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Potential of Chitinases as a Biopesticide against Agriculturally Harmful Fungi and Insects.

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Review Article

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ABSTRACT

Due to an increasing sensibility and pressure of public and environmental agencies against the application of chemical based pesticides and their long lasting adverse effects on ecosystems and human health, has motivated the search for non hazardous alternatives. The most trustworthy substitute of chemical pesticides is considered as biocontrol agents. These agents could be formulations of bacteria, fungi, viruses, plant extracts or antibiotics. Inhibition or killing of harmful pests by biocontrol agents is environmentally safe and without creating any soil pollution. In last two decades, chitinases have received the attention of researchers for their anti-insects and antifungal biocontrol activities. This review critically focused on the successful studies (inside and outside of laboratory) has been done for the evaluation of biocontrol potential of chitinases from different sources.

INTRODUCTION

Since the time (mid of sixties) of green revolution, utilization of chemical pesticides is considered as a reliable and beneficial choice for the inhibition of pests (fungi, insects, herbs and rodents). But an excessive and irrational use of pesticides brought unprecedented toxicity and negative effects to soil and ground water all over the world. Some pesticides called as persistent pesticides, bind strongly to soil particles and stays immobile for long time. Cleanup of immobile pesticides from soil and groundwater is tough and costliest effort. Belief on hazardous chemical pesticides for future agricultural purposes would mean further loss in soil quality, possibilities of water contamination and unsustainable burden on the fiscal system. On the other hand increasing use of azole-based agricultural chemicals has been implicated as a factor underpinning the increase in frequency of multiple-triazole-resistant (MTR) isolates of *Aspergillus fumigatus* infecting humans [1,2]. All these complications have driven the search for less or non harmful alternatives for pest control. Many biocontrol agents (bacteria, fungi and their spores, protozoans and plant extracts etc.) with specific fungal and insect targets have been reported. But they have their own limitations such as relatively short shelf life and inconsistent performance during field applications [3].

Chitinases belongs to the class of hydrolytic enzymes with a potential to inhibit or degrade the chitin containing pathogens like fungi, insects and their larva's. The use of chitinases as a bio control agent is one of the attractive and environmentally safe strategies. Potent chitinolytic enzymes irrespective of their production source can hydrolyze the fungal cell wall and integument of insects [4, 5, 6]. Chitin ($C_8H_{13}O_5N$) n is polysaccharide and semi-transparent material present throughout the nature. It is composed of units of *N*-acetylglucosamine (GlcNAc) or NAG, which are linked by β-1, 4 glycosidic bonds. Chitinases occurred in a wide range of organisms including bacteria, fungi, plants, insects, and animals. Chitinases from bacteria and fungi are extremely important for maintaining a balance between the large amount of carbon and nitrogen trapped in the biomass as insoluble chitin

in nature. Chitinases are needed by fungi to disrupt their existing cell walls during cell division and chitinases also played an important role in some plants and essential for the inhibition of fungal diseases. In case of insects and crustaceans, chitinases are associated with degradation of old cuticle. At present, biological control should be viewed seriously as an important component of integrated disease management (IDM), if a permanent reduction of chemical pesticide usage is our goal.

Antifungal and insecticidal mechanism of chitinases

To understand why the chitinases showed different antifungal activity on different pathogenic fungi, Yan et al. [7] elucidated the mechanism by studying the antifungal effect of purified recombinant rice chitinase from *Pichia pastoris*. He observed rice chitinase could efficiently inhibit the growth of *Rhizopus stolonifer* and *Botrytis squamosa* but had no significant inhibitory effect on *Aspergillus niger* and *Pythium aphanidermatum*. Outcome from this study is that chitinase exhibited different spectrum of antifungal activities due to the surface microstructure and the proportion of chitin in the fungal cell wall. Chitinase can easily interacts with chitin present in fungal cell wall when the scale-shaped materials are arranged in a manner allowing exposure of chitin fiber bundles on the surface of fungal cell wall. The more easily chitinase contacts its substrate in the fungal cell wall, then higher the velocity of chitin hydrolysis and inhibition of the phytopathogenic fungi.

The peritrophic membrane and exoskeleton of insects act as physicochemical barriers to environmental hazards and predators. Both are composite materials and made up of chitin, protein, lipids, catecholamine metabolites, minerals, and other minor components. However, some entomopathogenic fungi such as *Metarhizium anisopliae*, *Beauveria bassiana*, and *Aspergillus flavus* have overcome these kinds of barriers by producing multiple extracellular degradative enzymes, including chitinolytic and proteolytic enzymes that help the pathogens to penetrate the barriers and expedite fatal infection [4]. Mosquito-larvicidal activity of chitinase of *Aeromonas hydrophila* SBK1 was dependent on enzyme dose and duration of exposure. Microscopic view revealed that the enzyme lysed the musculatures and internal canals, while exo-skeleton remained intact. The chitinase attacked on internal organs of the mosquito [8].

Biocontrol potential of bacterial chitinases

Over the years, several bacterial species have been investigated for production and characterization of chitinases for their different applications. Chitinases from prokaryotes vary widely in their size, ranging from as low as 20 to about 90 kDa. They are found to be active over a wide range of temperature and pH, depending on the source of bacteria from which they have been isolated [9]. Wang *et al.* [10] isolated the *Bacillus amyloliquefaciens* V656 from the soil of northern Taiwan. This bacterium has the ability to produce two antifungal chitinases (FI and FII) with molecular weight of 14.4 and 16.9 kDa respectively. Antifungal potential of enzymes was observed against *Fusarium oxysporum.* Inhibition of *F oxysporum* was consistent at neutral pH but reduced significantly below pH 5. 0 at 37◦C. *Serratia plymuthica* strain IC14 was isolated from the surroundings of melon roots and noticed for chitinase production. Foliar application of strain IC14 protected the cucumber plant against *Botrytis cinerea* (gray mold) and *Sclerotinia sclerotiorum* (white mold) diseases of leaves under greenhouse conditions. An endochitinase had molecular mass of 58 kDa was speculated to be the main secreted chitinolytic enzyme [11]. *Enterobacter* sp. NRG4 produced high yield, 72.0 and 49 Uml^{-1} of chitinase when it was grown in presence of fungal cell wall of *Candida albicans* and *Fusarium moniliforme* respectively. Further this chitinase showed an inhibitory potential against the growth of hyphal tips of *Aspergillus niger, Fusarium moniliforme, Mucor rouxi* and *Rhizopus nigricans* by disc diffusion method on potato dextrose agar media at pH 5.4, 25◦C after 24h [12]. *Streptomyces coelicolor* A3(2) has 13 chitinase encoding genes, 11 of them were translating chitinases belonging to family 18 and two chitinases belonging to family 19 chitinases. Among them Chi19F inhibited the hyphal extension of *Trichoderma reesei, Trichoderma viride, Mucor javaniccus,* and *Fusarium solani*, significantly on PDA media at pH ~5.5, 30◦C. The antifungal activities of family 18 chitinases were not significant, but Chi18bA slightly inhibited the growth of *Trichoderma reesei, Trichoderma viride, and Mucor javaniccus* [13]. Kamil et al. [14] isolated *Bacillus licheniformis, Stenotrophomonas maltophilia,* and *Bacillus thuringiensis* from the soil of rhizosphere of undisclosed plant. In vitro *Bacillus licheniformis* was the most active bacterium for the suppression of fungal (*Rhizoctonia solani, Macrophomina phasiolina, Fusarium culmorum*, *Pythium* sp*, Alternaria alternata* and *Sclerotium rolfsii*) hyphal growth on PDA media at 28◦C. In green-house experiment *B. licheniformis* was also significantly reduced the damping off disease of *Helianthus annus* caused by *Rhizoctonia solani*. Development of an effective biocontrol approach against Phytophthora blight of pepper, Kim et al. [15] reported three chitinolytic bacteria, *Serratia plymuthica* strain C-1, strongly antagonistic to *Phytophthora. capsici*, *Chromobacterium* sp. strain C-61, strongly antagonistic to *Rhizoctonia solani* and *Lysobacter enzymogenes* strain C-3 antagonistic to *R. solani* and *Fusarium* sp. During pot studies they observed combining of three bacterial strains effectively suppressed *Phytophthora* blight more than application of any single bacterial strain. *Streptomyces* sp. DA11 was isolated from the marine sponge *Craniella australiensis* and investigated in vitro for its antifungal potential against *Aspergillus niger* and *Candida albicans.* Discs contained the purified chitinase were placed on the beef extract peptone plates and incubated with fungi and yeast at 28°C for 5 days. Growth inhibition of fungal hyphae and yeast was appeared around the perimeter of the discs containing purified chitinase [16]. Tu et al. [17] reported the isolation of chitinase

producing *Serratia marcescens* GEI from gut of Chinese honey bee *Apis cerana.* Three chitinase (ChiA, ChiB and ChiC1) genes of *S. marcescens* GEI were expressed by recombinant *Escherichia coli*. Two of them, ChiA and ChiB were acted synergistically against *Varroa destructor* (parasite of western honey bee *Apis mellifera*) with 100% mortality of mite in 5.0 days. Liu et al. [18] isolated *Aeromonas veronii* strain CD3 from pond sediments with an extracellular chitinase production. *A. veronii* CD3 has the potential to utilized myxospores as a carbon source in selective medium. This study emphasized, chitinase from this bacterium has the ability to control the myxozoan disease of fishes by degrading their shell valves. Due to the concerns about food safety issues and limited knowledge of Myxozoa life cycle and fish immune system, no chemicals, antibiotics or immune modulators are available to control myxozoa infection. *Aeromonas hydrophila* SBK1 has the stronger chitinolytic activity and mosquitocidal impact on *Culex quinquefasciatus*. The crude chitinase preparation from *A. hydrophila* SBK1 was tested against *C. quinquefasciatus* larvae. larvicidal effect was highest at 35◦C and pH 7.0. With an increasing of enzyme concentration, mortality percentage was also increased. *C. quinquefasciatus* is a major vector in India as well as in other tropical regions of the world causing 80 million annual lymphatic filariasis of which 30 million cases are chronic [8].

Biocontrol potential of fungal chitinases

From the earlier reports Vyas and Dehspandey [19] evaluated the biocontrol potential of *Myrothecium verrucaria* to control soil-borne plant pathogenic fungi *Sclerotium rolfsi* and *Fusarium* sp. They noticed the release of NAG from dried mycelia of fungi when incubated with chitinase of *M. verrucaria* at pH 5.0 for 1h at 50°C. *Monascus purpureus* CCRC31499 is a mold which produced an antimicrobial chitinase when it was grown on a shrimp and crab shell powder (SCSP). Chitinase of *M. purpureus* CCRC31499 inhibited the growth of *F. oxysporum* and *F. solani* [10]. Crude chitinase extract from *Trichoderma harzianum* showed an antifungal activity against *Aspergillus*, *Rhizopus* and *Mucor* sp. with significant antagonism against *Aspergillus niger* (NCIM 563) when observed on PDA media petriplates [20]. Binod et al. [21] evaluated the larvicidal potential of chitinase from *Trichoderma harzianum* against the *Helicoverpa armigera* (pest of cotton crop). Chitinase of *T. harzianum* worked as an effective antifeedant as it reduced the feeding rate and body weight of the larvae. Goettel et al. [22] reported fungi belong to the genus *Lecanicillium* (earlier classified as the single species *Verticillium lecanii*) are prominent insect pathogens and some have been used as commercial biopesticides in agricultural practices. *Lecanicillium* spp. uses both mechanical forces and hydrolytic enzymes like chitinases for penetration to the insect integument. Mishra et al. [23] isolated the chitinase producing fungi (*Conidiobolus coronatus* NFCCI 1235, C*. couchii* NFCCI 719, *C. coronatus* NFCCI 718, *Basidiobolus haptosporus* NFCCI 1922 and *Basidiobolus haptosporus* NFCCI 1923) of two different genera *Conidiobolus* and *Basidiobolus*. These genera belong to the saprophytic entomophthorales order of fungi i.e. most neglected group of mycological research. *Basidiobolus haptosporus* NFCCI 1922 utilized mycelia of tested fungal isolates as a major carbon source for the production of chitinase. Significant hydrolysis of fungal mycelia was observed with mycelium of *Aspergillus niger* after 72 h incubation. Recently, Kumar et al. [24] noticed the mycelial growth inhibition of *Fusarium oxysporum, F. ciceri, F. solani* and *F. udum* by chitinase from *Trichoderma asperellum* UTP-16 during well diffusion assay method. Antifungal activity of *Aspergillus niger* LOCK 62 was noticed on Petri plates made of Czapek Dox medium at 25◦C for 72h. Both crude and purified chitinases of *A. niger* LOCK 62 showed growth antagonism against *Fusarium culmorum*, *Fusarium solani*, and *Rhizoctonia solani* [25].

Biocontrol potential of plant chitinases

Plants do not contain immune system and thus are vulnerable to pathogens, resulting in significant crop loss globally. In order to protect themselves from pathogens, plants have evolved a number of defense responses that are elicited during their life cycle in response to developmental signals and pathogen attack. Plants expressed a wide variety of pathogenesis-related (PR) protein encoding genes of which the best characterized are those encode the chitinase [26]. Roberts and Selitrennikoff, [27] observed the difference between plant and bacterial chitinases on the basis of their antifungal activity. Chitinases were extracted from the grains of wheat, barley and maize and compared with chitinases from *Serratia marcescens, Streptomyces griseus* and *Pseudomonas stutzeri* for antifungal potential by inhibition of hyphal extension of *Trichoderma reesei* and *Phycomyces blakesleeanus.* Fungal hyphal inhibition was noticed with as little as 1 µg of each of the grain chitinases, whereas none of the bacterial chitinases had any effect on hyphal extension even at 50 µg chitinase used per assay. One of the plant chitinase was purified from yam, *Dioscorea opposita* and first time used as biofungicide against the control of *Sphaerotheca humuli* causative fungus of powdery mildew disease of strawberry plant. During the microscopic and eye investigation it was found that *S. humuli* was degraded by the spray of plant chitinase and disease did not appear again for more than two weeks [28]. A 30-kDa thermostable chitinase was extracted from the pericarpial portion of *Ficus awkeotsang* inhibited the spore germination of *Colletotrichum gloeosporioides* (common post-harvest pathogen of jelly fig). Chitinase gene from wheat plant was subcloned and expressed in *Escherichia coli*. Purified (33 kDa) recombinant wheat chitinase exerted (at 100µg conc.) a broad-spectrum antifungal potential against *Colletotrichum falcatum* (red rot of sugarcane) *Pestalotia theae* (leaf spot of tea), *Rhizoctonia solani* (sheath blight of rice), *Sarocladium oryzae* (sheath rot of rice) *Alternaria* sp. (grain discoloration of rice) and

Fusarium sp. (scab of rye). Under light microscope hyphae of inhibited fungi showed mycelial deformations such as poorly developed mycelium with swollen margins [26].

Biocontrol potential of insect chitinases

A 46 kDa *Manduca sexta* (tobacco bornworm) chitinase was isolated from the leaves of transgenic tobacco plants. Chitinase encoding gene of *M. sexta* was expressed in leaves, flowers, stems and roots. To determine the biocontrol potential of this chitinase, administered orally at a 2.0% concentration, caused 100% larval mortality of the merchant grain beetle, *Oryzaephilis Mercator* [29]. Purified chitinase (75kDa) from *Bombyx mori* was evaluated for its biocontrol potential against the Japanese pine sawyer (JPS) (belongs to genus of longhorn beetles) *Monochamus alternatus*. Oral ingestion of purified chitinase (11µg/50µl) caused high mortality in JPS as well as significant decrease in feed consumption and slight reduction of body weight. Fluorescence and microscopic observations confirmed that chitin present in peritrophic membrane of JPS was degraded by the action of orally ingested purified chitinase [30]. Zhang et al. [31] purified the chitinase from *Bombyx mori* (silkworm) 88 kDa and *Helicoverpa armigera* (bollworm) 75 kDa and compared their antifungal potential on PDA plates at 30^{O}C for 72 h. *Saccharomyces cerevisiae* and *Penicillium*, were significantly inhibited by *H. armigera* chitinase. Whereas *B. mori* chitinase was found to be relatively less effective inhibitor of spore germination in case of *Penicillium* sp.

Synergism between chitinases and Bacillus thurigiensis (Bt) as a potent environmentally safe anti insects biocontrol

Bacillus thuringiensis is a gram positive spore forming bacterium that forms parasporal (toxic proteins around the spores) crystals during sporulation. A diverse range of crystallized toxic proteins are produced by different strains of *B. thuringiensis* that acted as an insecticidals for different species of insects larvae. The insecticidal potential of crystal proteins can be enhanced by synergistic action between crystal proteins and chitinase enzyme [4]. An increased toxic effect towards *Spodoptera littoralis* was noticed when a combination of low concentrations of a truncated recombinant Bt toxin and *Serratia marcescens* endochitinase were incorporated into insect diet [32]. Crude chitinase preparations from *B. circulans* enhanced the toxicity of Bt *kurstaki* towards diamondback moth larvae [33]. Guzzo and Martins [34] reported the application of a commercial formulation of Bt to coffee leaves caused local and systemic inductions of both chitinase and β-1,3-glucanase. Two- to threefold increase in enzyme activities were observed. Liu et al. [35] reported three *B. thuringiensis* strains with chitinolytic activity can increase the insecticidal toxicity of *B. thuringiensis* DL5789 against *Spodoptera exigua larvae*. Arora et al. [36] noticed chitinase was produced constitutively by *Bacillus thuringiensis* HD-1A and purified (36 kDa) from the culture supernatant. The chitinase from *B. thuringiensis* HD-1A potentiated the insecticidal activity of insecticidal protein (Vip) when used against neonate larvae of *Spodoptera litura*. They observed potentiation of insecticidal activity of Vip in the presence of chitinase offers environmentally safe option to enhance the application of Bt proteins. Ding et al. [37] reported the co-fusion of *cry1A*c (encoding insecticidal toxin) gene of *B. thuringiensis* and tobacco (*Nicotiana tabacum*) endochitinase recombination is a promising approach to introduce co-fused genes into the *B. thuringiensis acrystalliferous* strains for the insecticidal control of *Helicoverpa armigera Hubner.*

Synergism between chitinase and glucanase as a possible potent environmentally safe antifungal biocontrol

The cell wall of fungi is complex composition of chitin, 1, 3-β- and 1,6-β-glucan units, although fungal cell wall components frequently varies markedly between species to species of fungi. [38]. Mauch et al. [39] purified the chitinase and *β*-1, 3-glucanase from fungal (*Fusarium* sp.) infected peas. Prompt lyses of pathogenic fungal hyphal tips were observed by the combined application of chitinase and β-1, 3-glucanase as compared to the application of chitinase or glucanase alone. Arlorio et al. [40] reported chitinases and β-1,3-glucanases works synergistically for the inhibition of *Trichoderma longibrachiatum* hyphal growth as evidenced by fluorescence and electron microscopy. There was a dilution of chitin and glucan from the hyphal apex of *Trichoderma* cell wall which causes the tip to swell and bursting. Arora et al. [41] observed the inhibition of fungal plant pathogens *Phytopathora capsici* and *Rhizoctonia solani* by *Pseudomonas* GRC3 as a result of collective antifungal impact of chitinase and β-1,3-glucanase.

Conclusion and futuristic considerations

Appropriate and target specific inhibition of harmful pests (phytopathogenic fungi and undesirable insects) depends upon the availability of highly active and stable preparations of chitinases, but with an economical cost. In future, advanced protein engineering may have open the door of new possibilities of generating the chitinases with robust functioning as a biocontrol agent even at harsh and extreme environmentally conditions [42]. The synergistic mechanism of chitinases with glucanases against fungi biocontrol is well-established approach. But still there is a need of dedicated and committed research efforts to understand, how to prepare effective and highly stable formulations of both catalysts that should be more progressive in fugal plant disease control.

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