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Heterologous expression of *Helicoverpa armigera* chitinase gene in *Pichia pastoris*

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Abstract
Insects' growth and development is due to the constant deposition and remodelling of the cuticle which is made up of chitin. Initial separation of new cuticle from old cuticle takes place during the apolysis stage of insects' growth cycle by the accumulation of moulting fluid. Moulting fluid contains high concentrations of chitinolytic and proteolytic enzymes. The chitinolytic and chitin synthase enzymes are responsible for periodic shedding and resynthesizing of chitin containing exoskeleton. Metabolism of chitin, can be a target for selective pest control management. Consequently, insect chitinases and respective genes are gaining importance as a biopesticide. *Helicoverpa armigera* being a polyphagous pest worldwide, its chitinase gene was considered for expression. The gene of size 1737bp was cloned into *E. coli* DH5α using pTZ57R/T vector and characterised by PCR and sequenced. Further the chitinase gene was subcloned into yeast transfer vector (pPICZαB) for expression. Linearised recombinant vector containing chitinase gene was introduced into yeast (*Pichia pastoris*, X-33) by electroporation and the recombinant yeast clones were identified by PCR analysis. The recombinant protein expressed in yeast (yeild of 1.2 mg per litre) was confirmed by SDS- PAGE and Western blotting. These results show that *P. pastoris* is a convient system for the production of heterologous chitinase that can be used as biopesticide.

Keywords: *Helicoverpa armigera, Pichia pastoris,* electroporation, chitinase, biopesticide

Introduction
Helicoverpa armigera Hubner is a highly polyphagous and one of the most serious pests of horticultural and agricultural crops that includes cotton, tomatoes, sunflower, beans, maize and several cucurbitous and citrus crops comprising of 182 plant species [34]. This serious pest has been reported from various countries like USA, Mexico, Manitoba, Australia and Peru. It is also widely distributed in East and South Asia viz., India, China and Pakistan. It is a multivoltine species with 3-4 generations requiring multiple control interventions every year. *Helicoverpa amigera* causes a great extent of damage to the crop as they always ingest the nitrogen rich part of the plants i.e., seeds and reproductive part [9,17] and the damage vary 50-90% depending upon the crop [27,32].
Chemical pesticides, a dominant tool of farmers, have been used from long time wherein the pest is controlled by spraying insecticides 10-12 times during crop period. Unfortunately, the insects have developed manifold resistance and cross resistance to organophosphates, carbamates and synthetic pyretheroids [3]. In the period of 50 years between 1930 and 1980, the amount of pesticides released into the environment is raised by about 1900% [13]. The result of which is unacceptable destruction of the environment that has raised the issues in matter of public health.
Alternate strategies such as pupa busting, pheromone traps and use of plant derived agents that has been tried extensively for control of this pest has shown limited success especially in large land holdings. *Bt* and its formulations was encouraged to control pest in the form of transgenic crops expressing cry proteins. Though this crop is grown in various parts of the world, concerns have repeatedly been raised about the continued utility of these toxins specifically in relation to development of resistance by insects to these insecticidal proteins. Few reports have even confirmed the development of resistance to cry proteins by *Helicoverpa amigera* [5, 42].

Increased resistance to chemical insecticides and recent evolution of the pest's resistance to the Bacillus thuringensis toxins has necessitated for the search of safer and environment friendly management tools. Enzyme based biopesticide, which might work by degradation of the vital structures, such as the peritrophic membrane and cuticle of insects or the cell wall of fungal pathogens can be tried to control the pest. Chitinase is one such enzyme which is gaining importance as an effective biopesticide in the recent years as the exoskeleton of the insect is made up of 40% of chitin [20].

Complete enzymatic hydrolysis of chitin is performed by a chitinolytic system that comprises of chitinase, chitobiose and β-N-acetylglucosaminidase, the action of which is known to be synergistic and consecutive. Chitinase, a chitin catabolic enzyme degrades chitin to low molecular weight, soluble and insoluble oligosaccharides such as chitotetraose, chitotriose, and chitobiose [28]. Chitinase inhibitor, allosamidin was used to disrupt larval ecdysis and increased mortality of two lepidopteran pests *Bombyx mori* and *Lacanobia separate* [31]. As per Blattner *et al.* (1997) reported increased mortality of the blowfly *Lucilia cuprina* with feeding tests and severe morphological alterations leading to larval mortality in webbing clothes moth *Tineola bisselliella* [4]. Though allosamidin are potential chitinase inhibitors none are used commercially because of difficult in their synthesis and high production costs [15]. Some researchers took a different approach, targeting the substrate instead of the enzyme, using the same enzyme as a tool to disrupt the growth process.

Preventing the formation of the chitin exoskeleton and thereby preventing moulting and in turn growth of the insects using chitinase, microbial as well as insect, has been found to be successful. Bacterial chitinases proved to be insecticidal showed enhanced activity in combination with *Bacillus thuringiensis* spores against *Choristoneura fumiferana* [37, 22, 23] and *Spodoptera exigua* [21] and in combination with baculovirus against *Lymantria dispar* [33]. Similarly, fungal chitinase of *Metarhizium anisopliae* transformed in to tomato plant was able to induce toxicity to *Tricholpsia ni* while failed against *Heliothis virescens* [10, 11].

Thomas and workers (2000) demonstrated the role of chitinase in liquefaction of baculovirus infected larvae through mutation of AcMNPV-*Autographa californica* multiple nuclear polyhedrosis viral chitinase genes which failed to bring about liquefaction of infected *T. ni* larvae [38]. Taking a cue from this work another group used AcMNPV chitinase to show the loss of integrity in the peritrophic membrane, in turn mortality, in *Bombyx mori* larvae [26].

Further, insect chitinases too have been tested against some of the insects wherein recombinant chitinases of heterologous expression have been used. The use of insects own product to arrest their growth might lessen the probability of developing resistance to chitinase, because chitinolysis associated moulting is essential for insect development.

Cloning an insect chitinase gene- *Manduca sexta* by Kramer and group in 1993 followed cloning and characterization of chitinase genes in number of insects [19, 18, 35, 43, 1, 8, 41]. Initially Ding and group potentiated the role of insect chitinase cloned in tobacco plant in killing tobacco budworms *Heliothis virscens* larvae and also emphasised the synergistic effect along with *Bt* toxin in decreasing the larval weight of tobacco hornworm *Manduca sexta* [7]. The same *Manduca sexta* chitinase mediated through AcMNPV was able to kill the larvae of fall armyworm *Spodoptera frugiperda* in approximately three quarters of the time required for the wild-type virus to kill the larvae [12].

Insect chitinase expressed in *Pichia pastoris* has shown 100% mortality in case of tomato moth larvae-*Laconobia oleraceae* at a low dose of 2.5µg/g [8] as the protein was expressed in the form of functional protein. The success report on yeast expressed recombinant proteins as reported by Fitches and group on *Lacobonia oleraceae* and on expressing chitinase of *Ostrinia furnacalis* as reported by other group prompted us to select yeast system to express *Helicoverpa armigera* chitinase gene [8,41]. Besides yeast system has the technical advantages of site specific integration, high amount of protein expression, leader sequence for the secretion of heterologous protein in the medium which can be purified easily less sophistication for the maintenance of cells and easy to scale up using large scale fermentation system. It also helps in proteolytic processing, folding, disulphide bond formation and glycosylation. Thus, many proteins that end up as inactive inclusion bodies in bacterial systems are produced as biologically active molecules in *P. pastoris*. The *P. pastoris* system is also generally regarded as being faster, easier, and less expensive to use than expression systems derived from higher eukaryotes such as insect and mammalian tissue culture cell systems and usually gives higher expression levels.

Materials and Methods

Isolation of *Helicoverpa armigera* chitinase gene

Helicoverpa armigera (NBAII–MP-NOC-001) mother stock procured from National Bureau of Agriculturally Insect Resource, Bengaluru, India, was reared on a modified semi synthetic diet in the laboratory [36]. Day-3 sixth instar larval integuments were dissected under physiological saline to isolate total RNA as per protocol described by Kingston *et al.* with necessary modifications [29]. The isolated RNA was used to obtain, initially, ss cDNA, at 42°C, for 1 hour using MMLV- RT (Moloney Murine Leukemia Virus Reverse Transciptase, Fermentas Life Sciences) and oligodT primer; later, ds cDNA of size ~1.8kb using gene specific primers employing standard PCR under conditions of 94°C for 1 minute, 60°C for 1 minute, extension at 72°C for 1.5 minutes and final extension at 72°C for 15 minutes. Primers were designed based on the *Helioverpa armigera* chitinase sequence from Genebank NCBI (Accn. No.: AY325496) including *EcoRI* and *Xba*I restriction sites with the sequence of forward primer and reverse primer respectively as indicated below for cloning and expression studies. Care was taken to remove stop codon toward the end of the gene to facilitate protein purification after expression using histidine tag.

Forward primer 5´ cagtcagaattcatgaga gtgatactagcgacg t 3´
Reverse primer 5´ tgacactctagaaggcgtcctgttcatgag 3´

Cloning and transformation

The resulting PCR product of size ~1.8kb was gel purified, quantified and ligated into pTZ57R/T in the ratio of 1:3 and transformed into *E.coli* DH5α through heat shock method. The transformants were plated on Luria Bertani plates containing 25µg of ampicillin, 4µl of IPTG and 40µl of X-gal and the positive white colonies were selected for sequencing. Further confirmation was done by restriction analysis and PCR of recombinant plasmid.

Construction of recombinant pPICZαB with *Helicoverpa armigera* chitinase

The *E coli* DH5α (Novagen, Germany) used as host for DNA manipulation were cultured in Luria Bertani medium (Himedia, India) that was supplemented with 25µg/25ml ampicillin for the selection of transformants and the recombinant plasmid was isolated using alkali lysis method. The recombinant plasmid was restricted using *Eco*RI and *Xba*I and the insert was released. The gene was further sub cloned into pPICZαB yeast transfer vector at *Eco*RI and *Xba*I sites which has AOX I promoter and *Saccharomyces cerevisiae* alpha factor secretion signal. Use of *Eco*RI and *Xba*I recognition sequences in primers facilitated the insertion of chitinase gene into pPICZalpha B yeast transfer vector. The alpha secretory signal present in the vector upstream to the chitinase gene enables the target protein to secrete into the medium. The recombinant vector was mobilized into *E.coli* DH5α competent cells. The positive bacterial transformants were selected through restriction digestion of plasmid DNA using *Eco*RI and *Xba*I enzymes and PCR analysis.

Transformation of yeast with pPICZalphaB containing chitinase gene and screening of *Pichia pastoris* expression strains through genomic DNA PCR.

Approximately 10 µg of recombinant expression plasmid pPICZalphaB containing chitinase gene was linearized by digesting with *Sac*I enzyme and used to transform into competent *P. pastoris* X-33 cells (Invitrogen, USA) by electroporation using Multiporator Eppendorf electroporator at 1,500V for 5milliseconds. After transformation, cells were plated on YPDS agar plates (1% Yeast extract, 2% Peptone, 2% Dextrose, 1M Sorbitol and 2% Agar) with Zeocin™-100µg/ml (Invitrogen, USA) and incubated at 30°C for 3-7 days until colonies appeared. The parent pPICZalphaB without insert, linearized with *Sac*I was also transformed that served as negative control. The colonies obtained were restreaked on fresh YPDS agar plates at higher concentration of Zeocin™ (300µg/ml). Transformants bearing the chromosomally integrated copies of the pPICZalphaB containing chitinase gene were then detected by a genomic PCR assay using the gene specific primers. Genomic DNA isolation was conducted following the protocol described by Harju and group [14]. Genomic DNA extracted from putative positive colonies was subjected to PCR using gene specific primers along with appropriate control samples.

Induction and Protein Expression

A positive colony was inoculated into 25ml of YPD-Yeast Extract Peptone Dextrose taken in a sterile 250 mL flask and incubated at 30°C in a shaking incubator (250-300 rpm) until culture reached an OD_{600}-6. As the cells reached log-phase growth, the culture was centrifuged at 4500rpm for 5 minutes at room temperature and the pellet was washed twice with BMMY- Buffered methanol Complex Medium to remove traces of YPD medium. To induce expression, the cell pellet was re-suspended to an OD_{600} -1 in BMMY medium and the culture was transferred to 1 litre baffled flask and incubated at 30ºC. At an interval of every 24 hours for 5 days, 0.5% methanol was added to maintain induction. Samples were withdrawn regularly every 24 hours for analysis of intracellular and extracellular expression by SDS- PAGE.

Purification of the target protein

In order to achieve the purification and concentration of the secreted proteins for further use, single colony of yeast transformant showing secreted expression was cultured under optimal expression conditions and the culture supernatant was collected after methanol induction. The recombinant protein containing polyhistidine tag was purified from the secreted media by Affinity column chromatography technique using Ni-NTA (Nickel-Nitrilotriacetic acid, Novagen, Germany) resin that uses polyhistidine tag from the fusion protein. The final concentration of the protein in the purified solution was estimated by The Lowry's method utilizing the Folin-Ciocalteau reagent.

Confirmation of the expressed pPICZalphaB **containing chitinase through Western blot**

The secretory expression of chitinase was confirmed with chitinase positive serum (raised by using the bacterial expressed chitinase) through Western blot. Briefly, proteins were transferred from the gel onto nitrocellulose membrane. After transfer, the membrane was probed with 1:3000 dilution of the chitinase specific monoclonal antibody raised in rabbit. *H. armigera* chitinase was expressed in prokaryotic system using pET32a and Rosetta DE3 and the protein was of approximately 76kDa because of the presence of Trx.Tag and this protein was used to raise antibodies (Bhat Biotech India Private Limited, Bengaluru).Pro Rec A (1:1,000) was used as secondary antibody. Reactivity of the recombinant protein was done with anti-chitinase antibodies using blocking reagent and Tris buffered saline [TBS containing 0.05% (v/v) Tween-20] and the substrate diamino benzidine (DAB). Bacterial expressed *H. armigera* chitinase sample was also processed for western blotting.

Results and Discussion

Isolation of *Helicoverpa armigera* chitinase and construction of pPICZalphaB containing chitinase recombinant vector

Total RNA isolated from the integument of day three sixth instar larvae resulted in the synthesis of first strand cDNA synthesis using M-MuLV RT (Moloney Murine Leukemia Virus Reverse Transciptase)**.** M-MuLV RT is an ideal enzyme for RT-PCR, and is the preferred reverse transcriptase for long mRNA templates especially for > 500 base pair limits, because the RNaseH activity of M-MuLV RT is weaker than the commonly used AMV RT (Avian Myeloblastosis Virus Reverse transcriptase) and thereby protecting the RNA template degradation. PCR amplicon of size 1.8kb was observed on agarose electrophoresis with gene specific primers and first strand cDNA as the template (Figure 1).

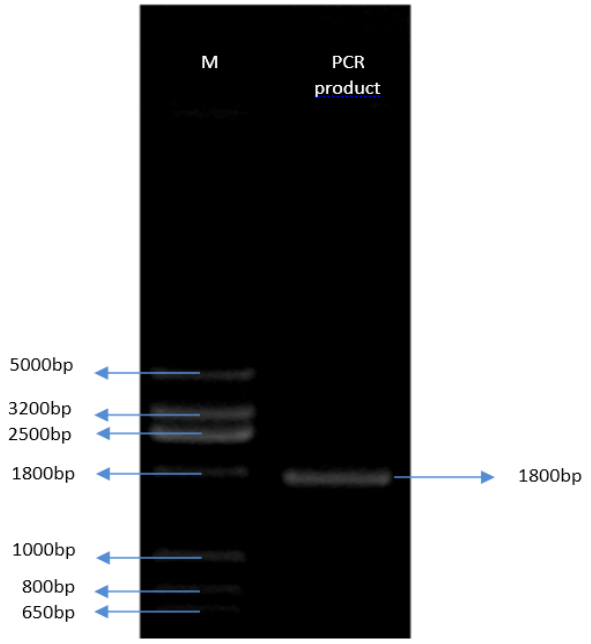

Fig 1. PCR amplification of *H. armigera* chitinase. PCR amplicon seen as a single band of 1.8kb.Lane M- DNA molecular weight marker (Hyperladder TM 1)

The band size was similar to the expected band of *Helicoverpa armigera* chitinase (AY325496). The chitinase gene was cloned into pTZ57R/T vector to generate a recombinant plasmid (pTZ57R/T + HA chi) which was confirmed by sequencing. Further restriction analysis of the recombinant plasmid pTZ57R/T+HA chi with *Eco*RI and *Xba*I resulted in 2 bands corresponding to vector backbone of 3 kb and the insert size of 1.8kb (Figure 2).

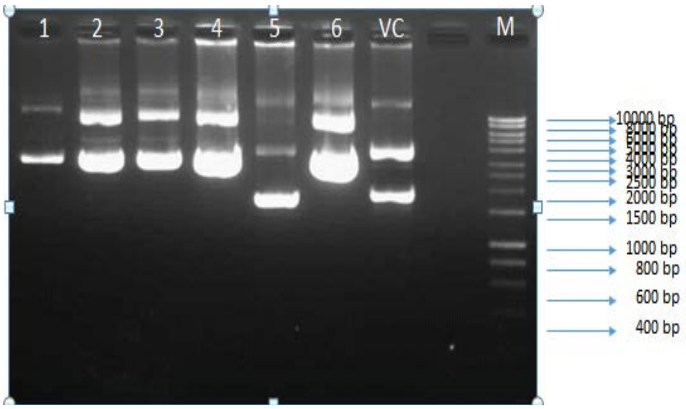

Fig 2. Cloning of PCR amplicon into pTZ57R/T. Lane 1-6 Recombinant plasmids (pTZ57R/T + HA chi), Lane 7, Vector control, Lane 8, intentionally left blank, Lane 9, Hyperladder TM 1 DNA marker. Recombinant plasmids are identified based on the band shift as compared to vector control. Lane 5, has not taken the insert.

Plasmid PCR analysis of recombinant plasmid pTZ57R/T+HA chi resulted in the amplification of 1.8kb insert (Figure 3).

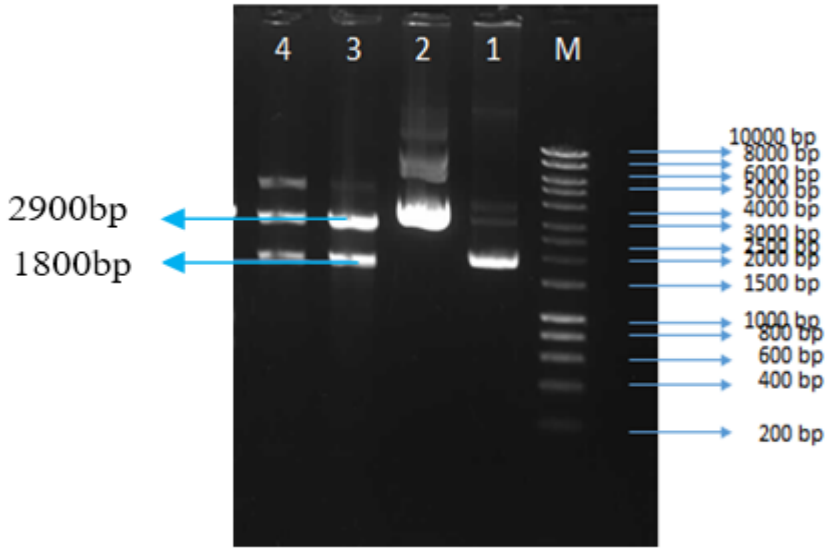

Fig 3. Restriction analysis of pTZ57R/T + HA chi using *Eco*RI and *Xba* I. Lane M, Hyperladder TM 1 DNA marker, Lane 1, Vector Control, Lane 2, Recombinant plasmid (pTZ 57R/T+ HA chi), Lane 3 and 4, Digested recombinant plasmid.

Sequencing result was compared with the National Centre for Biotechnology Information (NCBI) GeneBank data by basic local alignment search tool (BLAST). Gene sequence analysis showed that the gene had terminated beforehand at 1737bp rather at 1767bp as compared to the reference gene [1]. The gene when subjected to ExPASy translation tool gave rise to protein of 578aa instead of 588 amino. Thereby a new reverse primer (5´tga cac tct aga acg gtc ggc gtt gt 3´) with the restriction site *Xba*I was designed to obtain the product of size 1737bp. Cloning and transformation was carried out for the PCR product as described earlier.

Though the gene was terminated beforehand at 578 amino acids, sequence analysis still revealed the presence of highly conserved region of GH18 domain-glycohydrolase, for insect chitinases as suggested by many workers [19, 6, 39, 40, 2]. Next to the GH18 domain, a less conserved domain PEST domain (proline glutamate serine threonine) was observed and CBD (Chitin Binding) domain was found [16, 20, 30]. The presence of these three domains a tripartitate structure typical for insect chitinases, in spite of early termination, confirms that the gene belongs to group I chitinases of insects, as these domains are characteristic of the group.

The recombinant plasmid from positive clones (pTZ57R/T) was digested with *Eco*RI and *Xba*I and the DNA released was ligated into pPICZαB and recombinant pPICZαB+HA chi was produced (Figure 4).

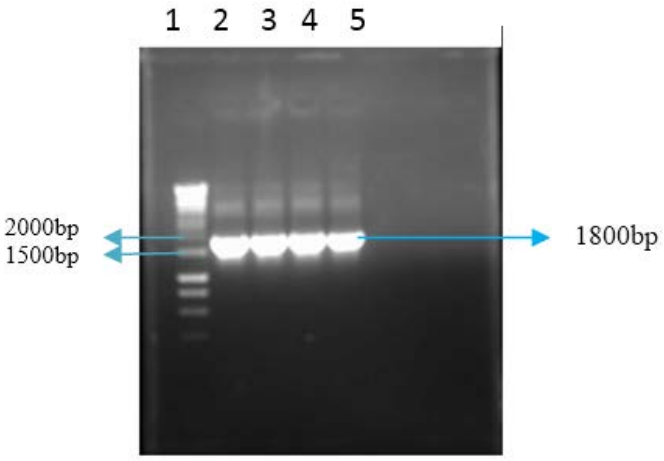

Fig 4. Plasmid PCR analysis of pTZ57R/T. Lane 1, Hyperladder ™ 1 DNA Marker, Lane 2-5 Plasmid PCR amplicons of sixe approximately 1.8kb

This can be observed by the DNA band shift towards the well as compared to the control vector (Figure 5).

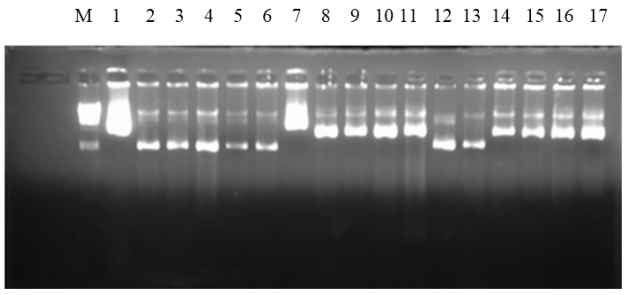

Fig 5. Transformation of pPICZαB+ HA chi into *E.coli* DH5α. Lane M, Marker λ DNA /Hind III. Lane 8, 9,10,11,14,15,16,17 transformed plasmids, Lane 1, 7, 12 vector control and Lane 2,3,4,5,6,13 untransformed plasmids. Change in the position of the band shift between vector control and transformed colonies confirm the presence of insert

Digestion of recombinant pPICZαB+HA chi with the *Eco*RI and *Xba*I resulted in two DNA fragments sized 3600bp and 1800bp respectively indicating that the clone had the insert (Figure 6).

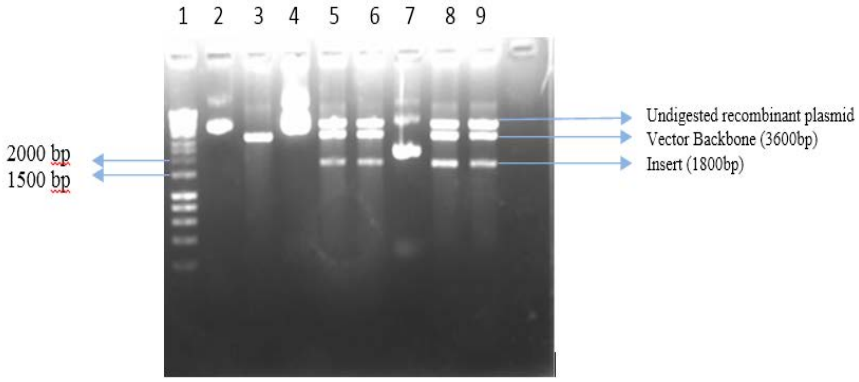

Fig 6. Restriction analysis of pPICZα B+ HA chi using *Eco*RI and *Xba*I. Lane 1, Hyperladder ™ 1 DNA marker, Lane 2, recombinant plasmid, Lane 3, Vector control, Lane 4 and 7, recombinant plasmid, Lane 5, 6, 7 and 8 digested recombinant plasmid. Digestion of the recombinant plasmid resulted in the vector backbone and the release of insert.

Transformation of *P. pastoris* and selection of transformants

Successful linearization of the recombinant plasmid was observed as a single band of size approximately 5500bp and thereby the linearized plasmid was transformed into competent X-33 (Figure 7).

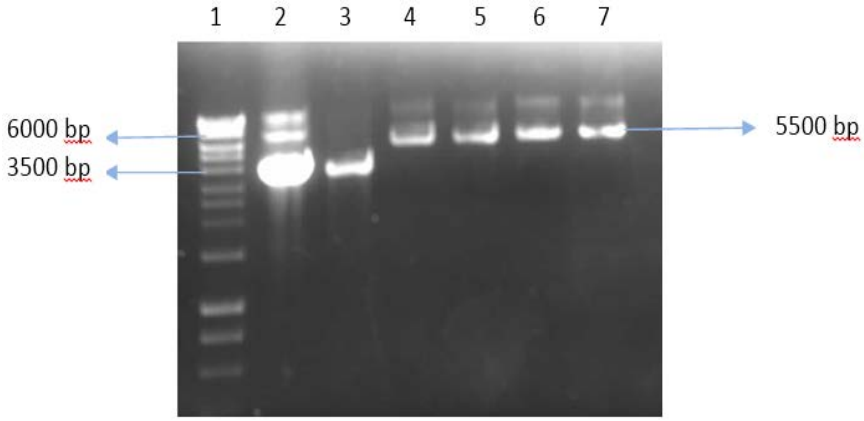

Fig 7. Linearistion of Recombinant Plasmid (pPICZα + HA chi) using *Sac*I. Lane 1, Hyperladder ™ 1Marker, Lane 2,Vector (pPICZαB), Lane 3 linearised vector (pPICZαB), Lane 4,5,6,7, linearised recombinant plasmid (pPICZαB+ HAchi). Lower band indicates linearisarion of the recombinant plasmid pPICZαB+ HAchi.

The transformants with copy inserts were screened on YPDS media harbouring Zeocin™ (100µg/ml). Around 40-50 colonies were observed on the transformed plate. Colonies when restreaked on YPDS plates with higher concentration of Zeocin™ (300µg/ml), only 8 colonies showed growth on a higher concentration of antibiotic. The integration of chitinase gene in the genome of *P. pastoris* was confirmed by genomic DNA PCR.

Genomic DNA was isolated from yeast transformants by lysis of cells with alternate freezing and heating at -80ºC and 95ºC respectively, a slight deviation from the protocol described by Harju and group [14] wherein they have used dry ice ethanol bath for freezing (Figure 8).

The isolated sample amplified expected DNA of 1737bp, indicating the successful isolation of genomic DNA, along with known positive control. Whereas negative sample did not show any bands (Figure 9).

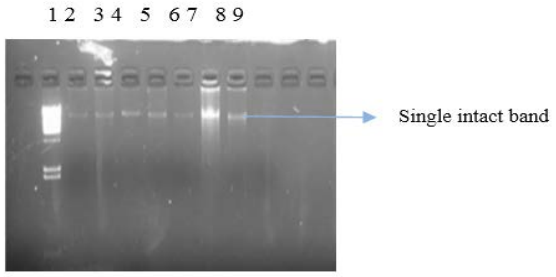

Fig 8. Genomic DNA isolation. Lane 1, λ DNA/Hind III DNA Marker, Lane 2-9 Genomic DNA isolation. Single band towards the well indicates genomic DNA.

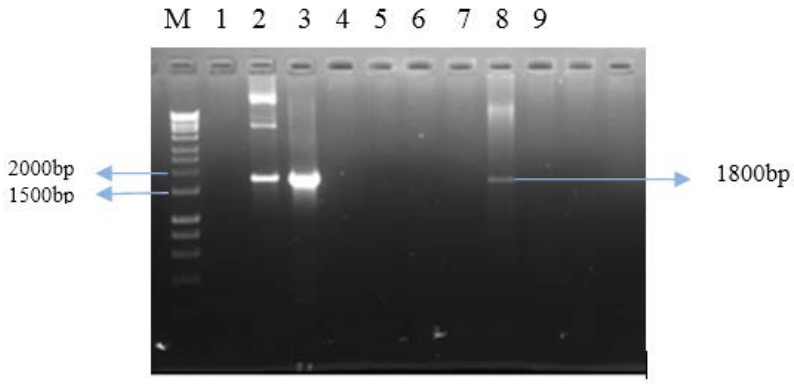

Fig 9. Genomic DNA PCR of Pichia pastoris transformed clones. Lane M, Marker. Lane1, Negative contol, Lane 2, Positive Control, Lane 3 – 9 Genomic DNA PCR amplicons. Lane 3 and 8 indicated the presence of insert in transformed colonies.

Analysis of the expressed product through SDS-PAGE

Four colonies that were found positive with genomic DNA PCR were selected for protein expression of the target gene. Protein expression was indicated by the presence of an extra band as compared to the uninduced culture. Protein band of approximately 66 kDa was observed after 48hr post induction in the supernatant sample indicating that the protein was extracellular because of the presence of α-signal secretion factor present in the vector. Studies conducted to optimise the expression level revealed the protein expression until 120 hrs of induction period with 0.5% methanol concentration (Figure 10).

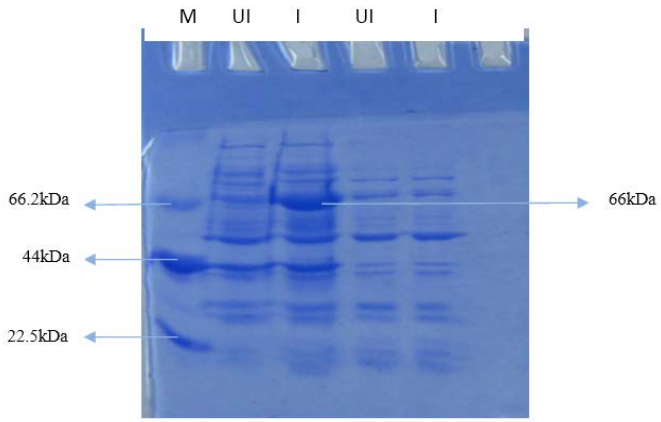

Fig 10. SDS-PAGE analysis of induced pPICZαB+HA chi clones. Lane M, Medium range protein marker, Lane UI, uninduced samples, Lane I, induced samples with methanol. An extra band at 66kDa in the induced sample as compared to the uninduced sample indicates protein expression.

Western Blotting

Pichia expressed *H. armigera* purified chitinase protein reacted positively with anti chitinase antibodies. Similarly *E.coli* expressed *H. armigera* chitinase also reacted positively with anti chitinase antibodies (Figure 11).

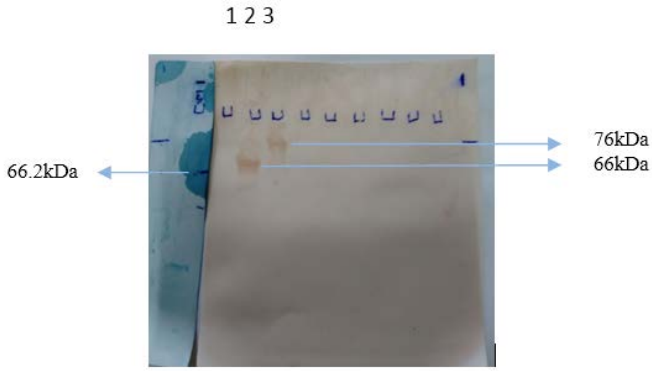

Fig 11. Western blotting of expressed *H. armigera* chitinase using anti chitinase antibodies which was raised in rabbit. Lane M, Medium range protein marker, Lane1, Negative control, Lane 2, *Pichia* expressed *H. armigera* chitinase, Lane 3, *E. coli* expressed *H. armigera* chitinase.

Conclusion

Though, many insect chitinases have been sequenced and characterized, expression work is limited to only few insects *i.e., Manduca sexta* [7,12], *Spodoptera litura* and *Helicoverpa armigera*[1] and *Lacanobia olaracea* [8], *Ostrinia furnacalis* [41] of which insect chitinase of *Lacanobia oleracea* and

Ostrinia furnacalis has been expressed successfully in *Pichia pastoris*. Present work involving the expression of *Helicoverpa armigera* chitinase in *Pichia pastoris* indicates that the yeast system can be used for insect chitinase expression studies.

The protein expression level was found to be 1.2mg/L which is closer to the range (0.5-2mg/L) reported by Fitches and group (2004) in case of *Lacanobia olaracea* chitinase. However, for high expression one can try different host-vector combinations or codon optimisation.

The present work reveals that the gene isolated is a variant of the one reported earlier by Ahmad and group in 2003[1] and the gene sequence is submitted to data bank under Accession No.KT894380.

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