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Microbial Synthesis of Chitinase in Solid Cultures and Its Potential as a Biocontrol Agent Against Phytopathogenic Fungus *Colletotrichum gloeosporioides*

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Abstract

Antifungal activity of chitinase can be effectively utilized in biologic pest control strategies. Because solid-state cultivation has been termed a cost-effective means for fungal growth and metabolite production, chitinase production by *Trichoderma harzianum* was studied using wheat bran-based solid medium containing 1% colloidal chitin. Chitinase synthesis was found to be growth associated because maximum enzyme (5.4 U/g of dry substrate) and biomass production occurred at 72 h. Substrate moisture had a critical impact on chitinase production; five grams of medium having an initial moisture content of 68.4% when incubated for 72 h increased the enzyme yield to 9.3 U/g of dry substrate. Optimization of colloidal chitin concentration showed that improvements in chitinase yield and maximum activity were attained with a 2% (w/w) concentration. Supplementation of additional nitrogen sources also influenced enzyme production, and the best yield was obtained with yeast extract. The effect of crude chitinase on hyphal morphology of the phytopathogenic fungus *Colletotrichum gloeosporioides* was swelling as well as lysis of hyphal wall, depending on the age of the mycelium. Studies of pH and thermal stability showed that crude culture filtrate was

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active over pH 4.0–6.0 and retained about 48.2% activity after 40 min of incubation at 40°C.

Index Entries: Biologic control; chitinase; solid-state cultivation; antifungal activity; pH stability; thermal stability.

Introduction

Plant protection is a major challenge to the agricultural field worldwide, with fungi being one of the main causes of significant loss of yield. Reliance on chemical pesticides/fungicides as the primary control strategy has resulted in serious environmental problems, such as the destruction of ecosystems and pollution in various levels of the food chain, and has been widely criticized in recent years. The public concern about the safety of these chemicals has also led to more rigorous regulatory requirements. The removal of these chemical pesticides/fungicides from the market, coupled with the emergence of herbicide- and insecticide-resistant pests, has heightened interest in the development of biologically based pest control strategies *(1)*.

Because pathogenic fungi and insects contain chitin in their protective covers, induction of chitinases is the main defense response in plants. Chitinases (EC 3.2.1.14) belong to a group of enzymes capable of degrading chitin directly to low molecular mass products. The enzymatic degradation of chitin appears to occur in two steps, which are similar in prokaryotes and eukaryotes *(2)*. An endochitinase reduces the polymer to oligomers, which are subsequently degraded to monomers by exochitinase; this leads to growth inhibition and death of microorganisms. Moreover, the chitin oligomers released are positively charged and possess, among others, antibacterial and antimutagenic properties.

Chitinases have been detected in bacteria; fungi; plants; and the digestive systems of coelenterates, nematodes, polychaetes, mollusks, and arthropods. In a recent review, Sandhya et al. *(3)* discussed the importance of microbial chitinases as biocontrol agents, strategies for improving their production, and the mechanism for their activity. Chitinase-producing organisms could be used directly in biologic control of fungi, or indirectly by using purified protein, or through gene manipulation *(4)*. The success of biologic control depends on large-scale production, along with shelf life and the establishment of bioagents against a specific target. Although intensive activity toward the development of biologic control is taking place, commercial products are few and costly. Thus, one of the important issues related to chitinase synthesis is the reduction of production costs.

Solid-state fermentation is a low-cost fermentation technology process utilizing the agroindustrial residues generated in large quantities throughout the world, such as wheat bran, rice bran, oil cakes, and corncob, in which generally filamentous fungi are exploited for their ability to grow on solid substrates under low moisture conditions and produce extracellular enzymes, among several other products *(5–9)*. Currently, most of the

agroindustrial residues are either not utilized or underutilized. Better utilization of agroindustrial residues by biotechnological means assumes social, economic, and industrial importance. Matsumoto et al. *(10)* and Rattanakit et al. *(11)* employed solid-state fermentation for utilizing waste chitinous materials as substrates for chitinase production. Nampoothiri et al. *(12)* reported chitinase production in solid-state fermentation using wheat bran as substrate. However, not much information is available on solid-state fermentation for fungal chitinase synthesis.

Trichoderma sp. is known to have extensive growth and sporulation in a variety of solid substrates such as cereal grains, meal, brans, straw, and plant residues. Among these, *Trichoderma harzianum* is used as a biologic control agent against a wide range of economically important airborne and soilborne plant pathogens *(3,13–16).*

The objective of the present work was to study the synthesis of chitinase by *T. harzianum* TUBF 691 under solid-state fermentation and optimize process parameters for its enhanced production. Studies were also carried out to investigate the in vitro antifungal activity of crude chitinase against phytopathogenic fungi and its characterization for pH and thermal stabilities.

Materials and Methods

Microorganism and Preparation of Inoculum

T. harzianum TUBF 691 was obtained from Technical University of Budapest, Hungary. The microorganism was maintained on potato dextrose agar slants and subcultured regularly every 2 wk. The spores from a fully sporulated slant were dispersed in 10 mL of 0.1% Tween-80 by dislodging them with a sterile loop under aseptic conditions. The spore suspension was used as inoculum. Viable spores present in the suspension were determined by serial dilution followed by plate count (which showed 2×10^{10} spores/mL).

Preparation of Colloidal Chitin

Colloidal chitin was prepared as described previously *(17).*

Solid-State Fermentation

Five grams of wheat bran (of mixed particle size ranging between 100 and 400 μm) containing 1% (w/w) colloidal chitin was moistened with salt solution and water to achieve the desired initial moisture content in a 250-mL Erlenmeyer flask. The substrate after thorough mixing was autoclaved, cooled, and inoculated with 1 mL of spore suspension and incubated at 30°C for a desired period of time.

Enzyme Extraction

One hundred milliliters of distilled water containing 0.1% Tween-80 was added to fermented substrate and mixed on a rotary shaker at 180 rpm

for 1 h. The mixture was then centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was collected and used for chitinase assay.

Chitinase Assay

Chitinase assay was based on the estimation of reducing sugars released during the hydrolysis of colloidal chitin. The reaction mixture, containing 0.5 mL of the crude enzyme, 0.5 mL of 1% colloidal chitin, and 1.0 mL of citrate phosphate buffer (pH 5.6), was kept in a water bath at 50°C for 10 min. Reducing sugars released were estimated by the method of Miller *(18)*. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of reducing sugars/(mL·min) under the reaction conditions. All the experiments were done in triplicates, and the data are presented as the mean value ± SD.

Determination of Fungal Biomass

The fungal biomass in solid medium was estimated by determining the *N*-acetyl glucosamine released by acid hydrolysis of chitin present in the cell wall of the fungi *(19)*. Glucosamine released from the chitin by acid hydrolysis was mixed with 1 mL of acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, ethanol (6 mL) was added followed by the addition of 1 mL of Erlich's reagent and incubated at 65°C for 10 min. After cooling, the optical density at 530 nm was taken against a reagent blank. *N*-acetyl glucosamine was used as a standard.

Estimation of Mycelial Chitin Content

A phytopathogenic fungus, *Colletotrichum gloeosporioides*, was cultured on potato dextrose broth (PDB) and mycelia of different ages were harvested, washed thoroughly with distilled water, and dried at 60°C. Chitin content was estimated as described for the estimation of fungal biomass.

Influence of Solid-State Fermentation Parameters on Chitinase Production

Different incubation periods (24, 48, 72, 96, 120 h) were employed to study the growth and chitinase production by *T. harzianum*. Optimum initial moisture content was determined by varying the volume of distilled wate–Üò | the medium so as to achieve a 50–80% initial moisture level. To optimize colloidal chitin concentration, solid-state fermentation medium was supplemented with different concentrations of colloidal chitin (0.5, 1.0, 1.5, 2.0, and 2.5% [w/w]). The effect of additional nitrogen sources was studied by adding organic and inorganic nitrogen sources (yeast extract, malt extract, tryptone, peptone, urea, corn steep solid, ammonium nitrate, ammonium sulfate, and diammonium hydrogen phosphate) at a 1% (w/w) level.

In Vitro Antifungal Assay

Phytopathogenic fungus (*C. gloeosporioides*) was grown in culture tubes (20 × 150 mm) containing 2 mL of PDB by incubating at 30°C and 130 rpm for 24, 48, and 72 h. PDB containing fungal mycelia of different ages was treated with crude chitinase (14.4 U/g of dry substrate). The resultant media was then incubated at 30°C for 24, 48, and 72 h. The variation in hyphal morphology was observed using a light microscope (Leica DMLS) at respective intervals of time with a magnification of ×40. Simultaneously, a control was carried out by treating the fungal mycelia with heat-killed enzyme (100°C for 15 min).

pH and Thermal Stability of Crude Chitinase Preparation

The pH stability of the crude enzyme was studied by incubating the enzyme in different buffers of varying pH (glycine HCl buffer: pH 2.6 and 3.6; acetate buffer: pH 4.2, 4.8, and 5.4; citrate phosphate buffer: pH 4.6, 5.6, and 6.6; phosphate buffer: pH 6.0, 7.0, and 8.0; glycine NaOH buffer: pH 9.0 and 10.6) at 4°C for 24 h and measuring the residual chitinase activity at pH 5.6 (assay pH). The thermal stability was determined by heating the crude enzyme at temperatures ranging from 30 to 70°C for different intervals of time up to 1 h using citrate phosphate buffer (0.1 *M*, pH 5.6). After heating for the required time, an aliquot of the enzyme was estimated for residual chitinase activity.

Results and Discussion

Effect of Incubation Time on Chitinase Production

Figure 1 presents the results of biomass growth and chitinase production by *T. harzianum* TUBF 691. A gradual increase in chitinase production was observed in solid-state fermentation medium having an initial moisture content of 61.8% from 48 h onward, and maximum activity was attained after incubating for 72 h (5.4 U/g of dry substrate). However, longer periods of cultivation resulted in a decrease in chitinase activity. Chitinase production by fungal culture generally has been found to be maximum between 72 and 96 h by other investigators as well. For example, Suresh and Chandrasekharan *(20)* reported 72 h as the optimum period for maximum chitinase production by the marine fungus *Beauveria bassiana* in a wheat bran–based medium. In addition, Nampoothiri et al. *(12)* reported 96 h as the optimum cultivation period for chitinase production by *T. harzianum* TUBF 781. There are several other reports describing maximum enzyme titers in 72–96 h from filamentous fungi grown in solid-state fermentation using agroindustrial residues for other enzymes also, such as glucoamylase *(21)*, α-amylase *(22)*, and phytase *(23)*.

In fermentation processes, metabolite production is very often correlated and is proportional to the quantity of biomass. Therefore, to optimize metabolite production, it is necessary to optimize microbial

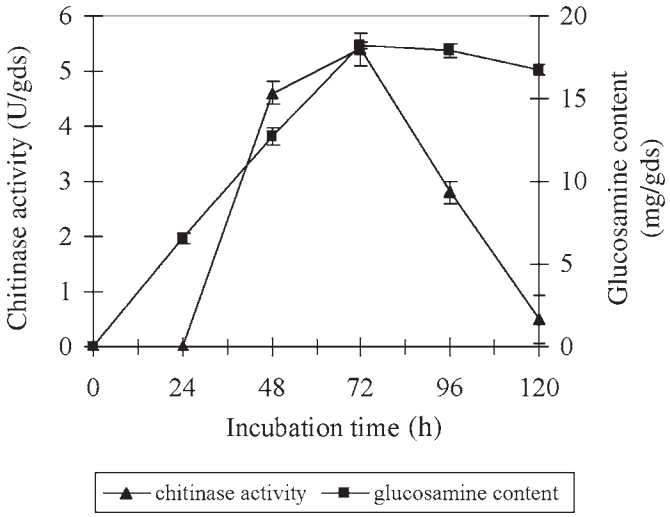

Fig. 1. Effect of incubation time on growth and chitinase production by *T. harzianum* TUBF 691. gds, grams of dry substrate.

growth. Glucosamine, a constituent of fungal mycelium, is considered a good biomass indicator for monitoring biomass in solid-state fermentation *(9,24)*. In the present study, glucosamine content in the fermented samples at different incubation periods clearly illustrated the growth–associated chitinase production by *T. harzianum* TUBF 691. By contrast, Vaidya et al. *(25)* observed a non-growth-associated production of chitinase by *Alcaligenes xylosoxydans* through submerged fermentation. The drastic reduction in chitinase yield after an optimum incubation time was probably owing to a reduced growth rate resulting from fast depletion of nutrients available to the fungus, and also could be owing to the production of secondary metabolites resulting in lower enzyme yield.

Influence of Initial Moisture Content

The initial moisture content of the fermentation medium is a critical factor that determines microbial growth and product yield in solid-state fermentation *(5,26,27)*. Different moisture levels were achieved in the substrate by altering the volume of distilled water added to the solid. The results presented in Fig. 2 show maximum enzyme production with an initial moisture content of 68.4% (9.3 U/g of dry substrate). Enzyme production increased almost 72% over that of the previous experiment (5.4 U/g of dry substrate), which clearly indicates the critical impact of moisture in solid-state fermentation. Any further increase in the initial moisture content reduced the enzyme production. Nampoothiri et al. *(12)* observed a similar result for chitinase synthesis. Rattanakit et al. *(11)* reported 58–65% water (w/v) for shrimp shellfish waste medium to be effective in enhancing chitinase production by *Aspergillus* sp. Usually in

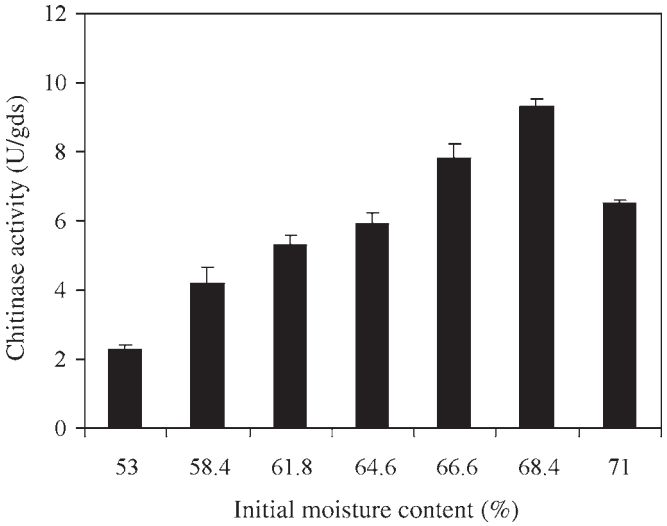

Fig. 2. Influence of initial moisture content on chitinase synthesis by *T. harzianum* TUBF 691. gds, grams of dry substrate.

solid-state fermentation using agroindustrial residues, optimum moisture content for growth and substrate utilization has been between 40 and 70%, depending on the organism and substrate for cultivation *(5,9)*. Interestingly, there could be a variation in the optimum moisture level for different strains of the same species of bacteria and mold *(27)*.

Moisture causes swelling and thereby facilitates better utilization of substrate by microorganisms *(28)*. However, lower moisture levels lead to reduced solubility of the nutrients in the solid substrate, lower degree of substrate swelling, and higher water tension *(29)*. Similarly, higher moisture contents were reported to cause decreased porosity, loss of particle structure, development of stickiness, reduction in gas volume, decreased gas exchange, and enhanced formation of aerial mycelium *(30)*.

Optimization of Colloidal Chitin Concentration

Figure 3 presents the results obtained on the synthesis of chitinase with different concentrations of colloidal chitin added to the solid medium. Apparently, this was extremely useful for the fungal culture, and chitinase titers reached 10.7 U/g of dry substrate in medium supplemented with 2% (w/v) colloidal chitin. There was a gradual increase in chitinase synthesis by the fungal culture with an increase in the concentration of colloidal chitin, but above 2% there was a sharp decline in the enzyme titers. It is known that an ideal substrate concentration in any fermentation process results in higher conversion efficiencies and optimum substrate utilization *(20)*. This could be the reason for a peak yield in enzyme synthesis with 2% (w/v) colloidal chitin and a decline in enzyme yield beyond this limit.

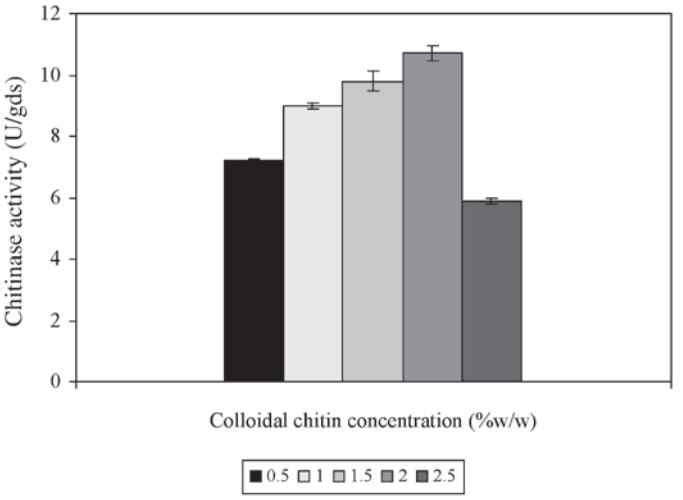

Fig. 3. Optimization of colloidal chitin concentration for chitinase synthesis by *T. harzianum* TUBF 691. gds, grams of dry substrate.

Chitinases can be synthesized in the absence of substrate (constitutive) or in its presence (adaptative). However, the addition of chitin to culture medium greatly enhances enzyme production *(17)*. There are reports showing that chitinases were produced by *T. harzianum* when grown in the presence of chitin or isolated fungal cell walls *(31–33)*. In most cases, chitin concentration in the range of 1.0–1.5% was found to be suitable for chitinase production *(34)*. In solid-state fermentation, among the different sources of chitin, colloidal chitin was found to be the best for chitinase production by *T. harzianum (14)*.

Effect of Additional Nitrogen Sources

The effect of nitrogen sources was examined by the addition of an organic and an inorganic nitrogen source (1% [w/w]) to the solid-state fermentation medium; the results are shown in Fig. 4. There was a mixed impact on enzyme synthesis by the fungal culture because some of the compounds, irrespective of organic or inorganic nature, harmfully affected the enzyme production and some resulted in an increase; some others had no or a marginal impact (Fig. 4). Urea was the most harmful compound: it resulted in 1.95 U/g of dry substrate chitinase, which was almost 5.3 times less than that of the control. Ammonical Nitrogen in general was not suitable. However, Vaidya et al. *(25)* found ammonium sulfate useful for chitinase production by *Aspergillus* sp. Yeast extract exerted the most desirable impact on the fungal culture, increasing the enzyme yield by 38.4% when compared with the control. Yeast extract has been reported as a versatile compound for microbial growth and activity by several investigators *(31,35)*, whose studies demonstrated an increase in enzyme titers such as chitinase and glucoamylase when yeast extract was used in fermentation

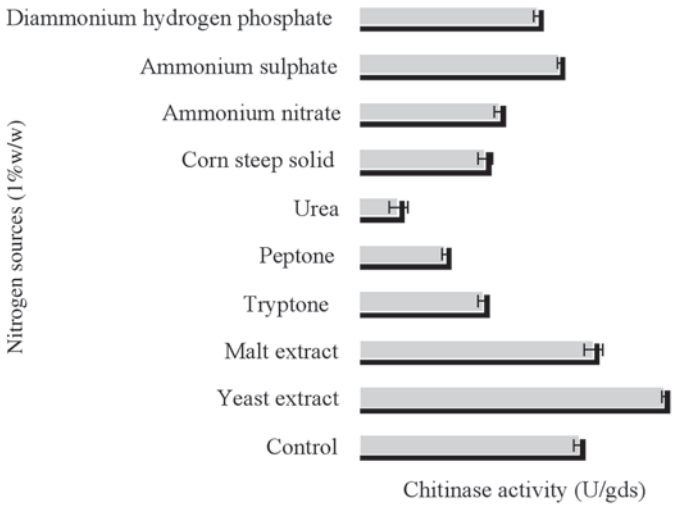

Fig. 4. Effect of additional nitrogen sources on chitinase synthesis by *T. harzianum* TUBF 691. gds, grams of dry substrate.

medium. In view of the significant results obtained with yeast extract, we decided to identify its most suitable concentration (ranging between 0 and 2% [w/w]). The results obtained showed 1% (w/w) as the most suitable (data not shown).

In Vitro Antifungal Assay

The effect of crude chitinase on the hyphal morphology of phytopathogenic fungus (*C. gloeosporioides)* of different ages was studied using a light microscope. From Fig. 5 it is evident that hyphae of *C. gloeosporioides* grown in the absence of chitinase (control) did not show any growth aberrations, whereas 24-h-old mycelia treated with chitinase showed lysis of fungal cell wall after 48 h of incubation, and extensive dissolution of cell wall was observed after 72 h. A similar result was also observed with 48-h-old mycelia (Fig. 6). In the case of 72-h-old mycelia, swelling of the hyphal wall was observed after 24 h of incubation and onward, followed by complete degradation after 72 h of incubation (Fig. 7).

Glucosamine content in the fungal cell wall varies with mycelial age and growth conditions. Hence, glucosamine concentration was determined for different ages of the mycelia. It was found that glucosamine concentration was negligible for 24- and 48-h-old mycelia, whereas 72-h-old mycelia contained 1.64 mg/g of dry wt. This showed that enzyme in the crude extract preferentially degraded chitin present in the cell wall. Almost all of the reported chitinase-producing microorganisms used chitin or colloidal chitin as a substrate or an elicitor for chitinase production, and chitinases thus produced were presumably responsible for their antifungal activities *(36–39)*. The involvement of chitinase in the control of *Sclerotium rolfsii* has also been established *(40)*. On coculturing of *Rhizoctonia solani* with

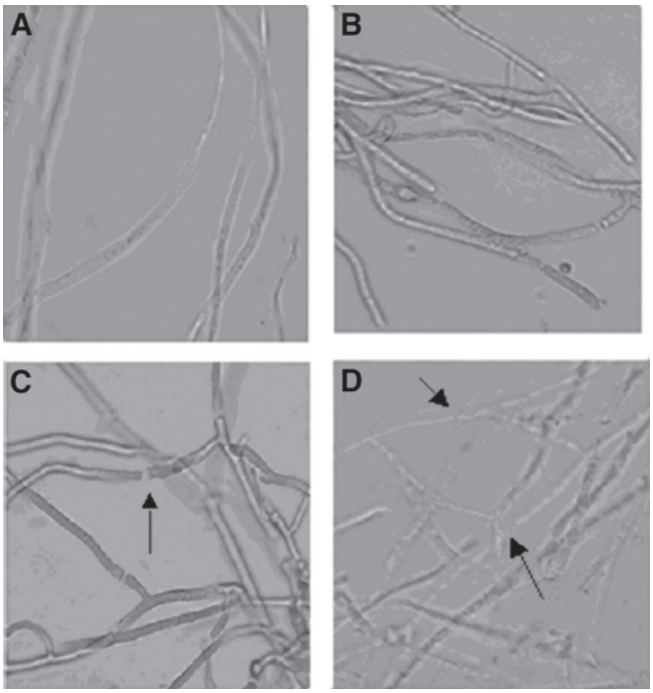

Fig. 5. Effect of crude chitinase on hyphal morphology of 24-h-old mycelia of phytopathogenic fungus *C. gloeosporioides* for different incubation periods: **(A)** control; **(B)** incubated for 24 h; **(C)** incubated for 48 h; **(D)** incubated for 72 h. Arrows indicate zone of lysis of cell wall by the action of chitinase.

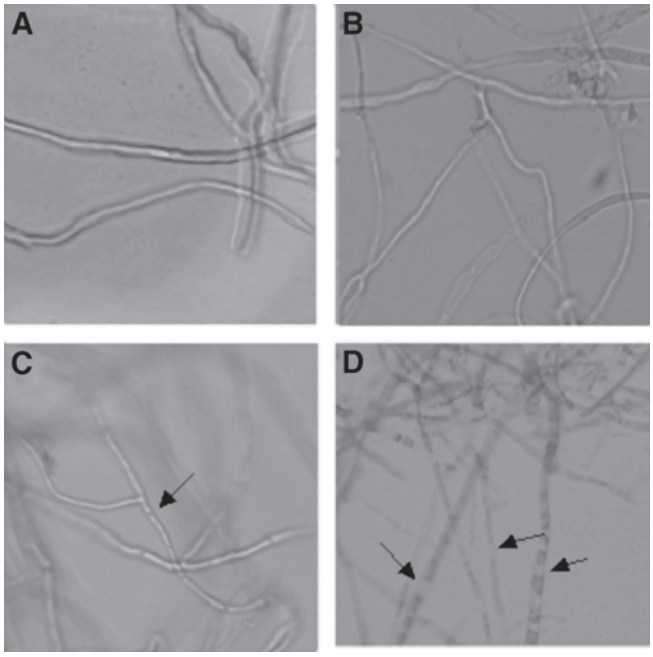

Fig. 6. Effect of crude chitinase on hyphal morphology of 48-h-old mycelia of phytopathogenic fungus *C. gloeosporioides* for different incubation periods: **(A)** control; **(B)** incubated for 24 h; **(C)** incubated for 48 h; **(D)** incubated for 72 h. Arrows indicate zone of lysis of cell wall by the action of chitinase.

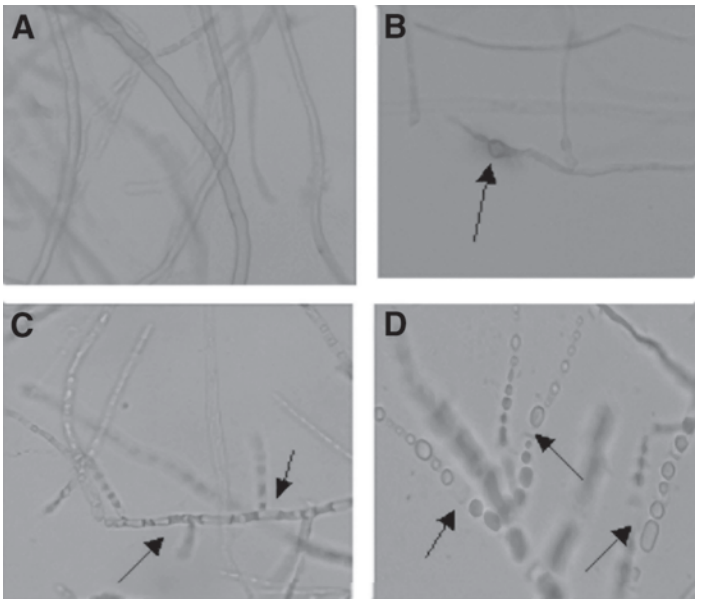

Fig. 7. Effect of crude chitinase on hyphal morphology of 72-h-old mycelia of phytopathogenic fungus *C. gloeosporioides* for different incubation periods: **(A)** control; **(B)** incubated for 24 h; **(C)** incubated for 48 h; **(D)** incubated for 72 h. Arrows indicate zone of lysis of cell wall by the action of chitinase.

Paenibacillus illinoisensis KJA-424, abnormal swelling and deformation of *R. solani* hyphae were observed, where the release of *N*-acetyl glucosamine was detected, which suggested that endochitinases secreted by *P. illinoisensis* KJA-424 played a role in hyphal swelling and lysis of fungal cell walls of *R. solani (41)*.

Several mechanisms have been proposed for the action of antifungal compounds on pathogenic fungi: interference with spore germination, inhibition by abnormal hyphae swelling, lysis and complete degradation of the hyphal tip, and suppression by competition for nutrients. In the present study, we observed swelling as well as degradation of the phytopathogenic fungal hyphae by chitinase preparation from solid culture. A similar result was observed when pathogenic *Botrytis cinerea* was treated with endochitinase produced by *T. harzianum (42)*.

pH and Thermal Stability

The study of pH stability of crude enzyme using different buffers of varying pH showed that chitinase from *T. harzianum* TUBF 691 was stable at about pH 4.0–6.0 (Fig. 8). Vyas and Deshpande *(43)* also observed that chitinase from *Myrothecium verrucaria* was active over a pH range of 4.0–6.5. This enzyme was used for fungal mycelial degradation and had five to six times more chitinase activity than lytic enzymes. Purified chitinase from *Streptomyces* RC 1071 also showed pH stability in the range of 4.0–9.0 *(44)*.

Residual chitinase activity was determined after incubating the enzyme at 30–70°C for different intervals of time using colloidal chitin as

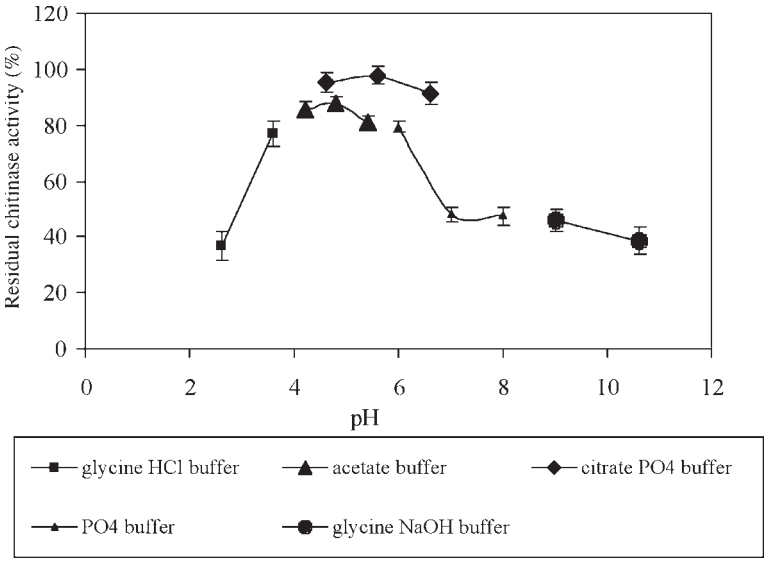

Fig. 8. pH stability of crude chitinase extract from *T. harzianum* TUBF 691.

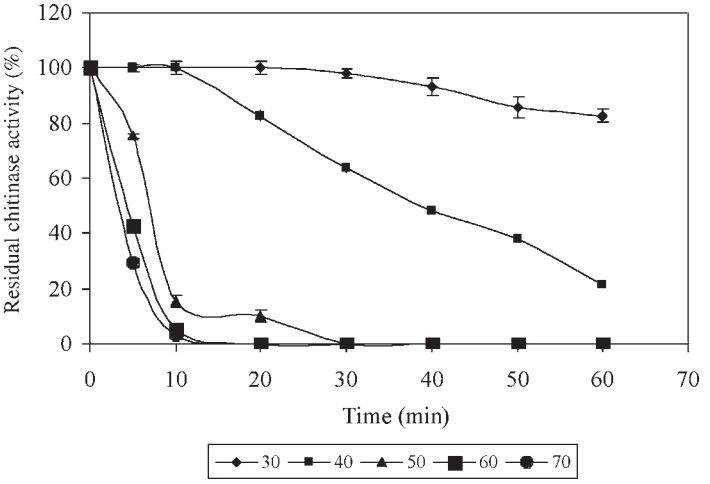

Fig. 9. Temperature stability of crude chitinase extract from *T. harzianum* TUBF 691.

substrate (Fig. 9). Its thermostable nature makes colloidal chitin a good prospect for use as a biocontrol agent. It was found that at 40°C the enzyme retained 48.2% activity after 40 min of incubation; however, the residual activity decreased significantly above 40°C. Higher thermal stabilities have also been observed for antifungal proteins of other origins *(45,46)*.

Our study showed that solid-state fermentation could be an effective technique for the production of chitinase-based biocontrol agents. There have been several reports regarding chitinase production under submerged fermentation, but its production in solid-state fermentation is very limited. Table 1 provides some studies regarding chitinase production under solid-

Table 1
Chitinase Production Under Solid-State Fermentation

Organism	Substrate	Chitinase activity (U/g of dry substrate)	Reference
Trichoderma harzianum TUBF 781	Wheat bran + colloidal chitin	3.18	*12*
Beauveria bassiana BTMF S10	Wheat bran + colloidal chitin	246.6	*20*
Aspergillus sp.	Shellfish waste	2.0	*48*
Penicillium aculeatum NRRL 2129	Wheat bran + chitin flakes	1.65	*47*
Verticillium lecanii ATCC 26854	Sugarcane pith bagasse with grasshopper cuticle	37.4	*49*
Verticillium lecanii ATCC 46578	Sugarcane pith bagasse with grasshopper cuticle	13.4	*49*

state fermentation. Solid-state fermentation has been considered of good potential for the use of agroindustrial residues such as rice husk, wheat bran, and sugarcane bagasse. Wheat bran supplemented with chitinaceous material was reported to be one of the best medium for chitinase production *(20,47)*. Rattanakit et al. *(48)* reported the use of shellfish waste for chitinase production in solid-state fermentation.

Conclusion

The results presented herein indicate that solid-state fermentation is an effective technique for the production of chitinase using a cheaper substrate. The effective utilization of wheat bran will further enhance the value of the present findings for chitinase production. Process parameters such as incubation time, initial moisture content, colloidal chitin concentration, and supplementation of additional nitrogen sources were found to have a profound influence on chitinase production by *T. harzianum*. The antifungal activity of crude enzyme extract was evidenced by in vitro antifungal assay. Extensive lysis and degradation was observed in 72-h-old mycelium of the fungal phytopathogen *C. gloeosporioides*. The thermal and pH stabilities of crude extract find great potential in plant pathogen control. Purification and characterization are necessary for a complete understanding about the nature of the enzyme before application in the field.

Acknowledgments

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