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BIOCONTROL POTENTIAL OF FUNGAL CHITINASE FROM HIGH YIELDING *TRICHODERMA VIRIDE* AGAINST *CORCYRA CEPHALONICA* (Stainton)

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ABSTRACT

In this study we reported the screening of ten different fungal isolates for higher chitinase yield and its effect against the stored pest *Corcyra cephalonica* as natural bio-control agent without toxic effects. Different fungal strains were cultured and screened the chitinase activity for the production of chitinase enzyme. The majority of the farmers profoundly rely on synthetic pesticides for the eradication of stored food pests. These synthetic pesticides are leaving a very large amount of toxic residue in the environment which is harmful to human beings. Due to these toxic effects of synthetic pesticide we attempted to find the biological control agent against stored pests. From the fungal populations, ten different fungal species were used to screen for the higher production of chitinase. The chitinase was partly purified by ammonium sulfate precipitation method and was evaluated for its anti-feedant and growth inhibitory activities against the stored food pest *Corcyra cephalonica* Stainton at 62.5, 125, 250, 500 and 1000 ppm enzyme concentrations. *Trichoderma viride* shows a maximum chitinase activity of 2.75±0.029 $U.mL^{-1}$ colloidal chitin on the sixth day. Next to *Trichoderma viride, Metarhizium anisoplea* and *Candida albicans* shows a chitinase activity of 2.51±0.031 and 2.23±0.070 $U.mL^{-1}$ Colloidal chitin respectively. Treatment of *Corcyra cephalonica* with the partially purified chitinase from *Trichoderma viride* reduced the normal larval development according to a dose-dependent manner. This study shows *Trichoderma viride* chitinase can affect the growth of *Corcyra cephalonica* larvae. The outcome necessitates further insect bioassays and detection of underlying mechanisms.

KEYWORDS: ***Corcyra cephalonica, Trichoderma viride,*** **DNS method, Biopesticide, Colloidal chitin**

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INTRODUCTION

Nowadays, synthetic pesticides are applied in agricultural segment (pre/post harvesting) for protecting the crops and grains from the harmness of insect pests[1-2]. The continuous use of these pesticides also results in momentous detrimental effects to public health and the environment. In general, the quantity of pesticides released into the environment which has risen drastically in the last few decades. On the other hand, the researchers continually try to find for an effectual and eco-friendly technique of controlling insect pests[3]. *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae), well-known as "rice moth", is one of the most widespread and serious insect pest of stored food grains which grounds to damage rigorously. The significance of *C. cephalonica* is that they develop more quickly on whole grains and in powder forms. The entire developmental period of *C. cephalonica* ranges is about 47-59 days and it infests depending on the products. Within this short span of life, *C. cephalonica* makes a sturdy economic loss. For the abolition of this alarming insect pest, synthetic pesticides such as phosphine fumigants and methyl bromide are considered as the most proficient treatment experienced for preceding decades. On the other hand, these pesticides have ozone-depleting nature[4-5], develop pest resistance by distracting biological system[6]. Scientific world is in hunt of alternative measures to lessen insect influx. It is projected that about 2.5 million tons of pesticides are used on crops per annum leading to $100 billion damage worldwide due to its elevated toxicity and non bio-degradable properties[7]. For this reason, a lot of the synthetic pesticides have been banned or are under assessment. Chitinases (E.C 3.2.2.14) are hydrolytic enzymes with a wide size varying from 20 kDa to 90 kDa[8]. They are present in almost all the organisms such as actinomycetes, arthropods, bacteria, fungi, humans, plants and yeasts. Chitin is the second abundant biopolymer present in the earth, next to cellulose. Chitin can be degraded to low molecular weight chito-oligomers by chitinases[9], which serve a broad range of biotechnological applications, agricultural, industrial and as therapeutics (anti-inflammatory drug for the treatment of ulcerative colitis and other gastrointestinal inflammation disorders)[10]. Especially, chitinases received an increasing attention in agricultural fields as a potent biocontrol agent against fungal phytopathogens and dreadful insect pest[3, 1]. An immense perceptive of the biological roles of different chitinases would aid to develop food preservatives and in the same way, increasing the shelf life of the foods[8]. Fed-batch fermentation, liquid batch fermentation and continuous fermentation were mainly employed for the microbial chitinase production[11]. Extracellular chitinase production was influenced by media components (micro and macro nutrients) such as nitrogen sources, carbon sources and agricultural residues such as rice bran and wheat bran[12].Ten different fungal strains are screened which secretes to a large amount of extracellular chitinase and chitinase from the fungal strain which shows a higher chitinase activity was partially purified. In the present study, chitinase from *Trichoderma viride* (MTCC No. 5532) was partially purified and tested against *C. cephalonica*.

MATERIAL AND METHODS

Culture and maintenance of fungal strains

Fungi isolates *Ampelomyces quisqualis* (MTTC No. 5683), *Aspergillus fumigatus* (MTCC No. 3376), *Aspergillus nidulans* (MTCC No. 6599), *Aspergillus terreus*, *Candida albicans* (MTCC No. 3017), *Metarhizium anisopliea* (MTCC No. 4104), *Penicillium oxysporum, Trichoderma harzianum* (MTCC No. 10730), *Trichoderma virens* (MTCC No. 5661) and *Trichoderma viride* (MTCC No. 5532) obtained from the Biogenix Research Center, Thiruvananthapuram, Kerala, India, were used in the present study. These cultures were maintained on Potato-Dextrose Agar (PDA) medium and sub cultured every bi-weekly. Fungi were grown on PDA slants at 37 ± 2 °C for 8 days. After sporulation, the spores were collected using sterile distilled water which contains 0.1% Tween-20 and which acts as the innoculum. The spores collected from different fungal isolates were inoculated to potato-dextrose broth (PDB) which contains chitinase production enhancing micro and macro nutrients (K_2HPO_4, 1 $g.L^{-1}$; $MgSO_4$, 0.5 $mg.L^{-1}$; $FeSO_4.7H_2O$, 0.2 $mg.L^{-1}$; $ZnSO_47H_2O$, 1 $mg.L^{-1}$; $Na_2MoO_4.2H_2O$, 0.02 $mg.L^{-1}$; $CuSO_4.5H_2O$, 0.02 $mg.L^{-1}$ and $MgCl_2.6H_2O$, 0.02 $mg.L^{-1}$) along with 2 g of colloidal chitin as a substrate in an Erlenmeyer flask[13].

Preparation of Colloidal chitin

The substrate (Colloidal chitin), was modified from the method of Sandhya *et al* (2004)[14] as described below. Ten grams of commercially available chitin flakes (HiMedia) were added with 250 mL of concentrated HCl and kept overnight under stirring (300 rpm, 4 °C). The mixture was added with 600 ml of pre-chilled double distilled water by constant mixing and allowed to settle down the precipitate. The precipitate was taken and centrifuged (13, 000 rpm, 10 min, 4 °C). The suspension was washed by pre-chilled distilled water until colloidal chitin acquired a neutral pH.

Preparation of crude chitinase enzyme

For the crude enzyme preparation, the fungal isolates were grown in the medium having chitinase production enhancing micro and macro nutrients along with the substrate colloidal chitin. These were cultured in 100 mL medium in 250 mL Erlenmeyer flask and incubated under shaking condition for eight days. The cultured fluids were centrifuged at 12, 000 rpm, 10 min at 4 °C. From the collected supernatant, 1 mL was taken and added with 1 mL of 10% wet colloidal chitin in phosphate buffer (0.2 M, pH 6.5). The above mentioned suspension was placed in a boiling water bath (50 °C) for 1 hr. After the incubation, 1 mL of 1% NaOH was added, mixed thoroughly and centrifuged (6000 rpm, 10 min, 4°C). The supernatant was allowed for ammonium sulphate precipitation, allowed to stand overnight. The pellet was centrifuged (10, 000 rpm, 4 °C, 20 min). Then the precipitate were dissolved in small amount of 20 mM citrate phosphate buffer (pH 7.8) and dialyzed against the same buffer. This partially purified chitinase was stored in -20°C and it was used for further assays.

Estimation of chitinase enzyme

Di-nitro salicylic acid method (DNS) was employed to make sure the chitinase activity. The reaction mixture contained 1 mL of enzyme solution and 1 mL of 10% colloidal chitin in 0.2 M phosphate buffer (pH 6.5) of different fungal strains independently. After incubation (50 °C, 30 min), the mixture was positioned in a boiling water bath for 5 min and centrifuged at 3000 rpm to get rid of non-degraded chitin and the supernatant was used for further assays. An equivalent volume of the supernatant and DNS solution (v/v) were mixed together and located in a boiling water bath for 15 min at 50 °C. After cooling to room temperature the absorbance was read at 535 nm using spectrophotometer (Agilent Cary 60)[14]. The activity was calculated from a standard curve using known concentration of N-acetyl glucosamine (GluNAC). One enzyme unit was defined as the amount of enzymes that produces 1 µmol of reducing sugar per hour under reaction condition.

Insect Bioassay

The *C. cephalonica* eggs were obtained from Department of Entomology, Tamil Nadu Agricultural University, Coimbatore, India. *C. cephalonica* were raised under laboratory conditions (28±1°C; 65% relative humidity; 12 h light/12 h dark cycle) and inoculated on a medium (pulverized pearl millet, broken up groundnut kernel, yeast, sulphur along with antibiotic) with slight alteration[15-16]. The food media were subjected to UV-exposure in a laminar air flow chamber prior than inoculating egg. Bioassays were succeeded with the IV instar *C. cephalonica* larvae by means of sorghum grains incorporated with 62.5, 125, 250, 500 and 1000 ppm of crude chitinase. Control larvae received only in sterilized distilled water. A group of 10 larvae per concentration was used for all the treatments with three replicates. The larvae were starved for three hours before feeding with the crude chitinase integrated pearl millet grains. Deformities in the larvae were recorded.

RESULTS

A total of 10 different fungal strains were screened for a hyper producing culture of chitinase (Figure 1). The fungus grew copiously and consistently all the way through the potato-dextrose medium supplemented with micro and macro nutrients along with colloidal chitin as a substrate. It is known that a perfect substrate in any fermentation process consequences in elevated conversion efficiencies and optimum substrate utilization[17].

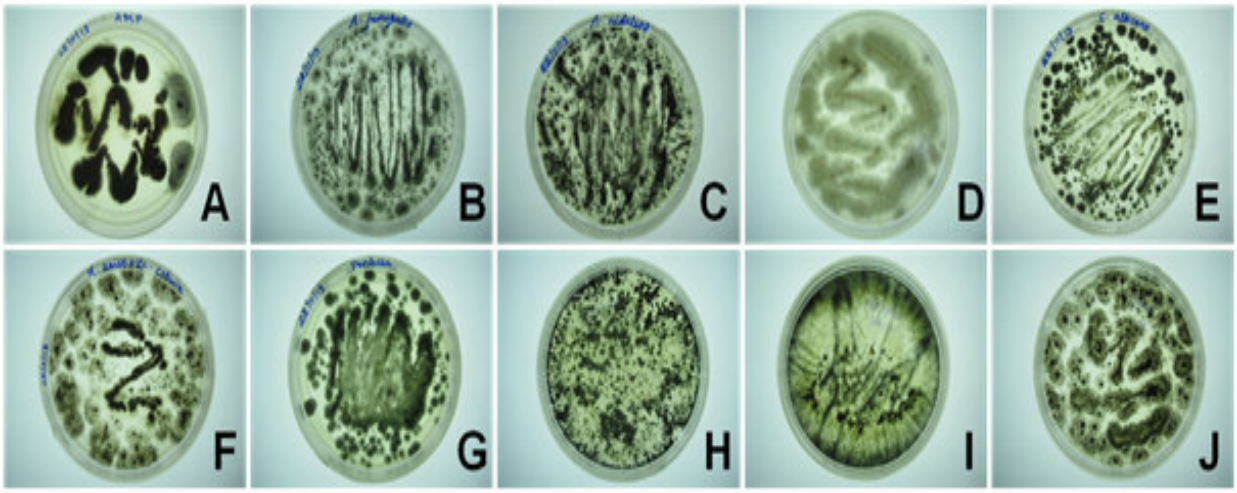

Figure 1
Photomicrographs representing the ten different fungal strains selected for screening

(A) *Ampelomyces quisqualis* (B) *Aspergillus fumigatus* (C) *Aspergillus nidulans* (D) *Aspergillus terreus* (E) *Candida albicans* (F) *Metarhizium anisopliea* (G) *Penicillium oxysporum* (H) *Trichoderma harzianum* (I) *Trichoderma virens* (J) *Trichoderma viride*

Chitinase activity was checked colorimetrically for the partially purified enzyme using colloidal chitin as a substrate. The chitinase activity of fungi selected for the present investigation is presented in the Table 1. *T. viride* was chosen for its capability to produce chitinase at elevated levels on the sixth day. When growing in the chitinase production enhancing medium (micro and macro nutrients) along with the suitable substrate colloidal chitinase, produced extracellular chitinase from two to eight days. The chitinase production increased from 2.536±0.030 to 2.75±0.029 U.mL^{-1} Colloidal chitin. Maximum activity was obtained after 3 and 4 days of incubation with no significant difference after which it decreased strikingly. The chitinase production showed growth relatedness as the incubation period progressed and maximum enzyme production was observed after 7 days. Growth rate and enzyme production pattern were generally dependant on the duration of incubation. After 7 days the production started to decrease as the growth of the organism might have reached a stage, from which it could no longer balance its steady growth with the availability of nutrient resources. Next to *T. viride*, *M. anisopliea* and *C. albicans* showed good chitinase activity on the sixth day of 2.51±0.031 and 2.23±0.070 U.mL^{-1} Colloidal chitin.

Table 1
Chitinase activity of crude extract of ten different fungal strains

Organisms	Chitinase Activity (U.mL^{-1} Colloidal chitin)			
	Second day	Forth day	Sixth day	Eighth day
Ampelomyces quisqualis	1.16±0.015	1.62±0.015	1.62±0.0550	1.806±0.0134
Aspergillus fumigatus	0.59±0.02	0.8±0.026	0.77±0.0584	0.94±0.049
Aspergillus nidulans	0.34±0.006	1.97±0.001	2.12±0.084	1.863±0.0138
Aspergillus terreus	1.036±0.018	1.31±0.028	1.773±0.035	1.85±0.01
Candida albicans	**0.156±0.018**	**2.45±0.038**	**2.23±0.070**	**2.18±0.0134**
Metarhizium anisopliea	**0.77±0.028**	**2.296±0.023**	**2.51±0.031**	**2.3±0.005**
Penicillium oxysporum	0.543±0.016	0.33±0.031	0.693±0.008	1.09±0.071
Trichoderma harzianum	2.083±0.021	1.713±0.02	1.636±0.051	1.74±0.021
Trichoderma virens	0.833±0.010	1.396±0.028	1.636±0.016	2.023±0.028
Trichoderma viride	**2.536±0.030**	**2.74±0.037**	**2.75±0.029**	**2.176±0.020**

*Each data represents the means ± S.E. of three replicates.

Mortality/ malformations of larvae because of the exposure to chitinase were monitored from the first day of the treatment itself. The larvae exposed to chitinase were found to be very lethargic, restraining themselves from feeding and appreciably reduced the growth of the larvae which was proportionate to the concentration of chitinase treated (Figure 2). The partially purified chitinase from *T. viride* did not show any mortality in the IV instar larvae. It was noted that the treated larvae were physiologically affected and malformed when compared to control larvae. As a result of the integration of the feed with that of partially purified chitinase, the percentage of pupation in larvae decreased in a dose-dependent manner. The larvae fed with chitinase incorporated feed showed delayed development, morphological and body color alterations (Figure 2B, C, D, E and F) in contrast with that of the control ones. The control larvae performed better in terms of growth as pointed out by the body weight and color (Figure 2A). It might be hypothesized that feeding on chitinase incorporated feed might have led to the obliteration of gut lining (peritrophic membrane), which eventually lead to the larvae being not able to feed well and leads to slow growth rate. In certain larvae the pupation was occurred and it was not properly pupated and finally it undergoes die (data not shown). Taken as a whole, the results had given clear cut validation that in an elevated dosage of chitinase, the feeding of larvae was reducing and in that way it leads to inappropriate pupation and thus the possibility of the next generation.

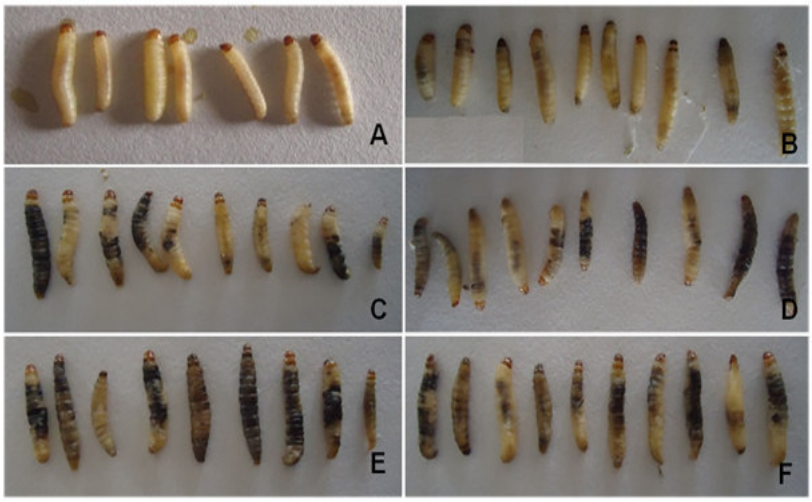

Figure 2
Effects of partially purified chitinase from *Trichoderma viride* on IV instar *Corcyra cephalonica* larvae
(A) Control (B) 62.5 ppm (C) 125 ppm (D) 250 ppm (E) 500 ppm (F) 1000 ppm

DISCUSSION

Enzymes are commonly used in numerous eco-friendly industrial sectors. With the development in biotechnology, particularly in the area of protein engineering which paved the way to a new epoch of application of enzymes in various industrial progressions for instance in agricultural sectors [11]. As per Pandey *et al.,* (1999)[18], as an alternative of animals/ plants, bacteria and fungus are generally preferred as an excellent enzyme source, because the enzyme content of micro-organisms are more predictable and controllable and of low production costs. Chitinase is an enzyme which is able to hydrolyze insoluble chitin monomeric/ oligo components. This enzyme is present in both higher and smaller organisms such as animals, plants, bacteria and fungus as their natural protectant[19]. Microbial chitinase has been produced by liquid batch fermentation, continuous fermentation and fed-batch fermentation. Chitinase produced from microorganisms is inducible in nature. Bushan (1998)[12] and Dahiya *et al.,* (2005)[20] described that the extracellular production of extracellular chitinase is mainly influenced by the macro/ micro nutrients and the suitable substrate that we are supplying. A lot of strains of the genera *Aspergillus* [21-24], *Candida*[25], *Pencillium*[26], *Metarhizium*[27] and

Trichoderma[28] have been isolated and checked for their chitinase activity. Preceding researches evidenced that the production of chitinase under submerged state by means of colloidal chitin as a sole source of carbon[29]. Chitin and chitinolytic enzymes such as chitinase are in advance for their biotechnological applications, in particular chitinase in the field of agriculture to control plant pathogens and insect pests[30, 8]. From the previous studies it was clear that chitinase from microorganisms has been used as a potent insecticide[31, 2]. The success in make use of chitinases for different facets depends on the delivery of extremely active products at rational cost. The exoskeleton and gut linings of insects were made up of chitin. Chitinase, an insect molting enzyme, has been described from *Bombyx mori, Corcyra cephalonica, Manduca sexta* and quite a lot of other species[30]. In our study, larvae exposed to the partially purified chitinase from *T. viride* showed some malformalities because they were restrained from feeding. Chandrasekaran *et al.,* (2012)[2] isolated and purified the chitinase from *Bacillus subtilis* and showed that it has an elevated insecticidal property, even at lower concentration of 6 µM within a time period of 48 hr against the *Spodoptera litura* Fab. Chitinase shared a major role in the morphogenesis of insects[11]. There have been many research reports on the degradation of insect gut lining (peritrophic membrane). The perforations caused by chitinase in the gut lining aids to the entry of pathogens into tissues and hemocoel of susceptible insects[32]. Similarly Ragev *et al.,* (1996)[33] proposed that ChiAll, a recombinant endochitinase encoded by *Serratia marcescens* incorporated leaves were fed to V instar *Spodoptera litura* also exhibited perforations in the gut lining and thereby reduced growth.

CONCLUSION

The present study carried out for checking the effectiveness of partly purified chitinase from *T. viride* as a biocontrol agent against the stored food pest *C. cephalonica.* The rice moth shows that the chitinase has the capacity to affect the growth and metamorphosis of the larvae; when use it as an antifeedant. It can be hypothesized that the adults rising from chitinase-treated larvae possibly unusual and incapable of normal life.

CONFLICT OF INTEREST

Conflict of interest declared none.

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