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Comparative biocontrol ability of chitinases from bacteria and recombinant chitinases from the thermophilic fungus *Thermomyces lanuginosus*

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Microbial chitinases (EC 3.2.1.14) are known to hydrolyse the chitinous gut epithelium of insects and cell walls of many fungi. In this study, seven chitinases from different bacteria and fungi were produced, characterized and their biocontrol abilities against graminaceous stem borers *Eldana saccharina*, *Chilo partellus* and *Sesamia calamistis* were assessed. All chitinases were stable over broad ranges of pH and temperature, however, recombinant fungal chitinases were more acid-stable than the bacterial counterparts. Chitinases from the thermophilic filamentous fungi *Thermomyces lanuginosus* SSBP (Chit1) and from *Bacillus licheniformis* (Chit lic) caused 70% and 80% mortality, respectively, in second instar larvae of *E. saccharina*. Six of the seven partially-purified microbial chitinases inhibited *Aspergillus niger*, *A. flavus*, *A. alliaceus*, *A. ochraceus*, *Fusarium verticillioides* and *Mucor* sp. Overall, microbial chitinases show promise as biocontrol agents of fungi and stalk–boring lepidopterans.

[**Key words:** Chitinases; Biocontrol; *Eldana saccharina*; *Chilo partellus*; *Sesamia calamistis*]

The cost-competitive, global sugar industries make an important contribution to international markets, given its agricultural and industrial investments, foreign exchange earnings, high employment and linkages with major suppliers, support industries and customers. South Africa is the leading sugar-producer in Africa and ranks within the top 15 of the 120 sugar-producing countries. However, agricultural losses due to insect pests such as the stalk–borers *Eldana saccharina* Walker (Lepidoptera: Pyralidae), *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae), *Chilo partellus* Swinhoe (Lepidoptera: Crambidae), white grubs (Coleoptera: Scarabaeidae), sugarcane thrips (Thysanoptera: Thripidae) and grasshoppers (Orthoptera) is a major concern (1,2). Aside from the adverse effect of stalk–borers, sugar production is directly linked to the occupational health of workers in sugar industries. Sugarcane workers are routinely exposed to microorganisms growing on sugar-rich substrates. Fungal contamination, especially exposure to fungal spores, is of prime concern, which contributes to several respiratory problems among workers such as rhinitis, lung function impairment, and bronchial hyperresponsiveness. Presently, a broad range of chemicals are used to counter the stalk–borers and fungal contamination in sugar industries.

Global use of chemical pesticides has severe environmental and health implications. Pesticides consumption worldwide is estimated to be two million tonnes annually (3). South Africa is ranked as one of the four leading importers of pesticides in sub-Saharan Africa (4,5). The devastating effects of chemical pesticides such as contamination of groundwater and riparian habitats, emergence of pesticide resistant organisms, constant depletion of biodiversity and increasing risk of pesticide related diseases (6) have resulted in a need to develop new approaches such as biocontrol. Biocontrol is a broad term encompassing several strategies that use natural organisms and their products to control or suppress pests.

Biocontrol of insects and fungi by targeting their chitinous morphological framework is a promising approach. The peritrophic membrane/matrix of insect gut is composed of 3–13% chitin — a linear polymer of β-1,4-*N*-acetylglucosamine (GlcNAC), while insect cuticles can contain up to 40% chitin on a dry mass basis (7). Fungal cell walls, on the other hand, consist of approximately 80% polysaccharides which also include chitin. The chitin β-(1,4) linkage-hydrolyzing chitinases (EC 3.2.1.14) are gaining importance as potential biocontrol agents due to their biocontrol activity against chitin-containing pathogenic insects, fungi, and nematodes. Chitinases are a diverse group of enzymes with a huge variation in their size, three-dimensional structure, substrate specificity and mode of action (8). While fungal chitinases have putative roles during the cell wall remodelling phases of life cycle, especially during hyphal growth, branching, hyphal fusion and autolysis, bacterial chitinases are predominantly involved in utilization of chitin in nature as carbon and nitrogen sources (9). Despite numerous reports on fungal and bacterial chitinases and their use in biocontrol of fungi (10–12), reports on the use of microbial chitinases to control insect pests are limited (13–16). To use chitinases for biocontrol and other possible applications, there is a need to study the comparative efficiency of bacterial and fungal chitinases.

In this study, chitinases were produced from several bacterial strains and recombinant yeast strains of *Pichia pastoris* harbouring chitinase genes of the thermophilic filamentous fungus *Thermomyces lanuginosus*. The enzymes were characterized and

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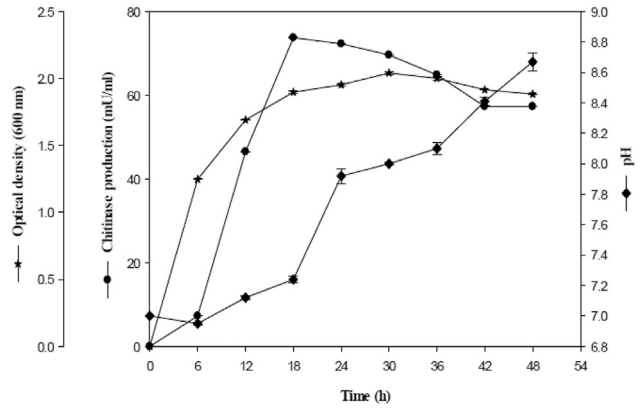

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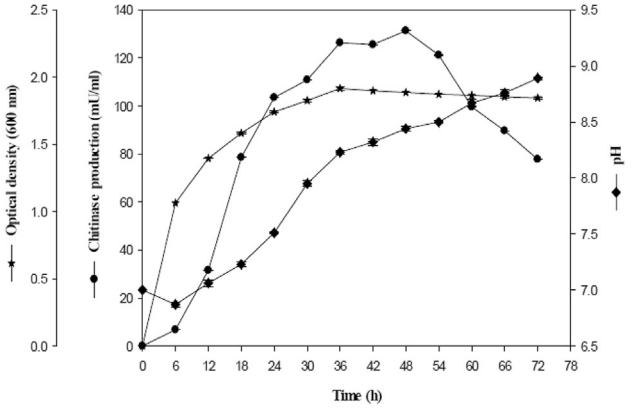

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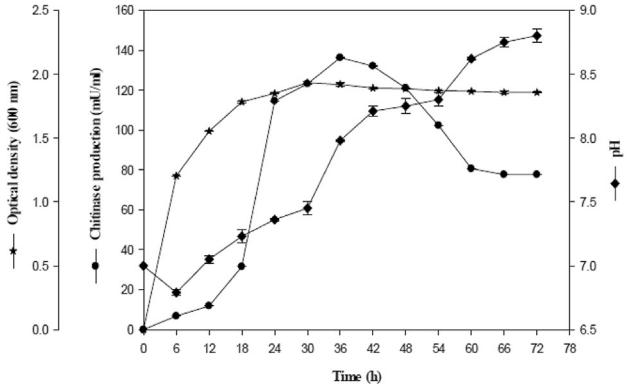

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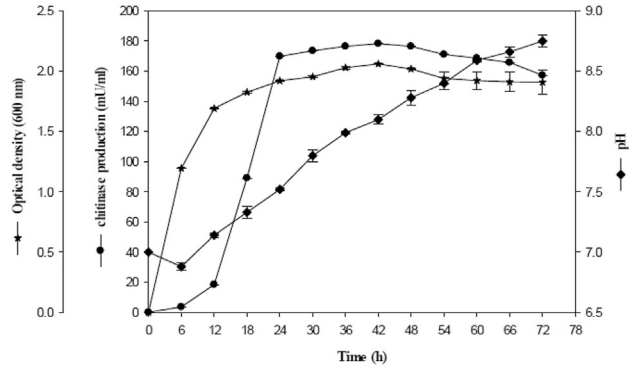

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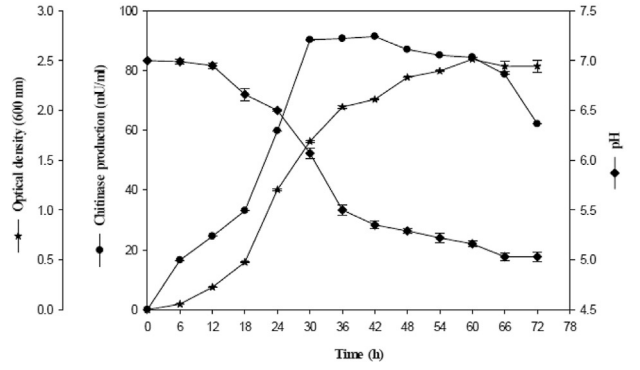

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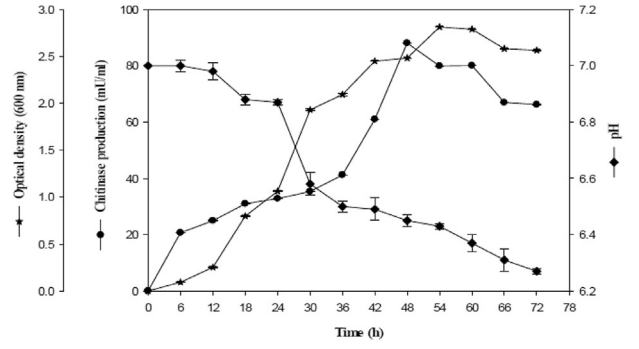

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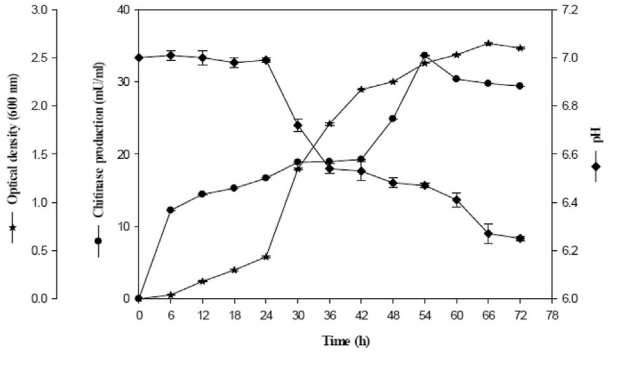

(g)

FIG. 1. Comparative production profile of chitinase from (a) *B. licheniformis*, (b) *B. firmus* 1489, (c) *B. firmus* 1505, (d) *Streptomyces* sp., (e) *P. pastoris* harboring *chit1,* (f) *P. pastoris* harboring *chit2,* and (g) *P. pastoris* harboring *chit1′*.

their biocontrol efficiencies were compared against the three insect pests identified above. Similarly, the biocontrol ability of these enzymes was also studied against an array of fungi, namely *Mucor* sp., *Fusarium verticillioides*, *Aspergillus niger*, *A. alliaceus*, *A. ochraceus* and *A. flavus*.

MATERIALS AND METHODS

Microorganisms, insect larvae and culture conditions This study included previously-isolated chitinase-producing microbial strains, namely *Bacillus firmus* 1489 producing Chit 1489, *B. firmus* 1505 producing Chit 1505, *Bacillus licheniformis* producing Chit lic and *Streptomyces* sp. producing Chit Strpt. All bacterial cultures were obtained from the CICIM culture collection, Wuxi, Jiangsu, China. Three recombinant strains of *P. pastoris* harbouring chitinase genes *T. lanuginosus* SSBP (*chit1* and *chit2*) and *T. lanuginosus* DSM (*chit1′*) were used to produce Chit1, Chit2 and Chit1′ (17). The bacterial cultures were maintained on nutrient agar slants enriched with 1% colloidal chitin, while yeasts were maintained on slants consisting of 1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar and 100 μg/ml of zeocin. Additionally, all microbial cultures were also preserved as glycerol stocks at −80°C. All the reagents, media and chemicals used in this research were of analytical grade and procured from Sigma–Aldrich (St. Louis, MO, USA).

Biocontrol efficiencies of chitinases were tested on second instar larvae of *E. saccharina*, *C. partellus* and *S. calamistis* supplied by South African Sugarcane Research Institute (SASRI), Mount Edgecombe, South Africa. *C. partellus* and *S. calamistis* larvae were maintained at 27 ± 2°C, with 8 h light and 16 h dark photo phase and 65 ± 5% relative humidity, while *E. saccharina* larvae were maintained at 28 ± 1.5°C, 75 ± 5% relative humidity with 8 h light and 16 h dark photo phase in the SASRI insect rearing unit. To ensure proper consumption of the enzyme-supplemented diet, the larvae were starved for 24 h prior to feeding.

Antifungal activities of chitinases were tested on six fungal cultures. *A. flavus* PPRI 5065, *A. ochraceus* PPRI 4687 and *A. alliaceus* PPRI 3185 were obtained from the culture collection of Plant Protection Research Institute (PPRI), Pretoria, South Africa (WDCM Number 351), while *A. niger*, *Mucor* sp. and *F. verticillioides* were sourced from the culture collection of the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa. Stock cultures were maintained on potato dextrose agar slants at 4°C.

Enzyme production and assay Chitinases from different microbial sources were produced in Erlenmeyer shake-flasks to study the characteristics and biocontrol activities of the enzymes. Recombinant chitinases from *T. lanuginosus* were produced by inoculating single colonies of *P. pastoris* into 20 ml YPG medium (1% yeast extract, 2% peptone, 1% glycerol) with zeocin (100 μg/ml) at 30°C, 200 rpm for 24 h and 1 ml of seed cultures (1% inoculum) were added to a 100 ml YPG medium with zeocin (100 μg/ml) in a 250 ml conical flask and incubated at 30°C, 200 rpm for 72 h. Similarly, different species of chitinase-producing *Bacillus* strains were spot inoculated on Luria Bertani agar plates with 1% colloidal chitin for 24 h and the colonies were inoculated into 20 ml Luria Bertaini broth containing 0.3% colloidal chitin. One milliliter of each seed culture was inoculated into separate conical flasks containing 100 ml of Luria Bertaini broth containing 0.3% colloidal chitin and incubated at 37°C, 180 rpm for 48 h to study the chitinase production profile of *B. lichenformis* and for 72 h for *B. firmus* strains 1489 and 1505. Similarly, the chitinase production profile of *Streptomyces* sp. was studied in chitin–yeast extract–salts medium containing (g/100 ml): chitin, 1.0; yeast extract, 1.5; K_2HPO_4, 0.3; $MgSO_4$, 0.2; and $FeSO_4 \cdot 7H_2O$, 0.1, at 30°C, 180 rpm for 72 h.

The crude enzymes were separated from cells by centrifugation at 18,200 × *g* for 10 min at 4°C and concentrated using ammonium sulphate precipitation followed by dialysis. Enzymes were further concentrated using 10 kDa molecular weight cut-off membranes. Microbial chitinases were assayed using colloidal chitin as a substrate. Colloidal chitin was prepared from shrimp shell chitin powder (Sigma–Aldrich) according to the method of Lee et al. (18). Enzyme solutions from different microbial sources (0.5 ml each) were added to 0.5 ml 1% (w/v) colloidal chitin suspension in appropriate buffers (0.1 M citrate phosphate buffer pH 4 for Chit1 and Chit Strpt; 0.1 M citrate phosphate pH 5 for Chit 2, 0.2 M phosphate buffer pH 6 and 7 for Chit1′ and Chit lic, respectively, and 0.2 M acetate buffer pH 5 for Chit 1489 and Chit 1505. The reaction mixtures were incubated for 30 minutes in a shaking water bath (80 rpm) at 40°C (for Chit1, Chit1′ Chit Strpt, Chit 1489 and Chit 1505), and at 50°C for Chit lic. The reaction was stopped by heating in boiling water for 15 minutes and centrifugation at 16,099 × *g* for 10 minutes and the supernatants was used for sugar analysis. Reducing sugars were estimated by the Schales' method (19,20) in which 0.6 ml of 0.5 M sodium carbonate solution containing 0.5 g/l potassium ferricyanide was added to 450 μl of chitooligosaccharide sample. The samples were incubated in boiling water for 15 min, cooled on ice and the absorbances were read at 420 nm. One unit of chitinase activity was defined as the amount of enzyme that liberates reducing sugar corresponding to 1 μmol of *N*-acetyl-D-glucosamine per minute. Protein concentration was determined using Bio-Rad protein dye reagent (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard.

Characterization of microbial chitinases The optimum pH and temperature of chitinases were determined at different pH values (pH 3.0–10.0) and temperatures (30°C–100°C), under standard assay conditions using colloidal chitin as the substrate. The buffers used were 100 mM citrate phosphate buffer (pH 3–5), phosphate buffer (pH 6–8), and glycine–NaOH buffer (pH 9–10). The optimum temperature for enzyme activity was measured using the standard assay in the range of 30°C–100°C at intervals of 10°C.

Thermostabilities of the microbial chitinases were determined after incubating enzymes at different temperatures for 2 h. Samples were withdrawn at 15 min intervals and assayed for chitinase activity under standard assay conditions. Similarly, pH stabilities of enzymes were studied by incubating 100 μl enzyme solutions in 900 μl buffers at different pH values for 2 h and calculating residual activities of samples at 15 min intervals.

Purification, kinetics and thermodynamics of Chit1 and Chit1′ Fungal chitinases, which showed higher biocontrol efficiencies against insect stem–borers, were purified to homogeneity using anion-exchange and gel-filtration methods using an AKTA purifier (GE Healthcare, Uppsala, Sweden). Briefly, concentrated and desalted samples of Chit1 and Chit1′ were loaded onto a HiTrap DEAE FF (GE Healthcare) anion-exchange column, previously equilibrated with 50 mM of respective buffers. The column was eluted with a linear gradient of 0–1 M NaCl at a flow rate of 2 ml/min. The active fractions were pooled and applied to a Superdex 200 Increase 10/300 column (GE Healthcare), and eluted with 50 mM respective buffers at 0.75 ml/min. Elution of the proteins was monitored at 280 nm. The active fractions were pooled and concentrated using Biomax Mr. 10 kDa molecular weight cut-off membranes (Millipore, Billerica, MA, USA). SDS–PAGE was carried out in a Mini-PROTEAN gel electrophoresis unit (Bio-Rad Laboratories) using a 12% polyacrylamide resolving gel. Zymograms were performed on 12% SDS-PAGE gels containing 0.01% (w/v) glycol chitin prepared from glycol chitosan (Sigma) using the method of Trudel and Asselin (21). SDS was removed by washing gels in appropriate buffers with 1% (v/v) Triton X-100 for 2 h at room temperature. The gel was then stained with freshly prepared 0.01% (w/v) Calcofluor white M2R (Sigma,) in 500 mM Tris-HCl (pH 8.9). After 5 min, the brightener solution was removed and the gel was destained for 1 h using distilled water at room temperature. Lytic zones were visualized by placing the gels on a light source (Gel Doc XR system, Bio-Rad Laboratories).

Purified Chit1 and Chit1′ were used for kinetic studies using chitooligosaccharides (CHOS; Megazyme, Bray, Ireland) as substrates. Standard curves of chitobiose, chitotriose, chitotetraose and chitohexaose were prepared by applying 0.2–1 μM CHOS on an Aminex column (Bio-Rad Laboratories) and elution using 5 mM sulphuric acid at a flow rate of 0.6 ml/min. The substrates were measured by absorption at 210 nm using a PDA detector linked to an HPLC (Prominence UFLC; Shimadzu, Kyoto, Japan). One and a half enzyme units of purified chitinases were added to the substrate solutions and the reaction mixtures were incubated at 40°C for Chit1′ and at 50°C for Chit1 for 6 h. An aliquot of the reaction mixture was withdrawn every 2 h and mixed with an equal volume of 0.5 M sodium hydroxide to stop the enzymatic reaction. The resultant samples were analysed by HPLC and the peak areas of CHOS obtained from the chromatograms were converted into CHOS concentrations, using the standard curves. Enzyme kinetic parameters (K_m, V_{max} and k_{cat}) with respect to chitotetraose, chitopentaose and chitohexaose were investigated by incubating the two enzymes at the appropriate temperature with varied substrate concentrations (0.06–1.0 μM). Initial rate velocities were used to plot hyperbolic curves using SigmaPlot 12 software (Systat Software Inc, San Jose, CA, USA), while kinetic parameters were computed from Michaelis–Menten curves and Lineweaver-Burk plots.

The thermodynamic parameters such as the activation energy (E_a, kJ mol), change in enthalpy (ΔH, kJ/mol), change in free energy (ΔG, kJ/mol), and change in entropy (ΔS, J/mol-K) for thermal denaturation of Chit1 and Chit1′ were determined using the following equations:

$$E_a = R \cdot \ln\left(\frac{V1}{V2}\right) \cdot \left(\frac{1}{T1} - \frac{1}{T2}\right) \quad (1)$$

$$\Delta H = E_a - RT \quad (2)$$

$$\ln\left(\frac{V_{max}}{T}\right) = \ln\left(\frac{KB}{h}\right) + \frac{\Delta S}{R} - \frac{\Delta S}{R} \cdot \frac{1}{T} \quad (3)$$

where *R*, *h*, *KB* and *T* is the gas constant, Planck constant, Boltzmann constant and absolute temperature, respectively.

Biocontrol activities of chitinases: insect bioassay Twenty larvae each of second, third and fourth instars of *C. partellus*, *E. saccharina* and *S. calamistis* were placed in separate 30 ml sterilized vials containing 8 ml of diet as supplied by SASRI (Tables S1 and S2), and mixed with 2, 6 and 8 units of each of the partially-purified chitinases as well as a cocktail of all the chitinases. One larva was placed in each vial, resulting in a total of 60 vials for each instar of larvae tested in triplicate. The same number of larvae and replicates of each insect pest were placed separately in vials containing diets without chitinases and these served as controls. Three different enzyme cocktails were prepared by mixing all tested enzymes and mixed to the diet with 2, 6 and 8 units of enzyme cocktails in vials. Results obtained from the screening step led to the selection of the most responsive instar of larvae and the

most effective enzyme dosage. The most effective enzyme dosage was again tested against the most responsive instar of larvae to verify the results. Further, only the most responsive larvae and the most effective enzyme dosage were used to determine the concentration that caused 50% mortality (LC_{50}). To determine the LC_{50}, 20 larvae of the most responsive species and instar were each placed in separate diet vials containing 8 ml of diet mixed with chitinases. In this case, a wider range of enzyme dosages was used (4–20 U/ml). The experiments were done in triplicate and average mortality determined. The same number of larvae and replicates of each insect pest were placed separately in vials containing diets mixed with distilled water containing Tween 80 (0.1% v/v) and these served as controls. The larvae were inspected for mortality and delay in developmental stages (pupation) every 24 h for 30 days and compared to the controls. First instar larvae were not included due to their high mortality attributed to their fragile nature. To determine LC_{50} for the most responsive larvae, data obtained was subjected to probit analysis (22,23).

Antifungal activity Zone of inhibition assays (24) were conducted using the agar plate well diffusion method. One millilitre of spore suspension (containing 1.5×10^4 spores/ml) of *A. flavus*, *A. ochraceus*, *A. alliaceus*, *A. niger*, *F. verticillioides* and *Mucor* sp. were uniformly spread on PDA plates in triplicate. Wells 5 mm in diameter were punched in the seeded PDA plates using a sterile cork borer. Five enzyme units of partially-purified enzymes were separately added in wells. All plates were incubated at 30°C for 72 h in triplicate. Antifungal activity of microbial chitinases was evaluated by measuring the diameter of inhibition zones. Antifungal activities were also confirmed by determining the percentage loss of fungal biomass when fungi were grown in media supplemented with chitinase. An inoculum of 1.5×10^4 spores of each of the aforementioned fungi was transferred separately into 250 ml conical flasks containing 100 ml potato dextrose broth and 10 enzyme units of each partially-purified chitinase. These were incubated for 5 days at 30°C in an orbital shaker at 200 rpm. All experiments were done in triplicate and the average biomass was recorded. Fungal cultures without chitinase were used as controls. Mycelia were harvested by filtration using pre-weighed filter paper (Whatman No. 1) that had been dried in the oven at 70°C. Harvested mycelia was dried to completion at 100°C and weighed to determine the percentage loss of biomass. Finally, mycolytic activity of chitinase was measured by the analysis of reducing sugars. An enzyme solution containing 5 units of each chitinase enzyme in optimum buffer was supplemented with 1% (w/v) air-dried mycelium and incubated at optimum temperature for 3 h. The concentration of the reducing sugars produced was determined by the Shales' procedure (19,20).

RESULTS AND DISCUSSION

Chitinase production profile All microorganisms used in this study exhibited microbial growth curves that consist of lag phases, log/exponential phases, and stationary phases (Fig. 1). While the recombinant yeast cells gradually multiplied to reach exponential and stationary phases, bacterial cells rapidly multiplied after inoculation, reaching the stationary phase after 18 h. This can be explained by the tendency of bacterial cells to rapidly multiply *via* binary fission when conditions are favourable while yeast cells take a bit longer (approximately 2 h) to begin budding (25).

The chitinase production by bacteria belonging to the *Bacillus* genus was comparable to previous reports. For instance, Narasimhan and Srividya (26) reported 1 U/ml chitinase production using *Bacillus subtilis*, while Eman (27) reported optimal chitinase production from *B. licheniformis* and *Bacillus thuringiensis* as 1.20 U/ml and 1.28 U/ml at pH 8 and 7, respectively. Also, Anuradha and Revathi (28) reported two chitin-hydrolyzing strains of *B. subtilis* and *B. atrophaeus*, that produced chitinase optimally at pH 6 (0.16 U/ml) and pH 8 (0.07 U/ml), respectively. There are variable reports on chitinase production from different species of *Streptomyces*. While Saadoun et al. (29) reported a *Streptomyces* sp. (strain S242) that produced 162 U/ml chitinase on the 4th day, *Streptomyces* strain P4 could only produce 0.00093 U/ml of chitinase (30). Chitinase production by a terrestrial *Streptomyces* sp. ANU 6277 was 7 U/ml (31). It should be noted that the amount and time of enzyme production is dependent on several nutritional and physical parameters. Any change in significant parameters can result a major change in enzyme production levels (32,33). In this study, rapid chitinase production was observed by bacterial cultures within the first 18–24 h. Maximum production time for bacterial chitinases were obtained as follows: after 48 h for *B. firmus* 1489 (131 mU/ml, Fig. 1c), at 36 h for *B. firmus* 1505 (136 mU/ml, Fig. 1d), 18 h for *B. licheniformis* (73 mU/ml, Fig. 1a) and at 42 h for *Streptomyces* sp. (strain U49) (178 mU/ml, Fig. 1e). Enzyme production gradually decreased in all strains after optimum production. This decrease in production may be attributed to proteolytic enzymes that hydrolysed chitinases, an increase of pH because of carbonate salts and protein released into the fermentation medium or accumulation of GlcNAc resulting from chitin decomposition (27). For the yeast cells, maximum chitinase production from *P. pastoris* with recombinant Chit1 (91 mU/ml) was at 42 h, while a production of 88 mU/ml and 33 mU/ml of recombinant Chit2 and Chit1′ was observed after 48 and 54 h, respectively. Enzyme production gradually decreased in all strains after their optimum production similar to chitinase production by bacteria. This is probably due to production of inhibitory metabolites or depletion of nutrients (34). Similarly, maximum chitinase production by *Microbispora* sp. V2 occurred after 48 h of incubation, after which it subsequently declined (35). *Bacillus cereus*, *B. alvei* and *B. sphaericus* produced highest chitinase after 48 h of incubation (36), while Faramarzi et al. (37) reported maximum chitinase production after 36 h by *Massilia timonae*. However, chitinase production in fungi is generally delayed, and was observed in *Penicillium aculeatum* (13) and *Trichoderma harzianum* (38) after 72 h of incubation.

Characterization of chitinases The bacterial chitinases were active over a broad range of pH (3.0–10.0). However, optimal activity for Chit 1489 (1.40 U/ml), Chit 1505 (1.30 U/ml) and Chit Strpt (1.45 U/ml) was observed at pH 6.0 (Table 1), whereas Chit lic (0.80 U/ml) was optimally active at pH 7.0. Similarly, recombinant chitinases were active at a broad pH range (pH 3.0–10.0). However, Chit1 (1.0 U/ml) and Chit2 (0.9 U/ml) were optimally active at slightly more acidic conditions compared to the bacterial chitinases (pH 5.0 and 4.0, respectively), while Chit1′ (0.4 U/ml) was optimum at pH 7.0. Bacterial chitinase were active from 30°C to 100°C and exhibited maximum activity at the following temperatures: 60°C for Chit Strpt (1.85 U/ml), Chit lic (0.9 U/ml) and Chit 1489 (1.45 U/ml) while the optimum for Chit 1505 (1.40 U/ml) was at 40°C. Recombinant chitinases were equally active over a broad temperature range (30–70°C). However, unlike bacterial chitinases they lost activity at 80°C. Chit1 was optimally active at 40°C (0.55 U/ml), Chit1 at 50°C (0.95 U/ml), and Chit2 at 40°C (0.95 U/ml). Chitinase DAU101 from *Bacillus* sp.

TABLE 1. pH and temperature characteristics of microbial chitinases.

Source	Designation	Optimum pH	Optimum temperature	Thermostability ($T_{1/2}$ in min)	pH stability ($T_{1/2}$ in min)	Shelf-life (days)
Bacillus licheniformis	Chit lic	7	60	346	231	21
T. lanuginosus DSM	Chit1′	7	40	115	77	30
T. lanuginosus SSBP	Chit1	5	50	630	213	30
T. lanuginosus SSBP	Chit2	4	40	577	117	30
Bacillus firmus 1489	Chit 1489	6	60	86	86	30
Bacillus firmus 1505	Chit 1505	6	40	231	693	30
Streptomyces sp. U49	Chit Strpt	6	40	86	30	30

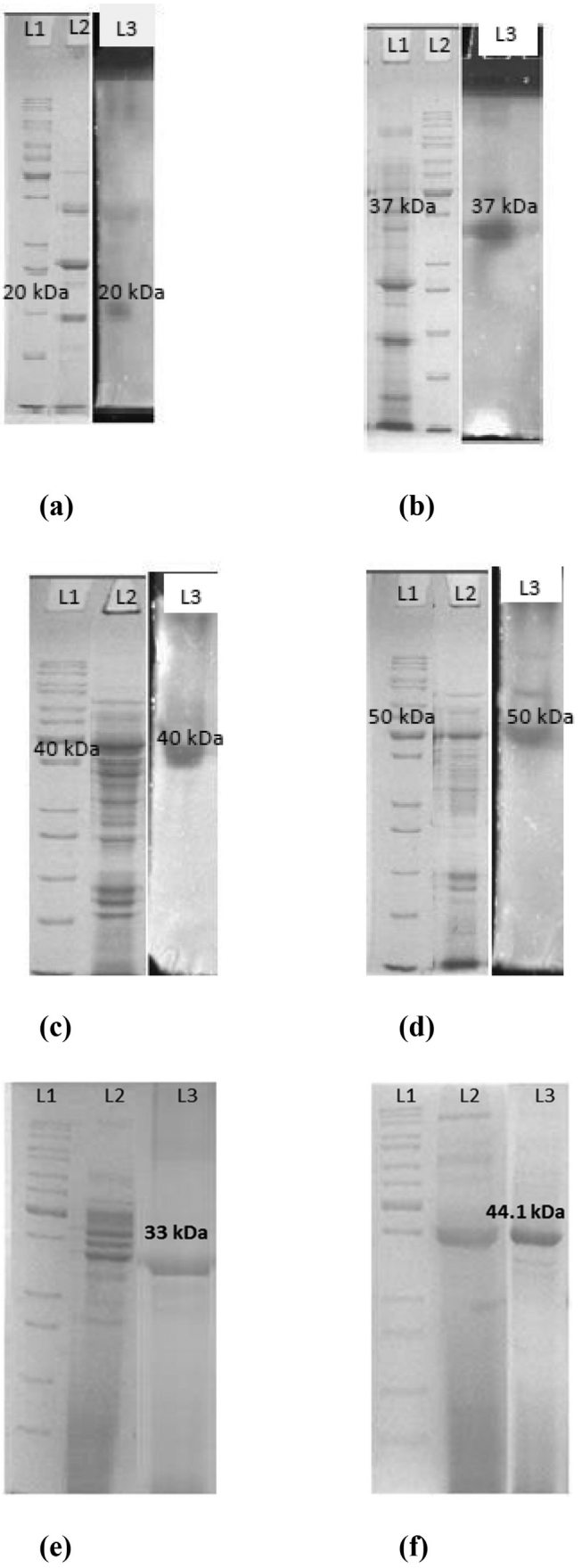

FIG. 2. Zymogram and SDS–PAGE of partially-purified bacterial chitinases (a–d) and purified fungal chitinases (e, f): (a) L1, Thermo Scientific page ruler unstained protein ladder (200 kDa); L2, SDS-PAGE of Chit Strpt (approximately 20 kDa); L3, zymogram of Chit Strpt; (b) L1, SDS-PAGE of Chit 1505 (approximately 37 kDa); L2, protein marker; L3, zymogram of Chit 1505; (c) L1, protein marker; L2, SDS-PAGE of Chit lic (approximately 40 kDa); L3, zymogram of Chit lic and (d) L1, protein marker; L2, SDS-PAGE of Chit 1489 (approximately 50 kDa); L3, zymogram of Chit 1489. (e) SDS-PAGE of purified Chit1′ from *T. lanuginosus* DSM: L1, marker; L2, partially purified enzyme; L3, purified enzyme. (e, f) SDS-PAGE of purified Chit1 from *T. lanuginosus* SSBP: L1, marker; L2, partially purified enzyme; L3, purified enzyme.

exhibited optimum activity at a pH 7.5 at 60°C (39). Chitinase NCTU2 from *Bacillus* sp. showed optimum activity in the temperature range of 50–60°C at pH 7.0 (40). Chitinase CH2 from *B. cereus* showed optimum at 40°C and at a pH of 7.1 (41). Chit Strpt used in this study was optimally active at 60°C. Chitinase produced by *T. lanuginosus* SY2 showed optimum catalytic activity at pH 4.5 and at a temperature of 55°C. The enzyme was stable at 50°C and had a half-life of 25 min at 65°C (42). Molecular masses of partially-purified recombinant chitinases was within a narrow range, approximately between 31 kDa and 44 kDa, whereas that of bacterial chitinases exhibited a wider range between 20 kDa and 50 kDa (Fig. 2a–d). The purified Chit1 was a 44.1 kDa protein while Chit1′ was 33 kDa, based on SDS-PAGE analysis (Fig. 2e, f). This is expected as Chit1′ is a truncated version of Chit1. Apart from the key chitinases secreted by the bacterial chitinases, as indicated by the thick bands on the zymogram gels in Fig. 2, bacterial cultures also produced other chitinases, but at lower levels. This is evidenced by presence of a few faint zones of hydrolysis on the gels. Thus, chitinase production by bacterial strains used in this study may consist of a chitinolytic system of chitinases that work synergistically. Comparable results have been reported by several other research groups that have used SDS-PAGE to analyse chitinases. Sakai et al. (43) reported that *Bacillus* sp. produced chitinases with molecular masses ranging between 25 and 80 kDa while chitinases from *Streptomyces* strain 68 had molecular masses of 25, 35, 67 and 200 kDa. On the other hand, chitinase NCTU2 from *Bacillus* sp. had a molecular mass of 36.5 kDa (31), while a 15 kDa chitinase from *B. cereus* strain CH2 was also reported (41).

Results obtained from the time course experiments with different CHOS inferred that Chit1 and Chit1′ showed both endo and exo activities due to the presence of $(GlcNAc)_4$, $(GlcNAc)_3$, $(GlcNAc)_2$ and GlcNAc as hydrolysis products. Both enzymes showed low activity on chitobiose. Chit1 released small amount of GlcNAc after the 6 h, whilst Chit1′ could not hydrolyze chitobiose even after 6 h of incubation. Both chitinases hydrolyzed chitotriose yielding chitobiose and GlcNAc, however, Chit1′ activity was lower than Chit1. Similar observations were made with chitotetraose where enzyme hydrolysis yielded $(GlcNAc)_3$, $(GlcNAc)_2$ and GlcNAc. However, both chitinases rapidly hydrolyzed $(GlcNAc)_5$ and $(GlcNAc)_6$ such that after 2 h of reaction chitopentaose and chitohexaose were fully converted to $(GlcNAc)_5$, $(GlcNAc)_4$, $(GlcNAc)_3$, $(GlcNAc)_2$ and GlcNAc. The major product of Chit1′ hydrolysis was chitotriose while Chit1 yielded mostly chitobiose. Additionally, it was observed that Chit1 yielded a higher concentration of GlcNAc compared to Chit1′. This was due to the slow activity of Chit1′ on chitobiose. Generally, the rate of hydrolysis increased with increase in chain length of CHOS.

It was observed that the V_{max} values of Chit1 for CHOS were higher than those Chit1′ (Table 5). The values increased with increase in length of the CHOS. K_m values for both Chit1 and Chit1′ for $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$ were low (0.021–0.086 μM), which was indicative of high substrate affinity. The k_{cat}/K_m values show that Chit1 is much more efficient than Chit1′ in terms of catalysis.

In thermodynamic studies, Chit1′ activation energy values for $(GlcNAc)_4$ and $(GlcNAc)_5$ were lower than E_a values of Chit1 (data

TABLE 2. Effect of 8 enzyme units on second instar larvae of *C. partellus*, *E. saccharina* and *S. calamistis*.

Enzyme	*Eldana saccharina*			*Sesamia calamistis*			*Chilo partellus*		
	Pupation (%)	Mortality (%)	Delayed pupation (%)	Pupation (%)	Mortality (%)	Delayed pupation (%)	Pupation (%)	Mortality (%)	Delayed pupation (%)
Control	97 ± 1.7	3 ± 1.7	0 ± 0.0	72 ± 1.5	0 ± 0.0	28 ± 0.6	73 ± 1.5	0 ± 0.0	27 ± 0.6
Chit2	84 ± 1.7	0 ± 0.0	16 ± 0.6	33 ± 1.5	0 ± 0.0	67 ± 1.0	50 ± 1.5	2 ± 1.7	48 ± 0.3
Chit1	72 ± 1.5	11 ± 1.7	17 ± 0.0	41 ± 0.3	15 ± 0.3	44 ± 0.9	42 ± 1.5	0 ± 0.0	58 ± 1.2
Chit1′	17 ± 1.7	70 ± 1.7	13 ± 0.3	13 ± 1.5	0 ± 0.0	87 ± 0.7	28 ± 1.5	7 ± 1.7	65 ± 1.7
Chit Strpt	62 ± 1.5	12 ± 1.5	26 ± 0.6	33 ± 1.6	3 ± 1.7	64 ± 0.3	27 ± 1.5	5 ± 2.9	68 ± 0.9
Chit 1489	43 ± 1.5	33 ± 1.7	24 ± 0.9	0 ± 0.0	10 ± 0.3	90 ± 0.9	13 ± 1.5	3 ± 1.7	84 ± 1.2
Chit 1505	49 ± 1.5	32 ± 1.7	19 ± 0.0	22 ± 1.5	0 ± 0.0	78 ± 0.3	44 ± 1.7	0 ± 0.0	56 ± 0.9
Chit lic	3 ± 1.6	78 ± 3.3	19 ± 0.0	0 ± 0.0	8 ± 1.7	92 ± 0.9	23 ± 1.7	7 ± 3.3	70 ± 0.3
Cocktail	77 ± 1.7	8 ± 1.7	15 ± 0.0	64 ± 1.9	3 ± 1.7	33 ± 0.9	63 ± 0.0	0 ± 0.0	37 ± 1.2

Data shown above are mean of experiments ± SE.

not shown). However, it was observed that Chit1 reactions on CHOS were quicker than Chit1′ regardless of the high activation energy. The enthalpy change values of Chit1′ and Chit1 when exposed to $(GlcNAc)_4$ and $(GlcNAc)_5$ were positive, indicating that the reactions were endothermic. Entropy changes were found to be negative for Chit1 and positive for Chit1′. Negative entropy changes in biocatalytic systems are due to compaction of enzyme molecules as well as changes arising from formation of charged particles and ordering of solvent molecules. Change in Gibbs free energy was positive for both Chit1 and Chit1′, which is indicative of non-spontaneous and endergonic reactions of chitinases with $(GlcNAc)_4$ and $(GlcNAc)_5$.

The mode of enzyme action of Chit1′ and Chit1 can be considered to be essentially the same. However, the difference in the speed of hydrolysis of the substrate (CHOS) could be attributed to the presence of additional carbohydrate binding modules in Chit1 that could be lacking in Chit1′. Carbohydrate binding modules can bind to carbohydrate ligands and influence the catalytic machinery. Other plausible reasons include correct positioning of the catalytic domains, decrystallization of the substrate thus enhancing substrate affinity and catalytic efficiency of the enzymes.

Biocontrol activities The effect of 8 enzyme units on second instar larvae of three insect pests were tested (Table 2). The efficacy of Chit1′ and Chit lic in terms of their LC_{50} values and probit values are shown in Table 3. Feeding larvae with chitinase-supplemented diet (especially second instar larvae) might have led to hydrolysis of the peritrophic membrane, causing disturbance in selective permeability properties. The damage might have resulted in nutritional imbalances due to several affected processes such as food acquisition, water retention or excretion and digestion, causing slow growth rate and eventually death. Higher enzyme dosages (6 and 8 units) might have been responsible for delayed pupation that became more apparent with reduction of larval stage. Chit1′ and Chit lic were the most effective chitinases against second instar larvae of *E. saccharina*, while Chit Strpt and Chit 1489 could only delay pupation by 26% and 24%, respectively. Appreciable mortality could not be achieved against second instar larvae of *S. calamistis* and *C. partellus* and most of the tested chitinases could only delay pupation with a maximum of 70–92%. Purified chitinase from *Pseudomonas fluorescens* MP-13 was used to achieve a 100% mortality of *Helopeltis* spp. under *in-vitro* conditions (44). Difference in mortality and pupation of insect larvae in this investigation can be explained on the basis of presumably different structural variations of these proteins and their orientations during interaction with chitinous peritrophic membranes. Less than 10% mortality in controls could be attributed to well-maintained parameters in SASRI insect rearing units. Similar reports on chitinases hydrolyzing insect gut peritrophic membrane as well as affecting insect growth and development are available. Infiltration of peritrophic membranes occurred *in vivo* after *Spodoptera littoralis* fifth instar larvae were fed a diet supplemented with recombinant endochitinase from *Serratia marcescens* (45). On the other hand, Binod et al. (13) evaluated larvicidal activity of a chitinase from *T. harzianum* against the cotton insect pest (*Helicoverpa armigera*). Chitinase from *T. harzianum* was observed to effectively decrease the feeding rate as well as body weight (antifeedant) of the cotton insect pest larvae (*H. armigera*). Lowest pupation percentage was observed when second instar larvae of *H. amigera* were treated with a higher dose (2000 U/ml) of chitinase from *Penicillium ochrochloron* (46). Similarly, Halder et al. (47) reported strong chitinolytic activity and mosquitocidal impact of *Aeromonas hydrophila* SBK1 on *Culex quinquefasciatus*. Additionally, microbial chitinases have been used in synergism experiments to enhance the potency of entomopathogenic microorganisms and microbial insecticides. A chitinase mixture from *Streptomyces albidoflavus* containing three endochitinases, two 1,4-β-D-chitobiosidases (exochitinase) and one glucosaminidase with ability to hydrolyze chitin affected the growth, development and survival of *Hypothenemus hampei* (coffee berry borer), *Bemisia argentifolii* (whitefly), *Trichoplusiani* (looper), *Myzus persicae* (potato aphid) and *H. virescens* (budworm) (48). Habeeb et al. (49) achieved 95.3–100% mortality of the camel tick *Hyalomma dromedarii* eggs using a combination of fungal chitinase and protease enzymes.

It was apparent that most of the microbial chitinases inhibited (to various extents as some produced wider zones of inhibition compared to others) the tested fungal strains (Fig. 3 and Table 4). However, chitinase from *Streptomyces* sp. could only inhibit *A. ochraceus* and *F. verticillioides*. The high quantity of reducing sugars released by the microbial chitinases with all tested fungi (except for Chit 1489) could be attributed to the easy accessibility of the chitin in weakened air-dried and reconstituted mycelia by the enzyme *vis a vis* mycelia in its native state as used in the zone of inhibition and dry mass assays. This was observed by the relatively high quantity of sugars released by Chit Strpt (data not shown) that had previously exhibited low antifungal activity in other antifungal

TABLE 3. LC_{50} data obtained for Chit1′ and Chit lic against second instar larvae of *E. saccharina*.

Mortality (%) ± SE	Probits	Enzyme dose (U/ml)	log (enzyme dose)
Chit1′ from *T. lanuginosus*			
78 ± 0.90	5.77	20.00	1.30
77 ± 0.30	5.74	15.00	1.18
67 ± 0.60	5.44	10.00	1.00
65 ± 1.50	5.39	8.00	0.90
43 ± 0.80	4.45	6.00	0.78
20 ± 1.20	3.82	4.00	0.60
Chit lic from *B. licheniformis*			
83 ± 0.90	5.95	20.00	1.30
82 ± 0.70	5.92	15.00	1.18
78 ± 1.80	5.77	10.00	1.00
77 ± 1.80	5.71	8.00	0.90
58 ± 1.90	5.2	6.00	0.78
30 ± 1.20	4.48	4.00	0.60

TABLE 4. Zone of inhibition due to action of microbial chitinases on different strains of fungi.

Fungi	Zone of inhibition (mm), mean ± SE							
	Chit1	Chit2	Chit1+Chit2	Chit1′	Chit strpt	Chit 1489	Chit1 1505	Chit lic
A. flavus	30 ± 1.8	17 ± 0.6	29 ± 0.7	30 ± 0.3	–	25 ± 0.6	30 ± 0.6	21 ± 0.6
A. niger	14 ± 1.2	16 ± 0.7	20 ± 0.5	24 ± 0.6	–	20 ± 0.5	30 ± 0.9	20 ± 1.0
A. ochraceous	31 ± 0.6	30 ± 1.5	29 ± 0.8	35 ± 0.3	22 ± 0.6	28 ± 0.7	40 ± 1.2	30 ± 0.8
A. alleaceous	27 ± 1.2	30 ± 0.3	23 ± 0.6	30 ± 1.2	–	30 ± 0.7	28 ± 0.5	25 ± 0.3
Mucor sp.	19 ± 1.2	18 ± 0.9	20 ± 0.5	28 ± 0.3	–	13 ± 0.3	25 ± 1.0	22 ± 0.7
F. verticillioides	26 ± 1.7	30 ± 0.6	26 ± 0.7	20 ± 0.6	18 ± 0.5	28 ± 0.8	30 ± 0.4	14 ± 0.9

The diameters of growth inhibition zones were measured on the third day of fungal incubation on agar media at 28°C.

TABLE 5. Summary of kinetic and thermodynamic parameters of purified Chit1 and Chit1' from *T. lanuginosus*.

Enzyme	CHOS	V_{max} (μM/min•mg)	K_m (μM)	k_{cat} (S^{-1})	k_{cat}/K_m (μM^{-1} S^{-1})	ΔH (kJ/mol)	ΔS (kJ/mol)	ΔG° (kJ/mol)
Chit1'	$(GlcNAc)_4$	0.690	0.021	0.000	0.017	14.02	−0.20	68.65
Chit1	$(GlcNAc)_4$	8.200	0.152	0.003	0.020	94.16	0.06	77.77
Chit1'	$(GlcNAc)_5$	1.700	0.025	0.001	0.034	2.54	−0.24	40.78
Chit1	$(GlcNAc)_5$	159.200	0.027	0.059	2.184	149.64	0.26	78.62
Chit1'	$(GlcNAc)_6$	0.760	0.086	0.000	0.004	–	–	–
Chit1	$(GlcNAc)_6$	199.900	0.021	0.074	3.526	–	–	–

assays. Generally, Chit1′ showed the best antifungal activity while Chit Strpt was least active against fungi. These differences in antifungal activities of microbial chitinases could be due to different subsite structures in the binding cleft (50) as well as the amount and surface microstructure of chitin. A scale shaped arrangement of chitin material in the fugal cell wall that allows exposure of chitin fibre on the fungal cell wall's surface facilitates easy chitinase–chitin interaction. Easy substrate access of chitinase in the fungal cell wall leads to a higher rate of chitin hydrolysis and inhibition of disease-causing fungi. This observation of differential antifungal activity on various pathogenic fungi was also noticed by Yan et al. (51) while investigating the antifungal activity of a purified rice chitinase produced by *P. pastoris*. They observed that although this chitinase efficiently inhibited *Botrytis squamosa* and *Rhizopus stolonifer*, it had no significant activity against *Pythium aphanidermatum* and *A. niger*. Microbial chitinases production is also increased in the vicinity of certain fungi. A high yield of 49 and 72.0 U/ml of chitinase from *Enterobacter* sp. NRG4 was achieved when the bacteria were grown in the vicinity of fungal cell walls of *Fusarium moniliforme* and *Candida albicans*, respectively. Further, this chitinase arrested the growth of hyphal tips in *A. niger*, *F. moniliforme*, *Mucor rouxii* and *Rhizopus nigricans* at pH 5.4, 25°C after 24 h (52). Of the three bacteria (*B. thuringiensis*, *Stenotrophomonas maltophilia* and *B. licheniformis*) isolated by Kamil et al. (53) from the soil rhizophere, *B. licheniformis* displayed the best activity against hyphal growth of several fungi (*Sclerotium rolfsii*, *Rhizoctonia solani*, *Alternaria alternata*, *Macrophomina phasiolina*, *Pythium* sp. and *Fusarium culmorum*) on PDA plates at 28°C. Further, Kim et al. (54) gave an account of three bacteria, namely *Serratia plymuthica* strain C-1, *Chromobacterium* sp., and *Lysobacter enzymogenes* strain C-3, that exhibited robust activity against *Phytophthora capsic*, *R. solani* and *Fusarium* sp. as well as *R. solani*. Chitinase Chi19F from *Streptomyces coelicolor*

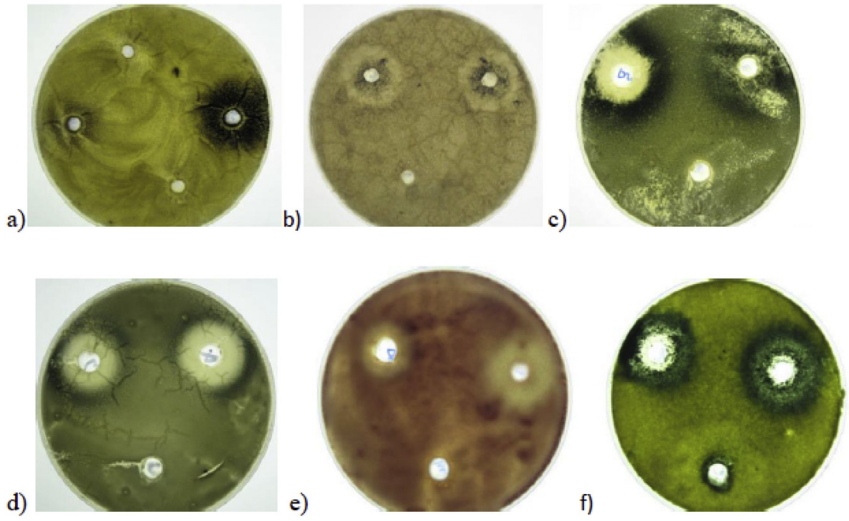

FIG. 3. Agar plates assays showing inhibition of fungi by chitinases. (a) *A. niger*: right well, Chit1; left well, Chit1; top well, Chit Strpt and bottom well, buffer control. (b) *Mucor* sp.: left well-, Chit1; right well, Chit lic; bottom well, buffer control. (c) *A. ochraceus*: right well, Chit 1505; left well, Chit1; bottom well, buffer control. (d) *A. alliaceus:* right well, Chit1; left well, Chit 1489; bottom well, buffer control. (e) *F. verticillioides*: right well, Chit 1505; left well, Chit Strpt; bottom well, buffer control. (f) *A. flavus*: right well, Chit1; left well, Chit lic; bottom well, buffer control.

A3(2) inhibited *Fusarium solani*, *Trichoderma reesei*, *Mucor javaniccus* and *Trichoderma viride* significantly on PDA plates at 30°C and at a pH of 5.5 (53).

In conclusion, tested chitinases significantly limited the growth of *E. saccharina*, *S. calamistis* and *C. partellus* through enhanced mortality and delay in pupation of, especially, the second instar larvae. High mortality was particularly observed in second instar larvae of *E. saccharina* when 6 and 8 units of Chit lic and Chit1′ were used, while delayed pupation was observed in second instar larvae of *S. calamistis*. Both bacterial and fungal chitinases used in this study exhibited remarkable antifungal activity against various tested fungi. Attempts are underway to study the kinetic and thermodynamic parameters of the purified chitinases.

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References

1. **Singels, A., Leslie, G. W., Mcfarlane, S. A., Sithole, P., Ferrer, S. R. D., and Maher, G.:** Review of South African sugarcane production in the 2010/2011 season from an agricultural perspective, Proc. S. Afr. Sug. Technol. Ass., **84**, 66–83 (2011).
2. **Manosathiyadevan, M., Bhuvaneshwari, V., and Latha, R.:** Impact of insects and pests in loss of crop production: a review, pp. 57–67, in: Dhnarajan, A. (Ed.), Sustainable agriculture towards food security. Springer, Singapore (2017).
3. **De, A., Bose, R., Kumar, A., and Mozumdar, S.:** Targeted delivery of pesticides using biodegradable polymeric nanoparticles, pp. 59–81. Springer, India, New Delhi (2014).
4. **Quinn, L. P., Fernandes–Whaley, M., Roos, C., Bouwman, H., Kylin, H., Pieters, R., and Van Den Berg, J.:** Pesticide use in South Africa: one of the largest importers of pesticides in Africa, pp. 49–96, in: Pesticides in the modern world-pesticides use and management. InTech (2011).
5. **Hatting, J. L., Moore, S. D., and Malan, A. P.:** Microbial control of phytophagous invertebrate pests in South Africa: current status and future prospects, J. Invertebr. Pathol., https://doi.org/10.1016/j.jip.2018.02.004 (2018).
6. **Nieder, R., Benbi, D. K., and Reichl, F. X.:** Health risks associated with pesticides in soils, pp. 503–573, in: Soil components and human health. Springer, Dordrecht (2018).
7. **Toprak, U., Erlandson, M., and Hegedus, D.:** Peritrophic matrix proteins, Trends Entomol., **6**, 23–51 (2010).
8. **Hamid, R., Khan, M. A., Ahmad, M., Ahmad, M. M., Abdin, M. Z., Musarrat, J., and Javed, S.:** Chitinases: an update, J. Pharm. Bioallied Sci., **5**, 21–29 (2013).
9. **Bhattacharya, D., Nagpure, A., and Gupta, R. K.:** Bacterial chitinases: properties and potential, Crit. Rev. Biotechnol., **27**, 21–28 (2007).
10. **Neeraja, C., Anil, K., Purushotham, P., Suma, K., Sarma, P. V. S. R. N., Moerschbacher, B. M., and Podile, A. R.:** Biotechnological approaches to develop bacterial chitinases as a bioshield against fungal diseases of plants, Crit. Rev. Biotechnol., **30**, 231–241 (2010).
11. **Russo, P., Arena, M. P., Fiocco, D., Capozzi, V., Drider, D., and Spano, G.:** *Lactobacillus plantarum* with broad antifungal activity: a promising approach to increase safety and shelf-life of cereal-based products, Int. J. Food Microbiol., **247**, 48–54 (2017).
12. **Shehata, A. N., El Aty, A. A. A., Darwish, D. A., Wahab, W. A. A., and Mostafa, F. A.:** Purification, physicochemical and thermodynamic studies of antifungal chitinase with production of bioactive chitosan-oligosaccharide from newly isolated *Aspergillus griseoaurantiacus* KX010988, Int. J. Biol. Macromol., **107**, 990–999 (2018).
13. **Binod, P., Sukumaran, R. K., Shirke, S. V., Rajput, J. C., and Pandey, A.:** Evaluation of fungal culture filtrate containing chitinase as a biocontrol agent against *Helicoverpa armigera*, J. Appl. Microbiol., **103**, 1845–1852 (2007).
14. **Berini, F., Caccia, S., Franzetti, E., Congiu, T., Marinelli, F., Casartelli, M., and Tettamanti, G.:** Effects of *Trichoderma viride* chitinases on the peritrophic matrix of Lepidoptera, Pest Manag. Sci., **72**, 980–989 (2016).
15. **Rishad, K. S., Rebello, S., Shabanamol, P. S., and Jisha, M. S.:** Biocontrol potential of halotolerant bacterial chitinase from high yielding novel *Bacillus pumilus* MCB–7 autochthonous to mangrove ecosystem, Pestic. Biochem. Physiol., **137**, 36–41 (2017).
16. **Berini, F., Katz, C., Gruzdev, N., Casartelli, M., Tettamanti, G., and Marinelli, F.:** Microbial and viral chitinases: attractive biopesticides for integrated pest management, Biotechnol. Adv., **36**, 818–838 (2018).
17. **Zhang, M., Puri, A. K., Govender, A., Wang, Z., Singh, S., and Permaul, K.:** The multi-chitinolytic enzyme system of the compost-dwelling thermophilic fungus *Thermomyces lanuginosus*, Process Biochem., **50**, 237–244 (2015).
18. **Lee, Y. G., Chung, K. C., Wi, S. G., Lee, J. C., and Bae, H. J.:** Purification and properties of a chitinase from *Penicillium* sp. LYG 0704, Protein Expr. Purif., **65**, 244–250 (2009).
19. **Schales, O. and Schales, S. S.:** A simple method for the determination of glucose in blood, Arch. Biochem., **8**, 285–292 (1945).
20. **Imoto, T. and Yagishita, K.:** A simple activity measurement of lysozyme, Agric. Biol. Chem., **35**, 1154–1156 (1971).
21. **Trudel, J. and Asselin, A.:** Detection of chitinase activity after polyacrylamide gel electrophoresis, Anal. Biochem., **178**, 362–366 (1989).
22. **Finney, D. J.:** Probit analysis, 3rd ed. Cambridge University Press, London (1971).
23. **Ibarra, J. E. and Federici, B. A.:** An alternative bioassay employing neonate larvae for determining the toxicity of suspended particles to mosquitoes, J. Am. Mosq. Contr. Assoc., **3**, 187–192 (1987).
24. **Zarei, M., Aminzadeh, S., Zolgharnein, H., Safahieh, A., Daliri, M., Noghabi, K. A., Ghoroghi, A., and Motallebi, A.:** Characterization of a chitinase with antifungal activity from a native *Serratia marcescens* B4A, Braz. J. Microbiol., **42**, 1017–1029 (2011).
25. **Soifer, I., Robert, L., and Amir, A.:** Single-cell analysis of growth in budding yeast and bacteria reveals a common size regulation strategy, Curr. Biol., **26**, 356–361 (2016).
26. **Narasimhan, A. and Srividya, S.:** Optimization of chitinase produced by a biocontrol strain of *Bacillus subtilis* using Plackett–Burman design, Eur. J. Exp. Biol., **2**, 861–865 (2012).
27. **Eman, Z. G.:** Chitinase production by *Bacillus thuringiensis* and *Bacillus licheniformis:* their potential in antifungal biocontrol, J. Microbiol., **50**, 103–111 (2012).
28. **Anuradha, V. and Revathi, K. J.:** Purification and characterization of chitinase from two *Bacillus* sp. isolated from crustacean shells, Microbiol. Biotechnol. Res., **3**, 160–167 (2013).
29. **Saadoun, I., Al–Omari, R., Jaradat, Z., and Ababneh, Q.:** Influence of culture conditions of *Streptomyces* sp. (strain S242) on chitinase production, Pol. J. Microbiol., **58**, 339–345 (2009).
30. **Julaluk, T. and Hataichanoke, N.:** Chitinase production and antifungal potential of endophytic *Streptomyces* strain P4, Maejo Int. J. Sci. Technol., **6**, 95–104 (2012).
31. **Narayana, K. J. P. and Vijayalakshmi, M.:** Chitinase production by *Streptomyces* sp. ANU 6277, Braz. J. Microbiol., **40**, 725–733 (2009).
32. **Jha, S. and Modi, H. A.:** Statistical optimization of chitinase production by *Streptomyces rubiginosus* SP24 and efficacy of purified chitinase to control root-knot nematode infection in *Vigna radiata* under controlled conditions, Chem. Biol. Technol. Agric., **5**, 20 (2018).
33. **Rishad, K. S., Rebello, S., Nathan, V. K., Shabanamol, S., and Jisha, M. S.:** Optimised production of chitinase from a novel mangrove isolate, *Bacillus pumilus* MCB-7 using response surface methodology, Biocatal. Agric. Biotechnol., **5**, 143–149 (2016).
34. **Nochur, S. V., Roberts, M. F., and Demain, A. I.:** True cellulase production by *Clostridium thermocellum* grown on different carbon sources, Biotechnol. Lett., **15**, 641–646 (1993).
35. **Nawani, N. N., Kapadnis, B. P., Das, A. D., Rao, A. S., and Mahajan, S. K.:** Purification and characterization of a thermophilic and acidophilic chitinase from *Microbispora* sp. V2, J. Appl. Microbiol., **93**, 965–975 (2002).
36. **Wang, S. L. and Hwang, J. R.:** Microbial reclamation of shellfish wastes for the production of chitinases, Enzyme Microb. Technol., **28**, 376–382 (2001).
37. **Faramarzi, M. A., Fazeli, M., Tabatabaei, Yazdi M., Adrangi, S., Jami, A. L., Ahmadi, K., Tasharrofi, N., and Aziz, M. F.:** Optimization of cultural conditions for production of chitinase by a soil isolate of *Massilia timonae*, Biotechnology, **8**, 93–99 (2009).
38. **Sandhya, C., Binod, P., Nampoothirini, K. M., Szakaes, G., and Pandey, A.:** Microbial synthesis of chitinase in solid cultures and its potential as a biocontrol agent against phytopathogenic fungus *Colletotrichum gloesporoorioides*, Appl. Biochem., **127**, 1–15 (2005).
39. **Lee, Y. S., Park, I. H., and Yoo, J. S.:** Cloning, purification, and characterization of chitinase from *Bacillus* sp. DAU101, Bioresour. Technol., **98**, 2734–2741 (2007).
40. **Wen, C. M., Tseng, C. S., Cheng, C. Y., and Li, Y. K.:** Purification, characterization and cloning of a chitinase from *Bacillus* sp. NCTU2, Biotechnol. Appl. Biochem., **35**, 213–219 (2002).

41. **Li, J. G., Jiang, Z. Q., Xu, L. P., Sun, F. F., and Guo, J. H.:** Characterization of chitinase secreted by *Bacillus cereus* strain CH2 and evaluation of its efficacy against *Verticillium* wilt of eggplant, BioControl, **53**, 931–944 (2008).
42. **Guo, R. F., Shi, B. S., Li, D. C., Ma, W., and Wei, Q.:** Purification and characterization of a novel thermostable chitinase from *Thermomyces lanuginosus* SY2 and cloning of its encoding gene, Agric. Sci. China, **7**, 1458–1465 (2008).
43. **Sakai, K., Yokota, A., Kurokawa, H., Wakayama, M., and Moriguchi, M.:** Purification and characterization of three thermostable endochitinases of *Bacillus* noble strain MH-1 isolated from chitin containing compost, Appl. Environ. Microbiol., **64**, 3397–3402 (1998).
44. **Suganthi, M., Senthilkumar, P., Arvinth, S., and Chandrashekara, K. N.:** Chitinase from *Pseudomonas fluorescens* and its insecticidal activity against *Helopeltis theivora*, J. Gen. Appl. Microbiol., **63**, 222–227 (2017).
45. **Regev, A., Keller, M., Strizhov, N., Sneh, B., Prudovsky, E., Chet, I., Ginzberg, I., and Koncz–Kalman, Z.:** Synergistic activity of a *Bacillus thuringiensis* delta–end toxin and a bacterial endochitinase against *Spodoptera littoralis* larvae, Appl. Environ. Microbiol., **62**, 3581–3586 (1996).
46. **Nilambari, S. P. and Jyoti, P. J.:** Significance of *Penicillium ochrochloron* chitinase as a biocontrol agent against pest *Helicoverpa armigera*, Chemosphere, **128**, 231–235 (2015).
47. **Halder, S. K., Maity, C., Jana, A., Pati, B. R., and Keshab, C. M.:** Chitinolytic enzymes from the newly isolated aeromonas *Hydrophila* SBK1: study of the mosquitocidal activity, Biocontrol, **57**, 441–449 (2012).
48. **Broadway, R. M., Gongora, C., Kain, W. C., Sanderson, J. P., Monroy, J. A., Bennet, K. C., Warner, J. B., and Hoffman, M. P.:** Novel chitinolytic enzymes with biological activity herbivorous insects, J. Chem. Ecol., **24**, 985–998 (1998).
49. **Habeeb, S. M., Ashry, H. M., and Saad, M. M.:** Ovicidal effect of chitinase and protease enzymes produced by soil fungi on the camel tick *Hyalomma dromedarii* eggs (Acari: Ixodidae), J. Parasit. Dis., **41**, 268–273 (2017).
50. **Sasaki, C., Yokoyama, A., Itoh, Y., Hashimoto, M., Watanabe, T., and Fukamizo, T.:** Comparative study of the reaction mechanism of family 18 chitinases from plants and microbes, J. Biochem., **131**, 557–564 (2002).
51. **Yan, R., Hou, J., Ding, D., Guan, W., Wang, C., and Wu, Z.:** In vitro antifungal activity and mechanism of action of chitinase against four plant pathogenic fungi, J. Basic Microbiol., **48**, 293–301 (2008).
52. **Dahiya, N., Tewari, R., Tiwari, R. P., and Hoondal, G. S.:** Chitinase from *Enterobacter* sp. NRG4: its purification, characterization and reaction pattern, Electron. J. Biotechnol., **8**, 134–145 (2005).
53. **Kamil, Z., Rizk, M., Saleh, M., and Moustafa, S.:** Isolation and identification of rhizosphere soil chitinolytic bacteria and their potential in antifungal biocontrol, Global J. Mol. Sci., **2**, 57–66 (2007).
54. **Kim, Y. C., Jung, H., Kim, K. Y. K., and Park, S. K.:** An effective biocontrol bioformulation against *Phytophthora* blight of pepper using growth mixtures of combined chitinolytic bacteria under different field conditions, Eur. J. Plant Pathol., **120**, 373–382 (2008).