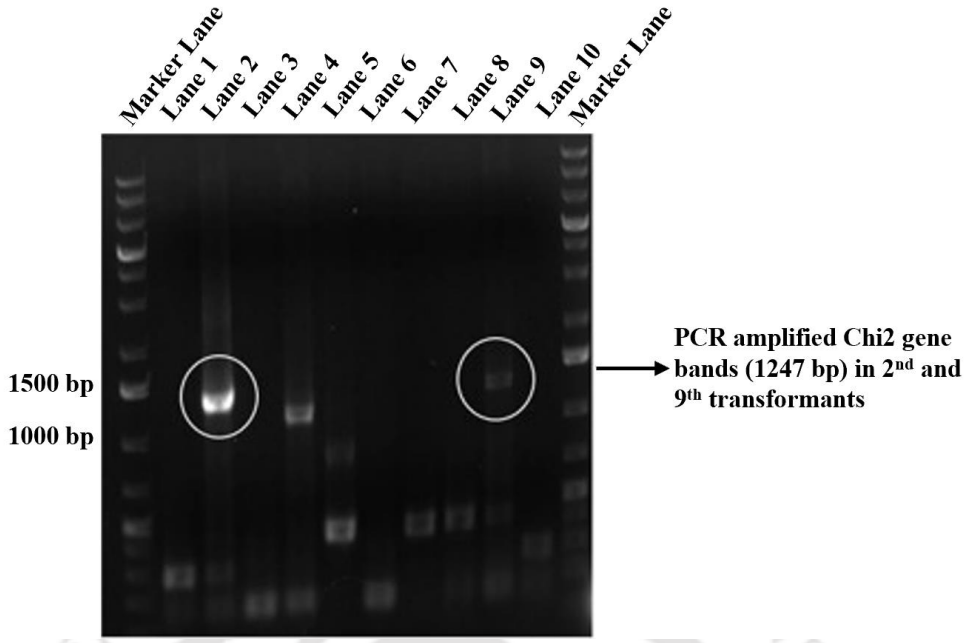

Fig 3.3.12 - Agarose gel analysis showing PCR amplified Chi2 gene (1247 bp) fragments. from Chi2 clones.
PCR confirmation for positive clones was done using Taq DNA polymerase and Chi2 gene-specific primers, utilizing plasmid DNA isolated from Chi2 clones 1st to 10th as templates.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 2 & 9 – 2nd and 9th plasmids are showing the Chi2 (1247 bp) gene insert.

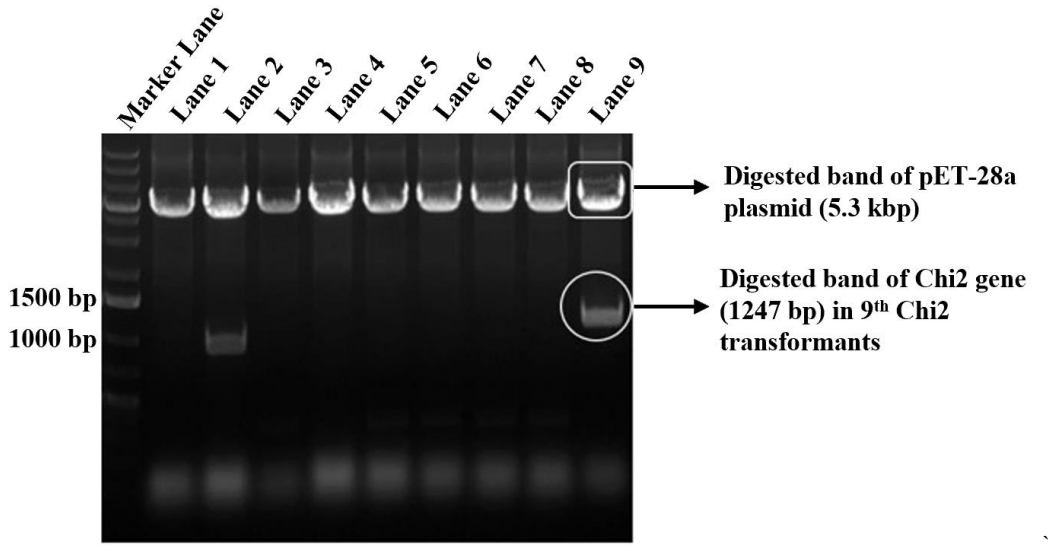

Fig 3.3.13 - Agarose gel electrophoresis image of Chi2 clone confirmation analysis by restriction enzyme digestion.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Plasmids (pET-28a – Chi2) isolated from transformants were digested with NcoI and XhoI enzymes.
Lane 9 –9th Digested Recombinant plasmid showing the Chi2 gene insert (1247 bp).

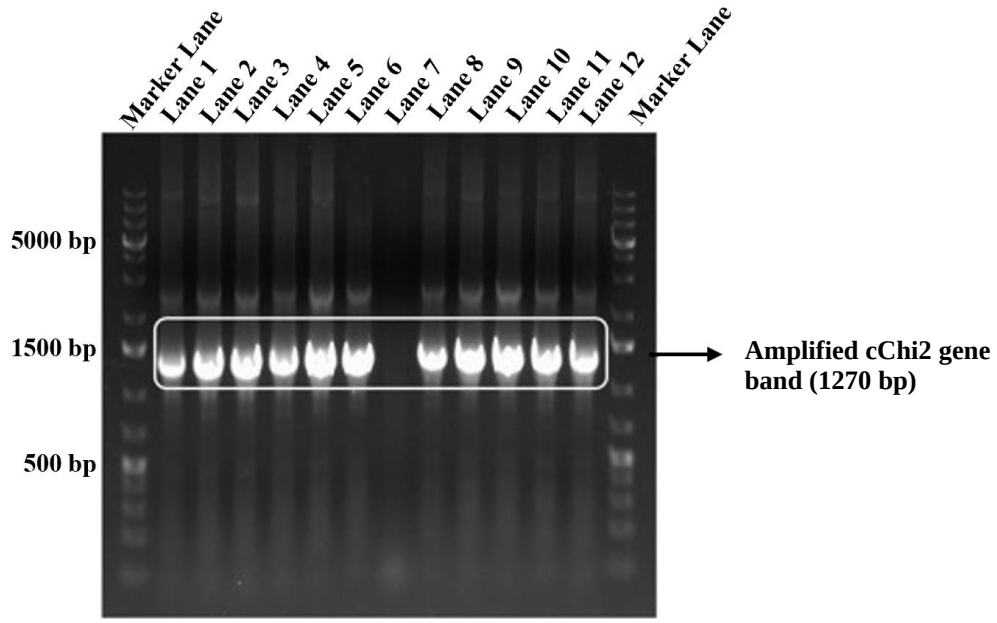

Fig 3.3.14 - Agarose gel analysis showing PCR amplified cChi2 gene (1270 bp) fragments from cChi2 clones.
PCR confirmation for positive clones was done using Taq DNA polymerase and cChi2 gene-specific primers, utilizing plasmid DNA isolated from cChi2 clone's 1st to 12th as templates.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 6, 8 to 12 – Except the 7th plasmid all 10 plasmids are showing the cChi2 gene (1270 bp) insert.

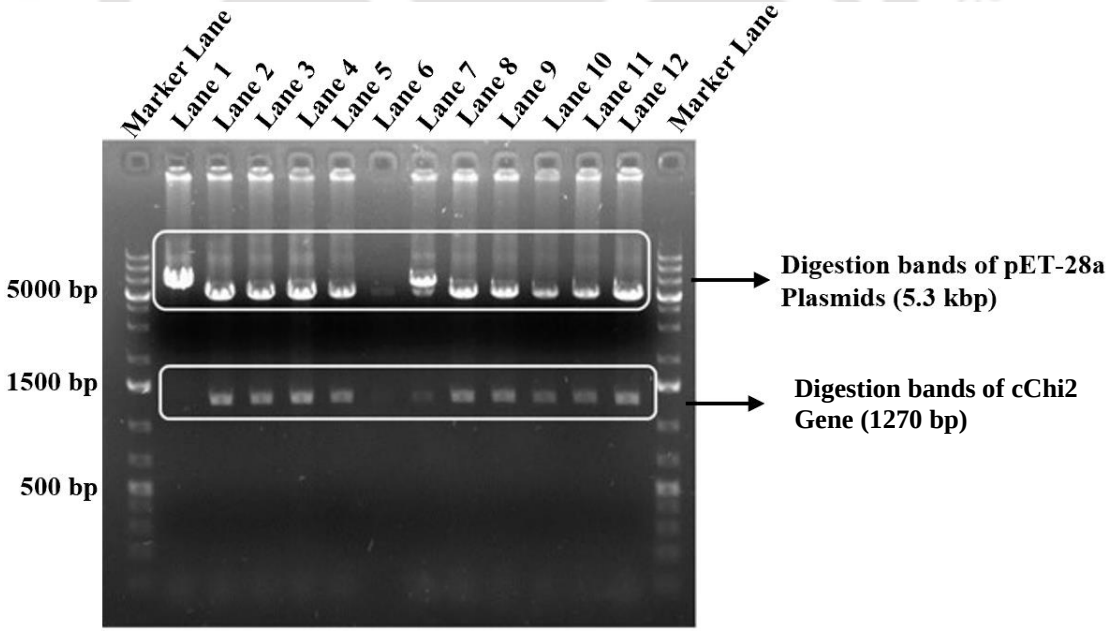

Fig 3.3.15 - Agarose gel electrophoresis image of cChi2 clone confirmation analysis by restriction enzyme digestion.
Plasmids (pET-28a-cChi2) isolated from transformants digested with NcoI and XhoI enzymes.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 12 –- Digested Recombinant plasmids (2nd to 5th & 7th to 12th is showing the cChi2 (1270 bp) gene insert.

3.3.5 Expression, affinity purification of wild-type (Chi2) and chimeric (cChi2) proteins

The recombinant pET-28-Chi2 and pET-28- cChi2 constructs containing the genes encoding Chi2 and cChi2, respectively, were transformed into *E. coli* BL21(DE3) competent cells. Post-transformation of purified recombinant plasmids into *E. coli* BL-21(DE3)- competent cells, the colonies were randomly chosen and cultured in 5 ml of LB medium supplemented with 50 µg/ml kanamycin. During the mid-exponential stage of growth, the cells were induced to overexpress the Chi2-$(His)_6$ and cChi2-$(His)_6$ proteins. SDS-PAGE gels were run and observed to assess protein expression by loading various fractions of induced cells containing the recombinant proteins into adjacent wells. Chi2 and cChi2 proteins were overexpressed and purified using immobilized metal ion affinity chromatography (**Fig. 3.3.16**). The Chi2 and cChi2 protein expression was optimized in LB Broth at 16°C. For final purification, Ni-NTA affinity chromatography was used. The Chi2-$(His)_6$ and cChi2-$(His)_6$ proteins were expressed in the supernatant as soluble fractions. The molecular weights of the purified Chi2 and cChi2 proteins were approximately 42 kDa and 44 kDa. Purified Chi2 and cChi2 proteins were quantified using the Bradford assay. The purified protein and other fractions eluted from the column were analyzed on 12% (SDS-PAGE) Sodium Dodecyl Sulfate - Poly Acrylamide Gel electrophoresis **(Fig. 3.3.16)**. The Bio-Rad Gel Doc EZ gel documentation system was used for gel imaging, and the Image Lab 4.0 software was used for analysis.

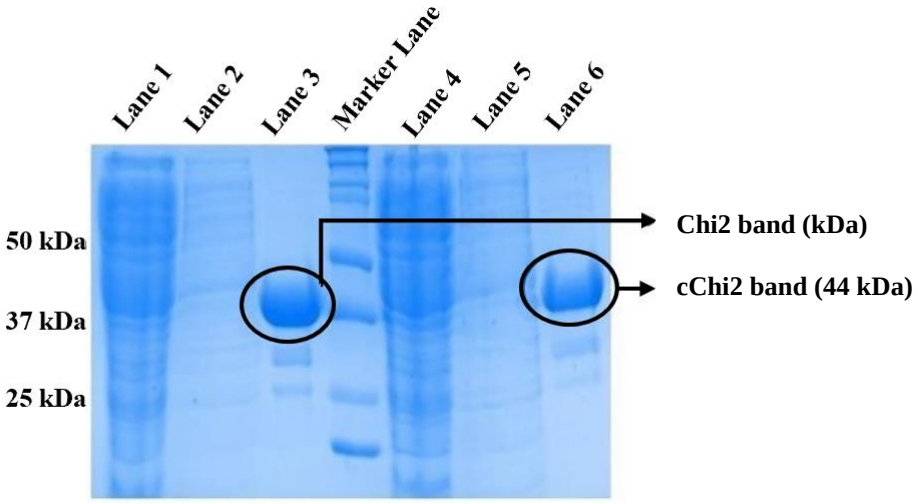

Fig 3.3.16 - Purification profile of recombinant Chi2-$(His)_6$ and cChi2-$(His)_6$ proteins on 12% (w/v) polyacrylamide gel. SDS-PAGE was used to resolve the proteins and Coomassie Brilliant Blue wasused to stain them. Marker Lane: Precision Plus protein ladder, (Biorad 1610374), lane 1: cell pellet after sonication, lane 1: flow-through (Chi2), lane 2: last column wash (Chi2), lane 3: purified Chi2 protein (42 kDa) cell-free medium supernatant after sonication, lane 4: flow-through (cChi2) lane 5: last column wash (cChi2), lane 6: purified cChi2 protein (44 kDa).

3.3.6 Binding assays

The swapping of the chitin-binding domain of the Chi2 chitinase of *Metarhizium anisopliae* with the Chitin-binding domain of the insect (*Bombyx mori*) chitinase led to the creation of a chimeric chitinase known as cChi2. The successful expression of this chimeric chitinase in *Escherichia coli* was confirmed through SDS-PAGE analysis. Subsequently, both Chi2 and cChi2 chitinases were purified using affinity chromatography on a Ni-NTA column and employed for chitin-binding and chitinase enzyme assays. The introduction of the new chitin-binding domain in the chimeric chitinase (cChi2) positively influenced its binding capacity and enzymatic efficiency and increased the affinity of the chimeric chitinase (cChi2) for the silkworm's cuticle. In reference to wild type chitinase, the chimeric chitinase cChi2 demonstrated considerably higher binding affinity. Compared to the wild-type Chi2 chitinase (24.41 µg), a 19.11% increase in binding efficiency (30.18 µg) of chimeric chitinase cChi2 was observed with the powdered cuticle. **(Fig 3.3.17)**.

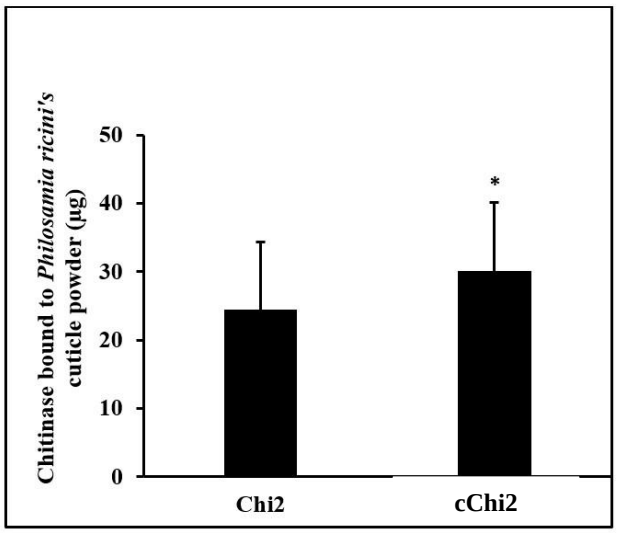

Fig 3.3.17 - Cuticle binding analysis of wild-type Chi2 and chimeric cChi2 chitinase
The percent protein bound was determined from assay mixtures containing 5 mg *Philosamia ricini's* cuticle powder in 50 mM potassium phosphate buffer (pH 6.0) plus 100 µg chitinase.
Chi2 - the amount of Chi2 protein bound (24.41 µg), with the powdered cuticle.
cChi2- the amount of cChi2 chimeric protein bound (30.18 µg) with the powdered cuticle.
*- The results of the student t-test are statistically significant (P < 0.02) and represent the mean values from three independent experiments.

3.3.7 Chitinase enzyme assays

In order to evaluate whether swapping the chitin-binding domain of Chi2 with Bmcbd could enhance the digestion of chitin components in the insect cuticle, we performed the chitinase enzyme assays experiments where we incubated the purified Chi2 and cChi2 proteins with Eri (*Philosamia ricini*) silkworm cuticles in a reaction mixture. Following this, we quantified the amount of N-Acetylglucosamine released in the supernatant. The Chimeric chitinase gave substantially more enzyme activity than wild-type chitinase in both 1 min and 4 mins enzyme assays. About 31.5 % increase in 1 min enzymeassays and 32.9 % in 4 mins enzyme assays has been seen in cuticle enzyme activities of chimeric chitinase cChi2 (72.93 U/mL, 1 min & 31.76 U/mL, 4 mins) compared to wild- type chitinase (49.97 U/mL, 1 min & 21.28 U/mL, 4 mins). Significant levels of cuticle degradation were observed at P < 0.05 with the enzymes **(Fig. 3.3.18 & Fig. 3.3.19)**.

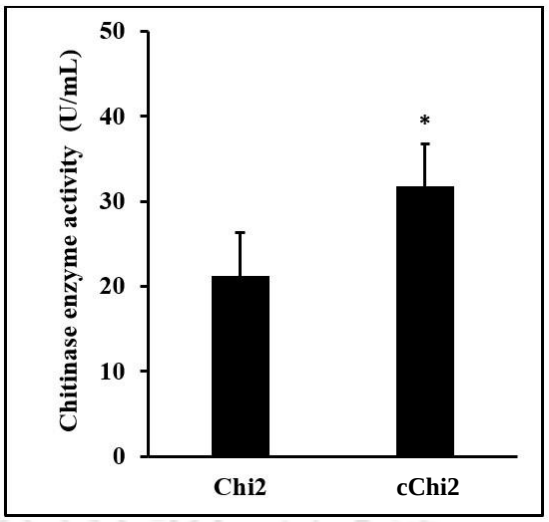

Fig 3.3.18 - Comparative enzymatic analysis (4 min) with wild-type Chi2 chitinase and chimeric cChi2 chitinase using *Philosamia ricini's* cuticle powder as substrate.
Chi2 – Enzyme activity of wild-type Chi2 enzyme (21.28 U/mL).
cChi2 - Enzyme activity of chimeric cChi2 enzyme (31.76 U/mL).
The chimeric chitinase cChi2 exhibited significantly higher enzyme activity compared to the wild-type chitinase.
*- The results of the student t-test are statistically significant ($P < 0.02$) and represent the mean values from three independent experiments.

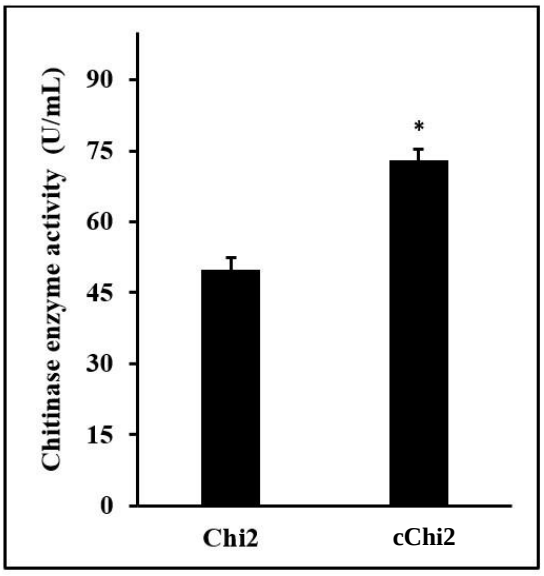

Fig 3.3.19 - Comparative enzymatic analysis (1 min) with wild-type Chi2 chitinase and chimeric cChi2 chitinase using *Philosamia ricini's* cuticle powder as substrate.
Chi2 – Enzyme activity of wild-type Chi2 enzyme (49.97 U/mL).
cChi2 - Enzyme activity of chimeric cChi2 enzyme (72.93 U/mL).
The chimeric chitinase cChi2 exhibited significantly higher enzyme activity compared to the wild-type Chi2 chitinase.
*- The results of the student t-test are statistically significant ($P < 0.05$) and represent the mean values from three independent experiments.

3.3.8 Homology Modeling

The web server PHYRE2 was utilized for the homology modeling of Chi2 and cChi2 proteins. For structure prediction of Chi2, Phyre2 used the most appropriate model structure of chitinase from *Hevea brasiliensis* (PDBe-2hvm) with 100% confidence and 77% coverage **(Fig. 3.3.20),** and protein structure of bmcbd was modeled against an avirulence protein4 (avr4) from *Cladosporium fulvum* with 99.4% confidence and 96% coverage. The percentage of different secondary structures was predicted through i-tasser, which showed 67.33 % random coils, 10.72 % β-strands, and 21.94 % helix in wild-type Chi2 chitinase and 68.59 % random coils, 11.81 % β-strands, and 19.6 % helix in chimeric cChi2 Chitinase.

The 3D protein structures of Chi2 and bmcbd were fused using UCSF chimera software in order to generate a 3D protein structure of the cChi2 enzyme **(Fig. 3.3.21)**. The conserved domains in the chitinase of *M. anisopliae* were found using the NCBI conserved domain search-NIH tool. GH-18 chitinase-like superfamily domain and CBM1, Fungal cellulose binding domain, were found to be two highly conserved domains. The Chi2 Chitinases of the GH18 (glycosyl hydrolase, family 18) type II digests chitin, an abundant polymer of beta-1,4-linked N-acetylglucosamine (GlcNAc) is a major component of fungi cell walls and arthropod exoskeletons. The GH18 domain has an eight-stranded beta/alpha barrel structure with a prominent active-site cleft at the C-terminus of the beta-barrel. The plant chitinases of types III and V, endo-beta-N-acetylglucosaminidases, hevamine, chitotriosidase, chitobiase, and chitolectins belong to the GH18 family.

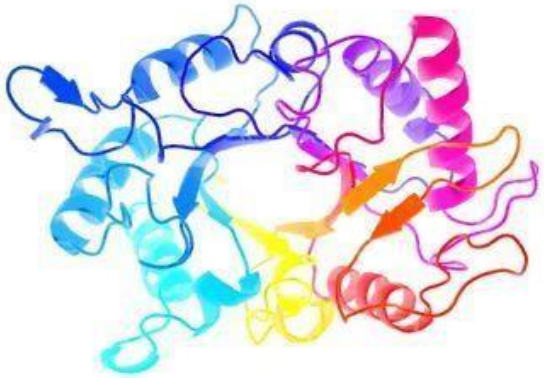

Fig. 3.3.20 - The 3D protein structure of wild-type Chi2 chitinase was modeled by the PHYRE2 web server. The predicted model attains a 100.00% confidence with 77% coverage and 36% identity to the template d2hvma (PDBe-2hvm) with a PDBe title of Hevamine a at 1.8-angstrom resolution.

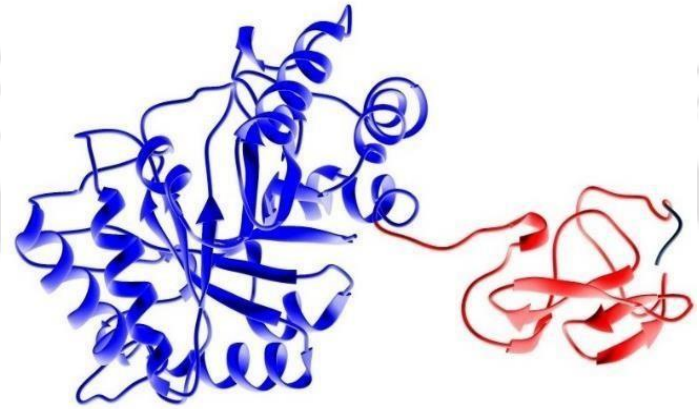

Fig. 3.3.21 - The structure of Bmcbd (*Bombyx mori* chitin-binding domain) was generated by the PHYRE2 web server. The predicted model attains a 99.4 % confidence with 96 % coverage and 35 % identity to the template c6bn0C (PDBe- 6bn0) Avirulence protein 4 (Avr4) from *Cladosporium fulvum* bound to the hexasaccharide of chitin. The 3D protein structure of chimeric cChi2 was generated by merging the modeled structure of Chi2 and Bmcbd using UCSF-Chimera software. Blue part - Chi2 (without chitin-binding domain), Red part – *Bombyx mori* chitin-binding domain.

3.3.9 Sequence analysis

Multiple sequence alignment was done using clustal omega, and a phylogenetic tree was generated from MSA values using Mega 11 software **(Fig. 3.3.22)**. Using the BlastP program, the homology of the *M. anisopliae* Chi2 chitinase gene with chitinase genes reported from other Organisms was investigated. The Chitinase gene of *M. anisopliae* revealed 82 % homology with the sequence of chitinase-3 of *Pochonia chlamydosporia 170.* It was 73.79 % identical to the endochitinase-2 of *Tolypocladium ophioglossoides*. It also shared 81.96 % protein identity with

chitinase -2 of *Purpureocillium lilacinum* and 79.44 % protein identity with *Cordyceps militaris CM01*. The identities of other chitinase genes from different organisms with the Chi2 gene were found as *Lachnellula suecica* (64.09 %), *Scytalidium lignicola* (68.51 %), *Xylaria nigripes* (62.59 %), *Daldinia childiae* (71.75 %) and Hyaloscypha sp. PMI 1271 (65.48 %).

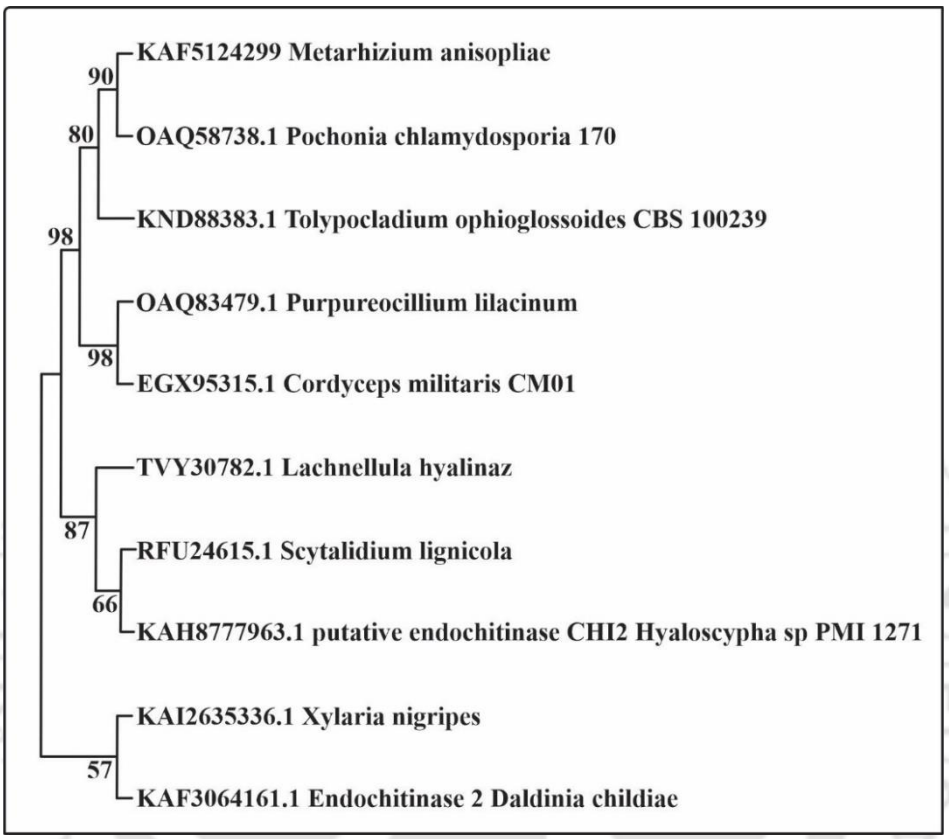

Fig. 3.3.22 - Phylogenetic tree depicting the evolutionary relationship of *M. anisopliae* chitinase (Chi2) with chitinase from different organisms.

Phylogenetic tree depicting the evolutionary relationship of *M. anisopliae* Chi2 chitinase with chitinases from different organisms. The construction of this phylogenetic tree was carried out using the MEGA version 11 software, a robust and widely used tool for conducting evolutionary analysis and constructing phylogenetic trees. Neighbor-joining approach with the Poisson model for multiple amino acid substitution and 1000 random bootstrap repeats was used.

High bootstrap values (close to 100%) indicate strong support for the branches. Alignment of all sequences was done using clustal omega. Binomen genus-species names are used. The evolutionary relationship revealed that *Metarhizium anisopliae's* chitinase was most closely related to the chitinase-3 of *Pochonia chlamydosporia* 170.

3.4 Discussion

Chitin is the second most abundant polymer in nature. The exoskeleton of insects, fungi, yeast, and algae, as well as the internal organs of other vertebrates, all contain chitin (85). Chitinases have drawn researchers' interest in the past two decades for their abilities to biologically control insects. Chitinases are chief components of biopesticides and they have also been used as biocontrol agents for a long time because of their ability to digest the integument of insect pests (134, 135).

The chitinases secreted by the entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana*, act directly on the cuticle of the insect's pests which initiates the intrudence of the fungus inside the insect (131, 91, 9). The Chi2 chitinase plays a direct role during the penetration phase of infection and significantly contributes to fungal virulence. The constitutive overexpression of the Bbchit-1 gene of *Beauveria bassiana* resulted in a 50 % reduction in the lethal concentration and lethal time of the transformants compared to the wild-type fungus (9). The carbohydrate-binding domain is responsible for recognizing and attaching the enzyme to insoluble substrates. This domain allows the enzyme to anchor itself firmly to the substrate by positioning the catalytic domain in close proximity to the substrate. Consequently, the enzymatic hydrolysis of the carbohydrate polymers occurs more efficiently, breaking down the insoluble polymers into smaller and soluble oligomer/monomer fragments (99). Chimeric enzymes are frequently created by combining the catalytic domain (GH domain) from one species with another domain, such as a Carbohydrate-Binding Module (CBM) from a different species. Several studies have demonstrated that chimeric enzymes can enhance catalytic efficiency, thermostability, and substrate specificity compared to their individual components (138, 139, 140).

Eliminating the substrate-binding domain from chitinases or cellulases reduces their activities on insoluble substrates (141). Aron Paek et al engineered a hybrid chitinase MbLCL-

BtChBD by swapping the bacterial and lepidopteran insect's domains. This hybrid chitinase showed greater binding affinity to chitin with a simultaneous increase in catalytic activity (142). Insects produce chitinases to facilitate the digestion of their cuticles during their molting phase. These chitinases typically possess a chitin-binding domain that aids in the binding of the chitinase to the chitin present in the cuticle and efficient degradation of chitin. Chitin-binding domains are widely recognized for facilitating the development of chimeric chitinases with improved catalytic efficiency and binding ability. Chimeric Chit 42 (ChC) chitinase was created by fusing the chitin-binding domain ChBD of *Serratia marcescens* chitinase B with *Trichoderma atroviride's* fungal chitinase Chit 42. Chimeric Chit42 (ChC) and Chit42 showed comparable activities towards colloidal chitin but (ChC) showed about a 70 % increase in its binding efficiency towards insoluble chitin with simultaneous enhancement in its catalytic efficiency. The chimeric chitinase (ChC) also showed an increase in chemical and thermal stability (143). According to a study done by Yanhua Fan et al, a hybrid chitinase was constructed by fusing the Bbchit 1 chitinase gene from *Beauveria bassiana* with the *Bombyx mori* chitin-binding domain BmChBD. When this hybrid chitinase construct (Bbchit1-BmChBD) was transformed into *B. bassiana* and constitutively expressed under a fungal promoter (gpd-Bbchit1-BmChBD), it increased the virulence of the fungus by a reduction in the time to death of insect pests *Myzus persicae* (12). By swapping the chitin-binding domain of Chi2 chitinase from the fungal insect pathogen *Metarhizium anisopliae* with the chitin-binding domain of *Bombyx mori* insect, we implemented a strategy to enhance the chimeric chitinase's capacity to bind with the insoluble cuticle of insect. This modification is aimed at enhancing the catalytic activity of the chitinase.

3.5 Conclusion

In summary, our study found that the chimeric chitinase, created by replacing the chitin-binding domain of *Metarhizium anisopliae's* wild-type chitinase with that *of Bombyx mori*, exhibited significant improvements. Binding assays revealed a 19% increase in binding efficiency and approximately 32% augmentation in the catalytic efficiency of the chimeric chitinase cChi2 compared to the wild-type chitinase Chi2.

These findings collectively demonstrate that the manipulation of chitin-binding domains of chitinase not only enhances the catalytic activity of the enzyme but also improves its substrate affinity, thereby offering promising prospects for the development of enzymes with improved chitin-degrading capabilities. This study contributes valuable insights into the potential applications of chimeric chitinases in *Metarhizium*-based biopesticides. The transformation of this chimeric chitinase construct in *Metarhizium anisopliae* and its overexpression under a constitutive promoter could significantly enhance the virulence of the fungi.

Table 3.2.1: List of primers used for the amplification of Chi2 and cChi2 genes.

Primer	Sequence
Chi2 Exon I FP_ NcoI_pET28a_Over hang	5'- CTTTAAGAAGGAGATATACCATGGTGCCCTTGACTGACAAGATATCAGTCAAGCC – 3'
Chi2 Exon I RP_Chi2 Exon II_overhang	5'- GACGTGCTGATGTAGCATGATTGGCCTATAGTGCCTGAAGGTATGTTGC – 3'
Chi2 Exon II FP_ Chi2 Exon I_overhang	5'- GGCAACATACCTTCAGGCACTATAGGCCAATCATGCTACATCAGCAC – 3'
Chi2 Exon II RP_Chi2 Exon III_overhang	5' – GACCGGAATAGCCCTCGCCGCCGCACTGACCCCACTGGGGAAC – 3'
Chi2 Exon III FP_Chi2 Exon II overhang	5' – CGGCACTGTTCCCCAGTGGGGTCAGTGCGGCGGCGAGGCTAT – 3'
Chi2 Exon III_His Tag_XhoI_pET28a_Overh ang	5' – GGTGGTGGTGGTGCTCGAGTTAATGATGATGATGATGATGCCGGCATGACGACCACCAA – 3'
Chi2bm FP_ NcoI_pET28a_Over hang	5' – CTTTAAGAAGGAGATATACCATGGTGCCCTTGACTGACAAGATATCAGTCAAGCC – 3'
Chi2bm RP_Bmcbd_overhang	5' - GTCCTCAGAGTTACACACATC GGGAACAGTGCCGCCGGT – 3'
Bmchi2 FP_Chi2_overhang	5' – CCGGCGGCACTGTTCCCGATGTGTGTAACTCTGAGGAC – 3'
Bmchi2 _RP_His Tag_XhoI_pET28a_Overh ang	5' – GGTGGTGGTGGTGCTCGAGTTAATGATGATGATGATGATGAGGCCAATCGCAAACGTTAAG – 3'

Table 3.2.2: Components of PCR mixture for amplification of Chi2 exon I, II, and III from the genomic DNA of *Metarhizium anisopliae*.

PCR components	Volume (µl)	Final concentration
Nuclease-free water	30.0	--
5x reaction buffer	10.0	1x
dNTP mix (10 mM)	1.0	0.2 mM
Forward primer (10 µM)	2.5	0.5 µM
Reverse primer (10 µM)	2.5	0.5 µM
DMSO	1.5	3%
Template (Genomic DNA) (50 µg/mL)	2.0	100.0 ng
Phusion DNA polymerase (2 U/µl)	0.5	0.02 U/µl
Total	50.0	--

Table 3.2.3 PCR cycles conditions for amplification of Chi2 exon I from *Metarhizium anisopliae*

Steps		Time
		2 min
I. Denaturation at 98°C		
II. 30 cycles of	i) Denaturation at 98°C	10 s
	ii) Annealing at 64°C	30 s
	iii) Extension at 72°C	15 s
III. Final extension at 72°C		10 min

Table 3.2.4 PCR cycles conditions for amplification of Chi2 exon II from *Metarhizium anisopliae*

Steps		Time
		2 min
I. Denaturation at 98°C		
II. 30 cycles of	i) Denaturation at 98°C	10 s
	ii) Annealing at 66°C	30 s
	iii) Extension at 72°C	30 s
III. Final extension at 72°C		10 min

Table 3.2.5 PCR cycles conditions for amplification of Chi2 exon III from *Metarhizium anisopliae*

Steps		Time
		2 min
I. Denaturation at 98°C		
II. 30 cycles of	i) Denaturation at 98°C	10 s
	ii) Annealing at 70°C	30 s
	iii) Extension at 72°C	15 s
III. Final extension at 72°C		10 min

Table 3.2.6 Components of PCR mixture for amplification of whole Chi2 gene of *Metarhizium anisopliae.*

PCR components	Volume (µl)	Final concentration
Nuclease-free water	30.0	--
5x reaction buffer	10.0	1x
dNTP mix (10 mM)	1.0	0.2 mM
Forward primer (10 µM)	2.5	0.5 µM
Reverse primer (10 µM)	2.5	0.5 µM
DMSO	1.5	3%
cDNA (30.0 µg/mL)	2.0	60.0 ng
Phusion DNA polymerase (2 U/µl)	0.5	0.02 U/µl
Total	50.0	--

Table 3.2.7 PCR cycles conditions for amplification of whole Chi2 gene from *Metarhizium anisopliae*

Steps		Time
I. Denaturation at 98°C		2 min
II. 30 cycles of	i) Denaturation at 98°C	10 s
	ii) Annealing at 72°C	30 s
	iii) Extension at 72°C	40 s
III. Final extension at 72°C		10 min

Table 3.2.8 Components of PCR mixture for amplification of Chi2bm fragment.

PCR components	Volume (µl)	Final concentration
Nuclease-free water	42.8	--
5x reaction buffer	14.0	1x
dNTP mix (10 mM)	1.4	0.2 mM
Forward primer (10 µM)	3.5	0.5 µM
Reverse primer (10 µM)	3.5	0.5 µM
DMSO	2.1	3%
Template DNA (50.0 µg/mL)	2.0	100.0 ng
Phusion DNA polymerase (2 U/µl)	0.7	0.02 U/µl
Total	70.0	--

Table 3.2.9 PCR cycles conditions for amplification of Chi2bm fragment

Steps		Time
		2 min
I. Denaturation at 98°C		
II. 30 cycles of	i) Denaturation at 98°C	10 s
	ii) Annealing at 60°C	30 s
	iii) Extension at 72°C	30 s
III. Final extension at 72°C		10 min

Table 3.2.10 Components of PCR mixture for amplification of Bmchi2 fragment.

PCR components	Volume (µl)	Final concentration
Nuclease-free water	30.0	--
5x reaction buffer	10.0	1x
dNTP mix (10 mM)	1.0	0.2 mM
Forward primer (10 µM)	2.5	0.5 µM
Reverse primer (10 µM)	2.5	0.5 µM
DMSO	1.5	3%
Template DNA (45.0 µg/mL)	2.0	90.0 ng
Phusion DNA polymerase (2 U/µl)	0.5	0.02 U/µl
Total	50.0	--

Table 3.2.11 PCR cycles conditions for amplification of Bmchi2 fragment

Steps		Time
		2 min
I. Denaturation at 98°C		
II. 30 cycles of	i) Denaturation at 98°C	10 s
	ii) Annealing at 70°C	30 s
	iii) Extension at 72°C	15 s
III. Final extension at 72°C		10 min

Table 3.2.12 Composition of restriction enzymes digestion reaction for Chi2 and cChi2 clone's confirmation

Components	Volume (µl)
10x cut smart buffer	2.0
Nuclease-free water	7.0
Plasmid DNA (40 ng/µl)	10.0
*Nco*I (20 U/µl)	0.5
*Xho*I (20 U/µl)	0.5
Total	20.0

Table 3.3.1: Secondary structure analysis of Chi2 and cChi2 proteins

Protein	α helix	β strands	Random coils
Chi2	21.94 %	10.72 %	67.33 %
cChi2	19.6 %	11.81 %	68.59 %

CHAPTER-4

Cloning and expression of the fusion protein f-cPr1a- cChi2 (f-PC) into *E. coli*

Chapter-4

4.1 Introduction

Beauveria bassiana and *Metarhizium anisopliae* are two prominent groups of filamentous fungal insect pathogens. These lineages act as effective biological control agents against arthropod pests and serve as primary sources for the development of fungal insecticides and acaricides (144, 145, 146). Infection by these insect pathogens occurs through the conventional route of cuticular penetration, where hyphae from germ tubes of conidia adhere to the insect surface and penetrate inside the insect host via cuticle. The successful infection of the insect host depends on two key factors: the secretion of extracellular enzymes (proteolytic, chitinolytic, and lipolytic) that degrade the cuticle, and the targeted application of mechanical pressure by specialized hyphal structures, such as appressoria. These factors play a crucial role in achieving effective host infection (147, 148, 78, 149). In recent decades, the subtilisin-like Pr1a proteases and Chi2 chitinase of *Metarhizium anisopliae* have been the focus of considerable attention among the enzymes secreted during fungal invasion. This is primarily due to their potential roles in influencing the fungal ability to cause mycosis which eventually leads to the death of the host (146, 148, 150, 91). The engineered *M. anisopliae* strains, which involved the overexpression of the cloned Pr1a gene, demonstrated increased virulence (8). This study provided compelling evidence supporting the notion that Pr1 protease is a crucial virulence factor (78, 151,152). Leger et al. successfully enhanced the potency of *Metarhizium anisopliae* by creating an engineered fungal strain that continuously expressed an endogenous subtilisin-like protease gene (Pr1a). As a result of this genetic modification, the engineered strain demonstrated a 25% faster ability to kill caterpillars (*Manduca sexta*) compared to the wild-type strain (8). Likewise, the overexpression of a chitinase gene (Bbchit1) augmented the virulence of *Beauveria bassiana* against aphids (*Myzus persicae*) (9). The overexpression of the chitinase and protease genes, in these strains, highlighted the promising application of these genes to enhance the efficacy of the fungi. These genetically modified strains showed improved abilities to penetrate and infect their

target hosts more effectively. The increased virulence demonstrated by these genetically modified strains provides valuable insights into potential strategies for developing more potent and environmentally friendly biocontrol agents against arthropod pests. Based on in vitro investigations, it was discovered that the proteases and chitinases secreted by insect pathogenic fungi work together synergistically to break down the cuticle (10). As a result, the researchers hypothesized that simultaneously overexpressing both protease and chitinase could amplify their effects, leading to a more significant enhancement in the virulence of the pathogenic fungi compared to the impact of each activity individually. In order to verify this hypothesis, a fusion gene construct was designed by Fang W et al by fusing Bbchit1 and a Pr1a-like cuticle degrading protease gene (CDEP1) from *B. bassiana* (Genbank accession number: AY040532). The transformants of *B. bassiana* expressing this hybrid enzyme exhibited significantly higher virulence compared to the wild type (WT) or to transformants that overexpressed either CDEP1 or Bbchit1 alone (11). When compared to the wild type (WT) or BbCDEP1 transformants (which overexpress CDEP1), the expression of CDEP1:Bbchit1 or Bbchit1 accelerated the process of cuticular penetration by *B. bassiana*.

In alignment with the principle followed in the research study conducted by Fang et al., we attempted to fuse chimeric protease (cPr1a) and chimeric chitinase (cChi2) to construct a chimeric protease-chitinase fused gene cassette in order to get a single chimeric fusion protein (f-cPr1a- cChi2) exhibiting dual enzyme nature which could be more efficient than either of chimeric protease and chimeric chitinase in degrading the insect cuticle.

4.2 Material and Methods

4.2.1 Bacterial strains and reagents

The *E. coli* Top-10 strain was used for cloning and expression vector maintenance. The f-cPr1a- cChi2 fusion protein was expressed in *E. coli* (BL21 DE3)) competent cells (NewEngland Biolabs, USA). All restriction enzymes, Phusion DNA polymerase, and ligation enzymes were procured from New England Biolabs, USA. All of the primers were synthesized by GCC Biotech (I) Pvt. Ltd., India. Plasmid DNA and gel extraction kits were obtained from Nucleospin, Macherey Nagel, GmBH.

4.2.2 Amplification of chimeric protease cPr1a and chimeric cChi2 with compatible overhangs

cPr1a was amplified using cPr1a clones as a template and gene-specific old forward primer and new reverse primer to introduce an overhang at the 3' end. The sequence of the DNA primers used for the amplification of cPr1a and cChi2, the composition of the PCR mixture, and the cycling conditions for amplification of the two chimeric protease and chimeric chitinase genes are described in **Table 4.2.1** to **Table 4.2.4**. cChi2 was amplified using cChi2 clones as a template and gene-specific new forward primer and old reverse primer in order to add an overhang at its 5' end.

4.2.3 Construction and cloning of f-cPr1a- cChi2 (f-PC)

cPr1a and cChi2 genes were reamplified using new sets of primers in order to introduce compatible ends in both genes. The cPr1a gene was fused with the cChi2 gene using the Gibson assembly method. Amplified products were segregated on 1% agarose gel and purified using a Nucleospin gel extraction kit (Macherey Nagel), GmBH. NcoI and XhoI restriction enzymes were used to digest the pET-28a plasmid (New England Biolabs, U.S.). Digested plasmid

products were separated on 1% agarose gel electrophoresis and purified using a Nucleospin gel extraction kit. The f-cPr1a- cChi2 construct was cloned into NcoI and XhoI digested pET-28a plasmid using the Gibson assembly method, and the reaction was performed at 50ºC for 60 min.

4.2.4 *E. coli* (Top-10) Competent cells Preparation

E. coli competent cell preparation was done following the protocol already described in section **2.2.12** of Chapter 2.

4.2.5 *E. coli* Top-10 Transformation

The construct of cPr1a and cChi2 was made using the Gibson assembly method and immediately transformed into the Top-10 competent cells after incubation. Screening of positive clones was done on LB agar plates supplemented with kanamycin (50 μgmL^{-1}).

4.2.6 Plasmid DNA isolation

Plasmid DNA isolation was done following the protocol previously mentioned in the section **2.2.214** of Chapter 2.

4.2.7 f-cPr1a- cChi2 clone confirmation by PCR method

The PCR method with gene-specific primers was used to confirm positive clones harboring f-cPr1a- cChi2 constructs. Plasmids were isolated from colonies selected from the Petri plates and incubated at 37ºC for 12 h after transformation. Taq DNA polymerasewas used to perform the PCR (New England Biolabs). The PCR-amplified products were analyzed on a 1% agarose gel. The composition of the PCR mixture and cycling conditions forthe amplification of the f-cPr1a- cChi2 gene are given in Tables **4.2.5** and **4.2.6**.

4.2.8 f-cPr1a- cChi2 clone confirmation by restriction digestion method

A restriction digestion reaction was performed with 2 µg of plasmids isolated from PCR-

confirmed positive f-cPr1a- cChi2 clones. The components of the restriction digestion reaction are described in **Table 4.2.7**. The reactions were carried out with NcoI (HF)and XhoI restriction enzymes (NEB) in a cut smart reaction buffer at 37 ºC for 2 h. On 1% agarose gel, digested products were analyzed. The ChemiDoc XRSTM gel documentation system was used for gel imaging (Bio-Rad, USA).

4.2.9 *E. coli* (BL21 DE3) competent cells preparation

E. coli (BL21 DE3) competent cells were prepared using the same protocol as described in section **2.2.6**.

4.2.10 Transformation of pET-28a-f-cPr1a- cChi2 construct into the expression host

The plasmid pET-28a-f-cPr1a- cChi2 was chemically transformed into the bacterial protein expression host *E. coli* (BL21 DE3) The selection was done on LB agar plates supplemented with kanamycin (50 µg m^{-l}) and incubated at 37ºC for 12-14 h.

4.2.11 f-cPr1a- cChi2 Protein expression

A single colony of *E. coli* (BL21 DE3) was used to inoculate 5 mL of LB broth supplemented with kanamycin (50 µg mL^{-l}) and incubated overnight at 37ºC at 180 rpm. The expression medium was inoculated with 1% of overnight grown culture. At 37ºC, the bacterial culture was grown till the OD600 reached 0.6. LB Broth (Tryptone 12 g l-1, yeast extract 5 g l-1, NaCl 10 g l-1) was used for the expression of the f-cPr1a- cChi2 protein. The protein induction was performed with 1.0 mM isopropylthiogalactopyranoside (IPTG), and the cultures were further incubated for 16 h at 16°C in a refrigerated incubator shaker at 180 rpm for expression of the protein. The protein was purified using Ni-NTA affinity chromatography.

4.2.12 f-cPr1a- cChi2 Protein affinity purification

The cell pellet was harvested after incubation by centrifuging the culture at 10,000 rpm for 30 minutes at 4ºC, and the pellet was resuspended in binding buffer A (40 mM Tris-Cl, 400 mM NaCl, 10 mM Imidazole, 10% Glycerol). The cells were sonicated using the Vibra cell sonicator with 4 seconds on and 20 seconds off pulse conditions. The lysate was subjected to centrifugation at 13,000 rpm for 1 h at 4ºC before being loaded into a Nickel-Nitrillo Triacetic Acid (Ni-NTA) affinity chromatography column pre-equilibrated with binding buffer A. Elution buffer containing 400 mM imidazole was used to elute different fractions, which were then analyzed using 15% Sodium Dodecyl Sulfate-Poly Acrylamide Gel electrophoresis (SDS-PAGE). Biorad Gel Doc EZ gel documentation system was used for gel imaging, and Image Lab software 4.0 from Biorad was used for analysis.

4.2.13 Protease enzyme assay

The protease enzyme assay method has previously been described in section **2.2.25** of Chapter 2.

4.3 Results

4.3.1 Cloning of cPr1a &cChi2 genes into the pET-28a vector

To create the fused cPr1a- cChi2 gene, the procedure entails combining two chimeric genes: cPr1a and cChi2. For successful construction, it is crucial that the 3' end of the cPr1a gene and the 5' end of the cChi2 gene have compatible ends. To introduce a cChi2 overhang at the 3’ end of the cPr1a gene, a reamplification of cPr1a was performed using a new set of primers. Similarly, to introduce a cPr1a overhang at the 5’ end of the cChi2 gene, the Chimeric chitinase gene was reamplified using a new pair of primers. Based on the agarose gel electrophoresis analysis, the PCR-amplified cPr1a gene was estimated to have a size of around 1030 bp, while the cChi2 gene was estimated to be approximately 1297 bp in size **(Fig 4.3.1 and Fig 4.3.2).** Following gel electrophoresis, the cPr1a and cChi2 genes were isolated from the gel using the Nucleospin gel extraction kit. Subsequently, the purified bands of cPr1a (1030 bp) and cChi2 (1297 bp) genes were then visualized on another agarose gel to verify their successful extraction **(Fig 4.3.3)**. The Gibson assembly method was employed to fuse and clone the cPr1a and cChi2 genes into a NcoI and XhoI digested pET28a vector.

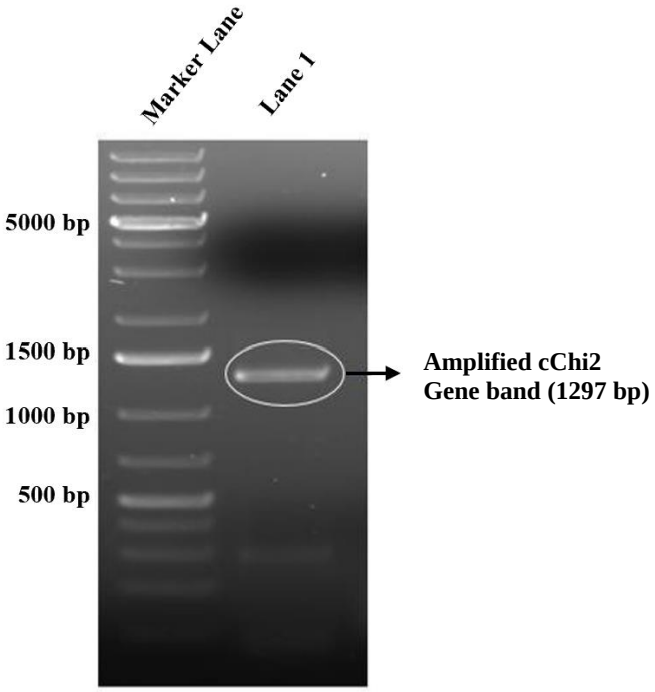

Fig 4.3.1 - Agarose gel electrophoresis analysis showing PCR amplified cChi2 (1297 bp) gene amplified using Chi2 gene as template of *Metarhizium anisopliae* employing polymerase chain reaction with gene-specific primers at annealing temperature 64°C, using Chi2 forward primer and Bmcbd reverse primer with cPr1a overhang at 5'end.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 – PCR amplified cChi2 gene (1297 bp).

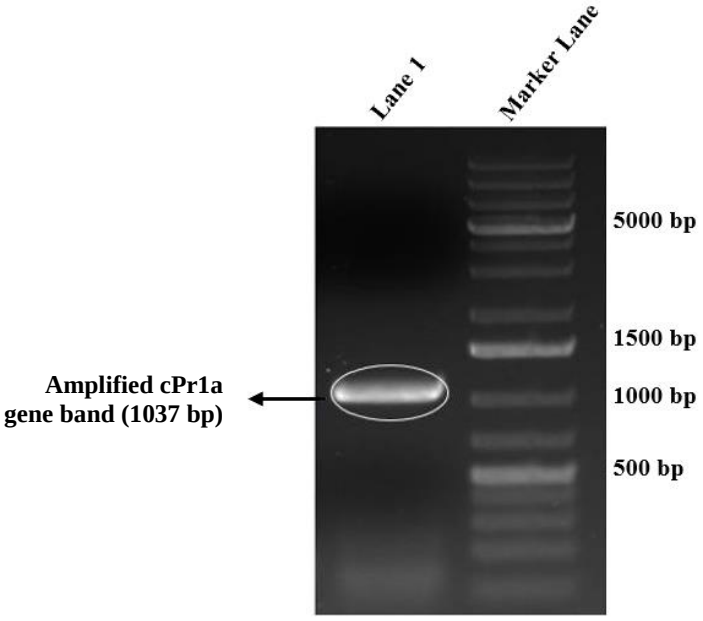

Fig 4.3.2 - Agarose gel electrophoresis analysis showing cPr1a (1037 bp) gene amplified from the cDNA of Metarhizium anisopliae using polymerase chain reaction at annealing temperature 66°C with Pr1a forward primer and Bmcbd reverse primer with cChi2 gene overhang at 3' end.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 – PCR amplified cPr1a gene (1037 bp).

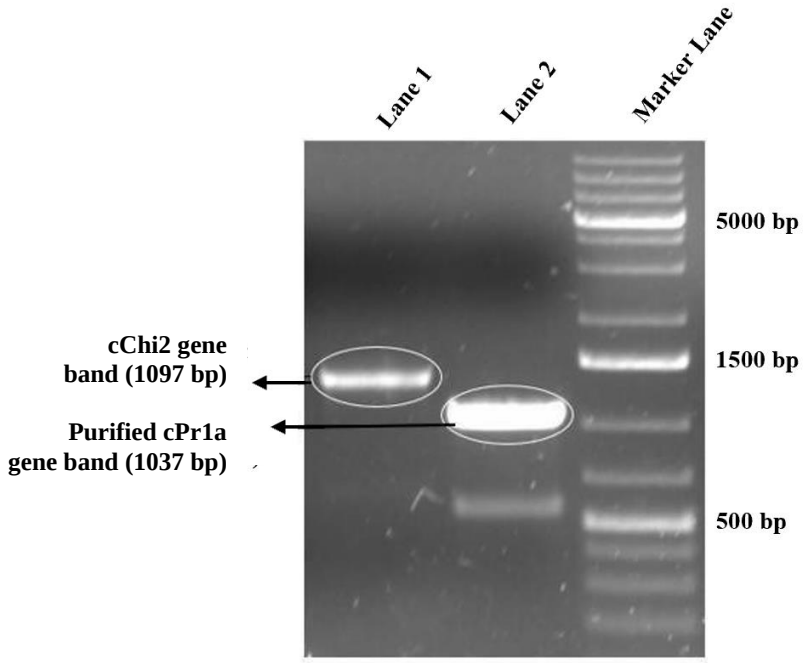

Fig 4.3.3 - Agarose gel electrophoresis analysis of gel-purified DNA fragments of cPr1a and cChi2 chimeric genes.
1% agarose gel was used for electrophoresis to resolve the gel-purified DNA fragments of cPr1a and cChi2 chimeric genes.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 – Gel-purified cChi2 gene band (1297 bp).
Lane 2 – Gel-Purified cPr1a gene band (1037 bp).

4.3.2 Plasmid DNA isolation from transformants and confirmation of f-cPr1a- cChi2 (f-PC) clones

The plasmids isolated from the f-PC transformant colonies were subjected to analysis using agarose gel electrophoresis **(Fig 4.3.4)** & **(Fig. 4.3.5).** To confirm the successful cloning of f-cPr1a- cChi2 (f-PC) into the pET-28a vector, the recombinant plasmids isolated from the f-PC transformant colonies were subjected to restriction digestion using the NcoI and XhoI restriction enzymes. Following restriction digestion of f-PC recombinant plasmids, an agarose gel analysis was performed. The results revealed that the plasmids obtained from the 2nd, 3rd, 4th, 5th, 7th, 8th, 10th, 11th, 14th, 15th, 17th, and 18th f-PC transformant colonies exhibited four DNA bands with sizes equivalent to the pET-28a vector (5369 bp) and f-cPr1a- cChi2 (217 bp, 486 bp, and 1577 bp). The 217 bp and 486 bp bands correspond to the cPr1a bands and the 1577 bp band

corresponds to the cChi2 gene and some portion of the cPr1a gene. This confirms the successful cloning of the f-PC gene into the pET28a vector **(Fig. 4.3.6)** & **(Fig. 4.3.7).** For the second verification of positive clones, the polymerase chain reaction (PCR) method was employed. The plasmids obtained from the f-PC transformants were used as templates for the subsequent steps. Upon conducting agarose gel analysis of the amplified products, it was observed that all the plasmids from the 1st to the 20th, except for the 5th, 6th, 14th, and 19th plasmids, contained the f-PC gene insert **(Fig 4.3.8 & Fig. 4.3.9).** The f-PC colonies carrying the positive clones were cultured in 10 ml LB media. Subsequently, their plasmids were extracted and preserved at -20 °C for further protein expression experiments.

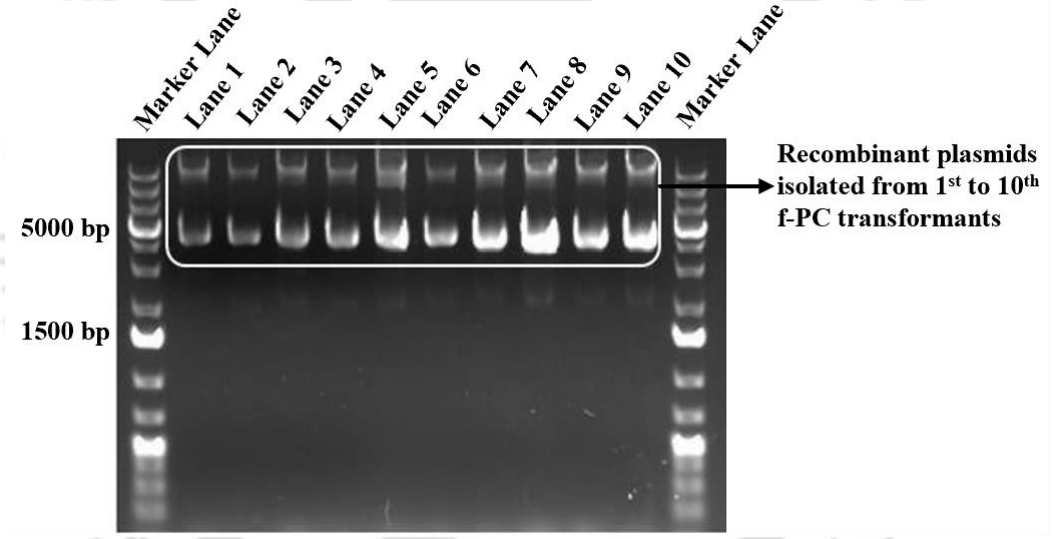

Fig. 4.3.4 - Agarose gel electrophoresis analysis showing recombinant plasmids isolated from f-PC 1st to 10th transformants.
E. coli Top 10 cells were transformed with the pET-28a-f-cPr1a- cChi2 (f-PC) plasmids and selected on LB agar plates containing kanamycin.
Plasmids were isolated from selected f-PC transformants and resolved using 1 % agarose gel electrophoresis.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 10 – Recombinant plasmids isolated from 1st to 10th f-PC transformants.

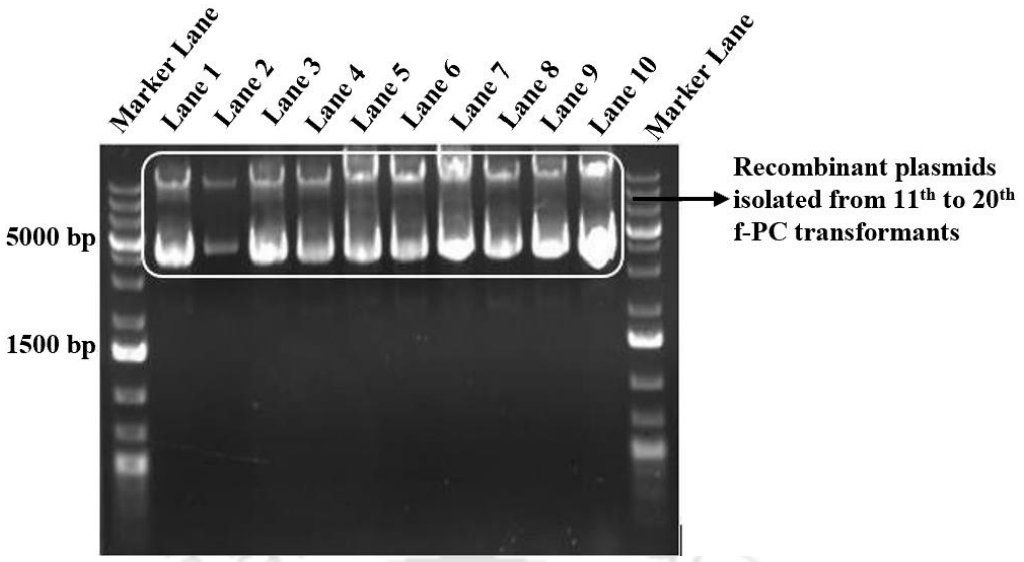

Fig. 4.3.5 - Agarose gel electrophoresis analysis showing recombinant plasmids isolated from f-PC transformants.
E. coli Top 10 cells were transformed with the pET-28a-f-cPr1a- cChi2 (f-PC) plasmids and selected on LB agar plates containing kanamycin.
Plasmids were isolated from selected f-PC 11th to 20th transformants and resolved using 1 % agarose gel electrophoresis.
Lane 1 to 10 – Recombinant plasmids isolated from 11th to 20th f-PC transformants.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 10 – Recombinant plasmids from 11th to 20th f-PC transformants.

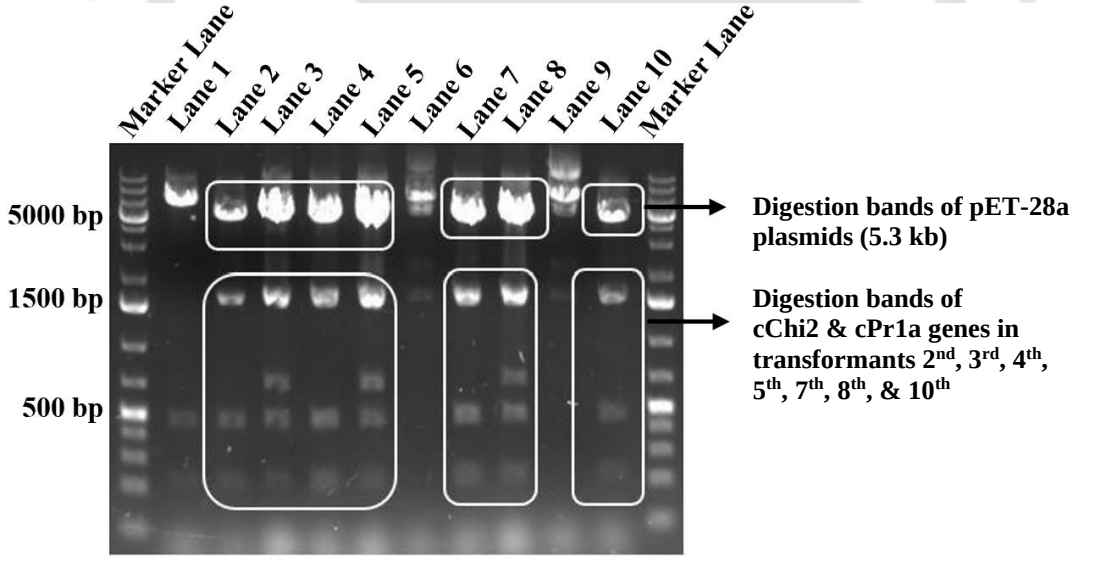

Fig. 4.3.6 - Agarose gel electrophoresis image of f-PC clone confirmation analysis by restriction enzyme digestion.
The plasmids (pET-28a -cPr1a- cChi2) from f-PC transformants 1st to 10th were digested with NcoI and XhoI enzymes.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 2 to 5, lanes 7, 8 & 10 - 2nd, 3rd, 4th, 5th, 7th, 8th & 10th digested recombinant plasmids showing the bands of chimeric chitinase cChi2 and chimeric protease cPr1a gene inserts (1577 bp, 467 bp & 217 bp).
The cPr1a gene has two NcoI restriction sites at 213 bp and 680 bp, giving three bands 467 bp, 217 bp, and 1577 bp instead of a single fused gene (2280) bp band.

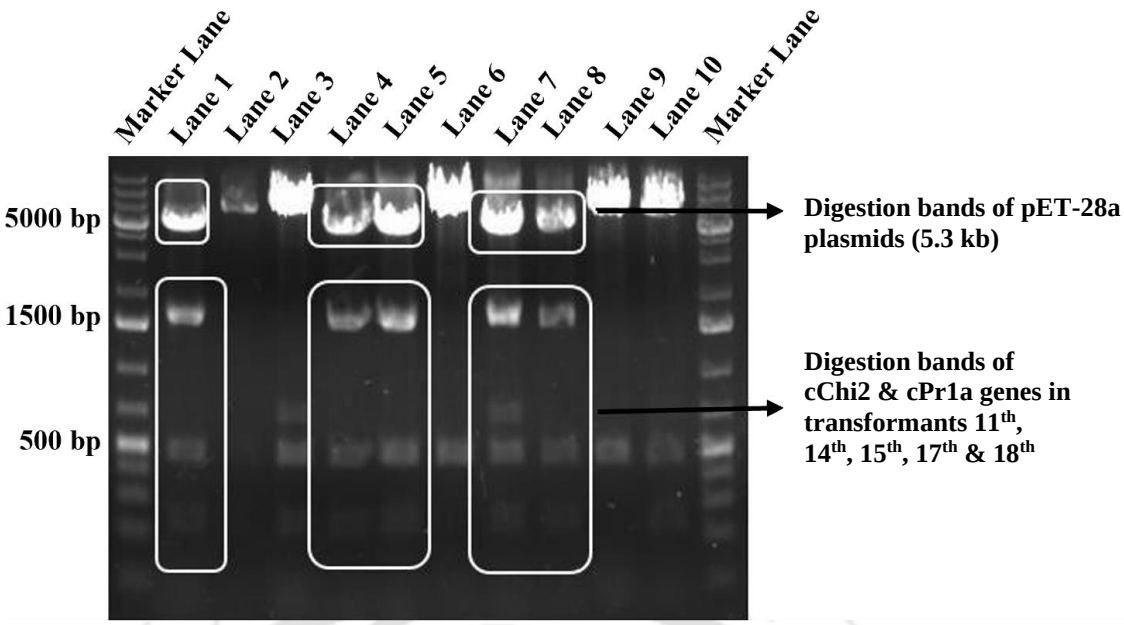

Fig 4.3.7 - Agarose gel electrophoresis image of f-PC clone confirmation analysis by restriction enzyme digestion.

The plasmids (pET-28a - cPr1a- cChi2) from f-PC transformants 11th to 20th were digested with NcoI and XhoI enzymes.

Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).

Lane 1, 4, 5, lanes 7 and 8 – 11th,14th,15th, 17th & 18th digested recombinant plasmids showing the bands of chimeric chitinase cChi2 and chimeric protease cPr1a gene inserts (1577 bp, 467 bp & 217 bp).

The cPr1a gene has two NcoI restriction sites at 213 bp and 680 bp, giving three bands 467 bp, 217 bp, and 1577 bp instead of a single fused gene (2280) bp band.

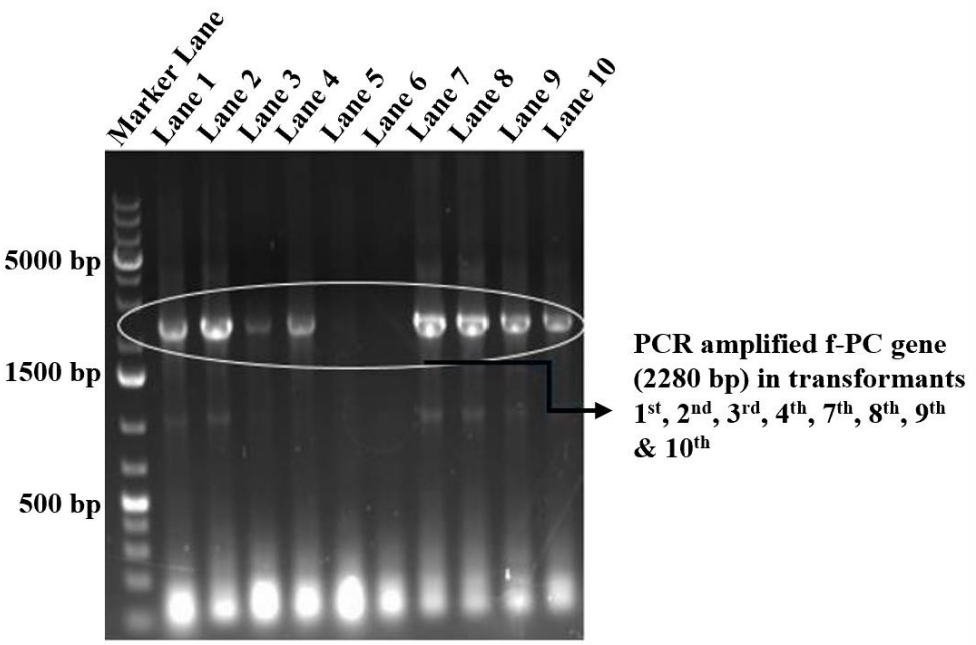

Fig. 4.3.8 - Agarose gel analysis showing PCR amplified f-PC gene fragments from f-PC clones.

PCR confirmation for positive clones was done using Taq DNA polymerase and f-cPr1a- cChi2 (f-PC) gene-specific primers, utilizing plasmid DNA isolated from f-PC clone's clones 1st to 10th as templates.

Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).

Lane 1 to 4 & 7 to 10 – PCR amplified f-PC gene (2280 bp) band in 1st, 2nd, 3rd, 4th, 7th, 8th, 9th and 10th clones.

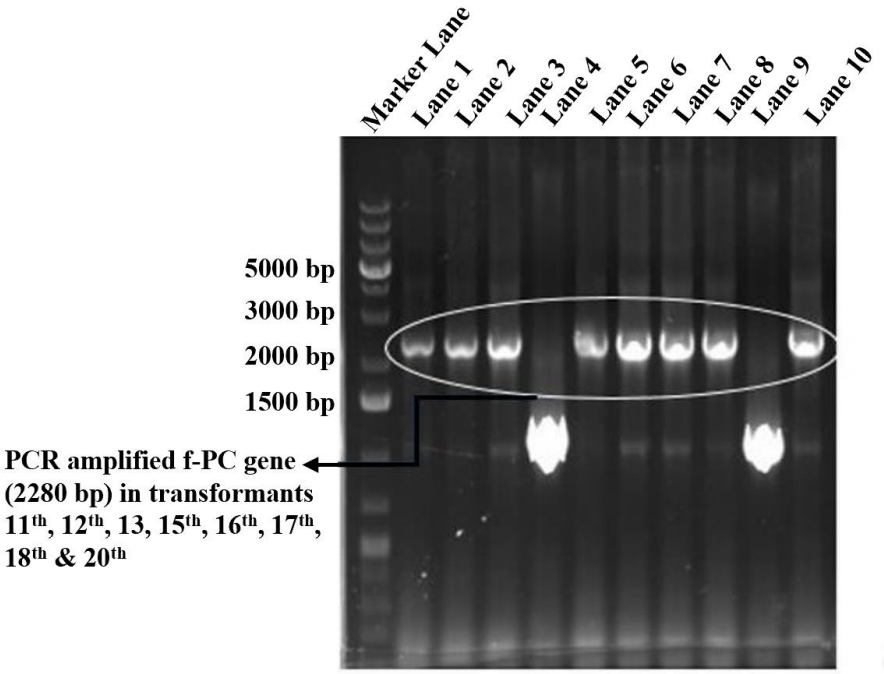

Fig. 4.3.9 Agarose gel analysis showing PCR amplified f-PC gene fragments from f-PC clones 11th to 20th.
PCR confirmation for positive clones was done using Taq DNA polymerase and f-cPr1a-cChi2 (f-PC) gene-specific primers, utilizing plasmid DNA isolated from f-PC clone's clones 11th to 20th as templates.
Marker lane – 1 Kb plus DNA Ladder (Thermo Scientific).
Lane 1 to 3, 5 to 8 & 10 – PCR amplified f-PC gene insert (2280 bp) band in 11th, 12th, 13th,15th,16th,17th, 18th and 20th clones.

4.3.3 Expression, purification of the f-cPr1a- cChi2 fusion protein

Post-transformation of the recombinant pET-28a-f-cPr1a- cChi2 constructs, which contain the fused chimeric genes encoding the chimeric protease-chitinase fusion protein, into *E. coli* BL21(DE3) competent cells. The f-cPr1a- cChi2 transformants were grown on LB agar plates containing kanamycin. The expression was first optimized on a tube-level 5 ml culture. The primary culture was grown overnight using the single isolated colony as a starting culture. The 5ml secondary media was inoculated with 1 % of overnight grown primary culture. At the mid-exponential stage of growth, the cells were induced to overexpress the f-cPr1a- cChi2-$(His)_6$ fusion protein. The f-PC protein expression was carried out under the optimized conditions of 1.0 mM IPTG concentration, 16 h of induction time, and 16°C temperature. The f-cPr1a- cChi2

fusion protein was successfully overexpressed and subsequently purified using immobilized metal ion affinity chromatography. For the final purification step, Ni-NTA affinity chromatography was employed and the binding, washing, and elution steps were followed to purify the protein while it was on the ice. The different fractions of the protein eluted from the column were analyzed on 12% (SDS-PAGE) Sodium Dodecyl Sulfate - Poly Acrylamide Gel electrophoresis (Fig 4.3.10). Gel imaging was done using a Bio-Rad Gel Doc EZ gel documentation system and analyzed with Image Lab software 4.0 from Bio-Rad. The f-cPr1a-cChi2-$(His)_6$ protein was expressed in the supernatant as soluble fractions. The purified fusion protein was observed to have a molecular weight of approximately 39 kDa, which is half of the expected 78 kDa. This result aligns with the sequencing data of thef-cPr1a- cChi2 (f-PC) clone, which revealed the presence of a stop codon in the cChi2 gene few bases away from the end of cPr1a. Consequently, the protein was truncated, leading to the observed lower molecular weight. Despite the truncation leading to the absence of the His tag, a very faint protein band was still observed, possibly attributed to the presence of histidine amino acids within the protein sequence. The quantification of the truncated purified f- f-cPr1a- cChi2 protein was performed using the Bradford assay. The recombinant fusion protein yield, as measured by the Bradford assay, ranged 8 mg per liter of *E. coli* culture.

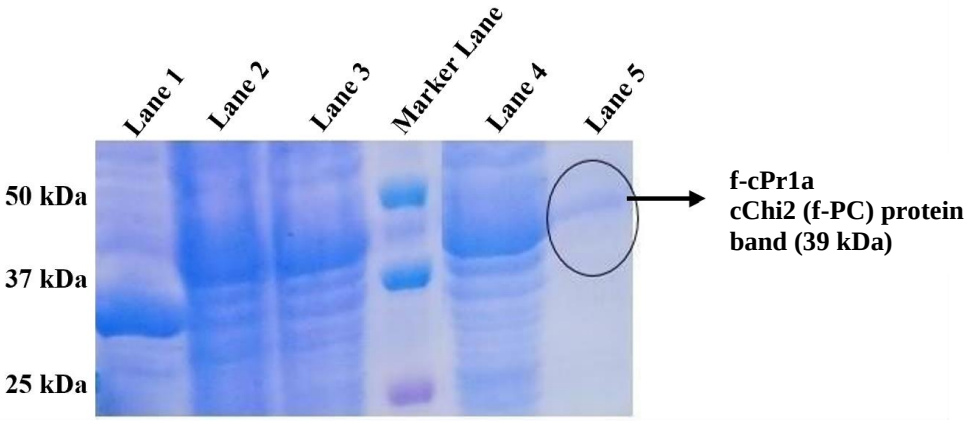

Fig. 4.3.10 - Purification profile of recombinant f- f-cPr1a- cChi2-$(His)_6$ protein on 12% (w/v) polyacrylamide gel. SDS-PAGE was used to resolve the proteins and Coomassie Brilliant Blue was used to stain them. Marker Lane: Precision plus protein ladder, (Biorad 1610374), lane 1: cell pellet after sonication, lane 2: cell free medium supernatant after sonication, lane 3: flow- through, lane 4: last column wash, lane 5: purified truncated f-cPr1a- cChi2 protein.

4.3.4 Enzyme assay

Protease assay - The aim of the protease enzyme assays was to assess how the fusion of the chimeric chitinase (cChi2) to the chimeric protease (cPr1a) influenced its cuticle degrading potential. The protease enzyme activity of the truncated f-cPr1a- cChi2 protein exhibited an approximately 86.48% increase, reaching 873.09 U/ml at 1 minute, in comparison to the enzyme activity of 117.98 U/ml observed at 4 minutes **(Fig. 4.3.11)**. The enzyme assay results demonstrated a significantly higher protease enzyme activity for the truncated f- f-cPr1a- cChi2 fusion protein when compared to both the wild-type Pr1a protease and the chimeric cPr1a protease. The protease activity of the f f-cPr1a- cChi2 fusion protein was significantly increased by 62.7% compared to the wild-type protease Pr1a (44.94 U/ml) and by 51.06% compared to the chimeric protease cPr1a (59.06 U/ml) **(Fig. 4.3.12).** The data obtained from the results of the student t-test for both enzyme assays is statistically significant ($P < 0.001$) and represents the mean value of three independent experiments.

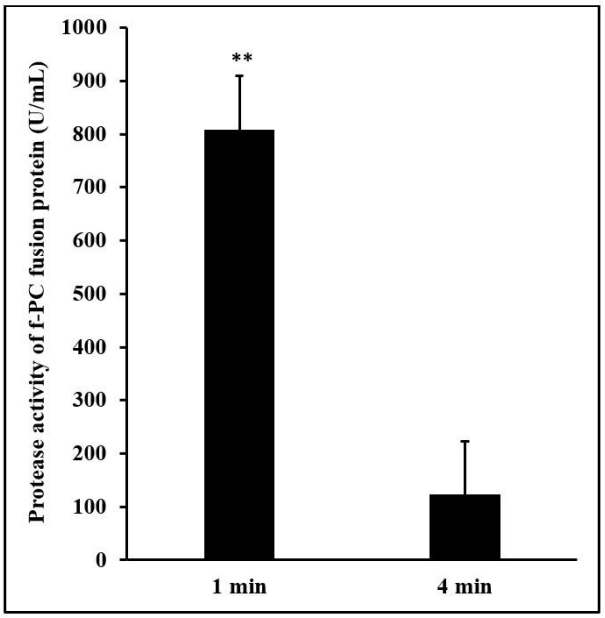

Fig. 4.3.11 - Comparative enzymatic analysis protease of f-PC truncated fusion protein using *Philosamia ricini* cuticle as substrate.
1 min – Protease enzyme activity of the fused truncated protein in the 1-minute assay (873.09 U/ mL).
4 mins – Protease enzyme activity of the fused truncated protein in the 4-minute assay (117.98 U/mL).
The protease enzyme activity of the fused truncated protein in the 1-minute assay exhibited significantly higher enzyme activity than in the 4-minute assay.
** - The results of the student t-test are statistically significant ($P < 0.001$) and represent the mean values from three independent experiments.

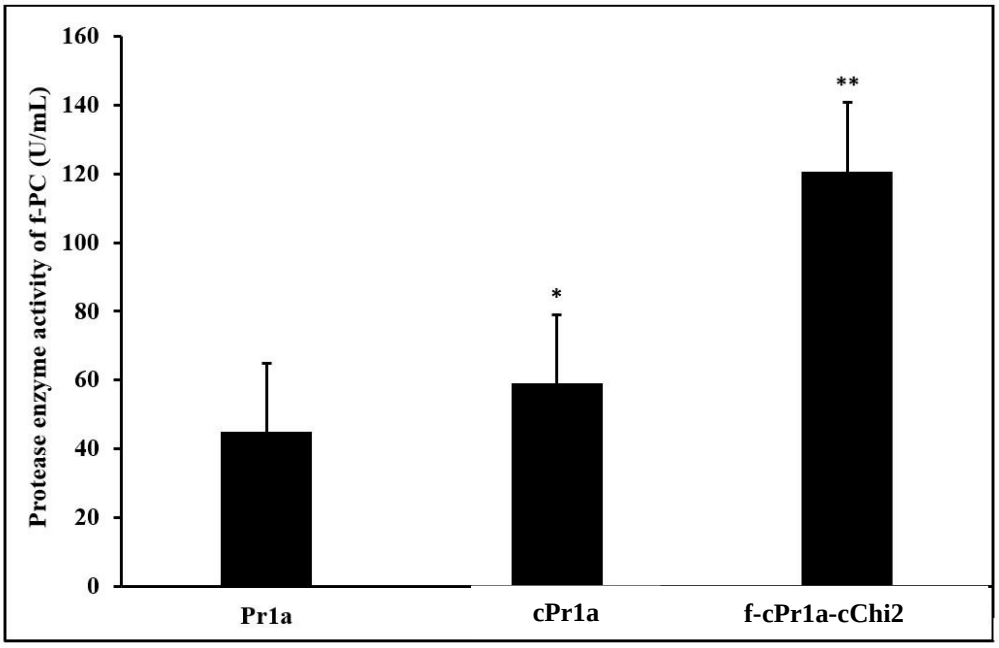

Fig. 4.3.12 - Comparative enzymatic analysis of protease activity of wild-type (Pr1a), chimeric protease (cPr1a), and truncated f- f-cPr1a- cChi2 (f-PC) fusion protein using *Philosamia ricini* cuticle as substrate in the 4-minute assay.
Pr1a – Protease enzyme activity of wild-type Pr1a enzyme (44.94 U/mL).
cPr1a - Protease enzyme activity of chimeric cPr1a enzyme 59.06 U/mL).
f-cPr1a- cChi2– Protease enzyme activity of truncated protease-chitinase fusion protein (120.69 U/mL).
* - The results of the student t-test are statistically significant ($P < 0.02$) and represent the mean values from three independent experiments.
**- The results of the student t-test are statistically significant ($P < 0.001$) and represent the mean values from three independent experiments.

4.4 Discussion

Fusion proteins were constructed as a class of novel biological molecules with versatile properties as a consequence of recombinant DNA technology (RDT). The fusion protein can be developed using a genetic engineering approach by fusing two or even more different protein domains together, carrying the distinct characteristics derived from each of their constituent moieties. In order to achieve effective degradation and biotransformation of lignocellulose, which requires synergistic actions of three different types of cellulase enzymes for its complete degradation, a new chimeric cellulase enzyme with β-glucosidase, exoglucanase, and endoglucanase activities was developed for the degradation of cellulose. The chimeric cellulase, which was expressed in *Saccharomyces cerevisiae*, has shown an augmentation in the specific activities of all three kinds of enzymes (exoglucanase, glucosidase, and endoglucanase) with respect to the single catalytic domain and the highest increase was seen in the activity of glucosidase (46.27%) (153). In a recent study, a CHACry fusion protein was fabricated by merging the catalytic domain of the chitinase gene, pachi of *Pseudomonas aeruginosa*, with Cry21Aa toxin of Bacillus thuringiensis, which showed enhanced solubility and increased nematicidal activity against *C.elegans* (154). Chitin and protein are important components of the body structure of insect cuticles, and the biodegradation of chitin and protein necessitates the activity of two enzymes viz chitinase and protease. Merging chitinase and protease to create a single enzyme system with dual enzyme characteristics can be of a great deal and will be capable of degrading chitin and protein-rich biomass proficiently. A recombinant fusion protein produced by combining the chitinase and alkaline protease in a single protein showed the capability to degrade both chitin and protein. This fusion protein, with the activities of two entirely distinct enzymes, can efficaciously digest intricate chitin and protein polymers. Optimized enzyme activities of the fusion protein with respect to individual enzymes enable more rapid biodegradation than single-enzyme biodegradation (155).

Metarhizium anisopliae secrete proteases and chitinases that work synergistically to break down insects' cuticles. By acting in tandem, proteases and chitinases efficiently target and hydrolyze the major components of the cuticle, such as proteins and chitin, facilitating the penetration and colonization of the host insect by the fungi. This synergistic action enhances the overall virulence and pathogenicity of the entomopathogenic fungi, making them effective biocontrol agents against various insect pests.

Previous research study has demonstrated that the overexpression of either the Pr1a protease or the Bbchit1 chitinase led to accelerated degradation of the insect cuticle with simultaneous enhancement in fungal virulence (8, 109, 9).

In a study by Fang W *et al*., they discovered that a combination of the *B. bassiana* Pr1a homolog (CDEP1) and Bbchit1 destroyed insect cuticles in vitro with greater efficacy than either CDEP1 or Bbchit1 separately. The fusion protein (CDEP1:Bbchit1) secreted by *B. bassiana* transformants breached the cuticle considerably quicker than the wild-type transformants overexpressing either Bbchit1 or CDEP1 (11).

The fusion protein was created by merging the chimeric protease and chimeric chitinase using the Gibson assembly method. The construction of the f- f-cPr1a- cChi2 protein by fusion of the chimeric protease & chimeric chitinase was attempted, but the truncated fusion protein of 39 kDa, instead of the full 78 kDa fusion protein was confirmed by the SDS-PAGE. but this truncated fusion protein is able to digest the protein present in the cuticle in a proficient manner.

The truncated fusion protein (f-t-cPr1a-cChi2) showed about 62.7% and 51.06% increase in protease enzyme activity with reference to the wild-type (Pr1a) and chimeric protease (cPr1a).

This enhancement in protease activity suggests that the fusion of a portion of chimeric chitinase (cChi2) with the chimeric protease (cPr1a) has a positive effect on the overall enzymatic performance of the protein. The fusion of some amino acids (some of the amino acids got mutated naturally during the process of construction of the fusion protein) from the chimeric chitinase to the chimeric protease may have resulted in improved catalytic efficiency or altered

substrate specificity, contributing to the observed increase in enzyme activity. Further investigations and detailed characterization would be valuable to understand the underlying molecular mechanisms and potential applications of this enhanced fusion protein.

4.5 Conclusion

A fusion protein with protease and chitinase dual enzyme activities has been constructed, and this truncated f-cPr1a-cChi2 fusion protein was proven to be more effective than the wild-type and chimeric protease enzyme. The protease–chitinase fusion construct could drastically improve the speed of killing the insect pests when transformed into the *Metarhizium anisopliae* fungi and hence can be a potent component of fungal biopesticide.

Table 4.2.1 List of primers used for the amplification of cPr1a and cChi2.

Primer	Sequence
cPr1a_FP_NcoI_pET28a_Overhang	5'- CTTTAAGAAGGAGATATACCATG GGCATCACTGAGCAGAGCGG – 3'
cPr1a_RP_ cChi2_Overhang	5'-CTGATATCTTGTCAGTCAAGGGCAC AGGCCAATCGCAAACGTTAAGTTTCAC-3'
cChi2_FP_ cPr1a _Overhang	5'- GTGAAACTTAACGTTTGCGATTGGCCTGTGCCCTTGACTGACAAGATATCAG- 3'
cChi2_RP_His tag_XhoI_pET28a_Overhang	5'-GGTGGTGGTGGTGCTCGAGTTAATGATGATGATGATGATG AGGCCAATCGCAAACGTTAAG- 3'

Table 4.2.2 Components of PCR mixture for amplification of gene encoding cPr1a and cChi2

PCR components	Volume (µl)	Final concentration
Nuclease-free water	30.0	--
5x reaction buffer	10.0	1x
dNTP mix (10 mM)	1.0	0.2 mM
Forward primer (10 µM)	2.5	0.5 µM
Reverse primer (10 µM)	2.5	0.5 µM
DMSO	1.5	3%
Template (Genomic DNA) (50 µg/mL)	2.0	100.0 ng
Phusion DNA polymerase (2 U/µl)	0.5	0.02 U/µl
Total	50.0	--

Table 4.2.3 PCR cycles conditions for amplification of cPr1a gene

Steps		Time
I. Denaturation at 98°C		2 min
II. 30 cycles of	i) Denaturation at 98°C	10 s
	ii) Annealing at 64°C	40 s
	iii) Extension at 72°C	15 s
III. Final extension at 72°C		10 min

Table 4.2.4 PCR cycles conditions for amplification of cChi2

Steps		Time
		2 min
I. Denaturation at 98°C		
II. 30 cycles of	i) Denaturation at 98°C	10 s
	ii) Annealing at 66°C	30 s
	iii) Extension at 72°C	45 s
III. Final extension at 72°C		10 min

Table 4.2.5 Components of PCR mixture for amplification of f-cPr1a-cChi2 fragment.

PCR components	Volume (µl)	Final concentration
Nuclease-free water	36.8	--
10x reaction buffer	5.0	1x
dNTP mix (10 mM)	1.0	0.2 mM
Forward primer (10 µM)	1.0	0.2 µM
Reverse primer (10 µM)	1.0	0.2 µM
25 mM $MgCl_2$	3.0	1.5 mM
Plasmid DNA (50.0 µg/mL)	2.0	100.0 ng
Neb Taq DNA polymerase (5 U/µl)	0.2	0.02 U/µl
Total	50.0	--

Table 4.2.6 PCR cycles conditions (Taq DNA polymerase) for amplification of f-cPr1a- cChi2

Steps		Time
		2 min
I. Denaturation at 95°C		
II. 30 cycles of	i) Denaturation at 95°C	10 s
	ii) Annealing at 70°C	30 s
	iii) Extension at 68°C	15 s
III. Final extension at 95°C		10 min

Table 4.2.7 Composition of restriction enzymes digestion reaction for f-cPr1a-cChi2 clone's confirmation

Components	Volume (µl)
10x cut smart buffer	2.0
Nuclease free water	7.0
Plasmid DNA (40 ng/µl)	10.0
*Nco*I (20 U/µl)	0.5
*Xho*I (20 U/µl)	0.5
Total	20.0

MAJOR FINDINGS

5. MAJOR FINDINGS

- Chimeric protease, cPr1a, showed significant enhancement (about 21.59 %) in binding efficiency compared to wild-type Pr1a protease when tested against *Philosamia ricini's* cuticle.

- About a 23.98 % increase in enzyme activity or cuticle digesting capacity of chimeric cPr1a protease has been evaluated.

- Chimeric chitinase, cChi2, has revealed a marked rise (19.12 %) in its binding efficiency when analogized with wild-type Chi2 chitinase.

- The chimeric cChi2 chitinase showed a 32.48 % increase in enzyme activity (in 4 min enzyme assays) and 31.46 % (in 1 min enzyme assays) compared to wild-type Chi2.

- The protease activity exhibited by fusion protein f-cPr1a- cChi2 in (4min enzyme assays) showed about 62.7 % and 51.06 % increase when compared to wild-type Pr1a protease and chimeric cPr1a protease, respectively.
- f-cPr1a- cChi2 protein has shown about an 87.36 % increase in its protease enzyme activity (879.93 U/ mL at 1 min) when compared with enzyme activity (111.2 U/mL at 4 mins).

FUTURE PROSPECTS

6. FUTURE PROSPECTS

- Overexpression of Pr1a, cPr1a, Chi2, cChi2, and f-cPr1a- cChi2 in *Metarhizium anisopliae*.

- Evaluation of virulence of the fungus expressing recombinant fusion proteins through insect bioassays.

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APPENDIX

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Reference       AGCACCCCGGTGTCGATTTCATTGAGAAGGACGCTGTGATGCGTATCAGCGGCATCACTG       60
Pr1aF           ----------------------------------------------GGCATCACTG       10
                                                               **********

Reference       AGCAGAGCGGTGCTCCCTGGGGTCTTGGGCGCATCTCTCACCGCAGTAAGGGAAGCACCA      120
Pr1aF           AGCAGAGCGGTGCTCCCTGGGGTCTTGGGCGCATCTCTCACCGCAGTAAGGGAAGCACCA       70
                ************************************************************

Reference       CCTATCGCTACGATGATAGTGCTGGTCAGGGTACTTGCGTATATATCATTGACACTGGTA      180
Pr1aF           CCTATCGCTACGATGATAGTGCTGGTCAGGGTACTTGCGTATATATCATTGACACTGGTA      130
                ************************************************************

Reference       TTGAGGCCTCCCACCCCGAGTTTGAGGGTCGCGCCACTTTTCTTAAGAGCTTCATCAGCG      240
Pr1aF           TTGAGGCCTCCCACCCCGAGTTTGAGGGTCGCGCCACTTTTCTTAAGAGCTTCATCAGCG      190
                ************************************************************

Reference       GTCAAAACACTGATGGCCACGGCCATGGGACTCACTGCGCTGGTACCATTGGTAGCAAGA      300
Pr1aF           GTCAAAACACTGATGGCCACGGCCATGGGACTCACTGCGCTGGTACCATTGGTAGCAAGA      250
                ************************************************************

Reference       CCTACGGTGTTGCCAAAAAGGCTAAGCTCTATGGTGTCAAGGTTCTTGACAACCAGGGCA      360
Pr1aF           CCTACGGTGTTGCCAAAAAGGCTAAGCTCTATGGTGTCAAGGTTCTTGACAACCAGGGCA      310
                ************************************************************

Reference       GTGGTTCCTACTCCGGTATCATCAGTGGCATGGACTACGTTGCACAGGACTCCAAGACCC      420
Pr1aF           GTGGTTCCTACTCCGGTATCATCAGTGGCATGGACTACGTTGCACAGGACTCCAAGACCC      370
                ************************************************************

Reference       GCGGCTGCCCCAACGGCGCCATTGCTTCCATGAGCCTGGGAGGTGGCTACTCGGCGTCCG      480
Pr1aF           GCGGCTGCCCCAACGGCGCCATTGCTTCCATGAGCCTGGGAGGTGGCTACTCGGCGTCCG      430
                ************************************************************

Reference       TCAACCAAGGTGCTGCTGCTTTGGTCAATTCTGGTGTCTTCCTTGCCGTCGCCGCTGGCA      540
Pr1aF           TCAACCAAGGTGCTGCTGCTTTGGTCAATTCTGGTGTCTTCCTTGCCGTCGCCGCTGGCA      490
                ************************************************************

Reference       ACGATAACCGGGATGCCCAGAACACCTCTCCCGCTTCCGAGCCTTCTGCCTGCACTGTTG      600
Pr1aF           ACGATAACCGGGATGCCCAGAACACCTCTCCCGCTTCCGAGCCTTCTGCCTGCACTGTTG      550
                ************************************************************

Reference       GTGCCTCTGCGGAAAATGACAGCCGATCTTCCTTCTCCAACTACGGCAGAGTTGTCGATA      660
Pr1aF           GTGCCTCTGCGGAAAATGACAGCCGATCTTCCTTCTCCAACTACGGCAGAGTTGTCGATA      610
                ************************************************************

Reference       TTTTCGCTCCTGGTAGCAATGTTCTTTCCACCTGGATTGTTGGCCGCACAAACTCCATCT      720
Pr1aF           TTTTCGCTCCTGGTAGCAATGTTCTTTCCACCTGGATTGGTGGCCGCACAAACACCATCT      670
                ************************************ ************* ******

Reference       CTGGTACCTCCATGGCTACTCCCCATATTGCCGGTCTGGCTGCCTACCTCAGTGCGCTCC      780
Pr1aF           CTGGTACCTCCATGGCTACTCCCCATATTGCCGGTCTGGCTGCCTACCTCAGTGCGCTCC      730
                ************************************************************

Reference       AAGGCAAGACTACCCCTGCCGCTCTTTGCAAGAAGATCCAGGACACTGCTACCAAGAACG      840
Pr1aF           AAGGCAAGACTACCCCTGCCGCTCTTTGCAAGAAGATCCAGGA-----------------      773
                ****************************************

Reference       TGCTCACCGGTGTTCCCTCTGGCACTGTCAACTACCTTGCCTACAACGGTGCC             893
Pr1aF           -------------------------------------------------             773
```

DNA sequencing of Cloned Pr1a gene. Alignment of sequencing data with the reference sequence (pr1A, M73795.1) available on the NCBI database. pr1AF represents the sequence obtained from N-terminal sequencing of pET-28a with T7 forward primer.

```
Reference       ------------------------------------GGCATCACTGAGCAGAGCGGT      21
Pr1aR           AAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCATCACTGAGCAGAGCGGT      60
                                                    ********************

Reference       GCTCCCTGGGGTCTTGGGCGCATCTCTCACCGCAGTAAGGGAAGCACCACCTATCGCTAC      81
Pr1aR           GCTCCCTGGGGTCTTGGGCGCATCTCTCACCGCAGTAAGGGAAGCACCACCTATCGCTAC      120
                ************************************************************

Reference       GATGATAGTGCTGGTCAGGGTACTTGCGTATATATCATTGACACTGGTATTGAGGCCTCC      141
Pr1aR           GATGATAGTGCTGGTCAGGGTACTTGCGTATATATCATTGACACTGGTATTGAGGCCTCC      180
                ************************************************************

Reference       CACCCCGAGTTTGAGGGTCGCGCCACTTTTCTTAAGAGCTTCATCAGCGGTCAAAACACT      201
Pr1aR           CACCCCGAGTTTGAGGGTCGCGCCACTTTTCTTAAGAGCTTCATCAGCGGTCAAAACACT      240
                ************************************************************

Reference       GATGGCCACGGCCATGGGACTCACTGCGCTGGTACCATTGGTAGCAAGACCTACGGTGTT      261
Pr1aR           GATGGCCACGGCCATGGGACTCACTGCGCTGGTACCATTGGTAGCAAGACCTACGGTGTT      300
                ************************************************************

Reference       GCCAAAAAGGCTAAGCTCTATGGTGTCAAGGTTCTTGACAACCAGGGCAGTGGTTCCTAC      321
Pr1aR           GCCAAAAAGGCTAAGCTCTATGGTGTCAAGGTTCTTGACAACCAGGGCAGTGGTTCCTAC      360
                ************************************************************

Reference       TCCGGTATCATCAGTGGCATGGACTACGTTGCACAGGACTCCAAGACCCGCGGCTGCCCC      381
Pr1aR           TCCGGTATCATCAGTGGCATGGACTACGTTGCACAGGACTCCAAGACCCGCGGCTGCCCC      420
                ************************************************************

Reference       AACGGCGCCATTGCTTCCATGAGCCTGGGAGGTGGCTACTCGGCGTCCGTCAACCAAGGT      441
Pr1aR           AACGGCGCCATTGCTTCCATGAGCCTGGGAGGTGGCTACTCGGCGTCCGTCAACCAAGGT      480
                ************************************************************

Reference       GCTGCTGCTTTGGTCAATTCTGGTGTCTTCCTTGCCGTCGCCGCTGGCAACGATAACCGG      501
Pr1aR           GCTGCTGCTTTGGTCAATTCTGGTGTCTTCCTTGCCGTCGCCGCTGGCAACGATAACCGG      540
                ************************************************************

Reference       GATGCCCAGAACACCTCTCCCGCTTCCGAGCCTTCTGCCTGCACTGTTGGTGCCTCTGCG      561
Pr1aR           GATGCCCAGAACACCTCTCCCGCTTCCGAGCCTTCTGCCTGCACTGTTGGTGCCTCTGCG      600
                ************************************************************

Reference       GAAAATGACAGCCGATCTTCCTTCTCCAACTACGGCAGAGTTGTCGATATTTTCGCTCCT      621
Pr1aR           GAAAATGACAGCCGATCTTCCTTCTCCAACTACGGCAGAGTTGTCGATATTTTCGCTCCT      660
                ************************************************************

Reference       GGTAGCAATGTTCTTTCCACCTGGATTGTTGGCCGCACAAACTCCATCTCTGGTACCTCC      681
Pr1aR           GGTAGCAATGTTCTTTCCACCTGGATTGGTGGCCGCACAAACACCATCTCTGGTACCTCC      720
                *************************** ************* *****************

Reference       ATGGCTACTCCCCATATTGCCGGTCTGGCTGCCTACCTCAGTGCGCTCCAAGGCAAGACT      741
Pr1aR           ATGGCTACTCCCCATATTGCCGGTCTGGCTGCCTACCTCAGTGCGCTCCAAGGCAAGACT      780
                ************************************************************

Reference       ACCCCTGCCGCTCTTTGCAAGAAGATCCAGGACACTGCTACCAAGAACGTGCTCACCGGT      801
Pr1aR           ACCCCTGCCGCTCTTTGCAAGAAGATCCAGGACACTGCTACCAAGAACGTGCTCACCGGT      840
                ************************************************************

Reference       GTTCCCTCTGGCACTGTCAACTACCTTGCCTACAACGGTGCC------------------      843
Pr1aR           GTTCCCTCTGGCACTGTCAACTACCTTGCCTACAACGGTGCCCATCATCATCATCATCAT      900
                ****************************************

Reference       ----------------------------------------                         843
Pr1aR           TAACTCGAGCACCACCACCACCACCACTGAGATCCGGCTG                          940
```

DNA sequencing of Pr1a. Alignment of sequencing data with the reference sequence (Pr1a, M73795.1) available on the NCBI database. Pr1aR represents the sequence obtained from C-terminal sequencing of pET-28a with T7 reverse primer.

Metarhizium anisopliae cuticle-degrading (Pr1a) mRNA, complete cds, GenBank: M73795.1

GGCATCACTGAGCAGAGCGGTGCTCCCTGGGGTCTTGGGCGCATCTCTCACCGCAGTAAGGG
AAGCACCACCTATCGCTACGATGATAGTGCTGGTCAGGGTACTTGCGTATATATCATTGACA
CTGGTATTGAGGCCTCCCACCCCGAGTTTGAGGGTCGCGCCACTTTTCTTAAGAGCTTCATCA
GCGGTCAAAACACTGATGGCCACGGCCATGGGACTCACTGCGCTGGTACCATTGGTAGCAA
GACCTACGGTGTTGCCAAAAAGGCTAAGCTCTATGGTGTCAAGGTTCTTGACAACCAGGGCA
GTGGTTCCTACTCCGGTATCATCAGTGGCATGGACTACGTTGCACAGGACTCCAAGACCCGC
GGCTGCCCCAACGGCGCCATTGCTTCCATGAGCCTGGGAGGTGGCTACTCGGCGTCCGTCAA
CCAAGGTGCTGCTGCTTTGGTCAATTCTGGTGTCTTCCTTGCCGTCGCCGCTGGCAACGATAA
CCGGGATGCCCAGAACACCTCTCCCGCTTCCGAGCCTTCTGCCTGCACTGTTGGTGCCTCTGC
GGAAAATGACAGCCGATCTTCCTTCTCCAACTACGGCAGAGTTGTCGATATTTTCGCTCCTG
GTAGCAATGTTCTTTCCACCTGGATTGTTGGCCGCACAAACTCCATCTCTGGTACCTCCATGG
CTACTCCCCATATTGCCGGTCTGGCTGCCTACCTCAGTGCGCTCCAAGGCAAGACTACCCCT
GCCGCTCTTTGCAAGAAGATCCAGGACACTGCTACCAAGAACGTGCTCACCGGTGTTCCCTC
TGGCACTGTCAACTACCTTGCCTACAACGGTGCCTAA

The deduced sequence of Pr1a (*Metarhizium anisopliae* 892)

GGCATCACTGAGCAGAGCGGTGCTCCCTGGGGTCTTGGGCGCATCTCTCACCGCAGTAAGGG
AAGCACCACCTATCGCTACGATGATAGTGCTGGTCAGGGTACTTGCGTATATATCATTGACA
CTGGTATTGAGGCCTCCCACCCCGAGTTTGAGGGTCGCGCCACTTTTCTTAAGAGCTTCATCA
GCGGTCAAAACACTGATGGCCACGGCCATGGGACTCACTGCGCTGGTACCATTGGTAGCAA
GACCTACGGTGTTGCCAAAAAGGCTAAGCTCTATGGTGTCAAGGTTCTTGACAACCAGGGCA
GTGGTTCCTACTCCGGTATCATCAGTGGCATGGACTACGTTGCACAGGACTCCAAGACCCGC
GGCTGCCCCAACGGCGCCATTGCTTCCATGAGCCTGGGAGGTGGCTACTCGGCGTCCGTCAA
CCAAGGTGCTGCTGCTTTGGTCAATTCTGGTGTCTTCCTTGCCGTCGCCGCTGGCAACGATAA
CCGGGATGCCCAGAACACCTCTCCCGCTTCCGAGCCTTCTGCCTGCACTGTTGGTGCCTCTGC
GGAAAATGACAGCCGATCTTCCTTCTCCAACTACGGCAGAGTTGTCGATATTTTCGCTCCTG
GTAGCAATGTTCTTTCCACCTGGATTGGTGGCCGCACAAACACCATCTCTGGTACCTCCATG
GCTACTCCCCATATTGCCGGTCTGGCTGCCTACCTCAGTGCGCTCCAAGGCAAGACTACCCC
TGCCGCTCTTTGCAAGAAGATCCAGGACACTGCTACCAAGAACGTGCTCACCGGTGTTCCCT
CTGGCACTGTCAACTACCTTGCCTACAACGGTGCCTAA

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Reference       ATGCATCATCTACGCGCTCTCGTCGGCGTCGGCCTTGCTGGTCTGGCGGCCGGGGTGCCC       60
Chi2F           ----------------------------------------------------GTGCCC       6
                                                                   ******

Reference       TTGACTGACAAGATATCAGTCAAGCCACGACAAGCACCCGGAGCGCAAAATGTCGTCTAC       120
Chi2F           TTGACTGACAAGATATCAGTCAAGCCGCGACAAGCACCCGGAGCGCAAAACGTCGTCTAT       66
                ************************* ********************** ********

Reference       TGGGGGCAGAACGGCGGCGGTACGATCGAAAATAACGACCTCGCGGCGTATTGCCAGCCC       180
Chi2F           TGGGGGCAGAATGGCGGCGGTACGATCGAGAATAACGACCTCGCGGCGTATTGCCAACCC       126
                *********** ***************** ************************** ***

Reference       AATTCCGGAATCGACGTTTTGGTTCTTGCATTTCTGTATCAATTTGGAAATGGCGGCAAC       240
Chi2F           AATTCCGGAATCGACGTTTTGGTTCTTGCATTTCTGTATCAATTTGGAAATGGCGGCAAC       186
                ************************************************************

Reference       ATACCTTCAGGCACTATAGGCCAATCATGCTACATCAGCACGTCCGGCCAGGGTCAAAAC       300
Chi2F           ATACCTTCAGGCACTATAGGCCAATCATGCTACATCAGCACGTCCGGCCAGGGTCAAAAC       246
                ************************************************************

Reference       TGCGAAGCCCTCACCGCAGCCATACACACCTGCCAGTCCGCCGGTGTCAAGATCGTCCTG       360
Chi2F           TGCGAAGCCCTGACCGCAGCCATACACACCTGCCAGTCCGCCGGTGTCAAGATCATCCTG       306
                *********** ****************************************** *****

Reference       TCCCTCGGCGGCGCGACGAGCTCCTACTCCCTCCAGACGCAGGCGCAAGCCGAGCAAATA       420
Chi2F           TCCCTCGGCGGCGCGACGAGCTCCTACTCCCTCCAGACGCAGGCGCAGGCCGAGCAAATA       366
                ********************************************** ************

Reference       GGCCAGTACCTATGGGACTCGTACGGCAACTCTGGAAACAAGACGGTGCAGCGGCCGTTT       480
Chi2F           GGCCAGTACCTATGGGATTCATACGGCAACTCTGGAAACAAGACGGTGCAGCGGCCGTTT       426
                ****************** ** ****************************************

Reference       GGCAGCAACTTTGTCAACGGGTTCGACTTCGACATTGAAGTCAACGGCGGCAGCAGCCAG       540
Chi2F           GGCAACAACTTTGTCAACGGATTCGACTTCGACATTGAAGTCAACGGCGGCAGCAGCCAG       486
                **** *************** ****************************************

Reference       TACTACCAGTACATGATTGCCAAGCTGCGCGCCAACTTTGCGTCGGACAAGTCCAACACG       600
Chi2F           TACTACCAGTACATGATTGCCAAGCTACGCTCCAACTTTGCGACGGACAAGTCCAACACG       546
                ************************** *** *********** *****************

Reference       TACCTGATTACCGGCGCGCCGCAGTGCCCCATCCCCGAGCCCAACATGGGCGTCATCATC       660
Chi2F           TACCTGATTACCGGCGCGCCGCAGTGCCCCATCCCCGAGCCCAACATGGGCGTCATCATC       606
                ************************************************************

Reference       AGCAACTCCGTCTTTGACCATCTCTACGTCCAGTTCTACAATAATAACAACTACACGGTC       720
Chi2F           AGCAACTCCGTCTTCGACCATCTCTACGTCCAGTTCTACAACAACAACAACTACACGGTC       666
                ************** ************************ ** ***************

Reference       CCCTGCGCGCTGGGCATCAACGGCAACGCCCCCTTCAACTACAACAACTGGACCTCCTTT       780
Chi2F           CCCTGCGCGCTGGGCATCAACGGCAACGCCCCCTTCAACTACAACAACTGGACGTCCTTT       726
                ************************************************** ******

Reference       ATCGCCGACACGCCGTCGGCCGGTGCCAAGATCTTCATTGGCGTGCCGGCCTCGCCGCTC       840
Chi2F           ATCGCCGATACGCCGTCGGCCGGTGCCAAGATCTTCATTGGCGTGCCGGCCTCGCCGCTG       786
                ******** ************************************************

Reference       GCGTCGACGGGGACGCCCAGCGGCGCGCAGTACTACGCCGCGCCGGAGCAACT-GGCCGC       899
Chi2F           GCGTCGACGGGGACGCCCAGCGGCGCGCAGTACTACGCCGCGCCGGAAGAACTGGGCCGC       846
                ********************************************  **** ******

Reference       CATTGTCGGCGAGTACAGGAGCGACGCCCATTTCGGCGGCATCATGATGTGGAGCGCGGG       959
Chi2F           CATTGTCGGCGAATACAGGAGCGACGCCCATTTCGGCGGCATCATGATGTGAAGCGCGGG       906
                ************ ************************************* ********

Reference       ATTTTCGGATGCCAATGTCAACGACGGGTGCACGTATGCGCAGCAGGCAAAGAGTATCCT       1019
Chi2F           CTTCTCGGATGCCAATGTCAACGACGGGTGCACGTATGCGCAGCAGGCAAAGAG------       960
                 ** ************************************************
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Reference       CGTCAACGGGGCGCCTTGCCCATCGTCGGGGCCTCCGAGTTCTACGCCGGCAACAGCCCC       1079
Chi2F           ------------------------------------------------------------       960

Reference       GGCCCCGACCGCGACCACGATGCCATCTTCGACTTCGGTGTCCTCTCCGACAGCGTCGCC       1139
Chi2F           ------------------------------------------------------------       960


Reference       TACCGGCGGCACTGTTCCCCAGTGGGGTCAGTGCGGCGGCGAGGGCTATTCCGGTTCTAC       1199
Chi2F           ------------------------------------------------------------       960


Reference       CCAGTGCGTTCCCCCTTACCAATGTGTCAAGCAAGGCGATTGGTGGTCGTCATGCCGGTG       1259
Chi2F           ------------------------------------------------------------       960


Reference       A                                                                  1260
Chi2F           -                                                                  960
```

DNA sequencing of Chi2. Alignment of sequencing data with the reference sequence (Chi2, Dq011663.2) available on the NCBI database. Chi2F represents the sequence obtained from N-terminal sequencing of pET-28a with T7 forward primer.

```
Reference       AGCCAGTACTACCAGTACATGATTGCCAAGCTGCGCGCCAACTTTGCGTCGGACAAGTCC      60
Chi2R           AGCCAGTACTACCAGTACATGATTGCCAAGCTACGCTCCAACTTTGCGACGGACAAGTCC      60
                **********************************.*** ***********:***********

Reference       AACACGTACCTGATTACCGGCGCGCCGCAGTGCCCCATCCCCGAGCCCAACATGGGCGTC      120
Chi2R           AACACGTACCTGATTACCGGCGCGCCGCAGTGCCCCATCCCCGAGCCCAACATGGGCGTC      120
                ************************************************************

Reference       ATCATCAGCAACTCCGTCTTTGACCATCTCTACGTCCAGTTCTACAATAATAACAACTAC      180
Chi2R           ATCATCAGCAACTCCGTCTTCGACCATCTCTACGTCCAGTTCTACAACAACAACAACTAC      180
                ******************* ************************** ** *********

Reference       ACGGTCCCCTGCGCGCTGGGCATCAACGGCAACGCCCCCTTCAACTACAACAACTGGACC      240
Chi2R           ACGGTCCCCTGCGCGCTGGGCATCAACGGCAACGCCCCCTTCAACTACAACAACTGGACG      240
                ************************************************************

Reference       TCCTTTATCGCCGACACGCCGTCGGCCGGTGCCAAGATCTTCATTGGCGTGCCGGCCTCG      300
Chi2R           TCCTTTATCGCCGATACGCCGTCGGCCGGTGCCAAGATCTTCATTGGCGTGCCGGCCTCG      300
                ************** ******************************************

Reference       CCGCTCGCGTCGACGGGGACGCCCAGCGGCGCGCAGTACTACGCCGCGCCGGAGCAACTG      360
Chi2R           CCGCTGGCGTCGACGGGGACGCCCAGCGGCGCGCAGTACTACGCCGCGCCGGAGGAACTG      360
                ***** ********************************************** *****

Reference       GCCGCCATTGTCGGCGAGTACAGGAGCGACGCCCATTTCGGCGGCATCATGATGTGGAGC      420
Chi2R           GCCGCCATTGTCGGCGAATACAGGAGCGACGCCCATTTCGGCGGCATCATGATGTGGAGC      420
                ****************.*******************************************

Reference       GCGGGATTTTCGGATGCCAATGTCAACGACGGGTGCACGTATGCGCAGCAGGCAAAGAGT      480
Chi2R           GCGGGCTTCTCGGATGCCAATGTCAACGACGGGTGCACGTATGCGCAGCAGGCAAAGAGT      480
                *****.** ************************************************

Reference       ATCCTCGTCAACGGGGCGCCTTGCCCATCGTCGGGGCCTCCGAGTTCTACGCCGGCAACA      540
Chi2R           ATCCTCGTCAATGGAGCGCCTTGCGCATCGTCGGGGCCTCCGAGTTCTACGCCGGCAACA      540
                *********** **.********* ************************************

Reference       GCCCCGGCCCCGACCGCGACCACGATGCCATCTTCGACTTCGGTGTCCTCTCCGACAGCG      600
Chi2R           GCTCCGGCCCCGACCGCGACCACGATGCCATCTTTGACC------TCCTCTCCAGCAGCG      594
                ** ******************************* ***       ********..*****

Reference       TCGCCTACCGGCGGCACTGTTCCCCAGTGGGGTCAGTGCGGCGGCGAGGGCTATTCCGGT      660
Chi2R           TCGCCTACCGGCGGCACTGTTCCCCAGTGGGGTCAGTGCGGCGGCGAGGGCTATTCCGGT      654
                ************************************************************

Reference       TCTACCCAGTGCGTTCCCCCTTACCAATGTGTCAAGCAAGGCGATTGGTGGTCGTCATGC      720
Chi2R           TCTACCCAGTGCGTTCCCCCTTACCAATGTGTCAAGCAAGGCGATTGGTGGTCGTCATGC      714
                ************************************************************

Reference       CGGTGA----------------------------------------------------      726
Chi2R           CGGCATCATCATCATCATCATTAACTCGAGCACCACCACCACCACCACTGAGATCCGG      772
        *** .:
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DNA sequencing of Chi2. Alignment of sequencing data with the reference sequence (Chi2, Dq011663.2) available on the NCBI database. Chi2R represents the sequence obtained from C-terminal sequencing of pET-28a with T7 reverse primer.

Metarhizium anisopliae var. *anisopliae* putative chitinase (Chi2) gene, complete cds, GenBank: DQ011663.2

ATGCATCATCTACGCGCTCTCGTCGGCGTCGGCCTTGCTGGTCTGGCGGCCGGGGTG
CCCTTGACTGACAAGATATCAGTCAAGCCACGACAAGCACCCGGAGCGCAAAATGT
CGTCTACTGGGGGCAGAACGGCGGCGGTACGATCGAAAATAACGACCTCGCGGCGT
ATTGCCAGCCCAATTCCGGAATCGACGTTTTGGTTCTTGCATTTCTGTATCAATTTGG
AAATGGCGGCAACATACCTTCAGGCACTATAGGCCAATCATGCTACATCAGCACGT
CCGGCCAGGGTCAAAACTGCGAAGCCCTCACCGCAGCCATACACACCTGCCAGTCC
GCCGGTGTCAAGATCGTCCTGTCCCTCGGCGGCGCGACGAGCTCCTACTCCCTCCAG
ACGCAGGCGCAAGCCGAGCAAATAGGCCAGTACCTATGGGACTCGTACGGCAACTC
TGGAAACAAGACGGTGCAGCGGCCGTTTGGCAGCAACTTTGTCAACGGGTTCGACTT
CGACATTGAAGTCAACGGCGGCAGCAGCCAGTACTACCAGTACATGATTGCCAAGC
TGCGCGCCAACTTTGCGTCGGACAAGTCCAACACGTACCTGATTACCGGCGCGCCGC
AGTGCCCCATCCCCGAGCCCAACATGGGCGTCATCATCAGCAACTCCGTCTTTGACC
ATCTCTACGTCCAGTTCTACAATAATAACAACTACACGGTCCCCTGCGCGCTGGGCA
TCAACGGCAACGCCCCCTTCAACTACAACAACTGGACCTCCTTTATCGCCGACACGC
CGTCGGCCGGTGCCAAGATCTTCATTGGCGTGCCGGCCTCGCCGCTCGCGTCGACGG
GGACGCCCAGCGGCGCGCAGTACTACGCCGCGCCGGAGCAACTGGCCGCCATTGTC
GGCGAGTACAGGAGCGACGCCCATTTCGGCGGCATCATGATGTGGAGCGCGGGATT
TTCGGATGCCAATGTCAACGACGGGTGCACGTATGCGCAGCAGGCAAAGAGTATCC
TCGTCAACGGGGCGCCTTGCCCATCGTCGGGGCCTCCGAGTTCTACGCCGGCAACAG
CCCCGGCCCCGACCGCGACCACGATGCCATCTTCGACTTCGGTGTCCTCTCCGACAG
CGTCGCCTACCGGCGGCACTGTTCCCCAGTGGGGTCAGTGCGGCGGCGAGGGCTATT
CCGGTTCTACCCAGTGCGTTCCCCCTTACCAATGTGTCAAGCAAGGCGATTGGTGGT
CGTCATGCCGGTGA

The deduced sequence of Chi2 (*Metarhizium anisopliae* 892)

GTGCCCTTGACTGACAAGATATCAGTCAAGCCGCGACAAGCACCCGGAGCGCAAAA
CGTCGTCTATTGGGGGCAGAATGGCGGCGGTACGATCGAGAATAACGACCTCGCGG
CGTATTGCCAACCCAATTCCGGAATCGACGTTTTGGTTCTTGCATTTCTGTATCAATT
TGGAAATGGCGGCAACATACCTTCAGGCACTATAGGCCAATCATGCTACATCAGCA
CGTCCGGCCAGGGTCAAAACTGCGAAGCCCTGACCGCAGCCATACACACCTGCCAG
TCCGCCGGTGTCAAGATCATCCTGTCCCTCGGCGGCGCGACGAGCTCCTACTCCCTC
CAGACGCAGGCGCAGGCCGAGCAAATAGGCCAGTACCTATGGGATTCATACGGCAA
CTCTGGAAACAAGACGGTGCAGCGGCCGTTTGGCAACAACTTTGTCAACGGATTCG
ACTTCGACATTGAAGTCAACGGCGGCAGCAGCCAGTACTACCAGTACATGATTGCC
AAGCTACGCTCCAACTTTGCGACGGACAAGTCCAACACGTACCTGATTACCGGCGC
GCCGCAGTGCCCCATCCCCGAGCCCAACATGGGCGTCATCATCAGCAACTCCGTCTT
CGACCATCTCTACGTCCAGTTCTACAACAACAACTACACGGTCCCCTGCGCGCT
GGGCATCAACGGCAACGCCCCCTTCAACTACAACAACTGGACGTCCTTTATCGCCGA
TACGCCGTCGGCCGGTGCCAAGATCTTCATTGGCGTGCCGGCCTCGCCGCTGGCGTC
GACGGGGACGCCCAGCGGCGCGCAGTACTACGCCGCGCCGGAGGAACTGGCCGCC
ATTGTCGGCGAATACAGGAGCGACGCCCATTTCGGCGGCATCATGATGTGGAGCGC
GGGCTTCTCGGATGCCAATGTCAACGACGGGTGCACGTATGCGCAGCAGGCAAAGA
GTATCCTCGTCAATGGAGCGCCTTGCGCATCGTCGGGGCCTCCGAGTTCTACGCCGG
CAACAGCTCCGGCCCCGACCGCGACCACGATGCCATCTTTGACCTCCTCTCCAGCA
GCGTCGCCTACCGGCGGCACTGTTCCCCAGTGGGGTCAGTGCGGCGGCGAGGGCTA
TTCCGGTTCTACCCAGTGCGTTCCCCCTTACCAATGTGTCAAGCAAGGCGATTGGTG
GTCGTCATGCCGGTGA

```
Reference       AGCACCCCGGTGTCGATTTCATTGAGAAGGACGCTGTGATGCGTATCAGCGGCATCACTG      60
 cPr1aF         ------------------------------------------------GGCATCACTG      10
                                                                **********

Reference       AGCAGAGCGGTGCTCCCTGGGGTCTTGGGCGCATCTCTCACCGCAGTAAGGGAAGCACCA     120
 cPr1aF         AGCAGAGCGGTGCTCCCTGGGGTCTTGGGCGCATCTCTCACCGCAGTAAGGGAAGCACCA      70
                ************************************************************

Reference       CCTATCGCTACGATGATAGTGCTGGTCAGGGTACTTGCGTATATATCATTGACACTGGTA     180
 cPr1aF         CCTATCGCTACGATGATAGTGCTGGTCAGGGTACTTGCGTATATATCATTGACACTGGTA     130
                ************************************************************

Reference       TTGAGGCCTCCCACCCCGAGTTTGAGGGTCGCGCCACTTTTCTTAAGAGCTTCATCAGCG     240
 cPr1aF         TTGAGGCCTCCCACCCCGAGTTTGAGGGTCGCGCCACTTTTCTTAAGAGCTTCATCAGCG     190
                ************************************************************

Reference       GTCAAAACACTGATGGCCACGGCCATGGGACTCACTGCGCTGGTACCATTGGTAGCAAGA     300
 cPr1aF         GTCAAAACACTGATGGCCACGGCCATGGGACTCACTGCGCTGGTACCATTGGTAGCAAGA     250
                ************************************************************

Reference       CCTACGGTGTTGCCAAAAAGGCTAAGCTCTATGGTGTCAAGGTTCTTGACAACCAGGGCA     360
 cPr1aF         CCTACGGTGTTGCCAAAAAGGCTAAGCTCTATGGTGTCAAGGTTCTTGACAACCAGGGCA     310
                ************************************************************

Reference       GTGGTTCCTACTCCGGTATCATCAGTGGCATGGACTACGTTGCACAGGACTCCAAGACCC     420
 cPr1aF         GTGGTTCCTACTCCGGTATCATCAGTGGCATGGACTACGTTGCACAGGACTCCAAGACCC     370
                ************************************************************

Reference       GCGGCTGCCCCAACGGCGCCATTGCTTCCATGAGCCTGGGAGGTGGCTACTCGGCGTCCG     480
 cPr1aF         GCGGCTGCCCCAACGGCGCCATTGCTTCCATGAGCCTGGGAGGTGGCTACTCGGCGTCCG     430
                ************************************************************

Reference       TCAACCAAGGTGCTGCTGCTTTGGTCAATTCTGGTGTCTTCCTTGCCGTCGCCGCTGGCA     540
 cPr1aF         TCAACCAAGGTGCTGCTGCTTTGGTCAATTCTGGTGTCTTCCTTGCCGTCGCCGCTGGCA     490
                ************************************************************

Reference       ACGATAACCGGGATGCCCAGAACACCTCTCCCGCTTCCGAGCCTTCTGCCTGCACTGTTG     600
 cPr1aF         ACGATAACCGGGATGCCCAGAACACCTCTCCCGCTTCCGAGCCTTCTGCCTGCACTGTTG     550
                ************************************************************

Reference       GTGCCTCTGCGGAAAATGACAGCCGATCTTCCTTCTCCAACTACGGCAGAGTTGTCGATA     660
 cPr1aF         GTGCCTCTGCGGAAAATGACAGCCGATCTTCCTTCTCCAACTACGGCAGAGTTGTCGATA     610
                ************************************************************
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DNA sequencing of cloned cPr1a gene. Alignment of sequencing data with the reference sequence (M73795.1 sequence + fepM03A03, chitin-binding sequence) available on the NCBI database. cPr1aF represents the sequence obtained from N-terminal sequencing of pET-28a with T7 forward primer.

```
Reference       CAGAGTTGTCGATATTTTCGCTCCTGGTAGCAATGTTCTTTCCACCTGGATTGGTGGCCG     60
 cPr1aR         CAGAGTTGTCGATATTTTCGCTCATGGTAGCAATGTTCTTTCCACCAGGATTGGTGGCCG     60
                ************************ ********************** ************

Reference       CACAAACACCATCTCTGGTACCTCCATGGCTACTCCCCATATTGCCGGTCTGGCTGCCTA     120
 cPr1aR         CACAAACACCATCTCTGGTACCTCCATGGCTACTCCCCATATGGCCGGTCTGGCTGCCTA     120
                ************************************** *****************

Reference       CCTCAGTGCGCTCCAAGGCAAGACTACCCCTGCCGCTCTTTGCAAGAAGATCCAGGACAC     180
 cPr1aR         CCTCAGTGCGCTCCAAGGCAAGACTACCCCTGCCGCTCTTTGCAAGAAGATCCAGGACAC     180
                ************************************************************

Reference       TGCTACCAAGAACGTGCTCACCGGTGTTCCCTCTGGCACTGTCAACTACCTTGCCTACAA     240
 cPr1aR         TGCTACCAAGAACGTGCTCACCGGTGTTCCCTCGGGCACTGTCAACTACCTTGCCTACAA     240
                ******************************** ***************************


Reference       CGGTGCCGATGTGTGTAACTCTGAGGACGACTACGTACCAGACAAGAAAGAGTGTAGCAA     300
 cPr1aR         CGGTGCCGATGTGTGTAACTCTGAGGACGACTACGTACCAGACAAGAAAGAGTGTAGCAA     300
                ************************************************************

Reference       GTATTGGCGATGTGTGAACGGCGAGGGAGTTCAATTCTCGTGTCAACCGGGGACAATCTT     360
 cPr1aR         GTATTGGCGATGTGTGAACGGCGAGGGAGTTCAGTTCTCGTGTCAACCGGGGACAATCTT     360
                ******************************** ***************************

Reference       CAACGTGAAACTTAACGTTTGCGATTGGCCT-----------------------------     391
 cPr1aR         CAACGTGAAACTTAACGTTTGCGATTGGCCTCATCATCATCATCATCATTAACTCGAGCA     420
```

DNA sequencing of cloned cPr1a gene. Alignment of sequencing data with the reference sequence, created by the fusion of M73795.1 sequence & fepM03A03 (chitin-binding sequence) available on the NCBI database. cPr1aR represents the sequence obtained from C-terminal sequencing of pET-28a with T7 reverse primer.

```
Reference       ------------------------------------------------------------  0
 cChi2F         AAAATTGAAGTTAGCGGAAACATTCCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGA  60

Reference       -----------GTGCCCTTGACTGACAAGATATCAGTCAAGCCGCGACAAGCACCCGGAG  49
 cChi2F         GATATACCATGGTGCCCTTGACTGACAAGATATCAGTCAAGCCGCGACAAGCACCCGGAG  120
                           **********************************************

Reference       CGCAAAACGTCGTCTATTGGGGGCAGAATGGCGGCGGTACGATCGAGAATAACGACCTCG  109
 cChi2F         CGCAAAACGTCGTCTATTGGGGGCAGAATGGCGGCGGTACGATCGAGAATAACGACCTCG  180
                ************************************************************

Reference       CGGCGTATTGCCAACCCAATTCCGGAATCGACGTTTTGGTTCTTGCATTTCTGTATCAAT  169
 cChi2F         CGGCGTATTGCCAACCCAATTCCGGAATCGACGTTTTGGTTCTTGCATTTCTGTATCAAT  240
                ************************************************************

Reference       TTGGAAATGGCGGCAACATACCTTCAGGCACTATAGGCCAATCATGCTACATCAGCACGT  229
 cChi2F         TTGGAAATGGCGGCAACATACCTTCAGGCACTATAGGCCAATCATGCTACATCAGCACGT  300
                ************************************************************

Reference       CCGGCCAGGGTCAAAACTGCGAAGCCCTGACCGCAGCCATACACACCTGCCAGTCCGCCG  289
 cChi2F         CCGGCCAGGGTCAAAACTGCGAAGCCCTGACCGCAGCCATACACACCTGCCAGTCCGCCG  360
                ************************************************************

Reference       GTGTCAAGATCATCCTGTCCCTCGGCGGCGCGACGAGCTCCTACTCCCTCCAGACGCAGG  349
 cChi2F         GTGTCAAGATCATCCTGTCCCTCGGCGGCGCGACGAGCTCCTACTCCCTCCAGACGCAGG  420
                ************************************************************

Reference       CGCAGGCCGAGCAAATAGGCCAGTACCTATGGGATTCATACGGCAACTCTGGAAACAAGA  409
 cChi2F         CGCAGGCCGAGCAAATAGGCCAGTACCTATGGGATTCATACGGCAACTCTGGAAACAAGA  480
                ************************************************************

Reference       CGGTGCAGCGGCCGTTTGGCAACAACTTTGTCAACGGATTCGACTTCGACATTGAAGTCA  469
 cChi2F         CGGTGCAGCGGCCGTTTGGCAACAACTTTGTCAACGGATTCGACTTCGACATTGAAGTCA  540
                ************************************************************

Reference       ACGGCGGCAGCAGCCAGTACTACCAGTACATGATTGCCAAGCTACGCTCCAACTTTGCGA  529
 cChi2F         ACGGCGGCAGCAGCCAGTACTACCAGTACATGATTGCCAAGCTACGCTCCAACTTTGCGA  600
                ************************************************************

Reference       CGGACAAGTCCAACACGTACCTGATTACCGGCGCGCCGCAGTGCCCCATCCCCGAGCCCA  589
 cChi2F         CGGACAAGTCCAACACGTACCTGATTACCGGCGCGCCGCAGTGCCCCATCCCCGAGCCCA  660
                ************************************************************

Reference       ACATGGGCGTCATCATCAGCAACTCCGTCTTCGACCATCTCTACGTCCAGTTCTACAACA  649
 cChi2F         ACATGGGCGTCATCATCAGCAACTCCGTCTTCGACCATCTCTACGTCCAGTTCTACAACA  720
                ************************************************************

Reference       ACAACAACTACACGGTCCCCTGCGCGCTGGGCATCAACGGCAACGCCCCCTTCAACTACA  709
 cChi2F         ACAACAACTACACGGTCCCCTGCGCGCTGGGCATCAACGGCAACGCCCCCTTCAACTACA  780
                ************************************************************

Reference       ACAACTGGACGTCCTTTATCGCCGATACGCCGTCGGCCGGTGCCAAGATCTTCATTGGCG  769
 cChi2F         ACAACTGGACGTCCTTTATCGCCGATACGCCGTCGGCCGGTGCCAAGATCTTCATTGGCG  840
                ************************************************************

Reference       TGCCGGCCTCGCCGCTGGCGTCGACGGGGACGCCCAGCGGCGCGCAGTACTACGCCGCGC  829
 cChi2F         TGCCGGCCTCGCCGCTGGCGTCGACGGGGACGCCCAGCGGCGCGCAGTACTACGCCGCGC  900
                ************************************************************

Reference       CGGAGGAACTGGCCGCCATTGTCGGCGAATACAGGAGCGACGCCCATTTCGGCGGCATCA  889
 cChi2F         CGGAGGAACTGGCCGCCATTGTCGGCGAATACAGGAGCGACGCCCATTTCGGCGGCATCA  960
                ************************************************************

Reference       TGATGTGGAGCGCGGGCTTCTCGGATGCCAATGTCAACGACGGGTGCACGTATGCGCAGC  949
 cChi2F         TGATGTGGAGCGCGGGCTTCTCGGATGCCAATGTCAACGACGGGTGCACGTATGCGCAGC  1020
                ************************************************************
```

DNA sequencing of cloned cChi2 gene. Alignment of sequencing data with the reference sequence, created by the fusion of Chi2 sequence (without chitin-binding domain) (DQ011663.2) & Bmcbd sequence (fepM03A03) available on the NCBI database. cChi2F represents the sequence obtained from N-terminal sequencing of pET-28a with T7 forward primer.

```
Reference       CTTTGCGACGGACAAGTCCAACACGTACCTGATTACCGGCGCGCCGCAGTGCCCCATCCC     60
 cChi2R         CTTTGCGACGGACAAGTCCAACACGTACCTGATTACCGGCGCGCCGCAGTGCCCCATCCC     60
                ************************************************************

Reference       CGAGCCCAACATGGGCGTCATCATCAGCAACTCCGTCTTCGACCATCTCTACGTCCAGTT     120
 cChi2R         CGAGCCCAACATGGGCGTCATCATCAGCAACTCCGTCTTCGACCATCTCTACGTCCAGTT     120
                ************************************************************

Reference       CTACAACAACAACAACTACACGGTCCCCTGCGCGCTGGGCATCAACGGCAACGCCCCCTT     180
 cChi2R         CTACAACAACAACAACTACACGGTCCCCTGCGCGCTGGGCATCAACGGCAACGCCCCCTT     180
                ************************************************************

Reference       CAACTACAACAACTGGACGTCCTTTATCGCCGATACGCCGTCGGCCGGTGCCAAGATCTT     240
 cChi2R         CAACTACAACAACTGGACGTCCTTTATCGCCGATACGCCGTCGGCCGGTGCCAAGATCTT     240
                ************************************************************

Reference       CATTGGCGTGCCGGCCTCGCCGCTGGCGTCGACGGGGACGCCCAGCGGCGCGCAGTACTA     300
 cChi2R         CATTGGCGTGCCGGCCTCGCCGCTGGCGTCGACGGGGACGCCCAGCGGCGCGCAGTACTA     300
                ************************************************************

Reference       CGCCGCGCCGGAGGAACTGGCCGCCATTGTCGGCGAATACAGGAGCGACGCCCATTTCGG     360
 cChi2R         CGCCGCGCCGGAGGAACTGGCCGCCATTGTCGGCGAATACAGGAGCGACGCCCATTTCGG     360
                ************************************************************

Reference       CGGCATCATGATGTGGAGCGCGGGCTTCTCGGATGCCAATGTCAACGACGGGTGCACGTA     420
 cChi2R         CGGCATCATGATGTGGAGCGCGGGCTTCTCGGATGCCAATGTCAACGACGGGTGCACGTA     420
                ************************************************************

Reference       TGCGCAGCAGGCAAAGAGTATCCTCGTCAATGGAGCGCCTTGCGCATCGTCGGGGCCTCC     480
 cChi2R         TGCGCAGCAGGCAAAGAGTATCCTCGTCAATGGAGCGCCTTGCGCATCGTCGGGGCCTCC     480
                ************************************************************

Reference       GAGTTCTACGCCGGCAACAGCTCCGGCCCCGACCGCGACCACGATGCCATCTTTGACCTC     540
 cChi2R         GAGTTCTACGCCGGCAACAGCTCCGGCCCCGACCGCGACCACGATGCCATCTTTGACCTC     540
                ************************************************************

Reference       CTCTCCAGCAGCGTCGCCTACCGGCGGCACTGTTCCCGATGTGTGTAACTCTGAGGACGA     600
 cChi2R         CTCTCCAGCAGCGTCGCCTACCGGCGGCACTGTTCCCGATGTGTGTAACTCTGAGGACGA     600
                ************************************************************

Reference       CTACGTACCAGACAAGAAAGAGTGTAGCAAGTATTGGCGATGTGTGAACGGCGAGGGAGT     660
 cChi2R         CTACGTACCAGACAAGAAAGAGTGTAGCAAGTATTGGCGATGTGTGAACGGCGAGGGAGT     660
                ************************************************************

Reference       TCAATTCTCGTGTCAACCGGGGACAATCTTCAACGTGAAACTTAACGTTTGCGATTGGCC     720
 cChi2R         TCAGTTCTCGTGTCAACCGGGGACAATCTTCAACGTGAAACTTAACGTTTGCGATTGGCC     720
                *** ********************************************************

Reference       TTCGTCATGCCGG-----------------------------------------------     733
 cChi2R         TTCGTCATGCCGGCATCATCATCATCATCATTAACTCGAGCACCACCACCACCACCACTG     780
                *************
```

DNA sequencing of cloned cChi2 gene. Alignment of sequencing data with the reference sequence, created by the fusion of Chi2 sequence (without chitin-binding domain) (DQ011663.2) & Bmcbd sequence (fepM03A03) available on the NCBI database. cChi2R represents the sequence obtained from C-terminal sequencing of pET-28a with T7 reverse primer.

```
Reference       ------------------------------------------------------------      0
fPCF            AAAAGGGATGTGAGCGGGATACATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAG      60


Reference       -------------GGCATCACTGAGCAGAGCGGTGCTCCCTGGGGTCTTGGGCGCATCTC      47
fPCF            GAGATATACCATGGGCATCACTGAGCAGAGCGGTGCTCCCTGGGGTCTTGGGCGCATCTC      120
                             *********************************************


Reference       TCACCGCAGTAAGGGAAGCACCACCTATCGCTACGATGATAGTGCTGGTCAGGGTACTTG      107
fPCF            TCACCGCAGTAAGGGAAGCACCACCTATCGCTACGATGATAGTGCTGGTCAGGGTACTTG      180
                ************************************************************


Reference       CGTATATATCATTGACACTGGTATTGAGGCCTCCCACCCCGAGTTTGAGGGTCGCGCCAC      167
fPCF            CGTATATATCATTGACACTGGTATTGAGGCCTCCCACCCCGAGTTTGAGGGTCGCGCCAC      240
                ************************************************************


Reference       TTTTCTTAAGAGCTTCATCAGCGGTCAAAACACTGATGGCCACGGCCATGGGACTCACTG      227
fPCF            TTTTCTTAAGAGCTTCATCAGCGGTCAAAACACTGATGGCCACGGCCATGGGACTCACTG      300
                ************************************************************


Reference       CGCTGGTACCATTGGTAGCAAGACCTACGGTGTTGCCAAAAAGGCTAAGCTCTATGGTGT      287
fPCF            CGCTGGTACCATTGGTAGCAAGACCTACGGTGTTGCCAAAAAGGCTAAGCTCTATGGTGT      360
                ************************************************************


Reference       CAAGGTTCTTGACAACCAGGGCAGTGGTTCCTACTCCGGTATCATCAGTGGCATGGACTA      347
fPCF            CAAGGTTCTTGACAACCAGGGCAGTGGTTCCTACTCCGGTATCATCAGTGGCATGGACTA      420
                ************************************************************


Reference       CGTTGCACAGGACTCCAAGACCCGCGGCTGCCCCAACGGCGCCATTGCTTCCATGAGCCT      407
fPCF            CGTTGCACAGGACTCCAAGACCCGCGGCTGCCCCAACGGCGCCATTGCTTCCATGAGCCT      480
                ************************************************************


Reference       GGGAGGTGGCTACTCGGCGTCCGTCAACCAAGGTGCTGCTGCTTTGGTCAATTCTGGTGT      467
fPCF            GGGAGGTGGCTACTCGGCGTCCGTCAACCAAGGTGCTGCTGCTTTGGTCAATTCTGGTGT      540
                ************************************************************


Reference       CTTCCTTGCCGTCGCCGCTGGCAACGATAACCGGGATGCCCAGAACACCTCTCCCGCTTC      527
fPCF            CTTCCTTGCCGTCGCCGCTGGCAACGATAACCGGGATGCCCAGAACACCTCTCCCGCTTC      600
                ************************************************************


Reference       CGAGCCTTCTGCCTGCACTGTTGGTGCCTCTGCGGAAAATGACAGCCGATCTTCCTTCTC      587
fPCF            CGAGCCTTCTGCCTGCACTGTTGGTGCCTCTGCGGAAAATGACAGCCGATCTTCCTTCTC      660
                ************************************************************


Reference       CAACTACGGCAGAGTTGTCGATATTTTCGCTCCTGGTAGCAATGTTCTTTCCACCTGGAT      647
fPCF            CAACTACGGCAGAGTTGTCGATATTTTCGCTCCTGGTAGCAATGTTCTTTCCACCTGGAT      720
                ************************************************************


Reference       TGGTGGCCGCACAAACACCATCTCTGGTACCTCCATGGCTACTCCCCATATTGCCGGTCT      707
fPCF            TGGTGGCCGCACAAACACCATCTCTGGTACCTCCATGGCTACTCCCCATATTGCCGGTCT      780
                ************************************************************


Reference       GGCTGCCTACCTCAGTGCGCTCCAAGGCAAGACTACCCCTGCCGCTCTTTGCAAGAAGAT      767
fPCF            GGCTGCCTACCTCAGTGCGCTCCAAGGCAAGACTACCCCTGCCGCTCTTTGCAAGAAGAT      840
                ************************************************************


Reference       CCAGGACACTGCTACCAAGAACGTGCTCACCGGTGTTCCCTCTGGCACTGTCAACTACCT      827
fPCF            CCAGGACACTGCTACCAAGAACGTGCTCACCGGTGTTCCCTCTGGCACTGTCAACTACCT      900
                ************************************************************


Reference       TGCCTACAACGGTGCCGATGTGTGTAACTCTGAGGACGACTACGTACCAGACAAGAAAGA      887
fPCF            TGCCTACAACGGTGCCGATGTGTGTAACTCTGAGGACGACTACGTACCAGACAAGAAAGA      960
                ************************************************************


Reference       GTGTAGCAAGTATTGGCGATGTG-TGAAC-GGCGAGGGAGTTCAA-TTCTCGTGTCAACC      944
fPCF            GTGTAGCCAGTATTGGCGATGTTGTGAACGGGCGAGGGAGTTCAGCTCCTCGTGTCAACC      1020
                *******.**************  ***** **************. * ************
```

```
Reference       GGGGAC--AATCTTCAACGTGAAACTT--AACGT-TTGCGA-TTGGCCTGTGCCCTTGAC      998
fPCF            GGGGGACAATTCTTCAACGTGGAAACTTAAACGTTTTGCGAATTGGCCTTCGGTCATGCC      1080
                ****..  *:***********.**. *  ***** ****** *******  *  *:**.*

Reference       TGACAAGAT----ATCAGTC-----AAGCCGCGACAAGCACCCGGAGCGC-AAAACGTCG      1048
fPCF            GGGTGACATCATCATCATTAACTCCGAGCAACCACAACCACCACACTTGAGAATCCGGCT      1140
                 *. .* **    **** *.     .***..* **** ****. ..  *. **:.** *

Reference       TCTATTGGGGGCAGAATGGCGGCGGTACGATCGAGAATAACGACCTCGCGGCGTATTGCC      1108
fPCF            GCTTACAAAGCCCGAAAGGAGCTG--------GAGTTTG---GCC-------TGCTTGCC      1182
                 **:: ...* *.***:**.*  *        ***::*.   .**         .*****

Reference       AACCCAATTCCGGAATCGACGTTTTGGTTCTTGCATTTCTGTATCAATTTGGAAATGGCG      1168
fPCF            ACCGCT-----GGAA-C--------AATACTAG--------------------------      1201
                *.* *:     **** *        ..*:**:*
```

DNA sequencing of cloned f- cPr1a-cChi2 gene. The alignment of sequencing data with the reference sequence created by the fusion of the cPr1a gene sequence & the cChi2 gene sequence. fPCF represents the sequence obtained from N-terminal sequencing of pET-28a with T7 forward primer.

```
Reference       CCAGGGCAGTGGTTCCTACTCCGGTATCATCAGTGGCATGGACTACGTTGCACAGGACTC      60
fPCR            CCAGGGCAGTGGTTCCTACTCCGGTATCATCAGTGGCATGGACTACGTTGCACAGGACTC      60
                ************************************************************

Reference       CAAGACCCGCGGCTGCCCCAACGGCGCCATTGCTTCCATGAGCCTGGGAGGTGGCTACTC      120
fPCR            -AAGACCCGCGGCTGCCCCAACGGCGCCATTGCTTCCATGAGCCTGGGAGGTGGCTACTC      119
                 ************************************************************

Reference       GGCGTCCGTCAACCAAGGTGCTGCTGCTTTGGTCAATTCTGGTGTCTTCCTTGCCGTCGC      180
fPCR            GGCGTCCGTCAACCAAGGTGCTGCTGCTTTGGTCAATTCTGGTGTCTTCCTTGCCGTCGC      179
                ************************************************************

Reference       CGCTGGCAACGATAACCGGGATGCCCAGAACACCTCTCCCGCTTCCGAGCCTTCTGCCTG      240
fPCR            CGCTGGCAACGATAACCGGGATGCCCAGAACACCTCTCCCGCTTCCGAGCCTTCTGCCTG      239
                ************************************************************

Reference       CACTGTTGGTGCCTCTGCGGAAAATGACAGCCGATCTTCCTTCTCCAACTACGGCAGAGT      300
fPCR            CACTGTTGGTGCCTCTGCGGAAAATGACAGCCGATCTTCCTTCTCCAACTACGGCAGAGT      299
                ************************************************************

Reference       TGTCGATATTTTCGCTCCTGGTAGCAATGTTCTTTCCACCTGGATTGGTGGCCGCACAAA      360
fPCR            TGTCGATATTTTCGCTCCTGGTAGCAATGTTCTTTCCACCTGGATTGGTGGCCGCACAAA      359
                ************************************************************

Reference       CACCATCTCTGGTACCTCCATGGCTACTCCCCATATTGCCGGTCTGGCTGCCTACCTCAG      420
fPCR            CACCATCTCTGGTACCTCCATGGCTACTCCCCATATTGCCGGTCTGGCTGCCTACCTCAG      419
                ************************************************************

Reference       TGCGCTCCAAGGCAAGACTACCCCTGCCGCTCTTTGCAAGAAGATCCAGGACACTGCTAC      480
fPCR            TGCGCTCCAAGGCAAGACTACCCCTGCCGCTCTTTGCAAGAAGATCCAGGACACTGCTAC      479
                ************************************************************

Reference       CAAGAACGTGCTCACCGGTGTTCCCTCTGGCACTGTCAACTACCTTGCCTACAACGGTGC      540
fPCR            CAAGAACGTGCTCACCGGTGTTCCCTCTGGCACTGTCAACTACCTTGCCTACAACGGTGC      539
                ************************************************************

Reference       CGATGTGTGTAACTCTGAGGACGACTACGTACCAGACAAGAAAGAGTGTAGCAAGTATTG      600
fPCR            CGATGTGTGTAACTCTGAGGACGACTACGTACCAGACAAGAAAGAGTGTAGCAAGTATTG      599
                ************************************************************

Reference       GCGATGTGTGAACGGCGAGGGAGTTCAATTCTCGTGTCAACCGGGGACAATCTTCAACGT      660
fPCR            GCGATGTGTGAACGGCGAGGGAGTTCAGTTCTCGTGTCAACCGGGGACAATCTTCAACGT      659
                ***************************.********************************

Reference       GAAACTTAACGTTTGCGATTGGCCTGTGCCCTTGACTGACAAGATATCAG-------TCA      713
fPCR            GAAACTTAACGTTTGCGATTGGCCTTCGTCAT-GCCGGTGACATCATCATCATTAACTCG      718
                ************************  * *.* *.* *: *..: ****        **.

Reference       AGC--CGCGACAAGCACC-CGGAGCGCAAAACGTCGTCTATTGGGGGCAGAATGGCGGCG      770
fPCR            AGCACCACCACCACCACCACTGAG----ATCCGGCTGCTAACAAAGCCCGAAAGGAAGCT      774
                ***  *.* **.* **** * ***    *:.** *  ***: ...* *.***:**..**

Reference       GTACGATCGAGAATAACGACCTCGCGGCGTATTGCCAACCCAATTCCGGAATCGACGTTT      830
fPCR            GAGTT-----GGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCC----------CTT      819
                *:.       *..*.. *.*. *** *.* *:*..*:* *.:*: **           **

Reference       TGGTTCTTGCATTTCTGTATCAATTTGGAAATGGCGGCAACATACCTTCAGGCACTATAG      890
fPCR            GGGG-------------CCTCTAAACGGGTCTGAGGGTTCC----CC-------------      849
                 **               .**:*:: **.:.**. ** :.*    *
```

DNA sequencing of cloned f- cPr1a-cChi2 gene. The alignment of sequencing data with the reference sequence created by the fusion of the cPr1a gene sequence & the cChi2 gene sequence. fPCR represents the sequence obtained from C-terminal sequencing of pET-28a with T7 reverse primer.

RESEARCH OUTPUT

9. RESEARCH OUTPUT

Manuscript under preparation

Neha Maurya and Gurvinder Kaur Saini**,** Development of chimeric protease (cPr1a) for enhanced enzyme activity.

Neha Maurya and Gurvinder Kaur Saini, Bioengineering of chimeric chitinase (cChi2) for increased bioactivity against cuticle of insects.

VITAE

10. VITAE

The author was born on April 30, 1988, in the South Mumbai District of Maharashtra, Colaba, India. In the academic years 2003–2005, she passed the higher and senior secondary examinations from Kendriya Vidyalaya No.2 in Faridabad, Haryana, administered by the Central Board of Secondary Education. She completed her B.Sc. (Hons) in Chemistry from Pt. J.L.N. Government College, Faridabad in 2011 and M.Sc. in Biotechnology from Himachal Pradesh University, Shimla in the year 2013.

Mrs. Neha Maurya joined the Ph.D. program at the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India, in the year 2014. She successfully presented her thesis work to the Doctoral Committee during the open seminar (Ph.D. Synopsis) she gave in June 2022. In October 2023, she submitted the Ph.D. thesis.