Penicillium expansum Lr, ex Thom, a soft rot pathogen of apple fruits was isolated from rotted fruits collected from the local markets. On inoculation of the isolate, caused the typical penicillial soft rot symptoms. The decayed portion was sharply demarcated from the healthy tissue. The spots, which showed variation in size and colour, were shallow at first, but later extended much deeper into the fruit. The rotten area did not become sunken. A musty odour accompanied the rotting. The fungus was maintained on FDA slants.

The bacterial pathogen Erwinia carotovora pv. atroseptica (Hellmer and Dowson) Dye, which causes black leg of potato was kindly supplied by Dr. S.H. De Boer, Research Station, Vancouver, B.C., Canada. Various staining and biochemical diagnostic tests were performed by the methods as given in Table 1. It is a gram-ve, motile rods, which on nutrient agar forms round, white, smooth, glistening and butyrous colonies. It grew at 25°C but not at 37°C, liquified gelatin, utilized citrate as the sole source of carbon, reduced nitrate to nitrite and was able to produce hydrogen sulphide but not indole.
<table>
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<th>Sr. No.</th>
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<td>1.</td>
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<td>Catalase test</td>
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<td>11.</td>
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or phenyl pyruvic acid, Also it was able to ferment glucose, sucrose, maltose, fructose, mannitol, with the production of acid. Voges-proskauer test was +ve while the methyl red test was -ve.

These pathogens were tested for their potentials in the production of the cell wall degrading enzymes.

**ENZYME SAMPLE PREPERATIONS:**

The pathogens were grown on media with different carbon sourced for enzyme production. The carbon sources used were, pectin, carboxy methyl cellulose, filter paper pulp, cotton fibers and xylan. The two common growth media used were Czapek and Richards. 'Analar' grade 'Sigma' chemicals were used. 25ml of the media were dispersed in 100 ml Erlenmeyer conical flasks and autoclaved at 15 psi for 20-30 minutes. The pH (pH 5 for fungus; pH 7 for bacteria) of the growth media were adjusted before autoclaving. The flasks were inoculated with fixed amount of inoculum from cultures of the two organisms. The inoculated flasks were incubated without shaking at 25°-27°C. After the desired growth period the culture media were filtered or centrifuged at 4°C. For the dry weight of fungus, the previously dried and weighed filter paper in which the medium was filtered, was put in oven for 48h at 80°C. Increase in weight
of the filter paper was noted. For the bacterial culture
the turbidity of the growth medium was measured at 610 nm.
After filtration or centrifugation (at 12,000 rpm for
15 min) the culture filtrates were used as the enzyme
samples. In order to store the samples, theocmersal at
1% concentration was added to the culture filtrates as a
preservative for contamination.

ASSAY OF PECTOLYTIC ENZYMES:

PME (Pectimethyl esterase) assay:

PME was assayed by the method of Mc Colloch and
Kertesz (1947) in which the amount of acid produced
in the reaction mixture is measured by titration using
0.01N NaOH to pH 7. The reaction mixture consists of
5ml of the pectin solution, 5 ml of enzyme sample and 0.5 ml
of 2N NaCl. The pH was immediately adjusted to 7. Few
drops of toluene were added to the reaction mixture which
was then incubated at 30°C. At every 15 min, the pH was
checked and if any loss in pH, it was adjusted to the
original level (pH 7) by titrating the reaction mixture
with 0.02N NaOH. The amount of NaOH needed to bring back
the pH to original level were noted. Controls were run
simultaneously with boiled enzyme sample. The activity was
obtained by taking the difference in amounts of NaOH needed
for the active enzyme sample and the control.
Assay of chain splitting pectolytic enzymes:

(A) Determination of nature of split:

The cleavage of pectic substances into smaller units of galacturonic acids is mediated by hydrolytic or trans-eliminative mechanisms. These cleavages can be known only by spectrophotometer. The hydrolytic and trans-eliminative reaction products can be distinguished by their reaction with thiobarbituric acid (TBA) (Neukom, 1960; Ayers et al., 1966; Sherwood, 1966). A colour reaction between an acid reagent solution of TBA and enzyme-substrate reaction have characteristic absorption peaks for the hydrolytic and trans-eliminative action. The unsaturated product of trans-eliminase action forms a chromogen which absorbs maximally at 550 nm (or 547 nm) while the product of hydrolase action shows maximum absorption at 510 nm. The unsaturated products released by lyases can also be detected by the absorption peaks at 230 and 235 nm (Albersheim et al., 1960b; Starr and Moran, 1962; Nagel and Anderson, 1965). The reaction products released hydrolytically (galacturonic acid) do not absorb at these wavelengths.

For the enzyme-substrate reaction, pectin or NaPP were used to prepare substrates at different pHs. For maintaining the pHs, the buffers used were, McIlvaines'
buffer (0.05M phosphate-citrate buffer) for pH between 4-7, 0.02M Tris buffer for pH between 7.5-9 and 0.05M Borax-NaGH buffer for pH 9.5-11. The reaction mixture consisted of 2 ml of the enzyme sample and 4 ml of 1.2 per cent NaPP or pectin solution (1:2 ratio of enzyme and substrate) taken in scratchless capped tubes and incubated at 30°C. After 4h of incubation 1 ml of the reaction mixture was taken and mixed with 15 ml of acidified TBA solution (5ml of 0.5N HCl and 10 ml of 0.01M TBA) in a culture tube and held in boiling water for one hour to allow the colour development by reaction of the reaction product and TBA (the TBA solution was prepared by dissolving 288 mg of thiobarbituric acid in 200 ml of glass distilled water). After cooling, the original volume was restored by adding glass distilled water. The absorption spectrum was taken over the wavelength range of 480-580 nm on a Shimadzu UV-double beam spectrophotometer. For the controls at O.D.=0, either boiled enzyme-substrate reaction mixture incubated for same period viz. 4h or active enzyme reaction mixture incubated for Oh, was used in place of the reaction mixture. Both gave similar results but it was found that the latter was more convenient and therefore, was used as the control. The TBA test of production of unsaturated compounds as a consequence of lyase action has proved very
convenient and is as reliable as the one taking peak in the UV region. However, for checking up this method was occasionally employed. The reaction mixture consisting of 5 ml of 0.2 per cent NaPP (at different pH), 2 ml of appropriate buffer and 2 ml of enzyme sample was incubated at 30°C for 2h in a scratchless capped culture tube in a water bath. 2 ml of 5 per cent TCA solution was added to precipitate out the proteins which were then centrifuged and separated. The absorption spectrum was then examined at 225-235 nm, using a clear aliquot withdrawn at 0h of the reaction time to adjust the optical density to 0. Absorption peak at 239 nm for lyase products has also been reported by Albersheim and Killas (1962) and Bush and Codner (1970).

A very good feature of the TBA test is the simultaneous detection of the two modes of cleavage if present together in the same test sample (Sherwood, 1966). On one substrate (pectin or pectic acid) at the same pH only one peak appears in the spectrum and the other enzyme active on another pH is masked. Then the heights of the peaks were proportional to the enzyme activity as indicated in viscosity loss experiments (to be described latter).

Enzyme specificity was examined by seeing the enzyme
activity on pectic acid and pectin for PAL/PG and PL/PMG respectively. Thus, we could detect whether the enzyme attacked the chain hydrolytically or in trans-eliminative manner.

(B) **Random or Terminal Site of Enzyme:**

The release of only monomeric products suggests terminal bond cleavage which can be known by measuring the increase in reducing sugar in the reaction product over the control. This was detected also chromatographically (Smith, 1958).

The random cleavage results in production of chains of shorter lengths which shows up in viscosity loss of the polymeric pectic as well as cellulolytic substrates in association of enzyme sample. A Fenske-Ostwald viscometer was used for measuring the depolymerizing activity of endo-forms of pectolytic enzymes and cellulases. A rapid loss in viscosity suggests random cleavage while a low or no viscosity reduction is indicative of terminal cleavage. Rombouts (1972) has quantified the endo-enzyme assay by viscosity loss by determination of the per cent of C1,4 bonds split at a given per cent of viscosity loss. Endo-enzymes will normally induce a 50 per cent viscosity loss with only 0.5-3.0 per cent hydrolysis.
where as 20 per cent hydrolysis or more is required for an exo-enzyme to induce a similar viscosity loss. Unlike the exohydrolases which release monomeric reaction products, exo-lyase enzymes normally release unsaturated dimers (Okamoto et al., 1964).

For the viscosity loss measurements one ml of the enzyme sample was mixed with 10 ml of the substrate (1.2 per cent pectin or NaPP and buffered with citric acid and NaOH or Tris buffer, depending on the pH requirement) to form the reaction mixture. 10 ml of 1.2 per cent the substrate (NaPP or pectin) was placed in the viscometer and allowed to equilibrate in a constant temperature tank (Toshniwal) at desired temperature. One ml of the enzyme sample was mixed with the substrate in viscometer by drawing up air through the large arm. The efflux time of the reaction mixture in seconds (time taken by the meniscus to fall from the upper to the lower line of the viscometer) was noted after 5, 10, 15, 20, 25 and 30 minutes, after the enzyme sample was added to the substrate. The per cent loss in viscosity was calculated by the formula:

\[
\frac{To - Tt}{To - Tw} \times 100
\]
Where, $T_0$ is the flow time immediately after addition of the enzyme sample, $T_t$ is the flow time after $t$ min and $T_w$ is the flow time for distilled water.

The viscosity loss was given a quantitative value by calculating the relative enzyme activity which is the reciprocal of the time in min for 50 per cent loss in viscosity multiplied by 1000. Relative activity was calculated by the formula:

\[
\frac{1}{\text{Time for 50 per cent loss in viscosity}} \times 1000
\]

The liberation of reducing sugars by action of exoenzymes was determined by Dinitrosalicylic acid (DNS) method using Miller's (1959) modified reagent. The DNS reagent was prepared as detailed below:

**Solution A** consisted of 300 ml of 4.5 per cent (w/v) NaOH, 800 ml of one per cent (W/v) DNS and 255 g of Potassium sodium tartrate (Rochelle salt).

**Solution B** 10g of crystalline phenol was mixed with 22ml of 10 per cent (W/v) NaOH and both were dissolved in glass distilled water and made up to one litre in a volumetric flask.
To 69 ml of solution B was added 6.9g of sodium bisulphite which was then mixed with solution A and stirred till the Rochelle salt was dissolved. The reagent thus prepared was kept in tightly-stoppered bottles at 4°C.

Before using pectin or NaPP for reducing sugars experiment, they were washed three times with 70 per cent ethanol to remove soluble sugars (Ishii and Yokotosuka, 1975). Then the pectin or NaPP solution was made by dissolving 0.625gms of these ethanol washed compounds in 100 ml glass distilled water. To determine the release of reducing sugars, 9 ml of 0.625% NaPP or pectin solution was taken in test tube and to this 1 ml of buffer of required pH and 1 ml of enzyme sample were added and incubated for 1h. The control was taken in the same way, but for boiled enzyme. After the incubation time, 1 ml of reaction mixture was withdrawn and added to 3 ml of DNS reagent and immediately kept in boiling water bath for 5 minutes. The yellow DNS in presence of NaOH (which is incorporated in the reagent solution) gets reduced to form a dark, reddish-brown compounds, 3-amino-5-nitrosalicylic acid. The tubes having enzyme activity show marked colour change in the solution. The tubes were cooled in chilled water and diluted by adding 10 ml of glass distilled water.
The optical density (O.D.) was determined for each diluted solution at the wavelength of 575 nm (as recommended by Summer and Somars, 1944) in Systronic spectrophotometer. The samples containing active enzymes were read against their paired blank containing boiled enzyme. The reading power of the test solution expressed in ug was extrapolated from a previously prepared standard curve for known concentrations of galacturonic acid.

One ml solution of various concentrations of galacturonic acid (viz. 1000µg/ml, 500µg/ml, 200µg/ml, 100µg/ml) mixed with 3 ml of the DNS reagent was taken in a tube and the O.D. was found out as detailed above. The O.D. (Y axis) was plotted against the concentrations (µg of galacturonic acid/ml) as the X axis. A straight line is obtained by joining the best fits among the different points. Enzyme activity was calculated as the difference in ug of reducing substances (galacturonic acid) between samples containing the active and boiled enzyme samples.

The chromatographic analysis of the released monomers, dimers and oligomers gave a picture of the site of bond cleavage. If the monogalacturonic acid appeared first in the sample of reaction mixture
spotted on the chromatogram, the enzyme activity was at the terminal end. However, if mixtures of oligogalacturonoids (tri or tetrauronoids) appeared before the release of monogalacturonic acids, random cleavage could be the type of cleavage which could easily be corroborated with the viscosity loss experiments.

For spotting, purification and concentration of the enzyme sample was essential. The enzyme sample was concentrated to one tenth in volume at room temperature in vacuo. Three volumes of cold acetone were added and the precipitate formed was allowed to settle overnight before it was collected (or alternatively centrifuged at 5°C) and dried under vacuum. One per cent solution of the dried precipitate was dialyzed against distilled water for 12h. The dialyzed solution served as the enzyme sample. This was done to avoid 'tailing' of spots on the chromatograms.

The enzyme sample mixed with 1.2 per cent NaPP solution at known pH was incubated at desired temperature. Aliquots from the reaction mixtures were withdrawn from time to time to see the sequence of release of the products of enzyme activity. D-galacturonic acid was taken and used as standard. Chromatograms were run in Butanol, acetic acid and water (in the ratio 4:1:5 v/v) in descending
manner. The 'run' chromatograms were dried by moving air and subsequently sprayed with aniline diphenylamine phosphate reagent (Buchan and Savag, 1952).

Spraying reagent:

i) Two grams of diphenylamine phosphate dissolved in 50 ml of butanol.

ii) 2 ml of aniline in 50 ml of butanol.

(i) & (ii) solutions were mixed and 10 ml of $H_3PO_4$ was added to it and shaken well. The mixture was filtered and stored in coloured bottle.

The sprayed chromatograms were dried at room temperature and in order to develop colour they were then kept in electric oven for 1 min at 100°C.

ASSAY OF CELLULASES:

The method of enzyme preparation was same as described for the pectolytic enzymes. The substances which served as the carbon sources were carboxymethyl cellulose (CMC), cotton fibers, filter paper pulp, cellobiose and glucose.

The enzyme activity was measured in terms of release of reducing sugars as glucose by the dinitro-salicylic acid (DNS) method as described by Miller (1959).
One ml samples were treated with 1 ml DNS solution and heated for 5 min in boiling water bath. The tubes were cooled for 5 min in chilled water. Ten ml of distilled water was added and the optical density was measured at 540 nm. The standard curve used here was prepared using different concentrations of glucose. Protein content in the enzyme samples was measured by Lowry's method (1951) using crystalline bovine serum albumin as the standard.

The cellulases activities viz. endoglucanase, exoglucanase and β-glucosidase were defined and measured according to Mandels et al. (1974) and Mandels (1977).

Endoglucanase or carboxymethyl cellulase (CMCase) or EC 3.2.1.4:

0.5 ml of enzyme sample was mixed with 0.5 ml of 1% CMC in citrate-phosphate buffer of pH 4.8 (for P. expensum) or pH 7 (for E. carotovora pv. atroseptica) and incubated for 30 min at 50°C in constant temperature tank. The reducing sugars were measured as glucose equivalents by the DNS method. The enzyme activity was also measured viscometrically, which according to Goksoyr and Erikson (1981) is the most sensitive method for endoglucanase assay. The activity of the enzyme was measured by the
loss in viscosity of CMC (0.5%) with passage of time in Fenske-Ostwald viscometer upto 30 min at 50°C. The per cent loss in viscosity and relative activity were calculated as given earlier for pectolytic enzymes.

Exoglucanase or cotton degrading activity (CA) or EC 3.2.1.91:-

Phosphoric acid swollen cellulose or avicel, which are cellulosics having low degree of polymerization, are preferred substrates. The reaction mixture consisted of 50 mg of absorbent cotton, 1 ml enzyme sample and 1 ml of citrate buffer at pH 4.8. This was incubated for 24h and the release of reducing sugars were determined as the glucose equivalents. The incubation was done at 50°C in constant temperature water tank.

β-glucosidase EC 3.2.1.21:-

The test reaction mixtures consisted of 0.5 ml enzyme sample and 0.5 ml of 1% salicin dissolved in citrate buffer at pH 4.8. The incubation was carried out for 30 min at 50°C in constant temperature water tank. The release of reducing sugars were determined as glucose equivalents.
ASSAY OF HEMICELLULASE (XYLANASES)

Endoxylanase EC 3.2.1.8:

The action of endoxylanase was determined viscometrically. 10 ml of 2% xylan (Sigma, USA) in citrate buffer at pH 5, was taken in a Penske-Ostwald viscometer and to this 1 ml of enzyme sample was added, then the flow time was noted at every 5 min for 30 min. % viscosity loss and relative activity were calculated as given before in pectolytic enzymes.

Exoxylanase:

The exoxylanase activity was determined by the release of reducing sugars in the reaction mixture (0.5 ml enzyme sample, 0.5 ml 1% xylan in citrate buffer at pH 5) incubated for 30 min at 50°C. The reducing sugars were calculated as xylose equivalents. In order to identify the action of this enzyme, chromatography was also done where xylose was taken as control and the spots were made on the sheet from the culture filtrate at every alternate day or at every 3h of reaction time. The chromatography experiment was done as detailed in the pectolytic enzymes, and instead of galacturonic acid xylose was taken as the control.