
Production of cellulolytic and pectic enzymes by isolates of *Phomopsis vexans* (Sacc. & Syd.) Harter in culture and in brinjal fruits

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Cellulolytic and pectic enzymes activity of isolates of *Phomopsis vexans* was studied *in vitro* and *in vivo*. The pH optima of cellulase (Cx) and pectic enzyme were 5.5 and 8.5 respectively at 30°C. The alkaline pH optima suggested involvement of a lyase type pectic enzyme. *in vitro* Cx and pectic enzyme activity of 3 isolates (25F> 17L> 10) were correlated with their virulence levels. Highest cellulase and pectic enzyme activity was noted at 9th day after inoculation of brinjal fruits over a span of 0-12 days.

Key words : Cellulolytic enzyme, Pectic enzyme, virulence, *Phomopsis vexans*

INTRODUCTION

Enzymes are regarded as major biochemical weapons in pathogenesis. Many economically important plant pathogens produce cellulolytic and pectic enzyme *in vitro* and *in vivo* (Bateman and Basham, 1976).

A close look into the conspicuous systems of brinjal fruit caused by *Phomopsis vexans* (Sacc. & Syd.) Harter suggested the possibility of involvement of cell wall degrading enzymes which has not been worked out so far. The present experiment was aimed at a comparative study of pectic enzyme production *in vitro* by 3 isolates of *P. vexans* having different degree of virulence in causing fruit rot of brinjal (*S. melongena*).

MATERIALS AND METHODS

Preparation of enzyme source in vitro

Three isolates (25 F> 17L> 10S in order of relative virulence level) of the test organism were grown in 250 ml Erlenmeyer flasks containing 50 ml Czapeck-Dox

broth (containing 0.5% sucrose) fortified with 1% CMC-Na salt for Cx and 1% Na-polypectate for pectic enzyme, respectively. The flasks were inoculated with 4 mycelial discs (5.0 mm dia.) and incubated at $30 \pm 1^\circ\text{C}$ for 15 days. The culture medium was passed through folds of filter paper, centrifuged at 5°C at 4000 g for 20 min. in a refrigerated centrifuge. The decant was dialysed at 4°C for 15-20 hr. with repeated changes of several litres of deionised water. The dialysed extract was used immediately as enzyme source.

Preparation of enzyme source in vivo

Tender brinjal fruits (cv. Pusa cluster) were artificially infected with the test organism. Two fruit discs (10 mm dia and 3 mm thickness) were cut at two points on the fruit. 2-3 mycelia discs (5 mm dia.) were placed in the grooves and finally plugged by the fruit discs. The inoculated portion was covered by cellotape and incubated at $30 \pm 1^\circ\text{C}$ for specified days. Fifty gram diseased tissue was carved out and macerated in a prechilled glass mortar adding 20 ml buffer soln (citrate-phosphate and tris-HCl buffer at pH 5.5 and 8.5 for Cx and pectic enzyme respectively). The extract was filtered, centrifuged and dialysed as in *in vitro* experiment.

Assay of enzyme activity

For Cx, viscosimetric method was adopted as described by Bateman (1966). The enzyme : buffered substrate (1% CMC-Na salt in citrate-phosphate buffer at pH 5.5) mixture in 1:4 ratio was taken in an Ostwald-Fenske viscosimeter and the flow time was recorded after specific time intervals.

For pectic enzyme the viscosimetric method adopted was essentially that described by Bell *et al.* (1955) with modifications as suggested by Hancock *et al.* (1964) using enzyme : buffered substrate (1.2% Na polypectate in tris-HCl buffer at pH 8.5) mixture in 1:4 ratio.

The pH optima for Cx and pectic enzyme was determined by adjusting the substrate (1% CMC-Na-salt for Cx and 1.2% Na-polypectate for pectic enzyme) to different pH (3.0 to 10.0) using different buffers (citrate-phosphate : 3.0-6.5; phosphate : 6.5-7.5 and Tris-HCl : 7.5-10.0) and incubating the reaction mixture (enzyme+substrate at 30°C in a thermostatic water bath for 60 min and that for temperature optima at a fixed pH (5.5 and 8.5 for Cx and pectic enzyme respectively) at varying temperatures (10- 30°C).

The percent reduction in viscosity was against time and the time required for 50% reduction in flowtime (PDFT₅₀) was calculated.

RESULTS

Cx and pectic enzyme activity and pH

Maximum Cx activity was recorded at pH 5.5. At acidic pH range on both side of this optima, the enzyme activity declined, being minimum at pH 4.5; with further lowering of pH from 4.5 to 3.0, a little increase in Cx activity was noted. At alkaline pH range, the enzyme activity showed a mild peak at pH 8.5 compared to peak at pH 5.5 and thereafter declined and become negligible at pH 10.0 (Fig. 1).

For pectic enzyme, the peak activity was recorded at pH 8.5. The enzyme activity sharply declined on both side of this optima and became negligible at pH 10.0. More or less a low level of enzyme activity was recorded over the acidic range of pH tried upto 4.5, becoming further lower thereafter (Fig. 1).

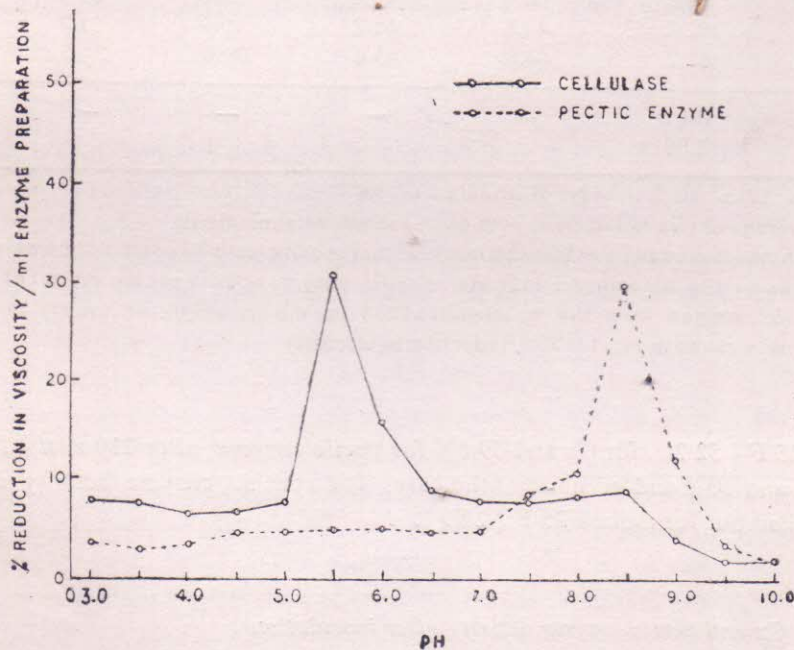


Fig 1. pH Optima of cellulase and pectic enzyme activity in cultural filtrate of *Phomopsis vexans*

Cx and pectic enzyme activity and temperature

The temperature optima for both Cx and pectic enzyme appeared to be at 30°C.

In vitro Cx and pectic enzyme activity in virulence level

All the isolates tested showed low pectic enzyme and Cx activity but except isolate 25 F (Table 1). Percent reduction in viscosity of substrate was maximum in

Table 1. *In vitro* cellulase and pectic enzyme activity of three isolates of *P. vexans*

Incubation period (min)	% Reduction in viscosity**					
	at pH 5.5 (Cx)			at pH 8.5 (pectic enzyme)		
	25 F *	17 L *	10 S *	25 F *	17 L *	10 S *
30	20.0	17.6	16.8	15.0	10.4	8.0
60	30.8	25.4	23.3	28.2	18.0	15.6
90	38.3	32.9	29.4	32.6	24.2	20.4
120	45.9	37.6	33.3	35.3	29.8	23.2
150	48.5	40.8	37.6	38.1	30.6	25.0
180	51.3	43.5	40.8	39.3	31.2	26.2
210	52.2	44.6	41.2	39.9	31.2	26.3
240	52.2	44.9	41.8	39.8	31.0	26.4
PDFT ₅₀ (Min.)	165.0 (2.99) a	—	—	—	—	—

* 25F > 17L > 10 S in order of virulence levels

** An average of two replications with two separate determinations

(—) Denotes low enzyme activity so much so that specific activity could not be determined

a Figures in the parenthesis indicate specific enzyme activity per mg dry wt of mycelial mat, determined from the equation $1/t \times 1000$ per mg dry weight of mycelial mat, where t = time required in min for 50% reduction in viscosity

isolate 25 F (52.2% for Cx and 39.8% for pectic enzyme after 210 min.) followed by 17 L and 10 S and in none of the cases the specific enzyme activity could be determined but for isolata 25 F (2.99).

In vitro Cx and pectic enzyme activity after inoculation

Maximum Cx and pectic enzyme activity under '*in vivo*' condition was recorded at the 9th day of inoculation (Table 2). Of the 4 specified incubation period (3,5,9 and 12 days) an increase in enzyme activity (both Cx and pectic enzyme) from 3 to 9 days was followed by a decline from 9-12 days and while the peak pectic enzyme activity declined sharply after 9th day but the Cx activity was maintained upto 12th day at a moderate level (Table 2).

Table 2. *In vivo* cellulase and pectic enzyme activity of isolate 25 F of *P. vexans* as a function of days after inoculation of brinjal fruit

Incubation period (min)	% Reduction in viscosity * recorded days after inoculation							
	Cellulase (pH 5.5)				Pectic enzyme (pH 8.5)			
	3rd day	5th day	9th day	12th day	3rd day	5th day	9th day	12th day
30	3.6	5.6	38.9	19.8	1.5	11.6	17.4	3.4
60	6.7	10.8	56.4	34.3	4.3	18.9	29.8	11.0
90	6.9	22.1	63.5	44.8	6.0	26.4	38.4	13.3
120	7.1	29.5	65.0	51.2	6.7	32.6	45.3	16.6
150	7.9	33.4	67.8	56.3	6.9	33.3	49.0	19.4
180	8.2	33.5	68.2	58.1	7.0	33.6	51.9	20.2
210	8.5	33.9	68.5	58.7	7.1	33.8	52.0	21.3
PDFT ₅₀ (Min.)	—	—	49.0	115.0	—	—	159.0	—

* An average of two replications with two separate determinations

(—) signifies PDFT₅₀ could not be determined due to low enzyme activity

In vivo Cx and pectic enzyme activity in virulence level

Assay of enzyme activity under '*in vivo*' condition exhibited a high Cx activity but the pectic enzyme activity at a low level. Isolate 25 F showed maximum Cx activity (60% after 90 min.) followed by 17 L and 10 S, like *in vivo* experiment but a different trend was noted for pectic enzyme activity where in highest enzyme secretion of the isolate 25 F (37.6% after 90 min) was followed by 10 S and 17 L (Table 3).

Table 3. *In vivo* cellulase and pectic enzyme activity * of three isolates of *P. vexans* different in virulence levels

Isolates	% Reduction in viscosity after a fixed period of incubation **	
	Cellulase (pH 5.5) after 90 min	Pectic enzyme (pH 8.5) after 90 min
25 F	60.0 (51.0)***	37.6 (—)
17 L	43.8 (—)	21.6 (—)
10 S	33.2 (—)	29.8 (—)

* Enzyme activity was determined 9 days after inoculation of brinjal fruits

** An average of two replications with two separate determinations

*** Figure in the parenthesis indicates PDFT₅₀ value in minute

(—) signifies PDFT₅₀ could not be determined due to low enzyme activity

DISCUSSION

The peak activity for pectic enzyme in this study was recorded at pH 8.5 and 30°C and that for cellulase at pH 5.5 and 30°C. The activity of the pectic enzyme at 8.5 apparently indicates that the enzyme involved here was of a lyse (transeliminase) type which in general is most active under alkaline conditions (Bateman 1966; Hall and Wood, 1970, Rambouts and Pilnik, 1972). Although no information is available on the nature of enzyme activity for this pathogen. Chon and Sackston (1971) have reported increased polygalacturonate transeliminase activity at pH 8.5 and 30°C for another pycnidial pathogen *Macrophomina phaseolina*. In the present study the test organism showed a low level of pectic enzyme activity over a wide pH range and the results are inconformity with the observations made by Goel and Mehrotra (1973-74) for *M. phaseolina*. The pathogen showed a high level of Cx activity compared to pectic enzyme and similar observation has been made by Pan and Sen (1984) for other pathogens.

Among the 3 isolates, 25 F showed highest Cx and pectic enzyme activity over the two other isolates assayed. A direct correlation between virulence and potency of enzyme secretion was noticed with a little deviation in case of isolate 10 S which at the later stage superseded pectic enzyme activity of isolate 17 L. Similar ambiguity with the virulence rating and enzyme secretion has been reported for other pathogens (Goel and Mehrotra, 1973-'74 Dhingra *et al.*, 1974).

In the present study, the highest Cx and pectic enzyme activity was noted at 9th day after infection. Batman and Basham (1976) emphasized that the ability of a pathogen to produce a given enzyme 'in vitro' or the detection of an enzyme in diseased tissue provides only circumstantial evidence of a role in pathogenesis. Results of the present study gives an idea on the ability and type of enzyme secretion by isolates of *P. vexans*. A detailed study demonstrating specific changes in the structural frame work of host cell in response to specific enzyme may explain the varying reaction of isolates with respect to enzyme secretion and virulence vis a-vis disease development.

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