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Antifungal Activity of Purified Hydrolase Complex from *Trichoderma hamatum*

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Abstract*: The use of chemical compounds to eradicate plant pathogens proved successful but the toxicological and environmental effects of these chemical compounds have been of great concern. However, there is the need for an alternative that is eco friendly. This work aimed at isolating Trichoderma species that has the potential to eradicate plant pathogens. Trichoderma hamatum was isolated from decayed wood sample and the production of hydrolytic enzymes from it was carried out using submerged fermentation under static and shaking incubation conditions. The enzymes produced were tested singly and in combination for their abilities to reduce the growth of pathogenic fungi (Aspergillus niger, Aspergillus flavus, Aspergillus terrus and Fusarium oxysporum). The enzymes were purified and the activities of the three enzymes produced were maximal on the third day of incubation. All enzymes (crude and partially purified) singly and in combination successfully reduced the growth of the test isolates. The partial purification of the enzymes using ammonium sulphate precipitation result in increase in the enzyme activities.*

Keywords: *Antifungal, Hydrolase, Submerged fermentation, Test organisms, Trichoderma hamatum.*

I. Introduction

Soil borne pathogens annually create major economical losses in many important crops. The use of chemical compounds to eradicate plant pathogens proved successful but the toxicological and environmental effects of these chemical compounds have been of great concern. Also, the high cost associated with the use of chemicals to control diseases caused by these pathogens is a limiting factor in the profitability of crop production[1]. These have led to drastic reduction in the availability of efficient chemical compound and appearance of new strains of pathogens. Hence, there is a need for a alternative that is eco-friendly.

Fungi are known to be biocontrol agents that secrete a wide array of enzymes that have been found very useful in preventing plant pathogens. Biological controls of plant diseases have been found as an alternative to the use of chemical compounds to control plant pathogens because it is cost effective, environmentally safe and once established, persists in the soil for a longer period and offers disease protection even in consecutive crop seasons[2].

The genus *Trichoderma*, a saprophytic fungus found in almost all soil, possess the ability to act as a biocontrol agent against fungal diseases of plant[3, 4]. The antifungal activity displayed by *Trichoderma* species against phytopathogenic fungi is as a result of multidegradation of the cell walls of the fungal host in the process of mycoparasitism[5]. *Trichoderma* produces an array of enzymes which are industrially important. Several works have been carried out on enzyme production by *Trichoderma* spp. For example, exo-β-1-3-glucanase has been produced from *Trichoderma reesei*[6]; exo-β-1-3-glucanase and cellulase have been produced from *Trichoderma viride*[7,8]; protease, cellulase, endo-β-1-3-glucanase and chitinase have also been produced from *Trichoderma harzianum*[9,10,11,12]. Xylanase was also produced from *Trichoderma longibratum*[13].

II. Materials And Methods

2.1 Samples collection

2.1.1 Sample

Soil samples were collected from the nursery of the Department of Microbiology, University of Ibadan and National Institute of Horticulture (NIHORT), Jericho, Ibadan, Oyo State. Decayed wood samples were collected from a decaying felled tree in front of Environmental Microbiology Laboratory, University of Ibadan using a sterile container and brought to the laboratory for analysis within 24 hours.

2.1.2 Test Organism

Aspergillus flavus and *Fusarium oxysporum* were obtained from the culture of collection of the Microbial Physiology and Biochemistry Laboratory, Department of Microbiology, University of Ibadan. *Aspergillus niger* were obtained from International Institute of Tropical Agriculture (IITA) Moniya, Ibadan. *Aspergillus terrus* was obtained from Kappa Scientific Institute, Trans Amusement Park, Bodija Ibadan. The organisms were maintained on Potato Dextrose Agar slants and preserved at 4^{o}C .

2.2 Isolation of Organisms

Standard suspensions of the samples were prepared by dissolving 1gram of each sample in 10mls of sterile distilled water. Different ten-fold dilutions were prepared from the stock solution according[14] and plated out in duplicate on Potato Dextrose Agar containing 50ppm streptomycin using pour plate method and incubated at 30^{o}C for 5 days. The organisms were subcultured to obtain pure cultures which were maintained on Potato Dextrose Agar slants and preserved at 4^{o}C .

2.3 Identification of Isolates

The isolated fungi were identified according to their micro-morphology, colour and morphology of the sporulating structures[14].

2.4 Inoculum preparation

Inoculum was prepared by adding 10ml of distilled water with 0.1% Tween 80 to the isolate on an agar slant[15].

2.5 Enzyme Production and Assay

2.5.1 Celluase

Cellulase was produced in a medium containing (g/l; w/v): $(NH_4)_2SO_4$, 1.4; Urea, 0.3; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.3; $CaCl_2$, 0.3; yeast extract, 0.1[16] and assayed[17].

2.5.2 Amylase

Amylase was produced in a medium containing (g/l; w/v): NH_4NO_3, 10; KH_2PO_4, 1.4; $MgSO_4.7H_2O$, 0.1; KCl, 0.5; starch, 20[18] and assayed[19].

2.5.3 Protease

Protease was produced in Czapek-Dox medium[20] and assayed using the method of Kunitz[21].

2.6 Protein Estimation

The protein estimation of the enzymes' filtrates were carried out according to the method of Lowry[22]

2.7 Enzymes purification

The crude enzymes were precipitated using ammonium sulphate[23]

2.8 Antagonistic activity assay of the crude and purified enzymes of *Trichoderma hamatum* against the test isolates

The antagonistic activity of the crude and purified enzymes were tested against different fungal isolates according to the method of Negash[24] and the mycelial growth reduction determined according to the formula: Mycelial growth reduction = Co – T[25,26]; where Co is the radial growth measurement of the test isolate in the control plate; T is the radial growth measurement of the test isolate in the test plate.

III. Results

The organism isolated grew in a circular pattern with the conidial area whitish green and no discoloration of the media was observed. The organism was identified to be *Trichoderma hamatum*.

Table 1 and Table 2 show the effect of time of incubation on the production of enzymes using two different incubation conditions (shaking and static). Amylase increased progressively with time under shaking condition while it attained its maximum activity at 48hours under the static condition. Cellulase produced under both incubation conditions increased progressively while Protease activity reached its maximum at 72 hours and then begins to decrease

TABLE 1 Effect of time of incubation on the activity (Units/ml) of the enzymes produced under shaking incubation condition

Time (hour)	Amylase	Cellulase	Protease
24	0.107±0.00	0.0001±0.00	0.00123±0.00
48	0.24±0.01	0.0006±0.00	-
72	0.28±0.01	0.0051±0.00	0.0186±0.00
120	-	-	0.0163±0.00
168	-	-	0.0123±0.00

Note: each value is the mean value ± standard error of mean
- means no assay of enzyme activity

TABLE2 Effect of time of incubation on the activity (Units/ml) of the enzymes produced under static incubation condition

Time (hour)	Amylase	Cellulase	Protease
24	0.026±0.00	0.0003±0.00	0.00123±0.00
48	0.24±0.01	0.0026±0.00	-
72	0.214±0.00	0.0062±0.00	0.0163±0.00
120	-	-	0.015±0.00
168	-	-	0.005±0.00

Note: each value is the mean value ± standard error of mean
- means no assay of enzyme activity

Table 3 and 4 show the effect of time of incubation on the dry mycelial weight obtained during enzyme production by *Trichoderma hamatum* under two different incubation conditions. The dry mycelia weight obtained during the production of amylase and cellulase increased with time while the dry mycelial weight of protease increased but decreased after 72hours for both incubation conditions.

TABLE3 Effect of time of incubation on the dry mycelial weight (g) obtained during enzymes production by *Trichoderma hamatum* under shaking incubation condition

Time (hour)	Amylase	Cellulase	Protease
24	0.61±0.00	0.50±0.05	0.10±0.01
48	0.79±0.00	0.76±0.03	-
72	0.82±0.00	1.11±0.08	0.36±0.01
120	-	-	0.22±0.01
168	-	-	0.20±0.01

Note: each value is the mean value ± standard error of mean
- means no dry mycelial weight determined

TABLE4 Effect of time of incubation on the dry mycelial weight (g) obtained during enzymes production by *Trichoderma hamatum* under shaking incubation condition

Time (hour)	Amylase	Cellulase	Protease
24	0.17±0.00	0.85±0.01	0.21±0.01
48	0.32±0.00	0.91±0.03	-
72	0.58±0.00	1.20±0.07	0.30±0.01
120	-	-	0.16±0.01
168	-	-	0.10±0.01

Note: each value is the mean value ± standard error of mean
- means no dry mycelial weight determined

Table 5 and 6 show the effect of time of incubation on the protein content of the enzyme produced by *Trichoderma hamatum*. The protein content of amylase and cellulase produced under shaking incubation condition increases with time while that of the static incubation condition also increases but decreases at 72hours. The protein content of protease also increases with time but decreases at 168 and 120 hours respectively.

TABLE5 Effect of time of incubation on the total protein content (mg/ml) of the enzymes produced by *Trichoderma hamatum* under shaking incubation condition

Time (hour)	Amylase	Cellulase	Protease
24	0.25±0.01	0.16±0.01	0.25±0.01
48	0.31±0.01	0.22±0.01	-
72	0.45±0.02	0.32±0.03	0.33±0.02
120	-	-	0.23±0.01
168	-	-	0.19±0.01

Note: each value is the mean value ± standard error of mean
- means no dry mycelial weight determined

TABLE6 Effect of time of incubation on the total protein content (mg/ml)of the enzymes produced by Trichoderma hamatum under static incubation condition

Time (hour)	Amylase	Cellulase	Protease
24	0.36±0.04	0.08±0.01	0.29±0.01
48	0.41±0.06	0.23±0.02	-
72	0.30±0.08	0.09±0.01	0.35±0.01
120	-	-	0.23±0.01
168	-	-	0.19±0.01

Note: each value is the mean value ± standard error of mean
- means no dry mycelial weight determined

The ammonium sulphate precipitation of the enzymes produced gave 1.15, 1.26 and 1.10 folds increase in the activities of amylase, cellulase and protease respectively.

Tables 7 -12 show the antagonistic activity assay of the crude culture filtrates and partially purifies enzymes of *Trichoderma hamatum* against the test isolates.

TABLE7 Mycelial growth reduction (mm) of the *Aspergillus flavus* by the crude filtrate (singly and in combination) of *Trichoderma hamatum* Incubation period (days)/mycelial growth (mm)

	Incubation period (days)/mycelial growth (mm)		
Enzymes	Day 1	Day 3	Day 5
A_1	6.50^{l}	8.25^{m}	11.50^{m}
A_2	7.25^{j}	8.75^{l}	11.25^{n}
A_3	7.75^{i}	10.75^{j}	15.50^{l}
C_1	12.50^{c}	43.25^{a}	51.00^{b}
C_2	13.50^{a}	22.00^{e}	34.25^{g}
C_3	9.25^{h}	17.50^{h}	35.00^{f}
P_1	6.25^{m}	NR	NR
P_3	11.00^{e}	31.00^{c}	41.50^{c}
P_5	3.50^{0}	NR	NR
P_7	7.75i	NR	NR
AC_1	13.25^{b}	43.25^{a}	64.50a
AC_2	13.50^{a}	22.25^{d}	25.75^{j}
AC_3	10.50^{f}	19.50^{g}	38.50^{e}
CP_1	5.50^{n}	10.50^{k}	21.50^{k}
CP_3	9.25^{h}	12.50^{i}	31.25^{i}
AP_1	11.50^{d}	NR	NR
AP_3	6.75k	NR	NR
ACP1	6.25^{m}	20.50^{f}	32.00^{h}
ACP3	9.75^{g}	39.75^{b}	40.00^{d}

Means with the same superscript within the same column are not significantly different following Duncan's Multiple range Test
Note:1 – 24hours; 2 – 48hours; 3 – 72hours, 5 – 120 hours, 7 – 168hours
A – Amylase; C – Cellulase; P - Protease
NR- No reduction

TABLE8 Mycelial growth reduction (mm) of the *Fusarium oxysporum* by the crude filtrate (singly and in combination) of *Trichoderma hamatum*

	Incubation period (days)/mycelial growth (mm)		
Enzymes	Day 1	Day 3	Day 5
A_1	NR	NR	NR
A_2	2.50^{m}	0.50^{e}	NR
A_3	5.00^{g}	2.50^{b}	NR
C_1	3.00^{k}	0.50^{e}	NR
C_2	NR	NR	NR
C_3	2.75^{l}	NR	NR
P_1	3.00^{k}	NR	NR
P_3	8.00^{e}	4.25^{a}	NR
P_5	3.25^{j}	1.50^{d}	NR
P_7	3.50^{i}	NR	NR
AC_1	4.75^{h}	NR	NR
AC_2	1.00^{n}	NR	NR
AC_3	NR	NR	NR
CP_1	6.50^{f}	NR	NR
CP_3	8.50^{d}	1.50^{d}	NR
AP_1	9.00^{c}	2.50^{b}	NR
AP_3	1.00^{n}	NR	NR

Means with the same superscript within the same column are not significantly different following Duncan's Multiple range Test
Note:1 – 24hours; 2 – 48hours; 3 – 72hours, 5 – 120 hours, 7 – 168hours

TABLE9 Mycelial growth reduction (mm) of the *Aspergillus niger* by the crude filtrate (singly and in combination) of *Trichoderma hamatum*

	Incubation period (days)/mycelial growth		
Enzymes	Day 1	Day 3	Day 5
A_1	1.50^{e}	5.50^{d}	10.25^{d}
A_2	2.00^{d}	7.25^{a}	12.50^{c}
A_3	2.50^{c}	6.25^{b}	12.75^{b}
C_1	3.00^{b}	NR	NR
C_2	2.00^{d}	NR	NR
C_3	3.00^{b}	5.75^{c}	8.50^{e}
P_1	NR	NR	NR
P_3	1.00^{f}	1.25^{g}	2.25^{h}
P_5	NR	NR	NR
P_7	NR	NR	NR
AC_1	4.00^{a}	1.25^{g}	NR
AC_2	NR	NR	NR
AC_3	0.50g	NR	NR
CP_1	1.00^{f}	NR	NR
CP_3	3.00^{b}	4.25^{e}	6.25^{f}
AP_1	1.50^{e}	NR	NR
AP_3	1.50^{e}	2.25^{f}	2.25^{h}
ACP1	2.00^{d}	NR	NR
ACP3	3.00^{b}	6.25^{b}	15.50^{a}

Means with the same superscript within the same column are not significantly different following Duncan's Multiple range Test
Note:1 – 24hours; 2 – 48hours; 3 – 72hours, 5 – 120 hours, 7 – 168hours
A – Amylase; C – Cellulase; P - Protease
NR- No reduction

TABLE10 Mycelial growth reduction (mm) of the *Aspergillus flavus* by the partially purified enzymes (singly and in combination) of *Trichoderma hamatum*

	Incubation period (days)/mycelia growth (mm)		
Enzymes	Day1	Day 3	Day 5
A	0.50[b]	4.25[d]	12.50[g]
C	1.50[a]	7.50[b]	28.50[c]
P	NR	NR	20.00[e]
AC	NR	2.75[f]	17.50[f]
AP	NR	5.25[c]	22.25[d]
CP	NR	3.00[e]	40.00[b]
ACP	NR	8.00[a]	45.00a

Means with the same superscript within the same column are not significantly different following Duncan's Multiple range Test Note:1 – 24hours; 2 – 48hours; 3 – 72hours, 5 – 120 hours, 7 – 168hours A – Amylase; C – Cellulase; P - Protease
NR- No reduction

TABLE11 Mycelial growth reduction (mm) of the *Aspergillus niger* by the partially purified enzymes (singly and in combination) of *Trichoderma hamatum*

	Incubation period (days)/mycelial growth (mm)		
Enzymes	Day1	Day 2	Day 3
A	NR	3.25[d]	7.50[g]
C	NR	NR	16.50[e]
P	NR	10.00[b]	26.50[c]
AC	NR	NR	10.50[f]
AP	NR	7.50[c]	21.25[d]
CP	NR	16.00[a]	44.00[a]
ACP	NR	3.00[e]	33.50[b]

Means with the same superscript within the same column are not significantly different following Duncan's Multiple range Test Note:1 – 24hours; 2 – 48hours; 3 – 72hours, 5 – 120 hours, 7 A – Amylase; C – Cellulase; P - Protease
NR- No reduction

TABLE 12 Mycelial growth reduction (mm) of the *Fusarium oxysporum* by the crude filtrate (singly and in combination) of *Trichoderma hamatum*

	Incubation period (days)/mycelia growth (mm)		
Enzymes	Day1	Day 3	Day 5
A	NR	3.50[g]	10.50[g]
C	21.50[a]	29.50[a]	39.50[a]
P	14.50[d]	17.00[d]	22.00[d]
AC	10.50[e]	12.25[f]	17.50[f]
AP	7.75[f]	15.25	21.75[e]
CP	19.50[b]	25.00[c]	28.50[c]
ACP	15.50[c]	29.00[b]	33.00[b]

Means with the same superscript within the same column are not significantly different following Duncan's Multiple range Test Note:1 – 24hours; 2 – 48hours; 3 – 72hours, 5 – 120 hours, 7 – 168hours A – Amylase; C – Cellulase; P - Protease
NR- No reduction

TABLE13 Mycelial growth reduction (mm) of the *Aspergillus terrus* by the partially purified enzymes (singly and in combination) of *Trichoderma hamatum*

	Incubation period (days)/mycelia growth (mm)		
Enzymes	Day 1	Day 3	Day 5
A	NR	0.50[a]	5.50[g]
C	NR	NR	19.50[d]
P	3.00[a]	12.75[a]	25.00[c]
AC	NR	NR	7.75[f]
AP	NR	8.25[b]	12.75[e]
CP	NR	6.50[c]	35.50[b]
ACP	0.50[b]	6.50[c]	39.50[a]

Means with the same superscript within the same column are not significantly different following Duncan's Multiple range Test Note:1 – 24hours; 2 – 48hours; 3 – 72hours, 5 – 120 hours, 7 – 168hours A – Amylase; C – Cellulase; P - Protease
NR- No reduction

Aspergilus flavus is a highly aggressive seed colonizer toxigenic plant pathogen[27]. Tables 7 and 10 show the mycelial growth reduction of *Aspergilus flavus*. Its growth was reduced by the culture filtrates (singly and in combination) with the most significant reduction of 64.50mm on the fifth day with amylase and cellulase

in combination, a most significant reduction of 45mm was also observed with the combination of the partially purified amylase, cellulase and protease on the fifth day. The study of the influence of non volatile substances produced by *Trichoderma* isolates are able to suppress the growth of *Aspergilus flavus* in vitro by dual culture method with the least growth reduction[27,28].

]Tables 8 and 12 show the growth reduction of *Fusarium oxysporum* by the crude culture filtrates and the partially purified enzymes respectively. The partially purified enzymes significantly reduced the growth of *Fusarium oxysporum. Fusarium oxysporum* is known to be both seed and soil borne facultative saprophyte and can survive in the soil up to six years in the absence of a susceptible host[29] . 79.9% reduction of *Fusarium oxysporum* has been recorded during the dual culture plate assay of *Trichoderma hamatum* and *Fusarium* spp[30]. *Trichoderma hamatum* has been shown by other works that it can be used to control and inhibit the growth of *Fusarium* spp[31,32].

The antagonistic activities of the crude culture filtrates and partially purified enzymes of *Trichoderma hamatum* reduced the growth of *Aspergillus niger*. The crude culture filtrates (singly and in combination) did not significantly reduced the growth of *Aspergillus niger*, a reduction of 15.50mm was observed on the fifth day with the combination of the three enzymes (amylase, cellulase and protease) as shown in table 9 while the partially purified enzymes significantly reduced the growth of *Aspergillus niger* with the most significant reduction of 44mm observed with the combination of cellulase and protease on the fifth day as shown in table 11. The least reduction shown by the crude culture filtrate could be due the presence of impurities in the culture medium thus inhibiting the antagonist potentials of the enzymes. The efficacy of *Trichoderma hamatum*, *Trichoderma harzianum, Trichoderma viride, Trichoderma longibrachiatum* to control and reduce the infection of *Aspergilus niger* under greenhouse conditions has been reported by Kishore[33].

Apart from *Fusarium* spp and *Aspergillus* spp, *Trichoderma* has also been reported to inhibit the growth of other organisms e.g *Phytophthora megakarya*[34], *Cladosporium herbarum*[35], *Ceractocystis paradoxa*[36,37] e.t.c.

IV. Conclusion

The combination of all the three enzymes produced yield an hydrolase complex which reduced the growth of the test isolate indicating that the hydrolytic enzymes of *Trichoderma* can be used to control pathogenic fungi and thus reduce the dependence on synthetic chemicals because it is natural and environmentally acceptable alternative to the existing chemical methods.

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