Stability studies of cuticle degrading and mycolytic enzymes of *Myrothecium verrucaria* for control of insect pests and fungal phytopathogens

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Myrothecium verrucaria produced extracellularly hydrolytic enzymes which can hydrolyse the insect cuticle as well as fungal cell wall. The addition of polyols, such as glycerol, sorbitol, xylitol (1 M) during ultra-filtration or freeze- drying of enzyme mixture increased the recovery of the enzymes in a concentrated form. Polyols (5 M) increased the temperature and pH stability of the enzymes, in the presence of glycerol chitinase , β-1,3-glucanase, lipase and protease retained 50-60% of initial activities at 40°C after 3 h. While xylitol (5 M) was effective in stabilizing activities at pH 5.0 and 7.5 at 25°C for 7 d. In the freeze-dried powder form, > 90% at 4°C for 1 year and 80-85% at 25°C for 2 months enzyme activities were retained. The addition of glycerol (1 M) to the enzyme mixture protected enzyme activities under sunlight (60-65% activity at RT) for 5 d. Because of glycerol (1 M), the efficacy of *M. verrucaria* enzyme preparation to control *Helicoverpa armigera* infestation in chick pea was increased to 70 ± 19%. While without glycerol the efficacy was 55 ± 23%. The germination of peanut seeds infected with *Sclerotium rolfsii* was observed to be increased (70 ± 5%) in a pot irrigated with enzyme mixture.

Keywords: CDE/ME complex, *Myrothecium verrucaria*, *Helicoverpa armigera*, polyols, *Sclerotium rolfsii*

Introduction

The protective covers of insect pests (cuticle) and fungal pathogens (cell wall) are mainly composed of chitin, β-1,4-linked *N*-acetylglucosamine (GlcNAc) polymer. Other components of insect cuticle are protein and lipid. While fungal cell wall has glucans and mannans in addition to chitin, protein and lipid components. Therefore, it is advantageous to use hydrolytic enzymes against both the groups. The cuticle-degrading enzymes (CDEs) include proteases, lipases, and chitinases, which are the main components of the mycolytic enzyme (ME) complex too[1-4]. *Myrothecium verrucaria*, an ascomycetous fungus, extracellularly produced the cuticle degrading and mycolytic enzymes[5-6]. The number of enzyme activities present in the mixture are: chitinase (endo-chitinase, EC 3.2.1.14 and *N*-acetylglucosaminidase, NAGase, EC 3.2.1.52), chitin deacetylase (CDA, EC 3.5.1.41), chitosanase (EC 3.2.1.132), β-1,3-glucanase (EC 3.2.1.39), endoglucanase (EC3.2.1.4), mannanase (EC 3.2.1.78), protease (EC 3.4.21.62) and lipase (EC 3.1.1.3)[7-8]. The CDE/ME activities were

successfully used singly and in combination with conidia of *Metarhizium anisopliae* for the control of different insect pests and pathogens in the agriculture field[5,9,10]. While addressing the issues of commercial feasibility of using enzymes, in particular and natural product, in general, in the field the major challenges, the cost and the stability (shelf life and operational) need due consideration[9]. In the present investigations various parameters affecting the stability of this hydrolytic enzyme complex have been studied.

Materials and Methods

Organism and Growth Conditions

Myrothecium verrucaria (MTCC 5191) was maintained on potato dextrose agar (PDA) slants at 28°C with 70-80% (relative humility) for 7 days. For long term maintenance it was routinely sub-cultured after 1 month and the mother cultures were maintained at 8°C until used.

Production of Cuticle Degrading/Mycolytic Enzymes of *M. verrucaria*

The extracellular CDE/ME activities of *M. verrucaria* were produced in a medium containing (g/L): chitin, 5; KH_2PO_4, 3; K_2HPO_4, 1; $(NH_4)_2SO_4$, 1.4; $MgSO_4$, 0.7; NaCl, 0.5; $CaCl_2$, 0.5; yeast extract, 0.5; bacto-

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peptone, 0.5; urea, 0.3; oxgall, 1 and trace metal solution, 1 ml (contained: $FeSO_4$, 5 mg; $MnSO_4$, 1.56 mg; $ZnSO_4$, 3.34 mg; $CoCl_2$, 2 mg); pH 6 under shaking condition (180 rpm) at 28°C for 7 d as described by Vyas and Deshpande (1989)[6]. The culture supernatant obtained after centrifugation (5000 × g) was adjusted to pH 5.0 with citric acid (1.0 M).

Downstream Processing of Cuticle Degrading/Mycolytic Enzymes of *M. verrucaria*

Membrane concentration using hollow fiber polysulfone membrane (10 KDa) of crude culture supernatant of *M. verrucaria* was carried out for different time intervals. Other methods used for concentration were freeze- drying of the enzyme mixture to dryness, spray- drying and $(NH_4)_2SO_4$ saturation (90%). The concentrated enzyme preparations were stored at -20°C until use.

Effect of Polyols on Thermostability of Cuticle Degrading/ Mycolytic Enzymes of *M. verrucaria*

The effect of different polyols (1-5 M, ethylene glycol, glycerol, xylitol and sorbitol) on the temperature (4°C, 25° C and 40° C) stability of the enzyme activities was studied. At regular intervals, aliquots of the enzyme mixture were removed and the residual enzyme activities in the sample were then estimated. The enzyme mixture without the addition of polyol was used as a control.

pH Stability of Cuticle Degrading/Mycolytic Enzymes of *M. verrucaria*

The pH stability of the enzyme mixture was studied by incubating crude enzyme mixture at 25°C for 7 day in a buffer of desired pH. The following buffers were used: acetate buffer (50 mM, pH 4.0-5.0), phosphate buffer (50 mM, pH 6.0-7.0), Tris-HCl buffer (50 mM, pH 8) and carbonate-bicarbonate buffer (50 mM, pH 9.0-10.0). At regular intervals, aliquots of the enzyme mixture were removed and the residual enzyme activities in the sample were then estimated.

UV Stability of Cuticle Degrading/Mycolytic Enzymes of *M. verrucaria*

The crude culture filtrate of *M. verrucaria* (10 ml) in a glass Petri plate was irradiated with 12 W electric UV lamp from 20 cm distance at 25°C for 5 h. The enzyme sample kept in a glass plate with lid at same distance was used as a control. After every hour aliquots were taken for assays.

The cumulative effect of sunlight and temperature on the stability of enzymes of *M. verrucaria* was also studied in the presence and absence of glycerol (1M) for 7 d. The enzyme samples incubated in dark at room temperature and at 4°C were served as controls.

Adsorption and Stability of Cuticle Degrading/Mycolytic Enzymes of *M. verrucaria* in Presence of Soil Constituents

A pot containing soil was drenched with *M. verrucaria* enzyme mixture. The 100 mL enzyme preparation was drenched/pot (containing chitinase, 50 U; β-1,3-glucanase, 7 U; lipase, 40 U and protease, 3 U) and the adsorption of enzymes on soil organic matter was determined. The soil samples (10 g) were taken from different portions of the pot (top 1-3 cm, middle 4-6 cm and bottom 7-9 cm) and enzyme activities were estimated by repeated washing (2-3 times) of the soil with acetate buffer (50 mM, pH 5.0).

Enzyme Assays

Chitinase Assay

Acid swollen chitin was used to estimate total chitinase activity in *M. verrucaria* culture filtrate as described by Vyas and Deshpande (1989)[6]. The acid-swollen chitin was prepared using crab shell chitin (Sigma) as described earlier[6]. The swollen chitin was then dialyzed at 4º C against distilled water. After homogenization in a waring blender for 1 min, the concentration of acid swollen chitin was adjusted to 7 mg/mL with acetate buffer (50 mM, pH 5.0).

The reaction mixture containing 1 mL acid swollen chitin, 1 mL of acetate buffer (50 mM, pH 5.0) and 1 mL of enzyme was incubated at 50° C for 1 h and then centrifuged (9000 x g, 5 min) and 0.5 ml of the clear aliquot was mixed with 0.1 mL borate buffer (50 mM, pH 9.2) and kept in boiling water bath for 3 min to stop the reaction. The *N*-acetylglucosamine (GlcNAc) produced was estimated colorimetrically at 585 nm with p-dimethyl amino benzaldehyde (DMAB) according to Reissig *et al* (1955)[11] and also as a reducing sugar by method of Somogyi (1952)[12]. One unit was defined as the activity which produced 1 μmol of GlcNAc /mL/min.

Endochitinase Assay

Endochitinase activity was estimated using ethylene glycol chitin as a substrate, as described earlier[6]. The reaction mixture containing 0.5 mL 1% ethylene glycol chitin in acetate buffer (50 mM, pH 5.0) and 0.5 ml of enzyme was incubated at 50º C for 30 min. The GlcNAc produced was measured as above. One international unit was defined as the activity which produced 1 μmol of GlcNAc/mL/ min.

N-Acetylglucosaminidase Assay

The *N*-acetylglucosaminidase activity was determined according to Nahar *et al* (2008)[13]. The activity was carried out by measuring the amount of *p*-nitrophenol released in a reaction mixture containing 0.9 ml of 1 mg/mL of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in acetate buffer (50 mM, pH 5.0) and 0.1 mL of enzyme, incubated at 50°C for 30 min. The reaction was terminated by adding 2 ml Na_2CO_3 (200 mM). The *p*-nitrophenol released was measured at 410 nm. One international unit was defined as enzyme activity that produced 1 μmol of *p*-nitrophenol/mL/ min.

β- 1,3-Glucanase Assay

The β-1,3-glucanase was estimated using laminarin as a substrate[6]. An aliquot of 0.5 mL enzyme was mixed with 0.5 mL of 1% laminarin prepared in acetate buffer (50 mM, pH 5.0) and incubated at 50º C for 30 min. Reducing sugars were estimated as glucose equivalents by Somogyi method[12]. One international unit was defined as the enzyme activity that produced 1 μmole of glucose/mL/min.

Protease Assay

Protease activity was measured using Hammerstein casein as a substrate[14]. The reaction mixture contained 100 µL of enzyme solution, 1 mL Hammerstein casein (1%) and 1 mL of sodium carbonate buffer (50 mM, pH 9.7). Enzyme reaction was carried out at 35ºC for 20 min and terminated by the addition of 3 mL trichloroacetic acid (TCA) (2.6 mL of 5% TCA+ 0.4 mL of 3.3N Hcl). The absorbance of the TCA soluble fraction was measured at 280 nm. One unit of enzyme released 1 μmol of tyrosine/mL/min.

Lipase Assay

Lipase activity was determined as described by Pignede *et al* (2000)[15]. The substrate emulsion was prepared with gum arabic (165 mL, 10% w/v, Sigma), olive oil (20 mL) and 15 g crushed ice. The reaction mixture contained 1 mL enzyme solution, 5 mL substrate emulsion and 2 mL of phosphate buffer (50 mM, pH 6.8). The reaction was carried out with shaking (80 rpm) at 37º C for 1 h. The reaction was terminated with 4 mL of acetone-ethanol (1:1 v/v) containing phenolphthalein (0.09 %) as an indicator. Enzyme activity was determined by titration of the fatty acids released with 50 mM NaOH. One unit of lipase is the amount of enzyme that released 1 μmole of fatty acids/mL/ min (measured as μmoles of NaOH required to neutralize under specific experimental condition).

Protein Estimation

Protein was estimated according to Lowry *et al* (1951)[16], using bovine serum albumin as a standard.

Testing of Cuticle Degrading/Mycolytic Enzymes for the Control of *Helicoverpa armigera* and *Sclerotium rolfsii*

The performance of enzyme preparation of *M. verrucaria* was studied against *H. armigera* in chickpea fields. *M. verrucaria* enzyme preparation mixed with glycerol (1M) was also used for experiment. The experimental layout was a randomised complete block design (RCBD) with seven treatments having three replications[14]. In view of the evaluation of enzyme preparation in the field the details of other treatments are not included in the present manuscript.

The root pathogen *S. rolfsii* was grown in YPG medium (100 ml), under shaking (180 rpm) at 28°C for 72 h. The soil: sand (2:1) mixture (25 kg) was sterilized by autoclaving. The soil-sand mixture was allowed to cool and infested with the broth containing mycelial mass (1000 mL) of *S. rolfsii*. Peanut seeds (2 nos.) were sown per pot (10 pots/ treatment) containing 200 g soil. The effect of enzyme mixture (100 mL enzyme mixture/ pot: chitinase, 50 U; β-1,3-glucanase, 7 U; lipase, 40 U and protease, 2 U) of *M. verrucaria* against *S. rolfsii* was studied. The drenching of enzyme preparation was done at an interval of 7 d (0, 7 and 14th d) while on other days the enzyme treated pots were irrigated with sterile distilled water (100 mL/pot). The results were compared with chemical fungicide, carzim (0.8 % w/v) treatment. Sterile soil and pathogen infested soil were also kept for comparison. All other pots were irrigated with sterile distilled water (100 mL/pot) every alternate day. Plants were allowed to grow up to 21 d. Percent peanut seed germination was recorded.

Results

Extracellular Cuticle Degrading/Mycolytic Enzyme Activities Produced by *M. verrucaria*

M. verrucaria produced extracellularly hydrolytic enzymes which were useful in the degradation of insect cuticle as well as fungal cell wall. As chitin is the main component of protective cover of pest and pathogens, the main carbon source in the medium was chitin. On chitin medium *M. verrucaria* produced

main enzyme activities under shaking condition on 7th d (U/mL) as: chitinase, 1.7 ± 0.1 (2.0 ± 0.1 by Somogyi method); endochitinase, 0.08 ± 0.02 (0.5 ± 0.03 by Somogyi method); *N*-acetylglucosaminidase, 5.9 ± 0.1 ; β-1,3-glucanase, 0.19 ± 0.01; protease, 0.12 ± 0.01; lipase, 1.58 ± 0.08. Other activities in the crude broth also showing role in the biocontrol as reported earlier[5] were (U/mL): chitosanase, 1.5 ± 0.08; chitin deacetylase, $1.0 \times 10^{-3} \pm 0.04$; endoglucanase, 0.3 ± 0.01; mannanase, 0.019 ± 0.005.

Downstream Processing Cuticle Degrading/Mycolytic Enzymes of *M. verrucaria*

The main concern for the application of enzymes in the field is transport of active preparation to the field. In this regard, the extracellular CDE/ME mixture produced by *M. verrucaria* was concentrated using different methods such as membrane concentration (Hollow fiber membrane, 10kDa), freeze drying, spray drying and ammonium sulphate (90%) precipitation. In case of spray drying 35-45% recovery of enzyme activities and protein was noted (data not shown). While in membrane concentration 70-90% and freeze drying 50-90% recovery of enzyme activities were observed (Table 1). Furthermore, the addition of polyol (1M) during the process increased the % recovery of all the activities (Table 1). As the crude culture filtrate had low protein (0.6-0.8 mg/ ml), the ammonium sulphate (90 %) saturation did not give any appreciable recovery of the enzyme activities. Therefore, the precipitation was carried out with the addition of different proteinaceous material. In the presence of skimmed milk powder (0.4%) the recovery of enzyme activities was increased to 60- 85% (Table 1).

Further studies were carried out with a freeze-dried powder (1 g) reconstituted in 35 mL of acetate buffer (50 mM, pH 5.0) having following activities (U/ mL): chitinase, 5.0 ± 0.2 (6.0 ± 0.2 by Somogyi method); endochitinase, 0.22 ± 0.02 (1.29 ± 0.03 by Somogyi method); *N*-acetylglucosaminidase, 15.88 ± 0.62 ; β-1,3-glucanase, 0.6 ± 0.04; protease, 0.3 ± 0.01; lipase, 4.32 ± 0.1.

Optimum Temperature and pH of Enzyme Complex of *M. verrucaria*

The optimum activities of *Myrothecium* chitinase (acid swollen chitin degrading activity, 5.0 ± 0.2 U/mL), endochitinase (0.22 ± 0.02 U/mL), *N*-acetyl-glucosaminidase (15.88 ± 0.62 U/mL) and β-1,3-glu-canase (0.60 ± 0.04 U/mL) were exhibited at 50°C, while lipase (4.32 ± 0.1 U/mL) and protease (0.30 ± 0.01 U/mL) showed maximum activity at 37°C.

Chitinolytic enzyme activities as well as β-1,3-glucanase activity of *M. verrucaria* had optimum pH 5.0. Lipase showed maximum activity at pH 7.0 while protease at pH 10.0.

Effect of Polyols on the Temperature Stability of Enzyme Complex of *M. verrucaria*

Stability of *M. verrucaria* enzyme mixture was carried out at 4°C, 25°C and 40°C with and without polyols such as ethylene glycol, glycerol, xylitol and sorbitol (5M). The Fig. 1 depicts retention of the enzyme activities with and without polyol at 40° C after 3 h. Glycerol (5M) showed maximum protection for chitinase, β-1,3-glucanase and lipase while maximum stability of protease was observed in the presence of sorbitol (5M). In the presence of glycerol

Additive	Final volume (%)	Residual activity (%)		
		Chitinase	Protease	Lipase
Ultra filtration	10			
No additive		91.2 ± 4.2	73.0 ± 3.0	75.0 ± 8.0
Glycerol (1 M)		95.9 ± 0.2	91.9 ± 4.9	79.4 ± 4.5
Lyophilization	Dry powder			
No additive		86.1 ± 3.6	50.8 ± 2.7	50.0 ± 4.8
Glycerol (1 M)		90.9 ± 3.8	94.4 ± 3.0	99.0 ± 1.0
Sorbitol (1 M)		86.8 ± 3.0	91.3 ± 3.1	99.0 ± 1.0
Mannitol (1 M)		90.8 ± 3.5	75.9 ± 2.9	99.0 ± 1.0
Ammonium sulphate (90%) precipitation	30-40			
No additive		35.7 ± 3.2	ND	0.6 ± 0.2
Defatted soya (0.3%)		70.9 ± 2.8	47.6 ± 3.8	71.9 ± 4.1
Skimmed milk powder (0.4%)		86.9 ± 2.4	74.7 ± 3.1	59.9 ± 2.9
Wheat bran (0.35%)		56.6 ± 4.5	47.5 ± 5.2	20.1 ± 3.6

Table 1 — Downstream processing of CDE/ME complex of *Myrothecium verrucaria*

chitinase, endochitinase, *N*-acetylglucosaminidase and protease retained 50-52% of initial activity at 40°C after 3 h. While lipase and β-1,3-glucanase retained 56-60% of initial activities.

Effect of glycerol concentrations less than 5 M up to 1 M on chitinase and other enzymes of *M. verrucaria* was also studied (Fig. 2). As the concentration of glycerol decreased the protective effect was also decreased for all the enzymes.

In the present study, glycerol was found to be suitable polyol for protection of chitinases and other enzymes of *M. verrucaria* at 40°C. Therefore the stability of enzyme mixture in liquid and in powder form was tested with and without glycerol at 4°C, 25°C and 40°C (Table 2). In presence of glycerol (5M) under liquid conditions all the enzymes retained 60-70% activity at 4°C for 6 m. In absence of glycerol, activities of these enzymes were 15-22%. In presence of glycerol (5M) all the enzymes showed 48-60% activity at 25°C up to 3 weeks. In lyophilized powder 70-75% enzyme activities were observed at 4°C after
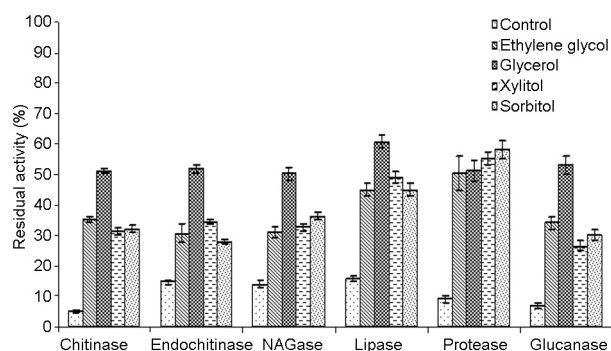

Fig. 1 — Effect of polyols on the temperature stability of CDE/ME activities of *Myrothecium verrucaria*. Enzyme activities were measured after incubation at 40°C with (5 M) and without polyols for 3 h. The initial enzyme activities of the reconstituted lyophilized powder were as described in the text.
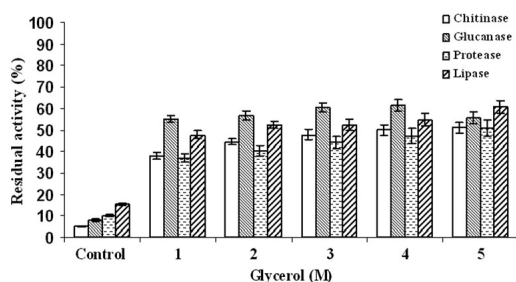

Fig. 2 — Effect of different concentration of glycerol on the temperature stability of CDE/ ME activities of *Myrothecium verrucaria*. Enzyme activities after incubation at 40°C with (1-5 M) and without glycerol for 3 h. The initial enzyme activities of the reconstituted lyophilized powder were as described in the text.

1 year, while > 90% enzyme activities were obtained in lyophilized powder mixed with glycerol (1 g enzyme powder mixed with 5 g glycerol). The protective effect of glycerol was more promising at 25°C and 40°C.

Effect of Different pHs on CDE/ME Complex of *M. verrucaria*

The pH stability of chitinase and other enzymes of *M. verrucaria* was studied by incubating the crude enzyme mixture at different pHs (4 to 10) at 25°C for 7 d. Chitinase, endochitinase and *N*-acetylglucosaminidase were stable in the pH range (5 to 7) with 59-72% residual activities at 25°C after 7 d. These enzymes also retained 50% of initial activities at pH 4 and 8 at 25°C after 7 d. The protease from *M. verrucaria* was more stable in the pH range of 6-10, with 71-79% residual activities at 25°C after 7 d. It also exhibited 60% residual activity at pH 5. Lipase from *M. verrucaria* was stable in the pH range 5-7, with 65-74% residual activities at 25°C after 7 d. It also exhibited 45% and 43% residual activities at pH 4 and 8, respectively. β-1,3-glucanase from *M. verrucaria* was stable in the pH range 4-6, with 70-79% residual activities at 25°C after 7 d. It also exhibited 65% and 48% residual activity at pH 7 and 8.

Effect of Polyols on the pH Stability of Enzyme Complex of *M. verrucaria*

The *M. verrucaria* chitinase, protease and lipase activities were estimated after the incubation at pH 5 and pH 7.5 (usual soil pH) at 25°C with and without polyols such as glycerol, xylitol and sorbitol (5M) for 7 d. It can be seen from Table 3 that the addition of polyols (5M) increased the residual activities after 7 d

Table 2 — Effect of glycerol on the temperature stability of CDE/ME activities of *Myrothecium verrucaria*

Temperature (°C)	Residual enzyme activities (%)		Time
	No addition	Glycerol*	
Culture filtrate			
4	15-22	60-70	6 months
25	<15	48-60	3 weeks
40	<15	51-61	3 h
Dry powder			
4	70-75	>90	1 year
25	60-65	80-85	2 months
40	40-50	57-65	1 week

*In Culture filtrate 5M glycerol (final concentration) was added while to lyophilized dry powder (1 g) glycerol (5 g) was added. Enzyme activities such as chitinase, endochitinase, *N*-acetylglucosaminidase, glucanase, protease and lipase were measured as described under Materials and Methods.

Table 3 — Effect of polyols on the pH stability of enzyme complex of *Myrothecium verrucaria*

Polyol (5 M)	Residual activity (%)		
	Chitinase	Protease	Lipase
pH 5			
Without addition	76.5 ± 0.8	57.8 ± 1.8	70.3 ± 2.2
Glycerol	95.1 ± 1.4	73.6 ± 2.2	86.2 ± 1.1
Sorbitol	92.1 ± 2.7	60.0 ± 2.7	90.8 ± 1.5
Xylitol	97.1 ± 1.3	99.0 ± 0.8	87.2 ± 1.0
pH 7.5			
Without addition	84.6 ± 0.8	75.5 ± 1.9	78.3 ± 1.3
Glycerol	92.2 ± 1.2	85.0 ± 2.3	84.6 ± 1.2
Sorbitol	99.7 ± 1.2	99.0 ± 0.7	84.4 ± 1.3
Xylitol	97.3 ± 2.5	97.2 ± 1.7	85.9 ± 1.2

incubation at pH 5 and 7.5. Among the three polyols tested, in the presence of xylitol all the residual activities were > 85% (Table 3).

UV Stability of Chitinase and Other Enzymes of *M. verrucaria*

For foliar spray, due consideration is necessary for the effect of ultraviolet radiation, if any, on biological preparation sprayed for control of pest and pathogens. In the presence of ultraviolet radiation the chitinase and other enzymes retained 85-90% residual activities at 25°C up to 5h (data not shown).

Usually natural sunlight especially the UV portion of the spectrum UV-B (280-310 nm) and UV-A (320-400 nm) were responsible for inactivation of different proteins. The cumulative effect of sunlight and temperature on the stability of chitinase and other enzymes of *M. verrucaria* was also studied in presence of glycerol (1M) for 7 d. The enzyme samples incubated in dark at room temperature (RT) and at 4°C were served as controls. Chitinase and other enzymes retained 41- 46% activity in presence of sunlight after 5 d. The enzymes incubated in dark retained 51-55% activity at RT after 5 d and at 4°C, 88-92% activities were observed. From above results it can be seen that only slight decrease (15 %) in the enzyme activities of *M. verrucaria* due to sunlight was observed. The yellowish-brown colour of the crude enzyme mixture might be protecting the enzyme activities. The addition of glycerol (1M) to the enzyme mixture protected enzyme activities under sunlight (60-65% activity at RT after 5 d) (data not shown).

Adsorption and Stability of Cuticle Degrading/Mycolytic Enzymes of *M. verrucaria* in Presence of Soil Constituents

During the pot experiment studies, a pot containing soil was drenched with *M. verrucaria* enzyme mixture. The 100 mL enzyme preparation (containing chitinase, glucanase, lipase and protease) was drenched/pot and the adsorption of enzymes and protein on soil organic matter was determined. Maximum enzyme activities were present in the top portion (50-52%) of the soil, while adsorption in the middle portion was 20-23% and at the bottom 10-17% adsorption was observed. This was correlated with adsorption of protein suggesting that there was no drastic differential adsorption of enzyme activities which could be released by washing (data not shown) (not required). This indicated that the soil application of the enzyme mixture can be used to control soil pathogens at different layer of soil by the slow release of enzyme activities from the organic matter.

Testing of CDE/ME Complex for the Control of *Helicoverpa armigera* and *Sclerotium rolfsii*

Under field conditions, the efficacy of *M. verrucaria* enzyme preparation measured as chitinase 2 U/mL to control *H. armigera* infestation was 55 ± 23%. The incorporation of glycerol (1M) in enzyme preparation of *M. verrucaria* showed increased efficacy (70 ± 19%) against *H. armigera* in the chickpea field. Furthermore, due to addition of glycerol pod damage decreased from 27 ± 5 % to 19 ± 4% while yield was increased by 27.9 %.

In the present investigations, the performance of enzymes (100 mL preparation/pot, chitinase, 50 U; β-1,3-glucanase, 7 U; lipase, 40 U and protease 2 U) of *M. verrucaria* was tested against *S. rolfsii* in pot experiment. Each treatment contained 10 pots with 2 seeds % pot. The germination of peanut seeds was 70 ± 5% treated with enzyme mixture of *M. verrucaria* in *S. rolfsii* infected pots. In the control treatment (pots containing sterile soil) irrigated with distilled water 90 ± 5% germination was seen while only 10 ± 5% germination was observed in *S. rolfsii* infested pots drenched with sterile distilled water. The pots treated with carzim showed 65 ± 10% germination of peanut seeds in *S. rolfsii* infected soil.

Discussion

Cuticle of insect and fungal cell wall are two protective covers and sometimes some insects like woolly aphids and mealy bugs have additional waxy coating too. The lipase activity produced by *M. verucaria* was useful to hydrolyze the additional waxy coating[5,6,10].

The temperature and pH optima chitinse enzymes were not drastically different from other reports in the

literature[17-19]. For instance, the temperature optima for chitinases in general are >40° C. While protease and lipase activities are usually optimal around 40° C. While pH optima for these activities were reported to be different from different sources. For example, that the optimum pH for the chitinase from *Beauvaria bassiana* was alkaline (pH 9.2) while that from *Bacillus* and *P. aeruginosa* strains pH optima were nearly neutral (pH 7)[20-23]. The chitinases from *Aeromonas* sp. exhibited higher activity in acidic condition[24]. Similarly in the present investigations, chitinases and glucanase activities of *M. verrucaria* were found optimum at pH 5.0. The optimum pH for protease from *B. cereus* was between 8-9 which was lower than *Myrothecium* protease activity[25-27].

M. verrucaria* enzymes retained 5-15% activity at 40° C after 3 h Fig. 1. In the present studies, glycerol (5M) showed maximum protection for lipase, chitinase and β-1,3-glucanase while maximum stability of protease was observed in the presence of sorbitol (5M) (Fig. 1). Bhushan (2000) showed that glycerol stabilized chitinase activity of *Bacillus* sp (>75 % activity after 8 weeks)[29]. The maximum stability of protease from *Bacillus cereus* at 60°C was reported to be with sorbitol (5 M). Matsumoto *et al* (1999) studied the influence of various polyols such as ethylene glycol, glycerol, xylitol and sorbitol on the thermostability of lipase from *Candida cylindracea*[30]. Maximum protection of lipase was observed in the presence of sorbitol[30]. Moreover, the concentration dependent effect of polyol was seen for lipase from *C. cylindracea* and same also in the present investigations Fig. 2. While studying thermostability of purified xylanase of *Aspergillus niger* the effect of addition of polyols (1M) such as

Lipase from *M. verrucaria* was stable in the pH range 5-7, with 65-74% residual activities at 25°C after 7 d. While the lipase activity of the crude extract from *P. aurantiogriseum*[28] remained stable in the pH range of 5.0 - 9.0. However, fungal lipases are, in general, quite stable in the pH range from 4 to 7 and unstable in alkaline pH. Most of the soils under agriculture fields have pH towards neutral or alkaline side. In that case the enzyme activities at that pH would contribute significantly in the biocontrol activities against soil pathogens. In this regard, most of the enzymes except lipase which has optimum pH 7 exhibited effectively 80-90 % activities at alkaline pH.

ethylene glycol (2C), glycerol (3C) and sorbitol (6C) was reported[31]. It was suggested that the thermo-stabilizing effect was proportional to the molecular size of polyol, which can further be correlated with the number of hydroxyl groups per polyol molecule. The sorbitol was found to have maximum protecting effect. However, in the case of *M. verrucaria* enzymes glycerol (3C) also was found to be an effective protectant. The protective effect of polyols can be attributed to hydrophobic interactions among the non-polar amino acid residues of the enzyme making the protein more resistant to unfolding and thermal denaturation[32]. Furthermore, the addition of polyols (5M) increased the residual activities of *M. verrucaria* at 25°C after 7 d incubation at pH 5 and 7.5 (Table 3).

The insecticidal activity of *B. thuringiensis* on crops following exposure to direct sunlight was reported to be reduced. *B. thuringiensis* applied to spruce trees lost 50% of the insecticidal activity in 8 d in shade, compared with 50% loss in 2 d in sunlight[33]. In case of *M. verrucaria* enzyme preparation, chitinase and other enzymes retained only 41-46% activities in presence of sunlight after 5 d. The addition of glycerol (1 M) was found to protect activities under sunlight significantly for 5 days.

Earlier Shternshis *et al* (2002) reported that foliar application of microbial chitinases was effective for the control of raspberry midge blight in Siberia[34]. The combination of chitinase and *B. thuringiensis* increased the mortality of *P. sticticalis*[35]. Duzhak *et al* (1995) observed that the insecticidal activity of two granulosis viruses and NPV was significantly increased by the addition of bacterial chitinase preparation[36]. The crude enzyme mixture of *M. verrucaria* in presence of glycerol (1M) was found to be effective against *H. armigera* in the chickpea field.

Biocontrol of soil born pathogen involves antibiosis, competition or hyperparasitism. Among them, hyperparasitism relies on lytic enzymes for the degradation of cell walls of pathogenic fungi[37]. Chitinase and β-1,3-glucanase when acting alone or synergistically, were shown to inhibit the growth of pathogenic fungi by degradation or lysis of fungal cell walls[38-40]. The use of crude enzyme mixture of *M. verrucaria* against *S. rolfsii* in pot experiment was also effective as fungicide.

In conclusion, the development of broad spectrum biocontrol agent which can target insect pest and fungal pathogens in single crop system can alleviate

the problem of high cost of application of biocontrol agent(s) in the field[7]. Use of fungi like *Trichoderma, Verticillium,* and *Metarhzium* having dual specificity against insect pest and fungal pathogens is one of the approaches for broad spectrum activity which was due to mainly cuticle degrading and mycolytic enzymes produced by these organisms[1]. One of the advantages of *Myrothecium* preparation is a range of enzymes produced when chitin was used as a sole carbon source. All these enzymes, cuticle degrading and mycolytic, play significant role in biocontrol of insect pests and fungal plant pathogens. In future the same preparation can be fortified with enzymes with different substrate specificities from different sources which can be useful to develop high volume low cost product, applied singly and in combination with other agents, for agricultural practices.

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References

1   Deshpande M V, Mycopesticides production by fermentation: potential and challenges, *Crit Rev Microbiol*, 25 (1999) 229-243.

2   Kaur G & Padmaja V, Relationships among activities of extracellular enzyme production and virulence against *Helicoverpa armigera* in *Beauveria bassiana*, *J Basic Microbiol*, 49 (2009) 264-274.

3   Mishra P, Kshirsagar P R, Nilegaonkar S S & Singh S K, Statistical optimization of medium components for production of extracellular chitinase by *Basidiobolus ranarum*: a novel biocontrol agent against plant pathogenic fungi, *J Basic Microbiol*, 52 (2012) 539-548.

4   Sandhya C, Adapa L K, Nampoothiri K M, Binod P, Szakacs G *et al*, Extracellular chitinase production by *Trichoderma harzianum* in submerged fermentation, *J Basic Microbiol*, 44 (2004) 49-58.

5   Chavan S, Kolomiets E, Kuptsov V, Mandrik M, Kulkarni M *et al*, Significance of cuticle degrading enzymes with special reference to lipase in biocontrol of sugarcane woolly aphids, *J Mycol Plant Pathol*, 39 (2009) 118-123.

6   Vyas P & Deshpande M, Chitinase production by *Myrothecium verrucaria* and its significance for fungal mycelia degradation, *J Gen Appl Microbiol*, 35 (1989) 343-350.

7   Vidhate R & Deshpande M V, Biocontrol of plant pathogenic fungi and insects pests: Hydrolytic enzyme as biological control agents, *SBSI Biopestic News*, 17 (2013) 3-5.

8   Vyas P & Deshpande M V, Enzymatic hydrolysis of chitin by *Myrothecium verrucaria* chitinase complex and its

utilization to produce SCP, *J Gen Appl Microbiol*, 37 (1991) 267-275.

9   Chavan S B & Deshpande M V, Chitinolytic enzymes: An appraisal as a product of commercial potential, *Biotechnol Prog*, 29 (2013) 833-846.

10 Patil R S, Deshpande A M, Natu A A, Nahar P, Chitnis M *et al*, Biocontrol of root infecting plant pathogenic fungus, *Sclerotium rolfsii* using mycolytic enzymes and chitin metabolism inhibitors singly and in combination, *J Biol Control*, 15 (2001) 157-164.

11 Reissig J L, Strominger J L & Leloir LF, A modified colorimetric method for the estimation of N-acetylamino sugars, *J Biol Chem*, 217 (1955) 959-966.

12 Somogyi M J, Notes on sugar determination, *J Biol Chem*, 195 (1952) 19-23.

13 Nahar P B, Kulkarni S A, Kulye M S, Chavan S B, Kulkarni G *et al*, Effect of repeated *in vitro* sub-culturing on the virulence of *Metarhizium anisopliae* against *Helicoverpa armigera* (Lepidoptera: Noctuidae), *Biocontrol Sci Technol*, 18 (2008) 337-355.

14 Kulkarni S A, Ghormade V, Kulkarni G, Kapoor M, Chavan S B *et al*, Comparison of *Metarhizium* isolates for biocontrol of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea, *Biocontrol Sci Technol*, 18 (2008) 809-828.

15 Pignede G, Wang H, Fudale F, Gaillardin C, Seman M *et al*, Characterization of Extracellular lipase encoded by LIP2 in *Yarrowia lipolytica*, *J Bacteriol*, 182 (2000) 2802-2810.

16 Lowry O H, Rosebrough N J, Farr A L & Randall R J, Protein measurement with the Folin phenol reagent, *J Biol Chem*, 193 (1951) 265-275.

17 Binod P, Pusztahelyi T, Nagy V & Sandhya C, Production and purification of extracellular chitinases from *Penicillium aculeatum* NRRL 2129 under solid-state fermentation, *Enz Microb Technol*, 36 (2005) 880-887.

18 Revathi M, Saravanan R & Shanmugam A, Production and characterization of chitinase from *Vibrio* species, a head waste of shrimp *Metapenaeus dobsonii* (Miers, 1878) and chitin of *Sepiella inermis* (Orbigny, 1848), *Adv Biosci Biotechnol*, l3 (2012) 392-397.

19 Natsir H, Patong A R, Suhartono M T & Ahmad A, Production and characterization of chitinase enzymes from Sulili hot spring in South Sulawesi, *Bacillus* sp. HSA, 3-1a, *Indonesian J Chem*, 10 (2010) 263-267.

20 Suresh P V & Chandrasekaran M, Impact of processes parameters on chitinase production by an alkalophilic marine *Beauveria bassiana* in solid state fermentation, *Process Biochem*, 34 (1999) 257-267.

21 Yuli P E, Suhartono M T, Rukayadi Y H, Jae K & Pyun Y R, Characteristics of thermo stable chitinase enzymes from the Indonesian *Bacillus* sp. 13.26, *Enz Microb Technol*, 35 (2004) 147-153.

22 Wang S L & Chang W T, Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in shrimp and crab shell powder medium, *Appl Environ Microbiol*, 63 (1997) 380-386.

23 Sakai K, Yokota A, Kurokawa H, Wakayama M & Moriguchi M, Purification and characterization of three thermostable endochitinases of a noble *Bacillus* strain, MH-1, isolated from chitin-containing compost, *Appl Environ Microbiol*, 64 (1998) 3397-3402

24 Ueda M & Arai M, Purification and some properties of chitinases from *Aeromonas* sp. No. 10S-24, *Biosci Biotechnol Biochem*, 56 (1992) 460-464.

25 Ghorbel B, Kamoun A S & Nasri M, Stability studies of protease from *Bacillus cereus* BG1, *Enz Microb Technol*, 32 (2003) 513-518.

26 Nilegaonkar S S, Zambare V P, Kanekar P P, Dhakephalkar P K & Sarnaik S S, Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326, *Bioresour Technol*, 98 (2007) 1238-1245.

27 Wang S L, Chao C H, Liang T W & Chen C C, Purification and characterization of protease and chitinase from *Bacillus cereus* TKU006 and conversion of marine wastes by these enzymes, *Marine Biotechnol*, 11 (2009) 334-344.

28 Lima V M G, Krieger N, Mitchell D A & Fontana J D, Activity and stability of a crude lipase from *Penicillium aurantiogriseum* in aqueous media and organic solvents, *Biochem Eng J*, 18 (2004) 65-71.

29 Bhushan B, Production and characterization of a thermostable chitinase from a new alkalophilic *Bacillus* sp. BG-11, *J Appl Microbiol*, 88 (2000) 800-808.

30 Matsumoto M, Kida K & Kondo K, Effects of polyols and organic solvents on thermostability of lipase, *J Chem Technol Biotechnol*, 70 (1999) 188-192.

31 Pal A & Khanum F, Characterizing and improving the thermostability of purified xylanase from *Aspergillus niger* DFR-5 grown on solid-state-medium, *J Biochem Technol*, 2 (2010) 203-209.

32 Hahn-Hägerdal B, Water activity: a possible external regulator in biotechnical processes, *Enz Microb Technol*, 8 (1986) 322-327.

33 Ragaei M, Laboratory evaluation of shellac as ultraviolet screen for the *Bacillus thuringiensis* var. *entomocidus* against *Spodoptera littoralis* larvae, *Anzeiger für Schädlingskunde, Pflanzenschutz, Umweltschutz*, 71 (1998) 132-134

34 Shternshis M V, Beljaev A A, Shpatova T V, Bokova J V & Duzhak A B, Field testing of Bacticide®, Phytoverm® and chitinase for control of the raspberry midge blight in Siberia, *Biocontrol*, 47 (2002) 697-706.

35 Shternshis M, Biopreparations for plant protection in Siberia: application and enhancement of activity, *J Agric Technol*, 1 (2005) 1-18.

36 Duzhak A B, Salganik R I, Shternshis M V, Ermakova N I, Tsvetkova V P *et al*, The influence of enzyme preparation of chitinase on biological insect control, *Siberian J Ecol*, 2 (1995) 457-461.

37 Chet I, Ordentlich A, Shapira R & Oppenheim A, Mechanisms of biocontrol of soil-borne plant pathogens by rhizobacteria, *Plant Soil*, 129 (1990) 85-92.

38 Mauch F, Mauch-Mani B & Boller T, Antifungal hydrolases in pea tissue II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase, *Plant Physiol*, 88 (1988) 936-942.

39 Wang S L, Yieh T C & Shih I L, Production of antifungal compounds by *Pseudomonas aeruginosa* K-187 using shrimp and crab shell powder as a carbon source, *Enz Microb Technol*, 25 (1999) 142-148.

40 Pradeep G C, Hah Y Y, Seung S C, Yun H C & Jin C Y, An extracellular chitinase from *Streptomyces* sp. CS147 releases N-acetyl-D-glucosamine (GlcNAc) as principal product, *Appl Biochem Biotechnol*, (2014) 1267-1266.