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EXTRACELLULAR ENZYMES OF YAM ROT-CAUSING PATHOGENS

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ABSTRACT

The range of produceable extracellular enzymes on solid media by the yam rot-causing pathogens was investigated, and the pathogens were found to produce abundant cellulase, polygalacturonase, amylase and urease. All pathogens, except *Botryodiplodia theobromae* also produced abundant lipase. Although all the pathogens produced phosphatase and pectate lyase, the production was low in *Botryodiplodia theobromae* and *Fusarium moniliforme.* Only *Aspergillus niger* did not produce protease or DNase.

INTRODUCTION

Previous studies (Ogundana et al, 1971) on the soft rot of yams in storage employing the liquid medium technique (Husain & Rich, 1958; Akinrefon, 1968) showed that the yam rot-causing pathogens produced extracellular cellulolytic and pectic enzymes presumably involved in the disintegration of the host cell wall (Husain & Dimond, 1960). The author believes that the possibility of the production of other enzymes apart from the cellulase and Polygalacturonase previously implicated in yam tissue maceration cannot be ruled out and therefore seeks for other method of estimation of enzyme production.

The use of solid media to investigate the production of extracellular enzymes by micro-organisms has been employed by many workers (Jeffries et al. .1957; Berkenkamp, 1973; Fessehatzion, 1978). Dingle, Reid & Solomons (1953 used solid medium technique for determination of enzyme activity in microfungi and suggested the use of the method for quantitative estimation of enzymes while Hankin & Anagnostakis (1975) employed solid media technique to screen large population of fungi for the presence or absence of specific enzymes. The present study, employing the use of solid medium technique, to detect other produceable enzymes by the yam rot-causing pathogens was therefore undertaken.

MATERIALS AND METHODS

All growth media were sterilised by autoclaving at 1.05 kg/cm^2 for 15 min. Inoculation was carried out in an ultraviolet irradiated sterile room. Standard inoculum discs were cut out with a sterilised 5 mm cork borer from the advancing edge of 3-day old cultures of the pathogens. Culture plates were incubated at 28^{o}C for 2-5 days, depending on rate of growth and flooded with appropriate reagent to give rise to a zone of specific activity at the periphery of the mycelium colony. The production of each of the following enzymes was assessed.

(i) Amylase: Amylolytic activity was based on the principle that starch degradation by the pathogens indicates their ability to produce amylase (Hankin & Anagnostakis, 1975). The medium

used therefore consisted of 0.2% soluble starch in nutrient agar, pH 6.0 and plates were flooded with 1% iodine solution after the incubation period. A yellow zone produced round the colony was taken to indicate the hydrolysis of starch.

(ii) Protease: The medium used was 0.4% gelatin in nutrient agar. After incubation the plates were flooded with an aqueous saturated solution of NH_4SO_4 to precipitate intact gelatin to give a clear zone round the fungal colony against an opaque background.

(iii) Lipase: Sorbitan monolaurate (Tween 20) was used as the lipid substrate. The medium adjusted to pH 6.0 (Sierra, 1957) contained Difco peptone 10g; NaCl, 0.5g; $CaCl_2.2H_2O$, 0.1g; agar, 20g; distilled water, 1000 ml. Lipolytic activity would show either as a visible precipitate due to the formation of crystals of calcium salt of the lauric acid liberated by the enzyme or as a clearing of such a precipitate around the colony due to complete degradation of the salt of the fatty acid.

(iv) Urease: The medium used contained 1% urea in Difco nutrient agar. After incubation, an overlay containing an indicator was poured over the surface of the colony. The overlay consisted of 100 ml of 0.1 M phosphate buffer (pH 6.0); urea, 1g; agar, 1g; and 0.5 ml of 1% bromo thymol blue as indicator (Hopwood, 1964). The colour of the overlay which should be orange to yellow would change to blue with the production of urease.

(v) Phosphatase: Test substrate was aqueous solution of 0.01 M phenolphthalein di-phosphate sodium salt. After incubation the plates were opened and inverted over NH_4OH solution. Utilization of the substrate would result in liberation of phenolphthalein in presence of ammonia and would be detected as pink to red colouration in the growth medium.

(vi) DNase: The medium (pH 6.0) used consisted of tryptone, 20g; DNA, 2g; NaCl, 0.5g; agar, 15g; distilled water, 1000 ml (Jeffries et al. .1957). Incubated plates were flooded with IN HCL to react with intact nucleate salts in the medium to form free nucleic acid as cloudy precipitate. Activity of DNase would then show as a clear zone round the colony against an opaque background.

(vii) RNase: The test substrate was Torula Yeast RNA, and was added to a basal medium (pH 6.4) made up of D-Glucose, 5g; NaCl, 2g; KH^2PO_4, 5g; $FeSO_4$, 0.05g; $MgSO_4.7H_2O$, 0.5g; vitamin free casamino acid, 5g; agar, 15g; distilled water, 1000 ml. Plates were flooded with IN HCl and examined for clear zones around the colonies against an opaque agar background.

(viii) Pectolytic enzymes: An improved solid medium (Hankin et al. 1971) to detect different pectolytic enzymes at appropriate acidic and alkaline conditions was employed. By this method, pecting polyglacturonase would be detected at pH 5.0 and pectate lyase at pH 7.0.

(ix) Cellulase: The substrate was sodium carboxymethyl cellulose in acetate buffer; the plates were developed with a 10% (w/v) solution of copper acetate, when clear zones appeared against an opalescent background to indicate cellulase activity.

RESULTS

Table 1 gives the results of enzyme production by the pathogens on solid media. The results show that the pathogens were able to produce amylase, urease, polygalacturonase and cellulase very abundantly. Phosphatase and pectate lyase were also abundantly produced by *A. niger* but weakly produced by *B. theobroma* and *F. moniliforme*. *P. sclerotigenum* produced phosphatase and pectate lyase very appreciably. Lipase production in all the pathogens except *B. theobromae* was also satisfactory. There were no RNase produced at all and where DNase was produced, it was weakly positive; and only *A. niger* failed to produce protease.

TABLE 1

Production of extracellular enzymes by the yam rot-causing pathogens on solid media.

Enzyme	Yam rot-causing pathogens			
	*A	B	F	P
Amylase	+++	++	++	++
Cellulase	+++	+++	+++	+++
D Nase	–	+	+	+
Lipase	+++	+	++	+++
Pectate lyase	+++	+	+	++
Phosphatase	+++	+	+	+++
Polygalacturonase	+++	+++	+++	++
Protease	–	+++	+	+++
RNase	–	–	–	–
Urease	++	++	+++	+++

Key:
+++ Very positive result with wide zone of enzyme activity round the pathogen colony
++ Satisfactory positive result
+ Weakly positive result with very narrow zone of enzyme activity
– No zone of activity detected
*A = *Aspergillus niger*
B = *Botryodiplodia theobromae*
F = *Fusarium moniliforme*
P = *Penicillium sclerotigenum*

DISCUSSION

The production of polygalacturonase and cellulase in the present studies serves as a confirmation of previous work (Ogundana et al., 1971) where the enzymes were detected by loss in viscosity of their substrates. The present investigation has shown that other enzymes, apart from those involved in tissue maceration, are produced by the pathogens. Some of these other enzymes might even release suitable substrates to those of the cell macerating types.

This method serves a useful purpose because a wide screening of fungi can be carried out as

to what type of extracellular enzymes can be produced. The assay method would therefore be of particular use in studies of microfungi, where large numbers of assay of limited accuracy are required. Dingle et al. (1953) reported that the method has in fact been used successfully for screening 120 cultures of fungi for polygalacturonase, lipase, protease, amylase, and cellulase activity. Further studies would now be carried out to ascertain whether these enzymes are produced *in vivo*, and how they affect rotting process.

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