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Production, partial purification and optimization of a chitinase produced from *Trichoderma viride*, an isolate of maize cob

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Abstract

Trichoderma viride obtained from maize cob was investigated for chitinase production. Effects of temperature, pH and some metal ions on the chitinase produced were determined. The enzyme was then purified by ammonium sulphate precipitation, DEAE-cellulose ion-exchange chromatography and sephadex G-100 gel filtration. There was progressive increase in the chitinolytic activity of *T. viride* from zero hour to the 50th hour. The optimum temperature and pH for chitinolytic activity of *T. viride* was 50°C and 5 respectively. There was reduction in the relative activity of chitinase produced by *T. viride* when EDTA and $MnCl_2$ were used as metal ions. *Trichoderma viride* was most sensitive to EDTA followed by $MnCl_2$. The activity was maximum when $CaCl_2$ was used. The chitinase produced by *T. viride* was stable at temperatures of 40 and 50°C.The chitinase produced was also stable at pH 6 and 7.

Keywords – chitinolytic activity – metal ions – stable – *T. viride*,

Introduction

Trichoderma species have been described as versatile mould, a nuisance to humans, useful fungi for industry and biocontrol and a bane to other fungi. They are present in nearly all soils and other diverse habitats. They are the most prevalent culturable fungi in soils (Mishra et al. 2011). They are common inhabitant of rhizosphere and contribute to control of many soil borne plant diseases caused by fungi (Chet et al. 1997, Sharma et al. 2011). Some genes of *Trichoderma* species can be used to provide resistance to the biotic and abiotic stresses such as salt, heat and drought (Kuc 2001).

Trichoderma species are used widely as biocontrol agents because they have more benefits to plant growth such as promoting plant growth, increasing the nutrient uptake from the soil, and decreasing the activity of the soil borne pathogens that ultimately affect the growth of the plant (Harman et al. 2004). The major biocontrol process involves antibiosis, providing plant nutrition and mycoparasitism (Janisiewicz & Korsten 2002). *Trichoderma* species are known for their production of cell wall degrading enzymes among which are chitinases.

Chitinases are enzymes that hydrolyze chitin, a polymer of β-1,4-N-acetylglucosamine. They are produced by various microorganisms including *Trichodema viride* and are known play

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important physiological and ecological roles (Duo-Chan 2006). Chitinase has a wide range of, biotechnological applications such as in the manufacturing of pharmaceutically important chitooligosaccharides and N acetyl-D-glucosamine (Kuk et al. 2005, Pichyangkura et al. 2002, Sørbotten et al. 2005), isolation of protoplasts from fungi and yeasts (Dahiya et al. 2005), production of single-cell protein (Vyas & Deshpande 1991), control of phytopathogenic fungi (Mathivanan et al. 1998) as well as in the treatment of waste containing chitin (Wang & Hwang 2001). The present investigation was undertaken in order to investigate the capability of *T. viride* isolated from maize cob to produce chitinase as well as optimize and partially purify the chitinase produced.

Materials & Methods

Trichoderma viride used for enzyme production was obtained from the Department of Microbiology, Federal University of Technology, Akure (FUTA). This was fungus was isolated from maize cob obtained within FUTA environ and was maintained on malt extract agar. Also, chitin (obtained from shrimp shell) used as substrate for enzyme production was purchased from Sigma Aldrich, Germany.

Preparation of crude enzyme

A spore suspension of *T. viride* was inoculated into 100 mL flask containing 20 ml of sterile minimal synthetic medium made up of $MgSO_4$. $7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH4NO_3, 1.0; $FeSO_4$, 0.002 and $ZnSO_4$ 0.002 supplemented with chitin. The cultures were cultivated at 30°C for 5 days on a rotary shaker. Culture filtrate was centrifuged and the supernatant was used immediately for enzyme activity and served as the enzyme solution (Mishra 2010, Gajera & Vakharia 2012). A 1 ml chitin solution was mixed with 1ml of enzyme solution and then incubated for 30 minutes. The reaction was stopped by boiling for 3 min in water bath by the addition of 1ml of dinitrosalicylate (DNS) reagent. The absorbance of reaction mixture was measured in a spectrophotometer (6850 UV/vis Jenway) at 520 nm.

Protein estimation

Protein determination was done according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Effects of temperature and pH on chitinase produced from T. viride

The methods of Omumasaba et al. (2001) and Aida and Taghreed (2014) was adopted with slight modifications. Spore suspension of *T. viride* was inoculated on 10ml of sterile minimal synthetic medium supplemented with chitin as the sole carbon source and then incubated at different temperature ranges viz; 30-90 °C. The culture filtrate was harvested and then studied for enzyme activity up to 72 hours. The same procedure was repeated for the effect of pH on chitinase production by *T. viride* and this done by varying the pH of the minimal synthetic medium from pH 3 to 10.

Effect of different metal ions on chitinase produced from T. viride

Spore suspension of *T. viride* was inoculated on sterile minimal synthetic medium supplemented with chitin and different metal ions viz $ZnCl_2$, $MnCl_2$, $CaCl_2$, EDTA and KCl. The culture filtrate was harvested and then studied for enzyme activity according to the method of Jenifer et al. (2014).

Partial purification of the chitinase produced from T. viride

Crude enzyme obtained from *T. viride* was precipitated by adding to 60% saturation. The mixture was centrifuged at 10,000rpm for 15 minutes at 4°C. The precipitates were then resuspended in 20mM phosphate buffer (pH6.5) according to Omumasaba et al. (2001). The sample was then pre-wet with membrane phosphate buffer (20mM, pH 6.5) and loaded into dialyzing

device for 1-2 hours at room temperature. Dialyzed enzyme solution was concentrated with 4 M sucrose solution.

The dialyzed sample was then loaded on a pre-equilibrated DEAE –cellulose column chromatography and washed with acetate buffer. The proteins were eluted in a stepwise manner on NaCl (0-1M) at a flow rate of 24mL/h at room temperature. The fractions were collected and the absorbance read at 280nm on a spectrophotometer. The fractions having chitinase activity were combined and dialyzed and concentrated by lyophilization. The protein containing fractions were assayed for chitinase activity (Hammami et al. 2013).

The protein pellet obtained after dialysis was loaded onto a column of Sephadex G-100 equilibrated with phosphate buffer (20mM, pH 6.5). The column was eluted a flow rate of 60mL/h at room temperature with a 1:1 volume gradient on NaCl (0.1-1M) in the same buffer. The elution fractions which contain protein were collected and assayed for chitinase activity. Fractions containing chitinase activity were pooled together and concentrated (Omumasaba et al. 2001; Jenifer et al. 2014).

Effect of temperature, pH and metal ions on stability of partially purified enzyme from T. viride

Optimum temperature for enzyme activity was determined by incubating the reaction medium at different temperature levels ranging from 30to 90°C and absorbance was taken at each temperature level in order to obtain the chitinase activity. Thermostability of the enzyme was performed in eppendorf tube containing 1ml of partially purified enzyme in 50mM Tris –HCl buffer (pH 9) and then incubated at temperatures 30-90°C for 2 hours. Aliquots were withdrawn every 30 minutes and residual chitinase activity was determined using 1% chitin as the substrate and the absorbance read. Control was at 0°C. The relative activity was determined according to the method of Jenifer et al. (2014).

Effects of pH on enzyme activity was carried out with three different buffers; acetate buffer (pH 3 to 5), phosphate buffer (pH 6to 7) and Tris-HCl buffer (pH 8 to 9) for 1 hour at 37°C. The residual chitinase activity was determined by standard assay. The stability of the enzyme was evaluated by incubating enzyme solution at pH 3 to 9 for 2 hours. Residual chitinase activity and relative activity was determined according to the method of Jenifer et al. (2014).

Effects of metal ions on enzyme stability was determined by incubating 1ml of enzyme with the previously stated metal ions (0.5ml) at 37°C for 1 hour. Enzyme activity without any metal served as control. Residual chitinase activity and relative activity was determined according to the method of Jenifer et al. (2014).

Results

Chitinase production by T. viride

There was progressive increase in the chitinolytic activity of *T. viride* from zero hour to 50th hour. Both the protein content and cell mass increased from zero hour to the 70th hour of incubation (Figure 1).

Effect of temperature and pH on chitinase produced by T. viride

The optimum temperature for chitinolytic activity of *T. viride* was 50°C. There was reduction in the activity from 60 to 90°C (Figure 2). The optimum pH for chitinolytic activity of *T. viride* was 5 after which reduction set in (Figure 3).

Effect of metal ions on chitinase produced by T. viride

There was reduction in the relative activity of chitinase produced by *T. viride* when EDTA and $MnCl_2$ were used as metal ions. *Trichoderma viride* was most sensitive to EDTA followed by $MnCl_2$. The activity was maximum when $CaCl_2$ was used (Figure 4).

Table 1 Yield of chitinase after purification

Step	Vol. (mL)	CA (U)	PC (mg)	TCA (U/ml)	TP (mg/ml)	SP (U/mg)	Yield (%)	Purification (Fold)
Crude extract	78.00	81.04	36.25	6321.35	2827.50	2.24	100.00	1.00
Ammonium precipitation/concentration	22.40	75.05	24.13	1681.18	540.40	3.11	26.60	1.39
Ion exchange	12.50	112.76	10.25	1409.44	128.13	11.00	22.30	4.92
Gel filtration	6.30	118.75	6.25	748.10	39.38	19.00	11.84	8.50

CA: Chitinase activity; PC: Protein concentration; TCA: Total chitinase activity; TP: Total protein; SP: Specific activity

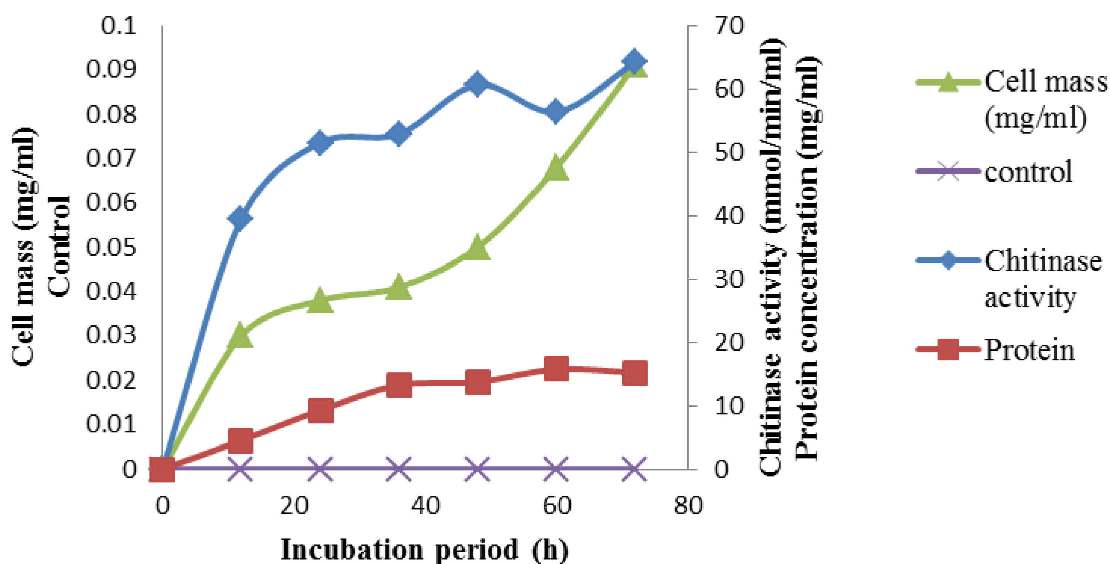


Fig. 1 – Chitinolytic activity of *Trichoderma viride* isolated from maize cob

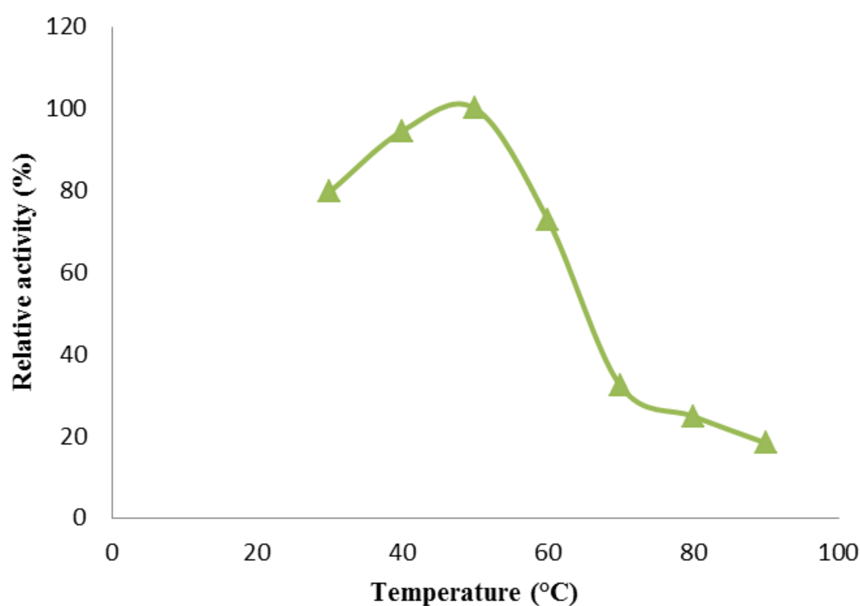


Fig. 2 – Effect of temperature on the chitinolytic activity of *T. viride*

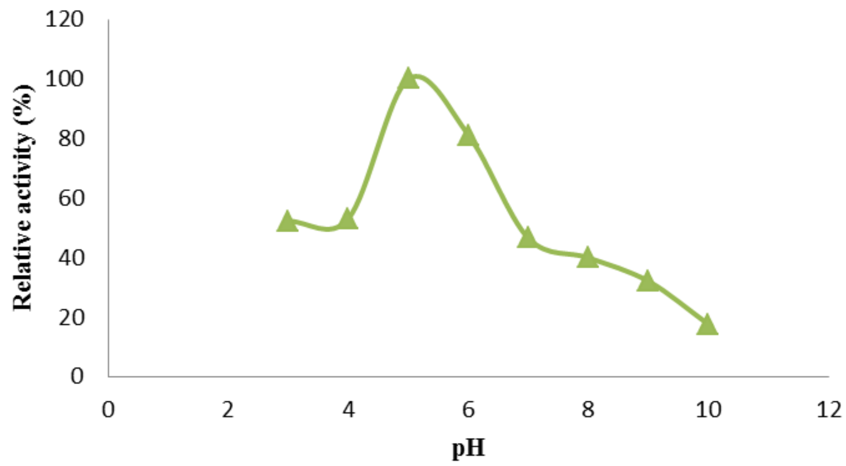
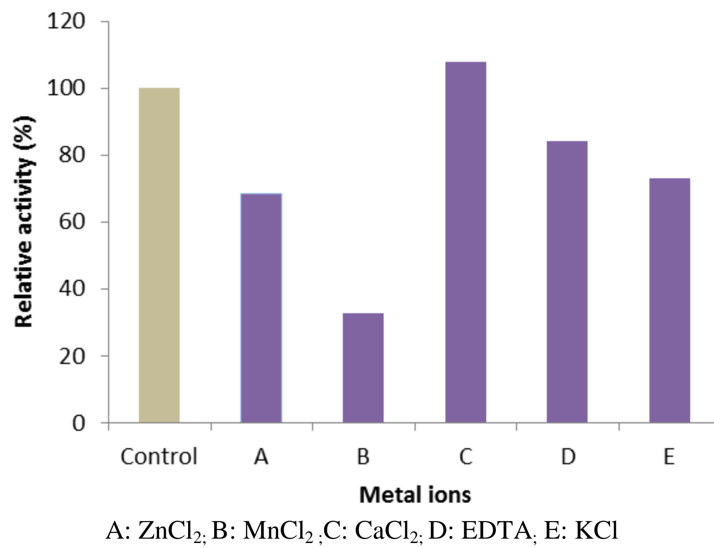


Fig. 3 – Effect of pH on the chitinolytic activity of *T. viride*



A: $ZnCl_2$; B: $MnCl_2$; C: $CaCl_2$; D: EDTA; E: KCl

Fig. 4 – Effect of metal ions on chitinolytic activity of *T. viride*

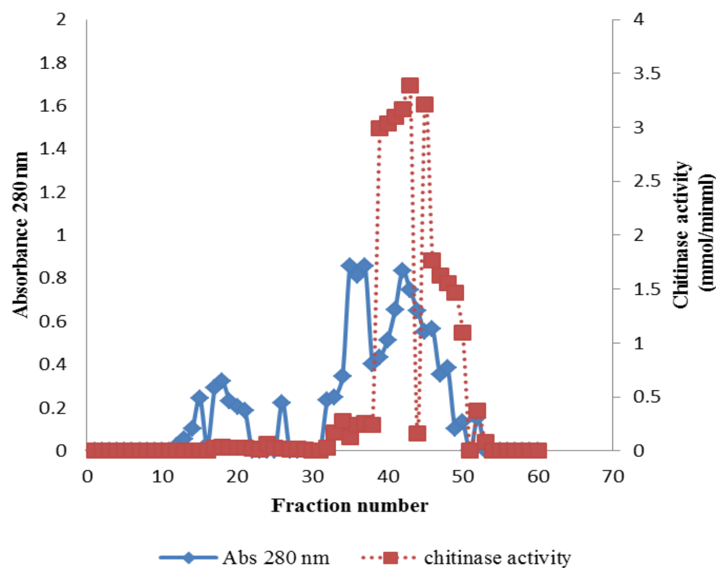


Fig. 5 – Purification of chitinase produced by *T. viride*

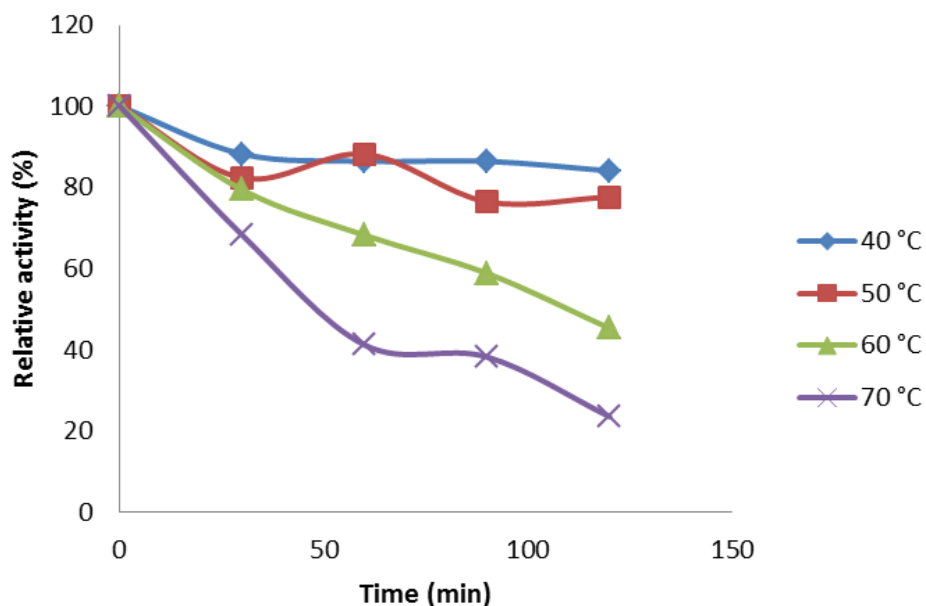


Fig. 6 – Effect of temperature on stability of chitinolytic activity of *T. viride*

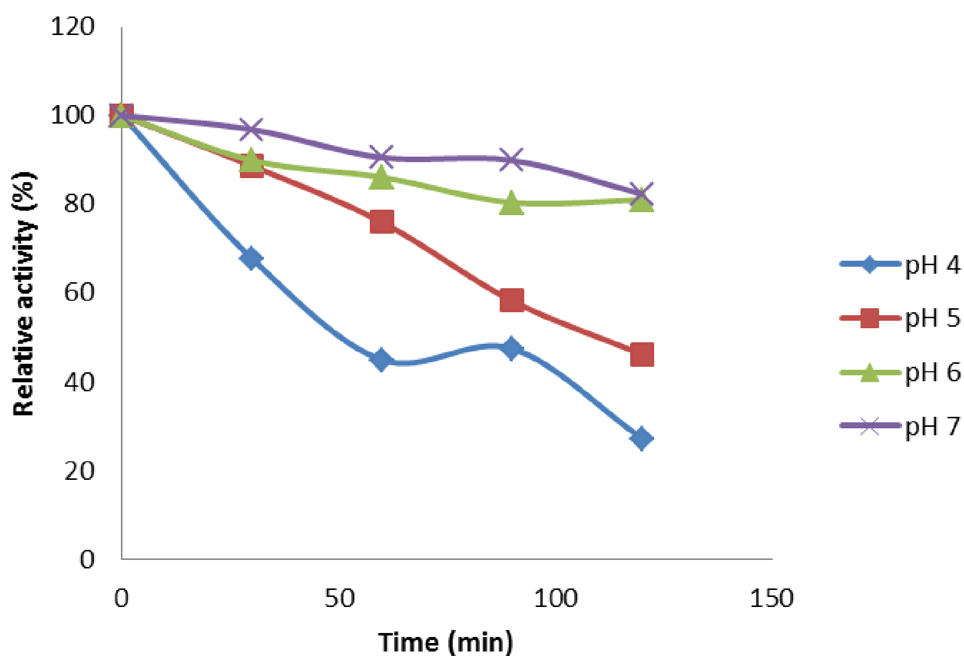


Fig. 7 – Effect of pH on the stability of chitinolytic activity of *T. viride*

Partial purification of crude chitinase produced by T. viride

Partially purified chitinase with ammonium sulphate concentration followed by DEAE cellulose column as well as Sephadex G-100 chromatography yielded 26.60, 22.30, 11.84 recovery with 1.39, 4.92, 8.50fold respectively (Figure 5 and Table 1).

Effect of temperature and pH on enzyme stability

The chitinase produced by *T. viride* was stable temperatures of 40 and 50°C. There was a decline from 60 to 90°C as shown in figure 6. Also, chitinase produced was stable at pH 6 and 7 (Figure 7).

Discussion

It is well known that *Trichoderma* spp. have the potential to produce cell wall degrading enzymes such as chitinase by using the materials that are present in the growth medium (Harman et al. 2004) and several studies have indicated the production of chitinase from *T. viride.* However, to the best of our understanding, this is the first report of producing chitinase from *T. viride* obtained from maize cob. Marco et al. (2003) noted that two isolates of *T. harzianum* (39.1 and 1051*)* produced and secreted on induction substantial amounts of chitinolytic enzymes and it increased within 72 h and maximal activity (0.39 U.ml-1) was produced by isolate 1051. This might be one of the reasons for its antagonistic potential against several phytopathogens (Mishra, 2010). The optimum temperature and pH for chitinolytic activity of *T. viride* were 50°C and 5 after which there was drop in enzyme activity. These are in agreement with the report of Aida & Taghreed (2014). A drop in the activity might be due to heat inactivation of the enzyme according to Aida & Taghreed (2014). Nampoothiri et al. (2004) and Jenifer et al. (2014) observed that the relative activity for enzyme production was highest at 40°C contrary to the present investigation. However, the effect of pH on enzyme was similar to the reports of Lee et al. (2000) and Jenifer et al. (2014). The metal ion EDTA was most inhibitory to the enzyme produced followed by Mn^{2+} while the relative activity was maximum when Ca^{2+} was used as the metal ion. Jenifer et al. (2014) observed that Mn^{2+} was completely inhibitory to enzyme produced while Ca^{2+} was not. The chitinase produced by *T. viride* was stable at temperatures of 40 and 50°C. Also, chitinase produced was stable at pH 6 and 7. Omumasaba et al. (2001) observed that chitinase produced from *T. viride* was stable at 45°C and pH ranges 3-5-6.0.

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