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THE INDUCTION OF SOME COMMON HYDROLYTIC ENZYMES AND ANTIBIOTICS IN *TRICHODERMA HARZIANUM AND FUSARIUM OXYSPORUM* USING SOME FOOD WASTES

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ABSTRACT

The excretion of some common hydrolytic enzymes and antibiotics by *Trichoderma harzianum* and *Fusarium oxysporum*, when grown on some food wastes was investigated. The food wastes, ripe plantain, unripe plantain, cassava, yam, sweet potato, orange and cocoyam peels were used to prepare liquid media in which the organisms were grown and the enzymes and antibiotics extracted and assayed. Only three out of the five enzymes under investigation were recovered. Polygalacturonase, C1-cellulase and Cx-cellulase showed varying rates of activity from the various wastes and organisms. Orange peel and cassava peel were the preferred food wastes for optimum productivity and activity of the three enzymes in *T. harzianum* and *F. oxysporum* respectively. There was no antibiotic recovered.

KEY WORDS: Hydrolytic enzymes, food wastes, assay.

INTRODUCTION

The competitive saprophytic ability (CSA) of any organism is dependent on the motley of hydrolytic enzymes secreted by it - both in quality and in quantity. Also the antagonism of any organism is dependent on the hydrolytic enzymes and/or antibiotics secreted by it in response to available substrates and the organism antagonized.

Fusarium oxysporum (FO) Schlecht emend. Sny. and Hans has been reported to excrete polygalacturonase, depolymerase and pectinmethylesterase which degrade pectin (Domsch and Gam, 1969), cellulose but not chitin (Jackson, 1965) and lignin (Sutherland and Crawford, 1981). *Trichoderma harzianum* (TH) Rifai is able to degrade starch (Franz, 1975) and cellulose (Park, 1976); it excretes extracellular mycolytic enzymes in addition to cellulases, lipases and proteases (Elad *et al.*, 1982a). Chet *et al.*, (1979), Chet and Baker (1981), Elad *et al.*, (1982a) and Haran *et al.*, (1995) reported the production of β-1,3-glucanase and chitinase by TH when grown on the cell wall of *Rhizoctonia solani* or laminarin and chitin; and it is also known to produce volatile and non-volatile antibiotics (Dennis and Webster, 1971a and b; Ghisaberti *et al.*, 1993) which are common to other members of the genus *Trichoderma* (Hadar *et al.*, 1979a, Chet and Baker, 1980, Harman *et al.*, 1980).

Godwin-Egein (1997) has observed the growth of these organisms on foodwastes, but the enzymes involved in the growth complex were not determined. The present study sets out to investigate the excretion of some common enzymes and antibiotics by the organisms when grown on these foodwastes.

MATERIALS AND METHODS

SOURCE OF THE ORGANISMS: The organisms used for the investigation were *Trichoderma harzianum* Rifai (TH) and *Fusarium oxysporum* (FO) Schlecht emend. Sny. and Hans. TH was isolated from the dark brown loamy soil (with high organic matter) of the Botanical garden of the University of Port Harcourt, Nigeria. It was isolated from the first 15cm depth of the soil by using the standard dilution plate method with the Trichoderma selective medium (TSM) developed by Ppavizas and Lumsden (1982). FO was isolated from a rubbish dump, where domestic wastes, the effluent and other solid wastes of a grinding mill were dumped, where diseased maize seedlings grew. It was isolated from the rhizosphere of the diseased seedlings using the standard dilution series on a Fusarium selective medium (FSM) (Komada, 1975) and from the root parts of the diseased seedlings on PDA and FSM. Axenic cultures of the organisms were maintained on PDA.

THE FOOD WASTES AND MEDIA PREPARATION: The food wastes, ripe and unripe plantain (*Musa paradisaca* L.), cassava (*Manihot esculentus* Crantz), yam (*Dioscorea rotundata* Poir), sweet potato (*Ipomea batatas* (L.) Lam), orange (*Citrus sinensis* (L.) Osbeck) and cocoyam (*Xanthosoma sagittifolium*) peels were oven dried (at 60°C for 24 h) to crisps and cumminuted in a Moulinex grinder-mill (France) to powder form. The powder was sifted to remove coarse particles and the media were prepared with the

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following composition:- peel powder 10g; K_2HPO_4 1.4g; distilled water 1000ml; $MgSO_4$ $7H_2O$ 0.2g; $NaNO_3$ 2.1g; yeast extract 0.1g of agar (Rees and Levinsons 1952). Before generating the peels, the tubers, cormel, hesperidium and fingers were surface sterilized by washing in 2% sodium hypochlorite solution. The peels were chipped and further soaked in a fresh 2% sodium hypochlorite solution for 4 h before drying. Ripe plantain peel dextrose broth (RPPDB), unripe plantain peel dextrose broth (UPPDB), cassava peel dextrose broth (CPDB), yam peel dextrose broth (YPDB), sweet potato peel dextrose broth (SPPDB), orange peel dextrose broth (OPDB) and cocoyam peel dextrose broth (CoPDB) were the seven liquid media prepared for the antibiotic study.

ENZYME STUDIES: Polygalacturonase (PG), pectinmethylesterase (PME), C_1-cellulase, Cx-cellulase, β-1,3-glucanase and chitinase secretion and activity in both organisms (FO & TH) were investigated.

CULTURE FILTRATE PREPARATION: Two discs (2 mm) of 2 days old cultures of the organisms on potato dextrose agar (PDA) were introduced into Erlenmeyer flasks (250 ml capacity) each containing 50 ml of the media above. The flasks were incubated for 7 days at 25°C. The mycelia were removed by filtration using several layers of muslin cloth and the filtrates were centrifuged at 10,000 rpm for 30 min. The samples were decanted and stored in the refrigerator. This was the crude *in vitro* enzymes of the organisms. Control flasks were inoculated with PDA discs. In addition laminarin and dead mycelia of the organisms replaced the peel powder in some flasks for β-1,3-glucanase and chitin and dead mycelia of the organisms replaced the peel powder for chitinase in some flasks. The mycelia of the organisms were harvested following Hader *et. al.* (1979) and were killed by autoclaving.

PARTIAL PURIFICATION OF ENZYMES:- As a first step in the fractionation of proteins in the solutions, solid $(NH_4)_2SO_4$ was added to the crude culture filtrate. Saturation levels of the salt were obtained at 40, 60, 80, 90 and 100% and the quantity added was calculated from tables (Green and Hughes, 1955). The salt was added to the solution in a water bath at 25°C. Precipitates obtained at each level was removed by centrifugation at 10,000 rpm for 30 min, after leaving to stand for 30 min. The supernatants were used for the next higher level saturation. The precipitate at each level was dissolved in small volume (5 ml) of water and dialysed against distilled water and stored in the refrigerator.

ENZYME ASSAY:-

PG ASSAY: The cup-plate (Dingle *et al.*, 1953) method was used. One gramme of Sodium polypectate (NaPP) was dissolved in 50 ml of water and the pH adjusted to 5.0, by adding an equal volume of 0.2 M sodium citrate buffer to give 1% NaPP in 0.1 M citrate buffer. The mixture was autoclaved for 10 min at 121°C, after 1g of Oxoid No3 agar was added. Twenty ml aliquots were transferred to sterile plastic Petri plates. and allowed to set before 4 mm diameter wells were cut out of the agar with sterile cork borer. An aliquot of 0.02 ml of enzyme sample was then pipetted into each well aseptically. The plates were incubated at 25°C for 24 h at the end of which the agar surface was flooded with 5 N HCl. The appearance of a white precipitate around the perimeter of the wells within 5 min was noted. The diameter of the precipitate (if present) was then measured as an indicator of enzyme activity, with the width of the zone being proportional to enzyme activity.

PME ASSAY: PME activity was determined by measuring the change in pH of the reaction mixture as used by Wick and Shroeder (1982). The reaction mixture was prepared with 10ml of the culture filtrate from Reese and Levinson's (1952) medium, 10ml of citrus pectin and 1ml of methyl red solution. The mixture was adjusted with 0.1M NaOH until faint yellow and stood at 30°C in a water bath. When the mixture turned red on standing, it was titrated back to yellow with 0.1M NaOH. Enzyme activity was expressed as mm of precipitate zone and measured as volume of 0.1M NaOH required to titrate the enzyme pectin mixture to extinction point with methyl red as indicator. The experiment was repeated 3 times.

C_1-CELLULASE ASSAY - RELEASE OF REDUCING SUGAR (Reese and Mandels, 1963):- An assay medium of 9 ml 1% carboxylmethyl-cellulose (CMC), 1 ml 0.2M acetate buffer, pH 5, 1 ml of enzyme and 1 ml distilled water in a 100 ml Erlenmeyer flask was kept at 30°C in a water bath to allow enzyme reaction to proceed. At 1 h interval, 1 ml of the reaction mixture was withdrawn and added rapidly to 2 ml dinitrosalicylic acid (DNSA) and heated for 5 min in boiling water. The mixture was cooled, made up to 5 ml with distilled water and the optical density read at 540 mm in a spectrophotometer. The amount of reducing sugar was then read off a glucose calibration curve.

Cx-CELLULASE ASSAY - VISCOMETRY: The reaction mixture composed 4 ml 0.5% CMC, 2 ml enzyme sample and 1 ml water. Cx-cellulase was expressed in relative viscometric units (RVU) or percentage loss in viscosity defined as 1000/t, where t = time for 50% loss in viscosity of the reaction mixture (Arinze, 1985a & b). Loss in viscosity was measured using size 300 Ostwald Fenske Viscometer (Galenkamp, England). Flow time for water represented 100% loss in viscosity. Boiled enzyme sample formed control.

CHITINASE ASSAY:- Chitinase activity was determined by following the release of N-acetylglucosamine according to Reissing *et al.*, (1955). Specific activity was expressed as μmoles N-acetylglucosamine/mg protein/h. The reaction mixture (Hader *et al.*, 1979) contained 2.0 ml of 0.1M phosphate buffer (pH 5.1), 1.6 mg colloidal chitin or FO cell walls and 10 ml culture filtrate. The reaction was carried out in a water bath at 37°C for 2 h and

stopped by immersing the tubes in boiling water for 15 min.

β-1, 3-GLUCANASE ASSAY: The activity of β-1, 3-glucanase was determined by following the release of free glucose, using the glucose oxidase reagent according to Elad *et. al.* (1983c). Specific activity was expressed as µmole glucose/mg protein/h. The reaction mixture contained 2 ml of 0.1M citrate buffer (pH 4.7) 1.6 mg soluble laminarin or cell wall of FO and 10 ml of culture filtrate, The reaction, carried out for 2 h at 45°C, was stopped by immersing the tubes in a boiling water bath for 15 min.

ANTIBIOTIC STUDY: Antibiotic production and activity of the organisms was investigated using the method described by Yeh and Sinclair (1980). Agar discs (4 mm diameter) cut from 5-day-old PDA cultures of the organisms were used to inoculate 50 ml of sterile liquid media (RPPDB, UPPDB, CPDB, YPDB, SPPDB, OPDB AND CoPDB) in 250 ml Erlenmeyer flasks. After 3 weeks of incubation at 25°C, the culture was filtered through a Buchner funnel containing three layers of filter paper (Whatman No 1). The culture filtrates were incorporated into 2% water agar to concentrations of 20% by volume after the water agar was autoclaved for 15 min. at 121°C and cooled to about 50°C. Water agar without the culture filtrate served as control. After, 20 ml of the treatments were poured into 9 cm diameter Petri dishes. Agar discs (5 mm) of the organisms were then placed in the centre of each plate. The treatments were replicated 4 times. Colony diameters in cm, were measured and recorded after 7 days incubation at 25°C. The experiment was repeated 3 times.

RESULTS

PG ASSAY: Table 1 shows the assessment of PG by the cup-plate method and indicates that PG was recovered from all media for both organisms. Furthermore, wider rings were generally formed by TH than FO in OP, and it was in this medium that there was the biggest significant difference observed between the two organisms in PG activity. On the average, least enzyme activity of TH was observed in SPP where no significant difference was observed between the two organisms. Optimum enzyme activity was observed for FO in CP and this was lower than that of TH in the same medium. There was no enzyme activity observed in the control flasks.

PME ASSAY: Results of PME assay were inconsistent and proved negative.

C_1-CELLULASE ASSAY: Results on Table 2 showed that reducing sugars were released from the dialysed culture filtrates at the various incubation periods from 1 h to 5 h. The release of reducing sugar was an indication of the presence of C_1-cellulase. There was no significant difference observed between the two organisms regarding the activity of the enzymes recovered from the various media, except in OP, where the activity of C_1-cellulase from TH was much higher than that of FO. FO enzyme from RPP, UPP, SPP and CoP were more active than that from TH while TH enzyme from CP, YP and OP were more active than those of FO.

Cx-CELLULASE ASSAY: Cx-cellulase was present in all culture filtrates, as shown on Table 3. The trend of recovery was the same as observed in the assessment of C_1-cellulase, with the least activity in OP for FO and optimum activity still in OP for TH. Filtrates of TH cultures from CP, YP and OP media were more active than those of FO.

Table 1: PG activity of TH and FO assessed as amount of precipitate formed around wells in the agar holding the enzyme solutions.

MEDIUM	PG ACTIVITY/ORGANISMS (mm. zone of activity)	
	FO	TH
Control	0.00	0.00
RPPDB	1.80C	2.30B
UPPDB	2.00C	1.87C
CPDB	3.10A	3.21A
SPPDB	1.95C	1.86C
YPDB	2.80B	3.21A
OPDB	1.60D	3.51A
CoPDB	2.11B	2.41B
TOTAL	15.36F	18.37E
MEAN	2.19B	2.62B

Values followed by the same letter do not differ significantly at P=0.05.

CHITINASE ASSAY: Results showed that no chitinase was secreted by both organisms when grown on the food wastes as shown on Table 4. Results however showed that TH excreted extracellular chitinase when grown on chitin and the cell walls of FO and that the chitinase activity was more with culture filtrates from chitin-based medium than that of FO-cell-wall-based medium.

β-1, 3-GLUCANASE ASSAY: Results of β-1, 3-glucanase assay are as shown on Table 4. There was no extracellular excretion of β-1, 3-glucanase by the organisms when grown on any of the food wastes in the study, but the results showed that TH excreted extracellular β-1, 3-glucanase when grown on laminarin and the cell walls of FO.

Table 2: C_1 cellulase actvity of TH and FO determined by the amount of reducing sugars produced during the degradation of filter paper substrate. Measure was in mg ml^{-1}.

MEDIUM	MEAN	
	TH	FO
Control	0.00	0.00
RPPDB	0.079C	0.084B
UPPDB	0.074C	0.077C
CPDB	0.094A	0.093A
SPPDB	0.066D	0.071C
YPDB	0.087B	0.083B
OPDB	0.094A	0.065D
CoPDB	0.069D	0.078C

Values followed by the same letter do not differ significantly at P = 0.05.

Table 3: Cx-cellulase activity in TH and FO.

MEDIUM	ORGANISM/ACTIVITY (RVU of activity by:)	
	TH	FO
Control	0.0	0.0
RPPDB	75B	83A
UPPDB	71B	79B
CPDB	88A	86A
SPPDB	56C	69C
YPDB	87A	85A
OPDB	90A	53C
CoPDB	60C	70B
TOTAL	527	18.37E
MEAN	75.29B	74.29B

Figures on the Table represent relative viscometric units (RVU) determined viscometrically with CMC as substrate. Figures followed by the same letter do not differ significantly at P = 0.05.

ANTIBIOTIC STUDY: No antibiotic activity was detected towards the organisms as the organisms grew normally in the plates.

DISCUSSION

TH and FO are known to excrete extracellular PG, PME, pectinase, cellulase and lignin degrading enzymes. In addition TH is known to excrete extracellular mycolytic enzymes (Chet and Baker, 1981; Haran *et al.*, 1995) like β-1, 3-glucanase and chitinase. It was reported by Elad *et al.*, (1983c) that the levels of hydrolytic enzymes produced by TH differ when different substrates are involved. This latter point was evident in the results of the present work, where it was observed that different food wastes gave different excretion levels and TH's excretion was different from that of FO even on the same food waste, in most cases. This may be the reason for the variable CSA of the two organisms and antagonistic performance in different media. For instance, significant differences were observed on OP, between the two organisms on the production of PG, and cellulase, where TH's production level far outstripped that of FO. On YP and CP, no significant difference was observed between the two organisms on enzyme production and this only shows that both organisms do well on them (YP and CP). The apparent difference (in performance of the organisms) between RPP and UPP cannot be understood, because the difference between them (RPP and UPP) is not in substrate content for PG and cellulases, but protein and mineral ion contents (Oyenuga, 1978). So it could be speculated that some of these proteins

Table 4: Chitinase and β-1,3-glucanase activities of TH, determined by the release of free glucose and N-acetylglucosamine respectively.

CARBON SOURCE	ORGANISM/ACTIVITY	
(Medium)	CHITINASE	GLUCANASE
Control	0.00F	0.00F
RPP	0.00F	0.00F
UPP	0.00F	0.00F
CP	0.00F	0.00F
SPP	0.00F	0.00F
YP	0.00F	0.00F
OP	0.00F	0.00F
CoP	0.00F	0.00F
Laminarin	0.00F	37.56A
Chitin	1.13D	0.00F
FO cell wall	0.17E	9.24B

Figures on the Table are expressions of chitinase activity as μmoles N-acetylglucosamine/mg protein/hr and β-1,3-glucanase activity as μmoles glucose/mg protein/hr. Values, on the Table, followed by the same letter do not differ significantly at P = 0.05.

and/or ions may play some role in the induction of hydrolytic enzyme excretion etc. From the foregoing, it can be concluded that, TH and FO excrete only PG and cellulases and not β-1, 3-glucanase, PME, chitinase and antibiotics, when grown on the food wastes in this investigation. Furthermore the preferred food wastes for optimum productivity and activity of the three enzymes are OP and CP in *T. harzianum* and *F. oxysporum* respectively.

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